THE ANTI-MYCOBACTERIAL PROPERTIES OF SOUTH AFRICAN MEDICINAL PLANTS

A Dissertation

Presented to the Faculty of the Graduate School

of Cornell University

in Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

by

Tyi Lindsey McCray

January 2013

© 2013 Tyi Lindsey McCray

THE ANTI-MYCOBACTERIAL PROPERTIES OF SOUTH AFRICAN MEDICINAL PLANTS

Tyi Lindsey McCray, Ph. D.

Cornell University, 2013

Nature holds innumerable and often unexplored sources of chemical structures that have great potential for pharmacological applications. This project investigated plant-derived compounds for their potential use as new tuberculosis (TB) drugs. The challenges of TB drug development are attributed to the complexity of the disease: *Mycobacterium tuberculosis* (Mtb), the causal agent of TB, can reside in human tissues for decades without replicating, but with the potential of resuming growth and developing into active TB. Currently, there are no antibiotics that specifically target non-replicating Mtb. This project uses ethno-botanical knowledge to navigate the chemical diversity found in South African medicinal plants and to identify plant derived compounds with inhibitory activity against Mtb. While no plant extracts have previously been tested against non-replicating Mtb, our data demonstrates that compounds isolated from *Warburgia salutaris* (Canellaceae) are effective in killing both replicating and non-replicating Mtb. Furthermore, the compound class drimane sesquiterpenes, characteristic of the Canellaceae family, was confirmed as being responsible for the demonstrated biological activity. Microarray studies were also completed to gain insight into the mechanism of action of drimane sesquiterpenes compounds in *Mycobacteria*. The data suggests that drimane compounds increase the expression of those genes involved in the general stress response of the bacteria. Additionally, some of the microarray data suggests that these compounds may be targeting the cell wall.

BIOGRAPHICAL SKETCH

Tyi Lindsey McCray is a proud native of the Bedford Stuyvesant section of Brooklyn. She lived with her family on an amazing, landmarked cul-de-sac with great neighbors. In Brooklyn she attended the best public schools including, P.S. 11, Clara Cardwell Public School 308, Phillipa Schuyler Middle School and Brooklyn Technical High School. She attended the University of Maryland Baltimore County where she obtained a Bachelor's of Science in Chemistry.

Tyi entered a doctoral program in Plant Biology at Cornell University. Here she explored her interest in plants and how communities worldwide have used plants as medicine. Tyi first became interested in ethnobotany after participating in the Minority Health and Health Disparities International Research Training Program in Peru, the Dominican Republic and Venezuela in 2004, under the direction of Dr. Eloy Rodriguez. It turned out to be a transformative trip, during which she developed a love for plants and made lifelong friends.

While science has always been Tyi's primary interest, she is extremely committed to a life of service, both in and out of the classroom and laboratory. To that end, she has volunteered in the Cornell Prison Education Program at the Auburn Correctional Facility, in addition to carrying a hefty teaching load at Cornell as a chemistry recitation and laboratory teaching assistant and tutor.

DEDICATION

To Maida ‘Lovey’ Springer-Kemp and General ‘Buddy’ Washington

ACKNOWLEDGMENTS

I would like to thank my parents, Melvin and Jan for giving me the passion for learning and the imperative to succeed at everything I do. My sister, Jamē and my brother, Melvin served as my very first community of fellow scientists. Jamē's love of science was infectious and so, here I am.

I would like to also thank my advisor, Tom Owens and the other members of my committee, William Crepet and Melissa Luckow, for encouraging this line of inquiry, and for the countless hours spent discussing my research, and how to communicate it to the broad audiences to whom it would be applicable. This work was undeniably a collaborative labor of love, and I could not have gotten it done without their assistance

This dissertation would not have been possible without many members of the Cornell community, faculty, staff and collaborators, all of whom provided support over the years. I would especially like to acknowledge Terry Plater, Ralph Christy and John Terry. Peter Smith, Tracy Seaman, and Eliya Madikane at the University of Cape Town Medical School and Pakamani Xaba at the Kirstenbosh Botanical Garden were generous with their time and resources while I was in South Africa. Robert Abramovitch and David Russell at Cornell's Veterinary School were gracious with their time and intellectual guidance. Carl Nathan at Weil Cornell Medical School Department of Microbiology and the incredibly helpful members of his lab, Jean Schneider and Tamutenda Chidawanykia were instrumental to this project. I would be remiss to not acknowledge Albert M. Morrishow, Kurt Reynertson at Weill Cornell Medical School and the custodial engineer person who so shrewdly brought us together at the most critical point! There are scores of others, too many people to name individually, who were of great help during my Ph.D. program.

Lastly, I'd like to acknowledge: Nia Tyler, Felicia Grosvenor, Michael Chen-Illamos, Marie Cox and Steven Thomas, whose friendship over the decades is my most valued possession.

TABLE OF CONTENTS

APPENDICIES

LIST OF TABLES

LIST OF FIGURES

LIST OF FIGURES CONTINUED

LIST OF ABBREVIATIONS

2CS	Two Component Systems
ABC	ATP-Binding Cassette
BCG	Bacillus Calmette-Guérin
BSA	Bovine Serum Albumin
BSL2	Biosafety Level 2
BSL3	Biosafety Level 3
Cca	Concanamycin A
CDC	Center For Disease Control
DCM	Dichloromethane
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoixde
Dots	Direct Observation Of Treatment, shortcourse
ETH	Ethambutol
Etoac	Ethyl Acetate
HPLC	High Performance Liquid Chromatography
IC_{50}	Half Maximal Inhibitory Concentration
ICL	Isocitrate Lyase
INH	Isoniazid
Inos	Inducible Enzyme Nitric Oxide Synthase
LTBI	Latent TB Infection
MDR-TB	Multidrug Resistant TB
Meoh	Methanol
MIC	Minimum Inhibitory Concentrations
Mmpl5	Mycobacterium Membrane Protein Large
Mmps5	Mycobacterium Membrane Protein Small
MS	Malate Synthase
Mtb	*Mycobacterium tuberculosis*

LIST OF ABBREVIATIONS Cont'd

NCI	National Cancer Institute
NHS	N-Hydroxysuccinimide
NMR	Nuclear Magnetic Resonance
NO	Nitric Oxide
OADC	Oleic Acid Albumin Dextrose Catalase-Enriched
ORF	Open Reading Frames
PE	Proline-Glutamate
PGRS	Polymorphic GC-Rich Sequence
POA	Pyrazinoic Acid
PPE	Proline-Proline Glutamate
PZA	Pyrazinamide
RIF	Rifampicin
RND	Resistance Nodulation Division
RNI	Reactive Nitrogen Intermediates
SAR	Structure Activity Relationship
SDS	Sodium Dodecyl Sulfate
TB	Tuberculosis
TCA	Tricarboxylic Acid
UV	Ultra Violet
WHO	World Health Organization
XDR-TB	Extensively Drug Resistant

CHAPTER 1

Introduction

1.1. Overview

Tuberculosis (TB) is the leading cause of death worldwide from a single human pathogen, *Mycobacterium tuberculosis* (Mtb). Mtb is transmitted when an infected individual generates aerosol droplets, often through coughing or sneezing, which are inhaled by new hosts (Dye et al., 2005). The pathology of the disease is complex and not all those who are infected will develop the disease. It is estimated that one-third of the world's population, 2.2 billion people, are currently infected with the bacterium Mtb (Russell, Barry and Flynn, 2010a). Each year, approximately 9 million people develop the disease TB and nearly 2 million people die (Rivers and Mancera, 2008). While there are treatments available, these statistics illustrate the urgent need to develop more effective treatment options that are capable of eradicating the disease (Field et al., 2012).

Nature is a source of innumerable, complex and highly varied chemical structures. In the past, natural products have been successfully developed into pharmaceutical drugs, especially in the treatment of infectious diseases (Pan et al., 2010). This dissertation research project will use ethnobotanical knowledge, the study of the traditional uses of plants, to navigate the chemical diversity found in nature to identify individual compounds or classes of compounds with anti-Mtb properties. This project will further focus on South Africa, which is known for its plant diversity and for its population's uses of these plants in the treatment of disease. While several places fit these parameters, the country of South Africa is particularly suited for such this inquiry due to its high rates of TB infection (Dye, 2005).

The principal objective of this project is to use the unique knowledge of medicinal plants in South Africa to yield novel perspectives on the types of chemical compounds that should be considered further in the development of anti-TB therapies. This project will first address the

question: are there South African medicinal plants that are effective against replicating or non-replicating *Mycobacterium*? Secondly, this project will identify the specific compound(s) or classes of compounds responsible for the biological activity. Finally, this project will gain insight into the cellular targets of the active compounds. The results of this project will contribute to treatment of one the oldest and most difficult to treat epidemics.

1.2. Epidemiology

Mtb is an ancient human pathogen that has existed since the beginning of human history (Harries and Dye, 2006). There is genetic evidence that the bacterium has coevolved with humans since the time of early hominids between 2.6 and 2.8 million years ago (Gutierrez et al. 2005). Evidence of Mtb has been found in Egyptian mummies and descriptions of TB are found in ancient texts, such as "Of the Epidemics" by Hippocrates in 400 B.C. (Harries and Dye 2006; Mathema et al., 2006). Documentation of TB continued throughout history; however, the worst mortality rate was throughout the Industrial Revolution. During this time TB was responsible for one in four deaths in Europe (Lonnroth et al., 2009). TB was even referred to by writer John Bunyan as "the captain of all these men of death" (Bunyan, 1680).

Even before the first drugs were introduced, TB incidence began to decline throughout the early 20th century due to economic growth and the resulting improvements in nutrition and living conditions (Lonnroth et al., 2009). The introduction of the first TB therapies, beginning in the 1940's, led to a dramatic decline in TB morbidity and mortality (Grange et al. 2009). Industrialized countries were able to control the disease with large-scale screenings to identify new TB cases and effective treatment. By the 1980's developed countries considered TB a conquered disease (Raviglione and Pio, 2002).

The success of TB control in Western nations was not shared by less developed countries. The strategies of mass testing and specialized case management did not work in resource poor countries that lacked the health care infrastructure necessary to provide services to the their

entire population (Grange et al., 2009). Furthermore, TB drugs were not affordable to the most affected nations. This public health failure eventually converged with the HIV crisis and led to the current TB epidemic (Fatkenheuer et al. 1999). In 1993, the World Health Organization (WHO) officially declared TB a global health emergency (Zaman, 2010). Today, TB is the leading cause of death worldwide. The WHO reports that in 2009 there were 9.4 million incident cases of TB and 1.7 million deaths due to the disease (Zaman, 2010). TB continues to disproportionately affect resource-poor countries; South-east Asia, Africa and regions of the Western Pacific make up 35%, 30% and 20% of TB cases respectively (Dye et al., 2010).

1.3. Pathology

1.3.1 Disease Progression

Mtb is transmitted from person to person by inhalation of small droplets containing the bacterium (Harries and Dye, 2006). However, the presence of the pathogen does not necessarily lead to the development of the disease; two other outcomes are possible. A person can develop a non-symptomatic, non-contagious form of the disease termed latent TB infection (LTBI). The third outcome represents rare and largely uncharacterized cases in which the bacterium is completely destroyed by the host's innate responses. The path that the infection takes depends on several dynamic interactions between the host and pathogen. The narrative is not simple or perfectly linear; the interactions of several types of immune cells must be considered over different time scales (Young, Stark and Kirschner, 2008).

After initial exposure, the bacteria that reach the alveoli (the terminal air spaces of the lung) are phagocytized by macrophage and dendritic cells of the host immune system (Young et al., 2008). Dendritic cells are then used to signal T-cells, a type of lymphocyte, anywhere within 5 days to a few weeks post-infection, (Banchereau and Steinman, 1998; Cooper, 2009). T-cells are central in cell-mediated immunity and are responsible for stimulating inflammation and the activation of the macrophages (Stewart, Robertson and Young, 2003). T-cells migrate to the site

of the infection and surround infected macrophages, forming a granuloma or tubercule (Cooper, 2009). The structure of the granuloma serves to segregate the infection from the rest of the lung and provide a localized environment for the immune cells to act against the infection (Russell et al., 2010a).

In approximately 5-10 % of people infected with Mtb, the immune system of the host is not able to control the infection (Harries and Dye, 2006). When granulomas are not adequately formed, there is no encapsulation of the infection. The bacteria actively replicate and enlarging areas of necrosis of the lung tissue develop (Jagirdar and Zagzag, 1996). Over the course of one year, disease symptoms develop, including coughing blood, sputum containing viable bacteria, chest pains, fever and fatigue (Boshoff and Barry, 2005). TB is primarily a pulmonary disease; however, the bacteria can also spread to other organs (Flynn and Chan, 2001). The presence of these symptoms and actively replicating Mtb are what characterize the disease TB, sometimes referred to as active TB (Boshoff and Barry, 2005). Infection with HIV, various medical treatments, aging, and alcohol or drug abuse can negatively affect the immune response and increase an individual's chance at developing active TB (Flynn and Chan, 2001; Wayne and Sohaskey, 2001).

The most common result of Mtb infection, representing almost ninety-five percent of cases, is called a latent tuberculosis infection (LTBI). LTBI describes an individual that has been exposed to Mtb, the infection has been established and an immune response develops to control but not eliminate the pathogen (Parrish, Dick and Bishai, 1998). LBTI is effectively a stalemate between the host and the bacteria. The expression LTBI is widely used, although the term dormancy and non-replicating persistent bacteria are also used to describe the bacteria present in a person who has an Mtb infection but has no clinical evidence of the disease tuberculosis (Wayne and Sohaskey, 2001).

The immune response of the host to the pathogen is crucial in controlling the infection, preventing a latent Mtb infection from developing into active TB. In most cases, this response is sufficient to forestall active disease for a lifetime, but because the bacteria are not eradicated, the

infection is chronic (Flynn and Chan, 2001; Wayne and Sohaskey, 2001). Viable bacilli have been isolated from granulomas in the lungs of persons with clinically inactive tuberculosis, and even from cadavers, indicating that Mtb can persist in humans for many decades (Wayne and Sohaskey, 2001). Non-replicating bacteria can reactivate years later if the host immune response fails (Wayne and Sohaskey 2001; Pieters, 2008).

1.3.2 Understanding the Challenges of Latent Tuberculosis Infections (LTBI)

The understanding of how the immune system is able to control, but not eradicate Mtb, is nascent and far from complete. In response to immune activation, Mtb enters a non-replicating state characterized by growth arrest and altered metabolic activity (Flynn and Chan, 2001). Investigations have revealed many of the conditions that induce latency including, but not limited to: low pH, reduced carbon availability, hypoxia, and the presence of reactive nitrogen intermediates (RNI) (Wayne and Sohaskey, 2001). Not all of these conditions pertain to all the Mtb present in the host due to heterogeneity between different granulomas and even heterogeneity within the same granuloma (Russell et al., 2010b). The evidence for each of these conditions is described below.

- Low pH- In the host, Mtb resides primarily in macrophages, more specifically in the macrophage's phagosome, the vacuole where all ingested materials initially reside. In the phagosome of an activated macrophage, the pH ranges from a pH of 6.2 to 4.5 depending on the activation state but a pH below 5.5 is associated with cessation of growth of pathogens *in vitro* (Vandal, Nathan and Ehrt, 2009). Further evidence that Mtb is exposed to an acidic environment is that pyrazidamide, an antibiotic that kills Mtb in vitro only under acidic pHs, is effective *in vivo* (Vandal et al., 2009). Also, the expression of acid responsive Mtb genes is increased during the infection of macrophages (Rohde, Abramovitch and Russell, 2007).

• Reactive nitrogen- Mtb-infected macrophages in humans contain the inducible enzyme nitric oxide synthase, which produces nitric oxide (NO), a lipid- and water-soluble radical gas (MacMicking, Xie and Nathan, 1997). NO reacts in water with oxygen and yields other radicals, including higher oxides, and unstable peroxides. The production of these reactive radicals by activated macrophages is considered to be a major anti-microbial response of the host defense (Flynn and Chan, 2001). MacMicking et al. (1997) describe the contribution of nitric oxide and its derivatives to the functions of the activated macrophage as including: 1) the formation of nucleophilic adducts that deaminate nucleosides and cause mutation and 2) the ability to form species that oxidize thiol groups, lipids, DNA, and nitrated tyrosines. Other studies demonstrate that the products of RNI exert a bacteriostatic effect at low concentrations and a bactericidal effect at high concentrations (Firmani and Riley, 2002).

• Hypoxia- It is widely accepted that Mtb in macrophages exist in a low oxygen environment based on the relative absence of blood vessels in TB lesions and the poor oxygen permeability of necrotic tissues (Wayne and Sohaskey, 2001). Furthermore, *in vitro* models involving controlled reduction of oxygen have shown that Mtb establishes a non-replicating state. The bacteria can begin replicating again when conditions become more favorable (Russell et al., 2010b). Mtb grown under hypoxic conditions was also found to be less susceptible to commonly used antibiotics such as isoniazid, rifampin and ethambutol.

• Carbon source - The sequestration of the bacteria primarily in macrophages limits the bacteria's access to sources of carbon. In turn, Mtb switches to the utilization of lipids and fatty acids as alternate carbon sources (McKinney et al., 2000). The full significance of these alterations in lipid metabolism is not known; however, there are several observations have been made regarding the use of lipids as a carbon source within the host. It is known that Mtb uses the glyoxylate cycle to retain carbon when growing on fatty acids as the limiting carbon source. The unique enzymes of this route are isocitrate lyase (ICL) and malate synthase (MS). ICL cleaves

isocitrate to glyoxylate and succinate, and MS converts glyoxylate and acetyl-CoA to malate. The end products can be used for gluconeogenesis and other biosynthetic processes (Dunn, Ramirez-Trujillo and Hernandez-Lucas, 2009). It is believed that tubercle bacilli catabolize a wide range of fatty acids, since they possess a large number of genes involved in this metabolism (Cole et al., 1998). Additionally, bacteria defective in different genes important for lipid metabolism, and therefore unable to make this metabolic shift, are shown to be avirulent (Russell et al., 2010b)

The conditions that Mtb experience *in vivo* are important because it is within the context of these different environments that TB drugs ideally need to function (Russell et al., 2010b). During LTBI, most Mtb are non-replicating and during active TB the some bacteria are replicating and some are non-replicating (Nathan et al., 2008; Boshoff and Barry, 2005). Conventional bioassays used in developing antibiotics, including those used to produce the currently used TB drugs, assay bacteria under growth sustaining conditions and assay for inhibition of that growth (Walsh, 2003; Nathan, 2004). Drugs created using these parameters are not as effective on non-replicating cultures (Stewart et al., 2003).

1.4. Tuberculosis Treatment

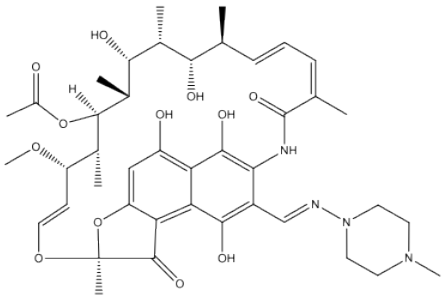
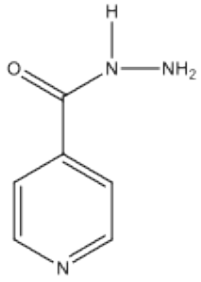
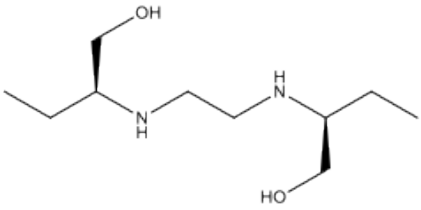
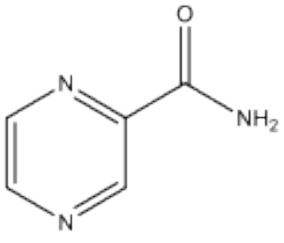
1.4.1 Current Treatment Options

The current TB treatment regimens as described by the WHO and the Center for Disease Control involve a combination of three or four antibiotics taken daily for two months during an initial intensive treatment phase, followed by two drugs taken for an additional 4-7 months in the continuation or sterilizing phase (Blumberg et al., 2003; Duncan, 2004). This 6-9 month regiment is called ‘short-course therapy’ in comparison with the original regiments of the 1950’s that took 12-18 months (Blumberg et al., 2003). However current treatments are still considered lengthy, cumbersome, and must be completed in order to be successful in eradicating LTBI. The

four antibiotics prescribed for the initial treatment of TB, named first-line drugs, are rifampicin (RIF), isoniazid (INH), pyrazinamide (PZA) and ethambutol (ETH) (Table 1.1). RIF and INH are effective with low dose requirements, easily administered orally and are always included in the initial 2-month intensive treatment phase (Blumberg et al., 2003).

- The rifamycins are a class of broad-spectrum antibiotics isolated from the bacterium *Amycolatopsis mediterranei* (formerly *Streptomyces mediterranei*) in 1959 and introduced into therapeutic use in 1968 (Sensi, 1983). This compound class, which includes the drug rifampicin, specifically inhibits bacterial RNA polymerase, the enzyme responsible for prokaryotic DNA transcription, by forming a stable drug-enzyme complex (Campbell et al., 2001). DNA-dependent RNA polymerase is an enzyme with an α2, β, β‘, σ subunit structure. The binding site for rifampicin has been located at the β subunit encoded by the bacterial rpoB gene (Floss and Yu, 2005). Bacterial resistance to RIF is caused by mutations leading to a change in the structure of the β subunit of RNA polymerase. There are over 35 amino acid substitutions that can lead to RIF-resistance (Shin et al., 2005).
- Vital Chorine, a French doctor, observed that nicotinamide, a water-soluble vitamin in the vitamin B group inhibited Mtb (Fox, 1952). This led to the development of structural analogues of nicotinamide including INH. INH is a prodrug, metabolized by the endogenous mycobacterial enzyme catalase-peroxidase via peroxidation to produce a range of carbon-, oxygen- and nitrogen-centered free radical species (Mdluli et al., 1996). The free radicals of INH form adducts with both NAD+ and NADP+ and the resulting products are inhibitors of a long-chain enoyl-acyl carrier protein reductase, InhA (Rozwarski et al., 1998). InhA is involved in the synthesis of mycolic acids, the unique and important component of the mycobacterial cell wall (He, Alian and Ortiz de Montellano, 2007).

Table 1.1. First-Line Tuberculosis Drugs

Rifampicin	
Isoniazid	
Pyrazinamide	
Ethambutol	

• Pyrazinamide (PZA), like INH, is an analogue of nicotinamide. Originally, PZA showed negligible activity against Mtb in laboratory cultures, but was found to be highly effective against mice infected with TB (McKenzie et al., 1948). It was later found that PZA has the ability to kill non-replicating Mtb that resides in acidic environments (Zhang et al., 2003). It has taken sixty years since the discovery of PZA for major progress to be made in determining the mode of action of the drug. It was known that PZA is a prodrug hydrolyzed intracellularly to pyrazinoic acid. Recently, Shi et al. (2011) determined that POA ultimately inhibits trans-translation, the process that removes stalled translational complexes, recycling functional ribosomes for subsequent use (Keiler, 2008). It is believed that the ability of PZA to inhibit the trans-translation process interferes with survival of Mtb under stress conditions (Shi et al., 2011). The introduction of PZA into clinical use played a major role in shortening the duration of TB therapy.

• Ethanbutol (EMB) is a first-line drug, however it makes a modest contribution to the fight against TB compared to the other first-line drugs. It is primarily used to treat people who may have multi-drug resistant Mtb, until their exact bacterial strain has been identified (Jonsson et al., 2011). Even as recently as the comprehensive review by Karakousis (2009), the mechanism of action of EMB was not completely understood. It is believed that EMB functions primarily through the inhibition of arabinogalactan biosynthesis, a structural component of the mycobacterial cell wall (Takayama and Kilburn 1989; Mikusova et al. 1995).

To increase the likelihood of proper and complete treatment of TB, the WHO in 1995 initiated 'direct observation of treatment, short-course' (DOTs) where trained personnel supervise the dispensation of medication to help increase patient compliance with treatment programs (Glaziou et al., 2011). The DOTs program has improved treatment outcomes, but alone it cannot create the progress needed to control the disease. The lack of completion of the treatment protocols has contributed heavily to the development of resistant strains. This is

because resistant strains that develop spontaneously then become established in the bacterial population (Goldman, Plumley and Laughon, 2007).

There are Mtb strains that are resistant to both INH and RIF, called multi-drug resistant TB (MDR-TB). In these cases, there are six classes of drugs that can be used as second option, called second-line drugs. The treatment of MDR-TB can require antibiotics to be administered for up to two years. MDR-TB is prevalent in countries of the former Soviet Union, South Africa, and China and is currently responsible for an estimated 490,000 incident cases of TB and 110,000 deaths worldwide each year (Banerjee, 2008). In 2006, the term extensively drug resistant TB (XDR-TB) was used to describe bacteria classified as MDR-TB that are also resistant to three or more of the second-line drugs (Goldman et al., 2007).

1.4.2 New Treatment Options

No new TB drugs have entered into clinical use in 40 years and structural modifications to first-line drugs have not allowed scientists to stay ahead of resistance (Mills, 2006). New drugs are needed to shorten the treatment of active disease by addressing the challenge of LTBI as well as addressing resistance to improve treatment of MDR-TB and XDR-TB with new modes of action (Lange et al., 2007). There are new drugs on the horizon at various stages of clinical trials (Glickman et al., 2006). For example, fluoroquinolones are antibiotics used in the treatment of several diseases because it has the ability to inhibit DNA gyrase, an essential bacterial enzyme that maintains super-helical twists in DNA (Wolfson and Hooper, 1985). This compound class is being investigated for its potential in TB therapy. There are other compounds, including diarylquinoline and nitramidazoles that represent compound classes with new mechanisms of action. These two drugs are being tested in clinical trials and are briefly discussed below. However, the mechanisms of action and targets of the other potential new drug classes, such as diamine and pyrrole, have not been fully established. Despite these new possibilities, the need for new antibiotics will continue as inevitable mutations will eventually allow Mtb to overcome each new agent synthesized.

• Diarylquinolines, particularly TMC207 (formerly R207910), is a compound class that offers a new mechanism of action by specifically targeting the proton pump of mycobacterial ATP synthase (Spigelman, 2007). *In vitro*, TMC207 potently inhibits drug-sensitive and drug-resistant Mtb isolates and is also bactericidal against non-replicating tubercule bacilli.

• Nitromidazoles do not represent a new drug class, however, they represent a new mechanism of action in Mtb. Within this compound class PA 824 and OPC67683 are in clinical development (Spigelman, 2007). The nitroimidazole class is derived from a natural product called azomycin (2-nitroimidazole), isolated from a streptomycete in the 1950s. The mechanism of action of this class is not well understood, however it is believed to acts as a prodrug requiring reductive activation of the aromatic nitro group to exert its antibacterial effect (Spigelman, 2007).

1.5. Natural Products, a Source of New Pharmaceuticals:

1.5.1 Past History of Natural Products in Drug Development

The innovative chemical structures found in nature can be used as a resource in the development of novel antibiotics, including those compounds with anti-mycobacterial properties. Drugs of natural origin are defined as compounds: 1) in their original, natural form; 2) derived semi-synthetically from natural products; or 3) synthetic products based on natural product models (Cragg, Newman and Snader, 1997). While this discussion focuses on plant-derived compounds, natural products can be isolated from a variety of sources, including bacteria, fungi, plants and animals. Plants have been successfully used in the treatment of disease throughout history. Indeed, written evidence of plants being used as analgesics dates back 2300 years when Theophrastus wrote about ‘the juice of poppy’, referring to opium poppy, *Papaver somniferum*

(Newman, Cragg and Snader, 2000). The isolation of morphine from opium was the first reported isolation of a natural product for medicinal purposes (Huxtable and Schwarz, 2001).

Natural products have been essential to the development of medicines. The WHO compiled a list of what it considers to be essential medicines, approximately 300 distinct drugs considered to be essential for a basic healthcare system (Reidenberg, 2010). This list includes nearly 210 small molecules and 139 are classified as natural products, 44 are unmodified natural products, 25 are semi-synthetic derivatives of natural products and over 70 are synthetic drugs based on natural products. Notable examples of the past success of natural products include aspirin derived from the willow tree, morphine from opium poppy and digitalis, a heart medicine extracted from the *Digitalis* genus (Mahdi, Mahdi and Bowen, 2006; Hauptman and Kelly, 1999). In treatment for cancer and infectious diseases, 79% and 75% of these drugs, respectively, are of natural origin (Newman, Cragg and Snader, 2003; Newman and Cragg, 2012).

1.5.2 Where Does The Chemical Diversity In Nature Come From?

The chemical diversity found in plants comes from secondary metabolites, low molecular weight, organic compounds that are not involved in nutrition or essential metabolic processes (Maplestone, Stone and Williams, 1992). Over one hundred thousand compounds have been identified and extracted from 400,000 flowering plant species, and this represents a small fraction of the total universe of compounds that are thought to exist (Goossens et al., 2003). There were several theories proposed to explain why secondary metabolites evolved in plants. The most widely accepted view is that secondary metabolites arose stochastically, via enzymes with broad specificity and chemical reactions that yield multiple products (Firn and Jones, 2003). The end result is the production of some compounds that are useful and others with no apparent function. This hypothesis is based on the observation that biological activity appears to be a rare property for any secondary product to have. However, some compounds do have adaptive significance including floral scent volatiles, pigments used to attract insect pollinators, and toxic chemicals to protect the plant from pathogens and herbivores (Stamp, 2003). It can be concluded

that evolution has favored those organisms that can generate and retain chemical diversity at low cost. Those organisms that survived are those that can produce a compound or compounds, amongst the many, that meet the challenges of their environment. Different ecological environments pose different challenges, and the secondary metabolites are likely to vary extensively from species to species and in different ecological environments (Firn and Jones, 2000).

1.5.3 How Can Researchers Generate Chemical Diversity?

Scientists trying to identify a chemical structure with a desired biological activity often follow the example of nature and attempt to assemble large numbers of compounds with a range of structural diversity. One method of doing this is to sample the chemical diversity found in nature through the collection of plant extracts and subsequent isolation of compounds with demonstrable properties. The establishment of chemical libraries has been successful in identifying natural products effective in the development of drug treatments. For example, the National Cancer Institute (NCI) developed a large chemical library of natural products and natural product derivatives screening program with the goal of identifying new anti-cancer compounds (Cragg et al., 1993). From 1960 to 1981, NCI collected and screened approximately 35,000 plant species. The Pacific Yew (*Taxus brevifoli*) was included in this screening program, leading to the identification of paclitaxel, commonly known as Taxol®, one of the most important chemotherapeutic agents against breast and ovarian cancers (Nicolaou et al., 1994). Camptothecin, isolated from the tree *Camptotheca acuminate*, is also an anti-cancer drug used in traditional Chinese medicine, and discovered as a result of the NCI screening program (Wall et al., 1966; Wall and Wani, 1996). These two drugs alone represent one third of the drugs used globally to inhibit growth of malignant cells (Jones, Chin and Kinghorn, 2006).

Another method of amassing a large chemical library is through the creation of a combinatorial library. Combinatorial libraries are created through the reaction of a small number of starting compounds with a larger number of reagents, and subsequently recycling the products

from each iteration with the same reagents, exponentially increasing the number of compounds (Lam, Lebl and Krchnak, 1997). This method was considered easier than isolating natural products. However, this method has not met expectations, and there are questions regarding the effectiveness of combinatorial libraries in drug creation (Horton, Bourne and Smythe, 2003). To date, only one *de novo* combinatorial compound in 25 years has been developed into a drug, Nexavar, used in kidney cancer treatment (Grabowski, Baringhaus and Schneider, 2008).

1.5.4 Why Are Natural Products 'Privileged' Structures?

Several studies have thoroughly compared the characteristics of natural products with that of synthetic compound libraries in an attempt to better understand the advantages of compounds from nature. The failure of combinatorial libraries has been attributed to the lack of biological relevance of the synthetic scaffolds (Kaiser et al., 2008). In contrast to synthetic compounds, natural products are considered 'privileged' structures because they were created with the ability to bind to biological macromolecules (Koehn and Carter, 2005; Firn and Jones, 2003). Spatial structure and fold types of proteins are fairly conserved throughout evolution; natural products may have the ability to bind to a variety of protein domains and folding motifs across many species and have a range of functions (Koch et al., 2004; Bon and Waldmann, 2010).

Koehn et al. (2005) explored the chemical scaffolds and pharmacologic properties of natural products to understand the physical differences that may be the basis of 'privileged structures'. Their paper points out that natural products were typically shown to have a greater number of chiral centers and increased steric complexity compared to either synthetic drugs or those compounds produced in combinatorial libraries. Natural products were also found to have a broader distribution of molecular properties (i.e. molecular mass, octanol-water partition coefficient and diversity of ring systems).

1.6. Using Ethnobotany to Navigate Chemical Space

While nature is a rich source of novel chemicals with pharmacological potential, identifying natural products with biological activity is fraught with difficulties (Spellberg et al., 2004). The organic chemist Friedrich Wöhler described organic compounds:

> "Organic chemistry [is] enough to drive one mad. It gives me the impression of a primeval forest full of the most remarkable things, a monstrous and boundless thicket, with no way of escape, into which one may well dread to enter."

Wöhler correctly alluded to the large number of compounds that exist in nature, the marvelous diversity of their structures and the trepidation many researchers feel when trying to make sense of the vast chemical space. Notwithstanding the obvious advantage of natural products, it is not feasible to sample all existing natural compounds. There is a need for a focused approach to identify a compound or compound class relevant to a particular biological phenomena (Bon and Waldmann, 2010). One such method is the use of ethnobotany, a term coined by the American botanist William Harshberger, to describe the study of how different human populations use plants, including but not limited to the use of plants as medicine (Heinrich and Bremner, 2006).

Some prominent examples of medicinal plants that have been developed into drugs are: the previously mentioned Pacific Yew (*Taxus brevifolia*) which was used by American Indians to treat arthritis and other ailments and *Artemisia* species, which are popular medicinal plants in many parts of the world that have been developed into treatments of malaria (Wani et al., 1971; Tan, Zheng and Tang, 1998). In fact, it has been noted that among all the drugs that have plant origins, 77% of the plants were used in traditional medicine (Rollinger, Langer and Stuppner, 2006). The means by which these and other plants were originally selected by humans for treating various illnesses are not known (Fabricant and Farnsworth, 2001). The treatment choices in traditional medicine are complex and involve social, cultural, historical and linguistic

considerations that are not fully understood by those outside the culture (Heinrich and Bremner, 2006). Furthermore, many ethnobotanical studies describe the uses of medicinal plants based on discussions with a shaman or herbalist who combines the roles of pharmacist and medical doctor with cultural and religious beliefs. Many of these herbalists believe that, along with divine elements, a potpourri of ingredients serves to provide the overall activity and clinical efficacy cannot be attributed to a single ingredient (Phillipson, 1994).

The fusion of the scientific and the spiritual causes many laboratory-based scientists to view ethnobotany as an exotic and exciting area, but not one they consider to be of relevance to their research (Heinrich and Bremner, 2006). Other scientists regard traditional belief systems as magic or mysticism, thus lacking credibility (Fabricant and Farnsworth, 2001). Even those scientists that fall in the middle of the spectrum have fundamental differences with the traditional holistic approach to medicine because they believe that traditional remedies should be investigated to identify a single active ingredient (Wang et al., 2012). No matter where on the scale one may fall, it is agreed that the continued use of certain plants over centuries empirically demonstrates that they contain compounds with biological activity in humans. According to the WHO, almost 65% of the world's population has incorporated traditional remedies in to their primary modality of medical care (Fabricant and Farnsworth, 2001).

