Design and Synthesis of Inhibitors of dTDP-D-glucose 4,6-
dehydratase (RmlB), an Enzyme Required for dTDP-L-Rhamnose
Production in M.Tuberculosis

by
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Abstract

The purpose of this work is to probe the dTDP-L-rhamnose pathway in an effort to develop small molecule inhibitors that could act as therapeutics for Mycobacterium tuberculosis. The necessity for newer, more effective treatments for tuberculosis is growing, as the bacteria evolve resistance to traditional treatments.

In an effort to develop more effective and perhaps more abbreviated courses of treatment, a plan was developed to investigate a pathway involved in cell wall biosynthesis as a promising target: the dTDP-L-rhamnose pathway. This pathway plays an essential role in linking the peptidoglycan and arabinogalactan portions of the mycolic acid-arabinogalactan-peptidoglycan complex, a significant part of the mycobacterial cell wall. The mounting level of biochemical understanding of this pathway and its importance in bacterial cell wall biosynthesis indicates that it is not only a relevant target but also an accessible one.

Of the four enzymes crucial to this biosynthetic pathway, one was chosen as the primary focus: dTDP-D-glucose 4,6-dehydratase (RmlB). There are 3 steps in the reaction mechanism of RmlB: oxidation of the C4 position of dTDP-D-glucose to form a 4-keto structure, dehydration of the C6 position via the elimination of water and a subsequent reduction to result in a 6-deoxy product. Crystal structures of this particular enzyme, dTDP-D-glucose 4,6-dehydratase (RmlB), complexed with single substrates or substrate analogs have provided a foundation for these studies, enabling the rational design of a small library of potential inhibitors. Twelve mechanism-based inhibitors of RmlB are proposed. These compounds reflect the current understanding of the mechanism and mimic the sugar portion of the sugar-nucleotide substrate at various steps throughout the reaction mechanism. Each of the proposed inhibitors is designed to inhibit one of the specific steps of the mechanism. While the intention of this project is to synthesize each compound in this library from commercially available starting materials in 15 steps or less, the primary goal of this particular dissertation is to synthesize 3 of the 12 proposed inhibitors from the commercially available starting material 1,5-anhydro-D-glucitol.

The long term goal of this work is to produce these compounds in significant amounts in order to test their efficacy in an animal model of mycobacterial infection.

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# Table of Contents

Design and Synthesis of Inhibitors of dTDP-D-glucose 4,6-dehydratase (RmlB), an Enzyme Required for dTDP-L-Rhamnose Production in M. Tuberculosis ......................................................... 1

Abstract ........................................................................................................................................ 2

Acknowledgements .......................................................................................................................... 3

Table of Contents .............................................................................................................................. 5

List of Figures ....................................................................................................................................... 7

List of Abbreviations ........................................................................................................................ 8

Chapter 1. Introduction ......................................................................................................................... 10

1.1 Overview ................................................................................................................................. 10

1.2 History and Epidemiology of TB Infection Worldwide ........................................................... 11

1.3 Infection with Mycobacterium Tuberculosis .......................................................................... 15

1.3.1 Characteristics of *Mycobacterium Tuberculosis* ..................................................................... 15

1.3.2 The Mycobacterial Cell Wall ..................................................................................................... 16

1.3.3 Transmission, Infection and Disease Progression ...................................................................... 17

1.4 Host-Pathogen Interactions and the Immune Response ............................................................ 18

1.4.1 Macrophage Activation and Response ..................................................................................... 19

1.4.2 Factors increasing susceptibility .............................................................................................. 24

1.5 Current Treatments and the BCG Vaccine ................................................................................. 25

1.5.1 Isoniazid .................................................................................................................................. 25

1.5.2 Rifampin ................................................................................................................................. 26

1.5.3 Ethambutol ............................................................................................................................ 26

1.5.4 Pyrazinamide .......................................................................................................................... 27

1.5.5 Streptomycin ......................................................................................................................... 27

1.5.6 Second line drugs .................................................................................................................. 27

1.5.7 Bacillus Calmette-Guérin Vaccine ........................................................................................... 28

1.5.8 The Need for New Therapeutics ............................................................................................ 28

Chapter 2. Target Selection and Project Design ................................................................................. 30

2.1 Target Exploration: Narrowing Down the Possibilities ............................................................ 30

2.2 Target Exploration: Focus on Cell Wall Biosynthesis and the MAPs ....................................... 33

2.2.1 Peptidoglycan Biosynthesis .................................................................................................... 33

2.2.2 Mycolic Acid Biosynthesis ........................................................................................................ 34

2.2.3 Arabinogalactan Biosynthesis ................................................................................................. 34

2.3 Target Identification: The d-TDP-L-Rhamnose Pathway .......................................................... 35

2.4 RmlB ......................................................................................................................................... 36

2.4.1 The Structure of RmlB .............................................................................................................. 36

2.4.2 The Active Site ......................................................................................................................... 37

2.4.3 Mechanism of Action .............................................................................................................. 38

2.5 Design of Inhibitors ................................................................................................................... 39

2.5.1 Inhibition of Oxidation ............................................................................................................. 41

2.5.2 Inhibition of Dehydration ........................................................................................................ 41

2.5.3 Inhibition of Reduction ........................................................................................................... 42

2.5.4 Inhibition of Experimental Focus ............................................................................................ 42

Chapter 3. Experimental Methods ..................................................................................................... 43

3.1 General ....................................................................................................................................... 43

3.2 Synthesis of 6-deoxy-1,5-Anhydro-D-glucitol (10). ................................................................. 43

2,3-O-Acetyl-4,6-O-Benzylidene-1,5-Anhydro-D-glucitol (19). .................................................... 43

2,3-O-Acetyl-4-O-Benzyl-1,5-Anhydro-D-glucitol (20). .................................................................. 44

2,3-O-Acetyl-4-O-Benzyl-6-Deoxy-6-Iodo-1,5-Anhydro-D-glucitol (21). ................................... 45

---5---
List of Figures

Figure 1. WHO statistics on global tuberculosis infection ................................................................. 64
Figure 2. Scanning electron micrographs of Mycobacterium tuberculosis........................................ 65
Figure 3. Schematic representation of the cell wall of M. TB ............................................................. 66
Figure 4. Stages of the immune response ......................................................................................... 67
Figure 5. The immune response against M. TB .................................................................................. 68
Figure 6. Chemical structures of first-line drugs ............................................................................. 69
Figure 7. The glyoxylate pathway as it relates to the citric acid cycle .............................................. 70
Figure 8. Biosynthesis of the mycolic acid-arabinogalactan-peptidoglycan complex .... 71
Figure 9. Biosynthesis of dTDP-L-rhamnose from glucose-1-phosphate ....................................... 72
Figure 10. The Structure of RmlB .................................................................................................. 73
Figure 11. The active site of RmlB ................................................................................................... 74
Figure 12. Proposed mechanism of action of RmlB ......................................................................... 75
Figure 13. The twelve inhibitors designed for RmlB ......................................................................... 76
Figure 14. Inhibitors designed against the oxidation step of the mechanism ............................... 77
Figure 15. Inhibitors designed against the dehydration step of the mechanism .......................... 78
Figure 16. Inhibitors designed against the reduction step of the mechanism ............................. 79
Figure 17. Retrosynthetic analysis using a protected glucal as the starting material ........................ 80
Figure 18. Retrosynthetic analysis using D-glucose as the starting material ................................. 81
Figure 19. Synthesis of inhibitors 10, 9 and 12 ............................................................................. 82
Figure 20. Suggested mechanism for the regioselective benzylidene ring opening reaction ............ 83
Figure 21. 1H Spectrum of Compound 19, 2,3-O-acetyl-4,6-O-benzylidene-1,5-anhydro-D-glucitol ................................................................. 84
Figure 22. 13C Spectrum of Compound 19 ..................................................................................... 85
Figure 23. gCOSY spectra of Compound 19 ................................................................................... 86
Figure 24. HETCOR spectrum of Compound 19 .......................................................................... 87
Figure 25. 1H spectrum of Compound 20, 2,3-O-acetyl-4-O-benzyl-1,5-anhydro-D-glucitol .......... 88
Figure 26. 13C spectrum of Compound 20 ..................................................................................... 89
Figure 27. gCOSY spectra of Compound 20 ................................................................................... 90
Figure 28. HETCOR spectrum of Compound 20 .......................................................................... 91
Figure 29. 1H Peak assignments for compounds 19 and 20 ............................................................ 92
Figure 30. 1H spectrum of Compound 21, 2,3-O-acetyl-4-O-benzyl-6-deoxy-6-ido-1,5-anhydro-D-glucitol ................................................................. 93
Figure 31. 13C spectrum of Compound 21 ..................................................................................... 94
Figure 32. 1H spectrum of Compound 22, 2,3-O-acetyl-6-deoxy-1,5-anhydro-D-glucitol ............ 95
Figure 33. 13C spectrum of Compound 22 ..................................................................................... 96
Figure 34. 1H spectrum of Compound 10, 6-deoxy-1,5-anhydro-D-glucitol .................................... 97
Figure 35. 13C spectrum of Compound 10 .................................................................................... 98
Figure 36. gCOSY spectrum of Compound 10 .............................................................................. 99
Figure 37. HETCOR spectrum of Compound 10 .......................................................................... 100
List of Abbreviations

BCG bacillus Calmette-Guérin

CTLs cytotoxic T-lymphocytes

DOTS Directly Observed Treatment, Short-course

dTTP deoxythymidine triphosphate

EMB ethambutol

FAS fatty acid synthase

ICL isocitrate lyase

IFN-γ interferon gamma

IL-12 interleukin 12

INH isoniazid

KO knock out

M. TB Mycobacterium Tuberculosis

MAPc mycolic acid-arabinogalactan-peptidoglycan complex

MDR-TB Multi-Drug Resistant Tuberculosis

MS malate synthase

NO nitric oxide

PI phosphatidyl inositol

PIA pyrazinoic acid

PPD purified protein derivative

PZA pyrazinamide

RIF rifampin

RmlB dTDP-D-glucose 4,6-dehydratase

ROI reactive oxygen intermediates

RNI reactive nitrogen intermediates
SDR short-chain dehydrogenase/reductase
TACO tryptophan aspartate containing coat
TB Tuberculosis
TLRs Toll-Like Receptors
TNF-α Tumor Necrosis Factor alpha
UDP-Galf UDP-galactofuranose
UDP-Galp UDP-galactopyranose
UDP-MurNAc UDP-N-acetyl muramic acid
WHO World Health Organization
Chapter 1. Introduction

1.1 Overview

Tuberculosis (TB), the disease caused by *Mycobacterium tuberculosis* (M.TB), is currently a serious health problem in the developing world, while its threat in developed countries continues to grow every year. Each year, over two million people die from TB, including a large number of HIV-positive individuals who are unable to fight the infection.\(^1\) Approximately two billion people are currently infected with M. TB, a number that is constantly growing, as around nine million new cases are identified each year.\(^2\) This growing rate of infection translates into a new person infected with TB every second; almost 1% of the total world population is newly infected each year. Of the one-third of the world population infected with TB, 5-10% will become sick or infectious during their lifetime.\(^1\) The rates of infection are growing at over 2% per year, despite implementation of health strategies worldwide.

Complicating matters is the sheer difficulty in treating the infection. Current therapy consists of a six to nine month barrage of four or more different medications, with different dosages and side effects. Despite this rigorous regimen, treatment can still leave latent TB infection in the lungs. Adding to the current crisis is the growing concern of multi-drug resistant (MDR) TB, which is defined as strains that are resistant to both isoniazid and rifampin, the two most effective current TB drugs.

The World Health Organization (WHO), along with numerous health experts, believe that the world is currently experiencing a public health crisis with respect to TB, and that this problem will continue to persist unless measures are taken to better control the spread of this disease. Unfortunately, 60% of TB-related deaths occur in the
poorest 20% of the global population, and, consequently, there is a serious dearth in the amount of funding put towards profit-driven diagnostic and therapeutic development.

