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Some Aspects of Nucleic Acids Chemistry

BY

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Abstract

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This thesis is divided into two parts based on a total of 8 papers: Part 1: *Synthesis, physicochemical and biochemical studies of chemically modified oligonucleotides and their duplexes and triplexes*. Potency of the chromophore conjugated DNA oligonucleotides as antigene and antisense gene repressors was evaluated. The effect of geometry, bulk and π -electron density of a series of chromophores, tethered at the 5'-end of oligonucleotides, as well as the effect of the linker nature, length and the attachment site of the chromophore to the oligo were explored based on the stability of the duplexes and triplexes. A dramatic improvement in the triplex stability with *ara*-U linked phenazine oligo (potent antigene) was achieved ($\Delta T_m = 16.5^\circ \text{C}$). A number of selected phenazine and dipyrrophenazine tethered antisense oligos (AONs) and their phosphorothioate analogues were shown to form the AON/RNA hybrid duplexes with enhanced thermal stability. CD experiments revealed that these duplexes have the global structure unaltered from that of the native counterpart. RNase H degradation studies on three RNA targets having different degrees of folded structures showed that tethering of phenazine and dipyrrophenazine increases the hydrolysis rates (potent antisense) of the target RNA, and that chemical nature of the chromophore influences the RNase H cleavage pattern. Further investigation at the RNA saturated conditions revealed that 3'-tethered chromophores influence the substrate recognition, and the kinetics of the cleavage by RNase H. Conjugation of different chromophores, charged polyaromatic systems and metal complexes with polyaromatic ligands at different sites of the AON revealed that RNase H is very sensitive to any modifications in the middle region of the AON/RNA duplex. On the contrary, any modification at the 3'-end of the AON regardless of the bulk of the substituent or presence of positive charge can be easily tolerated by the enzyme. Sensitivity of the RNase H towards the local structural changes in the AON/RNA hybrid was probed with a number of AONs containing a single 1-(1',3'-*O*-anhydro- β -D-psicofuranosyl)thymine with locked 3'-*endo* sugar conformation at different sites of AON. RNase H degradation studies revealed that the local conformational changes brought by the constrained nucleoside, although invisible by CD, span in the hybrid as far as 5 nucleotides toward the 5'-end of the AONs (3'-end of RNA), showing the unique transmission of the structural distortion from a single modification site. The results also showed that the structural requirements for the substrate binding and substrate cleavage by RNase H appear to be different. Part 2: *Preparation of biologically important isotope labelled oligo-RNAs for the NMR structure determination in solution*. Synthesis of the non-uniformly $^{13}\text{C}_5$ labelled 29mer HIV-1 TAR RNA was achieved by solid-phase synthesis using $^{13}\text{C}_5$ labelled ribonucleosides (from $^{13}\text{C}_6$ -D-glucose). Two hammerhead forming RNAs (16mer and 25mer) were synthesized according to the Uppsala NMR-window strategy, where the sugar residues of the nucleosides forming stem I, II and the loop of the stem III of the resulting hammerhead complex were deuterated. UV melting and high resolution NMR structural studies showed that the 16mer RNA under quasiphsiological condition folds to a very stable hairpin structure, which prevents formation of a hammerhead RNA with the 25mer, primarily owing to thermodynamic reasons.

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THE ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to by the Roman numerals.

- I. Puri, N.; Zamaratski, E.; Sund, C.; Chattopadhyaya, J. Synthesis of 5'-Polyarene-Tethered Oligo-DNAs and the Thermal Stability and Spectroscopic Properties of Their Duplexes and Triplexes. *Tetrahedron* **1997**, *53*, 10409-10432
- II. Zamaratski, E.; Chattopadhyaya, J. Synthesis of Phenazine-Tethered Arabino and Xylofuranosyl Oligonucleotide Conjugates: The Thermal Stability and Fluorescence Properties of Their Duplexes (DNA-DNA and DNA-RNA) and Triplexes. *Tetrahedron* **1998**, *54*, 8183-8206
- III. Ossipov, D.; Zamaratski, E.; Chattopadhyaya, J. Dipyrido[3,2-a:2',3'-c]phenazine-Tethered Oligo-DNA: Synthesis and Thermal Stability of Their DNA-DNA and DNA-RNA Duplexes and Triplexes. *Helv. Chim. Acta* **1999**, *82*, 2186-2200
- IV. Zamaratski, E.; Ossipov, D.; Pradeepkumar, P.I.; Amirkhanov, N.; Chattopadhyaya, J. The 3'-Modified Antisense Oligos Promote Faster Hydrolysis of the Target RNA by RNase H than the Natural Counterpart. *Tetrahedron* (in press, 2000).
- V. Amirkhanov, N. V.; Zamaratski, E.; Chattopadhyaya, J. The Recognition and Cleavage of RNA in the Antisense Oligo-RNA Hybrid Duplexes by RNase H. *Tetrahedron Lett.* (in press, 2000).
- VI. Pradeepkumar, P.I.; Zamaratski, E.; Földesi, A.; Chattopadhyaya, J. Transmission of the Conformational Information in the Antisense /RNA Hybrid Duplex Influence the Pattern of the RNase H Cleavage Reaction. *Tetrahedron Lett.* **2000**, *41*, 8601-8607
- VII. Milecki, J.; Zamaratski, E.; Maltseva, T. V.; Földesi, A.; Adamiak, R. W.; Chattopadhyaya, J. The First Example of Sequence-Specific Non-Uniformly $^{13}\text{C}_5$ Labelled RNA: Synthesis of the 29mer HIV-1 TAR RNA with ^{13}C Relaxation Window. *Tetrahedron* **1999**, *55*, 6603-6622.
- VIII. Zamaratski, E.; Trifonova, A.; Acharya, P.; Isaksson, J.; Maltseva, T.; Chattopadhyaya, J. Do the 16mer, 5'-GUGGUCUGAUGAGGCC-3' and the 25mer, 5'-GGCCGAAACUCGUAAGAGUCACCAC-3', Form a Hammerhead Ribozyme Structure in Physiological Condition? An NMR and UV Thermodynamic Study. *Nucleosides, Nucleotides and Nucleic Acids* (in press, 2000).

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Abbreviations

A	adenin-9-yl or adenosine
Ac	acetyl
ANA	arabinonucleic acid
Anth	9-(<i>N</i> -(2-oxyethyl)- <i>N</i> -methyl)aminomethyl anthracene
AON	antisense oligonucleotide
B	any nucleobase
C	cytosin-1-yl or cytidine
CD	circular dichroism
Ce	2-cyanoethyl
COSY	correlation spectroscopy
CPG	control pore glass
T _m	melting temperature
2D	two dimensional
DMF	dimethylformamide
DMTr	4,4'-dimethoxytrityl
DNA	deoxyribonucleic acid
Dppz	dipyridophenazine
DQF-COSY	double quantum filtered COSY
ds	double stranded
EDTA	ethylenediaminetetraacetic acid
ΔF	fluorescence enhancement
Flo	9-(<i>N</i> -(2-oxyethyl)- <i>N</i> -methyl)aminofluorene
G	guanin-9-yl or guanosin
HPLC	high performance liquid chromatography
HNA	hexitol nucleic acid
HSQC	heteronuclear single quantum correlation
i-Pr	isopropyl
K	Kelvin
LNA	locked nucleic acid
mRNA	messenger RNA
α-Napth	1-(2-oxyethoxy)naphthalene
β-Napth	2-(2-oxyethoxy)naphthalene
NMR	nuclear magnetic resonance
NOESY	nuclear Overhauser effect spectroscopy
NPhn	nitro 9-(2-oxyethoxy)phenanthrene
NPyr	nitro 1-(4-oxybutyl)pyrene
ON	oligonucleotide
Pac	phenoxyacetyl
PAGE	polyacrylamide gel electrophoresis
Phn	9-(2-oxyethoxy)phenanthrene
Phnbu	9-(4-oxybutoxy)phenanthrene
PNA	peptide nucleic acid
PO	phosphodiester
PS	phosphorothioate
Pyr	1-(4-oxybutyl)pyrene
Pzn	2-(<i>N</i> -(2-hydroxyethyl)- <i>N</i> -methyl)aminophenazine
Pznm	2-[(<i>N</i> -(2-oxyethyl)- <i>N</i> -methyl)amino]-9-ethylphenazinium ethylsulphate
RNA	ribonucleic acid
RNase H	ribonuclease H
ROESY	rotating frame nuclear Overhauser effect spectroscopy

RT-PCR	reverse transcription-polymerase chain reaction
SVPDE	snake venom phosphodiesterase
T	thymine-1-yl or thymidine
TBAF	n-tetrabutylammonium fluoride
THF	tetrahydrofuran
TOCSY	total correlation spectroscopy
Tol	4-toluidyl
ss	single stranded
U	uracil-1-yl or uridine
UV	ultraviolet

1. **Synthesis, physicochemical and biochemical studies of chemically modified oligonucleotides and their duplexes and triplexes.**

1.1 *Introduction*

Antisense oligonucleotides are designed to modulate the information transfer from the gene to the protein *via* altering the metabolism of RNA, which provides an effective tool to shut down the synthesis of offending proteins. The idea of using oligonucleotides (ON) to target nucleic acids in a sequence-dependent manner and, therefore, specifically inhibit synthesis of the chosen target protein was demonstrated by Zamecnik and co-workers in 1978.^{1,2} Oligonucleotides based therapeutics allow the specific recognition of the targeted sequence in the nucleic acids, which derives from the selectivity of Watson-Crick³ (duplex formation) or Hoogsteen^{4,5} (triplex formation) types of base pairing. Targeting of genomic sequences *via* the triplex formation with ds DNA (antigene approach) or targeting mRNA *via* hybrid duplex formation with antisense oligonucleotide (AON) (antisense approach) are efficient ways to selectively control gene expression on the transcriptional and translational levels, respectively.⁶⁻⁹ Targeting to the gene itself presents several advantages: the targeted gene is present in just two alleles whereas there may be thousands of copies of a mRNA; inactivation of mRNA (antisense approach) does not prevent the corresponding gene from repopulating the RNA pool. To get a desirable pharmacological effect at physiological conditions the triplex forming ONs must possess high binding affinity to the target. Higher affinity will also mean more successful competition of ON with nucleic acids tertiary structures and nucleic acids binding proteins. In the early 1980s, Letsinger and co-workers¹⁰ attached a phenanthridinium derivative to the deoxythymidine dinucleotide, which resulted in the formation of very stable complex with poly(rA). Later, residues of acridine,¹¹⁻¹⁴ phenazinium,¹⁵⁻¹⁸ ethidium^{16,19,20} and ellipticine²¹ were incorporated to the oligonucleotides. It was shown that chemical nature of the tethered chromophore dictates the strength of the π -stacking interactions between the chromophore and adjacent base pair of the duplex, which becomes the driving force for the duplex stabilization in aqueous solution.^{22,23} Large number of different functionalities including anthracene,^{24,25} anthraquinone,²⁶⁻³⁰ azidoethidium,²⁰ imidazole,²⁹ stilbene,³⁰ peptides,³¹⁻³⁵ Hoechst 33258^{36,37} and many others,³⁸⁻⁵⁵ have been conjugated to the oligonucleotides, and were shown to improve the stability of the complementary complexes *via* the intercalation or groove-binding mechanism. The stabilization effect for each particular tether is sensitive to the neighboring nucleotide residues and depends on the position of the attachment.⁴ Generally, conjugation of the chromophores at the 5'-end of an oligonucleotide leads to the formation of more stable complexes compared to the 3'-conjugation.⁴ The position of the intercalator

containing linker arm within the oligonucleotide is also of central importance. Intercalating agents such as *N*-hydroxyethyl-phenazinium⁵⁶, 2-methoxy-6-chloro-9-aminoacridine⁵⁷, ethidium⁵⁸, daunomycine⁴¹ have been conjugated to the ONs through terminal phosphates. Groove-binding agent such as Hoechst 33258 has been introduced at the 5'-end³⁷ and in the middle⁵⁹ of the oligo-DNAs, showing enhancement of the stability of duplexes and triplexes. Intercalating derivatives have also been attached to the *N*-2 of purine and *C*-5 of pyrimidine bases in the oligonucleotides.^{24,60-62} Binding properties of the resulting ON conjugates have shown⁶² to be dependent on the location of the modified blocks within the ON chain. Internucleotidic phosphate has also been used⁶³ for incorporation of acridine into the middle of the deoxyoligonucleotide. Intercalating agents such as pyrene,⁶⁴ anthracene⁶⁴ and phenazine⁶⁵ have been linked to the *C*-1' position of the 3'-deoxypsicosofuranosyluridine located at the interior^{64,65} or at the ends⁶⁵ of an oligonucleotide sequence to study the stabilities of duplexes^{64,65} and triplexes⁶⁵. The 2'-hydroxy group of ribonucleosides has also been used for tethering different polyaromatic systems, *e.g.* 2'-*O*-(1-anthraquinonylmethyl)uridine²⁷ and 2'-*O*-(1-pyrenylmethyl)uridine,⁶⁶ which were introduced at different positions of ONs by solid-phase synthesis. Acridine has been incorporated at the 5'-end of the ON chain using an abasic phosphoramidite⁶⁷ and to the internal abasic spacers by post-synthetic⁶⁸ tethering to the depurination site of a deoxy-ON. All these studies show that the stabilizing properties of intercalators are sequence-specific, and they also strongly depend on the chemical character and length of the linker. Probably in each particular case the proper balance has to be found for the length of the linker to be long enough to provide the delivery and optimal orientation of the stacker in the base pair region on the one hand, and on the other hand, it should be short to ensure the lowest increase in the entropy brought by this flexible linker.

A few reports have demonstrated that triplex forming ONs can indeed reach the nucleus, and form a complex with the target.⁶⁹⁻⁷¹ Accessibility of nuclear DNA to an antigenic ONs was shown on a sequence present in the integrated HIV-1 proviral genome targeted with prosalen tethered ONs⁷² and on a sequence in a human endogenous gene coding for the chemokine receptor CCR5 targeted with an ON conjugated to an alkylating reagent⁷³. However, the main limitations of the triplex strategy are as follows⁷⁴: (i) requirement for an oligopurine target sequence in DNA, which limits the choice of potential target sequences, and (ii) competition with DNA binding proteins in the nucleus (the chromatin structure, for example, is a parameter which is largely unknown for most of the potential target sequences).

The alternative to antigene approach in regulation of gene expression is to alter the intermediary metabolism of RNA (antisense approach – AON is designed to bind to the mRNA). Initially the antisense effect was envisioned as direct competition between the AON/RNA duplex and the macromolecular complex responsible for the translation of the mRNA. If the AON binds to the target RNA strongly enough, it will prevent gene expression by steric blocking of the ribosomal machinery.⁷⁵⁻⁷⁸ It was shown that in order to achieve hybrid-arrest translation the AON strand should cover part of the RNA extending from the cap site to about 15 nucleotides downstream of the initiation AUG.⁷⁹ However, once fully assembled, the ribosome has an ability to melt out any AON/RNA complexes formed along the mRNA, which means that steric blocking can prevent only the initiation step of translation. The fact that target mRNA did not always remain intact during this process suggested that AONs could induce the degradation of the RNA part of the AON/RNA hybrid by RNase H.^{80,81} The RNase H-mediated effects of the AONs on translation have been demonstrated in cell-free extracts^{82,83} and eukaryotic cells.⁸⁴⁻⁸⁶ These findings showed that it is possible not only to block specifically viral mRNA, but also to irreversibly destroy it. RNase H is an endogenous enzyme, which hydrolyses the RNA strand in an RNA/DNA hybrid duplex in a catalytic manner.^{87,88} It produces short oligonucleotides with 5'-phosphate and 3'-hydroxy groups as final products.⁸⁹ Bivalent cations as Mg^{2+} and Mn^{2+} are found to be necessary cofactors for enzymatic activity.^{88,90,91} The enzyme is widely present in various organisms,⁸⁹ including retroviruses, as a domain of the reverse transcriptase.⁹² The RNase H1 from *Escherichia coli* is the most characterized enzyme in this family.⁹³⁻⁹⁵ Even though the physiological functions of *E. coli* RNase H1 have not been understood clearly, it has been suggested to be involved in DNA replication and repair.⁹⁶ This enzyme was found to be required for the initiation of Col E1 DNA replication *in vitro*.^{97,98} It was suggested that after RNA has served as a primer, it is eliminated from product by RNase H. This enzyme is also involved in the chromosomal DNA replication.⁹⁹⁻¹⁰²

AON mediated RNase H degradation of the target mRNA can occur at any stage of gene expression: splicing, translation or reverse transcription. Inhibition of gene expression by this mechanism has obvious advantages over hybrid arrest: permanent inactivation of unwanted genetic information, and catalytical mechanism of action where the AON strand freed from the cleaved RNA is able to bind a second mRNA molecule and induce its cleavage. RNase H promoted cleavage became one of the strategies to treat viral infections.^{103,104} Recent isolation of the human RNase H1 and RNase H2 highlights the importance of the development of the antisense drugs utilizing this mechanism of action.¹⁰⁵⁻¹⁰⁸

To become a successful drug candidate, AON should meet a number of requirements. It should have high affinity to the target RNA and the resulting RNA/DNA hybrid duplex must be a suitable substrate for RNase H. Another issue for nucleic acid based therapeutics is the fate of AON in the cellular media.¹⁰⁹⁻¹¹¹ It was shown that removal of bases from the 3' end of the ON is the major pathway for metabolic degradation in plasma.¹¹²⁻¹¹⁵ Therefore in order to reach maximal therapeutic effect without using high concentration doses (which can result in toxic and immunostimulatory effects¹¹⁶⁻¹²¹) the stability of AON towards 3'-exonucleases should be improved.

2'-Deoxyphosphorothioates (PS), the first generation of AON in clinical trials,¹²² possess higher nuclease resistance compared to the phosphodiester oligonucleotides (PO) and activate RNase H to cleave the target RNA. Nevertheless PS-AONs have a few major drawbacks¹²²: (i) the stability of the RNA/DNA duplexes decreases by 0.5-1°C with introduction of each PS linkage, resulting in poor duplex formation at low AON concentrations. (ii) administration of higher concentrations or use of a longer PS-AONs can cause negative side effects, including inhibition of RNase H, because of their potency for non-specific binding to proteins.¹²³

In pursuit of improved binding affinity of AONs, modified internucleotide linkers, heterocyclic bases, or sugar moieties have been introduced: different 2'-*O*-alkyl¹²⁴ and 2'-*O*-aminoalkyl^{122,125} as well as 2'-fluoro¹²⁶ modifications in ribose resulted in significant increase of the duplex stability (about 2°C per modification) and locked nucleic acids (LNA) demonstrated an unprecedented ΔT_m of 46°C for a 9 base pairs long DNA/LNA duplex relative to native DNA/RNA hybrid.¹²⁷ Unfortunately all these modifications drive the sugar into the C3'-*endo* conformation typical for the A-type RNA/RNA duplex¹²⁸ which results in complete loss of the RNase H activity. However, arabinonucleic acids (ANA) and 2'-deoxy-2'-fluoro-arabinonucleic acids (2'F-ANA) were shown¹²⁹⁻¹³¹ to be substrates for RNase H. RNase H activity of the duplexes formed by 2'F-ANA was comparable with the corresponding non-modified substrates, and lower activity observed for ANA/RNA duplexes was attributed to their lower thermodynamic stability.¹²⁹ AONs possessing various 4'-*C* and 5'-*C* modifications in 2'-deoxyribose moiety have been reported to serve as substrates for RNase H with efficiency comparable with those of the non-modified DNAs irrespective of their lower affinity to the RNA target.^{132,133} Very recently cyclohexene nucleic acids (CeNA) were shown to have enhanced affinity to the target RNA, and resulting CeNA/RNA duplexes were susceptible to RNase H cleavage, although to a slightly lower extent compared to the native counterpart.¹³⁴ Although morpholino oligonucleotides,¹³⁵ AONs consisting of α -nucleotides,¹³⁶ HNA¹³⁷ as well as PNA¹³⁸ exhibited strong binding ability towards RNA, their, however, were not found to be suitable substrates for RNase H.

Among various backbone modifications, only phosphorothioates^{139,140} and boranophosphates¹⁴¹ were shown to support RNase H hydrolysis. Other modifications like methylphosphonates,^{142,143} phosphoro-*N*-morpholidates¹⁴⁴, phosphoro-*N*-butylamides,¹⁴⁴ methylenemethyl-imines¹⁴⁵ and N3'→P5' phosphoramidates¹⁴⁶ did not elicit RNase H activity. The high affinity of the above AONs towards RNA could still be exploited. The first approach arises from the RNase H-independent mechanism of action of AONs.¹²² If strongly bound to the target RNA such AON can interfere with metabolic processes associated with the mRNA (translation arrest). The second approach¹²² resulted in appearance of the second generation of AONs – chimeric oligonucleotides with terminals consisting of modified nucleotides to provide high affinity towards target RNA and high nuclease resistance, and middle part of the oligo (PO or PS-backbone) to support RNase H cleavage.¹⁴⁷⁻¹⁵⁰ Oligonucleotides containing 2'-deoxyuridine and 2'-deoxycytidine nucleosides bearing propyne, butyne or dimethylthiazole moieties at C5¹⁵¹ as well as the recently reported 9-(aminoethoxy)phenoxazine analogue of cytosine (G-clamp)¹⁵² showed enhanced binding affinity towards RNA but did not improve the ability to recruit RNase H compared to non-modified antisense oligo-DNAs.

In this context, any modifications which can allow the use of relatively short AO without loss of DNA/RNA duplex stability and preserved RNase H competency would be of considerable interest. The use of short AONs could provide a cost-effective solution for nucleic acids based therapeutics. In mammalian cell an AON as short as 11mer or perhaps shorter could identify and bind to an unique RNA sequence.^{153,154} Gene-selective, mismatch sensitive and RNase H-dependent inhibition of SV40 large T antigen was reported for heptamer antisense phosphorothioate oligonucleotides.¹⁵⁵ However, shorter AONs have lower affinity to the target. Conjugation of various chromophores and hydrophobic moieties to AONs can dramatically improve binding properties of the AONs toward RNA. However, only few of these conjugated AONs have been investigated on their ability to activate RNase H. Acridine-conjugated AONs, targeted to β -globin mRNA, were tested for RNase H potency in wheat germ extract which has the RNase H activity, and it was found that the acridine-linked 11mer AON was more potent inhibitor of β -globin synthesis than the unmodified counterpart.¹⁵⁶ Cholesterol residue was conjugated to the 3'- or 5'-end of the AON, targeted to 27bp fragment of *Ha-ras* oncogene mRNA, and tested for their RNase H eliciting power.¹⁵⁷ Results showed that at low concentration, the conjugated AONs were able to promote higher extent of target RNA hydrolysis by RNase H compared to the non-conjugated counterpart. It was shown¹⁵⁸ that tethering of chromophores to the 5'-end of the AONs increased the affinity of the AONs to the RNA target, and yet the heteroduplexes were found to retain global structures very similar to the corresponding native AON/RNA duplexes. The RNA component in these 5'-modified AON/RNA

heteroduplexes was found to be improved substrate of the RNase H hydrolysis compared to the native counterpart. Still there is very little known about how the chemical nature of the chromophore and its location within the hybrid AON/RNA duplex will influence the rate of RNase H promoted hydrolysis of RNA moiety and the cleavage pattern.

