ABSTRACT

Coenzymes for *in Vitro* Selection of DNA Enzymes

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Over the past 10 years a number of catalytically active single-stranded DNA molecules (deoxyribozymes) have been isolated from combinatorial libraries (pools) of randomized oligonucleotides. These enzyme-like DNAs customarily utilize divalent metal ions as cofactors in their catalysis. The metal ion cofactors are important because they can participate in both the correct folding of the deoxyribozymes and in the acid/base catalysis. In a new approach, we have designed and synthesized a group of potential small molecule coenzymes, designated as the IDA-oligonucleotide coenzyme, the *bis*-histidine aromatic coenzymes and the *bis*-imidazole peptide coenzymes, which could be used by deoxyribozymes in the hydrolysis of a target RNA phosphoester. It was hoped that careful design would provide these molecules with a balance of solubility, binding, and catalytic power. These synthetic coenzymes were included in combinatorial selection experiments aimed at isolating coenzyme-dependent deoxyribozymes from a randomized pool of DNA. Kinetic analysis of deoxyribozymes obtained in these experiments showed a rate enhancements that were dependent on the coenzyme used in the selection. Results of pH-dependent activity profiles of several

deoxyribozymes/coenzyme systems suggest a catalytic role for the coenzyme imidazoles. In conclusion, we have demonstrated that combinatorial selection techniques can be used to select for coenzyme-dependent deoxyribozymes. This convenient methodology provides the opportunity to utilize the power of synthetic organic chemistry for the invention of novel coenzymes optimized for desired transformations. In addition, the methodology described in this dissertation complements rational design approaches, and provides a unique opportunity for the study of various aspects of enzyme/coenzyme design.

Synthetic Coenzymes for *in Vitro* Selection of DNA Enzymes

by

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A Dissertation

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*To my parents Dorn N. and Aurelia,*
*and to the memory of my grandparents:*
*Santusa, Tarcila, Eulogio and Lorenzo,*
*whose only dream and goal in life was my success*

# CHAPTER ONE

## Background

### *Discovery of Ribozymes and the RNA World*

During the 1960s proteins were considered by the scientific community as the only biological catalysts available in nature. One of the biggest concerns of that time was that primitive DNA synthesis would have required the presence of specific enzymes and at the same time these enzymes would have required the genetic information in DNA and posterior translation of that information into the amino acid sequence of the protein enzymes. All this gave rise to the classic "chicken-and-egg" problem, proteins are required for DNA synthesis and DNA is required for protein synthesis.

It was not until 1967 that Woese's studies of ribosomal RNA led him to suggest that RNA could catalyze its replication and potentially give rise to the first DNA-based living cells.[1] Crick and Orgel further speculated about the possibility of simple polymers (RNA) or self-replicating proteins being responsible for the production of the initial genetic material of living systems.[2,3]

In the 1980s, Dr. Thomas R. Cech had been studying RNA splicing in the ciliated protozoan *Tetrahymena thermophila* when he discovered that an unprocessed RNA molecule could splice or process itself.[4] Cech observed that even in the complete absence of a protein enzyme, the pre-rRNA of the *Tetrahymena* was removed and the residual RNA pieces ligated correctly. The sole requirement for this reaction to proceed was the presence of both magnesium ions and guanosine. Based on these findings, Cech proposed that RNA molecules were not restricted to being passive carriers of genetic

information, but that instead, they could additionally have catalytic functions and could participate in cellular reactions. From this time on, the term "ribozymes" was applied to these catalytic RNA molecules.[4] In 1989, Thomas Cech and Sidney Altman won the Nobel Prize in Chemistry for their "discovery of catalytic properties of RNA." Another Nobel Laureate in Chemistry, Walter Gilbert, coined the phase “The RNA World” in 1986.[6] This phrase refers to a hypothetical primitive stage in the origin of life in earth, in which RNA carried out both a genetic and a catalytic role, without the participation of the later-formed DNA and proteins.

### *Natural Functional Oligonucleotides (Ribozymes)*

The discoveries by Cech and the RNA world postulate of Gilbert have been further supported by the discovery of additional ribozymes.[5] To date, seven naturally occurring classes of ribozymes have been characterized: group I, group II, RNase P, hammerhead, hepatitis delta virus, hairpin and Neurospora Varkud satellite.

Understanding of the structure and mechanisms of these main classes of natural ribozymes has been essential for establishing the concept of functional oligonucleotides. For example, one common characteristic of these natural ribozymes is that they catalyze chemical transformations primarily at the phosphate esters (Figure 1). The group I, group II and ribonuclease P ribozymes, generally considered complex ribozymes, catalyze site-specific RNA phosphoester cleavage by a mechanism that involves nucleophilic attack on the phosphorous center. These highly folded RNA molecules function by orienting a nucleophile and the phosphate for an efficient reaction.[5] Similarly, hammerhead, hepatitis delta virus, hairpin and Neurospora Varkud satellite ribozymes, considered small self-cleaving ribozymes, participate in phosphoester transfer reactions within RNA

where a 2' oxygen is directed and activated for nucleophilic attack at its adjacent phosphate.[5]

| Category | Number sequenced | Biological sources | Reaction performed (reaction product) |
|---|---|---|---|
| Self-splicing RNAs | | | transesterification (3′-OH) |
| Group I | >500 | Eukaryotes (nuclear and organellar), prokaryotes, bacteriophage | |
| Group II | >100 | Eukaryotes (organellar), prokaryotes | |
| Self-cleaving | | | |
| Group I-like | 6 | *Didymium, Naeglaria* | Hydrolysis (3′-OH) |
| Small self-cleavers | | | transesterification (2′, 3′>p) |
| hammerheads | 11 | Plant viroids and satellite RNAs, newt | |
| hairpin | 1 | Satellite RNA of tobacco ringspot virus | |
| HDV | 2 | Human hepatitis virus | |
| VS | 1 | *Neurospora* mitochondria | |
| RNase P RNAs | >100 bacterial | Eukaryotes (nuclear and organellar), prokaryotes | Hydrolysis (3′-OH) |

Figure 1. Characteristics of the main classes of natural ribozymes.[6] (Directly reproduced from reference 6).

*The Hammerhead Self-Cleaving Ribozyme*

A hammerhead ribozyme is a small catalytic RNA motif that catalyzes self-cleavage reaction.[8-10] Its name comes from a secondary structure which resembles a hammer. The hammerhead ribozyme is involved in the replication of a type of viroid and some satellite RNAs and contains three base-paired stems and a highly conserved core of residues required for cleavage. Stem I, II and III are formed by base pairing of complementary nucleotides through Watson-Crick hydrogen bonding (Figure 2). Stem II is capped by a four base loop at the top of the branch and stem III is connected by an irrelevant loop. The base sequences of the three stems can vary, depending on the

hammerhead ribozyme under consideration (obtained from different species). The only sequence restriction is that Watson-Crick base complementarity is maintained so that stem hydrogen bonding can form. On the contrary, the central core sequence is highly conserved. The core contains two domains: Domain 1 of the core comprises a CUGA sequence, the uridine turn, that follows Stem I, while domain 2 is formed by non-Watson Crick base pairing, and connects Stem II and Stem III. C17 is the most conserved residue and the position at which strand cleavage takes place.

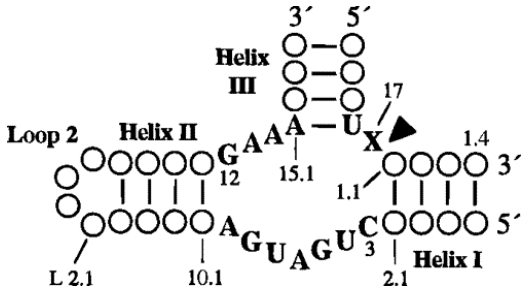



Figure 2. Secondary structure and conserved core of the hammerhead ribozyme. Arrow shows cleavage position.[16] (Directly reproduced from reference 16).

The cleavage reaction proceeds by an attack of a 2' hydroxyl oxygen of a catalytic site cytosine on the phosphorus atom attached to the 3' carbon of the same residue, thereby breaking the sugar phosphate backbone and producing a 2', 3' cyclic phosphate.[11] This mode of catalysis is commonly called an in-line mechanism, and a wide body of evidence supports the mechanism, including information generated from sulfur substitution for the scissile phosphate oxygen.[9,12] The reaction absolutely requires divalent cations such as $Mg^{2+}$.[13,14] pH studies reveal that the reaction rate linearly increases as the pH is raised from pH 5 to neutrality, suggesting the activation of the nucleophile (2'-OH) directly by a hydroxide ion or indirectly by metal ion coordinated to the 2'-OH that is then attacked by a hydroxide ion.[13,14] The resulting nucleophilic

oxygen then attacks the scissile phosphate. The configuration of the reaction intermediate is a trigonal bipyramid in which the apical positions are occupied by the leaving group (5'-OH) and the attacking group (2'-OH) (Figure 3). Therefore, the free 2'-OH on nucleotide C17 and the divalent cations are essential for the catalysis.[15] In *in vitro* experiments hammerhead ribozyme is capable of cleaving in *cis* (self-cleavage) or in *trans* (intermolecular reaction with any sequence targeted for cleavage)[16] with a rate constant of 1 $min^{-1}$ in presence of 10 mM of $Mg^{2+}$ metal ion.

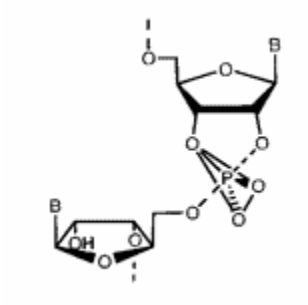


Figure 3. Trigonal bipyramid conformation in internal nucleophilic attack by hammerhead and hairpin ribozyme.[18] (Directly reproduced from reference 18).

*The Hairpin Self-Cleaving Ribozyme*

The hairpin ribozyme is another small catalytic RNA, which acts as an endonuclease, catalyzing a reversible sequence-specific cleavage reaction within RNA satellites of plant viruses.[9,10] Although the hammerhead and the hairpin ribozymes perform similar reactions, they do not share the same structure. The hairpin ribozyme contains four helices (H1, H2, H3 and H4) organized in two principal domains (A and B) (figure 4). The RNA substrate is located in a loop between helices 1 and 2, and its cleavage is reversible. The folding of the ribozyme casuses the two domains to interact side by side, creating a buried active site. This ribozyme is stabilized by interactions

between the loops which are highly conserved sequences. On the other hand, helical regions can largely be changed in sequence, provided that Watson–Crick base pairing is maintained.

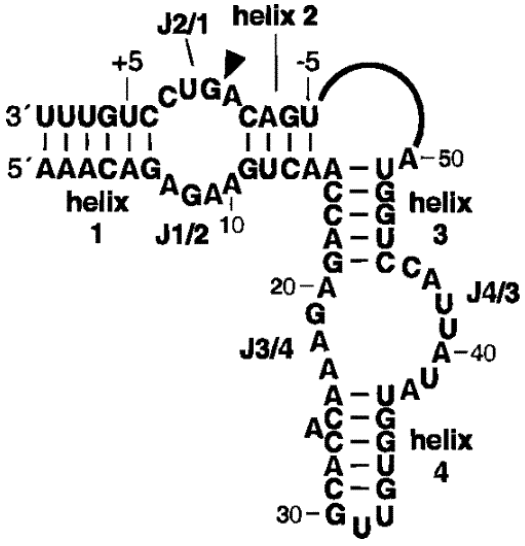



Figure 4. Secondary structure the hairpin ribozyme. Arrow shows cleavage position.[19] (Directly reproduced from reference 19).

The hairpin ribozyme effectively uses metal ions such as $Mg^{2+}$. The cleavage reaction generates products with 5'-hydroxyl and 2',3'-cyclic phosphate termini, which are analogous to those produced by three other natural ribozymes that are part of circular satellite RNAs:[20,21] the hammerhead, the hepatitis delta virus (HDV) and the Varkud satellite (VS) ribozymes. The cleavage rate of the hairpin ribozyme is not affected by pH changes between pH 5.5 and 8,[19] therefore excluding the 2'-OH activation as the rate-determining step. Other studies such as phosphorothioate substitution and reaction in presence of chelating agents, suggest that metal ions do not directly participate in the active site of the hairpin ribozyme and instead play only a structural role.[22-24] Further catalytic studies in presence of saturating concentrations of monovalent metal ions

showed a similar rate constant, suggesting that the nucleobases are the key elements of the catalytic process.[25]

*The Hepatitis Delta Virus (HDV) Ribozyme*

The HDV ribozyme is a small self-cleaving RNA molecule located in the hepatitis delta virus (HDV) satellite of the RNA of hepatitis B virus (HBV). It composed of 85 contiguous nucleotides that undergoes self-cleavage and generates unit-length monomers from the concatameric product of rolling circle replication.[26,27]

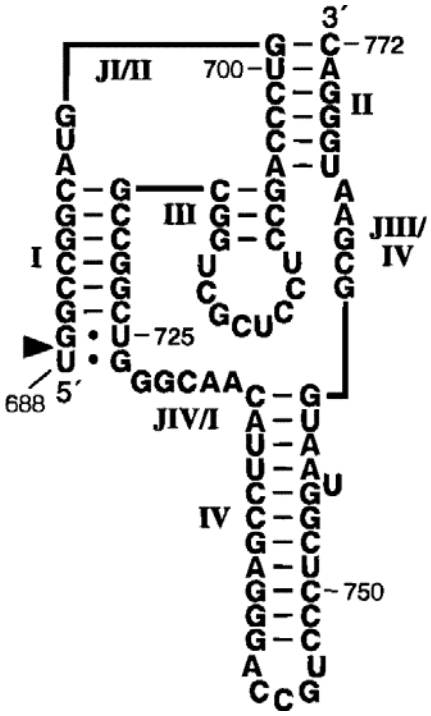



Figure 5. Secondary structure HDV ribozyme. Arrow shows cleavage position. (Directly reproduced from reference 29).

HDV is composed of 4 stems of highly conserved bases and variable sequence loops that connect the stems. The RNA substrate is localized in stem I. The central part of the HDV ribozyme model consists of a hairpin loop (loop III) with seven nucleotides

which are all important for activity. Stems IV and II play a crucial role in creating tertiary interactions that favor the correct conformation to the HDV ribozyme (Figure 5).[28,29] As seen with the hammerhead and hairpin ribozymes, the HDV self-cleavage reaction produces RNA containing 2',3'-cyclic phosphate and 5'-hydroxyl termini. Low concentrations of divalent metal ions are important for HDV ribozyme function,[25] and the cleavage rate of HDV ribozyme is constant between pH 5 and 9, suggesting that nucleobases, rather than hydroxide ions, activate the 2-OH nucleophile.[30] Mutation and crystallographic studies have shown that C75 of the HDV ribozyme could act as a general base when deprotonating the 2'-OH or act as a general acid protonating the oxygen leaving group (Figure 6).[31] In contrast to the hairpin ribozyme, which can operate in absolute absence of divalent metal ions, HDV requires at least low concentrations of metal ion cofactors for the formation of the active site.[32-34]

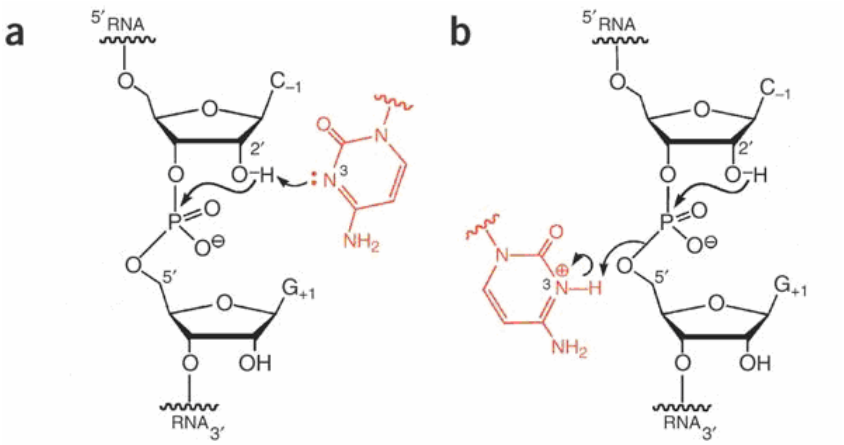



Figure 6. HDV ribozyme transesterification mechanism. (a) Cytosine acts as a general base, mediating proton transfer from the 2'-hydroxyl. (b) Alternatively, protonated cytosine acts as a general acid, mediating proton transfer to the 5'-oxygen leaving group.[35] (Directly reproduced from reference 35).

*Group I and II Ribozymes*

Group I and II ribozymes and ribonuclease P are considerably larger and more complex molecules than the self-cleaving ribozymes. They are most commonly found in mitocondrial genome of yeast and chloroplast of plants while the group I ribozyme is also localized in the genome of lower eukaryotes.[36] The mechanism by which Group I and II ribozymes mediate the splicing of the RNA is a series of two concerted phosphodiester bond cleavage–ligation (transesterification) reactions. Group I ribozymes self-splice in two steps to ligate flanking 5' and 3' exons, producing mature RNA transcripts. Group II ribozymes also cleave and insert themselves into double-stranded DNA in a reaction that requires the cooperation of the ribozyme active site and a protein moiety.[37]

Group I ribozyme, 421 nucleotides long, contains two primary structural domains. One domain of ~200 bases forms the conserved catalytic core that interacts with a less conserved peripheral domain.[38,39] The catalytic core is composed of helices designated P5-P4-P6 and P7-P3-P8, which form a cleft along which the P1 substrate helix binds (Figure 7). Crystallography and mutagenesis studies suggest that RNA folding, through core and peripheral interactions, is required for the function of large ribozymes.[36] In addition, sulfur substitution experiments have shown that magnesium ions are coordinated to the catalytic site, activating the 2-OH nucleophile and stabilizing the leaving group.[40-42] The group I ribozyme also employs an external guanosine nucleoside or nucleotide cofactor as nucleophile for the transesterification reaction. In a first step of transesterification, the 3'-hydroxyl of the guanosine acts as the nucleophile for an $S_N2$ attack on the phosphate of the target internucleotide linkage (exon-intron linkage). In a second step, the newly formed 3'-hydroxyl of the cleaved 5'-exon acts as nucleophile in

the subsequent attack at the second splice-site junction (intron-exon linkage), which results in the contiguous protein coding exon-exon segement, and extension of the intron sequence by one nucleotide (guanine) after cleavage (Figure 8).[43,44]

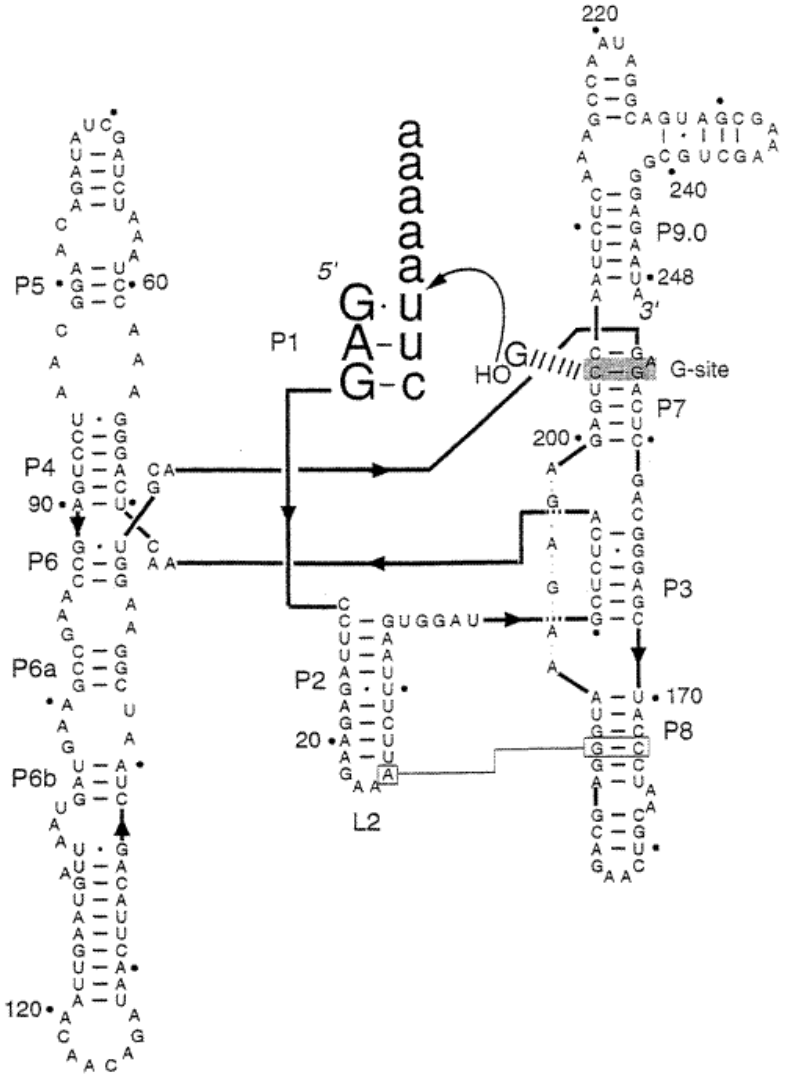



Figure 7. Secondary structure of the group I ribozyme (Anabaena ribozyme). Arrows show cleavage site.[36] (Directly reproduced from reference 36).

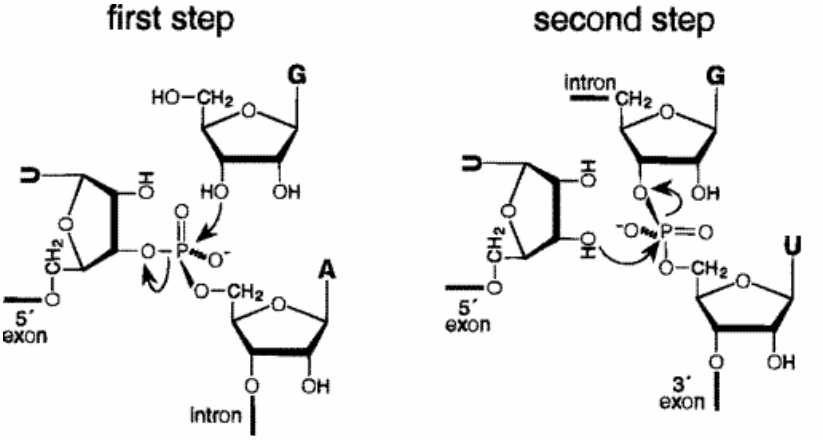



Figure 8. Two step mechanism of phosphate transfer by the group I ribozyme. First step shows attack by guanosine and second step attack by the newly formed 3'-OH exon.[45] (Directly reproduced from reference 45).

The group II ribozyme is composed of six domains (Figure 9). Only domain 1 and domain 5 are essential for minimal catalytic activity of the ribozyme, being domain 5 (33 nucleotides hairpin) the central core of group II ribozymes. Other elements such as domain 3 and the base of domain 2 significantly enhance reactivity by tertiary interactions. The group II ribozyme does not require free guanosine as cofactor, but instead it exploits the 2'-hydroxyl of a specific internal adenosine.[46-48] Transesterification occurs in fashion analogous to the group I ribozyme. Concentration dependence studies with metal ions have shown that both magnesium and potassium ions are essential for proper folding of the ribozyme and that this ions are actively involved in the catalytic reactions.[49]

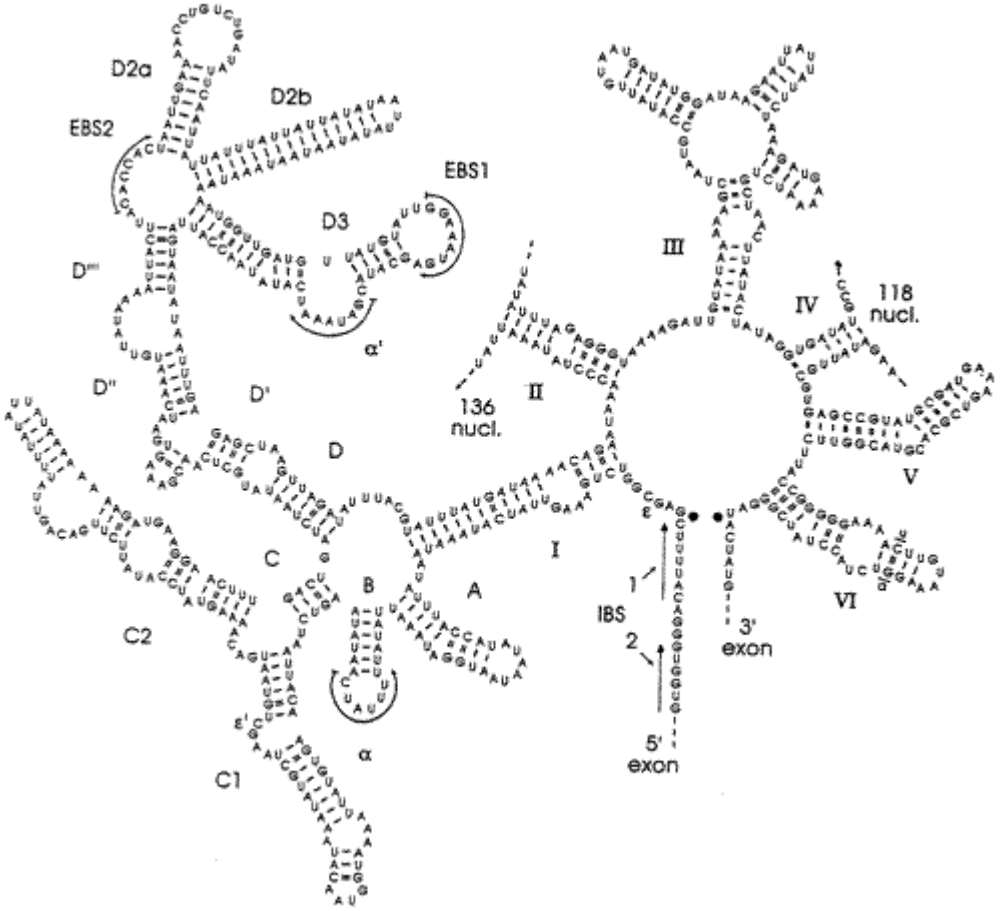



Figure 9. Secondary structure the group II ribozyme (yeast mitochondrial cytochrome oxidase). Roman numbers show different domains.[36] (Directly reproduced from reference 36).

*RNase P*

RNAse P is a ribonucleoprotein complex involved in processing tRNA. The RNase P is unique in that it consists of one RNA of typically 350–400 nucleotides associated in 15 domains (Figure 10) and one small basic protein of ≏14 kDa.[50] Although both subunits are essential in vivo, conditions of high $Mg^{2+}$ and monovalent salt concentrations activate catalysis by the RNA subunit alone *in vitro*.[51]

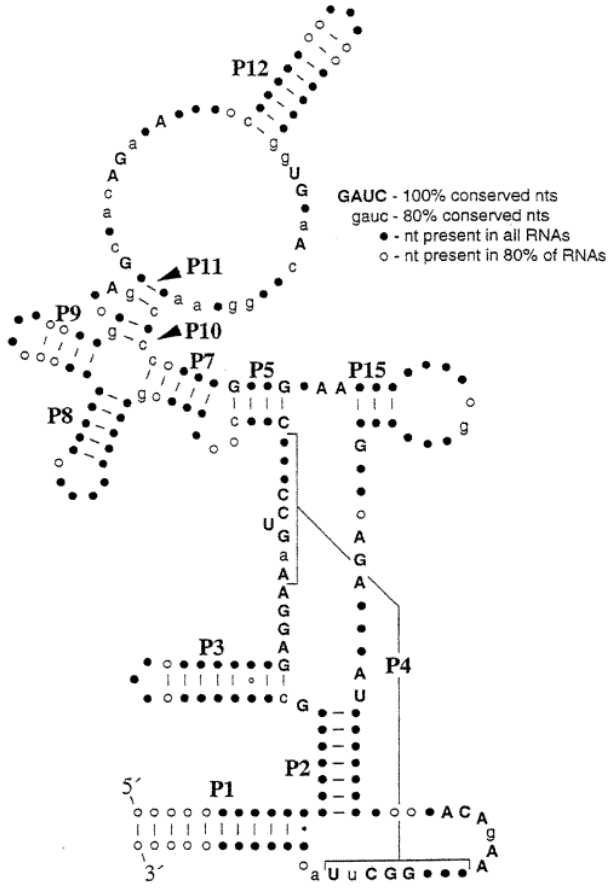



Figure 10. Secondary structure of the consensus bacterial RNase P sequence.[50] (Directly reproduced from reference 50).

Unlike most other ribozymes, the mechanism of phosphodiester cleavage by RNase P is hydrolysis rather than transesterification. Several divalent metal ion, including $Mg^{2+}$ are required for the cleavage activity, both for structural purposes, and to coordinate and activate a water molecule for attack at the scissile phosphodiester.[52] (Figure 11). In a proposed mechanism, one of the required hydrated magnesium ions is coordinated to the pro-S oxygen of the scissile phosphodiester, while two of the coordinated water molecules from this catalytic metal ion are involved in hydrogen bonding interactions-one to the attacking hydroxide, and the second to the adjacent 2'-OH.[53,54]

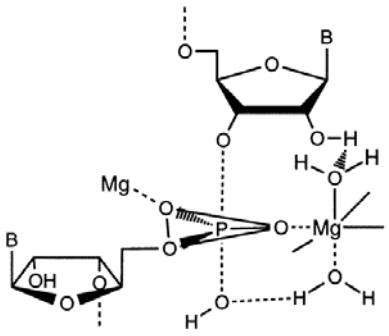



Figure 11. Proposed transition state of RNase P stabilized by $Mg^{2+}$.[19] (Directly reproduced from reference 19).

*Combinatorial Chemistry and the Selection of Artificial Functional Oligonucleotides*

The original idea of a combinatorial process can be found in nature. For example, a combinatorial process is used by nature both to generate the genomic information for all possible primary antibodies and to identify those antibodies that bind tightly to a given target molecule. Thus, the variable DNA sequence of a unique antibody is assembled from a predefined set of 100–1000 different V, J and D gene segments on the chromosomal DNA — a combinatorial principle, similar a to multi-component condensation reaction. In the primary immune response to antigen, a whole library of antibodies is generated. Out of this initial population, antibodies are selected that bind with a certain affinity to the given antigen. Starting with this binding sub-population, new antibodies, which are not encoded on the chromosomal DNA, are generated by somatic hypermutations. The entire process is repeated several times until high-affinity antibodies are generated. This evolutionary immune response is therefore an affinity-driven feedback cycle that yields antibodies with high affinity to the target antigen.[55]

The idea of generating a large number of diverse small molecules by combinatorial chemistry was first demonstrated by Ivar Ugi in 1961,[56] who used a four-

component reaction and systematically varied of the starting materials (now called the Ugi reaction). Several researchers have built upon this idea and explored the possibility of generating mixtures containing millions of individual compounds and screening them as a mixture for affinity to a receptor.[57-59]

Similarly, molecular biologists and biochemists use combinatorial chemistry techniques routinely to generate libraries containing millions of compounds as mixtures in their work. These millions of molecules (oligonucleotides or polypeptides) are screened (combinatorially selected) towards the binding or catalytic activity for a particular ligand[60, 61] or target reaction,[62,63] respectively. Similarly to chemical libraries, combinatorial nucleic acid libraries, for example, can be generated with enormous structural diversity (randomization of nucleotides), and opposed to chemical libraries, combinatorial nucleic acid libraries can be easily replicated using the polymerase chain reaction (PCR). Thus, nucleic acid libraries can be considered chemical combinatorial libraries in the broadest sense of the term.

One problem observed in both chemical and nucleic acids combinatorial libraries is an inability to cover the universe of all possible structures. In chemical libraries this is a well-known synthetic design problem, whereas in nucleic acid libraries the problem lies in the sample size. For example, a nucleic acid pool that has 40 randomized positions potentially contains $4^{40}$ or about $10^{24}$ different sequences, each of which will have a unique associated structure. In practice, working with one copy of each of those sequences would mean handling 12 kg of nucleic acids (Table 1). Therefore, working with 1 pmol of this combinatorial nucleic acid library represents only ~$10^{11}$ molecules. While it is clear that these incomplete libraries may or may not contain the best possible

number of molecules, additional randomization and mutation of these combinatorial nucleic acid libraries increase the total number of species that are examined during the course of a combinatorial selection.

Table 1. Number of random residues and combinatorial nucleic library.

| no. of random residues | no. of different compound in library | molecular weight based on no. of random residues |
|---|---|---|
| 1 | 4 | ~ 300 Da |
| 4 | 256 | ~ 1200 Da |
| 10 | $10^6$ | ~ 3000 Da |
| 20 | $10^{12}$ | ~ 6000 Da |
| 30 | $10^{18}$ | ~ 9000 Da |
| 40 | $10^{24}$ | ~ 12 kDa |
| 50 | $10^{30}$ | ~ 15 kDa |
| 80 | $10^{50}$ | ~ 24 kDa |

*SELEX and Nucleic Acid Aptamers*

One such combinatorial selection process is the systematic evolution of ligands by exponential enrichment (SELEX). The SELEX process is a combinatorial methodology that allows the rapid isolation of unusual oligonucleotide sequences (aptamers), from large random single-stranded sequence libraries of RNA or DNA (typically greater than $10^{15}$ potential molecules), that recognize virtually any chemical or biological entity with high affinity and specificity.[61, 64-86] Similar to protein antibodies, nucleic acid aptamers can be used as molecular recognition elements for analytical applications.[87] However, aptamers offer several advantages over antibodies since they exhibit superior biological stability, are easily obtained and synthesized at low cost.

SELEX technology was introduced in the 90s by Tuerk and Gold who were targeting the bacteriophage T4 DNA polymerase.[61] At the same time, Ellington and

Szostak discovered that relatively short RNA molecules (20-40 nucleotides, 8-15 kDa) could be selected for binding to synthetic small organic dyes,[69] and they called these RNA molecules "aptamers." The potential of aptamers has been amply demonstrated and significant progress made over the last 15 years; for example, these particular oligonucleotides are in these days extraordinary diagnostic tools and potential therapeutic agents.[88,89] The SELEX technology has been extended to oligonucleotides containing structural modifications, primarily through post-selection modifications.[69,90] Aptamer technology has been applied to various targets including proteins,[61, 70-72] antibodies,[73] enzymes,[74-76] as well as peptides.[77] Aptamers have also been identified that recognize small molecules such as dyes,[79] amino acids,[78-80] nucleotides,[81,82] and drugs.[83]

In its most basic form the SELEX process may be defined by the following series of steps (Figure 12):[91]

1. A combinatorial pool of nucleic acid sequences is prepared (steps 1 and/or 2). Each member of the combinatorial pool generally includes regions of fixed sequences (needed for a given structural arrangement or for posterior PCR amplification) and the regions of randomized sequences (providing diversity).
2. The combinatorial pool (steps 1 and/or 2) is exposed to a selected target (step 3) under conditions favorable for binding between the target and the members of the combinatorial pool. Under these conditions, the interaction between the target and the nucleic acids of the pool can be considered as forming nucleic acid–target pairs between the target and those nucleic acids having the strongest affinity for the target.

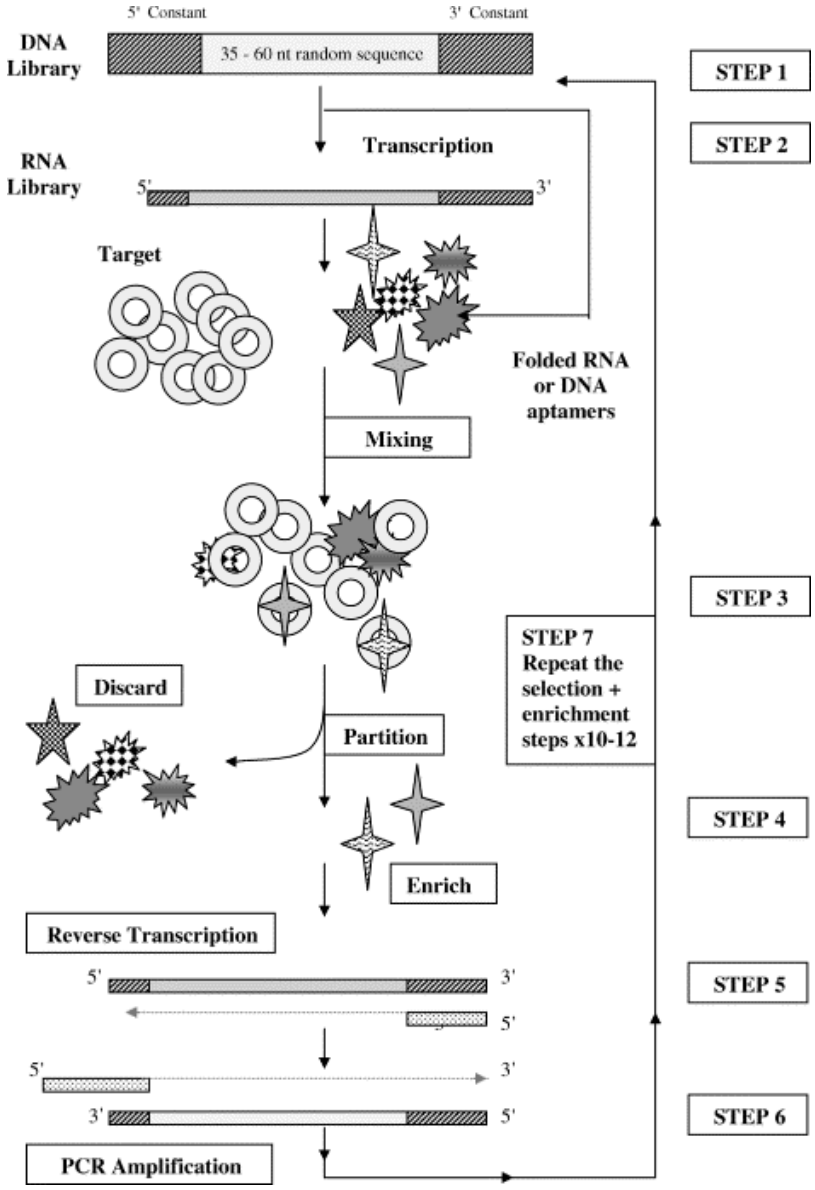



Figure 12. Systematic Evolution of Ligands by EXponential enrichment (SELEX).[91] (Directly reproduced from reference 91).

3. The nucleic acids with the highest affinity for the target are partitioned (step 4) from those nucleic acids with lesser affinity to the target. Because only an extremely small number of sequences corresponding to the highest affinity nucleic acids exist in the combinatorial pool, it is suggested to set a partitioning

criteria so that a significant amount of nucleic acids in the pool (approximately 5–50%) are retained during partitioning.

4. Those nucleic acids selected during partitioning are PCR amplified, step 5 (for RNA aptamers) and step 6 (in all cases), to create a new pool which is enriched in the nucleic acid having a relatively high affinity for the target.

By repeating these steps several times the combinatorial pool contains fewer unique sequences, and the average degree of affinity of those retained will generally increase. Taken to its extreme, the SELEX process will yield a final mixture containing one or more (likely to be less than 10) unique oligonucleotides representing those nucleic acids from the original combinatorial pool having the highest affinity to the target molecule.

*in Vitro Selection of RNA and DNA Enzymes*

By time that Tuerk and Gold were working in the process named by them as SELEX (61), Ellington and Szostak reported[92] a similar selection approach that they called "*in vitro* selection." Almost at the same time, this approach was also applied by Joyce to isolate a variation of the group I intron ribozyme capable of cleaving RNA.[93]

Since then, *in vitro* selection has been used by others to modify natural ribozymes structurally and functionally, and to create artificial ribozymes with new functions.[94-107] More recently, the use of *in vitro* selection has been extended to select another type of catalytic oligonucleotides known as deoxyribozymes[108- 129] and catalytic aptamers known as allosteric nucleic acid enzymes, a hybrid of an enzyme and aptamer in which upon binding to a ligand, the oligonucleotide becomes catalytic.[130-139]

*In vitro* selection is a combinatorial methodology in which RNA or DNA molecules with catalytic properties can be isolated from a large combinatorial library over the course of multiple cycles of selection and amplification (Figure 13). A successful *in vitro* selection depends on an effective strategy and control of certain factors, such as combinatorial library size and reactive oligonucleotide structural design.

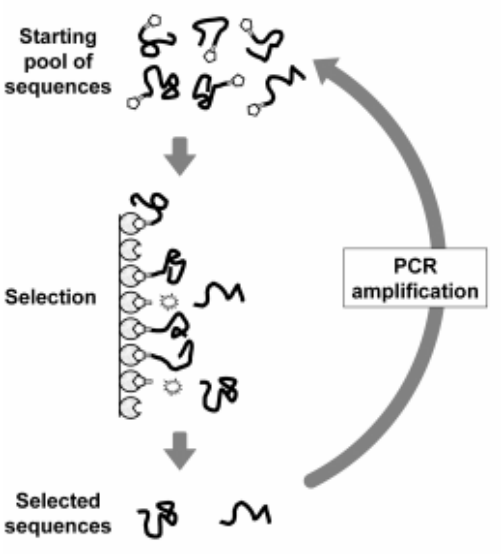



Figure 13. General *in vitro* selection process.[140] (Directly reproduced from reference 140).

The size of the combinatorial library, an important factor to consider in *in vitro* selection, depends on the number of nucleotides in the random-sequence domain and the amount sample of material that can be practically taken (for example, 1 nmol of 40 random-bases, equivalent to $6 \times 10^{14}$ molecules and equivalent to 12 μg of nucleic acid material). Furthermore, since the random-sequence domain contains the potential catalytic domain (of the same or shorter length), the length of the random-sequence is chosen based on the experience of previously found ribozymes or deoxyribozymes. For example, the catalytic domains of the RNA-cleaving deoxyribozymes 10-23 and bipartite

II are 15 and 20 nucleotides, respectively.[112,120] The probability of finding similar contiguous sequences in a 100-nucleotides random sequence would much higher than finding it in a 40-nucleotides element. In general, a smaller random-sequence domain may be limited by the number of diverse structures that it can produce, and a larger random-domain, although favorable for accessing diverse structures and more catalytic active molecules, may be also limited by the small fraction of total available sequence space (~$10^{14}$ of $10^{50}$ possible sequences in a 80 random-sequence) that can be actually sampled in any one experiment. Nucleic acid combinatorial libraries of 40 or 50 random nucleotides are preferred and can be chemically synthesized under standard methods in a commercial nucleic acid synthesizer. For this, instead of using four different solutions of each of the four phosphoramidites (dA, dC, dG and dT), a single solution containing a mixture of all four DNA phosphoramidite monomers (1:1:1:1) is used. Pools of random ribonucleotides are created by reverse transcription and then PCR amplification of chemically synthesized DNA pool or by direct amplification in an isothermal reaction employing both a reverse transcriptase and a DNA-dependent RNA polymerase.[141]

The library can be constructed to contain only random-sequence domains, or each member of the library can be designed to carry specific structural elements that, for example, create substrate binding sites or self organization binding sites (Figure 14).[143]

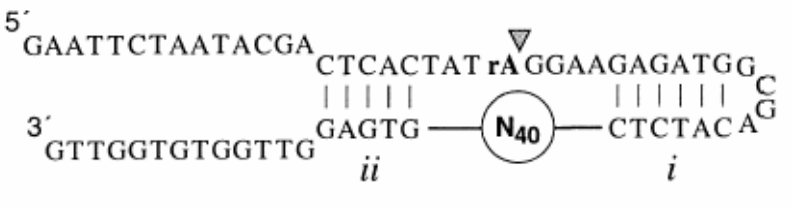



Figure 14: Design of a self-cleaving oligonucleotide. Substrate binding or pairing elements *i* and *ii*, $N_{40}$ denotes the random-sequence domain (potential catalytic domain), arrow indicates substrate reaction site.[143] (Directly reproduced from reference 143).

In order to screen catalytic nucleic acids, one must have a way of identifying those molecules that facilitate the desired chemical transformation from those that are inactive. Perhaps the most straightforward manner in which to achieve this goal is to identify or isolate nucleic acids that undergo self-modification. Currently, many of the catalytic polynucleotides that have been created by *in vitro* selection carry out reactions with nucleic acid substrates (phosphoester cleavage or ligation).

An effective *in vitro* selection strategy consists on a general algorithm composed of the following steps: 1) Generate a large number of different molecules (combinatorial library); 2) Select those molecules that meet some set of criteria (i.e., self-modification); 3) Amplify the selected molecules (PCR); and 4) repeat from (1) until catalytically active population of oligonucleotides is obtained.

Commonly, *in vitro* selection methods that have been successfully implemented start with a so called “naïve pool” of different DNA sequences containing the random-sequence domain. These DNA molecules are often biotinylated and bound to an avidin or streptavidin solid support. The equilibrium constant for avidin and its biotin is ~$10^{15}$ $M^{-1}$, making this an efficient method for solid-phase immobilization of nucleic acid pools or for isolating exceedingly rare sequences that acquire a biotin "tag." Active molecules are then eluted by adjusting the solvent conditions (concentration of cofactor in reaction buffer) or by limiting the reaction time to the permissive reaction conditions (selective pressure by defined incubation times). Once selected, the reactive molecules are amplified to enrich for progeny of molecules that have the desired property and to restore the original population size (i.e., ~$10^{14}$ molecules for a 40-random sequence domain). Frequently, the number of molecules recovered during the selection is fairly small. This

problem is solved by the use of sensitive amplification methods such as the polymerase chain reaction (PCR) to amplify the recovered molecules. This method generally uses primers that can recognize the fixed regions (Figure 1) within the eluted molecule with high sensitivity and specificity. This enzymatic reaction will make copies of the selected molecule. Repetitive PCR cycles yields a new pool of molecules that are derived from the selected molecules of the previous pool. Since PCR amplification is not a perfect reaction, passive mutations ($1 \times 10^{-4}$ mistakes per base pair per cycle) are introduced to the selected pools. This minimizes the loss of latent DNA enzymes during the initial rounds of selection when the population diversity is especially vulnerable to random experimental loses.[142]

The process described above, commonly known as "round of selection," is repeated many times (*in vitro* selection) until a catalytic efficiency limit is reached and the initial combinatorial library is minimized to a small subset of tens or hundreds of variants (enriched pool) that best meet the applied challenge.

At the completion of the selection process the individual sequences of the enriched pools are cloned and characterized as to the catalysis that had been selected. In many cases, the initial combinatorial library may contain only sequences that perform the desired catalytic process poorly, which is reflected low activity in the enriched pool. In those cases a mutagenesis procedure is used to generate a new pool based on a poor enzyme and the selection process repeated of this pool of related sequences. It is hoped that this will compensate for those sequences not initially present or lost in the selection steps. This optimization process is commonly referred as '*in vitro* evolution."

*In vitro selected self-cleaving ribozymes.* The concept of RNA world stimulated many researchers to find out all the possible reactions in which ribozymes can participate. Artificial ribozymes have been obtained by a combinatorial technique called *in vitro* selection. These new ribozymes, having completely new structures and catalytic activities other than self-cleavage (phosphoanhydride formation, amide bond formation, glycosidic bond formation, DNA and RNA ligation, phosphorilation and many more),[45] have been key elements in supporting the RNA world hypothesis.

The first reported artificial self-cleaving ribozyme was selected from a pool of partially mutagenized yeast $tRNA^{Phe}$. After few rounds of *in vitro* selection a $Pb^{2+}$-dependent self-cleaving ribozyme was isolated that cleaved at any individual site within the RNA molecule.[97] In a similar study, different degrees of mutation were applied to nucleotides involved in the tertiary structure formation of $tRNA^{Phe}$. After six rounds of parallel selection with these pools in presence of lead, a remarkable number of $Pb^{2+}$ self-cleaving ribozymes were obtained with a rate enhancement of 1000-fold over the rate of RNA phosphoester cleavage in the presence of metal alone.[98] Mechanistic studies with one of the self-cleaving ribozymes obtained during this selection, showed a two-step hydrolytic process[100] rather than transesterification, commonly seen in natural ribozymes.

Similarly, a group of $Mg^{2+}$-dependent self-cleaving ribozymes was isolated from a pool of ribonucleotides that contained a 100-random sequence domain.[101] After seven rounds of *in vitro* selection several classes of ribozymes were obtained with a rate enhancement of 1000-fold compared to the rate of RNA cleavage in presence of $Mg^{2+}$ alone. The calculated rate ($3 \times 10^{-3}$ $min^{-1}$) constant for the best ribozyme that was

obtained from this selection experiment, was still 1000-fold slower than that obtained for the hammerhead ribozyme under similar reaction conditions.

As seen, phosphodiester cleavage has been a widely studied reaction when selecting ribozymes artificially. The reason for this lies in the ease of performing the reaction and the general interest to obtain simpler structures that mimic the natural ribozymes. Selecting phosphodiester-cleaving ribozymes involves conceiving a large pool of potentially active molecules and selecting those with a self-modification capability (self-cleavage) (Figure 14). Molecules selected after a repetitive process of selective amplification became highly efficient for cleaving, but in a one to one catalysis that involves a single turnover. In order to function as true catalyst a ribozyme should enhance the rate of a reaction without itself being consumed or modified. This issue has been resolved by separating self-cleaving ribonucleotides into two components, a catalyst and a substrate, that recognize each other by base pairing and that are theoretically capable of multiple cycles of catalysis (multiple turnover). The self-splicing group I intron from *T. thermophila* was the first engineered ribozyme able to perform RNA cleavage under multiple turnover conditions.[144] In addition, many recently discovered ribozymes have been successfully transformed into multiple turnover RNA enzymes providing interesting information about their kinetics and thermodynamics.[105,145-149] One common characteristic evidenced by these engineered ribozymes with multiple turnover is the slow product release (product retained by base pairing) which limits the overall turnover. A solution was sought by introducing mismatches or shortening the ribozyme's recognition strand.[95,150] In this manner, unnatural ribozymes have been added to the list of true catalysts among enzymologists.

*In vitro selected RNA-cleaving DNA enzymes.* At present, no naturally occurring DNA enzymes (also referred as deoxyribozymes, DNAzymes or catalytic DNAs) have been discovered. DNA enzymes are single-stranded DNA molecules with catalytic capabilities discovered by *in vitro* selection from a combinatorial library composed of a population (pool) of random-sequence DNA sequences. The first isolated DNA enzyme was reported by Joyce and Breaker.[108] This catalytic DNA molecule catalyzed the $Pb^{2+}$-dependent cleavage of a single RNA phosphodiester. Beginning with a population of $10^{14}$ different DNA sequences followed by five rounds of selective amplification they were able to obtain an enriched catalytic population of DNA able to carry out as a whole the self-cleavage reaction at a rate of 0.2 $min^{-1}$. The uncatalyzed rate of reaction was about $10^{-4}$ $min^{-1}$, measured in the presence of 1 mM PbOAc and 10 mM $MgCl_2$ at pH 7.0 and 23°C. This corresponded to a rate enhancement of ~$10^3$-fold over the reaction catalyzed by the catalytic DNA enzyme population.

Following the same *in vitro* selection procedure an RNA-cleaving DNA enzyme that depends on $Mg^{2+}$ for its catalytic activity[109] was obtained. In this case the uncatalyzed rate of RNA cleavage is about $10^{-7}$ in presence of $Mg^{2+}$. Six rounds of *in vitro* selection were carried out in presence of 1 mM $MgCl_2$. After six rounds of selection, thirty individual DNA sequences were isolated from the population and found to have very similar sequences. The average catalytic rate of this sequences was found to be 0.002 $min^{-1}$ which represented a rate enhancement of $10^4$-fold over the uncatalyzed reaction. The most active of these catalytic DNAs was mutagenized at a degeneracy of 15% and subjected to seven additional rounds of selective amplification, which led to improve the catalytic activity by 10-fold. Similarly, *in vitro* selection was used to create

additional catalytic DNA pools that require $Zn^{2+}$ and $Mn^{2+}$ for the cleavage of a ribonucleotide phosphoester substrate. Active catalysts were obtained after six rounds of parallel *in vitro* selection with similar cleavage efficiency to the $Mg^{2+}$ dependent DNA enzymes but with lack of specificity on the particular metal used in the selection.

In another attempt to develop novel DNA enzymes, Sen was able to obtain a deoxyribozyme that could catalyze the cleavage of a ribonucleotide phosphoester substrate without the assistance of a divalent metal.[151] After 12 rounds of *in vitro selection*, individual DNA sequences were isolated from the enriched population and found to undergo self-cleavage in the presence of 1 M NaCl with a rate constant of about $10^{-3}$ $min^{-1}$. The reaction rate was not changed by adding divalent-metal-chelating agent or by replacing $Na^{+}$ with a other monovalent cation such as potassium. The rate enhancement for these DNA enzymes was calculated to be ~$10^{7}$-fold faster than the spontaneous rate of cleavage of a ribonucleotide phosphoester in absence of divalent metal ions. Re-selection from a partially randomized DNA pool afforded a second generation DNA enzymes that self-cleaved at a rate 10-fold faster than the first generation DNA enzymes.

With the objective to develop a histidine-dependent DNA enzyme Famulok performed an *in vitro* selection experiment with a mixture of excess histidine and low concentration of $MgCl_2$.[152] After seven rounds of *in vitro* selection, non of the resulting eight classes of deoxyribozymes obtained depend on histidine for the cleavage reaction. They either depended on $Mg^{2+}$ or even accelerated the phosphoester cleavage in absence of metal ion. The dominant sequence had $Mg^{2+}$-dependent, RNA-cleavage activity, but surprisingly was about 10-fold faster in the presence of $Ca^{2+}$.

Breaker was able to select a DNA enzyme that in fact was dependent on L-histidine and no divalent metal.[143] After 11 rounds of *in vitro* selection in absence of divalent metal ions, the enriched DNA population showed RNA-cleavage activity in the presence of histidine. DNA sequences isolated from the population showed four classes of DNA enzymes (4 different sequences). One such individual (designated HD1) was mutagenized and subjected to additional rounds of selection, reducing the concentration of histidine to 5 mM in the selection reaction. After the last round, individual sequences again were isolated from the population, and one of these (designated HD2) showed a rate enhancement of nearly a million-fold over the uncatalyzed substrate cleavage rate. It was suggested that the L-histidine cofactor is loosely bound to the DNA enzyme which positions the imidazole ring to serve as a general base.

In summary, several DNA enzymes have been obtained with catalytic rate enhancement similar to that of natural RNA enzymes. Catalytic cofactors, which generally are divalent metal and one instance histidine, help the DNA in its catalytic task. It was proved that a DNA enzyme can function without the assistance of a catalytic cofactor, as showed by the monovalent ion-dependent DNA enzyme. It seems likely, however, that cofactors or coenzymes will be required to achieve substantial catalytic rate enhancements, especially for other not yet anticipated chemical transformations.

### *Mechanistic Aspects of RNA-Cleaving Ribozymes and Deoxyribozymes*

Four distinct catalytic strategies have been suggested to accelerate the otherwise slow spontaneous rate of RNA cleavage (hydrolysis or transesterification) (Figure 15).[153] These four general catalytic strategies haven been designated as: α catalysis, in line-nucleophilic attack, β catalysis, neutralization of a negative charge of a non-bridging

phosphate oxygen, γ catalysis, deprotonation of a 2'-hydrolxyl group, and δ catalysis, neutralization of a negative charge on the 5'-oxygen atom.

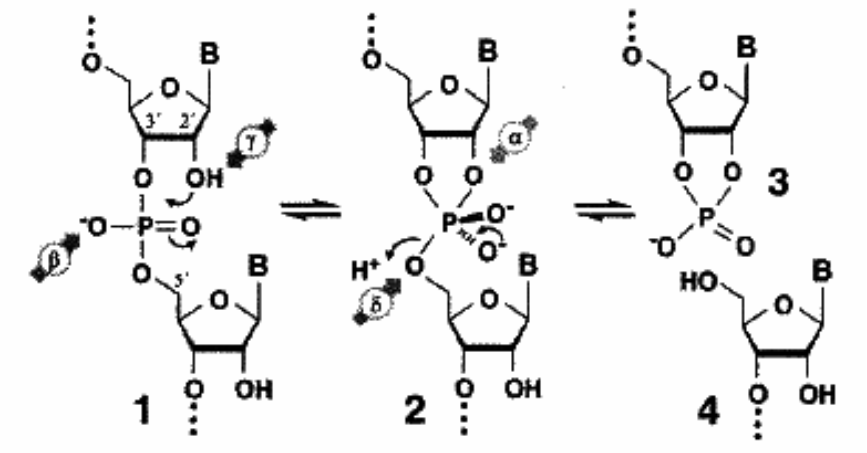



Figure 15. Catalytic strategies for phosphodiester transfer. (Greek lettering and arrow). α catalysis, in line-nucleophilic attack, β catalysis, neutralization of a negative charge of a non-bringing phosphate oxygen, γ catalysis, deprotonation of a 2'-hydrolxyl group, and δ catalysis, neutralization of a negative charge on the 5'-oxygen atom.[153] (Directly reproduced from reference 153).

Breaker has studied and compared a set of RNA-cleaving nucleic acid enzymes based on their maximum observed rate constant ($k_{obs}$) under single turnover conditions.[153] Multiple turnover conditions were excluded on these studies, since potential rate limiting effects would have to be taken into account (substrate association and product release). It was previously found that the rate constant for the uncatalyzed RNA transesterification was $10^{-7}$-$10^{-8}$ $min^{-1}$ under physiological conditions.[154] Natural ribozymes (hammerhead, harirpin, HDV, and others) can reach a maximum rate constant of 1 $min^{-1}$, which represents almost $10^{8}$-fold rate enhancement over the uncatalyzed reaction. Protein phosphoesterases on contrary can improve the rate of the same reaction by $10^{13}$-fold.[155] It was observed in Breaker's study that simple artificial nucleic acid enzymes limit their rate constant when using just one catalytic strategy (γ catalysis) (~2 x $10^{-2}$ $min^{-1}$). This

rate constant was before observed by Li and Breaker when measuring the RNA transesterification upon total deprotonation of the 2'-hydroxyl group at pH 14.5.[154] The histidine-dependent deoxyribozymes, for example, reveals a pH dependent profile that linearly increases the $k_{obs}$ from pH 5 until reaches a maximum at pH 7.4 ($k_{obs}$ = 1.7 $min^{-1}$). This rate constant approximately 100 – 1000-fold faster than that observed under γ catalysis, suggest an additional catalysis strategy to surpass the $10^{-2}$ $min^{-1}$ rate constant limit. It was speculated that histidine-dependent deoxyribozyme could be using and additional α catalysis, in which a 2'-oxyanion nucleophile is favorably positioned for in-line attack on the adjacent phosphorous center. It is important to mention that the first generation histidine ribozyme showed a rate constant of 0.01 $min^{-1}$ (γ catalysis),[142] and after optimization of this enzyme (mutagenesis and additional round of selection under more stringent conditions) the rate constant reached the 1 $min^{-1}$ (α catalysis). It can then be that the additional rounds of selection are responsible for creating this conformational effect in the catalytic site. Therefore, nucleic acid enzymes that use a combination of γ and α catalysis should show a theoretical maximum rate constant in the range of 1 $min^{-1}$. The in-line attacked by the 2'-oxyanion has been demonstrated with the extensive study of small self-cleaving natural ribozymes (hammerhead and hairpin) that show a maximum rate constant of 1 $min^{-1}$. In enzymes in which a non fitting pH profile (base catalysis) is seen, it could be suggested that perhaps other catalytic strategies, particularly combined with cofactor binding and complex folding, might be used by nucleic acid enzymes to match the αγ catalysis.[153]

Other types of catalysis such as the β catalysis were studied by Breaker's team.[153] By replacing the non-bridging oxygen by sulfur at the scissile phosphoester, they could

show that none of the αγ ribozymes (MR2, MR4 and MR11) used in this study was using a cofactor (metal ion) to neutralize the negative charge of the non-bringing phosphate oxygen. Evaluation of a previously found and optimized ribozyme that resembled some structural features of the naturally occurring hammerhead ribozyme (figure 16), designated as the X-motif ribozyme,[156] showed a $k_{obs}$ of 7 $min^{-1}$ using $Mg^{2+}$ as cofactor.

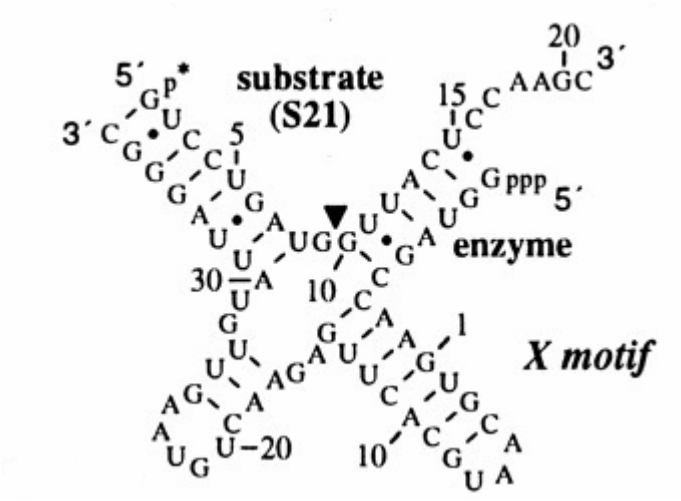



Figure 16. Secondary structure of the X motif ribozyme.[156] (Directly reproduced from reference 156).

This clearly superior maximum rate constant, when compared to the αγ catalysis, suggested that an additional catalytic strategy was being used by the artificial ribozyme. Sulfur replacement studies of the non-bridging oxygen at the targeted phosphoester demonstrated that indeed, X-motif ribozyme was using the β catalysis. Similarly, a largely studied and optimized (by repetitive rounds of selective amplification) DNA enzyme known as a the 10-23 deoxyribozyme (Figure 17)[157] has achieved a maximum catalytic rate (5 $min^{-1}$ in presence of $Mg^{2+}$ cofactor) that exceeds the αγ speed limit. However, the 10-23 deoxyribozyme didn't exhibit a thio effect, suggesting that this

enzyme most likely reaches this catalytic rate without coordination of the non-bridging oxygen.[157]

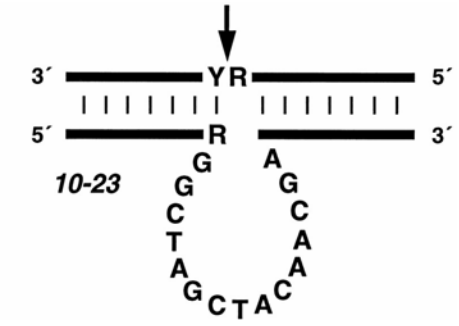



Figure 17. Catalytic domain of the 10-23 deoxyribozyme.[157] (Directly reproduced from reference 157).

| Catalytic strategy combination | | | | Maximum rate enhancement (neutral pH) | Speed limit ($min^{-1}$) |
|---|---|---|---|---|---|
| | No catalysis | | | — | $10^{-8}$ |
| α | | | | $10^{2}$ | $10^{-6}$ |
| | β | | | $10^{5}$ | $10^{-3}$ |
| | | γ | | $10^{6}$ | $10^{-2}$ |
| | | | δ* | $\geq 10^{6}$ | $\geq 10^{-2}$ |
| α | β | | | $10^{7}$ | $10^{-1}$ |
| α | | γ | | $10^{8}$ | $10^{0}$ |
| α | | | δ* | $\geq 10^{8}$ | $\geq 10^{0}$ |
| | β | γ | | $10^{11}$ | $10^{3}$ |
| | β | | δ | $\geq 10^{11}$ | $\geq 10^{3}$ |
| | | γ | δ* | $\geq 10^{12}$ | $\geq 10^{4}$ |
| α | β | γ | | $10^{13}$ | $10^{5}$ |
| α | | γ | δ* | $\geq 10^{14}$ | $\geq 10^{6}$ |
| α | β | | δ | $\geq 10^{13}$ | $\geq 10^{5}$ |
| | β | γ | δ | $\geq 10^{17}$ | $\geq 10^{9}$ |
| α | β | γ | δ | $\geq 10^{19}$ | $\geq 10^{11}$ |

Figure 18. Catalytic strategies and 16 possible combinations for enzymatic mechanism of RNA cleavage. The first entry is the state of no catalysis and is the background rate constant from which the remaining speed limits are calculated (23°C, 250 mM K+). (*) denote those speed limits for δ catalysis without β catalysis that are predicted not to be chemically permitted.[158] (Directly reproduced from reference 158).

