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Targeting RNA by the Antisense Approach and a Close Look at RNA Cleavage Reaction

JHARNA BARMAN

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Abstract
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This thesis summarizes the results of studies on two aspects of nucleic acids. Chemically modified antisense oligonucleotides (AONs) have been evaluated with regards to their suitability for mRNA targeting in an antisense approach (**Paper I – III**). The chemically modified nucleotidic units 2'-*O*-Me-T, 2'-*O*-MOE-T, oxetane-T, LNA-T, azetidine-T, aza-ENA-T, carbocyclic-ENA-T and carbocyclic-LNA-T were incorporated into 15-mer AONs and targeted against a 15-mer RNA chosen from the coding region of SV-40 large T antigen. The comparative study showed that a single modified nucleotide in the AON with *North-East* locked sugar (oxetane-T and azetidine-T) lowered the affinity for the complementary RNA whereas *North* locked sugars (LNA-T, aza-ENA-T, carbocyclic-ENA-T, and carbocyclic-LNA-T) significantly improved the affinity. A comparative RNase H digestion study showed that modifications of the same type (*North-East* type or *North* type) in different sequences gave rise to similar cleavage patterns. Determination of the Michaelis-Menten parameters by kinetic experiments showed that the modified AONs recruit RNase H resulting in enhanced turnover numbers (k_{cat}) although with weaker enzyme-substrate binding ($1/K_m$) compared to the unmodified AON. The modified AONs were also evaluated with regards to resistance towards snake venom phosphodiesterase and human serum to estimate their stability toward exonucleases. The aza-ENA-T and carbocyclic-ENA-T modified AONs showed improved stability compared to all other modified AONs. In general, the modified AONs with *North* type nucleotides (except LNA-T) were found to be superior to the *North-East* type as they showed improved target affinity, comparable RNase H recruitment capability and improved exonuclease stability.

The second aspect studied in this thesis is based on physicochemical studies of short RNA molecules utilizing NMR based pH titration and alkaline hydrolysis reactions (**Paper IV – V**). The NMR based (^{1}H and ^{31}P) pH titration studies revealed the effect of guaninyl ion formation, propagated electrostatically through a single stranded chain in a sequence dependent manner. The non-identical electronic character of the internucleotidic phosphodiesters was further verified by alkaline hydrolysis experiments. The internucleotidic phosphodiesters, which were influenced by guaninyl ion formation, were hydrolyzed at a faster rate than those sequences where such guaninyl ion formation was prevented by replacing G with $N^{1\text{-Me}}$-G.

Keywords: mRNA targeting, antisense oligonucleotides, target affinity, RNase H, Michaelis-Menten kinetics, exo-nuclease stability, NMR, pH titration, alkaline hydrolysis

Jharna Barman, Department of Biochemistry and Organic Chemistry, Avdelningen för organisk kemi

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To my parents

List of Original Publications

This thesis is comprised of the following original publications, which will be referred in the text by their Roman numerals.

I. Honcharenko, D.; Barman, J.; Varghese, O. P.; Chattopadhyaya, J. Comparison of the RNase H cleavage kinetics and blood serum stability of the north conformationally constrained and 2'-alkoxy modified oligonucleotides
Biochemistry **2007**, *46*, 5635 – 5646

II. Varghese, O. P.; Barman, J.; Pathmasiri, W.; Plashkevych, O.; Honcharenko, D.; Chattopadhyaya, J. Conformationally constrained 2'-N,4'-C-ethylene-bridged thymidine (aza-ENA-T): synthesis, structure, physical, and biochemical studies of aza-ENA-T modified oligonucleotides
J. Am. Chem. Soc. **2006**, *128*, 15173 – 15187

III. Srivastava, P.; Barman, J.; Pathmasiri, W.; Plashkevych, O.; Wenska, M.; Chattopadhyaya J. The five- and six-membered conformationally-locked 2',4'-carbocyclic ribo-thymidine: synthesis, structure and biochemical studies
J. Am. Chem. Soc. **2007**, *129*, 8362 – 8379

IV. Acharya, S.; Barman, J.; Cheruku, P.; Chatterjee, S.; Acharya, P.; Isaksson, J.; Chattopadhyaya, J. Significant pKa perturbation of nucleobases is an intrinsic property of the sequence context in DNA and RNA
J. Am. Chem. Soc. **2004**, *126*, 8674 – 8681

V. Barman, J.; Acharya, S.; Zhou, C.; Chatterjee, S.; Engstroem, A.; Chattopadhyaya, J. Non-identical electronic characters of the internucleotidic phosphates in RNA modulate the chemical reactivity of the phosphodiester bonds
Org. Biomol. Chem. **2006**, *4*, 928 – 941

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List of publications, not included in this thesis

VI. Honcharenko, D.; Varghese, O. P.; Plashkevych, O.; Barman, J.; Chattopadhyaya, J. Synthesis and structure of novel conformationally constrained 1',2'-azetidine-fused bicyclic pyrimidine nucleosides: their incorporation into oligo-DNAs and thermal stability of the hetero-duplexes
J. Org. Chem. **2006**, *71*, 299 – 314

VII. Bogucka, M.; Naus, P.; Pathmasiri, W.; Barman, J.; Chattopadhyaya, J. Facile preparation of the oxetane-nucleosides
Org. Biomol. Chem. **2005**, *3*, 4362 – 4372

VIII. Isaksson, J.; Plashkevych, O.; Pradeepkumar, P. I.; Chatterjee, S.; Barman, J.; Pathmasiri, W.; Shrivastava, P.; Petit, C.; Chattopadhyaya, J. Oxetane locked thymidine in the Dickerson-Drew dodecamer causes local base pairing distortions - an NMR structure and hydration study
J. Biomol. Struct. Dyn. **2005**, *23*, 299 – 330

IX. Isaksson, J.; Acharya, S.; Barman, J.; Cheruku, P.; Chattopadhyaya, J. Single-stranded adenine-rich DNA and RNA retain structural characteristics of their respective double-stranded conformations and show directional differences in stacking pattern
Biochemistry **2004**, *43*, 15996 – 16010

X. Opalinska, J. B.; Kalota, A.; Gifford, L. K.; Lu, P.; Jen, Kuang-Yu; Pradeepkumar, P. I.; Barman, J.; Kim, T. K.; Swider, C. R.; Chattopadhyaya, J.; Gewirtz, A. M. Oxetane modified, conformationally constrained, antisense oligodeoxyribonucleotides function efficiently as gene silencing molecules
Nucl. Acids Res. **2004**, *32*, 5791 – 5799

Contribution Report

Paper I Prepared the 2'-*O*-alkoxy modified nucleotides and the corresponding modified oligonucleotides. Characterized all the small molecules and oligonucleotides by MALDI-TOF mass spectrometry. Actively contributed to the UV based melting studies. Also planned and standardized the kinetic experiments. Actively contributed to the calculations, analysis of the results, and manuscript writing.

Paper II – III Characterized all small molecules and oligonucleotides by MALDI-TOF mass spectrometry. Actively contributed to the UV and CD experiments. Planned and performed all enzymatic studies in Paper II and contributed significantly for the same in Paper III.

Paper IV – V Prepared all the short nucleotide sequences. Actively partici pated in characterization and analysis of the results. Planned and performed all the alkaline hydrolysis experiments, which also includes isolation (HPLC), characterization (MALDI-TOF), and quantification of the products in Paper V. Also contributed significantly in manuscript writing of Paper V.

Contents

Abbreviations

A	Adenin-9-yl or adenosine
Ac	Acetyl
ANA	Arabino nucleic acid
AON	Antisense oligonucleotide
Aza-ENA	2'-*N*,4'-*C*-Ethylene bridged nucleoside
Azetidine	1',2'-Aminoethylene bridged nucleoside
B	Any nucleobase
Bn	Benzyl
BNA	Bridged nucleic acid
C	Cytosin-1-yl or cytidine
CD	Circular dichroism
CeNA	Cyclohexene nucleic acid
CMV	Cytomegalovirus
DNA	Deoxyribonucleic acid
ds	Double stranded
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
ENA	2'-*O*,4'-*C*-Ethylene bridged nucleic acid
FANA	2'-Deoxy-2'-fluoro-β-D-arabino nucleic acid
G	Guanin-9-yl or guanosine
HDV	Hepatitis delta virus
HNA	Hexitol nucleic acid
HOMO-DNA	Hexapyranosyl nucleic acid
K	Kelvin
k_{cat}	Turnover number
K_m	Michaelis constant
LNA	Locked nucleic acid
MALDI-TOF	Matrix-assisted laser desorption/ionization-time of flight
MOE	2'-*O*-Methoxyethyl
MF	Morpholino
mRNA	Messenger RNA
MS	Modification site
NMR	Nuclear magnetic resonance
ODN	Oligodeoxynucleotide
ON	Oligonucleotide
Oxetane	(1',3'-*O*-Anhydro-β-D-psicofuranosyl) nucleosides

PAGE	Polyacrylamide gel electrophoresis
PNA	Peptide nucleic acid
PO	Phosphodiester
PS	Phosphorothioate
RNA	Ribonucleic acid
RNase H	Ribonuclease H
siRNA	Small interfering ribonucleic acids
ss	Single strand
SVPDE	Snake venom phosphodiesterase
T	Thymin-1-yl or thymidine
TFO	Triplex forming oligonucleotide
T_m	Melting temperature
U	Uracil-1-yl or uridine
UV	Ultraviolet
V_{max}	Maximum velocity
VS	Varkud satellite

1 Introduction

1.1 General introduction to nucleic acids

Nucleic acids are essential biopolymers in the transmission, expression, and conservation of the genetic information. Deoxyribonucleic acid (DNA) acts mainly as the carrier of genetic information whereas ribonucleic acid (RNA) transports the genetic information from the DNA to the ribosome where another essential biopolymer, the protein, is manufactured by means of translation. In this process RNA has many functions. It brings the genetic information in the form of messenger (mRNA) to the ribosome which is also largely made up of RNA (called rRNA). It delivers the amino acids according to the genetic code triplet on the mRNA in the form of tRNA. This flow of genetic information constitutes the central dogma of molecular biology.[1]

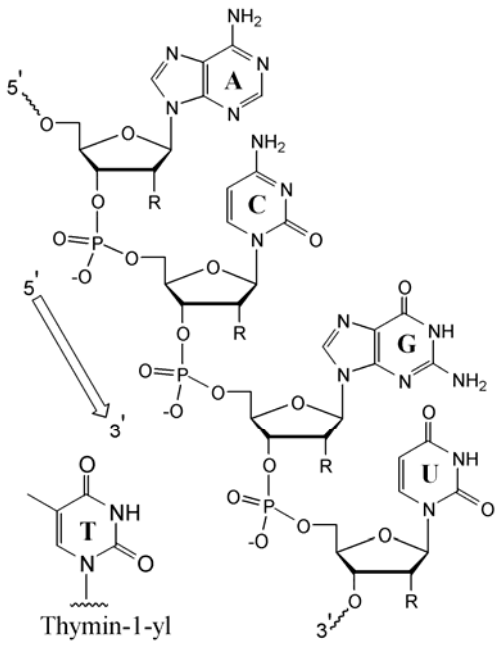

Figure 1. Schematic representation of the primary structure of DNA and RNA. R = OH for RNA, R = H and U is replaced by T for DNA

RNA also acts as a carrier of genetic information in RNA viruses and retroviruses. Apart from this, certain RNA molecules are known to act as enzymes either individually or in combination with proteins. This diverse activity of the RNA molecule led to the 'RNA World'[2,3] hypothesis which considers RNA as the only molecule, in a primordial age, that can serve not only as a carrier of genetic information from generation to generation, but also can carry out complex catalytic activities needed for their own replication and other complex reactions.[4,5,6]

1.1.1 Structure of nucleic acids

The primary structure of nucleic acids contains three parts, a pentofuranose sugar, phosphate and nucleobases. A 2'-deoxy-β-D-ribose (in DNA) or β-D-ribose (in RNA) sugar ring attached to a heterocyclic aromatic nucleobase through an N-glycosidic bond [*Figure 1*] is refered to as a nucleoside. The nucleoside units are connected through phosphodiester linkages from the 3'-carbon atom of one sugar unit to the 5'- carbon of the next sugar. The nucleobases found in DNA are the pyrimidines cytosine (C) and thymine (T) and the purines adenine (A) and guanine (G) whereas in RNA thymine is replaced by uracil (U) [*Figure 1*]. The sequence of nucleobases designates the primary structure of the nucleic acids.