1.2 Present work

1.2.1 Synthesis of 5'-tethered AONs (Paper I)

The strength of the π - π interactions between aromatic systems in aqueous solutions has been shown to be influenced by the surface area, electrostatics, polarisability and hydrophobicity of these interacting

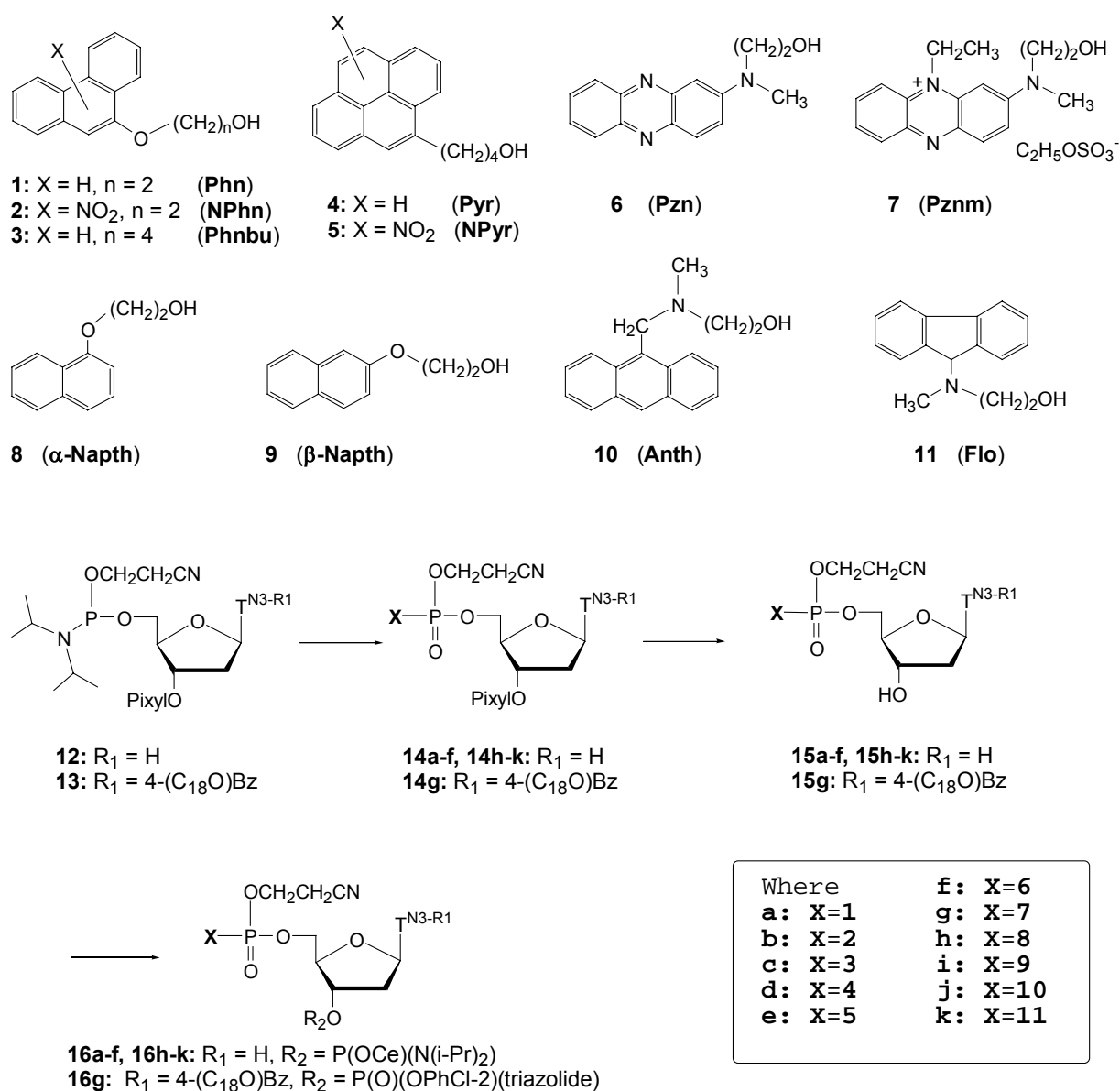


Figure 1: Synthesis of the chromophore-tethered blocks for the ONs synthesis

systems.^{22,159,160} To investigate the influence of changes in the character of the tethered fluorophore on the physicochemical properties of their duplexes (DNA/DNA and DNA/RNA) and triplexes, a series of planar polycyclic aromatic hydroxyalkylated fluorophores **1-11** (Figure 1) with different geometry, bulk, electron density, polarisability as well as hydrophobicity were conjugated to the oligodeoxynucleotides. Hydroxyalkyl tethers were used for the attachment of the chromophores through the terminal phosphate group at the 5'-end of the AONs. For synthesizing Phn (**1**), Phnbu (**3**), α -Naph (**8**) and β -Naph (**9**), the corresponding commercially available polycyclic phenols (9-hydroxyphenanthrene, 1-naphthol and 2-naphthol respectively) were alkylated with 1-*O*-DMTr-(CH₂)_n-CH₂-Cl (*n* = 1 or 3), in presence of equimolar amount of potassium *tert*-butoxide, followed by removal of the DMTr protecting group with acetic acid. The Pznm derivative (**7**) was synthesized by coupling of 9-*N*-ethylated phenazine⁵⁶ with 2-(*N*-methylamino)ethanol. For preparation of the Pzn derivative, after coupling of 9-*N*-methyl phenazinium¹⁶¹ with 2-(*N*-methylamino)ethanol, the 9-*N*-methyl group was removed with aqueous ammonia to afford **6**. 9-(chloromethyl)anthracene or 9-bromofluorene was reacted with 2-(*N*-methylamino)ethanol to give Anth (**10**) or Flo (**11**). Mono-nitration of Phn (**1**) or Pyr (**4**) was achieved by treatment of their acetyl-protected derivatives with HNO₃ in acetic acid and acetic anhydride at cold, giving NPhn (**2**) and NPyr (**5**) respectively, each one as a mixture of three different mononitro isomers.

Table 1: Sequences of the 5'-chromophore tethered AONs and their targets

Duplex studies		Triplex studies
5'-d(TCCAAACAT)-3'-p-X	Modification	5'-d(TCT ₆ CT ₆ CTT)-3'-p-X
17	-	31
18	X=1 (Phn)	32
19	X=2 (NPhn)	33
20	X=3 (Phnbu)	34
21	X=4 (Pyr)	35
22	X=5 (NPyr)	36
23	X=6 (Pzn)	37
24	X=7 (Pznm)	38
25	X=8 (α -Naph)	39
26	X=9 (β -Naph)	40
27	X=10 (Anth)	41
28	X=11 (Flo)	42
Single strand targets		Duplex targets
29 5'-d(CATGTTTGGG)-3'		43 5'-d(GCGTCT ₆ CT ₆ CTTGGC)-3'
30 5'-d(CATGTTTGGAC)-3'		5'-d(CGCAGA ₆ GA ₆ GAACCG)-3'

Resulting hydroxyalkyl chromophores were condensed with nucleoside 5'-phosphoramidite blocks (**12**, **13**) followed by standard oxidation procedure giving the corresponding phosphotriesters (**14a-k**).

Compounds **14a-k** were subsequently deprotected and converted to the corresponding 3'-amidites which were used in the solid phase synthesis (Table 1).

1.2.2 Thermal denaturation and fluorescence studies on duplexes and triplexes formed by the 5'-tethered ONs (Paper I)

All chromophore tethered AONs formed more stable duplexes compared to the native counterpart (Table 2). It is seen that angular Phn provided higher duplex stabilization ($\Delta T_m = 11.9^\circ \text{C}$, with target **30**) than the linear Anth ($\Delta T_m = 8^\circ \text{C}$, with target **30**) with both targets. Of the two naphthalene derivatives the β -isomer gave slightly higher stabilization ($\Delta T_m = 6.6^\circ \text{C}$, with target **29**) compared to the α -isomer ($\Delta T_m = 6.1^\circ \text{C}$, with target **29**). Flo showed the poorest stabilization effect on both targets ($\Delta T_m = 4$ and 4.8°C). Pzn provided the duplex stabilization ($\Delta T_m = 10$ and 11.5°C) close to that of Phn derivatives. The Pznm chromophore, which has lower electron density compared to the Pzn demonstrated slightly higher stabilization. Also nitration of Phn system resulted in marginal increase of the duplex stability. However, nitration of Pyr had negative effect on the duplex stability ($\Delta \Delta T_m \sim -1.5^\circ \text{C}$). Increase of the duplex length by two methylenes for the Phn chromophore resulted in decrease of the stabilization effect.

Table 2: Melting temperatures of the AON/DNA duplexes formed with targets **29** and **30**

9mer AONs	10mer target (29)		11mer target (30)	
	T_m	ΔT_m	T_m	ΔT_m
17	26.2	-	25.9	-
18	38.0	11.8	37.8	11.9
19	40.0	13.8	38.2	12.3
20	33.4	7.2	34.2	8.3
21	35.1	8.9	34.0	8.1
22	33.7	7.5	32.2	6.3
23	37.7	11.5	35.9	10.0
24	36.5	10.3	38.1	12.2
25	32.3	6.1	31.0	5.1
26	32.8	6.6	32.5	6.6
27	33.0	6.8	33.9	8.0
28	31.0	4.8	30.2	4.3

Triplex forming pyrimidine-rich 18mer AONs were expected to form Hoogsteen base pair in a parallel orientation^{162,163} to the complementary purine-rich duplex target. The stabilizing effect of the polyaromatic tethers on triplex was poorer compared to that on duplex (Table 3). Here also the angular Phn was more efficient stacker compared to linear Anth ($\Delta T_m = 4$ and 0.5°C respectively). Decrease of electron density of the Phn ring system by nitration made triplex more stable at all pHs. The same was

found when the pyrene chromophore ($\Delta T_m \sim 2.5\text{-}4^\circ \text{C}$) was converted to the mononitropyrene ($\Delta T_m \sim 4.5\text{-}5.5^\circ \text{C}$). Increasing the linker length on Phn resulted in increase of the triplex stability. Pzn tethered AON generated triplexes with the stability comparable to that obtained with Phn. Interestingly, no triplex formation was observed with the Pznm tether at any pH.

These results clearly show that linkers of different sizes will be beneficial for the optimal triplex stabilization compared to the duplex. Four atoms between the chromophore ring and the phosphorous of the 5'-phosphate gave higher duplex stability than the linker containing 6 atoms. The reverse was observed for the triplexes. Also, increasing bulk and hydrophobicity of the chromophore produced some

Table 3: Melting temperatures of the triplexes formed with duplex target **43**

18mer AONs	pH 7.3		pH 6.5		pH 6.0	
	T_m	ΔT_m	T_m	ΔT_m	T_m	ΔT_m
31	13.5	-	21.5	-	25.6	-
32	17.5	4.0	24.8	3.3	30.0	4.4
33	18.2	4.7	25.6	4.1	31.0	5.4
34	18.7	5.2	25.0	3.5	n.d.	-
35	16.0	2.5	23.9	2.4	29.5	3.9
36	17.9	4.4	25.9	4.4	31.0	5.4
37	17.4	3.9	24.3	2.8	30.3	4.7
38	n.d.	-	n.d.	-	n.d.	-
39	12.3	-1.2	19.6	-1.9	22.5	-3.1
40	14.3	0.8	21.4	-0.1	25.6	0.0
41	14.0	0.5	19.2	-2.3	26.9	1.3
42	13.0	-0.5	21.8	0.3	24.9	-0.7

duplex stabilization (compare Pyr (**21**) and Phn (**20**)) with butyl linker, however, no such correlation was observed with triplexes. Changes in the geometry of the chromophore showed that our angular polyaromatic tethers are better stackers compared to the linear systems. Decrease of the electron density of the stacker by nitration increased the stabilization effect, probably due to the more efficient π - π electron interactions with nuclear bases (more efficient donor-acceptor charge transfer) and less π - π electron repulsion. It is noteworthy, that practically no difference in stability of the duplexes formed by the targets **29** and **30** were observed.

Fluorescence properties of the chromophores were shown to be affected by their interaction with the nucleic acids, the nature of the chromophore, oligonucleotide sequence, the way the chromophore is tethered, the mode of binding^{24,37,164,165} as well as the hydration.¹⁶⁶ Both quenching and enhancement of the fluorescence have been observed with different chromophores upon the duplex or triplex formation.^{25,24,61,167-173} Large changes in the fluorescence intensity (ΔF) have been correlated to strong

intercalation of the chromophore between the nucleic bases^{14,170} or deep embedment into the duplex minor groove,^{36,172} while small or moderate changes have been attributed to exterior binding of the chromophore to the stacked nucleobases. Therefore, fluorescence measurements provide a tool for qualitative understanding of the mode in which the chromophore binds to the nucleic acids structures.

Very moderate changes in fluorescence was found upon the formation of the duplexes, showing only minute changes in the microenvironment of the chromophore. This suggests stacking of the chromophores with nucleic bases of the opposite strand of the duplex. The exception was observed for the Pznm tethered AON (**24**), where 5-fold decrease of ΔF was monitored. This result is in agreement with reported NMR studies,¹⁷ shown that phenazinium ion indeed intercalates between the nucleotide residues. No correlation between the ΔT_m and ΔF values of the corresponding duplexes was established, which is consistent with earlier observations suggesting that ΔF reflects more the change in the microenvironment of the chromophore rather than thermal stability of the complex.¹⁴

Comparison of the fluorescence intensities of the chromophore tethered triplexes also demonstrated only moderate changes in ΔF , indicating weak exterior binding of the chromophores to the nucleobases. The only exception was found for the pyrene conjugate, where 30-fold increase of ΔF was found.

1.2.3 *Synthesis of the AONs tethered with Pzn through the arabino- and xylo-nucleotides* (Paper II)

The 2-(*N*-(2-hydroxyethyl)-*N*-methyl)aminophenazine (**6**) was synthesized from 9-methyl phenazinium methylsulphate (**44**).^{161,174} A minor modification of this procedure gave Pzn derivatives with butyl and hexyl linkers, as in **45** and **46**, which were converted to the corresponding phenoxyacetyl (Pac) derivatives **47** and **48** (Figure 2). These derivatives were coupled to the corresponding *ara*- and *xylo*-uridine amidites (**51**) and (**63**). Pzn was introduced to the 5'-end of the oligonucleotides using the phosphoramidite **49** (Figure 2), which was prepared from 2-(*N*-(2-hydroxyethyl)-*N*-methyl)aminophenazine **49** in the usual way.¹⁷⁵

Preparation of the *ara*-uridine derivatives was carried out starting from (1,1,3,3-tetraisopropyl-1,3-disiloxanediyl)*ara*-uridine **50**.¹⁷⁶ The amidite **51** was then condensed with 2-(*N*-(2-hydroxyethyl)-*N*-methyl)aminophenazine **6**, 2-(*N*-(4-hydroxybutyl)-*N*-phenoxyacetyl)aminophenazine **45** and with 2-(*N*-(6-hydroxyhexyl)-*N*-phenoxyacetyl)aminophenazine **46** (Figure 2) through tetrazole activation,¹⁷⁷ followed by oxidation by iodine to give the phosphotriester blocks **52a-c** respectively. These compounds

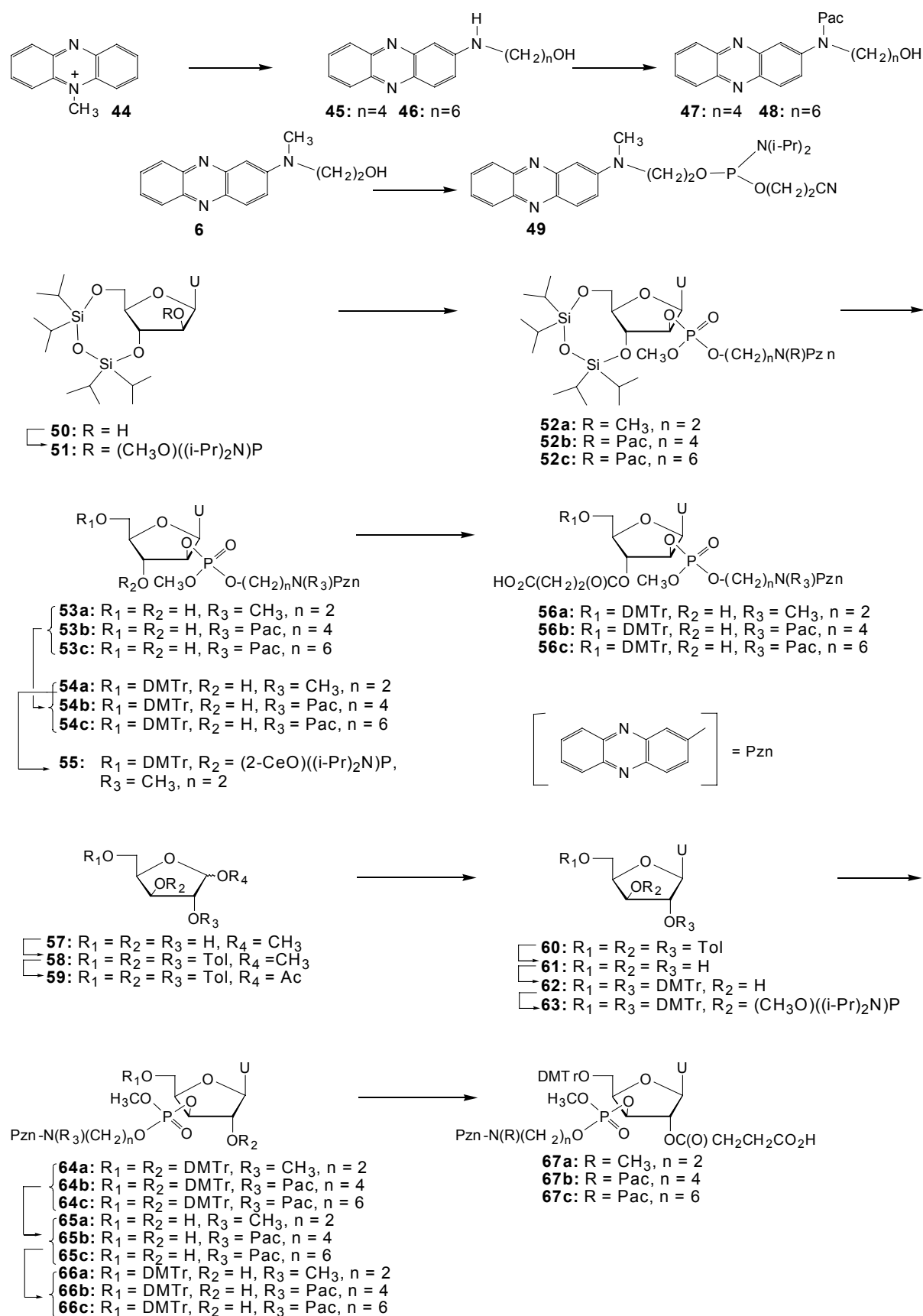


Figure 2: Synthesis of Pzn tethered *ara*-U and *xylo*-U blocks

were treated with *n*-tetrabutyl ammonium fluoride (TBAF) in THF to give the corresponding stable 2'-*ara* phosphotriester blocks, which were converted to their corresponding 5'-*O*-DMTr-derivatives **54a-c** and subsequently to the 3'-*O*-amidite (**55**) or 3'-*O*-succinyl derivatives (**56a-c**). Succinates were coupled to the 3-aminopropyl-CPG support to enable 3'-end derivatization of the oligonucleotides.

Synthesis of Pzn-tethered *xylo*-uridine derivatives started from an easily available mixture of α - and β -methyl-D-xylofuranosides **57**.¹⁷⁸ This anomeric mixture was *p*-toluoylated, converted to the corresponding 1-*O*-acetyl derivative **59**, and used for glycosylation with silylated uracil to give 1-(2', 3', 5'-tri-*O*-(*p*-toluyl)- β -D-xylofuranosyl)uracil **60**. Compound **60** was deprotected with NH₃/MeOH to give fully deprotected *xylo*-uridine **61**, which was converted to an isomeric mixture of 2', 5'-di-*O*-DMTr-*xylo*-U and 3', 5'-di-*O*-DMTr-*xylo*-U in 8:1 ratio. From this pure 2', 5'-di-*O*-DMTr-derivative **62** (79 %) was isolated. Compound **62** was converted to the corresponding amidite **63** and coupled to Pzn-linkers **6**, **47** or **48** to give the corresponding triester-blocks **64a-c**. Deprotection of these derivatives with trichloroacetic acid in dichloromethane-methanol mixture (9:1, v/v) gave 3'-*O*-Pzn tethered *xylo*-U-blocks **65a-c**. The 5'-*O*-DMTr-derivatives **66a-c** and their 2'-*O*-succinates **67a-c** were made in the same way as for compounds **54a-c** and **56a-c**, respectively (Figure 2).

