Synthesis, characterization and biological properties of branched RNA fragments containing chiral (R_p and S_p) 2',5'-phosphorothioate linkages

by

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To my parents Souad & Hanna Mourani
for all their love, support and sacrifices.

ABSTRACT

A method to synthesize a diastereomeric mixture of 2',5' phophorothioate A(p_sG) dimers in solution was developed. The sulfurizing reagent "EDITH" allowed for the synthesis of the diastereomeric mixture of dimers with minimal formation of oxidized side products. The separation of the two isomers was carried out using silica gel flash chromatography to afford the stereoisomerically pure R_p and S_p dimers. The orthogonal solution-phase coupling of the individual dimers to the appropriately protected monomers allowed for the creation of the corresponding branched trimers bearing vicinal 2',5'-phophorothioate and 3',5'-phosphodiester linkages.

Conversely, a convergent solid-phase strategy applicable to the synthesis of branched oligonucleotides was employed to construct a symmetrical branched phosphodiester trimer, A(pG)pG, using an adenosine bisphosphoramidite synthon. This compound served as a positive control substrate, relative to both the S_p and R_p-phophorothioate V-trimers, in the investigation of the stereochemical requirements of the yeast debranching enzyme (yDBR) at the 2',5'-phosphodiester linkage.

Spectroscopic methods, HPLC analysis, gel electrophoresis, mass spectroscopy, UV absorption and NMR were utilized in the characterization of the synthesized oligomers. In addition, circular dichroism spectroscopy (CD) was used in order to obtain pertinent insight into the three-dimensional structure of the branched oligonucleotides. Characterization of the molecules and assignment of the absolute stereochemistry of their phosphorothioate bonds was afforded *via* enzymatic digests with snake venom phosphodiesterase (SVPDE).

Branched oligonucleotides were labeled with radioactive phosphorus (^{32}P), however with significant difficulty, owing to the steric inaccessibility of the 3'-hydroxyl units as a result of the compact nature of their structures. The labeled molecules were subjected to the specific 2',5'-phosphodiesterase, yDBR, and the resultant products of debranching separated by gel electrophosresis (PAGE), visualized by autoradiography, and analyzed. The phosphodiester V-trimer control, A(pG)pG, the S_p-phosphorothioate trinucleotide, A(p_sG)pC, and S_p-phosphorothiate tetranucleotide ApA(p_sG)pC were all cleaved by the yDBR, although not nearly as efficiently as a control wild-type branched

18-mer yeast lariat intron mimic. In contrast, the R_p-isomer of the trinucleotide, A(p_sG)pC, was not cleaved at all by the yDBR enzyme, even after 24 h incubation.

RÉSUMÉ

Une méthode pour synthétiser un mélange diastéréomérique de dimères phosphothioriques A(p_sG) en solution a été développés. L'agent sulfurant "EDITH" permet la synthèse d'un mélange de dimères diastéréomériques avec une production minimale de dérivés secondaires oxidés. La séparation des deux isomères a été faite avec la chromatographie flash sur gel de silice ce qui a permis l'isolation de dimères R_p et S_p stéréoisomériquement pures. Le couplage orthogonale de la phase-solution des dimères individuels aux monomères protégés de manières appropriés, a permis la création de trimères branchés correspondants, portant des liaisons proximales 2',5'-phosphothioriques et des liaisons 3',5'-phosphodiesters.

Par contre, une stratégie phase-solide convergente applicable dans la synthèse d'oligonucléotides branchés a été utilisée pour synthétiser un trimère phosphodiester, A(pG)pG, en utilisant un synthon adénosine biphosphoamidite. Ce réactif a servi comme substrat positif de contrôle, à l'encontre des trimères-V S_p et R_p-phosphothioriques, dans l'étude des besoins stéréochimiques de l'enzyme de débranchement (yDBR) appartenant à la levure, au niveau de la liaison 2',5'-phosphodiester.

Les méthodes spectroscopiques telles que: l'analyse par CLHP, l'électrophorèse de gel de polyacrylamide (EGPA), la spectroscopie de masse, l'absorption par UV et RMN ont été utilisées dans la caractérisation des oligomères de synthèse. En plus, la spectroscopie de dichroïsme circulaire a été aussi utilisée pour obtenir les connaissances pertinentes sur la structure tridimensionnelle des oligonucléotides branchés. La caractérisation des molécules et la détermination de la stéréochimie absolue de leurs liens phosphothioriques a été obtenue par digestion enzymatique avec la phosphodiesterase de venin de serpent (SVPDE).

Cependant, les oligonucléotides branchés ont été marqués avec du phosphore radioactif (^{32}P) étant donné la difficulté importante de l'inaccessabilité stérique des groupes 3'-hydroxyle conséquante de la nature compact de leur structure. Les molécules

marquées ont été sousmises à l'action d'une 2',5'-phosphodiesterase spécifique, yDBR, et les produits résultants du débranchement furent séparés par EGPA, visualisés par autoradiographie et analysés. Le contrôle trimère-V phosphodiester, A(pG)pG, le trinucléotide phosphothiorique, A(p_sG)pC, et le tétranucléotide S_p-phosphothiorique ApA(p_sG)pC ont tous été clivés par le yDBR, mais pas aussi efficacement que le control branché de 18-mères de type sauvage mimiquant l'intron de la levure lariat. Par contre, l'isomère R_p de trinucléotide, A(p_sG)pC, n'a pas été clivé par l'enzyme yDBR, même après 24 heures d'incubation.

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TABLE OF CONTENTS

DEDICATION ii

ABSTRACT iii

RESUME iv

ACKNOWLEDGMENTS vi

TABLE OF CONTENTS vii

LIST OF FIGURES xi

LIST OF SCHEMES xiii

LIST OF TABLES xiii

ABBREVIATIONS xiv

CHAPTER 1: INTRODUCTION 1

1.1 Importance of Nucleic Acids 1

1.2 Flow of Genetic Information 4

1.3 RNA Splicing 5

1.4 The RNA Lariat Debranching Enzyme 7

1.5 Chemical Synthesis of Nucleic Acids 10

1.6 Solid Phase Synthesis of Oligonucleotides 14

1.7 Synthesis of Branched Oligomers 15

1.8 Phosphorothioates 18

1.9 Objectives 21

CHAPTER 2: SOLUTION SYNTHESIS OF PHOSPHOROTHIOATE BRANCHED OLIGONUCLEOTIDES 22

2.1 Monomer Synthesis 22

2.1.1 Synthesis of Both the Adenosine 2′- and 3′-Phosphoramidites 24

2.1.2 Synthesis of N^2-iBu-O^6-NPE-2',3'-di-Bz-Guanosine 25

2.1.3 Benzoylation of ($^{DMT}G^{N\text{-}iBu}$) (2.2) 26

2.1.4 Flash Chromatography Purification of ($^{DMT}{}_{Bz}G^{iBu,\ NPE}{}_{Bz}$) (2.4) 31

2.1.5 Synthesis of the Cytidine 5′-Phosphoramidites 31

2.2 Oligomer Synthesis 33
2.2.1 Sulfurization of the 2',5'-RNA Dimer (Beaucage *vs.* EDITH Reagent) (4.1) 34
2.2.2 Ratio of the Two Isomers in the 2',5'-A(p_sG) Dimer 36
2.2.3 Purification and Separation of the Protected Phosphorothioate Isomers 37
2.2.4 Deprotection of the Cyanoethyl Protecting Group 39
2.2.5 Desilylation of Dimers R_p-A(p_sG) (4.3a) and S_p-A(p_sG) (4.3b) 41
2.2.6 Synthesis of the V-Trimers (4.4a and 4.4b) 42
2.2.7 Detritylation of the V-Timers (4.5a and 4.5b) 44
2.2.8 Synthesis of the S_p-Y-Tetramer (4.7b) 45
2.2.9 Synthesis of the R_p-Y-Tetramer (4.7a) 46

CHAPTER 3: DEPROTECTION, PURIFICATION AND CHARACTERIZATION OF OLIGONUCLEOTIDES 48
3.1 Deblocking of Solution Synthesized Oligonucleotides 48
3.2 Deblocking of Solid-Phase Synthesized Oligonucleotides 48
3.3 Removal of TBAF Salts Using Dowex-Na^+ Exchange Chromatography 49
3.4 Purification of Linear Oligomers and V-Trimers Using Anion Exchange HPLC 49
3.5 Purification of the Y-Tetramer Using Polyacrylamide Gel Electrophoresis (PAGE) 50
3.6 Separation of the Isomers of the Deprotected Dimers A(p_sG) Using Reverse Phase HPLC 51
3.7 Desalting the Oligomers Using SEP-PAK 51
3.8 Quantitative and Qualitative Analysis Using UV Spectroscopy 52
3.9 Circular Dichroism (CD) 54

CHAPTER 4: BIOLOGICAL ASSAYS 58
4.1 Enzymatic Digestion of Oligonucleotides Using Snake Venom Phosphodiesterase (SVPDE)/Alkaline Phosphatase (AP) 58
4.2 3'-End Labeling with ^{32}P 62
4.3 Debranching Studies Using DBR from HeLa Extract 65
4.4 Debranching Studies Using His-Tagged yDBR 68

CHAPTER 5: EXPERIMENTAL 71
5.1 General Methods 71
5.1.1 Reagents and Chemicals 71
5.1.2 Chromatography 72
5.1.3 Instruments 72
5.2 Monomer Synthesis 74
5.2.1 N^6-Benzoyladenosine (1.1) 74
5.2.2 N^6-Benzoyl-5'-O-(4 4'-Dimethoxytrityl) Adenosine (1.2) 75
5.2.3 N^6-Benzoyl-5'-O-(4,4'-Dimethoxytrityl)-3'-O-*tert*-Butyldimethylsilyladenosine (1.3a) and N^6-Benzoyl-5'-O-Dimethoxytrityl-2'-O-*tert*-Butyldimethylsilyladenosine (1.3b) 75
5.2.4 N^6-Benzoyl-5'-O-(4,4'-Dimethoxytrityl)-3'-O-*tert*-Butyldimethylsilyladenosine -2'-O-[β-Cyanoethyl N,N-Diisopropyl] Phosphoramidite (1.4a) 76
5.2.5 N^6-Benzoyl-5'-O-(4,4'-Dimethoxytrityl)-2'-O-*tert*-Butyldimethylsilyladenosine -3'-O-[β-Cyanoethyl N,N-Diisopropyl] Phosphoramidite (1.4b) 76
5.2.6 N^2-Isobutyrylguanosine (2.1) 77
5.2.7 N^2-Isobutyryl-5'-O-(4,4'-Dimethoxytrityl) Guanosine (2.2) 77
5.2.8 N^2-Isobutyryl-2',3'-O-di-Benzoyl-5'-O-(4,4'-Dimethoxytrityl) Guanosine (2.3) 78
5.2.9 N^2-Isobutyryl-O^6-*p*-Nitrophenylethyl- 2',3'-O-di-Benzoyl-5'-O-(4,4'-Dimethoxytrityl) Guanosine (2.4) 78
5.2.10 N^2-Isobutyryl-O^6-*p*-Nitrophenylethyl- 2',3'-O-di-Benzoylguanosine (2.5) 79
5.2.11 N^4-Benzoylcytidine (3.1) 79
5.2.12 N^4-Benzoyl-5'-O-(4,4'-Dimethoxytrityl) Cytidine (3.2) 80
5.2.13 N^4-Benzoyl-2',3'-O-di-Benzoyl-5'-O-(4,4'-Dimethoxytrityl) Cytidine (3.3) 80
5.2.14 N^4-Benzoyl-2',3'-O-di-Benzoylcytidine (3.4) 81
5.2.15 N^4-Benzoyl-2',3'-O-di-Benzoylcytidine-5'-O-[β-Cyanoethyl N,N-Diisopropyl] Phosphoramidite (3.5) 81
5.3 Oligomer Synthesis 82
5.3.1 Solution Phase Synthesis 82
5.3.1.1 Solution Synthesis of 2',5'-Phosphorothioate Dimers (Ap_sG) (4.1a,b) 82

5.3.1.2 Removal of the Cyanoethyl-Protecting Group from the Phosphorothioate Linkage in the R_p Dimer (4.2a) 83
5.3.1.3 Removal of the Cyanoethyl-Protecting Group from the Phosphorothioate Linkage in the S_p Dimer (4.2b) 83
5.3.1.4 Removal of the Silyl Group (TBDMS) in the Presence of Dimethoxytrityl (DMT) and p-Nitrophenylethyl (NPE) Protecting Groups (4.3a) 83
5.3.1.5 Removal of the Silyl Group (TBDMS) in the Presence of Dimethoxytrityl (DMT) and p-Nitrophenylethyl (NPE) Protecting Groups (4.3b) 84
5.3.1.6 Solution Synthesis of the R_p-V-Trimer (A(p_sG)pC) (18a) 84
5.3.1.7 Solution Synthesis of the S_p-V-Trimer (A(p_sG)pC) (18b) 85
5.3.1.8 Removal of the Cyanoethyl-Protecting Group from the Phosphodiester Linkage in the V-Trimers (19a,b) 85
5.3.1.9 Detritylation of the R_p-V-Trimer (A(p_sG)pC) (20a) 85
5.3.1.10 Detritylation of the S_p-V-Trimer (A(p_sG)pC) (20a) 86
5.3.1.11 Solution Synthesis of the S_p-Y-Tetramer (ApA(p_sG)pC) (21b) 86
5.3.2 Solid Phase Synthesis 88
5.3.2.1 CPG Derivatization 88
5.3.2.2 Solid-Phase RNA Synthesis 88
5.4 Total Deprotection of the Fully Protected Oligomers 89
5.5 Oligonucleotide Purification, Desalting and Analysis 90
5.5.1 High Performance Liquid Chromatography (HPLC) 90
5.5.2 Polyacrylamide Gel Electrophoresis (PAGE) 91
5.5.3 C18 SEP-PAK™ Reverse Phase Chromatography 92
5.6 Enzymatic Studies 93
5.6.1 Enzymatic Digestion via Snake Venom Phosphodiesterase (SVPDE) 93
5.6.2 3'-End ^{32}P Labeling 93
5.6.3 Debranching of Synthetic Oligomers Using HeLa Extract 94
5.6.4 Debranching of Synthetic Oligomers Using yDBR 95
CONTRIBUTION TO KNOWLEDGE AND FUTURE STUDIES 96
BIBLIOGRAPHY 98
APPENDIX 104

LIST OF FIGURES

Figure

1	(a) Primary Structures of Heterocyclic Bases of DNA and RNA. (b) Common Chemical Constituents and their Connectivities in DNA, RNA, and 2',5'-Linked RNA.	3
2	Flow of Genetic Information.	5
3	An Illustration of the Splicing Reaction.	6
4	Structure of R_p and S_p Phosphorothioate Stereoisomers.	7
5	Illustration of the Debranching Reaction of a Lariat RNA.	9
6	Coupling Reactions Using Phosphoramidite and Tetrazole.	14
7	Solid-Phase Synthesis of bRNA Using the Convergent-Growth Approach.	16
8	Solid-Phase Synthesis of bRNA Using the Divergent-Growth Approach.	17
9	Solid-Phase Synthesis of bRNA Using Regioselective Convergent-Growth Approach.	17
10	Synthesis of Dinucleoside Phosphorothioates Using the Triester Method.	20
11	Sulfurization of a Dinucleoside Phosphite Triester by the Beaucage Reagent.	20
12	Structure of the R_p and S_p-Branched Oligonucleotide Isomers.	21
13	^{1}H-NMR and Mass Spectra of the Product of Benzoylation of $^{DMT}G^{N\text{-}iBu}$ Using 6 Equivalents of BzCl.	29
14	^{1}H-NMR and Mass Spectra of the Product of Benzoylation of $^{DMT}G^{N\text{-}iBu}$ Using 2 Equivalents of BzCl.	30
15	Structures of the Sulfurizing Reagents Beaucage and EDITH.	35
16	^{31}P-NMR Spectra of Crude A(p_sG) Dimers Synthesized Using Two Different Sulfurizing Reagents.	35
17	^{31}P-NMR Spectrum of Crude A(p_sG) Dimer (mixture of R_p and S_p isomers).	37
18	^{31}P-NMR Spectra of the Isomerically Pure A(p_sG) Dimers.	38
19	^{31}P-NMR Spectra of the Isomerically Pure A(p_sG) Dimers After the Removal of the Cyanoethyl Protecting Group.	39
20	Desilylation of the 3′-OH in the Presence of a Vicinal 2′,5′-Linked	

Phosphorothioate Triester Linkage. 40
21 ^{31}P-NMR Spectrum of the Deprotected R_p A(p_sG) Dimer. 41
22 ^{31}P-NMR Spectra of R_p-V-Trimer A(p_sG)pC, Before and After the Removal of the Cyanoethyl Protecting Group. 43
23 24% Denaturing PAGE of Branched Trimers and Tetramer (S_p & R_p isomers). 47
24 24% Preparative PAGE to Purify the Crude S_p-Y-Tetramer ApA(p_sG)pC. 50
25 Reverse-Phase HPLC Analysis of the Deprotected and Desalted Mixture of R_p and S_p-A(p_sG) Isomers 51
26 UV Profiles of Cytidine 5′-Phosphoramidite, A(p_sG) Dimer and A(p_sG)pC Trimer. 53
27 CD Spectra of Dinucleoside Monophosphates, Branched Phosphorothioate Trinucleotides and Tetranucleotides in H_2O. 55
28 CD Spectra of Dinucleoside Monophosphates and Branched Trinucleotides in H_2O. 57
29 HPLC Analysis of the Enzymatic Digest of Both S_p (a) and R_p (b) Isomer of the Dimer A(p_sG) Using Snake Venom Phosphodiesterase (SVPDE) and Alkaline Phosphatase (AP). 60
30 HPLC Analysis of the Enzymatic Digest of Both S_p (a) and R_p (b) Isomer of the V-Trimer A(p_sG)pC Using Snake Venom Phosphodiesterase (SVPDE) and Alkaline Phosphatase (AP). 61
31 Trial to 3′-End Label Small-Branched Oligonucleotides Using 5'-^{32}P-Cytosine-3',5'-Biphosphate on a Small Gel Apparatus. 63
32 Images of Autoradiograms of a Gel Analysis of ^{32}pCp and ^{32}pCp with T4 RNA Ligase, Both with the Absence of any Substrate. 63
33 3′-End Labeling of Small-Branched Oligonucleotides Using ^{32}pCp, Large Gel Apparatus. 65
34 Images of Autoradiograms of Assay for Substrate Recognition by Debranching Activity in HeLa Extracts. 67
35 Images of Autoradiograms as Evidence of Linear Oligonucleotide Degradation by Other Nucleases Present in the HeLa Extract. 67

36 Images of Autoradiograms of Assay for Y-Shaped Oligonucleotide, 19mer, Recognition by yDBR. 69
37 Images of Autoradiograms of Assay for Control V-Trimer Recognition by yDBR. 70
38 Images of Autoradiograms of Assay for Substrate Recognition by (yDBR). 70

LIST OF SCHEMES

Scheme

1 General Procedure for the Regiospecific Synthesis of the Branched Oligonucleotides. 23
2 Synthesis of Both the Adenosine 2′- and 3′-Phosphoramidites. 25
3 Synthesis of Protected Guanosine. 27
4 Partial Benzoylation of an Isobutyrylated Guanine. 28
5 Synthesis of the Cytidine 5′-Phosphoramidites. 32
6 Solution Phase Synthesis of 2′,5′-Phosphorothioate A(p_sG) Dimer. 34
7 Solution Phase Synthesis of V-Trimer. 44
8 Solution Phase Synthesis of Y-Tetramer. 45
9 3'-End Labelling V-Trimers with ^{32}P. 62
10 Debranching Reaction of 3'-^{32}P-End Labelled V-Trimer. 66

LIST OF TABLES

Table

1 Examples of Protecting Groups used in Nucleic Acid Synthesis. 13
2 R_f Values of Solution Synthesized Phosphorothioate Oligomers. 87
3 ^{32}P-NMR Data of Solution Synthesized Phosphorothioate Oligomers. 87

ABBREVIATIONS

A	adenosine
Å	angstrom
A_{260}	UV absorbance measured at 260 nm
A(pG)	the bracket annotation indicates a 2′,5′-linkage, *i.e.*, A2′p5′G
Ap_sG	the "p_s" indicates a phosphorothioate linkage
Ad^{Bz}	N^6-benzoylriboadenosine
Ad	adenine
AP	alkaline phosphatase
B	base
BIS	N,N′-methylene-bis(acrylamide)
BNA	branched nucleic acid
BPB	bromophenol blue
bRNA	branched RNA
Bz	benzoyl
C	cytosine
°C	degrees Celsius
CD	circular dichroism
CE	cyanoethyl
CH_3CN	acetonitrile
Cl	chloride
ca.	approximately (circa)
CaH_2	calcium hydride
Calc.	calculated
COSY	correlated spectroscopy
CPG	controlled pore glass
Cy	cytidine
d	doublet
dd	doublet of doublets
DCC	1,3-dicyclohexylcarbodiimide

DCE	1,2-dichloroethane
DCM	dichloromethane
DEAD	diethyl azodicarboxylate
DEC	1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride
DIPEA	diisopropylethylamine
DMAP	N,N-dimethyl-4-aminopyridine
DMF	n,n-dimethylformamide
DMSO	dimethylsulfoxide-d_6
DMT	dimethoxytrityl
DNA	2′-deoxyribonucleic acid
E.coli	*Escherichia coli*
EDTA	disodium ethylenediaminetetraacetate dihydrate
ESI	electospray ionization
EtOH	ethanol
EtOAc	ethyl acetate
FAB-MS	fast atom bombardment mass spectrometry
eq	equivalent(s)
G	guanosine
g	gram(s)
gCOSY	gradient COSY
Gu	guanine
$^{DMT}Gu^{ibu}$	N^2-isobutyryl-5'-O-(4,4'-dimethoxytrityl) riboguanosine
$^{HO}{}_{Bz}Gu^{ibu,NPE}{}_{Bz}$	N^2-isobutyryl-O^6-*p*-nitrophenylethyl- 2',3'-O-di-benzoylriboguanosine
h	hour(s)
hDBR	human (HeLa) debranching enzyme
HOAc	glacial acetic acid
HPLC	high performance liquid chromatography
Hz	Hertz
i.e.	that is
i-,n-,t-	iso, normal, tertiary
i-Bu	isobutyl

i-Pr	isopropyl
IVS	intervening sequence
LCAA-CPG	long-chain alkylamine controlled pore glass
M	molar
m	multiplet
MALDI TOF	matrix assisted laser desorption ionization time of flight
max	maximum
m/c	mass to charge ratio
MeOH	methanol
min	minute(s)
mL	milliliter
μL	microliter
mg	milligram
mM	millimolar
μM	micromolar
mol	mole
mRNA	messenger RNA
MS	mass spectroscopy
MW	molecular weight
NBA	nitrobenzyl alcohol
NEt_3	triethylamine
nm	nanometer
NMR	nuclear magnetic resonance
NPE	*p*-nitrophenylethyl
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PG	protecting group
P_2O_5	phosphorus pentoxide
ppm	parts per million
pre	precursor
Pu	purine

Py	pyrimidine
q	quartet
R_f	(TLC mobility) retardation factor
RNA	ribonucleic acid
rRNA	ribosomal RNA
r.t.	room temperature
s	singlet
S. cerevisiae	*Saccharomyces cerevisiae*
S. pombe	*Schizosaccharomyces pombe*
sec	second(s)
SnRNP	small nuclear ribonucleoprotein
SVPDE	snake venom phosphodiesterase
t	triplet
TBAF	tetra-*n*-butylammonium fluoride
TBDMS	*t*-butyldimethylsilyl
TCA	trichloroacetic acid
TEA	triethylamine
TEAA	triethylamine ammonium acetate
TEMED	N,N,N′,N′-tetramethylethylenediamine
THF	tetrahydrofuran
TLC	thin layer chromatography
TMSCl	trimethylchlorosilane
TRIS	2-amino-2-(hydroxymethyl)-1,3-propanediol
TREAT HF	triethylamine trihydrofluoride
tRNA	transfer RNA
TSA	toluenesulfonic acid
UV	ultraviolet
UV-VIS	ultraviolet-visible
v/v	volume by volume
yDBR	yeast debranching enzyme
XC	xylene cyanol

CHAPTER 1: INTRODUCTION

1.1 Importance of Nucleic Acids

Biological chemistry is a relatively new field that encompasses founding principles from a broad and well-established scope of more traditional disciplines. More specifically, this multidisciplinary integration of ideas has shaped the field of nucleic acid chemistry into a very influential discipline, marked by significant progress and remarkable developments in the past decade. This is not surprising since nucleic acids are considered to be the fundamental molecules of life, and operate through relatively well understood base recognition events. Additionally, the synthetic methodology for constructing small oligonucleotides is attractive, primarily because of its simplicity and automation as a result of the invention of DNA/RNA synthesizers over two decades ago. Deoxyribonucleic acid, DNA, is the molecule responsible for storing and transmitting genetic information[1]. However, it is the ribonucleic acid, RNA, which is responsible for carrying out the instructions encoded in the DNA template and can serve as a direct progenitor to protein synthesis.

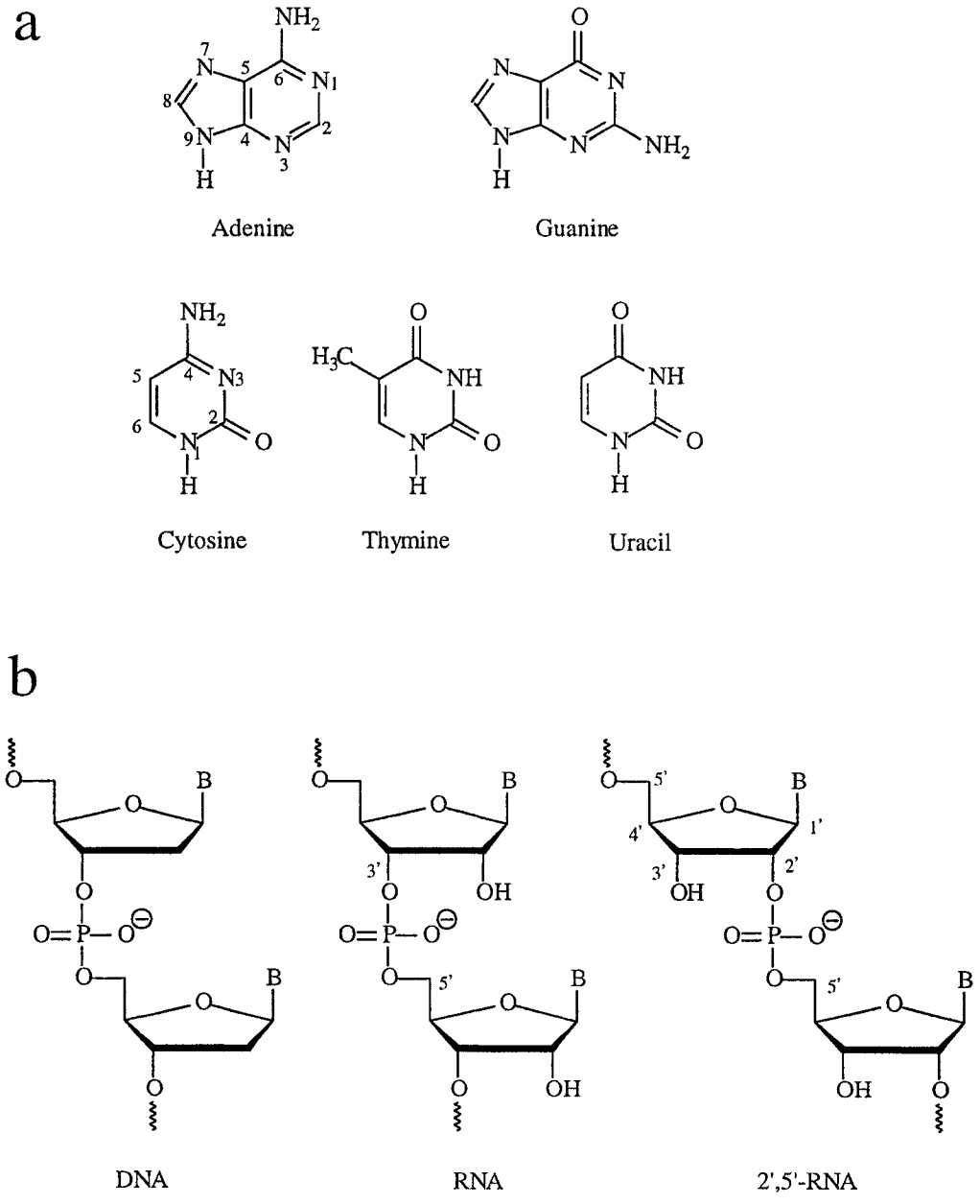
DNA and RNA are polymers of deoxyribonucleotide and ribonucleotide units, respectively. A nucleotide consists of a furanose sugar, a nitrogenous base and a phosphate moiety. The nitrogen heterocyclic bases can be divided into two classes, the bicyclic purines (Adenine and Guanine) and the monocyclic pyrimidines (Uracil, Cytosine and Thymine). The pentose sugars consist of β-D-deoxyribofuranose for DNA, and β-D-ribofuranose for RNA. The furanose sugars are attached to the heterocyclic bases through a C1'-N9 β-N-glycosidic bond for purines and a C1'-N1 β-N-glycosidic bond for pyrimidines (Figure 1). The corresponding nucleosides in turn are interconnected via phosphodiester linkages between the C3' hydroxyl of one nucleoside and the C5' hydroxyl of a neighbouring nucleoside.

Although DNA and RNA are very similar in their primary structure, subtle structural differences between these two classes manifest dramatically different functional consequences in the respective polymers. For example, the sugar units in DNA lack the 2'-hydroxyl groups, rendering DNA much more stable than RNA[2,3] both

chemically and enzymatically. This is because these 2'-hydroxyl groups on RNA can act as internal nucleophiles cleaving the RNA chain by forming 2′,3′-cyclic phosphates. Furthermore, DNA is typically double stranded with a helical formation whereas RNA is primarily single stranded. This pairing of the DNA to a complementary strand renders it less accessible and consequently more resilient towards chemical or enzymatic digestion, thermal decomposition or other harsh environmental conditions.

Accordingly, the generally greater level of stability of DNA as opposed to RNA, make the former class of molecules aptly suited for preservation of the genetic material while the latter serve to transmit this information and actively direct vital cellular functions.

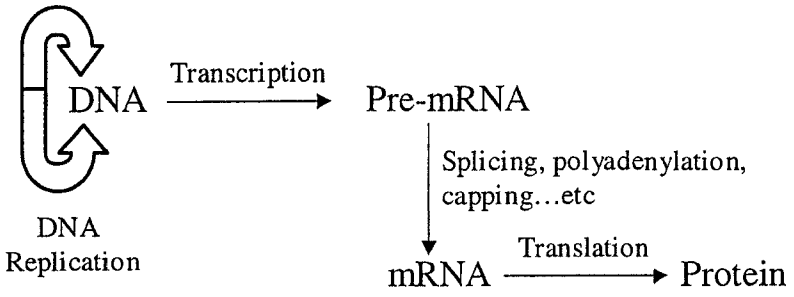
Figure 1: (**a**) Primary Structures of Heterocyclic Bases of DNA and RNA. (**b**) Common Chemical Constituents of DNA and RNA and their Connectivities in DNA, RNA, and 2',5'-Linked RNA, where B Represents the Heterocyclic Bases.



1.2 Flow of Genetic Information

The flow of genetic information is initiated by the partial unwinding of the double-stranded DNA followed by the transfer - or transcription - of its encoded message by an RNA polymerase to produce a messenger RNA (mRNA). In eukaryotes this mRNA, which is made in the nucleus, migrates to the cytoplasm and with the help of two other types of RNA, transfer RNA (tRNA) and ribosomal RNA (rRNA), is translated into a functional protein. For some time, this channeling of genetic information from DNA to proteins was widely believed to occur from a single continuous message, indeed the scenario for most prokaryotic organisms. However, the independent discoveries of P. A. Sharp[4] and R. Roberts[5] in 1977, proved that most eukaryotic RNAs contain segments that do not actually contribute to the final translation product *per se*. As such, the transcription of a particular genomic region does not directly produce the fully functional mRNA, but instead yields a precursor mRNA (pre-mRNA). This is because the nascent transcript consists of various 'expressed' or coding regions (*i.e.* exons) that may be interrupted by one or more non-coding or intervening sequences, called introns. It then has to undergo post-transcriptional processing prior to being translated into protein. These intricately controlled events allow for even greater diversity in the mRNA pool as well, since several different types of mature transcripts can be made from a single precursor, depending on how the information on the precursor is arranged during post-transcriptional processing. Introns have also been identified in genes coding for tRNA and rRNA, suggesting a rather important and perhaps well-defined role for these segments in RNA metabolism[6].

Figure 2: Flow of Genetic Information



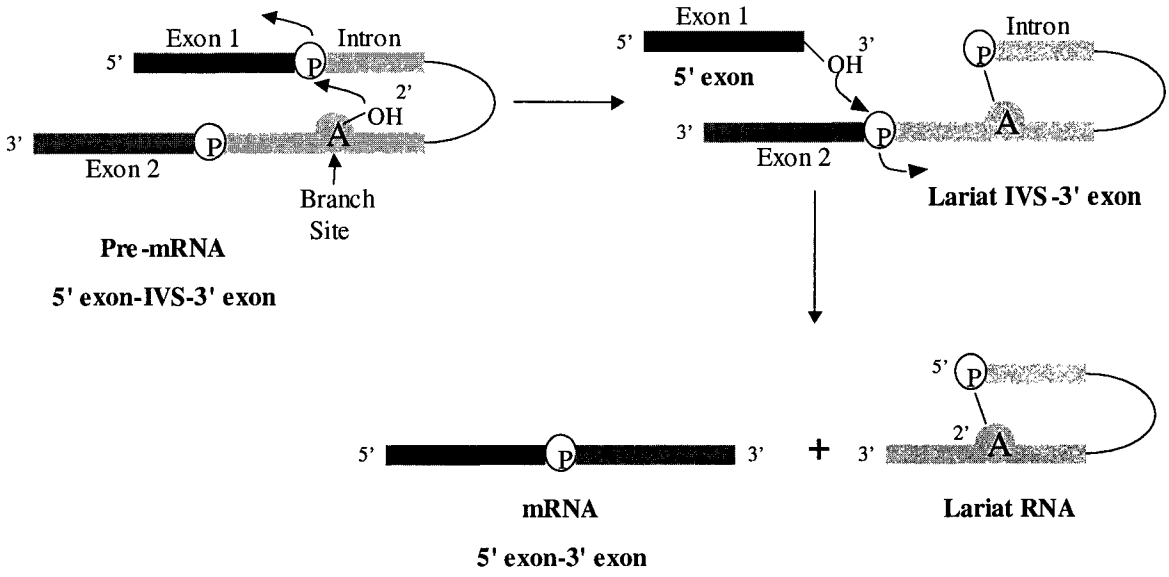
1.3 RNA Splicing

The cellular transformation of a pre-mRNA into its mature or functional form, mRNA, proceeds by way of a *splicing* reaction, in which the exons are ligated, or joined together, to form the mature linear RNA and the introns (intervening sequences, IVS) are excised in the shape of small circles with tails, or lariats[7].

Briefly, RNA splicing occurs by two transesterification reactions. The first step proceeds by an *intramolecular* nucleophilic attack of the 2'-hydroxyl of a specific "intronic" adenosine residue on the phosphate linkage at the 5'-site of the intron (Figure 3). This cleaves the 5'-exon/intron junction, creating a 2',5'–phosphodiester bond and liberating the first exon. Alternatively, the second step is characterized by an *intermolecular* nucleophilic reaction in which the 3'-hydroxyl of a terminal sugar residue in the free exon now attacks the phosphate at the remaining intron-exon junction. This joins both exon fragments through a regular 3',5'–phosphodiester bond and simultaneously cleaves the 3'-exon/intron junction, essentially transferring the 3'-exon to the 5'-exon with concomitant release of the intron from the former as lariat RNA.

In many eukaryotic organisms, these events are not autocatalytic and occur in a multiprotein complex, called the spliceosome[8,9,10]. The spliceosome consists of small nucleolar ribonucleoprotein units (snRNPs) which themselves are intricate assemblages of polypeptides and small nuclear RNAs (snRNAs). Assembly of these factors on the pre-mRNA occurs in a multistep fashion that involves binding and association of the snRNPs along the transcript to fold and orient the strand while holding the reactive centers in close proximity to aid in the subsequent transesterification steps.

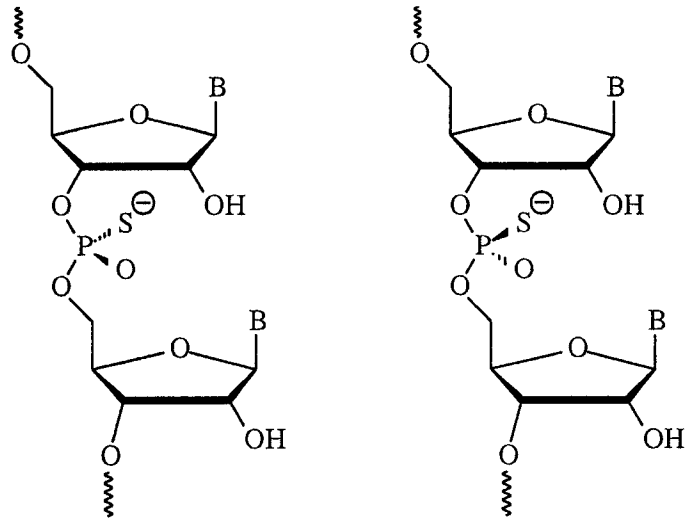
Figure 3: An Illustration of the Splicing Reaction.



where 'p' is a phosphate group, and 'A' represents the adenosine residue precursor used in lariat branch point formation.

Interestingly, the first step of splicing takes place with inversion of configuration at the phosphorus center, in accord with the transesterification model[11,12]. This was readily demonstrated by Maschhoff and Padgett[13], whose studies on the mechanism of splicing reactions provided the first substantial evidence of switched chirality around phosphorus upon formation of the branched structures. In their work, the natural phosphodiester linkage at the 5'-splice site of a pre-mRNA transcript was replaced with a phosphorothioate analogue (Figure 4). This substitution of a non-bridging oxygen by sulfur effectively confers chirality on the phosphorus center at this position. As such, analysis of the isolated products by snake venom phosphodiesterase digest was consistent with an in-line attack of phosphorus by the adenosyl hydroxyl group and thus no apparent covalent bond participation of the substrate with other factors in the spliceosomal complex. Further, only one of the two phosphorothioate stereoisomers was processed by the splicing apparatus, yet the stereochemical properties that enabled this selectivity were never further explored in the subsequent debranching of the chiral lariat products.

Figure 4: Structure of R_p and S_p phosphorothioate stereoisomers.



R_p S_p

1.4 The RNA Lariat Debranching Enzyme

As mentioned previously, the excised lariat RNA molecules have a unique structure where they have a circular core of variable nucleotide length attached to a stem of single stranded oligonucleotides. Specifically, the lariat topology is constrained in this form via a 'branch' point adenosine residue comprised of vicinal 2',5' and 3'5'-phosphodiester linkages (Figure 5). These branched molecules were first isolated from total nuclear RNA by Wallace and Edmonds in 1983[14]. Their low abundance relative to the mature mRNA suggested that the molecules were being hydrolyzed soon after their formation, probably to yield the corresponding linearized RNA.

Accordingly, a novel 2',5'-phosphodiesterase activity was observed by Ruskin and Green in 1985 in HeLa cell extracts. This "debranching" activity specifically hydrolyzes 2',5'-phosphodiester linkages in branched molecules to generate linear RNA species. Furthermore, only when the *in vitro* synthesized RNA lariats were deproteinized and added back to the extract was debranching observed, suggesting that the 2',5'-

phosphodiester linkages in these lariats are protected from cleavage by the lariat debranching enzyme during the normal *in vitro* splicing events[15].

