NOTE TO USERS

Synthesis and Biological Evaluation of Oligonucleotides containing 3'- and 5'-S-Phosphorothiolate Internucleotide Linkages

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Dedicated to my beloved parents Antonietta and Antonio Tedeschi for all their love, encouragement and sacrifices.

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TABLE OF CONTENTS

ABBREVIATIONS AND SYMBOLS

A	**adenosine**
Å	**angstrom**
A_{260}	**UV absorbance measured at 260 nm**
AcOH	**acetic acid**
ACN	**acetonitrile**
AIDS	**acquired immunodeficiency syndrome**
AON	**antisense oligonucleotide(s)**
ANA	**arabinonucleic acid**
2'F-ANA	**2'-fluoro-2'-deoxy-arabinonucleic acid**
APS	**ammonium persulphate**
ara	**arabino**
ATT	**6-aza-2-thiothymine**
AZT	**3'-azido-3'-deoxythymidine**
bp	**base pairs**
BIS	**N,N'-methylene-bis(acrylamide)**
BPB	**bromophenol blue**
Bz	**benzoyl**
°C	**degree Celsius**
CD	**circular dichroism**
CPG	**controlled-pore glass**
d	**doublet**
D4I	**2',3'-didehydro-2',3'-dideoxyinosine**
D4T	**2',3'-didehydro-2',3'-dideoxythymidine**
DCI	**4,5-dicyanoimidazole**
DCM	**dichloromethane**
dd	**doublet of doublets**
ddA	**2',3'-dideoxyadenosine**
ddC	**2',3'-dideoxycytidine**
ddT	**2',3'-dideoxythymidine**
ddI	**2',3'-dideoxyinosine**
DEPC	**diethyl pyrocarbonate**
DIPEA	**N,N'-diisopropylethylamine**
DMTr	**dimethoxytrityl**
DNA	**2'-deoxyribonucleic acid**
dNTP	**deoxyribonucleotide triphosphate**
ds	**double stranded**
DTT	**dithiothreitol**
E. coli	***Escherichia coli***
EDTA	**disodium ethylenediamine tetraacetate**
e.g.	**for example**
EtOH	**ethanol**
ETT	**5-ethylthiotetrazole**
eq	**equivalent(s)**
FAB-MS	**fast atom bombardment mass spectrometry**

3'-FT	**3'-deoxy-3'-fluorothymidine**
g	**gram(s)**
G	**guanosine**
ΔH°	**standard enthalpy change**
HIV-1	**human immunodeficiency virus type 1**
HMDS	**hexamethyldisilazane**
HPLC	**high performance liquid chromatography**
h	**hours**
Hz	**Hertz**
J	**coupling constant**
λ	**wavelength**
LCAA-CPG	**long-chain alkylamine controlled-pore glass**
M	**molar**
m	**multiplet**
MALDI-TOF	**matrix assisted laser desorption ionization time of flight**
MBO	**mixed-backbone oligonucleotide**
m/c	**mass to charge ratio**
MeOH	**methanol**
min	**minute(s)**
mL	**milliliter**
mg	**milligram**
mM	**millimolar**
μM	**micromolar**
MAST	**[Bis(2-methoxyethyl)amino]sulfur trifluoride**
MMTr	**monomethoxytrityl**
mol	**mole**
mRNA	**messenger RNA**
MS	**mass spectroscopy**
MW	**molecular weight**
N	**northern**
NBA	***p*-nitrobenzyl alcohol**
nm	**nanometer**
NMI	**N-methylimidazole**
NMR	**nuclear magnetic resonance**
OD	**optical density**
ODN	**oligodeoxynucleotides**
PAGE	**polyacrylamide gel electrophoresis**
ppm	**parts per million**
®	**register trademark**
R_f	**retardation factor (TLC mobility)**
RNA	**ribonucleic acid**
2'F-RNA	**2'-fluoro-2'-deoxyribonucleic acid**
2'OMe-RNA	**2'-O-methoxy-2'-deoxyribonucleic acid**
RNase H	**ribonuclease H**
rRNA	**ribosomal RNA**
RT	**reverse transcriptase**

rt	**room temperature**
s	**singlet (NMR)**
S	**southern**
ss	**single stranded**
$\Delta S°$	**standard entropy change**
sec	**second**
SEC	**size exclusion chromatography**
SVPDE	**snake venom phosphodiesterase**
T	**thymidine**
t	**triplet (NMR)**
TBAF	**tetra-*n*-butylammonium fluoride**
TBE	**TRIS/boric acid/ EDTA buffer**
TCA	**trichloroacetic acid**
TEA	**triethylamine**
TEMED	**N,N,N',N'-tetramethylethylenediamine**
THF	**tetrahydrofuran**
TLC	**thin layer chromatography**
T_m	**thermal melt transition temperature (melting temperature)**
™	**trademark**
TREAT-HF	**triethylamine trihydrofluoride**
TRIS	**2-amino-2-(hydroxymethyl)-1,3-propanediol**
tRNA	**transfer RNA**
U	**uridine**
UV	**ultraviolet**
UV-VIS	**ultraviolet-visible**
v/v	**volume by volume**
vs	**versus**
WC	**Watson-Crick**
w/v	**weight by volume**
XC	**xylene cyanol**

LIST OF FIGURES

CHAPTER I

CHAPTER II

CHAPTER III

LIST OF TABLES

LIST OF SCHEMES

ABSTRACT

The use of chemically modified oligonucleotides (AON) to selectively inhibit the expression of genes involved in pathological processes, offers a rational approach for the prevention and treatment of some disorders. Phosphorothioate oligonucleotides (PS-DNA), in which one of the non-bridging oxygen atoms of the internucleotide linkage is replaced by a sulfur atom, have been extensively studied and are among the most widely used "antisense" oligonucleotides (AON). However, the isomeric analogue, in which one of the two bridging oxygen atom is replaced by sulfur has not been extensively investigated, as such, this prompted our interest towards the construction of oligonucleotides containing the modified 3'-S- and 5'-S-phosphorothiolate linkages. An oligodeoxynucleotide (DNA) strand containing a single and three consecutive 3'-and 5'-S-phosphorothiolate internucleotide linkages as well as a chimeric oligonucleotide, composed of 2'-OMe-RNA 'wings' with a central hexanucleotide ("gap") composed of 3'-deoxy-3'-thiothymidine ($T_{3'S}$) residues were successfully prepared via the phosphoramidite approach. UV thermal melting and circular dichroic (CD) studies were carried out to ascertain the thermodynamic stability and helical conformation of the duplexes formed between these modified oligomers with their complementary DNA and RNA targets. Hybridization studies revealed that the incorporation of the modified 3'-S-phosphorothiolate linkages in oligonucleotides results in enhanced binding affinity towards a single stranded RNA or DNA target. Conversely, duplexes containing the 5'-S-phosphorothiolate linkages exhibit lower thermodynamic stability compared to the unmodified duplexes (DNA/DNA and RNA/DNA). Moreover, the oligonucleotide chimera containing the internal $T_{3'S}$ gap, showed a dramatic increase in binding affinities for single stranded RNA and DNA targets compared to the unmodified DNA oligonucleotide.

The ability of the AON/RNA hybrids to elicit RNase H activity was also evaluated. While the 3'S modification was detrimental to the enzyme activity, the 5'S-modification was well tolerated by the enzyme. This is consistent with the conclusions reached from the CD and UV studies that 3'S and 5'S modifications mimic, respectively, the native RNA and DNA structures. Thus, a DNA/RNA hybrid containing the 5'S-modification

within the DNA strand more closely resembles the normal DNA/RNA substrate of the enzyme.

Another goal was to synthesize 2',3'-dideoxy-2'-fluoro-3'-thioarabinonucleic acids (2'F-3'S-ANA) and evaluate their potential as an antisense agent. Our efforts towards the preparation of the required nucleoside monomer, 2'-deoxy-2'-fluoro-3'-S-phosphorothioamidite, led to the discovery of 2',3'-α-epi-thiothymidine, a novel [3.1.0]-fused 2',3'-modified nucleoside with potential antiviral activity.

RÉSUMÉ

L'utilisation d'oligonucléotides chimiquement modifiés pour l'inhibition sélective des gènes impliqués dans les processus pathologiques, offre une approche rationnelle pour la prévention et le traitement de certains troubles. Les oligonucléotides phosphorothioate (PS-ADN), dans lesquels un des atomes d'oxygène (mono-lié) de la liaison internucléotides est remplacé par un atome de soufre, ont été étudiés de manière intensive et font partie des oligonucléotides "antisens" (ONA) les plus utilisés.

Toutefois, l'analogue isomérique dans lequel un des deux atomes d'oxygène en pontage est remplacé par un atome de soufre n'a pas été étudié, ce qui a suscité notre intérêt vers l'élaboration d'oligonucléotides contenant des liaisons phosphorothiolate modifiées en 3'-S- et 5'-S-. Un brin d'oligodéoxynucléotide (ADN) contenant une et trois liaisons internucléotides consécutives de 3'-S- et 5'-S-phosphorothiolate, ainsi qu'un oligonucléotide chimère composé d'ailes de 2'-OMe-ARN entourant un hexanucléotide central ("brèche") qui consiste de résidus 3'-deoxy-3'-thiothymidine ($T_{3'S}$) ont été préparés avec succès par l'approche phosphoramidite. Des études thermales UV et de dichroïsme circulaire (DC) ont permis d'établir la stabilité thermodynamique et la structure hélicoïdale des duplex formés entre ces oligomères modifiés et leurs cibles complémentaires d'ADN et ARN. Les études d'hybridisation ont révélé que l'incorporation des liens modifiés 3'-S-phosphorothiolate dans les oligonucléotides a pour effet d'augmenter l'affinité de liaison avec une cible monocaténaire ADN ou ARN. Toutefois, les duplex contenant la liaison 5'-S- phosphorothiolate sont moins stables thermodynamiquement que les duplex non-modifiés (ADN/ADN et ARN/ADN). De plus, l'oligonucléotide chimère qui contient la brèche $T_{3'S}$ a montré une affinité de liaison largement accrue pour des cibles monocaténaires ADN et ARN, par rapport à l'oligonucléotide ADN non-modifié.

La capacité des hybrides ONA/ARN à provoquer l'activité ribonucléase H a aussi été évaluée. Tandis que la modification 3'-S est détrimentale à l'activité enzymatique, la modification 5'-S a bien été tolérée par l'enzyme. Ce résultat appuie les conclusions, obtenues lors des études DC et UV, que les modifications 3'-S et 5'-S-mimiquent, respectivement, les structures natives ADN et ARN. Par conséquent, un hybride

ADN/ARN contenant la modification 5'-S dans le brin d'ADN ressemble plus particulièrement au substrat normal ADN/ARN de l'enzyme.

Notre intérêt s'est aussi porté sur la synthèse d'acides 2',3'-bidésoxy-2'-fluoro-3'-thioarabinonucléiques (2'F-3'S-ANA) et l'évaluation de leur potentiel d'agents antisens. Nos efforts envers la préparation du monomère nucléoside désiré, le 2'-désoxy-2'-fluoro-3'-S-phosphorothioamidite, nous ont conduits vers la découverte du 2',3'-α-épi-thiothymidine, un nouveau [3.1.0]-nucléoside fusionné modifié en 2',3'-, qui possède une activité antivirale potentielle.

CHAPTER I: INTRODUCTION

1.1 INTRODUCTION TO NUCLEIC ACIDS[1,2,3]

The chemistry of nucleic acids can be considered to be the core chemistry of life. Nucleic acids are the most essential constituents of all living cells as they govern a number of cell's functions including metabolism. They also have vital roles fundamental to the storage and transmission of the genetic information within cells and also from one generation to the next.

There are two chemically distinct types of nucleic acids in a cell: 2'-deoxyribonucleic acid (DNA) and ribonucleic acid (RNA). Both DNA and RNA are composed of a linear sequence of four different monomers called nucleotides which are linked via 3'-5'-phosphodiester linkages. Nucleotides are the phosphate esters of nucleosides and consist of three components: a nitrogen heterocyclic base, a pentose sugar (five-carbon sugar) and a phosphate group, as depicted in **Figure 1.1**. In RNA, the pentose sugar is D-ribose whereas in DNA it is 2-deoxy-D-ribose. The heterocyclic bases are planar and divided into two classes; the purines which are fused heterocyclic rings and pyrimidines which are monocyclic. The presence of the nitrogen atom in these heterocycles gives the molecules a basic character; at physiological pH the natural heterocyclic bases are not protonated. These bases are comprised of adenine (A), guanine (G) and cytosine (C) (which are found in both DNA and RNA) whereas, thymine (T) is found only in DNA and uracil (U) in RNA. The 1' carbon of the sugar, either D-ribose or 2-D-deoxyribose, is linked via a N-glycosidic linkage to the nitrogen (9 or 1) atom in purines and pyrimidines, respectively. The configuration of this linkage is β, as the base lies on the same side as the C5' hydroxymethyl group. A nucleic acid strand has a chemical orientation or "directionality": one end of the chain has a free 5'-hydroxyl (OH) group and the other end a free 3'-OH.

A monumental discovery was accomplished by Watson and Crick in 1953, when they proposed the three-dimensional structure of DNA. They elucidated the structure via analysis of x-ray diffraction images obtained by Rosalyn Franklin and Maurice Wilkins

as well as model building. Their discovery revolutionized the study of genetics and molecular biology which ultimately led to the recombinant DNA techniques used by today's biotechnology industry.

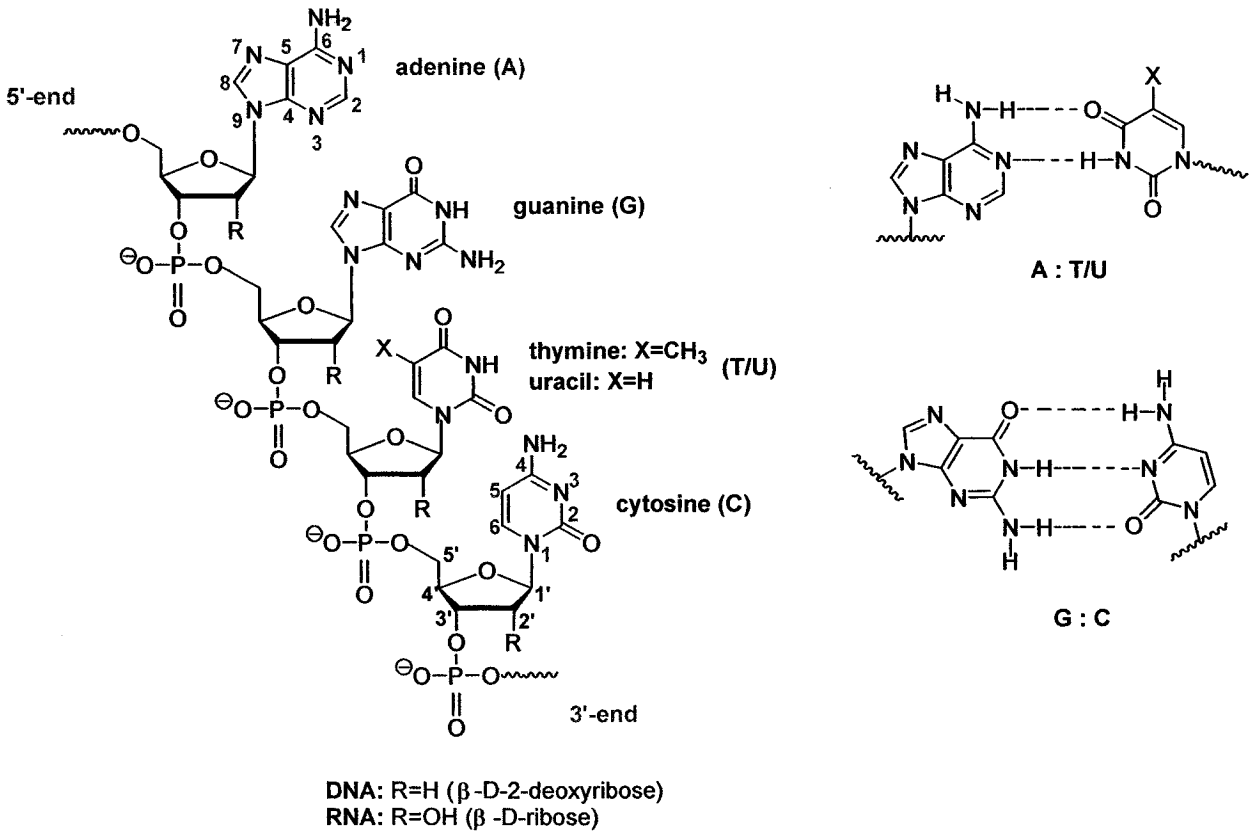


Figure 1.1: Primary structure of 2'-deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) and their constituent nucleosides; A, G, T(orU), and C. Hydrogen-bonding between bases ocuurs via Watson-Crick complementarity (A:T and G:C) and is demonstrated on the right. Each 3'-5'-phosphodiester linkage is generated by joining the 5' carbon in one nucleotide to the 3' carbon in the adjacent nucleotide resulting in the chemical polarity of the polynucleotide chain.

Watson and Crick suggested that DNA takes the shape of a double helix, an elegantly simple structure that resembles a "gently twisted ladder." The "rails" of the ladder consist of two anti-parallel sugar-phosphate strands which wind around a central axis in a rigid, right-handed helical arrangement. The strands are connected by "rungs" each composed of purine and pyrimidine base pairs held together by a variety of hydrogen-bonds and base-base stacking interactions among the flanking nucleotide resides at the interior of the helix. Specifically, adenine base pairs with thymine via two hydrogen bonds, whereas

guanine base pairs with cytosine via three hydrogen bonds. The second major interaction is hydrophobic and results in the vertical stacking of pi (π) electron systems of the coplanar heterocyclic bases along the vertical axis of the double helix. These two base-derived stabilizing interactions impart on the DNA molecules stability necessary to preserve life.

RNA bears the same sugar-phosphate backbone as DNA with the exception of an additional hydroxyl group at the C2' position of the sugar moiety. As a result, RNA is more labile or sensitive towards hydrolysis due to participation of the 2'OH in the degradation pathway of the sugar-phosphate backbone (both chemically and enzymatically). Unlike DNA which is usually in the double stranded form (preserves genetic information), RNA molecules exist predominantly in single stranded (ss) form in the cell. RNA molecules are synthesized by a process known as DNA transcription, where one of the two strands of DNA acts as a template. The resulting RNA transcript retains all of the information of the DNA sequence from which it was copied as well as the base-pairing properties of DNA. Three major types of RNA transcripts exist: messenger RNA (mRNA), transfer RNA (tRNA) and ribosomal RNA (rRNA).

1.2 DNA REPLICATION[1,4]

DNA was proven to be the carrier of genetic information which must be copied and transmitted from each cell to all of its progeny. Watson and Crick proposed that the mechanism of DNA replication occurs in a semi-conservative fashion in that each newly made strand is copied from the one of the parental strands and the product of replication are two molecules, each containing one parental strand and one newly synthesized strand.

Although the principle underlying gene replication is both elegant and simple, the actual machinery by which this copying is carried out in the cell is complicated and involves a complex of proteins that form a 'replication machinery'. Replication is initiated with the local separation of the two complementary DNA strands. Each strand then acts as a template for the formation of the new DNA molecule by the sequential addition of deoxyribonucleotide triphosphates (dNTP's). The fundamental reaction in which the enzyme DNA polymerase catalyzes the addition of dNTP's to the 3' end of the DNA chain is illustrated in **Figure 1.2**, in which each addition is followed by the concomitant release of pyrophosphate and its subsequent hydrolysis, which provides the energy for the DNA replication reaction rendering it effectively irreversible. The nucleotide added at each step is selected by a process which requires it to form a complementary base pair with the next nucleotide in the template strand, thereby generating a new DNA strand which is complementary in sequence to the template strand. Eventually, the genetic information is duplicated in its entirety, yielding two complete DNA double helices, each identical in nucleotide sequence to the parental DNA helix that served as the template.

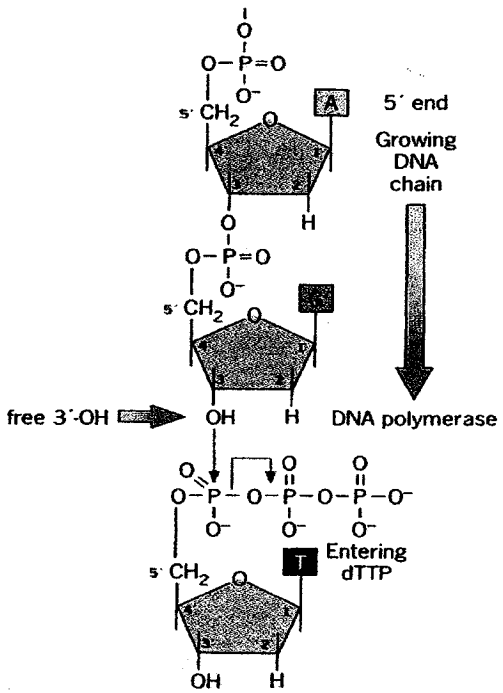


Figure 1.2: Schematic representation of the fundamental reaction by which DNA is synthesized within the cell. Figure adapted from Snustad, D. P.; Simmons, M.J. and Jenkins, J. B..[4]

DNA chain growth can be blocked by the incorporation of certain nucleotide analogs that have been modified in the sugar portion of the nucleotide. For instance, removal of the hydroxyl group from the 3'-carbon of the deoxyribose ring or conversion of the deoxyribose to another sugar for example, arabinose, prevents further chain elongation by blocking the formation of a 3'-5'-phosphodiester linkage in DNA synthesis. By inhibiting DNA replication, these nucleotide analogs slow the division of rapidly growing cells and viruses.

1.2.1 NUCLEOSIDES AS ANTIVIRAL AGENTS

Since the human immunodeficiency virus type I (**HIV- I**) was found to be the causative agent of AIDS, the search for potential agents against the virus has greatly stimulated studies on structurally modified nucleosides in the past years, most of this work being devoted to nucleoside analogues lacking the 3'-hydroxyl group. Several 2',3'-dideoxynucleosides, *e.g.* 2',3'-dideoxycytidine (**DDC**), 2',3'-dideoxyadenosine (**DDA**), 3'-deoxythymidine (**DDT**), 2',3'-dideoxyinosine (**DDI**), 3'-azido-3'-deoxythymidine

(AZT), 3'-fluoro-3'-deoxythymidine (**3'FT**), 2',3'-didehydro-3'-deoxythymidine (**D4T**) and 2',3'-didehydro-2',3'-dideoxyinosine (**D4I**), as depicted in **Figure 1.3,** have been successfully employed as chemotherapeutic agents due to their ability to selectively inhibit the HIV specific reverse transcriptase (RT), and consequently, suppressing the replication of HIV.[5]

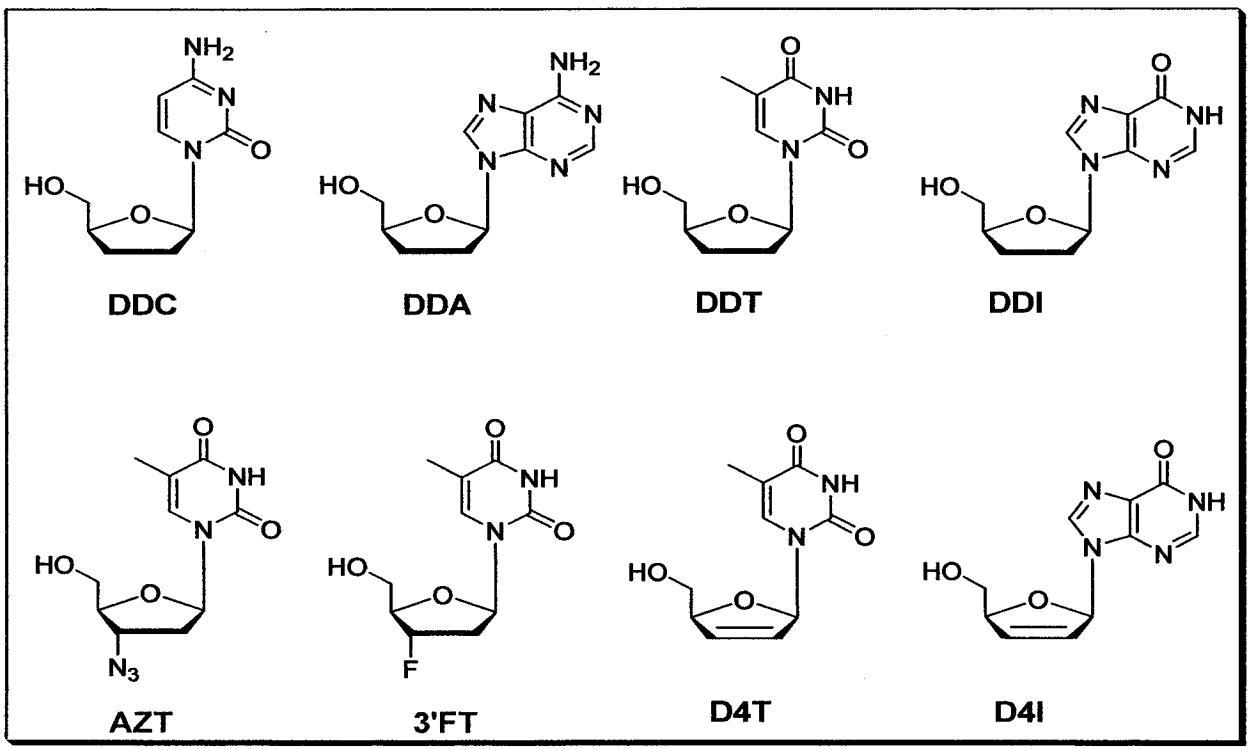


Figure 1.3: Biologically active nucleoside analogs. These compounds show promise as chemotherapeutic agents because of their ability to inhibit selectively HIV-1 reverse transcriptase.

These nucleoside analogues are initially converted to active 5'-triphosphates by cellular kinases which subsequently compete with the natural substrates during DNA chain elongation by HIV-1 reverse transcriptase (RT) and are incorporated into DNA. Since they lack a 3'-hydroxyl for elongation of the DNA chain and given that RT has no 3'-5' proofreading ability, DNA synthesis is irreversibly halted causing chain termination.[6,7] In addition, these nucleoside inhibitors are valuable as anti-HIV therapeutics since the corresponding triphosphates are much better substrates for HIV-1 RT than they are for cellular DNA polymerase (Pol α). This implies that these nucleoside analogues can selectively inhibit RT without disrupting the host DNA synthesis.[8]

Moreover, it has been reported that [3.1.0]-fused 2',3'-modified nucleosides, such as, 2',3'-β-epi-thiouridine, 2',3'-β-epi-iminouridine and 2',3'-β-epoxyuridine (**Figure 1.4**) are active against lymphotropic retroviruses (HTLV), especially HTLV-I, HTLV-II and HIV[9], and therefore are considered good candidates as therapeutic antiviral agents.

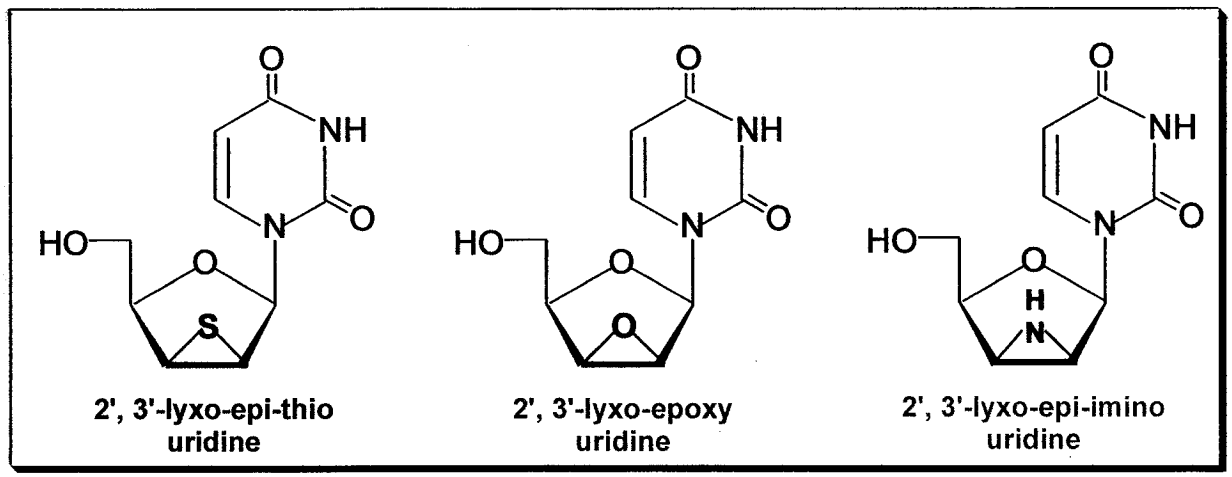


Figure 1.4: Structure of biologically active [3.1.0]-fused 2', 3'-modified nucleosides.

1.3 FLOW OF GENETIC INFORMATION[1,4]

The flow of genetic information from gene to protein begins in the cell's nucleus by the formation of a transcription initiation complex at the promoter region of the gene. This complex consists of protein transcription factors-RNA polymerase II complex and is responsible for unwinding a local region of the DNA duplex, exposing the nucleotides on a short stretch of DNA on each strand. One of the two exposed DNA strands acts as template for complementary base-pairing with incoming ribonucleoside triphosphate monomers, two of which are joined together by the polymerase to begin an RNA chain. The polymerase catalyzes the processive elongation of the RNA chain in the 5'→3' direction by moving stepwise along the DNA, by continuously unwinding the DNA double helix ahead of the polymerization site. This exposes a new region of the template strand for complementary base-pairing while rewinding the two DNA strands behind this site to displace the nascent RNA chain from the DNA template strand, as illustrated in **Figure 1.5**. The chain elongation process continues until the RNA polymerase encounters a stop (termination) signal, whereupon the polymerase halts releasing both the DNA template and the newly synthesized RNA chain. The final RNA product is known as the primary transcript or precursor RNA (pre-mRNA) and is a complementary copy of one on the two DNA strands. The pre-mRNA is then subjected to post-transcriptional processing which is important in the stabilization of the mRNA against the rapid enzymatic degradation once it leaves the nucleus. These modifications include: a). 5' "Capping" of the pre-mRNA which involves addition of a unique 7-methyl guanosine (7-MG) cap structure at the 5'-end of the primary transcript, which facilitates initiation of translation upon its entry into the cytoplasm; b). the addition of approximately 200 adenylic residues (poly-A tail) at the 3'-end of the pre-mRNA facilitates their exit from the nucleus; and c). maturation of the pre-mRNA occurs in the nucleus prior to transport to the cytosol by removal (splicing) of intervening (introns) RNA sequences that do not code for proteins and ligation of the remaining coding sequences (exons) to form the mature mRNA. The entry of mature mRNA in the cytoplasm signals the small ribosomal subunit to bind at the 5' end of the mRNA molecule at the start codon (AUG) which is recognized by a unique initiator transfer RNA (tRNA) molecule. The large ribosomal

subunit then binds to complete the ribosomal complex in order for the elongation phase of protein synthesis to proceed. During this phase aminoacyl tRNAs, each bearing a specific amino acid, sequentially bind to the corresponding codon in mRNA by forming complementary base pairs with the tRNA anticodon. Each amino acid is added to the carboxyl-terminal end of the growing polypeptide chain by means of a cycle of three sequential steps which include: aminoacyl-tRNA binding, followed by peptide bond formation and lastly, ribosome translocation in order to begin the next cycle of chain elongation, as demonstrated in **Figure 1.5**. The ribosome progresses from codon to codon in the 5'→3' direction along the mRNA molecule until a stop codon is reached. A release factor then binds to the stop codon, terminating translation and releasing the completed polypeptide from the ribosome. This channeling of biological information from DNA to RNA to protein has been coined the "Central Dogma of Molecular Biology" and is the inevitable pattern by which all genes are expressed in living organisms.

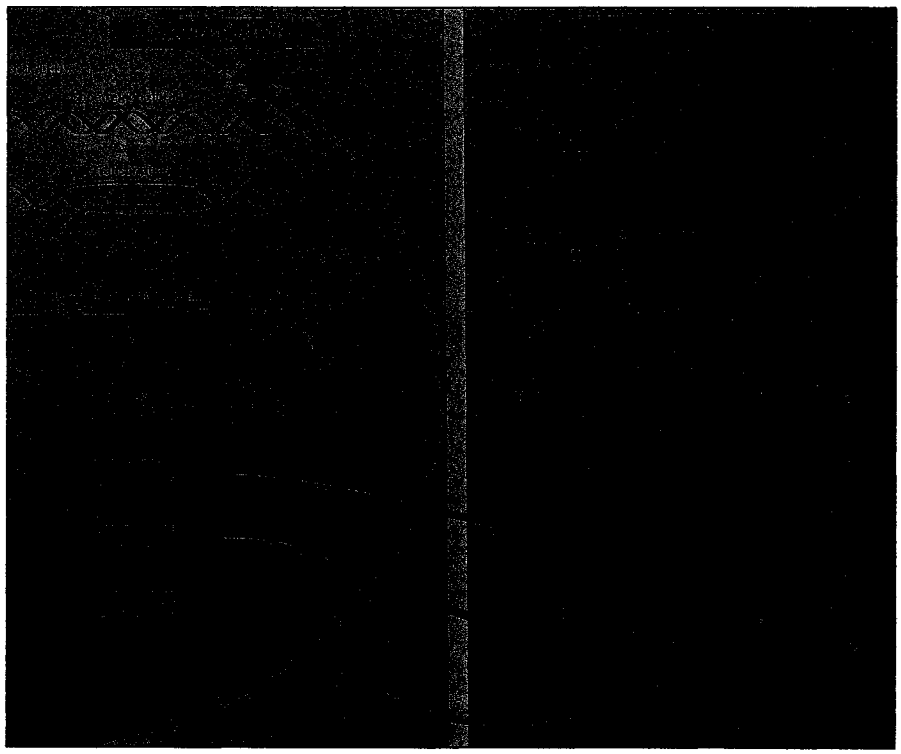


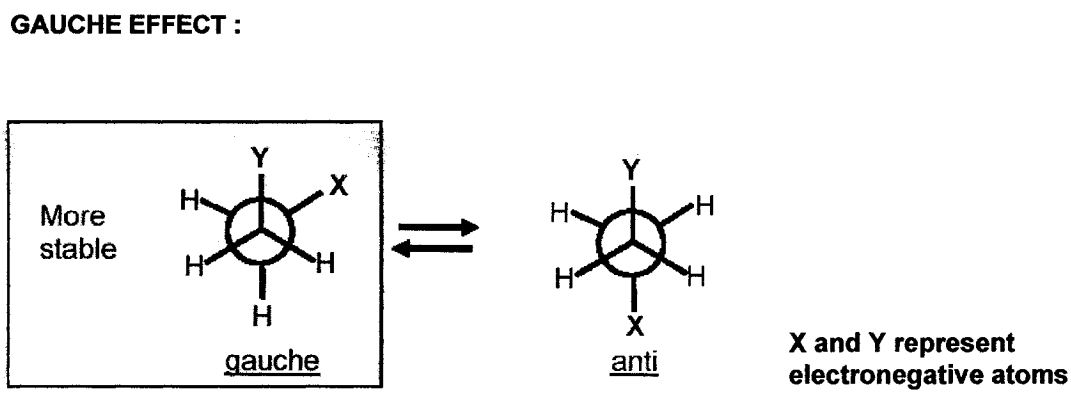
Figure 1.5: The flow of genetic information in eukaryotic cells. Transcription of the pre-mRNA followed by post-transcriptional processing takes place in the cell nucleus. The processed RNA is then exported to the cytoplasm, where the ribosomal machinery directs protein synthesis from the mRNA template. Figure adapted from Snustad, D. P.; Simmons, M.J. and Jenkins, J. B..[4]

1.4 NUCLEIC ACID SECONDARY STRUCTURE

The thermodynamic parameters, ΔH and ΔS work against each other during formation of duplex DNA. The enthalpy change is extremely favorable due to base stacking interactions and hydrogen bond formation, however, the entropy term is highly unfavorable due to the loss of rotational and translational freedom upon hybridization of two separate polynucleotide strands. By rigidifying the backbone by chemical modifications (section 1.5) in such a way that the single stranded state (random coil) is pre-organized into a helical base stacked conformation prior to hybridization, it is possible to reduce the loss of entropy upon duplex formation. The net effect is an increase in the thermodynamic stability of the duplex (T_m).[10]

Nucleotide units themselves in the helix structure can adopt a variety of different conformations depending on the constraints imposed by the sugar-phosphate backbone and the conformational space accessible to the base-pairs (*e.g.* π-π interactions).[11] The conformational structures of the nucleotides are defined by a series of torsion angles in the phosphate backbone, the furanose ring and in the glycosidic bond.[11] The sugar "puckers" in order to minimize the number of non-bonded ecplising interactions (steric and torsional strain) between the substituents on the furanose ring. The specific pucker adopted by the sugar is described by identifying the major displacement of the C2'- and C3'-carbons from the median plane of C1'-O4'-C4'.[12] In solution, the sugar rings in nucleic acids generally exist in an equilibrium between two extreme conformers, the so-called C2'-*endo* (south) and C3'-*endo* (north) puckers. The equilibrium between these two preferred conformational states is dictated by various steric and stereoelectronic effects of the sugar moiety (gauche effect) and the nucleobase (anomeric effect)[13,14], as depicted in **Figure 1.6.** Thus, the presence of the gauche effect of the 5'-OH and 3'-OH groups with respect to the O4' in 2'-deoxyribonucleosides, drives the pseudorotational equilibrium towards the C2'-*endo* pucker (**Figure 1.7**). In ribonucleotides, however, the interplay of stereoelectronic effects is more complex due to the additional gauche effects involving the 2'-OH.[13] The presence of the 2'-hydroxyl drives the pseudorotational equilibrium towards the C3'-*endo* pucker due to a gauche effect between 2'-O and 4'-O (ring oxygen) combined with an Edward-Lemieux anomeric effect at the glycosidic bond,

as depicted in **Figure 1.7**. This anomeric effect of the nucleobase originates from an optimal overlap between the non-bonding (O4') and the antibonding σ^* (C1'-N) orbitals; this is optimized in a purely C3'-*endo* (north) conformation.



