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ARTIFICIAL RIBONUCLEASES: OLIGONUCLEOTIDES CONJUGATED WITH METAL ION CHELATES OF AZACROWNS

Teija Niittymäki

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University of Turku

Faculty of Mathematics and Natural Sciences
Department of Chemistry
Laboratory of Organic Chemistry and Chemical Biology

Custos

Professor Harri Lönnberg
Department of Chemistry
University of Turku
Turku, Finland

Reviewed by

Professor Doctor Michael Göbel
Institute of Organic Chemistry
and Chemical Biology
Goethe University
Frankfurt, Germany

Professor Jacek Jemielity
Institute of Experimental Physics
University of Warsaw
Warsaw, Poland

Opponent

Professor Jouko Vepsäläinen
School of Pharmacy
University of Eastern Finland
Kuopio, Finland

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ABSTRACT

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Ribonucleic acid (RNA) has many biological roles in cells: it takes part in coding, decoding, regulating and expressing of the genes as well as has the capacity to work as a catalyst in numerous biological reactions. These qualities make RNA an interesting object of various studies. Development of useful tools with which to investigate RNA is a prerequisite for more advanced research in the field. One of such tools may be the artificial ribonucleases, which are oligonucleotide conjugates that sequence-selectively cleave complementary RNA targets. This thesis is aimed at developing new efficient metal-ion-based artificial ribonucleases. On one hand, to solve the challenges related to solid-supported synthesis of metal-ion-binding conjugates of oligonucleotides, and on the other hand, to quantify their ability to cleave various oligoribonucleotide targets in a pre-designed sequence selective manner.

In this study several artificial ribonucleases based on cleaving capability of metal ion chelated azacrown moiety were designed and synthesized successfully. The most efficient ribonucleases were the ones with two azacrowns close to the 3´-end of the oligonucleotide strand. Different transition metal ions were introduced into the azacrown moiety and among them, the Zn^{2+} ion was found to be better than Cu^{2+} and Ni^{2+} ions.

Key words: RNA, oligonucleotide conjugate, metal ion chelate, azacrown, synthesis, cleaving agent

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Väitöskirja, 107 s.
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Marraskuu 2014

RNA:lla eli ribonukleiinihapolla on monia biologisia tehtäviä soluissa: se on mukana muun muassa geenien koodauksessa, säätelyssä ja ilmenemisessä. Tämän lisäksi RNA-molekyylit voivat toimia katalyytteinä lukuisissa biologisissa reaktioissa. Nämä ominaisuudet tekevät RNA:sta hyvin mielenkiintoisen tutkimuskohteen. RNA-tutkimuksen edistyminen edellyttää käyttökelpoisten työkalujen kehittämistä. Yksi tällainen työkalu voisi olla keinotekoinen ribonukleaasi eli oligonukleotidikonjugaatti, joka pilkkoo RNA:ta selektiivisesti. Tämän väitöskirjan tarkoituksena on kehittää uusia tehokkaita metalli-ionikelaatteihin perustuvia keinotekoisia ribonukleaaseja. Tarkoituksena on ensinnäkin valmistaa metalli-ioneja sitovia oligonukleotidikonjugaatteja ja toiseksi tutkia niiden kykyä pilkkoa sekvenssi-selektiivisesti erilaisia RNA-kohteita.

Työssä suunniteltiin ja valmistettiin onnistuneesti useita keinotekoisia ribonukleaaseja, jotka perustuvat atsakruunun metalli-ionikelaatin pilkkomiskykyyn. Tehokkaimmat valmistetuista ribonukleaaseista olivat oligonukleotideja, joiden 3´-päässä oli kaksi atsakruunukelaattia. Atsakruunuun kelatoitiin myös erilaisia siirtymämetalleja; näiden joukosta Zn^{2+}-ioni osoittautui paremmaksi kuin Cu^{2+}- ja Ni^{2+}-ionit.

Avainsanat: RNA, oligonukleotidikonjugaatti, metalli-ionikelaatti, atsakruunu, synteesi

PREFACE

This thesis is based on the experimental work carried out in the Laboratory of Organic Chemistry and Chemical Biology at the Department of Chemistry, University of Turku. The financial support from the Academy of Finland, University of Turku and Jenny and Antti Wihuri Foundation are gratefully acknowledged.

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Turku, October 2014

Teija Niittynen

CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications referred to in the text by their Roman numerals (I-IV):

I Niittymäki, T., Kaukinen, U., Virta, P., Mikkola, S. and Lönnberg, H. Preparation of Azacrown-Functionalized 2´-*O*-Methyl Oligoribonucleotides, Potential Artificial RNases. *Bioconjugate Chem.* **2004**, *15*, 174-184.

II Niittymäki, T. and Lönnberg, H. Sequence-Selective Cleavage of Oligoribonucleotides by 3d Transition Metal Complexes of 1,5,9-Triazacyclododecane-Functionalized 2´-*O*-Methyl Oligoribonucleotides. *Bioconjugate Chem.* **2004**, *15*, 1275-1280.

III Niittymäki, T., Virta, P., Ketomäki, K. and Lönnberg, H. Di(azacrown) Conjugates of 2´-*O*-Methyl Oligoribonucleotides as Sequence-Selective Artificial Ribonucleases. *Bioconjugate Chem.* **2007**, *18*, 1583-1592.

IV Niittymäki, T., Burakova, E., Laitinen, E., Leisvuori, A., Virta, P. and Lönnberg, H. Zn^{2+} Complexes of 3,5-Bis[(1,5,9-triazacyclododecan-3-yloxy)methyl]phenyl Conjugates of Oligonucleotides as Artificial RNases: The Effect of Oligonucleotide Conjugation on Uridine Selectivity of the Cleaving Agent. *Helvetica Chimica Acta* **2013**, *96*, 31–43.

ABBREVIATIONS

A	adenosine
Ac	acetyl
AcOH	acetic acid
AIDS	acquired immune deficiency syndrome
All	allyl
Boc	*tert*-butyloxycarbonyl
C	cytidine
CPG	controlled pore glass
DCC	*N*,*N*´-dicyclohexylcarbodiimide
DEAD	diethyl azodicarboxylate
DMAP	4-dimethylaminopyridine
DME	1,2-dimethoxyethane
DMF	*N*,*N*-dimethylformamide
DMTr	4,4´-dimethoxytrityl (4,4´-dimethoxytriphenylmethyl)
DNA	deoxyribonucleic acid
ESI	electrospray ionization
G	guanosine
HBTU	*O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluoro-phosphate
HEPES	4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid
HIV	human immunodeficiency virus
HOBt	1-hydroxybenzotriazole
HPLC	high performance liquid chromatography
Lev	levulinoyl
mRNA	messenger RNA
MS	mass spectrometry
NMR	nuclear magnetic resonance
ODN	oligodeoxyribonucleotide
ORN	oligoribonucleotide
PhtN	phthalimido
PMB	4-methoxybenzyl
PNA	peptide nucleic acid
Py	pyridine
RNA	ribonucleic acid
RP	reversed phase
T	thymidine
T_m	melting temperature
TBD	1,5,7-triazabicyclo[4.4.0]dec-5-ene
TBDMS	*tert*-butyldimethylsilyl
Tfa	trifluoroacetyl
TFA	trifluoroacetic acid
THF	tetrahydrofuran
TOM	triisopropylsilyloxymethyl
Tr	trityl (triphenylmethyl)
Ts	tosyl (*p*-toluenesulfonyl)

1. INTRODUCTION

1.1 RNA

RNA, like DNA, is composed of a long chain of nucleosides linked together via phosphodiester bonds (**Figure 1**). The difference lies in the structure of the sugar moiety (β-D-ribofuranosyl *vs.* 2´-deoxy-β-D-*erythro*-pentofuranosyl) and in one base (U *vs.* T). The uniqueness of RNA/DNA primary structure is determined by the sequence of its bases. The secondary structure is formed via complementary Watson-Crick base-pairing between two chains of RNA/DNA. Since binding of U/T to A is mediated by two and binding of C to G by three hydrogen bonds, CG-pairs are stronger than UA- or TA-pairs. Short sequences of RNA and DNA are called oligoribonucleotides (ORN) and oligodeoxyribonucleotides (ODN), respectively.

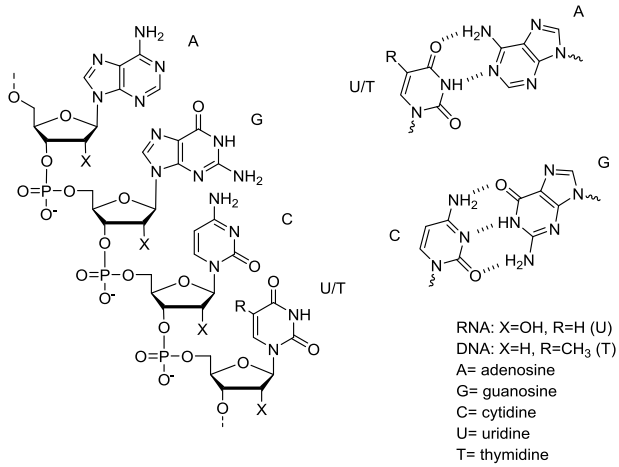


Figure 1. The structure of RNA and DNA and the Watson-Crick base-pairing.

The cleavage of phosphodiester bonds of RNA in a sequence selective manner is in cells catalyzed by both protein enzymes, ribonucleases, and catalytic RNA molecules. Naturally existing RNA molecules exhibiting catalytic activity are called ribozymes.[1] The use of these as research tools for cleaving RNA phosphodiester bonds is, however, cumbersome and in many cases impossible. There clearly is a demand for manipulation of RNA by simpler agents, and development of synthetic mimics of ribozymes has been the goal of many studies during the past two decades.[2-5] This kind of artificial ribonucleases, as they are called, can improve our understanding of mechanisms of enzyme action and they may also help to develop new biotechnological tools. These tools may, for

example, be artificial restriction enzymes, probes for structural studies of RNA and nucleic acid-targeting therapeutics. Although there have been many advances in the field, still the natural systems are far away as come for the catalytic activity.

Restriction enzymes that recognize and cleave DNA sequences very specifically are familiar and widely used tools in molecular biology.[6] Nevertheless corresponding restriction enzymes for the RNA scission on the same scale are not known. More widely useful sequence-selective manipulation of RNA by artificial restriction enzymes would bring about more advanced research in biotechnology.

Since the double stranded regions of RNA are much more resistant to cleavage than the single stranded regions,[7] cleaving agents can be used as probes in structural research of RNA.[8] The sites where the cleavage takes place upon treatment with such a probe are accessible to the cleaving agent, while absence of the cleavage indicates that the targeted site has not been reached. This structural analysis helps to understand the biological role of the three-dimensional structure of various RNAs.

Oligonucleotides which specifically inhibit unwanted genes by blocking messenger RNA (mRNA) activity are called antisense oligonucleotides. Their potential applications in chemotherapy have attracted many research groups in the area. The first thoughts about this antisense approach, as it is called, were published by Zamecnik and Stephenson in 1978.[9,10] In antisense approach the disease is silenced at an earlier stage than with the traditional drugs (**Figure 2**). In cell, DNA is first transcribed to mRNA which is translated to proteins. If a particular mRNA encodes some disease, the resulting proteins are typically the immediate cause of the disease, and traditional drugs (circle in the figure) are targeted towards them. Antisense drugs are oligonucleotides that hybridize with mRNA and, hence, the translation is inhibited and ideally the proteins which cause the disease are not expressed at all. There are many challenges that antisense drugs must overcome before they can be used as drugs in reality. If the antisense oligonucleotide is unmodified, its hybridization with complementary mRNA activates the intracellular enzyme RNase H which degrades the target RNA. This releases antisense oligonucleotide and makes the reaction catalytic as antisense oligonucleotide can be reused. Unfortunately most of the modified oligonucleotides do not activate RNase H, so their antisense effect remains stoichiometric blocking. The modification of antisense oligonucleotide is, however, necessary because otherwise oligonucleotide cannot penetrate through cell membrane and it is destroyed by nucleases. Modification can also improve hybridization efficiency or pharmacokinetic properties.

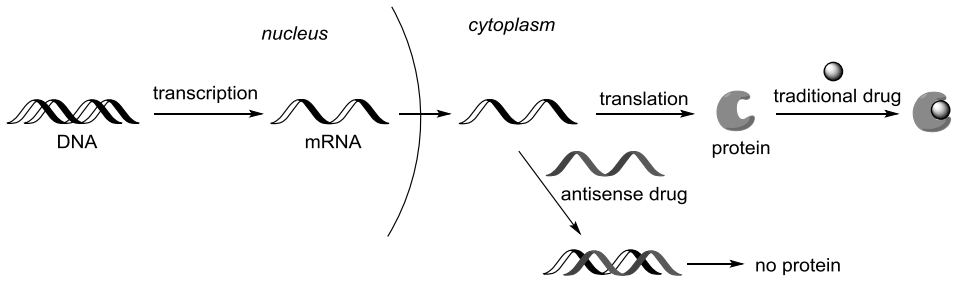


Figure 2. Schematic presentation of the antisense strategy. DNA encodes a disease, which is inhibited by the action of the antisense drug. The most common way the traditional drug works is also represented.

This thesis covers the main strategies how to cleave target RNA without the help of RNase H. This is achieved with the aid of different catalytic groups attached to oligonucleotides. These oligonucleotide conjugates are called artificial ribonucleases and they work as represented in **Figure 3**. They consist of two parts: oligonucleotide part which takes care of the recognition of the target RNA and the catalytic part (triangle in the figure) which cleaves the target. The aim is that the artificial ribonuclease is released in the reaction and it can be recycled.

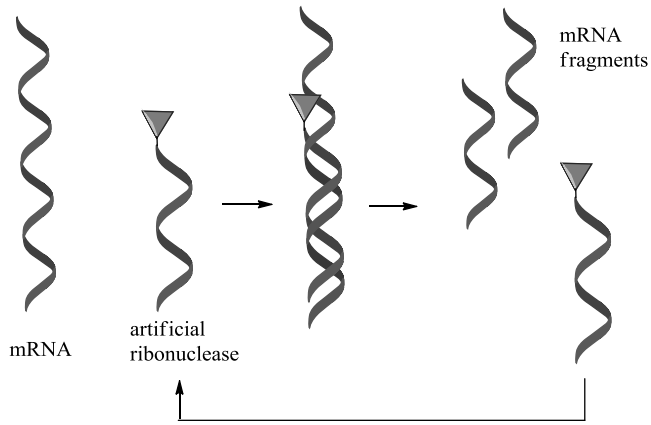


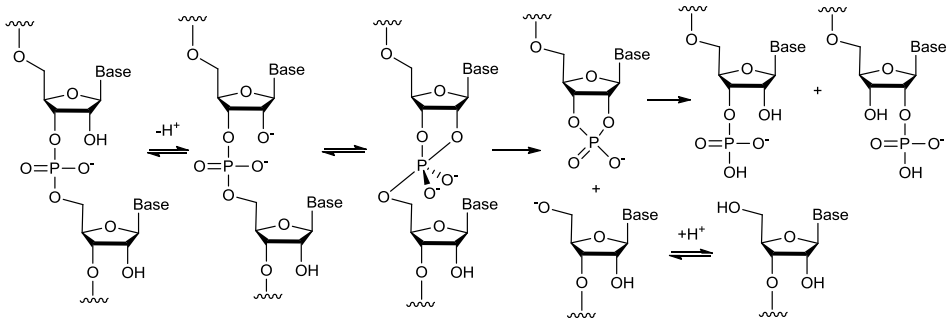
Figure 3. The use of an artificial ribonuclease. The triangle represents the catalytic group which cleaves the mRNA.

The first drug based on antisense technology came on the market in 1998. It was Vitravene, a phosphorothioate oligonucleotide targeted for the treatment of an eye disease, cytomegalovirus retinitis, of patients suffering from AIDS.[11] Owing to improved therapies for patients having HIV, the need for Vitravene has diminished and nowadays it has left the market. In 2013, another phosphorothioate oligonucleotide drug, Kynamro, was approved for clinical use. It lowers

cholesterol in patients with homozygous familial hypercholesterolemia (HoFH).[12] In addition there are more than 40 antisense oligonucleotides in clinical trials.[13,14] Three drug candidates for cancer treatment are already in phase III in clinical trials, so maybe the breakthrough is coming.

1.2 Cleavage of RNA

RNA molecules are or can be degraded at a certain speed which is often a critical factor for many biological processes, including those involved in processing of genetic information. Although RNA is, because of the 2´-hydroxy group, hydrolytically labile compared to DNA, it still is quite a stable molecule chemically. The half-life for the cleavage of a single phosphodiester bond is over 100 years under physiological conditions.[15] Cleavage of RNA can occur through oxidative or nucleophilic cleavage. In this thesis, only the nucleophilic cleavage is covered. As known already from the 1950s,[16] the nucleophilic cleavage is initiated by the attack of the neighboring 2´-hydroxy group on the phosphorus atom leading to the departure of the 5´-linked nucleoside and simultaneous formation of a 2´,3´-cyclic phosphate (**Scheme 1**).[17] This reaction proceeds through a pentacoordinate phosphorane intermediate or transition state in which the attacking nucleophile (2´-O^-) and departing alkoxide (5´-O^-) must occupy the apical positions, obeying the rules of Westheimer.[18] The cyclic 2´,3´-phosphate is then rapidly hydrolyzed to a mixture of 2´- and 3´-phosphates.

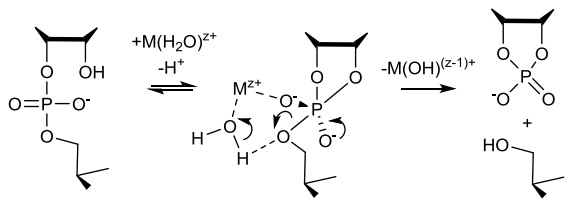


Scheme 1. Cleavage of the phosphodiester bond of RNA.