The example of Taxol® and other drugs demonstrate an important fact about the contribution of ethnobotany: the traditional uses of a plant are not always germane to the modern therapeutic indication for the derived drugs (Durzan, 2009). Therefore the knowledge obtained as result of an ethnobotanical study should not be limited to the traditional uses of the plant (Heinrich and Bremner, 2006). The information regarding indigenous uses of plants can be used as a tool to help scientists navigate 'the boundless thicket', steering them in the direction of plants that are of pharmacological value (Jones et al. 2006).

1.7. South Africa: A Case Study for Plants Used Ethnobotanically in the Treatment of Tuberculosis

Even when ethnobotanical information is used to systematically explore plants with pharmacological potential, there is still a chemical space too vast to completely explore. Researchers often limit investigations of ethnobotanically used plants to a specific region of the world. An ideal location would be a region known for its plant diversity and one that has a population adept at using these plants as medicine. The country of South Africa is particularly suited for such an inquiry into ethnobotanical treatments for TB, due to not only its biodiversity, but also its high rate of TB infection.

1.7.1 Plant Species Diversity

South Africa is the third most biologically diverse country on earth, with some 18,625 vascular plant species within its boundaries (comprising 7.5% of the world's total), of which 80% are endemic (Kepe, Saruchera and Whande, 2004; Cowling and Rundel, 1998). This plant diversity and endemism is largely attributed to the diverse ecological conditions of southern Africa relative to the rest of the continent. South Africa is divided into nine biomes, with a wide range of ecosystems, including areas that range from extreme desert to subtropical and have extreme heterogeneity of geology, soils and elevations (Egoh et al., 2009). South Africa is home to the succulent Karoo biome, which has the highest diversity of succulent plants in the world and is the most species-rich semi-desert. Also of note is the summer rainfall Grassland biome that is home to a wealth of species limited to southern Africa (Mucina and Rutherford, 2006). This diversity of biomes is remarkable for a relatively small country of 471,011 sq mi, roughly twice the size of Texas.

1.7.2 Ethnobotanical Knowledge

The diversity of plant species in South Africa is equally matched by a rich cultural diversity. Each of South Africa's ethnic groups has developed a tradition of using plants in the treatment of disease for more than four centuries. Black Africans account for 79% of the total population, with the largest ethnic groups being the Zulus, the Xhosa, the Northern Sotho and the Southern Sotho (McLaughlin, 2007). White South Africans, who are mainly descendants of British and Dutch settlers, are the second largest group. The remainder of the population identify themselves as mixed race at 8.9%, and Asian descendants of Indian, East Indian and Chinese indentured laborers at 2.5%. The tradition of using plants as medicine by all South Africans is still prevalent today. Currently, over half of the population use traditional healers as their primary source of care (van Wyk, 1997). Traditional healers have considerable stature and are formally recognized by the government alongside national healthcare providers (van Niekerk, 2012).

1.7.3 High Incidence of Tuberculosis

In addition to a rich history of using plants in the treatment of disease, South Africa also has one of the highest incidences of TB in the world (Dye, 2005). This is the unfortunate result of political and social norms that combined to create circumstances that promotes the spread of TB. Centuries of colonialism, followed by nearly fifty years of racial segregation resulted in overcrowded squatter settlements, the creation of a migrant labor class, and underdeveloped health services for black South Africans (Karim et al., 2009). When migrant workers, who temporarily lived in the cities, made regular visits to families in rural homelands, they spread TB and sexually transmitted diseases across the country. South Africa has 400,000 new cases of tuberculosis each year and in some regions over 10% of the cases are MDR-TB strains (Karim et al., 2009). The TB crisis is exacerbated by the HIV epidemic because it creates an immune-suppressed population that is particularly vulnerable to infection and unable to combat

replication of Mtb. South Africa also has a rich history of using plant medicine in the treatment of diseases including TB.

1.8. Challenges in Natural Products Research

While nature is a potential source of new compounds with pharmacological properties, identifying natural products with specific biological activity is fraught with difficulties. There are technical barriers that accompany any natural products research effort. Taxol® can be considered a success story, demonstrating how ethnobotany can assist in the identification of the biological resources to be found in nature. However, it also can be considered a cautionary tale, and examples from its discovery process will be used to illustrate some of the difficulties in natural products research.

1.8.1 The Plant Extraction and Fractionation Process

The investigation of plants for biological activity is a lengthy, multi-step extraction process followed by the identification of active compounds required to investigate a plant for biological activity (Bueno Sanchez and Kouznetsov, 2010). The steps of a plant extraction have changed little since being described in the first issue of the *Journal Plant Physiology* almost a century ago (Newton, Brown and Martin, 1926). While there are variations, the main steps involve the drying or lyophilization of the plant material, sequential extraction with solvents of different polarity and evaporation under vacuum pressure. This process is not easily modified to fit a high throughput model, making it unattractive for time sensitive projects and/or profit driven models.

The active compound(s) of a plant extract are identified via bioassay-guided fractionation, where the biological activity of an extract is assessed after alternating steps of fractionation and biological testing. The fractionation steps can be chemical and/or chromatographic. Fractionation is labor intensive and requires large quantities of plant material

because each step of the process significantly reduces the material yield. The initial isolation of the active component from the pacific Yew took four years; subsequent isolations took nine months to a year (Cragg et al., 1993). Fifteen thousand pounds of bark were required to yield 1.3 kg of the Taxol®. In 1991 alone, over 1.6 million pounds of bark was harvested for Taxol® production, causing concern about the survival of the Pacific Yew population (Wall and Wani, 1995).

1.8.2 Access and Supply of Plant Material

Population growth, urbanization and the unrestricted collection of medicinal plants from the wild has resulted in the over-exploitation of some species (Zschocke et al., 2000). There are concerns about the loss of biological diversity and the continued availability of medicinal plant resources (Hamilton, 2004). Ethnobotanist Walter Lewis made the following comment that is still true today (Lewis and Elvin-Lewis, 1995):

> "Serious dangers exist for the survival of such peoples and their cultures, and the ecosystems which nurture them and provide Western and traditional medicines with novel plant products for human well being everywhere."

Human behavior is believed to have caused the current and sixth known period of mass extinction with 8% of all plants threatened with extinction, including 4000 species used in traditional medicine (Rahbek and Colwell, 2011; Schippmann, Leaman and Cunningham, 2002). While one solution is large-scale cultivation, it is not viable in the short term to supply the quantities of plants required for both research and traditional uses (Zschocke et al., 2000). There are few cultivated medicinal plants. China is probably the country with the greatest cultivation of medicinal plants. In South Africa, 99% of those plants sold for traditional medicine are collected from the wild (Hamilton, 2004).

There are also restrictions to the availability of plant material due to intellectual property concerns. At the moment, most laws and regulations regarding intellectual property rights are not able to protect traditional knowledge and indigenous peoples (Kartal, 2007). This is because it is difficult to define intellectual property with regards to plant knowledge and to establish ownership (Kartal, 2007). Traditional knowledge of the uses of plants can be based on years, perhaps millennia, of use and experimentation. In the past, scientists, research institutes and commercial enterprises have taken samples of without the proper permission or on ethically unacceptable terms. It is often argued by proponents of indigenous rights that scientists or pharmaceutical companies cannot claim to be the sole inventors of any resulting product (Hamilton, 2004). Unfortunately, without a practical solution, some countries have reacted to the threat of theft of intellectual property by creating such tight access restrictions that they are likely to cause setbacks to research (Gupta, Gabrielsen and Ferguson, 2005). Many researchers afraid of a legal battle have avoided this type of research.

1.8.3 Difficulties and Solutions in Compound Identification

The occurrence of natural products as a mixture of structurally related compounds has historically made the isolation and identification of active compounds a challenge (Koehn and Carter, 2005). In the past, the identification of new natural products relied on the ability to isolate the compound of interest with a high degree of purity (Jaroszewski, 2005). Nuclear magnetic resonance (NMR) could only be used for structural identification after successful separation of sufficient amounts of a given compound. However, technological advances have made it so that less compound is needed and that complete separation is no longer necessary (Koehn, 2008).

There have been technological advances that have made the identification of compounds easier. Most notably, progress in NMR has allowed for the elucidation of the structure of compounds that would previously not have been possible. First, there have been improvements in the sensitivity of NMR through the use of stronger magnetic fields. This has dramatically

reduced the amount of material needed for structural analysis. Secondly, the use of pulse sequences in NMR can be used to produce diagnostic signals that provide scalar (through bond) and dipolar (through space) molecular connectivity correlations that can be used to determine the structure of the compound (Eisenreich and Bacher, 2007). To date, thousands of pulse sequences have been developed; each designed to ascertain a different type of physical information about a compound. Thirdly, the application of these technologies has led to the development of techniques that make it possible to identify compounds in increasingly complex mixtures (Schroeder et al., 2007). Although the determination of structures is technically challenging and resource intensive, the aforementioned advances make compound identifications possible that previously were not.

1.9. Plant-derived Compounds with Anti-mycobacterial Activity: Limitations in Previous Studies

A survey of the literature suggests that there are approximately of 400 reports of plants of global origin, and thousands of isolated phytochemicals that have been tested in some capacity for anti-tubercular activity (Newton et al., 2000; Pauli et al., 2005). The discussion to follow will highlight the limitations of these studies and consider improvements that can be made for future inquiries. More consideration is needed in the following areas: design and implementation of the biological assays used to evaluate the plant extracts and compounds, addressing the issue of latent TB and the inclusion of target analysis and structure activity relationships (SAR) studies (Thomson et al., 2004).

1.9.1 Biological Assays: *Mycobacterium* species

The genus *Mycobacterium* is divided into fast-growing species and slow-growing species. Most slow-growing members of the genus, including Mtb, are pathogenic to humans (McGaw, Lall, Meyer and Eloff, 2008). Research involving Mtb requires the Bio-safety Level 3

facilities to safely handle airborne human pathogens (Heifets and Desmond, 2004). Many institutions do not have the resources to work with Mtb and as a result choose to work with *M. smegmatis*, a fast-growing species that is non-pathogenic and requires no special safety considerations. The results of experiments using fast-growing *Mycobacterium* species are not always an accurate indicator for how effective a compound is against Mtb (McGaw et al., 2008). The better alternative is to use *M. bovis* BCG which is closely related in genetic composition, pathogenic to cattle but not to humans, and only requires a class 2 biosafety cabinet, which most institutions can accommodate (Gutierrez et al., 2005). The drawback of work with *M. bovis* BCG is that it is a slow-growing bacterium, with a doubling time of 15-20 hours; many experiments require a minimum of two weeks to obtain results.

1.9.2 Addressing the Challenge of Latent Tuberculosis Infections

The goal of current TB research is to identify antibiotics that are effective against non-replicating Mtb in order to reduce the duration of therapy. Such a compound would have to be effective under the growth conditions that Mtb encounters in the host. Those conditions include, but are not limited to, a reduced pH, the presence of RNI, decreased oxygen and limited carbohydrate availability. Past research into anti-mycobacterial compounds derived from plants still uses screening methods that involve *in vitro* conditions that sustain rapid growth and assaying for inhibition of that growth (Nathan 2004). Subsequently, most antibiotics, including those currently used to treat TB, target biosynthetic processes that bacteria need to replicate and increase their biomass. Only two of the first-line treatments for TB, rifampicin and pyrazidamide, have any efficacy under conditions that prevent the bacteria from replicating (Somoskovi, Parsons and Salfinger, 2001). New assays are available and have been used by microbiologists. However, it has not permeated into natural product studies (Parrish, Dick, and Bishai, 1998; Wayne and Sohaskey, 2001). Furthermore, no publications were found that discussed the inhibitory activity of plant extracts against non-replicating Mtb.

1.9.3 Structure Activity Relationships (SAR) Studies

Drug discovery efforts begin with compounds that have demonstrated inhibition in the micro-molar range progress in drug discovery efforts (Nathan, 2004). This parameter eliminates many plant-derived compounds from consideration since few isolated principles have activity below the micro-molar range. To improve inhibitory activity, structure activity relationship (SAR) studies are needed. The process involves a systematic comparison of structural modifications to active molecules in relation to their biological activity. SAR studies have the potential to create structural analogues whose inhibitory activity is increased by two orders of magnitude when compared to the original structure (Pauli et al. 2005). SARs can also be used to improve biological activity such as membrane permeability or increase solubility (Terstappen, et al., 2007). Camptothecin, an anti-leukemic and anti-tumor drug, progressed in the drug discovery process because of SAR results (Wall and Wani, 1995). Clinical trials were originally restricted because of insolubility, but extensive structure-activity studies identified analogs of camptothecin with equal or better anti-tumor activity and with better solubility properties.

Unfortunately, most natural products research projects do not include SAR studies (Pauli et al., 2005). SAR for natural products can be arduous. It can take a team of medicinal chemists years to make modifications that improve potency due to the complex structure and multiple chiral centers of many natural products (Nathan, 2004). In lieu of these investigations, inferences can be based on comparisons of the biological activity of available compounds that are in the same structural class (De Souza et al., 2007). These conclusions are limited, especially if they are based on heterogeneous protocols.

1.9.4 Investigation into the Mechanism of Action

To adequately address the problem of resistance, compounds with new mechanisms of action are needed. However, with few exceptions, analysis of molecular targets or intercellular mode of action is almost completely absent from natural product studies (Pauli et al., 2005). One approach to identifying the target of antibacterial compounds with an unknown mode of action is

to isolate spontaneously resistant mutants and identify the genetic basis of resistance (Mills, 2006). Recently developed genetic tools are facilitating this line of inquiry through improved bioinformatics and automated genome sequencing (Bansal, 2005). With these tools it is possible to identify a gene that confers resistance and develop an understanding about its function. Another approach is the use of DNA microarray analysis to look at the transcriptional response of the bacteria to the drug (Rosamond and Allsop, 2000). Comparing the differential expression of genes as a result of exposure to a compound of interest can help identify the genes and pathways that are important for bacteria to persist when exposed to an inhibitor (Waddell and Butcher, 2007).

Investigations into the mode of action can also serve additional purposes. These studies can also be used to augment inhibitory data. There was not much initial interest in Taxol® because it only had modest activity and was insoluble in water (Kingston, 2007). Taxol® only advanced through clinical trials after Susan Horwitz discovered its mechanism of action (Schiff, Fant and Horwitz, 1979). The identification of a molecular target can be used in conjunction with SAR to make calculated changes to the compound structure. The use of the molecular target is the best way to rationally optimize a compound.

1.10 Conclusion

New drugs are needed in the treatment of TB that address the challenge of non-replicating bacteria, and that improve treatment of MDR-TB and XDR-TB. The diversity of natural products found in plants represents a vast resource in the search for new anti-TB compounds. Plants have been utilized as medicines throughout history. The ethnobotanical knowledge underlying this use was thoroughly gathered through trial and error, the results of which have been passed down through generations. Today, this information can be applied to TB drug discovery. Plants can provide active compounds that are leads suitable for optimization by

medicinal and synthetic chemists. Even more than synthetic compounds, natural products contain diverse structures that have been shown to be active in drug discovery efforts.

This project uses the ethnobotanical knowledge of South African medicinal plants to identify plant-derived compounds with activity against replicating and non-replicating Mtb. Compounds of interest can be identified using an interdisciplinary approach that involves identifying plants of interest, preparation of plant extracts, biological screening using pharmacologically relevant assays. While the investigation of natural products presents many challenges, it is a worthwhile effort that can provide new and urgently needed treatment options for TB.

REFERENCES

Banchereau, J. and R. M. Steinman (1998) Dendritic cells and the control of immunity. *Nature*. 392, 245-52.

Banerjee R, Schecter GF, Flood J and Porco TC. (2008) Extensively drug-resistant tuberculosis: new strains, new challenges. *Expert Rev Anti Infect Ther*. 6(5), 713-24.

Bansal, A. K. (2005) Bioinformatics in microbial biotechnology--a mini review. In Microb Cell Fact, 19.

Bon, R. S. and H. Waldmann (2010) Bioactivity-guided navigation of chemical space. *Acc Chem Res*. 43, 1103-14.

Boshoff, H. I. M. and C. E. Barry (2005) Tuberculosis - Metabolism and respiration in the absence of growth. *Nature Reviews Microbiology*. 3, 70-80.

Blumberg HM, Burman WJ, Chaisson RE, Daley CL, Etkind SC, Friedman LN, Fujiwara P, Grzemska M, Hopewell PC, Iseman MD, Jasmer RM, Koppaka V, Menzies RI, O'Brien RJ, Reves RR, Reichman LB, Simone PM, Starke JR, Vernon AA; American Thoracic Society, Centers for Disease Control and Prevention and the Infectious Diseases Society. 2003. American Thoracic Society/Centers for Disease Control and Prevention/Infectious Diseases Society of America: treatment of tuberculosis. *Am J Respir Crit Care Med*. 167(4):603-62.

Bueno Sanchez, J. G. and V. V. Kouznetsov (2010) Antimycobacterial susceptibility testing methods for natural products research. Brazilian Journal of Microbiology, 41, 270-277.

Bunyan, J. 1680. The Life and Death of Mr Badman.

Campbell, E. A., N. Korzheva, A. Mustaev, K. Murakami, S. Nair, A. Goldfarb and S. A. Darst. (2001) Structural mechanism for rifampicin inhibition of bacterial RNA polymerase. *Cell*. 104(6), 901-12.

Cole, S. T., R. Brosch, J. Parkhill, T. Garnier, C. Churcher, D. Harris, S. V. Gordon, K. Eiglmeier, and S. Gas (1998) Deciphering the biology of Mycobacterium tuberculosis from the complete genome sequence. *Nature*. 393, 537-44.

Cooper, A. M. (2009) Cell-mediated immune responses in tuberculosis. *Annu Rev Immunol*. 27, 393-422.

Cowling, R. M., P. W. Rundel, P. G. Desmet and K. J. Esler (1998) Extraordinary high regional-scale plant diversity in southern African arid lands: Subcontinental and global comparisons. Diversity and Distributions, 4, 27-36.

Cragg, G. M., D. J. Newman and K. M. Snader. (1997) Natural products in drug discovery and development. *J Nat Prod*. 60, 52-60.

Cragg, G. M., S. A. Schepartz, M. Suffness and M. R. Grever (1993) The taxol supply crisis: New NCI policies for handling the large-scale production of novel natural product anticancer and anti-HIV agents. *Journal of Natural Products*. 56, 1657-1668.

De Souza, A. O., F. C. S. Galetti, C. L. Silva, B. Bicalho, M. M. Parma, S. F. Fonseca, A. J. Marsaioli, A. C. L. B. Trindade, R. P. F. Gil, F. S. Bezerra, M. Andrade-Neto and M. C. F. De Oliveira (2007) Antimycobacterial and cytotoxicity activity of synthetic and natural compounds. *Quimica Nova*. 30, 1563-1566.

Duncan, K. (2004) Identification and validation of novel drug targets in tuberculosis. *Curr Pharm Des*. 10, 3185-94.

Dunn, M. F., J. A. Ramirez-Trujillo and I. Hernandez-Lucas. (2009) Major roles of isocitrate lyase and malate synthase in bacterial and fungal pathogenesis. *Microbiology*. 155, 3166-75.

Durzan, D. J. (2009) Arginine, scurvy and Cartier's "tree of life". *J Ethnobiol Ethnomed*, 5, 5.

Dye C, Watt CJ, Bleed DM, Hosseini SM, Raviglione MC. (2005) Evolution of tuberculosis control and prospects for reducing tuberculosis incidence, prevalence, and deaths globally. *JAMA*. 293(22):2767-75.

Egoh, B., B. Reyers, M. Rouget, M. Bode and D. Richardson (2009) Spatial congruence between biodiversity and ecosystem services in South Africa. *Biological Conservation*. 142, 553-562.

Eisenreich, W. and A. Bacher. (2007) Advances of high-resolution NMR techniques in the structural and metabolic analysis of plant biochemistry. *Phytochemistry*, 68(22-24), 2799-815.

Fabricant, D. S. and N. R. Farnsworth. (2001) The value of plants used in traditional medicine for drug discovery. Environ Health Perspect, 69-75.

Fatkenheuer, G., H. Taelman, P. Lepage, A. Schwenk and R. Wenzel. (1999) The return of tuberculosis. Diagn Microbiol Infect Dis, 34(2), 139-46.

Field, S. K., D. Fisher, J. M. Jarand and R. L. Cowie. (2012) New treatment options for multidrug-resistant tuberculosis. *Ther Adv Respir Dis*. Eprint:1753465812452193.

Firmani, M. A. and L. W. Riley (2002) Reactive nitrogen intermediates have a bacteriostatic effect on Mycobacterium tuberculosis in vitro. *J Clin Microbiol.* 40, 3162-6.

Firn, R. D. and C. G. Jones. 2000. The evolution of secondary metabolism - a unifying model. Mol Microbiol, 37(5), 989-94.

Firn, R. D. and C. G. Jones (2003) Natural products--a simple model to explain chemical diversity. *Nat Prod Rep*. 20, 382-91.

Floss, H. G. and T. W. Yu (2005) Rifamycin-mode of action, resistance, and biosynthesis. *Chem Re.*, 105, 621-32.

Flynn, J. L. and J. Chan. (2001) Immunology of tuberculosis. Annu Rev Immunol, 19, 93-129.

Fox, H. (1952) Synthetic Tuberculistats.I. Pyridine Carboxylic Acid Derivatives. *J. Org. Chem*. 17, 542–546.

Glaziou, P., K. Floyd, E. L. Korenromp, C. Sismanidis, A. L. Bierrenbach, B. G. Williams, R. Atun and M. Raviglione. (2011) Lives saved by tuberculosis control and prospects for achieving the 2015 global target for reducing tuberculosis mortality. *Bull World Health Organ.* 89(8), 573-82.

Glickman, S. W., E. B. Rasiel, C. D. Hamilton, A. Kubataev and K. A. Schulman. (2006) Medicine. A portfolio model of drug development for tuberculosis. *Science,* 311(5765): 1246-7.

Goldman, R. C., K. V. Plumley and B. E. Laughon (2007) The evolution of extensively drug resistant tuberculosis (XDR-TB): history, status and issues for global control. *Infect Disord Drug Targets*. 7, 73-91.

Goossens, A., S. T. Hakkinen, I. Laakso, T. Seppanen-Laakso, S. Biondi, V. De Sutter, F. Lammertyn, A. M. Nuutila, H. Soderlund, M. Zabeau, D. Inze and K. M. Oksman-Caldentey (2003) A functional genomics approach toward the understanding of secondary metabolism in plant cells. *Proc Natl Acad Sci U S A.* 100, 8595-600.

Grabowski, K., K. H. Baringhaus and G. Schneider (2008) Scaffold diversity of natural products: inspiration for combinatorial library design. *Nat Prod Rep.* 25, 892-904.

Grange, J. M., N. Kapata, D. Chanda, P. Mwaba and A. Zumla. (2009) The biosocial dynamics of tuberculosis. *Trop Med Int Health*.14(2), 124-30.

Gupta, R., B. Gabrielsen and S. M. Ferguson (2005) Nature's medicines: traditional knowledge and intellectual property management. Case studies from the National Institutes of Health (NIH). *Curr Drug Discov Technol*. 2, 203-19.

Gutierrez, M. C., S. Brisse, R. Brosch, M. Fabre, B. Omais, M. Marmiesse, P. Supply and V. Vincent (2005) Ancient origin and gene mosaicism of the progenitor of Mycobacterium tuberculosis. PLoS Pathog, 1, e5.

Hamilton, A. C. (2004) Medicinal plants, conservation and livelihoods. 13,1477-1517.

Harries, A. D. and C. Dye (2006) Tuberculosis. *Ann Trop Med Parasitol*, 100, 415-31.

Hauptman, P. J. and R. A. Kelly (1999) Digitalis. *Circulation.* 99, 1265-1270.

He, X., A. Alian and P. R. Ortiz de Montellano. (2007) Inhibition of the Mycobacterium tuberculosis enoyl acyl carrier protein reductase InhA by arylamides. *Bioorg Med Chem*. 15(21) 6649-58.

Heifets, L. and E. Desmond. 2004. Tuberculosis and the Tubercle Bacillus. American Society of Microbiology Press.

Heinrich, M. and P. Bremner (2006) Ethnobotany and ethnopharmacy--their role for anti-cancer drug development. *Curr Drug Targets*. 7, 239-45.

Horton, D. A., G. T. Bourne and M. L. Smythe (2003) The combinatorial synthesis of bicyclic privileged structures or privileged substructures. *Chem Rev*. 103, 893-930.

Huxtable, R. J. and S. K. Schwarz. 2001. The isolation of morphine--first principles in science and ethics. Mol Interv, 1(4), 189-91.

Jagirdar J and Zagzag D. Pathology and Insights into Pathogenesis of Tuberculosis. In: Rom WN, Garay S, editors. Tuberculosis. Little, Brown and Company; 1996. pp. 467-82.

Jaroszewski, J. W. (2005) Hyphenated NMR methods in natural products research, part 1: direct hyphenation. *Planta Med*.71, 691-700.

Jones, W. P., Y. W. Chin and A. D. Kinghorn (2006) The role of pharmacognosy in modern medicine and pharmacy. *Curr Drug Targets*. 7, 247-64.

Jonsson, S., A. Davidse, J. Wilkins, J. S. Van der Walt, U. S. Simonsson, M. O. Karlsson, P. Smith and H. McIlleron. (2011) Population pharmacokinetics of ethambutol in South African tuberculosis patients. Antimicrob Agents Chemother, 55(9),4230-7.

Kaiser, M., S. Wetzel, K. Kumar and H. Waldmann (2008) Biology-inspired synthesis of compound libraries. *Cell Mol Life Sci*. 65, 1186-201.

Karim, S. S. A., G. J. Churchyard, Q. A. Karim and S. D. Lawn (2009) HIV infection and tuberculosis in South Africa: an urgent need to escalate the public health response. Lancet (British edition), 374, 921-933.

Karakousis PC. Mechanisms of action and resistance of antimycobacterial agents. In: Mayers DL, editor. Antimicrobial Drug Resistance. New York: Humana Press; 2009. pp. 271–291.

Kartal, M. (2007) Intellectual property protection in the natural product drug discovery, traditional herbal medicine and herbal medicinal products. *Phytother Res*. 21, 113-9.

Keiler, K. C. (2008) Biology of trans-translation. *Annu Rev Microbiol.* 62, 133-51.

Kepe, T., Saruchera, M., and W. Whande (2004). Poverty alleviation and biodiversity conservation: a South African perspective. 38(02), 143-145.

Kingston, D. G. 2007. The shape of things to come: structural and synthetic studies of taxol and related compounds. Phytochemistry, 68(14)1844-54.

Koch, M. A., L. O. Wittenberg, S. Basu, D. A. Jeyaraj, E. Gourzoulidou, K. Reinecke, A. Odermatt and H. Waldmann. 2004. Compound library development guided by protein structure similarity clustering and natural product structure. *Proc Natl Acad Sci U S A*, 101(48),16721-6.

Koehn, F. E. (2008) High impact technologies for natural products screening. *Prog Drug Res*, 65, 175, 177-210.

Koehn, F. E. and G. T. Carter. 2005. The evolving role of natural products in drug discovery. INat Rev Drug Discov, 206-20.

Lam, K. S., M. Lebl and V. Krchnak. 1997. The "One-Bead-One-Compound" Combinatorial Library Method. Chem Rev, 411-448.

Lange, R. P., H. H. Locher, P. C. Wyss and R. L. Then (2007) The targets of currently used antibacterial agents: Lessons for drug discovery. *Current Pharmaceutical Design*. 13, 3140-3154.

Lewis, W. and M. Elvin-Lewis (1995) Medicinal plants as sources of new therapeutics. *Annals of the Missouri Botanical Garden.* 82, 16-24.

Lonnroth, K., E. Jaramillo, B. G. Williams, C. Dye and M. Raviglione. (2009) Drivers of tuberculosis epidemics: the role of risk factors and social determinants. Soc Sci Med, 68(12), 2240-6.

MacMicking, J., Q. W. Xie and C. Nathan (1997) Nitric oxide and macrophage function. *Annu Rev Immunol.* 15, 323-50.

Mahdi, J. G., A. J. Mahdi and I. D. Bowen (2006) The historical analysis of aspirin discovery, its relation to the willow tree and antiproliferative and anticancer potential. *Cell Proliferation*. 39, 147-155.

Maplestone, R. A., M. J. Stone and D. H. Williams. (1992) The evolutionary role of secondary metabolites--a review. *Gene*, 115(1-2) 151-7.

Mathema, B., N. E. Kurepina, P. J. Bifani and B. N. Kreiswirth. 2006. Molecular epidemiology of tuberculosis: current insights. Clin Microbiol Rev. 19(4), 658-85.

McGaw, L. J., N. Lall, J. J. Meyer and J. N. Eloff. 2008. The potential of South African plants against Mycobacterium infections. J Ethnopharmacol, 119(3), 482-500.

McKenzie, D., L. Malone, et al. (1948) The effect of nicotinic acid amide on experimental tuberculosis of white mice. *J Lab Clin Med*. 33, 1249-53.

McLaughlin, E. (2007) Beyond the Racial Census: The Political Salience of Ethnolinguistic Cleavages in South Africa. *Comparative Political Studies*. 40, 435-456.

Mdluli, K., D. R. Sherman, M. J. Hickey, B. N. Kreiswirth, S. Morris, C. K. Stover and C. E. Barry, 3rd (1996) Biochemical and genetic data suggest that InhA is not the primary target for activated isoniazid in Mycobacterium tuberculosis. *J Infect Dis*. 174, 1085-90.

Mikusova, K., R. A. Slayden, G. S. Besra and P. J. Brennan (1995) Biogenesis of the mycobacterial cell wall and the site of action of ethambutol. *Antimicrob Agents Chemother.* 39, 2484-9.

Mills, S. D. (2006) When will the genomics investment pay off for antibacterial discovery? *Biochem Pharmacol*, 71(7), 1096-102.

Mucina, L. and C. Geldenhuys. 2006. The vegetation of South Africa, Lesotho and Swaziland. Pretoria: South African National Biodiversity Institute.

Nathan, C. (2004) Antibiotics at the crossroads. *Nature*. 431, 899-902.

Nathan, C., B. Gold, G. Lin, M. Stegman, L. P. de Carvalho, O. Vandal, A. Venugopal and R. Bryk. 2008. A philosophy of anti-infectives as a guide in the search for new drugs for tuberculosis. Tuberculosis (Edinb), 88 Suppl 1,S25-33.

Newman, D. J. and G. M. Cragg (2012) Natural products as sources of new drugs over the 30 years from 1981 to 2010. *J Nat Prod*. 75, 311-35.

Newman, D. J., G. M. Cragg and K. M. Snader (2000) The influence of natural products upon drug discovery. *Nat Prod Rep*. 17, 215-34.

Newman, D. J., G. M. Cragg and K. M. Snader (2003) Natural products as sources of new drugs over the period 1981-2002. Journal of Natural Products, 66 (7), 1022-1037.

Newton, R., W. R. Brown and W. Martin (1926) The extraction of plant tissue fluids and their utility in physiological studies. *Journal of Plant Physiology*. 1, 57-65.

Nicolaou, K. C., Z. Yang, J. J. Liu, H. Ueno, P. G. Nantermet, R. K. Guy, C. F. Claiborne, J. Renaud, E. A. Couladouros, K. Paulvannan and et al. (1994) Total synthesis of taxol. Nature, 367, 630-4.

Pan, S.-Y., S. Pan, Z.-L. Yu, D.-L. Ma, S.-B. Chen, W.-F. Fong, Y.-F. Han and K.-M. Ko (2010) New Perspectives on Innovative Drug Discovery: An Overview. *Journal of Pharmacy and Pharmaceutical Sciences*. 13, 450-471.

Parrish, N. M., J. D. Dick and W. R. Bishai (1998) Mechanisms of latency in Mycobacterium tuberculosis. *Trends in Microbiology*. 6, 107-112.

Pauli, G. F., R. J. Case, T. Inui, Y. Wang, S. Cho, N. H. Fischer and S. G. Franzblau (2005) New perspectives on natural products in TB drug research. *Life Sciences*. 78, 485-494.

Phillipson, J. D. (1994) Natural products as drugs. *Trans R Soc Trop Med Hyg*, 88 Suppl 1, S17-9.

Pieters, J. (2008) Mycobacterium tuberculosis and the macrophage: maintaining a balance. *Cell Host Microbe*, 3(6), 399-407.

Rahbek, C. and R. K. Colwell. (2011) Biodiversity: Species loss revisited. *Nature*, 473(7347), 288-9.

Raviglione, M. C. and A. Pio. (2002) Evolution of WHO policies for tuberculosis control, 1948-2001. Lancet, 359(9308), 775-80.

Reidenberg MM. (2007) World Health Organization program for the selection and use of essential medicines. *Clin Pharmacol Ther*. 81(4):603-6.

Rivers, E. C. and R. L. Mancera. (2008) New anti-tuberculosis drugs in clinical trials with novel mechanisms of action. *Drug Discov Today*. 13(23-24), 1090-8.

Rohde, K. H., R. B. Abramovitch and D. G. Russell. (2007) Mycobacterium tuberculosis invasion of macrophages: linking bacterial gene expression to environmental cues. *Cell Host Microbe*, 2(5), 352-64.

Rollinger, J. M., T. Langer and H. Stuppner (2006) Strategies for efficient lead structure discovery from natural products. *Curr Med Chem*. 13, 1491-507.

Rosamond, J. and A. Allsop (2000) Harnessing the power of the genome in the search for new antibiotics. *Science*. 287, 1973-1976.

Rozwarski, D. A., G. A. Grant, D. H. Barton, W. R. Jacobs, Jr. and J. C. Sacchettini (1998) Modification of the NADH of the isoniazid target (InhA) from Mycobacterium tuberculosis. *Science*. 279, 98-102.

Russell, D. G., C. E. Barry, III and J. L. Flynn (2010a) Tuberculosis: What We Don't Know Can, and Does, Hurt Us. *Science*. 328, 852-856.

Russell, D. G., B. C. VanderVen, W. Lee, R. B. Abramovitch, M. J. Kim, S. Homolka, S. Niemann and K. H. Rohde. 2010b. Mycobacterium tuberculosis wears what it eats. In Cell Host Microbe, 68-76.

Schiff, P. B., J. Fant and S. B. Horwitz (1979) Promotion of microtubule assembly in vitro by taxol. *Nature*. 277, 665-7.

Schippmann, U., D. J. Leaman and A. B. Cunningham. 2002. Impact of Cultivation and Gathering of Medicinal Plants on Biodiversity: Global Trends and Issues. In Biodiversity and the Ecosystem Approach in Agriculture, Forestry and Fisheries. Rome: FAO.

Schroeder, F. C., D. M. Gibson, A. C. Churchill, P. Sojikul, E. J. Wursthorn, S. B. Krasnoff and J. Clardy (2007) Differential analysis of 2D NMR spectra: new natural products from a pilot-scale fungal extract library. *Angew Chem*. 46, 901-4.

Sensi, P. (1983) History of the development of Rifampin. *Rev Infect Dis*. 5 Suppl 3:S402-6.

Shi, W., X. Zhang, X. Jiang, H. Yuan, J. S. Lee, C. E. Barry, 3rd, H. Wang, W. Zhang and Y. Zhang. (2011) Pyrazinamide inhibits trans-translation in Mycobacterium tuberculosis. *Science*, 1630-2.

Shin, S. S., V. Naroditskaya, A. Sloutsky, B. Werner, R. Timperi, J. Bayona, P. E. Farmer and M. C. Becerra (2005) rpoB gene mutations in clinical isolates of multidrug-resistant Mycobacterium tuberculosis in northern Lima, Peru. *Microb Drug Resist*. 11, 26-30.

Somoskovi, A., L. M. Parsons and M. Salfinger (2001) The molecular basis of resistance to isoniazid, rifampin, and pyrazinamide in Mycobacterium tuberculosis. *Respir Res*. 2, 164-8.