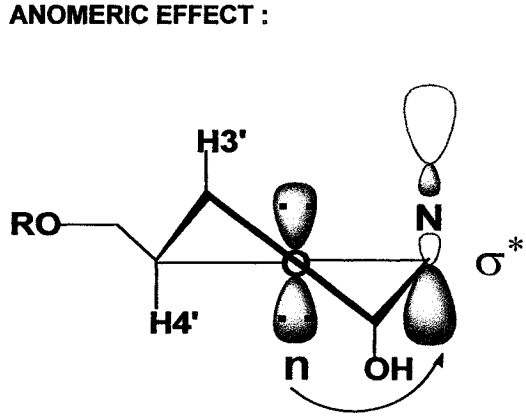


Figure 1.6: The gauche and anomeric effects are two major types of stereoelectonic factors which govern the furanose ring geometry.[15]

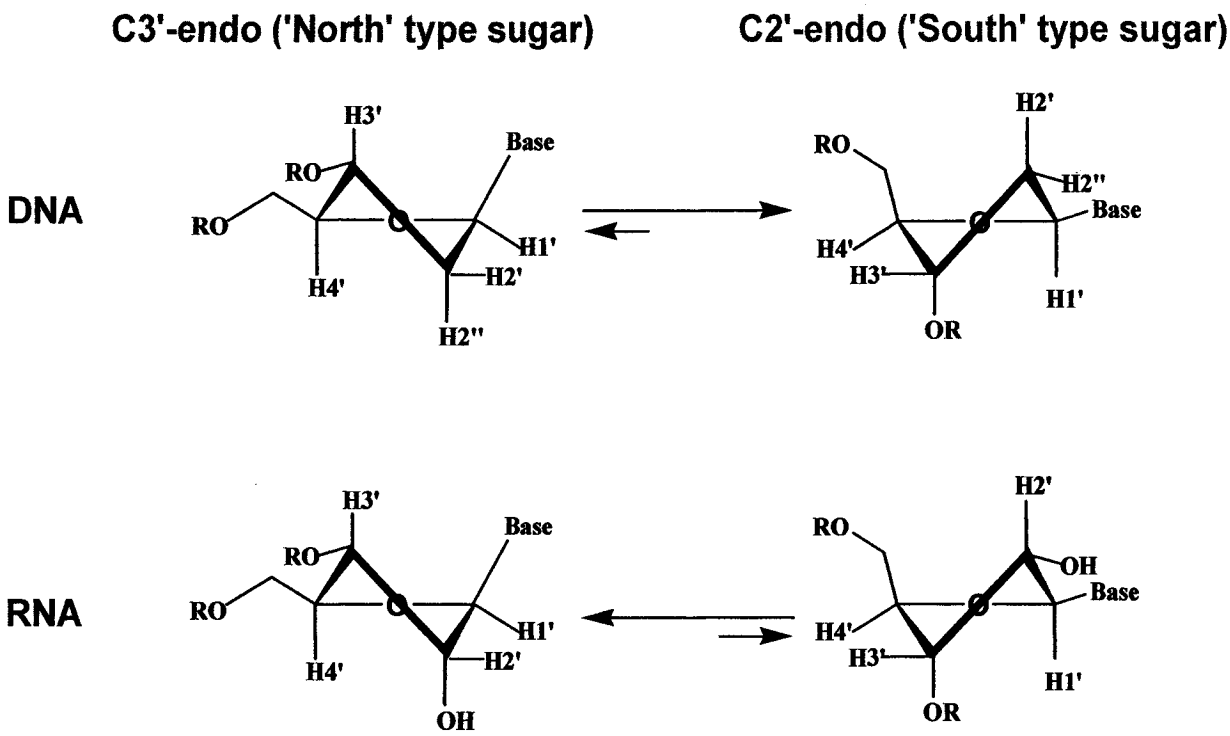
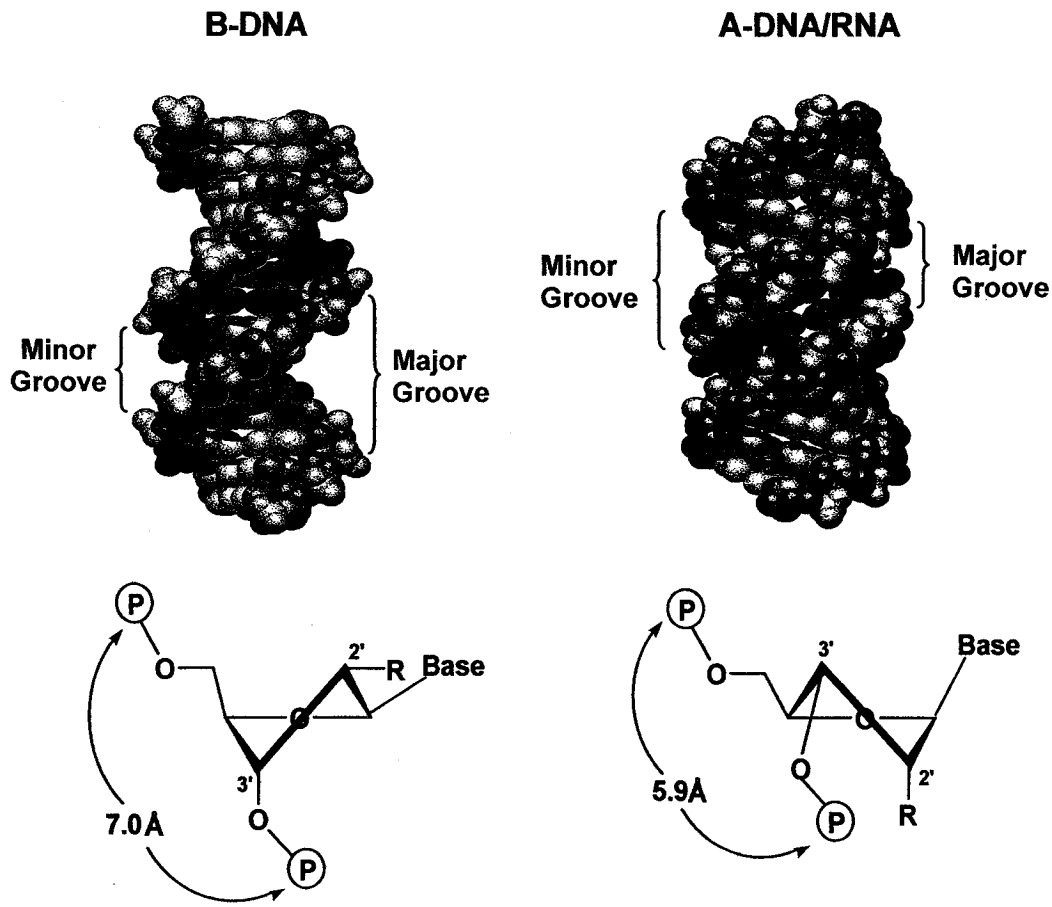


Figure 1.7: Conformations adopted by the furanose sugars of DNA and RNA.

Due to the innate flexibility of the sugar ring, which allows for the dynamic equilibrium between sugar conformations, two different categories of polynucleotide conformations, known as the A- and B-type families, have emerged. At low humidity (or high salt concentration) the favored form is the highly crystalline A-form whereas at high humidity (or low salt concentration) the dominant structure is B-form. A-DNA, like B-DNA, adopt a right-handed double helix made up of antiparallel strands held together by Watson-Crick base-pairing.[16] In B-form DNA, a C2'-*endo* sugar puckering mode is observed which pushes adjacent phosphates of one polynucleotide chain about 7 Å apart, giving rise to overwound helices. The distance between the individual base-pairs along the helix axis is 3.4Å with a length of approximately 10 base-pairs per turn of the helix (e.g. 34Å long). In addition, the base pairs sit directly on the helix axis, thereby producing major and minor grooves of similar depth. The major and minor groove widths are wide and narrow respectively in the B-form helical arrangement, as demonstrated in **Figure 1.8.** In contrast, the A-DNA polymorph, which closely resembles the conformation of RNA, is defined by a shorter internucleotide phosphate-phosphate distance (5.9 Å) compared to

B-DNA owing to the adopted C3'-*endo* sugar pucker in the backbone. The bases are displaced from the helical axis by 4.5 Å, thereby creating a hollow core down the center of the axis. As a result of the closer packing of the constituent residues, there are 11 bases in each turn of 28 Å, which gives a vertical rise of 2.6 Å per base-pair. Moreover, in order to maintain the normal van der Waals separation of 3.4 Å, the stacked bases are tilted sideways. As a result of these features, the major groove of A-DNA is remarkably deep yet narrow whereas, the minor groove is extremely shallow and broad[16] **(Figure 1.8)**. In both A and B helical arrangements, the glycosidic torsion angle places the bases in an anti-conformation, consequently, positioning the bulky portions of the nucleobases, namely the six-membered ring in purines and the O2 in pyrimidines, away from the pentose ring, resulting in a more energetically favored conformation is these structures.



	B-Form	A-Form
Helical sense:	Right	Right
Sugar conformation :	C2'-*endo*	C3'-*endo*
Helix Diameter:	23.7 Å	25.5 Å
Residues/Turn:	10.4	11
Twist/base-pair:	36	32.7
Rise/base-pair:	3.3-3.4 Å	2.9 Å
Base-Pair displacement from axis center:	-0.2 to -1.8 Å	4.5 Å
Major Groove width:	11.7 Å	2.7 Å
depth:	8.8 Å	13.5 Å
Minor Groove width:	5.7 Å	11 Å
depth:	7.5 Å	2.8 Å

Figure 1.8: Global helical conformations adopted by DNA and RNA nucleic acids and their corresponding average structure parameters. The preferred sugar conformation of the A-form and the B-form structures are depicted with their internucleotide phosphate-phosphate distances. The averaged structure parameters were obtained from Blackburn, G. and Gait, M., 1996.[16]

1.5 OVERVIEW OF THE ANTISENSE STRATEGY

The therapeutic use of antisense oligonucleotides represents a new paradigm for drug discovery. The idea of oligonucleotide-based antisense therapy is appealing and dates back to the 1960s when Belikova and colleagues proposed that RNA sequences serve as endogeneous inhibitors of gene expression in prokaryotes.[17] In 1977, Paterson and co-workers, reported that gene expression could be altered through the use of exogeneous, single-stranded nucleic acids which inhibit translation of a complementary RNA in a cell free system.[18] The following year, Zamecnik and Stephenson demonstrated the potential of short DNA oligomer (13-mer) to interact with the mRNA of the Rous sarcoma virus, thereby inhibiting viral replication in cell culture.[19] On the basis of this work, Zamecnik & Stephenson are widely credited for having first suggested the therapeutic utility of synthetic nucleic acids, commonly referred to as "antisense" oligonucleotides.

Specifically, Antisense Oligonucleotides (AONs) are short synthetic stretches of DNA or RNA and their analogs (12-30 nucleotides) which are designed to bind to their complementary mRNA sequence by Watson-Crick base-pairing once they enter the cells, subsequently leading to inhibition of expression of the corresponding disease-relevant protein encoded by the mRNA. Inhibition of translation by antisense oligomers was shown to occur by an active and/or passive mechanism upon hybridization between the complementary helices[20], as illustrated in **Figure 1.9**. Passive mechanism results from the hybridization between the AON and its target RNA. This interaction is strong enough to prevent gene expression by steric blocking or "translational arrest". In this approach, AONs are designed to bind to either the 5'-untranslated region or the AUG initiation region of the mRNA, thereby interfering with ribosomal binding and assembly and hence physically blocking the initiation and elongation steps of protein translation. In the active mechanism, binding of a DNA-like AON to the mRNA allows for the activation of RNase H, a constitutive and ubiquitous enzyme which specifically cleaves the mRNA strand of a DNA/RNA duplex via its endo- and 3'-5' exonuclease activities, leaving the AON intact to hybridize with yet another mRNA target. Cleavage of target mRNA by RNaseH is probably the most important mechanism of antisense action and underlies the activity of all the oligonucleotides successfully tested in clinical trials so far.

The antisense oligonucleotide (AON) approach to drug design has, in principle, two advantages over conventional drugs, firstly, the mRNA of the disease target gene has a defined sequence and secondly, the antisense oligonucleotide interacts with the target gene by Watson-Crick base pairing, providing specificity and affinity and are less likely to cause side effects (toxicity). Furthermore, most conventional drugs target proteins rather than mRNA. Once protein synthesis begins, millions of copies of a given protein can be produced, the amount depends on the gene coding for it, all of which have to be deactivated. However, compared with the protein it produces, the amount of mRNA is trivial, therefore, if a drug is designed to block the mRNA rather than a protein, it has a much easier task ahead of it in terms of amount of target to be deactivated.

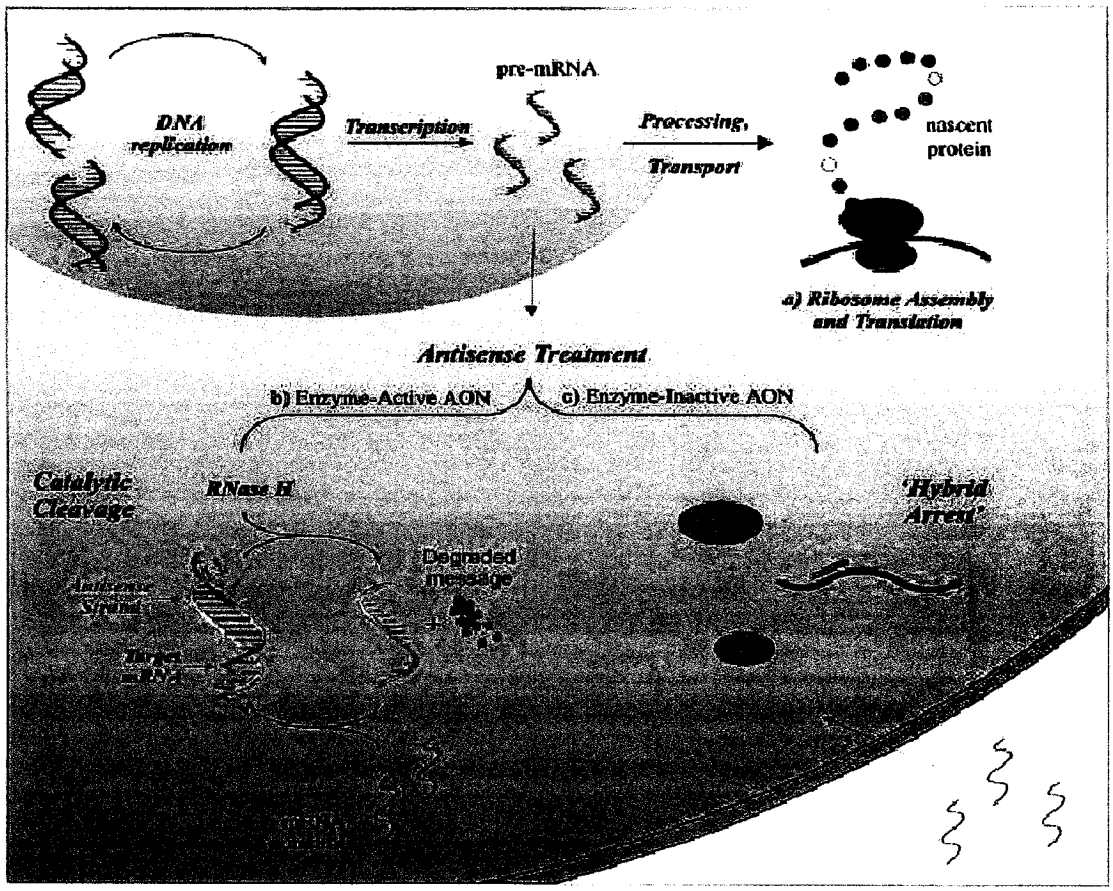


Figure 1.9: Fate of an mRNA transcript under (a) normal cellular conditions; (b). upon hybridization with an enzyme-inducing AON in the presence of ribonuclease H; and (c). mRNA inactivation by steric block of the message via duplex formation. Figure adapted from Mangos *et al.* 2002.[21]

Conventional phosphodiester oligonucleotides are generally not suitable for antisense use in intact cells or living organisms due to the susceptibility of the natural phosphodiester backbone by extra and intracellular nucleases present in biological fluids. As a result, this severely limits the potential therapeutic utility and *in vivo* diagnostic applications of phosphodiester oligonucleotides. Several carbohydrate, nucleobase and backbone modifications (**Figure 1.10**) have been investigated in order to develop more stable analogs of oligonucleotides while maintaining other desirable antisense properties.

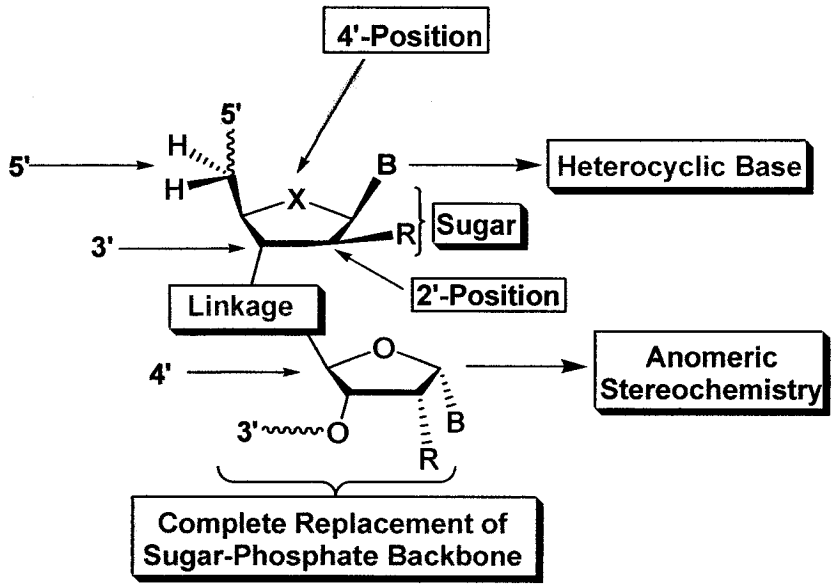


Figure 1.10: Possible chemical modifications of (a) phosphate groups, (b) bases (c) sugar groups of natural oligonucleotides.

The following minimal attributes are desirable for the use of synthetic oligonucleotides in antisense application: (a) resistance to nucleolytic degradation in cells and body fluids; (b) high affinity to the complementary mRNA target; (c) high selectivity in binding with the complementary mRNA target; (d) the ability to activate RNase H that selectively degrades the RNA strand of the AON/RNA complex; (e) efficient cell permeability; and (f) favorable pharmacokinetic and pharmacodynamic attributes. Many of these properties can be achieved by the choice of oligonucleotide modifications.[22] However, very strict rules have to be adhered to with the AON in order for the AON:RNA hybrid to be amenable to degradation by RNase H. As a consequence, only very few, of the numerous synthetic oligonucleotides tested for RNase H activity possess this feature.[23]

First-generation antisense molecules were designed to make the internucleotide linkages more resistant to nuclease attack. This was accomplished primarily by replacing one of the non-bridging phosphate oxygens with sulfur, methyl ($-CH_3$), borane ($-BH_3$) providing phosphorothioates[24], boranophosphates[25] and methylphosphonates[26], respectively (**Figure 1.11**). Although these analogs overcome the stability problem, all of these modifications result in relatively poor binding affinities towards complementary RNA targets. It has been suggested that this destabilization is caused by diastereoisomerism due to the chirality at the phosphorus centre.[27]

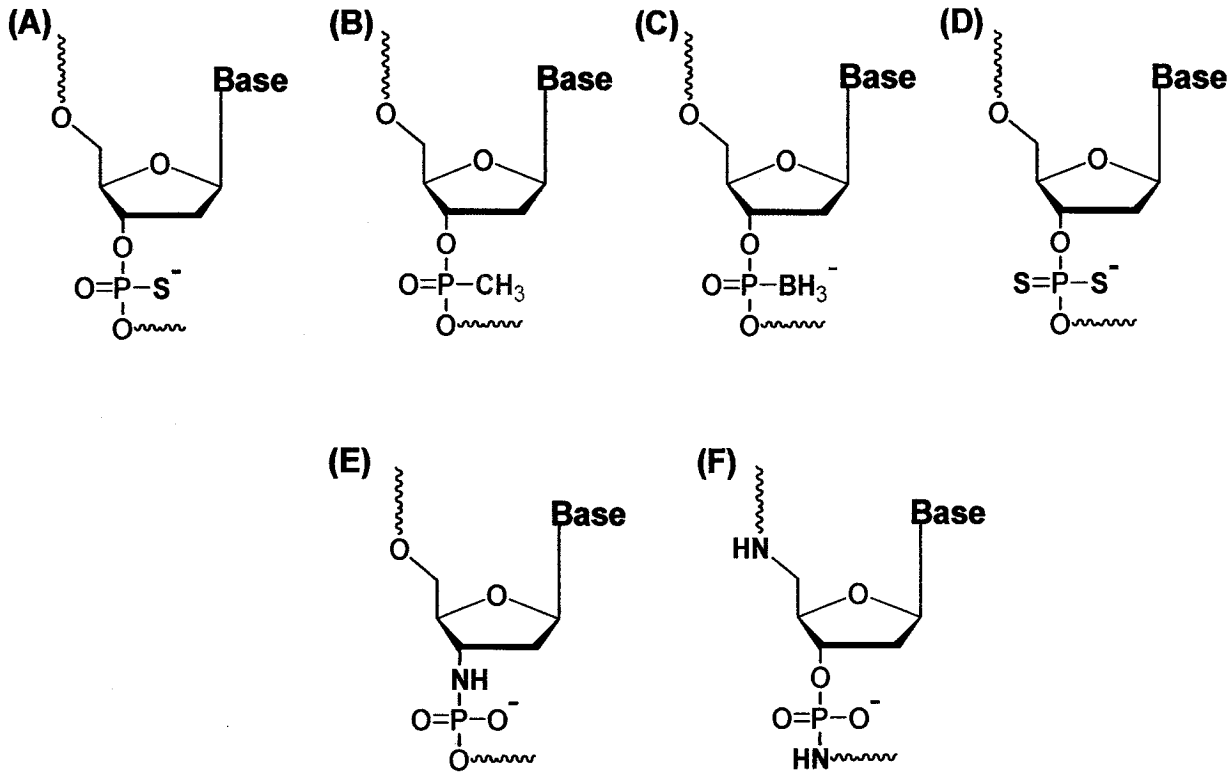


Figure 1.11: Examples of modified oligonucleotides containing internucleotide linkage modifications. (A) chiral phosphorothioate; (B) chiral methyl phosphonate; (C) chiral boranophosphate; (D) phosphorodithioate; (E) N3'-P5' phosphoramidate; (F) N5'-P3' phosphoramidate.

The methylphosphonate internucleotidic linkage is nonionic in contrast to the polyanionic natural phosphodiester backbone and have been developed to overcome the problems associated with the low ability of charged molecules to traverse the cell membranes. However, fully modified methylphosphonate oligonucleotides are unable to activate

RNase H. In contrast, boranophosphate DNA is isoelectronic to the natural phosphodiester linkage and a particularly interesting discovery is that these modified oligomers can activate RNase H and induce cleavage of RNA which significantly increases the likelihood that this modification will prove useful in antisense applications. Among these modifications, the introduction of phosphorothioate linkages profoundly influences the properties of AONs and as such have been extensively tested in various human clinical trials against numerous targets. This has culminated in a new drug, Vitravene, a phosphorothioate-based oligonucleotide, designed to inhibit the human cytomegalovirus (CMV) induced retinitis in AIDS patients and is the first antisense drug that has so far been approved for marketing.[28] PS-DNA was first synthesized by Eckstein and colleagues in the 1960s[29] and were first used as AONs for the inhibition of HIV replication by Matsukura and coworkers.[30] PS-DNA exhibits certain desirable properties which makes them potential antisense agents such as, enhanced nuclease resistance of the oligomer, attractive pharmacokinetic properties by promoting binding to serum proteins greatly increasing in vivo half-life, carry a negative charge for cell delivery, and activate RNase H. Despite these remarkable properties, PS-oligos have a tendency to induce non-specific effects, through binding to extracellular and cellular proteins, as well as cleavage of non-target mRNAs, that are only partially complementary, due to activation of RNase H.[31]

Moreover, the phosphorodithioate linkage where both of the nonbridging oxygens of the phosphodiester backbone are replaced by sulfur atoms have also been extensively studied.[32] These compounds are isostructural and isopolar to the natural nucleic acid backbone and unlike phosphorothioates, they retain the achiral nature of the phosphorus atom. Phosphorodithioate oligonucleotides have demonstrated resistance to a variety of nucleases and retain RNase H mediated cleavage of the mRNA message. However, the introduction of modified phosphorodithioate linkages in an oligonucleotide reduces its binding to a complementary target, induces secondary structures in single-stranded oligomers and enhances binding of modified oligonucleotides to proteins.[33]

Another series of oligonucleotides have been prepared through the replacement of either the 3'-or the 5'-bridging oxygen of the phosphodiester internucleotide linkage with

nitrogen (**Figure 1.11** E and F). Oligonucleotides containing N3'-P5' phosphoramidate[34] or N5'-P3' phosphoramidate[35] linkages where either the 3'- or the 5'-oxygen is respectively exchanged with nitrogen exhibit interesting biophysical and biochemical properties. Both N3'-P5' and N5'-P3' phosphoramidate oligonucleotides are resistant to various nucleases, do not bind to cellular proteins and form very stable complexes with the single-stranded complementary mRNA target. This has been attributed to the tendency of the sugar moieties to adopt a C3'-*endo* conformation when the 3'-O is replaced with an amine rendering it more structurally similar to RNA than DNA. The anomeric effect and a weaker gauche effect, N3'-C3'-C4'-O4', serve to preorganize the N3'-P5' phosphoramidate DNA into the required conformation of stable A-type duplexes. In addition, the H-bonding qualities present in the phosphoramidate group can serve as both donor and acceptor increasing the hydration of the backbone and coordination to anions.[10] This collectively contributes to the conformational rigidity of the backbone and the stability of phosphoramidate modified duplexes. Although these properties make them suitable antisense agents, they do not invoke the RNase H cleavage mechanism.

Other second-generation molecules have been developed to improve the safety and effectiveness of phosphorothioate oligonucleotides. An increasingly common oligonucleotide modification involves the alkylation and halogenation of the 2'-α-position of the sugar moiety[36] as illustrated in **Figure 1.12**. Placement of an electronegative substituent at the 2'-α-position of the furanose ring, such as 2'-O-methyl[37], 2'-O-ethyl[38], 2'-O-propyl[39], 2'-O-methoxyethyl[37] and 2'-F[40], has a profound effect on the sugar conformation which can be explained by the propensity of these substituents to shift the conformational equilibrium of the sugar towards the C3'-*endo* pucker via a O2' (or F2')-C2'-C1'-O4' gauche effect. This results in a pre-organized structure of the single-stranded oligomers which favors an RNA-like conformation and, upon complexation with the complementary RNA target, an A-form duplex is generated. In addition, AONs containing the aforementioned modifications, show great improvements in binding affinities towards the target RNA. A correlation was established between the C2'-substituent size and the stability of a DNA:RNA duplex.

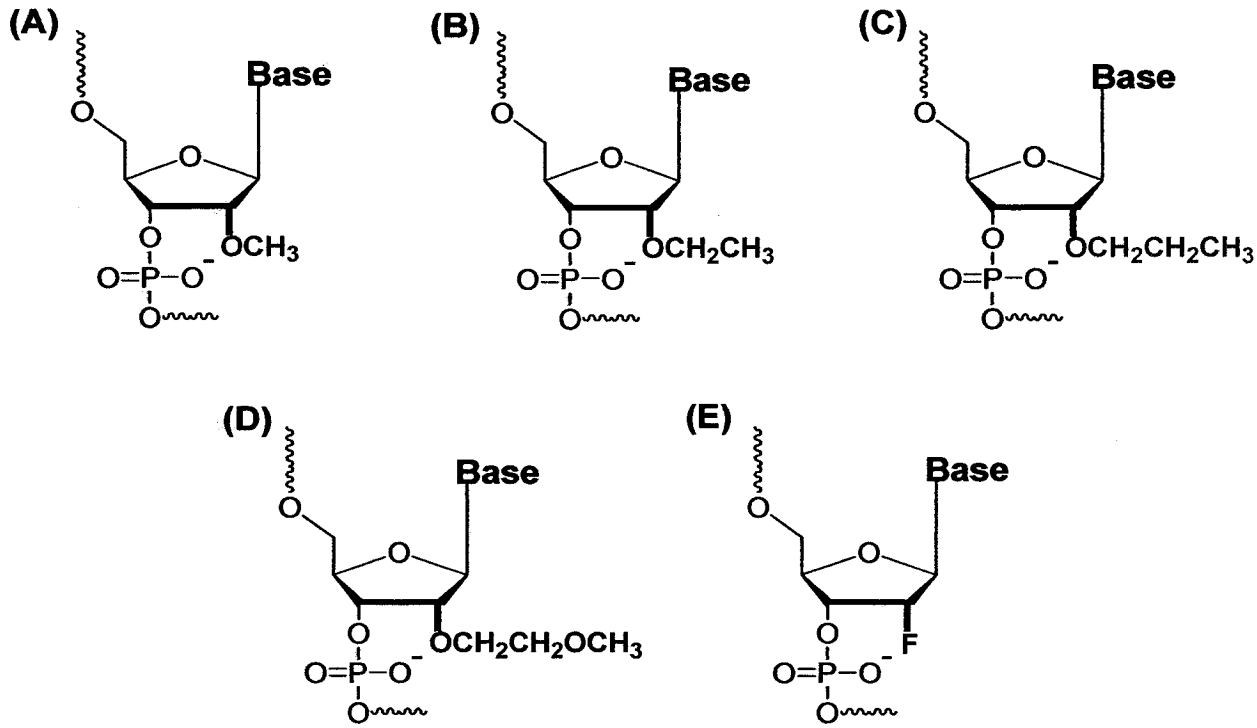


Figure 1.12: Examples of 2'-modified oligoribonucleotides. (A) 2'-O-methyl; (B) 2'-O-ethyl; (C) 2'-O-propyl; (D) 2'-O-methoxyethyl (2'-O-MOE); (E) 2'-fluoro.

Among the 2'-O-alkyl modified analogs, short substituents no larger than propyl have been shown to stabilize the duplex whereas, longer substituents (above 4-carbon chain) destabilizes the duplex. It was reasoned that a longer alkyl-chain may not be accommodated easily in the limited space of the minor groove of a hybrid duplex.[41] The exception is the 2'-O-MOE modification.

The increased thermal stability observed in the 2'-O-methyl and 2'-O-ethyl modified analogs is not only due to a shift in the S→N equilibrium but is also due to a purported 'hydrophobic effect' in which the 2'-substituents are suggested to force the nucleobase in a pseudoaxial position in order to avoid steric clashing with the vicinal phosphate and consequently, resulting in increased base stacking.[21,42] The enhanced affinity with the 2'-O-MOE modification was attributed to (a) the favorable gauche orientation of around the central linkage of the –O-C-C-O- side chain and (b) additional solvation of the alkoxy substituent in water.[21,26] In addition, these 2'-modified groups adopt exclusive gauche

orientations that maximize the interatomic distances with adjacent 3'-phosphates, thereby resulting in the disposition of the 2'-substituents in the minor groove which in turn adversely affects optimal recognition of the duplex by RNase H.[21,43] Even though, these 2'-modified sugars abrogate the oligonucleotide's ability to activate RNase H yet they confer many pharmacological advantages to oligonucleotides such as resistance to degradation by cellular nucleases, deceased toxicity compared to the PS-DNAs and hybridize specifically to their target mRNA with higher affinity than the isosequential phosphodiester or phosphorothioate.[31] However, due to the inability of 2'-modified oligomers to recruit RNase H, their antisense effect can only be attributed to steric block of translation.[44] This problem has been addressed by generating mixed-backbone oligonucleotides (MBOs) which are generally composed of two segments: (a) an oligonucleotide segment consisting of C3'-*endo* biased nucleotide units placed at the 3' and 5' termini and designed to exhibit nuclease stability and enhanced binding affinity for the mRNA complement; (b) an oligonucleotide segment which remains compatible with RNase H activation, *e.g.*, a phosphorothioate DNA gap situated in the centre of the oligomer.[45] With this modification, the 'gap' of the resulting chimeric duplex has a hybrid-like conformation which is then efficiently recognized by the enzyme. MBOs have increased affinity for the target RNA, increased uptake into cells, increased half-life of degradation, activate RNase H and show increased biological activity compared to the corresponding phosphorothioates DNA.[46]

1.5.1 ARABINONUCLEIC ACIDS

A structural modification that has been explored in our laboratory involves a switch in chirality of the C2'-OH group of the furanose ring from the α to the β-configuration as shown in **Figure 1.13**. Arabinonucleic acid (ANA)[47], the 2'-stereoisomer of RNA, and the corresponding 2'-deoxy-2'-fluoro-β-D-arabinonucleic acid analog (2'F-ANA)[48] were the first uniformly 2'-sugar-modified AONs reported to induce RNase H cleavage of a bound RNA molecule.

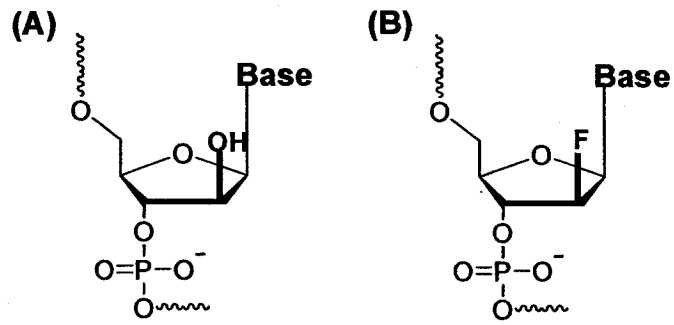


Figure 1.13: Structure of arabinonucleic acids. Inversion of configuration at the 2'-position of RNA and 2'F-RNA gives the corresponding epimeric arabinonucleic acids (A) ANA and (B) 2'F-ANA.

Inversion of the stereochemistry at the C2' position enforces a shift in the conformational equilibrium of the furanose sugar pucker from C3'-*endo*(RNA) to C2'-*endo*/O4'-*endo* ('DNA-like') pucker.[49] The O4'-*endo* (east) sugar pucker lies halfway between the C2'-*endo* (South, B-DNA) and C3'-*endo* (North, A-DNA) (**Figure 1.14**). This pucker, combined with the β-orientation of the C2'-substituents, projects the arabinose 2'-substituents into the major groove. Presumably, the O4'-*endo* conformation adopted by the ANA and 2'F-ANA strands in the duplex is due to a balance between stereoelectronic effects (gauche effect) and steric effects caused by the local environment of the 2'-substituent.[49] Additionally, CD studies reveal that the global geometries adopted by ANA/RNA and 2'F-ANA/RNA hybrids mimic quit closely that of the native DNA/RNA hybrids.[50]

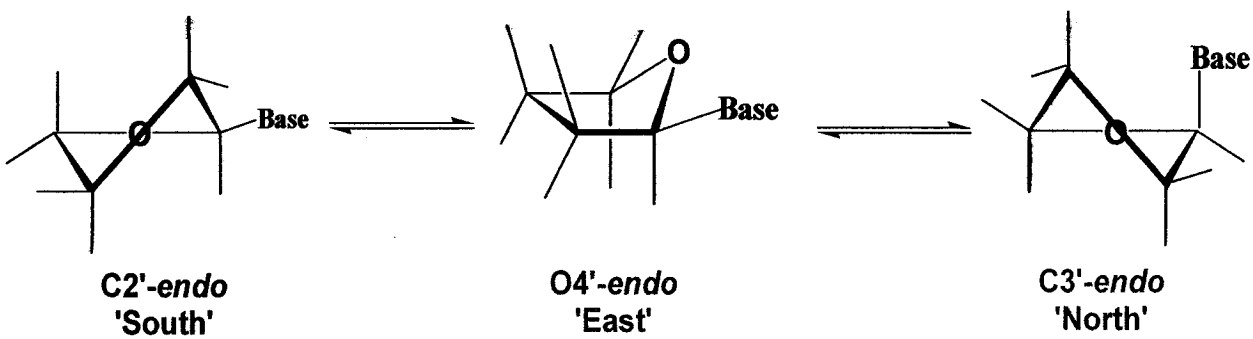


Figure 1.14: Interconversion pathway of the furanose ring through 3 lowest energy conformations.

ANA strands of mixed-base composition have shown to display weak binding affinity with complementary RNA target relative to native heteroduplexes with RNA. This destabilization is presumed to originate from the differential hydration pattern of the helices and from the steric interference by the β-C2'-OH group, which is oriented into the major groove of the helix where efficient base stacking is likely to be disrupted (e.g. unstacking). Molecular dynamics simulations of ANA:RNA duplexes have indicated that the sugar puckers of ANA strands adopt the C2'-*endo* geometry due to a combination of stereoelectronic effects and the formation of an intramolecular hydrogen bonding between the C2'-OH group and the C5'-oxygen of the arabinose sugar. This intrasugar hydrogen bond helps 'lock' the conformation in the C2'-endo form consequently, confering to the rigidity of the ANA strand.[47] However, the solution NMR structure of an ANA/RNA hybrid was determined and shows no evidence of such a C2'-OH/O5' interaction. Moreover, the hybrid ANA strand clearly appears to occupy an Eastern or O4'-*endo* pseudorotational conformation.[49]

Replacing the ara-2'-OH group with a smaller 2'-F atom resulted in compounds with elevated duplex stabilities towards target RNA. NMR studies suggest that the native 2'-deoxyribose sugars are 'flexible' and exist in a dynamic equilibrium between various conformers, namely C2'-*endo*, C3'-*endo* and O4' *endo*. Upon the incorporation of the "up" fluorine atom, the sugar becomes 'rigid' as a result of a strong gauche effect between the 2'F atom and the ring oxygen (*e.g.* [O4'-C1'-C2'-F]) causing the equilibrium to favor the eastern or O4'-*endo* conformation.[49] In addition, this O4'-*endo* geometrical preference occurs in order to relieve steric interactions between the 2'-F atom and the proximal heterocyclic base protons (specifically C6-H6 in pyrimidines; C8-N8 in purines) which is well accommodated by adopting a more eastern geometry as illustrated in **Figure 1.15.**

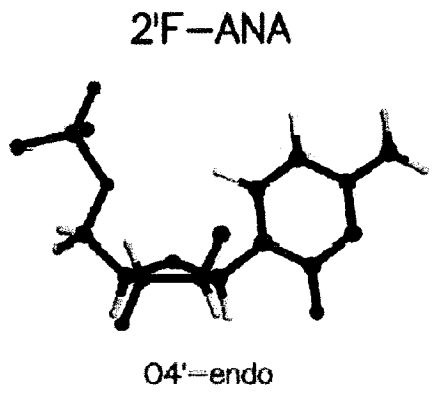


Figure 1.15: Illustration of the O4'-*endo* conformation adopted by the arabinose sugar of 2'F-ANA. The fluorine atom (green), above the ring, is pseudoaxial and gauche with respect to the ring oxygen.[51]

A comparison of ANA/RNA versus 2'F-ANA/RNA hybrids further reveals that the increased Tm for the latter may originate from the differential hydration pattern of these helices, namely, different extents of hydration of the 2'-F versus 2'-OH groups as well as the reduced steric interactions of the F atoms (vs. OH groups).[50] Additionally, the increased stability of 2'F-ANA/RNA hybrids relative to DNA/RNA is attributed not only to the conformational pre-organization of the sugar moieties but also from the clathrate-like ordered water molecules around the fluorine atoms.[50] Moreover, NMR and modeling analyses confirm a potential H-bonding interaction between the 'up' 2'-fluorine atom and the base protons (H6 or H8) may provide additional pre-organization to the 2'F-ANA strand.[50]

1.6 PROJECT OBJECTIVES

Modified oligonucleotides with 3'S- or 5'S-phosphorothiolate internucleotide linkage, where the bridging oxygen at 3'- or 5'- position is replaced by sulfur have also generated considerable interest. For instance, oligonucleotides containing the 3'S or 5'S-phosphorothiolate modified linkages have found widespread utility in the probing of specific interactions of proteins, enzymes and metals with nucleic acids.[52] In addition, the introduction of sulfur at the 3' and 5' bridging position of the phosphate linkage of DNA and RNA has been central to many mechanistic studies of various catalyzed RNA or DNA cleavage reactions due to their leaving group ability.[52] As these modifications had not yet been exploited for therapeutic antisense application, one of our objectives was to synthesize modified oligomers containing 3'-S and 5'-S-phosphorothiolate linkages. Of most interest to us were the 2'-fluoro-3'-S-arabinonucleic acid derivatives (**Figure 1.16**).