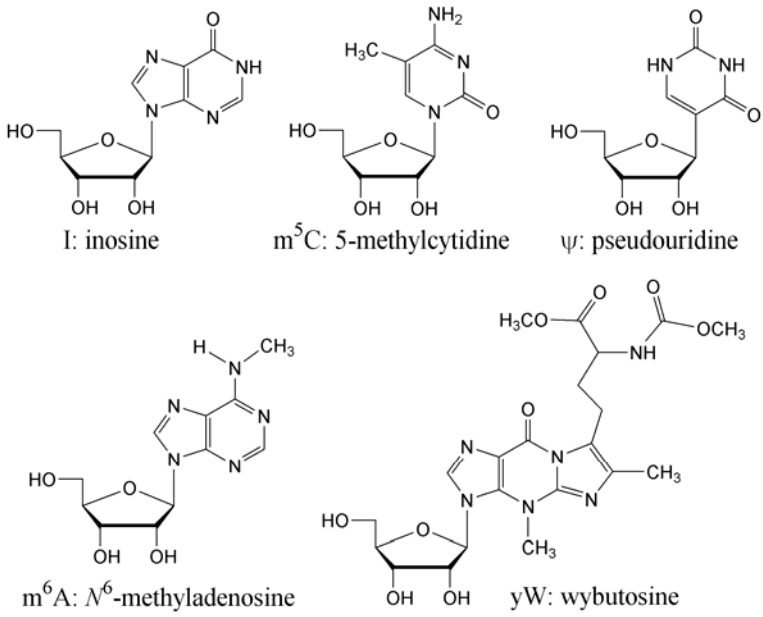

Figure 2. Schematic representation of a few naturally occuring modified nucleobases

Apart from the aforementioned nucleobases, a large number of modified nucleobases are also found in naturally occurring nucleic acids, mostly in RNAs[7,8] and particularly in transfer RNAs (tRNA) [*Figure 2*]. Some are modifications of the most common natural nucleobases whereas others are hypermodified.

The molecular components of the nucleic acids have rather compact shapes and structures. The conformational details are accurately defined by the torsion angles i.e., α, β, γ, δ, ε, and ζ in the sugar-phosphate backbone; θ_0 to θ_4 in the sugar furanose ring; and χ for the glycosidic bond [*Figure 3*]**.**[9,10,11] The torsion angle about the glycosidic bond ranges from *syn* (−90 º≤ χ ≤ 120 º) to *anti* (90 º≤ χ ≤ 270 º), *anti* being the most common type.[9,10,11]

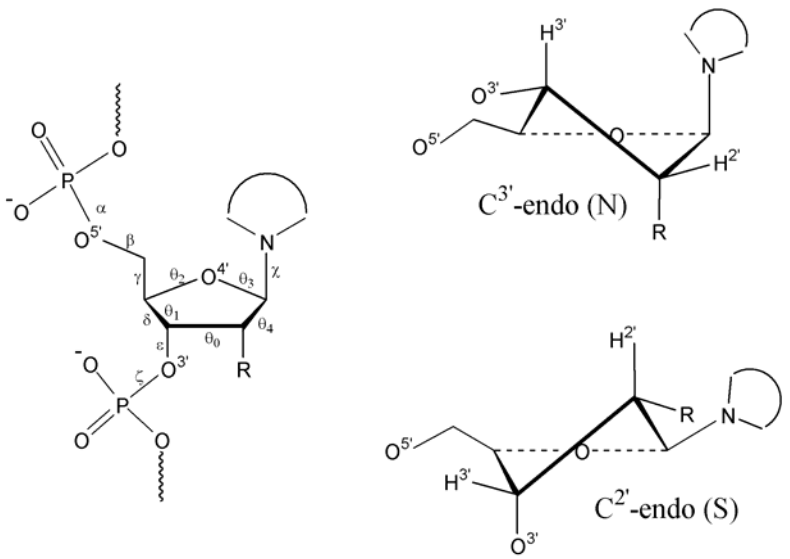

Figure 3. Schematic representation of the torsion angle notations and structures for the $C^{2'}$-endo (S) and $C^{3'}$-endo (N) sugar puckerings

In the pentofuranose ring, the planar form is not energetically favored and it is puckered to relieve strain.[12] It can adopt either the $C^{3'}$- endo (often referred to as *North* or N) or the $C^{2'}$-endo (*South* or S) conformations [*Figure 3*]. Only a few $O^{4'}$-endo (*East* or E) sugar conformation are known even though the pseudorotation cycle[13,14] predicts a variety of possibilities including the $O^{4'}$-exo (*West* or W) conformation. The $C^{2'}$-endo sugar conformation is most commonly found in the 2'-β-D-deoxyribonucleosides and $C^{3'}$-endo in the 3'-β-D-ribonucleosides. The nature of nucleobases,[15,16] and sugar modifications[17,18,19] can also greatly influence the pentofuranose sugar conformation to adopt predominantly one conformation over the other.

DNA predominantly exists a in duplex form where two anti parallel strands are held together by hydrogen bonding and stacking. In the duplex

region nucleic acids generally form Watson-Crick base pairs.[20] A number of non-Watson-Crick base pairs (also called non-canonical base pairs) such as Hoogsteen,[21] reverse Hoogsteen,[22,23] Wobble,[24,25] reverse Wobble[25,26] are also found [*Figure 4*].

Depending on factors such as hydration and counter ion concentration, DNA duplexes adopt A, B or Z-type helical structures, with B being the most common. Amongst these, A and B-types are right handed helices with characteristic groove widths and depths and typical helical parameters.[9,10] Also, A and B- type duplexes can be made from any sequence of nucleobases. On the other hand Z-type duplexes are left handed helices and have $(\text{pyrimidine-purine})_n$ tandem repeats of altering conformation with its own characteristic groove width, depth and helical parameters.[9,10]

Alternative structures are also known such as single stranded, circular, hairpin, triplex, and quadruplex DNA. On the other hand, RNA with its diverse functionality organizes itself in diverse global structures. Several secondary structures are quite commonly found in RNA including A form double stranded stems, stem loops, hairpin, and pseudoknots etc.[9,10,11]

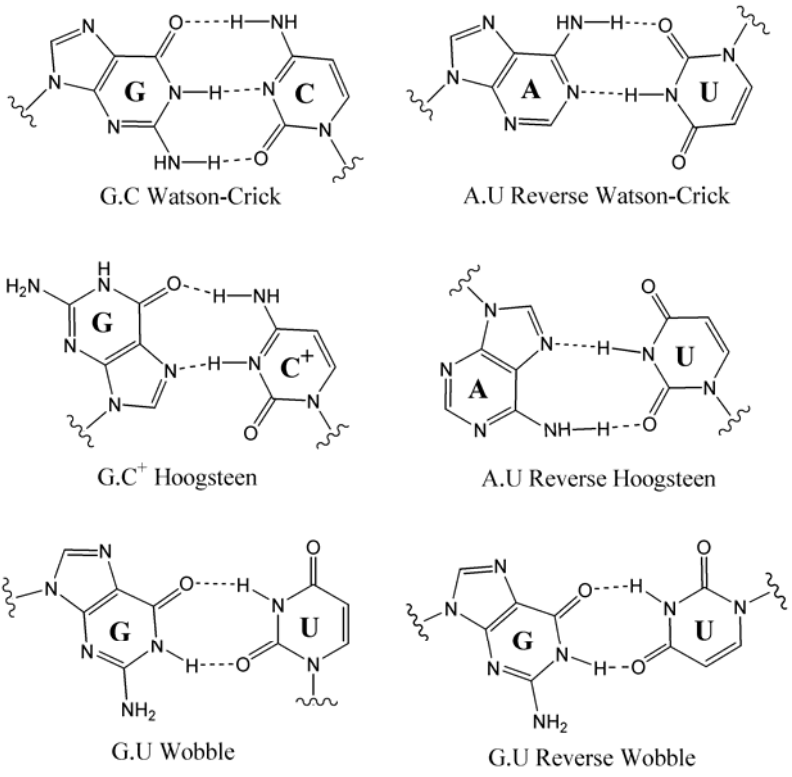

Figure 4. Schematic representation of canonical (Watson-Crick) and non-canonical base pairs

1.1.2 Quantification and analysis

The nucleobases have UV absorption maxima near 260 nm with extinction coefficients of about 10,000 M^{-1} cm^{-1}. Interactions among the nucleobases such as stacking causes up to 40% reduction of the absorption for double stranded polynucleotides and 25% reduction for single stranded polynucleotides compared to that of the sum of the absorptions of the nucleotide units.* The extinction coefficient of a single stranded polynucleotide is estimated from the sum of its components taking into consideration the nearest neighbor approximation.[27] The equation requires that the extinction coefficients of the nucleotides and the component dinucleotide phosphates are known and is used routinely with an estimated error of ±10% accuracy [*Equation 1*].

$$\varepsilon(\text{ApCpGpUp...ApG}) = 2[\varepsilon(\text{ApC}) + \varepsilon(\text{CpG}) + \varepsilon(\text{GpU}) + \ldots\varepsilon(\text{ApG})] - [\varepsilon(\text{Cp}) + \varepsilon(\text{Gp}) + \varepsilon(\text{Up})\ldots + \varepsilon(\text{Ap})]$$

Equation 1

The extinction coefficient of a double stranded nucleic acid is estimated with the help of a quadratic equation which involves Adenine-Thymidine (A.T) or Adenine-Uridine (A.U) base composition fraction, $f^{A.T}$.[9]

This method of quantification had been a very useful tool for routine concentration measurements throughout the studies presented in this thesis and was extensively used for the concentration correction of the small nucleotidic fragments in the alkaline hydrolysis experiments (discussed in the later part of the thesis).

Apart from the quantification of nucleic acids in solution, UV spectroscopy is often used for monitoring their melting behavior. A plot of UV absorption versus temperature is known as a melting curve and is usually cooperative in nature. The inflection point of the sigmoidal melting curve is known as the melting temperature.

Though the effect of structure on the UV absorption of oligonucleotides is complex and only the basic interpretations can be made, UV spectroscopy is commonly used to study the effects of chemical modifications on the thermal stability and the higher order structures of nucleic acids.[10,11] Thus UV based thermal melting studies has been very useful for the interpretation of the target affinity of an antisense oligonucleotide (AON) towards the complementary RNA, discussed in this thesis.

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* An absorbance in a polymer that is weaker than the sum of the absorbances of the components is known as hyperchromism.

Other spectroscopic techniques such as fluorescence, circular dichroism (CD), infrared, Raman, and nuclear magnetic resonance (NMR) as well as X-ray diffraction are also well used in nucleic acid structural analysis. Amongst these, fluorescence spectroscopy is increasingly being utilized in nucleic acids based detection techniques and diagnostics. On the other hand NMR spectroscopy and X-ray diffraction techniques are extensively used in nucleic acid structure determination and analysis of interactions with small molecules, proteins etc.

In the works presented in this thesis, NMR spectroscopy (^{1}H, ^{13}C and ^{31}P) is extensively used not only for the characterization and analysis but also for the studies of physicochemical properties of nucleotides and small oligonucleotides.

1.1.3 Reactivity of nucleic acids

The groups that are directly involved in chemical reactions of the nucleic acids are the nucleobase (the site for hydrogen bonding and stacking), the 2'-OH group of RNA (the site for transesterifications in the splicing reaction, and RNA catalysis, also discussed in the later part of the introduction), and the internucleotidic phosphodiesters of RNA (the site of cleavage reactions).

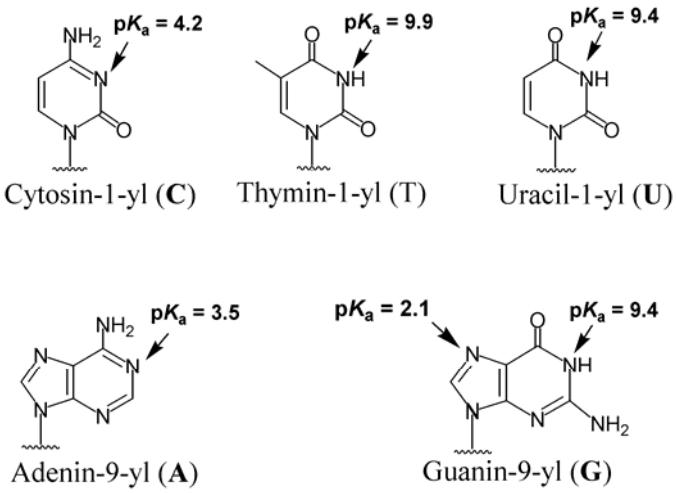
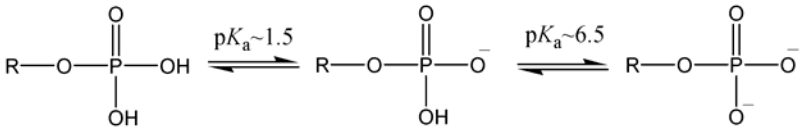


Figure 5. The pK_a values of nucleobases

All these different reaction sites contain ionizable groups with a variety of pK_a values. The wide range of pK_a values for the nucleobases is shown in *Figure 5*. The 2'-OH group of RNA has a pK_a value of around 12.0 – 14.0[28,29] whereas the internucleotidic phosphate group has a pK_a of 1.5[30,31] and the terminal phosphate has pK_a values of 1.5 and 6.5 [*Equation 2*].[10]



Equation 2

The reactivity of nucleic acids is greatly influenced by the primary structure and the environmental factors such as temperature, pH, salt concentration etc. This is because the primary structure in combination with the environment, and metal ion cofactors determine the secondary and tertiary structures, which in tern influences the molecular recognition as well as the reactivity.

