The isolation of anti-mycobacterial compounds from South African medicinal plants

Eliya Vukani Madikane

Medicinal bark, fruits and leaves of *Warburgia salutaris*

Thesis presented for the degree of

**Doctor of Philosophy in Pharmacology**

University of Cape Town

Supervisors: Assoc. Prof. P.J. Smith, Dr W.E. Campbell, and Assoc. Prof. G.B. Elisha

August 2005
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Warburgia salutaris juvenile tree grows next to the fallen “parent” tree on the foreground.

“This thesis is in memory of my late mother Nomvuyo Margaret Mkiva-Madikane and in appreciation of Ntombexhego Nosidima Clever-girl Mkiva-Kobese who nurtured me.”
DECLARATION

The isolation and characterization of anti-mycobacterial compounds
from South African medicinal plants

I, Eitya Vukan Madikane hereby declare that the work on which this thesis is
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otherwise) and that neither the substance nor any part of this work has been,
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This thesis is presented for the examination for the degree of Doctor of
Philosophy.

Signed: .............................................. in Cape Town on the
Date: .............................................. 2005
ACKNOWLEDGEMENTS

It is my greatest pleasure to mention and thereby acknowledge the following individuals who have contributed, directly or indirectly towards the completion of this thesis:

Without the guidance and support of Associate Professor Peter Smith, Associate Professor Gay Elisha, Professor Edith Sim and Dr Bill Campbel in academic matters, this thesis would not be possible. I am very much indebted to Gay for believing in me and for her tutelage; together with Edith and Peter she has been the wind beneath my juvenile wings.

I would like to express my gratitude to T. Doctor George Rhesha of Queenstown, who supplied me with a sample of the bark of Warburgia salutaris, which later became the focus of this research and to Dr Nell Crouch of the National Botanical Institute (NBI) for supplying me with the authenticated research material.

The initial anti-tubercular activity of Warburgia salutaris bark extract was identified with the most appreciated help of Mrs. Vanessa January and Ms. Monica Chojaacki of the Groote Schuur Hospital TB diagnostic laboratory.

My greatest thank you goes to three individuals at Oxford University, Dr Angela Russell for helping me with setting up the HPLC system, to Dr Sanjib Bhakta who's tutelage in microbiological techniques was most valuable and to Dr Timothy Claridge for performing the NMR experiments and helping with the structural elucidation.
ACKNOWLEDGEMENTS

I would like to thank the following colleagues for their emotional and social support during my Ph.D:

1. In the Department of Pharmacology, University of Cape Town - Denise Saravanakumar, Carmen Lategan, Malefa Tselanyane, Dr. Bennedict Bapela and Jessica Petersen.

2. In the Department of Pharmacology, Oxford University – Dr. James sandy, Dr. Akane Kawamura, Dr. Isaac Westwood, Matthew Anderton, Dr. Larissa Wakefield, Hillary Long and Liz Fulham and Mimi Mo.

My highest appreciation goes to Zolile Kobese for being my father. To my sisters Nosisa Madikane, Nothemba Madikane, Nontembiso Madikane, my nephews, nieces, aunts and cousins for their understanding and support. Mrs Nomthunzi Mkiva-Silere has been a pillar and a dear friend and I cannot show enough appreciation. Without the support of Mpumelelo Silinga I would have struggled a lot, I thank you my friend for having such a beautiful soul.

It was as a result of continued financial support from the South African Medical Research Council, the Sainsbury/Linbury Trust, the Oxford University Isis Innovation Fund and the University of Cape Town that this research project has advanced to completion. I thank you.
ABSTRACT

Drug-resistant tuberculosis is a problem for which novel drugs that target novel enzymes inside the tubercle bacillus are sought. South Africa has an abundance of biodiversity that is a valuable resource from which novel antitubercular agents could be extracted.

An Ethno-medicinal survey of medicinal plants in 3 provinces of South Africa was conducted using an expanded diagnostic criterion. Subsequent laboratory investigations revealed that a crude dichloromethane extract of Warburgia salutaris bark exhibited anti-mycobacterial action at a concentration of 50µg/ml but not against Escherichia coli, a gram-negative bacterium. The crude extract also inhibited the activity of the recombinant arylamine N-acetyltransferase from Mycobacterium smegmatis, a novel target for antitubercular intervention. A Bioassay-guided fractionation of this crude extract involved a combination of Solid Phase Extractions, analytical and preparative High Performance Liquid Chromatography.

Two anti-mycobacterial compounds, 1 and 2, which affected growth of M. bovis BCG (50µg/ml) were isolated for the first time in this species. Compound 1 is a novel drimane sesquiterpenoid lactone (C_{17}H_{24}O_{6}) that specifically targeted the arylamine N-acetyltransferase in M. bovis BCG while compound 2 is a known drimane sesquiterpenoid dialdehyde, cinnamodial (C_{17}H_{24}O_{5}). Chemical synthesis of these natural compounds would be a favorable source as yields obtained from the plant material were low, 0.015% for compound 1 and 0.002% for compound 2.
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<th>Description</th>
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<tbody>
<tr>
<td>1D</td>
<td>One dimensional</td>
</tr>
<tr>
<td>2-AF</td>
<td>2-aminoflourene</td>
</tr>
<tr>
<td>2D</td>
<td>Two dimensional</td>
</tr>
<tr>
<td>3D</td>
<td>Three dimensional</td>
</tr>
<tr>
<td>5-ASA</td>
<td>5-aminosalicylic acid</td>
</tr>
<tr>
<td>ACP</td>
<td>Anoyl acyl carrier protein</td>
</tr>
<tr>
<td>ADC</td>
<td>(Albumin, Dextrose, Catalase; Difco)</td>
</tr>
<tr>
<td>AFBs</td>
<td>Acid Fast Bacilli</td>
</tr>
<tr>
<td>AG</td>
<td>Arabinogalactan</td>
</tr>
<tr>
<td>BCG</td>
<td>Bacillus Calmette-Guerin</td>
</tr>
<tr>
<td>CH₂Cl₂</td>
<td>Dichloromethane</td>
</tr>
<tr>
<td>COSY</td>
<td>Proton-proton Correlation Spectroscopy</td>
</tr>
<tr>
<td>DIM</td>
<td>Phthiocerol dimycocerosates</td>
</tr>
<tr>
<td>DMAP</td>
<td>3,3'-dimethylallyl pyrophosphate</td>
</tr>
<tr>
<td>DOTS</td>
<td>Directly Observed Treatment Short-course</td>
</tr>
<tr>
<td>DTNB</td>
<td>5,5-Dithio-bis(2-nitrobenzoic acid)</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol; Cleland's reagent</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EC</td>
<td>Eastern Cape</td>
</tr>
<tr>
<td>FAMES</td>
<td>Fatty acid methyl esters</td>
</tr>
<tr>
<td>FHP</td>
<td>Farnesyl hydroxyphosphate</td>
</tr>
<tr>
<td>FPP</td>
<td>2E,6E-farnesyl pyrophosphate</td>
</tr>
<tr>
<td>FS</td>
<td>Free State province</td>
</tr>
<tr>
<td>HIV</td>
<td>Human immunodeficiency virus</td>
</tr>
<tr>
<td>HMBC</td>
<td>Heteronuclear Multiple Bond Correlation</td>
</tr>
<tr>
<td>HMG-ScoA</td>
<td>Hydroxymethylglutaryl-SCoA</td>
</tr>
<tr>
<td>HPLC</td>
<td>High Pressure Liquid Chromatography</td>
</tr>
<tr>
<td>HSQC</td>
<td>Heteronuclear Multiple Quantum Correlation</td>
</tr>
<tr>
<td>HTS</td>
<td>High-throughput screening</td>
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<tr>
<td>IKS</td>
<td>Indigenous knowledge systems</td>
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# List of Abbreviations

<table>
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<tr>
<td>INH</td>
<td>Isoniazid</td>
</tr>
<tr>
<td>IP</td>
<td>Intellectual property</td>
</tr>
<tr>
<td>IPP</td>
<td>Isopentenyl pyrophosphate</td>
</tr>
<tr>
<td>IPR</td>
<td>Intellectual property rights</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropyl β-D-thiogalactopyranoside</td>
</tr>
<tr>
<td>IR</td>
<td>Infra red</td>
</tr>
<tr>
<td>LAM</td>
<td>Lipoarabinomannan</td>
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<tr>
<td>LC-MS</td>
<td>Liquid Chromatography-Mass Spectroscopy</td>
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<tr>
<td>mAGP</td>
<td>Mycolyl-arabinogalactan-peptidoglycan</td>
</tr>
<tr>
<td>MAMES</td>
<td>Mycolic acid methyl esters</td>
</tr>
<tr>
<td>MDR-TB</td>
<td>Multi-drug resistant tuberculosis</td>
</tr>
<tr>
<td>MeCN</td>
<td>Acetonitrile</td>
</tr>
<tr>
<td>MeOH</td>
<td>Methanol</td>
</tr>
<tr>
<td>MIC</td>
<td>Minimum inhibitory concentration</td>
</tr>
<tr>
<td>MS</td>
<td>Mass Spectrometry</td>
</tr>
<tr>
<td>MSNAT</td>
<td>Mycobacterium smegmatis arylamine N-acetyltransferase</td>
</tr>
<tr>
<td>NAT</td>
<td>Arylamide N-acetyltransferase</td>
</tr>
<tr>
<td>NC</td>
<td>Northern Cape Province</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear Magnetic Resonance Spectroscopy</td>
</tr>
<tr>
<td>NOESY</td>
<td>Nuclear Overhauser Effect Spectroscopy</td>
</tr>
<tr>
<td>OD</td>
<td>Optical Density</td>
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<tr>
<td>p-ABA</td>
<td>p-aminobenzoic acid</td>
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<tr>
<td>PG</td>
<td>Peptidoglycan</td>
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<tr>
<td>P-GlcNAc-Rha</td>
<td>Phosphoryl-N-acetylglucosaminylo-rhamnosyl</td>
</tr>
<tr>
<td>PIM</td>
<td>Phosphatidylinositol mannoside</td>
</tr>
<tr>
<td>Pv</td>
<td><em>Pittosporum viridiflorum</em></td>
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<tr>
<td>SATMERG</td>
<td>South African Medicines Research Group</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis</td>
</tr>
<tr>
<td>SPE</td>
<td>Solid Phase Extractions</td>
</tr>
<tr>
<td>TB</td>
<td>Tuberculosis</td>
</tr>
<tr>
<td>TDM</td>
<td>Trihalose dimycolate</td>
</tr>
<tr>
<td>T. Dr.</td>
<td>Traditional Doctor</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>TFA</td>
<td>Trifluoroacetic acid</td>
</tr>
<tr>
<td>TLC</td>
<td>Thin Layer Chromatography</td>
</tr>
<tr>
<td>TNB</td>
<td>Thionitrobenzoate</td>
</tr>
<tr>
<td>TRAMED</td>
<td>Traditional medicine</td>
</tr>
<tr>
<td>UK</td>
<td>United Kingdom</td>
</tr>
<tr>
<td>UN-ECOSOC</td>
<td>United Nations - Economic and Social Council</td>
</tr>
<tr>
<td>UV/VIS</td>
<td>Ultra Violet/Visible</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organisation</td>
</tr>
<tr>
<td>WHO/IUATLD</td>
<td>World Health Organisation / International Union Against TB and Lung Disease</td>
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<tr>
<td>WHOSIS</td>
<td>World Health Organisation statistical information system</td>
</tr>
<tr>
<td>Wsa</td>
<td>Warburgia salutaris</td>
</tr>
<tr>
<td>Wso</td>
<td>Withania somnifera</td>
</tr>
</tbody>
</table>
Chapter 1

Introduction: TB, drug targets and hit compounds
1.1. Global burden of tuberculosis

In 1882 on the 24th of March, Robert Koch, a German scientist, announced in front of the Physiological Society of Berlin that he had found the cause of tuberculosis in man, the tubercle bacillus (Chadwick 1982; Grange 1996) now known as *Mycobacterium tuberculosis*. The discovery of streptomycin and isoniazid and the introduction of chemotherapy in the early 1950s gave hope that tuberculosis was to be eradicated from the face of the earth.

However, in 1991 a seminal paper by Arata Kochi was published in *Tubercle* (Kochi, 1991), highlighting the global burden of tuberculosis (Table 1.1.1): a third of the world’s population is infected with the bacilli, 2.9 million people die of tuberculosis each year around the world and 80% of the 8 million new cases of infection per annum, occur in the developing countries (Kochi, 1991).

**Table 1.1.1: The Global Toll of Tuberculosis**

<table>
<thead>
<tr>
<th>Region</th>
<th>People infected (Millions)</th>
<th>New Cases</th>
<th>Deaths</th>
</tr>
</thead>
<tbody>
<tr>
<td>Africa</td>
<td>171</td>
<td>1 400 000</td>
<td>660 000</td>
</tr>
<tr>
<td>Americas</td>
<td>117</td>
<td>560 000</td>
<td>220 000</td>
</tr>
<tr>
<td>Eastern Mediterranean</td>
<td>52</td>
<td>594 000</td>
<td>160 000</td>
</tr>
<tr>
<td>South-East Asia</td>
<td>426</td>
<td>2 480 000</td>
<td>940 000</td>
</tr>
<tr>
<td>Western Pacific</td>
<td>574</td>
<td>2 560 000</td>
<td>890 000</td>
</tr>
<tr>
<td>Europe and other industrialized countries</td>
<td>382</td>
<td>410 000</td>
<td>40 000</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>1 722</strong></td>
<td><strong>8 004 000</strong></td>
<td><strong>2 910 000</strong></td>
</tr>
</tbody>
</table>

Data from Kochi (1991)

Kochi’s paper challenged the WHO and governments to devise strategies to detect and combat tuberculosis, hence on the 24th of March 1993, 111 years after the identification of the causative agent, the WHO declared tuberculosis a global emergency (WHO, 1994a). The re-emergence of tuberculosis in the
late 1980s had been exacerbated by increasing HIV infection (Selwyn et al., 1989; Glynn et al., 2000; Raviglione et al., 1997) and poor socio-economic status (Holtgrave and Crosby, 2004; WHO, 2002a & 2002b). The latest WHO TB report (WHO, 2005) below shows clearly that the highest TB incidence rates are in the developing countries, especially in sub-Saharan Africa (Figure 1.1.1).

