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THE SIGNIFICANCE OF HYDROGEN BONDING INTERACTIONS IN THE CLEAVAGE OF RNA

by

Heidi Korhonen

TURUN YLIOPISTO
UNIVERSITY OF TURKU
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From the Department of Chemistry
University of Turku
Turku, Finland

Custos

Professor Satu Mikkola
Department of Chemistry
University of Turku
Turku, Finland

Reviewers

Professor Roger Strömberg
Department of Biosciences
Karolinska Institutet
Huddinge, Sweden

Professor Tamás Gajda
Department of Inorganic and Analytical Chemistry
University of Szeged
Szeged, Hungary

Opponent

Professor Anthony J. Kirby
Department of University Chemical Laboratory
University of Cambridge
Cambridge, UK

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ABSTRACT

RNA is essential for all living organisms. It has important roles in protein synthesis, controlling gene expression as well as catalyzing biological reactions. Chemically RNA is a very stable molecule, although in biological systems many agents catalyze the cleavage of RNA, such as naturally occurring enzymes and ribozymes.

Much effort has been put in the last decades in developing highly active artificial ribonucleases since such molecules could have potential in the therapeutic field and provide tools for molecular biology. Several potential catalysts have emerged, but usually detailed cleavage mechanism remains unresolved. This thesis is aimed at clarifying mechanistic details of the cleavage and isomerization of RNA by using simpler nucleoside models of RNA. The topics in the experimental part cover three different studies, one concerning the mechanism of catalysis by large ribozymes, one dealing with the reactivity of modified and unmodified RNA oligonucleotides and one showing an efficient catalysis of the cleavage and isomerization of an RNA phosphodiester bond by a dinuclear metal ion complex.

A review of the literature concerning stabilization of the phosphorane intermediate of the hydrolysis and isomerization of RNA phosphodiester bond is first presented. The results obtained in the experimental work followed by mechanistic interpretations are introduced in the second part of the thesis. Especially the significance of hydrogen bonding interactions is discussed.

PREFACE

This thesis is based on experimental work carried out in the Laboratory of Organic Chemistry and Chemical Biology at the Department of Chemistry, University of Turku during August 2005 – March 2010.

I wish to express my deepest gratitude to Professor Harri Lönnberg for giving me the opportunity to work as a Ph. D. student as well as a teaching assistant. I am also very grateful to Professor Satu Mikkola for patient guidance throughout these years. Her valuable advices, excellent knowledge of reaction kinetics and skills in scientific writing have made this thesis to become reality.

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My greatest gratitude belongs to my family. First of all my husband Teemu with whom I have built a life, including a house. And my precious children Suvi, Ville and Veera. I´m truly blessed because I have you. Thank you for all the love, happiness and joy you have brought to me. You are my everything!

Turku, April 2011

CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications (referred in the text with roman numerals):

I Ora, M., Linjalahti, H. and Lönnberg, H. Phosphodiester Cleavage of Guanylyl-(3´,3´)-(2´-amino-2´-deoxyuridine): Rate Acceleration by the 2´-Amino Function, *J. Am. Chem. Soc.* **2005**, *127*, 1826–1832.

II Linjalahti, H. and Mikkola, S. Intramolecular and Intermolecular Interactions Influence the Reactivity of RNA Oligonucleotides, *Chem. Biodiv.* **2007**, *4*, 2938-2947.

III Linjalahti, H., Feng, G., Mareque-Rivas, J. C., Mikkola, S. and Williams, N. H. Cleavage and Isomerization of UpU Promoted by Dinuclear Metal Ion Complexes, *J. Am. Chem. Soc.* **2008**, *130*, 4232-4233.

IV Korhonen, H., Mikkola, S. and Williams, N. H. The Mechanism of Cleavage and Isomerization of RNA Promoted by an Efficient Dinuclear Zn^{2+} Complex, *Chem. Eur. J.* **2011** (submitted).

ABBREVIATIONS

2´,3´-cAMP	2´,3´-cyclic adenosine monophosphate
2´,3´-cUMP	2´,3´-cyclic uridine monophosphate
CZE	capillary zone electrophoresis
DMAP	4-dimethylaminopyridine
DME	dimethyl ether
DMF	*N*,*N*-dimethylformamide
DMSO	dimethyl sulfoxide
DMTr	4,4´-dimethoxytrityl
DNA	deoxyribonucleic acid
MS	mass spectroscopy
His	histidine
HPLC	high performance liquid chromatography
HPNP	2-hydroxypropyl-4-nitrophenyl phosphate
Lys	lysine
MeCN	acetonitrile
mRNA	messenger RNA
NMR	nucleic magnetic resonance
ODN	oligodeoxyribonucleotide
PNA	peptide nucleic acid
Py	pyridine
RNA	ribonucleic acid
RNase	ribonuclease
RP	reversed phase
TAR	trans-activation responsive
TBAF	tetrabutylammonium fluoride
TBDMS	*tert*-butyldimethylsilyl
THF	tetrahydrofuran
Thr	threonine
TOM	triisopropylsilyloxymethyl
tRNA	transfer RNA
VS	Varkud Satellite

1. INTRODUCTION

1.1. Ribonucleic acid

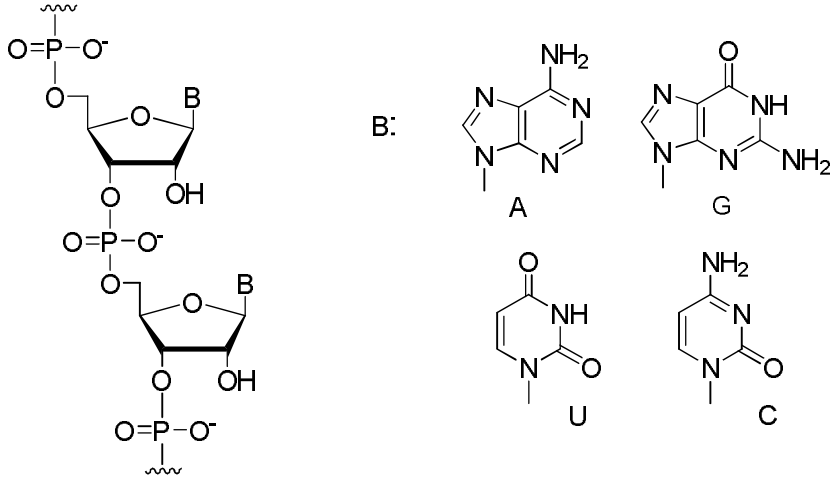
Ribonucleic acid (RNA) is a biologically important molecule with many roles varying from protein synthesis and gene regulation to chemical catalysis. At neutral pH and room temperature RNA is very stable with a half-life of approximately 100 years.[1] RNA can undergo an intramolecular transesterification reaction which results in either a cleavage or an isomerization of a phosphodiester bond. Numerous agents may catalyze the cleavage of RNA, including specific acids and bases, metal ions and their complexes not to mention naturally occurring enzymes.[2-4] Nature has optimized the structure of the active sites of RNA-cleaving enzymes and ribozymes to achieve selective and efficient phosphodiester hydrolysis. Metal ions, amino acid residues and even nucleobases can cooperate in activating the attacking nucleophile and stabilizing the pentacoordinated transition state and the leaving group by hydrogen bonds, electrostatic stabilization and/or proton transfer. Artificial mimics of enzymes and ribozymes have been an ambitious goal for many research groups. Modelling the active site of an enzyme by smaller molecules may provide more detailed information about the mechanism of action. Such compounds could also have various applications in the fields of medicinal chemistry and molecular biology. Challenges are, above all, sequence specificity and high catalytic activity, and accurate information of the mechanism is needed to improve the catalysts. Sequence specific cleavage is generally obtained by combining a cleaving agent and a sequence-recognition molecule such as an oligodeoxynucleotide. To date, all designed artificial nucleases are quite inefficient compared to natural enzymes and there is still much to be done.

This thesis concentrates on hydrogen bonding interactions involved in the transesterification reactions of RNA phosphodiester bond, especially those which stabilize the pentacoordinated phosphorane intermediate/transition state. First, a short introduction to the structure and reaction mechanisms of RNA is represented. The next chapters outline both natural and artificial systems which catalyze the cleavage of RNA, concentrating on examples utilizing hydrogen bonding interactions. Finally, the Results and Discussion section describes three studies where hydrogen bonding interactions are suggested to operate in stabilizing the transition state of the cleavage of RNA phosphodiester bond.

1.1.1. Structure of RNA

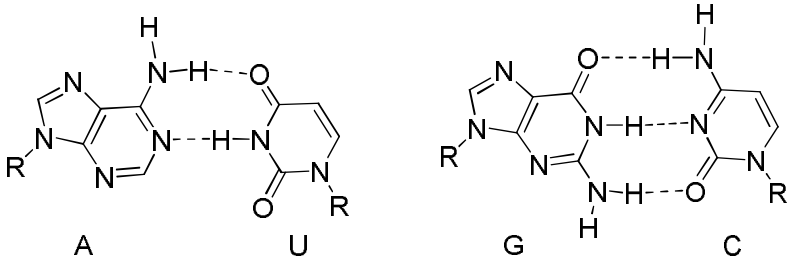
RNA is a polymer which consists of nucleosides joined with 3´,5´-phosphodiester bonds. The predominant nucleotide bases, adenine, guanine, cytosine and uracil are attached to C1 of D-ribose with a glycosidic bond (Scheme 1).

Scheme 1.



Long RNA molecules adopt complex, protein-like three dimensional structures stabilized by specific tertiary contacts including electrostatic interactions and base stacking. Secondary structures such as hairpins, bulges and loops are formed through complementary Watson-Crick base pairing. Accordingly, adenine binds uracil with two hydrogen bonds and guanine binds cytidine with three hydrogen bonds (Scheme 2).

Scheme 2.



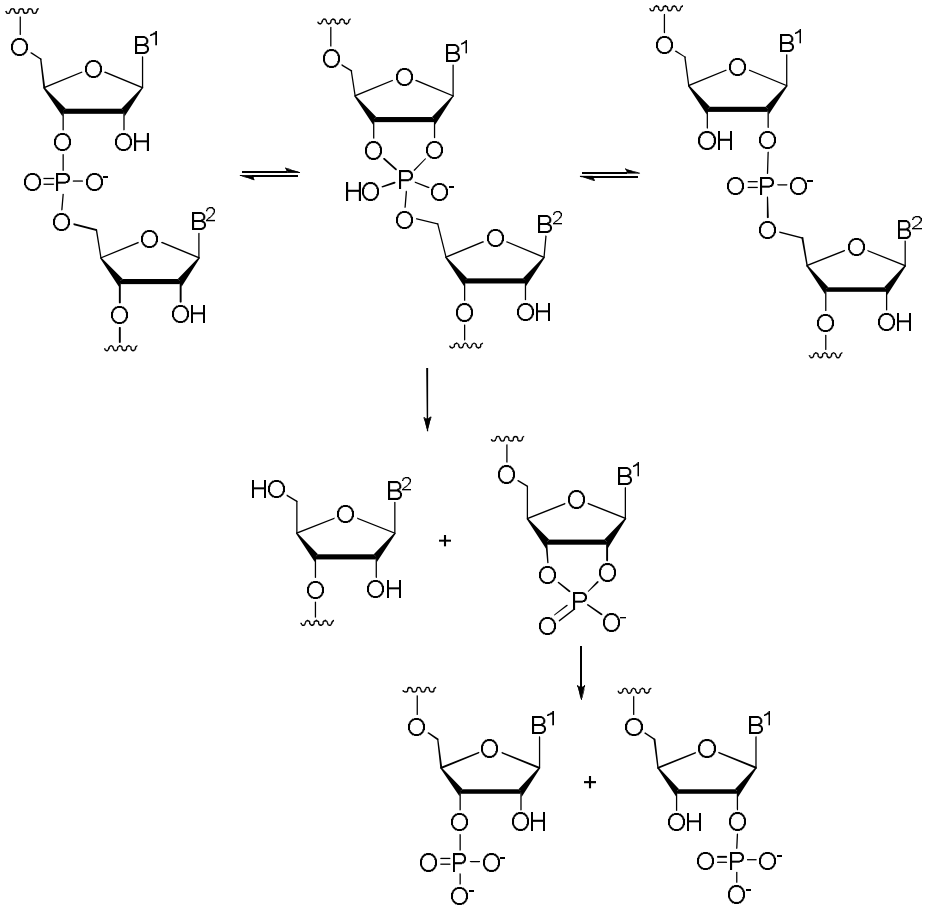
Undoubtedly G-C and A-U canonical Watson-Crick base pairs have the strongest influence on RNA structure. However, several non-canonical base pairs have been reported, including the A-U Hoogsten base pair and the G-U wobble pair, which are often found in RNA crystal structures.[5,6]

1.1.2. Hydrolytic reactions of RNA

It has been known since 1950's that the 2´-hydroxyl group serves as an intramolecular nucleophile in RNA transesterification reactions.[7] Accordingly, 2´-hydroxyl group

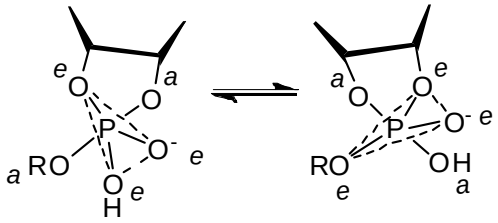
attacks the phosphate and a pentacoordinated intermediate/transition state is formed.[2,8,9] An in-line orientation of the entering 2´-oxygen, phosphorous and departing 5´-oxygen is believed to be essential for the cleavage reaction.[10] The departure of 5´-linked nucleoside initially produces 2´,3´-cyclic phosphate, which is rapidly hydrolyzed to a mixture of 2´- and 3´-terminal phosphate groups (Scheme 3).

Scheme 3.



Alternatively, a reversible intramolecular transesterification to a 2´,5´-phosphodiester bond can occur.[9] According to Westheimer's rules, nucleophiles may enter and leave the intermediate through apical positions only.[11] In addition, electronegative ligands prefer an apical position and electropositive ligands an equatorial position.[11] The attacking hydroxyl group is initially in an apical position and, hence, the neighbouring sugar oxygen is equatorial in the resulting five-membered ring. Ligands are reorganized in pseudorotation where two equatorial ligands become apical and the originally apical ligands equatorial, while one of the equatorial ligands remains equatorial (Scheme 4). This enables the breaking of P-O3´ bond. Consequently, pseudorotation is a prerequisite for isomerization.

Scheme 4.



a = apical
e = equatorial

Reaction conditions, especially pH, affect the course of the reaction. Both hydronium and hydroxide ions catalyze the cleavage of UpU, a simple dinucleoside monophosphate fragment of RNA, while only acid catalyzed and pH-independent isomerization are observed.[8] In addition, an acid catalyzed depurination is a competing reaction in the case of purine bases. The pH-rate profile for the cleavage and isomerization of UpU is represented in Figure 1.[8] The shape of the pH-rate profile implies that the cleavage of UpU proceeds via four different ionic forms, phosphodiester monocation [SH_3^+], neutral phosphodiester [SH_2], phosphodiester monoanion [SH^-] and dianion [S^{2-}] while only three forms, SH_3^+, SH_2 and SH^- are involved in isomerization reaction throughout the pH range studied (Scheme 5);[2,8]

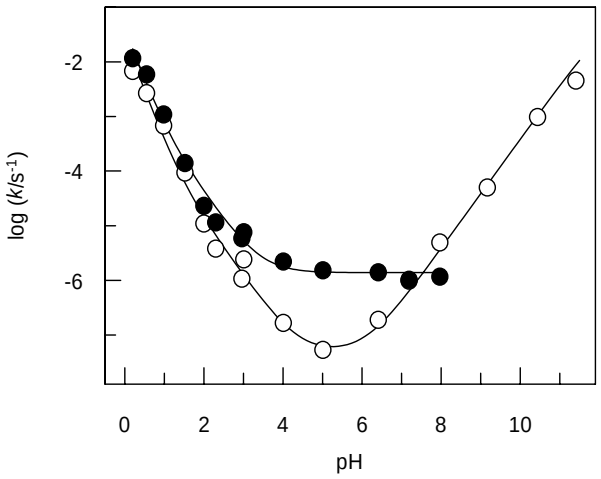
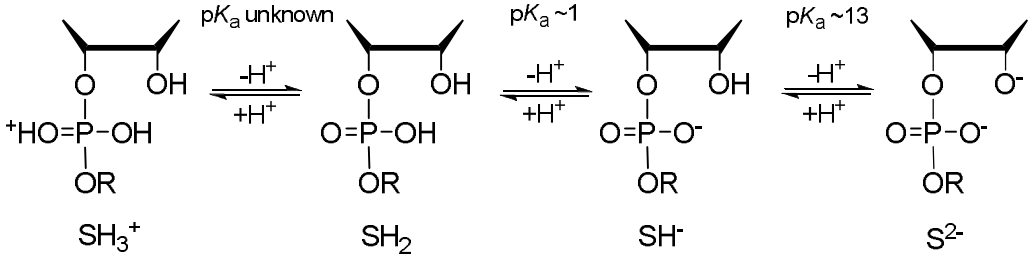


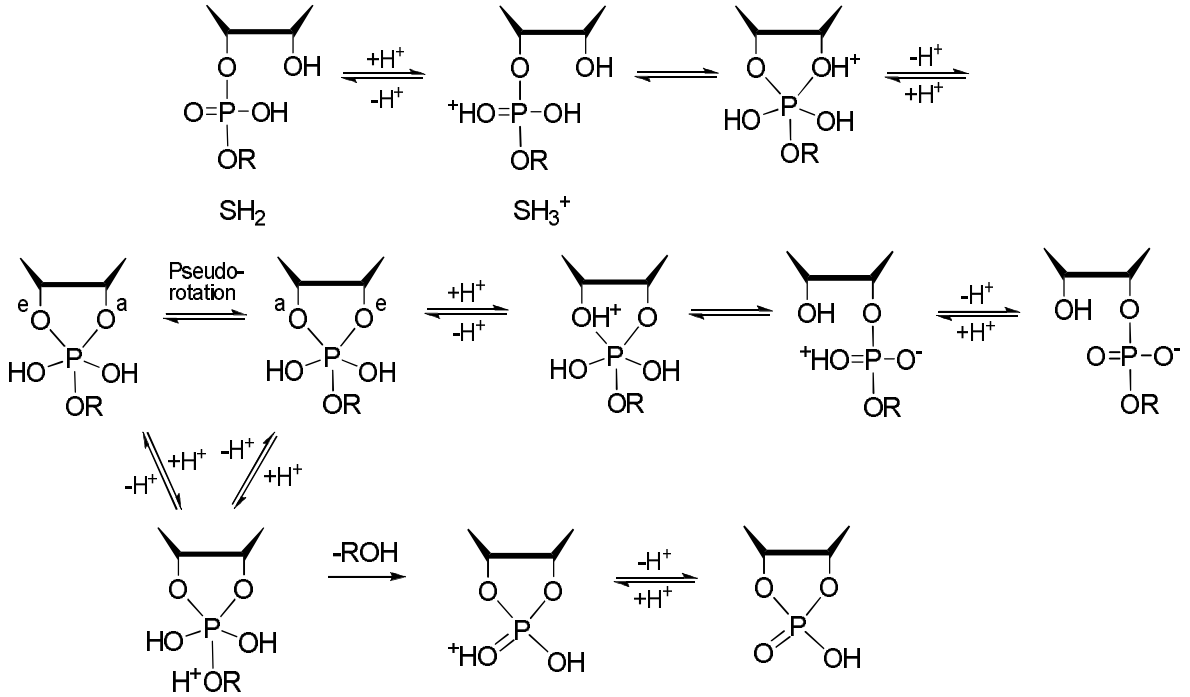
Figure 1. The pH-rate profiles for the cleavage (○) and isomerization (●) of UpU at 90 °C.[8]

Scheme 5.



