Selecting and improving the functionality of DNAzymes

by

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Abstract

DNAzymes are strands of catalytic DNA first discovered in 1994. These species are isolated through in vitro selection and are capable of catalyzing many different types of reactions. RNA-cleaving DNAzymes are one subset that have many biological implications; however, more work needs to be done to make them suitable for therapeutic in vivo applications. Modifying DNAzymes with protein functionalities represents a promising strategy to evolve efficient cleavage in vivo.

Chapter 2 described the syntheses of five modified 2'-deoxyuridine triphosphates and the enzymatic incorporation of these modified dUTPs. The modifications were introduced at the 5'-position, and consist of a carboxylate group, indole group and napthyl group. The enzymatic incorporation of these modified nucleotide triphosphates evaluated their suitability for use in an in vitro selection. It was found that Vent (exo-) DNA polymerase was able to incorporate all the modified dUTPs successfully.

In Chapter 3 and Chapter 4, two all-RNA-cleaving DNAzyme selections were described. DNAzyme clone 25 was selected in Chapter 3 against an HIV RNA target, which had a self-cleavage rate constant of 3.3 min\(^{-1}\). However, when the DNAzyme was tested for intermolecular cleavage activity, the result was unsatisfactory. It was found that the maximum rate constant had not been reached under 2 µM substrate, indicating a low substrate binding affinity. With this disappointing result, DNAzyme clone 25 was not considered for in vivo studies.

Conversely, DNAzyme clone 11 was selected in Chapter 4 and displayed a robust trans-cleavage activity and a high binding affinity towards a c-Myc oncogene target sequence. DNAzyme clone 11 was obtained from this process, which had a self-cleavage rate constant of 0.84 min\(^{-1}\). The intermolecular cleavage study showed that it had a cleavage \(k_{\text{max}}\) of 4.3 min\(^{-1}\) and \(K_m\) of 297 nM. The DNAzyme was then shown to be highly sequence-specific. Solid-phase
synthesis of the modified DNAzyme was attempted, and the crude oligonucleotide mixture obtained showed \textit{trans}-cleavage activity. Lastly, Chapter 5 described several failed DNAzyme selections in which no promising active strands were obtained.
Preface

The experiments in this dissertation were designed primarily by Dr. David M. Perrin and myself, and experiments were performed by myself unless otherwise indicated.

Chapter 2 was written by myself and the grammar was assisted by MSc. Abid Hasan. The synthesis of the 5-position-modified 2′-deoxyuridine triphosphate was conducted according the literature report, any modification of the synthesis was included in this thesis. The enzymatic incorporation was performed by myself and have been included in this chapter. A manuscript is preparing by Dr. David Perrin and myself and will be submitted for publication.

Chapter 3 was written by myself and the grammar was assisted by MSc. Jerome Lozada. The selection of RNA-cleaving DNAzyme clone 25 was done by myself and aided by Ms. Yajun Wang. The cloning of this selection was aided by Ms. Yajun Wang. The kinetic study was performed by myself. A manuscript is preparing by Dr. David Perrin and myself and will be submitted for publication.

Chapter 4 was written by myself and the grammar was assisted by Ms. Emily Miller. The selection of RNA-cleaving DNAzyme clone 11 was done by myself and aided by Ms. Yajun Wang. The cloning of this selection was aided by Ms. Yajun Wang. The kinetic study was performed by myself. The solid-phase synthesis was conducted by our collaborator Dr. David Sabatino. A manuscript is preparing by Dr. David Perrin and myself and will be submitted for publication.

Chapter 5. All the experiments were performed by myself.
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List of Abbreviations

A   adenosine
Ac   acetyl
Ac2O   acetic anhydride
AIBN  2,2’-azo-bis-isobutyrylnitrile
ATP   adenosine triphosphate
BNPP  bis(p-nitrophenyl)phosphate
C    cytidine
calcd. calculated
d   doublet
dA   2’-deoxyadenosine
dATP  2’-deoxyadenosine triphosphate
dC   2’-deoxycytidine
DCC   N,N’-dicyclohexylcarbodiimide
DCM   dichloromethane
DEPC  diethylpyrocarbonate
dcTP  2’-deoxycytidine triphosphate
dd   doublet of doublets
ddATP dideoxyadenosine triphosphate
ddTTP dideoxythymidine triphosphate
da'^TP  8-(2-(4-imidazolyl)aminoethyl)-2’-deoxyadenosine triphosphate
da'^homoTP  8-(3-(4-imidazolyl)aminopropyl)-2’-deoxyadenosine triphosphate
dc'^aTP  5-aminoallyl-2’-deoxycytidine triphosphate
dU'^aTP  5-aminoallyl-2’-deoxyuridine triphosphate
dG    2’-deoxyguanosine
dGTP  2’-deoxyguanosine triphosphate
DMA   N,N-Dimethylacetamide
DMF   dimethylformamide
DMTr  4,4’-dimethoxytrityl
DNA   deoxyribonucleic acid
dsDNA  double-stranded deoxyribonucleic acid
ssDNA single-stranded deoxyribonucleic acid
DNAzyme DNA enzyme
dNTPs 2’-deoxynucleoside triphosphates
dT    2’-deoxythymidine
DTT   (2S),(3S)-dithiothreitol
dTTP  thymidine triphosphate
dxTP  any one of the four dNTPs
dU    2’-deoxyuridine
dUTP  2’-deoxyuridine triphosphate
E. coli  Escherichia coli
EDC   1-ethyl-3-(3-dimethylaminopropyl) carbodiimide
EDTA  ethylenediaminetetraacetic acid
equiv. equivalents
ESI   electrospray ionization
Et    ethyl
G    guanosine
HIV   human immunodeficiency virus
HPLC  high performance liquid chromatography
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
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<tr>
<td>HPPNP</td>
<td>2-hydroxypropyl-4-nitrophenyl phosphate</td>
</tr>
<tr>
<td>IDT</td>
<td>Integrated DNA Technologies</td>
</tr>
<tr>
<td>IPTG</td>
<td>isopropyl β-D-thiogalactoside</td>
</tr>
<tr>
<td>$k_{\text{cat}}$</td>
<td>catalytic rate constant (multiple turnover number)</td>
</tr>
<tr>
<td>$k_{\text{max}}$</td>
<td>maximum rate constant</td>
</tr>
<tr>
<td>$K_m$</td>
<td>Michaelis constant of a substrate</td>
</tr>
<tr>
<td>$k_{\text{obs}}$</td>
<td>observed rate constant</td>
</tr>
<tr>
<td>LNA</td>
<td>locked nucleic acid</td>
</tr>
<tr>
<td>LTR</td>
<td>long terminal repeat</td>
</tr>
<tr>
<td>M</td>
<td>molecular weight, used in the description of molecular ion adducts from mass spectrometry, i.e. [M+H]</td>
</tr>
<tr>
<td>m</td>
<td>multiplet</td>
</tr>
<tr>
<td>MALDI</td>
<td>matrix assisted laser desorption ionization</td>
</tr>
<tr>
<td>Me</td>
<td>methyl</td>
</tr>
<tr>
<td>MeCN</td>
<td>acetonitrile</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>MS</td>
<td>mass spectrometry</td>
</tr>
<tr>
<td>Myc</td>
<td>Myelocytomatosis</td>
</tr>
<tr>
<td>N$_{30}$</td>
<td>random 30 nucleobases DNA</td>
</tr>
<tr>
<td>N$_{40}$</td>
<td>random 40 nucleobases DNA</td>
</tr>
<tr>
<td>NAPS Unit</td>
<td>Nucleic Acid Protein Services Unit</td>
</tr>
<tr>
<td>NBS</td>
<td>N-bromosuccinimide</td>
</tr>
<tr>
<td>NHS</td>
<td>N-hydroxysuccinimide</td>
</tr>
<tr>
<td>NMR</td>
<td>nuclear magnetic resonance</td>
</tr>
<tr>
<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>pKa</td>
<td>negative log of acid dissociation constant</td>
</tr>
<tr>
<td>PNA</td>
<td>peptide nucleic acid</td>
</tr>
<tr>
<td>PNK</td>
<td>polynucleotide kinase</td>
</tr>
<tr>
<td>py</td>
<td>pyridine</td>
</tr>
<tr>
<td>rC</td>
<td>cytidine (as opposed to the 2′-deoxycytidine)</td>
</tr>
<tr>
<td>Rf</td>
<td>thin layer chromatography retention factor</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RNase</td>
<td>ribonuclease</td>
</tr>
<tr>
<td>rRNA</td>
<td>ribosomal ribonucleic acid</td>
</tr>
<tr>
<td>s</td>
<td>singlet</td>
</tr>
<tr>
<td>SELEX</td>
<td>systematic evolution of ligands by exponential enrichment</td>
</tr>
<tr>
<td>siRNA</td>
<td>small interfering ribonucleic acid</td>
</tr>
<tr>
<td>T</td>
<td>thymidine</td>
</tr>
<tr>
<td>t</td>
<td>triplet</td>
</tr>
<tr>
<td>$Taq$</td>
<td>family A DNA polymerase from <em>Thermus aquaticus</em></td>
</tr>
<tr>
<td>TCA</td>
<td>trichloroacetic acid</td>
</tr>
<tr>
<td>TEN</td>
<td>tris, EDTA, NaCl buffer</td>
</tr>
<tr>
<td>Tfl</td>
<td>DNA polymerase derived from Thermus flavus</td>
</tr>
<tr>
<td>Theminator</td>
<td>DNA polymerase engineered form of the native DNA polymerase from thermococcus species 9°N-7</td>
</tr>
<tr>
<td>THF</td>
<td>tetrahydrofuran</td>
</tr>
<tr>
<td>TLC</td>
<td>thin layer chromatography</td>
</tr>
<tr>
<td>$T_m$</td>
<td>melting temperature</td>
</tr>
<tr>
<td>Tris</td>
<td>tris(hydroxymethyl)aminomethane</td>
</tr>
<tr>
<td>tRNA</td>
<td>transfer ribonucleic acid</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>Tth</td>
<td>DNA polymerase derived from Thermus thermophilus HB-8</td>
</tr>
<tr>
<td>U</td>
<td>uridine</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
<tr>
<td>Vent</td>
<td>family B DNA polymerase derived from <em>Thermococcus</em></td>
</tr>
<tr>
<td>X-Gal</td>
<td>5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside</td>
</tr>
<tr>
<td>δ</td>
<td>chemical shift</td>
</tr>
<tr>
<td>$\lambda_{\text{max}}$</td>
<td>wavelength of maximum absorbance</td>
</tr>
</tbody>
</table>
Acknowledgements

I would like to thank my supervisor Dr. David M. Perrin for his support and guidance. He was critical to my growth and development as a researcher. Without his support, it would be impossible that I can go this far. I would also like to thank all the past and present members of the Perrin Lab. It was a pleasure to share a workspace with every one of them. In particular, Dr. Ying Li, Ms. Yajun Wang, Ms. Emily Miller, MSc. Abid Hasan, Dr. Marleen Renders were all excellent lab mates who were never hesitant to give me advice or assistance. Also graduate students Dr. Curtis Lam, Dr. David Dietrich, Dr. Christopher Hipolito, Dr. Liang Zhao, MSc. Wenbo Liu, Dr. Zhibo Liu, MSc. Jerome Lozada, MSc. Daniel Walker, were all present during the early and late period of my graduate research. Thanks must also be given to my undergraduate student Mr. Dan Cojocaru, Ms. Emily Song.

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Dedication

A person's ability may be great or small, but if he/she has the spirit, he/she is already
noble-minded and pure, a person of moral integrity and above vulgar interests, a person
who is valuable to the people.
Chapter 1: Introduction

1.1 Nucleic acid

1.1.1 General introduction

Nucleic acids are one of the most important biomacromolecules in Nature and have been thoroughly investigated throughout most of the past century. Important findings include the discovery of nucleic acid structure in 1950s, research on central dogma in 1960s, the development of DNA recombinant technology in 1970s, ribozyme studies in 1980s and Human Genome Project in 1990s. Initially the role of nucleic acids was thought to be restricted to storage of genetic information in the form of deoxyribonucleic acids (DNA), and the transfer of genetic information in the form of ribonucleic acids (RNA). In 1980s, researchers discovered that ribonucleic acids possess a variety of abilities that extend beyond pure information transfer. In Nature, ribozymes (catalytic RNA) were discovered to be capable of catalyzing reactions, such as RNA cleavage, RNA self-splicing, and amide bond formation. Because of these catalytic activities, a hypothesis known as the “RNA world” was proposed, which suggested that life started with RNAs that played roles of both information storage and catalysis, two roles normally reserved to DNA and proteins respectively. The discovery of new ribozymes by combinatorial selections also supports this hypothesis. The technique of combinatorial selection will be addressed in section 1.3. Although RNAs have been found to have such diverse functions in Nature, DNAs are used solely for genetic information storage. While there are few chemical differences between DNA and RNA, the main difference is the presence or absence of a 2'-hydroxyl group. This difference mainly imparts enormous stability of DNA compared to RNA. Such stability underscores the importance of DNA as a genetic information storage material.

DNA was first isolated in 1869 from the chromosomes of cells. The stability of DNA is well-suited for its role as long-term information storage material. Aside from the stability due to
the lack of 2'-hydroxyl group, the genetic information itself is encoded within the sequence of the nucleobases. In Nature, there are two types of heterocycles that enable genetic coding: pyrimidines and purines. Thymine (T) and cytosine (C) are pyrimidines; adenine (A) and guanine (G) are purines. Guanine and cytosine form base pairing via 3 hydrogen bonds whereas thymine and adenine form via 2 hydrogen bonds (Figure 1.1). While the genes of all living things in Nature were encoded with these four nucleobases, uracil (U) as a pyrimidine was also found in the DNAs of some bacteriophages. In Nature DNA is commonly found as antiparallel double-stranded structure, which can minimize the non-specific hydrogen bonding by the nucleobases to other biomolecules. The stability of the double-stranded structure is improved by π-stacking and hydrophobic interactions of the nucleobase. The structure of double-stranded DNA was suggested by Watson and Crick as a double-helix model, and confirmed by X-ray diffraction technology.

Figure 1.1 Nucleobases of DNA and RNA, base pairing

There are three forms for double-stranded DNA as double helix, the right-handed helical twisted A-DNA and B-DNA forms and the left-handed helical twisted Z-DNA form. B-DNA is the most common structure, which is characterized by 10.5 nucleotides per turn as well as a major groove and a minor groove. The major groove is shallow and about 22 Å wide while the minor groove is deep and about 12 Å wide. Due to this difference, many proteins bind to the major groove instead of the minor groove. The minor groove generally exhibits a higher electronegative potential than the major groove. This difference is derived from the disposition of phosphate groups at the base edges; specifically AT-rich sequences display greater negative
electrostatic potentials.\textsuperscript{17} A-DNA and Z-DNA are rarely observed under physiological conditions. Besides double-stranded DNA, there are branched DNA\textsuperscript{18} and G-quadruplexes\textsuperscript{19}. With the stability and programmable and predictable characters of DNAs, DNA nanotechnology or DNA origami has provided many interesting structures, and represent a very hot research topic.\textsuperscript{20} However, the biological functions of these interesting structures are still under investigation.\textsuperscript{18,21-23}

Whereas DNA is reserved exclusively for genetic information storage, ribonucleic acids can transfer genetic information as messenger RNA (mRNA), carry amino acids on transfer RNAs (tRNAs) and catalyze the biosynthesis of proteins on the ribosome (rRNA). There are also many small-RNA species found to play roles of regulation in gene expression.\textsuperscript{24} Although RNAs play many roles in cellular environment, they are more chemically reactive due to the presence of the 2'-hydroxyl group. In addition, under physiological conditions, ribonucleases as well as ribozymes can rapidly degrade or cleave RNA.\textsuperscript{25} The nucleobases of RNA are adenine, guanine, cytosine and uracil (Figure 1.1). The difference between uracil and thymine is one methyl group at the 5-position. In terms of hydrogen bonding, DNA and RNA are essentially identical and not unexpectedly, RNA can form double helices as well. However, double-stranded RNA is not commonly found in cells. Although many short stem loops are composed of antiparallel double strands, most aforementioned RNAs are single-stranded RNAs. Very short interfering RNAs (siRNAs) do form double-stranded RNAs can interfere with gene expression.\textsuperscript{26} One illustrative RNA structure is shown in Figure 1.2. Several types of RNA structures are shown, and these structures are formed by the base pairing of AU or GC that leave clusters of unpaired nucleobases. These unpaired nucleobases form different loops that can interact. These contacts are called kissing interactions and provide some of the diverse three-dimensional structures found in RNA.\textsuperscript{27-29} Additionally, divalent metal cations play important roles to support the three dimensional structure of RNAs and give rise to catalytic activity.\textsuperscript{30} All the ribozymes were
thought to be divalent metal-dependent enzymes, until some were found to maintain activity in the absence of divalent metal cations in 1998 (section 1.2.3).\textsuperscript{31}

![RNA structure diagram](image)

**Figure 1.2** An stylized illustration of an RNA structure. 1, hairpin loop; 2, internal loop; 3, double strand; 4, junction; 5, single strand; 6, bulge

### 1.1.2 Development of catalytic nucleic acids

Catalytic nucleic acids include catalytic RNAs and catalytic DNAs. Catalytic RNAs as mentioned above are known as ribozymes while catalytic DNAs are called DNA enzymes, deoxyribozymes or DNAzymes. Ribozymes were found in Nature, but all the known DNAzymes have been discovered by combinatorial selection. Ever since the development of this combinatorial method, a considerable number of publications on ribozymes and DNAzymes has been conducted. Natural ribozymes can only catalyze a very limited number of reactions (ligation, self-splicing, peptide bond formation and RNA cleavage). Many other reactions were discovered to be catalyzed by ribozymes. Szostak and coworkers discovered the first self-polymerization ribozyme,\textsuperscript{32} Holliger *et al.* discovered RNA polymerase ribozymes with a novel in-ice evolution.\textsuperscript{33,34} Unrau and coworkers selected ribozymes that can catalyze ribonucleotide synthesis.\textsuperscript{35-37} Both Eaton and Jäschke groups discovered ribozymes that can catalyze carbon-carbon bond formation,\textsuperscript{38-40} Suga and coworkers developed ribozymes for alcohol
dehydrogenation and reduction. Such a wide reaction range also supports the “RNA world” hypothesis.

Although the combinatorial selection methods were generally recognized in 1990 following these seminal reports from the Gold, Szostak and Joyce labs, the first DNAzyme that was elected to cleave RNA was discovered in 1994 by Breaker and Joyce. Since this seminal work appeared, numerous research papers were published on distinct DNAzymes that can catalyze various reactions, such as DNA cleavage, RNA ligation, DNA phosphorylation, DNA adenylation, the Diels-Alder reaction and nucleopeptide linkage formation. DNA is much more stable than RNA, and therefore DNAzyme would have potentially increased advantages compared to ribozyme. In addition, solid-phase synthesis for DNA is more efficient and cheaper than RNA.

1.1.3 DNAzyme as a catalyst but not a template

Some reactions have been found to be catalyzed by DNAzymes, but the mechanisms of these reactions are rarely studied in detail. Two of the most comprehensive papers on this subject were undertaken by the Perrin lab. It is known that protein enzymes generally employ several mechanistic strategies that contribute to lower the transition-state energy; these include appropriate positioning of the reactive groups and imparting binding energy to recognize group states that enable specificity. When DNAzymes appropriately position its substrates, the catalysis process is often confused with templation-mediated rate enhancement. Indeed, DNA-templated synthesis (DTS) is a technique of controlling the reactivity of small molecules by increasing the effective molarity. It is important to distinguish DTS from true catalytic action (Figure 1.3). DTS can greatly increase the effective molarity of reactive small molecules from nM to µM or even high mM. The rate enhancement of DTS is evaluated by the ratio of $k_{\text{templated}}$ and $k_{\text{untemplated}}$, where $k_{\text{templated}}$ is a pseudo-first-order rate constant, and the $k_{\text{untemplated}}$ is a second-order rate constant. Therefore, the units for rate enhancement are given in simple units of
concentration. Yet because the units do not change between DTS and DNA catalysis, these are often confused as previously noted. Hence, DNA catalysis can be considered as any rate enhancement that is superior to that afforded by DTS. The rate enhancement can be evaluated by the ratio of $k_{\text{cat}}$ and $k_{\text{templated}}$, both pseudo-first-order rate constants. In addition, DNAzymes have an extended catalytic motif that would typically not be needed for a DTS template. The DTS product will associate with the template afterwards, and therefore has no capacity for multiple-turnover catalysis. In contrast, certain DNAzymes have capacity for multiple-turnover catalysis, and can be studied with Michaelis–Menten kinetics. However, some DNAzymes are incapable of multiple-turnover catalysis, because they are unable to dissociate from the catalysts and bind to new substrates. The binding region of DNAzyme as shown in Figure 1.3 can be adjusted according to different substrates. The length of the binding region directly affects the binding affinity of a given DNAzyme. However, the $k_{\text{cat}}$ is often less influenced by the binding region.

$$\begin{align*}
5' & A \quad B \quad 3' \\
5' & k_{\text{untemplated}} \\
3' & k_{\text{templated}} \\
5' & k_{\text{cat}}
\end{align*}$$

**Figure 1.3** DNA-templated synthesis versus DNA catalysis. a Untemplated reaction; b DTS; c DNAzyme catalysis. ( ) indicates the binding regions, ( ) indicates the catalytic region.

### 1.2 RNA phosphodiester bond cleavage

Of all the reactions that DNAzymes are known to catalyze, RNA cleavage was the first one reported and is still the most interesting reaction. RNA cleavage fundamentally is a transesterification reaction of phosphodiester bond. From a chemical viewpoint, the significance of fast transesterification is that it can be used in pesticide and chemical welfare agents control, because both pesticide and chemical welfare agents contain organophosphate function groups. The biological significance of RNA cleavage DNAzyme will be outlined in section 1.6.
1.2.1 Chemistry of phosphodiester bond cleavage

The transesterification of phosphodiesters via anchimeric assistance is a well-studied reaction. Due to the relative lassitude of uncatalyzed RNA cleavage, much work has been done with model substrates including RNA dinucleotides, DNA dinucleotides, activated 2-hydroxypropyl-4-nitrophenyl phosphate (HPPNP) and bis(p-nitrophenyl)phosphate (BNPP). The HPPNP and BNPP contain better leaving groups.\textsuperscript{65-67} The transesterification of HPPNP is shown in Figure 1.4.

![Transesterification of HPPNP](image)

Figure 1.4 Transesterifications of 2-hydroxypropyl-4-nitrophenyl phosphate (a) and RNA (b)

The transesterification is a substitution reaction at phosphorus. +Transesterification has several pathways, including a dissociative, an associative and a concerted pathway.\textsuperscript{68} Here only the associative pathway is shown in Figure 1.4. The hydroxyl group attacks the tetrahedral phosphorus and a pentacoordinated oxyphosphorane intermediate is formed. The intermediate then breaks down to a cyclic phosphate and 5'-hydroxyl group. This is generally considered to be
the pathway for RNA cleavage under physiological conditions. The pentacoordinated intermediate has a trigonal bipyramidal (TBP) geometry. RNA cleavage is one of the most well-studied reactions. Researchers have established that during cleavage the 2'-hydroxyl group necessarily attacks from the apical position and the 3'-oxygen must be equatorial, otherwise the energy of the intermediate is too high (Figure 1.4b). The pKa of 2'-hydroxyl group is normally about 13. The pKa of phosphoric acid is about 1. Extremely high or extremely low pH conditions can promote the cleavage reaction and the pH-rate profile for the cleavage is a U-shaped curve.\textsuperscript{69,70} It reaches the lowest point at about neutral pH conditions. In order to catalyze the cleavage reaction under neutral pH conditions, several strategies can be adopted: neutralizing the negative charge on the non-bridging phosphate oxygen, facilitating the deprotonation of the 2'-hydroxyl group, stabilizing the negative charge on the pentacoordinated intermediate, protonating the leaving oxygen and facilitating the in-line nucleophilic attack. These strategies were adopted to cleave the phosphodiester bond by different enzymes. To date, the fastest-known phosphodiester bond cleavage reaction is catalyzed by RNase-A, a protein enzyme with a maximum rate constant of $>80,000 \text{ min}^{-1}$. The best self-cleaving ribozymes and DNAzymes cleave RNA with a observed rate constant $10^0$-$10^1 \text{ min}^{-1}$.\textsuperscript{59,71} This difference in catalytic efficiency has significant implications to the structural difference between the building blocks for proteins and nucleic acids.

1.2.2 Protein enzymes that cleave RNA and DNA

Protein enzymes that cleave a RNA phosphodiester bond are known as ribonucleases (RNases). There are several sub-classes and of these, RNase-A is one of the most well-studied.\textsuperscript{72} RNase-A is a small protein enzyme with only 124 amino acid residues. The catalytic efficiency of RNase-A is close to the diffusion controlled limit, which is remarkable. However, the cleavage of RNA substrates is not sequence-specific.\textsuperscript{73,74} Although there are research on generating sequence-specific nuclease, the difficulty is incredibly hard and all the researches in this area have focused
on DNA-cleaving nuclease for the purpose of genome editing.\textsuperscript{75} RNase-A does not need divalent metal cations for activity. Three amino acid residues are critically involved in the catalytic reaction (Figure 1.5): histidine 12, histidine 119 and lysine 41. Histidine 12 acts as a general base and deprotonates the 2'-hydroxyl group. Histidine 119 acts as a general acid and provides a proton to the 5'-oxygen. Lysine 41 stabilizes the negative charge on the phosphate oxygen in ground state and also stabilizes the negative charge that accumulates in the transition state and the short-lived phosphorane intermediate.\textsuperscript{76-78} All aforementioned strategies are utilized by RNase-A, which provides for a remarkable efficiency. Unlike amino acids, building blocks of nucleic acids are poorly functionalized, or at least hypothesized to be poor compared to proteins. Significant efforts have been directed towards mimicking RNase-A with modified nucleic acids.

![Mechanism of phosphodiester bond cleavage by RNase-A](image.png)

**Figure 1.5** Mechanism of phosphodiester bond cleavage by RNase-A

### 1.2.3 Ribozyme

In the previous section (section 1.12), the development of a ribozyme by combinatorial selection was briefly discussed. The most important and well-studied ribozymes are still natural ribozymes, and in most cases these ribozymes are RNA-cleaving, self-splicing ribozymes. They play significant roles in living cells. The first natural ribozyme, Tetrahymena Group I intron ribozyme, was discovered by Cech and coworkers in 1982, for which Cech shared the Noble Prize in Chemistry at 1989. Since then, researchers have found several other classes of ribozymes
that can hydrolyze or cleave RNAs. Two examples, the Hammerhead ribozyme and the Hairpin ribozyme, were studied in great detail in terms of how ribozymes cleave RNA. Insight in terms of catalytic efficiency and mechanism was achieved through this work.\textsuperscript{79}

Initially, all ribozymes were thought to be divalent metal-ion dependent. Divalent metal cations such as Mg\textsuperscript{2+} are required to facilitate folding and catalysis yet in some cases addition of high molar concentration of monovalent cations can maintain catalytic activity suggesting that the M\textsuperscript{2+} is only needed for folding, not catalysis within the active site.\textsuperscript{80} For instance, Hammerhead ribozyme and Hairpin ribozyme are found to be divalent metal-independent ribozymes, as long as high concentrations of monovalent metal cations are present, presumably to induce the appropriate folding.\textsuperscript{31,78} Indeed divalent metal cations are essential for most of ribozymes and even more so for DNAzymes. Both ribozymes catalyze the cleavage reaction reversibly.\textsuperscript{81,82} The 2', 3'-cyclic phosphate terminus and the 5'-OH terminus can be ligated together without exogenous energy sources.\textsuperscript{81,82}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{image.png}
\caption{Structure of the minimal Hammerhead ribozyme and the minimal Hairpin ribozyme}
\end{figure}
Structurally the core of the hammerhead ribozyme consists of three stem loops separated by short linkers of conserved sequences, and are classified based on the location of the 5' and 3' termini. The minimal Hammerhead ribozyme (type III) is shown in Figure 1. The catalytic rate constant of Hammerhead ribozyme can reach a maximum rate constant $10^{0-10^1}$ min$^{-1}$ under mM Mg$^{2+}$. The cleavage mechanism is still under debate. One mechanism involves Mg$^{2+}$ as a cofactor. The other mechanism doesn’t utilize divalent metal cations directly within the catalytic core. These two mechanisms are shown in Figure 1. In both mechanisms nucleobase G-12 plays a general base role, and it activates the 2'-hydroxyl group. The 2'-hydroxyl group of nucleobase G-8 plays a general acid role, and it delivers a proton to the 5'-oxygen leaving group. This process is facilitated by Mg$^{2+}$ or a bridging water molecule. Studies using 5'-thio RNA substrates to achieve one order of magnitude rate enhancement show that the rate-limiting step is the departure of the 5'-oxygen. The 2'-hydroxyl group of G-8 appears to play a very important role in the catalytic step. However, DNA lacks the 2'-hydroxyl group, and this shortage makes the selection of RNA-cleaving DNAzyme in the absence of divalent metal cations a more challenging project.

Figure 1.7 Two proposed mechanisms of phosphodiester bond cleavage by Hammerhead ribozymes. a, mechanism using a hydronium; b, mechanism using a Mg$^{2+}$. 

11
Hairpin ribozymes have only been found in RNA satellites of plant viruses. The minimal Hairpin ribozyme is shown in Figure 1.6. The active form is the bent status between loop A and loop B. The mechanism of the Hairpin ribozyme also does not appear to require divalent metal cations. Although the divalent metal cations greatly facilitate the folding, the catalytic core is comprised of the nucleobases alone. As shown in Figure 1.6, when loop A and B contact with each other, nucleobase G-8 and A-38, which are crucial for catalytic activity, act as general base and general acid respectively to cleave the target. The pH-rate profile showed that one group with a pKa of ~6.2 and the other with a pKa of >9, indicating that the N1 of nucleobase the A-38 (green colour, Figure 1.8) is in a protonated state under physiological conditions (pH 7.4). The proposed transition state is shown in Figure 1.8. The protonated N1 of nucleobase A-38 provides a proton to the 5'-oxygen. The N1 of nucleobase G-8 (purple colour) may play the general base role, and deprotonate the 2'-OH. This catalytic mechanism is very similar to that of RNase-A: the A-38 plays the role of His-119, and the G-8 plays the role of His-12, as shown in Figure 1.5. The Hairpin ribozymes are not as efficient as protein enzymes (rate constants) in that they do have some similarities. The N1 of nucleobase G is involved the catalytic processes of several related ribozymes and it acts as a general base to deprotonate the 2'-OH, such as in the Hammerhead ribozyme in Figure 1.7 and that of Hairpin ribozymes in Figure 1.8. This catalytic method involving nucleobases can be adopted by DNAzymes as well.
1.2.4 Artificial nucleases

Besides protein enzymes and ribozymes, efforts have been made to mimic metallonucleases. It is rational to hypothesize that these artificial nucleases are capable of cleaving RNA. Complexes with divalent, trivalent, even tetravalent metal cations were studied and some strategies were utilized to make the catalysts work site-selectively. In many publications, a dimetallic or trimetallic complex was used to achieve catalytic efficiency. Examples are shown in Figure 1.9. Generally speaking, these artificial nucleases can be divided into two types; hydrolytic and oxidative.

**Figure 1.8** Proposed transition state of Hairpin ribozyme active site
Most artificial nucleases cleave phosphodiester bonds via a hydrolytic mechanisms. The metallic core can be quite varied. A majority of these nucleases have been only tested with model molecules, and the catalytic efficiency varies with rate constant $10^{-4}$-$10^0$ min$^{-1}$. A zinc complex catalyzing HPPNP hydrolysis is shown in Figure 1.10. The nuclease mimic contains a metallic core and a guanidine group to activate a water molecule and the phosphate respectively. The metal complex lowers the pKa of the bound water and turns the water into a coordinated hydroxide ion, which then deprotonates the 2'-hydroxyl group. Simultaneously, the guanidine group stabilizes the negative charge of the phosphate oxygen and the intermediate.
Oxidative cleavage is generally adopted by redox-active coordination complexes.\(^{95,102}\)

The metallic core typically is copper (II) yet in a few cases ferric/ferrous cations are used.\(^{97,104}\)

The sugar ring is oxidized to result in the RNA cleavage. The product is different from that obtained via the hydrolytic pathway. **Figure 1.11** shows one proposed mechanism.\(^{95,102}\)

The process starts with 4'-hydrogen atom abstraction. Then dissolved molecular oxygen combines with the radical to form an organoperoxide, which undergoes a Baeyer–Villiger-like oxidation to yield a lactone product, which quickly hydrolyzes into 3 species as 2'-hydroxypropenal with a base, 5'-phosphate and 3'-phosphoglycolate.\(^{95,102}\)

Other mechanistic variations start with H-atom abstraction at different positions and give different scission products.\(^{102}\)

**Figure 1.11** One proposed mechanism of RNA oxidative cleavage under aerobic conditions

### 1.3 *In vitro* selection & evolution

In Nature only RNAs have been found with catalytic activity. DNAs as enzymes have been developed by *in vitro* selection or evolution.\(^{105,106}\)

The idea was to apply the principles of Darwinian evolution to chemical molecules.\(^{106}\)

Dr. Sol Spiegelman published a paper entitled “An extracellular Darwinian evolution experiment with a self-duplicating nucleic acid molecule” in 1967.\(^{107}\)

However, that RNA, known as Spiegelman’s monster, is not considered to be a “self-duplicating” molecule. It was amplified by RNA replicase in a sequence and structure-specific
manner. In the experiments, some genomic RNA were added to a mixture of NTPs, salt and RNA replicase. After the RNA was replicated, a small portion of the reaction was transferred into a fresh reaction mixture for the next amplification. This process was repeated 74 rounds with an increased time stringency. The original 4500mer genomic RNA ended up with a much shorter 218mer RNA. Spiegelman’s work represents a pioneering work in \textit{in vitro} selection area, and this study and the following research have had a great impact on chemical biology.\textsuperscript{62,106,108,109}

The \textit{in vitro} selection of nucleic acid was initially reported in 1990 from the separate labs of Szostak, Joyce and Gold independently.\textsuperscript{42-44} In these separate articles, selection as a general method was described and the idea of Darwinian evolution was first realized in a chemical set-up.\textsuperscript{42-44} Selection has a very similar general process to Darwinian evolution (\textbf{Figure 1.12}). The process includes the following steps: (1) A library pool was initially built up for the selection. (2) The selection pressure was applied upon the library. (3) The survival species were amplified, new species were introduced due to mutations, and all the species formed a new library for the next cycle. (4) The final species were isolated and cloned after the desired goal was achieved.

