THE SYNTHESIS OF BRANCHED NUCLEIC ACID ANALOGS: PROBING THE SUBSTRATE SPECIFICITY OF THE YEAST DEBRANCHING ENZYME

by

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Dedicated to my parents Maria & Raffaele Liscio and
to my husband Franco Calabrese
for all their love and support

ABSTRACT

Branched nucleic acid analogs were synthesized in order to investigate the substrate specificity of the yeast debranching enzyme (*i.e.* specific cleavage at the 2', 5'-phosphodiester bond). In order to synthesize the branched nucleic acid analog dAA(G)TBDPSi (where the bracketed nucleoside is found at the 2'-position of A), the branch point nucleoside, N^6-Bz-5'-O-MMTr-3'-O-TBDPSi-adenosine-2'-O-[N,N-diisopropyl(cyanoethyl)] phosphoramidite was first synthesized. The branched trimer, dAA(G)TBDPSi was then assembled via solid phase synthesis using an automated DNA synthesizer. Another nucleic acid analog, dAA(G)PO_4^{2-} was also synthesized as was its corresponding expected debranched product, dAA(OH)PO_4^{2-}. The known yDBR enzyme substrate, dAA(G)G was synthesized as well, along with its debranched product dAA(OH)G so that it could be used as a positive control in yDBR enzyme studies. MALDI-TOF MS and PAGE were utilized to characterize the synthesized oligonucleotides.

dAA(G)PO_4^{2-} and dAA(G)G were radiolabelled and subjected to the yDBR enzyme and the autoradiograph was analyzed. dAA(G)PO_4^{2-} was in fact cleaved by the yDBR enzyme, although not nearly as efficiently as the fully branched dAA(G)G tetramer.

RÉSUMÉ

Des analogues d'acides nucléiques ramifiés ont été synthétisés dans le but d'étudier la spécificité des substrats de l'enzyme de levure non-ramifiée (i.e. le clivage spécifique au niveau de la liaison 2', 5'-phosphodiester). Pour réaliser la synthèse de l'analogue de l'acide nucléique ramifié dAA(G)TBDPSi (dans lequel le nucléoside entre parenthèse se trouve en position 2' de A), le nucléoside N^6-Bz-5'-O-MMTr-3'-O-TBDPSi-adenosine-2'-O-[N,N-diisopropyl(cyanoéthyle)] phosphoramidite situé à l'embranchement, a d'abord été synthétisé. Le trimère ramifié dAA(G)TBDPSi, a ensuite été synthétisé sur phase solide en utilisant le synthétiseur d'ADN automatisé. Un autre analogue d'acide nucléique, dAA(G)PO_4^{2-}, ainsi que le produit non-ramifié correspondant, dAA(OH)PO_4^{2-} ont également été synthétisés. Le substrat enzyme yDBR, dAA(G)G et le produit non-ramifié dAA(OH)G ont été synthétisés pour être utilisé comme contrôle positif pour les études portant sur l'enzyme yDBR. Les oligonucléotides synthétisés ont été caractérisés en utilisant les techniques de MALDI-TOF MS et de électrophorèse sur gel de polyacrylamide.

Finalement, dAA(G)PO_4^{2-} et dAA(G)G ont été marqué radioactivement; l'analyse du radiogramme a démontré que dAA(G)PO_4^{2-} était en fait clivé par l'enzyme yDBR, mais pas si bien que le substrat dAA(G)G.

ACKNOWLEDGMENTS

First and foremost, I would like to express my gratitude to Dr. Masad Damha for his guidance, patience and enthusiasm during the course of my work in his laboratory. His devotion to research and his expertise in the field of nucleic acids is admirable.

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A special thanks to my husband, Frank, for his computer expertise and for his continual love and encouragement throughout my university years.

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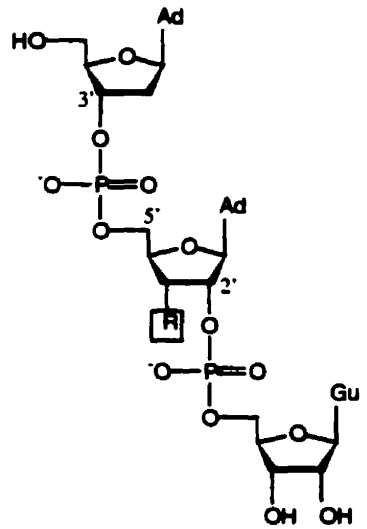
ABBREVIATIONS AND SYMBOLS

A	adenosine
Å	angstrom
A_{260}	UV absorbance measured at 260 nm
Ac	acetyl
Ad	adenine
AP	alkaline phosphatase
APS	ammonium persulphate
B	base
bDNA	branched DNA
BIS	N,N'-methylene-bis(acrylamide)
bNA	branched nucleic acid
bp	base pair
BPB	bromophenol blue
bRNA	branched RNA
Bz	benzoyl
C	Celsius
Cl	chloride
CaH_2	calcium hydride
CPG	controlled pore glass
DCE	1,2-dichloroethane
DCM	dichloromethane
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
dN	2'-deoxynucleotides
DNA	2'-deoxyribonucleic acid
DTT	dithiothreitol
E.coli	*Escherichia coli*
EtOH	ethanol
EtOAc	ethyl acetate
eq	equivalent(s)
G	guanosine
g	gram(s)
Gu	guanine
hDBR	human (HeLa) debranching enzyme
HIV-1	human immunodeficiency virus type 1
hnRNA	heterogeneous nuclear RNA

h	hours
Hz	Hertz
i-,*n*-,*t*-,	iso, normal, tertiary
i-Bu	isobutyryl
i-Pr	isopropyl
LCAA-CPG	long-chain alkylamine controlled pore glass
M	molar
MALDI TOF	matrix assisted laser desorption ionization time of flight
max	maximum
m/c	mass to charge ratio
MeOH	methanol
min	minute(s)
mL	millilitre
mg	milligram
mM	millimolar
µM	micromolar
MMTr	monomethoxytrityl
mol	mole
mRNA	messenger RNA
MS	mass spectrometry
NEt_3	triethylamine
nm	nanometre
NMR	nuclear magnetic resonance
nt	nucleotides
OD	optical density
PAGE	polyacrylamide gel electrophoresis
P_2O_5	phosphorus pentoxide
ppm	parts per million
pre	precursor
Pu	purine
Py	pyrimidine
py	pyridine
R_f	(TLC mobility) retardation factor
Rf	radio-frequency
rN	ribonucleotide
RNA	ribonucleic acid
RNase	ribonuclease
rRNA	ribosomal RNA
rt	room temperature
S. cerevisiae	*Saccharomyces cerevisiae*
sec	second
SnRNP	small nuclear ribonucleoprotein particles
T	thymidine
TBDMS	*t*-butyldimethylsilyl
TBDPS	*t*-butyldiphenylsilyl

TBAF	tetra-*n*-butylammonium fluoride
TBE	TRIS/boric acid/EDTA buffer
TCA	trichloroacetic acid
TEA	triethylamine
TEMED	N,N,N',N'-tetramethylethylenediamine
Th	thymine
THF	tetrahydrofuran
TLC	thin layer chromatography
TMSCl	chloro-trimethylsilane
TRIS	2-amino-2-(hydroxymethyl)-1,3-propanediol
TREAT •3HF	triethylamine trihydrofluoride
tRNA	transfer RNA
U	uridine
Ur	uracil
UV	ultraviolet
UV-VIS	ultraviolet-visible
v/v	volume by volume
w/v	weight by volume
yDBR	yeast debranching enzyme
XC	xylene cyanol

ABBREVIATIONS OF KEY STRUCTURES

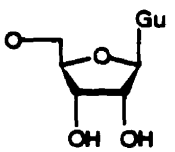


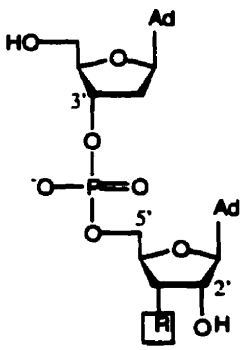
dAA(G)OH, R=OH

dAA(G)TBDPSi, R=OSi(Ph)$_2$t-Bu

dAA(G)PO$_4^{2-}$, R=OPO$_3^{2-}$

dAA(G)G, R=



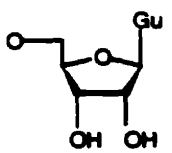


dAA(OH)TBDPSi, R=OSi(Ph)$_2$t-Bu

dAA(OH)PO$_4^{2-}$, R=OPO$_3^{2-}$

dAA(OH)OH, R=OH

dAA(OH)G, R=



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Introduction

1.1 The Structure and Functions of Nucleic Acids[1,2,3,4]

Due to the wide application of nucleic acids in chemistry, biochemistry, biology, medicine and material science, there is a broad interest among chemists to develop and apply the chemistry of this family of molecules. There are two types of nucleic acids: deoxyribonucleic acid (DNA) - found in the nuclei of cells and ribonucleic acid (RNA) - found largely in the cytoplasm of cells. DNA is the molecule responsible for the storage and transmission of genetic information in all cellular life forms and in many viruses. DNA directs both its own replication during cell division, and transcription (the process whereby mRNA is synthesized from specific sequences of DNA). RNA on the other hand, has varied biological functions: (i) messenger RNA directs the ribosomal synthesis of protein via a process known as translation; (ii) the ribosomal RNAs (rRNA) have both functional and structural roles; (iii) transfer RNAs (tRNAs) deliver amino acids to the ribosome during protein synthesis; (iv) certain RNAs associate with specific proteins to form ribonucleoproteins which participate in post-transcriptional processing of precursor-mRNA (pre-mRNA); (v) in several viruses, the carrier of genetic information is RNA as opposed to DNA.

Nucleotides are the building blocks of nucleic acids. A nucleotide is composed of three parts: a heterocyclic nitrogenous base, a pentose sugar and a phosphoryl group. The general structure and H-bonding motifs are shown in Figure 1.1. There are two types of nucleotide base (also termed nucleobases): The pyrimidines are composed of a single six-membered heterocyclic ring containing two nitrogen atoms where the N-1 of the pyrimidine is attached to the C-1' of the pentose sugar. Purines consist of a bicyclic ring

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[1] G. Zubay, "*Biochemistry*", 3rd edition, William C. Brown Publishing Company, 2, 3, (1993).
[2] D. Voet, J. Voet, "*Biochemistry*", John Wiley & Sons Inc., (1990).
[3] M.Gait, M. Blackburn, "*Nucleic Acids in Chemistry and Biology*", Oxford University Press, 2, (1996).
[4] C. Starr, R. Taggart, "*Biology - The Unity and Diversity of Life*", Wadsworth Publishing Company, Belmont, California, p. 217- 230, (1992).

system where a six-membered pyrimidine ring is fused to a 5-membered imidazole ring, where the glycosidic linkage is between the N-9 of the purine and C-1' of the pentose. Adenine and guanine are purine bases whereas uracil, thymine and cytosine are pyrimidine bases. These bases are common to both DNA and RNA except for uracil which is solely found in RNA and thymine which is uniquely found in DNA. In nucleotides the bases are attached to the C-1' of the pentose in a β-configuration. Thus the base is *cis* relative to C-5' and *trans* relative to C-3'-OH. A nucleoside lacks a phosphoryl group therefore it consists of a base linked to a sugar residue. The structural difference between RNA and DNA is the presence of an additional hydroxy group at the 2' position of the sugar ring in RNA. This feature makes RNA much more unstable than DNA both chemically and enzymatically. RNA is highly susceptible to base-catalyzed hydrolysis as shown in Figure 1.2. The base-induced deprotonation of the 2'-OH group facilitates its nucleophilic attack on the 3'-phosphorus thereby cleaving the RNA phosphodiester backbone. This is most likely why DNA as opposed to RNA evolved as the carrier for genetic information.

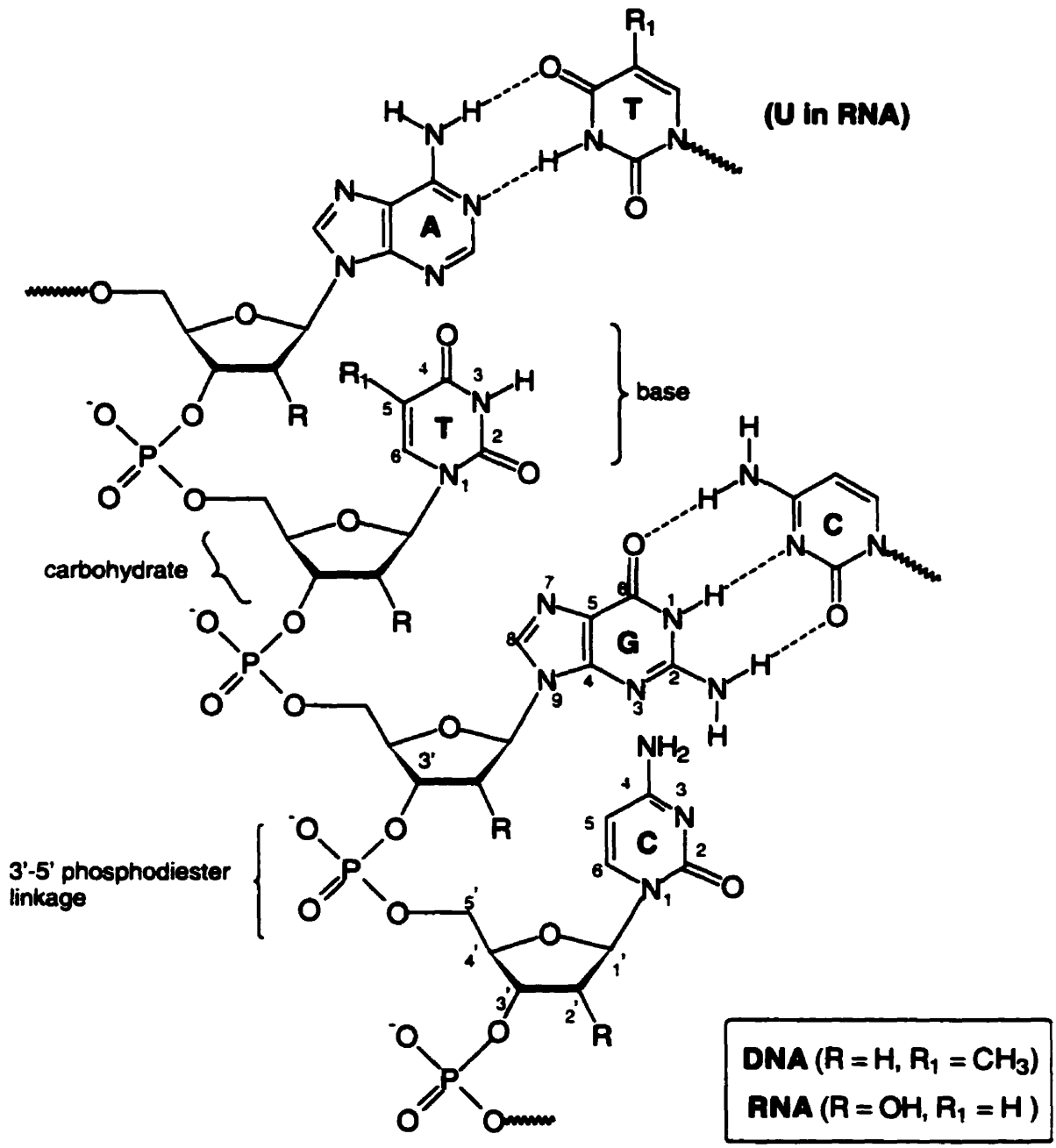

Figure 1.1 The Structure of 2'-Deoxyribonucleic Acid and Ribonucleic Acid
Numbering for pyrimidines (C,T,U), purines (A,G) and carbohydrates is given. DNA contains the carbohydrate β-D-2-deoxyribofuranose while RNA contains β-D-2-ribofuranose. The AT and GC Watson Crick base pairs are also illustrated.

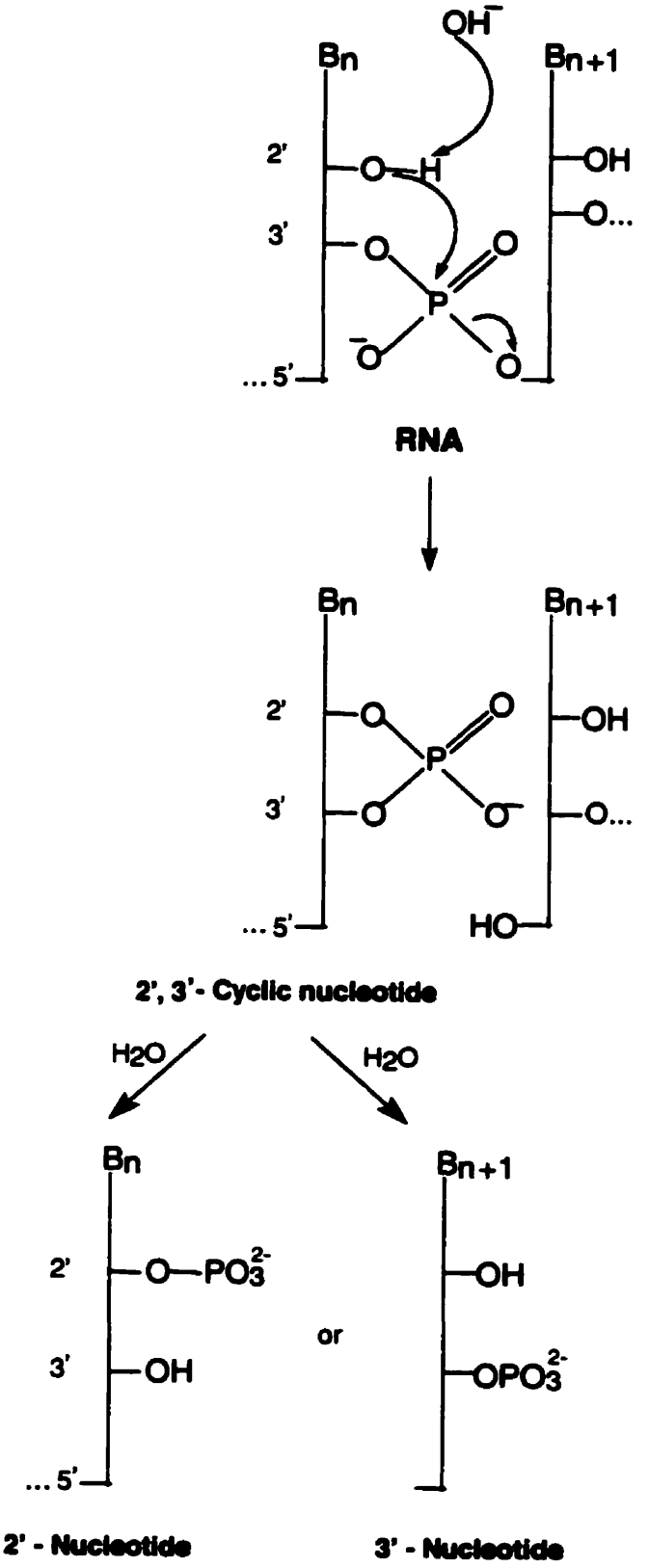

Figure 1.2 The Mechanism of Base-Catalyzed RNA hydrolysis

Nucleic acids adopt highly ordered three-dimensional structures. The factors that determine the conformation of nucleic acids are: the negative charge imparted by the phosphate groups, the noncovalent affinities between the purine and pyrimidine bases and the limitations imposed by the stereochemistry of the polynucleotide chains. X-ray diffraction studies have revealed that DNA is helical. It is composed of two polynucleotide strands that wind about a common axis and run antiparallel to each other[1]. The core of the helix is composed of base pairs which are held together via Watson-Crick hydrogen bonds (see Figure 1.1) and favorable stacking interactions between the planes of adjacent base pairs. Two hydrogen bonds form between adenine and thymine, whereas three hydrogen bonds form between guanine and cytosine. This phenomenon is known as complementary base pairing. DNA exists in several conformations known as A, B, and Z DNA. These different conformations arise due to puckering of the furanose ring and several rotatable single bonds per residue (six in the sugar-phosphate backbone and one in the C-1' N-glycosidic linkage). The biologically relevant form of DNA is that of the right handed B form double helix. The furanose rings adopt the C-2'-endo conformation (see figure 1.3) and the base pairs are nearly perpendicular to the helical axis. There are 10 bp per helical turn and the rise per turn, or helix pitch, is 34Å. The surface of B DNA is characterized by two grooves of unequal size namely, the major and minor grooves.

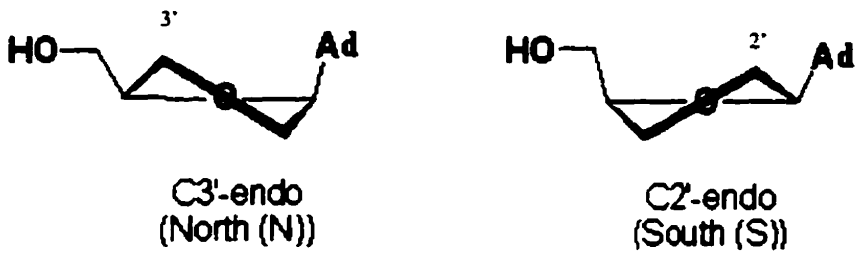

Figure 1.3 Two Pucker Conformations of the Furanose Rings of Ribose and Deoxyribose
The furanose rings in RNA have a stronger preference for the C-3'-endo conformation, whereas the sugars in DNA can adopt the C-2' and C-3'-endo conformation. In solution, DNA adopts the C-2'-endo pucker conformation.

A gene is a region of DNA that calls for the assembly of specific amino acids into a polypeptide chain. Two major processes occur in the transformation of a gene to a protein, namely transcription and translation. In eukaryotic transcription, single-stranded molecules of RNA are assembled on a DNA template in the nucleus of the cell. Genes are transcribed into three different types of RNA molecules: ribosomal RNA, messenger RNA (mRNA) and transfer RNA. Only mRNA goes on to translation, rRNA combines with protein to form the ribosome, and tRNA delivers specific amino acids to the growing polypeptide chain during translation. In translation, the RNA molecules are transported to the cytoplasm through nuclear pores in the nuclear membrane, where they are used as templates for assembling polypeptide chains. (In bacterial cells which have no nucleus, translation begins while RNA molecules are still peeling off the DNA). The polypeptide chains then fold into a three-dimensional protein. The central dogma of molecular biology which describes the flow of information in cells is shown in Figure 1.4. A circular relationship exists between DNA, RNA and protein. DNA is transcribed to RNA, RNA directs the synthesis of protein and proteins have structural and functional roles in cells. Although proteins have roles in the transmission of genetic information as an array of enzymes and proteins are involved in the processes of transcription and translation, they do not serve as templates for the synthesis of mRNA and DNA. RNA however, can be 'reverse transcribed' into DNA, a process that characterizes the so-called 'retroviruses' such as HIV-1.