1.2.4 Thermal denaturation and fluorescence studies on duplexes and triplexes formed by the Pzn-tethered ONs (Paper II)

All oligonucleotides modified with Pzn-tethered *ara*-U block (ONs **68-71** in Table 4) showed enhanced affinity to the target, excluding the middle-modified ON **73**, which failed to form any duplex (Table 4). The Pzn-tethered *ara*-U moiety at the 5'-end of the oligonucleotide **69** provided slightly less stabilization ($\Delta T_m = +8.2^\circ \text{C}$) compared to the previously designed 5'-Pzn-tethered dT block (ON **23**, $\Delta T_m = +10.0^\circ \text{C}$)¹⁷⁴. It was also observed that the effect of the Pzn-tethered *ara*-U block at the 3'-end of ON is slightly higher (ON **68**, $\Delta T_m = +8.7^\circ \text{C}$) than that at the 5'-end (ON **69**, $\Delta T_m = +8.2^\circ \text{C}$). As expected, employment of the Pzn-tethered *ara*-U block at both 3'- and 5'-termini of oligo-DNA (**70**), or Pzn-tethered *ara*-U block at 3'-end along with Pzn-tethered dT block at the 5'-end (**71**) resulted in further enhancement in duplex stability ($\Delta T_m = +11.4$ and $+14.4^\circ \text{C}$, respectively). Pzn-tethered *ara*-U moiety, conjugated at the 3'-end of the triplex forming 18-mer ON (**74**) provided remarkable triplex stabilization ($\Delta T_m = +16.1^\circ \text{C}$ in Table 4), which was much higher than 5'-end *ara*-U (ON **75**, $\Delta T_m =$

+3.3° C) or previously designed 5'-Pzn dT (ON **76**, $\Delta T_m = +3.9^\circ \text{C}$) modifications. Consistent together with dT block at the 5'-end of with our above observation on duplex studies, Pzn-tethered *ara*-U block

Table 4: Sequences of the ONs with their targets and melting temperatures of the corresponding complexes

Duplexes				Triplexes			
Oligo	Oligo sequence and place of modification	T_m (°C)	ΔT_m (°C)	Oligo	Oligo sequence and place of modification	T_m (°C)	ΔT_m (°C)
17	5'-d(TCCAAACAT)-3'	25.9	-	31	5'-d(TTCT ₆ CT ₆ CT)-3'	13.5	-
68	5'-d(TCCAAACA <u>U</u>)-3'	34.6	8.7	74	5'-d(TTCT ₆ CT ₆ C <u>U</u>)-3'	29.6	16.1
69	5'-d(<u>U</u> CCAAACAT)-3'	34.1	8.2	75	5'-d(<u>U</u> TCT ₆ CT ₆ CT)-3'	16.8	3.3
70	5'-d(<u>U</u> CCAAACA <u>U</u>)-3'	37.3	11.4	76	5'-d(<u>T</u> TCT ₆ CT ₆ CT)-3'	17.4	3.9
23	5'-d(<u>T</u> CCAAACAT)-3'	35.9	10.0	77	5'-d(<u>T</u> TCT ₆ CT ₆ C <u>U</u>)-3'	33.1	19.6
71	5'-d(<u>T</u> CCAAACA <u>U</u>)-3'	40.3	14.4	78	5'-d(<u>U</u> TCT ₆ CT ₆ C <u>U</u>)-3'	24.8	11.3
72	5'-d(TCCATACAT)-3' ^a	22.4	-	79	5'-d(TTCT ₆ C <u>U</u> T ₅ CT)-3'	n.d.	-
73	5'-d(TCCA <u>U</u> ACAT)-3' ^a	n.d.	-	43	5'-d(GCCAAGA ₆ GA ₆ GACGC)-3'	Duplex target	
30	5'-d(CATGTTTGGAC)-3'	ss DNA Target			5'-d(CGGTTCT ₆ CT ₆ CTGCG)-3'		

Conditions: 1μM each strand concentration in 20 mM PO₄³⁻ and 100 mM NaCl, pH 7.3. U = Pzn-tethered *ara*-U block, T = Pzn tethered to the 5' of dT. ^a Were hybridized with target **80**: 5'-d(CATGTATGGAC)-3'

at the 3'-end oligonucleotide, as in **77**, gave considerably improved stability of the triplex ($\Delta T_m = +19.6^\circ \text{C}$). As with duplexes, no triplex formation was detected with 18-mer ON having Pzn-tethered *ara*-U block in the middle (ON **79**).

Having obtained these data, we decided to explore the possibility to improve our results by optimization of the chain length of the tether, and changing the site of the sugar moiety of the nucleoside, which is used for anchoring the tethered intercalator, *i.e.* substitution of the *arabino*-uridine by the *xylo*-uridine. Since the oligo-DNAs modified with the Pzn-tethered *ara*-U block at the 3'-terminal formed the most stable double and triple helixes, we prepared a number of duplex and triplex forming ONs modified at the 3'-ends with *ara*-U and *xylo*-U nucleosides tethered to the Pzn through the butyl and hexyl linkers.

Table 5: Melting temperatures of the duplexes and triplexes formed by the 3'-end modified ONs with Pzn moiety tethered through the linkers of different length

Duplex Study: 5'-d(TCCAAACA <u>U</u>)-3'				Triplex study: 5'-d(TTCT ₆ CT ₆ C <u>U</u>)-3'			
Oligo	Tether	T_m (°C)	ΔT_m (°C)	Oligo	Tether	T_m (°C)	ΔT_m (°C)
81	53b	30	4.1	86	53b	26.2	12.7
82	53c	31.6	5.7	87	53c	30.0	16.5
83	65a	31.8	5.9	88	65a	21.5	8.0
84	65b	29.9	4.0	89	65b	25.8	12.3
85	65c	28.2	2.3	90	65c	24.4	10.9

All duplexes as well as triplexes formed by ONs modified with Pzn-tethered *xylo*-U blocks showed weaker stability than those formed by ONs modified with *ara*-analogs with the same length of the linker arms (Table 5). In case of both *ara*-U and *xylo*-U modified ONs increase of the linker size to the butyl and the hexyl decreased duplex stability compared to the ethyl linker. However, in the case of the triplex forming ONs the greatest stabilization was achieved with butyl linker (ON **87**, $\Delta T_m = 16.5^\circ \text{C}$). Further chain elongation resulted in reduced duplex stability.

All homo duplex forming ONs, prepared in this work, were also tested for their ability to act as AON to form stable hybrid duplexes with complementary RNA target. Higher affinity of the modified oligos to the RNA compared to the native counterpart would suggest their potential usefulness in the antisense approach in inhibiting viral RNA translation and expression.

Table 6: Melting temperatures of the AON/RNA hybrid duplexes

DNA/RNA hybrid	T_m ($^\circ\text{C}$)	ΔT_m ($^\circ\text{C}$)	DNA/RNA hybrid	T_m ($^\circ\text{C}$)	ΔT_m ($^\circ\text{C}$)
17 + 91	20.4	-	81 + 91	25.0	4.6
72 + 92	25.7	-	82 + 91	27.1	6.7
68 + 91	29.1	8.7	83 + 91	25.6	5.2
69 + 91	25.8	5.4	84 + 91	25.1	4.7
70 + 91	31.3	10.9	85 + 91	21.8	1.4
23 + 91	29.3	8.9	73 + 92	n.d.	-
71 + 91	32.6	11.7			

Where RNA target **91**: 5'-r(CAUGUUUGGAC)-3' and RNA target **92**: 5'-r(CAUGUAUGGAC)-3'

All tested modifications were shown to enhance the thermodynamic stability of the corresponding AON/RNA hybrid duplexes (Table 6). The results showed that, similar to the DNA/DNA duplexes, the best stabilization of the AON/RNA hybrids can be achieved with the short ethyl linker for *ara*-U (**68**) and *xylo*-U (**83**) modified AONs ($\Delta T_m = +8.7$ and 5.2°C respectively). Also the AONs modified with Pzn tethered *ara*-U block provided greater stabilization compared to *xylo*-U analogues with all linker sizes.

Fluorescence experiments showed absence of any significant changes in the chromophores fluorescence intensities upon the DNA/DNA and DNA/RNA duplex as well as triplex formation. Enhancement of thermal stability of duplexes by phenazine without any significant change in its fluorescent character (no changes in its microenvironment upon the complex formation) suggests that phenazine moiety is binding weakly to the exterior of the heterocyclic bases of nucleic acids complexes.

The above results show that tethering Pzn through the 2'-hydroxyl of the *ara*-U nucleoside to the 3'-terminal of the ONs provides a significant increase in triplex stability and is much more efficient

compared to the previously used 5'-end tethering. Also this type of modification increases thermal stability of the corresponding DNA/RNA hybrid duplexes, suggesting such ONs as potential antigene (high affinity to the double-stranded DNA target through the triplex formation) and antisense (improved affinity towards the target RNA) therapeutics.

1.2.5 Synthesis of dipyridophenazine-tethered AONs (Paper III)

To be able to attach dipyridophenazine (Dppz) to the linkers (in collaboration with Mr D. Ossipov) dipyrido[3,2-a;2',3'-c]phenazine-11-carboxylic acid (**94**)¹⁷⁹ was prepared by condensation of 1,10-

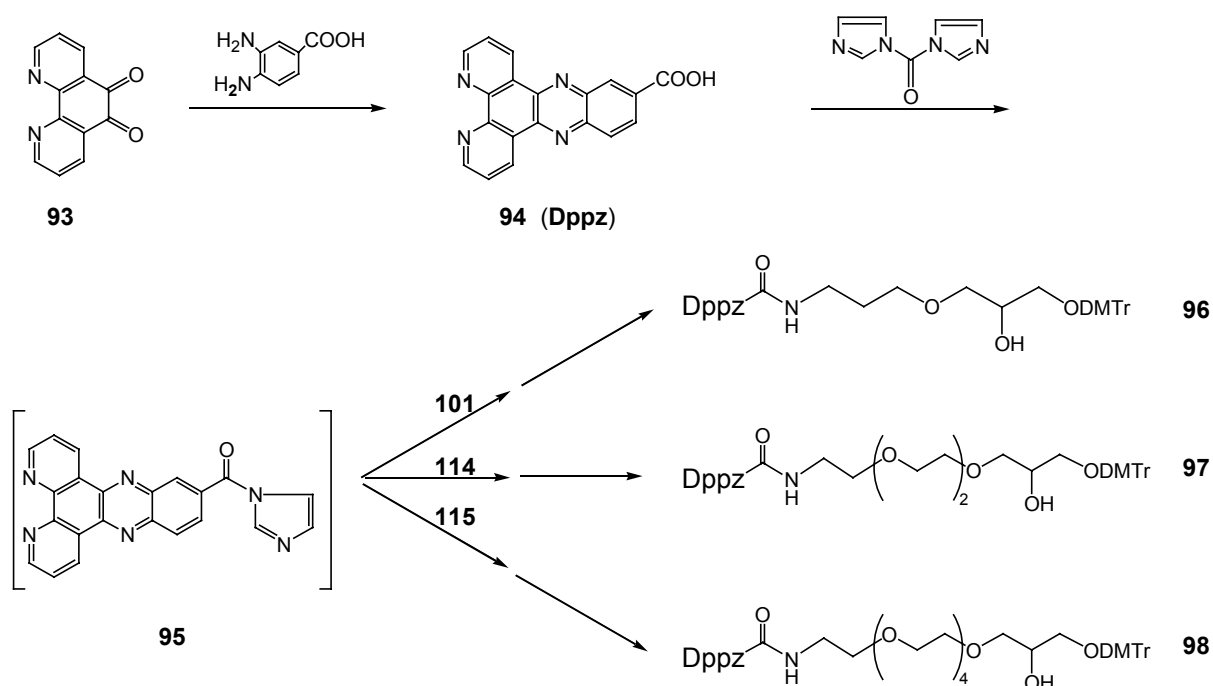


Figure 3: Synthesis of Dppz-tethered amidites with linkers of different length

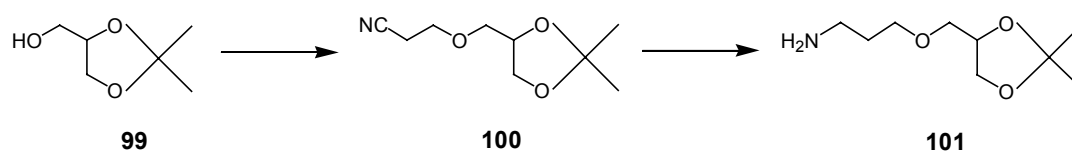
phenanthroline-5,6-dione **93** with 3,4-diaminobenzoic acid in ethanol. Heating a suspension of 11-dipyridophenazine carboxylic acid and *N,N'*-carbonyldiimidazole in pyridine led to the quantitative formation of acylimidazole **95**, which upon reaction with the amino function of the linkers (**101**, **114**, **115**) gave the corresponding amides (**96**, **97**, **98**).

Linkers based on the glycerol moiety extended with ethylene glycol spacers of different length were chosen for the attachment of the Dppz to the oligonucleotides because of their conformational flexibility and good aqueous solubility. The shortest propane-1,3-diyl linker arm^{180,181} was prepared from readily available solketal (2,2-dimethyl-1,3-dioxolane-4-methanol) **99**, which was converted to the 3-

(alkyloxy)propanenitrile **100**. The latter was reduced with NaBH₄ (cobalt(II) chloride as catalyst) to afford corresponding amino derivative **101**.

To prepare longer arms, monotosylates of tri and penta(ethylene glycol) **104** and **105** were treated with phthalimide in presence of 1,8-diazabicyclo[5.4.0]undec-7-ene at 80° C to afford the protected precursors (**106** and **107**) of the amino linkers. The reaction of **106** and **107** with allyl bromide in the presence of sodium hydride in THF-DMF afforded the corresponding olefins **108** and **109**, which were then oxidized with permanganate to the respective diols **110** and **111**. The primary hydroxyls of **110** and **111** were then protected with a 4,4-dimethoxytrityl and resulting compounds then subjected to the aminolysis with methylamine to give finally amines **114** and **115**.

Short linker



Long linkers

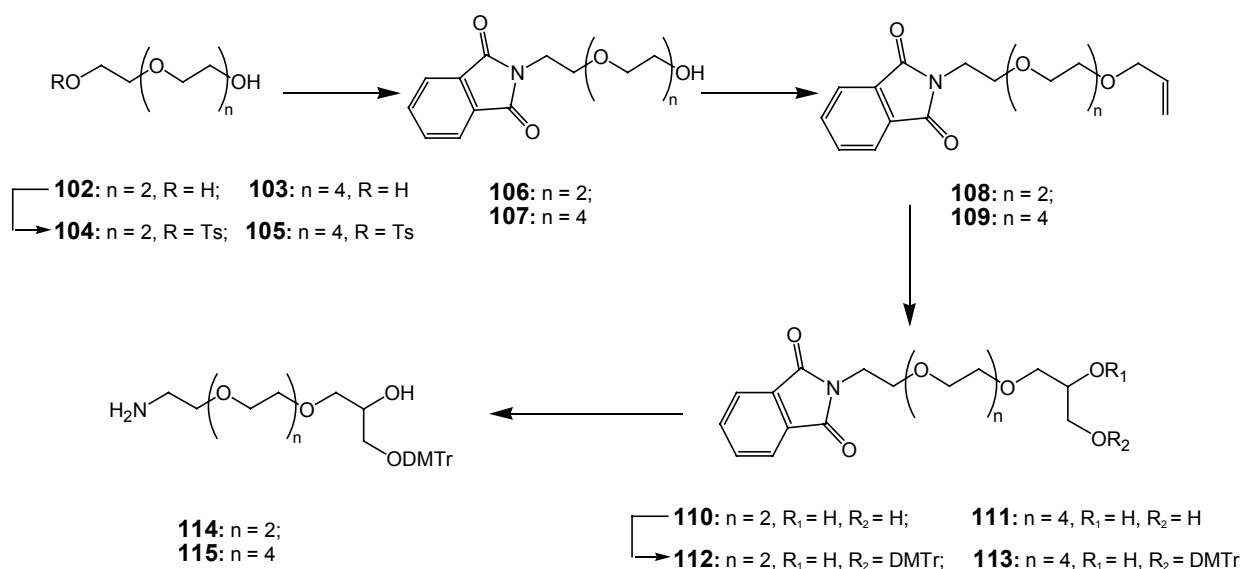


Figure 4: Synthesis of linkers

1.2.6 Thermal stability of the duplexes and triplexes formed by the Dppz-tethered ONs (Paper III)

DNA/DNA and DNA/RNA duplexes as well as triplexes (Table 7) were formed by mixing the appropriate ONs with their targets in 1:1 ratio (1 μ M each strand concentration). The melting temperatures obtained (in collaboration with Mr D. Ossipov) for these complexes (Table 7) showed that

Table 7: Sequences of the Dppz-tethered ONs and melting temperatures of the corresponding ON/DNA, AON/RNA duplexes and triplexes

Duplexes						
Oligo	Oligo sequence and place of modification (M)	Linker type	DNA/DNA duplex		DNA/RNA duplex	
			T _m (°C)	Δ T _m (°C)	T _m (°C)	Δ T _m (°C)
17	5'-d(TCCAAACAT)-3'	-	25.0	-	20.6	-
116	5'-d(MTCCAAACAT)-3'	96	33.5	7.6	25.2	4.6
117		97	36.8	10.9	26.1	5.4
118		98	33.2	7.3	25.2	4.9
119	5'-d(TCCAAACATM)-3'	96	34.3	8.4	27.1	6.5
120		97	33.2	7.3	25.2	4.5
121		98	35.6	9.7	28.0	7.4
122	5'-d(TCCAMAACAT)-3'	96	29.0	3.1	19.2	-1.5
123		97	30.1	4.2	21.5	0.9
124		98	26.4	0.5	21.1	0.4
125	5'-d(TCCAMACAT)-3'	96	14.7	-11.2	n.d.	n.d.
126		97	14.6	-11.3	n.d.	n.d.
127		98	n.d.	n.d.	n.d.	n.d.
Triplexes						
Oligo	Oligo sequence and place of modification (M)	Linker type	DNA/DNA/DNA Triplex			
			T _m (°C)	Δ T _m (°C)		
31	5'-d(TTCT ₆ CT ₆ CT)-3'	-	13.5	-		
128	5'-d(MTTCT ₆ CT ₆ CT)-3'	96	24.6	11.1		
129		97	20.9	7.4		
130		98	17.3	3.8		
131	5'-d(TTCT ₆ CT ₆ CTM)-3'	96	22.6	9.1		
132		97	20.3	6.8		
133		98	20.5	7.0		
134	5'-d(TTCT ₆ MCT ₆ CT)-3'	96	n.d.	n.d.		
135		97	20.6	7.1		
136		98	n.d.	n.d.		
137	5'-d(TTCT ₆ MT ₆ CT)-3'	96	15.8	2.3		
138		97	n.d.	n.d.		
139		98	n.d.	n.d.		
30	Single-strand DNA target		5'-d(CATGTTTGGAC)-3'			
91	Single-strand RNA target		5'-d(CAUGUUUGGAC)-3'			
43	Duplex target		5'-d(GCCAAGA ₆ GA ₆ GACGC)-3'			
			5'-d(CGGTTCT ₆ CT ₆ CTGCG)-3'			

n.d. = not determined. Conditions: 1 μ M each strand concentration in 20 mM PO₄³⁻ and 100 mM NaCl, pH 7.3.

tethering of Dppz moiety at the 5'- or 3'-end of the ON increases stability of both ON/DNA and AON/RNA duplexes, and in general the overall stability and stabilizing effect of the Dppz tethers were greater for the ON/DNA duplexes compared to AON/RNA hybrids. In the case of the 5'-tethered ONs maximal stability was achieved with 12-atom linker (AON **117**, $\Delta T_m = 10.9^\circ \text{C}$ for DNA/DNA duplex and 5.4°C for DNA/RNA duplex). However, when tethered to the 3'-end of the oligo, 7- and 18-atom linkers (AONs **119** and **121**) provide greater stability for both DNA/DNA and DNA/RNA duplexes. Incorporation of the Dppz in the middle region of the AON (as in **122**, **123** and **124**) might have slight stabilizing as well as destabilizing effect ($\Delta T_m = -1.5$ - 4.2°C) depending on the linker size, showing how sensitive the middle part of the duplex is towards the structural distortions brought by the flexible (with high degree of entropy) tethers. All triple helices were stabilized by introduction of Dppz to the 5'- or 3'-terminal of the ON as compared to the non-modified counterpart, yet there were significant variations in the triplex stabilities depending on the place of attachment (3'- or 5'-end of the ON) and the length of the linker arm (Table 7). The greatest stabilization ($T_m = 24.6^\circ \text{C}$, $\Delta T_m = 11.1^\circ \text{C}$) was observed with ON **128**, where Dppz moiety was introduced at the 5'-end of the oligo through the short 7-atom linker. Increase of the linker length (ON **130**) led to the significant drop in triplex stability ($\Delta T_m = 3.8^\circ \text{C}$). The same pattern was observed for the 3'-modified ONs (**131-133**). Probably the increase of entropy with long linker arms plays destabilizing role in the Dppz modified triplex complexes. In the case of middle modified triplex forming ONs, only two of them demonstrated enhanced stability: ON **137** ($\Delta T_m = 2.3^\circ \text{C}$) and AON **135** ($\Delta T_m = 7.1^\circ \text{C}$). This again shows that for triplexes as also in the case of the middle-modified ON/DNA and AON/RNA duplexes, introduction of a conformationally unconstrained tether in the center of the nucleic acids complex might have significant destabilizing effect, and the nature along with the length of the linker arm play a crucial role in thermal stability of such complexes.

1.2.7 *Physicochemical characterization of the RNA targets used in evaluation of the antisense potency of the Pzn and Dppz tethered AONs (Paper IV)*

Folding of the target mRNA in aqueous solution is one of the critical factors in evaluating the antisense potency of the AONs. An oligo-RNA, depending upon the size/length and the specific sequence, is known to form various structural motifs (duplex, triplex, hairpin, pseudoknots etc),¹⁸²⁻¹⁸⁵ resulting from either intramolecular or intermolecular self-assembly processes. Stability of such folded structures will dictate the kinetic and thermodynamic accessibility of the target RNA sequence to the AONs. The question remains which part of the complex mRNA structure - the single-stranded (loop) part or the duplex part - should be targeted to provide the best availability for the AONs to form the hybrid duplex,

followed by RNase H assisted cleavage. In few reports AONs were targeted to the mRNA and accessible sites were identified by RT-PCR assays.^{186,187} Many of those reports failed to establish a clear correlation between the features of the local RNA structure in the target and activity of the AONs to elicit RNase H response. Recent work¹⁸⁸ has failed to show the correlation between the folded structures of RNA predicted by computational methods^{189,190} and the RNase H response on the resulting AON/RNA hybrid duplexes. Although a practical solution for the problem of finding the optimal antisense sequence for the target RNA has been already suggested through the assay based on combinatorial AONs arrays,¹⁸⁶ a better understanding of the mechanism of the RNase H/substrate (*i.e.* RNase H/AON/RNA hybrid duplex) interactions is necessary to understand and predict the *kinetic* accessibility of the target RNA.