![World map of TB incidence rates](image)

**Figure 1.1.1.** Estimated incidence rates per 100 000 of all forms of TB in 2003 (WHO, 2005)

### 1.2. Multi-drug resistant tuberculosis

The prevalence of multi-drug resistant strains of tuberculosis, resistant to more than the two frontline drugs, isoniazid and rifampicin (Espinal, 2003) has made therapy difficult to achieve. The WHO suggests that prevention of MDR-TB should be prioritized as it occurs due to man-made factors. Out of 77 countries that submitted their TB drug resistance data to the WHO/IUATLD, 74 have reported drug resistance in new cases. The prevalence of single drug resistance in new cases ranged from 0% to 57.1%, Kazakhstan having the
highest percentage (WHO/IUATLD, 2004). South Africa was also among the countries which were studied and the prevalence of resistance to any one TB drug was generally less than 9.9%, except in the Kwazulu-Natal province where the prevalence was in the range of 10% to 19.9% (WHO/IUATLD, 2004).

1.2.1. TB “Hot Spots”

TB “Hot Spots” are countries where the prevalence of new MDR-TB cases exceeds 3% (WHO/IUATLD, 2000). These countries are illustrated in figure 1.2.1 and they are areas where emphasis of the DOTS-plus should be given to prevent the spread of MDR-TB. Dots-plus is a supplement to the standard DOTS programme (see section 1.3) and it extends to the use of second-line anti-TB drugs specifically in areas of high MDR-TB incidence (Newsletter of the Global Partnership Movement to Stop TB, 2002); MDR TB in South Africa is not an obvious problem.

Figure 1.2.1: The prevalence of MDR-TB in new cases of TB. There are six “HOT SPOT” countries or regions that had rates over 10%: Kazakhstan (25.3%); Karakalpakstan, Uzbekistan (19.8%); Israel (14.6%); Estonia (13.4%); Tomsk Oblast, Russian Federation (13.1%); and Latvia (10.3%). [WHO/IUATLD, 2004].
1.3. TB control

The milestones of the DOTS strategy (developed by Dr. Karel Styblo and adopted by the WHO) were to increase the case detection rates of new smear positive TB cases to at least 70% and TB treatment success to at least 85% by the year 2005 (WHO, 1994b). The DOTS programme entails 4 main levels of intervention, at a technical level, logistical level, operational level (Table 1.3.1) and political level. Within these levels, emphasis is put on 5 major areas crucial for TB control. They are: political commitment, case detection using sputum microscopy among persons seeking care for prolonged cough, standardized short course chemotherapy under proper case-management conditions including directly observed treatment, regular drug supply, and a standardized recording and reporting system that allows assessment of individual patients as well as overall programme performance (WHO, 1999).

<table>
<thead>
<tr>
<th>TECHNICAL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Case detection and diagnosis</td>
</tr>
<tr>
<td>Standardized short-course treatment</td>
</tr>
<tr>
<td>Direct observation at least during the initial phase</td>
</tr>
<tr>
<td>Recording and reporting of progress and cure</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>LOGISTICAL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dependable drug supply to the patient level</td>
</tr>
<tr>
<td>Laboratories for microscopy</td>
</tr>
<tr>
<td>Supervision and training of health care workers</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>OPERATIONAL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flexibility in implementation of technical aspects</td>
</tr>
</tbody>
</table>

(WHO, 1999)
In 2002, only 1 of the 22 high burden countries (Table 1.3.2) that have adopted the DOTS programme, Vietnam, had achieved both a detection rate above 70% and a treatment success rate of above 85% (WHO, 2001; WHO, 2005). Countries like Afghanistan, Cambodia, China, India, Indonesia and Philippines reached a treatment success rate of greater than 85% despite having low case detection rates than was recommended; and these high treatment success rates might not be real but could be as a direct consequence of under-reporting. South Africa did not implement DOTS fast enough and was underperforming in 2001 but did improve case detection dramatically in 2002 (WHO, 2005); and an improvement in treatment success rates is expected in South Africa in the future.

Table 1.3.2: Countries that have a high burden of TB

<table>
<thead>
<tr>
<th>COUNTRY</th>
<th>Year of WHO report</th>
</tr>
</thead>
<tbody>
<tr>
<td>Afghanistan</td>
<td>19</td>
</tr>
<tr>
<td>Bangladesh</td>
<td>29</td>
</tr>
<tr>
<td>Brazil</td>
<td>10</td>
</tr>
<tr>
<td>Cambodia</td>
<td>55</td>
</tr>
<tr>
<td>China</td>
<td>30</td>
</tr>
<tr>
<td>DR Congo</td>
<td>57</td>
</tr>
<tr>
<td>Ethiopia</td>
<td>36</td>
</tr>
<tr>
<td>India</td>
<td>31</td>
</tr>
<tr>
<td>Indonesia</td>
<td>27</td>
</tr>
<tr>
<td>Kenya</td>
<td>46</td>
</tr>
<tr>
<td>Mozambique</td>
<td>45</td>
</tr>
<tr>
<td>Myanmar</td>
<td>65</td>
</tr>
<tr>
<td>Nigeria</td>
<td>13</td>
</tr>
<tr>
<td>Pakistan</td>
<td>13</td>
</tr>
<tr>
<td>Philippines</td>
<td>62</td>
</tr>
<tr>
<td>Russia</td>
<td>6.9</td>
</tr>
<tr>
<td>South Africa</td>
<td>105</td>
</tr>
<tr>
<td>Tenzania</td>
<td>43</td>
</tr>
<tr>
<td>Thailand</td>
<td>65</td>
</tr>
<tr>
<td>Uganda</td>
<td>46</td>
</tr>
<tr>
<td>Viet Nam</td>
<td>88</td>
</tr>
<tr>
<td>Zimbabwe</td>
<td>47</td>
</tr>
</tbody>
</table>

C.d.r - Case detection rate, T.S - Treatment success (Data; WHO, 2005)
1.3.1. Current Global TB situation

The 2005 WHO report on tuberculosis has revealed that globally there is a reduction in deaths caused by tuberculosis. Only 1.7 million deaths were due to tuberculosis in 2003 (WHO, 2005), a reduction of 58% since Kochi’s paper, which estimated a 50% reduction in deaths due to tuberculosis over a period of 10-12 years, particularly in developing countries, if the DOTS strategy was adopted world-wide (Kochi, 1991). The increase in smear positive cases (from 1.7 million in 2001 to 3.9 million in 2003) and the increase in new cases (from 8 million in 1991 to 8.8 million in 2003) was as a result of increased coverage and notification (WHO, 2005). The countries that have adopted the DOTS strategy have increased dramatically from 171 in 2001 to 199 in 2003, showing increased notification of TB cases in the world.

1.3.2. Increasing deaths caused by TB in South Africa

Despite the Global decline of deaths due to TB and low MDR-TB incidence in South Africa, of the total reported South African annual deaths, respiratory tuberculosis was responsible for 6% and 4% of deaths in males and females older than 24 years, respectively, compared to the contribution of less than 2% and less than 1% in British male and female deaths, respectively (WHOSIS, 1996 data). The latest death statistics for South Africa indicate that there is an increase in deaths caused by TB; in 1997, 1999 and 2001 the percentage of the total annual deaths caused by tuberculosis in males and females was 7.8% and 5.8%, 10% and 7.7% and 12.3% and 10.1%, respectively (Statistics South Africa, 2005).
It has been shown that there is a high mortality rate due to tuberculosis in Sub-Saharan Africa where there is a high prevalence of HIV-1 infection (Raviglione et al., 1997). In KwaZulu Natal province of South Africa between 1991 and 1998, TB in adults increased by 360% with 65% of these being HIV-1 infected in 1997 (Wilkinson and Davies, 1997). In addition to this problem, HIV-1 positive TB-infected individuals have higher mortality rates when treated with isoniazid, streptomycin and thiacetazone than those treated with rifampicin-based treatment (Nunn et al., 1992; Okwera et al., 1994; Elliot et al., 1995). It was shown that recurrence of TB amongst HIV-1 positive patients after rifampicin-based treatment can occur (Fitzgerald et al., 2000). New effective TB drugs that would not interact with HIV drugs and that are less toxic to the host are needed.

1.3.3. The need for novel drugs

The increase in MDR-TB and deaths caused by TB has emphasized the necessity of novel cures to deal with the tubercle load that causes disease in the host. There are two key areas in which a novel agent has to show competency, at a molecular level and at a pharmacokinetic level. A drug that acts either on a novel target or a series of novel target proteins in the M. tuberculosis cell would be attractive. It would require more than one mutation to render the bacillus resistant to this new agent if it affects multiple components. A global alliance for TB drug discovery (http://www.tballiance.org) has been formed to help develop affordable and fast-acting drugs that would help fight tuberculosis. Affordable, fast-acting and
long-lasting drugs will ensure better pharmacokinetics and patient compliance and hence improve therapy.

However, an effective control measure will be one that tackles both the biomedical and also the social aspects of tuberculosis (Gandy and Zumla, 2002). Social issues that lead to the spread of tuberculosis such as poverty, overcrowding, inequality and access to quality medical care should be dealt with by governments.

In line with the intentions of this project, the following sections will focus only on the biomedical aspects of TB control with special reference to the *M. tuberculosis* cell wall lipids and the targeting of enzymes involved in mycobacterial lipid biosynthesis for anti-tubercular drug discovery.

1.4. The *tubercle bacillus*

1.4.1. General characteristics

The *tubercle bacillus* of the genus mycobacterium belongs to a family of mycobacteriaceae of the order Antinomycetales (Chadwick 1982; McMurray 1991). The word "mycobacterium" which means fungus-bacterium was used because the *tubercle bacillus* grew as mould-like pellicles on the surface of liquid agar (Grange, 1996). Under microscopy, the bacilli are rod-shaped gram-positive aerobic bacteria that tend to form branched filaments. The bacilli are about 0.2 to 0.6 μm X 1 to 10 μm in size (Murray et al., 2002). There are three main features that distinguish mycobacteria from other gram-positive
bacteria. Firstly, mycobacteria strongly retain carboluxsin dye when decolorized with acid-ethanol, hence, are termed acid-fast bacilli (Murray et al., 2002).

Secondly, mycobacteria have lipid rich cell walls that form hydrophobic barriers that render them resistant to disinfectants. Prominently, the presence of long chain mycolic acids of carbon lengths C₆₀-C₉₀ (McMurray 1991) in mycobacteria distinguishes them from other gram-positive bacteria such as Nocardia that have shorter mycolic acids, C₄₀-C₅₀ in length (McMurray 1991). Lastly, mycobacteria contain distinctively and unusually high contents of guanine and cytosine (Murray et al., 2002) that are greater than 60% in their DNA, compared to other gram-positive bacteria.

1.4.2. The unique mycobacterial cell wall

Cell walls of mycobacteria (Figure 1.4.2.1) are more complex than those of other gram-positive organisms (Slayers and Whitt, 1995). The inner cytoplasmic membrane is overlayed with a thick layer of peptidoglycans (PG), a polymer of N-acetylglucosamine and N-acetylmuramic acid (Crick et al., 2001; Reichman and Hershfield, 1993) and there is no outer membrane. The muramic end of the peptidoglycan layer is covalently linked to polysaccharides, known as arabinogalactan (AG) via a phosphoryl-N-acetylglucosamisoyl-rhamnosyl (P-GlcNAc-Rha) linkage unit.
Figure 1.4.2.1: The ultra-structure of the *M. tuberculosis* cell wall. The outermost layer consists of a polysaccharide-rich capsule. A mixture of complex phospholipids (orange) and trehalose mono- and dmycolates (TMM and TDM, green) are intercalating the mycolic acids (m, Black). The mycolic acids are esterified to the terminal arabinofuranosyl residues (blue) of arabinogalactan (AG). The galactan (yellow) residues of arabinogalactan are in turn linked to peptidoglycans (PG, burgundy) via phosphoryl-N-acetylglicosaminoisyl-rhamnosyl (P-GlcNAc-Rha) linkage units to form the mycolyl-arabinogalactan-peptidoglycan complex (mAGP) that overlays the plasma membrane (modified from Dover *et al.*, 2004).

The termini of AG are esterified to high molecular weight mycolic acids (Murray *et al.*, 2002) of an α-alkyl and β-hydroxy form (Crick *et al.*, 2001). The resulting mycolyl-arabinogalactan-peptidoglycan (mAGP) complex is termed the cell wall core (Brennan, 2003). The outer layer of this core is composed of a variety of immunologically important peptides, proteins, peptidoglycolipids and a polysaccharide-rich capsule, fueling the promise for new drug targets (Kremer and Besra, 2002). These surface components also include sulfated acyl trehaloses and trihalose dmycolates or “cord factor” that might be involved in virulence (Reichman and Hershfield, 1993).
This complex structure of lipids forms a highly impermeable barrier to antibiotics. The synthetic pathway of this structure has been targeted for antitubercular intervention. Various successful antimicrobial and antifungal drugs, such as isoniazid (INH), triclosan, thiolactomycin and cerulenin target this pathway (Heath et al., 2002). Isoniazid is used below as an example to illustrate the idea of targeting proteins involved in lipid synthesis. In addition, isoniazid is related to an enzyme of interest described later in this chapter.

Isoniazid is a front-line anti-TB drug; the enzymes that are targeted by the active form of INH, have been identified. Isoniazid is a prodrug and needs to be activated inside the cell by the mycobacterial catalase-peroxidase enzyme, KatG (Pierattelli et al., 2004). A 3D structure of the catalase-peroxidase of M. tuberculosis showing a hydrophobic pocket into which INH is likely to bind has been elucidated (Bertrand, et al., 2004). The structure of the activated form of INH is believed to be a very reactive acyl radical (Pierattelli et al., 2004). In early experiments of M. tuberculosis killing by INH; cell death correlated with the inhibition of mycolic acid biosynthesis (Takayama et al., 1972). There is still no consensus as to which enzyme is the primary target of the activated form of INH. It is believed that two enzymes that play a key role in fatty acid biosynthesis, the anoyl acyl carrier protein (ACP) reductase, InhA and the β-ketoacyl-ACP synthase, KasA (Slayden et al., 2000; Mdluli et al., 1998) are the primary targets of the activated form of INH [Figure 1.4.2.2].
Figure 1.4.2.2: Mycobacterial fatty acid biosynthetic pathway identified through use of isoniazid. There is evidence suggesting that isoniazid primarily targets the \textit{inhA} and/or \textit{kasA} gene products (Barry III \textit{et al.}, 1998).