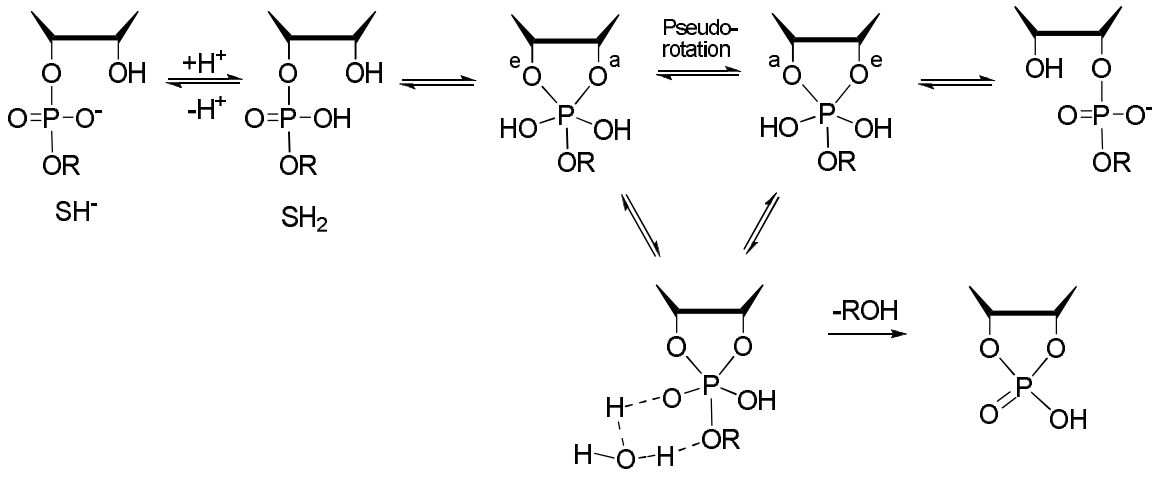
pH < 2. Under highly acidic conditions (at pH < 1) the reaction proceeds by a rapid protonation of the nonbridging oxygen prior to the attack of a 2´-hydroxyl group on the phosphate (Scheme 6). A monocationic phosphorane formed may either collapse back to the starting material or deprotonate to a neutral form. Protonation of the 5´-oxygen followed by the cleavage of a P-O5´ bond leads to cleavage products. Alternatively, pseudorotation may place the 3´-oxygen to an apical position and a subsequent protonation of the 3´-oxygen followed by the cleavage of a P-O3´ bond results in isomerization. β_{lg} (i.e. the sensitivity of the rate of the reaction to the pK_a of the leaving group) values of -0.12 for the cleavage reaction and -0.18 for isomerization, obtained in studies with uridine 3´-alkylphosphates (0.25 M HCl, 25 °C), are consistent with the mechanism where the leaving group is protonated.[12] No significant deuterium solvent isotope effects were found in a study of RNA models, 2-methylbenzimidazole ribonucleoside alkylphosphates, from acidic to neutral conditions.[13] The rate of both the cleavage and isomerization show a first order dependence on the hydronium ion concentration below pH 1.[8] Second order kinetics observed in 1 < pH < 2 implies that the reactive species is SH_3^+ even though the predominating ionic form is SH^-.

Scheme 6.



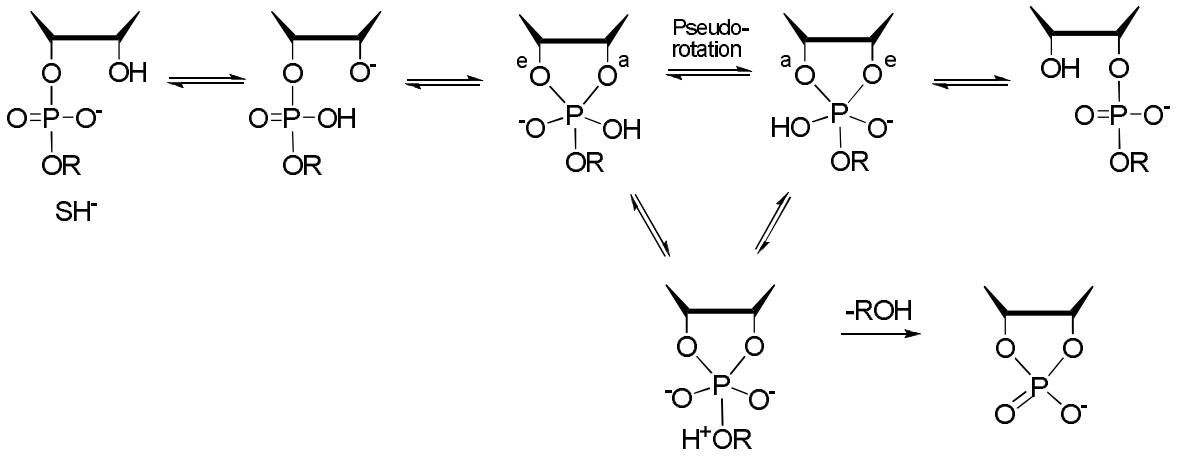
pH 2-4 Around pH 3 the predominating ionic form is SH^-. However, the rate of the cleavage and isomerization show first order dependence on the hydronium ion concentration over this narrow pH range. Consistently, the SH^- is initially protonated and the reaction proceeds by an attack of the 2´-hydroxyl group on neutral phosphate, followed by a water mediated proton transfer to the leaving group oxygen (Scheme 7). β_{lg} value of -0.19 obtained for the cleavage reaction is consistent with this mechanism.[14]

Scheme 7.



pH 4-6 The isomerization becomes pH-independent above pH 4. In contrast, the cleavage is pH-independent only in a narrow range around pH 5. pH-independent isomerization is much faster than cleavage; relative rates are 1 and 0.04, respectively. The reaction most probably proceeds by an attack of a 2´-oxyanion of a rare tautomer of SH^- on a neutral phosphate rather than by the attack of an undissociated 2´-hydroxyl on the phosphorane monoanion (Scheme 8).[9] The breakdown of the monoanionic phosphorane including water-mediated proton transfer leads to isomerization or cleavage products. β_{lg} value of -0.59 is reported to pH independent cleavage of uridine 3´-alkylphosphates.[14]

Scheme 8.

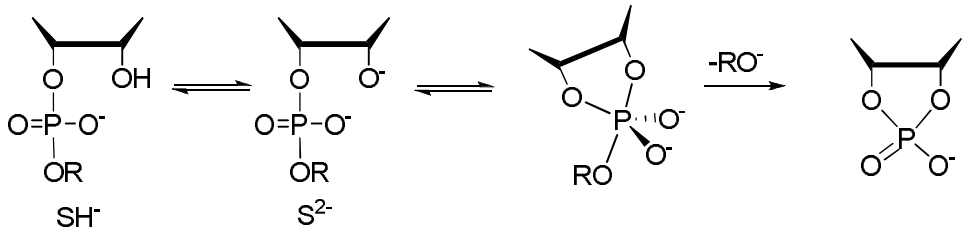


pH > 6 While the rate of the isomerization is pH-independent in the pH range 4-8, the cleavage becomes hydroxide ion catalyzed at pH 6. The first order dependence of the cleavage on the hydroxide-ion concentration continues up to pH 12. A rapid deprotonation of the 2´-hydroxyl group followed by the attack of the 2´-oxyanion on

the phosphate monoanion produces a dianionic phosphorane intermediate (Scheme 9). β_{lg} value of -1.28 suggest that the leaving group departs as an alkoxide ion.[12]

Isomerization is not observed because the dianionic phosphorane is unable to pseudorotate. It would require that negatively charged phosphoryl oxygen adopts an apical position, which is energetically unfavourable.[11] In contrast to the reaction under neutral and acidic conditions, the alkaline cleavage of phosphodiester bonds with an alkyloxy leaving group is characterized by a large solvent deuterium isotope effect value of 7.24, which can mainly be accounted for by the difference in the pK_a value of the attacking nucleophile.[13]

Scheme 9.

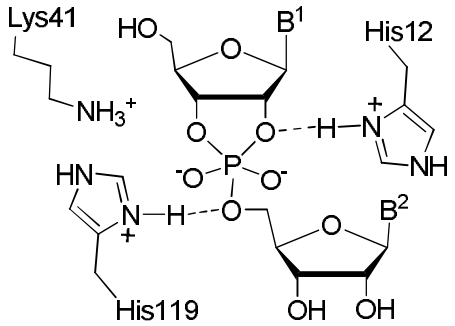


1.2. Systems involving hydrogen bonding interactions stabilizing the phosphorane

1.2.1. RNAse A

RNase A is a small protein with only 124 amino acid residues. It catalyzes both the cleavage of a P-O5´ bond of an RNA strand and the hydrolysis of a 2´,3´-cyclic phosphodiester to a 3´-phosphomonoester.[4,15,16] There is a clear pyrimidine specificity due to steric interactions and a specific hydrogen bonding to a pyrimidine base from Thr45.[17,18] In the generally accepted mechanism, the side chain of His12 acts as a base and deprotonates the 2´-hydroxyl group. His119 acts as an acid and protonates the leaving group (Scheme 10).[4,19] Studies with a stable transition state analog, uridine vanadate, have shown that Lys41 also stabilizes the phosphorane through electrostatic[20,21] or hydrogen bond interactions,[22] or even participates in the reaction by acting as a general base.[23]

Scheme 10. Transition state stabilization in the active site of RNaseA.[4]



The importance of these groups at the active site has been shown, for example, by replacing either His12 or His119 with alanine.[24] As a result, the affinity of the enzyme for the transition state decreased 10^4-fold in the cleavage of poly(C) and UpA. When Lys41 was replaced with an arginine residue, the activity of the enzyme was reduced about 98 %.[25] In addition, replacing Lys41 with alanine results in 10^5-fold rate decrease.[26]

1.2.2. Catalytic RNA

It has been known since early 1980's that specific, naturally occurring RNA sequences are able to catalyze the cleavage of certain phosphodiester bonds.[27,28] Compared to enzymes, these so called ribozymes have relatively few functional groups to participate in catalysis. Most ribozymes require the presence of divalent metal ions, usually Mg^{2+} ions, in order to be catalytically active.[29-31] Several different types of ribozymes are found in nature and they are divided into two groups on the basis of their size and catalytic mechanism: the small and large ribozymes.

1.2.2.1. Small ribozymes

Small ribozymes (50-150 nucleotides) include hairpin, hammerhead, hepatitis delta virus and *Neurospora* VS ribozymes.[32] They are self-splicing molecules all of which catalyze a reversible transesterification reaction using an internal nucleophile. Even though the structures of these four ribozymes are not strictly related, the initial cleavage products in all cases are the 2´,3´-cyclic phosphate and free 5´-hydroxyl group.[32-34] The reaction is, hence, analogous to the nonenzymatic cleavage of RNA. Rate constants of the cleavage are typically in the region of 1 min^{-1}, which means about 10^6-fold rate acceleration compared to the uncatalyzed reaction.[32,35] The catalysis by hairpin and hammerhead ribozymes is discussed below in more detail.

The hairpin ribozyme (Figure 2) catalyzes a sequence-specific, reversible transesterification of an RNA substrate and the reaction does not require the participation of divalent metal ions in catalysis.[34] The hairpin ribozyme is fully active in the presence of an exchange-inert complex $[Co(NH_3)_6]^{3+}$ and in the absence of

additional metal ions.[36] This small ribozyme achieves therefore its catalytic activity by utilizing only nucleobases.

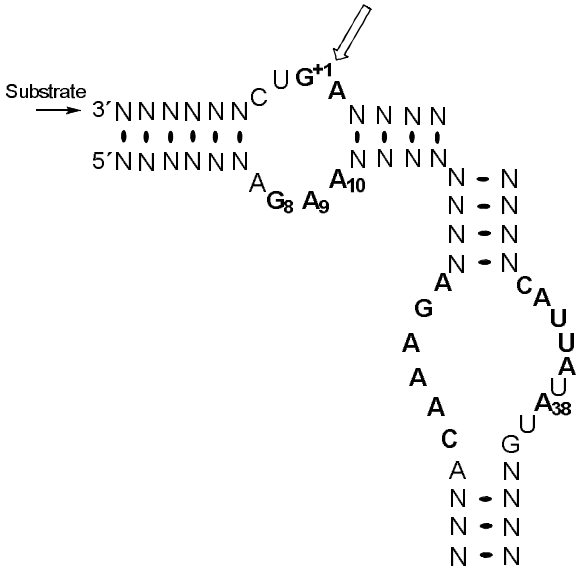
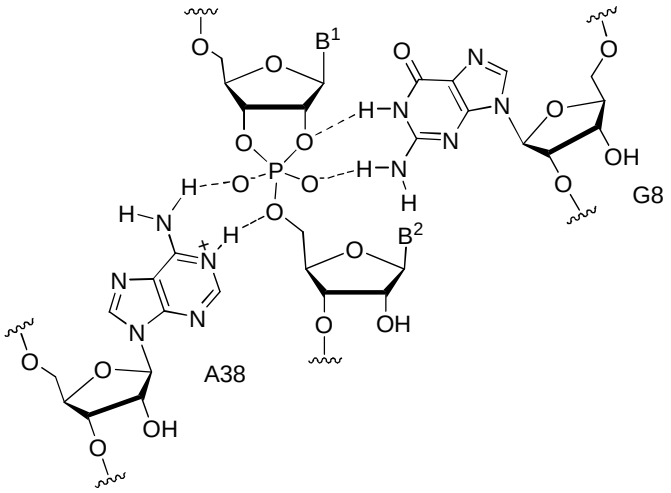


Figure 2. Secondary structure of a hairpin ribozyme. Bold letters refer to consensus nucleotides, N is any nucleotide and the large arrow points to the scissile bond.[36,37]

It has been suggested that at least three nucleobases, G8, A9 and A38, stabilize the transition state by donating multiple hydrogen bonds.[38,39] Hydrogen bonding interactions are also maximized in the transition state.[38] An abasic substitution of A38 reduces the rate of the cleavage 14000-fold.[40] Removing G8 from the catalytic center reduces the activity about 350-fold, but the loss of A9 does not significantly affect the catalysis.[41,42] The proposed mechanism of transition state stabilization by nucleobases is depicted in Scheme 11.[19,38,40] In addition, ordered water molecules in the catalytic center may contribute to catalysis by stabilizing the transition state electrostatically or by hydrogen bonding.[39,43] Kinetic solvent isotope experiments suggest a two-proton mechanism where there is a concerted deprotonation of the attacking nucleophile and a protonation of the leaving group at the rate limiting step.[44]

Not long ago the contribution of the N1 of A38 was proved by using an inert isostere of adenosine, N1-deazaadenosine.[45,46] The cleavage rate was reduced approximately 10^7-fold. Moreover, experiments on the acidity of the N1 of A38 with Raman crystallography,[45] extensive molecular dynamics simulations[47] and nucleotide analogue interference mapping using fluoroadenosine analogues[48] have shown that the A38 base is protonated prior to the transition state. A38 can, hence, have roles such as promoting the formation of a transition state and stabilizing it, protonating the 5´-leaving group and lowering the pK_a of the 2´-nucleophile.

Scheme 11. Proposed mechanism of the transition state stabilization in the cleavage of RNA by hairpin ribozyme.[19,38,40]



In hammerhead ribozyme, at least eleven consensus nucleotides form the catalytic core (Figure 3).[49,50] Nucleotides A9, G8 and G12 are at a hydrogen bonding distance to C17.[51] The sequence specificity at the cleavage site is reported to be GUC, as conserved trinucleotide, or generally NUX, where N is any nucleotide and X is any nucleotide but G.[50,52,53] A single[54-56] or a two-metal ion[57-61] mechanism has been proposed to operate in the cleavage reaction. Hydrogen bonding interactions may well contribute in the transition state, but they are less direct than in the case of the hairpin ribozyme.