Cleavage by the RNase H is a three-component process, which requires the presence of the all three participants: the RNA target, the AON and the RNase H. Experimental dissection of this complex process in to separate simplified steps [*i.e.* (i) binding of the AON to the target RNA and then (ii) the study of RNase H cleavage reaction] might result in misleading conclusions. This becomes clear from some recent reports, showing that the RNase H is the active participant in the AON/RNA duplex formation.¹⁹¹

To evaluate how the tertiary structures of the target RNA^{158,191,192} affect the binding to our AONs, we have chosen one 11mer RNA (**91**) and two longer 17mer RNA targets (**140**) and (**141**) with different self-folding capacities. The self-aggregation of these RNA targets was examined by temperature-dependent CD experiments and by UV thermal denaturation studies.

RNA targets:

(91):	5'-r(CAUGUUUGGAC)-3'	(non-aggregated)
(140):	5'-r(ACUCAUGUUUGGACUCU)-3'	(low-aggregated)
(141):	5'-r(UAACAUGUUUGGACUCU)-3'	(highly-aggregated)

Figure 5: Sequences of the RNA targets used to evaluate the antisense potency of the AONs

Thermal denaturation studies with the 11mer RNA (**91**) at 1 and 5 μ M concentrations showed the absence of any structural transition for this target. Melting experiments with low-aggregated 17mer RNA (**140**) did not show a clear sigmoidal transition although some hyperchromic effect was observed. In contrast, typical monophasic melting behavior was observed for the highly-aggregated 17mer RNA (**141**), which was found to be concentration dependent, and allowed us to calculate the thermodynamics of its self-aggregation process: $\Delta H^\circ = -342$ kJ/mol, $-\Delta S^\circ = -303.9$ kJ/mol and $\Delta G^\circ_{298} = -38.3$ kJ/mol.

Temperature-dependent CD experiments were performed to explore the tertiary structure formation of the RNA targets. Nucleic acids ellipticity is modulated by disorienting (melting) or reorienting (formation of structural forms) nucleic acid chromophores.¹⁹³ Thus it can be used for qualitative justification of the extent of the structural assembly. This was particularly valuable in the case of low-

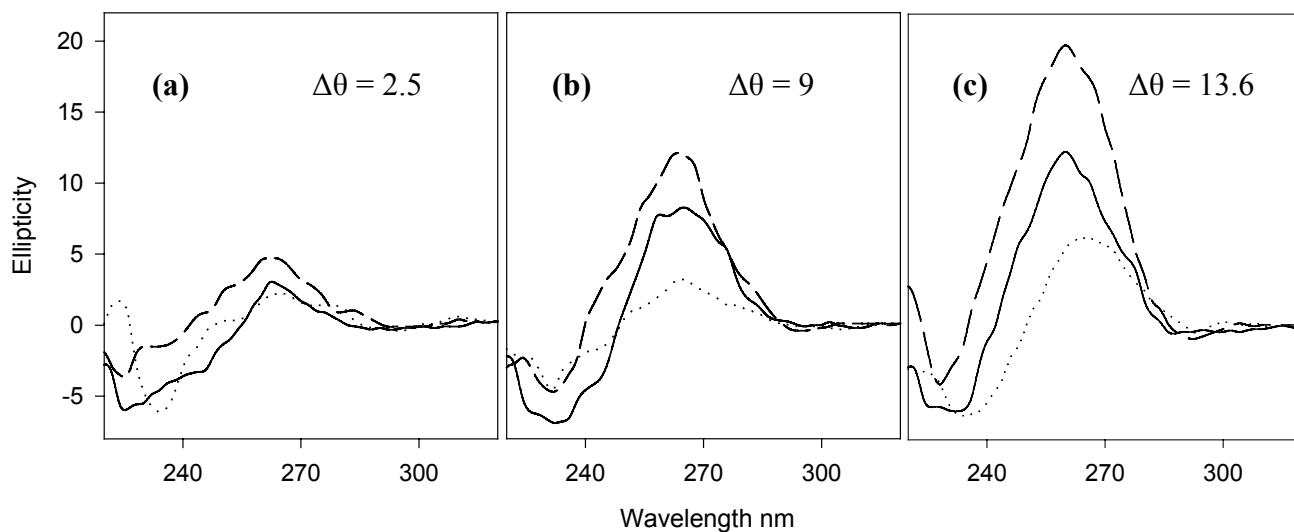


Figure 6: Temperature dependent CD spectra of (a) 11mer RNA (**91**), (b) low-aggregated 17mer RNA (**140**) and (c) highly-aggregated 17mer RNA (**141**) targets at (---) 6°C, (—) 20°C and (·····) 50°C. The extent of the RNA folding is reflected in the value of $\Delta\theta$ (in 20×10^{-2} mdeg), which is calculated as $\theta_{6^\circ\text{C}}$ minus $\theta_{50^\circ\text{C}}$.

aggregated 17mer RNA (**140**), which failed to show any clear transition in the UV melting experiments. The CD spectra of all three RNA targets were recorded at three different temperatures. Ellipticity of the 11mer RNA target (**91**) remained almost unchanged upon heating (Figure 6a), suggesting an absence of any significant tertiary structure. Ellipticity of the highly-aggregated RNA target (**141**) displayed very strong temperature dependency (Figure 6c), suggesting a high degree of structural organization. Although the low-aggregated 17mer RNA (**140**) did not show any clear transition in UV melting experiments, it did, however, exhibit a definite (but less pronounced compared to **141**) temperature dependency of ellipticity (Figure 6b). Consistent with the earlier observation,¹⁹⁴ temperature-dependent CD has been also found in this work to be a more sensitive tool to monitor structural transitions in nucleic acids than the UV. Clear temperature-dependent change of ellipticities at 265 nm were observed for both low- and high-aggregated 17mer RNA targets **141** and **142** (Figures 7a and c) by CD. The melting temperatures found by CD showed strong concentration dependency: the T_m values at 1 and 2.5

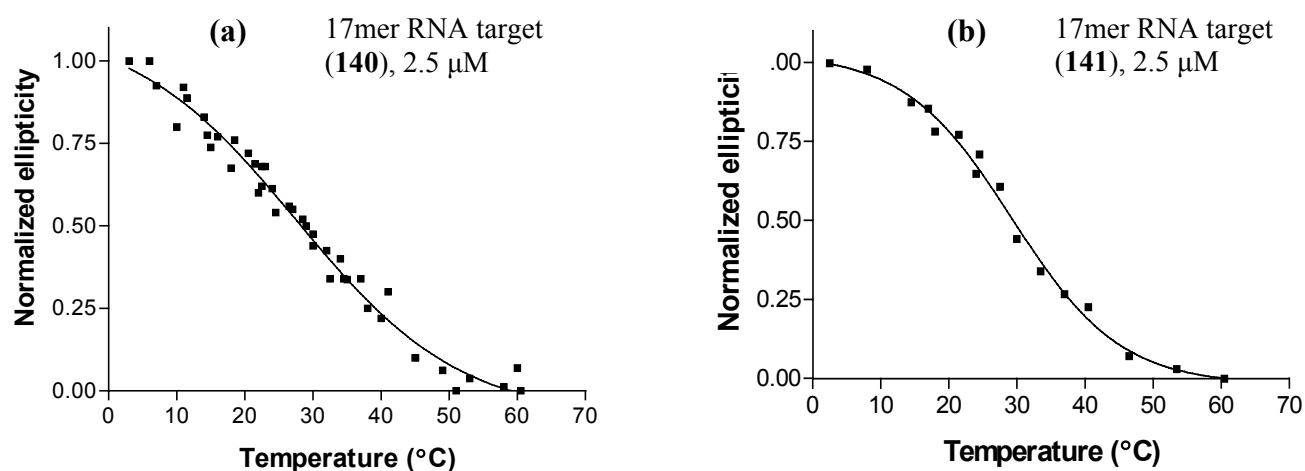


Figure 7: Plots (a) and (b) are typical melting curves for the low- (140) and highly-aggregated (141) RNA targets respectively, obtained by fitting the sigmoidal function with variable slopes into the ellipticity vs temperature plots.

μM RNA concentrations were found to be 24.7 and 27.7 °C for the target (140), and 28.8 and 29.8 °C for the target (141), showing that the self-aggregation process to form RNA/RNA duplexes in solution by these target RNAs indeed results from the bimolecular interstrand interactions (Figure 8).

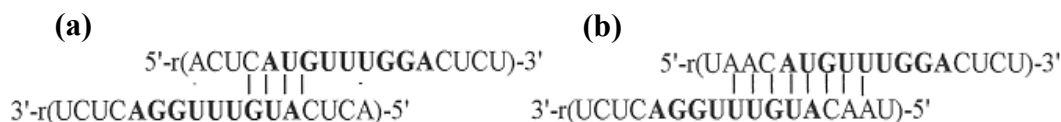


Figure 8: Proposed structures of RNA/RNA duplexes formed by (a) low-aggregated 17mer RNA target (140) and (b) highly-aggregated 17mer RNA target (141), resulting from intermolecular self-aggregation in solution. Bold letters represent the sequence complementary to the AONs

1.2.8 Thermodynamic characteristics of the AON/RNA hybrid duplexes formed by Pzn and Dppz tethered AONs with phosphodiester (PO) and thiophosphodiester (PS) backbone (Paper IV)

The use of the AONs producing more stable AON/RNA hybrid duplexes compared to the native counterpart will provide a possibility to achieve complete target RNA hybridization at much lower AON concentrations. Also, such AONs are expected to be more successful in competing with RNA folded structures to gain more access to the target sequence. All chromophore-conjugated (both PO and PS, Figure 9) AON/RNA duplexes formed with non-aggregated RNA target (91) demonstrated higher stability compared to the non-conjugated counterparts. In all cases the value of pairing entropy (ΔS°), which is a measure of the conformational constraints of the oligo chain, was found to be higher than that

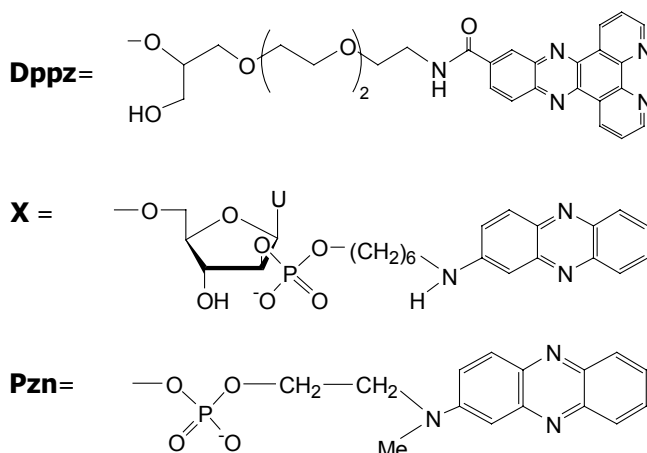
Antisense DNA oligonucleotides (AON):

Where:

Phosphodiester backbone (PO):

(17): 5'-TCCAAACAT-3'**(23):** **Pzn**-5'-TCCAAACAT-3'**(117):** **Dppz**-p-5'-TCCAAACAT-3'**(82):** 5'-TCCAAACAX-3'**(120):** 5'-TCCAAACAT-3'-**Dppz**

Phosphorothioate backbone (PS):

(142): 5'-TsCsCsAsAsCsAsT-3'**(143):** **Pzn**-5'-TsCsCsAsAsCsAsT-3'**(144):** **Dppz**-p-5'-TsCsCsAsAsCsAsT-3'**(145):** 5'-TsCsCsAsAsCsAsX-3'**(146):** 5'-TsCsCsAsAsCsAsT-3'-**Dppz****Figure 9:** Pzn and Dppz tethered AONs

for non-modified duplexes (Table 8), which can be attributed to the effect of the conformationally flexible tethers employed for the chromophore attachment. On the other hand, the additional π - π stacking between aromatic rings of the chromophore and base pairs of the duplex provides a significant gain in enthalpy (ΔH°). The 3'-modifications with both Pzn and Dppz resulted in more stable AON/RNA hybrids compared to the 5'-counterparts. Among all the duplexes tested, 3'-Dppz-AON/RNA hybrids (**120** among PO- and **146** among PS-AONs) were found to be the most stable, which was reflected in the net free energy of stabilization $\Delta\Delta G^\circ_{298}$ of -8.1 kJ/mol for PO and -3.9 kJ/mol for PS oligonucleotides, showing the better stacking capability of the Dppz group compared to the Pzn when it was attached to the 3'-end of oligonucleotide. When the Dppz group was tethered at the 5'-end (AONs **117** and **144**), the net free energy of duplex stabilization was lower ($\Delta\Delta G^\circ_{298} = -4.9$ kJ/mol for PO-oligo and -1.0 kJ/mol for PS-oligo) compared to the 5'-Pzn-AONs ($\Delta\Delta G^\circ_{298} = -6.0$ kJ/mol for PO-AON **23** and -1.7 kJ/mol for PS-AON **143**).

When the low-aggregated 17mer RNA target (**140**) was hybridized with the 9mer PO-AONs, clear helix-to-coil transitions were observed. All duplexes formed by modified PS-AONs with the low-aggregated 17mer RNA target (**140**) had T_m values very close to those of duplexes formed with the 11mer RNA target (**91**) (Table 9). The T_m of the duplex formed by the native AON (**17**) and the low-aggregated 17mer RNA target (**140**) was 2° C lower compared to that with the 11mer RNA target (**91**). The T_m s of the duplexes formed by the tethered PS-AONs and the low-aggregated 17mer RNA target (**140**) were

Table 8: Thermodynamic characteristics of the AON/RNA hybrid duplexes formed by PO- and PS-AONs with 11mer RNA target (**91**)

PO-AONs							
AON	T_m , (°C)	ΔT_m , (°C)	ΔH° , (kJ·mol ⁻¹)	ΔS° , (e.u)	$-T\Delta S^\circ$, (kJ·mol ⁻¹)	ΔG°_{298} , (kJ·mol ⁻¹)	$\Delta\Delta G^\circ_{298}$, (kJ·mol ⁻¹)
(17)	22.1	-	-226±8	-0.65±0.03	192.2	-33.8±0.5	-
(23)	28.7	6.5	-298±4.5	-0.87±0.01	258.0	-39.8±0.1	6
(117)	27.6	5.5	-286±20	-0.83±0.07	248.1	-38.7±2.8	4.9
(82)	28.6	6.5	-256±10	-0.73±0.03	-216.8	-39.2±1.5	5.4
(120)	30.6	8.5	-316±23	-0.92±0.07	274.5	-41.9±3.2	8.1
PS-AONs							
(142)	16.6	-	-220±14	-0.64±0.05	190.5	-29.5±1.6	-
(143)	19.1	2.4	-245±20	-0.72±0.06	214.0	-30.9±2.7	1.4
(144)	19.3	2.7	-279±14	-0.83±0.05	248.7	-30.6±1.3	1.1
(145)	19.7	3.1	-267±3.5	-0.79±0.01	236.1	-31.3±0.6	1.8
(146)	22.0	5.3	-257±15	-0.75±0.05	223.5	-33.4±1.5	3.9

somewhat lower compared to the T_m s of the corresponding duplexes with the 11mer RNA (**91**). No distinct transition was observed with the blank PS-AON (**142**) and the low-aggregated 17mer RNA target. The hybrids of the PS-AONs and the native AON (**17**) with 11mer RNA (**91**) had lower ΔG°_{298} of duplex formation (between 29.5 and 33.8 kJ/mol) compared to ΔG°_{298} of the tethered PO-AONs, therefore compete less effectively with RNA self-aggregation, which results in two competing structures, *i.e.* AON/RNA and RNA/RNA duplexes, in comparable concentrations in solution. This shows that the tethered chromophore thermodynamically assists in the shift of the two competing equilibria between the RNA/RNA and AON/RNA structures towards the AON/RNA hybrid duplex formation.

When AONs were hybridized with highly-aggregated 17mer RNA target (**141**), all the T_m s observed by UV were close to the T_m of the target itself (Table 9), which made it impossible to estimate the extent of the AON/RNA duplex formation. The free-energies of the duplex formation for all the tethered AONs with non-aggregated target were comparable (between 38.7-41.9 kJ/mol) with ΔG°_{298} for the RNA/RNA duplex formation of the 17mer RNA target (**141**) ($\Delta G^\circ_{298} = -38.4$ kJ/mol). This means that only a fraction of AON strands was able to form the desirable AON/RNA hybrid duplex, while a considerable portion of the RNA remained in the aggregated state.

Table 9: The T_{ms} (°C) observed when low-aggregated 17mer RNA target (**140**) and highly-aggregated 17mer RNA target (**141**) were hybridized with Pzn and Dppz tethered AONs.

AON/RNA	(17)	(23)	(117)	(82)	(120)	(142)	(143)	(144)	(145)	(146)
(140)	20.2	28.5	27.7	28.1	30.7	11-14 ^a	15.7	18.2	15.9	20.9
(141)	28.6	30.5	29.2	30.4	30.3	29.3	28.9	29.1	29.2	29.2

^aVery broad transition

These data show that involvement of the RNA in the formation of any tertiary structures affects the thermodynamic accessibility of the target sequence by the AONs, which should result in decreased potency of the AONs targeted to the aggregated RNA structures compared to the single-stranded counterpart.

1.2.9 Circular dichroism (CD) studies on the AON/RNA hybrid duplexes formed by Pzn and Dppz tethered AONs (Paper IV)

The CD spectroscopy is a very convenient way to determine the global helical structure of the nucleic acids complexes.¹⁹³ The special features of the CD spectra (the CD band intensities, wavelength of the CD bands maxima and minima as well as the crossover points) allow fast and easy classification of any nucleic acids complexes with respect to the canonical A (RNA/RNA) and B (DNA/DNA) structural classes. In agreement with X-ray,¹⁹⁵⁻¹⁹⁷ NMR,¹⁹⁸⁻²⁰² and other methods²⁰³⁻²⁰⁵ CD suggests^{206,207} that the conformation of DNA/RNA hybrid duplexes is intermediate between A- (RNA/RNA) and B- (DNA/DNA) type geometry, where the RNA strand is of the A-type and the DNA strand has a conformation close to the B-type.^{198,199} Shifts in the absorption bands or changes in the relative magnitudes of the Cotton effect reflect the change brought into the helical structure of the duplex upon any modification (nucleoside or backbone modifications in the AON strand), thereby allowing us to qualitatively estimate the alterations of the minor groove of the hybrid duplex, which has been assumed as one of the crucial factors responsible for the substrate recognition by the RNase H.^{198,199,208-210}

To investigate the effect of the tethered chromophores on the AON/RNA helix conformation, the CD spectra of duplexes formed by 9mer AONs (Figure 9) with the 11mer RNA target (**91**) were recorded at 16° C under identical conditions. All modified duplexes exhibited spectra supporting an intermediate structure between the A-type RNA/RNA and the B-type DNA/DNA hybrids, mimicking those of natural DNA/RNA hybrids (Figure 10). Thus the duplexes formed by modified AONs with 11mer RNA target (**91**) had CD spectra very similar to the blank duplex formed by the native AON (**17**) and RNA target (**91**), rather than those of the native DNA/DNA or RNA/RNA duplexes. The spectra of the modified duplexes had a positive band at 260-266 nm, a negative band at 234-240 nm and a

crossover point at 247-248 nm (the corresponding parameters for the non-modified duplex were 264, 232 and 248 nm, respectively). Changing the PO-backbone to PS did not alter the global conformation of the AON/RNA duplexes, which is in agreement with previous reports.²¹¹ For all tethered PS-AON/RNA duplexes, positive CD bands were observed at 262-265 nm, negative CD bands at 234-240 nm and crossover points at 252-254 nm, which were very close to the corresponding parameters found

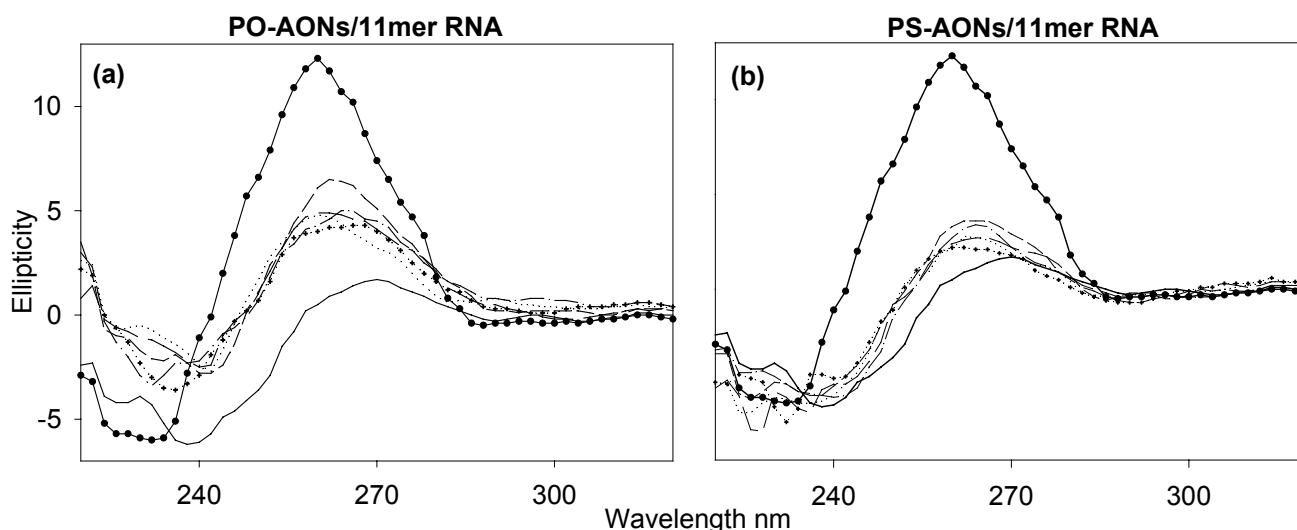


Figure 10: CD spectra of duplexes formed by AONs with 11mer RNA target (**91**) in RNase H digestion buffer at 16°C. **(a)** PO-AON/RNA hybrids: (---) blank oligo (**17**), (.....) (**23**), (•••••) (**117**), (— —) (**82**), (-----) (**120**). **(b)** PS-AON/RNA hybrids: (---) blank oligo (**142**), (.....) (**143**), (•••••) (**144**), (— —) (**145**), (-----) (**146**), (——) DNA/DNA (B-type) duplex formed by blank AON (**17**) and complementary 11mer DNA – 5'-d(CATGTTTGGAC)-3', (—•—•—) RNA/RNA (A-type) duplex formed by the highly-aggregated 17mer RNA (**141**)

for the natural hybrid duplex (264, 240 and 254 nm, respectively). Altogether these data showed that neither 3' nor 5' modifications altered the global helical structure of DNA/RNA hybrid, which suggested that the hybrid duplexes formed by the chromophore-tethered AONs had the potential to be as good substrates for RNase H as the natural DNA/RNA hybrids.