Enhancement of the reaction can be achieved by facilitating the deprotonation of the attacking 2´-OH, stabilizing the phosphorane intermediate, orienting the entering and departing nucleophiles properly and stabilizing or facilitating the protonation of departing 5´-O^-. Molecules that can work as artificial ribonucleases, *i.e.* enzymes capable of cleaving RNA, must therefore fulfill one or preferably several of the above mentioned rate-enhancing factors. It has been

estimated that the deprotonation of 2´-OH and protonation of 5´-O$^-$ both accelerate the cleavage reaction by 10^6-fold.[19] Neutralization of the negative charge on the non-bridging phosphoryl oxygen can enhance the reaction by a factor of 10^5 and allowance to the in-line orientation of the phosphorane intermediate can achieve the maximum of 100-fold acceleration. Understanding of the detailed mechanism of the cleavage reaction and factors affecting to it is the basic necessity for developing efficient and multifunctional catalysts.[20]

The cleavage of phosphodiester bonds of RNA is catalyzed by several metal-ion-dependent ribonucleases and ribozymes,[21] but also metal-ion-independent biocatalysts are known.[22] The most extensively studied example of the latter is RNase A, whose histidine residue deprotonates the attacking 2´-OH and protonates the departing 5´-O$^-$. In addition, the dianionic phosphorane intermediate is stabilized by hydrogen bonding with lysine residue. This thesis concentrates mostly on the metal-ion-dependent cleavage. The exact details of the mechanism of the metal ion promoted reactions have not been sufficiently clarified, even though the reaction itself has been known since 1950s.[23] There are various interpretations for the reaction, but usually only the cleavage of the 3´,5´-bond, not the isomerization to the 2´,5´-bond, is accelerated.[24] Only one metal ion participates in the reaction, except for the lanthanide ions where the polynuclear assemblies are the best catalysts.[25] The reaction appears to proceed as illustrated in **Scheme 2**. First, there is a pre-equilibrium formation of a dianionic phosphorane, which is stabilized by a coordinated metal aqua ion. This pre-equilibrium stage is followed by the rate-limiting step of the actual cleavage reaction in which the departing 5´-oxygen atom is protonated. 3d transition metal ions catalyze this reaction faster than alkaline earth metal ions.[21] Zn^{2+} ion belongs to the best metal ion catalysts, for example the poly(U) is degraded 1900 times more rapidly with 5 mM concentration of Zn^{2+} ion than without any metal ion (pH 5.6, 90 ºC).[26]



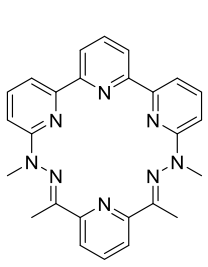
Scheme 2. Metal ion promoted cleavage of RNA.

1.3 Cleaving agents

Lots of efforts have been put on finding the cleaving agents that catalyze the cleavage of phosphodiester bonds efficiently. The catalysts fall mainly into two categories: a metal-ion-based catalysts and metal free systems which usually are nitrogen containing organic molecules.

In order to get metal ion promoted cleavage sequence selective, metal ions must be coordinated to some ligand which, in turn, can be attached to oligonucleotide. Lanthanide ions are exceptionally good cleaving catalysts as compared to other metal ions.[27,28] The most promising candidates of lanthanide-ion-based cleaving agents are the chelates of macrocyclic ligands, pyridine cyclophane **1**[29-32] and texaphyrin **2**.[33-35]

Another group of metal ions often used as cleaving agents is 3d transition metal ions, especially Cu^{2+} and Zn^{2+} ions. One significant advantage of these ions over lanthanides is their natural presence in intracellular fluids, hence, the ligands binding Cu^{2+} or Zn^{2+} may be expected to appear as Cu^{2+} or Zn^{2+} complexes even in intracellular environment. Especially Zn^{2+} ion, because of its total intracellular concentration is in micromolar range.[36] The Cu^{2+}-based cleaving agents have usually been terpyridine **3**[37-40] or 2,9-dimethylphenanthroline **4**[41,42] ligands. At the beginning of the research for this thesis, metal complexes used as sequence selective cleaving catalysts were mostly the above mentioned lanthanide and Cu^{2+} ion complexes. The first Zn^{2+} chelate was reported for the 2,9-dimethyl-phenanthroline **4**[41] and at the same time with our study, the group of Strömberg investigated quite thoroughly the Zn^{2+} complexes of **4**.[43-45]



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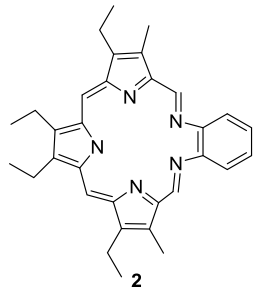

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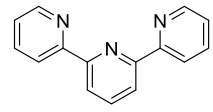

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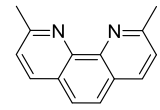
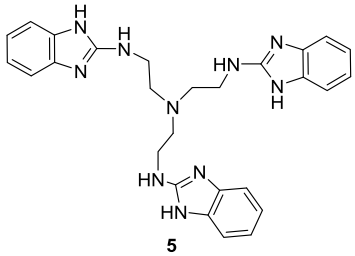


4

Although this thesis focuses on cleaving agents of metal ion complexes, main observations concerning metal ion independent agents[46] are briefly discussed here. The cleaving activity of the non-metallic catalysts has usually remained rather low. Guanidinium group constructs have shown the best activities, the most efficient being tris[2-(benzimidazol-2-yl)ethyl]amine (**5**)[47] and its attachment to an ODN has given quite effective sequence specific artificial

ribonuclease.[48] Another unusually powerful group of non-metallic cleaving agents is offered by imidazole containing constructs. These structures mimic the catalytic center of enzyme RNase A which bears histidine residues.[22] Several imidazole containing cleaving agents have been prepared and tested by the group of Vlassov.[49-53] Among many conjugates tested, the most efficient ones were oligonucleotides bearing structure **6**. Conjugates bearing several such groups and different linker arms were also studied.[52,53] Simple oligoamine conjugates have also been investigated, but they exhibit rather modest activity.[25,54] All the best cleaving agents of metal free systems have been designed during my research on Zn^{2+}-based agents, so at the time I started there were not many efficient artificial ribonucleases reported.



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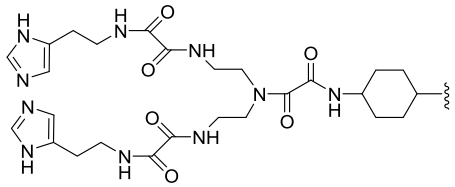

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1.4 Synthesis of oligonucleotides and attachment of catalytic agents

Artificial ribonucleases are typically synthesized by covalent attachment of the cleaving agent to a synthetic oligonucleotide strand (ODN, ORN or their analog) via some linker. ODN and 2´-*O*-alkyl-ORN strands are the most frequently used recognition domains. 2´-*O*-Alkyl-modification provides increased stability towards degradation by natural nucleases and higher binding affinity to the target RNA. Peptide nucleic acid (PNA) oligomers have also been used for this purpose; they have some desirable qualities, especially remarkable stability in biological fluids, that makes them good candidates.[55] They also form stable hybrids with target RNA and are nowadays easily synthetized by peptide chemistry.

The synthesis of the ODN/ORN strands are carried out on support and assembled on an automated synthesizer using either the more applied phosphoramidite strategy[56] or the H-phosphonate strategy[57] (**Figure 4**). The first nucleoside is anchored to the support, which is most commonly amino-functionalized controlled pore glass (CPG)-support, through the 3´-hydroxy group via a succinate linker. The 5´-hydroxy function is usually protected with an acid-labile 4,4´-dimethoxytrityl group. After its removal, the next nucleotide is coupled using a suitably protected building block with phosphoramidite or H-

phosphonate functionality together with appropriate activator. For the phosphoramidite chemistry the most used activators are 1*H*-tetrazole-based but also 4,5-dicyanoimidazole is used, especially for ORN, and for the H-phosphonate chemistry the activator is pivaloyl chloride. The main difference between the two strategies is the oxidation step; oxidation is carried out in every cycle on using the phosphoramidite strategy, while it is done only once after completion of the chain elongation in the H-phosphonate strategy. The capping is needed to terminate the chain elongation if the coupling is incomplete. On preparing ORN, the 2´-hydroxy function must be protected as silyl ether, for example, which withstands both acidic and basic conditions. The ammonolysis step releases oligonucleotide from the support and at the same time removes the base and phosphate protections.

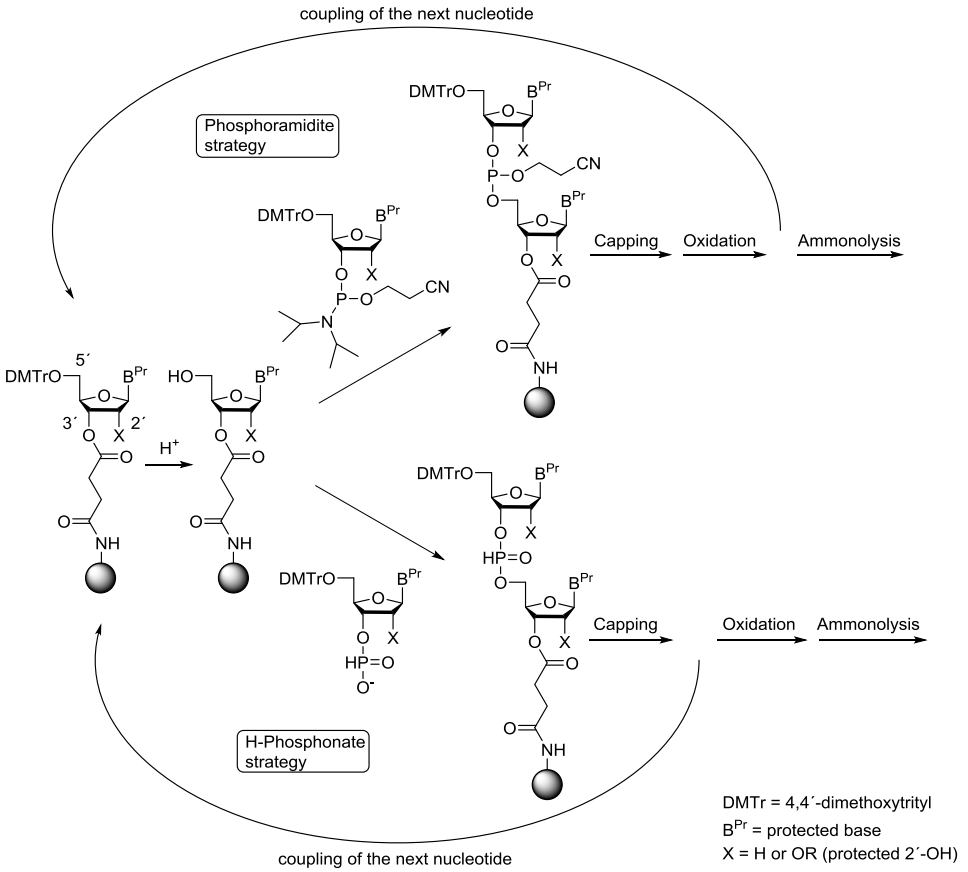
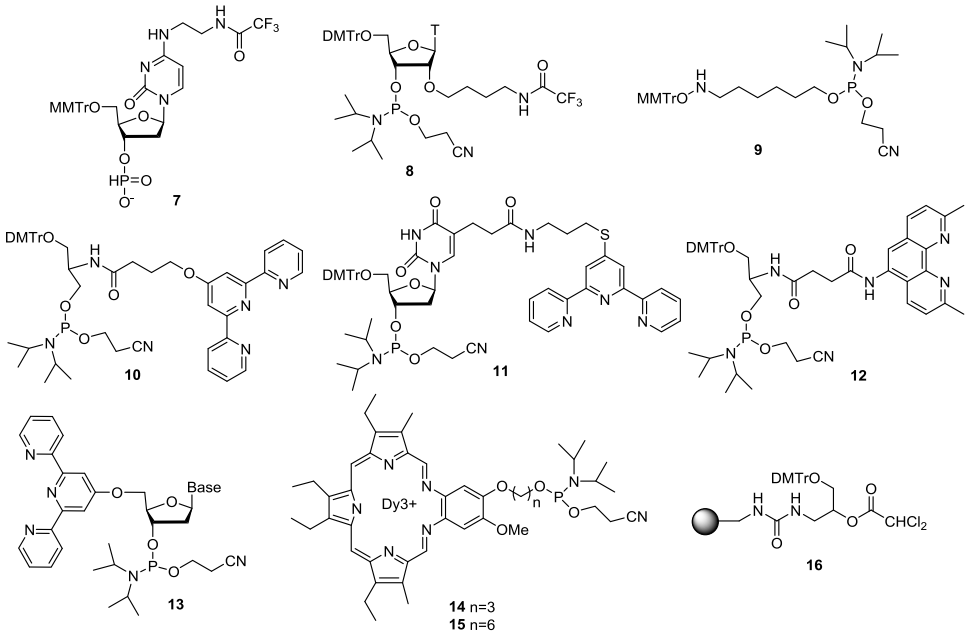


Figure 4. The solid-supported synthesis of oligonucleotide by the phosphoramidite and the H-phosphonate strategy.

On considering the conjugation of the catalyst to the oligonucleotide strand, one of the main things is the compatibility of the protecting groups used. The oligonucleotide synthesis has conventionally acid labile 5´-protecting groups and base labile protections on the phosphate (only in phosphoramidite strategy) and base moieties; in addition, the chain is released from the support with a basic nucleophile. If the catalytic moiety is attached before completion of the oligonucleotide synthesis, any protections on it must withstand acid treatment repeated in every synthesis cycle and the catalytic moiety itself must withstand the base treatment at the end of the synthesis. If, on the other hand, the attachment is done after the chain assembly, the compatibility of the protecting groups on oligonucleotide and on the catalyst must still be taking into consideration.

Two approaches are generally used for the conjugation of the catalytic group to the oligonucleotide; the attachment is done either in solution or on support. On applying the solution based approach, the oligonucleotide must bear a functionality where to attach the catalyst. In most cases that have been an amino group introduced to the oligonucleotide on support. For example, compounds **7**, **8** and **9** with protected amino functions have been used to tether the catalyst in the middle of the chain through modification of a base or sugar moiety[32,44] or at the 5´-end of the chain through a commercially available aminohexyl-linker.[33,58] Reaction of the amino-containing oligonucleotide in solution with a catalyst functionalized as a *N*-hydroxysuccinimide ester,[32,33,58] isothiocyanate[32,58] or phenylcarbamate[44] have yielded the desired oligonucleotide conjugates (**Figure 5**). Since the oligonucleotide is anyway assembled on support, it is only logical to carry out the conjugation on the same support. The notable advantage of the synthesis on support compared to that in solution is a much easier purification; most of the impurities can be simply washed away while the oligonucleotide conjugate is still anchored to the support. Another appealing feature is the possibility to do the whole synthesis automatically on synthesizer. This on-support-approach requires conversion of the catalyst to an appropriate building block which can be used directly on automated synthesis of the oligonucleotide. Building blocks for the phosphoramidite strategy have been introduced for the terpyridine (**10** and **11**)[37,38,40] and 2,9-dimethylphenanthroline ligands (**12**).[41] They bear both the phosphoramidite and the dimethoxytrityl functions, thus they can be incorporated into any position of the oligonucleotide chain as in **Figure 4** (building block of a catalyst is used instead of a nucleotide). Few reports are available for the conjugation of the catalyst (**13**-**15** having phosphoramidite but not the dimethoxytrityl function) only to the 5´-end of the ODN.[35,39] Since oligonucleotides are normally synthesized in the 3´→5´direction, coupling of the catalyst to the 5´-end is straightforward and can be done in the final step. 3´-Conjugates are more complex to construct but nowadays commercially available

universal support **16** (Glen Research, Universal Support III) allows utilization of appropriate building blocks of the catalyst in the first step of the chain elongation.[59,60]



PNA-based artificial ribonucleases have been synthesized on support by the Fmoc-chemistry. The catalyst have been incorporated either directly to the desired site as a PNA building block **17**[61] or indirectly to a diaminopropionic acid unit **18**, which contains a protected amino group to react, after exposure, with the phenylcarbamate activated catalyst, as indicated in **Figure 5**.[62,63]

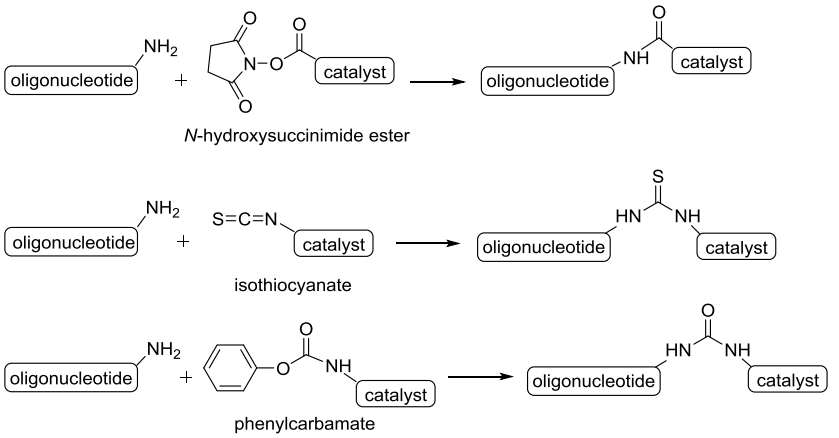
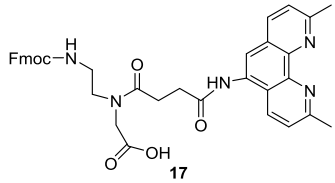


Figure 5. Attachment of the catalyst to the oligonucleotide chain in solution.



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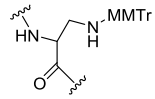

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1.5 Targeting of ribonucleases

The oligonucleotide strand serves as the recognition domain of the ribonuclease, and the cleaving agent as the active site. Because of the specificity of complementary Watson-Crick base pairing, the oligonucleotide strand may be made to direct the RNA cleavage toward any RNA target of known base sequence. Artificial ribonucleases are hoped to cleave the target RNA at a single desired position. If the metal-ion-promoted cleavage is aimed at being carried out in a sequence specific manner, the metal ion must be tightly bound to the ligand structure that allows its attachment to a predefined position within the oligonucleotide chain.

One requirement for artificial ribonucleases is the fast release of the cleaved RNA fragments from the ribonuclease to avoid product inhibition. In other words, in a first step, the target and ribonuclease must form a stable duplex in order to have the cleavage reaction to take place in a desired position, and after that, the duplex between cleaved fragments and the ribonuclease must be sufficiently destabilized to allow release of the ribonuclease. Thus the ribonuclease can bind and cleave another complementary RNA target sequence, and work as a real catalyst.