1.2 Molecular recognition of nucleic acids

1.2.1 Interactions with metal ions and small molecules

Many small molecules, synthetic and naturally occuring, as well as metal ions interact strongly with nucleic acids. Metal ions being positively charged interact electrostatically with the negatively charged phosphate oxygens of the backbone of nucleic acids in a nonspecific manner. In addition, atom specific, group specific, base specific, sequence specific, as well as secondary/tertiary structure specific binding of metal ions to nucleic acids are known. Metal ion binding is also known to be involved in nucleic acid biosynthesis, processing and degradation, as well as genetic information transfer and gene expression, mutagenesis, chromosomal abnormalities, carcinogenesis etc.[10,11,32]

A number of small organic molecules are known for reversible interactions with the nucleic acids such as electrostatic interactions along the exterior of the helix, direct interactions with the edges of the base pairs (known as groove binding), and intercalation between the base pairs. On the other hand, the intrinsic chemical nature of the building units, as discussed above, makes nucleic acids susceptible to various chemical reactions such as oxidation/reduction, hydrolysis, metallation, and photochemical reactions.[11,32]

Interactions with small molecules and metal ions can lead to inactivation or destruction of nucleic acids. Understanding interactions between nucleic acids and small molecules and metal ions can lead to improvement of existing chemotherapeutic treatments by antiviral, antitumor, and anticancer agents.[11,32]

Apart from the aforementioned important aspects, this area of research fueled the development of artificial nucleases,[33] usually acting in a combination of metal ions with small organic molecules with the aim to destruct targeted nucleic acids.

1.2.2 Interactions with proteins

Many biological processes such as transcription, replication, and recombination depend on interactions between nucleic acids and proteins, both specific and non-specific. DNA sequences that are active when bound to proteins in sequence specific manner can be classified into two groups, those bound to gene regulatory proteins[34,35,36] and those that are substrates for polymerase, topoisomerase, recombinase. [37,38]

RNA can interact with proteins through hydrophobic stacking, hydrogen bonding, and salt bridge formation with high sequence specificity. Specific uridine rich ssRNA binding to sex-lethal protein,[39] 3'-mRNA poly(A) tail recognition by poly(A) binding protein (PABP),[40] and the ssRNA region which binds to tRNA binding attenuation protein (TRAP)[41] are examples of such sequence-specific interactions.

1.2.3 Interactions with short oligonucleotides

Nucleic acids have received much attention as potential therapeutic agents. They can be used, in principle, to replace defective nonfunctioning genes and also to prevent the expression of unwanted genes or the specific mRNA which produces unwanted proteins. The specific interactions found between complementary strands, by means of Watson-Crick base pairing, suggests that oligonucleotides can be used as therapeutic agents to target DNA (antigene) or RNA, particularly mRNA, (antisense). With the discoversy of solid phase synthesis, together with already existing possibilities of chemical manipulation, it became practically possible to screen modified AONs in a cost effective manner.

In the antigene approach, an artificial short DNA sequence, a triplex forming oligonucleotides or TFOs, is used to target double stranded genomic DNA through major groove binding by formation of a triple helix or by strand invasion.[42,43,44] The TFO typically utilizes Hoogsteen or reverse Hoogsteen base pair [*Figure 4*] in order to bind to the DNA double strand. In spite of the apparent simplicity in the concept of controlling gene expression directly, there has been limited success until now. This is because of the difficulties in target accessibility due to the proteins associated with the genome and the need for homopurine-homopyrimidine tracts for triplex formation.[45]

1.3 Targeting mRNA

There are mainly three types of anti-mRNA strategies known. The first one utilizes single stranded antisense oligonucleotides (AON), is the most validated approach and was first discovered by Zamecnik and Stephenson.[46,47] The second approach is based on catalytically active oligonucleotides referred to as ribozymes, first discovered by Thomas Cech[48,49] and Sidney Altman.[50] RNA interference,[51,52] induced by small interfering RNA (siRNA) molecules, is the third method which has caught much attention recently.[53,54,55] Further details of the RNA interference is beyond the scope of this introduction.

Apart from the above mentioned strategies, DNAzymes[56,57] (DNA molecules acting catalytically) represent an increasingly interesting field of research toward the nucleic acid based therapeutics.

1.3.1 The antisense strategy

The antisense mechanism exploits an endogeneous enzyme, RNase H, as a tool to degrade the mRNA.[58,59,60] RNase H specifically cleaves the RNA moiety of an DNA:RNA heteroduplex.[61] The AONs which do not utilize RNase H, can inhibit protein translation by means of steric blocking.[62,63] However, RNase H recruitment remains as more attractive pathway to be exploited for the antisense approach since there is, at least in theory, the possibility of utilizing AONs in catalytic amounts as the RNase H cleaves the RNA in the AON:RNA hybrid and leaves the AON unaffected to find new targets. Thus, a small dosage is required for therapy by this strategy [*Figure 6*].[64]

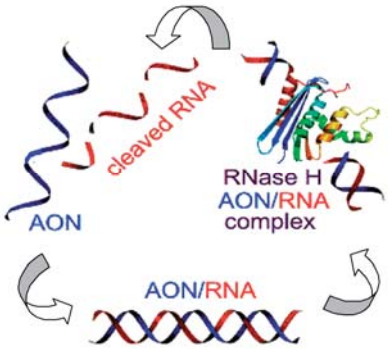


Figure 6. Schematic representation of mechanisms of antisense action

1.3.1.1 Chemically modified nucleotides developed for the antisense strategy

A synthetic short oligonucleotide to be used successfully as a therapeutic agent must fulfill a number of criterions. It must have high target affinity, a property related high target specificity. It must have high nuclease stability which is related to low toxicity and low effective dosage. It must also show good bioavailability which includes good cellular uptake and favorable pharmacokinetics.[65,66]

Since unmodified oligonucleotides are degraded very rapidly inside the cell, chemical modifications have been attempted to increase the resistance of the AONs to enzymatic degradation. In oligonucleotides, the nucleobase,

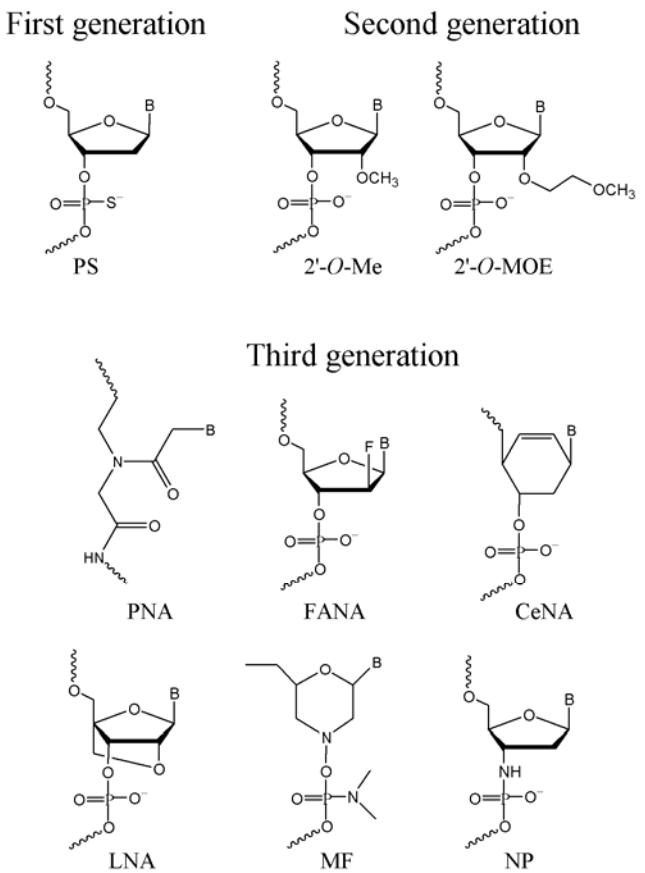


Figure 7. Nucleotide analogues corresponding to the first, second, and third generation of AONs

the sugar and the phosphate backbone are the most common targets for chemical modifications. In spite of the increased target affinity achieved

with modified nucleobases[67] when incorporated into AONs, their therapeutic potential *in vivo* have not been reported.[65]

The backbone modified phosphorothioate AONs (PS) where the non-bridging oxygen atom in the phosphodiester linkage was replaced by sulfer atom,[68,69] showed increased resistance toward nucleases. However, lower target affinity compared to the unmodified oligonucleotide,[70] non-specific interactions with proteins causing to unwanted side effects lead to the development of the second generation of modified AONs [*Figure 7*].

The second generation of modified AONs contains 2'-*O*-alkylated sugar moieties.[18] The combination of a phosphorothioate (PS) backbone with a 2'-*O*-alkylated sugar moiety lead to increased nuclease resistance and enhanced target affinity.[18] However, these modified AONs failed to recruit RNase H because of their RNA like sugar conformation that induced an A type duplex structure in the AON:RNA heteroduplex. This gave rise to the 'gap-mer' strategy in which the modified nucleotides are incorporated in a gap of 6 to 8 (or suitable) 2'-deoxyribonucleotide units.[18,71,72]

The lessons from first and second generations lead to the third generation of modified AONs with increased target affinity, high nuclease stability and good RNase H recruiting properties. Peptide nucleic acids (PNA),[73,74,75] arabino nucleic acids (ANA and FANA),[76,77,78] hexitol nucleic acids (HNA),[79] hexapyranosyl nucleic acids (HOMO-DNA),[80,81] cyclohexene nucleic acids (CeNA),[82] conformationally constrained nucleic acids (bridged/locked nucleic acid, BNA[83]/LNA;[84] ethylene bridged nucleic acids ENA[85,86] etc.), morpholino modified oligonucleotides (MF),[87,88,89] N3'-P5' phosphoramidates (NP),[90,91] and tricyclic DNA (tcDNA)[92,93] are important examples of third generation modified AONs. In spite of their many favorable properties, the challenges still remaining are to improve the cellular uptake and identifying the accessible sites[94] in the target mRNA. However, the delivery issue has been addressed by several strategies based on, for example, liposomes, dendrimers, nano particles, viral vectors etc.[95] On the other hand; methods utilizing oligonucleotide microarrays[96] and oligonucleotide libraries[97] are increasingly being applied to accessibility issue.

Nucleic acid based therapeutics have yielded two FDA approved drugs so far, Vitravene®, to treat the inflammatory viral infection of the eye caused by cytomegalovirus (CMV) and Macugen®, for age-related macular degeneration (AMD).[95,98] Another drug, Genasense®, targeting Bcl-2, a protein expressed in cancer cells to protect against chemotherapy, is waiting for FDA approval.[95,99] Though the "real" mechanism of action of these drugs is still debated,[100] more than 30 antisense based drugs are in different phases of clinical trials.[95,98]

1.3.2 RNA catalysis

The fact that RNA can act as catalyst was first discovered in the early 80's.[48,49,50] Several RNA sequences with enzymatic activities are known today, Group I and Group II introns, hepatitis delta virus (HDV), hairpin, hammerhead, Varkud satellite (VS) ribozymes are few examples.[3,101] Many investigations have been undertaken in order to understand how RNA with its fewer reactive groups compared to those of proteins can act as a biocatalyst. Ribozyme reactions can be broadly classified into two types on the basis of the termini generated during self-cleavage reactions. Ribozymes that use an exogenous nucleophile leave 2',3'-cis-diol and 5'-phosphate termini [*Figure 8*]; the group I intron and RNase P are examples of this type. Ribozymes that use the vicinal 2'-hydroxyl as the nucleophile leave 2',3'-cyclic phosphate and 5'-hydroxyl termini [*Figure 8*].[101,102]

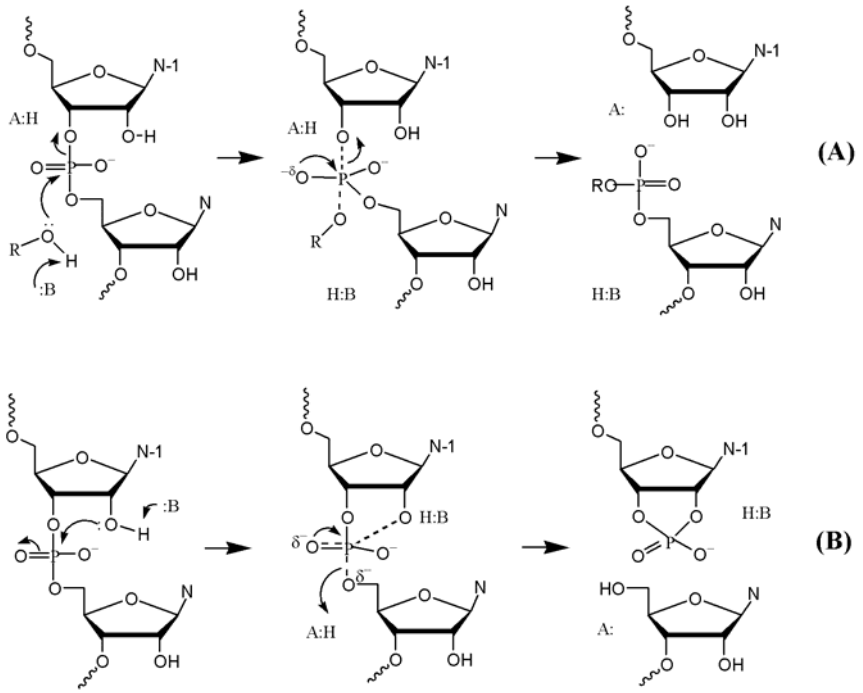


Figure 8. Mechanism of phosphodiester cleavage in large **(A)** and small ribozymes **(B)**

An increasing number of RNAs wih catalytic activities and a developing understanding of their mechanism of action have generated much interest for harnessing their catalytic activity for hydrolyzing RNA molecules.

1.4 Overview of the thesis

This thesis presents the biochemical evaluation of chemically modified short oligonucleotides in terms of antisense based RNA targeting. Chemical modifications at pentofuranose sugar have been used for this purpose. The modifications are 2'-*O*-Me-T, 2'-*O*-MOE-T, oxetane-T, LNA-T, azetidine-T, aza-ENA-T, carbocyclic-ENA-T, and carbocyclic-LNA-T.