The yeast debranching enzyme (yDBR) was later isolated and characterized by Boeke and Chapman in 1991[16]. Upon mutating the gene coding for the debranching enzyme, *dbr1*, the introns were found to be quite stable and accumulated to levels that exceeded those of the corresponding mRNA.

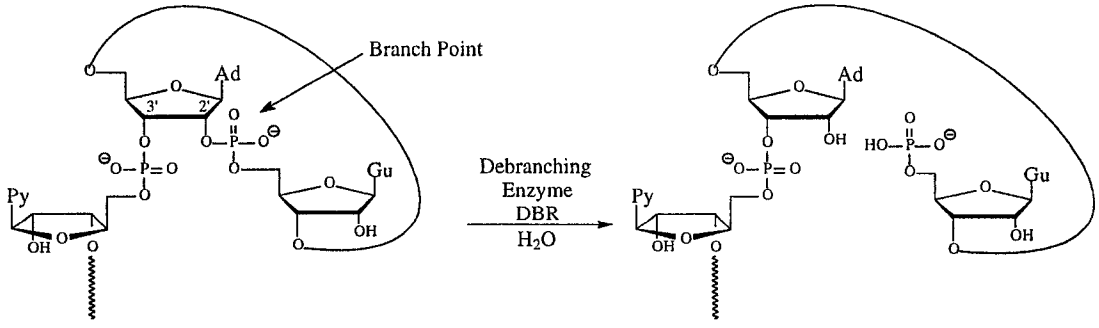
The only role the intronic part of the RNA was thought to possess was to provide protection by preventing nucleases from inducing damage to the messenger RNA. Also, the only role the lariat debranching enzyme was believed to have was to provide a method for linearizing the lariats for further degradation. However, recent studies have shown that these introns, along with the debranching enzyme, appear to play more essential and vital roles[17,18,19]. For example, small nucleolar RNAs (snoRNAs) play an important role in splicing by participating in processing pre-rRNA and modification of rRNA nucleotides. Some of these eukaryotic snoRNA are encoded in introns of protein genes and most mammalian snoRNAs known are intronic[18]. The production of these intronic snoRNAs was found to be dependent on the RNA lariat-debranching enzyme in *S. cerevisiae,* where it was shown that *dbr1* mutants produced the intronic snoRNA as "trapped" lariat forms, and the accumulation of mature intronic snoRNAs was reduced[17]. The function of DBR may be more crucial in higher eukaryotic cells, which contain a greater number of larger introns in most genes. In a study by Boeke and Nam, in which the gene coding for DBR was mutated, it was found that *S. pombe* mutants showed severe growth defects and altered cell morphologies relative to the wild type yeast strains. Alternatively, *S. cerevisiae*, a lower eukaryote, did not show any significant phenotypic defects, presumably because of its simpler genomic organization– *i.e.*, the latter species has much lower intron content, *ca.* 40% and 2.5%, respectively[19]. Conversely, *S. pombe* organisms appear to be much more dependent on nucleoside recycling via an intron degradation pathway. All these reasons make the DBR an essential enzyme, especially for higher eukaryotic organisms and probably even more crucial toward mammalian cell viability.

Although few studies have been carried out on the DBR enzyme, it is believed that there is probably a connection between this enzyme and the well characterized

Ser/Thr phosphatase family due to two reasons: (1) sequencing the gene coding for the DBR enzyme showed that it contains the GD/GNH signature motif which is characteristic of this class of enzymes, where the letters refer to the invariant amino acid residues of this class[20]. (2) As in the case of the Ser/Thr phosphatase family, the DBR exhibits enhanced activity in the presence of divalent cations such as Mg^{2+}, which was shown through collaboration between our group and Boeke *et al.* Further, yDBR was shown to digest various branched nucleic acids, but exhibited a strong preference for purines over pyrimidines at the branch point as well as the 2'-position. Conversely, the nucleotide at the 5'-position had only a negligible influence on debranching efficacy, since it was observed that branched oligonucleotides lacking the 5'-nucleotide were still highly susceptible to efficient debranching by the enzyme[21].

In a separate study examining the role of substrate stereochemistry on debranching activity, Padgett and coworkers[13] showed an inability for the HeLa cell RNA debranching enzyme to hydrolyze a branched oligonucleotide containing an R_p phosphorothioate at the 2'-5' linkage (Figure 4). It is noteworthy to mention that many enzymes are able to recognize and process only one isomer of a phosphorothioate substrate but not both.

Figure 5: Illustration of the Debranching Reaction of a Lariat RNA



1.5 Chemical Synthesis of Nucleic Acids

Many advances have been made to the chemical synthesis of RNA and DNA, without which the success that the nucleic acid field has been experiencing would not have been possible. The discovery of DNA is credited to the German scientist, Friedrich Miescher, although he never isolated it pure of proteins. Following this, there have been many discoveries from various disciplines that have contributed to a better appreciation of nucleic acid structure and function, and have greatly enriched the field. The discovery of the DNA helical structure occurred in 1953 when an X-ray crystallography of DNA fibers was taken by Wilkins and Franklin, and interpreted by Watson and Crick[22]. Two years later, the chemical synthesis of the first 3',5'-thymidine dinucleotide was reported by Michelson and Todd[23]. The synthetic pathways followed by Sir Alexander Todd and co-workers set some founding principles that were used for subsequent methodologies, such as phosphotriester and H-phosphonate approaches. Gobing Khorana employed the "phosphodiester approach" for oligonucleotide synthesis using carbodiimide reagents, such as DCC, to form the internucleotidic linkages in 1953[24]. Although this method provided an improvement to the existing methods, it still suffered the disadvantage of dealing with the ionic phosphodiester backbone. Introduction of the phosphate blocking group, the β–cyanoethyl moiety, to the phosphite triester approach by Letsinger and coworkers[25-29] along with the development of phosphoramidite synthons by Caruthers[30], overcame the disadvantage of the phosphodiester method to introduce the popular phosphotriester approach. Finally, an alternative method was introduced by Stawinski and coworkers in 1985 using the H-phosphonate approach, which offers the advantage of performing the oxidation of phosphite triester linkages only once at the end of the synthesis instead of the stepwise oxidation. However, this method is not as widely used as the phosphite triester method due to less efficient stepwise coupling, which is an important aspect especially when synthesizing long oligonucleotides.

Some of the major obstacles faced in synthesizing RNA/DNA oligomers are the undesired reactivities of the free exocyclic amines of the bases and the free hydroxyl groups of the sugar units that may occur during the coupling of one residue to another. The exocyclic amines are susceptible to the coupling reagents and can cause side

reactions, while the free hydroxyl groups on the sugar are susceptible to nonselective coupling and may yield a 3',3', or a 5',5'-phosphodiester linkage in addition to the desired 3',5'-linkage in DNA synthesis. In RNA synthesis, the presence of the 2'-hydroxyl group complicates the regiospecific addition of a monomer to the growing polymer even more. These reasons makes it essential to mask or protect these active sites prior to coupling; thus, protecting groups play a vital role in nucleic acid chemistry. The exocyclic amines may suitably be protected using acyl protecting groups. For example, adenosine and cytidine residues are typically protected in the form of benzoyl[31-32] (Bz) amides whereas guanosine is protected with an isobutyryl[33-34] (*i*-Bu) function. These protecting groups are stable against mild acidic or basic conditions and so are compatible with the overall oligomer synthesis. They can, however, be removed in a single step following oligonucleotide assembly using ammonium hydroxide.

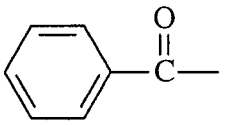
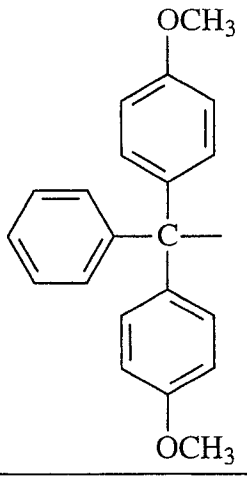
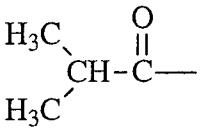
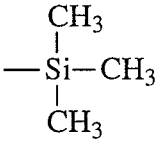
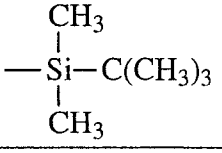
Conversely, a suitable strategy for differentiating among the hydroxyl groups of the monomeric sugars is not as trivial. Conventional approaches involve selective protection of the 5'-hydroxyl function, simply based on the fact that it is the only primary hydroxyl group, and therefore the most accessible. For this protection a bulky trityl group is used [dimethoxytrityl (DMT) or monomethoxytrityl (MMT)], which was introduced to nucleic acid chemistry by Khorana and coworkers[35]. The trityl protecting group not only masks the 5'-OH and renders it inert to reactions, but also imparts considerable lipophilicity to the nucleosides themselves and facilitates their dissolution in many organic solvents. During oligomer preparation, the protecting group is quickly removed under acidic conditions to liberate a trityl cation that is then quantitated by UV/Vis spectroscopy. Hence, this serves the added benefit of providing a good estimation of the coupling efficiency of each monomer addition of the multistep synthesis.

As already mentioned, RNA synthesis is more challenging than that of DNA as a consequence of the additional 2'-hydroxyl group on the sugar residues of the former. Thus, the 2'-hydroxyl must also be protected when synthesizing RNA oligomers in order to avoid intronucleotide cleavage and/or generating a random mixture of 2',5'- and 3',5'-internucleotide linkages during chain assembly. Conversely, if the 2',5'-linkage is desired, then only the 3'-hydroxyl in the nucleoside monomers is masked. The general protecting group for the 2', and/or 3'-hydroxyl moieties is the bulky *tert*-

butyldimethylsilyl (TBDMS) group, which was first used by Corey[36], but introduced to the nucleic acid area by Ogilvie and coworkers[37-39]. Using this protecting group yields mainly a monoprotected product as a mixture of the 2'- and 3'-monosilylated isomers (Scheme 2 in Results and Discussion). The resulting silyl ethers are stable under acidic conditions in which the trityl group is removed, but are rather labile toward fluoride and are easily displaced with triethylamine tris(hydrogen fluoride) or tetra-*n*-butylammonium fluoride (*i.e.* TBAF). Importantly, the mild conditions that are used to selectively remove this group disfavor isomerization and/or cleavage of the phosphodiester linkages.

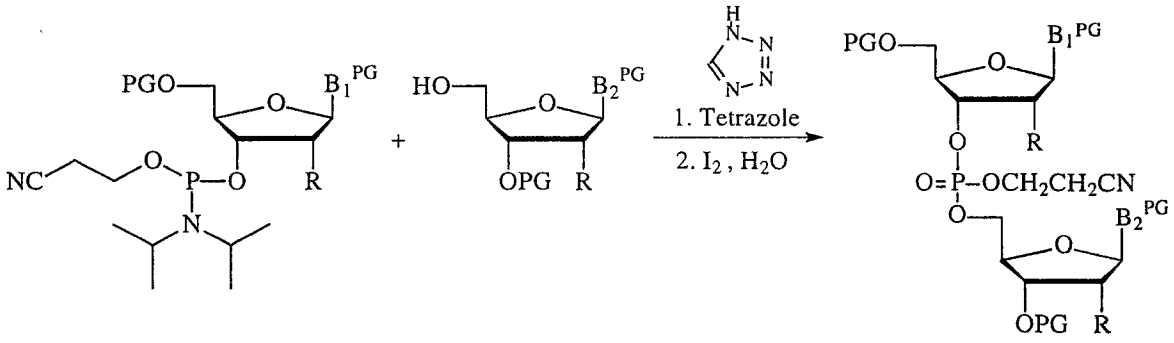
A relatively recent RNA synthesis method using 5'-O-Silyl-2'-O-orthoester protecting groups has been developed by Scaringe *et al*[40]. Both 5'- and 2'-protecting groups are stable toward RNA synthesis conditions, where the 5'-O-silyl ethers are labile to fluoride ions under neutral conditions, and the 2'-O-bis(2-acetoxyethoxy)methyl ether (ACE) is cleaved under mild acidic conditions. This 5'-O-SIL-2'-O-ACE chemistry enables clean synthesis of RNA oligonucleotides in high yields and is especially useful for longer oligonucleotides.

Table 1: Examples of Protecting Groups used in Nucleic Acid Synthesis

Protecting Group	Symbol	Structure	Protecting Position
Benzoyl	Bz		N^6-amino group of adenine N^4-amino group of cytosine
Dimethoxytrityl	DMT		5′-oxygen of sugar
Isobutyryl	*i*-Bu		N^2-amino group of guanine
Trimethylsilyl	TMS		2′, 3′-oxygens sugar
Tert-Butyl dimethylsilyl	TBDMS		2′ and/or 3′-oxygen of sugar

The last consideration in the chemical synthesis of DNA and RNA concerns the chemical nature of the internucleotide linkage. In 1975, this problem was addressed by Letsinger and coworkers, whose revolutionary contribution to the chemical synthesis of oligonucleotides resulted in the inception of the "phosphite triester" methodology[25-26,41]. Based on Letsinger's work, Caruthers and Beaucage introduced the use of nucleoside N,N-dialkylphosphoramidites to improve the synthesis of oligonucleotides[26,30,42]. This approach involves activating a nucleoside phosphoramidite by a weak acid such as tetrazole (pKa 4.9), leading it to couple quickly and almost quantitatively to a second suitably protected nucleoside or to a terminal residue of a growing chain (Figure 6). The phosphoramidite synthon is easily purified and stored as a stable powder at –20°C for long periods of time.

Figure 6: Coupling Reactions Using Phosphoramidite and Tetrazole.



nucleoside phosphoramidite

Where R=H in DNA, and OTBDMS in RNA

1.6 Solid Phase Synthesis of Oligonucleotides

The convenient implementation of nucleoside phosphoramidites as synthons in virtually all types of nucleic acid chemistries has made it possible to successfully commercialize the automated DNA/RNA synthesizer[43]. The main concept behind the "gene machine" involves the coupling of an excess of phosphoramidite, to achieve high coupling yields, to a protected nucleoside anchored to a solid support. The key steps of solid phase synthesis are: (1) attachment of a nucleoside to a solid support; (2) assembly

of the oligomer in the 3'- to 5'-direction; (3) removal of the oligonucleotide from the solid support; (4) complete deprotection of the synthesized oligonucleotide; and (5) purification of the deprotected oligonucleotide[44]. The second step (*i.e.* chain assembly) consists of: (I) Acetylation or "capping" of the support-bound protected nucleoside with acetic anhydride to mask any active sites and thereby prevent potential side reactions; (II) acidic detritylation of the 5'-DMT protecting groups, where both the reagents and the trityl cations are subsequently washed away; (III) addition and tetrazole activation of the 3'-phosphoramidites to extend the oligonucleotide; (IV) a final capping step to block unreacted hydroxyl termini and minimize the growth of shorter, incomplete sequences; and (V) oxidation of the intermediate phosphite triesters to yield the phosphate triesters. Steps (II) through (V) are repeated until the desired oligonucleotide is synthesized.

1.7 Synthesis of Branched Oligomers

Reports of solution-phase synthesis of branched oligomers first appeared in the literature soon after the initial identification of branched RNA in 1983. Most of these reports demonstrated the difficulties present in synthesizing vicinal 2',5'- and 3',5'-phosphodiester linkages in a stepwise manner.

Indeed, the chemical synthesis of bRNA/bDNA has been a topic of interest and careful investigation by Damha and coworkers[45-47]. Their initial work proved especially important since it set the foundations for solid phase synthesis of branched nucleic acids[45,48]. Using solid phase synthesis, three main methods were established to synthesize bNA.

The first method, which comprises the 'convergent growth' strategy[49] (Figure 7), involves growing two strands of oligomers and then joining them using a bisphosphoramidite branching synthon, introduced by Damha and coworkers[48], which can be further grown to yield a Y-structured RNA. The second method (Figure 8) involves the attachment of the first 5' residue to the solid support by its 5'-hydroxyl group and the growth of the 5' residues until the branched point residue in the reverse direction (5' to 3' direction)[50]. Two strands are then grown from the 2' and 3'-hydroxyls of the branched point by addition of 5'-phosphoramidites. Thus the molecule is grown in the

reverse direction (5' to 3' direction). These two methods, although straightforward, are limited in that the base sequences of the 2' and 3' tails are invariably identical to each other. The third method, although the most difficult to carry out (Figure 9), is also the most versatile[51]. In this method, one strand is grown in the regular 3' to 5' direction, where one of the residues consists of a uniquely 2'-protected sugar that may be selectively deprotected at any point in the synthesis to liberate the hydroxyl group. The deprotected residue can effectively serve as a branchpoint by initiating chain growth from the 2'-OH upon its unmasking. This method allows for more flexibility in the specific base composition of the 2' and 3' tails.

Figure 7: Solid-Phase Synthesis of bRNA Using the Convergent-Growth Approach[49].

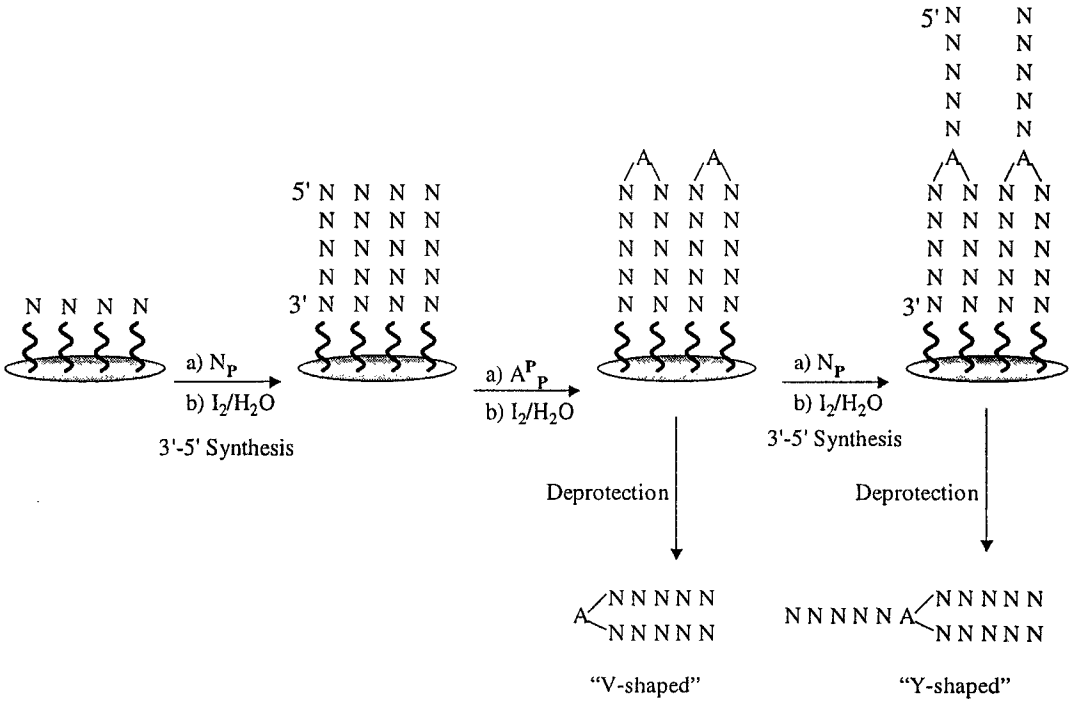


Figure 8: Solid-Phase Synthesis of bRNA Using the Divergent-Growth Approach[50].

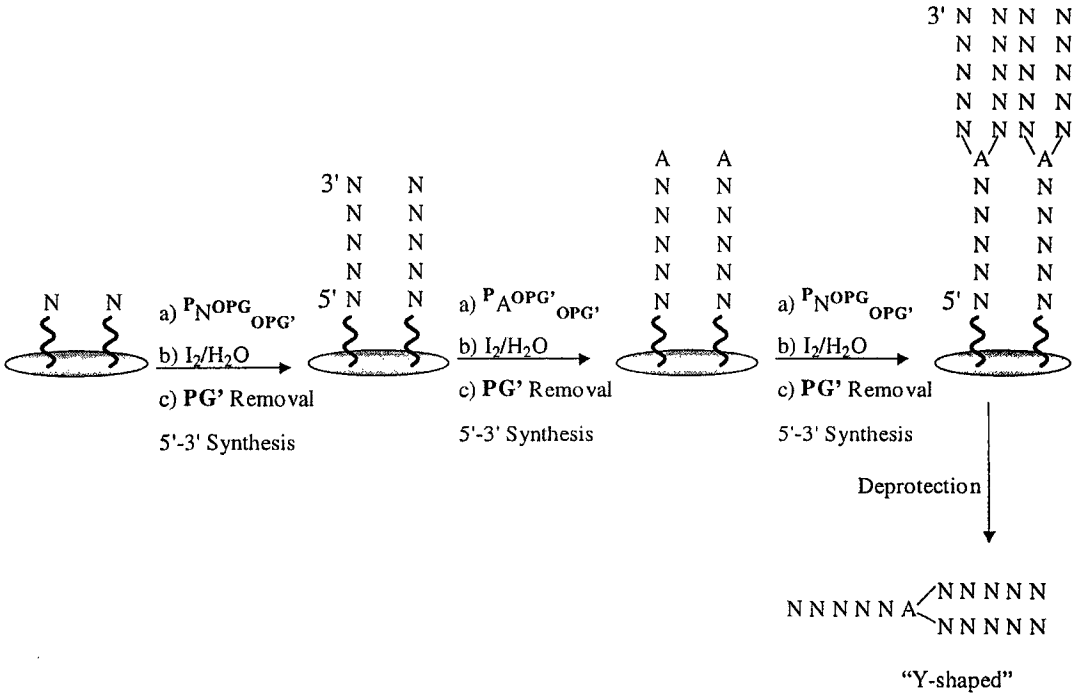
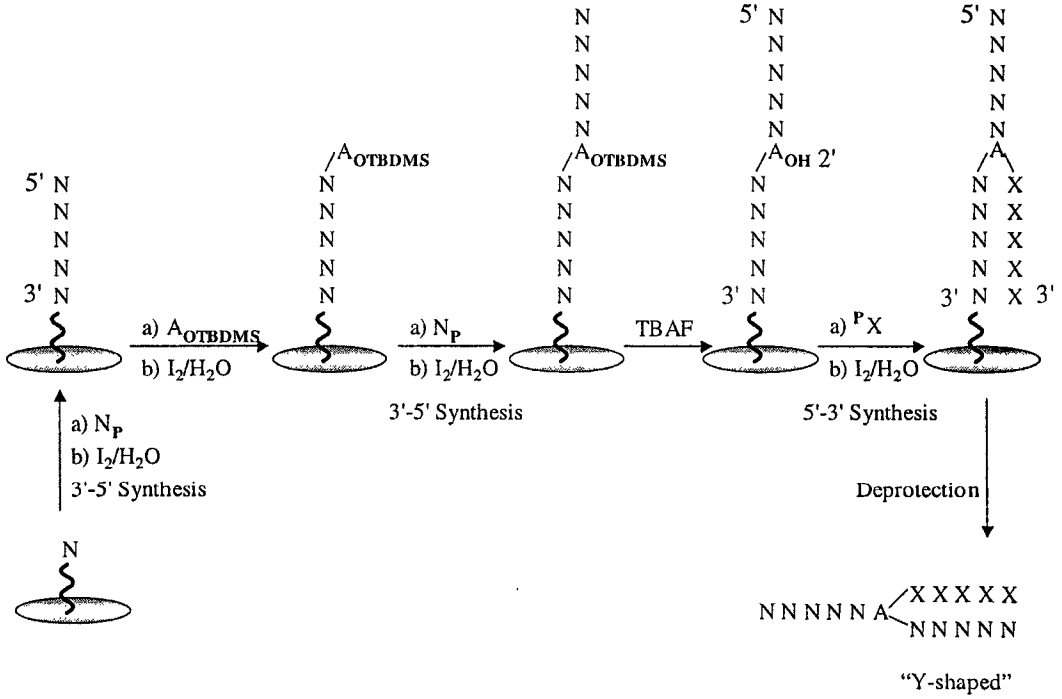


Figure 9: Solid-Phase Synthesis of bRNA Using Regioselective Convergent-Growth Approach[51].



1.8 Phosphorothioates

As mentioned above, a phosphorothioate linkage is an analogue of a phosphodiester in which a non-bridging oxygen atom is replaced with a sulfur atom (Figure 4)[52]. These analogues are very important and powerful tools that are used to unravel enzyme functions and mechanisms, and have potential application as reversible and irreversible inhibitors and spectroscopic probes of enzyme function. Exchanging the non-bridging oxygen with sulfur confers chirality on that particular phosphorus, generating at least a pair of diastereomers, without imposing a dramatic change to the properties of that atom.

The presence of phosphorothioates in RNA substrates has an effect on many enzymatic reactions, either by slowing them down as in the case of the hydrolysis of an R_p-phosphorothioate by Snake venom exonucleolysis or even entirely abolishing activity in some cases as in the case of hydrolysis of an S_p-phosphorothioate by the Klenow enzyme, *E. coli* DNA polymerase I[53]. Other examples of these enzymatic reactions include ribonucleases[54-55] and splicing reactions[12,13,56]. The phosphorothioate modification is likely to have significant effects on the debranching efficiency of the substrates prepared for this study as well. Thorough insights into the mechanistic properties of the debranching enzyme should be feasible using these compounds as models, and based on the general reasoning that oxygen substitution with sulfur should not significantly alter the structure of oligonucleotide. Indeed, the similarities between the two types of linkages were first realized in x-ray crystallographic analyses of a hexamer containing phosphorothioate linkages at every other linkage (R_p configuration)[57], and NMR studies of two (R_p)-PS-ODN/oligonucleotide hybrids[58-59].

Attempts to explain the inability of many enzymes to efficiently hydrolyze various phosphorothioates have often been proposed, despite the lack of any apparent difference in structure between the phosphorothioate oligonucleotides and their parent compounds. A general explanation for this difference of enzymatic reactivity was proposed based on the x-ray structure of the guanosine γ-thio-triphosphate (GTPγS), which is resistant to hydrolysis by transducin[60], a signal-coupling protein in visual excitation. The bulky sulfur atom induces a higher energy of activation for the substrates

to reach the transition state, accompanied by greater steric hindrance around phosphorus, which prevents water from reaching the γ-phosphate for nucleophilic attack. Other work by Brautigam and Steitz in 1998[61] on the crystallographic properties of the S_p isomer of a phosphorothioate oligonucleotide, which had been shown to be resistant to hydrolysis by *E. coli* DNA polymerase I, was carried out. The x-ray structure showed that the sulfur atom in the S_p isomer had displaced the two metal ions at the active site that were essential for activity, thus providing another explanation for this enzymatic discrimination.

Phosphorothioate oligonucleotides can be prepared following a similar procedure as that for the phosphodiester oligonucleotides using both the phosphotriester and the phosphite triester method. An earlier method for phosphorothioate synthesis using the phosphotriester approach was carried out by condensing β-cyanoethyl S-phosphorothioate with the 3'- and 5'-hydroxyl groups of the appropriately protected nucleosides followed by alkaline removal of the protecting groups (Figure 10)[62]. However, simultaneously with the β-elimination reaction to remove the β-cyanoethyl group, the undesired direct nucleophilic attack of water on phosphorus led to the loss of the sulfur, as high as 40%. The phosphite triester method is quite simple and attractive, since it is very similar to the procedure used for the synthesis of phosphodiester bonds. The only difference is the replacement of the oxidation step using I_2/H_2O by the addition of a "sulfurization reagent" to the phosphite triester to obtain the sulfur transfer (Figure 11)[63]. Currently, there are many successful sulfur transfer reagents that can be used to synthesize phosphorothioate bonds. Examples of these reagents are (1) 3*H*-1,2-Benzodithiole-3one 1,1-Dioxide, or the Beaucage reagent, which is one on the most common reagents used these days[64], and (2) 3-ethoxy-1,2,4-dithiazoline-5-one, or the EDITH reagent, which is a relatively new yet efficient sulfurizing reagent[65-67].

Figure 10: Synthesis of Dinucleoside Phosphorothioates Using the Triester Method.

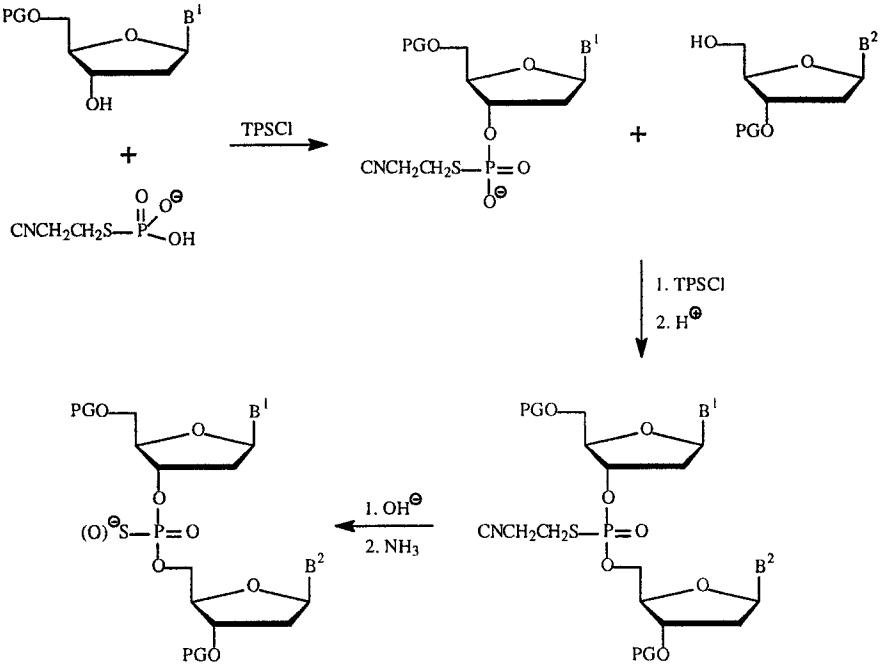
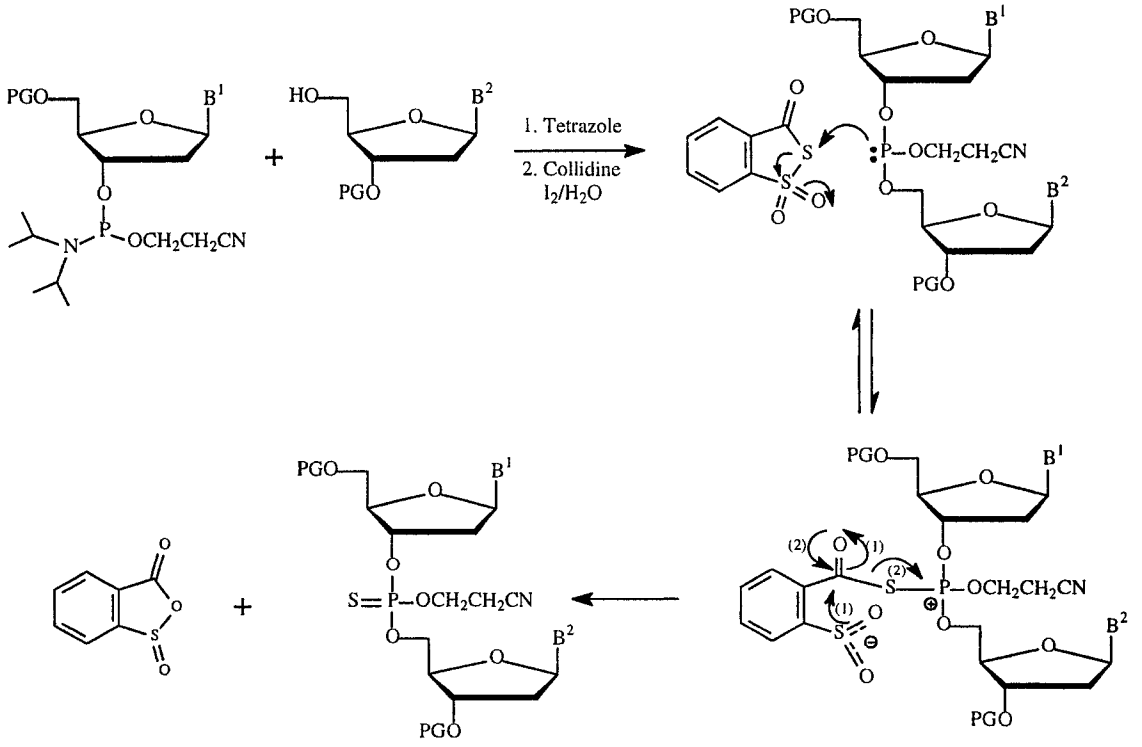


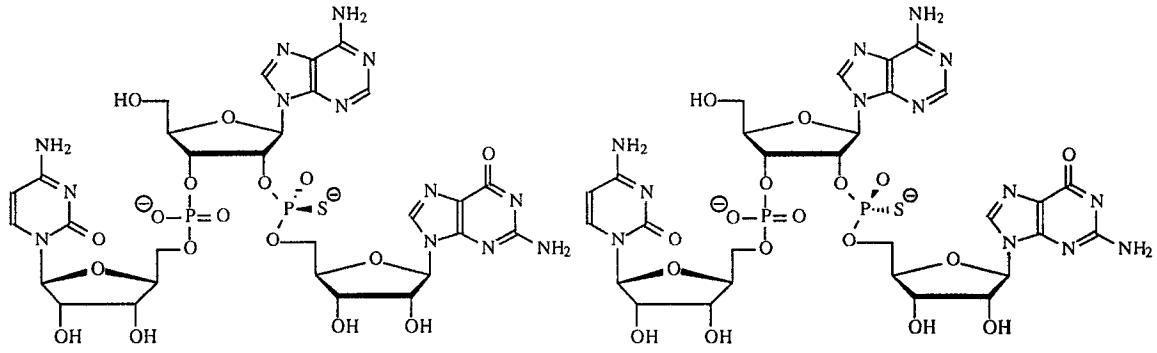
Figure 11: Sulfurization of a Dinucleoside Phosphite Triester by the Beaucage Reagent.



1.9 Objectives

With the recent studies and discoveries on DBR, more is known and appreciated about its importance and essential role, especially in higher eukaryotic cells. However, not much information currently exists regarding its precise mechanism of action, structure or substrate specificity. It is thus the focus of this thesis to explore the solution syntheses of the first branched RNA fragments with specified stereochemistry at the 2',5'-phosphorothioate linkage (Figure 12). Both isomers of the branched phosphorothioate will be used as substrates to further elucidate the particular substrate specificity of this important enzyme. Ultimately, this study serves to enhance previous yDBR investigations, and our knowledge about this unique polypeptide, such as its stereochemical preferences and possibly its X-ray structure, if one of the isomers proves to be an inhibitor or substrate that is capable of impeding the rate of reaction as compared to the natural homologue.

Figure 12: Structure of the R_p and S_p-Branched Oligonucleotide Isomers.



S_p-branched trinucleotide isomer

R_p-branched trinucelotide isomer

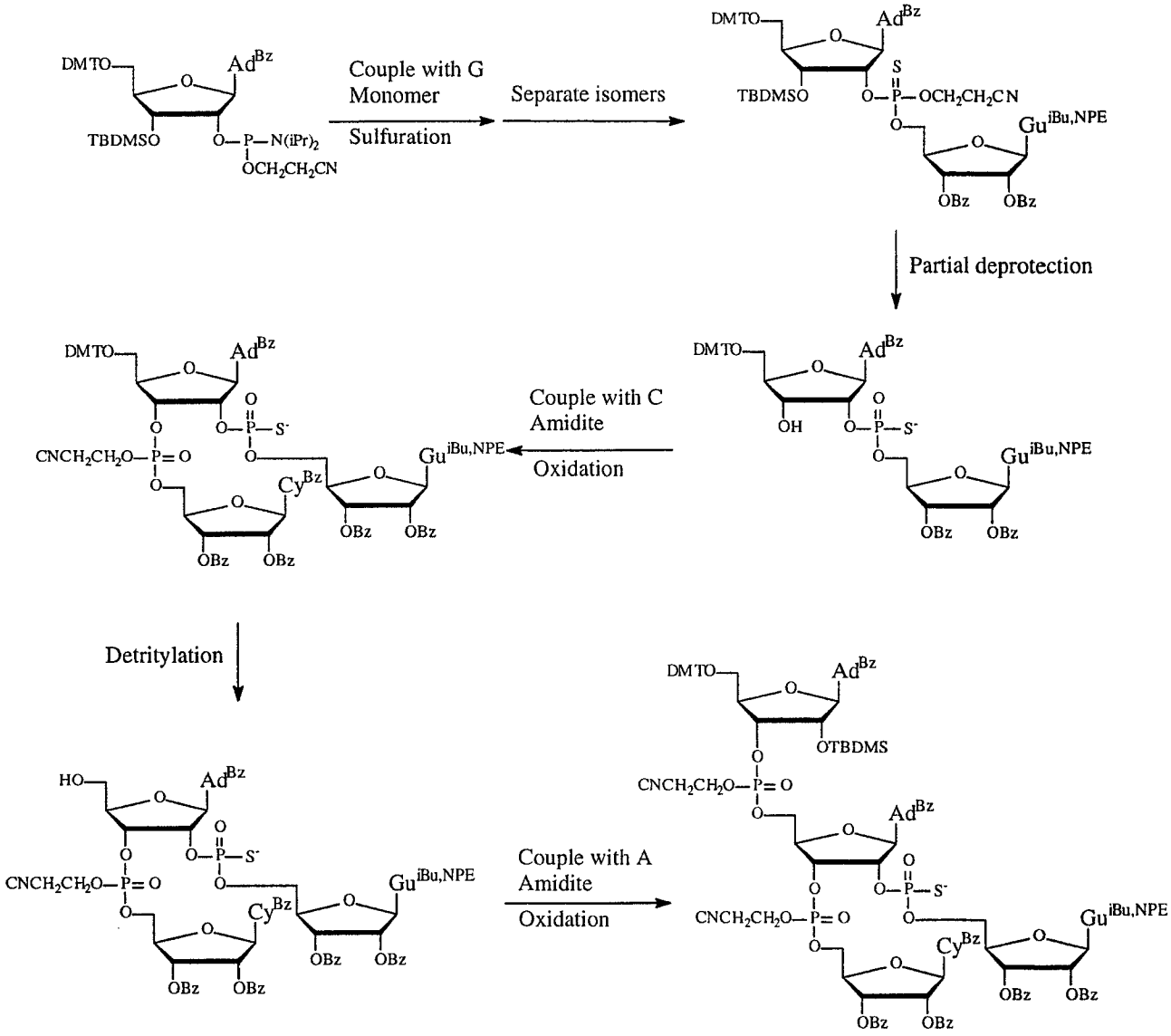
CHAPTER 2: SOLUTION SYNTHESIS OF PHOSPHOROTHIOATE BRANCHED OLIGONUCLEOTIDES

The study carried out by Padgett and coworkers[13] revealed that an RNA lariat containing an R_p 2′,5′-phosphorothioate linkage was not susceptible to hydrolysis with the HeLa cell RNA debranching enzyme. Since the S_p diastereoisomer was not available to them, it is not known whether the enzyme can process S_p 2′,5′-phosphorothioate linkages. The RNA lariat debranching enzyme appears to be present in all eukaryotic organisms, from yeast (*S. cerevisiae*) to mammalian cells. In order to further our understanding and insight into this interesting enzyme, the importance of which has only been discovered recently, some small branched oligonucleotides with 2′,5′-phosphorothioate linkages, were synthesized and tested as possible substrates. Such branched oligonucleotides allow us to probe the enzyme's substrate specificity, mechanism of action and may potentially yield information about its active site. Furthermore, if the R_p stereoisomer were to act as a competitive inhibitor and bind to the enzyme without being debranched, it would be a promising candidate for the future co-crystallization of enzyme: substrate complexes.