The precise cleavage site within the target RNA is also important. The single stranded regions of target are cleaved much more efficiently than the double stranded ones.[7] Accordingly, either the cleaving agent should be situated at the end of the oligonucleotide or if situated in an intrachain position, the ribonuclease and the target should be forced to hybridize with each other in such a manner that the RNA target forms a bulge which is susceptible to the cleavage.[31,32,64] Within a duplex region, the 2´-hydroxy function is not suitably oriented for the cleavage reaction, whereas in a bulged region there is enough conformational freedom for proper orientation.[31] The bulge can additionally be regarded as a pocket for the cleaving agent. Also the base sequence of the target RNA has an influence on the reactivity.[65]

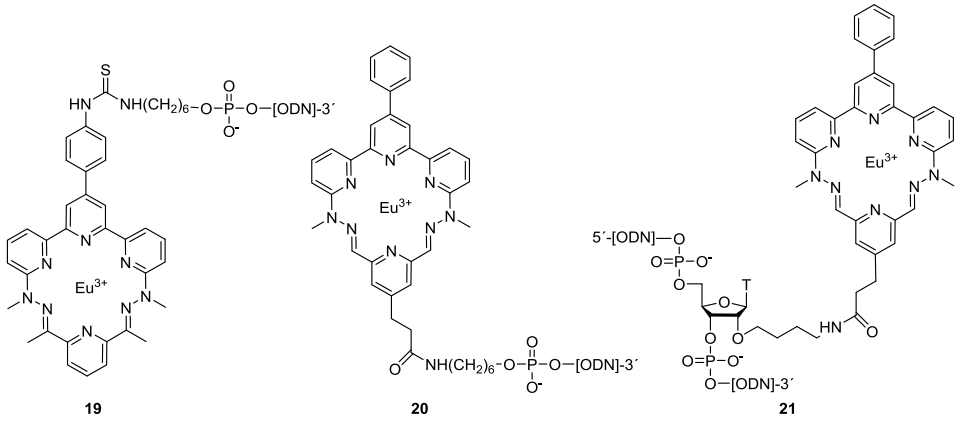
1.6 Kinetic measurements

Research groups have used slightly different experimental conditions when analyzing the action of artificial ribonucleases. Accurate comparison of the results is, hence, difficult. An approximate comparison can be found from literature.[3] The cleavage reactions have generally been carried out at only one temperature, pH and ionic strength, the most frequently used conditions being 37 ºC, pH 7.5 and 0.1 M ionic strength. There is only one study where the pH dependence of the cleavage rate has been studied.[40] The concentration of metal ion compared to that of the oligonucleotide-bound chelate has also varied from one study to another. The lanthanide ion based artificial ribonucleases have been synthesized by attachment of the preformed metal complex to the oligonucleotide probe. In other words, the concentration of the lanthanide ion has been the same as that of the oligonucleotide conjugate in the cleavage reaction.[35,58] The Cu^{2+} and Zn^{2+} based artificial ribonucleases, on the other hand, have been formed by addition of the metal ion post-synthetically to the oligonucleotide-bound chelate and the concentration of the metal ion in the cleavage reaction has varied from 1 equivalent[66] to excess of the metal ion.[43,61,67] The dependence of the rate on the concentration of Cu^{2+} ion[40,60] or Zn^{2+} ion[43] has been explored. Concentrations of the artificial ribonucleases and the targets have been different in various studies which may also influence on the rate of the cleavage and thus complicate the comparison. Still one complication is that various methods have been applied to the analysis of the aliquots withdrawn from the reaction mixture. Most of the cleavage rates have been obtained by densitometric analysis of autoradiograms of gel electrophoresis, the quantification of which is rather susceptible to experimental errors. Nevertheless, approximate first-order rate constants may be obtained by this method when several samples are analyzed as a function of time.[34,40] Many cleavage studies by gel electrophoresis are, however, based on a single aliquot at late stage of the reaction.[32,66] Only one group has used a more quantitative method by analyzing the samples with either RP HPLC[43] (in case of 2´-*O*-methyl-ORN based cleaving agents) or anion exchange HPLC[62] (in case of PNA based agents). This provides more accurate first-order rate constants. The anion exchange HPLC gave higher resolution than the RP HPLC.[62]

1.7 Metal-ion-based artificial ribonucleases

1.7.1 Lanthanide ion complexes

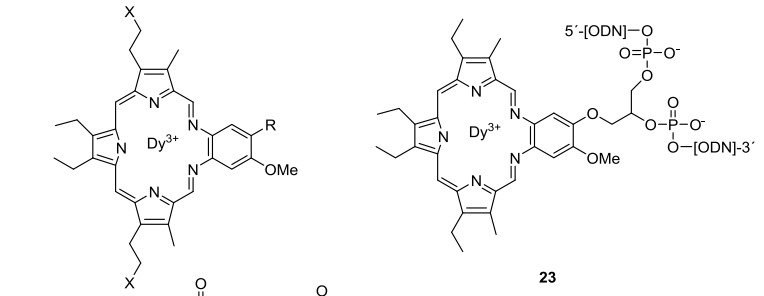
Lanthanide ion complexes attached to ODN have been shown to be effective sequence-selective artificial ribonucleases. For example, Eu^{3+} complexes of macrocyclic pyridine cyclophanes **19** and **20** have been used as ligands.[58] The target was a 29-mer ORN and after 16 h, at an excess of conjugate **19** or **20**, 51 % or 88 % of the target oligonucleotide was cleaved, respectively, at 37 ˚C and pH 7.4. The difference in the activity of these conjugates is believed to derive from the nature of the linker used to tether the lanthanide chelate to the oligonucleotide moiety. An intra-chain conjugated version of **20** was additionally prepared to ensure turnover.[32] This conjugate **21** was used with two types of targets, one with a fully complementary sequence and the other that formed a bulge upon hybridization with the conjugate. The two targets were incubated for 16 h at 37 ˚C and pH 7.4 in the excess of conjugate **21**. The fully matched target was cleaved only to a minor extent (7%), whereas the bulged target was cleaved almost quantitatively (92%). The efficient turnover was also observed with a related Eu^{3+}-complex on using 2´-*O*-methoxyethyl oligoribonucleotides instead of ODN as a sequence recognizing moiety.[68] Sequence-specific cleavage of large RNA target was tested using a conjugate similar to **19**, but again having the ODN replaced with the 2´-*O*-methoxyethyl-ORN.[29] The targets were 571 and 2977 nucleotides long *c-raf-1* RNA transcripts and the conjugates were 12 or 14 bases in length. The cleavage efficiency of 60–70 % was obtained within 4 h at 37 ˚C and pH 7.5 using 2-fold excess of the conjugate over the RNA target.



19 **20** **21**

Another macrocyclic ligand used as a cleaving agent with lanthanide ions, *viz.* with Dy^{3+}, is a texaphyrin complex conjugated to the 5´-end of ODN (**22a-e**).[35]

The cleavage reactions were carried out at 37 ˚C and pH 7.5 in excess of the conjugate. The most efficient cleavage was obtained with conjugate **22c**, which cleaved the target RNA at half-life of 2.1 h. The Dy^{3+} texaphyrin complex was additionally attached to an internal site of the ODN (**23**) and the conjugate obtained was reported to show turnover.[34] Under conditions of 10-fold excess of the target RNA, conjugate **23** cleaved 67 % of the total RNA.



22a: X=CH_2OH, R=OCH_2—$NH(CH_2)_6$—O—P(=O)(O-)—O—[ODN]-3´

22b: X=CH_2OH, R=$O(CH_2)_6$—O—P(=O)(O-)—O—[ODN]-3´

22c: X=CH_2OH, R=$O(CH_2)_3$—O—P(=O)(O-)—O—[ODN]-3´

22d: X=H, R=$O(CH_2)_6$—O—P(=O)(O-)—O—[ODN]-3´

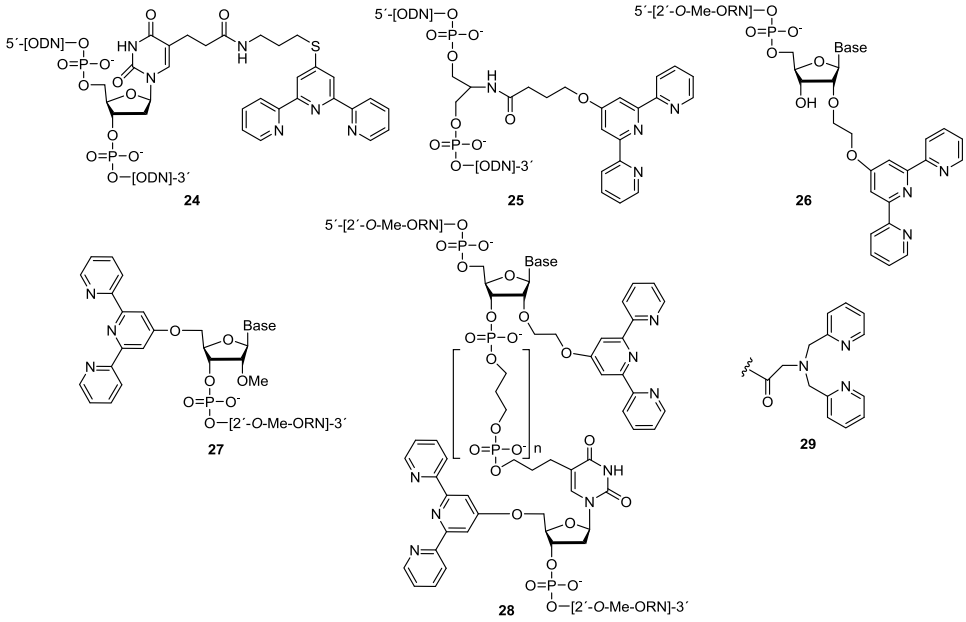
22e: X=H, R=$O(CH_2)_3$—O—P(=O)(O-)—O—[ODN]-3´

1.7.2 Transition metal ion complexes

The earliest transition metal-ion-based artificial ribonucleases date back to the same time as lanthanide ion based catalysts. The first one was Cu^{2+} chelate of terpyridine conjugated to an intra-chain position of a 17-mer ODN (**24**).[37] Its target was a 159-mer RNA sequence derived from a conserved region of the *gag*-mRNA of HIV. The amount of specific cleavage was 11 % at 37 ˚C and 18-25 % at 45 ˚C (both at pH 7.5) in excess of the conjugate over the target. The cleavage took place within the duplex region, not at a bulge. The flexibility of the target RNA strand was increased by replacing the nucleotide bearing the terpyridine complex by serinol.[38,41] This serinol-terpyridine ODN **25** placed the cleaving agent directly opposite to the unpaired nucleobase and thus increased the level of conformational freedom and made the cleavage much more susceptible resulting in a greater than 3-fold increase in overall RNA cleavage efficiency in comparison to **24**. Subsequently a series of ribozyme mimics were made, which contained the same serinol-terpyridine complex but now with propane-1,3-diol spacer(s) at either or both sides of the terpyridine unit.[66] This created a bulge

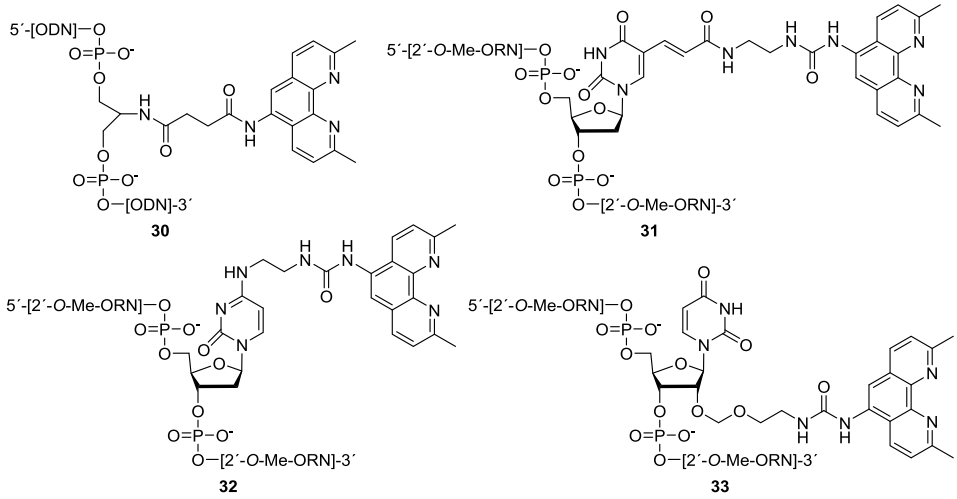
opposite to the catalyst, which eased the cleavage. The most active ribonuclease mimic among this series was the conjugate with one spacer on both sides of the terpyridine complex. It cleaved up to 67 % of the total RNA target at pH 7.4 and 45 ºC and showed turnover even in 10-fold excess of the target.

The cooperative reactivity was achieved for 3´- and 5´-tethered Cu^{2+}-terpyridine chelates **26** and **27**.[39,59] The 3´-conjugate alone was inactive and 5´-conjugate showed only modest activity (18 % of target was cleaved at pH 7.5 and 37 ºC in 20 h), but when they were used together in a tandem fashion, 92 % cleavage was reached under the same conditions. When the two conjugates were connected to each other via a flexible linker (to get the conjugate **28**), the cleavage was further enhanced twofold.[40] The most efficient cleavage was afforded by conjugate **28** with one linker unit (n=1). The reaction was fastest at pH 7.5 and showed turnover. Inspired by the cooperativity of the two cleaving agents mentioned above, a combinatorial approach was applied to searching the most efficient artificial ribonucleases amongst all combinations of four different cleaving agents.[60] Three out of the four cleaving agents contained the Cu^{2+}-terpyridine chelate and one the Cu^{2+} chelate of *N*,*N*-bis(2-pyridylmethyl)glycyl moiety (**29**). One of the combinations appeared to be clearly most efficient cleaving over 80 % of the target at pH 7.4 and 37 ºC in 20 h with turnover.

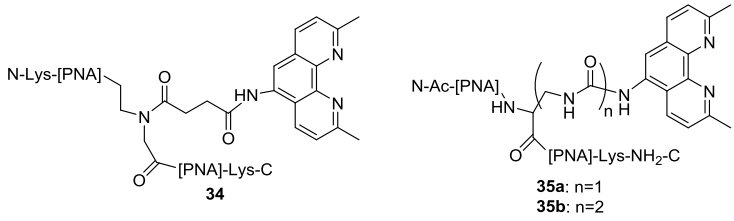


Another widely studied ligand for transition metal ions is 2,9-dimethylphenanthroline. The first such construct was tethered to the serinol unit incorporated in the middle of an ODN chain (**30**).[41] Its Cu^{2+} complex cleaved 65 % of the target RNA in 15 h, at pH 7.4 and 37 ºC. Comparisons to the corresponding terpyridine conjugate **25** showed about five-fold enhancement of cleaving activity. The reason for the lower activity of the terpyridine complex may be its tendency to dimerize and become inactive in that way.[42] Methyl substituents of 2,9-dimethylphenanthroline inhibit the formation of dimers,[69] which enhances its catalytic activity. The cleaving activity of the Zn^{2+} chelate of **30** is about 40 % of that of its Cu^{2+} counterpart.[41]

Parallel to our research on artificial ribonucleases, quite extensive studies were carried out with Zn^{2+} chelates of the 2,9-dimethylphenanthroline conjugates.[43,44,70] The influence of the linker structure and the site of tethering, as well as the structure of the target RNA, on the efficiency of the cleavage was investigated with conjugates of 11-mer 2´-*O*-methyl-ORN (**31**-**33**). The catalytic group was tethered either to the base moiety, *i.e.* C-5 of deoxyuridine or N-4 of cytidine, or to the 2´-position of the sugar moiety for evaluation of the effect of the attachment site on the cleaving activity. The Zn^{2+} chelates of the base moiety tethered conjugates **31** and **32** cleaved the target slightly faster than the Zn^{2+} chelate of the 2´-tethered conjugate **33**, the most efficient being **31** with the half-life of 11h at pH 7.4 and 37ºC.[43,44] The cleavage reactions showed turnover. The targets were designed to form bulges of 0–5 nucleotides opposite the cleaving complex, when hybridized to the conjugates. 3- And 4-nucleotide bulges were normally cleaved easier than 2- or 5-nucleotide bulges, and the cleavage of 1- and non-bulge combinations could hardly be detected at all. All phosphodiester bonds within the bulged regions were cleaved, although some preference among the cleavage sites could be in some cases observed. For example, with conjugate **31** and a target forming a 3-nucleotide bulge, almost 70 % of the cleavage took place at one site.[43] The cleaving activity fell down when the catalytic group was attached to the 5´-end of the 2´-*O*-methyl-ORN.[43,44] Further investigation on whether the target sequence could be changed revealed that the bulged part largely determined the cleavage rate, while the sequence of the duplex stem can be replaced with the desired one.[45]



The first PNA-based ribonuclease mimic reported contained Zn^{2+} chelate of 2,9-dimethylphenanthroline as the cleaving catalyst (**34**) and showed only modest activity.[61] The efficiency of such conjugates was afterwards improved by another research group who used Zn^{2+} and Cu^{2+} chelates of conjugates **35a** and **35b**.[62,67] The Zn^{2+} chelates of PNA based conjugates **35a** and **35b** were tested against targets forming 4- and 3-nucleotide bulges when hybridized. Combination of the conjugate **35a**, containing a shorter linker, and the target which formed a 4-nucleotide bulge turned out to be most active, exhibiting the half-life of 11 h (pH 7.4 and 37ºC).[62] The cleaving activity was comparable to that of the corresponding 2´-*O*-methyl-ORN based systems.[44] The same PNA conjugate **35a** was next studied with Cu^{2+} ions.[67] It was found to be considerably faster than the Zn^{2+} dependent counterpart; the best conjugate/target combination exhibited the half-life of 30 min under the same conditions. It also displayed efficient turnover and high selectivity of the cleavage site. With this result, the Cu^{2+} chelate of conjugate **35a** is the best artificial ribonuclease reported so far.