The RNase H recruitment properties of these modified oligonucleotides, duplexed with the target RNA, have been evaluated along with the thermal melting studies as a measure of target affinity. Also, the modified oligonucleotides were subjected to studies for resistance towards snake venom phosphodiesterase (SVPDE) and human serum.

In another study, the physicochemical properties of unmodified model short RNA sequences were addressed. It was demonstrated how changes in primary structure can modulate the intrinsic properties along a single strand. Finally, alkaline hydrolysis was used in a model chemical cleavage study, to study the impact of small structural changes on the reactivity of the internucleotidic phosphodiester bonds.

2 Evaluation of Modified AONs (Paper I-III)

The most used and validated antisense mechanism is that of RNase H recruitment and a synthetic AON has to show RNase H eliciting properties in addition to high affinity, good bioavailability and nuclease resistance. The PS oligonucletides were found to be toxic and also showed non specific interaction with proteins.[72] The second generation of modified AONs containing 2'-*O*-Me and 2'-*O*-MOE were attempted were found to be less toxic and also showed slightly higher target affinity towards complementary RNA.[18] Pradeepkumar et al. earlier developed *North-East* conformationally constrained 1',2'-oxetane modified nucleosides, which did not show increased target affinity towards complementary RNA (ΔT_m ~–5 to –3 °C with pyrimidine nucleotides and almost no decrease with purine nucleotides).[103,104,105,106]

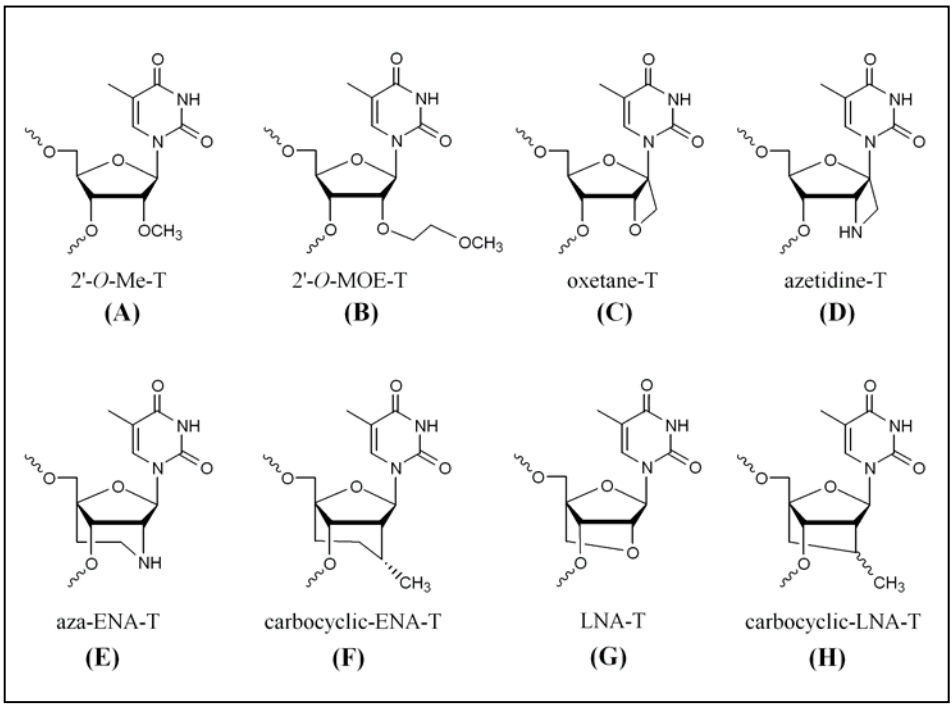


Figure 9. Schematic representation of nucleoside analogues incorporated into antisense oligonucleotides used in the present study

In the present work 15-mer AONs were synthesized, with single modified thymidine [*Figure 9*] incorporated into them. This AON sequence was designed to target a 15 mer RNA, chosen from the coding region of SV-40 large T antigen.[107]

The modifications used in the present study include well known modifications from the literature such as 2'-*O*-Me-T (**A)**, 2'-*O*-MOE-T (**B)**, and LNA-T (**G)**. The other modifications used were, oxetane-T (**C)**, by Pradeepkumar et al.; azetidine-T (**D)**, by Honcharenko et al.; aza-ENA-T (**E),** by Varghese et al.; carbocyclic-ENA-T (**F)**, and carbocyclic-LNA-T (**H),** by Srivastava et al. The present work describes the preliminary steps for the evaluation of antisense potential of these chemically modified AONs for therapeutic applications.

2.1 Thermal stability

The thermal stability (T_m) of the AON:RNA duplexes were measured by means of UV spectroscopy, utilizing the hypochromic property of nucleic acids. The AONs containing the 1',2'-azetidine-T (AONs **5** – **8**), the amino analogue of oxetane-T, showed slightly better target affinity than those of the oxetane-T modified AONs, but less than the native AON (Table 1). The 2'-*O*-Me-T and 2'-*O*-MOE-T, owing to their RNA like sugar conformations (*North*-type), showed a slightly more favorable duplex formation than the native sequence (AONs **2** and **3** in Table 1).

Incorporation of aza-ENA-T into the 15 mer AON (AONs **9** – **12**) showed enhanced T_m values (ΔT_m = + 2.5 °C to + 4 °C) compared to that of the native counterpart depending on the position of the modification. A similar trend of increase in T_m value was observed with the incorporation of the carbocyclic-ENA-T (AONs **13** – **16**, ΔT_m = + 1.5 °C), and the carbocyclic-LNA-T (AONs **21** – **24**, ΔT_m = + 3.5 °C to + 5 °C).

Table 1. Thermal denaturation[a] of modified and the native AONs, duplexed with the complementary RNA and DNA strands. The target RNA is 5'-r(GAAGAAAAAAUGAAG)-3', and the target DNA is 5'-r(GAAGAAAAAAUGAAG)-3'.

AON	Sequence	T_m/°C with RNA	ΔT_m	T_m/°C with DNA	ΔT_m*
1	3'-d(CTTCTTTTTTACTTC)-5'	44	…	45	…
2	3'-d(CTTCTTTTTT-*O*-Me ACTTC)-5'	44.5	+0.5	41	–4
3	3'-d(CTTCTTTTTT-*O*-MOE ACTTC)-5'	44.5	+0.5	40.5	–4.5
4	3'-d(CTTCTTTTTT-oxe ACTTC)-5'	39	–5	40.5	–4.5
5	3'-d(CT-azeCTTTTTTACTTC)-5'	38.5	–5.5	41	–4
6	3'-d(CTTCT-azeTTTTACTTC)-5'	40	–4	39.5	–5.5
7	3'-d(CTTCTTTT-azeTTACTTC)-5'	40	–4	39	–6
8	3'-d(CTTCTTTTTT-azeACTTC)-5'	40	–4	41	–4
9	3'-d(CT-aza-ENACTTTTTTACTTC)-5'	48	+4	44.5	–0.5
10	3'-d(CTTCT-aza-ENATTTTACTTC)-5'	46.5	+2.5	42.5	–2.5
11	3'-d(CTTCTTTT-aza-ENATTACTTC)-5'	47.5	+3.5	42	–3
12	3'-d(CTTCTTTTTT-aza-ENAACTTC)-5'	48	+4	42	–3
13	3'-d(CT-6-carboCTTTTTTACTTC)-5'	45.5	+1.5	43.5	–1.5
14	3'-d(CTTCT-6-carboTTTTACTTC)-5'	45.5	+1.5	39.5	–5.5
15	3'-d(CTTCTTTT-6-carboTTACTTC)-5'	45.5	+1.5	40.0	–5.0
16	3'-d(CTTCTTTTTT-6-carboACTTC)-5'	45.5	+1.5	39.5	–5.5
17	3'-d(CT-LNACTTTTTTACTTC)-5'	48	+4	47	+2
18	3'-d(CTTCT -LNATTTTACTTC)-5'	49	+5	46.5	+1.5
19	3'-d(CTTCTTTT-LNATTACTTC)-5'	49	+5	45.0	0
20	3'-d(CTTCTTTTTT-LNAACTTC)-5'	49	+5	46	+1
21	3'-d(CT-5-carboCTTTTTTACTTC)-5'	47.5	+3.5	45	0
22	3'-d(CTTCT-5-carboTTTTACTTC)-5'	49	+5	44	–1
23	3'-d(CTTCTTTT-5-carboTTACTTC)-5'	48	+4	44	–1
24	3'-d(CTTCTTTTTT-5-carboACTTC)-5'	47.5	+3.5	43.0	–2

[a] T_m values were measured as the maximum of the first derivative of the melting curve (A_{260} vs temperature) recorded in medium salt buffer (60 mM Tris-HCl at pH 7.5, 60 mM KCl, 0.8 mM $MgCl_2$, and 2 mM DTT) in the temperature range 20-70 °C using 1 μM concentrations of the two complementary strands. $\Delta T_m = T_m$ relative to RNA complement, and $\Delta T_m^* = T_m$ relative to DNA complement. T-6-carbo = carbocyclic-ENA-T; T-5-carbo = carbocyclic-LNA-T.

2.2 RNase H recruitment properties

As mentioned above, to function in the classical antisense mechanism, AONs have to be able to recruit RNase H. Therefore, all the AON:RNA duplexes containing modified AONs were subjected to digestion by *Escherichia coli* RNase H1. All of the modified AONs hybridized with the complementary RNA were found to recruit the *E. coli* RNase H1 with varying potential and patterns. The target RNA in the unmodified native AON **1**:RNA duplex was cleaved rather randomly but with a slight preference after A-8 position [*Figure 10* and *Figure 11*].

2.2.1 RNase H footprint pattern induced by AONs modified with 1',2'-locked nucleotide

The pattern of RNase H mediated cleavage of azetidine-T modified AON **5**:RNA was found to be quite similar to the pattern of the

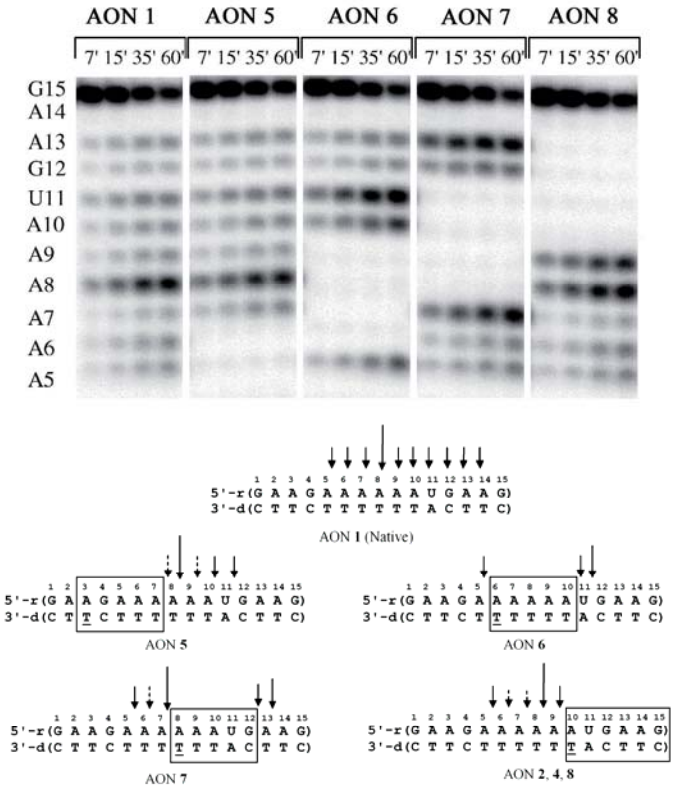


Figure 10. E. coli RNase H1 mediated cleavage footprints of the complementary RNA when duplexed with the native and the azetidine-T modified AONs

native AON **1**:RNA duplex except that there was no cleavage at the A5 and A6 positions. In the azetidine-T modified AONs **6** – **7**, duplexed with RNA, five nucleotides toward the 3'-end from the site opposite to the modification site (MS) were found not to be cleaved by RNase H. On the other hand for the AON **8**/RNA duplex, six nucleotides toward the 3'-end from the site opposite to the MS showed resistance toward RNase H mediated cleavage. Interestingly, all the azetidine-T modified AON **5** – **8** duplexed with RNA, showed similar cleavage patterns as those of the isosequential oxetane-T modified AON:RNA duplexes as reported earlier.[103,104]

2.2.2 RNase H footprint pattern induced by AONs modified with 2',4'-locked nucleotides

The modified AONs with 2',4'-locked nucleotides [AONs **9**-**12** with aza-ENA-T, AONs **13**-**16** with carbocyclic-ENA-T, AONs **17**-**2** with LNA-T and AONs **21**-**24** with carbocyclic-LNA-T] in iso-sequential positions, gave rise to uniquely similar cleavage footprinting. However, the footprint pattern