It has been shown that overexpression of \textit{InhA} results in high levels of resistance to INH, suggesting that this enzyme is involved in modulation of mycobacterial sensitivity to INH. However, it has not been shown yet in 3D by X-ray crystallography or even in 2D NMR analysis that INH interacts with the enoyl-ACP reductase, making it difficult to conclude that this enzyme is indeed
the target (Kremer et al., 2003). It has been shown experimentally that
isoniazid does not affect the InhA alone but also the KasA, in a complex
interrelated manner (Slayden et al., 2000). The importance of KasA on INH
antimycobacterial action is also questionable. Kremer et al. (2003) and Larsen
et al. (2002) in collaboration have shown that overexpression of KasA does not
result in INH resistance, suggesting that this enzyme is not involved in
modulation of mycobacterial sensitivity to INH.

However, if one considers that upon INH treatment, there is an accumulation
of saturated fatty acids, not unsaturated fatty acids, a consequence of
inhibiting a ketoacyl synthase (Takayama et al., 1975) then it would be more
plausible to say that KasA is the primary target. Whole genome microarray
analysis has shown that both these enzymes are upregulated significantly,
upon drug treatment (Wilson et al., 1999). A clear identification of the structure
of the activated form of INH and the 3D X-ray data of it, bound to its primary
target or targets, will help in the rational design of potent drugs with improved
pharmacodynamics to combat tuberculosis.

1.4.3. Novel drug targets of the cell wall of *M. tuberculosis*

The genome of *M. tuberculosis* encodes about 250 enzymes involved in lipid
metabolism in comparison with *Escherichia coli* genome that encodes only
about 50 of such enzymes (Cole et al., 1998). This suggests that the
synthesis of fatty acids in *M. tuberculosis* has a profound significance on the
survival and perhaps virulence of this organism. The cell wall of *M.*
tuberculosis, a focal point of interaction between the host and the bacterium, is made up of fatty acids amounting to 60% of whole dry weight (Daffe and Draper, 1998).

The biosynthetic pathway of these fatty acids that include various free surface lipids and polysaccharides such as the lipoarabinomannan (LAM), phosphatidylinositol mannoside (PIM), phtiocerol dimycocerosates (DIM), trehalose mono- and dimycolates (cord factor) and glycopeptidolipids needs to be further investigated so as to identify enzymes that could be potential drug targets. A mannosyltransferase of mycobacteria, PimF, involved in the biosynthesis of LAM (Alexander et al., 2004) has been identified. Studies to investigate the endogenous role of PimF and its importance on the survival or virulence of the M. tuberculosis are necessary. Mutations in the genes of Mycobacterium smegmatis encoding proteins involved in the biosynthesis of C-type glycopeptidolipids, result in the loss of the permeability barrier (Etienne et al., 2002). These findings could have implications for effective antibiotic entry into the M. tuberculosis cell.

Enzymes involved in the biosynthetic pathway of the mAGP complex (Figure 1.4.3.1) could be attractive tools for the identification of chemicals that could specifically and selectively inhibit this process in M. tuberculosis.
Figure 1.4.3.1: Biosynthesis of mAGP. Arabinogalactan and its ligation to peptidoglycan then mycolic acids. The step involved in the ligation of peptidoglycan to the arabinogalactan subunit is targeted for therapeutic intervention (Yagi et al., 2003) [figure from Crick et al., 2001]

It has been shown that inhibition of the mycobacterial rhamnosyltransferase, known as WbbL or Rv3265c (Figure 1.4.3.1), which is needed for the formation of the AG-P linker, leads to the irreversible loss of viability of the cells (Mills et al., 2004) and therefore makes for an attractive target. The last
enzymatic step in the biosynthesis of the peptidoglycan-arabinogalactan (PG-AG), which is the ligation of the newly formed AG to the PG (Figure 1.4.3.1), has also been identified as an attractive target for anti-tubercular drug discovery (Yagi et al., 2003). Other enzymes that are involved in the biosynthetic pathway of the mAGP complex have been identified (Table 1.4.3.1).

<table>
<thead>
<tr>
<th>Name</th>
<th>Function</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>DXS</td>
<td>Rv2682c</td>
<td>1-deoxy-D-xylulose 5-phosphate synthase</td>
</tr>
<tr>
<td></td>
<td>Rv1086</td>
<td>E,E-farnesyl diphosphate synthase</td>
</tr>
<tr>
<td></td>
<td>Rv2361c</td>
<td>Decaprenyl diphosphate synthase</td>
</tr>
<tr>
<td>RmlA</td>
<td>Rv0334</td>
<td>α-D-glucose-1-phosphate thymidyl transferase</td>
</tr>
<tr>
<td>RmlB</td>
<td>Rv3464</td>
<td>dTDP-D-glucose 4,6-dehydratase</td>
</tr>
<tr>
<td>RmlC</td>
<td>Rv3465</td>
<td>dTDP-4-keto-6-deoxy-D-glucose 3,5 epimerase</td>
</tr>
<tr>
<td>RmlD</td>
<td>Rv3266c</td>
<td>dTDP-ribofuranose synthetase</td>
</tr>
<tr>
<td>Gif</td>
<td>Rv3809c</td>
<td>UDP-Galp mutase</td>
</tr>
<tr>
<td>GifT</td>
<td>Rv3808c</td>
<td>UDP-Galf transferase</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MurA</td>
<td>Rv1315</td>
<td>Phosphoenolpyruvate:UDP-GlcNAc enolpyruvate MurC transferase</td>
</tr>
<tr>
<td>MurC</td>
<td>Rv2152c</td>
<td>L-alanine ligase</td>
</tr>
<tr>
<td>MurD</td>
<td>Rv2155c</td>
<td>D-glutamate ligase</td>
</tr>
</tbody>
</table>

(Crick et al., 2001)

The endogenous roles of all these enzymes and others that have not yet been identified need to be validated. Once the significance of these enzymes on the survival or virulence of *M. tuberculosis* has been established, they could be
incorporated into high-throughput screening (HTS) methods in order to identify their ligands and inhibitors, which could have significance for novel anti-tubercular drug discovery.

The search for an effective cure and vaccine against *M. tuberculosis* started soon after its discovery. It took almost seven decades before the most effective drug against tuberculosis was discovered: isoniazid (Fox, 1952; Bernstein *et al.*, 1952). Used in combination with other drugs, isoniazid became the backbone for the most effective tuberculosis chemotherapy. However, it was realized that the major route of isoniazid inactivation in humans was through acetylation (Evans *et al.*, 1960; Ellard and Gammon, 1976). The enzyme that is responsible for this biotransformation was found to be the polymorphic arylamine *N*-acyetyltransferase (NAT), which has recently been shown to be important in mycolic acid biosynthesis in mycobacteria (Bhakta *et al.*, 2004).

1.5. Arylamine *N*-acetyltransferases

1.5.1. Background on NATs

Arylamine *N*-acetyltransferases (NATs) are drug-metabolizing enzymes responsible for the biotransformation of various arylamines and heterocyclic amines, including carcinogens by transfer of an acetyl moiety from acetyl-CoA in a double ping-pong mechanism (Riddle *et al.*, 1971; Butcher *et al.*, 2002); meaning that the enzyme binds the acetyl-CoA first before it transfers the acetyl group to the substrate. NAT homologs have been found in both
eukaryotes (Ohtomi et al., 1989; Grant et al., 1989; Short et al., 1988; Andres et al., 1988; Ohsako et al., 1988; Deguchi et al., 1988) and prokaryotes (Hasmann et al., 1986; Dolomenie et al., 2001) including M. tuberculosis (Payton et al., 1999). There are two polymorphic isozymes of NAT in humans, NAT1 and NAT2 (Blum et al., 1990). NAT2 is responsible for interindividual variation in INH susceptibility of tuberculosis patients, having alleles conferring slow and fast acetylator status that differ by single nucleotide polymorphisms within its coding region (Grant et al., 1989).

The active site of all NATs consists of a hydrophobic pocket that contains a catalytic triad of 3 amino acids, a cysteine, a histidine and an aspartate (Sinclair et al., 2000) such as those of serine proteases. The cysteine has been shown to be crucial for the ping-pong catalytic activity of the NAT; in addition, glycine\(^{126}\) has been shown to be equally as important in the binding to acetyl-CoA in the NATs from Salmonella typhimurium and M. smegmatis [MSNAT] (Sandy et al., 2005).

The NAT in M. tuberculosis might be involved in modulating the sensitivity of the tubercol bacilli to isoniazid (Payton et al., 1999). NAT from mycobacteria acetylates isoniazid and induce resistance to this drug when over-expressed in E. coli (Payton et al., 1999). Recently, it has also been shown by a combination of 1D and 2D TLC analysis that deleting (in-frame) the nat gene in Mycobacterium bovis BCG, a model organism for M. tuberculosis, results in the absence of various cell wall lipids including the mycolic acids (Bhakta et al., 2004; figure 1.5.1.1 and 1.5.1.2).
Bhakta et al. (2004), by using Transmission Electron Microscopy and Scanning Electron Microscopy, showed that *M. bovis* BCG Δnat had cell morphology different from that of the wild type and that the trihalose dimycolate (TDM) or cord factor was also missing [Figure 1.5.1.3].
Even though this mutation was not lethal to the mycobacterium, it was clear that the growth of *M. bovis* BCG Δnat was affected, as the lag phase was extended; its susceptibility to antibiotics such as gentamicin, which have little activity against mycobacteria, was increased and that the intracellular killing of *M. bovis* BCG Δnat inside the macrophages was also increased (Bhakta *et al.*, 2004). This evidence has implications for anti-tubercular intervention; if NAT could be knocked out, chemically, in the bacilli; it would render the bacilli more susceptible to isoniazid (this prodrug will be free to be activated by KatG) and to other antibiotics (such as gentamicin). This would be the same notion as that of using amoxicillin/clavulanic acid for the treatment of amoxicillin resistant beta-lactamase producing bacteria (White *et al.*, 2004; File *et al.*, 2004) or using verapamil for resistance reversal in chloriquone resistant *Plasmodium falciparum* (Martiney *et al.*, 1995).

NAT is indirectly involved in modulation of INH activation by KatG and is directly involved in mycolic acid synthesis (Figure 1.5.1.4).
Figure 1.5.1.4: Inactivation of INH by NAT. Involvement of arylamine N-acetyltransferase in mycolic acid biosynthesis and also in the process of INH inactivation through acetylation using acetyl-CoA (Sim, 2004).

1.5.2. Targeting the bacterial NAT

Bacterial NATs just like human NAT2 cannot acetylate p-aminobenzoic acid (p-ABA), which is a specific substrate for the human NAT1 isozyme (Brooke et al., 2003a; Delomenie et al., 2001). It is important for bacterial NATs not to metabolize p-ABA as it is an intermediate in folate metabolism in bacteria and plants, a pathway that is crucial for bacterial survival but does not occur in eukaryotes. The sulphonamide, sulfamethazine (Similar to p-ABA an inhibitor of folate synthesis) and isoniazid are specific substrates for the NAT2 isozyme and are readily acetylated by bacterial NATs (Brooke et al., 2003a; Delomenie et al., 2001).

This suggests that bacterial NATs are catalytically similar to human NAT2. However, there is only 30% amino acid sequence identity between the
mycobacterial NATs and the human NAT2 (Payton et al., 1999). Moreover, some compounds such as 2-aminoflourene (2-AF) are substrates for both human NAT enzymes and are also significantly acetylated by bacterial NATs (Brooke et al., 2003a; Delomenie et al., 2001). Another NAT1 substrate, 5-aminocylisyl acid (5-ASA), is a good substrate for bacterial NATs. There seem to be different but overlapping substrate specificities between eukaryotic and prokaryotic NATs. The C-terminus of NATs has been implicated to have a controlling role on substrate specificity (Mushtaq et al., 2002) and the lack of sequence identity at the C-terminus “reflects a difference in endogenous roles of the different NAT homologues” (Sandy et al., 2005) [Figure 1.5.2.1] suggesting that specific inhibitors of bacterial NATs are likely to be selective.

![Figure 1.5.2.1](image)

The obvious difference between the eukaryotic NATs and the prokaryotic NATs is that the eukaryotic NATs have a conserved interdomain loop (Figure 1.5.2.2), which is absent in prokaryotic NATs (Kawamura et al., 2005). Specific
and selective inhibitors of the mycobacterial NATs are sought for anti-
tubercular intervention.

![Figure 1.5.2.2: The inter-domain loop of eukaryotic NATs. NAT from M.
smegmatis (A) does not have an extra loop (*), which is clearly visible in the
hamster NAT2 (B), human NAT1 (C) and human NAT2 (D) [Kawamura et
al., 2005].](image)

Selective inhibitors of bacterial NATs that are natural compounds or have
been derived from natural compounds would be attractive tuberculosis drugs.
The next section (section 1.6) gives an overview of the drug developmental
process.

**1.6. Drug discovery**

**1.6.1. Drug Discovery process**

The traditional drug discovery process starts with a particular disease process,
of which patho-physiology and molecular basis needs to be thoroughly
investigated (Figure 1.6.1.1). This would help identify and validate important targets (Tornell and Snaith, 2002) that can be used in High Throughput Screening (HTS) methods for the identification of hit compounds. A hit compound is defined as an agent that exhibits minor unspecified effects on the desired target (Carr and Jhoti, 2002).

![Figure 1.6.1.1: An Overview of the drug developmental process showing the 5 main activities (target identification and validation, lead generation, lead optimization, early clinical development and late clinical development) that overlap (Wess et al., 2001).](image)

The hit compound is developed into a lead agent by fulfilling the following additional criteria to the established *in vitro* efficacy: a clear and positive demonstration of its *in vivo* efficacy, establishment of structure-activity relations, elucidation of the mechanism of action, understanding of its pharmacokinetics, determination of its selectivity; its chemico-physical properties together with establishment of its synthetic route to help modify during the subsequent lead optimization stage.

