EXPANDING THE CHEMISTRY OF IN VITRO SELECTIONS AGAINST EXTRACELLULAR MYCOBACTERIAL GLYCOLIPIDS

by

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The final copy of this thesis has been examined by the signatories, and we
find that both the content and the form meet acceptable presentation standards
of scholarly work in the above mentioned discipline.
Despite the drastic decline in tuberculosis-related mortality due to the development of a vaccine and the discovery of effective anti-mycobacterial antibiotics during the middle part of the twentieth century, there has been a resurgence of the disease since the 1980’s primarily caused by the emergence of HIV/AIDS and drug-resistant *Mycobacterium tuberculosis* strains. The need for improved diagnostic tests that are capable of greater specificity, sensitivity, and rapidity than the current tests is apparent and such tests would contribute significantly to the number of lives saved annually. A DNA aptamer-based biosensor has the potential to meet these challenges; aptamers have proven to have lower dissociation constants and longer binding times than many antibodies, and therefore may be capable of increasing the specificity and sensitivity of a biosensor. In addition, DNA may be modified with various functional groups capable of forming covalent bonds to target molecules, thus creating a new classification of target-binding oligonucleotides termed “reactamers.” Such an addition would significantly reduce dissociation of the reactamer from the target. The systematic evolution of ligands by exponential enrichment (SELEX) process is commonly used to screen large pools of random oligonucleotides for the few sequences that can fold into a structure that is capable of binding a particular molecule. Aptamers and reactamers discovered in this way may be used to selectively remove the mycobacterium or antigen from a biological matrix, as well as serve as the detection
molecule via conjugation with a reporter in a similar fashion as that of a sandwich-ELISA test. Using this process, aptamers and reactamers for a mycobacterial extracellular surface antigen, lipoarabinomannan (LAM), were isolated. These aptamers and reactamers represent initial, promising steps toward future development of oligonucleotides that can bind carbohydrates, which are notoriously difficult targets, with high affinity and specificity. The research therefore serves not only as an attempt to improve the tuberculosis diagnostic assay status quo, but also as an exploration of the multi-faceted nature of oligonucleotides and their various recognition, binding, and catalytic capabilities.
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CHAPTER I

COMPREHENSIVE INTRODUCTION

Tuberculosis remains one of the most widespread and lethal of all human diseases; among deaths caused by infectious disease it is second only to human immunodeficiency virus/ acquired immunodeficiency syndrome (HIV/AIDS). A staggering 2 billion people, one-third of the world population, are currently infected with *Mycobacterium tuberculosis*, and the worldwide rate of infection is more than one new case every second. Although 90% of these cases will remain latent and never develop into active tuberculosis, the remaining fraction still accounts for an estimated 9 million new active tuberculosis cases per year, with around 2 million fatalities per year.\(^1\)

The World Health Organization (WHO) had set aggressive goals in order to more effectively manage the world burden of tuberculosis: diagnose 70% of new active tuberculosis cases and cure 85% of these cases by 2005. These targets have proven to be optimistic, as only 53% of new cases were being successfully diagnosed in 2004.\(^2\) To better meet these goals, the need for a revised detection method with improved diagnostics is evident. According to decision-tree modeling studies conducted to simulate the introduction of new and better diagnostic tests, a “perfect” diagnostic test (rapid, 100% sensitive, 100% specific, and accessible to everyone) would be projected


to save 625,000 lives annually, which accounts for about 37% of the current tuberculosis-caused fatalities. Even a new test that was 80% specific and showed 80% improved sensitivity over the current tests would save an estimated 100,000 lives annually if accessible to everyone, and would save 20,000 lives if administration of the test was necessarily restricted to tuberculosis clinics (Figure 1-1).³

**Figure 1-1.** Projected lives saved annually (in thousands) if sensitivity and specificity of diagnostics are improved and a) accessible to everyone or b) administered only in tuberculosis clinics. This figure was taken from Keeler, E.; et al. Reducing the global burden of tuberculosis: the contribution of improved diagnostics. *Nature*, **2006**, *444* suppl. 1, 49-57.

Novel diagnostic tests must compete with the current technique primarily used for the detection of active *M. tuberculosis*: sputum smear microscopy (SSM). Based on the differential staining (using the acid-fast Ziehl-Neelson staining procedure) of mycobacteria in a sputum sample, this technique is laborious, requires access to

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biosafety level 3 (BSL-3) facilities, and depends upon expensive reagents and specifically trained personnel. In addition, the detection limit of SSM is ≥10,000 bacilli per milliliter of sputum, which is unacceptably high and subsequently leads to successful detection of not more than 65-70% of new active tuberculosis cases. Since the SSM test relies directly on the appearance of bacilli in the lung sputum of patients, it follows that extrapulmonary tuberculosis cases, as well as pulmonary cases in which the bacterial load in the lungs is less than 10,000 bacilli per milliliter, will not be accurately diagnosed. Moreover, *M. tuberculosis* do not represent the only acid-fast staining bacteria; all of the mycobacterial species, including non-pathogenic saprophytic soil-dwelling mycobacteria such as *M. smegmatis*, as well as the Nocardia species will also be stained and may lead to a false positive diagnosis. However, since results can be achieved in a few hours, the test is sufficiently expeditious that patients do not have to return to the testing site at a later date for follow-up, which in the case of the tuberculin skin test (TST) leads to significant reductions in treatment efficacy due to patients who never return.

Culture methods in which patient samples are inoculated into media and grown to more detectable densities improve the sensitivity and specificity of diagnosis.

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substantially; however, such methods require a time delay of several days to weeks or longer before results become available due to the slow-growing nature of *M. tuberculosis*. The extended culture time needed to grow this mycobacterium arises from the waxy, thick, non-fluidic cell wall which coats the organism and hinders the diffusion of molecules into the cell, and also from the abnormally low concentration of porin proteins that would normally allow inward diffusion of polar nutrients. Nevertheless, many different culture-based methods are widely used, especially in relatively resource-rich areas. For example, the BACTEC™ mycobacterial-growth indicator tube (MGIT) manufactured by Becton Dickinson reduces the test time required (to 4-14 days) by utilizing the aerobic metabolism of the mycobacterium. A fluorophore that is quenched in the presence of dissolved oxygen is used to detect the consumption of oxygen by the respiration of the bacteria; when a specified amount of fluorescence is detected, the culture can then be pulled and further tested using SSM or subjected to polymerase chain reaction (PCR) and various restriction fragment-length polymorphism (RFLP) or DNA-hybridization probe assays that can unambiguously confirm the presence of *M. tuberculosis*, since the entire genome of the mycobacterium is known. The sensitivities of such nucleic acid amplification tests are around 92%, with specificities around 99%.

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10 Palicova et al. Susceptibility testing of *Mycobacterium tuberculosis* to anti-tuberculosis drugs: BACTEC MGIT 960 vs BACTEC 460TB system. 100th General Meeting of the American Society for Microbiology, 2000.

For the diagnostic methods currently in place, accuracy (a consequence of sensitivity and specificity) and accessibility (a consequence of rapidity and availability of resources) seem to be mutually exclusive. Recently, however, new discoveries of *M. tuberculosis*-specific antigens excreted in the urine of patients with active tuberculosis may be potential targets for biosensors that may be able to combine the desirable qualities of accuracy and accessibility. Several excreted proteins have been discovered, a few of which are specific to *M. tuberculosis*, namely a MoA-related protein coded for by the MT_1721 gene. Additionally, the major lipoglycan of the mycobacterial cell wall, lipoarabinomannan (LAM), was also found to be excreted in the urine of mice injected with LAM and in human patients with active tuberculosis.  

Anti-LAM IgG antibodies have been raised and used in an ELISA test (test time of 2 days) which successfully identified 14 out of 15 patients with active tuberculosis disease, a sensitivity of 93%.  

LAM is ubiquitous among all mycobacterial species. The structure of a LAM molecule consists of the following: a mannosyl-phosphotidyl-*myo-*inositol (MPI) anchor which is integrated into the fatty acids that comprise the outer leaflet of the cell wall; a mannan core which is composed of 30-35 mannose units in glycosidic linkage; arabinan branches which sum to a total of about 60 arabinose units; and caps on the

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non-reducing ends of the arabinan branches which are variable among mycobacterial species (Figure 1-2).¹⁴

Figure 1-2. Lipoarabinomannans: from structure to biosintesis. *Biochimie, 2003*, 85, 153-166. Structure of *M. tuberculosis* LAM and placement in the cell wall. This figure was taken from Nigou, J.; et al.

The mannan and arabinan components of LAM are relatively invariable among mycobacterial species; however, the caps on the non-reducing ends of the arabinan branches are composed of additional mannose units in the case of the *M. tuberculosis* complex (ManLAM), while those of *M. smegmatis* consist of phospho-myo-inositol (PILAM). In fact, it is the identity of the capping molecules that has been shown to be highly significant in determining the virulence of the species.

All pathogenic species of mycobacteria contain ManLAM, which serves several anti-inflammatory functions during the infection of these species into their hosts, such as the phagocytosis into and inactivation of macrophages, inhibition of release of pro-inflammatory cytokines, and inhibition of macrophage apoptosis. In contrast, the PILAM molecules of non-pathogenic species such as *M. smegmatis* are pro-inflammatory, and the bacilli are easily recognized and removed by the immune system. This difference in cellular response toward the two types of LAM is accounted for by differing surface receptor affinities resulting in unique signal transduction cascades: PILAM binds the cluster of differentiation 14 (CD14) receptor, while ManLAM is capable of binding a myriad of receptors such as the dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN) and mineralocorticoid (MR) receptors.\(^\text{15}\)

Although LAM from non-pathogenic species of mycobacteria differs chemically from that of the members of the *M. tuberculosis* complex, the anti-LAM antibodies raised thus far do not have the ability to differentiate between the species and therefore bind to all forms of LAM with a low specificity.\(^\text{16}\) Antibodies have long been the traditional bioanalytical detection reagent; however, an increasingly popular alternative to antibodies are aptamers, oligonucleotides that fold into structures capable of recognizing and binding to targets in a manner analogous to proteins. Figure 1-3 illustrates an example aptamer, in this case RNA, that has been selected to bind the small molecule neomycin B. The stem-loop secondary structure forms a

\(^{15}\) Nigou et al. Lipoarabinomannans: from structure to biosíntesis. *Biochimie*, 2003, 85, 153-166.

binding pocket for the target, and noncovalent interactions between the bases and neomycin B contribute to the specificity and affinity of the aptamer.\textsuperscript{17}

\textbf{Figure 1-3.} Aptamer-based carbohydrate recognition. \textit{Curr. Pharm. Des.}, \textbf{2010}, \textit{16}, 2269-2278. RNA aptamer for neomycin B demonstrating a) stem-loop secondary structure and b) specific binding to its neomycin B target. This figure was taken from Sun, W.; et al.

Aptamers have been proven to be frequently highly specific, and it is possible that they may be better able than antibodies to differentiate between mycobacterial

species, a requirement for any diagnostic test. Because the complementarity determining regions (CDRs) of antibodies are composed of β-sheet domains, the specificity of the antibody may be limited by this general structure, while oligonucleotides may fold into a greater variety of motifs. Moreover, DNA is inherently a stable molecule and may not require the controlled temperature environment upon which protein antibodies depend. This hypothesis provides the rationale behind performing the SELEX protocol to evolve DNA aptamers capable of specifically identifying the presence of *M. tuberculosis*, and the subsequent successful diagnosis of active tuberculosis disease.

In order to demonstrate the feasibility of evolving a *Mycobacterium tuberculosis*-specific DNA aptamer, *Mycobacterium smegmatis* was initially chosen as a model organism. *M. smegmatis* is a non-pathogenic, saprophytic mycobacterial species that dwells in soils, but bears strong resemblances to *M. tuberculosis* despite its lack of virulence. Both mycobacterial species contain large quantities of the lipoglycan LAM on the outer surface of their cell walls, which accounts for about 15% of the dry weight of the cell.\(^{18}\) The PILAM present on the surface of *M. smegmatis* cells, however, differs from ManLAM in that it contains negatively-charged phosphate group caps on the ends of the arabinan branches. After selecting for PILAM aptamers, the feasibility of applying the same SELEX protocol to discover ManLAM aptamers will be explored.

To evolve an aptamer that has the greatest potential of being useful in a biosensor, two different selections for LAM were performed. The first utilized pre-purified LAM molecules from mycobacteria as the target ligand, while the second targeted the whole, intact mycobacteria cell. This approach will allow for a broader

category of possible biosensors to be developed, including those sensors that would
detect LAM in the urine of patients, and those that would aid in the removal of the
etire microbe from sputum. In addition, the whole-cell selection may evolve
aptamers for cell-surface molecules other than LAM and may lead to the discovery of
additional biomarkers.

The SELEX process will be used in both the pre-purified LAM and whole-cell
selections. Figure 1-4 diagrams the steps involved: 1) a random ssDNA library of
approximately $10^{14}$ different sequences is synthesized (using phosphoramidite
chemistry developed by the Caruthers group) with fixed-sequence primer-binding
regions at the ends and a 40 nucleotide random region in the interior of each strand 2)
the ssDNA is subjected to PCR using a biotinylated 3’ primer 3) the now biotinylated
dsDNA is bound to streptavidin-coated beads 4) the non-biotinylated strand is eluted
with NaOH and washed away (at pH 12, which is sufficient to deprotonate the
nitrogenous bases and break the inter-strand hydrogen bonds) 5) the biotinylated,
bead-bound ssDNA template is subjected to primer extension using modified
nucleotides, which creates a modified complementary strand 6) the modified ssDNA is
eluted using NaOH 7) the modified ssDNA is incubated with either the pre-purified
LAM or the whole cell 8) the unbound ssDNA is washed away and 9) the bound ssDNA
is subjected to PCR to begin the cycle again.\textsuperscript{19} If successful, repetition of this cycle 8-9
times will gradually enrich the pool of ssDNA with those oligonucleotides that
specifically bind the target.

Successful evolution of aptamers with high affinity and specificity depends on the ability of the oligonucleotide to contain various functional groups that can interact with the target. It has been proven to be advantageous to modify some of the nitrogenous bases in order to provide more functionality and opportunities for interaction with the target. Figure 1-5 describes some of the deoxyuridine modifications that have been previously synthesized by the Eaton laboratory.\textsuperscript{20}

Figure 1-5. Various deoxyuridine modifications previously synthesized by the Eaton lab. This figure was taken from Vaught, J.D.; et al. Expanding the chemistry of DNA for in vitro selection. *J. Am. Chem. Soc.*, **2010**, *132*, 4141-4151.

In both selections, the thymine base normally found in DNA will be replaced with uracil that has been linked to a functional group via an amide linkage. Such modification adds another hydrogen bond acceptor and donor, as well as adding additional hydrophobic or other properties to the base. The partial double bond character of the amide bond also serves to confine the modification to a limited number of degrees of freedom defined by the equilibrium between amide bond isomers. In addition to providing functional groups, the modifications also may fold the aptamer into unique tertiary structures not encountered with unmodified DNA due to noncanonical base pairing facilitated by the modified uridines. Aptamers evolved with
these modified bases have proven to have lower dissociation constants and longer binding times than aptamers without modifications, and even some antibodies.

Once DNA aptamers have been selected and used to remove the target from a biological matrix such as urine or sputum, the challenge of detecting the LAM or mycobacterial cell still remains. A potential detection technique may involve the use of biomineralizing peptides that have been conjugated to the aptamer and allowed to bind the captured target in a fashion similar to a sandwich ELISA. Gold or silver nanoparticles formed by the peptide would allow detection either visually or spectroscopically. Alternatively, a detection method could involve tagging the aptamer with a fluorescent molecule, or with an enzyme capable of producing a colorimetric or chemiluminescent signal via a substrate.