1.2.10 RNase H degradation studies on the AON/RNA hybrid duplexes formed by Pzn and Dppz tethered AONs (Paper IV, in collaboration with Mr D. Ossipov)

In previous reports^{192,212-215} as well as in this work, the tertiary structures of the RNA targets have been shown to affect the binding of the AONs. However, little information is available on the RNase H dependent antisense activity of AONs when they are targeted to the self-aggregated RNAs. Therefore, the ability of the chromophore-tethered AONs to promote RNase H cleavage was examined on three different RNAs having various degree of folded structures. 5'-³²P labeled RNAs were hybridized with

complementary AON strands and incubated with *Escherichia coli* RNase H. The cleavage progress was monitored by PAGE.

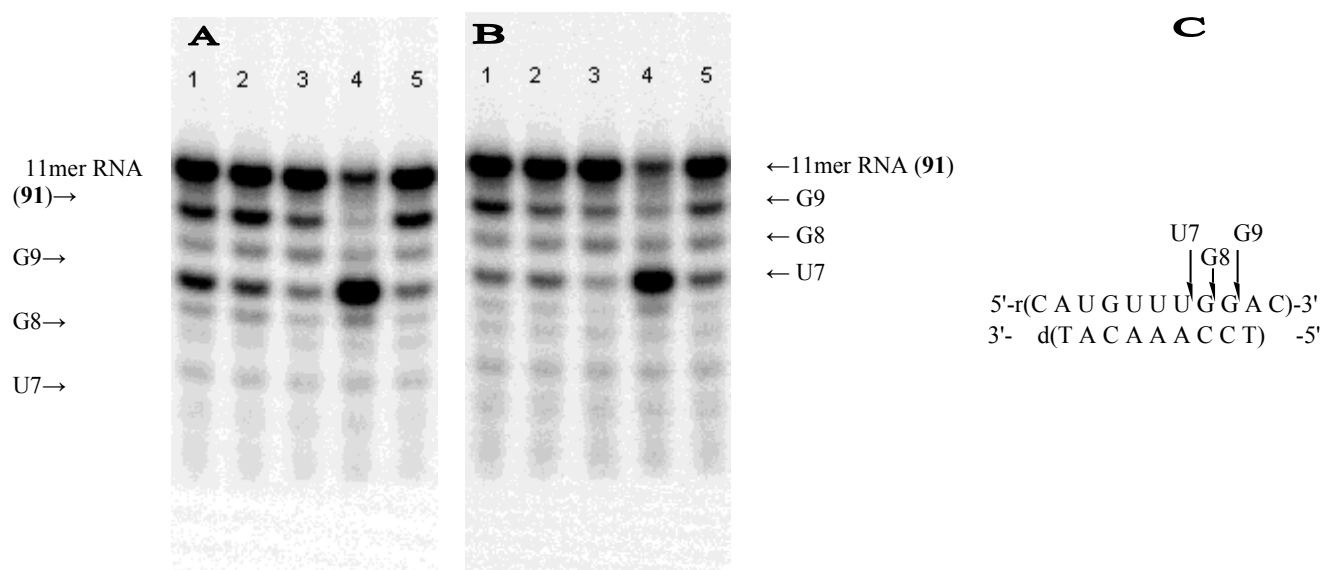


Fig 11: (A) RNase H hydrolysis of 11mer RNA (91) after 15 min of incubation when hybridized with PO-AONs: Lanes 1-5 correspond to the reactions with oligos (117), (23), (17), (120), (82) respectively. (B) RNase H hydrolysis of 11mer RNA (91) after 15 min of incubation when hybridized with PS-AONs: Lanes 1-5 correspond to the reactions with oligos (144), (143), (142), (146), (145) respectively (C) RNase H degradation pattern of 11mer RNA hybridized with modified and native 9mer AONs. Arrows indicate the major cleavage sites.

With short non-aggregated RNA target all modified PO-AONs promoted (after 2h of incubation with the enzyme) higher degree of RNA hydrolysis (72-92 %) compared to the natural counterpart (60 %). This is in good agreement with the thermodynamic stabilities (Table 8) of the corresponding duplexes, where all modified AON/RNA hybrids had higher stability compared to the native counterpart. It is noteworthy, that the 3'-Dppz-modified AON/RNA duplex was the most stable amongst all modified AON/RNA hybrids, and also caused the most rapid hydrolysis of the RNA strand. As evident from the PAGE pictures of the aliquots taken after 15 min of incubation with the enzyme (Figure 11a), the 3'-Dppz modified AON (120) not only promoted the highest extent of RNA hydrolysis, but also the fastest hydrolysis rate, reaching 83 % of the cleaved target RNA compared to 27 % for the blank duplex and 35-41% for the other modified substrates. It is noteworthy that all modified AON/RNA duplexes had the same cleavage sites (Figure 11C) as the native counterpart. The exception was found for the 3'-Dppz-conjugated hybrid, which showed site-specific hydrolysis, and had only one single cleavage site at the U7 position (Figure 11C). The extent of hydrolysis for PS-AON/RNA substrates (AONs (142-146) in Fig 11B) was slightly lower compared to the PO analogues as expected from the lower thermodynamic stability of these duplexes. As in the case of PO-analogues, the 3'-Dppz modification provided the

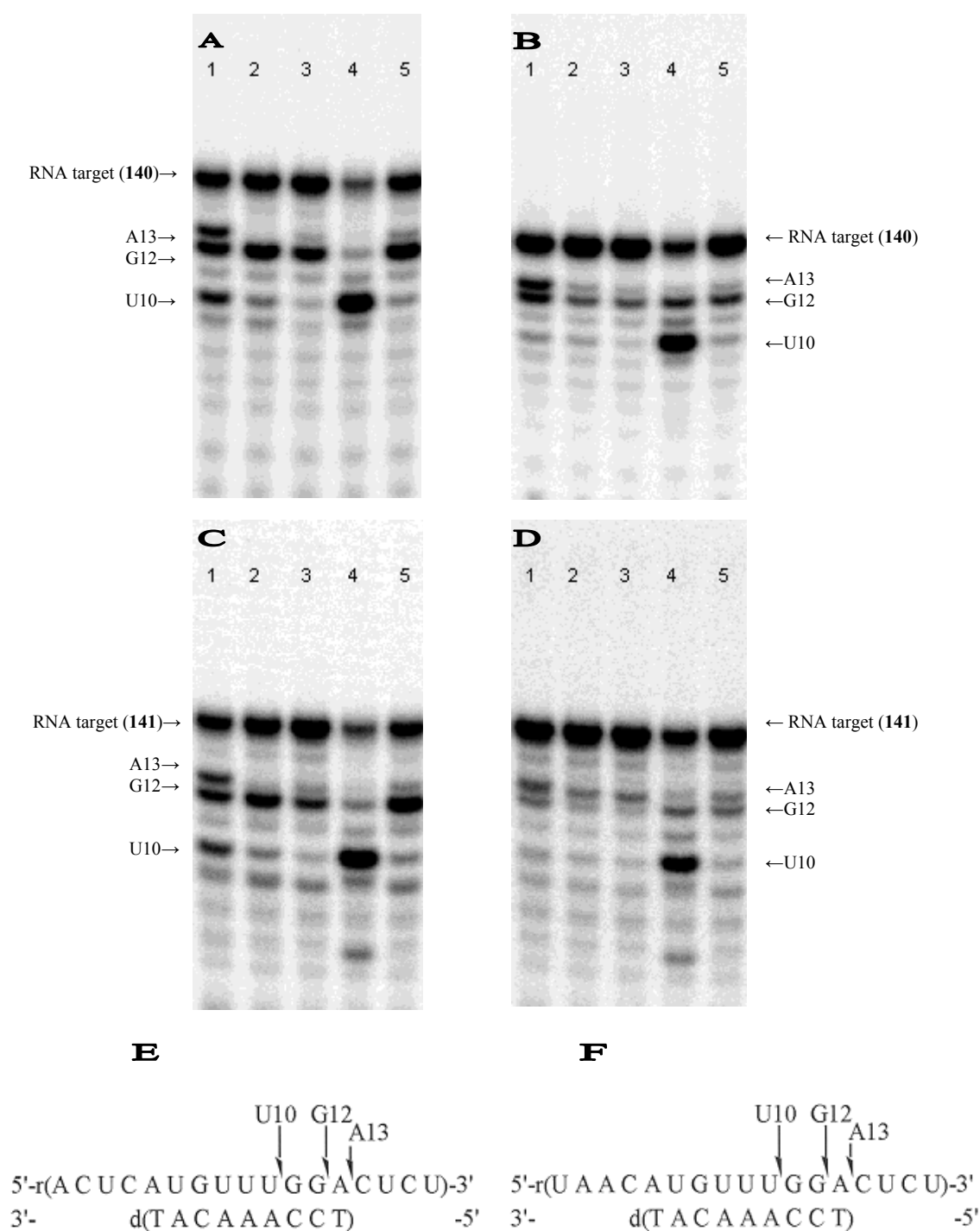


Fig 12: (A) RNase H hydrolysis of the low-aggregated 17mer RNA (140) hybridized with PO-AONs. (B) RNase H hydrolysis of the low-aggregated 17mer RNA (140) hybridized with PS-AONs. (C) RNase H hydrolysis of the highly-aggregated 17mer RNA (141) hybridized with PO-AONs. (D) RNase H hydrolysis of the highly-aggregated 17mer RNA (141) hybridized with PS-AONs. On each gel picture lanes 1-5 correspond to the reactions with oligos (117), (23), (17), (120), (82) (PO-AONs) or (144), (143), (142), (146), (145) (PS-AONs) respectively. RNase H degradation pattern of the low- (E) and the highly- (F) aggregated 17mer RNA targets hybridized with modified and native 9mer AONs. Arrows indicate the major cleavage sites.

highest degree of digestion (91 %) and the highest relative rate of hydrolysis. Changing the backbone from PO to PS did not alter the cleavage pattern for blank as well as for the modified duplexes. Again for the 3'-Dppz modified PS-AON/RNA duplex only a single cleavage site was observed at the same position as for PO counterpart (Figure 11b). Interestingly, when PO-AONs were hybridized with the low-aggregated or the highly-aggregated 17mer RNAs (**140**, **141**), hydrolysis rates remained approximately the same as those with the non-aggregated 11mer RNA (**91**). In the case of the PS-AONs a considerable decrease in antisense activity was observed only with the highly-aggregated 17mer RNA target (**141**) and not with the low-aggregated 17mer target RNA (**140**). This observation suggests that the rate of RNase H assisted conversion of the folded RNA structures to the single-stranded form, and subsequently its kinetic accessibility to drive the hybrid AON/RNA duplex formation is much faster than the RNase promoted cleavage rate of the RNA moiety in the hybrid, and the subsequent RNase H promoted cleavage of the hybrid is the slowest *i.e.* the rate-determining step of the process.

Both aggregated 17mer RNA targets (**140**) and (**141**) had fewer and common RNase H cleavage sites in contrast with the short non-aggregated 11mer RNA target (**91**) (Figures 12E and F), which shows that given similar nucleobase composition, different extent of the self-aggregation but identical AON hybridization sequence, the single strand accessibility of two differently folded 17mer RNAs (**140**) and (**141**) is the same in contrast to the relatively unfolded 11mer RNA target (**91**), where more single strand sites are accessible.

The important aspect of this work is that it shows that the general drawback of PS-AONs (relatively poorer thermodynamic stability of their duplexes with RNA compared to the native counterpart) as antisense drugs can be effectively compensated by tethering a suitable chromophore at the 3'- or at the 5'-end of the PS-AONs.

The fact that the hybrid duplexes with 3'-Dppz AONs and non-aggregated RNA target as well as 3'- and 5'-Dppz AONs with longer aggregated targets have different cleavage pattern compared to the non-modified and Pzn-modified AONs also shows that the nature of the chromophore directly contributes to the antisense effect of the AONs and suggests that, probably, factors other than thermodynamic stability of the AON/RNA duplex are also responsible for the enhancement of the RNase H potency observed with conjugated AONs (alteration of the duplex/RNase H interactions, for example).

1.2.9 Nuclease resistance of the 3'-Pzn and 3'-Dppz modified AONs (Paper IV)

To be suitable for pharmaceutical application, the AONs are required to be resistant to the nucleic-acids degrading enzymes.^{216,217} Natural oligonucleotides are very easily degraded by nucleases present in the cellular media with half-lives ranging from 10 min to 2 h.¹⁰⁹⁻¹¹⁵ A 3'-exonuclease activity resulting in sequential removal of the nucleotide bases from the 3'-end of the oligo was found to be responsible for most of the AON degradation in serum,¹¹²⁻¹¹⁵ therefore, modification of the 3'-end is expected to protect AON against degradation and increase its half-life in the cell. Introduction of methylphosphonate linkages²¹⁸ or 3'-conjugation of an inverted nucleotide unit²¹⁹ was shown to retard the exonuclease activity. Significant reduction of the extent of degradation was achieved by tethering different lipophilic and aromatic residues at the 3'-end of AON.^{220,221}

To explore the protective properties of 3'-Pzn and 3'-Dppz modifications, AONs (**82**), (**120**), (**145**) and (**146**) as well as non-tethered PO-AON (**17**) and PS-AON (**142**) were incubated with snake venom phosphodiesterase. Under experimental conditions non-modified PO-AON (**17**) had a half-life of 4 min, however, 3'-Pzn (**82**) and 3'-Dppz (**120**) PO-AONs did not show any sign of degradation even after 2 h of incubation (Fig 13A). Similar enhancement of stability was observed with the phosphorothioate analogues: the half-life for the non-modified PS-AON (**142**) was 30 min (higher concentration of snake venom phosphodiesterase compared to PO-AONs was used), when modified PS-AONs (**145**) and (**146**) remained more than 90 % intact after 2 h of incubation.

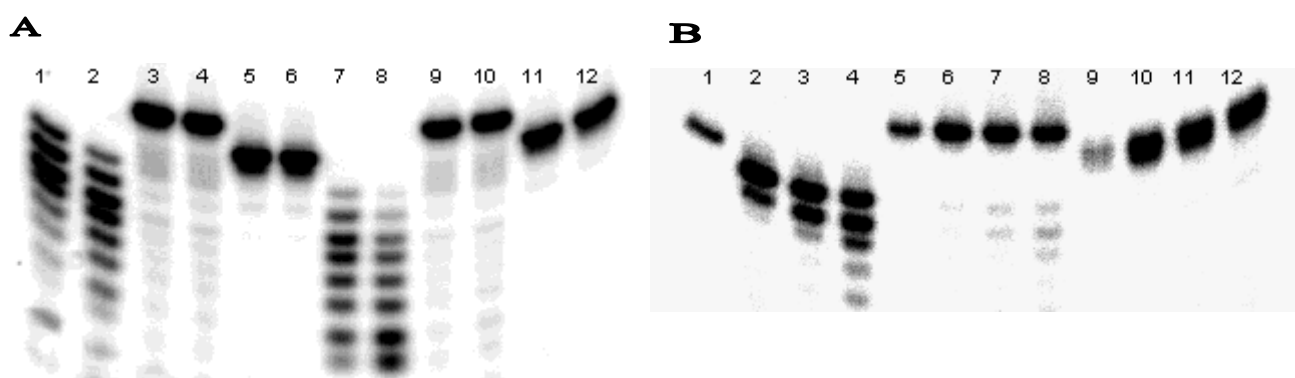


Fig 13: (A) Snake venom PDE degradation of the 3'-Pzn- (**82**) and 3'-Dppz-modified (**120**) and native (**17**) PO-AONs. In lanes 1 – 6 the concentration of 17.5 ng/μl of PDE was used: Lane 1: AON (**17**), 20 min; lane 2: AON (**17**), 120 min; lane 3: AON (**120**), 20 min; lane 4: AON (**120**), 120 min; lane 5: AON (**82**), 20 min; lane 6: AON (**82**), 120 min. Lanes 7 - 12 represent the same set of the reactions but with double amount of the enzyme (35 ng/μl). (B) Snake venom PDE degradation of the 3'-Pzn- (**145**) and 3'-Dppz-modified (**146**) and non-modified (**142**) PS-AONs: Lane 1: AON (**142**), 0 min; lane 2: AON (**142**), 10 min; lane 3: AON (**142**), 30 min; lane 4: AON (**142**), 120 min; lane 5: AON (**146**), 0 min; lane 6: AON (**146**), 10 min; lane 7: AON (**146**), 30 min; lane 8: AON (**146**), 120 min; lane 9: AON (**145**), 0 min; lane 10: AON (**145**), 10 min; lane 11: AON (**145**), 30 min; lane 12: AON (**145**), 120 min.

We suggest that the steric hindrance caused by the tethered polyaromatic system as well as the flexible linker prevent binding of the enzyme to the oligonucleotide, and results in the improved resistance observed with 3'-modified AONs. Interestingly, for both PO and PS-AONs the 3'-Pzn modification resulted in approximately 6 times higher stability compared to 3'-Dppz modifications. This additional resistance shown by 3'-Pzn AONs could be attributed to the presence of the unnatural *ara*-U nucleotide at the 3'-end of these oligonucleotides. Arabinonucleic acids¹²² were shown to be more nuclease-resistant than native DNA and therefore the 3'-*ara*-U moiety could contribute to the stability of these modified DNAs.

These data show that our 3'-modifications dramatically improve the stability of AONs towards the 3'-exonucleases (especially when the nucleotide with artificial sugar moiety is used), which should result in prolonged life-time in the cell media. Also, the lipophilic moieties conjugated to the AONs affect the biodistribution properties of antisense oligonucleotides and could influence the fraction of the drug delivered to different tissues, as it was shown for the cholesterol-conjugated oligonucleotides.²²²

1.2.9 *Influence of the 3' tether of the AON on the kinetics of the RNase H cleavage reaction* (Paper V, in collaboration with Dr N. V. Amirkhanov)

It has been shown above that 3'-chromophore conjugated AONs form more stable AON/RNA hybrid duplexes and promote faster RNase H hydrolysis of the target RNA compared to the native counterpart. However, the question remains if the improved thermodynamic stability of the tethered hybrid duplex is exclusively responsible for the higher rates of the RNase H promoted cleavage of such substrates. The fact that the chemical nature of chromophore might influence the cleavage pattern suggested that, possibly, factors other than thermodynamic stability (such as recognition of the substrate by the enzyme for example) might be involved in the cleavage enhancement demonstrated by tethered AONs. To explore this possibility, native AON (**17**) and the AONs conjugated to the Pzn (AON **147** in Figure 14) and Dppz (**120**) at the 3'-ends using the identical glycerol-diethylene glycol linkers were used to compare their ability to cleave the target RNA (**140**) at different AON concentrations. When RNA was hybridized with AONs in 1:1 ratio modified AONs demonstrated significantly higher initial hydrolysis rates compared to the non-modified counterpart: 1.9 times faster in the case of Pzn-AON and 5.2 times for the Dppz-AON. These data seem to be in agreement with the relative stability of the corresponding AON/RNA duplexes: the free energy of stabilization (ΔG°_{298}) were found to be -32.2 kJ/mol for the

native AON/RNA (**17** + **140**, $T_m = 20.1^\circ \text{C}$), -38.4 kJ/mol for Pzn'-AON/RNA (**147** + **140**, $T_m = 28.6^\circ \text{C}$) and -41.7 kJ/mol for Dppz-AON/RNA (**120** + **140**, $T_m = 30.6^\circ \text{C}$) hybrid duplexes.

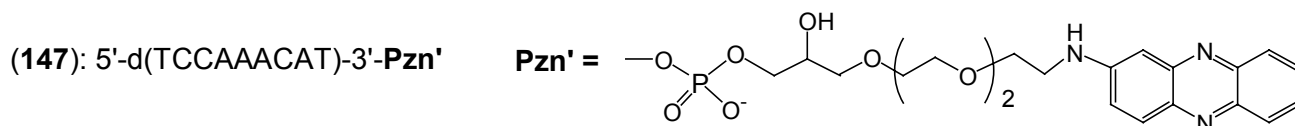


Figure 14: 3'-end modified 9mer AON containing Pzn moiety attached through the 12-atom linker

To exclude the effect of thermodynamics of the DNA/RNA hybrid formation on the cleavage rate, the RNase H hydrolysis was also performed under a condition where the target RNA is quantitatively converted to the corresponding AON/RNA hybrid with each AON (**17**, **120** or **147**) by using a large excess of AON. Under such an RNA saturation condition (using 10-10000 times excess of AON relative to RNA), the starting initial concentration of each AON/RNA hybrid duplex in the RNase H promoted cleavage reaction is the same and equal to the RNA concentration. This means that contribution of the first step (K_{d1} in Figure 15) of the RNase H cleavage reaction characterizing the hybrid duplex formation is bypassed.

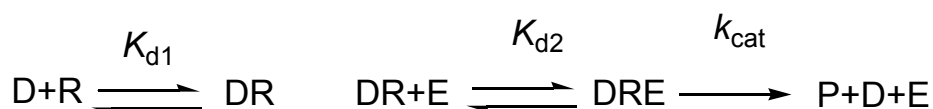


Figure 15: Kinetic scheme of the RNase H hydrolysis; D: AON; R: target RNA; K_{d1} : equilibrium constant of dissociation of a duplex DR; K_{d2} : equilibrium constant of dissociation of the substrate-enzyme complex DRE.