Agents of low molecular weight are favored for two major reasons: firstly, most of the currently successful drugs in the World Drug Index have an average molecular weight in the low 300s (Oprea, 2001). Secondly, the lead optimization process is estimated to add at least an additional molecular weight of about 80 (Oprea et al., 2001). However, there is no definite formula (Wess
et al., 2001), which the agent properties must obey to work best in vivo during a disease process in the host, except for the Lipinski rule of 5 (Lipinski et al., 2001). This rule suggests that the molecular weight of a compound should not be more than 500, should not have more than 5 H-bond donors, should not have more than 10 H-bond acceptors and the lipophilicity measure (LogP) must be less than 5; the compound would then diffuse well and be absorbed readily.

Once the criteria of a lead compound have been met, the newly recognized lead is optimized for parameters such as pharmacokinetics and selectivity. Additional parameters must be fulfilled; these include an acceptable Cyp450 profile, selectivity in a wide range of systems (such as other enzymes or microorganisms), solubility and clean toxicity profile. Only then will this compound be a pre-clinical candidate. The pre-clinical candidate will go into various early clinical investigations primarily in primates and subsequently in a few healthy human volunteers before it can be considered for the late phase II and III clinical investigations on a few patients and then on a large large number of patients respectively. At this stage of drug development various ethical issues need to be considered such as whether the agent under investigation will be compared to placebo for instance. The question being is it morally correct to give something that does not work to patients suffering from the disease in the name of medical advancement, etc. On average the drug development process takes at least 12 years and the phase III clinical stage is most extensive and expensive (http://www.FDAreview.org). Only when it is shown to be effective and safe enough to be used widely, will it be taken further for registration, production, marketing and distribution as a drug. Even
when a drug is already in clinical use, its short term and long-term effects should be monitored and reported and if anything undesirable about it manifests then it should be withdrawn from the market.

There are four approaches used to discover new hit compounds, namely the empirical screening method, the rational design method, drug metabolism studies and clinical observations. I shall only discuss briefly the rational design method; the empirical screening method as a starting point to rational design will be presented in great detail.

1.6.2. Identifying hits using rational design

The understanding of the molecular processes of bacteria or fungi that lead to disease in the host and the definition of the targets, proteins or receptors, is key for rational drug design (Lindsay, 2003). The 3D structure of the target protein, for example the NAT, showing the molecular structure and nature of the active site needs to be established using X-ray crystallography or NMR (Carr and Jhoti, 2002). Co-crystallization of the target protein (NAT) and the hit compound (inhibitor) should be done in order to understand the active site interactions between the hit compound and the enzyme. Once this information is known, better ligands can be modeled using application specific computer software (Figure 1.6.2.1, an example) before they are physically created in the test tube (van Dongen et al., 2002). This method can be applied in the development of anti-tubercular hit compounds of plant origin that are NAT ligands or inhibitors.
Computer-based Rational drug design alone without the supporting data from whole cell bioassays could lead to false positive and low success rates. Rational design based on natural products that are ligands or inhibitors might be more attractive as natural products are more physiologically acceptable than synthetically derived agents (Brohm et al., 2002, Tan et al., 1998). In fact, a commercially available library of 4000 pure non-redundant natural products has been created by Aventis and AnalytiCon Discovery, of which 39% are novel compounds (Bindseil et al., 2001).

**Figure 1.6.2.1:** Target-based drug design. A designed better ligand (green) from data of a low affinity hit (black) compound [van Dongen et al., 2002]. Anti-tubercular hit compounds can be enhanced by modifying their interactions with the bacterial NAT.

### 1.6.3. Hits derived from traditional screens

The traditional screening method involves screening of various different chemical compounds from synthetic chemical libraries, historical compound libraries, combinatorial libraries and natural products (Harvey, 1999) using various in vitro HTS bioassays. The diversity provided by natural products in particular increases the probability of finding a hit compound (Terret et al.,
1995). Though the findings and formulas of Lipinski et al. (2001) and Oprea et al. (2001) of what constitutes a successful drug are biased towards natural products, 61% of the 877 small-molecule new chemical entities introduced as drugs worldwide during 1981-2002 can be traced to or have been inspired by natural products (Newman et al., 2003).

In addition, the role that natural products have played in the discovery of drugs against infectious diseases and cancer cannot be underestimated. Between 1983 and 1994, 63% and 60% of approved anti-infective and anti-cancer drugs, respectively were of natural origin (Cragg et al., 1997). There is a wide range of exploitable natural products that can be investigated for the discovery of more novel chemical entities and they are listed in table 1.6.3.1 (Harvey, 2000).

**Table 1.6.3.1: Natural products from which drugs can be sourced**

<table>
<thead>
<tr>
<th>Strategy</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untapped geographical sources</td>
<td>Plant-based diversity has been</td>
<td>Concerns sustainability of natural collecting</td>
</tr>
<tr>
<td></td>
<td>historically successful</td>
<td></td>
</tr>
<tr>
<td>Preparation of libraries of</td>
<td>More compatible than mixtures for</td>
<td>Cost of production</td>
</tr>
<tr>
<td>isolated natural compounds</td>
<td>HTS</td>
<td></td>
</tr>
<tr>
<td>Marine resources</td>
<td>Unusual chemistry</td>
<td>Identification of source organisms. Recollection</td>
</tr>
<tr>
<td></td>
<td></td>
<td>difficult on large scale</td>
</tr>
<tr>
<td>Insects</td>
<td>Little studied</td>
<td>Scale-up potentially difficult</td>
</tr>
<tr>
<td>Plant tissue culture</td>
<td>Control over genetic pathways</td>
<td>Concerns over access to genetic material sourced</td>
</tr>
<tr>
<td></td>
<td></td>
<td>from developing countries</td>
</tr>
<tr>
<td>Combinatorial genetics</td>
<td>Convenient production using</td>
<td>Restricted range of structures</td>
</tr>
<tr>
<td></td>
<td>fermentor technology</td>
<td></td>
</tr>
</tbody>
</table>

(Harvey, DTT vol.5, no. 7 July 2000)
There is a promise that natural products will yet again provide us with the chemical diversity (Bindseil et al., 2001) that we could exploit for the reversal or abolition of problematic disease processes. There are two main methods in which natural products containing therapeutic agents can be identified: the random screening method and the ethno-medicinal approach. When discussing mainly the ethno-medicinal approach, as opposed to random screening, special reference will be paid to the discovery of therapeutic compounds from terrestrial plants.

1.6.4. The Ethno-medicinal approach to drug discovery

The ethno-medicinal approach is more successful than random screening because plants identified through this process have been tried and tested for the treatment of diseases for centuries by local peoples of the regions in which these plants exist. Thus there is a higher probability that a hit compound will be produced when the ethno-medicinal method is used (Slish et al., 1999). There are about 250,000 species of terrestrial plants in the world (Pezzuto, 1997) and only a small proportion of these have been investigated. Almost 10% of the world’s higher plants (van Wyk et al., 1997) grow in South Africa and these plants are valuable resources to screen for new useful chemicals. Chapter 2 of this thesis, in a narrative manner, describes how we used the ethno-medicinal process to search for medicinal plants of South Africa from which anti-tubercular agents can be sourced.
1.6.5. Anti-tubercular plant-derived agents

Natural products have yielded many different drugs in the past for the treatment of diseases; therefore, the fact that they contain therapeutic agents is not new. However, the efficacy, spectrum of activity and safety of most of these products have not been fully investigated. More than 30 000 plant species are found in South Africa (van Wyk et al., 1997) representing about 10% of the world’s higher plants. South African plants are a valuable resource from which anti-tubercular compounds could be isolated.

Several drugs on the market, such as the cancer drug, taxol, and the malaria drug artemisinin have been isolated from plants. There are several reports of agents that exhibit potent anti-tubercular activity. A lactone derivative of oleic acid, (−)-Z-9-octadecene-4-olide isolated from the stem bark of Micromelum hirsutum, has been shown to possess potent in vitro anti-tubercular activity against H37Rv (Ma et al., 2005). Two anti-tubercular glycosides from an organic soluble extract of the leaves of a native North American prairie plant Ipomoea leptophylla, showed in vitro activity against Mycobacterium tuberculosis (Barnes et al., 2004). Various plant derived quinolinedes (Jain et al, 2003) and terpenoids (Cantrell et al., 2001) have been shown to possess anti-tubercular action and we hope that South African plants would provide novel anti-tubercular compounds, as they have never been explored for drug discovery in the past.
1.7. Scope of this project

The increase in levels of drug resistance and HIV infection worldwide is a problem for TB control. Novel drugs that are potent, fast acting and long lasting are sought. Drugs that target novel enzymes in the tubercle bacilli would circumvent cross-resistance with existing drugs. A novel target for anti-tubercular intervention, the arylamine N-acetyltransferase, has been identified (Bhakta et al., 2004). The aim of the work presented in this thesis project is to select certain medicinal plants of South Africa, and screen them for antimycobacterial activity. Plant extracts showing anti-mycobacterial activity will subsequently be tested against NAT to identify inhibitors of this enzyme. Agents that possess these activities will be purified and characterized in order to identify a possible anti-tubercular compound that acts by specifically targeting the arylamine N-acetyltransferase. The thesis describes the extent to which the following aims have been met.
Aims

Specific Aims
The Aim of this study

The overall aim of this study is to identify anti-tubercular agents from medicinal plants of South Africa and determine their mode of action.

Specific aims

1. To conduct a field survey on plants used traditionally by healers of South Africa to treat symptoms associated with respiratory ailments such as those used for coughs, colds, influenza, and chest complaints.
2. To determine whether organic extracts of these plants affect the growth of mycobacteria.
3. To identify whether the extracts that inhibit the growth of mycobacteria also inhibit the activity of the Arylamine N-acetyltransferase.

Following on from the results of specific aims 1-3, the subsequent aims were established

4. To compare the purification of NAT inhibitory activity and the inhibitory activity against mycobacteria, to determine whether these two activities co-purify.
5. To determine the effect of purified fractions on the morphology of mycobacterial cells.
6. To elucidate the chemical structures of purified compounds isolated from those fractions that posses either or both of the activities defined in 3.
Chapter 2

Ethno-medicinal investigations for hit discovery
2.1. An Overview of Traditional Medicine in South Africa

Traditional medicine (TRAMED) is described as a set of "health practices, approaches, knowledge and beliefs incorporating plant, animal and mineral based medicines, spiritual therapies, manual techniques and exercises, applied singularly or in combination to treat, diagnose and prevent illnesses or maintain well-being" (WHO Traditional Medicines Strategy, 2002-2005). In South Africa it is estimated that there are 200 000 traditional healers who practice traditional medicine (A Special Report of the South African Medical Research Council, the Department of Health and the Centre for Science and Industrial Research: A Model for South Africa – The National Reference Centre for African Traditional Medicines.

The ratio of health care provider to population is 1:500 for traditional healers compared to 1:1400 for western trained doctors (Abdool et al., 1994). Almost 70% of black South Africans consult traditional healers for their primary health care needs (van Wyk, 1997), especially in the rural areas where hospitals might be kilometers away. The WHO estimates that in general, up to 80% of Africans use traditional medicine (WHO Traditional Medicines Strategy, 2002-2005). There is a clear indication that traditional healers are more accessible than western trained doctors in South Africa and that traditional medicine plays a vital role in the well-being of the nation.
2.1.1. Who are the healers?

Traditional healers possess the knowledge to practice traditional medicine. There are different types of traditional healers depending on ability, experience and training (Gelfand, 1956). In general, there are two main types of traditional healers, those who practice divination, the diviners and those who merely prescribe traditional medicine, the herbalists (Ndubani and Hojer, 1999). The diviner, or the igqirha as known by the isi-Xhosa speaking people of South Africa, would be an equivalent of the "spiritually based" practitioner while the herbalist or the inyanga as known by the isi-Zulu speaking people would be a "non-spiritual" practitioner (Tsey, 1997). The diviner is not only knowledgeable in the treatment of physical ailments but also in the illnesses of mind and soul (Tsey, 1997).

2.1.2. How do healers qualify?

The diviners are usually "called" to this profession in various ways. Some inherit the skill from their dead relations who were also healers when they were alive (Gelfand, 1956). Lack of consent to the "calling" could result in sickness or even madness, a process called ukuthwasa [to -thwasa means to be mad] (Hammond-Tooke, 1989). The only cure would be to consent to the "call" or accept the illness and go through an initiation process. To refuse could result in chronic madness, deformity or even death (Hammond-Tooke, 1989). [This process of consent is called "imvuma kufa" in isi-Xhosa. It is in actual fact in contradiction to the primary reason to consent in the first place, which is to be cured from the "ukuthwasa". To -vuma means to consent and -kufa means
death, meaning that one needs to "consent to death" in order to get cured, obviously this is not meant in the literal sense but it is an interesting contradiction, academically. One can go on by saying that if you refuse the "calling" and hence the "consent to death or to die", then you might die. I am not ridiculing this phenomenon here but am fascinated by it even with the objective eyes of an insider. It might be that the process of "consent to death" in the spiritual sense means that one needs to let go of his or her old self and be a new cleansed person]. An advanced traditional healer would be consulted, who would perform necessary rituals to cure the ukuthwasa and to cleanse the new initiate followed by an intensive training process to become a healer over a period of time.

The herbalist, on the other hand, is inspired and then decides to learn the practice by being apprenticed to an advanced healer for a period of time, or from a family member who is also a healer or has the knowledge of traditional medicine (Ndubani and Hojer, 1999), usually from a young age. The herbalist is more of a pharmacist than a diagnostian.

The most highly qualified healer would be the one who has been abducted for a period of time by people or the mermaids living under the waters, a process known as ukuthwetyulwa (Gelfand, 1956). ["Ukuthwetyulwa" is a Xhosa word that means to be abducted especially in the supernatural sense]. It is claimed that when he or she comes out of the water, he or she would be properly dressed in traditional healer garb symbolizing his or her high status, with all the
necessary knowledge of traditional medicine for the treatment of all illnesses. 
Gelfand (1956) claims that "He (or She) is able to cure all illnesses and his 
prophecies always eventuate".