2.1 Monomer Synthesis

In RNA synthesis, blocking (or "protecting") the reactive hydroxyl and exocyclic amino groups is essential and a key element for a successful synthesis. The protecting groups must be stable throughout chain assembly and labile enough during post-synthesis deprotection while maintaining the integrity of the RNA chain. The developed and commonly used method for RNA solid-phase synthesis is adequate for oligonucleotides containing 3′,5′-phosphodiester linkages. However, in order to synthesize a branched molecule with a 2′,5′-phosphorothioate bond a different method was followed (Scheme 1). The adequately protected monomers ("building blocks") were synthesized via solution-based methods as shown in Schemes 2, 3 & 5.

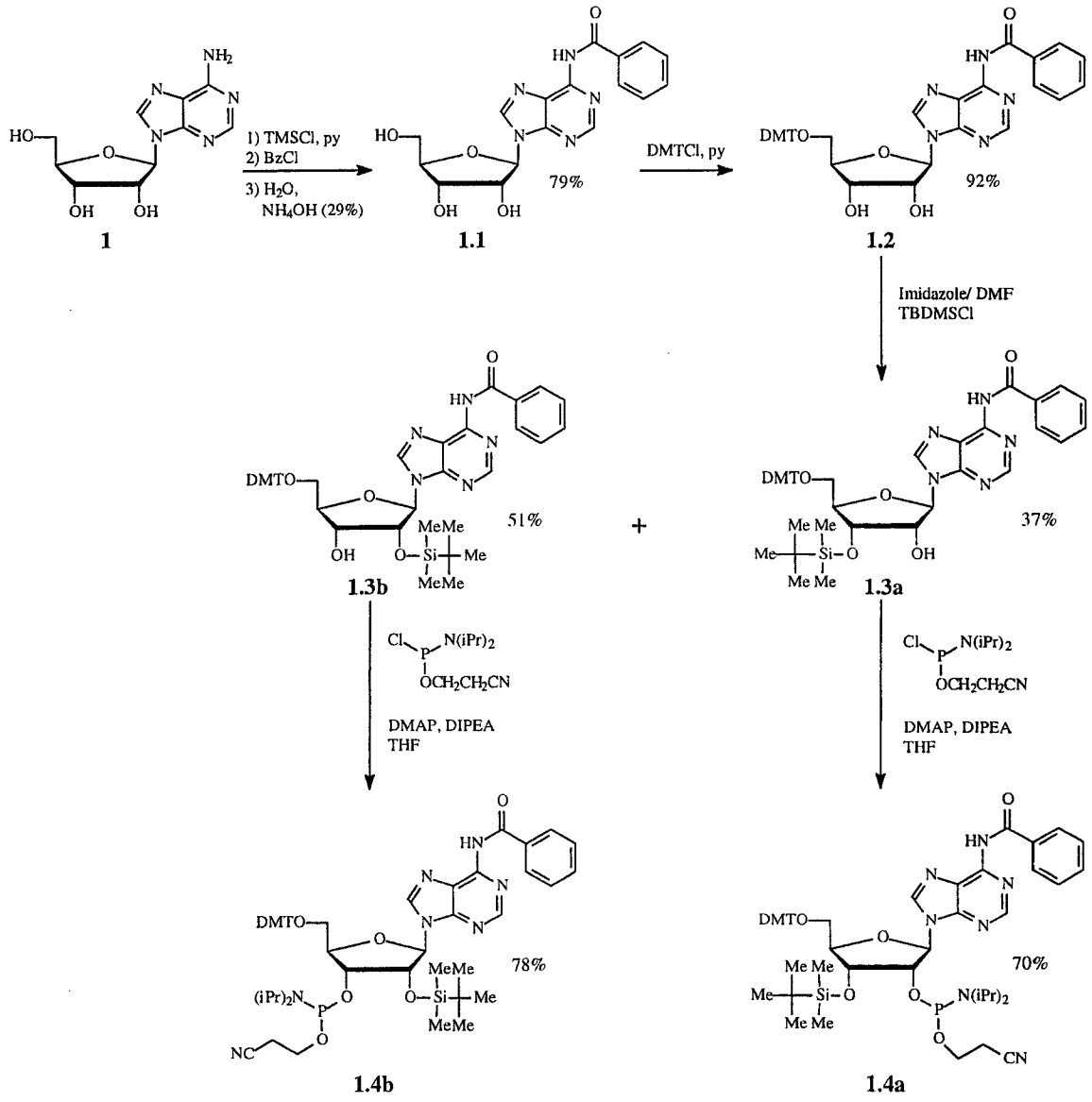
Scheme 1: General Procedure for the Regiospecific Synthesis of the Branched Oligonucleotides.



2.1.1 Synthesis of Both the Adenosine 2′- and 3′-Phosphoramidites:

Two of the required monomers utilized in the synthesis of the Y-tetramer are the regioisomeric adenosine phosphoramidites **1.4a** and **1.4b**. The exocyclic amino group of adenosine is first benzoylated (Bz) using the transient procedure developed by Jones *et al*[68]. This is followed by the protection of the primary hydroxyl group, 5′-OH, using the bulky 4′,4′-dimethoxytrityl group (DMT). The 2′- and 3′-hydroxyls are then protected using *t*-butyldimethylsilyl group (TBDMS) to yield a mixture of 2' and 3'-monosilylated nucleosides. Separation of the two isomers is possible by way of flash chromatography, with the 2′-silylated isomer traveling faster on the silica gel than the 3'-isomer. Although the DMF-imidazole silylation procedure proposed by Ogilvie *et al.* does not give the same level of selectivity for the 2′-TBDMS isomer as some other procedures[37b,69,70], it was the method of choice since both silylated isomers are required in the study. Each isomer was then phosphitylated separately to yield the corresponding phosphoramidite derivative (Scheme 2). These were obtained as white foams and were stored at –20°C before use.

Scheme 2: Synthesis of Both the Adenosine 2′- and 3′-Phosphoramidites.



2.1.2 Synthesis of N^2-iBu-O^6-NPE-2',3'-di-Bz-Guanosine:

The exocyclic amino group of the unprotected guanosine is first acylated with an isobutyryl protecting group (*i*Bu) using a transient procedure similar to that proposed by Jones *et al.*[68], however, isobutyric anhydride was used in the place of benzoyl chloride. This is followed by the protection of the 5′-primary hydroxyl group using the bulky 4′,4′-

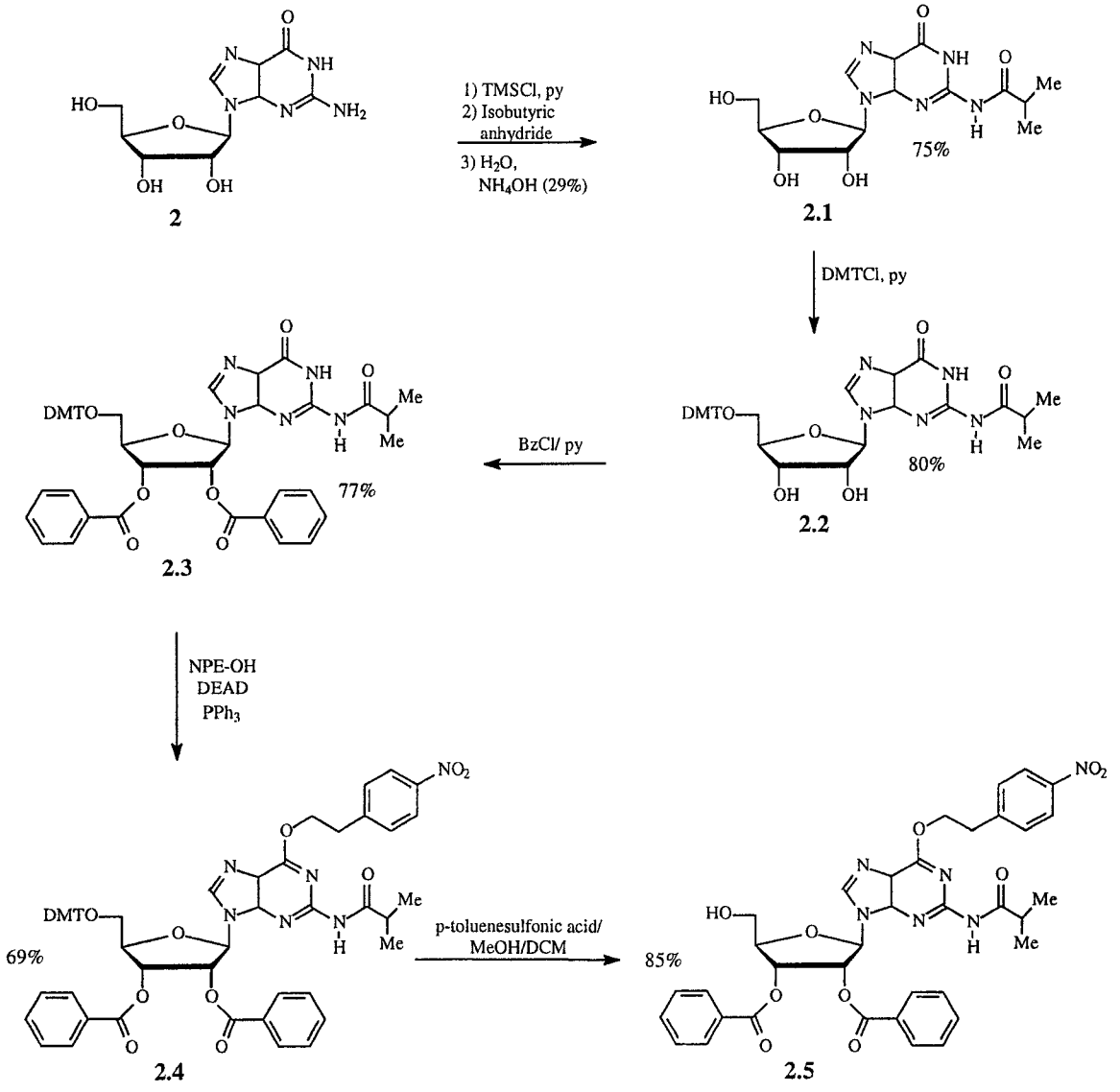
dimethoxytrityl group (DMT). The 2′- and 3′-hydroxyls were both protected using a benzoyl protecting group. This protecting group was used since it is not as bulky as the TBDMS moiety, and as such will provide the di-protected nucleoside in higher yields. It also provides an orthogonal protecting group to the TBDMS present on the adenosine phosphoramidite, consequently enabling us to carry out selective deprotections.

2.1.3 Benzoylation of ($^{DMT}G^{N\text{-}iBu}$) (**2.2**):

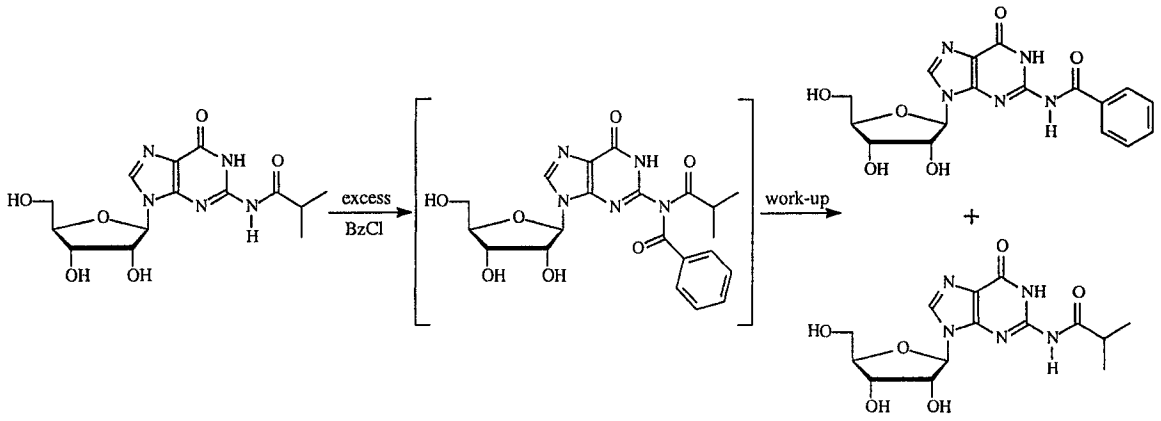
The benzoylation of the 2′,3′-hydroxyl groups of ($^{DMT}G^{iBu}$) was initially carried out by using a 6-fold excess of (BzCl) in order to drive the reaction to completion and obtain the di-benzoylated product (Scheme 3). Analysis of the ^{1}H-NMR spectrum of the isolated product revealed the presence of two very similar compounds (Figure 13B). One of these two products corresponds to the 2',3'-di-benzoylated nucleoside bearing the desired isobutyryl (*i*Bu) protecting group at the exocyclic amino position (indicated with an * in Figure 13B). The other product (indicated with a • in Figure 13B) bares a benzoyl (Bz) group at the amino position. These results were confirmed using mass spectroscopy where molecular weights of 863 g/mol and 897 g/mol were observed. The ratio of the N-isobutyrylated to the N-benzoylated nucleosides, calculated from the integration of the ^{1}H-NMR signals, was *ca.* 2: 1.

However the ratio of the above two derivatives changed when the amount of BzCl used was decreased from 6 to 2 equivalents. Analysis of the mass spectra of the products detected the presence of two N-benzoylated groups, consistent with the formation of N^2-isobutyryl-2',3'-O-di-benzoyl-5'-O-(4,4'-dimethoxytrityl) guanosine and N^2-benzoyl-2',3'-O-di-benzoyl-5'-O-(4,4'-dimethoxytrityl) guanosine (Figure 14). This suggested that, in addition to benzoylating the 2' and 3' hydroxyl groups, the isobutyrylated amine on the base also underwent partial benzoylation. In the basic workup step, one of those two protecting groups falls off leaving either the isobutyrylated or the benzoylated amine in the observed 2:1 ratio (Scheme 4).

Scheme 3: Synthesis of Protected Guanosine.



Scheme 4: Partial Benzoylation of an Isobutyrylated Guanine.

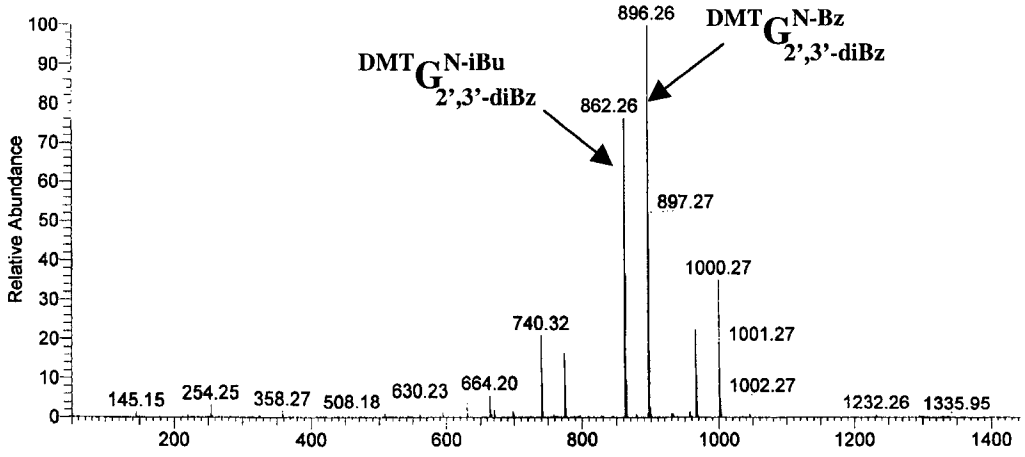


Nucleic acid chemists favor using *i*Bu, to protect the exocyclic amino function of guanosine, instead of Bz protection because the former is more labile. The results obtained from the reaction of base protected guanosine with 6 equivalents of BzCl indicate the presence of approximately 30% N-benzoylated and 70% N-isobutyrylated nucleosides. The inability to yield a pure 100% N-benzoylated nucleoside suggests that the presence of two protecting groups, on the tertiary amine, could somehow be facilitating the removal of the benzoyl group compared to the isobutyryl one.

Since at the end of the RNA branched oligonucleotide synthesis the protecting groups will all be removed, both products should converge to one. Therefore, we reasoned that having these two different amino protecting groups should not cause a problem and that the spectral signals of the N-isobutyryl and N-benzoyl groups (2:1 ratio) can be used as internal references during the solution synthesis of bRNA.

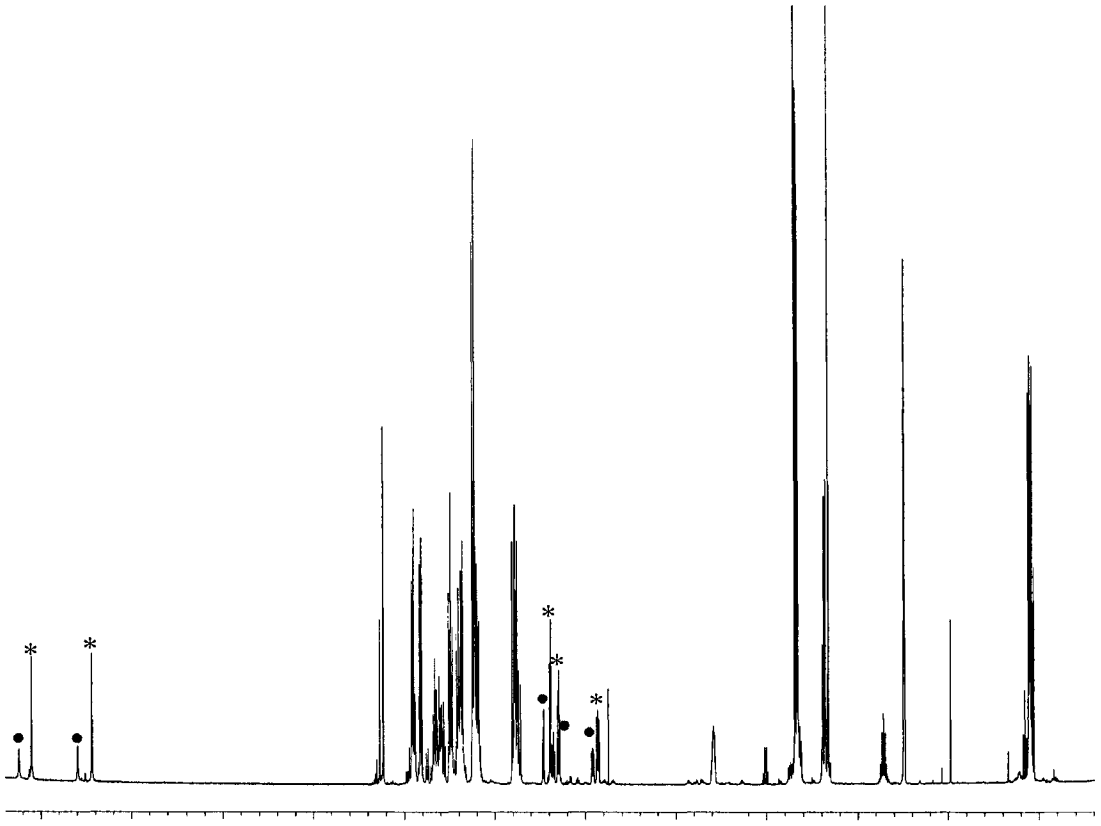
Figure 13: ^{1}H-NMR and Mass Spectra of the Product of Benzoylation of $^{DMT}G^{N\text{-}iBu}$ **(2.2)** Using 6 Equivalents of BzCl.

a



FAB Mass Spectrum (negative mode) of the products isolated after benzoylation of $^{DMT}G^{iBu}$.

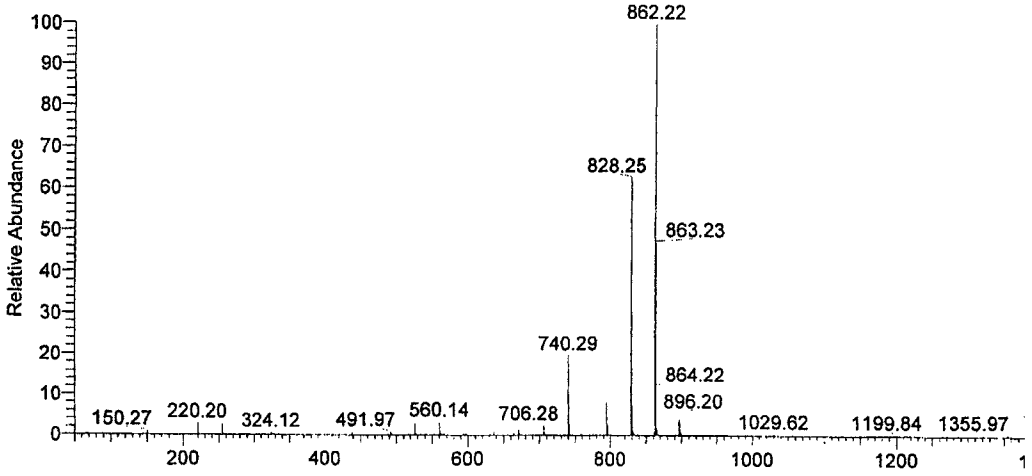
b



^{1}H-NMR Spectrum of the product, where * indicates the peaks corresponding to the desired N-iBu nucleoside and • indicates the peaks corresponding to the N-Bz nucleoside.

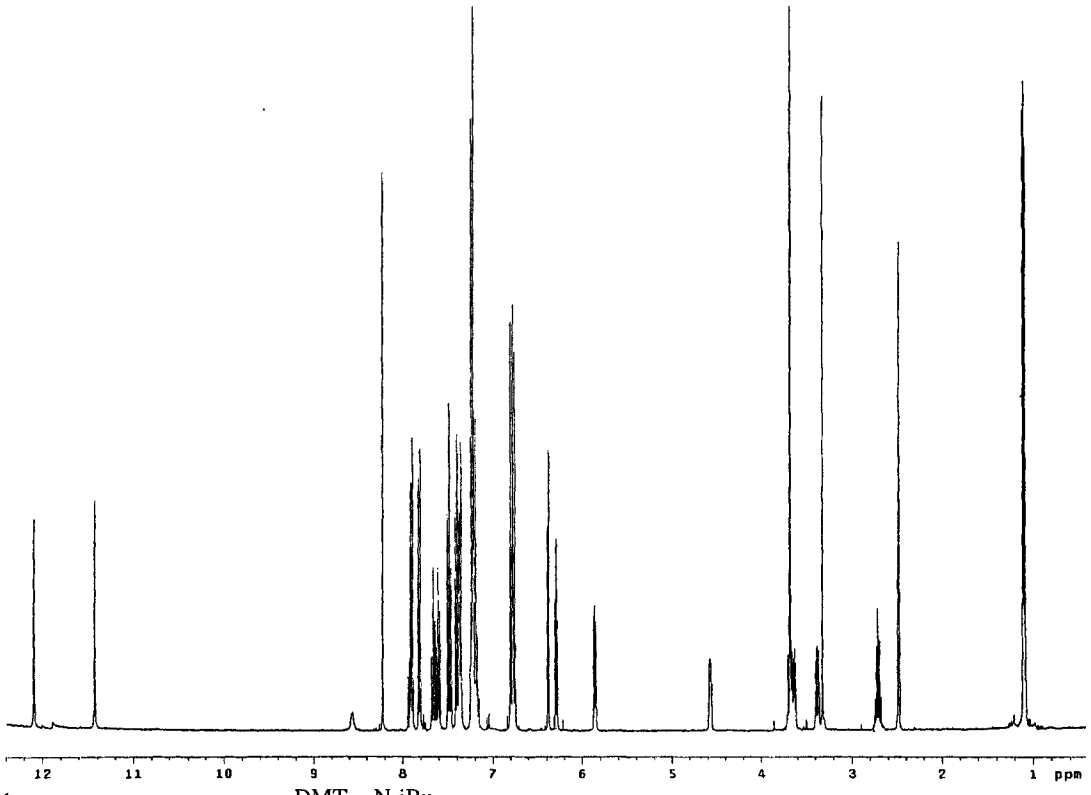
Figure 14: ^{1}H-NMR and Mass Spectra of the Product of Benzoylation of $^{DMT}G^{N\text{-}iBu}$ **(2.2)** Using 2 Equivalents of BzCl.

a



FAB Mass Spectrum (negative mode) of the products isolated after benzoylation of $^{DMT}G^{iBu}$, where the peak at 862.2 corresponds to the desired N-iBu nucleoside.

b



^{1}H-NMR Spectrum of $^{DMT}G^{N\text{-}iBu}_{2',3'\text{-}diBz}$ nucleoside.

2.1.4 Flash Chromatography Purification of ($^{DMT}_{Bz}G^{iBu,\ NPE}_{Bz}$) (**2.4**):

The O^6-position on the guanosine heterocycle was found to react with phosphoramidites during coupling reactions in solution and solid-phase syntheses[71-72]. However, Pon *et al.*[73] pointed out that any possible phosphitylation of the guanine ring at O^6 was readily cleaved by water or acetate anion to regenerate the free O^6. In solid phase synthesis, the capping step, which is carried out prior to the oxidation step, utilizes a mixture of acetic anhydride, 2,6-lutidine, and DMAP, which is a source of acetate ions. However, it is essential to protect this reactive site during solution synthesis since the "capping step" is not used. The protecting group of choice is the p-nitrophenylethyl (NPE) group introduced by variations of published protocols[74] (Scheme 3). The O^6-*p*-nitrophenylethyl protected guanosine was purified by flash silica gel chromatography using a gradient solvent system (methylene chloride/methanol). However, a UV active impurity, which was more polar than the product (9:1 CH_2Cl_2/MeOH), elutes first followed by a mixture of the product and the impurity. Further TLC analysis using CH_2Cl_2 as the solvent shows a reversal of relative polarity between the product and the impurity since this is the initial solvent used to pack the silica gel column. The polarity switch makes it difficult to purify the product using a methanol gradient. Therefore, the product was purified by flash gel chromatography using first CH_2Cl_2 until all the impurity, 4-nitrophenylethyl alcohol, had eluted followed by a 1-3% MeOH/CH_2Cl_2 gradient to elute the desired product.

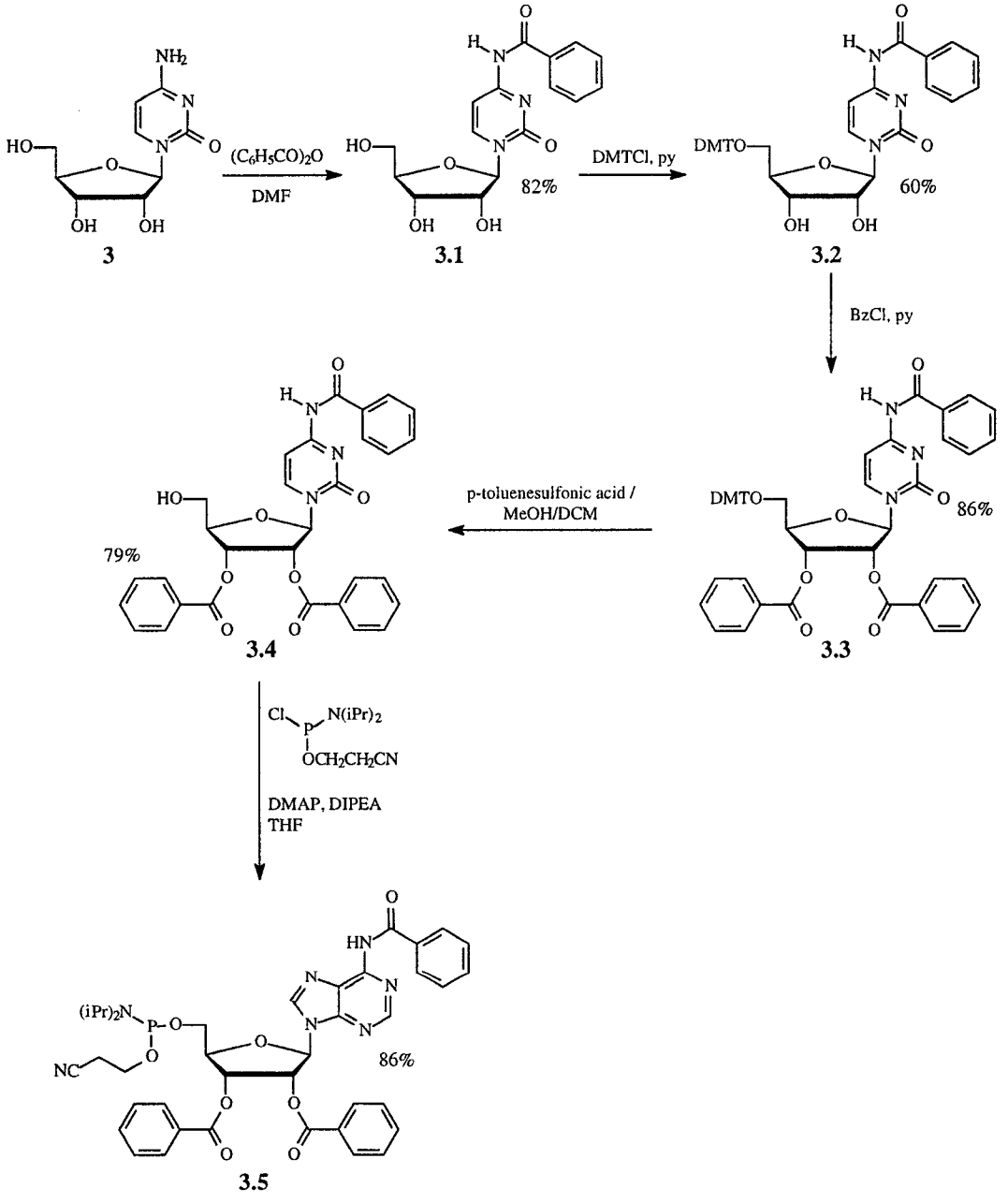
The trityl-protecting group was then removed using p-toluenesulfonic acid/MeOH/DCM to yield nucleoside **2.5** (Scheme 3).

2.1.5 Synthesis of the Cytidine 5′-Phosphoramidites:

Contrary to the previous phosphoramidites synthesized en route to the Y-tetramer products, the cytidine building block synthesized (**3.5**) contained a 5′-phosphoramidite moiety that would be coupled to a free 3′-hydroxyl of the suitably protected A(p_sG) dimer, to yield the V-trimer A(p_sG)pC . The exocyclic amino group of the cytidine is masked with a benzoyl protecting group (Bz) using the convenient N-acylating procedure

proposed by Bhat *et al.*[75] This is followed by the protection of the primary hydroxyl group, 5′-OH, using the bulky 4′,4′-dimethoxytrityl group (DMT). The 2′- and 3′-hydroxyls were both benzoylated and the trityl protecting group removed under acidic conditions to yield **3.4**. Finally, the free 5′-hydroxyl group was phosphitylated to yield the desired 5′-phosphoramidite derivative **3.5** (Scheme 5).

Scheme 5: Synthesis of the Cytidine 5′-Phosphoramidites



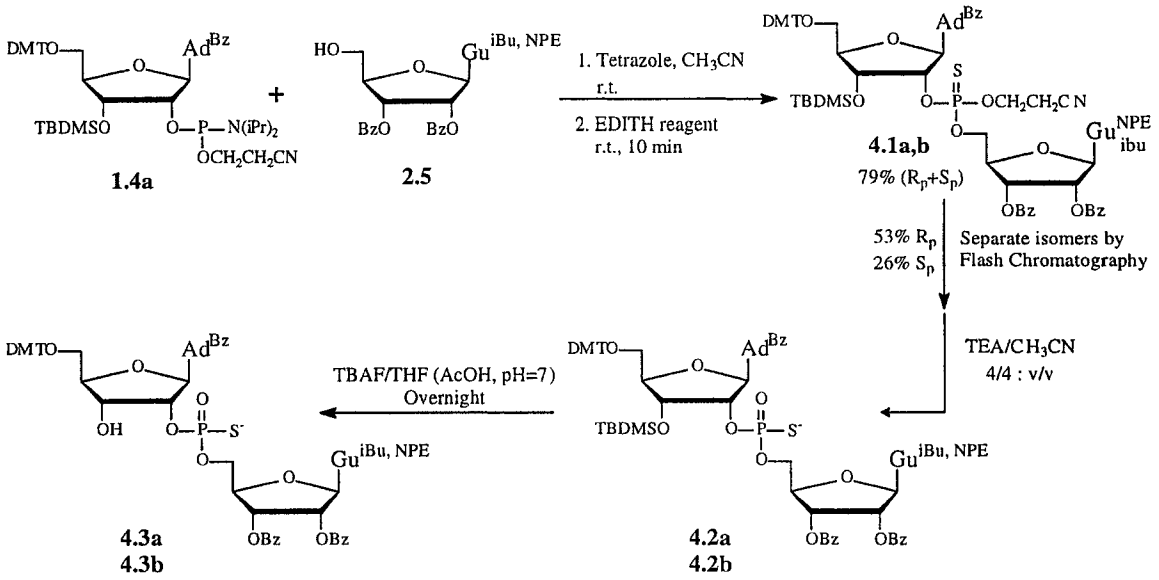
2.2 Oligomer Synthesis

Solid-phase RNA synthesis is more efficient and convenient than solution phase RNA synthesis. Primarily, it conserves time, owing to the fact that excess reagents can be washed away after each coupling step thereby eliminating the need for purification during synthesis of the RNA chain. However, the synthesis of branched oligonucleotides containing 2′,5′- and 3′,5′-phosphodiester linkages in which the 2′,5′-linkage, is a stereoisomerically pure phosphorothioate isomer, presents a challenge that has never been carried out by solid phase methods. An earlier attempt to synthesize branched oligonucleotides, comprising of a mixture of R_p and S_p 2′,5′-linked phosphorothioate isomers, by solid phase has been undertaken in our laboratory by Dr. J. Chen. Running a polyacrylamide gel (PAGE) of the products revealed the presence of multiple nucleotides. The unpredictable gel electrophoretic mobility of small bRNAs in addition to these products bearing similar molecular weights made the characterization of the products difficult. Solution phase synthesis will be employed in order to synthesize the desired branched products. Although the course is more laborious and time consuming, it provides an alternative method to synthesize the branched oligomers containing 2′,5′-linked phosphorothioate, separate its two isomers (R_p and S_p), and characterize the isomerically pure products.

The general scheme for the synthesis of the branched oligonucleotides commences with the coupling and sulfurization of the adenosine 2′-amidite **(1.4a)** with the properly protected guanosine monomer **(2.5)** to yield the 2′,5′-phosphorothioate dimer $A(p_sG)$. After separating the two isomers, the TBDMS is removed selectively from the 3′-hydroxyl adenosine. The cytidine 5′-amidite **(3.5)** is then coupled to the dimers (the R_p and the S_p isomers separately), followed by oxidation to yield a 3′,5′-phosphodiester linkage yielding the V-trimers $A(p_sG)pC$. The 5′-DMT is then removed from the branched adenosine and the trimer is coupled to the adenosine 3′-amidite **(1.4b)** to yield the Y-tetramer (Scheme 1).

The first step in assembling the branched oligonucleotide is synthesizing the 2′,5′-A(p_sG) phosphorothioate dimer, since separating phosphorothioate isomers at dimer level has already been established[76]. Phosphorothioate synthesis utilizes the same standard phosphoramidite coupling chemistry already well-established for the synthesis of phosphodiester linkages. However, while oxidation of the intermediary phosphite triester with I_2/H_2O yields phosphodiester bonds after deprotection, synthesis of internucleotide phosphorothioate linkages involves sulfurization of the triester using a sulfur delivering or "sulfurizing" reagent such as the 3H-1,2-benzodithiol-3-one-1,1-dioxide (Beaucage reagent) or 3-ethoxy-1,2,4-dithiazoline-5-one (EDITH reagent) (Scheme 6).

Scheme 6: Solution Phase Synthesis of 2′,5′-Phosphorothioate A(p_sG) Dimer.

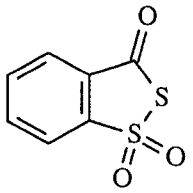


2.2.1 Sulfurization of the 2',5'-RNA Dimer (Beaucage *vs.* EDITH Reagent) (**4.1**):

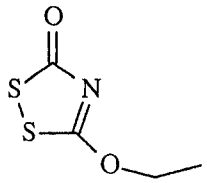
After the coupling step of **A (1.4a)** and **G (2.5)** to form the 2′,5′-linkage (Scheme 6), the dimer was sulfurized *in situ* to form a phosphorothioate bond. The two reagents used to sulfurize the dimer were the Beaucage and EDITH reagents. The Beaucage reagent afforded 64% of the desired phosphorothioate dimer and 22% of the undesired oxidized (P=O) phosphotriester dimer in a ratio of 3:1. This is not very surprising since

the by-product formed from the Beaucage reagent during sulfurization, 3*H*-2,1-benzoxathiolan-3-one-1-oxide, is a potent oxidizing reagent (Figure 11)[64,77]. The EDITH reagent, on the other hand, afforded clean sulfurized dimer.

Figure 15: Structures of the Sulfurizing Reagents Beaucage and EDITH.



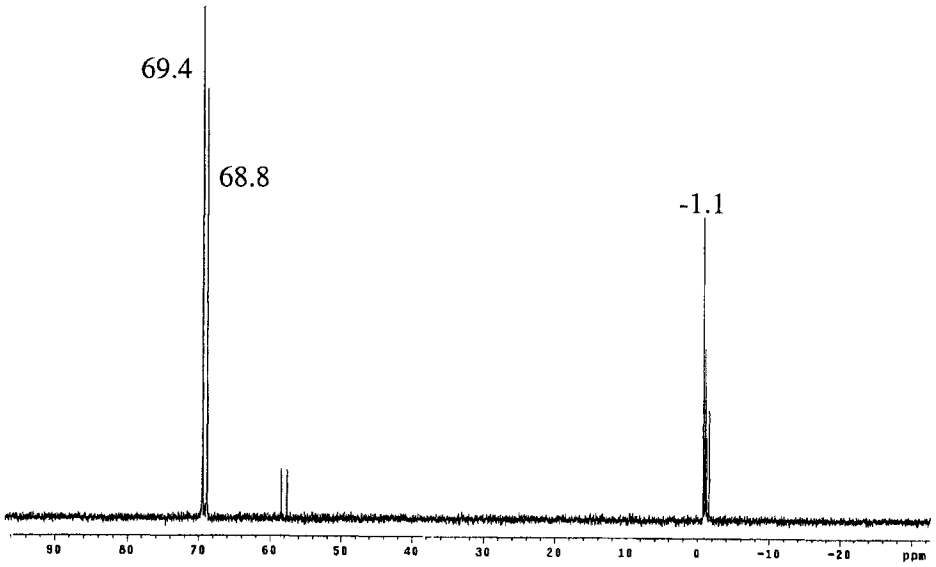
Beaucage
3H-1,2-benzodithiol-3-one-1,1-dioxide



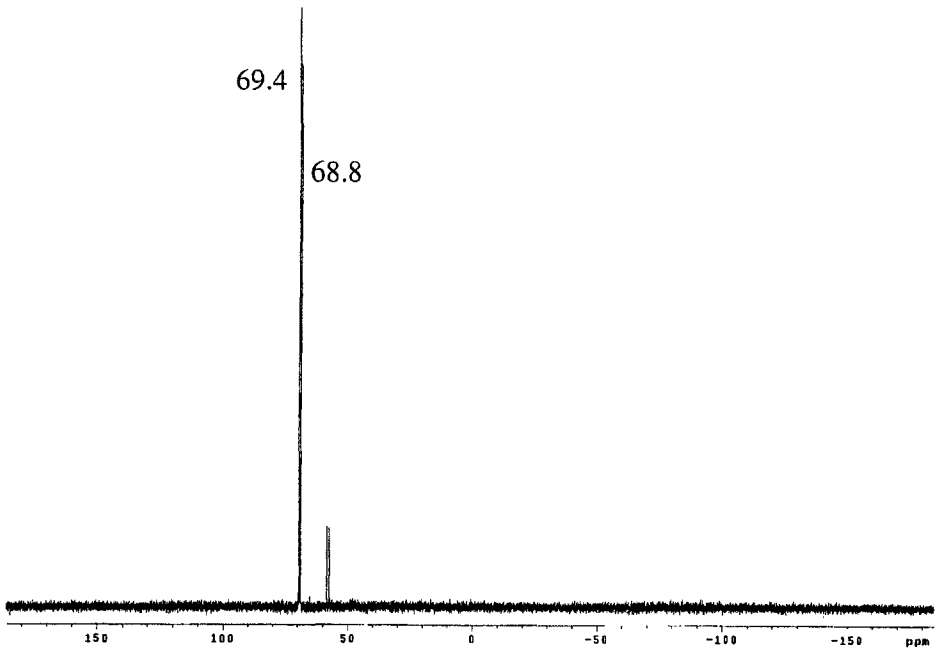
EDITH
3-ethoxy-1,2,4-dithiazoline-5-one

Although the EDITH reagent requires a longer reaction time, it is still fairly fast, necessitating a 10 min reaction time instead of the 5 min required for the Beaucage reagent. In addition to being a cleaner sulfurizing reagent it was found to be more stable than the Beaucage reagent in our coupling reactions (Figure 16). The two signals at ca. 58 ppm are from the dimers lacking the CE protecting groups at the phosphate sites. Therefore, EDITH was the sulfurizing reagent of choice (Scheme 6).