This project is derived from a novel approach to the development of therapeutics for TB. Instead of focusing on an established project in an existing lab, the problem of TB was analyzed in terms of identifying a new “ideal” target and developing a viable method for altering its biological activity. To achieve this goal, the deficits in natural and synthetic means to fight off the infection were examined. This was accomplished through an analysis of both the mechanisms by which the mycobacteria are able to overcome the human immune system as well as the shortcomings of existing therapeutics. There are a large number of possible drug targets in mycobacteria. While certain targets could be considered for further study, the main focus of this project will be the selection of one particular enzyme as a target, and the remainder of this dissertation will discuss the rational design of small molecule inhibitors of this protein. In an effort to develop more effective and perhaps more abbreviated courses of treatment, the target of interest is a particular enzyme, dTDP-D-glucose 4,6-dehydratase (RmlB), involved in the dTDP-L-rhamnose pathway in cell wall biosynthesis. Using the crystal structures of the enzyme complexed with single substrates or substrate analogs as a foundation for the studies, the intentions of this project were to conduct the rational design, synthesis and screening of a small library of sugar analogs that could be potential therapeutics for TB.

1.2 History and Epidemiology of TB Infection Worldwide

The problem of TB is nothing new. TB is clearly a significant worldwide killer, and has been for centuries; in fact, it has been referred to as the “white plague” due to the pale appearance of its victims. Robert Koch, who discovered the TB bacilli, wrote in 1880, “If the number of victims which a disease claims is the measure of its
significance, then all diseases, particularly the most dreaded infectious disease, must rank far behind tuberculosis. Statistics teach that one-seventh of all human beings die of tuberculosis, and that, if one considers only productive middle-age groups, tuberculosis carried away one-third and often more of these...” As it was in the 19th century and before, TB continues to be a problematic infection.

While cases of TB consistently dropped once therapeutics were introduced in the 1950’s and 1960’s, since the 1980’s the number has been growing once again. There are numerous possible reasons for this increase, but one of the main contributing factors to an increase in TB infections worldwide is the HIV/AIDS epidemic, as those afflicted with HIV/AIDS are much more susceptible to active TB infections than other individuals. About one-third of those afflicted with HIV/AIDS are infected with TB. Due to their compromised immune systems, HIV/AIDS victims are unable to fight the infection, resulting in high rates of reactivation TB and high rates of fatalities (raised by 5-20% over the normal 15%) due to TB. Other possibilities for the increase in TB infections worldwide could include increased poverty, higher levels of global travel, and significantly higher levels of crowding in big cities, as well as institutions such as prisons and hospitals. Lack of available treatment is another concern in the developing world, as there is a 50% fatality rate for untreated cases—a number which rises to 80% in HIV/AIDS patients. Whatever the reasons for this increase, it is clear that the problem is only getting worse at the status quo. During the 1990’s, it was approximated that there were ninety million new cases and around thirty million deaths due to TB. However, it is estimated that over the next twenty years, approximately one billion more people will become infected, with more than a hundred and fifty million people experiencing symptoms and over thirty six million of those succumbing to the infection. The infection is currently the leading cause of death due to infectious disease among people older than five.
Regionally, the distribution of TB is quite striking, as the rates of TB infection worldwide vary tremendously. The prevalence of TB is much higher in developing countries, as is the death rate due to the infection. TB accounts for 20% of adult deaths and 6% of infant deaths, and is considered to be the cause of 26% of all avoidable deaths in the developing world. It is hypothesized that the developing world is home to 95% of the world’s TB, and 98% of the deaths caused by the disease; overall, approximately 7% of all deaths in the developing world are attributed to TB. There is a relationship between lower standards of living and higher rates of TB, as it has a much higher incidence in poorer countries and large, crowded cities. Of the twenty two countries with the highest occurrence of TB, only one (Brazil) was not classified as a low income or low middle income country, according to the per capita income designations set by WorldBank.

The continents of Asia, Africa and South America are hardest hit, yet the top five infected countries (India, China, Indonesia, Bangladesh and Nigeria) are almost all in Asia (Figure 1). About three million cases per year occur in south-east Asia, while around two million cases per year are present in sub-Saharan Africa, an area of increasing concern because of the growing relationship between HIV/AIDS and TB. Forty percent of the global incidence occurs in India and China, the two countries with the largest populations worldwide. India has an estimated 1.8 million cases while China reports approximately 1.4 million. Both countries claim significantly high levels of treatment success (79% and 96%, respectively), yet it is clear their National TB programs are not as extensive and treatment is not as readily available as it should be.

DOTS or Directly Observed Treatment, Short-course is the primary strategy endorsed by the WHO to achieve high levels of treatment success. The strategy encompasses several goals that involve government commitment in TB control activities, case detection by sputum smear microscopy, standardized chemotherapy
regimens for eight weeks, an uninterrupted supply of essential anti-TB drugs, and finally, a recording/reporting system allowing for assessment of each individual patient and the TB program overall. It has been rated as an extremely cost-effective way of efficiently treating large numbers of patients, yet the distribution of DOTS enforcement is not as high as desired. The countries listed above are not the countries with the highest rates of incidence per 100,000 people. Cambodia, Zimbabwe, South Africa, Afghanistan and Uganda are the countries with very high rates of disease and these countries have very low therapeutic effectiveness. The enforcement of DOTS in most of these countries is minimal or non-existent, due to funding and personnel shortages, leading to the further spread of disease.

Problems with effective TB therapy and DOTS enforcement have led to the spread of multi-drug resistant (MDR) forms of TB. Due to the long course of treatment, patient compliance is a serious issue, as patients tend to feel better after two months of treatment. However, discontinuing treatment at that stage leads to the development of strains of MDR-TB. Poor availability of therapeutics along with incorrect single-drug treatment regimens also contribute to the growing problem of MDR-TB. There are cases of MDR-TB on every continent, yet there are certain countries with particularly high levels. Latvia (14.4%), Estonia (10.2%), Dominican Republic (6.6%), Ivory Coast (5.3%), Argentina (4.4%) and Russia (4.0%) are among those with the most significant incidences of MDR-TB. In countries not implementing DOTS, rates of MDR-TB greater than 2% were much more common—54% in non-DOTS countries versus 22% in DOTS countries. It is estimated that up to fifty million people may already be infected with MDR strains of TB, and this number continues to rise.
1.3 Infection with *Mycobacterium Tuberculosis*

Infections due to M.TB pose a significant public health concern, yet it is the lack of understanding of the complexities of the bacterium itself that frustrates the efforts to contain the disease. The unique features of the pathogen pose as much of a challenge in the development of therapeutics now as they have for the past 100 years. Despite the vast knowledge base currently available regarding M. TB, there are a considerable number of questions that remain. However, there have been recent developments in understanding the interactions between the pathogen and the host that present a beautiful scientific problem.

1.3.1 Characteristics of *Mycobacterium Tuberculosis*

*Mycobacterium Tuberculosis* possesses a variety of unusual characteristics that contribute to its virulence as a pathogen. The bacterium has a Gram-positive type cell wall, yet cannot be detected by the conventional Gram stain due to the waxy outer coating of numerous lipids. The rod-shaped bacteria (ranging between 1 to 4 μm in length and 0.3 to 0.6 μm in width) can be detected using an acid-fast stain, a harsh stain that involves heating the bacteria. The primary method of diagnosis in most of the world is through the culture of sputum samples and detection using this stain (Figure 2). The slow generation time of the bacterium (18-24 hours) is attributed to the hydrophobic cell surface, which may cause the clumping together of bacteria, thereby preventing the entry of nutrients into the cell.² The organism is an obligate aerobe, and, as expected, it prefers the high oxygen content of organs such as the lungs, despite having the capacity to change its metabolism and survive in a microaerophilic environment.
1.3.2 The Mycobacterial Cell Wall

While *M. TB* is considered Gram-positive, the organism possesses cell-wall features that are characteristic of both Gram-positive and Gram-negative bacteria, resulting in a particularly unique structure. The mycobacterial cellular envelope consists of three components: 1) a plasma membrane, 2) a covalently linked mycolic acid-arabinogalactan-peptidoglycan complex (MAPc) and 3) a polysaccharide-rich capsule-like material (Figure 3). The lipid content of the cellular envelope is so dramatic that it is estimated to make up 30-40% of the total weight of the bacilli and could account for characteristics such as low permeability, growth in clumps, physical strength and a decreased response to certain toxic substances.⁹

The plasma membrane serves as the initial protection of the mycobacterium from the outside environment. It appears to have characteristics quite similar to normal bacterial membranes, as does the peptidoglycan component of the MAPc. The MAPc consists of the cross-linked peptidoglycan which is bound covalently to arabinogalactan chains through a phosphoryl-N-acetylglucosaminosyl-rhamnosyl linker. The arabinogalactan is additionally esterified to a variety of α-alkyl and β-hydroxy mycolic acids.¹⁰ In particular, the arabinogalactan-peptidoglycan component of the cell wall has been implicated in the viability of mycobacteria; the relatively recent elucidation of biosynthesis pathways has resulted in a number of targets for small molecule enzyme inhibition. Certain pathways are becoming increasingly well-characterized, but the specific roles of many enzymes involved in these pathways remain elusive. There are a number of lipids that are non-covalently attached to the MAPc, and beyond this layer of lipids lies a loosely attached layer of polysaccharides and proteins, forming the outer capsule around the mycobacteria.⁹ Overall, this extensive cell wall decoration plays a role in the behavior of mycobacteria in the host
environment, and could affect processes ranging from intracellular growth, to the immune response, to reactions of the bacilli to therapeutics.

1.3.3 Transmission, Infection and Disease Progression

The disease itself is spread primarily by aerosolized droplets of tubercle bacilli containing three or less organisms; these droplet nuclei float in the air, and can remain airborne for long periods of time after the fluid evaporates. A patient with pulmonary TB can infect another while coughing, exhaling, sneezing, talking or spitting; it is estimated that 10-15 people each year are infected by one infected individual.4

Once inhaled, the bacteria travel to the alveoli, as infection in the upper respiratory tract is unlikely. Smaller droplets of bacilli can reach the alveoli, where they are taken up by alveolar macrophages which attempt to phagocytose the bacteria. Once the bacteria are taken up into the macrophages in a phagosome, the macrophage attempts to lyse the bacteria by fusing the phagosome and the lysosome. This fusion is often blocked by mycobacterial mechanisms, resulting in a pulmonary infection that consists of active bacteria living within inactivated macrophages.11 The mycobacteria are able to grow and spread, and can even enter systemic venous circulation at times, so they may re-enter the lungs and be deposited at additional locations within the lungs or other organs. At this phase there are three possibilities for the mycobacteria and the host effector cell: the macrophage can kill the mycobacteria, the mycobacteria can cause the cell death of the macrophage, or the macrophage can become a host cell for the mycobacteria and propagate infection.11 It is at this point that an active immune response is key in determining the outcome of infection. Poor T-cell immunity at this phase can lead to a serious progression of the disease, as well as transmission to others. The symptoms of an infection consist primarily of a fever, coughing for over three weeks (often with bloody sputum), chest pain, loss of energy and weight loss, in
addition to progressive (irreversible) lung destruction. In most cases, the immune response fails to completely clear the bacteria, which can continue to grow within alveolar macrophages.²

When phagocytes fail to clear the infection, other T-cells and macrophages amass around the bacterial growth. Macrophages can fuse to form giant cells, resulting in a layer consisting of macrophages and T-cells that surrounds the bacteria. This layer often forms a self-contained lesion, consisting of a mass of bacteria surrounded by a coat of fibrin.² This lesion, called a tubercle or granuloma, has a cheese-like consistency (*caseous necrosis*) at first, yet as bacterial growth continues and more phagocytes surround the area, the lesion becomes more liquid. Latent TB consists of mycobacteria contained in these lesions, and these liquefied lesions can form infectious aerosols, burst to reactivate an active TB infection or travel to other organs, resulting in miliary TB, which is often fatal. The chances of reactivation TB are 2-23% over a lifetime, but this number can increase depending on immune system factors.²

1.4 Host-Pathogen Interactions and the Immune Response

When encountering any pathogen, the human immune system attempts to mount an effective response to kill the invader. The case of M. TB is no different; there are a number of mechanisms by which the innate and adaptive immune systems can clear the infection. However, what makes mycobacteria perhaps more complex than certain other pathogens is their ability to evade damage by these intervention systems and thus render immune system processes ineffective. This section will discuss some of the interactions between mycobacteria and various human immune system components in order to highlight the major players at the cellular and molecular levels.
1.4.1 Macrophage Activation and Response

Once phagocytosed bacteria are living within a macrophage, there are a number of pathways that determine the fate of both the mycobacteria and macrophage. The complexity of M. TB lies in its ability to modulate normal immunological activity in its favor, despite a variety of mechanisms that are intended to clear the infection through the various stages of infection (Figure 4).