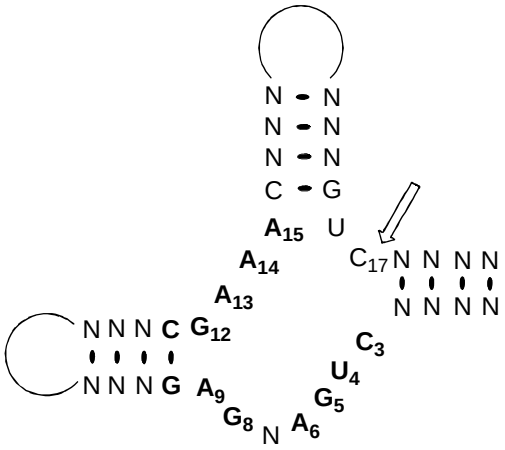
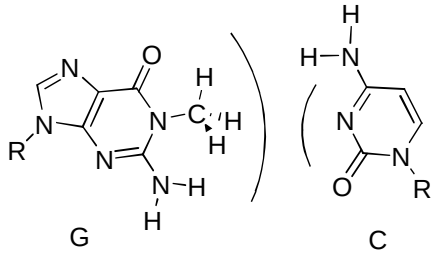


Figure 3. Secondary structure of a self cleaving hammerhead ribozyme. Bold letters refer to consensus nucleotides, N is any nucleotide and the large arrow points to the scissile bond.[31]

Mutations in the central core have revealed that all the conserved residues are needed for an efficient cleavage.[50] If the N1 group of a conserved guanosine residue G5, G8 or G12 in hammerhead ribozyme is methylated, the molecule becomes inactive.[62] A methyl group introduces steric bulk which prevents hydrogen bonding in Watson-

Crick base pairs (Scheme 12) and, therefore, important interactions at the active site are reduced. A substitution of G12 with analogs, such as inosine, may cause a 10^{-3}-fold rate reduction.[63,64] 2´-*O*-methylcytidine at the cleavage site prevents the cleavage, as can be expected.[65]

Scheme 12

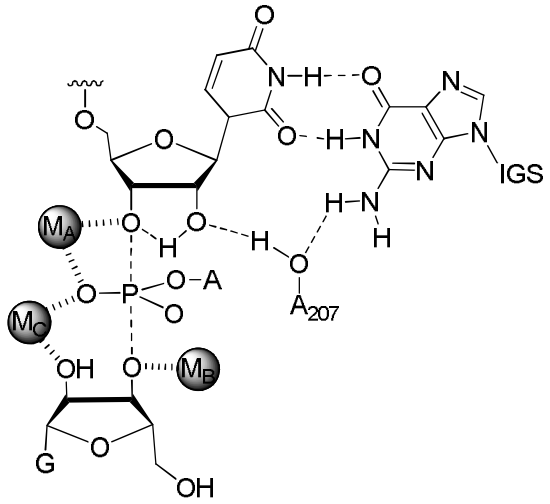


1.2.2.2 Large ribozymes

The group I and group II introns and the RNA subunit of RNase P are categorized as large ribozymes, as they consist of hundreds of nucleotides. The transesterification is catalyzed by an attack of an external nucleophile on the scissile phosphodiester bond. The attacking nucleophile is the 3´-OH of an external guanosine, a 2´-OH group of an internal adenosine or a water molecule in the cases of group I introns, group II introns and RNase P ribozyme, respectively.[66] Phosphorous atom undergoes an inversion of configuration which suggests a direct in-line reaction with a pentacoordinated transition state.[67-69] Large ribozymes are considered as obligate metalloenzymes.[67-73] Among the large ribozymes, the *Tetrahymena* group I intron is best known and studied so the following paragraph makes a short survey to the catalytic properties of this ribozyme.

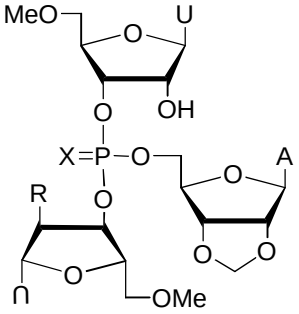
Three metal ions are suggested to operate in the catalytic core of *Tetrahymena* ribozyme, as demonstrated with multiple site-specific thio substitutions.[71,74,75] A hydrogen-bonding triad *via* A207 has been reported to stabilize the transition state (Scheme 13).[76] The cleavage site 2´-hydroxyl group is proposed to form a hydrogen bond to the adjacent 3´-oxygen. The suggestion is supported by numerous studies with modifications to the 2´-position.[77-79] For example, a substrate with a 2´-methoxy group reacts 10^5-fold slower than an unmodified one.[77] Results obtained with 2´-*O*-methyl-3´-thioguanosine as a nucleophile imply that the guanosine 2´-hydroxyl group is not similarly hydrogen bonded to the adjacent 3´-oxygen as is the case in the cleavage site.[80]

Scheme 13. Transition state interactions at the *Tetrahymena* ribozyme active site.[75,76]



In order to elucidate in more detail the mechanism utilized by large ribozymes, particularly the role of the neighbouring 2´-hydroxyl group as a hydrogen bond donor, small model systems consisting of trinucleoside 3´,3´,5´-monophosphates and -phosphorothioates have been studied (Scheme 14).[81-85] The results strongly suggest that the 2´-hydroxyl group stabilizes the phosphorane intermediate by donating a hydrogen bond to one of the phosphoryl oxygens rather than to the departing 3´-oxyanion. Methylation of one of the 2´-hydroxyl groups of **1**, resulting in compound **2**, retards the rate of the cleavage 27-fold under alkaline conditions, but the product distribution is not altered.[82]

Scheme 14



1; R = OH, X = O
2; R = OMe, X = O
3; R = OH, X = S
4; R = OMe, X = S
5; R = NHTfa, X = O

In the precence of Zn^{2+}, phosphorothioate triesters **3** and **4** are cleaved at comparable rates. Most likely the thiophilic metal ion binds to the sulphur and, hence, hydrogen bonding interactions to thiophosphorane intermediate are prevented.[84] In contrast, 2´-trifluoroacetamido group in the trinucleoside model **5** may be involved in stabilizing the departing 3´-oxyanion by hydrogen bonding.[85] The hydroxide-ion catalyzed cleavage is observed to occur exclusively by the rupture of the 3´-linked nucleoside containing the trifluoroacetamido moiety. Steric interactions may also contribute to the orientation of the intramolecular hydrogen bond.[85]

1.2.3. Weak interactions influencing the reactivity of oligoribonucleotides

The effect of base moieties (purine/pyrimidine) in the cleavage of simple dinucleoside monophosphates is only modest.[8] In contrast, the base sequence in a linear single-stranded oligoribonucleotides can have a significant contribution to the reactivity.[86] Both rate accelerations and retardations compared to reactions of dinucleoside monophosphates have been observed (Table 1).[86] In particular, the scissile UpA phosphodiester bond has showed enhanced reactivity.[86-88] Base stacking has often been proposed to affect the reactivity.[87,89-91] Stronger stacking causes retarded cleavage rates presumably due to restricted in-line conformation.[90] While base stacking is a well understood explanation for the rate retardations, the rate acceleration is more challenging to realize. The transition state is somehow stabilized possibly by a hydrogen bonding network around the scissile phosphodiester bond and the neighbouring bases may also participate in the reaction.[89]

Table 1. Pseudo first order rate constants for the cleavage of oligonucleotides in pH 8.5 and at 90 °C.[86]

Substrate	k_{cl} / 10^{-7} s^{-1}
UpU	2.2 ± 0.1
5´-GGGUA**U**-AAGUGC-3´	14.6 ± 0.2
5´-GGGUA**U**-AUGUGC-3´	43.8 ± 0.2
5´-GUGUA**U**-AAGUGC-3´	1.2 ± 0.1
5´-GGGUA**A**-AAGUGC-3´	<0.2[a]

[a] No reaction after 3 months

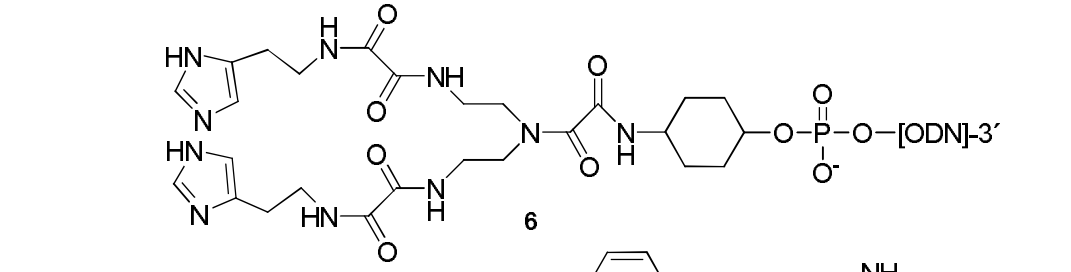
1.2.4. Metal-free catalysts for the cleavage of RNA

A wide range of metal-ion independent artificial catalysts capable of cleaving RNA molecules have been designed and studied. These include, for example, polyamine, guanidine and imidazole derivatives which can be conjugated with oligonucleotides or PNA. Much effort has been put to achieving a catalytic activity, at least close to that of naturally occurring enzymes, by mimicking the active center of enzymes. Indeed, many potential and intriguing catalysts have been developed. They may be general acid/base catalysts or operate by forming hydrogen bonds. These methods are not always strictly distinguishable.

As mentioned in Chapter 1.2.1., RNase A utilizes two histidine residues in the catalytic center. Thus, numerous mono- and diimidazole containing catalysts has been studied. The pK_a of imidazole is close to 7, so at physiological conditions both imidazole and imidazolium ions are present. An imidazole buffer catalyzes the cleavage of RNA phosphodiester bond, although quite modestly.[92-96] Several oligodeoxyribonucleotide conjugates of imidazole residues have emerged, among

them construct **6** which cleaves $tRNA^{Phe}$ with a half-life of 1.5 hours (pH 7.0 at 37 °C).[97]

Spermidine and other biogenic polyamines catalyze the cleavage of single stranded RNA.[87,88,98] At least two protonated secondary amino groups are needed for the catalysis. One binds probably to the oligoribonucleotide while the other participates in the reaction. Polyamine-imidazole conjugates (for example **7**) have been studied as site-specific RNA scission catalysts.[99,100] Spermine-imidazole constructs in the presence of free imidazole cleave single-stranded regions of $tRNA^{Asp}$ with a marked preference for pyrimidine-A sequences.[100] The location of the imidazole residue(s) and the number and the distance of nitrogen atoms have a significant influence on cleavage rates and selectivity. The polycationic molecule interacts with the negatively charged phosphate backbone and the catalysis is suggested to involve a co-operation of one of the amino groups, which is protonated at a physiological pH, and of the imidazole group, which acts as a base.[100]



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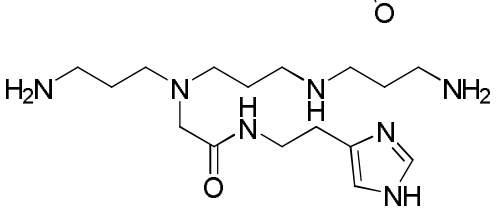

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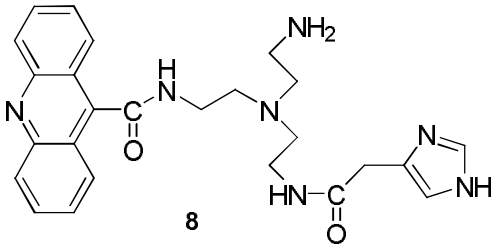

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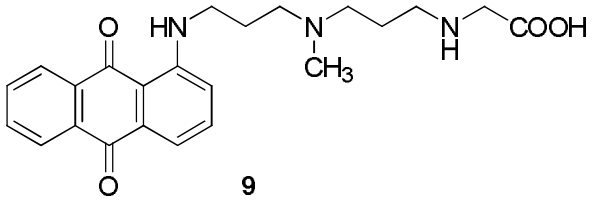

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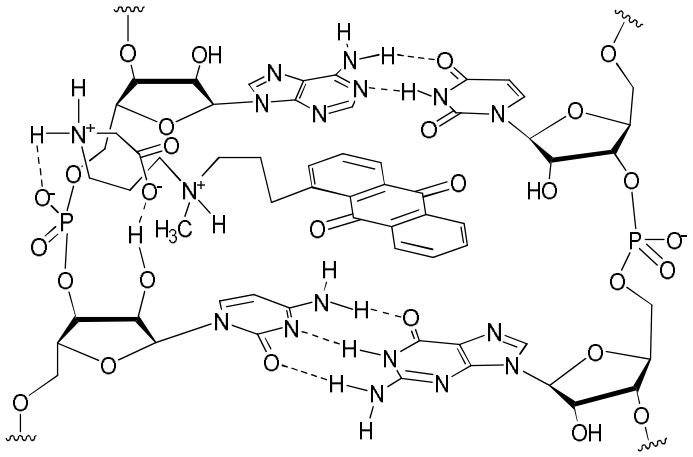
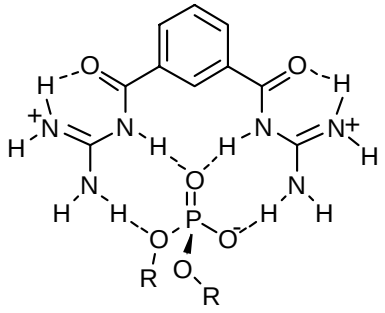
A conjugation of a catalyst and an intercalating agent has also been reported.[101-103] For example, acridine and anthraquinone intercalate to double stranded regions of nucleic acids. One of the most active catalysts, compound **8** cleaves almost 60 % of 16s/23s ribosomal RNA substrate in one hour (pH 7.4, 37 °C).[102] The reaction shows a bell shaped pH-rate profile which suggests a general acid-base catalytic mechanism. The conjugate of glycine and anthraquinone (**9**) has been reported to hydrolyze yeast $tRNA^{Phe}$.[103] The mechanism is suggested to involve a direct participation of carboxylate group, but not as a nucleophile, since a DNA substrate is not cleaved. The proposed mechanism is represented in Scheme 15. It is supposed that the carboxylate group activates the 2´-hydroxyl group for an intramolecular attack and the ammonium ion operates as a general acid catalyst.[103]

Scheme 15. Proposed mechanism for the hydrolysis of $tRNA^{Phe}$ by an intercalating catalyst.[103]

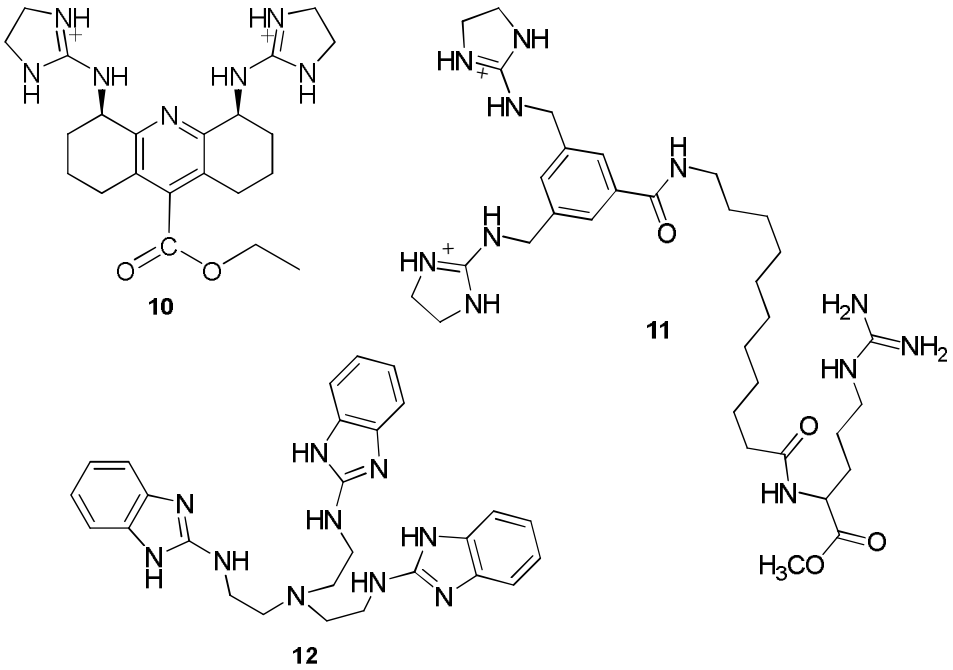


The essential amino acid arginine contains a guanidinium group in the side chain. Due to the fact that a guanidinium group can form multiple hydrogen bonds, many compounds containing such a group have been studied. Especially bis(guanidinium) compounds have shown catalytic activity, at least in nonaqueous solutions.[104,105] For example, a bis(acylguanidinium) compound shown in Scheme 16 catalyzes both inter- and intramolecular phosphodiester cleavage. Properly positioned guanidinium groups may provide stabilization via four hydrogen bonds. [104,105]

Scheme 16. Proposed hydrogen bond stabilization provided by two guanidinium groups.[104,105]



A water-soluble guanidium-based catalyst has been obtained by using aminoimidazoline groups as mimics of guanidinium groups.[106-108] Compound **10** cleaves mRNA at micromolar concentrations in the presence of imidazole.[106] In the proposed mechanism, imidazole deprotonates the 2´-hydroxy function and the transition state is stabilized by the two aminoimidazoline groups. Compound **11** has been reported to degrade HIV-1 mRNA TAR substrate (incubation 12 hours at pH 7 and 37 °C).[107]



Another guanidinium analogue, 2-aminobenzimidazole, has been found to have potential catalytic properties (most probably due to pK_a shift from 14 of guanidinium to 7 of 2-aminobenzimidazole).[109] A tris(2-aminobenzimidazole) molecule **12** cleaves single stranded RNA with a half-life of 120 min, thus being, when conjugated with antisense oligonucleotides, a candidate for efficient site specific catalyst.[109]

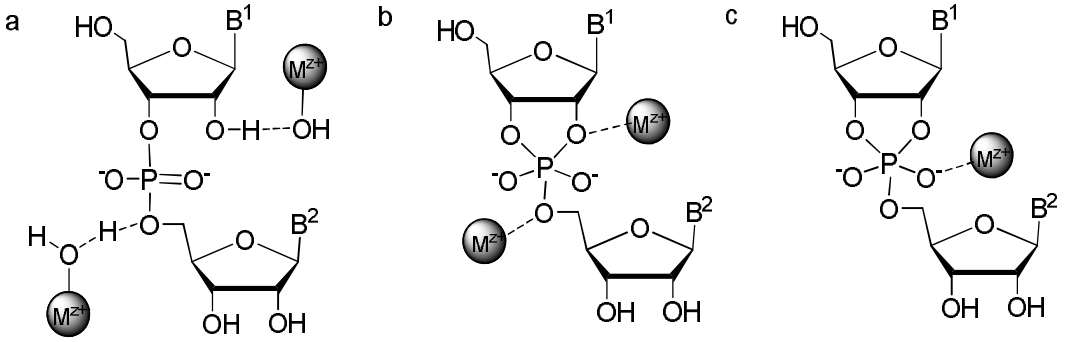
1.3. Catalysis of the cleavage of RNA by metal ion complexes

1.3.1. General background

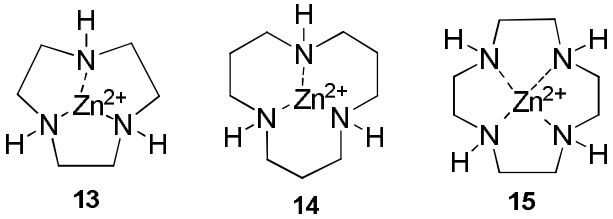
Natural RNA cleaving enzymes utilize the cooperation of metal ions and amino acid residues and many ribozymes are also metalloenzymes.[19,30,31] By imitating the active sites of enzymes, numerous artificial catalysts containing metal centres combined with general acids or bases have been explored. In order to design a good metal ion catalyst, one should try to optimize the interactions between a catalyst and a substrate, that is, the orientation of the catalyst in respect to the substrate, and interactions of the catalyst with the transition state of the reaction.