Spellberg, B., J. H. Powers, E. P. Brass, L. G. Miller and J. E. Edwards, Jr. (2004) Trends in antimicrobial drug development: implications for the future. *Clin Infect Dis*, 38(9), 1279-86.

Spigelman, M. K. (2007) New tuberculosis therapeutics: a growing pipeline. *J Infect Dis*. 196, S28-34.

Stewart, G. R., B. D. Robertson and D. B. Young (2003) Tuberculosis: a problem with persistence. *Nat Rev Microbiol*. 1, 97-105.

Tan, R. X., W. F. Zheng and H. Q. Tang (1998) Biologically active substances from the genus Artemisia. Planta Medica, 64, 295-302.

Takayama, K. and J. O. Kilburn (1989) Inhibition of synthesis of arabinogalactan by ethambutol in Mycobacterium smegmatis. *Antimicrob Agents Chemother*, 33, 1493-9.

Terstappen, G. C., C. Schlupen, R. Raggiaschi and G. Gaviraghi. (2007) Target deconvolution strategies in drug discovery. Nat Rev Drug Discov. 6(11), 891-903.

Thomson, C. J., E. Power, H. Ruebsamen-Waigmann and H. Labischinski. (2004) Antibacterial research and development in the 21(st) Century--an industry perspective of the challenges. *Curr Opin Microbiol*, 445-50.

van Wyk, B. E., B. van Oudtshoorn and N. Gericke. 1997. Medicinal Plants of South Africa. Pretoria, South Africa: Briza Publications.

van Niekerk, J. (2012) Traditional Healers formalized? South African Journal of Medicine, 102, 105-106.

Vandal, O. H., C. F. Nathan and S. Ehrt (2009) Acid Resistance in Mycobacterium tuberculosis. Journal of Bacteriology, 191, 4714-4721.

Waddell, S. J. and P. D. Butcher (2007) Microarray analysis of whole genome expression of intracellular Mycobacterium tuberculosis. *Current Molecular Medicine* (Hilversum), 7, 287-296.

Wall, M. E. and M. C. Wani (1995) Camptothecin and Taxol- Discovery to Clinic- 13th Bruce F. Cain Memorial Award Lecture. *Cancer Research*. 55, 753-760.

Wall, M. E. and M. C. Wani (1996) Camptothecin and taxol: from discovery to clinic. Journal of Ethnopharmocology, 51, 239-254.

Wall, M. E., M. C. Wani, C. E. Cook, K. H. Palmer, A. T. McPhail and G. A. Sim (1966) Plant Antitumor Agents. I. The Isolation and Structure of Camptothecin, a Novel Alkaloidal Leukemia and Tumor Inhibitor from Camptotheca acuminata. *J. Am. Chem. Soc.* 88, 3888-3890.

Wani, M. C., H. L. Taylor, M. E. Wall, P. Coggon and A. T. McPhail (1971) Plant antitumor agents. VI. The isolation and structure of taxol, a novel antileukemic and antitumor agent from Taxus brevifolia. J Am Chem Soc, 93, 2325-7.

Walsh, C. (2003) Where will new antibiotics come from? *Nat Rev Microbiol*. 1, 65-70.

Wang, Y., X. Fan, H. Qu, X. Gao and Y. Cheng. (2012) Strategies and techniques for multi-component drug design from medicinal herbs and traditional chinese medicine. *Curr Top Med Chem*, 12(12), 1356-62.

Wayne, L. G. and C. D. Sohaskey (2001) Nonreplicating persistence of Mycobacterium tuberculosis. *Annual Review of Microbiology*. 55, 139-163.

Wolfson, J. S. and D. C. Hooper (1985) The fluoroquinolones: structures, mechanisms of action and resistance, and spectra of activity in vitro. *Antimicrob Agents Chemother*, 28, 581-6.

Young, D., J. Stark and D. Kirschner. (2008) Systems biology of persistent infection: tuberculosis as a case study. *Nat Rev Microbiol*. 6(7), 520-8.

Zaman, K. (2010) Tuberculosis: A Global Health Problem *J Health Popul Nutr*. 28(2): 111–113.

Zhang, Y., M. M. Wade, A. Scorpio, H. Zhang and Z. Sun. (2003) Mode of action of pyrazinamide: disruption of Mycobacterium tuberculosis membrane transport and energetics by pyrazinoic acid. *J Antimicrob Chemother*. 790-5.

Zschocke, S., T. Rabe, J. L. Taylor, A. K. Jager and J. van Staden. (2000) Plant part substitution--a way to conserve endangered medicinal plants? *J Ethnopharmacol*, 71(1-2), 281-92.

CHAPTER 2

Collection and Testing of South African Medicinal Plants

2.1 Overview

The plant diversity of South Africa has great potential to offer metabolites that may be effective against *Mycobacterium tuberculosis* (Mtb). This chapter describes how South African medicinal plants were selected and evaluated for inhibitory activity against Mtb. Two biological assays were used to evaluate plant extracts, one that assesses inhibitory activity against replicating Mtb and another that evaluates the inhibitory activity against Mtb that is in a non-replicating state. Eradicating non-replicating Mtb is one of the major challenges in the treatment of tuberculosis (TB). While some of the plants tested have been previously investigated in some capacity for antimicrobial activity, none have been tested for activity against non-replicating Mtb. The results of this study indicate that the plant *Warburgia salutaris* demonstrates inhibitory activity against Mtb under both test conditions.

2.2 Introduction

Ethnobotanical knowledge of medicinal plants has been successfully used to identify plants with pharmacological potential. This study focuses on plants with inhibitory activity against Mtb. Past screening projects and current pharmaceutical drugs have shown that plants used in traditional medicine have a higher percentage of pharmacologically active compounds than plants selected at random (Hostettmann et al., 1996). Among all the drugs that have plant origins, 77% of them were used in traditional medicine (Rollinger, Langer and Stuppner, 2006). Since it is not plausible to test all medicinal plants, this project limited the investigation to South Africa, a country known for its plant diversity and with a population that has a strong tradition, which persist today, of using these plants in the treatment of disease.

2.2.1 Ethnobotany in South Africa

The African continent is believed to have the oldest known human settlements and a strong tradition of using plants as medicine (Vrba, 1996). However, the knowledge of African plants used in the treatment of disease is limited. First, the study of African medicinal plants was traditionally an oral tradition and is not as well documented as some other cultures, such as Ayurvedic or Chinese traditional medicine (Makunga, 2008). Secondly, the use of medicinal plants is situated within larger cultural frameworks that are often poorly understood (Iwu, 1993). The prevalent image of the African medicine man is that of a witch doctor muttering incomprehensible incantations, while the reality is far more complex. The original documents that describe the use of plants by African ethnic groups were prepared by Europeans and these compilations often do not reflect cultural intricacies. They are also filtered through the cultural perspective of each author; inevitably information has been omitted or altered (Makhubu, 1998).

It is estimated that between 12 and 15 million South Africans use approximately 3000 plant species in the treatment of disease (van Wyk, van Oudtshoorn and Gericke, 1997). Although more recent data is not available, by the end of the 1990's the total number of traditional healers in South Africa was estimated to be 350,000, outnumbering those who practice modern medicine (Kale, 1995; Peltzer, 2009). Traditional medicinal practices in South Africa, like the rest of the continent, have been dominated by oral tradition (Light et al., 2005). Early documentation of the uses of plants as medicine date back to the late nineteenth century (Hutchings et al., 1996). These early contributors were often missionaries, anthropologists, linguists and botanists. Information in these original records is sparse and does not always include details about the dosage, preparation methods, and plant identifications are not reliable (Hutchings et al., 1996).

Because documentation is incomplete, this study would have benefited from new discussions with traditional healers that currently use plants in the treatment respiratory ailments. Unfortunately, the existing policies in South Africa regarding medicinal plants are not adequate to ensure the equitable sharing of benefits that may result from the use of ethnobotanical

information (Makhubu, 1998). Also, there is no well-defined way to assign ownership to the knowledge and obtain informed consent. Without a way to protect the rights of prospective volunteers, this project did not include any surveys or discussions with people who practice traditional medicine. The focus, therefore, was only on those plants that had been discussed in previously published reports and were considered information already in the public domain.

2.2.2 The Plant Selection Process

Plants were selected for this study primarily based on their traditional medicinal usage as described in books and monographs. Due to the lack of documentation, two ethnobotanical inventories were heavily relied on to select plants for this study. The first was the reference text, "The Medicinal and Poisonous Plants of Southern and Eastern Africa" by Watt and Breyer-Brandwijk (1962), which has become the canonical documentation of indigenous botanical medicines of southern and eastern Africa, originally published in 1932. The second was the book "Medicinal Plants of South Africa", a monograph by van Wyk et al. (1997), which is a photographic guide to the most commonly used South African plant medicines. These two books, as well as peer- reviewed journals, were surveyed for any plant described in the treatment of respiratory ailments including cough cold, flu, bronchitis and tuberculosis. These broad criteria were used because it could not be assumed that TB was being accurately diagnosed and there was no way to verify the correctness of translated disease names.

Another consideration in the selection of plants is that the traditional uses of a plant are not always germane to the modern therapeutic indication for the derived drugs (ie. Taxol®). With this in mind, two species were included in this study, not because of their reported anti-therapies, but because they were readily available and of interest to other researchers at the University of Cape Town where the plant extraction for this research was taking place. These plants had been only previously reported in traditional treatments of malaria, and laboratory investigations into their biological activity were limited.

After a review of the literature, there were two factors that determined which plants were ultimately collected and used in this study. First, there was concern regarding the conservation status of the medicinal plants. Due to over-harvesting and habitat destruction, many valued indigenous plant species are now near extinction (Williams, Balkwill and Witkowski, 2000) and their collection is restricted. Secondly, some plants would have required extensive and costly travel to collect and were ultimately omitted from the study. Fortunately, with the assistance of Pakamani Xaba, botanist and curator at Kirstenbosch Botanical Garden in Cape Town, permission was given to collect most of the plants from the Kirstenbosch Botanical Garden. The South Africa National Biodiversity Institute has established small-scale cultivation of many medicinal plants within the botanical garden to promote the sustainable use and conservation of the country's plant biodiversity (Driver, 2005).

The logistics of transporting plant samples from South Africa into the United States also influenced the number of plants that were included in this study. Permits are required from the USDA Animal Plant Health Inspection Service for shipments of plant parts into the United States. In South Africa it would have also been necessary to obtain 'Phytosanitary Certificates of Inspection' issued by the National Plant Protection Service of South Africa. To circumvent this regulatory labyrinth, all collected plant parts were processed in South Africa and the dried plant extracts were shipped to Cornell University. While this facilitated the delivery of plant samples, it introduced a new time constraint. The laboratory facilities at the University of Cape Town had solvents of unknown purity that required simple, but time demanding, purification before they could be used for plant extractions. Ultimately, nine plants were successfully extracted and shipped for testing.

2.2.3 Recent Investigations into South African Medicinal Plants Used in the Treatment of TB

A 2008 review by McGaw et al. focuses on South African plants used to treat TB-related symptoms and the laboratory investigations into their biological activity. In South Africa, 180

plants are reported as used in the treatment of TB related symptoms, broadly defined as coughing, chest complaints and other respiratory ailments. McGaw et al. highlight the shortcomings of the past studies and this review helps illustrate how this project differs from these past studies.

The McGaw et al. review explains that a major emphasis of the ethnopharmacological research in South Africa is to validate traditional remedies. With this goal, most studies stop at analysis of crude extracts and do not progress to compound identification; demonstrating any inhibitory activity of the plant extract is sufficient to provide some scientific rationale for the traditional use. In contrast, the goal of the current project is not to validate traditional practices. Rather, it is to identify a compound or compound class that should be considered further in the development of new anti-TB therapies.

Past studies into the inhibitory activity of plant extracts commonly report minimum inhibitory concentrations (MIC) as high as 10 mg/mL. MICs are often calculated as the lowest concentration of drug that inhibits growth by at least 99% (Andrews, 2001). In natural products research initial MICs refer to crude extracts that likely contain hundreds of compounds, of which the active constituents represent only a small portion. Pharmaceutical drug development usually begins with pure compounds that have MICs that are measured in nanogram concentrations (Nathan, 2004). This restriction cannot be easily applied to natural products research. Plant extracts are complex chemical matrices that contain many compounds that do not contribute to the activity. Therefore the threshold for activity for plant extracts should understandably be more lenient. Additionally, isolated naturally occurring compounds have not had the benefit of being modified by medicinal chemists, who can increase the inhibitory activity by several orders of magnitude. While the threshold of activity for plants should be more than standards applied to pharmaceutical drugs, the parameters used by ethnobotanist must be more stringent than many previous studies (McGaw et al., 2008).

Only 30% of the plants documented in the McGraw study as being used in the treatment of respiratory ailments have been tested for activity against any Mycobacterium species.

Additionally, most investigations involve testing against fast-growing, non-pathogenic species, the results of which are not always an accurate indicator for how effective a compound will be against Mtb (McGaw et al., 2008). In the host, immune activation creates an heterogeneous environment that causes Mtb to enter a non-replicating state characterized by growth arrest and altered metabolic activity (Flynn and Chan, 2001). Many of the conditions that induce latency are believed to include, but may not be limited to, any combination of the following: low pH, reduced carbon availability, hypoxia and the presence of reactive nitrogen intermediates (Wayne and Sohaskey, 2001). It is within the context of these conditions that anti-TB compounds need to function. To date, no plant extracts have been tested under the conditions that prevent Mtb from replicating.

2.3 Ethnobotanical Description of Tested Medicinal Plants

• ***Artemisia afra*** Willd. (Asteraceae) is a highly aromatic perennial shrub, widely distributed throughout South Africa. *Artemisia afra* is one of the most widely used traditional medicines in South Africa. It is used to treat coughs, colds influenza, fever, and gastrointestinal disorders (van Wyk et al., 1997; Scott, Springfield and Coldrey, 2004). Often fresh leaves are inserted directly into the nostril or the leaves are boiled in water and the fumes inhaled. The volatile oil of *A. afra* contains camphor and thujone as major constituents (Graven et al., 1992). Ethanol extracts from *A. afra* have been shown to inhibit the growth of the non-pathogenic *Mycobacterium smegmatis* at 1.56 mg/mL (Mativandlela et al., 2008).

• ***Caesalpinia volkensii*** (Caesapiniaceae) occurs in forests and in tropical regions of southeast Africa (Bosch, 2008). Herbalists prescribe a hot aqueous extract of the leaves or root bark to treat malaria, diarrhea, and to fight pain during pregnancy (Njoroge and Kibunga, 2007). Aqueous and dichloromethane extracts of *C. volkensii* have been shown to be active against chloroquine–resistant and chloroquine-sensitive strains of malaria (Kuria et al., 2001). While this

plant is not commonly used to treat respiratory ailments, it was included in this study because it was of interest to many researchers at the University of Cape Town and there are very few laboratory investigations into its biological activity.

• ***Mentha longifolia*** (L.) Huds. (Lamiaceae), also known as wild mint, is a perennial herb of temperate Europe and North Africa. While it is not native to South Africa, it is grown there for its therapeutic uses. All parts are highly aromatic with a strong mint smell. The volatile oils that contain the monoterpenoids menthol and methone have been used as a decongestant and to treat many respiratory ailments including coughs, colds, and asthma (Oyedeji and Afolayan, 2006; Al-Bayati, 2009). It is also used for headache, fever, and indigestion (van Wyk et al., 1997).

• ***Polygala fruticosa*** Berg. (Polygalaceae) is a shrublet less than 0.5m in height and known for its attractive purple flowers. The plant occurs along the costal parts of the country in three provinces: Western Cape, Eastern Cape and KwaZulu-Natal (Levyns, 1955). Various parts of the plant are used in preparing treatments for a wide variety of ailments including tuberculosis, poor circulation, gonorrhea and sinusitis (van Wyk et al., 1997). The unusual coumarin, frutinone, has been isolated from the plant and has been shown to have antifungal activity (Di Paulo et al., 1989).

• ***Rhamnus staddo*** (Rhamnaceae) is primarily found in the eastern parts of Africa and is used primarily in the treatment of malaria. In laboratory investigations, extracts prepared from the leaf and bark of *R. staddo* showed significant antimalarial activity against chloroquine resistant *Plasmodium berghei* (Muregi et al., 2007). This plant was included in this study because researchers at the University of Cape Town were investigating its anti-malarial properties and there are very few laboratory investigations into its biological activity.

• ***Syzygium cordatum*** Hochst. ex C. Krauss subsp. cordatum (Myrtaceae) is a medium sized tree that can grow up to 15 meters in height. This plant has wide distribution in the eastern and northeastern parts of South Africa (Palmer and Pitman, 1972). The thick bark and occasionally the leaves are used to treat stomach complaints, diarrhea and respiratory ailments including tuberculosis, (van Wyk et al., 1997). It has been tested for inhibitory activity against fifteen gram-negative and gram-positive species. The acetone and methanol extracts of the stem bark and leaves had MICs varying between 0.31 and 6 mg/mL (Samie et al., 2005). The ethanol extract of the leaves demonstrated inhibitory activity against the non- pathogenic *M. smegmatis* at concentrations of 6.25 mg/mL (Mativandlela et al., 2008). There have been no additional studies into its activity against pathogenic *Mycobacterium*.

• ***Tarchonanthus camphoratus*** L. (Asteraceae) is an evergreen shrub or small tree. The trichomes on the leaves give the leaves a greyish appearance. *Tarchonanthus camphoratus* has a wide distribution across southern and east Africa and it is found in almost every part of the South Africa (Palgrave, 2002). Traditionally, the leaves and twigs are used to prepare aqueous extracts to treat a wide variety of diseases including: abdominal pain, headache, asthma, bronchitis, and inflammation (Watt and Breyer-Brandwijk, 1962). Laboratory investigations have determined that the active ingredients are monoterpenes, a high percentage of which are oxygenated monoterpenes; fenchol, cineole, terpineol. These compounds have been shown to have antibacterial activity against gram-negative and gram-positive bacteria (Matasyoh et al., 2007).

• ***Tulbaghia violacea*** var. violacea Harv. (Alliaceae), also known as wild garlic, is a bulbous plant with narrow hairless leaves. The plant has limited distribution in the eastern parts of the country in the provinces of Easter Cape and Southern Kwa-Zulu Natal. The bulbs and leaves are the primarily used in the traditional remedies for stomach ailments, fever, colds, and tuberculosis. Studies have shown that extracts of this plant exhibit antibacterial and antifungal activity (Kubec, Velisek and Musah, 2002). When the extract of *T. violacea* is applied to the soil

of plants that are prone to infections by the fungal pathogens *Botrytis cinerea*, *Pythium ultimum* and *Rhizoctonia solani*, there was an increasing plantlet survival (Lindsey and Van Staden, 2004). The plants in the Alliaceae family, including true garlic, *Allium sativum*, are generally characterized by the presence of low molecular mass sulfur compounds, such as alliin, which have also been isolated from *T. violacea* (Burton and Kaye 1992).

• ***Warburgia salutaris*** (Bertol. f.) Chiov. (Canellaceae) is a small tree, 5-10 m in height but reaching 20 m in some areas. Its medicinal uses date back several centuries, and it is listed as one of the plants traded when Arab dhows first reached the coast of East Africa (Hollmann, 1996). *Warburgia salutaris* has been reported in the treatment of over a dozen ailments. It is used in Zulu and Xhosa medicine for the treatment of venereal diseases, headache and gastric ulcers (van Wyk et al., 1997). The Venda people in South Africa use the plant to treat sores and ulcers (Mashimbye, Maumela and Drewes, 1999). A tea is prepared and used for respiratory ailments including colds, congestion, influenza, and other chest complaints. A dried powder is applied to sores (Rabe and van Staden, 1997). There are laboratory investigations into the biological activity of this plant, including two studies into the anti-mycobacterial properties of the plant. These studies showed that dichloromethane (DCM) extracts have inhibitory activity against replicating Mtb and *M. bovis* BCG (Clarkson et al., 2007; Madikane et al., 2007). This will be discussed in detail in chapter 3.

2.4 Methods

2.4.1 Plant Collection

Plants were collected in September 2007 at the Kirstenbosch Botanical Garden in Cape Town with the consent and assistance of Phakamani Xaba, botanist and curator of the Medicinal and Useful Plants Garden at the Kirstenbosch National Botanical Garden. Mature bark of

Warburgia salutaris (Bertol. f.) Chiov. (Canellaceae) is difficult to obtain and a sample of the dried, macerated bark, previously harvested at the Silver glen Nursery in Durban June 2002, was provided by Dr. Peter Smith at the University of Cape Town, South Africa. Plants were allowed to air dry at room temperature until they were extracted.

2.4.2 Plant Extraction

Plants were extracted using the laboratory facilities of Dr. Peter Smith at the University of Cape Town Medical School, South Africa. Plant material was dried in open air and macerated using an industrial blender. Powdered plant material was extracted sequentially using solvents of increasing polarity (dichloromethane, ethyl acetate, and methanol) over a period of 72 hours with agitation at room temperature. This extraction procedure does prevent the possibility that some monoterpenes and volatiles may be lost during drying and extraction. Not all plants were extracted with all solvents. The extracts were concentrated under vacuum, followed by drying under a stream of nitrogen. Dried plant extracts were shipped to Cornell University.

2.4.3 Mtb Bioassay

The biological assays used to screen the plant extracts were adapted from the procedure described by Bryk et al. (2008). Testing of these extracts was performed using the laboratory facilities of Dr. Carl Nathan at the Weill Cornell Medical College, in the Department of Microbiology. Extracts were tested for activity against Mtb under growth-sustaining and growth-inhibiting conditions. These biological screens used a lysine-pantothenate auxotroph of the wild-type Mtb H37Rv strain, Mtb mc26020 (ΔlysAΔpanCD). This multiple auxotroph was used because its reduced virulence makes it suitable for biosafety-level 2 (BSL2) handling, instead of the BSL3 facilities required for wild-type Mtb (Sambandamurthy et al., 2002).

Mtb cells were cultivated in Middlebrook 7H9 broth, pH 6.6-8 with 0.2% glycerol, 0.05% Tween 80 and 10% ADN (0.5% BSA, 2% dextrose, 0.85% NaCl) (Larsen, Biermann and Jacobs, 2007). Experiments were performed using an inoculum consisting of mid- to late-log-phase

cultures of Mtb (optical density at 580 nm, 0.5 – 0.8 AU). To assess bactericidal activity, growth was assessed in 96 well plates. Reaction wells were prepared with 98 µL of growth medium and 100 µL inoculum. The plant extracts to be tested were prepared in DMSO and 2 µL volumes were added to reaction well to yield a total reaction volume of 200 µL and a final concentration of 200 µg/mL. Rifampicin, an antibiotic currently used in TB treatment, was used as a positive control and DMSO, used to solubilize the extracts, and was included as a vehicle control.

The conditions that cause Mtb to enter a non-replicating state include, but are not limited to, low pH, reduced carbon availability, hypoxia, and the presence of reactive nitrogen intermediates (Wayne and Sohaskey, 2001). These physiological conditions were simulated *in vitro* to mimic the conditions that Mtb encounters the in macrophages that are believed to prevent replication. The experimental conditions are described below.

Growth inhibiting conditions: Midlog phase cells (A580 0.8–1.0) of Mtb, previously grown in growth sustaining media, were prepared in 7H9 at pH 5.5, 0.5 mM nitrite $NaNO_2$ and a modified Sauten's media with butyrate as a carbon source (0.1% NH_4Cl, 0.005% ferric ammonium citrate) with 10% AN (0.5% BSA, 0.85% NaCl), 0.02% tyloxapol, 0.05% butyrate). Each 96 well plate was kept at 37 °C and 1% O_2 in environmental chambers (BD Biosciences) (Bryk et al., 2008).

Growth sustaining conditions: Midlog phase cells (A580 0.8–1.0) were prepared as single cell suspensions in 7H9 at pH 6.8. These test conditions include ambient levels of O_2 (20%), pH 6.8, and 7H9 medium enriched with glycerol used as a carbon source.

The inhibitory activity of the plant extracts tested under growth-sustaining conditions was determined by measuring optical density (Molecular Devices, SpectraMaxPlus Absorbance Microplate Reader) after 7 days. For the growth-inhibiting conditions, following the 7-day exposure, killing was assessed by dilution into growth-sustaining media and outgrowth after 10 days was determined via optical density.

2.5 Results and Discussion

Nine different plants, used to prepare a total of nineteen separate extracts, were tested for inhibitory activity against Mtb under growth-sustaining conditions. All plant extracts were evaluated at a concentration of 200 μg/mL. The selection of 200 μg/mL recognizes that the minimum threshold for activity applied to pure compounds may be too stringent. However, even at this high concentration, only one extract, *W. salutaris*, showed greater than fifty percent inhibition of growth, (Table 1). The activity of *W. salutaris* agrees with a previous report by Madikane et al. (2007) which states that at 200 μg/mL the DCM extract of the mature bark completely inhibits the growth of replicating Mtb. The specific experimental method used in their study was not specified.

The DCM extract of the mature bark of *W. salutaris* was also the only extract that demonstrated inhibition of more than fifty percent under growth-inhibiting conditions, (Table 2). Additionally, when the inhibitory activities of all the plant extracts for both test conditions are compared, the percent inhibition was either equivalent or higher against replicating Mtb. One plant extract, *W. salutaris* leaves, appeared to have greater inhibitory activity under growth-inhibiting conditions, with 33% inhibition under growth-sustaining conditions and 42% under growth inhibiting conditions. However, subsequent t-test analysis reveals that there was no statistically significant difference between the results of the two test conditions.

The activity of *W. salutaris* is particularly notable since, to date, no plant extracts have been tested for inhibitory activity against Mtb under growth-inhibiting conditions or any other conditions intended to replicate conditions present in the host. This demonstrates the necessity of an interdisciplinary effort in natural products research to ensure that plant extracts are evaluated using the most relevant bioassays. There are tuberculosis drugs, such as rifampicin and pyrazinamide, effective against non-replicating Mtb. However, these drugs have been in use for four decades and there is an urgent need for new drugs with new mechanisms of action to combat resistance. Subsequent experiments are focused on the identification of those compounds

in *W. salutaris* that were responsible for this activity and gaining insight into the possible mechanisms of action.

Table 2.1. Plant extracts tested for inhibitory activity against Mtb under growth-sustaining conditions. Percent inhibition is normalized against the positive and negative controls, RIF and DMSO respectively.

Plant Name (Solvent)	Percent Inhibition
A. afra (EtOAc)	0
A. afra (MeOH)	0
C. volkensii (DCM)	8.71
M. longifolia (DCM)	4.0
M. longifolia (EtOAc)	14.0
P. fruticosa (EtOAc)	0
P. fruticosa (MeOH)	18.0
R. studdos (DCM)	22.6
S. cordatum leaves (MeOH)	0
S. cordatum leaves (DCM)	14.4
S. cordatum stem bark (DCM)	35.0
T. camphoratus (DCM)	43.7
T. camphoratus (EtOAc)	31.4
T. camphoratus (MeOH)	0
T. violacea (DCM)	0
T. violacea (MeOH)	6.31
W. salutaris, leaves (DCM)	33.0
W. salutaris, leaves (MeOH)	0
W. salutaris, mature bark (DCM)	91.9

Table 2.2. Plant extracts tested for inhibitory activity against Mtb under growth-inhibitory conditions. Percent inhibition is normalized against the positive and negative controls, RIF and DMSO respectively.

Plant Name (Solvent)	Percent Inhibition
C. volkensii (DCM)	3.3
M. longifolia (EtOAc)	5.6
M. longifolia (DCM)	5.6
P. fruticosa (MeOH)	1.5
R. studdos (DCM)	16.6
S. cordatum leaves (DCM)	19.4
S. cordatum stem bark (DCM)	10.8
T. camphoratus (DCM)	28.3
T. camphoratus (EtOAc)	33.7
T. violacea (MeOH)	0.52
W. salutaris, leaves (DCM)	42.3
W. salutaris, mature bark (DCM)	90.6

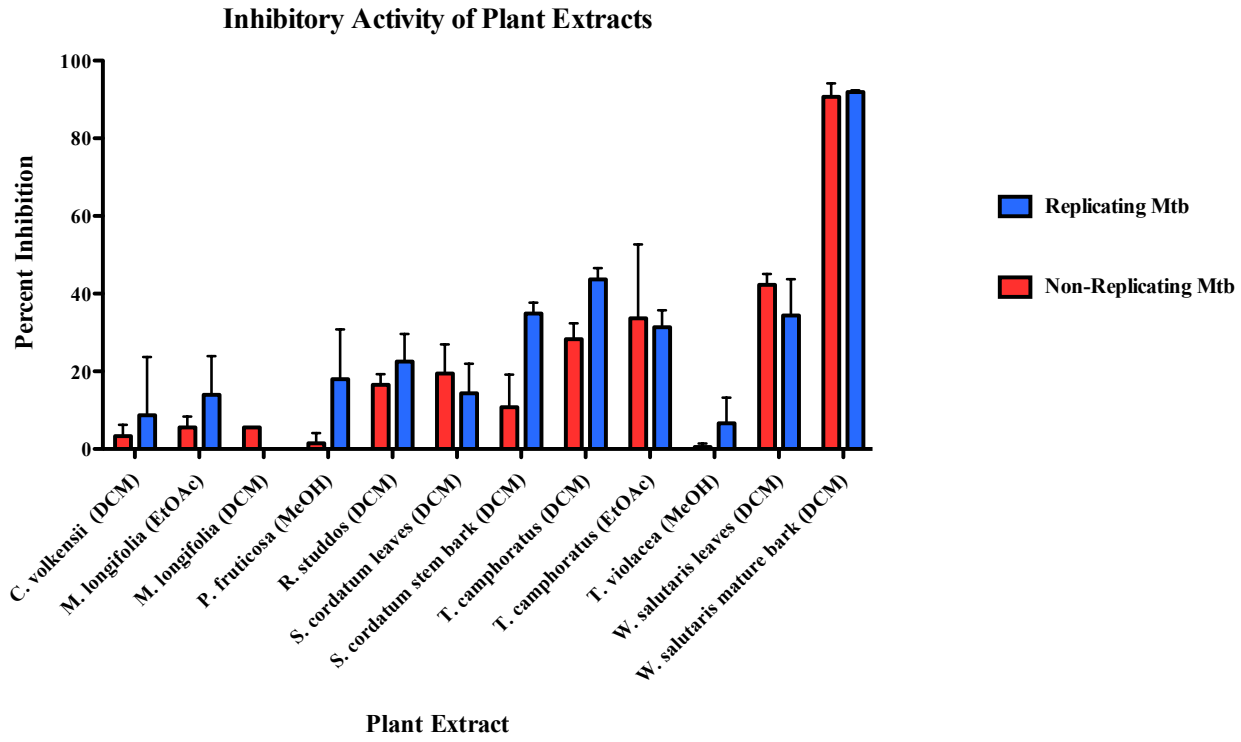


Figure 2.1. Inhibitory activity of South African plant extracts against replicating and non-replicating Mtb.

REFERENCES

Al-Bayati, F. A. (2009). Isolation and identification of antimicrobial compound from Mentha longifolia L. leaves grown wild in Iraq. *Ann Clin Microbiol Antimicrob*. 8, 20.

Andrews, J. (2001) Determination of minimum inhibitory concentrations. *Journal. Antimicrob. Chemother*. 48, Supplement S1,5-16.

Bosch, C.H., 2008. Caesalpinia volkensii. In: Schmelzer, G.H. and Gurib-Fakim, A. (Editors). Plant resources of tropical Affrica 11(1). Medicinal Plants 1. Prota Foundation, Wageningen, Netherlands/ Backhuys Publishers, Leiden, Netherlands. pp133.

Bryk, R., B. Gold, A. Venugopal, J. Singh, R. Samy, K. Pupek, H. Cao, C. Popescu, M. Gurney, S. Hotha, J. Cherian, K. Rhee, L. Ly, P. J. Converse, S. Ehrt, O. Vandal, X. Jiang, J. Schneider, G. Lin and C. Nathan (2008). Selective killing of nonreplicating mycobacteria. *Cell Host and Microbe*. 3, 137-145.

Burton, S. G. and P. T. Kaye (1992). Isolation and Characterisation of Sulphur Compounds from Tulbaghia violacea. *Planta Med*. 58, 295-6.

Clarkson, C., E. V. Madikane, S. H. Hansen, P. J. Smith and J. W. Jaroszewski (2007). HPLC-SPE-NMR characterization of sesquiterpenes in an antimycobacterial fraction from Warburgia salutaris. *Planta Medica*. 73, 578-584.

Di Paulo, E., M. Hamburger, H. Stoeckli-Evans, B. Rogers and K. Hostettmann (1989). A New Antifungal Chromonocoumarin and its Derivatives from Polygala fruticosa. *Planta Medica*. 55, 615-616.

Driver, A. 2005. National spatial biodiversity assessment 2004. In: Priorities for biodiversity conservation in South Africa'. Pretoria: South African National Biodiversity Institute.

Flynn, J. L. and J. Chan. 2001. Immunology of tuberculosis. *Annu Rev Immunol*. 19, 93-129.

Graven, E. H., S. G. Dean, K. P. Svoboda, S. Mav and M. G. Gundidza (1992) Antimicrobials and antioxidative properties of the volatile (essential) oil of Artemisia afra Jacq. *Flavour and Fragrance Journal*. 7, 121-3.

Hollmann, J. (1996) Portrait of a medicinal tree. *Veld and Flora*. 82, 115-116.

Hostettmann, K., J. L. Wolfender, S. Rodriguez and A. Marston. 1996. Chemistry, biological and pharmacological properties of African medicinal plants. In: Proceedings of the first International IOCD-Symposium Victoria Falls, Zimbabwe, February 28-25, 1996., eds. K. Hostettmann, F. Chinyanganya, M. Maillard and J. L. Wolfender, 20-42. Harare, Zimbabwe: University of Zimbabwe Publications.

Hutchings, A., A. Scott, G. Lewis and A. Cunningham. 1996. *Zulu Medicinal Plants, an Inventory*. University of Natal Press. South Africa.

Iwu, M. 1993. Handbook of African Medicinal Plants. Boca Raton, Florida: CRC Press.

Kale, R. (1995). South Africa's Health: Traditional healers in South Africa: a parallel health care system. *British Medical Journal*, 310, 1182-1185.

Kubec, R., J. Velisek and R. A. Musah (2002) The Amino Acid Precursors and Odor Formation in Society Garlic (Tulbaghia violacea Harv.). *Phytochemistry*. 60, 21-25.

Kuria, K. A. M., S. D. Coster, G. Muriuki, W. Masengo, I. Kibwage, J. Hoogmartens and G. M. Laekeman (2001). Antimalarial activity of Ajuga remota Benth (Labiatae) and Caesalpinia volkensii Harms(Caesalpiniaceae): in vitro confirmation of ethnopharmacological use. *Journal of Ethnopharmacology*. 74, 141-148.

Larsen, M. H., K. Biermann and W. Jacobs (2007) Laboratory Maintenance of Mycobacterium tuberculosis. Current Protocols in Microbiology, 6.

Levyns, M. (1955) Some geographical features of the family Polygalaceae in South Africa. *Transaction of the Royal Society of South Africa*, 34, 379-386.

Light, M. E., S. G. Sparg, G. I. Stafford and J. van Staden. (2005). Riding the wave: South Africa's contribution to ethnopharmacological research over the last 25 years. *J Ethnopharmacol*, 127-30.

Lindsey, K. L. and J. E. Van Staden (2004). Growth inhibition of plant pathogenic fungi by extracts of Allium sativum and Tulbaghia violacea. *South African Journal of Botany*. 70, 671-673.