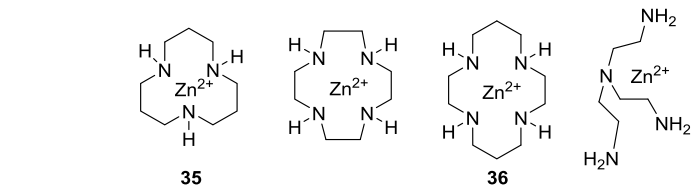


2. AIMS OF THE THESIS

RNA has many biological roles in cells: it takes part in coding, decoding, regulating and expressing of the genes as well as has the capacity to work as a catalyst in numerous biological reactions. These properties make RNA an interesting object of various studies. Development of useful tools with which to investigate RNA is a prerequisite for more advanced research in the field. One of such tools may be the artificial ribonucleases, which can be targeted to cleave any sequence of RNA. The present study is aimed at developing new efficient and sequence-selective metal-ion-based artificial ribonucleases. On one hand, to solve the challenges related to solid-supported synthesis of metal-ion-binding conjugates of oligonucleotides, on the other hand, to quantify their ability to cleave various oligoribonucleotide targets in a pre-designed sequence selective manner.

3. RESULTS AND DISCUSSION

Our group has previously discovered that certain metal ion complexes of macrocyclic polyamines, the so called azacrowns, significantly enhance the cleavage of RNA phosphodiester bonds. These azacrowns are also known to form remarkably stable complexes with 3d transition metal ions. The stability constants for the Ni^{2+}, Cu^{2+} and Zn^{2+} complexes of cyclic triamines range from 10^8 to 10^{16} M^{-1},[71] whereas those for the corresponding 2,9-dimethylphenanthroline complexes, for example, fall between 10^3-10^6 M^{-1}.[72] Tight binding of metal ions to ligands is advantageous under intracellular conditions, where the concentration of metal ions is low. The influence of metal ion complexes of azacrowns on the cleavage of phospohodiester bond(s) of dinucleoside monophosphates,[73] 2´,3´-cyclic monophosphates[73,74] and oligoribonucleotides[26,64,75] has been investigated in our laboratory. The cleavage rates for poly(U) with certain Zn^{2+} chelates is shown in **Figure 6**.[26] As seen from the cleavage rates, Zn^{2+} complex of 1,5,9-triazacyclododecane (**35**) exhibited the highest activity. It was also shown that the metal chelates promoted the cleavage without enhancing the isomerization of 3´,5´-phosphodiester bonds of poly(U) to 2´,5´-bonds. The cleaving capability of chelate **35** was studied in more detail with targets forming hairpin loops[75] and bulges.[64] Even small nucleotide bulges were found to allow reasonably fast cleavage, although increasing the bulge size and thus also the flexibility accelerated the cleavage. Within the duplex region, the chain was not cleaved. The chelate **35**, when anchored to oligonucleotide, can be considered to be a good candidate as an artificial ribonuclease. The present study focuses on preparing azacrown-functionalized oligonucleotides and evaluation of their applicability to cleave target RNA.



k / 10^{-6} s^{-1}	270	11	3.0	3.0

Figure 6. The first-order rate constants for the cleavage of phosphodiester bonds of poly(U) by Zn^{2+} chelates (2 mM) at pH 6.2, 90 ºC and I = 0.1 M.[26]

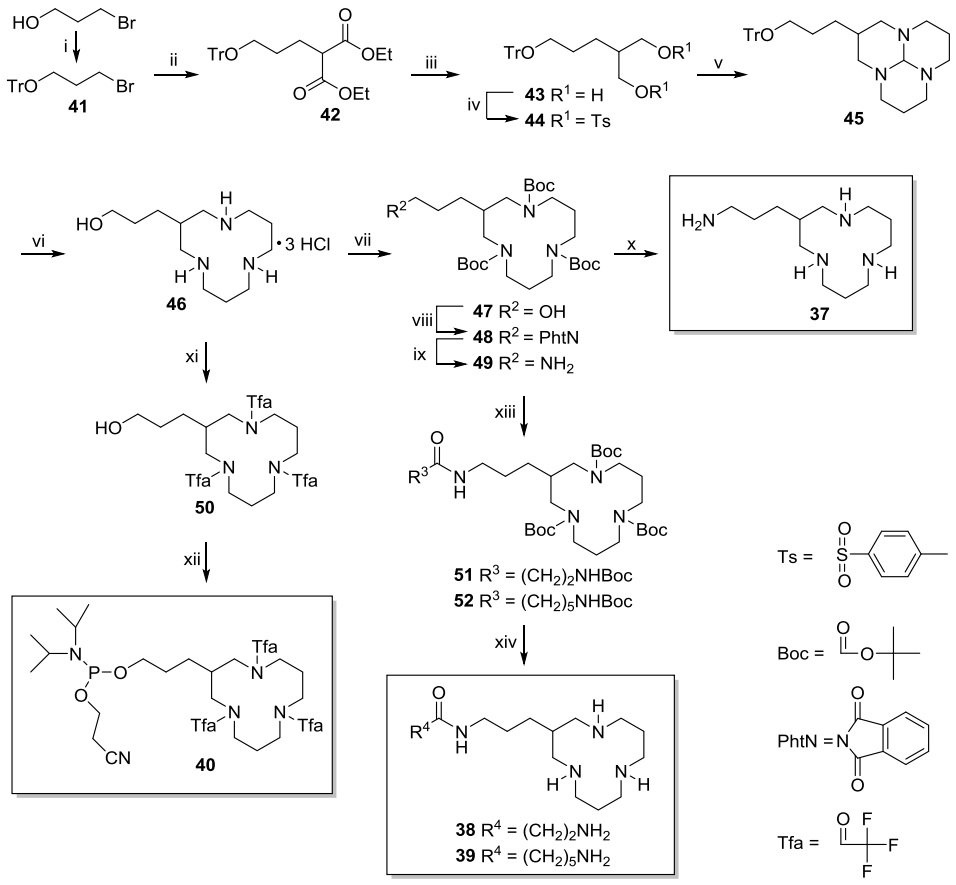
3.1 Synthesis of the artificial ribonucleases

We decided to synthesize the artificial ribonucleases by an on-support approach for the reasons considered in section 1.4. Thus building blocks derived from the azacrown were needed, as well as a method for their attachment to the solid-supported oligonucleotides.

3.1.1 Synthesis of the azacrown building blocks

Azacrowns are usually linked to other molecules via a ring-nitrogen atom. This may, however, affect the ability to form metal ion complexes. For example, methylation of all the nitrogen atoms of the 1,4,8,11-tetraazacyclotetradecane (**36** shown in **Figure 6**) destabilizes the formation of the Zn^{2+} complex by five orders of magnitude.[76] Therefore, attachment of the azacrown to the oligonucleotide through carbon atom of the ring seems more appealing and this approach was used in the present study, even though the synthesis of the azacrown bearing a side arm in carbon was somewhat challenging. The syntheses of 3-(3-aminopropyl)-1,5,9-triazacyclododecane (**37**), 3-[*N*-(3-aminopropanoyl)-3-aminopropyl]-1,5,9-triazacyclododecane (**38**), 3-[*N*-(6-aminohexanoyl)-3-aminopropyl]-1,5,9-triazacyclododecane (**39**) and 2-cyanoethyl 3-[1,5,9-tris(trifluoroacetyl)-1,5,9-triazacyclododecan-3-yl]propyl *N*,*N*-diisopropylphosphoramidite (**40**) used for conjugation of azacrown(s) to oligonucleotide are outlined in **Scheme 3**.[I,III] Azacrown **37** has been prepared previously, but the first step of that multistep synthesis was low yielding.[77] We decided, hence, to modify the synthesis of **37** as illustrated in **Scheme 3**.[I] Commercially available diethyl malonate was deprotonated to *C*2 carbanion which was used to alkylate 3-trityloxypropyl bromide (**41**). The obtained diethyl 2-(3-trityloxypropyl)malonate (**42**) was reduced to diol **43** and reacted with *p*-toluenesulfonyl chloride (TsCl) to form 1,3-ditosyloxy-2-(3-trityloxypropyl)propane (**44**). For the introduction of the azacrown structure, a previously reported method was utilized.[78,79] Accordingly, 1,5,7-triazabicyclo[4.4.0]dec-5-ene (TBD) was reacted with ditosylate **44** and subsequent $NaBH_4$-treatment afforded the orthoamide **45**. Acid-catalyzed hydrolysis of the orthoamide function and simultaneous removal of the trityl group gave the 3-(3-hydroxypropyl)-1,5,9-triazacyclododecane as a trihydrochloride salt (**46**). Nitrogen atoms of the ring were then protected with *tert*-butoxycarbonyl (Boc) groups and the hydroxy function was converted to amino function by displacement with a phthalimido (PhtN) group by Mitsunobu reaction, followed by a hydrazine treatment. Finally, the Boc-protections were removed with hydrogen chloride and the product obtained as a hydrochloride salt was converted to a free base **37** by passing it through an anion-exchange resin

(HO$^-$ form). All reactions gave good yields, usually about 80 % without optimization.



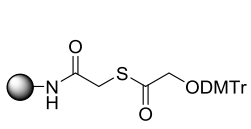
Scheme 3. Syntheses of the azacrown building blocks **37**–**40**. Reagents and conditions: (i) TrCl, Et_3N, THF; (ii) diethyl malonate, NaOEt, EtOH; (iii) $LiAlH_4$, Et_2O; (iv) TsCl, Py; (v) 1. TBD, DME, 2. $NaBH_4$, DME; (vi) 6 M aq HCl; (vii) Boc_2O, NaOH, H_2O, MeCN; (viii) PhtNH, DEAD, Ph_3P, THF; (ix) NH_2NH_2, EtOH; (x) 1. 6 M aq HCl, MeOH, 2. Dowex 2x8, HO$^-$; (xi) TfaOMe, NaOMe, MeOH; (xii) 2-cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite, Et_3N, CH_2Cl_2; (xiii) BocNH$(CH_2)_n$COOH (n=2 or 5), DCC, HOBt, dioxane; (xiv) 1. TFA, CH_2Cl_2, 2. Dowex 2x8, HO$^-$.

The linker aimed at connecting the azacrown to the oligonucleotide was elongated by acylating the primary amino group of compound **49** with *N*-Boc-protected β-alanine or 6-aminohexanoic acid (**Scheme 3**).[I] After removal of the Boc groups and conversion to a free base the azacrowns having *N*-(3-amino-propanoyl)-3-aminopropyl (**38**) and *N*-(6-aminohexanoyl)-3-aminopropyl (**39**) tethers were obtained.

The azacrown building block **40** (**Scheme 3**) was synthesized for the preparation of oligonucleotide conjugates bearing two azacrown moieties.[III] The previously prepared[I] **46** was protected by acylating the nitrogen atoms with trifluoroacetyl (Tfa) groups, after which the hydroxy function was phosphitylated with 2-cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite.

3.1.2 Synthesis of the building blocks for the oligonucleotide conjugation

Monofunctionalized oligonucleotides were prepared by using a previously developed thioester method that enables formation of an amide bond between the azacrown and oligonucleotide. The azacrown was attached to the 3´- and 5´-ends of the oligonucleotide as well as in the middle of the chain. For these purposes, the thioester function was constructed on the support **53**[80] to obtain the 3´-conjugates and introduced into the non-nucleosidic phosphoramidite reagents **54**[80] and **55**[81] for the preparation of the 5´- and intrachain conjugates, respectively. The support **53** was an aminoalkylated CPG-support acylated with 5-(4,4´-dimethoxytrityloxy)-4-oxo-3-thiapentanoyl groups. The phosphoramidite reagents **54** and **55** contained 2-benzylthio-2-oxoethyl groups for conjugation.



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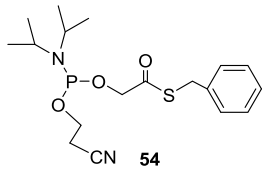

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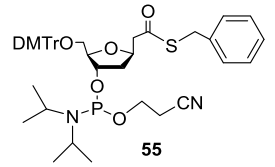
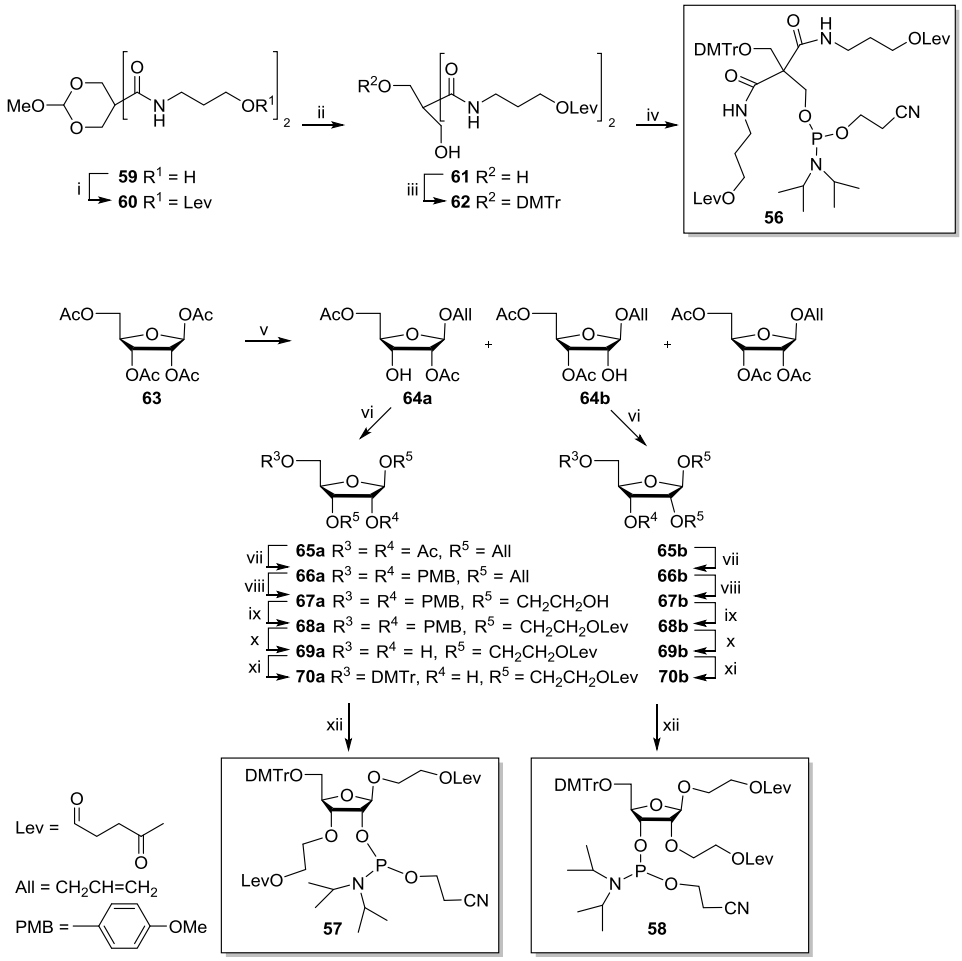


55

Difunctionalized oligonucleotides were prepared by coupling azacrown phosphoramidite **40** to a single non-nucleosidic building block bearing two hydroxy functions.[III] Levulinic acid esters (Lev) were used as protecting groups of these functions, because they can be conveniently removed on-support by fast treatment with hydrazinium acetate in pyridine.[82,83] The syntheses of the three building blocks, *i.e.* 2-cyanoethyl 3-(4,4´-dimethoxytrityloxy)-2,2-bis[*N*-(3-levulinoyloxypropyl)carbamoyl]propyl *N*,*N*-diisopropylphosphoramidite (**56**), 2-cyanoethyl [(2-levulinoyloxyethyl) 5-*O*-(4,4´-dimethoxytrityl)-3-*O*-(2-levulinoyloxyethyl)-β-D-ribofuranoside-2-yl] *N*,*N*-diisopropylphosphoramidite (**57**) and 2-cyanoethyl [(2-levulinoyloxyethyl) 5-*O*-(4,4´-dimethoxytrityl)-2-*O*-(2-levulinoyloxyethyl)-β-D-ribofuranoside-3-yl] *N*,*N*-diisopropylphosphoramidite (**58**) are outlined in **Scheme 4**.



Scheme 4. Syntheses of the building blocks **56**–**58** for the double conjugations. Reagents and conditions: (i) Lev_2O, DMAP, Py; (ii) aq 80 % AcOH; (iii) DMTrCl, Py; (iv) 2-cyanoethyl *N*,*N*,*N*´,*N*´-tetraisopropylphosphorodiamidite, tetrazole, MeCN; (v) AllOH, $BF_3{\cdot}Et_2O$, CH_2Cl_2; (vi) AllO(CO)OMe, $Pd(OAc)_2$, PPh_3, THF; (vii) 1. NaOMe, MeOH, 2. NaH, PMBCl, DMF; (viii) 1. OsO_4, $NaIO_4$, H_2O, dioxane, 2. $NaBH_4$, EtOH, CH_2Cl_2; (ix) Lev_2O, DMAP, Py; (x) H_2, Pd/C, EtOH; (xi) DMTrCl, Py; (xii) 2-cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite, Et_3N, CH_2Cl_2.

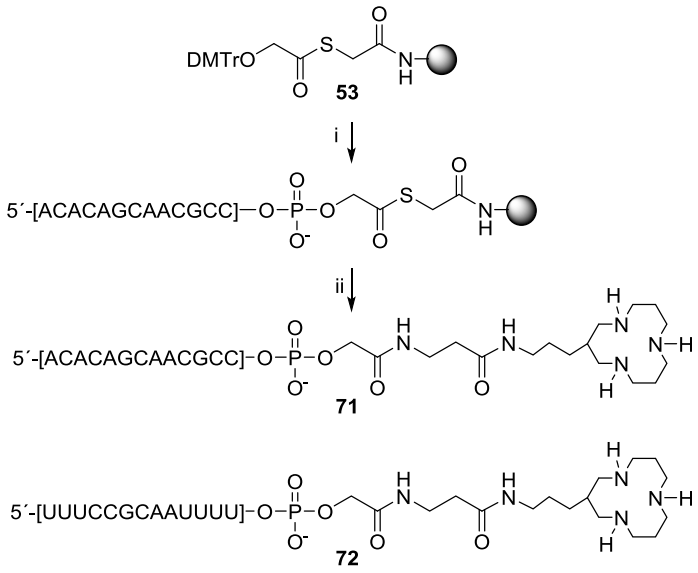
The starting material for the building block **56**, *N*,*N*´-bis(3-hydroxypropyl)-2-methoxy-1,3-dioxane-5,5-dicarboxamide (**59**), was prepared as described previously.[84] The hydroxypropyl arms of **59** were esterified with levulinic anhydride and the resulting compound **60** was treated with acetic acid to expose the other two hydroxy functions.[III] One of the hydroxy groups of **61** was protected as DMTr-ether and the other was phosphitylated to obtain the desired building block **56**.