2.1.3. How do healers diagnose disease?
It is said that the cause of disease dictates cure. According to Gelfand (1956), 
the diviners are termed the diagnosticians while the herbalists are termed the 
therapists. The diviners throw the bones to call on ancestors for enlightenment, 
to tell them through the bones, what the problem with the patient is and what 
medicines or rituals would help. During the diagnosis the healer would ask for 
as much information about the patient's life, social relationships and 
environment. The healer digs deep into the symptoms and any psychological 
phenomena trying to determine "why" the disease happened at the time it did. 
They also look at the patient's present and past activities focusing on the 
behavior that would most likely be the cause of conflict with others or 
ancestors. The diagnosis might take some time involving several visits until the 
bones "speak". If the bones do not speak, the patient might decide to consult 
another healer or the current healer might refer them to another or to a 
western trained doctor (as it happens nowadays). The sickness is usually 
assumed to be caused by spiritual activity or by witchcraft. The activity is 
usually identified as being due to the anger of the ancestors, which causes an 
imbalance in the patient's own or family life.
2.1.4. How do healers treat disease?

Healing, in African terms, as Hewson (1998) notes in the Annals of Internal Medicine, is balancing the imbalance caused by angry ancestors or witchcraft. The healing process that traditional healers use encompasses the person as a whole rather than treating the disease as a separate entity. The healer would treat the whole family, the household to ward away bad spirits, the livestock for reproduction and the people for well-being and wealth. This is the basis for the holistic approach the traditional healers apply in their healing methods. The traditional healer can distinguish between the illnesses of the mind, those of the body and those that have both elements. That is the reason why they mostly administer herbal preparations together with rituals. Gelfand (1956) explains by saying that the medicine is to cure the physical damage already endured by the body, while the ritual is for the psychological health. Medicines are prepared as decoctions, tinctures, inhalants, enemas, and infusions or as ointments. They are mostly taken orally but rectal, nasal and topical administration is common. The healer prepares these medicines, but sometimes, he or she could prescribe the patients to make the preparations themselves, as he or she sees fit.

In the following paragraphs the ethno-medicinal survey and findings will be described.
2.2 The Field Trip

An ethno-medicinal approach to drug discovery was applied; we visited three provinces of South Africa, the Northern Cape (NC), the Free state (FS) and the Eastern Cape (EC) [see map 2.2.1]. We used a non-structured qualitative research method. We did not know whom we were going to meet and we had no previous backgrounds of the traditional healers we knew by name. We were referred to most of them by people we met along the road. We introduced ourselves as students from the Department of Pharmacology of the University of Cape Town. We told them that as part of the vision of the South African Traditional Medicines Research Group (SATMERG), my project was concerned with identifying South African medicinal plants exhibiting anti-tubercular action, followed by the isolation and characterization of the specific molecules responsible for this activity. Using an “expanded diagnostic criteria” we asked for information on terrestrial plants that are being used traditionally by healers to treat coughs, cold, fever, influenza, pneumonia, respiratory ailments and tuberculosis. Where possible, we asked for samples of the plant parts they mentioned, some of the healers gave us the samples and some did not.
MAPS 2.2.1 B, C and D: A geographic representation of South Africa showing the route (black line) of the field trip and the towns visited (red dots). We visited three provinces of South Africa, the Northern Cape, the Free State and the Eastern Cape. Map A illustrates the whole region of South Africa, including all 9 of her provinces. Amongst the cities or villages visited, are those (red dots in maps B, C and D) where we met healers who gave us either information and/or samples of plant material they use in traditional medicine. These are, Van Rynscorp, Kubus, Upington, Kimberly, Bloemfontein, Queenstown, Mdantsane (East London), Keiskammahoek, and Uitenhage.
We recorded the information they gave us after verbal consent was established. We told the healers that the samples they gave us once they reached the laboratory would be subjected to biological testing. We assured them that they would be informed if any pharmaceutical lead with a potential for commercialization was discovered and that ways of compensation would be worked out amongst all the people involved. We told them that this process could take several years before we could be certain that a compound isolated from a plant had the potential for further clinical testing, and that we would communicate with them accordingly. This was done so that no false hopes were instilled and also to allow the necessary ease for research to take place. This was in line with the recommendations of the Convention on Biological Diversity with respect to transparency in communication and benefit sharing.

The questions we posed and the way we asked them varied from one healer to another and the interviews were conversational. However, they were formal, as we had to introduce a level of professionalism. We spoke to many different individuals from different backgrounds and, in an anthropological sense; an understanding was required as was an appreciation and acknowledgment of the healers' knowledge, opinions and beliefs. Without this crucial set of mind, trust could never be attained and the interviews might have been jeopardized. It is important at this point to give a general view of traditional healing in South Africa.
2.2.1. Traditional healing in the Northern Cape

Most of the people we spoke to in the Northern Cape were self-medicating and were not necessarily practicing as traditional healers. Indigenous healing therefore seemed to be a family or a community based non-commercial entity. The people of this province were predominantly of Dutch or Khoisan descent and spoke Afrikaans, Nama or both. In a small village of Kubus we arrived at a hall where the community members were meeting regarding a pending court case related to ways of compensation for being forcefully removed from their land, by diamonds mining giants who operated in that land. We had a chance to speak to them after this meeting and a translator was present, as they could not speak English. Amongst them was an elderly woman who seemed to know most about the herbs they use to treat illnesses such as coughs, colds, influenza, and sores. We did not manage to get samples here as we decided not to ask for them given the nature of the meeting they just had. It would be advisable for plants used by the Nama people to be investigated, hopefully by somebody who knows the culture and who is perhaps an insider.

Those who have adopted the Rastafarian way of life, which has a characteristic usage of substances from herbal sources, were selling medicinal plants on the streets. It was such an overwhelming experience when we met Rastaman Benjamin (not his real name) in his house in a village of VanRhynsdorp. When he arrived home with a sack full of various medicinal herbs that he had just collected, it was time for the family to meet and engage in the cannabis smoking ritual that has religious importance to the Rastafarian people. We
participated in the ritual, however, without overindulging and compromising our state of mind. This created an environment of trust and allowed him to be free to share what he knew about medicinal plants. From the onset of our expedition, we realized that there were problems between the national conservation authorities and those who harvested and sold plants for medicinal purpose. We did not dwell on that issue, as our mission was to learn about the medicinal usage of the plants in this region.

2.2.2. Traditional healing in the Free State and Eastern Cape

In contrast to the Northern Cape, traditional healers in the Free State and the Eastern Cape are common and traditional healing is commercialized. The healers we spoke to were of Sesotho and isi-Xhosa backgrounds. They usually practiced at their homes and some of them (The herbalists) in their herbal stores. It was in these two provinces that we had our first glimpse of the healer's perception of tuberculosis; what causes it, how they diagnose and treat it. There was a general feeling that academic institutions were exploiting traditional healers in the name of scientific advancement and some of them even mentioned names of people who they encountered before. We did not ask or get any sample from the healers who had these views; we sympathized with them and promised that in the future processes would be carried out according to the United Nations resolutions of the Convention on Biological Diversity.
2.2.2.1. The general perception of tuberculosis disease process in the EC and FS

Tuberculosis is seen as a poison that dirties the blood. Usually this poison is introduced by witchcraft through drinks, food and other inexplicable means. The poison infests the lungs and makes holes in them and hence haemoptysis (coughing up blood from the respiratory tract; usually indicating a severe infection of the bronchi or lungs). There seems to be an understanding that once the poison has caused disease, transmission of the disease can occur. One healer told us that during the umqombothi (African traditional beer) drinking ceremony the pot is passed around for everyone to drink, in turns. If one of the participants is infected and the other has a compromised immune system, then transmission can occur. There is also a belief that the poison can be harmless for years inside the body until it is activated by factors that suppress ones bodily functions and its crucial defense mechanisms such as alcohol abuse (Kovacs and Jerrells, 2004) and smoking.

Human Immunodeficiency Virus (HIV) infection also increases the risk of TB infection (Sonnenberg et al., 2005). It is interesting to note that the notion of activation of the dormant poison is analogous to the phenomenon of reactivation of latent or dormant tuberculosis (Parrish et al., 1998). Parish et al. (1998) illustrate the scale of latent TB infection that might be activated sometime in life, showing that HIV infection exacerbates the reactivation rate, which becomes a regular yearly process (Figure 2.2.2.1.1).
Figure 2.2.2.1.1: HIV infection that defects the immune system increase the risk of latent TB reactivation. A similar phenomenon in traditional medicine involves alcohol abuse and smoking, factors known to suppress the immune system. These factors activate the poison in the lungs of an individual infected with TB.

2.2.2.2. Diagnosis of tuberculosis in the EC and FS

One healer from Kimberly claims to give his patients an emetic potion made from the ground tuber of *Dioscorea sylvatica*. This healer would determine the cause of the disease and especially confirming his suspicion of TB by assessing the consistency of the resulting vomit. Most healers we spoke to mentioned that besides the throwing of bones they looked at symptoms such as coughing blood, wasting, loss of appetite and fever as an indication of TB infection.
2.2.2.3. A referral system between nurses and traditional doctors

We found a “pseudo” hospital in Uitenhage, near Port Elizabeth that had a resident medical doctor, a resident spiritual healer and a resident isangoma or igqirha (The word “pseudo” is used with caution; it means “not genuine but having the appearance of” and hopefully does not underestimate this wonderful initiative). Tuberculosis or any other illness would be confirmed by diagnosis at the local hospital. After the patient had finished treatment, the success of such treatment would be confirmed at the local hospital with the help of the doctor who facilitates this referral system. “Mama (Mother) Keiti” in Keiskammahoek area of King William’s Town and “Tata (Father) Hlathi” in Mdantsane Township, East London, both throw bones for diagnosis and would also refer patients to the hospital and vice versa with the help of a senior nurse. We saw a photograph album full of pictures of patients “Tata Hlathi” had helped within this referral system. “Mama Keiti” would be called by the hospital to come and help with cases that the western trained doctors had difficulty dealing with.

In a study conducted in the district of Hlabisa in Kwa-Zulu Natal province, the traditional healers were asked to assist in the TB DOTS programme that is community based (community-based DOTS programme) and all patients that the traditional healers observed during their TB treatment did finish treatment, were cured and happy with the service they received. Traditional healers could play a bigger role in the South African health care system.
2.2.2.4. Treatment of tuberculosis in the EC and FS

The healers could either use one plant or a combination of plants; mixtures are prepared mostly in boiling water to allow the plant material to seep in it. The debris might be strained and discarded; the liquid that contains the active metabolites would be drunk, sometimes on an empty stomach and sometimes before eating. Where a mixture of plants have been extracted, each has a specific purpose, such as cleansing the blood, killing the germ or destroying the poison that causes the illness and strengthening the blood so that the disease does not come back or no other diseases might attack.

In Queenstown, a herbalist, Traditional Doctor (T. Dr.) George Rhesha gave us a sample of 3 barks that he used to treat tuberculosis. They are: the bark of Withania somnifera (Wso), the bark of Warburgia salutaris (Wsa) and the bark of Pittosporum viridiflorum (Pv). He prescribed to us a method of preparation, which was to grind the 3 barks separately into fine powder and then add each powder using a teaspoon, in a ratio of 2:1:2 (Wso:Wsa:Pv) in 750ml of boiling water and allow it to extract overnight, then strain and drink ½ a cup (ca. 100ml) of this mixture three times everyday on an empty stomach for 6 months. He said, with a very intriguing voice, “This one (Warburgia salutaris) is the strong one”, hence the small dose used in the mixture. This preparation and use of the mixture of these three plants was not generally used in South Africa for the treatment of tuberculosis. Within the scope and geographical scale of this exploration, this preparation and its use, uniquely belongs to T. Dr. George Rhesha.
2.3. Indigenous Knowledge Systems (IKS) and Intellectual Property (IP)

Throughout history there are examples of indigenous peoples using natural products for the treatment of diseases. In many instances, the indigenous people's knowledge of the healing properties of natural products has never been documented and could be lost completely if nothing is done to protect and preserve it. The implementation of the United Nations Convention on Biological Diversity (Appendix 1: United Nations Convention on Biological Diversity, J. Ethnopharm. 1996), which was agreed on at the 1992 Earth Summit in Rio de Janeiro and the legally binding International Labour Organization Convention 169 (Convention No. 169 concerning Indigenous and Tribal Peoples in Independent Countries, 1991) has set the ground for national governments to devise measures for the protection and preservation of indigenous knowledge and biodiversity. Governments should support research and development on useful natural products for the purposes of sustainability, innovation and commercial benefit of their nations.

It is important to explain the concept of "indigenousness"; the definition of indigenous peoples is not easy (Posey, 2002). Basically, indigenous relates to pre-colonial minorities who are culturally distinct and are determined to preserve their heritage and ancestral lands (UN ECOSOC, 1986). These people will usually instill a sense of communal solidarity and ownership of natural resources that exist in their environments (Del, 2000). In South Africa, it becomes difficult to apply this term, because black people who lived
here in the pre-colonial era and who practice traditional medicine were and remain the majority. In addition, the South African population is culturally diverse and is developing. Cultural “fusion” or “pollination” happened years ago and continues to flourish. There has been a lot of inter-cultural and recently, to some extent, inter-racial marriages. Due to urbanization, many black South Africans stay in cities; in addition to their local African way of life, they have adopted a western lifestyle. The knowledge of various cultural or pre-colonial lifestyle systems, such as agricultural practices, healthcare, judicial systems, just to name a few, have been pollinated by western values. One cannot easily and exclusively identify indigenousness in this context.

However, when it comes to the pre-colonial healthcare system, especially with respect to traditional healers and the way they qualify, diagnose and treat illnesses, one has to agree that the knowledge of traditional medicine is indeed culturally or perhaps spiritually distinct and should be preserved and protected. The knowledge required for the preparation of certain natural products, whether of plant or animal origin to treat disease should be an individual property only if no other healer or person uses the same recipe. However, the natural resources that are usually used in traditional medicine, such as plant or animal material are or perhaps should be of a communal or national ownership. The recognition of the individual ownership of cultural knowledge is one of the reasons why Intellectual Property Rights (IPR) laws are seen as inadequate; amongst other things, they are said to ignore
collective or communal rights to property, require an "invention" and stimulate commercialization (Posey, 2002).