Madikane, V. E., S. Bhakta, A. J. Russell, W. E. Campbell, T. D. W. Claridge, B. G. Elisha, S. G. Davies, P. Smith and E. Sim (2007). Inhibition of mycobacterial arylamine N-acetyltransferase contributes to anti-mycobacterial activity of Warburgia salutaris. *Bioorganic and Medicinal Chemistry.* 15, 3579-3586.

Makhubu, L. (1998) Bioprospecting in an African Context. *Science*. 282, 41-42.

Makunga, N.P. (2009). Turning folklore into an ethnomedicinal catalogue. *S. Afr. j. sci*. 105, no.7-8.

Mashimbye, M., M. Maumela and S. Drewes (1999). A drimane sesquiterpenoid lactone from Warburgia salutaris. *Phytochemistry*, 5, 435-438.

Matasyoh , J., J. Kiplimo , N. Karubiu and T. Hailstorks (2007). Chemical composition and antimicrobial activity of essential oil of Tarchonanthus camphorates. *Food Chemistry*. 101, 1183-1187.

Mativandlela, S. P., J. J. Meyer, A. A. Hussein, P. J. Houghton, C. J. Hamilton and N. Lall (2008). Activity against Mycobacterium smegmatis and M. tuberculosis by extract of South African medicinal plants. *Phytother Res*. 22, 841-5.

McGaw, L. J., N. Lall, J. J. Meyer and J. N. Eloff. (2008). The potential of South African plants against Mycobacterium infections. *J Ethnopharmacol*. 119(3), 482-500.

Muregi, F. W., A. Ishih, T. Miyase, T. Suzuki, H. Kino, T. Amano, G. M. Mkoji and M. Terada (2007). Antimalarial activity of methanolic extracts from plants used in Kenyan ethnomedicine and their interactions with chloroquine (CQ) against a CQ-tolerant rodent parasite, in mice. *Journal of Ethnopharmacology*. 111, 190-195.

Nathan, C. (2004). Antibiotics at the crossroads. *Nature*. 431, 899-902.

Njoroge, G. and J. Kibunga (2007). Herbal medicine acceptance, sources and utilization for diarrhea management in a cosmopolitan urban area (Thika, Kenya). *African Journal of Ecology*. 45, 65-70.

Oyedeji, A. and A. Afolayan (2006). Chemical composition and antibacterial activity of the essential oil isolated from South African Mentha longifolia (L.) sep. capensis (Thunb.) Briq. *Journal of Essential Oil Research.* 18, 57-59.

Palgrave, M. C. 2002. *Keith Coates Palgrave Trees of Southern Africa,* edn 3. Struik Publishers, Cape Town.

Palmer, E. and Pitman N. 1972. *Trees of Southern Africa*. vol 2. A. A. Balkema Cape Town

Peltzer, K. (2009). Utilization and Practice of Traditional/Complementary/Alternative Medicine (TM/CAM) in South Africa. *Afr J Tradit Complement Altern Med*. 6, 175-185.

Rabe, T. and J. van Staden (1997). Antibacterial activity of South Africa plants used for medicinal purposes. *Journal of Ethnopharmacology*. 56, 81-87.

Rollinger, J. M., T. Langer and H. Stuppner (2006) Strategies for efficient lead structure discovery from natural products. *Curr Med Chem*. 13, 1491-507.

Sambandamurthy, V. K., X. Wang, B. Chen, R. G. Russell, S. Derrick, F. M. Collins, S. L. Morris and W. R. Jacobs (2002). A pantothenate auxotroph of Mycobacterium tuberculosis is highly attenuated and protects mice against tuberculosis. *Nature Medicine*. 8, 1171-1174.

Samie, A., Obi, C. L., P. O. Bessong and L. Namrita (2005) Activity profiles of fourteen selected medicinal plants from Rural Venda communities in South Africa against fifteen clinical bacterial species. *African Journal of Biotechnology*. 4, 1443-1451.

Scott, G., E. P. Springfield and N. Coldrey (2004). A Pharmacognostical Study of 26 South African Plant Species Used as Traditional Medicines. *Pharmaceutical Biology*. 42, 186-213.

van Wyk, B. E., B. van Oudtshoorn and N. Gericke. 1997. Medicinal Plants of South Africa. Pretoria, South Africa: Briza Publications.

Vrba, E. (1996) Climate, heterochrony and human evolution. *Journal of Anthropological Research.* 52, 1-28.

Watt, J. M. and M. Breyer-Brandwijk. 1962. *The medicinal and poisonous plants of southern and eastern Africa*. Edinburgh: E. and S. Livingstone.

Wayne, L. G. and C. D. Sohaskey (2001) Nonreplicating persistence of Mycobacterium tuberculosis. *Annual Review of Microbiology*. 55, 139-163.

Williams, V. L., K. Balkwill and E. T. F. Witkowski (2000). Unraveling the commercial market for medicinal plants and plant parts on the Witwatersrand, South Africa. *Economic Botany*. 54, 310-327.

CHAPTER 3

Bioassay Guided Fractionation of *Warburgia salutaris* and Biological Activity of Synthetic Drimane Compounds

3.1 Overview

Nine South African medicinal plants were tested for inhibitory activity against both replicating and non-replicating Mtb. The dichloromethane (DCM) extract of the mature bark of *Warburgia salutaris* demonstrated inhibitory activity against Mtb under both test conditions. *Warburgia salutaris* was included in this screen because it is used in South African traditional medicine to treat a variety of illnesses, including tuberculosis (TB). To date, no plant extract has been tested for inhibitory activity against non-replicating *Mycobacterium tuberculosis* (Mtb). This finding is important because potential new TB treatments are needed that are effective against non-replicating Mtb to shorten treatment courses.

This chapter will describe 1) the method of used to isolate and identify the compounds responsible for the biological activity 2) the biological assays used to assess the products of each fractionation step and 3) the investigation of synthetic drimane compounds that are structurally similar to those compounds isolated from *W. salutaris*. The result of the bioassay-guided fractionation identified the drimane sesquiterpenes class, specifically the compounds warburganal and muzigadial, as contributing to the biological activity. Naturally occurring drimanes are not readily available in large quantities and it was therefore important to identify synthetic analogues for use in subsequent testing. Thirty-one synthetic drimane sesquiterpenes were tested at 50 μg/mL and eight had percent inhibitions of greater than 65%.

3.2 Introduction

3.2.1. Taxonomy

The Canellaceae family is a small family of 5 genera. The genus *Warburgia* is exclusively African and includes four species: *W. salutaris* Berto F. Chiov, *W. elongata* Verd., *W. stuhlmannii* Engl. and *W. ugandensis* Sprague. *Warburgia salutaris* has limited distribution and is found in southern Africa (South Africa, Lesotho, Swaziland, Mozambique). The other plants of this genus: *W. elongata* and *W. stuhlmannii* are found along the East African coastline and *W. ugandensis* is found in the east African highlands of Uganda, Kenya and Tanzania (Muchugi et al., 2008).

There has been some controversy regarding the organization of species within the *Warburgia* genus. In 1956, Verdcourt reported only three species in the genus, *W. stuhlmannii*, *W. ugandensis* and *W. elongata* (Verdcourt, 1956). In 1976, *W. salutaris* was included in the genus (Ross, 1976). Other works have debated whether *W. salutaris* is the same as *W. ugandensis* subsp. ugandensis but the two have been kept separate based on differences in the number of ovules and the difference in fruit size (Hollmann, 1996). *Warburgia ugandensis* has 30 ovules and fruits up to 5cm in diameter, while *W. salutaris* has 15-20 ovules with fruits 1.5-2.5 cm in diameter (Ross, 1976; Verdcourt, 1956). A recent study by Muchugi et al. (2008) analyzed the genetic diversity of wild populations of the genus and determined that there is considerable similarity between *W. salutaris* and *W. stuhlmannii* populations from Tanzania, despite their distinct morphological characteristics. Muchugi et al. (2008) speculate that *W. salutaris* and *W. stuhlmannii* are in fact the same species that have adapted to different ecological environments.

3.2.2. Ecology and Conservation of *W. salutaris*

Warburgia salutaris is found along the eastern seaboard of South Africa in the indigenous forests, the smallest biome in the country, and the recently classified eastern Albany

thicket biome (Mucina and Geldenhuys, 2006). Most forests in South Africa occur as areas no larger than 10 hectares imbedded within larger scale biomes (Mucina and Geldenhuys, 2006). The indigenous forest in South Africa represents only 0.27% of the total land surface and is the country's second most species-rich biome (Lawes, Eeley and Piper, 2000).

Warburgia salutaris is threatened due to anthropogenic disturbances of its habitat and the widespread medicinal uses of the plant. In 1995, members of the Natal National Parks Board found that in all of the naturally occurring localities *W. salutaris* trees had been debarked and there were few mature specimens remaining (Johnson and Scott-Shaw, 1995). Even though this plant is considered fast growing with vigorous coppicing ability there are still concerns the plant will become extinct in unprotected areas (Botha, Witkowski and Shackleton, 2004). There are efforts to cultivate *W. salutaris*; however, the current level of cultivation does not meet the level of demand (Botha et al., 2004).

3.2.3. Biological Activity of *W. salutaris* and Drimane Sesquiterpenes

The biological activity of *W. salutaris* has been documented as far back as 1904 when the *Transvaal Agricultural Journal* reported the trees to be more resistant to than any other trees in South Africa's Transvaal region (Simpson, 1905). Laboratory investigations into the insect anti-feedant properties of this plant and other members of the Canellaceae family have identified drimane sesquiterpenes as the compound class responsible for this biological activity (Gols, van Loon and Messchendorp, 1996). Members of this compound class all contain the drimane chemical scaffold, shown in Figure 3.1. While drimane sesquiterpenes are considered chemosystematic markers of the Canellaceae family, these compounds are not restricted to plants. Drimane sesquiterpenes can be found in fungi, the defense secretions of the marine slug *Dendrodoris limbata* and in sponges in the *Dysidea* genus (Zapata et al., 2009; D'Ischia, Prota and Sodano, 1982; Montagnac et al., 1996).

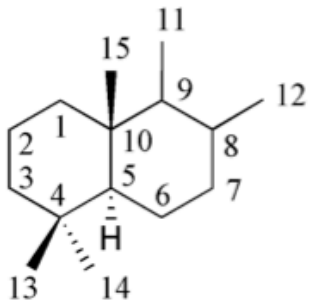
Drimane sesquiterpenes have always been identified as the compound class responsible for the biological activity in every investigation of plants from the *Warburgia* genus. Some of

these prior studies into biological activities of drimane sesquiterpenes will be discussed in this section.

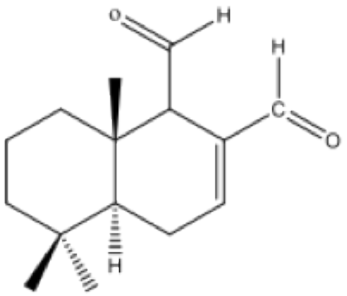
The initial investigations of the biological activity of drimane sesquiterpenes were its insect anti-feedant properties. The application of two naturally occurring drimanes, polygodial and warburganal to the surface of leaves, resulted in the decreased feeding of Colorado potato beetle larvae *Leptinotarsa decemlineata*, on leaf discs (Messchendorp, vanLoon and Gols, 1996). The topical application of these two compounds on the insect's cuticle also decreased food intake of untreated leaf discs, indicating that besides deterrent effects, these molecules influence feeding behavior. Polygodial, also found in *Warburgia* species, has also been shown to decrease the transmission of the potato Y virus, even by aphids considered to be highly resistant to insecticides (Asakawa et al., 1988).

The antimicrobial activities of isolated drimane compounds from *Warburgia* have also been studied. Isa Kubo and colleagues reported antifungal activity against the yeasts *Candida albicans* and *Saccharomyces cerevisiae* (Kubo and Taniguchi, 1988; Kubo, Fujita and Lee, 2001). Rabe et al. (1997) reported that the aqueous extracts of *W. salutaris* have inhibitory activity against *Escherichia coli* while others reported that the DCM extracts of the plant did not inhibit growth of *E. coli* (Rabe and van Staden, 1997; Clarkson et al., 2007). The disparity in these results is likely due to the different compounds that are present in plant extract prepared with different solvents.

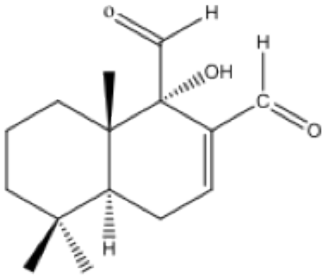
The anti-mycobacterial activities of extracts *Warburgia* species have also been previously evaluated. The DCM extract of the bark of *W. ugandensis* was found to be active against the fast growing species *M. aurum, M. fortuitum, M. phlei* and *M. smegmatis*, with minimum inhibitory concentration (MIC) values ranged from 4 to 128 µg/ml (Wube et al., 2010). Another study investigated the activity of the crude DCM extract of bark of *W. salutaris* against Mtb and *M. bovis* BCG (Madikane et al., 2007). This study reported that at 100 µg/ml the crude DCM extract completely inhibited the growth of replicating *M. bovis* BCG (Clarkson et al., 2007). The



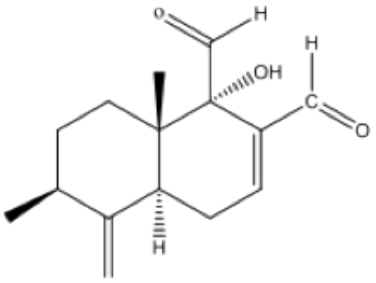
Drimane



Polygodial



Warburganal



Muzigadial

Figure 3.1. The chemical structures of drimane sesquiterpenes. Drimane shown here is the chemical skeleton characteristic of all drimane compounds, also shown is the numbering system used for this ring system. Polygodial is a common naturally occurring drimane. Warburganal and Muzigadial were isolated in this study from the active extract of *W. salutaris*.

Clarkson study also reports that at 200 µg/ml the DCM extract of the mature bark completely inhibits the growth of Mtb. However, neither the data nor the testing methods were published. Therefore conclusions cannot be drawn about the differences in the inhibition between the two *Mycobacterium* species.

This dissertation project differs from past investigations into the inhibitory activity of *W. salutaris*. First, these past studies used the disc diffusion method to evaluate the inhibitory activity. This method has not proven to be the best way to make quantitative measurements of the MIC. Additionally, none of these studies looked at the inhibitory activity of Mtb under growth inhibiting conditions.

3.3 Methods

The steps of the plant extraction process and the bioassay-guided fractionation are outlined in the schematic diagram in Figure 3.2 and discussed in detail below.

3.3.1. Plant Material and Plant Extraction

The mature bark of *W. salutaris* was obtained from Parceval Pharmaceuticals (Ltd), Wellington South Africa. Small pieces of bark were ground with an industrial blender. 250 g of powdered plant material was extracted with 1.5 L DCM over a period of 72 hours with agitation at room temperature. The extract was then vacuum filtered from the remaining plant material (Figure 3.2).

3.3.2. High Performance Liquid Chromatography Pretreatment

Liquid-Liquid extraction: An equal volume of hexane was added to the DCM plant extract. This caused colloidal particles to fall out of solution and the resulting clear, light brown liquid was collected. This liquid was then evaporated to dryness using a rotary evaporator.

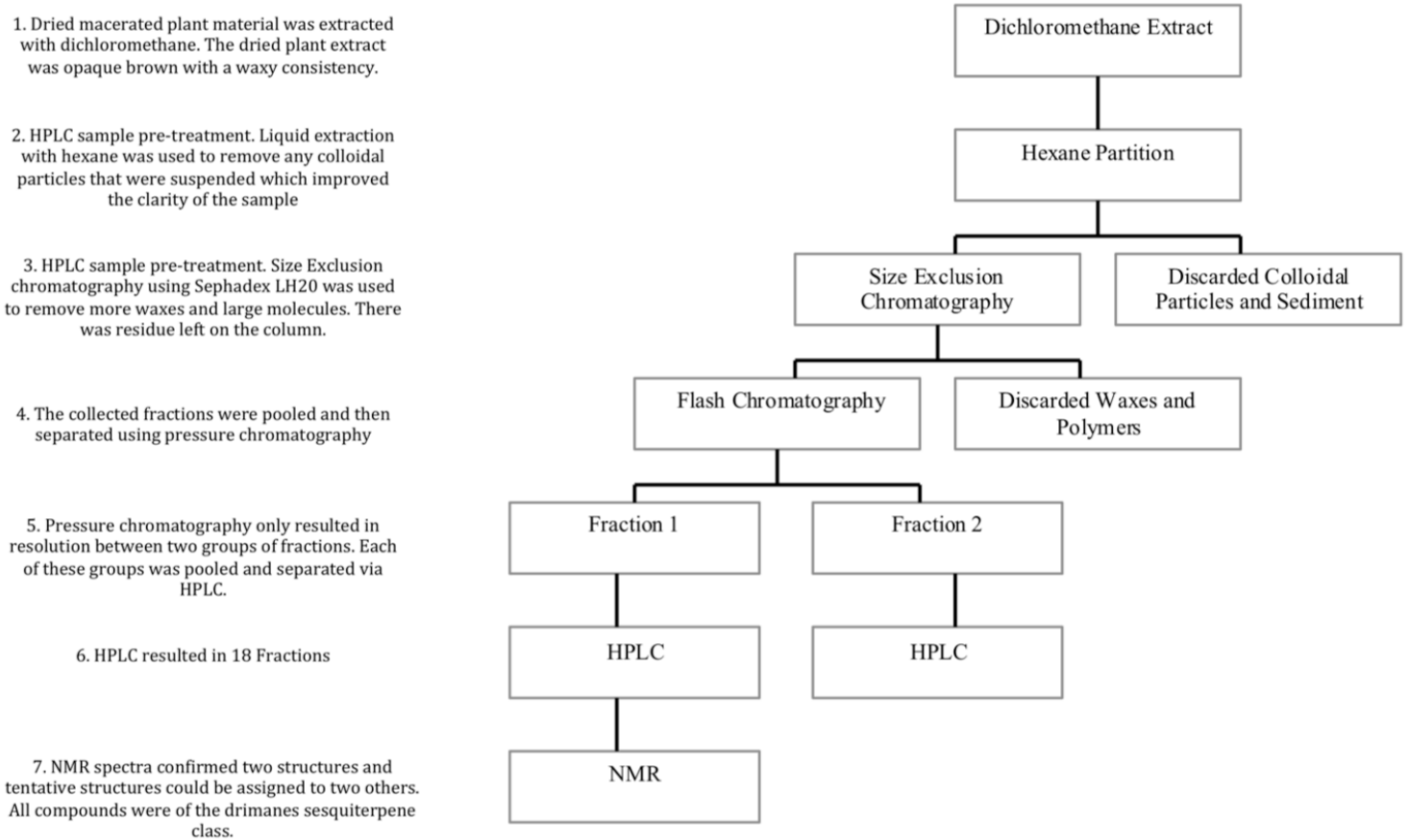


Figure 3.2 Schematic diagram of the bioassay guided fractionation of *W. salutaris*

Size exclusion media, Sephadex LH20, was allowed to swell for approximately 3 hours in acetonitrile and then poured into a Fischer glass column. The dried crude plant extract was dissolved in acetonitrile and applied to the column. Acetonitrile was delivered manually at room temperature. All eluate was pooled and rotary evaporated to dryness.

3.3.3. Reverse phase flash chromatography

Flash chromatography was performed on the product obtained from the size exclusion chromatography. The 55.8 mg sample was loaded onto Celite 545 with acetonitrile. The celite was added to the 12 g silica gel loading column (RediSep Rf Gold™). A solvent gradient of water progressing to 100% acetonitrile was used. Ultra violet (UV) absorption was used to monitor the separation of the compounds at 230 nm and 260 nm, the absorbance maxima of several drimane compounds (Clarkson, 2008). The fraction collector is coupled to absorbance detection and fractions are only collected when the absorbance levels becomes greater than the baseline. Portions of the sample that did not contain compounds with absorbance's at these wavelengths were also collected separately and saved.

3.3.4. High Performance Liquid Chromatography (HPLC)

The dried samples which resulted from the flash chromatography were dissolved in ~200 µl of acetonitrile and injected into the HPLC (Agilent 1100 C18 column). A solvent gradient using water and acetonitrile (a gradient decreasing from 95% H_2O to 0% H_2O for 40 minutes followed by 10 minutes of 100% acetonitrile) with a flow rate 3.6 mL/min and a pressure of 400 bar. A diode array detector recorded the absorbance of four wavelengths (210, 230, 260, 320 nm) corresponding to the ultraviolet absorbance maxima of several known drimane sesquiterpenes compounds (Clarkson, 2008). An initial run was used to determine the boundaries between each fraction with the intention to include the minimum number of peaks in each fraction. A total of eighteen fractions were collected over the entire preparative run. The boundaries of the 18

fractions were designed to include the minimum number of peaks, and therefore the minimum number of compounds in each fraction.

3.3.5. NMR Identification of Isolated Drimanes

HPLC fractions were dried under nitrogen gas and dissolved in deuterated chloroform (Cambridge Isotope Laboratories). Samples were placed in microscale NMR tubes (Shigemi Inc.). NMR spectra were recorded on a Varian INOVA 600 NMR (600 MHz for ^{1}H, 151 MHz for ^{13}C) using a HCN indirect-detection probe. NMR spectra were processed using Varian VNMR and MestreLabs MestReC software packages, with the assistance of the Cornell University NMR facility director Dr. Ivan Keresztes.

3.3.6. Evaluating Anti-Mycobacterial Activity

Cultures of *M. bovis* BCG were grown from -80 °C frozen stocks to midlog phase in Middlebrook 7H9 broth (Difco Laboratories). The bacteria were grown at 37 °C in Middlebrook 7H9 broth, supplemented with 0.2% glycerol, 0.05% Tween 80, 0.5% bovine serum albumin (BSA), 0.2% dextrose, and 0.085% NaCl (Larsen, Biermann and Jacobs, 2007). Experiments were performed using mid- to late-logphase cultures (optical density measured at 580 nm, 0.5 – 0.8 absorbance units (AU).

To assess bactericidal activity, the reaction wells of standard 96 well plates were prepared with 98 μL of 7H9 medium and 100 μL *M. bovis* BCG inoculum. The fractions to be tested were dried and dissolved in DMSO and 2 μL volumes were added to the reaction well to yield a final volume of 200 μL. Rifampicin (RIF) at an experimental concentration of 0.5 μg/ml and dimethyl sulfoxide (DMSO) were included as positive and negative controls respectively.

- **Growth inhibiting conditions**: Midlog phase cells (A580 0.8–1.0 AU) of *M. bovis* BCG, previously grown in growth sustaining media, were prepared in 7H9 at

pH 5.5, 0.5 mM $NaNO_2$ with 10% solution composed of (0.5% BSA, 0.85% NaCl), 0.02% tyloxapol, 0.05% butyrate) (Bryk et al., 2008).

- **Growth sustaining conditions**: Midlog phase cells (A580 0.8–1.0 AU) were prepared in 7H9 at pH 6.8. These test conditions include 20% O_2, pH 6.8, and 7H9 medium enriched with glycerol as a carbon source. (Bryk et al., 2008).

After 3 days, killing was assessed either by measuring the optical density or by plating the cultures on solid medium. For the growth inhibiting conditions following the 3-day exposure, killing was assessed by dilution into growth sustaining media and outgrowth after 4 days was determined via optical density (Tecan SPECTRAFLUOR Plus). To plate on solid media, growth media was supplemented with 10% oleic acid-albumin-dextrose-catalase-enriched (OADC) and incubated at 37 °C. Colony forming units were counted after 14-21 days.

3.3.7. Synthetic Drimane Compounds

Synthetic drimane compounds were obtained from Dr. Duy Hua, a collaborator from the University of Kansas, Department of Chemistry. The structures of all 31 compounds are shown in Appendix A.

3.3.8. Calculating IC_{50}

A nonlinear regression analysis was performed on the dose-response data to calculate the half maximal inhibitory concentration (IC_{50}). The vehicle control DMSO and the positive control RIF were used to calculate normalized percent inhibition of each concentration. The percent inhibition vs. log concentration was plotted and GraphPad Prism software, using the method of least squares, determined the values of the following equation:

$$\text{Percent Inhibition} = \text{Bottom} + \frac{(\text{Top}-\text{Bottom})}{(1+10^{((\text{LogEC50}-X)*\text{HillSlope})})}$$

In this this fitting the slope was not assumed to be one; rather the slope was determined as one of the fit parameters.

3.4. Results and Discussion

3.4.1. Fractionation of *W. salutaris*

HPLC is a technique often used in natural products research to separate the individual components of a crude extract. Here HPLC was used to obtain fractions with simplified chemical compositions so that the subsequent NMR spectra could be more easily analyzed. HPLC requires that samples be particle free and completely soluble (Uihlein, 1988). The crude extract of *W. salutaris*, did not initially meet these requirements; it was an opaque liquid and when dried it was a very waxy substance. Two pretreatment steps were needed before HPLC could be preformed. The pretreatment steps were intended to remove substances that would damage an HPLC column, while minimizing loss of those compounds responsible for the biological activity.

The first pretreatment step was a liquid extraction with hexane. The purpose was to remove any unfiltered solids, especially colloidal particles. Small colloidal particles were not easily removed from the solution with conventional filtration methods. Once hexane was added, the overall density of the solution was reduced so that the colloids, which were denser, settled out of solution and could be more easily removed. This method was employed instead of waiting sufficient time, perhaps several days, for coagulation and settling or using several costly filters. The product of this procedure was more transparent than the initial crude extract.

The second pretreatment step performed was size exclusion chromatography using the adsorbent media Sephadex LH20. Size exclusion chromatography can be used as a fractionation step but it can also be used to remove high molecular weight and polymeric material from a sample (Ghisalberti, 1993; Vihma et al., 1990). The size exclusion product of the *W. salutaris* extract was transparent yellow and had improved solubility compared to the previous product of

hexane treatment. Unfortunately, residue was visible on the column that could not be removed even after several sequential washes with DCM and acetonitrile.

The inhibitory activity of the initial DCM crude extract, the liquid extraction product and the size exclusion chromatography eluate, were evaluated in a dose dependent manner against replicating *M. bovis* BCG. This bacterium is a slow growing *Mycobacteria* that is pathogenic to cattle but not to humans. *M. bovis* BCG is considered a suitable test species because there is extensive genetic similarity between the two species, with only 0.01%–0.03% synonymous variation (Gutierrez et al., 2005). Additionally, *M. bovis* BCG was used because it only requires a level 2 safety cabinet. Non-linear regression analysis was used to calculate concentration that is required for 50% inhibition *in vitro* (IC_{50}). The IC_{50} of the DCM crude extract, the liquid extraction product and the size exclusion chromatography product were determined to be 6.0 µg/mL (± 4.8 -7.4), 5.3 µg/mL (± 4.1 -7.0) and 8.0 µg/mL (± 7.2 -8.7) respectively (Table 3.1, Figure 3.3).

Table 3.1. Summary of IC_{50} analysis of the different fractionation steps of *W. salutaris*

	Crude DCM Extract (ug/ml)	**DCM/Hexane Extract** (ug/ml)	**Size Exclusion Product** (ug/ml)
Best-fit values			
IC_{50}	6.0	5.3	8.0
HillSlope	2.7	1.4	4.1
95% Confidence Intervals			
IC_{50}	4.8 to 7.4	4.1 to 7.0	7.2 to 8.7
HillSlope	1.2 to 4.2	0.85 to 2.0	3.0 to 5.4
Goodness of Fit			
R square	0.9778	0.9794	0.9972

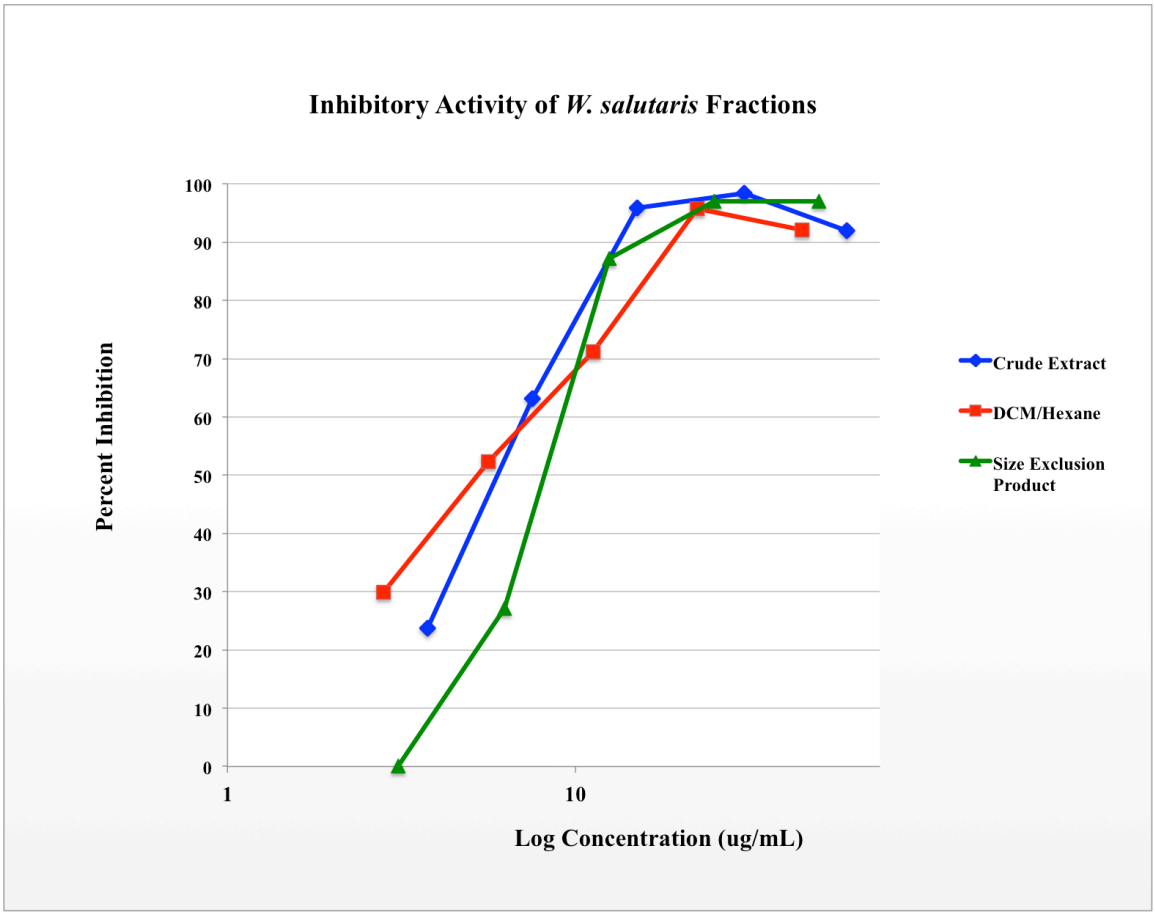


Figure 3.3. Inhibitory activity of *W. salutaris* fractions. The initial crude DCM extract ( ), after the crude DCM extract was treated with hexane ( ), the product of the size exclusion product ( )

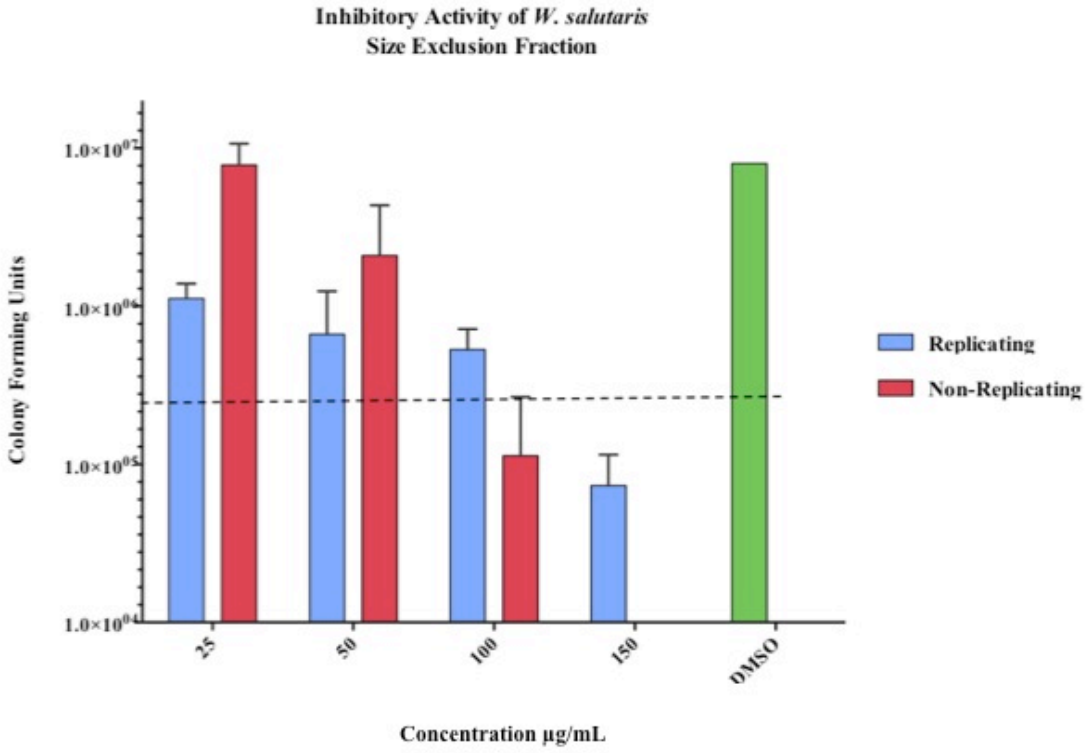


Figure 3.4. The product of the size exclusion fraction was evaluated for activity against *M. bovis* BCG under both growth-inhibiting and growth-sustaining conditions. The dashed line represents 50 percent inhibition based on the vehicle control.

The removal of colloidal particles and other insoluble materials that contributed to the overall weight of the extract, but not to the biological activity, should have decreased the IC_{50} with each fractionation step. However, the relatively identical IC_{50} values of 6.0 and 5.3 indicate that some compounds with biological activity were also removed (Figure 3.3 and Table 3.1). The particles that were removed were colloidal particles or residue that remained on the column and it was not possible to test them for biological activity. The decision to continue with the bioassay-guided fractionation, despite the apparent loss of some active components, was also influenced by the limited access to additional plant material. *Warburgia salutaris* is a threatened species and the availability of plant material is limited and expensive. This made it impossible to perfect the separation procedures. It would have been ideal to isolate every compound in the plant that may have contributed to the activity. However, the benefits of the improved solubility and suitability of the sample for HPLC was thought to be more important than preventing the loss of some of the components of the extract. In plants there are thousands of secondary metabolites and that are often distributed among a few compounds classes. It is likely that any biologically active compounds that were lost were from the same compound class as the active compounds in the fraction that remained.

The product that resulted from the size exclusion chromatography had an IC_{50} of 8.0 (± 7.2 - 8.7), which is slightly higher than the previous fractionation steps. This indicates an additional loss of biologically active compounds. This fraction was tested against non-replicating *Mycobacterium* and results demonstrate that the pooled eluate from the size exclusion column still contains inhibitory activity against *M. bovis* BGC in a dose dependent manner under both conditions (Figure 3.4).

Flash chromatography was used to create a less complex mixture for subsequent HPLC separation (Ghisalberti, 1993). In flash chromatography, the precise conditions (including the solvent used and the gradient elution) are not standard and are typically determined for each sample. In this case, a step gradient of water and acetonitrile was used similar to previous publications of *Warburgia* fractionation (Clarkson, 2008). The absorbance of the eluate was

monitored at two UV wavelengths (230, 260 nm) that drimane compounds are known to absorb. Fractions were only collected when the UV absorbance was above the baseline. There were portions of the sample that didn't absorb light at the wavelengths chosen. These were collected and saved in the event that they needed to be analyzed in the future.