It is thus appropriate to say that perhaps at one time ribozymes and deoxyribozymes could reach higher rates (Figure 18) as those observed in protein ribonucleases, which practically combine all four catalytic strategies, by using different cofactors and *in vitro* selection methods that allow copying the catalytic strategies suggested.

### *Coenzymes for Nucleic Acid Enzymes*

It has been presented so far that, even though divalent metal ions play a useful role in nucleic acid catalysis, they are not necessarily essential for such process. Many researchers have envisioned ways to enhance the catalytic efficiency of nucleic acids by using cofactors other than divalent metal ions.

The incorporation of prosthetic groups (imidazoles and cationic amines) onto oligonucleotides and their phophoramidate precursor monomers has been a remarkable way for enhancing the catalytic potential of nucleic acid enzymes.[159-162] Using this approach, Joyce and Barbas were able to select a DNA enzyme that catalyses the cleavage of RNA substrates by incorporating imidazole-modified deoxyuridine monomers in place of thymidines.[163] Even though very low concentrations of the divalent metal ion zinc were still required to give a rate constant of 4 $min^{-1}$, no catalytic enhancement was observed over the unmodified DNA enzyme selected in presence of 2 mM $Mg^{2+}$ or in presence of 100 uM $Zn^{2+}$ in absence of magnesium.[164] In a similar approach, Perrin has exploited the use of two modified nucleotides that deliver both electrostatic complementarity (cationic amines) and general acid/base catalysis at pH 7 (imidazole) as part of his design in order to select a metal-independent DNA enzyme for RNA cleavage.[165] After nine rounds of selection and characterization of clones, a DNA

enzyme was obtained ($9_{25}$-11) with a rate constant of 0.04 $min^{-1}$ that is not affected by the presence of divalent metal ions. Another proposed approach to increasing the functionality of nucleic acids for an enhanced catalysis had been the post-modification of a previously selected unmodified ribozyme.[166,167] However, results from these studies showed a loss of the selected activity.

Vaguely mentioned by some[168,169] but never described in the literature is the potential use of separate cofactor molecules (coenzymes) that the nucleic acid enzymes can use in catalysis. This viability of this approach has been demonstrated by the use of the natural amino acid L-histidine as coenzyme for the HD2 deoxyribozyme.[142] Recently, Soukup has shown, for the first time, that the natural ribozyme, *glmS* ribozyme (Figure 19), uses a coenzyme (glucosamine-6-phosphate, 10 mM) to promote self-cleavage with a rate constant of 1.1 $min^{-1}$ and with an apparent $K_d$ of 0.03 mM.[170]

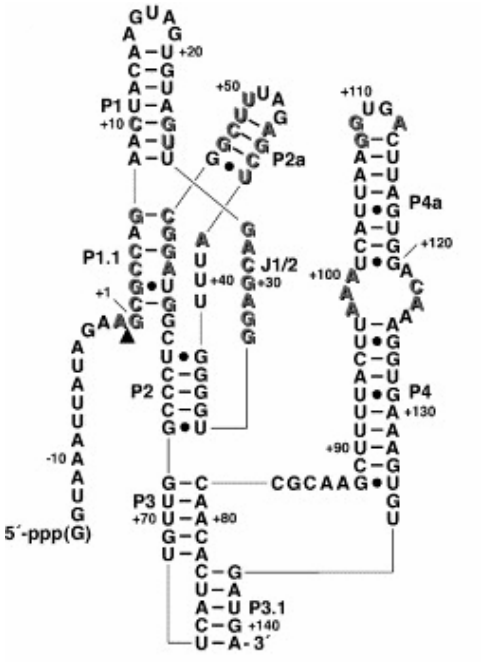



Figure 19. Secondary structure of *glmS* Ribozyme.[171] (Directly reproduced from reference 171).

Although self-splicing group I ribozymes exploit exogenous guanosine as a nucleophilic substrate, catalysis is attributable to intrinsic RNA structure and metal ion cofactors. The *glmS* ribozyme cleavage products possess 5'-hydroxyl and 2',3'-cyclic phosphate termini that do not incorporate glucosamine-6-phosphate (GlcN6P).[172] Study of the reactivity of *glmS* ribozyme cleavage with some GlcN6P analogs (Figure 20) has demonstrated that, rather than allosteric effector, glucosamine-6-phosphate participates directly in catalysis, facilitating the transfer of a single proton upon binding to the ribozyme.

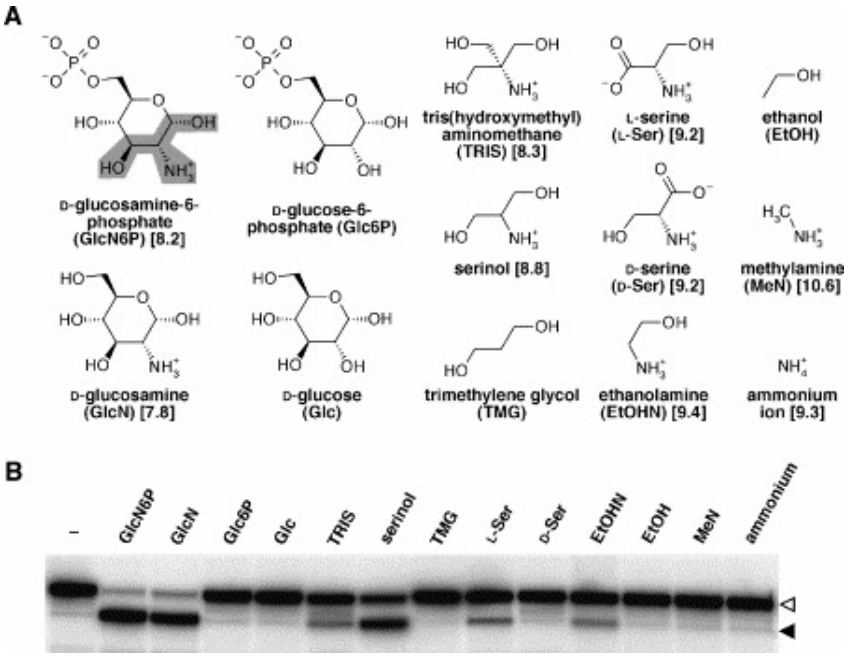



Figure 20. Dependence of *glmS* Ribozyme Self-Cleavage on Amine-Containing Analogs of GlcN6P.[170] (Directly reproduced from reference 170).

As mentioned before, in an allosteric nucleic acid enzyme, the ligand would regulate the catalytic activity of a deoxyribozyme or ribozyme by binding to it at a binding site is that is spatially distinct from that of the catalytic site. In other words, this binding process regulates the folding of the enzyme for a favorable or unfavorable

catalysis. Although a number of allosteric effectors for nucleic acid enzymes have been carefully studied,[173] none of them resembles the *glmS* ribozyme mechanistically. The phosphate functionality of GlcN6P was observed to participate in binding to the *glmS* ribozyme while the amine functionality unexpectedly has no effect on binding.

This recent finding and the robust combinatorial techniques applied to nucleic acids support our research hypothesis that novel coenzymes can be designed and synthesized for a given catalytic task hoping to enhance the catalytic competency of nucleic acid enzymes.

# CHAPTER TWO

## Iminodiacetic Acid-Oligonucleotide Coenzymes for DNA enzymes

### *Introduction*

Even though nucleic acids don't have diverse functional groups like the amino acids in proteins, they are still able to participate in catalysis. Commonly, their functional capacities are improved through the use of divalent or monovalent metal ion cofactors. The activity of all known nucleic acid enzymes (natural and artificially made) is either completely dependent upon or greatly enhanced by these metal ion cofactors. In most cases, the metal ion is thought to either participate directly in catalysis or to play an indirect role by contributing to the structural integrity of the enzyme.[21, 174] A different approach for expanding the repertoire of functional groups in nucleic acid enzymes is using chemically modified nucleotides.[175] The modified nucleotides normally have covalently attached functional groups such as metal chelators, and are integrated in the oligonucleotide using DNA polymerases that tolerate the functionalized nucleotide. Although this strategy has been successful it holds many practical disadvantages. For example, it is complicated to introduce the modified nucleotides site-specifically using a regular polymerase or to incorporate them in vivo experiments.[163]

In the present study, we sought to develop a coenzyme for DNA enzymes that was composed of a small trinucleotide bearing an iminodiacetic acid appendix (5'-IDA-TCC-3') (Figure 21). The trinuclotide element of this potential coenzyme would non-covalently interact with the DNA enzyme in most cases simply by normal base pair interactions. The metal chelator, iminodiacetic acid, was predicted to enhance the

reactivity of the catalytic site by providing good binding site for metal ions. This coenzyme design would be incorporated in an *in vitro* selection experiment aiming to obtain a coenzyme-depended DNA enzyme.

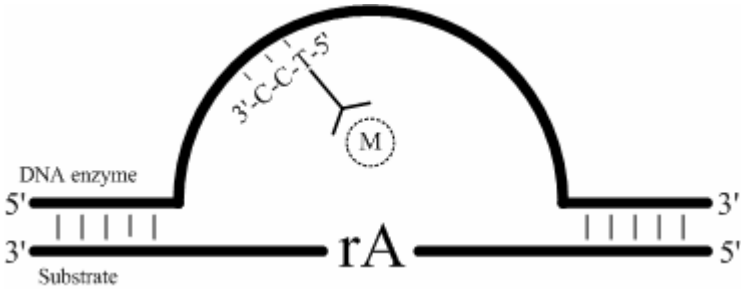



Figure 21. Coenzyme 5'-IDA-TCC-3'design. Coenzyme base paring the DNA enzyme and coordinating a metal ion, (M), metal ion and rA, ribonucleotide moiety in substrate.

To begin this research, extensive preliminary work was done with the goal of understanding the theoretical and practical concepts of the *in vitro* selection process as well as the various techniques involved. Many well-established *in vitro* selection model experiments[108,109] were followed and reproduced using $Pb^{+2}$ and $Zn^{+2}$ as metal ion cofactors. These preliminary selection experiments helped us optimize the polymerase chain reactions and the selection conditions required for a fruitful outcome. The data from these experiments is not shown but was critical in the development of our *in vitro* selection experimentation.

Iminodiacetic acid (IDA) is a known divalent and trivalent metal ion chelator. In a previous work done in Dr. Kane's research laboratory, IDA was incorporated via modified phosphoramidate esters of iminodiacetic acid thymidine into DNA oligonucleotides.[176] We selected 5'-IDA-TCC-3', one of these modified DNA oligonucleotides, for use as a potential coenzyme. This system was incorporated in an *in vitro* selection experiment aiming to obtain a 5'-IDA-TCC-dependent DNA enzyme

system for the cleavage of a ribonucleotide phosphoester substrate. Our rationale is that the oligonucletotide part of the coenzyme (5'-TCC-3') would interact and bind to the DNA enzyme and the iminodiacetic acid would coordinate and position available divalent and trivalent metal ions to the target phosphoester in the catalytic site. By excluding the participation of free metal ions we would be able to prove the participation of our coenzyme system (5'-IDA-TCC-3'). For this, negative selections and careful preparation of a 1:1 mixture of 5'-IDA-TCC-3':Metal ion would be considered.

*In vitro* selection was started with a naïve pool of DNA composed of a core of 40 randomized nucleotides that provided a total of $10^{24}$ different DNA sequences, which was expected to include some potentially catalytic molecules. Since metal ions ($Zn^{+2}$, $Pb^{+2}$, $Ca^{+2}$, $Mg^{+2}$, etc) have a good chance to participate as simple coenzymes,[109] a negative selection –incubation of the DNA strands with metal ion prior to the actual selection reaction– was applied in order to eliminate the possibility of isolating DNA strands that simply use the metal as cofactors. After ten rounds of *in vitro* selection using 5'-IDA-TCC-3' and $Pb^{+2}$ as the potential coenzyme system, an enriched DNA pool was obtained. Individual members of this pool were subcloned, sequenced and assayed for their ability to use the proposed coenzyme for the cleavage of a ribonucleotide phosphate substrate.

Similarly, ten rounds of *in vitro* selection were performed using a reaction buffer containing 5'-IDA-TCC-3' as coenzyme along with the metal ion lutetium ($Lu^{3+}$). Recently, much research has focused on the use of lanthanides as general catalysts for RNA hydrolysis and in the development of artificial nucleases.[177-182] Ample evidence now exists to support the view that the coordination number of lanthanides is greater than six and probably 8, 9 or 10;[183] so that the $Lu^{3+}$-IDA complex would be expected to be still

partially hydrated in solution ($Lu(IDA)(OH_2)_6$).[184] This characteristic is very desirable since many reports suggest the need of hydrated metal ions as true catalysts in some nucleic acid catalysis.[19, 185]

## *Materials and Methods*

### *General Section*

Primers, DNA oligonucleotide template and other oligonucleotides were synthesized by Midland Certified Reagent Co. (Midland, TX) according to our specifications. Sequences for template and primers are shown in Table 2. Biotinylated derivatives of primers were used for immobilization of the DNA onto affinity column. UV quantification of DNA oligonucleotides was accomplished using a GeneQuant Pro spectrophotometer from Amershan Pharmacia, using a 5 mm pathlength 10 μL volume spectrosil cell.

Table 2. Primers and oligonucleotides used in *in vitro* selection.

| Primers | Sequences |
|---|---|
| Template (97 mer): **T** | 5'-CTAATACGACTCACTATAGGAAGAGATGGCGACAT CTC$(N)_{40}$GTGAGGTTGGTGTGGTTG-3' |
| Primer 1 (18 mer): **P1** | 5'-CAACCACACCAACCTCAC-3' |
| Primer B2 (23 mer): **PB2** | 5'-Biotin-GAATTCTAATACGACTCACTATrA-3' |
| Primer 2 (38 mer): **P2** | 5'-GAATTCTAATACGACTCACTATAGGAAGAGATGGCGAC-3' |
| Primer **B1** (18 mer): **PB1** | 5'-Biotin-CAACCACACCAACCTCAC-3' |
| Primer B**B1** (23 mer): **PBB1** | 5'-GAATTCTAATACGACTCACTATrA-3' |
| Substrate (23 mer): **S** | 5'-CGACTCACATATrAGGAAGAGATG-3' |

*Taq* DNA polymerase, dNTPs, 10X PCR Buffer and 15 mM $MgCl_2$ solution were obtained from Promega Corporation. Distilled and sterile water was obtained from Dr.

Christopher Kearney's molecular genetics laboratory. PCR reactions were carried out using a RoboCycler® Thermocycler from Stratagene. DNA samples were centrifuged using an Eppendorf model 5415R refrigerated microcentrifuge.

Streptavidin AffiniTip™ Columns were obtained from Genosys Biotechnologies, Inc. High quality buffer components were obtained from FisherBiotech. pH was adjusted using Corning pH Meter 430 that was calibrated at pHs 4, 7 and 10 with certified buffer solution from Fisher Scientific. Agarose gel electrophoresis was performed using a Minicell® Primo™ Thermo EC320 gel apparatus from Fisher Scientific. Polyacrylamide gel electrophoresis was performed using Bio-Rad Mini-Protean 3 cell electrophoresis system. Southern blots were performed using Mini Trans-Blot electrophoretic transfer cell from Bio-Rad. Electrophoresis was run with a VWR 105 power supply. DNA gel purification was monitored using VWR UV transilluminator model LM-20E. Certified™ low range ultra agarose, Criterion TBE polyacrylamide urea gels (10% and 15%) and 10X TBE Buffer were obtained from Bio-Rad Laboratories. 10X TAE Buffer was obtained through VWR. TBE–urea sample buffer (89 mM Tris·HCl/89 mM boric acid/2 mM EDTA/7 M urea/12% Ficoll/0.01% Bromophenol Blue/0.02% Xylene Cyanol FF) was obtained from Bio-Rad. MassRuler™ DNA Ladder and 6X mass loading dye solution was obtained from Fermentas. Southern Blot was done using Zeta-Probe blotting membrane from Bio-Rad and North2South chemiluminescent detection kit form Pierce Biotechnology, Inc. Gels were photographed using Fluor-S™ Multilmager and Quantity One® quantification software version 4.0 from Bio-Rad. Cloning was done using TA cloning kit from Invitrogen Corpotation. Plasmid extraction was done using GeneElute Plasmid Miniprep kit from Sigma. Thermal cycle

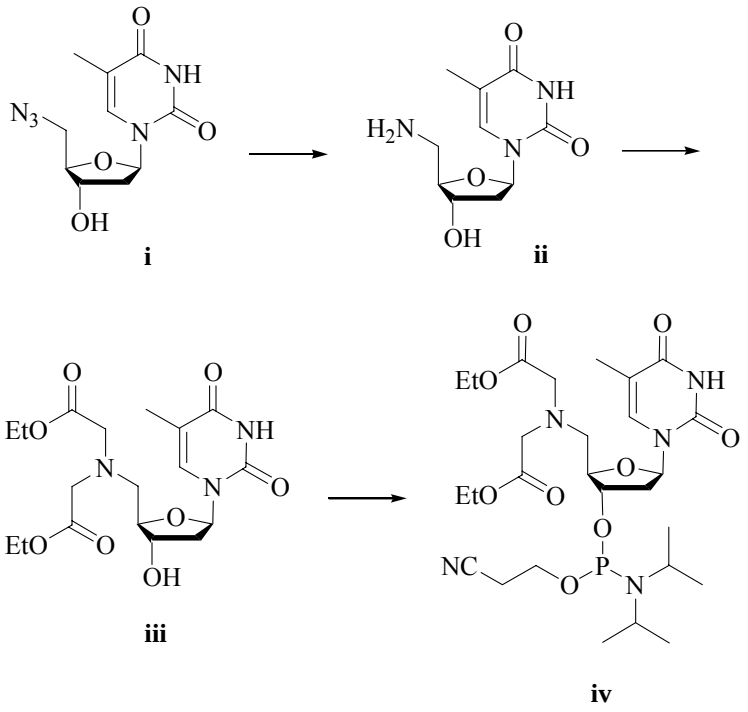
sequencing reaction was done with a RoboCycler® thermocycler from Stratagene and products separated and detected using CEQ™ 8000 Genetic Analysis System running CEQ 2000 software V5.0 from Beckman Coulter.

Radioactive probes (Adenosine 5′-Triphosphate, [$\gamma$-32P] Ultratide™, 6000 Ci/mmol; 222 TBq/mmol) were purchased from ICN Biomedicals. Radiolabeled samples were purified using Mini Quick Spin Oligo Columns from Roche. T4 Polynucleotide Kinase was obtained from Fermentas. Liquid radioactive samples were quantified in Bio-Safe II liquid scintillation cocktail from Research Products International, Inc. using a Beckman Coulter model LS 6500 Multi-Purpose Scintillation Counter. Gels were dried using Gel Dryer FBGD45, a vapor trap and a Maxima® C Plus Vacuum Pump from Fisher Scientific. Gels were photographed using a Fluor-S™ Multlmager, Personal Molecular Imager FX™ System, Kodak Phosphor storage K screen and Quantity One® Quantification Software Version 4.0 from Bio-Rad.

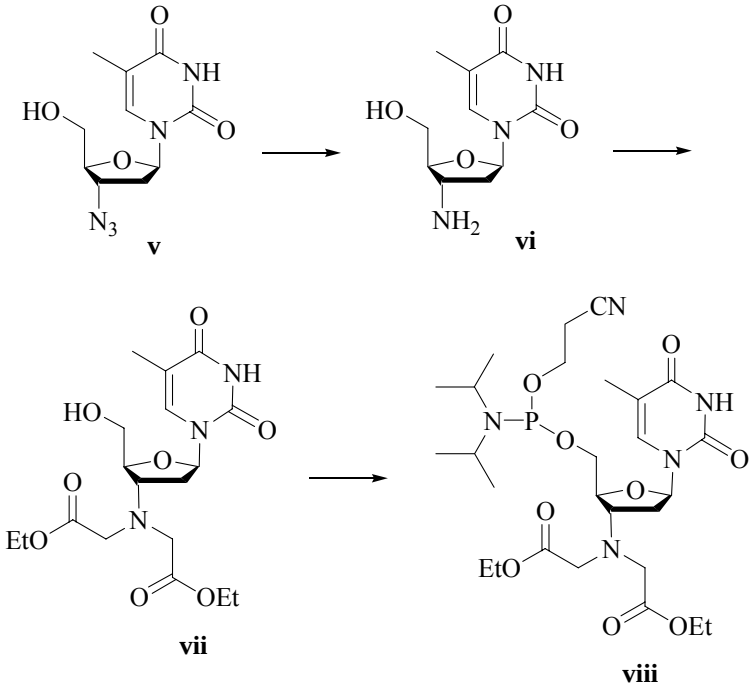
*Synthesis of Potential IDA-Oligonucleotide Coenzymes*

Iminodiacetic acid (IDA) phosphoramidate intermediates were synthesized by Dr. Jonklaas according to Schemes 1 and 2.[176] Potential IDA-oligo coenzymes were synthesized by Midland Certified Reagent Co. (Midland, TX) according to our specifications.

Compound **iv** (5'-IDA-Thy-Phosphoramidate), was incorporated in a normal direction solid phase DNA synthesis in order to afford compounds of the type 5'-IDA-NNN-3' (Midland Certified Reagent Co.).




Scheme 1. Synthetic Scheme for 5'-IDA-Thy-Phosphoramidate (**iv**).[176] (Directly reproduced from reference 176).




Scheme 2. Synthetic Scheme for 3'-IDA-Thy-Phosphoramidate (**viii**).[176] (Directly reproduced from reference 176).

Compound **viii** (3'-IDA-Thy-Phosphoramidate), was incorporated in a reverse orientation solid phase DNA synthesis in order to afford compounds of the type 5-NNN-IDA-3' (Midland Certified Reagent Co.).

For the present research work, compound 5'-IDA-TCC-3' (**OG01**) (Figure 22) was synthesized and purified by Midland Certified Reagent Co. (Lot Number, 072198-338) and introduced in an *in vitro* selection experiment.

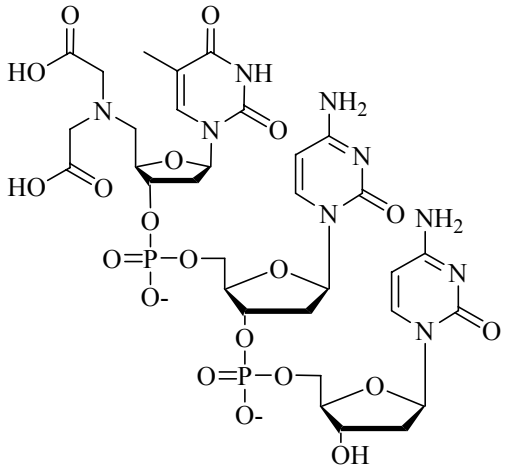


Figure 22. Structure of 5'-IDA-TCC-3' (**OG01**).

*In Vitro Selection Using **OG01** Coenzyme*

*Selection using **OG01**-$Pb^{2+}$ coenzyme.* A DNA template (20 pmol) was initially extended by PCR reaction with primers **1** (32 pmol) and **B2** (32 pmol) in a 200 μL PCR cocktail using four thermocycles of 94 °C (15 s), 50 °C (30 s), 72 °C (30 s). The extended DNA template, containing biotin and ribonucleotide moiety, was then ethanol precipitated at -20 °C for 4 h. The desired 101 bp DNA product was gel purified (1.5% low range ultra agarose) and suspended in 60 μL binding buffer (50 mM HEPES, 0.5 M NaCl, 0.5 M KCl, 0.5 mM, pH 7.5 at rt). The DNA solution was loaded on an AffiniTip column, which had been previously equilibrated with 5 × 100 mL binding buffer. The

column was incubated for a period of 30 min at room temperature, and then the unbound DNA was washed off with 5 × 100 mL binding buffer. The non-biotinylated complementary DNA strands were eluted from the column with 5 × 100 mL freshly prepared 0.2 N NaOH, and the column was immediately neutralized with 5 × 100 mL binding buffer. Column matrix, containing adenosine ribonucleotide incorporated DNA, was incubated in binding buffer for 1 h (negative selection) and then carefully eluted with 10 × 100 mL binding buffer. Reaction was then started by adding 3 × 20 µl of reaction buffer (binding buffer containing 1 mM **OG01** and 1 mM $Pb^{+2}$, pH 7.5 at room temperature) to the column matrix over a period of 1 h. DNA molecules eluted from the column by reaction buffer were ethanol precipitated overnight at -20 °C and then amplified with primers **1** (20 pmol) and **2** (20 pmol) in a 100 µL PCR cocktail with twenty cycles of 92 °C (10 s), 50 °C (30 s), 72 °C (30 s). 10 µL of the PCR product were extended by PCR reaction with primers **1** (80 pmol) and **B2** (80 pmol) in a 200 µL PCR cocktail with six thermocycles of 94 °C (15 s), 50 °C (30 s), 72 °C (30 s). The extended DNA product was ethanol precipitated at -20 °C for 4 h. The desired 101 bp DNA products were gel purified (1.5% low range ultra agarose) and the isolated DNA was used for the next round of selection. In rounds 5 to 10, the stringency of the selection was increased by reducing the time of reaction as follows: round 5 (45 min), round 6 (45 min), round 7 (30 min), round 8 (30 min), round 9 (15 min) and round 10 (15 min). After 10 rounds of selection a first generation DNA pool (G1-OG01-$Pb^{2+}$) was obtained.

*Cloning and sequencing of G1-OG01-$Pb^{2+}$.* 1 µL of 1/10 dilution of the PCR product (amplified with primers **1** and **2**) from the $10^{th}$ round of selection were again PCR amplified with primers **1** (20 pmol) and **2** (20 pmol) in a 50 µL PCR cocktail with ten

cycles of 92 °C (10 s), 50 °C (30 s), 72 °C (30 s). Amplified DNA sequences were subcloned into the vector pCR2.1 (TA cloning kit, Invitrogen). 1 µL of the fresh PCR product was ligated into PCR®2.1 vector using T4 DNA ligase in an overnight reaction at 14 °C. Ligation reaction was then mixed TOP10F competent cells, and the mixture heat shocked for 30 s at 42 °C to allow transformation of individual the vectors into the cells. After 1 h incubation at 37 °C, 10 and 100 µL of the preparation were spread on LB plates that had been previously prepared with ampicillin and IPTG. After the liquid was absorbed the plates were incubated at 37 °C for 18 h. After incubation, 24 colonies showing a pink coloration were picked up and grown overnight at 37 °C in 5 mL of LB medium broth. Plasmid DNA from colonies was isolated and purified using GeneElute Plasmid Miniprep kit from Sigma (see Appendix A). The purified plasmids were quantified using *XhoI* restriction enzyme and then subjected to a PCR reaction in order to generate copies of the insert (see Appendix A). The PCR products were purified by agarose gel electrophoresis, quantified and sequenced using the traditional fluorescent ddNTP sequencing method of Sanger (see Appendix A). The sequencing reaction products were separated and detected on a CEQ 8000 model capillary electrophoresis sequencing system from Beckman by Dr. Robert Adams at the Biology Department.

*Trans-cleavage reaction of G1-OG01-$Pb^{2+}$ individual sequences.* Sequences obtained from G1-OG01-$Pb^{2+}$ were chemically synthesized by Midland Certified Reagents (Midland, TX) according to our specifications (20 nmol scale syntheses and gel filtration grade) and each designated as G1-TX (X being the assigned clone number). Independently, a 5' biotinylated substrate (**S**) containing an embedded ribonucleotide was synthesized by Midland Certified Reagents. Before the reaction was started, 1 µL of 1

μM solution of the biotinylated substrate in 5 mM Tris buffer at pH 7.5 was mixed with 4 μL of 2.5 μM solution of each of the synthesized sequences 5 mM Tris buffer at pH 7.5. The tubes were heated for 30 sec at 92 °C and slowly cooled down to room temperature. The *trans*-cleavage reaction was started by adding 5 μL of 2 mM **OGC01** and 2 mM $Pb^{2+}$ in 2X buffer (50 mM HEPES, 0.5 M NaCl, 0.5 M KCl, pH 7.5 at rt) to each tube. After 4 h, the reaction was stopped with 10 μL of TBE-urea sample buffer. The tubes were then heated to 90 °C for 5 min before loading on a warmed 15% precast polyacrylamide TBE-urea gel that had been prerun for 30 min. Samples were loaded on the gel, run and subjected to a southern blotting. DNA products were transferred to nylon membrane and detected by chemiluminescence with North2South Hybridization and Detection Kit (see Appendix A).

*In Vitro Selection of DNAzymes Using **OG01**-$Lu^{3+}$ Coenzyme*

*Selection using **OG01**- $Lu^{3+}$ coenzyme.* A DNA template (20 pmol) was initially extended by PCR reaction with primers **1** (32 pmol) and **B2** (32 pmol) in a 200 μL PCR cocktail using four thermocycles of 94 °C (15 s), 50 °C (30 s), 72 °C (30 s). The extended DNA template, containing biotin and ribonucleotide moiety, was then ethanol precipitated at -20 °C for 4 h. The desired 101 bp DNA product was gel purified (1.5% low range ultra agarose) and suspended in 60 μL binding buffer (50 mM HEPES, 0.5 M NaCl, 0.5 M KCl, pH 7.5 at rt). The DNA solution was loaded on an AffiniTip column, which had been previously equilibrated with 5 × 100 mL binding buffer. The column was incubated for a period of 30 min at room temperature, and then the unbound DNA was washed off with 5 × 100 mL binding buffer. The non-biotinylated complementary

DNA strands were eluted from the column with 5 × 100 mL freshly prepared 0.2 N NaOH, and the column was immediately neutralized with 5 × 100 mL binding buffer. Column matrix, containing adenosine ribonucleotide incorporated DNA, was incubated in binding buffer containing 0.1 mM $Lu^{3+}$ for 1 h (negative selection) and then carefully eluted with 10 × 100 mL binding buffer. Reaction was then started by adding 3 × 20 µl of reaction buffer (binding buffer containing 1 mM **OG01** and 1 mM $Lu^{3+}$, pH 7.5 at room temperature) to the column matrix over a period of 1 h. DNA molecules eluted from the column by reaction buffer were ethanol precipitated overnight at -20 °C and then amplified with primers **1** (20 pmol) and **2** (20 pmol) in a 100 µL PCR cocktail with twenty cycles of 92 °C (10 s), 50 °C (30 s), 72 °C (30 s). 10 µL of the PCR product were extended by PCR reaction with primers **1** (80 pmol) and **B2** (80 pmol) in a 200 µL PCR cocktail with six thermocycles of 94 °C (15 s), 50 °C (30 s), 72 °C (30 s). The extended DNA product was ethanol precipitated at -20 °C for 4 h. The desired 101 bp DNA products were gel purified (1.5% low range ultra agarose) and the isolated DNA was used for the next round of selection. In rounds 5 to 10, the stringency of the selection was increased by reducing the time of reaction as follows: round 5 (45 min), round 6 (45 min), round 7 (30 min), round 8 (30 min), round 9 (15 min) and round 10 (15 min). After 10 rounds of selection a first generation DNA pool (G1-OG01-$Lu^{3+}$) was obtained.

*Cloning and sequencing of G1-OG01-$Lu^{3+}$.* 1 µL of 1/10 dilution of the PCR product (amplified with primers **1** and **2**) from the 10$^{th}$ round of selection were again PCR amplified with primers **1** (20 pmol) and **2** (20 pmol) in a 50 µL PCR cocktail with ten cycles of 92 °C (10 s), 50 °C (30 s), 72 °C (30 s). Amplified DNA sequences were subcloned into the vector pCR2.1 (TA cloning kit, Invitrogen). 1 µL of the fresh PCR

product was ligated into PCR®2.1 vector using T4 DNA ligase in a overnight reaction at 14 °C. Ligation reaction was then mixed TOP10F competent cells, and the mixture heat shocked for 30 s at 42 °C to allow transformation of individual the vectors into the cells. After 1 h incubation at 37 °C, 10 and 100 μL of the preparation were spread on LB plates that had been previously prepared with ampicillin and IPTG. After the liquid was absorbed the plates were incubated at 37 °C for 18 h. After incubation, 24 colonies showing a pink coloration were picked up and grown overnight at 37 °C in 5 mL of LB medium broth. Plasmid DNA from colonies was isolated and purified using GeneElute Plasmid Miniprep kit from Sigma (see Appendix A). The purified plasmids were quantified using XhoI restriction enzyme and then subjected to a PCR reaction in order to generate copies of the insert (see Appendix A). The PCR products were purified by agarose gel electrophoresis, quantified and sequenced using the traditional fluorescent ddNTP sequencing method of Sanger (see Appendix A). The sequencing reaction products were separated and detected on a CEQ 8000 model capillary electrophoresis sequencing system from Beckman by Dr. Robert Adams at the Biology Department.

*Trans-cleavage reaction of G1-OG01-$Lu^{3+}$ individual sequences.* Sequences obtained from G1-OG01-$Lu^{3+}$ were chemically synthesized by Midland Certified Reagents (Midland, TX) according to our specifications (20 nmol scale syntheses and gel filtration grade) and each designated as G1-TX (X being the assigned clone number). Independently, a substrate (**S**) containing an embedded ribonucleotide was synthesized by Midland Certified Reagents, and 5' labeled with [$\gamma$-32P]ATP in our laboratory (see Appendix A). The radiolabeled substrate was purified by gel filtration using Sephadex G-50 columns and beta emission quantified using liquid scintillation counter (see

Appendix A). Before the reaction was started, 1 μL of 1 μM solution of the biotinylated substrate in 5 mM Tris buffer at pH 7.5 was mixed with 4 μL of 2.5 μM solution of each of the synthesized sequences 5 mM Tris buffer at pH 7.5. The tubes were heated for 30 sec at 92 °C and slowly cooled down to room temperature. The *trans*-cleavage reaction was started by adding 5 μL of 2 mM **OGC01** and 2 mM $Lu^{3+}$ in 2X buffer (50 mM HEPES, 0.5 M NaCl, 0.5 M KCl pH 7.5 at rt) to each tube. After 4 h, the reaction was stopped with 10 μL of TBE-urea sample buffer. The tubes were then heated to 90 °C for 5 min before loading on a warmed 15% precast polyacrylamide TBE-urea gel that had been prerun for 30 min. Samples were loaded on the gel, run and analyzed under standard methods (see Appendix A).

## *Results and Discussions*

### *In vitro Selection using **OG01**-$Pb^{2+}$ coenzyme*

To investigate the participation of **OG01** as a coenzyme for a DNA enzyme, we considered a simple experimental protocol previously established by Breaker and Joyce[108] that consisted of two main steps: 1) several rounds of *in vitro* selection to establish an enriched DNA pool from a random-sequenced pool followed by; 2) the cloning and sequencing of a number of clones to find a catalytic DNA molecule able to perform the proposed reaction dependent on our coenzyme. The *in vitro* selection experiment was started with a DNA template (**T**) molecule that contained 40 random nucleotides equivalent to $4^{40}$ or ~$10^{24}$ potential sequences. It was very important to use a DNA pool with a capacity to yield diverse structures from which at least one or more DNA molecules will posses some catalytic tendency for the reaction proposed (cleavage

of a ribonucleotide phosphate). It has been observed that smaller segments of random nucleotides limit the number of diverse structures and catalytic active molecules, and 50 or 40 random nucleotides have been determined to be an adequate number to start an *in vitro* selection process.[112, 142] PCR extension of 20 pmol of this DNA template (~ $10^{15}$ different DNA molecules) with primers **1** and **B2** afforded a 101 bp DNA product designated as a "naïve pool." Each extended DNA duplex in the naïve pool contained a potential self-cleaving strand and a non-catalytic complementary strand. The self-cleaving strands were composed (from 5' to 3') of a biotin moiety, a fixed sequence of deoxyribonucleotides, an adenosine ribonucleotide, 40 random deoxyribonucleotides and a second fixed sequence of deoxyribonucleotides. Each fixed sequence of deoxyribonucleotides served as a primer recognition site for PCR amplification, and as a base pairing design for DNA enzyme-substrate recognition binding (Figure 23).

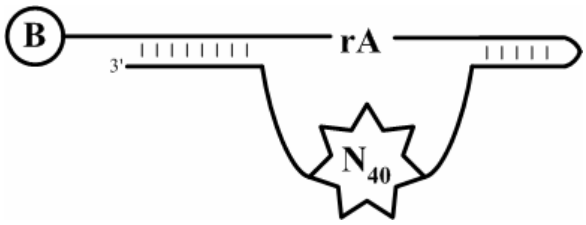



Figure 23. Self-cleavage design of the amplified template (**T**) for *in vitro* selection. (B) biotin; $N_{40}$, random nucleotides; and rA, embedded ribonucleotide cleavage site.

Extended DNA molecules (101 bp) were gel purified, following standard methods, to get rid of undesired PCR products or biotinylated primers. Elimination of this step would reduce the number of random-sequence molecules attached to the streptavidin column because of competition for the binding sites with other biotinylated molecules present in the sample. The purified DNA pool was loaded onto a Streptavidin protein gel column. After 1 h of incubation, unbound DNA molecules were eluted off the

column. The non-biotinylated complementary strands were eluted off the column by alkaline denaturation with 0.2 N NaOH solution. Each column was then rapidly neutralized and equilibrated with buffer (50 mM HEPES, 0.5 M NaCl, 0.5 M KCl, pH 7.5 at rt). The column matrix, containing potential self-cleaving DNA molecules (Figure 24), was then subjected to a negative selection in presence of binding buffer (50 mM HEPES, 0.5 M NaCl, 0.5 M KCl, pH 7.5 at rt). Negative selection would disfavor the isolation of DNA enzymes that use buffer components (HEPES, NaCl, KCl and potential divalent metal ions present) as cofactors. After 1 h of negative selection was performed the column was thoroughly washed and re-equilibrated with binding buffer. The DNA molecules with coenzyme-dependent RNA phosphoester cleavage activity were then eluted at room temperature over a period of 1-h reaction with reaction buffer (binding buffer containing 1 mM of **OG01** and 1 mM $Pb^{2+}$) (Figure 24). We decided to use 1 mM of coenzyme and 1 hour of selection reaction based on previous reports of *in vitro* selection of DNA enzymes in presence of metal ion cofactors,[108] and expecting to observe comparable results.

The eluted molecules were ethanol precipitated and then PCR amplified with primers **1** and **2** to generate an adequate amount of copies of the selected DNA molecules. This PCR amplification was used as a qualitative inspection of the progress of the selection process. The eluate containing DNA molecules cleaved as product of the selection reaction were PCR amplified in parallel to a blank that contained a non-reacted eluate (column incubated with buffer without coenzyme). Presence of the expected size DNA product (101 bp) showed us that some DNA product had been collected as product of the selection reaction possibly favored by the coenzyme. Blank amplification showed

incorrect size amplified products (~50 bp) as a result of a possible primer-primer extension.

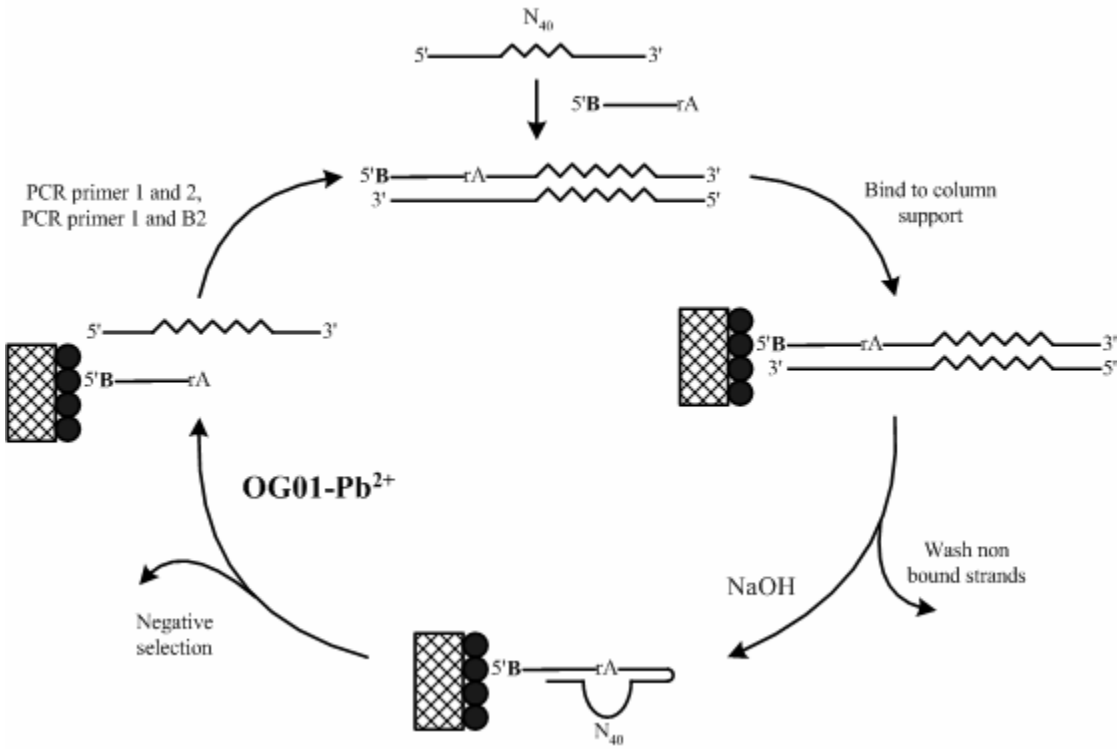



Figure 24. *In vitro* selection using **OG01**-$Pb^{2+}$.

10 μL of the PCR product (with primer 1 and 2) were additionally PCR amplified with primers **1** and **B2** in order to reintroduce the biotin and the adenosine ribonucleotide moieties to the DNA strands. The extended PCR product (101 bp DNA molecules) was gel purified and saved for the next round of selection (Figure 24). Decreasing reaction times for rounds 6-10 were chosen hoping that this selection pressure would have a positive impact of enhancing the catalytic rate of the selected DNA population.

After 10 rounds of selection an enriched DNA pool was expected (G1-OG01-$Pb^{2+}$). An average of 10 rounds of selective amplification had been chosen based on reports that suggest this number of rounds as sufficient to establish an enriched

population of DNA enzymes with consensus sequences.[143] We then decided to clone and sequence a sample of G1-OG01-$Pb^{2+}$ aiming to study the individual DNA molecules present in this pool. Cloning was done in collaboration with Dr. Christopher Kearney at the Molecular Genetics Laboratory at Baylor University. Using TA cloning from Promega and following standard methods 16 clones were obtained. Sequencing of these 16 clones was done in collaboration with Dr. Robert Adams at the Biology Department at Baylor University, and using the standard fluorescent ddNTP Sanger method (see Appendix A). Unexpectedly, all 16 clones shared the same exact sequence (R10) (Table 3). Evaluation of the pools obtained after the 8th and 5th round of selection gave sequences (R8-1, R8-2, R8-3, R5-1 and R5-2) to some extent related to the sequence obtained at the 10th round (Table 3).

Table 3. DNA sequences obtained after *in vitro* selection with **OG01**-$Pb^{2+}$.

| Name | Sequence |
|---|---|
| R10 | 5'-ATCTCGACACTTGGAAATTAGATTAGAGGCTAATGTGCTTCCATAGTGAG-3' |
| R8-1 | 5'-ATCTCGGGTATTCTAGATGATTTAAATTCCCATCAGAGCACCATAGTGAG-3' |
| R8-2 | 5'-ATCTCGGCCGACGCTCGTGACTGGCTTAAACATTATCTCCGAATAGTGAG-3' |
| R8-3 | 5'-ATCTCGCGTCCGGACTGACTATACACACTGAGAAACGCCTAGGTAGTGAG-3' |
| R5-1 | 5'-ATCTCGGAATATGGCGCGATACTCCCTGTCATTACAATATGAGGGGTGAG-3' |
| R5-2 | 5'-ATCTCGGAAACTCGTCAACCCGACATTTTCATGTCCTGATGCTGGGTGAG-3' |

These sequences were incorporated in an intermolecular format reaction (*trans*-cleavage reaction) with a 5' biotinylated DNA substrate embedded with an adenosine ribonucleotide (**S**). *Trans*-acting DNA strand were chemically synthesized by Midland Certified Reagents (Midland, TX). Each DNA strand contained (from 5' – 3') a base pairing or substrate recognition domain, the catalytic moiety (40 nucleotides) and a second substrate recognition domain (Figure 25). The *trans*-reaction was used as a quick

screening to determine which catalytic moiety has the most effective coenzyme-dependent ribonucleotide phosphoester cleavage activity.

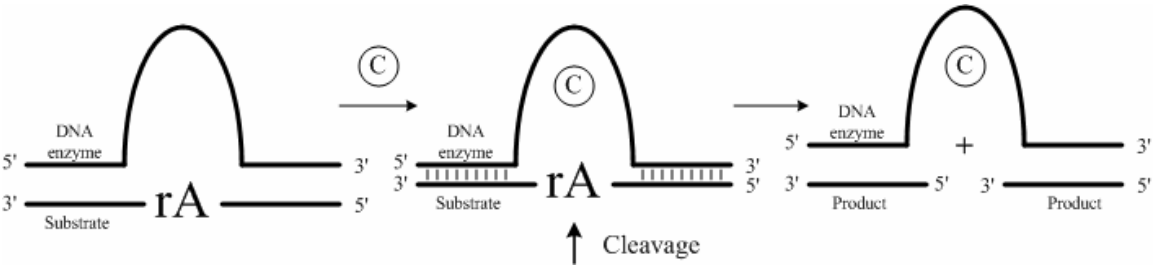



Figure 25. *Trans*-cleavage reaction. Intermolecular reaction of a DNA enzyme and substrate. (C) coenzyme and rA ribonucleotide cleavage site.

The catalytic assay was conducted in presence of 1 mM **OG01** and 1 mM $Pb^{2+}$ in buffer (50 mM HEPES, 0.5 M NaCl, 0.5 M KCl, pH 7.5 at rt) and in a single-turnover fashion: trace amount of substrate oligonucleotide (1 pmol) and excess of DNA catalyst (10 pmol). Reaction products were separated in 10% denaturing polyacrylamide gel, transferred to a nylon membrane (Southern blotting) and detected by chemiluminescence (see Appendix A). After many attempts, we observed that even though this technique is sensitive, the small product (11 bases) was not retained by the nylon membrane (Zeta-Probe blotting membrane, Bio-Rad) and therefore not detected.

*In vitro Selection of DNAzymes using **OG01**-$Lu^{3+}$ coenzyme*

After properly attaching the biotinylated strands of the naïve pool to a Streptavidin affinity column, a negative selection was performed in presence of binding buffer (50 mM HEPES, 0.5 M NaCl, 0.5 M KCl, pH 7.5 at rt) containing 0.1 mM $Lu^{3+}$. Negative selection would disfavor the isolation of metal-dependent deoxyribozymes or

catalytic DNA molecules that use buffer components (HEPES, NaCl, KCl, $Lu^{3+}$ itself and potential divalent metal ions present) as cofactors.

The DNA molecules, with coenzyme-dependent RNA phosphoester cleavage activity, were then eluted at room temperature over a period of 1-h reaction with reaction buffer (binding buffer containing 1 mM of **OG01** and 1 mM $Lu^{3+}$). Very careful attention was put in preparing the reaction buffer. Since we desire **OG01** and $Lu^{3+}$ to work as a complex ($Lu(IDA)(OH_2)_6$),[184] they were carefully measured and mixed in an 1:1 ratio in order to avoid the presence of free $Lu^{3+}$ in the reaction buffer. The eluted molecules were ethanol precipitated and then PCR amplified with primers **1** and **2** to generate an adequate amount of copies of the selected DNA molecules. 10 μL of the PCR product were additionally PCR amplified with primers **1** and **B2** in order to reintroduce the biotin and the adenosine ribonucleotide moieties to the DNA strands. The extended PCR product (101 bp DNA molecules) was gel purified and saved for the next round of selection (Figure *in vitro* selection). Decreasing reaction times for rounds 6-10 were chosen hoping that this selection pressure would have a positive impact of enhancing the catalytic rate of the selected DNA population. After 10 rounds of selection according Figure 26 an enriched DNA pool (G1-OG01-$Lu^{3+}$) was expected.

This DNA pool was prepared in order to separate the self-cleaving strands (Figure 27). The preparation of the G1-OG01-$Lu^{3+}$ for self-cleavage was based on the same process used to bind the self-cleaving DNA to the affinity column for *in vitro* selection.

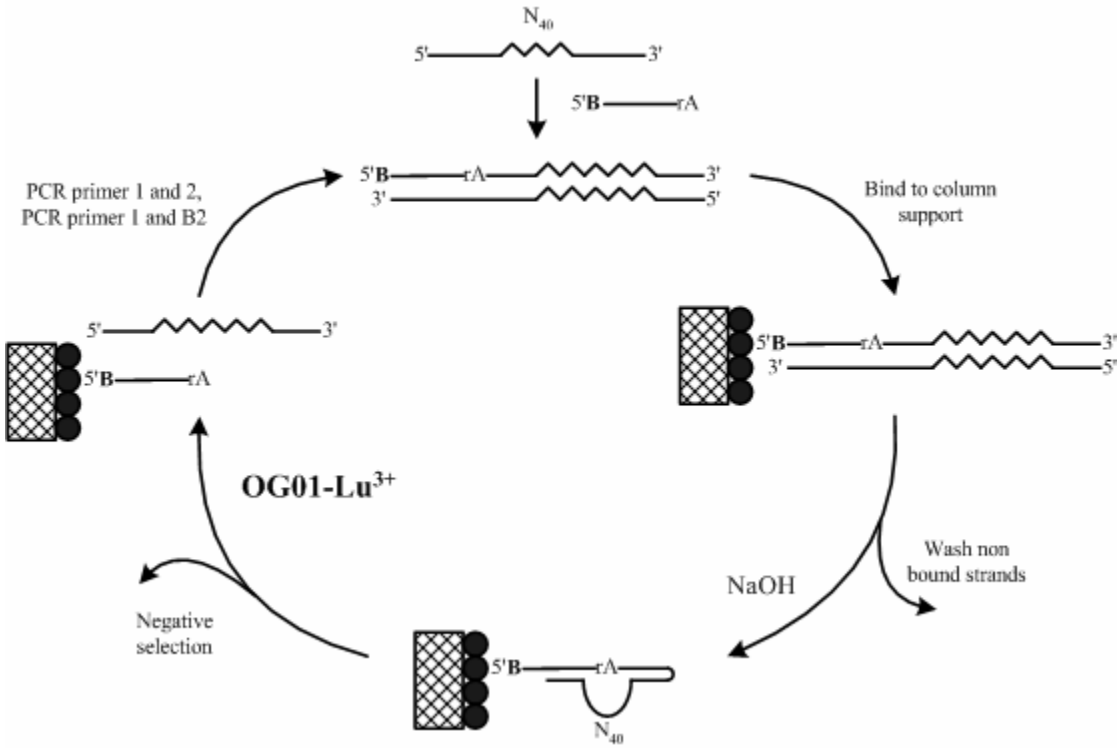



Figure 26. *In vitro* selection using **OG01**-$Lu^{3+}$.

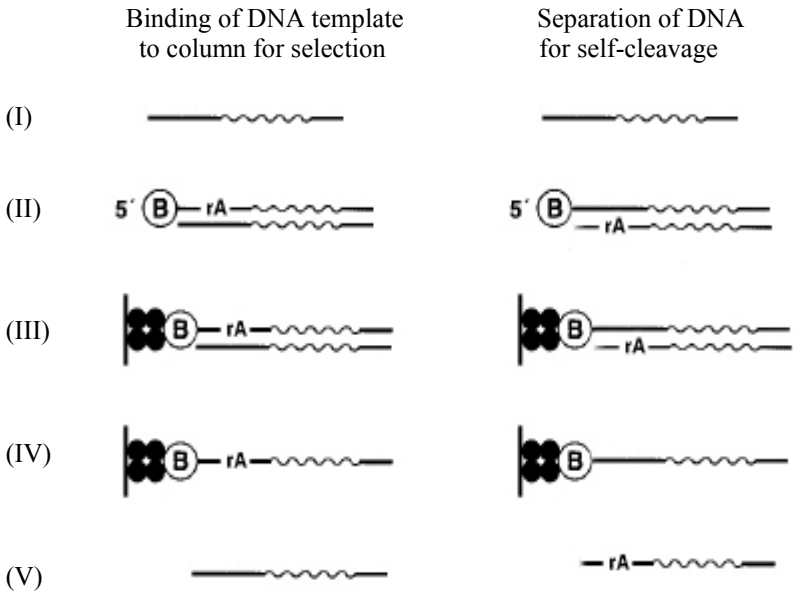



Figure 27. Preparation of DNA for self-cleavage. (I) template; (II) DNA extension with primers 1 and B2 (normal amplification), and primers **B1** and **B1** (inverse amplification); (III) extended DNA binding to neutravidin column; (IV) alkaline denaturation and elution of complementary strand; (V) DNA strand isolation.

DNA pool was PCR extended with primers **B1** and **B1**. PCR reaction with these primers would generate a biotinylated strand and a complementary non-biotinylated self-cleaving strand. After the double-stranded DNA was bound to the affinity column the complementary strand was eluted by alkaline denaturation (0.2 N NaOH). The collected DNA molecules were precipitated, suspended in sterile distilled water, quantified by standard methods and stored at -20 °C.

Before starting the self-cleavage reaction (Figure 28), DNA molecules were heat denatured for 30 sec at 90 °C. It was observed that initial denaturation and slow refolding before interaction with the cofactor or coenzyme improved deoxyribozymes activity.[151] The reaction was then started by adding the coenzyme to a final concentration of 1 mM. After 4 h of reaction at room temperature products were separated in 15% denaturing polyacrylamide gel and detected by standard methods. Many attempts to quantify the self-cleavage efficiency of the selected pool were hidden by the lack of an appropriate detection method. Ethidium bromide staining was inadequate to detect the short single-stranded products (11 nucleotides). Other detection methods (SYBR dyes) didn't give any suiTable results either.

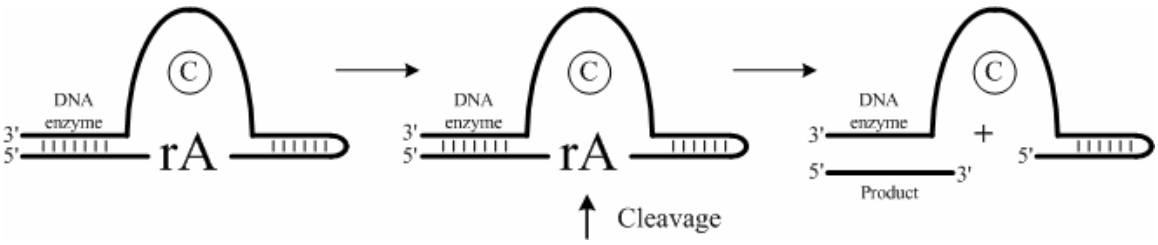



Figure 28. Self-cleavage (*cis*) reaction. Intramolecular reaction of a DNA enzyme embedded with a ribonucleotide phosphoester substrate. (C) coenzyme and rA ribonucleotide cleavage site.

We then decided to clone and sequence a sample of enriched DNA at the 9th and 10th round of selection. Cloning was done in collaboration with Dr. Christopher Kearney at the Molecular Genetics Laboratory at Baylor University. Using TA cloning from Promega and following standard methods 16 clones were obtained. Sequencing of these 16 clones was done in collaboration with Dr. Robert Adams at the Biology Department at Baylor University, and using ddNTPs (see Appendix A).

A group of sequences (4 for 10th round, and 4 for 9th round) with closely related sequences at the catalytic region ($N_{40}$) were obtained (Table 4). Alignment of these sequences using CLUSTALW program showed the very few differences among the sequences (Figure 29).

Table 4. DNAsequences obtained after *in vitro* selection with **OG01**-$Lu^{3+}$.

| Name | 40-bases catalytic segment underlined |
|---|---|
| R10-1 | 5'-ATCTCGTGACGCATTCACTGTTTGTTATAATCCAATTATTATCATGTGAG-3' |
| R10-2 | 5'-ATCTCGTGACGCGATTACTGTTCCTTATAATCCAATTATTATCATGTGAG-3' |
| R10-3 | 5'-ATCTCGTGACGCGATTACTGTTTGTTATAATCCAATTATTATCATGTGAG-3' |
| R10-4 | 5'-ATCTCGTAGCTTGGATAAAGTTTGTTCTGGAAGGGGCATTGTGTCGTGAG-3' |
| R9-1 | 5'-ATCTCTTGACTCCGTTACTGTCCGATCTAAATATCTGACTGTGTTGTGAG-3' |
| R9-2 | 5'-ATCTCGTGACGCCATTACTGTTTGTTATAATCCAATTATTATCATGTGAG-3' |
| R9-3 | 5'-ATCTCGTGACGCATTCACTGTTTGTTATAATCCAATTATTATCATGTGAG-3' |
| R9-4 | 5'-ATCTCTTGACGTCGTAGTGGTTTGTTATAATCCAATTATTATCATGTGAG-3' |

```
#R10-1 ATCTCGTGA CGCATTCACT GTTTGTTATA ATCCAATTAT TATCATGTGA G
#R10-2 ......... ...GA.T... ...CC..... ......... ......... .
#R10-3 ......... ...GA.T... ......... ......... ......... .
#R10-4 .......AG .TTGGAT.AA .......C.G GAAGGGGC.. .G.GTC.... .
#R9-1  .....T... .T.CG.T... ..CC.A.C.. .ATATC.G.C .G.GT..... .
#R9-2  ......... ...CA.T... ......... ......... ......... .
#R9-3  ......... ......... ......... ......... ......... .
#R9-4  .....T... ..TCG.AGTG ......... ......... ......... .
```

Figure 29. Alignment of G1-OG01-$Lu^{3+}$ obtained sequences using CLUSTALW program.

These sequences were incorporated in a *trans*-cleavage reaction with a 5' radiolabeled DNA substrate embedded with an adenosine ribonucleotide (**S**). *Trans*-acting DNA strand were chemically synthesized by Midland Certified Reagents (Midland, TX). Each DNA strand contained (from 5' – 3') a base pairing or substrate recognition domain, the catalytic moiety (40 nucleotides) and a second substrate recognition domain (Figure 23). The substrate **S** was 5' labeled with [$\gamma$-32P]ATP and purified according to standard methods. The *trans*-reaction was used as a quick screening to determine which catalytic moiety has the most effective coenzyme-dependent ribonucleotide phosphoester cleavage activity.

The catalytic assay was conducted in presence of 1 mM **OG01** and 1 mM $Lu^{3+}$ in buffer (50 mM HEPES, 0.5 M NaCl, 0.5 M KCl, pH 7.5 at rt) and in a single-turnover fashion: trace amount of substrate oligonucleotide (0.2 pmol) and excess of DNA catalyst (10 pmol). Reaction products were separated in 15% denaturing polyacrylamide gel and detected by autoradiography (Figure 30).

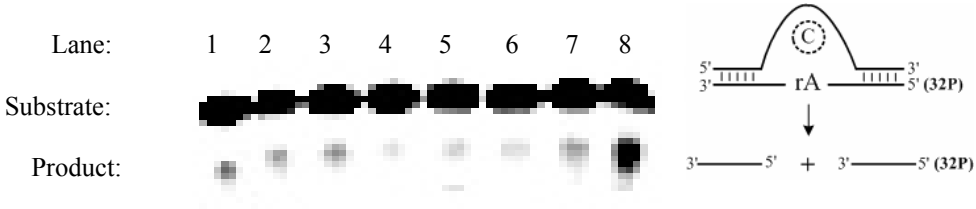



Figure 30**.** *Trans*-cleavage reaction of individual sequences of G1-OG01-$Lu^{3+}$ with **OG01**/$Lu^{3+}$. Lane 1, R9-01; Lane 2, R9-02; Lane 3, R9-03; Lane 4, R9-04; Lane 5, R10-01; Lane 6, R10-02; Lane 7, R10-03; Lane 8, R10-04; 1 mM OGC01 and 1 mM $Lu^{3+}$, 0.25 uM S, 2.5 uM catalyst. 4 h reaction, pH 7.5, rt. 15% TBE-urea PAGE autoradiography.

Sequence R10-4 showed an apparent cleavage product. Judging from the sequences, R10-4 has the most dissimilar compared to the group (R9-01, R9-02, R9-03,

R9-04, R10-01, R10-02, R10-03 and R10-04). A *trans*-cleavage reaction with the group of sequences in presence of $Lu^{3+}$ and not **OG01**, showed a very particular result (Figure 31). R10-4 showed a similar cleavage efficiency compared to the reaction with **OG01** and $Lu^{3+}$. In addition all the other sequences showed variable amounts of products. This may be an indication that the system **OG01**-$Lu^{3+}$ is not required for the catalysis with the selected DNA enzyme and that $Lu^{3+}$ itself is causing the catalytic effect.

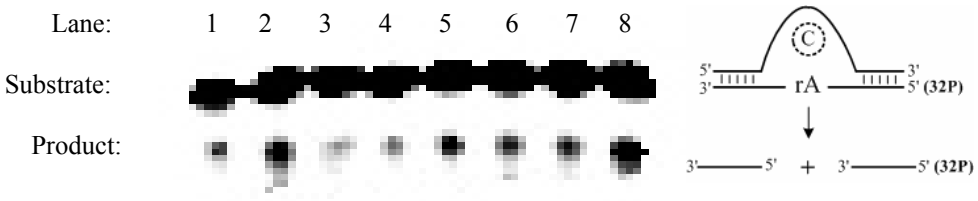



Figure 31**.** *Trans*-cleavage reaction of individual sequences of G1-OG01-$Lu^{3+}$ with $Lu^{3+}$. Lane 1, R9-01; Lane 2, R9-02; Lane 3, R9-03; Lane 4, R9-04; Lane 5, R10-01; Lane 6, R10-02; Lane 7, R10-03; Lane 8, R10-04; 1 mM $Lu^{3+}$, 0.25 μM **S**, 2.5 μM catalyst. 4 h reaction, pH 7.5, rt. 15% TBE-urea PAGE autoradiography.