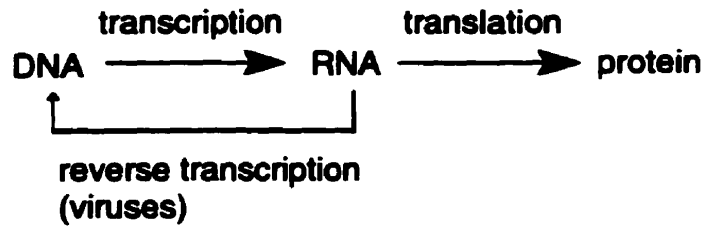

Figure 1.4 The Central Dogma of Molecular Biology

1.2 Historical Perspectives on mRNA Splicing

In prokaryotes, most primary mRNA transcripts proceed to translation without modification. This is not the case with eukaryotes in which the primary mRNA transcripts undergo extensive post-transcriptional processing before being transported from the nucleus to the cytoplasm (refer to figure 1.5). The RNA splicing hypothesis[5] emerged in the late 1970's. This hypothesis explained the results from early investigations on eukaryotic gene transcription which found primary transcripts to be quite heterogeneous in length (*i.e.* hnRNA). The length ranged from approximately 2000 to well over 20 000 nucleotides - a result inconsistent with the known size of eukaryotic proteins[2]. However, both the long hnRNAs and the shorter mRNAs were found to have a cap consisting of a 7-methylguanosine residue linked to the 5' end by a triphosphate bond[6], as well as stretches of adenosine residues at the 3' end known as a poly(A) tail - typically 150 to 200 residues in length[7]. Work on Adenovirus led to the realization that hnRNA transcribed from diverse cellular genes was processed by RNA splicing into cytoplasmic mRNA[8] . Subsequent to this, several other cellular genes were found to have noncoding intervening sequences (introns). For instance, the chicken ovalbumin gene was found to be 7700 bases long with 7 introns and 8 exons (coding sequences). Once the introns are removed and the exons are spliced together, the final product that remains is 1872 nucleotides long[9]. Comparing the sequences of exon-intron junctions from a diverse group of eukaryotes revealed a high degree of homology. The intron's 5' boundary was found to be invariably GU, the intron's 3' boundary was invariably found to be AG. The 3' splice site was also found to be preceded by a tract of 11 predominantly pyrimidine nucleotides[10]. Vertebrate, plant and yeast cells were found to have these consensus sequences in

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[5] S.M. Berget, C. Moore, P.A. Sharp, *Proc. Natl. Acad. Sci. USA*, **74**, 3171-3175, (1977).
[6] Y. Furuichi, M. Morgan, S. Muthukrishnan, A.J. Shatkin, *Proc. Natl. Acad. Sci.USA*, **72**, 362-366, (1975).
[7] M. Edmonds, M.H. Vaughan, H. Nakazoto, *Proc. Natl. Acad. Sci.USA*, **68**, 1336-1340, (1972).
[8] P.A. Sharp, *Cell*, **77**, 805-815, (1994).
[9] P. Chambon, *Scientific American*, **244**, 60-66, (1981).
[10] R. Breathnach, P. Chambon. *Annu. Rev. Biochem.*, **50**, 349-384, (1981).

common[11]. The importance of the consensus sequence will be further discussed in the next section.

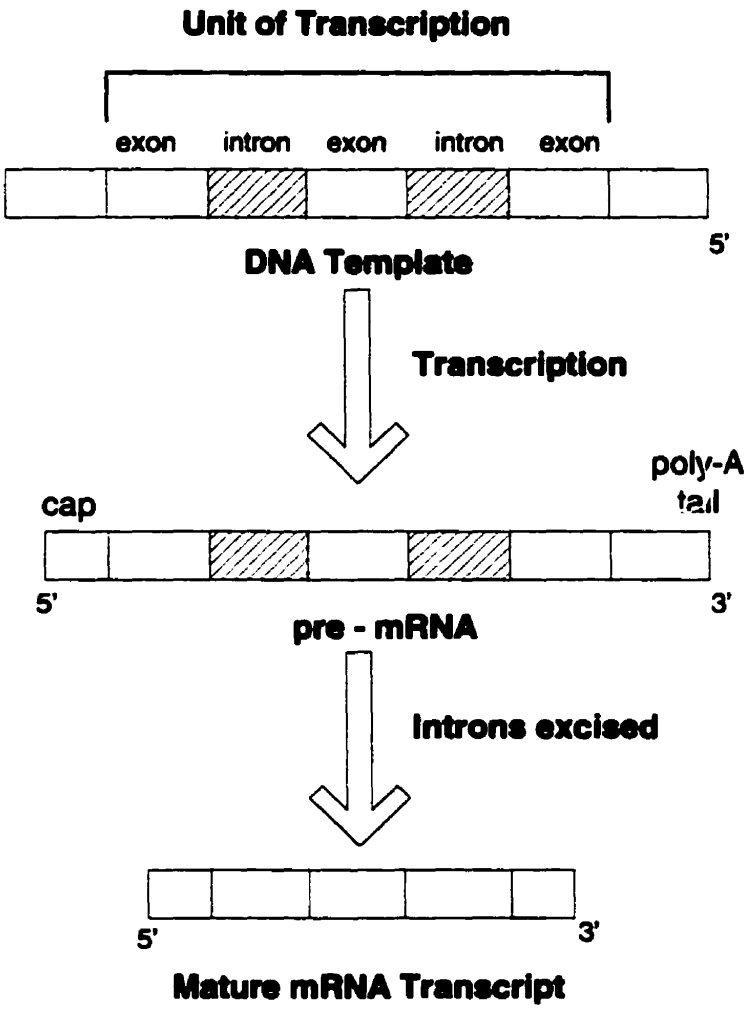

Figure 1.5 Transcription and Modification of Newly Formed mRNA in the Nucleus of Eukaryotic Cells. Once a particular region of DNA is transcribed, several modifications take place, namely: (a) transcript is capped at one end and polyadenylated at the other, (b) introns are snipped out and degraded, (c) exons are spliced together into a mature mRNA transcript. The mature mRNA transcript is then transported to the cytoplasm for translation.

[11] R. A. Padgett, P. J. Grabowski, M. M. Konarska, S. Seiler, P. A. Sharp, *Annu. Rev. Biochem.*, **55**, 1119-1150, (1986).

1.3 The Splicing Reaction

As explained in the previous section, eukaryotic genes consist of alternating expressed (exons) and unexpressed noncoding intervening sequences (introns). Following the capping and polyadenylation of the primary transcript, a process termed gene splicing occurs whereby introns are excised and their flanking exons are connected to yield the mature mRNA. Intron excision occurs in a two step reaction. The first step of the reaction involves formation of a "lariat" RNA structure with concomitant excision of the 5' exon (Figure 1.6). This occurs when the 2'- hydroxy group of a specific adenosine residue (found within the intron) attacks a phosphate at the 5' end of the intron. An unusual 2'-5' phosphodiester bond is formed, where the intron in lariat configuration is now linked to the 3' exon. The second step of the reaction occurs when the 3' hydroxy group of the 5' exon attacks the 5' phosphate of the 3' exon. Thus, another transesterification reaction occurs whereby the two exons are spliced together and the RNA intron is released in lariat form[1]. The intermediate state, whereby two exons are held in position for the second step of the reaction, is indicative of a complex holding the exons in place. This multicomponent complex is known as the spliceosome[12,13,14]. The complex is composed of small nuclear ribonucleoprotein particles (snRNPs; pronounced "snurps") which consist of many polypeptides as well as the small nuclear RNAs (snRNAs) namely, U2, U4, U5, U6, and U1. The snRNAs in the spliceosome form the catalytic sites for both reactions, whereas the proteins serve to precisely orient the snRNAs with the pre-mRNA so as to allow the reactions to take place. The consensus sequence at the 5' splice site appears to be complementary to the U1 snRNA and this interaction is essential for splicing[15]. The consensus sequence around the branch site is complementary to a conserved sequence in U2 snRNA - which has also been shown to be essential for

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[12] P.J. Grabowski, S.R. Seiler, P.A. Sharp, *Cell*, **42**, 345-353, (1985).
[13] E. Brody, J. Abelson, *Science*, **228**, 963-967, (1985).
[14] D. Frendewey, W. Keller, *Cell*, **42**, 355-367, (1985).
[15] J. Rogers, R. Wall, *Proc. Natl. Acad. Sci. USA*, **77**, 1877-1879, (1980).

splicing[16]. U4 and U6 snRNAs have complementary regions therefore they bind to each other[17]. The spliceosome is formed when this bi-snRNP complex binds to U5 snRNP forming a complex with the other components[18]. Once the spliceosome is formed, rearrangements of the snRNAs follow before the first step of splicing occurs. Further rearrangements occur between the first and second steps of splicing as well[8].

[16] R. Parker, P.G. Siliciano, C. Guthrie, *Cell*, **49**, 229-239, (1987).
[17] C. Guthrie, B. Patterson, *Annu. Rev. Genet.*, **22**, 387-419, (1988).
[18] M. M. Konarska, P. A. Sharp, *Cell*, **49**, 763-774, (1987).
[19] T. R. Cech, *Cell*, **43**, 713-716, (1985).

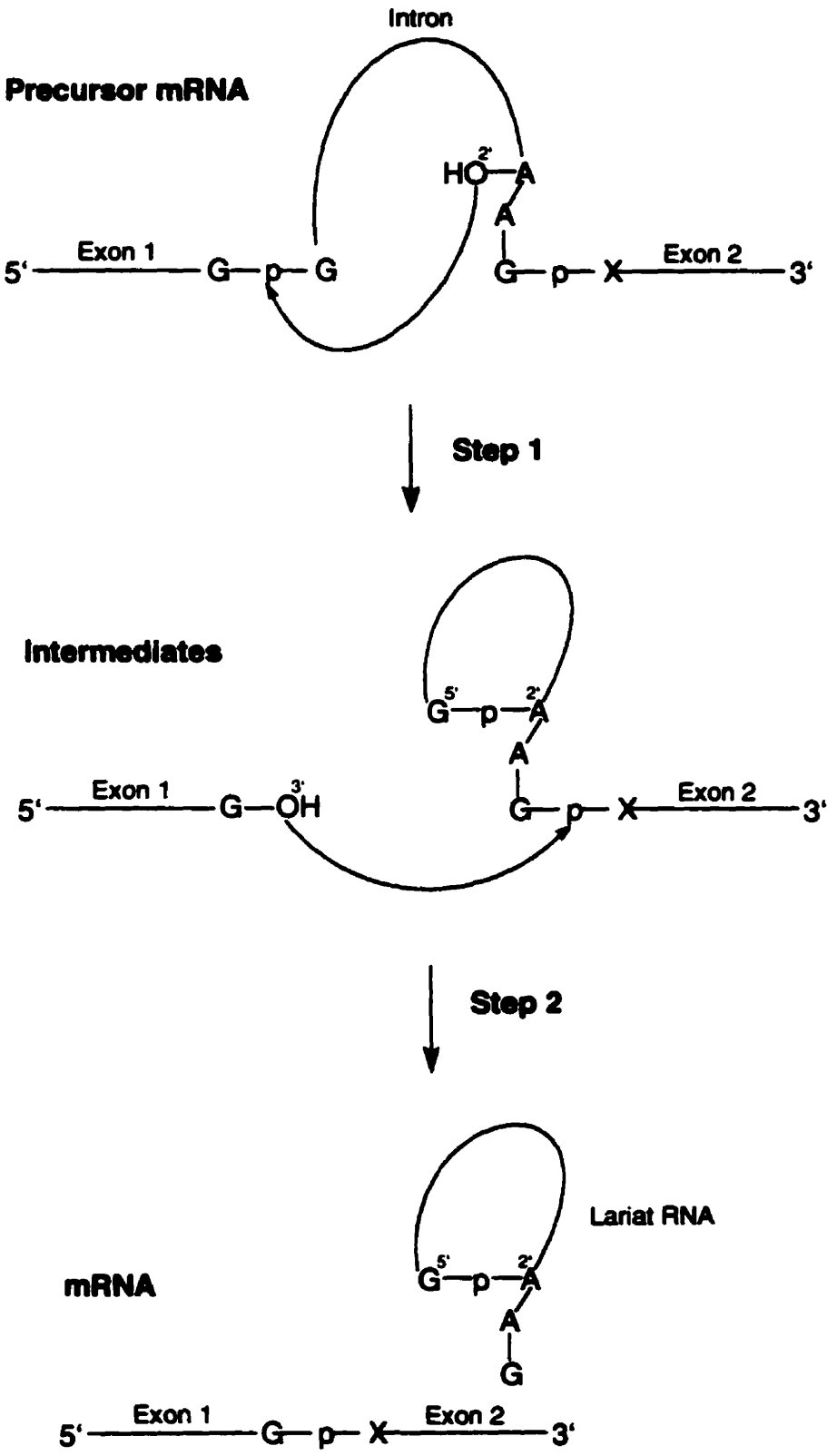

Figure 1.6 The Splicing of Pre-mRNA

The mechanism of splicing by the spliceosome may be related to the self-splicing mechanism of group I and II introns. In fact, it has been suggested that these reactions are evolutionarily related. Group I introns occur in the protozoan *Tetrahymena* during the processing of rRNA. The mechanism shown in figure 1.7 involves a 3' hydroxyl group of a guanosine cofactor attacking the phosphodiester bond at the 5' splice site. The second step is also a transesterification reaction whereby the exons are joined and the intron is released[19]. The mechanism for group II introns (commonly found in mitochondria and chloroplasts) is very similar to that of the spliceosomal process. However, proteins do not catalyze the reactions - RNA structures within the intron are responsible for catalysis[20].

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[20] P. S. Perlman, C. L. Peebles, C. Daniels, "*Intervening Sequences in Evolution and Development*", Oxford University Press, NY, NY, 112-161, (1990).

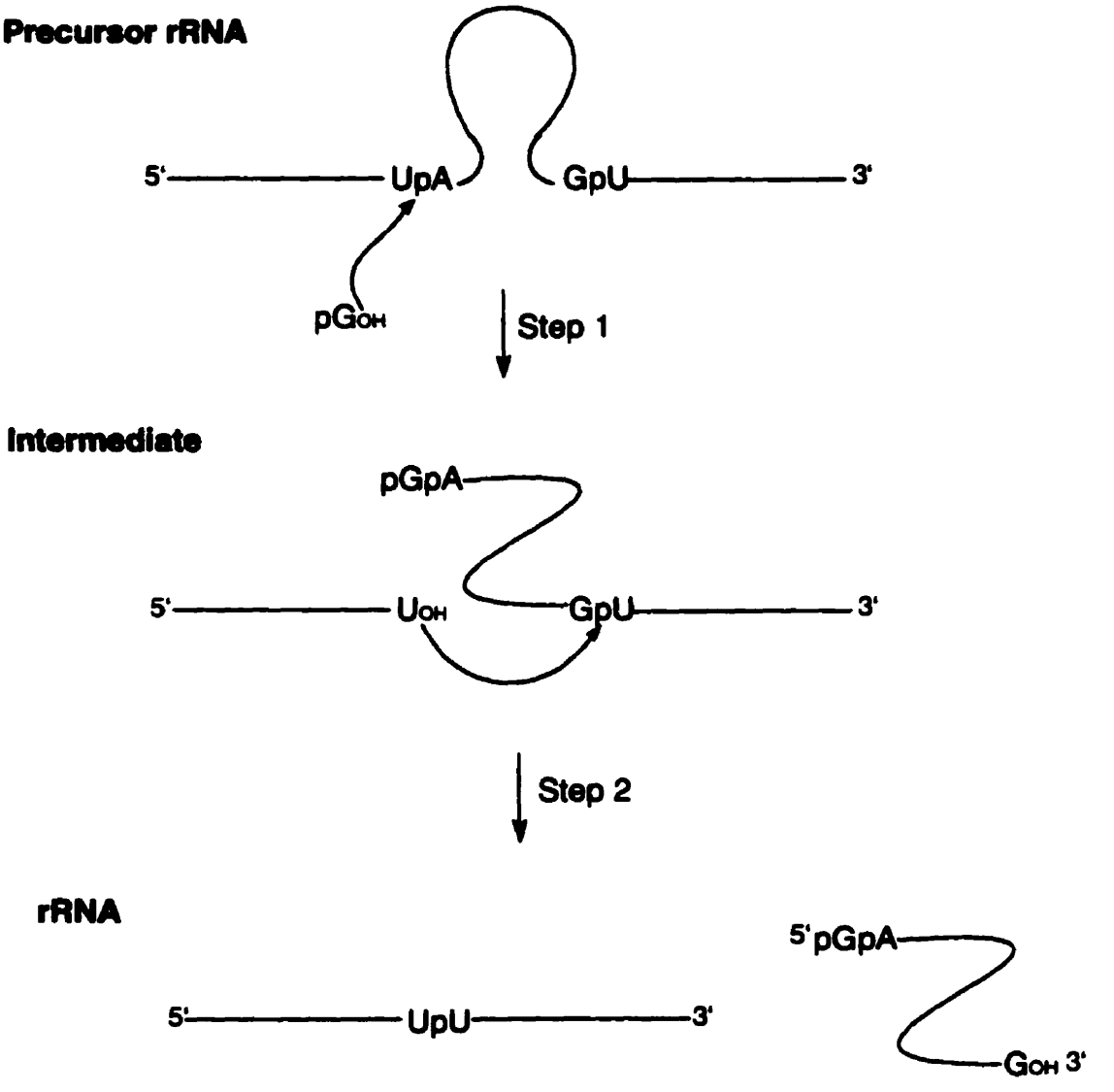

Figure 1.7 Self-splicing from the Protozoan *Tetrahymena*.

1.4 The Importance of Splicing

Several diseases in humans are associated with mRNA splicing reactions and spliceosome formation. Defective mRNA splicing may be one cause of the human disease β^+-thalassemia. Mutations in the intron of the β-globin mRNA precursor causes inefficient splicing. For instance, cleavage at the 5' splice site and lariat formation do not occur when the precursor contains a β^+-thalassemia deletion which removes the polypyrimidine stretch and AG nucleotides at the 3' splice site[21]. These mutations lead to very low levels of mature mRNA thereby producing a β-globin deficiency. Another disease that is associated with defective mRNA splicing is systemic lupus erythematosus - an often fatal disease. Patients afflicted with this condition produce anti-U1-snRNP antibodies. The antibodies destroy U1-snRNP sequences which as previously described are complementary to the 5' splice junction - thus inhibiting splicing[2].

Alternative splicing is a regulatory phenomenon that occurs in eukaryotic viruses such as SV40 and polyoma. It is also seen in many eukaryotic genes that contain several exons in their nascent transcripts[22]. A transcript with three or more exons can therefore be spliced in such a way that all the exons are included in the mature mRNA or as few as two. A particularly striking example of the control of gene expression by RNA-processing involves a complex transcription unit isolated from rat DNA[22]. This DNA segment encodes calcitonin - a hormone produced in the thyroid gland. The calcitonin primary transcript was also found to be present in pituitary cells. This transcript contains two poly A sites. Cleavage at the first site followed by exon splicing results in the calcitonin mRNA in the thyroid gland. When cleavage occurs at the second poly A site and the calcitonin coding exon is spliced out, a mRNA coding for the neuropeptide CGRP (calcitonin-gene-related protein) results. Thus two protein hormones are produced from the same transcript in different tissue[22]. Alternative splicing is crucial for the

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[21] R. Reed, T. Maniatis, *Cell*, **41**, 95-105, (1985).
[22] J.E. Darnell, "*Readings from Scientific American*", W. H. Freeman and Company, New York, 26-36 (1985).

viability of many vertebrate organisms. It is estimated that 1 of every 20 genes is expressed via alternative paths of splicing in different cell types or growth periods[8].

1.5 The RNA Lariat Debranching Enzyme

After splicing, the RNA "lariat" intron containing the unusual 2',5'-phosphodiester bond vicinal to the 3', 5'- phosphodiester bond is debranched. The RNA lariat debranching enzyme is responsible for specifically cleaving (hydrolyzing) the 2', 5'-phosphodiester linkage, thus linearizing the lariat intron. This enzyme was first noted in mammalian HeLa cell extracts[23] - it will be referred to as hDBR. The gene encoding the yeast RNA lariat debranching enzyme (yDBR) was later isolated in *Saccharomyces cerevisiae*[24]. A genetic screen was used to identify cellular factors involved in the transposition frequency of the retrotransposon Ty1 in yeast. A mutation in a gene, now referred to as the DBR1 gene, was found to reduce the transposition frequency. Interestingly, the dbr1 mutant cells were also found to contain high levels of intron lariats. DBR1 was found to encode the debranching enzyme. Although the enzyme is essential for intron turnover, it is not essential for cell viability.

The yDBR also digests other branched structures derived from different species which include multicopy single-stranded DNA (msDNA) and trans-splicing intermediates. msDNA found in *Myxococcus xanthus*[25] (a Gram-negative bacterium) and *E.coli*[26] consists of a 162-base single stranded DNA which is linked to an internal rG of a 77-base RNA via a 2'-5'-phosphodiester linkage. The Y-like trans-splicing intermediate is formed upon maturation of pre-mRNAs when the joining of independent transcripts occurs - this method of splicing is unique to the trypanosomes, a family of protozoa[27]. It is believed that the minimum requirement for the debranching enzyme is a 2'-5'-phosphodiester bond vicinal to a 3'-5'-phosphodiester bond which is common to both msDNA and the trans-splicing intermediate. Determining whether or not there is a

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[23] B. Ruskin, M. R. Green, *Science*, **229**, 135-140, (1985).
[24] K. B. Chapman, J. D. Boeke, *Cell*, **65**, 483-492, (1991).
[25] T. Yee, T. Furuichi, S. Inouye, *Cell*, **38**, 203, (1984).
[26] B. C. Lampson, J. Sun, M. Y. Hsu, J. Vallejo-Ramirez, S. Inouye, M. Inouye, *Science*, **243**, 1033, (1989).

sequence requirement around the branch point that can affect the debranching reaction is of interest. msDNA from various *E. coli* strains, as well as several synthetic branched RNAs have been used to allow mechanistic and structural studies of the yDBR[28]. The naturally occurring substrates of the yDBR have an $A^{2'5'}G$ linkage (where A is the branch point of the lariat intron). msDNAs on the other hand, have a G branch point residue rather than an A residue, as well as a deoxyribonucleotide at the 2'-position; nevertheless they were still found to be good substrates for the yDBR. When msDNAs with a purine versus a pyrimidine at the 2'-position were compared, the results indicated that a 2'-purine was needed for efficient cleavage[28]. Y-shaped 18mers consisting of hexanucleotides joined at a branch point have also been studied[28]. These substrates had a limitation, as the 2' and 3' substituents were identical in sequence. However, the results still revealed that the efficiency of debranching was much higher when the first nucleotide at the 2' position was a purine as opposed to a pyrimidine. Although varying the branch point A residue to a C residue while maintaining a purine at the 2'-position caused a decrease in the rate of debranching, this decrease was not as significant as that caused by a pyrimidine at the 2'-position (with a branch point A residue). Two sets of tetramers have also been synthesized in order to study the specificity of cleavage by the yDBR[29] - they are 5'-AA^{N}_{N}-3' and NA^{G}_{G}, where N = G, A, C, U. These probable substrates were radiolabelled with ^{32}P and incubated with the yDBR for 10, 50 and 100 minutes. Their gel mobility was subsequently compared to that of the corresponding linear trimers (expected debranched products) in order to determine the efficiency of debranching of the different tetramers. This study also suggested that the yDBR had a strong preference for a 2' purine over a pyrimidine. In terms of substrates with a 2' purine residue, those with a 2'G residue, were cleaved faster than those with a 2'A residue. A strong preference for the 5'- nucleotide was not as evident, however, tetramers with a 5' purine were found to be the best substrates. Thus AA^{G}_{G} and GA^{G}_{G} are the best substrates

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[27] N. Agabian, *Cell*, **61**, 1157-1160, (1990).
[28] K. Nam, R. H. E. Hudson, K. B. Chapman, K. Ganeshan, M. J. Damha, J. D. Boeke, *The Journal of Biological Chemistry*, **269**, 20613-20621, (1994).
[29] R. H. E. Hudson, Ph.D. Thesis, University of Toronto, Canada, (1995); with M. J. Damha

for the debranching enzyme, UA^{G}_{G} and CA^{G}_{G} are also good substrates, however, tetramers with a pyrimidine at the 2' position are very poor substrates.

Whereas AA^{G}_{G} is cleaved efficiently, the linear trinucleotide A3'p5'A2'p5'G (AA^{G}) is not a substrate for the yDBR enzyme. This indicates that for the debranching reaction to occur, the 2',5'-phosphodiester linkage has to be adjacent (vicinal) to a 3',5'-phosphodiester bond. It is not known, however, whether the enzyme only requires the presence of a 3'-phosphate group, or the entire 3'-phosphate-nucleoside residue, or simply, a bulky group at the 3'-position.