The flash chromatography spectrum showed that there was poor resolution between components of the extract (Figure 3.5). However, with limited sample there was not an opportunity to further evaluate the elution properties and optimize the experimental conditions. This method was only marginally effective in separating the compounds contained in the extract, which appeared to be of similar polarity. The flash chromatography fractions were therefore pooled into three samples, (Figure 3.5). HPLC was performed on the sample containing fractions 41-49 because it was more soluble than fractions 51-56, making it easier to test biologically, and larger than fractions 37-39.

HPLC separation was monitored using UV-visible spectra of four wavelengths that drimane compounds are known to absorb. The HPLC chromatogram showed well-defined peaks, according to the samples absorbance spectra at 210, 230, 260 and 320 nm, (Figure 3.6). All fractions were tested for biological activity at 100 μg/mL against replicating *M. bovis* BCG (Figure 3.7). The data suggests that each fraction contained some compound(s) that contributed to the overall biological activity of the extract.

3.4.2. NMR Identification of Drimane Compounds

The 18 dried HPLC fractions were all below half a milligram in weight. Some portion of each sample was used in biological testing and was therefore unrecoverable. Consequently, it was difficult to prepare NMR samples that had high enough concentrations for ^{13}C spectra. Microscale Shigemi NMR tubes were used to reduce the needed volume of the sample from approximately 600 μL to about 250 μL, effectively doubling the concentration. Additionally, the use of the solvent DMSO in biological testing was difficult to completely remove from samples. The presence of trace amounts of DMSO resulted in prominent peaks in the NMR spectra that

obscured peaks of the sample (Gottlieb, Kotlyar and Nudelman, 1997). Two fractions could be prepared at a high enough concentrations (HPLC fraction 7 and 14, Figure 3.6) and ^{13}C NMR spectra were obtained.

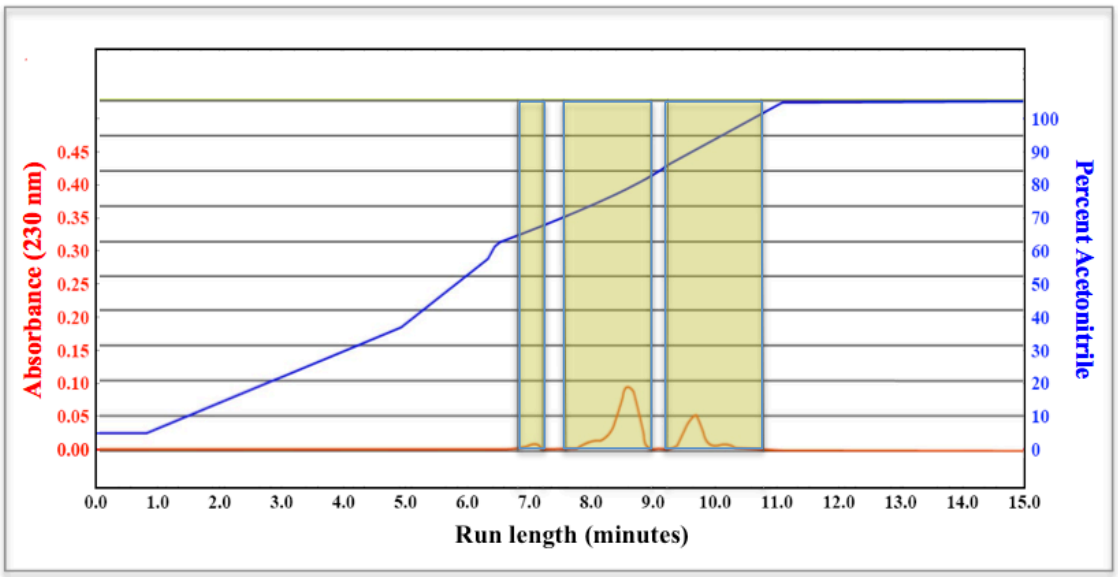


Figure 3.5. Flash Chromatography of the size exclusion product. The three shaded regions represent the pooled fractions 37-39, 41-49, and 51-56.

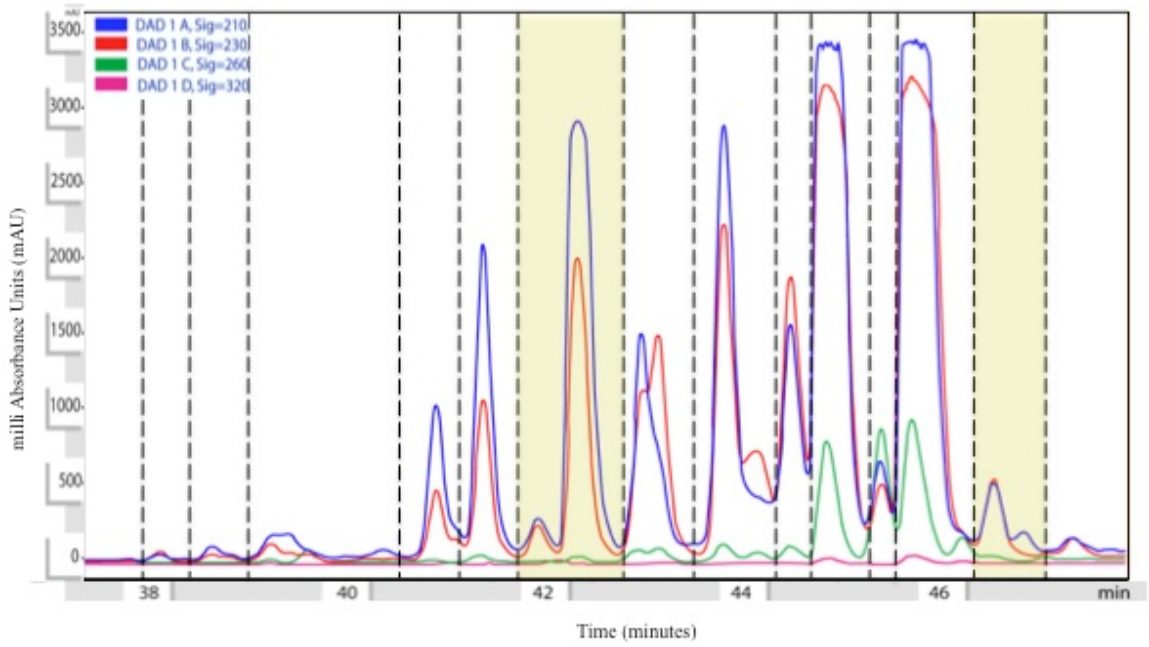


Figure 3.6. HPLC of flash chromatography fractions 41-49. The separation of the sample was monitored via UV wavelengths that drimane compounds are known to absorb, 210nm ( ), 230nm ( ), 260 ( ), 320nm ( ). 18 samples were collected. Fractions 7 and 14 are shaded ( ).

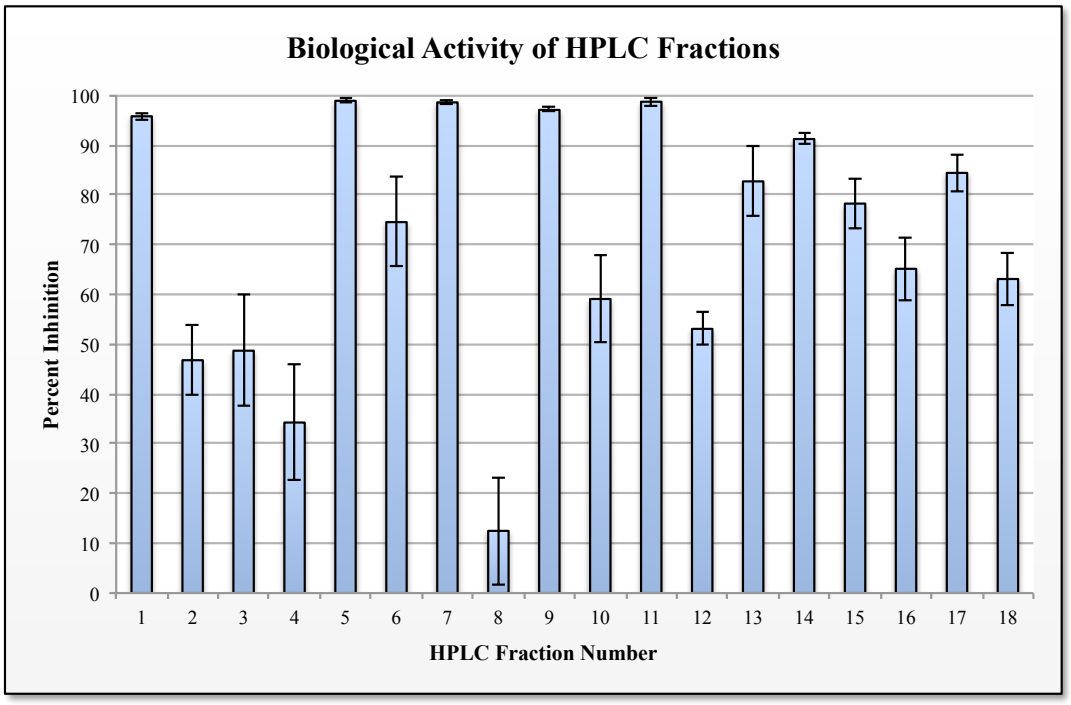


Figure 3.7. Percent inhibition of each of the HPLC fractions against *M. bovis* BCG under growth sustaining conditions. The percentages are normalized against the positive and vehicle control RIF and DMSO respectively.

One dimensional (1D) ^{1}H-NMR (600MHz) spectra were acquired for HPLC fraction 7, the sample that eluted between 41.48 to 42.48 min. The HPLC chromatogram suggests that this fraction contains two distinct compounds. However, there was enough information to obtain the structure of the most prominent compound in this sample was elucidated. The ^{1}H-NMR spectrum showed signals of two aldehyde groups (δ = 9.59, doublet, J = 0.8 Hz, H-11; 9.38, singlet, H-12) that were further confirmed by carbonyl signals observed in the 13C-NMR spectra at 201.12 and 192.56. Also identified were an olefinic hydrogen (δ = 7.19, d, J = 4.7, 3.2 Hz, H-7), a hydroxyl group (δ = 4.11, doublet, 11-OH), two methyl groups (δ = 1.02, H-14; 0.81, H-15), a methine resonance (δ = 2.01, d, J = 4.7 Hz, H-5), and excocyclic methylene protons (δ = 4.88 and 4.70, triplet, J = 1.8,18.8 Hz, H-13). The compound was identified as muzigadial, (1S, 4aS, 8a S)-1-hydroxy-5,5,8a-trimethyl-1,4,4a,5,6,7,8,8a octahydronaphthalene-1,2-dicarbaldehyde), Figure 3.1. This compound was previously isolated from *W. salutaris* and *W. ugandensis*. The NMR spectra obtained were in agreement with observed shifts in previous publications (Table 3.2, Appendix A) (Clarkson et al., 2007; Kioy, Gray and Waterman, 1990; Mashimbye, Maumela and Drewes, 1999).

There was enough information to determine the structure of the most abundant compound in HPLC fraction 14 (t_R = 46.18- 47.0 min). This spectrum exhibited two aldehyde resonances (δ = 9.7, doublet, J = 1.5 Hz, H-11; 9.39, singlet, H-12) and a hydroxyl group (δ = 4.08, doublet, J = 1.5 Hz, 11-OH) similar to those of muzigadial. Three methyl groups were identified at (δ = 0.92, H-13; δ =0.97, H-14, δ = 1.07, H-15). The ^{13}C NMR assignments are in agreement with those reported previously and by comparison of structurally related compounds, Table 3.3 (Wube et al., 2005; Clarkson et al., 2007). The compound was identified as warburganal, (1S)-1,4,4aα,5,6,7,8,8a- Octahydro-1α-hydroxy- 6β, 8aβ- dimethyl-5-methylene-1,2-naphthalenedicarbaldehyde, Figure 3.1.

Table 3.2. H^1 and C^{13} NMR spectra data for warburganal (HPLC Fraction 14)

Carbon No.	δ, ppm	Proton δ, ppm	Position	Splitting	J_{HH}, Hz
C-1	31.38	1.67	ax	ddd	12.8,12.5, 5.9
		1.01	eq	ddd	12.8, 5.1, 3.2
C-2	17.75	1.47	ax	m	
		1.51	eq	m	
C-3	41.37	1.24	ax	ddd	13.2, 12.9, 5.1
		1.42	eq	dddd	13.2,3.2, 3.2, 1.8
C-4	32.92				
C-5*	41.54	1.87	ax	dd	11.9, 5.3
C-6*	25.92	2.32	ax	ddd	20.9, 2.6
		2.56	eq	dd	20.9, 5.3, 5.3
C-7	157.46	7.24		ddd	5.3, 2.6
C-8	140.21				
C-9	77.55				
C-10	41.4				
C-11	202.13	9.7		d	1.5
C-12	192.64	9.39		s	
C-13	33	0.92		s	
C-14	22.08	0.97		d	0.7
C-15	17.02	1.07		d	0.8
9-OH		4.08		d	1.5

*J's measured in gCOSY

Table 3.3. H^{1} and C^{13} NMR spectra data for muzigadial (HPLC Fraction 7)

Carbon No.	δ, ppm	Proton δ, ppm	Position	Splitting	J_{HH}, Hz
1*	30.77	1.95	ax	ddd	14, 13, 4.3
		1	eq	ddd	13, 3.3, 3.3
2[1]	31.53	1.07	ax	dddd	13, 13, 13, 3.7
		1.64	eq	dddd	13, 3.5, 3.5, 3.5
3	38.09	1.94	ax	m	
4	151.51				
5[2]	39.9	2.51		m	
6[3]	27.48	2.46		m	
		2.59		m	
7	155.6	7.19		dd	4.7, 3.2
8	139.62				
9	77.36				
10	42.12				
11	201.12	9.59		d	0.8
12	192.56	9.38		s	
13	105.97	4.88		t	1.8
		4.7		t	1.8
14	18.3	1.02		d	6.6
15	15	0.81		s	
9-OH		4.11		d	0.8

*J's measured in gCOSY
[1]Three similar couplings averaging to 3.5
[2]1H from gCOSY, 13C from HMBC due to overlap w/ DMSO (2.55/40.9)
[3]1H from gCOSY due to overlap w/ DMSO (2.55/40.9)

3.4.3. Biological Activity of Synthetic Drimane Compounds

Each of the thirty-one synthetic drimane compounds (Appendix A) were tested at 50 μg/mL. Nine compounds inhibited the growth of Mtb by more than 65%, (Compounds **4**, **6**, **8**, **9**, **15**, **18**, **19**, **23** and **24**, Figure 3.8). The 31 synthetic compounds were tested at a single concentration due to limited compound availability. These structures are difficult to synthesize and each compound has multiple chiral centers. The discussion to follow focuses on common features of the natural and synthetic compounds that may contribute to biological activity (Figure 3.8, Appendix A). The biological activity of a molecule reflects a combination of different physical interactions. Any comprehensive analysis would require a larger number of compounds than the 31 compounds available. Additionally, these compounds would have to be more similar in structure than the synthetic compounds tested.

- **Functional groups at the C9 position**

Previous publications have indicated that the unsaturated dialdehyde functionality at the C8- C9 position as the source of the biological activity. This is based investigations of the antibiotic properties, the hot taste to humans, and antifeedant activity of these compounds (Jansen, de Groot 2004). Tangiguchi et al. (1998) also investigated the inhibitory activity of naturally occurring drimane compounds against the yeast *Saccharomyces cerevisiae*. They reported that when the C9 aldehyde groups were reduced the inhibitory activity was lost (Tangiguchi et al., 1988). While these conclusions were not based on investigations into the anti-mycobacterial activity, it did prompt a closer look at the functional groups that the C9 position.

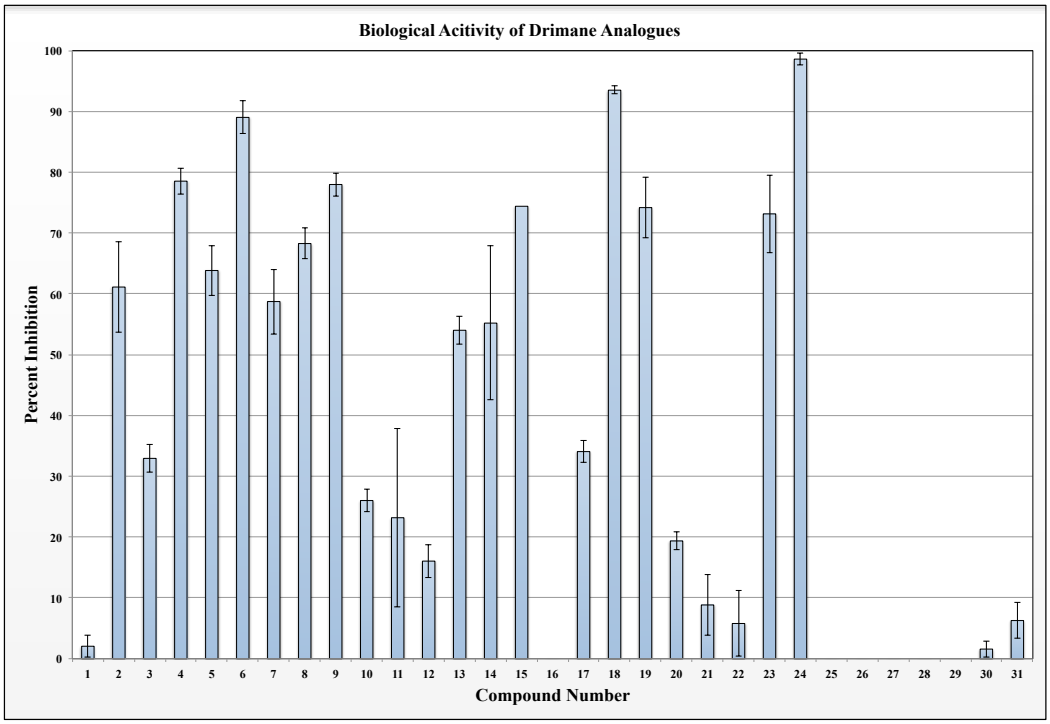


Figure 3.8 Percent inhibition of each of the synthetic drimane compounds against *M. bovis* BCG under growth sustaining conditions. The percentages are normalized against the positive and vehicle control RIF and DMSO respectively.

The aldehyde functional group at the C9 position is largely absent from the synthetic drimanes tested in this study. In fact, only one of the nine synthetic compounds with inhibitory activity above 65% had an aldehyde at this position (Compound **23**). Some of the synthetic compounds contained other oxygenated substituents at this position such as primary alcohols (Compound **6**, **9**, **19**), secondary alcohols (Compound **4** and **8**) and an ester (Compound **18**) (Appendix A). This does suggest that the C9- aldehyde group may not be necessary for activity of the synthetic drimane compounds.

It is interesting to compare the activity of compound **7** and **20**, whose only structural difference is that compound **7** has a primary alcohol at the C9 position and compound **20** has an aldehyde at this position. Compound **7** demonstrated nearly 60% percent inhibition while compound **20** inhibited less than 20 percent of bacterial growth. Additionally, two compounds, **15** and **24**, do not contain any oxygen atoms at this location, and still showed inhibitory activity above 90%. Two possible inferences can be drawn; either the presence of an aldehyde group at the C9 position is not specifically necessary for the biological activity or the mechanism of action of drimane compounds in *Mycobacteria* differs from the proposed mechanism of drimane compounds in yeast.

- **Degree of Saturation**

The degree of saturation is important to the biological activity of the molecule; it can change the orientation of a molecule and affect its activity and selectivity. Many drimanes isolated from plants contain a saturated C7-C8 bond. This was not the case in the synthetic drimane compounds; only one out of the nine biologically active synthetic compounds (compound **9**) had a double bond at this position. Compounds **6** and **9** have the same structure except that one member of the pair has a C7-C8 double bond. Both compound **6** and **9** were active with 89% and 77% inhibition, respectively. This suggests that a double bond at this location is not needed for activity.

Another pair of compounds that differ in saturation is compounds **22** and **23**. Compound **22** has a C7-C8 double bond with only 5.8% inhibition and compound **23** has a C8-C12 bond double bond with 73% inhibition. This also suggests that the presence of a double bond does not necessarily confer activity.

- **Polycyclic structures**

Many synthetic drimanes have a third or a fourth ring at C8-C9 position. For example, compounds **4** and **8** have lactone and compounds **13-17** have hexane ring. Compound **16**, which has a diether ring at C3 position and **17** are not active. Compounds **25-31** all have at least two additional six member rings at this position. This suggests that one ring at this position does not affect activity, but any more ring structures at this position makes the compound too large to interact with its target.

- **Inconclusive structural attributes**

There are other structural features of the synthetic compounds are noteworthy, although they cannot always be used to draw concrete conclusions regarding their contribution to the biological activity.

i. There are no substituents on C1, C2, C6, C7 (with the exceptions of compound **12** which has a substituent on C6). It is not clear whether the absence of a constituent is necessary for activity.
ii. All compounds have a dimethyl at the C4 position except compound **12**, which has nothing and **13**, which has one methyl group. Additionally, naturally occurring drimanes have some variation at this carbon. For instance polygodial and warburganal have the dimethyl group, while muzigadial has an alkene group. There seems to be no apparent contribution of to the biological activity of this carbon.

iii. All of the synthetic compounds have a hydrogen atom in the same orientation (pointing out of the plane) at the C5 position, except compound **12** which has a different orientation and compound **13** which does not have a compound here. This data suggests that there is no apparent contribution of this carbon to the biological activity.

iv. All the synthetic compounds and the naturally occurring drimanes have a methyl group at the C10 position, except compound **12**. There is no apparent contribution of this carbon.

3.4.4. Selection of a Synthetic Drimane Analogue

The results of the bioassay-guided fractionation of *W. salutaris* identified two drimane sesquiterpenes, warburganal and muzigadial, as constituents that contribute to the biological activity of the DCM plant extract. Access to additional plant material was limited due to the threatened status of *W. salutaris* and it was therefore necessary to find a suitable synthetic analogue. Synthetic compounds that are based on natural product scaffolds can present a challenge because these compounds are difficult to synthesize. They have multiple chiral centers and the synthesis can be almost a hundred steps with low yields (Jansen and de Groot, 2004). Therefore, without the benefit of a medicinal chemist to synthesize the most potent analogue, the availability of the synthetic drimane compounds had to be considered. While, compound **8** was not the most potent of the synthetic molecules it was structurally similar to other naturally occurring drimane compounds and readily available. For these reasons it was selected as a synthetic analogue for subsequent testing.

Compound **8** was investigated for dose dependent activity against *M. bovis* BCG under growth sustaining and growth inhibiting conditions and was found to have an IC_{50} of 26.9 and 36.2 µg/mL respectively, (Figure 3.9, Table 3.4). Based on these results, compound **8** will be used for subsequent investigations into the mechanism of action of drimanes in *Mycobacteria*.

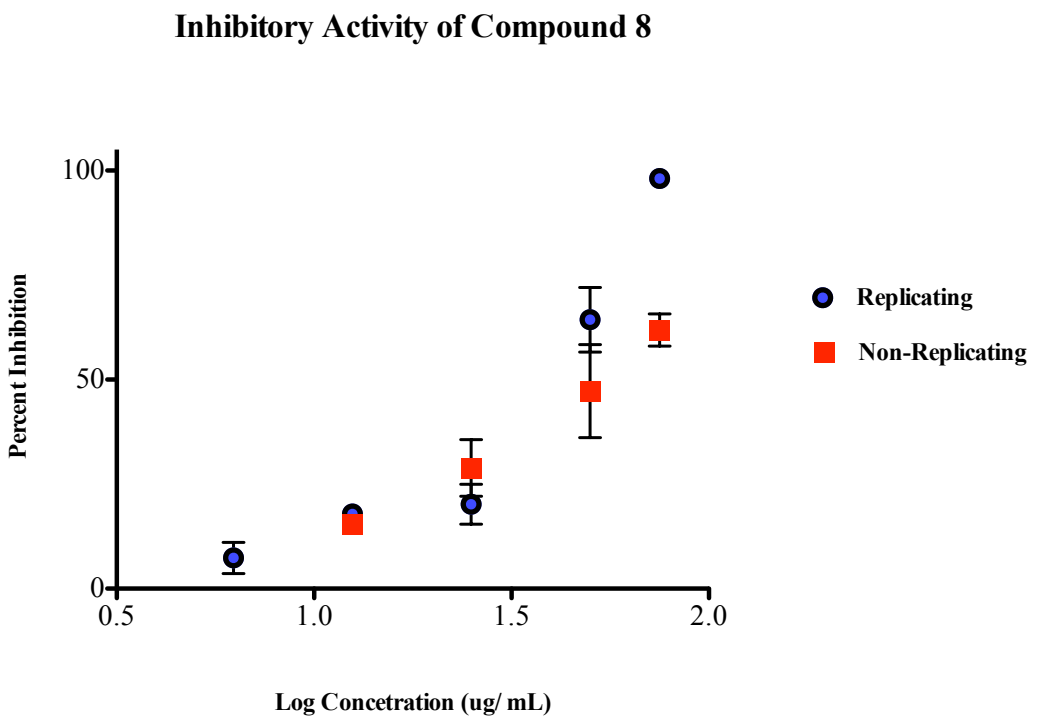


Figure 3.9. Dose dependent inhibition of compound **8** against replicating *M. bovis* BGC (pH 6.7), non-replicating *M. bovis* BCG (pH5.5, 0.5mM $NaNO_2$)

Table 3.4. IC_{50} of Compound **8**, when tested against replicating and non- replicating *M. bovis* BCG.

Best-fit values	**Replicating**	**Non-replicating**
IC_{50} (μg/mL)	26.7	36.2
Hill Slope	1.9	3.0
95% Confidence Intervals		
IC_{50} (μg/mL)	14.5 to 49.0	21.1 to 62.2
HillSlope	0.46 to 3.3	-0.89 to 6.9
Goodness of Fit		
R square	0.98	0.94

3.5 Conclusion

The screening of plants used in traditional medicine has great potential in the search for novel anti-mycobacterial compounds. This project was able to demonstrate that the DCM extract of the *W. salutaris* was biologically active against both replicating and non-replicating Mtb. This project differs from other investigations into the anti-mycobacterial activity of South African medicinal plants in that these previous studies rarely tested for inhibitory activity against slow-growing pathogenic species and to date no plant extracts have been screened for inhibitory activity against Mtb under non-replicating conditions.

While this project was able to go further than past similar studies, it was not without its limitations. One challenge was the availability of plant material. Without additional plant material it was not possible to optimize extraction methods to ensure that no compounds that were contributing to the biological activity were lost during the extraction process. The relatively constant IC_{50} values between the fractionation steps suggest that some biologically active compounds were indeed lost. The lack of plant material also limited the number of compounds that could be identified from the active fraction. The structures of the two compounds, muzigadial and warburganal, which were present in sufficient concentrations were elucidated with NMR spectroscopy. This underscores the need for the sustainable use of medicinal plants and programs that supply plant material for scientific research.

To facilitate future studies, the biological activity of synthetic compounds was evaluated. Plant-derived compounds have been used as the basis of chemical scaffolds for synthetic or semi-synthetic compounds. This diminishes the need for plant material and the isolation of every bioactive compound from plants, since the ultimate goal is to create synthetic derivatives. This project did not have the benefit of a medicinal chemist to synthesize derivatives of the compounds isolated from *W. salutaris*. Thirty-one synthetic compounds that were similar in structure were available and nine were found to inhibit growth of Mtb by more than 65%. Compound **8** was a biologically active synthetic compound that demonstrated inhibitory activity

in a dose dependent manner with an IC_{50} of 26.9 μg/mL and 36.2 μg/mL against replicating and non-replicating *M. bovis* BCG respectively. Compound **8** was selected to be used in subsequent investigations into the mechanism of action of drimane compounds in *Mycobacteria*.

The experiments discussed demonstrate the potential that ethnobotanical knowledge has in leading the search for new chemicals with inhibitory activity against Mtb. This study also demonstrates that the difficulties of natural products research are unavoidable and investigations with natural products are resource intense multi-disciplinary endeavors. Notwithstanding, drugs of natural origin are essential to modern pharmacopeias and research into plant-derived antibiotics should continue.

REFERENCES

Asakawa, Y., G. W. Dawson, D. C. Griffiths, J. Y. Lallemand, S. V. Ley, K. Mori, A. Mudd, M. Pezechkleclaire, J. A. Pickett, H. Watanabe, C. M. Woodcock and Z. N. Zhang (1988). Activity of drimane antifeedants and related-compounds against aphids, and comparative biological effects and chemical-reactivity of (-)-polygodial and (+)-polygodial. *Journal of Chemical Ecology*. 14, 1845-1855.

Botha, J., E. Witkowski and C. M. Shackleton (2004). The impact of commercial harvesting on Warburgia salutaris ('pepper-bark tree') in Mpumalanga, South Africa. *Biodiversity and Conservation*. 13, 1675-1698.

Bryk, R., B. Gold, A. Venugopal, J. Singh, R. Samy, K. Pupek, H. Cao, C. Popescu, M. Gurney, S. Hotha, J. Cherian, K. Rhee, L. Ly, P. J. Converse, S. Ehrt, O. Vandal, X. Jiang, J. Schneider, G. Lin and C. Nathan (2008). Selective killing of nonreplicating mycobacteria. *Cell Host and Microbe*. 3, 137-145.

Clarkson, C., E. V. Madikane, S. H. Hansen, P. J. Smith and J. W. Jaroszewski (2007). HPLC-SPE-NMR characterization of sesquiterpenes in an antimycobacterial fraction from Warburgia salutaris. *Planta Med*. 73, 578-84.

D'Ischia, M., Prota, G. and G. Sodano (1982). Reaction of polygodial with primary amines: an alternative explanation to the antifeedant activity. *Tetrahedron Letters*. 23, 3295-3298.

Ghisalberti, E. 1993. *Bioactive Natural Products: Detection, isolation and structural determination*. Boca Raton: CRC Press.

Gols, G. J. Z., J. J. A. van Loon and L. Messchendorp (1996). Antifeedant and toxic effects of drimanes on Colorado potato beetle larvae. *Entomologia Experimentalis et Applicata*. 79, 69-76.

Gottlieb, H. E., V. Kotlyar and A. Nudelman (1997). NMR Chemical Shifts of Common Laboratory Solvents as Trace Impurities. *J Org Chem.* 62, 7512-7515.

Gutierrez, M. C., S. Brisse, R. Brosch, M. Fabre, B. Omais, M. Marmiesse, P. Supply and V. Vincent (2005). Ancient origin and gene mosaicism of the progenitor of Mycobacterium tuberculosis. 1, e5.

Hollmann, J. (1996). Portrait of a medicinal tree. *Veld and Flora.* 82, 115-116.

Jansen, B. J. M. and A. de Groot (2004). Occurrence, biological activity and synthesis of drimane sesquiterpenoids. *Natural Product Reports*. 21, 449-477.

Johnson, D. and R. Scott-Shaw (1995). The pepper bark tree of Zululand. *Veld and Flora*. 81, 16.

Kioy, D., A. I. Gray and P. G. Waterman (1990). A comparative study of the stem-bark drimane sesquiterpenes and leaf volatile oils of Warburgia ugandensis and W. Stuhlmannii. *Phytochemistry*. 29, 3535-3538.

Kubo, I., K. Fujita and S. H. Lee (2001). Antifungal mechanism of polygodial. *J Agric Food Chem*. 49, 1607-11.

Kubo, I., K. Fujita, S. H. Lee and T. J. Ha (2005). Antibacterial activity of polygodial. *Phytother Res*. 19, 1013-7.

Kubo, I. and M. Taniguchi (1988). Polygodial an antifungal potentiator. *Journal of Natural Products*. 51, 22-29.

Larsen, M. H., K. Biermann and W. Jacobs (2007). Laboratory Maintenance of Mycobacterium tuberculosis. *Current Protocols in Microbiology.* 6, Unit 10A.1.

Lawes, M. J., H. Eeley and S. E. Piper (2000). The relationship between local and regional diversity of indigenous forest fauna in KwaZulu-Natal Province, South Africa. *Biodiversity and Conservation.* 9, 683-705.

Madikane, V. E., S. Bhakta, A. J. Russell, W. E. Campbell, T. D. W. Claridge, B. G. Elisha, S. G. Davies, P. Smith and E. Sim (2007). Inhibition of mycobacterial arylamine N-acetyltransferase contributes to anti-mycobacterial activity of Warburgia salutaris. *Bioorganic and Medicinal Chemistry*. 15, 3579-3586.

Mashimbye, M., M. Maumela and S. Drewes (1999). A drimane sesquiterpenoid lactone from Warburgia salutaris. *Phytochemistry.* 5, 435-438.

Messchendorp, L., J. J. A. vanLoon and G. J. Z. Gols (1996). Behavioural and sensory responses to drimane antifeedants in Pieris brassicae larvae. *Entomologia Experimentalis Et Applicata*. 79, 195-202.

Montagnac, A., M. T. Martin, C. Debitus and M. Pais (1996). Drimane Sesquiterpenes from the Sponge Dysidea fusca. *Journal of Natural Products.* 59, 866-868.

Muchugi, A., G. M. Muluvi, R. Kindt, C. A. C. Kadu, A. J. Simons and R. H. Jamnadass (2008). Genetic structuring of important medicinal species of genus Warburgia as revealed by AFLP analysis. *Tree Genetics and Genomes.* 4, 787-795.

Mucina, L. and C. Geldenhuys. (2006). The vegetation of South Africa, Lesotho and Swaziland. Pretoria: South African National Biodiversity Institute. Strelitzia 19, 738-747.

Rabe, T. and J. van Staden (1997). Antibacterial activity of South Africa plants used for medicinal purposes. *Journal of Ethnopharmacology*. 56, 81-87.

Ross, J. H. 1976. Canellaceae. Pretoria: Department of Agricultural Technical Services.

Simpson, C.B. (1905) How to destroy termites or white ants. *Transvaal Agricultural Journal.*3, 765-768.

Tangiguchi, M., Y. Yano, E. Tada, K. Ikenishi, S. Oi, H. Haraguchi, K. Hashimoto and I. Kubo (1988). Mode of action of polygodial, antifungal sesquiterpene dialdehyde. *Agric. Biol. Chem.* 52, 1409-1414.

Uihlein, M. (1988) Sample pretreatment for HPLC. *Chromatographia*. 25, 244.

Verdcourt, B. 1956. Canellaceae. In: Flora of Tropical East Africa, eds. W. B. Turrill and E. Milne-redhead. London.

Vihma, V., H. Adlercreutz, A. Tiitinen, P. Kiuru, K. Wähälä and M. J. Tikkanen (1990). Quantitative Determination of Estradiol Fatty Acid Esters in Human Pregnancy Serum and Ovarian Follicular Fluid. *Clinical Chemistry*. 47, 1256-1262.

Wube, A. A., F. Bucar, S. Gibbons and K. Asres (2005). Sesquiterpenes of Warburgia ugandensis and their antimycobacterial activity. *Phytochemistry*. 66, 2309-2315.

Wube, A. A., F. Bucar, S. Gibbons, K. Asres, L. Rattray and S. L. Croft (2010). Antiprotozoal Activity of Drimane and Coloratane Sesquiterpenes towards Trypanosoma brucei rhodesiense and Plasmodium falciparum In Vitro. *Phytotherapy Research.* 24, 1468-1472.

Zapata, N., F. Budia, E. Vinuela and P. Medina (2009). Antifeedant and growth inhibitory effects of extracts and drimanes of Drimys winteri stem bark against Spodoptera littoralis (Lep., Noctuidae). *Industrial Crops and Products*. 30, 119-125.