## Conclusions

The synthetic small molecule **OG01** in combination of metal ions was chosen as candidate coenzyme for the DNA enzyme cleavage of a ribonucleotide phosphoester substrate because of the potential for the trinucleotide moiety to function as non-covalent DNA binder and the metal chelator iminodiacetic acid moiety to function as metal ion provider. After 10 rounds of *in vitro* selection, carried out as suggested by Joyce and Breaker,[108,109] with this potential coenzyme in presence of equivalent amounts of $Pb^{2+}$ resulted in the isolation of an enriched DNA population with individual members all having the same sequence. DNA populations selected under similar procedures in presence of divalent metal ions or the amino acid histidine have previously shown from 4 to 10 unique sequences in samples of 24 clones.[108,109,143,151] On the other hand, very high

sequence similarity has been observed in experiments in which negative selection has been employed.[186] Negative selection as part of the *in vitro* selection experiments can be considered to more rapidly reduce the diversity of the enriched DNA population. Sequence analysis of earlier populations (rounds 5 and 8) showed some diversity among the sequences which is indicative of the selection progress.[142] The detection methods (southern blotting and chemiluminesce) initially used to evaluate the cleavage efficiency of the individual sequences obtained after cloning and sequencing were unsuccessful. Since the design of the self-cleavage or *trans* experiments produced an 11-bases long product, ethidium bromide or southern blotting/chemiluminesce detection methods were insufficient to detect such tiny quantities of the small oligonucleotides. Although the catalytic efficiency of the selected molecules was not determined, the experience gained with this experiment was extremely valuable for the development of this project.

Similarly, **OG01** and equivalent amounts of $Lu^{3+}$ were used in an *in vitro* selection experiment aiming to obtain a ribonucleotide phosphoester-cleaving DNA enzyme. As reported,[184] it was hoped that the iminodiacetic acid of **OG01** would coordinate $Lu^{3+}$ and leave some lutetium coordination bonds, which could potentially coordinate water and form hydroxyl ions necessary for the 2'OH activation in the phosphoester cleavage reaction. This activation event with lanthanides has been previously studied in the cleavage of a ribonucleotide phosphoester bond with a terbium-Lead DNA enzyme system.[187] After 10 rounds of selective amplification alternated with negative selections, four individuals with very high consensus sequence were obtained. Cloning and sequencing analysis of the $9^{th}$ round showed an additional group of individuals with sequences very similar among themselves and with the sequences

obtained in the 10$^{th}$ round. This finding supports our previous suggestion concerning the low diversity found in enriched DNA populations when negative selection is considered. Interesting, sequences found in the selection with **OG01**-$Lu^{3+}$ completely differed to the sequences obtained after rounds of selection with **OG01**-$Pb^{2+}$. This observation resembles the different sequences found when DNA enzymes are selected in presence of different divalent metal ions,[45] and suggests that the trinucleotide coenzyme element of **OG01** didn't create any unique structural feature in the DNA enzyme beyond the simple base pairing. Analysis of the catalytic region ($N_{40}$) of the sequences obtained after 10 rounds of selection with **OG01** and metal ions showed a complementary (5'-GGA-3') trinucleotide sequence (Figure 32) for the unique sequence obtained with **OG01**-$Pb^{2+}$ and for the R10-4 sequence obtained with **OG01**-$Lu^{3+}$.

In order to study the catalytic efficiency of sequences obtained with **OG01**-$Lu^{3+}$ we decided this time to label a ribonucleotide-embedded DNA substrate with a radioactive probe ([$\gamma$-32P]ATP). The time invested in learning this technique was worthwhile as we could now detect cleavage product and could see that R10-04 (selected with **OG01**-$Lu^{3+}$ and having the complementary sequence to **OG01**) had the best apparent cleavage efficiency. The similar cleavage efficiency of this catalytic DNA sequence in presence of $Lu^{3+}$ only (no **OG01**) was very disappointing, since this suggested that free $Lu^{3+}$ was the primary catalyst, and that **OG01** did not participate together with $Lu^{3+}$.

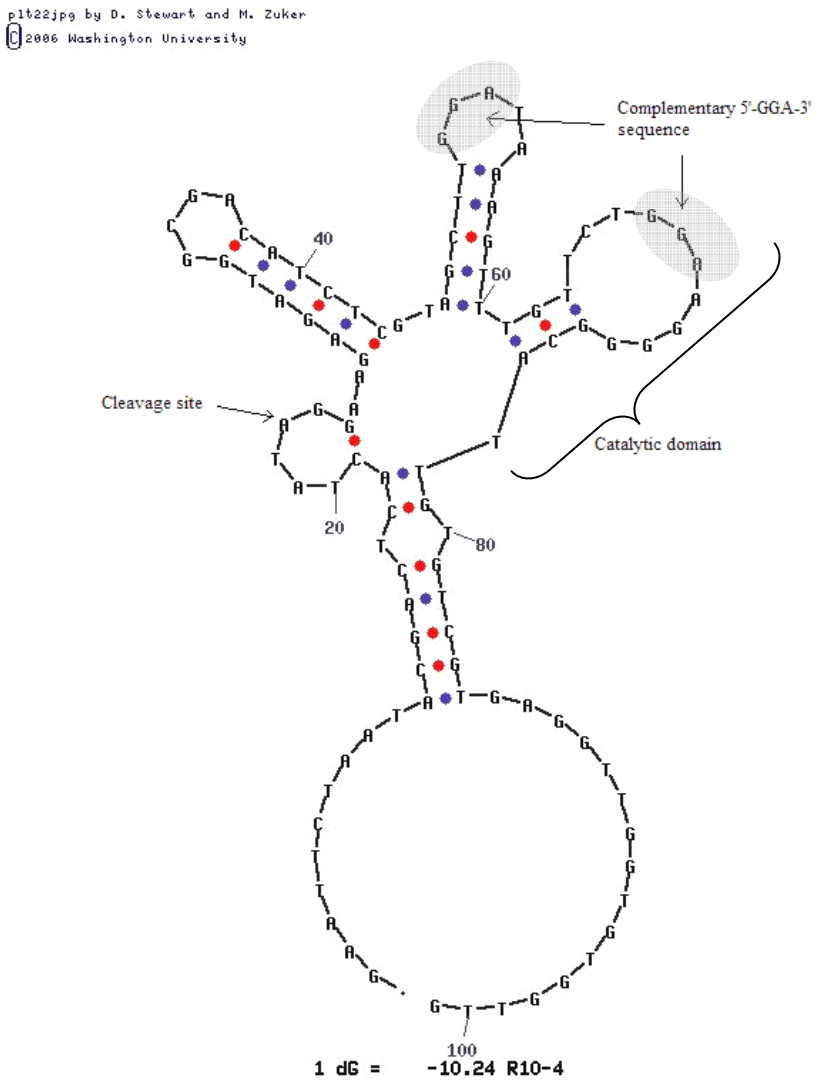



Figure 32. Secondary structure of R10-4. Highlighted is the 5'-GGA-3' sequence complementary to the trinucleotide of **OG01** (5'-IDA-TCC-3'). Cleavage site at position 23.

Those findings and the enormous experience gained (DNA manipulation, *in vitro* selection, single stranded DNA isolation, cloning, sequencing and DNA radiolabeling) drove us to look for simpler coenzyme designs in which the participation of metal ions could be completely excluded.

# CHAPTER THREE

## *Bis*-Histidine Aromatic Coenzymes for DNA enzymes

### *Introduction*

Several approaches to improve the catalytic power of DNA enzymes using different metal ion cofactors have been recently reported.[188] Among those, the use of specific divalent metal ions ($M^{2+}$) as cofactors have been the most studied but not the most satisfactory.[152,168] In order to improve the catalytic power of DNAzymes, several groups have incorporated covalently attached prosthetic groups such as imidazoles, guanidines or cationic amines into DNAzymes.[159-163] Although this strategy has been moderately successful, it faces many limitations such as the use of modified nucleotides that are recognized by polymerase, resulting in the creation of globally modified DNAzymes.[175] In a different approach, the Breaker group successfully selected for a histidine-dependent DNAzyme.[143] This experiment demonstrated that DNAzymes can effectively utilize non-metal cofactors, and suggested that combinatorial selection could be used to identify DNAzymes that utilize these small-molecules.

In order to prove the participation of these synthetic small molecules as coenzyme a model reaction had to be chosen. RNA cleavage has been the most studied reaction *in vitro* selection experiments of DNA enzymes and ribozymes because of the simplicity of the reaction and need for sequence-specific artificial ribonucleases. To design our prospective small molecule coenzymes an extensive literature investigation of artificial ribonucleases was done with the purpose to find a poorly effective and non sequence-specific artificial ribonuclease whose characteristic could be used in our design and

whose performance perchance enhanced. Potential catalytic structures composed of peptides,[189-191] oligoamines[192] or imidazole-based compounds[193-195] equipped with imidazole, carboxyl and/or amino groups that normally constitute the catalytic domains of natural enzymes were conjugated to structures capable of interacting with RNA. As affinity domains intercalating dyes,[193] cationic structures,[194-196] or oligonucleotides[197-201] have been used. Some of the oligonucleotide-based artificial ribonucleases were shown to cleave RNA at target sequences,[202,203] although with poor efficiency. Therefore, further progress in this area may be possible with the use of novel strategies such as the cooperative participation of synthetic ribonucleases with DNA enzymes to ensure effective and specific RNA cleavage.

In an effort to demonstrate the participation of synthetic small molecules as coenzymes for the DNA catalyzed cleavage of a ribonucleotide phosphoester substrate in the absence of catalytic metal ions, we designed and synthesized a group of potential coenzymes each containing two histidines connected via amide bonds to a simple aromatic diacid core (Figure 33). Although each of these compounds contains different aromatic cores, the common imidazole residues have significant potential in the acid/base catalysis of RNA hydrolysis, as can be seen in the protein enzyme ribonuclease A.[204] Imidazole residues have also been used to enhance the hydrolysis of RNA by DNA enzymes, both as a covalently attached prosthetic group[163,205-207] and as a simple coenzyme (histidine).[143] It was hoped that our synthetic design would provide a balance of solubility, binding, and catalytic power so as to serve as efficient coenzymes.

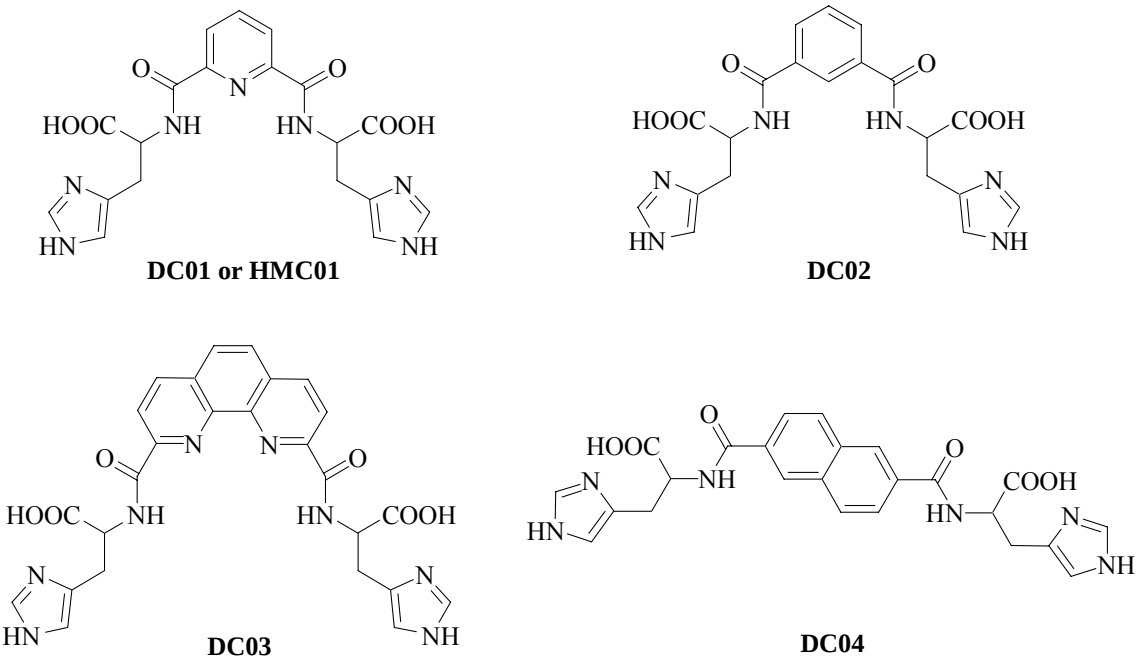



Figure 33. *Bis*-histidine aromatic coenzymes.

A preliminary study of the participation of synthetic small molecules as potential DNA enzyme coenzymes was performed with **HMC01**. This potential coenzyme was incorporated in an *in vitro* selection experiment modeled after those previously reported by Joyce and Breaker.[108, 109] The goal was to obtain a coenzyme-depended DNA enzyme system for the cleavage of a ribonucleotide phosphate substrate. Negative selection, before the actual selection reaction, would disfavor the isolation of metal-dependent deoxyribozymes or catalytic DNA molecules that use buffer components (HEPES, NaCl, KCl, EDTA and potential divalent metal ions present) as cofactors. EDTA, a chelating agent, was included in our buffer in order to minimize the participation of possibly present trace amount of divalent metal ions (Mg, Zn, Pb, Ca, Mn and Cu) during the reaction. After 10 rounds of selection, self-cleavage reactions with the enriched DNA

pool would show whether or not the coenzyme favors the catalysis and at which extent (cleavage efficiency).

Cloning and sequencing of the catalytic DNA pool would provide individual enzyme sequences. Evaluation of these sequences for the cleavage of a ribonucleotide phosphoester in presence of **HMC01**, would suggest the most efficient catalytic DNA molecule. Mutagenesis of this DNA molecule would generate a subset of similar strands (new pool) or progeny. This new pool of DNA strands would then be incorporated in a reselection experiment with more stringent conditions (smaller selection reaction times and concentration) aiming to obtain more efficient coenzyme-DNA enzyme systems. In the case that the re-selected DNA molecules don't exceed the catalytic rate obtained for the first selected pool, a third selection (second reselection) could potentially be performed with even more stringent conditions.

With the experience and information gained with **HMC01**, four potential coenzymes designated as pyridine coenzyme (**DC01**), benzene coenzyme (**DC02**), phenanthrolin coenzyme (**DC03**) and naphthalene coenzyme (**DC04**) were incorporated in parallel *in vitro* selection for ribonuclease activity aiming to rapidly obtain four distinct coenzyme-depended DNA enzyme systems. After 10 rounds of selection, we expected to obtain four families of DNA enzymes that would each specifically use its cognate coenzyme for the accomplishment of the planned reaction. Studies of cross-reactivity, kinetic, pH and binding with each obtained coenzyme-DNA enzymes systems were anticipated to provide us with some insights about their mechanism.

## *Materials and Methods*

### *General Section*

Solvents (ethyl acetate and hexane) were obtained from the Baylor Science Building stockroom and distilled prior to use. Triethylamine obtained from Aldrich was distilled and stored under $N_2$. Amino acids and ester-activated amino acids were obtained from Bachem Co. All other reagents were purchased from Acros Organics, Alfa Aesar and VWR and were used as received. Reactions were monitored by thin layer chromatography (TLC) (silica gel 60 F254) and chromatographic purifications were performed by flash column chromatography with silica gel (230-400 mesh) obtained from EM Science.

A Bruker DPX 300 MHz NMR spectrometer running XWIN-NMR 3.1 was used for $^{1}H$ (300 MHz) and $^{13}C$ NMR (75 MHz). Chemical shifts are expressed in ppm (δ), peaks are listed as singlet (s), doublet (d), triplet (t), or multiplet (m), and coupling constants (*J*) are expressed in Hz. High resolution mass spectrometry was carried out by the Mass Spectrometer Laboratory of University of California, Riverside. Reverse phase HPLC was carried out with a Beckman HPLC system running 32 Karat Software 5.0 with a model 126 solvent module, a model 168 diode array detector and a AquaSep C8 5 μm 100Å Column (ES Industries, Berlin, Germany). Ultrapure water and acetonitrile grade HPLC were obtained from Alfa Aesar. HPLC running buffers A (0.1% TFA in HPLC $H_2O$) and B ($CH_3CN$:$H_2O$ with 0.1% TFA (9:1)). The standard method was 1 mL/min with simple gradient of 5-95% buffer B over 7 min. Sample volume was 10 μl and peaks were detected between 168 and 254 nm.

Primers, DNA oligonucleotide template and other oligonucleotides were synthesized by Midland Certified Reagent Co. (Midland, TX), according to our specifications. Sequences for template and primers are shown in Table 2. Biotinylated derivatives of primers were used for immobilization of the DNA onto affinity column. UV quantification of DNA oligonucleotides was accomplished using a GeneQuant Pro spectrophotometer from Amershan Pharmacia, using a 5 mm pathlength 10 µl volume spectrosil cell.

*Taq* DNA polymerase, dNTPs, 10X PCR Buffer and 15 mM $MgCl_2$ solution were obtained from Promega Corporation. Sterile distilled water was obtained from Dr. Christopher Kearney's molecular genetics laboratory. PCR reactions were carried out using a TC-312 Thermal Cycler from Techne Inc. DNA samples were centrifuged using an Eppendorf model 5415R refrigerated microcentrifuge.

Immobilized NeutrAvidin™ Protein gel and Handee™ Mini-Spin Column Kit were obtained from Pierce Biotechnology, Inc. High quality buffer components were obtained from FisherBiotech. pH was adjusted using Corning pH Meter 430 that was calibrated at pHs 4, 7 and 10 with certified buffer solution from Fisher Scientific. Agarose gel electrophoresis was performed using a Minicell® Primo™ Thermo EC320 gel apparatus from Fisher Scientific. Polyacrylamide gel electrophoresis was performed using Bio-Rad Mini-Protean 3 cell electrophoresis system. Electrophoresis was run with a VWR 105 power supply. DNA gel purification was monitored using VWR UV transilluminator model LM-20E. Certified™ low range ultra agarose, Criterion TBE polyacrylamide urea gels (10% and 15%) and 10X TBE Buffer were obtained from Bio-Rad Laboratories. 10X TAE Buffer was obtained through VWR. TBE–urea sample

buffer (89 mM Tris·HCl/89 mM boric acid/2 mM EDTA/7 M urea/12% Ficoll/0.01% Bromophenol Blue/0.02% Xylene Cyanol FF) was obtained from Bio-Rad. MassRuler™ DNA Ladder and 6X mass loading dye solution was obtained from Fermentas. Cloning was done using TA cloning kit from Invitrogen Corpotation. Plasmid extraction was done using GeneElute Plasmid Miniprep kit from Sigma. Thermal cycle sequencing reaction was done with a RoboCycler® thermocycler from Stratagene and products separated and detected using CEQ™ 8000 Genetic Analysis System running CEQ 2000 software V5.0 from Beckman Coulter. Cloning and sequencing experiments were also performed by Molecular Cloning Laboratories (MCLAB) in San Francisco, CA, and by Fisher's SeqWrite facility in Houston, TX, according to our specifications.

The radioactive probe (Adenosine 5′-Triphosphate, [$\gamma$-32P] Ultratide™, 6000 Ci/mmol; 222 TBq/mmol) was purchased from ICN Biomedicals. Radiolabeled samples were purified using Mini Quick Spin Oligo Columns from Roche. T4 Polynucleotide Kinase was obtained from Fermentas. Liquid radioactive samples were quantified in Bio-Safe II liquid scintillation cocktail from Research Products International, Inc. using a Beckman Coulter model LS 6500 Multi-Purpose Scintillation Counter. Gels were dried using Gel Dryer FBGD45, vapor trap and Maxima® C Plus Vacuum Pump from Fisher Scientific. Gels were photographed using a Fluor-S™ Multlmager, Personal Molecular Imager FX™ System, Kodak Phosphor storage K screen and Quantity One® Quantification Software Version 4.0 from Bio-Rad.

*Synthetic Procedures*

Bis-histidine pyridine coenzyme (**HMC01**) (Figure 33) was initially synthesized and provided by Dr. Hui Ming Chang, postdoctoral fellow in Dr. Robert R. Kane's

Research Laboratory in the Chemistry and Biochemistry Department of Baylor University (Waco, TX). A set of new bis-histidine aromatic coenzymes containing pyridine, benzene, phenanthrolin and naphthalene core (**DC01**, **DC02**, **DC03** and **DC04**) were synthesized under standard solution phase peptide synthesis using BOP coupling reagent. The final products were obtained by standard deprotection methods under acidic conditions. The purity of each coenzyme was confirmed by spectroscopic analysis and full spectrum HPLC analysis.

*2-({6-[1-Carboxy-2-(1H-imidazol-4-yl)-ethylcarbamoyl]-pyridine-2-carbonyl}-amino)-3-(1H-imidazol-4-yl)-propionic acid* (***DC01)***. 2,6-pyridinedicarboxylic acid (1 equivalent, 0.5 mmol, 0.083 g) and the BOP reagent (2 equivalents, 1 mmol, 0.444 g) were added to 12 mL of anhydrous DMF at 0 °C. The solution was placed under dry $N_2$ and stirred for 10 min. H-His(1-Trt)-OMe·HCl (2 equivalents, 1 mmol, 0.447 g) and triethylamine (4 equivalents, 2 mmol, 0.28 mL) were then added, and the reaction was allowed to continue stirring for 24 h at room temperature. The reaction solution was then poured onto 200 mL of ice water to give a white suspension. The solid was recovered by vacuum filtration, washed with large amounts of cold water (200 mL) and dried under vacuum. The crude product was separated by flash chromatography (silica gel, 50:50 hexanes:ethyl acetate) to afford the protected **DC01** (0.247 g, 0.26 mmol, 52%). The acid labile protecting groups (Trt and methyl ester) were then removed under reflux in 2 mL 1N HCl:Acetone solution (l:1.5) for 24 h. After the reaction was cooled down, acetone was removed under vacuum, and the resulting suspension was filtered. The collected solution was evaporated to dryness *in vacuo* to afford **DC01** (0.103 g, 0.234 mmol, 90%) (Figure 34) as a white solid: $^1$H NMR (300 MHz, $D_2O$) $\delta$ ppm 8.41 (s, 2H),

8.02 (m, 3H), 7.14 (s, 2H), 4.92 (dd, $J$ = 9.4, 4.9 Hz, 2H), 3.38 (dd, $J$ = 15.5, 4.9 Hz, 2H), 3.20 (dd, $J$ = 15.5, 9.4 Hz, 2H); $^{13}$C NMR (75 MHz, $D_2O$) $\delta$ ppm 172.49, 168.72, 146.38, 146.19, 133.94, 127.38, 127.21, 117.65, 53.55, 25.74; $^{+}$ev-FAB-HRMS *m/z* 442.1475 [M + H]$^{+}$ ($C_{19}H_{20}N_7O_6$, calcd 442.1475, $\Delta$ = 0 ppm); HPLC (standard method) $t_R$ = 5.56 min, purity >93%

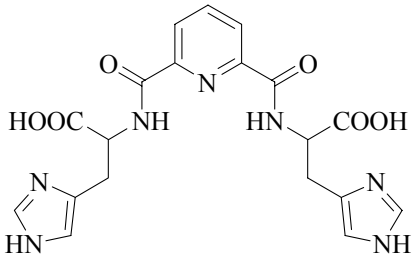



Figure 34. Structure of **DC01**.

*2-{3-[1-Carboxy-2-(1H-imidazol-4-yl)-ethylcarbamoyl]-benzoylamino}-3-(1H-imidazol-4-yl)-propionic acid (**DC02**)*. Isophthalic acid (1 equivalent, 0.5 mmol, 0.083 g) and the BOP reagent (2 equivalents, 1 mmol, 0.444 g) were added to 12 mL of anhydrous DMF at 0 °C. The solution was placed under dry $N_2$ and stirred for 10 min. H-His(1-Trt)-OMe·HCl (2 equivalents, 1 mmol, 0.447 g) and triethylamine (4 equivalents, 2 mmol, 0.28 mL) were then added, and the reaction was allowed to continue stirring for 24 h at room temperature. The reaction solution was then poured onto 200 mL of ice water to give a white suspension. The solid was recovered by vacuum filtration, washed with large amounts of cold water (200 mL) and dried under vacuum. The crude product was separated by flash chromatography (silica gel, 50:50 hexanes:ethyl acetate) to afford the protected **DC02** (0.318 g, 0.33 mmol, 67%). The acid labile protecting groups (Trt and methyl ester) were then removed under reflux in 2 mL 1N HCl:Acetone solution (l:1.5)

for 24 h. After the reaction was cooled down, acetone was removed under vacuum, and the suspension obtained was filtered. The collected solution was evaporated to dryness *in vacuo* to afford **DC02** (0.124 g, 0.28 mmol, 86%) (Figure 35) as a white solid: $^{1}$H NMR (300 MHz, $D_2O$) $\delta$ ppm 8.46 (s, 2H), 7.88 (t, $J$ = 1.8 Hz, 1H), 7.74 (dd, $J$ = 7.9, 1.8 Hz, 2H), 7.46 (t, $J$ = 7.9 Hz, 1H), 7.18 (s, 2H), 4.81 (dd, $J$ = 9.1, 5.2 Hz, 2H), 3.34 (dd, $J$ = 15.2, 5.3 Hz, 2H), 3.17 (dd, $J$ = 15.3, 9.1 Hz, 2H); $^{13}$C NMR (75 MHz, $D_2O$) $\delta$ ppm 165.08, 161.00, 125.18, 124.89, 122.59, 121.08, 120.58, 117.95, 108.92, 44.11, 17.84; $^{+}$ev-FAB-HRMS *m/z* 441.1522 $[M + H]^{+}$ ($C_{20}H_{21}N_6O_6$, calcd 441.1523, $\Delta$ = 0.2 ppm); HPLC (standard method) $t_R$ = 5.25 min, purity >95%.

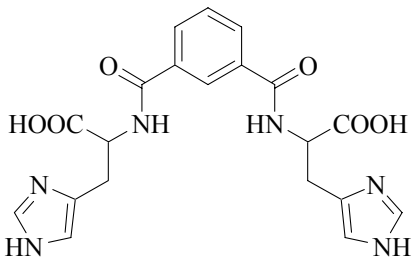



Figure 35. Structure of **DC02**.

*2-({9-[1-Carboxy-2-(1H-imidazol-4-yl)-ethylcarbamoyl]-[1,10]phenanthroline-2-carbonyl}-amino)-3-(1H-imidazol-4-yl)-propionic acid (**DC03**)*. 2,9-[1,10]phenanthrolinedicarboxylic acid was previously synthesized in our lab from 2,9-Dimethyl-1,10-phenanthroline (Acros) by $SeO_2$ oxidation followed by 80% $HNO_3$ reflux[1]. 2,9-[1,10]phenanthrolinedicarboxylic acid (1 equivalent, 0.5 mmol, 0.134 g) and the BOP reagent (2 equivalents, 1 mmol, 0.444 g) were added to 12 mL of anhydrous DMF at 0 °C. The solution was placed under dry $N_2$ and stirred for 10 min. H-His(1-Trt)-OMe·HCl (2 equivalents, 1 mmol, 0.447 g) and triethylamine (4 equivalents, 2

mmol, 0.28 mL) were then added, and the reaction was allowed to continue stirring for 24 h at room temperature. The reaction solution was then poured onto 200 mL of ice water to give a light brown suspension. The solid was recovered by vacuum filtration, washed with large amounts of cold water (200 mL) and dried under vacuum. The crude product was separated by flash chromatography (silica gel, 50:50 hexanes:ethyl acetate) to afford the protected **DC03** (0.189 g, 0.18 mmol, 35%). The acid labile protecting groups (Trt and methyl ester) were then removed under reflux in 2 mL 1N HCl:Acetone solution (l:1.5) for 24 h. After the reaction was cooled down, acetone was removed under vacuum, and the suspension obtained was filtered. The collected solution was evaporated to dryness *in vacuo* to afford **DC03** (0.081 g, 0.15 mmol, 88%) (Figure 36) as a light yellow solid: $^{1}$H NMR (300 MHz, $D_2O$) $\delta$ ppm 8.46 (s, 2H), 8.01 (d, $J$ = 8.5 Hz, 2H), 7.81 (d, $J$ = 8.5 Hz, 2H), 7.35 (s, 2H), 7.23 (s, 2H), 4.92 (dd, $J$ = 6.0, 7.9 Hz, 2H), 3.44 (dd, $J$ = 15, 6.0 Hz, 2H), 3.28 (dd, $J$ = 15, 7.9 Hz, 2H); $^{13}$C NMR (75 MHz, $D_2O$) $\delta$ ppm 173.54, 165.99, 147.76, 142.62, 138.64, 133.41, 130.37, 129.01, 127.80, 121.40, 117.15, 52.52, 26.57; $^{+}$ev-FAB-HRMS *m/z* 543.1740 $[M + H]^+$ ($C_{26}H_{23}N_8O_6$, calcd 543.1741, $\Delta$ = 0.2 ppm); HPLC (standard method) $t_R$ = 6.11 min, purity >99%.

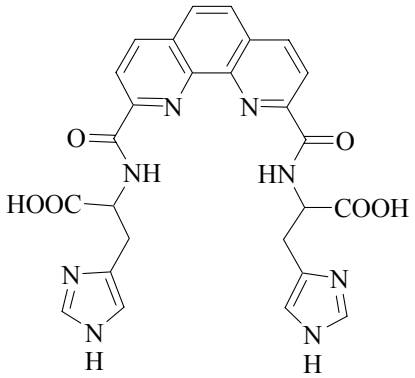



Figure 36. Structure of **DC03**.

*2-({6-[1-Carboxy-2-(1H-imidazol-4-yl)-ethylcarbamoyl]-naphthalene-2-carbonyl}-amino)-3-(1H-imidazol-4-yl)-propionic acid (**DC04**)*. 2,6-naphthalenedicarboxylic acid (1 equivalent, 0.5 mmol, 0.108 g) and the BOP reagent (2 equivalents, 1 mmol, 0.444 g) were added to 12 mL of anhydrous DMF at 0 °C. The solution was placed under dry $N_2$ and stirred for 10 min. H-His(1-Trt)-OMe·HCl (2 equivalents, 1 mmol, 0.447 g) and triethylamine (4 equivalents, 2 mmol, 0.28 mL) were then added, and the reaction was allowed to continue stirring for 24 h at room temperature. The reaction solution was then poured onto 200 mL of ice water to give a light brown suspension. The solid was recovered by vacuum filtration, washed with large amounts of cold water (200 mL) and dried under vacuum. The crude product was separated by flash chromatography (silica gel, 50:50 hexanes:ethyl acetate) to afford the protected **DC04** (0.305 g, 0.30 mmol, 61%). The acid labile protecting groups (Trt and methyl ester) were then removed under reflux in 2 mL 1N HCl:Acetone solution (l:1.5) for 24 h. After the reaction was cooled down, acetone was removed under vacuum, and the suspension obtained was filtered. The collected solution was evaporated to dryness *in vacuo* to afford **DC04** (0.130 g, 0.26 mmol, 89%) (Figure 37) as a light yellow solid: $^{1}$H NMR (300 MHz, $D_2O$) $\delta$ ppm 8.43 (s, 2H), 7.95 (s, 2H), 7.71 (d, $J$ = 8.5 Hz, 2H), 7.49 (d, $J$ = 8.5 Hz, 2H), 7.15 (s, 2H), 4.80 (dd, $J$ = 9.0, 5.2 Hz, 2H), 3.31 (dd, $J$ = 15.5, 5.2 Hz, 2H), 3.15 (dd, $J$ = 15.5, 9.0 Hz, 2H); $^{13}$C NMR (75 MHz, $D_2O$) $\delta$ ppm 173.56, 169.79, 133.55, 133.37, 131.69, 129.57, 128.94, 127.59, 124.16, 117.10, 52.45, 26.24; $^{+}$ev-FAB-HRMS *m/z* 491.1679 [$MH^+$] ($C_{24}H_{23}N_6O_6$, calcd 491.1679, $\Delta$ = 0 ppm); HPLC (standard method) $t_R$ = 5.76 min, purity >99%.

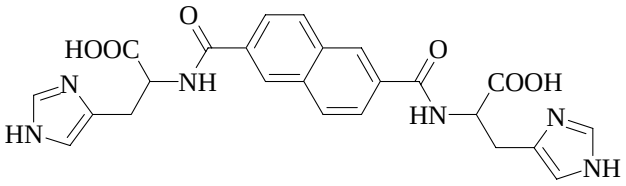



Figure 37. Structure of **DC04**.

*In vitro Selection of Catalytic DNA Strands that Use Bis-Histidine Pyridine Coenzyme (**HMC01**)*

*Selection using **HMC01***. A DNA template (20 pmol) was initially amplified by PCR reaction with primers **1** (32 pmol) and **B2** (32 pmol) in a 200 µL PCR cocktail using four thermocycles of 94 °C (15 s), 50 °C (30 s), 72 °C (30 s). The amplified DNA template, containing biotin and ribonucleotide moiety, was then ethanol precipitated at -20 °C for 4 h. The desired 101 bp DNA product was gel purified (1.5% low range ultra agarose) and suspended in 60 µL binding buffer (50 mM HEPES, 0.5 M NaCl, 0.5 M KCl, 0.5 mM EDTA, pH 7.5 at rt). The resulting DNA solution was loaded on a neutravidin column, which had been previously equilibrated with 5 × 100 mL binding buffer. The column was incubated for a period of 30 min at room temperature, and then the unbound DNA was washed off with 5 × 100 mL binding buffer. The non-biotinylated complementary DNA strands were eluted from the column with 5 × 100 mL freshly prepared 0.2 N NaOH, and the column was immediately neutralized with 5 × 100 mL binding buffer. Column matrix, containing adenosine ribonucleotide incorporated DNA, was incubated in binding buffer for 1 h (negative selection) and then carefully eluted with 10 × 100 mL binding buffer. Reaction was then started by adding 3 × 20 µl of reaction buffer (binding buffer containing 50 mM coenzyme, pH 7.5 at room temperature) to the column matrix over a period of 1 h. DNA molecules eluted from the

column by reaction buffer were ethanol precipitated overnight at -20 °C and then amplified with primers **1** (20 pmol) and **2** (20 pmol) in a 100 μL PCR cocktail with twenty cycles of 92 °C (10 s), 50 °C (30 s), 72 °C (30 s). 10 μL of the PCR product were amplified by PCR reaction with primers **1** (80 pmol) and **B2** (80 pmol) in a 200 μL PCR cocktail with six thermocycles of 94 °C (15 s), 50 °C (30 s), 72 °C (30 s). The amplified DNA product was ethanol precipitated at -20 °C for 4 h. The desired 101 bp DNA products were gel purified (1.5% low range ultra agarose) and the isolated DNA was used for the next round of selection. In rounds 5 to 10, the stringency of the selection was increased by reducing the time of reaction as follows: round 5 (45 min), round 6 (45 min), round 7 (30 min), round 8 (30 min), round 9 (15 min) and round 10 (15 min). After 10 rounds of selection a first generation DNA pool (G1-HMC01) was obtained.

*Self-cleavage reaction of G1-HMC01*. 10 μL of PCR product (amplified with primers **1** and **2**) from the $10^{th}$, $9^{th}$ and $8^{th}$ round of selection were extend by PCR reaction with primers **B1** (80 pmol) and **B2rA** (80 pmol) in a 200 μL PCR cocktail with six thermocycles of 94 °C (15 s), 50 °C (30 s), 72 °C (30 s). The amplified DNA products were ethanol precipitated at -20 °C for 4 h. The desired 101 bp DNA products were gel purified (1.5% low range ultra agarose) and suspended in 60 μL of binding buffer (50 mM HEPES, 0.5 M NaCl, 0.5 M KCl, 0.5 mM EDTA, pH 7.5 at rt). Each DNA solution was loaded on a separate neutravidin column, which had been previously equilibrated with 5 × 100 mL of binding buffer. Columns containing DNA solution were incubated for 30 min at room temperature, and then the unbound DNA was washed off with 5 × 100 mL of binding buffer. The non-biotinylated complementary DNA strands were eluted from each column with 3 × 100 mL of freshly prepared 0.2 N NaOH, quickly

mixed with 60 μL of 3M sodium acetate pH 5.2 and ethanol precipitated overnight at -20 °C. Each precipitated DNA pool was suspended in 20 μL of sterile distilled water and 5' labeled with [$\gamma$-32P]ATP and T4 polynucleotide kinase (see Appendix A). Radiolabeled DNA pools were purified using Sephadex G-50 columns, beta emission quantified using liquid scintillation counter (see Appendix A) and each diluted in sterile distilled water to the same number counts per milliliter (cpm/ml).

Each radiolabeled DNA pool was subjected to a self-cleavage reaction in presence of 50 mM solution of its cognate coenzyme in buffer (50 mM HEPES, 0.5 M NaCl, 0.5 M KCl, 0.5 mM EDTA, pH 7.5 at rt). For each round DNA pool, 1 μL of radiolabeled DNA pool and 4 μL of sterile distilled water were added to a microcentrifuge tube. Samples were heat denatured for 30 s at 90 °C and slowly cooled to room temperature. Reaction was started by adding 5 μL of reaction buffer (100 mM coenzyme in 2X binding buffer) and stopped after 4 h with 10 μL of TBE-urea sample buffer. Reaction products were heated to 90 °C for 5 min before loading on a warmed 10% precast polyacrylamide TBE-urea gel that had been prerun for 30 min. Samples were loaded on the gel, run and analyzed under standard methods (see Appendix A).

*Cloning and sequencing of G1-HMC01*. 1 μL of 1/10 dilution of the PCR product (amplified with primers **1** and **2**) from the 10$^{th}$ round of selection were again PCR amplified with primers **1** (20 pmol) and **2** (20 pmol) in a 50 μL PCR cocktail with ten cycles of 92 °C (10 s), 50 °C (30 s), 72 °C (30 s). The PCR products were subcloned using TA cloning kit from Promega (see Appendix A). 24 plasmids containing the desired insert were purified using GeneElute Plasmid Miniprep kit from Sigma (see Appendix A). The purified plasmids were quantified using XhoI restriction enzyme and

then subjected to a PCR reaction in order to generate copies of the insert (see Appendix A). The PCR products were purified by agarose gel electrophoresis, quantified and subjected to a sequencing reaction using ddNTPs (see Appendix A). The sequencing reaction products were separated and detected by Dr. Robert Adams at the Biology Department.

*Trans-cleavage reaction of G1-HMC01 individual sequences.* Sequences obtained from G1-HMC01 were chemically synthesized by Midland Certified Reagents (Midland, TX) according to our specifications (20 nmol scale syntheses and gel filtration grade) and each designated as G1-TX (X being the assigned clone number). Independently, a substrate (**S**) containing an embedded ribonucleotide was synthesized by Midland Certified Reagents, and 5' labeled with [$\gamma$-32P]ATP in our laboratory (see Appendix A). The radiolabeled substrate was purified by gel filtration using Sephadex G-50 columns and beta emission quantified using liquid scintillation counter (see Appendix A). Before the reaction was started, 1 μL of the radiolabeled substrate was mixed with 4 μL of each of the synthesized sequences (20 μM) in separate tubes. The tubes were heated for 30 sec at 92 °C and slowly cooled down to room temperature. The *trans*-cleavage reaction was started by adding 5 μL of 100 mM **HMC01** in 2X buffer (50 mM HEPES, 0.5 M NaCl, 0.5 M KCl, 0.5 mM EDTA, pH 7.5 at rt) to each tube. After 4 h, the reaction was stopped with 10 μL of TBE-urea sample buffer. The tubes were then heated to 90 °C for 5 min before loading on a warmed 15% precast polyacrylamide TBE-urea gel that had been prerun for 30 min. Samples were loaded on the gel, run and analyzed under standard methods (see Appendix A).

*Self-cleavage reaction of G1-* ***HMC01*** *best catalytic strand*. The most active sequence obtained from G1-HMC01 was chemically synthesized by Midland Certified Reagents (Midland, TX) according to our specifications (20 nmol scale synthesis and gel filtration grade). 10 pmol of this sequence was amplified by PCR reaction with primers **B1** (80 pmol) and **B2rA** (80 pmol) in a 200 μL PCR cocktail with six thermocycles of 94 °C (15 s), 50 °C (30 s), 72 °C (30 s). The amplified DNA product was ethanol precipitated at -20 °C for 4 h. The desired 101 bp DNA product was gel purified (1.5% low range ultra agarose) and suspended in 60 μL of binding buffer (50 mM HEPES, 0.5 M NaCl, 0.5 M KCl, 0.5 mM EDTA, pH 7.5 at rt). The DNA solution was loaded on a neutravidin column, which had been previously equilibrated with 5 × 100 mL of binding buffer. The column containing the DNA solution was incubated for 30 min at room temperature, and then the unbound DNA was washed off with 5 × 100 mL of binding buffer. The non-biotinylated complementary DNA strands were eluted from the column with 3 × 100 mL of freshly prepared 0.2 N NaOH, quickly mixed with 60 μL of 3M sodium acetate pH 5.2 and ethanol precipitated overnight at -20 °C. The precipitated DNA was suspended in 20 μL of sterile distilled water and 5' labeled with [$\gamma$-32P]ATP and T4 polynucleotide kinase (see Appendix A). Radiolabeled DNA was purified using Sephadex G-50 column and beta emission quantified using liquid scintillation counter (see Appendix A).

Radiolabeled DNA sequence was subjected to a self-cleavage kinetic reaction in presence of 50 mM solution of **HMC01** in buffer (50 mM HEPES, 0.5 M NaCl, 0.5 M KCl, 0.5 mM EDTA, pH 7.5 at rt). 5 μL of the radiolabeled DNA and 20 μL of sterile distilled water were added to a microcentrifuge tube. Sample was heat denatured for 30 s

at 90 °C, slowly cooled to room temperature, and reaction was started by adding 20 μL of reaction buffer (100 mM coenzyme in 2X binding buffer). After designated times (15 min, 4 h, 12 h and 24 h), 10 μL of the reaction solution was taken and stopped by mixing with 10 μL of TBE-urea sample buffer. Reaction products were heated to 90 °C for 5 min before loading on a warmed 10% precast polyacrylamide TBE-urea gel that had been prerun for 30 min. Samples were loaded on the gel, run and analyzed under standard methods (see Appendix A).

*Mutagenesis of G1-HMC01 best catalytic sequence.* A mutagenized library, based on the best strand obtained from G1-HMC01, was chemically synthesized by Midland Certified Reagents (Midland, TX) according to our specifications (20 nmol scale synthesis and gel filtration grade). In the mutagenized strand, each base position of the catalytic domain was present with 85% probability, and each of the other three bases was present with 5% probability (0.15 degeneracy per position).

*Reselection using **HMC01***. The mutagenized DNA (20 pmol) was initially amplified by PCR reaction with primers **1** (32 pmol) and **B2** (32 pmol) in a 200 μL PCR cocktail using four thermocycles of 94 °C (15 s), 50 °C (30 s), 72 °C (30 s). The amplified DNA template, containing biotin and ribonucleotide moiety, was then ethanol precipitated at -20 °C for 4 h. The desired 101 bp DNA product was gel purified (1.5% low range ultra agarose) and suspended in 60 μL binding buffer (50 mM HEPES, 0.5 M NaCl, 0.5 M KCl, 0.5 mM EDTA, pH 7.5 at rt). The resulting DNA solution was loaded on a neutravidin column, which had been previously equilibrated with 5 × 100 mL binding buffer. The column was incubated for a period of 30 min at room temperature,

and then the unbound DNA was washed off with 5 × 100 mL binding buffer. The non-biotinylated complementary DNA strands were eluted from the column with 5 × 100 mL freshly prepared 0.2 N NaOH, and the column was immediately neutralized with 5 × 100 mL binding buffer. Column matrix, containing adenosine ribonucleotide incorporated DNA, was incubated in binding buffer for 1 h (negative selection) and then carefully eluted with 10 × 100 mL binding buffer. Reaction was then started by adding 3 × 20 μl of reaction buffer (binding buffer containing 5 mM coenzyme, pH 7.5 at room temperature) to the column matrix over a period of 1 h. DNA molecules eluted from the column by reaction buffer were ethanol precipitated overnight at -20 °C and then amplified with primers **1** (20 pmol) and **2** (20 pmol) in a 100 μL PCR cocktail with twenty cycles of 92 °C (10 s), 50 °C (30 s), 72 °C (30 s). 10 μL of the PCR product were amplified by PCR reaction with primers **1** (80 pmol) and **B2** (80 pmol) in a 200 μL PCR cocktail with six thermocycles of 94 °C (15 s), 50 °C (30 s), 72 °C (30 s). The amplified DNA product was ethanol precipitated at -20 °C for 4 h. The desired 101 bp DNA products were gel purified (1.5% low range ultra agarose) and the isolated DNA was used for the next round of selection. In rounds 5 to 10, the stringency of the selection was increased by reducing the time of reaction as follows: round 5 (30 min), round 6 (30 min), round 7 (15 min), round 8 (15 min), round 9 (5 min) and round 10 (5 min). After 10 rounds of reselection a DNA pool (G2-HMC01) was obtained.

*Self-cleavage reaction of G2-HMC01.* 10 μL of PCR product (amplified with primers **1** and **2**) from the $10^{th}$, $9^{th}$ and $8^{th}$ round of reselection were extend by PCR reaction with primers **B1** (80 pmol) and **B2rA** (80 pmol) in a 200 μL PCR cocktail with six thermocycles of 94 °C (15 s), 50 °C (30 s), 72 °C (30 s). The amplified DNA

products were ethanol precipitated at -20 °C for 4 h. The desired 101 bp DNA products were gel purified (1.5% low range ultra agarose) and suspended in 60 μL of binding buffer (50 mM HEPES, 0.5 M NaCl, 0.5 M KCl, 0.5 mM EDTA, pH 7.5 at rt). Each DNA solution was loaded on a separate neutravidin column, which had been previously equilibrated with 5 × 100 mL of binding buffer. Columns containing DNA solution were incubated for 30 min at room temperature, and then the unbound DNA was washed off with 5 × 100 mL of binding buffer. The non-biotinylated complementary DNA strands were eluted from each column with 3 × 100 mL of freshly prepared 0.2 N NaOH, quickly mixed with 60 μL of 3M sodium acetate pH 5.2 and ethanol precipitated overnight at -20 °C. Each precipitated DNA pool was suspended in 20 μL of sterile distilled water and 5' labeled with [$\gamma$-32P]ATP and T4 polynucleotide kinase (see Appendix A). Radiolabeled DNA pools were purified using Sephadex G-50 columns, beta emission quantified using liquid scintillation counter (see Appendix A) and each diluted in sterile distilled water to the same number counts per milliliter (cpm/ml).

Each radiolabeled DNA pool was subjected to a self-cleavage reaction in presence of 5 mM solution of its cognate coenzyme in buffer (50 mM HEPES, 0.5 M NaCl, 0.5 M KCl, 0.5 mM EDTA, pH 7.5 at rt). For each round DNA pool, 1 μL of radiolabeled DNA pool and 4 μL of sterile distilled water were added to a microcentrifuge tube. Samples were heat denatured for 30 s at 90 °C and slowly cooled to room temperature. Reaction was started by adding 5 μL of reaction buffer (10 mM coenzyme in 2X binding buffer) and stopped after 4 h with 10 μL of TBE-urea sample buffer. Reaction products were heated to 90 °C for 5 min before loading on a warmed 10% precast polyacrylamide TBE-

urea gel that had been prerun for 30 min. Samples were loaded on the gel, run and analyzed under standard methods (see Appendix A).

*Cloning and sequencing of G2-HMC01.* 1 μL of 1/10 dilution of the PCR product (amplified with primers **1** and **2**) from the 10$^{th}$ round of reselection were again PCR amplified with primers **1** (20 pmol) and **2** (20 pmol) in a 50 μL PCR cocktail with ten cycles of 92 °C (10 s), 50 °C (30 s), 72 °C (30 s). The PCR products were subcloned using TA cloning kit from Promega (see Appendix A). 24 plasmids containing the desired insert were purified using GeneElute Plasmid Miniprep kit from Sigma (see Appendix A). The purified plasmids were quantified using XhoI restriction enzyme and then subjected to a PCR reaction in order to generate copies of the insert (see Appendix A). The PCR products were purified by agarose gel electrophoresis, quantified and subjected to a sequencing reaction using ddNTPs (see Appendix A). The sequencing reaction products were separated and detected by Dr. Robert Adams at the Biology Department.

*Trans-cleavage reaction of G2-HMC01 individual sequences.* Sequences obtained from the G2-HMC01 were chemically synthesized by Midland Certified Reagents (Midland, TX) according to our specifications (20 nmol scale syntheses and gel filtration grade) and each designated as G2-TX (X being the assigned clone number). Independently, a substrate (**S**) containing an embedded ribonucleotide was synthesized by Midland Certified Reagents, and 5' labeled with [$\gamma$-32P]ATP in our laboratory (see Appendix A). The radiolabeled substrate was purified by gel filtration using Sephadex G-50 columns and beta emission quantified using liquid scintillation counter (see

Appendix A). Before the reaction was started, 1 μL of the radiolabeled substrate was mixed with 4 μL of each of the synthesized sequences (20 μM) in separate tubes. The tubes were heated for 30 sec at 92 °C and slowly cooled down to room temperature. The *trans*-cleavage reaction was started by adding 5 μL of 10 mM **HMC01** in 2X buffer (50 mM HEPES, 0.5 M NaCl, 0.5 M KCl, 0.5 mM EDTA, pH 7.5 at rt) to each tube. After 4 h, the reaction was stopped with 10 μL of TBE-urea sample buffer. The tubes were then heated to 90 °C for 5 min before loading on a warmed 15% precast polyacrylamide TBE-urea gel that had been prerun for 30 min. Samples were loaded on the gel, run and analyzed under standard methods (see Appendix A).

*Self-cleavage reaction of G2-HMC01 best catalytic sequence*. The most active sequence obtained from G2-HMC01 was chemically synthesized by Midland Certified Reagents (Midland, TX) according to our specifications (20 nmol scale synthesis and gel filtration grade). 10 pmol of this sequence was amplified by PCR reaction with primers **B1** (80 pmol) and **B2rA** (80 pmol) in a 200 μL PCR cocktail with six thermocycles of 94 °C (15 s), 50 °C (30 s), 72 °C (30 s). The amplified DNA product was ethanol precipitated at -20 °C for 4 h. The desired 101 bp DNA product was gel purified (1.5% low range ultra agarose) and suspended in 60 μL of binding buffer (50 mM HEPES, 0.5 M NaCl, 0.5 M KCl, 0.5 mM EDTA, pH 7.5 at rt). The DNA solution was loaded on a neutravidin column, which had been previously equilibrated with 5 × 100 mL of binding buffer. The column containing the DNA solution was incubated for 30 min at room temperature, and then the unbound DNA was washed off with 5 × 100 mL of binding buffer. The non-biotinylated complementary DNA strands were eluted from the column with 3 × 100 mL of freshly prepared 0.2 N NaOH, quickly mixed with 60 μL of 3M

sodium acetate pH 5.2 and ethanol precipitated overnight at -20 °C. The precipitated DNA was suspended in 20 μL of sterile distilled water and 5' labeled with [$\gamma$-32P]ATP and T4 polynucleotide kinase (see Appendix A). Radiolabeled DNA was purified using Sephadex G-50 column and beta emission quantified using liquid scintillation counter (see Appendix A).

Radiolabeled DNA sequence was subjected to a self-cleavage kinetic reaction in presence of 5 mM solution of **HMC01** in buffer (50 mM HEPES, 0.5 M NaCl, 0.5 M KCl, 0.5 mM EDTA, pH 7.5 at rt). 5 μL of the radiolabeled DNA and 20 μL of sterile distilled water were added to a microcentrifuge tube. Sample was heat denatured for 30 s at 90 °C, slowly cooled to room temperature, and reaction was started by adding 20 μL of reaction buffer (10 mM coenzyme in 2X binding buffer). After designated times (30 min, 12 h and 24 h), 10 μL of the reaction solution was taken and stopped by mixing with 10 μL of TBE-urea sample buffer. Reaction products were heated to 90 °C for 5 min before loading on a warmed 10% precast polyacrylamide TBE-urea gel that had been prerun for 30 min. Samples were loaded on the gel, run and analyzed under standard methods (see Appendix A).

*Mutagenesis of G2-HMC01 best catalytic sequence.* A mutagenized library, based on the best strand obtained from G2-HMC01, was chemically synthesized by Midland Certified Reagents (Midland, TX) according to our specifications (20 nmol scale synthesis and gel filtration grade). In the mutagenized strand, each base position of the catalytic domain was present with 85% probability, and each of the other three bases was present with 5% probability (0.15 degeneracy per position).

*Second reselection using **HMC01***. The mutagenized DNA (20 pmol) was initially amplified by PCR reaction with primers **1** (32 pmol) and **B2** (32 pmol) in a 200 μL PCR cocktail using four thermocycles of 94 °C (15 s), 50 °C (30 s), 72 °C (30 s). The amplified DNA template, containing biotin and ribonucleotide moiety, was then ethanol precipitated at -20 °C for 4 h. The desired 101 bp DNA product was gel purified (1.5% low range ultra agarose) and suspended in 60 μL binding buffer (50 mM HEPES, 0.5 M NaCl, 0.5 M KCl, 0.5 mM EDTA, pH 7.5 at rt). The resulting DNA solution was loaded on a neutravidin column, which had been previously equilibrated with 5 × 100 mL binding buffer. The column was incubated for a period of 30 min at room temperature, and then the unbound DNA was washed off with 5 × 100 mL binding buffer. The non-biotinylated complementary DNA strands were eluted from the column with 5 × 100 mL freshly prepared 0.2 N NaOH, and the column was immediately neutralized with 5 × 100 mL binding buffer. Column matrix, containing adenosine ribonucleotide incorporated DNA, was incubated in binding buffer for 1 h (negative selection) and then carefully eluted with 10 × 100 mL binding buffer. Reaction was then started by adding 3 × 20 μl of reaction buffer (binding buffer containing 0.1 mM coenzyme, pH 7.5 at room temperature) to the column matrix over a period of 1 h. DNA molecules eluted from the column by reaction buffer were ethanol precipitated overnight at -20 °C and then amplified with primers **1** (20 pmol) and **2** (20 pmol) in a 100 μL PCR cocktail with twenty cycles of 92 °C (10 s), 50 °C (30 s), 72 °C (30 s). 10 μL of the PCR product were amplified by PCR reaction with primers **1** (80 pmol) and **B2** (80 pmol) in a 200 μL PCR cocktail with six thermocycles of 94 °C (15 s), 50 °C (30 s), 72 °C (30 s). The amplified DNA product was ethanol precipitated at -20 °C for 4 h. The desired 101 bp DNA

products were gel purified (1.5% low range ultra agarose) and the isolated DNA was used for the next round of selection. In rounds 5 to 10, the stringency of the selection was increased by reducing the time of reaction as follows: round 5 (30 min), round 6 (30 min), round 7 (15 min), round 8 (15 min), round 9 (1 min) and round 10 (1 min). After 10 rounds of reselection a DNA pool (G3-HMC01) was obtained.

*Self-cleavage reaction of G3-HMC01.* 10 μL of PCR product (amplified with primers **1** and **2**) from the $10^{th}$, $9^{th}$ and $8^{th}$ round of second reselection were extend by PCR reaction with primers **B1** (80 pmol) and **B2rA** (80 pmol) in a 200 μL PCR cocktail with six thermocycles of 94 °C (15 s), 50 °C (30 s), 72 °C (30 s). The amplified DNA products were ethanol precipitated at -20 °C for 4 h. The desired 101 bp DNA products were gel purified (1.5% low range ultra agarose) and suspended in 60 μL of binding buffer (50 mM HEPES, 0.5 M NaCl, 0.5 M KCl, 0.5 mM EDTA, pH 7.5 at rt). Each DNA solution was loaded on a separate neutravidin column, which had been previously equilibrated with 5 × 100 mL of binding buffer. Columns containing DNA solution were incubated for 30 min at room temperature, and then the unbound DNA was washed off with 5 × 100 mL of binding buffer. The non-biotinylated complementary DNA strands were eluted from each column with 3 × 100 mL of freshly prepared 0.2 N NaOH, quickly mixed with 60 μL of 3M sodium acetate pH 5.2 and ethanol precipitated overnight at -20 °C. Each precipitated DNA pool was suspended in 20 μL of sterile distilled water and 5' labeled with [$\gamma$-32P]ATP and T4 polynucleotide kinase (see Appendix A). Radiolabeled DNA pools were purified using Sephadex G-50 columns, beta emission quantified using liquid scintillation counter (see Appendix A) and each diluted in sterile distilled water to the same number counts per milliliter (cpm/ml).

Each radiolabeled DNA pool was subjected to a self-cleavage reaction in presence of 0.1 mM solution of **HMC01** in buffer (50 mM HEPES, 0.5 M NaCl, 0.5 M KCl, 0.5 mM EDTA, pH 7.5 at rt). For each round DNA pool, 1 μL of radiolabeled DNA pool and 4 μL of sterile distilled water were added to a microcentrifuge tube. Samples were heat denatured for 30 s at 90 °C and slowly cooled to room temperature. Reaction was started by adding 5 μL of reaction buffer (0.2 mM coenzyme in 2X binding buffer) and stopped after 4 h with 10 μL of TBE-urea sample buffer. Reaction products were heated to 90 °C for 5 min before loading on a warmed 10% precast polyacrylamide TBE-urea gel that had been prerun for 30 min. Samples were loaded on the gel, run and analyzed under standard methods (see Appendix A).

*Cloning and sequencing of G3-HMC01.* Cloning and sequencing experiments were performed by Molecular Cloning Laboratories (MCLAB) (San Francisco, CA). 0.1 μL of the PCR product (amplified with primers **1** and **2**) from the $10^{th}$ round of selection were again PCR amplified with primers **1** (20 pmol) and **2** (20 pmol) in a 50 μL PCR cocktail with ten cycles of 92 °C (10 s), 50 °C (30 s), 72 °C (30 s). The PCR product was sent to MCLAB without additional treatment for cloning and sequencing (24 clones) according to our specifications.

*Trans-cleavage reaction of G3-HMC01 individual sequences*. Sequences obtained from the G3-HMC01 were chemically synthesized by Midland Certified Reagents (Midland, TX) according to our specifications (20 nmol scale syntheses and gel filtration grade) and each designated as G3-TX (X being the assigned clone number). Independently, a substrate (**S**) containing an embedded ribonucleotide was synthesized by

Midland Certified Reagents, and 5' labeled with [$\gamma$-32P]ATP in our laboratory (see Appendix A). The radiolabeled substrate was purified by gel filtration using Sephadex G-50 columns and beta emission quantified using liquid scintillation counter (see Appendix A). Before the reaction was started, 1 μL of the radiolabeled substrate was mixed with 4 μL of each of the synthesized sequences (20 μM) in separate tubes. The tubes were heated for 30 sec at 92 °C and slowly cooled down to room temperature. The *trans*-cleavage reaction was started by adding 5 μL of 0.2 mM **HMC01** in 2X buffer (50 mM HEPES, 0.5 M NaCl, 0.5 M KCl, 0.5 mM EDTA, pH 7.5 at rt) to each tube. After 4 h, the reaction was stopped with 10 μL of TBE-urea sample buffer. The tubes were then heated to 90 °C for 5 min before loading on a warmed 15% precast polyacrylamide TBE-urea gel that had been prerun for 30 min. Samples were loaded on the gel, run and analyzed under standard methods (see Appendix A).

*Self-cleavage reaction of G3-HMC01 best catalytic sequence*. The most active sequence obtained from G3-HMC01 was chemically synthesized by Midland Certified Reagents (Midland, TX) according to our specifications (20 nmol scale synthesis and gel filtration grade). 10 pmol of this sequence was amplified by PCR reaction with primers **B1** (80 pmol) and **B2rA** (80 pmol) in a 200 μL PCR cocktail with six thermocycles of 94 °C (15 s), 50 °C (30 s), 72 °C (30 s). The amplified DNA product was ethanol precipitated at -20 °C for 4 h. The desired 101 bp DNA product was gel purified (1.5% low range ultra agarose) and suspended in 60 μL of binding buffer (50 mM HEPES, 0.5 M NaCl, 0.5 M KCl, 0.5 mM EDTA, pH 7.5 at rt). The DNA solution was loaded on a neutravidin column, which had been previously equilibrated with 5 × 100 mL of binding buffer. The column containing the DNA solution was incubated for 30 min at room

temperature, and then the unbound DNA was washed off with 5 × 100 mL of binding buffer. The non-biotinylated complementary DNA strands were eluted from the column with 3 × 100 mL of freshly prepared 0.2 N NaOH, quickly mixed with 60 µL of 3M sodium acetate pH 5.2 and ethanol precipitated overnight at -20 °C. The precipitated DNA was suspended in 20 µL of sterile distilled water and 5' labeled with [$\gamma$-32P]ATP and T4 polynucleotide kinase (see Appendix A). Radiolabeled DNA was purified using Sephadex G-50 column and beta emission quantified using liquid scintillation counter (see Appendix A).

Radiolabeled DNA sequence was subjected to a self-cleavage kinetic reaction in presence of 0.1 mM solution of **HMC01** in buffer (50 mM HEPES, 0.5 M NaCl, 0.5 M KCl, 0.5 mM EDTA, pH 7.5 at rt). 5 µL of the radiolabeled DNA and 20 µL of sterile distilled water were added to a microcentrifuge tube. Sample was heat denatured for 30 s at 90 °C, slowly cooled to room temperature, and reaction was started by adding 20 µL of reaction buffer (0.2 mM coenzyme in 2X binding buffer). After designated times (5 min, 30 min, 1 h, 2 h, 3 h and 4 h), 10 µL of the reaction solution was taken and stopped by mixing with 10 µL of TBE-urea sample buffer. Reaction products were heated to 90 °C for 5 min before loading on a warmed 10% precast polyacrylamide TBE-urea gel that had been prerun for 30 min. Samples were loaded on the gel, run and analyzed under standard methods (see Appendix A).

*In vitro Parallel Selection of Catalytic DNA Pools that Use Bis-Histidine Aromatic Coenzymes (**DC01**, **DC02**, **DC03** and **DC04**)*

*Parallel selection using **DC01**, **DC02**, **DC03** and **DC04**.* A DNA template (80 pmol) was initially amplified by PCR reaction with primers **1** (128 pmol) and **B2** (128

pmol) in a 800 μL PCR cocktail using four thermocycles of 94 °C (15 s), 50 °C (30 s), 72 °C (30 s). The amplified DNA template, containing biotin and ribonucleotide moiety, was then ethanol precipitated at -20 °C for 4 h. The desired 101 bp DNA product was gel purified (1.5% low range ultra agarose) and suspended in 240 μL binding buffer (50 mM HEPES, 0.5 M NaCl, 0.5 M KCl, 0.5 mM EDTA, pH 7.5 at rt). The resulting DNA solution was equally distributed into four tubes and each solution loaded on a separate neutravidin column, which had been previously equilibrated with 5 × 100 mL binding buffer. The columns were incubated for a period of 30 min at room temperature, and then the unbound DNA was washed off with 5 × 100 mL binding buffer. The non-biotinylated complementary DNA strands were eluted from each column with 5 × 100 mL of freshly prepared 0.2 N NaOH, and the columns were immediately neutralized with 5 × 100 mL binding buffer. Columns matrices, containing ribonucleotide incorporated DNA, were incubated in binding buffer for 1 h (negative selection) and then carefully eluted with 10 × 100 mL binding buffer. Reactions were then started by adding 3 × 20 μL of each reaction buffer (binding buffer containing 50 mM coenzyme, pH 7.5 at rt) to a column over a period of 5 h. DNA molecules eluted from each column by reaction buffer were ethanol precipitated overnight at -20 °C and then amplified with primers **1** (20 pmol) and **2** (20 pmol) in a 100 μL PCR cocktail with twenty cycles of 92 °C (10 s), 50 °C (30 s), 72 °C (30 s). 10 μL of each of the PCR products were amplified by PCR reaction with primers **1** (80 pmol) and **B2** (80 pmol) in a 200 μL PCR cocktail with six thermocycles of 94 °C (15 s), 50 °C (30 s), 72 °C (30 s). The amplified DNA products were ethanol precipitated at -20 °C for 4 h. The desired 101 bp DNA products were gel purified (1.5% low range ultra agarose) and the isolated DNA was used for the next

round of selection. In rounds 5 to 10, the stringency of the selection was increased by reducing the time of reaction as follows: round 5 (120 min), round 6 (60 min), round 7 (30 min), round 8 (15 min), round 9 (5 min) and round 10 (1 min). After 10 rounds of selection four enriched DNA pools (pool DC01, pool DC02, pool DC03 and pool DC04) were obtained.