Lanthanide ions effectively catalyze the cleavage of HPNP (**16**) and UpU.[110,111] Transition metals, as simple aquo ions, promote also the cleavage of phosphodiester bonds, but the rate acceleration is quite modest.[111] Metal ions can have a variety of functions in promoting phosphodiester hydrolysis. A metal ion coordinated water molecule can act as a general acid and a metal ion bound hydroxide ion as a general base (Scheme 17 (a)). Metal ions can also provide direct electrophilic activation by coordinating to 2´-oxygen, to 5´-oxygen of the leaving group or to the non-bridging oxygen (Scheme 17 (b) and (c)).[66,112]

Scheme 17. Metal ion as (a) a general acid/base catalyst, (b) a Lewis acid and (c) an electrophilic catalyst.[66]



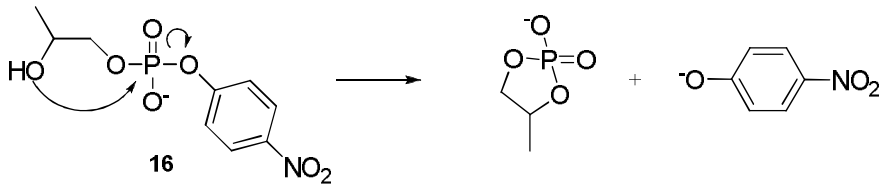
Metal ions bound to an appropriate ligand usually bind the substrate more strongly and show a higher resistance to precipitation.[111,113] Macrocyclic amine ligands, for example those in complexes **13-15**, are known to maintain Zn^{2+} ions in solution at neutral or even mildly alkaline pH.[113,114] Appropriate ligands enable also the formation of site-specific cleaving agents where the catalytic group is attached to a sequence recognition agent, such as an oligodeoxynucleotide.[3]



Dinucleoside monophosphates or activated phosphodiesters, such as HPNP (**16** in Scheme 18), are usually used in kinetic experiments as simple models of RNA. HPNP reacts rather fast even at room temperature and its reactions are convenient to follow. However, an efficiently promoted cleavage of HPNP is not a warranty of an efficient cleavage of RNA. Often a quite different rate enhancement is observed for nucleoside substrates and substrates with a good leaving group. The main thing that accounts for the difference is the distinction in the rate-limiting steps, which for aryl esters is the formation of the phosphorane intermediate and for RNA, the breakdown of the

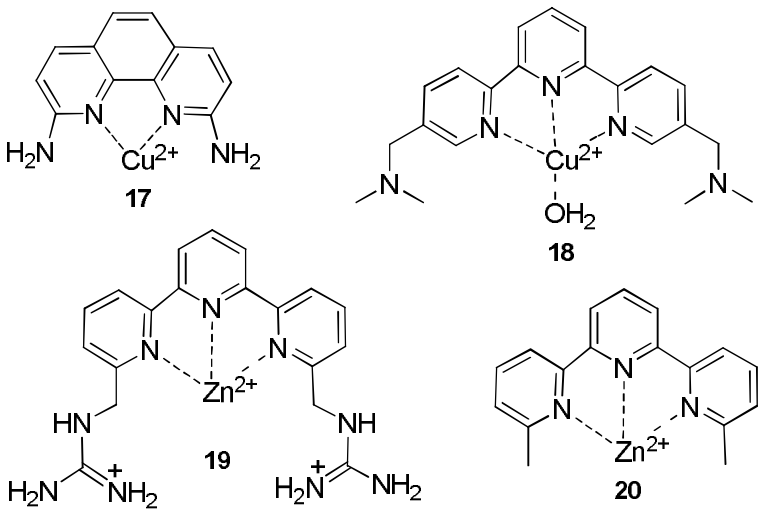
intermediate.[3,115] Some of the differences may be also attributed to steric interactions.[116]

Scheme 18.



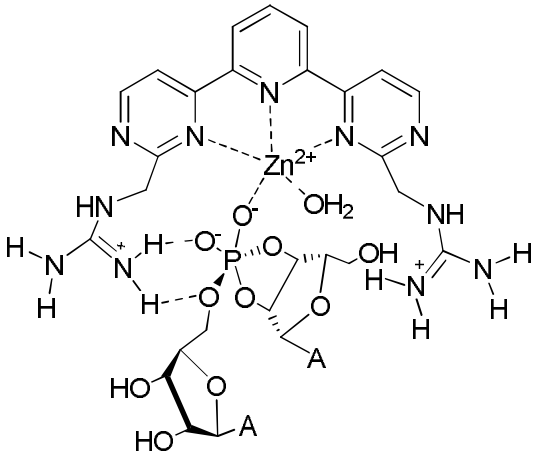
1.3.2. Mononuclear metal ion complexes containing auxiliary functional groups

The cooperativity of a metal ion and ammonium or guanidinium groups has been studied and many potential catalysts have emerged. Complex **17**, for example, catalyzes the hydrolysis of 2´,3´-cAMP about 2×10^4 times more efficiently than a catalyst lacking the amino groups, and it provides over 10^9 -fold rate acceleration over the background reaction.[117] Very likely the amino group participates in the reaction by donating hydrogen bonds. Terpyridine based metal ion complexes are reported to effectively catalyze the transesterification of RNA under physiological conditions.[118] Complex **18** with tertiary amino groups attached to terpyridine catalyzes the cleavage of HPNP 7 times more efficiently than a complex without the amino groups, presumably due to deprotonation of the attacking nucleophile by an amino group.[119]

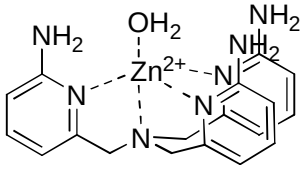


Considerable rate acceleration has been reported by complex **19**. The rate constant of the hydrolysis of ApA at pH 7.4 and 37 °C is about 2×10^{-5} s^{-1}.[120] This complex is more than 3000 times more effective than complex **20**. The results strongly suggest an efficient cooperation between the metal ion and the guanidinium groups. The proposed mechanism includes phosphate activation by the zinc ion and one of the guanidinium groups (Scheme 19).

Scheme 19. A possible mechanism for transition state stabilization in the hydrolysis of ApA by **19**.[120]



Complex **21** cleaves HPNP almost 750 times faster than a complex without the hydrogen bond donating amino groups.[121] A rate enhancement of four orders of magnitude in the cleavage of HPNP with respect to the background reaction is observed with complex **22**.[122] However, in this case the amino groups are responsible for only a 13-fold rate acceleration and the effect of the hydroxyl group to the rate of the reaction is negligible. Computer-assisted calculations have revealed that complex **23** utilizes hydrogen bond interactions from the NH_3^+ group in addition to hydrogen bonds from the pyridine amino groups in stabilizing the phosphorane transition state.[123] The authors claim that complex **23** is the most active monometallic Zn^{2+} complex in cleaving HPNP so far reported.[123]



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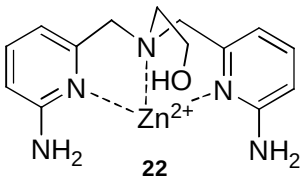

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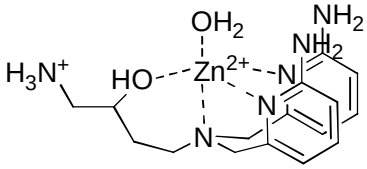

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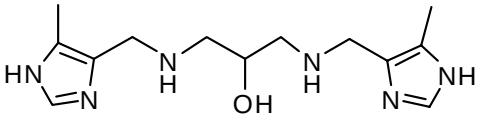
1.3.3. Di- and trinuclear metal ion complexes

Dinuclear metal ion systems have gained much interest since a double metal ion system is generally involved also in the reactions of natural nucleases and ribozymes.[29,124,125] The cooperation of two metal ions in a complex can efficiently enhance the hydrolysis of RNA and smaller phosphodiester model compounds. It is generally believed that the catalytic efficiency of dinuclear metal ion complexes has its origin in a double Lewis acid activation where two metal ions bind the two phosphoryl oxygens.[126,127] The interaction of phosphoryl oxygens with metal ions makes the phosphate group more susceptible towards the nucleophilic attack by the 2´-hydroxy group.

1.3.3.1. Co-operation of the two metal ion centres

The double Lewis acid activation and a general base catalysis are combined in the catalysis by a dinuclear Zn^{2+} complex of dinucleating bis-imidazole ligand **24**.[128] The best catalytic activity is achieved at a metal-to-ligand ratio of 2:1. Both the transesterification of HPNP and the hydrolysis of 2´,3´-cUMP are accelerated 10^4 -fold (pH 8, 37 °C). Moreover, it has been reported that between pH 6-11, an equimolar solution of Cu^{2+} and ligand **25** does not catalyze the cleavage of HPNP.[129] However, the rate of the cleavage increases above 1:1 ratio and the maximum activity is reached with two metal ions per a ligand molecule (Figure 4).



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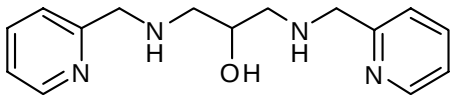

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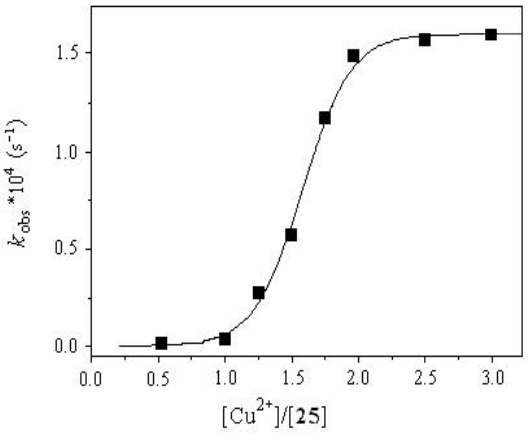
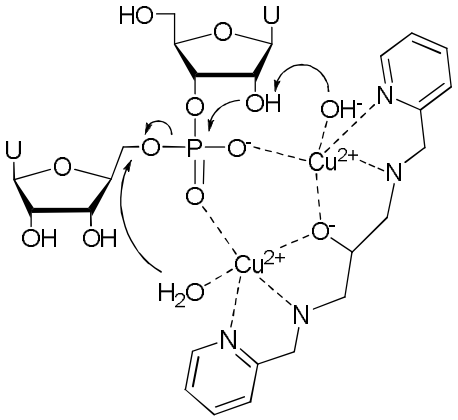


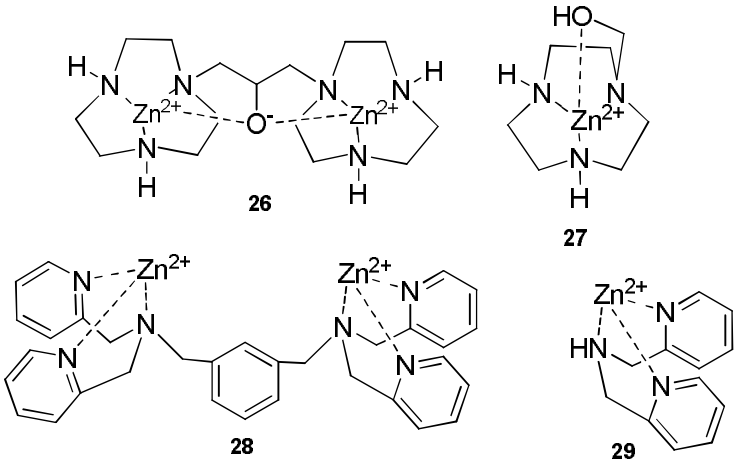
Figure 4. Dependence of the rate of the cleavage of HPNP on the [Cu^{2+}]/[**25**] ratio. ([**25**] = 0.4 mM, pH = 7.5). [129]

It has been proposed that the mechanism of the catalysis involves an activation of the attacking nucleophile by a metal-bound hydroxide ion in addition to the double Lewis-acid activation (Scheme 20).[129] A metal bound water molecule may act as a general acid by protonating the leaving group.

Scheme 20. The proposed mechanism for the transesterification of UpU by a dicopper complex of ligand **25**.[129]

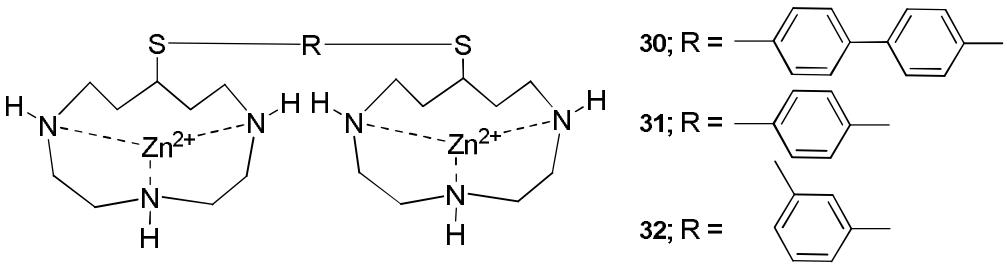


There are many examples where bimetallic systems are clearly more efficient catalysts for the cleavage of a phosphodiester bond than their monometallic counterparts.[126,130-136] For example, dinuclear metal ion complex **26** cleaves HPNP 120 and nearly 200 times faster than related mononuclear analogs **13** and **27**, respectively.[131,132] In addition, dinuclear metal ion complex **28** cleaves dinucleoside monophosphate substrates approximately 10-fold faster than mononuclear complex **29**.[136]

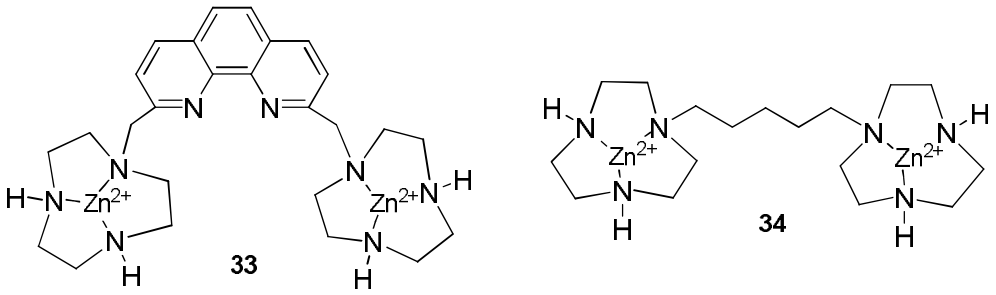


1.3.3.2. Structural factors influencing the activity

The geometry and the length of the spacer, which determines the distance between two metal ions, contributes to the activity of bimetallic complexes.[130,132,137] Geometric requirements have been explored in studies with complexes **30**-**32**.[130] The cleavage of UpU is catalyzed two times more efficiently by complex **30** than by **31** with a shorter linker. Furthermore, complex **32** with a 1,3-disubstituted linker catalyzes the cleavage of UpU less effectively than complex **31** with a 1,4-disubstituted linker.