Thus a fixed concentration of the target RNA (**140**) was mixed with each AON at different concentrations (from 10^{-8} to 10^{-5} M), and the maximal extent of hydrolysis (after 2 h of incubation) of the resulting hybrid by RNase H was determined for each concentration. The results of these titrations (Figure 16) clearly showed that 100% duplex formation could be achieved with much lower Dppz-AON (**120**) concentration compared to Pzn-AON (**147**) or the native counterpart (as ΔG°_{298} of AON/RNA duplex increases, proportionally less amount of AON was required to saturate the target RNA). However, at RNA saturated conditions one should expect the same degree of extent of RNA hydrolysis at t_∞ for all the samples provided all the hybrid duplexes were equally recognized by the enzyme. Figure 16 shows that we have different extent of the RNA cleavage at t_∞ (shown at the plateau) for different AON-RNA duplexes. This means that the catalytic activity of RNase H toward the RNA component in various AON/RNA hybrid duplexes are indeed different, suggesting that kinetic parameters of the RNase H promoted cleavage of the tethered AON/RNA hybrids are different from those of the

unmodified counterpart. Because the relative global helical conformation of all three AON/RNA hybrids are identical as evident from their CD spectra, these differences in the catalytic activities must be due to

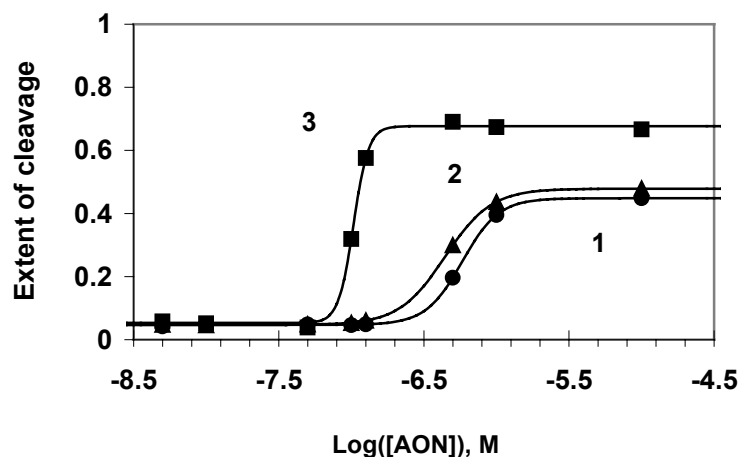


Figure 16: Extent of hydrolysis of the target RNA (**140**) (1 nM) in the AON-RNA hybrids by RNase H as a function of the logarithm of AON concentration (the concentrations of AONs range from 10^{-8} to 10^{-5} M). Curves 1, 2 and 3 correspond to the hybrid duplexes formed by native 9mer AON (**17**), Pzn'-AON (**147**) and Dppz-AON (**120**) respectively.

the preferential recognition of the tethered Dppz chromophore in **120** *vis-a-vis* Pzn in **147** compared to the native counterpart.

These results confirm that the change of the aromatic nature of the 3'-tethered chromophore changes the substrate recognition and catalysis by RNase H. The mechanism of this phenomenon is not yet understood, but we suggest that interactions of the large polyaromatic tether with hydrophobic core of the enzyme might result in higher affinity of Dppz-tethered AON/RNA hybrid duplex to the RNase H. This shows that changing the properties of the tethered chromophore gives us an efficient tool to steer the AON/RNA hybrid recognition to effectively engineer the AONs with higher RNase H potency.

1.2.13 RNase H sensitivity towards the modifications at different sites of the AON/RNA hybrid duplex (Paper IV, in collaboration with Dr N. V. Amirkhanov and Mr D. Ossipov)

Although, structural requirements for the DNA/RNA hybrids to be suitable substrates for RNase H are well described,^{198,199,208-210} the tolerance level of this enzyme towards the modifications, which do not alter the helicity of the duplex is not clearly understood. It was recently reported that AONs conjugated at the 3'-end to the polyethylene glycol moieties of different structure and molecular weight stimulate the hydrolysis of RNA by RNase H at the same sites and to the same extent as the native oligo-DNA.²²³ As it was shown on the example of 3'-Pzn and 3'-Dppz tethered AONs (see chapter 1.2.12) different tethers with various structural parameters (lipophilicity, bulk, presence of charges etc.) affected the binding affinity of the enzyme to the hybrid duplex and, therefore, the cleavage rates. Our studies also showed

that various tethers at the 3'-end of AON can be very easily tolerated by the enzyme. Bulky Dppz group when attached to the 3'-end of AON provided higher RNase H activity of the corresponding AON/RNA

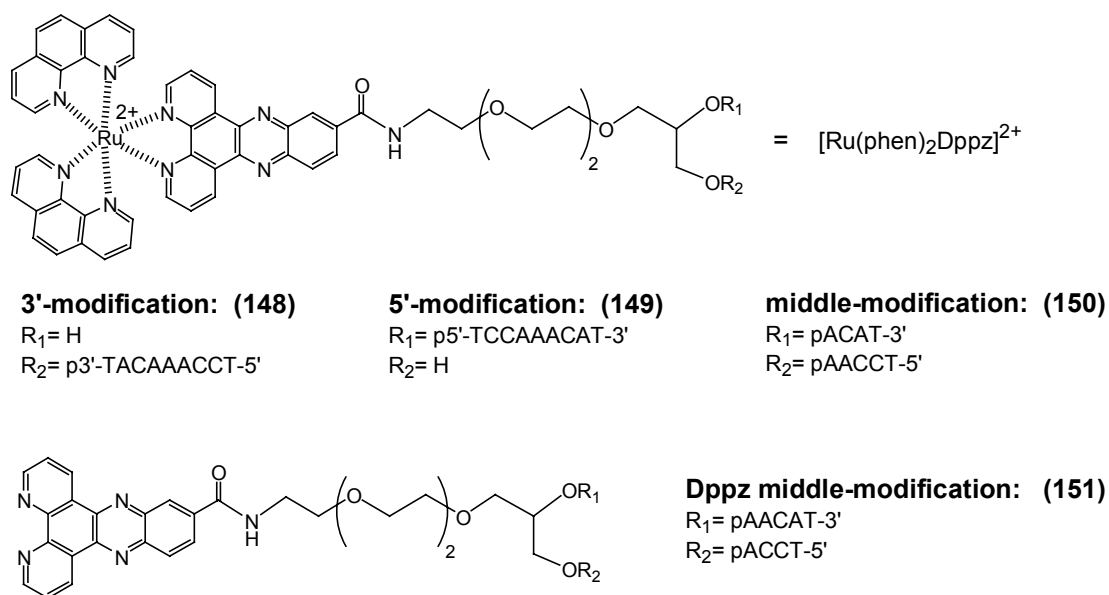


Figure 17: Composition of the 3' and 5'-end $[\text{Ru}(\text{phen})_2\text{Dppz}]^{2+}$ modified AONs, and Dppz and $[\text{Ru}(\text{phen})_2\text{Dppz}]^{2+}$ middle modified AONs

duplexes compared to the hybrid duplexes modified with rather small Pzn group. Even when bulky and non-planar 3'- $[\text{Ru}(\text{phen})_2\text{Dppz}]^{2+}$ complex (AON **148** in Figure 17) was attached to the 3'-end of the AON the extent of RNase H cleavage (Table 10) remained the same as for the Dppz modified duplex and much higher than for the native counterpart. This change of the steric and electronic properties of the 3'-group did not have any influence on the cleavage pattern, revealing the same single site of cleavage as for the 3'-Dppz-AON (**120**).

Table 10: Dependence of the extent of RNase H hydrolysis of the low-aggregated 17mer RNA target (**140**) hybridized with 9mer PO-AONs from the bulk of the modification and the tethering site (the extent of hydrolysis for non-modified AON (**17**) was 60 %)

Modification at the 3'-end	Extent of hydrolysis	Modification at the 5'-end	Extent of hydrolysis	Modification in the middle	Extent of hydrolysis
Dppz (120)	91%	Dppz (117)	75%	Dppz (151)	50%
Ru-complex (148)	92%	Ru-complex (149)	49%	Ru-complex (150)	4%

In order to investigate the effect of the positive charge of the tethered ligand (as in $[\text{Ru}(\text{phen})_2\text{Dppz}]^{2+}$ complex) at the 3'-end of the AON without seriously altering the size of the tether, we have compared the extent of cleavage promoted by 3'-phenazinium-AON **153** and 3'-phenazine-AON **152** (Figure 18),

having the modifications of almost the same size, but where phenazinium moiety possessed a positive charge because of the nitrogen alkylation at the N9 position. These AONs were shown to promote the RNase H cleavage of single-stranded and aggregated RNAs with very similar rates. These results clearly show that neither of such factors as steric bulk or charge of the substituent tethered at the 3'-end of AON can interfere with RNase promoted cleavage.

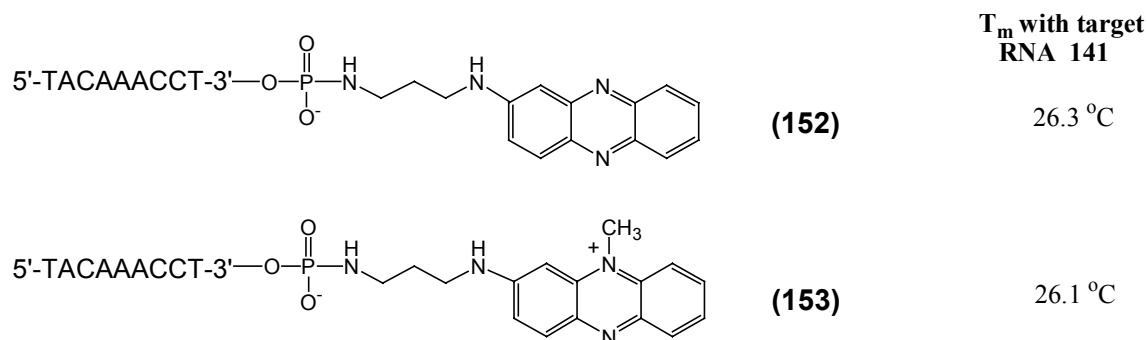


Figure 18: Composition of the 3'-phenazine and 3'-phenazinium tethered 9mer AONs

In contrast, the 5'-end of AON was more sensitive to the steric bulk of the tether. Substitution of the 5'-Dppz moiety in AON (**117**) with 5'-[Ru(phen)₂Dppz]²⁺ complex (AON **149** in Figure 17) dramatically reduced the extent of the cleavage (49 %) to a level which was even poorer than for the native hybrid (60 %). In our examples, the cleavage of the target RNA in the AON/RNA duplex takes place at the sites opposite to the 5'-end of the AON segment, and presence of any tether at this position (especially as large as [Ru(phen)₂Dppz]²⁺ complex) might interfere with the cleavage. This could be the reason why relatively small intercalators like Pzn or Dppz were tolerated at the 5'-end by the enzyme but not the [Ru(phen)₂Dppz]²⁺ modification.

When Dppz or [Ru(phen)₂Dppz]²⁺ modifications were introduced in the middle (AONs **151** and **150** respectively in Figure 17) of the duplex formed with 11mer RNA target (**91**), no hydrolysis by RNase H was observed. When these middle-modified AONs were targeted to the 17mer RNA (**140**), hydrolysis with Dppz modification (AON **151**) was observed although to the less extent compared to the native AON/RNA duplex (Table 10), but no significant hydrolysis was achieved with middle-[Ru(phen)₂Dppz]²⁺-modified AON (**150**). As an endonuclease, RNase H is supposed to bind within the DNA/RNA hybrid before any cleavage can take place. Presumably, our modifications placed in the middle prevent the enzyme from binding, which results in poorer or even complete loss of RNase H activity of such AONs.

These results show that the tolerance level of the RNase H towards various tethers is indeed dependent on the location of the modification in the hybrid duplex. The 3'-end of the AON seems to be the most suitable for the introduction of different chemical functions because it can easily accommodate bulky or charged moieties without interfering with the RNase H cleavage. The middle of the short AON/RNA duplex is the least favorable place for the modification.

1.2.14 *RNase H sensitivity to the local conformational changes in the AON strand of the AON/RNA hybrid duplex* (Paper VI, in collaboration with Mr P.I. Pradeepkumar)

In the previous chapter we have discussed the effect of the tethers, which did not alter the helical conformation of the AON/RNA duplex on the rate and the pattern of the RNase H promoted cleavage. Structural disturbances brought to the hybrid duplex by different chemical modification might have negative effect on the rate of hydrolysis. Therefore, the information about the sensitivity of the RNase H towards the minor conformational changes is very important in the design of novel AONs utilizing the RNase H dependent mechanism of action. To investigate the response of the RNase H to the local structure alterations in the AON/RNA duplex, we introduced a single bicyclic psiconucleoside, 1-(1',3'-*O*-anhydro- β -D-psicofuranosyl)thymine²²⁴ moiety, **1** (**154** in Figure 19a), with locked 3'-*endo* sugar conformation (N-form) at different sites of AON sequence targeted to the coding region of the SV-40 large T Antigen.²²⁵ It is now quite clear^{122,226} that all modifications that lead to preferential North-type sugar, including its constrained form, in an AON lead to the higher affinity to the target RNA, but result in the loss of RNase H activity, because they resemble RNA/RNA duplex, which is not a substrate for the enzyme. However, the question is still unanswered how far the conformational disturbances brought by a single N-type nucleoside will be transmitted in the hybrid duplex and where the balance is for accomplishing tighter binding and target specificity to the RNA without losing the RNase H potency. Recently, structural studies²²⁷ have been carried out by NMR and CD spectroscopy on hybrid duplex formed by AON, containing one locked nucleic acid (LNA) block to examine if there have been any conformational changes in the microenvironment around the modification site. It was found that 3'-nucleotide flanking the modification site adopts a clear N-type conformation. Notable difference was also observed in CD, and it was concluded that overall A-type conformation existed in the AON strand of the duplex stretching only one nucleotide both at 3'- and 5'-ends of the LNA nucleotide modified site, which means that this part of the duplex takes up a more A-RNA/A-RNA type helix. No report of the

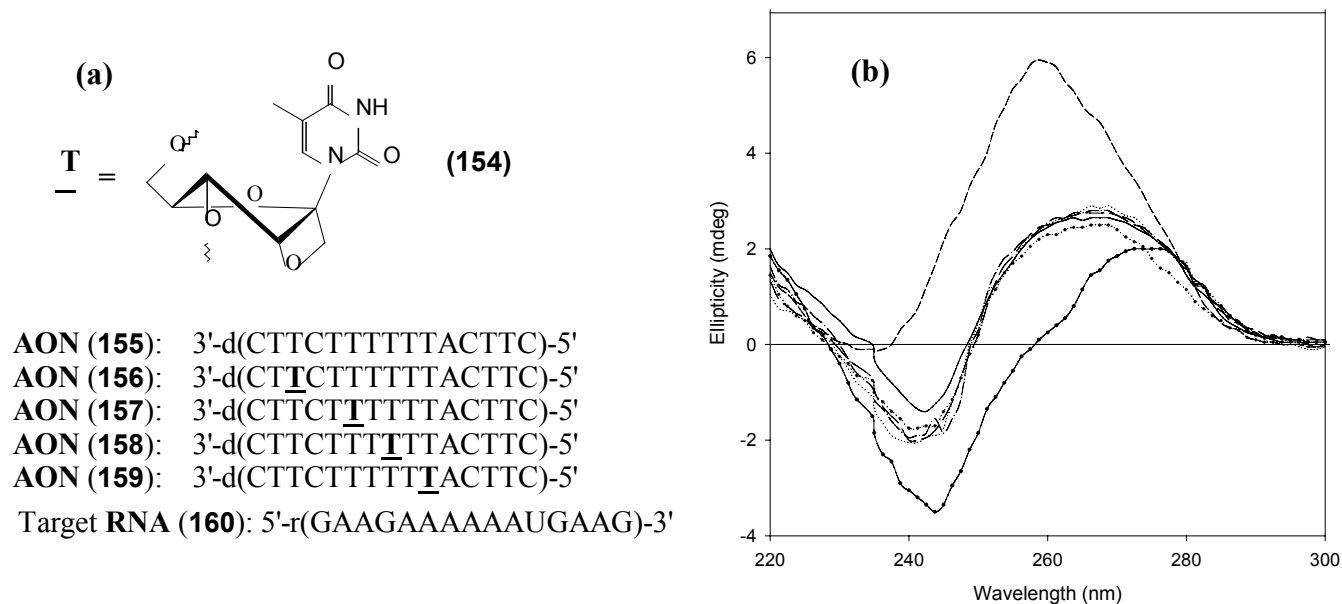


Figure 19: (a) Sequences of the AONs and their RNA target (b) CD spectra of the duplexes formed by the AONs (155) – (159) and RNA target (160): (·····) (155), (—) (156), (----) (157), (·+·+·+·) (158) and (— · — · —) (159). For comparison, typical B-type and A-type spectra are presented: (— · — · —) DNA/DNA duplex formed by AON (155) and complementary DNA: 5'-d(GAAGAAAAAATGAA)-3', (—) RNA/RNA duplex formed by self-complementary 17mer RNA: 5'-r(UAACAUGUUUGGACUCU)₂-3' (141)

RNase H cleavage pattern of the above single LNA-modified AON has been however available to assess how far the local structural deformation has taken place compared to what has been observed by NMR. Clearly, the cleavage pattern of the RNase H degradation of these duplexes should provide an answer about the extent and direction of the structural changes from the "enzyme point of view", and only those changes are of importance for the further development of the antisense technology.

Incorporation of 1-(1',3'-O-anhydro-β-D-psicofuranosyl)thymine in to the AONs resulted in reduced thermodynamic stabilities of the corresponding AON/RNA duplexes (T_m s: 44.5° C for AON (155)/RNA (160), 37.7° C for AON (156)/RNA (160), 39.5° C for AON (157)/RNA (160), 39.7° C for AON (158)/RNA (160) and 39.3° C for AON (159)/RNA (160)), showing that the 2', 3'-oxymethylene bridged nucleoside moiety, T, has most probably introduced a local conformational heterogeneity¹²² in the AON/RNA hybrid structure in view of the fact that a mismatch in a DNA/RNA duplex normally results in a 12-18° C loss of T_m .²²⁸

Circular dichroism studies on these AON/RNA hybrid duplexes revealed that all of them had very similar global helical conformation, mimicking that of natural DNA/RNA hybrid duplexes, having a positive band at 263-267 nm, negative band at 240-245 nm and crossover point at 247-250 nm. This

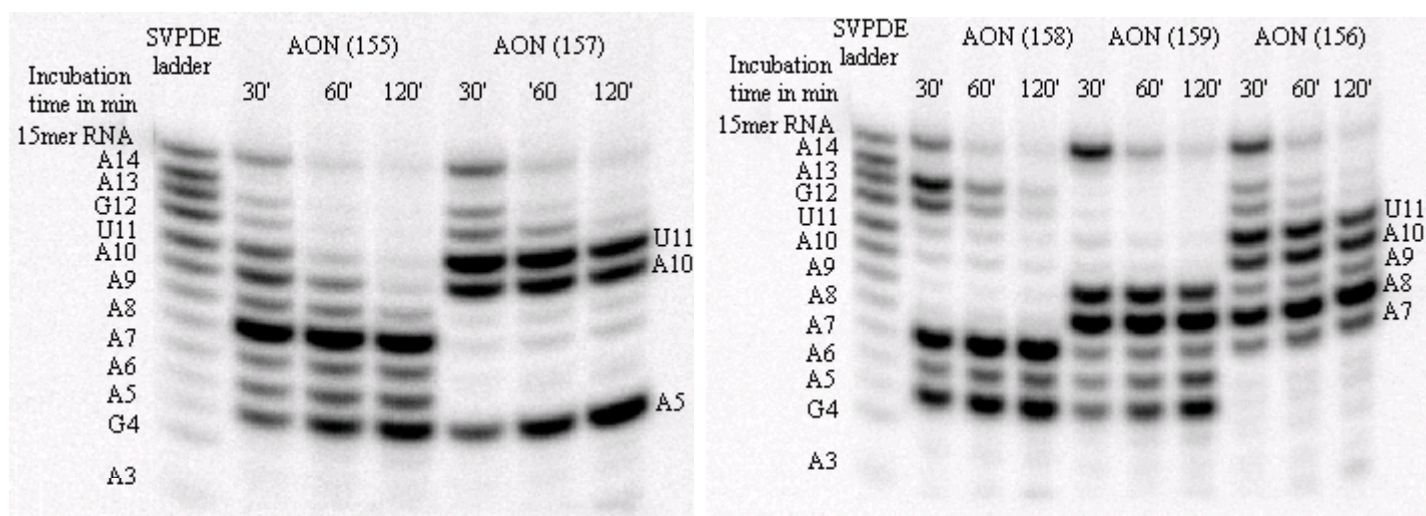


Figure 20: The PAGE analysis of RNase H hydrolysis of the hybrid duplexes formed by AONs (**155**) – (**159**) and RNA (**160**), after 30, 60 and 120 min of incubation. The exact footprint and the assignment of the RNA cleavage sites in the hybrid duplexes can be deduced by the partial digest of the target RNA by snake venom phosphodiesterase (SVPDE)

suggests that single modification by conformationally constrained **T** nucleotide at any position in the AON strand produces only minor structural changes in the AON/RNA hybrid duplex. The RNase H degradation studies showed that all modified duplexes were good substrates for the enzyme, which was indicated by very similar rates of hydrolysis. However, comparison of the cleavage pattern revealed the systematic change in the RNase H recognition of the duplex depending on the position of the modification. The cleavage activity of the enzyme in all modified duplexes was suppressed within a 5 base pairs long region towards the 3'-end of the RNA in the modified AON/RNA hybrids, starting from the base opposite to the modified **T** nucleotide in the AON strand. Thus, in the native hybrid duplex (**155** + **160**) the whole region from A5 to U11 of the RNA was accessible for RNase H promoted cleavage, but in the case of the duplex with AON (**156**), having modification opposite to A3 of the complementary RNA, made the region A3-A7 not accessible for RNase H cleavage which resulted in the loss of the cleavage sites at A5 and A6. Instead, major sites at A10 and U11 appeared in addition to the preserved sites A7, A8 and A9. This shift of the cleavage sites shows that the A3-A7 region, although not accessible to the cleavage, can serve as a binding site for the enzyme. In the duplex with modification opposite to A6 of RNA (with AON **157**) complete loss of RNase H promoted cleavage occurred in the region A6-A10 and the only sites accessible for the cleavage were at A5, A10 and U11 situated on the edges of the A6-A10 region. This again proves that the site of the duplex, which is resistant to the hydrolysis, serves perfectly as the binding site for the enzyme. Further shift of the