*Preparation of DNA pool for catalytic studies.* 10 μL of each PCR product (amplified with primers **1** and **2**) from the $10^{th}$ round of selection were amplified by PCR reaction with primers **B1** (80 pmol) and **B2rA** (80 pmol) in a 200 μL PCR cocktail with six thermocycles of 94 °C (15 s), 50 °C (30 s), 72 °C (30 s). The amplified DNA products were ethanol precipitated at -20 °C for 4 h. The desired 101 bp DNA products were gel purified (1.5% low range ultra agarose) and suspended in 60 μL of binding buffer (50 mM HEPES, 0.5 M NaCl, 0.5 M KCl, 0.5 mM EDTA, pH 7.5 at rt). Each DNA solution was loaded on a separate neutravidin column, which had been previously equilibrated with 5 × 100 mL of binding buffer. Columns containing DNA solution were incubated for 30 min at room temperature, and then unbound DNA was washed off with 5 × 100 mL of binding buffer. The non-biotinylated complementary catalytic DNA strands were eluted from each column with 3 × 100 mL of freshly prepared 0.2 N NaOH, rapidly mixed with 60 μL of 3M sodium acetate pH 5.2 and ethanol precipitated overnight at -20 °C. Each precipitated DNA pool was suspended in 20 μL sterile distilled water and 5' labeled with [$\gamma$-32P]ATP and T4 polynucleotide kinase (see Appendix A). Radiolabeled DNA pools were purified using Sephadex G-50 columns, beta emission quantified using liquid scintillation counter (see Appendix A) and each diluted in sterile distilled water to the same number of counts per milliliter (cpm/ml). Radiolabeled DNA

pools were stored at 0 °C and used in catalytic experiments. The naïve DNA pool (before *in vitro* selections) was prepared and radiolabeled under the same conditions.

*Self-cleavage reaction of DNA pool.* Each radiolabeled DNA pool was subjected to a self-cleavage reaction in presence of 50 mM solution of its cognate coenzyme in buffer (50 mM HEPES, 0.5 M NaCl, 0.5 M KCl, 0.5 mM EDTA, pH 7.5 at rt) (Table 5). Positive control consisted of the alkaline hydrolysis of the ribonucleotide moiety, and negative control consisted of the reaction in buffer in absence of coenzyme. For each DNA pool, 1 µL of radiolabeled DNA pool and 4 µL of sterile distilled water were added to two microcentrifuge tubes (A and B). Samples were heat denatured for 30 s at 90 °C and slowly cooled to room temperature. Reaction was started by adding 5 µL of 2X binding buffer to tube A (negative control) and 5 µL of reaction buffer (100 mM coenzyme in 2X binding buffer) to tube B. Tube C was prepared by mixing 1 µL of each radiolabeled DNA pool and 5 µL of 0.5 N NaOH (positive control). After 4 h of reaction, tube C was neutralized with 5 ul of 0.5 N HCl, and 10 µL of TBE-urea sample buffer was added to each tube. Tubes were then heated to 90 °C for 5 min before loading on a warmed 10% precast polyacrylamide TBE-urea gel that had been prerun for 30 min. Samples were loaded on the gel, run and analyzed under standard methods (see Appendix A).

Table 5. DNA pools (DC01, DC02, DC03 and DC04) self-cleavage experiment design.

| 10% denaturing polyacrylamide gel | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Lane | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| Conditions | A | B | C | A | B | C | A | B | C | A | B | C |
| | Pool DC01 | | | Pool DC02 | | | Pool DC03 | | | Pool DC04 | | |
| 50 mM coenzyme, pH 7.4, 4 h, rt conditions (A = Neg. rxn, B = Cognate coenzyme, C = Alkaline hydrolysis) | | | | | | | | | | | | |

*Cross-reactivity of DNA pool.* Radiolabeled DNA from each catalytic DNA pool was cross-reacted with its cognate coenzyme and other three coenzymes used in the parallel selection (Table 6). For example, DNA pool DC05 was reacted with **DC01**, **DC02**, **DC03** and **DC04**. For each pool, 1 μL of radiolabeled pool and 4 μL of sterile distilled water were added to each of four microcentrifuge tubes (A, B, C and D). Samples were heat denatured for 30 s at 90 °C and slowly cooled to room temperature. Reaction was started by adding 5 μL of reaction buffer (2X binding buffer containing 100 mM **DC01**) to tube A, 5 μL of reaction buffer (2X binding buffer containing 100 mM **DC02**) to tube B, 5 μL of reaction buffer (2X binding buffer containing 100 mM **DC03**) to tube C and 5 μL of reaction buffer (2X binding buffer containing 100 mM **DC04**) to tube D. After 4 h of reaction, 10 μL of TBE-urea sample buffer was added to each tube. Tubes A, B, C and D for each DNA pool were heated to 90 °C for 5 min before loading on a 10% precast polyacrylamide TBE-urea warmed gel that had been prerun for 30 min. Samples were loaded on the gel, run and analyzed under standard methods (see Appendix A). The naïve DNA pool (before *in vitro* selections) was reacted under the same conditions described for the selected pools.

Table 6. Cross-reactivity (pools DC01, DC02, DC03 and DC04) experiment design.

| 10% denaturing polyacrylamide gel | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Lane | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 |
| Conditions | A | B | C | D | A | B | C | D | A | B | C | D | A | B | C | D | A | B | C | D |
| | Pool DC01 | | | | Pool DC02 | | | | Pool DC03 | | | | Pool DC04 | | | | Naïve Pool | | | |
| 10 mM coenzyme (A = **DC01**, B = **DC02**, C = **DC03**, D = **DC04**), 1 h, pH 7.4, rt | | | | | | | | | | | | | | | | | | | | |

*Kinetic study of DNA pool.* Radiolabeled DNA from each DNA pool was reacted in presence of its cognate coenzyme for different time periods in order to obtain its rate

constant ($k_{obs}$) (Table 7). 15 µl of each radiolabeled DNA pool was mixed with 60 µl of sterile distilled water. The samples were heat denatured for 30 s at 90 °C and slowly cooled to room temperature. The reactions were started by adding 75 µl of reaction buffer (2X binding buffer containing 100 mM of coenzyme, pH 7.4) to each of the four microcentrifuge tubes. After designated times (0 min, 5min, 15min, 30min, 45min, 1 h, 2 h, 4 h, 8 h, 12 h, 24 h and 48 h), 10 µl aliquots were taken from each tube, mixed with 10 µl denaturing solution (95% formamide, 1mM EDTA and 0.05% each of xylene cyanol and bromophenol solution) and stored at 0 °C. After the last reaction time sample was taken, the tubes were heated to 90 °C for 5 min before loading on a warmed 10% precast polyacrylamide TBE-urea gel that had been prerun for 30 min. Samples were loaded on the gel, run and analyzed under standard methods (see Appendix A).

Table 7. Rate constant (pools DC01, DC02, DC03 and DC04) experiment design.

| 10% denaturing polyacrylamide gel | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Lane | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 |
| Reaction time | 0 min | 5 min | 15 min | 30 min | 45 min | 1 h | 2 h | 4 h | 8 h | 12 h | 24 h | 48 h |
| Aliquot of self-cleaving DNA pool and 10 mM of cognate coenzyme per well, pH 7.4, rt | | | | | | | | | | | | |

*Binding study of DNA pool.* Radiolabeled DNA from each DNA pool was reacted in presence of different concentrations of its cognate coenzyme in order to obtain an approximate enzyme-coenzyme affinity constant (Kd) (Table 8). For each DNA pool, 1 µl of radiolabeled DNA and 4 µl of sterile distilled water were added to a battery of 10 tubes. Samples were heat denatured for 30 s at 90 °C and slowly cooled to room temperature. Reactions were started by adding 5 µl of serial dilutions of **DC03** (0.02 mM, 0.2mM, 2 mM, 20mM and 200 mM) 2x binding buffer at pH 7.4 to each battery of tubes (each point was prepared twice). After 4 h of reaction, 10 µl of TBE-urea sample

buffer was added to each tube. Tubes were heated to 90 °C for 5 min before loading on a warmed 10% precast polyacrylamide TBE-urea gel that had been prerun for 30 min. Samples were loaded on the gel, run and analyzed under standard methods (see Appendix A).

Table 8. Binding study (pools DC01, DC02, DC03 and DC04) experiment design.

| 10% denaturing polyacrylamide gel | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Lane | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 |
| Conditions | 0.001 mM coenzyme | | 0.01 mM coenzyme | | 0.1 mM coenzyme | | 1 mM coenzyme | | 10 mM coenzyme | | 100 mM coenzyme |
| Aliquot of self-cleaving DNA pool per well, 4 h, pH 7.4, rt | | | | | | | | | | | |

*pH profile of DNA pool.* Radiolabeled DNA from each DNA pool was reacted in presence of its cognate coenzyme at different pHs in order to study the efficiency of the cleavage (Table 9). For each DNA pool, 1 µl of radiolabeled DNA and 4 µl of sterile distilled water were added to a battery of 10 tubes. Samples were heat denatured for 30 s at 90 °C and slowly cooled to room temperature. Reactions were started by adding 5 µL of reaction buffer (2X binding buffer containing 100 mM of coenzyme) at pHs 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5 and 9 to each of the four battery of tubes. After 4 h of reaction, 10 µl of TBE-urea sample buffer was added to each tube. Tubes were heated to 90 °C for 5 min before loading on a warmed 10% precast polyacrylamide TBE-urea gel that had been prerun for 30 min. Samples were loaded on the gel, run and analyzed under standard methods (see Appendix A).

Table 9. pH profile (pools DC01, DC02, DC03 and DC04)experiment design.

| 10% denaturing polyacrylamide gel | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Lane | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| Conditions | pH 5.0 | pH 5.5 | pH 6.0 | pH 6.5 | pH 7 | pH 7.5 | pH 8.0 | pH 8.5 | pH 9 |
| | Aliquot of self-cleaving DNA pool and 10 mM of cognate coenzyme per well, 1 h, rt | | | | | | | | |

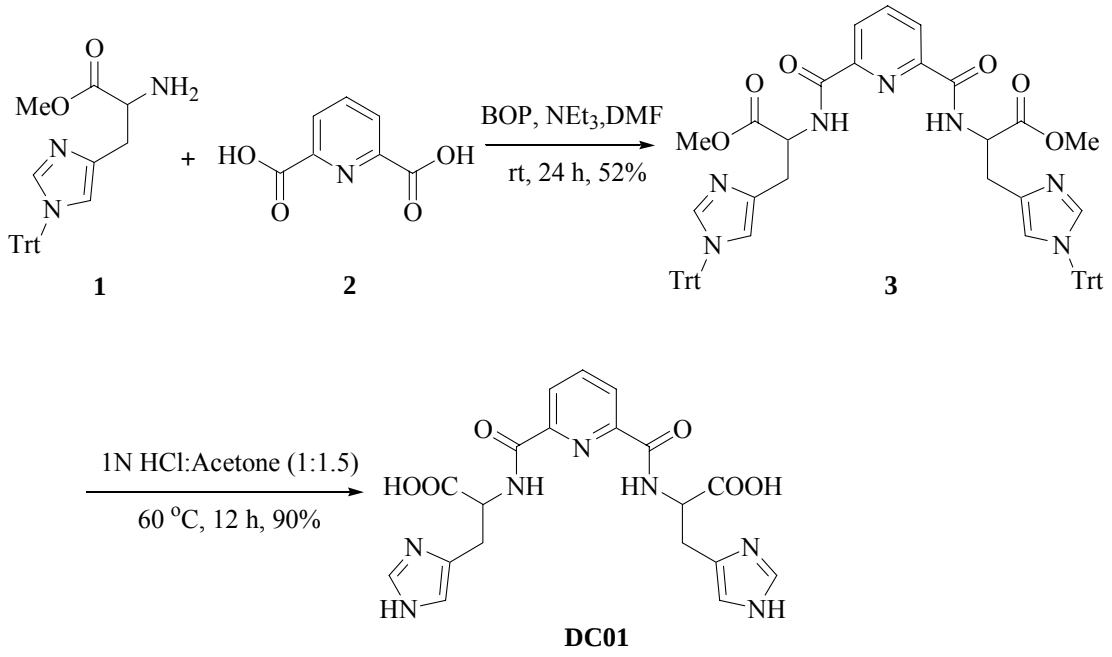
*Cloning and sequencing of DNA pool.* Cloning and sequencing experiments were performed by Molecular Cloning Laboratories (MCLAB) (San Francisco, CA). 0.1 μL of the PCR product (amplified with primers **1** and **2**) from the $10^{th}$ round of selection were again PCR amplified with primers **1** (20 pmol) and **2** (20 pmol) in a 50 μL PCR cocktail with ten cycles of 92 °C (10 s), 50 °C (30 s), 72 °C (30 s). The PCR product was sent to MCLAB without additional treatment for cloning and sequencing (24 clones) according to our specifications.

## *Results and Discussions*

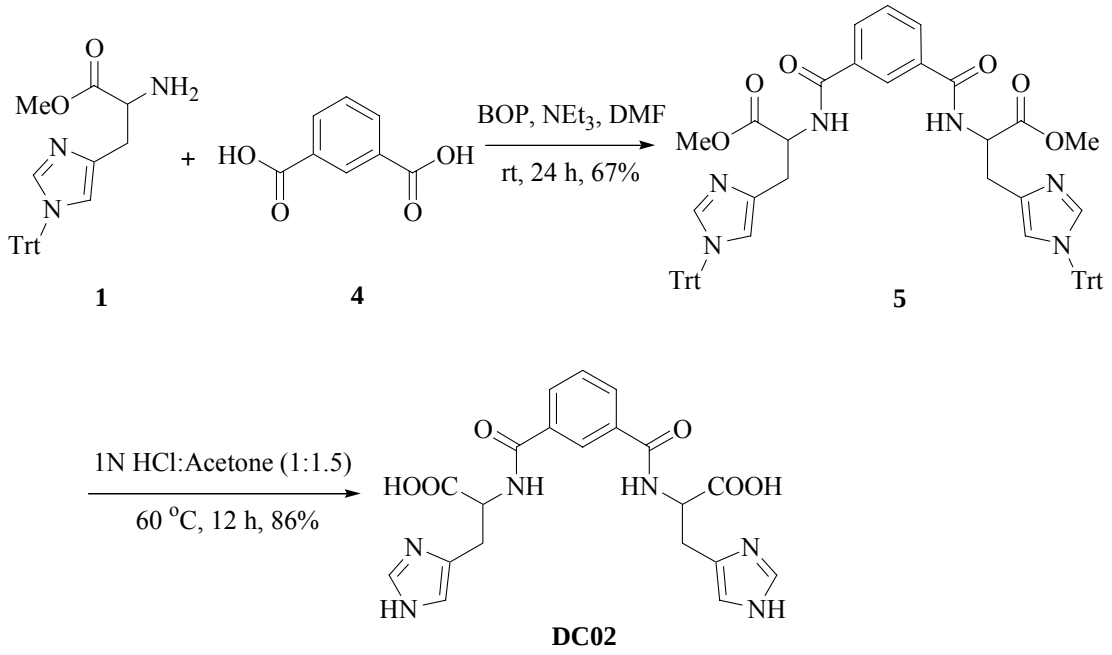
### *Bis-Histidine Aromatic Coenzymes Syntheses*

BOP-mediated coupling reaction of a protected histidine (His-(1-Trt)-OMe) with aromatic diacids followed by acidic deprotection provided the desired diamides coenzymes **DC01**, **DC02**, **DC03** and **DC04** in good yields. This coenzyme design was intended to provide the necessary solubility (due to carboxylate residues and H-bonding sites) and DNA binding (via intercalation and/or H-bonding), while providing functionality able to significantly enhance the catalytic power of the 'naked' DNA enzymes. While the coenzymes have several potential catalytic groups, the imidazole residues are expected to have exceptional potential for the catalysis of RNA hydrolysis.

*Synthesis of 2-({6-[1-Carboxy-2-(1H-imidazol-4-yl)-ethylcarbamoyl]-pyridine-2-carbonyl}-amino)-3-(1H-imidazol-4-yl)-propionic acid* (***DC01)***. Pyridinedicarbonyl, 2,6-bis(histidine) coenzyme (**DC01**) was prepared according to Scheme 3. The commercially available protected histidine (H-His(Trt)-OMe·HCl) **1** was coupled with 2,6-pyridinedicarboxylic acid **2** using BOP as the condensating agent furnishing the protected coenzyme after 24 h reaction (monitored by TLC). Precipitation of the crude product in ice water followed by filtration and further purification by flash chromatography afforded **3** in 52% yield. Removal of the acid labile protecting groups (Trt and methyl ester) under reflux for 12 h in 1 N HCl:Acetone (1:1.5) afforded **DC01** in 47% overall yield (two steps).




Scheme 3. Synthetic Scheme for **DC01**.

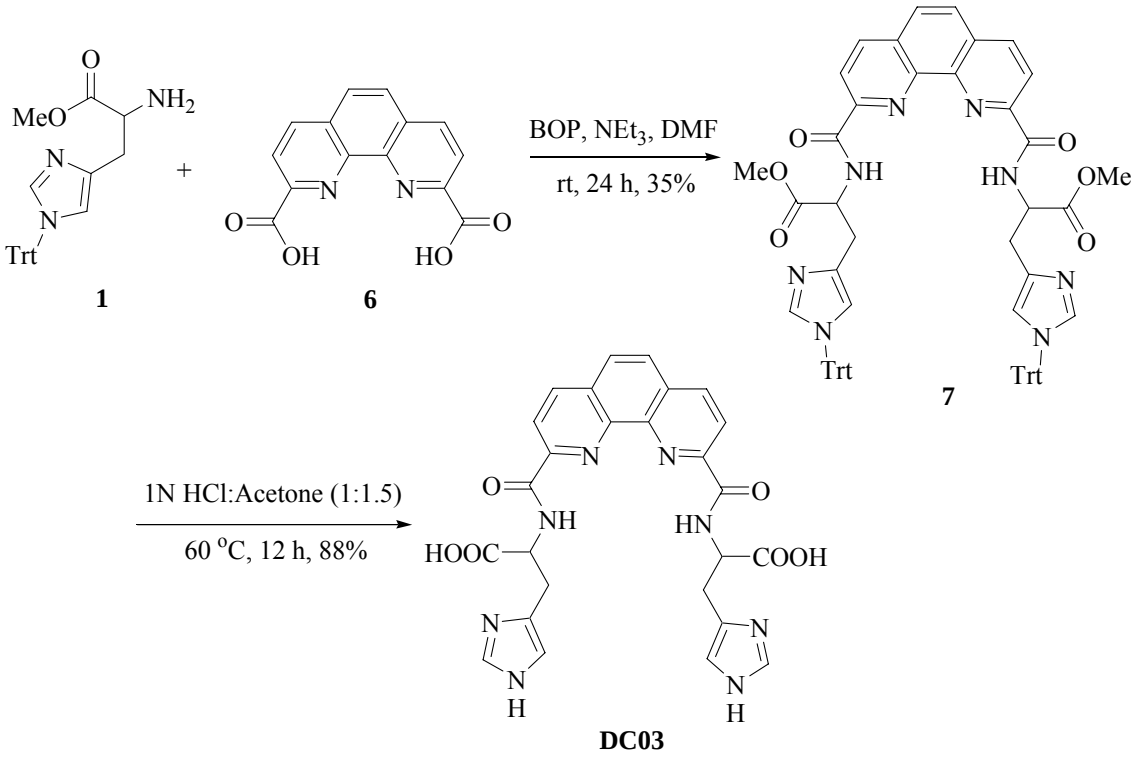



Scheme 4. Synthetic Scheme for **DC02**.

*Synthesis of 2-{3-[1-Carboxy-2-(1H-imidazol-4-yl)-ethylcarbamoyl]-benzoylamino}-3-(1H-imidazol-4-yl)-propionic acid (**DC02**)*. Benzenedicarbonyl, 1,5-bis(histidine) coenzyme (**DC02**) was prepared according to Scheme 4. The commercially available protected histidine (H-His(Trt)-OMe·HCl) **1** was coupled with isophthalic acid **4** using BOP as the condensating agent furnishing the protected coenzyme after 24 h reaction (monitored by TLC). Precipitation of the crude product in ice water followed by filtration and further purification by flash chromatography afforded **5** in 67% yield. Removal of the acid labile protecting groups (Trt and methyl ester) under reflux for 12 h in 1 N HCl:Acetone (1:1.5) afforded **DC02** in 57% overall yield (two steps).

*Synthesis of 2-({9-[1-Carboxy-2-(1H-imidazol-4-yl)-ethylcarbamoyl]-[1,10]phenanthroline-2-carbonyl}-amino)-3-(1H-imidazol-4-yl)-propionic acid (**DC03**)*. [1,10]phenathrolindicarbonyl, 2,9-bis(histidine) coenzyme (**DC03**) was prepared

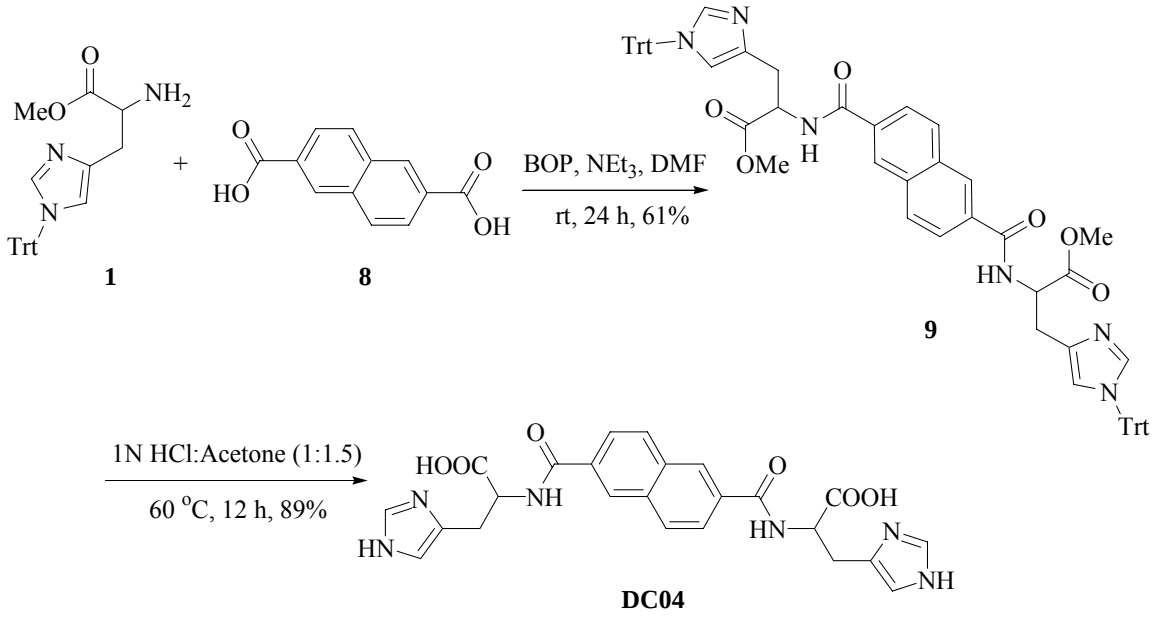
according to Scheme 5. The commercially available protected histidine (H-His(Trt)-OMe·HCl) **1** was coupled with 2,9-[1,10]phenanthrolinedicarboxylic acid **6** using BOP as the condensating agent furnishing the protected coenzyme after 24 h reaction (monitored by TLC). Precipitation of the crude product in ice water followed by filtration and further purification by flash chromatography afforded **7** in 35% yield. Removal of the acid labile protecting groups (Trt and methyl ester) under reflux for 12 h in 1 N HCl:Acetone (1:1.5) afforded **DC03** in 30% overall yield (two steps).




Scheme 5. Synthetic Scheme for **DC03**.

*Synthesis of 2-({6-[1-Carboxy-2-(1H-imidazol-4-yl)-ethylcarbamoyl]-naphthalene-2-carbonyl}-amino)-3-(1H-imidazol-4-yl)-propionic acid (**DC04**)*. Naphthalene, 2,6-bis(histidine) coenzyme (**DC04**) was prepared according to Scheme 6. The commercially available protected histidine (H-His(Trt)-OMe·HCl) **1** was coupled

with 2,6-naphthalenedicarboxylic acid **8** using BOP as the condensating agent furnishing the protected coenzyme after 24 h reaction (monitored by TLC). Precipitation of the crude product in ice water followed by filtration and further purification by flash chromatography afforded **9** in 61% yield. Deprotection of the acid labile protecting groups (Trt and methyl ester) under reflux for 12 h in 1 N HCl:Acetone (1:1.5) afforded **DC02** in 54% overall yield (two steps).




Scheme 6. Synthetic Scheme for **DC04**.

*In Vitro Selection of Catalytic DNA Strands that Use Bis-Histidine Pyridinedicarbonyl Coenzyme (**HMC01**)*

**HMC01** was initially synthesized in our laboratory and incorporated in a previously employed and well established *in vitro* selection experiment. The *in vitro* selection experiment was started with a DNA template (**T**) molecule that contained 40 random nucleotides equivalent to $4^{40}$ or $10^{24}$ potential sequences. PCR extension of 20 pmol of this DNA template (~$10^{15}$ different sequences) with primers **1** and **B2** afforded a

101 bp double-stranded DNA product designated as a "naïve pool." Each double-stranded DNA molecule in the naïve pool contained a potential self-cleaving strand and a non-catalytic complementary strand. The self-cleaving strands were composed (from 5' to 3') of a biotin moiety, a fixed sequence of deoxyribonucleotides, an adenosine ribonucleotide, 40 random deoxyribonucleotides and a second fixed sequence of deoxyribonucleotides. The fixed sequences of deoxyribonucleotides served as a primer recognition site and a base pairing design for DNA enzyme-substrate recognition binding (Figure 23).

After appropriately attaching the biotin-tagged strands of the naïve pool to a neutravidin affinity column and eluting the complementary strands, a negative selection in presence of binding buffer (50 mM HEPES, 0.5 M NaCl, 0.5 M KCl, 0.5 mM EDTA, pH 7.5 at rt) was performed. Negative selection would disfavor the isolation of metal-dependent deoxyribozymes or catalytic DNA molecules that use buffer components (HEPES, NaCl, KCl, EDTA and potential contaminant divalent metal ions present) as cofactors. EDTA, a chelating agent, was also included in our buffer in order to minimize the participation of trace amounts divalent metal ions (Mg, Zn, Pb, Ca, Mn and Cu) during the selection reaction. 1 h of negative selection was performed after which the column was thoroughly washed and re-equilibrated with binding buffer.

The DNA molecules, with coenzyme-dependent RNA phosphoester cleavage activity, were then eluted at room temperature over a period of 1-h reaction with reaction buffer (biding buffer containing 50 mM of **HMC01**). We decided to use 50 mM of coenzyme and 1 hour of selection reaction based on the report of a histidine-dependent catalytic polynucleotide[143] and expecting to observe comparable results. The eluted

molecules were ethanol precipitated and then PCR amplified with primers **1** and **2** to generate an adequate amount of copies of the selected DNA molecules. 10 µL of the PCR product were additionally PCR amplified with primers **1** and **B2** in order to reintroduce the biotin and the adenosine ribonucleotide moieties to the DNA strands. The amplified PCR product (101 bp DNA molecules) was gel purified and saved for the next round of selection (Figure 38). Decreasing reaction times for rounds 6-10 were chosen hoping that this selection pressure would have a positive impact of enhancing the catalytic rate of the selected DNA population.

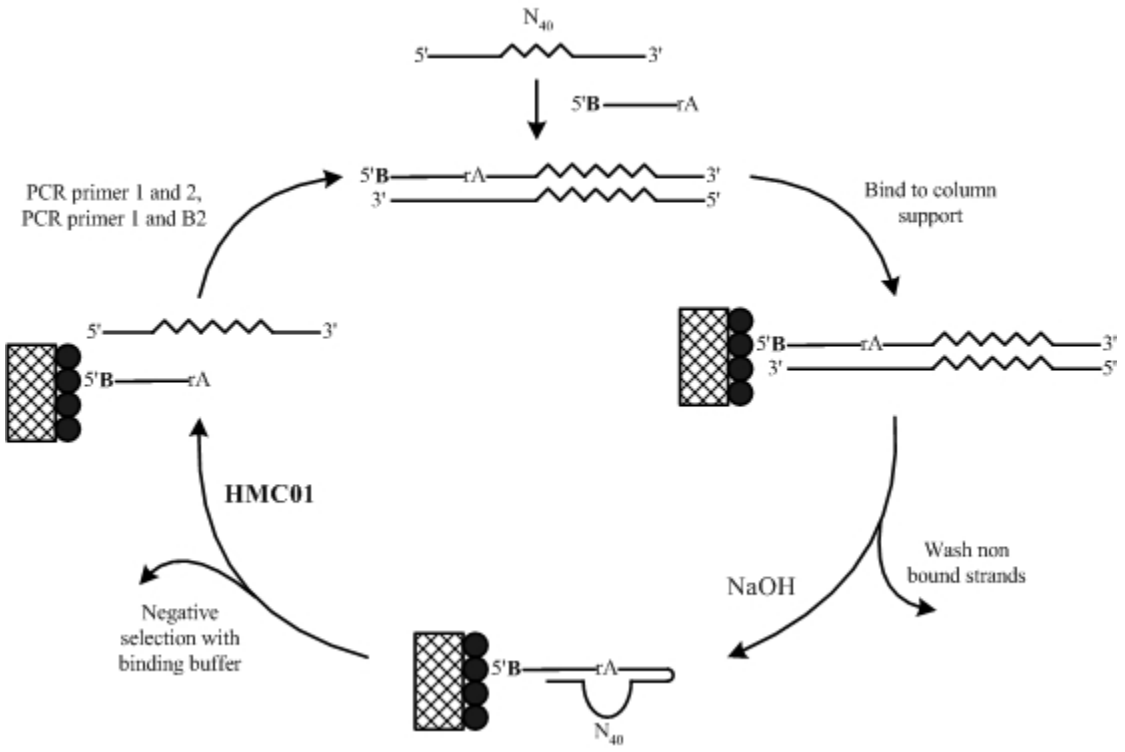



Figure 38. *In vitro* selection using **HMC01**.

After 10 rounds of selection, catalytic strands from the enriched DNA pool from rounds 8th, 9th and 10th were isolated, terminally radiolabeled using standard methods and assayed for self-cleavage under conditions equal to those employed during the *in vitro*

selection (50 mM HMC-01 in binding buffer). Self-cleavage experiments with these enriched DNA pools gave a preliminary indication of the presence of catalytic DNA molecules with coenzyme-dependent ribonucleotide phosphoester cleavage activity (Figure 39). Similar cleavage product in rounds 8, 9 and 10 suggested a peak activity and that additional rounds of selection were unnecessary.

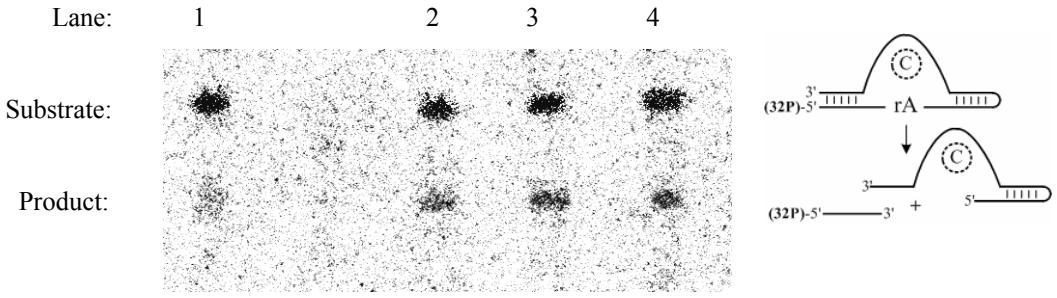



Figure 39**.** Self-cleavage reaction of G1 pools (rounds 8, 9 and 10) selected with **HMC01**. Lane 1, reaction without **HMC01**; Lane 2, 8th round DNA pool with 50 mM **HMC01**; Lane 3, 9th round DNA pool with 50 mM **HMC01**; Lane 4, 10th round DNA pool with 50 mM **HMC01**; 4 h reaction, pH 7.5, rt. 10% TBE-urea PAGE autoradiography.

DNA pool from the 10th round of selection (G1-HMC01) was subcloned using TA cloning kit (Promega), and individual molecules of this pool were sequenced in collaboration with Dr. Robert Adams (Baylor Biology Department, Baylor University). Evaluation of 24 of these clones gave 5 distinct sequences from which G105 appeared 8 times and the others in very low frequency (2-3 times) (Table 10). Comparative sequence analysis done by phylogeny construction showed two main groups of consensus sequences, one composed of G1-01, G1-04 and G1-05, and the other composed of G1-02 and G1-03 (Figure 40).

Table 10. G1-HMC01 individual sequences.

| Name | Frequency | Catalytic sequence (underlined corresponding to $N_{40}$) |
|---|---|---|
| G1-01 | 2 | 5'-ATCTCATTGCGTGTGATAAATTATTTAGAAGTCGTCAAGGCGGGGGTGAG-3' |
| G1-02 | 3 | 5'-ATCTCCTGCAGAGAATAAGCCTACTTGACTTCCCGAGTCACCGAGGTGAG-3' |
| G1-03 | 2 | 5'-ATCTCCTGCAGAGAATAGAGCCTACTTGACTTCCCGAGTCACCGGGTGAG-3' |
| G1-04 | 3 | 5'-ATCTCCTCTTCCATTTTCAATTCAGAAGAACTCGTCAAGAAGGCGGTGAG-3' |
| **G1-05** | **8** | 5'-ATCTCCTCGCTCGATGCGATGTTTCGCTTGGTGGTCGAGGGCAGGGTGAG-3' |

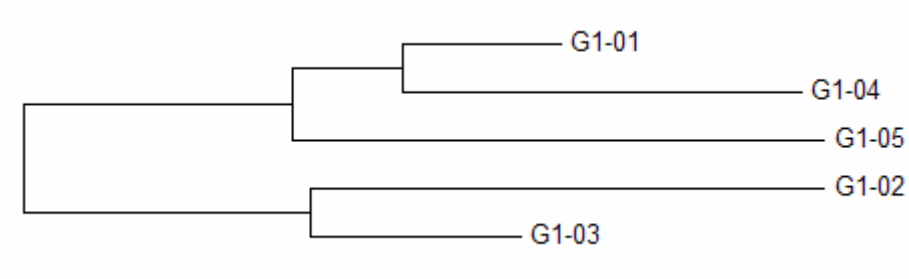



Figure 40. Artificial phylogeny construction of G1-HMC01.

Sequences obtained were chemically synthesized and incorporated in an intermolecular reaction (*trans*-reaction) with a DNA substrate embedded with an adenosine ribonucleotide (**S**). Each catalytic DNA strand contained (from 5' – 3') a base pairing or substrate recognition domain, the catalytic moiety (40 nucleotides) and a second substrate recognition domain, and was identified as (G1-T01, G1-T02, G1-T03, G1-T04 and G1-T05). The substrate **S** was 5' labeled with [$\gamma$-32P]ATP and purified according to standard methods. The *trans*-reaction was used as a quick screening to determine the 40-bases catalytic moiety with the most effective coenzyme-dependent ribonucleotide phosphoester cleavage activity. The catalytic assay was conducted in presence of 50 mM **HMC01** in buffer (50 mM HEPES, 0.5 M NaCl, 0.5 M KCl, 0.5 mM EDTA, pH 7.5 at rt) and in a single-turnover fashion: trace amount of substrate oligonucleotide (0.2 pmol) and excess of DNA catalyst (10 pmol). Reaction products

were separated in 15% denaturing polyacrylamide gel and detected by autoradiography (Figure 41).

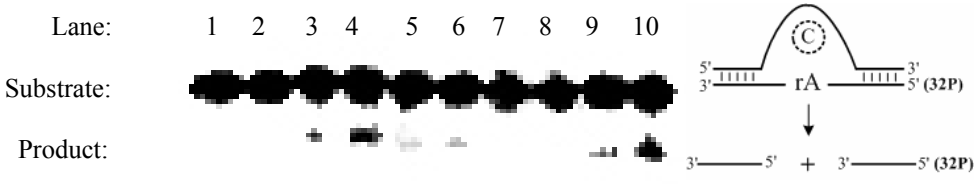



Figure 41**.** *Trans*-cleavage reaction of individual sequences of G1-HMC01. Lane 1 and 2, G1-T01; Lane 3 and 4, G1-T02; Lane 5 and 6, G1-T03; Lane 7 and 8, G1-T04; Lane 9 and 10, G1-T05; Lanes 1, 3, 5, 7 and 9, buffer without **HMC01**; Lanes 2, 4, 6, 8 and 10, reaction with 50 mM **HMC01**. 4 h reaction, pH 7.5, rt. 15% TBE-urea PAGE autoradiography.

Strands G1-T02 and G1-T05, each belonging to a different consensus sequence group, showed an apparent dependence on **HMC01** and a cleavage efficiency of 8-9%, others didn't show any detecTable cleavage product. G1-T02 and G1-T05 were further studied in order to see if the cleavage efficiency is improved with amplified reaction times. Amplified reaction times didn't improve the cleavage efficiency of either G1-T02 or G1-T05, while G1-T05 did always show a slightly higher cleavage product (12%).

A variation of G1-T05 was chemically synthesized as a ribonucleotide-containing self-cleaving DNA strand (G1-C05) according to Figure 23. This strand was subjected to a self-cleavage reaction (*cis*-reaction) in presence of **HMC01** in order to study whether or not these reaction conditions would improve upon the cleavage efficiency previously observed in the *trans*-cleavage reaction. This reaction was also considered as a model to give preliminary information about the rate constant of this coenzyme-DNA enzyme system. G1-C05 was 5' labeled with [$\gamma$-32P]ATP and purified according to standard methods. The *cis*-cleavage reaction was conducted in presence of 50 mM **HMC01** in

buffer (50 mM HEPES, 0.5 M NaCl, 0.5 M KCl, 0.5 mM EDTA, pH 7.5 at rt), products separated in 10% denaturing polyacrylamide gel and detected by autoradiography (Figure 42).

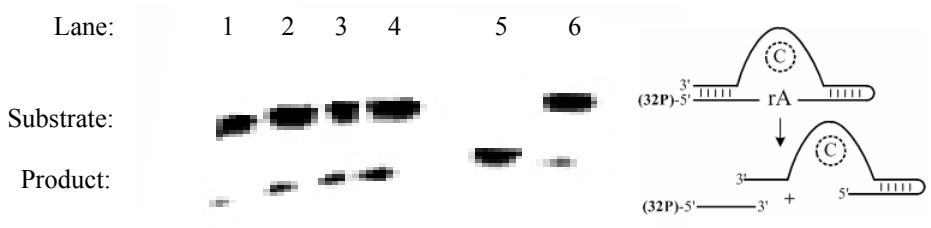



Figure 42**.** Self-cleavage reaction of G1-C05. Lane 1, 50 mM HMC01 reaction for 15 min; Lane 2, 50 mM HMC01 reaction for 4 h; Lane 3, 50 mM HMC01 reaction for 12 h; Lane 4, reaction for 24 h; Lane 5, alkaline hydrolysis with NaOH; Lane 6, reaction without **HMC01** for 24 h. pH 7.4 and rt. 10% TBE-urea PAGE autoradiography.

Self-cleavage reaction carried out with G1-C05 showed 12% maximum cleavage efficiency with an apparent rate constant ($k_{obs}$) of ~ 1 x $10^{-4}$ $min^{-1}$ and no apparent product in absence of the coenzyme HMC01 even at a longer reaction time. A secondary structure prediction of G1-05 using DNA *mfold* program (Dr. Michel Zuker, Washington University) provided a particularly unique structure (Figure 43) with a $\Delta G$ = -18.28 kcal/mol. This secondary structure prediction shows a non-paired adenosine ribonucleotide cleavage site (nucleotide number 23), the two base paring or recognition elements and the catalytic loop (from nucleotide 44 to 83). The catalytic moiety was recognized to contain regions of Watson-Crick pairing in different positions.

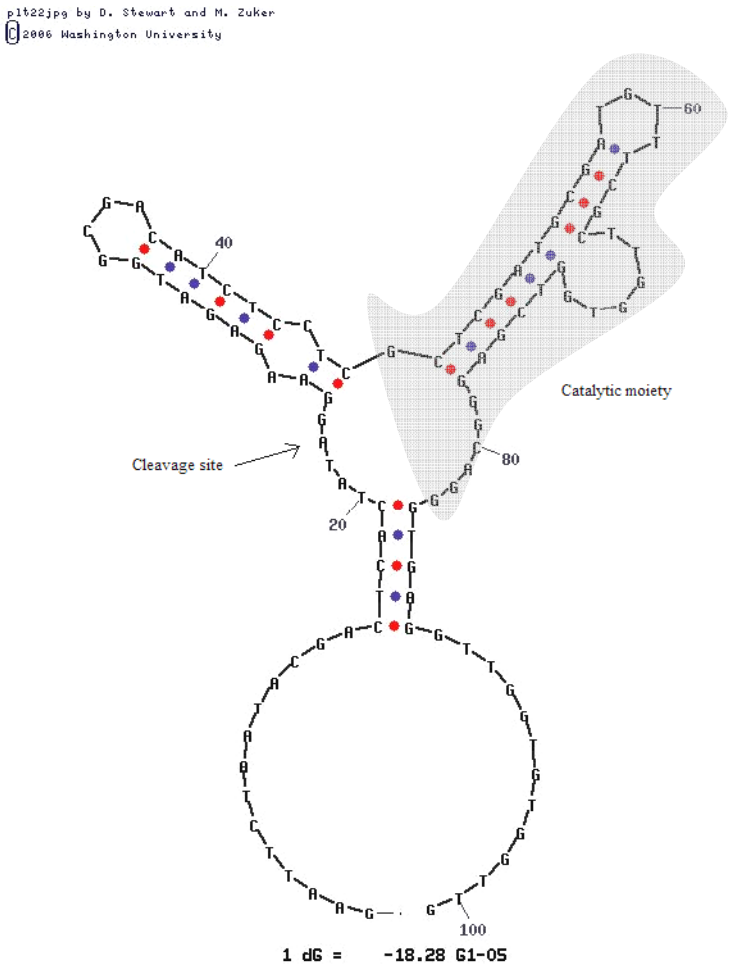



Figure 43. The most sTable secondary structure of G1-05. Predicted by the DNA *mfold* program (Dr. Michael Zuker, Washington University).

In an attempt to improve the enzymatic rate of the reaction catalyzed by G1-05 and our coenzyme **HMC01**, we chose to optimize the enzyme's sequence by reselection of a partially mutagenized G1-05. This randomly mutagenized family was expected to provide a new universe of DNA molecules that perchance were not present in the first selection given that only a restricted number of strands were taken ($10^{15}$ out of $10^{24}$ possible strands). The new DNA pool was synthesized based on the sequence of G1-05 with a degeneracy of 0.15 per position. The new molecules had an 85% probability of

keeping the original nucleotide, and a probability of mutation to the other three nucleotides at 5% each.[151]

20 pmol of the new library (mutagenized G1-C05) was subjected to an additional ten rounds of selection under progressively more stringent conditions. The concentration of the coenzyme **HMC01** was reduced to 5 mM, and in contrast to the first selection the reaction times were more drastically decreased:[151] rounds 1, 2, 3, 4 (60 min), round 5 (30 min), round 6 (30 min), round 7 (15 min), round 8 (15 min), round 9 (5 min) and round 10 (5 min). After 10 rounds of reselection a DNA pool (G2-HMC01) was obtained.

The different populations of DNA molecules obtained after the 8th, 9th and 10th round of reselection were assayed for self-cleavage activity under conditions identical to those used during the *in vitro* selection (5 mM HMC01) (Figure 44). For this assay, the catalytic strands from enriched DNA pools were isolated and terminally radiolabeled using standard methods.

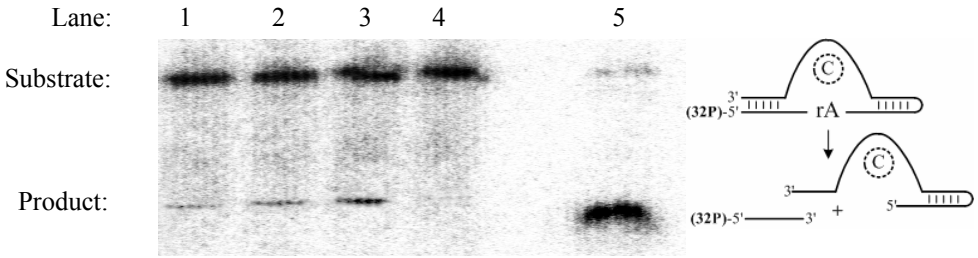



Figure 44**.** Self-cleavage reaction of G2 pools (rounds 8, 9 and 10) selected with **HMC01**. Lane 1, selected DNA 8th round with 5 mM **HMC01**; Lane 2, selected DNA 9th round with 5 mM **HMC01**; Lane 3, selected DNA 10th round with 5 mM **HMC01**; Lane 4, selected DNA 10th round without **HMC01**; Lane 5, selected DNA 10th round with NaOH. 4 h reaction, pH 7.5, rt. 10% TBE-urea PAGE autoradiography.

The second generation selected pool G2-HMC01 exhibited a modest level of activity (~ 10% cleavage). Self-cleavage reaction with G2-HMC01 in absence of the coenzyme **HMC01** didn't show any appreciable product. G2-HMC01 was subcloned

using TA cloning kit (Promega), and individual molecules of this pool were sequenced in collaboration with Dr. Robert Adams (Baylor Biology Department, Baylor University). Evaluation of 24 of these clones gave 5 distinct sequences, and the most abundant sequence G2-04 was present 9 times (Table 11). Comparative sequence analysis done by phylogeny construction showed that G1-05 (mutagenized sequence) was most closely related to G2-01 and G2-04 (Figure 45).

Table 11. G2-HMC01 individual sequences**.**

| Name | Presence | Catalytic sequence (underlined) corresponding to $N_{40}$ |
|---|---|---|
| G2-01 | 2 | 5'-CATCTCCTCGCTAGATGCGATATTACGCATGGGGGTCGAGGGCAAGGTGAG-3' |
| G2-02 | 2 | 5'-CATCTCACTGGGGTAGAATTAAATTGGGACCAAGGCTCTCTAGTGTGTGAG-3 |
| G2-03 | 2 | 5'-CATCTCGGTACCCAAGAAGGGCAACACATCTTCCGACATTCGTATTGTGAG-3' |
| **G2-04** | **9** | 5'-CATCTCATACAGTGTGATGAATACTTTGAATTGGGCGCGGGTGGGGTGAG-3' |
| G2-05 | 4 | 5'-CATCTCGTTGACTCCTTACTGTCCGATCTAAATATCTGACTGTGTTGTGAG-3' |

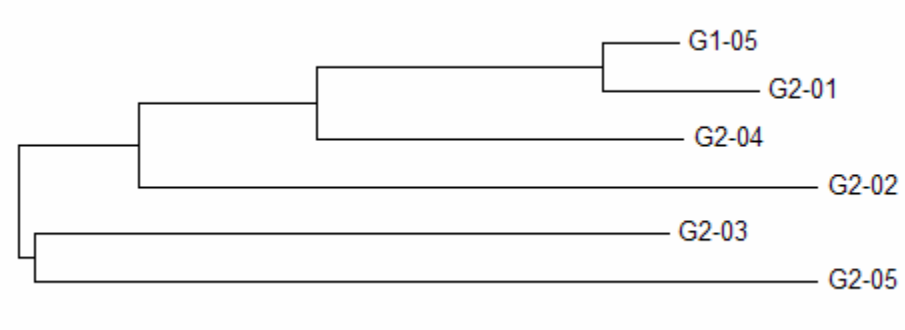



Figure 45. Artificial phylogeny construction of G2-HMC01 and G1-05.

Sequences obtained were chemically synthesized and incorporated in an intermolecular reaction (*trans*-reaction) with a DNA substrate embedded with an adenosine ribonucleotide (**S**). Each catalytic DNA strand contained (from 5' – 3') a base pairing or substrate recognition domain, the catalytic moiety and a second substrate

recognition domain and was identified as (G2-T01, G2-T02, G2-T03, G2-T04 and G2-T05). The substrate **S** was 5' labeled with [$\gamma$-32P]ATP and purified according to standard methods. The *trans*-reaction was used as a quick screening to determine the 40-bases catalytic moiety with the most effective coenzyme-dependent ribonucleotide phosphoester cleavage activity. The catalytic assay was conducted in presence of 5 mM **HMC01** in buffer (50 mM HEPES, 0.5 M NaCl, 0.5 M KCl, 0.5 mM EDTA, pH 7.5 at rt) and in a single-turnover fashion: trace amount of substrate oligonucleotide (0.2 pmol) and excess of DNA catalyst (10 pmol). Reaction products were separated in 15% denaturing polyacrylamide gel and detected by autoradiography (Figure 46).

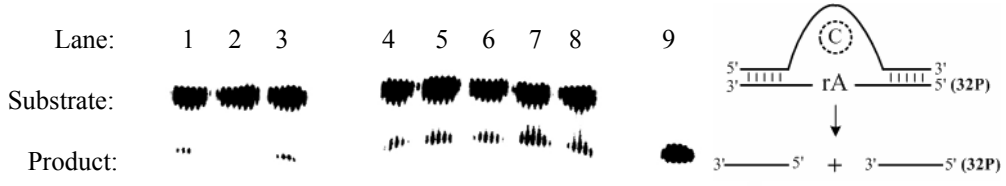



Figure 46**.** *Trans*-cleavage reaction of individual sequences of G2-HMC01. Lane 1, substrate in buffer; Lane 2, substrate with G2-T04 only; Lane 3, substrate with **HMC01** only; Lane 4, 5 mM HMC01 with G2-T01; Lane 5, 5 mM HMC01 with G2-T02; Lane 6, 5 mM HMC01 with G2-T03; Lane 7, 5 mM HMC01 with G2-T04; Lane 8, 5 mM HMC01 with G2-T08; Lane 9, substrate alkaline hydrolysis with NaOH. 4 h reaction, pH 7.5, rt. 15% TBE-urea PAGE autoradiography.

All the strands showed an apparent dependence on **HMC01** and a cleavage efficiency of 8-18%, with G2-T04 exhibiting a slightly better cleavage efficiency (18%). This molecule was also the one with the larger presence among the sequenced clones of G2-HMC01 (Table 11).

Sequence analysis of the 40-bases catalytic segment of G2-04 (5'-ATACAGTG TGATGAATACTTTGAATTGGGCGCGGGTGGG-3', the most predominant sequence after reselection) and G1-05 (5'-CTCGCTCGATGCGATGTTTCGCTTGGTGGTCGA

GGGCAGG-3', the most predominant and active sequence after selection) revealed some similarities in the base content of both: A guanidine rich segment toward the 3' end and a approximate 60% CG content. Although original naïve pool and G1-05 random-sequence domain included 40 nucleotides, G2-04 contains only 39 nucleotides that correspond to this region. This single nucleotide deletion was carefully analyzed. It could possibly be associated to a problem in the sequencing reaction related to a highly organized single-stranded DNA that wouldn't efficiently unfold under standard PCR denaturing methods. This single nucleotide deletion has also been observed by others[143] and has been attributed to the *in vitro* selection itself.


Figure 47. Self-cleavage reaction of G2-C04. 1, substrate without **HMC01**; 2, 30 min reaction; 3, 12 h reaction; 4, 24 h reaction; 4, substrate in NaOH. Substrate 2 pmol, 5 mM **HMC01** and buffer. 10% TBE-urea PAGE autoradiography.

A variation of G2-T04 was chemically synthesized as a ribonucleotide-containing self-cleaving DNA strand (G2-C04) according to Figure 23. This strand was subjected to a self-cleavage reaction (*cis*-reaction) in presence of **HMC01** in order to study whether or not these reaction conditions influence the cleavage efficiency previously observed in the *trans*-cleavage reaction. This reaction was also considered as a model to give preliminary information about the rate constant of this catalytic DNA strand. G2-C04 was 5' labeled with [$\gamma$-32P]ATP and purified according to standard methods. The *cis*-cleavage reaction was conducted in presence of 50 mM **HMC01** in buffer (50 mM

HEPES, 0.5 M NaCl, 0.5 M KCl, 0.5 mM EDTA, pH 7.5 at rt), products separated in 10% denaturing polyacrylamide gel and detected by autoradiography (Figure 47).

Using 5 mM of the coenzyme **HMC01** and after 24 h of self-cleavage reaction, G2-C04 showed 26% of cleavage efficiency and apparent $k_{obs}$ of 1.8 x $10^{-4}$ $min^{-1}$. This rate constant is very similar to the apparent rate constant calculated for the G1-C05 ($k_{obs}$ of 1 x $10^{-4}$ $min^{-1}$), but with G2-C04 exhibiting a higher cleavage efficiency (26 %). A secondary structure prediction of G2-04 using DNA *mfold* program (Dr. Michel Zuker, Washington University) provided two interesting structures (Figures 48 and 49) with ΔGs of -8.46 kcal/mol (A) and -9.28 kcal/mol (B). Structure A showed an unpaired adenosine ribonucleotide cleavage site (nucleotide number 23), the two base paring or recognition elements and the catalytic loop (from nucleotide 44 to 82) containing regions of Watson-Crick pairing in different positions. Structure B showed an unpaired adenosine ribonucleotide cleavage site (nucleotide number 23), three new base paring or recognition elements and a new catalytic loop (from nucleotide 44 to 50). Although structure A keeps the original self-cleaving design, structure B showed a completely different organization with new Watson-Crick paring sites, and the ribonucleotide substrate still available for cleavage (unpaired substrate).

A reselection was performed with a DNA pool composed of mutagenized strands on the basis of the sequence of G2-C04. A randomly mutagenized DNA pool was synthesized based on the sequence of G2-04 with a degeneracy of 0.15 per position. The new molecules had an 85% probability of keeping the original nucleotide, and a probability of mutation to the other three nucleotides at 5% each.[151]

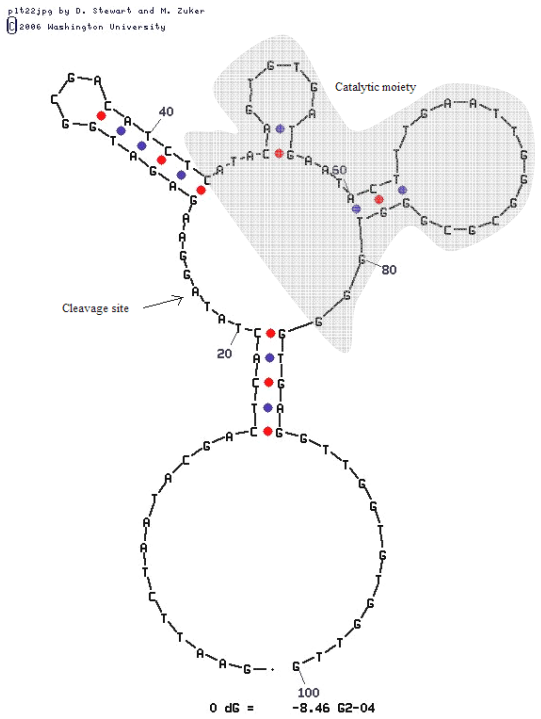



Figure 48. A secondary structure of G2-04 (A). Predicted by the DNA *mfold* program (Dr. Michael Zuker, Washington University).

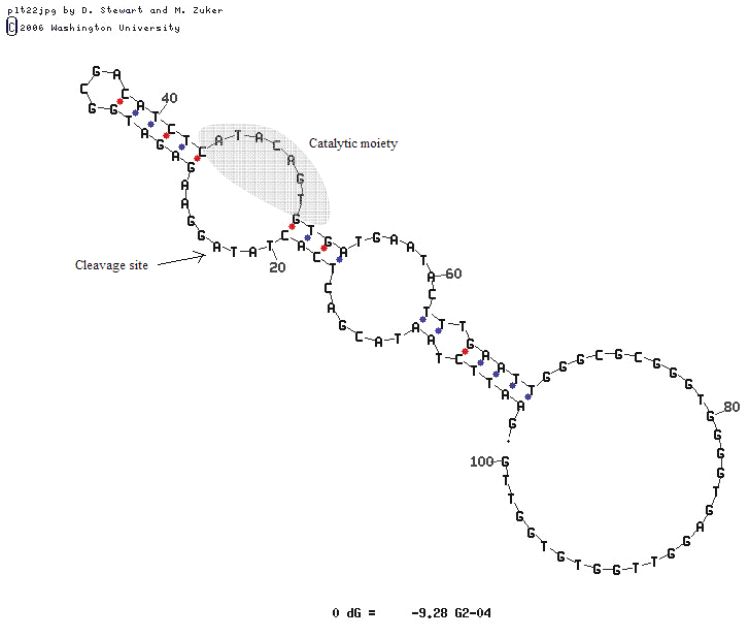



Figure 49. A secondary structure of G2-04 (B). Predicted by the DNA *mfold* program (Dr. Michael Zuker, Washington University).

20 pmol of the new library (mutagenized G2-C04) was subjected to an additional ten rounds of second reselection under progressively more stringent conditions. The concentration of the coenzyme **HMC01** in the selection reaction was reduced to 0.1 mM, and in contrast to the initial *in vitro* selection the reaction times were more drastically decreased:[151] rounds 1, 2, 3, 4 (60 min), round 5 (30 min), round 6 (30 min), round 7 (10 min), round 8 (10 min), round 9 (1 min) and round 10 (1 min). After 10 rounds of reselection a DNA pool (G2-HMC01) was obtained.

The different populations of DNA molecules obtained after the 8th, 9th and 10th round of reselection were assayed for self-cleavage activity under conditions identical to those used during the *in vitro* selection (0.1 mM HMC01) (Figure 50). For this assay, the catalytic strands from the enriched DNA pools were isolated and terminally radiolabeled using standard methods.

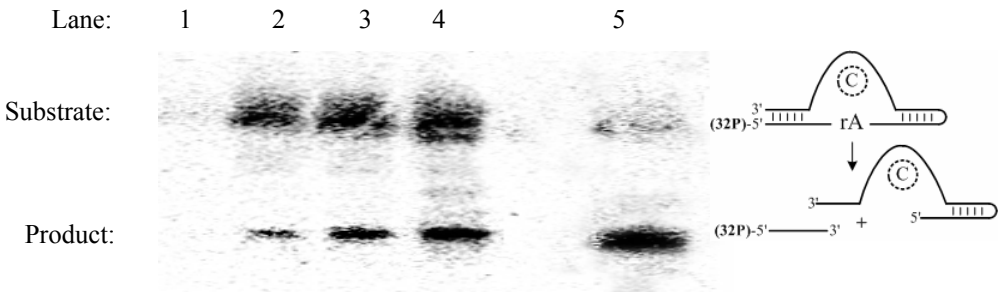



Figure 50**.** Self-cleavage reaction of G3 pools (rounds 8, 9 and 10) selected with **HMC01**. Lane 1, selected DNA 8th round with 5 mM **HMC01**; Lane 2, selected DNA 9th round with 5 mM **HMC01**; Lane 3, selected DNA 10th round with 5 mM **HMC01**; Lane 4, selected DNA 10th round without **HMC01**; Lane 5, selected DNA 10th round with NaOH. 4 h reaction, pH 7.5, rt. 10% TBE-urea PAGE autoradiography.

The G3-HMC01 (DNA pool obtained after the 10th round of second reselection) exhibited a moderate level of activity (~ 24% cleavage). G3-HMC01 was subcloned using TA cloning kit (Promega), and individual molecules of this pool were sequenced by

MCLAB (San Francisco, CA). Evaluation of 24 of these clones gave 19 complete nucleotide sequences. Of these 19 sequences, all were unique and with very small differences (4 to 5 bases different) among them (Table 12).

Table 12. G3- **HMC01** individual sequences.

| Name | Presence | Catalytic sequence (underlined) corresponding to $N_{40}$ |
|---|---|---|
| G3-01 | 1 | 5'-ATCTCAAACATTGTGATGAATACATTGAATTGGGAGTGGGCGGGGTGAGG-3' |
| G3-02 | 1 | 5'-ATCTCATACATTGTGCGGTATACTTTGAATTGGGTGCGGGTGGGGTGAGG-3' |
| G3-03 | 1 | 5'-ATCTCATAAAGTGAGAAGAATATTTCGAATTGGGTGCGGGTGGGGTGAGG-3' |
| G3-04 | 1 | 5'-ATCTCATACAGGGTGATCAATACTTGGAATTGGGCGTGGGTGGGGTGAGG-3' |
| G3-05 | 1 | 5'-ATCTCATACAGTGTGAAGAATACTTTGAATTGGGCGCGGGCGGGGTGAGG-3' |
| G3-06 | 1 | 5'-ATCTCCTACCGTGTGATGAATGCTTAGAATTGGGTGCGGGCGGGGTGAGG-3' |
| G3-07 | 1 | 5'-ATCTCATACACTGTGATGAATACTGTGAATTGGGTGCGGGTGGGGTGAGG-3' |
| G3-08 | 1 | 5'-ATCTCATACAGTGTGAGGAATACTATGAATTGGGCGCGGGTGGGGTGAGG-3' |
| G3-09 | 1 | 5'-ATCTCATACTGTGTTATGAATACTTTGAATTGGGCGCGGGTGGGGTGAGG-3' |
| G3-10 | 1 | 5'-ATCTCCTACAGGGTCTTGAATACTTAGAATTGGGCGCGGGTGGGGTGAGG-3' |
| G3-11 | 1 | 5'-ATCTCATACTGTGTGAATAATACTTTGAATTGGGAGTGGGTGGGGTGAGG-3' |
| G3-12 | 1 | 5'-ATCTCACACTGTGTGAGGCATACTTTGAATTGGGCGCGGGTGGGGTGAGG-3' |
| G3-13 | 1 | 5'-ATCTCATACAGTGTGATGAATCCTTTGAATTGGGCGAGGGTGGGGTGAGG-3' |
| G3-14 | 1 | 5'-ATCTCAGACAGTGTGATGAATACTTAGAATTGGGCGCGGGTGGGGTGAGG-3' |
| G3-15 | 1 | 5'-ATCTCGTACAGTGTGATGAATACTTTGAATTGGGAGCGGGTGGGGTGAGG-3' |
| G3-16 | 1 | 5'-ATCTCACAGACTGTGAGGAATACTGTGAATTGGGTGCGGGTGGGGTGAGG-3' |
| G3-17 | 1 | 5'-ATCTCATACCGTGTGATGAATAATTTGAATTGGGCGCGGGTGGGGTGAGG-3' |
| G3-18 | 1 | 5'-ATCTCATACAGGGTCAGGAATACTTTGAATTGAACGCCCCTCGGGTGAGG-3' |
| G3-19 | 1 | 5'-ATCTCATTCAATAGGATGGATCATTTGAATTGGGCGCGGGTGGGGTGAGG-3' |

Alignment of these sequences using MEGA 3.1 (Molecular Evolutionary Genetics Analysis Software) provided by Dr. Eric Baker (Baylor University) showed the highly conserved DNA sequences obtained after this second reselection process and their resemblance to their progenitor (G2-04) (Figure 51).

```
#G2-04 ATCTCATACA GTGTGATGAA TACTTTGAAT TGGGCGCGGG TGGGGTGAGG
#G3-01 ......A... T......... ...A...... ....A.T... C.........
#G3-02 .......... T....CG.T. .......... ....T..... ..........
#G3-03 ........A. ...A..A... ..T..C.... ....T..... ..........
#G3-04 .......... .G.....C.. .....G.... ......T... ..........
#G3-05 .......... ......A... .......... .......... C.........
#G3-06 .....C...C .......... .G...A.... ....T..... C.........
#G3-07 .......... C......... ....G..... ....T..... ..........
#G3-08 .......... ......G... ....A..... .......... ..........
#G3-09 .........T ....T..... .......... .......... ..........
#G3-10 .....C.... .G..CT.... .....A.... .......... ..........
#G3-11 .........T ......AT.. .......... ....A.T... ..........
#G3-12 ......C..T ......G.C. .......... .......... ..........
#G3-13 .......... .......... .C........ ......A... ..........
#G3-14 ......G... .......... .....A.... .......... ..........
#G3-15 .....G.... .......... .......... ....A..... ..........
#G3-16 ......C.G. C.....G... ....G..... ....T..... ..........
#G3-17 .........C .......... ..A....... .......... ..........
#G3-18 .......... .G..C.G... .......... ..AA...CCC .C........
#G3-19 .......T.. A.AG....G. .CA....... .......... ..........
```

Figure 51. Alignment of sequences obtained in G3-HMC01.