The first detection method, biomineralization, is not only frequently encountered in nature, but it has the potential to become a method of nanoparticle and nanostructure fabrication for industrial uses. Specifically, a 12-amino acid-long peptide (A3) has been created that can control the growth of gold nanoparticles in a solution in which HAuCl₄ is reduced to Au(s), possibly by passivating the surface of the particle nuclei. Gold particles that are small in diameter (5-10 nm) have the ability to scatter light differently than those of larger diameter due to surface plasmon resonance, and a solution of small particles will appear red in color, as opposed to the

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blue or metallic gold color of larger particles or bulk gold, respectively. A small diameter particle produces surface plasmon polaritons (electromagnetic waves at the metal/dielectric interface) with shorter wavelengths and higher frequencies than larger particles, leading to the absorption of photons with matching frequencies. Bulk materials thus resonate at frequencies in the infrared range while nanoparticles resonate in the ultraviolet-visible range, and the absorption of these higher frequency photons creates the visible color associated with nanoparticle suspensions. Several diagnostic tests involving gold nanoparticles are already in use or are currently being developed, such as certain brands of home pregnancy test and disposable nucleic acid sensors.25

In contrast to gold nanoparticles, fluorescent molecules have sharp emission bands, and their sensitivity may be altered by modifying the structure of the compound. Fluorescent or enzymatic tagging of antibodies (or aptamers) for sandwich ELISA assays is currently commonplace and would represent the most straightforward approach to detection in a diagnostic test. Either may be directly attached to the antibody by covalent cross-linking chemistry or indirectly via addition of a biotin-streptavidin interaction. Although direct labeling with fluorophores requires fewer protocol steps during an ELISA, enzymatic generation of a chemiluminescent signal can be much more sensitive. One such example of an optimized chemiluminescence-detection system has been developed by ThermoScientific. In this system, the detection antibody is biotinylated prior to the sandwich ELISA protocol. Next, a solution of horseradish peroxidase that has been covalently coupled to streptavidin is applied, forming a strong bond between the enzyme and antibody. Finally, an

optimized solution of luminol and peroxide is applied to the well, and the enzyme catalyzes the oxidation of the luminol into an excited-state product that subsequently decays with the emission of light. The catalysis of many product molecules from one enzyme serves to amplify the signal, leading to increased sensitivity of the detection system. Figure 1-6 illustrates the chemiluminescent reaction.

Figure 1-6. Light-generating reaction for chemiluminescent ELISA detection. This figure was taken from “SuperSignal ELISA Pico Chemiluminescent Substrate.” Piercenet. 2014. Web. 17 July 2014.

Therefore, a potential biosensor may be imagined to work in the following way: 1) a LAM-specific aptamer would be fixed to a substrate 2) the matrix containing the target would be incubated on the substrate 3) a second LAM-specific aptamer that had been conjugated to a biomineralizing peptide, fluorophore, or enzyme would be allowed to bind and then the unbound molecules would be washed away and 4) the reagents needed to produce nanoparticles or chemiluminescent signal would be added. A summary of a potential sandwich-detection assay is described in Figure 1-7.
Figure 1-7. A potential sandwich assay for the detection of LAM in biological samples.

The feasibility of such a diagnostic is confirmed by the recent development of the Determine™ TB-LAM urine test strip by Alere. Anti-LAM antibodies absorbed onto a nitrocellulose strip compose a portable, inexpensive immunochromatographic assay capable of diagnosing patients with tuberculosis by detecting LAM excreted into urine. The test has ample room for improvement, however, as one clinical trial resulted in
only a 45% sensitivity and 66% specificity for accurate detection of tuberculosis-positive patients. Figure 1-8 shows a photograph of the test.

**Figure 1-8.** Photograph of a Determine™ TB-LAM urine test strip by Alere. This figure was taken from Lawn, S. D. Point-of-care detection of lipoarabinomannan (LAM) in urine for diagnosis of HIV-associated tuberculosis: a state of the art review. *BMC Infectious Diseases*. 2012, 12, 103.

Due to the inherent stability of DNA, the high affinity and specificity of base-modified aptamers, and the abundance of LAM as a marker for active tuberculosis, the feasibility of creating a novel biosensor from these components that is capable of much improved performance over current diagnostic tests seems promising, and the number of lives that may be saved annually provide the motivation for exploring this area of research.

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CHAPTER II

IN VITRO SELECTION AGAINST INTACT CELLS OF MYCOBACTERIUM SMEGMATIS USING EXISTING BENZYL DEOXYURIDINE MODIFICATION

Introduction

In order to develop a selection protocol for the discovery of LAM aptamers, the model organism *M. smegmatis* was chosen for its similarity to *M. tuberculosis*, yet lack of virulence. On the extracellular surface, *M. smegmatis* differs from *M. tuberculosis* mainly in that its glycolipid coating is composed of PILAM instead of ManLAM. The differences between these two types of LAM are summarized in Figure 2-1.
Figure 2-1. General differences between PILAM and ManLAM. The most striking variability is observed on the ends of the arabinan branches.

A consequence of the phosphate group-capping of the PILAM arabinan branches is the presence of negative charges at neutral pH, which ManLAM lacks. The negative charges serve to break up the electron cloud monotony of a neutral carbohydrate, but pose a challenge for the binding of negatively-charged DNA. Possible mechanisms for aptamer binding in this case may include shielding of charge with metal cations, or pKa perturbation of a base or bases via a specific sequence run, a phenomenon that has been observed in other labs.\(^{27}\) A more detailed examination of the LAM capping structures is given in Figure 2-2.

Figure 2-2. Capping structures at the ends of the arabinan branches for a) PILAM (M. smegmatis) and b) ManLAM (M. tuberculosis).

Because immediate access to M. tuberculosis cells was unavailable, selection protocol parameters such as partitioning method of the unbound aptamer pool from the bound population, washing stringency, and incubation shaking frequency can be optimized with M. smegmatis before continuing on to M. tuberculosis.

Selections

Selection for a PILAM Aptamer Using Intact Mycobacterium Smegmatis Cells

Design. The scheme for the selection of PILAM aptamers using M. smegmatis cells is loosely based on procedures outlined in Vaught et al for the enzymatic steps.28 Incubation, washing and partitioning, however, are different and were optimized in order to minimize background contamination of sequences that bound to the cells in a

non-specific manner. Figure 2-3 illustrates the general steps for partitioning during the selection using intact cells (refer to Figure 1-4 in chapter I for a scheme including the enzymatic steps).

**Figure 2-3.** Partitioning steps for the selection scheme using intact cells.

Synthesis of a starting pool of $10^{14}$ random sequences can be accomplished synthetically by phosphoramidite chemistry using a DNA/RNA synthesizer. A general procedure for oligonucleotide preparation in such a manner is described in Figure 2-4.
A controlled-pore glass bead support is covalently coupled to the starting nucleoside that will form the 3' end of the oligonucleotide. The dimethoxytrityl protecting group is removed by dichloroacetic acid to free the 5' hydroxyl. A solution of phosphoramidite is applied and mixed with dicyanoimidazole to form an activated intermediate that is susceptible to nucleophilic attack. After addition of the phosphoramidite to the oligonucleotide chain, any strands that have failed to react

**Figure 2-4.** Cycle in the synthetic construction of an oligonucleotide by phosphoramidite chemistry. This figure was taken from Applied Biosystems. Models 392 and 394 DNA/RNA Synthesizers User's Manual. 1991, Revision C, 6-4.
with the phosphoramidite are capped by acetylation to prevent further growth of the chain. In the last step of the cycle, the phosphite linkage is oxidized to a phosphotriester with iodine and water. Addition of dichloroacetic acid in the next step begins the cycle again, and the process is repeated for each base that composes the oligonucleotide. The completed oligonucleotide is cleaved from the solid support with concentrated ammonium hydroxide, which also removes the protecting groups on the exocyclic amines of the bases and the cyanoethyl groups on the phosphates.

To obtain a random pool of \(10^{14}\) different sequences, one nanomole of the synthetic DNA is purified by polyacrylamide gel electrophoresis (PAGE) and one cycle of polymerase chain reaction (PCR) is performed with a biotinylated 3' primer (see Figure 1-4 in chapter I). The biotinylated, double-stranded product is bound to streptavidin-coated beads to provide a facile method for washing and strand separation, and the non-biotinylated strand is eluted with sodium hydroxide at pH 12. After washing the beads with buffer, primer extension is then performed with the modified deoxyuridine replacing the normal deoxythymidine in the reaction. The modification chosen for this selection was benzyl, shown as molecule 6a in Figure 2-5.
The modified strand is then eluted with sodium hydroxide, the sample is neutralized, and quantified by absorbance spectrophotometry.

Before incubation with the target, the modified DNA strands must be folded into three dimensional structures. This can be accomplished by heating the sample to boiling to remove incorrectly-formed interactions between the bases and return the strand to a linear form. The sample is then cooled slowly to room temperature to allow correct folding to take place.

After folding, the DNA aptamers can be incubated with the target, in this case intact cells, at room temperature. The cells are shaken during the incubation to prevent settling. Removal of the unbound sequences after the incubation period can
be accomplished either by centrifuging the cells into a pellet and removing the supernatant, or filtering the cells through a 0.2 µm cellulose acetate spin column. The bound sequences can be eluted by raising the pH of the solution so that the hydrogen bonds between the bases that dictate the structure of the aptamer are disrupted by deprotonation, causing linearization of the strand and removal of the aptamer from the target into the solution. The supernatant is then removed and subjected to PCR to amplify sequences that correspond to folded structures that bind the target. A primer extension step, as above, begins a new cycle and the procedure is repeated many times until the majority of the pool binds the target. During later rounds of the cycle, harsher parameters are applied to the partitioning step in order to select for sequences that can bind to the target with the most specificity and affinity, such as increased number and duration of washings, competitors, and shorter incubation times. When acceptable conditions for good binders have been reached, the selection can be ended and the remaining pool of sequences subjected to sequencing. After classification of the sequences into families based on similarity, representative members are chosen, synthesized, and analyzed for binding affinity.

Characterization of the sequences for specificity and binding affinity can be accomplished by a variety of assays, including PAGE gel-shift, cell-binding, and fluorescence polarization anisotropy (FPA). PAGE gel-shift, as illustrated by Figure 2-6, utilizes the propensity of larger molecules and complexes to migrate more slowly in a polyacrylamide gel matrix, causing an upward shift of the complex as compared to the unbound aptamer.
Figure 2-6. Prototypical PAGE gel-shift assay in which the aptamer-target complex migrates more slowly in the gel matrix than the unbound aptamer.

In addition to gel-shift assays, binding of the aptamer to PILAM can be assessed by cell-binding experiments in which the aptamer is tagged with a fluorescent molecule, the aptamer is incubated with the cells, and the unbound aptamer is removed by filtration or cell pelleting in a centrifuge and washing. The amount of fluorescent signal present in both the eluate and retained on the cells can be measured to determine binding affinity.

Yet another characterization technique commonly employed to assay aptamer binding is FPA, fluorescence polarization anisotropy. An aptamer, antibody, or other molecule of interest is tagged with a fluorescent molecule. In solution at a given temperature, the tagged aptamer will possess a rotational velocity, $V_{r1}$. Polarized light
of wavelength in the excitation region of the fluorophore is transmitted through the sample and transforms the fluorophore into its excited state. As the fluorophore relaxes with the emission of light, the aptamer rotates according to $V_{r1}$, and the emitted light is depolarized proportionately as the orientation of the fluorophore with respect to the detectors becomes increasingly random. This depolarization is measured by the instrument via polarization filters as perpendicular and parallel light intensities relative to the original excitation plane. An anisotropy value, $r$, is calculated according to Equation 2-1.

$$
 r = \frac{I_\parallel - I_\perp}{I_\parallel + 2I_\perp}
$$

**Equation 2-1.** Anisotropy, $r$, from the emission intensity parallel to the excitation plane, $I_\parallel$, and the emission intensity perpendicular to the excitation plane, $I_\perp$.

When the aptamer is bound to the target in a complex, the rotational velocity becomes $V_{r2}$, which is lower than the unbound velocity $V_{r1}$. The slower rotational velocity leads to less depolarization of emitted light due to the fluorophore having traversed a smaller distance and retained more similarity to its original orientation. Each FPA assay must be optimized, and the fluorophore must be appropriately chosen such that its lifetime allows sufficient, but not excessive, rotation of the complex before emission takes place. Lifetimes that are either too short or too long lead to reduction in signal intensity relative to background noise.

A diagram for the general concept of FPA is shown in Figure 2-7.
**Figure 2-7.** Illustration of a general FPA assay. An aptamer or other receptor is tagged with a fluorescent molecule. A) the unbound aptamer rotates with a rotational velocity $V_{r1}$, and after excitation with polarized light, the fluorophore emits light that is depolarized proportionately to $V_{r1}$. B) The bound aptamer-target complex rotates with a rotational velocity $V_{r2}$ that is slower than $V_{r1}$, resulting in less depolarization of the emitted light.

**Results and Discussion.** The selection as described above was continued for ten rounds, and was monitored by noting the RT-PCR cycle at which the amplification signal displayed an inflection point. The RT-PCR traces indicated that the selection was progressing well, as evidenced by a signal that appeared generally around PCR cycle 15. Appearance of signal at earlier cycles would suggest that the diversity of the pool was not decreasing rapidly enough, and that numerous sequences were capable of binding to the cells with specificity and affinity. Appearance of signal at later cycles
would suggest that the selection parameters might have been too harsh, and that the pool was not capable of binding the cells with specificity and affinity. The RT-PCR traces for all selection rounds can be found in Appendix A.

The selection parameters applied at each round are summarized in Table 2-1.

<table>
<thead>
<tr>
<th>Round #</th>
<th>Incubation Time (min.)</th>
<th># of Cells</th>
<th>Competitor Buffer</th>
<th>Wash Duration (min.)</th>
<th>Dextran Sulfate</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>180</td>
<td>3 x 10⁹</td>
<td>No</td>
<td>5</td>
<td>No</td>
</tr>
<tr>
<td>2</td>
<td>180</td>
<td>4.5 x 10⁷</td>
<td>Yes</td>
<td>5</td>
<td>No</td>
</tr>
<tr>
<td>3</td>
<td>60</td>
<td>4.5 x 10⁷</td>
<td>Yes</td>
<td>5</td>
<td>Yes</td>
</tr>
<tr>
<td>4</td>
<td>30</td>
<td>1 x 10⁷</td>
<td>Yes</td>
<td>5</td>
<td>Yes</td>
</tr>
<tr>
<td>5</td>
<td>15</td>
<td>1 x 10⁷</td>
<td>Yes</td>
<td>15</td>
<td>Yes</td>
</tr>
<tr>
<td>6</td>
<td>15</td>
<td>1 x 10⁷</td>
<td>Yes</td>
<td>20</td>
<td>Yes</td>
</tr>
<tr>
<td>7</td>
<td>15</td>
<td>1 x 10⁷</td>
<td>Yes</td>
<td>40</td>
<td>Yes</td>
</tr>
<tr>
<td>8</td>
<td>15</td>
<td>1 x 10⁶</td>
<td>Yes</td>
<td>40</td>
<td>Yes</td>
</tr>
<tr>
<td>9</td>
<td>20</td>
<td>1 x 10⁵</td>
<td>Yes</td>
<td>40</td>
<td>Yes</td>
</tr>
<tr>
<td>10</td>
<td>25</td>
<td>5 x 10⁴</td>
<td>Yes</td>
<td>40</td>
<td>Yes</td>
</tr>
</tbody>
</table>

Table 2-1. Selection parameter summary for selection against intact *M. smegmatis* cells.

In the experiments summarized in Table 2-1, the competitor buffer was composed of 1 µM prothrombin, 1 µM casein, 0.3% w/v human serum albumin, and 1 µM yeast mannan. The competitor buffer was added to the solution during incubation
in order to provide a variety of proteins and saccharides to serve as competitors for the binding of the aptamers to the cell. In this way, aptamers that non-specifically bind to irrelevant molecules were selected against. Wash duration refers to the time that each wash (three washes in total) was allowed to incubate, to allow aptamers with fast off-rates to be selected against. In addition to using long washing durations, dextran sulfate was added prior to the wash steps to select against aptamers with fast off-rates. Dextran sulfate, a polyanion that is used for many passivating and coating applications, strongly binds to targets in the absence of other binders, and will prevent any aptamers that have dissociated from the target from rebinding.

Further reduction in cell number (i.e., \( \leq 10,000 \)) resulted in a complete disappearance of RT-PCR signal after partitioning, signaling that the selection could not be advanced past this point. Sanger sequencing was performed on the DNA pool from round 10, and the sequences were categorized into families based on sequence similarity using the software program, Daughter of Sequence Alignment (DOSA).\(^{29}\) DOSA results for all selections are given in Appendix B. Analysis revealed that the sequences could be grouped into three major families, the largest characterized by the presence of an identical 6-mer: “CCTTTT” at variable locations within the 40N random region. The second family contained a 5-mer composed entirely of cytidine bases, and a third family contained an 8-mer of the sequence “CCTCTTCC”. In this analysis, a single orphan could not be categorized as belonging to any of the above families, hereafter known as “WCMS35”.