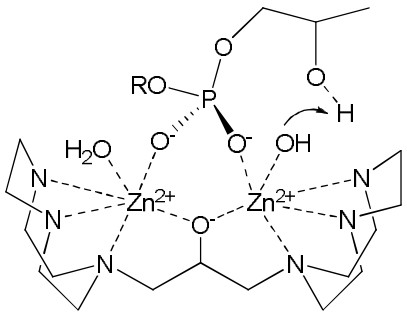


An alkoxy function in the spacer can provide an enhanced catalytic activity.[116,128,129,132,139] An alkoxy function has been suggested to diminish the repulsive electrostatic interactions between two metal ions allowing them to orientate closer to each other. A dinuclear metal ion complex **26**, for example, reduces the half-life of UpU to 500 hours (pH 7 at 25 °C).[139] Complexes **33** and **34** are clearly less active most probably due to the fact that the structures of the ligands do not enable an appropriate cooperation of the metal ions.[132]

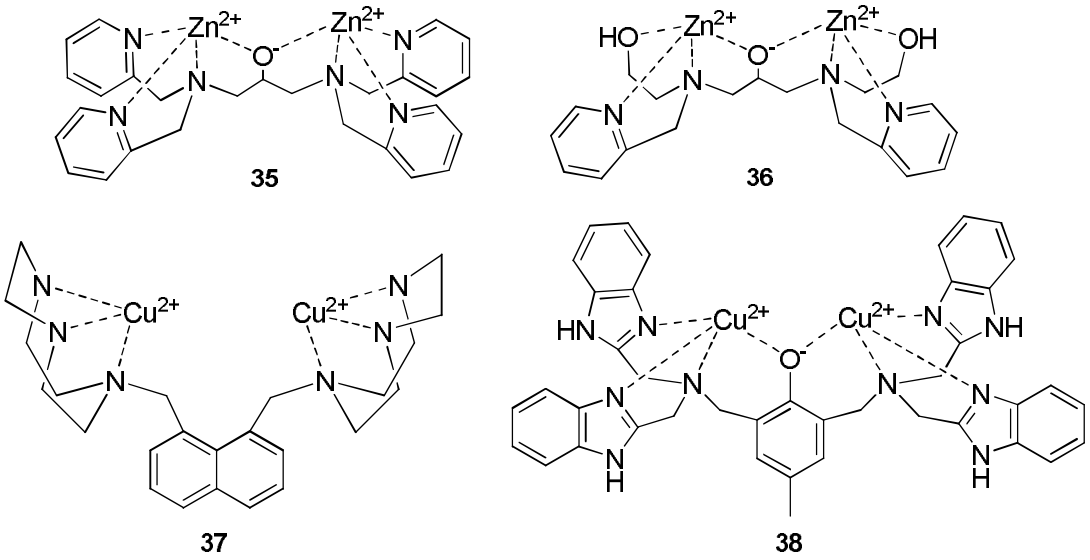


As mentioned, the proposed mechanism is usually a double Lewis-acid activation,[126] where both phosphoryl oxygens of a phosphate diester are coordinated to a dinuclear metal ion complex as shown in Scheme 21.[140] One metal ion can also participate in activating the attacking nucleophile, while the other stabilizes the leaving group. The lack of a solvent deuterium isotope effect has been proposed to indicate that most or all of the rate acceleration is due to electrostatic stabilization of the dianionic transition state by metal dications.[140]

Scheme 21. Electrostatic stabilization of the transition state in the cleavage of HPNP by dinuclear metal ion complex **26**.[132]

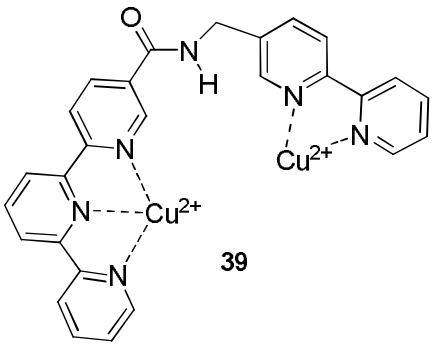


Dinuclear Zn^{2+} complex **35** has been shown to hydrolyze ApA with a half-life of approximately 4 hours (pH 6.9 at 50 °C) indicating a good cooperation of the two metal ions.[141,142] If the two pyridylmethyl groups of complex **35** are replaced with hydroxyethyl groups, producing complex **36**, the catalytic activity increases 4-fold.[135] The intracomplex alcohol moieties are proposed to act as acid catalysts rather than as nucleophiles. In addition to bimetallic zinc complexes, dicopper complexes have also proven to markedly accelerate the cleavage of phosphodiester bonds. For example complexes **37** and **38** provide a rate acceleration of 5-6 orders of magnitude for the transesterification of HPNP and ApA.[1,127,143]



1.3.3.3. Base selectivity

Even though an efficient catalysis has been observed in the previous examples, no marked base selectivity has been detected. Still, there are reports on complexes with a notable selectivity. A good example of a relatively simple complex where the highly effective phosphodiester cleavage is combined with a considerable base selectivity is dinuclear Cu^{2+} complex **39**.[133] It provides over a 10^5-fold rate acceleration for the hydrolysis of ApA compared to the background rate. In addition, the hydrolysis of ApA is 12, 17 and 87 times faster than that of CpC, of UpU and of GpG, respectively. A strong stacking interaction between adenine and the ligand rather than a hydrogen bonding or a metal ion coordination to nucleobases is suggested to contribute to the base selectivity.[133] Trinuclear complex **40** hydrolyzes dinucleoside monophosphates more effectively than the dinuclear complex does. The catalysis by complex **40** shows also a considerable dependence on the substrate; relative rate constants for the cleavage of CpA, ApA, GpA, UpA and GpC are 1, 0.69, 0.4, 0.21 and 0.11, respectively.[142] Additionally, the formation of 3´-monophosphate products is preferred over 2´-monophosphate products (> 90 % in most cases) which make the complex very unique.



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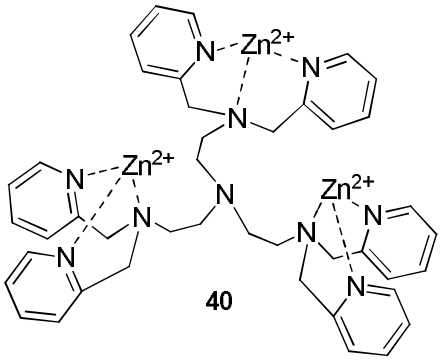

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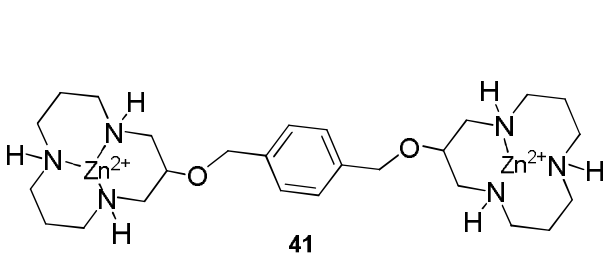
A marked base selectivity in the cleavage of dinucleoside monophosphates has been observed also in studies with zinc complexes of di- and tri-nucleating azacrown ligands, **41** and **42**.[144,145] The dinuclear complex cleaves ApU and UpA up to 100 times faster than UpU or ApA.[144] One of the azacrown moieties is proposed to anchor the uracil base by forming a mixed-ligand complex by displacing the N3 proton and second azacrown complex acts as a catalyst.



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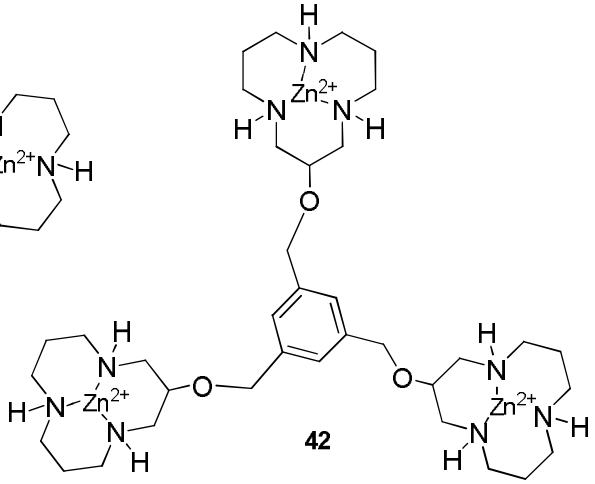
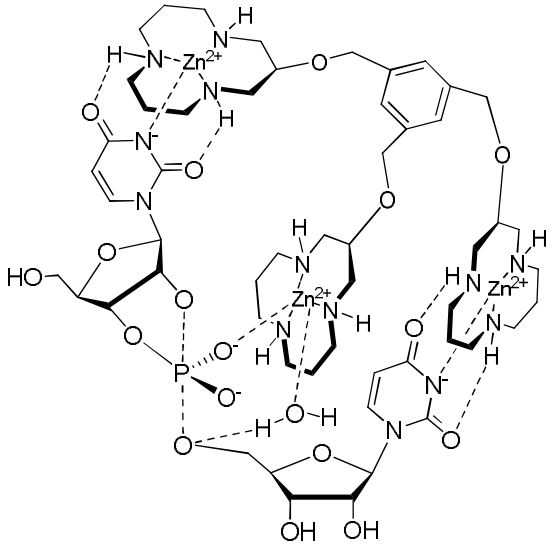


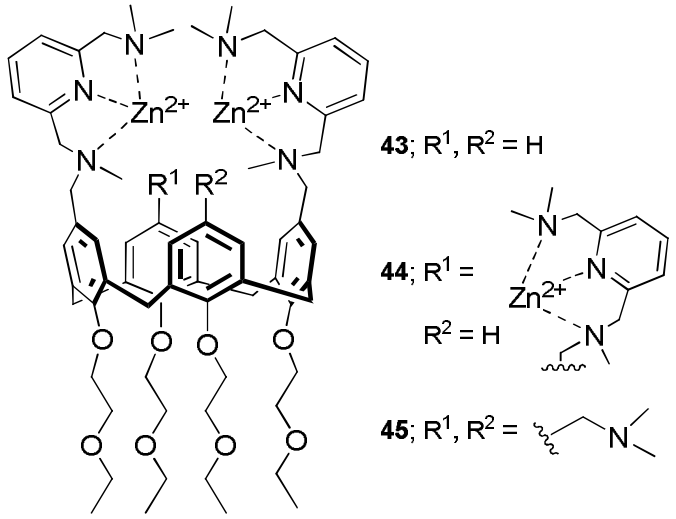
42

In the case of UpU, both azacrown moieties are bound to nucleobases which prevents the catalysis. In contrast, in the case of ApA, the catalytic inactivity is possibly due to too weak interactions between the substrate and the cleaving agent. Guanine base may also anchor the azacrown moieties but the interaction is weaker.[145] By introducing a third azacrown group to the cleaving agent, the cleavage of UpU is achieved. Accordingly, two azacrown moieties are proposed to anchor the base moieties, while the third catalyzes the cleavage (Scheme 22).

Scheme 22. Proposed transition state interactions in the cleavage of UpU by trinuclear Zn^{2+} complex **42**.[144]



A calix[4]arene derivatives with two or three 2,6-bis(aminomethyl)pyridine units (**43** and **44**, respectively) cleave HPNP 23000 and 32000 times faster compared to the background reaction, respectively (in 50 % CH_3CN/H_2O, pH 7.0 and at 25 °C).[146,147] A dinuclear complex is also 50 times more reactive than its mononuclear analog which suggests an efficient cooperation of the two metal ion centers. Binding and catalytic activity of complex **45** containing two dimethylamino groups are lower, possibly due to steric interactions, but one of the dimethylamino groups may act as a general base deprotonating the substrate.[148] Interestingly, only trinuclear complex **44** has been observed to have any considerable catalytic activity in cleaving RNA dinucleoside monophosphate substrates.[149,150] Kinetic experiments with a series of dinucleoside monophosphates have showed also significant nucleobase specificity for **44**.[149]

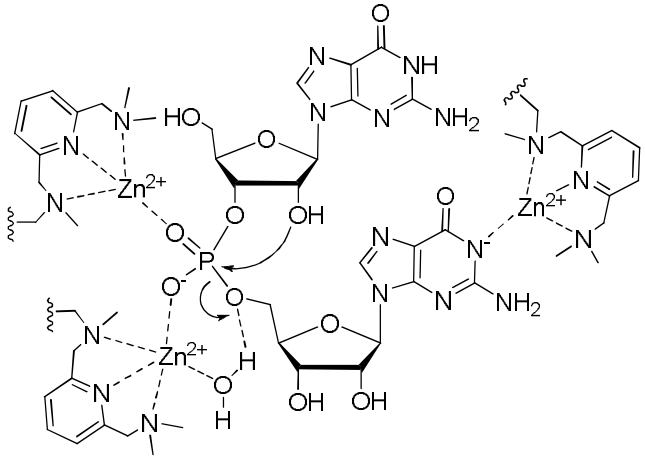


Observed pseudo-first order rate constants for the cleavage of GpG, UpU, CpC and ApA by **43** and **44** are shown in Table 2.[149] Complex **44** catalyzes the cleavage of GpG 8.5 times faster than that of UpU and 160 times faster than the cleavage of ApA. It has been suggested that the complex prefers to bind GpG and UpU rather than ApA because guanine and uracil base moieties have an acidic NH group which can be deprotonated by the complex resulting in a formation of a stable nucleobase-zinc complex and, hence, may orientate the substrate in an appropriate position prior to the cleavage. The proposed mechanism is represented in Scheme 23.[149]

Table 2. Pseudo-order rate constants for the cleavage of RNA dinucleoside monophosphates (in 35 % EtOH/H_2O, pH 8.0 at 50 °C).[149]

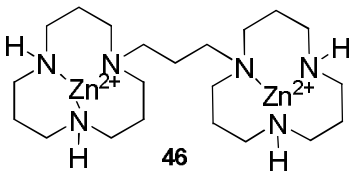
Substrate	**43** k_{obs} / 10^{-5} s^{-1}	**44** k_{obs} / 10^{-5} s^{-1}
GpG	0.45	72
UpU	0.45	8.5
CpC	0.58	6.1
ApA	0.28	0.44

Scheme 23. The proposed interactions in the cleavage of GpG by **44**.[149]



1.3.3.4. Medium effects

It has recently been reported that a medium effect can have an enormous influence in phosphoryl transfer reactions.[151-154] The study of a methanolytic cleavage of HPNP with dinuclear Zn^{2+} complex **46**, for example, revealed 10^{12} –fold rate acceleration relative to the background reaction.[151] Evidently the cooperative role of metal ions is more pronounced in less polarized solvents. The bridging oxyanion in the linker is not essential for the catalytic activity.[153] For example, complex **47** is 160 times less reactive than complex **28** in cleaving HPNP in methanol. One should bear in mind that very likely there are differences in the mechanisms of reactions in water and in organic solvents.[154] Conclusions about the catalytic activity in water should not be drawn on the basis of results obtained in methanol. They can, however, provide important detailed information of the reaction mechanisms of divalent metal ion systems.



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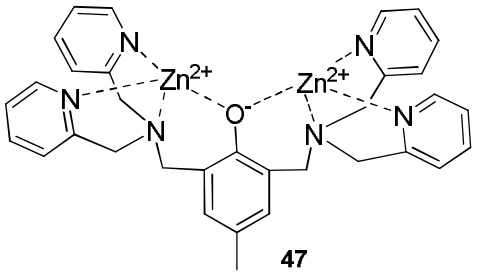

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2. AIMS OF THE THESIS

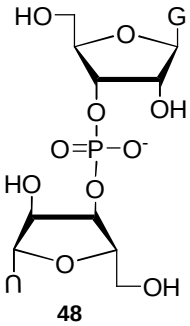
This thesis consists of three different case studies where the goal is to demonstrate the presence of weak interactions that are involved in stabilizing the phosphorane intermediate/transition state in the cleavage of RNA phosphodiester bond. To achieve this goal, kinetic experiments followed by mechanistic inspections have been performed. The subjects include the synthesis and cleavage of a small model system mimicking the mechanism of catalysis by large ribozymes, the reactivity of modified and unmodified RNA oligonucleotides as well as the catalysis by a dinuclear metal ion complex acting as an artificial ribonuclease. In each case, the aim of the study is described in more detail in the beginning of the chapter.

3. RESULTS AND DISCUSSION

3.1. Hydrogen bonding interactions of a 2´-amino function in a 3´,3´-linked ribonucleoside

This study is aimed at elucidating the reaction mechanism of large ribozymes. As mentioned in the introduction, the cleavage mechanism of large ribozymes proceeds by an attack of an external nucleophile on the phosphorus atom which gives a pentacoordinated transition state that is decomposed by a departure of the 3´-linked nucleoside. No cleavage of the 5´-linked nucleoside has ever been observed.[66] The 2´-hydroxy function has been suggested to stabilize the departing 3´-oxyanion as a hydrogen-bond donor.[73-76,78] Hydrogen bonding of the 2´-OH to the departing O3´ and/or to a nonbridging phosphorane oxygen atom may result in an enhanced cleavage rate. The rate-acceleration effect has been previously studied by comparing the cleavage rates of guanylyl-(3´,3´)-uridine (3´,3´-GpU, **48**) and guanylyl-(3´,3´)-(2´,5´-di-*O*-methyluridine) (3´,3´-Gp^{Me}U, **49**). [155] The hydroxide-ion catalyzed cleavage of 3´,3´-GpU (**48**) takes place 46 times as fast as 3´,3´-Gp^{Me}U (**49**).[155] Since there are two scissile bonds in **48** and only one in **49**, the overall rate acceleration is only 23-fold. A larger influence may be expected if the hydrogen-bond donor, the 2´-OH, simultaneously serves as a hydrogen-bond acceptor, which increases its acidity. The 2´-ammonium ion substituent may be used to mimic such a hydroxy group exhibiting a markedly enhanced acidity. Thus, comprehensive kinetic studies with a model system, guanylyl-(3´,3´)-(2´-amino-2´-deoxyuridine) (**50**), have been done and the interpretation of the results focus on the possible stabilization of the transition state via an intramolecular hydrogen bonding by the 2´-amino group.

The results clearly indicate that the 2´-amino group in a 3´,3´-linked ribonucleoside markedly accelerates the cleavage of the phosphodiester bond by accepting a hydrogen bond from the phosphorane hydroxyl ligand and/or by donating a hydrogen bond to the phosphorane oxyanion ligand. When the results obtained in the present study are compared with results obtained previously[155] with 3´,3´-GpU (**48**) and its dimethyl derivative (3´,3´-Gp^{Me}U, **49**), it can be seen that the ability to form hydrogen bonds apparently has an influence on the reactivity. This observation implies that such a hydrogen bond interaction may also be involved in the reactions of large ribozymes.