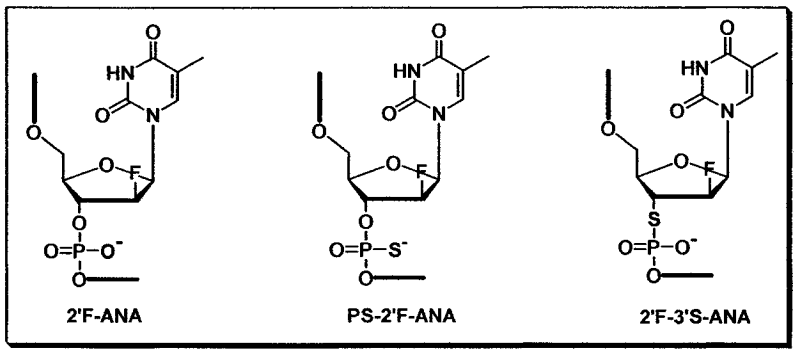


Figure 1.16: Structures of modified oligonucleotides.

While this work was is progress, Cosstick and co-workers established, using high-resolution NMR spectroscopy, the conformational consequences upon the introduction of a *single* 3'-S-phosphorothiolate linkage in the DNA strand of a DNA:RNA dodecamer duplex.[53,54,55] These studies revealed that the furanose ring conformation is altered in AONs containing the 3'-S-phosphorothiolate modification, with a large increase in the percentage of C3'-*endo* puckering. Presumably, the replacement of the 3'-oxygen of DNA with the more electropositive and larger sulfur atom, reduces the contribution of the gauche effect between the 3'-substituent and O4' of the ring oxygen and pushes the equilibrium to the C3'-*endo* or northern hemisphere as illustrated in **Figure 1.17.** This

allows the anomeric effect between the lone pair on the O4' and the C1'-N glycosidic bond to stabilize the C3'-*endo* conformation.

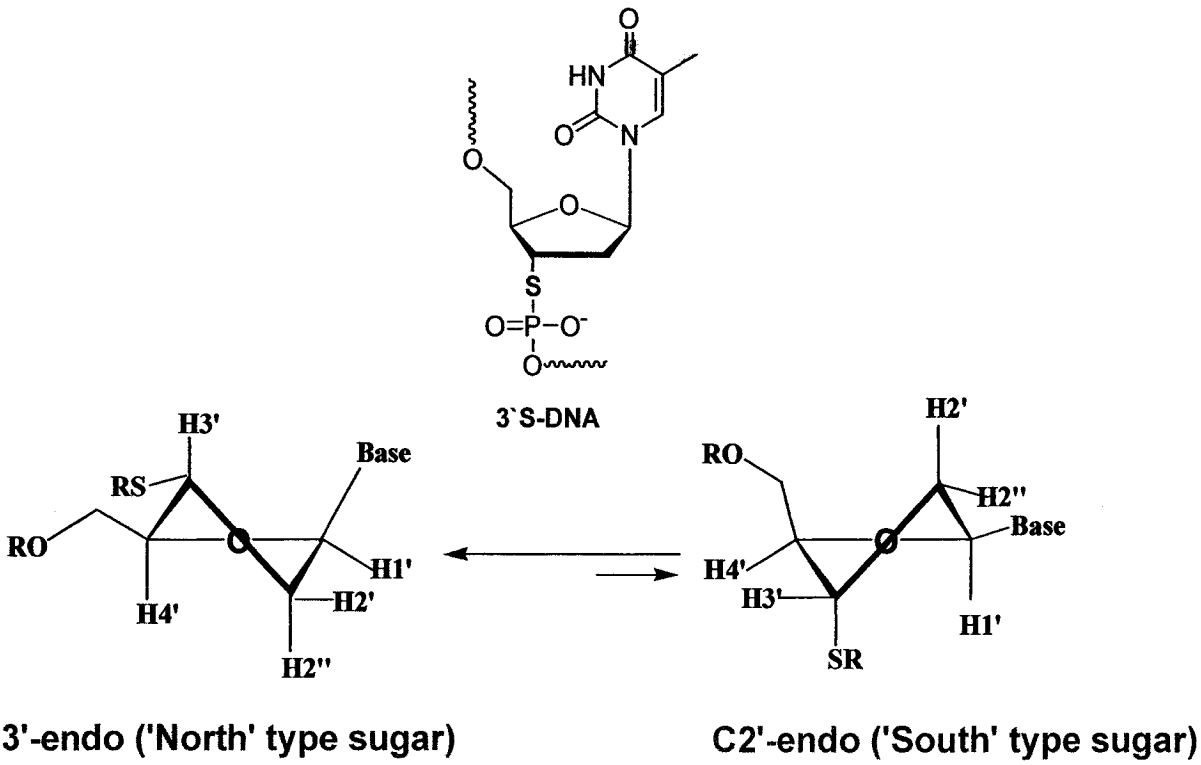


Figure 1.17: A schematic representation of the 3'-S-phosphorothiolate linkage. Substitution of the 3'-oxygen atom in a phosphodiester linkage with a less electronegative and larger sulfur atom pushes the conformational equilibrium of the attached sugar towards the north conformer.

Moreover, this shift from the C2'-*endo* (south) to C3'-*endo* (north) conformational equilibrium was found to be transmitted to the subsequent (n+1) sugars; the 5'-terminal sugar is affected most and is pushed to a predominantly C3'-*endo* conformation whereas, the 3'-terminal sugar is affected to a lesser degree, resulting in a more equal distribution of C2' and C3'-*endo* conformers.[53] Moreover, UV thermal melting studies have also been conducted by Cosstick *et al.*, in order to probe the thermodynamic behaviour of a series of singly and doubly 3'-S-phosphorothiolate-modified duplexes. An enhanced thermal stability was observed upon complexation of the AON with the RNA target.

Modified oligomers containing 5'-S-phosphorothiolate linkages have been less extensively studied. The introduction of the modified 5'-S-phorothiolate internucleotide

linkage in oligonucleotides is not expected to have a major impact on the conformational parameters of the deoxyribose moiety typically conferring an DNA-like sugar puckering (C2'-*endo*) of the AON (**Figure 1.18**).

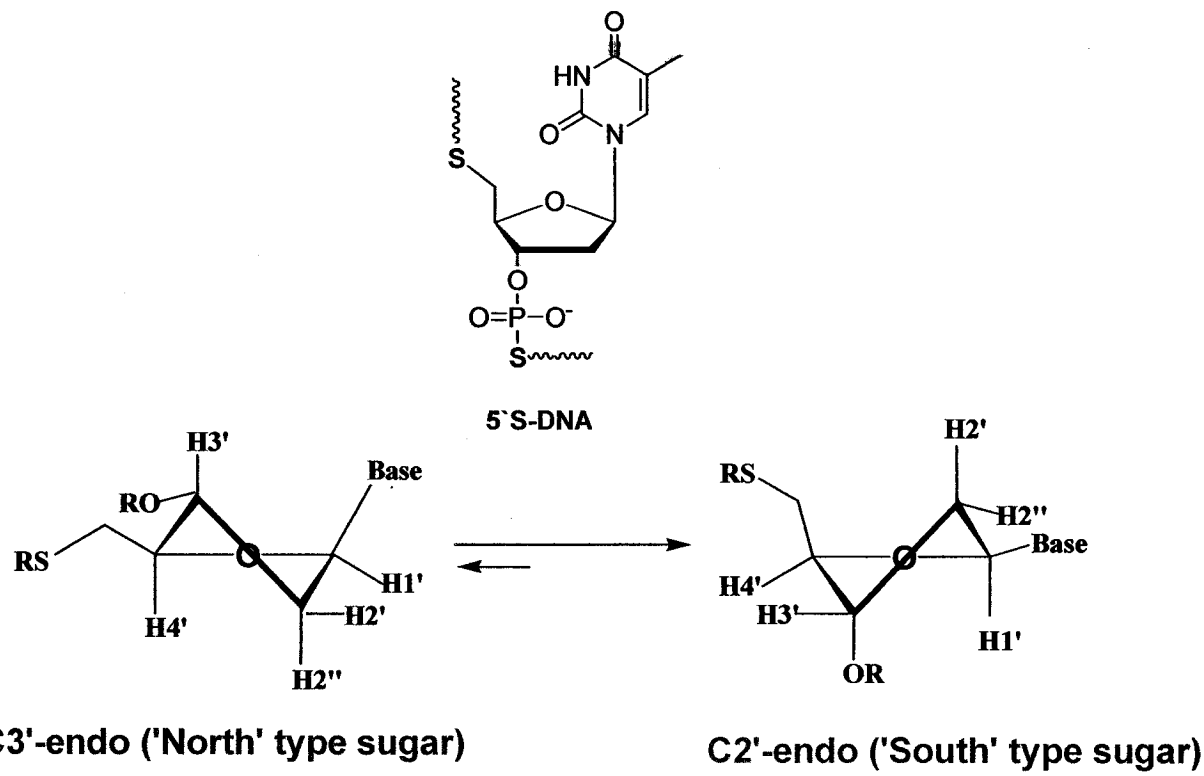


Figure 1.18: A schematic representation of the 5'-S-phosphorothiolate linkage and the corresponding north and south sugar puckers.

Our laboratory has investigated numerous nucleic acid analogues, the most promising for antisense purposes has been the arabinose-derived AON analogues, namely, ANA (the 2'-epimer of RNA)[47] and 2'F-ANA[48] (see section 1.5.1, pp 22). Our laboratory has recently shown that 2'F-ANA oligomers bind with high affinity to the RNA complement and are capable of eliciting RNase H activity. In addition, this laboratory has evaluated the antisense activity of phosphorothioate-linked 2'F-ANA oligonucleotides (PS-2'F-ANA).[56] These uniformly modified oligonucleotides were somewhat less efficient in directing RNase H cleavage of target RNA compared to their phosphorothioate-linked DNA counterparts and showed only weak antisense inhibition of cellular target expression. However, the isomeric analogue, in which one of the two bridging oxygen

atoms (3'O or 5'O) of the phosphodiester linkage is replaced with a sulfur atom, has not been evaluated for its potential as an antisense agent (**Figure 1.16**). This prompted our interest towards the synthesis of modified oligonucleotides containing 2'-3'-dideoxy-2'-fluoro-3'-thioarabinonucleic acid (2'F-3'S-ANA) residues and subsequently, evaluating its biological and physicochemical properties. It has been well documented that in 2'-F-arabinonucleosides the presence of a strong gauche effect between the highly electronegative fluorine atom and the O4' oxygen locks the five-membered ring into the O4'-*endo* sugar pucker (eastern conformation).[51] However, as mentioned previously, the 3'-S modified residues are characterized by a C3'-*endo* (northern) antipodal preference. Therefore, in a nucleoside comprising both (2'-F and 3'-S) modifications one would expect a "competition" between these two different conformations.

This thesis describes our attempts to synthesize 2'F-3'S-ANA oligonucleotides. While the synthesis of the required nucleoside building blocks failed miserably, we were successful at synthesizing 5'S-DNA and 3'S-DNA, *e.g.*, oligonucleotides comprising 3'- and 5'-S-phosphorothiolate linkages. We also evaluated their physicochemical properties, namely, their affinity towards the complementary RNA target (T_m), their ability to activate RNase H and their conformational properties when bound to the RNA complement (circular dichroism). During the course of these studies we isolated and characterized a novel [3.1.0]-fused 2',3'-α-epi-thionucleoside derivative with potential antiviral properties.

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CHAPTER II: SYNTHESIS OF 2',3'-DIDEOXY-2',3'-α-EPI-THIOTHYMIDINE AND THE PHOSPHORAMIDITE DERIVATIVES OF 3'-DEOXY-3'-THIOTHYMIDINE AND 5'-DEOXY-5'-THIOTHYMIDINE.

2.1 INTRODUCTION

Oligonucleotide analogues have become essential tools for probing structural and mechanistic aspects of nucleic acid biochemistry and have been extensively investigated as potential therapeutic antisense agents. Unmodified oligonucleotides are susceptible to nuclease degradation at both the 3'- and the 5'- positions of the internucleotide linkage. Consequently, attempts to impart nuclease resistance to therapeutic antisense oligonucleotides have been directed towards modifying the internucleotide linkage. Success has been achieved primarily with respect to modification of the 'non-bridging' oxygen atom in the naturally occurring phosphodiester linkage. Examples of such a modification include phosphorothiote[1,2]- modified oligonucleotides in which a single non-bridging oxygen is substituted with a sulfur atom as well as phosphorodithioate[3]-modified oligonucleotides in which both of the non-bridging oxygen atoms have been substituted with sulfur atoms. Phosphorothioate deoxyribonucleotides (**PS-DNA**; **Figure 2.1**) exhibit desirable antisense properties such as enhanced nuclease resistance, improved bioavailability and the ability to induce RNase H mediated hydrolysis of target RNA.[4] **Vitravene™**, a 21-mer phosphorothioate-DNA, has shown very promising antisense activity and has been approved for clinical use in the USA for the treatment of patients suffering from cytomegalovirus (**CMV**)-induced retinitis.[5] Unfortunately, PS-DNA possesses a relatively low binding affinity for target RNA which hinders its potency in antisense application. Our laboratory has recently demonstrated that 2'-deoxy-2'-fluoroarabinonucleic acids (**2'-F-ANA**) have both high-affinity RNA binding and are able to elicit RNase H degradation of the target RNA.[6,7] In addition, the antisense activity of phosphorothioate linked 2'-deoxy-2'-fluoro-arabinonucleic acids (**PS-2'F-ANA**; **Figure 2.1**) have been evaluated and have also shown to induce RNase H cleavage of target RNA.[4] However, the isomeric analogue in which a sulfur atom replaces one of the two bridging oxygen atoms, have not yet been investigated as

potential antisense agents. Given this, we became interested in the synthesis of 2',3'-dideoxy-2'-fluoro-3'-thioarabinothymidine in order to construct 2'F-ANA containing the 3'-S-phosphorothiolate internucleotide linkages (**2'F-3'S-ANA**; **Figure 2.1**).

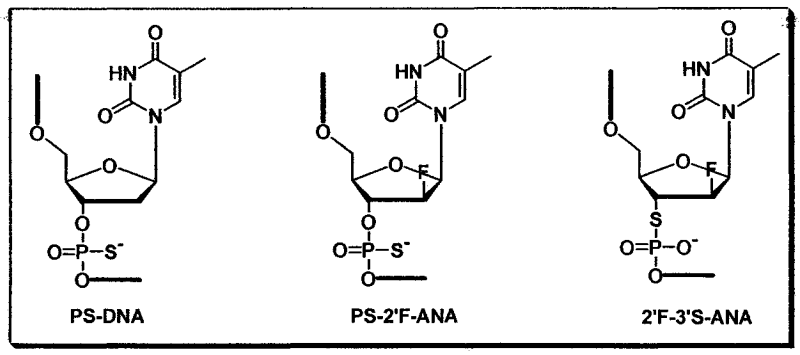


Figure 2.1: Structure of modified oligonucleotides.

The 3'O to 3'S substitution is synthetically more challenging to achieve and as a result has been less extensively investigated. Given that there have been no reports describing the preparation of 2'-fluoro-3'-thioarabinonucleosides our aim was to synthesize the 3'-S-phosphorothioamidite derivative of 2'-fluoroarabinothymidine for incorporation into oligonucleotides via solid-phase phosphoramidite method.

Recently, oligoribonucleotides containing a few 3'-S- and 5'-S- phosphorothiolate units have been used extensively as model compounds in studying the mechanisms of cleavage of RNA by enzymes. In particular, the incorporation of 3'- and 5'-thiolate ribonucleotide units into RNA has proven to be very useful in studying the role of metal ions involved in RNA self-splicing catalysis[8,9] and cleavage by hammerhead ribozymes.[10,11] In addition, oligodeoxynucleotides containing this linkage have been used to investigate cleavage processes by the ***Eco* RV** restriction endonuclease[12], the DNA repair enzyme **T4** endonuclease **4V**[13], the *Tetrahymena* ribozyme[14], the Klenow fragment 3'-5' exonuclease[15] and the resolution of Holliday junctions by RuvC.[16] These 3' and 5'-sulfur analogues permit the identification of metal binding sites in biological systems. However, to date, data concerning the applicability of oligomers constructed from either 3'-S or 5'-

S phosphorothiolate internucleotide linkages for antisense therapy does not exist (**Figure 2.2**).

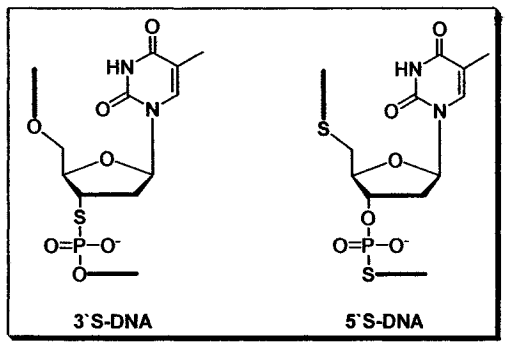


Figure 2.2: Structure of modified DNA oligonucleotides containing 3'- and 5'-S-phosphorothiolate internucleotide linkages.

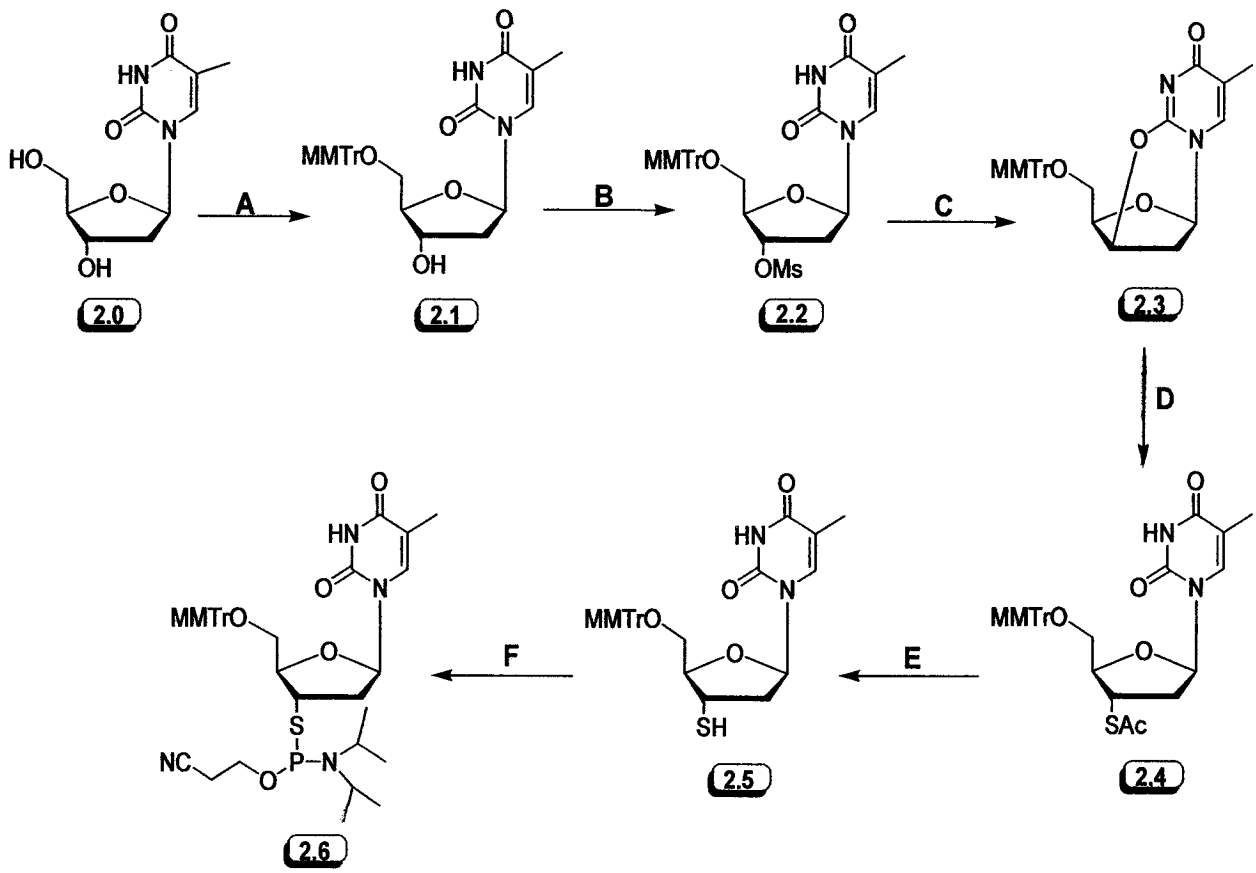
Oligonucleotides containing this modification are particularily attractive since they are isopolar and isosteric with the natural congener and unlike the linkages of PS-DNA (Figure 1) are achiral.[17] Moreover, their increased nuclease resistance and the ability of the 3'-thio substitution to push the conformational equilibrium of the deoxyribose ring predominantly towards the C3'-*endo* (north) conformation and consequently obtaining more stable DNA:RNA hybrids, renders 3'S-DNA a good candidate for antisense therapy.[18,19] Therefore, another of our goals was to synthesize nucleoside 3'-S and 5'-S-phosphorothioamidite derivatives and subsequently, incorporate them into oligonucleotides. The following sections describe the synthesis and the characterization of the aforementioned monomers.

2.2 SYNTHESIS OF 3'-DEOXY-3'-THIOTHYMIDINE DERIVATIVES

In recent years the synthesis of DNA dinucleotides[20,21,22] and DNA oligonucleotides[23,24] containing one or two 3'-S-phosphorothiolate linkages have been reported. They have been synthesized using a variety of approaches including phosphoramidite[24] and phosphate triester[25] chemistry and methods based on the Michaelis-Arbusov reaction.[26,27] These approaches have also been extended to the incorporation of the 3'-thio analogues of ribonucleotides into RNA as ribodinucleotides and oligoribonucleotides[28] containing this modified linkage.

The synthesis of 2',3'-dideoxy-3'-thioadenosine, 3'-deoxy-3'-thiouridine and 2',3'-dideoxy-3'-thiocytidine have previously been reported.[14,22,23] In addition, the preparation of 3'-deoxy-3'-thiothymidine was first reported by Cosstick and co-workers in 1988, starting from the xylo-configured nucleoside by the displacement of the 3'-sulfonate ester with sodium thiobenzoate.[20,21] Recently, they reported a more immediate route towards the preparation of 3'-deoxy-3'-thiothymidine which involves the direct ring-opening of a 2,3'-anhydrothymidine with thiobenzoate.[23,24] Our synthetic scheme towards the preparation of 3'-deoxy-3'-thiothymidine and its phosphorothioamidite derivative is depicted in ***Scheme 2.1***, and is based on a previously reported procedure.[29] Thymidine was chosen as a relatively cheap and convenient starting material. Synthesis starts with regioselective 5'-hydroxyl tritylation of thymidine affording 5'-O-(4-monomethoxytrityl)thymidine (**2.1**) in 98% yield. Subsequent conversion into the 3'-O-mesyl derivative by treatment with excess methanesulfonyl chloride in dry pyridine solution afforded the mesylate (**2.2**) in 97% yield. The latter when heated under reflux, with an excess of triethylamine in ethanol solution generated the 5'-O-(4-monomethoxytrityl)-2,3'-anhydronucleoside (**2.3**) in 87 % yield with net inversion of stereochemistry at the C3' position. Nucleophilic ring-opening of the 2,3'-anhydronucleoside with a thionucleophile, sodium thioacetate, in dry dioxane at 80°C, afforded thymidine thioester derivative (**2.4**)[30], in 95 % yield. Deacetylation of (**2.4**) was accomplished using 10 M NaOH in ethanol solution at 5°C for 1 hour. The reaction

mixture was subsequently neutralized using 1 M HCl, evaporated in vacuo and purified by flash column chromatography yielding the free thiol (**2.5**) in 70% yield and only minor quantities (10%) of the corresponding disulfide (see experimental section for detailed characterizations). The 3'-thionucleoside was converted to the desired 3'-S-phosphorothioamidite by overnight reaction with 2-cyanoethyl *N,N*-diisopropylaminochlorophosphoramidite in the presence of *N,N*-diisopropylethylamine, as acid scavenger and catalytic amounts of 1-methylimidazole. Purification by flash column chromatography gave the desired thymidine 3'-S-phosphorothioamidite derivative (**2.6**) in 95% yield.



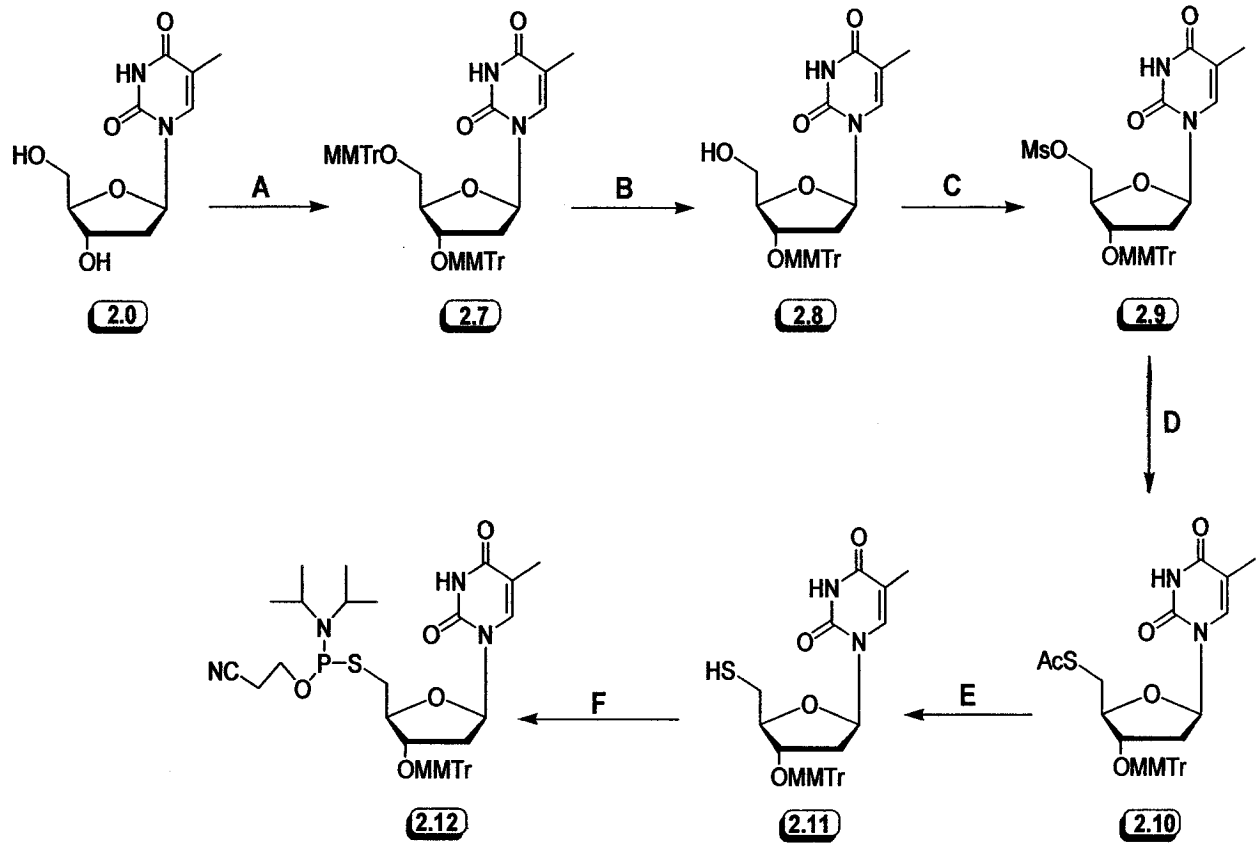
Scheme 2.1: Synthesis of 3'-deoxy-3'-thiothymidine and its 3'-S-phosphorothioamidite derivative. A. MMTr-Cl (1.5 eq.), pyridine, r.t., overnight (98%); B. MsCl (3.0 eq.), pyridine, r.t., overnight (97%); C. Et_3N, EtOH, reflux, 16h (87%); D. NaSAc, dioxane, reflux, overnight (95%); E. 10M NaOH, EtOH, 1h (70%); F. 2-cyanoethyl-N,N-diisopropylchlorophosphoramidite (1.2 eq.), 1-methylimidazole (0.6 eq.), N,N-diisopropylethylamine (5.0 eq.), THF, r.t., overnight (95%).

2.3 SYNTHESIS OF 5'-DEOXY-5'-THIOTHYMIDINE DERIVATIVES

The synthesis of modified dinucleotides[31,32,33,34] and oligonucleotides[35,36,37] containing a 5'-thio-bridged substitution has also been reported in the context of deoxyribonucleotides and ribonucleotides and their synthesis is amenable to phosphoramidite chemisty. DNA dinucleotides containing the 5'-S-phosphorothiolate linkage were first reported by Cook[33] and subsequently by Nagyvary *et al.*[31] in 1970. These analogues have initially been prepared in solution via nucleophilic displacement of a 5'-tosyl- or 5'-iodonucleosides with 3'-phosphorothioate nucleosides. Subsequently, methods have been developed for incorporating a 5'-S-phosphorothiolate linkage which can be compatible with automated DNA synthesis via the phosphoramidite chemistry.[35,36,37] This involves the synthesis of the appropriate building block, 5'-S-trityl-5'-deoxythymidine-3'-phosphoramidite, by displacement of the 5'-arylsulfonyl group from the 5'-O-p-toluenesulfonyl-thymidine derivative with sodium triphenylmethylmercaptide.[35] Moreover, this synthetic strategy has also been extended to the preparation of suitably protected adenosine amidites.[36]

An alternative strategy for the introduction of a 5'-bridging sulfur atom into oligonucleotides was developed by Rodriguez *et al.*[37]. which involves the synthesis of 5'-deoxy-5'-thiothymidine and the corresponding 5'-S-phosphorothioamidite derivative with subsequent incorporation of the latter into an oligonucleotide via conventional solid phase synthesis. The preparation of 5'-deoxy-5'-thiothymidine was accomplished by reacting thymidine with thiolacetic acid in a regio-selective Mitsunobu coupling reaction. This was followed by the 3'-OH protection of the 5'-S-(thioacetyl)-nucleoside intermediate with dimethoxytrityl chloride and subsequent removal of the 5'-S-acetyl group by treatment with base to afford the 5'-deoxy-3'-dimethoxytrityl-5'-thiothymidine. The synthetic route we adapted for the preparation of 5'-deoxy-5'-thiothymidine is a modification of a previously reported procedure by Reist *et al.*[38],
whereby the free 5'-hydroxyl group of the 3'-monomethoxytritylthymidine is initially converted to the 5'-mesylate, which is subsequently being displaced by a thionucleophile. This synthetic strategy is outlined in ***Scheme 2.2***, again using thymidine as the starting

material. The first step involves the dimethoxytritylation of thymidine affording 3',5'-ditritylated thymidine derivative (**2.7**) in 85% yield. Subsequently, the 5'-monomethoxytrityl group was regioselectively cleaved using $ZnBr_2$ in CH_3NO_2 at 0°C for 45 min yielding the desired 3'-monomethoxytritylthymidine (**2.8**) in 80% overall yield.[39] Activation of the 5'-hydroxyl group by treatment of (**2.8**) with excess methanesulfonyl chloride (MsCl) in dry pyridine gave the 5'-mesylate (**2.9**) in 98% yield. Displacement of the 5'-mesyl group was accomplished by refluxing (**2.9**) with a large excess of sodium thioacetate in dry dioxane at 80°C (overnight) yielding the desired 3'-monomethoxytrityl-5'-acetylthiothymidine (**2.10**) in 85% yield. The 5'-thioester was subsequently deacetylated using an argon saturated solution of 10 M NaOH in aqueous ethanol at 5°C affording the 5'-deoxy-5'-thiothymidine derivative (**2.11**) in 80% yield and the corresponding dimeric disulfide (10%). It was also noted that prolonged exposure to these conditions gave the disulfide quantitatively. Phosphitylation of the latter using 2-cyanoethyl *N,N*-diisopropylaminochlorophosphoramidite under standard conditions produced the desired 5'-S-phosphorothioamidite derivative (**2.12**) in a 92% yield.



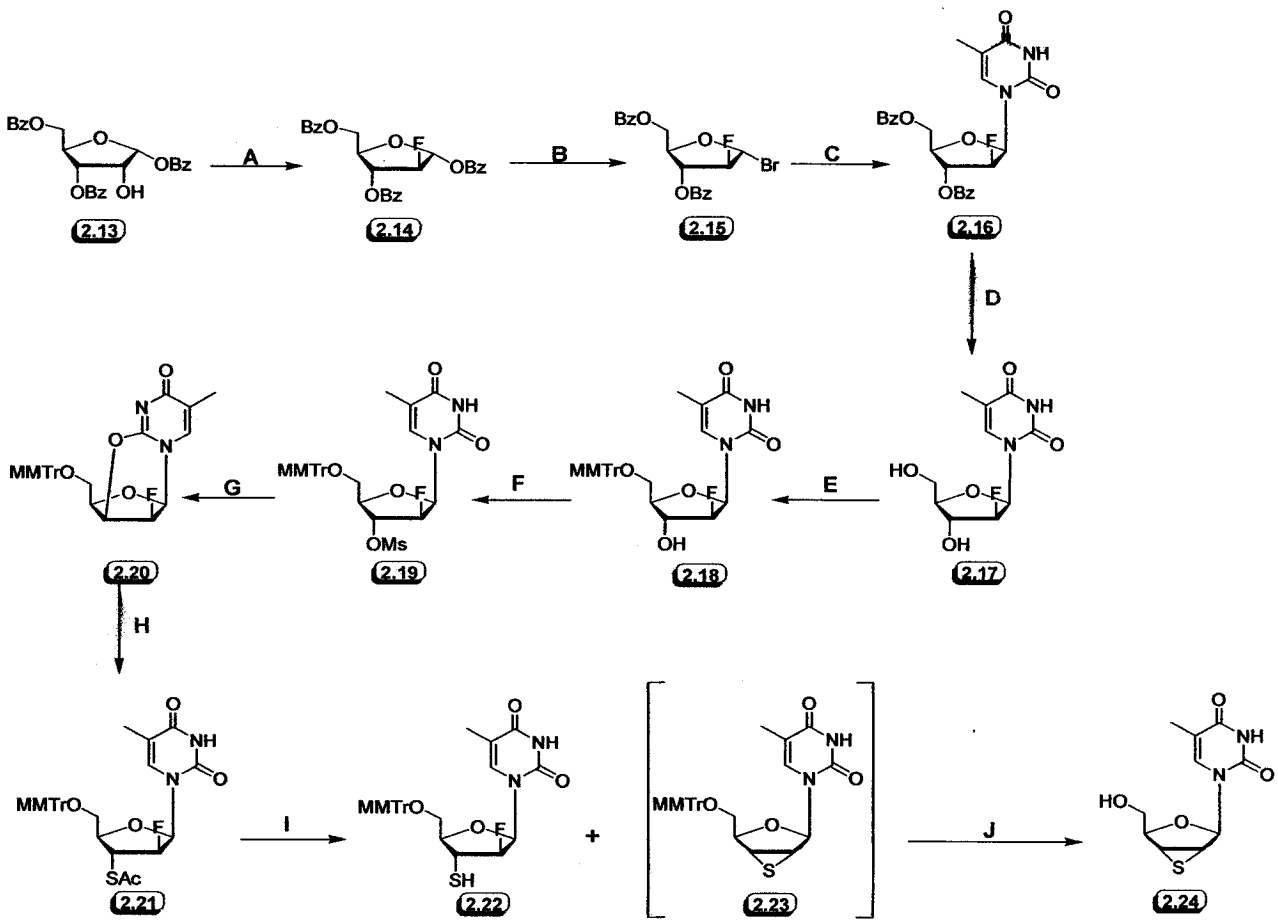
2.0 2.7 2.8 2.9 2.10 2.11 2.12

Scheme 2.2: Synthesis of 5'-deoxy-5'-thiothymidine and its 5'-S-phosphorothioamidite derivative. A. MMTr-Cl (2.0 eq.), pyridine, 60°C, overnight (85%); B. $ZnBr_2$, CH_3NO_2, 0°C, 45 min (80%); C. MsCl (3.0 eq.), pyridine, r.t., overnight (98%); D. NaSAc, dioxane, reflux, overnight (85%); E. 10M NaOH, EtOH, 1h (80%); F. 2-cyanoethyl-N,N-diisopropylchlorophosphoramidite (1.2 eq.), 1-methylimidazole (0.6 eq.), N,N-diisopropylethylamine (5.0 eq.), THF, r.t., overnight (92%).