CHAPTER 4

Microarray Analysis of *Mycobacterium tuberculosis* Treated with Synthetic Drimane Compound 8

4.1 Overview

Previous chapters have described the selection and screening of South African medicinal plants for inhibitory activity against *Mycobacterium tuberculosis* (Mtb), the causal agent of the tuberculosis (TB). These experiments showed that the dichloromethane (DCM) extract of the mature bark of *Warburgia salutaris* demonstrated inhibitory activity against Mtb under both replicating and non-replicating conditions. Subsequently, the drimane class of compounds was confirmed as being responsible for this activity. The synthetic drimane compound 1-Hydroxy-3a,6,6,9a-tetramethyldecahydronaphtho[2,1-b]furan-2(1H)-one (compound **8**) was used here to investigate the intracellular target(s) of this compound class in Mtb using microarray analysis.

Microarray analysis can be used to identify those genes that are differentially regulated in response to environmental stress, including an organism's response to antibiotics. In this experiment, we studied the *in vivo* transcriptional profiles of Mtb treated with compound **8** with that of untreated cells. There were 125 genes upregulated more than 1.5 times untreated cells in Mtb exposed to compound **8** measured under growth-inhibiting conditions in macrophages. The subsets of genes found to be upregulated include: two transporter loci, genes in the PhoP regulon, genes induced after treatment with a surfactant, and genes regulated in response to low pH. Many of these genes represent a general stress response of the bacteria and others suggest that the cell wall may be the target of these compounds.

4.2 Introduction

Attempts to eradicate TB are exacerbated by long-term persistence of non-replicating Mtb in macrophages of the host and the emergence of multidrug-resistant (MDR) Mtb strains (Onozaki and Raviglione, 2010). In the past, the primary method used to combat the development of resistance of bacteria to antibiotics was to modify the chemical structures of existing drugs (Hughes, 2003). However, this is only a short-term solution since the same mechanism that confers resistance to the parent molecule will eventually give rise to resistance in the derivative (Brazas and Hancock, 2005). What is needed are antibiotics that have new modes of action, and consequently an understanding of the mechanism of action is a compulsory part of any drug discovery effort. To demonstrate that plant-derived compounds are candidates that should be considered for further drug discovery research, it is important that this project go beyond the identification of active compounds. These microarray data can be used to provide insights into the cellular target of the drimane sesquiterpenes in Mtb.

4.2.1 How can microarray data be used to determine the mode of action?

Microarray analysis can be used to identify regulatory pathways and to provide the metabolic context of different observed responses to environmental perturbations, including drug treatment (Rohde, Abramovitch and Russell, 2007). When an organism is treated with a given compound, each compound potentially affects the expression of a distinct set of genes, often reflective of the primary target of that compound class, and termed the ‘gene signature’ (Brazas, 2005). Past studies have successfully correlated the expression profile of drug-treated yeast with the expression profile of a yeast mutant, which then led to the identification of the target of the antifungal compound. Theoretically, when you compare the gene expression induced by novel compounds with those of compounds whose mechanism of action is more understood, you can validate or identify the mechanism of novel inhibitors.

This rather straightforward approach used in yeast studies is not as easily applicable to Mtb. In Mtb, microarrays were first reported to describe those genes induced after Mtb was treated with isoniazid (INH) treatment. The genes identified encoded proteins related to the mode of action. Interestingly this study also identified other genes, including those that were involved in the mycobacterial response to the toxicity of the INH such as efflux proteins (Wadell et al., 2004). The microarray data obtained from Mtb studies reflect a complex host-pathogen interaction and this makes interpreting the microarray data challenging. Thus far in Mtb, microarray profiling after exposure to drugs has enabled compounds to be classified into similar groups based on mode of action. Complete elucidation of the mechanism of action would require complimentary biochemical and genetic studies, thus microarrays are generally most useful as hypothesis generating tools. (Wadell et al., 2004).

Another challenge in interpreting the transcriptional response to a compound is that the microarray data will also include expression changes in genes that may have no relationship, direct or otherwise, to the mode of action of the antibiotic. There are those genes that are indirectly affected by inhibition of the primary target. These often represent genes mediating the response of the antibiotic those genes that are involved in general stress responses. Secondly, expression data may include genes that modulate bacterial metabolism by altering transcription patterns. Studies have shown that antibiotics with different chemical structures and different modes of inhibitory action activate or repress a wide variety of promoters in *Escherichia coli* and *Pseudomonas*. While these examples are not Mtb, it does suggest that antibiotics have additional roles other than inhibition of growth by the inhibitors of specific target functions. There are also genes that are differentially regulated in response to the downstream consequences of target inhibition. These kinds of genes are included in the expression profiles are complicating components in understanding microarray data.

Comparing microarray data with previously published experiments is not conclusive. However, this method can be used to provide important information that will eventually lead to elucidating the mode of action of drimane compounds against Mtb. When the results of our

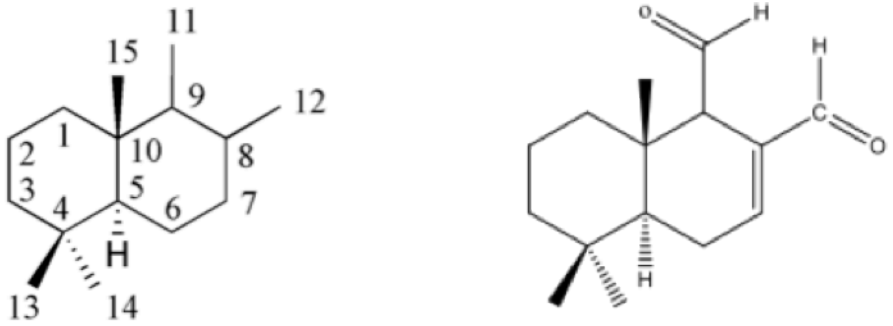
experiment were compared to a compendium of existing data, several subsets of genes were shown to be upregulated in common. These data include *in vitro* models of infection where responses to individual stimuli can be more controlled as well as expression studies in well-characterized mutants. There was no measured effort to ignore those genes that were down regulated. However in focusing on previously reported studies, only upregulated genes were highlighted. It is also important to note that while two different time points were tested, there wasn't enough information to make a time dependent analysis, nor was there sufficient statistical resolution to draw any conclusions about these temporal changes in expression.

4.2.2 Previous studies of the mode of action of Drimanes.

The drimane sesquiterpenes compound class was originally identified for their insect antifeedant properties (Kubo and Taniguchi, 1976). Drimane sesquiterpenes were later found to have potent antifungal activity especially against the yeast *Saccharomyces cerevisiae* and *Candida albicans* (Taniguchi et al., 1988). These studies prompted investigations into the cellular target of drimane sesquiterpenes, in particular polygodial, in yeast. Polygodial is a drimane sesquiterepene structurally similar to compound **8** used in our microarray study (see figure 4.1). Electron micrographs showed that when *S. cerevisiae* cells were treated with 50 μg/ml of polygodial, the cell membrane was fragmented and the nuclear membranes also were more irregular in appearance (Kubo, Fujita and Lee, 2001). A separate study by Forsby et al. (1992) showed that when human neuroblastoma cells were treated with low concentrations of polygodial, there was leakage of cellular constituents. The conclusion of these studies is that polygodial acts as a surfactant that nonspecifically disrupts the lipid-protein interface in membranes (Kubo et al., 2005).

Previous chapters have discussed the anti-mycobacterial properties of drimane compounds isolated from *W. salutaris*. *Warburgia salutaris* is a popular South African medicinal plant and previous studies have investigated its anti-mycobacterial properties. One investigation into the cellular target used transmission electron microscopy to show the affect of a *W. salutaris*

extract on the cellular morphology of *M. bovis* bacillus Calmette-Guérin (BCG). The results showed that *M. bovis* BCG when treated with a crude extract of *W. salutaris* (50 µg/mL) had an altered cell wall structure (Madikane et al., 2007). The mode of action of drimane compounds against Mtb has not been investigated using microarrays.



Drimane **Polygodial**

Figure 4.1. The chemical structures of drimane sesquiterpenes. Drimane shown here is the chemical skeleton characteristic of all drimane compounds, also shown is the numbering system used for this ring system. Polygodial is a common naturally occurring drimane.

4.2.3 The *Mycobacterium* Cell wall

A short discussion of the cell wall of Mtb is prudent since many of the genes upregulated in this study are associated with some aspect of the cell wall. The cell wall is one of three components of the cell envelope, which also includes the plasma membrane and a capsule (Figure 4.2). The Mycobacterium cell wall lies adjacent to the plasma membrane and includes a covalently linked layered core comprised of (moving out from the plasma membrane) peptidoglycan, galactofuran, highly branched arabinofuran, and finally mycolic acids (Figure 4.2; Brennan, 2003) The low permeability of the mycobacterium cell envelope is attributed to the properties of the cell wall. In experiments where cell envelopes were disrupted with various solvents, the outer most layer, also call the capsule, consisting of free lipids and proteins are solubilized. However, the mycolic acid–arabinogalactan–peptidoglycan complex remains an insoluble residue (Brennan, 2003).

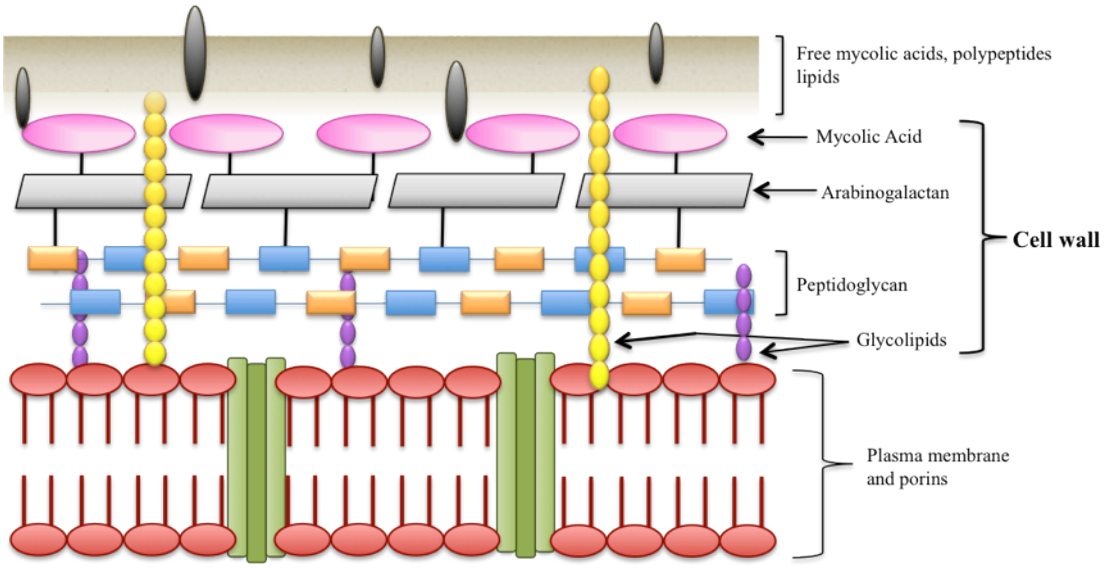


Figure 4.2 A schematic of the mycobacterial cell envelope, adapted from Ouellet Johnston, and de Montellano, 2010.

4.3 Methods

4.3.1 Bacterial strains and cells

Microarray experiments were performed by Dr. Robert Abramovitch at the College of Veterinary Medicine, Cornell University according to a previously described procedure (Rohde et al., 2007). In order to increase the genetic diversity of Mtb genes in this study, two strains of Mtb, CDC1551 a recent clinical isolate, and H37Rv, the wild type Mtb strain, were used. Both were routinely cultured in 7H9-OADC medium without shaking in 75 cm^2 vented tissue culture flasks. Bone marrow-derived macrophages were isolated from C57BL/6 mice and grown in Dulbecco's Modified Eagle Medium DMEM media (Rohde et al., 2007).

4.3.2 Macrophage Infections and RNA Isolation

Prior to infections, Mtb were declumped by repeated passages through a 21-gauge needle and resuspended in infection buffer DMEM with 5% fetal calf serum, 10mM HEPES, pH 7.4). Macrophage infections were initiated by centrifugation (1000xg, 10 min) of bacteria onto confluent macrophage monolayers followed by incubation at 37° C. Macrophages infected with Mtb were treated with compound **8** dissolved in minimal DMSO at a final concentration of 100 μg /mL for either 4 or 24 hrs. The experimental control was bacteria treated only with DMSO. Addition of a lysis buffer (4M guanidine thiocyanate, 0.5% Na N-lauryl sarcosine, 25 mM sodium citrate, and 0.1M β-mercaptoethanol) selectively lysed the macrophages, leaving Mtb cells intact while at the same time stopping Mtb transcription and RNA degradation (Butcher et al., 1998; Mangan et al., 2002). Samples were vortexed and passed repeatedly through a 21-gauge needle to separate clumps of macrophages and reduce viscosity. Intracellular Mtb were recovered by centrifugation at 3500 rpm for 30 minutes. Three technical replicates were completed.

4.3.3 Linear Application of Mycobacteria RNA

Pelleted Mtb were digested with 5 μg/ml lysozyme before being lysed in Trizol at 65^{o} C using a BeadBeater and 0.1mm silicon beads. Total RNA was isolated from Trizol lysates by chloroform extraction followed by addition of ethanol and direct application to Qiagen RNeasy column purification. Residual DNA contamination was removed using Turbo DNAfree DNase (Ambion).

4.3.4 Microarray Fabrication

Oligonucleotide microarrays consisted of 4295 open reading frames (ORF), representing 3924 ORFs from Mtb strain H37Rv and 371 ORFs from CDC1551. Oligos were spotted in duplicate on Corning UltraGAP (amino-silane coated) slides (Rohde et al., 2007).

4.3.5 Microarray Hybridization

Total RNA was reverse transcribed, using indirect labeling (to ensure that probe populations were labeled with equivalent efficiencies), into amino-allyl cDNA using Superscript III (Invitrogen Life Technologies), 5'-amine modified random hexamers (Integrated DNA Technologies) (Xiang et al., 2002), and amino-allyl (aa) d- UTP (Sigma). CyDye fluors were conjugated to aa-cDNA using CyDye Post-Labelling Reactive Dye reagents (Amersham) containing N-hydroxysuccinimide ester derivatives of Cy3 and Cy5. After quenching the labeling reactions with 4 M hydroxylamine, excess reactive dyes were removed by QiaQuick purification (Qiagen).

5-10 μg of Cy-labeled cDNA from paired samples (treated and untreated cells) was dried using a Speedvac and resuspended in 50 μl of hybridization buffer (5X Saline-sodium citrate (SSC) 25% formamide, 0.1% SDS, and 25 μg salmon sperm DNA). Samples were denatured at 95^{o} C for 5 min and briefly cooled to 60^{o} C before being applied to arrays under a glass LifterSlip (Erie Scientific). Slides were prehybridized for 1 hr in 25% formamide, 5X SSC, 0.1% SDS, 1% BSA and washed with H_2O and isopropanol. Labeled targets were hybridized to microarrays in

humidified slide chambers (Corning) at 45° C for 16-18 hr. Arrays were washed sequentially with buffer 1 (2X SSC, 0.1% SDS) pre-warmed to 45° C, buffer 2 (0.2X SSC, 0.1% SDS), buffer 3 (0.2X SSC), and buffer 4 (0.05X SSC). Finally, slides were dipped in deionized ultra-filtered water before being dried by centrifugation.

4.3.6 Microarray Data Analysis

Microarrays were scanned with a GenePix 4000B (Axon Instruments, Inc.) with image analysis, spot intensity determination, background measurements and spot quality assessment conducted using Imagene software (version 6.0, Biodiscovery). Spots with signal intensities fewer than three standard deviations above background were excluded from further analysis. Subsequent normalization, statistical analysis, and visualization of array data were performed with Genespring 7.3 (Agilent). Genes with significant changes in expression levels relative to controls were identified based on fold change (1.5-fold).

4.4 Results

In the Mtb treated with compound **8**, 125 genes were upregulated more than 1.5 times compared to the expression of untreated cells after both 4 and 24 hours, (see Appendix B for full gene list). These genes are a subset of a total of 4295 open reading frames that were examined and 3528 (82%) that were expressed above statistical background.

4.4.1 Genes regulated by the PhoP regulon are induced

Bacteria use surface exposed signal transduction systems as a mechanism to monitor and adapt to changing environmental conditions. PhoPR is a two-component signal transduction system, containing an environmentally responsive histidine kinase, PhoR, and a response regulator, PhoP, which mediate cellular responses through differential expression of genes

(Ryndak, Wang and Smith, 2008; Mascher, Helmann and Unden, 2006). In this microarray study many genes regulated by PhoP where upregulated after treatment by drimane compound **8**.

The *Phop* gene was identified as differentially expressed between the avirvulent Mtb H37Ra strain and the virulent H37Rv strain (Ryndak et al., 2008). PhoP has been shown to regulate the expression of genes essential for survival inside macrophages and also genes that are involved in lipid metabolism (Ryndak et al., 2008). The ability to survive under the variety of growth conditions experienced in the macrophages is one of the factors of virulence. Walters et al. (2006) compared the gene expression in Mtb strain H37Rv with gene expression in the *phoP* mutant and showed that 44 genes had a higher expression in the virulent wild type strain compared to the *phoP* mutant. More than half of the 44 genes identified in the Walters et al. study are annotated to encode proteins involved in lipid metabolism, lipid secretion and synthesis of components of the cell envelope. It was also found that the *phoP* mutant has a structurally altered cell envelope; complex lipids that are normally present in wild type are absent in the mutant (Walters et al., 2006). In our investigation 21 of the 44 genes regulated by PhoP were upregulated more than 1.5 fold in Mtb exposed to compound **8** as compared to untreated cells (Figure 4.3).

While the function of the majority of the genes regulated by Phop is unknown, others are annotated to be involved in polyketide synthesis, such as pks2, pks3 and pap1. The mycobacterial cell wall contains a number of polyketide-derived complex lipids (Mohanty, Sankaranarayanan and Gokhale, 2011). It is known that pks2 and msl3 encode proteins responsible for the biosynthesis of sulphatides and acyltrehaloses, respectively (Gonzalo Asensio et al., 2006). These cell wall components are unique to pathogenic *Mycobacteria*. Both the pks2 and msl3 genes were up regulated in Mtb exposed to compound **8**, as compared to untreated cells.

Other genes regulated by *Phop* are annotated to encode proteins that are localized to the cell envelope, including *Mmpl1, Mmpl3, Mmpl8*, Rv2576, Rv2373. The mycobacterium membrane protein large (Mmpl) family of proteins are proposed to be involved in the transport

of lipids and other components of the cell wall (Domenech, Reed and Barry, 2005) and will be discussed in Section 4.4.4. Since the upregulation of Phop is a key to survival while exposed to the many stress conditions *in vivo*, it would suggest that the increased upregulation of these genes in Mtb exposed to compound **8** as compared to untreated cells indicated that these bacteria are responding to and additional stress. These genes are not necessarily indicative of a particular mode of action.

The microbial cell wall acts as the interface between the infecting bacilli and the host environment therefore it is not surprising to find genes involved in the synthesis and modification of cell wall associated fatty acids upregulated *in vivo*. It is not clear whether these lipid moieties are part of a response to strengthen the lipid-rich mycobacterial cell wall or play a different role in the response of the pathogen to stress (Wadell and Butcher, 2011). The utilization of fatty acids appears to be common to all infection models and therefore may represent a fundamental adaptation to intracellular growth (Wadell et al., 2007).

4.4.2 PE/PPE Protein Family

Our microarray data indicate that ten genes upregulated by Mtb exposed to compound **8** are identified as belonging to the PE and PPE family of proteins (see Appendix B). These genes are named for their highly conserved residues near the start of their encoded proteins. The PE (proline-glutamate) and PPE (proline-proline glutamate) gene families represent almost 200 genes, which is 10% of the coding capacity of the Mtb genome (Cole et al., 1998, Sampson, 2011). Many researchers have interpreted this large percentage to be indicative of the importance of these genes in the pathogenesis of Mtb, and many of these genes are duplicated (Cole et al., 1998). Originally, it was suggested that the purpose of this gene family was to promote antigenic diversity (Okkels et al., 2003). However, since these genes are unique to *Mycobacterium*, and they are only present in the pathogenic species of the genus, it is believed that these genes also play an essential role in virulence (Mukhopadhyay and Balaji, 2011). Additionally, the

inactivation of two PE genes resulted in decrease survival of Mtb in granulomas (Ramakrishnan, Federspiel and Falkow, 2000).

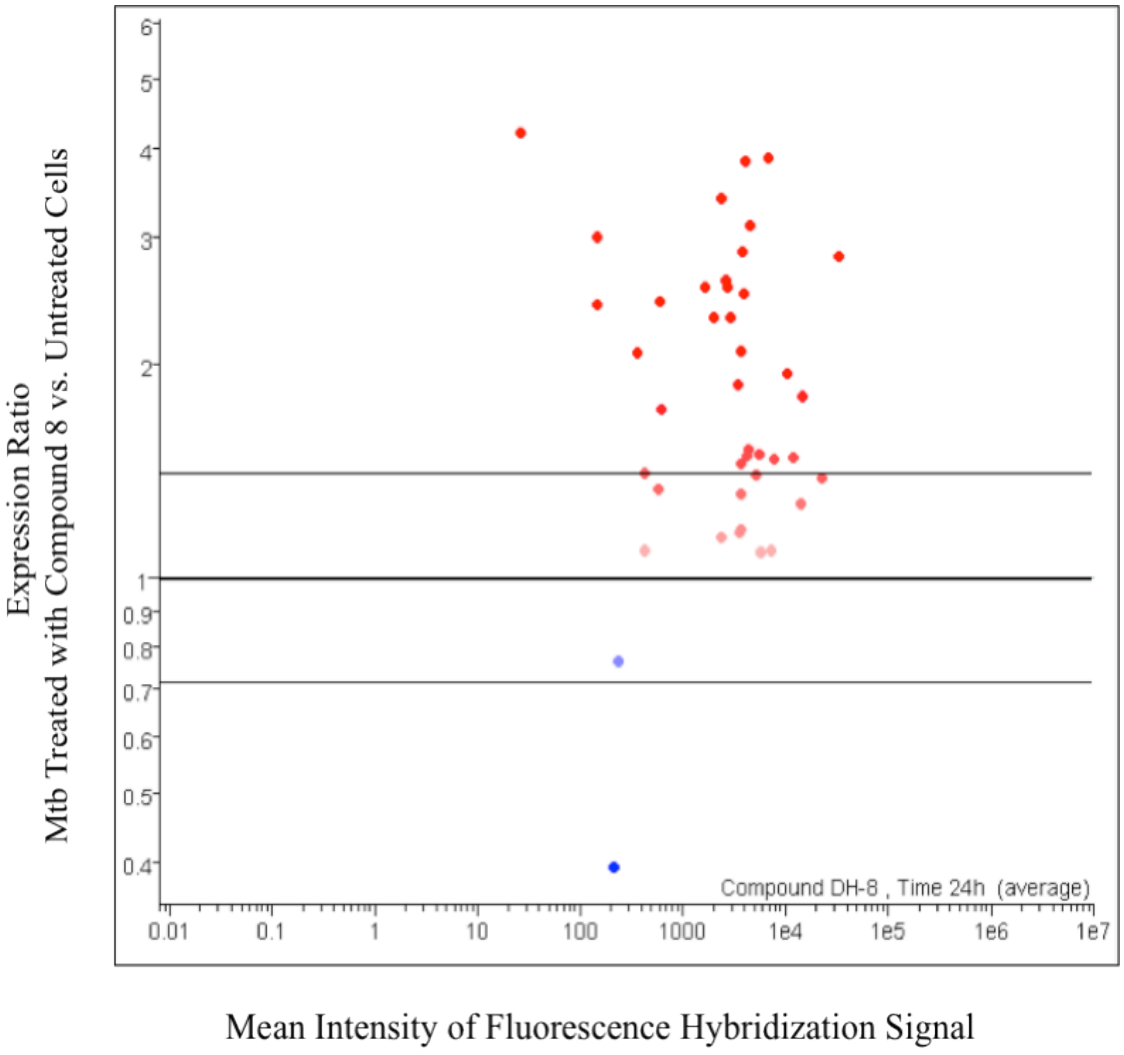


Figure 4.3 Expression ratio of Phop regulated genes, as identified by Walters et al. (2006), Mtb treated with drimane compound **8** versus untreated cells.

Table 4.1 Genes that where identified as regulated by PhoP according to the Walters et al. (2006) study and their expression in Mtb exposed to drimane compound **8** vs. expression in a DMSO control.

Systematic	24h	4h	Description
pks2 (Rv3825c)	3.884	2.884	Probable polyketide synthase
pks3 (Rv1180)	3.844	1.863	Probable polyketide synthase beta-ketoacyl synthase domain,
Rv1184c	3.406	1.893	Hypothetical proteins
Rv3767c	3.118	1.534	Unknown
papa3 (Rv1182)	3.009	2.423	Hypothetical proteins
papa1 (Rv3824c)	2.863	2.115	Probable polyketide synthase associated protein, sulpholipid biosynthesis
Rv3615c	2.823	1.682	Unknown
Lipf (Rv3487c)	2.616	1.222	Unknown
mez Rv2332	2.566	1.323	Catalyzes the oxidative decarboxylation of malate into pyruvate
Rv2331	2.560	1.289	Unknown
fadd21 (Rv1185c)	2.513	1.776	Probable acyl-coAsynthase
rv1179c	2.445	1.102	Unknown
mmpl10 (Rv1183)	2.318	1.371	Probable transmembrane protein
mmpl8 (Rv3823c)	2.082	1.358	Probable conserved integral membrane transport protein
Rv2633c	1.795	1.323	Function unknown, hypothetical protein
Rv1639c	1.518	1.074	Function unknown, probable hypothetical membrane protein
uvra (Rv1638)	1.501	1.268	Involved in nucleotide excision repair
Rv2396	1.938	0.823	Member of PE family, PGRS subfamily of proteins
Rv3479	1.725	0.840	Unknown
Rv3613c	1.490	0.781	Unknown
nark1 (Rv2329c)	1.485	0.745	Probable nitrite extrusion protein
Rv2376c	1.479	1.007	Unknown, predicted outer membrane protein
Rv2632c	1.467	1.290	Function unknown, hypothetical protein
cdh (Rv2289)	1.449	1.008	Involved in phospholipid biosynthesis
leud (Rv2987c)	1.402	0.657	Involved in leucine biosynthesis at the second step
Rv3477	1.398	0.627	Member of PE family of proteins
Rv3616c	1.379	1.100	Unknown
rfe (Rv1302)	1.334	1.098	Involved in AG biosynthesis
Rv0116c	1.312	1.232	Unknown
Rv3614c	1.273	0.875	Unknown
Rv3686c	1.172	0.810	Unknown
Rv3478	1.160	1.123	Member of PPE family of proteins
Rv1361c	1.142	0.926	Member of the PPE family of proteins
Rv3312c	1.096	0.935	Unknown
mmpl3 (Rv0206c)	1.094	0.665	Possible membrane transport protein
fadd9 (Rv2590)	0.764	0.587	Involved in lipid degradation, probable fatty acid Co-A ligase
MT3135	0.652	0.866	Hypothetical protein
Rv2576c	0.556	0.857	Unknown
fbpa (Rv3804c)	0.395	0.895	32 kDa antigen protein 85-A precursor, mycolyltransferase

The PE/PPE genes are notable in response to drimane compounds because a recent review indicates that their products are localized to the cell wall (Sampson, 2011). Cell fractionation and immunoelectron microscopy studies similarly concluded that all PE proteins contain a functional domain responsible for localization to the mycobacterial cell wall (Cascioferro et al., 2007). Additionally, genes belonging to the PE-PGRS (polymorphic GC-rich sequence) subfamily have been shown to specifically localize to the cell wall of Mtb (Sani et al., 2010). These genes have not been shown to be involved in the general stress response of Mtb. However, since the function of these genes is not clear, it is premature to suggest that the upregulation of these may indicate that the cellular target of these drimane compounds is the cell envelope.

4.4.3 Genes that respond to the surfactant SDS

Manganelli et al. (2001) used microarrays to compare the gene expression profiles of the wild type Mtb strain H37Rv after exposure to sodium dodecyl sulfate (SDS), an anionic detergent commonly used to denature proteins and disrupt membranes (Tan et al., 2002). A total of 62 genes were upregulated in wild type Mtb H37Rv after exposure to SDS as compared to untreated cells. In our study 20 of the 62 genes identified as being SDS-sensitive were upregulated by more than 1.5 times after both 4 hours and 24 hours of exposure to drimane compound **8** as compared to untreated cells (Figure 4.4).

Many of the genes up regulated after exposure to SDS were found to be involved in fatty acid degradation. In the macrophage, Mtb switches to the utilization of lipids and fatty acids as alternate carbon sources (McKinney et al., 2000). One gene central to fatty acid metabolism and found to be upregulated here is *aceA*, which encodes for the enzyme isocitrate lyase (Icl), an enzyme essential for the metabolism of fatty acids. Icl is the first enzyme in the glyoxylate cycle, and is upregulated in non-replicating of Mtb. It has also been observed that an *icl1* null strain of Mtb was not able to sustain the chronic, persistent infection seen in mice infected with wild-type Mtb (McKinney et al., 2000). Moreover, bacteria lacking both *icl1* and *icl2* are unable to grow

on fatty acids or in macrophages, and are rapidly cleared from the lungs of infected mice (Muñoz-Elías et al., 2005). These observations suggest that adaptation to the host environment involves a metabolic shift in the carbon source used by the bacteria to the acetyl-CoA generated by the b-oxidation of fatty acids (Gould et al., 2006).

Much of this work on the upregulation of genes associated with fatty acid metabolism focuses on changes in the metabolic pathways required for survival within the activated macrophages of the host. Mtb preferentially utilizes fatty acids and this preference is thought to be due to the increased availability of lipids (Mckinney et al., 2000). However, there is growing evidence that indicate this metabolic change is a part are part of innate programming in response to persistence, not the carbon source. In Mtb exposed to stress conditions that prevent replication and in media that contained different carbon sources, the switch of carbon metabolism from sugars to fatty acids was independent of the carbon source or the stress response (Shi et al., 2010). This would suggest that the upregulation of these genes may be a response of the bacteria to the stress induced by compound **8**.

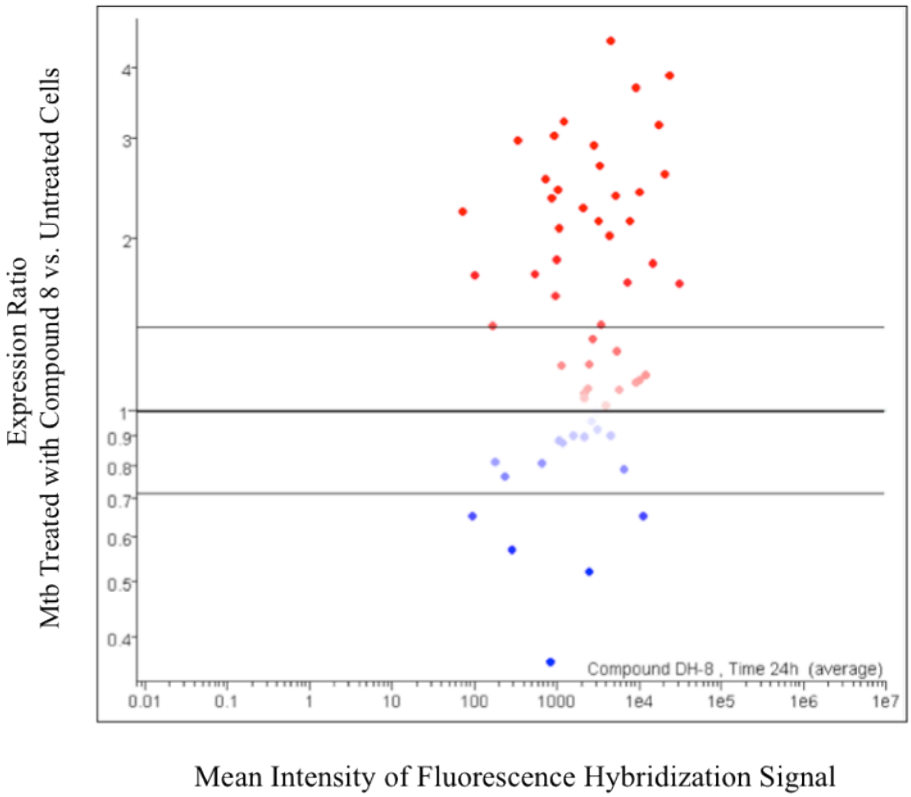


Figure 4.4 Managanelli (2001) identified genes in Mtb differentially expressed after exposure to SDS. The expression of these genes were investigated in Mtb exposed to drimane compound **8**. This figure shows the expression ratio of SDS regulated genes in Mtb treated with drimane compound 8 compared to untreated cells.

Table 4.2 The expression ratio of genes differentially regulated in response to exposure with SDS.

Systematic	24h	4h	Description
35kd_Ag (Rv2744c)	1.674	1.813	Unknown
Acea (Rv0467)	2.351	1.574	Probable isocitrate lyase, involved in glyoxylate bypass
Atsa (Rv0711)	2.684	1.798	Arylsulfatase, involved in the mineralization of sulfates
Hsp (Rv0251c)	2.405	1.763	Possibly heat shock protein belonging to HSP20 family
Htpx (Rv0563)	2.379	1.906	Probable heat shock protein X, likely transmembrane)
Iles (Rv1536)	2.017	1.104	Involved in the catalytic activity of isoleucine
Mmpl5 (Rv0676c)	3.157	2.951	Probable transmembrane protein involved in fatty acid transport
Mmps5 (Rv0677c)	3.855	3.970	Possible membrane protein
Rv0146	2.080	1.526	Possible methyltransferase involved in lipid metabolism
Rv0465c	2.541	1.593	Possible transcriptional regulator
Rv0678	3.667	5.241	Unknown
Rv0981	1.731	2.390	Transcriptional regulatory protein of a two component system
Rv0997	2.140	2.196	Unknown
Rv1057	2.913	2.774	Unknown
Rv1168c	1.586	0.905	Member of the PPE-family of proteins
Rv1169c	1.836	0.977	Member of the PE family of proteins
Rv1806	4.450	2.036	Member of the PE family of proteins
Rv1807	2.427	1.560	Member of the PE family of proteins
Rv1808	3.024	1.698	Member of PPE family of proteins
Rv1809	3.201	1.749	Member of PPE family of proteins
Rv2050	2.142	1.904	Unknown
Rv2745c	1.664	1.847	Possible transcriptional regulatory protein
Rv3406	2.256	2.241	Unknown
Cysd (Rv1285)	0.884	0.833	Probable sulfate adenylate transferase subunit 2
Cysn (Rv1286)	0.807	0.843	Probable sulfate adenylate transferase,
Ding (Rv1329c)	1.334	1.024	Probable ATP-dependent helicase
Fada (Rv0859)	1.048	0.751	Function unknown, probable involvement in lipid metabolism
Fadb (Rv0860)	1.267	0.857	Involved in fatty acid degradation
Fadb2 (Rv0468)	1.091	1.378	Probable 3-hydroxyacyl-coa dehydrogenase
Fadd9 (Rv2590)	0.764	0.587	Involved in lipid degradation, probable Acyl-CoA synthetase

Table 4.2 Continued

Systematic	24h	4h	Description
Fade23 (Rv3140)	1.131	1.510	Involved in lipid degradation, putative acyl-CoA dehydrogenase
Fade24 (Rv3139)	0.955	1.645	Probable acyl-CoA dehydrogenase
Glta1 (Rv1131)	0.901	1.058	Probable citrate synthase
Nada (Rv1594)	1.115	1.204	Quinolinate synthetase.
Nadb (Rv1595)	0.923	1.252	Probable L-aspartate oxidase
Nadc (Rv1596)	0.361	2.022	Involved in de novo biosynthesis of NAD and NADP
Rv0516c	0.996	0.974	May be involved in regulating sigma factor
Rv0712	1.069	1.503	Unknown
Rv0789c	0.901	1.239	Unknown
Rv1129c	0.878	0.774	Unknown
Rv1130	0.786	1.181	Involved in methyl citrate cycle
Rv1195	0.898	0.920	Member of the PE family of proteins
Rv1196	1.087	0.883	Member of PPE family of proteins
Rv1199c	1.194	1.288	Possibly required for the transportation of insertion elements
Rv2053c	1.409	1.432	Probable transmembrane protein
Rv2743c	1.201	1.413	Unknown
Rv3023c	0.811	1.072	Probable transposase
Rv3115	0.569	0.772	Probable transposase
Rv3854c	0.650	1.153	Possible monooxygenase
Rv3855	0.520	0.970	Possible transcriptional regulatory protein
Sige (Rv1221)	1.153	1.285	Alternative sigma factor of extracytoplasmic function (ECF) family

4.4.4 Efflux systems

The genes associated with two efflux pumps were upregulated in Mtb exposed to compound **8**. Both eukaryotic and prokaryotic organisms have efflux pumps that remove molecules from within the cell (De Rossi, Ainsa and Riccardi, 2006). Many of these efflux systems have broad substrate profiles that allow structurally diverse compounds to be exported from the cell, including antibiotics of different chemical classes (Ryan et al., 2001). Efflux systems have been identified as a resistance mechanism in many bacteria, including Mtb, to antibiotics. It is believed that each transporter originally evolved to transport a physiological substrate or group of related substrates and the ability of these transporters to efflux toxins is merely an opportunistic side effect (De Rossi et al., 2006). The ability of these proteins to extrude antibiotics and other toxins from the cell is likely a serendipitous result of the ability of these pumps to transport a wide range of substrates (Piddock, 2006). It is believed that genes encoding efflux pumps are not specific to a given antibiotic and the upregulation is believed to be a response to environmental stress (Brazas and Hancock, 2005).