The syntheses of the ribose-based branching units **57** and **58** started from the commercial 1,2,3,5-tetra-*O*-acetyl-β-D-ribofuranose (**63**).[III] A BF_3 promoted glycosidation with allyl alcohol gave a mixture of allyl 2,5- and 3,5-di-*O*-acetyl-β-D-ribofuranosides **64a** and **64b** together with allyl 2,3,5-tri-*O*-acetyl-β-D-ribofuranoside.[85] The free hydroxy groups of **64a** and **64b** were then allylated,[86] after which the isomers **65a** and **65b** were separated by silica gel chromatography. The base-labile acetyl protections were replaced with 4-methoxybenzyl (PMB) group and the allyl group was first converted to aldehyde with $OsO_4/NaIO_4$ and then reduced to a 2-hydroxyethyl group with $NaBH_4$, giving compounds **67a** and **67b**. The hydroxy group was levulinoylated and the PMB protections were removed by Pd/C-catalyzed hydrogenolysis. Among the exposed hydroxy functions of **69a** and **69b**, the primary hydroxy group was subjected to dimethoxytritylation, and the remaining secondary hydroxy group to phosphitylation, which gave the building blocks **57** and **58** in the form required for the oligonucleotide synthesis.

3.1.3 Assembly of the azacrown-conjugated oligonucleotides

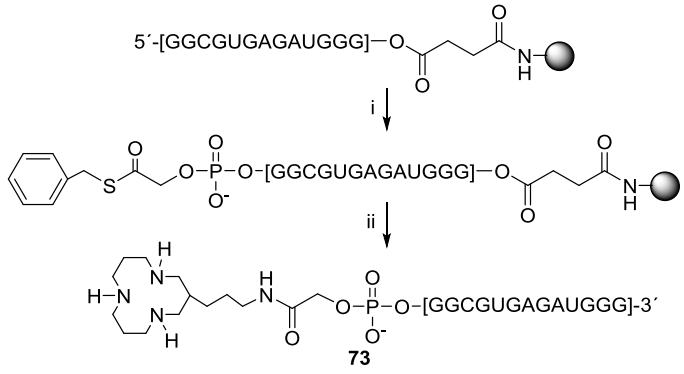
2´-*O*-Methyl ORN strand was used as a recognition domain of the artificial ribonucleases. This choice was based on the good stability towards naturally existing nucleases and the high binding affinity to the target RNA. 2´-*O*-Methyl ORNs were synthesized in 1 μmol scale by the conventional phosphoramidite method on an automated DNA/RNA synthesizer following the standard RNA coupling protocol. All azacrown conjugated 2´-*O*-methyl ORNs were purified by ion-exchange HPLC or RP HPLC and characterized by mass spectrometry.

The oligonucleotide conjugates bearing one azacrown unit at the 3´- or 5´-end or in the middle of the chain were prepared by forming an amide bond by the thioester approach. Accordingly, 3´-tethered conjugates were synthesized by assembling the desired 2´-*O*-methyl ORN chain on the modified support **53** bearing the thioester linker (**Scheme 5**).[I] After the chain assembly, the linker was cleaved by using an amino-functionalized azacrown derivative **38** as an attacking nucleophile. The deprotection of the 3´-tethered conjugate **71** was completed by ammonolysis in solution. Conjugate **72** having a different base sequence was synthesized similarly.



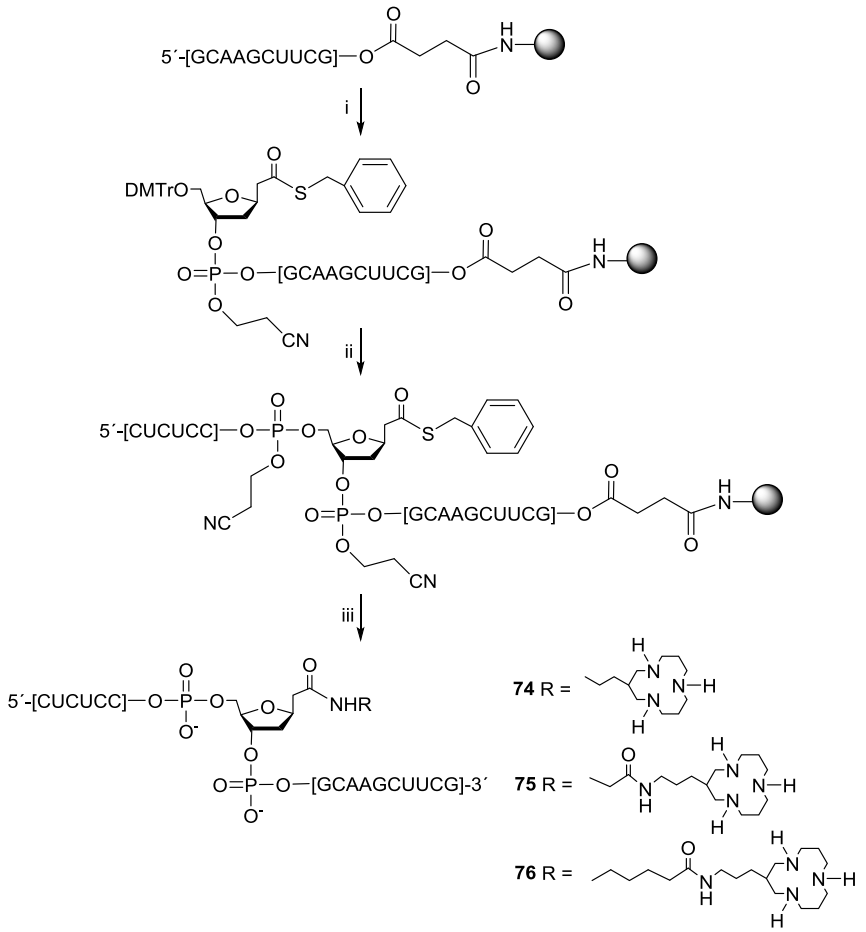
Scheme 5. Synthesis of the 3´-tethered conjugates **71** and **72**. Reagents and conditions: (i) oligonucleotide synthesis; (ii) 1. **38**, H_2O, 2. aq NH_3.

Conjugation to the 5´-end was accomplished with a non-nucleosidic phosphoramidite reagent **54**, which also contained the thioester function for the azacrown attachment.[I] The standard phosphoramidite chemistry was used for the 2´-*O*-methyl ORN chain assembly and reagent **54** was employed in the last coupling cycle (**Scheme 6**). The amino functionalized azacrown **37** was then reacted with the thioester group, and the deprotection of conjugate **73** and its release from the support was completed with ammonia.



Scheme 6. Synthesis of the 5´-tethered conjugate **73**. Reagents and conditions: (i) **54**, tetrazole, MeCN; (ii) 1. **37**, H_2O, 2. aq NH_3.

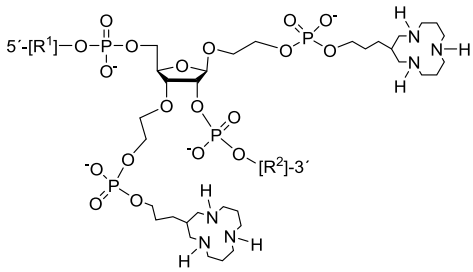
The intrachain conjugation was performed using a non-nucleosidic building block **55** in a desired position within the chain (**Scheme 7**).[I] After the oligonucleotide synthesis, the thioester bond was cleaved with three different azacrown derivatives **37**, **38** or **39** to obtain intrachain conjugates **74**, **75** or **76** having 4-, 8- or 11-atom long tether to the azacrown. The synthesis was completed with ammonolysis.



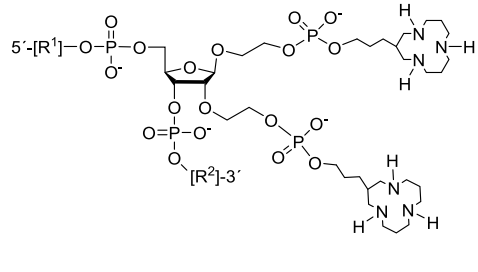
Scheme 7. Synthesis of the intrachain conjugates **74**–**76**. Reagents and conditions: (i) standard oligonucleotide synthesis cycle with **55**; (ii) oligonucleotide synthesis; (iii) 1. **37**, **38** or **39**, H_2O, 2. aq NH_3.

The oligonucleotide conjugates containing two azacrowns in the middle of the chain or close to the 3´-terminus were prepared using a phosphodiester linkage between the azacrown and the oligonucleotide strand. For this purpose, levulinoyl-protected hydroxy functions were incorporated into the 2´-*O*-methyl ORNs with the aid of branching units **56**, **57** or **58**.[III] **Scheme 8** illustrates, as an example, the synthesis of oligonucleotide conjugates **77** and **78**. After the chain

assembly, the levulinoyl protections were removed on-support with hydrazinium acetate in pyridine and the azacrown building block **40** was coupled by two consecutive coupling cycles. Finally, the conjugate was released into solution and deprotected with ammonia treatment. Conjugates **79**–**82** were prepared similarly. The conventional phosphoramidite chemistry with RNA coupling protocol was used for the oligonucleotide synthesis, except that two coupling cycles had to be used to couple **56**. For this building block, also a prolonged detritylation time was needed, consistent with earlier studies.[84,87,88] Commercially available 5´-*O*-acetylated nucleotide building block was used in a last coupling cycle to protect the terminal hydroxy function at the time the azacrown building block was coupled to the other hydroxy groups.



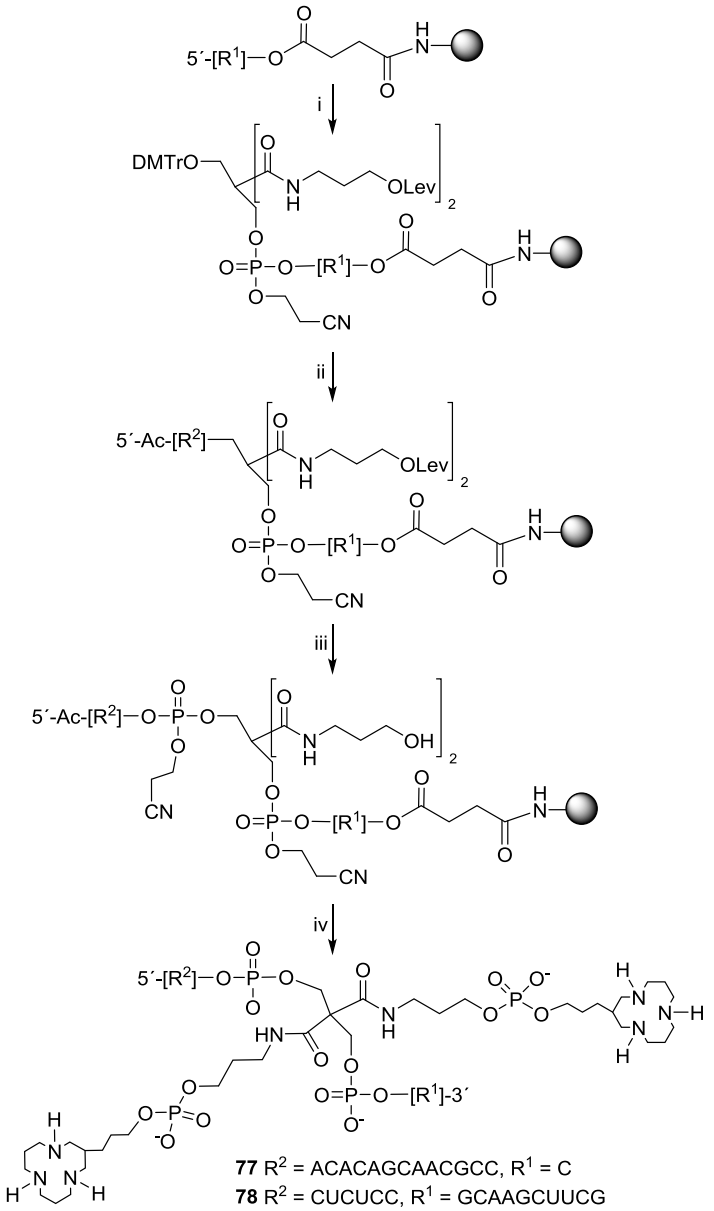
79 R^1 = ACACAGCAACGCC, R^2 = C
80 R^1 = CUCUCC, R^2 = GCAAGCUUCG



81 R^1 = ACACAGCAACGCC, R^2 = C
82 R^1 = CUCUCC, R^2 = GCAAGCUUCG

3.2 Synthesis of the targets

The chimeric ribo/2´-*O*-methyl ORNs used as targets in the kinetic studies were synthesized on an automated DNA/RNA synthesizer from commercially available 2´-*O*-methyl and 2´-*O*-triisopropylsilyloxymethyl (TOM)-protected 2-cyanoethyl-*N*,*N*-diisopropylphosphoramidite building blocks.[I-IV] The standard RNA coupling protocol for the conventional phosphoramidite strategy was used on a 1 μmol scale. The targets were purified by ion-exchange HPLC and characterized by mass spectrometry. Sterilized water and equipment were used for all solutions and handling of the targets.



Scheme 8. Synthesis of the difunctionalized conjugates **77** and **78**. Reagents and conditions: (i) oligonucleotide synthesis cycle with **56**; (ii) oligonucleotide synthesis; (iii) $H_2NNH_2 \cdot H_2O$, Py, AcOH; (iv) 1. two consecutive standard phosphoramidite couplings with **40**, 2. aq NH_3.

3.3 Kinetic studies

The cleaving activities of the conjugates bearing one azacrown at the 3´-end (**71** and **72**), 5´-end (**73**) or within (**74**–**76**) the 2´-*O*-methyl ORN chain, as well as the activities of the conjugates bearing two azacrowns at the penultimate site at 3´-end (**77**, **79** and **81**) or within (**78**, **80** and **82**) the chain, were determined.[I-III] Because preliminary studies showed only very low activity for the 5´-tethered conjugate **73**, we decided to leave that conjugate from further investigations.[I] The more accurate cleavage studies were done with other conjugates by using chimeric ribo/2´-*O*-methyl ORNs **83**–**87** (**Figure 7**) as targets.[I-III] The sequence of 2´-*O*-methylribonucleotides within these chimeric targets facilitated the synthesis and ensured efficient hybridization with the complementary artificial ribonuclease, whereas the ribonucleotide sequence offered the potentially scissile phosphodiester bonds. The base sequence of 3´-tethered conjugate was fully complementary with the 3´-terminal sequence of its target (**83**, **84**) and the base sequences of the intrachain conjugate and its targets (**85**–**87**) were designed to form either a tri- or pentanucleotide bulge upon hybridization.

83 3´-UGUGUCGUU**GCGGACAACAA**-5´

84 3´-AAAGGCGUU**AAAAUCUUCAU**-5´

85 3´-GAGAG**GAAACG**UUCGAAGC-5´

86 3´-GAGAG**GAAAAACG**UUCGAAGC-5´

87 3´-GAGAG**GUUUCG**UUCGAAGC-5´

Figure 7. Structures of the targets **83**–**87**. The bold letters refer to ribonucleotides, the rest to 2´-*O*-methylribonucleotides.

All the cleavage reactions were carried out in 0.1 M HEPES buffer at pH 7.3 and 35 ºC and the ionic strength was adjusted to 0.1 M with $NaNO_3$. The progress of the reactions was followed by analyzing the composition of the samples withdrawn at suitable intervals from the reaction mixtures by capillary electrophoresis. To ease the quantification of the samples, *p*-nitrobenzenesulfonate was used as internal standard. As is discussed in section 1.6, the conditions of the cleavage reactions and the methods used for the analysis of the samples differ significantly between various research groups. The capillary electrophoresis may be regarded as an accurate method, compared, for example, to the extensively applied gel electrophoresis.

An illustrative example of monitoring the cleavage reaction is given in **Figure 8**, which shows electropherograms obtained in the beginning, in the middle and in the end of the cleavage reaction. The followed reaction was the cleavage of the

target **85** by the Zn^{2+} complex of the oligonucleotide **82** conjugated with two azacrowns.[III] As seen from the electropherograms, all oligonucleotides and internal standard appeared at different retention times, and the target disappeared and two product oligonucleotides were formed as the reaction proceeded. The first-order rate constant was obtained for the disappearance of the target by comparison of the peak area to that of the internal standard.

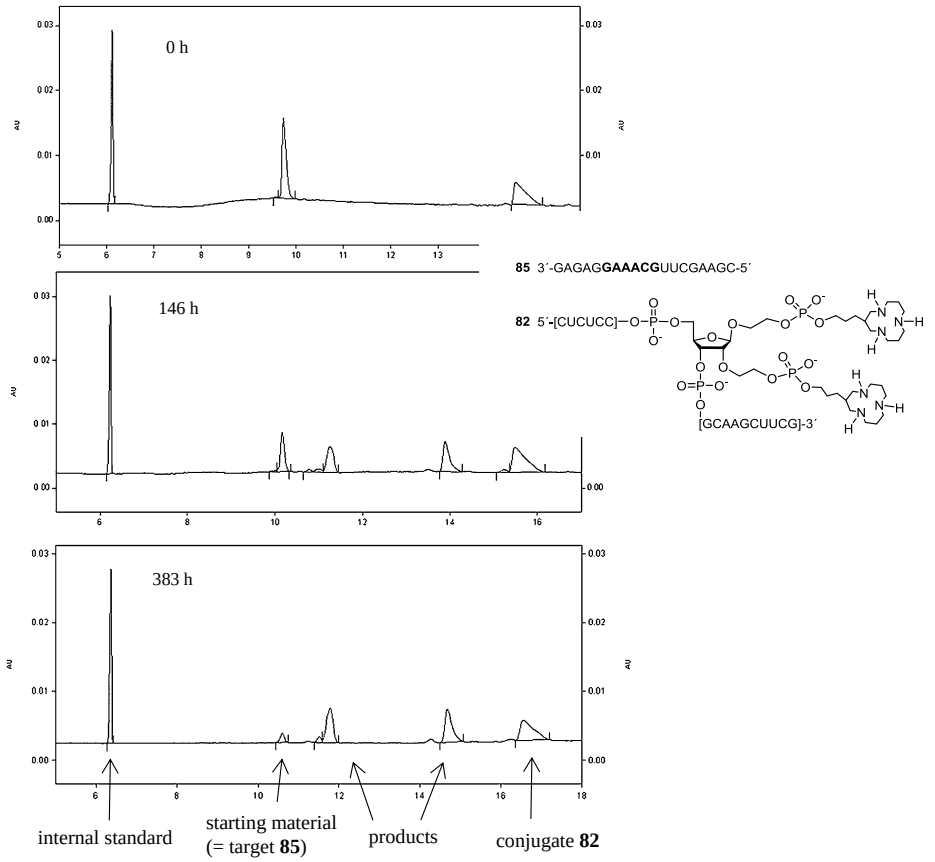


Figure 8. The electropherograms obtained at times 0, 146 and 383 h for the cleavage of the target **85** by the Zn^{2+} complex of the conjugate **82**. The reaction was carried out in HEPES buffer (0.1 M, I = 0.1 M with $NaNO_3$) at pH 7.3 and 35 ºC. The initial concentration of both the target and the conjugate was 18 μM.