Figure 16: ^{31}P-NMR Spectra of Crude A(p_sG) Dimers Synthesized Using Two Different Sulfurizing Reagents.



A. Sulfurization using Beaucage reagent.

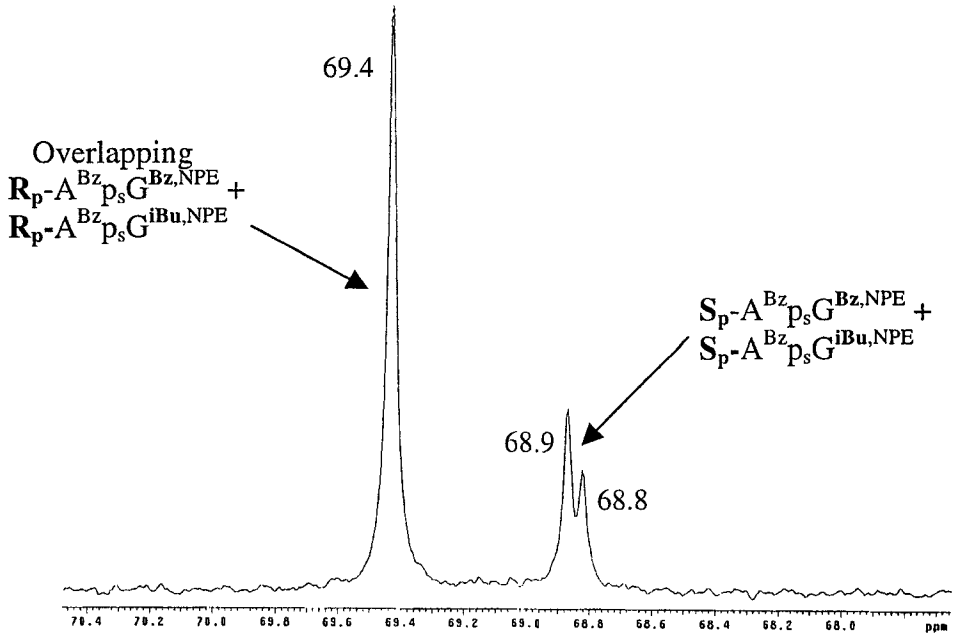


B. Sulfurization using EDITH reagent.

2.2.2 Ratio of the Two Isomers in the 2',5'-A(p_sG) Dimer:

To date several successful methods have been developed for stereoselective synthesis of phosphorothioate bonds utilizing chiral auxiliaries[78,79]. However, the synthesis of the 2',5'-A(p_sG) dimer was carried out using a non-stereoselective method since both isomers were required for the study of the stereospecificity of the yeast lariat debranching enzyme. Although, a non-stereoselective method was employed for the synthesis of the phosphorothioate dimer, the R_p isomer (**4.1a**) was produced to a greater extent than S_p isomer (**4.1b**) (*ca.* 2:1 respectively). The reaction was repeated three times and similar ratios were obtained for each attempt (Figure 17). The ^{31}P-NMR signal of the R_p isomer has a higher chemical shift than the S_p isomer, where the stereochemistry of the two isomers were assigned enzymatically using snake venom phosphodiesterase (SVPDE) as will be discussed in section 4.1. The ^{31}P-NMR signal of each isomer, although it is more apparent for the S_p isomer, consist of two close signals of the two derivatives, the $A^{Bz}p_sG^{Bz,NPE}$ and the $A^{Bz}p_sG^{iBu,NPE}$ (Figure 17).

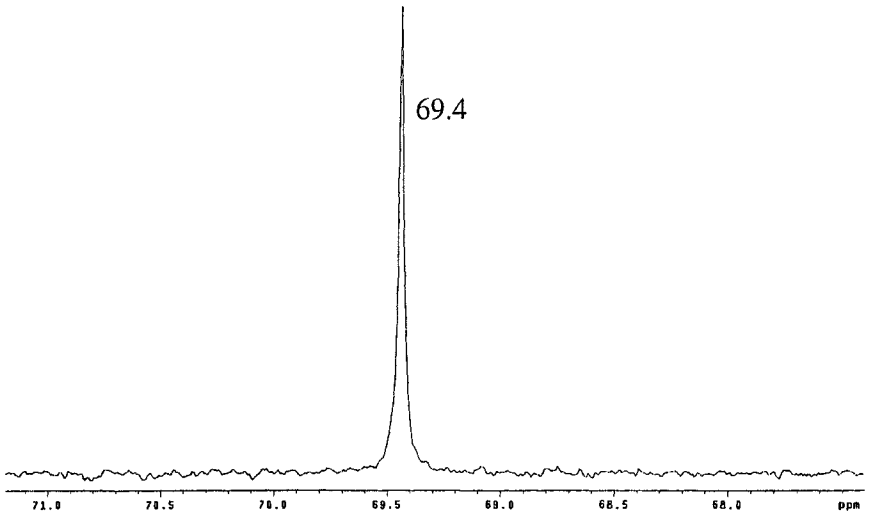
Figure 17: ^{31}P-NMR Spectrum of Crude A(p_sG) Dimer (mixture of R_p and S_p isomers).



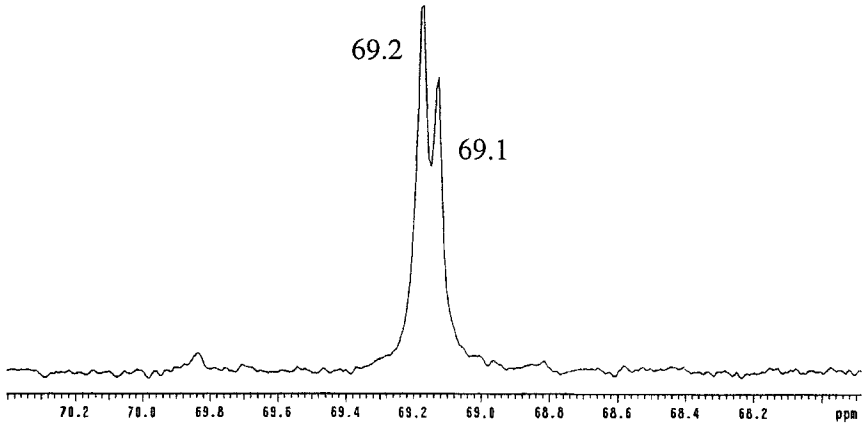
2.2.3 Purification and Separation of the Protected Phosphorothioate Isomers:

The fully protected dimers were observed to lose their cyanoethyl-protecting group at room temperature, which was ascertained by both TLC and ^{31}P-NMR. Even at -20°C, after one week, some loss of the cyanoethyl protecting groups was detected. The two isomers of the dimer were purified and separated as fast as possible since they are more polar and thus more difficult to separate after the loss of the cyanoethyl group. The solvent system used to separate the isomers via flash silica gel chromatography was CH_2Cl_2/EtOEt (1:1). Nevertheless, in the case of the reactions with the Beaucage reagent, where some of the dimers underwent oxidation, the slower eluting S_p isomer eluted with the oxidized dimer in the above solvent system. As an alternative means, both the phosphorothioate and oxidized dimers were separated on a preparative TLC using CH_2Cl_2/acetone (8.5:1.5) solvent system.

Figure 18: ^{31}P-NMR Spectra of the Isomerically Pure A(p_sG) Dimers.



A. R_p-A(psG) dimer, both $A^{Bz}p_sG^{Bz,NPE}$ + $A^{Bz}p_sG^{iBu,NPE}$ derivatives are present.



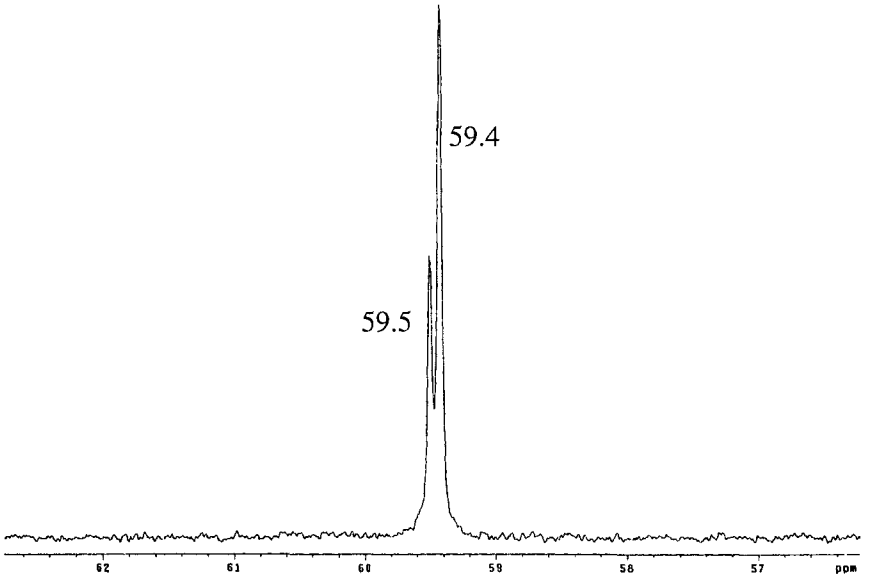
B. S_p-A(p_sG) dimer, both $A^{Bz}p_sG^{Bz,NPE}$ + $A^{Bz}p_sG^{iBu,NPE}$ derivatives are present.

2.2.4 Deprotection of the Cyanoethyl Protecting Group:

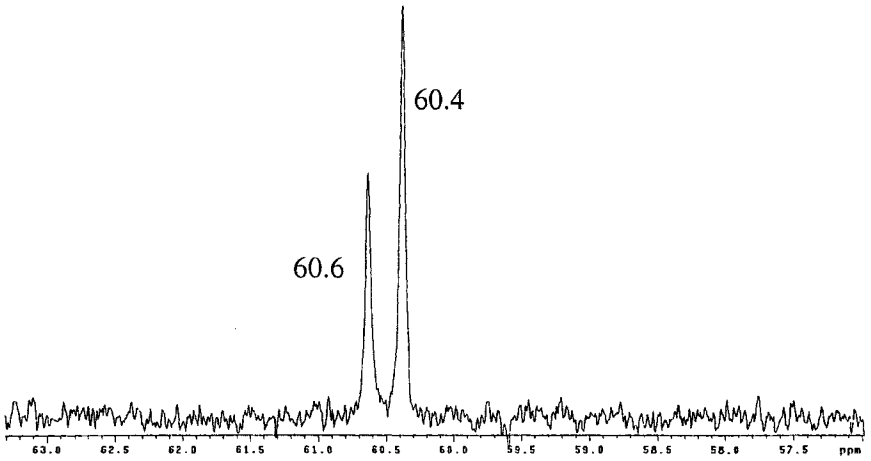
The cyanoethyl protecting group was removed from the dimer (**4.1**) under basic conditions (TEA/CH_3CN), subsequent to the separation of the two isomers. The structures were confirmed by FAB MS in addition to ^{31}P-NMR.

It is crucial to remove the cyanoethyl protecting group prior to the deprotection of the TBDMS group from the 3′-OH, since the 3'-OH could then act as an internal nucleophile resulting in the fissure of the internucleotide linkage (Figure 20). This occurs by way of a highly unstable 2′,3′-cyclic phosphorothioate triester intermediate. Removing the cyanoethyl group renders the phosphorus atom less electrophilic due to the presence of the negative charge, thereby reducing the possibility of the nucleophilic attack. The coupling of the cytidine 5′-amidite (**3.5**) to the free 3′-OH of the dimer was then carried out as fast as possible to mask the free 3′-hydroxyl group at the adenosine branch point.

Figure 19: ^{31}P-NMR Spectra of the Isomerically Pure A(p_sG) Dimers After the Removal of the Cyanoethyl Protecting Group.

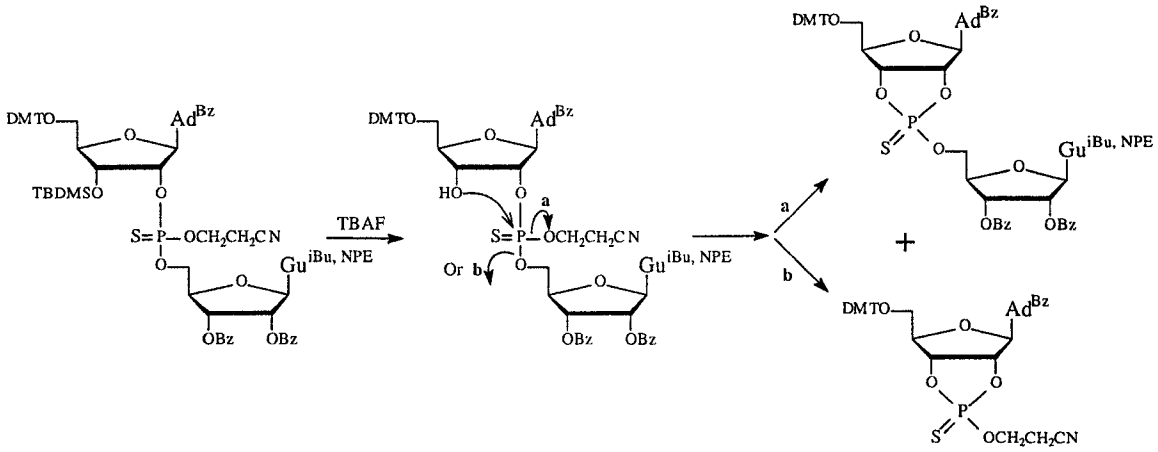


A. R_p-A(p_sG) dimer (no cyanoethyl protecting group), both $A^{Bz}p_sG^{Bz,NPE}$ + $A^{Bz}p_sG^{iBu,NPE}$ derivatives are present.



B. S_p-A(p_sG) dimer (no cyanoethyl protecting group), both $A^{Bz}p_sG^{Bz,NPE}$ + $A^{Bz}p_sG^{iBu,NPE}$ derivatives are present.

Figure 20: Desilylation of the 3′-OH in the Presence of a Vicinal 2′,5′-Linked Phosphorothioate Triester Linkage.

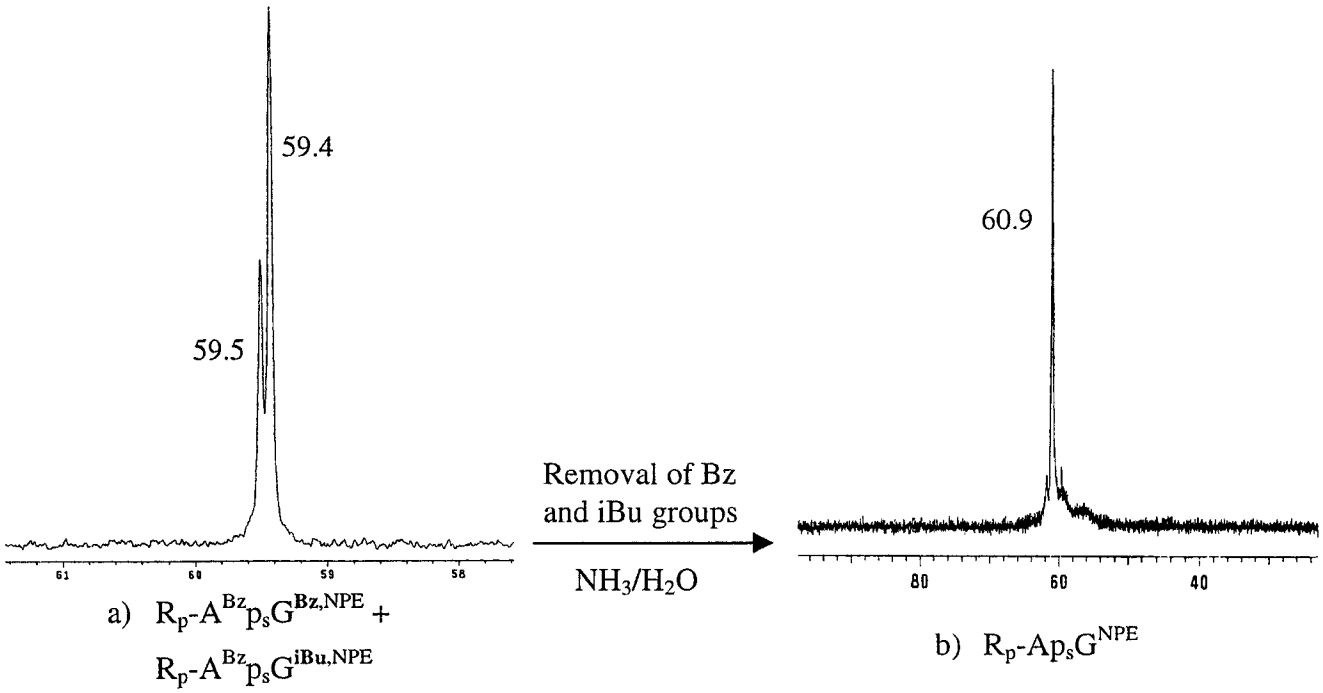


The R_p- and S_p-configured phosphorus nuclei of either isomerically pure dimer each exhibit two distinct chemical shifts in the proton-decoupled ^{31}P NMR spectrum, respectively, which suggests the presence of both isobutyryl and/or benzoyl moieties at the N^6 position of the guanosine terminal nucleotide (Figure 19). Thus, the nature of the protecting group at the guanine exocyclic nitrogen appears to remotely influence the

electronic properties of the nearby internucleotide linkage. Furthermore, only one peak is observed in the ^{31}P NMR spectrum following sample ammonolysis and removal of the i-bu and Bz groups with NH_4OH/EtOH (Figure 21).

Additional evidence for the presence of both chemical moieties in each of the two chiral samples is provided via mass spectrometric analysis, which further substantiates this hypothesis.

Figure 21: ^{31}P-NMR Spectrum of the Deprotected R_p A(p_sG) Dimer.



2.2.5 Desilylation of Dimers R_p-A(p_sG) (**4.3a**) and S_p-A(p_sG) (**4.3b**):

The desilylation of the dimers had to be carried out without the removal of any of the other protecting groups. The most common reagent used to remove a silyl-protecting group is tetra-*n*-butylammonium fluoride (TBAF) as a solution in THF. However, treatment of dimer (**4.2**) with this rather basic reagent also leads to the removal of the O^6-p-nitrophenylethyl protecting group. Another very common reagent typically used for the desilylation of nucleotides is triethylamine trihydrofluoride (TREAT-HF), however this

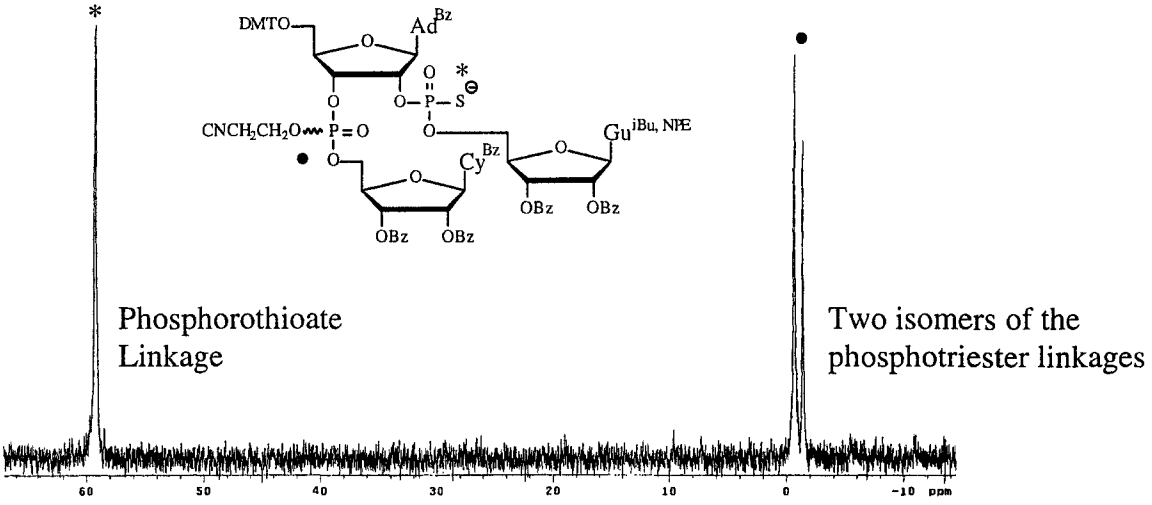
reagent is acidic enough to promote the cleavage of the dimethoxytrityl-protecting group. The removal of the NPE group most likely proceeds via an $F^{\ominus}$-assisted β-elimination reaction. A relatively new method has been developed by Wincott *et al.* allowing for the removal of silyl groups in the presence of trityl groups[80]. In our hands, this reaction proved to be dependent on the freshness of the TREAT-HF, since carrying out the desilylation using old reagent generated many side products. When fresh TREAT-HF was utilized, the reaction was much cleaner, however, the molecular weight of the product obtained (MW=1440 g/mol) did not correspond to the expected product (MW=1461 g/mol). Thus, an alternative method had to be developed in order to remove the silyl group without removing the 5′-DMT or O^6-NPE protecting groups. Since the trityl group is removed in an acidic media (*e.g.* HF) and the nitrophenylethyl group is removed under basic conditions ($F^{\ominus}$), neutral conditions are most appropriate to remove the silyl group. The method of choice was to incubate the dimer with a preneutralized solution of 1.0M TBAF in THF using AcOH. This method was very clean, quantitative, straightforward, and as confirmed by mass spectroscopy (ESI), led to the selective removal of the TBDMS group.

2.2.6 Synthesis of the V-Trimers (**4.4a** and **4.4b**):

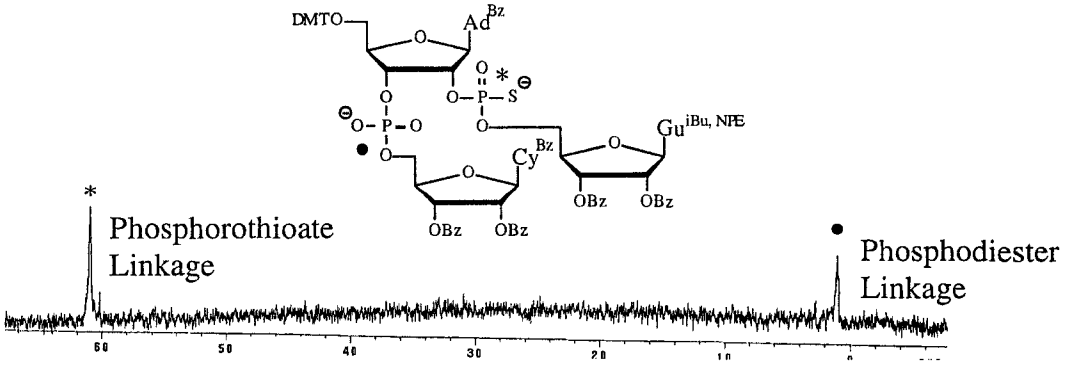
After the coupling and the oxidizing steps required in the synthesis of the V-trimer (**4.4**) (Scheme 7), the crude product had to be purified as fast as possible since it appeared to detritylate overnight, even at –20°C. Loss of the trityl group renders the molecules very polar and thus difficult to purify and separate from the excess cytidine 5′-phosphate monomer, a by-product of the reaction owing to oxidation of the cytidine 5′-phosphoramidite monomer used. This fast detritylation is most probably due to the acidic nature of the residual tetrazole (pKa 4.8), which is used in the coupling step, in addition to the significant detritylation occurring on the silica gel. To avoid this problem triethylamine (0.5% v/v) was added to the chromatography solvent system. Unfortunately, the base promoted the removal of the 2-cyanoethyl phosphate protecting group to yield the V-trimer **4.5** whose R_f value nearly coincided with the R_f value of unreacted dimer **4.3** (Scheme 7). This complication was minimized by carrying out the

coupling reaction in a nitrogen glove box with an excess of phosphoramidite reagent **3.4** thus forcing the consumption of dimer **4.3**. The ^{31}P-NMR of the V-trimer with the protected phosphotriester **4.4** exhibits two separated signals for the phosphotriester linkage. However, after the removal of the cyanoethyl, the V-trimer **4.5** exhibits only one ^{31}P-NMR signal for the phosphodiester bond (Figure 22). The two close derivatives [$A^{Bz}(p_sG^{Bz,NPE})pC^{Bz}$ + $A^{Bz}(p_sG^{iBu,NPE})pC^{Bz}$] appear to have very close ^{31}P-NMR signals since they do not show separate sets of signals (Figure 22).

Figure 22: ^{31}P-NMR Spectra of R_p-V-Trimer A(p_sG)pC, Before and After the Removal of the Cyanoethyl Protecting Group.

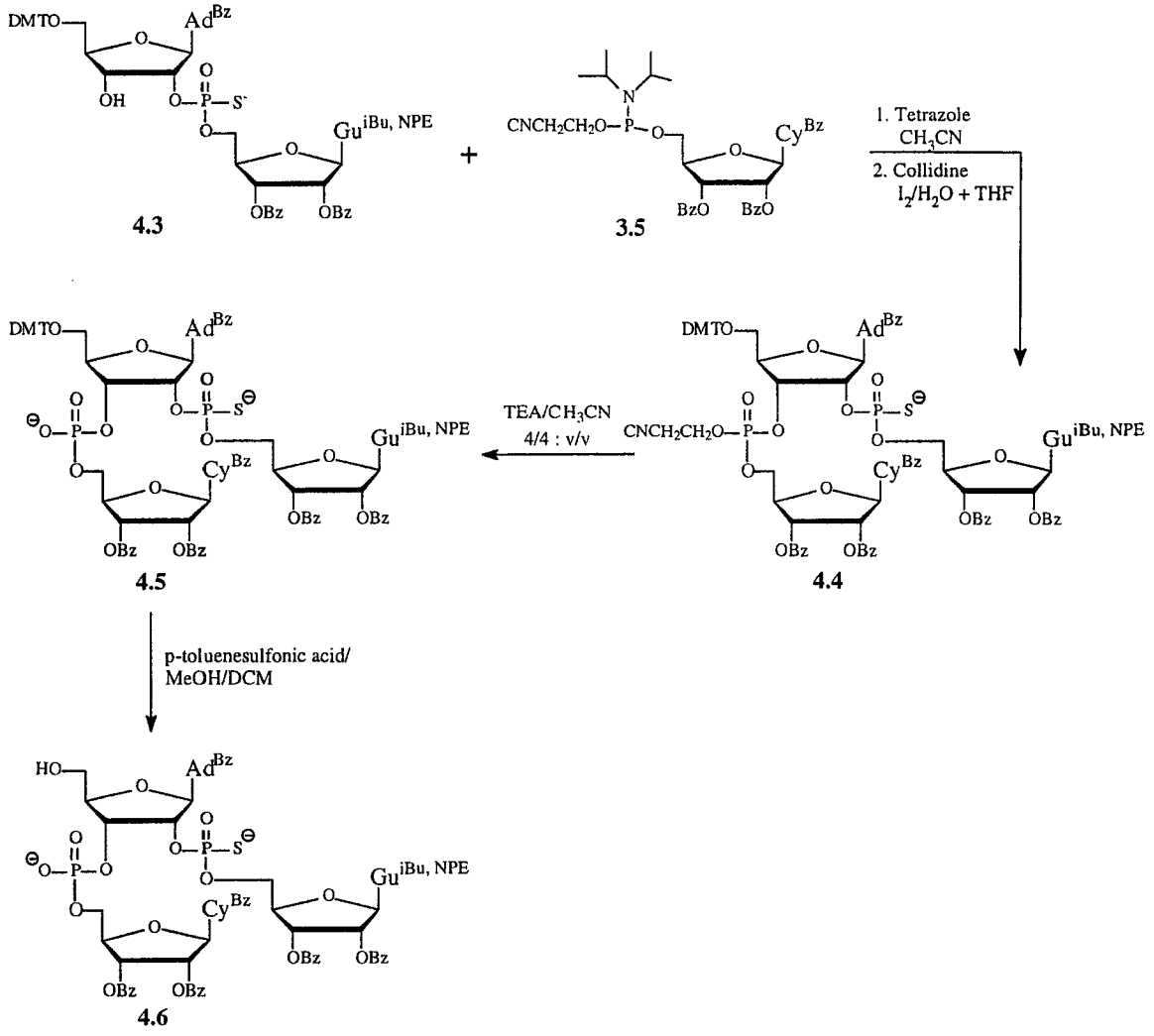


A. Branched R_p-trimer A(p_sG)pC with the cyanoethyl protecting group.



B. Branched R_p-trimer A(p_sG)pC without the cyanoethyl protecting group.

Scheme 7: Solution Phase Synthesis of V-Trimer.

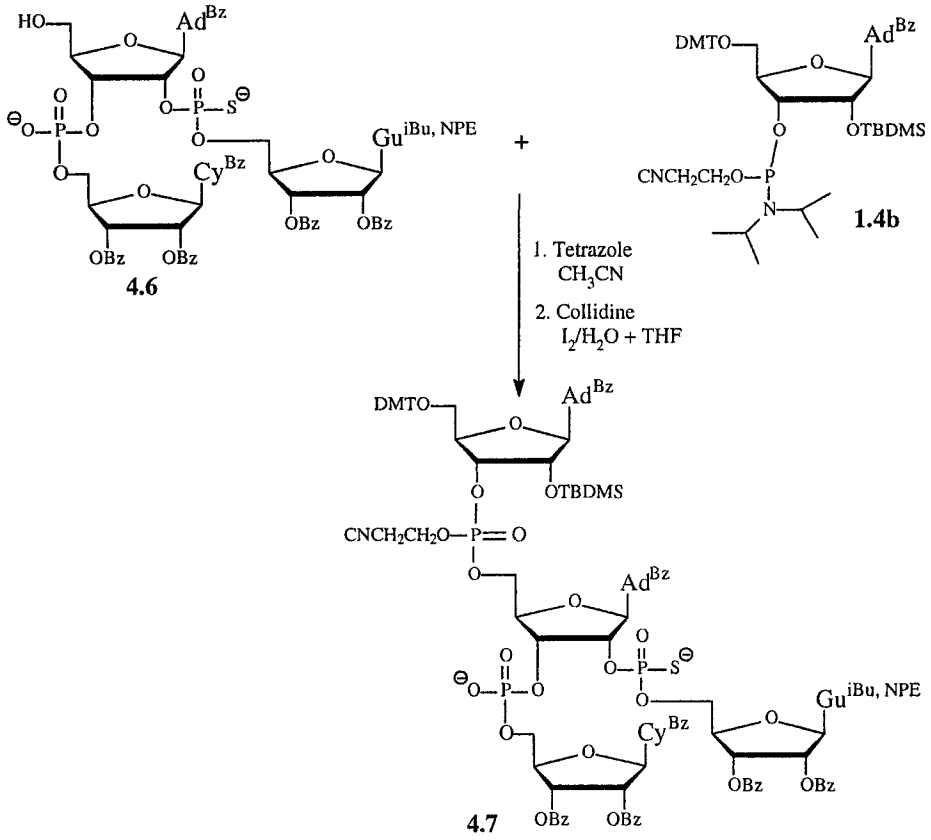


2.2.7 Detritylation of the V-Trimers (**4.5a** and **4.5b**):

The V-trimer was detritylated before coupling it to the adenosine phosphoramidite to produce the Y-tetramer. This was attempted by exposing V-trimer **4.5** to 15% trifluoroacetic acid (TFA) in n-butanol for 40min at r.t. Purification by flash silica gel chromatography yielded a product with a molecular weight of 1813 g/mol instead of the expected 1775 g/mol. When the detritylation was conducted using toluene sulfonic acid (TSA) the expected product with a molecular weight of 1775 g/mol was obtained.

However, purification of this material by flash gel chromatography led to partial conversion to the material with the molecular weight of 1815 g/mol. Drying this mixture and storing it at –20°C for 48 h led to the detection of an even larger amount of the material with the molecular weight of 1815 g/mol. The ^{1}H-NMR spectrum of the 1775/1815 'mixture' exhibited a broad polymer-like spectrum. Since the detritylated trimers (**4.6**) appeared to be "changing" rapidly, the coupling reaction to synthesize the Y-tetramer was carried out as fast as possible after the detritylation reaction.

Scheme 8: Solution Phase Synthesis of Y-Tetramer.



2.2.8 Synthesis of the S_p-Y-Tetramer (**4.7b**):

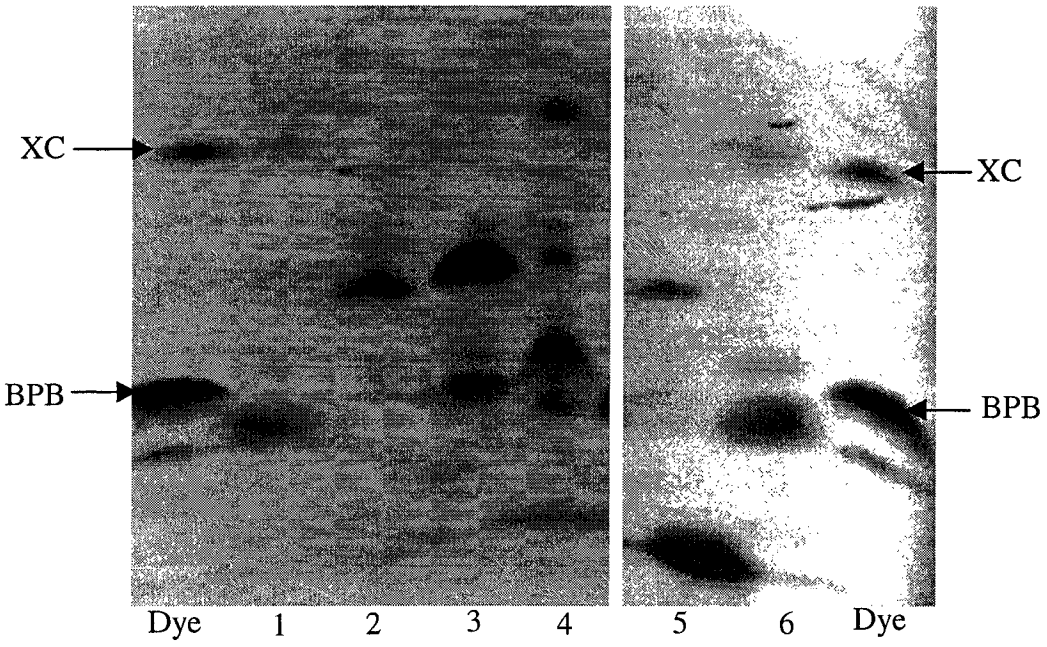
The dimethoxytrityl protecting group, which is present in the tetramer but not the trimer, usually turns orange upon cleavage by heating on a TLC plate. This feature was very convenient as it allowed easy monitoring of the formation of the Y-tetramer **4.7b**

(Scheme 8). Because the R_f of the starting material (V-trimer **4.6b**) and the product (Y-tetramer **4.7b**) were very similar; it was not possible to determine by TLC whether all of the V-trimer had been consumed. However, HPLC runs after the deprotection of the products confirmed the nearly complete consumption of V-trimer **4.6b**. Purification of Y-tetramer **4.7b** using flash silica gel chromatography, proved quite difficult since the compound is very polar and has an R_f value similar to that of the adenosine monomer used in excess during the coupling reaction. Purification by selective precipitation in stirring Hexanes did not work either since other polar impurities precipitated out as well. Thus, partial purification was carried out using flash silica gel chromatography, while final and total purification was carried on the fully deprotected Y-tetramer using polyacrylamide gel electrophoresis (PAGE) (see chapter 3, pp. 49).

2.2.9 Synthesis of the R_p-Y-Tetramer (**4.7a**):

Attempts to couple the corresponding R_p-V-trimer to the adenosine-3′-amidite to produce the R_p-Y-tetramer were carried out according to the procedure described above. After total deprotection and analysis by PAGE, it was concluded that the coupling reaction did not proceed successfully as was the case for S_p-Y-tetramer **4.7b**, most probably due to the presence of moisture. The debranching reaction in the study carried out by Maschhoff *et al.*[13] had been carried out only on a lariat with an R_p 2′,5′-phosphorothioate, which did not debranch. Therefore, synthesizing the R_p-Y-tetramer was not essential since the S_p isomer, which is the isomer in question, was synthesized successfully. Furthermore, both the R_p and the S_p isomers of the V-trimer were synthesized and were available for debranching assays in order to demonstrate the necessity of a residue at the 5'-position of the adenosine branch point for substrate recognition by the enzyme.

Figure 23: 24% Denaturing PAGE of Branched Trimers and Tetramer (S_p and R_p Isomers). Bands Visualized by UV Shadowing. Lane Numbers are at the Bottom of the Gel. *Lanes 1, 6:* Adenosine 3′-monophosphate, *lane 2:* Pure R_p-V-trimer, *lane 3:* Control crude Y-tetramer ApA(pG)pG, *lane 4:* Crude R_p-Y-tetramer, *lane 5:* Crude S_p-Y-tetramer.



CHAPTER 3: DEPROTECTION, PURIFICATION AND CHARACTERIZATION OF OLIGONUCLEOTIDES

3.1 Deblocking of Solution Synthesized Oligonucleotides

Solution-synthesized oligonucleotides necessitate the abolition of three different "classes" of protecting groups, namely the exocyclic amino, exocyclic and sugar oxygen, and phosphate protecting groups. This also applies to branched RNA fragments. The first step was the removal of the dimethoxytrityl from the 5'-hydroxyl using acidic treatment, such as TFA. The second step involved the removal of the exocyclic amino-protecting groups, as well as the benzoyl-protecting groups from the 2', and 3'-hydroxyls. This was carried out by treating the oligonucleotides with a 3:1 solution of NH_4OH/EtOH for 24 h in general, or 48 h to effect the complete elimination of isobutyryl protecting groups on the guanine bases. Finally, the oligomers were treated with TBAF in THF for 4 h to remove the TBDMS as well as the NPE-protecting groups.

3.2 Deblocking of Solid-Phase Synthesized Oligonucleotides

The linear controls and the phosphodiester V-trimer A(pG)pG were synthesized using automated synthesizers. Syntheses conducted by this approach enable the choice of keeping the trityl group on or removing it at the end of the synthesis; the latter method was adopted. The first post-synthetic step is the treatment of the support-bound oligonucleotide with a 3:1 solution of NH_4OH/EtOH to concurrently deprotect the bases, phosphates and cleave the oligonucleotide from the support. Furthermore, in opposition to solution-phase oligomers synthesis, the solid-phase methodology does not employ the use of NPE protecting groups on the oxygen of the heterocyclic guanine base. As such, the desilylating reagent TREAT-HF effects the complete eradication of the TBDMS protecting groups. Moreover, the use of TREAT-HF as a desilylating reagent compared with TBAF is much more desirable as it is easily removed by evaporation and produces fewer by-product salts.