Screening the sequences for affinity to PILAM was accomplished by the gel-shift method. Because the target of the selection was an entire \( M. \) \textit{smegmatis} cell, it is

\(^{29}\) Software from Dom Zichi, personal communication.
possible that some of the sequences could be aptamers for extracellular proteins. Despite the fact that there are approximately 5 million PILAM molecules coating the surface of each cell, the hydrophobic benzyl modifications may predispose the aptamers to bind proteins, which may contain significant hydrophobic regions. To eliminate protein-binding aptamers and screen for the best PILAM-binding aptamer, native PAGE was run using 26 of the sequences. Figure 2-8 shows the results of the gel-shift assay.
Figure 2-8. Gel-shift assay screening 26 sequences from round 10 of the *M. smegmatis* selection. Red lines divide each sequence with the sequence number (assigned during the sequencing procedure) above each partition. Within each sequence partition, the left lane was run with only the DNA aptamer present, while the right lane included both the DNA aptamer and extracted PILAM.
As can be seen from the gels, about 80% of the partitions show a lighter band in the right lane as compared to the left, with the most pronounced difference for sequence 35. This difference suggests that the aptamer (the molecule being stained in this case) is binding to the PILAM and shifting upward, causing the area to which the unbound aptamer migrates to be lighter due to the presence of less aptamer. In typical gel-shift assays that analyze aptamer-protein binding, a band higher in the gel corresponding to the bound complex is usually observed in addition to the lightening of the lower band that corresponds to the unbound aptamer. In this case, however, the target PILAM does not possess a discrete molecular weight, but is composed of a Gaussian-like distribution of PILAM molecules with variable numbers of sugar residues, as well as chain lengths and number of lipid anchors. Such a distribution, when run on a gel, presents as a dilute, smeared band. Therefore, it is likely that any bound aptamer will also be distributed throughout the band, and the localized concentration in the gel region would be too dilute to be visualized by this staining method. Because sequence 35 appears to present the greatest gel shift, it was chosen for further characterization, and will hereafter be termed “WCMS35” (whole-cell mycobacterium smegmatis 35).

Further characterization of WCMS35 was attempted through a filter-partitioned cell-binding assay. After primer extension, the aptamer was tagged via terminal transferase with an Alexa-488 fluorophore. Various numbers of *M. smegmatis* cells were incubated for 30 minutes in 10 nM aptamer solutions. After incubation, the cells were washed by filtration through 0.2 µm filters, and the relative fluorescent units of both the retentate on the filter and eluate through the filter were recorded. As can be seen from Figure 2-7, the results describe an opposite trend than would be expected.
An aptamer with an affinity for PILAM on the surface of *M. smegmatis* would be expected to bind the cells, and increasing numbers of cells would therefore show increased fluorescence. Conversely, the fluorescence of the eluate should decrease as the cell numbers increase due to higher quantities of aptamer being bound up by the cells. The results, however, present an opposite trend in each case, a confusing observation that contradicts the gel-shift results in Figure 2-6. The experiment was repeated with cell pelleting and washing as the partitioning step with the same results.
A third characterization test, FPA, was attempted to assay binding of WCMS35 to PILAM. Table 2-2 shows the anisotropy response as PILAM was gradually added to a constant quantity of WCMS35.

<table>
<thead>
<tr>
<th>PILAM (nM)</th>
<th>Anisotropy</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.14</td>
</tr>
<tr>
<td>0.1</td>
<td>0.10</td>
</tr>
<tr>
<td>0.5</td>
<td>0.10</td>
</tr>
<tr>
<td>1</td>
<td>0.10</td>
</tr>
<tr>
<td>2</td>
<td>0.10</td>
</tr>
<tr>
<td>10</td>
<td>0.08</td>
</tr>
<tr>
<td>50</td>
<td>0.08</td>
</tr>
<tr>
<td>100</td>
<td>0.08</td>
</tr>
<tr>
<td>500</td>
<td>0.08</td>
</tr>
<tr>
<td>1000</td>
<td>0.08</td>
</tr>
</tbody>
</table>

**Table 2-2.** Anisotropy response to gradual addition of PILAM to WCMS35 (66 nM).

The results shown in Table 2-2 for the FPA experiment indicate that the anisotropy value decreases with increasing concentration of target, which is opposite to typical FPA protein-binding experiments that generally produce a positive anisotropy change. However, FPA with nucleic acids, which often contain primer-binding regions that are uninvolved with target recognition, have been frequently observed to produce negative anisotropy changes. This phenomenon is hypothesized to be due to the localized motion of the fluorophore, which in the case of
oligonucleotides is attached at the end of one of the primer regions. Before binding, the primer region, and thus the fluorophore, may be folded into the structure of the aptamer and the rotational velocity restricted. After binding, the target may be displaced by either aptamer conformational changes or replacement of the primer by the target in the binding site. Therefore, after binding, the primer region and attached fluorophore would experience an increase in localized rotational velocity that leads to a decrease in the anisotropy value.\textsuperscript{30,31}

The gel-shift and FPA assays provide evidence that WCMS35 binds to PILAM with some affinity. However, the lack of binding to whole \textit{M. smegmatis} cells is confusing; in addition, the results of the gel-shift and FPA assays are not reliably reproducible. It is possible that the buffer conditions in which the aptamer was selected are not ideal for binding, but sufficient to allow a minimal number of the sequence to survive the selection rounds and be amplified. Incubation of the aptamer pool with the cells for several hours resulted in complete recovery of full-length DNA, eliminating the possibility of cell-based DNA degradation. Adjusting salt and surfactant concentrations, pH, temperature or other variables may allow binding of WCMS35 to PILAM to be optimized, however, because the selection against \textit{M. smegmatis} cells was intended as a preliminary proof-of-concept pilot, it was decided to focus project efforts on the more important goal of finding an \textit{M. tuberculosis} aptamer.

\textbf{Experimental.} All chemicals were purchased from Sigma Aldrich unless otherwise stated. DNA phosphoramidite chemistry synthesis reagents were purchased

\textsuperscript{30} Zhang et al. Fluorescence anisotropy reduction of allosteric aptamer for sensitive and specific protein signaling. \textit{Analytical Chemistry}. 2012, 84, 3070-3074.

DNA synthesis. DNA synthesis-grade acetonitrile was further dried for 1-2 days with activated molecular sieve packs. Phosphoramidite bottles (1 gram) were diluted to 100 mM and dicyanoimidazole was prepared at 0.25 M with the dried acetonitrile. Dichloroacetic acid (13 mL) was transferred into a heat-dried synthesizer bottle and approximately 500 mL of anhydrous dichloromethane was canulated to the bottle under argon. Both the dicyanoimidazole and dichloroacetic acid solutions were dried for 1-2 days with activated molecular sieve packs. Oxidizer, Cap A, and Cap B solutions were used as purchased without further drying. A bottle for the random region was prepared by hand-mixing the phosphoramidites in a glove box under an argon atmosphere. The random region bottle was corrected for phosphoramidite incorporation bias by mixing the phosphoramidites in the ratio 0.28 A: 0.20 G: 0.32 C:
0.20 T. All bottles were exchanged onto the synthesizer and a 90N random library was synthesized with a 40N random region flanked by primer binding regions. The DNA was cleaved from the column with fresh ammonium hydroxide for 1 hour at room temperature, and then placed in a glass vial at 55 °C for 18 hours. The ammonium hydroxide was cooled, evaporated, and the resulting solid was subjected to PAGE purification.

Polymerase Chain Reaction. PCR reactions were performed with the following 5X PCR Mix: 10 µM each forward and reverse primers, 25 mM magnesium chloride, 5X as-purchased KOD XL buffer, trace amount of Sybr Green I Nucleic Acid stain, 1 mM each dNTP, and 0.075 U/µl KOD XL polymerase. To 48 µl of sample were added 12 µl of 5X PCR Mix, and the reaction was cycled as follows; cycle 1: 96 °C for 15 seconds, 55 °C for 10 seconds, 71 °C for 30 minutes; cycle 2-31: 96 °C for 15 seconds, 71 °C for 1 minute. The PCR reaction was monitored in real time and the traces were recorded.

Primer Extension. Primer extension reactions were performed with the following 1X Primer Extension Mix: 2.5 µM forward primer, 120 mM Tris-HCl at pH 7.8, 10 mM potassium chloride, 6 mM ammonium sulfate, 7 mM magnesium sulfate, 0.1% Triton X-100, 0.1 mg/mL bovine serum albumin, 0.5 mM each dNTP, and 0.015 U/µl KOD XL polymerase. Biotinylated PCR product or single-stranded synthetic, biotinylated DNA was incubated with an appropriate quantity of streptavidin-coated, magnetic MyOne Dynabeads with shaking at 1000 rpm for 15 minutes. The beads were washed 3X with dH₂O and if double-stranded PCR product was used, 20 mM sodium hydroxide was applied for 30 seconds. The beads were washed again 3X with dH₂O and the Primer Extension Mix was added. The reaction was shaken at 1000
rpm at 71 °C for 30 minutes. After incubation, the beads were again washed 3X with dH₂O and and 85 µl of 20 mM sodium hydroxide was added. After 30 seconds, 80 µl of sodium hydroxide are removed and neutralized with 20 µl of 80 mM hydrochloric acid, 25 mM HEPES, and 0.05% Tween 20.

Cell Culture of *M. smegmatis*. Mycobacteria were cultured in aseptic conditions for approximately 1 week to late log phase in Middlebrook 7H9 broth prepared according to the manufacturer's instructions. After culture, plating and cell density calculations were performed on Middlebrook 7H11 agar.

Selection Incubation and Partitioning. Approximately 10 pmol of modified primer extension product was buffered in 40 mM HEPES, 102 mM sodium chloride, 5 mM potassium chloride, 5 mM magnesium chloride, and 0.05% Tween 20 at pH 7.5. The modified DNA was folded by heatcooling with the following sequence: 96 °C for 5 minutes, 70 °C for 5 minutes, 48 °C for 5 minutes, and 37 °C for 5 minutes. In later rounds (see Table 2-1) Protein Competitor Buffer was added to 1 µM prothrombin, 1 µM casein, 0.3% w/v human serum albumin, and 1 µM yeast mannan. For rounds that included dextran sulfate, it was included at a concentration of 5 mM after incubation and before washing. During incubations at room temperature, the cells were kept suspended by shaking at 1000 rpm. Partitioning was either accomplished by pelleting the cells via centrifuge at 1000 rpm and aspirating the supernatant or filtering the cells through a 0.2 µm cellulose acetate spin column via centrifuge at 1000 rpm. After washing, the bound sequences were eluted for 5 minutes with 2 mM sodium hydroxide, and then neutralized with 2 mM hydrochloric acid 125 mM Tris-HCl at pH 7.5.
Cloning and Sequencing. Cloning of sequences was accomplished as per the manufacturer's instructions by transforming chemically competent *E. coli* with plasmid vectors inserted with sequences from the ending selection pool. The bacteria were plated, transformed colonies were chosen by blue/white screening, and individual colonies were grown overnight in separate tubes. Plasmids were extracted and sent for sequencing at Genewiz. After sequencing, the company shipped back the plasmids to facilitate screening of isolates. Plasmids were subjected to PCR and primer extension to create a matrix of modified DNA aptamer isolated sequences.

Gel-Shift Assay. Modified DNA aptamer isolates were folded according to the heatcooling protocol described above. Deacylated PILAM (0.125 μg) was incubated with 1 pmol of each DNA sequence for 1 hour. The reactions were run, along with an additional 1 pmol of DNA without PILAM, on a native 8% polyacrylamide gel at 150 V in Tris-borate buffer. The gels were visualized by staining with Sybr Green I Nucleic Acid Stain and scanning on a gel scanner with a 473 nm laser.

Cell-binding Assay. Modified DNA aptamer isolates were tagged in 14 μl of water, to which was added 2 μl 10X as-purchased terminal transferase buffer, 2 μl 10X as-purchased cobalt chloride solution, 1 μl of 1 mM Alexa 488-dUTP, and 1 μl terminal transferase. The reactions were incubated at 37 °C for 1 hour, 14 mM EDTA was added, and the aptamers were purified by dialysis with a 10 kDa membrane cassette. The aptamers were folded according to the heatcooling protocol described above. *M. smegmatis* cells were incubated with 1 pmol of WCMS35 for 30 minutes, with shaking at 1000 rpm, at room temperature. The samples were added to the wells of a 96-well, 0.2 μm PVDF filter plate and centrifuged at 1500 rpm for 1 minute, with the eluate collected in a solid-bottomed 96-well plate placed beneath the filter plate.
The cells were washed 3X with selection buffer and the fluorescent signal of each fraction was recorded on a plate reader.

Fluorescence Polarization Anisotropy. FPA experiments were performed with aptamers folded and tagged as described above. WCMS35 was filled into a quartz cuvette at a concentration of 66 nM and a volume of 250 µl in selection buffer. Deacylated PILAM was gradually added to the cuvette and pipette-mixed. After each addition, the sample was incubated for 15 minutes at room temperature, and the anisotropy value was recorded.
CHAPTER III

EXPANSION OF IN VITRO SELECTION CHEMISTRY WITH THE ADDITION OF A PHENYLBORONIC ACID MOIETY

Introduction

In the previous chapter, a selection for a mycobacterial glycolipid was described in which the modified deoxyuridine used was already in existence and has been proven to produce aptamers with high affinity to protein targets. As revealed by x-ray crystallography, the high affinities of modified DNA aptamers (SOMAmers) for their targets are due in part to the large amount of hydrophobic interactions along the binding interface that are made possible by the addition of benzyl, or other hydrophobic, modifications. Such modifications increase the diversity of interactions between the aptamer and target; SOMAmers utilize not only the hydrogen-bonding provided by the natural bases, but have the added advantage of potential interaction with hydrophobic target areas. Because protein targets generally contain regions of hydrophobicity, these interactions can be exploited to produce aptamers that are able to bind proteins in a manner analogous to antibodies, and therefore they have lower dissociation constants than their unmodified counterparts.

In contrast to proteins, however, the hydrophobic portions of glycolipids such as LAM not only comprise a relatively small part of the molecule, but are “poor in information.” While the extent of branching and type of sugar residues in the carbohydrate part of LAM differs among LAM type and even among mycobacterial

strains of the same species, the lipid portion is largely identical, being composed of only palmitic, stearic and tuberculostearic fatty acids. Such a structure requires that any aptamer that is specific for LAM bind to the more “information rich” carbohydrate chains. Because the carbohydrate chains are composed of sugar residues that possess hydroxyl groups, this portion of LAM is unlikely to interact strongly with hydrophobic moieties, and it seems likely that the contribution of such deoxyuridine modifications as benzyl would be mainly to assist with folding the aptamer into unique tertiary structures via inter-base interactions that would form the hydrogen-bonding, natural bases into a binding pocket.

This hydrophobic interaction-determined folding appears to have yielded aptamers for the negatively-charged, phosphorylated PILAM on the surface of M. smegmatis cells, but seems to be inadequate for the largely neutral M. tuberculosis ManLAM. Selections were attempted against extracted, biotinlyated ManLAM using a variety of hydrophobic deoxyuridine modifications such as benzyl, napthyl, and tryptoaminyl, all of which showed little or no progress compared to the negative control. Due to the neutral nature of the target, it is possible that stronger interactions than those provided by the natural or hydrophobic-modified bases are needed to assist binding of the aptamer. In general, detection of low concentrations of carbohydrates in aqueous solutions through noncovalent interactions has proven difficult due to the necessity of overcoming the solvation energies of both the receptor and target. Davis and Wareham sum up the issue in a review on carbohydrate detection methods in which they assert that “the typical carbohydrate largely resembles a water cluster” and “with binding constants for protein-monosaccharide complexes apparently limited to about $10^7\text{ M}^{-1}$, even Nature finds carbohydrate
To compensate for the high degree of solvation, the energy “payoff” on binding must be great, and stronger interactions provided by different modifications are needed.

In order to enable the aptamers to form stronger interactions with LAM, different modifications that are capable of either forming stronger noncovalent bonds or covalent bonds with sugar residues are necessary. Upon review of the literature, two molecules seemed to be appropriate: pyridine and boronic acid. Both molecules have been extensively proven to interact with sugar molecules, and incorporation of these moieties into DNA aptamers could be accomplished via three different scenarios, which are outlined briefly in Figure 3-1.

Figure 3-1. Summary of three binding scenarios involving pyridyl and/or boronic acid-modified aptamers binding to LAM. In Arrangement 1, the pyridyl groups are scattered along the body of the aptamer via modification of deoxyuridine, and form hydrogen bonds to the sugar residues in LAM. In Arrangement 2, the pyridyl groups are also scattered along the body of the aptamer via modification of deoxyuridine, and form hydrogen bonds to the sugar residues in LAM, but in addition, they form an adduct with a boronic acid modification on the 5' end of the aptamer, which forms covalent bonds with the vicinal diols on the sugar residues of LAM. In Arrangement 3, the deoxyuridine bases are modified with a boronic acid moiety, which forms covalent bonds with the vicinal diols on the sugar residues of LAM (at basic pH).