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{in_vitro_selection_scheme.png}
\caption{A general \textit{in vitro} selection scheme}
\end{figure}

The selection process that was used to isolate the RNA-cleaving modified DNAzyme 925-11 is demonstrated in \textbf{Figure 1.13}.\textsuperscript{110} First, a library of DNA oligonucleotide sequences
containing a random region flanked by two primer binding regions is produced. The two primer binding regions are employed for the amplification step. The length of random region is changeable according to needs. The number of possible sequences is $4^{20}$ (4 different nucleobases, 20 is the length of random region). However, researchers conduct practical selections with approximately picomole of molecules. The total number of molecules in the selection is typically $10^{11}$-$10^{13}$. Thus, if a library has more than 22 random bases, the total number of sequences will not be fully presented in the selection. The library is generally synthesized by solid-phase DNA synthesis. The randomness is achieved by coupling the mixture of the four DNA phosphoramidite building blocks in the solid-phase synthesis, so that all the four nucleobases can be equally presented in the synthesized DNA sequence. To guarantee the equal incorporation in the coupling step, the ratio of the phosphoramidites in the mixture is determined by the relative reactivity.
Second, a biotinylated RNA-containing primer is annealed to the oligonucleotides library, the library of oligonucleotides is transcribed into modified DNA library by elongation in the presence of modified dUTP and dATP (Figure 1.18), canonical dCTP and dGTP, buffers and DNA polymerase. If a selection needs an RNA library, DNA can be transcribed into RNA first. Once a desired library is available (either DNA or RNA), a selection for binding or catalysis is launched. In this RNA-cleaving modified DNAzyme selection, the library template is removed by mildly basic washes (0.1 M NaOH) to obtain a single-stranded library. Third, a selection buffer is added to the single-stranded oligonucleotides (Figure 1.13). The cleavage reaction is incubated for variable time and conditions and then the cleaved strands were collected. The important part of selection is to separate the active species from inactive ones, which essentially involves physical separation of cleaved strands that represent active one from uncleaved strands that represent inactive ones. Several strategies can be considered, such as streptavidin-biotin technique and polyacrylamide gel electrophoresis (PAGE). In most of the cases, both techniques were employed cooperatively to optimize the quality of the selection. Finally, following the separation, the strands that are isolated and presumed to be active are amplified by polymerase chain reaction (PCR). Then the next round of selection is ready to begin. The selection process is repeated typically for 5-15 rounds. If the aim is achieved, the selection pool will be cloned and sequences can be identified. Selection stringency and introduction of mutation are the other two important aspects that need be concerned during the selection.62,106,111

1.4 RNA-cleaving DNAzyme

RNA cleavage was the first one of several activities selected for and is still the most interesting reaction. Many RNA-cleaving DNAzymes require a divalent metal cation cofactor, such as Mg^{2+}, to be active. The divalent metal cofactors are so important to DNAzymes that the rate constant of the fastest-known divalent metal-dependent RNA-cleaving DNAzymes can reach
values on the order of $10^1$ min$^{-1}$. This rate constant is more than 1000 times higher than the rate constant of the fastest divalent metal-independent ones (less than $10^{-2}$ min$^{-1}$).\textsuperscript{112,113} Whereas the substrates of DNazymes are RNAs, a single embedded ribonucleotide linkage is often used in many selections to control the site of cleavage. Hence most selected DNazymes have no or a little activity when subject to all-RNA targets.\textsuperscript{45,110,114-116}

### 1.4.1 Divalent metal-dependent DNazyme

The first RNA-cleaving DNazyme was a lead (II) dependent DNazyme. The DNazyme was selection with a single embedded ribonucleotide. When the substrate for this lead (II) dependent DNazyme is replaced with an all-RNA target, the catalytic activity was only $10^5$ times increase compared to the uncatalyzed reaction.\textsuperscript{45} Meanwhile lead (II) normally is not present in cellular environment. Shortly thereafter, Breaker and Joyce identified a magnesium-dependent RNA-cleaving DNazyme.\textsuperscript{114} This DNazyme E2 has a rate constant 0.01 min$^{-1}$ at 1 mM magnesium and 0.08 min$^{-1}$ in a saturated solution of magnesium cation. Although it is a slow cleaver compared to protein nucleases and even some ribozymes, the DNazyme nevertheless operates in the presence of magnesium (II), which is available in cellular environment.\textsuperscript{114} A calcium-dependent DNazyme was discovered by Famulok and coworkers with a rate constant of 0.1 min$^{-1}$.\textsuperscript{117} Compared to some ribozymes, in terms of the catalytic efficiency, these DNazyme are not efficient. Additionally, DNazyme E2 and Ca$^{2+}$-dependent DNazyme cannot cleave all-RNA target.

In a later selection, Santoro and Joyce discovered the famous all-RNA-cleaving magnesium-dependent DNazymes 10-23 and 8-17(Figure 1.14).\textsuperscript{63} Since their report, these two DNazymes have been the two most commonly-used RNA-cleaving DNazymes to date.\textsuperscript{63} In the selection they did not place pre-programmed primer binding region between the all-RNA substrate and the DNazyme. The all-RNA substrate was directly presented to an N$_{50}$ DNA library.\textsuperscript{63} Both of the DNazymes are relatively small motifs. The DNazyme 10-23 has a first-
order rate constant of 0.15 min\(^{-1}\) and \(K_m\) of 0.5 nM, and a second-order rate constant of 10\(^8\) min\(^{-1}\)M\(^{-1}\) in the presence of 2 mM magnesium. Therefore, the catalytic efficiency is comparable with that of the Hammerhead ribozyme. Although it still has a slower maximum rate constant than RNase-A (80,000 min\(^{-1}\)), its \(K_m\) is more favourable than that of RNase-A (10\(^{-4}\)-10\(^{-5}\) M\(^{-1}\)).\(^{63}\) The capability of the divalent metal-dependent DNAzymes may reach the limit.

Figure 1.14 Illustrative scheme of DNAzyme 10-23 and DNAzyme 8-17 (lower case represents the RNA nucleobase)

### 1.4.2 Divalent metal-independent DNAzyme

Geyer and Sen reported the selection of the first divalent metal-free DNAzyme, named Na8, that was found to cleave one embedded ribonucleotide with a rate constant of 0.007 min\(^{-1}\) in the presence of 0.5 M NaCl (pH 7.0) at 25 °C. Although the value of the rate constant is much smaller than that of DNAzyme 10-23, considering the uncatalyzed rate in the absence of divalent metal cations, the rate enhancement of \(k_{cat}/k_{uncat}\) is remarkable.\(^{113}\) In another study, Famulok and coworkers selected several RNA-cleaving DNAzymes, which are also divalent metal-independent and are related to DNAzyme Na8 in terms of sequence homology.\(^{118}\) Breaker and coworkers selected a histidine-dependent DNAzyme HD2 that has a rate constant of 1 min\(^{-1}\). In this selection histidine was used as the cofactor instead of a divalent metal cation. Apparently this idea was inspired by divalent metal-independent protein enzymes, and the pH-rate profile suggested a general base role for the imidazole group of histidine.\(^{119}\)
1.5 Modified DNAzymes

1.5.1 Modified oligonucleotides

The selected DNAzymes and natural ribozymes reach remarkable catalytic efficiencies of $10^8 \text{ min}^{-1}\text{M}^{-1}$, this mainly is due to that the $K_{m}$ and is much lower than $K_{m}$ values of protein enzymes.\textsuperscript{71} However, the observed catalytic rate constant is still 3-4 orders of magnitude lower than those of many protein enzymes.\textsuperscript{108} The major reason for this discrepancy is hypothesized to be that the functional groups of nucleic acids are much poorer compared with the functional groups of proteins. Twenty-one amino acids provide more functional groups than nucleic acids. The idea of grafting functionalities into nucleic acids to enhance the catalytic activity or expand the function was remarkable and important in both the selection field and the synthetic biology.\textsuperscript{120}

Beyond functional groups, nucleic acids have two major parts, including the ribose moieties with the phosphodiester linkages and the nucleobases. Modifications to the ribose moiety and the phosphodiester linkage have been carried out to address the cellular stability and permeability of the oligonucleotides (Figure 1.15).\textsuperscript{121,122} Most modifications can enhance the nuclease resistance as well as the stability in cellular environment. Some modifications can eliminate the negative charge of the nucleotides and improve the cellular permeability. The modifications predominantly will destabilize the base pairing interactions with natural DNA or RNA; however, the triazole as backbone modified DNA has a higher melting temperature due to the positive charge on the backbone.\textsuperscript{123} Among these modified oligonucleotides, very few can be synthesized by PCR. For some of the modifications it is extremely hard to obtain a relatively long nucleotide that is entirely modified.\textsuperscript{121,122} PNA and LNA are two well-studied examples. PNA (peptide nucleic acid) utilizes the neutral N-(2-aminoethyl)-glycine linkage to replace the phosphate backbone, the nucleobases are attached to the glycine amino group though methylene carbonyl linkages.\textsuperscript{124} PNA so far is not found to be digested by nucleases or proteases.\textsuperscript{125} LNA, locked nucleic acid, contains a 2'-O, 4'-C-methylene bridge. This bridge can restrict the sugar
rings and the structure conformation is locked.\textsuperscript{126} LNA was found to form highly stable base pairing with natural DNAs or RNAs.\textsuperscript{126}

\textbf{Figure 1.15} Sugar modifications and backbone modifications

Over the decades, nucleobase modifications have been extensively investigated with very diverse modifications that have been introduced according to different requirements and purposes.\textsuperscript{120,127} Some modifications can lead to the loss of base pairing, or create a whole new set of base pairs. Several types of modifications are shown in \textbf{Figure 1.16}.\textsuperscript{120,128-130} These nucleobases containing hydrophobic aromatic rings were employed as fluorophores to image genetic sequences.\textsuperscript{130} Polymerases were discovered that did not need Watson-Crick base pairing
to copy a base pair. Some of these modified bases were even used in vivo, and amplified and transcribed by enzymes in cell. The other modifications do not break the natural Watson-Crick base pairing, so that modifications are often found to be at the 5-position of pyrimidines, the 8-postion of purines, and the 7-postion of deazapurines. Uridines modified at the 5-position have been used in aptamer selections. Adenines modified at the 8-position as well as 7-deazaguanine have been used in RNA-cleaving DNAzyme selections (Figure 1.16).
1.5.2 Issues of using modified dNTPs in selection

To select modified DNAzymes (or aptamers for that matter) from pools of modified nucleotides, a few issues must be addressed. First, modified dNTPs are often poor substrates of DNA polymerases. Second, the transcription of a modified DNA template might be problematic. Hence, modified dNTPs must be incorporated by polymerases to generate modified DNA libraries in order to start selections. Generally speaking, the modified dNTPs will probably never be as good substrates for polymerases as natural dNTPs. However, as long as the DNA library is not so long such that full-length modified oligonucleotides can still be obtained, then the selection should be able to proceed. The incorporation may be further improved by optimizing the pH, salt concentrations, and nucleotide concentrations as well. Equally important: the modified DNA template must be able to be transcribed into unmodified DNA, such that the sequence information can be preserved during the selection rounds. Although only a few studies have addressed the amplification bias of modified DNA, it is generally accepted that the evolution done by Spiegelman showed that the amplification step will prefer the shorter strands.107,134,135 By the same token, the selection process may prefer the less modified species and keep a balance between catalytic activities and the extent of modification. It is also important to note that when a modified dXTP replaces the appropriate natural dXTP, it must replace all instances of the modified dXTP.

1.5.3 Modified RNA-cleaving DNAzyme

Use of modified nucleotides in selection was first performed by Latham in 1994.136 An aptamer containing a 5-pentynyl-uridine was obtained from this selection, and the modification was shown to be crucial for aptamer function.136 This selection proved that a modified nucleotide can be utilized in *in vitro* selection. The first modified RNA-cleaving DNAzyme was discovered by Joyce and coworkers who used a dUTP functionalized with imidazole group (Figure 1.17).137 An N50 library was employed, the substrate was all-RNA substrate, and pre-programmed binding
strategy was not used. DNAzyme 16.2-11 was selected, which can cleave RNA in the presence of Zn$^{2+}$. The DNAzyme was made by solid-phase synthesis, and the rate constant of trans-cleavage reaction can reach 3.1 min$^{-1}$ under 30 µM Zn$^{2+}$ at pH 7.5 and 37 ºC. In the initial N$_{50}$ structure there were 11 modified nucleotides of which 7 modified nucleotides were found in the binding arm regions and were irrelevant to activity. Three (red letter in Figure 1.17) modified nucleotides were crucial for activity.$^{137}$

![DNAzyme 16.2-11 and modified nucleotide](image)

**Figure 1.17** DNAzyme 16.2-11 and modified nucleotide (lower case represents the RNA nucleobase)

Perrin and coworkers discovered the first divalent metal-independent modified DNAzyme 9$_{25}$-11, which was selected with two modified nucleotides, 8-[2-(4-imidazolyl)aminoethyl]-dATP and 5-aminoallyl-dUTP (Figure 1.18).$^{110}$ The selection was expected to select an RNase-A mimic, and it was conducted in the absence of divalent metals with a N$_{20}$ random library. DNAzyme 9$_{25}$-11 has a self-cleaving rate constant of 0.044 min$^{-1}$ at pH 7.5 and 24 ºC.$^{110}$ Although the catalytic rate constant was modest, the rate enhancement $k_{obs}/k_{uncat}$ of $10^9$ was remarkable and the idea of mimicking RNase-A with two functional groups that are found at the active site of RNase-A provided an important addition to the field of biomimetic catalysis. Following selection, the DNAzyme was synthesized by solid-phase synthesis and it was capable of catalyzing the trans-cleavage reaction.$^{138}$ The mechanistic study by Perrin et al. showed that this DNAzyme is a true RNase-A mimic.$^{139}$ In this study, the 2'-OH at the substrate cleavage site, which would normally be deprotonated by a general base, was replaced with an electrophilic bromoacetamide moiety.$^{139}$ The highly electrophilic 2'
bromoacetamide probe was well positioned to alkylate the DNAzyme in particular, to facilitate its identification (Figure 1.18).\textsuperscript{139} The mechanistic investigation implicated two modified imidazoles in general acid and base catalysis and one cationic amine in stabilizing the anionic phosphorane transition state.\textsuperscript{139} The chemical functionality in this case expanded the diversity of catalysis strategies for nucleic acid enzymes. With \textit{in vitro} selection methodology, the expanded chemical functionality gave a biomimetic catalyst which is different from natural nucleic acid enzymes and more like a protein enzyme.

\textbf{Figure 1.18} DNAzyme 9\textsubscript{25-11} with modified nucleotides and the mechanistic study and the proposed mechanism

Very similar modifications were used by Williams \textit{et al.} to select modified DNAzymes.\textsuperscript{133} Unlike the selection of DNAzyme 9\textsubscript{25-11}, in this selection the amine group was attached to 7-deaza-dATP, and the imidazole modified dUTP was the same one used by Joyce and coworkers.\textsuperscript{137} An all-RNA substrate was used in this selection, and the rate constant of this DNAzyme is 0.07 min\textsuperscript{-1} in the absence of divalent metal cations. However, when both modified
nucleotides, or either one was replaced by one or both natural nucleotides, the DNAzyme still retained considerable catalytic activity. Thus, the role of these modifications was not clearly understood. Moreover that DNAzyme cannot be converted to a trans-cleaver for multiple turnover.\textsuperscript{133}

Perrin and coworkers took the selection of modified DNAzyme even further with the selection of DNAzymes 9-86, 10-66 (\textbf{Figure 1.19}) and 12-91.\textsuperscript{115,116,140} Among these DNAzymes, a third modification, a positive charged guanidine group, was introduced besides these two functional groups in DNAzyme 9\textsubscript{25}-11. DNAzyme 9-86 has a catalytic rate constant of 0.134 min\textsuperscript{-1} at 24 °C, and is a divalent metal-independent DNAzyme as well. DNAzyme 10-66 was selected with a N\textsubscript{40} library. The catalytic rate constant is about 0.6 min\textsuperscript{-1} at 24 °C. DNAzyme 10-66 was examined for its trans-cleavage activity, and its derivative DNAzyme 10-66t is capable of cleaving at a single embedded RNA linkage with multiple turnover. DNAzyme 12-91 was selected with an all-RNA substrate, and it can cleave an all-RNA substrate with a rate constant of 0.06 min\textsuperscript{-1} in the absence of divalent metal cations, and cleave an embedded RNA substrate with a rate constant of 0.26 min\textsuperscript{-1} under optimal conditions. Although these DNAzymes have quite good activities in the absence of divalent metal cations, only DNA 10-66 has trans-cleavage activity and its second-order rate constants is not comparable with DNAzyme 10-23 and ribozymes under their optimal conditions. More efforts are needed in the field of modified DNAzyme to fulfill the requirement of pharmaceutical applications.
1.6 Pharmaceutical applications

The most important potential application of fast RNA-cleaving DNAzymes is to use DNAzymes \textit{in vivo} to down-regulate the gene expression.\textsuperscript{63} The therapeutic application of RNA-cleaving nucleic acids has been proposed and studied at various levels ever since the discovery of highly efficient DNAzymes. DNAzyme 10-23 and DNAzyme 8-17 have been mainly chosen for \textit{in vivo} study, because their low $K_m$ value imply high binding affinity, and the DNAzymes cofactor, Mg\textsuperscript{2+}, exists in cellular environment at a considerable concentration.\textsuperscript{141} Many studies have been conducted with these two DNAzymes to control the gene expression \textit{in vivo} and some studies have achieved some positive results such as tumor suppressions.\textsuperscript{142-144} Compared with other oligonucleotide-based gene silencing strategies, there are several advantages for using DNAzymes. First, DNAzymes do not require assistance from other enzymes in cells, and they can bind to and cleave RNA target in an RNase-independent manner (antisense technology...
require RNase-H for cleaving RNA). Second, DNAzymes are more physiologically stable than ribozymes and other RNA-based counterparts. Third, DNAs have economical advantage compared to RNAs. One disadvantage is that delivery of DNAzymes into cells has yet been well developed. Although no clinical trials have been reported till now, DNAzyme 10-23 has been utilized *in vivo* to cleave different RNA targets, such as vascular endothelial growth factor receptor 2, isocitrate lyase of *Mycobacterium tuberculosis*, hepatitis C virus.

The delivery of DNAzymes into cells was reported in a few articles. In one study, DNAzyme was encapsulated in microspheres that were made of poly-lactic acid and poly-glycolic acid. Poly-propylene imine was used in another study, these positively charged dendrimers can neutralize the negative charged DNA backbone. One promising method was reported that using nanoparticles made of carbohydrate-based polymer chitosan. The study showed apoptosis with DNAzyme, and the nanoparticles showed non-toxicity. Besides the delivery problem, DNAzyme must be stable in cellular environment. The DNAzyme is stable in term of self-degradation, but protein nucleases can digest DNAzyme in a very rapid fashion. To address this stability issue of DNAzyme in cellular environment, modified nucleotides can be used at two terminuses. The 2'-omethyl modified nucleotides at 5' terminus and the inverted nucleotides at 3' terminus were essentially utilized. These two modifications will increase the stability of oligonucleotides under physiological conditions and were widely used in antisense therapy to increase the stability of oligonucleotides as well.

**1.7 Research plan**

The object of this thesis is to develop all-RNA-cleaving DNAzymes with modified nucleotides that can be used in therapeutic application, as the previously selected DNAzymes discussed in section 1.5.3 are not suitable for this purpose. To develop a DNAzyme that cleaves an all-RNA substrate with a great $k_{cat}$ and a low $K_m$ value in the absence of divalent metals still represents a very important but challenging project. The same three modified nucleotides used in
the selection of the DNAzyme 10-66 will be used in this thesis. In Chapter 2, several modified
dUTPs were synthesized and the enzymatic incorporation of these modified dUTPs were
performed. The suitability of using these modified dUTPs in aptamer and DNAzyme selection
was evaluated. In Chapter 3, the selection of all-RNA-cleaving DNAzyme against a HIV RNA
target was performed and the evaluation of the selected DNAzyme via self-cleavage kinetic
studies was conducted. The \textit{trans}-cleavage reaction was conducted as well. In Chapter 4, another
all-RNA-cleaving DNAzyme selection against a c-Myc oncogene target was conducted, the
selected DNAzyme was evaluated by both self- and \textit{trans}-cleavage studies. The potential of using
this DNAzyme \textit{in vivo} was evaluated and the solid-phase synthesis of this DNAzyme was
attempted. In Chapter 5, a few novel selections of DNAzymes were conducted, however, no
DNAzyme was isolated successfully. In Chapter 6, the significance of the projects completed in
this thesis was summarised and the future possibilities of this work were discussed.
Chapter 2: Synthesis and enzymatic incorporation of modified dUTPs

2.1 Introduction

Applications and issues involved in in vitro selections with modified nucleotides were discussed in Chapter 1 (section 1.6). In the present chapter, the synthesis, enzymatic incorporation or selection of 5-position modified dUTPs will be discussed and described. These modified dUTPs can be classified in several categories, based on the linkage at the 5-position vinylic (2.1), alkynyl (2.2), alkylamino (2.3) or amide (2.4) (Figure 2.1).

![Figure 2.1 5-position modified 2'-deoxyuridine triphosphates](image)

The syntheses of modified dUTPs started with different precursors based on which linkage was to be installed. For instance, the allyl (2.1) and alkynyl (2.2) category can be synthesized via a coupling reaction between a 5-iodouridine and a suitably-protected alkene or alkyne. This robust synthetic methodology has been employed for the syntheses of 5-aminoallyl-deoxyuridine triphosphate and 5-aminopropargyl-deoxyuridine triphosphate, and both are commercially available. Furthermore, the amino group can serve as a handle for further synthetic functionalization of the pendant arm. These two types of modified nucleotides (2.1, 2.2) are generally good substrates for DNA polymerases, and widely used in modified DNAzyme selections. As an example, Perrin and coworkers pioneered the use of 5-aminoallyl-dUTP in
the selection of DNAzyme 9_{25-11}, which is the first divalent metal-independent modified DNAzyme (section 1.6).^{110}

\[ \text{Figure 2.2} \text{ Modified dUTPs synthesized by others} \]

Barbas and coworkers treated 5-aminoallyl-2′-deoxyuridine triphosphates with functionalized activated esters and made several modified nucleotide triphosphates (2.5).^{152} A brief incorporation test showed these modified dUTPs are good substrates for thermostable DNA polymerases and reverse transcriptase, and are suitable for \textit{in vitro} selection studies.^{152} One of these aminoallyl modified nucleotides afforded, in collaboration with Joyce’s group, was used to select the first modified DNAzyme (16.2-11) that was capable of cleaving RNA in the presence of Zn^{2+} (section 1.6).^{137} Williams \textit{et al.} employed the same modified dUTP (2.5) and selected for one divalent metal-independent RNA-cleaving DNAzyme.^{133} Sawai and coworkers further modified the aminoallyl linkage, culminating in the synthesis of amides 2.6, 2.7 and 2.8; furthermore, the enzymatic incorporation of the analogous triphosphates was studied with KOD Dash DNA polymerase.^{135,154} Their results showed that the DNA polymerase accepted the uridine analogues bearing sterically bulky and cationic groups at the 5-position, leading to the corresponding PCR product.^{154} However, a carboxyl group modified dUTP at the 5-position appeared to be poor substrate and the corresponding PCR products could not be obtained.^{154} Researchers also made modified dUTPs by Pd-mediated coupling with an alkynye-derived
functional group. Wang et al. made 2-cyanobenzothiazole (CBT) modified dUTP (2.9), which was then incorporated in DNA for post-synthetic modification. The CBT group can condense with cysteine, which affords a thiazole, which has numerous biological implications and is the basis for cross-linking reaction to proteins or other bioconjugates. Sugimoto and coworkers attached an N°-adeninylethyl group to the modified dUTP (2.10) and utilized it in aptamer selection. From this was selected, an aptamer which can detect the cytotoxic alkaloid camptothecin at sub-micromolar concentration.

Examples of alkylamino- and amide-based linkages (examples 2.3 and 2.4 in Figure 2.1) are much sparser in the literature. The first amide-linked dUTP was only reported in 2010, and the synthesis of the alkyl-linkage dUTPs is confounded by synthetic challenges. The synthesis of 5-aminomethyl-deoxyuridine triphosphate (2.3) was developed by Kolpashchikov et al. The amide can be built from the corresponding amine, by reacting 5-aminomethyl-deoxyuridine triphosphate with an activated ester (Figure 2.3a).

![Figure 2.3](image)

**Figure 2.3** Synthetic methods for 2.3 and 2.4

The synthesis of the amide functional group within the amide-linked d-UTPs (2.4; Figure 2.3b) was completed in one step from the corresponding 5-iodouridine via a coupling with tetrakis (triphenylphosphine) palladium(0), carbon monoxide and the desired amine by Eaton and
coworkers. The resulting nucleoside was subjected to triphosphorylation under standard “Eckstein” conditions (Figure 2.6). The Family B polymerases, Deep Vent and KOD XL, were found to be the most successful in generating full-length DNA with this type of modified dUTP. The modified DNA template could also be used in primer extension using dTTP to create unmodified DNA suitable for standard PCR using these polymerases. Gold et al. employed these modified dUTPs with hydrophobic functional groups in aptamer selections, and obtained aptamers possessing nanomolar binding affinities to a plethora of proteins.

As shown in this chapter, the modification at the 5-position may afford robust handles for modification of dUTP, both synthetically and biologically. Successful DNAzyme and aptamer selections have been demonstrated with 5-position modified dUTPs. Based on such ample precedence, the synthesis of several tryptophan, carboxylate and hydrophobic group functionalized dUTPs was undertaken. These efforts are detailed in the remainder of this chapter.

### 2.2 Objective

The synthesis of five modified dUTPs (Figure 2.5) is reported in this chapter, each commencing from 5-aminomethyl dUTP. These triphosphates were utilized in an enzymatic incorporation test to ascertain the potential of these modified dUTPs for selection. The functional groups appended were chosen for structural diversity, as well as similarity to the hydrophobic amino acids, phenylalanine (in the case of compound 2.11A and compound 2.11D) and tryptophan (compound 2.11B and compound 2.11E) and as well as the anionic amino acid aspartate (compound 2.11C). The terminal carboxylate group (compound 2.11C) is hypothesized to be a good ligand for Zn$^{2+}$, the divalent metal of choice for some nucleases which provide for very efficient catalysts for DNA cleavage.
2.3 Synthesis of modified dUTPs

2.3.1 Precursor synthesis

For the synthesis of the modified dUTPs, 5-aminomethyl-2′-deoxyuridine triphosphate was first synthesized according to previously described protocols. This robust synthesis, outlined in Figure 2.5, was originally developed by Kolpashchikov et al.\textsuperscript{153,161} Commercially available deoxythymine was subjected to DMT-protection of the 5′-OH group, followed by acetylation of the 3′-OH. The resulting intermediate was treated with \textit{N}-bromosuccinimide to afford the alkyl bromide, which was directly converted to the amine in one pot. This reaction typically shows a poor yield (~30%) due to conjugate elimination of the bromide, as well as undesired cleavage of the acid-labile DMT group. Trifluoroacetamide protection, followed by DMT deprotection afforded 2.12. Compound 2.12 was then subjected to triphosphorylation and followed by deprotection to afford 2.13.
2.3.2 Triphosphorylation

With the nucleoside 2.12 in hand, two triphosphorylation methods were attempted to obtain 2.13. Initially, I attempted a one pot, four-step synthesis developed by Ludwig and Eckstein (Figure 2.6). The nucleoside was first reacted with 2-chloro-4\textit{H}-1,3,2-benzodioxaphosphorin-4-one, followed by addition of bis-tributylammonium pyrophosphate to displace the salicylate intermediate. The resulting trivalent phosphite centre was oxidized by molecular iodine. Finally, the cyclic triphosphate was linearized, and global deprotection by aqueous ammonium hydroxide afforded the desired dUTP.

Each reaction in the for mentioned scheme was monitored by $^{31}$P-NMR spectroscopy, which confirmed the identity of the intermediates (Figure 2.7). The phosphorinone and pyrophosphate reagents were found at 147.4 ppm and $-9.3$ ppm, respectively, whereas the phosphorinone on the 5'-hydroxyl group manifested as a set of singlets at 126.5 and 125.5 ppm. The tributylammonium pyrophosphate addition gave rise to a triplet at 108.5 ppm and two multiplets for the phosphate group at $-19.7$ and $-20.2$ ppm. The crude product was purified by preparative TLC as previous reported.\(^1\)
The second method differs from the aforementioned one in that the reaction orders are inverted (Figure 2.8). The 2-chloro-4H-1,3,2-benzodioxaphosphorin-4-one was first reacted with bis-tributylammonium pyrophosphate, which was subjected to double displacement to give a triphosphate ring. The first advantage of this method was that the 2'-OH of nucleoside did not
require protection. Secondly, if the first reaction failed, which could be determined by $^{31}$P-NMR, the expensive modified nucleoside would not be wasted on the following reaction.

The $^{31}$P-NMR of the triphosphate ring exhibited a triplet at 98.5 ppm and two multiplets at -19.7 and -20.2 ppm for the phosphates. The suitably protected nucleoside was then reacted with the triphosphate ring, upon which the phosphite centre shifted to 108.5 ppm. The subsequent reaction iodine oxidation, followed by global deprotection was performed similarly to the previously described protocol, and the crude triphosphate was purified by TLC.

Figure 2.8 Triphosphorylation method-2 and $^{31}$P-NMR spectroscopy. A Reaction after the addition of phosphorinone (98.5 ppm triplet for phosphite, -20 ppm triplet for the phosphates). B Reaction after the addition of nucleoside (108.5 ppm triplet for phosphite, -20 ppm triplet for the phosphates).
2.3.3 Synthesis of modified dUTPs

With 2.13 in hand, the five desired amides were synthesized next by coupling with respective NHS-activated esters (Figure 2.9).\textsuperscript{164} These activated esters were in turn synthesized by the EDC-mediated coupling of NHS and the appropriate carboxylic acid. While these esters of 11A, 11B, 11D and 11E were used without further purification, significant di-activated material was observed in the case of the ester of 11C, which necessitated silica column chromatography. Compound 2.13 was coupled with each NHS ester separately, with 0.8 M aqueous sodium bicarbonate in water/DMF solution. After completion of each reaction as indicated by TLC, preparative TLC or HPLC were employed for purification.

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{synthesis_of_modified_dUTPs.png}
\caption{Synthesis of modified dUTPs}
\end{figure}

2.4 Enzymatic incorporation

After the synthesis and characterization of these five modified dUTPs, the incorporation test with a few DNA polymerases was conducted to evaluate the potential of the modified dUTPs for an \textit{in vitro} selection. A single nucleotide incorporation study with the Sequenase Version 2.0 DNA polymerase was conducted first. First, this polymerase was chosen due to its mild incorporation temperature of 37 °C. Second, Sequenase can incorporate 8-histaminyl dATP successfully, which is hypothesized to be responsible for the activity of RNA-cleaving DNAzymes (section 1.6).

To my dismay, all of the modified dUTPs incorporated equally poorly in the single nucleotide incorporation experiment, with minimal incorporation of even two modified dUTPs.
within a single sequence. To improve the incorporation of the modified nucleotides, I sought to employ one modified dUTP for screening against alternative DNA polymerases. From this experiment, the Therminator DNA polymerase was found to be optimal at incorporating the modified dUTP. However, this polymerase is known to have poor fidelity of incorporation and was deemed unsuitable for a selection.\textsuperscript{165}

For this reason, I redoubled my attention to Sequenase, thereby modifying the buffer pH and reaction temperature to favour the incorporation of modified dUTPs. Finally, an elongation in the presence of 5-allylamino dCTP, 8-histaminyl dATP, each of the modified dUTPs, as well as natural dGTP, in order to evaluate the feasibility of selection with multiple modifications.

### 2.4.1 Single nucleotide incorporation

In five separate single nucleotide incorporation experiments, each of the five modified nucleoside triphosphates was separately incorporated with primer and Template 1 in the presence of Sequenase (Figure 2.10). Template 1 contains five adenines consecutively, which requires the polymerase to elongate five modified dUTPs, making this a very challenging sequence to recopy into modified DNA. The results from this study, shown in Figure 2.9, indicated that Sequenase is only able to elongate one or two modified nucleotides, before stalling regardless of which modified dUTP is used. In the case of napthyl- and carboxylate-modified dUTPs (Lanes 4, 8 and 6, respectively, corresponding to compounds 6A, 6C and 6D), the incorporation of only one nucleotide was observed. To my dismay, the indole-modified 2.11B and 2.11E (lanes 5 and 7) showed incorporation of two modified nucleotide triphosphates, albeit as minor products, as indicated by the higher-running bands in the PAGE.
Figure 2.10 Single nucleotide triphosphates incorporation test with primer and Template 1. Lane 1, primer; lane 2, ddTTP; lane 3, dTTP; lane 4, 2.11A; lane 5, 2.11B; lane 6, 2.11C; lane 7, 2.11D; lane 8, 2.11E

2.4.2 DNA polymerases screening and optimization

DNA polymerases screening

In light of the failure of incorporating more than two modified dUTPs in the presence of Sequenase, alternative polymerases were sought as a remedy. Ideally, I desired a polymerase that would be active at physiological temperature, would have the ability to incorporate 8-histaminyl dATP as well as modified dCTP, with minimal truncation during incorporation, and good fidelity. Because the worst incorporation was observed with the napthyl modified dUTPs (in particular 2.11A), a screening for polymerases with 2.11A was commenced under standard conditions. The results are outlined in Figure 2.11, and indicate that, similar to Sequenase (Section 2.4.1 and lane 11 in Figure 2.11), most of the polymerases screened in this study only incorporated one modification (lanes 4-6 and 10).

Most notably, Therminator DNA polymerase, known for its broad substrate scope, did incorporate at least five modified nucleotides (lane 8). Similarly, Vent (exo-) DNA polymerase elongated five consecutive modified nucleotides (lane 7). KOD (lane 9) which incorporated two or three modifications.
Figure 2.11 DNA polymerases screening with 2.11A and Template 1. Lane 1, primer only; lane 2, ddTTP; lane 3, dTTP; lane 4, Tth; lane 5, Tfl; lane 6, Thermosequenase; lane 7, Vent(exo-); lane 8, Therminator; lane 9, KOD; lane 10, Klenow; lane 11, Sequenase Version 2.0

Although promising results were obtained with Vent (exo-), the inability of this polymerase to incorporate 8-histaminyl dATP (in contrast to Sequenase) would prohibit maximal its utility in an in vitro selection that features this modified dATP. As previously mentioned, Therminator DNA polymerase’s poor fidelity precludes its usage in selections,\textsuperscript{165} whereas KOD requires a non-physiological temperature for optimal activity.

Finally, I opted to employ Sequenase Version 2.0 DNA polymerase to optimize the conditions for incorporation, because of the limitations associated with each of the other polymerases that were screened. Despite the failure met in obtaining a polymerase that was better at incorporating dUTP, I envisaged that the results from this study could be used for a selection with only modified dUTP (this was attempted, albeit unsuccessfully).

**Concentration optimization**

Optimization studies commenced with the optimization of concentration on Template 1 (Figure 2.12). As observed in Figure 2.12, the polymerase is better able to incorporate the modified nucleotide at higher concentrations. However, as expected, no full-length product was observed due to the challenging template sequence.
Instead of using the challenging Template 1, I opted to employ the less demanding Template 2 for the optimization of concentration. This resulting sequence would contain eight modified nucleotide triphosphates in the full-length product, with only two consecutive modifications (Figure 2.13). In this experiment, it was found that the first truncation by Sequenase could be alleviated by using higher concentrations of modified nucleotide triphosphates. However, significant truncation was still observed at the position where two Us were incorporated. To confirm that the major product was the two-Us truncate, the gel contrast in Figure 2.13A was increased. This way, the truncation on each base position can be clearly observed (lane 7), and thus it is certain that the two consecutive truncations were causing stalling of the polymerase. To my delight, at 250 μM concentration of substrate, trace amounts of full-length product was indeed observed. Then the enzyme concentration was altered (Figure 2.13B). Although not as effective as increasing substrate concentration, increasing the enzyme concentration has led to more incorporation of the modified nucleotide.
Figure 2.13 Substrate and Sequenase concentration optimization with Template 2. A Substrate concentration optimization. Lane 1, primer; lane 2, ATGC(20 μM each); lane 3, 10 μM; lane 4, 25 μM; lane 5, 50 μM; lane 6, 100 μM; lane 7, 250 μM (20 μM AGC each). B Polymerase concentration optimization. Lane 1, primer; lane 2, ATGC (20 μM each, 2 units polymerase); lane 3, 1 unit; lane 4, 2 units; lane 5, 3 units; lane 6, 4 units; lane 7, 5 units (20 μM AGC + 50 μM compound 2.11A).

Temperature, time and reaction pH optimization

It has been shown that in some cases, the optimal temperature for incorporation of modified nucleotides using Sequenase is actually not the commercially prescribed 37 ºC. For instance, for the incorporation of 8-histaminyl dATP, 32 ºC was found to be the optimal
temperature (unpublished result). In line with these findings, the temperature for this incorporation was altered from the optimum temperature of 37 ºC. However, modification of the temperature in this case did not significantly impact the level of incorporation (Figure 2.14) and greater amounts of truncated product were produced compared to the full-length product. The optimal temperature was found to be 37 ºC.