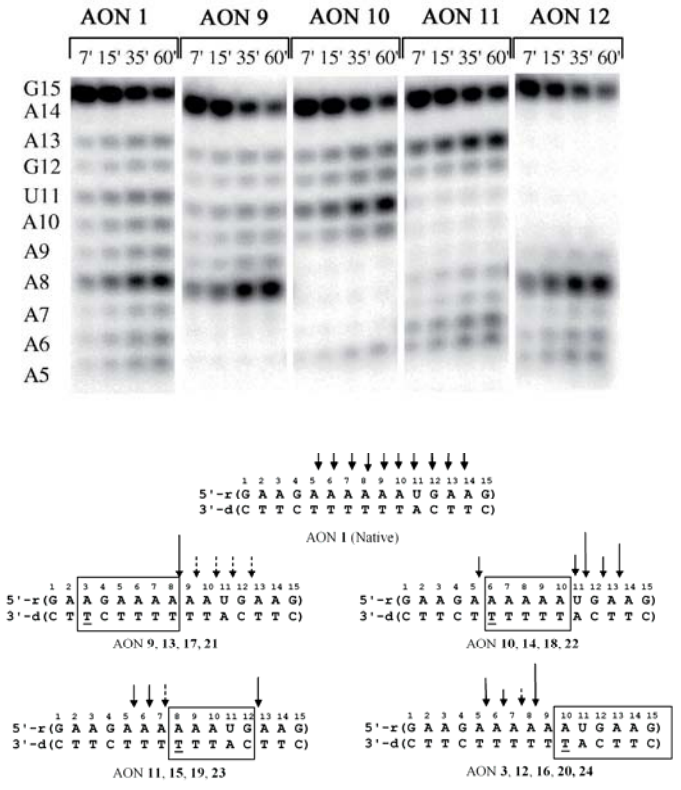


Figure 11. E. coli RNase H1 mediated cleavage footprinting of the complementary RNA when duplexed with the native and the aza-ENA-T modified AONs

was found to be different from those of iso-sequential AONs with 1',2'-locked nucleotides [AONs **5**-**8** with azetidine-T, and oxetane-T[103,104]]. For the AONs with modifications at position T3 from 3'-end [AONs **9**, **13**, **17**, **21**], the main cleavage site was found at A13 position of the complementary RNA unlike the iso-sequential azetidine-T modified AON **5** and iso-sequential oxetane-T[103,104] modified AONs, which showed additional strong cleavage at A7 and G12 positions. In the case of modification at position T10 from 3'-end [AONs **12**, **16**, **20**, **24**], seven nucleotides toward the 3'-end from the site opposite to the modification were found to be insensitive toward RNase H promoted cleavage [*Figure 11*].

Interestingly, the RNase H enzyme could discriminate the subtle differences between the flexibility of the modified sugars exhibited by the *North*-type 2',4'-locked system [aza-ENA, carbocyclic-ENA, LNA, and carbocyclic-LNA] and *North-East*-type 1',2'-locked system [oxetane-T, and azetidine-T]. On the other hand it could not differentiate between the hydrophobic [carbocyclic-ENA, carbocyclic-LNA] and hydrophilic [aza-ENA and LNA] character of the modified nucleotides unlike the exonucleases i.e., snake venom phophodiesterase (SVPDE) and human serum. Studies with exonucleases are discussed toward the end of this chapter.

The 2'-*O*-alkylated nucleotides [2'-*O*-Me-T and 2'-*O*-MOE-T], known to confer *North*-type conformation to the sugar,[18] were expected to have similar effects as 2',4'-locked nucleotides, on RNase H mediated cleavage of the target RNA. The 2'-*O*-MOE-T modified AON **3** indeed imparted similar resistance toward the RNase H mediated cleavage of the target RNA as the iso-sequential AON counterparts containing 2',4'-locked nucleotides [AONs **12**, **16**, **20**, and **24**]. However, the RNase H cleavage pattern of 2'-*O*-Me-T modified AON **2** was rather like iso-sequential AON with 1',2'-locked nucleotide, AON **8**, but with very little cleavage at A9 position. It is noteworthy that an extra strong cleavage at A9 position was the only difference between iso-sequential AONs with 2',4'-locked nucleotide [AONs **12**, **16**, **20**, and **24**] and 1',2'-locked nucleotide [AONs **4**[103,104] and **8**].

2.3 RNase H cleavage kinetics

2.3.1 Pseudo first order cleavage rates

The pseudo first order cleavage rate constants of RNase H digestion were determined by densitometric quantification of gels and subsequently plotting of the uncleaved RNA fraction as a function of time. The reaction rate constants were obtained by fitting the degradation curves to single-exponential decay functions.[108] The relative cleavage rates were found to be very similar to that of the native counterpart irrespective of the type and the site of modi-

fication in the AON strand [**Papers I**, **II** and **III**]. However, pseudo first order cleavage kinetics is a rather crude simplification to an enzymatic reaction. Therefore, a detailed Michaelis-Menten kinetics study was performed for selected AONs.

2.3.2 Michaelis-Menten kinetics

We chose iso-sequentially modified AONs [**2**, **3**, **8**, **12**] for the detailed Michaelis-Menten kinetic studies in order to understand the impact of the modifications on the binding affinity and the catalytic activity of the RNase H [**Paper I**]. Kinetic parameters were subsequently compared with those of the native AON 1 and earlier reported oxetane-T modified AON **4**.[105]

The catalytic constant (k_{cat}), which represents the maximum number of substrate molecules converted to products per active site per unit time,[109] is greater for the reaction with the modified AON:RNA duplexes [1.4 – 1.6 fold for 2'-*O*-Me-T (AON **2**), 2'-*O*-MOE-T (AON **3**), and azetidine-T (AON **8**) and ~2.6-fold for aza-ENA-T (AON **12**)] than for that with the native AON **1:**RNA duplex. However, the catalytic effectivity (k_{cat}/K_m) for the hydrolysis of all chemically modified substrates was found to be lower than that for the native substrate, without exception [~1.1 fold less for 2'-*O*-Me-T (AON **2**), ~2.6-fold less for 2'-*O*-MOE-T (AON **3**), ~1.4 fold less for oxetane-T (AON **4**), ~2.7 fold less for azetidine-T (AON **8**), and ~3.2 fold less for aza-ENA-T (AON **12**)] [Table 5, Figure 7 in **Paper I**]. This was due to the fact that introduction of a modified moiety to the AON decreased the enzyme binding affinity ($1/K_m$) [1.3 – 1.7 fold less for 2'-*O*-Me-T (AON **2**) and oxetane-T (AON **4**), 3.8 – 4.2 fold less for azetidine-T (AON **8**) and 2'-*O*-MOE-T (AON **3**), and ~8.3 fold less for aza-ENA-T (AON **12**)].

2.4 Resistance to nuclease degradation

Native DNA is degraded very fast in a cellular system. Replacement of the phosphodiester (PO) backbone with the phosphorothioates (PS) was shown to prevent the AONs from fast degradation inside the cell. However, these modifications were probably less stable than they were initially believed[66] and moreover led to toxicity problems related to non-specific target binding.[95] Therefore, one of the major problems to be resolved in order to allow successful applications of AONs in therapeutics is their stability in living cells.

It is primarily the exonucleases, with modest contributions from endonucleases, which degrade the AONs.[66] Therefore, the modified AONs were treated with snake venom phosphodiesterase (SVPDE) from *Crotalus adamanteus*, which is a 3'-exonuclease, and human blood serum, which contains several 3'-exonucleases.

2.4.1 Resistance of modified AONs to snake venom phosphodiesterase degradation

The unmodified native AON **1** was completely degraded within 1 h, not to our surprise. Also the 2'-*O*-Me-T modified AON **2**, 2'-*O*-MOE-T modified AON **3**, and all LNA-T modfied AONs **17** – **20** showed similarly poor resistance towards SVPDE hydrolysis. The azetidine-T containing AONs **5** – **7**, showed relatively higher stability, compared to those of the aforementioned unmodified native and modified AONs. The azetidine-T containing AON **8** degraded as fast as the native AON **1** [*Figure 12*].

All the other modified AONs containing aza-ENA-T, carbocyclic-ENA-T, and carbocyclic-LNA-T nucleotides were stable for more than 48 h in presence of SVPDE. It is important to note that the full length modified AONs were hydrolyzed within the initial hours of treatment with SVPDE. The stable fractions were found to be the ones cleaved on the 3'-side of the modification site (MS) in the case of azetidine-T containing AONs **5** – **7**, and at the next phosphodiester bond on the 3'-side of the MS in the case of aza-ENA-T, carbocyclic-ENA-T, and carbocyclic-LNA-T containing AONs **9** – **12**, **13** – **16**, and **21** – **24** respectively. However, aza-ENA-T modified AONs were further hydrolyzed at the phosphodiester on the 3'-side of the MS with increasing time. It can be concluded that the carbocyclic modifications in general imparted high stability to the AONs against SVPDE.

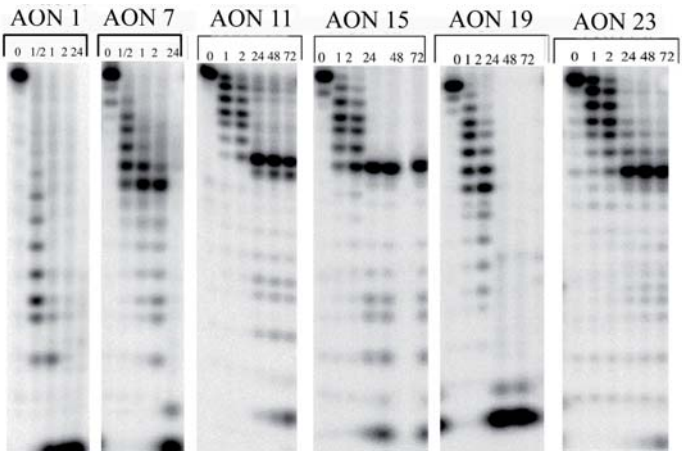


Figure 12. Autoradiograms of 20% denaturing PAGE showing degradation pattern of 5'-^{32}P-labeled AONs in SVPDE. The numbering of the lanes represents the time of incubation with enzyme (h)

2.4.2 Stability in human serum

The native AON **1** and the 2'-*O*-Me-T modified AON **2** were found to be degraded completely within 4 h of treatment with human serum. The 2'-*O*-MOE-T modified AON **3**, the azetidine-T modified AON **8** and all the LNA-T modfied AONs **17** – **20** were somewhat more stable than the native counterpart but completely degraded within 12 h of treatment with human serum. All other modified AONs **5** – **7**, **9** – **12**, and **21** – **24** showed good stability, and were not completely degraded by 48 h. The cleavage pattern was found to be comparable to those found after the treatment with SVPDE for all the stable AONs. However, presence of phosphodiesterases in the human serum cleaved the radio labeled 5'-phosphate and made the quantification impossible.

2.5 Conclusions and implications

As stated above, extensive comparative studies are very important in order for step-by-step evaluation of the chemical modifications necessary in the development of successful therapeutic agents. The isosequential AONs with 1',2'-locked nucleotides showed lower T_m with complementary RNA. This was expected as a 1',2'-lock in the sugar residue is known to impose strain in the glycosidic torsion (χ) and thus it disrupts the linearity required for hydrogen bonding.[110] On the other hand, a 2',4'-lock in the sugar moiety arrests the sugar in the absolute *North*-type conformation and does not impose such strain in the glycosidic torsion. Therefore AONs with 2',4'-lock nucleotides showed much improvement in T_m when duplexed with complementary RNA. The order of stability was found to be LNA-T ≥ carbocyclic-LNA-T ≥ aza-ENA-T > carbocyclic ENA-T > OMe-T ≈ OMOE > native > azetidine-T > oxetane T, in the AON:RNA duplexes.

The RNase H could uniquely differentiate between different types of modification in the AON:RNA hybrid duplexes, even though the global duplex structure remained unchanged, measured by CD spectroscopy.[103,104,105] The 1',2' locked oxetane and azetidine modified AONs showed identical cleavage pattern, and the 2',4'-locked aza-ENA-T, LNA-T carbocyclic LNA-T and carbocyclic ENA-T showed similar pattern. Another interesting result was that the chemical difference between the carbocyclic-LNA-T and LNA-T, nucleotide with a 2'-carbon atom linked to an exocyclic methyl group instead of 2'-oxygen, could cause a significant difference in nuclease stability of the AONs. This can be an important achievement from the pharmacological perspective as enhanced life-time of the AONs in blood serum could mean reduced dosage.

It is evident from the above results that small structural and chemical changes can bring about significant differences in the intrinsic physico-

chemical properties which are well-recognized by the enzymes. Therefore it is important to understand the subtle changes at the molecular level. The changes may apparently be small but they are intriguing in the way they modulate the chemical reactivity of the nucleic acids. This aspect is addressed in the next section of this thesis.