In the three arrangements presented in Figure 3-1, the pyridyl group serves two functions: the formation of hydrogen bonds with the hydroxyl groups on the sugar residues, and the formation of an adduct with the boronic acid modification. The interaction of pyridyl with boronic acids will be discussed later. Figure 3-2 reviews the structure of the pyridyl-modified deoxyuridine that has been previously synthesized by the Eaton laboratory and is described in a paper by Vaught et al.
Figure 3-2. Molecule 6e represents the “R” group attached to the deoxyuridine via an amide bond to form the pyridyl-modified deoxyuridine. This figure was taken from Vaught, J.D. Expanding the chemistry of DNA for in vitro selection. *J. Am. Chem. Soc.* **2010**, *132*, 4141-4151.

Pyridine has been shown to form hydrogen bonds to carbohydrates, especially in conjunction with amide bonds, and is being researched as a possible component in saccharide detectors.\(^{34}\) Figure 3-3, from a paper by Mazik et al., describes the hydrogen bonding between pyridine, an amide bond, and a hydroxyl group on a sugar molecule.

Because pyridine has already been proven to form strong hydrogen bonds with sugars, it seems likely that its addition as a functional moiety to DNA aptamers would make the binding of the aptamer to LAM more favorable.

Although pyridine is likely to increase the hydrogen bonding of the aptamer to LAM, the formation of covalent bonds between the two would be even more desirable. If the equilibrium between a free aptamer and its bound state is represented by an on-rate, $k_1$, and an off-rate, $k_{-1}$, then the addition of a second, covalent bond-forming step would introduce another, catalytic rate constant, $k_2$, that would be irreversible (or at least much less reversible) than the first binding equilibrium determined solely by noncovalent interactions, as described in Figure 3-4.
Alternatively, the theoretical rate of covalent bond formation between an aptamer and its target could be described with Michaelis-Menton enzyme saturation kinetics, given by Equation 3-1, in which the turnover number, $k_{\text{cat}}$, cannot exceed one.

$$v = k_{\text{cat}} [A] \frac{[T]}{K_m + [T]}$$

**Equation 3-1.** Enzyme reaction rate according to Michaelis-Menton saturation kinetics, where $v$ is reaction rate, $k_{\text{cat}}$ is turnover number in maximum number of product complexes formed per aptamer molecule per second, $A$ is aptamer concentration, $T$ is target concentration, and $K_m$ is the Michaelis constant representing the target concentration at which the reaction rate is half-maximum.
Because of this second, “irreversible” equilibrium, a molecule that can form covalent bonds to sugars, such as boronic acid, would be a good addition to a DNA aptamer, since it is well known that boronic acids can bind to molecules containing vicinal diols.

Boronic acids, unlike their carbon analogs, carboxylic acids, are not found in nature. Dennis G. Hall, author of *Boronic Acids: Preparation, Applications in Organic Synthesis and Medicine*, describes them as being regarded as “peculiar and rather neglected compounds” until their recent resurgence in popularity in the past few decades. Boronic acids represent the second stage of oxidation of boron (Figure 3-5) and are relatively stable, exhibit low toxicity, and because they will eventually oxidize to innocuous boric acid, are considered “green” compounds.35

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Figure 3-5. Various oxygenated boron-containing compounds. This figure was taken from Hall, D.G. *Boronic Acids: Preparation, Applications in Organic Synthesis and Medicine*, 1st ed.; Wiley: New York, 2005.

The low toxicity of boronic acid is demonstrated by its use in the new anticancer drug Velcade®, which is the first approved drug containing a boronic acid moiety.\(^{36}\)

The “boronic ester” illustrated in Figure 3-5 demonstrates why boronic acids are useful for the handling and detection of carbohydrates. The hydroxyl groups of the starting boronic acid can react with vicinal 1,2 or 1,3 diols, such as those on many types of sugars, to form covalent ester bonds (the mechanism of this formation will be detailed later). A large amount of research is now being focused on the development of saccharide detectors, and boronic acid-based separation of sugars via column chromatography is currently commonplace. One example of current research into

saccharide detection involving boronic acids, among many, is that of the Hamachi group, which is working on a hybrid biochemistry/boronic acid biosensor for carbohydrates. The group coupled a fluorescent boronic acid moiety to concanavalin-A, a lectin known to bind to monosaccharides. In this way, they can alter a naturally-occurring sugar-binding system with a synthetic modification, increasing the binding affinity and possibly the specificity of the protein, in addition to providing a fluorescent readout for detection.\textsuperscript{37}

The peculiar nature of boronic acids is due to boron’s vacant, unhybridized p orbital and electron deficient outer valence comprised of only 6 valence electrons. For this reason, boronic acids are Lewis acids, and only very rarely do they display Brønsted acidity via the proton on the hydroxyl groups. The boron center is sp\textsuperscript{2} hybridized, and boronic acids generally assume a trigonal planar geometry, with the axis of the p orbital orthogonal to the plane of the molecule. Because their derivatives are more readily incorporated into biopolymers, phenylboronic acids will be the only type of boronic acids considered.

In the structure of phenylboronic acid molecules, the electrons in the \pi orbital of the phenyl ring, as well as the lone pair in the non-orthogonal sp\textsuperscript{3} orbital of each oxygen atom, are partially delocalized into the vacant p orbital of neighboring boron. Such electron sharing causes a partial double bond character in the two boron-oxygen bonds, as well as a partial \pi character in the boron-carbon bond, shortening and strengthening them considerably, and also accounting for the considerable effect of ring substituents on the Lewis acidity of the boronic acid. When the delocalization is

broken by coordination of a fourth atom to boron, the p orbital is no longer vacant and the electron delocalization vanishes. Evidence for this phenomenon is provided by the bond lengths: after coordination of the fourth atom, the lengths of the boron-carbon and boron-oxygen bonds increase by about 0.045 Å and 0.10 Å respectively. In fact, the bond energy difference between the tricoordinated and the tetracoordinated boronic acid for the boron-oxygen bond can be as high as 50 kJ/mol. The partial electron sharing within the phenylboronic acid molecule is illustrated in Figure 3-6.

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Figure 3-6. Typical phenylboronic acid molecule. The electrons from neighboring atoms partially fill boron’s vacant p orbital, causing a partial double bond character in the B-O bonds and partial π character in the B-C bond.

Another noteworthy aspect of boronic acids is the observation that boronate esters possess more Lewis acidity than their boronic acid counterparts. An explanation for this observation has been proposed in which the relief of bond angle strain is identified as the cause of the increased acidity. As a trigonal planar boronic acid, the bond angles are around 120°. When the boronate ester is formed, however, the oxygen-boron-oxygen bond angle is forced into a 108° angle, which more closely resembles that of a tetrahedral geometry, and induces the molecule to become tetracoordinated, and thus sp³ hybridized, more easily.⁴²

During the resurgence in popularity of boronic acids as synthetic intermediates in the past few decades, a question arose concerning the mechanism of boronate ester formation (for example, to sugar vicinal diols) from the starting boronic acid (described in Figure 3-7): does the trigonal boronic acid form the ester bonds first, and then the resulting increase in Lewis acidity leads to the formation of a stabilizing tetracoordinated adduct ($K_{\text{trig}}$ followed by $K_a$), or does the tetrahedral boronic acid form first, with the formation of the ester bonds as the second step ($K_a$ followed by $K_{\text{tet}}$)?

![Equilibrium between all possible species of boronic acid during boronate ester formation.](image)

**Figure 3-7.** Equilibrium between all possible species of boronic acid during boronate ester formation. This figure was taken from Hall, D.G. *Boronic Acids: Preparation, Applications in Organic Synthesis and Medicine*, 1st ed.; Wiley: New York, 2005.

The former seemed more intuitive, as the vacant p orbital on the boron was hypothesized to be necessary for nucleophilic attack on the boron. Pizer and Tihal
answered this question with an important study that examined the kinetics of boronate ester formation, and they arrived at a more complicated, counterintuitive conclusion. Using the temperature-jump relaxation method, they determined that the rate constants for formation of the trigonal boronate ester (about 0.1 M\(^{-1}\)s\(^{-1}\)) were much slower than those for the formation of the corresponding tetrahedral boronate ester. They concluded that if, in fact, the tetrahedral boronic acid reacted to form its boronate ester through a trigonal intermediate, then that must be the rate limiting step and the overall rate would be comparable to that of the ester formation of the trigonal boronic acid (see Figure 3-8).

Figure 3-8. Illustration of the mechanism of boronate ester formation according to experimental kinetic measurements. a) formation of the trigonal boronate ester b) formation of the tetrahedral boronate ester c) formation of the tetrahedral boronate ester through a trigonal boronic acid intermediate, an impossibility due to the presence of a slower rate-determining step than the overall reaction rate.
However, because the rate of reaction for the tetrahedral boronic acid complex was much faster, it must be reacting directly with the diols for the formation of the boronate ester. In fact, the tetracoordinated boronic acid reacted about $10^3$-$10^4$ times faster than its tricoordinated counterpart, and even as much as $10^6$ times faster if upper limits were considered. The authors hypothesized that the longer, weaker boron-oxygen bond in the tetracoordinated complex versus the tricoordinated complex (recall that the addition of a fourth atom to boron abolishes the partial double bond character between boron and oxygen) transformed each boronic acid hydroxyl group into a better leaving group, allowing the non-acidic hydroxyl groups of the ligand to successfully participate in the formation of ester bonds.\(^{43}\)

Pizer and Tihal suggested that final evidence for the possibility of their proposed mechanism is provided by the successful isolation and characterization of pentacoordinated boronic acid compounds. A catechol boronic acid ester (Figure 3-9) was analyzed by X-ray crystallography and found to contain a hypervalent boron, evidenced by the rigid ether groups, held in place by a “two-electron, three-atom center.”\(^{44}\)

![Catechol boronic acid ester demonstrating boron hypervalence.](image)

**Figure 3-9.** Catechol boronate ester demonstrating boron hypervalence.


As previously stated, the Lewis acidity of boronic acids can result in the coordination of basic molecules to the boron, transforming it from tricoordinated to tetracoordinated, and facilitating formation of boronate ester bonds. Figure 3-10 illustrates such a scenario as it would apply to the arrangements in Figure 3-1, with pyridine as the basic molecule.

Figure 3-10. Coordination of pyridine to boronic acid to form a tetracoordinated adduct. After coordination, the boronic acid would form covalent ester bonds with the vicinal diols on a LAM sugar residue.

In another scenario, at basic pH, the body of the aptamer could contain deoxyuridines modified with a phenylboronic acid moiety instead of pyridyl, and the hydroxide ions present in the solution of high pH would act as the basic molecules needed to form an adduct with the boron and thus induce the formation of ester bonds to sugar vicinal diols.

**Synthesis**

As shown in Figure 3-2, deoxyuridine modified with a pyridyl group has already been synthesized by the Eaton lab. Boronic acid modifications, however, had not yet
been synthesized with an amide bond linker directly connecting the boronic acid moiety to the deoxyuridine base. The Wang group has synthesized several varieties of boronic acid-modified deoxythymidine, both with and without fluorescent properties, using “click” chemistry (Figure 3-11).

![Figure 3-11](image)

**Figure 3-11.** An example of a boronic acid-modified deoxythymidine synthesized by the Wang group. This figure was taken from Cheng, Y.; et al. Design, synthesis, and polymerase-catalyzed incorporation of click-modified boronic acid –TTP analogues. *Chem. Asian J.* 2011, 6, 2747-2752.

For arrangement 3 (refer to Figure 3-1), a boronic acid-modified deoxyuridine is needed. In order to simplify the structure and minimize the number of rotatable bonds, the attachment of a phenylboronic acid moiety directly to the deoxyuridine base via an amide bond would be desirable. Such a structure is illustrated in Figure 3-12.

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Figure 3-12. Structure of a boronic acid-modified deoxyuridine in which a phenylboronic acid moiety is attached to the deoxyuridine base via an amide bond linker.

For arrangement 2 (Figure 3-1), the boronic acid moiety need only be attached to the 5' end of a DNA primer. This primer would then be used in a primer extension reaction that generates the rest of the DNA aptamer (see previous chapter). Since the DNA primers used throughout this thesis are synthetically prepared using phosphoramidite chemistry, the boronic acid moiety can be attached through the preparation of a boronic acid-modified phosphoramidite (Figure 3-13).

Figure 3-13. Structure of a boronic acid-modified phosphoramidite for synthetic attachment of the phenylboronic acid moiety to the 5’ end of a DNA primer.
Synthesis of a Boronic Acid-Modified Deoxyuridine: “Click” Chemistry Pathway

**Design.** This first scheme for the preparation of a boronic acid-modified deoxyuridine (hereafter referred to as BA-dUTP) follows the procedure outlined in a paper previously published in the Eaton lab by Vaught et al. Scheme 3-1 details the preparation of a deoxyuridine containing a triple bond-terminated linker attached to the uridine base. The triple bond would then be used to attach an azidophenylboronic acid moiety via “click” chemistry as shown in Scheme 3-2.

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Scheme 3-1. Preparation of a triple bond-terminated linker attached to the uridine base. Procedure based on a protocol from Vaught et al.
Scheme 3-2. Attachment of boronic acid to deoxyuridine via “click” chemistry. Scheme is based on a protocol from Jena Bioscience.\(^{47}\)

In Scheme 3-1, the starting material 5-iodo-2’-deoxyuridine can be purchased. To protect the hydroxyl groups on the deoxyribose, dimethoxytrityl (DMT) and acetate groups are added to the 5’ and 3’ positions, respectively, generating 3-3. Next, a carboxyamidation reaction is performed under pressurized carbon monoxide and heat, using tetrakis[triphosphene]palladium(0) as the catalyst. This procedure is a

one-step method that adds the carbonyl group to the 5 position of the uridine base and then attaches the propargylamine group via an amide bond, forming 3-4. The 5’ hydroxyl group is then deprotected using trichloroacetic acid, and a one-pot, three-step procedure phosphorylates the structure into the triphosphate 3-8. The 3’ hydroxyl group is then deprotected with concentrated ammonium hydroxide.

The final structure in Scheme 3-1, 3-9, is carried into Scheme 3-2, where a 4-azidomethylphenylboronic acid is attached via “click” chemistry. The scheme is based on a standard copper(I)-catalyzed “click” chemistry protocol and is based on a procedure from Jena Bioscience.

The final structure in Scheme 3-2, 3-12, is a slight departure from the ideal molecule shown in Figure 3-12 in that a triazole group separates the phenylboronic acid moiety from the amide bond linker. However, the molecule still represents a significant reduction in the size of the nucleotide and the number of rotatable bonds, and the ease of synthesis assumed to accompany “click” reactions is a compelling motivator for the compromise.

**Results and Discussion.** Scheme 3-1 was performed up through the carboxyamidation reaction, skipping the hydroxyl group-protecting steps, as compound 3-3 was already in the possession of the laboratory. During silica column purification of putative compound 3-4, elution fractions were run on silica thin layer chromatography (TLC) plates, and a strange observation was noted. Under short wave-UV light, one of the product spots glowed bright blue, in contrast to the dark spots of the starting material and other product elution fractions against the greenish background fluorescence of the TLC plate. Upon exposure to acid, the spot displayed a bright orange color indicative of DMT cation release, evidence that the nucleoside
was present. The elution fraction was sent for mass spectrometry analysis, and an observed mass was found at 676.2262 amu, compared to the expected mass for compound 3-4 of 676.2265 amu.

Despite finding the expected mass for compound 3-4, it was hypothesized that the triple bond of the propargylamine had caused the formation of an unknown cyclized product capable of fluorescence, as the structure of 3-4 would not be expected to possess fluorescent properties. It was therefore decided that the carboxyamidation reaction should be carried out in two steps to avoid complications with such a reactive molecule as propargylamine.

**Experimental.** All chemicals were purchased from Sigma Aldrich and used without further purification unless otherwise noted. Triethylamine, dimethylacetamide, and tetrahydrofuran were dried over molecular sieves and degassed by the “freeze-pump-thaw” method. Tetrakis[triphenylphosphine]palladium(0) was purchased from Strem Chemicals and used without further purification. Mass spectrometry analysis was performed by Old Dominion University, Norfolk, VA.