3.3 Removal of TBAF Salts Using Dowex-Na^+ Exchange Chromatography

Immediately after the desilylation step using TBAF, the oligomers, which have an oily appearance, are applied to a sterile Dowex-Na^+ column (6 x 2 cm) and eluted using sterile double-distilled water[69]. The eluant was collected in small fractions in polypropylene tubes, which were lyophilized to dryness. Quantitation using UV spectroscopy indicates the presence of most of the oligomers in the first fraction. This method removes tetrabutylammonium ions, which greatly interfere with the HPLC analysis and purification step possibly because they are not very soluble water. Drying the oligomers after the column gives a powdery material, which dissolves quite readily in water.

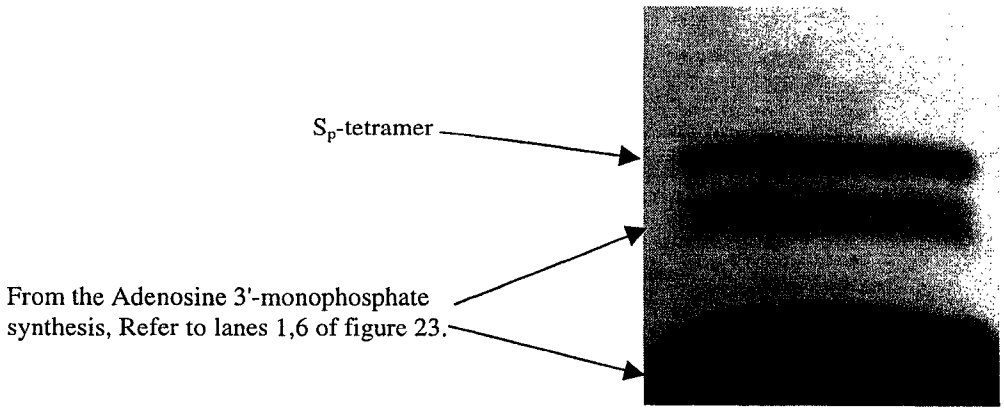
3.4 Purification of Linear Oligomers and V-Trimers Using Anion Exchange HPLC

Anion exchange high-performance liquid chromatography (HPLC) separates species by charge primarily through electrostatic interactions between the negatively charged oligonucleotides, due to the phosphate backbones, and the surface charge of the anion exchange resin. Therefore, HPLC was employed to purify the deprotected linear oligomers as well as the V-trimers synthesized via solution and solid-phase methods (Refer to appendix for HPLC profiles). Running an analytical HPLC of the crude solution-synthesized phosphorothioate Y-tetramer [ApA(p_sG)pC] showed, as expected, the adenosine 3′-monophosphate peak to be the major component. This was verified by running an HPLC on a synthesized adenosine 3′-monophosphate. The significant abundance of the adenosine 3'-monophosphate peak, in relation to the other compounds in the mixture, made the chromatogram difficult to resolve and rendered the tetramer non-isolable. Therefore, a different method had to be used to purify the tetramers.

3.5 Purification of the Y-Tetramer Using Polyacrylamide Gel Electrophoresis (PAGE)

Polyacrylamide gel electrophoresis (PAGE) differs from anion-exchange HPLC in that it separates species based on their sizes as well as charges or conformation, where the compounds migrate through a polymer of a finite size according to a mass to charge ratio. The analysis and isolation of the S_p-phosphorothioate isomer of the Y-tetramer [ApA(p_sG)pC] was realized via analytical PAGE. Under UV-shadowing, the crude S_p-Y-tetramer appeared to contain species corresponding to the monophosphate monomer and one more species running close to the V-trimer (Figure 23). The analytical PAGE of the crude R_p-Y-tetramer, however, showed too many spots and the one corresponding to the possible tetramer was minor. Since the S_p-Y-tetramer is the more crucial isomer and the one that had not been tested against debranching activity, it was purified using preparative PAGE (Figure 24). The S_p-Y-tetramer was thereby purified via PAGE and consequently subjected to analysis on anion-exchange HPLC. Analytical HPLC results proved that the oligomer is not a trimer since it elutes with a higher retention time, confirming that it most probably is a tetramer. Mass spectroscopy (MALDI-TOF III) was employed to measure the molecular weight of the oligomer, where the calculated molecular weight of the Y-tetramer is 2626 g/mol. The molecular weight found corresponded to the Y-tetramer, providing a second proof that the Y-tetramer synthesis was successful.

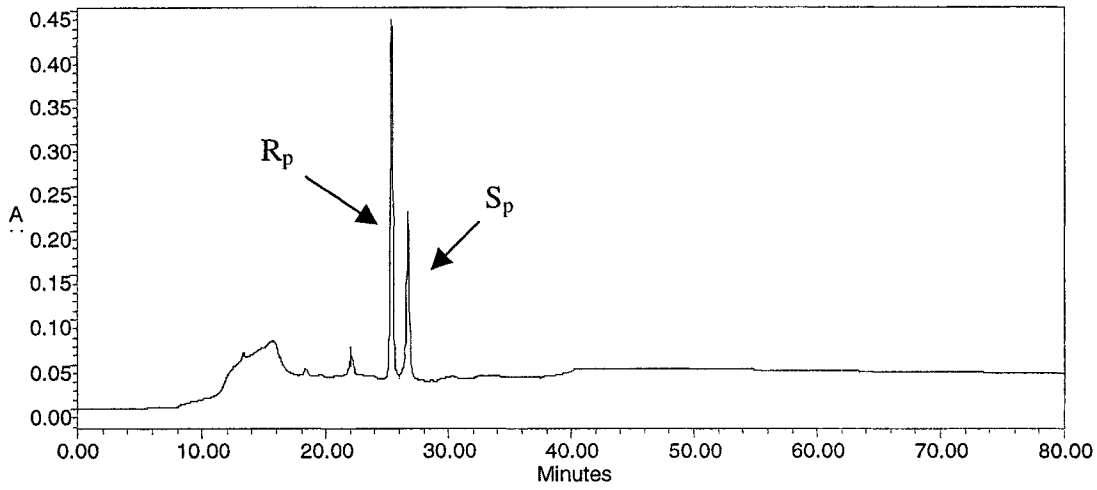
Figure 24: 24% Preparative PAGE to Purify the Crude S_p-Y-Tetramer ApA(p_sG)pC (*lane 3* of Figure 23).



3.6 Separation of the Isomers of the Deprotected Dimers A(p_sG) Using Reverse Phase HPLC

A second efficient method of separating the R_p and the S_p isomers of the completely deprotected dimers, other than the flash gel chromatography, is reverse-phase HPLC. The HPLC solvent gradient used in the analysis of the enzymatic degradation products is capable of separating the R_p and S_p isomers of the dimer. This gradient consists of 0-10% acetonitrile in 50 mM TEAA for 10 min followed by 10-35% acetonitrile for 15 min. However, for preparative runs, where injecting larger amounts of oligomers might cause an overlap in peaks with similar retention times, a different gradient was needed to obtain significant peak resolution. In this case, the gradient used to separate the isomers is 0-20% acetonitrile in 50 mM TEAA (60 min).

Figure 25: Reverse-Phase HPLC Analysis of the Deprotected and Desalted Mixture of R_p and S_p-A(p_sG) Isomers.



3.7 Desalting the Oligomers Using SEP-PAK

Oligomer purifications conducted by anion exchange HPLC and PAGE contain a multitude of salts in conjunction with the purified oligonucleotide as a result of either the mobile phase used, or the considerable number of salts present in the gel matrix. The most commonly utilized method for the rapid desalting of oligonucleotides is to

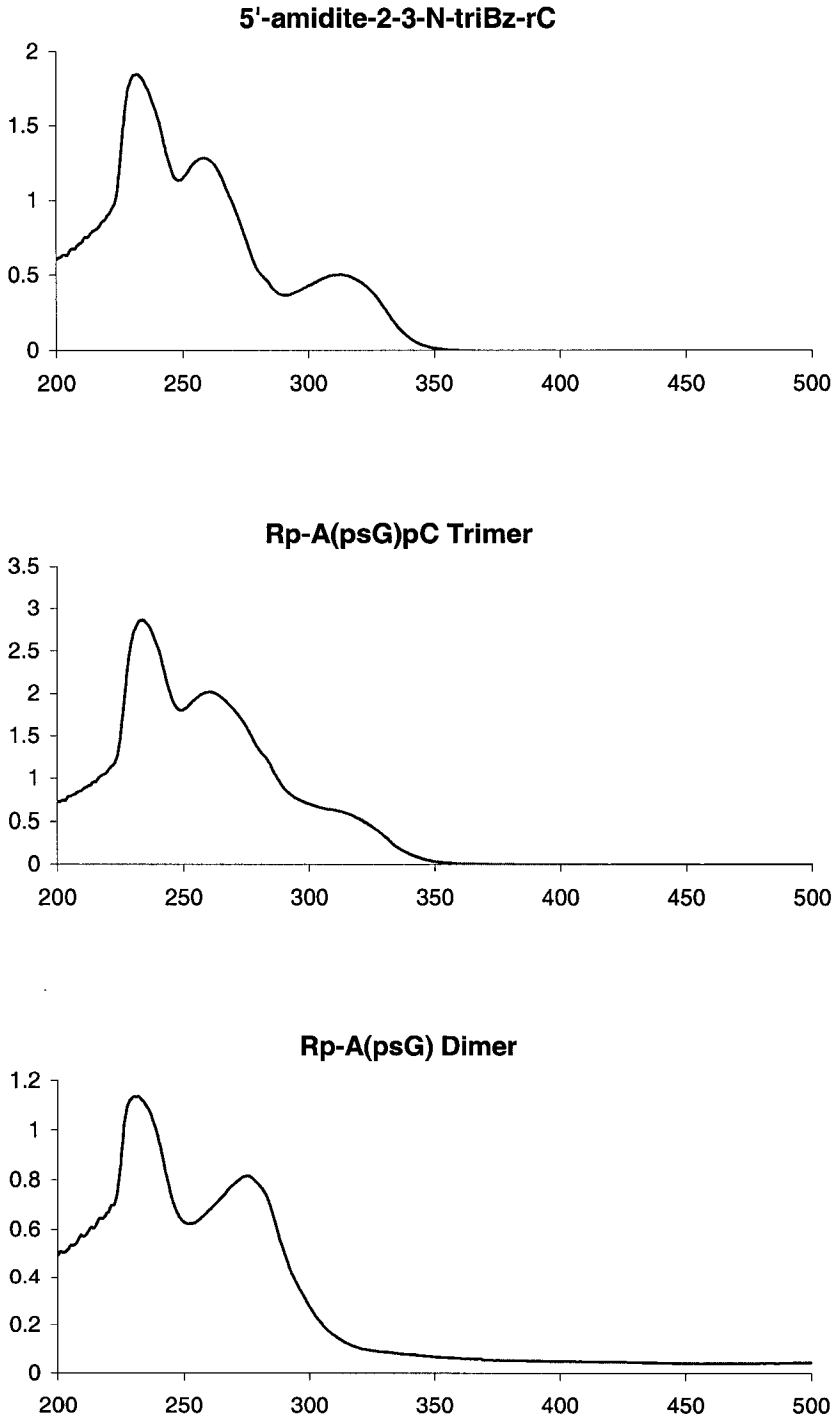
precipitate them from a slightly hydrophobic alcohol such as propanol or butanol. However, in the case of these small oligomers, the backbone negative charges are not sufficient to make them polar enough to precipitate out. As a viable alternative, the small branched oligomers were desalted using reverse-phase chromatography on C_{18} SEP-PAK™ cartridges. This method proved to be very efficient for the desalting of oligomers of such small sizes. Oligomers purified by means of reverse-phase HPLC, such as the phosphorothioate dimers A(p_sG), did not need to be desalted since this method does not utilizes salts in the solvent system.

3.8 Quantitative and Qualitative Analysis Using UV Spectroscopy

Using Beer's Law, the intensity of the absorbance of a solution of oligonucleotide, measured using ultraviolet spectroscopy, provides direct information about the concentration of a given oligonucleotide in a sample. However, numerous by-product salts produced during the deprotection stages contain absorbance profiles, which may sometimes overlap with the oligomers of interest's peak. As such, many of the samples must be desalted prior to quantitating them by UV spectroscopy (see above).

On the other hand qualitative information can be provided from the shape of the spectra, since different nucleotides have different absorption profiles. For example the phosphorothioate dimer which contains adenosine and guanosine, A(p_sG), absorbs at two wavelengths, *ca.* 230 and 275 nm, while N-4 benzoylated cytidine absorbs at three different wavelengths, *ca.* 230, 260 and 312 nm. This provides a good additional characterization tool to differentiate between the A(p_sG) dimer and the A(p_sG)pC trimer after the coupling reaction of the dimer to the cytidine amidite (Figure 26).

Figure 26: UV Profiles of Cytidine 5′-Phosphoramidite, A(p_sG) Dimer and A(p_sG)pC Trimer.



3.9 Circular Dichroism (CD)

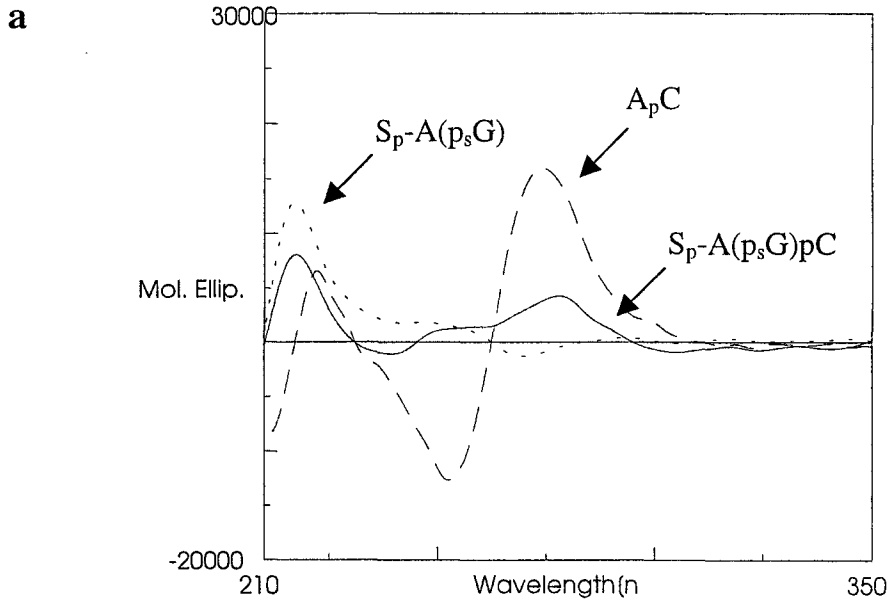
Circular dichroism is a powerful optical tool to obtain information about base interactions in oligonucleotides[81-82]. CD experiments measure the differential absorbance of left- and right-handed circularly polarized light as a function of irradiation frequency[83]. A region of rapid change with respect to wavelength, which is termed the *Cotton effect*, is usually the main feature in CD spectra. Both the sign and absolute intensity in Cotton effects are affected by molecular stereochemistry providing information about the structure[85].

The CD spectra of the phosphorothioate oligonucleotides (V-trimers, and S_p-Y-tetramer), and some of the corresponding 2'-5' and 3'-5' dinucleotide monophosphates and trinucleotide diphosphates were obtained and analyzed to gain further information about possible base interactions. The CD spectra of the two isomers of A(p_sG) have similar peak positions to the corresponding A(pG) phosphodiester dinucleotide reported by Damha[85], but are less intense in magnitude (Figure 28). The CD spectra of the phosphorothioate V-trimers, both R_p and S_p isomers, show a non-conservative cotton effect (Figure 27 a and b), where the sum of the rotational strength (peak area of Cotton effect) is not equal to zero as can be seen from the positive band at 275 nm and the shoulder at 255 nm. These types of non-conservative CD spectra are usually exhibited by base-stacked dimers containing guanosine bases due to the presence of at least two overlapping $\pi \rightarrow \pi^*$ bonds from the guanosine momomers in the spectra region measured (210-300 nm)[87,88]. Therefore, interpreting CD spectra of oligomers containing guanosine can prove quite difficult. However, this observation indicates that the 2'-guanine residues in the phosphorothioate V-trimers are involved in base stacking interactions.

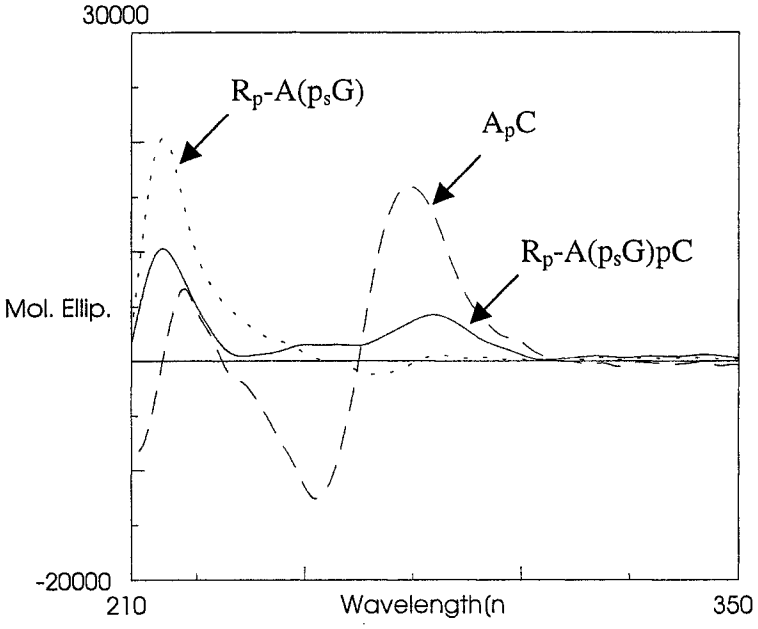
The low intensity detected in the 2'-5' A(p_sG) optical activity is retained in these branched phosphorothioate trimers, which possess similar CD patterns but lower band intensities than the phosphodiester V-trimers A(pG)pC reported by Damha (Figure 28)[85]. This low intensity observation is also true in the phosphorothioate Y-tetramer's case (Figure 27 c), S_p-ApA(p_sG)pC. This similarity in intensity between the branched phosphorothioate oligomers and the 2'-5' A(p_sG) is possibly a second indication to the involvement of the 2'-guanosine residues in stacking reactions.

The shapes of the CD patterns of both phosphorothioate V-trimers appear to be intermediate between the corresponding 2'-5' and 3'-5' dinucleotide monophosphates, which is also the case for the phosphorothioate S_p-Y-tetramer. This suggests that two relatively equal populations exist for the phosphorothioate V-trimers, where the branched adenine base forms a stack with the chromophore of the 2'-residues in one population, and with the 3'-residues in the second. This observation is not seen in the normal phosphodiester V-trimers where the dominant population is the adenine-2'-residues stacking[85].

Figure 27: CD Spectra of Dinucleoside Monophosphates, Branched Phosphorothioate Trinucleotides and Tetranucleotides in H_2O (pH= 7.4).



b


30000
R_p-A(p_sG)
A_pC
R_p-A(p_sG)pC
Mol. Ellip.
-20000
210
Wavelength(n
350

c

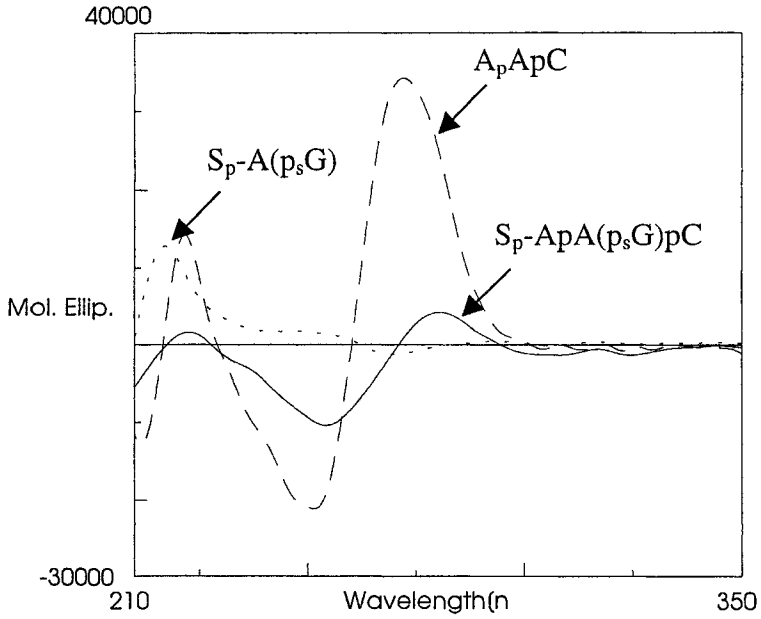
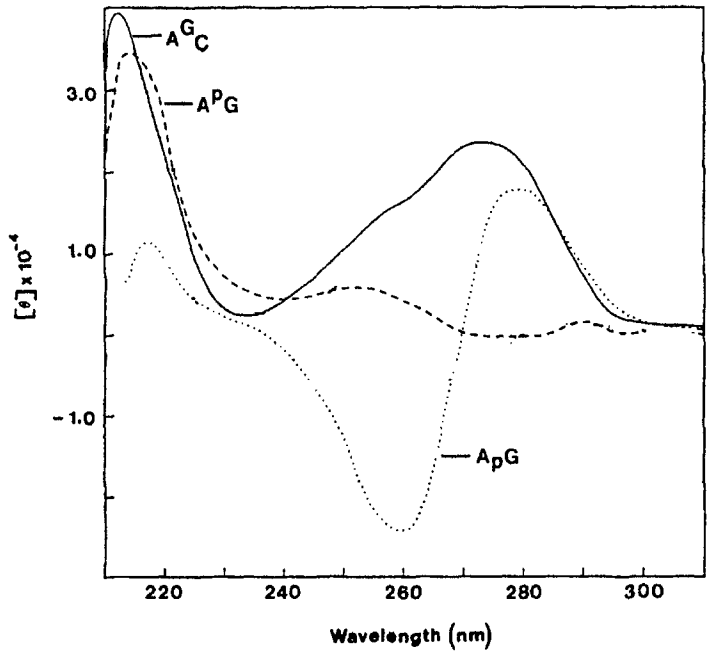

40000
A_pApC
S_p-A(p_sG)
S_p-ApA(p_sG)pC
Mol. Ellip.
-30000
210
Wavelength(n
350

Figure 28: CD Spectra of Dinucleoside Monophosphates and Branched Trinucleotides in H_2O (pH= 7.4)[85].



CHAPTER 4: BIOLOGICAL ASSAYS

4.1 Enzymatic Digestion of Oligonucleotides Using Snake Venom Phosphodiesterase (SVPDE)/Alkaline Phosphatase (AP)

Snake venom exonuclease (Phosphodiesterase I) can be used for sequence studies of ribo- and deoxyribo-oligonucleotides and for stereochemical assignment of phosphorothioates. The enzyme successively hydrolyzes 5'-mononucleotides from 2'- and 3'-OH-terminated oligonucleotides. It has stereoselective hydrolytic activity towards the R_p isomer of phosphorothioates for both 3',5'- and 2',5'-phosphodiester linkages[76,86], which can be used to determine the absolute configuration of phosphorothioate linkages. Since little is known about the SVPDE's behavior toward branched phosphorothioate oligonucleotides, the enzymatic characterization was first carried out on the two separated isomers of the 2',5' Ap(s)G dimer.

Commercially available standards consisting of unprotected nucleosides (rA, rG, rC, rI) were analyzed by reverse-phase HPLC using an appropriate solvent system to resolve the individual components and identify their retention times. Inosine (rI) was run as a standard due to the nature of the enzymatic digestions performed. Since the SVP enzyme produces 5'-monophosphate nucleosides as its reaction products, the 5'-phosphate must be subsequently removed for analysis. This is performed using the enzyme alkaline phosphatase. A possible contaminant in this enzyme is another enzyme, adenosine deaminase. This enzyme catalyzes the conversion of adenosine nucleosides into inosine. The same HPLC conditions can be used to separate the two isomers of the A(p_sG) dimer, where the faster eluting isomer on the HPLC column is also the faster eluting isomer on flash gel chromatography.

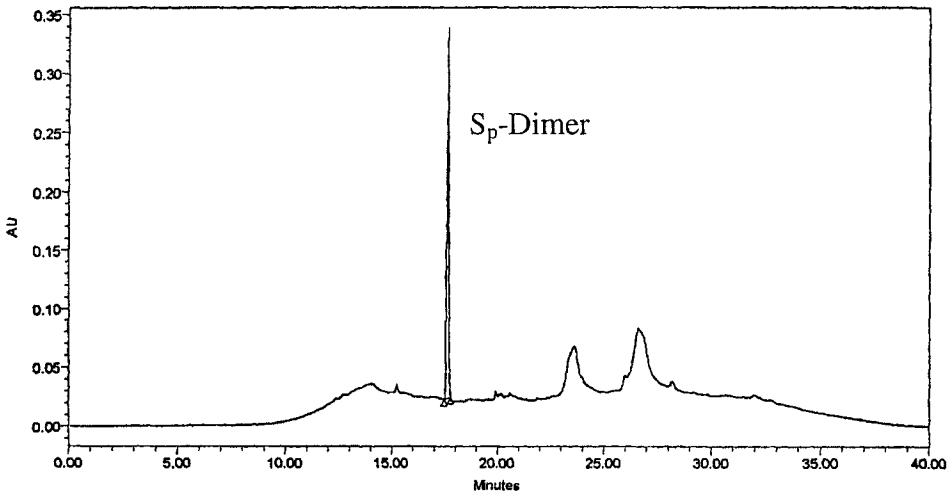
The SVPDE/AP assays were carried out on a regular ApG and the two isomers of A(p_s)G. The phosphodiester linkage of ApG was fully hydrolyzed within 4 h. However, the faster eluting dimer showed more than 50% hydrolysis after 4 h, while the slower eluting dimer showed no sign of hydrolysis even after 24 h (Figure 29). Thus, the faster eluting dimer has an R_p configuration, since cleavage of a phosphorothioate bond by SVPDE is R_p selective even in a 2',5'-linkage[76], and the slower eluting dimer has an S_p configuration.

The SVPDE/AP assays were carried out on the V-trimers [5'-A(p_sG)pC] in order to verify the stereochemistry of the two isomers (S_p and R_p), and to check if the enzyme is able to hydrolyze branched oligoribonucleotides. The same conclusions, as in the dimers' case, were reached in the V-trimers. The S_p-V-trimer shows full hydrolysis of the 3',5' phosphodiester bond after 2 h, yielding the C monomer and the S_p isomer of the Ap_sG dimer. The accumulation of the S_p dimer demonstrates that it is not a substrate for the SVPDE enzyme, and substantiates the previous hydrolysis studies at the dimer level (Figure 30). The R_p-V-trimer HPLC spectra exhibits a more complex chromatogram with more peaks corresponding to the R_p dimer, the C monomer, the A monomer and the G monomer. The noisy baseline, which is characteristic of enzymatic digestions, and the small amounts used resulted in noisy HPLC chromatograms. This is especially evident in the case of the R_p-V-trimer where the incomplete hydrolysis resulted in numerous but less intense peaks.

These enzymatic assays have determined the stereochemistry of the phosphorothioate linkages and demonstrated the ability of SVPDE to hydrolyze phosphodiester linkages of branched molecules.

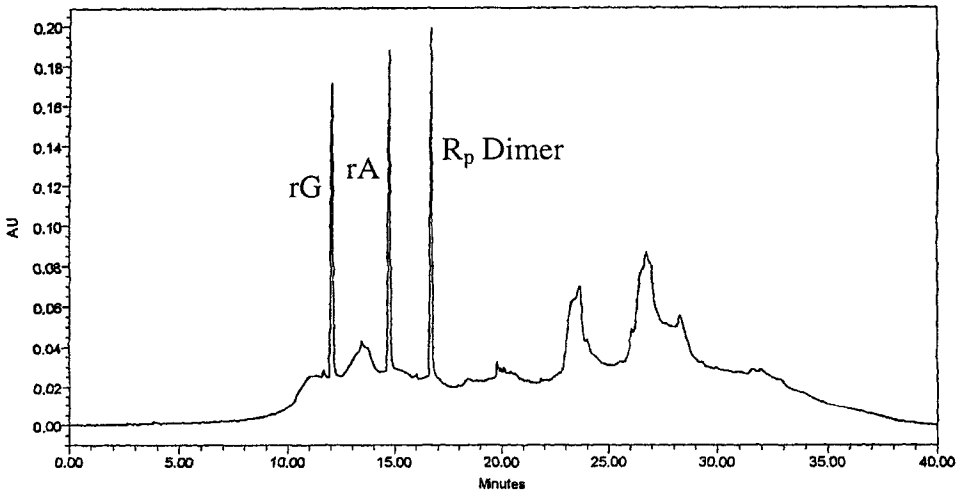
Figure 29: HPLC Analysis of the Enzymatic Digest of Both S_p (**a**) and R_p (**b**) Isomer of the Dimer A(p_sG) Using Snake Venom Phosphodiesterase (SVPDE), which Preferentially Hydrolyzes R_p-Phosphorothioates Regardless of Connectivity (*i.e.* 3′-5′, as well as 2′-5′ linkages), and Alkaline Phosphatase (AP).

a



S_p-dimer [A(p_sG)] + SVPDE/AP

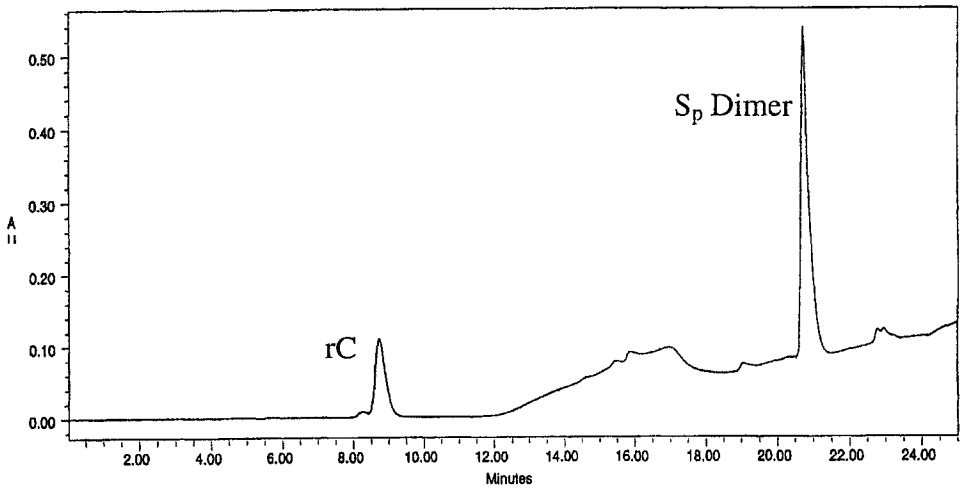
b



R_p-dimer [A(p_sG)] + SVPDE/AP

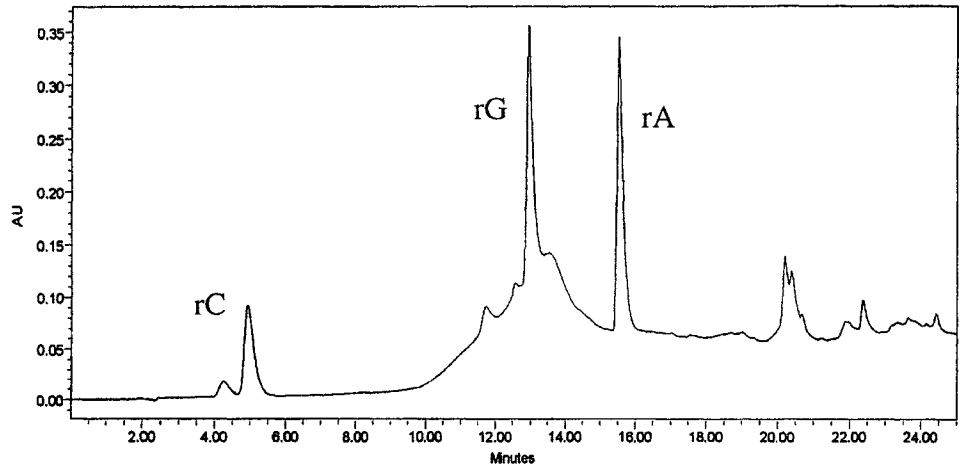
Figure 30: HPLC Analysis of the Enzymatic Digest of Both S_p (**a**) and R_p (**b**) Isomer of the V-Trimer A(p_sG)pC Using Snake Venom Phosphodiesterase (SVPDE), which Preferentially Hydrolyzes R_p-Phosphorothioates Regardless of Connectivity (*i.e.* 3′-5′, as well as 2′-5′ linkages), and Alkaline Phosphatase (AP).

a



S_p-trimer [A(p_sG)pC] + SVPDE/AP

b

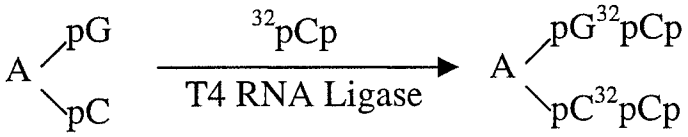


R_p-trimer [A(p_sG)pC] + SVPDE/AP

4.2 3'-End Labeling with ^{32}P

Typically, efficient labeling of oligonucleotides is conducted using the enzyme T4 polynucleotide kinase and γ–^{32}P-[ATP] as a phosphate source. Previous studies[51] have demonstrated the inutility of such a method for the labeling of small and large V-shaped oligonucleotides, as they lack a 5'-nucleotide tract, thereby not rendering them as choice substrates for the enzyme. As a practical alternative, such small branched oligonucleotides may be labeled at their 3'-ends with the enzyme T4 RNA ligase and 5'-^{32}P-cytosine-3',5'-biphosphate (*i.e.* ^{32}pCp) (Scheme 9). T4 RNA ligase is an enzyme specific to the ligation of residues containing 5'-phosphates and 3'-hydroxyl units. This method is also quite practical for the purpose of the debranching studies, as it allows one to follow the release of both the 2'5'-linked and 3',5'-linked fragments produced by the hydrolysis of the 2',5'-phosphodiester (or phosphorothioate) bond by the enzyme.

Scheme 9: 3'-End Labeling V-Trimers with ^{32}P.



Initially, the labeling reactions were carried out on small amounts of oligomers (5 pmol) and excess amount of ^{32}pCp, according to the procedure used in our laboratory[21]. For the linear control substrates and linear expected products of the enzyme hydrolysis reaction, 1.2 equivalents of ^{32}pCp were added, while for the branched molecules 2.4 equivalents were added to insure full labeling of both 2'-linked and 3'-linked residues (Scheme 9). Once the labeling reaction was complete, the labeled oligomers were analyzed on small 24% acrylamide gels. However, the results demonstrated similar patterns for all the reactions, where many bands were present. The linear 3',5'-ApC dimer was the only oligomer that appeared to have an extra, slower moving band that was not present in the rest of the reactions (Figure 31, Lane 1). Comparing these results with a positive control containing only the radioactive ^{32}pCp and T4 RNA ligase, a similar pattern was evident. When a negative control was run,

containing only the ^{32}pCp, more than two bands were apparent in the gel, indicating that the commercial sample of ^{32}pCp itself was not pure. Incubating the ^{32}pCp with T4 RNA ligase and buffer in the absence of a substrate yielded numerous bands which were observed in the positive control and the labeling assays (Figure 32).

Figure 31: Trial to 3′-End Label Small-Branched Oligonucleotides Using 5'-^{32}P-Cytosine-3',5'-Biphosphate on a Small Gel Apparatus. Lane numbers are at the Bottom of the Gel. *Lane 1: Linear ApC, lane 2:* Branched S_p-Y-tetramer ApA(p_sG)pG, *lane 3:* Branched S_p-V-trimer A(p_sG)pG , *lane 4:* Branched R_p-V-trimer A(p_sG)pG, *lane 5:* Control V- trimer A(pG)pG, *lane 6:* Linear ApCpC trimer, *lane 7:* Linear S_p-dimer A(p_sG), *lane 8:* Linear R_p-dimer A(p_sG).

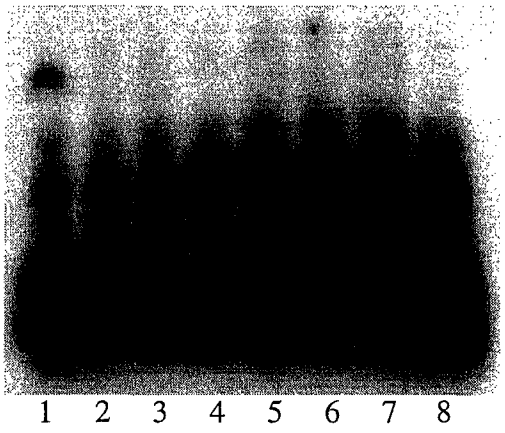
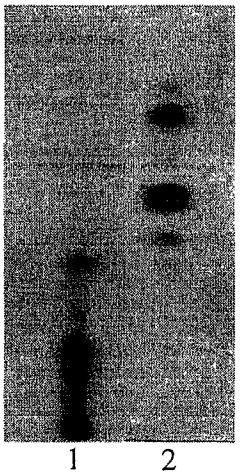
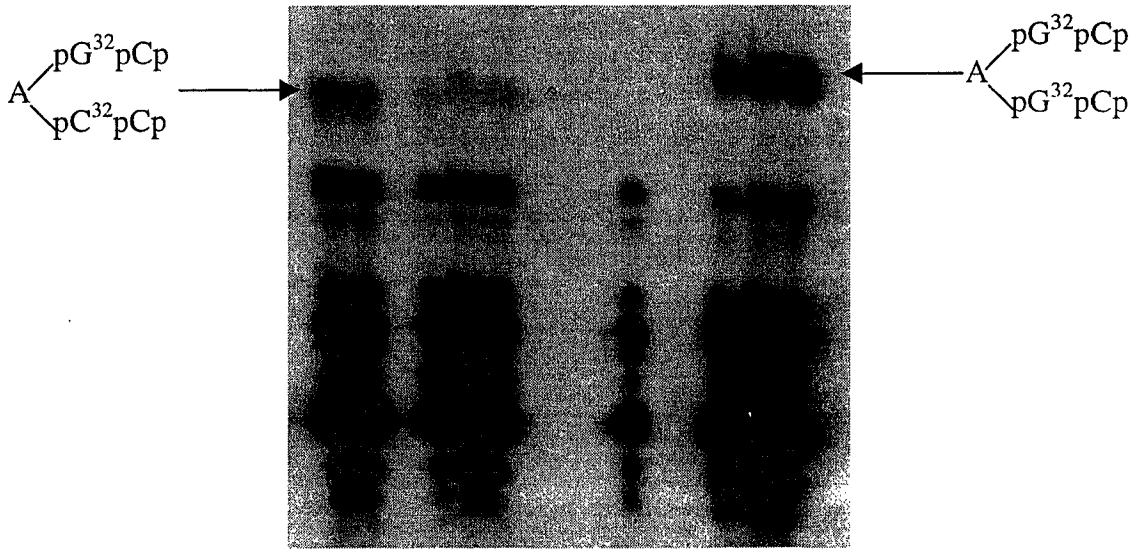


Figure 32: Images of Autoradiograms of a Gel Analysis of ^{32}pCp and ^{32}pCp with T4 RNA Ligase, Both with the Absence of any Substrate. *Lanes 1:* ^{32}pCp used directly from the bottle (-)ve, *lane 2:* ^{32}pCp incubated with T4 RNA and buffer (+)ve.