3.3.1 Cleavage by the mono(azacrown) conjugates

Figure 9 shows, as an example, the cleavage of target **83** by the 3´-tethered conjugate **71** under the conditions mentioned above.[II] The initial concentration of the target and the conjugate was 36 μM and that of Zn^{2+} ion somewhat higher to

ensure complete complexing. The influence of the Zn^{2+} ion concentration on the cleavage reaction was investigated by increasing the concentration from 50 μM to 150 μM, and this did not affect the cleavage rate. The cleavage site within target **83** was determined by spiking the product mixture with potential cleavage products. The major product was found to be 3´-UGUGUCGUU**GCGG**-5´, the minor product being 3´-UGUGUCGUU**GCGGA**-5´. Accordingly, the target **83** was mainly cleaved at the 5´-side of the last base-paired nucleotide and slightly cleaved at the phosphodiester bond one nucleotide towards the 5´-end. The reaction showed turnover in spite of the fact that the cleavage site is outside the complementary region of the target and the artificial nuclease. The target was entirely cleaved obeying first-order kinetics, even when present in 4-fold excess compared to the cleaving agent.

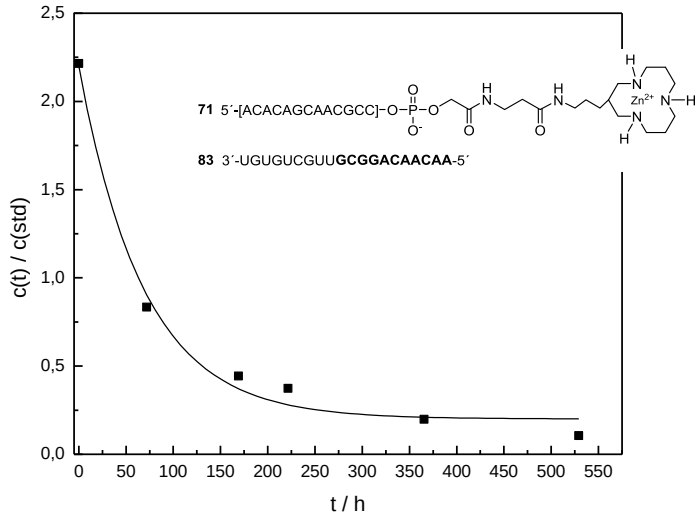


Figure 9. Cleavage of the target **83** by the Zn^{2+} complex of artificial nuclease **71** in HEPES buffer (0.1 M, I = 0.1 M with $NaNO_3$) at pH 7.3 and 35 ºC. The initial concentration of both the target and the nuclease was 36 μM.[II]

Table 1 records the first-order rate constants for the cleavage of targets **83** and **84** by cleaving agents **71** and **72**, respectively.[II] The **71**/**83** combination of the cleaving agent and the target evidently forms a more stable duplex than the **72**/**84** pair, as it contains 8 CG base-pairs compared to only 4 CG base-pairs in **72**/**84**. The less firmly hybridized conjugate **72** cleaved its target 5 times more efficiently than conjugate **71** under turnover conditions (4-fold excess of the target compared to the cleaving agent). The enhanced cleavage does not probably result from the reduced affinity to the target, but from the fact that 5´-UpA-3´

bond is cleaved instead of 5´-ApG-3´ bond. The 5´-UpA-3´ bond is known to be cleaved easier than the other phosphodiester bonds.[65] With the more stable pair **71**/**83**, the cleavage activity increased with increasing concentration of the cleaving agent, whereas the less stable pair **72**/**84** behaved quite in an opposite manner: increasing the concentration of the conjugate **72** retarded the cleavage. This may result from the tendency of conjugate **72**, containing two sequences of contiguous uracil bases, at higher concentration to undergo intermolecular association by mutual interaction between the Zn^{2+} chelate in one conjugate and a uracil base in another. The explanation is reasonable because the azacrown chelates have been shown to bind to uracil bases.[89]

Comparative measurements with Cu^{2+} and Ni^{2+} ions were also carried out.[II] Both of these metal ion chelated conjugates were less efficient than the Zn^{2+} ion chelates, as is seen from the **Table 1**.

Table 1. First-order rate constants for the cleavage of targets **83** and **84** by metal ion chelates of 3´-tethered mono(azacrown) conjugates **71** and **72**, respectively, in 0.1 M HEPES buffer at pH 7.3 and 35 ºC (I = 0.1 M with $NaNO_3$).

cleaving agent	target	M^{2+}	*c*(cleaving agent) / *μ*M	*c*(target) / *μ*M	*k* / 10^{-6} s^{-1}
71	**83**	Zn^{2+}	36	36	1.5 ± 0.2
			18	18	1.4 ± 0.1
			18	36	0.31 ± 0.02
			9	36	0.20 ± 0.02
		Cu^{2+}	18	18	0.49 ± 0.01
		Ni^{2+}	18	18	0.71 ± 0.03
72	**84**	Zn^{2+}	18	36	0.77 ± 0.06
			9	36	1.0 ± 0.1
		Cu^{2+}	9	36	0.62 ± 0.08
		Ni^{2+}	9	36	0.71 ± 0.03

Table 2 records the first-order rate constants for the cleavage of targets **85**–**87**, forming bulges of various size and base content by the intrachain conjugates **74**–**76** bearing linkers of different length.[II] The cleavage by these intrachain conjugates also showed turnover, *i.e.* the disappearance of the target was complete and obeyed first-order kinetics even in 4-fold excess of target. The length of the linker attaching the azacrown to the oligonucleotide had some effect on the cleavage rate. With target **85**, forming an A_3-bulge, the rate was enhanced

45 % on going from the longest to the shortest linker and with A_5-bulged target **86**, the rate enhancement was 30 %.

The influence of the bulge size created on the target upon hybridization was investigated by comparing the targets forming A_3- (**85**) and A_5-bulges (**86**). The more flexible A_5-bulge was cleaved 60-80 % more efficiently than the A_3-bulge with all the intrachain conjugates **74**–**76**. Interestingly, the U_3-bulge was remarkably more stable than the A_3-counterpart. The reason may be the tendency of the Zn^{2+} azacrown chelate to bind uracil base of the U_3-bulge and in that way prevent the catalytic action.[89]

Table 2. First-order rate constants for the cleavage of targets **85**–**87** by Zn^{2+} ion chelates of intrachain mono(azacrown) conjugates **74**–**76** in 0.1 M HEPES buffer at pH 7.3 and 35 ºC (I = 0.1 M with $NaNO_3$).

cleaving agent	target	*c*(cleaving agent) / *μ*M	*c*(target) / *μ*M	k / 10^{-6} s^{-1}
74	**85**	18	18	0.74 ± 0.06
	86	18	18	1.2 ± 0.08
	87	9	36	< 0.1
75	**85**	18	18	0.64 ± 0.06
		18	36	0.96 ± 0.05
		9	36	0.48 ± 0.02
	86	18	18	1.1 ± 0.06
		18	36	0.88 ± 0.04
		9	36	0.37 ± 0.02
	87	9	36	0.12 ± 0.02
76	**85**	18	18	0.51 ± 0.01
		18	36	0.60 ± 0.04
		9	36	0.46 ± 0.02
	86	18	18	0.92 ± 0.04
		18	36	0.96 ± 0.05
		9	36	0.41 ± 0.03
	87	9	36	0.11 ± 0.01

Table 3 lists the first-order rate constants for the cleavage by intrachain chelates of Cu^{2+} and Ni^{2+} ions as compared to Zn^{2+} ion.[II] The Cu^{2+} and Ni^{2+} chelates of conjugates **75** and **76** were less powerful cleaving agents than the Zn^{2+} chelates. The same was discovered for the 3´-tethered conjugates as well, but now with the intrachain conjugates, the Cu^{2+} chelate was more efficient than the Ni^{2+} chelate, while the situation was opposite with the 3´-tethered conjugates.

Table 3. First-order rate constants for the cleavage of targets **85** and **86** by metal ion chelates of intrachain mono(azacrown) conjugates **75** and **76** in 0.1 M HEPES buffer at pH 7.3 and 35 ºC (I = 0.1 M with $NaNO_3$).

cleaving agent	target	M^{2+}	*c*(cleaving agent) / *μ*M	*c*(target) / *μ*M	k / 10^{-6} s^{-1}
75	**85**	Zn^{2+}	18	36	0.96 ± 0.05
		Cu^{2+}	18	36	0.25 ± 0.01
		Ni^{2+}	18	36	0.17 ± 0.02
	86	Zn^{2+}	18	36	0.88 ± 0.04
		Cu^{2+}	18	36	0.55 ± 0.08
		Ni^{2+}	18	36	0.26 ± 0.02
76	**85**	Zn^{2+}	18	36	0.60 ± 0.04
		Cu^{2+}	18	36	0.20 ± 0.02
		Ni^{2+}	18	36	0.12 ± 0.01
	86	Zn^{2+}	18	36	0.96 ± 0.05
		Cu^{2+}	18	36	0.44 ± 0.06
		Ni^{2+}	18	36	0.14 ± 0.01

3.3.2 Cleavage by the bis(azacrown) conjugates

The cleaving activities of conjugates **77**, **79** and **81**, bearing two azacrowns close to the 3´-end of a 2´-*O*-methyl ORN, were determined under the same conditions as the activities for the monofunctionalized conjugates.[III] The cleavage of target **83** obeyed first-order kinetics with all the cleaving agents studied, as is seen from **Figure 10**. Interestingly, the catalytic activity differed significantly regardless of the structural similarity of the cleaving agents, conjugate **79** showing the best activity. The cleavage site was again defined by spiking the product mixture with

potential cleavage products and 3´-UGUGUCGUU**GCGGA**-5´ was found to be the product from reactions with all the cleaving agents **77**, **79** and **81.** In other words, the target was cleaved at the 5´-side of the nucleoside opposite to the non-nucleosidic unit holding the azacrown ligands.

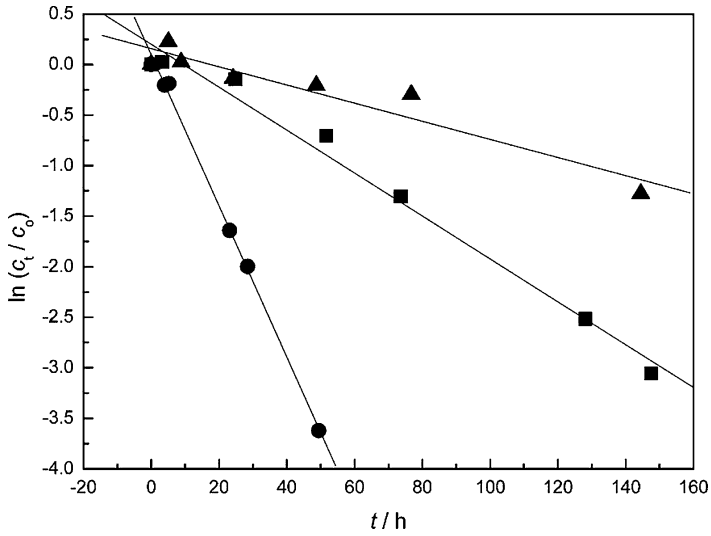


Figure 10. Cleavage of the target **83** by the Zn^{2+} complexes of the artificial ribonucleases **77**, **79** and **81** in HEPES buffer (0.1 M, I = 0.1 M with $NaNO_3$) at pH 7.3 and 35 ºC. The initial concentration of both the target and the nuclease was 18 μM. The logarithmic concentration of **83** is plotted against time. Notation: **77** (■), **79** (●) and **81** (▲).[III]

Table 4 records the first-order rate constants for the cleavage reactions by the conjugates bearing two azacrown ligands either close to the 3´-terminus (**77**, **79** and **81**) or in the middle of the chain (**78**, **80** and **82**).[III] The influence of the cleavage rate on the Zn^{2+} ion concentration was studied with conjugates **77** and **79** by increasing the concentration of the Zn^{2+} ion from 90 μM to 180 μM and keeping the other conditions unchanged. As is seen from the table, the rate constants obtained were equal within experimental errors, suggesting that the concentration of 90 μM is sufficient to achieve the maximal cleaving activity. The reaction showed turnover in spite of the fact that the cleavage took place outside the complementary region, as can be seen from the reactions where the target **83** was present in 4-fold excess compared to the cleaving agent **77** or **79**.

Table 4. First-order rate constants for the cleavage of target **83** by Zn^{2+} ion chelates of 3´-terminal bis(azacrown) conjugates **77**, **79** and **81** and for the cleavage of targets **85** and **86** by Zn^{2+} ion chelates of intrachain bis(azacrown) conjugates **78**, **80** and **82** in 0.1 M HEPES buffer at pH 7.3 and 35 ºC (I = 0.1 M with $NaNO_3$).

cleaving agent	target	*c*(cleaving agent) / *μ*M	*c*(target) / *μ*M	*c*(Zn^{2+}) / *μ*M	*k* / 10^{-6} s^{-1}
77	**83**	18	18	90	5.9 ± 0.3
79		18	18	90	20.8 ± 0.5
81		18	18	90	2.5 ± 0.3
77		18	18	180	6.3 ± 0.3
79		18	18	180	20.7 ± 0.9
77		9	36	90	0.27 ± 0.01
79		9	36	90	0.76 ± 0.08
78	**85**	18	18	90	0.9 ± 0.3
80		18	18	90	1.7 ± 0.1
82		18	18	90	1.1 ± 0.2
78	**86**	18	18	90	1.2 ± 0.1
80		18	18	90	1.7 ± 0.1
82		18	18	90	1.0 ± 0.1

The activity of the artificial ribonucleases **78**, **80** and **82** incorporating two azacrowns in the middle of the 2´-*O*-methyl ORN chain, was tested with targets **85** and **86**, forming upon hybridization an A_3- or A_5-bulge, respectively, opposite to the azacrown ligands.[III] All the ribonucleases cleaved the targets, but at a slower rate than the 3´-tethered counterparts. Again, the conjugate derived from the 1-*O*,3-*O*-difunctionalized ribofuranosyl building block (**80**) was the most efficient, but the catalytic activity was more than 1 order of magnitude lower than that of its 3´-terminal counterpart **79**. The reason for the higher activity of the 1-*O*,3-*O*-difunctionalized azacrown conjugates **79** and **80** over their 1-*O*,2-*O*-difunctionalized counterparts **81** and **82**, respectively, remains obscure. It may simply be that the orientation of the 3-*O*-tethered azacrown group is optimal for the cleavage.

3.3.3 Comparison of the mono- and bisconjugated artificial ribonucleases

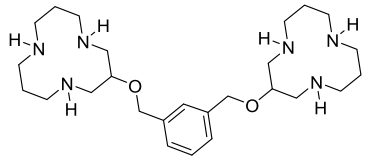
The cleavage of phosphodiester bonds within similar targets by monomeric Zn^{2+} complex of 1,5,9-triazacyclododecane (**35**) as a catalyst has been previously studied. The first-order rate constant, for example, for the cleavage of 5´-UpA-3´ bond within a linear single strand at pH 7.4 and 35 ºC is 4.2 x 10^{-6} s^{-1} on using the Zn^{2+} chelate at a 5 mM concentration.[64] The rate constant for the cleavage by the 3´-tethered mono(azacrown) cleaving agent **72**, which also cleaves the target at the 5´-UpA-3´ bond, is 1.0 x 10^{-6} s^{-1} at pH 7.3, 35 ºC and 9 μM concentration of the conjugate (**Table 1**).[II] Accordingly, the conjugation of one azacrown to the sequence-recognizing oligonucleotide probe accelerates the cleavage 130-fold. A similar comparison can be made for the cleavage of A_3-bulge; the monomeric chelate **35**[64] at the concentration of 5 mM cleaves the bulge at rate 4.5 x 10^{-6} s^{-1} and intrachain mono(azacrown) conjugate **74** at concentration of 18 μM cleaves the bulge at rate 0.74 x 10^{-6} s^{-1} (**Table 2**).[II] Accordingly, the cleavage of A_3-bulge is accelerated 46-fold when azacrown is attached to the oligonucleotide chain.

Attaching two azacrown units to the 3´-terminal site of oligonucleotide chain enhances further the cleavage capability. Mono(azacrown) conjugate **71** and bis-(azacrown) conjugates **77**, **79** and **81**, all of which have the same base sequence, cleave target **83** at the rates 1.4 x 10^{-6} s^{-1} (**Table 1**),[II] 5.9 x 10^{-6} s^{-1}, 20.8 x 10^{-6} s^{-1} and 2.5 x 10^{-6} s^{-1} (**Table 4**),[III] respectively, at the identical conditions. In other words, all the bisfunctionalized conjugates exhibit better catalytic activity than the monofunctionalized counterpart, the enhancement being most significant with conjugate **79**, 15-fold compared to **71**. If the best bis(azacrown) conjugate **79** is compared to the monomeric Zn^{2+} chelate **35** at the same concentration (18 μM), the rate acceleration is 1000-fold.[64]

The artificial ribonucleases **78**, **80** and **82** bearing two azacrowns in the middle of the chain accelerate the cleavage only slightly more efficiently than their mono-azacrown counterpart. The mono(azacrown) conjugate **75**, having approximately the same length of the tether as **78**, **80** and **82**, exhibit a first-order rate constant of 0.64 x 10^{-6} s^{-1} for the A_3-bulged target **85** (**Table 2**).[II] Under the same conditions, the rate constants referring to cleavage of the same target by the bis(azacrown) conjugates **78**, **80** and **82** are 0.9 x 10^{-6} s^{-1}, 1.7 x 10^{-6} s^{-1} and 1.1 x 10^{-6} s^{-1}, respectively (**Table 4**).[III] At its best, the acceleration is 2.5-fold. The A_5-bulged target **86** is cleaved approximately as readily by conjugates with one and two azacrowns.