Scheme 3-1. A solution was made under an inert argon atmosphere and in a thick-walled, Teflon-stoppered Schlenk flask of 0.7 mmol 5-iodo-5’-O-DMT-3’-O-acetyl-2’-deoxyuridine (3-1), 0.22 mmol propargylamine, 0.34 mmol triethylamine, approximately 0.2 mmol tetrakis[triphenylphosphine]palladium(0) and 15 mL of 1:1 DMA/THF. The flask was evacuated and filled with carbon monoxide three times, and then pressurized to 35 psi, heated to 65 °C, and stirred for approximately 120 hours. The product was evaporated under high vacuum to a dark brown, thick oil, and
purified via a flash silica gel column and 5% methanol/dichloromethane. The elution fractions were run on silica TLC plates with 5% methanol/dichloromethane.

Spectroscopic data for Scheme 3-1. HRMS (ESI-) expected 676.2265 amu, found 676.2262 amu.

Scheme 3-2. Scheme 3-2 was not attempted as the results of Scheme 3-1 necessitated a change in the plan.

**Synthesis of a Boronic Acid-Modified Deoxyuridine: MIDA Boronate Pathway**

**Design.** Due to the results of Scheme 3-1, it was decided that the carboxyamidation reaction would be performed in two steps to avoid complications with the reactive propargylamine. As a result of further literature review, it was discovered that the propargylamine and "click" chemistry step might be avoided altogether because of the advent of a new protecting group for boronic acids, methyliminodiacetic acid (MIDA). MIDA renders boronic acids unreactive due to its formation of ester bonds with the hydroxyl groups, as well as adduct formation via an amine (Figure 3-14).\(^\text{48}\)

\[ \text{Example of the protection of a boronic acid moiety by MIDA.} \]

Novel features of the MIDA protecting group now make it possible for molecules containing boronic acid moieties to be manipulated more easily. The MIDA group is not easily displaced by harsh chemical or physical conditions; even Jones oxidation conditions are well-tolerated and the boronic acid group remains protected throughout these types of reactions. Historic boronic acid-protecting groups, such as catechol, are unable to weather these harsh conditions. The remarkable resilience of the MIDA protecting group is thought to originate from the bulky carbonyl and methyl groups surrounding the boron atom, which provide steric hindrance against nucleophilic attack and subsequent hydrolysis. Removal of the MIDA protecting group, however, is very easy and can be accomplished with short incubations in mild, aqueous base. Again, this is in contrast to classical boronic acid protecting groups such as pinacol, which can be extremely difficult to remove completely under mild conditions.

The discovery of MIDA by Gillis and Burke in 2008 thus represents a milestone in boronic acid chemistry that will lead to great advancements in the use of these compounds, not only as synthetic intermediates, but in the development of saccharide detectors. In the present case, MIDA may facilitate the attachment of boronic acid to DNA strands by allowing harsher conditions during synthesis, such as exposure to oxidizing agents during nucleoside phosphorylation and synthetic DNA strand preparation via phosphoramidites. In addition, the gentle removal conditions for MIDA from the completed DNA strand are beneficial for maintenance of the DNA strand integrity. Such often mutually exclusive properties would be impossible with the repertoire of previously-used boronic acid protecting groups such as catechol and pinacol.

The unique properties of MIDA as a protecting group are exploited in the new plan for BA-dUTP synthesis, outlined in Schemes 3-3, 3-4, 3-5, and 3-6.
**Scheme 3-3.** Direct attachment of the boronic acid moiety to the deoxyuridine base. Scheme 3 combines a number of different protocols (see text).

**Scheme 3-4.** One option for preparation of a MIDA-boronate ester directly from aminomethylphenyl boronic acid.
Scheme 3-5. Another option for the preparation of 4-aminomethylphenylboronic acid, MIDA ester from a commercially-available brominated phenylboronic acid through an azide intermediate.

Scheme 3-6. A third option for the preparation of 4-aminomethylphenylboronic acid MIDA ester from a commercially-available MIDA boronate starting material through a series of intermediates.

In Scheme 3-3, the starting material 5’-DMT-5-iodo-3’-acetyl-2’-deoxyuridine, 3-13, can be prepared as previously described in Scheme 3-1 or purchased. A trifluoroethyl group and a carbonyl group are attached at the 5 position of the uridine
base via the catalyst trans-bis(benzonitrile)dichloropalladium(II) using a procedure described by Ito et al to form **3-14**. Structure **3-14** and **3-22** from Scheme 3-4 would then be reacted in a room temperature solution to form structure **3-15**. Detritylation of **3-15** is accomplished according to a facile procedure by Pathak et al in which a 5% solution of trifluoroacetic acid is added and the sample is immediately purified by silica column chromatography. The final steps to phosphorylate **3-16** are carried out in a one-pot, three-step procedure detailed by Ludwig and Eckstein.

In Scheme 3-4, starting material **3-21**, 4-aminomethylphenylboronic acid, can be purchased. Refluxing **3-21** and MIDA in Toluene/DMSO with azeotropic removal of water via a Dean-Stark apparatus should prepare **3-22**, the MIDA boronate.

In Scheme 3-5, another option is presented for the preparation of 4-aminomethylphenylboronic acid, MIDA ester, from 4-bromomethylphenylboronic acid, which can be purchased. Refluxing **3-23** and MIDA in Toluene/DMSO with azeotropic removal of water via a Dean-Stark apparatus should prepare **3-24**, the MIDA boronate. The amine can then be synthesized through an azide intermediate, in this case by the Staudinger reaction.

In Scheme 3-6, a third option is presented for the preparation of 4-aminomethylphenylboronic acid, MIDA ester, from 4-hydroxymethylphenylboronic acid, MIDA ester, which can be purchased. To prepare **3-27**, triphenylphosphine and

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imidazole catalyze the substitution of the hydroxyl group for iodide in a reaction from Gillis and Burke.\textsuperscript{53} The amine can then be synthesized through an azide intermediate, in this case by the Staudinger reaction.

**Results and Discussion.** Scheme 3-3 was performed up to the preparation of 3-14 with good results, and the compound was set aside to await the synthesis of the 4-aminomethylphenylboronic acid, MIDA ester, which proved much more challenging than first assumed. The most direct method, outlined in Scheme 4, was attempted first. Numerous attempts using both a Dean-Stark apparatus and activated molecular sieves for azeotropic removal of water were made. Aqueous precipitation of a lovely white solid product, as expected from the protocol from Gillis and Burke\textsuperscript{54} was obtained, but the compound yielded confusing, unexpected \(^1\)H NMR spectra, as shown in Figure 3-15.


**Figure 3-15.** Stacked NMR spectra comparing the starting material in Scheme 3-4 to the putative product. Of note is the disappearance of the aromatic peaks in the left-hand region of the lower spectrum.

As shown in the figure, the spectrum of the starting material 3-21 contains peaks in the region 7.0-8.0 indicative of the aromatic protons of the phenyl ring. The spectrum of the putative product, however, does not contain these aromatic peaks. The same result was obtained whether the procedure was performed using a Dean-Stark apparatus or molecular sieves. A comparison of the spectrum of the experimental product with the published spectrum of a similar MIDA boronate ester to the expected product is informative (Figure 3-16).
Figure 3-16. Comparison of $^1$H NMR spectra for a similar MIDA boronate species as the expected product in Scheme 3-4 and the actual product spectrum.

A quartet of peaks characteristic of the folded MIDA group around 4 ppm is present in the mysterious product that is not present in either the boronic acid starting material or the free MIDA starting material. Such a similarity in this portion of the spectrum may suggest that the MIDA group did in fact form an ester with the boronic acid, but perhaps the boron-containing part of the molecule detached from the phenyl group. A possible explanation for this phenomenon may be similar to the “Petasis Reaction”, which is illustrated in Figure 3-17.
Although not strictly equivalent, both reactions involve an amine, carbonyl group, and aryl boronic acid species, and the ability of boron to form carbon-carbon bonds has long been fundamental to its use as a synthetic intermediate.

These unexpected results from Scheme 3-4 led to an alternate plan to synthesize 4-aminomethylphenylboronic acid, MIDA ester. Scheme 3-5 was the next to be tried. Upon refluxing 3-23 for the preparation of 3-24, a dark brown, thick suspension formed, and ¹H NMR spectroscopy revealed a broad, ill-defined mass of peaks in the region of 7.0-7.5 ppm, indicating the presence of possible polymerization. It was decided that 3-23 was too reactive to survive the protocol, and Scheme 3-5 was abandoned in favor of Scheme 3-6.

Despite being composed of more steps than the previous schemes, the execution of Scheme 3-6 was much more successful. Conversion of 3-27 to 3-29 proceeded smoothly, and the standard Staudinger reaction was performed in an
attempt to convert azide 3-29 to amine 3-30. Initially, the results were confusing. TLC and NMR analyses both confirmed the complete disappearance of starting material 3-29 from the reaction, but purification of the expected product from the reaction mixture proved extremely difficult. Because the amount of triphenylphosphine added to the reaction (3 equivalents) was so large, it was difficult to determine if the difficult-to-remove excess triphenylphosphine reagent and triphenylphosphine oxide by-product was interfering with the successful purification of the product, since its TLC spot overwhelmed a large portion of the plate.

Attempts were made to scavenge the excess triphenylphosphine species with high-loading Merrifield resin,\textsuperscript{55} and also to perform the reaction with water-soluble triphenylphosphine trisulfonate, neither of which proved successful. Finally, the reaction was performed with triphenylphosphine bound to a solid, bead-like resin support that could be easily filtered from the reaction solution. Performing the reaction in this way revealed that the majority of the boronic acid compound appeared to be bound as the iminophosphorane intermediate, and that only a small portion (<5\%) was hydrolyzing into the desired amine. Boiling the resin in acidic aqueous solution did nothing to liberate more product.

Although the yield of 4-aminomethylphenylboronic acid, MIDA ester, from Scheme 3-6 was extremely low, enough product was isolated to prepare a small amount of 3-15 according to Scheme 3-3. \textsuperscript{1}H NMR spectroscopy revealed that a small amount of 3-15 had been successfully made. At this time, it was decided that project efforts would be shifted to the synthesis of the boronic acid-modified phosphoramidite, as synthesis of a sufficient quantity of 3-15 was proving difficult. More efficient

\textsuperscript{55} Lipshutz et al. Efficient scavenging of \textsubscript{3}P and \textsubscript{3}P=O with high-loading Merrifield resin.
methods for conversion of the azide group to an amine, such as catalyzed hydrogenation reactions, will be explored by the group in the future.

**Experimental.** All chemicals were purchased from Sigma Aldrich and used without further purification unless otherwise noted. NMR spectroscopy was done on a Bruker 300 MHz spectrometer.

Scheme 3-3. Starting material 3-13 (1.52 mmol) was placed in a 500 mL heavy-walled modified Schlenk flask, along with 430 µl triethylamine, 1.1 mL trifluoroethanol, 12 mg trans-bis(benzonitrile)dichloropalladium(II), and 15 mL acetonitrile. The flask was purged with carbon monoxide for 5 minutes and then pressurized to 13 psi, sealed, and heated to 60 °C. The reaction was stirred overnight, and then cooled and vented. A black precipitate was observed. The reaction was filtered through glass wool and the solvent was evaporated. The yellow oily residue was partitioned between ethyl acetate and water, and the organic layer was washed with brine and dried with sodium sulfate. The product was purified using gravity silica column chromatography with 10% methanol/1% triethylamine/chloroform, and 1.22 mmol of off-white crystalline solid was isolated for an 80% yield.

Spectroscopic data for Scheme 3-3. 3-13: $^1$H NMR (300 MHz, CHCl$_3$-d$_1$) δ 8.20 (s, 3H), 7.45-7.25 (m, 13H), 6.87 (d, $J = 9$ Hz, 1H), 6.39-6.32 (m, 1H), 5.45 (d, $J = 6$ Hz, 1H), 4.17 (s, 1H), 3.81 (s, 6H), 3.45 (dd, $J = 9$, 3 Hz, 1H), 2.74-2.67 (m, 1H), 2.40-2.25 (m, 2H). 3-14: $^1$H NMR (300 MHz, CHCl$_3$-d$_1$) δ 8.68 (s, 3H), 7.46-7.21 (m, 13H), 6.83 (d, $J = 9$ Hz, 1H), 6.21-6.17 (m, 1H), 5.23 (d, $J = 6$ Hz, 1H), 4.39-4.14 (m, 2H), 4.20 (s, 1H), 3.80 (s, 6H), 3.45 (dd, $J = 9$, 3 Hz, 1H), 2.74-2.67 (m, 1H), 2.40-2.29 (m, 2H).
Scheme 3-4. Starting material 3-aminomethylphenylboronic acid 3-21 (3.31 mmol) was placed in a 25 mL roundbottom flask, along with MIDA (3.7 mmol), DMSO (2 mL) and toluene (5 mL). The flask was attached to a Dean-Stark trap filled with toluene. The trap was fitted to a water-cooled reflux condenser. The stirred solution was refluxed for 3 hours and then cooled and concentrated under high vacuum to a yellow oil residue. Water was added to the residue (10 mL) and a lovely white solid was precipitated. The solid was washed with 5 mL of additional water and filtered. Coevaporations (2X) with acetonitrile were performed to remove residual water and 50 mg of product were isolated.

Spectroscopic data for Scheme 3-4. 3-21: $^1$H NMR (300 MHz, DMSO-$d_6$) δ 8.54 (s, 2H), 8.11 (s, 2H), 7.85-7.34 (m, 4H), 3.97 (d, $J = 6$ Hz, 2H). 3-22: $^1$H NMR (300 MHz, DMSO-$d_6$) δ 4.12 (dd, $J = 72$, 18 Hz, 4H), 2.50 (s, 3H).

Scheme 3-5. Starting material 4-bromomethylphenylboronic acid 3-23 (3.31 mmol) was placed in a 25 mL roundbottom flask, along with MIDA (3.7 mmol), DMSO (2 mL) and toluene (5 mL). The flask was attached to a Dean-Stark trap filled with toluene. The trap was fitted to a water-cooled reflux condenser. The stirred solution was refluxed for 3 hours and then cooled and concentrated under high vacuum to a dark brown, thick oil residue. Water was added to the residue (10 mL) and a small amount of brown solid was precipitated. Coevaporations (2X) with acetonitrile were performed to remove residual water.

Spectroscopic data for Scheme 3-5. 3-23: $^1$H NMR (300 MHz, DMSO-$d_6$) δ 8.11 (s, 2H), 7.83-7.30 (m, 4H), 4.70 (s, 2H). 3-24: $^1$H NMR (300 MHz, DMSO-$d_6$) δ 8.32 (s, 2H), 8.00-7.57 (m, 4H), 7.43-7.29 (m, 3H), 4.27 (dd, $J = 63$, 18 Hz, 4H).
Scheme 3-6. Starting material 4-hydroxymethylphenylboronic acid **3-27** (3.3 mmol) was placed in a 100 mL roundbottom flask, along with triphenylphosphine (3.7 mmol), iodine (3.7 mmol) and imidazole (5 mmol). The dry reagents were ground together with the round end of a glass stir rod until a paste formed (10 minutes) which was dark violet in color. Anhydrous dichloromethane was added (30 mL) and the mixture was stirred under an argon atmosphere for 1 hour. Water was added to the flask (40 mL) and the layers were separated. The aqueous layer was extracted 2X with 40 mL of each of dichloromethane. The dichloromethane fractions were combined and washed with a saturated solution of sodium thiosulfate (5 mL) and brine (10 mL). The dichloromethane solution was dried with sodium sulfate, filtered, and concentrated to a yellow residue. The residue was washed 3X with 10 mL of diethyl ether and allowed to air dry to a fluffy yellow powder. The powder was dissolved in dimethylformamide and 10 mmol of sodium azide was added. The reaction was stirred at room temperature for 2 hours. Water (10 mL) was added and a white precipitate was formed. Ethyl acetate (20 mL) was added, the layers were separated and the aqueous phase was extracted 2X with 20 mL of ethyl acetate each. The solvent was evaporated under high vacuum to yellow oil **3-29**, which was purified by silica chromatography using 10% methanol/chloroform to yield 2.7 mmol of yellow wax, for an 82% yield. For the standard Staudinger reaction, azide **3-29** (1.1 mmol) was again dissolved in 10 mL of dimethylformamide in a 100 mL roundbottom flask, and triphenylphosphine (3.3 mmol) were added. The solution was stirred for 2 hours until the disappearance of starting material via TLC was observed. Water was added to the flask (10 mL) which caused the precipitation of a white solid. Ethyl acetate (10 mL), the layers were separated, and the aqueous phase was extracted 2X with 10 mL each of ethyl acetate. The organic fractions were combined and the solvent was evaporated to a white
crystalline solid. Silica chromatography was attempted with 10% methanol/chloroform. For the resin-bound Staudinger reaction, azide 3-29 (10 mmol) was dissolved in 10 mL of dimethylformamide in a 100 mL roundbottom flask, and beads of resin-bound triphenylphosphine were added (43 mmol, 3.4 g). The solution was stirred for 2 hours until the disappearance of starting material via TLC was observed. The resin was filtered through glass wool to remove the resin beads, and water (30 mL) and ethyl acetate (30 mL) were added. The layers were separated, and the aqueous phase was extracted 2X with 30 mL each of ethyl acetate. The organic fractions were combined and the solvent was evaporated to a colorless film of approximately 1 mmol, for a 10% yield.