It appears that the small size of the oligonucleotides being labeled, in addition to their special, branched, conformation causes them to migrate on the gel similarly to the incubated ^{32}pCp, which makes them difficult to separate, especially using gel plates with small dimensions. Therefore, running the gels using larger gel apparatus with larger plate dimensions would ensure a higher degree of separation. Also, since the branched molecules are small and consist of only one residue at the 2'- and the 3'-end of the branched point, causing it to be more sterically hindered, the labeling of these hindered oligomers is not very efficient and too small to detect. Therefore, using a larger amount of oligomers and a smaller amount of ^{32}pCp would, in principle, drive the labeling reaction forward. Indeed, carrying out the labeling reactions using the above conditions and purifying these reactions using large gel apparatus appeared to render the gel easier to analyze and purify (Figure 33). As can be noted from the gel, the control phosphodiester V-trimer, A(pG)pG, is a better substrate of T4 RNA ligase than the phosphorothioate V-trimers. Although using the branched oligomers in excess causes the labeling to be either on the 2'-, the 3'- or both tails, all the products appear to migrate together and overlap on the gel, where only one spot appeared on the gel, making it easy to excise and purify them all together. Using these products for the debranching reactions should not affect the outcome of these enzymatic studies since the number of the final products will be the same and the only difference will be the intensity "amount" of these debranching products. Further studies should contemplate the use of Capillary Electrophoresis (CE), as an alternative method to study the debranching of these small branched molecules by yDBR[89]. Using this method helps in testing the molecules for debranching without handling radioactivity, and also in avoiding the problems of labeling these small branched and sterically hindered molecules.

Figure 33: 3′-End Labeling of Small-Branched Oligonucleotides Using ^{32}pCp, Large Gel Apparatus. The (+)ve control contains ^{32}pCp and ligase without any substrate.



4.3 Debranching Studies Using DBR from HeLa Extract

The debranching assays were initially carried out on the labeled branched molecules, the "V" control A(pG)pG, the R_p and the S_p-V-trimers and the S_p-Y-tetramer, using a HeLa cell extract containing debranching activity. A final concentration of 10 mM of EDTA was used to inhibit any other nuclease activity that might degrade the molecules[15]. The enzymatic activity was destroyed by heating the reactions after a 0.5, 1, 4, 8, and 24 h reaction time. In order to avoid the risk of losing any of the small radioactivity used in the assays, the samples were not extracted (phenol-chloroform) or precipitated (ethanol) prior to loading them on the gels. Such a method is typically conducted in order to devoid the samples of proteins which may cause the radioactivity to become lodged in the wells of the gel.

The debranching assays using HeLa extracts of both the branched A(pG)pG control and the S_p A(p_sG)pC thioate, reveal the appearance of three new peaks after 30 min of incubation with the extract. The first product, a very faint spot on the gel,

migrates faster than the branched starting material, which is where one of the debranched products ($ApG^{32}pCp$) is expected to run (Scheme 10). The other two products migrate slower than the starting material, where monomers are expected to migrate indicating a possibility of degradation occurring due to the presence of other nucleases in the extract (Figure 34). The S_p-Y-tetramer results, data not shown, exhibit a similar pattern as for the S_p-V-trimer, where the two slower moving bands are more separated. However, one of the two slower moving bands disappears after the 1 h point, which can be an additional indication of the possibility of degradation instead or along with debranching. The R_p-V-trimer, which is not expected to debranch, showed only the two products that migrate slower, but no indication of debranching since the faster moving products did not appear on the gel (Figure 34). Subsequent debranching assays were performed on linear molecules to test any possibilities of degradation that are due to other nucleases in the extract. The linear labeled trimer [$ApCpC^{32}pCp$] degraded when incubated with the HeLa extract and the EDTA for 20 h indicating the effect of the nucleases present in the HeLa extracts (Figure 35). Other experiments using the HeLa extract revealed degradation of the linear ApCpCp as fast as 1 h (data not shown). These results imply that the HeLa extract contains nucleases that degrade the oligomers and interfere with the possible debranching that could be taking place. Therefore, the extract would not be a suitable choice as a source for debranching activity as the competing degradation reactions, which arise, make it difficult to judge the extent of debranching occurring.

Scheme 10: Debranching Reaction of 3'-^{32}P-End Labeled V-Trimer.

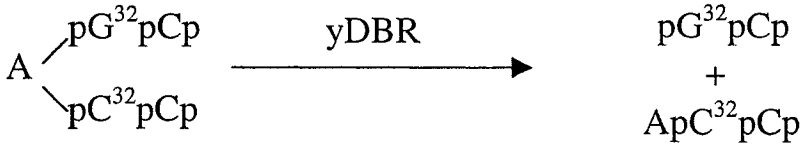


Figure 34: Images of Autoradiograms of Assay for Substrate Recognition by Debranching Activity in HeLa Extracts. The triangles indicate time of incubation and the negative control, (-)ve, contains only the oligomer.

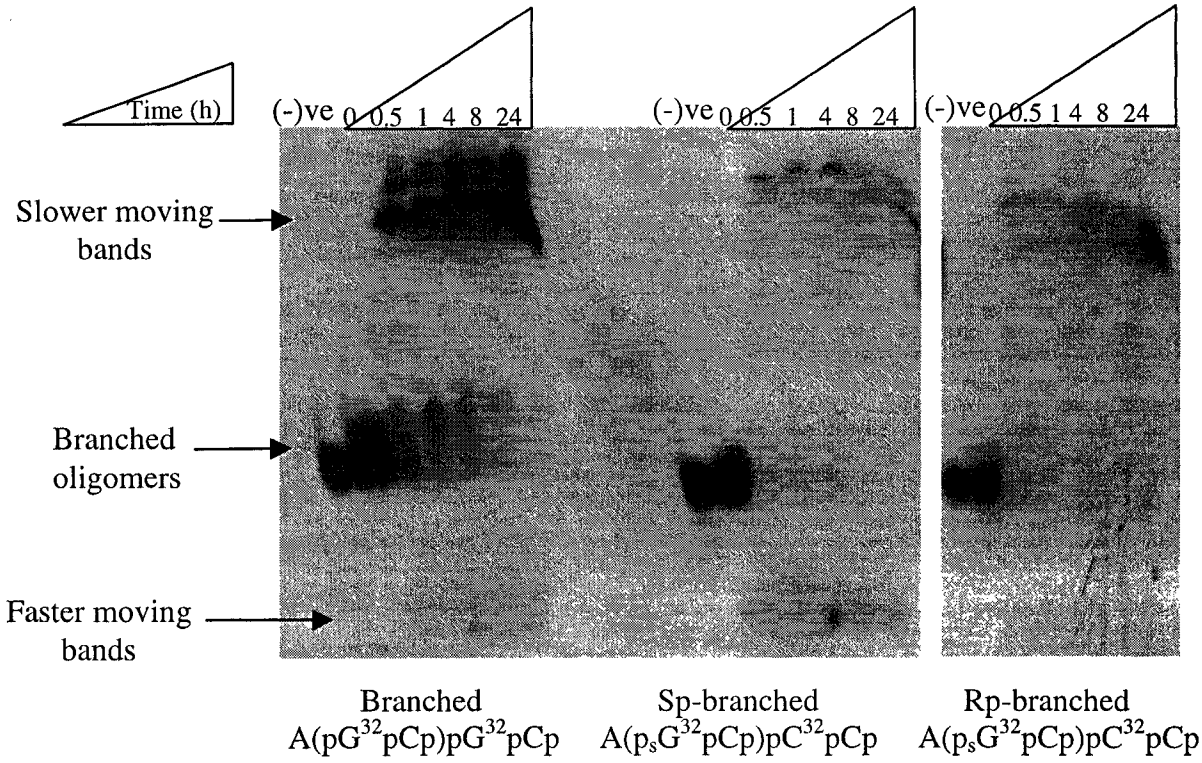
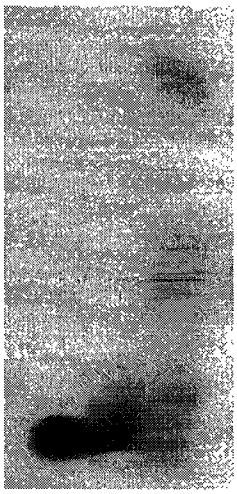


Figure 35: Images of Autoradiograms as Evidence of Linear Oligonucleotide Degradation by Other Nucleases Present in the HeLa Extract. The (-)ve control contains only the oligomer



(-)ve (+)ve 20h
linear ApC^{32}pCp

4.4 Debranching Studies Using His-Tagged yDBR

His-tagged purified yeast debranching enzyme (generously provided by Dr. Jef Boeke, Johns Hopkins University) was used to carry out debranching assays on the branched thioate oligomers, as well as some control branched phosphodiester oligomers. Subjecting a linear oligomer to yDBR showed no sign of degradation, as expected, in the presence of pure debranching enzyme (data not shown).

Typically, the debranching of the Y-shaped oligonucleotide, similar to the wild-type RNA lariat produced by the splicing of a yeast actin RNA transript (19mer), occurs within 30 minutes (see Figure 36). When the enzyme was used to debranch a Y-shaped 19mer, it was noted that after 30 min, more than half of the molecule had been debranched (Figure 36). The Y-shaped oligomers obtained from S. Carriero, which were 5'-end labeled, consisted of the sequence 5'-UACUAA(GUAUGU)GUAUGU, while the linear control 12mer, which is one of the debranching products and was also 5'-end labeled, had the sequence 5'-UACUAAGUAUGU.

Hypothetically, the scission of the 2',5' bond of the phosphorothioate V-trimers should yield two products; the first is the linear 3'-tail trimer [ApC^{32}pCp], which was synthesized separately as a control, while the second is the 2'-tail dimer [p_sG^{32}pCp] (Scheme 10). Moreover, the scission of the 2',5' bond of the phosphorothioate Y-tetramer should yield two products; the first is the linear 3'-tail tetramer [ApApC^{32}pCp], while the second is the same 2'-tail dimer as for the V-trimer, [p_sG^{32}pCp].

The debranching assays were carried out by incubating the phosphodiester V-trimer with the yDBR for 0.5, 6 and 24 h, and the phosphorothioate V-trimers and Y-tetramer for 0.5, 1, 6 and 24 h. Debranching studies of the control phosphodiester V-trimer, A(pG^{32}pCp)G^{32}pCp, revealed partial debranching at 6 h indicated by the appearance of a new faster moving band which migrates close to the linear ApC^{32}pCp. The small difference in the mobility between the debranched product and the linear trimer is due to the difference in one of the residues, where the trimer resulting from the debranching reaction is ApG^{32}pCp. The partial debranching appeared to increase at 24 h, but there were significant amounts of branched molecule still present (Figure 37). This

result indicates that small branched molecules, in this case trimers, might not be recognized as efficiently by the enzyme as large branched oligomers.

The debranching studies of the S_p-V-trimer yielded two bands that migrate faster than the starting material, one of which corresponds to the linear control trimer $ApC^{32}pCp$ (Figure 38). The new species were evident only after 6 h of incubation with the enzyme, where their intensities increased with time. Since incubation times with the enzyme were not conducted between the 1 and 6 h time points, it is not possible to draw any conclusion at the present time about the kinetic of the debranching reaction of small phosphorothioate oligomers.

The results of the debranching of the S_p-Y-tetramer show a possible debranching product, which co-migrates with one of the products of the V-trimer debranching assay, suggesting that this would be the linear 2'-tail dimer [$p_sG^{32}pCp$] (Figure 38). However, the presence of a second product band could not be clearly visualized, possibly due to the low amount of radioactivity used in the assay.

The R_p-V-trimer, however, showed no signs of debranching even after 24 h (Figure 38), as expected from the previous study conducted by Padgett[11].

The results indicated here reveal that the size of the substrate plays a significant role for efficient debranching, where the small substrates, V-trimers, were not cleaved as efficiently as the large branched 19-mer. Employing CE to study the debranching assays of the V-trimers could prove a good method to verify and corroborate the results obtained here in addition to avoiding the problems that arise from radiolabeling the substrates.

Figure 36: Images of Autoradiograms of Assay for Y-Shaped Oligonucleotide, 19mer, Recognition by yDBR. The (-)ve control contains only the oligomer.

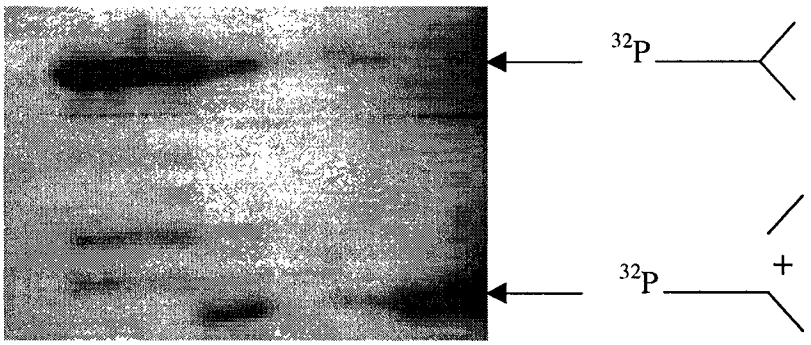


Figure 37: Images of Autoradiograms of Assay for Control V-Trimer Recognition by yDBR. The triangles indicate time of incubation, the (-)ve control contains only the oligomer.

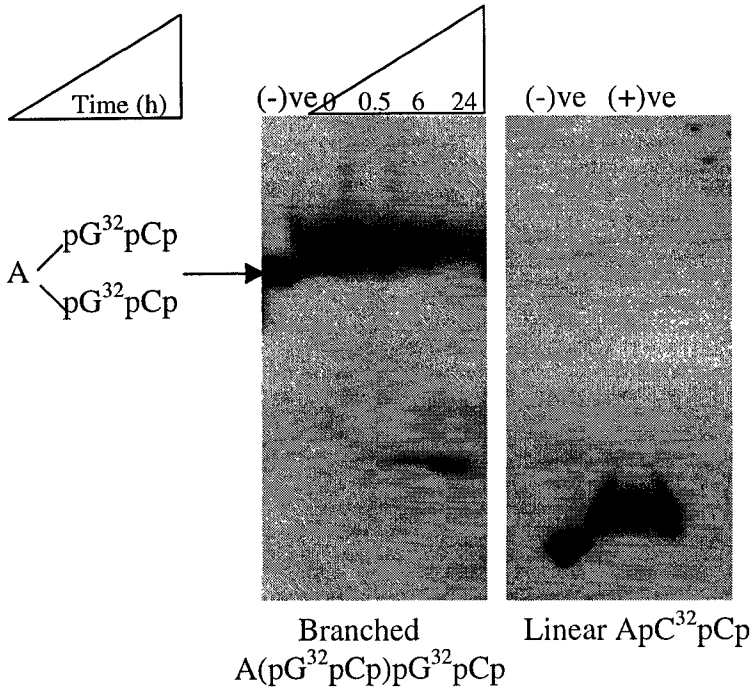
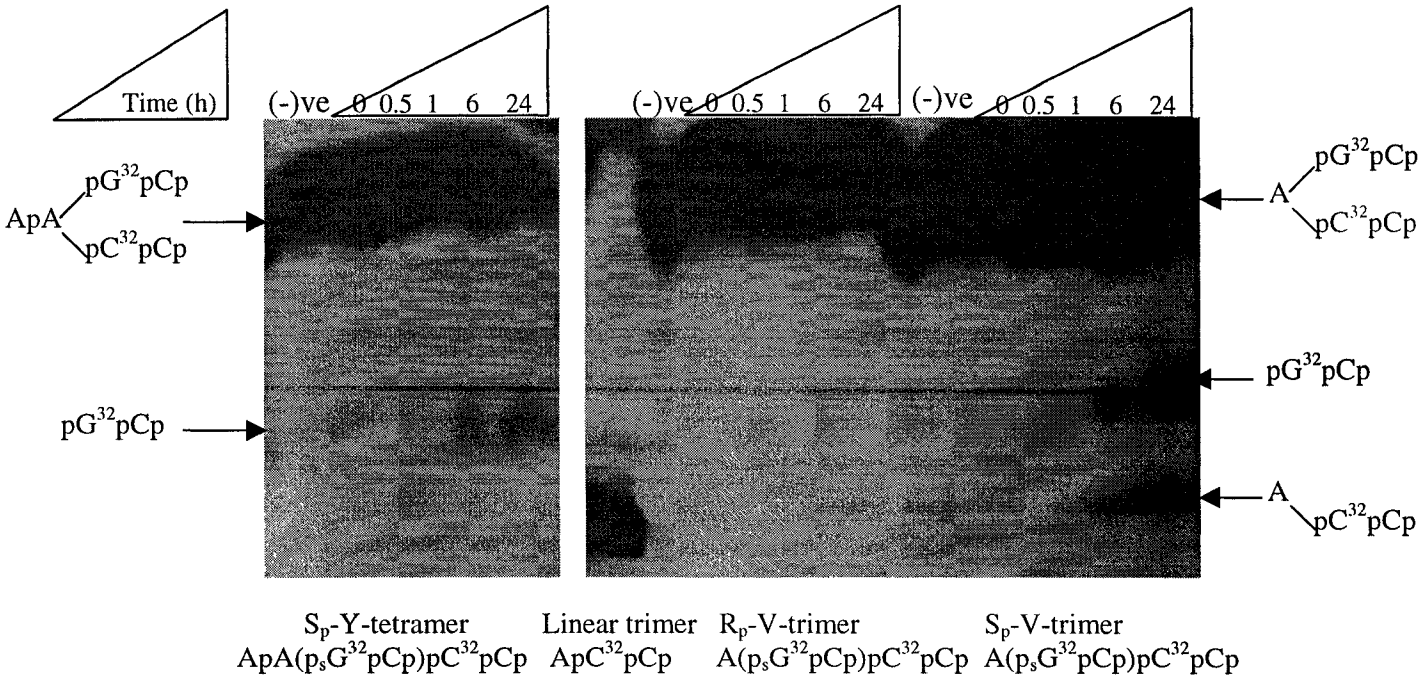


Figure 38: Images of Autoradiograms of Assay for Substrate Recognition by (yDBR). The triangles indicate time of incubation, the (-)ve controls contains only the oligomer.



CHAPTER 5: EXPERIMENTAL

5.1 General Methods

5.1.1 Reagents and Chemicals:

In general, high boiling point solvents used for reaction purposes were fractionally distilled under reduced pressure, according to standard protocols prior to use[90]. Pyridine was continuously refluxed over barium oxide under nitrogen and distilled prior to use. Tetrahydrofuran (THF) was refluxed over sodium and benzophenone under nitrogen until a purple color persisted. Acetonitrile (CH_3CN), used for organic synthesis, was refluxed over phosphorus pentoxide, P_2O_5, under inert atmosphere before usage, while acetonitrile used for nucleoside phosphoramidite coupling, in solution or on solid phase, was further refluxed and distilled over calcium hydride (CaH_2). Dichloromethane (DCM) was refluxed over CaH_2. N,N-dimethylformamide (DMF), collidine (2,3,6-trimethylpyridine), and diisopropylethylamine (DIPEA) were all dried by refluxing over calcium hydride (CaH_2), distilled under reduced pressure, and stored over 4 Å molecular sieves. Triethylamine (TEA) was distilled over calcium hydride (CaH_2) and was stored over 4 Å molecular sieves. Other solvents and reagents such as methanol, chloroform, diethyl ether, hydrochloric acid, glacial acetic acid, aqueous ammonia, anhydrous sodium sulfate, iodine, *para*-toluenesulfonic acid (TSA), imidazole, 1,2-dichloroethane, triethylamine tris(hydrofluoride) or TREAT HF, and tetra-*n*-butylammonium fluoride (TBAF) were used as purchased unless otherwise specified.

Moisture sensitive reactions, (*e.g.* silylation and phosphitylation of nucleosides), required that all solvents, reagents and glassware be dry. Glassware used in such reactions were oven-dried overnight and purged with argon before being sealed with rubber septum. The necessary reagents were allowed to warm to room temperature in a dessicator prior to use.

In order to prevent detritylation and/or premature activation nucleosides and their respective phosphoramidites, reactions containing traces of acidity were washed with 5% $NaHCO_3$, and subsequently dried over anhydrous sodium sulfate in the workup.

5.1.2 Chromatography:

Silica Gel Column Chromatography: Gravity and flash silica gel columns were performed on Merck Kieselgel 60 (200-400 mesh) silica gel (VWR).

Thin Layer Chromatography (TLC): Reactions and silica columns were analyzed and monitored using Merck Kieselgel 60F 254 silica analytical TLC aluminum sheets (0.2 x 20 cm x 20 cm). Nucleosides and their derivatives were visualized using a short wavelength UV light source (254 nm). Compounds bearing DMT protecting groups were visualized by heating the aluminum surface of TLC plates resulting in a strong orange color (DMT cation). A cysteine stain (16 mL sulfuric acid, 0.5 g cysteine and 100 mL water) was found to be a good method for preliminary characterization, where upon treating a TLC plate with it, it colors the compounds bearing DMT protecting groups with a strong orange color, and after heating the TLC plate it turns the compounds bearing sugar components into a dark brown color.

Thick Layer Chromatography: Preparative TLC to purify compounds was carried out using glass plates (20 cm x 20 cm) coated with a thick layer of Kieselgel 60 GF 254 silica gel.

Cation Exchange Chromatography: Ammonium salts produced by the desilylation reaction via the TBAF reagent, were removed using Dowex 50W-X8 cation exchange resin (Na^+ form, 20-50 mesh). The resin was regenerated by washing with 0.1 N HCl, followed by 0.1 N NaOH, followed by autoclaved H_2O until the filtrate was of neutral pH. It was then autoclaved and packed into a column (5 x 1.5 cm) and equilibrated with autoclaved H_2O prior to usage[69].

5.1.3 Instruments:

NMR Spectrophotometer: All spectra were obtained at ambient temperature, unless otherwise mentioned, using Varian XL-200, XL-300, XL-400, and XL-500 spectrophotometers. All ^1H and ^{31}P chemical shifts are reported in ppm downfield from their respective reference. For ^1H NMR, tetramethylsilane (TMS) was used as a

reference whereas 85% H_3PO_4 was used as an external reference for ^{31}P NMR (δ 0 ppm). The deuterated solvents used were acetone–d_6, MeOH-d_4, DMSO-d_6, and CH_2Cl_2-d_2.

UV Spectrophotometry: UV/VIS spectra were collected using a Varian CARY1 UV/VIS spectrophotometer at ambient temperature (Varian: Mulgrave, Victoria, Australia). The data was computed using Cary WinUV version 2 software (Varian Ltd.). Spectra were obtained in 95% ethanol for protected nucleoside derivatives, and water for the completely deprotected oligonucleotides.

Circular Dichroism: Circular dichroic spectra were recorded using a JACSO J-710 spectropolarimeter at ambient temperature[91]. The spectra were recorded using an average of five scans between 180 and 350 nm, obtained at a rate of 50 nm/min and sampling wavelength of 0.2 nm. The buffer used was 50 mM Na_2HPO_4, 100 mM HCl, at pH 7.25.

FAB-MS Spectrometry: Fast bombardment mass spectra were collected using KRATOS MS25RFA high-resolution spectrometer under Xenon gas with a beam energy of ~7 KV. Nitrobenzyl alcohol (NBA) matrix was used.

ESI, APCI: Both the electospray ionization and the atmospheric pressure chemical ionization spectra were collected using a Finnigan LCQ Duo, ThermoQuest. When spectra were obtained in the negative mode, these methods proved especially useful for the characterization of compounds bearing negative charges.

MALDI-TOF Mass Spectrometry: The MALDI was performed using a Kratos mass spectrometer. The oligomers (0.5 OD dissolved in 5 μl H_2O) were mixed with 5 μl of matrix, and 1 μl of the solution was used per analysis. The matrix used contained 10 mg/ml 6-Aza-2-thiothymine in a solution of 1:1 CH_3CN/ammonium acetate (20 mM) as described elsewhere[92].

HPLC: High performance liquid chromatography analysis and purifications were carried out on a Waters HPLC equipped with a Waters 486 detector and a 600 pump and a 600E system controller. The system is interfaced to a PC running Millennium® software version 3.20. For oligonucleotide purification, an anion exchange 7.5 x 75 mm Protein-Pak™ DEAE 5PW HPLC column was used. The mobile phases consisted of: Solvent A: H_2O, Solvent B: 1 M $LiClO_4$. Chromatograms were run using a gradient of 0-10% Solvent B over 60 min with a flow rate of 1.2 mL/min at ambient temperature. Enzymatic digestion analyses were performed using a reverse-phase HP Hypersil ODS-

C_{18} column (4.6 x 250 mm, 5 μm) with a flow rate of 1.2 mL/min. The mobile phases comprised of: Solvent A: 50 mM TEAA (triethylammonium acetate), Solvent B: CH_3CN. Chromatograms were obtained utilizing a gradient of 0-10% Solvent B over 10 min, followed by 10-35% Solvent B over 15 min[46]. The deprotected isomers of the phosphorothioate dimers were resolved on an HB Zorbax RX-C_{18} semi-preparative reverse-phase column (9.4 x 250 mm, 5 μm) at r.t. with a flow rate of 4 mL/min. The mobile phases consisted of, Solvent A: 50 mM TEAA (triethylammonium acetate), solvent B: CH_3CN. The gradient utilized 0-20% Solvent B over 60 min.

5.2 Monomer Synthesis

5.2.1 N^6-Benzoyladenosine (**1.1**):

Adenosine (29.9 mmol, 8 g) was dried by coevaporation with anhydrous pyridine (3 x 50 mL) prior to N-benzoylation according to the transient silylation method (Jones *et al.*)[68]. To a suspension of the dry adenosine in anhydrous pyridine (160 mL) was added trimethylchlorosilane (TMSCl) (7.5 eq, 224 mmol, 28 mL) and the mixture was stirred at r.t. for 15 min. To the silyl ether protected nucleoside was added benzoyl chloride (BzCl; 5 eq, 149.5 mmol, 17.4 mL) and the mixture was stirred at r.t. for an additional 2.5 h. The reaction was terminated by the addition of H_2O (20 mL) with constant stirring for 15 min over an ice bath, followed by 29% NH_4OH (60 mL). The mixture was left stirring for 30 min. The solvent was evaporated under reduced pressure and the crude product dissolved in H_2O and washed with ethyl acetate. Crystallization of the product from water was initiated after partial evaporation of the solvent, and completed by cooling the solution at 4°C overnight. Filtration of the resultant crystals allows for the isolation the pure product as a white powder (79 % yield, 8.8 g). TLC: CH_2Cl_2/MeOH 9:1, R_f 0.26. ^1H NMR (DMSO-d_6, 400 MHz): 8.75 (s, 1, H_8), 8.71 (s, 1, H_2), 8.04 (d, 2, benzoyl), 7.64 (m, 1, benzoyl), 7.55 (dd, 2, benzoyl), 6.03 (d, 1, $H_{1'}$), 5.56 (d, 1, $OH_{2'}$), 5.25 (d, 1, $OH_{3'}$), 5.13 (t, 1, $OH_{5'}$), 4.64 (m, 1, $H_{2'}$), 4.18 (m, 1, $H_{3'}$), 3.97 (dt, 1, $H_{4'}$), 3.67-3.58 (m, 2, $H_{5'}$, $H_{5''}$).

5.2.2 N^6-Benzoyl-5'-O-(4 4'-Dimethoxytrityl) Adenosine (**1.2**):

N^6-benzoyladenosine (**1.1**) (15.6 mmol, 5.8 g) was dried by coevaporation with anhydrous pyridine. Triethylamine (TEA) (~3 eq) was added to the dried Ad^{Bz} and the mixture was dissolved in anhydrous pyridine (80mL), and cooled to 0°C before adding dropwise a mixture of DMTCl (1.2 eq, 18.8mmol, 6.4 g) and DMAP (catalytic amount, 30 mg) dissolved in pyridine (25 mL) under a nitrogen atmosphere. The mixture was allowed to warm to r.t. and was stirred overnight before it was quenched with 5% $NaHCO_3$ (70 mL). The crude product was with ethyl acetate (3 x 50mL), and the combined organic layers dried over anhydrous Na_2SO_4. The solvent was removed under reduced pressure to afford a dark brown oil which was later purified by flash silica gel column chromatography using a gradient in CH_2Cl_2/MeOH/NEt_3 (gradient 99.5/0/0.5 to 96.5/3/0.5), in order to recover the product as a pure white foam (92% yield, 9.7 g). TLC: CH_2Cl_2/MeOH 9:1, R_f 0.47. MS (FAB-nitrobenzyl alcohol, calc. M^+: 673), found: MH^+: 674. ^1H NMR (DMSO-d_6, 400 MHz): 11.23 (s, 1, NH), 8.66 (s, 1, H_8), 8.58 (s, 1, H_2), 8.02 (d, 2, benzoyl), 7.63 (m, 1, benzoyl), 7.53 (dd, 2, benzoyl), 7.34-7.21-6.81 (overlapping m, 13, DMT), 6.05 (d, 1, $H_{1'}$), 5.62 (d, 1, $OH_{2'}$), 5.27 (d, 1, $OH_{3'}$), 4.76 (m, 1, $H_{2'}$), 4.31 (m, 1, $H_{3'}$), 4.09 (dt, 1, $H_{4'}$), 3.70 (s, 6, OCH_3), 3.22 (m, 2, $H_{5'}$, $H_{5''}$).

5.2.3 N^6-Benzoyl-5'-O-(4,4'-Dimethoxytrityl)-3'-O-*tert*-Butyldimethylsilyladenosine (**1.3a**) and N^6-Benzoyl-5'-O-Dimethoxytrityl-2'-O-*tert*-Butyldimethylsilyladenosine (**1.3b**):

To a stirred solution of N^6-benzoyl-5'-O-dimethoxytrityladenosine (**1.2**) (3.3 mmol, 2.2 g) and imidazole (2.6 eq, 8.6 mmol, 0.59 g) in anhydrous DMF (20 mL), was added TBDMS-Cl (1.3 eq, 4.2 mmol, 0.63 g) in one portion and stirred at r.t. for 20 h. The reaction was stopped by the addition of 5% $NaHCO_3$ (5 mL), and the solvent was evaporated under reduced pressure. The residue was taken up in CH_2Cl_2 (40 mL), washed with saturated brine (2 x 50 mL), and concentrated to a yellow foam. The crude product was purified and the two isomers were separated by flash silica gel column chromatography using an EtOAc/CH_2Cl_2 gradient (1:7 to 1:2), to yield 51% (1.3 g) of N-

Bz-5'-DMT-2'-TBDMS-rA and 37% (0.93 g) of N-Bz-5'-DMT-3'-TBDMS-rA as pure white foams. TLC: CH_2Cl_2/MeOH 9:1, R_f 0.81 for both isomers; CH_2Cl_2/EtOAc 2:1, R_f 0.42 and 0.6 for the 3'- and 2'-isomer, respectively. MS (FAB-nitrobenzyl alcohol, calc. M^+: 787), found: MH^+: 788, DMT^+: 303. Refer to appendix for the ^1H and gCOSY NMR spectra of both isomers.

5.2.4 N^6-Benzoyl-5'-O-(4,4'-Dimethoxytrityl)-3'-O-*tert*-Butyldimethylsilyladenosine-2'-O-[β-Cyanoethyl N,N-Diisopropyl] Phosphoramidite (**1.4a**):

To a stirred solution of N^6-Bz-5'-O-DMT-3'-O-TBDMS-rA (**1.3a**) (1.27 mmol, 1g) and diisopropylethylamine (DIPEA) (4 eq, 5.06 mmol, 0.88 mL) in anhydrous THF (16 mL), was added *N,N*-diisopropyl-β-cyanoethylphosphonamidic chloride (1.7 eq, 2.15 mmol, 0.5 mL) dropwise at 0°C over 5 min. The reaction proceeded at r.t., whereupon a white precipitate (diisopropylethylammonium hydrochloride) appeared, and stirring was continued for 1.5 h. The mixture was diluted with EtOAc (100 mL, prewashed with 5% $NaHCO_3$) and the crude product was washed with saturated brine solution (3 x 100 mL). The combined organic layers were dried over anhydrous Na_2SO_4, filtered and the solvent was removed under reduced pressure to afford a light yellow oil. The crude product was purified by flash silica gel column chromatography using EtOAc/Hexanes/NEt_3 (gradient 3:6.5:0.5 to 4:5.5:0.5), to provide a pure white foam (70% yield, 0.875g). TLC: CH_2Cl_2/MeOH 9/1, R_f 0.78; EtOAc/Hexanes/NEt_3 7/2.5/0.5, R_f 0.52. MS (FAB-nitrobenzyl alcohol, calc. M^+: 988), found: MH^+: 989, DMT^+: 303. Refer to appendix for ^{31}P NMR.

5.2.5 N^6-Benzoyl-5'-O-(4,4'-Dimethoxytrityl)-2'-O-*tert*-Butyldimethylsilyladenosine-3'-O-[β-Cyanoethyl N,N-Diisopropyl] Phosphoramidite (**1.4b**):

The phosphoramidite (**1.4b**) was synthesized using a similar procedure as for the synthesis of (**1.4a**) starting with the monomer (**1.3b**) (0.4 mmol, 0.32 g), yielding a pure white foam (78% yield, 0.31g). TLC: EtOAc/Hexanes/NEt_3 7:2.5:0.5, R_f value for the two diastereomers are 0.47 and 0.54; EtOAc/Hexanes/NEt_3 6:3.5:0.5, R_f 0.56 and 0.63.

MS (FAB-nitrobenzyl alcohol, calc. M^+: 988), found: MH^+: 989, DMT^+: 303. Refer to appendix for ^{31}P NMR.

5.2.6 N^2-Isobutyrylguanosine (**2.1**):

Guanosine (35.3 mmol, 10 g) was dried by coevaporation (anhydrous pyridine; 3 x 60 mL) and suspended in anhydrous pyridine (160 mL) to which chlorotrimethylsilane (TMSCl; 7.8 eq, 265 mmol, 33.6 mL) was added dropwise at 0°C. The mixture was stirred at r.t. for 1.25 h, cooled to 0°C and isobutyric anhydride (5 eq, 176.5 mmol, 29 mL) was added, after which stirring was resumed overnight at r.t. Workup of the reaction was initiated by adding cold H_2O (150 mL) followed by 28% NH_4OH (65 mL) at 0°C and the mixture was stirred at r.t. for 50 min at which time it was concentrated to an oil, dissolved in water and washed with ethyl acetate. Purification was carried out via crystallization from H_2O, to yield a white powder in **75%** isolated yield. ^1H NMR (DMSO-d_6, 400 MHz): 8.24 (s, 1, H_8), 5.77 (d, 1, $H_{1'}$), 4.40 (m, 1, $H_{2'}$), 4.41 (m, 1, $H_{3'}$), 3.88 (dt, 1, $H_{4'}$), 3.59-3.52 (m, 2, $H_{5'}$, $H_{5''}$), 2.75 (m, 1, CH-*i*Bu), 0.99 (dd, 6, CH_3-*i*Bu).

5.2.7 N^2-Isobutyryl-5'-O-(4,4'-Dimethoxytrityl) Guanosine (**2.2**):

N^2-isobutyrylguanosine (**2.1**) (24.4 mmol, 8.6 g), dried by coevaporation with anhydrous pyridine, was suspended in (80 mL) anhydrous pyridine. The mixture was cooled to 0°C before adding dropwise a mixture of DMTCl (1.2 eq, 29 mmol, 9.9 g) and DMAP (catalytic amount, 40 mg) in pyridine (25 mL) under a nitrogen atmosphere. The mixture was allowed to warm to r.t. and was stirred overnight before it was stopped by adding 5% $NaHCO_3$ (100 mL). The crude product was then extracted with ethyl acetate, and the combined organic layers dried over anhydrous Na_2SO_4. The solvent was removed *in vacuo* to afford a dark, brown oil. Purification was achieved via flash silica gel column chromatography of the crude mixture using a CH_2Cl_2/MeOH/NEt_3 gradient (99.5:0:0.5 to 96.5:3:0.5), to recover a pure white foam in 80% yield (12.7 g). TLC: CH_2Cl_2/MeOH 9:1, R_f 0.36. MS (FAB-nitrobenzyl alcohol, calc. M^+: 655), found: MH^+: 656, DMT^+: 303. ^1H NMR (DMSO-d_6, 400 MHz): 8.11 (s, 1, H_8), 7.31-7.20-6.80

(overlapping m, 13, DMT), 5.84 (d, 1, $H_{1'}$), 5.62 (d, 1, $OH_{2'}$), 5.19 (d, 1, $OH_{3'}$), 4.51 (m, 1, $H_{2'}$), 4.19 (m, 1, $H_{3'}$), 4.01 (dt, 1, $H_{4'}$), 3.70 (s, 6, OCH_3), 3.23-3.15 (m, 2, $H_{5'}$, $H_{5''}$), 2.74 (m, 1, CH-*i*Bu), 1.09 (dd, 6, CH_3-*i*Bu).

5.2.8 N^2-Isobutyryl-2',3'-O-di-Benzoyl-5'-O-(4,4'-Dimethoxytrityl) Guanosine (**2.3**):

N^2-isobutyryl-5'-O-(4,4'-dimethoxytrityl) guanosine (**2.2**) (3 mmol, 2 g) was dried by coevaporation with anhydrous pyridine. The dried $^{DMT}Gu^{iBu}$ was suspended in anhydrous pyridine (40 mL) and cooled to 0°C before adding BzCl (2.4 eq, 7.3 mmol, 0.85 mL) dropwise. The mixture was allowed to warm to r.t. and was stirred for 3.5 h before it was stopped by adding 20 mL of H_2O at 0°C and stirring at r.t. for 5 min. The crude product was dissolved in CH_2Cl_2 (100 mL) and washed with H_2O. The organic phase was dried over anhydrous Na_2SO_4, and the solvent was removed under reduced pressure. The crude product was purified by flash silica gel column chromatography using CH_2Cl_2/MeOH (gradient 0-3% MeOH), to yield a pure white foam (77%, 2 g). TLC: CH_2Cl_2/MeOH 9/:, R_f 0.54. MS (APCI-methyl alcohol, calc. M^+ for product with *i*Bu: 863, calc. M^+ for product with Bz: 897), found: M^+: 863, 896 in ratio of 10:1, respectively, DMT^+: 303. Refer to the results and discussion section for ^1H NMR.

5.2.9 N^2-Isobutyryl-O^6-*p*-Nitrophenylethyl- 2',3'-O-di-Benzoyl-5'-O-(4,4'-Dimethoxytrityl) Guanosine (**2.4**):

To a stirred solution of (**2.3**) (0.69 mmol, 0.6 g), triphenylphosphine (Ph_3P; 3 eq, 2.08 mmol, 0.53g) and 2-*p*-nitrophenylethanol (3 eq, 2.08 mmol, 0.34 g) in anhydrous THF, was added diethyl azodicarboxylate (DEAD; 2.25 eq, 1.56 mmol, 0.24 mL) dropwise and the reaction stirred overnight at r.t. The reaction was concentrated to an oil and extracted from 5% $NaHCO_3$ with CH_2Cl_2. The organic phase was dried over anhydrous Na_2SO_4, and the solvent removed under reduced pressure. Since the relative polarity of the product and the impurities (possibly Ph_3P=O) in 100% CH_2Cl_2 is opposite that in CH_2Cl_2/MeOH mixture, which would effect the separation using a gradient solvent system, the crude product was purified by flash silica gel column chromatography

using 100% CH_2Cl_2 until the impurities were flushed out before using a gradient containing 1-2% MeOH (69% yield, 0.48 g). TLC: CH_2Cl_2/MeOH 19:1, R_f 0.77; CH_2Cl_2/MeOH 29:1, R_f 0.6; Toluene/EtOAc 1:1, R_f 0.58; Et_2O/CH_2Cl_2/EtOH 21:76:3, R_f 0.79. MS (FAB-nitrobenzyl alcohol, calc. M^+ for product with *i*Bu: 1013, calc. M^+ for product with Bz: 1047), found: MH^+: 1014, 1048 in ratio of ca. 3:1, respectively.