**Oxidative burst**

Reactive oxygen intermediates (ROI) and reactive nitrogen intermediates (RNI) comprise the two major sets of effector molecules important in the macrophage defense against mycobacteria (Figure 5). These reactive intermediates are synthesized in parallel by two related pathways. Beginning with O$_2$ and L-arginine, the macrophage is able to form radicals and other damaging agents to use against the bacteria. ROI are a product descendent from O$_2$, as the enzyme phagocyte oxidase (phox) catalyzes the formation of superoxide, which is used in the synthesis of peroxide by superoxide dismutase. Hydrogen peroxide then undergoes radical mechanisms to result in hydroxyl and ferryl radicals. The importance of ROI in defense against mycobacteria is slightly controversial, yet they do seem to play some kind of role, despite the number of bacterial resistance genes and products that are used to neutralize this response. RNI are formed in a similar manner from guanidine-L-arginine, which is transformed into nitric oxide (NO) radicals due to nitric oxide synthase (iNOS or NOS2). These nitric oxide radicals can go on to form peroxynitrite (along with superoxide) or they can form NO$_2^*$, NO$_2$, S-nitrosothiols, dinitrosyl iron complexes and other reactive molecules. Similar to ROI, mycobacteria have developed a number of mechanisms involving molecular scavengers, antioxidant enzymes, repair systems and antioxidant regulons. Studies of inducible nitric oxide synthase (iNOS or NOS2) and phagocyte...
NADPH oxidase (phox) suggest that RNI are significantly more important in the defense against TB. Macrophages from actively infected individuals have been found to have high levels of NOS2, and the levels of NO exhaled in TB patients are higher than that of non-infected individuals. There appear to be more mechanisms by which ROI can be evaded, as many cell wall components such as lipoarabinomannan and phenolicglycolipid I are oxygen radical scavengers. However, despite the controversy over the relative importance of these two mechanisms, it is clear that they play a role in the macrophage defense.

Control of phagolysosome fusion

The response of macrophages to mycobacteria is to form a phagosome around the bacteria, and once the bacteria have been taken up into the cell, fuse the mycobacteria-containing phagosome with the acidic lysosome, thereby ensuring the death of the bacteria. However, one of the most complex mechanisms of mycobacterial survival is its ability to block this phagosome-lysosome fusion, allowing the bacilli to survive indefinitely within the macrophage.

There are a number of biochemical reasons for the arrest in phagolysosome maturation. The ability of the lysosome to degrade particles depends upon hydrolases that must be maintained at low pH levels, yet it has been demonstrated that mycobacteria-infected phagosomes possess a lack of vacuolar ATPases, which are usually responsible for acidification; the less acidic environment could be responsible for the lower amount of degradation by the vacuole. Another possible mechanism is the role of Rab GTPases; Rab5 helps in early endosome fusion while Rab7 assists in late endosomal membrane trafficking, yet it has been found that mycobacterial phagosomes utilize Rab5 while they exclude Rab 7. Calcium-binding calmodulin may be involved in the regulatory functions of membrane fusion, as phagocytosis usually leads to an
increase in calcium concentrations, yet mycobacteria are able to alter the signaling
cascade and prevent this increase.11 Another important protein could be the
tryptophan aspartate containing coat (TACO), an actin-binding protein that is
implicated in the early phagosome membrane. However, the TACO-membrane
association is expected to be transient, yet the retention of this association is postulated
to prevent phagolysosome fusion by delaying maturation of the phagosome;
mycobacteria that are somehow able to prevent the detachment of TACO are able to
survive longer within macrophages.12 There are other mechanisms that may explain
the lack of degradation of mycobacteria by macrophages; certain mycobacterial
sulfatides may be able to inhibit phagolysosomal fusion, while it is also possible that
high levels of ammonia production by mycobacteria may change saltatory movements
and even raise the pH of the compartment.13

CD8+ Cells

The activities of CD8+ cells, in protecting against mycobacteria are two-fold; the
cytotoxic functions of the cells enable them to kill intracellular mycobacteria directly in
addition to serving as a facilitator in lysing macrophages containing live bacteria
(Figure 5). Initially, the CD8+ cells are presented with mycobacterial lipid or peptide
antigens by antigen-presenting cells via molecules such as MHC Class I or CD1.14
Once these CD8+ cells come into contact with an infected macrophage, they can
activate the macrophage to kill the mycobacteria through the production of interferon-
gamma and tumor necrosis factor-alpha, two crucial cytokines that can activate
enzymes such as nitric oxide synthase. CD8+ cells are able to function as cytotoxic T
lymphocytes (CTLs) in order to facilitate lysing of infected macrophages. This lysing
can occur via two pathways: a perforin-dependent pathway and a Fas/FasL pathway.
The perforin dependent pathway involves granulysin: perforin forms a pore that
enables the uptake of granulysin, a cytolytic T cell granule protein, into the macrophage, resulting in the death of both the macrophage and its mycobacteria. However, if the mechanism of macrophage lysis is via the Fas/Fas ligand system, the macrophage is destroyed but the live mycobacteria are released.14

*CD4*<sup>+</sup>

*CD4*<sup>+</sup> cells are important in the presentation of mycobacterial antigens by MHC class II molecules. However, the primary effector function of *CD4*<sup>+</sup> cells is the active role in IFN-γ production, which is crucial in macrophage activation. These cells are also involved with NOS2 expression in the early phases of infection, but these cells play a role in later stages of the infection as well, as they have been found to be important in the priming and maintenance of CD8<sup>+</sup> T cell effector and memory functions. The influence of these cells in the immune response has been studied extensively. For example, it has been found that M. TB infected mice treated with an anti-CD4<sup>+</sup> antibody experienced a reactivation of active disease, demonstrating the key role of these cells. M. TB has been able to alter the activity of these cells through disrupting the macrophage-CD4<sup>+</sup> cell interaction. M. TB-infected macrophages are unable to present antigens to CD4<sup>+</sup> T cells, and infection by mycobacteria may inhibit recognition of macrophages by CD4<sup>+</sup> cells by down regulating the surface expression of MHC Class II molecules. This interference with normal function is yet one more mechanism by which mycobacteria can survive within the host successfully.13

*Interferon-gamma*

*Interferon-gamma* (IFN-γ) is perhaps the most important cytokine involved in M.TB infection. It plays a crucial role in macrophage activation by enhancing the Th1 response and increasing IL-12 production.13 It is produced by CD4<sup>+</sup> and CD8<sup>+</sup> cells,
along with NK cells, and has been found to be important in antigen-specific T-cell immunity. The functions of IFN-γ are quite diverse; it has been known to promote the production of opsonising antibodies, increase CTL activation as well as NK cell activity and increase macrophage MHC Class II activity. It may also trigger the movement of T cells into infected areas by facilitating a bond between endothelial cells and T cells and serve as a trigger to differentiate macrophages into active effector cells. It has been found that IFN-γ knock-out (KO) mice are most susceptible to virulent TB; additionally, it was found that KO mice had a defective macrophage response and low NOS2 expression.

**Tumor Necrosis Factor-α**

Tumor Necrosis Factor-α (TNF-α) is a cytokine that plays a role in the mediation of macrophage activation. During acute infection of M. TB, TNF-α expression is increased. Along with IFN-γ, TNF-α initiates an increase in NOS2 expression, enabling a stronger response to M. TB infection. TNF-α may also coordinate the migration and localization of cells within particular tissues, and it appears to be essential in granuloma formation, as it regulates expression of certain adhesion molecules. However, it has also been found that TNF-α is responsible for lung tissue damage, as well as the symptoms of weight loss experienced by TB patients.

**Interleukin-12**

Interleukin-12 (IL-12) is a pro-inflammatory regulatory cytokine which is primarily produced by phagocytes. The release of IL-12 is induced after phagocytosis, and is thought to initiate a Th1 response by stimulating IFN-γ production. The exact role of IL-12 is not completely understood, yet it has been
found that IL-12 KO mice are highly susceptible to M.TB infections,\textsuperscript{17} while treating TB infected mice with IL-12 in the early phase of infection increased the mean survival time and decreased the bacterial load.\textsuperscript{13} IL-12 serves as a mediator that can connect the response of phagocytes to that of T cells through its control over IFN-\(\gamma\).

\textit{Toll-Like Receptors}

Adaptive Toll-like receptors (TLRs) facilitate the activation of the immune response against M.TB. TLRs can recognize a number of mycobacterial antigens; the antigens most important in the immune response are the 19kDa lipoprotein, lipoarabinomannan and phosphatidylinositolmannan, all of which interact with TLR2. TLR2 is proposed to have a more significant role in defense against TB than TLR4; activation of TLR2 can result in an induction of macrophage apoptosis and the down regulation of MHC class II, two important anti-mycobacterial functions.\textsuperscript{18}

\textbf{1.4.2 Factors increasing susceptibility}

Most characteristics that have been identified as increasing a particular individual's susceptibility towards TB infection involve the response of the immune system; whether it is in the form of a genetic variation or other factors that decrease normal immune functions. A defect in IL-12 or IFN-\(\gamma\) causes an individual to be at a significantly heightened risk of TB, while other mutations, such as ones in the promoter regions of TNF-\(\alpha\), or the natural resistance-associated macrophage protein 1 (nRAMP), result in only slight increases in susceptibility.\textsuperscript{19}

A normal person infected with TB bacilli incurs a 10% risk of developing active TB throughout a lifetime. A weakening of the immune system, however, can increase this risk. The single largest factor to increase the chances of reactivation is HIV infection; the risk level is raised to 8-10% per year (as opposed to 10% per lifetime) due
to the immunocompromised state of the patients.\textsuperscript{4} Other factors include silicosis, renal disease, malignancy, diabetes, malnutrition or long-term treatment with immunosuppressive drugs.\textsuperscript{19}

\section*{1.5 Current Treatments and the BCG Vaccine}

From the above description of mycobacteria, it is quite clear that there are a number of complexities associated with successfully fighting infections of M. TB. There are a number of anti-mycobacterial agents in use, yet none are perfect. In fact, only four can be considered "first-line" treatments, while others are only used on an "as-needed" basis due to the rapid resistance that is developed by the mycobacteria. TB therapy is currently made up of an initial two-month phase of four drugs daily (Isoniazid, Rifampin, Pyrazinamide and Ethambutol) after which time the regimen solely consists of Isoniazid and Rifampin. In cases of MDR-TB, the so-called "DOTS-plus" strategy is employed, and the use of second-line drugs along with any viable first-line options is used. This section will summarize the currently available treatments and the problems associated with each.