Spectroscopic data for Scheme 3-6. 3-27: 1H NMR (300 MHz, CHCl₃-d₁) δ 7.48 (dd, J = 42, 6 Hz, 4H), 4.75 (d, J = 6 Hz, 2H), 3.84 (dd, J = 42, 15 Hz, 4H), 2.63 (s, 3H). 3-28: 1H NMR (300 MHz, CHCl₃-d₁) δ 7.45 (dd, J = 15, 6 Hz, 4H), 4.47 (s, 2H), 3.85 (dd, J = 42, 18 Hz, 4H), 2.60 (s, 3H). 3-29: 1H NMR (300 MHz, CHCl₃-d₁) δ 7.42 (dd, J = 66, 9 Hz, 4H), 4.35 (s, 2H), 4.01 (dd, J = 108, 15 Hz, 4H), 2.53 (s, 3H). 3-30: 1H NMR (300 MHz, DMSO-d₆) δ 7.31 (dd, J = 42, 9 Hz, 4H), 4.26 (d, J = 6 Hz, 2H), 4.21 (dd, J = 69, 18 Hz, 4H), 3.17 (s, 3H).

Scheme 3-3 continued. Structure 3-14 (0.3 mmol) was dissolved in 1.5 mL of dimethylformamide in a 10 mL roundbottom flask, along with structure 3-30 (1 mmol) from Scheme 6 and N,N-diisopropylethylamine (0.45 mmol). The solution was stirred at room temperature overnight. The mixture was concentrated under high vacuum and ethyl acetate (10 mL) was added. Water (10 mL) was added, the layers were separated, and the organic layer was washed with brine (10 mL). The organic layer was dried with sodium sulfate and concentrated to a yellow oil.
Spectroscopic data for Scheme 3-3 continued. **3-14:** $^1$H NMR (300 MHz, CHCl$_3$-d$_1$) δ 8.68 (s, 3H), 7.46-7.21 (m, 13H), 6.83 (d, $J = 9$ Hz, 1H), 6.21-6.17 (m, 1H), 5.23 (d, $J = 6$ Hz, 1H), 4.39-4.14 (m, 2H), 4.20 (s, 1H), 3.80 (s, 6H), 3.45 (dd, $J = 9$, 3 Hz, 1H), 2.74-2.67 (m, 1H), 2.40-2.29 (m, 2H). **3-15:** $^1$H NMR (300 MHz, CHCl$_3$-d$_1$) δ 9.18 (s, 3H), 7.41-7.14 (m, 17H), 6.85 (d, $J = 9$ Hz, 1H), 6.40-6.35 (m, 1H), 5.41 (d, $J = 6$ Hz, 1H), 4.25 (s, 1H), 4.70-4.57 (m, 4H), 4.02 (d, $J = 2$ Hz, 2H), 3.84 (s, 6H), 3.47 (dd, $J = 9$, 3 Hz, 1H), 2.70-2.55 (m, 1H), 2.45-2.33 (m, 2H).

**Synthesis of a Boronic Acid-Modified Phosphoramidite**

**Design.** The preparation of a boronic acid-modified phosphoramidite is described by Scheme 7.

![Scheme 3-7](image)

**Scheme 3-7.** Synthesis of a boronic acid-modified phosphoramidite from 2-cyanoethyl N,N-diisopropylchlorophosphoramidite and 4-hydroxymethylphenylboronic acid starting materials.

In Scheme 3-7, 2-cyanoethyl N,N-diisopropylchlorophosphoramidite is mixed with 4-hydroxymethylphenylboronic acid in extremely dry tetrahydrofuran, along with 3 equivalents of Hünig’s base. The modified phosphoramidite can then be introduced onto the DNA synthesizer and incorporated into a synthetic DNA strand.

**Results and Discussion.** The execution of Scheme 3-7 proceeded smoothly, despite the 4-hydroxymethylphenylboronic acid being only partially soluble in THF.
Between THF coevaporations for azeotropic removal of water from 3-32, the suspension was vigorously vortexed in attempts to break up the clumps of solid, but large clumps remained after the third coevaporation. In addition, oil 3-31 proved too viscous for aspiration with a microliter gastight glass syringe, so plastic pipette tips were used. Upon addition of acetonitrile to the reaction after introduction onto the DNA synthesizer, the phosphoramidite dissolved fully into solution. Despite these potential drawbacks, the yield of boronic acid-labelled primer after attachment of the moiety on the synthesizer was 30-70%.

**Experimental.** All chemicals were purchased from Sigma Aldrich and used without further purification unless otherwise noted. NMR spectroscopy was done on a Bruker 300 MHz spectrometer. Inductively-Coupled Plasma Mass Spectrometry was done by the Geology Department at the University of Colorado at Boulder. Electrospray Ionization (negative mode) Mass spectrometry was done by Dr. Ken Hill at Agilent, Boulder, CO.

Scheme 3-7. Starting material 3-32 was placed into a silanized J. Young tube (0.40 mmol) and THF that had been dried over sodium/benzophenone was transferred by vacuum to form a suspension. The suspension was vortexed, and the THF was removed under high vacuum. The procedure was repeated for a total of 3 coevaporations for the azeotropic removal of water. In a glovebox with an inert argon atmosphere, Hünig’s base (1.20 mmol) and 2-cyanoethyl N,N-diisopropylchlorophosphoramidite (0.36 mmol) were added to the J. Young tube, and the tube was sealed with the Teflon stopper and inverted several times. After recording the NMR spectra, the contents of the J. Young tube were transferred in a glovebox with an inert argon atmosphere to a commercially-available bottle designed
to attach to the DNA synthesizer. The DNA primer was then synthesized according to procedures outlined previously in chapter II.

Spectroscopic data for Scheme 3-7. 3-32: \( ^1H \) NMR (300 MHz, THF-\( d_8 \)) \( \delta \) 7.41 (dd, \( J = 45, 6 \text{ Hz}, 4\text{H} \)), 4.59 (d, \( J = 6 \text{ Hz}, 2\text{H} \)), 4.01 (dd, \( J = 69, 18 \text{ Hz}, 4\text{H} \)). 3-33: \( ^1H \) NMR (300 MHz, THF-\( d_8 \)) \( \delta \) 7.44 (dd, \( J = 42, 9 \text{ Hz}, 4\text{H} \)), 4.83-4.68 (m, 2H), 4.25 (dd, \( J = 105, 18 \text{ Hz}, 4\text{H} \)). 3-33: \( ^{31}P \) NMR (300 MHz, THF-\( d_8 \)) \( \delta \) 148 (s, 55,000 relative intensity), 13 (s, 10,000 relative intensity). 3-33 when attached to primer: ICP-MS found 7132 ppb phosphorous, 23 ppb boron. 3-33 when attached to primer: HRMS (ESI-) expected for triethylammonium salt 8570.55 amu, found 8570.28 amu.
CHAPTER IV

IN VITRO SELECTION AGAINST ISOLATED LIPOARABINOMANNAN AND INTACT CELLS OF MYCOBACTERIUM TUBERCULOSIS USING EXISTING PYRIDYL DEOXYURIDINE MODIFICATION AND NOVEL PHENYLBORONIC ACID MODIFICATION

Introduction

In the previous chapter, the synthesis of a phosphoramidite modified with a phenylboronic acid moiety was described. Synthetic attachment of the boronic acid component to the 5’ end of a DNA aptamer library via primer extension, while incorporating pyridyl-modified dUTP into the body of the strand provides the basis for arrangement 2 (see Figure 4-1).
Figure 4-1. Summary of three binding scenarios involving pyridyl and/or boronic acid-modified aptamers binding to LAM. In Arrangement 1, the pyridyl groups are scattered along the body of the aptamer via modification of deoxyuridine, and form hydrogen bonds to the sugar residues in LAM. In Arrangement 2, the pyridyl groups are also scattered along the body of the aptamer via modification of deoxyuridine, and form hydrogen bonds to the sugar residues in LAM, but in addition, they form an adduct with a boronic acid modification on the 5' end of the aptamer, which forms covalent bonds with the vicinal diols on the sugar residues of LAM. In Arrangement 3, the deoxyuridine bases are modified with a boronic acid moiety, which forms covalent bonds with the vicinal diols on the sugar residues of LAM (at basic pH).

Such an arrangement should facilitate aptamer binding to carbohydrates such as LAM. The catalysis of covalent bond formation by an aptamer relegates it to its own distinct class of molecules, hereafter referred to as reactamers.

As previously mentioned, unsuccessful attempts were made to discover aptamers modified with hydrophobic modifications to M. tuberculosis ManLAM. The PCR traces during these failed selections indicated that the aptamers eluted from their
target were not enriching with respect to a negative control that did not contain ManLAM. To assess whether addition of the boronic acid molecule, as well as the pyridyl modifications, would enable the isolation of reactamers to ManLAM, two selections will be performed using extracted ManLAM and intact, inactivated \textit{M. tuberculosis} cells. In order to determine the contribution of the boronic acid to binding, parallel selections were run in which pyridyl modifications were incorporated into the body of the DNA strands, but no boronic acid moieties were attached to the 5’ ends.

\textbf{Selections}

\textbf{Design.} Because several parameters had previously been explored during the \textit{M. smegmatis} selection in chapter II, much of the general process of selection remained the same, with a few notable differences.

Selection Against Extracted ManLAM. The first selection, in which extracted ManLAM would serve as the target, required that the ManLAM be tagged with a biotin handle, with which it could be isolated from solution and selectively manipulated during a partitioning step. Scheme 4-1 describes the addition of a biotin tag to ManLAM.
Scheme 4-1. Addition of a biotin tag to ManLAM via sodium periodate oxidation and hydrazide chemistry.

The covalent coupling of a biotin tag to ManLAM is accomplished in two steps. In the first, vicinal diols on some sugar residues are oxidized to aldehydes with sodium periodate, in the dark at 0 °C. To preserve as much of the LAM structure as possible, the oxidizer concentration and duration of the step is optimized so that only a few sugar residues on each LAM molecule are oxidized. Next, a biotin-hydrazide conjugate is incubated with the LAM at room temperature for several hours, during which the hydrazide group reacts with any available aldehydes.56

In addition to the biotinylated extracted ManLAM, a synthetic fragment made by the Chatterjee lab at Colorado State University was also targeted in parallel selections. The structure of the fragment was intended to mimic a branch point on the ManLAM molecule comprised of arabinose linkages unique to LAM and not likely to be found in

the background of patient samples. The fragment, which was biotinylated during synthesis, was termed Ara-6 and its structure is shown in Figure 4-2.

![PD Ara6-Biotin](image)

**Figure 4-2.** Structure of Ara-6 fragment synthesized by the Chatterjee lab at Colorado State University. Figure is also by Chatterjee lab.

After biotinylation of the ManLAM, the selection can be performed as described in chapter II with the modified partitioning step illustrated in Figure 4-3.
Figure 4-3. Partitioning step for a selection in which extracted ManLAM is the target. The ManLAM is biotinylated and bound to streptavidin-coated magnetic beads to facilitate washing and separation from solution.

In contrast to the whole-cell selections, the individual ManLAM molecules will be bound to streptavidin-coated magnetic beads via their biotin tag during partitioning. Once bound to the beads, the ManLAM molecules can be easily separated from solution and washed by placing the sample tube into a magnetic holder that collects the beads to one side of the tube so that the supernatant can be easily removed. To ensure that PCR amplification signal represents reactamers that are indeed binding to LAM and not the streptavidin on the beads, a counter-selection step was introduced in which the DNA pool was incubated with beads that did not contain LAM. The supernatant containing sequences that did not bind the beads were
then removed and used for the remainder of the selection round. To monitor the effectiveness of the counter-selection, control experiments would be included in each selection round in which the beads did not contain any bound LAM, but underwent the same incubation, washing and elution steps with half the pool of DNA. The control experiments would be subjected to PCR and the amplification signal difference would be used to measure the progress or decline of the selection.

The final difference for reactamer selections versus the previous selection in chapter II is the formation of covalent bonds between the boronic acid and the vicinal diols of the sugar residues. Because boronic acids form stable covalent bonds to sugars at basic pH (see chapter III), the elution step at pH 12 described for the \textit{M. smegmatis} selection cannot be used. Instead, an elution step in which the biotin-streptavidin bond holding the LAM molecule to the bead will be broken by resuspending the beads in deionized, unbuffered water at heating to 90 °C. Such conditions have been demonstrated to efficiently break the otherwise impervious biotin-streptavidin interaction.\textsuperscript{57}

Selection Against Intact \textit{M. tuberculosis} Cells. The reactamer selection against intact \textit{M. tuberculosis} cells is subject to the same elution requirements as above, but because there exists no biotin-streptavidin coupling, the bound sequences must be eluted under acidic conditions. Like the extracted LAM selection, a counter-selection step will be introduced in which the DNA pool is incubated with \textit{M. smegmatis} cells, and the supernatant containing the sequences that do not bind are removed and used for the selection round. The partitioning step can otherwise be accomplished similarly to the \textit{M. smegmatis} selection in that the cells can be pelleted or filtered during the

\textsuperscript{57} Holmberg et al. The biotin-streptavidin interaction can be reversibly broken using water at elevated temperatures. \textit{Electrophoresis}. \textbf{2005}, \textit{26}, 501-510.
partitioning step; however, because \textit{M. tuberculosis} is virulent, the cells must first be inactivated in order to safely carry out the necessary enzymatic, washing, and sequencing steps of the selection. Inactivation of bacteria is commonly accomplished via gamma irradiation, and it has been shown that doses of 2.4 megarads kills \textit{M. tuberculosis} to a degree of certainty of $10^{20}$. Alamar Blue tests are used to ensure that the cells are no longer metabolically active. Such treatment renders the cells safe to manipulate in biosafety level 1 conditions.\textsuperscript{58}

\textbf{Results and Discussion.}

Selection Against Extracted ManLAM. Selection progress was monitored by recording the RT-PCT traces (Appendix A). Initially, the difference between amplification cycles for boronic acid-modified versus pyridyl-only pools was substantial, and both pools also amplified much earlier than the control beads, which did not contain any bound ManLAM, as can be seen in Figure 4-4.

\textsuperscript{58} SOP PP004. Gamma Irradiation of \textit{M. tuberculosis}. Colorado State University.
Figure 4-4. RT-PCR traces for round 2 of the selection against extracted ManLAM. Blue traces correspond to the boronic acid-modified pool eluted off of the ManLAM on the beads. Red traces represent the boronic acid-modified pool eluted off of the Ara-6 fragment on the beads. Green traces represent the pyridyl only-modified pool eluted off of the ManLAM on the beads. Light blue, pink, and light green traces, respectively to their darker colors, correspond to the negative control pools eluted off of beads not containing any carbohydrate target. Brown signifies a PCR blank containing the PCR Mix (see chapter IV, experimental section) without DNA template.

Such large differences between the traces suggests that the pyridyl-only aptamers were binding the bound LAM significantly better than they were the streptavidin bead background, and that the boronic acid-modified reactamers were binding the LAM significantly better than the pyridyl-only aptamers. As the rounds progressed and selection pressure was increased, however, all traces began to collapse toward the control, although the boronic-acid traces were always earlier than the pyridyl. Several rounds were tried in which the concentration of LAM, competitors,
and incubation times were changed gradually, but the traces persisted in eventually collapsing toward the control with only one or two cycles of difference.

This phenomenon is commonly observed during aptamer selections due to the presence of a solid support that is capable of interacting with the pool. Eventually, despite the addition of counter-selection steps, the background binding can overwhelm binding to the intended target, and the selection cannot be progressed further. For this reason, a pool was chosen for sequencing that, although only five consecutive rounds from the beginning of the selection, still displayed significant separation between the control and experimental traces. The pool had been subjected to the lowest LAM concentration, highest competitor concentration, and shortest incubation period tested at which the PCR traces retained significant separation. The five consecutive rounds chosen as the most successful are outlined in Table 4-1.