- The efflux system encoded by mmpS5-mmpL5

The genes *MmpS5*, *MmpL5* and Rv0678 were found to be upregulated by Mtb exposed to compound **8** by more than 2 fold (Figure 4.5). The MmpL5 (mycobacterium membrane protein large) and MmpS5 (mycobacterium membrane protein small) have been previously identified to encode a hypothetical efflux system belonging to the resistance nodulation division (RND) family of transporters. The gene Rv0678 has been linked to the transcriptional regulation of these two transporter genes (Milano et al., 2009).

Pairwise alignment of amino acid residues of MmpL5 in Mtb and *Escherichia coli* AcrA have shown the two proteins to be analogous (De Rossi et al., 2006). Sequence similarity was also found between the *E. coli* gene *AcrB* and *mmpS5*, although to a lesser extent. To date, no analogous gene analogous to *TolC* has been identified in Mtb. The *E. coli* AcrAB-TolC is the typical example of the RND family tripartite system of transporters. This system includes 1) a

transporter (efflux) protein located in the inner membrane named AcrB, 2) an accessory protein located in the periplasmic space named AcrA and 3) an outer membrane protein, TolC (De Rossi et al., 2006).

The Mmps5-MmpL5 efflux pumps were originally identified in the resistance mechanism of Mtb to antibiotics belonging to the azole compound class (Milano et al., 2009). While the exact natural substrates of the mmps5-mmpl5 efflux pump remain unidentified, it has been suggested that these proteins are involved in the transport of fatty acids because they are flanked by genes involved in lipid metabolism and polyketide biosynthesis (Milano et al., 2009; Butcher, Mangan and Monahan, 1998).

- Genes Encoding the Putative Tetronasin ABC transporter

ATP-binding cassette (ABC) transporters represent a super family of multi-subunit permeases that transport molecules across biological membranes (Braibant, Gilot and Content, 2000). These transporters contain four domains; two hydrophobic membrane-spanning domains and two cytoplasmic nucleotide binding domains (Davidson et al., 2008). The Mtb gene Rv1218c shows sequence similarity to TnrB, an ABC transporter found in *Streptomyces longisporoflavus*. TnrB is known to confer resistance to the antibiotic tetronasin (Demetriadou et al., 1985). Studies by Linton et al. (1994) concluded that TrnB protects the cell from tetronasin by removing the antibiotic from the cell.

In Mtb treated with compound **8**, Rv1218c and the genes Rv1216c, Rv1217c and Rv1219c, which encode the other domains of this ABC-transporter, were upregulated in Mtb exposed to compound **8** (Figure 4.6). The natural substrate of this transporter is not known and studies suggest that this transporter is involved in mediating the efflux of a wide variety of chemical classes (Balganesh et al., 2010). There is not enough evidence to speculate on the role of this transporter. The upregulation of these genes could be the bacteria's attempt to remove the drimane compounds from the cell or an increased need to transport a different substrate.

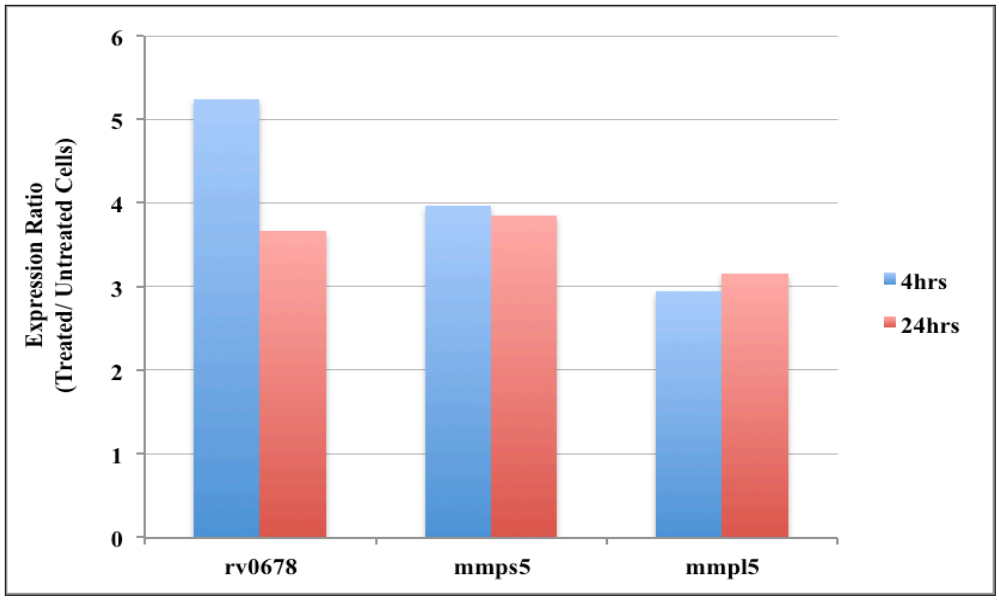


Figure 4.5. Expression ratio of genes *MmpL5, MmpS5*, Rv0678, in Mtb treated with compound **8** versus untreated cells after 4 and 24 hours.

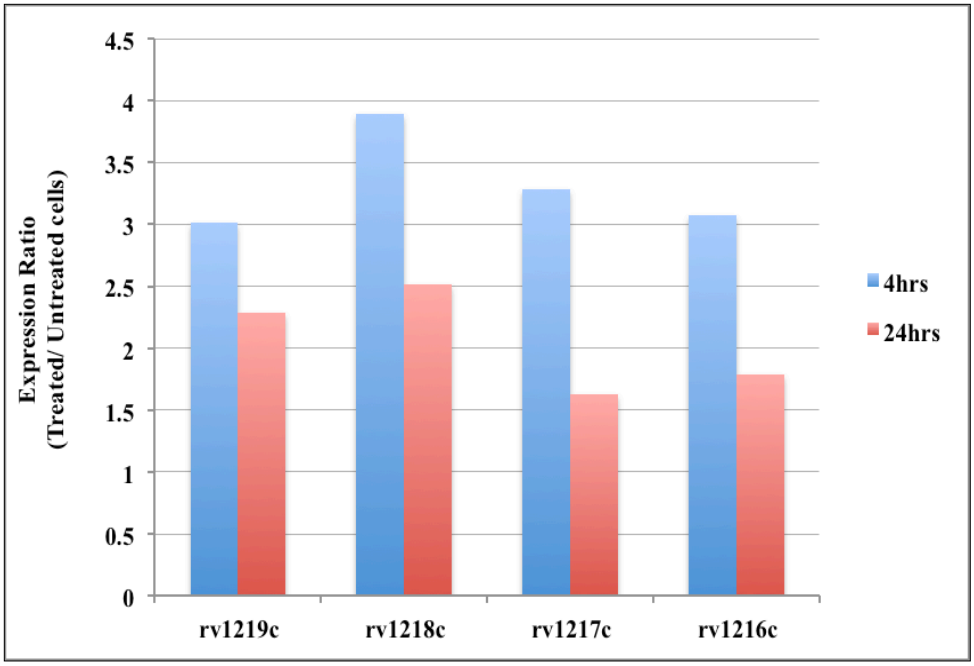


Figure 4.6 Expression ratio of genes Rv1216c, Rv1217c, Rv1218c, Rv1219c, in Mtb treated with compound **8** versus untreated Mtb, after both 4 and 24 hours.

4.4.5 Mtb Genes that respond to acidification are induced.

Two studies have investigated the transcriptional response of Mtb to an acidic environment, an important characteristic of the macrophage host. First, Yates et al. (2005) investigated the global transcription response of Mtb in macrophages treated with concanamycin A (CcA), which prevents the macrophage from acidifying as compared to Mtb in untreated macrophages. The Yates et al. study concluded that 44 genes were differentially regulated in response to an acidic pH in the macrophages. A separate study by Rodhe et al. (2007) investigated genes up regulated in response to an acidic pH *in vitro*. There are 16 genes that were identified in both the Yates et al. and Rodhe et al. studies as being responsive to an acidic environment.

Thirteen of these the genes identified as acid sensitive were upregulated more than 1.5 times in the Mtb treated by drimane compound **8** (Figure 4.7). The Rohde et al. study concluded that pH might serve as an environmental cue that the bacteria uses to initiate metabolic changes and enter a non-replicating state. Many of the 16 genes have been previously discussed in previous sections and are upregulated in response to other stress conditions. This supports the idea that these genes may not be responding directly to a decrease in pH, instead are upregulated in response to general stress conditions.

A gene known to be upregulated in response to a low pH is *WhiB3*. *WhiB3* is one of seven Mtb whiB-like genes that encode small proteins postulated to be transcriptional regulators (Geiman et al., 2006). Specifically, WhiB3 binds to the principal sigma factor, RpoV, and is thought to play a role in bacterial survival and tissue pathology late in infection (Steyn et al., 2002). *WhiB3* and many of the whiB-like genes are upregulated in response to a wide range of stressful stimuli. Some of these stimuli are encountered in the macrophages including oxidants, low pH and others stess stimuli such as sodium dodecyl sulfate, and heat shock are not (Rohde et al., 2007). This lends to the conclusion that these genes may be induced as a part of a general

stress response and the upregulation of these genes in Mtb treated with compound 8 as compared to the untreated cells is indicative of the additional stress these cells are experiencing.

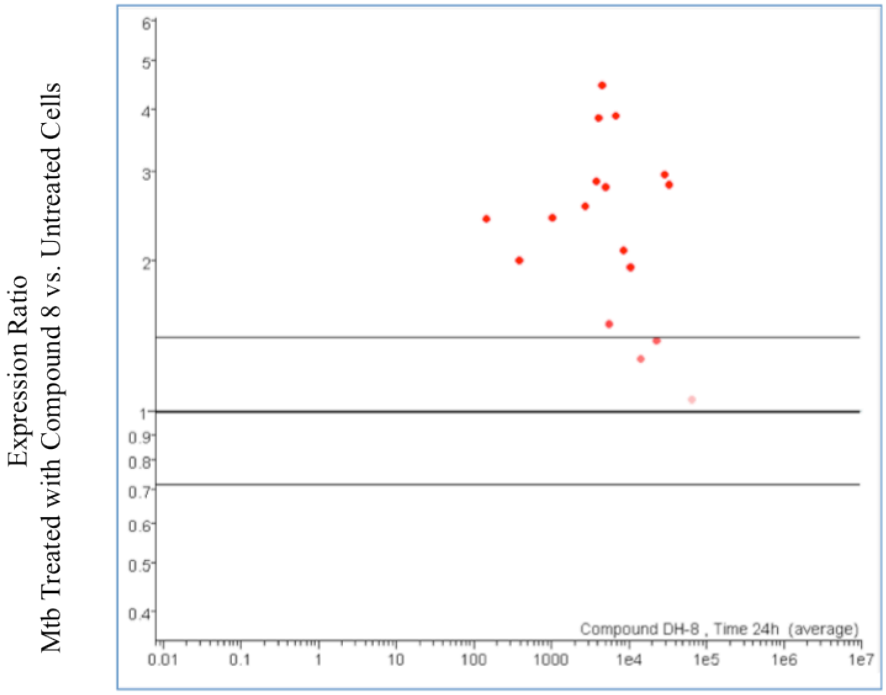


Figure 4.7. There are 16 genes that were identified in studies by both Yates et al. (2005) and Rodhe et al. (2007) as being responsive to an acidic environment. This figure shows the expression ratio of these pH sensitive genes in Mtb exposed to compound **8** versus untreated cells after 24hrs.

Table 4.3 The expression ratio of pH sensitive genes, both Yates et al. (2005) and Rodhe et al. (2007), in Mtb exposed to compound **8** versus untreated cells after both 4 and 24 hours.

Systematic	24h	4h	Description
Rv2632c	1.467	1.290	Unknown
Rv2633c	1.795	1.323	Unknown
Rv2628	0.548	1.440	Unknown
Rv2396	1.938	0.823	Member of PE family, PGRS subfamily of proteins
Rv2390c	1.995	1.183	Unknown
Rv2389c	1.267	0.819	Known to promote the growth of non growing cells
Pks2, (Rv3825c)	3.884	2.884	Probable polyketide synthase
Papa1 (Rv3824c)	2.863	2.115	Involved in sulpholipid biosynthesis
Rv3746c	1.626	1.232	Belonging to the PE family of proteins
Mt3223	0.744	0.458	Unknown
Rv3613c	1.490	0.781	Unknown
Whib3 (Rv3416)	2.096	1.671	Transcriptional regulatory protein
Lipf (Rv3487c)	2.616	1.222	Unknown
Rv1807	2.427	1.560	Member of the PE family of proteins
Rv1806	4.450	2.036	Member of the PE family of proteins
Rv2030c	0.657	0.943	Unknown
Fdxa (Rv2007c)	0.420	1.102	Involved in electron transfer during the production of ferredoxin
Pks4 (Rv1181)	2.797	1.495	Proposed to be involved in polyketide synthase
Pks3 (Rv1180)	3.844	1.863	Proposed to be involved in polyketide synthase
Papa3 (Rv1182)	3.009	2.423	hypothetical protein
Rv1403c	2.556	1.980	Unknown
Rv2627c	0.426	1.065	Unknown
Rv2626c	0.484	1.101	Unknown
Hspx (Rv2031c)	0.916	1.289	Belongs to the small heat shock protein family
Rv3130c	0.646	1.208	Involved in the synthesis of triacylglycerol

4.5 Conclusion

The microarray data from this investigation provides some insight into the effect of drimane compounds on the Mtb. Many of the genes upregulated in response to treatment with drimane compounds were also identified in previous publications. These previous studies described the transcriptional response of Mtb to a wide range of stresses. The upregulation of genes in our study and also in studies using unrelated compounds indicates that these genes are a part of a common cellular response to antimicrobial stresses and overlapping secondary targets. Some of the gene identified are also involved in fatty acid and lipid biosynthesis and are specifically localized to the cell wall.

This initial investigation into the gene expression of Mtb treated with drimane compounds is not conclusive. It is difficult from this data alone to separate the direct effects of drimane compounds on an individual target from the indirect effects. However, the data obtained can guide future inquiries. The gene expression of many antibiotics reflect more than just the inhibition of a single protein. Future experiments should be conducted to more fully understand the indirect and downstream effects of drimane compounds on Mtb. Three future microarray studies are proposed below:

- *In vitro Microarray Experiments*

  The understanding of microbial interactions with the host has been enhanced by expression studies using *in vitro* models to dissect out the complex interactions between pathogen and host and then compared to *in vivo* transcriptional data (Wadell and Butcher 2011). The interpretation of this data relied upon published studies to help isolate and identify specific signals. However it seems that in vitro experiments, that specifically look at the effects of drimane compounds on Mtb are needed to facilitate the interpretation of the complex data obtained from this broad study.

- *Analysis of gene expression over a wide range of times*

Understanding the role of temporal changes in gene expression is important to the interpretation of complex microarray analysis. mRNA transcript expression analysis of early time points after treatment with an antibiotic are not likely to include expression changes in the target itself. Differential expression of the target gene or of a gene encoding a functionally related protein will eventually occur as the organism attempts to compensate for the loss of the target protein (Brazas and Hancock, 2005).

- *Investigating Gene Expression Over a Range of Concentrations*

Subsequent microarray experiments should use a range of sub-inhibitory concentrations of the drimane compound. It has been suggested that when bacteria are treated with antibiotics at low concentrations, expression patterns may correlate more with direct target inhibition (Brazas and Hancock, 2005). Also, there are biological responses to environmental signals or stresses that are characterized by biphasic dose response relationships exhibiting low dose stimulation and high dose inhibition. The testing of the transcription at a range of concentrations will help isolate the effects on transcription that are in addition to their inhibitory properties.

Even with the proposed additional microarray studies, the cellular target or the mechanism of action cannot be elucidated solely by microarray studies. Another approach, although arduous and with uncertain results, used to identify the mechanism of action is to identify the mutants that are resistant to this compound. This can be done by growing Mtb on solid media that contains drimane compounds at a concentration above the MIC and subsequently isolating those colonies that are able to survive. This method was successful in the

identification of strains of Mtb resistant to diarylquinoline (Koen et al., 2005). These mutants were then fully sequenced and shown to have independent point mutations in a subunit of ATP synthase. This method is resource intense because it requires the ability to fully sequence bacterial genomes. While this method was not initially successful when tried in our lab using twice the MIC, it should be investigated again.

REFERENCES

Balganesh, M., S. Kuruppath, N. Marcel, S. Sharma, A. Nair and U. Sharma. (2010). Rv1218c, an ABC transporter of Mycobacterium tuberculosis with implications in drug discovery. *Antimicrob Agents Chemother*. 54(12), 5167-72.

Braibant, M., P. Gilot and J. Content. (2000) The ATP binding cassette (ABC) transport systems of Mycobacterium tuberculosis. *FEMS Microbiol Rev*. 24(4), 449-67.

Brazas, M. D. and R. E. Hancock (2005) Using microarray gene signatures to elucidate mechanisms of antibiotic action and resistance. *Drug Discov Today*, 10, 1245-52.

Brennan, P. J. (2003) Structure, function, and biogenesis of the cell wall of Mycobacterium tuberculosis. *Tuberculosis* (Edinb), 83(1-3), 91-7.

Butcher, P. D., J. A. Mangan and I. M. Monahan (1998) Intracellular gene expression. Analysis of RNA from mycobacteria in macrophages using RT-PCR. *Methods Mol Biol*. 101, 285-306.

Cascioferro, A., G. Delogu, M. Colone, M. Sali, A. Stringaro, G. Arancia, G. Fadda, G. Palu and R. Manganelli. (2007) PE is a functional domain responsible for protein translocation and localization on mycobacterial cell wall. *Mol Microbiol*, 66(6), 1536-47.

Cole, S. T., R. Brosch, J. Parkhill, T. Garnier, C. Churcher, D. Harris, S. V. Gordon, K. Eiglmeier, S. Gas, C. E. Barry, 3rd, F. Tekaia, K. Badcock, D. Basham, D. Brown, et al. (1998) Deciphering the biology of Mycobacterium tuberculosis from the complete genome sequence. *Nature*. 393, 537-44.

Daffe, M. and P. Draper (1998) The envelope layers of mycobacteria with reference to their pathogenicity. *Adv Microb Physiol*, 39, 131-203.

Davies, J., Spiegelman, G. and Grace Yim (2006) The world of subinhibitory antibiotic concentrations. *Current Opinion in Microbiology*. 9(5); 445–453.

Davidson, A. L., E. Dassa, C. Orelle and J. Chen. (2008) Structure, function, and evolution of bacterial ATP-binding cassette systems. *Microbiol Mol Biol Rev*. 317-64.

De Rossi, E., J. A. Ainsa and G. Riccardi. 2006. Role of mycobacterial efflux transporters in drug resistance: an unresolved question. FEMS Microbiol Rev. 72(2), 36-52.

Demetriadou, A. K., E. D. Laue, J. Staunton, G. A. F. Ritchie, A. Davies and A. B. Davies (1985) The polyether antibiotic ICI 139603 retains oxygen from acetate at C-1 and C-17, and from propionate at C-3 and C-21, after incorporation of [1-13C,1-18O2]acetate and [1-13C,1-18O2]propionate, and on this basis the tetrahydropyran and cyclohexane rings may be formed concertedly in a novel biosynthetic cyclisation. *J. Chem. Soc., Chem. Commun*. 408-410.

Dengler V, Meier PS, Heusser R, Berger-Bächi B, and McCallum N. (2011) Induction kinetics of the Staphylococcus aureus cell wall stress stimulon in response to different cell wall active antibiotics. *BMC Microbiol.* 20;11:16.

Domenech, P., M. B. Reed and C. E. Barry, 3rd. (2005) Contribution of the Mycobacterium tuberculosis MmpL protein family to virulence and drug resistance. *In Infect Immun*, 73(6): 3492-501.

Forsby, A., E. Walum and O. Sterner. (1992) The effect of six sesquiterpenoid unsaturated dialdehydes on cell membrane permeability in human neuroblastoma SH-SY5Y cells. *Chem Biol Interact*. 84(1), 85-95.

Geiman, D. E., T. R. Raghunand, N. Agarwal and W. R. Bishai. (2006) Differential gene expression in response to exposure to antimycobacterial agents and other stress conditions among seven Mycobacterium tuberculosis whiB-like genes. *Antimicrob Agents Chemother*. 50(8), 2836-41.

Gonzalo Asensio, J., C. Maia, N. L. Ferrer, N. Barilone, F. Laval, C. Y. Soto, N. Winter, M. Daffe, B. Gicquel, C. Martin and M. Jackson. (2006) The virulence-associated two-component PhoP-PhoR system controls the biosynthesis of polyketide-derived lipids in Mycobacterium tuberculosis. *J Biol Chem.* 281(3), 1313-6.

Gould, T. A., H. van de Langemheen, E. J. Munoz-Elias, J. D. McKinney and J. C. Sacchettini. (2006) Dual role of isocitrate lyase 1 in the glyoxylate and methylcitrate cycles in Mycobacterium tuberculosis. *Mol Microbiol*. 61(4), 940-7.

Hoffmann, C., A. Leis, M. Niederweis, J. M. Plitzko and H. Engelhardt. (2008) Disclosure of the mycobacterial outer membrane: cryo-electron tomography and vitreous sections reveal the lipid bilayer structure. *Proc Natl Acad Sci USA*. 105(10), 3963-7.

Hughes, D. (2003) Exploiting genomics, genetics and chemistry to combat antibiotic resistance. *Nat Rev Genet*. 4(6), 432-41.

Kaur, D., M. E. Guerin, H. Skovierova, P. J. Brennan and M. Jackson. (2009) Chapter 2: Biogenesis of the cell wall and other glycoconjugates of Mycobacterium tuberculosis. *Adv Appl Microbiol*. 69, 23-78.

Koen A., Verhasselt P., Guillemont J., Göhlmann HW, Neefs JM, Winkler H, Van Gestel J, Timmerman P, Zhu M, Lee E, Williams P, de Chaffoy D, Huitric E, Hoffner S, Cambau E, Truffot-Pernot C, Lounis N and Jarlier V. (2005). A diarylquinoline drug active on the ATP synthase of Mycobacterium tuberculosis. *Science*. 307(5707), 223-7.

Kubo, I., K. Fujita and S. H. Lee (2001) Antifungal mechanism of polygodial. *J Agric Food Chem*. 49, 1607-11.

Kubo, I., K. Fujita, S. H. Lee and T. J. Ha (2005) Antibacterial activity of polygodial. *Phytother Res.* 19, 1013-7.

Kubo, I. and M. Taniguchi (1976) Polygodial, an antifungal potentiator. *Journal of natural products*. 51, 22-9.

Linton, K. J., H. N. Cooper, I. S. Hunter and P. F. Leadlay (1994) An ABC-transporter from Streptomyces longisporoflavus confers resistance to the polyether-ionophore antibiotic tetronasin. *Mol Microbiol.* 11, 777-85.

Madikane, V. E., S. Bhakta, A. J. Russell, W. E. Campbell, T. D. W. Claridge, B. G. Elisha, S. G. Davies, P. Smith and E. Sim (2007) Inhibition of mycobacterial arylamine N-acetyltransferase contributes to anti-mycobacterial activity of Warburgia salutaris. *Bioorganic and Medicinal Chemistry*. 15, 3579-3586.

Manganelli, R., M. I. Voskuil, G. K. Schoolnik and I. Smith. (2001) The Mycobacterium tuberculosis ECF sigma factor sigmaE: role in global gene expression and survival in macrophages. *Mol Microbiol*. 41(2), 423-37.

Mascher, T., J. D. Helmann and G. Unden (2006) Stimulus perception in bacterial signal-transducing histidine kinases. *Microbiol Mol Biol Rev*. 70, 910-38.

Milano, A., M. R. Pasca, R. Provvedi, A. P. Lucarelli, G. Manina, A. L. Ribeiro, R. Manganelli and G. Riccardi. (2009) Azole resistance in Mycobacterium tuberculosis is mediated by the MmpS5-MmpL5 efflux system. *Tuberculosis (Edinb)*. 89(1), 84-90.

Mohanty, D., R. Sankaranarayanan and R. S. Gokhale. (2011) Fatty acyl-AMP ligases and polyketide synthases are unique enzymes of lipid biosynthetic machinery in Mycobacterium tuberculosis. *Tuberculosis (Edinb)*. 91(5), 448-55.

Mukhopadhyay, S. and K. N. Balaji. (2011) The PE and PPE proteins of Mycobacterium tuberculosis. *Tuberculosis (Edinb*). 91(5), 441-7.

Niederweis, M. (2003) Mycobacterial porins--new channel proteins in unique outer membranes. *Mol Microbiol*. 49(5), 1167-77.

Okkels, L. M., I. Brock, F. Follmann, E. M. Agger, S. M. Arend, T. H. Ottenhoff, F. Oftung, I. Rosenkrands and P. Andersen (2003) PPE protein (Rv3873) from DNA segment RD1 of Mycobacterium tuberculosis: strong recognition of both specific T-cell epitopes and epitopes conserved within the PPE family. *Infect Immun*. 71, 6116-23.

Onozaki, I. and M. Raviglione. (2010) Stopping tuberculosis in the 21st century: goals and strategies. *Respirology*. 15(1), 32-43.

Ouellet, H., Johnston, J., and Paul R. Ortiz de Montellano (2010). The Mycobacterium tuberculosis cytochrome P450 system. *Archives of Biochemistry and Biophysics*. 493(1), 82–95.

Piddock, L. J. 2006. Multidrug-resistance efflux pumps - not just for resistance. Nat Rev Microbiol, 629-36.

Rachman, H. and S. H. Kaufmann (2007) Exploring functional genomics for the development of novel intervention strategies against tuberculosis. Int J Med Microbiol, 297, 559-67.

Ramakrishnan, L., N. A. Federspiel and S. Falkow. 2000. Granuloma-specific expression of Mycobacterium virulence proteins from the glycine-rich PE-PGRS family. Science, 1436-9.

Rohde, K. H., R. B. Abramovitch and D. G. Russell. (2007) Mycobacterium tuberculosis invasion of macrophages: linking bacterial gene expression to environmental cues. *Cell Host Microbe*. 2(5), 352-64.

Ryan, B. M., T. J. Dougherty, D. Beaulieu, J. Chuang, B. A. Dougherty and J. F. Barrett (2001) Efflux in bacteria: what do we really know about it? *Expert Opin Investig Drugs.* 10, 1409-22.

Ryndak, M., S. Wang and I. Smith. (2008) PhoP, a key player in Mycobacterium tuberculosis virulence. *Trends Microbiol*. 16(11), 528-34.

Sampson, S. L. (2011) Mycobacterial PE/PPE proteins at the host-pathogen interface. *Clin Dev Immunol*. 20011: 497203.

Sani, M., E. N. Houben, J. Geurtsen, J. Pierson, K. de Punder, M. van Zon, B. Wever, S. R. Piersma, C. R. Jimenez, M. Daffe, B. J. Appelmelk, W. Bitter, N. van der Wel and P. J. Peters (2010) Direct visualization by cryo-EM of the mycobacterial capsular layer: a labile structure containing ESX-1-secreted proteins. *PLoS Pathog*, 6(3): e1000794.

Steyn, A. J., D. M. Collins, M. K. Hondalus, W. R. Jacobs, Jr., R. P. Kawakami and B. R. Bloom. (2002) Mycobacterium tuberculosis WhiB3 interacts with RpoV to affect host survival but is dispensable for in vivo growth. *Proc Natl Acad Sci USA* . 99(5), 3147-52.

Tan, A., A. Ziegler, B. Steinbauer and J. Seelig. (2002) Thermodynamics of sodium dodecyl sulfate partitioning into lipid membranes. *Biophys J.* 1547-56.

Taniguchi, M., Y. Yano, E. Tada, K. Ikenishi, O. I. Susumu, Haraguchih, K. Hashimoto and I. Kubo (1988) Mode of action of polygodial, an antifungal sesquiterpene dialdehyde. *Agricultural and Biological Chemistry*. 52, 1409-1414.

Waddell, S. J., Stabler, R.A., Laing, K, Kremer, L., Reynolds, R. C. and Besra, G,S. (2004)The use of microarray analysis to determine the gene expression profiles of Mycobacterium tuberculosis in response to anti-bacterial compounds. *Tuberculosis (Edinb)*. 84(3-4):263-74.

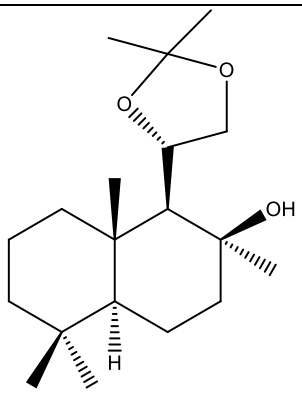
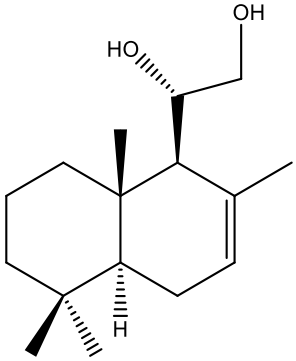
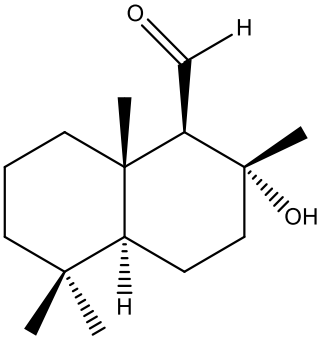
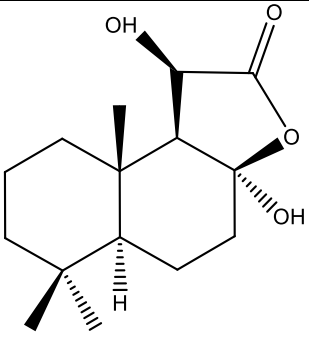
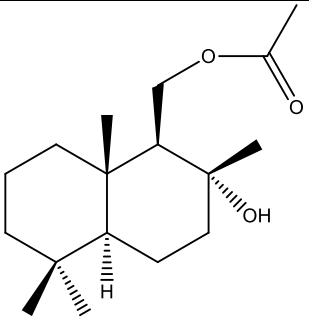
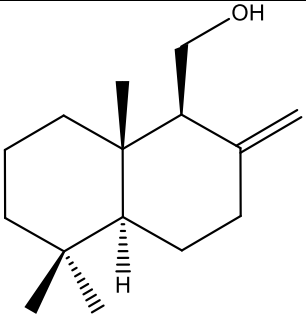
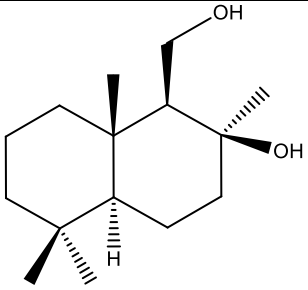
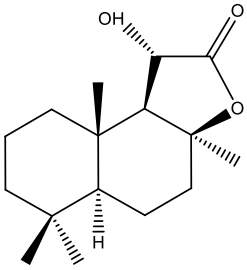
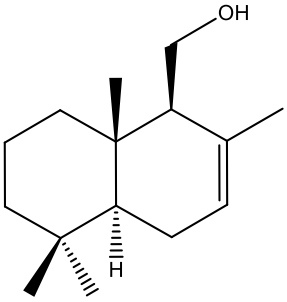
Waddell, S. J. and P. D. Butcher (2007) Microarray analysis of whole genome expression of intracellular Mycobacterium tuberculosis. *Current Molecular Medicine.* 7, 287-296.

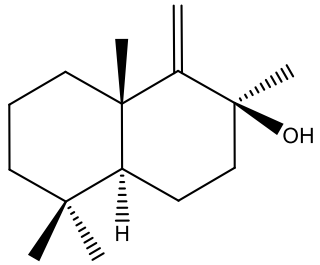
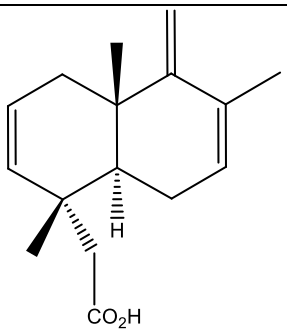
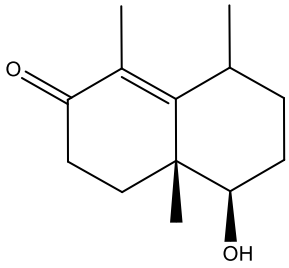
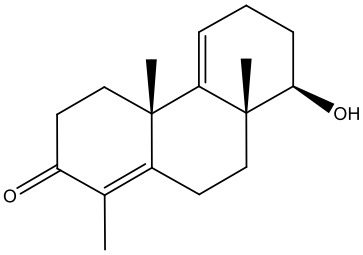
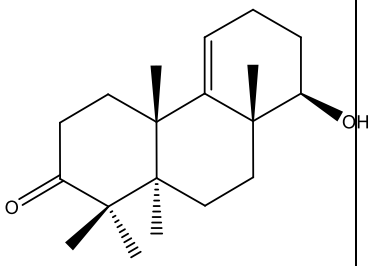
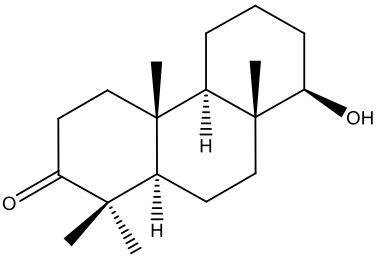
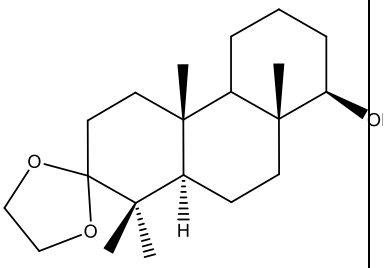
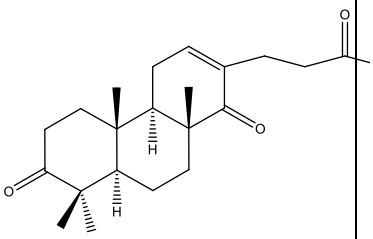
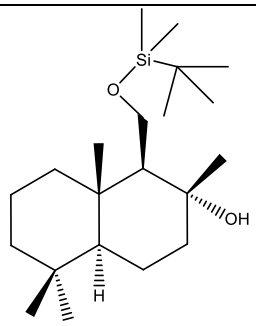
Walters, S. B., E. Dubnau, I. Kolesnikova, F. Laval, M. Daffe and I. Smith. (2006). The Mycobacterium tuberculosis PhoPR two-component system regulates genes essential for virulence and complex lipid biosynthesis. *Mol Microbiol*. 60(2), 312-30.