1.6 Objectives

In order to continue to probe the substrate specificity of the yDBR, "tetramers" of the type dAA(G)X will be synthesized. In order to determine the importance of the 3' residue, X will be modified to a bulky tertiary butyl diphenyl silyl group (TBDPSi) and to a phosphate group (PO_4^{2-}) as shown in figure 1.8. The "tetramer" dAA(G)TBDPSi will be synthesized as follows: the modified phosphoramidite: N^6-Bz-5'-O-MMTr-3'-O-TBDPSi adenosine-2'-O-[N,N-diisopropyl-(cyanoethyl)]phosphoramidite will be made and incorporated into the tetramer by standard solid phase oligonucleotide methods employing a DNA/RNA synthesizer. dAA(G)TBDMSi will also be prepared and converted into dAA(G)PO_4^{2-} by desilylation and subsequent phosphorylation. The corresponding linear compounds will also be synthesized as they are the expected products of the debranching reaction. All compounds will be characterized using MALDI-TOF MS as well as PAGE. dAA(G)PO_4^{2-} will be radiolabelled with ^{32}P using 3'-phosphatase free polynucleotide T4 kinase, incubated with yDBR enzyme and the autoradiograph of the gel analyzed to determine whether or not dAA(G)PO_4^{2-} is in fact cleaved by the enzyme. The outcome of these debranching experiments will help establish whether the yDBR enzyme absolutely requires a 3'-nucleotide residue, or whether only a charged 3'-phosphate is necessary.

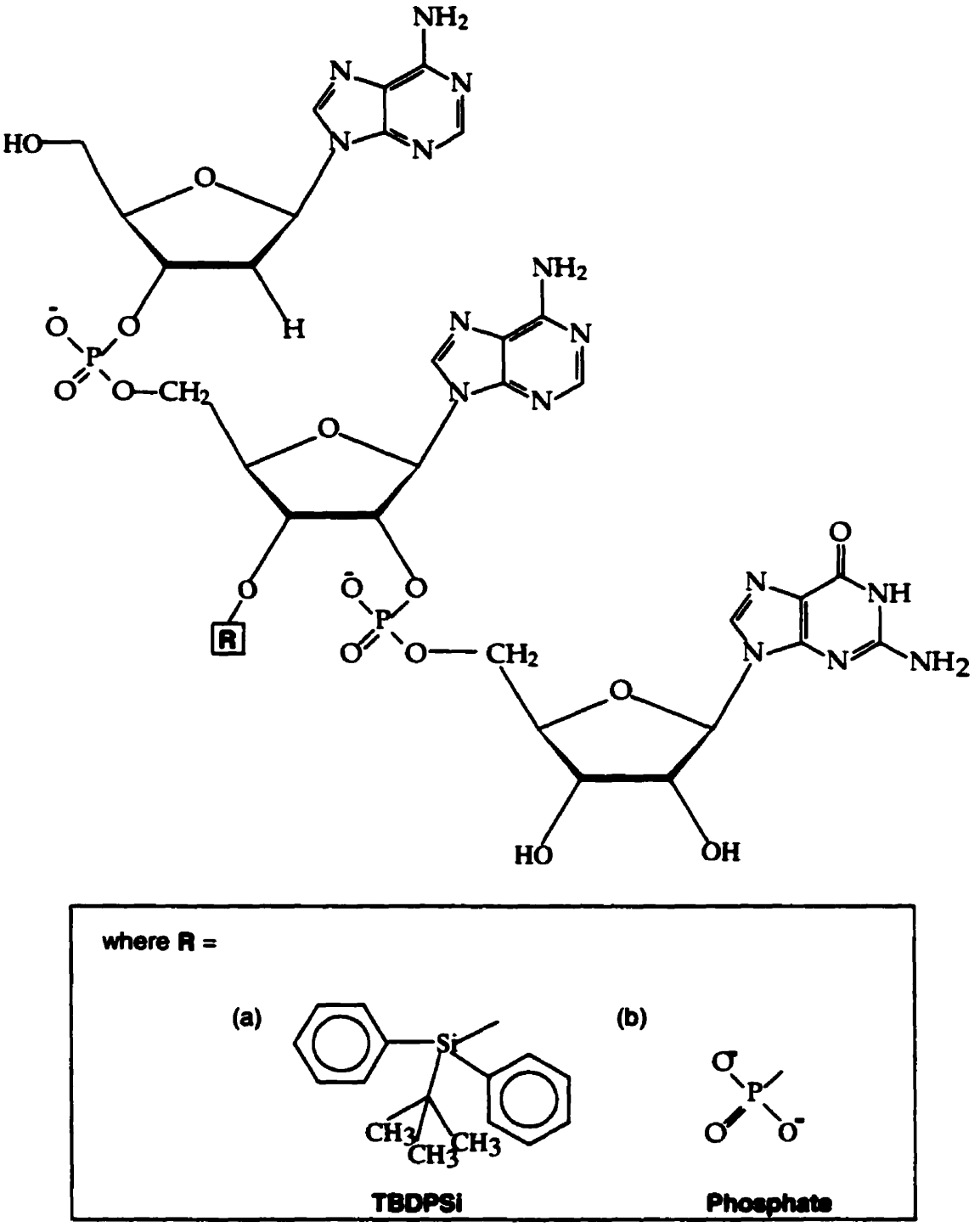


Figure 1.8 "Branched" Nucleic Acid Analogs
The 3'-position is modified to (a) a bulky tertiary butyl diphenyl silyl group, (b) a phosphate group.

Synthesis of Branched "Tetramers"

2.1 Synthesis and Characterization of a Modified Phosphoramidite

2.1.1. The Importance of Protecting Groups

In this section, the synthetic steps involved in the formation of a modified phosphoramidite will be explained and the importance of protecting groups will become evident. With the development of protecting groups, the synthesis of oligonucleotides has become trivial. Protecting groups are essential as there are several nucleophilic centers on nucleosides. These include the 5'-hydroxyl group, the 3'-hydroxyl group and the exocyclic amino groups of dA, dC and dG. To allow formation of the internucleoside 3'-5' phosphodiester bond, the nucleophilic groups that can potentially interfere with the exclusive formation of this bond are chemically protected (see Table 2.1). The 5'-hydroxyl group and the exocyclic amino group(s) of the 5'-nucleoside must be protected. The 3'-hydroxyl group and the heterocyclic base must be protected as well. Two classes of protecting groups exist, they are: temporary and permanent protecting groups. A temporary protecting group is removed after each coupling step - for instance, the 5'-hydroxyl group of nucleosides is often temporarily protected, with more labile functionalities. Permanent protecting groups, on the other hand, must remain in place until the end of the synthesis. The exocyclic amino groups and the phosphate are often protected with permanent protecting groups which are removed at the end of the synthesis, yielding the final deprotected oligonucleotide[3].

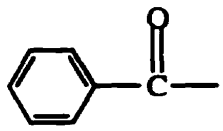
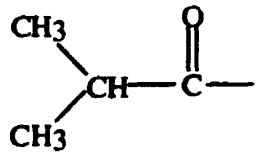
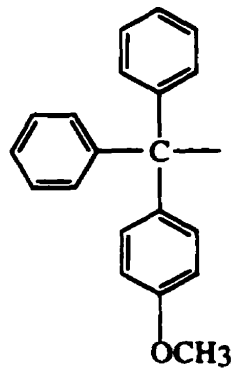
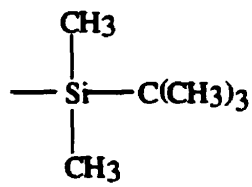
Protecting Group	Structure	Position
Benzoyl (Bz)		N^6-amino group on adenine and N^4-amino group on cytosine
Isobutyryl (i-bu)		N^2-amino group on guanine
Monomethoxytrityl (MMT)		5'- Oxygen of ribose sugar
tert-butyl dimethylsilyl (TBDMS)		2'- Oxygen of ribose sugar

Table 2.1 Commonly used Protecting Groups

2.1.2. Benzoylation of Adenosine

More than 30 years ago, Khorana and co-workers developed acyl permanent protecting groups for the exocyclic amino groups of adenine, cytosine and guanine in order to carry out the synthesis of oligonucleotides. Adenine and cytosine are protected by a benzoyl group, whereas guanine is protected by an isobutyryl group. These groups remain stable under mildly basic and acidic conditions and are removed with concentrated aqueous ammonia when the synthesis is complete. Acylation can be accomplished by per-acylation or the more recently developed 'transient' protection method shown in Figure 2.1. Per-acylation involves simultaneously acylating the hydroxyl and amino groups, followed by selective O-de-acylation thereby yielding the N-acylated nucleoside[30]. The transient protection method involves silylation of the hydroxy groups with trimethylchlorosilane followed by in-situ treatment with an acylating agent (Figure 2.1). Ammonia is then used to cleave the trimethylsilyl groups as well as the initially formed N,N-dibenzoyldeoxynucleoside derivative to give the desired N-acyl deoxynucleoside[31]. This latter method was used for the benzoylation of adenosine as the initial step for the synthesis of N^6 - Bz - 5' - O-MMTr - 3'- O - TBDPSi adenosine - 2' - O-[N,N diisopropyl(cyanoethyl)] phosphoramidite (Figure 2.1). The acylating agent used in this case was benzoyl chloride and the reaction was carried out at 0°. Compound 2.2 (rA^{Bz}) crystallized out of solution upon work-up and was of sufficient purity that further purification was unnecessary (yield of 84%).

2.1.3. Tritylation of Benzoyl Adenosine[32,33]

Protection of the 5'-OH group of compound 2.2, was accomplished by tritylation of A^{Bz} using monomethoxytrityl chloride (MMTCl), as earlier attempts at tritylation using dimethoxytrityl chloride (DMTCl) resulted in lower yields. Thus, compound 2.2 was coevaporated with absolute pyridine and subsequently suspended in this solvent. MMTCl was added and the resulting mixture allowed to stir at room temperature. The

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[30] M. J. Gait, "*Oligonucleotide Synthesis - a practical approach* ", IRL Press Limited, (1984).
[31] G. S. Ti, B. L. Gaffney, R. A. Jones, *J. Amer. Chem. Soc*, **104**, 1316-1319, (1982).
[32] G. H. Hakimelahi, Z.A. Proba, K.K. Ogilvie, *Canadian Journal of Chemistry*, **60**, 1106, (1982).
[33] M. J. Damha, K. K. Ogilvie, *Journal of Organic Chemistry*, **53**, 3710, (1988).

reaction was complete after 24 hours as revealed by TLC analysis. Work-up followed by chromatography afforded $^{MMT}rA^{Bz}$ (2.3) in 83% yield. The chromatographic property of 2.3 (R_f value) is shown in Table 2.2.

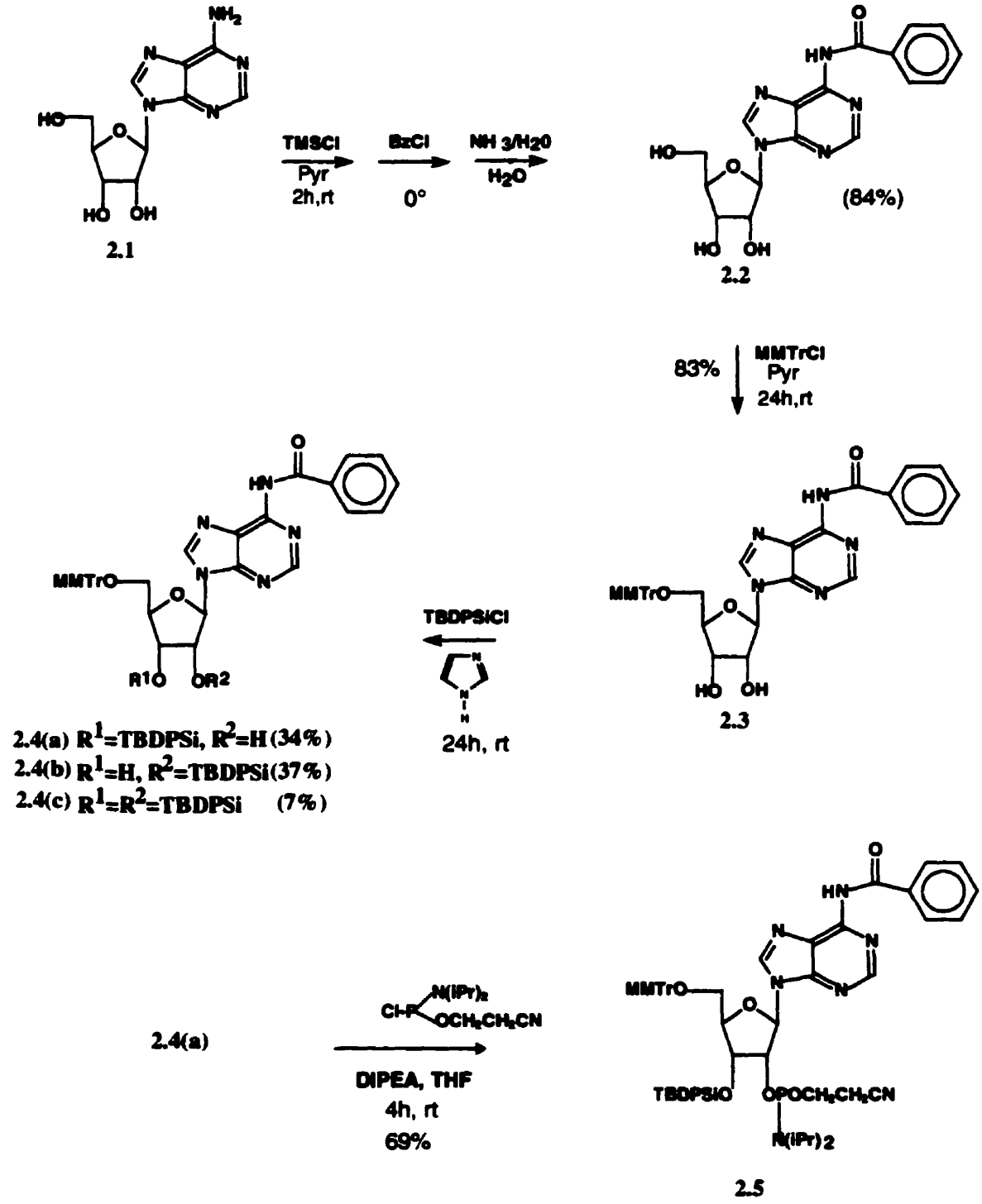

Figure 2.1 Synthetic Scheme for the Formation of a Modified Phosphoramidite

2.1.4. Silylation[34] of 5'-O-(Monomethoxytrityl)-N^6-Benzoylated-Adenosine

Compound 2.3 was coevaporated with absolute pyridine and suspended in a mixture of absolute acetonitrile and absolute pyridine. Imidazole (2.5 eq) was added, followed by *tert*-butyl diphenyl silyl chloride (TBDPSi-Cl) (1.25 eq) and the reaction stirred overnight at room temperature. After 16 hours, TLC analysis revealed the reaction was complete and the crude product was evaporated to dryness. The residue was diluted with dichloromethane and washed with 5% $NaHCO_3$ solution. The aqueous layer was re-extracted twice with dichloromethane for maximum recovery. The combined organic layer was dried over $MgSO_4$, filtered and evaporated to afford the crude product which was purified by flash chromatography using a gradient of ethyl acetate in toluene. Compounds 2.4(b) and 2.4 (c) eluted at a 4:1 ratio of toluene/ethyl acetate. For elution of compound 2.4(a), a 1:1 ratio of toluene/ethyl acetate was used. The R_f values for all intermediates are shown in Table 2.2. It is established that the 2'-silyl isomer travels faster than the 3'-isomer on silica gel gravity columns and on TLC[35].

2.1.5. Phosphitylation[35] of N^6- Benzoyl - 5' - O - (Monomethoxytrityl) - 3' - O - tert-Butyl diphenyl Silyl - Adenosine

Initially compound 2.4(a) was dissolved in absolute acetonitrile and phosphitylated using 1.2 eq of N,N-diisopropylamino cyanoethyl phosphochloridite and 2.4 eq of DIPEA. However, TLC analysis revealed a number of side products, presumably due to migration of the silyl ether linkage from the 3' to the 2' position. The procedure was modified - acetonitrile substituted with THF. Acetonitrile is an aprotic solvent with an appreciable dipole moment (μ=3.94). It can effectively solvate a cation causing an ion pair to be brought into solution. THF on the other hand, is a cyclic ether with a smaller dipole moment (μ=1.2) thus it is less likely to cause isomerization. Therefore, compound 2.4(a) was dissolved in THF, 0.12 eq of DMAP[36] added followed

[34] M. Wasner, PhD Thesis, Hartung-Gorre Verlag, Konstanz, Germany (1995).
[35] M.J. Damha, K.K. Ogilvie, Methods in Molecular Biology, Vol. 20: Protocols for Oligonucleotides and Analogs, Human Press Inc., Totowa, NJ, (1993).
[36] S. K. Chaudhary, O. Hernandez, Tetrahedron Letters, 2, 99-102, (1979).

by 4 eq of DIPEA (added dropwise over a 20 second period), and finally 1.2 eq of N, N-diisopropyl - amino cyanoethyl phosphochloridite. A white precipitate (diisopropylethyl-ammonium hydrochloride) indicated that the desired reaction had occurred. After 3 hours, TLC analysis revealed that the reaction had gone to completion and no isomerization was apparent. The crude product was then dissolved in dichloromethane and extracted with saturated NaCl/5% $NaHCO_3$ (4:1) and the aqueous phase re-extracted with CH_2Cl_2. The combined organic layer was dried over $MgSO_4$, filtered and evaporated. Flash chromatography ensued to purify the crude phosphoramidite. Rapid elution with CH_2Cl_2:Hexanes:NEt_3 (50:45:5) using a 3 cm diameter column, afforded the pure product (69% yield). All R_f values are shown in table 2.2.

Compound	% Yield	R_f value
2.2	84%	0.26 1:9 MeOH:CH_2Cl_2
2.3	83%	0.39 1:9 MeOH:CH_2Cl_2
2.4(a)	34%	0.45 1:1 toluene:ethyl acetate
 2.4(b)	37%	0.54 1:1 toluene:ethyl acetate
 2.4(c)	7%	0.71 1:1 toluene:ethyl acetate
2.5	69%	0.81, 0.72 (2 diastereomers) 1:1 ether:CH_2Cl_2

Table 2.2 Percentage Yield and Retardation Factor on TLC Plates for Synthesized Compounds

2.1.6. Characterization of the Monomer

^{31}Phosphorus NMR analysis of compound 2.5 was necessary in order to establish the certainty of it being a phosphoramidite. The spectrum is shown below. Two peaks centered around 152 ppm (referenced to H_3PO_4 in acetone) is diagnostic of this group of compounds (two diastereoisomers).

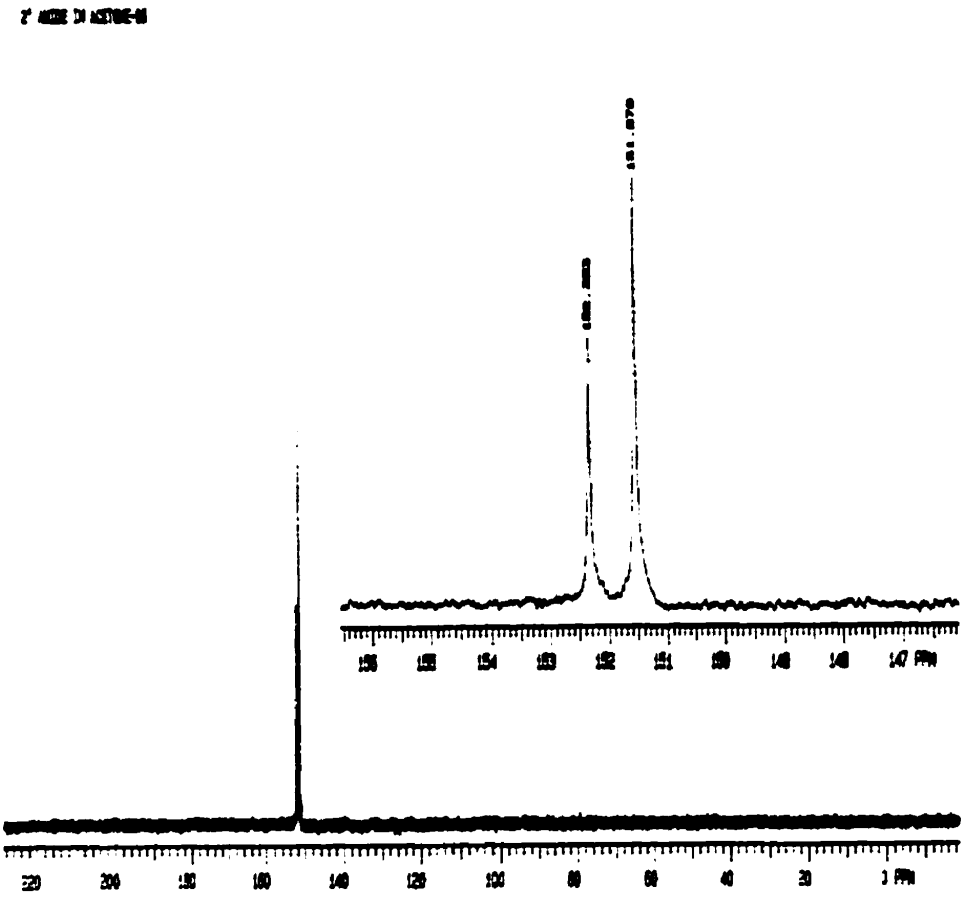

Figure 2.2 ^{31}Phosphorus NMR Spectrum of N^6-Bz-5'-O-MMTr-3'-O-TBDPSi adenosine-2'-O-[N,Ndiisopropyl(cyanoethyl)] phosphoramidite in Acetone-d6 (H_3PO_4 as reference).

2.2. Synthesis and Characterization of Branched "Tetramers" and Linear Trinucleotide Controls

2.2.1. Introduction to Solid Phase Oligonucleotide Synthesis[37]

After the discovery of DNA by Friedrich Miescher and the subsequent understanding that DNA is in fact hereditary material by several research groups (Griffith, Avery, MacLeod & McCarthy[38] and Hershey and Chase[39]), work on the structure of DNA began. Sir Alexander Todd and his group proved the structure of nucleotides by synthetic studies. Wilkins and Franklin clarified the DNA structure by X-ray crystallography. Watson and Crick[40] then proposed the structure of DNA to be double helical in nature. Thus the stage was set to begin work on the synthesis of polynucleotides. The first synthesis of a dinucleotide was accomplished by Michelson and Todd[41] in 1955 - making use of NCS (N-chlorosuccinimide) in the reaction scheme. In order to accomplish phosphorylation, Khorana and Todd introduced carbodiimide coupling reagents. This became the basis of the *phosphodiester* approach which posed some difficulty due to the negatively charged phosphodiester back-bone which caused branching to take place. Letsinger solved this dilemma by utilizing a cyanoethyl phosphate protecting group which gave way to the *phosphotriester*[42] approach resulting in higher yields. In 1975, Letsinger developed the phosphite-triester[43] approach - the basis of this approach was the generation of a nucleoside-3'-phosphochloridite which could then couple to a protected nucleoside. Beaucage and Caruthers[44] modified the phosphite-triester approach in the

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[37] M. J. Damha, Ph.D. Thesis, McGill University, Montreal, Canada, (1988); with K.K. Ogilvie.
[38] O. T. Avery, C. M. MacLeod, C. McCarthy, *J. Exp. Med*, **79**, 137-158, (1944).
[39] A. D. Hershey, M. Chase, *J. Gen. Physiol.*, **36**, 39-56, (1952).
[40] J. D. Watson, F. H. C. Crick, *Nature*, **171**, 737, (1953).
[41] Michelson, A. Todd, *J.Chem. Soc.* part 3, 2632-2638, (1955).
[42] (a) R.L.Letsinger, V. Mahadevan, *J. Amer. Chem. Soc.*, **87**, 3526, (1965). (b) R.L. Letsinger, V. Mahadevan, *J. Amer. Chem. Soc.*, **88**, 5319, (1966). (c) R.L. Letsinger, K.K. Ogilvie, *J. Amer. Chem. Soc.*, **89**, 4801, (1967). (d) R.L. Letsinger, P.S. Miller, *J. Amer. Chem. Soc.*, **91**, 3356 & 3360, (1969).
[43] (a) R.L. Letsinger, W.H. Lunsford, *J. Amer. Chem. Soc.*, **97**, 3278-3279, (1975). (b) R.L. Letsinger, W.H. Lunsford, *J. Amer. Chem. Soc.*, **98**, 3655-3661, (1976).
[44] M.H. Caruthers, *Science*, **230**, 281-285, (1985).

early 1980's with the use of nucleoside N, N-dialkylphosphoramidites. Upon activation by a weak acid (*i.e.* tetrazole) coupling with a protected nucleoside would ensue. With these developments in polynucleotide synthesis, automation became possible. In the early 1980's, Ogilvie[45] and coworkers developed the DNA Synthesizer ("Gene Machine") which is currently used in many laboratories to assemble DNA chains in a matter of hours.