2.3.1. Innovation from IKS and benefit sharing

I believe that innovation will lead to the advancement of nations in the developing world. Positive cultural ways of life that stimulate creativity will lead to innovation and inventions will make life easy and meaningful. Inventions emanating from local cultural backgrounds would give a sense of pride and wealth through commercialization [Assuming there is free trade amongst all nations and that all nations specialize in exploiting natural resources in their own regions for their own commercial gain].

A solution to the issues relating to IPR would be that governments of countries where exploitable natural resources exist, create an environment for local industries to exploit these resources for the development of medicines, food products, agrichemicals, cosmetics etcetera for local and international markets. They should take an initiative to invest in local research and development of natural resources into useful and patentable products. National or communal patents would make sure local people are protected from exploitation and would encourage them to cultivate and preserve their useful natural resources for sustainable development.

If the companies that exploit local knowledge are owned locally by governments and are selling developed products to local people at the lowest cost possible; the royalties and profits directed to community building
and support, then the people or the traditional healers would not feel exploited. They will be happy knowing that the profits will be ploughed back to making sure their children have the best education for an example. The healers could even be supplied with the designed medicinal products coming from their knowledge for them to use in their practices in a way to acknowledge, recognize, regulate and integrate (in parallel) their healing systems with the western ones.

This is the way I see benefit sharing as being completely fulfilled and resulting in local human development and poverty alleviation. Most importantly, the local people need to participate fully in the process of innovation through transparent consultations and should benefit readily (Iwu, 1996). Research into indigenous knowledge will help record and preserve this precious resource so that generations to come can benefit from it.

2.4. Governments acknowledge the role of traditional medicine

Governments have the duty to formulate national policies for proper utilization of traditional medicine and incorporate it into the national health care systems as endorsed by the WHO strategy on Traditional Medicines 2002-2005 (WHO, 2002). They must establish ways of regulating the testing, registration and hence use of natural products and create awareness about safe and effective traditional medicine therapies. They must also initiate ways of cultivating and conserving medicinal plants for instance to make sure that they are used in a
sustainable way. In South Africa, a traditional medicines bill has been approved by parliament (Traditional Healers Bill, 2003).

This bill will help register, regulate and control traditional health practitioners. The Biodiversity Act is also in place and will be made law soon. There is also a proposed bill on intellectual property and patents. We can say that inline with the South African National Research and innovation strategy; there is a promise that a patentable product of local plant origin emanating from local research could be available.

It would be more beneficial for South Africa not to combine the two healthcare systems but rather make sure they are regulated and co-exist equally in harmony and that there is a referral system between them. The traditional healers of South Africa can now write prescriptions and issue medical certificates. This is the starting point towards recognizing that traditional healing is an important healthcare system that has stood the test of time.

2.5. Plants mentioned during this field trip

In total a number of 18 different plant samples were collected during the field trip of which 15 have documented information on their traditional medicinal use, pharmacology and active metabolites (see Table 2.6.1a, b and c). A list of plants (Table 2.6.2a and b) [Vernacular names] mentioned mainly in the Northern Cape, which we did not get samples of, needs to be investigated further for any documented knowledge on their traditional medicinal uses, pharmacology and active metabolites before any biological activity is tested.
From this table (Table 2.6.2a and b) one can see that there is a great topical use of natural plant products for the treatment of sores in the Northern Cape. While you will find that, in the Eastern Cape for instance, sores would be treated internally using orally administered remedies.

The preparation of crude organic extracts of the plant parts we were supplied with and their biological testing is explained in chapter 3.
### Table 2.6.1a Potentially anti-tuberculosis plants collected using an ethno-medicinal approach to drug discovery

<table>
<thead>
<tr>
<th>Latin Name</th>
<th>Part Used</th>
<th>Medicinal Uses</th>
<th>Active Constituents</th>
<th>Known Pharmacology</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Canellaceae)</td>
<td></td>
<td>abdominal pains and constipation</td>
<td></td>
<td>Hemicytotoxic, Molluscidal</td>
<td></td>
</tr>
<tr>
<td>Isibhara (Xhosa)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Gunneraceae)</td>
<td></td>
<td>cancerous sores, stomach ailments, urinary stones, impotence, cold tonic</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Iphuzi (Xhosa)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Solanaceae)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ulavumiba (Zulu)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Apiaceae)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Iqwii (Xhosa)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Continued....
<table>
<thead>
<tr>
<th>Latin Name</th>
<th>Part Used</th>
<th>Medicinal uses</th>
<th>Active Constituents</th>
<th>Known Pharmacology</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hyacinthaceae</td>
<td></td>
<td></td>
<td>Homoisoflavones</td>
<td>Antispasmodic</td>
<td></td>
</tr>
<tr>
<td>Unmathungu (Xhosa)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pittosporaceae</td>
<td></td>
<td></td>
<td></td>
<td>Analgesic, Immunoregulatory</td>
<td></td>
</tr>
<tr>
<td>Umkhwenkwe (Xhosa)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dioscorea sylvestri</td>
<td>Tuber</td>
<td>Chest complaints, bronchiactasis, blood purifier, ritual emetic, swelling, rashes</td>
<td>Diosgenin</td>
<td>Contraceptive</td>
<td>van Wyk 2000, Kolmanson et al. 2002</td>
</tr>
<tr>
<td>Ugebeleweni (Zulu)</td>
<td></td>
<td></td>
<td></td>
<td>Anti-bacterial</td>
<td></td>
</tr>
<tr>
<td>Hypoxidaceae</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>African potato (English)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diospyros capensis</td>
<td>Leaves, twigs</td>
<td>Fever, colds, flu, hypertension, diarrhoea, cancers</td>
<td>Lactones</td>
<td>None yet</td>
<td>van Wyk et al. 1997</td>
</tr>
<tr>
<td>Astaraceae</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Koorsbos (Afrikaans)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Continued....
<table>
<thead>
<tr>
<th>Latin Name</th>
<th>Part used</th>
<th>Medicinal Uses</th>
<th>Active Constituents</th>
<th>Known Pharmacology</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ruta graveolens</td>
<td>Leaves, twigs</td>
<td>Fever, convulsions, fits, epilepsy, hysteria, respiratory ailments, heart disease, toothache, earache, ease child birth</td>
<td>Coumarins, furanocoumarine alkaloids, flavonoids</td>
<td>Analgesic</td>
<td>van Wyk et al: 1997</td>
</tr>
<tr>
<td>Rutaceae</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wynruit (Afrikaans)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Apiaceae</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Siekenroots (Afrikaans)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Publina natalensis</td>
<td>Root, fresh leaves</td>
<td>Wounds, burns, rashes, itching, ringworms, cracked lips, convulsions, herpes, venereal diseases, diabetes, rheumatism, urinary complaints, blood disorders</td>
<td>Anthraquinones, chrysophanol</td>
<td>Anti-bacterial</td>
<td>van Wyk et al: 1997</td>
</tr>
<tr>
<td>Asphodelaceae</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rooiwortel (Afrikaans)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Apiaceae</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vaarkoortje (Afrikaans)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
**Figure 2.6.2a:** Traditional preparations and administration of plants mentioned mainly in the Northern Cape (no samples obtained)

<table>
<thead>
<tr>
<th>Vernacular name</th>
<th>Part used/ preparation</th>
<th>Doses/administration /Uses</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kasteerboom</td>
<td>Seeds, crush into powder and braai/roast - use part oil: part water</td>
<td>1 tsp orally for Coughs. Rub on affected area for head, neck and chest pains</td>
</tr>
<tr>
<td></td>
<td>Leaves, crush fresh and use some as plaster</td>
<td>For wounds</td>
</tr>
<tr>
<td>Kigelia sp.</td>
<td>Pod, grind into fine powder</td>
<td>Put powder on tongue and down it with water for tuberculosis</td>
</tr>
<tr>
<td>Varkoor</td>
<td>Whole plant, dry and grind into fine powder</td>
<td>Put powder on sores. Used orally to clean blood</td>
</tr>
<tr>
<td>(Bakolie, Wynruit, varkoor, sick en troos)</td>
<td>Boil all plants in little water to generate an oil</td>
<td>Ointment for sores and as insect repellent</td>
</tr>
<tr>
<td>Artemisia afra</td>
<td>Boil plant is little water to generate an oil</td>
<td>Teaspoon when needed for different ailments</td>
</tr>
<tr>
<td>Snake leaf</td>
<td>Leaves, boil and drink a spoon in the morning</td>
<td>Emetic</td>
</tr>
<tr>
<td>Kankerbos, Marijuana, ½ snake leaves, ganiesbos and African potato</td>
<td>Unspecified</td>
<td>Eat raw for strength</td>
</tr>
<tr>
<td>Bloemblomsalie</td>
<td>Unspecified</td>
<td>Drink a glass 3X a day for belly cramps and for strengthening blood.</td>
</tr>
</tbody>
</table>

Continued.............
**Figure 2.6.2b:** Traditional preparations, administration of plants mentioned mainly in the Northern Cape (no samples obtained)

<table>
<thead>
<tr>
<th>Vernacular name</th>
<th>Part used/preparation</th>
<th>Doses/administration /Uses</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ganiesbos, kruntjie roor my nie</td>
<td>Leaves, boil in water</td>
<td>Wash wounds</td>
</tr>
<tr>
<td></td>
<td>Fry leaves till they are brown in petroleum jelly</td>
<td>Ointment for sores and hair grower</td>
</tr>
<tr>
<td>Lavender</td>
<td>Unspecified</td>
<td>Eczema</td>
</tr>
<tr>
<td>Swartbitterbos</td>
<td>Root, dry and grind into powder, pour boiling water</td>
<td>Drink a glass for colds, sores and diarrhoea. Chew fresh for same</td>
</tr>
<tr>
<td>Jantjiebarend</td>
<td>Leaves, prepare as tea</td>
<td>Drink for coughs</td>
</tr>
<tr>
<td>Bergsalle + loubes</td>
<td>Leaves, prepare as tea</td>
<td>Drink ½ a cup in the morning and evenings for coughs and colds</td>
</tr>
<tr>
<td>(pronounced as Qhwabes in Nama)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Olieboom</td>
<td>Seeds, roast and grind and mix with petroleum jelly</td>
<td>Apply on sores and put plaster on top</td>
</tr>
<tr>
<td>Seerverklein</td>
<td>Leaves, dry and grind and mix with petroleum jelly</td>
<td>Apply on painful sores.</td>
</tr>
<tr>
<td>Ngwebeba</td>
<td>Leaves</td>
<td>Use as plaster over Mathunga for sores</td>
</tr>
<tr>
<td>Sweetroot (or alone), Buchu</td>
<td>Steep in boiling water</td>
<td>Drink a cup occasionally or as needed for chest pains</td>
</tr>
<tr>
<td>Ysterhouttoppe</td>
<td>Unspecified</td>
<td>TB, Chest complaints</td>
</tr>
</tbody>
</table>
Chapter 3

Effect of crude extracts on the growth of mycobacteria
3.1. Introduction

A traditional drug screen, with respect to natural products involves the extraction of the plant material in various solvents in order to separate plant secondary metabolites of different polarities. Usually one would exhaustively extract the dried powdered material in solvents such as ethanol (EtOH), methanol (MeOH) or water (H₂O) in order to extract the polar components followed by another exhaustive extraction in solvents such as ethyl acetate (EtAc), dichloromethane (CH₂Cl₂) or n-hexane (n-hex) in order to extract the less polar and non-polar components.

3.2. Initial screens of collected plant samples

The 18 plant parts obtained during the field trip described in chapter 2 were air dried and ground before they were exhaustively extracted in methanol and then in dichloromethane to yield 36 crude organic extracts. An overnight culture of *Mycobacterium aurum* A⁺ was diluted in 2YT broth to a suspension that had an OD of 0.5 at 600nm. To determine the effect of the crude organic extracts on the growth of *Mycobacterium aurum* A⁺, an aliquot of the diluted culture (50µl) was added to each of 32 crude organic extracts (final concentration at 500µg/ml) in a 96-well plate format. Based on OD readings at 600nm, one of the crude extracts inhibited the growth of more than 90% of the organisms at this concentration, though others showed insignificant inhibition. The plant sample from which this active crude extract was prepared was supplied by Traditional Doctor (TDr.) George Rhesha, a herbalist from Queenstown in the Eastern Cape province. A literature review
using the vernacular names, isibhara and the pepper bark tree revealed the corresponding latin name, *Warburgia salutaris*. The taxonomy and use of *Warburgia salutaris* in traditional medicine is presented at the end of this chapter.

### 3.3. Authentication of *Warburgia salutaris* bark

The bark sample of *Warburgia salutaris* was authenticated with the help of Mr. Terry Trinder-Smith, a curator, against a voucher specimen of *Warburgia Salutaris* at the Bolus Herbarium, Department of Botany of the University of Cape Town. *Warburgia salutaris* bark from a well-characterized tree of about 15 years old was supplied with the generosity of Dr. Neil Crouch of the South African National Botanical Institute (NBI). This material was harvested at the Silver Glen Nursery in Durban in June of 2002, just after the flowering season. A voucher specimen has been deposited at the NBI’s herbarium in Durban under the name N. Crouch 947, NH.

### 3.4. *Warburgia salutaris* vs *M. tuberculosis* H37Rv

The plant material from Silver Glen Nursery was air dried and extracted to exhaustion in the same way as the initial sample was prepared, sequentially in methanol, thenafter in dichloromethane [see section 8.3]. To confirm the observed anti-mycobacterial activity of the crude dichloromethane extract of *Warburgia salutaris* bark, a sample was prepared [section 8.5] and submitted to the microbiology diagnostic laboratory at Groote Schuur Hospital in Cape Town (South Africa) for testing using the Bactec® 460 system [see section
8.5]. In the following sections we shall refer to the crude dichloromethane extract of *Warburgia salutaris* bark as "the crude extract".