3.4 Conjugates derived from a uracil selective cleaving agent

Base moiety selective cleavage of phosphodiester bonds has been achieved by small molecular cleaving agents. For example, the azacrown moieties when attached to an aromatic scaffold (**88**) cleave target RNA at the 5´-side and to a lesser extent at the 3´-side of uridines.[89-91] The selectivity arises from the tendency of the azacrown to bind to uracil base. Thus, one of the Zn^{2+}-azacrown groups anchors the cleaving agent **88** to the uracil base, while the other Zn^{2+}-azacrown group works as a catalyst and cleaves the phosphodiester bond of the target. The cleavage is two orders of magnitude faster at a uridine site than at an adenosine or a cytidine site, which may be enough for the short targets. For the longer sequences, random background cleavage, which is proportional to the number of phosphodiester bonds in oligomer, is too fast to allow controlled cleavage. This part of the thesis focuses on clarifying whether utilization of the anchoring ability to the uracil base can be used in synergy with the sequence recognition of the 2´-*O*-methyl ORN probe. Does that cooperation enhance the catalytic efficiency, resulting in cleavage precisely at a single phosphodiester bond? The principle behind the study is outlined in **Figure 11**. Accordingly, oligonucleotide conjugates bearing the dinuclear azacrown ligand **88** have been synthesized and their ability to cleave various RNA targets has been investigated.[IV]



88

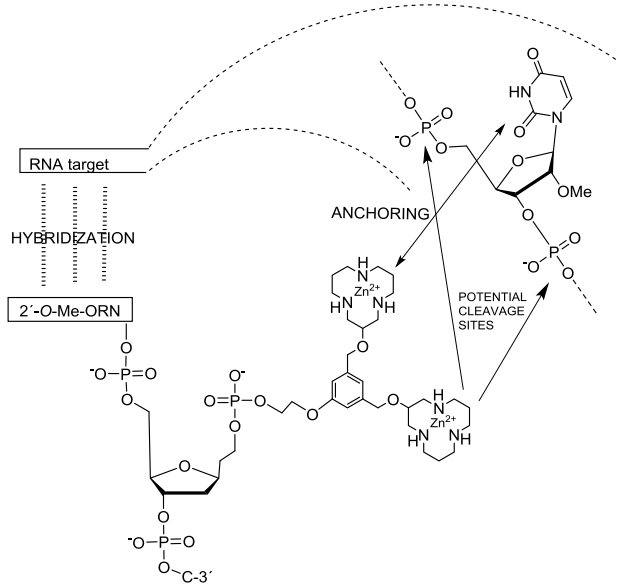
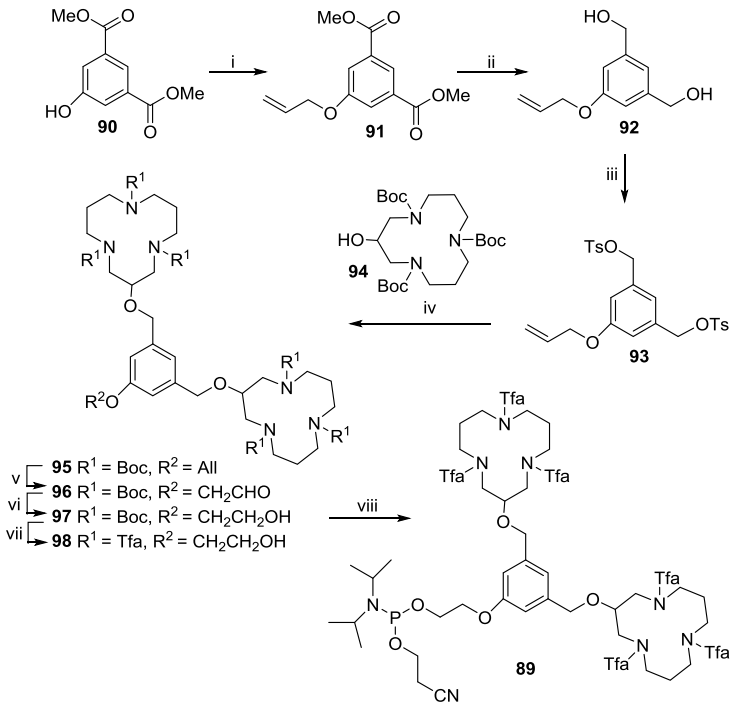


Figure 11. The principle of cooperative action of artificial ribonucleases.[IV]

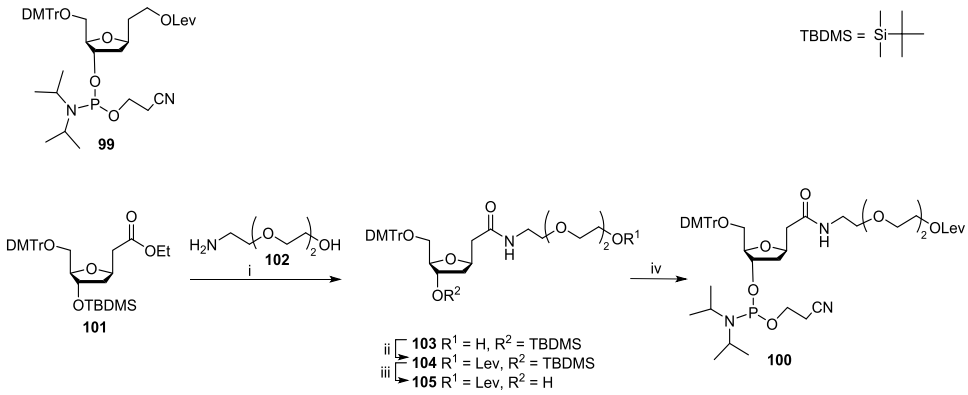
3.4.1 Synthesis of the oligonucleotide conjugates

Dinuclear azacrown ligand **88** was designed to incorporate a phosphoramidite group for the oligonucleotide conjugation. For this purpose 2-cyanoethyl 2-[3,5-bis({[1,5,9-tris(trifluoroacetyl)-1,5,9-triazacyclododecan-3-yl]oxy}methyl)phenoxy]ethyl *N*,*N*-diisopropylphosphoramidite (**89**) was prepared according to **Scheme 9**.[IV] Commercially available dimethyl 5-hydroxyisophtalate (**90**) was first treated with allyl bromide, and the ester groups of **91** were then reduced to hydroxy functions, which were converted to better leaving groups by tosylation. 1,5,9-Tris[(*tert*-butoxy)carbonyl]-1,5,9-triazacyclododecan-3-ol (**94**), prepared as described earlier,[89] was used as a nucleophile to replace the tosyl groups of **93**. The allyl function of the bis(azacrown) compound **95** was converted to aldehyde to obtain **96**, which was onward reduced to alcohol **97**. Finally, the Boc protections were replaced with Tfa groups and the hydroxy function was phosphitylated to yield the desired bis(azacrown) building block **89** to be used for the oligonucleotide conjugation.



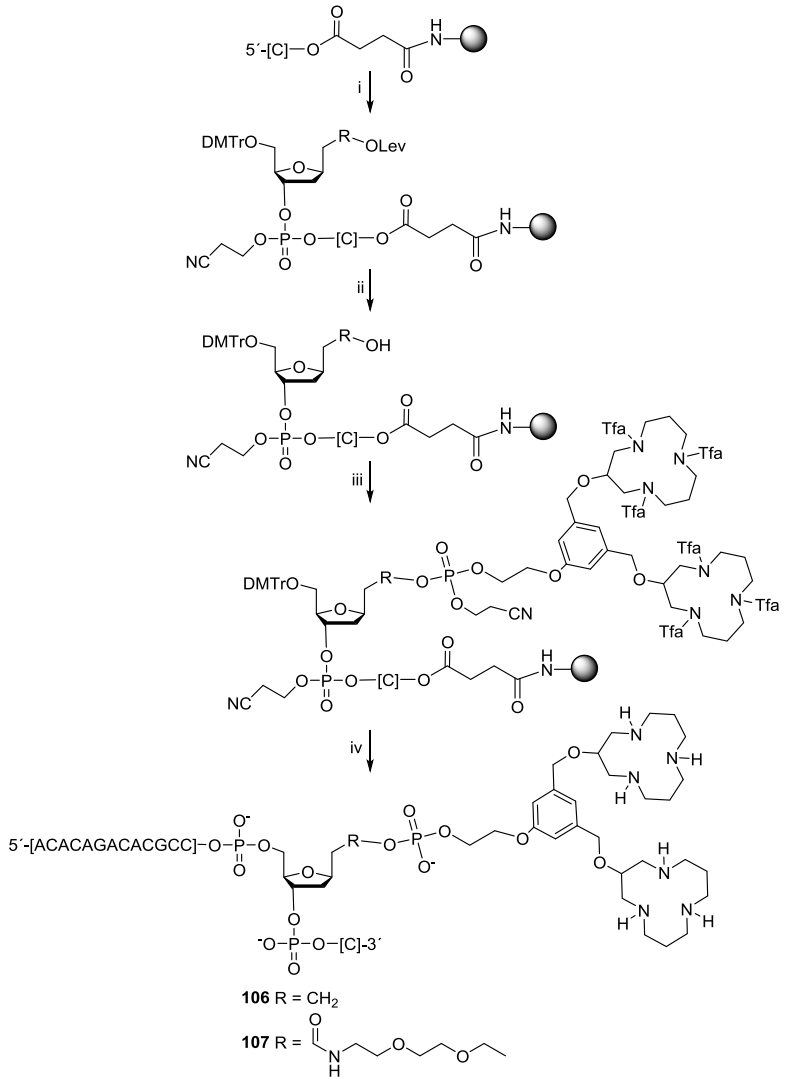
Scheme 9. Synthesis of the bis(azacrown) building block **89**. Reagents and conditions: (i) AllBr, $^{i}Pr_2EtN$, DMF; (ii) $LiAlH_4$, Et_2O; (iii) TsCl, NaOH, H_2O, dioxane; (iv) **94**, NaH, DMF; (v) OsO_4, $NaIO_4$, H_2O, dioxane; (vi) $NaBH_4$, EtOH; (vii) 1. TFA, CH_2Cl_2, 2. TfaOMe, Et_3N, MeOH, 3. $(Tfa)_2O$, Py, CH_2Cl_2, 4. Et_3N, MeOH; (viii) 2-cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite, Et_3N, CH_2Cl_2.

The non-nucleosidic building blocks **99** and **100** (**Scheme 10**), which allow the attachment of bis(azacrown) **89** to the oligonucleotide chain, were prepared as follows. 2-Cyanoethyl {(2*R*,3*S*,5*S*)-2-[(4,4´-dimethoxytrityl)oxymethyl]-5-(2-levulinoyloxyethyl)tetrahydrofuran-3-yl} *N*,*N*-diisopropylphosphoramidite (**99**) was synthesized as described earlier.[92] Its analog **100**, with a longer side chain, was synthesized from previously prepared compound **101**.[92] The aminolysis of **101** with 2-[2-(2-aminoethoxy)ethoxy]ethanol (**102**) yielded amide **103**, which was subjected to acylation of the hydroxy group with levulinic anhydride and then to removal of the silyl protection with Bu_4NF.[IV] Compound **105** was finally converted to the phosphoramidite building block **100** by conventional method.



Scheme 10. Non-nucleosidic building blocks **99** and **100**. Reagents and conditions: (i) 1. aq KOH, dioxane, 2. Dowex-50 (pyridinium form), Py, 3. **102**, HBTU, $^{i}Pr_2EtN$, DMF; (ii) Lev_2O, Py; (iii) Bu_4NF, THF; (iv) 2-cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite, Et_3N, CH_2Cl_2.

Incorporation of the above-mentioned building blocks to the 2´-*O*-methyl ORN strand is outlined in **Scheme 11**.[IV] The commercially available support with N^4-benzoyl-5´-*O*-(4,4´-dimethoxytrityl)-2´-*O*-methylcytidine was used. The 5´-*O*-protection was first removed and the non-nucleosidic building block, either **99** or **100**, was coupled manually using a prolonged coupling time. Then the levulinoyl protection was removed with hydrazinium acetate in pyridine and the bis-(azacrown) building block **89** was coupled manually using again a prolonged coupling time. The support bearing the bis(azacrown) block was loaded to the synthesizer, and the 2´-*O*-methyl ORN sequence was assembled by the standard phosphoramidite chemistry. The fully deprotected oligonucleotide conjugates **106** and **107** were obtained by ammonolysis.



Scheme 11. The synthesis of the bis(azacrown) oligonucleotide conjugates **106** and **107**. Reagents and conditions: (i) manual oligonucleotide synthesis cycle with **99** or **100**; (ii) $H_2NNH_2 \cdot H_2O$, Py, AcOH; (iii) manual oligonucleotide synthesis cycle with **89**; (iv) 1. oligonucleotide synthesis, 2. aq NH_3.

3.4.2 Hybridization with target sequences

Targets for the bis(azacrown) oligonucleotide conjugates **106** and **107** are shown in **Figure 12**. All the targets are 20-mer chimeric ribo/2´-*O*-methyl ORNs **108**–**113** having fully complementary 3´-terminal sequence and containing different 5´-terminal ribonucleotide overhangs. One uridine is located in various places

within the overhang. For comparative purposes, an unconjugated 2´-*O*-methyl ORN **114** was synthesized and hybridized with the same targets.

Targets:

108 3´-UGUGUCUGU**GCGGACAACAA**-5´

109 3´-UGUGUCUGU**GCGGUAAACAA**-5´

110 3´-UGUGUCUGU**GCGGAUAACAA**-5´

111 3´-UGUGUCUGU**GCGGAAUACAA**-5´

112 3´-UGUGUCUGU**GCGGAAAUCAA**-5´

113 3´-UGUGUCUGU**GCGGAAAAUAA**-5´

Reference oligonucleotide:

114 5´-ACACAGACACGCC-3´

Figure 12. Structures of the targets **108**–**113** and the reference oligonucleotide **114**. The bold letters refer to ribonucleotides, the rest to 2´-*O*-methylribonucleotides.

Table 5 records the melting temperatures for the duplexes of bis(azacrown) conjugate **107** and reference oligonucleotide **114** with several targets **108**–**113**.[IV] As can be seen from the data, the conjugate **107** hybridizes with all the targets even somewhat better than the reference oligonucleotide **114** containing no azacrown groups. The duplexes formed are clearly stable at the temperature of the cleavage reactions, 35 ºC. The presence of Zn^{2+} ion further stabilizes the duplexes, the stabilization being most evident with the target **109**, which has the uracil base directly after the complementary region of the conjugate **107** and the target. In this case, the temperature is increased by 5.3 ºC, while the increase is only 1.7 ºC with the reference oligonucleotide **114** and the same target. When conjugate **107** was hybridized with target **108**, containing no uracil within the overhang, the addition of Zn^{2+} ion increased the temperature only by 2.2 ºC. Evidently, the azacrown group of the conjugate **107** truly recognizes the uracil base of the target.

Table 5. Melting temperatures of the duplexes of oligonucleotide conjugate **107** and reference oligonucleotide **114** with targets **108**–**113** in the absence and presence of Zn^{2+} ion (10 μM) in 10 mM phosphate buffer at pH 7.0 (I = 0.1 M with NaCl). The concentrations of the oligomers were 2 μM.