Synthesis of DNA via the solid phase strategy involves coupling a protected nucleotide in solution with a residue bound to an insoluble support. The main features of the solid-phase approach will be reviewed in the following paragraphs as the synthesis of the "tetramers" of interest is discussed. It is important to mention that solid phase oligonucleotide synthesis occurs in the 3' to 5' direction rather than in the 5' to 3' direction which occurs in nature. Thus the 3'- nucleoside must initially be attached to a support (Figure 2.3). There are many solid supports available yet the most useful under all synthetic conditions is controlled pore glass (CPG). CPG beads are both rigid and non-swellable, and come in a variety of particle sizes and porosities. The CPG is functionalized by a long chain alkylamine (LCAA-CPG) - thus ensuring that reactive sites are away from the surface and thereby accessible to all reagents.

In the synthesis of dAA(G)X it was necessary to first derivatize the LCAA-CPG[46] support with the 2' nucleoside, $^{MMT}rG^{ibu}$. LCAA-CPG was first activated with acid($TCA/EtCl_2$) and then reacted with succinic anhydride as shown in Figure 2.3. The support 2.8 was then reacted with 5'-MMT rG^{ibu} in the presence of the coupling reagent 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (DEC). Any unreacted hydroxyl groups were capped with acetic anhydride (Ac_2O) to prevent formation of side products during oligoribonucleotide synthesis.

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[45] G.A. Urbina, G. M. Sathe, W.C. Liu, M.F. Gillen, P. D. Duck, R. Bender, K. K. Ogilvie, *Science*, **214**, 270-274, (1981).

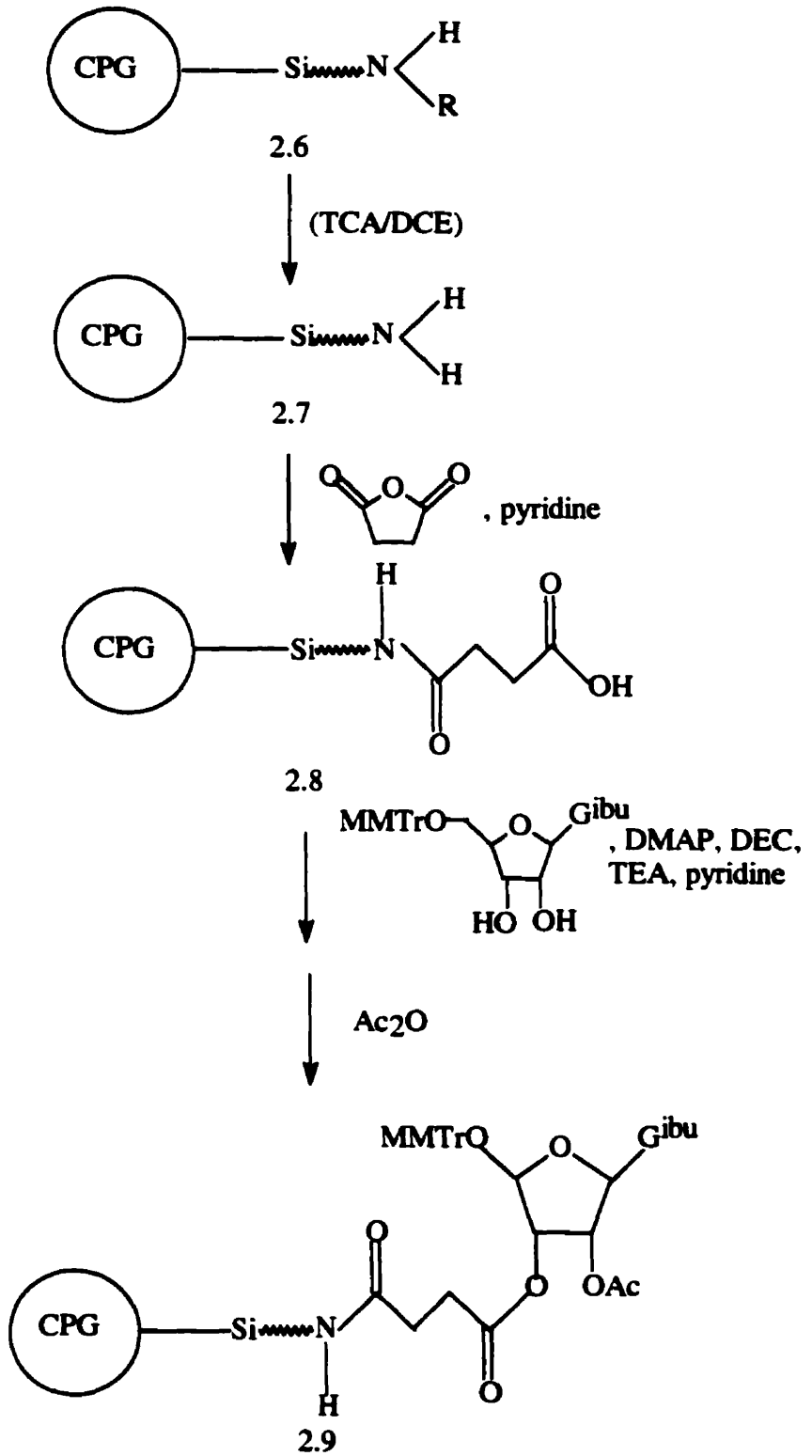

Figure 2.3 Derivatization of LCAA-CPG

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[46] R. T. Pon, "*Methods in Molecular Biology*", **20**, 465-496, (1993).

Machine-aided solid-phase synthesis of DNA and RNA is simply a cycle of four consecutive reactions[47]. They are: detritylation, coupling, capping and oxidation. The aforementioned will be discussed in greater detail in the following paragraphs.

The synthetic cycle begins with removal of the acid-labile MMT or DMT group of the support-bound protected nucleoside. Detritylation is effected using trichloroacetic acid (TCA) in dichloroethane. It is important to note that the trityl cation is highly reactive therefore, it can re-tritylate the nucleoside. To avoid this, it is necessary to remove the trityl cation from the column by continuous elution. The eluant is collected into tubes of a fraction collector as it is chanelled through the trityl collection port of the DNA Synthesizer. In order to obtain the coupling efficiency, the orange color (DMT cation; λ_{max} = 503 nm) or the yellow color (MMT cation) (λ_{max} = 478 nm) that is liberated in solution is compared to that of the previous cycle. The absorbance of the eluant is found spectrophotometrically. The molar amount of the DMT or MMT cation can be determined using Beer's Law: $A = \varepsilon Cl$,

where: A = absorbance

ε = extinction coefficient

C = concentration

l = path length

Before coupling of the phosphoramidite to the support-bound nucleoside, it is essential to make the support anhydrous - any remaining nucleophiles such as water will compete with the phosphoramidite thereby lowering coupling efficiency. It is for this reason, acetonitrile must be extensively delivered to the column, followed by drying by an argon reverse flush. Moreover, an acetonitrile wash to remove traces of TCA is essential so as to prevent decomposition of an incoming phosphoramidite. At this point, the mild acid, tetrazole is delivered. Depending on the oligonucleotide sequence, a specific phosphoramidite and tetrazole are delivered to the column where the two reagents mix. The tetrazole (excess) is responsible for activating the phosphoramidite by

transferring a proton to the nitrogen of the diisopropyl group on the phosphoramidite (Figure 2.4). The protonated amine is now a good leaving group and is displaced by tetrazolide anion resulting in tetrazolyl phosphoramidite. This reactive intermediate allows for the formation of the 5'-3' internucleotide linkage.

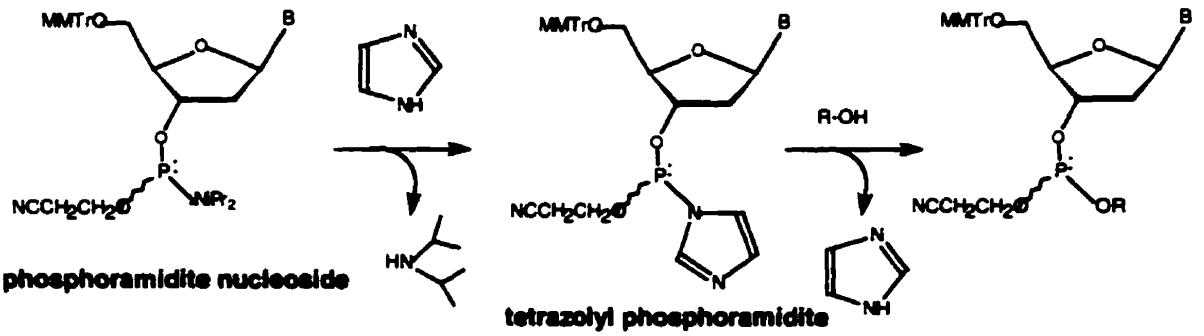

Figure 2.4 The Coupling Step
Phosphoramidites and tetrazole are delivered to the column which contains the support-bound nucleoside. The diisopropylamine is protonated and displaced by tetrazole. When the 5' OH couples to the phosphorus, a 5' to 3' internucleotide linkage is created.

Coupling is not always quantitative, in fact a small percentage of support-bound nucleotides fail to react with the activated tetrazolyl phosphoramidite. The result is failure or truncated sequences that remain attached to the support and unless they are 5'-acetylated, they will continue to propagate in subsequent coupling steps. Thus sequences with one base less than the product are generated and due to the small difference in their size, isolation of the product becomes more difficult. In order to avoid this problem, the so-called "capping step" is necessary. A powerful acetylating agent (see figure 2.5) is formed when equal volumes and equimolar amounts of acetic anhydride and 1-methylimidazole(NMI) are simultaneously delivered to the column. This agent acetylates the 5'- hydroxyl group of the failure sequences rendering them unreactive for the rest of the synthesis.

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[47] Applied Biosystems 381A DNA Synthesizer User's Manual, (1987).

The unstable phosphite linkage that has been formed must be oxidized to the more stable pentavalent phosphate triester. An iodine-water-pyridine-tetrahydrofuran mixture is used. It has been shown that better results are obtained when oxidation follows capping rather than the reverse. If capping were to follow oxidation, traces of water from the iodine solution could cause acetic anhydride to form acetic acid during capping. The acid formed would render the capping step less efficient. But most importantly, a capping step immediately following coupling reverses unwanted phosphitylation (branching) reactions occurring at the lactam function of guanines.

Oxidation signals the end of a synthetic cycle. The 5' end still has a DMT or MMT protecting group. In order to continue the synthesis, the 5'-trityl group is removed and another cycle of base addition occurs. The steps are repeated until the DNA or RNA of interest is fully synthesized.

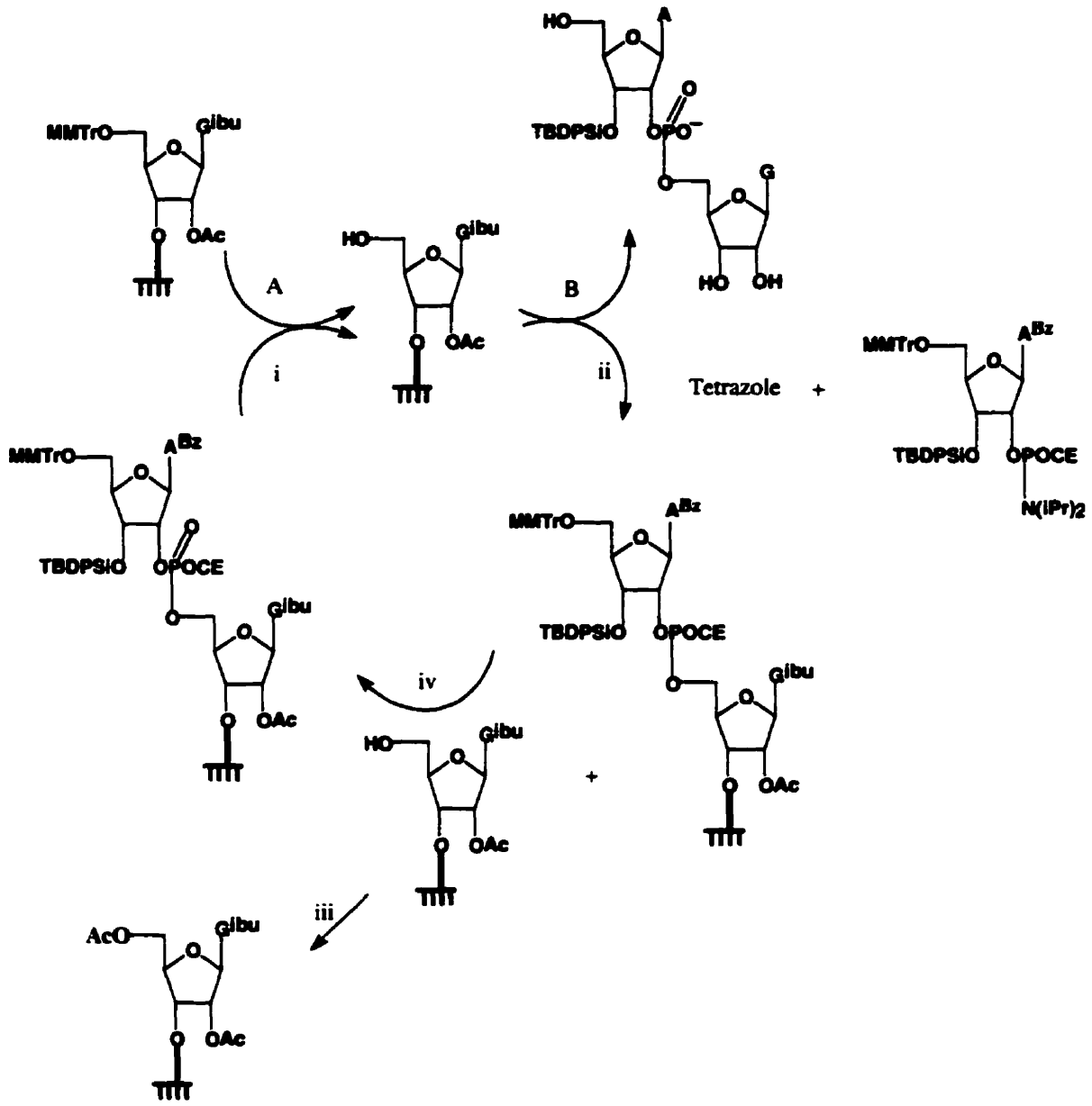

Figure 2.5 Solid Phase Synthesis : (A) Cycle Entry. (i) detritylation, (ii) coupling, (iii) capping, (iv) oxidation. (B) Cycle Exit - cleavage and deprotection

At the end of the synthesis, a choice can be made as to whether or not the trityl group at the 5' position remains. When the synthesized oligonucleotides are to be purified via gel electrophoresis or ion exchange HPLC, the trityl group at the 5' position is usually removed. When purifying by OPC (Oligonucleotide Purification Cartridge) or trityl-specific reverse phase HPLC, the trityl group is usually left on to help in the purification process.

Once the synthesis is complete, the product still carries base and phosphate protecting groups. Thus deprotection is necessary so as to yield biologically active oligonucleotide. As shown in Figure 2.6, for a dinucleotide, ammonium hydroxide (29% NH_4OH: ethanol) (3:1, v/v, room temperature, 48 h) removes the β-cyanoethyl, benzoyl and isobutyryl protecting groups and cleaves the dimer from the support. This also cleaves the acetyl caps from failure sequences. Once deprotection is accomplished, desalting and purification may be necessary before the oligonucleotides may be used in experiments.

Quantitation of the synthesized oligonucleotides is easily achieved by UV spectroscopy, measured at the UV absorbance maxima of nucleic acids (*i.e.* 260nm). A dilute solution of the oligonucleotide is measured by scanning the 200 - 350 nm region or by taking a single wavelength measurement. Beer's law is used to determine the concentration of the solution. Thus, the absolute quantity can be calculated. A useful approximation is the following; 1 odu of a single stranded oligonucleotide is about 33 micrograms.

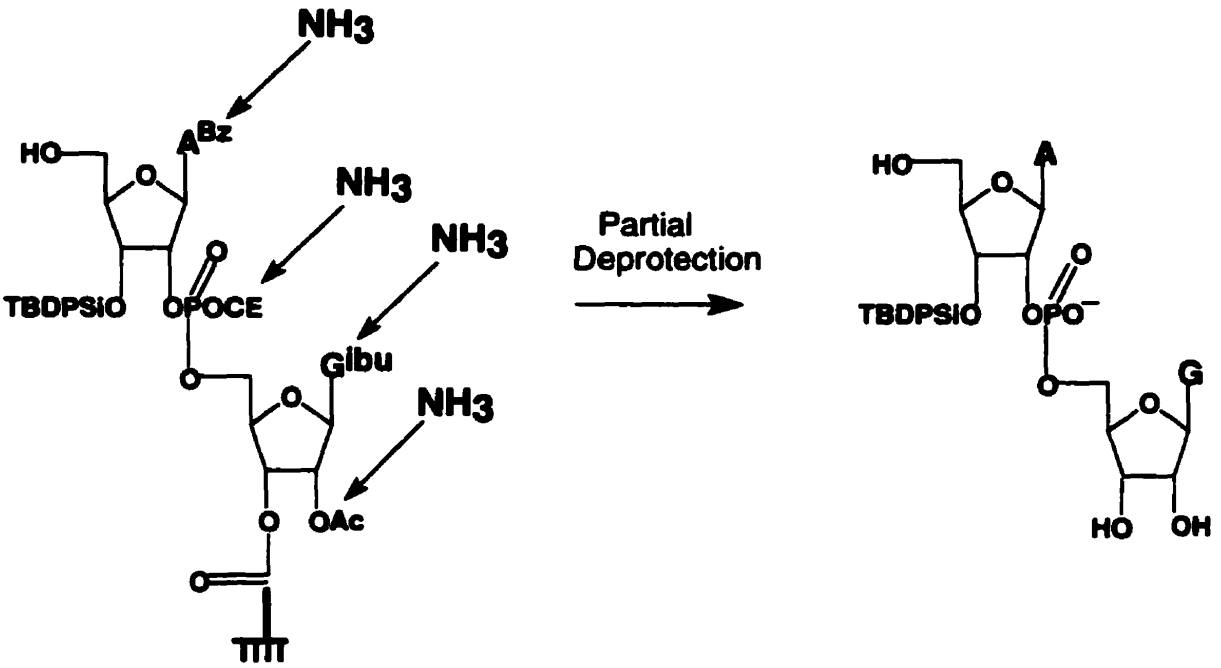

Figure 2.6 Deprotection and Cleavage of an Oligonucleotide

2.2.2. The Synthesis of dAA(G)TBDPSi

The aforementioned solid phase synthesis approach was utilized in order to synthesize the "tetramer" or branched analogue dAA(G)TBDPSi (see Figure 2.7). In order to accomplish this synthesis, it was necessary to attach the $^{MMT}rG^{iBu}$ nucleoside to the LCAA-CPG support as previously described (Figure 2.3). Once this was completed, the rG derivatized LCAA-CPG support (loading = 42 μmol nucleoside/g) was placed in a column on an automated DNA synthesizer and capped. The amidites were introduced dissolved in acetonitrile; the concentration was 0.15 M and 0.10 M for the modified rA (2.5) and dA, respectively. The standard 58-cycle phosphoramidite protocol utilized in the Damha laboratory was employed for the synthesis (1 μmol scale) of the branched nucleic acid analog. The only change in the standard cycle was an increase in the coupling wait time to 900 seconds for the modified rA, since it bears a large 3' TBDPSi protecting group vicinal to the 2'-phosphoramidite moiety. The coupling efficiency ranged from 90

to 98 %, based on the trityl cation released after coupling. Deprotection and cleavage with aqueous ammonia/ethanol (3:1, r.t. 48 hours) afforded the crude 'tetramer'.

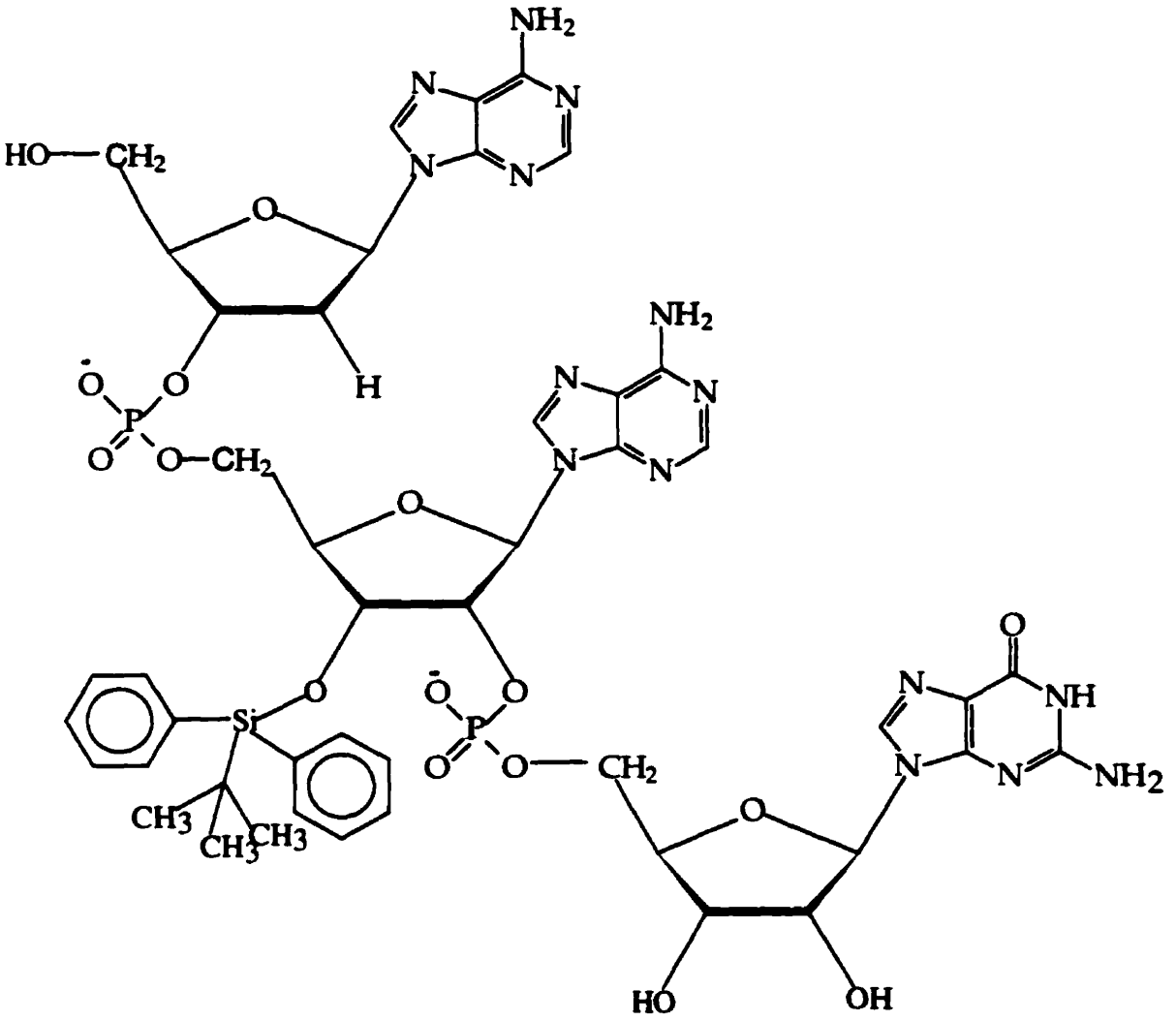

Figure 2.7 Structure of dAA(G)TBDPSi.