The sequences obtained were chemically synthesized and incorporated in an intermolecular reaction (*trans*-reaction) with a DNA substrate embedded with an adenosine ribonucleotide (**S**). Each catalytic DNA strand contained (from 5' – 3') a base pairing or substrate recognition domain, the catalytic moiety and a second substrate recognition domain and was identified as (G3-T01, G3-T02, G3-T03, G3-T04, G3-T05, etc). The substrate **S** was 5' labeled with [$\gamma$-32P]ATP and purified according to standard methods. The *trans*-reaction was used as a quick screening to determine the 40-bases catalytic moiety with the most effective coenzyme-dependent ribonucleotide phosphoester cleavage activity. The catalytic assay was conducted in presence of 0.1 mM **HMC01** in buffer (50 mM HEPES, 0.5 M NaCl, 0.5 M KCl, 0.5 mM EDTA, pH 7.5 at rt) and in a single-turnover fashion: trace amount of substrate oligonucleotide (0.2 pmol) and excess of DNA catalyst (10 pmol). Reaction products were separated in 15% denaturing polyacrylamide gel and detected by autoradiography (Figure 52).

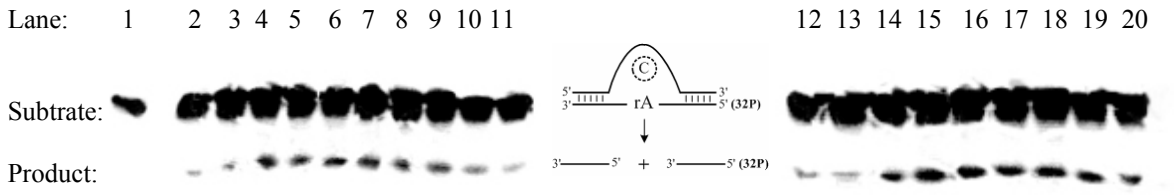



Figure 52. *Trans*-cleavage reaction of individual sequences of G3-HMC01. Lanes 2-20 represent sequences G3-01 to G3-19. Lane 1, DNA pool without **HMC01**. 15% TBE-urea PAGE autoradiography.

All the strands showed an apparent dependence on **HMC01** and many of them a cleavage efficiency of 10-20%, being G3-T14 the catalytic strand with the slightly better cleavage efficiency (20%). This strand was chemically synthesized as a ribonucleotide-containing self-cleaving DNA strand (G3-C14) according to Figure 23, and subjected to a self-cleavage reaction (*cis*-reaction) in presence of **HMC01** in order to learn whether or not these reaction conditions affect the cleavage efficiency previously observed in the *trans*-cleavage reaction. This reaction was also considered as a model to give preliminary information about the rate constant of this catalytic DNA strand. G2-C04 was 5' labeled with [$\gamma$-32P]ATP and purified according to standard methods. The *cis*-cleavage reaction was conducted in presence of 50 mM **HMC01** in buffer (50 mM HEPES, 0.5 M NaCl, 0.5 M KCl, 0.5 mM EDTA, pH 7.5 at rt), products separated in 15% denaturing polyacrylamide gel and detected by autoradiography (Figure 53).

Using 0.1 mM of the coenzyme **HMC01** and after 4 h of self-cleavage reaction with G3-C014, 28% of cleavage efficiency was observed and with an apparent $k_{obs}$ of 1.1 x $10^{-3}$ $min^{-1}$. This rate constant is 10-fold faster than the apparent rate constant calculated for the G1-C05 ($k_{obs}$ of 1 x $10^{-4}$ $min^{-1}$) and with similar cleavage efficiency.

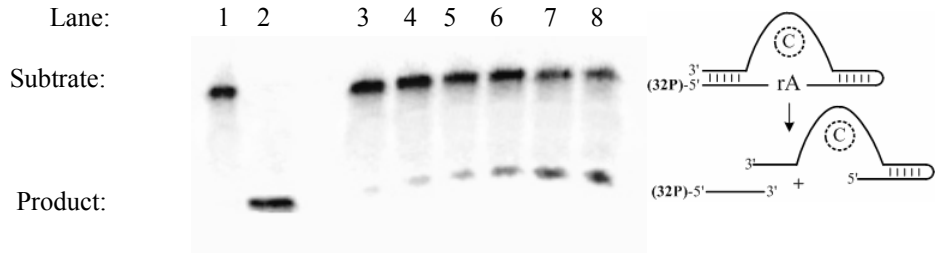



Figure 53. Self-cleavage reaction of G3-C14. Lane 1, substrate without **HMC01**; Lane 2, alkaline hydrolysis with NaOH, Lane 3, 5 min reaction; Lane 4, 30 min reaction, Lane 5, 60 min reaction; Lane 6, 120 min reaction; Lane 7, 180 min reaction; Lane 8, 240 min reaction. 0.1 mM **HMC01**, pH 7.5, rt.

A secondary structure prediction of G3-C14 using DNA *mfold* program (Dr. Michel Zuker, Washington University) provided a particularly unique structure (Figure 54) with a $\Delta G$ = -14.46 kcal/mol. This secondary structure prediction showed an unpaired adenosine ribonucleotide cleavage site (nucleotide number 23), two new base paring or recognition elements (completely different to the originally designed) and an apparent smaller catalytic loop (from nucleotide 44 to 50). Judging from the prediction and the base composition of G3-C14 and G2-C04, it is interesting to note that minimal modifications in the base composition of these selected catalytic molecules make a substantial change not only in their calculated free energies but also in their obtained experimental rate constants (G3-C14 $k_{obs}$ = 1.1 x $10^{-3}$ $min^{-1}$, and G2-C04 $k_{obs}$ = 1.8 x $10^{-4}$). Mutation and reselection with DNA pool originated from a mutagenized G2-C024 made possible to obtain G3-C04, a two-bases dissimilar and 10-fold faster catalytic DNA strand.

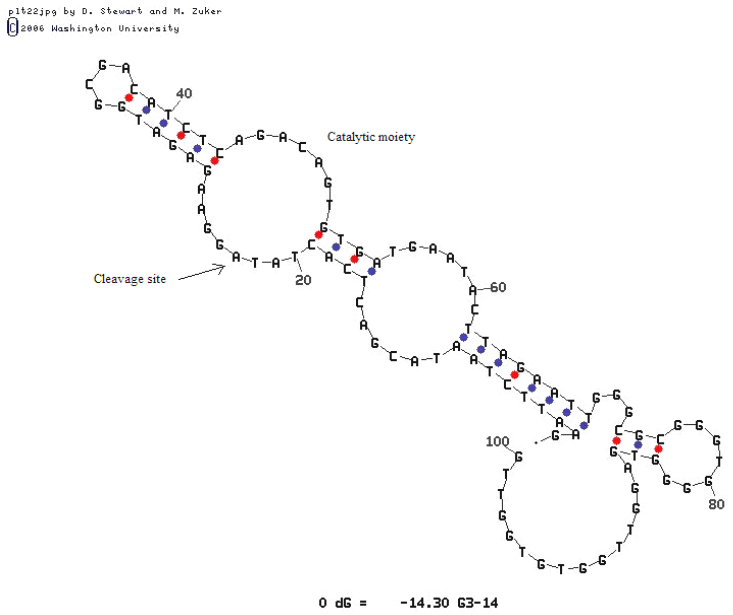



Figure 54. The most stable secondary structure of G3-14. Predicted by the DNA *mfold* program (Dr. Michael Zuker, Washington University).

*In vitro Parallel Selection of Catalytic DNA pools using bis-Histidine Aromatic coenzymes (**DC01**, **DC02**, **DC03** and **DC04**)*

In light of the previous results, we decided to explore more broadly the structure of the coenzyme **HMC01** by looking at a small panel of prospective coenzymes. A set of four potential coenzymes were synthesized and carefully characterized (**DC01, DC02, DC03 and DC04**). The aspirant coenzymes each contain two histidine residues connected via amide bonds to a simple aromatic diacid core (Figure 33). These aromatic cores (benzene, pyridine, phenanthrolin and naphthalene) would serve as potential DNA intercalators and would give to the coenzymes a way to interact with the catalytic DNA molecules. Simultaneously, these cores would generate a particular geometry in each coenzyme when distributing the two potentially catalytic histidines to specific positions. Four different catalytic DNA pools were expected after performing the parallel selection, and different catalytic activity expected for each coenzyme-DNA enzyme system. This

methodology represents a rapid way to produce catalytic DNA enzymes and to screen potential coenzymes.

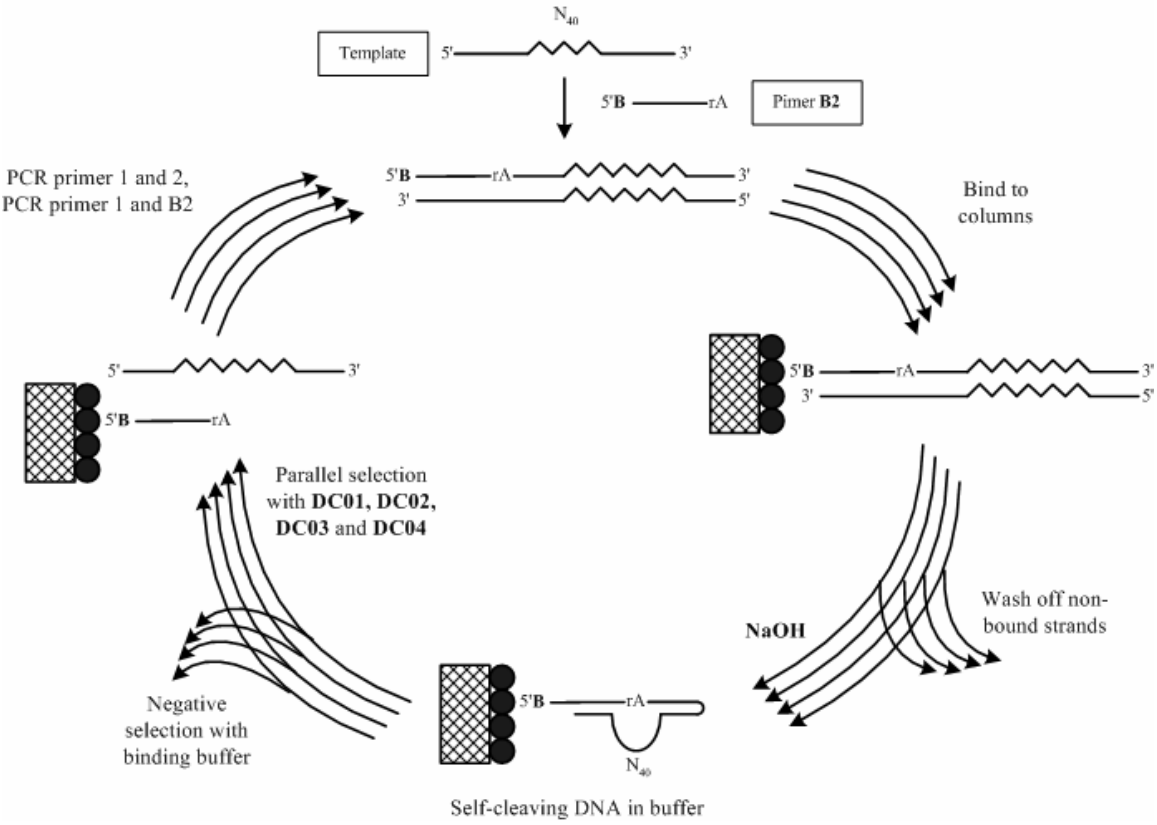



Figure 55. Parallel *in vitro* selection using **DC01**, **DC02**, **DC03** and **DC04**. Selection starts with the PCR amplification of the template, followed by the immobilization of the DNA in resin and exposure of the DNA to a designated coenzyme to favor hydrolysis of embedded ribonucleotide.

Compounds **DC01, DC02, DC03 and DC04** were in incorporated in a parallel *in vitro* selection experiment (Figure 55) aiming to obtain families (pools) of DNA enzymes that use these small molecules for the cleavage of a ribonucleotide phosphoester substrate.

As described for the *in vitro* selection using **HMC-01**, the *in vitro* selection was initiated with a DNA template (**T**) molecule that contained 40 random nucleotides

equivalent to $4^{40}$ or $10^{24}$ different DNA molecules. PCR extension of 80 pmol of this DNA template (~ $10^{15}$ different DNA molecules) with primers **1** and **B2** afforded a 101 bp DNA product designated as a "naïve pool." Each amplified DNA molecule in the naïve pool contained a self-cleaving strand (with embedded adenosine ribonucleotide and with a 5' biotin moiety) and a complementary non-biotinylated strand. Self-cleaving strands were composed (from 5' to 3') of a biotin moiety, a fixed sequence of deoxyribonucleotides, an adenosine ribonucleotide, 40 random deoxyribonucleotides and a second fixed sequence of deoxyribonucleotides. Each fixed sequence of deoxyribonucleotides served as a primer recognition site and a base pairing design for DNA enzyme-substrate recognition binding (Figure 23).

This naïve pool was equally divided in four parts and each immobilized on a solid support containing 100 μL of neutravidin protein gel. After removing the non-biotinylated DNA strands off the column under standard denaturing methods, the self-cleaving DNAs were incubated in binding buffer (50 mM HEPES, 0.5 M NaCl, 0.5 M KCl, 0.5 mM EDTA, pH 7.5 at rt) for 1 hour (negative selection). Negative selection would disfavor the isolation of metal-dependent deoxyribozymes or catalytic DNA molecules that use buffer components (HEPES, NaCl, KCl, EDTA and potential divalent metal ions present) as cofactors. EDTA, a chelating agent, was included in our buffer in order to minimize the participation of possibly present divalent metal ions (Mg, Zn, Pb, Ca, Mn and Cu) during the reaction. After negative selection the column was thoroughly washed and re-equilibrated with binding buffer. Each pool was then exposed to a single coenzyme (50 mM in binding buffer) for 4 h at room temperature (selection reaction). We decided to use the same concentration of coenzyme (50 mM) in the selection reaction

consistent with the procedures used to select the catalytic DNA molecules previously obtained with **HMC01**. The 4 h of initial incubation was used based on a recent report of the dynamics of vitro selection experiments.[142] This report suggested that longer initial selection reaction time avoids losing potential catalytic strands and favors the establishment of robust catalytic DNA populations. Reaction times for rounds 1 to 5 were consistent at 4 h. Decreasing reaction times for rounds 6-10 were chosen hoping that this selection pressure would have a positive impact of enhancing the catalytic rate of the selected DNA population (round 5 (2 h), round 6 (1 h), round 7 (30 min), round 8 (15 min), round 9 (5 min) and round 10 (1 min)). Four enriched DNA pools were obtained after 10 rounds of selection: pool DC01, pool DC02, pool DC03 and pool DC04 (selected with coenzymes **DC01**, **DC02**, **DC03** and **DC04**, respectively).

Catalytic sequences of the enriched DNA pools were isolated, 5' radiolabeled using standard methods and assayed for self-cleavage in reaction buffer containing 50 mM of each coenzyme (**DC01**, **DC02**, **DC03** and **DC04)**. The self-cleavage experiment was designed as shown in Table 5 and the reaction products separated on a 10% denaturing polyacrylamide gel (Figure 56).

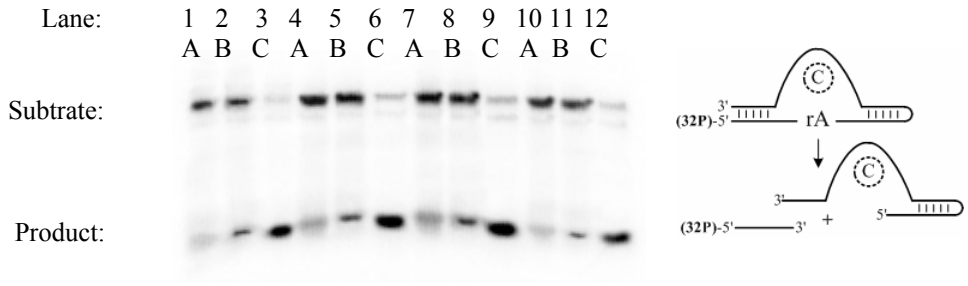



Figure 56. Self-cleavage reaction of the 10th round DNA pools selected with **DC01**, **DC02**, **DC03** and **DC04**. Pool DC01 (lanes 1, 2 and 3); Pool DC01 (lanes 4, 5 and 6) Pool DC01 (lanes 7, 8 and 9) Pool DC01 (lanes 10, 11 and 12). A, negative control; B, reaction in presence of associated coenzyme; C, chemical hydrolysis. Reaction conditions 1 hour. 10% TBE-urea PAGE autoradiography.

All four DNA pools showed an apparent activity in presence of their respective coenzyme with cleavage efficiency of 10-14%. Each DNA pool was found to be competent to self-cleave in the presence of its cognate coenzyme, and DNA pools DC01, DC02, and DC03 did not exhibit measurable cross-reactivity in the presence of the unmatched coenzymes (Figure 57). Calculations are presented in Table 13.

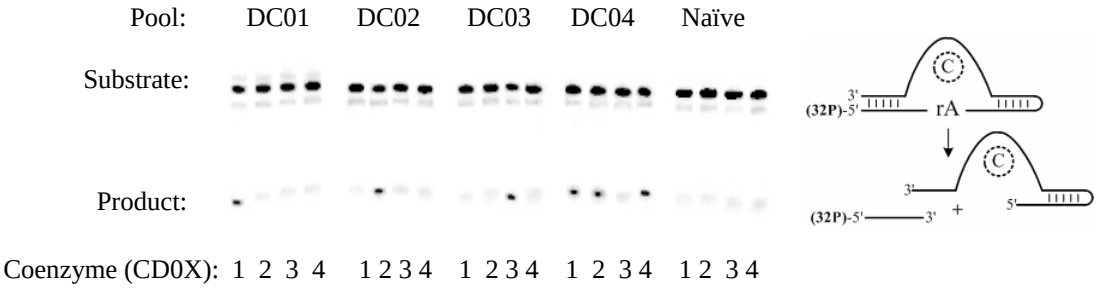



Figure 57. Cross-reactivity of the 10th round DNA pools selected with **DC01**, **DC02**, **DC03** and **DC04**. Each of the four DNA pools obtained after 10 rounds of selection were allowed to react with each of the potential coenzymes (10mM in buffer) for 4 h at room temperature. Pool DC01 was a DNA selected using coenzyme **DC01**, etc. Naïve pool is the naïve DNA library before selection. 10% TBE-urea PAGE autoradiography.

Table 13. Pools DC01, DC02, DC03 and DC04 cross-reactivity calculations for the cleavage efficiency.

| Pool | DC01 | DC02 | DC03 | DC04 | DC04 | DC04 |
|---|---|---|---|---|---|---|
| Coenzyme | **DC01** | **DC02** | **DC03** | **DC01** | **DC02** | **DC04** |
| Subst. (ODu*mm$^2$) | 0.8435 | 1.103 | 1.065 | 1.15 | 1.27 | 0.918 |
| Prod. (ODu*mm$^2$) | 0.0897 | 0.111 | 0.128 | 0.082 | 0.093 | 0.1375 |
| % | 10 | 9 | 11 | 7 | 7 | 13 |

Initial kinetic studies of the self-cleavage reaction revealed that in the presence of the appropriate coenzymes, catalytic DNA pools DC01, DC02, and DC03 catalyzed the desired hydrolysis reactions to a similar extent, while the more promiscuous catalytic DNA pool DC04 was less active (Tables 14-17, Figures 58-61).

Table 14. Kinetic data of pool DC01.

| Time (min) | Fraction cleaved | $k_{obs}$ $min^{-1}$ | Log($k_{obs}$) | Fraction uncleaved | Ln(fract. uncleaved) |
|---|---|---|---|---|---|
| 5 | 0.02 | 0.004 | -2.397 | 0.98 | -0.008 |
| 15 | 0.07 | 0.004 | -2.330 | 0.93 | -0.031 |
| 30 | 0.12 | 0.004 | -2.397 | 0.88 | -0.055 |
| 45 | 0.12 | 0.002 | -2.574 | 0.88 | -0.055 |
| 60 | 0.14 | 0.002 | -2.632 | 0.86 | -0.065 |

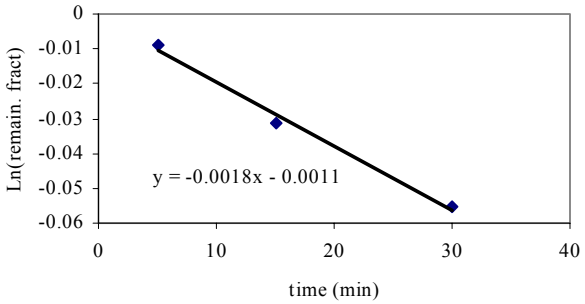



Figure 58. Calculation of rate constant for pool DC01.

Table 15. Pool DC02 kinetic data.

| Time (min) | Fraction cleaved | $k_{obs}$ $min^{-1}$ | Log($k_{obs}$) | Fraction uncleaved | Ln(fract. uncleaved) |
|---|---|---|---|---|---|
| 5 | 0.02 | 0.004 | -2.397 | 0.98 | -0.008 |
| 15 | 0.03 | 0.002 | -2.698 | 0.97 | -0.013 |
| 30 | 0.09 | 0.003 | -2.522 | 0.91 | -0.040 |
| 45 | 0.12 | 0.002 | -2.574 | 0.88 | -0.055 |
| 60 | 0.12 | 0.002 | -2.698 | 0.88 | -0.055 |

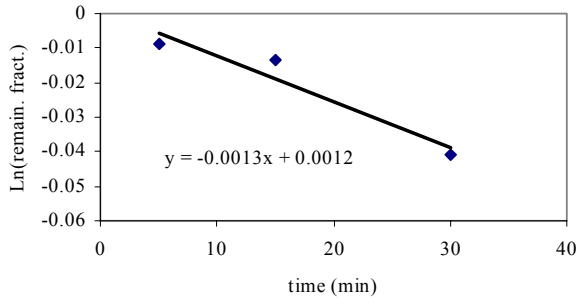



Figure 59. Calculation of rate constant for pool DC02.

Table 16. Pool DC03 kinetic data.

| Time (min) | Fraction cleaved | $k_{obs}$ $min^{-1}$ | Log($k_{obs}$) | Fraction uncleaved | Ln(fract. uncleaved) |
|---|---|---|---|---|---|
| 5 | 0.05 | 0.010 | -2.000 | 0.95 | -0.022 |
| 15 | 0.06 | 0.004 | -2.397 | 0.94 | -0.026 |
| 30 | 0.11 | 0.003 | -2.435 | 0.89 | -0.050 |
| 45 | 0.12 | 0.002 | -2.574 | 0.88 | -0.055 |
| 60 | 0.12 | 0.002 | -2.698 | 0.88 | -0.055 |

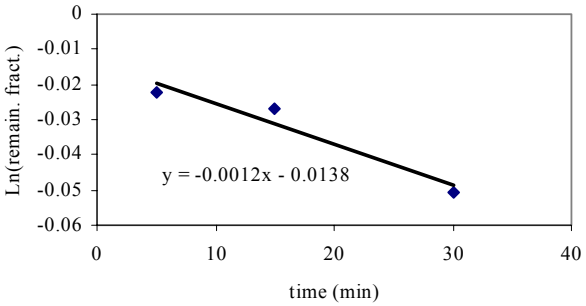



Figure 60. Calculation of rate constant for pool DC03.

Table 17. Pool DC04 kinetic data.

| Time (min) | Fraction cleaved | $k_{obs}$ $min^{-1}$ | Log($k_{obs}$) | Fraction uncleaved | Ln(fract. uncleaved) |
|---|---|---|---|---|---|
| 5 | 0.03 | 0.006 | -2.221 | 0.97 | -0.013 |
| 15 | 0.03 | 0.002 | -2.698 | 0.97 | -0.013 |
| 30 | 0.04 | 0.001 | -2.875 | 0.96 | -0.017 |
| 45 | 0.07 | 0.001 | -2.808 | 0.93 | -0.031 |
| 60 | 0.14 | 0.002 | -2.632 | 0.86 | -0.065 |

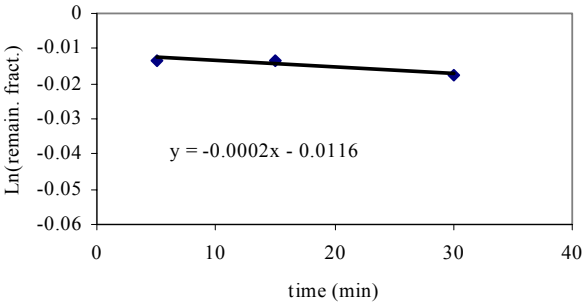



Figure 61. Calculation of rate constant for pool DC04.

The rate constant ($k_{obs}$) for each DNA pool was calculated as during the initial velocity of the reaction by plotting the natural logarithm of the fraction of substrate uncleaved over time where the negative slope of the line represent the $k_{obs}$. Instead of 60 min of initial reaction, we consider 30 min because of better adherence ($R^2 > 0.9$) to the linear regression trend line. The $k_{obs}$ for these enzymes are similar to rates previously measured for the DNA enzymes selected with $Mg^{2+}$ ($0.002min^{-1}$ after 6 rounds),[109] histidine ($0.0015min^{-1}$ after 11 rounds),[143] or in the absence of divalent metal-ions or added cofactors ($0.001min^{-1}$ after 12 cycles).[151] The rate of non-enzymatic cleavage of the initial naïve pool in the presence of each of the synthetic coenzymes was determined to be ~4 x $10^{-6}$ $min^{-1}$, or approximately ~10x faster than the rate previously observed for background cleavage of RNA by free histidine,[143] potentially due to increased molecular complexity and dimeric nature of the synthetic coenzymes. In any case, the rate enhancement by these enzyme families over the background reaction is less than the comparison DNA enzymes referred to previously, $Mg^{2+}$ (~2000 fold after 6 rounds)[109] or histidine (~4000 fold after 11 rounds).[143] In fact, the rate enhancements showed by DNA pools DC01-DC04 is similar to that seen in allosteric ribozymes.[208]

Accordingly, in order to discover whether compounds **DC01-DC04** served as functional coenzymes or as allosteric effectors a series of pH studies were performed. The pH profiles for enzyme/coenzyme systems **DC01** and **DC03** are strikingly similar to that previously observed for the histidine-dependent deoxyribozyme. The pH curves for **DC02** and **DC04** were more complex and not readily interpretable. When log($k_{obs}$) is graphed against pH for **DC01** and **DC03**, the rates initially increases until a plateau is reached. The slope of the linear part of each graph is ~1 (0.89 for **DC01**; 1.2 for **DC03**)

(Figures 62 and 63), suggesting the protonation of a single functional group, and the calculated residue p$K_a$'s (6.0 for **DC01**; 6.3 for **DC03**) suggest the involvement of the imidazole residues in the catalysis.

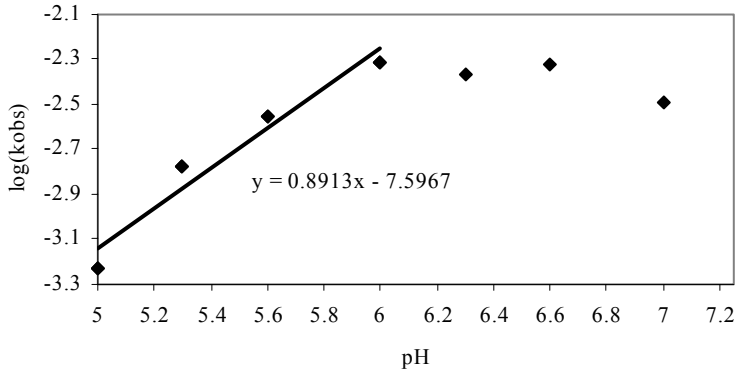



Figure 62. pH profile of pool DC01.

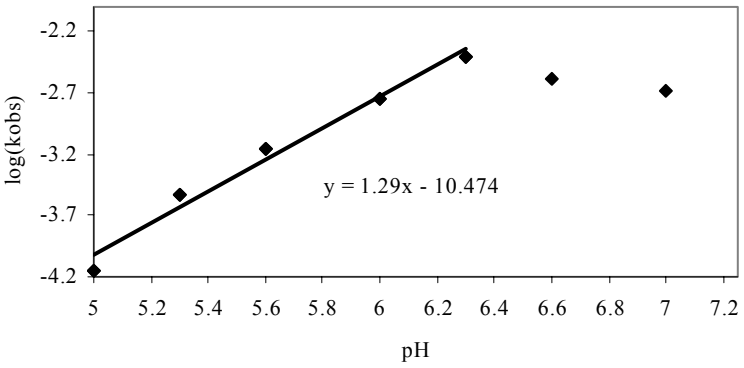



Figure 63. pH profile of pool DC03.

The coenzyme specificity observed for catalytic DNA pools DC01, DC02, and DC03 is notable. The observation that cleavage in pools DC01 and DC02 is not catalyzed by the non-cognate coenzyme (pool DC01 with coenzyme **DC02**, or pool DC02 with coenzyme **DC01**) is especially remarkable, as these coenzymes differ only by one atom. The promiscuity observed in DNA pool DC04 can be rationalized by

proposing that only one of the aryl-bound histidine residues in coenzyme **DC04** can occupy the enzyme at any given instant. Since coenzymes **DC01** and **DC02** can each present a single histidine attached through an aromatic molecule, it is possible that these coenzymes can occupy the binding site in catalytic DNA pool DC04. The amplified 'bent' structure of the aryl linker found in compound **DC03**, on the other hand, may preclude it's binding into the coenzyme **DC04** pocket. Finally, the study of the affinity of the catalytic DNA pools with their cognate coenzymes showed a binding pocket in DNA pool DC04 that was not saturated at concentrations as high as 100 mM while the apparent binding constant (Kd) for the other pools (DC01, DC02 and DC03) has been calculated to be ≈ 10 mM (Figures 64-67). This can be considered as another reason for the promiscuity observed in DNA pool DC04.

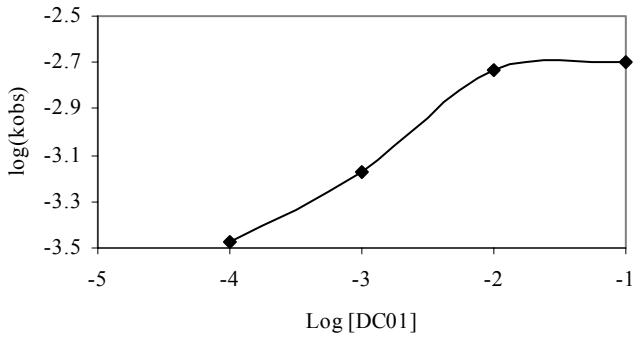



Figure 64. $k_{obs}$ versus concentration (M) of pool DC01.

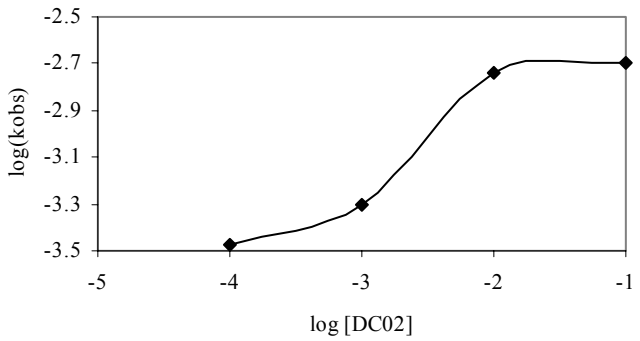



Figure 65. $k_{obs}$ versus concentration (M) of pool DC02.

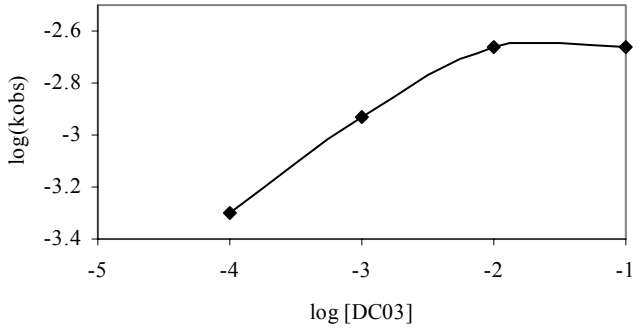



Figure 66. $k_{obs}$ versus concentration (M) of pool DC03.

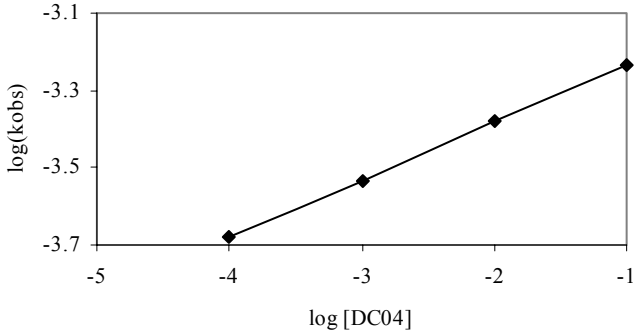



Figure 67. $k_{obs}$ versus concentration (M) of pool DC04.

*Conclusions*

Four prospective coenzymes for DNA enzymes have been designed and synthesized (**DC01**, **DC02**, **DC03** and **DC04**). We have demonstrated that combinatorial selection techniques, such as used parallel *in vitro* selection, can be used to rapidly select for coenzyme-dependent DNA enzymes.

**HMC01**, a potential coenzyme, was designed based on poorly effective artificial ribonucleases. **HMC01** was equipped with two histidines that normally constitute the catalytic domain of natural enzymes. Additionally, the *bis*-histidine containing **HMC01** was conjugated to a 2,6-dicarbonyl pyridine ring capable of interacting with RNA. *In vitro* selection of ribonucleotide phosphoester-cleaving DNA enzymes in presence **HMC01** was established as a model experiment to obtain catalytic DNA molecules that truly use a synthetic small molecule as coenzyme, with metal ions excluded by negative selection and EDTA chelating agent. After 10 rounds of *in vitro* selection using progressively reduced reaction times, a DNA population was obtained that cleaved a ribonucleotide phosphoester substrate in presence of **HMC01** with a cleavage efficiency of ~10 % in a *cis*-reaction (self-cleavage). Self-cleavage reaction is a rapid and convenient way to study the catalytic DNA population's catalytic parameters which very much resembles those of the individual sequences.[142] The percent product plateau has been previously observed in several reports and should be matter of further investigation. A ~1 x $10^{-3}$ $min^{-1}$ rate constant (*cis*-cleavage reaction) was observed for the most efficient sequence (G1-05) isolated from this catalytic pool (obtained by standard cloning and sequencing techniques). This rate represents 500-fold rate enhancement over the uncatalyzed reaction (~4 x $10^{-6}$ $min^{-1}$, ribonucleotide phosphoester substrate in presence

of **HMC01** without DNA catalyst). Mutagenesis of this sequence at a degeneracy of 15% and ten additional rounds of selective amplification under stringer conditions (lower concentration of coenzyme and shorter selection reactions), led to improved cleavage efficiency of the best sequence (G2-04) from second generation DNA sequence (25 % in a *cis*-reaction), but not the apparent catalytic rate ($1.8 \times 10^{-3}$ $min^{-1}$). In a third effort to improve the catalytic efficiency of this **HMC01**-dependent DNA enzyme, mutagenesis and ten extra rounds of selection afforded a DNA enzyme (G3-14) with an apparent rate constant of $1 \times 10^{-3}$ $min^{-1}$ in a *cis*-cleavage reaction, 500-fold faster than the uncatalyzed reaction ($\sim 4 \times 10^{-6}$ $min^{-1}$).

This experiment demonstrated that small molecule coenzymes can be designed, synthesized and incorporated in *in vitro* selection experiments to obtain DNA enzymes that are assisted by coenzyme. This has not been previously reported in the literature. Unfortunately, evolution of the selected DNA enzymes (mutagenesis and re-selection using stringer conditions) didn't noticeably improve the rate constant of the individuals in this case. Perhaps, after repetitive selections (30 rounds in total) under more and more stringent condition, an aptameric character (stronger binding) for the selected molecules dominated over the expected catalytic character.

In order to study the structural characteristics **HMC01**, a set of potential coenzymes were synthesized in which, additional to pyridine, a benzene, phenanthrolin and naphthalene aromatic rings were considered (**DC01**, **DC02**, **DC03** and **DC04**). Based on the vitro selection experiment model previously employed with **HMC01**, a parallel selection with the prospective coenzymes resulted to be an opportune and convenient way of: 1) screening the potential of small molecules as coenzymes for DNA

enzymes; 2) rapidly selecting catalytic DNA populations dependent on those coenzymes; 3) comparing the structural and mechanistic roles of the coenzymes; and 4) predicting the characteristics of novel coenzymes based on common structural features seen.

Table 18 shows a summary of the kinetic parameters calculated for the four catalytic DNA pools obtained after 10 rounds of selective amplification in presence of **DC01**, **DC02**, **DC03** and **DC04**.

Table 18. Kinetic parameters of catalytic pools DC01, DC02, DC03 and DC04.

| Catalytic DNA pool–Coenzyme | $k_{obs}$ x $10^3$ | Fold rate enhancement | Apparent Kd for coenzyme |
|---|---|---|---|
| DC01 | 1.8 | ~450 | 10 mM |
| DC02 | 1.4 | ~350 | 10 mM |
| DC03 | 1.2 | ~300 | 10 mM |
| DC04 | 0.2 | ~50 | 100 mM |
| N-DC01, N-DC02, N-DC03, or N-DC04 | ~0.004 | n/a | n/a |

All fours lineages showed catalytic activity in the presence of their cognate coenzyme with a rate constant ($k_{obs}$) in the range of $10^{-3}$min$^{-1}$. This rate represents 1000-fold improvement over the uncatalyzed reaction in this first generation DNA population which is similar to the rate obtained for the third generation DNA enzyme obtained with **HMC01** (1.1 x $10^{-3}$ min$^{-1}$). A simple variation in the *in vitro* selection that is noteworthy to mention, has been the scheme of reduced reaction times. For the first generation of DNA enzymes with **HMC01** (50 mM) the selection reaction time was reduced from 1 h to 15 min, then for the second generation (with 5 mM **HMC01**) the selection reaction time was reduced from 1 h to 5 min, and for the third generation (with 1 mM **HMC01**) the selection reaction time was reduced from 1 h to 1 min. For the parallel *in vitro* selection experiment with **DC01**, **DC02**, **DC03** and **DC04** (50 mM), the selection

reaction times were progressively decreased from 2 h to 1 min. Recent reports[142] suggested that relatively longer reaction time in the first rounds of selection avoids loosing potential enzymes and helps enriching the selected pool of them, and more reduced reaction times in the last rounds of selection reasonably improves the rate of the selected enzymes. The quenching method used to stop the selection reaction made it very difficult to reach selection times shorter than 1 min. A method able to stop the selection reaction at the second or millisecond level would potentially prove very helpful in improving the enzyme efficiency (a better rate constant).

Cross-reactivity studies with the selected DNA pools and coenzymes demonstrated the specificity of three of the selected pools towards their cognate coenzymes. This significant result shed light on how the coenzymes might be interacting with the DNA enzymes. All three non cross-reactive pools have been selected and operate in presence of coenzymes (**DC01**, **DC02** and **DC03**) in which the two imidazole moieties are not too distant. It could be speculated that both imidazole rings in each of the coenzymes are interacting with a pre-formed binding pocket or catalytic pocket. On the other hand, pool DC04 has been selected with a coenzyme (**DC04**) in which the two imidazole rings are significantly separated. This molecular coenzyme (**DC04**) shape could have created a single imidazole binding or catalytic pocket to which other similar imidazole-containing coenzymes had access. Additionally, the cross-reactivity found with pool DC04 was accompanied by poor affinity of **DC04** for the putative binding site of the DNA enzymes. A critical question is whether these coenzymes participate directly in the catalytic process or indirectly as simple structural elements in the folding of the

DNA enzyme. Detailed pH studies support the participation of **DC01** and **DC03** as general bases in the DNA enzyme catalyzed cleavage of a ribonucleotide phosphoester.

Information gained from the study of these *bis*-imidazole-containing coenzymes will aid in the design of new coenzymes for use in future *in vitro* selection experiments. However, further studies, including the detailed investigation of the sequence composition of the individual members of these enriched DNA pools, will help understanding specific molecular mechanistic aspects of these novel coenzyme-DNA enzyme systems and will offer a chance for their improvement.

# CHAPTER FOUR

## *Bis*-Imidazole Peptido Coenzymes for DNA enzymes

### *Introduction*

In a continuation of our investigation of the characteristics of useful synthetic coenzymes for the DNA catalyzed hydrolysis of a ribonucleotide phosphoester substrate, we decided to design and synthesize a new group of potential coenzymes with a common catalytic moiety but with simple structural distinctions at the DNA binding element. We expected that these molecules would cooperate differently with DNA, and therefore help us to understand what structural features are necessary for an enhanced catalytic activity in coenzyme-DNA enzyme systems.

The design of these new potential coenzymes was based on some non-specific imidazole-conjugated RNase mimics previously reported in the literature.[193] Those RNase mimics consist of a tripeptide containing two functional imidazole groups. Although these molecules were reported to have a poor catalytic efficiency and site-specificity for RNA hydrolysis, we believe that they represented a reasonable starting point for our design of synthetic coenzymes for DNA enzymes. In addition to the catalytic imidazole groups, we incorporated the following variable DNA interacting appendices, with characteristic functional group, in our coenzyme design (Figure 68): 1) a negatively charged appendix (glutamic acid) that could potentially provide a loose interaction with the negatively charged phosphate backbone of the DNA enzyme or generate hydrogen bonding with nucleobases of DNA enzymes; 2) a positively charged appendix (arginine) that could provide a strong interaction with the negatively charged

phosphate backbone of the DNA enzyme or generate hydrogen bonding with nucleobases of DNA enzymes; 3) an aromatic appendix (pyridine) that would potentially create base stack interactions or generate hydrogen bonding with nucleobases of DNA enzymes; and 4) no additional appendix (to serve as a reference for the study of the previously mentioned designs).

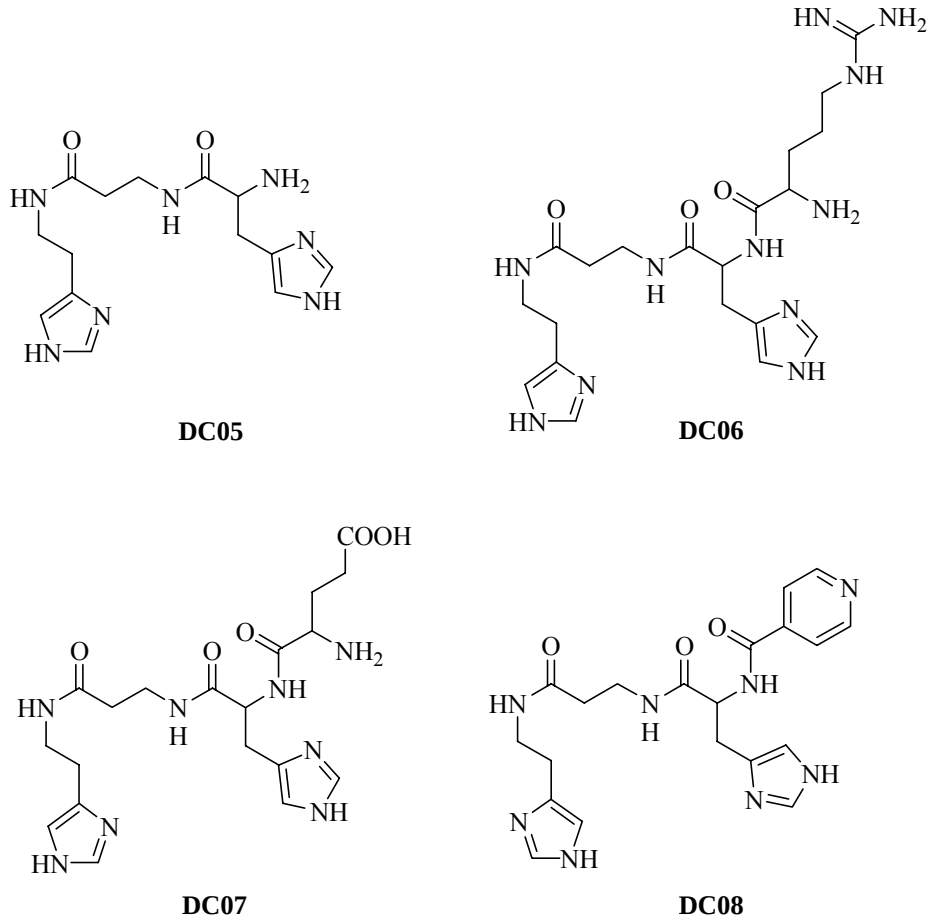



Figure 68. *Bis*-imidazole peptido coenzymes.

These four potential coenzymes, designated as intermediate coenzyme (**DC05**), arginine coenzyme (**DC06**), glutamic coenzyme (**DC07**) and aromatic coenzyme (**DC08**), were incorporated in a parallel *in vitro* selection for ribonuclease activity aiming to obtain coenzyme-depended DNA enzymes systems. After 10 rounds of selection, we expected

to obtain four pools of DNA enzymes that would specifically use their cognate coenzymes for the proposed reaction. Studies of cross-reactivity, kinetic, pH and binding with each obtained coenzyme-DNA enzymes systems was anticipated to provide us with some insights about their mechanism.

## *Materials and Methods*

### *General Section*

Solvents (ethyl acetate, hexanes and methanol) were obtained from the Baylor Science Building stockroom and distilled prior to use. Triethylamine obtained from Aldrich was distilled and stored under $N_2$. Amino acids and ester-activated amino acids were obtained from Bachem Co. All other reagents were purchased from Acros Organics, Alfa Aesar and VWR, and were used as received. Reactions were monitored by thin layer chromatography (TLC) (silica gel 60 F254) and chromatographic purifications were performed by flash column chromatography with silica gel (230-400 mesh) obtained from EM Science. A Bruker DPX 300 MHz NMR spectrometer running XWIN-NMR 3.1 was used for $^{1}H$ (300 MHz) and $^{13}C$ NMR (75 MHz). Chemical shifts are expressed in ppm (δ), peaks are listed as singlet (s), doublet (d), triplet (t), or multiplet (m), and coupling constants (*J*) are expressed in Hz. Mass spectrometric analysis was carried out in the analytical chemistry Laboratory of Dr. Kevin Chambliss at Baylor University on a Varian model 1200L triple-quadrupole mass analyzer (Varian, Inc., Palo Alto, CA, USA). Reverse phase HPLC was carried out with a Beckman HPLC system running 32 Karat Software 5.0 with a model 126 solvent module, a model 168 diode array detector and a AquaSep C8 5 μm 100Å Column (ES Industries, Berlin,

Germany). Ultrapure water and acetonitrile grade HPLC were obtained from Alfa Aesar. HPLC running buffers were A (0.1% TFA in HPLC $H_2O$) and B (9:1, $CH_3CN$:$H_2O$ with 0.1% TFA). The standard method was 1 mL/min with linear gradient of 5-95% buffer B over 17 min. Sample volume was 10 μL and peaks were detected between 168 and 254 nm. Primers, DNA oligonucleotide template and other oligonucleotides were synthesized by Midland Certified Reagent Co. (Midland, TX) according to our specifications. Sequences for template and primers are shown in Table 2. Biotinylated derivatives of primers were used for immobilization of the DNA onto affinity column.

UV quantification of DNA oligonucleotides was accomplished using a GeneQuant Pro spectrophotometer from Amershan Pharmacia, using a 5 mm pathlength 10 μL volume spectrosil cell. *Taq* DNA polymerase, dNTPs, 10X PCR Buffer and 15 mM $MgCl_2$ solution were obtained from Promega Corporation. Distilled and sterile water was obtained from Dr. Christopher Kearney's molecular genetics laboratory. PCR reactions were carried out using a TC-312 Thermal Cycler from Techne Inc. DNA samples were centrifuged using an Eppendorf model 5415R refrigerated microcentrifuge. Immobilized NeutrAvidin™ Protein gel and Handee™ Mini-Spin Column Kit were obtained from Pierce Biotechnology, Inc. High quality buffer components were obtained from FisherBiotech. pH was adjusted using Corning pH Meter 430 that was calibrated at pHs 4, 7 and 10 with certified buffer solution from Fisher Scientific. Agarose gel electrophoresis was performed using a Minicell® Primo™ Thermo EC320 gel apparatus from Fisher Scientific. Polyacrylamide gel electrophoresis was performed using Bio-Rad Mini-Protean 3 cell electrophoresis system. Electrophoresis was run with a VWR 105 power supply. DNA gel purification was monitored using VWR UV transilluminator

model LM-20E. Certified™ low range ultra agarose, Criterion TBE polyacrylamide urea gels (10% and 15%) and 10X TBE Buffer were obtained from Bio-Rad Laboratories. 10X TAE Buffer was obtained through VWR. TBE–urea sample buffer (89 mM Tris·HCl/89 mM boric acid/2 mM EDTA/7 M urea/12% Ficoll/0.01% Bromophenol Blue/0.02% Xylene Cyanol FF) was obtained from Bio-Rad. MassRuler™ DNA Ladder and 6X mass loading dye solution was obtained from Fermentas. Radioactive probes (Adenosine 5′-Triphosphate, [$\gamma$-32P] Ultratide™, 6000 Ci/mmol; 222 TBq/mmol) were purchased from ICN Biomedicals. Radiolabeled samples were purified using Mini Quick Spin Oligo Columns from Roche. T4 Polynucleotide Kinase was obtained from Fermentas. Liquid radioactive samples were quantified in Bio-Safe II liquid scintillation cocktail from Research Products International, Inc. using a Beckman Coulter model LS 6500 Multi-Purpose Scintillation Counter. Gels were dried using Gel Dryer FBGD45, vapor trap and Maxima® C Plus Vacuum Pump from Fisher Scientific. Gels were photographed using a Fluor-S™ Multlmager, Personal Molecular Imager FX™ System, Kodak Phosphor storage K screen and Quantity One® Quantification Software Version 4.0 from Bio-Rad.

*Synthetic Procedures*

A set of four *bis*-imidazole containing coenzymes were synthesized according the procedures that follow and were designated as **DC05**, **DC06**, **DC07** and **DC08**. All intermediate compounds were prepared under standard solution phase peptide synthesis using either PyBOP coupling reagent or isolated activated ester. The final products were produced by normal deprotection methods under acidic or basic conditions. The purity of

each coenzyme was confirmed by spectroscopic analysis and full spectrum HPLC analysis.

*Trityl-[2-(1-trityl-1H-imidazol-4-yl)-ethyl]-amine (**2**)*. Histamine dihydrochloride (1 equivalent, 1.8 g, 10 mmol) was dissolved in anhydrous chloroform (25 mL), and triethylamine (2 equivalents, 2.8 mL, 20 mmol) was subsequently added under dry $N_2$. The solution was vigorously stirred and refluxed for 15 min at 70 °C. After the solution was cooled down to room temperature, a solution of trityl chloride (4 equivalent, 11.1 g, 40 mmols) in anhydrous chloroform (2ml) was added drop wise. The reaction was allowed to stir at room temperature for 24 h. The reaction was stopped and washed several times with deionized water (3x 50 mL). Organic layer was then washed with brine (2x50 mL), dried over anhydrous sodium sulfate and concentrated *in vacuo*. Purification by flash chromatography (silica gel; ethyl acetate:hexanes, 50:50) afforded the desired product HA-(1-Trt)-Trt (**2**) (4.4 g, 7.4 mmol, 74%) (Figure 69) as a yellow solid. TLC (ethyl acetate:hexanes, 50:50): $R_f = 0.65$. $^1$HNMR ($CDCl_3$, 300 MHz): $\delta$ pmm 7.39-6.50 (m, 31 H), 6.50 (s, 1H), 2.73 (t, $J = 6.1$ Hz, 2H), 2.39 (t, $J = 6.1$ Hz, 2H), 1.78 (broad singlet, 1H).

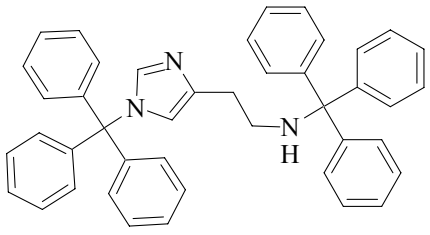



Figure 69. Structure of HA-(1-Trt)-Trt (**2**).[212]

*2-(1-Trityl-1H-imidazol-4-yl)-ethylamine (**3**)*. 5 mL of 5% trifluoroacetic acid in dichloromethane (v/v) was added to a flask containing protected histamine **2** (4.1 g, 7 mmol) until the solution turned deep yellow and the pH was around 4. The solution was allowed to stir for 30 min at room temperature, and completion of the deprotection was checked by TLC (ethyl acetate:hexanes, 50:50). Reaction was stopped by adding aqueous $NH_4OH$ 28% dropwise until the solution turned milky white and the pH was slightly basic. The solution was concentrated to dryness under vacuum. Purification by flash chromatography (silica gel; methanol:ethyl acetate, 10:90) afforded the desired product HA-(1-Trt)-$NH_2$ (**3**) (2.3 g, 6.5 mmol, 92%) (Figure 70) as a yellowish solid. TLC (methanol:ethyl acetate, 10:90): $R_f = 0.68$. $^1$HNMR (300 MHz, $CDCl_3$): $\delta$ ppm 7.4 (s, 1H), 7.36-7.29 (m, 9H), 7.14-7.09 (m, 6H), 6.64 (s, 1H), 3.28 (t, $J = 6.0$ Hz, 2H), 2.87 (t, $J = 6.0$ Hz, 2H), 1.44 (broad singlet, 2H).

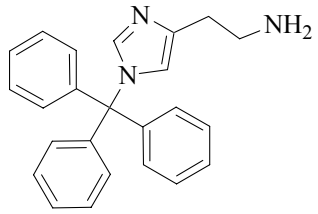



Figure 70. Structure of HA-(1-Trt)-$NH_2$ (**3**).[212]

*3-Amino-N-[2-(1-trityl-1H-imidazol-4-yl)-ethyl]-propionamide (**5**)*. Compound **3** (1 equivalent, 5 mmol, 1.76 g), Fmoc-β-Ala-OPfp (1 equivalent, 5 mmol, 2.38 g) and triethylamine (1 equivalent, 5 mmol, 0.14 mL) were dissolved in 10 mL anhydrous DMF and stirred for 24 h under dry $N_2$. The reaction was quenched with water (5 × 50 mL), washed with brine (2 × 50 mL), dried over sodium sulfate and concentrated *in vacuo*. The crude product was separated by flash chromatography (silica gel, 100% ethyl

acetate) to afford HA-(1-Trt)-β-Ala-Fmoc (2.2 g, 3.4 mmol, 68%). The Fmoc protecting group was removed by adding 10 mL of 50% piperidine in dichloromethane (v/v) and allowing it to react for 20 minutes at room temperature. Completion of the reaction was checked by TLC (methanol:ethyl acetate, 10:90) and reaction concentrated to dryness *in vacuo*. Purification by flash chromatography (silica gel; methanol:ethyl acetate, 10:90) afforded the desired product HA-(1-Trt)-β-Ala-$NH_2$ (**5**) (1.28 g, 3.0 mmol, 89%) (Figure 71) as a yellowish solid. TLC (methanol:ethyl acetate, 10:90): $R_f$ = 0.2. $^1$HNMR (300 MHz, $D_2O$): $\delta$ ppm 7.73 (s, 1H), 7.07-6.88 (m, 15H), 6.63 (s, 1H), 3.18 (t, $J$ = 6.4 Hz, 2H), 2.97 (t, $J$ = 6.8 Hz, 2H), 2.50 (t, $J$ = 6.4 Hz, 2H), 2.34 (t, $J$ = 6.8 Hz, 2H).

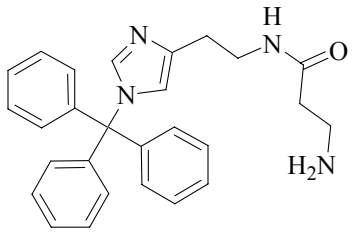



Figure 71. Structure of HA-(1-Trt)-β-Ala-$NH_2$ (**5**).

*2-Amino-3-(1-trityl-1H-imidazol-4-yl)-N-{2-[2-(1-trityl-1H-imidazol-4-yl)-ethylcarbamoyl]-ethyl}-propionamide (**7**)*. Compound **5** (1 equivalent, 3 mmol 1.28 g), Fmoc-His(1-Trt)-OPfp (1 equivalent, 3 mmol, 2.4 g) and triethylamine (1 equivalent, 3 mol, 0.14 mL) were dissolved in 10 mL anhydrous DMF and stirred for 24 h under dry $N_2$. The reaction was then washed with water (5 × 50 mL), brine (2 × 50 mL), dried over sodium sulfate and concentrated *in vacuo*. The crude product was separated by flash chromatography (silica gel, 100% ethyl acetate) to afford HA-(1-Trt)-β-Ala-His-(1-Trt)-Fmoc (1.8 g, 1.8 mmol, 60%). The Fmoc protecting group was removed by adding 5 mL of 50% piperidine in dichloromethane (v/v) and allowing the protected peptide to react

for 20 minutes at room temperature. Completion of the reaction was checked by TLC (methanol:ethyl acetate, 10:90) and reaction concentrated to dryness *in vacuo*. Purification by flash chromatography (silica gel, methanol:ethyl acetate, 10:90) afforded the desired product HA-(1-Trt)-b-Ala-His-(1-Trt)-$NH_2$ (**7**) (1.1 g, 1.4 mmol, 80%) (Figure 72) as a light yellow solid. TLC (methanol:ethyl acetate, 10:90): $R_f$ = 0.2. $^1$HNMR (300 MHz, $CDCl_3$): $\delta$ ppm 8.23 (t, $J$ = 5.9 Hz, 1H), 7.36-7.31 (m, 21H), 7.13-7.09 (m, 12H), 6.63 (s, 1H), 6.61 (s, 1H), 3.84 (broad singlet, 2H), 3.63 (dd, $J$ = 8.0, 4.2 Hz, 1H), 3.54-3.38 (m, 4H), 2.98 (dd, $J$ = 15, 4.2 Hz, 1H), 2.88 (dd, $J$ = 15, 8.0 Hz, 1H), 2.69 (t, $J$ = 6.3 Hz, 2H), 2.34 (t, $J$ = 6.8 Hz, 2H).

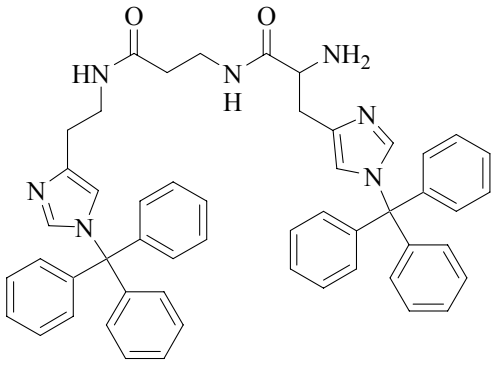



Figure 72. Structure of HA-(1-Trt)-b-Ala-His-(1-Trt)-$NH_2$ (**7**).

*2-Amino-3-(1H-imidazol-4-yl)-N-{2-[2-(1H-imidazol-4-yl)-ethylcarbamoyl]-ethyl}-propionamide (**DC05**).* Compound **7** (1 equivalent, 0.3 mmol, 0.25 g) was mixed with 1 mL 95% trifluoroacetic acid solution in dichloromethane and allowed to react for 1 h. Completion of the reaction was checked by TLC (methanol:ethyl acetate, 10:90) and reaction was concentrated *in vacuo* by coevaporation with ether. The crude product was dissolved in water, extracted with chloroform (3 × 50 mL) and dried to afford the desired product HA-β-Ala-His-$NH_2$ (**DC05)** (0.089 g, 0.28 mmol, 93%) (Figure 73) as a white

solid: $^{1}$H NMR (300 MHz, $D_2O$) $\delta$ ppm 8.54 (s, 1H), 8.41 (s, 1H), 7.26 (s, 1H), 7.09 (s, 1H), 4.07 (t, $J$ = 7.0, 1H), 3.29 (t, $J$ = 6.7 Hz, 2H), 3.24-3.20 (m, 2H), 3.18 (d, $J$ = 7.0 Hz, 2H), 2.74 (t, $J$ = 6.7 Hz, 2H), 2.22 (t, $J$ = 6.6 Hz, 2H); $^{13}$C NMR (75 MHz, $D_2O$) $\delta$ ppm 173.58, 167.71, 134.37, 133.09, 130.71, 125.81, 118.41, 116.17, 52.13, 38.01, 36.07, 34.68, 26.06, 24.01; (+) ESI-MS *m/z* 320.3 $[M + H]^+$, ($C_{14}H_{21}N_7O_2$, calcd 319.18); HPLC (standard method) $t_R$ = 3.58 min, purity > 95%.

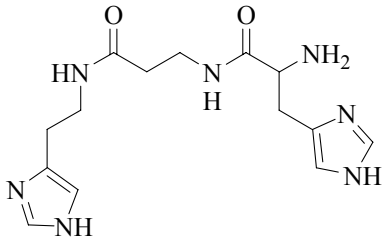



Figure 73. Structure of HA-β-Ala-His-$NH_2$ (**DC05**).