3 Sequence Dependent Physicochemical Properties of ssRNA (Paper IV – V)

The discovery that the RNA can act as an enzyme, a ribozyme, has been asubject of great interest to chemists. In the ribozyme action only some phosphodiester bonds in presence of several others, are hydrolyzed preferentially with the help of their intricate structural motifs. This can be induced by metal ion cofactors and enzymes.[111,112,113] The phosphodiester bond cleavage, both in ribonuclease mediated RNA cleavage (i.e. RNase H) and in ribozyme action, takes place via a transesterification reaction (ribozyme mechanism is discussed in the introduction). Recently, the base-promoted RNA transesterification reaction and cleavage has been studied in several small model systems in many laboratories in order to understand the mechanistic detail of ribozyme activity at the molecular level. Studies of alkali promoted hydrolysis experiments in small model systems, such as monomethyl and monoisopropyl esters of adenosine 2'- and 3'-monophosphates,[114,115] dimeric RNAs[116] were carried out previously in order to understand the cleavage mechanism. Various short 2'-*O*-methylated[117,118,119] and 2'-deoxy[120,121] chimeric oligonucleotide sequences containing only *one* reactive ribonucleotide have also been subjected to alkaline cleavage studies. Several mechanistic features have been identified as essential prerequisites for non enzymatic phosphodiester bond cleavage. The nucleophilicity of the 2'-OH (which is dependent on its pK_a),[122,120] the electrophilicity of the reacting phosphate,[123,124] the in-line conformation of the attacking 2'-oxyanion and the departing 5'-oxyanion,[125] and the leaving group capability of the 5'-oxyanion.[123,124] It has also emerged that the intramolecular environment with the hydrogen bonding network, stacking, nucleobase composition around the scissile bond also play important role.[126,127]

It was known that the electronic environment around the phosphate group in RNA is vital for protein-RNA interactions.[128] It was also known that the conformational change at the phosphate is important for facilitating the hydrolysis in ribozymes.[129] However, it was not known if some specific scissile phosphodiesters in RNA have variable charges in comparison with the other phosphates owing to some sequence-specific unique scaffolds or folding motifs. It was also not understood if the variable phosphate charges can potentially tune the chemical reactivity in the biologically ubiquitous transesterification reactions.

In the present work it has been shown in model short heptameric single stranded DNA and RNA sequences that the pK_a of the 9-guanine residue that ionizes to form a 9-guaninyl anion, varies in a sequence dependent manner. It has been demonstrated that the effect of 9-guaninyl ion formation can be seen in the neighboring bases toward 3'- and 5'- ends in both the ssDNA and ssRNA. It has also emerged that the electronic environments and thus the reactivity of the neighboring phosphodiesters in the ssRNA sequences were affected in a sequence specific manner whereas in the ssDNA sequences they were not.

3.1 Determination of pK_a values by ^{1}H NMR

It has been shown previously that the change in physicochemical character of a nucleobase can be followed by monitoring the pH dependent chemical shifts of the aromatic marker proton of the nucleobase itself as well as by monitoring the aromatic marker proton of neighboring nucleobases.[130,131] In the present work, heptameric ssDNA and ssRNA sequences were designed

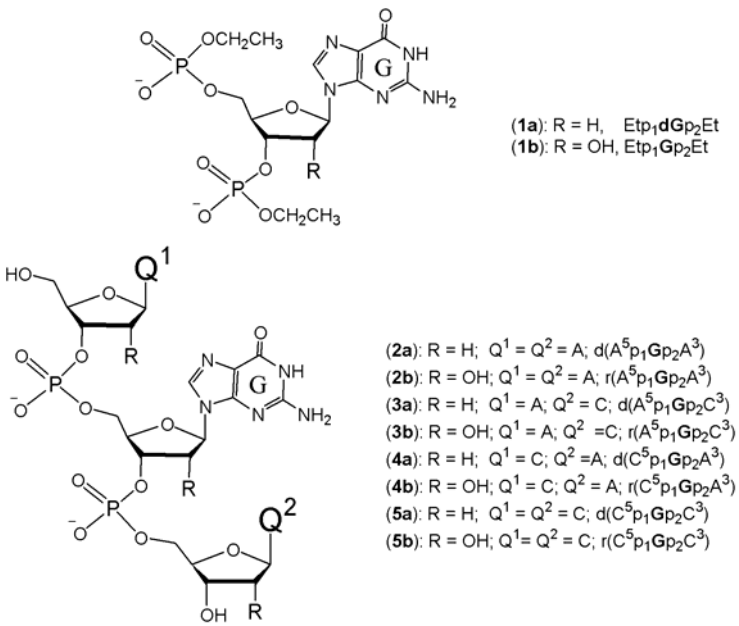


Figure 13. Schematic representation of the monomeric and trimeric sequences used in the present work

in such a way that, from among all the aromatic residues, only one gave rise to an anionic species under the alkaline pH. Only the **G** in the middle of the sequence was ionized. Similar studies were also carried out with reference

trimeric sequences [*Figure 13*], which were at the core of heptamers [*Figure 14*].

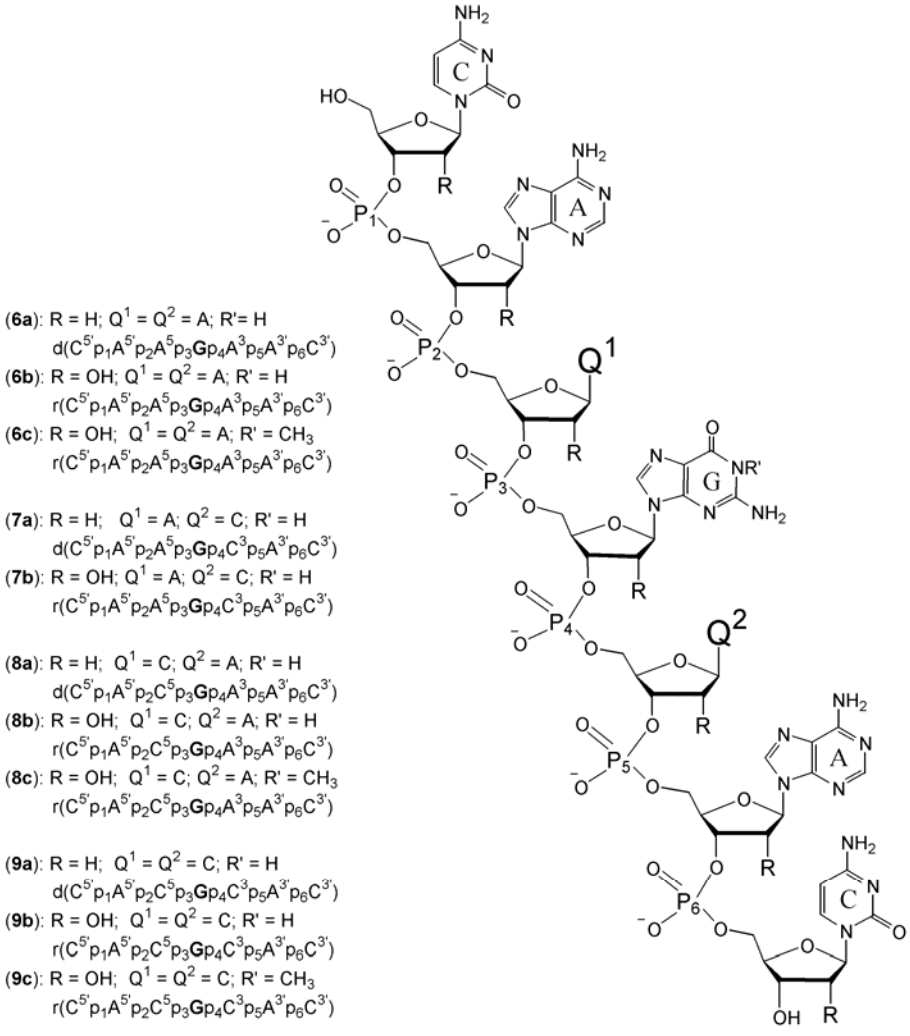


Figure 14. Schematic representation of the heptameric sequences used in the present work

3.1.1 Sequence dependent pK_a modulation a $\boldsymbol{G^-}$ ion formation

Titrations in the pH range 6.6 – 12.5, of the model heptameric sequences, **6a – 9a** and **6b – 9b** as well as of their reference trimeric sequences, **2a – 5a**

and 2b – 5b were performed. The apparent pK_{a1}[†] (for detailed specification see **paper IV**) of ***G*** in the group of four trimeric ssDNAs **2a – 5a** varied from 9.49 ± 0.01 to 10.24 ± 0.01 (ΔpK_{a1} = 0.75), whereas in the group of four heptameric ssDNAs **6a – 9a**, the apparent pK_{a1} of ***G*** varied from 10.39 ± 0.01 to 11.06 ± 0.01, ΔpK_{a1} = 0.67 [Table 2]. On the other hand, the apparent pK_{a1} of ***G*** in the four trimeric ssRNAs **2b – 5b** varies from 10.03 ± 0.01 to 10.25 ± 0.01 (ΔpK_{a1} = 0.22), while in a group of four heptameric ssRNAs **6b – 9b** the apparent pK_{a1} varied from 10.09 ± 0.02 to 10.58 ± 0.01 (ΔpK_{a1} = 0.49).

A variation in apparent pK_{a1} values of ***G*** was observed within the set of trimeric or heptameric ssDNA and ssRNA sequences. Though the phosphate charges and the pentose sugar units within each group of trimers or heptamers remain the same, the variation was observed most likely because the pseudoaromatic character of ***G*** within each group was changed due to different sequence context. On the other hand, when the apparent pK_{a1} variations of the ***G*** in ssDNA trimers were compared with those of the corresponding ssDNA heptamers and, the ssRNA trimers with those of the corresponding ssRNA heptamers, the effect of different microenvironments were evident. The differences in the microenvironments were due to the variable stacking owing to the respective nearest-neighbors and chain lengths.

[†] pK_a, as is used here, is of the monomeric unit, Etp(d/rG)pEt (1a/1b). Apparent pK_{a1} is the pK_a arising in presence of electrostatic interaction of G with additional neighboring electronic groups such as additional nucleobases, phosphates, and the pentose-sugar units across the single-stranded nucleic acids. Apparent pK_{a2} is the pK_a which is seen from the marker protons in the neighboring nucleobases (A or C) in the trimeric and heptameric ssDNA/ssRNA sequences.

Table 2. Comparison of pK_a[a] values of ***G*** in ssDNAs **2a – 9a** and in ssRNAs **2b – 9b** as well as in their monomeric counterparts EtpdGpEt (**1a**) and EtpGpEt (**1b**)

2'-Deoxy units		^{1}H	pK_a	2'-Ribo units		^{1}H	pK_a
(**1a**)		H8G	9.59 (± 0.01)	(**1b**)		H8G	9.29 (± 0.01)
(**2a**)	dA^5	H8A	10.33 (± 0.02)	(**2b**)	rA^5	H8A H2A	10.04 (± 0.01) 9.98 (± 0.01)
	dG	H8G	10.24 (± 0.01)		rG	H8G	10.03 (± 0.01)
	dA^3	H8A	10.45 (± 0.03)		rA^3	H8A H2A	9.97 (± 0.01) 10.03 (± 0.02)
(**3a**)	dA^5	H8A H2A	10.08 (± 0.01) 10.14 (± 0.06)	(**3b**)	rA^5	H8A H2A	10.08 (± 0.01) 10.04 (± 0.01)
	dG	H8G	10.10 (± 0.01)		rG	---	------
	dC^3	H5C H6C	10.07 (± 0.01) 10.11 (± 0.01)		rC^3	H5C	10.03 (± 0.01)
(**4a**)	dC^5	H5C H6C	9.91 (± 0.02) 9.95 (± 0.01)	(**4b**)	rC^5	H5C H6C	10.07 (± 0.01) 10.18 (± 0.01)
	dG	H8G	10.01 (± 0.01)		rG	H8G	10.25 (± 0.01)
	dA^3	H2A	9.98 (± 0.01)		rA^3	H8A	10.19 (± 0.01)
(**5a**)	dC^5	H5C H6C	9.42 (± 0.04) 9.50 (± 0.01)	(**5b**)	rC^5	H5C H6C	10.20 (± 0.01) 10.26 (± 0.02)
	dG	H8G	9.49 (± 0.01)		rG	H8G	10.13 (± 0.01)
	dC^3	H5C H6C	9.37 (± 0.01) 9.37 (± 0.01)		rC^3	H5C H6C	10.11 (± 0.01) 10.16 (± 0.01)
(**6a**)	$dC^{5'}$	---	------	(**6b**)	$rC^{5'}$	H6C	10.41 (± 0.01)
	$dA^{5'}$	H2A	11.19 (± 0.02)		$rA^{5'}$	H2A	10.40 (± 0.01)
	dA^5	H2A	11.13 (± 0.01)		rA^5	H8A H2A	10.58 (± 0.01) 10.35 (± 0.01)
	dG	H8G	11.06 (± 0.01)		rG	H8G	10.58 (± 0.01)
	dA^3	H8A H2A	10.86 (± 0.01) 11.21 (± 0.02)		rA^3	H2A	10.39 (± 0.01)
	$dA^{3'}$	H8A	11.15 (± 0.01)		$rA^{3'}$	H8A	10.46 (± 0.02)
	$dC^{3'}$	H5C H6C	11.13 (± 0.02) 11.01 (± 0.01)		$rC^{3'}$	---	------
(**7a**)	$dC^{5'}$	---	------	(**7b**)	$rC^{5'}$	H6C	10.53 (± 0.02)
	$dA^{5'}$	H2A	10.88 (± 0.02)		$rA^{5'}$	H2A	10.46 (± 0.03)
	dA^5	H8A H2A	10.67 (± 0.03) 10.76 (± 0.02)		rA^5	H8A	10.80 (± 0.04)
	dG	H8G	10.74 (± 0.02)		rG	---	------
	dC^3	H5C	10.80 (± 0.02)		rC^3	H6C	10.31 (± 0.02)
	$dA^{3'}$	H8A H2A	10.61 (± 0.03) 10.83 (± 0.02)		$rA^{3'}$	H8A	10.34 (± 0.03)
	$dC^{3'}$	H5C H6C	10.65 (± 0.03) 10.71 (± 0.02)		$rC^{3'}$	H5C H6C	10.31 (± 0.03) 10.87 (± 0.08)
(**8a**)	$dC^{5'}$	H5C H6C	10.85 (± 0.04) 10.78 (± 0.03)	(**8b**)	$rC^{5'}$	H6C	10.50 (± 0.02)
					$rA^{5'}$	H8A H2A	10.60 (± 0.01) 10.50 (± 0.02)
	$dA^{5'}$	---	------		rC^5	---	------
	dC^5	H5C H6C	10.71 (± 0.04) 10.64 (± 0.04)		rG	---	------
	dG	H8G	10.79 (± 0.04)		rA^3	H8A H2A	10.56 (± 0.01) 10.59 (± 0.01)
	dA^3	H8A	10.69 (± 0.04)				
	$dA^{3'}$	---	------		$rA^{3'}$	H8A H2A	10.37 (± 0.02) 10.17 (± 0.03)
	$dC^{3'}$	---	------		$rC^{3'}$	---	------
(**9a**)	$dC^{5'}$	H5C H6C	10.41 (± 0.01) 10.55 (± 0.01)	(**9b**)	$rC^{5'}$	H6C	10.60 (± 0.02)
					$rA^{5'}$	H8A H2A	10.69 (± 0.03) 10.59 (± 0.02)
	$dA^{5'}$	H2A	10.37 (± 0.02)				
	dC^5	H5C H6C	10.31 (± 0.01) 10.25 (± 0.01)		rC^5	H5C H6C	9.78 (± 0.02) 9.74 (± 0.02)
	dG	H8G	10.39 (± 0.01)		rG	H8G	10.09 (± 0.02)
	dC^3	H5C H6C	10.32 (± 0.01) 10.58 (± 0.01)		rC^3	H5C H6C	10.99 (± 0.03) 10.70 (± 0.04)
	$dA^{3'}$	H8A	10.42 (± 0.02)		$rA^{3'}$	H8A	10.47 (± 0.01)
	$dC^{3'}$	H5C H6C	10.36 (± 0.01) 10.45 (± 0.02)		$rC^{3'}$	H5C	10.37 (± 0.01)