2.4 SYNTHESIS OF 2',3'-DIDEOXY-2',3'-α-EPI-THIOTHYMIDINE

The introduction of a fluorine substituent in the sugar moiety of a nucleoside may influence its overall electronic property due to the strong electronegativity of the fluorine atom, resulting in the increased chemical stability of these fluorinated nucleosides (*e.g.*, towards hydrolysis of the N-glycosidic bond) which frequently leads to a dramatic change in their biological activity.[40] Fluorinated nucleoside analogues have shown promising therapeutic potential (mainly antiviral and antitumor activities); for instance, the presence of the fluorine substituent in the sugar alters the biological activities towards cellular, pathogenic and tumor specific enzymes in a number of different ways. As indicated above, our group has recently shown that phosphodiester-linked oligonucleotides comprised of 2'-deoxy-2'-fluoro-D-arabinonucleic acid (**2'F-ANA**) exhibit both high binding affinity for target RNA and is able to induce RNase H degradation of the target RNA suggesting that 2'F-ANA may demonstrate potent intracellular antisense activity. In addition, the antisense activity of phosphorothioate-linked 2'F-ANA oligonucleotides (**PS-2'F-ANA**) has also been evaluated by our research group and found that PS-2'F-ANA(fully arabino)/RNA hybrids are substrates of RNase H. To date, there have been no reports regarding the isomeric analogue in which one of the two bridging phosphate oxygen atoms of the phosphodiester linkage is replaced with a sulfur atom. Given that modified oligonucleotides comprised of 2',3'-dideoxy-2'fluoro-3'-thioarabinonucleic acids (**2'F-3'S-ANA**) and 2'-5'-dideoxy-2'fluoro-5'-thioarabinonucleic acids (**2'F-5'S-ANA**) have not yet been evaluated, our main objective was to synthesize modified oligonucleotides containing 2',3'-dideoxy-2'-fluoro-3'-thioarabinonucleic acids (**2'F-3'S-ANA**). In order to accomplish this, the desired 2'-deoxy-2'-fluoro-3'-S-phosphorothioamidite monomers must initially be prepared. Here, we report our attempts towards the synthesis of 2'-fluoro-3'-thioarabinothymidine and its 3'-S-phosphorothioamidite derivative suitable for automated incorporation into oligonucleotides. The developed synthetic strategy involves the preparation of the 2'-fluoroarabinothymidine intermediate (**2.17**)[41], by adapting the same synthetic protocol previously developed for the preparation of 3'-S-phosphorothioamidites via direct

nucleophilic ring-opening of a 2,3'-cycloanhydronucleoside intermediate. The synthetic route is outlined in ***Scheme 2.3***.



Scheme 2.3: Synthesis of 2',3'-dideoxy-2',3'-α-epi-thiothymidine. A. MAST/CH_2Cl_2, 50°C, overnight (93%); B. HBr/AcOH/ CH_2Cl_2, r.t., overnight (98%); C. silylated thymine, CCl_4, 80°C, 3d (90%); D. NH_4OH, EtOH, (1:1) r.t., overnight (97%); E. MMTr-Cl (1.5 eq.), pyridine, r.t., overnight (80%); F. MsCl (3.0 eq.), pyridine, r.t., overnight (98%); G. Et_3N, EtOH, reflux, 16h (97%); H. NaSAc, dioxane, reflux, overnight (41%); I. 10M NaOH, EtOH, 1h: 2'fluoro-3'-thioarabinothymidine (**2.22**) (45%) and the corresponding 5'-monomethoxytrityl-2',3'-dideoxy-2',3'-α-epi-thiothymidine (**2.23**) (20%); J. 3% TCA/ CH_2Cl_2, 10 min, (70%).

In the first step the 2'-hydroxyl group of the commercially available 1,3,5-tri-O-benzoyl-α-D-ribose (Syn Prep, Montreal, QC) was substituted with fluoride using [Bis(2-methoxyethyl)amino]sulfur trifluoride (MAST). Conversion of the C1'-benzoyl group to the bromide with hydrogen bromide (HBr) afforded exclusively the α-1-bromoarabinose (**2.15**) with retention of sugar C-1' configuration.[42] The α-bromo sugar was then used, without isolation, for S_N2-type glycosylation reaction with the persilylated thymine base in CCl_4 to yield the nucleoside (**2.16**) almost exclusively in the β-configuration. This high stereoselectivity may be due to the electronegative nature of the 2'-F atom which prevents the ionization of the C1'-leaving group to produce an oxonium ion intermediate; therefore, the glycosylation reaction mainly proceeds via the more energetically favored S_N2 pathway. Base catalyzed hydrolysis of the 3'-and 5'-benzoyl groups followed by tritylation of the 5'-hydroxyl group yielded compound (**2.18**). The latter nucleoside was then mesylated at the C3' position and subsequently converted to the 2,3'-anhydronucleoside (**2.20**). Direct ring opening of the 2,3'-anhydro linkage, in nucleoside (**2.20**), with sodium thioacetate, gave nucleoside (**2.21**) which was confirmed by proton NMR (**Figure 2.3**). Deacetylation of compound (**2.21**) under strong basic conditions *e.g.* 10M NaOH/EtOH solution afforded the thiol (**2.22**) as the major product (45%) along with a minor component (20%) which was characterized as 5'-monomethoxytrityl-2',3'-dideoxy-2',3'-α-epithiothymidine (**2.23**) (**Figure 2.4**). We decided to further investigate this reaction by deprotection of the 3'-acetyl group under various mild basic conditions (*e.g.* NH_4OH : EtOH (4:1); NH_4OH : EtOH (1:1); MeOH : NEt_3 : H_2O (2:1:1)) at room temperature. However, we observed that under all these conditions formation of the 2',3'-α-epi-sulfide (**2.23**) was favored, with little or no production of the desired 2'fluoro-3'-thioarabinothymidine (**2.22**) (see **Table 2.1**).

Figure 2.3: ^{1}H NMR spectrum of 5'-O-Monomethoxytrityl-2',3'-dideoxy-2'-fluoro-3'-acetylthioarabinothymidine (**2.21**). The spectrum was recorded on a Varian XL-400 (400 MHz) spectrometer using acetone as a solvent.

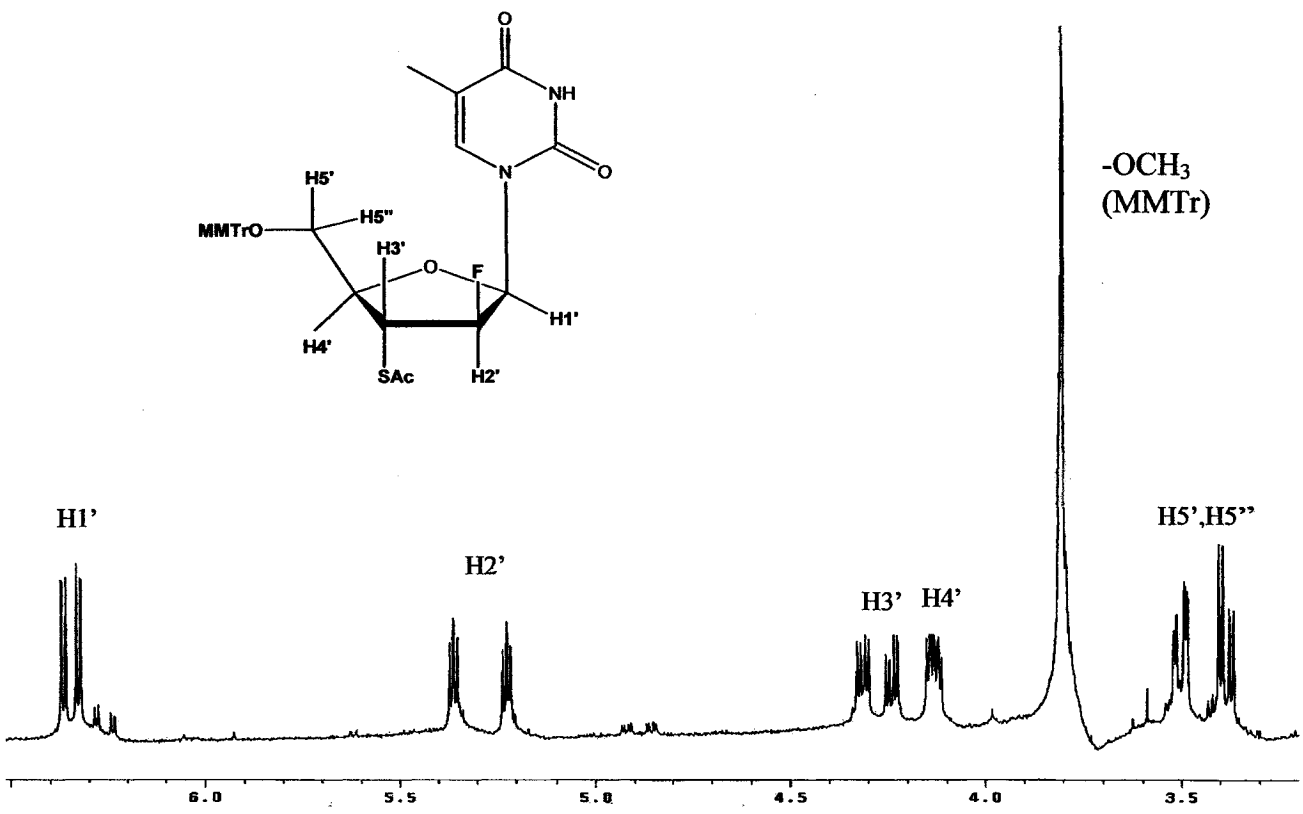


Figure 2.4: ^{1}H NMR spectrum of 5'-O-Monomethoxytrityl-2',3'-dideoxy-2',3'-α-epi-thiothymidine (**2.23**). The spectrum was recorded on a JEOL-270 (270 MHz) spectrometer using acetone as a solvent.

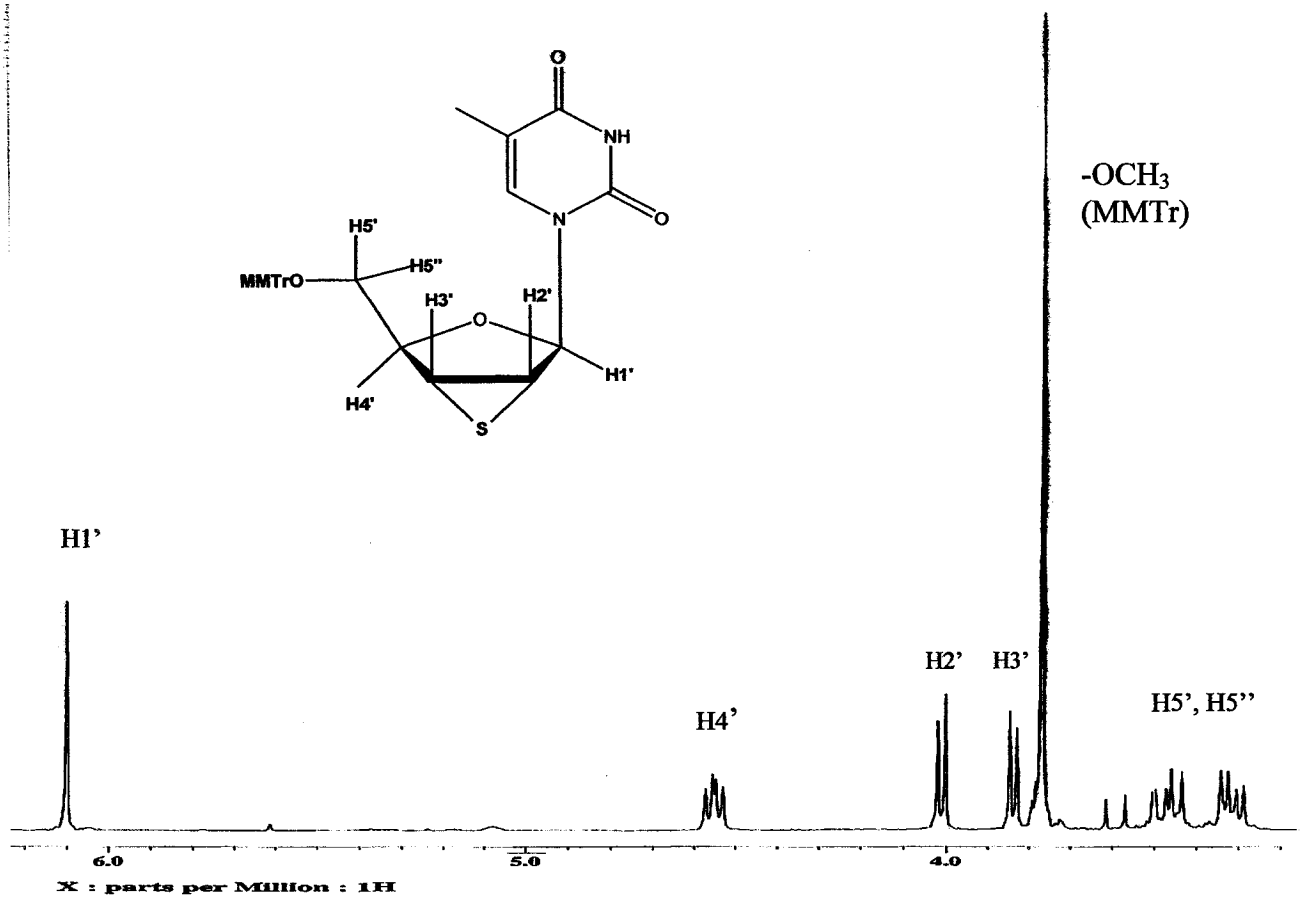
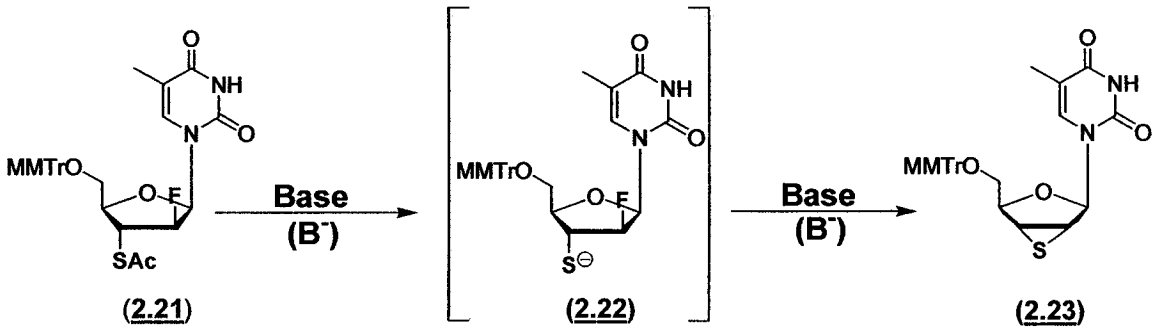


Table 2.1: % Recovery[a] of the products obtained from the deacetylation of nucleoside intermediate (2.21) under basic conditions.

BASIC CONDITIONS USED	% RECOVERY OF 2'-FLUORO-3'-THIO-ARABINOTHYMIDINE	% RECOVERY OF 2'-3'-α-EPI-THIO THYMIDINE	% RECOVERY OF 2'-FLUORO-3'-ACETYLTHIO-ARABINOTHYMIDINE
10 M NaOH /EtOH, (1h.)	45%	20%	10%
conc. aq. NH_3/EtOH (4:1), 10 min.	10%	70%	5%
conc. aq. NH_3/EtOH (1:1), 1d	-	80%	6%
MeOH : NEt_3 : H_2O (2:1:1), 10 min	-	74%	11%

[a] The crude mixture was purified by silica gel chromatography using a gradient of chloroform followed by chloroform/methanol (99:1) to elute the products. The reaction mixtures contained 0.42 mmol of (2.21) in 250ml volume of base and were conducted at r.t. conditions at specified reaction times.

The mechanism we propose for the deacetylation reaction under mild basic conditions is depicted in *Scheme 2.4*. Rapid removal of the 3'-acetyl group affords first (2.22) which undergoes rapid intramolecular cyclization to afford the 2',3'-α-epi-sulfide derivative (2.23).



(2.21) (2.22) (2.23)

Scheme 2.4: The proposed mechanism for the deacetylation reaction yields 5'-MMTr-2',3'-dideoxy-2',3'-α-epi-thiothymidine instead of the desired thiol intermediate.

This demonstrates how powerful a "soft" thiolate group can be, attacking the "soft" carbon center (C2') intramolecularly and displacing a relatively poor leaving group

(fluoride). Thus, Pearson's hard soft acid base (HSAB) principles[43,44,45] combined by the entropically favored cyclization reaction account for the rapid conversion of the 3'-thiol (**2.22**) into the 2',3'-episulfide (**2.23**). Deprotonation of the "soft" thiol group by the "soft" base (NH_3) generates the thiolate anion which then participates in a direct intramolecular S_N2 type displacement reaction on the vicinal carbon (C2') bearing the fluorine atom to afford the 2',3'-α-epi-sulfide. Under strong basic conditions (**2.22**) forms as the major product due to unfavorable interactions between the 'hard' base(OH^-) and "soft" sulfhydryl group, and consequently, retarding the formation of the corresponding 2'-3'-α-epi-sulfide.

The 2'-fluoro-3'-thioarabinothymidine (**2.22**) which we successfully isolated in low yields, was subjected to standard phosphitylating conditions (diisopropylethylamine, chlorophosphite reagent in THF, r.t.). Under these conditions, the thiol was slowly being converted to 5'-O-monomethoxytrityl-2',3'-dideoxy-2',3'-α-epi-thiothymidine (**2.23**), with little or no 3'-S-phosphorothioamidite obtained. In a previous attempt[46], we were able to isolate and characterize the desired 3'-S-phosphorothioamidite, unfortunately, we have been unable to reproduce these results. Due to these difficulties, 2'F-3'S-ANA-oligonucleotides could not be prepared. Nevertheless, our attempts led to the isolation of a novel nucleoside analogue (**2.23**). Detritylation of 5'-monomethoxytrityl-2',3'-α-epi-thiothymidine (**2.23**) in the presence of 3% TCA in dichloromethane for 15 min afforded the 2',3'-dideoxy-2',3'-α-epi-thiothymidine (**2.24**) in 70% yield. The resulting [3.1.0]-fused 2',3'-modified nucleoside is currently under evaluation as potential antiviral agent ("chain terminator" of viral DNA synthesis) owing to the lack of the 3'-hydroxyl group. Synthesis of 2',3'-ribo-epi-thio nucleosides has been limited to the uridine analogue.[47,48] In fact, there have been two reports describing the synthesis of 2',3'-α-epi-thiouridine. The preparation of 2',3'-ribo and lyxo-epithio nucleosides derived from uridine derivatives was first described by Ueda and co-workers in 1976[47] (**Figure 2.5**).

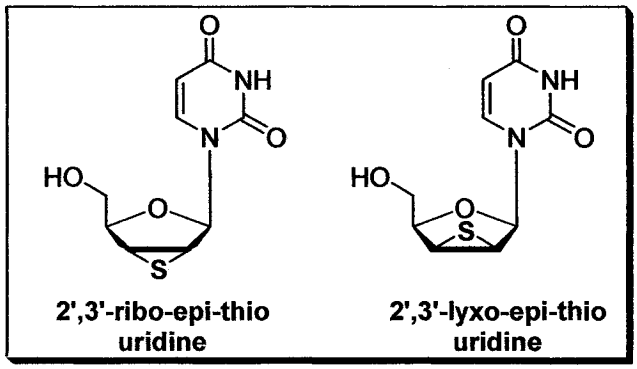
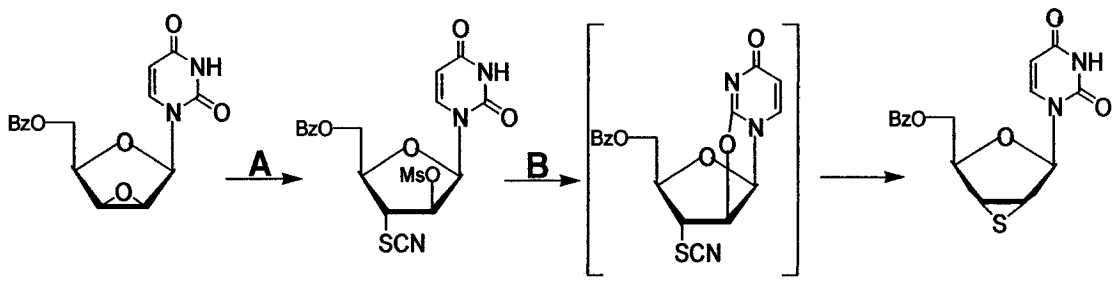


Figure 2.5: Structure of 2',3'-α- and β-epi-thiouridine. It is important to note that in the case of 2',3'-α-epi-thiouridine the three membered ring is fused in the exo orientation (α-face) whereas, the 2',3'-β-epi-thiouridine has the three-membered ring fused in the endo orientation (β-face).

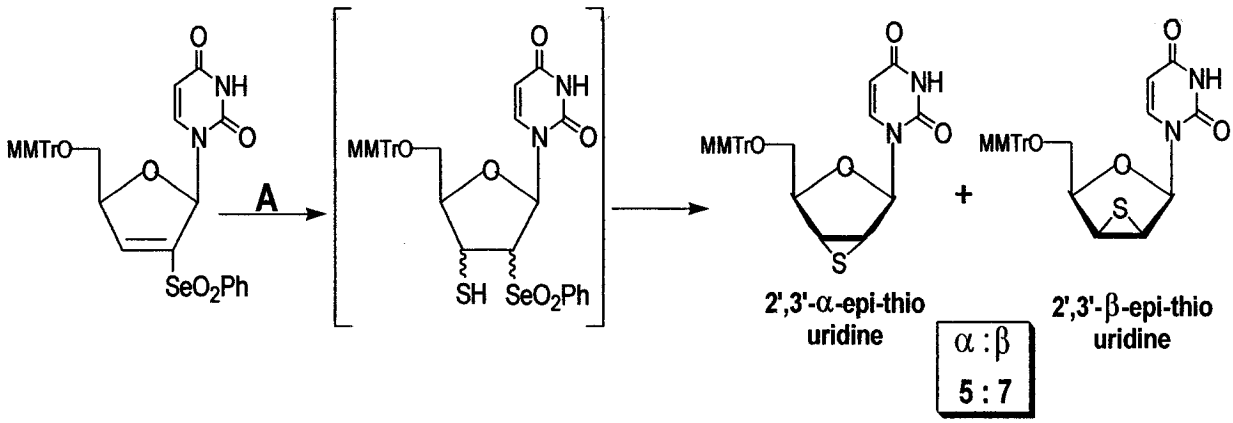
The procedure they developed starts from a 5'-O-benzoyl-2',3'-anhydro-epoxy uridine precursor which was treated with ammonium thiocyanate yielding the 3'-thiocyanato derivative, which upon mesylation, afforded the 2'-O-mesyl-3'-thiocyanato derivative (***Scheme 2.5***). The final conversion of the 2'-O-mesyl-3'-thiocyanato derivative to the desired 2',3'-α-epi-thiouridine was accomplished via a 2,2'-anhydro nucleoside intermediate which, upon basic treatment, afforded the desired nucleoside with the three-membered ring fused in the exo orientation (α-face) in quantitative yield.



Scheme 2.5: Synthesis of 5'-O-benzyl-2',3'-α -epi-thiouridine. A. i). HN_4SCN, dioxane, reflux, overnight, ii). MsCl, pyridine; B. KSAc, DMF, 5°C, overnight.

Chattopadhyaya and co-workers, subsequently prepared 2',3'-α-fused systems via 2',3'-ene-2'-phenylselenonyl nucleosides as substrates for Michael-type addition reactions using sulfur, nitrogen, oxygen and carbon nucleophiles.[48,49] Specifically, the 2',3'-ribo-

epithio nucleosides were synthesized by conjugate addition at the 3'-carbon of the 5'-O-MMTr-2',3'-ene-2'-phenylselenone (Michael acceptor) with hydrogen sulfide (a sulfur nucleophile) (***Scheme 2.6***). This occurs due to the strong electron-withdrawing nature of the 2'-selenonyl group connected to the 2',3'-double bond and consequently, activates the 2',3'-double bond towards nucleophilic addition from the α- and β-face of the C-3'. The resulting 2'-selenonyl-3'-substituted nucleoside intermediates (isomer mixtures) was found to be very unstable due to the leaving group character of the 2'-selenonyl substituent. Therefore, intramolecular S_N2 type displacement reaction at the 2'-carbon bearing the selenonyl group by the vicinal C3' sulfhydryl group gave access to 5'-O-MMTr-2',3'-α-epi-thiouridine and its β-stereoisomer (5:7 ratio, in favor of the α-isomer).



Scheme 2.6: Synthesis of 5'-O-MMTr-2',3'-α-epi-thiouridine. A. 0.2% H_2S in THF (w/v, 10 equiv.), NEt_3 (20 equiv), 20°C, overnight.

Moreover, the structural properties of 2',3'-α-epi-thiouridine have been reported by Chattopadhyaya *et al.*.[50] The optimized structure of the 2',3'-α-fused-cyclic nucleoside shows preferential anti-orientation of the uracil base and a nearly flat geometry of the furanoid ring with a slight O4'-endo puckering. Dr. Masad J. Damha has conducted molecular modeling on the 2',3'-α-epi-thiothymidine (molecular mechanics; Hyperchem softward version 7.0). The lowest energy conformation obtained consisted of a flat furanose ring with O4'-endo puckering, depicted in **Figure 2.6**, and is consistent with the proton NMR spectrum of (**2.23**) and (**2.24**) and previously mentioned results obtained by Chattopadhyaya *et al.*.[50]

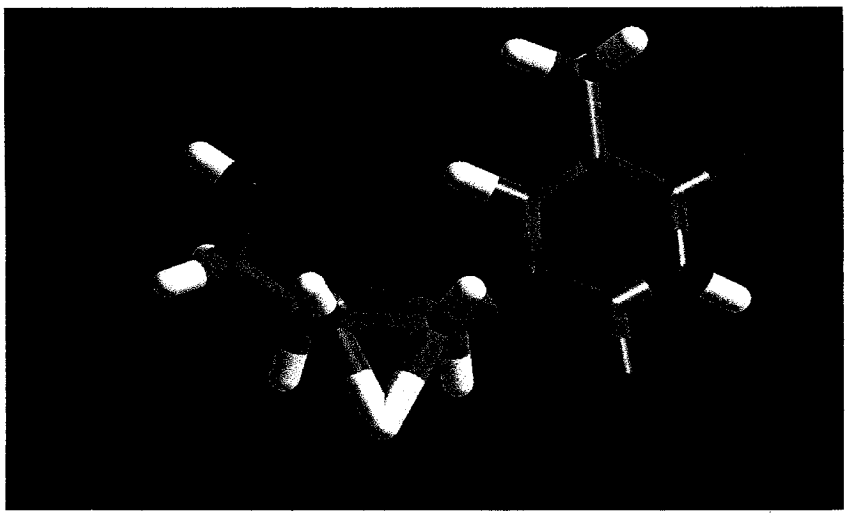


Figure 2.6: Lowest energy conformation of 2',3'-α-epi-thiothymidine. The drawing shows the flat geometry of the furanose ring with a slight O4'-*endo* puckering and the preferential pseudo-equatorial orientation of the thymine base.

2.5 CONCLUSIONS

The synthesis of phosphoramidite derivatives of 5'-deoxy-5'-thiothymidine and 3'-deoxy-3'-thiothymidine required for solid-phase 5'S and 3'S-DNA synthesis were prepared in very good yields. However, attempts to prepare the corresponding 2',3'-deoxy-2'-fluoro-3'-S-phosphorothioamidite derivative failed miserably. Our numerous attempts towards making this compound led to the discovery of 2',3'-dideoxy-2',3'-α-epi-thiothymidine (a class of [3.1.0]-fused 2',3'-nucleosides) via an unprecedented 3'-thiolate assisted cleavage of a carbon-fluorine bond. 2',3'-Epi-thio-ribonucleosides may find use as antiviral agents by their intracellular conversion to 5'-triphosphate derivatives and subsequent incorporation and termination of viral DNA synthesis.

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CHAPTER III: SYNTHESIS, PHYSICOCHEMICAL AND BIOLOGICAL EVALUATION OF OLIGONUCLEOTIDES CONTAINING 3'-S-AND 5'-S-PHOSPHOROTHIOLATE INTERNUCLEOTIDE LINKAGES

3.1 INTRODUCTION

As described in section 1.5, antisense technology has become one of the most attractive and increasingly popular therapeutic strategies used to prevent the progress of diseases such as cancer and infectious diseases through the inhibition of the specific gene expression. Antisense oligonucleotides (AON) are short chemically modified DNA constructs which are designed to hybridize to a specific sequence of a given mRNA target through Watson-Crick base-pairing interactions, thereby interfering with gene production of specific disease related proteins. An ideal antisense molecule should possess chemical stability, nuclease resistance, properties favorable for cell availability and good affinity for binding to the target RNA. Additionally, the inhibitory potency of antisense agents seem to correlate with their ability to elicit ribonuclease H (RNase H) degradation of the RNA target. RNase H is a ubiquitous cellular enzyme that specifically degrades the RNA strand of DNA/RNA hybrids, thereby inactivating the mRNA toward further cellular metabolic processes.[1,2,3,4,5]

Phosphorothioate deoxyribonucleotides (PS-DNA) are among the most widely used antisense inhibitors since they exhibit desirable properties important for antisense use. Unfortunately, PS-DNA possesses a relatively low binding affinity for RNA targets and non-specific binding to cellular proteins which impairs its potency in antisense application.[6] Accordingly, much effort in antisense development has been directed in developing new antisense oligonucleotides with improved binding affinity for target RNA while retaining the ability to activate RNase H.[5] The latter requirement is particularly important since RNase H potentiates the biological activity of antisense oligonucleotides by degrading the complementary mRNA portion of the AON-mRNA duplex thereby releasing the AON. Consequently, AONs possessing 2'-modifications of the sugar moiety have received considerable attention since these oligonucleotide analogs exhibit remarkable affinity towards target RNA, enhanced chemical stability and

nuclease resistance.[7,8] For instance, 2'-O-alkyl RNA (*e.g.* methyl, ethyl and propyl) bind more readily to target RNA compared to PS-DNA and the natural phosphodiester-linked DNA and RNA. Unfortunately, although 2'-substituted RNA analogs have certain desirable properties, the fact that they do not elicit RNase H activity limits their usefulness as antisense agents.[9] Attempts to take advantage of the beneficial properties of these modified oligomers while maintaining the substrate requirements for RNase H have led to the use of chimeric oligonucleotide analogs.[10,11,12,13,14] These analogs intermix deoxy and modified nucleotides within the oligomer in order to achieve the desired increases in metabolic stability and binding affinity while simultaneously supporting RNase H activity.[12] The most commonly used motif includes capping both the 3'-and 5'-ends of the modified oligomer with a contiguous string of modified nucleotide (wings) which permits the formation of stable duplexes with RNA and protects the compound from exonuclease degradation. Moreover, the central DNA core (gap) serves as a substrate for the enzyme, enabling RNase H degradation of target RNA[12] as depicted in **Figure 3.1.**

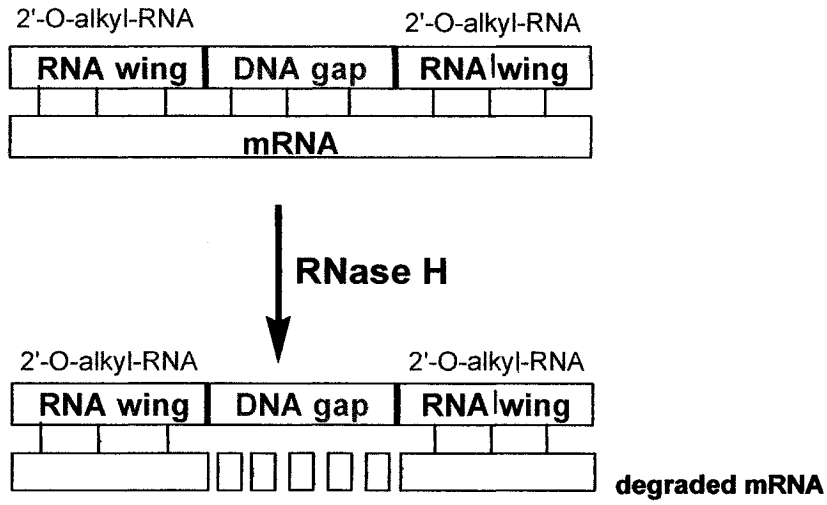


Figure 3.1: Mode of action of RNA-DNA-RNA "gapmers". The most widely used current AON technology makes use of chimeric oligonucleotides consisting of 2'-O-alkyl RNA (which does not invoke RNase H degradation of the target RNA) flanking a central core segment of DNA. This design provides enhanced RNA binding affinity and nuclease resistance via interaction with the 2'-O-alkyl RNA and elicits RNase H degradation of the target RNA via the DNA core.

To date, oligonucleotides containing phosphorothiolate modifications have been exploited to illuminate the roles of metals in nucleic acid catalysis[15] and have also enabled the engineering of new metal-dependent catalysts due to their ability to co-ordinate metal ions[16]; however, their biochemical use as antisense repressors has not yet been exploited. The conformational consequences upon the introduction of a single 3'-S-phosphorothiolate linkage in dinucleotides and in the DNA strand of a DNA:RNA dodecamer duplex have been established using high-resolution NMR spectroscopy by Cosstick and co-workers.[17,18,19] These studies revealed that the conformation of the sugar to which the sulfur is attached shifts to the north (C3'-endo) conformation as illustrated in **Figure 3.2.**

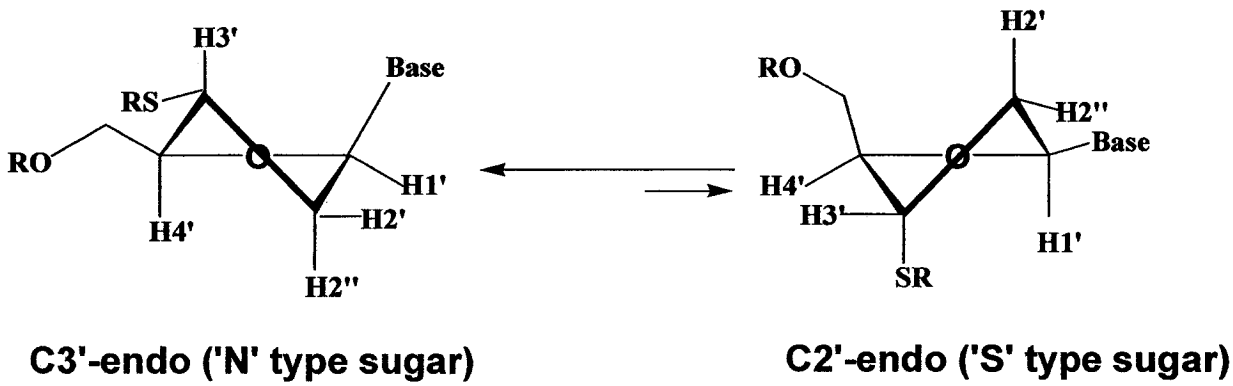


Figure 3.2: A schematic representation of the C3'-endo (northern) conformation adopted by the 3'-S-modified nucleosides.

In addition, this shift in the north-south conformational equilibrium was found to be transmitted to the subsequent (n+1) sugar, making them RNA mimetics and consequently, displaying enhanced RNA binding affinity. A series of singly and doubly 3'-S-phosphorothiolate-modified duplexes were investigated by UV melting studies and the enhanced stability that these conformational changes should bring when bound to the complementary mRNA target was confirmed.[18] Although there is very recent literature precedent concerning the 3'-S modification, we are unaware of the 5'-S modification having been previously investigated in this manner. In the current study we have evaluated the physiochemical properties of the different 3'- and 5'-S-phosphorothiolate modified oligomers, namely, their affinity towards complementary RNA and DNA

targets (T_m), their global helical conformation (CD studies) and their ability to activate RNase H.

3.2 SOLID PHASE SYNTHESIS AND THE PURIFICATION OF OLIGONUCLEOTIDES CONTAINING THE 3'- AND 5'-S-PHOSPHOROTHIOLATE LINKAGE

Modified oligonucleotides containing the 3'-S-phosphorothiolate linkages have been constructed using a variety of approaches including methods based on a Michaelis-Arbusov reaction[20,21,22,23] and using phosphotriester[20] and phosphoramidite chemistries.[15,24,25,26,27] The latter approach has gained favor due to its compatibility with standard solid-phase oligonucleotide synthesis; consequently, Cosstick and co-workers were the first to study the incorporation of 3'-deoxy-3'-thiothymidine into a growing oligonucleotide chain using phosphoramidite chemisty.[26,28,29] When the standard DNA synthesis protocol was used therefore keeping coupling times, activator (tetrazole) and thioamidite concentrations at their standard value, they observed no incorporation of the 3'-thioamidite (**2.6**) into the growing oligonucleotide chain. The standard protocol was modified in several ways: (a) *p*-nitrophenyltetrazole was used as an activator (pK_a 3.61) instead of the more commonly used tetrazole activator (pK_a 4.9) for the coupling reaction of (**2.6**); (b) the use of tetrabutylammonium periodate or tetrabutylammonium oxone rather than the standard oxidizing reagent which consists of iodine-pyridine-H_2O in THF; (c) the use of a five fold increase in the concentration of 3'-S-phosphorthioamidite (**2.6**) relative to the unmodified phosphoramidites; and (d) the coupling of the 3'-thioamidite (**2.6**) was performed manually by removing the reaction column from the DNA synthesizer and delivering the reagents (the 3'-thioamidite and the activator) via a syringe. This manual coupling procedure was inefficient as it made rigorous exclusion of moisture difficult, was time-consuming and gave inconsistent yields. Moreover, the 5-(*p*-nitrophenyl)tetrazole is poorly soluble in acetonitrile and not suited for conventional solid-phase synthesis. In order to overcome these problems, Cosstick and co-workers have recently re-optimized these conditions which involved the use of commercially available activators such as 5-ethylthiotetrazole (ETT) and 4,5-dicyanoimidazole (DCI) in 1M concentration, increasing the concentration of the 3'-S-phosphorthioamidite monomer to 100 mg/mL, extending coupling times to 15 min and using the standard oxidation conditions (iodine-pyridine-H_2O in THF). This resulted in coupling yields of

80-90% as determined by trityl release assay.[30] Both ETT and DCI were found to be potent activators, although DCI is preferred due to its reduced acidity and increased nucleophilicity. DCI as an activator resulted in 85-90% coupling yields with a single 15 min coupling procedure whereas with ETT the optimum coupling time was only 2.5 min (85-90% coupling). Postsynthetic deprotection and purification followed standard protocols.