An aliquot of the crude extract (200µg/ml) was injected in a Bactec 460® vial, which had been inoculated with *M. tuberculosis* H37Rv [see section 8.4]. At the same time, isoniazid (0.05µg/ml) or acetonitrile (2.5%) were added to separate vials containing *M. tuberculosis* H37Rv. Based on the release of radiolabelled $^{14}$CO$_2$ by viable organisms, growth indexes were obtained daily over a period of 12 days (Figure 3.4.1). In the absence of isoniazid, acetonitrile or extract, *M. tuberculosis* H37Rv grew exponentially after a lag phase period of 5 days (Figure 3.4.1). A similar growth pattern was observed when acetonitrile was added (Figure 3.4.1). In contrast, like INH, the crude extract inhibited growth of *M. tuberculosis* H37Rv (Figure 3.4.1), confirming the anti-tubercular activity of the crude dichloromethane extract of *Warburgia salutaris* bark.

**Figure 3.4.1:** Effect of crude dichloromethane extract of *Warburgia salutaris* bark on the growth of *M. tuberculosis* H37Rv. Similar to 0.05µg/ml of INH (solid triangles), 200µg/ml of the crude dichloromethane extract (open squares) inhibited the growth of *M. tuberculosis* H37Rv when compared to the untreated cells (open diamonds). Acetonitrile concentration (2.5%) used to dissolve the extract did not inhibit growth on its own (solid diamonds).
All subsequent experiments on the crude dichloromethane extract of *Warburgia salutaris* bark were conducted in the Department of Pharmacology, Oxford University using *M. bovis* BCG Pasteur as the test organism.

### 3.5. *Warburgia salutaris* vs *M. bovis* BCG

Experiments were carried out to study the effect of the crude extract on the growth of *M. bovis* BCG Pasteur. A culture of *M. bovis* BCG (OD=1.0) was diluted 1:100 in Middlebrook 7H9 broth in a 100ml tissue culture treated roller bottle [see section 8.6 for detailed method]. The crude extract was added to the prepared 1% cultures to give final concentrations of 20µg/ml and 100µg/ml of the crude extract. The bottles were incubated at 37°C in a roller incubator for 7 days. To obtain a growth curve, OD readings were taken every 12 hours at a wavelength of 600nm. Based on these OD readings, in the absence of the extract, cultures grew exponentially after a lag phase of 3 days (Figure 3.5.1). When tested at a low concentration of 20µg/ml, the crude extract had little effect on the growth of *M. bovis* BCG whereas a concentration of 100µg/ml inhibited growth completely (Figure 3.5.1).

To ascertain whether the cultures treated with 100µg/ml of the crude extract contained any viable cells after the 7-day incubation period, the cells were centrifuged at 3000g for 5 minutes. The supernatant was discarded and the cells were resuspended in 1ml of residual broth. The cells were inoculated into 100ml of fresh Middlebrook 7H9 broth and incubated at 37°C for another
7 days. OD readings were taken every 24 hours for 7 days and based on these, no growth was observed.

**Figure 3.5.1:** Effect of crude dichloromethane extract of *Warburgia salutaris* on the growth of *M. bovis* BCG. At a concentration of 100µg/ml, the crude extract inhibited *M. bovis* BCG (open circles) compared to the untreated organisms (open diamonds). Similar activity was not observed when a concentration of 20µg/ml (open triangles) was used. Growth was briefly halted after the crude extract was added to a culture at mid-log phase of growth (open squares) but eventually started to grow slowly.

To determine the effect of the crude extract (100µg/ml) on exponential phase cultures of *M. bovis* BCG, an appropriate volume (100µl) was added to a mid-log phase culture of *M. bovis* BCG (Day 3, figure 3.2.4.1). As shown, growth was arrested, indicating activity of the crude extract.

### 3.6. *Warburgia salutaris* vs morphology of *M. bovis* BCG

To study the effect of the crude extract on the morphology of *M. bovis* cells, a Ziehl–Neelsen hot staining procedure [section 8.8] was performed on the 7th day of incubation of treated (crude extract and acetonitrile) and untreated cultures. An aliquot (20µl) of the cultures was placed on a glass microscope slide, allowed to air dry, gently heat fixed and stained with the Z-N stain. The
cells were visualized using a light microscope (Olympus BH2) and were photographed using a Nikon digital camera [see section 8.8.1].

The preparation from the untreated cells showed a large number of Acid Fast Bacilli (AFBs), beading and clumping was also observed (Figure 3.6.1). In contrast, the crude extract treated cells were few in number, indicating that the crude extract inhibited cell multiplication. The solvent, acetonitrile (0.1%) that was used for the preparation of the crude extract, did not affect the number of the cells [Figure 3.6.1]. Importantly, bacterial contamination (gram positive and gram negative organisms) was not observed in the cultures.

![Figure 3.6.1](image_url)

**Figure 3.6.1:** The effect of the crude extract on cell morphology of *M. bovis* BCG as visualized by light microscopy (X1000 magnification) after a Ziehl–Neelsen hot staining procedure was performed. *M. bovis* BCG cultures were incubated for 7 days with extract (C), 0.1% acetonitrile (B) and untreated (A) as explained in section 3.2.5.
3.7. Crude extract vs a gram-negative bacterium

To determine whether the crude extract had activity against a gram-negative organism, *E. coli* JM109 was chosen. A 1:100 dilution of an overnight culture of *E. coli* JM109 (OD=1.9) was prepared in 3 separate sterile conical flasks containing 100ml of LB broth. The crude extract (100µl) was added into one of the flasks containing the 1% culture to the final concentration of 100µg/ml. An aliquot (100µl) of either LB broth or 100% acetonitrile was added separately in the two remaining flasks. The flasks were incubated at 37°C with shaking and the growth of *E. coli* JM109 was monitored by taking OD readings at 600nm every 30 minutes until the cultures reached stationary phase (7 hours).

Based on the OD readings, *E. coli* cells in the absence of the crude extract grew rapidly, the lag phase lasted for an hour and the cells divided exponentially for the next four hours and then after, began to grow slowly. Similar growth was observed in the cultures treated with the crude extract, or acetonitrile indicating no effect of the crude extract or acetonitrile (used to dissolve the crude extract) on the growth of *E. coli* JM109 (Figure 3.7.1). Whether this finding extends to other gram-negative organisms, is not known but is beyond the scope of this thesis.
3.8. Conclusion

Experiments described in this chapter indicated that the crude dichloromethane extract of *Warburgia salutaris* bark inhibited growth of *M. aurum* A+, *M. tuberculosis* H37RV and *M. bovis* BCG but not *E. coli*. Mindful of the importance of NAT in mycobacterial cell wall synthesis and morphology in *M. bovis* BCG [section 1.5.1], studies were carried out to determine the effect of the crude extract on NAT activity. These experiments are described in chapter 4.

3.9. The taxonomy of *Warburgia salutaris*

*Warburgia salutaris* tree belongs to the Canellaceae family also known as the white cinnamon family. This tree grows in dense evergreen environments of the north of Zambia and Malawi, from Zimbabwe in the north to Kwa-Zulu-
Natal in the south. Salutaris means "salutary to health" (van Wyk and Gericke, 2000).

The young bark is smooth but when matured it is rich brown, rough with yellowish cocky lenticels. The inner matured bark (Figure 3.9.1) is reddish in colour with a peppery smell and taste. Demand for *Warburgia salutaris* root bark in traditional medicine is very high. This has led to over-exploitation of this natural resource such that it is now endangered. Today most of the bark in the street market comes from Mozambique (van Wyk and Gericke, 2000). It is hoped that cultivation and harvesting of the stem and branch bark instead of the root bark would save the trees from destruction.

*Figure 3.9.1: Warburgia salutaris* bark is commonly used in traditional medicine. (Hollmann and van Der Schiff, 1996; van Wyk, 1997).

The leaves (Figure 3.9.2), even though they are not important in traditional medicine, are simple, arranged alternatively, elliptic to lanceolate and covered with clear dotted glands. The upper surface of the leaves is glossy and dark green with veins while the bottom is pale yellow also with veins. When
crushed the leaves smell and taste peppery too. The leaves make very good spice for various dishes and also can be made into a delicious peppery tea.

**Figure 3.9.2:** Flower (A), fruit (B) and leaves (C) of a *Warburgia salutaris* tree (van Wyk, 1997)

The fruit (Figure 3.9.2) is an oval berry with smooth leathery skin, 20-40mm in diameter. It is green turning to dark purple when matured and is covered in glands. The flowers (Figure 3.9.2) are solitary or in 3-flowered cymes in axils of leaves, green with 3 sepals, 10 obovate and gland dotted petals, 5 of which are inner, thinner, yellower than the outer 5. Stamens are joined to form a tube that forms a prominent structure at the centre of the flower. The wood is light yellow sapwood and dark yellowish brown heartwood. *Warburgia salutaris* flowers from April to May and fruits from October to January.
3.10. Use of *Warburgia salutaris* in traditional medicine

Medicinally the inner bark is used to treat colds, to clear sinuses and headaches; it is dried, ground and used as snuff, sometimes mixed with *Erythrophleum lasainthum* Corbisely (Hutchings *et al*., 1996). It is chewed for chest complaints or burned and the smoke is inhaled. When boiled in water with roots, the inner bark is good for malaria. Decoctions of powdered bark are added in porridge for abdominal pains. It is also used as an emetic to clear patches in the lungs (Venter and Venter, 1996).

The bark is used as an expectorant for coughs, and as a natural antibiotic to treat chest infections productive of purulent sputum, venereal diseases and constipation (van Wyk and Gericke, 2000). The bark is also used to treat cancer, rheumatism and stomach ulcers. It is applied to cuts on temples for headaches and has been used as an aphrodisiac (van Wyk and Gericke, 2000). Leaves of *Cannabis sativa* L. mixed with powdered bark of *Warburgia salutaris* are smoked for dry coughs (Hutchings *et al*., 1996). An ointment made from a mixture of pounded leaves of *Hibiscus surattensis* L. and the bark of *Warburgia salutaris* in animal fat is applied to the penis in cases of irritation, sores or inflammation of the urethra (Hutchings *et al*., 1996). Bark is used in Tanzania for toothache, the Vhenda people use it for skin complaints and even to make dogs alert and more ferocious (Hutchings *et al*., 1996).
Chapter 4

*Warburgia salutaris* vs mycobacterial NAT
4.1. Introduction

A high throughput microtitre plate assay, which can be used to identify novel compounds that are NAT ligands or inhibitors, has been developed (Brooke et al., 2003a; Brooke et al., 2003b) using a pure recombinant MSNAT (Sandy et al., 2002). The idea behind this assay is the transfer of an acetyl group by NAT from acetyl-CoA to a substrate. Ellman's reagent or 5,5-Dithio-bis(2-nitrobenzoic acid) [DTNB] is being used for the detection of the free CoA thiol produced. DTNB reacts with the free CoA in a 1:1 ratio to produce a symmetric coloured compound, thionitrobenzoate (TNB), which absorbs light maximally at a wavelength of 412nm. The reaction mechanism is as illustrated below in scheme 4.1.1.

![Scheme 4.1.1: Arylamine N-acetyltransferase catalyzed transfer of an acetyl group from Acetyl-CoA to an arylamine substrate, isoniazid. The NAT first binds to Acetyl-CoA, and in the presence of an arylamine substrate, free CoA is released and the acetyl group transferred to the arylamine. Upon its addition, DTNB reacts with the free CoA in a 1:1 ratio and TNB, which we observe spectrophotometrically, is formed.](image-url)
4.2. Screening the crude extract for anti-NAT activity

4.2.1. Protein expression and purification

The gene for the open reading frame of *Mycobacterium smegmatis* N-acetyltransferase containing an N-terminal hexa-histidine tag (Hexa-his-tag) was cloned in a pET28b vector which was transformed into *E. coli* BL21(DE3)pLysS cells for expression, as described (Payton et al., 1999). A culture of the transformed *E. coli* cells that had grown to a suitable suspension (OD$_{600nm}$ = 0.4 to 0.6) was induced with isopropyl β-D-thiogalactopyranoside (IPTG) so that protein synthesis could begin. These cells were incubated at 27°C overnight before they were lysed. The protein was purified using an affinity column (Ni-NTA agarose, Qiagen) and was subjected to SDS-PAGE to determine its purity [see chapter 8 for detailed methods].

![Figure 4.2.1.1:](image)

**Figure 4.2.1.1:** SDS-PAGE analysis of protein fractions from affinity column. The Biorad® molecular weight markers (lane 1) and fractions of each of the whole lysate (lane 2), unbound material (lane 3), 1mM (lane 4), 10mM (lane 5), 50mM (lane 6) and the 250mM (Lane 7) imidazole washes were electrophoresed. The pure recombinant MSNAT eluted in the 50mM imidazole wash (lane 6).
The protein band corresponding to the purified recombinant MSNAT protein eluted in the 50mM imidazole wash (Figure 4.2.1.1, lane 6).

4.2.1.1. Determination of Molecular weight

BioRad® low molecular weight markers are 6 components of known molecular weight. These components are Phosphorylase b, Bovine Serum albumin, Ovalbumin, Carbonic anhydrase, Soybean Trypsin inhibitor and Lysozyme. Their corresponding molecular weights are shown below in Table 4.2.1.1.1.

<table>
<thead>
<tr>
<th>SDS-PAGE Standard</th>
<th>MW</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphorylase B</td>
<td>97,400</td>
</tr>
<tr>
<td>Serum albumin</td>
<td>66,200</td>
</tr>
<tr>
<td>Ovalbumin</td>
<td>45,000</td>
</tr>
<tr>
<td>Carbonic Anhydrase</td>
<td>29,000</td>
</tr>
<tr>
<td>Trypsin Inhibitor</td>
<td>21,500</td>
</tr>
<tr>
<td>Lysozyme</td>
<td>14,400</td>
</tr>
</tbody>
</table>

A plot of the logarithm of the molecular weights of these known components versus their relative migration (Rr) on the gel [Figure 4.2.1.1, lane 1] gives a molecular weight standard curve [Figure 4.2.1.1.1].
The predicted molecular size corresponding to the 275 long amino acid sequence, coding for the MSNAT protein was calculated initially using an online proteomics tool, ProtParam, to be 30.2kDa (http://au.expasy.org). The molecular weight of the protein band corresponding to the purified recombinant NAT protein (Figure 4.2.1.1, lane 6) as calculated using the molecular weight standard curve was 32.3kDa. This weight includes the extra weight of the hexa-histidine tag, which has a molecular weight of about 2.1kDa. There are 20 extra amino acids on the N-terminus of the recombinant MSNAT protein due to the hexa-histidine tag [figure 4.2.1.1.2 A] (Sandy et al., 2002).
Figure 4.2.1.1.2: Recombinant MSNAT before (A) and after (B) the N-terminus hexa-histidine tag was removed. Before the removal of the his-tag (red shading), the recombinant MSNAT protein has 20 extra amino acids on its N-terminus including a thrombin cleavage site (green shading). The thrombin cuts between the Arg and the Gly, resulting in 3 non-authentic amino acids (Glu, Ser and His), remaining attached to the pure recombinant protein (B).