5.2.10 N^2-Isobutyryl-O^6-*p*-Nitrophenylethyl- 2',3'-O-di-Benzoylguanosine (**2.5**):

To N^2 - isobutyryl - O^6 -*p* – nitrophenylethyl - 2', 3' – O - di – benzoyl – 5' – O – (4,4'-dimethoxytrityl) guanosine (**2.4**) (0.56mmol, 0.48 g) was added *p*-toluenesulfonic acid-MeOH/CH_2Cl_2 (12 mL; prepared by stirring 3 g *p*-TSA, 30 mL MeOH and 120 mL CH_2Cl_2) at r.t. Under these conditions, complete cleavage of the DMT group was achieved at 15 min, at which time the reaction was diluted with CH_2Cl_2 and washed with 5% $NaHCO_3$ before drying of the combined organic layers over anhydrous Na_2SO_4, followed by solvent removal *in vacuo* to afford a dark yellow crude residue. The pure product was precipitated from stirred hexanes and filtered to give a white residue. (85% yield, 0.28 g). TLC: CH_2Cl_2/MeOH 9:1, R_f 0.59; CH_2Cl_2/Acetone 3:1, R_f 0.4. MS (FAB-nitrobenzyl alcohol, calc. M^+ for product with *i*Bu: 710, calc. M^+ for product with Bz: 744), found: MH^+: 711, 745 in ratio of 3:1, respectively. Refer to appendix for ^1H and gCOSY NMR.

5.2.11 N^4-Benzoylcytidine (**3.1**):

To a suspension of cytosine (41 mmol, 10g) in anhydrous N,N-dimethylformamide was added benzoic anhydride (1 eq, 41 mmol, 9.2 g) and the mixture was stirred at r.t. for 25 h. After removal of the solvent under reduced pressure, the residue was triturated with excess diethyl ether and the crystalline product was collected by filtration to afford a white product[75]. TLC: CH_2Cl_2/MeOH 9:1, R_f 0.21 (82% yield, 11.7 g).

5.2.12 N^4-Benzoyl-5'-O-(4,4'-Dimethoxytrityl) Cytidine (**3.2**):

N^6-benzoylcytidine (**3.1**) (14.9 mmol, 5.18g), dried by coevaporation with anhydrous pyridine, was suspended in anhydrous pyridine (60 mL), cooled to 0°C and flushed with N_2 prior to the dropwise addition of a solution consisting of DMTCl (1.2 eq, 17.9 mmol, 6.1 g) and DMAP (catalytic amount, 40 mg) in pyridine (25 mL). The mixture was allowed to warm to r.t. and stirred for 36 h before it was quenched with 5% $NaHCO_3$ (100 mL). The crude product was then extracted thrice with ethyl acetate, and the combined organic layers were dried (anhydrous Na_2SO_4), filtered, and the solvent removed *in vacuo* to afford a dark yellow oil. The crude residue was purified by flash silica gel column chromatography using CH_2Cl_2/MeOH/NEt_3 (gradient 99.5:0:0.5 to 96.5:3:0.5), to isolate the product as a white foam (60% yield, 5.8g). TLC: CH_2Cl_2/MeOH 9:1, R_f 0.45. MS (FAB-nitrobenzyl alcohol, calc. M^+: 649), found: MH^+: 650, DMT^+: 303, ($MH\text{-}DMT^+$): 346.

5.2.13 N^4-Benzoyl-2',3'-O-di-Benzoyl-5'-O-(4,4'-Dimethoxytrityl) Cytidine (**3.3**):

N^4-benzoyl-5'-O-dimethoxytritylcytidine (**3.2**) (5.0 mmol, 3.27 g) was dried by coevaporation (anhydrous pyridine), suspended in anhydrous pyridine (60 mL) and the mixture was cooled to 0°C prior to the dropwise addition of BzCl (3.5 eq, 17.6 mmol, 2.04 mL). The mixture was allowed to warm to r.t. and stirred for 3.5 h before it was quenched with H_2O (30 mL) at 0°C and stirring continued at r.t. for 5 minutes. The crude product was dissolved in CH_2Cl_2 (120 mL) and washed with H_2O. The organic phase was dried over anhydrous Na_2SO_4, filtered and the solvent was removed under reduced pressure. The crude product was purified by flash silica gel column chromatography using CH_2Cl_2/MeOH (gradient 0-3% MeOH), to recover a pure pale yellow foam (86% yield, 3.73g). TLC: CH_2Cl_2/MeOH 9:1, R_f 0.74. MS (FAB-nitrobenzyl alcohol, calc. M^+: 857), found: MH^+: 858, DMT^+: 303.

5.2.14 N^4-Benzoyl-2',3'-O-di-Benzoylcytidine (**3.4**):

To N^4 – benzoyl - 2' - 3' – O – di – benzoyl - 5' – O – (4,4'-dimethoxytrityl) cytidine (**3.3**) (3.76 mmol, 3.24g) was added a solution consisting of *p*-toluenesulfonic acid/MeOH/CH_2Cl_2 (76 mL), which was prepared by dissolving *p*-TSA (3 g) in MeOH (30 mL) and CH_2Cl_2 (120 mL) at r.t. After 15 min, the reaction was diluted with CH_2Cl_2 and washed with 5% $NaHCO_3$ before the combined organic layers were dried (anhydrous Na_2SO_4), filtered and the solvent removed *in vacuo* to afford a dark yellow crude residue. The pure product was precipitated from a cold solution of stirred hexanes and was filtered to yield a white residue. (79% yield, 1.51 g). TLC: CH_2Cl_2/MeOH 9.5:0.5, R_f 0.40; Hexanes/EtOAc/NEt_3 2.7:7:0.5, R_f 0.19. MS (FAB-nitrobenzyl alcohol, calc. M^+: 555), found: MH^+: 556. Refer to appendix for ^1H NMR.

5.2.15 N^4-Benzoyl-2',3'-O-di-Benzoylcytidine-5'-O-[β-Cyanoethyl N,N-Diisopropyl] Phosphoramidite (**3.5**):

To a stirred solution of N^4-benzoyl-2'-3'-O-di-benzoylcytidine (**3.4**) (2.71 mmol, 1.5 g), DIPEA (4 eq, 10.84 mmol, 1.86 mL) and DMAP (0.1 eq, 0.271 mmol, 33 mg) in anhydrous THF (24 mL) was added the N,N-diisopropylamino-β-cyanoethylphosphonamidic chloride reagent (1.2 eq, 3.25 mmol, 0.72 mL) dropwise at 0°C. The reaction was allowed to go to r.t. with the concomitant appearance of a white precipitate (diisopropylethylammonium hydrochloride), and was stirred for 2.5 h. The mixture was diluted with EtOAc (150 mL, prewashed with 5% $NaHCO_3$) and the crude product was washed with saturated brine solution (4 x 60 mL). The combined organic layers were dried over anhydrous Na_2SO_4 and the solvent removed under reduced pressure to afford a light yellow oil. Purification proceeded by flash silica gel column chromatography using EtOAc/Hexanes/NEt_3 (gradient 4:5.5:0.5 to 5:4.5:0.5), to afford a pure white foam (86% yield, 1.76g). TLC: EtOAc/Hexanes/NEt_3 7:2.5:0.5, R_f 0.38; CH_2Cl_2/MeOH 9.5:0.5, R_f 0.65. MS (FAB-nitrobenzyl alcohol, calc. M^+: 755), found: MH^+: 656. Refer to appendix for ^{31}P NMR.

5.3 Oligomer Synthesis

***NOTE*:** Refer to the results and discussion section for the NMR data of the dimers and trimers.

5.3.1 Solution Phase Synthesis:

*5.3.1.1 Solution Synthesis of 2',5'-Phosphorothioate Dimers (Ap_sG) (**4.1a,b**):*

To an Argon purged glass hypovial with an oven-dried magnetic stirring bar, was added compound **2.5** $^{HO}{}_{Bz}Gu^{iBu,NPE}{}_{Bz}$ (0.128 mmol, 91 mg), suitably protected adenosine-2'-phosphoramidite (**1.4a**; 1.2 eq, 0.154 mmol, 0.15 g) and tetrazole (4.4 eq, 0.565 mmol, 40 mg). The reaction was started by injecting anhydrous acetonitrile (1.5 mL) under nitrogen, and stirred at r.t. until complete consumption of the starting material was observed after 2 h. To this mixture was added a solution of the sulfurizing reagent, EDITH (1.2 eq, 0.154 mmol, 25 mg) in anhydrous acetonitrile (1 mL) under an inert atmosphere to sulfurize the phosphite triester intermediate. The mixture was stirred at r.t. for 10 min and the solvent was evaporated. The crude product was dissolved in CH_2Cl_2 and washed with 5% $NaHCO_3$. The organic phase was dried over anhydrous Na_2SO_4, and the solvent was removed under reduced pressure to obtain a yellow solid. The crude dimer was purified by flash silica gel column chromatography in order to separate the two isomers using CH_2Cl_2/Et_2O 5:5 (79% total yield, 53% or 0.109 g of the first isomer (R_p), 26% or 0.053 g of the second isomer (S_p)). TLC: CH_2Cl_2/Et_2O 9:1, R_f value for the two S_p and the R_p dimers are 0.16, 0.34; CH_2Cl_2/MeOH 9:1, R_f 0.47, 0.63; CH_2Cl_2/Acetone 3:1, R_f 0.4, 0.53, respectively. Refer to Tables 2&3 for the R_f and ^{31}P NMR values. MS (FAB-nitrobenzyl alcohol, calc. M^+ for product with *i*Bu: 1629, calc. M^+ for product with Bz: 1663), found for the R_p isomer: MH^+: 1630, 1664 in ratio of 2.3:1, respectively, and DMT^+: 303; and for the S_p isomer: MH^+: 1630, 1664 in ratio of 2.5:1, respectively, and DMT^+: 303.

*5.3.1.2 Removal of the Cyanoethyl-Protecting Group from the Phosphorothioate Linkage in the R_p Dimer (**4.2a**):*

The R_p dimer **(4.1a)** (0.062 mmol, 101 mg) was dissolved in 3.5 mL TEA/CH_3CN (4:6 v/v) and the reaction was stirred at r.t. for 4 h. The solvent was then evaporated and the crude dimer redissolved in CH_2Cl_2 and washed with 5% $NaHCO_3$. The organic phase was dried over anhydrous Na_2SO_4, and the solvent evaporated under reduced pressure generating the crude thio-phosphodiester which was used without further purification in the ensuing reaction (97% yield, 94mg). Refer to Tables 2&3 for the R_f and ^{31}P NMR values. MS (FAB-nitrobenzyl alcohol, calc. M^+ for product with *i*Bu: 1575, calc. M^+ for product with Bz: 1609), found: MH^+: 1576, 1610 in ratio of 2.8:1, respectively. DMT^+: 303.

*5.3.1.3 Removal of the Cyanoethyl-Protecting Group from the Phosphorothioate Linkage in the S_p Dimer (**4.2b**):*

The S_p dimer **(4.1b)** (0.031 mmol, 50 mg) was dissolved in 1.5 mL TEA/CH_3CN (4:6 v/v) and the reaction was stirred at r.t. for 3 h. The solvent was then evaporated and the crude dimer redissolved in CH_2Cl_2 and washed with 5% $NaHCO_3$. The organic phase was dried over anhydrous Na_2SO_4, and the solvent evaporated under reduced pressure generating the crude thio-phosphodiester which was used without further purification in the ensuing reaction (98% yield, 47.3mg). Refer to Tables 2&3 for the R_f and ^{31}P NMR values. MS (FAB-nitrobenzyl alcohol, calc. M^+ for product with *i*Bu: 1575, calc. M^+ for product with Bz: 1609), found: MH^+: 1576, 1610 in ratio of 1.7:1, respectively. DMT^+: 303.

*5.3.1.4 Removal of the Silyl Group (TBDMS) in the Presence of Dimethoxytrityl (DMT) and p-Nitrophenylethyl (NPE) Protecting Groups (**4.3a**):*

The protected thio-phosphodiester R_p dimer (**4.2a**), (0.049 mmol, 77 mg), was dissolved in excess TBAF solution (1.0 M in THF) that was preneutralized using acetic

acid, HOAc. The reaction was stirred at 45°C overnight before it was quenched with H_2O and the crude product extracted with CH_2Cl_2, and dried under reduced pressure. The crude dimer was purified by flash silica gel column chromatography using EtOAc/MeOH (gradient 0-2% MeOH) (70% yield, 50 mg). Refer to Tables 2&3 for the R_f and ^{31}P NMR values. MS (ESI-MeOH-negative mode, calc. M^+ for product with *i*Bu: 1461, calc. M^+ for product with Bz: 1495), found: M^-: 1460, 1494 in ratio of ca. 2.4:1, respectively.

*5.3.1.5 Removal of the Silyl Group (TBDMS) in the Presence of Dimethoxytrityl (DMT) and p-Nitrophenylethyl (NPE) Protecting Groups (**4.3b**):*

The protected thio-phosphodiester S_p dimer **(4.2b),** (0.03 mmol, 47.3 mg), was dissolved in excess TBAF solution (1.0 M in THF) that was preneutralized using acetic acid, HOAc. The reaction was stirred at 42°C for 19 h before it was quenched with H_2O and the crude product extracted with CH_2Cl_2, and dried under reduced pressure. The crude dimer was purified by flash silica gel column chromatography using EtOAc/MeOH (gradient 0-2% MeOH) (82% yield, 36 mg). Refer to Tables 2&3 for the R_f and ^{31}P NMR values. MS (ESI-MeOH-negative mode, calc. M^+ for product with *i*Bu: 1461, calc. M^+ for product with Bz: 1495), found: M^-: 1460, 1494 in ratio of ca. 1.3:1, respectively.

*5.3.1.6 Solution Synthesis of the R_p-V-Trimer (A(p_sG)pC) (**18a**):*

To an Argon purged glass hypovial with a magnetic stirring bar, was added the desilylated R_p dimer **(4.3a)** (0.027mmol, 40 mg), cytidine-5'-phosphoramidite **(3.5)** (6 eq, 0.164 mmol, 127 mg) and tetrazole (6 eq, 0.164 mmol, 12.6 mg). The reaction was initiated by injecting anhydrous acetonitrile (0.75 mL) and stirred at r.t. for 3 h in a dry nitrogen box. 2,4,6-collidine (6 eq, 0.164 mmol, 21.7 μL) was added followed by addition of aqueous iodine solution (0.1 M in THF/water 3:1.5, excess) in order to oxidize the phosphite triester intermediate. After 5 minutes the reaction was diluted with CH_2Cl_2 and washed with 5% sodium bisulfite. The organic phase was washed with 5% $NaHCO_3$, dried over anhydrous Na_2SO_4, and the solvent was removed under reduced pressure to obtain a foam. The crude V-trimer was purified by flash silica gel column

chromatography using CH_2Cl_2/MeOH/0.5%TEA (gradient 0-2% MeOH) (82% total yield, 47.5 mg). Refer to Tables 2&3 for the R_f and ^{31}P NMR values.

*5.3.1.7 Solution Synthesis of the S_p-V-Trimer (A(p_sG)pC) (**18b**):*

To an Argon purged glass hypovial with a magnetic stirring bar, was added the desilylated S_p dimer (**4.3b**) (0.021mmol, 30 mg), cytidine-5'-phosphoramidite (**3.5**) (6 eq, 0.123 mmol, 90 mg) and tetrazole (6 eq, 0.123 mmol, 8.7 mg). The reaction was initiated by injecting anhydrous acetonitrile (0.60 mL) and stirred at r.t. for 1.25 h in a dry nitrogen box. 2,4,6-collidine (6 eq, 0.123 mmol, 16.25 μL) was added followed by addition of aqueous iodine solution (0.1 M in THF/ water 3:1.5, excess) in order to oxidize the phosphite triester intermediate. After 5 minutes the reaction was diluted with CH_2Cl_2 and washed with 5% sodium bisulfite. The organic phase was washed with 5% $NaHCO_3$, dried over anhydrous Na_2SO_4, and the solvent was removed under reduced pressure to obtain a foam. The crude V-trimer was purified by flash silica gel column chromatography using CH_2Cl_2/MeOH/0.5%TEA (gradient 0-2% MeOH) (75% total yield, 32.2 mg). Refer to Tables 2&3 for the R_f and ^{31}P NMR values.

*5.3.1.8 Removal of the Cyanoethyl-Protecting Group from the Phosphodiester Linkage in the V-Trimers (**19a,b**):*

Refer to the procedure on the removal of the cyanoethyl-protecting group from the phosphorothioate linkage in the dimers. Refer to Tables 2&3 for the R_f and ^{31}P NMR values.

*5.3.1.9 Detritylation of the R_p-V-Trimer (A(p_sG)pC) (**20a**):*

The R_p-V-trimer (**19a**) (0.011 mmol, 23 mg) was dissolved in 2.5 mL CH_2Cl_2 and 2.5 mL (*p*-TSA/MeOH/CH_2Cl_2), prepared by stirring 0.5 mg *p*-TSA in 5 mL MeOH and 20 mL CH_2Cl_2, and the reaction was stirred at r.t. for 25 minutes. It was diluted with CH_2Cl_2, washed with 5% $NaHCO_3$, dried over anhydrous Na_2SO_4 and the solvent was

dried under reduced pressure. The crude V-trimer was purified by flash silica gel column chromatography using CH_2Cl_2/MeOH (gradient 0-2% MeOH). (>90% yield) Refer to Table 2 for the R_f values. MS (ESI-MeOH-negative mode, calc. M^+ for product with *i*Bu: 1775, calc. M^+ for product with Bz: 1809), found: M^-: 1775, 1809 in ratio of 2.75:1, respectively.

*5.3.1.10 Detritylation of the S_p-V-Trimer (A(p_sG)pC) (**20a**):*

The S_p-V-trimer (**19b**) (0.010 mmol, 22 mg) was dissolved in 3 mL CH_2Cl_2 and 0.6 mL (*p*-TSA/MeOH/CH_2Cl_2), prepared by stirring 0.5 mg *p*-TSA in 5 mL MeOH and 20 mL CH_2Cl_2, and the reaction was stirred at r.t. for 10 minutes. It was diluted with CH_2Cl_2, washed with 5% $NaHCO_3$, dried over anhydrous Na_2SO_4 and the solvent was dried under reduced pressure. The crude V-trimer was purified by flash silica gel column chromatography using CH_2Cl_2/MeOH (gradient 0-2% MeOH). (93% yield, 17.4 mg) Refer to Table 2 for the R_f values. MS (ESI-MeOH-negative mode, calc. M^+ for product with *i*Bu: 1775, calc. M^+ for product with Bz: 1809), found: M^-: 1775, 1809 in ratio of ca. 3:1, respectively.

*5.3.1.11 Solution Synthesis of the S_p-Y-Tetramer (ApA(p_sG)pC) (**21b**):*

To an Argon purged glass hypovial with a magnetic stirring bar, was added the detritylated S_p-V-trimer (**20b**) (0.01 mmol, 17.4 mg), adenosine-3'-phosphoramidite (**1.4b**) (6 eq, 0.059 mmol, 59 mg) and tetrazole (18 eq, 0.176 mmol, 11 mg). The reaction was commenced by injecting anhydrous acetonitrile (0.5 mL) and stirred at r.t. for 2.5 h in a dry nitrogen box. 2,4,6-collidine (6 eq, 0.059 mmol, 7.8 μL) was then added followed by addition of aqueous iodine solution (0.1 M in THF/H_2O 3:1.5, excess) to oxidize the phosphite triester intermediate. After 5 minutes the reaction was diluted with CH_2Cl_2 and washed with 5% sodium bisulfite. The organic phase was washed with 5% $NaHCO_3$, dried over anhydrous Na_2SO_4, and the solvent removed under reduced pressure to attain a foam. The crude Y-tetramer was partially purified by flash silica gel column chromatography using CH_2Cl_2/MeOH/0.5%TEA (gradient 0-2% MeOH). Refer to Table

2 for the R_f values. MS (MALDI-TOF III, calc. M^- for product without the cyanoethyl protecting group: 2626), found: M^-: 2633 = 2626 + 7 (Li cation).

Table 2: R_f Values of Solution Synthesized Phosphorothioate Oligomers.

Oligomer #	R_f value in CH_2Cl_2/MeOH, 9:1	
	R_p isomer	S_p isomer
4.1	0.47	0.63
4.2	0.4	0.42
4.3	0.35	0.33
18	0.44	0.45
19	0.28	0.31
20	0.26	0.27
21	0.25	0.29

Table 3: ^{32}P-NMR Data of Solution Synthesized Phosphorothioate Oligomers.

Oligomer # in NMR solvent	^{31}P NMR (ppm)	
	R_p isomer	S_p isomer
4.1 in acetone-d_6	69.4	68.8, 68.9
4.2 in acetone-d_6	59.4, 59.5	60.2, 60.5
4.3 in acetone-d_6	59.5, 59.8	---
18 in CH_2Cl_2-d_2	59.6 + (-0.3, -1.1)	---
19 in CH_2Cl_2-d_2	61.1 + 1.1	60.2 + 1.0

5.3.2 Solid Phase Synthesis:

5.3.2.1 CPG Derivatization:

LCAA-CPG (5 g) was activated by suspending it in 3% TCA/dichloroethane, and was stirred at r.t. for 16 h[70]. The CPG was then filtered and washed with 9:1 triethylamine:diisopropylethylamine, followed by dichloromethane and ether, and was then dried under vacuum over P_2O_5. The activated CPG (1 g) was added to succinic anhydride (2 mmol), DMAP (0.33 mmol) and anhydrous pyridine (6 mL), and the mixture was stirred at r.t. for 16 h in order to succinylate the free amines on the surface of the CPG. The CPG was then filtered and washed with pyridine followed by CH_2Cl_2 and ether. In order to derivatize the succinylated CPG with a specific nucleoside, the succinylated CPG (0.5 g), the required nucleoside (0.1 mmol), DEC (1 mmol), triethylamine (40 μL) and DMAP (50 μmol) were suspended in anhydrous pyridine (6 mL) and the solution was stirred at r.t. overnight. The CPG was then filtered and washed with pyridine, CH_2Cl_2 and ether and dried under vacuum over P_2O_5. Determination of the nucleoside loading on the solid support was conducted spectrophotometrically by measuring the amount of the trityl cations released upon the addition of 3% TCA in dichloroethane to a small amount of CPG.

5.3.2.2 Solid-Phase RNA Synthesis:

The solid support was placed in a Teflon column between two filters and the sides of the column were sealed using aluminum seals (Pierce). The active sites on the solid support were first capped using a standard programmed capping cycle (Ac_2O) on an automated DNA synthesizer (Applied Biosystems 381A). Refer to appendix for the solid-phase synthesis cycle. At the end of the synthesis, a 5 min argon reverse flush followed by a 10 sec block flush were applied in order to dry the column before cleavage of the support-bound oligomers and deprotection of the phosphate and exocyclic amino protecting groups.

5.4 Total Deprotection of the Fully Protected Oligomers

The general procedure can be illustrated by the deprotection of the S_p-V-trimer [A(p_sG)pC].

(a) **Removal of the Dimethoxytrityl groups**. The oligomers synthesized in solution were detritylated as part of the total deprotection method. Oligomers synthesized on the solid support were detritylated using a pre-programmed step as part of their synthesis cycle. The general procedure for the detritylation was carried out using two diverse methods which will be illustrated by the detritylation of the S_p-Y-tetramer [ApA(p_sG)pC], and the S_p-V-trimer [A(p_sG)pC].

(1) The S_p-V-trimer (5 mg) was dissolved in 500 μL CH_2Cl_2, followed by the addition of TFA (12 μL) at r.t. The solution turned orange immediately, indicating the presence of trityl cations. The reaction was diluted with CH_2Cl_2 after 7 minutes and was washed with 5% $NaHCO_3$ before the combined organic layers were dried over anhydrous Na_2SO_4 and the solvent was removed under reduced pressure to afford a dark yellow crude residue.

(2) The S_p-Y-tetramer (6.5 mg) was dissolved in 300 μL CH_2Cl_2 and *p*-toluenesulfonic acid-MeOH/CH_2Cl_2 (300 μL, the solution prepared by stirring 0.52g *p*-TSA, 5.2 mL MeOH and 20.8 CH_2Cl_2) was added at r.t. The solution turned orange immediately, indicating the presence of trityl cations. The reaction was diluted with CH_2Cl_2 after 10 minutes and was washed with 5% $NaHCO_3$ before the combined organic layers were dried over anhydrous Na_2SO_4 and the solvent was removed under reduced pressure.

(b) **Removal of the Benzoyl and Isobutyryl groups**. The detritylated trimer was dissolved in 1.5 mL NH_4OH and 0.5 mL EtOH, and the reaction was stirred at r.t. for 48 h. After cooling the sample in an acetone/dry ice bath, the ammonia solutions were removed using a low vacuum Speed-Vac® concentrator and the ethanol and H_2O were removed under pressure using a high vacuum Speed-Vac® concentrator.

(c) **Removal of the *tert*-Butyldimethylsilyl and *p*-Nitrophenylethyl groups**. The material obtained above was treated with 1.0 M TBAF/THF (40 eq, 0.056 mL) for 4 h at r.t. The reaction was diluted with H_2O and passed through a column of Dowex Na^+ ion exchange resin. The eluant (10 mL) was quantitated on a UV spectrometer to yield 42 A_{260} units (42 O.D.) of crude S_p-V-trimer [A(p_sG)pC].

5.5 Oligonucleotide Purification, Desalting and Analysis

The oligomers were purified using anion exchange and reverse phase HPLC, except for the tetramer, which was purified using polyacrylamide gel electrophoresis (PAGE).

5.5.1 High Performance Liquid Chromatography (HPLC):

A Waters HPLC instrument equipped with a W600E multisolvent delivery system, dual pump heads (225 μL), UK6 injector, a temperature controller and an M486 Tunable UV detector was used. The instrument was interfaced with to a computer running the software "Millenium 3.2 Chromatography Manager".

(1) **Reverse phase HPLC**: This technique was used to resolve and purify the two isomers of the Ap_sG dimer, and for the analysis of enzymatic digestion products generated by the enzyme SVPDE. Analytical and the preparative injections were performed on a reverse-phase C_{18} column (250 mm X 4.9 cm) from Waters. Acetonitrile and 50 mM TEAA were filtered through a 0.45 μm filter and degassed with helium prior to use.

(a) Purification and separation of the R_p and the S_p isomers of the dimer (Ap_sG) were conducted using a linear gradient of 0-20% 50 mM TEAA over 60 min at a temperature of 22°C, with a flow rate of 1.0 mL/min for the analytical injections and 4.0 mL per min for the preparative injections. For analytical runs, 0.3-0.7 O.D. of oligomers was injected, and for preparative runs, 3-7 O.D. was injected.

(b) The SVPDE enzymatic digests were analyzed by reverse-phase HPLC using a gradient of 0-10% acetonitrile in 50 mM TEAA over 10 min followed by 10% - 35% acetonitrile over a further 15 min. Under these conditions, the order of elutions of nucleosides is rC (5.9 min), rI (12.3 min), rG (12.7), and rA (15.2 min).

(2) **Anion exchange HPLC:** This technique was used to purify the linear and branched dimers and trimers. The anion-exchange column used was Protein Pak DEAE-5PW (7.5 mm X 7.5 cm) from Waters. Lithium perchlorate (1 M in water) and H_2O (doubly distilled and deionized using the Millipore system) were filtered through a 0.45 μm filter and degassed with helium prior to use. Purification was performed using a linear gradient

of 0-10% Lithium perchlorate over 60 min, with a flow rate of 1.0 mL/min and the temperature set to 22°C. For analytical runs, 0.3-0.7 O.D. of oligomers was injected, and for preparative runs, 3-7 O.D. was injected.

5.5.2 Polyacrylamide Gel Electrophoresis (PAGE):

The dimers, trimers and tetramers were run on analytical 24% polyacrylamide (7*M* urea) denaturing gels, and the S_p-Y-tetramer was purified using a preparative 24% polyacrylamide (7*M* urea) denaturing gel. To prepare the 24% polyacrylamide gel the following stock solutions were prepared and stored at 4°C.

(1) 50% acrylamide: Acrylamide (100 g) and BIS (5 g) were dissolved in 200 mL H_2O.

(2) 10 X TBE Buffer: EDTA (3.72 g), Boric acid (55.65 g) and TRIS-HCl (109 g) were dissolved in 1L H_2O.

(3) 24% Denaturing Gel: 7*M* Urea (105.1 g), and TRIS (25 mL) were dissolved in 50% acrylamide stock solution (120 mL) and H_2O to give a final volume of 250 mL.

An analytical gel was performed using 0.75 mm thick plate spacers and was prepared by sonicating 30 mL of 24% denaturing gel for 10 min in order to dislodge any air bubbles present. Ammonium persulfate (10% in water, 200 μL) was added to the 24% denaturing gel, followed by TEMED (20 μL). The gel was quickly poured between the glass plates. A sample comb for analytical gels was inserted at the top of the gel, which was left to polymerize for at least 1 h. The wells were washed with 1 X TBE buffer, prior to loading the gel, to remove any excess urea. Formamide loading buffer (10 μL of an 8:2 v/v mixture of deionized formamide to 10 x TBE buffer) was added to the samples, containing 0.5-1.5 O.D. of dry oligonucleotide, and the samples loaded onto the gel. The run was monitored by loading 10 μL of a mixture of visible dyes (2% w/v xylene cyanol and 2% w/v bromophenol blue in formamide loading buffer) into wells at both sides of the gel. The gel was run at constant voltage (500 V) until the fastest moving dye (bromophenol blue) reached the bottom of the gel. The gel was removed from the plates, covered with Saran Wrap® and photographed over a fluorescent TLC plate (20 x 20 cm)

illuminated by hand held UV lamp (254 nm) using Polaroid PolaPan® 4 x 5" Instant Sheet Film (#52, medium contrast, ISO 400/27°C; f4.5, 16 seconds) through a Kodak Wrattn gelatin filter (#58 green).

The preparative gel to purify the S_p-Y-tetramer was prepared and ran using a similar procedure as for the analytical gel except the spacers were thicker (1.5 mm) and 50 mL 24% denaturing gel was used with 350 mL ammonium persulfate and 35 µL TEMED. 35 µL of formamide loading buffer was added to the crude S_p-Y-tetramer (32 O.D.) before it was loaded to the gel. The separate bands were visualized under UV lamp (254 nm) and excised with a scalpel blade, and individually crushed in sterile 10 mL polypropylene tubes. Autoclaved H_2O (5 mL) was added to the tubes and they were shaken at r.t. for 24 h in order to extract the oligomers from the gel matrix. The tubes were centrifuged and the oligomers-containing supernatant evaporated under reduced pressure on a Speed-Vac® concentrator.

5.5.3 C18 SEP-PAK™ Reverse Phase Chromatography:

The oligomers purified above by anion exchange HPLC and PAGE were desalted using C_{18} SEP-PAK cartages. The cartridges were flushed with 10 mL MeOH then equilibrated with 10 mL of doubly distilled and autoclaved H_2O. The oligomers were dissolved in 1 mL of 50 mM TEAA (triethylammonium acetate) and were slowly loaded into the cartridges to collect the first fraction in a 1 mL microtube. The cartridge was flushed further with 50 mM TEAA (3 mL) in order to elute the salts. The oligomers were eluted from the column in 10 fractions of 1 mL each using a solution of 50mM TEAA: MeOH (7:3). The individual fractions were analyzed by diluting the samples in 1 mL of sterile water, and measuring the UV absorbance profile at 260 nm. Typically, the oligonucleotide of interest may be found in the first two or three fractions.

5.6 Enzymatic Studies

5.6.1 Enzymatic Digestion via Snake Venom Phosphodiesterase (SVPDE):

The enzyme SVPDE from *Crotalus adamanteus*, a 3'-exonuclease, (0.027 units/mg) was dissolved in 2 mL buffer A (0.11 M Tris, 0.11 M NaCl and 15 mM $MgCl_2$ with pH 8.9) to give a final concentration of 0.00135 U/μL. The residual nucleoside 5'-monophosphates were converted to their constituent nucleosides via *in situ* treatment with alkaline phosphatase from calf intestinal mucosa (AP, 2000 units/mL), which was kept in the storage buffer provided (10 mM tris-HCl (pH 8.0), 50 mM KCl, 1 mM $MgCl_2$, 0.1 mM $ZnCl_2$ and 50% glycerol. AP (5 μL) was added to 10 μL of its 10X buffer (100 mM Tris-acetate, 100 mM magnesium acetate, and 500 mM potassium acetate) and 85 μL of H_2O, to give a 20-fold dilution of enzyme. 90 μL of this solution was added to each assay. The oligomers (0.2 O.D. each) were dissolved in 30 μL buffer B (50mM Tris-HCl and 10 mM $MgCl_2$ with pH 8). The SVPDE (0.002 U) and AP (9 U) enzymes were added, and the reactions were incubated at 37° C for the allotted times before they were analyzed using HPLC (refer to the Reverse phase HPLC section in experimental for the procedure).

5.6.2 3'-End ^{32}P Labeling:

Branched oligomers that lack a 5'-oligonucleotide track, *i.e.* "V" shaped oligomers, are very poor substrates for T4 polynucleotide kinase and as such are not efficiently labeled at their 5'-termini[93]. Alternatively, 3'-end labeling of the branched oligonucleotides was chosen as a means for the introduction of a radioactive tag at a strategic point in the molecule. Since the branched oligonucleotides contain two 3'-termini/molecule (as a result of the vicinal 2',5'- and 3',5'-phosphodiester linkages), substrates in which the 2',5'-phosphodiester linkage have been hydrolyzed by the debranching enzyme would produce two labeled fragments of diverse mobility. This allows for the visualization and characterization of the products of the reaction with even greater certainty. Labeling of the branched oligomers and linear control molecules at their

3'-termini was conducted in a similar method to Uhlenbeck *et al.* using the enzyme, T4 RNA Ligase and [5'-^{32}P]cytidine 3',5'-biphosphate[94].

The oligomers (0.01OD units) were dissolved in 0.5 μL of the 10X T4 RNA Ligase buffer (500 mM Tris-HCl (pH 7.5), 100 mM $MgCl_2$, 100 mM DTT and 10 mM ATP) 0.5 μL deionized DMSO-d_6, 0.5 μL acetylated Bovine Serum Albumin (BSA; 0.1 mg/ml), 0.5 μL (5 U) T4 RNA Ligase (10 units/μL), 2 μL [5'-^{32}P]cytidine 3',5'-biphosphate (3,000 Ci/mmol), to a final volume of 5 μL with sterile water.

The reaction mixtures were incubated at 37° C overnight, and the enzyme inactivated by heating at 70° C for 10 min. Their labeling reactions were taken up in gel loading dye (5 μL), purified from any unlabeled material on a 24% denaturing polyacrylamide gel, and visualized by autoradiography (Kodak X-Omat AR film). The labeled oligonucleotides were excised from the gel and extracted by crushing and soaking the gel section in H_2O at 37° C overnight, and desalted via size-exclusion chromatography on Sephadex G-5 columns. The labeled oligonucleotides were stored as dry pellets at –20° C until needed.

5.6.3 Debranching of Synthetic Oligomers Using HeLa Extract:

Oligonucleotides labeled in the above manner were debranched using HeLa extracts containing debranching activity (Source: Generous gift by Dr. Andrew MacMillan, U. Alberta). The labeled oligomers were dissolved in 3.3μL HeLa extract debranching buffer (60 mM HEPES, pH 7.6, 375 mM KCl, 1.5 mM $MgCl_2$, 3 mM DTT, 30% glycerol), 1ml of 100 mM EDTA, 1 μL HeLa nuclear extract, and H_2O to obtain a final volume of 10 μL per reaction. The reactions were incubated in 37° C for the allotted times, terminated by heating at 70° C for 10 min, and lyophilized to dryness before loading dye was added and the mixtures analyzed via 24% denaturing PAGE and visualized by autoradiography.

5.6.4 Debranching of Synthetic Oligomers Using yDBR:

Oligonucleotides labeled in the same above manner were debranched using purified His-tagged yeast lariat debranching enzyme (yDBR) (Dr. J. Boeke, Johns Hopkins University). The labeled oligomers were dissolved in 2.5 μL yDBR debranching buffer (Final concentration of 20 mM HEPES, pH 7.6, 125 mM KCl, 0.5 mM $MgCl_2$, 1 mM DTT, 10% glycerol), 0.5-1 μL yDBR (50-100 ng), and H_2O to obtain a final volume of 7.5 μL per reaction. The debranching reactions were incubated at 37° C, inactivated by heating at 70° C for 10 min, and lyophilized before loading dye was added and the mixtures were analyzed using 24% denaturing PAGE and visualized by autoradiography.

CONTRIBUTION TO KNOWLEDGE AND FUTURE STUDIES

The synthesis of a diastereomeric mixture of 2',5' phophorothioate A(p_sG) dimers in solution with minimal oxidized side product was carried out using EDITH reagent. Sulfurization of the intermediary phosphite triester using the widespread Beaucage reagent resulted in 25% of oxidized side product. The two phosphorothioate isomers were separated at the phosphotriester stage using flash chromatography to yield the stereoisomerically pure dimers. A method to selectively remove a silyl protecting group from an oligomer in the presence of both a trityl (DMT) and a p-nitrophenylethyl (NPE) protecting group was developed using a pre-neutralized TBAF reagent. Orthogonal deprotection of the dimer protection groups and coupling to a suitable monomer at the 3'-position allowed for the synthesis of isomerically pure branched oligomers containing both 2',5' phosphorothioate and 3',5'-phosphodiester linkages. The synthesized oligomers were characterized by HPLC, gel electrophoresis, mass spectroscopy, NMR and enzymatic digests using SVPDE.

In order to elucidate the stereochemical requirements for hydrolysis of the 2',5'-linkage of a branched oligonucleotide by the yeast lariat debranching enzyme (yDBR), a variety of known substrates (phosphodiester V-trimer A(pG)pG, and branched wild-type lariat intron mimic 19-mer) and potential substrates (branched R_p & S_p trinucleotide phosphorothioates; A(p_sG)pC and branched S_p tetranucleotide phosphorothioate AA(p_sG)C) were prepared. The 2',5'-internucleotide linkage of the branched R_p trinucleotide, A(p_sG)pC, was not cleaved by the yDBR enzyme, suggesting that the yDBR enzyme is sensitive to changes carried out on the pro-R_p oxygen. The S_p phosphorothioate oligomers, along with the V-trimer [A(pG)pG] and 19-mer wild-type controls, were all substrates of the yDBR enzyme, albeit with diverse efficacies, as demonstrated by the appearance of a new oligonucleotide species of faster mobility when the radioactively labeled species were visualized by autoradiography. Furthermore, the small branched oligomers were not cleaved nearly as efficiently as the large branched 19-mer, suggesting that larger branched oligonucleotides are representative of superior substrates to the yDBR enzyme. The highlighted results shed more light on the yDBR enzyme and its substrate specificity, where it is obvious that changing the pro-R_p oxygen

at the 2',5'-phosphodiester bond averts hydrolysis by the enzyme. Also, the yDBR enzyme appears to have a stronger preference for larger substrates than branched trimers and tetramers.

Future studies using these solution synthesized phosphorothioate branched molecules can provide further insight towards the full understanding of the mechanism of specific 2',5'-phosphodiester linkage hydrolysis by the yDBR enzyme. Subjecting the S_p branched oligomer substrate to the yDBR and studying the stereochemistry of the debranching products would shed light on whether the cleavage of the 2′,5′-phosphodiester bond of RNA lariats proceeds with inversion or retention of configuration at the phosphorus atom. Inversion of configuration would indicate a direct attack of water on the 2′,5′-bond, while a retention of configuration indicates two inversions resulting from a formation of a covalent-enzyme-bRNA intermediate followed by a direct water attack on the 2′,5′-linkage. Moreover, competitive inhibition studies using both the S_p and the R_p branched oligomers can be carried out in order to ascertain if the R_p phosphorothioate (the substrate which is not hydrolyzed) is nonetheless recognized by the enzyme, and binds to its active site. Given that this isomer may potentially be an inhibitor of debranching, it may then be used in order to co-crystallize the enzyme, thus possibly allowing for the attainment of a much desired X-ray crystal structure.