Mag and co-workers first described the incorporation of a 5'-S-phosphorothiolate linkage in an oligodeoxynucleotide dodecamer using the solid phase phosphoramidite procedure.[31,32,33] The 5'-S-phosphorothiolate linkage was incorporated by using 5'-S-trityl-5'-deoxythymidine-3'-phosphoramidite, as the modified thymidine building block. The preparation of the modified oligonucleotide was accomplished with two different synthesis cycles. The first one was the standard DNA synthesis protocol used for the preparation of a heptamer containing the modified linkage. The second cycle consisted of modified detritylation and coupling conditions in order to facilitate the coupling between the 5'-SH terminus and standard phosphoramidite monomers enabling chain assembly to obtain a dodecamer. The deblocking step was performed manually by removing the column containing the support bound oligonucleotide from the instrument and treating it with 50 mM aqueous silver nitrate solution at r.t. for 15 min in order to cleave the triphenylmethyl (trityl) group protecting the thiol. This heavy metal cleavage reaction was necessary because the S-trityl group could not be easily cleaved by mild acids (*e.g.* AcOH, trichloroacetic acid) commonly used to deprotect 5'-O-dimethoxytrityl- or monomethoxytrityl-nucleosides. The column was then washed with water and subsequently treated with 50 mM dithiothreitol (DTT) for five minutes to minimize disulfide formation. The column was once again washed with water and dried by several washes with anhydrous acetonitrile. The synthesis cartridge, now containing the 5'-terminal thiol, was re-attached to the synthesis instrument and was allowed to couple with a tenfold excess of the next standard phosphoramidite monomer in the presence of tetrazole, increasing the coupling time to 300 s at r.t.. The P (III) linkage was subsequently oxidized to the corresponding phosphorothiolate by iodine/water.

Subsequent phosphoramidite monomers were added in the conventional manner until the desired modified dodecamer was obtained. However, this method is not compatible with the synthesis of modified oligonucleotides containing consecutive 5'-S-phosphorothiolate linkages because the deprotection reaction required to obtain the desired modified internucleotide linkage employs a silver salt that cleaves the trityl protecting from the 5'-thiol moiety. These salts may also cleave any previously formed P-S bond in the growing oligonucleotide chain.[33]

In addition, Rodriguez and co-workers have prepared 5'-thio-modified and 5'-dithio-modified oligonucleotides using traditional polymer-supported techniques[33] **(Figure 3.3).**

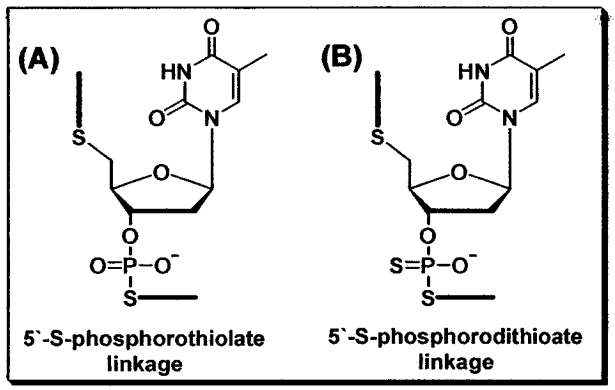


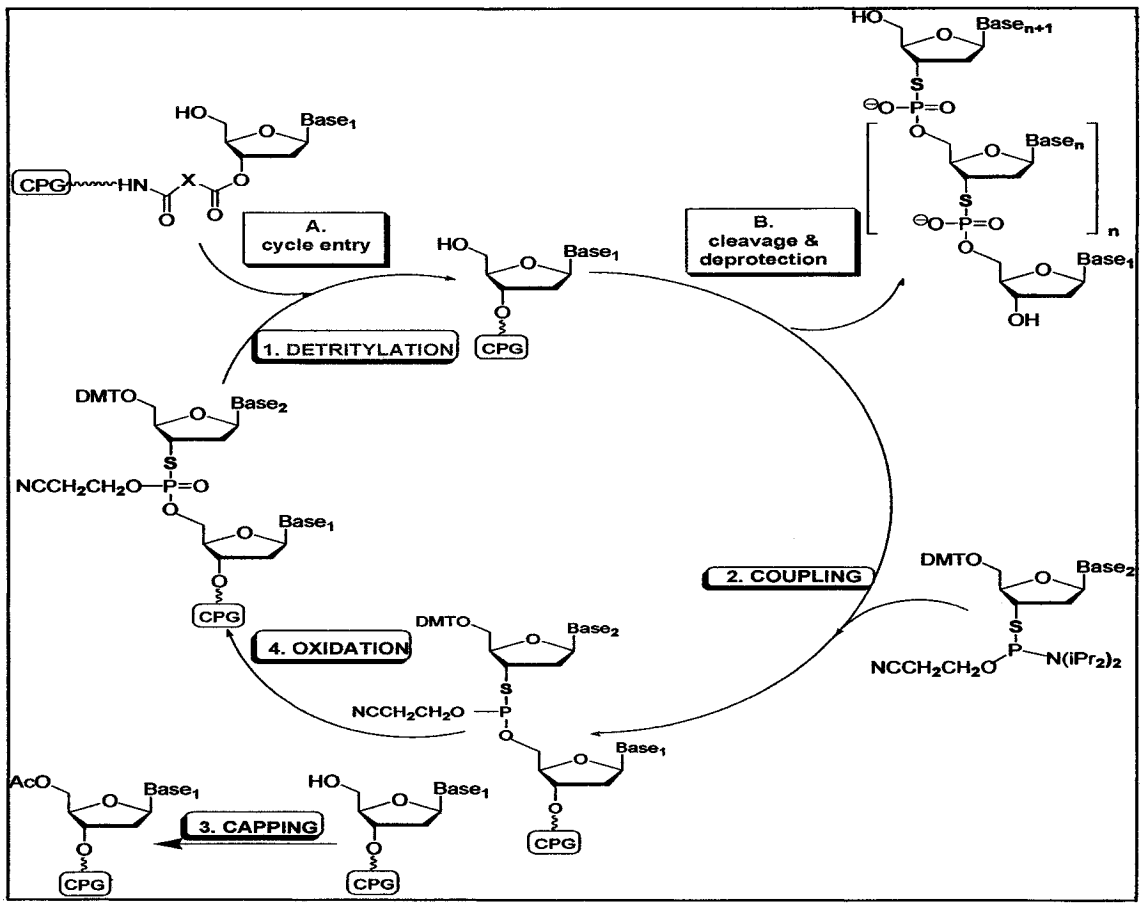
Figure 3.3: Modified oligonucleotides containing (A) a 5'-S-phosphorothiolate linkage where the 5'-bridging oxygen atom of the internucleotide linkage is replaced by a sulfur atom; and (B) a 5'-S-phosphorodithioate linkage where both the 5'-bridging and one of the non-bridging oxygen atoms of the phosphodiester linkage are replaced by sulfur atoms.

The incorporation of the above mentioned modified linkages in oligonucleotides was achieved by employing two different methods: (a) the traditional phosphoramidite method by unconventional (5'→ 3') direction of synthesis using the 5'-S-phosphorothioamidite synthon and (b) the H-phosphonate method by conventional (3'→5') direction of synthesis using a 3'-methylhydrogen-thiophosphonate nucleoside synthon. Thus, while the synthesis of 5'-thio and dithio-modified oligonucleotides utilizes traditional phosphoramidite technology, it proceeds in the less commonly used 5'→3' ("reverse") direction of synthesis using 5'-S-phosphorothioamidite monomeric

subunits and "reverse" 5'-nucleoside CPG as the solid support. The following modifications were made to the standard DNA synthesis cycle: (a) prior to the coupling of the modified amidite an extra trichloroacetic acid (TCA) addition was performed followed by CH_2Cl_2 washes and subsequently, double consecutive couplings (250 second wait each) for each modified linkage introduced in the growing oligonucleotide chain; (b) the oxidation conditions consisted of a 0.02 M iodine in tetrahydrofuran (THF)/pyridine/water which replaced the standard 0.1 M iodine oxidation solution inorder to minimize cleavage of the P-S bond thus allowing the synthesis of consecutive 5'-S-phosphorothiolate linkages; and (c) a 0.150 M solution of the pyrrolidino-thiophosphoramidites was used instead of the more commonly used diisopropylamino-thiophosphoramidites since better coupling yields were obtained due to the increased reactivity of the phosphorus center. Oxidation of the P(III) species with a freshly prepared solution of 5% (w/v) elemental sulfur (S_8) in carbon disulfide (CS_2)-pyridine 1:1 (v/v), containing 6% triethylamine generated the 5'-S-phosphorodithioate linkage.

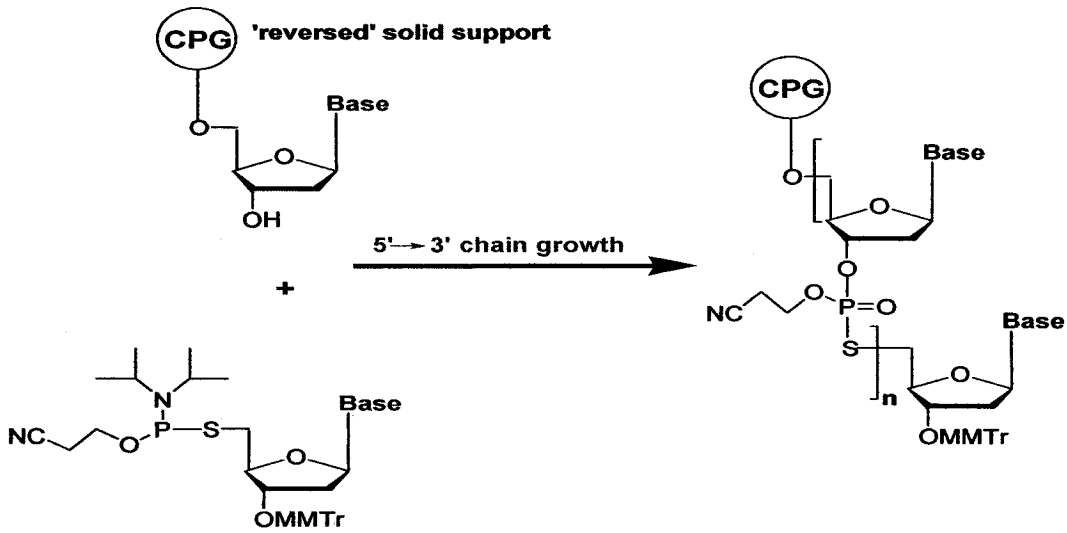
All oligonucleotides in our present studies were synthesized using an Applied Biosystems 381 automatic DNA synthesizer on a 1-μmol scale. The 3'- and the 5'- nucleoside linked solid supports were obtainted commercially. The incorporation of 3'-S-phosphoramidites in an oligonucleotide occurs in the most commonly used 3'-5'direction **(Figure 3.4),** however, introduction of the 5'-S-phosphoramidites proceeds in the less commonly used 5'-3' direction of synthesis as depicted in **Figure 3.5.** The synthetic cycle used for construction of modified oligonucleotides containing the 3'-S-phosphorothiolate linkages was similar to the one recently developed by Cosstick *et al.*[30] The procedure consists of a standard reaction cycle, standard capping, oxidation and deblocking reagents. The standard dT and 2'-OMe-rU amidites were prepared as 0.100M solutions in anhydrous acetonitrile; moreover, the modified 3'-and 5'-S-phosphoramidites were prepared as 0.150M solution in anhydrous acetonitrile. The activator used to carry out the couplings was DCI (4,5-Dicyanoimidazole) (1M in dry acetonitrile) and was chosen due to its

Figure 3.4: Illustration of automated solid-phase phosphoramidite synthesis of oligonucleotides containing 3'-S-phosphorothiolate linkages.



(A) Cycle entry with support bound and suitably protected nucleoside; (1) detritylation using 3%TCA in dichloromethane; (2) coupling of the free 5'-OH to a nucleoside 3'-S-phosphoramidite using an activating reagent (e.g. tetrazole); (3) capping of unreacted 5'-OH groups using acetic anhydride; (4) oxidation of the phosphite trimester with iodine solution; (B) Cycle exit along with cleavage and deprotection of oligonucleotide chain after n+1 cycles.

Figure 3.5: The solid-phase synthesis of oligonucleotides containing the modified 5'-S-phosphorothiolate linkage follows the same cycle as depicted in Figure 3.4, however, proceeds in the less commonly used 5'→3' direction.



reduced acidity. The only modifications made to Cosstick's protocol involved extended 3'-and 5'-S-phosphoramidite coupling times (20 min vs 15 min) and detritylation times (220 s vs 140 s required to remove the more stable 5'-monomethoxytrityl protecting group as opposed to the 5'-dimethoxytrityl group). In addition, the oxidation conditions were slightly modified to prevent possible oxidation at the 3'-S- position and consisted of 0.05M solution of iodine-pyridine-H_2O in THF instead of the more commonly used 0.1M solution. This may minimize the possible cleavage of the P-S linkage which usually occurs in the presence of iodine.[23,24] The sequences synthesized in this study are listed in below in **Table 3.1**. Coupling yields were determined by trityl assays and were found to be modest (75-80%) for the incorporation of one modified 3'-and 5'-S-phosphorothiolate linkage in the growing oligonucleotide chain. However, we found that upon subsequent incorporations for both the 3'- and 5'-S-phosphoramidites the coupling yields decreased substantially (50-60%). This may be attributed to the reduced reactivity of the 3'- and 5'-S-phosphoramidites compared to the corresponding standard 3'- and 5'-O-phosphoramidites. This led to the use of a more acidic activating agent in order to improve the coupling between the 3'- or 5'-S-phosphoramidite monomers and the growing oligonucleotide chain. However, it has been reported that coupling reactions

with 3'-S-phosphoramidites in the presence of a more acidic activating agent results in the displacement of 3'-thionucleoside from the phosphorus center and is accompanied by the formation of many side products.[25,26,28,29] In particular, Cosstick and co-workers have identified a symmetrical dimer (A) , which results from the liberated 3'-thionucleosides reacting with the 3'-S-phosphoramidite monomer, as well as a thiophosphorothioamidate (B) derived from the oxidation of the unactivated 3'-S-phosphoramidite monomer and the desired thiophosphate (C) as illustrated in **Figure 3.6.**

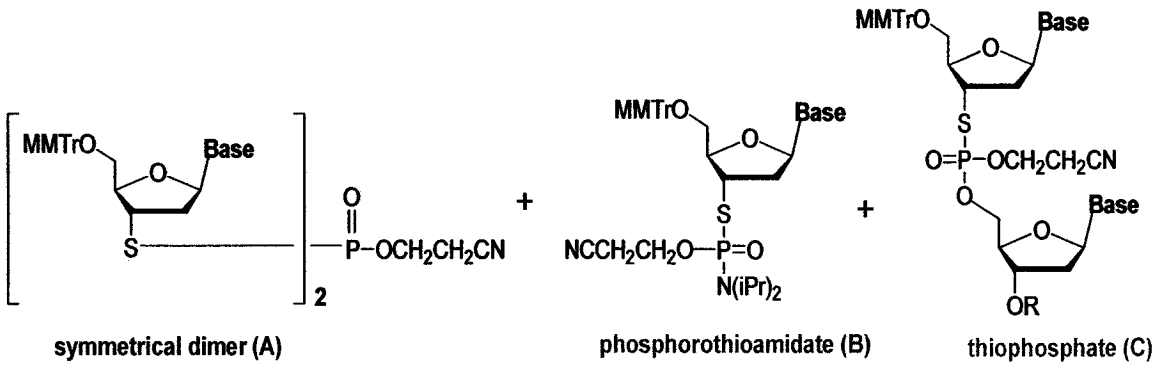


Figure 3.6: The reduced reactivity of 3'-S-phosphoramidites led to the use of a more acidic activating reagent, *p*-nitrophenyltetrazole. Unfortunately, the thioalkyl is particularily susceptible to displacement from the phosphorus center under these acidic conditions; consequently, coupling reactions are accompanied by the formation of many products, in particular, the aforementioned ones which have been isolated and characterized.

Although, it was not possible to prepare an all modified oligonucleotide containing 3'-S- and 5'-S-phosphorothiolate internucleotide linkages due the reduced reactivity of 3'-and 5'-S-phosphoramidite subunits, we did however manage to successfully synthesize an oligonucleotide chimera composed of 2'-OMe-rU "wings" with an internal hexanucleotide segment made out of 3'-deoxy-3'-thiothymidine units (**Table 3.1**). To our knowledge this represents the longest sequence of 3'S-DNA ever synthesized.

Table 3.1 : Modified oligonucleotides containing 3'-and 5'-S-phosphorothiolate linkages synthesized in this work.

SEQUENCES	Designation	ODs [a] crude	ODs pure
d (TTT TTT TT($T_{3'S}$) TTT TTT TTT)	**3.0**	149.7	75.0
d (TTT TTT TT($T_{3'S}$) ($T_{3'S}$) ($T_{3'S}$)T TTT TTT)	**3.1**	75.0	14.7
d (TTT TTT TTT ($^{5'S}$T)TT TTT TTT)	**3.2**	174.8	45.4
d (TTT TTT T($^{5'S}$T)($^{5'S}$T) ($^{5'S}$T)TT TTT TTT)	**3.3**	85.1	8.8
$U_{me}U_{me}U_{me}U_{me}U_{me}U_{me}(T_{3'S}T_{3'S}T_{3'S}T_{3'S}T_{3'S}T_{3'S})U_{me}U_{me}U_{me}U_{me}U_{me}U_{me}$	**3.4**	51.6	2.0

[a] An OD reading estimates the amount of DNA in solution by measuring the absorbance at 260 nm. Deoxthymidine (dT); 3'-deoxy-3'-thiothymidine ($T_{3'S}$); 5'-deoxy-5'-thiothymidine ($^{5'S}$T); and 2'-O-methyluridine (U_{me}).

Following chain assembly, oligonucleotides were cleaved from the solid support and deprotected as previously described.[34] The crude modified oligomers were purified by either (a) preparative gel electrophoresis (16% acrylamide) followed by desalting (Sephadex G-25) or (b) anion-exchange HPLC followed by desalting via size-exclusion chromatography (Sephadex G-25). Under these conditions, the desired full-length oligomer eluted last.

The sequences were characterized by MALDI mass spectrometry (**Figure 3.7**). **Table 3.2** shows the predicted and the experimentally determined mass values. All sequences are within 1% of the calculated molecular weight which offers proof of the composition of the sequences.

Table 3.2: MALDI results for the 3'-and 5'-S-phoshorothiolate modified oligonucleotides[a].

SEQUENCES	Designation	MW (calc.)	MW (exp.)
d (TTT TTT TT($T_{3'S}$) TTT TTT TTT)	**3.0**	5430	5433
d (TTT TTT TT($T_{3'S}$) ($T_{3'S}$) ($T_{3'S}$)T TTT TTT)	**3.1**	5462	5465
d (TTT TTT TTT ($^{5'S}$T)TT TTT TTT)	**3.2**	5430	5459
d (TTT TTT T($^{5'S}$T)($^{5'S}$T) ($^{5'S}$T)TT TTT TTT)	**3.3**	5462	5465
$U_{me}U_{me}U_{me}U_{me}U_{me}U_{me}$($T_{3'S}T_{3'S}T_{3'S}T_{3'S}T_{3'S}$)$U_{me}U_{me}U_{me}U_{me}U_{me}U_{me}$	**3.4**	5701	5702

[a] The oligonucleotides and matrices were diluted with ultrapure deionized water and HPLC grade solvents. Purified samples (1μL, 0.2-1 mmol/μL in water) were mixed with 1μL of saturated ATT/spermine (80 mg/mL ATT dissolved in 1:1 water/CH_3CN containing 25 mM spermine) and 1μL fucose (50 mM in water). The analyte/matrix mixture (1μL) was spotted in duplicate on a sample plate and allowed to dry under a warm stream of air. The crystalline spots were analyzed and the spectra processed using software supplied by Kratos.

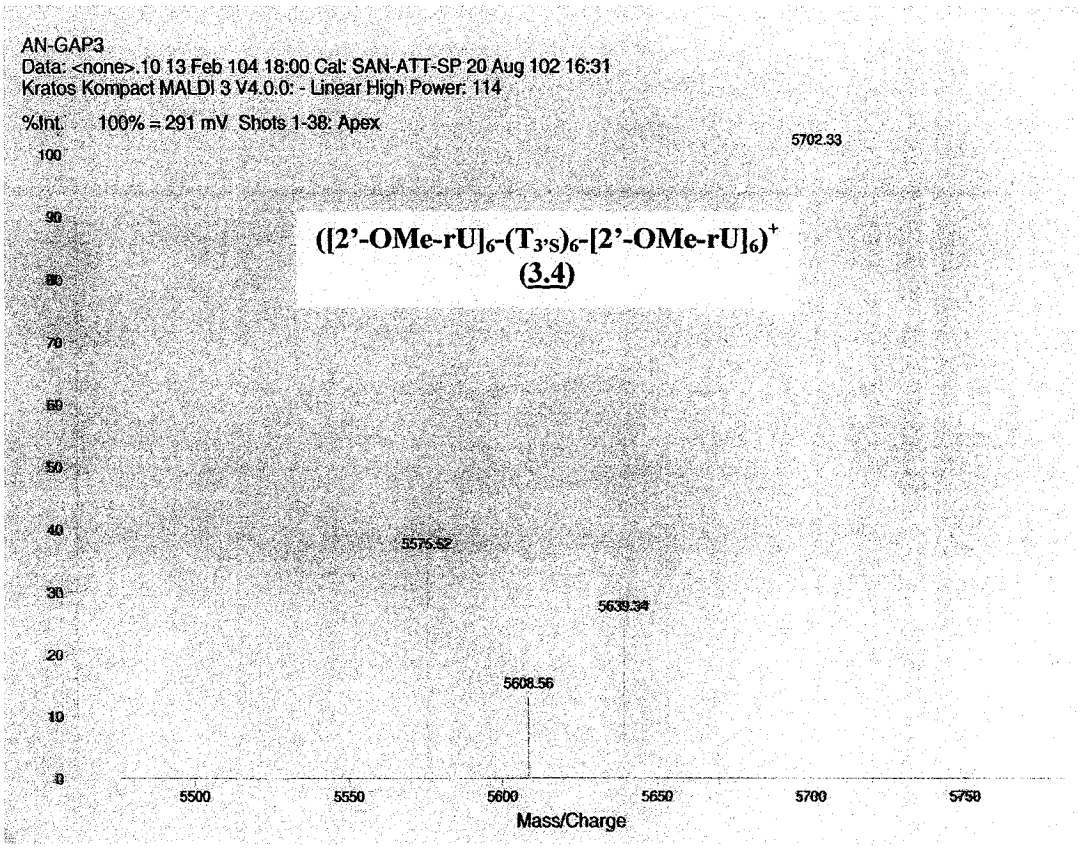


Figure 3.7: MALDI-TOF spectrum of the chimeric oligonucleotide $[2'\text{-OMe-rU}]_6\text{-}(T_{3'S})_6\text{-}[2'\text{-OMe-rU}]_6$ (**3.4**).

3.3 BINDING STUDIES OF OLIGOMERS CONTAINING 3'-AND 5'-S-PHOSPHOROTHIOLATE LINKAGES TOWARDS SINGLE STRANDED DNA AND RNA TARGETS

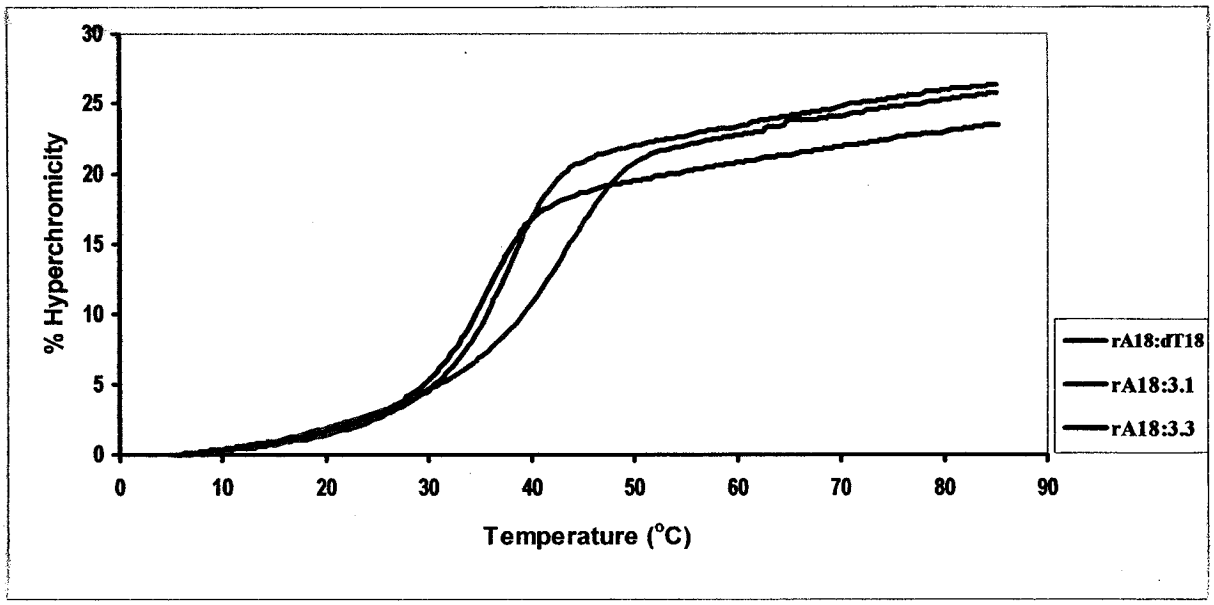
Duplex formation between the 18 nt modified oligomer (referred as "antisense" strands) with the respective DNA and RNA targets was monitored by both UV and CD spectroscopy. The homopolymers containing a single and multiple $dT_{3'S}$ and $^{5'S}dT$ units were used to assess the effect of 3'S and 5'S substitutions on the duplex stability of dT/rA and dT/dA hybrids. The melting curves obtained for the modified oligomers hybridized to DNA or RNA exhibited sharp monophasic transitions, indicative of formation of a single cooperative complex from which accurate T_m values were extracted. This cooperative nature indicates two-state melting behaviour for duplexes. The overall shapes of the curves were similar but, as expected, the T_m values varied. The results obtained are summarized in **Table 3.3.**

Table 3.3 : Melting temperatures (T_m) of hybrids formed between the modified oligonucleotides with the corresponding DNA or RNA target.

SEQUENCES	Designation	T_m (°C) DNA target	T_m (°C) RNA target
d (TTT TTT TTT TTT TTT TTT)	**3.5**	43.6	37.6
d (TTT TTT TT($T_{3'S}$) TTT TTT TTT)	**3.0**	43.4	37.7
d (TTT TTT TT($T_{3'S}$) ($T_{3'S}$) ($T_{3'S}$)T TTT TTT)	**3.1**	42.7	42.2
d (TTT TTT TTT ($^{5'S}$T)TT TTT TTT)	**3.2**	42.5	35.8
d (TTT TTT T($^{5'S}$T)($^{5'S}$T) ($^{5'S}$T)TT TTT TTT)	**3.3**	39.9	33.8

The melting temperature (T_m) were obtained from the maxima of the first derivative melting curves (A_{260} vs. temperature). Modified oligonucleotides and complementary targets (rA_{18} and dA_{18} oligomers) were mixed in equimolar ratios in 140 mM KCl, 1mM $MgCl_2$ and 5 mM Na_2HPO_4 buffer, pH 7.2, to provide a total duplex concentration of ca. 5 μM.

Figure 3.8: Thermal melting curves of oligonucleotides (AON) hybridized to complementary single-stranded RNA.



Refer to Table 3.3 for the corresponding base sequences.

A modified oligonucleotide comprised of a single 3'-S-phosphorothiolate linkage (**3.0**) showed no significant change in T_m when hybridized to a DNA or RNA target compared with the unmodified hybrid duplexes. However, the modified oligomer containing three consecutive 3'-S-phosphorothiolate linkages (**3.1**) displays an increased binding affinity towards single-stranded RNA (stabilization of 1.5 °C/residue; ΔT_m = +1.5 °C/mod.) whereas, a reduced binding affinity towards single stranded DNA was observed (destabilization of 0.3°C/residue; ΔT_m = - 0.3 °C/mod.). Recently, Costtick and co-workers have conducted similar UV thermal melting studies with DNA duplexes containing one or two 3'-S-modifications in the DNA strand in order to probe the impact of this modification on duplex stability.[18] When a single 3'-S-phosphorothiolate linkage was introduced a stabilization of 1.6-2.5°C/residue compared to the DNA/RNA hybrids was observed. A further increase in T_m (ΔT_m = +3°C/mod.) was noted when the modified link is incorporated in two adjacent residues. In addition, it was noted that when the 3'-S substitutions were separated by a single non-modified residue a further increase in T_m was observed (ΔT_m = +4°C/mod.). The enhanced thermal stability exhibited by the

DNA/RNA hybrids containing the 3'-S modification is attributed to the tendency of the thiosugar moieties to adopt an RNA-like, C3'-*endo* conformation due to the presence of a sterically large and electropositive sulfur atom at C3'.[17,18,19] This sugar pucker promotes pre-organization of the modified DNA strand to a more stable A-form geometry resulting in a hybrid that strongly resembles the native RNA/RNA duplexes both in structure and thermodynamic stability.

In contrast, modified DNA oligomers containing a single 5'-S-phosphorothiolate modified linkage (**3.2**) exhibited lower affinity towards DNA and RNA targets relative to the unmodified DNA strand. A destabilization of 1.1°C was observed with the modified DNA/DNA duplex and an even greater destabilization (1.8°C) was noted with the modified DNA/RNA hybrid. Additionally, oligonucleotide (**3.3**) which contains three consecutive 5'-S-phosphorothiolate modified linkages formed weaker complexes with both complementary single stranded DNA and RNA with a destabilization of 1.3°C per 5'-S linkage compared to the natural counterparts (**Figure 3.8**).

As described above, many sugar-phosphate backbone modifications have been made in an attempt to improve the binding affinity of the oligonucleotide for the target RNA. Ribose modifications (*e.g.*, alkylation) particularily at the 2'-position, have yielded oligonucleotide analogs with certain desirable properties, however, the fact that they do not elicit RNase H compromise their usefulness as antisense agents. Such a lack of recognition by RNase H may be overcome by creating mixed-backbone or chimeric oligonucleotides composed of 2'-O-substituted RNA 'wings' and a DNA 'gap' positioned in the middle of the chimera. This allows for enhanced binding affinities to target RNA via the 2'-O-alkyl RNA and elicits RNase H degradation of target RNA via the DNA core.[10,11,12,13,14]

Due to the enhanced binding affinity exhibited by oligomers containing the 3'-S modification towards the RNA target, a chimeric oligonucleotide was synthesized. The internal hexanucleotide segment was composed of 3'-deoxy-3'-thiothymidine ($T_{3'S}$) residues, flanked by 2'-O-methyluridine (2'-OMe-rU) 'wings' (**3.4**). The (2'-O-methylribose)$_6$-($T_{3'S}$)$_6$-(2'-O-methylribose)$_6$ 'gapmer' oligonucleotide displays an unprecedented affinity towards *both* single stranded RNA and DNA targets. A

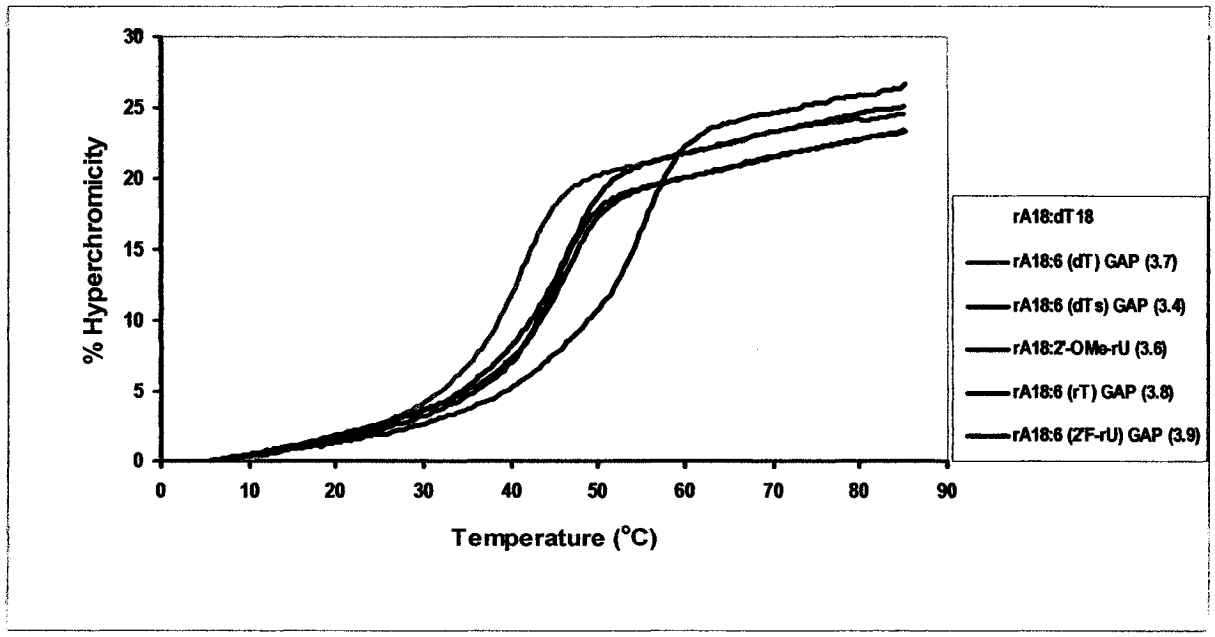
stabilization of 2.5°C per insertion was observed when bound to either the complementary DNA or RNA targets compared to the control chimeric oligomer (**3.7**). This finding is in accordance with the notion that the 3'-thio modification shifts the sugar pucker towards the northern conformation resulting in increased T_m value (**Table 3.4**). Furthermore, we studied chimeric oligonucleotides containing an internal gap composed of the following uridine analogues: 2'-OMe-rU, 2'-deoxy-2'-fluorouridine (2'-F-rU) and ribothymidine (rT). These RNA mimetics were found to form more thermodynamically stable duplexes with RNA compared to the corresponding DNA/RNA duplexes.[8] In fact, it has been well documented that the ratio of the 3'-*endo* conformer population increases linearly with the electronegativity of the 2'-pendant groups (i.e. 2'F >2'OMe >2'-OH) acting in conjunction with the gauche effect, resulting in preferential C3'-*endo* puckering, duplex stability and A-like character of the AON strand.[8] Consequently, the RNA-like antisense strand upon hybridization with target RNA results in duplex with increased thermal stability. Therefore, it was of interest to conduct further experiments in order to compare the thermal stabilities of these various modified chimeric oligomers with the chimeric oligonucleotide containing the $(T_{3'S})_6$ gap (**3.4**). The results obtained, summarized in **Table 3.4**, suggest that the chimeric oligonucleotide containing the 3'-thio modification provides the largest stabilization against RNA binding (**Figure 3.9**). Therefore, these modifications can be ranked on the basis of their ability to form thermodynamically stable duplexes with the corresponding RNA target as follows: 3'-S-modification (ΔT_m = +2.3 °C) > 2'F-RNA (ΔT_m = +0.9 °C) > rT (ΔT_m = +0.8 °C) > 2'OMe-RNA (ΔT_m = +0.5 °C) > dT (ΔT_m = Ø). Thus, we conclude that placing an electronegative group at the 2' position or a non-electronegative group (or less electronegative group than oxygen) at the 3' position is effective in shifting the sugar pucker towards the C3'-*endo* conformation and significantly improving binding affinities towards a target RNA compared to the unmodified DNA.

Table 3.4: Melting temperatures (T_m) of hybrids formed between oligonucleotide chimeras (gapmers) with the corresponding DNA or RNA targets[a].

SEQUENCES	Designation	Tm (°C) DNA target	Tm (°C) RNA target
d (TTT TTT TTT TTT TTT TTT)	**3.5**	43.6	37.6
($U_{me}U_{me}U_{me}$ $U_{me}U_{me}U_{me}$ $U_{me}U_{me}U_{me}$ $U_{me}U_{me}U_{me}$ $U_{me}U_{me}U_{me}$ $U_{me}U_{me}U_{me}$)	**3.6**	35.0	45.1
$U_{me}U_{me}U_{me}U_{me}U_{me}U_{me}$ d(TTT TTT) $U_{me}U_{me}U_{me}U_{me}U_{me}U_{me}$	**3.7**	32.0	42.1
$U_{me}U_{me}U_{me}U_{me}U_{me}U_{me}$ r(TTT TTT) $U_{me}U_{me}U_{me}U_{me}U_{me}U_{me}$	**3.8**	39.7	46.8
$U_{me}U_{me}U_{me}U_{me}U_{me}U_{me}$ r($U_fU_fU_f$ $U_fU_fU_f$) $U_{me}U_{me}U_{me}U_{me}U_{me}U_{me}$	**3.9**	37.1	47.2
$U_{me}U_{me}U_{me}U_{me}U_{me}U_{me}$ ($T_{3'S}T_{3'S}T_{3'S}$ $T_{3'S}T_{3'S}T_{3'S}$) $U_{me}U_{me}U_{me}U_{me}U_{me}U_{me}$	**3.4**	47.1	55.9

[a] Melting temperatures (T*m*) were obtained from the maxima of the first derivative melting curves (A_{260} vs. temperature). Modified oligonucleotides and complementary targets (RNA and DNA oligomers) were mixed in equimolar ratios in 140 mM KCl, 1mM $MgCl_2$ and 5 mM Na_2HPO_4 buffer, pH 7.2, to provide a total duplex concentration of *ca.* 5 µM. Deoxythymidine (dT); 2'-O-methyluridine (U_{me}); ribothymidine (rT); 2'-fluorouridine (U_f); and 3'-deoxy-3'-thiothymidine ($T_{3'S}$).

Figure 3.9: Thermal melting curves of the various chimeric oligonucleotides duplexed with the RNA target.

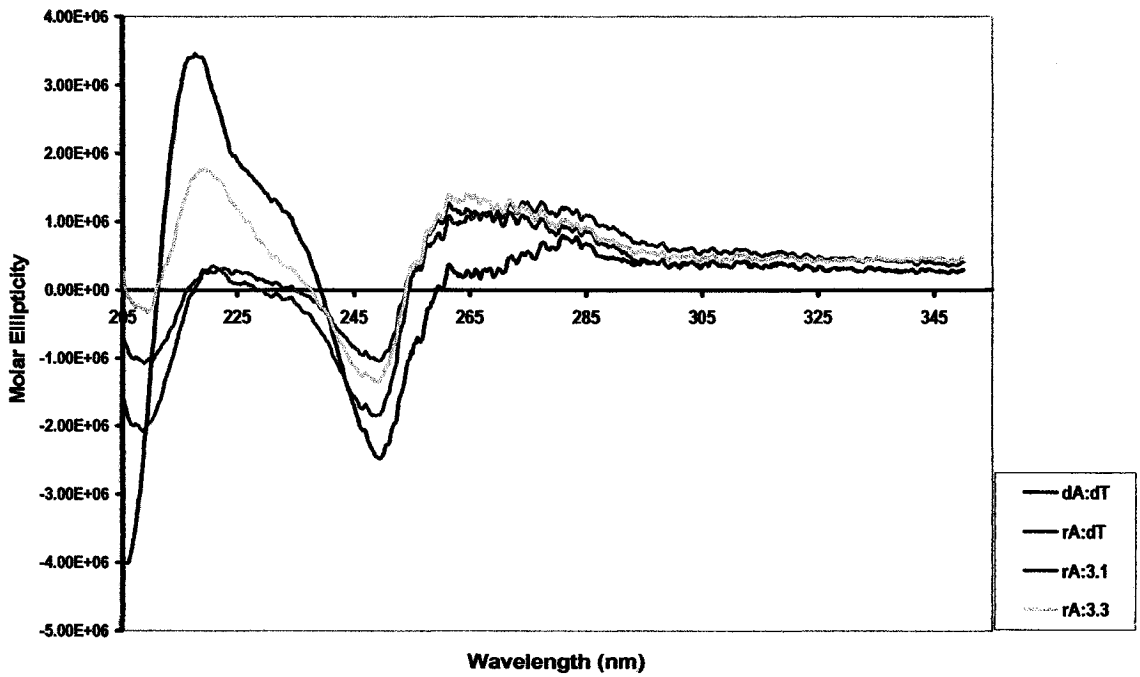


Refer to Table 3.4 for the corresponding base sequences.