\subsection*{1.5.1 Isoniazid}

Isoniazid (INH) has been the most important anti-mycobacterial agent in use since the 1950's, and it continues to dominate therapeutic regimens, aside from cases of MDR-TB (Figure 6). It is hypothesized that INH is a prodrug which is transformed into a number of reactive radical species by the mycobacterial catalase-peroxidase KatG. These reactive molecules can react with a number of mycobacterial targets, but the two most well-understood enzymes are InhA (an enoyl ACP reductase) and KasA (a beta-ketoacyl ACP synthase), both of which are involved in the cell wall mycolic acid biosynthesis. Resistance often arises to INH. Resistance to INH can be attributed to
mutations in katG; it is proposed that a particular serine is mutated into a threonine, resulting in a form of the catalase-peroxidase that does not react with INH but maintains enough normal activity to continue detoxification against antibacterial radicals.\textsuperscript{20} Another gene, ndh (NADH dehydrogenase), which increases the ratio of NADH/NAD\textsuperscript{+} in order to prevent a reaction between the isonicotinic acyl radical and an NAD radical, has also been implicated in INH resistance.\textsuperscript{21} Despite the fact that the activated INH can react with a number of mycobacterial cellular components, drug resistance is becoming a serious problem.\textsuperscript{21}

1.5.2 Rifampin

Rifampin (RIF), a compound with a very complex macrocyclic ring structure, works to inhibit transcription in mycobacteria via an interaction with the DNA-dependent RNA polymerase (Figure 6). The mode of action is fairly well understood, and crystallographic studies confirm the tight binding of the drug to RNA polymerase. The means by which mycobacteria develop resistance to rifampin is based upon mutations in an 81-base pair region of the gene encoding the B-subunit of RNA polymerase (rpoB).\textsuperscript{20}

1.5.3 Ethambutol

Ethambutol (EMB), a diamine derivative, is another first-line therapeutic used in defense against TB (Figure 6). The precise mechanism of action is not yet known, yet it is clear it is involved in the inhibition of cell wall biosynthesis. It has been shown to inhibit the incorporation of mycolic acid into the mycobacterial cell wall,\textsuperscript{22} and it has more recently been suggested that EMB blocks some part of the polymerization process involved in arabinogalactan and lipoarabinomannan biosynthesis.\textsuperscript{23} More support for an interaction between EMB and arabinosyl transferases is demonstrated by the gene
which confers resistance to EMB; mutations in embCAB, an operon encoding arabinosyl transferases, have been implicated in most cases of resistance to EMB.24

1.5.4 Pyrazinamide

Pyrazinamide (PZA) is a nicotinamide-like prodrug that reacts with pyrazinamidase to form pyrazinoic acid (POA), the active form of the drug, that can accumulate in the mycobacterial cytoplasm (Figure 6). Large amounts of POA can cause a lowering of the intracellular pH, and perhaps inactivates pH sensitive enzymes such as fatty acid synthase. When resistance to PZA occurs, it is usually the case that mycobacteria stop using pyrazinamidase. Most PZA-resistant mycobacteria possess a mutation in the promoter region of the gene that encodes pyrazinamidase (pncA).20

1.5.5 Streptomycin

Streptomycin, an aminocyclitol glycoside antibiotic, is considered as another first-line drug against TB, yet its use is limited by serious toxicity and its intramuscular delivery method (Figure 6). It inhibits protein synthesis by somehow interfering with translational proofreading, perhaps by interacting with the ribosomal S12 protein and 16SrRNA.21 Streptomycin increases the missense rate by suppressing proofreading and possibly disrupts the decoding of aminoacyl-tRNA, leading to incorrect translation. Resistance is attributed to point mutations in the S12 ribosomal protein encoded by RpsL, and mutations in the rrs operon encoding the 16S rRNA.25

1.5.6 Second line drugs

There are a number of second-line drugs that are used in the treatment of TB. These therapeutics are classified as second-line because of decreased efficacy against the bacilli, increased toxicity, increased rates of resistance or unfavorable pharmacokinetics. These drugs include ethionamide, kanamycin, cycloserine, para-
aminosalicylic acid, capreomycin, amikacin and the fluoroquinolones, all of which are used primarily in cases where resistance is developed against the first-line therapeutics. However, none of these are optimal therapeutic options for the treatment of TB infections.²

1.5.7 Bacillus Calmette-Guérin Vaccine

The vaccine available for TB, called bacillus Calmette-Guérin (BCG), is an attenuated strain of *Mycobacterium bovis* that is the most widely administered vaccine in the WHO Expanded Programme for Immunization. However, the efficacy of this vaccine is quite controversial. Protection from disseminated and meningeal TB in infants and children is quite reliable, but its value in preventing pulmonary TB in adults is questionable; studies on the rates of vaccine protection show dramatically different efficiencies, ranging from 77% in the UK to 0% in India.⁸ As it stands, the BCG vaccine does not do a successful job of providing immunity against TB, and is currently contraindicated in HIV patients, as it is a live vaccine.⁶ Additionally, vaccination with BCG results in a positive response to the purified protein derivative (PPD), and therefore a false-positive on the Mantoux diagnostic skin test, one of the most effective diagnostic tools used to identify TB-infected persons.⁶

1.5.8 The Need for New Therapeutics

In 1993, the WHO declared that TB was a global emergency, yet over the past ten years there has not been a significant increase in practical solutions to this problem. There has not been a new drug approved for TB in over thirty years, yet the current health crisis demonstrates a significant need for new developments in order to eradicate TB. The effective therapies currently in use are not optimal; due to nine-month long regimens, patient non-compliance is a serious issue, leading to the emergence of newer
drug-resistant strains. New anti-TB therapies are an absolute necessity for MDR-TB as well as latent TB infection, and the development of such novel therapies is the focus of the remainder of this thesis.
Chapter 2. Target Selection and Project Design

2.1 Target Exploration: Narrowing Down the Possibilities

In the process of choosing the dTDP-L-rhamnose pathway in TB as the therapeutic objective for rational drug design, a range of possibilities were considered based upon the current literature. The field of TB research is constantly evolving, and the validity of targets is constantly changing. The purpose of this section is to briefly explore various other possible targets and to explain reasons why the dTDP-L-rhamnose pathway was viewed most favorably as a target for novel TB therapeutics.

Isocitrate Lyase and Malate Synthase

The glyoxylate cycle is a shunt that is used to effectively convert acetate (degraded from fatty acids, amino acids or ethanol) into carbohydrates by bypassing the loss of CO₂ in the citric acid cycle in M. TB (Figure 7). The two enzymes involved in the cycle, isocitrate lyase (ICL) and malate synthase (MS) catalyze the formation of malate from isocitrate, via the intermediate glyoxylate; the cycle facilitates the formation of succinate from two acetate molecules (as acetyl-Coenzyme A), conserving carbon that would otherwise be released as carbon dioxide via the normal Krebs cycle enzymes. In conditions of latency, fatty acids may be the primary source of carbon and energy for mycobacteria, and it has been found that the ability of M. TB to persist in macrophages is dependent upon ICL but not necessarily MS.²⁷

The use of ICL as a possible target for anti-mycobacterial development bears further investigation, as not only is it an enzyme not present in humans, but it has been examined extensively through mechanistic and crystallographic studies. However, it was believed to an imperfect target because of the similarities between the substrate
and end products of the enzyme as compared to endogenous human substrates from the citric acid cycle. Any inhibitors would most likely lead to undesired side effects.

**Galactofuranose pathway**

Another particular pathway of interest was the pyranose pathway consisting of UDP-galactopyranose (UDP-Galp) mutase and galactofuranose (Galf) transferase, which together function to transform an UDP-galactopyranose into UDP-galactofuranose and transfer galactofuranose molecules onto the growing chain of the arabino-galactan complex, a component of the cell wall. The UDP-Galp mutase has been crystallized, although the mechanism by which the mutase achieves the unprecedented ring contraction of a nonreducing sugar is unclear. Galf transferase is even more complex; it has been suggested that there are two to four separate transferases involved, with the overall activity being significantly complicated. Nevertheless, the pathway of core galactofuran synthesis has been found to be essential for the viability of mycobacteria, and these galactofuranose units are not present in human tissues. Despite these important details, the lack of a clear mechanism renders these enzymes a less attractive target for the rational design of inhibitors.

**Antigen 85 complex**

The Antigen 85 complex is made up of three related mycolyl transferases, Antigen A, B and C. These three proteins are important in transferring a mycolic acid molecule between one trehalose monomycolate molecule and another, which leads to the formation of trehalose dimycolate, also known as the “cord factor,” and free trehalose, two important factors that are incorporated into the mycobacterial cell wall. The cord factor has been implicated in cell wall integrity, while the Antigen 85 complex itself may also foster interaction between the mycobacteria and the
macrophage. However, while there are some structures for this complex, the role of these proteins is not completely understood; the value of this complex as a drug target has been controversial, and thus it was decided to pursue alternatives.

*Phosphatidylinositol synthase*

Phosphatidylinositol (PI), along with other lipoglycan molecules such as PI mannosides, lipomannan and lipoarabinomannan, have been implicated in the cell wall integrity of M. TB, and may additionally be important in the response of the mycobacterium to the host immune system. Studies of PI synthase mutants have revealed that this enzyme is essential to mycobacterial survival and the mechanism is somewhat well understood, suggesting that it could be a viable therapeutic target. However, as humans also possess a version of PI synthase, inhibitors of this enzyme may lack specificity and could be quite harmful to human tissues.

*Other Targets*

The sheer number of possibilities for TB drug development is quite overwhelming. Due to the recent sequencing of the M.TB genome, there have been a number of targets identified, yet the mechanistic and structural knowledge about many of these is still in the early phases of discovery. Among targets that could be important are: the gated mechanosensitive ion channel, which has been implicated in ionic and osmotic regulation; InhA, the enoyl-acyl carrier protein reductase; glutamine synthase, which helps to control ammonia levels and thereby modulate phagosome-lysosome fusion; and the β-ketoacyl-ACP synthase, which is important in mycolic acid biosynthesis. Numerous other pathways such as those involving peptidoglycan synthesis specific to mycobacteria, and the β-oxidation processes important in fatty acid degradation could also reveal worthy targets. Another strategy employed to treat TB
involves the modulation of the host-pathogen interaction, in order to augment the
immune response, enabling the body to successfully fight the infection. It is clear,
however, that the knowledge base for this type of drug development is currently
lacking; not enough is known about such interactions to design appropriate
therapeutics effectively. Since further development is required before these strategies
can be successfully pursued, a more well-established pathway became the focus of this
project.

2.2 Target Exploration: Focus on Cell Wall Biosynthesis and the MAPc

As previously mentioned, the composition of the cellular envelope consists of
three components: 1) a plasma membrane, 2) a covalently linked mycolic acid-
arabinogalactan-peptidoglycan complex (MAPc) and 3) a polysaccharide-rich capsule
like material. The MAPc in particular is assembled via a complex set of biosynthetic
pathways; the three components are synthesized in parallel and joined together as the
final step.

2.2.1 Peptidoglycan Biosynthesis

Since peptidoglycan structures are fairly homologous between various bacterial
species, it is assumed that the peptidoglycan layer of M. TB is no different. Two
enzymes, MurA and MurB, are important in the synthesis of UDP-N-acetyl muramic
acid (UDP-MurNAc) from UDP-N-acetyl-glucose and enoylpyruvate. The UDP-
MurNAc is subsequently used to form a UDP-MurNAc-pentapeptide through the
linkage of an L-alanine, a D-glutamic acid residue, a diaminopimelic acid molecule and
finally a D-alanyl-D-alanine dipeptide, reactions catalyzed by MurC, MurD, MurE and
MurF. At this point, this peptide molecule is linked to an undecaprenyl phosphate and
bound to a N-acetyl glucose molecule to form a GlcNAc-MurNAc(pentapeptide)-
diphosphoryl-undecaprenol, also known as Lipid II, by MurG. This fragment is subsequently ready for transport to the inner cell membrane where the undecaprenyl diphosphate is released and the peptide crosslinks are formed. Each of these enzymes are potential targets for anti-bacterial agents, yet one disadvantage is that there would be no specificity for mycobacteria in particular.10

2.2.2 Mycolic Acid Biosynthesis

The mycolic acids decorating the surface of mycobacteria can be classified in a variety of different ways, yet most possess a linear carbon chain (between 60 and 90 carbons), with vicinal carboxyl and hydroxyl groups on carbons 23 and 24 (±2), and often contain cyclopropane rings in place of double bonds.34 Both fatty acid synthase (FAS) type I and type II systems are present in mycobacteria, and the synthesis of these mycolic acids proceeds like much of fatty acid synthesis; the fatty acid synthase system processes—β-ketoacyl synthase, β-ketoacyl reductase, β-hydroxyacyl dehydratase and enoyl reductase—perform the cyclic functions of condensation, ketone reduction, dehydration and enoyl reduction. However, while the system is relatively well-characterized, not only are structural details not fully known, but there is additionally a lack of specificity with regards to other bacteria (FAS II) or even humans (FAS I).

2.2.3 Arabinogalactan Biosynthesis

The portion of the MAPc that was of most interest was the branched-chain arabinogalactan component, which links together the peptidoglycan and mycolic acid layers. Two residues, a N-acetyl-glucose and a rhamnose, link the peptidoglycan and arabinogalactan layers together; the galactose moieties are added on to this rhamnose residue in succession, followed by the branching out of the layer by the addition of the arabinose groups (Figure 8). The synthesis of the galactose molecules has been
described earlier, and it is hypothesized that the arabinose sugars originate from the pentose phosphate pathway/hexose monophosphate shunt. While the synthesis of this layer has been somewhat characterized, the majority of the enzymes involved have not been crystallized or even distinctly isolated; it was decided that the optimal target for this project would be a structurally and mechanistically understood pathway, and for this reason many of the arabinogalactan component enzymes were eliminated.  

2.3 Target Identification: The d-TDP-L-Rhamnose Pathway

The arabinogalactan component of the cell wall is linked to the peptidoglycan part via an N-acetylglucosamine and a rhamnose. As rhamnose is not a sugar produced by humans, it is synthesized within the bacteria and transferred onto the growing chain. L-Rhamnose is a 6-deoxyhexose that is synthesized from glucose-1-phosphate and deoxythymidine triphosphate (dTTP) and found in the cell walls of numerous pathogenic bacteria. This pathway represents an interesting therapeutic target, as no analogs of this pathway have been elucidated in humans.