[a] All p*K*a values and the corresponding errors have been calculated from Hill plot analyses (**Figure S2** in the Supporting Information of **Paper IV**).

3.1.2 Differential electrostatic modulation toward the 3'- and the 5'- end

The cross-modulation of apparent pK_{a1} values of ***G***, induced by tandem nearest-neighbor electrostatic interactions, was also evident from the variation of the apparent pK_{a2} values, obtained from each marker protons in the neighboring nucleobase moieties, **A** and **C** in trimers **2a – 5a** and **2b – 5b** and in heptamers **6a – 9a** and **6b – 9b**. The trimeric sequences show similar electrostatic transmission in both the 3'- and 5'- direction from ***G*** for both ssDNA **2a – 5a** and ssRNA **2b – 5b**. In contrast, the electrostatic effect of the $\boldsymbol{G}^-$ formation was transmitted differently through the neighboring nucleobases across the strand in both the 3'- and 5'- directions, depending upon the sequence context in the heptameric ssDNA and ssRNA sequences. The distance up to which the electrostatic effect of $\boldsymbol{G}^-$ was transmitted is shown in *Figure 15*.

d-$^{5'}$(CAA**G**AAC)$^{3'}$ **(6a)** r-$^{5'}$(CAA**G**AAC)$^{3'}$ **(6b)**

d-$^{5'}$(CAA**G**CAC)$^{3'}$ **(7a)** r-$^{5'}$(CAA**G**CAC)$^{3'}$ **(7b)**

d-$^{5'}$(CAC**G**AAC)$^{3'}$ **(8a)** r-$^{5'}$(CAC**G**AAC)$^{3'}$ **(8b)**

d-$^{5'}$(CAC**G**CAC)$^{3'}$ **(9a)** r-$^{5'}$(CAC**G**CAC)$^{3'}$ **(9b)**

Figure 15. Propagation of the electrostatic interaction toward the 3'- and the 5'-ends as a result of $\boldsymbol{G}^-$ formation

The apparent pK_{a2} values of ***G*** determined from the marker protons of the neighboring nucleobases in both the 3'- and 5'- ends remain almost the same as the apparent pK_{a1} values [Table 2] for all the trimeric ssDNA (**2a – 5a)** and ssRNA (**2b – 5b**) sequences as well as for the heptameric ssDNA (**6a – 9a**) and ssRNA (**6b**) sequences. This is because the pseudoaromatic characters of **A** or **C** in those sequences are similarly modulated by the electrostatic interaction with the $\boldsymbol{G}^-$ anion. In contrast, the pK_{a2} values of ***G*** in the heptameric ssRNA sequences **7b**, **8b**, and **9b** in both the 3'- and 5'- ends, are further modulated by that of the pK_{a1} of ***G***. The apparent pK_{a2} values measured from different marker protons were found to be non uniform as A3' ≠ A5' ≠ A5 in **7b**, A3' ≠ A3 ≠ A5' in **8b**, and A3' ≠ A5' in **9b**; as well as C3' ≠ C3 ≠ C5' in **7b** and C3' ≠ C3' ≠ C5' ≠ C5 in **9b.** This is due to the difference

in partial ionic charges within the same nucleobase as well as due to the differences in intrinsic pseudoaromatic character depending upon their non identical microenvironments.

The heptameric ssRNA sequence **6b**, where ***G*** flanked by 5'-**A** and 3'-**A** showed comparatively poorer cross-modulation of pK_a of ***G*** (ΔpK_{a2} = 0.23) by its neighboring nucleobases, while the sequences 7**b**, where ***G*** is flanked by 5'-**A** and 3'-**C,** (ΔpK_{a2} = 0.56), **8b**, where ***G*** is flanked by 5'-**C** and 3'-**A,** (ΔpK_{a2} = 0.43), and **9b**, where ***G*** is flanked by 5'-**C** and 3'-**C** (ΔpK_{a2} = 1.25) show larger cross modulation of pK_a. [ΔpK_{a2} = {(pK_{a1} of ***G***)$_{\text{from δH8G in ssDNA or ssRNA}}$} − {(p$K_{a2}$ of ***G***)$_{\text{from marker protons (H8A/H2A/H5C/H6C) of ssDNA or ssRNA}}$}]. It is noteworthy that the pK_{a2} perturbation within the sequence was the largest when the neighboring nucleobases of ***G*** were pyrimidines, whereas it was lowest when the neighboring nucleobases of ***G*** were purines.

3.1.3 Correlation of ΔpK_a of ***G*** with δH8G and with the oligomerization shift

The apparent pK_{a1} of ***G*** as observed in heptameric ssDNAs **6a** – **9a** and ssRNAs **6b** and **9b** [Table 2] was always more basic by ca. 0.2 – 0.3 units from the pK_a of ***G*** in their monomeric counterparts EtpdGpEt and EtpGpEt, respectively. Comparing such modulation of pK_a of ***G*** [ΔpK_a = {(pK_a of ***G***)$_{\text{from δH8G in ssDNA or ssRNA}}$} − {(p$K_a$ of ***G***)$_{\text{from δH8G in EtpdGpEt or EtpGpEt}}$}] as a function of either the chemical shift of ***G*** [Figure 3A in **Paper IV**] or the oligomerization shift ($\delta_{monomer} - \delta_{oligomer}$) [Figure 3B in **Paper IV**], within the series of heptameric ssDNA and ssRNA sequences at neutral pH, showed the effect of the sequence context in their respective chemical environments. It became evident from the above correlation that the pK_a perturbation (ΔpK_a) of ***G*** in an oligomer increases with an increased stacking interaction with the nearest-neighbors which means that the heptamers with the middle 5'-purine(A)-***G***-purine(A)-3' sequences (**6a** and **6b**) are the most stacked, hence the pK_a is most modulated, and those having a middle 5'- pyrimidine(C)-***G***-pyrimidine(C)-3' sequences (**9a** and **9b**) are the least stacked, hence the pK_a is least modulated.

However, in the case of trimeric ssDNA and ssRNA sequences, such correlations could not be obtained, as their structures are presumably more random than those of the heptamers.

3.2 The ^{31}P chemical shifts of the internucleotidic phosphorous nuclei

The internucleotidic phosphodiesters (pK_a = 1.5) in ssDNA and ssRNA are fully ionized in the pH range (6.6 – 12.5) used in the present study (**Paper V**). Yet the ^{31}P resonances for each of the internucleotidic phosphates are shifted downfield due to the formation of $\boldsymbol{G}^-$ and show sigmoidal behavior giving an inflection point typical of a titration curve. The pK_a of $\boldsymbol{G}$ obtained from the pH dependent ^{31}P chemical shifts [Table 2] of various ^{31}P resonances in the trimeric ssDNAs and ssRNAs, and in the heptameric ssDNAs is a result of the through-space repulsive electrostatic interaction. The central $\boldsymbol{G}$ is gradually transformed to $\boldsymbol{G}^-$ upon titration which results in repulsive interaction with already negatively charged phosphates. In the case of heptameric ssRNA sequences ^{31}P chemical shifts are the results of electrostatic interactions between the phosphate and the 9-guaninyl anion as well as of the interaction between the phosphate anion and the 2'-oxyanion.

It is well known that the chemical shift is dictated by the screening of a nucleus, which in turn is directly correlated to the diamagnetic shielding by the neighboring electrons. This would normally mean that the phosphate ionization would be expected to shield the phosphorus to a higher field as for protons. However, it is well known that for various types of phosphates,[132,133,134] phosphonates,[135] and aminophosphonates[135] the resonances are shifted downfield in alkaline pH compared to those under neutral conditions. The downfield shift of the ^{31}P resonances reflects weaker screening of the ^{31}P nucleus owing to delocalization of charge into its dπ orbitals.[136]

This is also true for some of the internucleotidic phosphorus nuclei, in our short model sequences. The ^{31}P NMR shifts of those phosphorus atoms are shifted downfield with an increase in pH, because of the excess negative charge accumulation (charge repulsion occurs between the electron cloud in the outermost orbitals of phosphorus and the central 9-guanylate ion/2'-oxyanion) around the phosphorus nucleus leads to the delocalization of the excess negative charge into its own dπ orbitals through pπ–dπ orbital overlap.

3.2.1 Non-identical electronic environment around internucleotidic phosphodiesters in ssRNAs

3.2.1.1 The pK_a of central guanine residue obtained from ^{31}P NMR

It was possible to obtain the p$K_{a(31P)}$ [‡] values from almost all the internucleotidic phopsphorus nuclei in case of the trimeric sequences. However, in the

[‡] The pK_a values obtained from ^{1}H NMR is mentioned as p$K_{a(1H)}$ and that from ^{31}P NMR is mentioned as p$K_{a(31P)}$ in this thesis. They were denoted as pK_{a1} in paper IV and pK_{a2} in paper V respectively.

case of heptameric ssDNAs, p$K_{a(31P)}$ values were obtainable only from two of the neighboring intenucleotidic phosphate groups (p_3 and p_4 for sequences **6a**, **7a**, and **8a**; p_2 and p_4 for sequence **9a**) and those for heptameric ssRNAs were obtainable from three neighboring internucleotidic phosphate residues (p_2, p_3, and p_4).

The p$K_{a(31P)}$ values within the trimeric ssDNA, trimeric ssRNA as well heptameric ssDNA sequences were not appreciably different [Table 1 and Table S1 in **Paper V**]. However, for the heptameric ssRNA sequences, the p$K_{a(31P)}$ values within a specific sequence were noticeably different and they varied in a sequence dependent manner. The maximum difference [between p$K_{a(31P)}$ values obtained from p_3 and p_4] was found to be 0.76 pK_a unit in the case of ssRNA sequence **8b**, and the minimum difference [between p$K_{a(31P)}$ values obtained from p_3 and p_4] was 0.25 pK_a unit in the case of sequence **7b** [Table 1, and Table S1 in **Paper V**].

3.2.1.2 Comparison of p$K_{a(31P)}$ values with p$K_{a(1H)}$ values of δH8G

The p$K_{a(31P)}$ values from phosphate groups within a sequence of all trimeric ssDNA, trimeric ssRNA, all four heptameric ssDNA sequences and one heptameric ssRNA, **6a**, were comparable to the p$K_{a(1H)}$ values within the experimental errors. On the contrary, in the case of heptameric ssRNA sequence **9b**, the p$K_{a(31P)}$ value from p_2, was found to be as much as 0.84 pK_a unit higher than that of its p$K_{a(1H)}$ value, obtained from its pH dependent δH8G chemical shift. Unfortunately, similar p$K_{a(1H)}$ values could not be obtained for the heptameric ssRNA sequences **7b** and **8b**, therefore we can not make any comparison with the p$K_{a(31P)}$ values.