![Temperature optimization with Template 2 and Sequenase Version 2.0. Lane 1, primer; lane 2, natural ATGC elongation; lane 3, 42 ºC; lane 4, 41 ºC; lane 5, 39.4 ºC; lane 6, 37.6 ºC; lane 7, 34.7 ºC; lane 8, 32.2 ºC; lane 9, 30.7 ºC; lane 10, 30 ºC.](image)

**Figure 2.14** Temperature optimization with Template 2 and Sequenase Version 2.0. Lane 1, primer; lane 2, natural ATGC elongation; lane 3, 42 ºC; lane 4, 41 ºC; lane 5, 39.4 ºC; lane 6, 37.6 ºC; lane 7, 34.7 ºC; lane 8, 32.2 ºC; lane 9, 30.7 ºC; lane 10, 30 ºC.

Although the foregoing experiments had all been conducted with a two hours incubation time (this is commonly used in the literature), I had observed that the majority incorporation was finished within the first five minutes. A more thorough time course study of incorporation was undertaken at this point (Figure 2.15). Here, it was found that longer times did not lead to significant changes in the yield of full-length product. Thus, the two hours reaction time was found to be optimal for this incorporation.
Finally, the reaction pH was optimized from the commercially available reaction buffer, which is at pH 7.5. It was found that the optimal pH was 7.5, as well. Higher pH values led to more truncation, whereas lower pH did not promote the incorporation, either (Figure 2.16).

**Figure 2.15** Time optimization with Sequenase Version 2.0. Lane 1, primer; lane 2, natural ATGC elongation; lane 3, 5 minutes; lane 4, 10 minutes; lane 5, 35 minutes; lane 6, 65 minutes; lane 7, 90 minutes; lane 8, 120 minutes; lane 9, 180 minutes; lane 10, 240 minutes

**Figure 2.16** pH optimization with Sequenase Version 2.0. Lane 1, primer only; lane 2, pH 6.5; lane 3, pH 7.0; lane 4) pH 7.5; lane 5, pH 8.0; lane 6, pH 8.5; lane 7, ATGC

**Full-length elongation**

With the optimized conditions for incorporation in hand, I sought to conduct a full-length extension on Template 2 using each of the modified dUTPs that had been synthesized previously
Template 2 contains eight modified residues, and therefore I had anticipated a low melting point for such an oligonucleotide. A low \( T_m \) could promote undesired denaturation of the duplex, prior to completion of elongation, which would lead to truncation. Unfortunately, I found that none of the compounds \( 2.11A-E \) gave any full-length product in the optimized conditions with Sequenase (Figure 2.17). Although the lone-modified U is incorporated well, the incorporation of several consecutive-modified Us is challenging, and leads to significant truncation.

![Figure 2.17](image)

**Figure 2.17** Full-length incorporation with Template 2 and Sequenase Version 2.0 polymerase. Lane 1, ATGC; lane 2, primer only; lane 3, AGC + 2.11B; lane 4, AGC + 2.11A; lane 5, AGC + 2.11C; lane 6, AGC + 2.11D; lane 7, AGC + 2.11E

In light of these disappointing results, full-length elongation with Vent (exo-) DNA polymerase was conducted. Although it was known that modified dATP is a poor substrate for Vent (exo-), I nevertheless chose to pursue this polymerase in hopes of conducting a singly modified (dUTP) selection. From earlier experiments, it was known that Vent (exo-) DNA polymerase incorporates naphthyl 2.11A well (Figure 2.11, lane 7). Unsurprisingly, full-length product was observed for this incorporation (lane 3, Figure 2.18), with no truncation at all. Similar results were found for 2.11C and 2.11D (lanes 5 and 6, Figure 2.18) wherein no
truncation was found. Although 2.11B and 2.11E produced full-length product, some minor truncations were observed (lanes 4 and 7, Figure 2.18). In summary, Vent (exo-) DNA polymerase was found to be a good polymerase for the modified dUTPs synthesized in this project.

Figure 2.18 Full-length incorporation with Template 2 and Vent (exo-) DNA polymerase. Lane 1, ATGC; lane 2, primer only; lane 3, AGC + 2.11A; lane 4, AGC + 2.11B; lane 5, AGC + 2.11C; lane 6, AGC + 2.11D; lane 7, AGC + 2.11E.  

The underlying hypothesis of this thesis is that the inclusion of more than one modified nucleoside in a DNAzyme would result in greater structural diversity, thereby leading to improved catalytic activity in selections. To test this hypothesis, two other modified dNTPs, 8-histaminyl dATP and 5-allyl-amino dCTP (Section 1.6), were elongated in one primer elongation assay using each of the five synthetically accessed dUTPs (2.11A-E; Figure 2.19) in the presence of Sequenase. Unfortunately, none of the five dUTP modifications (2.11A-E) led to any full-length product (lanes 3-7). As expected for Sequenase, the major truncation was at the double-A position.

In light of the positive results in Figure 2.18, I hoped to use Vent (exo-) for a similar incorporation study. Although it was known that 8-histaminyl dATP is difficult to incorporate with this polymerase,166 I hoped that the benefits conferred by this polymerase would outweigh
the truncates expected at dATP. Unfortunately, no full-length product was found with this polymerase, either, and complete truncation at the modified A was observed (lane 8). This result confirms the earlier conclusion that Vent (exo-) is a poor polymerase for the incorporation of modified dATP.

Figure 2.19 Three modified nucleotide triphosphates elongation for potential selection (colour indicates modified NTPs). Lane 1, primer only; lane 2, ATGC; lane 3, 8-histaminyl dATP + 5-allyl-amino dCTP + dGTP + 2.11B; lane 4, 8-histaminyl dATP + 5-allyl-amino dCTP + dGTP + 2.11A; lane 5, 8-histaminyl dATP + 5-allyl-amino dCTP + dGTP + 2.11D; lane 6, 8-histaminyl dATP + 5-allyl-amino dCTP + dGTP + c2.11C; lane 7, 8-histaminyl dATP + 5-allyl-amino dCTP + dGTP + 2.11E; lane 8, Vent (exo-) with 8-histaminyl dATP + 5-allyl-amino dCTP + dGTP + 2.11B.

2.5 Discussion and conclusion

The purpose of the present chapter was to explore functionalized dUTPs for use in future selections. Five modified deoxyuridine triphosphates analogues containing various functional groups, which may be interesting for aptamer and DNAzyme selections, were synthesized. Initially, enzymatic incorporation was carried out by single nucleotide incorporation assays using 2.11A-E. The five modified nucleotide triphosphates were incorporated with Sequenase Version 2.0 DNA polymerase and the recommended reaction conditions. Unfortunately, each of the
substrates proved to be quite poor for Sequenase Version 2.0 DNA polymerase at the initially-tested conditions.

Thus, I chose to screen for other DNA polymerases to improve the incorporation of modified dUTPs. Thermally-stable DNA polymerases Therminator, KOD and Vent (exo-) were found to give improved incorporation, as compared to Sequenase Version 2.0. These results signify that a singly-modified dUTP selection could be conducted using these polymerases.

Nevertheless, as my goal was a three-modified nucleotide selection, and thermally stable polymerases, such as Vent (exo-) DNA polymerase, are known to incorporate of 8-histaminyl dATP poorly, we assumed that the utility of these nucleotides would be minimal especially if no other functionalities could be incorporated. Furthermore, there are concerns pertaining to the fidelity of thermally stable DNA polymerases and thus Theminator DNA polymerase was not chosen. Therefore, I again turned my attention to Sequence Version 2.0 DNA polymerase, and decided to further optimize the reaction conditions to improve incorporation. Indeed, it is known that 5-aminomethyl-dUTP modified with a tyrosine-like functional group is not a good substrate for Sequenase under commercially mandated reaction conditions. I opted to utilize only 2.11A for polymerase screening and optimization of conditions. Only one of the modified nucleotide triphosphates was chosen for optimization. I rationalized that each of the synthesized dUTPs are sterically similar to one another, and would be treated similarly by DNA polymerases. Despite my efforts in optimizing the reaction conditions, no full-length product was observed with Sequenase using any of the modified dUTPs.

Finally, the incorporation of modified dUTPs were tested in the presence of modified dATP and dCTP to prepare full-length product which would be highly modified. Unfortunately, multiple modifications’ incorporation presents a challenge for both Sequenase and thermally stable polymerases. In conclusion, a selection featuring multiple modifications under the conditions screened is not feasible.
2.6 Experimental section

2.6.1 Materials and methods

General: All starting materials were purchased from Sigma-Aldrich, Alfa Aesar and Fisher Scientific, and used without further purification, unless noted. NMR spectra were recorded on a Bruker Avance 300 Spectrometer, ESI mass spectra were recorded on Bruker Esquire-LC, and HPLC was performed on an Agilent 1100 series instrument. UV spectra were recorded on a Beckmann DU 800 spectrophotometer.

HPLC purification was performed on an Agilent 1100 system using a Phenomenex Jupiter 10μ C4 300A column at a flow rate 1 mL/min. HPLC solvents containing two buffer systems: A, 50 mM triethylammonium acetate (pH 7.0) in ACN/water (1:1); B, 50 mM triethylammonium acetate (pH 7.0) in water. The purification was conducted with a solvent gradient as outlined in Table 2.1.

Table 2.1 HPLC solvent system

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>0</th>
<th>10</th>
<th>18</th>
<th>19</th>
<th>24</th>
</tr>
</thead>
<tbody>
<tr>
<td>% ACN/water</td>
<td>0</td>
<td>1</td>
<td>25</td>
<td>50</td>
<td>50</td>
</tr>
</tbody>
</table>

2.6.2 Synthesis

![Chemical Structure](image)

Chemical Formula: C₁₄H₁₆F₃N₃O₇
Molecular Weight: 395.29

Compound 2.12 was synthesized follow the method developed by Kolpashchikov et al and modified by previous lab member.¹⁶¹ ¹H-NMR (300 MHz, CDCl₃, 25°C): δ=8.76 (s, 1H, H₄), 8.25 (s, 1H, H₃), 7.60–7.50 (m, 1H, H₁), 6.26 (m, 1H, H₅), 5.39–5.34 (m, 1H, H₇), 4.23–4.13 (m,
Compound 2.13 was synthesized by the following triphosphorylation methods.

Method 1: Method 1 followed the same procedure described by previous lab members.\(^{161}\) The synthesis and purification gave rise to 4 \(\mu\)mol (8% yield). Compound 2.13 can be quantified by the extinction coefficient of 8600 \(\text{cm}^{-1}\text{M}^{-1}\) at 268 nm.\(^ {153}\) MS (ESI\(^+\)): \(m/z=418.4\) (M+Na).

\[
\begin{align*}
\text{H}_2\text{N} & \quad \text{N} \\
\text{O} & \quad \text{NH}
\end{align*}
\]

2.13

Chemical Formula: \(\text{C}_{10}\text{H}_{18}\text{N}_3\text{O}_{14}\text{P}_3\)

Molecular Weight: 497.18

Method 2: Tri-\(n\)-butylamine (50 \(\mu\)L) was transferred to the NMR tube containing tributylammonium pyrophosphate (35.6 mg, 75 \(\mu\)mol) dissolved in anhydrous DMF (150 \(\mu\)L). The reaction mixture was added to 2-chloro-4-\(H\)-1,3,2-benzodioxaphosphorin-4-one (11 mg, 55 \(\mu\)mol) dissolved in DMF (150 \(\mu\)L) with vigorous stirring. After 30 minutes, \(^{31}\text{P}\)-NMR indicated the formation of a cyclic triphosphite product (98.5 ppm). A solution of compound 2.12 (19.8 mg, 50 \(\mu\)mol) dissolved in 1,4-dioxane (345 \(\mu\)L) and pyridine (115 \(\mu\)L) was added to the reaction mixture. The reaction was stirred for one hour, after which I\(_2\) (19.9 mg, 79 \(\mu\)mol) in H\(_2\)O/pyridine (1/49, 1000 \(\mu\)L) were added. The solution was stirred over 20 minutes, and excess I\(_2\) was quenched by the drop by drop addition of aqueous sodium bisulfite (5%) until the disappear of I\(_2\) color. The reaction was transferred into a 25 mL flask and evaporated to dryness. The resulting solid was resuspended in H\(_2\)O (5 mL) and left to stand at room temperature for 30 minutes. Ammonium hydroxide (10 mL, 30%) was added to the mixture. After stirring at room temperature for an additional 3 hours, the solvent was evaporated to dryness. Then the solid was
resuspended in a minimal amount of water. The crude product was purified by preparative-TLC using an eluent of dioxane/H₂O/NH₄OH (6:4:1) to afford the title compound which was quantified by UV spectroscopy (12% yield).

Compound **2.11A**: 2-Napthyl acetic acid (0.382 g; 2 mmol), N-hydroxysuccinimide (0.253 g; 2.2 mmol) and EDC.HCl (0.421g; 2.2 mmol) were added to a 25 mL round-bottom flask, followed by the addition of 15 mL of DCM. The reaction was stirred at room temperature overnight, after which TLC (EtOAc/hexane=1/1, Rf=0.4) analysis indicated completion. An additional 15 mL DCM was added, and the reaction mixture was washed with saturated NaHCO₃ (3×20 mL), 1M HCl (3×20 mL), dried over MgSO₄, filtered, and evaporated to dryness *in vacuo*. TLC indicated the purity of the compound, MS (ESI⁺): 306.6 (M+Na). The NHS ester (2.5 µmol) was added to 5 µL DMF, 250 nmol compound **2.13** in 10 µL H₂O, and 10 µL 0.8 M NaHCO₃. A mixture of the three solutions was stirred for 2 hours, after which TLC analysis (dioxane/H₂O/NH₃.H₂O=6/4/1) indicated completion, The reaction mixture was purified on preparative TLC (dioxane/H₂O/NH₃.H₂O=6/4/1; Rf=0.18). The synthesis and purification gave rise to 22 nmol (9%). Compound **2.11A** can be quantified by the extinction coefficient of 21000 cm⁻¹M⁻¹ at 280 nm. MS (ESI⁺): *m/z*=732.1 (M+3Na-2H), 754.0 (M+4Na-3H).
Compound 2.11B: Compound 2.11B was synthesized in the similar manner to compound 2.11A. Indole-3-acetic acid (0.350 g; 2 mmol) N-hydroxysuccinimide (0.253 g; 2.2 mmol) and EDC.HCl (0.421 g; 2.2 mmol) were used to synthesize the indole-3-acetic acid NHS ester. MS (ESI\(^{+}\)): \(m/z=295.3\) (M+Na). Then 250 nmol compound 2.13 in 10 \(\mu\)L H\(_2\)O, and 10 \(\mu\)L 0.8M NaHCO\(_3\) were reacted with 2.5 \(\mu\)mol the NHS ester in 5 \(\mu\)L DMF. The reaction mixture was loaded on preparative TLC; (dioxane/H\(_2\)O/NH\(_3\).H\(_2\)O=6/4/1; \(R_f=0.17\)). The synthesis and purification gave rise to 25 nmol (10% yield). Compound 2.11B can be quantified by the extinction coefficient of 14300 cm\(^{-1}\)M\(^{-1}\) at 279 nm. MS (ESI\(^{+}\)): \(m/z=721.1\) (M+3Na-2H), 743.0 (M+4Na-3H).

Compound 2.11C: Compound 2.11C was synthesized in the similar manner to compound 2.11A. Terephthalic acid (0.332 g; 2 mmol), N-hydroxysuccinimide (0.230 g; 2.0 mmol) and DCC (0.412 g; 2.0 mmol) were added together, followed by the addition of 15 mL of dioxane. The reaction was stirred at room temperature overnight, after which TLC
(EtOAc/hexane/MeOH=4/4/0.5) analysis indicated completion. The solvent was evaporated by rotovap. The solid was dissolved in 20 mL DCM and the undissolved solid was filtered. The solution was washed with 1M HCl (3×20 mL) and dried over MgSO₄. The solution was concentrated and purified by on a flash chromatography column (12.5×2.5 cm column). The product was eluted with EtOAc/hexane/MeOH (4:4:0.5). MS (ESI⁻): \( m/z = 262.4 \) (M-H). Then 250 nmol compound 2.13 in 10 µL H₂O, and 10 µL 0.8M NaHCO₃ were reacted with 2.5 µmol the NHS ester in 5 µL DMF. The reaction mixture was loaded on preparative TLC; (dioxane/H₂O/NH₃.H₂O=6/4/1; Rf=0.13). The synthesis and purification gave rise to 19 nmol (8% yield). Compound 2.11C can be quantified by the extinction coefficient of 17500 cm⁻¹M⁻¹ at 260 nm. MS (ESI⁺): \( m/z = 652.1 \) (M+Li), 674.1 (M+Na+Li).

![Chemical Structure](image)

**Chemical Formula:** C₂₂H₂₆N₃O₁₅P₃

**Molecular Weight:** 665.38

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**Compounds 2.11A**. 1-Naphtyl acetic acid (0.382g; 2 mmol), N-hydroxysuccinimide (0.253 g; 2.2 mmol) and EDC.HCl (0.421g; 2.2 mmol) were used to synthesize the 1-naphtyl-acetic acid NHS ester. MS (ESI⁺): \( m/z = 306.6 \) (M+Na). Then 250 nmol compound 2.13 in 10 µL H₂O, and 10 µL 0.8M NaHCO₃ were reacted with 2.5 µmol the NHS ester in 5 µL DMF. The reaction mixture was loaded on preparative TLC; (dioxane/H₂O/NH₃.H₂O=6/4/1; Rf=0.18). The synthesis and purification gave rise to 28 nmol (11% yield). Compound 2.11D can be quantified by the extinction coefficient of 20700 cm⁻¹M⁻¹ at 280 nm. MS (ESI⁻): \( m/z = 663.9 \) (M-H), 685.9 (M+Na-2H).
Compound 2.11E: Compound 2.11E was synthesized in a similar manner to compound 2.11A. Indole-3-propanoic acid (0.378g; 2 mmol), N-hydroxysuccinimide (0.253 g; 2.2 mmol) and EDC.HCl (0.421g; 2.2 mmol) were used to synthesize the indole-3-acetic acid NHS ester. MS (ESI⁺): \( m/z = 287.3 \) (M+H). Then 250 nmol compound 2.13 in 10 µL H₂O, and 10 µL 0.8M NaHCO₃ were reacted with 2.5 µmol the NHS ester in 5 µL DMF. The reaction mixture was purified by preparative TLC (dioxane/H₂O/NH₃.H₂O=6/4/1; Rf=0.17), to afford the title compound in 25 nmol (10% yield). Compound 2.11E can be quantified by the extinction coefficient of \( 14300 \text{ cm}^{-1}\text{M}^{-1} \) at 279 nm. MS (ESI⁻): \( m/z = 667.1 \) (M-1H), 689.1 (M+Na-2H).

2.6.3 Enzymatic incorporation

All incorporations were performed using autoclaved materials. Water was treated with diethyl pyrocarbonate (1 µl/10 mL) prior to autoclaving. Natural dNTPs and Klenow were purchased from Fermentas. T4 polynucleotide kinase, Vent (exo–), Therminator and Taq DNA polymerase were purchased from New England Biolabs. Sequenase version 2.0 and Thermosequenase DNA polymerase were purchased from Affymetrix. KOD DNA polymerase was purchased from EMD Millipore. Tfl and Tth DNA polymerase were purchased from Promega. \(^{32}\text{P}-\gamma\text{-ATP} \) was purchased from Perkin Elmer. Radioactivity was visualized using a Typhoon 9200 variable mode imager from GE Healthcare. All DNA oligonucleotides were purchased from Integrated DNA Technologies.

Solutions/buffers.
Loading solution: Formamide (27 mL), EDTA\textsubscript{(aq)} (3 mL, 0.5 M), xylene cyanol\textsubscript{(aq)} (300 μL, 0.05 %) and bromophenol blue\textsubscript{(aq)} (300 μL, 0.05 %).

Elution buffer: 1 % LiClO\textsubscript{4}, 10 mM Tris in water, pH 8.0

Oligonucleotides

Primer: 5'-TAATCGGGAAGGTCAGGGGGGAAAAGAAAA-3'

Template 1: 5'-CCTCGACAGGTAGAATCAATGAAAAATTTTCTTTTCCCCCCTGACCTTCCCGATT-3'

Template 2: 5'-CCTCGACATCTAGAATCAATGACCGGTTTTCTTTTCCCCCCTGACCTTCCCGATT-3'

Labelling of primer at the 5' end

Primer (2 nmol), polynucleotide kinase (PNK) buffer (4 μL, 10X), $^{32}$P-$\gamma$-ATP (25 μCi, 1 μL), PNK (1 μL) was diluted to a final volume of 40 μL. The reaction was incubated at 37°C for 2 hours and then terminated by heating at 65°C for 20 minutes. Loading solution (40 μL) was added to the reaction, and the resulting mixture was purified by denaturing PAGE (15 %). Product was identified by UV shadowing and cut out. Gel material was crashed into fine particle with a flame-sealed pipette tip and eluted with elution buffer at 65°C 10 minutes. The liquids were evaporated and ethanol precipitated. Finally, the isolated solid was re-suspended in 40 μL water and desalted with a G25 spin column, quantified by UV.

Primer extension

Reactions were prepared in a final incorporation volume of 10 μL. 5'-$^{32}$P-labelled primer (Primer, 3 pmol) was annealed with the template (3 pmol) in the presence of enzyme buffers. Pyrophosphatase (0.2 μL/reaction), and dithiothreitol (DTT) (0.4 μL/reaction) were added to the annealed oligonucleotides as a cocktail. The nucleotide triphosphates concentration was 50 μM unless indicated. Experiments were incubated at different temperature for different DNA polymerase. The enzymes were added last; 1–6 units of enzyme were used. After the incubate,
loading solution (20 μL) was added to each reaction and partial sample was loaded on denaturing PAGE (13 %) was carried out. The rest polymerases the elongation temperature are shown in Table 2.2.

**Table 2.2 DNA polymerase elongation temperature**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Tth</th>
<th>Tfl</th>
<th>Thermosequenase</th>
<th>Vent (exo-)</th>
<th>Therminator</th>
<th>KOD</th>
<th>Klenow</th>
</tr>
</thead>
<tbody>
<tr>
<td>$T_{\text{el}}$</td>
<td>70</td>
<td>74</td>
<td>74</td>
<td>75</td>
<td>75</td>
<td>75</td>
<td>37</td>
</tr>
</tbody>
</table>
Chapter 3: RNA-cleaving DNAzyme selection against HIV RNA target

3.1 Introduction

3.1.1 HIV mRNA as a therapeutic target

The RNA genome of the human immunodeficiency virus (HIV) type 1 contains about 9200 nucleobases, and encodes 15 different types of proteins.\textsuperscript{168} The RNA starts and ends with a long terminal repeat (LTR) which is the control element for gene expression of the viral proteins.\textsuperscript{168} The HIV-1 LTR, is used by the virus to enable it to insert its genetic material into the host genome.\textsuperscript{168} Since the HIV-1 LTR regulates the viral genome, suppressing the expression of LTR mRNA leads to the suppression of HIV viral life cycle, thus validating it as a good therapeutic target.\textsuperscript{169} Besides attacking LTR mRNA transcription, maturation or translation, methods of attacking other steps of HIV life cycle can lead to suppression.\textsuperscript{170} One effective method of treatments is antiretroviral therapy (ART) which has reduced a significant amount of mortalities of people with HIV virus and increased their life spans.\textsuperscript{171} The method can prevent the growth of HIV by blocking some steps of HIV life cycle. For example, one of the drug used in ART, Zidovudine, is a nucleoside reverse transcriptase inhibitor that blocks the reverse transcriptase enzyme that builds HIV DNA.\textsuperscript{172} This type of drugs belongs to a family of compounds of nucleoside analogues. The HIV RNAs of patients who were receiving antiretroviral therapy were suppressed to undetectable levels when measured using conventional clinical assays. However, the residual HIV RNAs were detectable with ultrasensitive assays.\textsuperscript{173} Other protocols have to be developed to achieve the goal of more effectively treating HIV. As was discussed the potential applications of DNAzymes in Chapter 1, a DNAzyme that can cleave HIV LTR RNA could provide an alternative way to suppress the HIV.
3.1.2 RNA-cleaving DNAzyme for potentially treating HIV

Chapter 1 briefly described the potential therapeutic applications of DNAzyme 10-23 (Section 1.6). Although there were a number of DNAzymes that can catalyze RNA cleavage, only DNAzyme 10-23 and DNAzyme 8-17 were considered to be potential therapeutic agents for the following reasons: First, unlike some DNAzymes that can only cleave embedded RNA target, DNAzyme 10-23 and DNAzyme 8-17 are all-RNA-cleaving DNAzymes. Second, in comparison to a Pb²⁺-dependent DNAzyme, DNAzyme 10-23 and DNAzyme 8-17 require Mg²⁺ cofactors that exist within cellular environment. Lastly, DNAzyme 10-23 and DNAzyme 8-17 have very strong binding affinities with their RNA targets, which gives them nanomolar $K_m$ values. Because the concentration of RNA target in cells can be very low, DNAzymes with low binding affinity have little potential therapeutic applications. However, both DNAzymes still rely on a considerably higher concentrations Mg²⁺ than exist within cellular environment. Modified DNAzymes were found that can cleave all-RNA target in the absence of divalent metal. Additionally, a modified DNAzyme that has the catalytic efficiency of DNAzyme 10-23 in the absence of divalent metal would be a significant first step to obtain a therapeutic DNAzyme.

3.2 Objective of this project

The goal of this project was to use combinatorial selection with modified nucleotide triphosphates to discover all-RNA-cleaving DNAzymes against the HIV-LTR RNA sequence. Once an efficient self-cleaving DNAzyme was obtained, the enhanced DNAzyme would be used to examine the trans-cleavage activity and the possibility of using it in vivo. To obtain the desired DNAzyme, three modified nucleotide triphosphates were considered: $dU^{\beta\beta\beta}TP$ (1), $dC^{\alpha\alpha\alpha}TP$ (2), and $dA^{\alpha\alpha\alpha}TP$ (3). Multiple assays were conducted to evaluate the catalytic efficiency of the DNAzyme.
Figure 3.1 Chemical structures of dUgaTP (1), dCaaTP (2), and dAimTP (3)

3.3 In vitro selection

3.3.1 Selection process

The selection of a RNA-cleaving DNAzyme was performed using the commonly-used streptavidin-biotin methodology as shown in Figure 3.2. A biotinylated primer (Primer 1) containing 17 nt RNA target was polymerized against a template containing 30 degenerate positions, in the presence of the three modified nucleotide triphosphates (Figure 3.1) and dGTP in order to synthesize a library of modified oligonucleotides with sequences of greater than $10^{13}$. The library had $4^{30}$ possible sequences which could form secondary structures that would support catalytic activity. The biotin binds extraordinarily strongly to streptavidin beads and allowed the primer extension products to be immobilized on streptavidin beads. The immobilized double-stranded nucleic acids were briefly treated with 0.1 M sodium hydroxide to remove the non-biotinylated template strand. The remaining single-stranded modified oligonucleotides were incubated in an environment of 50 mM sodium cacodylate buffer pH 7.4, 200 mM NaCl, and 1 mM EDTA. The EDTA ensured that no divalent metals were involved in the selection. Subsequently, the modified nucleotides were collected from the eluted selection buffer, purified by PAGE, and amplified using PCR. The 1st PCR amplification products were loaded on PAGE, which guaranteed the correct PCR products were produced. The 2nd PCR amplification synthesized enough material for next round of selection.
**Figure 3.2** RNA-cleaving DNAzyme selection. (•), RNA region; (N\textsubscript{30}), N\textsubscript{30} random library; ball (B), biotin; red colour N\textsubscript{30} library; pink colour, elongated DNA with modified nucleotide triphosphates.

In theory, the library initially contains very few specific sequences that have the desired activity, particularly during the earlier rounds of selection. If these inactive sequences are inefficiently removed then the number of selection rounds required is increased and some cases inefficiency may even prevent the selection from being successful. In selecting with modified nucleotides, the selection becomes even more complicated. It is generally known that modified oligonucleotides are poor substrates for polymerase used in the reamplification step.\textsuperscript{134}
Therefore, PAGE was used to increase the efficiency of selection by further separating the cleaved strand from any contaminating uncleaved strands that escaped streptavidin capture.

The selection was conducted by gradually reducing the incubation time and cleavage buffer composition in favour of lower ionic strength. Specifically, the reaction time was decreased from 60 minutes to 5 minutes over the selection course to increase the time stringency (Table 3.1). However, in all selection rounds the percentage of cleavage was examined at 60 minutes. The sodium chloride in the cleavage buffer was decreased from 200 mM to 10 mM to favour more stable catalysts that can operate in very low ionic strengths (Table 3.2). The incubation times and salt concentration in these two tables directly affect the percentage of cleavage in Figure 3.3. A total of 22 rounds of selection were performed.

**Table 3.1** Supernatant was collected after the indicated incubation time at each round of selection.

<table>
<thead>
<tr>
<th>Time (minutes)</th>
<th>5</th>
<th>10</th>
<th>60</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rounds</td>
<td>17-22</td>
<td>5-15</td>
<td>1-4, 16</td>
</tr>
</tbody>
</table>

**Table 3.2** Sodium chloride concentration used in each round of selection

<table>
<thead>
<tr>
<th>NaCl concentration (mM)</th>
<th>10</th>
<th>20</th>
<th>100</th>
<th>200</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rounds</td>
<td>16, 21</td>
<td>15</td>
<td>13, 14</td>
<td>1-12, 17-20, 22</td>
</tr>
</tbody>
</table>

Incubation time was generally employed as a selection tool to enrich the pool for a fast cleaver. In theory, when the supernatant was collected after a shorter incubation time, the percentage of cleavage at 60 minutes should increase in the following round. For instance, the time for cleavage was reduced (stringency was increased) in round 5, the percentage of cleavage after 60 minutes did not increase significantly in round 6; however, a significant increase was observed in round 7. In the same manner, the time stringency was increased in round 17, but for unknown reasons, no significant improvement was achieved in the following rounds. This failure to improve activity may be due to the fact that a stringent incubation of 5 minutes did not significantly differ from that of a 10 minutes incubation.

The monovalent metal cation concentration was also decreased to favour the appearance of better cleavers. In theory, during rounds conducted with low metal cation concentrations (10 or
20 mM), the percentage of cleavage after 60 minutes should be relatively low. However, the percentage of cleavage should increase significantly when the metal cation concentration is brought back to the initial level (200 mM). If the application of low salt is effective, changes should be observed when the sequences are exposed to low salt followed by high salt conditions. For example, from round 14 to 16, the salt concentration was reduced from 100 mM (round 14) to 20 mM (round 15) and then to 10 mM (round 16), therefore, the percentage of cleavage after 60 minutes decreased from ~11% (round 14) to 6% (round 15) and then to 5% (round 16). However, after the salt concentration was brought back to 200 mM in round 17, the percentage of cleavage increased appreciably (20%, Figure 3.3).

![Figure 3.3](image.png)

**Figure 3.3** Rounds of RNA-cleaving DNAzyme selection. The percentage of cleavage was examined after 60 minutes incubation time

### 3.3.2 Mutagenic PCR

A highly diverse library is important not just for the beginning of a selection, but also for when the selection starts showing certain amounts of activity. If the library has limited diversity, the selection would not improve. The disadvantage of modified oligonucleotides is that the more modified nucleobases that a sequence has, the less likely it will be amplified in 1st PCR.
amplification step. It is necessary to continually introduce new sequences, through mutagenic PCR, to compensate for the loss of diversity due to selection. Keeping a diverse library allows for a greater chance of highly active sequences. If the introduction of diversity is related to the active sequences in the library, this will lead to a preferential exploration of sequences that are more likely to result in an improved sequence. The more active sequences will have a larger number of copies and turn into a new generation of parental sequences.

Vent (exo-) DNA polymerase that was used in the 1st amplification and 2nd amplification steps has an intrinsic error rate of $10^{-6}$ to $10^{-9}$ per nucleotide. However, for short oligonucleotides (30mer), this error rate is too low to produce any new sequences that as mutants might exhibit increased activity. A good error rate should produce one mutation per copy. There are several standardized protocols that utilize either altered reaction conditions or a mutant thermostable DNA polymerase to promote mutations during elongation and PCR cycles. Ideally a few mutations should be or will be introduced at the PCR amplification step. The most commonly-used technique is error-prone PCR which relies on altered reaction conditions and unbalanced concentrations of the four dNTPs to achieve an error rate of 0.007 per nucleotide. An alternative protocol, hypermutagenic PCR, relies on unbalanced concentrations of four dNTPs and divalent metal cations. The error rate is about 0.1 per nucleotide and there is some substantial sequence bias.

Experiments were performed to introduce mutations using hypermutagenic PCR conditions during the selection process. Within the 2nd amplification step, one 100 μL non-mutagenic 2nd PCR and one 100 μL hypermutagenic PCR were prepared separately and were then combined to form a new library. However, in creating this new library, the mutagenic PCR reduced the copy number of active strands and the percentage of cleavage in next round of selection. Nevertheless, the percentage of cleavage would be expected to increase to the initial level or higher within the following rounds. If the mutagenic PCR and selection were effective,
this pattern of decreasing and increasing the percentage of cleavage should be observed. For example, mutagenic PCR was performed in round 7, then the cleavage percentage was observed to decrease in round 8 followed by an increase in the subsequent rounds (Figure 3.3). Additional mutagenic PCR were performed in rounds 12, 15, 18. With the assistance of stringent salt concentrations, time stringency, and increasing diversity, the cleavage increased to more than 20% at 60 minutes, at which point the selection was considered as a successful selection and terminated for cloning.

3.3.3 Sequences analysis

After completing 22 rounds of selection, the activities in rounds 18-22 were compared to determine the best generation from which individual sequences were to be cloned. Round 22 showed the fastest cleavage rate in both 5 minutes and 60 minutes was subsequently chosen for cloning. The sequences obtained from cloning are shown in Table 3.3. The average length of the random region was 30.4 nucleotides, and the average number of modified residues was 19.3. This is a moderate difference from the statistically random number of 22.9. The average number of modified As was 4.2, which is significantly lower than the statistically expected number of 7.6 modified As if the distribution had been entirely random. The average number of modified Ts (9.5) was in fact slightly higher than the expected average. This result is consistent with the hypothesis that the modified dATP is disfavoured in both the incorporation step and the transcription step by DNA polymerases. However, an average number of 9.5 modified Ts in the modified library indicated that the modified T was perfectly well incorporated and amplified by the DNA polymerase used in this selection.
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<th># of modification</th>
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Table 3.3 Clone sequences isolated from the selection

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By comparing all the clones in Table 3.4, several patterns were observed among the sequences. First, not many sequences were duplicated among all the clones. For instance, only clones 03, 16 and 51 were found to be identical; clones 07 and 55 shared the same sequence. This result showed that the selection library in round 22 was still very diverse, which was unexpected. Second, although almost all the clones were unique; a high degree of similarity was observed among some of the clones. Nearly one third of the sequences started with TTATAGT at the 5' terminus, while 10 additional sequences started with TTACTGT. The first 25 nucleobases were the same among clones 14, 37, 60 and 64. Besides these similarities, some sequences shared the same subsequence at the 3' terminus. More than ten sequences contained the GAGTTT at the 3' terminus, while several others contained the GTGTCCC at the 3' terminus. There were some sequences that did not share similarity with any other sequences. Some had aberrant sequences, which probably were inactive because of the irregularity and how these inactive sequences found their way through 21 rounds of selection remains unclear.
A kinetic analysis of a portion of the clones (5 time points per clone) was done to obtain some preliminary data (Table 3.4). Table 3.4 additionally shows cleavage yields for these clones after 180 minutes incubation time.

**Table 3.4** Clone sequences chosen for kinetic studies. Only the catalytic regions are shown. Clone 41 and 48 were added with one thymidine at the 5' terminus to form clone 41' and 48'.