3.4 CIRCULAR DICHROMISM (CD) OF DUPLEXES

Circular dichromism (CD) is a very powerful, qualitative technique used to gain insight on nucleic acid structure, particularly the helical arrangement of double helices.[35,36,37] CD spectral analysis was used to establish whether the incorporation of 3'-and 5'-S-phosphorothiolate linkages within DNA has a fundamental influence on the conformation of the duplex structure. B-form helices (DNA/DNA) display a positive band centered near 285 nm, a negative band near 250 nm and a crossover signature at ca. 260 nm and a fairly intense positive band positioned at 220 nm. In contrast, A-form duplexes (RNA/RNA) exhibit a strong band centered around 265 nm followed by two consecutive negative bands one positioned at 245 nm and the other at 210 nm. In addition, DNA forms a duplex with RNA which gives rise to a spectra that indicate a form intermediate between A and B but generally resembling the A-helical form. Structurally, this heteroduplex conformation is closer to the A-form of RNA/RNA duplexes and thus is commonly described as an 'A-like' helical structure. These CD spectra were recorded at 5°C where only the duplex and no single strands exist. As shown in **Figure 3.10,** the CD spectra of the AON/RNA hybrid containing three consecutive 3'-S-phosphorothiolate linkages closely resembles the spectra of the RNA/RNA duplex. This is likely due to the N-type sugar pucker adopted by the 3'-S modified sugars which may, in turn, perturb the remaining deoxyribonucleotides in the AON strand so as to shift their sugar conformation to a N-type pucker. This would drive the structure of the AON/RNA hybrid towards a pure A-type duplex geometry. In contrast, the CD spectra of the AON/RNA hybrid containing three contiguous 5'-S-phosphorothiolate linkages closely mimics the spectra of the normal DNA/RNA heteroduplex suggesting that the AON strand containing the 5'-S modified sugars adopt a DNA-like or C2'-*endo* type conformation allowing the modified AON/RNA duplex to retain the intermediate geometry of DNA/RNA hybrids.

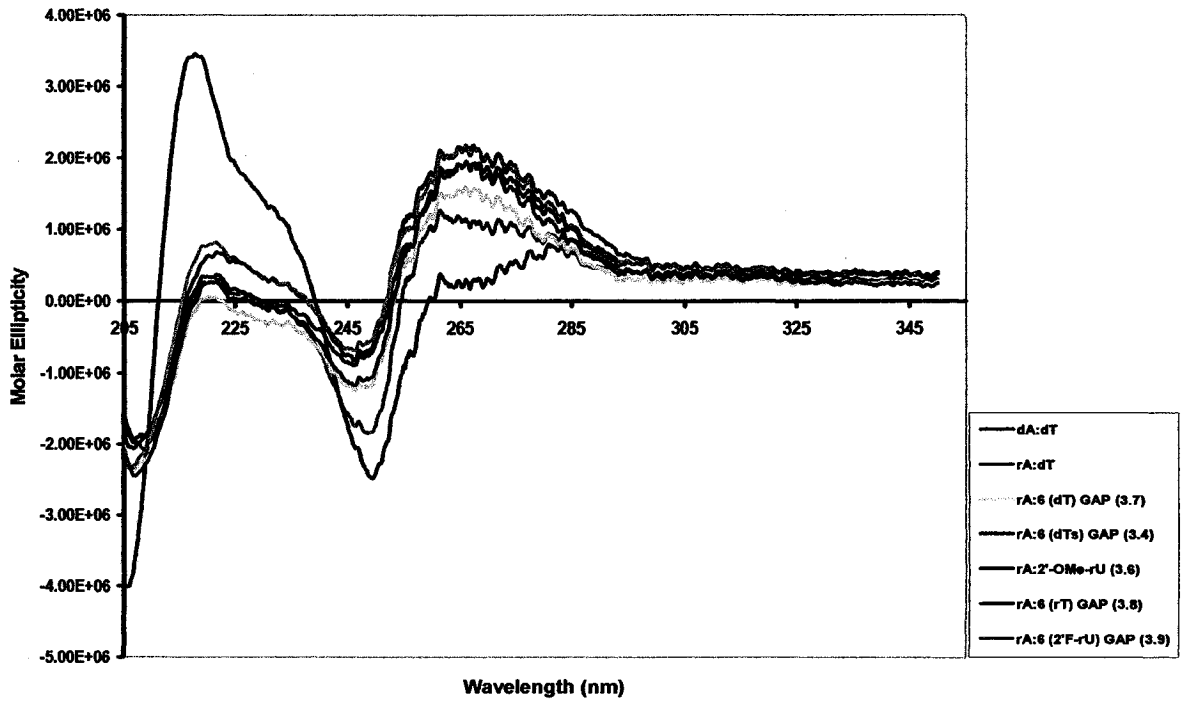
Figure 3.10: CD spectra of oligonucleotides hybridized to a common rA_{18} target at 5°C.



CD spectras were obtained using a medium ionic strength buffer: 140 mM KCl, 1mM $MgCl_2$, 5 mM Na_2HPO_4, pH 7.2.

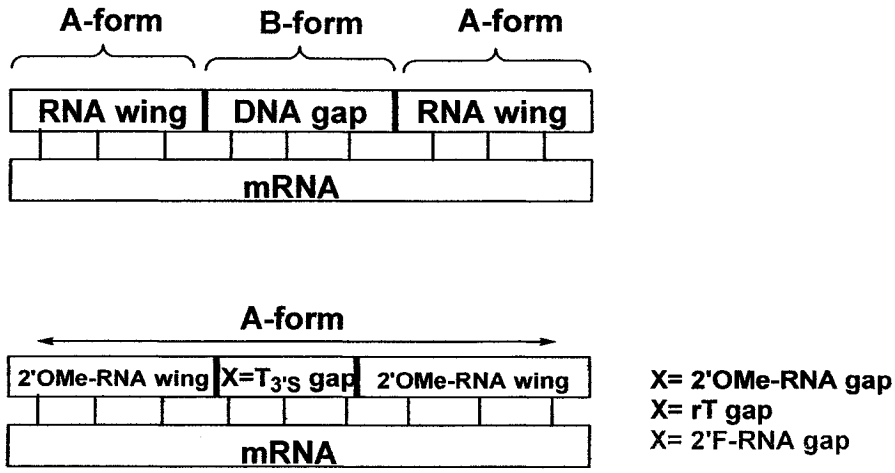
The CD spectra of the various oligonucleotide chimeras comprised of 2'-modified ribonucleotides gaps such as, $(2'OMe\text{-}rU)_6$, $(2'F\text{-}rU)_6$ and $(rT)_6$ hybridized with the single-stranded RNA target (rA_{18}) were also obtained (**Figure 3.11**). These modifications are expected to preorganize the AON into a pure C3'-*endo* (north) conformation resulting in a AON/RNA duplex that exhibits a characteristic A-form CD pattern. As expected, the use of combinations of north (ribo) and east/south (DNA) in the same strand, as in the case of the chimeric oligomer comprised of a $(dT)_6$ gap, is disruptive as they introduce A/B junctions along the AON/RNA duplex (**Figure 3.12**). Futhermore, the CD spectrum of the chimeric oligomer containing the 3'-S modified gap hybridized with the RNA target was characteristic of an A-form duplex. This again is consistent with a strong preference of the 3'S-residues to adopt an RNA-like or C3'-*endo* conformation.

Figure 3.11: CD spectra of various chimeric oligonucleotides hybridized to a common rA_{18} target at 5°C.



CD spectras were obtained using a medium ionic strength buffer: 140 mM KCl, 1mM $MgCl_2$, 5 mM Na_2HPO_4, pH 7.2.

Figure 3.12: Schematic representation of chimeric hybrid duplexes.



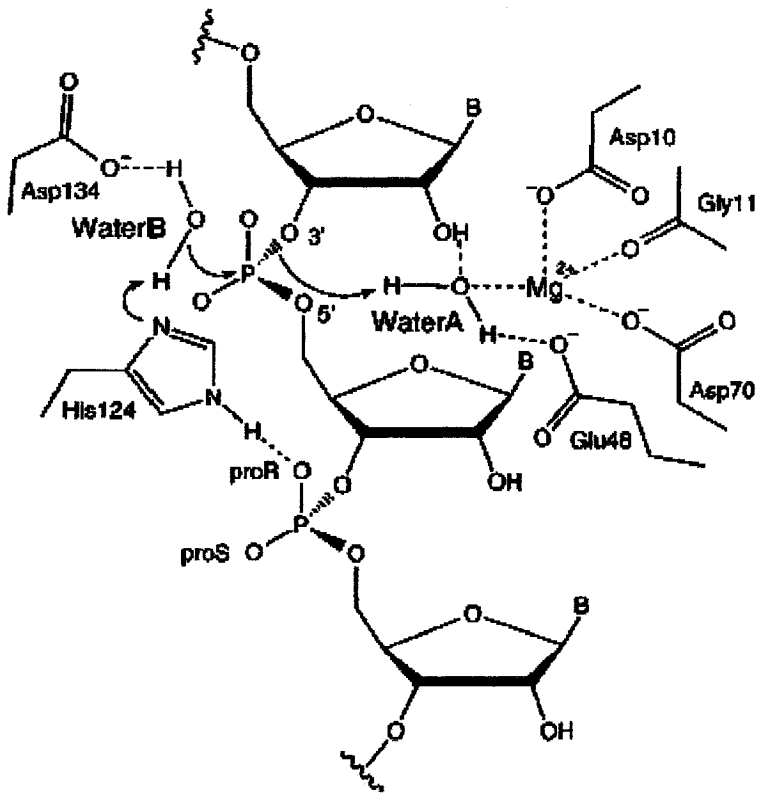
Hybrids of RNA and 2'-OMe-RNA/dT chimeras are comprised of 'A-B-A' conformational domains, whereas those between RNA and 2'-OMe-RNA/$T_{3'S}$ chimeras belong to the A conformational family.

3.5 OLIGOMER-INDUCED DEGRADATION OF RNA BY *E. coli* RNASE H

Antisense oligonucleotides (AON) duplexed with the target mRNA can inhibit gene expression gene via several putative mechanisms such as translational arrest mediated by the blockage of ribosomal read-through[38,39] and by the recruitment of an endogenous enzyme, RNase H, which specifically degrades the target RNA in the AON/RNA hybrid duplex.[40] While both of these mechanisms can successfully inhibit the protein synthesis encoded by the mRNA, elicitation of RNase H cleavage of target mRNA is considered to be the more efficient of the two mechanism for suppressing target gene expression. Despite the extensive studies conducted on RNase H, its biological role is not yet well defined. ***Escherichia coli*** (*E. coli*) RHase H1 has been shown to be implicated in many biological processes, including DNA replication and repair[41], in chromosomal DNA replication[42], transcription and viral reverse transcription events.[43] In addition, RNase H functions as an endonuclease which cleaves the RNA moiety of the RNA/DNA hybrid in the presence of divalent cations such as Mg^{2+} or Mn^{2+} leaving 5'-phosphate and 3'-hydroxyl oligonucleotide products.[44] Two alternative mechanisms have been proposed for the catalytic function of *E. coli* RNase H. The first is a two-metal ion mechanism in which one of the two metal ions activates the attacking hydroxide ion, and the second is a general acid-base mechanism where an amino acid residue, instead of the metal ion, activates the attacking hydroxide ion as shown in **Figure 3.13**. X-ray analysis, NMR and kinetic studies suggest that RNase H mediated hydrolysis occurs via the general acid-base mechanism and the key amino acids, namely, Asp10, Asp70, His124, Asp34 and Glu48 are required for catalysis as identified by site directed mutagenesis.[45]

Of the hundreds of modified antisense oligonucleotides that have been evaluated for RNase H, only a handful have the ability to activate RNase H when hybridized to RNA namely, DNA phosphorothioates[46], phosphorodithioates[47], boranophosphate[48], the 2'-arabino modifications such as arabinonucleic acids (ANA)[49] and 2'-deoxy-2'-fluoro-arabinonucleic acids (2'F-ANA)[50] and 'gapmer' oligonucleotides consisting of a non-

Figure 3.13: Schematic representation of the proposed catalytic mechanism of *E. coli* RNase H at the active site.



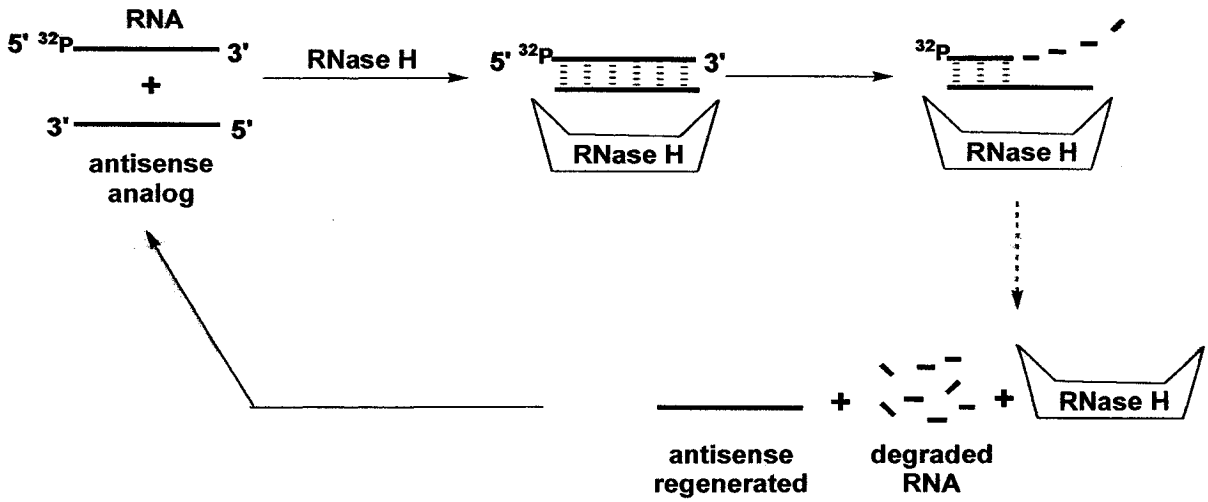
Asp10 and Asp70 are responsible for the binding of the Mg^{+2} ion to the correct position in the active site of the enzyme; His124 accepts a proton from the attacking water molecule, which acts as a general base; Asp134 holds this water molecule; Glu48 anchors the water molecule that acts as a general acid. The phosphate group 3' to the scissile phosphodiester bond has been shown to be implicated in the general acid-base mechanism for the hydrolysis of the P-O3' bond of RNA by E. coli RNase H. Note that Water B and Water A represent the water molecules that act as a general base and general acid, respectively. Figure adapted from Haruki *et al.* 2000.[45]

uniform backbone (**Figure 3.12**).[51] The fact that few AONs elicit RNase H activity demonstrates the sensitivity of this enzyme to the structure of the duplex substrate. Based on the studies reported thus far, the following criteria must be met by the substrate for efficient RNase H mediated hydrolysis to occur[52,53]: (a) a flexible conformation of the AON/RNA heteroduplex that is characterized by an A-type C3'-*endo* puckering for the RNA strand and a DNA-like or O4'-*endo* puckering for the AON strand[53]; (b) the AON/RNA duplex should have an intermediate minor groove width similar to that of

DNA/RNA-like hybrids; in fact, this feature is thought to allow RNase H to discriminate between DNA/RNA and RNA/RNA duplexes[54,55]; (c) the presence of the 2'-OH group on the target RNA which facilitates the formation of an outersphere complex[56,52,53] (**Figure 3.13**); and (d) no protrusions should be present in the minor groove or its proximity in order to preserve the availability of the 2'-OH of the RNA and the associated hydration pattern.[57,53]

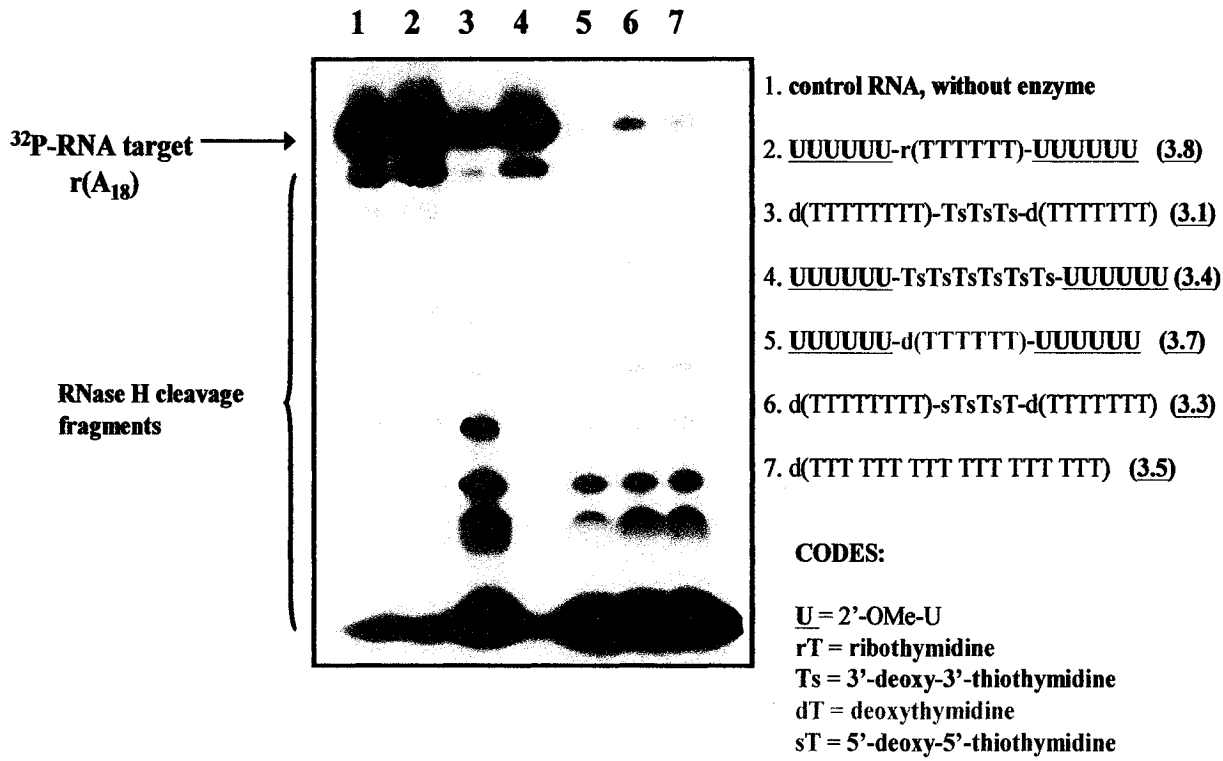
Investigations towards the use of 3'- and 5'-phosphorothiolate modified oligomers as antisense agents stems not only from our desire to develop an oligonucleotide with the desirable characteristics but also to better understand the substrate specificity of RNase H. Given the previous results indicating that the 3'S- and 5'S- residues respectively adopted RNA and DNA-like conformations, we expected these modifications to have constrasting behaviour with respect to RNase H activation. In fact, we predicted that the RNA character of the 3'S-modification would not elicit RNase H activity. Thus, the various modified oligomers were evaluated for their ability to stimulate RNase H activity by incubating them with ^{32}P labeled target RNA and RNase H. **Figure 3.14** shows the general assay used in testing RNase H cleavage. The reaction is initiated by the addition of *E. coli* RNase H. After a period of time the, an aliquot is removed and quenched by a buffer and heating to 100°C. The reaction products are resolved by gel electrophoresis and then visualized by autoradiography. The presence of a 'ladder' of cleavage products demonstrates that the RNA portion of the duplex has been degraded.

Figure 3.14: Schematic representation of the RNase H assay.



The effect of 3'-and 5'-S-phosphorothiolate modified linkages on the rate of RNase H cleavage was determined by gel analysis (**Figure 3.15**). We first examined the activity of AON containing three contiguous 3'- and 5'-S-phosphorothiolate internucleotide linkages (Fig. 3.15, lanes 3 and 6). This was done in conjuction with the natural DNA control (Fig. 3.15, lane 7). The results show that incorporation of the 3'-S-phosphorothiolate modified linkages attenuates the rate at which RNase H cleaves the AON/RNA hybrid (Fig. 3.15, lane 3 vs lane 7). By contrast, modified oligomers containing 5'-S-phosphorothiolate linkages have only a slight effect on the cleavage of the AON/RNA duplex (Fig. 3.15, lane 6 vs 7), consistent with the notion that the 5'-S modification has little impact on the conformation of the antisense strand. These results are consistent with the notion that the 3'- and 5'-S modification are RNA and DNA "mimics" respectively. The replacment of the 3'-oxygen atom in a phosphodiester linkage with a sulfur atom is expected to decrease the influence of the 3'-gauche effect shifting the conformational equilibrium of the sugar moiety in the single strand to adopt a C3'-*endo* conformation, making these analogs very good RNA mimics (**Figure 3.2**). However, in substituting the 5'-oxygen with a sulfur in the phosphodiester linkage is expected to have little effect on the conformational parameters of the deoxyribose ring and these analogs are considered DNA mimics. The close structural resemblance between the 5'-S modified and the unmodified hybrids is corroborated by their almost identical CD curves (**Figure 3.10**).

Figure 3.15: Electrophoretic gel analysis of *E. coli* RNase H1 mediated cleavage of ^{32}P-labeled target RNA duplexed with various AON.



The RNase H assay required 1 pmol of target 5'-[^{32}P]-rA_{18} and 3 pmol of test AON in 60 mM Tris HCl (pH 7.8) containing 60 mM KCl and 2.5 mM $MgCl_2$. Reactions were started by the addition of *E. coli* RNase H1 and were carried out at ca. 22°C. Bands were visualized by autoradiography.

Next, we turned our attention to the [2'-OMe-rU]$_6$-(xT)$_6$-[2'-OMe-rU]$_6$ "gapmers" and evaluated their ability to elicit RNase H activity. These studies were done in conjuction with a chimeric oligomer containing the corresponding deoxythymidine (dT)$_6$ and ribothymidine (rT)$_6$ 'gaps' which are used as controls. Studies have shown that if the gap is DNA, the size required to efficiently direct RNase H cleavage of target RNA is four to five residues, although more are required to confer significant biological activity both *in*

vitro and *in vivo*.[5,10,52] As expected neither chimeric oligonucleotides, [2'-OMe-rU]$_6$-(rT)$_6$-[2'-OMe-rU]$_6$ and [2'-OMe-rU]$_6$-(T$_{3'S}$)$_6$-[2'-OMe-rU]$_6$, served as substrates for RNase H whereas the chimeric oligonucleotide comprised of a central (dT)$_6$ segment induced efficient RNase H cleavage of target RNA (Fig. 3.15, lanes 2 and 4 vs lane 5). The inability of the (T$_{3'S}$)$_6$/RNA hybrid in activating RNase H further supports the notion that the (T$_{3'S}$)$_6$ segment adopt an RNA-like (C3'-*endo*) conformation.

3.6 CONCLUSION

The solid-phase synthesis of a series of modified oligomers containing a single and three consecutive 3'-and 5'-S-phosphorothiolate internucleotide linkages was accomplished using the phosphoramidite approach. In addition a chimeric oligonucleotide, composed of 2'-OMe-rU 'wings' with a central hexanucleotide segment containing the 3'-deoxy-3'-thiothymidine ($T_{3'S}$) modified residues was also successfully synthesized. Hybridization studies and biological investigations were conducted on these various AONs. UV thermal melting studies revealed that the incorporation of the modified 3'-S-phosphorothiolate linkages in oligonucleotides results in enhanced binding affinity towards single stranded RNA and DNA target. Conversely, the modified oligomers containing 5'-S-phosphorothiolate linkages exhibit lower thermodynamic stability when duplexed with the corresponding complementary DNA or RNA targets compared to the unmodified DNA/DNA and RNA/DNA hybrids. Moreover, the oligonucleotide chimera containing an internal $T_{3'S}$ gap, showed a dramatic increase in binding affinities for single stranded RNA and DNA targets. This unusually high thermal stability exhibited by the 3'-S modification prompted us to compare it to chimeric oligonucleotides comprised of 2'-modified ribonucleoside gaps such as, 2'OMe-rU, 2'F-rU and rT, which have previously been demonstrated to exhibit a pronounced increase in affinity towards RNA. The stabilizing effects of the 3'-S modification on chimeric AON/RNA duplexes were shown to be superior to those of the 2'-OMe-rU, 2'F-rU and rT substitutions. The relative order of stability of hybrid duplexes with RNA is as follows: 3'-S-modification > 2'F-rU > rT > 2'OMe-rU. The resultant duplex stabilization displayed by the 3'-S modification is likely due to a high population of its C3'-*endo* sugar pucker, which favors the formation of stable duplexes characteristic of RNA/RNA helices. Consistent with this notion, AON/RNA hybrids where the AON is 3'-S-DNA are not substrates for RNase H. In contrast, the duplex formed between RNA and the AON containing three consecutive 5'-S-phosphorothiolate linkages are geometrically similar to the native DNA/RNA substrates suggesting that the 5'-S nucleoside adopts an eastern/southern C2'-endo conformation. This predisposes the AON strand in the AON/RNA duplex to adopt a

geometry that closely mimic the A/B form of the native DNA/RNA substrates. Consequently, AON/RNA hybrids comprised of modified 5'-S-phosphorothiolate linkages serve as excellent substrates for *E. coli* RNase H, thereby, enabling degradation of target RNA.

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CHAPTER IV: EXPERIMENTAL MATERIALS AND METHODS

4.1 GENERAL EXPERIMENTAL

4.1.1 General Reagents

Dichloromethane (DCM), acetonitrile (CH_3CN), pyridine (Fisher) and dioxane (Fisher) were dried by refluxing over calcium hydride (CaH_2; Aldrich) for 24 h followed by distillation under inert atmosphere. The following solvents were distilled just prior to use and withdrawn from the septum port of a collection bulb via a dry syringe: Tetrahydrofuran (THF) was dried by continuous reflux over sodium metal and benzophenone under an inert atmosphere, distilled into a collection bulb and withdrawn from the septum port via a dry syringe. Triethylamine (Fisher) and N-methylimidazole (NMI; Aldrich) were dried by refluxing and distillation over CaH_2 and stored under inert atmosphere over activated molecular sieves. N,N-diisopropylethylamine (DIPEA; Aldich) was dried by mild heating and stirring over CaH_2 (50-60°C, 16h) followed by distillation under vacuum and stored over activated molecular sieves in septum-sealed bottles. Chloroform, acetone, ethanol, methanol, diethyl ether, nitromethane (CH_3NO_2; Aldrich) and anhydrous carbon tetrachloride (CCl_4; Aldrich) were used as obtained.

The following reagents were all purchased from Sigma-Aldrich Canada and used as received: [Bis(2-methoxyethyl)amino]sulfur trifluoride (MAST), hydrogen bromide in acetic acid (HBr, 30% (w/v)), sodium hydride (NaH), hexamethyldisilazane (HMDS), thymine, p-anisylchlorodiphenylmethane (MMTr-Cl, methanesulfonylchloride (MsCl), thioacetic acid, anhydrous zinc bromide ($ZnBr_2$) and trichloroacetic acid (TCA). The compound 1,3,5-Tri-*O*-benzoyl-α-D-ribofuranose was purchased from Pfanstiehl. Thymidine and β-Cyanoethyl-(N,N-diisopropylamino)phosphochloridite were purchased from ChemGenes Corp. (Ashland, MA). β-Cyanoethyl-(N,N-diisopropyl) phosphoramidous chloride was stored at -20°C and was warmed to room temperature in a dessicated environment before use. 4,5-Dicyanoimidazole (DCI, 99%; GLS Synthesis

Inc., Worcester, MA) and ethylthiotetrazole (ChemGenes Corp.) were dried over P_2O_5 for 24 h prior to use. Analytical reagent grade glacial acetic acid, ammonium hydroxide, ammonium acetate, sodium sulfate, sodium chloride and sodium hydroxide were obtained from Fisher.

4.1.2 Chromatography

Purification of compounds by flash column chromatography was carried out using Silicycle™ ultra-pure silica gel (230-400 mesh). Thin layer chromatography (TLC) was performed using Merck Kieselgel 60 F-254 aluminum-back analytical silica gel sheets. Compounds were visualized on TLC plate by illumination with a UV light source at 254 nm and/or by dipping the TLC plate in a sulfuric acid-based solution in order to visualize any trityl-bearing species.

4.1.3 Instrumentation

NMR Spectroscopy All NMR spectra were recorded using the JEOL 270 MHz and the 400 MHz and 500 MHz Varian Mercury spectrometers. For new compounds synthesized, all ^1H assignments were made using 2-D NMR experiment homonuclear correlated spectroscopy (COSY). Deuterated solvents used (acetone-D_6, chloroform-$CDCl_3$ and MeOH-CD_3OD) were purchased from Cambridge Isotope Laboratories.

UV Spectroscopy UV and visible absorbance spectra were collected on a Varian Cary 1 UV/Vis spectrophotometer (Varian: Mulgrave, Victoria, Austrilia). Thermal denaturation of oligonucleotides was followed in the ultraviolet spectrum using a CARY spectrophotometer equipped with a thermostated cell holder with data collection by software supplied by the manufacturer (Cary Win UV, version 2.00). Unless otherwise noted, all spectra were obtained in water or aqueous buffers.

FAB-Mass Spectrometry Mass spectrometric analysis was conducted on a Kratos MS25RFA high resolution mass spectrometer using a *p*-nitrobenzyl alcohol (NBA) matrix.

4.2 GENERAL AUTOMATED SOLID-PHASE OLIGONUCLEOTIDE SYNTHESIS

4.2.1 General Reagents

Reagents for the solid-phase synthesis of oligonucleotides were prepared from high quality synthesis grade materials. All solvents (*e.g.* acetonitrile, THF) were dried under inert atmosphere as previously described, distilled immediately prior to use and withdrawn from a septum-sealed collection bulb with a dry glass syringe and stainless steel needle. The phosphoramidite activation reagent consisted of 1.0 M 4,5-dicyanoimidazole (DCI) in anhydrous acetonitrile. Removal of the 3' or 5'-dimethoxytrityl or monomethoxytrityl groups was carried out with a solution of 3% (w/v) trichloroacetic acid (TCA) in dichloromethane (DCM). The reagents for the acetylation or "capping" of any unreacted hydroxyl groups bound to the solid support were prepared as follows: Cap A; 10% (v/v) acetic anhydride and 10% (v/v) dry 2,4,6-collidine in anhydrous THF, Cap B; 16% (v/v) dry NMI in anhydrous THF. Intermediate phosphate trimesters were oxidizedto the more stable phosphotriesters using a solution of 0.05 M iodine (1.23 g. of iodine (Aldrich) in THF/pyridine/water (75:20:2, v/v/v)).

All standard 5'-O-dimethoxytrityl-3'-O-(2-cyanoethyl)N,N-diisopropylphosphoramidite of the various 2'-deoxy and ribonucleosides as well as the "inverted" 2'-deoxy nucleoside, 3'-O-dimethoxytrityl-5'-O-(2-cyanoethyl)N,N-diisopropylphosphoramidites, were purchased from ChemGenes Corp. (Ashland, MA). These phosphoramidite reagents were stored at -20°C and dried over *in vacuo* overnight over P_2O_5 prior to use.

Long chain alkylamine controlled-pore glass (LCAA-CPG; 500 Å pore size) derivatized with the appropriate linker arm and the 3'- or 5'- anchored nucleosides were purchased from ChemGenes Corp. (Ashland, MA).

In addition, several oligonucleotide chimeras used in this study, namely, the 2'-OCH_3-RNA containing the corresponding $(2'\text{-}OCH_3)_6$, $(2'F)_6$ and $(rT)_6$ gaps, were obtained from the University of Calgary DNA Synthesis Laboratory (Calgary, AB, Canada).

4.2.2 Solid-Phase Synthesis of Oligonucleotides

a. Chain Assembly

Syntheses were carried out on an Applied Biosystems (ABI) 381A synthesizer using the standard β-cyanoethylphosphoramidite chemistry. Oligomers were prepared on a 1μmol scale using LCAA-controlled pore glass (500 Å) derivatized with 3'-or 5'- anchored nucleosides. Modified oligomers containing 3'-S-phosphorothiolate linkages were prepared by the more commonly used 3'-5' direction of synthesis using CPG supports containing 3'-anchored nucleosides; whereas, the synthesis of oligomers containing the modified 5'-S-phosphorothiolate internucleotide linkage proceeds in the less commonly used 5'-3'-direction and makes use of CPG with 5'-anchored nucleosides (thymidine). The standard cycle was revised to include the following: (1) Detritylation: solution of 3% TCA in DCM was continuously delivered for 140 seconds to the column for the removal of DMT-containing groups and 220 seconds for MMTr-containing groups. Monitoring of successive coupling efficiencies was conducted by measuring the absorbance of the trityl cation released during this step. The eluate was collected and the absorbance was measured by UV spectroscopic quantitation of trityl for $DMTr^+$ (λ=504 nm, deoxy and ribo) and $MMTr^+$ (λ=478 nm, modified oligomers), to determine coupling yields.

(2) Phosphoramidite Coupling: the coupling time or "wait" step was extended to 120 seconds for the 2'-deoxyribonucleoside phosphoramidites (dT, dA), 20 min for the modified nucleosides (3'-deoxy-3'-S-phosphorthioamidite ($dT_{3'S}$) and 5'-deoxy-5'-S-phosphorthioamidite ($^{5'S}dT$)) and 450 seconds for the ribonucleoside phosphoramidite (rA) (3). Capping: acetylation of the unreacted 5'-hydroxyl groups was accomplished by a 17 second delivery of Cap A and Cap B reagent followed by a 45 second "wait" step and repeated, (4). Oxidation: oxidant solution consisting of 0.05 M iodine solution in

THF/pyridine/H_2O was delivered to the column for 20 seconds followed by a 20 second "wait" step.

Prior to oligonucleotide assembly, the nucleoside-derivatized solid support (1 μmol CPG), was treated with a mixture of Cap A and Cap B reagents according to the pre-installed "capping" cycle provided by ABI. This step ensures that any undesired hydroxyl or amino groups on the CPG surface are masked by acetylation[1,2], and also eliminates any trace moisture at the beginning of the synthesis. Phosphoramidite reagents were dissolved in dry, freshly distilled acetonitrile, which was introduced via a dry syringe through the septum of a sealed glass bottle containing the appropriate monomer. The final concentrations of the monomers were 0.10 M for 2'-deoxyribonucleoside phosphoramidites and 0.15 M for the ribonucleoside phosphoramidites and the modified 3'-5'-S-phosphorothioamidites. The coupling efficiencies were monitored by the trityl assay.

b. Deprotection of Oligomers

Sterilized water was prepared by treating double distilled water (Millipore; Billerica, MA) with 0.1% (v/v) diethyl pyrocarbonate (DEPC; Aldrich) in a glass bottle and stirring it at room temperature for at least 2 h thus ensuring that any RNases were inactivated by covalent alkylation. Subsequently, it was autoclaved in an electric steam sterilizer at *ca.* 121°C (250 F, 15 PSI) for 1 h to eliminate residual DEPC. All plasticware, glassware and pipet tips were sterilized by autoclaving for 1 h at 121°C in the presence of DEPC. Following deprotection, gloves were worn at all times when handling the oligonucleotides in order to minimize contamination and degradation from nucleases present on the skin. Aqueous ammonia (29%), absolute ethanol, 1-butanol and triethylamine trihydrofluoride (TREAT-HF) were used as received.

CPG-bound oligonucleotides were transferred to a 1.5-mL microtube and suspended in 3:1 (v/v) aqueous ammonia /absolute ethanol and gently shaken at r.t. for 24 h to cleave the oligonucleotide from the support and deblock any phosphate and base protecting

groups. After centrifugation, the supernatant was collected, the CPG was washed with ethanol (3 x 0.5 mL) and the fractions dried in a Speed-Vac® concentrator under vacuum. Given that RNA oligomers bear an extra 2'-TBDMS protecting group, the resulting pellet was consequently treated with the desilylating reagent, TREAT-HF (5μL/crude A_{260} unit) for 48 h at r.t..[3] The solution was then either quenched with 1mL of sterile water and dried, or precipitated directly from the desilylation reaction by adding 25 μL of 3M sodium acetate (pH 5.5) followed by 1 mL of cold 1-butanol.[4] The precipitated material was then centrifuged at maximum speed for 10 min and the RNA pellet was washed with 70% ethanol and dried.

4.3 ANALYSIS AND PURIFICATION OF SYNTHETIC OLIGONUCLEOTIDES

4.3.1 Purification by Polyacrylamide Gel Electrophoresis (PAGE)

Fully deprotected oligonucleotides were analyzed and purified by denaturing PAGE using a vertical slab electrophoresis unit (Hoefer Scientific SE600; Fisher Scientific). Electrophoresis grade acrylamide, *N,N'*-methylene-bisacrylamide (BIS), ammonium persulfate (APS), *N,N,N',N'*-tetramethylethylenediamine (TEMED) and urea were obtained from Amersham. Bromophenol blue (BPB), xylene cyanol (XC), 2-Amino-2-(hydroxymethyl)-1,3-propanediol (Tris), boric acid and ethylenediamine tetraacetate dehydrate (EDTA) were purchased from Bio-Rad (Mississauga, ON).