2.2.3. Purification of dAA(G)TBDPSi *using Polyacrylamide gel electrophoresis (PAGE)*

PAGE[2,35] is a fast and economical method that can be used to check the purity of crude oligomers and can subsequently be used to purify the desired oligonucleotide. This technique separates oligonucleotides based on size, i.e., the gel retards the movement of large molecules relative to small ones. The gels are made by the polymerization of acrylamide and N,N'-methylene-bis-acrylamide (Bis) in Tris/Boric Acid/EDT buffer (TBE). The polymerization is induced by free radicals resulting from the chemical decomposition of ammonium persulfate ($S_2O_8^{2-} \longrightarrow 2SO_4^{-}\bullet$). N,N,N',N'-tetramethylethylenediamine (TEMED), is a free radical stabilizer which is added to the gel mixture. The format of choice for PAGE is the slab gel that consists of two glass plates which are held apart by two side spacers (the thickness of the gel depends on the thickness of the spacers). The acrylamide solution is poured in between the plates, a comb is inserted forming the wells where the oligomers are loaded. Once polymerized (1h), the samples are loaded (dissolved in TBE). Dyes such as bromophenol blue and xylene cyanol are run as indicators of the migration distance of the oligomers. The acrylamide concentration of the gel determines how fast the oligonucleotides move. Oligonucleotides that are 2-20 nucleotides in length are most commonly run on 24% polyacrylamide gels with the current set at 10 mA for the first 15 minutes followed by 20 mA to completion (~ 4h). The gel is then removed from its holder, wrapped in plastic wrap and placed over a fluorescent TLC plate and visualized with a UV lamp (254 nm). A permanent record can be made by photographing the gel. Once the desired sequence is located, the oligonucleotides can be purified by preparative PAGE using 1.5 mm thick, side spacers and combs that will form 1 or 2 lanes. After electrophoresis, the desired band is excised using a scalpel and placed in a 16 ml polypropylene tube with sterile water overnight, in order to elute the oligonucleotide. The tube is vortexed and centrifuged and the supernatant is transferred to another tube, dried down and then desalted. This was the procedure followed in the purification of the nucleic acid analog dAA(G)TBDPSi. Ten ODU of the crude sample were loaded. Reversed-phase chromatography using C_{18} Sep-

Pak[35] cartridges was used to desalt the sample, affording 3 ODU of pure dAA(G)TBDPSi (33% yield).

2.2.4. The Synthesis of dAA(G)P[48]

Next, dAA(G)P was synthesized (Figure 2.8). To effect the synthesis of the nucleic acid analog, dAA(G)P, the starting material, dAA(G)TBDMS was needed. The previously synthesized dAA(G)TBDPS could have also been used, but it was kept for analysis with the yDBR enzyme. The synthesis of dAA(G)TBDMS was accomplished using the previously described procedure for synthesis of dAA(G)TBDPS. The 5'-DMT-3'-TBDMSilyl-ribo Adenosine (N-Bz) cyanoethyl phosphoramidite was purchased from ChemGenes. The % coupling (trityl analysis) for the addition of rA and dA was 94% and 104%, respectively. At the end of the synthesis, the 5'-hydroxyl group was capped by running a capping cycle (see Figure 2.8). To assure acylation of the 5'-hydroxyl group, this step was repeated three times. At this point, the synthesis column was removed from the gene machine. In order to dry the solid support, argon was flushed through the column. To remove the phosphate protecting (cyanoethyl) groups, the support was treated with a solution of NEt_3/CH_3CN (10 ml, 4:6 v/v) over a 90 minute period. A syringe was used to push the solution through the column. The column was then washed with 30 ml of CH_3CN followed by 30 ml THF. The next step was removal of the 3'- silyl protecting group. This was effected by pushing 1 ml of a 1M solution of TBAF/THF through the column over a 10 minute period. The support was then washed with 30 ml THF followed by 30 ml CH_3CN. In order to achieve phosphorylation of the 3'-OH group, the synthesis column was reinstalled on the 'Gene Machine'. One addition ensued via the standard cycle for addition of a 5'-phosphoramidite. The only change that was made to the cycle was a more concentrated phosphoramidite solution (0.3 M) and a longer coupling time (30 minutes). The phosphoramidite utilized was 2-[2-(4,4'-Dimethoxytrityloxy) ethylsulfonyl] ethyl-(2-cyanoethyl)-(N,N-diisopropyl) phosphoramidite (or 5'-Phosphate-ON Reagent), which was obtained from ChemGenes (shown on the following page).

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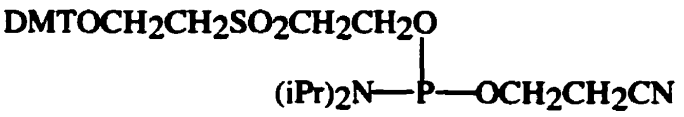
[48] R.S. Braich, M.J. Damha, *Bioconjugate Chemistry*, **8**, No. 3, 370-377, (1997).



Trityl assay analysis revealed 49% coupling of the 5'-Phosphate-ON Reagent. Deprotection was effected using aqueous ammonia/ethanol (3:1, r.t. 48 hours). PAGE was used for purification of the oligonucleotides – 18 ODU were loaded. Reversed-phase chromatography using C_{18} Sep-Pak[35] cartridges was used to desalt the oligonucleotides. Six ODU were recovered (33% yield). Subsequent to the synthesis of dAA(G)P, analytical gel analysis revealed that desilylation of dAA(G)TBDMS (during the synthesis of dAA(G)P) did not proceed to completion thereby resulting in a low yield of dAA(G)P.

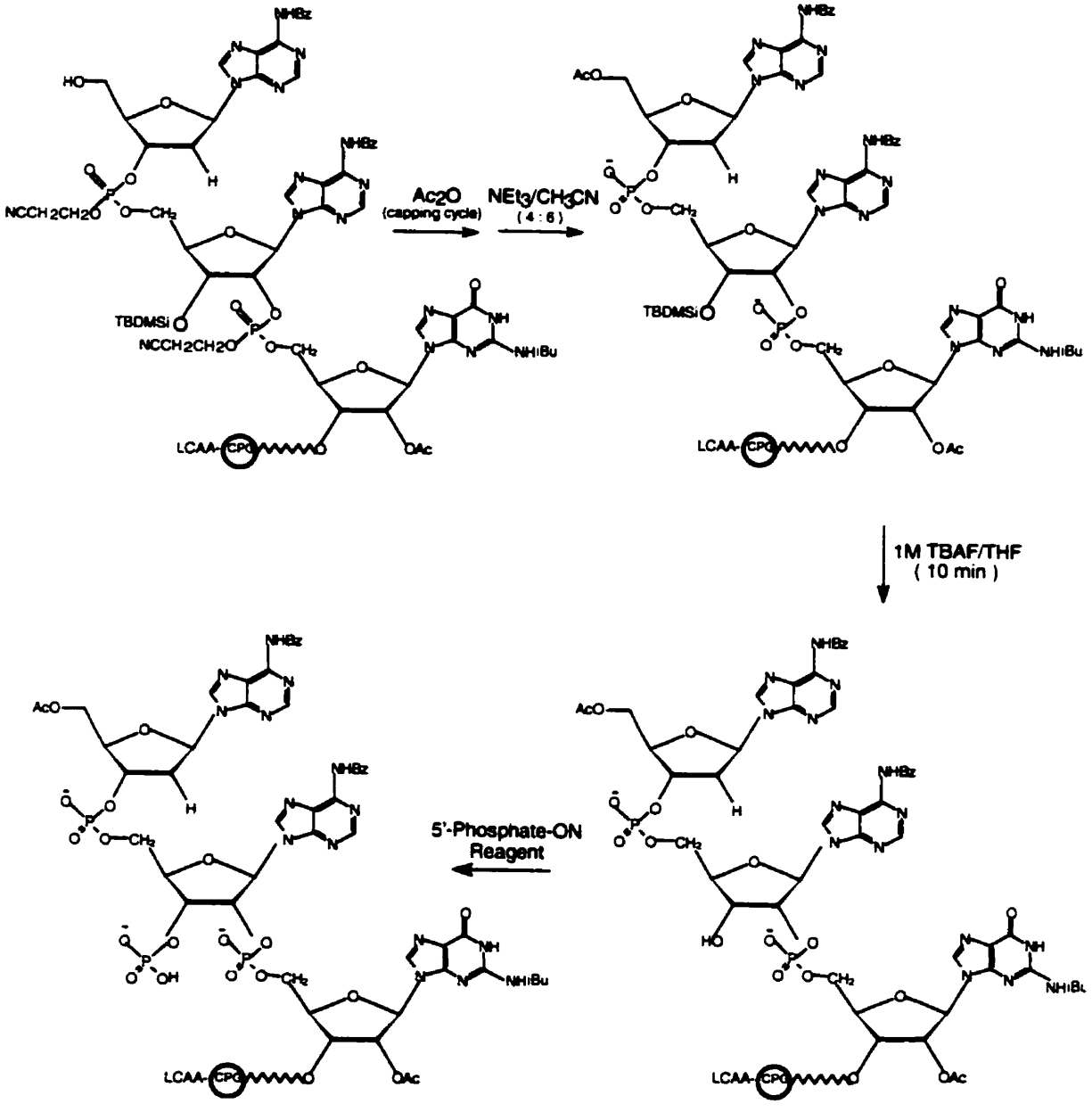
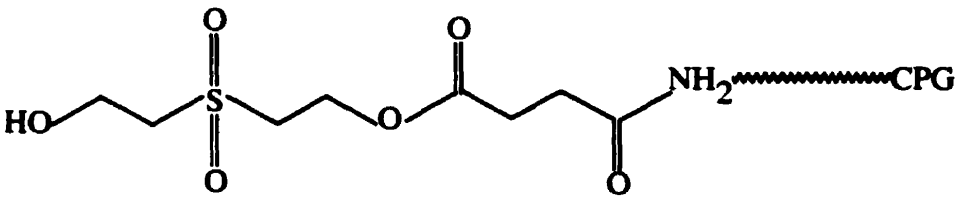


Figure 2.8 Synthesis of dAA(G)P

2.2.5. The Synthesis of dAA(OH)P

One of the presumed cleavage products of dAA(G)P by the yDBR enzyme is dAA(OH)P. Thus dAA(OH)P was synthesized by solid phase synthesis (1 μmol) for comparison and analysis in the cleavage assays. The synthesis of dAA(OH)P was accomplished using the following solid support (loading = 30 umol/g):



This support was a gift from Dr. Richard Pon (University of Calgary, Department of Medicine). Before addition of the first phosphoramidite (2'-silylated rA phosphoramidite), it was necessary to modify the 58 step cycle - the detritylation step was removed since the support did not contain a trityl group. The next addition was that of dA - this proceeded according to the standard 58 step cycle. The coupling (trityl analysis) was 91%. Deprotection was effected using aqueous ammonia/ethanol (3:1, r.t. 48 hours). The resulting oligonucleotide was dAA(Si)P, thus it was necessary to remove the silyl group at the 2' position (Figure 2.9). The crude oligonucleotide was placed in an eppendorf tube and desilylated by adding 25 μl of TREAT•3HF[49]. After 8h, 1 ml of water was used to quench the reaction. Preparative PAGE was used for purification of dAA(OH)P (10 ODU were loaded). Desalting was effected as previously described, affording 2 ODU of the desired product (20% yield).

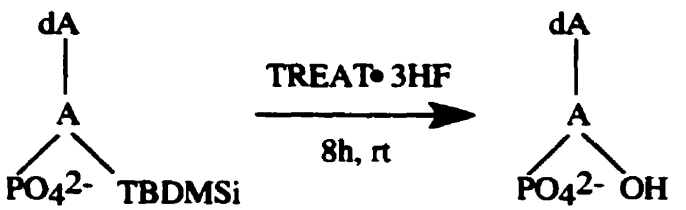


Figure 2.9 Desilylation of dAA(Si)P

2.2.6. The Synthesis of dAA(G)G

It has previously been established[29] that dAA(G)G is a substrate of the yDBR enzyme, therefore it was synthesized so that it could be used as a control in yDBR enzyme studies. The chemical synthesis of branched RNA has been described previously[50]. Briefly, the method consists of coupling an adenosine bis(phosphoramidite) reagent, to two adjacent support-bound oligonucleotide chains (see Figure 2.10)[51]. The support that was used in this synthesis was rG derivatized LCAA-CPG support (loading = 42 μmole nucleoside/g). The standard 58 step cycle was used (the only cycle change was a 900 sec wait step for bisA). The first addition was that of N^6-benzoyl-5'-O-dimethoxytrityladenosine 2',3'- bis(β-cyanoethylphoshoramidite) (0.03M in acetonitrile); this compound was provided by Dr. Sebastien Robidoux (McGill University, Dr. Damha's laboratory). The coupling of dA phosphoramidite followed, yielding 26 ODU of crude dAA(G)G. Deprotection and purification was performed as previously described. (20% yield).

---

[49] (a) M.C. Pirrung, S.W. Shuey, D.C. Leuer, L. Fallon, *Biorg. Med. Chem. Lett.*, 4, 1345, (1994). (b) E. Westman, R. Stromberg, *Nucleic Acids Research*, 22, 2430, (1994).

[50] (a) S. V. Zabarylo, M. J. Damha, *Tetrahedron Lett*, 30, 6295, (1989). (b) M. J. Damha, K. Ganeshan, R. H. E. Hudson, S. Zabarylo, *Nucleic Acids Research*, 20, 6565, (1992). (c) R. H. E. Hudson, M. J. Damha, *J. Am. Chem. Soc.*, 115, 2129, (1993).

[51] M. J. Damha, K.K. Ogilvie, *J. Org. Chem.*, 53, 3710, (1988).

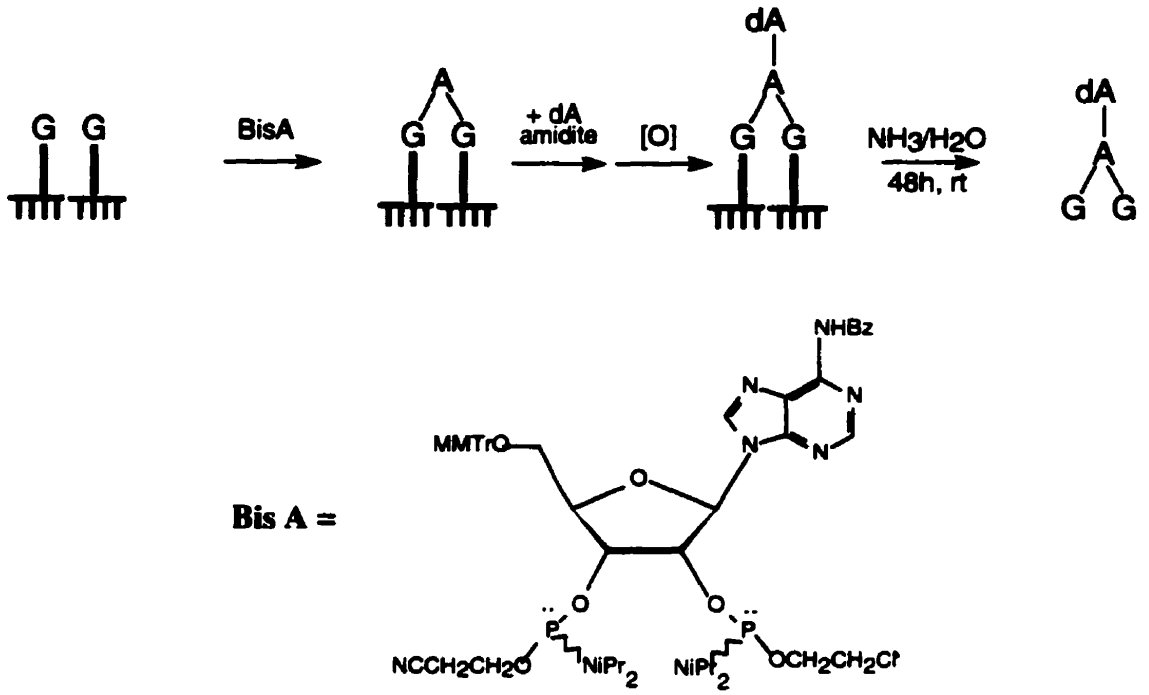


Figure 2.10 The Chemical synthesis of Branched RNA

2.2.7. Synthesis of dAA(OH)G

dAA(OH)G was synthesized as it is one of the presumed cleavage products of dAA(G)G by the yDBR enzyme. In order to synthesize dAA(OH)G, dAA(TBDMSi)G was first synthesized (1 µmol synthesis). It would then be desilylated to yield the compound of interest. The synthesis of dAA(TBDMSi)G was effected using the 58 step cycle on the DNA Synthesizer. The 2' silylated phosphoramidite was purchased from Chemgenes. A crude yield of 33 ODU was obtained. Ten ODU of dAA(TBDPSi)G was placed in an eppendorf and desilylated by adding 25 µl of TREAT•3HF. After 8h, 1 ml of water was used to quench the reaction. Purification yielded 4 ODU (40% yield).

2.2.8 Characterization

All compounds were characterized by MALDI-TOF MS and analytical PAGE.

MALDI-TOF-MS (Matrix-assisted laser desorption-ionization - time-of-flight - mass spectrometry)[52,53] is a recently developed soft ionization technique which has considerably expanded the application of mass spectrometry to the analysis of high-molecular weight biopolymers such as nucleic acids. MALDI generates intact, gas-phase ions of high molecular weight analytes by isolating analyte molecules in an appropriate crystalline matrix and irradiating the sample with a laser tuned to an absorption band of the matrix. Conventional time-of-flight mass analysis of these ions allows determination of the molecular mass. MALDI is useful in terms of characterization of modified oligonucleotides. Commonly, modified oligonucleotides behave similar to normal nucleic acids in MALDI and matrices that are used for normal oligonucleotides are just as effective with the modified nucleic acids.

The molecular weight of the following sequences were confirmed using MALDI: dAA(G)TBDPS, dAA(G)P, dAA(OH)P, dAA(G)G, dAA(OH)G. These are reported in Table 2.3 and are in agreement with the calculated values. The spectra are shown on the pages that follow.

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[52] Michael C. Fitzgerald, Lloyd M. Smith. *Annual Review Biophysical Biomolecular Structure*, **24**,117-40, (1995).

Compound	Calculated (Expected) MW ($gmol^{-1}$)	Observed* MW ($gmol^{-1}$) ± 0.1%	Interpretation
dAA(G)TBDPS	1162.1	1163.5, 1167	M, M + Li^+
dAA(G)P	1001.6	1000, 1028.9, 1049.1	M, M + Na^+, M + K^+
dAA(OH)P	657.4	659.9	M
dAA(G)G	1268	1274.3	M + Li^+
dAA(OH)G	923.7	923.4, 931.9	M + Li^+

Samples contained 0.2 mmol of oligonucleotide in a matrix of dithranol (10mg/ml), THF (10ml), LiBr/THF (5mg/µl).

* Both major and minor signals are indicated

Table 2.3 Calculated and Experimentally Determined Molecular Weights of the Oligonucleotides

[53] M. Karas, F. Hillenkamp., *Anal. Chem.*, **63**, 1193A-1203A, (1991).

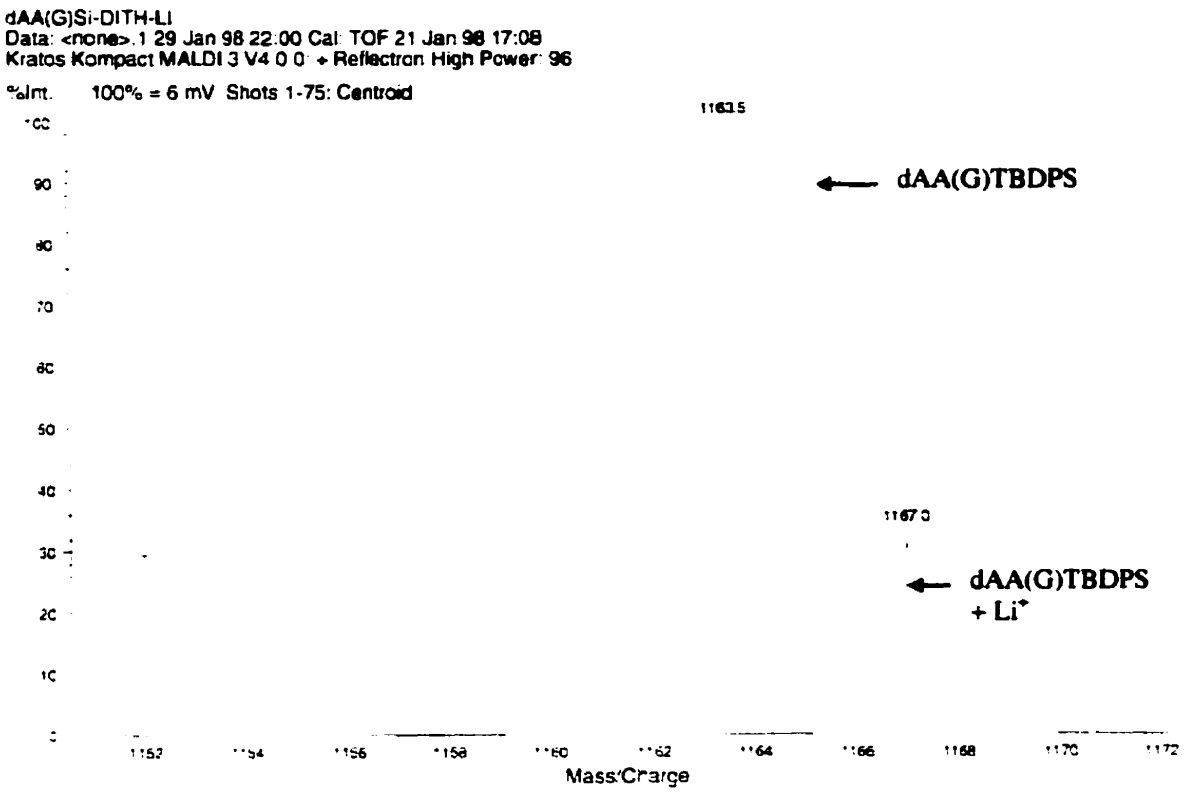

Figure 2.11 MALDI-TOF Spectrum of dAA(G)TBDPS
Matrix: 10 mg/ml Dithranol, THF (10 ml), LiBr/THF (5 mg/µl).