(NR; no reaction detected)

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<td>TTATAGTGTGGCCTGCTGCTGATGCTGCCGT</td>
<td>NR</td>
<td>0</td>
</tr>
<tr>
<td>57</td>
<td>TTATAGTGTGGCCTGCTGCTGATGCTGCCGT</td>
<td>NR</td>
<td>0</td>
</tr>
<tr>
<td>58</td>
<td>TTATAGTGTGGCCTGCTGCTGATGCTGCCGT</td>
<td>NR</td>
<td>0</td>
</tr>
<tr>
<td>59</td>
<td>TTATAGTGTGGCCTGCTGCTGATGCTGCCGT</td>
<td>NR</td>
<td>0</td>
</tr>
<tr>
<td>60</td>
<td>TTATAGTGTGGCCTGCTGCTGATGCTGCCGT</td>
<td>NR</td>
<td>0</td>
</tr>
<tr>
<td>61</td>
<td>TTATAGTGTGGCCTGCTGCTGATGCTGCCGT</td>
<td>NR</td>
<td>0</td>
</tr>
<tr>
<td>62</td>
<td>TTATAGTGTGGCCTGCTGCTGATGCTGCCGT</td>
<td>NR</td>
<td>0</td>
</tr>
<tr>
<td>63</td>
<td>TTATAGTGTGGCCTGCTGCTGATGCTGCCGT</td>
<td>NR</td>
<td>0</td>
</tr>
<tr>
<td>64</td>
<td>TTATAGTGTGGCCTGCTGCTGATGCTGCCGT</td>
<td>NR</td>
<td>0</td>
</tr>
<tr>
<td>65</td>
<td>TTATAGTGTGGCCTGCTGCTGATGCTGCCGT</td>
<td>NR</td>
<td>0</td>
</tr>
<tr>
<td>66</td>
<td>TTATAGTGTGGCCTGCTGCTGATGCTGCCGT</td>
<td>NR</td>
<td>0</td>
</tr>
</tbody>
</table>
Clones containing TTATAGT at 5' terminus were grouped, and arranged from the highest rate constant to the lowest constant (shown in Table 3.5). The fastest cleaver, clone 25, was chosen as a reference sequence (red, Table 3.5). The comparisons between clone 25 and other clones showed a rough pattern. The more similar a given clone was to clone 25, the higher rate constant it had. For clone 41 (green), adding one T at 5' terminus (41') increased the rate constant more than 1000 times, which may indicate that the T must be very crucial for the activity of the TTATAGT family.

Table 3.5 “TTATAGT” family. Only the bases that differ from clone 25 are shown. Bases that are identical notated with a dot. “_” was added where necessary to align the sequences with clone 25 properly. (NR; no reaction detected)

<table>
<thead>
<tr>
<th>Clone #</th>
<th>Catalytic region</th>
<th>$k_{obs}(\text{min}^{-1})$</th>
</tr>
</thead>
<tbody>
<tr>
<td>25</td>
<td>TTATAGTCGTTGCGAGTCTTTTGAGGTA</td>
<td>3.3</td>
</tr>
<tr>
<td>01</td>
<td>........A.........</td>
<td>1.44</td>
</tr>
<tr>
<td>52</td>
<td>........A...........CG..TGA..TT</td>
<td>1.28</td>
</tr>
<tr>
<td>41'</td>
<td>........A..A.....C....CG..TGA..TT</td>
<td>1.04</td>
</tr>
<tr>
<td>05</td>
<td>........A...........C.....G..TGA..TT</td>
<td>0.94</td>
</tr>
<tr>
<td>07/55</td>
<td>........T..........C.....G..TGA..TT</td>
<td>0.86</td>
</tr>
<tr>
<td>58</td>
<td>........G..A........G.A.G.A.GCCGTTG</td>
<td>0.015</td>
</tr>
<tr>
<td>13</td>
<td>........G..A....T.G.A.GCTTG..AT_</td>
<td>0.014</td>
</tr>
<tr>
<td>57</td>
<td>.................G.A.GCTCC..GTT</td>
<td>0.0084</td>
</tr>
<tr>
<td>63</td>
<td>........T.........G.A.G..TGTCGC_</td>
<td>0.0052</td>
</tr>
<tr>
<td>06</td>
<td>........G..A....T.GTA.G..TGCCCG_</td>
<td>0.0049</td>
</tr>
<tr>
<td>50</td>
<td>........G..A....TAGG.ACGT.T..CGGA</td>
<td>0.0022</td>
</tr>
<tr>
<td>41</td>
<td>........A..A.....C....CG..TGA..TT</td>
<td>0.00045</td>
</tr>
<tr>
<td>31</td>
<td>........T.......T....G.A.G...GCCGAT</td>
<td>NR</td>
</tr>
<tr>
<td>43</td>
<td>........G..A....G..ACTTTGGAGTA__</td>
<td>NR</td>
</tr>
<tr>
<td>66</td>
<td>........T.......T.G.ACGCATGT.AGT</td>
<td>NR</td>
</tr>
</tbody>
</table>

These clones employing TTACA at 5' terminus were grouped. A similar table (Table 3.6) was created in the same manner with Table 3.5 using clone 3 (red) as a template. Among these clones no similar pattern (TTATAGT family) was found. For instance, clone 30 only differs with clone 03 by two bases; however, clone 30 has 10^4 times slower rate constant.
Table 3.6 “TTACA” family. The table was created with the similar manner of “TTATAGT” family. (NR; no reaction detected)

<table>
<thead>
<tr>
<th>Clone #</th>
<th>Sequence</th>
<th>k_{obs}(min^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>03/51/16</td>
<td>TTACAGTGGTAGCGGCGTGACAGTGTAGTGGTT</td>
<td>0.71</td>
</tr>
<tr>
<td>30</td>
<td>........T........................................</td>
<td>0.000053</td>
</tr>
<tr>
<td>32</td>
<td>........T...T...TT..........................</td>
<td>NR</td>
</tr>
<tr>
<td>48</td>
<td>____________<strong>.T.G..T.C..CG..C</strong></td>
<td>NR</td>
</tr>
<tr>
<td>48’</td>
<td>____________<strong>.T.G..T.C..CG..C</strong></td>
<td>0.003</td>
</tr>
<tr>
<td>56</td>
<td><strong><strong>A.C</strong></strong>____.T.G..T.G..TCCC__</td>
<td>NR</td>
</tr>
<tr>
<td>39</td>
<td>......A.......A.G...TG.GTGA.G...............</td>
<td>0.000093</td>
</tr>
<tr>
<td>08</td>
<td>......C.T....T.G..T.C.GTGCTCC</td>
<td>0.015</td>
</tr>
<tr>
<td>20</td>
<td>______________.TT.GCACGGAG.GAGCC</td>
<td>0.017</td>
</tr>
</tbody>
</table>

3.4 Analysis of clone 25

3.4.1 Self-cleavage kinetics

The preliminary data (Table 3.4) showed that clone 25 had the fastest cleavage rate. A more detailed kinetic assay in which 14 time points were collected was done to determine a more precise cleavage rate constant. A notable feature of the data set when fitted to either a single-exponential equation or a double-exponential equation was that the pre-exponential term, which represented the fraction of DNAzyme cleaved compared to that approximated at infinity, had an apparent value that is significantly lower than unity. This indicated that a significant fraction of the DNAzyme was inactive, which may be due to misfolding or misincorporation of a modified base. Correction for these inactive DNAzymes should increase the amplitude of the graph and raise the value of the pre-exponential term to a value of one. Such a correction is commonly used in both ribozyme and DNAzyme kinetic analysis. However, fitting the amplitude to the experimental value did not affect the calculated rate constant. Rate constants were first determined using a single exponential equation (Equation 3.1). The single-exponential equation gave a correlation coefficient 0.88 indicating a bad fit.

\[ P = P_0 \left(1 - e^{-k_{obs} \cdot t}\right) \]  

\[ P = P_1 \left(1 - e^{-k_{obs \_1} \cdot t}\right) + P_2 \left(1 - e^{-k_{obs \_2} \cdot t}\right) \]  

Equation 3.1

Equation 3.2

Thus, the double-exponential equation (Equation 3.2) was employed to recalculate the rate constants for each phase. This exercise greatly improved the correlation coefficient (0.996),
indicating a much better fit. While the data appear to fit a double-exponential curve, indicating the reaction is biphasic, the majority of the active DNAzymes (~60%) are fast-cleaving species; the slow-cleaving species are only about 40% of the active DNAzyme. The fraction cleaved after 3 hours is approximately 71%. The rate constant of fast phase is 3.3±0.5 min\(^{-1}\), and slow phase is 0.13±0.04 min\(^{-1}\). Both the fast phase curve and slow phase curve are shown in Figure 3.4.

**Figure 3.4** Self-cleavage kinetics of DNAzyme clone 25. Experiments were performed in 50 mM sodium cacodylate pH 7.4, 200 mM NaCl, 1 mM EDTA at room temperature. Time points were collected at 0, 0.033, 0.083, 0.167, 0.333, 0.5, 1, 2, 3, 5, 10, 30, 60, 180 minutes. \(k_{obs}=3.3±0.5\) min\(^{-1}\) (R\(^2\) > 0.99). Red curve is the real date fitting curve. Green curve is the slow-phase cleavage curve. Blue curve is the fast-phase cleavage curve.
3.4.2 Predicted secondary structure of DNAzyme clone 25

The sequence of the self-cleaving species Clone 25 was put into mFOLD with the following parameters: 200 mM Na\(^+\) and 0.5mM Mg\(^{2+}\) at 30 °C. The secondary structure is shown in Figure 3.5, which gave the lowest folding energy. One notable point is that the four nucleobases of the designed cleavage site formed base pairs with the catalytic region. Nucleobases C8, C9 and G10 formed base pairs with G56, G55 and C54 respectively, and U11 formed a mismatch with G53 (Figure 3.5). It was found that the cleavage site was at the phosphate linkage between C8 and C9. One could imagine that these 3 G-C base pairings and one G-T mismatch may be crucial for DNAzyme to maintain cleavage activity. The four nucleobases GCGG (53-56) of the self-cleavage construct correspond to nucleobases 12-15 of the catalytic region, as shown in Figure 3.5.

![Predicted secondary structure of DNAzyme clone 25 by mFOLD](image)

**Figure 3.5** Predicted secondary structure of DNAzyme clone 25 by mFOLD. The secondary structure contains three stem loops – one engineered (I) and two arising from the catalytic region (II and III). Modified bases are indicated by coloured letters. The pink letters represent the all-RNA target.
3.4.3 Kinetics

pH-rate profile

The pH dependence of the DNAzyme clone 25 was investigated in order to understand the mechanism of catalysis and, thereby, infer the role the modifications could play. Two possible pH-rate profiles are seen for DNAzymes and ribozymes: log-linear and bell-shaped. For example, the hammerhead ribozymes have been found to be log-linear profiles which indicates a single proton transfer mechanism. Secondly, a bell-shaped pH-rate profile could indicate that the catalysis undergoes a two-step general acid/general base mechanism. Bell-shaped profiles were found in several DNAzymes discovered by the Perrin lab. The pH-rate profile study was subsequently performed at pH 6.5, 7.0, 7.5, 8.0, 8.5, and 9.0. The rate constants for the fast phase were calculated and are shown in Table 3.7. A graph was obtained by fitting the rate constants into Equation 3.3 (Figure 3.6). The corresponding bell-shaped profiles indicated the cleavage reaction undergo a general acid/base mechanism. The rate constant reaches maximum around pH 7.8. The pKa is 7.3 and the pKa' is 8.3.

Table 3.7, Catalysis rate constant under variant pH conditions.

<table>
<thead>
<tr>
<th>pH</th>
<th>6</th>
<th>6.5</th>
<th>7</th>
<th>7.5</th>
<th>8</th>
<th>8.5</th>
<th>9</th>
</tr>
</thead>
<tbody>
<tr>
<td>$k_{obs}$ (min$^{-1}$)</td>
<td>0.29</td>
<td>0.44</td>
<td>1.36</td>
<td>3.3</td>
<td>3.38</td>
<td>1.37</td>
<td>0.68</td>
</tr>
</tbody>
</table>

$$k_{obs} = \frac{K_{max}}{1 + 10^{(pKa - pH)} + 10^{(pH - pKa')} + 10^{(pKa - pKa')}}$$

Equation 3.3
Figure 3.6 The cleavage rate dependence on pH and temperature of DNAzyme clone 25. Experiments were performed in a selection cleavage buffer. $K_{\text{max}}=6.3$ min$^{-1}$ ($R^2=0.95$), $E_a=11.8$ kcal/mol

**Temperature profile**

Temperature dependence is a very important component for catalysis. By measuring rate constants under different temperatures, the activation energy can be determined by fitting the data to the Arrhenius equation (Equation 3.4).

$$\ln(k_{\text{obs}}) = \ln A - \frac{E_a}{R \cdot T}$$  \hspace{1cm} \textit{Equation 3.4}

$E_a$ is the activation energy and $A$ is the pre-exponential factor.

The selection was conducted at room temperature; however, as the final purpose work would be to use DNAzymes to cleave RNA at 37 °C. The temperature profile was conducted to evaluates the potential of using DNAzyme clone 25 in vivo. For a DNAzyme, the catalytic process involves hydrogen bonds between the two binding arm and forming two double strands at the pre-cleavage step. When the temperature rises to a point that is higher than the $T_m$ of these double strands, the cleavage will be disturbed. The temperature profile was conducted at 5 °C, 15 °C, 20 °C, 25 °C, 30 °C, 35 °C and 45 °C. The rate constants at different temperatures are shown in Table 3.8. A linear fitting was employed with the data between 5 °C to 25 °C, which gave an activation energy of 12±2 kcal M$^{-1}$ (Figure 3.6).
Table 3.8 Temperature profile

<table>
<thead>
<tr>
<th>T(K)</th>
<th>278.15</th>
<th>288.15</th>
<th>293.15</th>
<th>298.15</th>
<th>303.15</th>
<th>308.15</th>
<th>318.15</th>
</tr>
</thead>
<tbody>
<tr>
<td>1/T(K&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>0.0036</td>
<td>0.00347</td>
<td>0.00341</td>
<td>0.00335</td>
<td>0.0033</td>
<td>0.00325</td>
<td>0.00314</td>
</tr>
<tr>
<td>k&lt;sub&gt;obs&lt;/sub&gt;(min&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>1.00</td>
<td>1.80</td>
<td>3.3</td>
<td>4.22</td>
<td>3.85</td>
<td>2.24</td>
<td>2.10</td>
</tr>
<tr>
<td>ln(k)</td>
<td>0</td>
<td>0.59</td>
<td>1.19</td>
<td>1.44</td>
<td>1.35</td>
<td>0.81</td>
<td>0.74</td>
</tr>
</tbody>
</table>

Divalent metal profile

DNAzyme clone 25 was further characterized in terms of the effects that divalent metal cations would have on the self-cleavage reaction. Generally divalent metals can facilitate the formation of secondary structure in DNA, and if the secondary structure is catalytically active, it may increase the cleavage activity, leading to a higher rate constant. Conversely it is possible that divalent metal cations may bind to the imidazole group, which plays a very important role in the cleavage. This interaction may change the secondary structure and lead to the loss of activity.

Table 3.9 shows the rate constants under the selection buffer that included a selection of alkaline earth metals, transition metals, lanthanide cations and imidazole (each at 0.5 mM) individually. No significant promotion on the rate constants was observed with all the tested divalent metal cations, except for Cu<sup>2+</sup> and Hg<sup>2+</sup>, which were found to inhibit the self-cleavage. Since Hg<sup>2+</sup> and Cu<sup>2+</sup> can inhibit the reaction, the lowest concentrations that can be used to experimentally detection inhibition of cleavage were examined. It was found that at a concentration lower than 0.5 mM Cu<sup>2+</sup> or Hg<sup>2+</sup>, the cleavage was inhibited only partially (data not shown).
Table 3.9  Effect of metal cations on the cleavage rate of DNAzyme clone 25. (n.d. no cleavage was detected)

<table>
<thead>
<tr>
<th>Metal</th>
<th>$k_{obs}$ (min$^{-1}$)</th>
<th>$k_{rel}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer only</td>
<td>3.3</td>
<td>1</td>
</tr>
<tr>
<td>Mg$^{2+}$</td>
<td>3.18</td>
<td>0.97</td>
</tr>
<tr>
<td>Zn$^{2+}$</td>
<td>3.57</td>
<td>1.09</td>
</tr>
<tr>
<td>Ca$^{2+}$</td>
<td>4.27</td>
<td>1.30</td>
</tr>
<tr>
<td>Mn$^{2+}$</td>
<td>5.06</td>
<td>1.54</td>
</tr>
<tr>
<td>Cu$^{2+}$</td>
<td>n.d.</td>
<td>--</td>
</tr>
<tr>
<td>Hg$^{2+}$</td>
<td>n.d.</td>
<td>--</td>
</tr>
<tr>
<td>Ba$^{2+}$</td>
<td>4.63</td>
<td>1.41</td>
</tr>
<tr>
<td>Ni$^{2+}$</td>
<td>1.99</td>
<td>0.60</td>
</tr>
<tr>
<td>Co$^{2+}$</td>
<td>3.51</td>
<td>1.07</td>
</tr>
<tr>
<td>UO$_2^{2+}$</td>
<td>1.79</td>
<td>0.54</td>
</tr>
<tr>
<td>Fe$^{3+}$</td>
<td>4.31</td>
<td>1.31</td>
</tr>
<tr>
<td>Ce$^{3+}$</td>
<td>0.91</td>
<td>0.28</td>
</tr>
<tr>
<td>Imidazole*</td>
<td>1.76</td>
<td>0.53</td>
</tr>
</tbody>
</table>

Modified base replacement test

DNAzyme clone 25 was synthesized with the three modified nucleotide triphosphates. After the kinetic assays, the modified base replacement assays were investigated to examine whether the modifications were absolutely necessary for cleavage activity. First, the DNAzyme was synthesized by using two of the three modified nucleotides and the remaining two canonical nucleotide triphosphates, and subjected to self-cleavage test (three different combinations). Figure 3.7 shows that all the modifications are necessary for DNAzyme clone 25 to maintain the activity. Finally the DNAzyme was synthesized with modified uridines, modified cytosine, natural guanidine and 8-(3-(4-imidazolyl)aminopropyl)-2’-dATP (d$^{homo}$ ATP) (Figure 3.7C) and subjected to self-cleavage test. The d$^{homo}$ ATP contains an imidazole group that would be similar with the modified adenosine used in this selection. The synthesized DNAzyme clone 25 with d$^{homo}$ ATP showed no cleavage activity.
Figure 3.7 Self-cleavage of modified base replacement test. A Modified DNAzyme synthesized by single modification replacement. B Modified DNAzyme synthesized with dC\textsuperscript{sa}TP, dU\textsuperscript{pa}TP and dA\textsuperscript{homo}TP. C Structure of dA\textsuperscript{homo}TP. Six time points were collect at 0,1,3,10,30 and 60 minutes for all four tests.

Embedded RNA cleavage test

It is common that RNA-cleaving DNAzymes have lower rate constants when cleaving an all-RNA target compared with a target with only embedded-RNA linkage.\textsuperscript{116,180} In this case, the selection was conducted with an all-RNA target. Thus, it may be interesting to know whether DNAzyme clone 25 can cleave embedded RNA substrate with a higher rate constant. Self-cleavage of DNAzyme clone 25 was performed using an DNA target containing four embedded-ribophosphodiester linkages. The cleavage reaction was also determined to be a biphasic reaction,
in which the fast phase accounts for the majority of the reaction ($k_{obs}=1.16\ \text{min}^{-1}$). This rate constant differed significantly from the rate constant obtained with the all-RNA substrate. Additionally, the PAGE analysis indicated that cleavage proceeded at only one site instead of two sites observed for the all-RNA target (gel not shown), which may be one reason that DNAzyme 25 has a lower rate constant for embedded-RNA cleavage.

**Sequence specificity cleavage test**

In this chapter, HIV all-RNA target was utilized in the selection, and DNAzyme clone 25 was selected. Then it was engineered to cleave the Myc oncogene RNA target in Chapter 4. The gel image of self-cleavage reaction is shown in **Figure 3.8**. No cleavage product was detected after 120 minutes incubation time, which may indicate that the DNAzyme clone 25 is sequence-specific.

![Figure 3.8](image)

**Figure 3.8** Self-cleavage of DNAzyme clone 25 with a different all-RNA target. Time points were collected at 0, 1, 3, 10, 30, 60, 120 minutes.

**3.5 Trans-cleavage reaction study**

The trans-cleavage studies were conducted following the self-cleavage assays in order to evaluate the potential of using DNAzyme clone 25 in vivo. Multiple turnover studies were conducted to obtain the $k_{max}$ and $K_m$ of DNAzyme clone 25. Additional experiments involving
several divalent metals were examined to observe any inhibitory or additive effect. At the end, the trans-cleavage experiments were conducted at 37 °C.

**Multiple-turnover study**

The multiple-turnover studies were performed with 50 nM, 100 nM, 300 nM, 500 nM, 1000 nM, 2000 nM and 4000 nM substrate, and 25 nM DNAzyme in the presence of 500 µM Mg$^{2+}$ at 30 ºC. The calculated rate constants under different substrate concentrations are shown in Figure 3.9. At conditions 2000 nM all-RNA, the substrate started to show degradation (undesired cleavage) at several different sites, which prevented a precise rate constant being obtained. Due to this fact, no values for $k_{max}$ and $K_m$ could be calculated.

![Diagram](image)

**Figure 3.9** Multiple-turnover studies of DNAzyme clone 25. **A** Trans-cleavage construct. **B** Trans-cleavage at 2000 nM RNA substrate concentration. Time points were collected at 5, 10, 30, 60, 180, 300 and 1260 minutes. **C** Rate constants at different substrate concentrations
Trans-cleavage reactions were then conducted at 37 °C, which showed very limited cleavage. The self-cleavage reaction reached the maximum rate constant at 25 °C. It is understandable that the trans-cleavage rate constant will drop at 37 °C compared to that of 30 °C. Unfortunately, such low catalytic efficiency at 37 °C sabotaged the in vivo application of this DNAzyme.

Effects of divalent metal cations

As was seen in previous studies (Table 3.9), at concentration greater than 0.5 mM, Hg²⁺ and Cu²⁺ can inhibit the self-cleavage reaction. The trans-cleavage reaction was tested at 300 nM all-RNA substrate, 25 nM DNAzyme in the presence of various divalent metal cations (500 µM). The rate constants are shown in Table 3.10. Interestingly, Ca²⁺ and Mn²⁺ can significantly increase the rate constant.

Table 3.10 Effect of metal cations on the trans-cleavage rate of DNAzyme clone 25

<table>
<thead>
<tr>
<th>Metal (0.5 mM)</th>
<th>$k_{obs}$ ($10^{-2}$ mol⁻¹ min⁻¹)</th>
<th>$k_{rel}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mg²⁺</td>
<td>1.86</td>
<td>1.0</td>
</tr>
<tr>
<td>Mn²⁺</td>
<td>14.3</td>
<td>7.7</td>
</tr>
<tr>
<td>Zn²⁺</td>
<td>3.87</td>
<td>2.1</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>15.2</td>
<td>8.2</td>
</tr>
<tr>
<td>Cu²⁺</td>
<td>0.6</td>
<td>0.33</td>
</tr>
</tbody>
</table>

3.6 Discussion

3.6.1 General discussion

A selection with modified nucleotide triphosphates is more complicated in all steps of the selection. In the selection, the sequences that contained fewer modified nucleotides are more likely to survive in both the elongation and first amplification steps. Although dA¹mTP is a very poor substrate for polymerase, it was used in the selection. Any sequences containing three or more consecutive adenosines could not be produced by elongation. Additionally, it is known that modified oligonucleotides are poor templates for polymerase. The active species may have been lost in both the selection and amplification steps because of the abovementioned reasons. If the cleavage was detectable in the selection and the active species were enriched, the selection...
would be successful from this viewpoint. Notably the objective of this project was to obtain an all-RNA-cleaving DNAzyme with a higher rate constant than that of DNAzyme 12-91 and DNAzyme Clone 25 which had a significantly higher rate constant lead to achieving the goal of the project.116

3.6.2 Selection analysis

Ideally, all inactive species should be removed within the first round, resulting in a fair amount of activity in the following round. In practice, very faint activity was detected in the third round, which is still quite early. The fact that cleavage was observed in such early rounds, suggested that there were active sequences in the original library. The percentage of cleavage increased significantly in rounds 4 and 5. This rapid increase in activity may indicate either that the selection may produce a few highly active DNAzymes or the selection gene pool was mainly occupied by the moderately active species within a couple of rounds. This selection showed promising results in round 4. In the attempts to pursue fast cleaving species the time stringency was increased in round 5 and in round 7 the percentage of cleavage in 60 minutes doubled (Figure 3.3). Therefore, increasing time stringency proved to be very effective.

Because the correct secondary structure of active species could lead to the cleavage activity and metal cations can facilitate the folding of DNA sequence, if one active sequence could fold properly under low ionic strength, it was hypothesized that it could fold into the proper secondary structure faster under high ionic strength, which may provide a higher cleavage rate constant. Therefore, the metal concentration was introduced as a strategy in the selection. The sodium chloride concentration was decreased by a factor of 2 in round 13, and the percentage of cleavage within 60 minutes did not change significantly. A much lower metal cation concentration (10 mM) was used in round 16, in which the yield decreased to 5%. Even in the later stage of selection, the cleavage yield only reached 7% under low metal cation concentration. When the metal cation concentration was brought back to the initial level (200 mM), the cleavage
yield increased nearly fourfold from 5% to approximately 20%. This low metal cation stringency strategy certainly helped the selection proceed to a higher level of activity.

As was previously discussed (section 3.3.2), the diversity of library played a key role in selection. Mutagenic PCR was introduced in round 7 and the percentage of cleavage fell by more than 50% in round 8. Because the cleavage yield had reached 13% in round 7 and the highest cleavage yield was approximately 20% in the whole selection (Figure 3.3), it could not be known whether the active species was produced by mutagenic PCR. However, the mutagenic PCR did help the library maintain the diversity.

3.6.3 Secondary structure of DNAzyme clone 25

DNAzyme clone 25 contains three stem loop structures according to mFOLD (Figure 3.5). Loop I was deliberately engineered to distance the all-RNA target from the DNAzyme catalytic region. Loop II and Loop III are part of the N30 region and constitute the catalytic motif. The modified side chain cannot be considered by the mFOLD program, yet clearly the positively-charged groups of the side chains must have a significant influence on the DNAzyme structure. This secondary structure only provided some structural hypotheses of the sequence. To solve the real three-dimensional structure, much work needs to be done by using NMR or X-ray crystallography. The predicted structure showed that the designed cleavage site formed Watson-Crick base pairs with the catalytic region. It was also found that this sequence (GCGG) was highly conserved around bases 12-15 among almost all the screened clones. Clones that did not contain GCGG in this range were either very slow cleavers or had no activity. This phenomenon was not seen with DNAzyme 925-11, DNAzyme 10-66 or DNAzyme 12-91.110,116,140

3.6.4 Kinetics

Although the DNAzyme clone 25 had a bell-shape pH-rate profile, it had a slight difference from some modified DNAzymes exhibiting bell-shape pH-rate profiles, such as DNAzyme 9-86, DNAzyme 10-66, and DNAzyme 12-91.115,116,140 The pH-rate profile of
DNAzyme clone 25 contained a much sharper peak, resulting in the two calculated pKa values of ~7.3 and 8.3. Since the pKa of imidazole is approximately 7, the protonation states of the imidazole groups on the DNAzyme will vary between pH 6 and 8. The indicated two-step general acid/general base mechanism is likely to be played by two imidazole groups. It is worth noting that there are five imidazole groups in the catalytic region. Without more studies it cannot be concluded which imidazole acts as the general base or general acid. It might also be possible that the imidazole can facilitate the folding of the correct DNA conformation. The pH could change the charged status of imidazole leading to the decrease of catalytic activity. More kinetic studies can be done to address the role of imidazole in the catalysis. The guanidine group of the modified uridine and the amino group of the modified cytosine would be protonated in the range of pH 6-9. Therefore, besides facilitating the folding of the DNAzyme, these two groups could act as a general acids and provide a proton to the 5'-oxygen; however, the imidazole group would be more suited to play this role. Furthermore, since there are many guanidine and amino groups in the catalytic region and it is challenging to elucidate the role of every one.

DNAzyme clone 25 reach the maximum rate constant at 25 ºC, which is approximately the temperature the selection was conducted. Modified DNAzymes were found to have different optimal temperatures. The optimal temperature for DNAzyme 9-86 and DNAzyme 12-91 was determined to be 13 ºC. It was found that for DNAzyme 9-86 the optimal temperature was at 37 ºC. The enthalpy of activation calculated from temperature profile was slightly less than those of DNAzyme 9-86 (15.3 kcal/mol) and DNAzyme 12-91 (13.7 kcal/mol). The lower activation energy resulted a higher rate constant for DNAzyme clone 25. The modified nucleotide triphosphates used in selecting DNAzyme 9-86, DNAzyme 12-91 and DNAzyme clone 25 were identical. All three DNAzymes contain quite a significant number of positively charged groups, which were designed to stabilize the structure of DNAzyme. The thermostabilities of these three DNAzymes were not identical.
3.6.5 Nucleoside replacement studies

From the modified base replacement test (Figure 3.7), it can be concluded that the modifications are absolutely necessary for cleavage activity. From the three single modification replacement tests, it can be noticed that the elongation had a much better full-length product when not using dA\textsuperscript{imp}TP. Both the dC\textsuperscript{aa}TP and dU\textsuperscript{imp}TP did not cause significant amounts of truncation. These two charged modified groups were involved in folding the three-dimensional structure folding, the loss of these charged groups probably changed the three-dimensional structure and led to the loss of cleavage activity. When the dA\textsuperscript{imp}TP was replaced with the dA\textsuperscript{homo}TP, which had a longer linkage, the cleavage activity of DNAzyme clone 25 was lost entirely. A similar result was also observed with the nucleotide replace experiment of DNAzyme 9-86.\textsuperscript{115} However, for the other DNAzyme 20-49, the replacement of dA\textsuperscript{imp}TP with dA\textsuperscript{homo}TP did not lead to the loss of activity.\textsuperscript{116}

3.6.6 Trans-cleavage study

The overall results for the trans-cleavage studies were somewhat unsatisfactory. The rate constant of trans-cleavage was much lower than that of self-cleavage reaction. The binding affinity of DNAzyme clone 25 was quite low resulting in a relatively high $K_m$. The long loop I was specifically engineered to distance the all-RNA target from the catalytic region. Therefore, if the catalytic region is able to overcome the barrier of distance caused by loop I, bind to the all-RNA target and operate self-cleavage successfully, it is anticipated that the DNAzyme will be similarly successful with a trans-cleavage target. Although the self-cleavage rate constant reached 3.3 min\textsuperscript{-1}, the trans-cleavage performance was poor, which indicated that the loop design did not act as expected in this case. The reason of this poor trans-cleavage performance could due to the fact that the binding arm contain several modified nucleotides (Figure 3.5), which might destabilize the base pairing.
3.7 Conclusion

In this chapter a DNAzyme was successfully selected that can cleave all-RNA target with an apparent rate constant of 3.3 min\(^{-1}\). The DNAzyme had a bell-shape pH-rate profile and was inhibited by 0.5 mM Cu\(^{2+}\) or Hg\(^{2+}\). All the modifications were critical for DNAzyme to maintain the catalytic activity. The trans-cleavage studies showed that the DNAzyme was a bad trans-cleaver. The DNAzyme did not reach maximum rate constant under 2 µM and the all-RNA substrate started to show degradation at high substrate concentration. With these considerations DNAzyme clone 25 will not be considered for \textit{in vivo} studies.

3.8 Materials and methods

3.8.1 Materials and equipment

dA\(^{114}\)TP, dU\(^{233}\)TP and dA\(^{115}\)TP were prepared by previous members in the Perrin lab.\(^{161}\) dC\(^{108}\)TP was purchased from TriLink. All the oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA) and were purified by 10–15% 7 M urea denaturing PAGE. Ultrapure dNTPs were obtained from Fermentas. Sequenase Version 2.0 DNA polymerase was purchased from Affymetrix. Lambda exonuclease, Taq DNA polymerase, T4 polynucleotide kinase and Vent exo(-) DNA polymerase were obtained from New England Biolabs. Streptavidin magnetic particles were purchased from Roche. The nucleotide triphosphates \(^{32}\)P-\(\alpha\)-dGTP and \(^{32}\)P-\(\gamma\)-ATP were purchased from Perkin-Elmer. pGEM-T-Easy Vector Systems kit was obtained from Promega. Plasmid miniprep kit was from Invitrogen. LiClO\(_4\) was purchased from J.T. Baker. The rest chemicals were purchased from Sigma-Aldrich and Fisher Scientific. X-Gal and SOC media were purchased from Invitrogen. Ampicillin disodium salt was purchased from Sigma. Water used in all experiments was first treated with diethyl pyrocarbonate (DEPC. 5 µL per 100 mL) and autoclaved. Radioactive PAGEs were exposed to storage phosphor screens, and then the screen was scanned with a Typhoon 9200 Phosphorimager from GE Healthcare. Plasmid
concentrations were measured with NanoDrop 2000C (Thermoscitific). Temperature was adjusted with VWR temperature-controlled water bath.

### 3.8.2 Oligonucleotides

The following oligonucleotides were used:

El-1: 5'-/5Biosg/T40-CCC-3'

El-splint: 5'-AAAAACCCGGGAAAAA-3'

El-primer: 5'-GGGTTTTT(gegugccgucuguugg)TTTTGCGTCGGCCTGCGCCAACAG-3'

Primer 1:
5'-/5Biosg/T40-CCCGGGTTTTT(gegugccgucuguugg)TTTTGCGTCGGCCTGCGCCAACAG-3'

Primer 2: 5'-GCGCTCGCGCTCGCCAACAG-3'

Primer 3: 5'-/5Phos/GCGTCGGCCTCGCCAACAG-3'

Primer 4: 5'-TTTTTTTTTTTTTTTTTTTTTTGCGTCGGCCTGCGCCAACAG-3'

Primer 5: 5'-GCGCTCGCGCTCGCCAACAG-3'

Primer 6: 5'-/5Biosg/T40-
CCCGGGTTTTTGCCTGCG(cegu)CTCGTTGGTTTTGCGTCGGCCTGCGCCAACAG-3'

Template: 5'-GCGCTCGCGCTGCGCCAACAG(N30)CTGTTGGCGCAGGCCGACGC-3'

Biotinylated template clone 25:
5'/Biosg/GGCCTGCTACCTCAAAGGACTCCGCAACGACTATAACTGTTGGCGCAGGCCGACGC-3'

All-RNA substrate target 1: (gcgugccgucuguugg)3'

### 3.8.3 Buffers and cocktails

**Buffers**

Selection cleavage buffer: 50 mM sodium cacodylate (pH 7.4), 200 mM NaCl, 1 mM EDTA.

Elution buffer: 1% LiClO₄, 10 mM Tris-HCl (pH 8) in water.
Cleavage buffer (pH variance): 50 mM sodium cacodylate, 200 mM NaCl and 1 mM EDTA. The pH of all buffers was adjusted to 6.00, 6.50, 7.01, 7.50, 8.00, 8.50, or 9.00.

NaOH neutralization buffer: 25 mM sodium cacodylate (pH 6)

10x Cleavage buffer: 500 mM sodium cacodylate (pH 7.4), 2000 mM NaCl.

Loading solution: Formamide (27 mL), EDTA(aq) (3 mL, 0.5 M), xylene cyanol(aq) (300 μL, 0.05 %) and bromophenol blue (aq) (300 μL, 0.05 %).

Cocktails

5X 1st amplification cocktail: 9 nmol Primer 2, 7.5 nmol Primer 3, 11.5 μL 100 mM MgSO4, 115 μL 10X thermopol buffer, 3.45 μL 100 mM dNTPs (100 mM), added water to make a total 230 μL solution.

5X 2nd amplification cocktail: 10 nmol Primer 4, 7.5 nmol Primer 3, 11.5 μL 100 mM MgSO4, 115 μL 10X thermopol buffer, 3.45 μL 100 mM dNTPs (100 mM), added water to make a total 230 μL solution.

Cocktail for cloning PCR: 10 nmol Primer 2, 7.5 nmol Primer 5, 11.5 μL 100 mM MgSO4, 115 μL 10X thermopol buffer, 3.45 μL 100 mM dNTPs (100 mM), added water to make a total 230 μL solution.

1X mutagenic 2nd amplification cocktail: 1.3 nmol Primer 4, 1.7 nmol Primer 3, 20 μL 10X thermopol buffer, 4 μL 25 mM MnCl2, 0.4 μL 100 mM dGTP and dATP, 2 μL 100 mM dCTP and dTTP, added water to make a total 180 μL solution.