*2-Amino-6-carbamimidoyl-hexanoic acid (2-(1H-imidazol-4-yl)-1-{2-[2-(1H-imidazol-4-yl)-ethylcarbamoyl]-ethylcarbamoyl}-ethyl)-amide (**DC06**)*. Boc-Arg(Boc)$_2$-OH (1.1 equivalent, 0.33 mmol, 0.15 g) and PyBOP (1.1 equivalent, 0.33 mmol, 0.16 g) were mixed in 5 mL anhydrous DMF at 0 °C for 15 min. Compound **7** (1 equivalent, 0.3 mmol, 0.25 g) and triethylamine (1.1 equivalent, 0.33 mmol, 0.05 mL) were added and stirred at room temperature for 12 h under dry $N_2$. The reaction was then washed with water (5 × 50 mL), brine (2 × 50 mL), dried over sodium sulfate and concentrated *in vacuo*. The crude product was purified by flash chromatography (silica gel, 100% ethyl acetate) to afford HA-(1-Trt)-β-Ala-His-(1-Trt)-Arg(Boc)$_2$-Boc (0.29 g, 0.23 mmol, 77%). Protecting groups (Trt and Boc) were then removed by treatment with 1 mL 95% trifluoroacetic acid solution in dichloromethane for 2 h. Reaction completion was checked by TLC (methanol:ethyl acetate, 10:90) and then concentrated *in vacuo* by

coevaporation with ether. Product was dissolved in water, washed with chloroform (3 × 50 mL) and dried to afford the desired product HA-β-Ala-His-Arg (**DC06)** (0.096 g, 0.2 mmol, 88%) (Figure 74) as a white solid: $^{1}$H NMR (300 MHz, $D_2O$) $\delta$ ppm 8.47 (s, 1H), 8.42 (s, 1H), 7.17 (s, 1H), 7.09 (s, 1H), 4.47 (t, $J$ = 7.3 Hz, 1H), 3.89 (t, $J$ = 6.5 Hz, 1H), 3.29 (t, $J$ = 6.6 Hz, 2H), 3.26-3.15 (m, 2H), 3.05 (t, $J$ = 6.9 Hz, 2H), 3.03 (d, $J$ = 7.3 Hz, 2H), 2.74 (t, $J$ = 6.6 Hz, 2H), 2.21 (t, $J$ = 6.5 Hz, 2H), 1.80-1.69 (m, 2H), 1.52-1.39 (m, 2H); $^{13}$C NMR (75 MHz, $D_2O$) $\delta$ ppm 173.59, 170.95, 169.37, 156.74, 133.66, 133.10, 130.73, 128.01, 117.29, 116.16, 52.92, 52.56, 40.38, 38.02, 35.94, 34.90, 28.05, 26.43, 24.05, 23.50; (+) ESI-MS *m/z* 476.3 $[M + H]^+$ ($C_{20}H_{33}N_{11}O_3$, calcd 475.28); HPLC (standard method) $t_R$ = 3.85 min, purity > 97%.

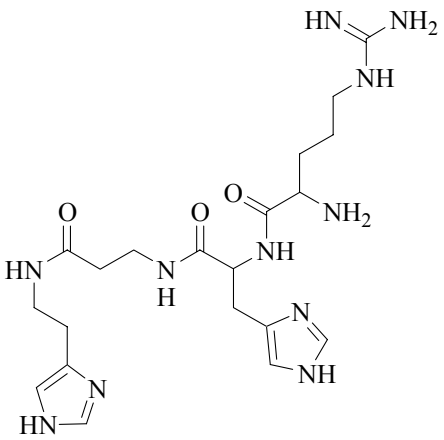



Figure 74. Structure of HA-β-Ala-His-Arg (**DC06**).

*4-Amino-4-(2-(1H-imidazol-4-yl)-1-{2-[2-(1H-imidazol-4-yl)-ethylcarbamoyl]-ethylcarbamoyl}-ethylcarbamoyl)-butyric acid (**DC07**)*. Compound **7** (1 equivalent, 0.3 mmol, 0.25 g) and Boc-Glu(OtBu)-OSu (1 equivalent, 0.3 mmol, 0.12 g) and triethylamine (1 equivalent, 0.3 mmol, 0.05 mL) were dissolved in 5 mL anhydrous DMF and stirred for 12 h under dry $N_2$. The reaction was then washed with water (5 × 50 mL),

brine (2 × 50 mL), dried over sodium sulfate and concentrated *in vacuo*. The crude product was purified by flash chromatography (silica gel, 100% ethyl acetate) to afford HA-(1-Trt)-β-Ala-His-(1-Trt)-Glu(OtBu)-Boc (0.26 g, 0.24 mmol, 81%). Protecting groups (Trt, Boc and OtBu) were removed by adding 2 mL of 1N HCl in Acetone (1:1.5) and allowing it to reflux 12 h at room 65 °C. After reaction was cooled, acetone was removed *in vacuo* and remaining aqueous solution was filtered. The water was evaporated *in vacuo* to afford the desired product HA-β-Ala-His-Glu (**DC07**) (0.102 g, 0.23 mmol, 95%) (Figure 75) as a white solid: $^{1}$H NMR (300 MHz, $D_2O$) $\delta$ ppm 8.44 (s, 1H), 8.39 (s, 1H), 7.14 (s, 1H), 7.06 (s, 1H), 4.43 (t, $J$ = 7.1 Hz, 1H), 3.90 (t, $J$ = 6.4 Hz, 1H), 3.25 (t, $J$ = 6.6 Hz, 2H), 3.17 (t, $J$ = 6.8 Hz, 2H), 3.01 (d, $J$ = 7.1, 3.4 Hz, 2H), 2.70 (t, $J$ = 6.6 Hz, 2H), 2.32 (t, $J$ = 7.4, Hz, 2H), 2.18 (t, $J$ = 6.8 Hz, 2H), 1.99-1.89 (m, 2H); $^{13}$C NMR (75 MHz, $D_2O$) $\delta$ ppm 175.87, 173.65, 170.80, 169.08, 133.63, 133.08, 130.72, 128.02, 117.32, 116.17, 52.92, 52.18, 38.03, 35.98, 34.96, 28.97, 26.40, 25.83, 24.03; (+) ESI-MS *m/z* 449.3 $[M + H]^+$ ($C_{19}H_{28}N_8O_5$, calcd 448.22); HPLC (standard method) $t_R$ = 4.08 min, purity > 98%.

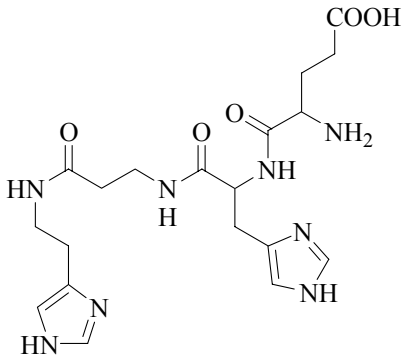



Figure 75. Structure of HA-β-Ala-His-(1-Trt)-Glu (**DC07**).

*N-(2-(1H-Imidazol-4-yl)-1-{2-[2-(1H-imidazol-4-yl)-ethylcarbamoyl]-ethylcarbamoyl}-ethyl)-isonicotinamide (**DC08**)*. Isonicotinic acid (1.1 equivalent, 0.33 mmol, 0.04 g) and PyBOP (1.1 equivalent, 0.33 mmol, 0.16 g) were mixed in 5 mL anhydrous DMF at 0 °C for 15 min. Compound **7** (1 equivalent, 0.3 mmol, 0.25 g) and triethylamine (1.1 equivalent, 0.33 mmol, 0.05 mL) were added and stirred at room temperature for 12 h under dry $N_2$. The reaction was then washed with water (5 × 50 mL), brine (2 × 50 mL), dried over sodium sulfate and concentrated *in vacuo*. The crude product was purified by flash chromatography (silica gel, 100% ethyl acetate) to afford HA-(1-Trt)-β-Ala-His-(1-Trt)-Isonicotinamide (0.19 g, 0.21 mmol, 65%). The trityl protecting group was removed by treatment with 1 mL 95% trifluoroacetic acid solution in dichloromethane for 2 h. Reaction completion was monitored by TLC (methanol:ethyl acetate, 10:90) and at reaction completion the mixture was concentrated *in vacuo* by coevaporation with ether. The crude product was dissolved in water and washed with chloroform (3 × 50 mL) and dried to afford the desired product HA-β-Ala-His-Isonicotinamide (**DC08)** (0.08 g, 0.19 mmol, 90%) (Figure 76) as a light yellow solid: $^1$H NMR (300 MHz, $D_2O$) $\delta$ ppm 8.79 (d, $J$ = 5.6 Hz, 2H), 8.47 (s, 1H), 8.40 (s, 1H), 8.15 (d, $J$ = 5.6 Hz, 2H), 7.18 (s, 1H), 7.08 (s, 1H), 4.68 (t, $J$ = 7.2 Hz, 1H), 3.25 (m, 4H), 3.20-3.11 (m, 2H), 2.72 (t, $J$ = 6.5 Hz, 2H), 2.23 (t, $J$ = 6.7 Hz, 2H); $^{13}$C NMR (75 MHz, $D_2O$) $\delta$ ppm 173.70, 170.87, 165.06, 149.24, 142.57, 133.68, 133.08, 130.71, 128.33, 125.58, 117.32, 116.16, 53.69, 38.02, 36.06, 35.04, 26.30, 24.03; (+) ESI-MS *m/z* 425.1 $[M + H]^+$ ($C_{20}H_{24}N_8O_3$, calcd 424.20); HPLC (standard method) $t_R$ = 5.35 min, purity > 92%.

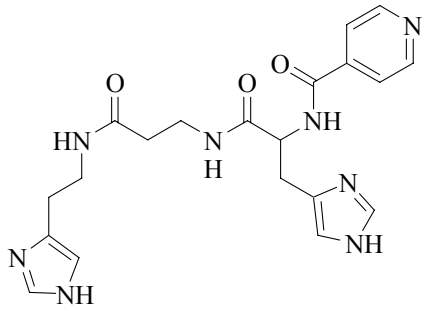


Figure 76. Structure of HA-β-Ala-His-Isonicotinamide (**DC08**).

*Parallel in Vitro Selection of Catalytic DNA Pools that Use Bis-Imidazole Peptido Coenzymes (**DC05**, **DC06**, **DC07** and **DC08**)*

A DNA template (80 pmol) was initially extended by PCR reaction with primers **1** (128 pmol) and **B2** (128 pmol) in a 800 μL PCR cocktail using four thermocycles of 94 °C (15 s), 50 °C (30 s), 72 °C (30 s). The extended DNA template, containing biotin and ribonucleotide moiety, was then ethanol precipitated at -20 °C for 4 h. The desired 101 bp DNA product was gel purified (1.5% low range ultra agarose) and suspended in 240 μL binding buffer (50 mM HEPES, 0.5 M NaCl, 0.5 M KCl, 0.5 mM EDTA, pH 7.5 at rt). The resulting DNA solution was equally distributed into four tubes and each solution loaded on a separate neutravidin column, which had been previously equilibrated with 5 × 100 mL binding buffer. The columns were incubated for a period of 30 min at room temperature, and then the unbound DNA was washed off with 5 × 100 mL binding buffer. The non-biotinylated complementary DNA strands were eluted from each column with 5 × 100 mL of freshly prepared 0.2N NaOH, and the columns were immediately neutralized with 5 × 100 mL binding buffer. Columns matrices, containing rA incorporated DNA, were incubated in binding buffer for 1 h (negative selection) and then carefully eluted with 10x100 mL binding buffer. Reactions were then started by adding 3 × 20 μL of each reaction buffer (binding buffer containing 50 mM coenzyme, pH 7.5 at

rt) to a column over a period of 5 h. DNA molecules eluted from each column by reaction buffer were ethanol precipitated overnight at -20 °C and then amplified with primers **1** (20 pmol) and **2** (20 pmol) in a 100 μL PCR cocktail with twenty cycles of 92 °C (10 s), 50 °C (30 s), 72 °C (30 s). 10 μL of each of the PCR products were extended by PCR reaction with primers **1** (80 pmol) and **B2** (80 pmol) in a 200 μL PCR cocktail with six thermocycles of 94 °C (15 s), 50 °C (30 s), 72 °C (30 s). The extended DNA products were ethanol precipitated at -20 °C for 4 h. The desired 101 bp DNA products were gel purified (1.5% low range ultra agarose) and the isolated DNA was used for the next round of selection. In rounds 5 to 10, the stringency of the selection was increased by reducing the time of reaction as follows: round 5 (120 min), round 6 (60 min), round 7 (30 min), round 8 (15 min), round 9 (10 min) and round 10 (5 min). After 10 rounds of selection four DNA pools (pool DC05, pool DC06, pool DC07 and pool DC08) were obtained.

*Preparation of DNA Pool for Catalytic Studies*

10 μL of each PCR product (amplified with primers **1** and **2**) from the $10^{th}$ round of selection were extended by PCR reaction with primers **B1** (80 pmol) and **B2rA** (80 pmol) in a 200 μL PCR cocktail with six thermocycles of 94 °C (15 s), 50 °C (30 s), 72 °C (30 s). The extended DNA products were ethanol precipitated at -20 °C for 4 h. The desired 101 bp DNA products were gel purified (1.5% low range ultra agarose) and suspended in 60 μL of binding buffer (50 mM HEPES, 0.5 M NaCl, 0.5 M KCl, 0.5 mM EDTA, pH 7.5 at rt). Each DNA solution was loaded on a separate neutravidin column, which had been previously equilibrated with 5 × 100 mL of binding buffer. Columns containing DNA solution were incubated for 30 min at room temperature, and then

unbound DNA was washed off with 5 × 100 mL of binding buffer. The non-biotinylated complementary catalytic DNA strands were eluted from each column with 3 × 100 mL of freshly prepared 0.2N NaOH, rapidly mixed with 60 μL of 3M NaOAc pH 5.2 and ethanol precipitated overnight at -20 °C. Each precipitated DNA pool was suspended in 20 μL sterile distilled water and 5' labeled with [$\gamma$-32P]ATP and T4 polynucleotide kinase (see Appendix A). Radiolabeled DNA pools were purified using Sephadex G-50 columns, beta emission quantified using liquid scintillation counter (see Appendix A) and each diluted in sterile distilled water to the same number of counts per milliliter (cpm/ml). Radiolabeled DNA pools were stored at 0 °C and used in catalytic experiments. The naïve DNA pool (before *in vitro* selections) was prepared and radiolabeled under the same conditions.

*Self-Cleavage of DNA Pool*

Each radiolabeled DNA pool was subjected to a self-cleavage reaction in presence of 50 mM solution of its cognate coenzyme in buffer (50 mM HEPES, 0.5 M NaCl, 0.5 M KCl, 0.5 mM EDTA, pH 7.5 at rt) (Table 19). Positive control consisted of NaOH chemical hydrolysis of the ribonucleotide moiety, and negative control consisted of the reaction in buffer in absence of coenzyme. For each DNA pool, 1 μL of radiolabeled DNA pool and 4 μL of sterile distilled water were added to two microcentrifuge tubes (B and C). Samples were heat denatured for 30 s at 90 °C and slowly cooled to room temperature. Reaction was started by adding 5 μL of 2X binding buffer to tube B and 5 μL of reaction buffer (100 mM coenzyme in 2X binding buffer) to tube C. After 4 h of reaction, 10 μL of TBE-urea sample buffer was added to tubes B and C. Tube A was prepared by mixing 1 μL of each radiolabeled DNA pool, 9 μL of binding buffer and 10

μL of TBE-urea sample buffer (time zero). Tubes A, B and C for each DNA pool were heated to 90 °C for 5 min before loading on a warmed 10% precast polyacrylamide TBE-urea gel that had been prerun for 30 min. Samples were loaded on the gel, run and analyzed under standard methods (see Appendix A). The naïve DNA pool was used as positive control by hydrolyzing it with 0.5 N NaOH and running on gel along with other samples. The self-cleavage reaction was also performed in presence of a final concentration of 10 mM of coenzyme following the same procedure described above.

Table 19. Self-cleavage (pools DC05, DC06, DC07 and DC08) experiment design.

| 10% denaturing polyacrylamide gel | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Lane | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 |
| Conditions | A | B | C | A | B | C | A | B | C | A | B | C | NaOH |
| | Pool DC05 | | | Pool DC06 | | | Pool DC07 | | | Pool DC08 | | | Naïve Pool |
| 50 mM Coenzyme, pH 7.4, 4 h, rt conditions (A = neg. rxn, B = time 0, C = cognate coenzyme) | | | | | | | | | | | | | |

*Cross-Reactivity of DNA pool*

Radiolabeled DNA from each catalytic DNA pool was cross-reacted with its cognate coenzyme and other three coenzymes used in the parallel selection (Table 20). For example, DNA pool DC05 was reacted with **DC05** and **DC06**, **DC07** and **DC08**. For each pool, 1 μL of radiolabeled pool and 4 μL of sterile distilled water were added to each of four microcentrifuge tubes (A, B, C and D). Samples were heat denatured for 30 s at 90 °C and slowly cooled to room temperature. Reaction was started by adding 5 μL of reaction buffer (2X binding buffer containing 100 mM **DC05**) to tube A, 5 μL of reaction buffer (2X binding buffer containing 100 mM **DC06**) to tube B, 5 μL of reaction buffer (2X binding buffer containing 100 mM **DC07**) to tube C and 5 μL of reaction buffer (2X binding buffer containing 100 mM **DC08**) to tube D. After 4 h of reaction, 10

μL of TBE-urea sample buffer was added to each tube. Tubes A, B, C and D for each DNA pool were heated to 90 °C for 5 min before loading on a 10% precast polyacrylamide TBE-urea warmed gel that had been prerun for 30 min. Samples were loaded on the gel, run and analyzed under standard methods (see Appendix A).

Table 20. Cross-reactivity (pools DC05, DC06, DC07 and DC08) experiment design.

| 10% denaturing polyacrylamide gel | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Lane | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 |
| Conditions | A | B | C | D | A | B | C | D | A | B | C | D | A | B | C | D |
| | Pool DC05 | | | | Pool DC06 | | | | Pool DC07 | | | | Pool DC08 | | | |
| 10 mM coenzyme (A = DC05, B = DC06, C = DC07, D = DC08), 1 h, pH 7.4, rt | | | | | | | | | | | | | | | | |

*Kinetic Study of DNA Pool DC07*

Radiolabeled DNA pool DC07 was reacted in presence of its cognate coenzyme (**DC07**) for different time periods in order to obtain its rate constant ($k_{obs}$) (Table 21). 15 μL of radiolabeled DNA pool DC07 was mixed with 60 μL of sterile distilled water. The sample was heat denatured for 30 s at 90 °C and slowly cooled to room temperature. The reaction was started by adding 75 μL of reaction buffer (2X binding buffer containing 100 mM **DC07**, pH 7.4). After designated times (0 min, 5min, 15min, 30min, 45min, 1 h, 2 h, 4 h, 8 h, 12 h, 24 h and 48 h), 10 μL aliquots were taken, mixed with 10 μL denaturing solution (95% formamide, 1mM EDTA and 0.05% each of xylene cyanol and bromophenol solution) and stored at 0 °C. Additionally, 1 μL of DNA pool DC07 was reacted in presence of 9 μL binding buffer (no coenzyme) for 48 h (negative control), and 1 μL of DNA pool DC07 was hydrolyzed with 0.5 N NaOH (positive control). After the last reaction time sample was taken, tubes were heated to 90 °C for 5 min before loading on a warmed 10% precast polyacrylamide TBE-urea gel that had been prerun for 30 min.

Samples were loaded on the gel, run and analyzed under standard methods (see Appendix A). Kinetic study of DNA pool DC07 was also performed in presence of histidine, as describe above for **DC07**.

Table 21. Kinetic study (pool DC07) experiment design.

| 10% denaturing polyacrylamide gel | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Lane | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 |
| Conditions | + rnx | - rnx | 0 min | 5 min | 15 min | 30 min | 45 min | 1 h | 2 h | 4 h | 8 h | 12 h | 24 h | 48 h |
| 10 mM **DC07**, pH 7.4, rt | | | | | | | | | | | | | | |

*Binding Study of DNA Pool DC07*

Radiolabeled DNA pool DC07 was reacted in presence of different concentrations of its cognate coenzyme (**DC07**) in order to obtain an approximate enzyme-coenzyme affinity constant ($K_d$) (Table 22). 1 µL of radiolabeled DNA pool DC07 and 4 µL of sterile distilled water were added to a battery of 10 tubes. Samples were heat denatured for 30 s at 90 °C and slowly cooled to room temperature. Reaction was started by adding 5 µL of serial dilutions of DC07 (0.02 mM, 0.2mM, 2 mM, 20mM and 200 mM) 2X binding buffer at pH 7.4 to the tubes (each point was prepared twice). After 4 h of reaction, 10 µL of TBE-urea sample buffer was added to each tube. Additionally, 1 µL of DNA pool DC07 was reacted in presence of 9 µL binding buffer (no coenzyme) for 4 h (negative control), 1 µL of DNA pool DC07 was hydrolyzed with 0.5 N NaOH (positive control), and 1 µL of DNA pool DC07 was mixed with 9 µL binding buffer and 10 µL TBE-urea sample buffer (time zero). Tubes were heated to 90 °C for 5 min before loading on a warmed 10% precast polyacrylamide TBE-urea gel that had been prerun for 30 min. Samples were loaded on the gel, run and analyzed under standard methods (see Appendix A).

Table 22. Binding study (pool DC07) experiment design.

| 10% denaturing polyacrylamide gel | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Lane | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 |
| Conditions | - rnx | Time 0 | 0.001 mM DC07 | | 0.01 mM DC07 | | 0.1 mM DC07 | | 1 mM DC07 | | 10 mM DC07 | | 100 mM DC07 | + rnx |
| 4 h, pH 7.4, rt | | | | | | | | | | | | | | |

*pH profile of DNA pool DC07*

Radiolabeled DNA pool DC07 was reacted in presence of its cognate coenzyme (**DC07**) at different pHs in order to study the efficiency of the cleavage (Table 23). 1 µL of radiolabeled DNA pool DC07 and 4 µL of sterile distilled water were added to a battery of 10 tubes. Samples were heat denatured for 30 s at 90 °C and slowly cooled to room temperature. Reaction was started by adding 5uL of reaction buffer (2X binding buffer containing 100 mM **DC07**) at pHs 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5 and 9. After 4 h of reaction, 10 µL of TBE-urea sample buffer was added to each tube. 1 µL of DNA pool DC07 was reacted in presence of 9 µL binding buffer at pH 7.4 for 4 h (negative control), 1 µL of DNA pool DC07 was hydrolyzed with 0.5 N NaOH (positive control), and 1 µL of DNA pool DC07 was mixed with 9 µL binding buffer at pH 7.4 and 10 µL TBE-urea sample buffer (time zero). Tubes were heated to 90 °C for 5 min before loading on a warmed 10% precast polyacrylamide TBE-urea gel that had been prerun for 30 min. Samples were loaded on the gel, run and analyzed under standard methods (see Appendix A).

Table 23. pH profile (pool DC07) experiment design.

| 10% denaturing polyacrylamide gel | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Lane | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 |
| Conditions | - rnx | Time 0 | pH 4.0 | pH 5.0 | pH 5.5 | pH 6.0 | pH 6.5 | pH 7 | pH 7.5 | pH 8.0 | pH 8.5 | pH 9 | + rnx |
| 10 mM **DC07**, 1 h, rt | | | | | | | | | | | | | |

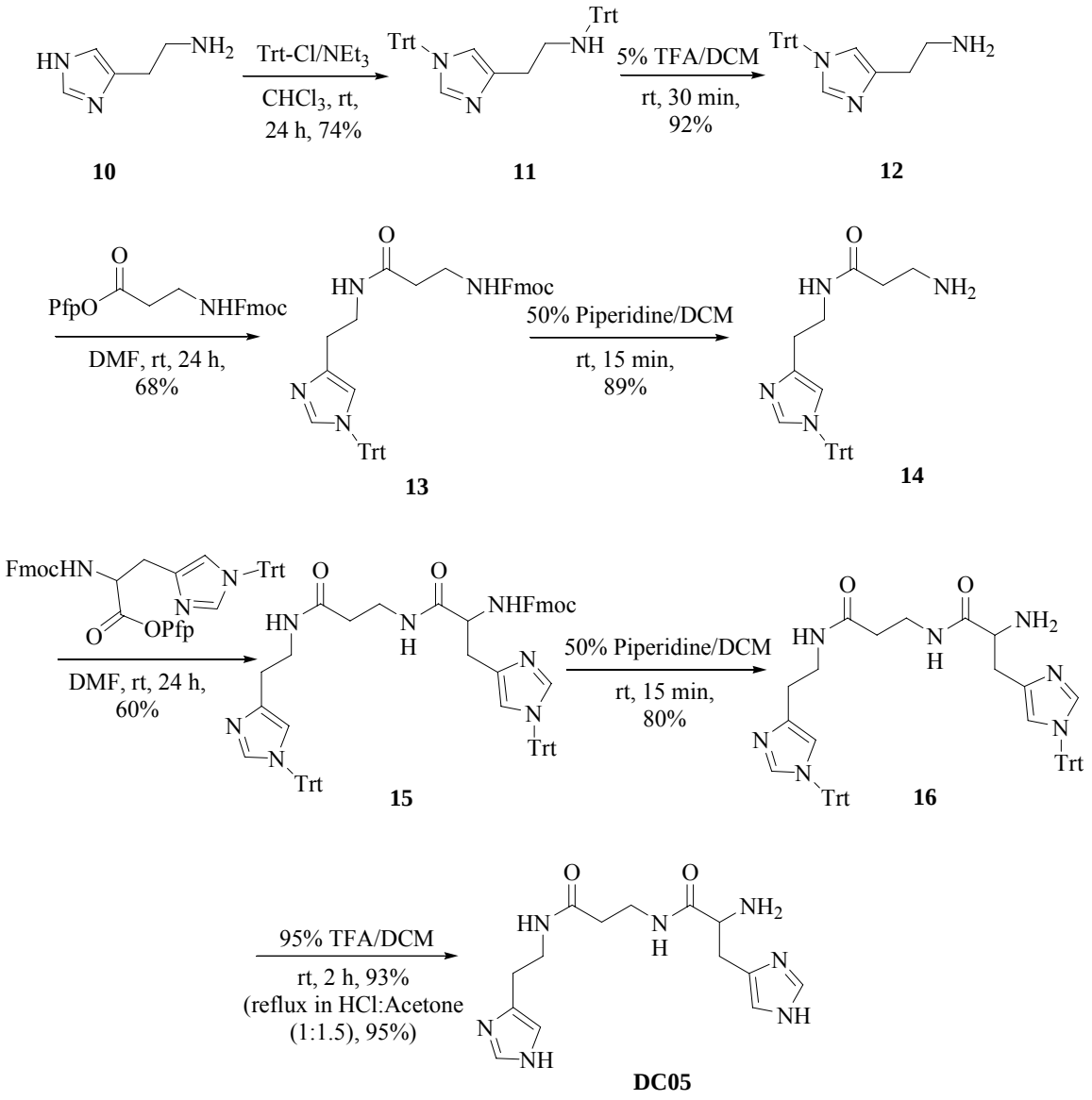
## *Results and Discussions*

### *Bis-Imidazole Peptido Coenzymes Synthesis*

*Synthesis of 2-Amino-6-carbamimidoyl-hexanoic acid (2-(1H-imidazol-4-yl)-1-{2-[2-(1H-imidazol-4-yl)-ethylcarbamoyl]-ethylcarbamoyl}-ethyl)-amide (**DC05**).* Diprotected histamine **11** was prepared by reacting histamine **10** in presence of excess equivalents of trityl chloride (74% yield).[212] The modification of several reaction conditions (equivalents of base, equivalents of trityl chloride and temperature) didn't result in an improvement of the reported yield for this reaction. Compound **11** was then selectively deprotected at the aliphatic amine with a 5% TFA solution.[212] Complete deprotection, observed by TLC, was achieved at 30 min at room temperature without observable deprotection of the imidazole ring. After neutralization and flash column purification, trityl monoprotected histamine **12** was obtained in 92% yield. Scale-up to 10 g of this reaction occurs without problems and with similar yields.

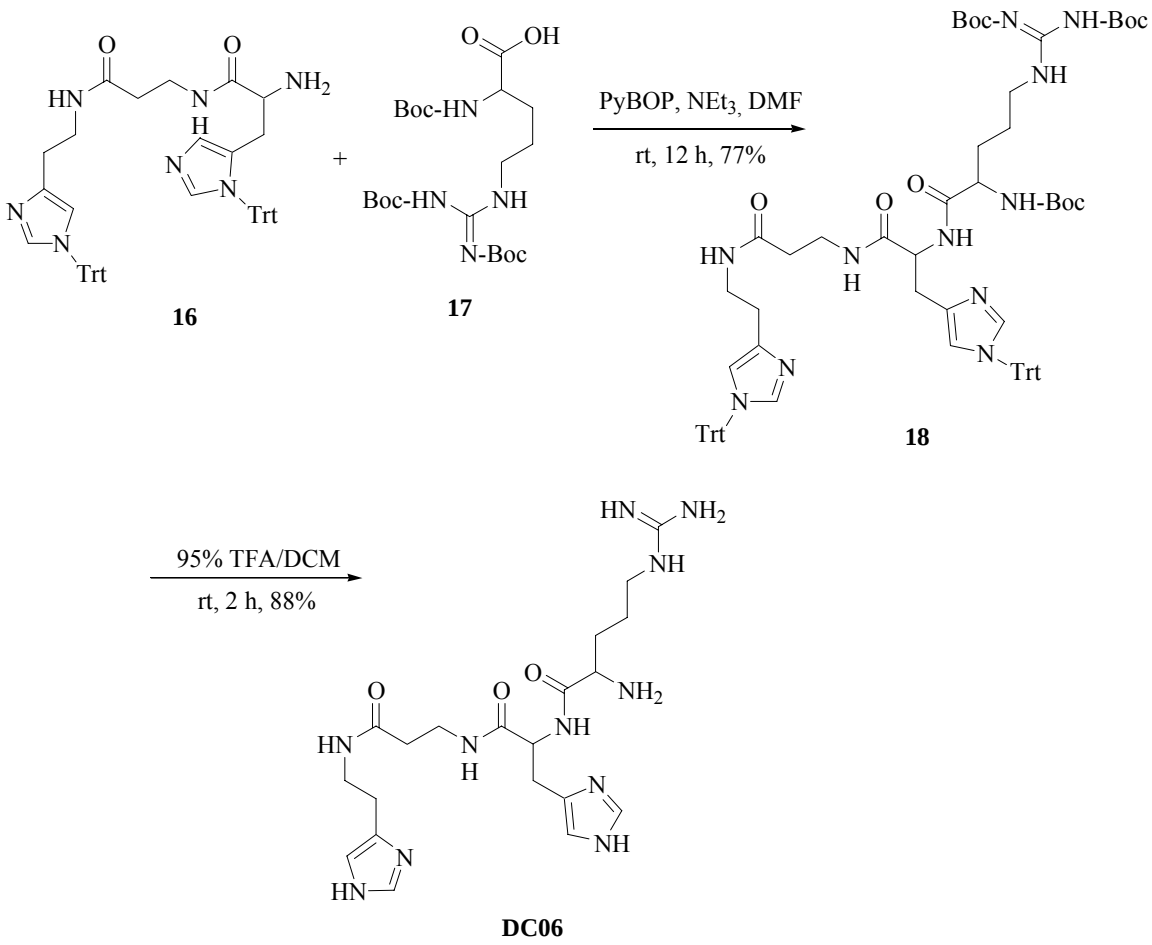
HA-(1-Trt)-β-Ala-Fmoc **13** was obtained by stirring equimolar amounts of **12** and the commercially available pentafluorophenol active ester of β-alanine (Fmoc-β-Ala-OPfp) for 12 at room temperature (68% yield). Selective deprotection of base-labile Fmoc group with a 50% piperidine solution in dichloromethane (v/v) and further purification with flash chromatography afforded HA-(1-Trt)-β-Ala-$NH_2$ **14** in good yield (89%). HA-(1-Trt)-β-Ala-His-(1-Trt)-Fmoc **15** was obtained after stirring equimolar amounts of **14** and commercially available pentafluorophenol active ester of histidine (Fmoc-His(1-Trt)-OPfp) at room temperature for 12 h (60% yield). Selective deprotection of base-labile Fmoc group with a 50% piperidine solution in dichlorometane

(v/v) and further purification by flash chromatography afforded HA-(1-Trt)-β-Ala-His(1-Trt)-$NH_2$ **16** in 80% yield. Additional removal of the acid-labile protecting group (Trt) with 95% TFA in dichloromethane (v/v) at room temperature for 2 h afforded intermediate cofactor **DC05** in 93% yield. Deprotection of **16** was also carried out at reflux in 1N HCl:Acetone (1:1.5) for 12 h, which afforded **DC05** in similar yield (Scheme 7).



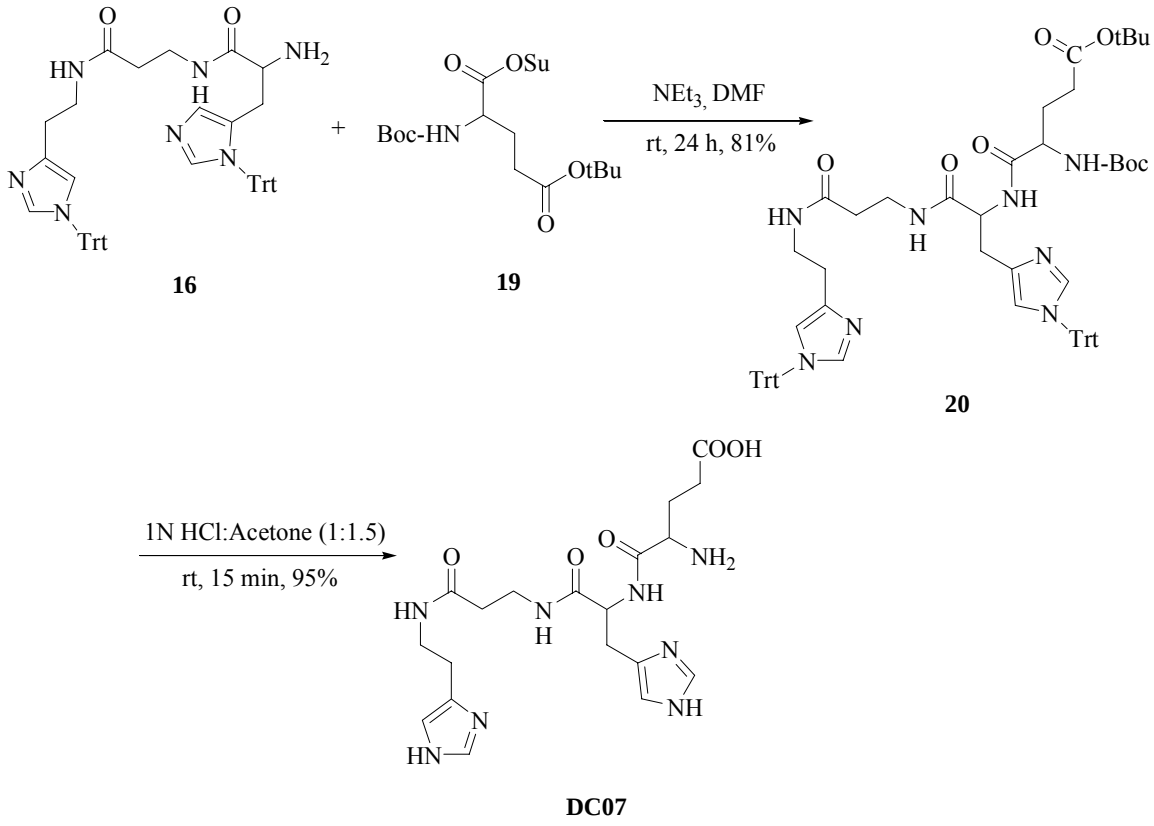

Scheme 7. Synthetic scheme for **DC05**.

*Synthesis of 2-Amino-6-carbamimidoyl-hexanoic acid (2-(1H-imidazol-4-yl)-1-{2-[2-(1H-imidazol-4-yl)-ethylcarbamoyl]-ethylcarbamoyl}-ethyl)-amide (**DC06**)*. HA-(1-Trt)-β-Ala-His-(1-Trt)-Arg(Boc)$_2$-Boc **18** was obtained after stirring equimolar amounts of **16**, PyBOP reagent and a commercially available protect analog of arginine (Boc-Arg(Boc)$_2$-OH) **17** at room temperature for 12 h (77% yield). Deprotection of the acid-labile protecting groups (Trt and Boc) with 95% TFA in dichloromethane (v/v) at room temperature for 2 h afforded Arginine Cofactor (**DC06**) in 88% yield (Scheme 8). Deprotection of **18** at reflux in 1N HCl:Acetone (1:1.5) for 12 h only produced a partially deprotected product.



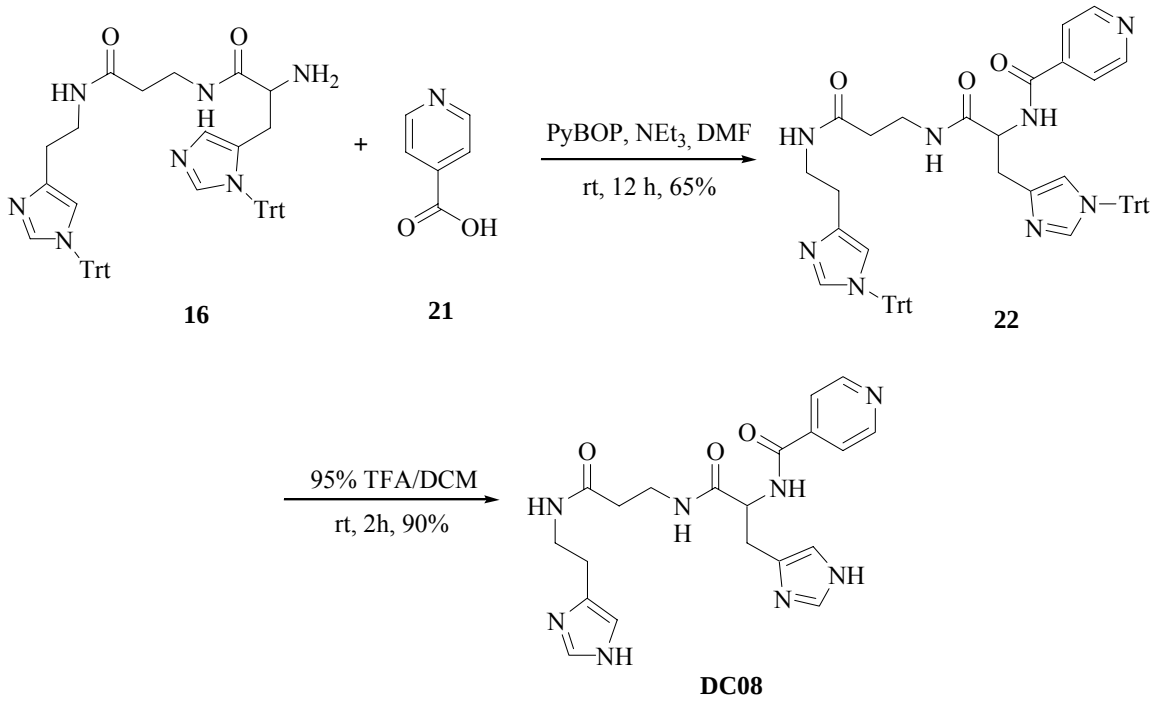

Scheme 8. Synthetic scheme for **DC06**.

*Synthesis of 4-Amino-4-(2-(1H-imidazol-4-yl)-1-{2-[2-(1H-imidazol-4-yl)-ethylcarbamoyl]-ethylcarbamoyl}-ethylcarbamoyl)-butyric acid (**DC07**).* HA-(1-Trt)-β-Ala-His-(1-Trt)-Glu(OtBu)-Boc **20** was obtained after stirring equimolar amounts of **16** and the commercially available succinimide active ester glutamic acid (Boc-Glu(OtBu)-OSu) **19** at room temperature for 12 h (81% yield). Deprotection of the acid-labile protecting groups (Trt and Boc) with a 95% TFA in dichloromethane (v/v) at room temperature for 2 h afforded glutamic cofactor (**DC07**) in 95% yield (Scheme 9). Deprotection of **20** at reflux in 1N HCl:Acetone (1:1.5) for 12 h afforded **DC07** in similar yield.




Scheme 9. Synthetic scheme for **DC07**.

*Synthesis of N-(2-(1H-Imidazol-4-yl)-1-{2-[2-(1H-imidazol-4-yl)-ethylcarbamoyl]-ethylcarbamoyl}-ethyl)-isonicotinamide (**DC08**).* HA-(1-Trt)-β-Ala-His-(1-Trt)-Isonicotinamide **22** was obtained after stirring equimolar amounts of **16**, PyBOP coupling reagent and Isonicotinic acid **21** at room temperature for 12 h (65% yield). Deprotection of the acid-labile protecting group (Trt) with a 95% TFA in dichloromethane (v/v) at room temperature for 2 h afforded aromatic cofactor (**DC08**) in 90% yield (Scheme 10). Deprotection of **22** at reflux in 1N HCl:Acetone (1:1.5) for 12 h afforded **DC08** in similar yield.




Scheme 10. Synthetic scheme for **DC08**.

*Parallel in Vitro Selection using **DC05**, **DC06**, **DC07** and **DC08**.*

Continuing with our study of coenzymes for *in vitro* selection of DNA enzymes, we decided to incorporate a set of newly designed and synthesized potential coenzymes

(**DC05**, **DC06**, **DC07** and **C08**) in a parallel *in vitro* selection experiment (Figure 77) aiming to obtain families (pools) of DNA enzymes that use these small molecules for the cleavage of a ribonucleotide phosphoester substrate. **DC05**, **DC06**, **DC07** and **C08** were each dissolved in buffer (50 mM HEPES, 0.5 M NaCl, 0.5 M KCl, 0.5 mM EDTA, pH 7.5 at rt) and demonstrated good solubility and stability (HPLC monitored).

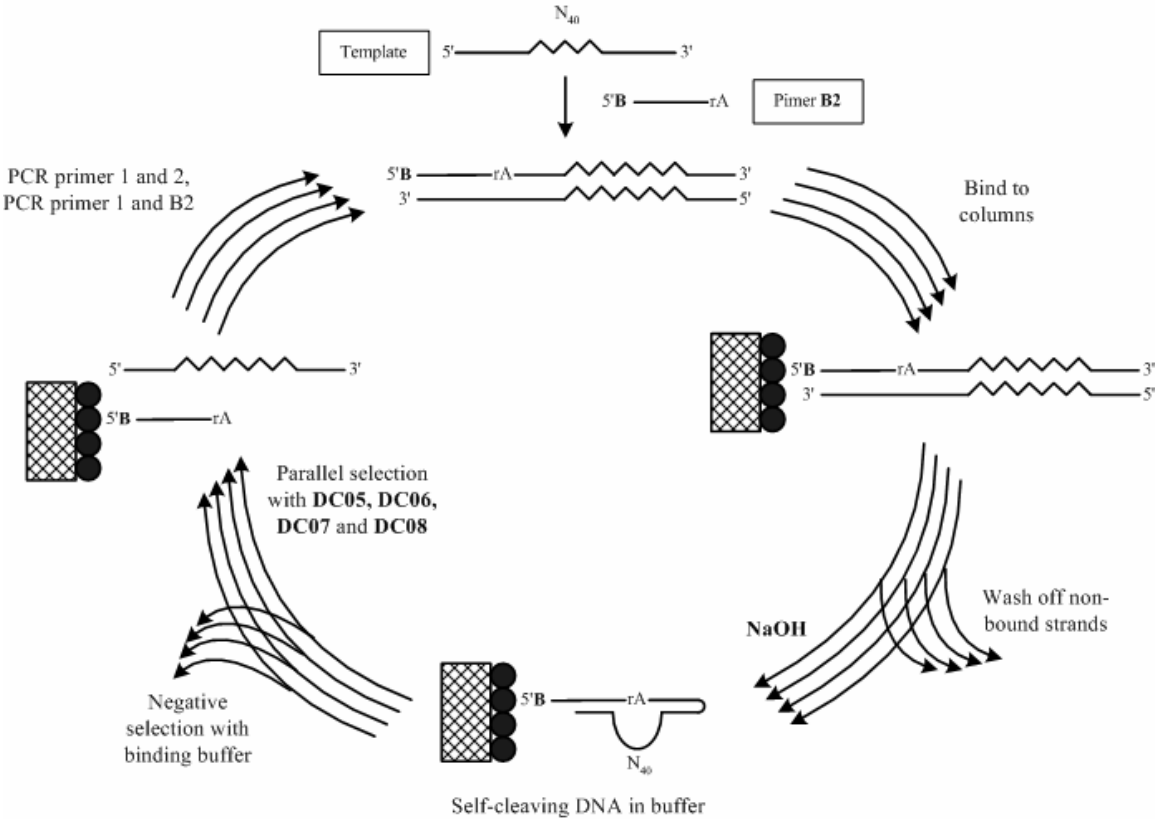



Figure 77. Parallel *in vitro* selection using **DC05**, **DC06**, **DC07** and **DC08**.

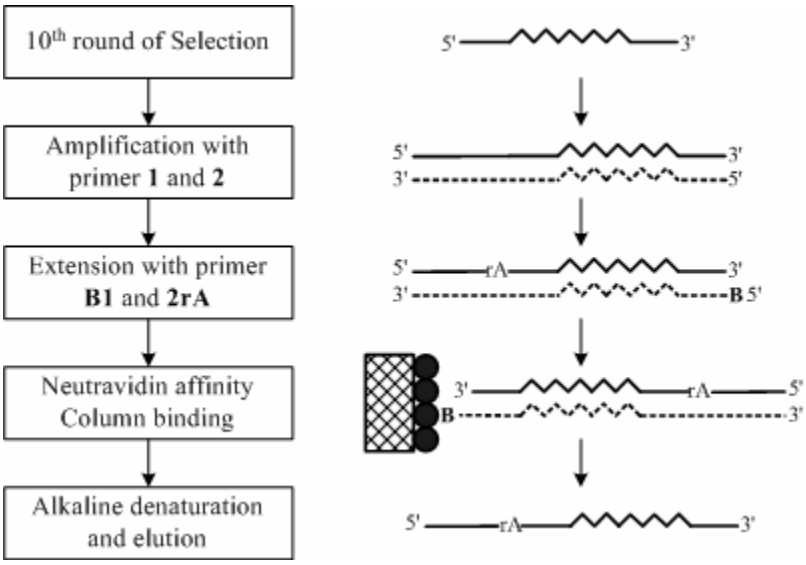
To select coenzyme depended DNA pools we modified a well-established model system[108,109] previously used to isolate DNA molecules that catalyze the cleavage of a ribonucleotide phosphodiester in presence of metal ion cofactors. We began the *in vitro* selection with a naïve DNA pool composed of sequences each containing 40 random deoxyribonucleotides from which at least some few DNA molecules were expected to be

competent for catalysis. Approximately $10^{15}$ of these sequences, equivalent to 80 pmol of DNA, were PCR extended to introduce to each one a 5' biotin moiety and a single ribonucleotide (rA). Extended DNA molecules (101 bp) were gel purified to get rid of undesired PCR products or biotinylated primers. The biotin moiety allowed us to immobilize the DNA molecules to a solid support (column) packed with neutravidin, which had a binding capacity of 100-150 pmol biotin/ml of gel (NeutrAvidin™ user's manual). The purified DNA pool (~40 pmol) was equally distributed between four separate columns (labeled as DC05, DC06, DC07 and DC08) each containing 100 μL of neutravidin protein gel. After 1 h of incubation, unbound DNA molecules were eluted off the column. The non-biotinylated complementary strands were eluted off the column by alkaline denaturation with 0.2 N NaOH solution. Each column was then rapidly neutralized and equilibrated with buffer (50 mM HEPES, 0.5 M NaCl, 0.5 M KCl, 0.5 mM EDTA, pH 7.5 at rt). The column matrix, containing potential self-cleaving DNA molecules, was then subjected to a negative selection. Negative selection would disfavor the isolation of deoxyribozymes or catalytic DNA molecules that use buffer components (HEPES, NaCl, KCl, EDTA and potential divalent metal ions present) as cofactors. EDTA, a chelating agent, was also included in our buffer in order to additionally minimize the participation of possibly present divalent metal ions (Mg, Zn, Pb, Ca, Mn and Cu) during the reaction. 1 h of negative selection was performed after which the column was thoroughly washed and re-equilibrated with binding buffer.

The selection reactions were then started by the addition of buffer containing 50 mM of the various coenzymes to the column matrix, which were then incubated for a period of 5 h. We decided to use 50 mM of as the coenzyme concentration based on the

report of a histidine-depended catalytic polynucleotide.[143] The 5 h of initial incubation was used based on a recent report of the dynamics of vitro selection experiments.[142] This report suggested that for the selection of RNA-cleaving deoxyribozymes, more than five hours is unproductive towards the establishment of a catalytic DNA population from a random-sequence pool, and that less than four hours would increase the risk of loosing potential catalytic strands and reducing the sequence diversity of the selected DNA pool. Reaction times for rounds 1 to 5 were consistent at 5 h. Decreasing reaction times for rounds 6-10 were chosen hoping that this selection pressure would have a positive impact of enhancing the catalytic rate of the selected DNA population. The shortest reaction time (1 min, at the $10^{th}$ round of selection) was limited by the method design. Regarding to this, automatic quenching methods would be necessary to select for catalytic DNA molecules with reaction times in the range of the seconds or milliseconds.

After 10 rounds of selection, the enriched DNA pools from each of the four experiments were isolated following Scheme 11. Pools were amplified with primer P1 and P2 and then an aliquot of the PCR products were reamplified with primers PB1 and P2rA. Self-cleaving strands were separated by alkaline denaturation in a streptavidin column affinity, terminally radiolabeled using standard methods and tested for self-cleavage in reaction buffer solutions containing coenzymes (**DC05**, **DC06**, **DC07** and **DC08**). Self-cleavage experiments with these *in vitro* selected DNA pools gave a preliminary indication of a ribonucleotide phosphoester cleavage activity in presence of 50 mM of cognate coenzyme in buffer (50 mM HEPES, 0.5 M NaCl, 0.5 M KCl, 0.5 mM EDTA, pH 7.5 at rt ).




Scheme 11. Isolation of catalytic DNA molecules from round of selection. Dashed line showing the complementary non-catalytic strand.

*Self-Cleavage Reaction of DNA Pool*

Prior to start the reaction, each radiolabeled DNA pool was heat denatured for 30 sec at 90 °C. It was observed that initial denaturation and slow refolding before exposure to a cofactor or coenzyme improved deoxyribozymes activity.[151] The reaction was then started by adding the coenzyme to a final concentration of 50 mM. A negative control, which was intended to give preliminary information about the dependence of the reaction to the coenzymes, was conducted under the same conditions stated above but in absence of coenzyme. After 4 h of reaction at room temperature products were separated in 10% denaturing polyacrylamide gel and detected by autoradiography. A positive control (chemical hydrolysis of the naïve DNA pool with NaOH solution) was performed to demonstrate the complete cleavage of the DNA pool substrate (100% cleavage efficiency). Self-cleavage was also performed with coenzyme at 10 mM final

concentration in buffer (Figure 78) and similar apparent cleavage efficiency (Figure 78) as in the reaction with 50 mM coenzyme was observed.

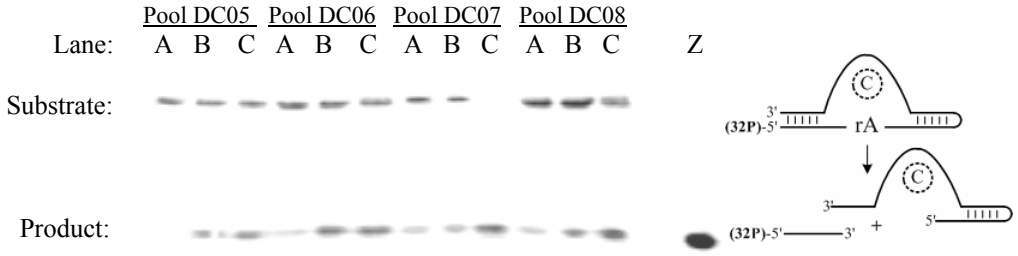



Figure 78**.** Self-cleavage reaction of the 10th round DNA pools selected with **DC05**, **DC06**, **DC07** and **DC08**. Pool DC05, DNA pool selected with **DC05**; Pool DC06, DNA pool selected with **DC06**; Pool DC07, DNA pool selected with **DC07**; Pool DC08, DNA pool selected with **DC08**. Lane A, reaction at time 0; Lane B, negative control reaction without coenzyme; Lane C, reaction in presence of cognate coenzyme. Lanes Z, chemical hydrolysis of ribonucleotide containing DNA pool as control for product mass marker and 100% cleavage. Reaction time 4 h, 10 mM coenzyme, buffer (50 mM HEPES, 0.5 M NaCl, 0.5 M KCl, 0.5 mM EDTA, pH 7.5 at rt).

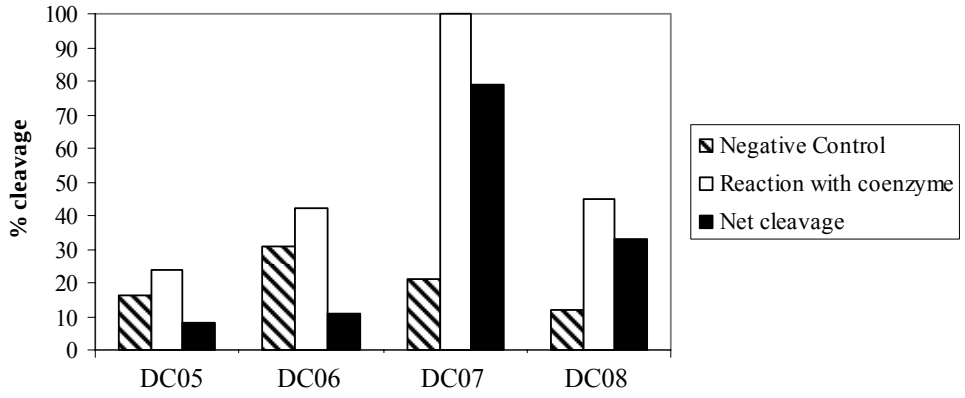



Figure 79. Self-cleavage efficiency DNA pools DC05, DC06, DC07 and DC08.

To report the actual cleavage efficiency of each DNA pool-coenzyme system, the negative control cleavage (cleavage due to the components of the buffer) was subtracted from the cleavage observed in presence of coenzyme in buffer. The self-cleavage experiment performed with this set of newly synthesized coenzymes (**DC05**, **DC06**,

**DC07** and **DC08**) clearly showed marked cleavage efficiency for the DNA pool-coenzyme DC07 of around 80% when subtracted 20% of negative control cleavage. The cleavage efficiency of the other systems (DC05, DC06 and DC08) was low and didn't surpass 30% when the negative control cleavage was subtracted (Figure 79). Approximately 10-60% of self-cleavage product formation was observed in DNA populations selected with divalent metal ions,[109] and 15% in DNA populations selected in presence of monovalent metal ions.[151] Extensive sequence diversity studies of *in vitro* selected DNA populations have shown more than 90% identical sequences (genotypic character) after seven rounds of selection under progressively reduced reaction times, and very similar catalytic rate (phenotypic character) between the selected DNA population and its individual clones.[209] These findings drove us to study the catalytic parameters of the selected pools instead of the individual clones.

*Cross-Reactivity of DNA Pool*

To establish the selectivity of selected pool for its cognate coenzyme, each radiolabeled DNA pool was reacted with its cognate coenzyme and other three coenzymes for 4 h in buffer (50 mM HEPES, 0.5 M NaCl, 0.5 M KCl, 0.5 mM EDTA, pH 7.5 at rt).

No coenzyme specificity was observed for the glutamic pool (pool selected with coenzyme **DC07**) (Figure 80) and its self-cleavage in presence of **DC05**, **DC06** and **DC08** (at comparable cleavage efficiency) may suggest that only a part of the coenzyme, such as a histidine or imidazole, can be interacting with the catalytic site. This is supported by the fact histidine alone has been seen to effectively participate in the self-cleavage of the glutamic pool. In addition the glutamic pool showed similar activity in

presence of N-acetylhistamine but not in presence of histamine. Primary amine of histamine may facilitate strong unspecific-site binding interaction of the small molecule to the catalytic DNAs and impeding the molecule to reach for the catalytic pocket.


Figure 80. Cross-reactivity of the 10th round DNA pools selected with **DC05**, **DC06**, **DC07** and **DC08**. Lanes A, reaction with **DC05**; Lanes B, reaction with **DC06**; Lanes C, reaction with **DC07**; Lanes D, reaction with **DC08**. 10 mM coenzyme, buffer (50 mM HEPES, 0.5 M NaCl, 0.5 M KCl, 0.5 mM EDTA, pH 7.5 at rt), 4 h at rt. 10% denaturing PAGE autoradiography.

These observations together with the catalytic efficiency of the selected pools drove us to further study the binding, rate constant and pH profile of the apparently more active and promiscuous DNA pool DC07 (glutamic pool) with its cognate coenzyme **DC07**. These studies would give more insights of the modus operandi of this particular system.

*Kinetic Study of DNA Pool DC07*

Radiolabeled glutamic pool was reacted in presence of different concentrations of **DC07** in order to obtain its rate constant ($k_{obs}$). Due to the design of the DNA pool for self-cleavage, the rate constant would correspond to a single turnover reaction. Denaturing polyacrylamide gel electrophoresis (Figure 81) shows a distinct self-cleavage product of the glutamic pool even after 5 min of incubation with the coenzyme **DC07.**

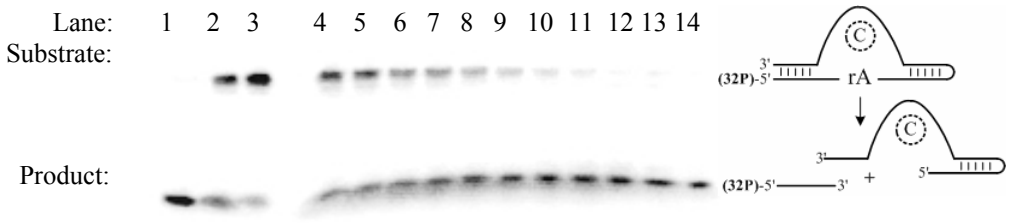



Figure 81. Kinetic assay with glutamic pool. Lane 1, chemical hydrolysis with NaOH; Lane 2, subtrate incubate in absence of coenzyme; Lane 3, reaction at time 0; Lane 4, reaction at 5 min; Lane 5, reaction at 15 min; Lane 6, reaction at 30 min; Lane 7, reaction at 45 min; Lane 8, reaction at 1 h; Lane 9, reaction at 2 h; Lane 10, reaction at 4 h; Lane 11, reaction at 8 h; Lane 12, reaction at 12 h; Lane 13, reaction at 24 h; Lane 14, reaction at 48 h. 10 mM coenzyme, pH 7.4, rt. 10% denaturing PAGE autoradiography.

The cleavage products increased linearly during the first hour of reaction and reached a plateau of ~ 85% after 5 h of reaction (Figure 82).

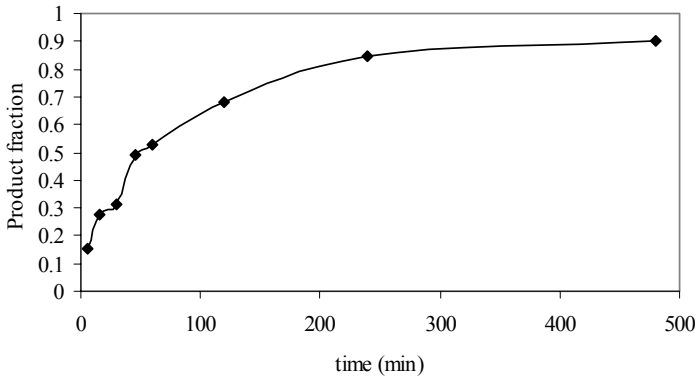



Figure 82. Fraction of cleaved product versus time (min) for glutamic pool.

The glutamic pool rate constant ($k_{obs}$) for self-cleavage was calculated as the negative slope of the natural logarithm of the fraction of the substrate remaining over the time.[143] $k_{obs}$ for the glutamic pool is approximately 1 x $10^{-2}$ $min^{-1}$ (Fig 83). This rate is ~$10^3$-fold faster than the spontaneous rate of cleavage under the same conditions (5 x $10^{-6}$ $min^{-1}$). In addition, the $k_{obs}$ for the glutamic pool is similar to rates previously measured

for the 'first-generation' DNA enzymes selected with $Mg^{2+}$ ($0.002min^{-1}$ after 6 rounds),[109] histidine ($0.0015min^{-1}$ after 11 rounds),[143] or in the absence of divalent metal-ions or added cofactors ($0.001min^{-1}$ after 12 cycles).[151]

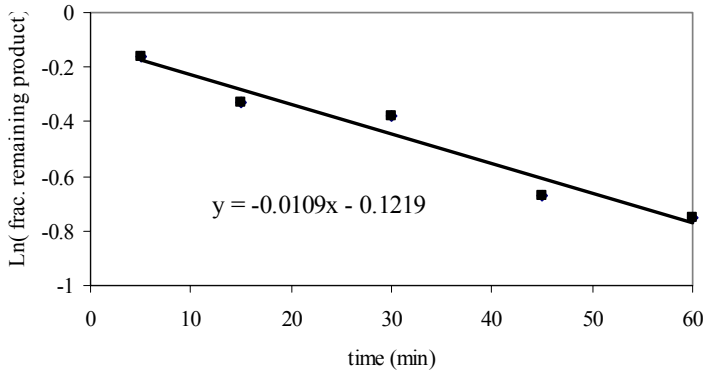



Figure 83. Rate constant calculation for glutamic pool. Calculated as the negative slope of the graph logarithm of the remaining substrate vs. time.

*Binding Study of DNA Pool DC07*

We also studied how different concentrations of glutamic coenzyme (**DC07**) would influence the cleavage efficiency of self-cleaving DNA pool DC07. Duplicate points of each concentration were considered in order to assist in the quantification of the cleaved products. Figure 84 shows the dependence of cleavage efficiency on coenzyme **DC07** concentration. At higher concentrations of coenzyme (100 mM) the cleavage efficiency decreased from a plateau. This large concentration of coenzyme could be significantly perturbing the pH of the reaction due to the high concentration of **DC07**. Reaction scale (10 μL) didn't allow measuring the pH of the reaction at these conditions although the large scale experiment showed a noticeable decrease of the solution pH at high concentrations of the coenzyme **DC07** (> 100 mM).