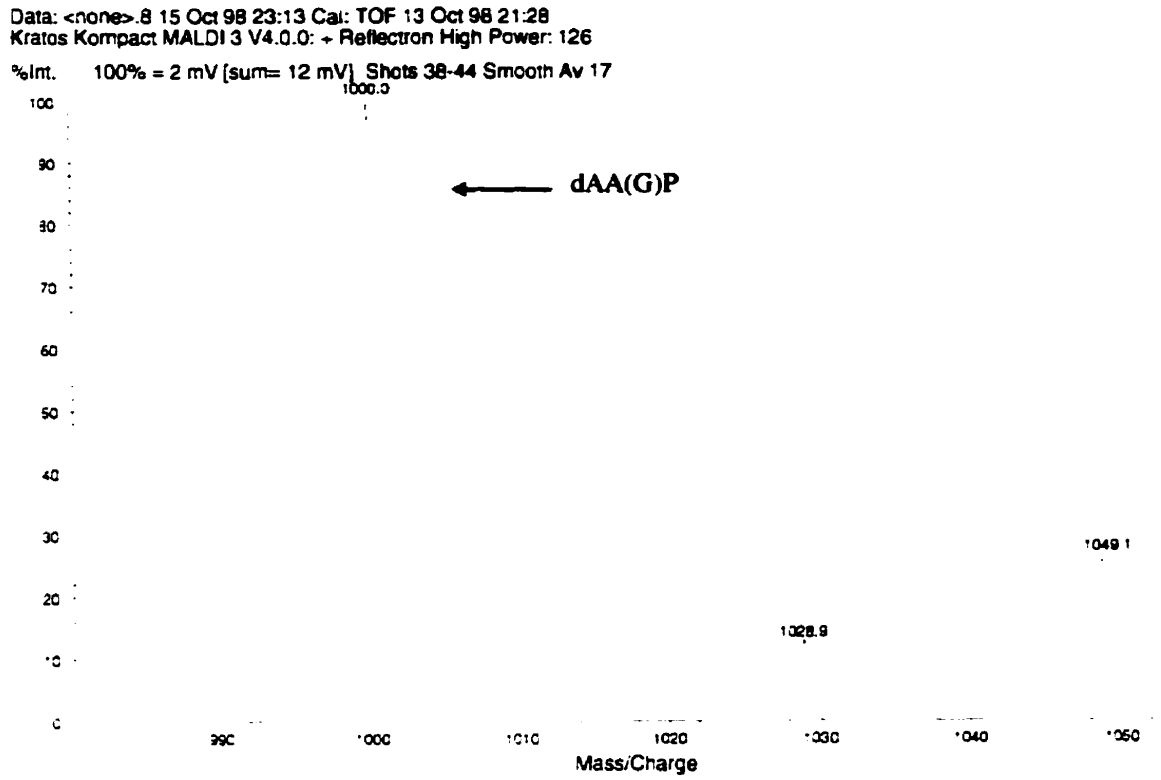

Figure 2.12 MALDI-TOF Spectrum of dAA(G)P
Matrix: 10 mg/ml Dithranol, THF (10 ml), LiBr/THF (5 mg/µl).

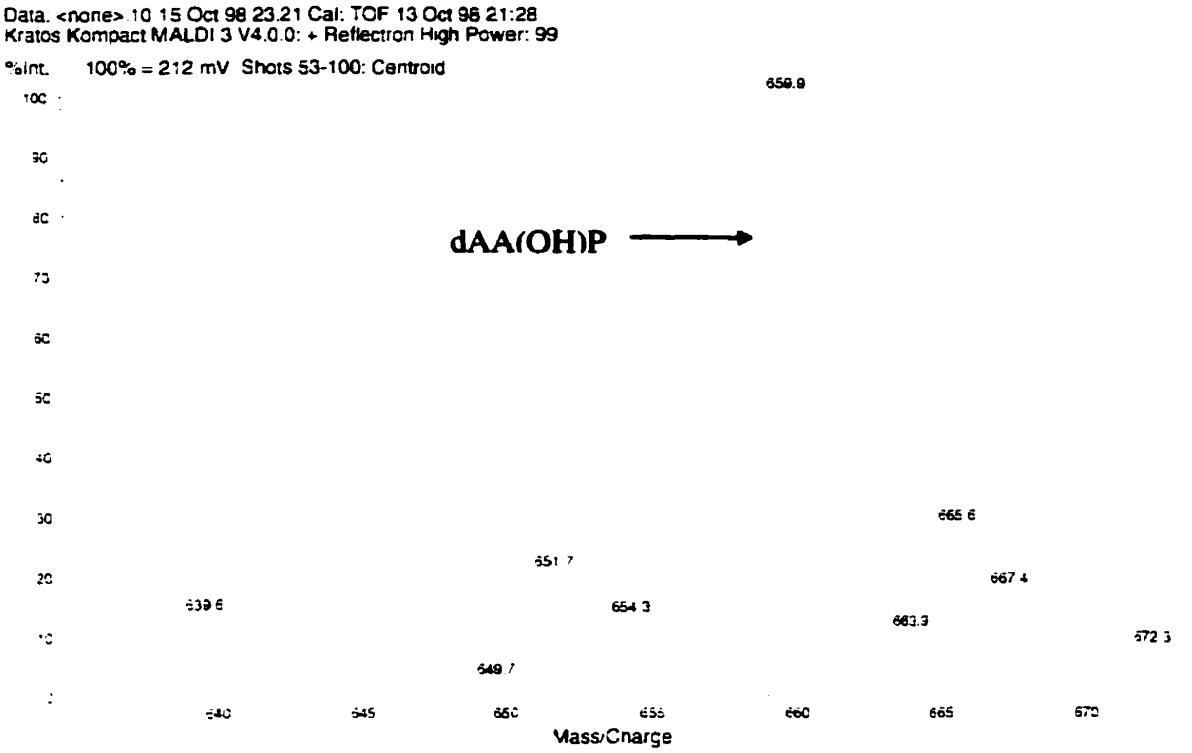

Figure 2.13 MALDI-TOF Spectrum of dAA(OH)P
Matrix: 10 mg/ml Dithranol, THF (10 ml), LiBr/THF (5 mg/µl).

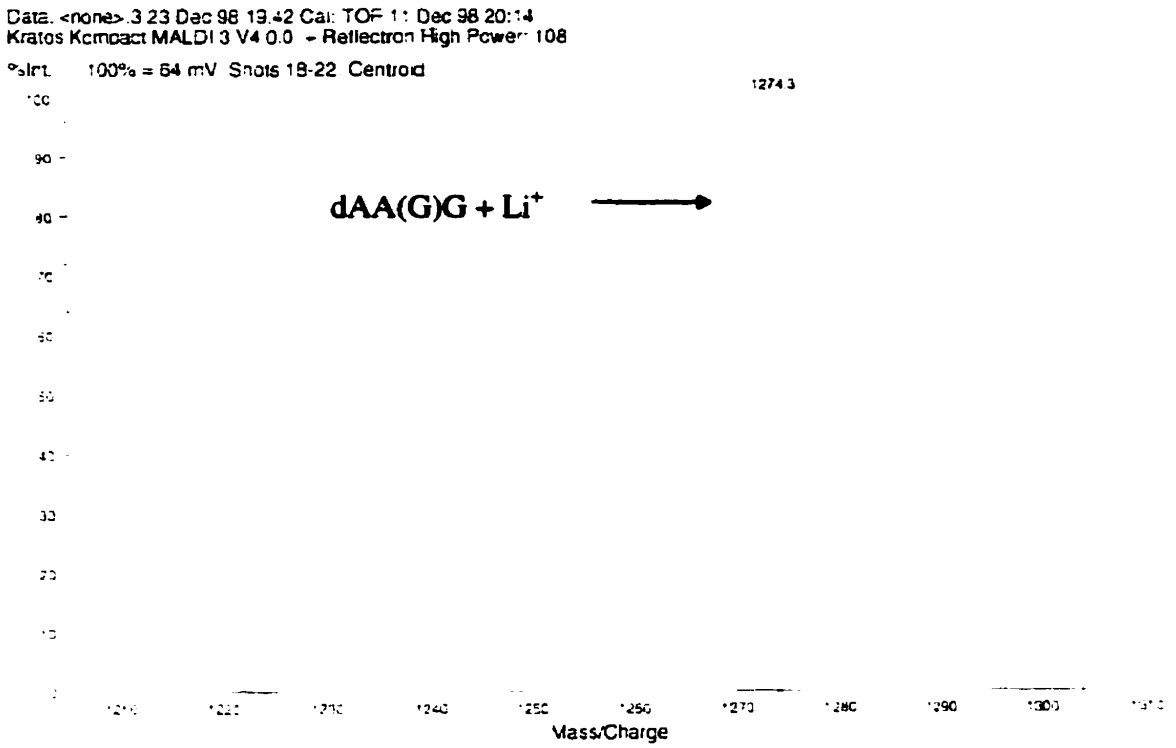

Figure 2.14 MALDI-TOF Spectrum of dAA(G)G

Matrix: 10 mg/ml Dithranol, THF (10 ml), LiBr/THF (5 mg/µl).

Note: Although the molecular weight of dAA(G)G is 1268 g/mol, the spectrum shows a peak at 1274 g/mol, this is due to the binding of a Lithium ion from the matrix.

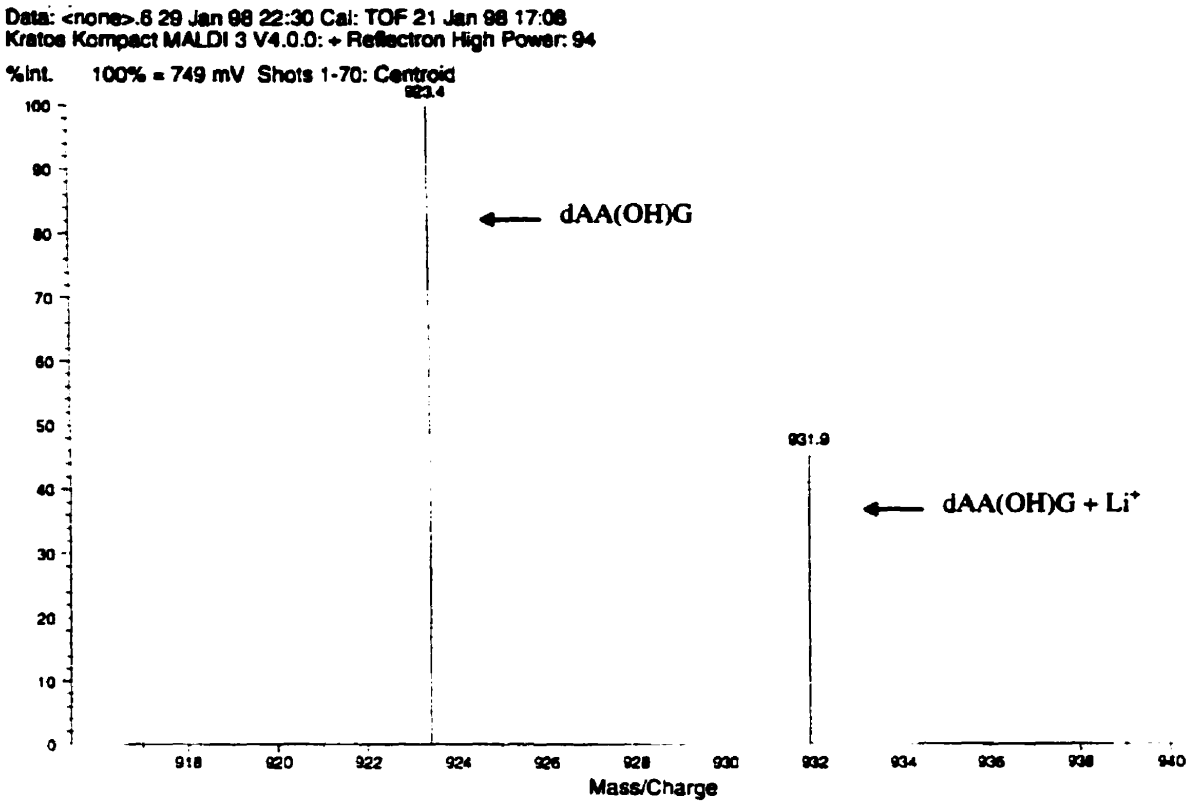

Figure 2.15 MALDI-TOF Spectrum of dAA(OH)G

Matrix: 10 mg/ml Dithranol, THF (10 ml), LiBr/THF (5 mg/μl).

Note: The molecular weight of dAA(OH)G is 923.7 g/mol, another peak in the spectrum which is evident is that at 931.9 g/mol - this is due to the binding of a Lithium ion from the matrix.

Analytical PAGE (24% acrylamide) in conjunction with MS (MALDI-TOF) were extremely useful in the characterization of the synthesized oligonucleotides. The PAGE gels shown in Figures 2.16 and 2.17 reveal the migration of the various synthesized compounds. Figure 2.16 reveals important information about the crude compounds dAA(G)G and dAA(G)P. In lane D, it is evident, in addition to the desired tetranucleotide dAA(G)G (slow moving band), a side product had formed. These are likely to be a mixture of regioisomeric dAA(P)G and dAA(G)P which are inevitably produced during the Damha branching reaction outlined in Figure 2.10. Consistent with this notion is the fact that these side products have identical mobility to an independently prepared sample of dAA(G)P (lanes B&C). In lanes B and C, the slow moving band is the starting material dAA(G)Si (as confirmed by comparison to lane A), which resulted from incomplete desilylation in the synthesis of dAA(G)P. The desired compound dAA(G)P is the fast moving band migrating on lanes B and C, as confirmed by MALDI-MS. Its faster mobility relative to dAA(G)OH (not shown) and dAA(G)Si is consistent with the fact that dAA(G)P has 2 additional negative charges at the 3'-monophosphate group. The identity of dAA(G)P is further established by subjecting a small amount of this compound to enzymatic hydrolysis with alkaline phosphatase[54] (AP). AP normally catalyzes the removal of 5'- or 3'-monophosphate groups from DNA and RNA. Indeed, in the case of dAA(G)P, we observed the formation of dAA(G)OH upon treatment with AP (J.D. Boeke; data not shown). The same enzymatic assay established the identity of dAA(OH)P, which yielded the expected dimer dAA(OH)OH upon treatment with AP (Figure 2.17).

[54] J. Sambrook, E.F. Fritsch, T. Maniatis. "*Molecular Cloning; A Laboratory Manual*", Cold Spring Harbor Laboratory, 2nd ed, (1989).

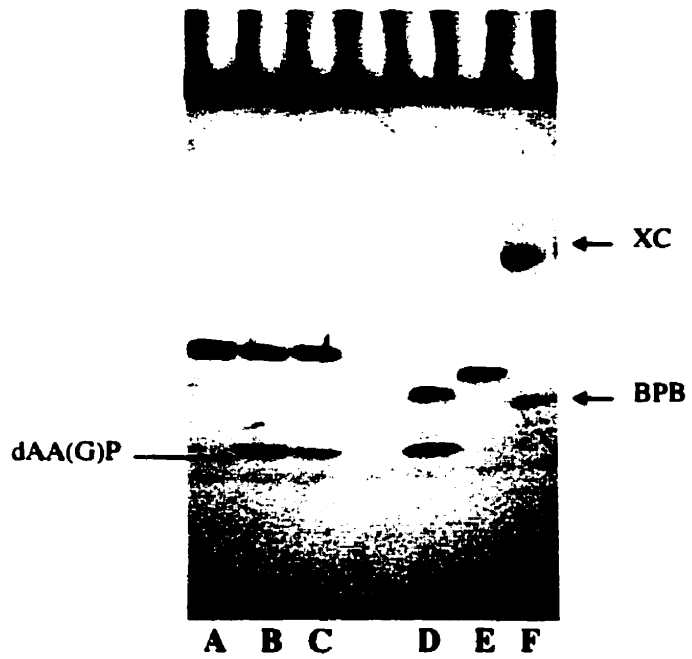

Figure 2.16 Analytical Polyacrylamide Gel Electrophoresis (24% Acrylamide):

Lane	Compound
A	Pure dAA(G)Si.
B, C	Crude dAA(G)P. This analysis reveals that the desired trimer dAA(G)P (fast moving band) is mixed with the starting material [dAA(G)Si].
D	Crude dAA(G)G. The tetramer is the slow moving band. The fast moving band was assigned to a mixture of isomeric dAA(G)P and dAA(P)G, which are characteristic side products of the Damha branching reaction (See Figure 2.9).
E	Pure A(G)G sample provided by Dr. Damha.
F	dyes; xylene cyanol (XC) and bromophenol blue (BPB)

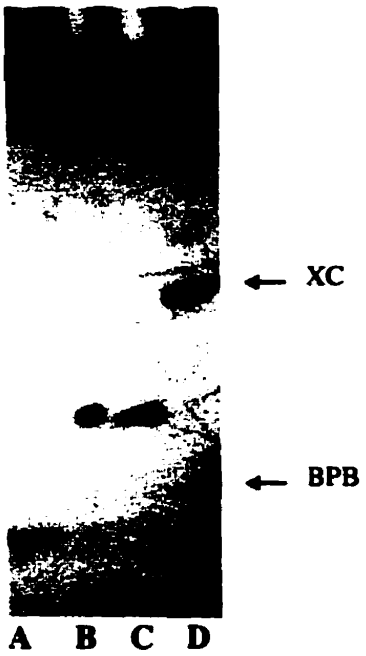

Figure 2.17 Analytical Polyacrylamide Gel Electrophoresis (24% Acrylamide):

Lane	Compound
A	Pure dAA(OH)P
B	dAA(OH)P + AP yields a slow moving compound with the same mobility as that of dAA(OH)(OH).
C	Authentic sample of dAA(OH)OH
D	dyes; xylene cyanol (XC) and bromophenol (BPB)

Results and Conclusion of yDBR Enzyme Studies

3.1 Introduction

The yDBR enzyme plays an important role - it carries out a rate-limiting step in the *in vivo* degradation of intron lariats (*i.e.* the cleavage of the 2'-5'-phosphodiester bond). The cleavage reaction yields a linearized lariat, allowing further degradation in the RNA turnover process to occur. The yDBR enzyme is significantly different as compared to other phosphodiesterases - it does not cleave 3'-5'-phosphodiester bonds nor does it cleave linear oligonucleotides containing 2'-5'-linkages[23].

Several examples of introns that are markedly stable (*in vivo*) have been reported. An interesting example, is a spliced human intron (IVS1) which is derived from the gene that encodes the β chain of the T cell receptor[55] (an essential element in the immune response). This revelation may imply that this important gene has a unique regulatory mechanism. Another example, is a transcript that is produced by the Herpes simplex virus (type 1). This latency-associated transcript (LAT) RNA regulates viral latency[56]; it has been found to accumulate in infected neurons and infected tissue culture cells. It has been postulated that these introns are stable because they adopt a structure that is resistant to debranching and/or they are associated with proteins that inhibit the debranching reaction. Therefore, studying the yeast debranching enzyme may ultimately lead to therapeutic applications

The gene (DBR1) that encodes the debranching enzyme in *S. Cerevisiae* was isolated and characterized in 1991, and later (1994) purified to 90% homogeneity[24]. Due to the availability of this purified enzyme, it could be studied further. However, the variety of natural yDBR enzyme substrates is limited - forked RNA or msDNA are not highly varied in their sequence and are difficult to produce. Fortunately, methods to produce branched RNA have been established by our research group thus, studying the substrate specificity of the enzyme is now possible.

---

[55] (a) L. Qian, M.N. Vu, M.S. Carter, M.F. Wilkinson, *Nucl. Acids Research*, **20**, 5345, (1992). (b) L. Qian, M.N. Vu, M.S. Carter, J. Doskow, M.F. Wilkinson, *J. Immunol.*, **151**, 6801, (1993).
[56] M.J. Farrell, A.T. Dobson, L.T. Feldman, Proc. *Natl. Acad. Sci.*, **88**, 790, (1991).

The natural substrates of the yDBR enzyme have $A^{2'5'}G$ linkage (where A is the branchpoint of the intron lariat). As described in Section 1.5, several branched oligonucleotides have been synthesized in the Damha research group, in order to learn more about the yDBR enzyme. Studies with synthesized Y-shaped 18-mers revealed that a purine was preferred over a pyrimidine at the 2'-position. A pyrimidine at the branch point as opposed to the branch point purine A residue also caused a decrease in the rate of debranching, yet the decrease was not as significant as a pyrimidine in place of a purine at the 2'-position. In the past, tetramers[28] have also been synthesized - these compounds are good substrates for T4 polynucleotide kinase thus, radiolabelling at the 5'-end can easily be effected. The two sets of tetramers that were studied are the following: 5'-$AA^{N}{}_{N}$-3' and $NA^{G}{}_{G}$, where N = G, A, C, U. Again, this study indicated that the enzyme had a strong preference for a purine over a pyrimidine at the 2' position (2'G being cleaved even faster than 2'A). In addition, tetramers with a 5' purine were found to be better substrates (however, this was not as significant as a purine at the 2' position). Therefore, the best substrates for the yDBR enzyme were found to be $AA^{G}{}_{G}$ and $GA^{G}{}_{G}$.

In this work, a branched nucleic acid analogs with a 3'-TBDPSi group ($dAA^{G}{}_{TBDPS}$) in place of a nucleoside was synthesized. This particular analog was chosen as it is a bulky group and steric hindrance at the 3'-position was proposed as a potential reason for the specific 2'-5' hydrolysis by the yDBR enzyme. It was necessary to use a dA residue at the 5'-position rather than a rA residue because the 3'-OH group of rA would have required silyl protection. Following synthesis, removal of the silyl group on the 5' residue would have also caused removal of the silyl group on the branch point A residue. The three-dimensional energy minimized structure of the oligonucleotide analog, $dAA^{G}{}_{TBDMS}$ revealed that the three-dimensional structure is quite similar to that of $AA^{G}{}_{C}$, a substrate of the yDBR enzyme (see Figure 3.1). Furthermore, monitoring of intramolecular base stacking interactions via NMR analysis and Nuclear Overhauser effects has revealed that the adenine ring of the branched oligoribonucleotides is involved in a base stacking interaction with the guanosine of the 2'-residue[57,58,59,60]. These studies

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[57] M. Lee, S. Huss, G. Gosseline, J.L. Imbach, J. A. Hartly, J. W. Lown, *J. Biomed. Struct. Dyn.*, **5**, 651, (1987).
[58] M. J. Damha, K. K. Ogilvie, *Biochemistry*, **27**, 6403, (1988).
[59] C. Sund, P. Agback, L. H. Koole, A. Sandstrom, J. Chattopadhayaya, *Tetrahedron*, **48**, 695, (1992).

suggest that a conformational distortion point may be present in naturally occuring RNA lariats. This base-stacking interaction was also seen in compound dAA^{G}_{TBDMS}, indicating that it may in fact be a substrate of the yDBR enzyme.