3.8.4 Methods

Elongation

Fifteen pmol of Primer 1 were annealed to 15 pmol of template DNA prepared by 2nd amplification PCR or mutagenic 2nd amplification PCR, and then enzymatically polymerized at 32 °C for 4 hours using 13 units of Sequenase in a mixture containing 5 mM DTT, 50 μM dA₃⁻₅TP, 20 μM of each dU₃⁺TP, dC₃⁻⁴TP, dGTP, and 5–15 μCi of 32P-α-dGTP to give a final volume of 40
μL. The reaction was overlaid with mineral oil. After incubation, the reaction was quenched by adding 1μL 500 mM EDTA (pH 8) solution.

Selection

50μL of magnetic streptavidin beads were transferred into a 1.5mL eppendorf tube, and washed with TEN buffer (3×100 μL). The elongation reaction mixture was added to the beads, and incubated at room temperature for 30 minutes. Then the beads with bound DNAs were magnetized and the supernatant was removed. The beads were washed with TEN buffer (2×100 μL), template stripping buffer (5×100 μL, no longer than 30 sec/wash), then NaOH neutralization buffer (1×200 μL) and water (1×100 μL). Then the modified single strand DNA was incubated with 160 μL varying cleavage buffer for varying time spans (Table 3.1, 3.2) at room temperature. After incubation, the beads were magnetized and the supernatant was transferred to a new eppendorf tube. Then 1% LiClO₄ in acetone (800 μL) and 5x 1ˢᵗ amplification cocktail (2 μL) were added to the supernatant. The eppendorf tube was centrifuged at 13x10³ rpm for 15 minutes. Then discarded the supernatant, and added 1mL of iced ethanol to the pellet in the eppendorf tube. The sample was agitated by vortex and centrifuged at 13x10³ rpm for 15 minutes. The ethanol was decanted and any residual ethanol was removed at 65°C for around 5 minutes. The pellet was dissolved in 5μL of dH₂O. 10 μL loading dye was added to the pellet, and then the sample was loaded onto 7% 7 M urea denaturing PAGE. The species corresponding to the cleaved product was cut and eluted using elution buffer and precipitated with ethanol. The precipitated pellet was dried, and resuspended in 60 μL water, and used in the 1ˢᵗ amplification PCR step.

1ˢᵗ amplification PCR

The cleavage product eluted from selection was PCR amplified using Primer 2 and Primer 3 and an internal label (10 μCi ³²P-α-dGTP) for 25 cycles (15 s at 58 °C, 40 s at 75 °C and 15 s at 95 °C). The reaction (40 μL) included 0.1 units/μL Vent (exo-) DNA polymerase, 20 mM Tris-HCl (pH 8.8 at 25 °C), 10 mM (NH₄)₂SO₄, 10 mM KCl, 3 mM MgSO₄, 0.1% gelatin, 7 μM
oligonucleotides and 0.3 mM of each natural dNTP. Before purification by 10% 7 M urea denaturing PAGE, the dsDNA was extracted with 40 μL phenol/chloroform/isoamyl alcohol (25:24:1), and precipitated with 400 μL ethanol. After agitating and 15 minutes of centrifuging the ethanol was decanted and residual amounts were evaporated. The resulting pellet was resuspended in 44 μL water, and was added with 5 μL 10x lambda exonuclease buffer and 1 μL lambda exonuclease (5 units). The sample was incubated at 37 ºC 3 hours. The phosphorylated strand was then digested. The resulting product was 50 μL phenol/chloroform/isoamyl alcohol (25:24:1), precipitated with 500 μL ethanol. After agitating and 15 minutes of centrifuging the ethanol was decanted and residual amounts were evaporated. The resulting pellet was resuspended in 20 μL water. 30 μL loading solution was added, and then the sample was purified by 10% 7 M urea denaturing PAGE. The corresponding band was cut out, eluted with elution buffer, precipitated with ethanol. The resulting pellet was resuspended in 25 μL water, and used in the 2nd amplification PCR step.

2nd amplification PCR

10 μL 1st amplification PCR product was added to 20 μL 5X PCR cocktail, 5 μL Vent (exo-) DNA polymerase (10 units), 65 μL water to give a 100 μL reaction. The amplification reaction was thermocycled using the same program for 30 cycles. After the amplification, the amplicon product was treated in the same manner with the 1st amplification product. After extraction, precipitation and resuspension, the DNA was also subjected to digestion by lambda exonuclease. The single-stranded DNA was dissolved in 1 M NaOH (20 μL) and loading solution (30 μL) prior to loading on a 10% 7 M urea denaturing mini-PAGE. The single-stranded DNA was identified by UV-shadowing, cut out, eluted with elution buffer, precipitated with ethanol. The resulting pellet was resuspended in 40 μL and desalted using a G25 column. The DNA was quantified by UV absorption and used in elongation step.
The N₃₀ library oligonucleotides from IDT were not used for elongation directly. A 2nd amplification was first done with the N₃₀ library. 300 pmol template was added to 20 μL 5X PCR cocktail, 5 μL Vent (exo-) DNA polymerase (10 units), 65 μL water to make a 100 μL reaction. The amplification reaction was thermocycled using the same program for 10 cycles. After the amplification, the amplicon product was treated in the same manner with the 2nd amplification including extraction, precipitation and resuspension, digestion, PAGE purification, G25 desalting. The single-stranded DNA was quantified by UV absorption and used in elongation step.

Mutagenic 2nd amplification PCR

5 μL 1st amplification PCR product was added to 90 μL 1X PCR cocktail and 5 μL Taq DNA polymerase (25 units). The mutation can be achieved by the unbalanced dNTPs concentrations. The amplification reaction was thermocycled using the same program for 30 cycles. After the amplification, the amplicon product was treated in the same manner with the 2nd amplification including extraction, precipitation and resuspension, digestion, PAGE purification, gel elution, G25 desalting, quantification by UV absorption and used in elongation step.

Gel elution

Gel bands containing the desired oligo were cut out and crushed into small gel particles with a flame-sealed pipette tip. 300 μL of elution buffer was added to the gel particles. The suspension was agitated by vortex, incubated at 65°C for 10 minutes, and centrifuged at 13x10³ rpm for 5 minutes. The supernatant was collected and transferred to a new 1.5 mL eppendorf tube. This elution process was repeated twice. The collected supernatants were combined and evaporated on a vacuum centrifuge at 30°C. After the elution was evaporated to about 100 μL, 1 mL of ethanol was added to the elution, and mixed by vortex, and then centrifuged at 13x10³ rpm for 15 minutes. The supernatant was discarded. Another 1 mL of ethanol was added to the elution, and mixed by vortex, and then centrifuged at 13x10³ rpm for 15 minutes. The ethanol was
decanted as well as residual amounts were evaporated. The resulting pellet was resuspended in water.

**Ligation**

4 nmol El-splint, 2 nmol El-1 and 2 nmol El-primer were annealed under 95°C for 30 seconds, cooled down to room temperature then cooled on ice. 5 μL 10X ligation buffer, 2 μL T4 DNA ligase (4000 units) and water were added to the annealed solution to give a 50 μL reaction. The reaction was incubated at 15°C for 16 hours. After the completion, the reaction was extracted 50 μL phenol/chloroform/isoamyl alcohol (25:24:1), and precipitated with 500 μL ethanol. After agitating and then 15 minutes of centrifuging, the ethanol was decanted and residual amounts were evaporated. The resulting pellet was resuspended in 20 μL water. 30 μL loading solution was added, and then the sample was purified by 10% 7 M urea denaturing PAGE. The corresponding band was cut out, eluted with elution buffer, and precipitated with ethanol. The resulting pellet was resuspended in 30 μL water. The ligation product was quantified by UV absorption and used in elongation step.

**TOPO TA cloning**

The product from the second PCR amplification was used as template for PCR with Taq DNA polymerase to produce double stranded material with overhanging 3′-dA’s. TA-cloning was then performed on this product using the pGEM-T-Easy Vector Systems kit. Using electroporation, the resulting plasmids were transformed into E. coli DH10B cells, and the cells were streaked onto lysogeny broth (LB) agar containing ampicillin (100 mg/L). Individual white colonies were picked and used to inoculate 1.5 mL SOC medium solution. The samples were cultured at 37 ºC for 16 hours. Plasmids were isolated with Plasmid miniprep kit using a centrifuge. The plasmids concentrations were measured with NanoDrop and were restriction digested in the presence of EcoRI to screen for the appropriately sized inserts. After that plasmids were submitted to the UBC NAPS Unit for sequencing.
Screening of the clones

Single-stranded templates for individual sequences obtained from the sequencing of the individual clones were ordered from IDT. To screen the clones for self-cleavage activity, 3 pmol of Primer 1 were annealed to 4 pmol of template and extended using dCαTP, dUβTP, dAδTP, natural dGTP and 32P-α-dGTP. Elongation reactions were quenched after 4 hours with the addition of 0.5 μL 0.5 M EDTA. 10 μL streptavidin beads were pre-washed with TEN buffer (3×30 μL). Extension reactions were incubated with the pre-washed streptavidin beads for 30 minutes at room temperature. After the incubation, the beads were washed with TEN buffer (2×30 μL), template stripping buffer (5×30 μL), NaOH neutralization buffer (1×60 μL), H2O (1×30 μL). Clones were incubated with 40 μL selection cleavage buffer at room temperature. At time points 0, 3, 10, 60 and 180 minutes, an aliquot (5 μL) was removed and quenched by adding loading solution. Cleaved and uncleaved oligonucleotides were resolved on a 10% denaturing PAGE, visualized using a Phosphorimager and rectangles were drawn around the bands corresponding to cleaved and uncleaved products. The data were fitted to a single-exponential equation using Origin Pro for an initial estimate of the observable rate constant.

Temperature, pH and divalent metal effect profile

For each temperature kinetic assay, 3 pmol elongation was carried out. The elongation was then incubated with 10 μL pre-washed streptavidin beads for 30 minutes at room temperature. The beads were treated in the same manner with screening of the clones step. 40 μL of cleavage buffer was added to the beads. At time points 0, 1, 3, 10, 30, 60 minutes, an aliquot (5 μL) was removed and quenched with an addition of 8 μL loading solution. Cleaved and uncleaved oligonucleotides were resolved on a 10% denaturing PAGE, visualized using a Phosphorimager and rectangles were drawn around the bands corresponding to cleaved and uncleaved products. The data were fitted to a single-exponential equation using Origin Pro for an initial estimate of the observable rate constant.
Elongation for *trans*-cleavage study

30 pmol of Primer 5 were annealed to 30 pmol of biotinylated template clone 25 DNA and the enzymatically polymerized at 32 °C for 4 hours using 13 units of Sequenase in a mixture containing 5 mM DTT, 50 μM dA<sup>imTP</sup>, 20 μM of each dU<sup>tpTP</sup>, dC<sup>haTP</sup>, dGTP, and 15 μCi of <sup>32</sup>P-α-dGTP to give a final volume of 40 μL. The reaction was overlaid with mineral oil. After incubation, the reaction was quenched by adding 1μL 500 mM EDTA (pH 8) solution.

Measuring the concentration of DNAzyme clone 25

A 1 μL aliquot of the resulting elongation solution was diluted 3x, 10x, 30x, 100x, 300x, 1000x and 2 μL of each dilution was spotted on a TLC-plate (appendices Chapter 3) to generate an autoradiographic calibration curve that relates autoradiographic density to volumes and ultimately the number of pmol of dGTP (specific activity). The logarithm of signal intensity of plotted against the logarithm of the dilution (appendices Chapter 3) and the data was fitted to a square line using linear regression. The rest of elongation product was immobilized on 50 μL of pre-washed streptavidin beads by incubating at room temperature for 30 minutes. After washes with TEN buffer (2×100 μL), then water wash (2×100 μL), the enzymatically synthesized modified DNAzyme was removed from the template by 0.1 M NaOH (2×30 μL) at room temperature for 30 seconds, and then neutralized with 5M HCl to pH 7-8. The collected oligonucleotides was desalted through a G25 column. A 1 μL aliquot of the resulting DNAzyme solution was diluted 10 times and 2 μL of the dilution was spotted on the same TLC-plate. The concentration of DNAzyme could then be determined using Equation 3.5 and 3.6

\[ x = (y - b)/a \]  
\[ [\text{DNAzyme}] = \frac{[\text{dGTP}] \times 10}{12 \times e^{-x}} \]

Where \( a \) is the slope and \( b \) the intercept of the linear regression of calibration curve where \( x \) is the logarithm of the dilution factor of the DNAzyme, \( y \) is the logarithm of signal intensity of the
diluted DNAzyme. The factor of 10 and 12 arise because of the 10 times dilution of the DNAzyme spotted on the TLC-plate and the presence of 12 dGs in the catalytic region of DNAzyme clone 25 and the left binding arm (Figure 3.5).

5'-End labelling of substrates

1 nmol of all-RNA substrate target 1 was 5'-end labelled with 30 μCi of \(^{32}\)P-\(\gamma\)-ATP by using 30 units of T4 polynucleotide kinase in a 40 μL volume reaction. The reaction mixture was incubated at 37 °C for 2 hours. Then the reaction was terminated by heating at 65 °C for 20 minutes. 30 μL loading solution was added to the reaction mixture, and the resulting mixture was loaded on a 20% PAGE. Product was identified by UV shadowing and cut out. Gel material was crushed into fine particle with a flame-sealed pipette tip and eluted with elution buffer at 65 °C under 10 minutes. The liquids were evaporated and ethanol precipitated. Finally, the isolated solid was re-suspended in 40 μL water and desalted with a G25 spin column, quantified by UV.

Multiple-turnover kinetics

0.5 pmol DNAzyme clone 25 was added to appropriate amount of radioisotope-labelled all-RNA substrate. The mixture was then denatured under 95 °C for 2 minutes, cooled to 0 °C on ice, followed by the addition of 2 μL 10x cleavage buffer (with or without divalent metal cations) to initiate the reactions, water was added to make a total volume of 20 μL. At each time point, a 2 μL potion was transferred into a tube containing 7 μL loading solution. Cleaved and uncleaved oligonucleotides were resolved on a 20% denaturing PAGE, visualized using a Phosphorimager, and rectangles were drawn around the bands corresponding to cleaved and uncleaved products. The rate constant was obtained for each substrate concentration from a fitting of the data point obtained over the first 10-20% of the reaction.
Chapter 4: RNA-cleaving DNAzyme selection against Myc oncogene target

4.1 Introduction

4.1.1 c-Myc is a therapeutic target

The c-Myc (myelocytomatosis) gene encodes one of the most important transcriptional regulators and it is involved in many aspects of cellular growth and metabolism. Many c-Myc target genes, such as cyclin A2, cyclin D2 and serine hydroxymethyl transferase, have been identified by several techniques. As a transcriptional activator, c-Myc supports the increase in the production of nucleic acids, proteins, and lipids necessary for cellular growth. However, c-Myc may also act as a transcriptional repressor; for example, it has been shown that entopic Myc can suppress transcriptional initiation of endogenous Myc in a dose-dependent manner. c-Myc is also an oncogene, and therefore it is important in tumor and cancer research. The distinction between normal physiological and oncogenic Myc function is whether its expression level is regulated. Dysregulated Myc expression is found in many commonly occurring human cancers and contributes to at least 40% of all human cancers.

Due to its important role in human tumor growth, the c-Myc gene is a promising therapeutic target. Regulating the metabolic pathways that relate to the c-Myc gene or transcribed protein represents a logical approach for cancer treatment. For instance, c-Myc is capable of transactivating genes involved with lactate dehydrogenase A (LDHA). Using antisense RNA, researchers were able to suppress the LDHA expression in several human lymphoid tumor cell lines, resulting in significantly decreased soft agar colony growth. Later on, researchers showed that stable interference RNA (shRNA) mediated knock-down of LDHA expression in mouse mammary tumor cells, which resulted in prolonged survival of tumor-inoculated animals as compared with those inoculated with control cells. In another approach, the phosphorodiamidate morpholino oligomer AVI-4126 was used to inhibit c-Myc translation in a
sequence-specific manner. A significant reduction in prostate tumor burden was observed with AVI-4126-treated animals compared with the randomized oligonucleotides and saline control group. A phase I clinical trial performed on AVI-4126 showed that patients who received high dose had a low angiographic restenosis compared with low dose and control. As was discussed in Chapter 1 (section 1.7), DNAzymes are another promising therapeutic approach because they can be used in regulating gene expression by cleaving RNA in vivo; however, so far no research has successfully used a DNAzyme to cleave c-Myc RNA. Obtaining a divalent metal-independent all-RNA-cleaving DNAzyme will be the first step toward a DNAzyme therapeutic application for targeting c-Myc.

4.1.2 An attempt to re-engineer DNAzyme clone 25

Recall that Chapter 3, I reported the discovery of an all-RNA-cleaving DNAzyme that can intramolecularly cleave an all-RNA target with a high rate constant. However, the trans-cleavage activity was relatively poor, and the modified DNAzyme showed poor adaptability toward different target sequences. An attempt to engineer DNAzyme clone 25 to specifically cleave a c-Myc all-RNA target was unsuccessful. Therefore, a new selection was required.

4.2 Objective of this project

The goal of this project was to study the selection of an all-RNA-cleaving DNAzyme selected from the three aforementioned modified nucleotide triphosphates against the c-Myc target sequence. Once an efficient self-cleaving DNAzyme was obtained, I would aim to use this enhanced DNAzyme to test the trans-cleavage activity and study the possibility of using it in vivo. To obtain the DNAzyme, I planned to use the same three modified nucleotide triphosphates, dUspTP (1), dCspTP (2), and dAimTP (3), that were used in Chapter 3. Lastly, I intended to conduct multiple assays to evaluate catalytic efficiency of the DNAzyme.
Figure 4.1 Chemical structures of dUgaTP (1), dCaaTP (2), and dAimTP (3)

4.3 In vitro selection

4.3.1 First selection

The selection of an RNA-cleaving DNAzyme was performed using the same streptavidin-biotin methodology that was described in Chapter 3 (as shown in Figure 3.2), but using a different oligonucleotide sequence set-up as shown in Figure 4.2.

Bio-T₄₀CCCCGGTTTTT(ccgccgacgaugcccuc) CGCGAGCGC TTTTGGCGCT CCGGGAGCGGT GTCGTCG

Figure 4.2 c-Myc RNA-cleaving DNAzyme selection structure

The selection followed the same strategy used in Chapter 3 and the stringency was increased by varying the incubation time and cleavage buffer composition used in the selection of self-cleaving strands. The reaction time was systematically decreased from 60 minutes down to 3 minutes over the course of selection (Table 4.1). In all of the selection rounds the cleavage percentage was examined after 60 minutes of incubation. The sodium chloride concentration in the cleavage buffer was varied from 200 mM to 10 mM in order to further increase the stringency.
of the reaction conditions (Table 4.2). A total of 24 rounds of selection were performed (Figure 4.3).

Table 4.1 Supernatant was collected after the indicated incubation time at each round of selection

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>3</th>
<th>5</th>
<th>10</th>
<th>60</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rounds</td>
<td>20-24</td>
<td>12-15,17,19</td>
<td>9-10,</td>
<td>1-8,11,16,18</td>
</tr>
</tbody>
</table>

Table 4.2 Sodium chloride concentration used in each round of selection

<table>
<thead>
<tr>
<th>Salt concentration (mM)</th>
<th>10</th>
<th>200</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rounds</td>
<td>16, 18,20</td>
<td>1-15,17,19, 21-24</td>
</tr>
</tbody>
</table>

Figure 4.3 Rounds of RNA-cleaving DNAzyme selection. The percentage of cleavage was measured after 60 minutes incubation time

In rounds 16 and 18, the selection reaction was incubated for 60 minutes instead of 5 minutes on purpose because the metal cation concentration had also been decreased to 10 mM. A longer incubation time allowed all the highly active species to survive these two rounds. The percentage of cleavage in 60 minutes decreased due to the low salt concentration, and when the salt concentration was brought back to 200 mM in rounds 17 and 19 to allow for recovery, the percentage of cleavage increased significantly. In rounds 20 and 21, the sequences were exposed to low salt followed by high salt conditions once again, but failed to respond to the metal cation
changes as no significant increase or decrease in the percentage of cleavage was measured. Overall, the selection appeared to work very well because the cleavage percentage continually increased until the end of selection, except for the rounds where the metal concentration was lowed.

**Mutagenic PCR**

“Hypermutagenic” PCR was conducted in rounds 13, 17 and 20. The technique appeared to be successful in round 14, but not in round 21 as judged by an increase in activity in subsequent generations. In round 18, the low metal cation concentration played a more important role on the low percentage of self-cleavage seen. The percentage was not much improved between 17 and 19. The improvement between round 20 and 21 was probably due to the increase of time stringency. Unexpectedly, the mutagenic PCR in round 21 did not negatively affect the cleavage percentage.

After 24 rounds of selection, rounds 22-24 were compared to determine the best generation to clone. Round 24 showed the fastest cleavage rate at both 3 minutes and 60 minutes, and hence sequences in this round were chosen for cloning. The sequences obtained are shown in Table 4.3. The average length of the random region was 29.8 nucleotides, and the average number of modified residues was 16.3, which is a significant difference from the statistically random number of 22.5 (30*3/4). It is understandable that the average number of modified residues found in the cloned sequences would be lower because the amplification and incorporation rates of modified nucleotides are poor compared to unmodified oligonucleotides. 

134
Table 4.3 Cloned sequences isolated from the selection (red sequences are the fastest and most abundant sequences in all clones)

<table>
<thead>
<tr>
<th>Clone #</th>
<th>Catalytic region</th>
<th>Length (base)</th>
<th># of modification</th>
<th># of times repeated</th>
</tr>
</thead>
<tbody>
<tr>
<td>01</td>
<td>AATCTCAGACCTTGCCGACGATGGTGATTA</td>
<td>30</td>
<td>23</td>
<td>1</td>
</tr>
<tr>
<td>02</td>
<td>TTGGTGTAGGCCGGATGGGTGGTGTTGGCGCGTGG</td>
<td>30</td>
<td>14</td>
<td>1</td>
</tr>
<tr>
<td>03</td>
<td>TTGGTGTAGGCCGGATGGGTGGTGTTGGCGCGTGG</td>
<td>30</td>
<td>15</td>
<td>1</td>
</tr>
<tr>
<td>04</td>
<td>TGCCTGAGTCTGCTGGTGGCGGTCCCTGGTGTCCGT</td>
<td>29</td>
<td>18</td>
<td>1</td>
</tr>
<tr>
<td>06</td>
<td>TTGGTGTAGGCCGGATGGGTGGTGTTGGCGCGTGG</td>
<td>30</td>
<td>14</td>
<td>10</td>
</tr>
<tr>
<td>08</td>
<td>TTGGTGTAGGCCGGATGGGTGGTGTTGGCGCGTGG</td>
<td>30</td>
<td>19</td>
<td>9</td>
</tr>
<tr>
<td>10</td>
<td>TTGGTGTAGGCCGGATGGGTGGTGTTGGCGCGTGG</td>
<td>30</td>
<td>16</td>
<td>3</td>
</tr>
<tr>
<td>11</td>
<td>TTGGTGTAGGCCGGATGGGTGGTGTTGGCGCGTGG</td>
<td>30</td>
<td>15</td>
<td>5</td>
</tr>
<tr>
<td>13</td>
<td>GGCAGTCGGGGGTCGGGATGGGTGGCGCGTGG</td>
<td>30</td>
<td>15</td>
<td>1</td>
</tr>
<tr>
<td>14</td>
<td>TGCCAGTCGGGGGTCGGGATGGGTGGCGCGTGG</td>
<td>30</td>
<td>18</td>
<td>1</td>
</tr>
<tr>
<td>18</td>
<td>TTGGTGTAGGCCGGATGGGTGGTGTTGGCGCGTGG</td>
<td>30</td>
<td>15</td>
<td>1</td>
</tr>
<tr>
<td>19</td>
<td>AATCTCAGACCTTGCCGACGATGGTGATTA</td>
<td>30</td>
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<td>1</td>
</tr>
<tr>
<td>20</td>
<td>TTGGTGTAGGCCGGATGGGTGGTGTTGGCGCGTGG</td>
<td>30</td>
<td>15</td>
<td>1</td>
</tr>
<tr>
<td>22</td>
<td>CGATGGTCTGGTGTGGCGCGCGGAGGTGGGTGGGGCGGG</td>
<td>30</td>
<td>16</td>
<td>1</td>
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<td>CGATGGTCTGGTGTGGCGCGCGGAGGTGGGTGGGGCGGG</td>
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</tr>
<tr>
<td>25</td>
<td>TTGGTGTAGGCCGGATGGGTGGTGTTGGCGCGTGG</td>
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<td>14</td>
<td>3</td>
</tr>
<tr>
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<td>16</td>
<td>2</td>
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<tr>
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<td>19</td>
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<td>18</td>
<td>1</td>
</tr>
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<td>TTGGTGTAGGCCGGATGGGTGGTGTTGGCGCGTGG</td>
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<td>14</td>
<td>1</td>
</tr>
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<td>46</td>
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<td>18</td>
<td>1</td>
</tr>
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<td>54</td>
<td>TTGGTGTAGGCCGGATGGGTGGTGTTGGCGCGTGG</td>
<td>30</td>
<td>15</td>
<td>1</td>
</tr>
<tr>
<td>65</td>
<td>TTGGTGTAGGCCGGATGGGTGGTGTTGGCGCGTGG</td>
<td>29</td>
<td>14</td>
<td>1</td>
</tr>
</tbody>
</table>

The sequences were found to have very high similarity. Among the 54 clones obtained, there were 24 unique sequences and the rest were degenerate. For example, clone 6 was repeated 10 times, and clone 8 was repeated 9 times. Among the 24 unique sequences there was also a high degree of similarity. To visualize the level of similarity, all 24 sequences were grouped and each base was highlighted with a different colour (refer to Table 4.4). It can be noticed that the library is very convergent. Eleven sequences shared a very high degree of similarity (red-coloured clones) Clone 4 and 8 only differed by one base (purple-coloured clones), and clones 14, 35 and 46 only by two bases (blue-coloured clones).
Table 4.4 Clone sequences grouped by similarity. The T is highlighted with yellow, the G with purple, and the A with green.

<table>
<thead>
<tr>
<th>Family #</th>
<th>Clone #</th>
<th>Catalytic region sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>02</td>
<td>TGGCTGTGCCCTGCTGCCTCCCTGGCC</td>
<td></td>
</tr>
<tr>
<td>06</td>
<td>TGGCTGTGCCCTGCTGCCTCCCTGGCC</td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>TGGCTGTGCCCTGCTGCCTCCCTGGCC</td>
<td></td>
</tr>
<tr>
<td>03</td>
<td>TGGCTGTGCCCTGCTGCCTCCCTGGCC</td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>TGGCTGTGCCCTGCTGCCTCCCTGGCC</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>TGGCTGTGCCCTGCTGCCTCCCTGGCC</td>
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<td>20</td>
<td>TGGCTGTGCCCTGCTGCCTCCCTG GCC</td>
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<td></td>
</tr>
<tr>
<td>30</td>
<td>TGGCTGTGCCCTGCTGCCTCCCTG GCC</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>TGGCTGTGCCCTGCTGCCTCCCTG GCC</td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>TGGCTGTGCCCTGCTGCCTCCCTG GCC</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>TGGCTGTGCCCTGCTGCCTCCCTG GCC</td>
<td></td>
</tr>
<tr>
<td>35</td>
<td>TGGCTGTGCCCTGCTGCCTCCCTG GCC</td>
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</tr>
<tr>
<td>46</td>
<td>TGGCTGTGCCCTGCTGCCTCCCTG GCC</td>
<td></td>
</tr>
<tr>
<td>37</td>
<td>TGGCTGTGCCCTGCTGCCTCCCTG GCC</td>
<td></td>
</tr>
<tr>
<td>08</td>
<td>TGGCTGTGCCCTGCTGCCTCCCTG GCC</td>
<td></td>
</tr>
<tr>
<td>04</td>
<td>TGGCTGTGCCCTGCTGCCTCCCTG GCC</td>
<td></td>
</tr>
<tr>
<td>41</td>
<td>TGGCTGTGCCCTGCTGCCTCCCTG GCC</td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>TGGCTGTGCCCTGCTGCCTCCCTG GCC</td>
<td></td>
</tr>
<tr>
<td>23</td>
<td>TGGCTGTGCCCTGCTGCCTCCCTG GCC</td>
<td></td>
</tr>
<tr>
<td>39</td>
<td>TGGCTGTGCCCTGCTGCCTCCCTG GCC</td>
<td></td>
</tr>
<tr>
<td>37</td>
<td>TGGCTGTGCCCTGCTGCCTCCCTG GCC</td>
<td></td>
</tr>
<tr>
<td>22</td>
<td>TGGCTGTGCCCTGCTGCCTCCCTG GCC</td>
<td></td>
</tr>
<tr>
<td>54</td>
<td>TGGCTGTGCCCTGCTGCCTCCCTG GCC</td>
<td></td>
</tr>
</tbody>
</table>

Most of the 24 clones were screened for cleavage activities. Clones 6 and 8 showed the best cleavage rate, with a half-life of 60 minutes. The rate constant showed no improvement compared with the reported DNAzyme 12-91.116 Clones 18, 20, 25, 14 and 46 had minimal activities. In round 24, the library was dominated by clone 6 and clone 8, causing a cleavage rate of 25% after 60 minutes. Since no individual clone showed satisfactory cleavage activity, another strategy was proposed to use to further pursue a better all-RNA cleaver.

4.3.2 Reselection

It was hypothesized that further mutating generation 24 by mutagenic PCR would not produce a better cleaver because of the low diversity of sequences. Therefore, a new library was
designed based on the sequences of clones 6 and 8 and used to conduct another selection, which was I refer to as “reselection”. This strategy guaranteed that the best clone in the reselection will be better than or equally active compared to clones 6 and 8, as these clones would be present in the pool, and would increase the chance of obtaining a better DNAzyme compared to a selection using a new completely randomized library.

To start, two separate libraries were designed based on clones 6 and 8. Each cloned DNAzyme sequence was turned into a template (Figure 4.4). Regarding the bases in the DNAzyme active region, 70% were conserved, and the remaining 30% were changed to 10% each of the other three bases. As an example, the last base in clone 8 can be considered: thymidine became adenosine in the template, and the library consisted of 70% A, and 10% each of C, G and T at that position (green letters in Figure 4.4). After two separate random libraries were obtained from clone 6 and 8, they were combined equally to form a single mixed library. Statistically, the library would contain 0.0011% of both clone 6 and 8 (0.730=0.0022%), and therefore it would be possible to select DNAzymes with some homology to these parent sequences.

**Figure 4.4** Random library design process with clone 8. 1 obtaining the template sequence and addition of primer region. 2 randomizing the N₃₀ region. Red colour represents the primer region, and the green letters represent the example referred to in the text.
The reselection was performed using the same streptavidin-biotin methodology previously described. The reselection was also done with the same strategy as in previous selections by varying the incubation time and cleavage buffer composition to increase the stringency. In all the selection rounds the cleavage percentage was examined after 60 minutes, it was examined after 5 minutes in the later selection rounds as well (rounds 4-9). The reaction time was decreased from 60 minutes down to 3 minutes (Table 4.5). The sodium chloride concentration in cleavage buffer was varied from 200 mM to 10 mM (Table 4.6). A total of 9 rounds of selection were performed (Figure 4.5). Hypermutagenic PCR was used in round 4, 6 and 8.

Table 4.5 Supernatant was collected after the indicated incubation time at each round of selection

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>3</th>
<th>5</th>
<th>60</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rounds</td>
<td>8,9</td>
<td>4-7</td>
<td>1-3</td>
</tr>
</tbody>
</table>

Table 4.6 Sodium chloride concentration used in each round of selection

<table>
<thead>
<tr>
<th>Salt concentration (mM)</th>
<th>10</th>
<th>200</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rounds</td>
<td>6,7</td>
<td>1-5,8,9</td>
</tr>
</tbody>
</table>

Figure 4.5 Rounds of RNA-cleaving DNAzyme selection. Percentage of cleavage was examined after 5 minutes (green) and 60 minutes (red) incubation time
4.3.3 Cloning and sequence analysis

After 9 rounds of selection, cloning was performed on the PCR amplified DNA obtained from generation 8 of the reselection. The sequences obtained are shown in Table 4.7, and an analysis of the sequences is shown in Table 4.7. The average length of the random region was 29.7 nucleotides, and the average number of modified residues was 18.5, which is a slight difference from the statistically random number of 22.5.