The first enzyme in the pathway is RmlA (glucose-1-phosphate thymidylyltransferase), which transfers a dTDP on to glucose-1-phosphate to make dTDP-glucose. RmlB, a dTDP-D-glucose 4,6-dehydratase, next converts the dTDP-glucose into dTDP-4-keto-6-deoxy-D-glucose. The third enzyme in the pathway, RmlC or dTDP-4-keto-6-deoxy-D-glucose, epimerizes the dTDP-4-keto-6-deoxy-D-glucose into dTDP-L-lyxo-6-deoxy-4-hexulose. Finally, RmlD catalyses the reduction of the C4 keto group to form dTDP-L-rhamnose (Figure 9). The dTDP-L-rhamnose is then transferred onto the N-acetylglucosamine that will become the arabinogalactan component. As an essential linker between the peptidoglycan and the arabinogalactan molecule, rhamnose serves an important purpose; the inhibition of this sugar pathway would theoretically prevent the assembly of the complex and prevent bacterial growth.
survival. It has been recently found that the formation of dTDP-L-rhamnose is essential for the growth of M.TB.

2.4 RmlB

RmlB, the second enzyme in the dTDP-L-Rhamnose pathway, is perhaps the best characterized in terms of structure and mechanism of the four enzymes. Of the four, it presents the best therapeutic target because of the high level of understanding of its function. Distinct crystal structures of the enzyme with its cofactor and the substrate have been solved, resulting in considerable insight into the positioning of these molecules within the active site.

Mechanistic studies have confirmed the presence of proposed intermediates, and the convergence of both mechanistic and structural analyses have resulted in general acceptance of the currently proposed mechanism. Additionally, the intermediates and product of this conversion are distinct from any human substrates, suggesting that selective targeting of the bacterial enzyme is possible. Therefore, structure-based inhibitor design will be used to produce small-molecule inhibitors that are hoped to selectively act upon this target enzyme.

2.4.1 The Structure of RmlB

RmlB is a member of the short-chain dehydrogenase/reductase extended family. It is a homodimeric enzyme; monomer association occurs through hydrophobic interactions via a four-helix bundle. Each monomer has two domains: a larger N-terminal Rossmann fold with seven β-strands and ten α-helices and a smaller C-terminal domain with six α-helices and four β-strands. These domains come together to form an active site cavity that encompasses both the sugar-nucleotide and cofactor binding sites. The larger domain binds the cofactor NAD⁺ and the smaller domain
binds dTDP-D-glucose. The binding site contains a three glycine motif present in most enzymes of the short-chain dehydrogenase/reductase (SDR) family and the SDR-characteristic active site residues of threonine, tyrosine and lysine form a catalytic triad that is essential for activity. The enzyme utilizes NAD\(^+\) but regenerates it at the end of the reaction. The structure of RmlB from \textit{S. enterica} with the cofactor NAD\(^+\) and the substrate bound has been reported at 2.57\(\AA\) (Figure 10).\(^{35}\)

2.4.2 The Active Site

Within the active site, the NAD\(^+\) adopts a syn conformation; the si face is exposed, leading to pro-\(S\) hydride transfer from the C4 sugar-nucleotide (Figure 11). There are both polar and hydrophobic residues important in binding the NAD\(^+\). The NAD\(^+\) diphosphate group is dependent upon the characteristic SDR GlyXGlyXXGly sequence for binding, while the adenine sits in a hydrophobic pocket around Ile21, Ala57, Val77, Ala81 and Leu107. The nicotinamide ring shares electrostatic interactions with Asp32, Thr35, Asp58, Thr99, Tyr167 and Lys171, positioning it in the proper way for the reaction mechanism.\(^{35}\)

Binding of the nucleotide-sugar is similarly stabilized by both hydrophobic and polar interactions with nearby residues. The thiamine ring is surrounded by a hydrophobic crevice created by Pro204, Leu207, Leu210, Val211, Pro222, Ile223, Val269 and Val270. Additionally the ring \(\pi\)-stacks with Tyr224. The sugar portion of the substrate is involved with Thr133, Asp134 and Glu135, each of which play important roles in the reaction mechanism. Thr133, along with Lys171, help to lower the pKa of Tyr167, which is situated next to the C4 hydroxyl group. Thr133 and Glu135 form hydrogen bonds with C4 while Asp134 interacts with the hydroxyl group located on C6. The sugar ring is on a parallel plane to the NAD\(^+\) nicotinamide ring; this ring also serves to lower the pKa of the active site base Tyr167.\(^{38}\)

\(~37~\)
2.4.3 Mechanism of Action

This enzyme catalyses the conversion of dTDP-D-glucose to dTDP-4-keto-6-deoxy-D-glucose through three primary steps: oxidation, dehydration and reduction (Figure 12).

**Oxidation**

The first step in the mechanism is the oxidation of the glucosyl C4 and the corresponding reduction of NAD+ to NADH. The process begins with a lowering of the pKa of Tyr167 to 6.41 due to hydrogen bonding to nearby Thr133 and the positive electrostatic fields created by nearby Lys206 and NAD+, allowing Tyr167 to act as the active site base. Once the substrate binds, the nicotinamide C4 is 3.5Å away from the glucosyl C4, positioning it for a hydride transfer. The tyrosinate deprotonates the hydroxyl group on C4, leading to the formation of the ketone and reduction of the NAD+ (compound 14 in Figure 12). This leads to a lowering of the pKₐ of the proton on C5, which is important in the dehydration.38

**Dehydration**

The dehydration step involves the removal of water across C5 and C6 and can proceed via two possible mechanisms: the enolization of the dTDP-4-ketoglucose intermediate, followed by elimination, or by a concerted elimination across the C5-C6 bond (compound 15 and 16 in Figure 12). It is not clear which mechanism is in effect, but recent evidence suggests the latter may be occurring. The key in this step is to protonate the leaving C6-hydroxyl group and deprotonate C5, allowing the formation of the double bond. It is believed that Asp134 donates a proton to the hydroxyl group while Glu135 abstracts the proton from C5.38
Reduction

In the last step, a hydride is transferred from NADH to C6, causing a regeneration of the original NAD+ molecule. The end result is an inversion in the configuration at C6, along with the addition of a proton back to C5 to make the final product, dTDP-4-keto-6-deoxy-D-glucose (Compound 17 in Figure 12). Studies on the distances between the C6 and the NADH nicotinamide ring suggest that they must be close together, implying that the sugar changes position after the elimination in order to be aligned properly for the reduction. The theory that the hexopyranosyl ring rotates slightly, while the dTDP portion of the substrate remains in place, is supported by experimental evidence. This movement would bring Tyr167 close enough (3.5 Å) to C5, indicating that it would be possible for Tyr167 to protonate C5 and renew itself as the active site base. It is also hypothesized that Glu135 is within 3.5 Å of Asp134, and the proton on Glu135 could shuttle back to Asp134 ending the catalytic cycle.

2.5 Design of Inhibitors

The library of compounds designed as proposed inhibitors of RmlB is derived from the current understanding of the mechanism (Figure 13). Each of the molecules proposed are sugar analogs; they possess no hydroxyl (or nucleotide group) at C1. The 1-deoxy form is preferred as the lack of nucleotide results in a smaller molecule overall, which is desirable in terms of cellular uptake and even drug delivery. In the event that potent compounds are identified from this library, further structure-activity studies will be proposed. The work discussed herein is therefore a starting point for the design of effective and therapeutically viable inhibitors of RmlB.

As each step of the mechanism is targeted, the question of whether changes and shifts within the enzyme active site during the reaction will affect the efficacy of these molecules. While there is a proposed rotation of the bond between the sugar moiety
and the nucleotide portion of the substrate during the course of the reaction, this supposed movement of the sugar-nucleotide during catalysis should not be an issue, as it is a change in the phosphate bond between the sugar and nucleotide and these proposed inhibitors do not have a nucleotide component. Therefore, it is hoped that these molecules will be easily accommodated by the active site of the enzyme regardless of which stage of the mechanism they are designed to mimic.

Liu et al. have synthesized an inhibitor, CDP-6-deoxy-6,6-difluoro-D-glucose against a related enzyme, CDP-D-glucose 4,6-dehydratase (isolated from Yersinia pseudotuberculosis), that has activity of 0.94 mM (K_i) against the enzyme and forms a covalent bond to the enzyme. This enzyme has an analogous mode of action to RmlB, and such a mechanism is purported to be similar in the formation of all 6-deoxyhexoses. However, it is not clear how closely related the active site of that enzyme is to RmlB, and therefore unclear as to whether these sorts of inhibitors would have similar activity as inactivators of RmlB. Additionally, as aforementioned, the analogs proposed do not have a nucleotide attached, as the nucleotide binding site is removed from the active site residues. The inclusion of a nucleotide group would increase the molecular weight of the inhibitor, which could lead to problems in cellular uptake and bioavailability, therefore initial inhibitor design will focus solely on modifications of the sugar moiety.

It remains unclear if these molecules will be taken up into mammalian cells, as glucose uptake into cells depends upon the specificity of the glucose transporter and molecules of glucose permease that allow passive diffusion into cells. However, since certain modified glucose molecules (such as deoxy and fluorine substituted analogs) are taken into cells, there is a high possibility that they can be taken up into macrophages and even through the GI tract (if administered orally, for example). Bacterial cells possess even more sugar transporters and permeases than mammalian cells, and are
therefore even more likely to take up these molecules. They depend on exogenous sugar molecules for survival, and these molecules closely resemble the proposed inhibitors, therefore it is possible that due to the carbohydrate-like nature of these inhibitors, mycobacterial uptake may not be a serious issue.

2.5.1 Inhibition of Oxidation

Compound 1 replaces the proton of C4 with a fluorine, which could prevent the initial oxidation in the first step of the mechanism (compound 14 in Figure 14) but may still resemble the substrate closely enough to bind well to the active site of RmlB. Compound 12 possesses an exocyclic carbon-carbon double bond in the place of a C4 hydroxyl; this may be a mechanism-based inactivator if the active site tyrosinate attacks the double bond.

2.5.2 Inhibition of Dehydration

Compound 2 substitutes an amine group instead of a hydroxyl group on C4. This compound could potentially be oxidized, but may not undergo imine formation and certainly would not incur a negative charge on the nitrogen during the dehydration step; however it retains the ability to form hydrogen-bonding interactions with the enzyme. In fact, it is quite likely that the amine would be protonated at physiological pH and therefore be even more drawn towards the tyrosinate ion. Compound 3 would prevent the dehydration step from occurring, as it replaces the proton on C5 with a fluorine substituent and would therefore not allow for proton abstraction. However, it would mimic compound 14 in the reaction mechanism catalyzed by RmlB. Compound 10 is similarly designed to allow for the oxidation step but not the subsequent dehydration step, as there is no water available to eliminate from the C6 position. Compounds 8 and 11, where the former possess an amine on the
C4 position and both contain fluorines on C6, are other variations intended to hinder further reaction by preventing the dehydration/elimination step.

Compounds 4, 5 and 6 are inhibitors of the dehydration step designed to mimic the geometry of an enolate, provided that the mechanism proceeds via an enolate formation, as this reaction may be concerted, as mentioned earlier. Both Cl and Br are suggested as oxygen atom mimetics, although the size of a chlorine atom is closer to that of oxygen. The 6-deoxy form, compound 6, is also suggested, as it combines features of both the dehydration and reduction step (Figure 15).

2.5.3 Inhibition of Reduction

Compounds 7 and 9 are end-product inhibitors using both fluorines and hydrogens, respectively, on C6. Compound 9 is the actual product (compound 17 in Figure 16) with hydrogens on C1 instead of a nucleotide group. As the enzyme theoretically returns to its initial state after catalysis, these derivatives can mimic the end product of the reaction and therefore should be accommodated by the active site.

2.5.4 Selection of Experimental Focus

While there were twelve different inhibitors designed originally, the experimental approach of this thesis focused on the synthesis of three specific compounds of the set. These three inhibitors, 9, 10 and 12, are proposed to resemble molecules at different steps in the reaction mechanism, and compose a basic framework for the initial evaluation of this strategy to inhibit RmlB.
Chapter 3. Experimental Methods

3.1 General

All reagents and solvents for syntheses and purification were obtained from commercial sources (primarily Sigma-Aldrich and Toronto Research Chemicals) and used without further purification. Reactions were followed by detecting compounds using Hanessian's Cerium Molybdate stain on thin-layer chromatography plates (TLC).