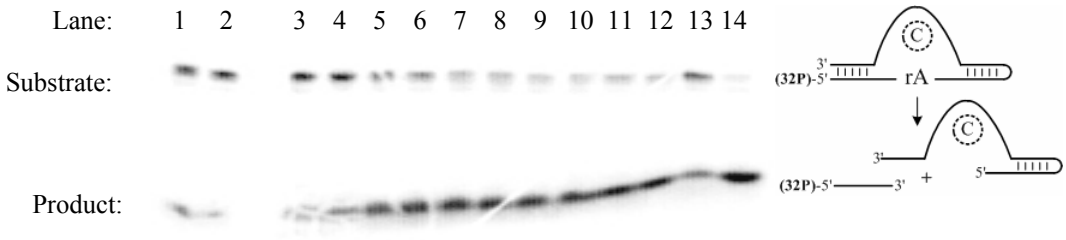



Figure 84**.** Binding assay with glutamic pool with **DC07**. Lane 1, substrate incubate in absence of coenzyme; Lane 2, reaction at time 0; Lanes 3 and 4 , reaction at 0.001mM coenzyme; Lane 5 and 6, reaction at 0.01mM coenzyme; Lane 7 and 8, reaction at 0.1mM coenzyme; Lane 9 and 10, reaction at 1mM coenzyme; Lanes 11 and 12, reaction at 10mM coenzyme; Lane 13, reaction at 100mM; Lane 14, chemical hydrolysis with NaOH. Reaction time 2 h, buffer (50 mM HEPES, 0.5 M NaCl, 0.5 M KCl, 0.5 mM EDTA, pH 7.5 at rt). 10% denaturing PAGE autoradiography.

A plot of the Log$k_{obs}$ versus Log[coenzyme] (Fig 85) revealed that glutamic pool catalytic site is saturated at a concentration of 1 mM **DC07** (apparent $K_d \approx 2 \times 10^{-4}$ M; max $k_{obs} = 1.5 \times 10^{-2}$ $min^{-1}$ at pH 7.5). The slope (0.5) of the curve Log$k_{obs}$ versus Log[coenzyme] before saturation possibly suggest that one imidazole per mol of coenzyme (that contains two imidazoles) is saturating the active site of one mol of catalytic DNA molecules (glutamic pool).

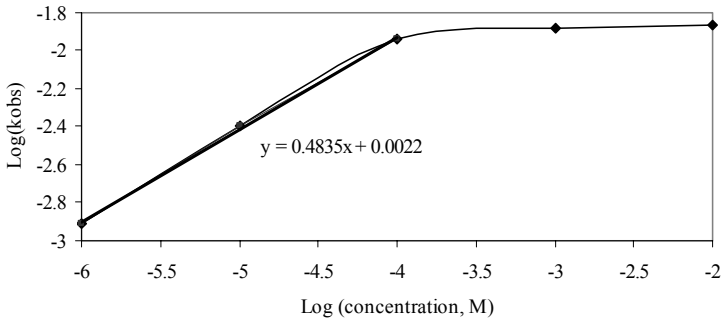



Figure 85. Log($k_{obs}$) versus Log(concentration, M) for glutamic pool and **DC07**. Log (concentration, M) = logarithm of the molar concentration of **DC07**.

To establish the possible presence of a glutamic pool catalytic site dependent simply on a imidazole ring we studied the histidine dependent self-cleavage reaction under the same reaction condition described for the glutamic pool-**DC07**. It was found that the cleavage efficiency associated to the glutamic pool-histidine system (70%) (Figure 86) with a maximum apparent $k_{obs}$ of 6 x $10^{-3}$ $min^{-1}$, which is similar to the reaction with **DC07** (apparent $K_d \approx 2 \times 10^{-4}$ M; max $k_{obs} = 1.5 \times 10^{-2}$ $min^{-1}$ at pH 7.5).

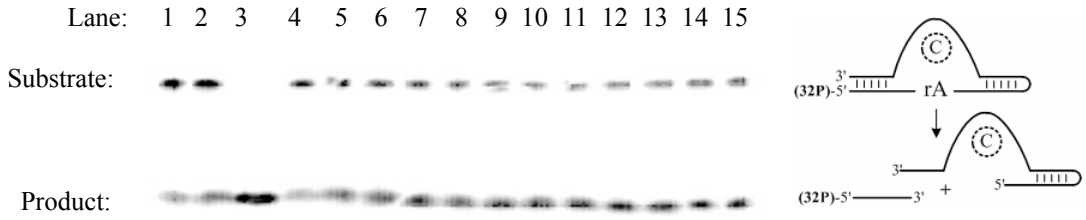



Figure 86. Binding assay with glutamic pool and histidine. Lane 1, reaction at time 0; Lane 2, substrate incubate in absence of coenzyme; Lane 3, chemical hydrolysis with NaOH; Lanes 4 and 5, reaction at 0.002mM histidine; Lanes 6 and 7, reaction at 0.02mM histidine; Lane 8 and 9, reaction at 0.2mM histidine; Lanes 10 and 11, reaction at 2mM histidine; Lanes 12 and 13, reaction at 20mM histidine; Lanes 14 and 15, reaction at 200mM histidine. Reaction time 2 h, buffer (50 mM HEPES, 0.5 M NaCl, 0.5 M KCl, 0.5 mM EDTA, pH 7.5 at rt). 10% denaturing PAGE autoradiography.

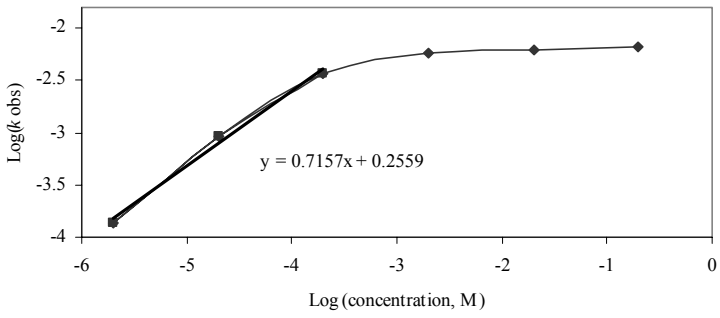



Figure 87. Log(Kobs) versus Log(concentration) for glutamic pool and histidine. Log (M), logarithm of the concentration of Histidine.

The gutamic pool self-cleavage efficiency was dependent on histidine concentration. The glutamic pool catalytic site was saturated at 1 mM of Histidine (apparent $K_d \approx 3 \times 10^{-4}$ M; max $k_{obs} = 6 \times 10^{-3}$ $min^{-1}$) which similar to that found for the glutamic pool-**DC07** system. A slope of 0.7 (~ 1) of the curve Log$k_{obs}$ versus Log[coenzyme] (Figure 87) before saturation may suggest that one mol of histidine saturates the binding site of one mole the catalytic DNA molecules (glutamic pool).

*pH Profile of DNA Pool DC07*

The functional role of the coenzyme **DC07** was studied by evaluating the cleavage efficiency of the glutamic pool – **DC07** system at different pH (5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5 and 9) in reaction buffer (10 mM **DC07**, 50 mM HEPES, 0.5 M NaCl, 0.5 M KCl, 0.5 mM EDTA) (Figure 88). Although the working highly effective pH range for HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffer is suggested to be 6.8 - 8.2,[210] some reports showed the successful use of HEPES buffer at pHs from 5 to 9.[211] In order to keep the same reaction conditions we decided to use HEPES buffer in all the indicated pHs.

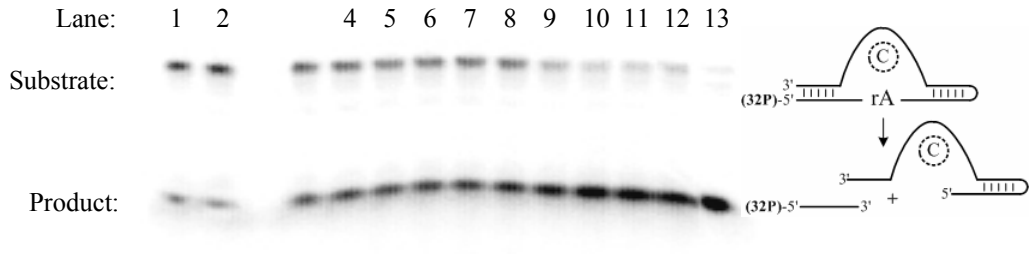



Figure 88. pH profile of glutamic pool. Lane 1, substrate incubate in absence of coenzyme; Lane 2, reaction at time 0; Lane 4, reaction at pH 5; Lane 5, reaction at pH 5.5; Lane 6, reaction at pH 6; Lane 7, reaction at pH 6.5; Lane 8, reaction at pH 7; Lane 9, reaction at pH 7.5; Lane 10, reaction at pH 8; Lane 11, reaction at pH 8.5; Lane 12, reaction at pH 9; Lane 13, chemical hydrolysis with NaOH. 2 h reaction time, 10 mM coenzyme. 10% denaturing PAGE autoradiography.

Cleavage efficiency reached an optimum level at pH ~7.5 (pH used in the selection process). The pH profile of the glutamic pool in absence of coenzyme was also studied under the same conditions described for **DC07**. This cleavage background was subtracted from the product resulting in the pH profile shown in Figure 87. Figure 89 shows that the $k_{obs}$ values were essentially dependent upon pH at 5-6. The log of the $k_{obs}$ plotted against pH yielded a slope of 0.67 (~ 1), similar to the $k_{obs}$ versus pH plots of both naturally occurring self-cleaving ribozymes (such as the hammerhead ribozyme)[13] and several artificial deoxyribozymes.[143,152] This linear dependence of $k_{obs}$ on pH is indicative of proton transfer in the rate-determining steps of catalysis in which our coenzyme **DC07** might be participating. The intersect of the linear increment of pH and the maximum rate constant is obtained at pH 6, suggesting an approximate pKa of 6 for the catalytic moiety of our coenzyme **DC07**. This pKa value corresponds in particular with that for the imidazole group of histidine, suggesting that if the chemical step of this enzymatic process is rate limiting, the deprotonated form of the imidazole moiety of **DC07** may serve as the true catalyst in this reaction.

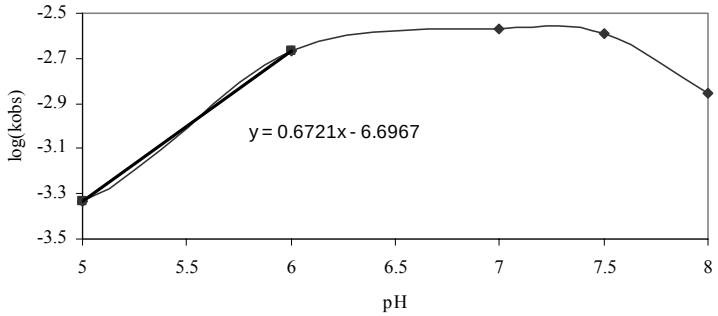



Figure 89. Dependence of the glutamic pool rate constant on pH. Background subtracted.

*Conclusions*

Several recent studies have been directed to the development of more competent (larger $k_{obs}$) DNA enzymes that use divalent metal ions as cofactors. A number of metal ion-dependent DNA enzymes such as the 10-23 and 8-17 deoxyribozyme,[112] capable of cleaving an RNA phosphoester, are well known, but present only moderate cleavage efficiency compared to the natural ribonuclease A. Few studies have been done in finding alternative cofactors,[143] such as small molecules, for DNA enzymes.

The results presented in this chapter again demonstrate the potential of synthetic small molecules as coenzymes for DNA enzymes that cleave a ribonucleotide phosphoester substrate. Four new coenzymes for the DNA enzymes have been designed based on previous reports of RNase A-mimicking molecules,[193] and properly synthesized and characterized.

After including these coenzymes in well-established parallel *in vitro* selection model used before, four enriched DNA pools were obtained. Self-cleavage experiments with the DNA pools selected in presence of the intermediate, arginine and aromatic coenzymes didn't show any significant cleavage efficiency.

As previously seen in ribonuclease mimics, we expected that the presence of either cationic or aromatic binding elements may strengthen the binding of the coenzyme to the DNA enzyme and therefore improve the catalytic efficiency. In contrast, the DNA pool selected in presence of the coenzyme holding a glutamic acid binding element (**DC07**), showed exceptional cleavage efficiency not seen before in our study with these coenzymes.

This first generation glutamic DNA pool showed a rate constant of $10^{-2}$ $min^{-1}$, which represents a ~$10^3$-fold rate enhancement compared to the uncatalyzed reaction. The rate constant of the other selected pools (DC05, DC06 and DC08) was comparable to that of the enzyme pools previously selected with the *bis*-histidine aromatic coenzymes (**DC01**, **DC02**, **DC03** and **DC04**).

We considered that the anionic charge of **DC07**, may be responsible for a "loose" interaction with the phosphate backbone of the DNA enzyme, and that this may significantly influence the catalytic efficiency. Binding studies with **DC07** and its catalytic pool of DNA gave evidence of this loose interaction (apparent $K_d$, ~1 mM).

pH studies showed a potential catalytic role for the imidazole of **DC07** as a general base (single proton transfer) that can activate the 2'-hydroxyl for the nucleophilic attack to the phosphate ester. This pH data together with the catalytic rate (1 x $10^{-2}$ $min^{-1}$) found, very much agrees with the γ catalysis proposed by Breaker.[158] Surprisingly, it was observed that the enzyme pool selected with **DC07** was also active in the presence of free histidine, with rate constant (1 x $10^{-3}$ $min^{-1}$, 10-fold slower than with **DC07**) and a similar binding constant. Similarly, other coenzymes (**DC05**, **DC06** and **DC08**) were found to cross-react with the glutamic pool, although with a lower efficiency.

An important lesson that emerges from this study is the suggestion that the use of poorly-bound coenzyme in the *in vitro* selection process may result in the selection of DNA enzymes that are ideal balance between coenzyme binding and coenzyme dependent catalysis.

Future work, including additional cloning and sequencing of the individual members of the pools will be necessary in order better characterize these DNA enzymes and to more clearly understand details of their mechanism.

# CHAPTER FIVE

## Conclusions and Future Directions

Eight potential coenzymes (**DC01**, **DC02**, **DC03**, **DC04**, **DC05**, **DC06**, **DC07** and **DC08**) for DNA enzymes have been synthesized, characterized, incorporated in *in vitro* selection experiments, and analyzed for ribonucleotide phosphoester cleavage activity. Additionally, **OG01**, a previously synthesized potential coenzyme, was studied in a same fashion. Our studies of these synthetic coenzyme-DNA enzyme systems reveal the desired RNA cleavage dependent upon the presence of the coenzyme. The results should aid in the choice of structural characteristics for the design of future improved DNA enzyme coenzymes. However, further studies are certainly necessary in order to more clearly understand details of catalytic mechanism of these coenzyme-DNA enzyme systems.

Although our initial studies with **OG01** did not result in the isolation of a coenzyme dependent DNA enzyme, they permitted us to establish a robust methodology for the evaluation of potential coenzymes for DNA enzymes based on a commonly used combinatorial technique known as *in vitro* selection.

DNA enzyme pools selected using the *bis*-histidine-containing aromatic coenzymes **DC01**, **DC02** and **DC03** have shown specificity for their cognate coenzymes with apparent dissociation constant of ~1 mM and rate constants of $1.5 \times 10^{-3}$ $min^{-1}$ for self-cleavage, representing a ~450-fold enhancement over the uncatalyzed reaction. In particular, pH studies with **DC01** and **DC03** suggest a single proton transfer as the rate limiting step with a calculated $pK_a$, 6 and 6.3 respectively, suggesting their participation

of the coenzyme imidazoles as general bases. While the coenzyme specificity observed for DNA enzyme pools **DC01**, **DC02**, and **DC03** is notable, lack of specificity of the pool selected with **DC04** to its cognate coenzyme, as well as higher dissociation constant, has been observed for this system. Future structural, kinetic and inhibition studies with individual sequences from the catalytic DNA pools will provide valuable insights into fundamental molecular interactions of these novel catalytic systems.

In further experimentation, DNA enzyme pools selected using *bis*-imidazole-containing peptidic coenzymes **DC05**, **DC06**, **DC07** and **DC08** demonstrated little specificity for their cognate coenzymes. In particular, the catalytic DNA pool obtained with **DC07**, which has a glutamic acid residue, was also active in presence of **DC05**, **DC06, DC08**, free histidine and N-acetylhistamine. Interestingly, the catalytic DNA pool obtained with **DC07** has a rate constant (in the presence of **DC07**) of 1 x $10^{-2}$ $min^{-1}$, which represents a ~5000-fold enhanced over the uncatalyzed reaction. This is higher than that found for the best enzyme/coenzyme pair in the more rigid aromatic series (**DC01**, **DC02**, **DC03** and **DC04**). The cross-reactivity of this obtained pool with imidazole-containing analogs may suggest the presence of a pre-organized catalytic pocket, which in presence of the coenzyme results in reaction. pH studies with this coenzyme-DNA enzymes pool system have revealed a potential mechanistic role for **DC07** involving a proton transfer that involves the imidazole as general base catalysis (~$pK_a$ = 6). The rate constant (1 x $10^{-2}$ $min^{-1}$) and general base catalysis reported for this coenzyme-DNA enzyme system may suggest a γ catalytic strategy[142] and the direct participation of **DC07** in catalysis. Evaluation of the individual sequences present in the catalytic DNA pool obtained with **DC07** will allow further characterization and

optimization of these novel DNA enzymes that apparently use a synthetic organic compound to attain catalytic competency.

We can conclude that in general, the *bis*-histidine aromatic coenzyme-DNA enzyme systems display good specificity but poor reaction completeness. On the other hand, systems containing *bis*-imidazole peptido coenzyme-DNA enzyme systems show limited specificity but good reaction progress.

The experimental methodology described in this dissertation provides the opportunity to utilize the tools of synthetic organic chemistry for the invention of novel coenzymes that can potentially provide enzymes with enhanced and/or unusual catalytic properties. This rapid and efficient combinatorial methodology of *in vitro* selection complements intuitive chemical design approaches, providing another approach to the creation of enzymes that utilize novel coenzymes, a unique opportunity for the study of a variety of aspects of coenzyme/enzyme design, and the pursuit of fundamental investigations into coenzyme/enzyme interactions.

# APPENDICES

# APPENDIX A

## Molecular Biology Protocols

# APPENDIX A

## Molecular Biology Protocols

### *PCR Amplification of DNA Template*

Reference: Kearney, C. Molecular Genetics Laboratory Manual, Baylor University

1. Take PCR components out of the -20 °C freezer to thaw, place them into a tube rack to thaw, and then into a "chiller box" to stay cold while assembling your PCR.
2. Prepare the following solutions:

   Template (**T**): 5 µM

   5'-CTAATACGACTCACTATAGGAAGAGATGGCGACATCTC$(N)_{40}$GTGAGGTTGGTGTGGTTG-3'

   Primer B2 (**PB2**): 10 µM

   5'-Biotin-GAATTCTAATACGACTCACTATrA-3'

   Primer 1 (**P1**): 10 µM

   5'-CAACCACACCAACCTCAC-3'
3. Prepare PCR cocktail following recipe below. Be sure to mix all tubes well (by shaking, then shake contents down into the bottom of the tube) before pipetting them.

| | |
|---|---|
| DNA template | 1 µL |
| 10X PCR buffer | 5 µL |
| 25 mM $MgCl_2$ | 3 µL |
| 2 mM dNTPs | 5 µL |
| 10 µMPrimer **1** | 8 µL |
| 10 µM Primer **B2** | 8 µL |

0.05 U/ul *Taq* Poly 1 µL

Sterile water to a total volume of 49 µL

4 cycles of 94 °C (15 s), 50 °C (30 s), 72 °C (30 s).

4. After the PCR is complete, run out samples on an agarose gel to purify or store in freezer at -20 °C until the gel is run.

*Preparation of Low Range Ultra Agarose Gel*

Reference: Kearney, C. Molecular Genetics Laboratory Manual, Baylor University

1. Take 30 mL of 1X TAE electrophoresis buffer for the gel casting tray apparatus. Take additional 400 mL 1X TAE electrophoresis buffer to run the gel.
2. Tape ends of casting tray and place comb. Make sure that there is a paper width of space between the comb and the bottom of the gel tray and that the comb is leveled. Make sure the gel holder is also level.
3. Prepare 1.5% Low Range ultra Agarose Gel by using 0.45 g of gel power in 30 mL of TAE buffer.
4. Combine in 125 mL flask. Cover top of flask with plastic wrap and punch a small hole in the top. Heat in the microwave at medium heat, 30 s/time until dissolved. Swirl after each heating. Be sure all agarose particles are completely melted.
5. Pour into gel casting tray (cool solution enough to touch to prevent warping of the tray).
6. Let gel solidify for 30 min before use.
7. Remove the tape on each end of the tray and put the gel in the electrophoresis apparatus. Cover the gel to a depth of 1-2 mm. with sufficient 1X TAE buffer. The gel may be stored overnight in this condition but no longer.

*Running DNA Samples in Agarose Gel*

Reference: Kearney, C. Molecular Genetics Laboratory Manual, Baylor University

1. Prepare each DNA sample by mixing 5 vol of DNA solution with 1 vol of 6X DNA loading dye solution (Fermentas).
2. Load your samples in separate wells of the gel. Load the samples in the wells using a P20 automatic pipette. Change pipette tips between samples. Place the tip of the pipette containing the sample to be loaded under the buffer, just at the opening of the well. Be careful not to stab a hole in the bottom of the well with the pipette tip.
3. Place the cover on the gel electrophoresis unit and plug the electrode leads into the power supply unit.
4. Turn on the power and run the gel at 95-105 V for 30-45 min or until the tracking dye is 2/3 to 3/4 of the distance to the end of the gel.
5. Turn off the power.
6. Unplug the electrode leads, remove the top of the unit, take out the gel and gel holder (caution, the gel can slide off the holder).
7. Slide the gel into ethidium bromide staining solution (0.5 μg/ml in water). Ethidium bromide is a mutagen and potential carcinogen, wear gloves all the time.
8. Stain for 15 min. Remove gel from stain and place on clear plastic container filled with distilled water.
9. Gels can be washed in water for additional 30 min to remove excess of staining solution.
10. Put gel on top of plastic wrap and cover.

11. Examine gel on a transilluminator using long-wavelength (302 nm) UV light, desired bands can be cut (see DNA extraction from agarose gel protocol) or photographed.

*DNA Extraction from Agarose Gel*

Reference: QIAEX II, Gel extraction kit, QIAGEN.

1. Excise the DNA band from the agarose gel with a clean sharp scalpel.
2. Put the gel in a 1.5 mL centrifuge tube and add 300 µL of Buffer QX1.
3. Resuspend QIAEX II by vortexing for 30 s. Add 10 µL of QIAEX II to the gel sample and mix.
4. Incubate at 50 °C for 10 min to solubilize the agarose and bind the DNA to the QIAEX crystal particles. Mix by vortexing every 2 min to keep QIAEX II in suspension.
5. Centrifuge the sample for 30 s at maximum speed (~13,000 rpm) and carefully remove supernatant with a pipette.
6. Wash the pellet with 500 µL of Buffer QX1 (vortex and centrifuge at max. speed).
7. Wash the pellet twice with 500 µL of Buffer PE (vortex and centrifuge at max. speed).
8. Air-dry the pellet for 10–15 min or until the pellet becomes white. Do not vacuum dry the pellet, it reduces the DNA extraction efficiency.
9. To extract DNA, add 20 µL of 10 mM Tris·Cl, pH 8.5 and resuspend the pellet by vortexing. Incubate 5 min at room temperature.

10. Centrifuge for 30 s and carefully pipette the supernatant into a clean tube. Repeat one more time for maximum DNA recovery.

Reference: Kearney, C. Molecular Genetics Laboratory Manual, Baylor University

1. In case QIAEX II, Gel extraction kit is not available this general method can be used.
2. Use a clean sharp scalpel to cut out the segment of the gel containing the band of interest, keeping the size of the gel slice the smallest possible.
3. Transfer the gel slice to a 1.5 mL centrifuge tube. Use a disposable pipette tip to crush the gel against the wall of the tube.
4. Calculate the approximate weight of the gel slice and add 3 volumes of gel NaI elution solution (8 M NaI, 0.2 M sodium sulfite in sterile distilled water) to the centrifuge tube.
5. Close the tube and incubate at 45-55 °C for 5 min, mixing every 1-2 min.
6. Add 5 µL glassmilk, mix at let incubate at room temperature fro 5 min. Mix every 1-2 min.
7. Centrifuge sample at maximum speed (13,000 rpm) for 5 s. Discard the supernatant, being extremely careful to avoid taking white sediment.
8. Resuspend pellet in 400 µL buffer (5 M NaCl, 1 M TrisHCl, 0.5 M EDTA, 50% v/v ethanol in water), centrifuge for 5 sec and pipette off the supernatant.
9. Repeat step 8 for 5 additional times.
10. Dissolve pellet in 10 µL of TE (pH 8.0) or water by vortexing.
11. Centrifuge at max speed for 30 s and take the supernatant containing DNA to a new tube.

*Ethanol Precipitation of DNA*

Reference: Sambrook and Russell, Molecular Cloning, Laboratory Manual, CSHL Press.

1. Mix DNA solution with 1/10 volume of 3M NaOAc pH 5.2 in a centrifuge tube.
2. Add 3 vol of 95% ethanol.
3. Precipitate DNA at -20 °C or 0 °C for 60 min to overnight.
4. Centrifuge at 4 °C at 13,000 rpm. for 20 min to pellet DNA.
5. Carefully remove the supernatant. Since DNA pellet is many times is not visible, don't touch the microcentrifuge tube inside walls or bottom.
6. Wash the pellet DNA with 70% ethanol and spin for 5 min.
7. Air-dry the DNA and resuspend in water or buffer.

*Quantification of DNA by UV Spectrophotometry*

Reference: GeneQuant pro DNA calculator, User Manual. Individual DNA samples' absorbance will be measured at 230, 260, 280 and 320 nm; DNA concentration, 260/280 and 260/230 ration will be calculated.

1. The 5 mm Quartz cuvette is washed with 1 mL 0.1 M NaOH, 1 mL sterile water, and 1 mL ethanol and wiped with a kimwipe.
2. The spectrophotometer is turned on.
3. When the display reads "Instrument Ready", "set up" is selected.
4. When the display reads "Setup-Base", "Base Type – DNA" is selected for PCR amplified DNA. "Base Type – Oligo" is selected when quantifying primers or DNA template.
5. The path length is set to 5 mm and the units to ng/μL.
6. "Yes" is selected for all absorbance options.

7. The dilution is entered if necessary: enter “1” if running an undiluted sample (“10” for 1:10, range 1-1000, etc).
8. The cuvette is filled with 10 µL same buffer that was used to dissolve the DNA. Make sure there are no bubbles inside the cuvette.
9. The cuvette is wiped with a kimwipe and inserted into the spectrophotometer with a black panel in front.
10. Set reference is selected for the background.
11. When the display reads “Instrument Ready”, the cuvette is removed.
12. The water is withdrawn from the cuvette with a P-10 pipette and filled with 10 µL of the DNA sample.
13. The cuvette is inserted and “DNA” or “Oligo” is selected.
14. The cuvette is removed and rinsed with 1 mL sterile water and then with 1 mL ethanol. The setup/”set ref” is not repeated unless settings or solvent are changed between samples.

*Preparation of Denaturing DNA Polyacrylamide Gel Electrophoresis*

Reference: Sambrook and Russell, Molecular Cloning, Laboratory Manual, CSHL Press.

1. Assemble the glass plates with spacers for casting the gel.
2. For a 10% TBE urea gel, prepare a gel solution by mixing 22.2 mL of acrylamide:bis solution (45%), 10 mL 10X TBE buffer, 32.5 mL water and 42g. urea.
3. Heat the solution in a 55 °C water bath for 3 min. and then allow it to cool at room temperature for 15 min. Degas the solution attaching a vacuum line.

4. Add 3.3 mL of freshly prepared 1.6% ammonium persulfate and swirl the gel solution gently to mix the reagents.
5. Add 50 μL of TEMED to the gel solution and mix gently.
6. Pour acrylamide gel solution mix between plates and insert appropriate comb. Allow gel to polymerize at room temperature for at least 30 min.
7. After polymerization of the gel is completed, remove comb and rinse sample wells with water.
8. Store gels in plastic bag in TBE buffer at 4 °C.

*Running DNA Samples in Denaturing DNA Polyacrylamide Gel*

Reference: Kearney, C. Molecular Genetics Laboratory Manual, Baylor University

1. Fill lower reservoir of the Mini-protean 3 cell gel tank with 1X of TBE buffer
2. Attach glass plates to the gel electrophoresis apparatus. Fill the upper buffer chamber with 1X TBE buffer so that the wells are covered and rinsed them with buffer.
3. Mix DNA samples with 1 volume of sample loading dye solution and load on gel.
4. Place the cover on the gel electrophoresis unit and plug the electrode leads into the power supply unit.
5. Turn on the power and run the gel at 190-200 V for 15-30 min or until the tracking dye is 2/3 to 3/4 of the distance to the end of the gel.
6. Turn off the power.
7. Unplug the electrode leads, remove the top of the unit, take out the gel and gel holder (caution, the gel can slide off the holder).

8. Slide the gel into ethidium bromide staining solution (0.5 μg/ml in water). Ethidium bromide is a mutagen and potential carcinogen; wear gloves all the time.
9. Stain for 15 min. Remove gel from stain and place on clear plastic container filled with distilled water.
10. Gels can be washed in water for additional 30 min to remove excess of staining solution.
11. Put gel on top of plastic wrap and cover. Gel can be dried (see Drying Polyacrylamide Urea Gel with radioactive DNA protocol for reference) or photographed under UV light.
12. Photograph gel on a transilluminator using long-wavelength (302 nm) UV light.

*Electroblotting of DNA*

Reference: Sambrook and Russell, Molecular Cloning, Laboratory Manual, CSHL Press.

1. Run the DNA samples in denaturing Polyacrylamide gel.
2. When electrophoresis is almost complete, cut a piece of nylon membrane sufficient in size to cover the relevant parts of the gel. Pour distilled water ~0.5 cm deep in a glass dish. Wet the membrane by floating it on the surface of the water, and then submerge it. Leave for 5 min.
3. When electrophoresis is complete, cut a piece of Whatman 3MM filter paper slightly larger than the gel. Lay the filter paper on the surface of the gel and remove trapped air bubbles by rolling a glass pipette overt the surface. The gel should adhere to the filter paper. Lift the gel off the glass plate by peeling the filter paper away.

4. Soak two 3M Scotch-brite pads in 0.5X TBE and remove air pockets by repeated squeezing and agitation. Cut seven pieces of Whatman paper 3MM paper to the same size as the gel and soak these for 15 to 30 min in 0.5X TBE.
5. Place the opened gel holder of the Trans-Blot cell in a shallow tray, with the grey panel resulting flat on the bottom, and place one of the saturated Scotch-brite pads on the inner surface of the grey panel. Place three soaked filter papers on the pad. To ensure that there are no trapped air bubbles, build up the stack of filter papers one by one, carefully searching for trapped bubbles and removing them by rolling a glass pipette over the surface of the top paper.
6. Flood the filter paper carrying the gel with 0.5X TBE and place on top of the filter-paper stack. Flood the surface of the gel with 0.5X TBE and place the prewetted membrane onto the gel.
7. Flood the surface of the membrane with 0.5X TBE and place the remaining four sheets of saturated Whatman 3MM paper on top, followed by the saturated Scotch-brite pad. Close the gel holder.
8. Half-fill the Trans-Blot cell with 0.5X TBE and place the gel holder in the cell with the grey panel facing towards the cathode. Fill the cell with 0.5X TBE and electroblot at 30V (~125 mA) for 4 h.
9. Switch off the power and remove the gel holder, denature the DNA by placing it for 10 min, DNA-side-up, on three pieces of Whatman 3MM paper soaked in 0.4M NaOH.
10. Rinse the membrane in 2XSSC, place on a sheet of Whatman 3MM paper, and allow drying.

11. Wrap the membrane in UV-transparent plastic wrap, place DNA-side-down on a UV transilluminator (254-nm wavelength) and irradiate for 30 s.
12. Store membranes dry between sheets of Whatman 3MM paper for several months at room temperature. For long-term storage, place membranes in a desiccator at room temperature.

*Detection of DNA by Chemiluminescent Hybridization*

Reference: North2South user's manual. Pierce

1. Add enough North2South™ Blocking Buffer to generously cover the membrane (nylon membrane or scotch-brite membrane); 0.25 mL of buffer is recommended per $cm^2$ of membrane. Incubate with constant, gentle agitation (shaking or rotating) for 15 min at room temperature at room temperature.
2. Pour off some of the North2South™ Blocking Buffer solution into a separate tube and add stabilized Streptavidin-HRP conjugate to the solution for a 1:300 final dilution. Ass the diluted conjugate to the membrane and incubate for 15 min at room temperature with agitation.
3. Dilute North2South™ Wash Buffer (4X) to 1X with sterile $dH_2O$. Wash the membrane 4 times for 5 min per wash with 1X North2South™ Wash Buffer at room temperature with gentle agitation. Place membrane into a clean wash container for the next step.
4. Incubate the membrane in the North2South™ Substrate Equilibrium Buffer (0.25 mL of buffer is recommended per $cm^2$ of membrane) for 5 min at room temperature with gentle agitation.

5. Mix equal volumes of the North2South™ Luminol/Enhancer solution and North2South™ Stable Peroxide solution. Prepare enough solution to completely cover the membrane (approximately 0.1 mL/cm$^2$).
6. Place the moist membrane on clear plastic wrap and cover with North2South™ Working Solution (use at least 0.1 mL/cm$^2$). Incubate for 5-10 min at room temperature.
7. Drain (decant) off the substrate from the membrane surface. Blot membrane briefly on clear paper tower or filter paper.
8. Transfer the moist membrane to sheet protector or wrap in clear plastic wrap. Carefully remove any trapped air bubbles or wrinkles within the plastic wrap. Dry the exterior of the sheets or plastic wrap with a clean paper towel.
9. Place the plastic-protected membrane into a film cassette and expose blot to film for 1 min.
10. Remove the film from the holder and develop the film using Bio-Rad Fluor-S™ Multlmager**.**

*Subcloning of DNA*

Reference: TA Cloning Kit user's manual, Invitrogen

*PCR Amplification.*

1. Prepare PCR cocktail following recipe below:

| | |
|---|---|
| DNA template | 10 ng |
| 10X PCR buffer | 5 µL |
| 2 mM dNTPs | 5 µL |

| | |
|---|---|
| 25 mM $MgCl_2$ | 3 µL |
| 10 µM Primer 1 | 2 µL |
| 10 µM Primer 2 | 2 µL |
| Taq Polymerase | 1 unit |
| Sterile water | to a total volume of 50 µL |

94 °C (30 s); 10 cycles of 92 °C (15 s), 50 °C (30 s), 72 °C (30 s).

*Cloning into pCR®2.1*

1. Centrifuge one vial of pCR® 2.1 to collect all the liquid in the bottom of the vial.
2. Use the formula below to estimate the amount of PCR product needed to ligate with 50 ng of pCR® 2.1 vector:
3. $$\text{x ng PCR product} = \frac{(\text{Y bp PCR product})(50 \text{ ng pCR}^{®}2.1 \text{ vector})}{(\text{size in bp of the pCR}^{®}2.1 \text{ vector: } \sim 3900)}$$

   where x ng is the amount of PCR product of Y base pairs to be ligated for a 1:1 (vector:insert) molar ratio. It is recommended that a 1:1 (vector:insert) ratio be used; however higher ratios (vector:insert) won't affect the reaction.
4. Using the concentration previously determined for the PCR sample, calculate the volume needed to give the amount determined in step 3. Use sterile water to dilute your PCR sample.
5. Set up the 10 µL ligation reaction as follows:

   a. Fresh PCR product x µL
   b. 10X ligation buffer 1 µL
   c. pCR®2.1 vector (25 ng/ul) 2 µL
   d. Sterile Water to a volume of 9 µL

e. T4 DNA ligase (4.0 Weiss units) 1 μL

f. Final volume 10 μL

6. Incubate the ligation reaction at 14 °C for a minimum of 4 h (preferably overnight).

7. Ligation reaction can be stored at -20 °C until ready for transformation.

*Transformation.*

1. Equilibrate a water bath to 42 °C

2. Bring the SOC medium to room temperature.

3. Take LB plates containing 50 μg/ml of kanamycin or 100 μg/ml ampicillin and equilibrate at 37 °C for 30 min. Spread 40 μL each of 100 mM IPTG and 40 mg/ml X-Gal onto the plates. Let the liquid soak into plates.

4. Centrifuge the vials containing the ligation reaction briefly and place them on ice.

5. Thaw on ice 50 μL vial of frozen One Shot® competent cells for each ligation/transformation.

6. Pipette 2 μL of each ligation reaction directly into the vial of competent cells and mix by stirring gently with the pipette tip.

7. Incubate the vials on ice for 30 min. Store the remaining ligation mixtures at -20 °C.

8. Heat shock for exactly 30 s in a 42 °C water bath. Do not mix or shake. Remove the vials form the 42 °C water bath and place on ice.

9. Add 250 μL of SOC medium (at room temperature) to each tube.

10. Shake the vials horizontally at 37 °C for 1 hour at 225 rpm in shaking incubator.

11. Spread 10 µL to 200 µL from each transformation vial on separate, labeled LB agar plates containing IPTG and X-Gal and antibiotic. Two different volumes are plated to ensure that at least one plate has well-spaced colonies.
12. Make sure the liquid is absorbed, then invert the plates and place them in a 37 °C incubator for at least 18 h. Shift plates to 4 °C for 2-3 h to allow proper color development.
13. Take pink isolated colonies and mix with 5 mL of sterile LB medium broth.
14. Incubate at 37 °C for 12 hour.

*Purification of Plasmid DNA*

Reference: Plasmid Miniprep kit technical bulleting. Sigma

1. Pellet cells from 1-5 mL overnight culture 1 min (1 mL from TB or 2xYT; 1-5 mL from LB medium). Discard supernatant.
2. Resuspend cells in 200 µL resuspension solution. Pipette up and down or vortex.
3. Add 200 µL of Lysis Solution. Invert gently to mix. Do not vortex. Allow to clear for ≤ 5 min.
4. Add 350 µL of Neutralization Solution (S3). Invert 4-6 times to mix.
5. Pellet debris 10 min at max speed.
6. Add 500 mL Column Preparation Solution to binding column in a collection tube.
7. Spin at ≥12,000 x g, 1 min. Discard flow-through.
8. Transfer cleared lysate into binding column.
9. Spin 30 s - 1 min. Discard flow-through.
10. Add 750 µL Wash Solution to column. Spin 30"-1 min. Discard flow-through.
11. Spin 1 min. to dry column.

12. Transfer column to new collection tube.

13. Add 100 µL Elution Solution. Spin 1 min.

14. Elute contains plasmid DNA.

*Quantification of Plasmid DNA*

Reference: Kearney, C. Molecular Genetics Laboratory Manual, Baylor University

1. Prepare the following reaction mixture:

| | |
|---|---|
| 10X BAS buffer | 1 µL |
| 2NE buffer | 1 µL |
| XhOI restriction enzyme | 0.5 µL |
| DNA sample | 2 µL |
| Sterile $dH_2O$ | to 10 µL |

2. Let it react for 1.5 h at 37 °C and stop by adding 1 volume of sample loading dye solution.

3. Run sample in 1% agarose gel.

*Amplification of DNA Insert from Vector*

Reference: Kearney, C. Molecular Genetics Laboratory Manual, Baylor University

1. Prepare PCR cocktail following recipe below.

| | |
|---|---|
| DNA template | 1 µL equivalent to 10-50 ng |
| 10X PCR buffer | 5 uL |
| 25 mM $MgCl_2$ | 3 µL |
| 2 mM dNTPs | 5 µL |
| 5 µM Primer **1** | 4 µL |

| | |
|---|---|
| 5 μM Primer M13 | 4 μL |
| Taq Polymerase | 0.5 μL (2.5 units) |
| Sterile water | to a total volume of 50 μL |

94 °C (5 min); 30 cycles of 94 °C (1 min), 56 °C (1 min), 72 °C (1 min); 72 (2 min).

2. Gel purify ~200 bp product with 1% agarose gel.

3. Use purified product for sequencing reaction.

*Sequencing of DNA*

Reference: Kearney, C. Molecular Genetics Laboratory Manual, Baylor University

1. Prepare DTCS premix

| | |
|---|---|
| 10X Sequencing Reaction Buffer | 200 μL |
| dNTPs Mix | 100 μL |
| ddUTP Dye Terminator | 200 μL |
| ddGTP Dye Terminator | 200 μL |
| ddCTP Dye Terminator | 200 μL |
| ddATP Dye Terminator | 200 μL |
| *Taq* Polymerase | 100 μL |
| Total volume | 1200 μL |

2. Prepare and run sequencing reaction as follow:

| | |
|---|---|
| DNA template | 0.5-6 μL (3-10 ng) |
| Primer (1,6 μM) | 2 μL |
| DTCS Premix | 12 μL |
| Sterile $dH_2O$ | to 20 μL |

30 cycles, 96 °C (20 s.), 50 °C (20 s.), 60 °C (4 min).

3. Sterilize and label a 0.5 mL microfuge tube.
4. Add to the tube 4 µL of stop solution (95% formamide) and 1 µL of 20mg/ml of glycogen. Transfer the sequencing reaction the 0.5 mL microfuge tube and mix.
5. Add 60 µL 95% cold ethanol and mix and immediately centrifuge at 14,000 rpm at 4 °C for 15 min. Remove supernatant with pipette.
6. Rinse pellet with 200 µL 70% ethanol and centrifuge at 14,000 rpm at 4 °C for 2 min. Remove the supernatant with pipette. Repeat this process one more time.
7. Vacuum dry the sample for 40 min.
8. Resuspend the pellet in 40 µL sample loading solution (SLS).

*Labeling of 5'-DNA end Using [γ-$^{32}$P]-ATP*

Reference: Protocol for Labeling 5'-termini of DNA by Forward Reaction, Fermentas

1. Prepare the following reaction mixture:

   Dephosphorylated DNA (DNA PCR product, chemically synthesized Template DNA or primer): 1-20 pmol of 5'-termini

   10X reaction buffer A: 2µL

   [gama-$^{32}$P]-ATP 20 pmol:

   Water, nuclease-free to 19 µL

   T4 Polynucleotide Kinase (10u): 1µL
2. Incubate at 37 °C for 30 min.
3. Add 1µL 0.5M EDTA (pH 8.0) and extract with an equal volume of chloroform.
4. Separate labeled DNA from unincorporated label by gel filtration on Sephadex G-50.

Reference: High Specific Activity Labeling with [γ-$^{32}$P]-ATP, Novagen.

1. Assemble the following reaction in a microcentrifuge tube:

   1-50 pmol of dephosphorylated DNA (DNA PCR product) or oligonucleotide.

   5 μL 10X Kinase Buffer.

   150 μCi [γ-$^{32}$P]-ATP (>3000 Ci/mmol, 10 mCi/ml, 50 pmol total).

   10 units T4 Polynucleotide Kinase.

   sterile distilled water to 50 μL.

2. Incubate at 37 °C for 10 min.
3. Stop the reaction by adding 2 μL of 0.5M EDTA and heat at 70 °C for 10 min.
4. Centrifuge briefly to collect the liquid at the bottom of the tube.
5. Separate labeled DNA from unincorporated label by gel filtration on Sephadex G-50.

Note: In order to work with radioactive material, Baylor Radiation Mandatory Training is required. Personal protection and precautions are found in the Baylor Radiation Safety Manual.

*Purification* of [γ-$^{32}$P]-*labeled DNA*

Reference: Quick spin column user's manual. Roche

1. Take sephadex column from storage at 4 °C and allow it to warm to room temperature.
2. Gently invert the column several times to homogenize the gel slurry.

3. Remove the top cap off the column, and then remove the bottom tip. The top cap must be removed first to avoid creating a vacuum and uneven flow of the column buffer.
4. Place column in a clean 1.5 mL microcentrifuge tube.
5. Centrifuge at 1,000x gravities for 2 min. Discard the collection tube and the eluted buffer.
6. Keeping the column in an upright position, very slowly and carefully apply the DNA sample (up to 50 µL) to the center of the column gel bed.
7. Keeping the column in an upright position, place the column in a second collection tube.
8. Centrifuge at 1,000x gravities for 4 min.
9. Save the eluate from the second collection tube. This contains your purified DNA sample.
10. Discard the column to the $\gamma$-$^{32}$P solid waste container.

Note: In order to work with radioactive material, Baylor Radiation Mandatory Training is required. Personal protection and precautions are found in the Baylor Radiation Safety Manual.

*Quantification of* [$\gamma$-$^{32}$P]-*labeled DNA by Liquid Scintillation*

Reference: Personal notes.

1. Fill scintillation vials with 3 mL of Bio-Safe II liquid scintillation cocktail using the Hirschmann Dispenser.
2. Place the blank sample in a glass scintillation vial in a position 1 of the sample rack.

3. Place all other samples individually into glass scintillation vials.
4. Attach the screw caps on each sample and shake to completely mix sample with liquid scintillation cocktail.
5. Attach use program card #9 (for H-3, C-14 and P-32).
6. A halt rack is provided inside the sample are of the instrument.
7. Select the "Main Menu" button on the instrument.
8. Select "Automatic Counting" from this menu.
9. Select "Start" button on the instrument.
10. The instrument will run the calibration program and then the samples.
11. After counting is complete, the samples should be disposed of in the $\gamma$-$^{32}$P liquid waste container

Note: In order to work with radioactive material, Baylor Radiation Mandatory Training is required. Personal protection and precautions are found in the Baylor Radiation Safety Manual.

*Drying of Radioactive Polyacrylamide Denaturing Gel*

Reference: Sambrook and Russell, Molecular Cloning, Laboratory Manual, CSHL Press.

1. Prepare 100 mL of gel-fixing solution that contains 10% ethanol and 10% glacial acetic acid in deionized water
2. Soak gel in the gel-fixing solution for 30 min.
3. Lift the gel from the fixation solution keeping it horizontal. Drain excess of solution and place gel on top a Whatman 3MM paper. Gel fixing-solution is disposed to $\gamma$-$^{32}$P liquid waste container
4. Cover the gel with Saran Wrap.

5. Place the system containing: 3MM paper in bottom, gel and plastic wrap on top (from bottom to top), in the gel dryer unit.
6. Start the solvent trap and vacuum pump. If the tramp is full the gel won't dry at all.
7. Set the dryer at 70 °C for 2 h. Do not preheat the platform.
8. Remove the gel from the dryer and peel off the Saran Wrap and proceed to develop.

Note: In order to work with radioactive material, Baylor Radiation Mandatory Training is required. Personal protection and precautions are found in the Baylor Radiation Safety Manual.

*Autoradiography and Reading of Radioactive Denaturing Gel*

Reference: Sambrook and Russell, Molecular Cloning, Laboratory Manual, CSHL Press.

1. Activate the phosphor screen under white light for 30 min.
2. Place dried gel in a spring-loaded metal cassette.
3. Cover the gel with phosphor screen.
4. Close the cassette and let the gel expose the screen for 2-3 min.
5. Turn the light of the room off. White light can activate the phosphor screen again and exposure information would be lost.
6. Transfer the exposed film inside the Personal Molecular Imager FX™ System.
7. Read de autoradiograph using Quantity One software from Biorad.

Note: In order to work with radioactive material, Baylor Radiation Mandatory Training is required. Personal protection and precautions are found in the Baylor Radiation Safety Manual.

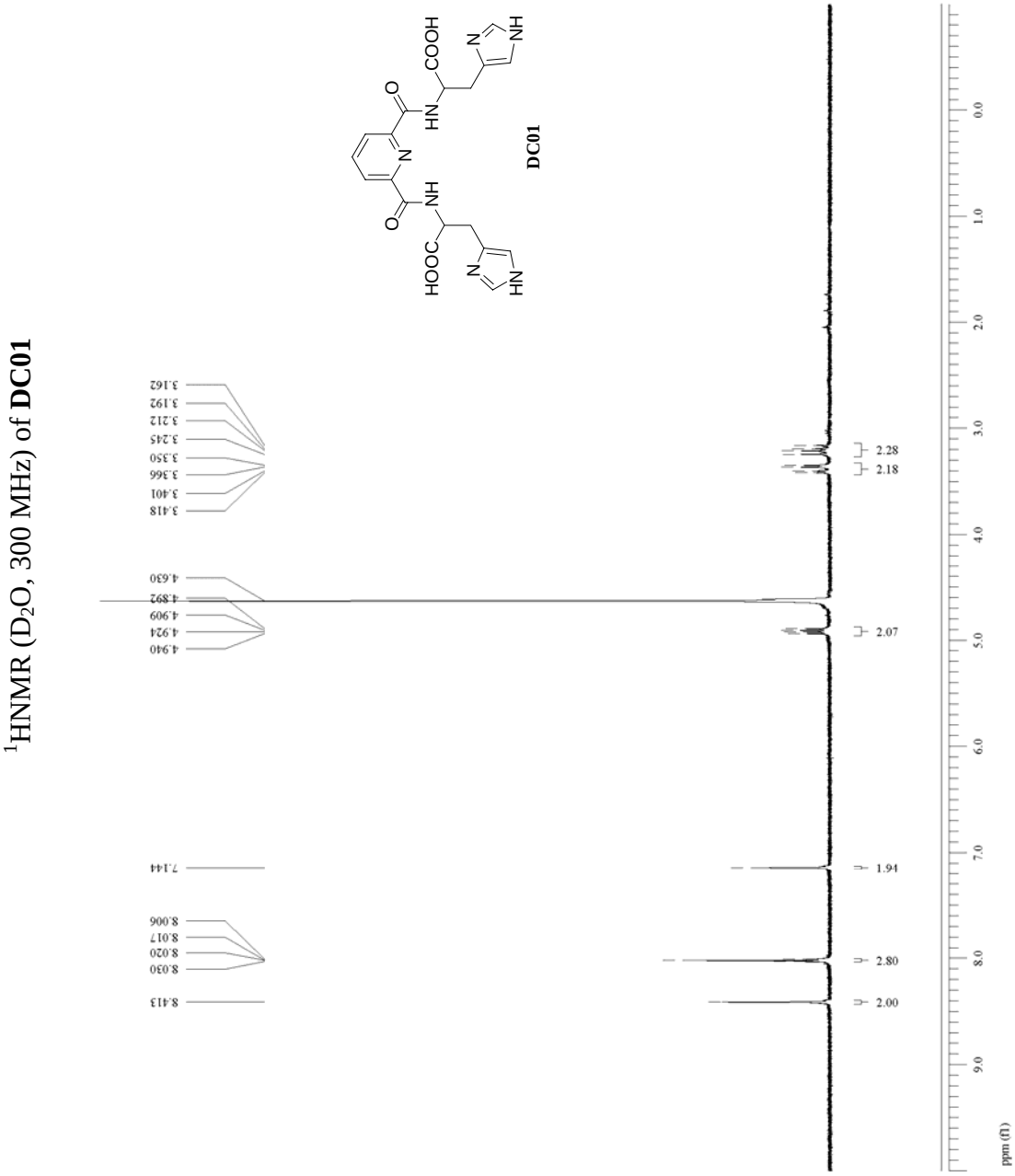
APPENDIX B

Selected NMR Spectra

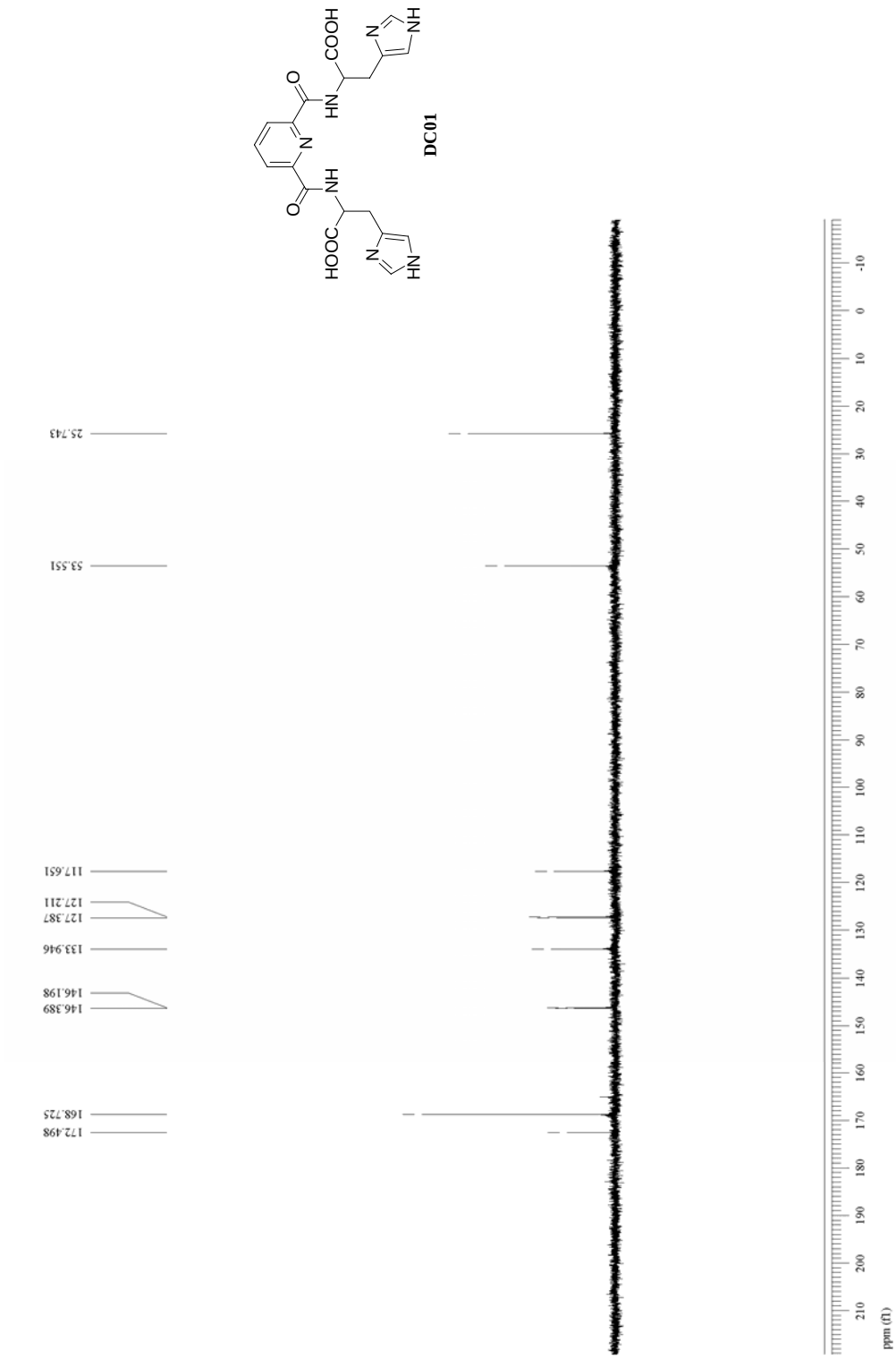
# APPENDIX B

## Selected NMR Spectra

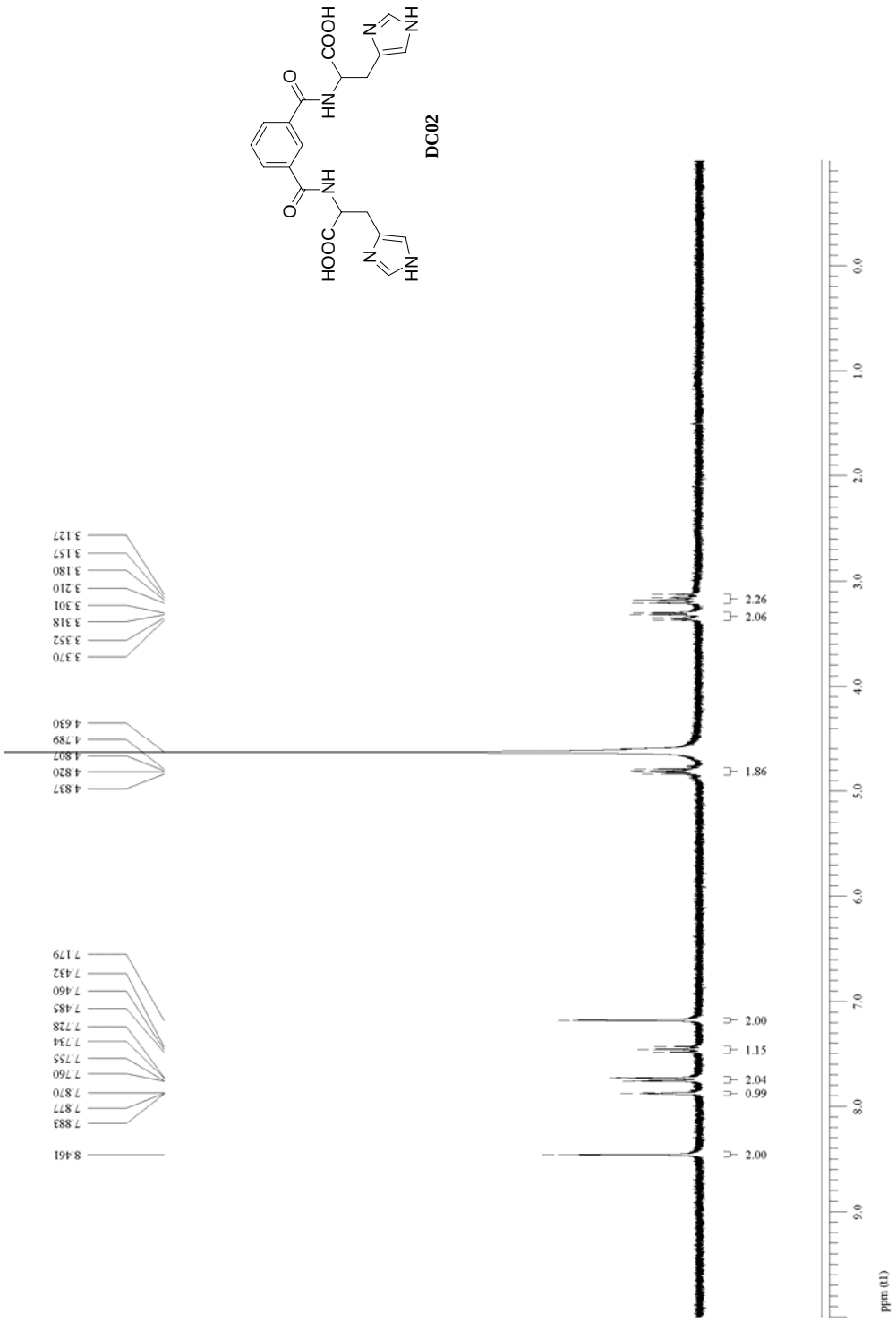
$^1$HNMR ($D_2O$, 300 MHz) of **DC01**



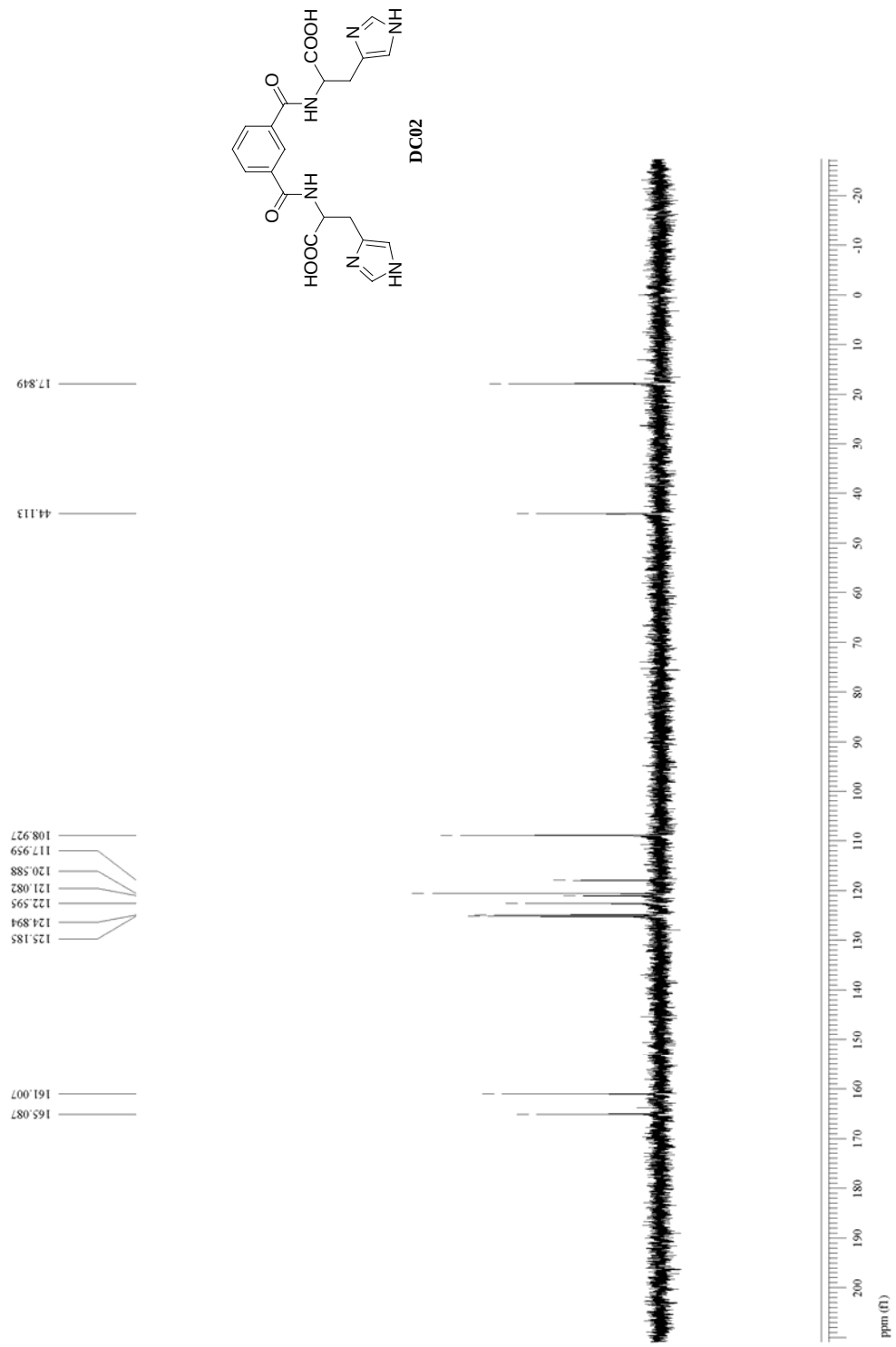
$^{13}$C-NMR ($D_2O$, 75 MHz) of **DC01**