Table 4.7 Clone sequences isolated from the selection

<table>
<thead>
<tr>
<th>Clone #</th>
<th>Sequence</th>
<th>Length (base)</th>
<th># of modification</th>
</tr>
</thead>
<tbody>
<tr>
<td>01</td>
<td>TTAGCAGCAGCTGTGCTGTTGCTGCCGTTG</td>
<td>29</td>
<td>17</td>
</tr>
<tr>
<td>02</td>
<td>TTAGCAGCAGCTGTGCTGTTGCTGCCGTTG</td>
<td>30</td>
<td>17</td>
</tr>
<tr>
<td>03/42/53</td>
<td>TTAGCAGCAGCTGTGCTGTTGCTGCCGTTG</td>
<td>30</td>
<td>20</td>
</tr>
<tr>
<td>04</td>
<td>TGGCTGTAGTCTATTCTGCCGTTGCTGTTG</td>
<td>30</td>
<td>22</td>
</tr>
<tr>
<td>05/09/44</td>
<td>TGGGAGTAGTCTGTGTTGCTGCCGTTG</td>
<td>30</td>
<td>20</td>
</tr>
<tr>
<td>06</td>
<td>TTTGTGTAGTCTGTGTTGCTGCCGTTG</td>
<td>30</td>
<td>22</td>
</tr>
<tr>
<td>07</td>
<td>GGTCTGAGTCCGTTGCTGCCGTTG</td>
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<td>18</td>
</tr>
<tr>
<td>08/18</td>
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<td>15</td>
</tr>
<tr>
<td>10</td>
<td>TTGCTGTGAGCTGCTGCTGCTGCTG</td>
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<td>16</td>
</tr>
<tr>
<td>11</td>
<td>TGGCTGTAGTCTGCTGCTGCTGCTG</td>
<td>28</td>
<td>19</td>
</tr>
<tr>
<td>12</td>
<td>TTTGTGTAGTCTGCTGCTGCTGCTG</td>
<td>30</td>
<td>22</td>
</tr>
<tr>
<td>13/37/46/56/59</td>
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<td>30</td>
<td>17</td>
</tr>
<tr>
<td>14</td>
<td>TTAGTGGGCCCAGGTAGCTGCTGCTG</td>
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<tr>
<td>15</td>
<td>TTGCTGTGAGCTGCTGCTGCTGCTG</td>
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</tr>
<tr>
<td>17</td>
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<tr>
<td>19</td>
<td>ATCTCAGCTCTGCTGCTGCTGCTG</td>
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<td>23</td>
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<tr>
<td>24</td>
<td>TGGGAGTAATCTTGCTGCTGCTGCTG</td>
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</tr>
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<td>25</td>
<td>TGGCTGTAGGCGAGGTGCTGCTGCTGCTG</td>
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<td>15</td>
</tr>
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<tr>
<td>28</td>
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<td>13</td>
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<td>29</td>
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<td>33</td>
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### Table 4.7 Clone sequences isolated from the selection

<table>
<thead>
<tr>
<th>Clone #</th>
<th>Sequence</th>
<th>Length (base)</th>
<th># of modification</th>
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<tbody>
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<td>34</td>
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</tr>
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</tr>
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<td>36</td>
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</tr>
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<td>39</td>
<td>GGTATGTAAGGCGAGTTAGTGTGCCATTGG</td>
<td>30</td>
<td>17</td>
</tr>
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<td>GCCGTAGTGGGCAGAGCTTCCGCTTGGT</td>
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<td>17</td>
</tr>
<tr>
<td>41</td>
<td>ATGGTGCAAGGCGAGTTAGTGTCCGGCTGG</td>
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</tr>
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<td>AATCTCAGACCTTGCCGACGCTGTGTTTA</td>
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</tr>
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</tr>
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</tr>
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<td>61</td>
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<td>30</td>
<td>16</td>
</tr>
</tbody>
</table>

A kinetic analysis of a portion of the clones (5 time points per clone) was performed to obtain some preliminary data (Table 4.8). In comparing all the clones, several patterns were observed in the sequences. First, there is much more diversity compared with the sequencing results from first selection. Second, two groups share a lot of similarity with the two templates derived from clones 6 and 8. These sequences likely were derived from the randomized reselection library and not from mutagenic PCR. The sequences that have high similarity with clone 8 from the first selection are shown in Table 4.8 with the rate constants presented in decreasing order. The sequences that have high similarity with clone 6 from first selection are also shown in Table 4.9, and are displayed in the same way. Some of the remaining sequences can also be grouped into families, and they are shown in Table 4.9.
### Table 4.8 Kinetic studies for the clone 08 family. For each sequence, only the bases that differ from clone 8 are shown. Bases that are identical notated with a dot. “_” was added where necessary to align the sequences with clone 8 properly. (NR; no reaction detected, NE; no elongation product)

<table>
<thead>
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<th>Clone 08 family</th>
<th>Sequence</th>
<th>$k_{obs}$ (min$^{-1}$)</th>
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<td>.TG.A.G.............CA.TT....................</td>
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</tr>
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<tr>
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<td>0.2990</td>
</tr>
<tr>
<td>05/09/44</td>
<td>..GGA.............T...CC..............T.T</td>
<td>0.2799</td>
</tr>
<tr>
<td>20</td>
<td>..GGA.............T...CC..............</td>
<td>0.2856</td>
</tr>
<tr>
<td>33</td>
<td>.CG.................T.C.CG..............T.C</td>
<td>0.2898</td>
</tr>
<tr>
<td>15</td>
<td>.TG...G.............CA.TT........T.C</td>
<td>0.2500</td>
</tr>
<tr>
<td>32</td>
<td>.CG.A................._C.G...........</td>
<td>0.1908</td>
</tr>
<tr>
<td>38</td>
<td>......AGTCG.A......CT..............T.C</td>
<td>0.1197</td>
</tr>
<tr>
<td>12</td>
<td>.TG...........T.C..CA..............T.T</td>
<td>0.08813</td>
</tr>
<tr>
<td>39</td>
<td>G.TA.............GC..A....T..............</td>
<td>0.0088</td>
</tr>
<tr>
<td>55</td>
<td>CCG.................T.C.CG..............T.C</td>
<td>0.0084</td>
</tr>
<tr>
<td>48</td>
<td>G.TT......G...A....T..............</td>
<td>0.00432</td>
</tr>
<tr>
<td>04</td>
<td>...G............AT.C.C..............T.C</td>
<td>0.00141</td>
</tr>
<tr>
<td>27/47/58</td>
<td>.CG.................T.C..C..............T.C</td>
<td>0.000175</td>
</tr>
<tr>
<td>24</td>
<td>.CGGA...A.....C...CCG...........</td>
<td>NR</td>
</tr>
<tr>
<td>16</td>
<td>.................T.......A......T.C...........</td>
<td>NR</td>
</tr>
<tr>
<td>45</td>
<td>G.GT..G...GC..A....T..............</td>
<td>NR</td>
</tr>
<tr>
<td>35</td>
<td>.................CT..............A...........</td>
<td>NR</td>
</tr>
</tbody>
</table>

### Table 4.9 Kinetic studies for the clone 06 family. For each sequence of 06 family, only the bases that differ from clone 6 are shown. Bases that are identical notated with a dot. “_” was added where necessary to align the sequences with clone 6 properly. The rest sequences are shown as well. (NR; no reaction detected, NE; no elongation product)

<table>
<thead>
<tr>
<th>Clone 06 family</th>
<th>Sequence</th>
<th>$k_{obs}$ (min$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>06 (selection)</td>
<td>TTGGTGAGGCGAGGCTGCGTTCGCCG</td>
<td>0.01</td>
</tr>
<tr>
<td>25</td>
<td>.................T..............</td>
<td>0.0300</td>
</tr>
<tr>
<td>34</td>
<td>.................TA.........</td>
<td>0.02093</td>
</tr>
<tr>
<td>61</td>
<td>A.................T.T..............</td>
<td>0.01841</td>
</tr>
<tr>
<td>50</td>
<td>A..............................</td>
<td>0.00776</td>
</tr>
<tr>
<td>10</td>
<td>.................AGTT..............</td>
<td>NR</td>
</tr>
<tr>
<td>41</td>
<td>A.....C.............TA........................</td>
<td>NR</td>
</tr>
<tr>
<td>31</td>
<td>.................CC.........</td>
<td>NR</td>
</tr>
</tbody>
</table>
Many sequences shared high degrees of similarity with clone 8; however, their rate constants were highly variable. Conversely, there were fewer clones in the clone 6 family and all had low rate constants. The rate constant for clone 8 and clone 6 was approximately 0.01 min\(^{-1}\), and the best clone from the reselection was clone 11 (from the clone 8 family), which had a rate constant 0.8376 min\(^{-1}\). This represented an 80-fold improvement, and therefore it could be concluded that the reselection was moderately successful and provided at least one clone for further characterization.

4.4 Analysis of clone 11

4.4.1 Self-cleavage kinetics

The preliminary data showed that clone 11 had the highest rate constant. A more detailed kinetic assay in which 11 time points were collected was done to determine a more precise rate constant \((\text{Table 4.10})\). When the data were fitted to either a single-exponential equation or a double-exponential equation, which represents the fraction of DNAzyme cleaved compared to that approximated at infinity, the total fraction cleaved had an apparent value that was lower than unity. This feature was also observed with the data of DNAzyme clone 25 in Chapter 3. However, fitting the amplitude to the experimental value obtained did not affect the value of the calculated rate constant. Rate constants were first calculated using a single exponential equation \((\text{Equation 3.1})\). The single-exponential equation fitting gave a correlation coefficient 0.89 indicating a bad fit. The rate constant was recalculated with a double-exponential equation \((\text{Equation 3.2})\), which gave a correlation coefficient 0.996, indicating a much better fit. The fast phase had a rate constant of 0.84±0.09 min\(^{-1}\), and the slow phase had a rate constant of 0.056±0.001 min\(^{-1}\) \((\text{the fast phase curve and slow phase curve are shown in Figure 4.6})\). While the data appear to fit a double-exponential rate process, suggesting that the reaction is a biphasic reaction, the majority of the active DNAzyme (~60%) are fast-cleaving species; the slow-cleaving species represents approximately 40% of the active DNAzyme. In order to ensure that the reaction had gone to
completion, another data set was collected over 20 hours instead of 3 hours. The cleavage percentage reached approximately 70%, indicating that cleavage is complete after 3 hours.

**Table 4.10** Self-cleavage kinetic data for clone 11

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>0</th>
<th>0.5</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>6</th>
<th>9</th>
<th>15</th>
<th>30</th>
<th>60</th>
<th>180</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fraction cleaved</td>
<td>0</td>
<td>0.15</td>
<td>0.24</td>
<td>0.34</td>
<td>0.38</td>
<td>0.48</td>
<td>0.50</td>
<td>0.54</td>
<td>0.61</td>
<td>0.64</td>
<td>0.68</td>
</tr>
</tbody>
</table>

**Figure 4.6** Self-cleavage kinetics of DNAzyme clone 11. Experiments were performed in 50 mM sodium cacodylate pH 7.4, 200 mM NaCl, 1 mM EDTA at room temperature. Red curve, real data fitting curve; green curve, slow-phase cleavage curve; blue curve, fast-phase cleavage curve. \( k_{obs} = 0.84 \pm 0.09 \text{ min}^{-1} \) (\( R^2 > 0.99 \))
4.4.2 Predicted secondary structure of DNAzyme clone 11

The sequence of the self-cleaving species Clone 11 was entered into mFOLD with the following parameters: 200 mM Na\(^+\) and 0.5mM Mg\(^{2+}\) at 30 °C. The predicted secondary structure is shown in Figure 4.7, which gave the lowest folding energy. The structure may not be accurate, because the three designed cleavage site nucleobases form hydrogen bonds with the catalytic region and this is very uncommon.

![Secondary structure predicted by mFOLD. The secondary structure contains two stem loops – one engineered (I) and one arising from the degenerate region (II). Modified bases are indicated by coloured letters. The pink letters represent the all-RNA target](image)

4.4.3 Modified base replacement test

DNAzyme clone 11, which has a rate constant of 0.84 min\(^{-1}\), was obtained with three modified nucleotide triphosphates. DNAzyme clone 11 was examined whether the modifications
were absolutely necessary for cleavage activity. In order to probe the importance of the modifications, different combinations of modified nucleotides were replaced by unmodified nucleotides. The elongation reactions in which at least one modified nucleotide was replaced with an unnatural nucleotide counterpart are shown Figure 4.8. It can clearly be observed that all the modifications are critical for the cleavage activity, as replacing any one of the three modified nucleotides with a natural nucleotide is shown to lead to the complete loss of cleavage activity.

**Figure 4.8** Self-cleavage of modified base replacement test. A+U, modified A and U with canonical G and C; A+C, modified A and C with canonical G and T; U+C, modified U and C with canonical A and G; A, modified A with canonical G, C and T. Time points were collected at 0, 1, 3, 10, 30 and 60 minutes. The lane on the far left is full-length elongation product treated with 1 M NaOH

### 4.4.4 Kinetics

A divalent metal profile, pH-rate profile, and temperature-rate profile were conducted to understand DNAzyme clone 11. Firstly, the divalent metal effect was evaluated on the rate of self-cleavage of DNAzyme clone 11. As was discussed in Chapter 3, divalent metals may affect the cleavage in two different manners (section 3.4). A selection of alkaline earth metals, transition metals, lanthanide cations and imidazole (each at 0.5 mM) were individually included in the selection buffer in order to determine their effects on the rate constants (**Table 4.11**). No
significant inhibition of the self-cleavage reaction was observed but Ce$^{3+}$ was found to increase the rate by a factor 5.1.

**Table 4.11** Effect of metal cations on the cleavage rate of DNAzyme clone 11

<table>
<thead>
<tr>
<th>Metal</th>
<th>$k_{obs}$ (min$^{-1}$)</th>
<th>$k_{rel}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer only</td>
<td>0.84</td>
<td>1</td>
</tr>
<tr>
<td>Mg$^{2+}$</td>
<td>1.34</td>
<td>1.6</td>
</tr>
<tr>
<td>Zn$^{2+}$</td>
<td>0.82</td>
<td>1.0</td>
</tr>
<tr>
<td>Ca$^{2+}$</td>
<td>0.68</td>
<td>0.8</td>
</tr>
<tr>
<td>Mn$^{2+}$</td>
<td>0.96</td>
<td>1.2</td>
</tr>
<tr>
<td>Cu$^{2+}$</td>
<td>0.55</td>
<td>0.7</td>
</tr>
<tr>
<td>Hg$^{2+}$</td>
<td>0.87</td>
<td>1.0</td>
</tr>
<tr>
<td>Ba$^{2+}$</td>
<td>0.77</td>
<td>0.9</td>
</tr>
<tr>
<td>Ni$^{2+}$</td>
<td>1.21</td>
<td>1.5</td>
</tr>
<tr>
<td>Co$^{2+}$</td>
<td>1.47</td>
<td>1.8</td>
</tr>
<tr>
<td>UO$_2$$^{2+}$</td>
<td>0.42</td>
<td>0.5</td>
</tr>
<tr>
<td>Fe$^{3+}$</td>
<td>2.01</td>
<td>2.4</td>
</tr>
<tr>
<td>Ce$^{3+}$</td>
<td>4.28</td>
<td>5.1</td>
</tr>
<tr>
<td>Imidazole*</td>
<td>0.84</td>
<td>1.0</td>
</tr>
</tbody>
</table>

**pH-rate profile**

The pH dependence of the DNAzyme clone 11 was investigated to understand the mechanism of the catalyst. Due to the fact that the pKa of the imidazole on the modified adenosine is around 7, the pH-rate profile may indicate what role the imidazole group plays in the mechanism. Generally either bell-shaped or log-linear pH-rate profiles are seen for DNAzymes and ribozymes. A bell-shaped profile supports a two-step general acid/general base mechanism. Many DNAzymes have this type mechanism, including several modified DNAzymes discovered by the Perrin group.$^{116,140,180,190}$ A log-linear pH-rate profile indicates a single proton transfer mechanism. The hammerhead ribozyme was found to have this type of pH-rate profile.$^{178}$ The self-cleavage assays were done at pH 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, and 9.0, and the rate constants for the fast phase were calculated (**Table 4.12**). A graph plotting pH versus observed rate constant was obtained, and the data appeared to be fitted into a log-linear equation (**Figure 4.9**). The log-linear fit supported the single proton transfer mechanism. Two possible single proton transfer
steps are shown in Figure 4.9B. When the experiment was conducted at pH 9.0, the fast phase cleavage reaction was so fast that accurate data became very difficult to obtain manually. Therefore, the true rate constant may be higher than calculated.

Table 4.12 pH-rate profile, rate constant under different pH

<table>
<thead>
<tr>
<th>pH</th>
<th>6.0</th>
<th>6.5</th>
<th>7.0</th>
<th>7.5</th>
<th>8.0</th>
<th>8.5</th>
<th>9.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>$k_{_{obs}}$ (min$^{-1}$)</td>
<td>0.031</td>
<td>0.16</td>
<td>0.52</td>
<td>0.84</td>
<td>1.13</td>
<td>2.16</td>
<td>2.37</td>
</tr>
</tbody>
</table>

Figure 4.9 pH-rate profile of DNAzyme clone 11 and proton transfer steps. A Self-cleavage rate dependence on pH of DNAzyme clone 11, measured in a buffer containing 50 mM sodium cacodylate (pH 6.0-9.0), 200 mM NaCl, 1 mM EDTA at room temperature. B One possible single proton transfer step is indicated with red coloured 1
Temperature profile

The final study investigated at the optimal temperature for self-cleavage, as well as the DNAzyme’s performance under physiological temperature. The activation energy for the cleavage reaction was also determined from the temperature profile. As was discussed in Chapter 3, the pre-cleavage step in which hydrogen bonding between the two binding arms and the RNA substrate strand form two partial double strands is highly temperature-dependent. When the temperature rises to a point that is higher than the melting point temperature of these double strands, the cleavage reaction will have a lower catalytic rate. The temperature profile was performed at 5 °C, 15 °C, 20 °C, 25 °C, 30 °C, 35 °C and 45 °C. The rate constants obtained at different temperatures are shown in Table 4.13, and was highest at around 30°C. The linear portion of the data set was fit to the Arrhenius equation (Equation 3.3) and gave an activation energy of 19±2 kcal M⁻¹ (Figure 4.10).

Table 4.13 Temperature profile data

<table>
<thead>
<tr>
<th>T(K)</th>
<th>278.15</th>
<th>288.15</th>
<th>293.15</th>
<th>298.15</th>
<th>303.15</th>
<th>308.15</th>
<th>318.15</th>
</tr>
</thead>
<tbody>
<tr>
<td>1/T(K⁻¹)</td>
<td>0.0036</td>
<td>0.00347</td>
<td>0.00341</td>
<td>0.00335</td>
<td>0.0033</td>
<td>0.00325</td>
<td>0.00314</td>
</tr>
<tr>
<td>k_{obs}(min⁻¹)</td>
<td>0.34</td>
<td>0.52</td>
<td>0.84</td>
<td>1.33</td>
<td>2.63</td>
<td>1.10</td>
<td>0.76</td>
</tr>
<tr>
<td>ln(k)</td>
<td>-1.07881</td>
<td>-0.59784</td>
<td>-0.07257</td>
<td>0.14842</td>
<td>0.34359</td>
<td>-0.0202</td>
<td>-0.18633</td>
</tr>
</tbody>
</table>
Figure 4.10 Temperature profile of DNAzyme clone 11. Experiments were conducted in a buffer containing 50 mM sodium cacodylate (pH 7.4), 200 mM NaCl, 1 mM EDTA at 5 °C, 15 °C, 20 °C, 25 °C, 30 °C, 35 °C and 45 °C (R²=0.97).

Sequence specificity cleavage test

DNAzyme clone 11 was engineered to cleave the HIV RNA target sequence in Chapter 3 to test for sequence specificity. The self-cleavage results are shown in Figure 4.11. No activity was detected when the DNAzyme clone 11 was tested against this target. The loss of cleavage activity could be due to the fact that the DNAzyme is site-specific.

Figure 4.11 Self-cleavage reactions of DNAzyme clone 11 with the HIV RNA target. Time points were collected at 0, 1, 3, 10, 30, 60, 120 minutes.
4.5 Trans-cleavage reaction studies

4.5.1 Multiple-turnover studies

In addition to the self-cleavage reaction studies, the trans-cleavage activities of clone 11 were investigated. The multiple-turnover reactions were firstly conducted at 30 °C with 500 µM Mg²⁺, 50 mM sodium cacodylate (pH 7.4) and 200 mM NaCl. The data were fitted to the Michaelis–Menten equation (the curve is shown in Figure 4.12). The $k_{max}$ and $K_m$ values were determined to be 4.32 min⁻¹ and 297 nM, respectively. Therefore, the second-order rate constant is $1.5 \times 10^7$ M⁻¹min⁻¹.

![Trans-cleavage construct](image1)

**Figure 4.12** Trans-cleavage construct (A) and multiple-turnover studies of DNAzyme clone 11 (B). DNAzyme concentration, 25 nM; substrate concentration 25, 50, 100, 300, 500, 1000, 2000 nM. $k_{max}$=4.32 min⁻¹, $K_m$=297 nM (R²=0.995).
4.5.2 Divalent metal effect

The multiple-turnover reaction was tested in the absence of Mg\(^{2+}\) as well as in the presence of various divalent metal cations. There was no significant change in the rate constants in the presence or absence of Mg\(^{2+}\), indicating Mg\(^{2+}\) plays no significant role in catalysis. At 500 µM, Zn\(^{2+}\), Mn\(^{2+}\) and Cu\(^{2+}\) showed no effect when compared to no divalent metal conditions. Ca\(^{2+}\) showed two-fold improvement. Partial inhibition was observed with 500 µM Pb\(^{2+}\) while a concentration of 500 µM Hg\(^{2+}\) fully inhibited the trans-cleavage reaction.

4.5.3 Trans-cleavage at 37 °C

The trans-cleavage reactions were conducted at 37 °C in order to evaluate the potential of using DNAzyme clone 11 in vivo. Although it was found that the rate constant decreased significantly at 37 °C compared to 30 °C for DNAzyme clone 25 in Chapter 3, this was not observed for DNAzyme clone 11. The multiple-turnover reactions were conducted at 37 °C to obtain the \(k_{\text{max}}\) and \(K_m\). The value of \(k_{\text{max}}\) and \(K_m\) at 37 °C did not differ significantly from that of at 30°C. Therefore, DNAzyme clone 11 was found to perform well at physiological temperature.

4.5.4 Cleavage of different base-base junctions

Trans-cleavage reaction of different base-base junctions were tested with DNAzyme clone 11. The original cleavage site junction is the rA-rU junction (\(U\), Figure 4.12); the rU was replaced with rA, rG or rC to form rA-rA, rA-rG or rA-rC junctions. Under the same reaction conditions, cleavage at the rA-rA junction showed a greater than 100-fold decrease, and the cleavage products of rA-rG and rA-rC junctions were barely detectable after a 24 hours incubation period (Figure 4.13). Therefore, the DNAzyme appears to be very target specific.
4.5.5 Solid-phase synthesis

Since trans-cleavage studies with enzymatically synthesized DNAzyme clone 11 showed promising results, the following step was to synthesize the DNAzyme by solid-phase synthesis. With the consideration that in vivo studies of this DNA may be pursued, three 2'-O-methyl-Cs at the 5' terminus and an inverted T at the 3' terminus were installed in order to increase the stability of oligonucleotides under physiological conditions. The appropriate phosphoramidites were made and sent to a collaborator for synthesis.

The synthesized oligonucleotides were globally deprotected, and a very small amount of was labeled with $^{32}$P-$\gamma$-ATP and loaded on 7% PAGE along with enzymatically synthesized DNAzyme to verify the solid-phase synthesis result (Figure 4.14). The 5'-$^{32}$P-labeled oligonucleotide appeared as a smear on the gel instead of a discrete band, indicating poor synthesis. While some of the solid-phase material was the same length as the enzymatically synthesized DNAzyme, it could not be concluded that these modified oligonucleotides were the desired product due to the overall poor quality and very low yield. The same sequence was also synthesized in three variations: the first contained all canonical nucleobases, the second contained a single modified A, and the third contained a single modified C and a single modified U (Figure 4.14). The synthesis of the all-natural oligonucleotide was successful as was the synthesis of the
modified CU sequence, Whereas the modified A synthesis had a truncation but was moderately successful. The modified A phosphoramidite is known to poorly couple in for solid-phase synthesis and hence a small degree of truncation was not unexpected.

![Image of gel with lanes labeled A and B with Truncation marker]

Sequence of DNAzyme clone 11: 5'-
\[\text{mC}^m\text{C}^m\text{CTCGGAGGGGCTGCCAGTATGGTGGCGTTGTTTGTTCGCGGCGGCGCT}\_\text{inv}^{-}3'\]

**Figure 4.14** Crude modified oligonucleotides from solid-phase synthesis. A lane 1, solid-phase synthesis result labeled by $^{32}\text{P}-\gamma$-ATP; lane 2, enzymatically synthesized DNAzyme clone 11. B lane 1, 53mer DNA; lane 2, DNAzyme clone 11 without modification; lane 3, DNAzyme clone 11 with only one modified A; lane 4, DNAzyme clone 11 with only one modified U and modified C each. (TGCGGAGGGGC), binding arm region; (TGCCAGTATGGTGGCGTTTGTTCGCGGCGGCG), modified As, Ts, Cs (G is not modified).

*Trans*-cleavage studies were conducted with the crude oligonucleotides that was obtained from solid-phase synthesis following deprotection, and the all-RNA substrate concentration was set at 1000 nM. Although the solid-phase synthesis was clearly messy, some cleavage activity was observed (**Figure 4.15 A**). Subsequently, a large fraction of the solid-phase synthesized oligonucleotide (30%) was loaded on a denaturing polyacrylamide gel subjected to purification. While the product once again appeared as a smear under UV shadowing, the position
corresponding to the correct full-length DNAzyme was cut out and eluted. The eluted modified oligonucleotides were tested for trans-cleavage (Figure 4.15 B).

Figure 4.15 Trans-cleavage study of solid-phase synthesis DNAzyme clone 11. A Trans-cleavage reaction with crude modified oligonucleotides at 0 hours, 20 hours (lanes 1-2). Lane 0, RNA substrate treated with 0.5% K₂CO₃ solution. B Trans-cleavage with purified modified oligonucleotides at 0, 30, 60, 120, 1200 minutes (lanes 1-5).

In order to try to purify the solid-phase synthesized oligonucleotides, a gel retardation assay was carried out in an attempt of purify the synthetic modified DNAzyme (Figure 4.16A). This involved the following procedure: a unmodified template of DNAzyme clone 11 was labeled by ³²P-γ-ATP first, then the template was annealed to solid-phase synthesis oligonucleotides mixture. The full-length modified sequences from the crude mixture preferentially formed a duplex with the template sequences and this duplex migrated more slowly than unbound truncated product (single-stranded undesired oligonucleotides). The duplex was cut out of the gel and purified by gel elution (Figure 4.16B). The sample was then divided into two aliquots. One aliquot was treated with lambda-exonuclease to digest the phosphorylated template, then purified and tested for trans-cleavage activity (Figure 4.16D). The other aliquot was tested directly without digestion (Figure 4.16C). Slightly more cleavage activity was observed with the
template-digested sample however this level of cleavage was still quite low (Figure 4.16D, lane 5).

Figure 4.17 Gel retardation assay and trans-cleavage reaction. A Experimental flow chart. B Gel retardation assay. lane 1, template; lane 2, template with modified oligonucleotides mixture. C Trans-cleavage reaction using modified oligonucleotides without digestion. Lane 0, RNA substrate treated with 0.5% K$_2$CO$_3$. Lanes 1-5, cleavage after 0, 30, 60, 120, 1200 minutes. D Trans-cleavage using modified oligonucleotides with digestion. Lanes 1-5, cleavage after 0, 30, 60, 120, 1200 minutes.
4.6 Discussion

4.6.1 General discussion

In this selection, because the same modified nucleotide triphosphates and similar protocol were utilized as in HIV RNA-cleaving DNAzyme selection, similar problems including poor modified dATP incorporation and poor amplification of modified template were encountered. These included low efficiency of dATMP incorporation and poor accuracy of first PCR amplification. From the initial selection, the selected DNAzymes clone 6 and 8 had disappointingly low activities when compared to previously reported DNAzymes, such as DNAzyme 12-91. However, the reselection process yielded much more promising results. The fastest clone had a rate constant of 0.84 min\(^{-1}\), which is a 14-fold improvement over DNAzyme 12-91. Moreover this DNAzyme showed excellent \textit{trans}-cleavage activities, and therefore may be a promising candidate for \textit{in vivo} study.

4.6.2 Selection and reselection analysis

The set-up for the initial selection was almost identical to the selection described in Chapter 3, with the exception of the primer, template and library sequences. Despite the similarities, the rate of enrichment was very sluggish compared to that reported in Chapter 3. No significant cleavage was seen until round 7, whereas cleavage was observed in round 4 as reported in Chapter 3. Additionally, in the following several rounds, the cleavage yield did not increase significantly, and reached only 5% in round 12. It is suspected that this poor selection performance was due to the fact that the library didn’t contain a good DNAzyme sequence. Alternatively it is thought that the desirable active sequences were poorly amplified compared to the undesirable inactive sequences, and therefore were extremely underrepresented in the selection pool. Time stringency was used to successfully improve cleavage yield to 7% in round 13, but this likely represented the best result without intervention and this was far poorer than the desired activity.
Therefore, mutagenic PCR was used to introduce more sequences to the library and the metal cation concentration stringency strategy was applied (section 4.3.2). With these changes, the cleavage yield increased 3 times, from 4% to approximately 15%. In round 20, application of a low monovalent metal cation concentration did not negatively affect the cleavage yield as expected. Therefore, it was thought that some very active DNAzymes had been selected that were able to operate under low metal cations concentrations, which was a highly desirable result. However, the subsequent sequencing analysis showed that the selected clones were highly similar to each other and each showed a low self-cleavage rate, which resulted in the high activity rate seen in the pool. This DNAzyme selection showed that with the initiatives introduced an active DNAzyme could be selected but high activity couldn’t be guaranteed.

The reselection showed significant enrichment at a much earlier stage compared to the initial selection. Cleavage was visible beginning in round 2 and round 3 and the cleavage yield increased dramatically to 20% in round 4. This phenomenon was seen only once in the selection of DNAzyme 10-66 conducted by the Perrin lab. It is thought that the fast progression was due to the fact that highly active DNAzymes existed in the library and were successfully propagated. Using mutagenic PCR, time stringency and metal cation concentration stringency, the cleavage yield was successfully increased to 40% at 60 minutes and 15% at 5 minutes. This library had a higher overall rate constant compared to the best clones from the initial selection. Therefore, it was hypothesized that a better DNAzyme existed in the library pool. DNAzyme clone 11 from this reselection had a rate constant of 0.84 min⁻¹, which is approximately 80 times higher than its parental clones 6 and 8.

4.6.3 Secondary structure of clone 11 and sequence analysis

DNAzyme clone 11 contains two stem loop structures according to the mFOLD predicted secondary structure (Figure 4.7). Loop I was specifically engineered to distance the all-RNA target from the catalytic region. Loop II is part of the N₃₀ region and constitutes the catalytic
motif. The predicted structure provided some basic understanding of the sequence. DNAzyme clone 11 contains 4 bases that form Watson-Crick base pairs with the catalytic region. Loop II has five Watson-Crick base pairs, which provide stability to the overall structure. These 4 bases and the loop II pairings are highly conserved among the clones that have a relatively high rate constant; only clone 13 has relatively high rate constant and a different structure pattern.

### 4.6.4 Kinetics

Varying the divalent metal cation had little effect on the relative rate constant with the exception of Ce$^{3+}$. The presence of Ce$^{3+}$ increased the rate constant by a factor of 5, which is significant. The reason for the increase is unknown. Other lanthanide metals were not examined, but one could imagine that other lanthanide metals may also be able to facilitate the reaction due to their similar chemical properties.

The effect of pH was studied within the range of pH 6.0-9.0. The observed increase in cleavage rate from pH 6.0 to 7.0 was much more robust than the increase observed from pH 7.0 to 9.0. Since the pKa of imidazole is approximately 7, at a pH range 6.0 to 7.0 the fraction of deprotonated imidazole should increase significantly. Deprotonated imidazole is crucial for cleavage activity, which is thought to be the reason that the rate increase was more significant in this range. At a pH higher than 7.0, the pH-rate profile is a log-linear profile with a relative high correlation. The log-linear pH-rate profile indicated that a single proton transfer step is the rate limiting step. This single proton transfer step could involve the removal of the 2'-OH proton by the imidazole group (Figure 4.9). If this is the rate limiting step, the rate constant will increase appreciably between pH 6.0-7.0. As this is what was observed, it may be concluded that the imidazole-mediated single proton transfer step is the rate limiting step.

DNAzyme clone 11 reaches maximum cleavage activity at around 30 °C. From a physiological perspective, this is more promising compared to the temperature optimum observed for DNAzyme clone 25 in Chapter 3. The enthalpy of activation (18.5kcal/mol) is slightly higher.
compared to DNAzyme 9-86 (15.3kcal/mol) and DNAzyme clone 25 (11.8kcal/mol). The reason could be that the cleavage target for DNAzyme clone 11 is completely different than the DNAzyme 9-86 and DNAzyme clone 25 target.

4.6.5 Sequence specificity cleavage test

In both Chapter 3 and 4, the selected modified DNAzymes could not be engineered into cleavers for a different target sequence. It is hypothesized that this is due to the modified groups of the catalytic region, as many unmodified DNAzymes have been successfully reengineered for new targets. In one experiment conducted with DNAzyme clone 11 99% of trans-cleavage activities were lost when the base junctions of the target sequence were changed, which may support this hypothesis. Although the high sequence-specificity could be interpreted as shortcoming of modified DNAzymes, it also means that the cleavage reaction is highly selective, and this could be advantageous in certain therapeutic applications.

4.6.6 Multiple-turnover study

The trans-cleavage study yielded promising results overall. The second-order rate constant of DNAzyme clone 11 is $10^7$ M$^{-1}$min$^{-1}$ under physiological conditions. It is comparable to those best DNAzymes that require high Mg$^{2+}$ concentrations for optimal activities. For instance, DNAzyme 10-23 has a second-order rate constant of $10^9$ M$^{-1}$min$^{-1}$ under 200 mM Mg$^{2+}$ conditions but this value falls to $10^8$ at 2 mM Mg$^{2+}$. It is plausible that at the 0.5 mM Mg$^{2+}$ concentration used in the trans-cleavage studies of DNAzyme clone 11, the cleavage rate of DNAzyme 10-23 would be even more depressed. However, the $K_m$ value of my DNAzyme is much higher than that of DNAzyme 10-23, likely because the modified DNAzyme binding arm did not derive a significant energetic benefit from hydrogen bonding with the RNA substrates. It is hypothesized that the modified binding arm did not involve the catalytic activity, therefore, if one can synthesize the DNAzyme clone 11 containing two unmodified binding arms using solid-
4.6.7 Solid-phase synthesis study

Although the solid-phase synthesis was not very successful, some positive results were achieved including detection of small amounts of catalytic activity in the crude synthetic product following global deprotection. The messy synthesis could be due to several reasons, such as low quality phosphoramidites, poor synthesis performance or poor deprotection. It is also possible that the modified phosphoramidites were poor substrates for solid-phase synthesis, which is known to be true for the modified A phosphoramidite (Figure 4.14 lane 3). However, since the sequence contains only two modified As this cannot fully account for the poor results. Despite the low quality of the solid-phase synthesis product, the trans-cleavage studies conducted using this material showed encouraging results. Both the crude and the full-length purified modified oligonucleotides showed trans-cleavage activity, as did the oligonucleotides that were isolated from the gel retardation assay.

4.7 Conclusion

In this chapter, an all-RNA-cleaving DNAzyme was successfully selected with high self- and trans-cleaving activities. DNAzyme clone 11 was obtained after a selection and reselection process from a library containing three modified nucleotides. This DNAzyme reached a rate constant of 0.84 min⁻¹ for the self-cleavage reaction, and a $k_{\text{max}}$ of 4.32 min⁻¹ and $K_m$ of 297 nM for the trans-cleavage reaction under 0.5 mM Mg²⁺. DNAzyme clone 11 is inhibited by 0.5 mM mercury in trans-cleavage reactions. Solid-phase synthesis of DNAzyme clone 11 was attempted, but no pure modified DNAzyme was synthesized. Nonetheless, the mixture of crude solid-phase synthesized oligonucleotides showed cleavage activity. Future work should focus on achieving a clean solid-phase synthesis product. Additionally, in vivo studies could be considered due to DNAzyme clone 11’s promising physiological properties.
4.8 Materials and methods

4.8.1 Materials and equipment

dA\textsuperscript{im-TP}, dU\textsuperscript{gs-TP} and dA\textsuperscript{homo-TP} were prepared by previous lab members.\textsuperscript{134,161} dC\textsuperscript{anr-TP} was purchased from TriLink. Guanidine-modified 2'-deoxyuridine phosphoramidite and allylamino-modified 2'-deoxycytosine phosphoramidite were synthesized according to literature procedures.\textsuperscript{161,191} Imidazole-modified 2'-deoxyadenine was synthesized by previous lab members. All oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA) and were purified by 10–15% 7 M urea denaturing PAGE. Ultrapure dNTPs were obtained from Fermentas. Sequenase Version 2.0 DNA polymerase was purchased from Affymetrix. Lambda exonuclease, Taq DNA polymerase, T4 polynucleotide kinase and Vent (exo-) DNA polymerase were obtained from New England Biolabs. Streptavidin magnetic particles were purchased from Roche. The nucleoside triphosphates $^{32}$P-\textalpha-dGTP and $^{32}$P-\textgamma-ATP were purchased from PerkinElmer. pGEM-T-Easy Vector Systems kit was obtained from Promega. Plasmid miniprep kits was from Invitrogen. LiClO\textsubscript{4} was purchased from J.T. Baker. The rest chemicals were purchased from Sigma-Aldrich and Fisher Scientific. X-Gal and SOC media was purchased from Invitrogen. Ampicillin disodium salt was purchased from Sigma. Water used in all experiments was first treated with diethyl pyrocarbonate (DEPC. 5 μL per 100 mL) and autoclaved. Radioactive PAGEs were exposed to storage phosphor screens, and then the screen was scanned with a Typhoon 9200 Phosphorimager from GE Healthcare. Plasmid concentrations were measured with NanoDrop 2000C (Thermo Scientific). Temperature was adjusted with VWR temperature-controlled water bath.

4.8.2 Oligonucleotides

Primer-Neo-2 and Primer-Neo-3 for this selections are the 1\textsuperscript{st} amplification PCR primers. Primer-Neo-4 and Primer-Neo-3 were used in the 2\textsuperscript{nd} amplification step. Primer-Neo-5 and Primer-Neo-2 were used in the cloning steps. Primer-Neo-1 was used in the elongation step. Biotinylated
template 11 was used in the elongation for trans-cleavage study. Phosphorylated template 11 was used in the gel retardation assay.