$^1$H and $^{13}$C NMR spectra were recorded on the Bruker AVANCE-400 NMR Spectrometer at the Department of Chemistry NMR Instrumentation Facility at MIT. The 2D spectra (gCOSY, HETCOR and DEPT) were obtained using the Varian Inova-500 NMR Spectrometer. For those $^1$H NMR spectra run in CDCl$_3$, the internal standard trimethylsilane was used as a reference point, while in other solvents, the residual protons were used (CD$_3$OD $\delta$ 4.89). For $^{13}$C NMR spectra run in CDCl$_3$, the peaks are assigned relative to the residual carbons ($\delta$ 77.23 for the central peak) while those run in CD$_3$OD are referenced to the central solvent signal at $\delta$ 49.15. The abbreviations used are s, d, dd, t, q, and m, which are used to describe a singlet, doublet, doublet of doublets, triplet, quartet and multiplet, respectively. All J coupling values are reported in Hz, while chemical shifts are given in ppm.

3.2 Synthesis of 6-deoxy-1,5-Anhydro-D-glucitol (10).

2,3-O-Acetyl-4,6-O-Benzylidene-1,5-Anhydro-D-glucitol (19). 1,5-anhydro-D-glucitol (18) (1 g, 6.1 mmol) was dissolved in DMF (5 mL). Benzaldehyde dimethyl acetal (2.5 mL, 18.1 mmol) and a catalytic amount of p-TsOH (.116 g, .61 mmol) were added and the reaction mixture was stirred at 40°C overnight. The reaction was monitored by TLC and stopped after approximately 20 hours by the addition of triethylamine (0.85 mL). The reaction was stirred for an additional 15 minutes and
allowed to cool to room temperature before being poured into H$_2$O (120 mL). The aqueous layer was washed with CHCl$_3$ (3 x 200 mL); the organic sections were combined, dried using anhydrous Na$_2$SO$_4$ and concentrated, resulting in a yellow oil. The mixture of compounds was carried on without further purification. The benzylidene-protected glucitol (1.5 g, 6.1 mmol) was dissolved in pyridine (8 mL) and acetic anhydride (20.0 mL) and heated at 80°C for 5 hours. The reaction mixture was cooled to room temperature and poured into a mixture of H$_2$O (150 mL) and EtOAc (150 mL); after extraction with H$_2$O (5 x 150 mL), the organic sections were dried using anhydrous Na$_2$SO$_4$ and concentrated. The desired product was purified by column chromatography (5:1 hexane: ethyl acetate; R$_f$=0.3), yielding 1.6 g of a pure white solid (80% yield). $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ 7.43 (in, 2H), 7.35 (m, 3H), 5.50 (s, 1H), 5.34 (t, J=9.6 Hz, 1H), 5.05 (m, 1H), 4.33 (dd, J=10.6 Hz, J=5 Hz, 1H), 4.13 (dd, J=11.2 Hz, J=5.6 Hz, 1H), 3.72 (t, J=10.2 Hz, 1H), 3.63 (t, J=9.6 Hz, 1H), 3.47 (m, 1H); 3.42 (t, J=8.2 Hz, 1H), 2.08 (s, 3H), 2.05 (s, 3H); $^{13}$C NMR (400 MHz, CDCl$_3$): $\delta$ 170.05, 169.92, 136.78, 128.95, 128.10, 125.99, 101.28, 78.63, 72.30, 71.31, 69.49, 68.44, 67.27, 20.75, 20.61.

2,3-O-Acetyl-4-O-Benzyl-1,5-Anhydro-D-glucitol (20). 2,3-O-acetyl-4,6-O-benzylidene-1,5-anhydro-D-glucitol (19) (400mg, 1.2 mmol) was dissolved into BH$_3$•THF (10 mL) and the reaction vessel was cooled to -10°C. A solution of .55 M TMSOTf in CH$_2$Cl$_2$ (1.1 mL) was added dropwise; the reaction was stirred for 1.5 hours at -10°C until there was a significant change in the TLC. The reaction was poured into ethyl acetate (50 mL), washed with H$_2$O (3 x 50 mL), dried with Na$_2$SO$_4$ and evaporated. The product was purified using column chromatography (2:1 hexane: ethyl acetate; R$_f$=0.3) to yield 365 mg of a fluffy white solid in 90% overall yield. $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ 7.25 (m, 5H), 5.18 (t, J=9.6 Hz, 1H), 4.83 (m, 1H), 4.56 (d,
J=1.6 Hz, 2H), 3.99 (dd, J=11.2 Hz, J=5.6 Hz, 1H), 3.80 (dd, J=12 Hz, J=2 Hz, 1H),
3.63 (dd, J=12 Hz, J=4 Hz, 1H), 3.58 (t, J=9.6 Hz, 1H), 3.29 (m, 1H), 3.24 (t, J=10.8
Hz, 1H), 1.97 (s, 1H), 1.95 (s, 3H), 1.90 (s, 3H); 13C NMR (400 MHz, CDCl3): δ 170.40,
170.34, 137.73, 128.69, 128.15, 128.11, 79.93, 75.90, 75.73, 74.99, 69.76,
66.81, 61.77, 21.06, 20.92.

2,3-O-Acetyl-4-O-Benzyl-6-Deoxy-6-Iodo-1,5-Anhydro-D-glucitol (21).
Triphenyl phosphine (233 mg, .89 mmol) and imidazole (120 mg, 1.8 mmol) were
added slowly to a stirring solution of 2,3-O-acetyl-4-O-benzyl-1,5-anhydro-D-glucitol
(20) (200 mg, .60 mmol) in toluene (10 mL). Iodine (210 mg, .83 mmol) was
gradually added and the reaction stirred at room temperature for 3 hours. The reaction
mixture was then dissolved in EtOAc (2 x 25 mL) and washed with a saturated solution
of sodium sulfite (25 mL) followed by water (2 x 25 mL) and a saturated solution of
sodium chloride (1 x 25 mL). The organic section was dried with anhydrous Na2SO4
and evaporated and the residue purified using column chromatography (4:1 hexane:
ethyl acetate; Rf=0.3) to yield 185 mg (69% overall) of the slightly yellowish-white
solid. 1H NMR (400 MHz, CDCl3): δ 7.25 (m, 5H), 5.19 (s, 1H), 4.89 (m, 1H), 4.65
(dd, J=15.6 Hz, J=4.4 Hz, 2H), 4.03 (dd, J=11.2 Hz, J=5.6 Hz, 1H), 3.47 (t, J=9.2 Hz,
1H), 3.42 (dd, J=11 Hz, J=3 Hz, 1H), 3.34 (m, 2H), 2.95 (m, 1H), 1.96 (s, 3H), 1.92 (s,
3H); 13C NMR (400 MHz, CDCl3): δ 170.34, 170.25, 137.58, 128.81, 128.34, 128.09,
79.78, 77.70, 75.71, 75.45, 69.60, 66.72, 21.11, 20.95, 7.12.

2,3-O-Acetyl-6-Deoxy-1,5-Anhydro-D-glucitol (22). 2,3-O-acetyl-4-O-benzyl,
6-deoxy-6-iodo-1,5-anhydro-D-glucitol (21) (120 mg, .27 mmol) was dissolved in a
1:1 methanol/dioxane solution (10 mL). A small amount of triethylamine (100 µL)
was added and the solution was degassed with argon for 10 minutes. Activated Pd/C
(10% by wt.) was added (120 mg) and a H2 balloon was placed over the reaction,
which was then allowed to stir for 24 hours. At the end of 24 hours, the reaction was filtered through Celite and evaporated to remove solvent and triethylamine. The resulting solid was dissolved in degassed ethanol (24 mL), Pd/C (10% by wt.) was once again added and the reaction stirred for an additional 24 hours. Once the mixture was filtered through Celite and evaporated, the compound was purified using column chromatography (2:1 hexane: ethyl acetate; Rf=0.3), resulting in 45 mg (71%) of a crystalline white solid. $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ 4.94 (m, 2H), 4.01 (m, 1H), 3.29 (m, 3H), 2.67 (s, 1H), 2.10 (s, 3H), 2.02 (s, 3H); $^{13}$C NMR (400 MHz, CDCl$_3$): $\delta$ 172.23, 170.26, 77.55, 76.91, 74.71, 69.45, 66.81, 21.15, 20.95, 17.91.

6-Deoxy-1,5-Anhydro-D-glucitol (10). The 2,3-O-acetyl-6-deoxy-1,5-anhydro-D-glucitol (22) (45 mg, .19 mmol) was dissolved in a .014 M solution of sodium methoxide in methanol (2 mL) and stirred for 2 hours. To stop the reaction, a small amount of Amberlite Resin (H+) was added and the pH was monitored until it returned to neutral pH was achieved. The resin was removed by filtration and the final product was recovered in pure form after the solution was evaporated to dryness. The final product was a white solid with a total yield of 20 mg (70%). $^1$H NMR (400 MHz, CD$_3$OD): $\delta$ 3.80 (dd, J=11.2 Hz, J=5.2 Hz, 1H), 3.42 (m, 1H), 3.21 (t, J=8.8 Hz, 1H), 3.17 (m, 1H), 3.12 (t, J=10.6 Hz, 1H), 2.93 (t, J=9.2 Hz, 1H), 1.2 (d, J=6 Hz, 3H); $^{13}$C NMR (400 MHz, CD$_3$OD): $\delta$ 79.77, 78.13, 77.31, 71.78, 71.08, 18.52.
Chapter 4. Results and Discussion

In order to synthesize compounds 10, 9 and 12 in large quantity, it was necessary to develop a synthesis that was reasonably short and efficient, based upon existing methodology within the field of synthetic carbohydrate chemistry.

4.1 Retrosynthetic Analysis

As part of the initial project, the synthesis of twelve different inhibitors was proposed, using a commercially available protected glacial as the starting material. For the three compounds focused upon in this project, the original scheme (Figure 17) involves a common intermediate and is quite analogous to the rationale described below; the hydroxyl on the 6 position of the glucal would be removed, and the 4 position could be oxidized or methylenated to arrive at the desired products. The use of a selectively protected glucal (Compound 25) as the starting material would make it possible to add substituents to the C1 position if desired, yet this double bond could also be removed (to result in a 2-hydroxy) through the selective opening of a Danishefsky epoxidation. However, it became obvious that this synthesis was not feasible; the cost of the protected glucal was significantly prohibitive and synthesizing the protected glucal from an unprotected glucal would have added five to six steps on to each synthesis. As a result, a different synthetic approach was necessary.

The goal of synthesizing 12 different inhibitors was judged to be unrealistic for an S.M. Thesis. Therefore, it was decided that a smaller number of inhibitors should be chosen to focus efforts upon and attempt to synthesize these lead compounds in gram quantities for animal antimicrobial experiments. The three inhibitors (10, 9 and 12) chosen were selected based on their synthetic accessibility, the possibility that they will interact with RmlB at different stages of its catalytic mechanism and their close

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structural relationship, which suggested they could be synthesized through a common intermediate.

The strategy for the synthesis of these three inhibitors was to make them from a common intermediate, Compound 30. Inhibitor 12 could conceivably be obtained from intermediate 29 through a Wittig reaction, followed by removal of residual protecting groups. Access to inhibitor 9 from this intermediate would proceed via a similar deprotection step; synthesis of this key intermediate could be achieved by a selective oxidation of the C4 position of another intermediate, Compound 30. This key intermediate, which could link inhibitors 10, 9 and 12, consists of a selectively protected version of inhibitor 10 that would enable the oxidation and methylenation of C4 (Figure 18).

One option used commonly in carbohydrate synthesis to achieve a selective protection is the benzylidene acetal protecting group. This group can simultaneously protect the hydroxyls on the C4 and C6 positions and, in this case, would be more useful than other conventional protecting groups (acetyl, benzyl, benzoyl or allyl) or the other common acetal protecting group, the isopropylidene acetal, which may protect the C4 and C6 positions but could protect any combination of adjacent hydroxyls. In this case, if the C4 and C6 positions were protected with the benzylidene acetal, the other two hydroxyl groups (on C2 and C3) could be protected with an orthogonal protecting group.