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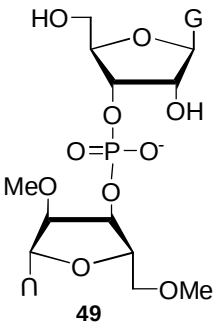

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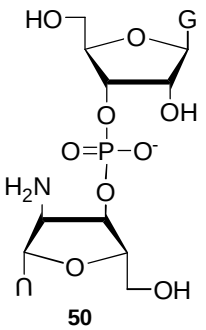


50

3.1.1. Product distribution and pH-rate profile of guanylyl-(3′,3′)-(2′-amino-2′-deoxyuridine)

Hydrolytic reactions of guanylyl-(3′,3′)-(2′-amino-2′-deoxyuridine) (**50**) were followed in the pH range 0-13 at 90 °C. Under acidic conditions (pH < 3) the starting material is partially isomerized to guanylyl-(2′,3′)-(2′-amino-2′-deoxyuridine) and both isomers are cleaved initially to guanosine 2′,3′-cyclic monophosphate and 2′-amino-2′-deoxyuridine. 2′,3′-cGMP is rapidly hydrolyzed to a mixture of guanosine 2′- and 3′-phosphates. Depurination at pH ≤ 2 yields guanine and non-chromophoric products.

The pH-rate profile was obtained by a non-linear fit of k_{obs} vs. $-\log[H^+]$ as described in detail in ref. I. The cleavage is first-order in acidity at pH < 2 and first-order in hydroxide ion concentration at pH > 8. Between these two pH values, two pH-independent plateaus are observed: slow pH-independent cleavage at pH 3-4 and 7-fold faster cleavage at pH 6-8. The isomerization and depurination are both first-order in hydronium ion concentration at pH < 3. Under less acidic conditions, the rate of the isomerization starts to level off to a zero-order dependence of rate on hydronium ion concentration. The pH-rate profiles for the partial reactions are depicted in Figure 5.

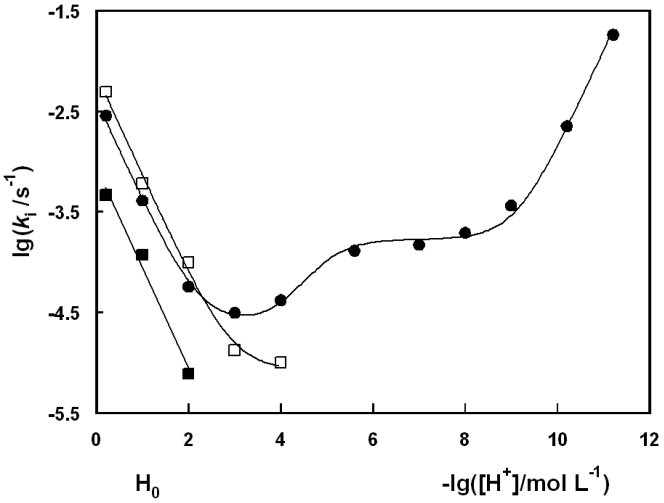


Figure 5. pH-rate profile for the cleavage (k_{cl}; ●), isomerization (k_{is}; □), and depurination (k_{dp}; ■) of guanylyl-(3′,3′)-(2′-amino-2′-deoxyuridine) at 90 °C (I = 0.1 M with NaCl).[I]

3.1.2. Mechanistic inspection and comparison of the results with 3′,3′-GpU and 3′,3′-Gp^{Me}U

To illustrate the effects that the replacement of the 2′-hydroxy function of the uridine moiety in 3′,3′-GpU with an amino group has on the cleavage rate, the pH-rate profiles obtained with unmodified 3′,3′-GpU (**48**)[155] and its dimethyl derivative 3′,3′-Gp^{Me}U (**49**) [155] are all also included in Figure 6.

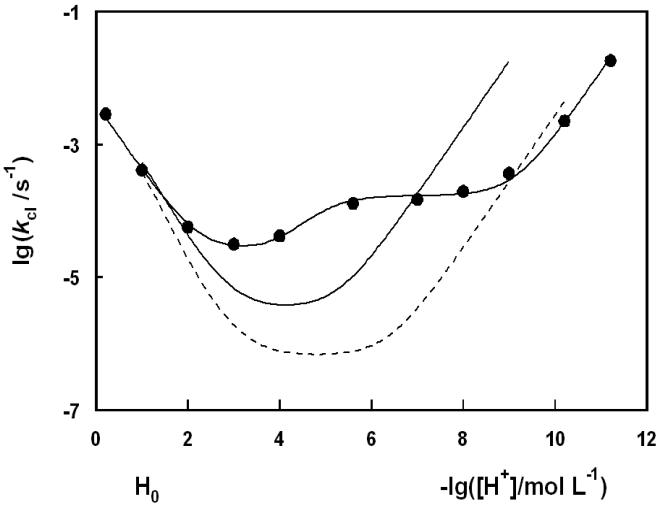
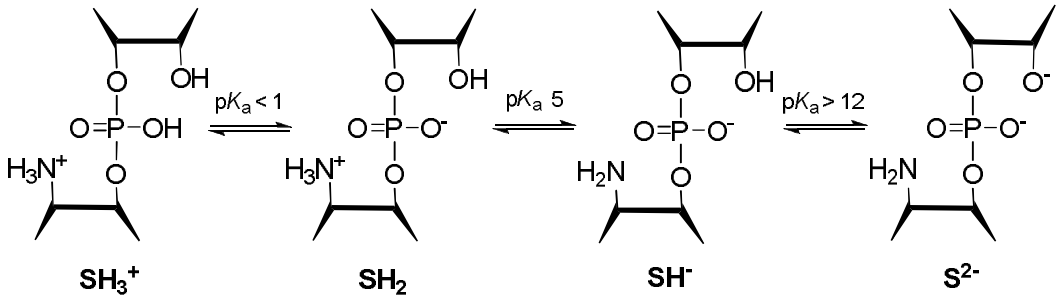


Figure 6. pH-rate profile for the cleavage (k_{cl}) of 3′,3′-Gp^{NH2}U (**50**) at 90 °C (I = 0.1 M with NaCl).[I] The solid and dotted lines show the corresponding curves for 3′,3′-GpU (**48**) and 3′,3′-Gp^{Me}U (**49**), respectively.[155]

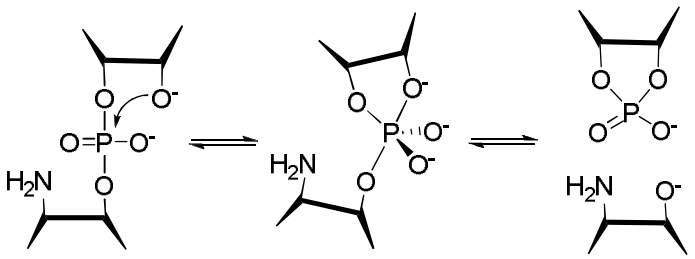
The shape of the pH-rate profile in Figure 5, together with the known pK_a value of the 2′-ammonium group of 2′-amino-2′-deoxyuridine, which may be expected to be around 5 at 90 °C, indicates that the cleavage of 3′,3′-Gp^{NH2}U (**50**) proceeds *via* four different ionic forms shown in Scheme 24.

Scheme 24



pH > 8. The cleavage proceeds by the attack of guanosine 2′-oxyanion on the phosphorous atom followed by an "in-line" departure of 3′-oxyanion of 2′-amino-2′-deoxyuridine from the dianionic phosphorane intermediate (Scheme 25). The relative cleavage rates of the 2′-amino-, 2′-methoxy-, and 2′-hydroxy-substituted compounds are 1, 2.2, and 75, respectively. Under these conditions, the 2′-amino group does not result in a rate-acceleration similar to that of the 2′-hydroxy group. Compound **50** is clearly less reactive than **48**. Evidently the amino group is too weakly acidic to stabilize either the phosphorane intermediate or the departing 3′-oxyanion by hydrogen bonding.

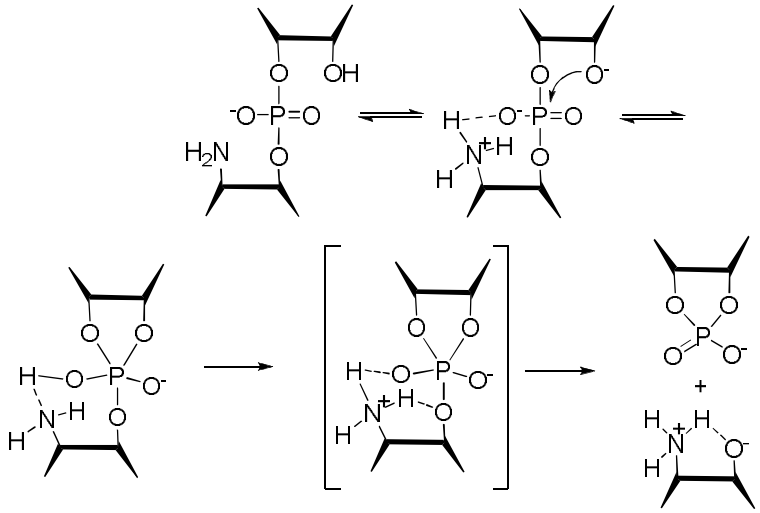
Scheme 25



pH 4-6 and 6-8. The hydroxide ion-catalyzed reaction in the pH range 4-6 results from passing the pK_a value (~ 5) of the 2´-ammonium ion, that is, when the 2´-NH_3^+-group is being deprotonated. At pH 6-8, the pH-independent reaction refers to uncatalyzed cleavage of the monoanion SH^-. The predominant ionic form of **50** at pH > 5 is the monoanion SH^-. The reactive species, however, is not the major tautomer indicated in Scheme 24, but most likely a minor tautomer having the amino group protonated and the 2´-hydroxy group of the guanine moiety deprotonated (Scheme 26). Firstly, 2´-oxyanion is a much better nucleophile than the 2´-hydroxy group[2,156-158] and secondly, the 2´-ammonium ion may be expected to stabilize the phosphorane intermediate and to facilitate inductively the cleavage of the P-O3´-bond.

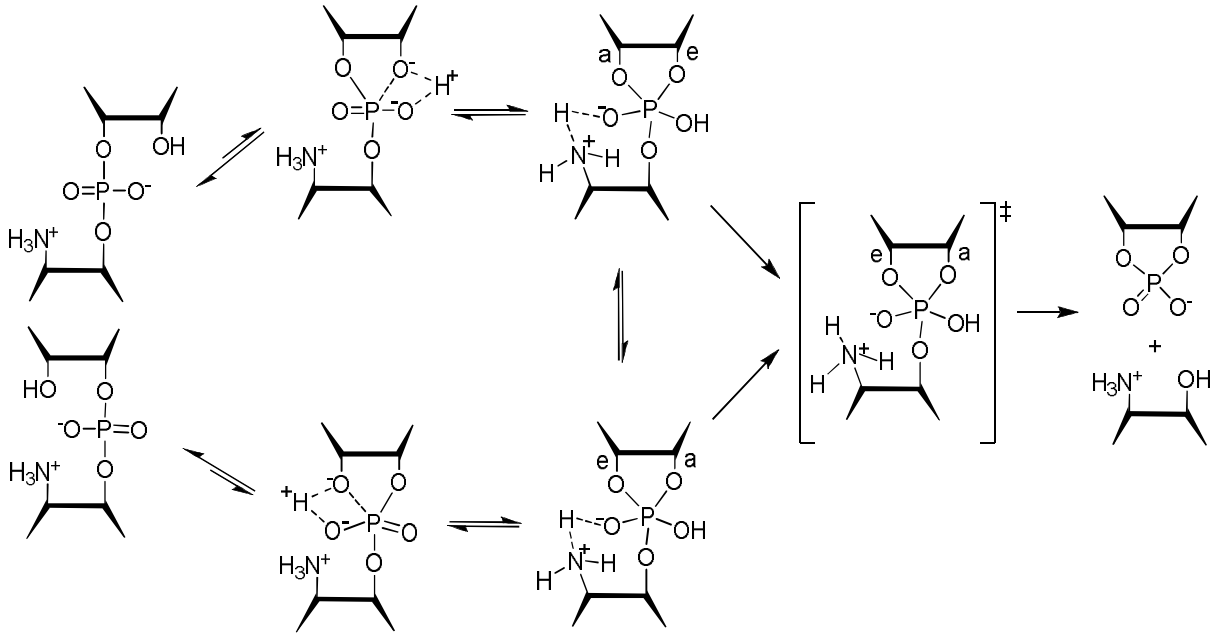
The protonated amino group markedly accelerates the cleavage reaction. For example, hydroxide ion catalyzed cleavage of 3´,3´-$Gp^{NH2}U$ (**50**) is more than 10^3 times faster than 3´,3´-$Gp^{Me}U$ (**49**). This rate acceleration may consist of three different contributions: (i) stabilization of the phosphorane intermediate electrostatically and/or by hydrogen bonding, (ii) stabilization of the leaving group by intramolecular hydrogen bonding of the ammonium group to the departing 3´-oxygen atom, and (iii) inductive reduction of electron density of the departing 3´-oxygen atom. It appears reasonable to assume that the 2´-ammonium group is initially weakly hydrogen bonded to the phosphoryl oxyanion, because of the low basicity of the phosphodiester group. However, as the 2´-oxyanion approaches the phosphorus atom, the basicity of the nonbridging oxygen atoms is increased, the hydrogen bonding of the ammonium group is strengthened, and when the phosphorane structure is completed, the proton has actually been transferred from the nitrogen atom to the oxygen atom. In principle, the amino group could also facilitate the breakdown of the phosphorane intermediate. When the leaving group starts to depart, the basicity of the nonbridging oxygen atom is decreased. Consequently, the hydrogen-bonded proton is transferred from the phosphorane oxygen atom to the amino nitrogen atom, and concomitant with strengthening of the ammonium ion character, hydrogen bonding to the departing 3′-oxygen atoms starts to play a role.

Scheme 26



pH 3-4. In this narrow pH-range, the reaction occurs by spontaneous cleavage of the neutral zwitterion of **50** (Scheme 27). A 16-fold and 42-fold rate acceleration for the cleavage reaction of **50**, as compared to the cleavage of compounds **48** and **49**, respectively, is observed. Under these conditions, the amino group is protonated and the resulting ammonium group may only serve as a hydrogen bond donor. The cleavage is about 7 times slower than at pH 6-8 when the amino group is not protonated. Under these conditions, the leaving group is at least partially protonated and the rate is, hence, not sensitive to the stability of the leaving group. The fact that zwitterionic 3´,3´-Gp^{NH2}U (**50**) is isomerized about 5 times more rapidly than 3´,3´-Gp^{Me}U (**49**) provides support for the suggestion of stabilization of the phosphorane intermediate by electrostatic or hydrogen bonding interactions between the phosphorane monoanion and the 2´-ammonium ion.

Scheme 27



pH < 2. The phosphorane intermediate is obtained most likely by an attack of the 2´-hydroxy group on neutral phosphate.[2] Under these conditions, the nonbridging oxygen is protonated and the leaving group departs as an alcohol. The rates of the reactions of 3´,3´-Gp^{NH2}U (**50**), 3´,3´-GpU (**48**), and 3´,3´-Gp^{Me}U (**49**) are comparable. Thus, stabilization of phosphorane intermediate or leaving group by intramolecular hydrogen bonding does not play a role. However, the pH-rate profile of 3´,3´-Gp^{NH2}U (**50**) differs in the sense that the cleavage and isomerization of 3´,3´-Gp^{NH2}U (**50**) exhibit a first-order dependence on acidity, while the reactions of 3´,3´-GpU (**48**) and 3´,3´-Gp^{Me}U (**49**) are second-order in the hydronium ion concentration. Evidently the presence of the positively charged 2´-ammonium group markedly retards the protonation of the phosphodiester linkage to a monocationic form, and the reaction via a monoprotonated (neutral) phosphodiester prevails over a wider pH range than in the case of 3´,3´-GpU (**48**) and 3´,3´-Gp^{Me}U (**49**).

3.2. Exocyclic amino group of the 5´-linked nucleoside acting as a hydrogen bond donor

This part intends to study further the source of the reactivity differences between different oligonucleotide sequences and explore the effect of possible intramolecular hydrogen bonding interaction on the reactivity of phosphodiester bonds. Nucleic acid bases have been shown to form hydrogen bonds to the scissile phosphodiester bond in ribozyme reactions and this interaction has been suggested to contribute to the efficiency of the reaction.[159] In addition, previous studies have shown that a base composition of even a short oligonucleotide can affect significantly the reactivity of phosphodiester bonds within the sequence.[86] Phosphodiester bonds between pyrimidine and adenosine have frequently been suggested to be particularly reactive.[87-89,100] Thus, the cleavage of heterosequence oligonucleotides with a UpA scissile bond has been studied and the reactivity has been compared to that of an oligo-uridine sequence and of an oligonucleotide containing an N^6,N^6-dimethyladenosine as the leaving group nucleoside.

The results show that under neutral conditions, oligonucleotides with an adenosine moiety as the 5´-linked nucleoside can be more than 200-fold more reactive than the reference oligonucleotide, a 13-mer oligo-uridine. Experiments with the modified oligonucleotide with N^6,N^6-dimethyladenosine as the 5´-linked nucleoside moiety suggest that the exocyclic amino group is involved in the reaction, interacting with the neighboring phosphodiester bond. The UpA bond in N^6,N^6-methylated oligonucleotide is up to 20 times less reactive in comparison to a UpA bond next to an unmodified adenosine. These results can, therefore, be regarded as evidence of an adenine base acting, most probably, as a hydrogen bond donor in interactions that stabilize the transition state of the cleavage of RNA, thereby enhancing the reaction.

3.2.1. The reactivity of unmodified oligonucleotides

In previous studies, substantial reactivity differences has been observed between oligonucleotides which contain different number of purine nucleotides and have, hence, different rigidities.[86] The reactivity of a phosphodiester bond was observed to depend not only on the neighboring nucleosides but, surprisingly, also on nucleosides further along the sequence.[86] Thus, few presumably interesting oligonucleotides were chosen for further study.

The sequences of the oligonucleotides studied are shown in Chart 1. Oligonucleotide **53** has been reported to react slower than UpU while **52** and **54** reacted significantly faster.[86] The oligonucleotides contain only one ribonucleoside, and consequently, only one scissile phosphodiester bond. The rest of the nucleoside moieties are 2´-*O*-methylated, which prevents the cleavage of the adjacent phosphodiester bond.