El-1: 5'-/5Biosg/T_40^-CCC-3'

El-splint: 5'-AAAAACCCGGGAAAAA-3'

El-neo-primer: 5'-GGGTTTTT(ccgcgaucgauccccuc)TTTTTCGTCGGCCTGCGGAGGGGC-3'

Primer-Neo-1: 5'/5Biosg/T_40-

CCCAGGGTTTTT(ccgcgaucgauccccuc)TTTTTCGTCGGCCTGCGGAGGGGC-3'

Primer-Neo-2: 5'-GCCTCGCCTCGCCGCACG-3'

Primer-Neo-3: 5'/5Phos/GCGTCGGCCTCGCCGAGGGG-3'

Primer-Neo-4: 5'-TTTTTTTTTTTTTTTTTTTTTTTTTTTTGCGTCGCGCGCCCGCGA-3'

Primer-Neo-5: 5'/GCCTCGCCTCGCCGAGGGG-3'

Template-Neo: 5'-GCCTCGCCTCGCCGAGGGG(AG_30)GCCCCTCCGACGCGACGC-3'

Primer-Neo-6: 5'/5Biosg/T_40-

CCCAGGGTTTTTCCGCGA(cgau)GCCCCTCTTTTTGCGTCGCGCGCCGAGGGG-3'

Biotinylated template 11: 5'-

/5Biosg/GCGCGCCGCGGAACAAACGAACGCCACAAGACTACTGGCAGCCCTCCCGCAG

GC-3'

Phosphorylated template 11: 5'-

/5Biosg/GCGCGCCGCGGAACAAACGAACGCCACAAGACTACTGGCAGCCCTCCCGCAG

-3'

All-RNA substrate target: 5'(ccgcgaucgauccccuc)T-3'

All-RNA substrate target AA: 5'(ccgcgaucgauccccuc)T-3'

All-RNA substrate target AG: 5'(ccgcgaucgauccccuc)T-3'

All-RNA substrate target AC: 5'(ccgcgaucgauccccuc)T-3'
4.8.3 Buffers and cocktails

Buffers

Selection cleavage buffer: 50 mM sodium cacodylate (pH 7.4), 200 mM NaCl, 1 mM EDTA.
Elution buffer: 1% LiClO₄, 10 mM Tris-HCl (pH 8) in water.
Cleavage buffer (pH variance): 50 mM sodium cacodylate, 200 mM NaCl and 1 mM EDTA. The pH of all buffers was adjusted to 6.00, 6.50, 7.01, 7.50, 8.00, 8.50, or 9.00.
NaOH neutralization buffer: 25 mM sodium cacodylate pH 6
10x Cleavage buffer: 500 mM sodium cacodylate (pH 7.4), 2000 mM NaCl.
Loading solution: Formamide (27 mL), EDTAₐq (3 mL, 0.5 M), xylene cyanolₐq (300 μL, 0.05 %) and bromophenol blueₐq (300 μL, 0.05 %).
6x gel shift binding buffer: 15% Ficoll®-400, 60mM EDTA, 20mM Tris-HCl, 0.12% bromophenol blue, pH 8.0

Cocktails

5x 1st amplification cocktail: 9 nmol primer-Neo-2, 7.5 nmol primer-Neo-3, 11.5 μL 100 mM MgSO₄, 115 μL 10x thermopol buffer, 3.45 μL 100 mM dNTPs (100mM), added water to make a total 230 μL solution.
5x 2nd amplification cocktail: 10 nmol primer-Neo-4, 7.5 nmol primer-Neo-3, 11.5 μL 100 mM MgSO₄, 115 μL 10x thermopol buffer, 3.45 μL 100 mM dNTPs (100mM), added water to make a total 230 μL solution.
Cocktail for cloning PCR: 10 nmol primer-Neo-2, 7.5 nmol primer-Neo-5, 11.5 μL 100mM MgSO₄, 115 μL 10x thermopol buffer, 3.45 μL 100 mM dNTPs (100mM), added water to make a total 230 μL solution.
1x mutagenic 2nd amplification cocktail: 1.3 nmol primer-Neo-4, 1.7 nmol primer-Neo-3, 20 μL 10x thermopol buffer, 4 μL 25 mM MnCl₂, 0.4 μL 100mM dGTP and dATP, 2 μL 100mM dCTP and dTTP, added water to make a total 180 μL solution.
4.8.4 Methods

Elongation

Fifteen pmol of Primer-Neo-1 were annealed to 15 pmol of template DNA prepared by 2nd amplification PCR or mutagenic 2nd amplification PCR, and then enzymatically polymerized at 32°C for 4 hours using 13 units of Sequenase in a mixture containing 5 mM DTT, 50 μM dA<sub>in</sub>TP, 20 μM of each dUTP<sub>in</sub>, dCTP<sub>in</sub>, dGTP, and 5–15 μCi of 32P-α-dGTP to give a final volume of 40 μL. The reaction was overlaid with mineral oil. After incubation, the reaction was quenched by adding 1μL 500 mM EDTA (pH 8) solution.

Selection

50μL of magnetic streptavidin beads were transferred into a 1.5 mL eppendorf tube, and washed with TEN buffer (3×100 μL). The elongation reaction mixture was added to the beads, and incubated at room temperature for 30 minutes. Then the beads with bound DNAs were magnetized and the supernatant was removed. The beads were washed with TEN buffer (2×100 μL), template stripping buffer (5×100 μL, no longer than 30 sec/wash), then NaOH neutralization buffer (1×200 μL) and water (1×100 μL). Then the modified single strand DNA was incubated with 160 μL varying cleavage buffer for varying time spans (Table 4.1, 4.2, 4.5, 4.5) at room temperature. After incubation, the beads were magnetized and the supernatant was transferred to a new eppendorf tube. Then 1% LiClO<sub>4</sub> in acetone (800 μL) and 5x 1<sup>st</sup> amplification cocktail (2 μL) were added to the supernatant. The eppendorf tube was centrifuged at 13x10<sup>3</sup> rpm for 15 minutes. Then the supernatant was discarded, and 1 mL of iced ethanol was added to the pellet in the eppendorf tube. The sample was agitated by vortex and centrifuged at 13x10<sup>3</sup> rpm for 15 minutes. The ethanol was decanted and any residual ethanol was removed at 65 °C for around 5 minutes. The pellet was dissolved in 5μL of H<sub>2</sub>O whereupon 10 μL loading dye was added to the pellet, and then the sample was loaded onto 7% 7 M urea denaturing PAGE. The species corresponding to the cleaved product was cut and eluted using elution buffer and precipitated.
with ethanol. The precipitated pellet was dried and resuspended in 60 μL water and used in the 1st amplification PCR step.

1st amplification PCR

The cleavage product eluted from selection was PCR amplified using primers-Neo-2 and primer-Neo-3 and an internal label (10 μCi $^{32}$P-α-dGTP) for 25 cycles (15 s at 58 °C, 40 s at 75 °C and 15 s at 95 °C). The reaction (40 μL) included 0.1 units/μL Vent (exo-) DNA polymerase, 20 mM Tris-HCl (pH 8.8 at 25 °C), 10 mM (NH$_4$)$_2$SO$_4$, 10 mM KCl, 3 mM MgSO$_4$, 0.1% gelatin, 7 μM oligonucleotides and 0.3 mM of each natural dNTP. Before purification by 10% 7 M urea denaturing PAGE, the dsDNA was extracted with 40 μL phenol/chloroform/isoamyl alcohol (25:24:1), precipitated with 400 μL ethanol. After agitating and 15 minutes of centrifuging the ethanol was decanted and residual amounts were evaporated. The resulting pellet was resuspended in 44 μL water, add 5 μL 10x lambda exonuclease buffer and 1 μL lambda exonuclease (5 units). The sample was incubated at 37 °C 3 hours. The phosphorylated strand was digested. The resulting product was 50 μL phenol/chloroform/isoamyl alcohol (25:24:1), precipitated with 500 μL ethanol. After agitating and centrifuging 15 minutes, the ethanol was decanted and residual amounts were evaporated. The resulting pellet was resuspended in 20 μL water. 30 μL loading solution was added, and then the sample was purified by 10% 7 M urea denaturing PAGE. The corresponding band was cut out, eluted with elution buffer, precipitated with ethanol. The resulting pellet was resuspended in 25 μL water and used in the 2nd amplification PCR step.

2nd amplification PCR

10 μL 1st amplification PCR product was added to 20 μL 5X PCR cocktail, 5 μL Vent (exo-) DNA polymerase (10 units), 65 μL water to give a 100 μL reaction. The amplification reaction was thermocycled using the same program for 30 cycles. After the amplification, the amplicon product was treated in the same manner with the 1st amplification product. After
extraction, precipitation and resuspension, the DNA was also subjected to digestion by lambda exonuclease. The single-stranded DNA was dissolved in 1 M NaOH (20 μL) and loading solution (30 μL) prior to loading on a 10% 7 M urea denaturing mini-PAGE. The single-stranded DNA was identified by UV-shadowing, cut out, eluted with elution buffer, precipitated with ethanol. The resulting pellet was resuspended in 40 μL and desalted using a G-25 column. The DNA was quantified by UV absorption and used in elongation step.

The N30 library from IDT was not used for elongation directly. Instead a 2nd amplification was performed first with the N30 library. 300 pmol template was added to 20 μL 5X PCR cocktail, 5 μL Vent (exo-) DNA polymerase (10 units), 65 μL water to make a 100 μL reaction. The amplification reaction was thermocycled using the same program for 10 cycles. After the amplification, the amplicon product was treated in the same manner with the 2nd amplification including extraction, precipitation and resuspension, digestion, PAGE purification, G25 desalting. The single-stranded DNA was quantified by UV absorption and used in elongation step.

**Mutagenic 2nd amplification PCR**

5 μL 1st amplification PCR product was added to 90 μL 1X PCR cocktail, 5 μL Taq DNA polymerase (25 units). The mutation was achieved by the unbalanced dNTPs concentrations. The amplification reaction was thermocycled using the same program for 30 cycles. After the amplification, the amplicon product was treated in the same manner with the 2nd amplification including extraction, precipitation and resuspension, digestion, PAGE purification, G25 desalting. The single-stranded DNA was quantified by UV absorption and used in elongation step.

**Gel elution**

Gel bands containing the desired oligo were cut out and crushed into small gel particles with a flame-sealed pipette tip. 300 μL of elution buffer was added to the gel particles. The suspension was agitated by vortex, incubated at 65 °C for 10 minutes, and centrifuged at 13x10³
rpm for 5 minutes. The supernatant was collected and transferred to a new 1.5 mL eppendorf tube. This elution process was repeated twice. The collected supernatants were combined and evaporated on a vacuum centrifuge at 30 °C. After the elution was evaporated to ~100 μL, 1 mL of ethanol was added to the elution, and mixed by vortex, and then centrifuged at 13x10^3 rpm for 15 minutes. The supernatant was discarded and another 1 mL of ethanol was added to the elution, and mixed by vortex, and then centrifuged at 13x10^3 rpm for 15 minutes. The ethanol was decanted as well as residual amounts were evaporated. The resulting pellet was resuspended in water.

**Ligation**

4 nmol El-splint, 2 nmol El-l and 2 nmol El-neo-primer were annealed under 95°C for 30 seconds, cooled down to room temperature then cooled on ice. 5 μL 10X ligation buffer, 2 μL T4 DNA ligase (4000 units) and water were added to the annealed solution result a 50 μL reaction. The reaction was incubated at 15°C for 16 hours. After the completion, the reaction was extracted 50 μL phenol/chloroform/isoamyl alcohol (25:24:1), precipitated with 500 μL ethanol. After agitating and 15 minutes of centrifuging the ethanol was decanted and residual amounts were evaporated. The resulting pellet was resuspended in 20 μL water. 30 μL loading solution was added, and then the sample was purified by 10% 7 M urea denaturing PAGE. The corresponding band was cut out, eluted with elution buffer, precipitated with ethanol. The resulting pellet was resuspended in 30 μL water. The ligation product was quantified by UV absorption and used in elongation step.

**TOPO TA cloning**

The product from the second PCR amplification was used as template for PCR with Taq DNA polymerase to produce double stranded material with overhanging 3’-dA’s. TA-cloning was then performed on this product using the pGEM-T-Easy Vector Systems kit. Using electroporation, the resulting plasmids were transformed into E. coli DH10B cells, and the cells
were streaked onto lysogeny broth (LB) agar containing ampicillin (100 mg/L). Individual white colonies were picked and used to inoculate 1.5 mL SOC medium solution. The samples were cultured at 37 °C for 16 hours. Plasmids were isolated with Plasmid miniprep kit using a centrifuge. The plasmids concentrations were measured with NanoDrop and were restriction digested in the presence of EcoRI to screen for the appropriately sized inserts which the plasmids were submitted to the UBC Nucleic Acid Protein Service Unit for sequencing.

**Screening of the clones**

Single-stranded templates for individual sequences obtained from the sequencing of the individual clones were ordered from IDT. To screen the clones for self-cleavage activity, 3 pmol of Primer-Neo-1 were annealed to 4 pmol of template and extended using dC*sup*TP, dU*sup*TP, dA*sup*TP, natural dGTP and *32*P-α-dGTP. Elongation reactions were quenched after 4 hours with the addition of 0.5 μL 0.5 M EDTA. 10 μL streptavidin beads were pre-washed with TEN buffer (3×30 μL). Extension reactions were incubated with the pre-washed streptavidin beads for 30 minutes at room temperature. After the incubation, the beads were washed with TEN buffer (2×30 μL), template stripping buffer (5×30 μL), NaOH neutralization buffer (1×60 μL), H2O (1×30 μL). Clones were incubated with 40 μL selection cleavage buffer at room temperature. At time points 0, 3, 10, 60 and 180 minutes an aliquot (5 μL) was removed and quenched by adding loading solution. Cleaved and uncleaved DNAzymes were resolved on a 10 % denaturing PAGE, visualized using a Phosphorimager and rectangles were drawn around the bands corresponding to cleaved and uncleaved products. The data were fitted to a single- or double-exponential equation using Origin Pro for an initial estimate of the observable rate constant.

**Temperature, pH and divalent metal effect profile**

For each temperature kinetic assay, a 3 pmol elongation reaction was carried out. The elongation reaction was then incubated with 10 μL pre-washed streptavidin beads for 30 minutes at room temperature. The beads were treated in the same manner as with screening of the clones.
40 μL cleavage buffer was added to the beads. At time points 0, 1, 3, 10, 30, 60 minutes, an aliquot (5 μL) was removed and quenched with an addition of 8 μL loading solution. Cleaved and uncleaved oligonucleotides were resolved on a 10 % denaturing PAGE, visualized using a Phosphorimager and rectangles were drawn around the bands corresponding to cleaved and uncleaved products. The data were fitted to a single-exponential equation using Origin Pro for an initial estimate of the observable rate constant.

**Elongation for trans-cleavage study**

30 pmol of Primer 5 were annealed to 30 pmol of biotinylated template clone 11 DNA, and then enzymatically polymerized at 32 °C for 4 hours using 13 units of Sequenase in a mixture containing 5 mM DTT, 50 μM dA[^32]TP, 20 μM of each dU[^32]TP, dC[^32]TP, dGTP, and 15 μCi of 32-P-α-dGTP to give a final volume of 40 μL. The reaction was overlaid with mineral oil. After incubation, the reaction was quenched by adding 1μL 500 mM EDTA (pH 8) solution.

**Measuring the concentration of DNAzyme clone 11**

A 1 μL aliquot of the resulting elongation solution was diluted 3x, 10x, 30x, 100x, 300x, 1000x and 2 μL of each dilution was spotted on a TLC-plate (appendices Chapter 4) to generate an autoradiographic calibration curve that relates autoradiographic density to volumes and ultimately the number of pmol of dGTP (specific activity). The logarithm of signal intensity of plotted against the logarithm of the dilution (appendices Chapter 4) and the data was fitted to a square line using linear regression. The rest of elongation product was immobilized on 50 μL of pre-washed streptavidin beads by incubating at room temperature for 30 minutes. After washes with TEN buffer (2×100 μL), then water wash (2×100 μL), the enzymatically synthesized modified DNAzyme was removed from the template by 0.1 M NaOH (2×30 μL) at room temperature for 30 seconds, and then neutralized with 5M HCl to pH 7-8. The collected oligonucleotides was desalted through a G25 column. A 1 μL aliquot of the resulting DNAzyme
solution was diluted 10x and 2 μL of the dilution was spotted on the same TLC-plate. The concentration of DNAzyme could then be determined using Equations 4.1 and 4.2

\[ x = (y - b)/a \]  \hspace{1cm} \text{Equation 4.1}

\[ [\text{DNAzyme}] = \frac{[\text{dGTP}]^{10}}{14 + e^{-x}} \] \hspace{1cm} \text{Equation 4.2}

Where \( a \) is the slope, \( b \) the intercept of the linear regression of calibration curve, \( x \) is the logarithm of the dilution factor of the DNAzyme, and \( y \) is the logarithm of signal intensity of the diluted DNAzyme. The factor of 10 and 14 arise because of the 10-fold dilution of the DNAzyme spotted on the TLC-plate and the presence of 14 dGs in the catalytic region of DNAzyme clone 11 and the left binding arm (Figure 4.12).

5'-End labelling of substrates

1 nmol of all-RNA substrate target 2 was 5'-end labelled with 30 μCi of \(^{32}\text{P}\)-γ-ATP by using 30 units of T4 polynucleotide kinase in a 40 μL volume reaction. The reaction mixtures were incubated at 37 °C for 2 hours. Then the reaction was terminated by heating at 65 °C for 20 minutes. 30 μL loading solution was added to the reaction mixture, and the resulting mixture was loaded on a 20% PAGE. Product was identified by UV shadowing and cut out. Gel material was crashed into fine particle with a flame-sealed pipette tip and eluted with elution buffer at 65 °C 10 minutes. The liquids were evaporated and ethanol precipitated. Finally, the isolated solid was re-suspended in 40 μL water and desalted with a G25 spin column, quantified by UV.

Multiple-turnover kinetics

0.5 pmol DNAzyme clone 11 was added to appropriate amount of radio-labelled all-RNA substrate. Then the mixture was denatured under 95 °C for 2 minutes, cooled to 0 °C on ice, followed by adding 2 μL 10x cleavage buffer (with/without divalent metal cation) to initiate the reactions, water was added to make a total volume of 20 μL. At each time point, 2 μL aliquots were transferred into 7 μL loading solution. Cleaved and uncleaved oligonucleotides were resolved on a 20 % denaturing PAGE, visualized using a Phosphorimager and rectangles were
drawn around the bands corresponding to cleaved and uncleaved products. The rate constant was obtained for each substrate concentration from a fitting of the data point obtained over the first 10-20% of the reaction. $k_{\text{max}}$ and $K_m$ values were determined by plotting the rate constants versus substrate concentrations and fitting the result curve to Michaelis-Menten equation.

**Synthesis**

![Chemical structure](image)

Chemical Formula: C$_{51}$H$_{56}$F$_3$N$_6$O$_9$P  
Molecular Weight: 985.01

The synthesis of allylamino-modified 2′-deoxycytosine phosphoramidite was synthesized by following the literature.$^{192}$ $^1$H-NMR (300 MHz, CDCl$_3$, 25°C): $\delta$=8.25 (m, 2H, H5, H24), 8.06 (m, 1H, H1), 7.60–7.30 (m, 14H, H17-H23), 6.89–6.80 (m, 4H, H16), 6.45-6.26 (m, 2H, H3, H6), 6.12-6.05 (m, 1H, H4), 4.75–4.65 (m, 1H, H8), 4.25–4.15 (m, 1H, H9), 3.75–3.45 (m, 6H, H2, H13, H11), 3.72 (s, 6H, H15), 3.50–3.30 (m, 2H, H10), 2.73 (m, 2H, H12), 2.45 (m, 2H, H7), 1.20–1.10 (m, 9H, H14), 1.10–1.00 (m, 3H, H14). $^{31}$P-NMR (121 MHz, CDCl$_3$, 25°C): 149.4 ppm. MS (ESI$^+$) $m/z$=1007.9 (M+Na).
The synthesis of guanidine-modified 2′-deoxyuridine phosphoramidite was synthesized by following the literature.\textsuperscript{161} \textsuperscript{1}H-NMR (300 MHz, CDCl\textsubscript{3}, 25 °C): δ=11.70 (s, 1H, H3), 8.42 (s, br, 1H, H8), 8.09 (m, 1H, H4), 7.76 (s, 1H, H9), 7.46–7.40 (m, 2H, H22), 7.35–7.22 (m, 7H, H19, H20, H21), 6.89–6.85 (m, 4H, H18), 6.40–6.31 (m, 2H, H6, H10), 5.54 (d, J=15.8 Hz, 1H, H7), 4.71–4.64 (m, 1H, H12), 4.32 (m, 2H, H23), 4.25 (m, 2H, H2), 4.23–4.13 (m, 1H, H13), 3.85–3.52 (m, 6H, H5, H15, H25), 3.79 (s, 6H, H17), 3.50–3.30 (m, 2H, H14), 2.76–2.72 (m, 4H, H1, H24), 2.63 (m, 1H, H26), 2.60–2.45 (m, 1H, H11), 2.46 (t, J=6.3 Hz, 1H, H26), 2.40–2.32 (m, 1H, H11) 1.20–1.13 (m, 9H, H16), 1.10–1.05 (m, 3H, H16). \textsuperscript{31}P-NMR (121 MHz, CDCl\textsubscript{3}, 25°C): 149.6 ppm. MS (ESI\textsuperscript{+}) m/z=1045.0 (M+Na).

The solid-phase synthesis was conducted by our collaborator on Applied Biosystem 3400 DNA synthesizer using standard phosphoramidite chemistry.\textsuperscript{193} The phosphoramidites were dissolved in acetonitrile at 0.15 mM. The coupling time was 90 seconds. The synthesis run report is shown in appendices.

**Global deprotection of solid-phase synthesis and purification of modified DNAzyme**

The oligonucleotides were deprotected by adding 200 μL 50% piperidine/water to the whole sample of the CPG that was removed from the cartridge. Then the reaction was incubated at room temperature for 24 hours. After the incubation, the reaction was frozen and lyophilised.
The resulting solid was resuspended in 500 µL fresh 28% ammonia solution, then incubated at 55 °C for 12 hours. The reaction was centrifuged at 13x10³ rpm for 5 minutes. The supernatant was collected. The remaining solid was resuspended with 500 µL water, and centrifuged again. The supernatant was collected and combined. The supernatant was frozen and lyophilised again. The resulting solid was dissolved in 100 µL 10 mM Tris-HCl, 1 mM EDTA, pH 7.8 buffer. 30% of the oligonucleotides was added to 30 µL loading dye and 1 µL 1 M NaOH and loaded on a 10% PAGE for purification.

**Trans-cleavage study with solid-phase synthesis modified DNAzyme**

Varied amounts of solid-phase synthesized oligonucleotides were added to an appropriate amount of radioisotope-labelled all-RNA substrate. Then the mixture was denatured under 95°C for 2 minutes, cooled to 0°C by ice, followed by the addition of 2 µL 10x cleavage buffer (containing 5 mM Mg²⁺) to initiate the reactions, water was added to make a total volume of 20 µL. At each time point, 2 µL reactions were transferred into 7 µL loading solution. Cleaved and uncleaved oligonucleotides were resolved on a 20% denaturing PAGE, visualized using a Phosphorimager.

**Gel-retardation assays**

25 pmol ³²P-γ-ATP labelled phosphorylated template 11 was added to 2 µL crude oligonucleotides solution following global deprotection, and the mixture was annealed together by heating to 95°C for 2 minutes and cooled to room temperature slowly. 6x gel shift binding buffer (2 µL) and water were added to result in a 12 µL solution. The solution was loaded onto a non-denaturing 10% 7M urea PAGE. After loading the samples, the gel was run at room temperature in 0.5X TBE buffer and was checked frequently to guarantee that no heat was generated to warm up the gel. The gel was exposed to X-ray film overnight at –20°C with an intensifying screen. Alternatively, the gel was analyzed using Phosphorimager.
Chapter 5: Failed selections

5.1 Introduction

5.1.1 DNAzymes catalyzed chemical reactions

As was discussed in Chapter 1, over the years DNAzymes have been selected to catalyze reactions apart from RNA cleavage including the Diels-Alder reaction, and nucleopeptide bond formation. However, DNA can also be used to catalyze chemical reactions or participate in larger catalytic constructs. For instance, it was discovered that the nitro-aldol (Henry) reaction could be catalyzed by unmodified double-stranded DNA derived from salmon testes. In this case, the DNA sequences tested were entirely arbitrary and were not selected for as in DNAzyme discovery. Double-stranded helical DNA strands have also been used as part of catalytic scaffolds in order to confer enantioselectivity to metal-catalyzed reaction and have been employed as a co-catalyst for asymmetric catalysis. Such pioneering studies were reported by Feringa and coworkers. All the ligands employed are aromatic and heterocyclic compounds, such as phenanthroline and its derivatives. In these reactions, the ligand and starting material first associate with copper, and DNA functions as a stereocontrol element that confers high enantioselectivity to the catalyzed reaction.

5.1.2 DNA catalyzed click reaction

As a frequently used bioorthogonal reaction, the copper catalyzed 2+3 dipolar cycloaddition reaction (known as the click reaction) is used to label biomolecules, such as proteins, DNAs, RNAs and sugars. Huisgen described the original process in which azide-alkyne cycloaddition results in the formation of triazole; however, in 2001, Sharpless and coworkers reported that this reaction can be highly improved in the presence of copper (I). Furthermore, certain ligands that can chelate with copper (I) were found to enhance the reaction rate and
stabilize the metal cation. Several of these ligands are shown in Figure 5.1.\textsuperscript{202,203} The method to label oligonucleotides by the click reaction commonly requires alkylated or azido oligonucleotides,\textsuperscript{204,205} and the copper (I) catalyzed click reactions are usually undertaken with copper (I) in complex with various ligands. The need for a suitable ligand for copper is underscored by the fact that the click reaction without ligand under aqueous conditions can generate peroxide as the side product, which can damage the biomolecular substrates that are undergoing click-type conjugation.\textsuperscript{206} In addition, the click reaction proceeds without selectivity, which means that with any biomolecule containing an azide or terminal alkyne residues can participate in the reaction. Since chemical reactions can be catalyzed with DNA scaffolds that are used as co-catalysts in the presence of copper chelators such as phenanthroline, it was hypothesized that a highly active metallo-DNAzyme, with added functionalities for chelation, could be selected to afford a DNA-based ligand with high affinity for copper along with catalytic activity. The idea of a DNAzyme selection based on copper (I) catalyzed click reaction is to use modified oligonucleotides to provide the scaffold and metal binding core, while the DNAzymes obtained from this method would be sequence-specific and provide reaction selectivity.

![Figure 5.1 Ligands for copper click reaction. a Tris(1,2,3-triazolyl)methyl amine (TBTA) and its tert-butyl analog (TTTA). b Sulfonated bathophenanthroline.](image)

**Figure 5.1** Ligands for copper click reaction. a Tris(1,2,3-triazolyl)methyl amine (TBTA) and its tert-butyl analog (TTTA). b Sulfonated bathophenanthroline

### 5.1.4 DNAzyme catalyze hydroamination

Whereas the bulk of this thesis concerned the application of modified dNTPs for the selection of RNA-cleaving DNAzymes that would be active under physiological conditions, it is
always interesting to expand the catalytic scope of modified DNAzymes. Hence, a selection to generate DNAzymes that can catalyze novel organometallic reaction was attempted. To choose an appropriate reaction for DNAzyme selection, a few guidelines were established: the reaction needed to be water and perhaps oxygen insensitive, transition metal cations had to be able to bind to the nucleobases or functional groups, and the reaction needed to be amenable to selection. Based on these criteria, it was hypothesized that C-N bond formation via hydroamination catalyzed by platinum or palladium would be a suitable reaction candidate. In addition, platinum (II) is known for its considerable affinity for the nucleobases of nucleic acids. Therefore, it was hypothesized that DNA would provide a robust catalytic core for platinum (II) in order to catalyze a hydroamination reaction. Furthermore as imidazole is known to be good ligand for palladium (II), both metals can be considered in the selection system to select DNAzymes for this reaction.

### 5.1.3 DNAzymes catalyzed DNA cleavage reaction

DNA hydrolysis has a half-life estimated to be millions of years under physiological conditions. It is difficult to accelerate this hydrolysis. It has been reported that the cleavage of DNA predominantly is via oxidation or depurination. Self-hydrolysis of G-quadruplex DNA has been found, but apparently has a very limited sequence scope. DNAzymes selected to catalyze DNA hydrolysis have only been reported by two groups to date. Silverman and coworkers discovered DNAzyme 10MD5 accidently. They initially attempted to select a DNAzyme that could cleave amide bonds of a tripeptide target using an N40 library region. Instead of amide bond cleavage, the selected DNAzyme 10MD5 was found to be capable of cleaving DNA and the cleavage site was found to be at the right-hand binding arm region. The DNAzyme requires both Zn\(^{2+}\) and Mn\(^{2+}\) as cofactors and the hydrolysis has a rate constant of 0.045 min\(^{-1}\). Although the rate constant is not significantly fast, the reported rate enhancement is as high as 10\(^{8}\). Breaker and coworkers discovered the DNAzyme I-R3 with a novel selection
method involving a library of sequences containing two random regions. The DNAzyme is a 
Zn\(^{2+}\)-dependent DNAzyme with an observed rate constant of 1 min\(^{-1}\), which is very impressive. However, it may be interesting to know whether modified DNAzymes can perform better in this challenging area.

5.2 Objectives in this chapter

Within this chapter, I took on two related goals that would expand the catalytic repertoire of DNA in terms of the reaction class that DNA could catalyze. In each case modified nucleotides were also used with the expectation that any resulting activity would be further enhanced by the metal binding properties. The goal of this project is to study the selection of DNAzymes contain modified nucleotides that can catalyze novel reactions, such as a copper-mediated click reaction, hydrolytic DNA cleavage and hydroamination. To select the desired DNAzymes, I used the same modified nucleotides; dC\(^{aa}\)TP, dU\(^{gp}\)TP, and dA\(^{im}\)TP as in Chapter 3 (Figure 5.2). An additional nucleotide, the thio modified dTTP was also utilized in the selection. For copper-mediated click reaction selection, I did two selections in parallel; the first one was conducted with dU\(^{gp}\)TP (1), dC\(^{aa}\)TP (2), dA\(^{im}\)TP (3) and natural dGTP, the other one with dC\(^{aa}\)TP (2), 4-thio-dTTP (4), dA\(^{im}\)TP (3) and natural dGTP. In addition, dU\(^{gp}\)TP, dA\(^{im}\)TP, canonical dCTP and dGTP were chosen for the hydroamination selection. Because dC\(^{aa}\)TP (2) presents a primary amine, I believed that this would not appropriate to use, because the selection substrate contains amino group.
5.3 In vitro selection

5.3.1 In vitro selection for click reaction

There are a few differences between this selection scheme and the RNA-cleaving DNAzyme selection scheme used in Chapter 3. The first difference is that the azide group is directly linked to biotin, therefore, the click reaction will not change the length of the oligonucleotides in the selection. Although the streptavidin-biotin methodology theoretically can separate the product from inactive oligonucleotides, changes of length between the product and the inactive oligonucleotides are preferred. Therefore, a ribose nucleotide has been introduced at the loop region (Figure 5.3). The click reaction product will be fished out using streptavidin beads from inactive oligonucleotides. The treatment of the click reaction product containing the catalytic domain with sodium hydroxide will cleave the embedded ribose, liberating the active oligonucleotides into solution. The active strands can then be PAGE purified and amplified for the next round of selection.
Figure 5.3 Illustrative scheme of the selection of DNAzyme catalyzing copper-mediated click reaction

The second difference is that the template is biotinylated and the 5'-terminus of the primer is modified with an alkyne group. Therefore, the template will bind to streptavidin beads, and the modified oligonucleotides library can be removed from the template and collected for later usage. Because a very basic solution (0.1 M NaOH) was used to break the hydrogen bonds of DNA duplex, thereby removing the modified strand, the resulting supernatant that contains the modified oligonucleotides must be neutralized for further use. The volume of the supernatant is too small to precisely adjust the pH using a pH meter. Moreover neutralization with HCl alone cannot guarantee a neutral pH as volumes are also small. Therefore, a rough pH adjustment is undertaken by adding 1 eq. of HCl in the presence of phosphate buffers. The initial reaction conditions were 1 µM alkylated oligonucleotides, 50 µM azido-biotin, 100 µM sodium ascorbate, 10 µM copper sulfate and 10 mM magnesium sulfate and the sodium chloride from neutralization. The reaction was incubated at room temperature for 1 hour.
For the selection using dUguTP, only 6 rounds were conducted, all under the initial reaction conditions. Because the reaction products had to be separated from the unreacted oligonucleotides before being loaded onto PAGE, it was impossible to quantitatively judge the improvement after each round of the selection. Therefore, the improvement of each round was estimated using the following method: at each round 1 µL of the neutralized selection solution containing the starting oligonucleotides was removed as a standard control (Lane 3 of Figure 5.4), and compared to the selection product on PAGE (Lane 5 of Figure 5.4). The gel images of selection round 6 using dUguTP is shown in Figure 5.4. The selection showed no promising result in the first 6 rounds, as it appeared that only the background reaction was observed in each round. Considering the possibility that the 4-thio-dTTP may form a better complex with copper (I), I gave up this selection after 6 rounds and focused on the selection with 4-thio-dTTP.

![Gel images of selection using dUguTP. R6, selection round 6. Lane 1, elongation reaction; lane 2, supernatant after the first incubation with streptavidin beads; lane 3, oligonucleotides removed from the biotinylated template; lane 4, the supernatant used in lane 3 treated with 1 M NaOH; lane 5, selection reaction after 1 hour incubation](image)

**Figure 5.4** Gel images of selection using dUguTP. R6, selection round 6. Lane 1, elongation reaction; lane 2, supernatant after the first incubation with streptavidin beads; lane 3, oligonucleotides removed from the biotinylated template; lane 4, the supernatant used in lane 3 treated with 1 M NaOH; lane 5, selection reaction after 1 hour incubation

The selection stringency with 4-thio-dTTP was controlled by varying the time for the click reaction to occur and the reaction buffer composition. The reaction time was decreased from 60 minutes to 10 minutes over the course of selection (Table 5.1), and the copper concentration...
in the reaction buffer was decreased from 10 µM to 3 µM (Table 5.2). A total of 16 rounds were performed.

**Table 5.1** Reaction time for each round of selection using 4-thio-dTTP.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>60</th>
<th>30</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rounds</td>
<td>1-7</td>
<td>8</td>
<td>9-16</td>
</tr>
</tbody>
</table>

**Table 5.2** Copper concentrations in each round of selection

<table>
<thead>
<tr>
<th>Cu²⁺ (µM)</th>
<th>10</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rounds</td>
<td>1-9,11</td>
<td>10,12-16</td>
</tr>
</tbody>
</table>

**Figure 5.5** Comparison between rounds 3 and 9 for selection using 4-thio-dTTP. Lane 1, elongation reaction of round 9; lane 2, supernatant after the first incubation with streptavidin beads of round 9; lane 3, oligonucleotides removed from the biotinylated template of round 9; lane 3', oligonucleotides removed from the biotinylated template of round 3; lane 4, sample for lane 4 treated with 1 M NaOH; lane 5, selection of round 9 after 1 hour; lane 5, selection of round 3 after 1 hour.

The selection with 4-thio-dTTP started to show some improvement in round 3. The elongation using 4-thio-dTTP was found to be cleaner than the elongation using dUTP. In round 3 the selection product was clearly observed (gel image not shown). In round 8, the click reaction was examined in the absence of copper (II). After 30 minutes, no product was found for the click reaction in the absence of copper (II). Additionally it also proved that any occurrence non-specific binding was not significant. In round 9, the selection result was compared with the selection from round 3. The gel image showed a significant difference between the two rounds of
selection, which may indicate that some species were enriched in this selection (Figure 5.5). This was further proven by the comparison between rounds 4, 7 and 11, and another comparison between rounds 3, 9 and 12 (gel images not shown).

In round 10 the selection was conducted with a lower copper (II) concentration (3 µM), and the appeared yield of selection products decreased significantly (Lane 5 of Figure 5.6). The selection using natural dTTP was carried out in parallel as well (Lane 5’ of Figure 5.6). The click reaction using 4-thio-dTTP did not show significantly more selection products compared to the click reaction using natural dTTP. The following selection rounds were undertaken using 3 µM copper (II) concentration as well. Unlike the RNA-cleaving DNAzyme selection, the appeared yield of the click reaction did not recover to previous level (gel images not shown). The comparison between using natural dTTP and modified dTTP for round 10 was repeated, and no significant difference was observed either (gel image not shown). At this point, the selection was considered to have failed and was abandoned.

**Figure 5.6** Gel images of selection using 4-thio-dTTP. **R10**, round 10; lane 1, elongation reaction; lane 2, supernatant after incubation with streptavidin beads; lane 3, oligonucleotides removed from the biotinylated template; lane 4, sample for lane 3 treated with 1 M NaOH; lane 5, selection reaction. lane 5’, selection reaction with natural dTTP instead of 4-thio-dTTP.