Typically, analytical (0.75 mm) and preparative (1.5 mm) PAGE were run on 12-24% (w/v) acrylamide gels containing 1xTBE running buffer (89 mM Tris/boric acid, 2.5 mM EDTA, pH 8.3). Deionized formamide (Sigma) was prepared by stirring over mixed bed ion-exchange resin (Bio-Rad AG 501-X8) for 30 minutes and filtered. Sample loading buffer consisted of 8:2 (v/v) deionized formamide: 10xTBE. Running dye markers contained 2% (w/v) of both XC and BPB dissolved in sample loading buffer. All aqueous electrophoresis solutions were prepared using double distilled water (Millipore).

Gel solutions (30 mL) were degassed by sonication (10 min) and polymerization was initiated by the addition of 10% (w/v H_2O) ammonium persulfate (APS; 200 μL) and TEMED (25 μL). Crude samples were dissolved in 15 μL or 50-100 μL of loading buffer for analytical and preparative gels respectively, and heated at 90°C for 5 min. Gels were run at 500 V for 30 min followed by 800 V until the faster moving BPB dye was 2 inches from the bottom of the gel (*ca.* 2.5 h). Following electrophoresis, the gel was wrapped in plastic film (*e.g.* Saran Wrap), placed over a fluorescent TLC plate and the resolved oligonucleotide bands were illuminated using a handheld UV lamp. The UV-illuminated gel images were digitally captured on a Kodak DC3800 camera.

For preparative gels, the desired oligomer band was excised from the gel with a scalpel blade, placed in a sterile culture tube, crushed to fine particles and soaked in autoclaved water (5 mL) for 24-48 h.[5] The slurry was then centrifuged to settle much of the gel debris and the supernatant was then dried. Subsequently, the purified oligomers were water-extracted from the crushed gel pieces and "desalted" by size-exclusion chromatography on Sephadex G-25® as discussed below.

4.3.2 Purification of Synthetic Oligonucleotides by Anion–Exchange HPLC

All modified oligonucleotides were analyzed and purified from the crude mixture by anion-exchange HPLC. HPLC grade lithium perchlorate ($LiClO_4$) was purchased from Aldrich. Solutions of $LiClO_4$ for HPLC analysis and purification were filtered through a 0.45 μM nylon membrane and degassed under vacuum prior to use.

Analyses and preparatory injections were conducted on a Waters Breeze® system consisting of a 1525 binary pump, a 2487 dual absorbance detector, a Rheodyne® manual injector, an internal column heater and a Waters Protein-Pak™ DEAE 5PW (7.5 mm x 75 mm Waters) column. The anion-exchange HPLC is prepared by setting the detector wavelength to 260 nm, heating the column to 50-60°C in order to denature any intramolecular or intermolecular interactions between self-complementary oligomers and finally, equilibrating with buffer A (deionized water) using a flow rate of 1 mL/min.

A linear gradient of deionized water (Buffer A) and 1M $LiClO_4$ (Buffer B) was used over a specified time: 0% to 20% buffer B (1M $LiClO_4$) in 70 min to elute the modified oligomer and then the column was washed and re-equilibrated.

Analytical injections typically contain 0.1-0.5 A_{260} units of oligonucleotide dissolved in 25-50 μL of water, whereas preparatory injections had 50-100 A_{260} units dissolved in 100-200 μL of water. Sample loading to the column depends on the quality of the crude mixture as well as the type of oligomer. Higher loadings were shown to overload the column and compromise separation of the desired oligomer from failure sequences present in the crude mixture. In the case of preparatory runs (*e.g.* purification), which usually consists of higher loadings, the absorbance was monitored at higher wavelengths (290 nm) to avoid saturation of the detector signal. Following a preparatory run, fractions corresponding to the peak of interest were collected in sterile culture tube. The retention time was similar to that obtained during routine HPLC analysis of the same oligomer. The fractions were dried down and then desalted by size exclusion chromatography on Sephadex G-25® as discussed below.

4.3.3 Desalting of Oligonucleotides

The desired oligonucleotides were separated from any low molecular weight organic species and water soluble salts by size exclusion chromatography (SEC), with Sephadex G-25®, a highly crosslinked oligosaccharide (*e.g.* dextran) matrix. Macromolecules, such as oligonucleotides, are completely excluded from the bead pores, whereas smaller molecules (*e.g.* salts) penetrate the beads to varying extents depending on the size of the molecule. Sephadex G-25® was purchased in its dry form from Amersham, and swollen in water for 2 h prior to autoclaving in the presence of DEPC (0.1% v/v) at 121°C for 1hour. The sephadex (10 mL) was poured into a (10 mL) sterile plastic syringe plugged with silanized glass wool and washed with 3 column volumes of DEPC water. The "salted" sample, dissolved in 1 mL of water, was loaded onto the column and eluted with water (10 mL). The eluted oligomer was collected in 1 mL fractions and the amount of oligomer in each was quantitated by UV spectroscopy at 260 nm. Typically the pure,

desalted oligonucleotide are collected in fractions # 4-7 (UV measurement). The fractions containing the oligomer were then pooled, dried, redissolved in 1 mL of sterile water and this stock solution was stored at -20°C.

4.3.4 Characterization of Oligonucleotides by MALDI-TOF Mass Spectrometry[6,7]

The purified and desalted oligomers were analyzed by MALDI-TOF-MS (matrix assisted laser desorption ionization time-of-flight mass spectrometry) on a Kratos Kompact-III instrument (Kratos Analytical Inc., New York) in either negative reflector mode (< 30 nucleotides) or linear mode (> 30 nucleotides). Samples must be desalted prior to analysis in order to minimize the adduction of cations, which shift the ion signal to higher *m/z* values, thus interfering with accurate determination of molecular weights. When a sample is ionized, normally singly charged ions of oligonucleotides are observed, which makes a MALDI spectra easy to interpret. Matrices and co-matrices such as: 6-aza-2-thiothymine (ATT), spermine and fucose were purchased from Sigma-Aldrich. The oligonucleotides and matrices were diluted with ultrapure deionized water and HPLC grade solvents. Purified samples (1μL, 0.2-1 mmol/μL in water) were mixed with 1μL of saturated ATT/spermine (80 mg/mL ATT dissolved in 1:1 water/CH_3CN containing 25 mM spermine) and 1μL fucose (50 mM in water). The analyte/matrix mixture (1μL) was spotted in duplicate on a sample plate and allowed to dry under a warm stream of air. The crystalline spots were analyzed and the spectra processed using software supplied by Kratos.

4.4 BIOPHYSICAL CHARACTERIZATION OF OLIGONUCLEOTIDES

4.4.1 Hybridization Studies: UV-Thermal Denaturation Studies

The hybridization characteristics of oligonucleotides were investigated by monitoring the change in UV-absorbance(λ=260nm) with increasing temperature, by generating UV denaturing profiles ("melting curves"). These studies were conducted using a Varian CARY 1 UV-Vis spectrophotometer (Varian; Mulgrave, Australia) equipped with a multiple cell holder, a Peltier thermal cell holder and a temperature controller. Spectra

were processed using Cary Win UV software (version 2.00). The hybridization buffer consisted of : 140 mM KCl, 1 mM $MgCl_2$, 5mM Na_2HPO_4, pH 7.2 and was stored at -20°C. Oligonucleotide extinction coefficients (ε_{260}) were calculated by applying the nearest-neighbor approximation of Puglisi and Tinoco[8] using an internet-based biopolymer calculator. Molar extinction coefficients for the modified oligomers were assumed to be the same as those of normal DNA strands. Moreover, the extinction coefficient of the oligonucleotide chimeras, consisting of two 2'OMe-rU_6 "wings" flanking a hexathymidylate "gap", namely, dT_6 and $(dT_{3'S})_6$, were assumed to be similar to the sum of the individual components.

Samples for thermal denaturation analysis were prepared by lyophilizing an equimolar mixture of complementary strands to dryness and then re-dissolving in 1 mL of buffer. Oligonucleotide mixtures were heated to 90°C for 10-15 min in order to dissociate any non-specifically bound regions, cooled slowly to room temperature for 2-3 h and then left at 4 °C overnight. The annealed samples were transferred to Hellma QS-1.000 (Cat # 114) quartz cells, sealed with a Teflon-wrapped stopper and degassed by sonication for 15 seconds. The complexed oligonucleotides were equilibrated to 5 °C in the cell holder of the spectrophotometer for 5 min prior to spectral acquisition. The absorbance at 260 nm was measured at 0.5 °C intervals at a temperature rate of 0.5 °C/minute. The thermal melting temperature (T*m*) values were calculated as the maximum of the first derivative plots of the absorbance *versus* temperature profiles and coincides with the point at which half of the complexed oligonucleotides are in their single-stranded state. Spectra were acquired in duplicate and the calculated T*m*'s were consistent within 0.5-1 °C of each other. The data obtained was transferred to spreadsheet software (Microsoft® Excel 97) for subsequent analysis and presentations. Comparative hyperchromicity values (*e.g.* changes in relative absorbance) were obtained by using the formula: $H=(A_T-A_O)/A_f$, where H is the hyperchromicity, A_T is the absorbance at any given temperature (T), A_O is the initial absorbance reading and A_f is the absorbance at the highest temperature.[8] This facilitates comparison on a more uniform basis, for parallel runs, especially at identical concentrations and in the same buffer. Hyperchromicity values (H%) are reported as the

percent increase in absorbance at the wavelength of interest with respect to the final absorbance and the T*m*s were calculated using the base-line method in accordance with that of Puglisi and Tinoco.[8]

4.4.2 Circular Dichroism Spectroscopy (CD)

CD spectra were collected on a JASCO J-710 spectropolarimeter. Samples were contained in a Hellma QS-1.000 (Cat# 114) fused quartz cell, which was cooled by an external circulating bath (VWR Scientific) at constant temperature (5 °C). Oligonucleotide solutions for CD measurements were prepared with the same buffer which was used for the UV melting. Before data acquisition, samples were allowed to equilibrate for 5-10 min in the instrument's cell holder to the appropriate sampling temperature. The spectra were representative of 3 individual scans recorded between 220-340 nm at 5 °C. Individual scans were at a rate of 50 nm/min using a sampling wavelength of 0.2 nm and a bandwidth of 1 nm. The acquired data was processed using the J-700 Windows software (Version 1.00) supplied by the manufacturer (Jasco Inc.). The CD spectra were background subtracted (i.e. buffer subtraction), smoothed and were corrected for concentration such that the molar ellipticity could be determined. The corrected data was exported to Microsoft® Excel spreadsheet software for further analysis and presentation.

4.5 BIOLOGICAL STUDIES

4.5.1 Induction of Ribonuclease H (RNase H) Activity by Oligonucleotides

RNase assays were carried out at room temperature (≈ 22°C). The ability of homopolymeric oligonucleotides to elicit RNase H degradation of target RNA was determined by assays (10 μL final volume) that comprised of 1 pmol of 5'-[^{32}P]-labeled complementary target RNA and 3 pmol of test oligonucleotide in 10 μL of 60 mM Tris-HCl (pH 7.8) containing 60 mM KCl and 2.5 mM $MgCl_2$, followed by heating at 90°C for 2 min and slow cooling to room temperature. Duplex substrate solutions were allowed to stand at room temperature for at least 1 h prior to use. Reactions were initiated by the

addition of *E. coli* RNase H and aliquots were removed at various times and quenched by the addition of loading buffer (98% deionized formamide containing 10 mM EDTA, 1 mg/mL bromophenol blue and 1mg/mL xylene cyanol). After heating at 100°C for 5 min, reaction products were resolved by electrophoresis on 16 % polyacrylamide sequencing gels containing 7 M urea and visualized by autoradiography. These experiments were conducted by Dr. Kyung-Lyum Min, a member of our group.

4.6 MONOMER PREPARATION

4.6.1 Synthesis of 3'-Deoxy-3'-thiothymidine [2.5] and its 3'-S-Phosphorothioamidite derivative [2.6]

5'-O-*p*-Monomethoxytritylthymidine [2.1]

Thymidine (8.58 g, 35.4 mmol) was co-evaporated with dry pyridine (3 x 30 mL) and then suspended in pyridine (350 mL). Then MMTr-Cl (16.4 g, 53.1 mmol) was added and the mixture was allowed to stir overnight. The mixture was then diluted with 200 mL of dichloromethane and the organic layer was washed with 200 mL of brine. The organic layer was then dried over $NaSO_4$, filtered and evaporated. The crude was purified on a silica gel column using chloroform followed by chloroform/methanol (99:1) to elute the product **[2.1]**. The product (21 g, 98%) was a colorless foam. $R_f(SiO_2)$: 0.22 (chloroform : methanol- 20:1)- ^{1}H-NMR (acetone-d_6, 270 MHz): δ = 9.97(s, 1H, N-H), 8.02(s, 1H, 6H), 7.48-6.90(m, 14H, trityl-H), 6.39-6.34(m, 1H, 1'-H), 4.61-4.57(m, 1H, 4'-H), 4.06-4.02(m, 1H, 3'-H), 3.79(s, 3H, OCH_3), 3.41-3.32(m, 2 H, 5'-H_a, 5'-H_b), 2.85 (s, 1H, 3'-OH), 2.44-2.26(m, 2H, 2'-H_a, 2'-H_b), 1.46(s, 3H, 5-CH_3). ESI-MS (positive); *m/z* : 537.2 $[M+Na]^+$.

3'-O-Methanesulphonyl-3'-deoxy-5'-(monomethoxytrityl)thymidine [2.2] and 2,3'-Anhydro-3'-deoxy-5'-(monomethoxytrityl)thymidine [2.3][9]

Methanesulfonyl chloride (4.02 mL, 51.9 mmol) was added dropwise over a period of 15 min to a cooled (ice-water-bath) solution of **[2.1]** (8.9 g, 17.3 mmol) in pyridine

(150 mL). The cooled reactants were then stirred at 0°C for 3 h and subsequently, stirred overnight at room temperature. The organic layer was then washed with $NaHCO_3$ (250 mL) and the product was extracted with dichloromethane (3 x 100 mL). The organic layer was then dried over $NaSO_4$, filtered and evaporated to yield the product **[2.2]** (10 g, 97%) as a brown foam. R_f (SiO_2): 0.74 (chloroform : methanol- 20:1)- ^{1}H-NMR (acetone-d_6, 270 MHz): δ = 10.07(s, 1H, N-H), 8.565(d, 1H, 6-H, *J*=2.7 Hz), 7.52-6.89(m, 14H, trityl-H), 6.36(t, 1H, 1'-H), 5.53-5.50(m, 1H, 4'-H), 4.35-4.31(m, 1H, 3'-H), 3.86(s, 3H, OCH_3), 3.53-3.43(m, 2 H, 5'-H_a, 5'-H_b), 3.18(s, 3H, SO_2CH_3), 2.70-2.64(m, 1H, 2'-H_a), 2.06-2.00(m, 1H, 2'-H_b), 1.46(s, 3H, 5-CH_3). ESI-MS (positive); *m/z* : 615.0 $[M+Na]^+$.

The above material (10 g), triethylamine (158 mL) and ethanol (420 mL) were heated together, under reflux, for 18 h. Upon completion, the mixture was cooled, washed with H_2O (200 mL) and the product was extracted with dichloromethane (2x150 mL). The organic layer was then dried over $NaSO_4$, filtered and evaporated to dryness to yield the product **[2.3]** (7.66 g, 87%) an off white foam. R_f (SiO_2): 0.12 (chloroform : methanol-20:1)- ESI-MS (positive); *m/z* : 519.2 $[M+Na]^+$.

3'-Acetylthio-3'-deoxy-5'-(monomethoxytrityl)thymidine [2.4][10]

Compound **[2.3]** (7.66 g, 15.4 mmol), in its crude form, was co-evaporated twice with dry pyridine (2 x 50 mL) and once with dry dioxane (1 x 50 mL) prior to use. The procedure according to Elzagheid *et al.* was then used to obtain the desired compound **[2.4]**; (8.43 g, 95%) as a pale yellow syrup. R_f (SiO_2): 0.45 (chloroform : methanol- 20:1)- ^{1}H-NMR ($CDCl_3$, 400 MHz): δ = 8.48(s, 1H, N-H), 7.68(s, 1H, 6-H), 7.44-7.25(m, 12H, trityl-H), 6.85(d, 2H, *J* = 9.0 Hz, *O*-anisyl H), 6.28(t, 1H, 1'-H), 4.65(m, 1H, 4'-H), 4.19(m, 1H, 3'-H), 3.79(s, 3H, OCH_3), 3.70(s, 3H, SCOCH_3), 3.50(m, 2H, 5'-H_a, 5'-H_b), 2.78(m, 1H, 2'-H_a), 2.45(m, 1H, 2'-H_b, 1.49(s, 3H, 5-CH_3). ESI-MS (positive); *m/z* : 595.2 $[M+Na]^+$, 611.1 $[M+K]^+$.

3'-Deoxy-3'-thio-5'-(monomethoxytrityl)thymidine [2.5][11]

Treatment of the above compound **[2.4]** (0.89 mmol) in argon saturated ethanol (89 mL) at 5 °C with 10 M NaOH (2.6 mL) gave complete deacetylation after 1 h. Upon completion, the reaction mixture was neutralized by slow addition of a 1M HCl solution until pH~6. The reaction was worked up by evaporating the solvent in vacuo and the crude product was purified on a silica gel column using a gradient of chloroform followed by chloroform/methanol (99:1) to elute the product. Appropriate fractions were combined and concentrated to dryness to afford **[2.5]** (0.33 g, 70%) as a pale yellow foam. R_f (SiO_2): 0.42 (chloroform : methanol- 20:1)- ^{1}H-NMR (acetone-d_6, 270 MHz): δ = 10.1(s, 1 H, N-H), 7.98(s, 1 H, 6-H), 7.67-7.24(m, 14H, trityl-H), 6.18(dd, 1H, 1'-H, $J_{1'\text{-}2'Ha}$ = 7.4 Hz, $J_{1'\text{-}2'Hb}$ = 3.2 Hz), 3.96-3.90(m, 2H, 3'-H, 4'-H), 3.77(s, 3H, OCH_3), 3.52(dd, 1H, 5'-H_a, $J_{5'\text{-}5''}$ = 10.8 Hz, $J_{5'\text{-}4'}$ = 2.5 Hz), 3.44(dd, 1H, 5'-H_b, $J_{5'\text{-}5''}$ = 10.8 Hz, $J_{5'\text{-}4'}$ = 3.9 Hz), 2.88(s, 1H, 3'-SH), 2.73-2.64(m, 1H, 2'-H_a), 2.46-2.34(m, 1H, 2'-H_b), 1.55(s, 3H, 5-CH_3). ESI-MS (positive); *m/z* : 553.2 $[M+Na]^+$.

The corresponding **Thymidine 3'-S-S-3'-Thymidine disulfide,** was also isolated from the column in about 10% yield. R_f (SiO_2): 0.26 (chloroform : methanol- 20:1)- ^{1}H-NMR (acetone-d_6, 500 MHz): δ = 10.0(s, 1H, N-H), 8.01(s, 1H, 6-H), 7.65-6.87(m, 14H, trityl-H), 6.19-6.17(m, 1H, 1'-H), 4.08-4.06(m, 1H, 4'-H), 3.89-3.84(m, 1H, 3'-H), 3.76(s, 3H, OCH_3), 3.50(dd, 1H, 5'-H_a, $J_{5'\text{-}5''}$ = 11 Hz, $J_{5'\text{-}4'}$ = 2.5 Hz), 3.34(dd, 1H, 5'-H_b, $J_{5'\text{-}5''}$ = 10.8 Hz, $J_{5'\text{-}4'}$ = 4.0 Hz), 2.62-2.56(m, 1H, 2'-H_a), 2.53-2.47(m, 1H, 2'-H_b), 1.50(s, 3H, 5-CH_3). ESI-MS (positive); *m/z* : 1081.2 $[M+Na]^+$.

5'-O-Monomethoxytritylthymidine-3'-S-phosphorothioamidite [2.6][12]

To 3'-deoxy-3'-thio-5'-(monomethoxytrityl)thymidine **[2.5]** (260 mg, 0.491mmol), was added dry TFH (10 mL), N,N-diisopropylethylamine (430 µL, 2.45 mmol), 1-methylimidazole (20 µL, 0.294 mmol) and β-cyanoethyl-N,N-di-isopropylchlorophosphoramidite (160 µL, 0.736 mmol). After 15 min the reaction mixture turned cloudy. The reaction was allowed to proceed at r.t. overnight after which

it was quenched with methanol (5 mL) and stirred for an additional 5 min. The reaction was worked up by evaporating the solvent in vacuo. The crude product was purified via flash column chromatography using triethylamine/chloroform (5:95) to yield **[2.6]** (340 mg, 95%) as a colorless foam. R_f (SiO_2): 0.53 (chloroform : methanol- 20:1)- ^{31}P-NMR (109.4 MHz, ppm, $CDCl_3$): 165.1, 160.5; ESI-MS (positive); *m/z* : 753.2 $[M+Na]^+$.

4.6.2 Synthesis of 5'-Deoxy-5'-thiothymidine [2.11] and its 5'-S-Phosphorothioamidite derivative [2.12]

3',5'-Bis-O-*p*-Monomethoxytritylthymidine [2.7]

Thymidine (10 g, 41.3 mmol) was co-evaporated with dry pyridine (3 x 50 mL) and then suspended in pyridine (450 mL). Then MMTr-Cl (31.9 g, 103 mmol) was added and the mixture was allowed to stir overnight at 60 °C. The mixture was then diluted with 250 mL of dichloromethane and the organic layer was washed with 300 mL of saturated aqueous sodium bicarbonate. It was then dried over $NaSO_4$, filtered and evaporated. The crude was purified by flash column chromatography using chloroform to afford the product **[2.7]** (27.5 g, 85%) as a pale yellow foam. R_f(SiO_2): 0.78 (chloroform : methanol- 20:1)- ^{1}H-NMR (acetone-d_6, 270 MHz): δ =9.97(s, 1H, N-H), 8.02(s, 1H, 6-H), 7.32-6.82(m, 28H, trityl-H), 6.38-6.34(m, 1H, 1'-H), 4.47-4.45(m, 1H, 4'-H), 3.97(m, 1H, 3'-H), 3.80(s, 3H, (OCH_3)$_a$), 3.77(s, 3H, (OCH_3)$_b$), 3.17(dd, 1H, 5'-H_a, $J_{5'-5''}$ = 10.5 Hz, $J_{5'-4'}$ = 2.5 Hz), 3.01(dd, 1H, 5'-H_b, $J_{5'-5''}$ = 10.4 Hz, $J_{5'-4'}$ = 3.2 Hz), 2.01-1.94(m, 1H, 2'-H_a), 1.87-1.85(m, 1H, 2'-H_b), 1.39(s, 3H, 5-CH_3).

3'-O-*p*-Monomethoxytritylthymidine [2.8][13]

3',5'-Bis-O-*p*-Monomethoxytritylthymidine **[2.7]** (7.24 g, 9.20 mmol) was co-evaporated with toluene (3 x 30 mL) and then dissolved in CH_3NO_2 (20 mL). The mixture was cooled to 0 °C and added to a stirred slurry of $ZnBr_2$ (10 g) in CH_3NO_2 (73 mL) at 0 °C. After 45 minutes, the reaction was quenched with 1 M ammonium acetate (300 mL). Dichloromethane (200 mL) was added to the organic phase, was washed with water and saturated sodium chloride, dried over sodium sulfate and evaporated to a gum *in vacuo*.

The crude was purified on a silica gel column using a gradient of chloroform to chloroform/methanol (98:2) to elute the desired compound **[2.8]** as a colorless foam (3.78 g, 80%). R_f (SiO_2): 0.25 (chloroform : methanol- 20:1)- ^{1}H-NMR (acetone-d_6, 500 MHz): δ = 9.91(s, 1H, N-H), 8.02(s, 1H, 6H), 7.52-6.91(m, 14H, trityl-H), 6.34(dd, 1H, 1'-H, $J_{1',2'Ha}$= 4.86 Hz, $J_{1',2'Hb}$= 2.97 Hz), 4.43(m, 1H, 4'-H), 3.89(m, 1H, 3'-H), 3.78(s, 3H, OCH_3), 3.54-3.52(m, 1 H, 5'-H_a), 3.36-3.34 (m, 1H, 5'-H_b), 2.84 (s, 1H, 5'-*OH*), 1.87-1.83 (m, 2H, 2'-H_a, 2'-H_b), 1.74(s, 3H, 5-CH_3); ESI-MS (positive); *m/z* : 537.1 $[M+Na]^+$.

5'-O-Methanesulphonyl-5'-deoxy-3'-(monomethoxytrityl)thymidine [2.9][9]

Methanesulfonyl chloride (0.93 mL, 12.0 mmol) was added dropwise over a period of 15 min to a cooled (ice-water-bath) solution of **[2.8]** (2.05 g, 4.00 mmol) in pyridine (100 mL). The cooled reactants were then stirred at 0 °C for 3 h and subsequently, stirred overnight at room temperature. The organic layer was then washed with $NaHCO_3$ (200 mL) and the product was extracted with dichloromethane (3 x 100 mL). The organic layer was then dried over $NaSO_4$, filtered and evaporated to yield the desired product **[2.9]** (2.31g, 98%) as a dark brown gum. R_f (SiO_2): 0.74 (chloroform : methanol- 20:1); ESI-MS (positive); *m/z* : 615.0 $[M+Na]^+$.

5'-Acetylthio-5'-deoxy-3'-(monomethoxytrityl)thymidine [2.10][10]

Compound **[2.9]** (2.96g, 5.0mmol) was co-evaporated twice with dry pyridine (2 x 25 mL) and once with dry dioxane (1 x 25 mL) prior to use. The procedure according to Elzagheid *et al.* was then used to obtain the desired compound **[2.10]** (2.43 g, 85%) as a pale yellow syrup. R_f (SiO_2): 0.60 (chloroform : methanol- 20:1)- ^{1}H-NMR (acetone-d_6, 270 MHz): δ = 9.97(s, 1H, N-H), 7.65-7.35(m, 14H, trityl-H), 6.28-6.24(m, 1H, 1'-H), 4.36-4.21(m, 1H, 4'-H), 4.05-4.02(m, 1H, 3'-H), 3.79(s, 3H, OCH_3), 3.58-3.52(m, 1H, 5'-H_a), 2.95-2.85(m, 1H, 5'-H_b), 2.80(s, 3H, SCOCH_3), 1.83-1.77(m, 2H, 2'-H), 1.76(s, 3H, 5-CH_3). ESI-MS (positive); *m/z* : 595.2 $[M+Na]^+$.

5'-Deoxy-5'-thio-3'-(monomethoxytrityl)thymidine [2.11][11]

Treatment of the above compound **[2.10]** (1.78 mmol) in argon saturated ethanol (178 mL) at 5°C with 10 M NaOH (5.2 mL) gave complete deacetylation after 1 h. Upon completion, the reaction mixture was neutralized with slow addition of 1M HCl solution until pH~6. The reaction was worked up by evaporating the solvent in vacuo and the crude product was purified on a silica gel column using a gradient of chloroform followed by chloroform/methanol (99:1) to elute the product. Appropriate fractions were combined and concentrated to dryness to afford **[2.11]** (0.74 g, 80%) as a pale yellow foam. R_f (SiO_2): 0.43 (chloroform : methanol- 20:1)- ^{1}H-NMR (acetone-d_6, 270 MHz): δ = 9.98(s, 1 H, N-H), 8.02(s, 1 H, 6-H), 7.51-6.91(m, 14H, trityl-H), 6.30(dd, 1H, 1'-H, $J_{1'\text{-}2'Ha}$ = 8.6 Hz, $J_{1'\text{-}2'Hb}$ = 6.2 Hz), 4.29-4.26(m, 1H, 4'-H), 4.01-3.96(m, 1H, 3'-H), 3.79(s, 3H, O*CH*$_3$), 2.85(s, 1H, 3'-*SH*), 2.51-2.43(m, 2H, 5'-H_a, 5'-H_b), 1.89-1.83(m, 2H, 2'-H_a, 2'-H_b), 1.77(s, 3H, 5-*CH*$_3$). ESI-MS (positive); *m/z* : 553.2 $[M+Na]^+$.

3'-O-Monomethoxytritylthymidine-5'-S-phosphorothioamidite [2.12][12]

To 5'-deoxy-5'-thio-3'-(monomethoxytrityl)thymidine **[2.11]** (300 mg, 0.566 mmol), was added dry TFH (10 mL), N,N-diisopropylethylamine (493 µL, 2.83 mmol), 1-methylimidazole (27 µL, 0.340 mmol) and β-cyanoethyl-N,N-di-isopropylchlorophosphoramidite (189 µL, 0.849 mmol). After 15 min the reaction mixture turned cloudy. The reaction was allowed to proceed at r.t. overnight after which it was quenched with methanol (5 mL) and stirred for an additional 5 min. The reaction was worked up by evaporating the solvent in vacuo. The crude product was purified via flash column chromatography using triethylamine/chloroform (5:95) to yield **[2.12]** (380 mg, 92%) as a colorless foam. R_f (SiO_2): 0.40 (chloroform : methanol- 20:1)- ^{31}P-NMR (109.4 MHz, ppm, $CDCl_3$): 165.2, 163.6; ESI-MS (positive); *m/z* : 753.3 $[M+Na]^+$.

4.6.3 Synthesis of 2',3'-Dideoxy-2', 3'-α-epi-thiothymidine [2.24]

2'-Deoxy-2'-fluoro-1',3',5'-tri-O-benzoyl-α-D-arabinofuranose [2.14][14]

This compound was prepared according to the procedure established by M. I. Elzagheid *et al.* To a stirred solution of 1,3,5-tri-O-benzoyl-α-D-ribofuranose (10 g, 21.6 mmol) in anhydrous dichloromethane (100 mL) was added dropwise MAST (8 mL, 43.39 mmol) and allowed to stir at 40 °C overnight. The reaction mixture was initially cooled and diluted with DCM (200 mL) and subsequently quenched with $NaHCO_3$ (300 mL). The organic layer was washed with water (2 x 200 mL), $NaHCO_3$ (200 mL), dried over anhydrous Na_2SO_4, filtered and evaporated. The compound was purified by column chromatography using $CHCl_3$ to yield the desired product **[2.14]** (9.33 g, 93%) was obtained as a colorless foam. R_f (SiO_2): 0.34 (dichloromethane)- ^{1}H-NMR (acetone-d_6, 400 MHz): δ = 8.1-7.4(m, 15H, Bz), 6.7(d, 1H, 1'H, $J_{1',2'F}$ = 9.2 Hz), 5.6(dd, 1H, 3'H, $J_{3',2'F}$ = 20 Hz, $J_{3',2'}$ = 3.6 Hz), 5.5-5.7(d, 1H, 2'H, $J_{2',2'F}$ = 48 Hz), 4.9 (m, 1H, 4'H), 4.7-4.8(m, 2H, 5'-H_a, 5'-H_b).

2'-Deoxy-2'-fluoro-3',5'-di-O-benzoyl-α-D-arabinofuranosyl bromide [2.15][14]

This compound was prepared according to the procedure established Elzagheid *et al.* A solution of compound **[2.14]** (9.33 g, 20.1 mmol) in anhydrous dichloromethane (125 mL) and HBr (30% in acetic acid, 17 mL) were allowed to stir at r.t. overnight. The reaction mixture was diluted with DCM (100 mL) and was subsequently washed with $NaHCO_3$ (200 mL). The organic layer was dried over Na_2SO_4, filtered and evaporated to yield the product **[2.15]** (8.34 g, 98%) as a brown oil. R_f (SiO_2): 0.54 (dichloromethane)- ^{1}H-NMR (CD_2Cl_2, 500 MHz): δ = 8.2-7.3(m, 10H, Bz), 6.8(d, 1H, 1'H, $J_{1',2'F}$ = 19 Hz), 5.6(d, 1H, 2'H, $J_{2',2'F}$ = 50 Hz), 5.4 (dd, 1H, 3'H), 4.8(m, 3H, 4'H, 5'-H_a and 5'-H_b).

2'-Deoxy-2'-fluoro-3',5'-di-O-benzoyl-β-D-arabinothymidine [2.16][14]

This compound was prepared according to the procedure established by Elzagheid *et al.* by initially protecting the base to obtain 2,4-Bis-O-(trimethylsilyl)-thymine. This

compound was prepared by dissolving a dry mixture of thymine (6.59 g, 52.5 mmol) and ammonium sulfate (659 mg) in anhydrous acetonitrile (200 mL). Hexamethyldisilazane (HMDS) (11 mL) is then added to the reaction mixture and it was then allowed to reflux for 4 h at 100 °C. Once the reaction was clear, it was allowed to cool and the excess hexamethyldisilazane was removed by evaporation to afford the bis-silated pyrimidine which was used for the preparation of the nucleoside.

Subsequently, the nucleoside **[2.16]** was prepared by dissolving 2'-deoxy-2'-fluoro-3',5'-di-O-benzoyl-α-D-arabinofuranosyl bromide **[2.15]** and the bis-silated thymine (2,4-Bis-O-(trimethylsilyl)-thymine) (8.34 g, 19.7 mmol), in anhydrous CCl_4 (90 mL). The mixture was allowed to reflux for 4 d at 80 °C. The reaction mixture was allowed to cool down to r.t. and was subsequently diluted with DCM (500 mL) and then washed with 1L water. The organic layer was then dried over anhydrous Na_2SO_4 and the solvent was evaporated to obtain the crude product as a slightly brown solid. The impure mixture was recrystallized from ethanol/dichloromethane to afford **[2.16]** (8.50 g, 90%) as a white solid. R_f (SiO_2): 0.49 (chloroform : methanol- 9:1)- ^{1}H-NMR (DMSO-d_6, 500 MHz): δ = 11.5(s, 1H, N-H), 8.0-7.4(m, 11H, 6H and Bz), 6.3(dd, 1H, 1'H, $J_{1',2'}$ = 4 Hz, $J_{1',2'F}$ = 19 Hz), 5.7(ddd, 1H, 3'H, $J_{2',3'}$ = 2 Hz, $J_{3',4'}$ = 4 Hz, $J_{3',2'F}$ = 19 Hz), 5.5(ddd, 1H, 2'H, $J_{1',2'}$ = 4 Hz, $J_{2',3'}$ = 2 Hz, $J_{2',2'F}$ = 53 Hz), 4.8-4.7(m, 2H, 5'-H_a, 5'-H_b), 4.6(m, 1H, 4'H), 1.6 (s, 3H, 5-CH_3).

2'-Deoxy-2'-fluoro-β-D-arabinothymidine [2.17][14]

This compound was prepared according to the procedure established by Elzagheid *et al.* To a suspension of 2'-deoxy-2'-fluoro-3',5'-di-O-benzoyl-β-D-arabinothymidine **[2.16]** (8.50 g, 18.2 mmol) in ethanol (250 mL) was added concentrated NH_4OH (250 mL) and the resulting mixture was allowed to stir for 3 d. The reaction was worked up by coevaporating the solvent and the desired product **[2.17]** (4.6 g, 97%) was obtained as a slightly brown solid. R_f (SiO_2): 0.49 (chloroform : ethanol- 3:1)- ^{1}H-NMR (CD_3OD, 500 MHz): δ = 7.7(s, 1H, 6H), 6.2(dd, 1H, 1'H, $J_{1',2'}$ = 4 Hz, $J_{1',2'F}$ = 17 Hz), 5.0(ddd, 1H, 2'H, $J_{1',2'}$ = 4 Hz, $J_{2',3'}$ = 2.5 Hz, $J_{1',2'F}$ = 50 Hz), 4.3(ddd, 1H, 3'H, $J_{2',3'}$ = 2.5 Hz, $J_{3',4'}$ =

4.5 Hz, $J_{1',2'F}$ = 20 Hz), 3.9(dd, 1H, 4'H, $J_{3',4'}$ = 4.5 Hz, $J_{4',5'and\,5''}$ = 4.5 Hz), 3.9-3.7(m, 2H, 5'-H_a, 5'-H_b), 1.87(s, 3H, 5-CH_3).

5'-O-Monomethoxytrityl-2'-deoxy-2'-fluoro-arabinothymidine [2.18][14]

This compound was prepared according to the procedure established by Elzagheid *et al.* The above compound **[2.17]** (2.3 g, 8.8 mmol) was coevaporated with anhydrous pyridine (2 x 50 mL) and then suspended in anhydrous pyridine (200 mL). Then MMTr-Cl (4.10 g, 13.3 mmol) was added and the resulting mixture was allowed to stir overnight. The mixture was diluted with chloroform (200 mL), washed with brine (200 mL) and the organic layer was dried over anhydrous Na_2SO_4. The solvent was evaporated and the crude product was purified by column chromatography using dichloromethane followed by dichloromethane/methanol (19:1) to elute the desired product **[2.18]** (3.77 g, 80%) as a colorless foam. R_f (SiO_2): 0.3 (chloroform : methanol- 9:1)- ^{1}H-NMR (DMSO-d_6, 500 MHz): δ = 11.5(s, 1H, N-H), 7.4-6.9(m, 15H, trityl-H and 6H), 6.1(dd, 1H, 1'H, $J_{1',2'}$ = 4.5 Hz, $J_{1',2'F}$ = 16 Hz), 5.0 (ddd, 1H, 2'H, $J_{1',2'}$ = 4 Hz, $J_{2',3'}$ = 2.5 Hz, $J_{2',2'F}$ = 53 Hz), 4.3 (ddd, 1H, 3'H, $J_{2',3'}$ = 2.5 Hz, $J_{3',4'}$ = 4.5 Hz, $J_{3',2'F}$ = 19 Hz), 3.9 (m, 1H, 4'H), 3.7(s, 3H, OCH_3), 3.3(m, 2H, 5'-H_a, 5'-H_b), 1.6(s, 3H, 5-CH_3).

5'-O-Monomethoxytrityl-2',3'-dideoxy-2'-fluoro-3'-methanesulfonylarabinothymidine [2.19] and 5'-O-Monomethoxytrityl-2',3'-dideoxy-2'-fluoro-2,3'-O-anhydroarabinothymidine [2.20][9]

Compound **[2.19]** was prepared according to the procedure used for the preparation of compound **[2.2]** using 5'-O-Monomethoxytrityl-2'-deoxy-2'-fluoro-arabinothymidine **[2.18]** (2.01 g, 3.78 mmol), methanesulfonyl chloride (0.88 mL, 11.3 mmol) in pyridine(100 mL). The desired product **[2.19]** (2.26 g, 98%) was obtained as a brown oil. R_f (SiO_2): 0.29 (chloroform : methanol- 20:1)- ^{1}H-NMR (acetone-d_6, 270 MHz): δ = 11.3(s, 1H, N-H), 8.61(d, 1H, 6-H, $J_{6,1'}$ = 4.2 Hz), 7.54-6.91(m, 14H, trityl-H), 6.41(dd, 1H, 1'-H, $J_{1',2'}$ = 4.2 Hz, $J_{1',2'F}$ = 17.4 Hz), 5.63(dm, 1H, 2'-H, $J_{2',3'}$ = 2.2 Hz, $J_{1',2'}$ = 4.2 Hz, $J_{2',2'F}$ = 27 Hz), 5.52-5.49(m, 1H, 3'-H), 4.37-4.34(m, 1H, 4'-H), 3.77(s, 3H, OCH_3), 3.60(dd, 1H, 5'-H_a, $J_{5',4'}$ = 3.5 Hz, $J_{5',5''}$ = 10.9 Hz), 3.53(dd, 1H, 5'-H_b,

$J_{5',4'}$ = 4.2 Hz, $J_{5'-5''}$ = 10.7 Hz), 3.24(s, 3H, SO_2CH_3), 1.68(s, 3H, 5-CH_3). ESI-MS (positive); *m/z* : 632.9 $[M+Na]^+$.