<table>
<thead>
<tr>
<th>Round #</th>
<th>Incubation Time (hr.)</th>
<th>[ManLAM] (nM)</th>
<th>Competitor Buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>24</td>
<td>250</td>
<td>No</td>
</tr>
<tr>
<td>2</td>
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<td>250</td>
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</tr>
<tr>
<td>3</td>
<td>16</td>
<td>250</td>
<td>Yes</td>
</tr>
<tr>
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<td>4</td>
<td>70</td>
<td>No</td>
</tr>
<tr>
<td>5</td>
<td>16</td>
<td>250</td>
<td>No</td>
</tr>
</tbody>
</table>

Table 4-1. Round parameters for selection against extracted ManLAM.
The Competitor Buffer for this selection contained the same proteins as in chapter II for the *M. smegmatis* selection (1 µM each prothrombin and casein, 0.3% w/v human serum albumin) with the addition of 1 µM yeast mannan and 10 µM each mannose and glucose. The addition of these carbohydrate competitors was intended to assist in the selection of reactamers specific for LAM that would not bind unrelated sugar residues.

After subjecting round 5 to Sanger sequencing, the sequences were classified into families as for the PILAM selection (see Appendix B). As in chapter II, attempts were made to screen the sequences for affinity to ManLAM by gel-shift assay, but they proved unsuccessful. Because the buffer used for the assay was Tris-borate, it is possible that the high concentration of borate served as a competitor for the binding of the reactamer to the LAM. Alternative buffer systems were tried, but proved challenging as the buffer could not be recirculated.

In order to characterize the sequences in a manner more similar to how they would be used in a diagnostic test, enzyme-linked immunosorbent assays (ELISAs) were used to screen several representative sequences. This assay was performed by the project collaborators at Colorado State University, Chatterjee lab, with reactamers provided by the Eaton lab. The results of the initial screening ELISA are presented in Figure 4-5.
Figure 4-5. Screening reactamer isolates from extracted ManLAM selection using ELISA. Several reactamer sequences (R#) were bound to LAM-coated plates, along with a LAM antibody (908.1b) positive control. Results were assayed by an alkaline phosphatase colorimetric detection method. LAM was coated onto the plates from either a buffer solution or urine, and proteinase K (pk) was used to treat one of the urine/LAM solutions prior to plate coating. Figure from Chatterjee lab at Colorado State University.

From Figure 4-5 it can be seen that the reactamer sequences bound LAM to a similar extent as the positive control LAM antibody, and that sequence 43, hereafter referred to as R43, bound marginally better than the other sequences.

To further characterize the sequences, FPA was performed on four isolates, R3, R21, R36 and R70. All four binding curves obtained from this assay appeared very similar. A representative example is given in Figure 4-6.
Figure 4-6. Binding curve for R21 obtained via FPA. The reactamer concentration was held constant at 1 nM while increasing amounts of either ManLAM or PILAM were added. The samples were allowed to incubate at room temperature for 45 minutes to allow binding equilibrium to be reached.

The FPA assay was restricted to a lower detection limit of about 1 nM of the tagged DNA due to the reduction of light intensity through the polarization filters. Because of this, the DNA concentration is too high for a $K_d$ to be calculated; however, from the results it is reasonable to conclude that the $K_d$ is most likely in the low nanomolar range, and that the reactamer does not significantly bind PILAM at concentrations up to 60 nM.

Although the initial ELISA and FPA results were promising, obtaining reproducible binding proved challenging. In fact, subsequent ELISAs done at Colorado
State University, as well as in-house, infrequently yielded positive results; the signal for the reactamer wells was at times indistinguishable from the negative controls. As in the case of WCMS35 (chapter II), it was hypothesized that binding conditions were not optimal for the sequences. All characterization tests had been carried out in the same buffer as the selection rounds. In an attempt to optimize binding and improve the irreproducibility, various parameters were systematically altered.

One buffer condition that was hypothesized to affect binding was salt concentration. Studies done by other groups on the thrombin aptamer indicated that decreasing the sodium chloride concentration of the original selection buffer had a positive effect on the binding affinity of the aptamer, presumably due to lowered shielding effects.\(^5^9\) ELISAs were attempted using R43 in selection buffer that contained 0%, 20%, 40%, 60% or 80% the original sodium chloride concentration in which the reactamers were selected. None of the altered buffers produced an increase in binding reproducibility.

Another putative explanation for the inconsistent results was proposed in which the pyridyl groups were causing aggregation of the reactamers due to the presence of trace amounts of heavy metal cations. Pyridine-containing molecules have been shown to be chelators of metal cations,\(^6^0,6^1\) and chelation could induce misfolding or aggregation of clumps of reactamers, causing spurious binding data. To eliminate this


possibility, ethylenediaminetetraacetic acid (EDTA) was added to the assay buffer at concentrations of 1 mM or 2.5 mM. Because the binding affinity for EDTA for heavier metal cations is many orders of magnitude larger than for magnesium, the 5 mM magnesium component of the selection buffer would not interfere with chelation of trace heavy metal cations by EDTA. Addition of EDTA to the assay buffer did not, however, improve reactamer binding.

In addition to the parameters above, an exhaustive screening of other variables defining the binding conditions was attempted. Factors such as Tween 20 surfactant concentration, buffer age, 96-well plate composition, incubation time, washing stringency, incubation temperature, DNA concentration and rate of cooling during folding, and plate coating techniques were examined, without success. To account for the possibility that the ELISA plate coating distorted the three dimensional motifs of the LAM molecules beyond recognition of the reactamer, cell-binding experiments similar to those in chapter II were attempted while varying the above parameters, but binding signal was again unreliable. Further exploration and optimization of binding conditions is needed during future work.

Selection Against Intact \textit{M. tuberculosis} Cells. The selection was continued for 15 rounds, and was monitored by recording the RT-PCR traces (Appendix A). Again, similar to the extracted ManLAM selection, the PCR traces suggested that the boronic acid-modified reactamers and the pyridyl-only aptamers both bound the \textit{M. tuberculosis} cells with higher affinity than the closely-related \textit{M. smegmatis} cells. In fact, a strong PCR amplification signal was still retained even under seemingly difficult binding conditions, including short incubation times, long washing times, high
concentrations of salts and competitors, and low DNA concentrations. Table 4-2 summarizes the parameters for each round of selection.
<table>
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<th>Round #</th>
<th>Incubation Time (min.)</th>
<th># of Cells</th>
<th>Competitor Wash</th>
<th>Wash Duration (hr.)</th>
<th>[DNA] (nM)</th>
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<td>10,000</td>
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<td>200</td>
</tr>
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</table>

**Table 4-2.** Round parameters for selection against intact *M. tuberculosis* cells. Composition of Competitor Buffer column entries designated with a ‘+’ is described in the text.
The *M. tuberculosis* cells as obtained were present as large aggregates of bacteria that settled immediately from suspension. Although clumping during culture is characteristic of mycobacteria in general, the *M. tuberculosis* samples were much more aggregated than their *M. smegmatis* relatives. As such, reducing the number lower than about 10 million cells proved risky, as much smaller numbers than intended may have been in the sample due to the cells not being monodispersed. However, every other parameter possible was pushed to extreme limits without disappearance of the PCR amplification signal.

By round 16, the incubation time of the DNA with the cells was a mere 5 minutes, at a reduced concentration of 10 nM DNA. For the majority of the rounds, the cells were washed three times with selection buffer, and then allowed to remain in solution overnight to allow the dissociation of weak binders. The next day, three additional washes were performed before elution of the bound sequences. In addition, until round 13, the same Competitor Buffer used in the previous selection was added during incubation. This solution was composed of 1 µM each prothrombin, casein, and yeast mannan, 0.3% w/v human serum albumin, as well as 10 µM each of mannose and glucose. Since the selection was progressing well, during round 14 additional components were added to the competitor buffer, including 20 mM additional potassium chloride, 81 mM additional sodium chloride, 160 mM urea, and 1 µM uromodulin. These additions were designed to more closely simulate the composition of urine, from which the reactamers were envisioned to isolate excreted ManLAM from patients in eventual diagnostic assays. Initially (see Appendix A), the PCR amplification signal difference between the *M. tuberculosis* cells and the control
M. smegmatis cells was greatly reduced after the additions to the Competitor Buffer, but the signal difference was observed to reappear again during round 15.

Round 17 was sequenced, this time by Next Generation sequencing, to provide greater detail than Sanger sequencing was able. In the case of Sanger sequencing, between 20-48 sequences were returned for each pool, as opposed to around 2 million with the Next Generation sequencing. The greater amount of data provided by the Next Generation sequencing constructs a more complete picture of the convergence of the selection. The boronic acid-modified and pyridyl-only pools were each submitted for sequencing, and about 2 million reads were returned for each. The 1,000 most populous sequences were then analyzed via DOSA and families were identified (Appendix B). Similarities among families, and even identical sequences, were observed between the two pools, perhaps indicating convergent evolution toward particular binding motifs. Again, however, cell-binding assays indicated that the binding of the isolates was sporadic and irreproducible. Future work is needed to explain the discrepancy between promising PCR trace results during the selections and the subsequent inconsistent binding of isolates.

In summary, an aptamer for PILAM was isolated, and it was shown that selection of aptamers against ManLAM with the commonly used hydrophobic modifications proves very challenging. A scheme for the synthesis of a boronic acid modified nucleotide was worked out, and results were obtained that show that introduction of basic or a novel boronic acid modification appears to improve the ability of DNA to bind ManLAM according to the data provided by the PCR traces.
Overall, the project represents initial and promising steps toward boronic acid-based reactamers. Although encouraging data has been generated by a multitude of different characterization methods, it has proven difficult to get the binding to be reproducible, which may be caused by a low percentage of reactamers in the pool that are capable of binding. Initially, it was imagined that introduction of a boronic acid or pyridine modification into a DNA strand might cause the reactamer to bind nonspecifically to every carbohydrate. However, the PCR trace data indicates that this has not been the case, and that reactamers can be easily selected that bind ManLAM with affinities many orders of magnitude greater than PILAM. In light of this, further work can be focused on making the boronic acid group more reactive toward sugars with the addition of electron-withdrawing groups to the phenyl ring, which may cause a greater percentage of the pool to bind.

**Experimental.** All chemicals were purchased from Sigma Aldrich unless otherwise stated. DNA phosphoramidite chemistry synthesis reagents were purchased from Glen Research, Sterling, VA. KOD XL polymerase was purchased from EMD Millipore, Darmstadt, Germany. Terminal transferase and dNTPs was purchased from New England Biolabs, Ipswich, MA. Alexa tags, Sybr Green I Nucleic Acid Stain, TOPO cloning kit and Dynabeads were purchased from Invitrogen, Carlsbad, CA. Biotin-LC-Hydrazide and HABA-avidin assay kit were purchased from Pierce Protein Products, Rockford, IL. All custom oligos that were not synthesized in-house were purchased from Integrated DNA Technologies, Coralville, IA. Pyridyl-modified dUTP had been previously synthesized by the Eaton laboratory. *M. tuberculosis* cells and extracted ManLAM samples were a generous gift from the Chatterjee lab, Colorado State University, Fort Collins, CO, and cultures were fully grown and irradiated before
delivery. Sanger sequencing was done by SeqWright, Houston, TX. Next Generation sequencing was done by the Biofrontiers Institute, University of Colorado at Boulder, Boulder, CO. RT-PCR was done on an iQ5 Bio-rad RT-PCR instrument, Bio-rad, Hercules, CA. FPA analysis was done on a Fluorolog-3, Horiba Jobin Yvon, Kyoto, Japan. DNA synthesis was done on a Model 394 DNA/RNA Synthesizer, Applied Biosystems, Inc., Foster City, CA. Gels were scanned using a Fujifilm FLA-5100 gel scanner, Fujifilm, Tokyo, Japan.

DNA synthesis. DNA synthesis-grade acetonitrile was further dried for 1-2 days with activated molecular sieve packs. Phosphoramidite bottles (1 gram) were diluted to 100 mM and dicyanoimidazole was prepared at 0.25 M with the dried acetonitrile. Dichloroacetic acid (13 mL) was transferred into a heat-dried synthesizer bottle and approximately 500 mL of anhydrous dichloromethane was canulated to the bottle under argon. Both the dicyanoimidazole and dichloroacetic acid solutions were dried for 1-2 days with activated molecular sieve packs. Oxidizer, Cap A, and Cap B solutions were used as purchased without further drying. A bottle for the random region was prepared by hand-mixing the phosphoramidites in a glove box under an argon atmosphere. The random region bottle was corrected for phosphoramidite incorporation bias by mixing the phosphoramidites in the ratio 0.28 A: 0.20 G: 0.32 C: 0.20 T. All bottles were exchanged onto the synthesizer and a 90N random library was synthesized with a 40N random region flanked by primer binding regions. The DNA was cleaved from the column with fresh ammonium hydroxide for 1 hour at room temperature, and then placed in a glass vial at 55 °C for 18 hours. The ammonium hydroxide was cooled, evaporated, and the resulting solid was subjected to PAGE purification.
Polymerase Chain Reaction. PCR reactions were performed with the following 5X PCR Mix: 10 µM each forward and reverse primers, 25 mM magnesium chloride, 5X as-purchased KOD XL buffer, trace amount of Sybr Green I Nucleic Acid stain, 1 mM each dNTP, and 0.075 U/µl KOD XL polymerase. To 48 µl of sample were added 12 µl of 5X PCR Mix, and the reaction was cycled as follows; cycle 1: 96 °C for 15 seconds, 55 °C for 10 seconds, 71 °C for 30 minutes; cycle 2-31: 96 °C for 15 seconds, 71 °C for 1 minute. The PCR reaction was monitored in real time and the traces were recorded.

Primer Extension. Primer extension reactions were performed with the following 1X Primer Extension Mix: 2.5 µM forward primer, 120 mM Tris-HCl at pH 7.8, 10 mM potassium chloride, 6 mM ammonium sulfate, 7 mM magnesium sulfate, 0.1% Triton X-100, 0.1 mg/mL bovine serum albumin, 0.5 mM each dNTP, and 0.015 U/µl KOD XL polymerase. Biotinylated PCR product or single-stranded synthetic, biotinylated DNA was incubated with an appropriate quantity of streptavidin-coated, magnetic MyOne Dynabeads with shaking at 1000 rpm for 15 minutes. The beads were washed 3X with dH₂O and if double-stranded PCR product was used, 20 mM sodium hydroxide was applied for 30 seconds. The beads were washed again 3X with dH₂O and the Primer Extension Mix was added. The reaction was shaken at 1000 rpm at 71 °C for 30 minutes. After incubation, the beads were again washed 3X with dH₂O and and 85 µl of 20 mM sodium hydroxide was added. After 30 seconds, 80 µl of sodium hydroxide are removed and neutralized with 20 µl of 80 mM hydrochloric acid, 25 mM HEPES, and 0.05% Tween 20.

Biotinylation of ManLAM. ManLAM (6 nmol) was dissolved in 250 µl of 100 mM sodium acetate, pH 5.5, and chilled on ice. Sodium periodate was added to a
concentration of 1 mM, and the reaction was incubated on ice for 5 minutes in the dark. After incubation, sodium sulfite (90 mM) was added and the reaction was incubated at room temperature for 10 minutes. The reaction was buffer exchanged into PBS, pH 7.4, using 10,000 MWCO Microcons. Biotin-LC-Hydrazide was dissolved into DMSO at a concentration of 50 µM and the stock was added to the LAM sample to a concentration of 15 µM. The reaction was incubated at room temperature for 3 hours, and then dialyzed into dH2O for 2 days. Quantitation of biotinylation was accomplished by the HABA-avidin fluorescence assay via the manufacturer’s instructions and was found to be 80.5%.

Selection Incubation and Partitioning. Approximately 10 pmol of modified primer extension product was buffered in 40 mM HEPES, 102 mM sodium chloride, 5 mM potassium chloride, 5 mM magnesium chloride, and 0.05% Tween 20 at pH 7.5. The modified DNA was folded by heatcooling with the following sequence: 96 °C for 5 minutes, 70 °C for 5 minutes, 48 °C for 5 minutes, and 37 °C for 5 minutes. The pool was incubated for 1 hour with either 10 µl of magnetic beads or 10 million M. smegmatis cells, and the supernatant was removed and used for the selection round. In later rounds (see Tables 4-1 and 4-2) Protein Competitor Buffer was added to 1 µM prothrombin, 1 µM casein, 0.3% w/v human serum albumin, and 1 µM yeast mannan for the selection against extracted ManLAM, with the addition of 20 mM additional potassium chloride, 81 mM additional sodium chloride, 160 mM urea, and 1 µM uromodulin for the selection against intact M. tuberculosis cells. Partitioning for the extracted ManLAM selection was accomplished by adding an appropriate number of MyOne Dynabeads to the sample according to the manufacturer’s instructions. The sample was shaken at 1000 rpm for 15 minutes, and the beads were washed by
placing the sample tube into a magnetic holder and aspirating the supernatant. The bound sequences were then eluted by breaking the biotin-streptavidin bond by resuspending the beads in deionized water and heating to 90 °C for 5 minutes. The supernatant was then quickly aspirated and cooled. During partitioning for the selection against intact *M. tuberculosis* cells, the cells were kept suspended by shaking at 1000 rpm. Partitioning was accomplished by pelleting the cells via centrifuge at 1000 rpm and aspirating the supernatant. After washing, the bound sequences were eluted for 5 minutes with 5 mM hydrochloric acid, and then neutralized with 20 mM sodium hydroxide.