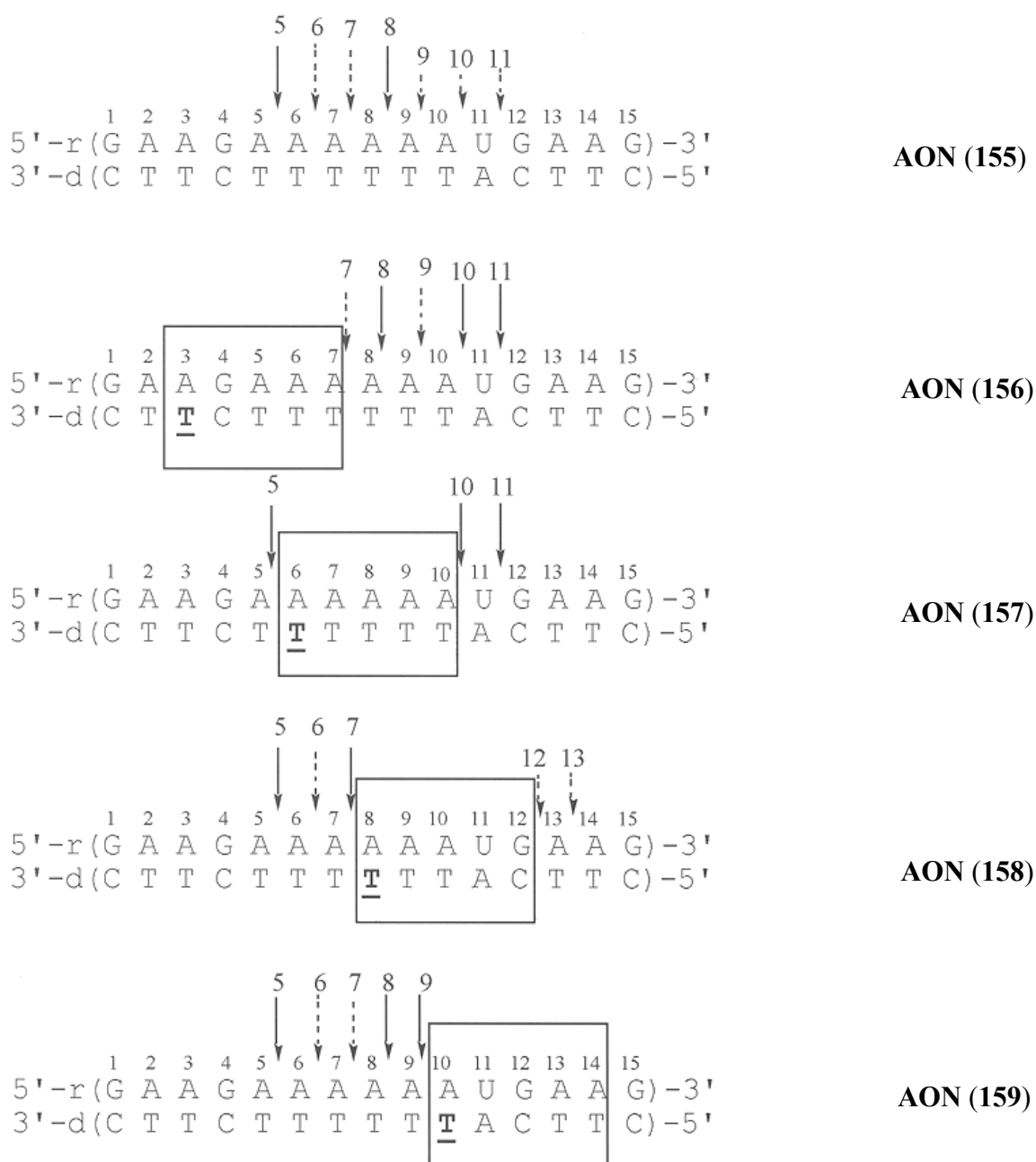


Figure 21: RNase H cleavage pattern of the hybrid duplexes formed by AONs **155** – **159** and RNA target **160**. Solid and dashed arrows represent the major and the minor cleavage sites respectively, at complete degradation (after 2 h of incubation). Short arrows demonstrate the sites accessible for the cleavage after 30 min of incubation. Boxes represent the parts of the RNA sequence insensitive towards RNase H cleavage

cleavage sites was observed in the duplex having the modification opposite to A8 site in RNA (with AON **158**). Here the regions A5 - A7 and A13 - G15 were accessible for the cleavage reaction. As

expected, the AON modification opposite to A10 of the complementary RNA, as in AON (**159**), resulted in the absence of any cleavage sites in the region between A10 to G15, while regaining all the cleavage sites from A5 –A9, present in the native hybrid duplex.

The above results suggest that introduction of a single 1-(1',3'-*O*-anhydro- β -D-psicofuranosyl)thymine modification into the AON produces very fine conformational changes in the substrate AON/RNA duplex, which are not possible to detect by CD, but which are significant enough for the RNase H recognition and catalytic properties. Interestingly, the local conformational changes in the hybrid span a total of 5 nucleotides toward the 5'-end of the AON (3'-end of RNA) in the case of all modified AONs, which shows how the duplex can transmit the structural distortion brought by the constrained nucleotide. The results also show that the structural requirements for the substrate binding and substrate cleavage by RNase H appear to be different (for example RNase H could bind to the A3 - A7 region in the AON (**156**)/RNA duplex to produce hydrolysis at A7, but the cleavage at position A5, present in the native duplex, was absent.

2. Preparation of biologically important isotope labeled oligo-RNAs for the NMR structure determination in solution.

2.1 Introduction

NMR is one of the most powerful means,²²⁹⁻²³⁶ in conjunction with various computational methods,^{237,238} to study the three-dimensional structure and intermolecular dynamics²³⁹ of DNA and RNA and their complexes in solution under quasiphenological conditions. A considerably large number of oligo-DNAs have been studied so far by NMR,^{235,236,240} compared to oligo-RNA.^{230,241-243} This is because of the fact that ribose-ring region (6.5-4.0 ppm²²⁹) of the oligo-RNA spectra is less dispersed, and because of the more problematic chemical synthesis of the RNA oligomers. For the determination of the three-dimensional structure of DNA and RNA molecules, two types of complementary information are required: (1) the conformation of the nucleotide units described in terms of torsional angles, which can be obtained from homonuclear proton-proton and heteronuclear proton-phosphorus, carbon-phosphorus coupling constants,²⁴⁴⁻²⁴⁶ and (2) interproton distance information (derived from the nuclear Overhauser effect (nOe)).²⁴⁷ Although this information is available from 2D and 3D NMR experiments for a smaller oligonucleotides, it is very difficult to collect this information for the large biologically functional DNA and RNA, so usefulness of NMR spectroscopy for structural studies of these molecules becomes very restricted. Besides overcrowding in the ribose region, there are other factors making the spectrum more

complicated: (1) associated line broadening arising from decreased T_2 relaxations,²²⁹ (2) decreased sensitivity caused by slower tumbling rate,²²⁹ (3) the spin diffusion preventing accurate nOe volume measurements.²⁴³

In order to tackle these problems, various isotope-labelling techniques have been developed. Uniformly ^{13}C -labelled blocks have been selectively incorporated into oligo-DNA to establish the connectivities of nucleobases to their sugar residues²⁴⁸ or to extract the $^3J_{\text{HH}}$ coupling information,²⁴⁹ whereas site-specific ^{15}N labelling²⁵⁰⁻²⁵³ has been used to investigate the structural properties of oligonucleotides. Uniformly or sequence specifically $^{13}\text{C}/^{15}\text{N}$ -labelled oligomers²⁵⁴⁻²⁵⁶ have been prepared by solid-phase chemical synthesis or by enzymatic synthesis^{243,257-259,260-262} (using NTPs²⁶³⁻²⁶⁵ and T7 RNA polymerase²⁶⁶ or dNTPs²⁶⁰⁻²⁶² and Klenow DNA polymerase), whereas nonuniformly $^{15}\text{N}/^{13}\text{C}$ -labelled oligo-RNA²⁶⁷ and -DNA²⁶⁸ molecules have been prepared by ligation of labelled and non-labelled stretches. The importance of the use of non-uniformly ^{13}C -labelled oligo-DNA²⁶⁹⁻²⁷⁴ has been well demonstrated for NMR studies in solution. The ^{13}C -labels have been mainly incorporated at C1'/5', but methodologies are also available to introduce the ^{13}C -labels at other sugar carbons.²⁷⁵ It was also found, that $^{13}\text{C}/^{15}\text{N}$ labelled oligonucleotides have several disadvantageous relaxation properties: (1) ^{13}C labels decrease proton T_2 relaxation decreasing the sensitivity of homonuclear J correlation techniques²⁵⁹ (2) the short ^{13}C T_2 relaxations for proton bearing carbons²⁷⁶ in nucleosides result in ^{13}C signal broadening and signal loss²⁵⁹ due to long pulse sequences of many heteronuclear experiments²⁵⁹ (3) relaxation between neighboring nuclei.²⁵⁹

The deuterium labelling of oligonucleotides is based on suppressing parts of the ^1H NMR spectra²⁷⁷⁻²⁸³ and extracting necessary information from the NMR-visible non-deuterated part. For the first time the deuteration approach was introduced in 1972 by Danyluk *et al.*²⁷⁷⁻²⁸³ Enzymatically prepared predeuterated building blocks turned out to be inadequate for assignment purposes because of residual proton signals (~90 atom % ^2H incorporation). The stereospecific chemical synthesis, which was used for the first time to introduce deuterium on the sugar carbons (>97 atom % ^2H incorporation), was developed in our laboratory in 1986.²⁸⁴⁻²⁸⁶ Subsequently, we have developed the Uppsala "NMR-window" concept,^{276,286-305} in which partially deuterated sugar residues were non-uniformly incorporated into either oligo-DNA^{286-289,291,295,301} or -RNA^{292,300} by the solid-phase synthesis protocol or enzymatic means²⁹³ for simplification of the spectral crowding^{286-289,291-294,296,300} and coupling patterns,^{291-294,296,300} increasing NOE intensities,^{291-294,296} probing dynamics by selective T_1 and T_2 measurements,^{276,297,298,300} reducing the spin diffusion^{291,296} as well as the line-broadening²⁹⁰ associated with ^1H dipolar relaxation. The use of combination of ^2H and ^{13}C labelling for assignment of the spectra of the oligonucleotides also

has been reported.^{297,298,300} The different schemes of deuterium incorporation have been widely applied for obtaining the structural information.²⁸⁶⁻³²⁸ Stereoselectively 2'(R)-deuterated 2'-deoxynucleoside blocks³⁰⁶⁻³⁰⁸ have been used for extraction of the $^3J_{H1',H2'}$ and $^3J_{H1',H2''}$ coupling constants from COSY-type experiments.³⁰⁹ Deuteration of C5/C6 positions of pyrimidines or C5-methyl of thymine and C8 of purine nucleobases³²³⁻³²⁶ was employed for simplification of the NOESY spectra of oligonucleotides. The diastereotopic H5'/5'' methylene resonances were assigned by incorporation of isotopomeric 5'(R/S) mixture of 2H -labelled nucleosides^{310-315,319-322} into oligo-DNA.^{316,317} $5'-^2H/^13C$ double-labelled DNA blocks provided information about vicinal 1H - ^{31}P coupling constants.³¹⁸ The spectral overcrowding of aromatic to H1' region in the NOESY spectra of an RNA duplex³²⁷ was decreased upon selective incorporation of C1'-deuterated nucleosides. $3',4',5',5''-^2H_4$ -Labelled nucleosides were uniformly incorporated into RNA and the effect of the specific deuteration on the spectral complexity and relaxation properties were studied.^{299,328}

2.2 Present work

2.2.1 Synthesis of sequence-specific non-uniformly $^{13}C_5$ labeled HIV-1 TAR RNA (Paper VII, in collaboration with Dr J. Milecki and Dr A. Földesi)

Development of protocol for non-uniform ^{13}C -labelling of large biologically important oligo-RNAs using solid-phase chemical synthesis will aid in the elucidation of structure, dynamics, and interactions of RNAs. This was exemplified by sequence selective introduction of the ^{13}C -labelled nucleosides into the 29mer RNA constituting the trans activator regulatory (TAR) element of HIV-1 with bulged hairpin structure, which plays critical role in the regulation of the viral transcription.³²⁹ The 29mer HIV-1 TAR RNA bulged hairpin has been labelled with four $1',2',3',4',5'-^{13}C_5$ ribonucleoside units specifically incorporated into the stem (^{27}A and ^{43}G), bulge (^{24}C) as well as in the loop (^{31}U) regions (Figure 22) as

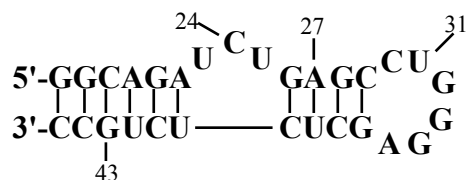


Figure 22: Sequence and secondary structure of the 29mer HIV-1 TAR RNA (numbering indicate $^{13}C_5$ -ribofuranose labeled nucleotides)

an illustration of the solid-phase approach compared to the enzymatic non-uniform labelling by residue-type.³³⁰⁻³³² The structure of this hairpin has been earlier investigated through uniform³³⁰⁻³³⁵ as well as by

the residue-type³³⁰⁻³³² $^{13}\text{C}/^{15}\text{N}$ labelling, which provided a direct comparison for the evaluation of our ^{13}C relaxation window.

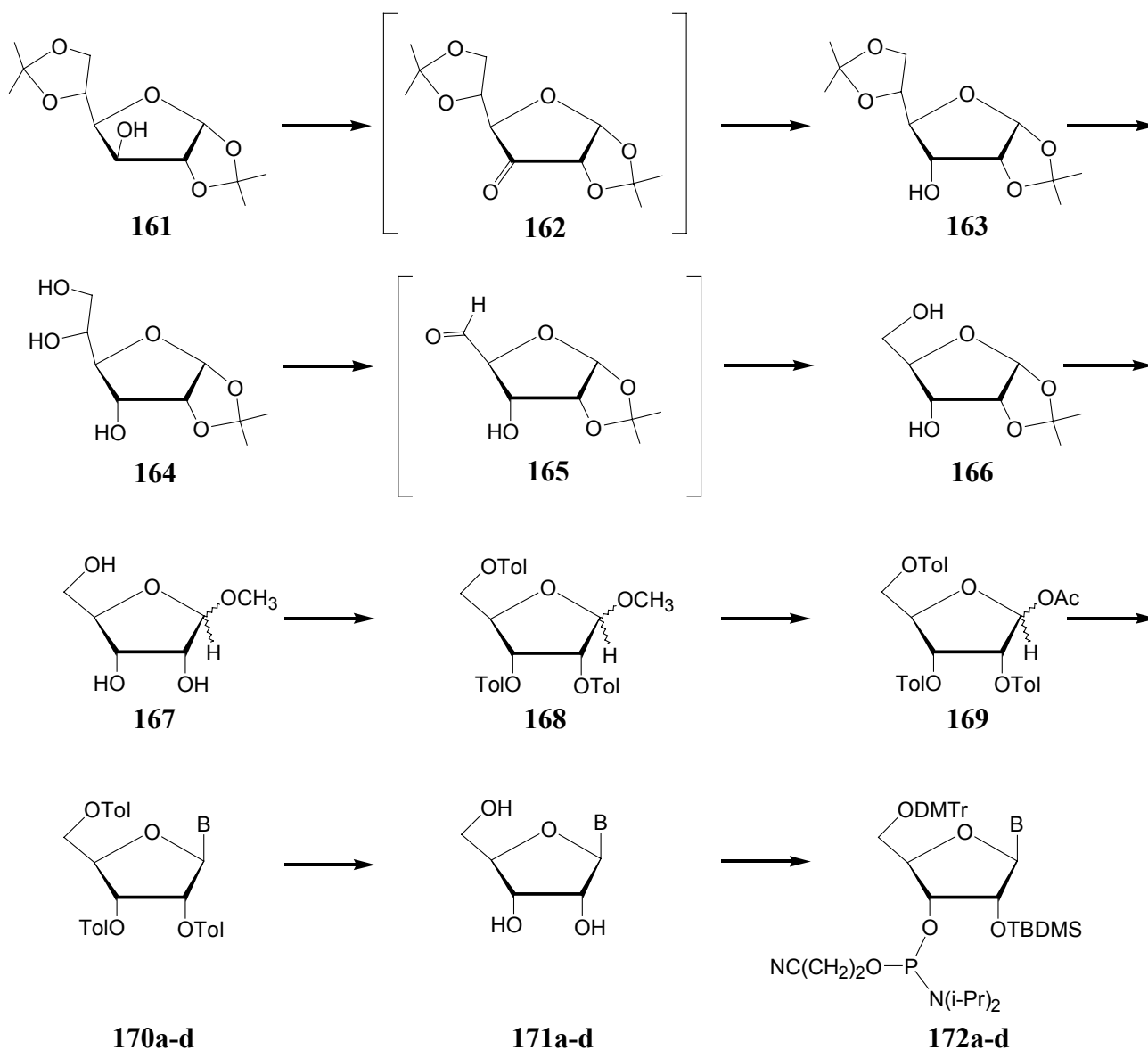


Figure 23: Synthesis of ribonucleoside- $^{13}\text{C}_5$ phosphoramidites, where B is: **a** - N^6 -benzoyladenine-9-yl, **b** - N^4 -benzoylcytidine-1-yl, **c** - uracil-1-yl, **d** - N^2 -acetyl- O^6 -diphenylcarbamoylguanin-9-yl or N^2 -isobutyryl-guanin-9-yl

$^{13}\text{C}_6$ -D-Glucose (99 atom% ^{13}C) was converted (in collaboration with Drs J. Milecki and A. Földesi) into 1,2:5,6-di-*O*-isopropylidene- α -D-glucofuranose (**161**)³³⁶⁻³³⁹ (Figure 23). Compound **161** was transformed³³⁹⁻³⁴¹ into *allo*-isomer **163**^{336,339} by oxidation/reduction reaction sequence without isolation of the intermediate ketone **162**. Selective hydrolysis of 5,6-*O*-isopropylidene group^{295,336,341} (**164**), followed

by oxidation with NaIO_4 ³⁴² (**165**) and reduction with NaBH_4 gave D-ribose derivative **166**.^{336,337,341} Acidic hydrolysis²⁹⁵ of the remaining isopropylidene group gave D-ribose,^{343,344} which was quantitatively converted into a mixture of its α/β -methyl furanosides **167**.²⁸⁷ This mixture was converted to 1-*O*-acetyl-2,3,5-tri-*O*-(4-toluoyl)- α/β -D-ribofuranose²⁸⁷ (compounds **169**, 42 % yield from $^{13}\text{C}_6$ -D-glucose). Condensation of **169** under Vorbrüggen's condition^{345,346} gave protected nucleosides **170**²⁸⁷ in 66-81% yields. Selective deprotection of the *O*-acyl groups from **170** was achieved with aqueous 2*N* NaOH-ethanol-pyridine,²⁸⁷ which allowed in case of adenosine and cytosine to keep the base protected. In case of guanosine, complete deprotection (with methanolic ammonia hydrolysis) and re-protection³⁴⁷ by isobutyryl group at *N*²-site was necessary. The *O*-deacylated nucleosides **171**^{336,348} were subjected to dimethoxytritylation as crude mixtures, giving high yields of 5'-*O*-dimethoxytrityl derivatives,^{336,349} which were 2'-*O*-protected with TBDMS^{336,350,351} group using the silver nitrate procedure,³⁵⁰ and subsequently phosphitylated by isomerisation-free procedure^{175,352} with 2-cyanoethyl *N,N*-diisopropylchloro-phosphoramidite to yield the desired building blocks **172**^{200,353} for oligonucleotide synthesis (see the original Paper VII for the details).