Since the use of a benzyl protecting group would not be suitable as an orthogonal protecting group as it is cleaved under the same conditions as the benzylidene group, the typical possibilities include the allyl group, the benzoyl group and the acetyl group. As allyl protecting groups are susceptible to cleavage under conditions of catalytic hydrogenation, this would not be optimal, as that is the method of removal of the benzyl group (resulting from benzylidene acetal cleavage). Between
the benzoyl and acetyl protecting groups, the acetyl group was chosen because of its slight advantage in terms of ease in formation and cleavage.

With the benzylidene acetal in place and the other hydroxyls suitably protected, the acetal could be regioselectively cleaved to protect the C4 hydroxyl group, leaving the C6 hydroxyl available for removal. There are a number of ways that could be employed to eliminate the hydroxyl group. However, since a hydrogenation must be used to remove the benzyl group on the C4 position, it seemed as though the most direct route would be to release the C6 hydroxyl with an iodine substituent that could be simultaneously cleaved with a hydrogenation step. Finally, the benzylidene and acetyl-group protected compound could be synthesized easily from 1,5-anhydro-D-glucitol. This latter compound is available from methyl α-D-glucopyranoside, an inexpensive commercially available reagent, through a reduction of the anomeric carbon (Figure 19).

4.2 Reduction of Methyl α-D-Glucopyranoside

The proposed first step in the synthesis of the three proposed inhibitors involved the reductive cleavage of methyl α-D-glucopyranoside. This step was attempted according to the procedure published by Bennek et al, yet there were significant differences in the results obtained and those reported. The procedure involved an aqueous workup that left the product in water, which was very difficult to fully remove. Additionally, there appeared to be some side reactions with the silylating reagents (trimethylsilyl trifluoromethansulfonate, triethylsilane and bis(trimethylsilyl) trifluoroacetamide) used that resulted in the formation of a polymer type material that was impossible to separate from the desired product. As a result, the yield was unquantifiable. The material was carried forward through the benzylidene acetal
formation and acetylation steps, yet the contaminants remained unable to be separated from the desired product despite multiple attempts at purification. In view of this outcome, other options were considered; it did not appear that there was a less problematic synthesis available for 1,5-anhydro-D-glucitol. However, a company (Toronto Research Chemicals) was found that sold the 1,5-anhydro-D-glucitol at a more reasonable price than Sigma-Aldrich, and after performing a cost analysis on the materials consumed during the course of the reductive cleavage versus the cost of the pure glucitol, it was decided to purchase the material instead of optimizing this step.

4.3 Benzylidene Acetal Formation and Acetyl Protection

The process of forming the benzylidene acetal according to the method of Ishii et al resulted in high yield. The reaction is carried out with a catalytic amount of acid and an excess of benzaldehyde dimethyl acetal, some of which remains unreacted upon termination of the reaction. However, attempts to purify the desired compound at this phase failed; the compound appeared to adhere strongly to the silica gel. Other attempts to remove the benzaldehyde dimethyl acetal were also unsuccessful: a distillation of the crude oil and evaporation at a high temperature (60°C) to remove the reagent resulted in decomposition of the desired product. Thus, it was decided to acetylate the crude reaction mixture without purification, which appeared to be quite successful. The acetylation was carried out in a standard fashion, and once the product was acetylated, purification was facile.\textsuperscript{42} The yield of 75% over these two steps is encouraging in the effort to synthesize larger quantities of material. The presence of the desired product was verified by $^1$H NMR, $^{13}$C NMR and MS, yet a concern at this stage was the inability to specifically assign the signals in the NMR spectra (Figures 21 and 22).
4.4 Assignment of NMR Spectra and the Regioselective Ring Opening Reaction

Perhaps the most complicated part of this synthesis was the regioselective opening of the benzylidene acetal ring. There are a number of methods that could be employed in the opening of this ring; the literature was explored for an option that had high yields and specificity yet did not use extremely harsh conditions or particularly hazardous chemicals. There were three possible procedures that were considered: one used dibutylboron trifluoromethanesulfonate, a fairly hazardous chemical, another required prolonged low temperature (-78°C) maintenance and the final one utilized trimethylsilyl triflate, a hazardous chemical, yet less so compared to the dibutylboron trifluoromethanesulfonate. Therefore, this latter procedure was chosen as the initial method. The procedure reported by Jiang et al. suggested that high levels of selectivity were possible, but it was necessary to confirm the selectivity of the ring opening. The reaction was attempted according to the reported procedure, yet there was clearly some formation of another isomer. It was unclear as to which isomer was formed predominantly, but there seemed to be significant regioselectivity (about 9:1) when the temperature of the reaction was approximately 0°C. When the reaction was repeated and the temperature warmed up to almost 10°C, the regioselectivity was greatly decreased, and there was a mixture of the two isomers, in perhaps a 60:40 ratio. Clearly, the regioselectivity of this reaction is temperature dependent. The optimal temperature appears to be around -10°C, at which point the reaction proceeds to form one isomer in a 97:3 yield.

The mechanism of this reaction is quite interesting. The selectivity in the ring opening process is most likely due to steric reasons rather than electronic considerations, as there is some of each isomer formed, and there is no clear electronic
difference between the two that favors opening towards one direction or another. The silicon from the trimethylsilyl trifluoromethanesulfonate has a partial positive charge, and, because of the bulky nature of the triflate and three methyl groups, the easiest approach is most likely on the side of the C6 oxygen rather than the C4 oxygen, which is shielded by the phenyl ring as well as the neighboring acetyl group on the C3 oxygen. This interaction results in bond cleavage between the C6 oxygen and the benzyl methylene carbon, resulting in a proposed resonance stabilized transition state structure (Figure 20). This proceeds to the final product, which consists of a free hydroxyl on the C6 position and a 4-O-benzyl protected hydroxyl group. Despite this rationale, it was clear that additional studies would be necessary to confirm that the primary isomer formed was the sought-after product.

In order to determine which isomer was formed, the most logical choice was to assign the peaks in the NMR spectra from the previous step and compare the changes to the ring-opened product. In this vein, gCOSY, HETCOR and DEPT experiments were carried out on both the pure 2,3-O-acetyl 4,6-O-benzylidene 1,5-anhydro-D-glucitol (Compound 19) and the 2,3-O-acetyl 4-O-benzyl 1,5-anhydro-D-glucitol (Compound 20) (Figures 21, 22, 23, 24, 25, 26, 27 and 28). In assigning the gCOSY spectra, published 1H NMR assignment for glucose was used as a reference. The peak at 5.34 ppm is attributed to H3. Using this as a reference, the remaining peaks were then assigned. It was clear that the singlet at 5.49 ppm belonged to the proton from the benzylidene acetal. The assignment of the peaks in the region between 3.00 and 5.50 ppm was significantly more complicated; the proton at 5.34 ppm splits with protons at 5.04 ppm, 4.12 ppm, 3.62 ppm and itself. While gCOSY spectra generally do not show long distance coupling, it became clear that there exists some strong long range coupling (coupling over several bonds) by protons in a similar region (Figure 23). It would make sense, in terms of the spatial configuration of the proposed compound, that
the H3 proton would split with the H2, but it is unclear what the other two sources of splitting could be. As H3 is an axial proton, protons in the general vicinity of the H3 area include H5 (also axial) and the axial proton off C1 (H1_{axial}). However, from the HETCOR spectra (Figure 24), it was noted that the proton signals at 4.33 ppm and 3.70 ppm and the set at 4.12 ppm and 3.38 ppm arise from the two methylene groups (C1 and C6). Therefore, it would appear that the set of protons at 4.12 ppm and 3.38 ppm are close to H3 (in particular the H1_{axial} proton), and are possibly the C1 protons. The peak at 5.04 ppm is a complex multiplet, splitting with the protons at 5.34, 4.33, 4.12 and 3.38 ppm; because of its spatial proximity to H3 and the fact that it splits both the protons from the C1 methylene group, this proton is assigned as H2. This leaves the protons at 3.62 ppm and the multiplet at 3.47 ppm as unassigned. As the H5 proton is more likely to be the proton splitting protons from both the C6 and C1 methylene groups in addition to H2, the multiplet at 3.47 ppm is most likely H5, and the triplet at 3.62 ppm is then the proton off C4. The protons in the 7.30-7.50 ppm region are those associated with the phenyl ring, while those at 2.04 and 2.06 ppm correspond to the two acetyl groups. To confirm the assignments of these protons, these values were compared to a similar molecule reported by Rye et al.\textsuperscript{44} The peak assignments appear to be almost identical to those reported, with the exception of the anomeric position which in the case of compound 19 is a methylene group. Additionally, the $^{13}$C NMR can be assigned based upon the proton assignments, through the use of HETCOR spectra. The 2 peaks at 170.22 and 170.09 ppm correspond to the carbonyl carbons from the acetyl groups while the carbons from the phenyl ring demonstrate chemical shifts at 137.00, 129.13, 128.29 and 126.19 ppm. The carbon from the acetal is displayed at 101.46 ppm, while the peaks at 20.94 and 20.80 ppm correspond to the methyl groups from the acetyl protecting groups. The other shifts are assigned based
upon the gCOSY assignments of peaks: C4 at 78.82 ppm, C3 at 72.51 ppm, C5 at 71.51 ppm, C2 at 69.69 ppm, C6 at 68.63 ppm, and C1 at 67.46 ppm.

Using these assignments, it is possible to analyze the specific changes in NMR spectra between the benzylidene acetal protected compound 19 and the ring opened product 20 to determine whether the benzyl group is in fact on the C4 oxygen or the undesired C6 position. The ¹H NMR peaks in the aromatic region for this compound are closer together than the precursor, while the acetal peak has disappeared as expected (Figure 25). The triplet for H3 and the multiplet for H2 have shifted upfield slightly to 5.23 and 4.90 ppm, respectively, as they are identifiable by their similar splitting patterns in the gCOSY spectrum (Figure 27). The peaks at 4.62 ppm do not split with any other protons, suggesting they belong to the methylene from the benzyl group. There is now only one doublet of doublets in the range between 4.00 and 4.50 ppm; the doublet of doublets appearing at 4.05 ppm corresponds to one of the H1 protons. It is expected that one of the H6 protons shifts significantly, if this product is the desired isomer, while the H4 proton would be expected to remain at nearly the same chemical shift as the previous compound. The chemical shift of H4 is 3.69 ppm compared to the 3.62 ppm from before, which is consistent with the idea that its chemical environment would not change drastically due to the change from benzylidene protected to benzyl protected. The remaining peaks for H5 and H1 are nearly the same as before, as is the H6 peak at 3.63 ppm. It is only a new doublet at 3.85 ppm that constitutes the major change, along with a peak at 2.25 ppm that corresponds to a hydroxyl proton. This change in the second H6 proton signal further confirms that the major product here is the 4-O-benzyl protected sugar, rather than the 6-O-benzyl protected compound. Therefore, it was determined that the reaction conditions used to obtain compound 20 did indeed result in formation of the desired isomer (Figure 29).
4.5 Iodination

There are numerous possible methods for the iodination of a 6-hydroxy group. The primary consideration in the determination of the optimal experimental approach was the selectivity of primary versus secondary alcohols, as it was desired to react only the 4-O-benzyl product and not the small amount (less than 5%) of 6-O-benzyl material, enabling complete purification of the desired product 21. As heating the reaction would most likely encourage the reaction of the secondary alcohol, an iodination reaction performed at room temperature was selected for use, despite this procedure\textsuperscript{45} having slightly lower yields than another procedure involving chlorodiphenylphosphine and a higher temperature instead of triphenylphosphine.\textsuperscript{46} The other option was to convert the free hydroxyl group into a triflate and then into a halogen, however this would have added an unnecessary step and would most likely have decreased yields.

The mechanism of the reaction in this case is relatively straightforward. The triphenylphosphine interacts with the free hydroxyl group, and binds to the oxygen, creating a good leaving group. This leaving group is displaced in an $S_N2$-type fashion by an iodine, resulting in the final product, Compound 21. While the reaction was successful in this case, the yields can most likely be improved, as the conditions were not optimized. However, the formation of the desired product has been confirmed by $^1$H and $^{13}$C NMR, and the assignment of the peaks from the synthetic precursors enables the analysis of these spectra. The major changes between the ring-opened product and this material are the loss of the hydroxyl proton peak and the shift of one of the H6 protons upfield (Figures 30 and 31).
4.6 Hydrogenation

The hydrogenation reaction was carried out according to a procedure by Takeo et al., described as successful in removing an iodide. This particular procedure was chosen over others because it suggested conditions that did not involve the use of a Parr apparatus (or any sort of high H₂ pressure), and it removed the iodide cleanly, which was thought to be a more difficult group to remove than the benzyl protecting group.