Cloning and Sequencing. For Sanger sequencing, cloning of sequences was accomplished by sending the sequencing company a TOPO vector containing the inserted DNA pool created according to the manufacturer’s instructions. The company performed cloning, colony picking, plasmid extraction and sequencing. After sequencing, the company shipped back the plasmids to facilitate screening of isolates. Plasmids were subjected to PCR and primer extension to create a matrix of modified DNA aptamer isolated sequences. For Next Generation sequencing, bar codes were incorporated into each DNA pool through modified PCR primers and sent to the company.

Gel-Shift Assay. Modified DNA aptamer isolates were folded according to the heatcooling protocol described above. ManLAM (0.125 µg) was incubated with 1 pmol of each DNA sequence for 1 hour. The reactions were run, along with an additional 1 pmol of DNA without ManLAM, on a native 8% polyacrylamide gel at 150 V in Tris-borate buffer. The gels were visualized by staining with Sybr Green I Nucleic Acid Stain and scanning on a gel scanner with a 473 nm laser.
Cell-binding Assay. Modified DNA aptamer isolates were tagged in 14 µl of water, to which was added 2 µl 10X as-purchased terminal transferase buffer, 2 µl 10X as-purchased cobalt chloride solution, 1 µl of 1 mM Alexa 488-dUTP, and 1 µl terminal transferase. The reactions were incubated at 37 °C for 1 hour, 14 mM EDTA was added, and the aptamers were purified by dialysis with a 10 kDa membrane cassette. The aptamers were folded according to the heatcooling protocol described above. *M. smegmatis* and *M. tuberculosis* cells were incubated with 1 pmol of each sequence for at least 30 minutes, with shaking at 1000 rpm, at room temperature. The cells were washed by pelleting 3X with selection buffer and the fluorescent signal of each fraction was recorded on a plate reader.

Fluorescence Polarization Anisotropy. FPA experiments were performed with aptamers folded and tagged as described above. Reactamers were filled into a quartz cuvette at a concentration of 10 nM and a volume of 250 µl in selection buffer. ManLAM was gradually added to the cuvette and pipette-mixed. After each addition, the sample was incubated for 45 minutes at room temperature, and the anisotropy value was recorded.

Enzyme-linked Immunosorbent Assay. In house ELISAs were done in the following general manner. ManLAM (0.5 µg) was dissolved in dH2O (50 µl) and applied to the well of a 96-well plate. The solution was allowed to air-dry overnight and the wells were blocked with either 1% BSA in PBS, pH 7.4 or fat free milk for 1.5 hours. The blocking solution was discarded, the wells were rinsed once with selection buffer and the biotinylated reactamer or aptamer sample (15 nM) was added. The plate was incubated at room temperature for 1-4 hours and the wells were washed three times with buffer. Neutravidin-horseradish peroxidase conjugate (1:30,000 dilution from
manufacturer stock) was applied and allowed to incubate for 30 minutes at room temperature. The wells were washed three times and SuperSignal Pico chemiluminescent substrate was mixed with a peroxide solution (1:1) in a kit provided by the manufacturer. The substrate solution was applied to the wells and luminescent signal was recorded by the plate reader within 5 minutes.
BIBLIOGRAPHY


Lipshutz, B.H.; Blomgren, P.A. Efficient scavenging of Ph₃P and Ph₃P=O with high-loading Merrifield resin.


Chapter II: Selection for a PILAM Aptamer Using Intact Mycobacterium Smegmatis Cells.

In all cases, red traces correspond to the aptamer pool eluted off of the M. smegmatis cells. Blue traces represent a PCR blank containing the PCR Mix (see chapter II, experimental section) without DNA template.

Round 1.
Round 2.

![PCR Amplitication vs Cycle: Date 29-Jan-11 2344.0.wm.png](image1)

Round 3.

![PCR Amplitication vs Cycle: Date 29-Jan-11 1102.0.wm.png](image2)
**Round 4.**

![Graph for Round 4.]

**Round 5.**

![Graph for Round 5.]
Round 6.

Round 7.
Round 8.

![Graph of Round 8](image1.png)

Round 9.

![Graph of Round 9](image2.png)
Round 10.

Chapter IV: Selection for a Reactamer Using Extracted ManLAM from Mycobacterium Tuberculosis Cells.

In all cases, blue traces correspond to the boronic acid-modified pool eluted off of the ManLAM on the beads. Red traces represent the boronic acid-modified pool eluted off of the Ara-6 fragment on the beads. Green traces represent the pyridyl only-modified pool eluted off of the ManLAM on the beads. Light blue, pink, and light green traces, respectively to their darker colors, correspond to the negative control pools eluted off of beads not containing any carbohydrate target. Brown signifies a PCR blank containing the PCR Mix (see chapter IV, experimental section) without DNA template. Yellow traces, when present, represent traces for unrelated PCR experiments that were run on the same plate.

Round 1.

A PCR trace for round 1 was not recorded, as the PCR was done directly off of the beads, which masked the signal.
Round 2.

Round 3.
Round 4.

Round 5.
Chapter IV: Selection for a Reactamer Using Intact Mycobacterium Tuberculosis Cells.

In all cases, blue traces correspond to the boronic acid-modified pool eluted off of the *M. tuberculosis* cells. Green traces represent the pyridyl only-modified pool eluted off of the *M. tuberculosis* cells. Light blue and light green traces, respectively to their darker colors, correspond to the negative control pools eluted off of *M. smegmatis* cells. Brown signifies a PCR blank containing the PCR Mix (see chapter IV, experimental section) without DNA template. Yellow traces, when present, represent traces for unrelated PCR experiments that were run on the same plate.

**Round 1.**

![Graph](PCR Amplification vs Cycle: 2016-07-15 13-09-00-00.png)

In the PCR traces above, the additional orange, turquoise and grey traces correspond to aptamer pools modified with tryptoaminyl, napthyl, and benzyl, respectively.
Round 2.

Round 3.
Round 4.

![Graph showing data over cycles]

Round 5.

![Graph showing data over cycles]
Round 6.

Round 7.
Round 8.

Round 9.
Round 10.

Round 11.
*Round 12.*

![Graph](PCR Amplification vs Cycle 12.png)

*Round 13.*

![Graph](PCR Amplification vs Cycle 13.png)
Round 14.

Round 15.
**Round 16.**

![Graph for Round 16](image1.png)

**Round 17.**

![Graph for Round 17](image2.png)
## Appendix B

### Categorization of Sequences Using Daughter of Sequence Alignment (DOSA) Analysis

#### Chapter II: Selection for a PILAM Aptamer Using Intact Mycobacterium Smegmatis Cells.

**Family 1**

| WCMS25  | AGC CCC GAT AAG ATC CGC CTT C CC TTT T TT ACC ACA CTC A |
| WCMS42  | TCG TGA CAT TAG ATC GTA AAT TGA C CC TTT CCC AAT TGC G |
| WCMS19  | ATT TCC CGA CCT CTT C CC TTT T TC CCC ACC ATC TCC TCG |
| WCMS3   | TAC TCC CCT TT A CCT TTT TCG CCA AAT ATA CCC TCA TCA T |
| WCMS39  | CTC AAC CCT TT G CAT AAC CTT TCC CCT ATT TTC ACC ACC ACT C |
| WCMS9   | CTC AAT CCT CCT CCT C CC TTT ATG CCT TAC TCT TCA CCC TTT |
| WCMS41  | C CC TTT CTA CCC T CC TTT T TA ACC CCA ATT ATA CCC CT |
| WCMS11  | TCC CGC CTT AAC TTC TCA CAC C CC TTT T TC AAC CTC CTC |
| WCMS29  | TTT GCC GTA TAC CCT CCA TTT ACC AC C CTT TT G ACC TCC |
| WCMS33  | GAT CCC CTC CCT TT A CGC TTT TAT ACC TAC TCC CCA CTT |
| WCMS17  | CCC TCC TTA CCT TT TCG CCT TAT CCT GAT CCG TTC AA |
| WCMS23  | TTT TTA ACC GCC TTA TAC CTC CCC C CC TTT CAG GCC TCA |
| WCMS12  | CCT TTT CCC CCT ATC CAA GCT TCC CCC CTC CTC CTA ATT |
| WCMS10  | TTC ACC TAT TCC C CC TTT T CC CGG CCC TCT CCC TTA TTG T |
| WCMS44  | GTT ATA AAC CCC TTC TTC TCC CCC C CC TTT T AC CAC CCC TGT |
| WCMS2   | CCT TTT TAA AAT CCC CCT CTT CCA CAT GCC CTG GCC TGC A |
| WCMS24  | TTC A CC TTT T TA CAG CAC CTA TCC CCC CCT AGC TTC TG |
| WCMS34  | TTT ATC C CC TTT T TA CCC GCC CTA TCC AAT ACA CCC CCT G |
| WCMS47  | CCC CCC TTA TC C CCT TT T AAC CGC AGA ACA TGC ACT TA |
| WCMS46  | TCT GAC CCT TTT TAC CTT GTT ATC C CC TTT CAT CAC CCC C |

**Family 2**

| WCMS28  | TTC CCT AAC CCT CTC CCC CQ C TAT TTT CCC CCT CTC CGC A |
| WCMS31  | TTT GAA CCC TAC CAC TTT TTA CCC CC T GAC ATT CTC ACC G |
Chapter IV: Selection for a Reactamer using ManLAM Extracted from Mycobacterium Tuberculosis Cells – Boronic Acid-Modified Reactamers

Family 1
ACACTATTTGC

Family 2
AAAA

Family 3
AAAA

Family 4
AAAA  GGAA
BALP47  G GG AA A GAG CTT GCA AAG AGC G AA AA A CCC AGA GGA GAT T

**Family 5**

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**Family 6**

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**Orphans**

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Chapter IV: Selection for a Reactamer using Ara-6 Fragment – Boronic Acid-Modified Reactamers

**Family 1**  

G**GTACCGTCAAA******ACACAG**

| BAAP32 | GAC TTA TTG A G T C GT ACC GTC AAA CCG TA A CAC AG T TCT T |
| BAAP48 | ATC G AT GTA CCG TCA AA G TAA C AC ACA G CG TAC CCA CCA T |

**Orphans**

| BAAP1 | CCG ATC GGC ATA AAC GAG CAC ACT TCG TGC CTA TAA CTA |
| BAAP2 | TAC GAC TAG GAG GTG CCG ACA AAC CCC TAC CCT CAG CCT |
| BAAP3 | ATC CCA CCC ATC CAC TTA GGC CAA AAT GAA GCG ATT GCC T |
| BAAP5 | AAA CTA CGA TCA ACC CAC CTT GCA AAG ATG ACG AGC ACA C |
| BAAP6 | GCT GTC GAG TCA CAC CAC ATG CCG TGA TAC GAA GGC GAT T |
| BAAP7 | TAT CCA AAA TGA CTG ACT CTA AAT GAC GCA AAT CTC AAG GGC |
| BAAP9 | CCG GAC ACT ACG CCT ATA ACG AGC AAT ATG CAC CAC GGC CC |
| BAAP10 | TGT ATT GCG GAT TTA TTG TAA ATG GAC TTA GAG TGG A |
| BAAP11 | TCT CCG CTA AGG ATT AAG CCT TAC AGG TAA TGG TTA AAC A |
| BAAP12 | CCC GCC CCA CAC ATC CCA ATC GCC TTA ACA AAC ACC AAG C |
| BAAP13 | GGC ATA CTT AGC CAG ACT AGA AGC ACA AAT AAC AAA ACG C |
| BAAP14 | ATC AGG CGG GCG GTA ACT GAC AGA GAA GCG TCT CTA ACA A |
| BAAP18 | GTG TAA CTA AAC CAG CGC AAA ATA TTA GCA TCA CGG GGG C |
| BAAP21 | ACA AAC ATA TAA ACG AAT CGC CCC TTA CCT GAT GAA AAG C |
| BAAP24 | GCA TTC AAC ACC CAA GAC TTG TCG ACG ACG TCA CGG TGA A |
| BAAP26 | AAA TTA GAC ACC ATG GGG TTG CAA GAT ATG TAA CCG T |
| BAAP27 | GTA ACG GAC AGA TGA CTA AGG AGT CTG CCA TGG GAC ATT C |
| BAAP30 | CCC TCA GAA TAG GCT AGG TCG CGA CGA AAG ACC GCT CTG G |
| BAAP31 | TTA TAT ACA TTC TCC CAC TAA GGG GCA CAA GGT TCA GGA G |
| BAAP34 | CCC GTC ACA CCC GGC ACG ACA CCC TGT AGG CTT CAG TAT |
| BAAP36 | ACA CTC GGT CTG ACT CCA GGA TCA TTA AAC AAG CCC CAC T |
| BAAP39 | TAC CGT GTT TTA GGC GCA TCC GTT ACT CCC CTT GGT T |
| BAAP43 | CTC TGA TGT TCA ACG GAC CGC ATC AAT CTG ACC GCT GTG A |
| BAAP44 | TCC CGG ACG AAG TTA TAC CCA CGC CAA TCA ACT TCA CCA C |

Chapter IV: Selection for a Reactamer using ManLAM Extracted from Mycobacterium Tuberculosis Cells – Pyridyl Only-Modified Aptamers

**Orphans**

| LP2   | CTA CCA GGT CGA CCA CTA GGC GGA TTG GCC ACG CTA GAA C |
Chapter IV: Selection for a Reactamer using Intact Mycobacterium Tuberculosis Cells – Boronic Acid-Modified Aptamers (Next Generation Sequencing, Top Twenty Motifs of Most Populous 1,000 Sequences)

Motif 1       TGCTTATGTCCCGCCCGGGG
Motif 2       CCGGGGGGGGTCC
Motif 3       GGGGTCCCTTGG
Motif 4       GTTGGGTATA
Motif 5       TCCCTGGGGTGGG
Motif 6       CTCCTCGGGGATCGA
Motif 7       AGGGGGTATGGCGACGAAGT
Motif 8       CGTGGCCCGGG
Motif 9       GCCGCCCAGG
Motif 10      GGTCCCGGGG
Motif 11      AGATTTGGTGAGCACAGTCG
Motif 12      AAAGTTGGGTAGTTCGAA
Motif 13      TTGGCCGTGAGACACAGTGCGGATTGGG
Motif 14      GGGAGTTGTTGTTGAT
Motif 15      GGATCGAGAGCAGTGCCCC
Motif 16      GGATGGTGAGACACAGTGCGGATTGGG
Motif 17      GCATTGGGAGGCACCGGAATG
Motif 18      GGCATCGGCCGCTGAG
Motif 19      TTACAGGACCCCGGGG
Motif 20      GGCAGGTTGACGATCGGCACGTAAG
Chapter IV: Selection for a Reactamer using Intact Mycobacterium Tuberculosis Cells – Pyridyl Only-Modified Aptamers (Next Generation Sequencing, Top Twenty Motifs of Most Populous 1,000 Sequences)

Motif 1 AGATGCGGGCCCGGG
Motif 2 GGCGGGGTTTGAATTGGATAGCTATCTG
Motif 3 GGCATCGAACGTGGGG
Motif 4 GGGGTTGACTTTGTTGGGAGTATGCC
Motif 5 TTGGGGGAAAGACATTGATCAAAGAAGGCA
Motif 6 AAGAAGGCAATCGACCT
Motif 7 GTGGTCGCCGG
Motif 8 GGGGCGGTT
Motif 9 AGTCGTTGAG
Motif 10 TTGTCTGCTTGAAGAGTAGTGGGCGGG
Motif 11 CCATTGGGTA
Motif 12 TAATGGGTA
Motif 13 TGGTTGTGCTTGGGCTAGGG
Motif 14 CTGATTGGGTAACAGAGGCAA
Motif 15 TTGGGTAAGCA
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