Target	T_m (107) / °C		T_m (114) / °C	
	without Zn^{2+}	with Zn^{2+}	without Zn^{2+}	with Zn^{2+}
108	76.1	78.3	73.8	74.9
109	76.7	82.0	73.3	75.0
110	76.7	79.7	73.5	76.1
111	77.5	79.8	75.0	74.6
112	78.2	78.5	74.5	74.4
113	75.9	77.9	75.5	74.4

3.4.3 Kinetic studies

It has been reported earlier that dinuclear Zn^{2+} complex of bis(azacrown) monomer **88** cleaves ORN targets 5´-CAAUAC-3´ and 5´-CAACAC-3´ at rates 7.70 x 10^{-6} s^{-1} and 0.24 x 10^{-6} s^{-1}, respectively.[91] In other words, the target with uracil base within the sequence is cleaved 32 times faster than the target without uracil residue. The obvious rate acceleration most likely results from the anchoring of one azacrown group of the monomer **88** to the uracil base, bringing the other azacrown group near the cleavable phosphodiester bond.[89,91] The melting temperature studies also indicate that the bis(azacrown) oligonucleotide conjugate **107** really recognizes the uracil base of the target **109**.[IV] For these reasons, one might expect this anchoring ability in synergy with the sequence recognition by the oligonucleotide probe to result in facilitated and selective cleavage of the targets containing uracil base near the cleavage site. However, this is not the case. **Table 6** records the rate constants for the cleavage of the targets **108**–**113** by the bis(azacrown) conjugates **106** and **107** and also, for comparison, by the bis(azacrown) monomer **88** in the presence of the reference oligonucleotide **114**.[IV] As can be seen, the target **108** containing no uracil was cleaved even better than the targets containing uracil. That is, tethering of the bis(azacrown) **88** to the sequence recognizing oligonucleotide does not speed up the cleavage reaction. On the contrary, the uracil specific cleavage is retarded. The reason for this observation may be that when the oligonucleotide moiety of

the bis(azacrown) conjugate **106** or **107** is hybridized with the target and at the same time the azacrown is anchored to the uracil base of the target, the rigidity of the structure prevents the remaining azacrown to reach the phosphodiester bond any more. Consistent with this assumption, the cleavage by the monomeric bis(azacrown) **88** together with the reference oligonucleotide **114** resulted at some point even higher cleavage rates than conjugates **106** and **107**. The earlier finding with the mono(azacrown) conjugates **74**–**76**, according to which uracil bulges were not cleaved in striking contrast to adenine bulges, lends additional support for this interpretation.[II]

Table 6. First-order rate constants for the cleavage of targets **108**–**113** by Zn^{2+} ion chelates of bis(azacrown) conjugates **106** and **107** and monomeric bis(azacrown) **88** in presence of reference oligonucleotide **114** in 0.1 M HEPES buffer at pH 7.3 and 35 ºC (I = 0.1 M with $NaNO_3$). The concentration of the oligomers and **88** were 18 μM and that of Zn^{2+} ion 90 μM.

target	k (106) / 10^{-6} s^{-1}	k (107) / 10^{-6} s^{-1}	k (88 + 114) / 10^{-6} s^{-1}
108	2.44 ± 0.07	1.66 ± 0.08	0.8 ± 0.1
109	1.09 ± 0.04	0.19 ± 0.03	0.9 ± 0.1
110	2.7 ± 0.1	0.44 ± 0.03	4 ± 1
111	0.39 ± 0.04	0.25 ± 0.02	0.5 ± 0.1
112		0.82 ± 0.05	0.8 ± 0.1
113		0.79 ± 0.08	1.5 ± 0.2

4. CONCLUSIONS

In this study several artificial ribonucleases based on cleaving capability of metal ion chelated azacrown moiety were designed and synthesized. The synthesis strategy is such that can be easily adapted to tethering of various groups other than azacrowns, to the desired position in oligonucleotide strands. The most efficient ribonucleases were the ones with two azacrowns close to the 3´-end of the 2´-*O*-methyl ORN, the best being 1-*O*,3-*O*-difunctionalized conjugate **79**.[III] Among the transition metal ions studied, the Zn^{2+} ion was found to be better than Cu^{2+} and Ni^{2+} ions.[II]

It is challenging to improve further the cleaving activity by simply bringing the cleaving agent near the cleavable site. Apparently, on achieving some kind of optimum structure for both the cleaving agent and the target, it may be possible to get even more potent artificial ribonucleases. 2,9-Dimethylphenanthroline, being more efficient cleaving agent than azacrown, may form together with the target more ideal structure or orientation for the cleavage.[67] It has, however, one major disadvantage over the azacrown; its stability constants for the Zn^{2+} and Cu^{2+} complexes are much weaker.[72] Thus, even though the 2,9-dimethyl-phenanthroline cleaves better in excess of metal ion, the situation is probably opposite under intracellular conditions where the metal ion concentration is lower. At this moment, the best cleaving agents may be fast enough for the biotechnological studies *in vitro*; for example for cutting of large RNA molecules or for analyzing the three-dimensional structures of RNAs. But before they can be more widely used *in vivo*, and thus also in therapeutic applications, the cleaving efficiency, and possibly also the complex stability, must be considerably improved.

5. EXPERIMENTAL

5.1 Synthesis and analysis of the compounds

The detailed synthetic methods of the compounds discussed in this thesis can be found in the original publications.[I-IV] All the oligonucleotides were synthesized on an Applied Biosystems 392 or 3400 DNA/RNA synthesizer by conventional phosphoramidite strategy. The monomeric compounds were analyzed by MS, ^{1}H NMR, ^{13}C NMR and ^{31}P NMR when needed and the oligonucleotide conjugates were analyzed by ESI-MS.

5.2 Kinetic measurements

The reactions were carried out in Eppendorf tubes immersed in a water bath of 35 ºC. The pH was adjusted with 0.1 M HEPES buffer to 7.3 and the ionic strength with $NaNO_3$ to 0.1 M. The concentrations of the azacrown conjugated oligonucleotides, targets and metal ions (added as nitrates) were as mentioned in the tables. 4-Nitrobenzenesulfonate ion was used as an internal standard. The total volume of the reaction mixture was 200 μl in each kinetic run. Aliquots of 20 μl were withdrawn at suitable intervals and the reaction was quenched by adding aq HCl and cooling to 0 ºC.

The samples were analyzed immediately by capillary zone electrophoresis (Beckman Coulter P/ACE MDQ CE System) using a fused silica capillary (inner diameter 50 μM, effective length 50 cm) with inverted polarity, citrate buffer (0.2 M, pH 3.1) and -30 kV voltage. The quantification was based on comparison of the UV absorptions at 254 nm of the target oligonucleotide and internal standard. The peak area was first normalized by dividing it by migration time. The first-order rate constants were calculated for the disappearance of the target according to the integrated first-order rate law.

5.3 Melting temperature studies

The melting curves (absorbance versus temperature) were measured at 260 nm on a Perkin-Elmer Lambda 35 UV/VIS Spectrometer using 10 mM potassium phosphate buffer (pH 7) containing 0.1 M NaCl. The concentrations of the oligonucleotides and their targets were 2 μM. The T_m values were determined as the maximum of the first derivative of the melting curve.

6. REFERENCES

1. Takagi, Y., Ikeda, Y. and Taira, K. *Top. Curr. Chem.* **2004**, *232*, 213-251.
2. Desbouis, D., Troitsky, I. P., Belousoff, M. J., Spiccia, L. and Graham, B. *Coord. Chem. Rev.* **2012**, *256*, 897-937.
3. Niittymäki, T. and Lönnberg, H. *Org. Biomol. Chem.* **2006**, *4*, 15-25.
4. Morrow, J. R. and Iranzo, O. *Curr. Opin. Chem. Biol.* **2004**, *8*, 192-200.
5. Trawick, B., Daniher, A. T. and Bashkin, J. K. *Chem. Rev.* **1998**, *98*, 939-960.
6. Roberts, R. J. *Proc. Natl. Acad. Sci. U. S. A.* **2005**, *102*, 5905-5908.
7. Kolasa, K. A., Morrow, J. R. and Sharma, A. P. *Inorg. Chem.* **1993**, *32*, 3983-3984.
8. Forconi, M. and Herschlag, D. *Methods Enzymol.* **2009**, *468*, 91-106.
9. Zamecnik, P. C. and Stephenson, M. L. *Proc. Natl. Acad. Sci. U. S. A.* **1978**, *75*, 280-284.
10. Stephenson, M. L. and Zamecnik, P. C. *Proc. Natl. Acad. Sci. U. S. A.* **1978**, *75*, 285-288.
11. Azad, R. F., Driver, V. B., Tanaka, K., Crooke, R. M. and Anderson, K. P. *Antimicrob. Agents Chemother.* **1993**, *37*, 1945-1954.
12. Hair, P., Cameron, F. and McKeage, K. *Drugs* **2013**, *73*, 487-493.
13. Singh, J., Kaur, H., Kaushik, A. and Peer, S. *Int. J. Pharmacol.* **2011**, *7*, 294-315.
14. Bennett, C. F. and Swayze, E. E. *Annu. Rev. Pharmacol. Toxicol.* **2010**, *50*, 259-293.
15. Williams, N., Takasaki, B., Wall, M. and Chin, J. *Acc. Chem. Res.* **1999**, *32*, 485-493.
16. Brown, D. and Todd, A. *Annu. Rev. Biochem.* **1955**, *24*, 311-338.
17. Oivanen, M., Kuusela, S. and Lönnberg, H. *Chem. Rev.* **1998**, *98*, 961-990.
18. Westheimer, F. *Acc. Chem. Res.* **1968**, *1*, 70-78.

19. Emilsson, G. M., Nakamura, S., Roth, A. and Breaker, R. *RNA* **2003**, *9*, 907-918.

20. Lönnberg, H. *Org. Biomol. Chem.* **2011**, *9*, 1687-1703.

21. Schnabl, J. and Sigel, R. K. O. *Curr. Opin. Chem. Biol.* **2010**, *14*, 269-275.

22. Raines, R. *Chem. Rev.* **1998**, *98*, 1045-1065.

23. Dimroth, K., Jaenicke, L. and Heinzel, D. *Justus Liebigs Ann. Chem.* **1950**, *566*, 206-210.

24. Mikkola, S., Kaukinen, U. and Lönnberg, H. *Cell Biochem. Biophys.* **2001**, *34*, 95-119.

25. Matsumura, K. and Komiyama, M. *J. Biochem.* **1997**, *122*, 387-394.

26. Kuusela, S. and Lönnberg, H. *J. Chem. Soc., Perkin Trans. 2* **1994**, 2301-2306.

27. Eichhorn, G. I. and Butzow, J. J. *Biopolymers* **1965**, *3*, 79-94.

28. Morrow, J. R., Buttrey, L. A., Shelton, V. M. and Berback, K. A. *J. Am. Chem. Soc.* **1992**, *114*, 1903-1905.

29. Canaple, L., Hüsken, D., Hall, J. and Häner, R. *Bioconjugate Chem.* **2002**, *13*, 945-951.

30. Häner, R., Hall, J. and Rihs, G. *Helv. Chim. Acta* **1997**, *80*, 487-494.

31. Hüsken, D., Goodall, G., Blommers, M., Jahnke, W., Hall, J., Häner, R. and Moser, H. E. *Biochemistry* **1996**, *35*, 16591-16600.

32. Hall, J., Hüsken, D. and Häner, R. *Nucleic Acids Res.* **1996**, *24*, 3522-3526.

33. Magda, D., Miller, R. A., Sessler, J. L. and Iverson, B. L. *J. Am. Chem. Soc.* **1994**, *116*, 7439-7440.

34. Magda, D., Wright, M., Crofts, S., Lin, A. and Sessler, J. L. *J. Am. Chem. Soc.* **1997**, *119*, 6947-6948.

35. Magda, D., Crofts, S., Lin, A., Miles, D., Wright, M. and Sessler, J. L. *J. Am. Chem. Soc.* **1997**, *119*, 2293-2294.

36. Bae, S. N., Lee, Y. S., Kim, M. Y., Kim, J. D. and Park, L. O. *Gynecol. Oncol.* **2006**, *103*, 127-136.

37. Bashkin, J. K., Frolova, E. I. and Sampath, U. J. Am. Chem. Soc. 1994, 116, 5981-5982.

38. Daniher, A. T. and Bashkin, J. K. *Chem. Commun.* **1998**, 1077-1078.

39. Inoue, H., Furukawa, T., Shimizu, R., Tamura, T., Matsui, M. and Ohtsuka, E. *Chem. Commun.* **1999**, 45-46.

40. Sakamoto, S., Tamura, T., Furukawa, T., Komatsu, Y., Ohtsuka, E., Kitamura, M. and Inoue, H. *Nucleic Acids Res.* **2003**, *31*, 1416-1425.

41. Putnam, W. and Bashkin, J. K. *Chem. Commun.* **2000**, 767-768.

42. Putnam, W., Daniher, A. T., Trawick, B. and Bashkin, J. K. *Nucleic Acids Res.* **2001**, *29*, 2199-2204.

43. Åström, H., Williams, N. H. and Strömberg, R. *Org. Biomol. Chem.* **2003**, *1*, 1461-1465.

44. Åström, H. and Strömberg, R. *Org. Biomol. Chem.* **2004**, *2*, 1901-1907.

45. Murtola, M. and Strömberg, R. *Arkivoc* **2009**, 84-94.

46. Koroleva, L. S., Serpokrylova, I. Y., Vlassov, V. V. and Silnikov, V. N. *Protein Pept. Lett.* **2007**, *14*, 151-163.

47. Scheffer, U., Strick, A., Ludwig, V., Peter, S., Kalden, E. and Göbel, M. W. *J. Am. Chem. Soc.* **2005**, *127*, 2211-2217.

48. Gnaccarini, C., Peter, S., Scheffer, U., Vonhoff, S., Klussmann, S. and Göbel, M. W. *J. Am. Chem. Soc.* **2006**, *128*, 8063-8067.

49. Vlassov, V., Abramova, T., Godovikova, T., Giege, R. and Silnikov, V. *Antisense Nucleic Acid Drug Dev.* **1997**, *7*, 39-42.

50. Beloglazova, N. G., Silnikov, V. N., Zenkova, M. A. and Vlassov, V. V. *FEBS Lett.* **2000**, *481*, 277-280.

51. Garipova, I. Y. and Silnikov, V. N. *Russ. Chem. Bull., Int. Ed.* **2002**, *51*, 1112-1117.

52. Beloglazova, N. G., Fabani, M. M., Zenkova, M. A., Bichenkova, E. V., Polushin, N. N., Silnikov, V. V., Douglas, K. T. and Vlassov, V. V. *Nucleic Acids Res.* **2004**, *32*, 3887-3897.

53. Beloglazova, N. G., Fabani, M. M., Polushin, N. N., Silnikov, V. V., Vlassov, V. V., Bichenkova, E. V. and Zenkova, M. A. *J. Nucleic Acids* **2011**, 1-17.

54. Komiyama, M., Inokawa, T. and Yoshinari, K. *J. Chem. Soc., Chem. Commun.* **1995**, 77-78.

55. Lundin, K. E., Good, L., Strömberg, R., Gräslund, A. and Smith, C. I. E. *Adv. Genet.* **2006**, *56*, 1-51.

56. Beaucage, S. L. and Caruthers, M. H. In *Current protocols in nucleic acid chemistry;* John Wiley & Sons, Inc: USA, **2000***; Vol. 1*, pp 3.3.1-3.3.20.

57. Strömberg, R. and Stawinski, J. In *Current protocols in nucleic acid chemistry;* John Wiley & Sons, Inc: USA, **2004***; Vol. 1*, pp 3.4.1-3.4.15.

58. Hall, J., Hüsken, D., Pieles, U., Moser, H. E. and Häner, R. *Chem. Biol.* **1994**, *1*, 185-190.

59. Inoue, H., Furukawa, T., Tamura, T., Kamada, A. and Ohtsuka, E. *Nucleosides Nucleotides Nucleic Acids* **2001**, *20*, 833-835.

60. Kalek, M., Benediktson, P., Vester, B. and Wengel, J. *Chem. Commun.* **2008**, 762-764.

61. Whitney, A., Gavory, G. and Balasubramanian, S. *Chem. Commun.* **2003**, 36-37.

62. Murtola, M. and Strömberg, R. *Org. Biomol. Chem.* **2008**, *6*, 3837-3842.

63. Sandbrink, J., Murtola, M. and Strömberg, R. *Nucleosides Nucleotides Nucleic Acids* **2007**, *26*, 1485-1489.

64. Kaukinen, U., Bielecki, L., Mikkola, S., Adamiak, R. and Lönnberg, H. *J. Chem. Soc., Perkin Trans. 2* **2001**, 1024-1031.

65. Kaukinen, U., Lyytikäinen, S., Mikkola, S. and Lönnberg, H. *Nucleic Acids Res.* **2002**, *30*, 468-474.

66. Trawick, B., Osiek, T. and Bashkin, J. K. *Bioconjugate Chem.* **2001**, *12*, 900-905.

67. Murtola, M., Wenska, M. and Strömberg, R. *J. Am. Chem. Soc.* **2010**, *132*, 8984-8990.

68. Häner, R., Hall, J., Pfützer, A. and Hüsken, D. *Pure & Appl. Chem.* **1998**, *70*, 111-116.

69. Linkletter, B. and Chin, J. *Angew. Chem. Int. Ed. Engl.* **1995**, *34*, 472-474.

70. Åström, H. and Strömberg, R. *Nucleosides, Nucleotides Nucleic Acids* **2001**, *20*, 1385-1388.

71. Zompa, L. J. *Inorg. Chem.* **1978**, *17*, 2531-2536.

72. Irving, H. and Mellor, D. *J. Chem. Soc.* **1962**, 5237-5245.

73. Kuusela, S. and Lönnberg, H. *J. Phys. Org. Chem.* **1993**, *6*, 347-356.

74. Kuusela, S. and Lönnberg, H. *J. Phys. Org. Chem.* **1992**, *5*, 803-811.

75. Zagorowska, I., Kuusela, S. and Lönnberg, H. *Nucleic Acids Res.* **1998**, *26*, 3392-3396.

76. Nakani, B. S., Welsh, J. J. B. and Hancock, R. D. *Inorg. Chem.* **1983**, *22*, 2956-2958.

77. Hovinen, J. *Bioconjugate Chem.* **1998**, *9*, 132-136.

78. Alder, R. W., Mowlam, R. W., Vachon, D. J. and Weisman, G. R. *J. Chem. Soc., Chem. Commun.* **1992**, 507-508.

79. Kim, R. D., Wilson, M. and Haseltine, J. *Synth. Commun.* **1994**, *24*, 3109-3114.

80. Hovinen, J., Guzaev, A., Azhayeva, E., Azhayev, A. and Lönnberg, H. *J. Org. Chem.* **1995**, *60*, 2205-2209.

81. Hovinen, J. and Salo, H. *J. Chem. Soc., Perkin Trans. 1* **1997**, 3017-3020.

82. Azhayev, A., Guzaev, A., Hovinen, J., Azhayeva, E. and Lönnberg, H. *Tetrahedron Lett.* **1993**, *34*, 6435-6438.

83. Guzaev, A., Azhayeva, E., Hovinen, J., Azhayev, A. and Lönnberg, H. *Bioconjugate Chem.* **1994**, *5*, 501-503.

84. Katajisto, J., Virta, P. and Lönnberg, H. *Bioconjugate Chem.* **2004**, *15*, 890-896.

85. Liu, M. Z., Fan, H., Guo, Z. W. and Hui, Y. *Carbohydr. Res.* **1996**, *290*, 233-237.

86. Guibe, F. and Saint M'leux, Y. *Tetrahedron Lett.* **1981**, *22*, 3591-3594.

87. Katajisto, J., Heinonen, P. and Lönnberg, H. *J. Org. Chem.* **2004**, *69*, 7609-7615.

88. Guzaev, A., Salo, H., Azhayev, A. and Lönnberg, H. *Bioconjugate Chem.* **1996**, *7*, 240-248.

89. Wang, Q. and Lönnberg, H. *J. Am. Chem. Soc.* **2006**, *128*, 10716-10728.

90. Wang, Q., Leino, E., Jancso, A., Szilagyi, I., Gajda, T., Hietamäki, E. and Lönnberg, H. *Chembiochem* **2008**, *9*, 1739-1748.

91. Laine, M., Ketomäki, K., Poijärvi-Virta, P. and Lönnberg, H. *Org. Biomol. Chem.* **2009**, *7*, 2780-2787.

92. Ketomäki, K. and Virta, P. *Bioconjugate Chem.* **2008**, *19*, 766-777.