Originally, it was supposed that this step would simultaneously remove the benzyl protecting group on the C4 position. However, when this reaction was attempted using 1:1 methanol/dioxane as a solvent and a small amount of triethylamine, it appeared that the iodide was removed, yet the benzyl group remained on the C4 hydroxyl group. When the reaction mixture was filtered and subsequently hydrogenated again in degassed ethanol without triethylamine, according to a method reported by Gray et al., the removal of the benzyl group progressed efficiently. At this stage, the goal of hydrogenating both portions of the compound simultaneously was still highly attractive, therefore the hydrogenation was attempted using normal ethanol and no triethylamine, and the hydrogenation did not progress well. It seems that removal of the iodide progresses neatly when triethylamine is present, which perhaps neutralizes the acid formed in the reaction. However, the reaction may be solvent-dependent, as the two hydrogenation conditions for iodo and benzyl removal seem to be optimized in slightly different solvent systems. It is also possible that the success of hydrogenation is based upon the degassing of the solvent or the H₂ pressure, as the reactions went to completion only after ethanol degassing and two balloons of H₂ instead of one. Whatever the case, it appears that the slight differences in the nature of these two hydrogenations necessitate setting up the reaction in two subsequent stages. The yields are not quite as quantitative as described in the literature, but the overall
yield from these two steps is 71%; it may be possible to further optimize these steps. In
the end however, the desired product is obtained, as confirmed by NMR studies (Figures
32 and 33). In the $^1$H NMR spectrum, the benzyl protons have disappeared, while a
chemical shift corresponding to a methyl group has appeared (for C6), as well as a
hydroxyl proton peak (for C4). The acetyl groups are present in the same region, yet
the remaining protons have less distinct splitting patterns. The $^{13}$C NMR spectrum
corresponds to the correct number of carbons in the appropriate regions.

4.7 Final Deprotection and Peak Assignment of Inhibitor 10

The deprotection of the two acetyl protecting groups is the final step in the
synthesis. The method employed was a standard procedure,$^{49}$ using a small amount of
sodium methoxide in methanol, and an acidic resin to quench the reaction. The
advantage of this procedure is that there is no need to purify the final product, as this
compound is most likely too polar to purify using a silica gel column. The yields thus
far for this step have been lower (70%) than expected (quantitative), but this is most
likely due to the small scale of the reaction; it is difficult to measure such small
quantities of product accurately.

The $^1$H and $^{13}$C NMR spectra were again studied using 2D NMR spectral
techniques for compound 10, as the assignment of the peaks was essential in
confirming the correct structure (Figures 34, 35, 36 and 37). The relative positions of
the sugar protons have changed, and although the exact chemical shift has changed
only slightly, there is an overall trend of movement upfield. The most downfield proton
is the doublet of doublets at $3.80$ ppm, which corresponds to one of the H1 protons,
similar to previous compounds. The multiplet at $3.43$ ppm fits the usual splitting
pattern of the H2 proton, as it splits with the H1 proton at $3.80$ ppm along with other
protons in the $3.10$-$3.30$ ppm range. The triplet, multiplet and triplet in the $3.10$-$3.30$
ppm range overlap slightly, but it appears that the multiplet at 3.16 ppm is the H5 proton, as it has a similar coupling pattern as before. The remaining three triplets are H1, H3 and H4, and the question of which signals correspond to which protons is revealed using the gCOSY spectrum. The peak at 3.12 ppm is clearly H1, as it splits with the other H1 proton and H2. The triplet at 3.21 ppm is most likely the H3 proton, as it couples with the proton at 2.92 ppm and the H2 proton, and the triplet at 2.92 ppm couples with the proton at 3.21 ppm and H5. Spatially, it is more intuitive that the H3 proton would couple with both H2 and H4 while the H4 proton would couple with H3 and H5. Additionally, the doublet at 1.21 ppm corresponds to the methyl group on C6; overall, the assignment of these peaks fits with the proposed product. Due to time constraints, the finalized products 9 and 12 are not described here, yet they are easily accessible from the successful synthesis of compound 10.

4.10 Conclusions and Future Studies

The long-term goals of this study are grounded in the mounting need for newer therapeutics for TB. However, the primary goal of this project was to develop and evaluate inhibitors for dTDP-D-glucose 4,6-dehydratase (RmlB). The efficacy of this rational inhibitor design will be tested as these inhibitors move into biological tests; the animal testing of these three inhibitors only touches the surface of the original goals of this project. Encouraging results from the animal tests, to be performed by Professor Eric Rubin at the Harvard University School of Public Health, could lead to the continuation of this project. Completing the synthesis of the 12 proposed inhibitors as well as expanding the compounds tested into a larger library would be the logical progression, and it is the sincere hope of the author that this is accomplished.

Another possibility for the progression of this project is to develop assays to determine levels of enzymatic inhibition, cellular uptake and bioavailability of these
derivatives. As the reproduction of the bacteria in macrophages is a key factor in the prolonged lifetime of the infection, an assay for studying the ability of these inhibitors to treat infected macrophages would also be implemented. The initial set of inhibitors will provide the framework for further library design and screening, perhaps using high-throughput screening techniques.

There are significant long-range goals in the motivation for this study. From a public health standpoint, TB is a serious killer: two million people each year die from TB and a new person is infected with TB every second. It is estimated that in the next twenty years almost one billion people will become infected, with over 200 million of these people manifesting the debilitating symptoms of TB; clearly this is a serious health problem that is not going away.

The dangers of a growing population of people infected with TB are largely ignored because of the disease's prevalence in primarily developing nations. However, people in the United States and the United Kingdom, particularly displaced or homeless people in urban areas such as San Francisco and London, are at risk as well. Overall risk is currently over 7% in the United States and at 13% in the UK; the rates of infection in poorer countries are much higher. TB is a global health emergency, and the most viable solution to this problem, especially considering the increase in multi-drug resistant TB, is to develop newer treatments that can match or surpass current TB drugs such as isoniazid and rifampin.4

The development of new therapies for TB is an exciting scientific problem, and this study hopes to take a step in the direction of meeting this challenge.
References


Figures.
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Figure 1. WHO statistics on global tuberculosis infection.
Figure 2. Scanning electron micrographs of *Mycobacterium tuberculosis*, courtesy of http://www.bact.wisc.edu/Bact303/atlas (top) and http://www.niaid.nih.gov/dir/labs/lhd/barry.htm (bottom).
Figure 3. Schematic representation of the cell wall of M. TB.
Early (innate)

TNF-a
IFN-g

Immature phagosome formed. TNF-a and IFN-g secreted to activate oxidative burst mechanisms.

Activated infected macrophage

Late (acquired)

Mycobacterial antigens presented by CD1 molecules to T cells. Lysis via granulysin is activated.

Granulysin
Cell Lysed

Chronic

Granuloma contains mycobacteria

Infected macrophage
Lymphocyte
Giant Foamy Cell
Macrophage

Figure 4. Stages of the immune response.
Figure 5. The immune response against M. TB.
Acetyl CoA

Oxaloacetate

Citrate

Malate

Glyoxylate

Isocitrate

Succinate

Oxaloacetate

Citrate

Malate synthase

Isocitrate lyase

Glyoxylate

Succinate

Other Fates

α-Ketoglutarate

Succinate

Succinyl-CoA
Figure 9. Biosynthesis of dTDP-L-rhamnose from glucose-1-phosphate.
Figure 10. The Structure of RmlB. Top: Ribbon diagram of entire enzyme. Bottom: Detailed view of active site and substrates.
Figure 11. The active site of RmlB. Top left: Space filling view of the substrate and cofactor within the active site. Top right: Parallel ring arrangement of NADH and dTDP-D-glucose in the active site. Bottom: Proximity of important active site residues to the substrate and cofactor.
Figure 12. Proposed mechanism of action of RmlB.
Figure 13. The twelve putative inhibitors designed for RmlB.
Figure 14. Putative inhibitors designed against the oxidation step of the mechanism.
Figure 15. Putative inhibitors designed against the dehydration step of the mechanism.
Figure 16. Putative inhibitors designed against the reduction step of the mechanism.
Figure 17. Retrosynthetic analysis using a protected glucal as the starting material.
Figure 18. Retrosynthetic analysis using D-glucose as the starting material.

\[
\begin{align*}
28 & \quad \leftrightarrow \quad 29 & \quad \leftrightarrow \quad 30 \\
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\text{H}_2\text{C} \text{CH}_3 \text{C} \quad \text{O} \\
\text{O}
\end{array} & \quad \rightarrow & \quad \begin{array}{c}
\text{PGO} \quad \text{OPG} \\
\text{O} \\
\text{CH}_3 \text{O}
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\text{PGO} \quad \text{OPG} \\
\text{O} \\
\text{CH}_3 \text{O}
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\text{PGO} \quad \text{OPG} \\
\text{O} \\
\text{HO} \text{CH}_3 \text{O}
\end{array} \\
\begin{array}{c}
\text{P} \text{G} \text{O} \quad \text{OPG} \\
\text{HO}
\end{array} & \quad \rightarrow & \quad \begin{array}{c}
\text{P} \text{G} \text{O} \quad \text{OPG} \\
\text{O}
\end{array} & \quad \rightarrow & \quad \begin{array}{c}
\text{P} \text{G} \text{O} \quad \text{OPG} \\
\text{O} \\
\text{HO} \text{HO} \text{OH}
\end{array} & \quad \rightarrow & \quad \begin{array}{c}
\text{P} \text{G} \text{O} \quad \text{OPG} \\
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\text{P} \text{G} \text{O} \quad \text{OPG} \\
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\text{HO} \text{HO} \text{OH}
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\end{align*}
\]

\[
31 & \quad \rightarrow \quad \text{D-glucose}
\]
Figure 19. Synthesis of putative inhibitors 10, 9 and 12.
Figure 20. Suggested mechanism for the regioselective benzylidene ring opening reaction.
Figure 21. $^1$H Spectrum of Compound 19, 2, 3-O-acetyl-4,6-O-benzylidene-1,5-anhydro-D-glucitol.
Figure 22. $^{13}$C Spectrum of Compound 19.
Figure 23. gCOSY spectra of Compound 19.
Figure 24. HETCOR spectrum of Compound 19.
Figure 25. $^1$H spectrum of Compound 20, 2,3-O-acetyl-4-O-benzyl-1,5-anhydro-D-glucitol.
Figure 26. $^{13}\text{C}$ spectrum of Compound 20.
Figure 27. gCOSY spectra of Compound 20.
Figure 28. HETCOR spectrum of Compound 20.
Figure 29. $^1$H Peak assignments for compounds 19 and 20.
Figure 30. $^1$H spectrum of Compound 21, 2,3-O-acetyl-4-O-benzyl-6-deoxy-6-iodo-1,5-anhydro-D-glucitol.
Figure 31. $^{13}$C spectrum of Compound 21.
Figure 32. $^1$H spectrum of Compound 22, 2,3-O-acetyl-6-deoxy-1,5-anhydro-D-glucitol.
Figure 33. $^{13}$C spectrum of Compound 22.
Figure 34. 1H spectrum of Compound 10, 6-deoxy-1,5-anhydro-D-glucitol.
Figure 36. gCOSY spectrum of Compound 10.
Biography

The author was born on August 8, 1981 in Bridgeport, Connecticut. She grew up in various cities in southern California and graduated from San Clemente High School in 1998. She attended the Massachusetts Institute of Technology and received an S.B. in Chemistry along with a minor in Brain and Cognitive Sciences in 2002. While an undergraduate, she worked in the laboratory of Dr. Ann Graybiel on neurochemical and behavioral modifications induced by dopaminergic agents. After graduation, she joined the lab of Professor John Essigmann in order to pursue this project, which she designed during the spring of her senior year. As part of her master’s, she accompanied MIT faculty to Bangkok, Thailand to teach a course on Biotechnology and Engineering at the Chulabhorn Research Institute. Upon completion of her master’s degree in Molecular and Systems Toxicology from MIT in June 2003, she plans on entering the University of California, Berkeley in pursuit of a doctorate in Chemistry.