Chart 1. Sequences of oligonucleotides studied. Bold letters refer to the single ribonucleoside in 2´-*O*-methylated oligonucleotides and the hyphen shows the position of the scissile phosphodiester bond. A^{me} refers to N^6,N^6-dimethyladenosine (2´-*O*-methylated).

51 5´-UUUUU**U**-UUUUUUU-3´
52 5´-GGGUA**U**-AUGUGC-3´
53 5´-GUGUA**U**-AAGUGC-3´
54 5´-GGGUA**U**-AAGUGC-3´
55 5´-GGGUA**U**-A^{me}UGUGC-3´
56 5´-GUGUA**U**-A^{me}AGUGC-3´

The formation of two products was observed in most cases (Figure 7). Independent of the substrate oligonucleotide, one product migrated very close to the starting material, whereas the migration time (t_m) of the other product was clearly longer. Spiking with an authentic sample showed that the product with a longer t_m is the oligomeric product with a free 5´-OH group.

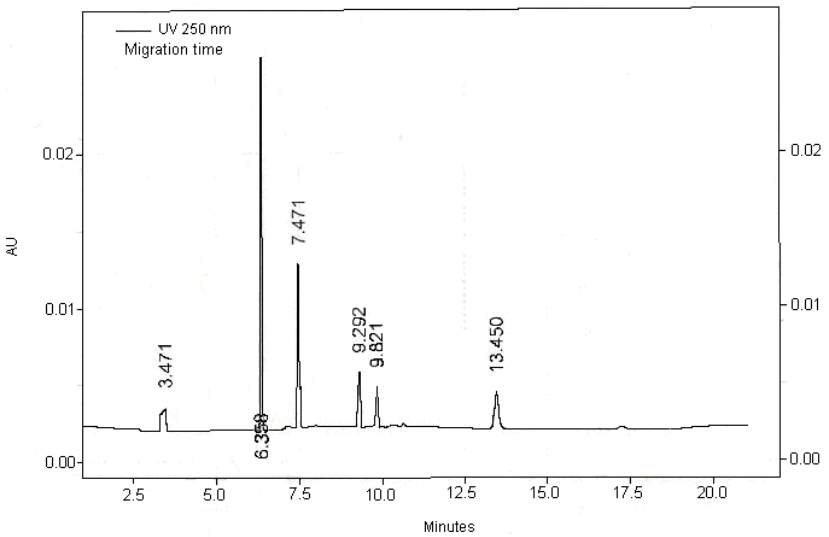


Figure 7. An example of CZE electropherogram of the cleavage of oligonucleotide **52** at pH 6.5.[II] The sample is taken at t = 140 hours. Background electrolyte: citrate buffer

(0.2 M, pH 3.1). Other conditions: fused silica capillary (50 μm i.d., 57 cm total length, 50 cm effective length], -30 kV applied voltage, 25 °C capillary temperature, direct detection at 254 nm. Migration times / min: potassium 4-nitrobenzenesulfonate, 6.350; oligo-uridine **51**, 7.471; oligonucleotide **52**, 9.821; product with free 5´-OH group, 13.450; product with a terminal monophosphate group, 9.292.

The rate constants of the cleavage of oligonucleotides **51-54** collected in Table 3 show that under acidic and basic conditions the reactivity of heterosequences **52-54** is similar to that of oligo-uridine **51**. Under neutral conditions the situation changes. Despite the careful control of reaction conditions, large variations in the rate constants were observed, and in Table 3, a rate constant range is given.

Table 3. Rate constants for the cleavage of the scissile phosphodiester bond of oligonucleotides **51**-**54** at 35 °C.[II] The observed rates varied in the given range and the number of experiments is indicated in parenthesis.

Substrate	k /10^{-6} s^{-1} pH 5.5	k /10^{-6} s^{-1} pH 6.5	k /10^{-6} s^{-1} pH 7.5	k /10^{-6} s^{-1} 10 mM NaOH	k /10^{-6} s^{-1} 10 mM HCl
51	< 0.01[a]	< 0.01[a]	< 0.01[a]	1.3 ± 0.1	1.3 ± 0.1
52	0.51-1.8 (3)[b]	0.11-4.5 (6)[b]	0.40-0.71 (2)[b]	1.4 ± 0.1	0.7 ± 0.1
53	0.7-2.8 (2)[b]	0.02-2.7 (3)[b]	0.02-0.04 (2)[b]	2.7 ± 0.1	1.1 ± 0.1
54	0.15-1.9 (4)[b]	1.2-1.4 (2)[b]	0.04-1.3 (2)[b]	1.2 ± 0.1	0.8 ± 0.1

[a] No reaction was observed in six months.
[b] Oligo-uridine was added in the same reaction medium as a standard.

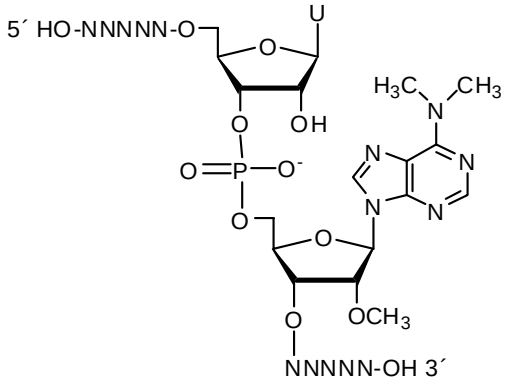
The reason for the peculiar behaviour is still not known. A complex equilibrium system involving intramolecular and intermolecular interactions within and between oligonucleotides is one possible explanation why the rate constants vary. However, even though the heterosequences were rapidly cleaved, oligo-uridine (**51**) in the same reaction medium remained intact; the heterosequences were always more reactive than oligo-uridine. Under neutral conditions, a UpA phosphodiester bond in the middle of a short oligonucleotide can be more than 200 times more reactive than a UpU bond in an oligo-uridine sequence. The difference is consistent with suggestions[159] that adenine base can be involved in the cleavage of a phosphodiester bond by forming hydrogen bonds to the phosphorane transition state.

3.2.2. The effect of methylation of the exocyclic amino group of the leaving group adenosine

The possibility of intramolecular hydrogen bonding was further studied with oligonucleotides where the exocyclic amino group of the leaving group adenosine was dimethylated (Scheme 28). The reactivity of methylated oligonucleotide **55** and

corresponding unmodified oligonucleotide **52** at pH 6.5 is compared in Figure 8. The methylated oligonucleotide is clearly less reactive than the unmodified oligonucleotide in the same reaction solution. Methylation renders the phosphodiester bond up to 20 – times less reactive in comparison to a UpA bond next to an unmodified adenosine.

Scheme 28



In the case of unmodified oligonucleotides **52** and **53**, out of altogether thirteen rate constants determined in the pH range 5.5 – 6.5, ten are above 5×10^{-7} s^{-1}. The five rate constants determined for the cleavage of the N^6,N^6-dimethylated oligonucleotides **55** and **56** at pH 5.5 to 6.5 vary in the range $(0.02 – 1.4) \times 10^{-7}$ s^{-1}. It is, therefore, clear that even though the rate constants are not quite reproducible, the methylation of the exocyclic amino group of the neighboring adenine reduces the reactivity of the phosphodiester bond. It can be concluded that the adenine base from 5´-linked nucleoside is involved in the reaction, acting probably as a hydrogen bond donor in interactions that stabilize the transition state of the cleavage of RNA.

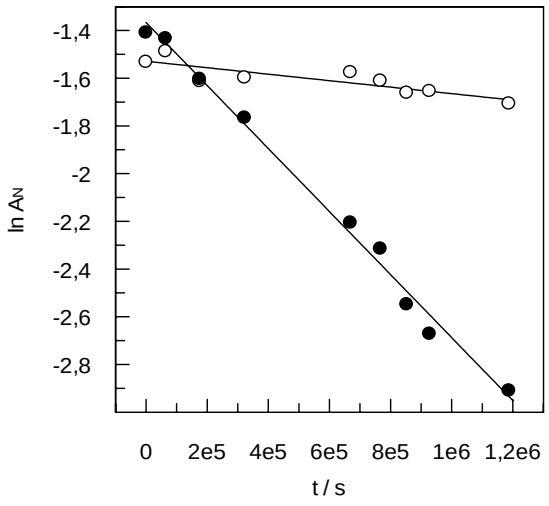


Figure 8. A comparison of the reactivity of N^6,N^6-dimethylated oligonucleotide **55** (○) and the corresponding non-methylated oligonucleotide **52** (●) at pH 6.5 and 35 °C.[II] The rate constants obtained from the plot are $(1.3 \pm 0.1) \times 10^{-6}$ s^{-1} and $(1.4 \pm 0.3) \times 10^{-7}$ s^{-1} for **52** and **55**, respectively.

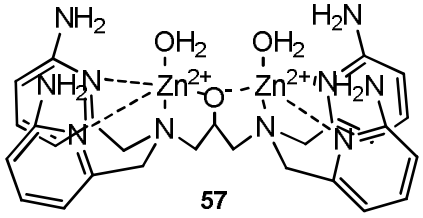
3.3. Aminopyridyl-based ligand provides efficient stabilization to pentacoordinated phosphorane intermediate

The third part of the thesis focuses on a dinuclear metal ion complex that catalyzes the cleavage of phosphodiester bonds. Preliminary kinetic studies have shown that the complex shows remarkable rate enhancement in the cleavage of both the activated substrate HPNP and UpU.[160] In order to find out the mechanistic details, a variety of substrates including different dinucleoside monophosphates and uridine 3´-alkylphosphates were exploited and, hence, a β_{lg} value for the reaction was obtained. In addition, reactions were studied in corresponding D_2O solutions and kinetic solvent isotope effect values were determined. All measurements were performed at pH 6.5. Undoubtedly a pH-rate profile would have provided more information, especially about the mechanism, but the complex was not stable at higher pH.

The rate enhancement on both the cleavage and isomerization of RNA phosphodiester bond by the complex is significant. The efficiency most probably comes from the cooperation of double Lewis acid activation by two Zn^{2+}-ions and the hydrogen-bonding environment provided by the ligand. Strong interactions enable the complex to act as an electrophilic catalyst; it neutralizes the charge on the phosphate and enhances the nucleophilic attack of the 2´-oxyanion on the phosphate. Tightly bound complex also enables the phosphorane intermediate to be sufficiently long-lived to pseudorotate and, consequently, the isomerization is observed. The results obtained in the present study provide the first examples of metal ion catalyzed phosphate diester isomerization close to neutral pH.

3.3.1. Structure of the dinuclear metal ion complex

The dinuclear metal ion complex **57** represent a system designed to effectively bind and catalyze the cleavage of phosphate esters. In addition to binding two metal ions, the ligand also introduces four aminopyridyl hydrogen bond donors. In X-ray studies it has been observed that when the phosphate binds to the complex, the phosphoryl oxygen atoms are positioned at a hydrogen-bonding distance of the amino groups.[160] Therefore the amino groups may assist the binding and stabilize the transition state.



57

3.3.2. Catalytic cleavage and isomerization of dinucleoside monophosphates

When UpU is incubated with 1 mM **57** at pH 6.5 at 25 °C, it is efficiently cleaved with a half-life of about 7 hours. This corresponds to a rate acceleration of ~10^6-fold,[161]

making this complex one of the most efficient Zn^{2+} based catalysts for promoting the cleavage of RNA phosphodiester bond under mild aqueous conditions, reported to date.[160,162] The extra stabilization provided by the four hydrogen bond donating amino groups is relevant. An analogous complex containing two pyridyl groups and two aminopyridyl groups was observed to cleave UpU about 40 times slower. Previous studies have shown, that in the presence of complex **35**, UpU is cleaved with half-life of approximately 25 hours (pH 7.0 and 50 °C).[136] The concentration of **57** does not influence the rate of the cleavage. Evidently the complex binds the monoanionic phosphate very strongly and saturation is achieved at the millimolar concentrations used.

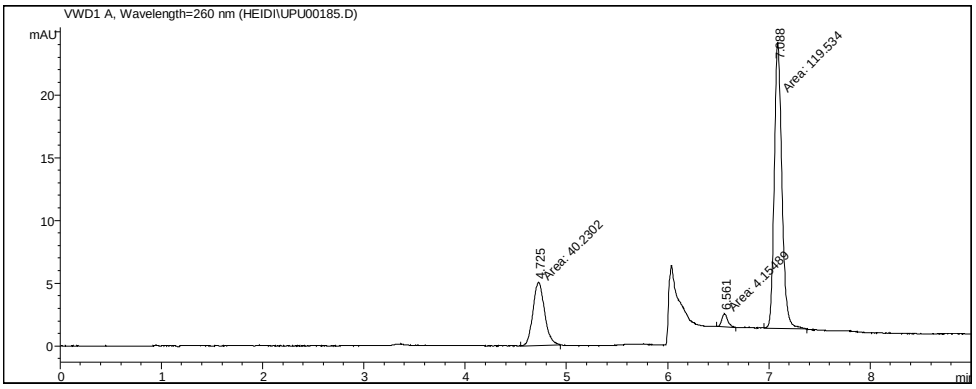
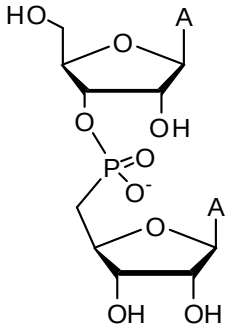


Figure 9. The reaction of UpU with 2 mM complex **57** at 25 °C and pH 6.5.[III] The sample is taken at t = 4 hours. The peaks are: 4.7 minutes, uridine; 6.6 minutes, 2´,5´-UpU; 7.1 minutes, 3´,5´-UpU. The peak at ~6 minutes is due to the step-wise elution method.

The reaction proceeds *via* the usual intramolecular transesterification reaction where 2´-oxygen attacks the phosphate and the initial cleavage products are 2´,3´-cUMP and uridine. Very unexpectedly, 2´,5´-isomer of UpU appeared in the chromatograms (Figure 9) which implies that the complex catalyzes also the isomerization of phosphodiester bonds. The signal area of the 2´,5´-isomer remained low throughout the whole reaction, so rate constants for the isomerization could not be calculated accurately. Nucleoside phosphonate **58** was used therefore as a substrate to verify the observation of isomerization. This substrate cannot be cleaved by an intramolecular transesterification, but previous studies have shown that it isomerizes in the same way as dinucleoside monophosphates do, that is, in pH-independent and acid-catalyzed reactions but not under alkaline conditions.[95,163,164]

Rate constants for the cleavage of dinucleoside monophosphates and isomerization of phosphonate **58** in the presence of complex **57** are collected in Table 4. Several late transition metal ions were screened for activity. The Co^{2+} and Zn^{2+} complexes were the most efficient catalysts in promoting the cleavage of UpU, with a 4-fold advantage in favour of Co^{2+}.



58

Table 4. Rate constants for the cleavage of dinucleoside monophosphates and isomerization of nucleoside phosphonate **58** in the presence of 1 mM complex **57** at pH 6.5 and 25 °C.[III,IV]

Substrate	k_{obs}/ 10^{-5} s^{-1}
3´,5´-UpU	2.6 ± 0.3
2´,5´-UpU	0.67 ± 0.01
3´,5´-ApA	4.2 ± 0.2
2´,5´-ApA	0.28 ± 0.01
3´,5´-UpA	1.64 ± 0.07
3´,5´-ApU	1.25 ± 0.03
3´,5´-GpG	3.64 ± 0.08
58	0.125 ± 0.002[a] 0.087 ± 0.003[b]

[a] Interconversion of 3´-isomer to 2´-isomer.
[b] Interconversion of 2´-isomer to 3´-isomer.

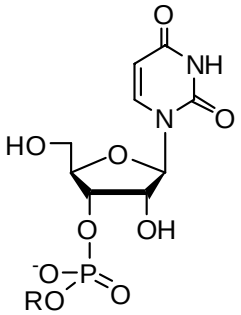
The rate enhancement for isomerization is modest in comparison to that observed for the cleavage reaction. The complex promoted isomerization still is about 500-fold faster than the uncatalyzed reaction and represents the first substantial metal ion catalytic acceleration of this process. It seems that even though the formation of the phosphorane intermediate is enhanced, it does not result in an efficient isomerization because the reaction is somehow inhibited. The most logical explanation is that pseudorotation is the rate limiting step of the isomerization. Considering that the rate-enhancement relies on the extensive interactions between the phosphate group and the complex, it can be suggested that pseudorotation is not favourable, because it would require severing those interactions. Consistent with this suggestion, the cleavage of 2´,5´-isomers UpU and ApA are slightly slower than that of their 3´,5´-isomers, which suggests that the interactions of the 2´,5´-isomer may be weaker (Table 4). In addition,

the rate enhancement shows only little dependence on the base composition as can be seen from the rate constants of the cleavage of various 3´,5´-dinucleoside monophosphates collected in Table 4.

Isomerization requires both the formation and the pseudorotation of the phosphorane intermediate. Even if the dianionic phosphorane was sufficiently stable to be a true intermediate, the pseudorotation is expected to be slow, since it would require that a negatively charged oxygen ligand adopts an apical position, which is energetically unfavourable.[11,165,166] The rate enhancement for the isomerization and the cleavage observed presumably results from the combination of the Lewis acid effect of the metal ions and hydrogen bond donors of the ligand stabilizing the anionic oxygens of the phosphorane.

3.3.3. Solvent isotope effects and β_{lg}

In order to elucidate the mechanism in more detail, kinetic studies were done with uridine 3´-alkylphosphates **59**-**64** and uridine 3´-phenylphosphate (**65**). Rate constants presented in Table 5 show that complex **57** promotes the cleavage of **59-64** and the rate depends strongly on the acidity of the leaving group. The isomerization of the alkyl phosphates is also enhanced. In most cases, the isomerization is slower than the cleavage, and the proportion of the 2´-isomer remains low. Isomerization is faster than the cleavage, if the esterified alcohol is a poor leaving group. The dependence of isomerization rate on the acidity of esterified alcohols is less significant than in the case of cleavage, and, consequently, the proportion of the isomerization decreases as the acidity of the leaving group increases. Kinetic solvent isotope effects of both the uncatalyzed and **57** -promoted reactions were observed to vary between 1 and 2.