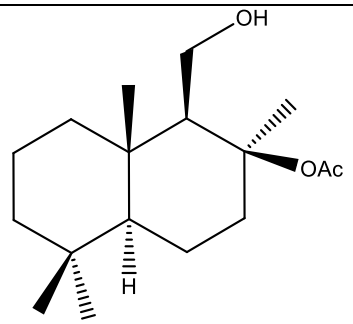
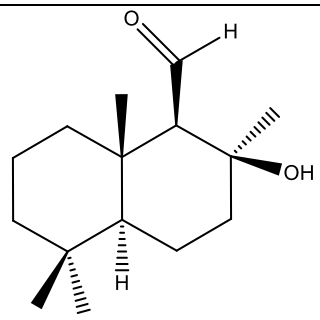
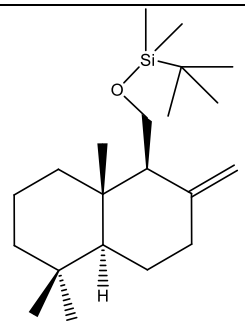
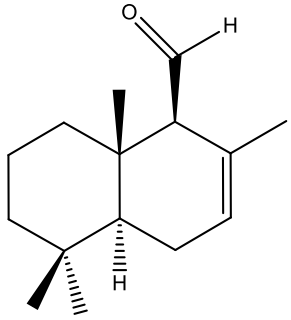
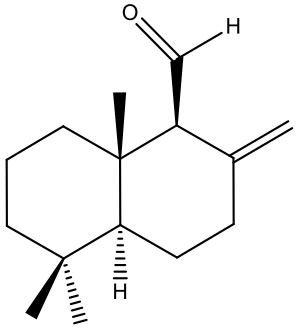
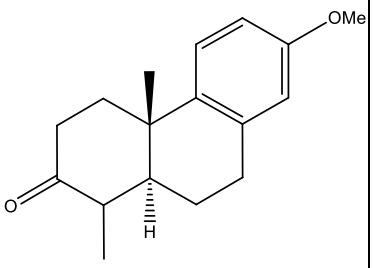
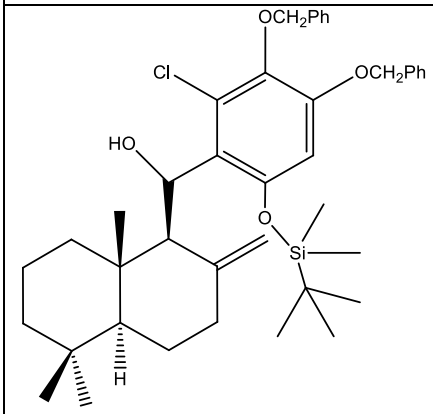
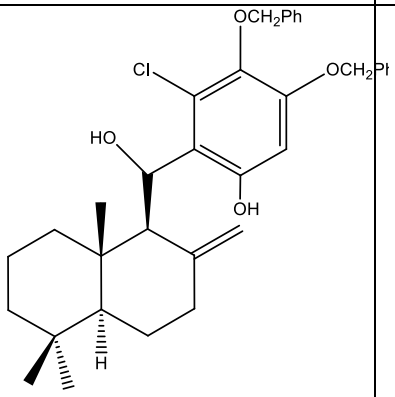
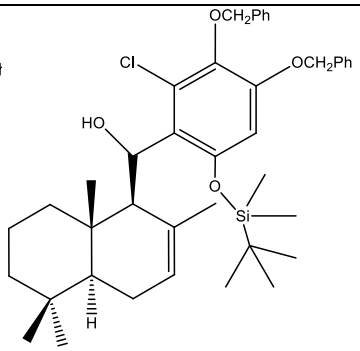
Yates, R. M., A. Hermetter and D. G. Russell. (2005) The kinetics of phagosome maturation as a function of phagosome/lysosome fusion and acquisition of hydrolytic activity. *Traffic*. 6(5), 413-20.

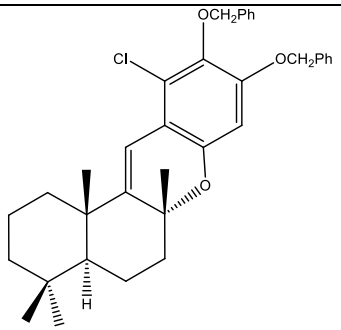
APPENDIX A

Structures of Synthetic Drimane Compounds

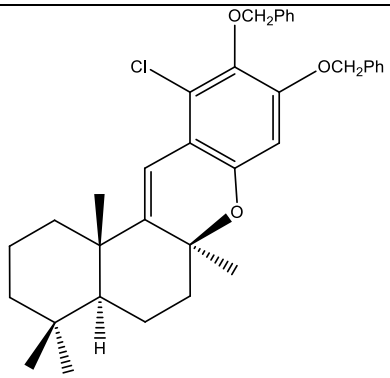
 **1**	 **2**	 **3**
 **4**	 **5**	 **6**
 **7**	 **8**	 **9**

 **10**	 **11**	 **12**
 **13**	 **14**	 **15**
 **16**	 **17**	 **18**

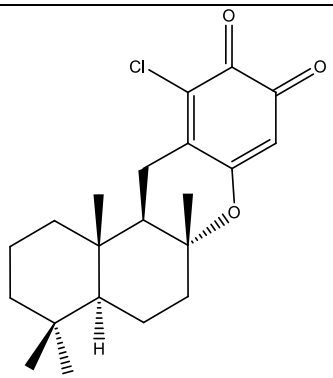
 **19**	 **20**	 **21**
 **22**	 **23**	 **24**
 **25**	 **26**	 **27**



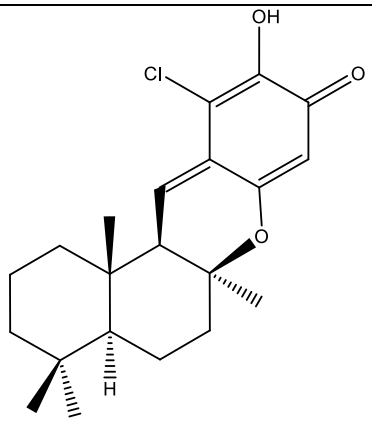
28



29



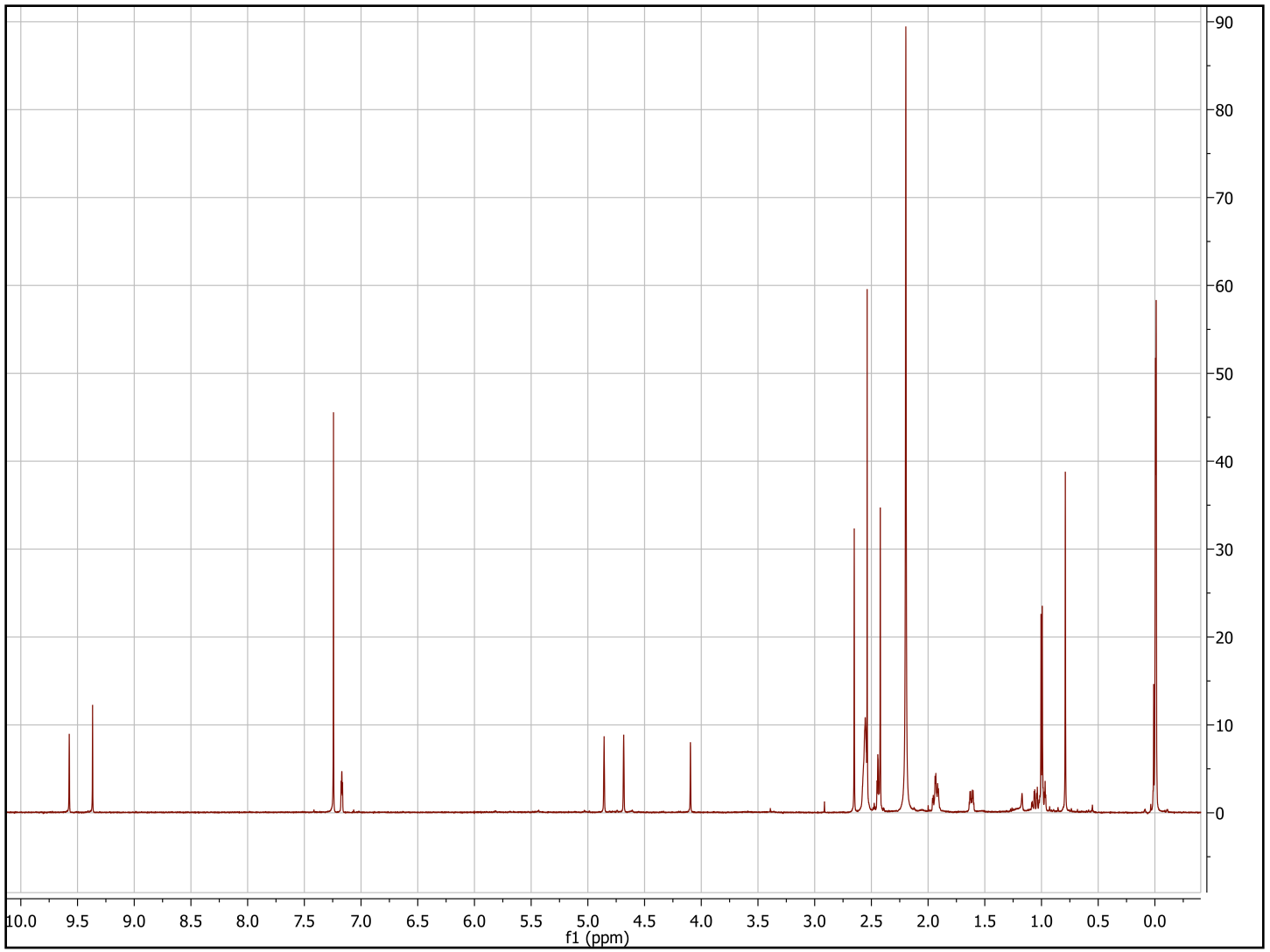
30



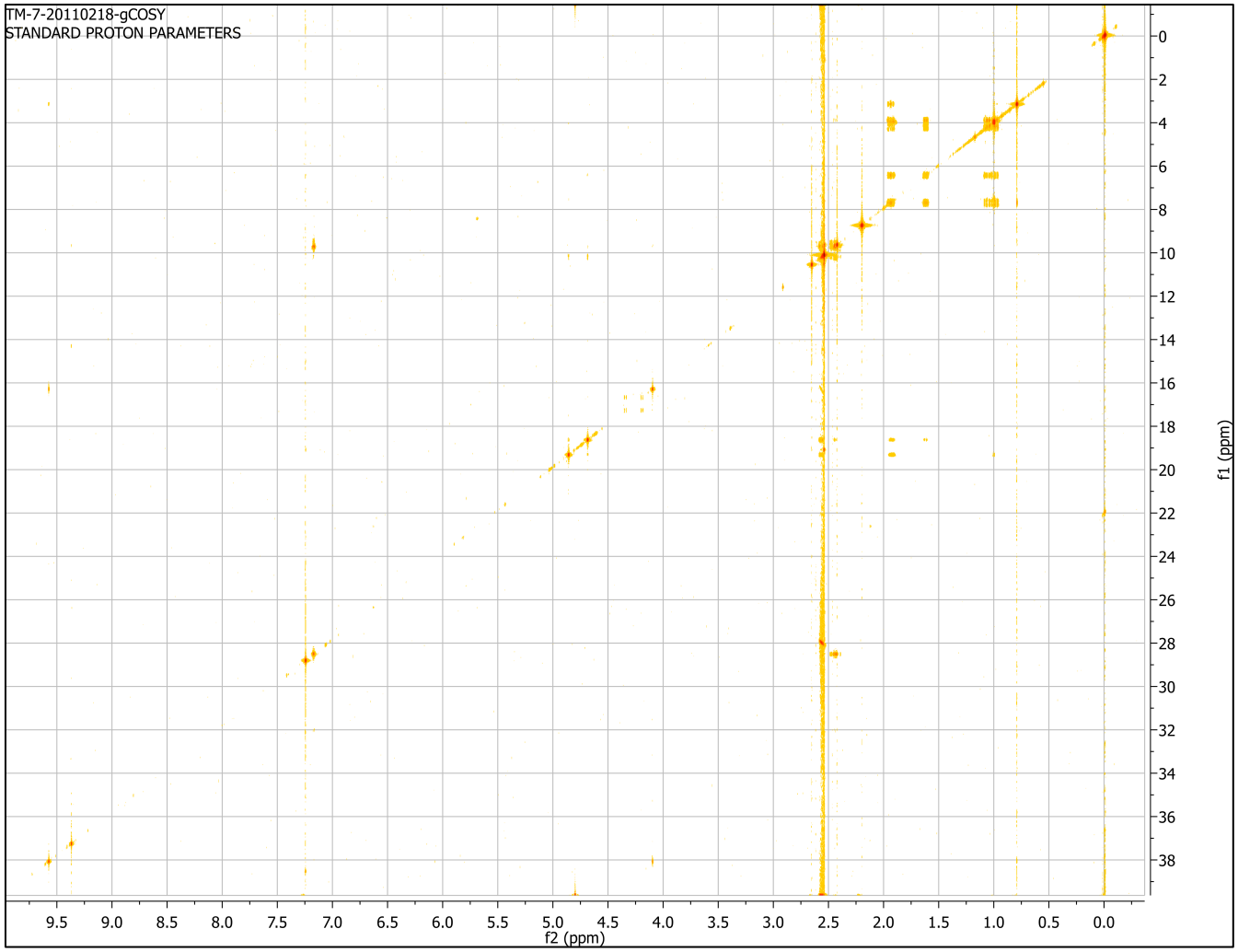
31

Appendix B

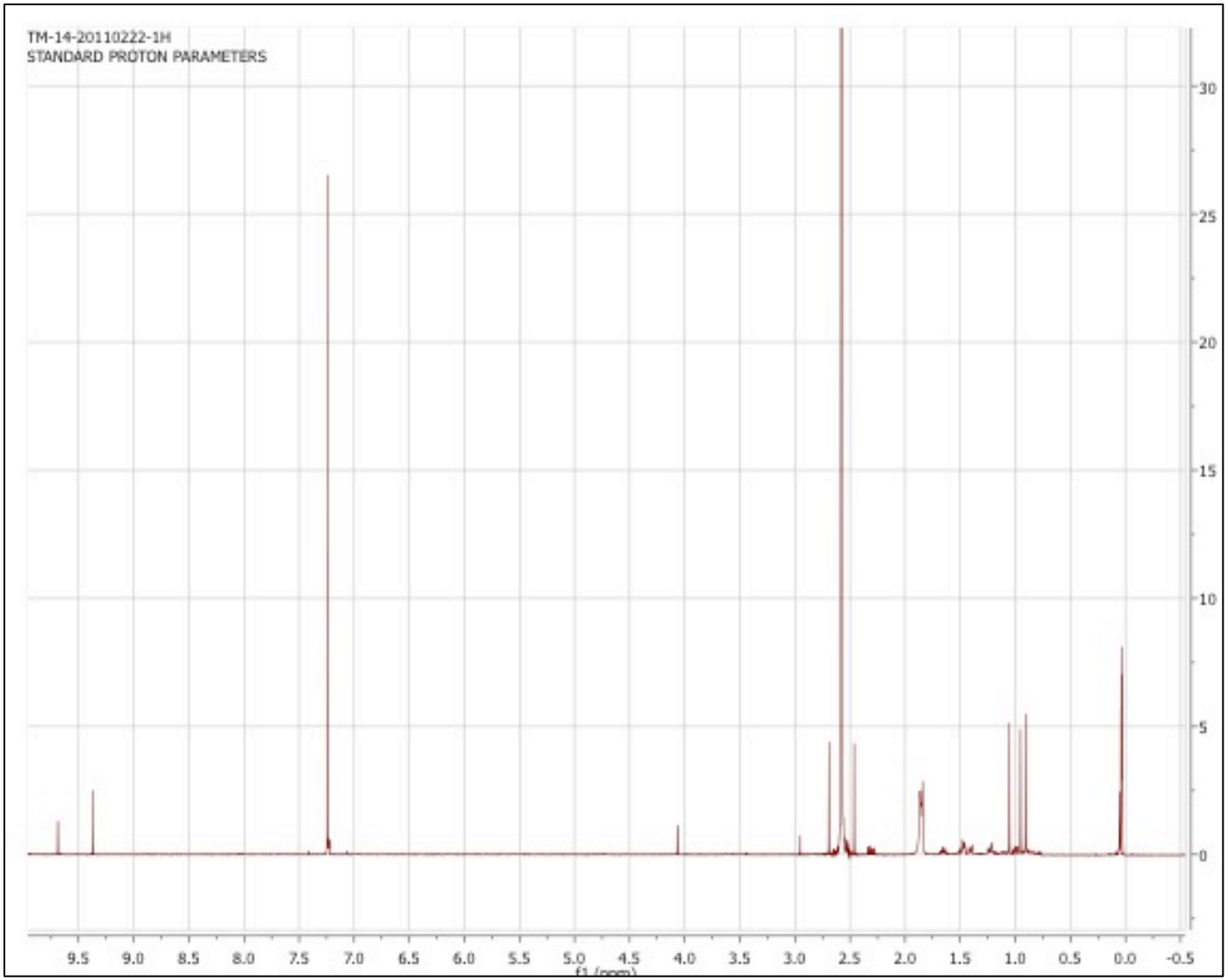
Selected NMR Spectra of Isolated Drimane Sesquiterpenes



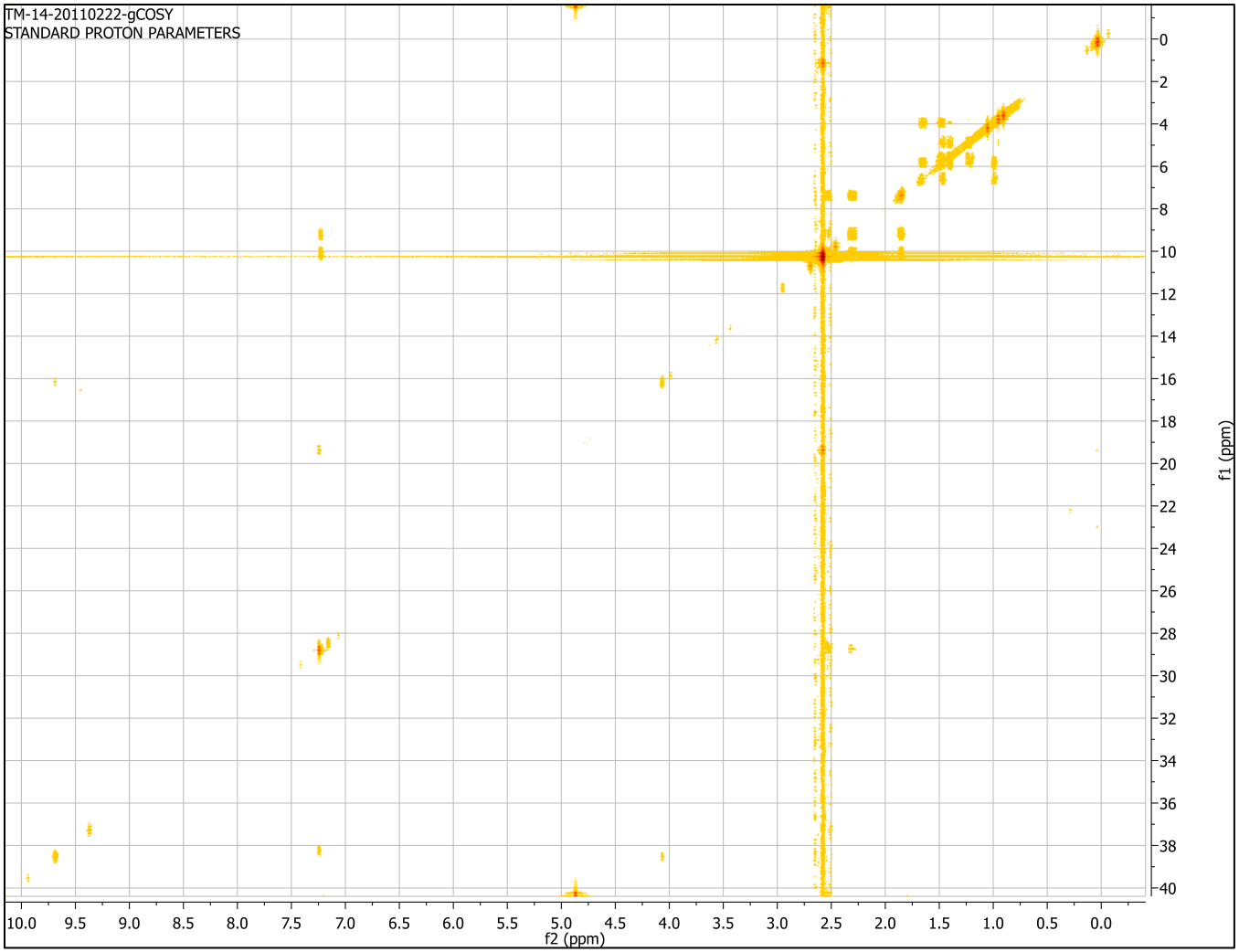
Appendix B1. ^{1}H NMR Spectra of HPLC fraction 7. This compound was identified to be drimane sesquiterpenesmuzigadial.



Appendix B2. gCOSY NMR Spectra of HPLC fraction 7. This compound was identified to be drimane sesquiterpene muzigadial.



Appendix B3. ^{1}H NMR Spectra of HPLC fraction 14. This compound was identified to be drimane sesquiterpene warburganal.



Appendix B4. gCOSY Spectra of HPLC fraction 14. The most abundant compound was identified to be drimane sesquiterpene warburganal.

APPENDIX C

Genes Upregulated More Than 1.5 Times In Microarray Analysis

Gene Name	4h		24h		Function
	Normalized	t-test	Normalized	t-test	
		P-value		P-value	
rv0026	2.14	0.04	2.79	0.19	HP
rv0027	1.65	0.04	1.77	0.01	Unknown function conserved hypothetical protein gene product
rv0048c	1.56	0.04	1.59	0.01	unknown possible membrane protein
rv0067c	3.17	0.01	2.41	0.05	possibly involved in transcritional regulatory mechanism
rv0068	3.48	0	4.13	0.02	Function unknown; probably involved in cellular metabolism
sdaa (rv0069c)	2.65	0.13	1.86	0	sdaA L-serine dehydratase SdaA
rv0132c	1.76	0.08	1.58	0.08	fgd2 f420-dependent glucose-6-phosphate dehydrogenase
rv0136	2.08	0.12	1.93	0.04	cytochrome P450 138
rv0137c	1.52	0.26	1.69	0.24	msrA, methionine sulfoxide reductase A
rv0146	1.53	0.01	2.08	0.23	Function unknown. Possible methyltransferase.
bgls (rv0186)	1.51	0.01	1.52	0.08	beta-glucosidase
rv0195	1.66	0.26	3.32	0.01	Part of two component system, probably LuxR
rv0213c	7.51	0	2.23	0	Methyltransferase
rv0219	2	0.01	3.11	0.02	Unknown
lpqi (rv0237)	1.77	0.24	2.47	0.23	Lipoprotein
fada2 (rv0243)	1.97	0.01	1.53	0.08	acetyl-CoA acetyltransferase

Gene Name	4h		24h		Function
	Normalized	t-test	Normalized	t-test	
		P-value		P-value	
fade5 (rv0244c)	1.51	0.02	2.26	0.04	acyl-CoA dehydrogenase
rv0250c	1.97	0.09	1.84	0	Function unknown
hsp (rv0251c)	1.76	0.05	2.4	0.05	heat shock protein
cobq (rv0255c)	1.99	0.05	2.99	no replicates	cobyric acid synthase
naru (rv0267)	2.09	0.02	2.68	0.02	Integral membrane nitrite extrusion protein
rv0327c	1.78	0.03	1.77	0.16	cytochrome P450
rv0331	2.05	0.12	1.83	0.22	Function unknown
clpb (rv0384c)	1.83	0.03	1.63	0.07	endopeptidase ATP binding protein
rv0416	1.93	0.01	2.53	0	sulfur carrier protein
rv0424c	1.68	0.07	1.5	0.13	Unknown function conserved hypothetical protein gene product
rv0452	1.67	0.01	2.16	0.03	Possible involved in a transcriptional mechanism
rv0465c	1.59	0.23	2.54	0.08	Possible involved in a transcriptional mechanism
acea (rv0467)	1.57	0.02	2.35	0.02	icl isocitrate lyase
rv0532	1.82	0.08	1.68	0.07	PE-PGRS family protein
htpx (rv0563)	1.91	0.09	2.38	0	heat shock protein
rv0585c	1.94	0.01	2.7	0.04	Probable conserved integral membrane protein

Gene Name	4h		24h		Function
	Normalized	t-test	Normalized	t-test	
		P-value		P-value	
rv0585c	1.94	0.01	2.7	0.04	Probable conserved integral membrane protein
mmpl5 (rv067c)	2.95	0.06	3.16	0.01	transmembrane transport protein MmpL5
mmps5 (rv0677c)	3.97	0.01	3.86	0.02	Conserved membrane protein
rv0678	5.24	0.02	3.67	0	Unknown function conserved hypothetical protein gene product
rv0690c	1.6	0	1.9	0.01	Unknown function conserved hypothetical protein gene product
rplc (rv0701)	1.83	0.01	1.59	0.04	50S ribosomal protein
atsa (rv0711)	1.8	0.03	2.68	0	arylsulfatase AtsA
xylb (rv0729)	1.78	0.04	3.12	0.06	xylB D-xylulose kinase XylB
rv0754	1.77	0.32	2.17	0	PE-PGRS family protein
rv0802c	1.51	0.01	1.64	0.02	Aceylation substrate unknown
rv0810c	1.7	0.09	2.03	0.03	Unknown function conserved hypothetical protein gene product
lpqr (rv0838)	1.79	0.02	2.03	0.06	lpqR lipoprotein
cysm3 (rv0848)	1.52	0.01	2.33	0	cysteine synthase A
rv0849	1.62	0	2.53	0.04	Thought to be involved in the transport of an unknown substrate across the membrane
rv0850	1.6	0.22	2.84	0.07	required for the transposition of an insertion element
rv0981	2.39	0.04	1.73	0.04	mprA two component response transcriptional regulatory protein MprA

Gene Name	4h		24h		Function
	Normalized	t-test	Normalized	t-test	
		P-value		P-value	
rv0997	2.2	0.02	2.14	0.3	Unknown function conserved hypothetical protein gene product
pabb (rv1005c)	1.88	0.01	2.08	0.05	aminodeoxychorismate synthase component I
rv1057	2.77	0.02	2.91	0.03	Function unknown
rv1073	1.86	0.04	2.35	0	Unknown function conserved hypothetical protein gene product
lytb' (rv1110)	1.63	0.02	1.61	0.07	LYTB-like protein
rv1174c	2.41	0	1.68	0.01	T-cell antigen
pks3 (rv1180)	1.86	0.08	3.84	0.05	polyketide beta-ketoacyl synthase PKS3
papa3 (rv1182)	2.42	0.34	3.01	0.3	polyketide synthase associated protein PapA3
rv1184c	1.89	0.11	3.41	0.07	hypothetical protein
fadd21 (rv1185c)	1.78	0.02	2.51	0.07	acyl-CoA synthetase
rv1200	1.61	0.07	1.89	0.02	Thought to be involved in a transport system across the membrane
rv1216c	3.08	0	1.79	0.02	Probable integral membrane protein
rv1217c	3.28	0.01	1.62	0	Thought to be involved in a transport system across the membrane
rv1218c	3.89	0.01	2.52	0	Thought to be involved in a transport system across the membrane
rv1219c	3.02	0.05	2.29	0.04	Involved in transcriptional mechanism
rv1347c	1.5	0	2.76	0.06	Acylation substrate unknonwn

Gene Name	4h		24h		Function
	Normalized	t-test	Normalized	t-test	
		P-value		P-value	
rv1353c	2.19	0.03	2.43	0.23	Probable trascritional regulatory protein
rv1390	1.61	0.13	2.45	0.11	DNA-directed RNA polymerase subunit
pria (rv1402)	1.59	0.28	1.55	0.11	priA primosome assembly protein
rv1403c	1.98	0.12	2.56	0.02	Putative methyl transferase
trxa (rv1470)	1.62	0.02	1.53	0.02	thioredoxin TRXA
rv1791	1.54	0.02	1.56	0.2	PE family protein
rv1792	3.07	0.05	2.19	0.16	Pseudo
rv1803c	1.7	0.03	2.42	0.03	PE-PGRS family protein
rv1806	2.04	0.03	4.45	0.02	PE family protein
rv1807	1.56	0.02	2.43	0.02	PPE family protein
rv1808	1.7	0.03	3.02	0.04	PPE family protein
rv1809	1.75	0.13	3.2	0.06	PPE family protein
rv1810	1.52	0.02	2.18	0.02	Unknown function conserved hypothetical protein gene product
rv1945	1.82	0.04	1.58	0.02	Unknown function conserved hypothetical protein gene product
rv2037c	2.01	0.2	2.62	0.24	Unknown, possible conserved trans-membrane protein
rv2050	1.9	0.13	2.14	0.12	Unknown function conserved hypothetical protein gene product
rv1353c	2.19	0.03	2.43	0.23	Probable trascritional regulatory protein
rv1390	1.61	0.13	2.45	0.11	DNA-directed RNA polymerase subunit

Gene Name	4h		24h		Function
	Normalized	t-test	Normalized	t-test	
		P-value		P-value	
pria (rv1402)	1.59	0.28	1.55	0.11	priA primosome assembly protein
rv1403c	1.98	0.12	2.56	0.02	Putative methyl transferase
trxa (rv1470)	1.62	0.02	1.53	0.02	thioredoxin TRXA
rv1791	1.54	0.02	1.56	0.2	PE family protein
rv1792	3.07	0.05	2.19	0.16	Pseudo
rv1803c	1.7	0.03	2.42	0.03	PE-PGRS family protein
rv1806	2.04	0.03	4.45	0.02	PE family protein
rv1807	1.56	0.02	2.43	0.02	PPE family protein
rv1808	1.7	0.03	3.02	0.04	PPE family protein
rv1809	1.75	0.13	3.2	0.06	PPE family protein
rv1810	1.52	0.02	2.18	0.02	Unknown function conserved hypothetical protein gene product
rv1945	1.82	0.04	1.58	0.02	Unknown function conserved hypothetical protein gene product
rv2037c	2.01	0.2	2.62	0.24	Unknown, possible conserved trans-membrane protein
rv2050	1.9	0.13	2.14	0.12	Unknown function conserved hypothetical protein gene product
rv2094c	1.6	0.01	1.95	0.01	arginine translocase protein A
rv2100	1.92	0.02	1.64	0.05	Unknown function conserved hypothetical protein gene product
rv2182c	1.59	0.01	1.72	0.01	transfer of fatty acyl groups

Gene Name	4h		24h		Function
	Normalized	t-test	Normalized	t-test	
		P-value		P-value	
rv2333c	1.86	0.03	2.2	0.06	Thought to be involved in a transport system across the membrane
rv2347c	3.67	0.02	4.51	0.07	ESAT-6 like protein
mbtd (rv2381c)	1.7	0.07	2.4	0.03	polyketide synthetase
trpe2 (rv2386c)	1.57	0.07	3.27	0.04	component I of anthranilate synthase
ahpc (rv1792)	1.55	0.15	1.93	0.05	alkyl hydroperoxide reductase subunit C
clpP2 (rv2460c)	1.85	0.02	2.05	0.02	ATP-dependent Clp protease proteolytic subunit
rv2541	2.51	0.02	1.85	0.01	Unknown
rv2694c	3.01	0.34	2.6	0.03	Unknown function conserved hypothetical protein gene product
rv2699c	1.67	0.02	2.73	0.09	Unknown function conserved hypothetical protein gene product
sigb (rv2710)	7.33	0.12	2.59	0.02	RNA polymerase sigma factor SigB
35kd_ag (rv2744c)	1.81	0.25	1.67	0.13	hypothetical protein
rv2745c	1.85	0.01	1.66	0.1	Possibly involved in transcriptional mechanism
rv2802c	1.73	0.01	1.86	0.12	Unknown
rv2824c	2.65	0.16	2.26	0.03	HP
gcpe (rv2868c)	1.5	0.08	1.56	0.08	biosynthesis of secondary metabolites
rv3054c	1.59	0.03	3.01	0.11	hypothetical protein

Gene Name	4h		24h		Function
	Normalized	t-test	Normalized	t-test	
		P-value		P-value	
rv3160c	4.62	0	3.23	0.04	Possibly involved in transcriptional mechanism.
rv3161c	2.51	0.01	2.37	0.09	Function unknown; probably involved in cellular metabolism
lipv (rv3203)	1.67	0.01	1.74	0.01	lipV lipase LipV
rv3402c	2.04	0.05	4.68	0.04	Unknown function conserved hypothetical protein gene product
rv3406	2.24	0.07	2.26	0.02	Function unknown; probably involved in cellular metabolism
whib3 (rv3416)	1.67	0.03	2.1	0.04	transcriptional regulatory protein WHIB-like
rv3430c	1.67	0.51	1.56	no replicates	Possible transposase
rv3520c	1.51	0.16	1.67	0.02	Function unknown probably involved in cellular metabolism
rv3539	1.56	0.04	1.68	0.02	PPE family protein
rv3588c	1.52	0.01	1.55	0.34	
fole (rv3609c)	2.09	0.05	1.78	0.02	GTP cyclohydrolase I
rv3615c	1.68	no replicates	2.82	0.14	Unknown function conserved hypothetical protein gene product
rv3765c	1.69	0.05	2.21	0.02	two component transcriptional regulatory protein
rv3766	1.66	0.03	1.82	0.03	Unknown function conserved hypothetical protein gene product
rv3767c	1.53	0.1	3.12	0	Function unknown. Possible methyltransferase.

Gene Name	4h		24h		Function
	Normalized	t-test	Normalized	t-test	
		P-value		P-value	
epib (rv3784)	1.51	0.01	1.82	0.02	Possibly involved in biosynthesis pathway of lipopolysaccharide biosynthesis
rv3802c	1.68	0	1.77	0.02	Unknown function conserved hypothetical protein gene product
papa1 (rv3824c)	2.12	0	2.86	0.1	polyketide synthase associated protein
pks2 (rv3825c)	2.88	0.01	3.88	0.09	polyketide synthase
rv3830c	1.54	0.05	3.63	0.01	transcriptional regulatory protein
rv3862c	1.86	0.03	2.27	0.02	transcriptional regulatory protein WHIB-like WHIB6
rv3880c	4.19	0.03	1.82	0.02	Unknown function conserved hypothetical protein gene product
MT2619	1.56	0.14	1.9	0.02	Lipoprotein
MT1746.1	1.68	no replicates	2.54	0.08	Unknown function conserved hypothetical protein gene product
MT3297	1.61	0.25	1.52	0.13	hypothetical protein
MT2467	1.98	0.19	2.96	0	PE-PGRS family protein
MT1025.2	1.75	0.02	1.83	0	hypothetical protein