During the synthesis, the identity and purity of the intermediates were ascertained by ^1H -NMR, which was complex owing to ~99 atom % ^{13}C isotope enrichment (^1H - ^{13}C spin-spin couplings and the line broadening). Identity of the intermediates however could be accurately assessed by calculating the middle-points of the J-coupled multiplicities of the sugar protons and comparing them with the spectra

Table 11: Examples of the ^1H -NMR chemical shifts (δ) of different ^{13}C -labeled nucleoside derivatives (in CDCl_3) in comparison with non-labeled counterparts

Comp.	H1'	H2'	H3'	H4'	H5'	H5''
170a ^a	6.82; 6.19 6.55; (6.51)	6.67; 6.09 6.37; (6.33)	6.51; 5.93 6.22; (6.23)	5.18; 4.62 4.83; (4.81)	5.11; 4.55 4.90; (4.90)	4.95; 4.40 4.68; (4.67)
170b ^a	6.83; 6.19 6.51; (6.52)	6.08; 5.51 5.84; (5.80)	6.19; 5.59 5.89; (5.87)	5.04; 4.48 4.76; (4.75)	5.11; 4.56 4.84; (4.85)	4.95; 4.41 4.68; (4.67)
170c ^a	6.59; 5.98 6.30; (6.35)	6.07; 5.50 5.66; (5.72)	5.96; 5.35 5.89; (5.85)	4.91; 4.34 4.62; (4.67)	5.02; 4.46 4.74; (4.72)	4.85; 4.26 4.55; (4.62)
170d ^a	6.64; 6.03 6.34; (6.35)	6.51; 5.92 6.30; (6.23)	6.51; 5.92 6.30; (6.22)	5.08; 4.54 4.86; (4.83)	5.08; 4.54 4.86; (4.88)	4.94; 4.42 4.68; (4.70)

^a obs. values, average, (^{12}C value)

of the non-labelled counterparts³⁵¹ (Table 11). The purity also could be estimated by looking at the resonances of the aromatic protons of the aglycone or the resonances arising from the protecting groups. In the HSQC-CT spectra of specifically $^{13}\text{C}_5$ -ribose labelled 29mer TAR RNA (Figure 24) all five

regions for ^1H - ^{13}C crosspeaks, [$^1\text{H}(1')\text{-}^{13}\text{C}(1')$, $^1\text{H}(2')\text{-}^{13}\text{C}(2')$, $^1\text{H}(3')\text{-}^{13}\text{C}(3')$, $^1\text{H}(4')\text{-}^{13}\text{C}(4')$, $^1\text{H}(5')\text{-}^{13}\text{C}(5')$], corresponding to four $^{13}\text{C}_5$ -ribose labelled residues in 29mer TAR RNA were clearly observed. Even at concentration of 0.23 mM, the experiment has been performed with 32 number of scans and was

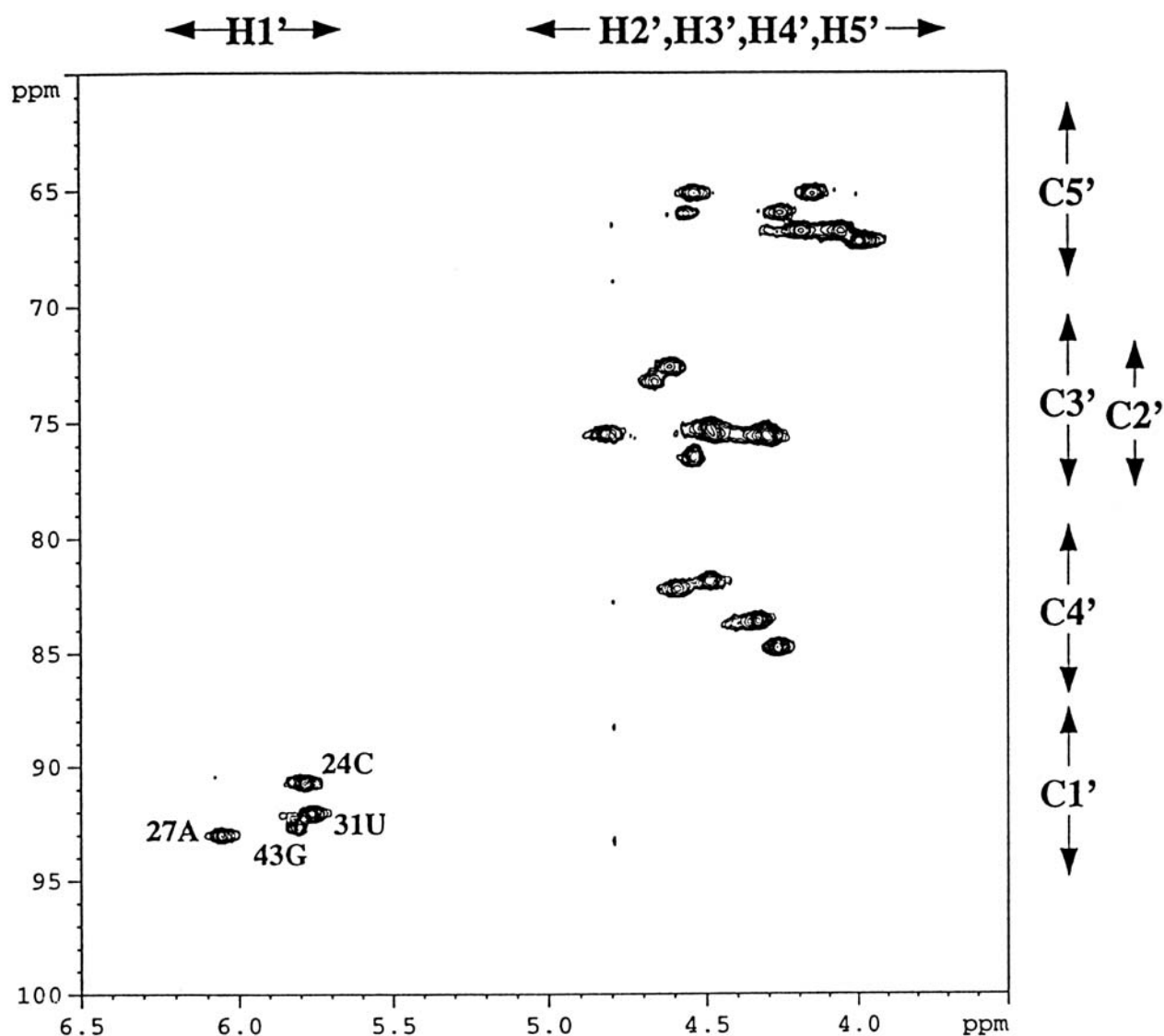


Figure 24: HSQC-CT spectra of specifically $^{13}\text{C}_5$ -ribose labelled (*i.e.* ^{24}C , ^{27}A , ^{31}U and ^{43}G residues) 29mer TAR RNA are shown. On the right ($\delta^{13}\text{C}$) and top ($\delta^1\text{H}$) of the spectra, the chemical shifts of different types of sugar-carbon or -proton regions are defined by arrows. For five carbon-proton regions, four sets of ^1H - ^{13}C crosspeaks corresponding to four different specifically labelled residues are clearly observed.

accomplished during 5 h, which gave an excellent opportunity to perform the relaxation studies of these selectively labelled residues in RNA, thereby overcoming the problem of overcrowding of chemical

shifts in ^1H - ^{13}C experiments usually encountered routinely with the uniformly ^{13}C labelled RNA³⁵⁴. Clearly, non-uniform specific labelling as in our 29mer RNA achieved by chemical synthesis would allow to perform full T_1 and T_2 relaxation studies (the "NMR relaxation window") of each type of sugar-carbons for all four strategically placed ^{13}C -labelled residues because of an optimal dispersion of the sugar ^{13}C resonances.

In our attempts to recover^{355,356} the excess of the labeled amidites during the synthesis, we found that after collection of the amidite solution from synthesizer and subsequent removal of tetrazole by water extraction, the remaining H-phosphonate diester could be hydrolyzed to the nucleoside with excess of DIPEA. This procedure allows to reduce the cost of such selective ^{13}C labelling.

2.2.2 *Influence of the RNA secondary structure on the hammerhead ribozyme formation in the solution at physiological conditions* (Paper VIII, in collaboration with A. Trifonova)

Revolutionary discovery of the first catalytic RNA molecules (group I intron³⁵⁷ and RNase P³⁵⁸) in the early 1980s showed that proteins are not the only catalytic moieties in the cell and that RNA can catalyze its own biochemical transformation. The RNA based enzymes (ribozymes) known to date include group I and II introns RNA subunit of RNase P, hammerhead, hairpin, hepatitis delta virus ribozyme and ribosomal RNA.³⁵⁷⁻³⁶³ Hammerhead ribozyme³⁶²⁻³⁶⁵ is the smallest natural ribozyme consisting of three short helices and universally conserved junction sequence and can be made from two or more separate RNAs.^{364,365} Need for understanding of the mechanism of ribozymes action and their potential use as therapeutic agents for inhibiting gene expression³⁶⁶⁻³⁷⁰ resulted in several attempts to determine detailed structure of the hammerhead ribozyme. These studies involve electrophoretic mobility assays,^{372,373} NMR,³⁷⁴⁻³⁸¹ fluorescence resonance energy transfer³⁸²⁻³⁸⁴ and X-ray diffraction³⁸⁵⁻³⁹⁰. There is only one crystal structure of the all-RNA hammerhead ribozyme (16mer RNA + 25mer RNA) available to date.³⁸⁶⁻³⁹¹ However, ribozyme of this sequence has been shown to have surprisingly low cleavage rate at physiological conditions.³⁹² It was also noticed that even crystallization at high salt concentration (1.8 M Li_2SO_4) resulted in formation of the co-crystals, which did not possess any catalytic activity.³⁹² All these data prompted us to undertake an UV melting and high resolution NMR structural study of this hammerhead RNA under physiological conditions (low salt concentration), to shed light on the dynamics of RNA folding and the ribozyme formation. To simplify NMR investigation, the hammerhead forming RNAs (16mer and 25mer) were synthesized according to the Uppsala NMR-window strategy (Figure 25), where the sugar residues of the nucleosides forming stem I,

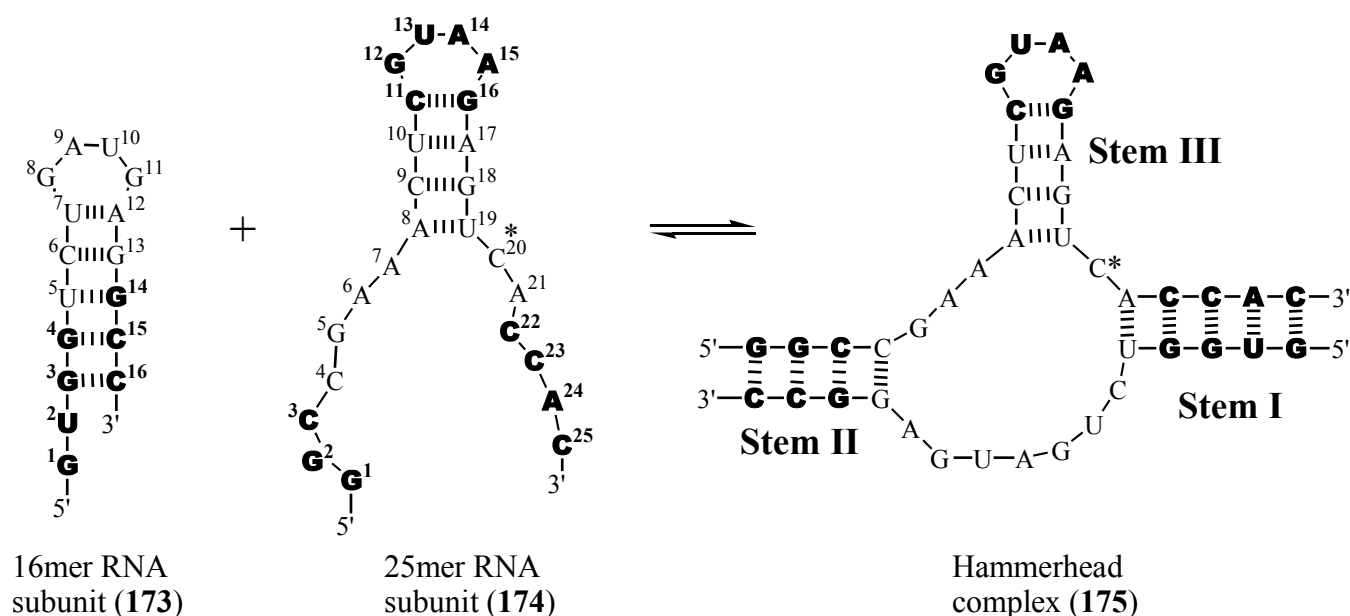


Figure 25: The secondary structures of the 16mer (173) and 25mer (174) RNA strands, and the hammerhead ribozyme construct (175) for which X-ray data is available. The deuterated residues are printed in bold.

stem II and the loop of the stem III of the resulting hammerhead complex were deuterated (Figure 25). Deuterated nucleosides were synthesized using the deuterated ribose prepared earlier,²⁸⁷ exploiting the synthetic route previously described.²⁸⁹ All pyrimidine residues were also C-5 deuterated and 2'-*O*-methyl cytidine³⁹³ was introduced at the active site to prevent the cleavage reaction.

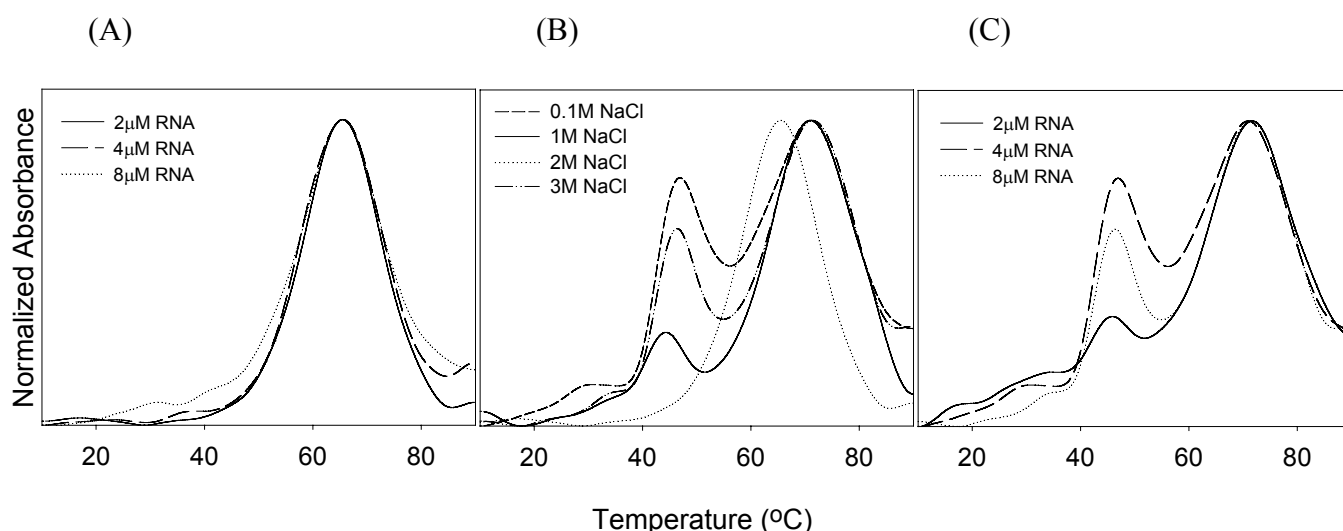


Figure 26: (A) First derivatives of the UV melting curves observed for 16mer RNA (173) in 2 M NaCl phosphate buffer (all buffers contain 20 mM phosphate pH = 7.5), show concentration independency of this transition. (B) The relative amount 16mer RNA going through the second transition is increasing with increased NaCl concentration in the phosphate buffer (the RNA concentration is 8 μM in all samples). (C) Population of the 16mer RNA tertiary structure as a function of RNA concentration: the high temperature transition is still RNA concentration independent ($T_m = 71.8 \pm 0.4^\circ \text{C}$), whereas the second transition appears to be a bimolecular process (each sample was recorded in the buffer containing 2 M NaCl).

Melting temperature of the main transition of the 16mer is not RNA concentration dependent at any salt concentration ($65.6 \pm 0.3^\circ \text{C}$ in 100 mM NaCl, $71.1 \pm 0.1^\circ \text{C}$ in 1 M NaCl and $71.8 \pm 0.4^\circ \text{C}$ in 2 M NaCl) in the range of the RNA concentrations from 0.5 to 24 μM (Figure 26a). This suggests that the high temperature transition (and the only transition at low salt) belongs to the hairpin-random coil interconversion (equilibrium 2 in Figure 27). Indeed, when the 16mer RNA has been fully assigned using NOESY/ROESY spectra in combination with DQF-COSY and HSQC (Figure 28A) in phosphate buffer alone (10 mM phosphate, pH 6.7), it was found to be in a hairpin conformation. Increase of the salt concentration (Figure 26b) results in appearance of the second – low temperature – transition. This transition was found to be RNA concentration dependent (Figure 26c) suggesting that it reflects a bimolecular process and can be assigned to the duplex-hairpin interconversion (equilibrium 1 in Figure 27). The 25mer RNA shows one concentration independent transition in 0.1 M NaCl phosphate buffer (pH 7.5). The imino, H6/H8 and the H1' protons of the 25mer could be assigned for the loop region (residues ^8A to ^{16}U), but flexibility of the ends and the existence of a secondary structure complicated full assignment of the 25mer (Figure 28B).

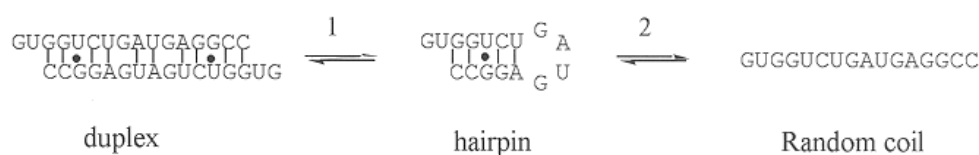


Figure 27: Equilibria occurring in the 16mer RNA (**173**) in aqueous solution

When the 16mer RNA and the 25mer RNA are mixed together in 0.1 M NaCl phosphate buffer (pH 7.5), the hairpin formed by the 16mer remains intact (Figure 28C), preventing the formation of any hammerhead-like structure to any significant extent to NMR. Thus, we conclude that the 16mer catalytic part and the 25mer substrate part of the ribozyme do not form the active hammerhead complex spontaneously in 10-30 mM phosphate buffer containing as much as 300 mM NaCl and 7.5 mM Mg^{2+} . This means that the hairpin formation under physiological condition is thermodynamically favored, and therefore the free energy of hammerhead formation in this media must be significantly higher than the -27.4 kJ/mol found for the hairpin. Further increment of the salt concentration (300 mM NaCl and 7.5 mM Mg^{2+}) and the addition of 500 mM Li_2SO_4 give rise to significant changes for most of the resonances in the imino- and H6/H8-H1' regions. This, together with the appearance of new imino resonances, allow us to suggest that the observed line-broadening (even the cancellation of some crosspeaks) is not only caused by the added salt, but could also be attributed to slow exchange between

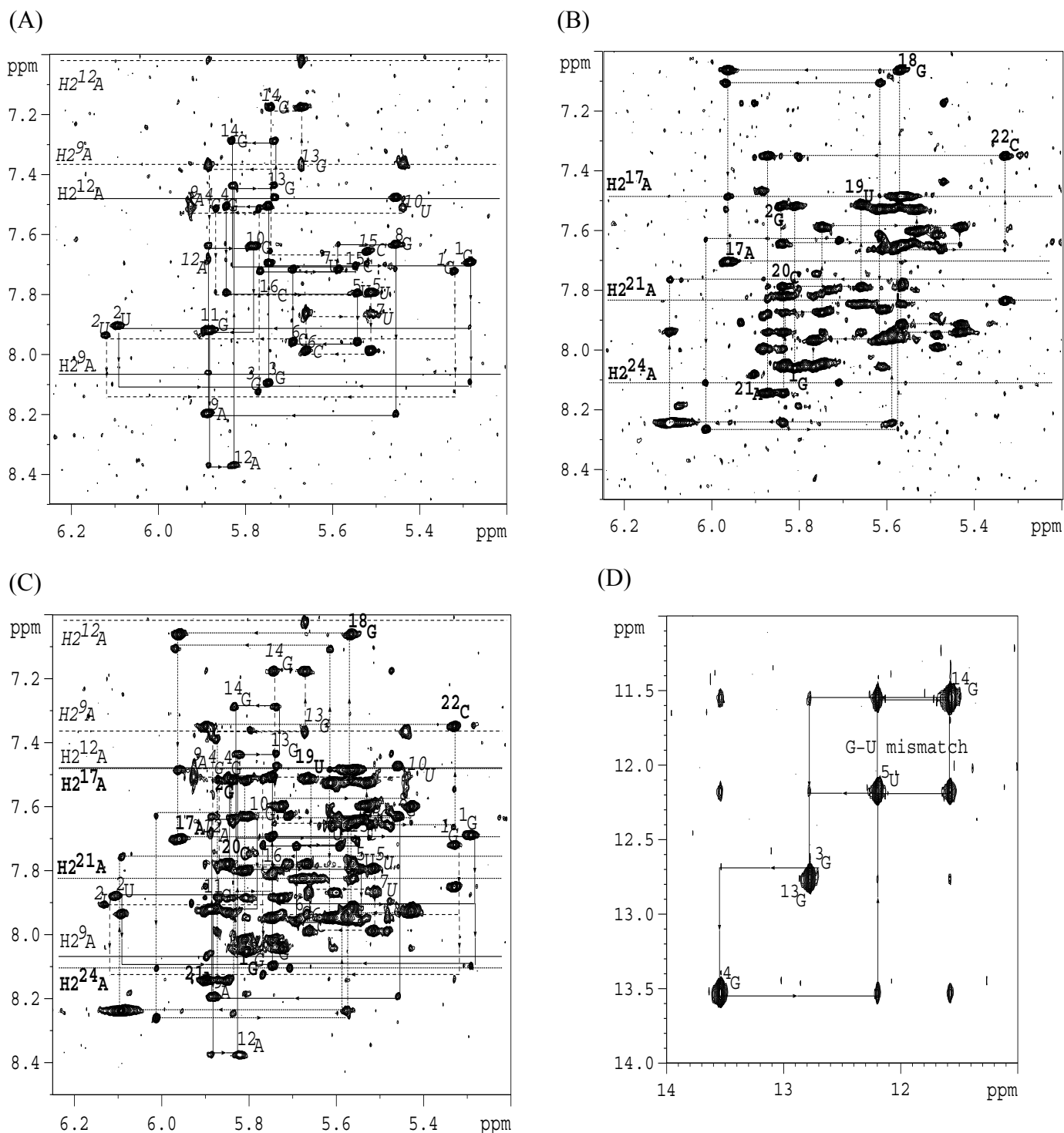


Figure 28: (A) 16mer (173) assignment at 600 MHz (20° C). The solid lines trace the aromatic-H1' connections in the 16mer hairpin structure. The dashed lines trace a secondary duplex structure that only exists to any large extent in salt concentrations above 100 mM NaCl and temperatures below 40° C. (B) The sequential H6/H8-H1' assignment of the 25mer RNA (174) at 20° C. A secondary uni-molecular structure, possibly a different fold, also exists in approximately 4:6 ratio. (C) The H6/H8-H1' region of the mixture of 16mer and 25mer resembles the superimposition of the spectra of the two components separately. (D) The imino region of the 16mer (173) at 0° C shows the internal base pairings, including the characteristic G-U mismatch.

the hairpin/duplex structure of the 16mer and a higher ordered structure.

The NMR study of this particular hammerhead structure was initiated on the basis that the crystal structures of the modified and non-modified all-RNA ribozyme of this sequence were already reported.³⁸⁶ The structures of the unmodified hammerhead RNA in the absence or presence of divalent cations at low pH (non-cleavable conditions) were found to be similar to the modified counterpart.³⁸⁷ Soaking such crystals in Mg^{2+} containing buffer of higher pH allows the cleavage reaction in the crystal, and flash-freezing of the crystal after short exposure to the cleavage solution (few minutes) gave the possibility to trap and investigate the structure of the catalytically active conformation of the ribozyme.³⁸⁷ This study revealed a significant conformational change in the region of the cleavage site resulting in positioning the scissile phosphate backbone toward the 2'-hydroxyl of the ribose to accommodate a geometry compatible with in-line attack. However, crystal growth and cleavage conditions were far from physiological - 1.8 M Li_2SO_4 , 25-100 mM MgSO_4 , 20 % glycerol. Authors also mentioned the possibility that captured active intermediate form might exist in high concentration only in the crystal (due to lattice contacts) and would not accumulate appreciable in the solution. Clearly, the solution structure of the hammerhead ribozyme in physiological conditions was required. This would provide a direct comparison between solution and solid-state structures as well as validity and applicability of the X-ray structural findings to solve the mechanism of the RNA self-cleavage *in vivo*. However, the NMR data on structure of the hammerhead ribozyme available today is very limited.^{377,380,394,395} Although some progress on structure and conformation has been made by assignment of imino protons of the complexes,³⁷⁷ and use of $^{13}\text{C}/^{15}\text{N}$ labeled RNAs in heteronuclear NMR experiments allowed to observe some intermolecular NOE signals of non-exchangeable protons,^{380,394,395} the complete assignment of the catalytic core of the hammerhead in solution under quasiphsiological condition is not yet available. Clearly, removal of the proton resonances from ribose moieties of the helical parts of the hammerhead by selective deuteration, as demonstrated in this work, through Uppsala NMR window,^{276,286-305} would greatly simplify the assignment of the catalytic part.

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