**5.3.2 In vitro selection for hydroamination**

The hydroamination selection shares several commonalities with the click reaction selection. In both selections, the length of the oligonucleotides did not change over the reaction
and the streptavidin-biotin methodology was used to separate active and inactive species. The modified oligonucleotides were collected after being removed from the template as well (Figure 5.7). However, this selection differed from the click reaction selection in a few aspects: first, a Sephadex G25 column was used to remove excess styrene-biotin in this selection instead of the 1% LiClO₄ acetone solution used in the click reaction selection. This is because there was a concern that the solid pellet containing most of the metals and oligonucleotides formed by the precipitation using 1% LiClO₄ acetone solution may affect the reaction. Second, under the hydroamination conditions, amide substitution may occur: the amino group on the primer may attack the amide bond rather the double bond of styrene. The resulting oligonucleotides would contain the biotin tag, and differentiation from the desired product of hydroamination would be impossible.

**Figure 5.7** Illustrative scheme of the hydroamination selection
The initial selection was conducted with 1 µM amino-oligonucleotides, 50 µM styrene-biotin, 1 µM K$_2$PtCl$_4$, 1 µM PdCl$_2$, 1 µM CuCl$_2$, 10 mM Na$_2$HPO$_4$, 10 mM MgSO$_4$ and the NaCl from the neutralization step at room temperature for 24 hours. A total of 8 rounds of selection were performed. Again it was not possible to quantitatively judge each round of selection so and the radioactivities of two samples were determined to have a rough reaction yield. One sample consisted of the oligonucleotides collected after using the Sephadex G25 column, while the other consisted of oligonucleotides bound to streptavidin beads after all the washes. The ratio of these two radioactivities was considered to roughly represent the yield. The radioactivity was simply measured using a survey meter and therefore the measurements are not highly accurate.

In round 1, the selection reaction was carried out in the presence and absence of catalytic divalent metals. A faint band showed up at the correct place when the selection was carried out in the presence of catalytic metals (gel image not shown). In round 2, two methods for removing the excessive styrene-biotin starting material were compared. LiClO$_4$ precipitation gave significantly more product than Sephadex G25 column separation. Two possible reasons were considered. First, hydroamination occurred in the precipitated pellet. Second, a portion of the small solid particles (containing most of oligonucleotide and metal cations from precipitation) had not dissolved in later steps and the wash steps did not remove these particles. In the third round, the selection was carried out with catalytic metals at the initial concentration and 100 times concentration in parallel. No significant difference was observed with higher metal cations concentration only that the little amount of modified oligonucleotides in selection migrated slower on PAGE. This result might be because multiple Pt cations became bound to one DNA molecule, reducing the negative charge on the DNA and decreasing the mobility of DNA significantly. Pt (II) may have also caused cross-linking between different nucleobases that then produced a different secondary structure, which may have led to the activity loss. The selection did not appreciably improve, since the radioactivity of oligonucleotides that remained on streptavidin beads remained at 0.5-2%
of the initial radioactivity in all the selection rounds. The selection gel image of round 4 is shown in Figure 5.8.

![Figure 5.8](image)

**Figure 5.8** Hydroamination selection gel images of round 4. R4, selection round 4. Lane 1, elongation reaction; lane 2, supernatant after the first incubation with streptavidin beads; lane 3, oligonucleotides removed from the biotinylated template; lane 4, sample for lane 3 treated with 1 M NaOH; lane 5, reaction supernatant after the second incubation with streptavidin beads; lane 6, selection after 24 hours.

Although it seems that the hydroamination reaction was not observed in the selection, some oligonucleotides may have become enriched for certain activity over the course of the selection. First, after round 2, the percentage of oligonucleotides eluted from the Sephadex G25 column gradually increased (Table 5.3). This gradual change may indicate some enrichment in the selection process. After 7 round, I abandoned this selection.

**Table 5.3** Radioactivity percentage of oligonucleotides eluted from Sephadex G25 column.

<table>
<thead>
<tr>
<th>Rounds</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Percentage</td>
<td>40%</td>
<td>&lt;30%</td>
<td>&lt;30%</td>
<td>50%</td>
<td>60%</td>
<td>60%</td>
<td>70%</td>
</tr>
</tbody>
</table>

**5.3.3 In vitro selection for DNA cleavage reaction**

The DNA cleavage selection was similar to the all-RNA cleavage selection described in Chapter 3. However, DNA cleavage is a much more challenging reaction due to the lack of a 2'-hydroxy group on the ribose. Moreover, in a DNA cleavage selection, the cleavage can theoretically occur at any location rather than the designed site. This was shown with the
selection method employed by Breaker and coworkers, which permitted DNA cleavage to occur at almost any location within each DNA construct and increased the likelihood of a successful selection outcome.\textsuperscript{48} In this selection I intend to select DNAzyme with modifications only cleave at the designed site.

A total of 7 rounds were performed(Figure 5.9). The initial selection conditions was 200 mM NaCl, 50 mM sodium cacodylate, 20 mM MgCl\textsubscript{2}, 1 mM ZnBr\textsubscript{2}, 1 mM CaCl\textsubscript{2} at pH 7.4. After round 3, 10 mM MnCl\textsubscript{2} was employed in the selection. The selection lane did not change much in all the selection rounds, but the elongation became much cleaner, and less truncation was produced over the course of selection. One particular truncation at that was observed at the very beginning appeared to increase over the courses of the selection (Figure 5.9 red arrow). Because many primer extension products of the same length were also found in the supernatant (Figure 5.9 red arrow), it was hypothesized that the truncation and the oligonucleotides in the supernatant were identical and were produced by template-directed elongation using the biotinylated primer as the template. Therefore, after round 5 the Primer 1 was replaced with the Primer 1’, which contains an alkane linker such that the template would not be elongated. In the following rounds 6 and 7, the truncation was almost eliminated entirely (gel images are not shown). No cleavage products were observed in the selection lane after any round. The selection was abandoned after 7 rounds.

Figure 5.9 DNA cleavage selection gel images of round 1. Lane 1, elongation reaction; lane 2, supernatant after incubation with streptavidin beads; lane 3, modified oligonucleotides stayed

\[152\]
with streptavidin beads; lane 4, sizing lane (for reference); lane 5, selection after 3 hours. (red arrows indicate the truncation mentioned above)

5.4 Discussion and conclusion

General discussion

Unlike the RNA-cleaving reactions in Chapter 3 and 4, the click reaction, the hydroamination reaction and the DNA cleavage attempted in this chapter are much more complicated and challenging. All the selections were not completed and sadly, no conclusive result was obtained. Although tremendous efforts were made with the click reaction selection, it was unclear whether there was any enrichment occurring and the designed modification did not act as expected. The hydroamination selection and DNA-cleaving selection were briefly attempted, and also failed to produce positive results. It was believed the hydroamination itself did not provide proper conditions a DNAzyme selection. Specific comments for each of the selections are found below.

Click reaction selection

As we discussed in previous content, the click reaction generates hydrogen peroxide in the presence of oxygen. Significant amounts of oxidized oligonucleotides were observed in the selection in the presence of copper; however, when copper was removed from the reaction, both click product and oxidized oligonucleotides were not observed. Although many protein enzymes employ metal catalytic cores and radical reaction mechanisms under aqueous conditions, it is much harder for DNA to mimic protein enzymes because the DNA scaffolds lack the ability to form a catalytic core that is capable of excluding oxygen. Unlike the RNA-cleaving reaction, which can be facilitated by water in many aspects, the DNA click reaction can be prevented by oxygen. Hydrogen peroxide produced by copper (I) and oxygen can attack the DNA skeleton at many points to provide oxidative cleavage products and nucleobase oxidation products. It was observed that the oxidative products did not change with the selection process, which might indicate that the oxidation observed was non-specific.
The selection with dUgTP was abandoned after 6 rounds of selection because it became evident that copper could only form a complex with the imidazole group. In addition, the modified dATP was a poor substrate for the polymerase. On the one hand, the polymerase prefers to incorporate oligonucleotides containing fewer modified As, but on the other hand, the modified A would likely be crucial for DNAzyme activity. This contradiction may have been a determining factor in the failure of this selection.

Much more information was obtained from the selection with 4-thio-dTTP. Some species were selected and enriched through this process. The selection started to show improvement at early rounds, and this improvement continued until round 10 with the same copper concentration (10 µM). When the copper concentration was lowered to 3 µM, the click reaction product decreased significantly and did not recover from this decrease. Initially, it was thought that the sodium ascorbate had slowly deteriorated over the course of the selection; however, using fresh sodium ascorbate did not solve the problem. Moreover, increasing the copper concentration in the following selection did not result in the recovery of high yield click product. Overall, the relative yield of the click reaction increased at the early stage and decreased at the late stages for unknown reasons. This result was also observed in a RNA-cleaving selection. To better understand these results, deep-sequencing could be conducted. Additionally, the reaction activity using natural dTTP in the selection did not significantly differ from that of 4-thio-dTTP, which weakened the value of the selection, as the role of modified nucleotides was unclear. As a result, further cloning was not carried out.

**Hydroamination selection**

Hydroamination with platinum (II) containing a multiple-step reaction mechanism is likely too complex to be carried out by DNA. Although platinum (ii) as a commonly-used catalyst is known for being capable of forming a very tight covalent bond with nucleobases, no nucleic acid enzymes catalyzed reaction involving a platinum catalytic core has been reported to date. It
is likely that other researchers tried and failed to carry out similar selections. Three direct interactions between nucleic acids and metals were described in Lippard and Berg’s book “Principles of Bioinorganic Chemistry”: the phosphate charge interaction, base covalent bond interaction and sugar covalent bond interaction. It was observed that DNAzyme catalyzed reactions with metals rely principally on the phosphate charge interaction. Besides being an inherently difficult reaction to catalyze, selection inefficiency may significantly increase the difficulty of this type of selection as well.

**DNA cleavage selection**

As was discussed in previous section (section 5.1.3), hydrolytic DNA cleavage selection is a challenging selection. The same set-up with RNA cleavage selection was employed, which has no advantage compared to Breaker’s method. The selection time was 3 hours, but in hindsight should have been longer to allow for more cleavage. To further increase the chance of success, lanthanide metals should probably be considered. Terbium (III) is known to facilitate DNAzymes activity in some cases, such as branched-RNA synthesizing DNAzyme and DNA-cleaving DNAzyme. Not much information was obtained from this selection due to the limited efforts to characterize the results, but the suggestions outlined above could prove helpful for future modified DNA-cleaving DNAzyme selections.

**5.5 Materials and methods**

**5.5.1 Materials and equipment**

All the chemicals were purchased from Sigma-Aldrich and Fisher Scientific. LiClO₄ was purchased from J.T. Baker. Styrene-biotin and Azido-biotin were prepared according to literature procedures.²¹⁸,²¹⁹ dA<sup>im</sup>TP and dU<sup>im</sup>TP were synthesized by previous lab members.¹³⁴,¹⁶¹ 4-Thio-dTTP and dC<sup>ac</sup>TP were purchased from TriLink. All oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA) and were purified by 10–15% 7 M urea denaturing PAGE. Ultrapure dNTPs and yeast tRNA were obtained from Fermentas. Sequenase Version 2.0
DNA polymerase was purchased from Affymetrix. Lambda exonuclease and Vent (exo-) DNA polymerase were obtained from New England Biolabs. Streptavidin magnetic particles were purchased from Roche. Sephadex G25 was purchased from GE Healthcare. The nucleoside triphosphates $^{32}\text{P}-\alpha$-dGTP and $^{32}\text{P}-\gamma$-ATP were purchased from Perkin-Elmer. Water used in all experiments was first treated with diethyl pyrocarbonate (DEPC. 5 μL per 100 mL) and autoclaved. Radioactive PAGEs were exposed to storage phosphor screens, and then the screen was scanned with a Typhoon 9200 Phosphorimager from GE Healthcare.

**Oligonucleotides:** Click reaction selection and hydroamination selection shared the same PCR amplification primer system. DNA cleavage selection and the selection in Chapter 3 shared the same PCR amplification primer system. Primer 2 and Primer 3 for all three selections are the 1st amplification PCR primers. Primer 4 and Primer 3 were used in the 2nd amplification of each selection. Primer 1 or Primer 1’ were used in the elongation step of each selection.

**Click reaction selection**

Primer 1: 5'-/alknyl/CTGTTGGTTTTTGCGGrUrCGGCTGCGCCAACAG-3'

Primer 2: 5'-GCGCTCGCGGCGGTGC-3'

Primer 3: 5'-/5Phos/GCGTCGGCCTGCGCCAACAG-3'

Primer 4: 5'-/Biosg/TTTTTTTTTTGCGCTCGCGGCGGTGC-3'

Template: 5'-GCGCTCGCGGCGGTGC(N40)CTGTTGGCGCAGGCCGACGC-3'

**Hydroamination selection**

Primer 1: 5'-/amino/CTGTTGGTTTTGCGGrUrCGGCTGCGCCAACAG-3'

**DNA cleavage selection**

Primer 1: 5'-/Biosg/T43TGCGTGCCCCTGCTGTTGGTTTTGCGTGCGCCTGCGCCAACA-3'

Primer 1': 5'-/Biosg/T43TGCGTGCCCCTGCTGTTGG/iSpC3/TTTTGCGTGCGCCTGCGCCAACA-3'

Template: 5'-GCGCTCGCGGCGGTGC(N30)CTGTTGGCGCAGGCCGACGC-3'
Primer 2: 5- GCGCTCGCGCGGCGTGC-3
Primer 3: 5- /5Phos/GCGTCGGCCTGCGCCAACAG-3
Primer 4: 5- TTTTTTTTTTTTTTTTTTGCCTCIGCGGCGGCGTGC-3

Buffers and cocktails

Elution buffer: 1% LiClO$_4$, 10 mM Tris-HCl (pH=8) in water.

5X 1st amplification cocktail; 9 nmol Primer 2, 7.5 nmol Primer 3, 11.5 μL 100 mM MgSO$_4$, 115 10X thermopol buffer, 3.45 μL 100 mM dNTPs (100 mM), added water to make a total 230 μL solution.

5X 2nd amplification cocktail; 10 nmol Primer 4, 7.5 nmol Primer 3, 11.5 μL 100 mM MgSO$_4$, 115 10X thermopol buffer, 3.45 μL 100 mM dNTPs (100 mM), added water to make a total 230 μL solution.

5X Hydroamination buffer: 5 μM PdCl$_2$, 5 μM K$_2$PtCl$_4$, 5 μM CuCl$_2$, 50 mM MgCl$_2$, 50 mM Na$_2$HPO$_4$, pH=7.0.

DNA cleavage reaction buffer: 50 mM sodium cacodylate, 200 mM NaCl, 20 mM MgCl$_2$, 1 mM ZnCl$_2$, 1 mM CaCl$_2$, 10 mM MnCl$_2$, pH=7.4.

5.5.2 Methods

The gel elution, 1st amplification PCR, 2nd amplification PCR and elongation of all three selections are similar to these in Chapter 3 and 4. Here only the elongation, 1st amplification PCR and 2nd amplification PCR are demonstrated respectively with click reaction selection. The selection step of all three selection are shown in details.

Elongation

Thirty pmol of Primer 1 (different primer for different selection) were annealed to 30 pmol of template DNA prepared by 2nd amplification PCR, and then enzymatically polymerized at 32°C for 4 hours using 13 units of Sequenase in a mixture containing 5 mM DTT, 50 μM dA$_{5^\text{nt}}$TP, 20 μM of each dU$_{5^\text{nt}}$TP (or 4-thio-dTTP), dC$_{5^\text{nt}}$TP (dCTP for hydroamination selection),
dGTP, and 15 μCi of $^{32}$P-α-dGTP to give a final volume of 40 μL. The reaction was quenched by adding 1 μL 500 mM EDTA (pH=8) solution.

**Click reaction selection**

50μL of magnetic streptavidin beads were transferred into a 1.5 mL eppendorf tube, and washed with TEN buffer (3×100 μL). The elongation reaction mixture was added to the beads, and incubated at room temperature for 30 minutes. Then the beads with bound DNAs were magnetized and the supernatant was removed. The beads were washed with TEN buffer (2×100 μL), water buffer (2×100 μL). Then the modified oligonucleotides were removed from the biotinylated template by two 0.1 M NaOH washes (2×40 μL). Each wash the supernatant was quenched by 0.4 M HCl and 0.5 M pH 8.0 phosphate buffer. The two supernatants were combined, pH was approximately 7-8, evaporated to a total volume of 25 μL. Azido-biotin (1.5 nmol) in 1 μL DMF, 0.3 μL copper sulfate (variable concentration), 2.7 μL 100 mM magnesium sulfate and 1 μL 9 mM sodium ascorbate were added to the 25 μL sample. The reaction was vortexed and incubated at room temperature for varying time span. After incubation, 1 μL 0.5 M EDTA was added to quench the reaction. 500 μL 1% LiClO$_4$ and 5x 1st amplification cocktail (2 μL) were added to the reaction. The eppendorf tube was centrifuged at 13x10$^3$ rpm for 15 minutes. Then discarded the supernatant, and added 1 mL of iced ethanol to the pellet in the eppendorf tube. The sample was agitated by vortex and centrifuged at 13x10$^3$ rpm for 15 minutes. The ethanol was decanted and any residual ethanol was removed at 65°C for around 5 minutes. The pellet was dissolved in 30μL of dH$_2$O. The dissolved oligonucleotides were incubated with 50 μL pre-washed magnetic beads. After 30 minutes, the supernatant was removed and beads were washed with TEN buffer(2×100 μL), template stripping buffer (3×100 μL, no longer than 30sec/wash). 1 M NaOH (5 μL) was added and sample was heated at 65°C for 5 minutes. 10 μL loading dye was added to the sample, and then the sample was loaded onto 7% 7 M urea denaturing PAGE. The species corresponding to the cleaved product was cut and eluted using...
elution buffer and precipitated with ethanol. The precipitated pellet was dried and resuspended in 60 μL water and used in the 1st amplification PCR step.

**Hydroamination selection**

50μL of magnetic streptavidin beads were transferred into a 1.5 mL eppendorf tube, and washed with TEN buffer (3×100 μL). The elongation reaction mixture was added to the beads, and incubated at room temperature for 30 minutes. Then the beads with bound DNAs were magnetized and the supernatant was removed. The beads were washed with TEN buffer (2×100 μL), water buffer (2×100 μL). Then the modified oligonucleotides were removed from the biotinylated template by two 0.1 M NaOH washes (2×40 μL). For each wash the supernatant was quenched by 0.4 M HCl and 0.5 M pH 8.0 phosphate buffer. The two supernatants were combined, pH was approximately 7-8, evaporated to a total volume of 23 μL. Styrene-biotin (1.5 nmol) in 1 μL DMF, 6 μL 5X hydroamination buffer were added to the 23 μL sample. The reaction was vortexed and incubated at room temperature for 24 hours. After incubation, 10 μg tRNA in 1 μL water was added to sample. Then the sample was run through a Sephadex G25 column to remove the unreacted styrene-biotin. Then the eluted sample was incubated with 50 μL pre-washed magnetic beads. After 30 minutes, the supernatant was removed and beads was washed with TEN buffer(2×100 μL), template stripping buffer (3×100 μL, no longer than 30sec/wash). 1 M NaOH (5 μL) was added and sample was heated at 65 °C for 10 minutes. 15 μL loading dye was added to the sample, and then the sample was loaded onto 7% 7 M urea denaturing PAGE. The species corresponding to the cleaved product was cut and eluted using elution buffer and precipitated with ethanol. The precipitated pellet was dried and resuspended in 60 μL water and used in the 1st amplification PCR step.

**DNA cleavage selection**

50μL of magnetic streptavidin beads were transferred into a 1.5 mL eppendorf tube, and washed with TEN buffer (3×100 μL). The elongation reaction mixture was added to the beads,
and incubated at room temperature for 30 minutes. Then the beads with bound DNAs were magnetized and the supernatant was removed. The beads were washed with TEN buffer (2×100 μL), template stripping buffer (5×100 μL, no longer than 30 sec/wash), then NaOH neutralization buffer (1×200 μL) and water (1×100 μL). Then the modified single strand DNA was incubated with 160 μL DNA cleavage buffer for 3 hours at room temperature. After incubation, the beads were magnetized and the supernatant was transferred to a new eppendorf tube. Then 1% LiClO₄ in acetone (800 μL) and 5x 1st amplification cocktail (2 μL) were added to the supernatant. The eppendorf tube was centrifuged at 13×10³ rpm for 15 minutes. Following removal of the supernatant, the pellet was washed with 1 mL of iced ethanol. The sample was agitated by vortex and centrifuged at 13×10³ rpm for 15 minutes. The ethanol was decanted and any residual ethanol was removed at 65°C for around 5 minutes. The pellet was dissolved in 5μL of dH₂O. 10 μL loading dye was added to the pellet, and then the sample was loaded onto 7% 7 M urea denaturing PAGE. The species corresponding to the cleaved product was cut and eluted using elution buffer and precipitated with ethanol. The precipitated pellet was dried and resuspended in 60 μL water and used in the 1st amplification PCR step.

1st amplification PCR

The product eluted from each selection was PCR amplified using Primer 2 and Primer 3 and an internal label (10 μCi dGTP α-[³²P]) for 25 cycles (15 s at 58 °C, 40 s at 75 °C and 15 s at 95 °C). The reaction (40 μL) included 0.1 units/μL Vent (exo-) DNA polymerase, 20 mM Tris-HCl (pH 8.8 at 25 °C), 10 mM (NH₄)₂SO₄, 10 mM KCl, 3 mM MgSO₄, 0.1% gelatin, 7 μM oligonucleotides and 0.3 mM of each natural dNTP. Before purification by 10% 7 M urea denaturing PAGE, the dsDNA was extracted with 40 μL phenol/chloroform/isoamyl alcohol (25:24:1), precipitated with 400 μL ethanol. After agitating and 15 minutes of centrifuging the ethanol was decanted and residual amounts were evaporated. The resulting pellet was resuspended in 44 μL water, add 5 μL 10x lambda exonuclease buffer and 1 μL lambda
exonuclease (5 units). The sample was incubated at 37 °C 3 hours. The phosphorylated strand was digested. The resulting product was 50 μL phenol/chloroform/isoamyl alcohol (25:24:1), precipitated with 500 μL ethanol. After agitating and 15 minutes of centrifuging the ethanol was decanted and residual amounts were evaporated. The resulting pellet was resuspended in 20 μL water. 30 μL loading solution was added, and then the sample was purified by 10% 7 M urea denaturing PAGE. The corresponding band was cut out, eluted with elution buffer, precipitated with ethanol. The resulting pellet was resuspended in 25 μL water and used in the 2nd amplification PCR step.

2nd amplification PCR

10 μL 1st amplification PCR product was added to 20 μL 5X PCR cocktail, 5 μL Vent (exo-) DNA polymerase (10 units), 65 μL water to result a 100 μL reaction. The amplification reaction was thermocycled using the same program for 30 cycles. After the amplification, the amplicon product was treated in the same manner with the 1st amplification product. After extraction, precipitation and resuspension, the DNA was then subjected to digestion by lambda exonuclease. The single-stranded DNA was dissolved in 1 M NaOH (20 μL) and loading solution (30 μL) prior to loading on a 10% 7 M urea denaturing mini-PAGE. The single-stranded DNA was identified by UV-shadowing, cut out, eluted with elution buffer, precipitated with ethanol. The resulting pellet was resuspended in 40 μL and desalted using a G-25 column. The DNA was quantified by UV absorption and used in elongation step.

The N30 library (N40 for click reaction and hydroamination selection) from IDT was not used for elongation directly. A 2nd amplification was done first with the N30 library. 300 pmol template was added to 20 μL 5X PCR cocktail, 5 μL Vent (exo-) DNA polymerase (10 units), 65 μL water to make a 100 μL reaction. The amplification reaction was thermocycled using the same program for 10 cycles. After the amplification, the amplicon product was treated in the same manner with the 2nd amplification including extraction, precipitation and resuspension, digestion,
PAGE purification, G25 desalting. The single-stranded DNA was quantified by UV absorption and used in elongation step.

5.5.3 Synthesis

5'-alkyne modified oligonucleotides (Primer 1): 5'-amino-modified oligonucleotide amino-primer (5 nmol in 10 µL water) diluted with 30 µL 0.1 M sodium phosphate buffer, pH 7.9 then added 30 fold molar excess of N-succinimidyl-5-hexynoate (0.15 µmol) in 5 µL DMF, which was synthesized according to the reference. The reaction mixture was incubated at room temperature for 3 hours. The terminal of the reaction was monitored by HPLC. Then the reaction was diluted with 100 µL water, extracted with 200 µL of chloroform. And precipitated by 500 µL 1% LiClO₄ in acetone, washed with 500 µL ethanol. Dry the pellet, add 50 µL water, then the oligonucleotide solution was desalted by G25 column. MALDI-TOF mass spectrometry: [M+H]^+ : calcd: 9802, found: 9811.
Chapter 6: Summary and future directions.

6.1 Summary

This thesis has explored the synthesis of modified nucleotide triphosphates, the use of modified nucleotides in the selection of RNA-cleaving DNAzymes and the selection of novel-reaction-catalyzing DNAzymes. In Chapter 2 I synthesized five modified 2'-deoxyuridine triphosphates and enzymatically incorporated the modified nucleotide triphosphates in template directed elongation reaction. The modifications contain a carboxylate group, indole group and napthyl group. They represent different functionalities; the carboxylate represents a metal binding ligand group, indole represents a tryptophan-like functional group, and the napthyl group modification represents a hydrophobic functional group. The enzymatic incorporation evaluated the suitability of the modified nucleotide triphosphates as substrates for polymerases. Although all the modified dUTPs were found to be relatively poor substrates for Sequenase Version 2.0 DNA polymerase, Vent (exo-) DNA polymerase was able to incorporate all the modified dUTPs successfully. In Chapter 3 and Chapter 4, two all-RNA-cleaving DNAzyme selections were conducted to select DNAzymes that can efficiently and intermolecularly cleave all-RNA substrate in the absence of divalent metal cations. In the DNAzyme selection against HIV RNA target in Chapter 3, I selected DNAzyme clone 25 that had a self-cleavage rate constant of 3.3 min\(^{-1}\). The DNAzyme was evaluated under different pH, temperature and divalent metal cations conditions. Overall, DNAzyme clone 25 is an excellent self-cleaving DNAzyme. However, when the DNAzyme was tested for trans-cleavage activity, the result was unsatisfactory. It was found to have a relatively low catalytic rate constant and a low substrate binding affinity. With this disappointing result, DNAzyme clone 25 is not likely to warrant consideration for \textit{in vivo} studies. However, in Chapter 4, I obtained a DNAzyme clone 11 that was found to have a high trans-cleavage rate constant and high binding affinity. The selection was conducted with a c-Myc
oncogene target sequence. Although the initial selection failed to produce an efficient DNAzyme, I redesigned the selection with the best clone from the initial selection and obtained DNAzyme clone 11 from this process. DNAzyme clone 11 had a self-cleavage rate constant of 0.84 min\(^{-1}\). The DNAzyme was evaluated at different pH, temperature and divalent metal conditions. The trans-cleavage study showed that it has a trans-cleavage \(k_{\text{max}}\) of 4.3 min\(^{-1}\) and \(K_m\) of 297 nM. The DNAzyme lost almost all cleavage activity when the cleavage junctions were changed to different nucleobases, indicating that it is highly sequence-specific. Solid-phase synthesis of the modified DNAzyme was attempted, and although no pure product was synthesized, the oligonucleotide mixture obtained showed trans-cleavage activity. Chapter 5 illustrated several failed DNAzyme selections. Although I did not successfully obtain any DNAzymes, many useful observations were made regarding the DNAzyme selection process that both add to the general knowledge of this class of enzymes and may aid future DNAzyme selections.

6.2 Future directions

Several results were achieved herein that led to the characterization of two DNAzymes that were studied in considerable depth in this thesis. My work addresses an unmet need from a therapeutic perspective: to this date there has not been an unmodified or modified DNAzyme discovered that has successfully used in a therapeutic application. It is hoped that others may follow up on these specific DNAzymes or at least derive encouragement to pursue this line of research to address the need for a divalent metal-independent RNA-cleaving DNAzyme. The DNAzyme clone 25 in Chapter 3 did not have a desired intermolecular cleavage activity, and therefore future studies with this clone are likely not worthwhile. However, clone 3, also obtained from this selection with different homology with clone 25, was found to have a relatively high rate constant for self-cleavage and preliminary intermolecular cleavage studies might yield promising results. The DNAzyme clone 11 in Chapter 4 definitely should be re-synthesized via solid-phase synthesis to obtain a better product, as this clone demonstrated excellent
intermolecular cleavage activity under physiological conditions. A pure solid-phase synthesized product would allow for the determination of a more precise trans-cleavage rate constant and would be suitable for in vivo studies. As an initial test, the DNAzyme could be test with a c-Myc-overexpressing cancer cell line. In addition, Following the work in Chapter 2, a protein binding aptamer selection with these modified nucleotide triphosphates could be conducted. There is some precedence of this type of selection, as Davies and coworkers have used the similarly functionalized 2'-deoxyuridine triphosphates in an aptamer selection and obtained some protein-binding aptamers which have nM binding affinity. Finally, the results from the novel selection attempted in Chapter 5 demonstrated that it is hard achieve success with complex reaction, such as the click reaction and hydroamination reaction. While the pursuit of novel DNAzyme-catalyzed reactions should not be abandoned, it is perhaps best to select simpler reactions in the future. Lastly, the DNA cleavage selection would be worthwhile to attempt this selection a second time.
References

(37) CHAPPLE, K. E.; BARTEL, D. P.; UNRAU, P. J. RNA 2003, 9, 1208.
(39) CHAPPLE, K. E.; BARTEL, D. P.; UNRAU, P. J. RNA 2003, 9, 1208.
(43) Tuerk, C.; Gold, L. Science 1990, 249, 505.


Appendices

A Appendix to Chapter 2: NMR, MS and UV Spectroscopy and HPLC purification

Chemical Formula: C$_{14}$H$_{16}$F$_{3}$N$_{3}$O$_{7}$
Molecular Weight: 395.29

MS (ESI$^+$)

$^1$H-NMR
Chemical Formula: \( \text{C}_{10}\text{H}_{18}\text{N}_{3}\text{O}_{14}\text{P}_3 \)
Molecular Weight: 497.18

MS (ESI)

Compound 2.11A: MS for S1, MS for compound 2.11A, HPLC, UV

Chemical Formula: \( \text{C}_{16}\text{H}_{13}\text{NO}_4 \)
Molecular Weight: 283.28

Chemical Formula: \( \text{C}_{22}\text{H}_{26}\text{N}_{3}\text{O}_{15}\text{P}_3 \)
Molecular Weight: 665.38
MS (ESI$^+$) for S1

MS (ESI$^+$) for 2.11A
HPLC for 2.11A

UV for 2.11A
Compound **2.11B**: MS for modified group, MS, HPLC, UV for compound **2.11B**

![Chemical structure of S2](image1)

Chemical Formula: C\textsubscript{14}H\textsubscript{12}N\textsubscript{2}O\textsubscript{4}
Molecular Weight: 272.26

![Chemical structure of 2.11B](image2)

Chemical Formula: C\textsubscript{20}H\textsubscript{25}N\textsubscript{4}O\textsubscript{15}P\textsubscript{3}
Molecular Weight: 654.35

**MS (ESI') for S2**

![MS (ESI') for S2](image3)

**MS (ESI') for 2.11B**

![MS (ESI') for 2.11B](image4)
HPLC for 2.11B

UV for 2.11B

Compound 2.11C: MS for modified group, MS, HPLC, UV for compound 2.11C

Chemical Formula: C_{18}H_{22}N_{3}O_{17}P_{3}
Molecular Weight: 645.30

Chemical Formula: C_{12}H_{9}NO_{6}
Molecular Weight: 263.21
MS (ESI) for S3

MS (ESI⁺) for 2.11C

HPLC for 2.11C
UV for **2.11C**

![UV spectrum for 2.11C]

**Compound 2.11D**: MS for modified group, MS, HPLC, UV for compound 2.11D

**Chemical Formula**: $C_{22}H_{26}N_3O_{15}P_3$  
**Molecular Weight**: 665.38

**Chemical Formula**: $C_{16}H_{13}NO_4$  
**Molecular Weight**: 283.28

MS (ESI⁺) for **S4**

![MS spectrum for S4]

180
MS (ESI-) for 2.11D

HPLC for 2.11D

UV for 2.11D
Compound 2.11E: MS for modified group, MS, HPLC, UV for compound 2.11E

Chemical Formula: C₁₅H₁₄N₂O₄
Molecular Weight: 286.29

Chemical Formula: C₂₁H₂₇N₄O₁₅P₃
Molecular Weight: 668.38

MS (ESI⁺) for S5

MS (ESI⁻) for 2.11E
HPLC for 2.11E

UV for 2.11E
B Appendix to Chapter 3: DNAzyme concentration measurement

Radioactivity percentage and volume for each dilution and the spotted TLC

A 1 μL aliquot of the resulting elongation solution was diluted 3x, 10x, 30x, 100x, 300x, and 2 μL of each dilution was spotted on a TLC-plate (spots 1-5) to generate an autoradiographic calibration curve that relates autoradiographic density to volumes and ultimately the number of pmol of dGTP (specific activity). The logarithm of signal intensity of plotted against the logarithm of the dilution and the data was fitted to a square line using linear regression. A 1 μL aliquot of the resulting DNAzyme solution was diluted 10 times and 2 μL of the dilution was spotted on the same TLC-plate (spot 6). The concentration of DNAzyme could then be determined using equation 1 and equation 2

The dilution time and radioactivity volume.

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The TLC plate:

\[ X = (Y - b)/a \]  
Equation 1
\[
[\text{DNAzyme}] = \frac{[d\text{GTP}] \times 10^{a}}{N \times 10^{b}}
\]  

Equation 2

X: log(dilution time), Y: log(volume), N: number of G incorporated in the DNAzyme, for this DNAzyme N=12, dGTP concentration: 20 µM

So, a=-1.075, b=7.719, \log(313288)=5.496, X=2.068

\[
[\text{DNAzyme}] = \frac{[20\mu\text{M}] \times 10^{a}}{12 \times 10^{b}} = 0.143 \mu\text{M}
\]
C Appendix to Chapter 4: DNAzyme concentration measurement and NMR and Mass spectroscopy

DNAzyme concentration measurement: the same method was employed here as in Chapter 3.

The dilution time and radioactivity volume.

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The TLC plate:

![TLC plate image](image)

So, \(a=-0.970\), \(b=7.255\), \(\log(313288)=5.341\), \(X=1.973\)

\[
\text{[DNAzyme]} = \frac{[20\mu M] \times 10}{14 \times 10^{1.973}} = 0.152\mu M
\]
NMR and MS for phosphoramidite U and C

Chemical Formula: C₅₁H₅₆F₃N₆O₉P
Molecular Weight: 985.01

Phosphoramidite C

³²P NMR for phosphoramidite C.
$^1$H NMR for phosphoramidite C.

MS (ESI$^+$) for phosphoramidite C

Chemical Formula: $C_{51}H_{60}N_9O_{12}P$
Molecular Weight: 1022.07
$^{32}$P NMR for phosphoramidite U.

$^1$H NMR for phosphoramidite U.

MS (ESI$^+$) for phosphoramidite U
AB 3400 DNA Synthesizer Run Report

Run Title: N30-11
Run Time: 18h, 20m, 4s
Instrument: 3400
Software: AB 3400 DNA Synthesizer 1.21
Cycle: 0.2umol-rna

Sequences

Column 1: N30-11 (Size=54mer)
5'> 888 TGC GGA GGG GC5 G66 7G5 7G5 655 G5G G6G 556 G55 5G5 56G 6GG 6G6 G6T <3'

Note: 8=2’-OMe rC
5=dUgua(ceac)
6=dCam(Tfa)
7=dAimid

Trityl Results

Trityl levels were monitored until they fell within 0% of baseline
Minimum acceptable average step-wise yield was 0%

Average step-wise yield for column 1 was 97.2%

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D Appendix to Chapter 5: Mass spectroscopy

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