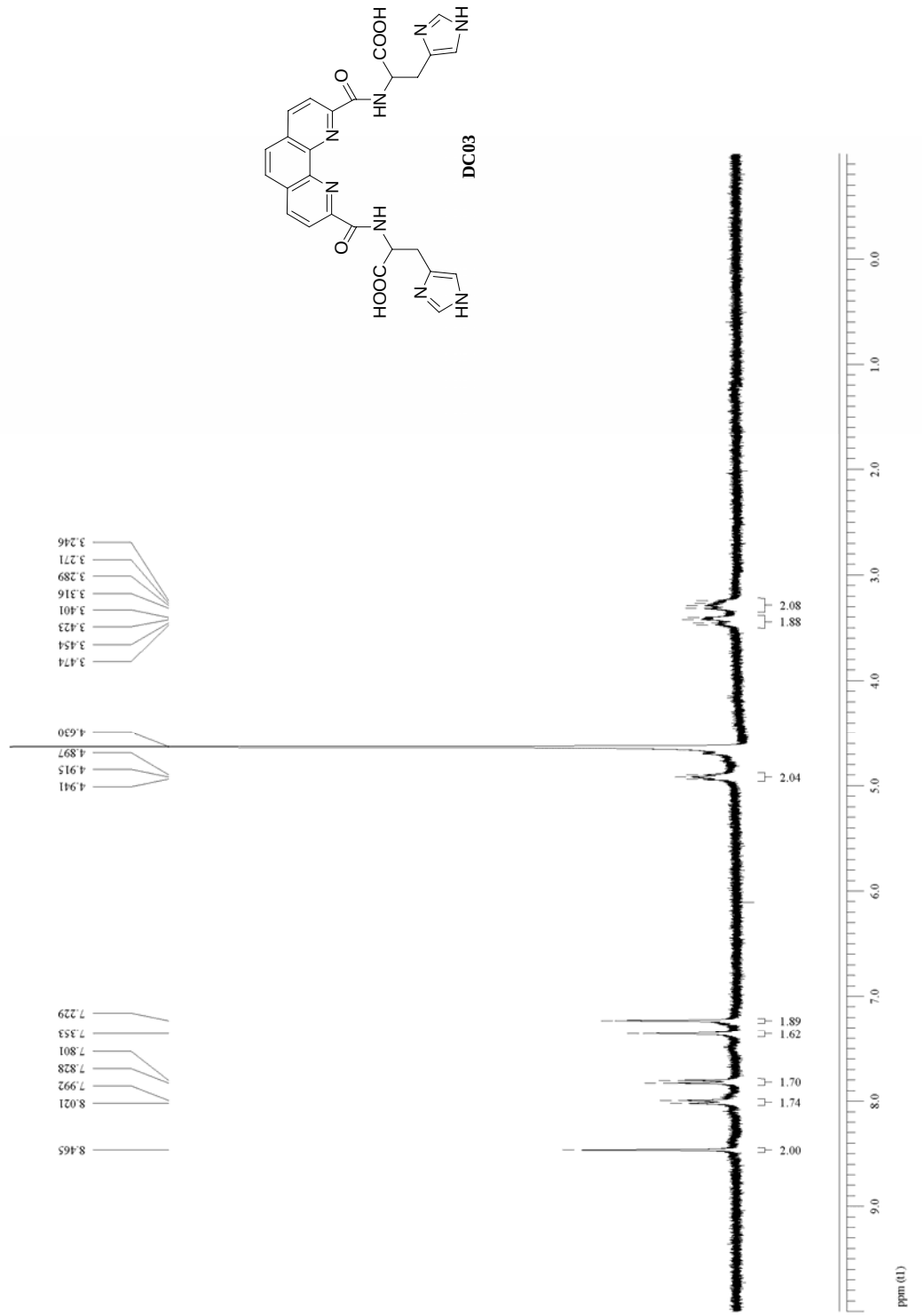
$^{1}$HNMR ($D_2O$, 300 MHz) of **DC02**



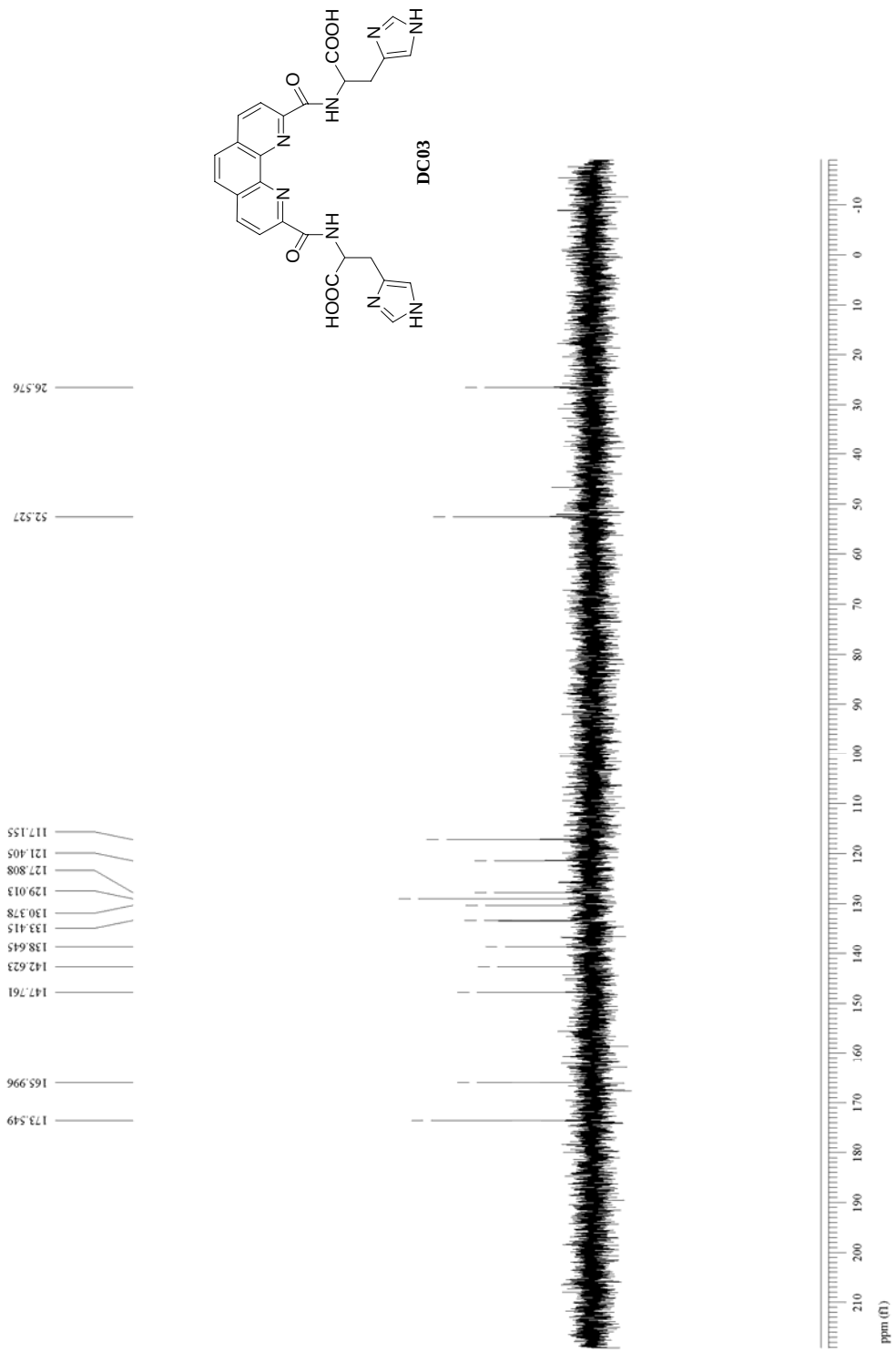
$^{13}$C-NMR ($D_2O$, 75 MHz) of **DC02**



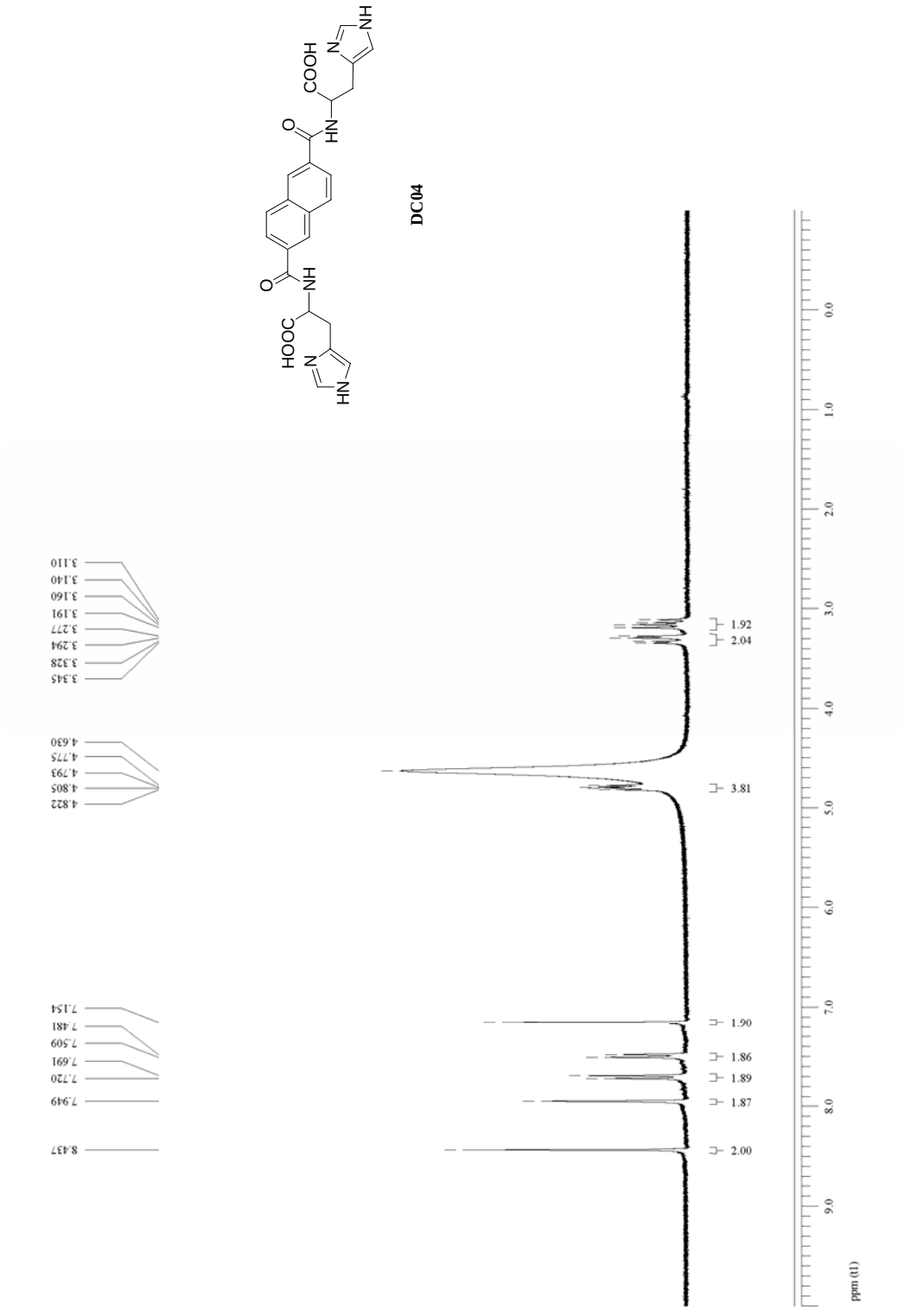
$^1$HNMR ($D_2O$, 300 MHz) of **DC03**



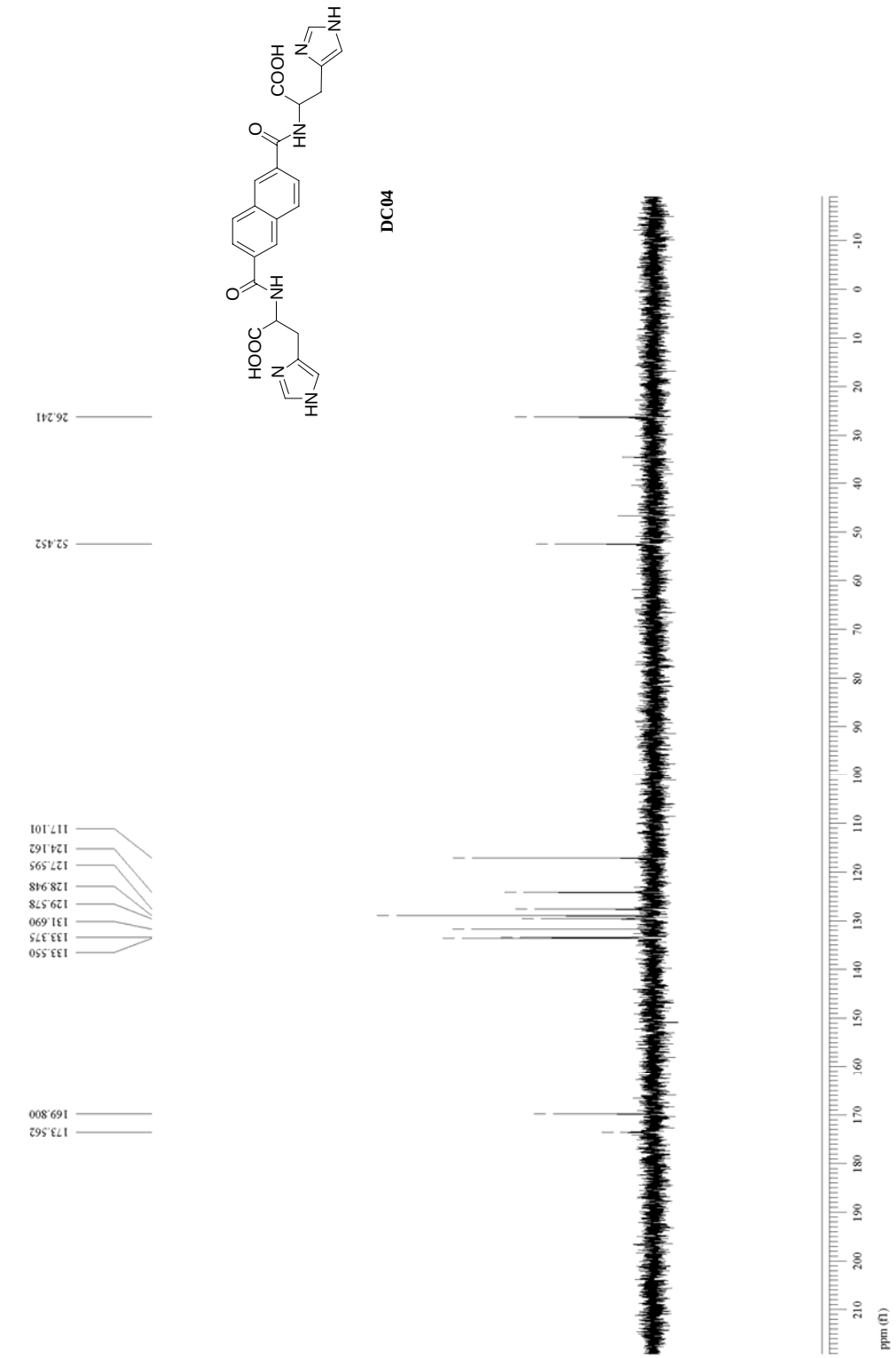
$^{13}$C-NMR ($D_2O$, 75 MHz) of **DC03**



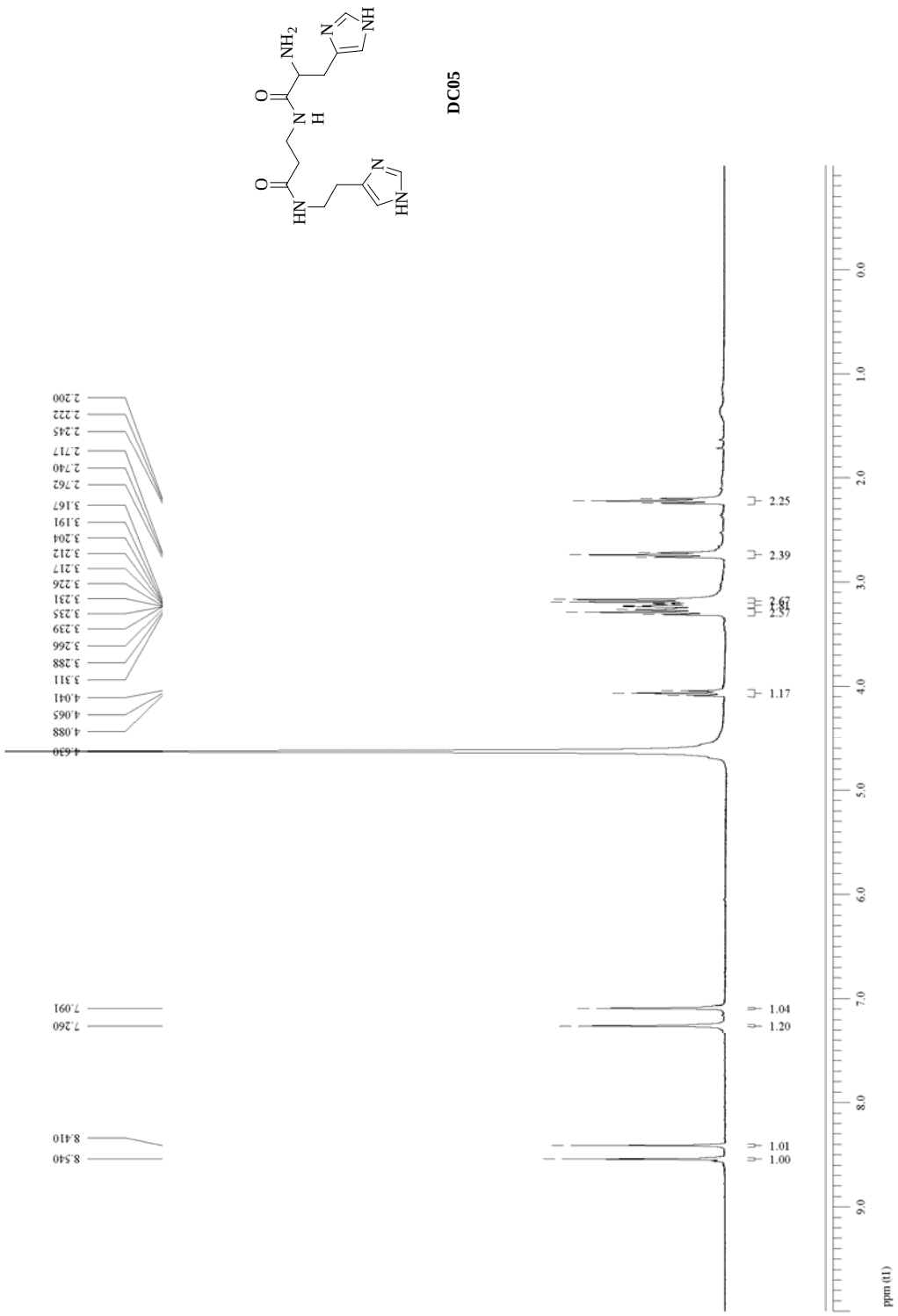
$^1$HNMR ($D_2O$, 300 MHz) of **DC04**



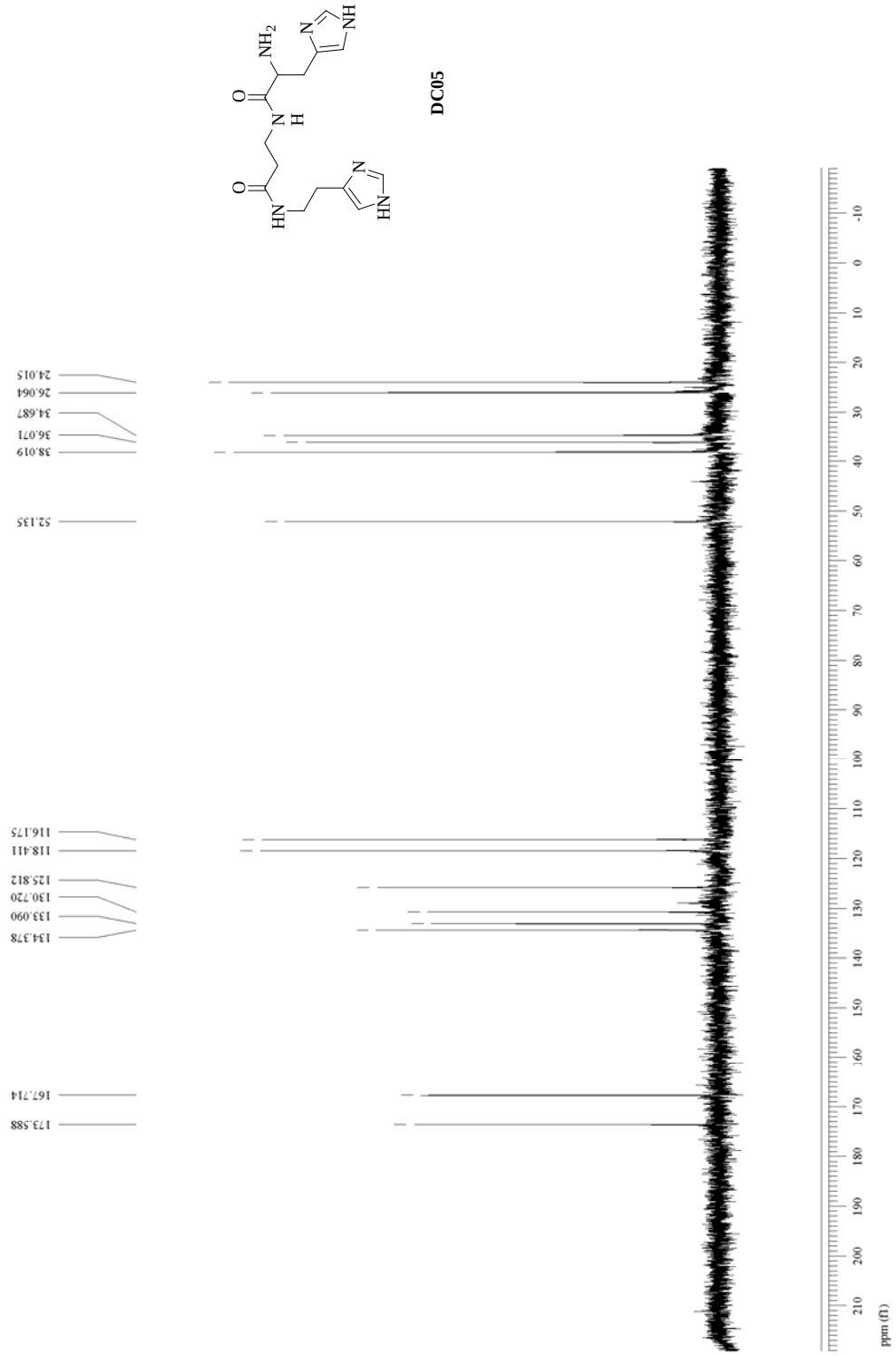
$^{13}$C-NMR ($D_2O$, 75 MHz) of **DC04**



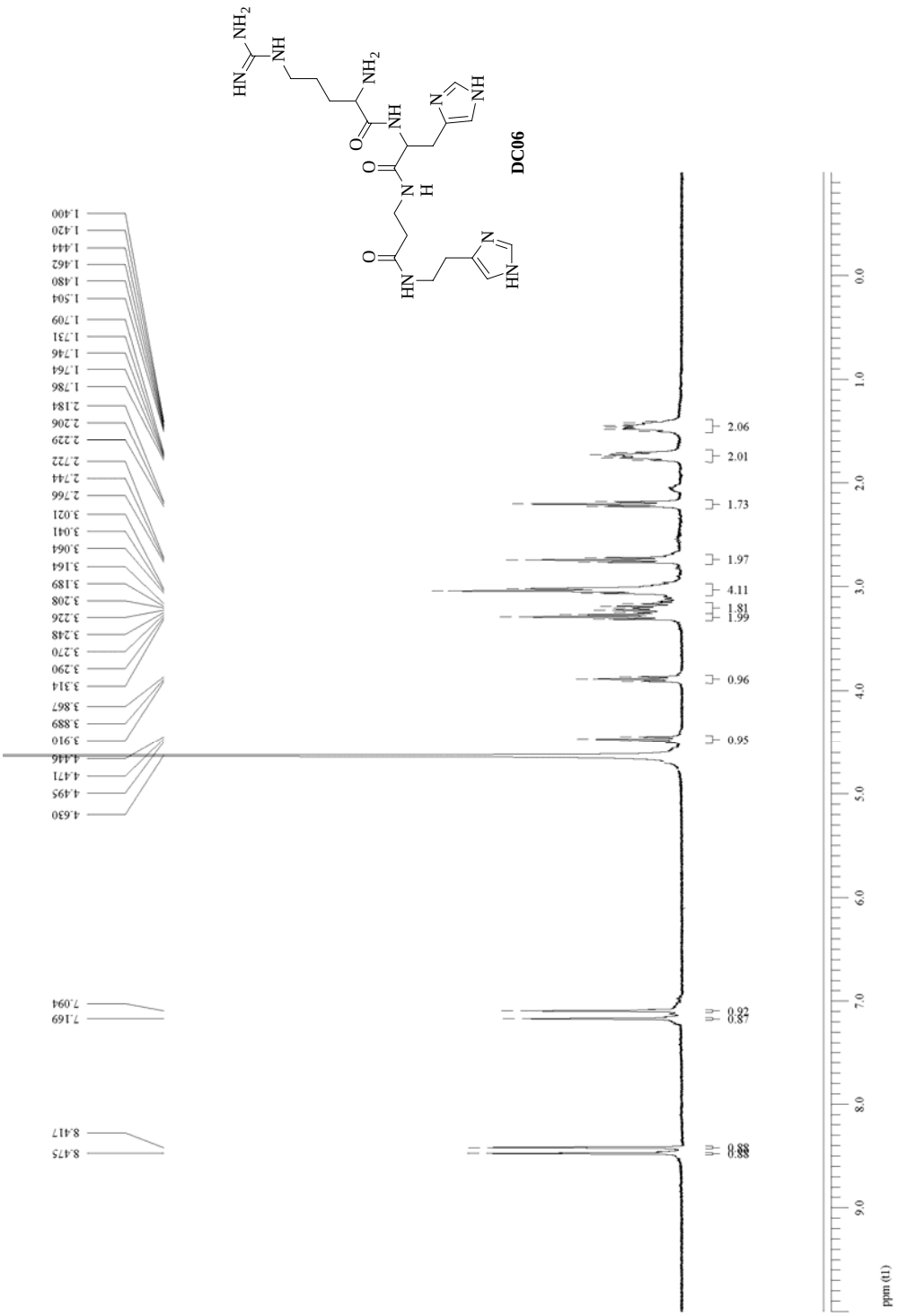
$^{1}$HNMR ($D_2O$, 300 MHz) of **DC05**



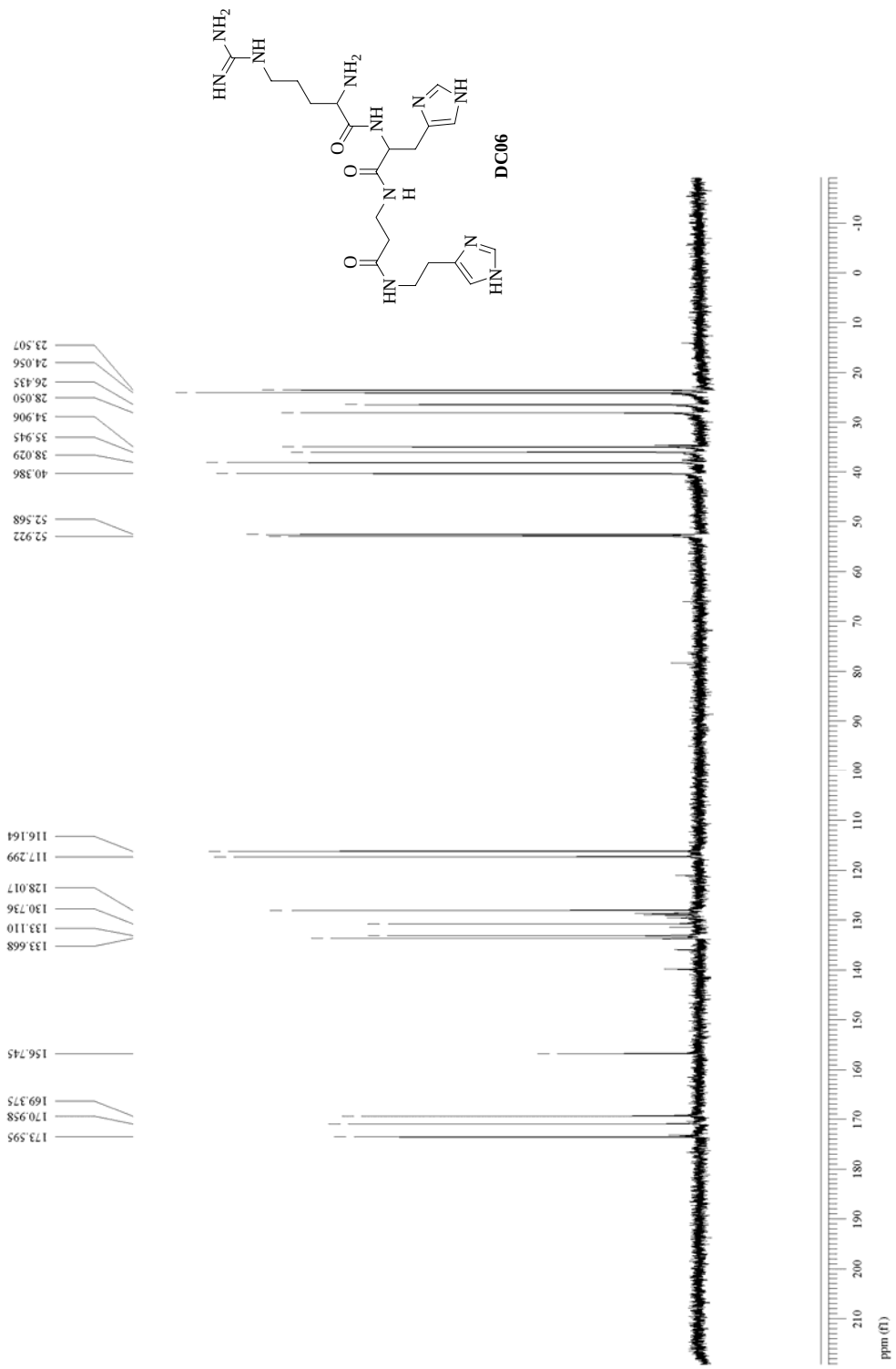
$^{13}$C-NMR ($D_2O$, 75 MHz) of **DC05**



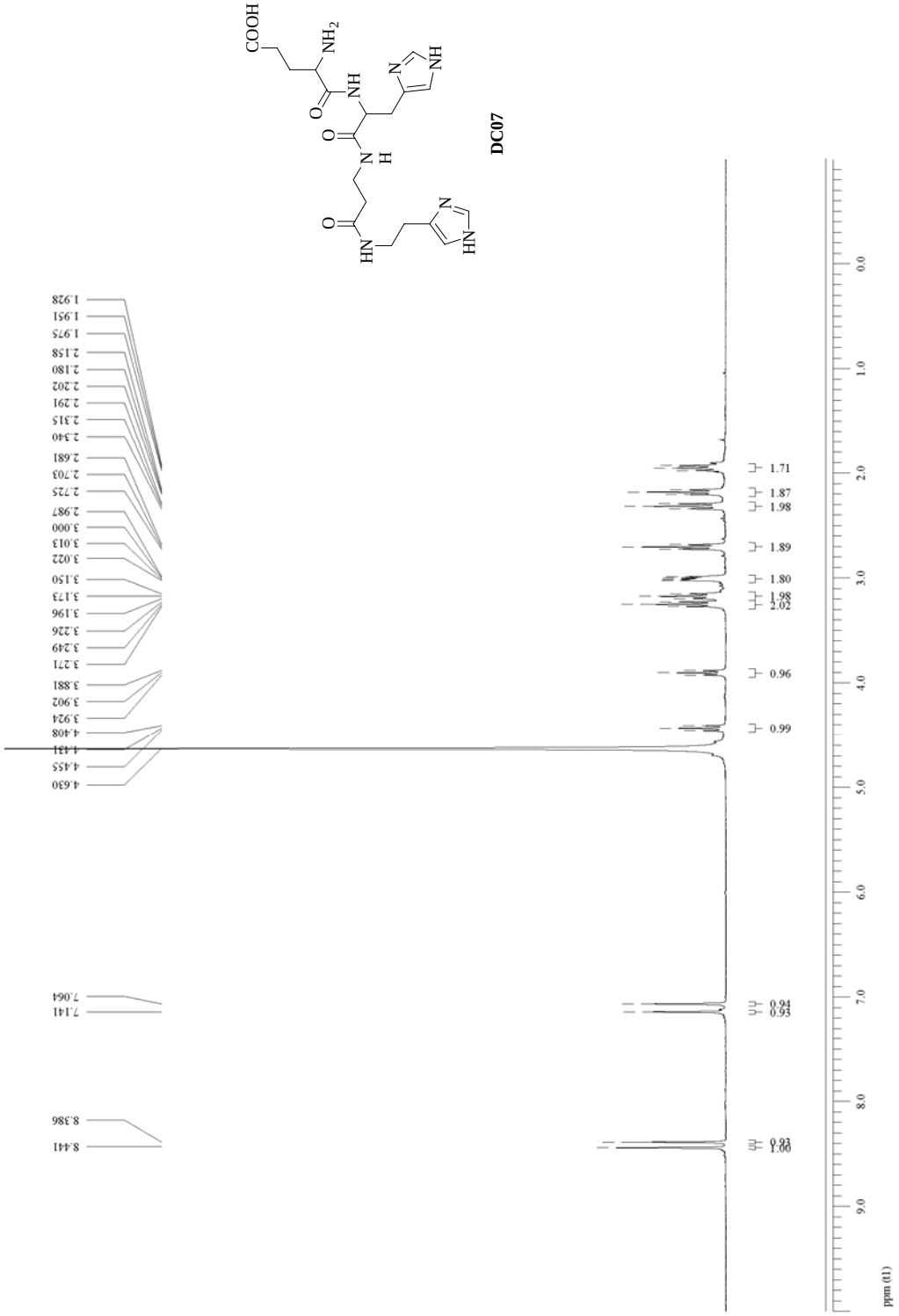
$^1$HNMR ($D_2O$, 300 MHz) of **DC06**



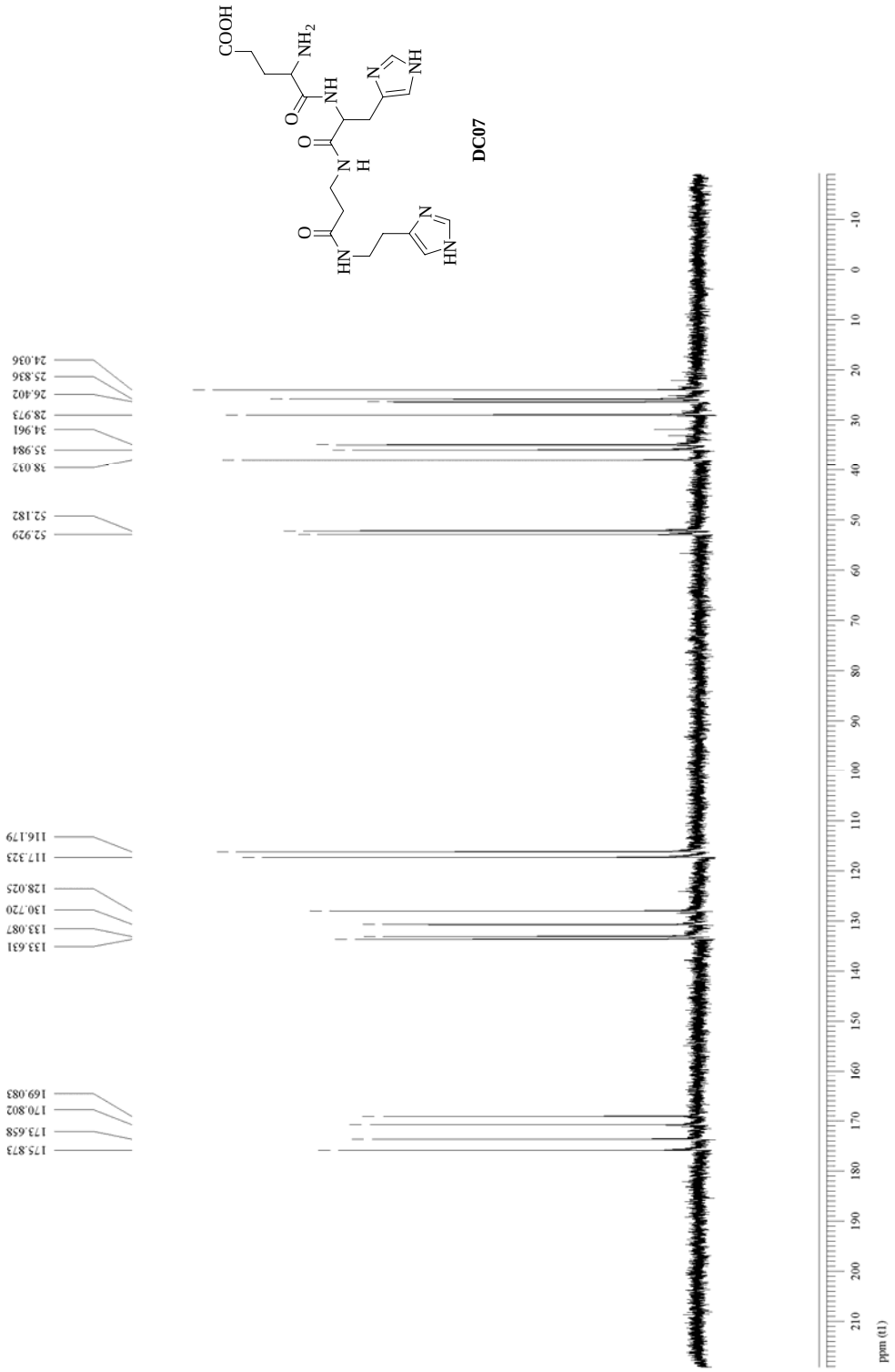
$^{13}$C-NMR ($D_2O$, 75 MHz) of **DC06**



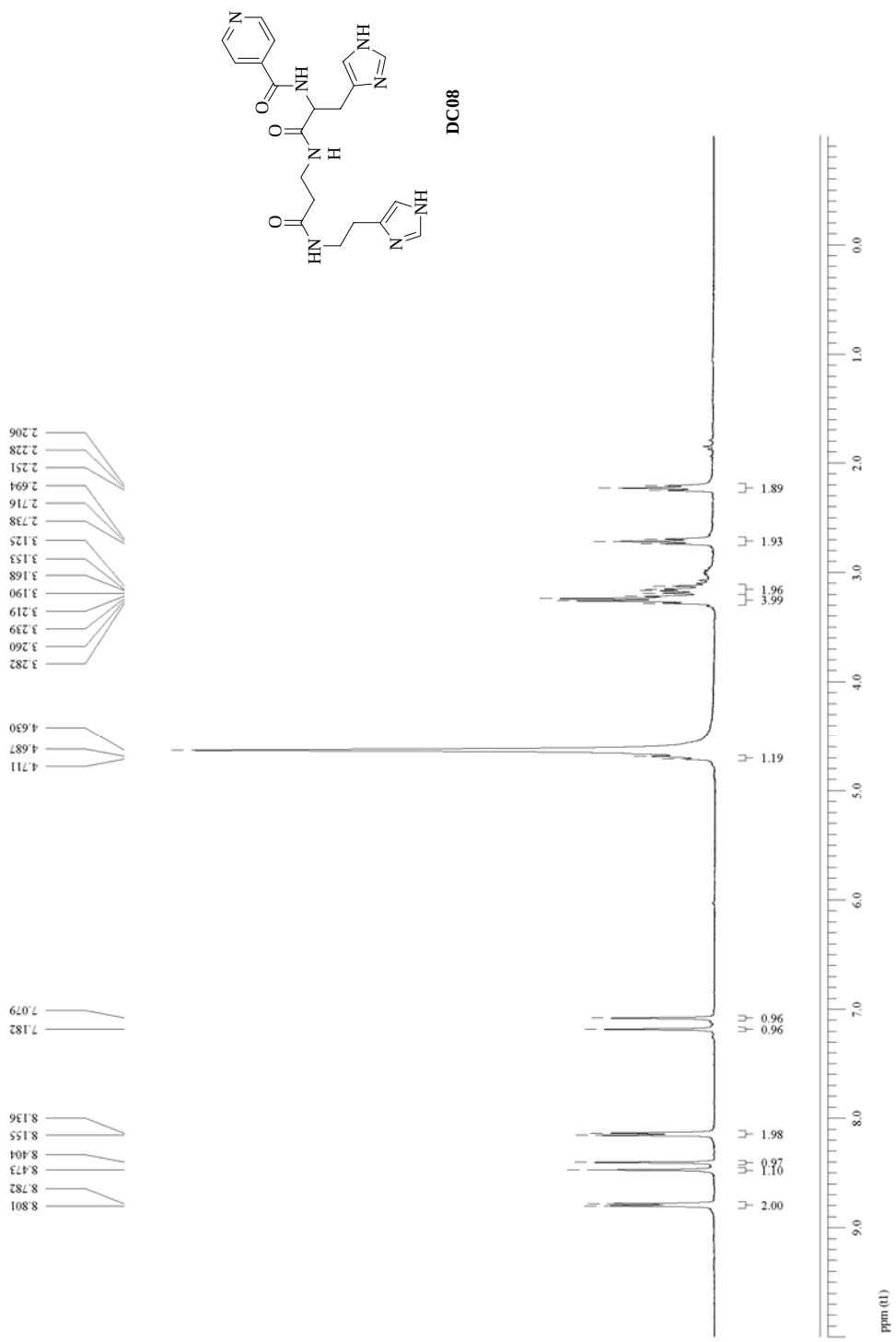
$^{1}$HNMR ($D_2O$, 300 MHz) of **DC07**



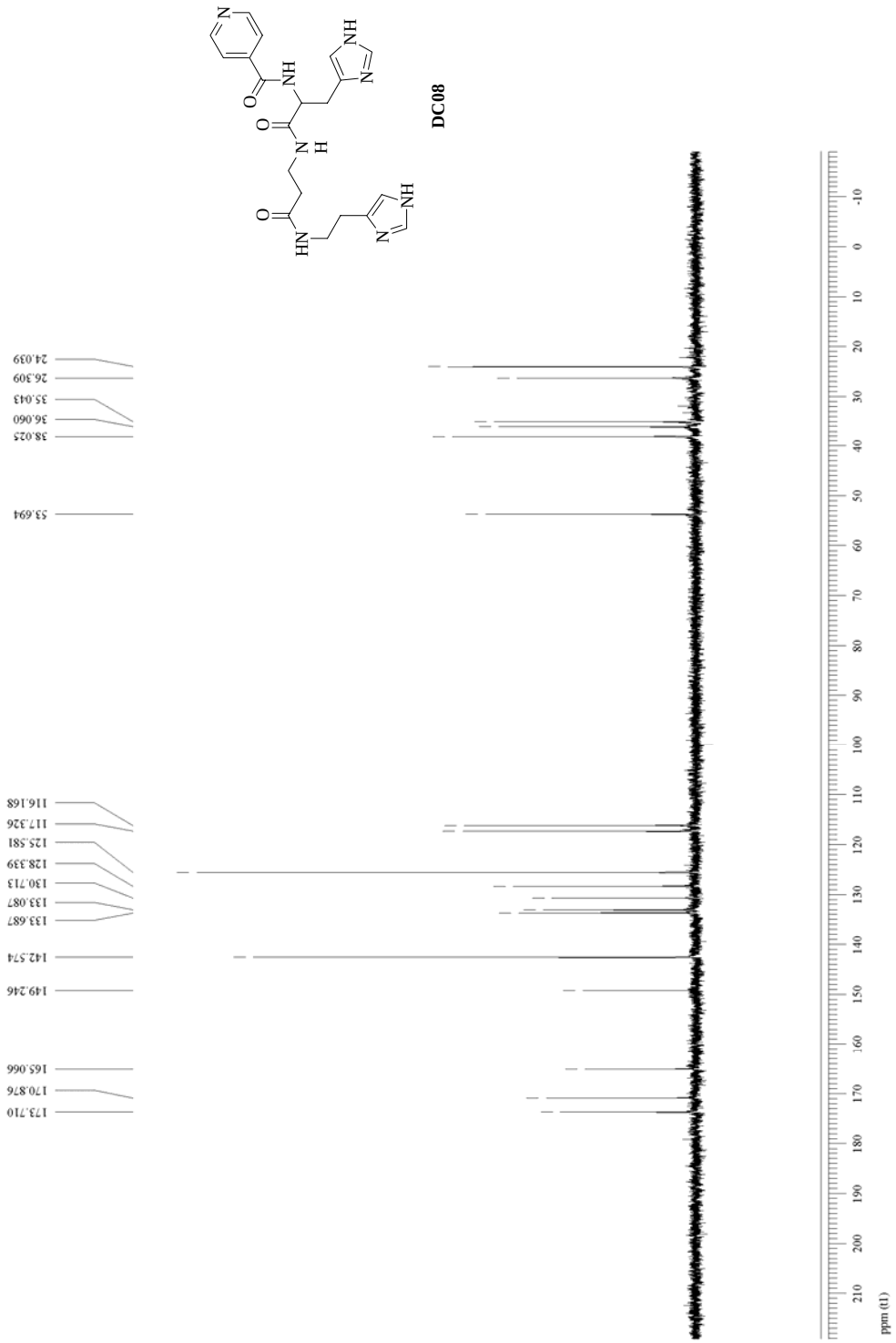
$^{13}$C-NMR ($D_2O$, 75 MHz) of **DC07**



$^1$HNMR ($D_2O$, 300 MHz) of **DC08**



$^{13}$C-NMR ($D_2O$, 75 MHz) of **DC08**



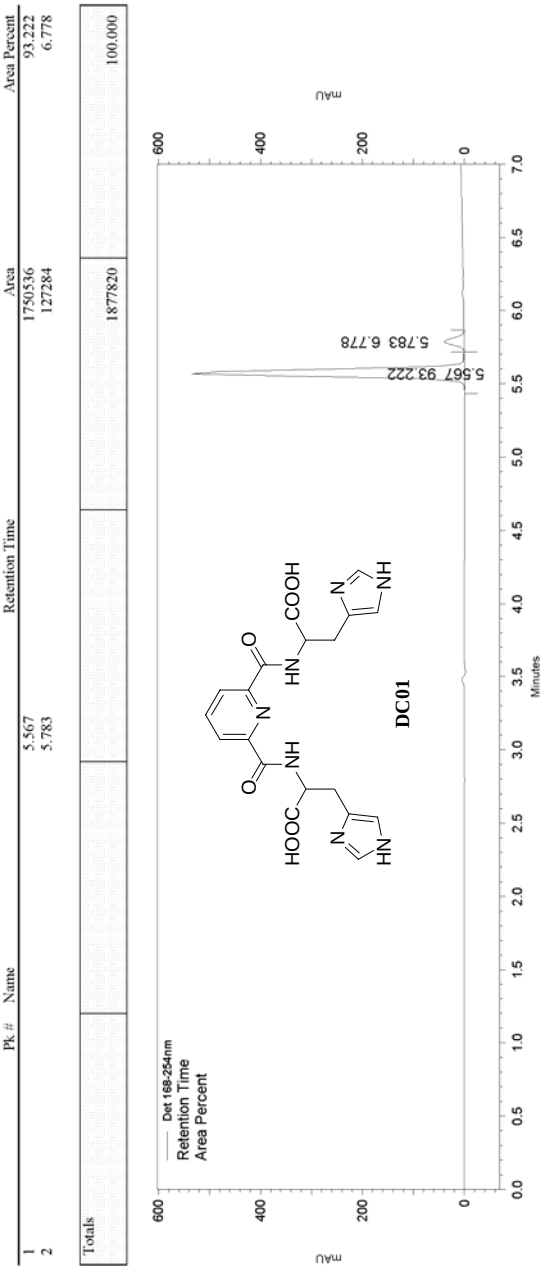
# APPENDIX C

## Selected HPLC Spectra

# APPENDIX C

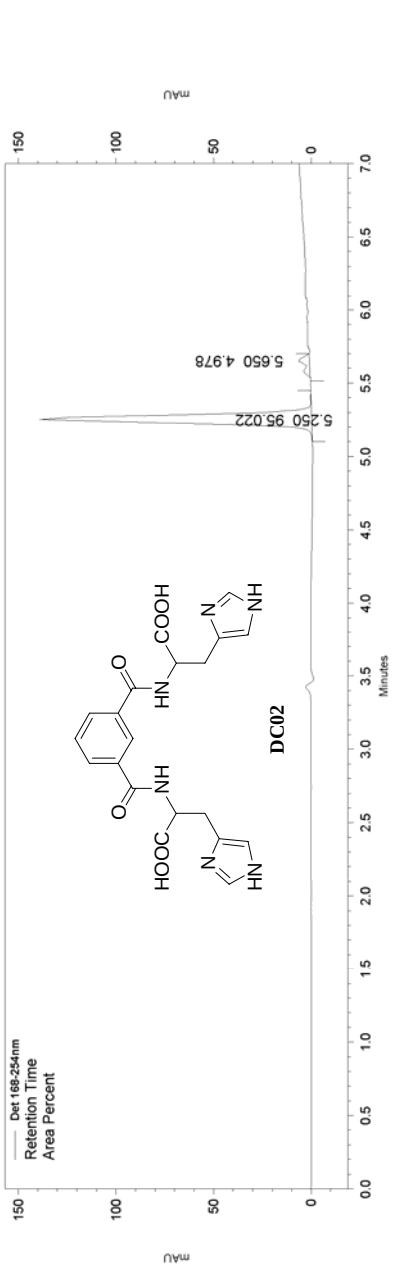
## Selected HPLC Spectra

HPLC for **DC01**

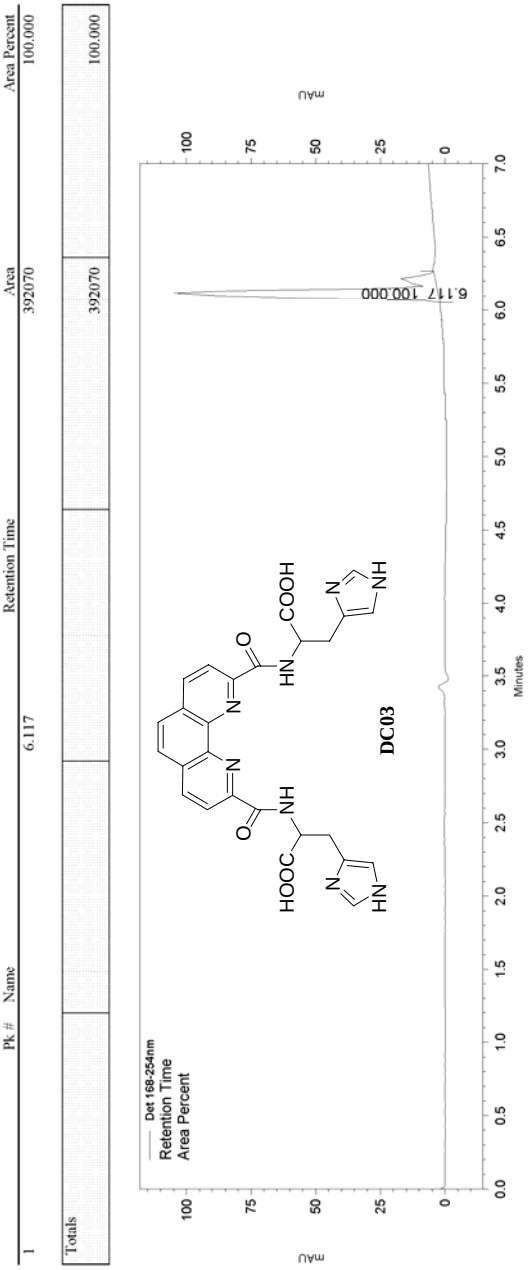


HPLC for **DC02**

| Pk # | Name | Retention Time | Area | Area Percent |
|---|---|---|---|---|
| 1 | | 5.250 | 507446 | 95.022 |
| 2 | | 5.650 | 26584 | 4.978 |
| Totals | | | 534030 | 100.000 |

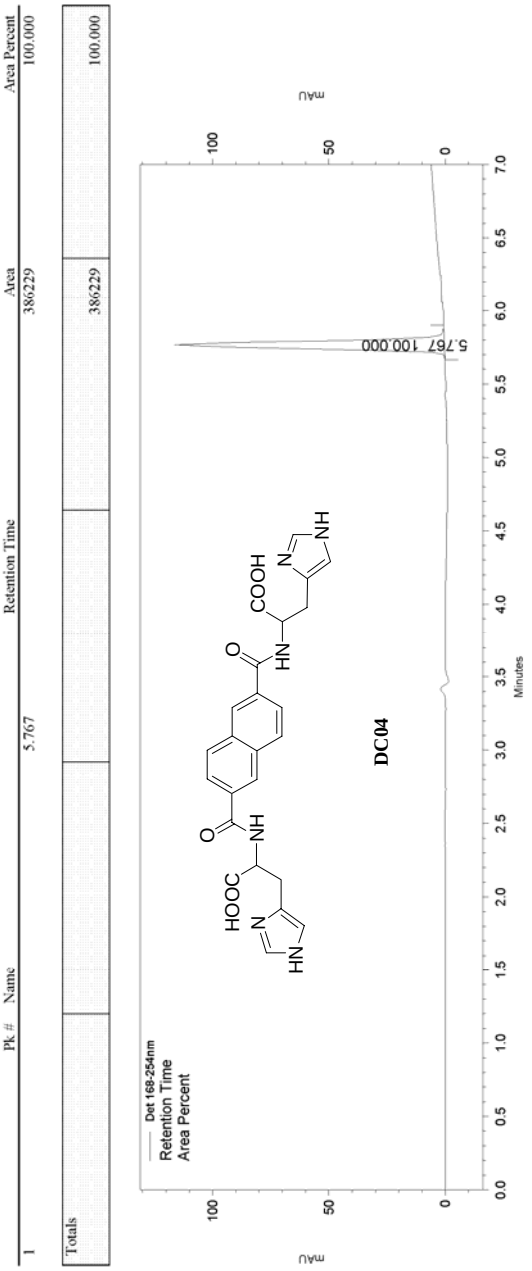


HPLC for **DC03**

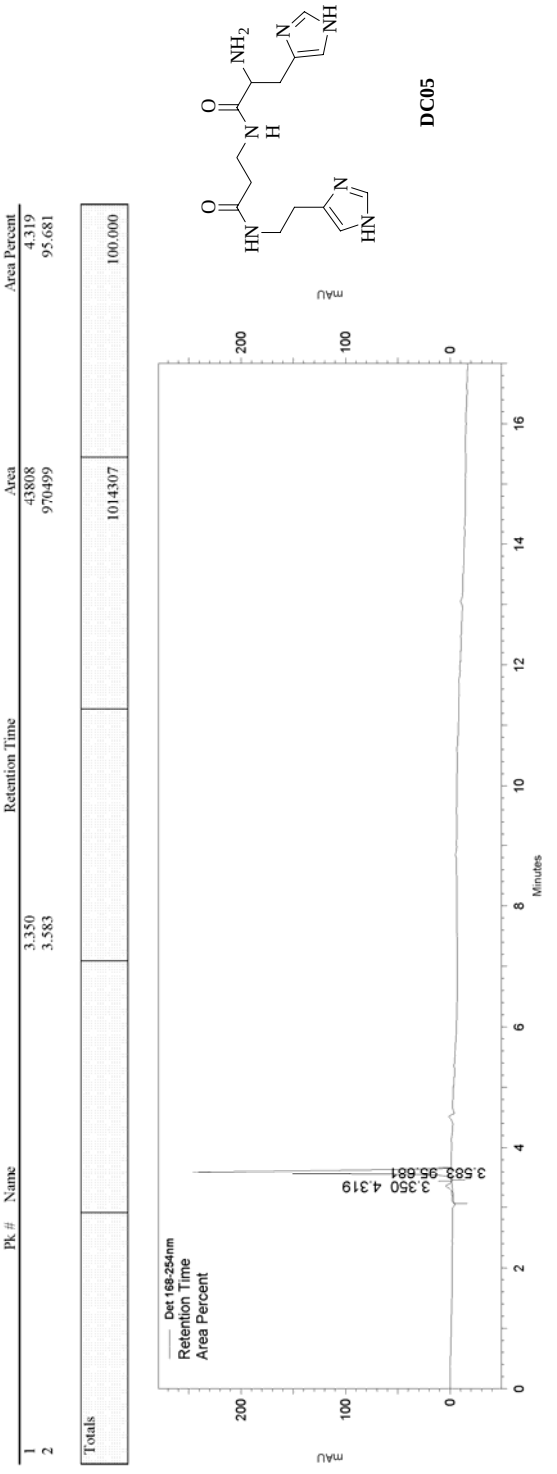



| Pk # | Name | Retention Time | Area | Area Percent |
|---|---|---|---|---|
| 1 | | 6.117 | 392070 | 100.000 |
| Totals | | | 392070 | 100.000 |

HPLC for **DC04**



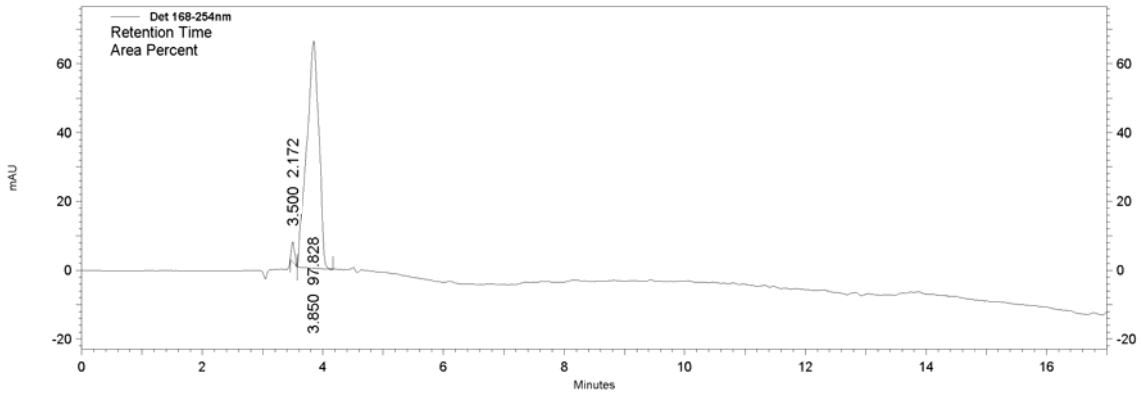
HPLC for **DC05**

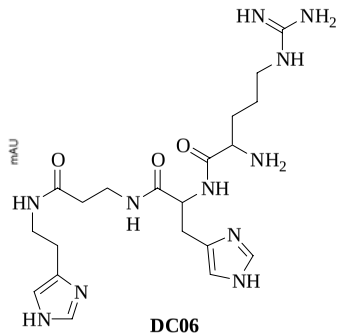



| Pk # | Name | Retention Time | Area | Area Percent |
|---|---|---|---|---|
| 1 | | 3.350 | 43808 | 4.319 |
| 2 | | 3.583 | 970499 | 95.681 |
| Totals | | | 1014307 | 100.000 |

$^{1}$HNMR for **DC06**

| Pk # | Name | Retention Time | Area | Area Percent |
|---|---|---|---|---|
| 1 | | 3.500 | 20754 | 2.172 |
| 2 | | 3.850 | 934986 | 97.828 |
| Totals | | | 955740 | 100.000 |

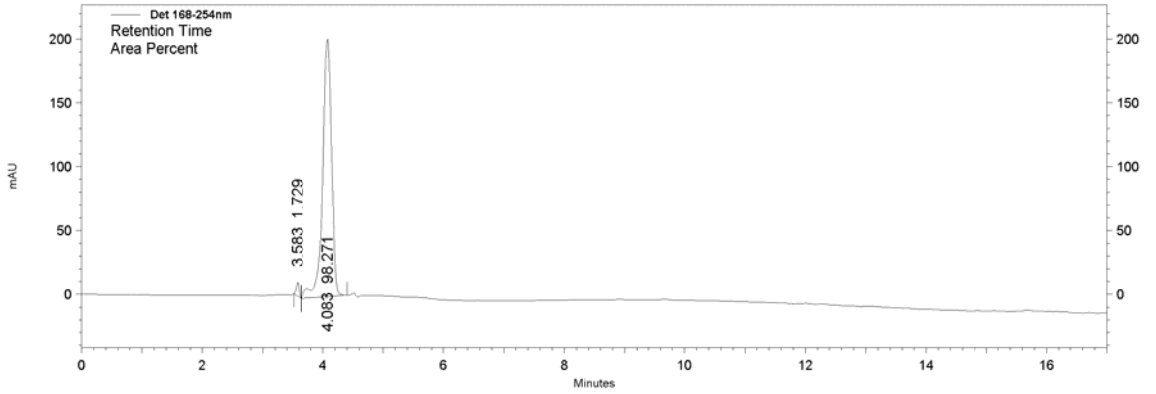


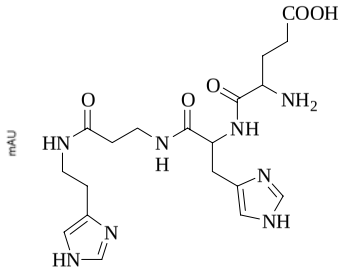



**DC06**

HPLC for **DC07**

| Pk # | Name | Retention Time | Area | Area Percent |
|---|---|---|---|---|
| 1 | | 3.583 | 36459 | 1.729 |
| 2 | | 4.083 | 2071616 | 98.271 |
| Totals | | | 2108075 | 100.000 |

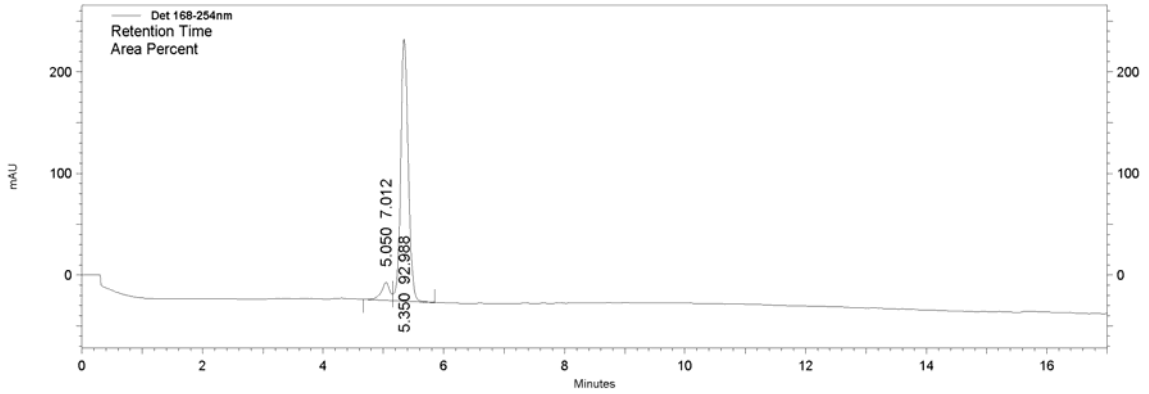


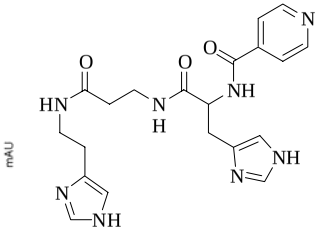




**DC07**

HPLC for **DC08**

| Pk # | Name | Retention Time | Area | Area Percent |
|---|---|---|---|---|
| 1 | | 5.050 | 169802 | 7.012 |
| 2 | | 5.350 | 2251656 | 92.988 |
| Totals | | | 2421458 | 100.000 |



**DC08**

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