Compound **[2.20]** was prepared according to the same procedure used for the preparation of compound **[2.3]**. The above material **[2.19]** (1.75 g, 2.87 mmol), triethylamine (30 mL) and ethanol (75 mL) were allowed to react and upon completion yielded the desired product **[2.20]** (1.43 g, 97%) as a brown solid. R_f (SiO_2): 0.22 (chloroform : methanol- 20:1)- ESI-MS (positive); *m/z* : 537.1 $[M+Na]^+$.

5'-O-Monomethoxytrityl-2',3'-dideoxy-2'-fluoro-3'-acetylthioarabinothymidine [2.21][10]

Compound **[2.20]** (2.71 g, 5.27 mmol) was co-evaporated twice with dry pyridine (2 x 30 mL) and once with dry dioxane (1 x 30 mL) prior to use. The procedure established by Elzagheid *et al.* was then used to obtain the desired compound **[2.21]** (1.28 g, 41%) as a pale yellow syrup. R_f (SiO_2): 0.50 (chloroform : methanol- 20:1)- ^{1}H-NMR (acetone-d_6, 400 MHz): δ = 10.15 (1H, s, N-H), 7.60-6.90 (16H, m, trityl-H and H-6), 6.33 (dd, 1H, 1'-H, $J_{1',2'}$ = 4.4 Hz, $J_{1',2'F}$ = 15.6 Hz), 5.3 (dt, 1H, 2'-H, $J_{2',3'}$ = 5.2 Hz, $J_{2',2'F}$ = 54.8 Hz), 4.3 (dm, 1H, 3'-H, $J_{3',2'}$ = 3.6 Hz, $J_{3',2'F}$ = 16.8 Hz), 4.1 (m, 1H, 4'-H), 3.79 (s, 3H, OCH_3), 3.36 (dd, 2H, 5'-H_a, 5'-H_b), 2.37 (s, 3H, SCOCH_3), 1.64 (s, 3H, 5-CH_3). ESI-MS (positive); *m/z* : 613.1 $[M+Na]^+$, 589.1 (M-1).

5'-O-Monomethoxytrityl-2',3'-dideoxy-2'-fluoro-3'-thioarabinothymidine [2.22][11]

Compound **[2.22]** was prepared according to the procedure used for the preparation of compound **[2.5]** using compound **[2.21]** (250 mg, 0.424 mmol), 10 M NaOH (1.24 mL) in ethanol (43 mL). The desired product **[2.22]** (100 mg, 45%) was obtained as a pale yellow foam. R_f (SiO_2): 0.41(chloroform : methanol- 20:1)- ^{1}H-NMR (acetone-d_6, 270 MHz): δ = 10.2(s, 1 H, N-H), 8.01(s, 1 H, 6-H), 7.54-6.86(m, 14H, trityl-H), 6.33(dd, 1H, 1'-H, $J_{1',2'}$ = 4.7 Hz, $J_{1',2'F}$ = 12.7 Hz), 5.23(dt, 1H, 2'-H, $J_{2',3'}$ = 4.7 Hz, $J_{1',2'}$ = 4.7 Hz, $J_{2',2'F}$ = 54 Hz), 4.06-4.02(m, 1H, 3'H), 3.79(s, 3H, OCH_3), 3.73-3.63(m, 1H, 4'-H), 3.44-

3.29(m, 2H, 5'-H_a, 5'-H_b), 2.85(s, 1H, 3'-*SH*), 1.64(s, 3H, 5-CH_3). ESI-MS (positive); *m/z* : 571.0 $[M+Na]^+$.

The corresponding **5'-O-Monomethoxytrityl-2',3'-dideoxy-2', 3'-α-epi-thiothymidine [2.23],** was also isolated from the column in about 20% yield. R_f (SiO_2): 0.47 (chloroform : methanol- 20:1)- ^{1}H-NMR (acetone-d_6, 270 MHz): δ = 10.2(s, 1 H, N-H), 8.00(s, 1 H, 6-H), 7.60-6.85(m, 14H, trityl-H), 6.10(s, 1H, 1'-H), 4.55-4.50(m, 1H, 4'-H), 4.01(d, 1H, 2'-H, $J_{2',3'}$ = 4.7 Hz), 3.83(d, 1H, 3'H, $J_{3',2'}$ = 5.0 Hz), 3.77(s, 3H, OCH_3), 3.50-3.44(m, 1H, 5'-H_a), 3.34-3.29(m, 1H, 5'-H_b), 1.57(s, 3H, 5-CH_3). ESI-MS (positive); *m/z* : 551.2 $[M+Na]^+$.

2',3'-Dideoxy-2', 3'-α-epi-thiothymidine [2.24]

The above compound **[2.23]** (30 mg, 0.057 mmol), was dissolved in a 3% TCA solution (prepared with dichloromethane) (2 mL) and the mixture was allowed to stir for 15 min. The reaction was worked up by evaporating the solvent in *vacuo* and the crude product was purified on a silica gel column using a gradient of triethylamine/chloroform (5:95) followed by triethylamine/chloroform/methanol (5:94:1) to elute the product. Appropriate fractions were combined and concentrated to dryness to afford **[2.24]** (10 mg, 70%) as an colorless oil. R_f (SiO_2): 0.38(chloroform : methanol- 20:1)- ^{1}H-NMR (CD_3OD, 400 MHz): δ = 7.82(s, 1 H, N-H), 7.79(d, 1 H, 6-H, $J_{6,1'}$ = 1.2 Hz), 6.00(s, 1H, 1'-H), 4.31(t, 1H, 4'-H, $J_{4',5'}$ = 4.4 Hz), 3.86(d, 1H, 2'-H, $J_{2',3'}$ = 5.2 Hz), 3.73-3.68(m, 3H, 3'-H and 5'-H), 2.61(s, 1H, 5'-*OH*), 1.82(d, 3H, 5-CH_3, $J_{5,6}$ = 1.2 Hz). ESI-MS (positive); *m/z* : 279.2 $[M+Na]^+$.

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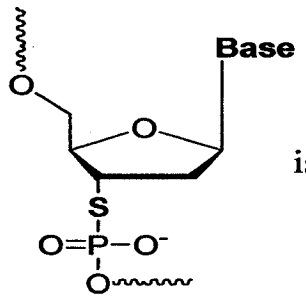
CONTRIBUTION TO KNOWLEDGE

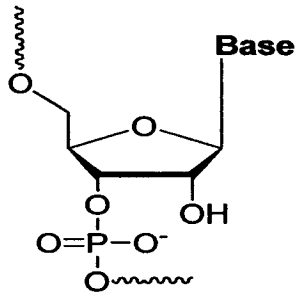
Modified oligonucleotides containing contiguous 3'- and 5'-S-phosphorothiolate linkages were successfully prepared via solid-phase synthesis and subsequently evaluated for their potential as antisense agents. UV melting studies were conducted in order to probe the impact of these modifications on DNA/RNA and RNA/RNA duplex stability. Incorporation of 3'-S modified residues in oligonucleotides resulted in enhanced binding affinities towards single stranded RNA and DNA targets. In contrast, modified oligomers containing the 5'-S modification exhibited lower thermodynamic stability when duplexed with their complementary RNA or DNA targets compared to the corresponding unmodified DNA/DNA and RNA/DNA duplexes. Due to the enhanced binding affinity displayed by the modified oligomers containing the 3'-S-phosphorothiolate internucleotide linkages, a chimeric oligonucleotide was constructed consisting of 2'-OMe-rU 'wings' with a central 3'-deoxy-3'-thiothymidine ($T_{3'S}$) hexanucleotide segment ('gap'). This 'gapmer' oligonucleotide (**3.4**) showed greater binding affinity towards a complementary RNA target relative to gapmers containing an internal hexanucleotide gap composed of the following uridine analogues: 2'-OMe-rU, 2'-F-rU and rT. All of these modifications have been previously demonstrated to form more thermodynamically stable duplexes with RNA compared to the natural DNA/RNA hybrid. We found that the stabilizing effects exhibited by the 3'-S modification on chimeric oligonucleotide/RNA duplex were superior to those of the 2'-OMe-rU, 2'F-rU and rT substitutions. The relative thermal stability of the duplexes formed between the 'gapmer' oligonucleotides and target RNA was as follows: 3'-S-modification > 2'F-rU > rT > 2'-OMe-rU. These results combined with the CD studies indicate that substitution of the normal DNA phosphodiester by the 3'-S-phosphorothiolate linkage strongly biases the nucleoside sugar puckering towards the C3'-*endo* (northern) conformational state. This C3'-*endo* (RNA-like) conformation serves to pre-organize the 3'S-phosphorothiolate DNA into the required conformation for the formation of stable A-type duplexes (CD studies). Although AONs containing the 3'-S modification exhibit certain desirable properties rendering them promising antisense candidates, unfortunately, they did not elicit RNase H activity. In contrast, the duplex formed between RNA and the AON containing three contiguous 5'-S-phosphorothiolate linkages are geometrically similar to the native

DNA/RNA substrates suggesting that the 5'-S nucleotides adopt a DNA-like (eastern/southern C2'-*endo*) conformation. This predisposes the AON strand in the AON/RNA duplex to adopt a more B-like geometry closely mimicking the native DNA/RNA substrates. Consequently, AON/RNA hybrids comprising 5'-S-phosphorothiolate linkages serve as substrates for *E. coli* RNase H, thereby, enabling degradation of target RNA.



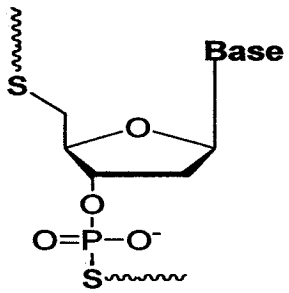
(3`S-DNA)

is conformationally equivalent to



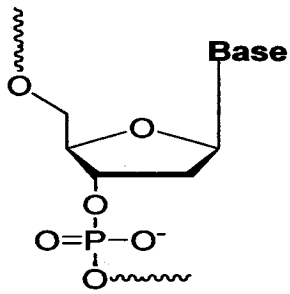
(RNA)

and,



(5`S-DNA)

more closely resembles the structure of



(DNA)

Our attempts towards the synthesis of 2',3'-dideoxy-2'-fluoro-3'-S-phosphorothioamidite precursors for the synthesis of 2',3'-dideoxy-2'-fluoro-3'-thioarabinonucleic acids led to the very first isolation and characterization of 2',3'-dideoxy-2',3'-α-epi-thiothymidine, a novel [3.1.0]-fused 2',3'-modified nucleoside. This class of compounds may find use as antiviral agents by their intracellular conversion to 5'-triphosphate derivatives and subsequent incorporation and termination of viral DNA synthesis.

APPENDIX :

NMR Spectra

Figure 1: ^{1}H NMR spectrum of 3'-Acetylthio-3'-deoxy-5'-(monomethoxytrityl)thymidine (2.4). The spectrum was recorded on a Varian XL-400 (400 MHz) spectrometer using chloroform as a solvent.

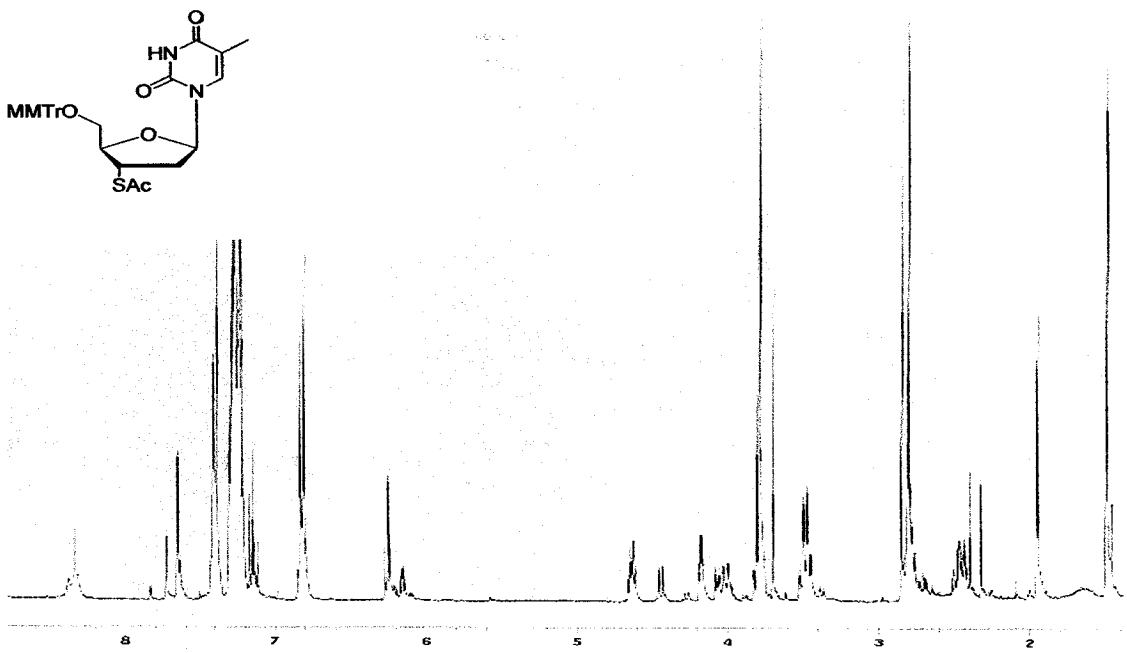


Figure 2: ^{1}H NMR spectrum of 3'-Deoxy-3'-thio-5'-(monomethoxytrityl)thymidine (2.5). The spectrum was recorded on a JEOL-270 (270 MHz) spectrometer using acetone as a solvent.

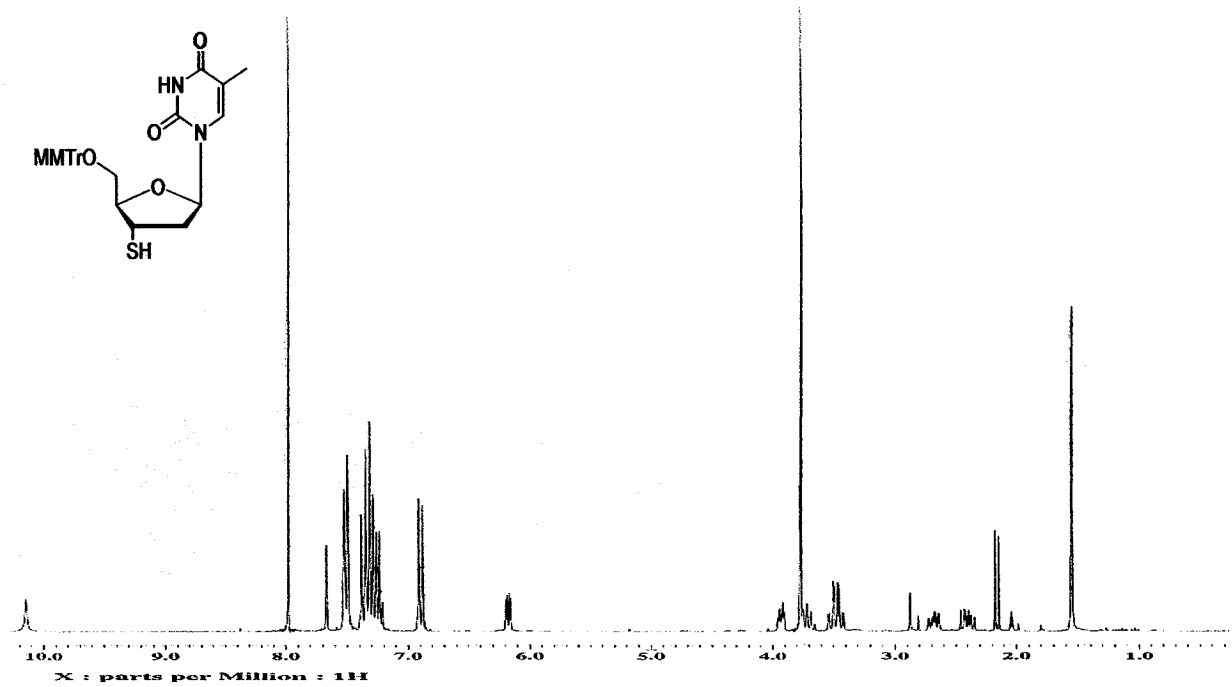


Figure 3: ^{31}P NMR spectrum of 5'-O-Monomethoxytritylthymidine-3'-S-phosphorothioamidite (**2.6**). The spectrum was recorded on a JEOL-270 (270 MHz) spectrometer using chloroform as a solvent.

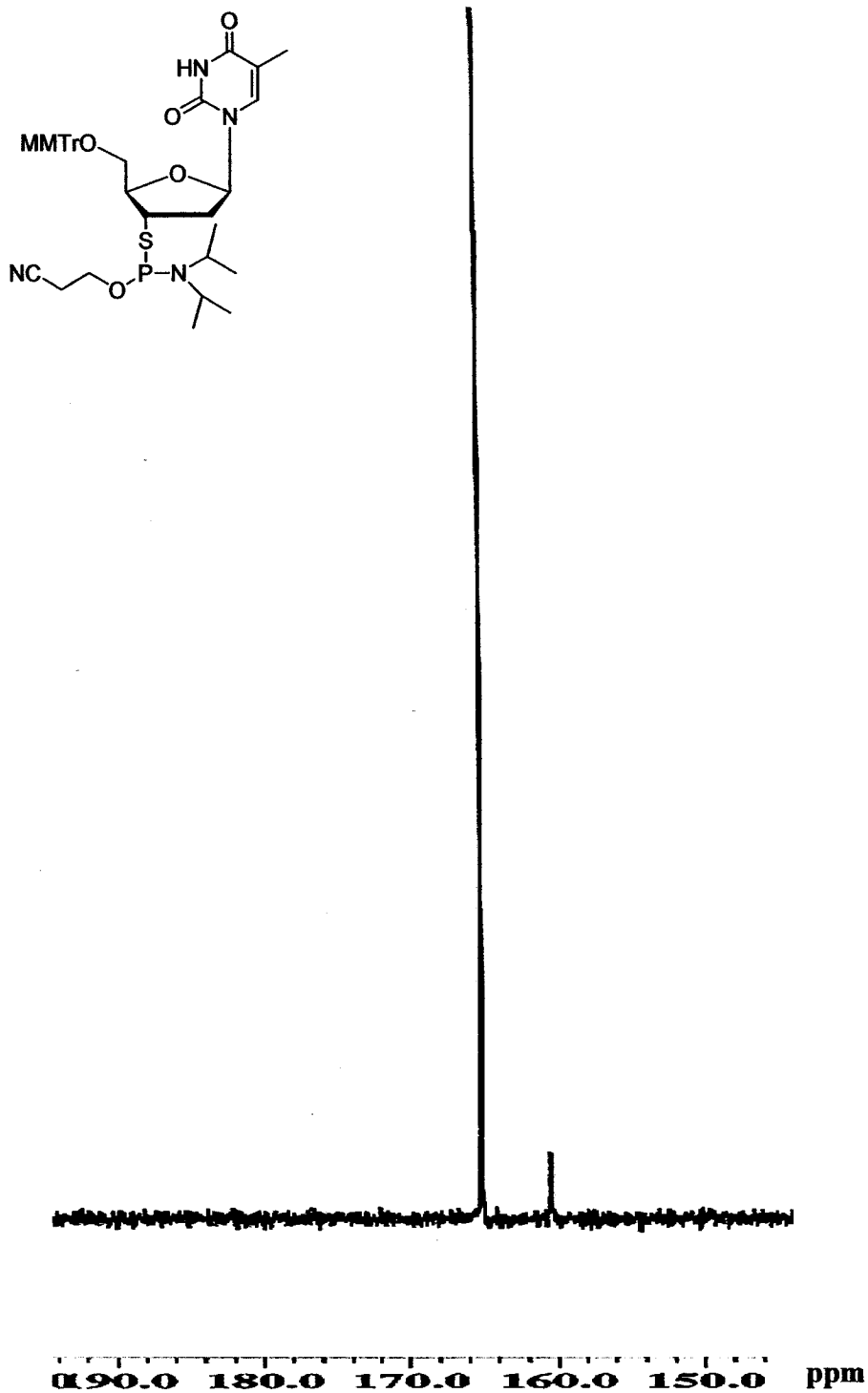


Figure 4: ^{1}H NMR spectrum of 5'-Acetylthio-5'-deoxy-3'-(monomethoxytrityl)thymidine (**2.10**). The spectrum was recorded on a JEOL-270 (270 MHz) spectrometer using acetone as a solvent.

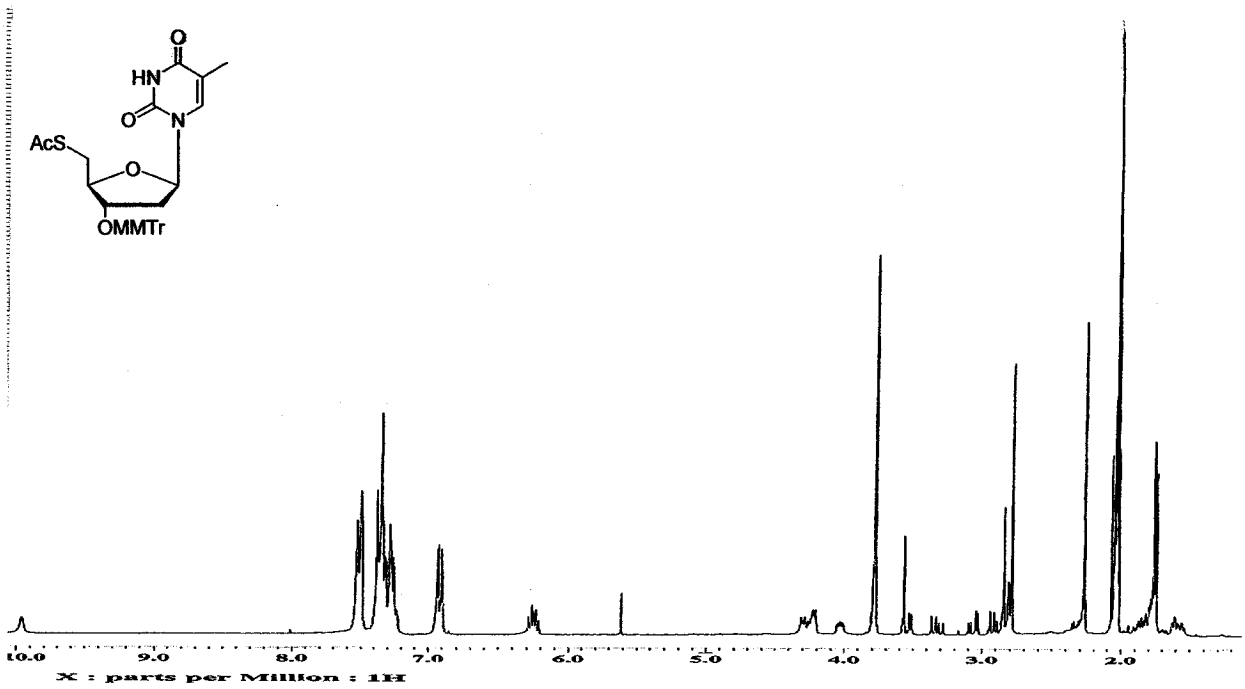


Figure 5: ^{1}H NMR spectrum of 5'-Deoxy-5'-thio-3'-(monomethoxytrityl)thymidine (**2.11**). The spectrum was recorded on a JEOL-270 (270 MHz) spectrometer using acetone as a solvent.

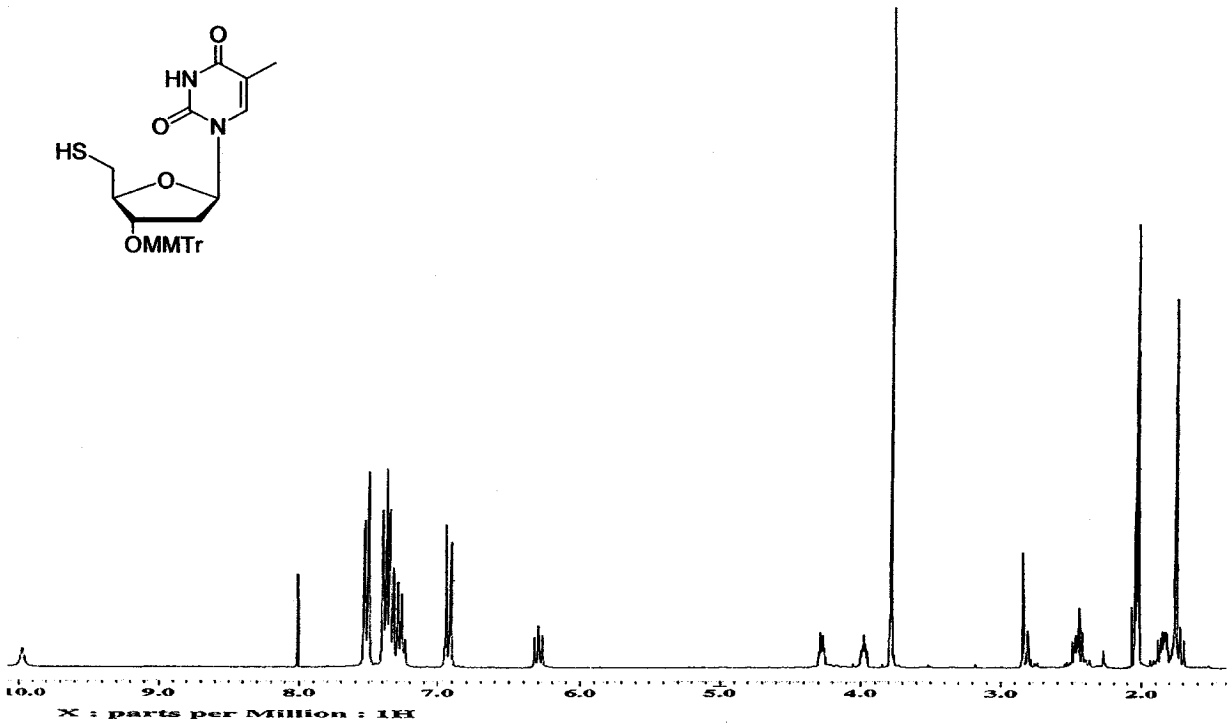


Figure 6: ^{31}P NMR spectrum of 3'-O-Monomethoxytritylthymidine-5'-S-phosphorothioamidite (**2.12**). The spectrum was recorded on a JEOL-270 (270 MHz) spectrometer using chloroform as a solvent.

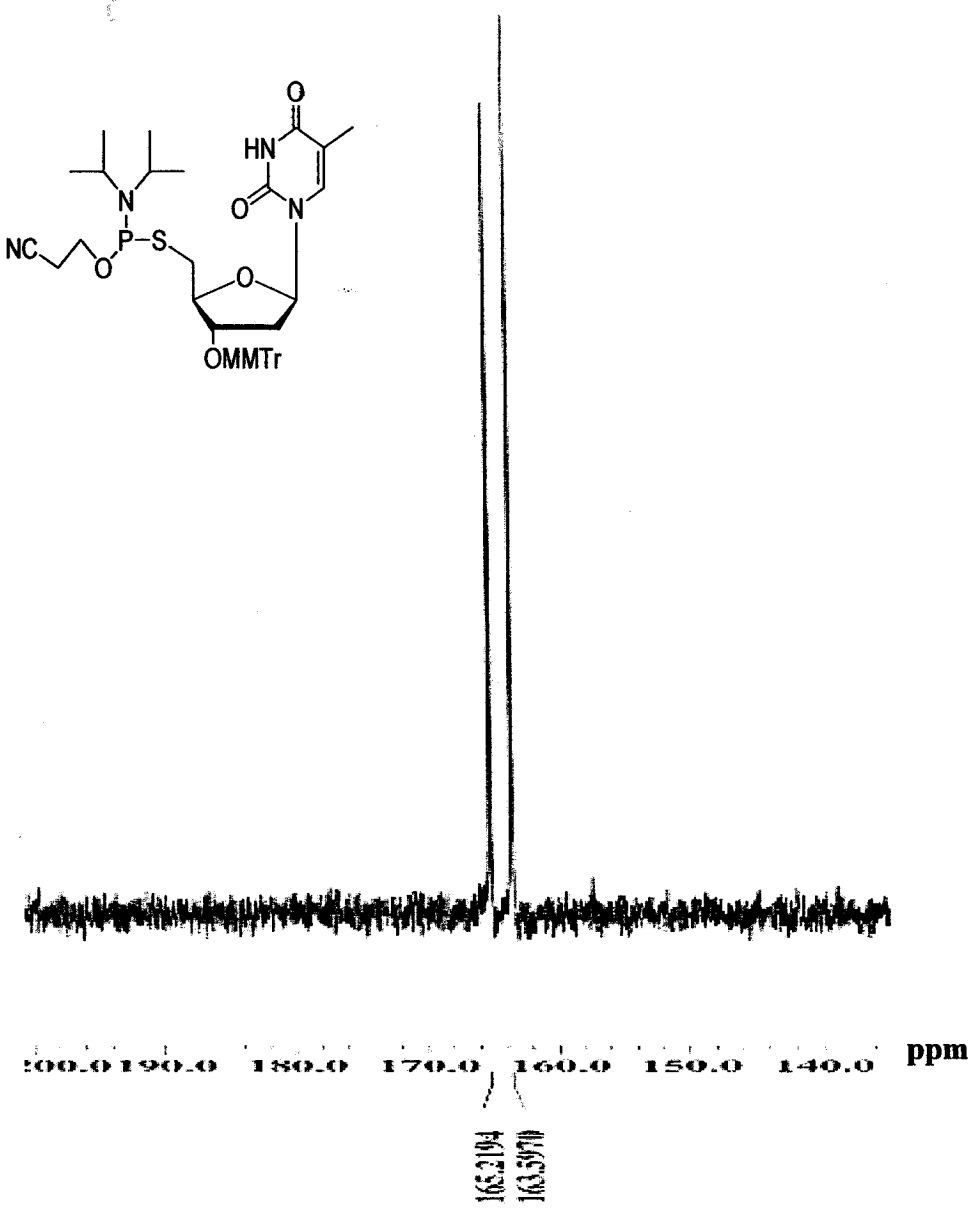


Figure 7: ^{1}H NMR spectrum of 5'-O-Monomethoxytrityl-2',3'-dideoxy-2'-fluoro-3'-acetylthioarabinothymidine (2.21). The spectrum was recorded on a Varian XL-400 (400 MHz) spectrometer using acetone as a solvent.

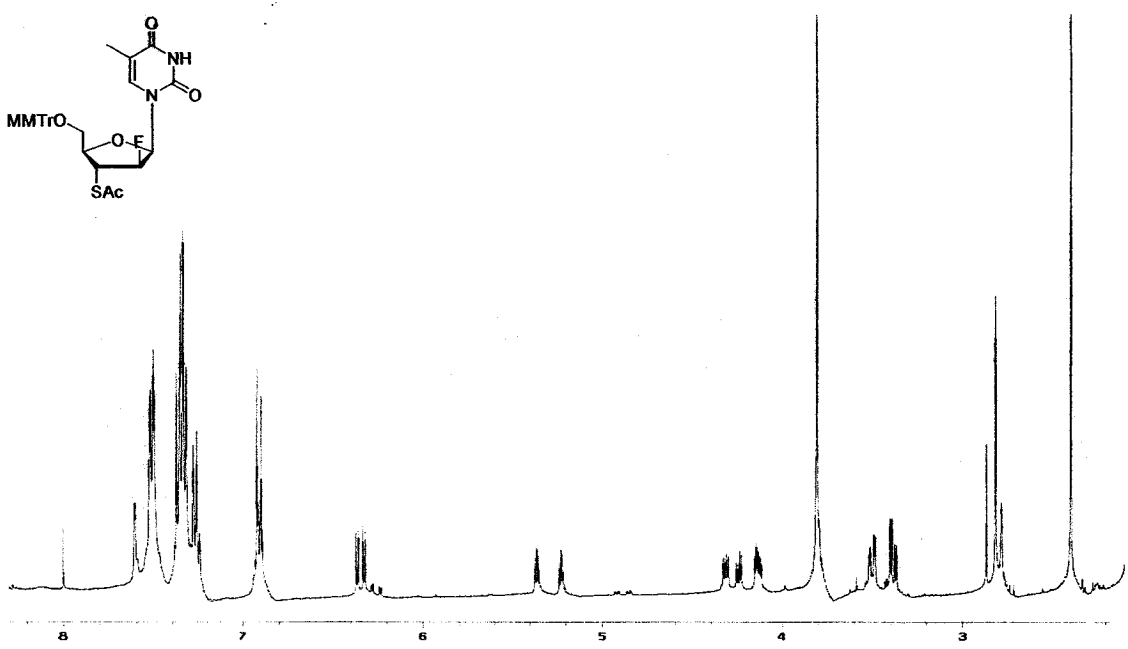


Figure 8: ^{1}H NMR spectrum of 5'-O-Monomethoxytrityl-2',3'-dideoxy-2'-fluoro-3'-thioarabinothymidine (2.22). The spectrum was recorded on the JEOL-270 (270 MHz) spectrometer using acetone as a solvent.

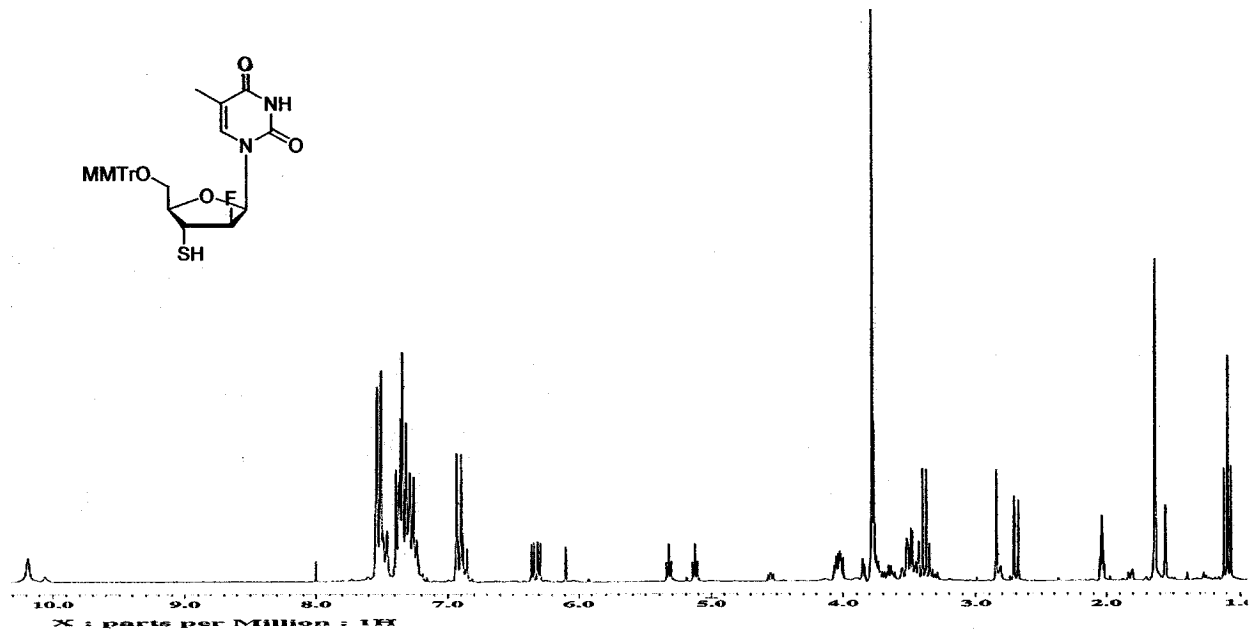


Figure 9: ^{1}H NMR spectrum of 5'-O-Monomethoxytrityl-2',3'-dideoxy-2',3'-α-epi-thiothymidine (2.23). The spectrum was recorded on a JEOL-270 (270 MHz) spectrometer using acetone as a solvent.

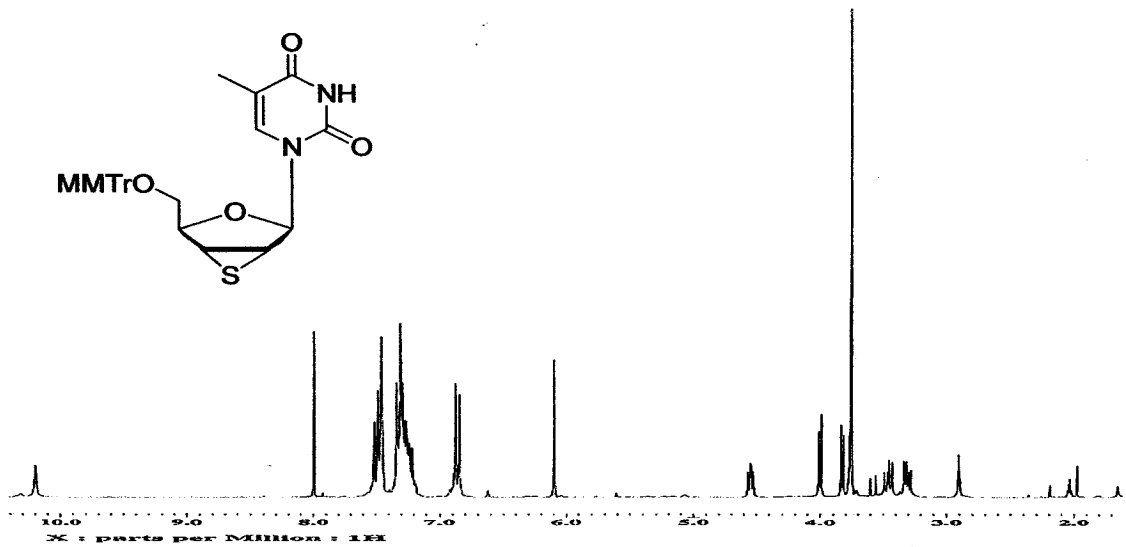


Figure 10: ^{1}H NMR spectrum of 2',3'-dideoxy-2',3'-α-epi-thiothymidine (2.24). The spectrum was recorded on a JEOL-270 (270 MHz) spectrometer using methanol as a solvent.