3.3 Alkaline hydrolysis of heptameric ssRNA sequences

3.3.1 Overall degradation

The electronic environments around the internucleotidic phosphates were found to be sequence dependent in our studies and alkaline hydrolysis experiments were carried out to investigate if the chemical reactivity of the internucleotidic phosphodiesters varied accordingly. All the heptamers were treated with 0.03 N aqueous NaOH, at pH 12.5 and 20 °C. It was found that the ssRNAs were degraded at different rates depending upon the sequence context. The overall degradation of the heptamers after 1 h was of 11.7% for **6b**, 7.8% for **7b**, 13.9% for **8b** and 11.3% for **9b** [*Figure 16*]**.**

3.3.2 Preferential cleavage

Even though there are six phosphodiester bonds in each of the four native heptameric ssRNAs (**6b**, **7b**, **8b** and **9b**), hydrolysis was preferred at the internucleotide phosphodiesters (p_2, p_3 and p_4, *Figure 16*) from which $pK_{a(31P)}$ values were obtained. Some other relatively minor cleavages were also observed at other internucleotide phosphodiesters, which did not show a $pK_{a(31P)}$, such as p_1 of Cp_1A- (in all ssRNAs) as well as at p_5 of the Cp_5A block in **7b** and **9b**, and in that of the Ap_5A block in **6b** and **8b** (no cleavage was observed at p_6 of any of the ssRNAs).

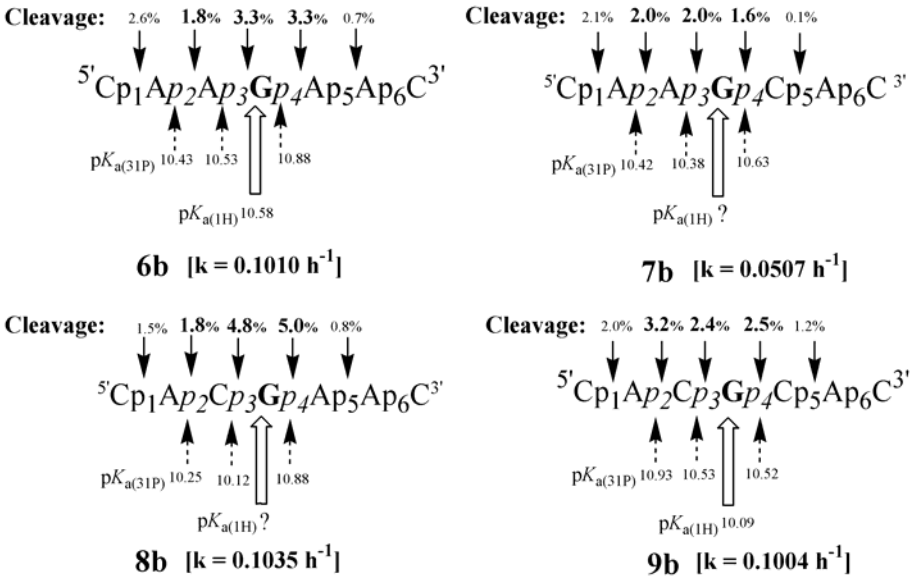


Figure 16. The alkaline hydrolysis at 1 h (in 0.03 N aqueous NaOH, pH 12.5, 20 ◦C) for the heptameric ssRNAs.

3.3.3 Fast cleavage at 5'-terminal Cp_1A

The cleavage rates at Cp_1A were quite comparable to or lower than the cleavage rates at the phosphodiesters (p_2, p_3 and p_4) from which the $pK_{a(31P)}$ values were obtainable. In contrast, the cleavage rates at Cp_1A were always higher than the cleavage rates at internucleotidic p_5, from which $pK_{a(31P)}$ value was not obtained. This higher cleavage rate at Cp_1A, outside the group of phosphodiesters with $pK_{a(31P)}$, is consistent with earlier observations that the 5'-r(CpA)-3' or 5'-r(UpA)-3' blocks undergo cleavage at a faster rate than other internucleotide linkages in the chimeric DNA/RNA[120,121]or 2'-*O*-

methylated-RNA/RNA[117,118,119]oligonucleotides as well as in natural RNA polymers.[137]

3.3.4 The relation between internucleotidic cleavage rate and p$K_{a(31P)}$

Comparison of the cleavage rates at p_2, which shows a p$K_{a(31P)}$ revealed [*Figure 16*] that the Ap_2C fragment in **9b** was cleaved at a much faster rate (3.2%) than that in **8b** (1.8%), whereas the relative cleavage rates at Ap_2A in **6b** (1.8%) and in **7b** (*ca.* 2.0%) were comparable. The cleavage at Ap_2C was quicker in **8b** (1.8%) and in **9b** (3.2%), while Ap_6C blocks in all the heptameric ssRNAs were completely resistant to hydrolysis (also, no p$K_{a(31P)}$ was obtainable from these). This highly preferential cleavage at the Ap_2C over the Ap_6C in **8b** or **9b** was remarkable, assuming that the 2'-OH of the adenosine in Ap_2C and Ap_6C have comparable pK_a values. Despite the fact that the internucleotide phosphodiester at Ap_2C was capable of sampling fewer in-line cleavage configurations than that of the 3'-terminal Ap_6C (because of its location in the strand), the cleavage was favored at Ap_2C. This suggests that the chemical character of the internucleotidic p_2 in **8b** or **9b**, were very special, perhaps due to their enhanced electrophilic character compared to those of p_6. A comparison of the chemical reactivity at the p_2 [Ap_2C in **8b** (1.8%) and **9b** (3.2%), Ap_2A in **6b** (1.8%) and **7b** (2.0%)] also showed that there was a complex set of stereoelectronic and conformational factors that was responsible for dictating the propensity for cleavage, depending upon the sequence context.

3.3.5 Comparison of the cleavage rates at p_2, p_3 and p_4

The cleavage rates at p_3 and p_4 were found to be faster than those of the cleavage at p_2 in –Ap_2Ap_3**G**p_4A– in **6b** and –Ap_2Cp_3**G**p_4A– in **8b**, whereas the cleavage at p_2 is preferred over p_3 and p_4 in –Ap_2Cp_3**G**p_4C– in **9b**. In contrast, the relative cleavages at p_2, p_3 and p_4 in –Ap_2Ap_3Gp_4C– in **7b** were comparable. This shows the importance of the sequence context in a hydrolysis experiment with many internucleotide phosphodiester, as in the heptameric ssRNA sequences.

3.4 Alkaline hydrolysis of $N^{1\text{-Me}}$-G containing ssRNA

The above studies on alakaline hydrolysis of the heptameric ssRNA sequences clearly suggested that due to the presence of a charged center $\boldsymbol{G}^{-}$ in close proximity, some of the internucleotidic phosphodiesters (p_2, p_3, and p_4)

were preferentially cleaved over others. We reasoned that replacing central **G** with $N^{1\text{-Me}}$-**G** would stop the $\boldsymbol{G}^{-}$ formation and therefore would affect the rate of hydrolysis at the internucleotidic phosphodiesters.

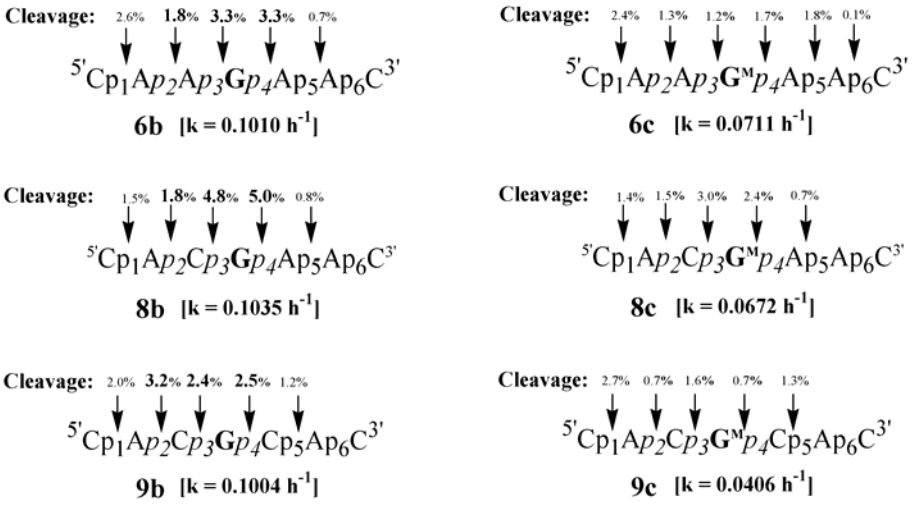


Figure 17. Comparison of the alkaline hydrolysis at 1 h (in 0.03 N aqueous NaOH, pH 12.5, 20 ◦C) for native (**6b**, **8b**, **9b**) and $N^{1\text{-Me}}$-G -containing heptameric ssRNAs.

3.4.1 Reduced cleavage rates due to the absence of $\boldsymbol{G}^{-}$

The overall alkaline degradation rates were found to be slower for $N^{1\text{-Me}}$-**G** containing heptameric ssRNAs (**6c**, **8c** and **9c**) compared to those of the corresponding native sequences, in general. After treatment with 0.03 N NaOH, at pH 12.5 and 20 °C, 8.5% of overall degradation was observed for $N^{1\text{-Me}}$-**G** containing **6c**. The corresponding native **7b** was cleaved 11.7%. Under the same condition $N^{1\text{-Me}}$-**G** heptamer **8c** was degraded 9.0%, (degradation of the native **8b** was 13.9%) and $N^{1\text{-Me}}$-**G** heptamer **9c** was degraded 7.0% (degradation of the native **9b** was 11.3%) [*Figure 17*].

The *preferential* cleavages at the internucleotidic phosphodiesters p_2, p_3 and p_4 were also considerably reduced in the case of the $N^{1\text{-Me}}$-**G** containing ssRNAs (**6c**, **8c** and **9c**) because of the disappearance of the electrostatic effect of $\boldsymbol{G}^{-}$.

3.5 Conclusions and implications

The studies in this section show how the physicochemical properties of the nucleobases are dependent on nearest neighbor electrostatic interactions. In

an RNA sequence $P^1Q^1\underline{N}Q^2P^2$, the physicochemical integrity of $\underline{N}$ is actually dictated by the characters of both neighboring Q^1 and Q^2. At the same time the properties of Q^1 and Q^2 are further tuned by the electronic nature of P^1 and P^2. Thus the pseudoaromatic character of $\underline{N}$ can have a number of possible variations in the nature depending on the nature of Q^1 and Q^2. These inequalities in the electronic environments brought in by the different sequence contexts can also lead to variable chemical reactivities of the internucleotidic phosphodiester bonds. Thus these studies offer insights into the mechanistic detail of enzymatic and non-enzymatic processes at a molecular level. Also, these insights can help designing new chemical modifications which can be used to tune and control the reactivity of RNA.

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Sammanfattning

Avhandlingen beskriver studier av viktiga egenskaper hos nukleinsyror avseende deras förmåga att fungera som läkemedel samt studier av grundläggande fysikalisk-kemiska egenskaper av betydelse för dess reaktivitet. Ett antal kemiskt modifierade nukleotider har inkorporerats i syntetiska oligonukleotider och dess egenskaper har utvärderats och jämförts med dem som förekommer i naturen. De syntetiska nukleotiderna 2'-*O*-Me-T, 2'-*O*-MOE-T, oxetane-T, LNA-T, azetidine-T, aza-ENA-T, carbocyclic-ENA-T och carbocyclic-LNA-T har inkorporerats i en sekvens om 15 nukleotider, en så kallad 15-mer, som binder till en komplementär 15-mer tagen ur en naturligt förekommande sekvens som kodar för ett naturligt T antigen hos organismen SV-40. En jämförande studie visar att 15-mererna med oxetane-T och azetidine-T har lägre affinitet för målfragmentet än den naturligt förekommande sekvensen. Dessa två baser kännetecknas av att sockerresten i nukleotiden låsts i en väldefinierad konformation som inte är kompatibel med stark bindning. De modifierade nukleotiderna LNA-T, aza-ENA-T, carbocyclic-ENA-T och carbocyclic-LNA-T band emellertid bättre än den naturliga sekvensen. En viktig del av läkemedelsstrategin är att syntetisera icke-naturliga fragment som kan binda till målsekvensen i en konformation som känns igen av enzymet RNAs H, så att det bryts ned av kroppsegna komponenter. De modifierade sekvenserna har därför undersökts med avseende på förmåga att binda till målfragmentet och rekrytera RNAs H. Modifierade sekvenser visade förmåga att rekrytera RNAs H med högre katalytisk aktivitet (k_{cat}) men med lägre affinitet ($1/K_m$) än de naturliga fragmenten. Vidare har motståndskraften emot enzymatisk nedbrytning av nukleaser, såsom fosfodiesteras från ormgift samt de som förekommer i humant serum, undersökts genom att nedbrytningshastigheten bestämts. De 15-merer som modifierats med aza-ENA-T and carbocyclic-ENA-T visade väsentligt förhöjd motståndskraft mot enzymatisk nedbrytning av nukleaser. Sammantaget så visade en grupp av modifierade oligonukleotider väsentligt förbättrade egenskaper i form av förhöjd affinitet, jämförbar förmåga att rekrytera RNAs H samt förhöjd förmåga att motstå enzymatisk nedbrytning, jämfört med den naturliga sekvensen.

NMR spektroskopiska studier och studier av alkalisk hydrolys av oligonukleotider har utförts för att undersöka effekten av elektrostatisk växelverkan på oligonukleotiders funktionella egenskaper. Jonisering av guanin resten kan monitoreras via NMR spektroskopiska studier av pK_a värden för angränsande grupper, t ex med hjälp av ^{31}P NMR spektroskopi av fosforkärnan i

den internukleotidiska fosfodiestern. De fosfodiestrar vars pK_a påverkas av guanins dissociation visade sig hydrolyseras snabbare är andra och den elektrostatiska växelverkan har därmed visats vara en faktor som påverkar reaktiviteten på ett sekvensberoende sätt.

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