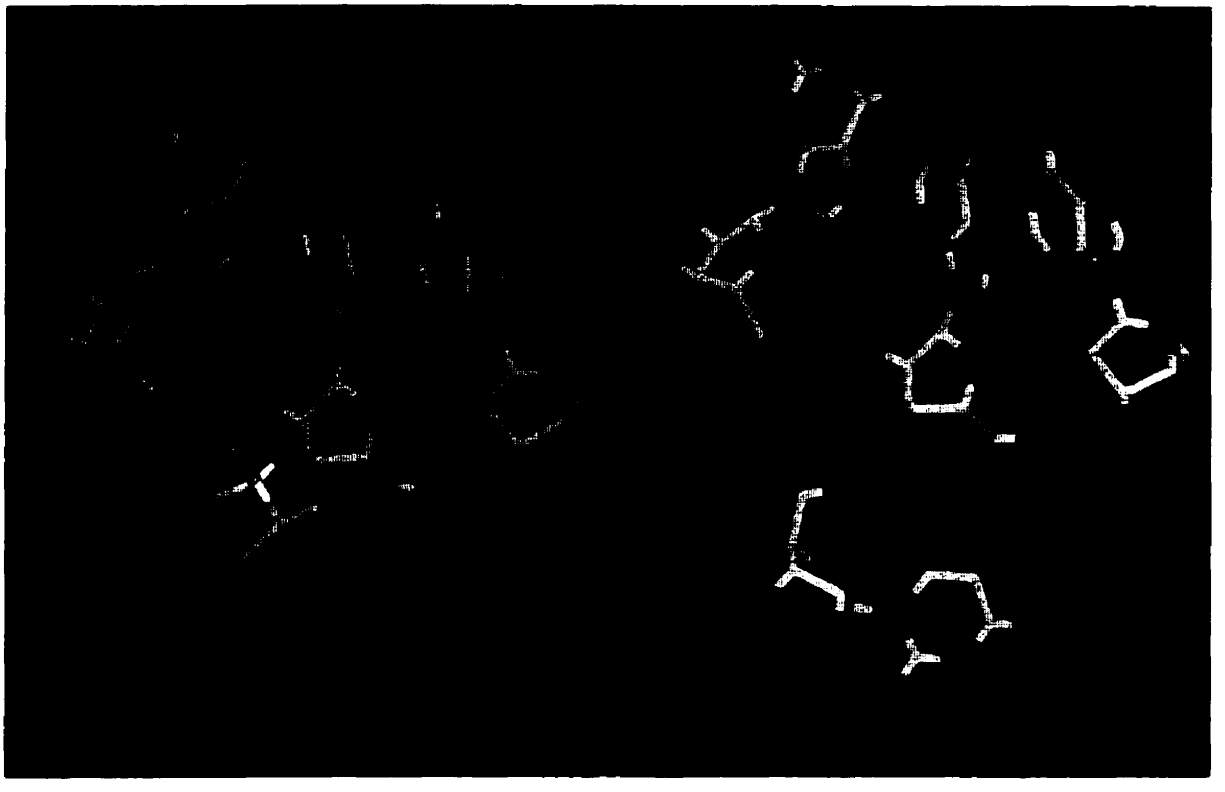

Figure 3.1 Three-dimentional image of dAA(G)TBDMSi (left) and dAA(G)C (right).

[60] R.H.E. Hudson, K. Ganeshan, M.J. Damha, *J. Am. Chem. Soc.*, **115**, 2119, (1993).

3.2 Results

In the previous sections, the synthesis of dAA(G)TBDPSi, dAA(G)P, dAA(G)G, along with their corresponding (yDBR enzyme) cleavage products, [dAA(OH)TBDPSi, dAA(OH)P and dAA(OH)G] was described. The oligonucleotide, dAA(G)TBDPSi, was synthesized so as to determine if steric hindrance is a factor in the hydrolysis of the 2'-5' phosphodiester bond. It will be used in further studies on the debranching enzyme by other members of the Damha laboratory.

If dAA(G)P is in fact a substrate of the debranching enzyme, the results may narrow down the search for the amino acids that lie in the active site of the enzyme.

dAA(G)P was subjected to cleavage studies with the yDBR enzyme. Initially, dAA(G)P was radiolabelled at the 5'-end with T4 polynucleotide kinase using [γ-^{32}P]ATP (see Figure 3.2). The radiolabelled oligonucleotide was then incubated with the yDBR enzyme (provided by Dr. Jeff Boeke, Johns Hopkins University). dAA(OH)P was also radiolabelled as this was the expected cleavage product. As we knew the tetramer dAA(G)G was in fact a good substrate for the yDBR enzyme, it was subjected to the yDBR enzyme so that it could serve as a positive control. The results were analysed via electrophoresis and autoradiography as shown in figure 3.3. From the gel we find that as expected ^{32}P-dAA(G)G was cleaved by the yDBR enzyme (16 hour incubation period) to yield ^{32}P-dAA(OH)G. The experiment also revealed that dAA(G)P was partially cleaved by the yDBR enzyme. As seen in Figure 3.3, there was some debranching after 4 hours (< 5%), and more so after 16 hours (ca. 5%). Clearly, it was not as good a substrate as the tetramer dAA(G)G. These results show that while the 3'-nucleotide is necessary for the efficient hydrolysis of the 2'-5' phosphodiester bond, its presence is not absolutely essential to observe some cleavage. It should also be noted that while dAA(G)P undergoes ~ 5% hydrolysis by the yDBR enzyme, the linear trimer dAA(G)OH (lacking a 3'-phosphate group) remains intact upon exposure to the yDBR enzyme.

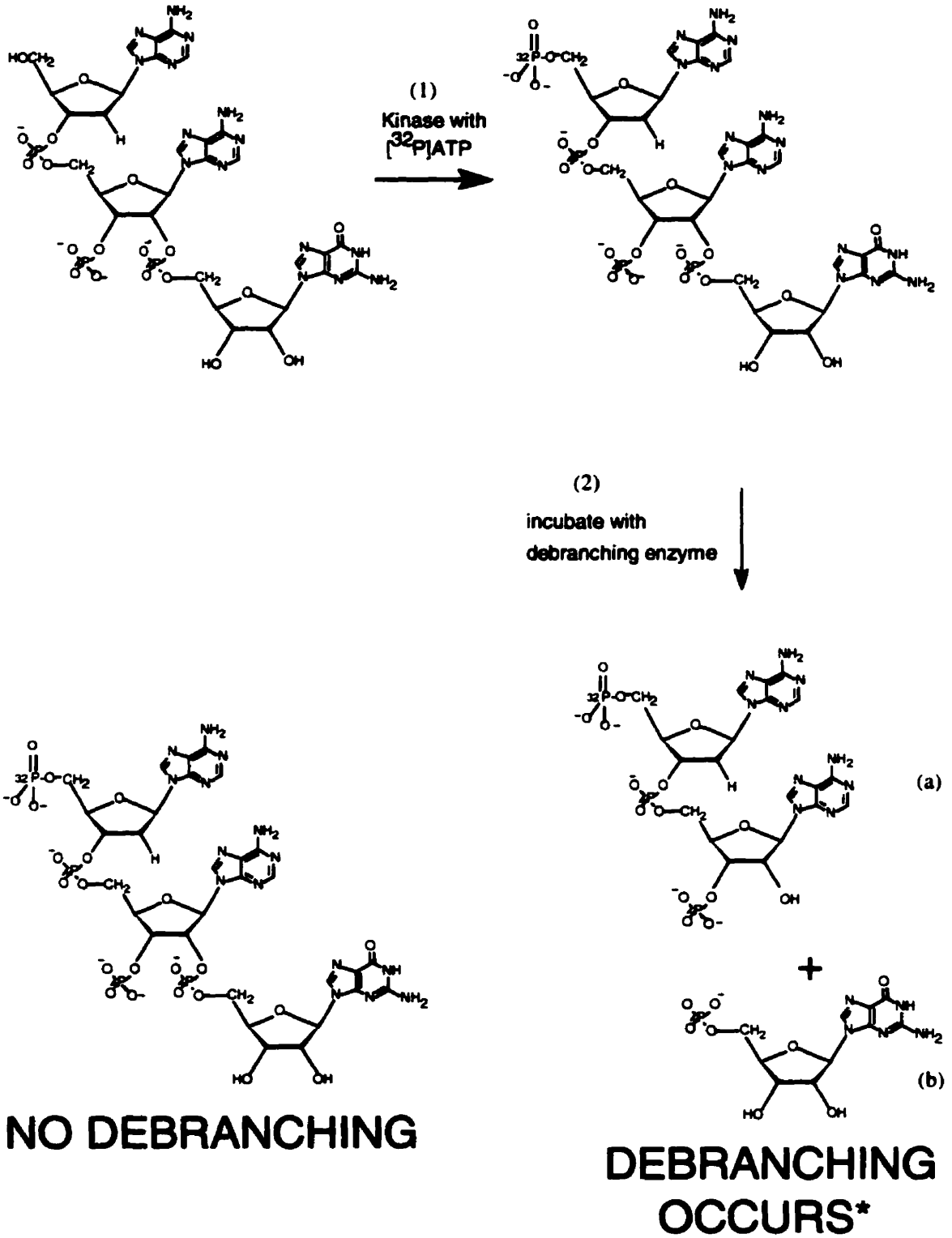

Figure 3.2 yDBR Enzyme Assay

(1) dAA(G)P was radiolabelled at the 5'-end with T4 polynucleotide kinase using [γ-^{32}P]ATP
(2) The radiolabelled oligonucleotide was incubated with the yDBR enzyme (4h, 16h).
* When debranching occurs, the labelled frament (a) is detected by PAGE, the non-labelled fragment (b) is not detected by PAGE.

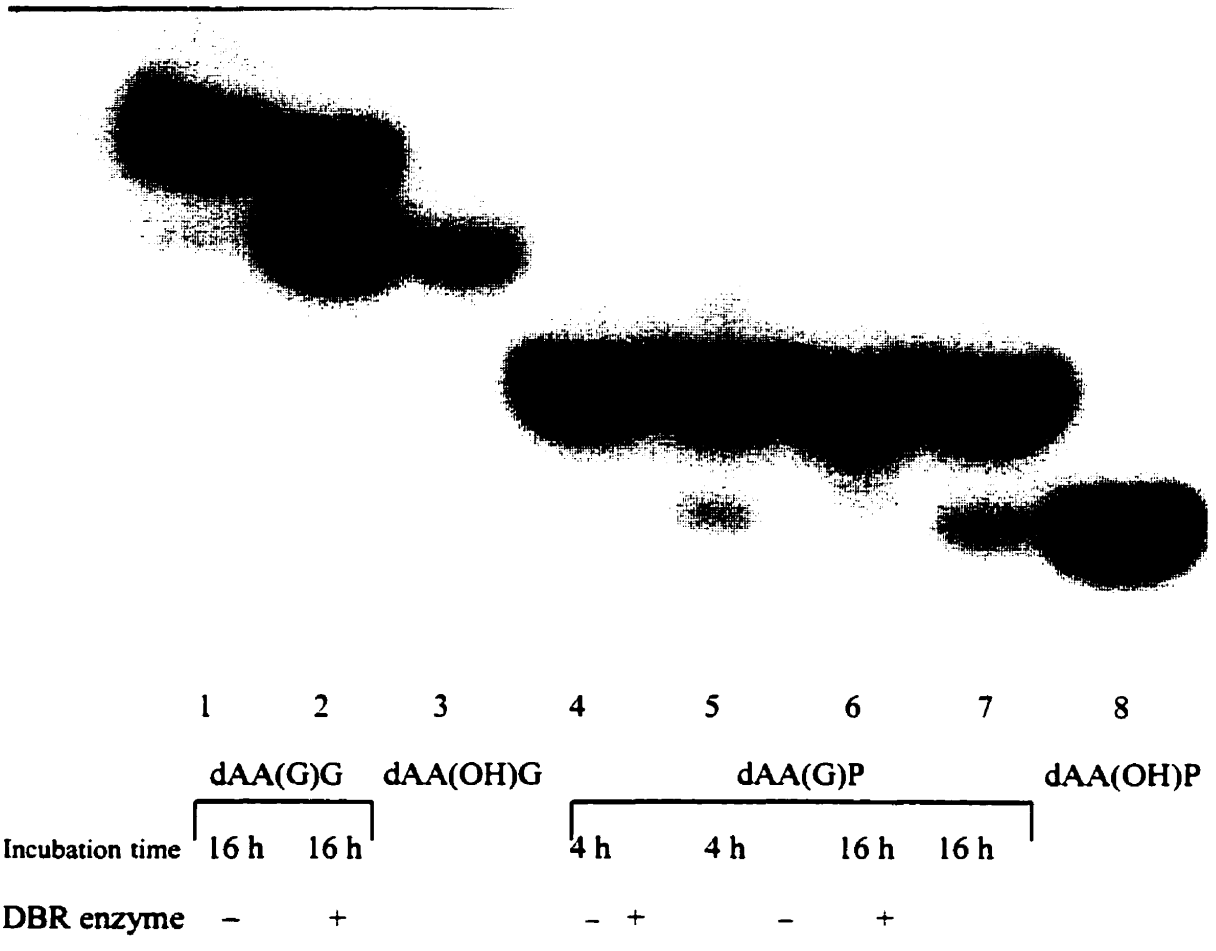

Figure 3.3 Autoradiograph of dAA(G)G and dAA(G)P Subjected to the yDBR Enzyme.

3.3 Contributions To Knowledge and Future Studies

The following compounds were successfully synthesized: dAA(G)TBDPSi, dAA(G)P, dAA(G)G, along with their corresponding linear expected debranched products; dAA(OH)TBDPSi, dAA(OH)P and dAA(OH)G. The compounds were characterized by polyacrylamide gel electrophoresis and MALDI-TOF MS.

dAA(G)P was subjected to the yDBR enzyme. Autoradiograph analysis revealed that this branched nucleic acid analog was in fact cleaved by the yDBR enzyme, although the cleavage was not as efficient as with the tetramer dAA(G)G. The results, however, are important, as it was evident that nucleic acid analogs are useful tools in determining the substrate specificity of the yDBR enzyme.

Future studies with the branched nucleic acid analog dAA(G)TBDPSi will prove useful as well. If dAA(G)TBDPSi is cleaved by the enzyme, it may become evident that steric hindrance at the 3' position rather than the presence of a 3'-nucleotide is an important requirement for specifically cleaving the 2'-5' phosphodiester bond. If it is not cleaved by the enzyme, this does not necessarily mean that dAA(G)TBDPSi does not bind to the enzyme's active site. Thus, competitive inhibition studies will be needed in order to determine if in fact it does bind to the active site of the yDBR enzyme. This type of study can open the door to novel inhibitors of the yDBR enzyme, isolation of cross-linked branched RNA-yDBR enzyme complexes and X-ray crystallography on the aforementioned complex.

Much remains to be learned about the yDBR enzyme. However, with the synthetic capability of producing substrates for the yDBR enzyme, the sequence and substrate specificity and the catalytic domains of the enzyme will soon be elucidated. With the potential therapeutic application of branched oligoribonucleotides, it is a challenge worth pursuing.

Aspects of the studies described in this thesis have been presented:

(1) A. Liscio, R.S. Braich, A. Noronha, R.H.E. Hudson, M.J. Damha, Yeast RNA Lariat Debranching Enzyme: Base Sequence and Substrate Specificity, 81st CSC Conference, Whistler, B.C., 05/1998.

(2) A. Liscio, M.J. Damha, Determining the Substrate Requirements of the Yeast RNA Lariat Debranching Enzyme, Chemistry & Biochemistry Graduate Research Conference, Concordia University, Montreal, Canada, 02/1998.

(3) A. Liscio, M.J. Damha, Probing The Substrate Specificity of the Yeast Debranching Enzyme, 8th Quebec/Ontario Minisymposium in Bioorganic and Organic Chemistry, Quebec City, Canada, 11/1997.

Experimental

4.1 General Methods

4.1.1 Reagents

High boiling point solvents were dried and subjected to fractional distillation under reduced pressure (standard protocols were followed)[61]. Anhydrous pyridine (obtained from Fischer Scientific, Toronto, ON) was refluxed for 16 h, distilled over barium oxide and then redistilled just prior to use.

Anhydrous tetrahydrofuran (Fisher Scientific) was refluxed overnight at room temperature over CaH_2. THF was then decanted, refluxed with sodium and benzophenone under nitrogen until a purple color persisted. Prior to use, THF was distilled and removed from a collection bulb using a syringe through a septum port.

Dimethoxytrityl chloride was purchased from Dalton Chemical Laboratories Inc. (Toronto, ON). *Tert*-Butyldimethylsilyl chloride and benzoyl chloride were purchased from Aldrich Chemical Company (Milwaukee, WI).

Imidazole (Aldrich) was recrystallized from THF and vacuum dried over P_2O_5 prior to use.

N, N', - diisopropylaminocyanoethyl phosphochloridite (Dalton) was stored at 20°C in 15 ml glass Hypo Vials (Pierce) sealed with a Viton serum cap (Chromatographic Specialties, Ontario, Canada).

4-Dimethylaminopyridine (Aldrich) was used as received.

N, N-diisopropylethylamine (Aldrich) was shaken with CaH_2 pellets and distilled before it was used.

Solutions of 1M tetra-*n*-butylammonium fluoride (TBAF) in tetrahydrofuran (Aldrich) were stored over 4 Å molecular sieves.

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[61] A.J. Gordon, R.A. Ford, "*The Chemist's Companion*", John Wiley & Sons, New York, NY, USA, 1972.

4.1.2 Silylation of Nucleosides

As this is a moisture sensitive reaction, solvents and reagents must be anhydrous. It is necessary for reaction vessels (round-bottom flasks), to be oven-dried, flushed with argon and sealed with a stopper. TBDMS-Cl and TBDPS-Cl reagents must be warmed to room temperature (in a desiccator) before use.

4.1.3 Phosphitylation of Nucleosides

Phosphitylation also required anhydrous conditions as detailed above. All glassware was necessarily dried overnight in an oven with minimum exposure to the atmosphere. DMAP was stored for at least 24 h before use (room temperature over P_2O_5 *in vacuo*). The reaction vessel (15 ml Hypo-Vial) sealed by a rubber septum contained the nucleoside - prior to starting it was purged with argon. The reagents were introduced via glass syringes, dropwise and generally at 0°C (ice bath). After addition the reaction was allowed to stir at room temperature until completion.

4.1.4 TLC Analysis

Merck Kieselgel 60F 254 silica gel analytical sheets (0.2 mm × 20 cm × 20 cm) (Merck #5735) were used for the analysis of tritylation, silylation and phosphitylation reactions. A 254 nm UV light source was used to detect various products.

4.2 Monomer Preparation

4.2.1 *Preparation of N^6-Bz-5'-O-MMTr-3'-O-TBDPSi adenosine-2'-O-[N,N-diisopropyl (cyanoethyl)] phosphoramidite (2.5)*

a. Benzoyl Adenosine (2.2)

The ribonucleoside adenosine (8g) was coevaporated with anhydrous pyridine (~40 ml) (3 times). To the dry adenosine, anhydrous pyridine (225 ml) and chloro-trimethyl silane (TMSCl) (24.44 g, 225 mM) was added. After stirring for 2 h at ambient temperature the mixture was cooled to 0°C and benzoyl chloride (BzCl) (12.65 g, 90 mmol) was added dropwise over a 30 min period. The mixture was allowed to warm to 25°C and stirred for an additional 2 h. At this point TLC analysis revealed the reaction was complete. To stop the reaction, 30 ml of H_2O at 0°C was added. It was stirred for a further 5 min. Concentrated ammonia was then added (60 ml). After stirring for 15 min, the ammonia was evaporated. At this point water was added (500 ml) and extracted with 100 ml of dichloromethane. The water was evaporated until crystallization occured. At this point it was filtered overnight. The R_f value was found to be 0.26 (1:9 MeOH: CH_2Cl_2). (84% yield, 9.30 g)

b. 5'-O-(Monomethoxytrityl)-N^6-Benzoyl-Adenosine (2.3)

Compound 2.2 (9.23 g, 24.85 mmol) was coevaporated with absolute pyridine and subsequently suspended in this solvent. MMTCl (9.21 g, 29.82 mmol) was added (all at once) and the resulting mixture was stirred at rt. TLC analysis revealed the reaction was complete after 24 h. The solution was poured into 3% $NaHCO_3/H_2O$ and CO_2 gas evolved. The resulting solution was then extracted with dichloromethane and the crude product purified by flash chromatography. The product (83% yield, 13.2 g) eluted from the column with 3% MeOH in CH_2Cl_2. The R_f value was found to be 0.39 (1:9 MeOH: CH_2Cl_2).

c. N^6- Benzoyl - 5' - O - (Monomethoxytrityl) - 3' - O - *tert*-Butyldiphenyl Silyl Adenosine (2.4a)

Compound 2.3 (6 g, 9.32 mmol) was coevaporated with absolute pyridine and suspended in a mixture of absolute acetonitrile and absolute pyridine. Imidazole (1.59 g, 23.35 mmol) was added and dissolved, followed by *tert*-butyl diphenyl silyl chloride (TBDPSi-Cl) (3.20 g, 11.65 mmol) and the reaction stirred overnight at room temperature. After 16 hours, TLC analysis revealed the reaction was complete and the crude product was evaporated to dryness. The residue was diluted with dichloromethane and washed with 5% $NaHCO_3$ solution. The aqueous layer was re-extracted twice with dichloromethane for maximum recovery. The combined organic layer was dried over $MgSO_4$, filtered and evaporated to afford the crude product which was purified by flash chromatography using a gradient of ethyl acetate in toluene. Compounds 2.4(b) (37% yield, R_f = 0.54, 1:1 toluene/ethyl acetate) and 2.4 (c) (7% yield, R_f = 0.71) eluted at a 4:1 ratio of toluene/ethyl acetate. For elution of compound 2.4(a) (34% yield, R_f = 0.45) a 1:1 ratio of toluene/ethyl acetate was used.

Compound 2.4 (a): ^{1}H-NMR (DMSO-d6, ppm): 11.20 (s, NH); 8.52, 8.51 (2s, H2, H8); 8.1-7.1 (m, aromatic); 6.75 (d, aromatic); 6.14 (d, H1', J= 5.9Hz); 5.74 (d,2'OH); 4.86 (m, H2'); 4.46 (m, H3'); 4.15 (m, H4'); 3.70 (s, CH3O-);3.01 (dd, H5'); 2.98 (dd, H5"); 1.03 (s, t-Bu).

Compound 2.4 (b): ^{1}H-NMR (DMSO-d6, ppm): 11.18 (s, NH); 8.37, 8.33 (2s, H2, H8); 8.1-7.1 (m, aromatic); 6.75 (d, aromatic); 6.14 (d, H1', J= 5.9Hz); 5.48 (d,2'OH); 4.97 (m, H2'); 4.16 (m, H3'); 4.07 (m, H4'); 3.69 (s, CH3O-);3.18 (dd, H5'); 3.07 (dd, H5"); 0.94 (s, t-Bu).

2D-J correlated 1H-NMR spectroscopy (1H-Relayed-COSY at 500 MHz) allowed us to distinguish between the 3'-silylated monomer 2.4(a) and its 2'-silylated regioisomer 2.4(b). The spectra of these compounds are shown in Figure 5.1 and 5.2, respectively (see Appendix). The chemical shifts are given in ppm. The spectrum of the 3'-silyl isomer (Figure 5.1) reveals proton H2' (4.8 ppm) is coupled to H1'(6.2 ppm), H3' (4.4 ppm) and a hydroxyl proton (i.e., 2'OH; 5.80 ppm). As expected, proton H4' (4.1 ppm) is coupled to H5' (3.0 ppm) and H5" (2.8 ppm). The spectrum of the 2'-silyl isomer

(Figure 5.2) reveals, as expected, coupling between H2' (5.0 ppm) and H1' (6.2 ppm), as well as H3' (4.1ppm). Furthermore, we observed coupling between H3' (4.1 ppm) and the 3'OH proton (5.5 ppm).

d. N^6-Bz-5'-O-MMTr-3'-O-TBDPSiadenosine-2'-O-[N,N-diisopropyl(cyanoethyl)] phosphoramidite (2.5)

Compound 2.4(a) (1.057 g, 1.198 mmol) was dissolved in THF, DMAP[36] (0.12 eq) was added followed by DIPEA (0.37 g, 2.88 mmol) added dropwise over a 20 second period, and finally N, N - diisopropyl-amino cyanoethylphosphochloridite (0.34 g, 1.43 mmol,). A white precipitate (diisopropylethylammonium hydrochloride) indicated that the desired reaction had occurred. After 3 hours, TLC analysis revealed that the reaction had gone to completion and no isomerization was apparent. The crude product was then dissolved in dichloromethane and extracted with saturated NaCl/5% $NaHCO_3$ (4:1) and the aqueous phase re-extracted with CH_2Cl_2. The combined organic layer was dried over $MgSO_4$, filtered and evaporated. Flash chromatography ensued to purify the crude phosphoramidite. Rapid elution with CH_2Cl_2:Hexanes:NEt_3 (50:45:5) using a 3 cm diameter column, afforded the pure product (69% yield, 0.9 g). The R_f values for the two diastereomers are 0.81, 0.72 (1:1 ether:CH_2Cl_2).