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APPENDIX

Figure I: ^{1}H and gCOSY NMR of $^{DMT}{}_{TBDMS}Ad^{Bz}$ (**1.3a**). The spectrum was recorded on a Varian XL-400 (400 MHz) spectrometer using DMSO as a solvent

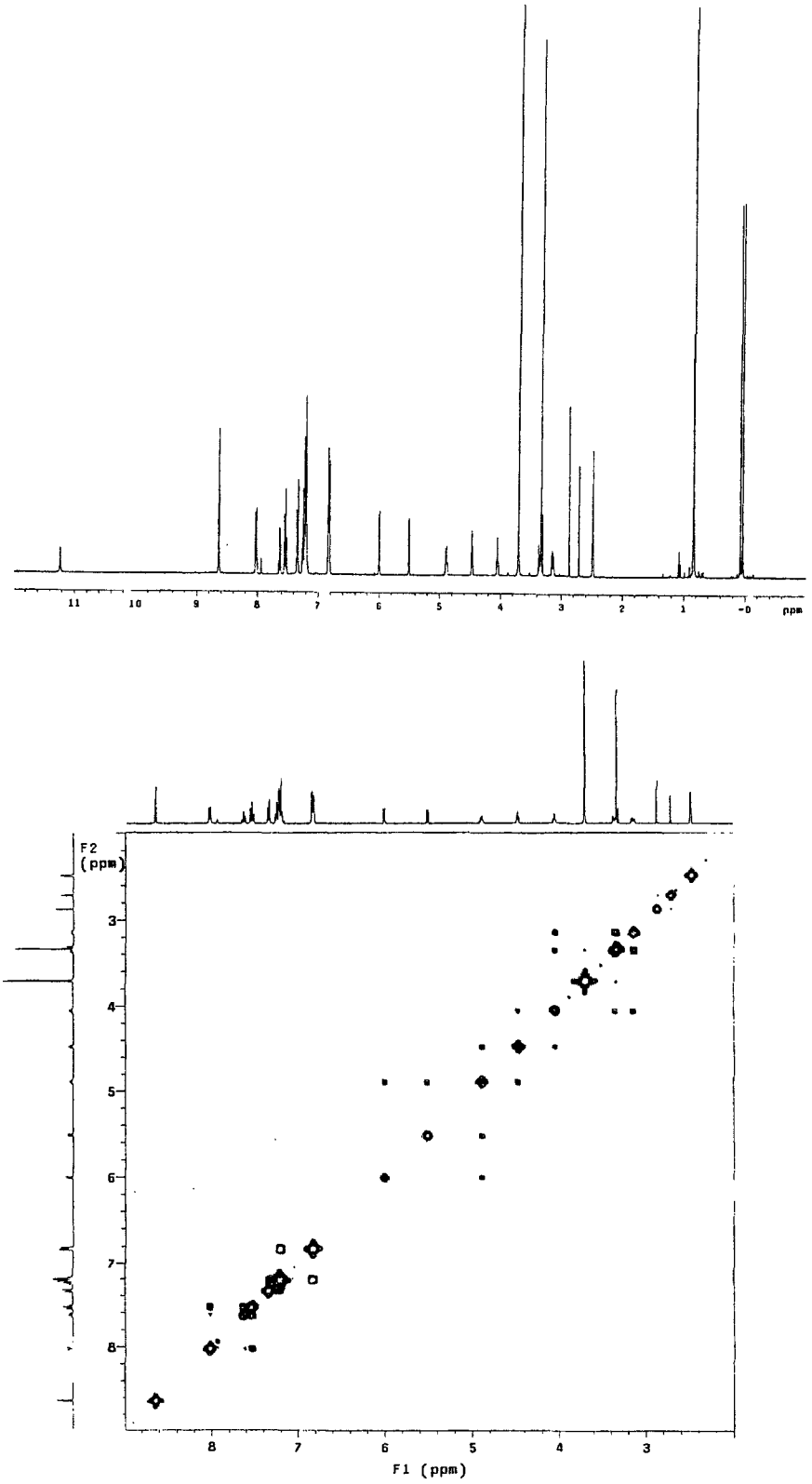


Figure II: ^{1}H and gCOSY NMR of $^{DMT}Ad^{Bz}_{TBDMS}$ (**1.3b**). The spectrum was recorded on a Varian XL-400 (400 MHz) spectrometer using DMSO as a solvent.

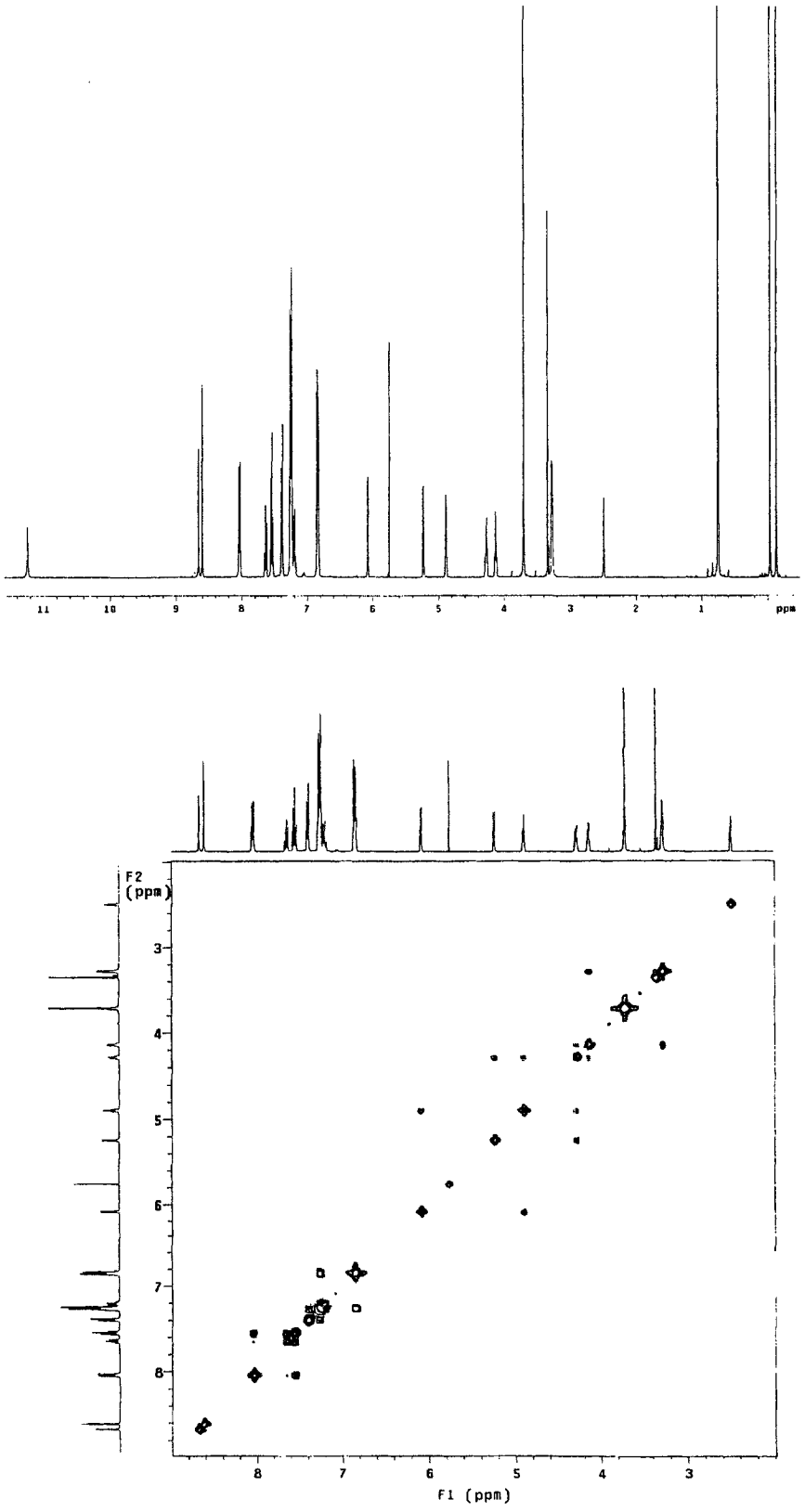


Figure III: ^{31}P NMR of Adenosine-2'-Amidite (**1.4a**) (Due to the chiral phosphorus center, this compound exists as a mixture of 2 diastereomers resulting in the two phosphorus signals). The spectrum was recorded on a Varian XL-200 (200 MHz) spectrometer.

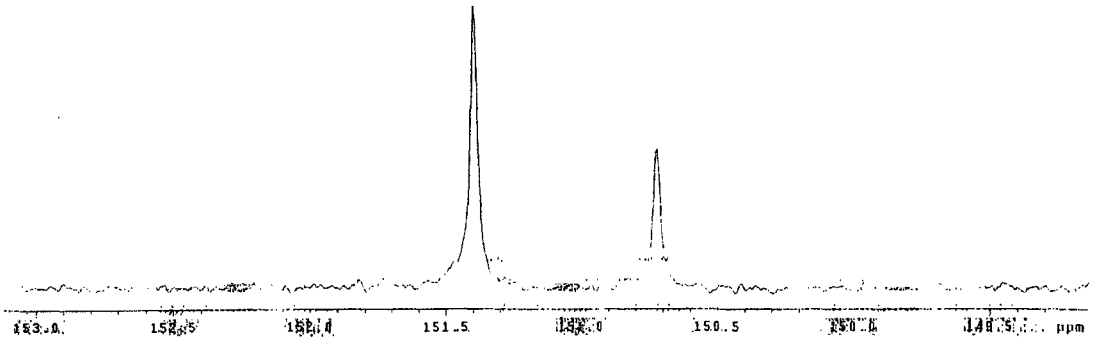


Figure IV: ^{31}P NMR of Adenosine-3'-Amidite (**1.4b**) (Due to the chiral phosphorus center, this compound exists as a mixture of 2 diastereomers resulting in the two phosphorus signals). The spectrum was recorded on a Varian XL-200 (200 MHz) spectrometer.

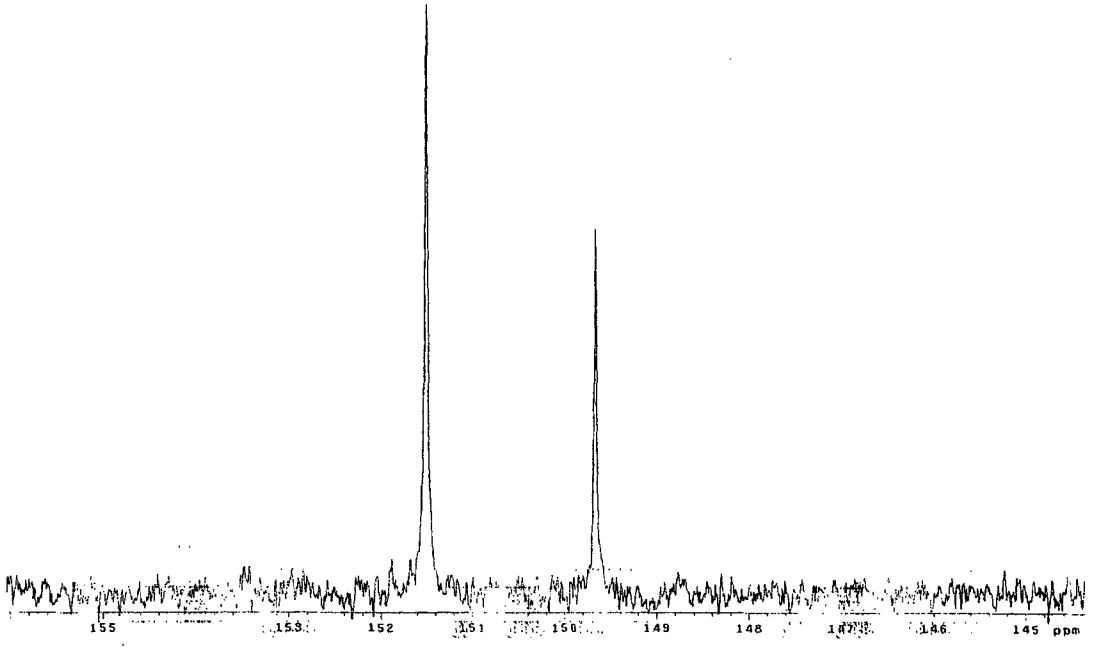
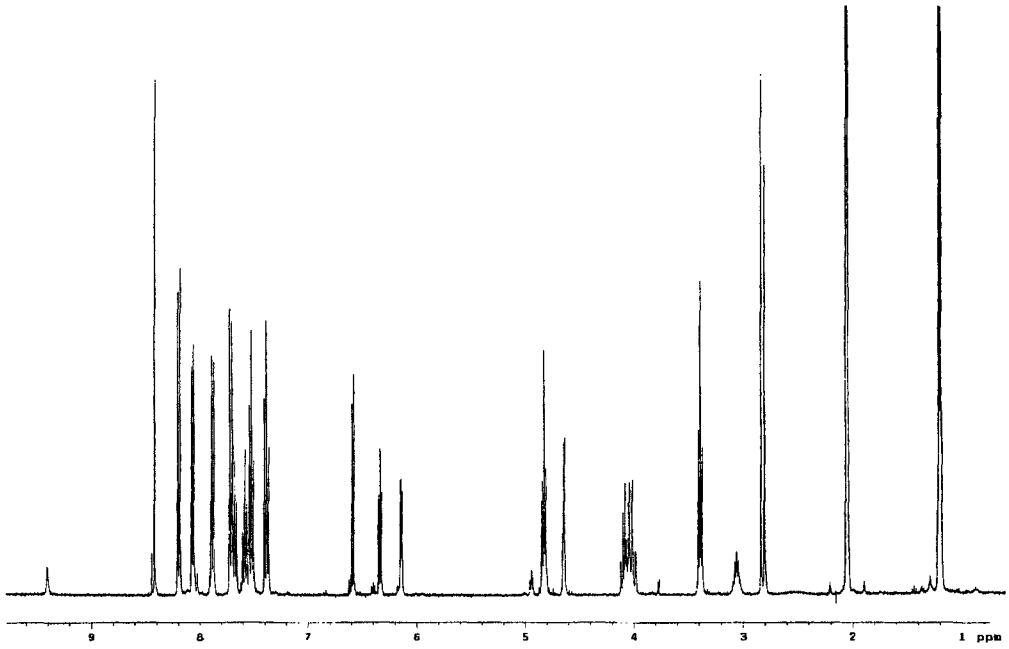


Figure V: ^{1}H and gCOSY NMR of $_{Bz}Gu^{iBu,NPE}{}_{Bz}$ (**2.5**). The spectrum was recorded on a Varian XL-400 (400 MHz) spectrometer using acetone as a solvent.



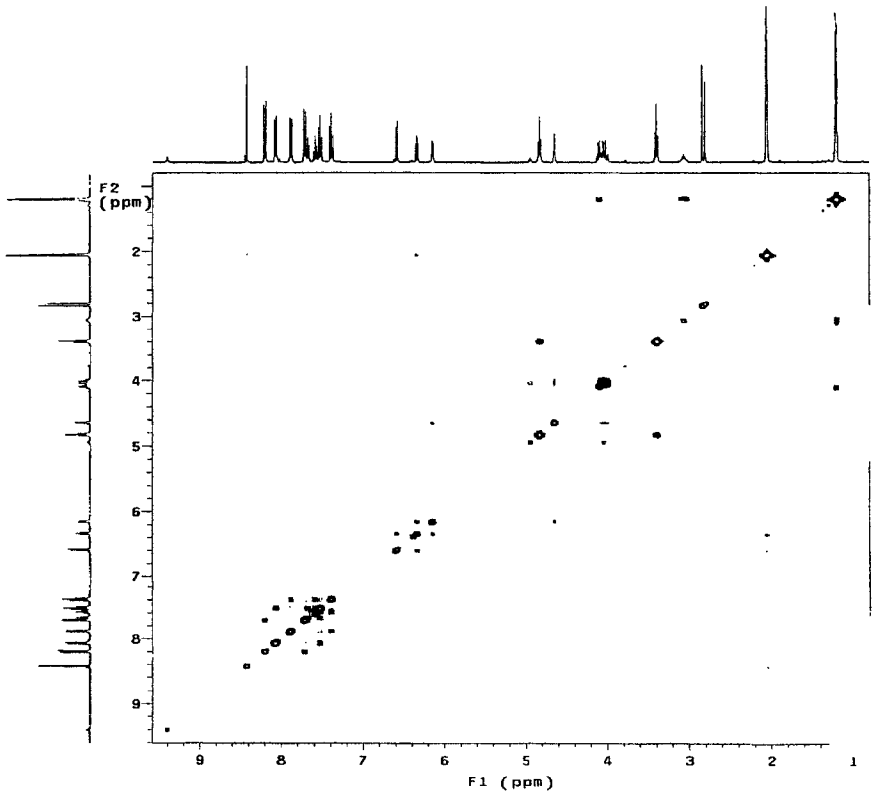


Figure VI: ^{1}H NMR of $_{Bz}Cy^{Bz}{}_{Bz}$ (**3.4**). The spectrum was recorded on a Varian XL-400 (400 MHz) spectrometer using acetone as a solvent.

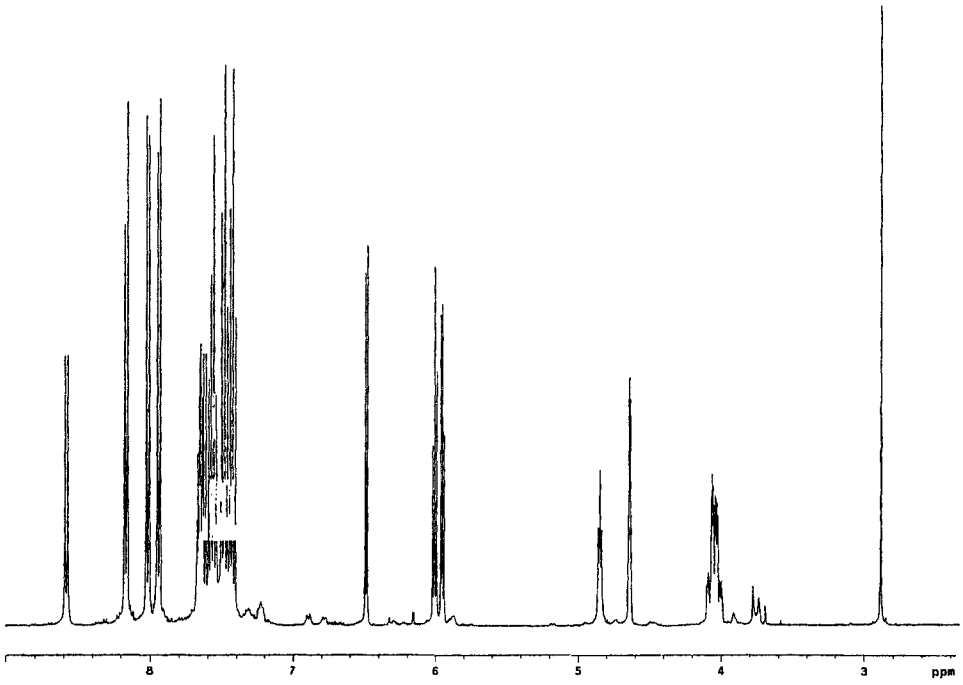


Figure VII: ^{31}P NMR of Cytidine-5'-Amidite (**3.5**) (Due to the chiral phosphorus center, this compound exists as a mixture of 2 diastereomers resulting in the two phosphorus signals). The spectrum was recorded on a Varian XL-200 (200 MHz) spectrometer.

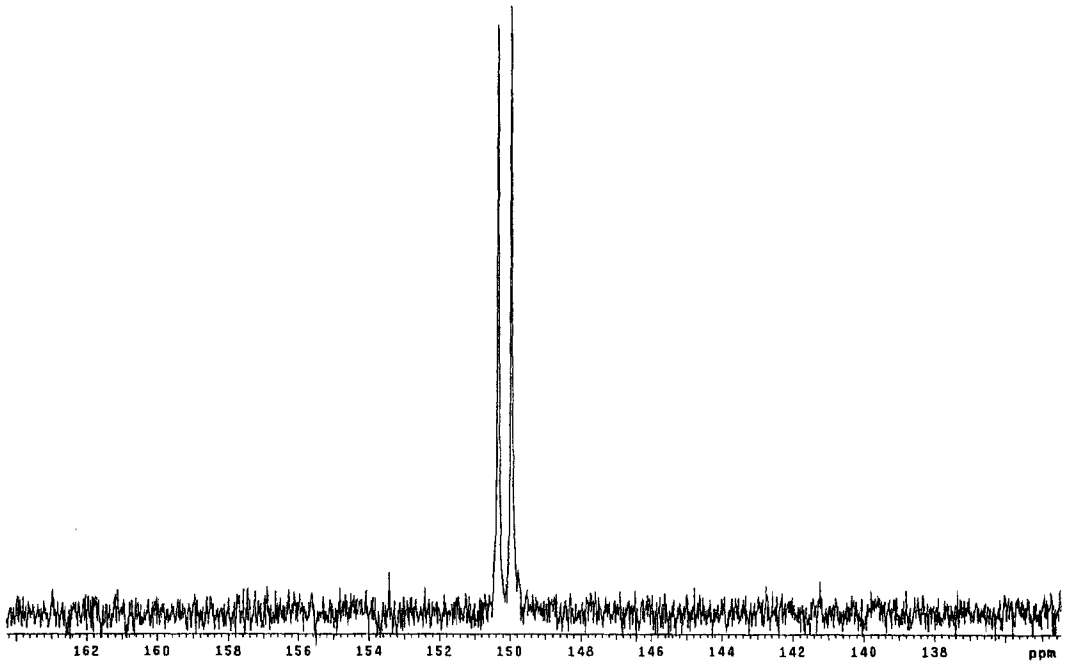


Figure VIII: PAGE 24% Polyacrylamide Analytical Gel of Phosphorothioate Dimers and V-Trimers (S_p and R_p isomers). Bands visualized by UV shadowing. Lane numbers are at the bottom of the gel. *Lanes 1:* Linear ApC with traces of ApApC, *lane 2:* Pure R_p A(p_sG) dimer, *lane 3:* Pure R_s A(p_sG) dimer, *lane 4:* Linear ApApC, *lane 5:* Pure V-trimer control A(pG)pG, *lane 6:* Crude R_p-V-trimer A(p_sG)pG, *lane 7:* Pure R_p-V-trimer A(p_sG)pG.

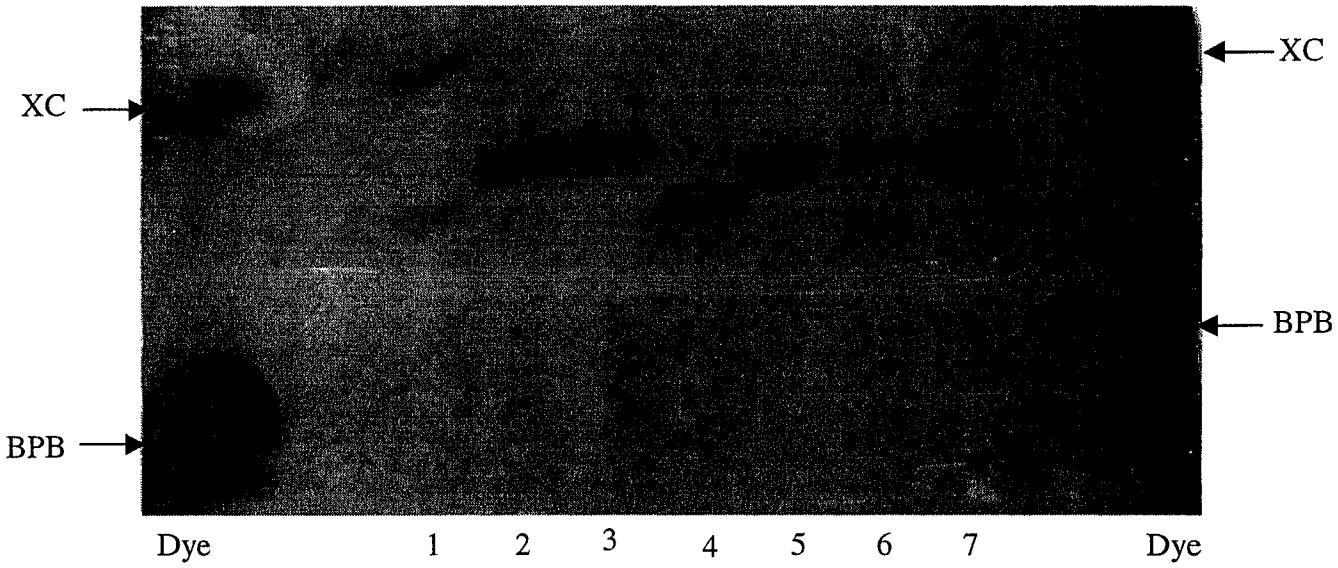


Figure IX: Reverse Phase HPLC Analysis of the Four Ribonucleic Acids. These conditions were used to separate the ribonucleic acids and to analyze the SVPDE digests of the phosphorothioate oligonucleotides.

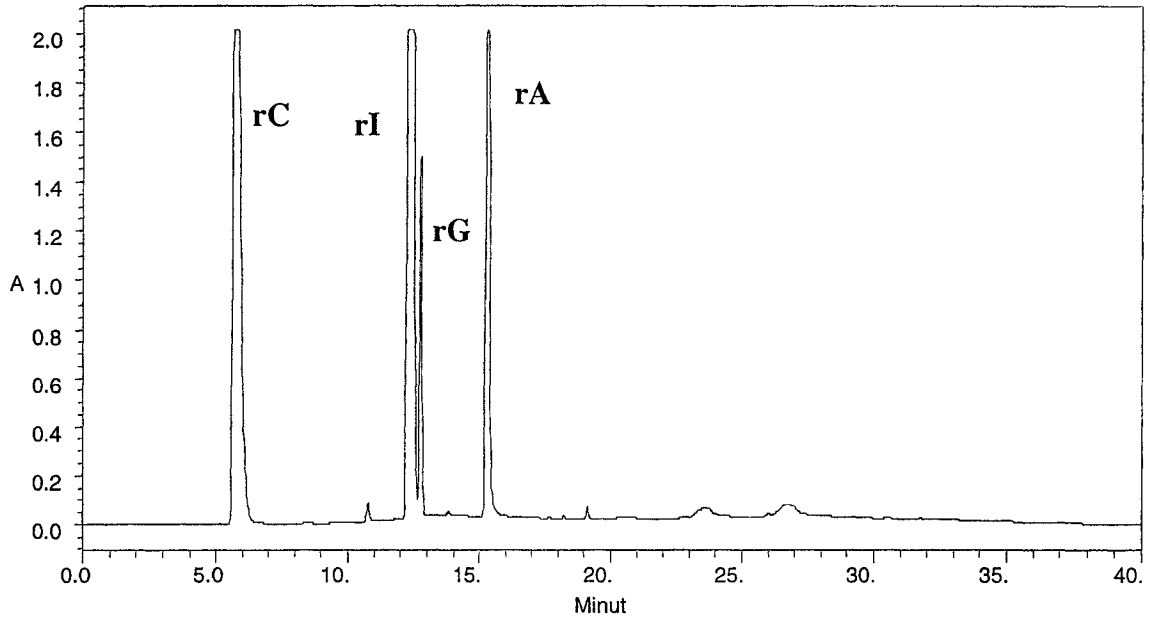


Figure X: Reverse Phase HPLC Analysis of the R_p Dimer A(p_sG). These conditions were used to analyze the SVPDE digests of the phosphorothioate oligonucleotides.

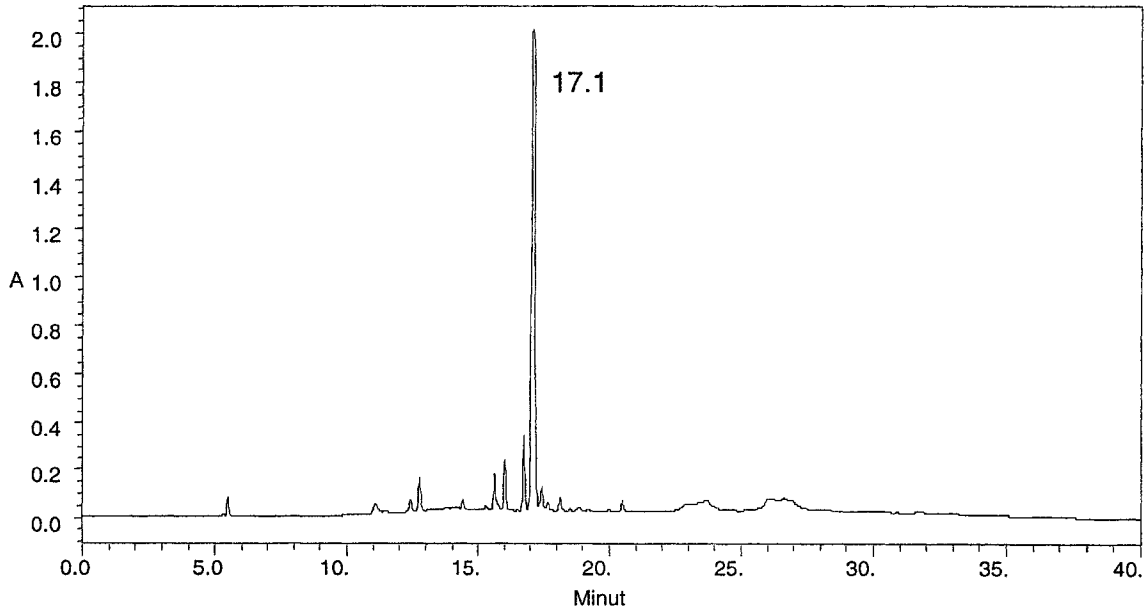


Figure XI: Reverse Phase HPLC Analysis of the S_p Dimer A(p_sG). These conditions were used to analyze the SVPDE digests of the phosphorothioate oligonucleotides.

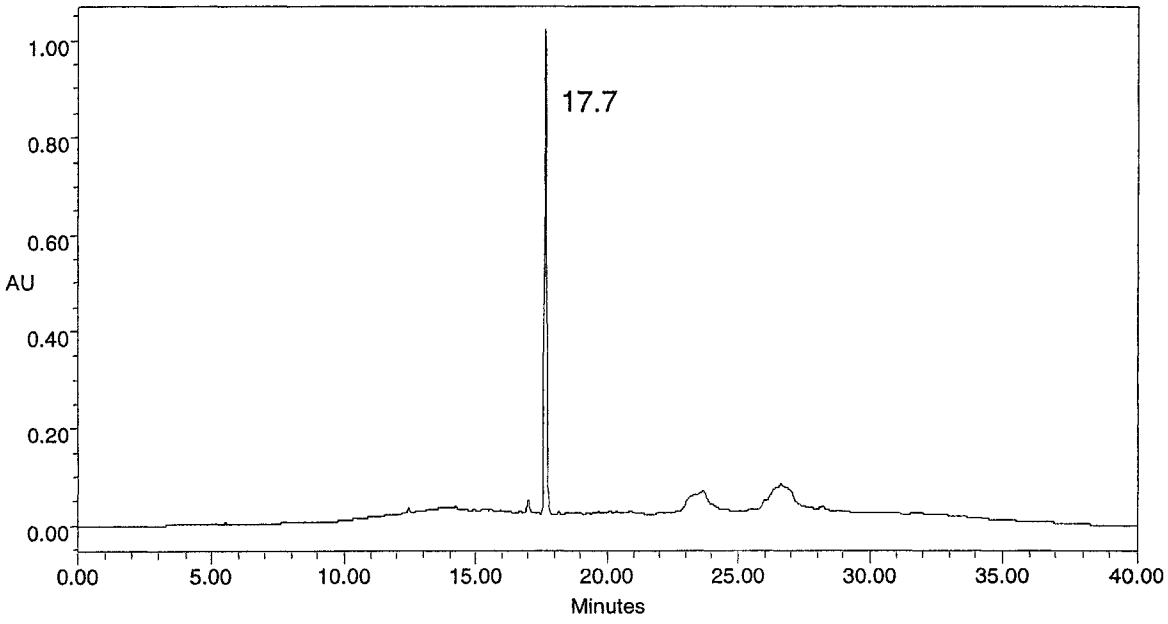


Figure XII: Reverse Phase HPLC Analysis of the R_p Trimer A(p_sG)pC. These conditions were used to analyse the SVPDE digests of the phosphorothioate oligonucleotides.

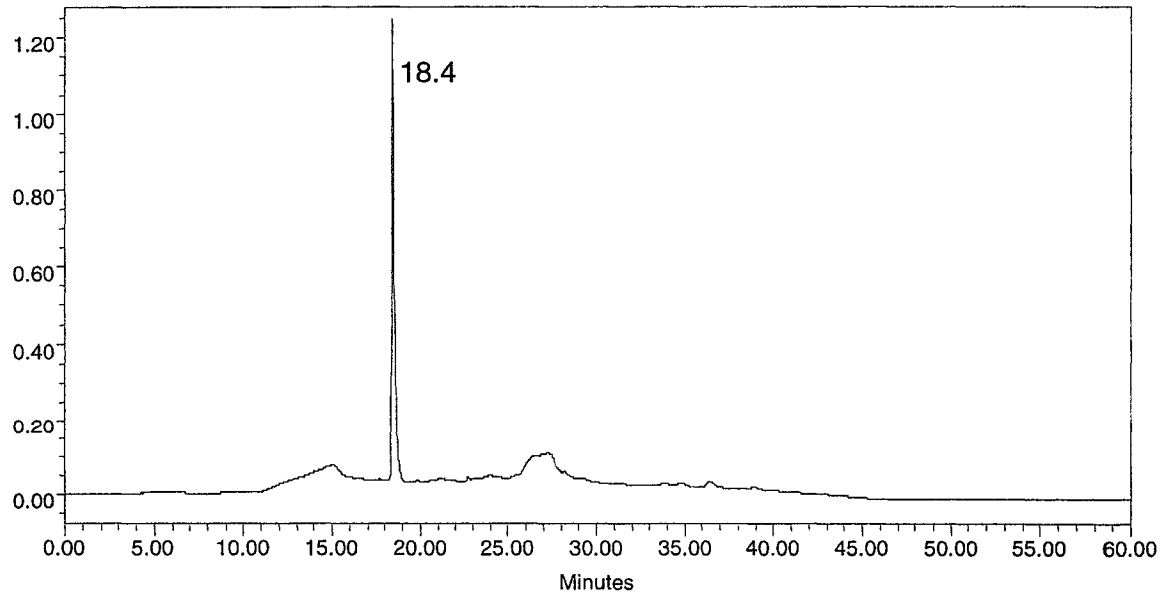
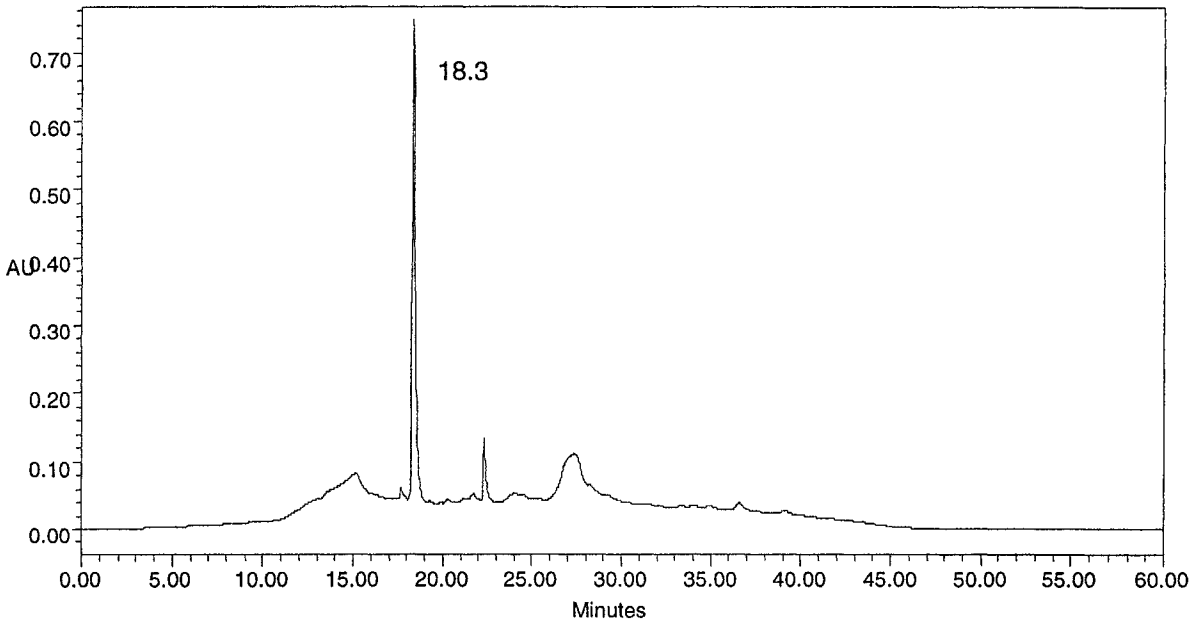


Figure XIII: Reverse Phase HPLC Analysis of the S_p-V-trimer A(p_sG)pC. These conditions were used to analyse the SVPDE digests of the phosphorothioate oligonucleotides.



Cycle of the Solid Phase Synthesis:

Table I: Automated Capping Cycle of Free CPG Bound Amino and Hydroxyl Groups Using an ABI 381A DNA Synthesizer.

Synthesis Step #	Function Name	Step Time (sec)
1	Acetonitrile to waste	5
2	Acetonitrile to column	60
3	Argon reverse flush	5
4	Argon block flush	5
5	Cap A + Cap B to column	15
6	Wait	300
7	Repeat steps 5 and 6	
8	Argon reverse flush	5
9	Acetonitrile to column	30
10	Repeat steps 8 and 9	
11	Argon reverse flush	5
12	Argon block flush	5

Where Capping reagent A (CAP A) is made of 10% (v/v) acetic anhydride/10% (v/v) dry 2,4,6-collidine in anhydrous THF.

Capping reagent B (CAP B) is made of 16% (v/v) dry N-methylimidazole in anhydrous THF.

Solid-phase oligonucleotide synthesis was conducted utilizing the standard 1.0 μmol DNA/RNA cycle provided by the manufacturer, and was used with little modifications. The coupling time was increased in the case of RNA phosphoramidites to 600 sec, and the detritylation step was increased to 200 sec. The concentration of RNA phosphoramidites in acetonitrile used is 0.15 M, and the concentration of bis-phosphoramidite used for the branched point is 0.03 M.

Table II: Automated 1 μmol Synthesis Cycle of Branched RNA Oligonucleotides Using an ABI 381A DNA Synthesizer.

Synthesis Step #	Function Name	Step Time (sec)
1	Acetonitrile to waste	5
2	Acetonitrile to column	45
3	Argon reverse flush	5
4	Argon block flush	5
5	Advance fraction collector	1
6	3% TCA to waste	10
7	3% TCA to column	140
8	Acetonitrile to column	30
9	3% TCA to column	60
10	Argon block flush	10
11	Acetonitrile to waste	5
12	Acetonitrile to column	120
13	Argon reverse flush	5
14	Argon block flush	5
15	Acetonitrile to waste	5
16	Acetonitrile to column	60
17	Argon reverse flush	5
18	Argon block flush	5
19	Phosphoramidite preparation	3

20	Activator to column	5
21	Phosphoramidite and activator to column	5
22	Repeat steps 20 and 21 (2 times)	
23	Activator to column	3
24	Wait	200
25	Argon reverse flush	5
26	Argon block flush	5
27	Cap A + Cap B to column	17
28	Wait	45
29	Repeat steps 27 and 28	
30	Acetonitrile to waste	5
31	Argon block flush	5
32	Acetonitrile to waste	5
33	Argon reverse flush	5
34	Argon block flush	5
35	Oxidant to waste	5
36	Oxidant to column	20
37	Acetonitrile to waste	5
38	Argon block flush	5
39	Wait	20
40	Acetonitrile to waste	5
41	Argon reverse flush	10
42	Argon block flush	5
43	Acetonitrile to waste	5
44	Acetonitrile to column	18
45	Argon reverse flush	5
46	Repeat steps 44 and 45 (6 times)	
47	Argon block flush	5