59 R = $CH(CH_3)_2$
60 R = Me
61 R = $CH_2CH_2OCH_2CH_3$
62 R = CH_2CHF_2
63 R = CH_2CHCl_2
64 R = CH_2CCl_3

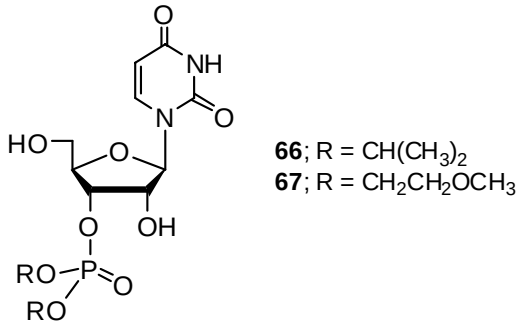
65 R = Ph

The catalysis on the cleavage of **65** by the complex is very efficient. **65** is cleaved too rapidly to determine rate constants by HPLC-method. The substrate **65** was completely cleaved in less than two minutes, which suggests that the rate constant of the cleavage is 10^{-2} s^{-1} or higher. The reactivity was confirmed using UV spectroscopy. The observed rate constant with 0.35 mM **57** is 0.023±0.005 s^{-1}.

Table 5. Rate constants of cleavage (k_{cl}) and isomerization (k_{is}) of the substrates **59**-**64** in the presence of 1 mM complex **57** at pH 6.5 and 25 °C.[IV]

Substrate	k_{cl} /10^{-6} s^{-1}	k_{is} /10^{-6} s^{-1}
59; R=$CH(CH_3)_2$	0.086 ± 0.006	0.28 ± 0.01
60; R=Me	5.8 ± 0.2	2.4 ± 0.1
61; R=$CH_2CH_2OCH_2CH_3$	4.4 ± 0.4	1.2 ± 0.2
62; R=CH_2CHF_2	450 ± 10	30 ± 10
63; R=CH_2CHCl_2	380 ± 10	40 ± 30
64; R=CH_2CCl_3	5030 ± 60	ND

If the rates of the reactions of **57** promoted cleavage of nucleoside 3´-alkyl phosphates and the previously reported reactivities of triesters **66** and **67** are compared, it is observed that the reactions resemble each other; The rate constants reported for the cleavage of uridine 3´-bis(isopropyl) phosphate **66** and uridine 3´-bis(methoxyethyl) phosphate **67** at 25 °C are 4.0 × 10^{-8} s^{-1} and 3.7 × 10^{-6} s^{-1}, respectively,[167] which are very close those values shown in Table 5 for complex **57** promoted cleavage of the isopropyl ester **59** and the ethoxyethylester **61**. The role of the coordinated metal ion complex can, hence, be considered to be more or less the same as that of the second esterified alcohol in triesters **66** and **67**. By neutralizing the charge, it makes the phosphate group more electrophilic and also enables the pseudorotation. The similarity of the reactions is also supported by the fact that the rate of the isomerization depends on the leaving group in both cases.



66; R = $CH(CH_3)_2$
67; R = $CH_2CH_2OCH_3$

β_{lg} value and kinetic deuterium solvent isotope effects provide further evidence of the triester-like mechanism. A previous report on the reaction of nucleoside 3´-dialkylphosphates show that at pH 6.5 and 25 °C, the predominant cleavage pathway is the spontaneous reaction.[167] This reaction has been proposed to involve a nucleophilic attack of the 2'-oxyanion on the phosphate, which results in a formation of a monoanionic intermediate. The departure of the leaving group is assisted by a

concerted proton transfer from the solvent to the leaving group oxygen. This reaction is characterized by β_{lg} value of -0.94. This value is rather negative, but a comparison to that determined under alkaline conditions suggests that a concerted protonation takes place as the leaving group departs.[167] β_{lg} value obtained in the present work is -0.92±0.07, which is well consistent with that obtained with nucleoside dialkyl phosphates. The kinetic solvent isotope effect values are also consistent with this mechanism; values of 1-2 can well be attributed to a reaction where the large value expected for the deprotonation of 2´-hydroxy group is compensated by a value resulting from the protonation of the leaving group.

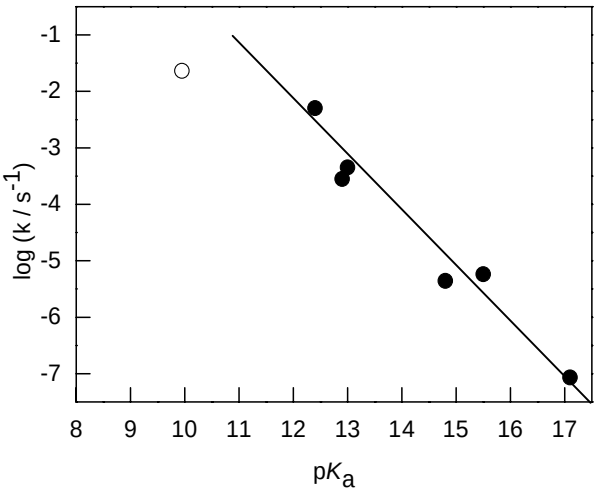


Figure 10. Logarithmic first-order rate constants for the cleavage of uridine 3´-alkylphosphates **59**-**64** plotted against the pK_a values of the leaving groups (●) and observed rate of cleavage of **65** (○).The data refer to 1 mM complex **57** at pH 6.5 and 25 °C. The line has a gradient of -0.92± 0.07.

The rate of reaction of **65** does not fall on the same line with the other data. This is consistent with previous report on the alkaline cleavage of uridine 3´-aryl and alkyl phosphates, where a clear break in linearity is observed at pK_a ~12.6.[115] As mentioned in Chapter 1.3.1., it is due to the difference in the rate-limiting steps. Furthermore, the break in linearity revealed that the reaction goes through a true intermediate.[115] Thus, the observation of the isomerisation reaction and the deviation of the cleavage rate of **65** from the other data (Figure 10) indicates that the cleavage proceeds most probably through a phosphorane intermediate stabilized by **57**. Significantly slower isomerisation suggests that the changes in the geometry weaken the interactions between **57** and the phosphorane intermediate.

4. CONCLUSIONS

The results presented here provide strong evidence of the presence of hydrogen bond interactions that stabilize the pentacoordinated phosphorane intermediate in different systems. Firstly, 2´-amino group in a 3´,3´-linked ribonucleoside markedly accelerates the cleavage of the phosphodiester bond. Guanylyl-(3´,3´)-(2´-amino-2´-deoxyuridine) is more than 2 orders of magnitude more labile than guanylyl-(3´,3´)-(2´,5´-di-*O*-methyluridine) under conditions where hydrogen bonding is to be expected. Secondly, studies with oligonucleotides where the exocyclic amino group of the leaving group adenosine was dimethylated suggest that the exocyclic amino group participates in the cleavage reaction by donating an intramolecular hydrogen bond. Finally, highly efficient phosphodiester cleavage is achieved with a dinuclear metal ion complex. Double Lewis acid activation through two Zn^{2+}-ions combined with the hydrogen-bonding environment provided by the ligand stabilize the phosphorane intermediate so markedly that the intermediate has enough lifetime to pseudorotate and, hence, also isomerization is observed.

5. EXPERIMENTAL

5.1. Materials and methods

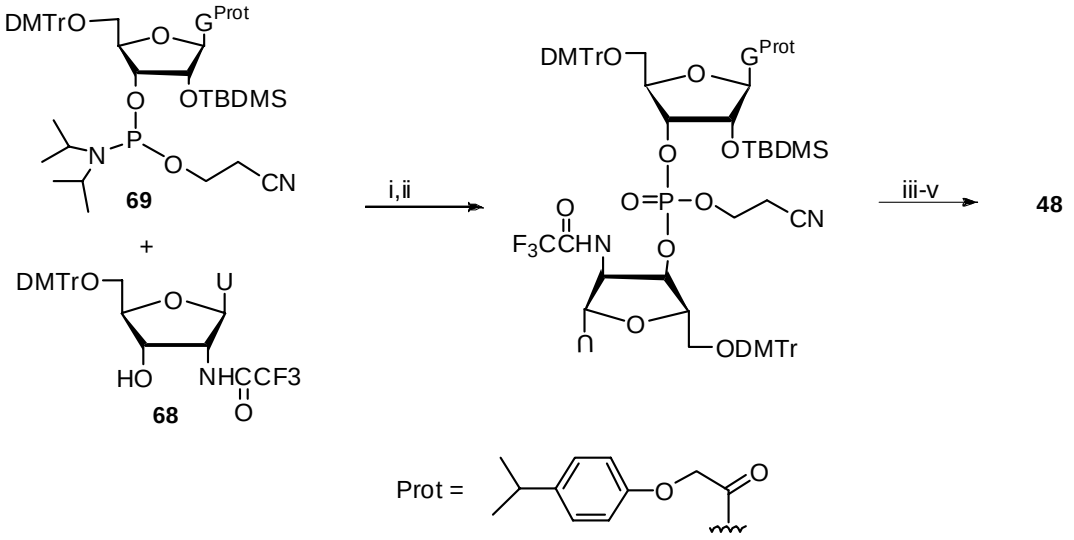
Materials. 3´,5´- and 2´,5´-dinucleoside monophosphates were commercial products of Sigma. 2´-Amino-2´-deoxyuridine was purchased from BIORON and was converted to the corresponding 5´-*O*-(4,4´-dimethoxytrityl)-2´-*N*-trifluoroacetyl-2´-amino-2´-deoxyuridine (**68**) as described previously.[168] 2´-*O*-methylated and 2´-*O*-TOM protected phosphoramidite building blocks were purchased from Glen Research. Ligand **57 (***N,N,N',N'*-[tetrakis-(6-amino-pyrid-2-ylmethyl)-1,3-diamino]-propan-2-ol) was synthesized as described previously.[160]

Methods. Oligonucleotides **51-56** were synthesized with ABI 3400 DNA/RNA Synthesizer. The NMR spectra were recorded on a Bruker AM 200, Bruker AV 500 or JEOL 400 spectrometer. The ^{1}H NMR chemical shifts refer to internal TMS, and the ^{31}P NMR shifts refer to external orthophosphoric acid. The mass spectra were acquired using a Perkin-Elmer Sciex API 365 triple quadrupole LC/MS/MS spectrometer.

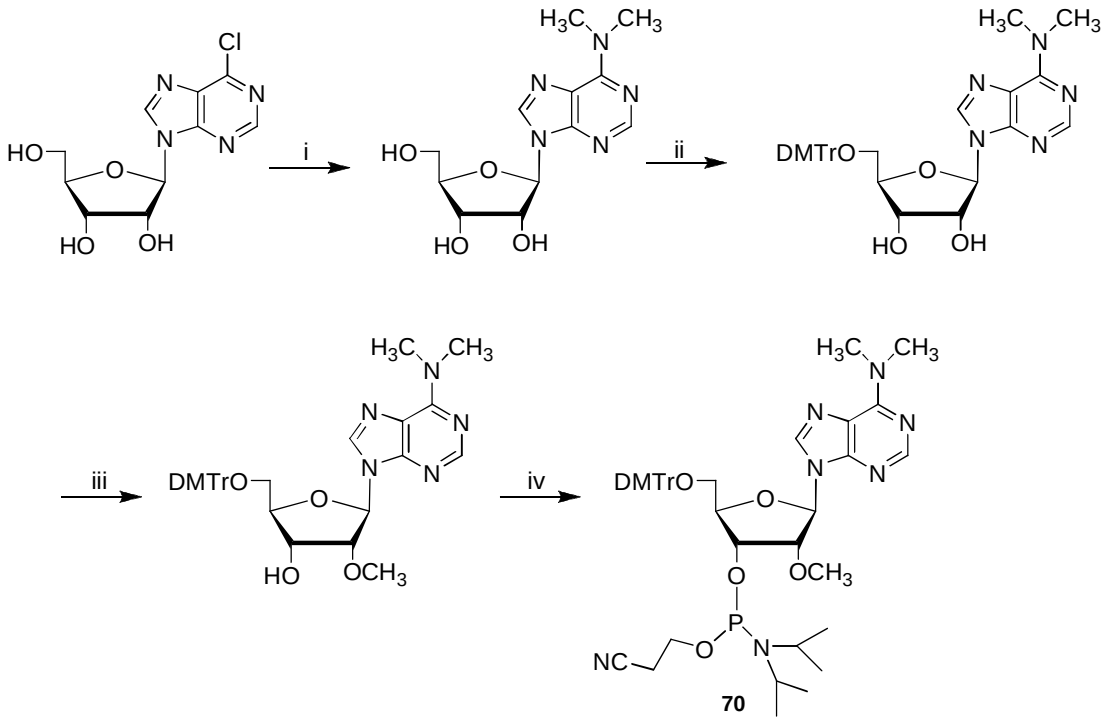
Synthesis of guanylyl-(3´,3´)-(2´-amino-2´-deoxyuridine) (48). Common phosphoramidite methodology was used to synthesize guanylyl-(3´,3´)-(2´-amino-2´-deoxyuridine) (**48**) as described previously.[155] Accordingly, commercially available guanosine phosphoramidite **69** and 5´-*O*-(4,4´-dimethoxytrityl)-2´-*N*-trifluoroacetyl-2´-amino-2´-deoxyuridine (**68**) were dissolved in a solution of tetrazole in dry acetonitrile (Scheme 29). The formed phosphate ester was oxidized with I_2. Dimethoxytrityl group was removed with 80 % aqueous acetic acid solution. Saturated methanolic ammonia was used to remove the 2´-*N*-trifluoroacetyl protecting group and protections in phosphate and base moieties. After removal of the silyl protecting group with tetrabutylammonium fluoride, the crude product was purified with RP HPLC and finally passed through a Na^{+}-form Dowex 50-W (100-200) mesh cation exchange column.

Synthesis of ribo/2´-*O*-methylribo oligonucleotides. Oligonucleotides **51-56** were synthesized from commercial 2´-*O*-methylated and 2´-*O*-TOM protected phosphoramidite building blocks by conventional phosphoramidite strategy, according to the standard RNA coupling protocol. Synthesis of N^6,N^6-dimethyladenosine building block **70** is depicted in Scheme 30. Saturated aqueous ammonia released the oligonucleotide products from solid support and removed the base-labile protecting groups. 2´-*O*-TOM group was removed by treating with triethylaminetrihydrofluoride in DMSO. Oligonucleotides were desalted by using cartridges and the crude oligonucleotide was then isolated by ion exchange HPLC and finally desalted by RP HPLC.

Scheme 29. Preparation of the 3´,3´-linked ribonucleoside **48**. i) tetrazole, MeCN; ii) I_2, H_2O/THF/2,6-lutidine (2:4:1); iii) 80 % AcOH, MeOH/H_2O (1:1); iv) NH_3/MeOH; v) TBAF/THF.[I]



Scheme 30. The synthesis of N^6,N^6-dimethyladenosine building block **70.** i) $HN(CH_3)_2 \cdot HCl$, Et_3N, DMF, RT, 1 h, ii) DMTrCl, Et_3N, Py (dry), RT, 3,5 h, iii) CH_2N_2/DME, $SnCl_2 \cdot 2H_2O$, DMSO, RT, 1 h, iv) $iPr_2NP(Cl)OCH_2CH_2CN$, DMAP, CH_2Cl_2, Et_3N.[II]



5.2. Kinetic measurements

The reactions were carried out in sealed glass tubes or Eppendorf tubes immersed in a thermostated water bath. Metal ion complexes were prepared by mixing appropriate amounts of the ligand, metal ion and buffer acid and then adjusting the pH by adding an appropriate amount of base. Aliquots were withdrawn at appropriate intervals to cover 1-3 half-lives. Aliquots were cooled down on an ice bath and either analyzed immediately or stored in a freezer until analysis. Analysis was carried out with RP-HPLC (using a Waters Atlantis^{TM} dC18 4.6 × 150 mm column or Thermo ODS Hypersil 250 × 4 mm column), or, in the case of oligonucleotides, *Beckman-Coulter P/ACE-MDQ* system equipped with a sample-cooling unit.

5.3. Calculation of rate constants

Guanylyl-(3´,3´)-(2´-amino-2´-deoxyuridine). At pH > 4, the pseudo first-order rate constants for the disappearance of **50** were obtained by applying the integrated first-order rate equation (Eq. 1) to the time-dependent diminution of the concentration of the starting material. $[\mathbf{50}]_0$ is the initial concentration of the substrate.

Eq. 1. $$\ln[\mathbf{50}]_t = -kt + \ln[\mathbf{50}]_0$$

At pH 0-2, the first order rate constants (k_{dp}) for the depurination of an isomeric mixture of **50** were calculated by applying Eq. 2, by bisecting the rate constant (k_{dec}) for the decomposition of the isomeric mixture of the starting material to the rate constants of parallel first-order reactions on the basis of the product distribution at the early stages of the reaction. Equation 3 was then applied to obtain the rate constants (k_{cl}) for the cleavage of isomeric Gp^{NH2}Us.

Eq. 2. $$k_{dp} = \frac{[\text{Guanine}]_t}{[\mathbf{50}]_0 - [\mathbf{50} + 2´,3´\text{-Gp}^{NH2}\text{U}]_t} k_{dec}$$

Eq. 3. $$k_{cl} = k_{dec} - k_{dp}$$

Oligonucleotides. The signals from CZE chromatograms were first divided by the corresponding migration time to yield areas directly proportional to the concentration. The signal areas for the starting material and products were then normalized by diving with the signal area for the internal standard (potassium 4-nitrobenzenesulfonate). Rate constants for the cleavage of the starting material were calculated on the basis of the decrease of the signal area as the function of reaction time using the integrated rate-law of first-order reactions.

Isomerization. First-order rate constants for isomerization (k_{is}) were calculated by the UFIT 1.0 program[95] using the concentrations of the isomers as a function of reaction time.

6. REFERENCES

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