4.3 Oligonucleotide Synthesis

4.3.1 CPG Derivatization

Long-chain alkylamine controlled-pore glass (LCAA-CPG, porosity: 500 Å, density: 0.4 g/ml) was obtained from CPG Inc.(Fairfield, NJ). Nucleosides that were not made were purchased from ChemGenes (Waltham, MA). Derivatization[62] of LCAA-CPG proceeded as follows: LCAA-CPG (5g) was stirred in a solution of 3% trichloroacetic acid in dichloroethane at rt for 16 h. The activated CPG was filtered and washed with 9:1 triethylamine:diisopropylethylamine (50 ml), followed by dichloroethane and ether. The

[36] S. K. Chaudhary, O. Hernandez, Tetrahedron Letters, 2, 99-102, (1979).

support was dried under vacuum over P_2O_5 before use. In order to succinylate the CPG, 1 g of activated CPG, succinic anhydride (2 mmol), DMAP (0.33 mmol) were placed in a 6 ml glass Hypo-vial and finally, anhydrous pyridine (6 ml) was added. The vial was shaken for 16 h at rt. The support was then filtered and washed successively with pyridine, methylene chloride and ether. In order to derivatize the support with a particular nucleoside, 0.5 g of succinylated CPG and nucleoside (0.10 mmol) were placed in a 6 ml Hypo-vial together with DEC coupling reagent (1 mmol), triethylamine (40µl), DMAP (50 µmol) and anhydrous pyridine (6 ml) and the mixture was shaken at room temperature overnight. The CPG was then filtered, washed successively with pyridine, dichloromethane and ether and dried. The trityl cation assay was used to determine nucleoside loading. The derivatized support was capped on an Applied Biosystems 381A synthesizer using the standard capping cycle. The capping reagents used were acetic anhydride/2,4,6 collidine/THF (1:1:8 v/v/v) and 16% N-methylimidazole/THF.

4.3.2 Solid Phase Synthesis

Oligonucleotide synthesis was carried out on an Applied Biosystems 381A synthesizer. An ISCO Retriever II fraction collector was attached to the synthesizer in order to collect the released trityl cation. The concentration of phosphoramidites was 0.1M and 0.15 M for DNA and RNA, respectively made up in appropriate amounts of anhydrous acetonitrile. 1 µmol scale synthesis was carried out for each oligonucleotide synthesized. The columns were packed by transferring an appropriate amount of nucleoside-derivatized support into a Teflon™ body (Applied Biosystems). The Teflon™ end fittings with filter were crimped into place using aluminum Hypo-Vial seals (Pierce).

Anhydrous acetonitrile was dried over phosphorus pentoxide (5 g/ L of CH_3CN), refluxed and then continuously refluxed over CaH_2 under N_2. It was distilled prior to use for synthesis and amidite preparation. Detritylation was effected using 3% trichloroacetic acid/1,2-dichloroethane (or dichloromethane). Activation ensued using 0.5 M tetrazole in

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[62] M. J. Damha, K. Ganeshan, H.H.E. Hudson, S.V. Zabarylo, *Nucleic Acids Research*, **20**, No. 24, 6565-6573, 1992.

acetonitrile. Capping follows with acetic anhydride/2,4,6-collidine/THF (1:1:8 v/v/v) and 16% N-methylimidazole/THF. [Note: N-methylimidazole was dried by fractional distillation from calcium hydride and stored over activated (400°C) molecular sieves (Type 4A)]. For the oxidation step, 0.1 M iodine in THF:pyridine:water (75:20:2, v/v/v) was used. The reagents used for oligonucleotide synthesis can also be purchased from Applied Biosystems.

4.3.3 Monitoring Synthesis Performance

A preliminary monitor of synthesis performance is obtained via trityl cation assay using trichloroacetic acid (TCA). Each TCA eluate is collected in a test tube and is diluted to 10 ml with 5% TCA/1,2-dichloroethane. The absorbance is measured at 504 nm for the DMT cation (ε = 76 ml cm^{-1} μmol^{-1}) or 478 nm for MMT cation (ε= 56 ml cm^{-1} μmol^{-1}). Comparison of each synthesis cycle to the previous one reveals the average % yield per cycle. Sometimes, better yields are obtained than would be expected from trityl assays. This occurs, particularly on humid days, as part of the trityl cation solution that is released gets hydrolyzed.

4.3.4 Ultraviolet Spectra

In order to measure coupling efficiency and to quantify oligonucleotides, Ultraviolet spectra were recorded using a Varian CARY 1 UV-Vis spectrophotometer. A personal computer and CARY supplied software (version 1.3e) was used in order to obtain the absorbance maxima for the oligonucleotides. (260 nm for oligonucleotides, 504 nm for DMT cation and 478 nm for MMT cation).

4.4 Deprotection

4.4.1 Cleavage and N-Deacylation

Removal of the benzoyl, isobutyryl and β-cyanoethyl protecting groups was accomplished using 29% NH_4OH:ethanol (3:1, v/v, rt, 48 h). This treatment also cleaves the synthesized oligonucleotides from the solid support.

4.4.2 Desilylation

To remove the TBDMS protecting group, the oligonucleotide is treated with a 1.0 M solution of tetrabutylammonium fluoride (TBAF/THF) at room temperature for 16 hours. In general, 50 equivalents of TBAF are used per TBDMS group. The reaction is quenched with ~ 5 ml of sterile water.

Another method of desilylation is to use triethylamine trihyrofluoride (TREAT•3HF). This reagent (25 µl) is added to 10-30 ODU of oligonucleotide (8 hours at room temperature). 1 ml of water is then added to quench the reaction. The TREAT• 3HF is conveniently removed by lyophilizing the sample under vacuum.

4.5 Purification

4.5.1 Autoclave

Oligoribonucleotides are extremely sensitive to ribonucleases found on hands, in sweat, in breath and in the air. Thus precautions must be taken while handling deprotected ribonucleotides by using sterile equipment and reagents where possible. Plasticware, glassware and water must be autoclaved with diethylpyrocarbonate (DEP)[63]. Double-distilled water was sterilized by adding DEP as a 1% solution and subsequently

[63] L. Ehrenberg, F. Hedorcsak, *Prog. Nucl. Acids Res.*, **16**, 189-262, 1976.

autoclaving at 120°C for 20 minutes. This water can be safely used for a period of two weeks. Small glassware and plasticware was sterilized by autoclaving for 20 min. at 120°C. Large glassware was washed with double-distilled autoclaved water and dried in an oven at 110°C. It is also essential to wear gloves during the handling of oligonucleotides.

4.5.2 Polyacrylamide Gel Electrophoresis (PAGE)

In general, 24% polyacrylamide gels were used for both analytical and preparative purification. The 24% gel matrix consisted of 50% acrylamide/2.5% bis-acrylamide in double-distilled water (24 ml), 10× TBE (0.9 M Tris-base borate, 93 mM EDTA, pH 8.3, 5 ml) and double-distilled water (21 ml); Prior to polymerization the solution [30 ml (analytical), 50 ml (preparative)] was degassed by attaching the 125 ml vacuum filtration flask to a house vacuum for 10 min. N,N,N',N'-tetramethylethylenediamine (TEMED) [20 µl (analytical), 30 µl (preparative)] and 10% ammonium persulfate [200 µl (analytical), 250µl (preparative)] were then added followed by thorough but brief mixing and the mixture poured between glass plates separated by appropriate spacers (0.75 mm and 1.5 mm for analytical and preparative gels, respectively). The wells were formed by insertion of the appropriate combs into the freshly poured gel matrix between the plates. After 1 h, the gel polymerized and the samples were loaded. 0.2 A_{260} U of oligonucleotides were added per lane (for analytical gels) and 10 to 20 A_{260} U were added (for preparative gels). The oligonucleotides were dissolved in 10 µl of 80% formamide loading buffer (deionized formamide in 10× TBE buffer) [Note: formamide is deionized by stirring with mixed bed AG 501-X8 resin (0.3 g/10 ml) for 2 h]. The gels were run at constant current (10 mA for the first 15 min, 20 mA to completion ~ 4h) using 0.09 M TBE buffer (pH 8.3). Markers (bromophenol blue and xylene cyanol) were run in the first and last lane (0.5% dye in 80% formamide loading buffer).

4.5.3 Visualization of Bands in Gel Electrophoresis

Subsequent to electrophoresis, the gels were wrapped in plastic Saran™ wrap, placed over a fluorescent TLC plate and visualized with a UV lamp (254 nm). In order to obtain a permanent record of the gels, they were photographed using Polaroid PolaPan™ 4 × 5 cm Instant Sheet Film (#52, medium contrast, ISO 400/21°C; f4.5, 16 second) through a Kodak Wratten green gelatin filter (#58).

4.5.4 Desalting

Desalting of oligonucleotides via reversed-phase chromatography was effected using C_{18} SEP-PAK® cartidges (Waters Associates). The procedure used is the following: 5 ml of MeOH (HPLC grade) was pushed through the SEP-PAK cartridge (using a syringe) followed by 5 ml of 5% MeOH/H_2O followed by 10 ml of H_2O. The oligonucleotide was dissolved in 1 ml H_2O and loaded on the cartridge and the eluant was collected. Water (5 ml) was then pushed through the cartridge twice (eluant collected). The pure oligonucleotide was extracted by pushing through 1 ml of 50% MeOH/H_2O (eluant collected) and finally, 1 ml of MeOH was pushed through and collected. The eluant from the last steps usually contain the desalted oligomers and these were lyophilized and quantitated (A_{260}).

4.6 Characterization

4.6.1 ^{31}P NMR

Phosphoramidite 2.5 was analyzed in Acetone-d6 using a Varian XL-300 NMR spectrophotometer equipped with a 5 mm broadband probe, tuned to 121.5 MHz. The spectrum was obtained at 25°C. Chemical shifts are relative to H_3PO_4 (external reference).

4.6.2 Enzymatic Digestion

Alkaline phosphatase (AP), from calf-intestine was obtained from Boehringer Mannheim as a suspension in 3.2 M $(NH_4)_2SO_4$/0.1 mM $ZnCl_2$, pH 7.0. The oligonucleotides dAA(G)P and dAA(OH)P (0.3 A_{260}) were digested at 37°C for 2 h with 1 µl of AP and 17 µl incubation buffer (0.1 M Tris-HCl/1 mM ZnCl2 (pH 7.2). Note: The incubation buffer was made up with autoclaved double-distilled water that was filtered through a 0.45 micron filter (buffer stored at -20°C prior to use).

4.6.3 MALDI-TOF Mass Spectrometry

Matrix-assisted laser desorption/ionization time of flight mass spectra (MALDI-TOF) were recorded on a Kratos Kompact-III TOF instrument with a minimum laser output of 6 mW at a wavelength of 337 nm light, 3 ns pulse width, 100 mm diameter spot. The MALDI instrument was operated in a positive mode. The matrix consisted of a mixture of Dithranol (1,8,9-Anthracenetriol; 10 mg/ml) and LiBr (5 mg/µl) in THF (10 ml).

4.7 Studies with the yDBR enzyme

4.7.1 ^{32}P Labelling

dAA(G)G (25 pmol) and dAA(OH)G (25 pmol) were end labeled with [^{32}P]ATP (0.5 µl) in 10 µl total volume using polynucleotide T4 kinase (New England Biolab) under reaction conditions specified by the manufacturer. dAA(G)P and dAA(OH)P were labeled with [^{32}P] ATP using 3'-phosphatase free polynucleotide T4 kinase (Roche Molecular Biochemicals). End labeling reactions were done at 37°C for 1 h. Equal volume (10µl) of 95% formamide loading dye was then added to the reacion. The reaction products were then resolved on 24% acrylamide gel. The gels were autoradiographed, the bands were excised and 300 µl of buffer (30 mM Tris.Cl pH 7.5, 300 mM NaCl, 3mM EDTA) was added for overnight elution at 4°C. The next day, 750 µl of 100% EtOH was added to the precipitate. Samples were incubated at 4°C for 15

min, followed by a 15 min centrifugation at 4°C. Samples were washed once in 70% EtOH, air dried, and resuspended in 10 µl of water.

4.7.2 Debranching Reactions

The yDBR enzyme was generously provided by Dr. Jeff Boeke (Johns Hopkins University). 2 µl (3-5 pmol) of the eluted oligonucleotides were used for the debranching reaction. The debranching reaction was carried out in 15 µl volume containing 20mM HEPES/KOH (pH 7.6), 125 mM KCl, 0.5 mM $MgCl_2$, 1 mM DDT, and 10% glycerol, along with 3-6 µl of enzyme (50 units total) for 4 - 16 hours at 30°C. The reaction was stopped by adding 15 µl of 95% formamide loading dye. The samples were run on a 24 % acrylamide gel (7M urea) and analyzed by autoradiography. Samples were exposed for 1-12 h at rt. (Note: One unit of the DBR enzyme is the amount required to hydrolyze 1.0 fmol of msDNA Ec86 to completion at 30°C for 30 min in the aforementioned reaction buffer[28]).

Appendix

381A Cycle/Procedure

Name: DNA Synthesis Cycle
Number of Steps: 58

Step numbers	Function #	Function Name	Step time
1	10	# 18 to waste	5
2	9	# 18 to column	60
3	2	reverse flush	5
4	1	block flush	5
5	28	phosphoramidite prep	3
6	90	tetrazole to column	5
7	19	Base + tetrazole to column	5
8	90	tetrazole to column	3
9	19	Base + tetrazole to column	5
10	90	tetrazole to column	3
11	19	Base + tetrazole to column	5
12	90	tetrazole to column	3
13	4	Wait	depends on sequence
14	4	Wait	depends on sequence
15	2	Reverse flush	5
16	1	Block flush	5
17	16	CAP prep	3
18	22	CAP to column	15
19	10	# 18 to waste	5
20	1	Block flush	5
21	10	# 18 to waste	5
22	2	Reverse flush	5
23	1	Block flush	5
24	81	# 15 to waste	5
25	13	# 15 to column	20
26	10	# 18 to waste	5
27	1	Block flush	5
28	4	Wait	10
29	4	Wait	10
30	10	# 18 to waste	5
31	2	Reverse flush	10
32	1	Block flush	5
33	10	# 18 to waste	5
34	9	# 18 to column	15

35	2	Reverse flush	5
36	9	# 18 to column	15
37	2	Reverse flush	5
38	9	# 18 to column	15
39	2	Reverse flush	5
40	9	# 18 to column	15
41	2	Reverse flush	5
42	1	Block flush	5
43	33	Cycle entry	1
44	10	# 18 to waste	5
45	9	# 18 to column	30
46	2	Reverse flush	5
47	1	Block flush	5
48	5	Advance fc	1
49	6	Waste port	1
50	82	# 14 to waste	5
51	14	# 14 to column	100
52	1	Block flush	5
53	10	# 18 to waste	5
54	9	# 18 to column	120
55	2	Reverse flush	5
56	1	Block flush	5
57	7	Waste bottle	1
58	34	Cycle end	1

Key:

# 18	Acetonitrile
# 15	0.1 M Iodine in THF:pyridine:water (75:20:2, v/v/v)
# 14	3% TCA/1,2-dichloroethane
CAP	acetic anhydride/2,4,6-collidine/THF (1:1:8, v/v/v)
fc	fraction collector

381A Cycle/Procedure

Name: Capping cycle
Number of steps: 17

Step Numbers	Function #	Function name	Step time
1	33	Cycle entry	1
2	10	# 18 to waste	5
3	9	# 18 to column	60
4	2	reverse flush	5
5	1	block flush	5
6	16	CAP prep	3
7	22	CAP to column	15
8	4	Wait	300
9	22	CAP to column	15
10	4	Wait	300
11	2	reverse flush	5
12	9	# 18 to column	30
13	2	reverse flush	5
14	9	# 18 to column	30
15	2	reverse flush	5
16	1	block flush	5
17	34	Cycle end	1

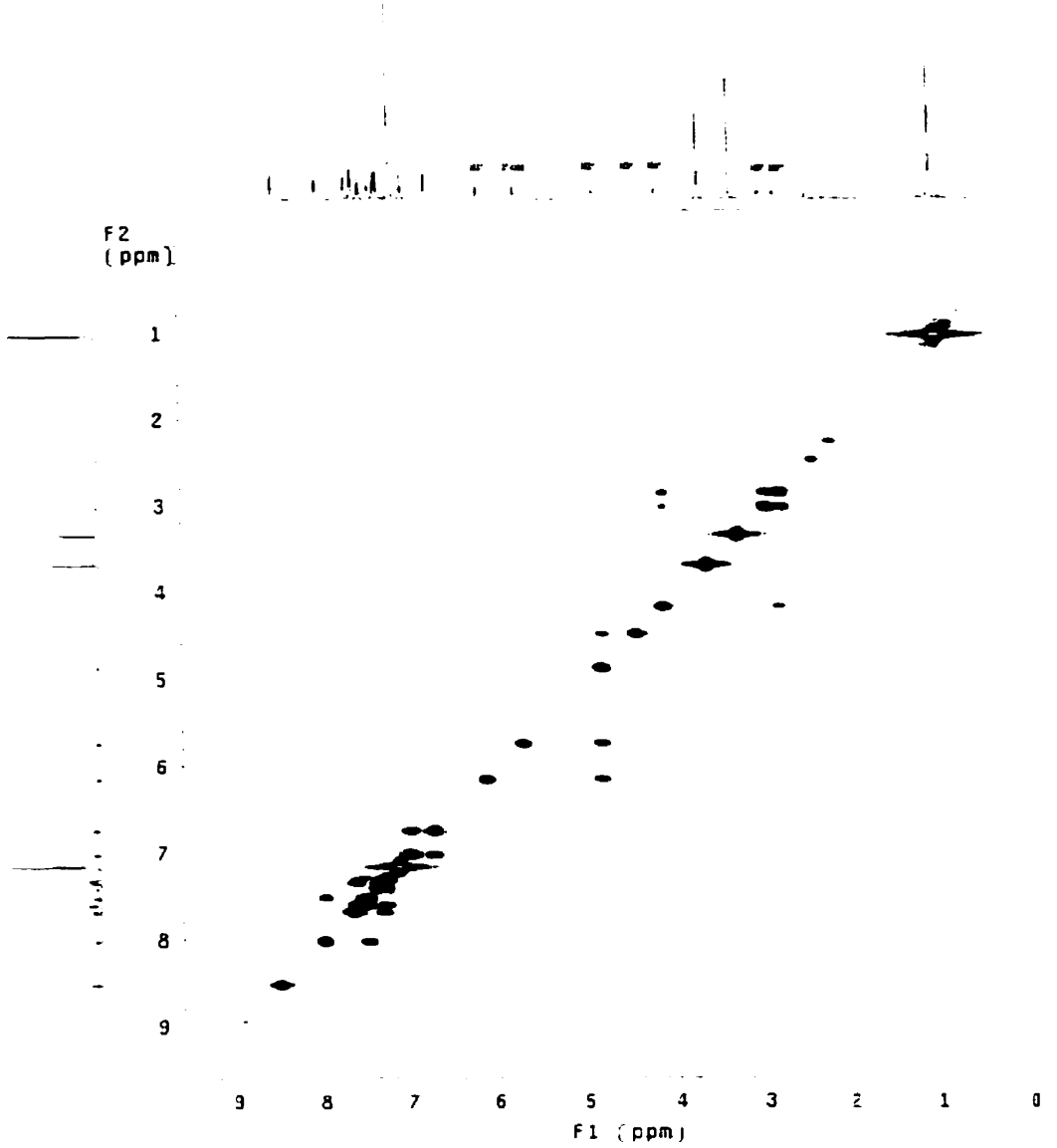

Figure 5.1 2D-J correlated 1H-NMR spectroscopy of N^6-Benzoyl-5'-O-(Monomethoxytrityl) - 3' - O - *tert*-Butyldiphenyl Silyl Adenosine

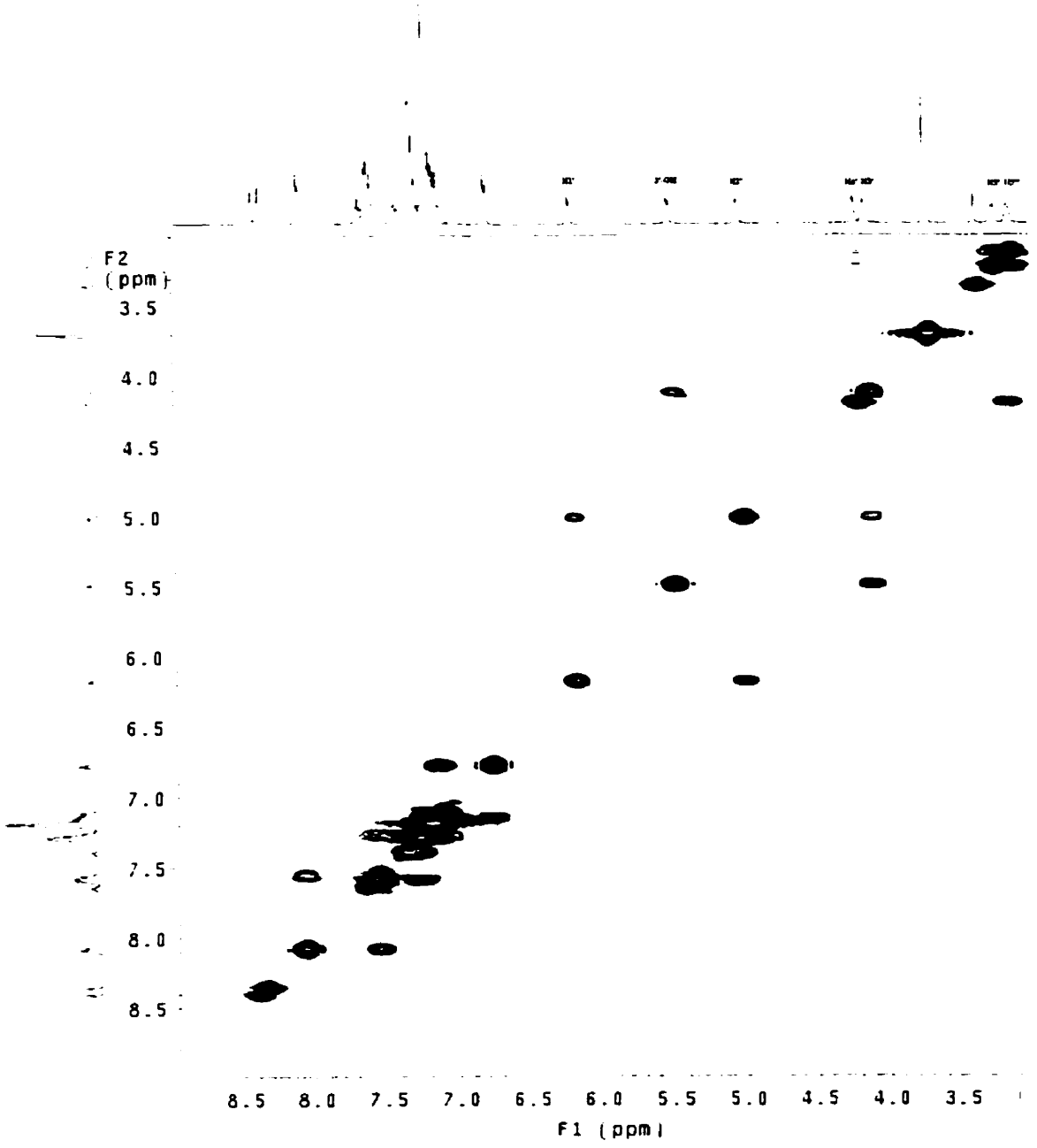

Figure 5.2 2D-J correlated 1H-NMR spectroscopy of N^6- Benzoyl -5' - O - (Monomethoxytrityl) - 2' - O - *tert*-Butyldiphenyl Silyl Adenosine