4.2.1.2 Cleavage of the hexa-His-Tag

To cleave the hexa-his-tag from the purified recombinant protein, a protease called thrombin was used and cleavage was confirmed by subjecting an aliquot of the cut and the uncut protein to SDS-PAGE analysis. The cut protein (Figure 4.2.1.1.2 B) has 17 less amino acids than the uncut protein (Figure 4.2.1.1.2 A) and hence the former traveled faster on the SDS gel (Figure 4.2.1.2.1).
Figure 4.2.1.2.1: SDS-PAGE analysis of protein with or without his-tag. Cleaved (2) and uncleaved (3) MSNAT enzyme were loaded on a gel and after electrophoresis it was evident that cleavage had taken place and that due to the extra weight the MSNAT with the hexa-his-tag traveled slower than the cleaved enzyme.

The $R_i$ value for the purified and cleaved MSNAT protein was found to be 0.65 (Figure 4.2.1.2.1, Lane 2) while the one containing the hexa-his-tag had an $R_i$ value of 0.63 (Figure 4.2.1.2.1, Lane 3). Hence, their molecular weights were found by interpolation on the standard curve (Figure 4.2.1.1.1) to be 30.9kDa and 32.3kDa respectively, as previously reported (Payton et al., 1999). This confirmed that the extra amino acids that are due to the hexa-his-tag are indeed removed. However, Thrombin cleavage leaves three non-authentic amino acids at the N-terminus (Figure 4.2.1.1.2 B), a glutamic acid, a serine and a histidine, making the protein 278 amino acids long (Sandy et al., 2002).

4.2.2. Enzyme assays

CoA, a product in the NAT reaction, is commercially available and has been used to detect acetyl-CoA hydrolysis in the presence of arylamine substrates and inhibitors (Brooke et al., 2003a). A plot of OD versus CoA concentration was prepared (Figure 4.2.2.1) as described in the section 8.1.2. This plot was
specifically used to determine the amount of CoA produced by MSNAT per one OD unit.

**Figure 4.2.2.1: CoA Standard Curve.**
Different concentrations of CoA ranging from 0-500 μM were prepared in 20mM Tris.HCl buffer pH 8.0. Then 25 μL of 5mM DTNB in 6.4M Guanidine.HCl, 0.1M Tris.HCl pH 7.3, was added. Absorbance was measured at a wavelength of 405nm. A plot of OD vs CoA concentrations was drawn. An OD of 1 gives a concentration of 242.2 μM of CoA.

### 4.2.2.1. Determination of MSNAT activity

Enzyme reactions that obey the Michaelis-Menten kinetics can be explained by the steady state approach. An enzyme (E) molecule binds a substrate (S) to give a product (P) as shown in scheme 4.2.2.1. Unlike the rapid equilibrium assumption, the Briggs-Haldane steady-state approach was based on the assumption that the concentration of the ES complex, [ES], does not necessarily need to be at equilibrium with the concentrations of E, [E] plus S, [S], but rather the formation of ES and its breakdown to E plus S or E plus P is constant (Fersht, 1985 and 1999). Once intermediates reach this steady state the reaction rate becomes
slower with time and velocity can be measured as enzymologists traditionally do. However, the velocity measured is an approximation since substrates do get depleted. If the measurement has been done in the shortest time possible, such that only the formation of the first few percentages of products are observed and that substrate depletion is negligible, then it would be a good approximation. Under these conditions the formation of product is generally linear with time. The product formed is proportional to the concentration of the ES complex (Segel, 1993) and the initial rate of the reaction (\( \nu \)) is the rate of the breakdown of the ES complex into E plus P, which is the rate of appearance of product.

\[
\begin{array}{c}
\frac{k_1}{k_2} \\
E + S \rightleftharpoons ES \rightarrow E + P \quad \text{...........Scheme 4.2.2.1}
\end{array}
\]

The corresponding Michaelis-Menten equation (Equation 4.1) for scheme 4.2.2.1 follows below and its derivation is described by Segel (1993) and Fersht (1999).\( K_m \) is the Michaelis constant, for reactions obeying the Michaelis-Menten kinetics, \( K_m \) is a measure of the substrate concentration required for effective catalysis to occur. \( V_{\text{max}} \) is the maximum velocity of the enzyme-catalyzed reaction.

\[
\frac{\nu}{V_{\text{max}}} = \frac{[S]}{K_m + [S]} \quad 4.1
\]
product formation is constant for both enzyme concentrations in the first 5 minutes of the assay (figure 4.2.2.2.1). However, for [E]_12, beyond 5 minutes, the appearance of the product is not constant with time. If the assay time is allowed to go beyond 10 minutes, it is also clear, even with [E]_11, that the reaction is no longer in the linear range, meaning that the velocities over 10 minutes are not really instantaneous and that product formation is not constant. It is very important to establish the assay conditions such that the appearance of product is linear with time and that true initial velocities are measured. Therefore, for the subsequent enzyme activity assays, it has been established that using 1μg/well of MSNAT enzyme and allowing the reaction to take place for 10 minutes, was ideal to maintain first order kinetics or linearity of product formation.

![Figure 4.2.2.2.1: Effect of Enzyme concentration on reaction rate. Either 2.04μg (solid squares) or 1.02μg (open squares) MSNAT enzyme was used in the MSNAT reaction (see section 4.3.2.2.). CoA produced during the reaction was determined and represented graphically. The higher the concentration of MSNAT used, the higher the concentration of free CoA produced per unit time.](image)
4.2.2.3. Activity of cleaved enzyme vs his-tagged enzyme

The amount of CoA produced by the cleaved MSNAT protein was slightly higher compared to the amount produced by the enzyme that contained the hexa-his-tag (Figure 4.2.2.1). However, statistically, with 95% confidence, there was no significant difference between the two enzymes ($p = 0.07$, $r = 0.99$). Only pure cleaved recombinant MSNAT enzyme was used for the assays.

![Figure 4.2.2.1: Effect of removing his-tag on reaction rate. 1 μg of either cleaved (solid triangles) or uncleaved (open triangles) MSNAT enzyme was used in the MSNAT reaction (see section 4.3.2.2.) CoA produced during the reaction was determined and represented graphically. Though the cleaved enzyme produces slightly more CoA per unit time, removing the his-tag does not significantly affect the reaction rate of the MSNAT enzyme.]

4.2.2.4. Acetylation reaction in the absence of substrate

In the absence of MSNAT, AcCoA or isoniazid, no reaction occurred. When the enzyme was incubated with AcCoA but no isoniazid, a slight colour change was observed which might be due to experimental noise (Figure 4.2.2.4.1), indicating that no enzymatic acetylation took place in the absence of the substrate.
4.2.2.5. v with respect to [S]

The reaction of the MSNAT enzymes with respect to isoniazid concentration obeys the Michaelis-Menten kinetics and is saturable. The relationship of velocity of an enzyme and its substrate concentration is hyperbolic in nature. In an in vitro situation, the true instantaneous velocity of a reaction is the slope of the tangent to the hyperbolic curve drawn from the origin (Alison and Purich, 1979) [as shown by the dotted line drawn on the CoA production curve in figure 4.2.2.5.1].
Figure 4.2.2.5.1: $v$ vs $[S]$. The plot of reaction rate versus substrate concentration shows that the enzyme is saturable with respect to the substrate. The true instantaneous velocity of the MSNAT catalyzed reaction with respect to isoniazid will be the slope of the tangent to the curve, drawn from the origin (as shown by the dotted line).

Graphically, this data can be represented using the Lineweaver-Burk plot. The plot of $1/v$ versus $1/[S]$ is called the Lineweaver-Burk and it allows for the direct determination of $K_m$ and $V_{max}$. The Lineweaver-Burk expression (see equation 4.4) was derived from the Michaelis-Menten equation through rearrangements and inverting. The curve will cut the y-axis at $1/V_{max}$ while the x-axis at $-1/K_m$. Subsequently, other kinetic constants that are important to the enzymologist, such as $K_{cat}$ (The catalytic constant) and $K_{cat}/K_m$ (The specificity constant) can be calculated (Fersht, 1999).

\[
\frac{1}{v} = \frac{K_m}{V_{max}} \frac{1}{[S]} + \frac{1}{V_{max}}
\]  
4.4
4.2.2.6. The kinetics of enzyme inhibition

When an inhibitor is present, the Michaelis-Menten equation can be written as follows:

\[
\frac{V}{V_{\text{max}}} = \frac{[S]}{K_m \left(1 + \frac{[I]}{K_i}\right) + [S]}
\]

4.5

The corresponding reciprocal is:

\[
\frac{1}{V} = \frac{K_m}{V_{\text{max}}} \left(1 + \frac{[I]}{K_i}\right) \frac{1}{[S]} + \frac{1}{V_{\text{max}}}
\]

4.6

The expression for the Michaelis-Menten equation when a competitive inhibitor is present is as shown in equation 4.5. When there is no inhibitor, the inhibitor concentration, [I], is equal to zero hence the normal Michaelis-Menten equation. When the inhibitor is added, the value of \(K_m\) increases by a value of \((1 + [I]/K_i)\). The corresponding reciprocal of equation 4.5 is as shown in equation 4.6.

4.2.2.7. Warburgia salutaris vs MSNAT

When different concentrations of the crude bark extract of Warburgia salutaris were added in the MSNAT reaction in the presence of isoniazid, the plot of \(1/V\) versus \(1/[S]\) (Figure 4.2.2.7.1) showed a typical trend indicative of the presence of
a competitive inhibitor. The increase in $K_m$ as the extract concentration increased and the lines almost meeting and cutting the y-axis at a single point of $1/V_{max}$ is a diagnostic indication of a presence of a competitive inhibitor in the crude extract. However, when the different concentrations of *Warburgia salutaris* crude bark extract were added, the calculations from the Lineweaver-Burk plot shows that, $K_m$ increased while $V_{max}$ also increased. However, whilst the increase in $V_{max}$ may be with experimental error it may suggest mixed inhibition.

**Figure 4.2.2.7.1:** Effect of the crude extract of *Warburgia salutaris* on the activity of the MSNAT enzyme. In the NAT assay described in section 4.3.2.2, crude extract [here showing 0μg/ml (triangles), 12.5μg/ml (open squares) and 100μg/ml (solid squares)] was added with isoniazid and the reaction was allowed to take place as normal. CoA produced during the reaction was determined and reaction rates were calculated. The plot of the inverse of reaction rates versus the inverse of substrate concentrations shows that the crude extract inhibits the MSNAT activity in a competitive fashion.
4.3. Summary

The crude extract of *Warburgia salutaris* bark behaves as a competitive inhibitor of NAT from *M. smegmatis*. This MSNAT inhibitory activity, together with the anti-mycobacterial activity described in chapter 3 stimulated further investigations of this crude extract, this time focusing on the isolation of the purified components that are responsible for both activities i.e. where the two activities coincide.
Chapter 5

Isolation and purification of compound 1 and 2
A number of natural compounds have been isolated from the Callaneceae family of Warburgia species, specifically, from *Warburgia ugandensis* and *Warburgia salutaris*. Active sesquiterpenoid lactones have been isolated from plant components extracted in various solvents such as ethyl acetate (Rabe and van Staden, 2000) and hexane (Gu et al., 2004) but mostly from dichloromethane extracts (Mashimbye et al., 1999; de Almeida Alves et al., 2001; Wonganuchitmeta et al., 2004). Sesquiterpenoid lactones that exhibit antitubercular action have been described (Cantrell et al., 2001) and are discussed in detail on chapter 6.

In this chapter, the isolation and purification of two anti-mycobacterial compounds from the crude dichloromethane extract of *Warburgia salutaris* bark is described. There are two bioassays that have been used to guide the identification of these active agents. They are: the inhibition of growth of *M. bovis BCG* assay and the inhibition of the recombinant *M. smegmatis* NAT enzyme assay. An overview of the purification process is illustrated in figure 5.1.1.
5.1. Overview of the purification process and yields

Figure 5.1.1: Overview of the purification of anti-mycobacterial compounds 1 and 2 from the crude dichloromethane extract of *Warburgia salutaris* bark. The fractions were screened against the growth of *M. bovis* BCG and against the activity of the MSNAT. The yellow blocks indicate where most of the NAT inhibitory activity and the *M. bovis* BCG growth inhibitory activity co-eluted. These fractions were fractionated further to yield small amounts of compound 1 and 2.
5.2. Solid Phase Extractions

SPE [see section 8.11] was used in order to separate the components of the crude dichloromethane extract of *Warburgia salutaris* bark into manageable mixtures for the subsequent process of High Performance Liquid Chromatography (HPLC). A solution of the crude extract was loaded onto the bed of an SPE C-18 reversed phase column, and the different components were eluted with increasing concentrations of highly pure acetonitrile (HPLC grade). Three fractions were generated namely Fr 30, Fr 60, and Fr 100 which eluted with 30%, 60% and 100% acetonitrile:water respectively. These fractions were prepared in the same way as for the crude extract [see section 8.10.8] and screened for inhibitory action against the MSNAT enzyme. The only difference in the interpretation of the data was that once the specific activities were determined using equation 4.3, the relative specific activities of the enzyme preparations treated with different concentrations of fractions in comparison with the untreated enzyme preparation (control) were calculated and plotted against extract concentration (figure 5.2.1). These fractions were also screened for activity against the growth of *M. bovis* BCG [see section 8.1.2].
The three fractions of the crude extract exhibited inhibitory action against the growth of *M. bovis* BCG (Figure 5.2.2) and also against the MSNAT activity (Figure 5.2.1). Fr 30 contained most of both these activities and hence further investigations were carried out on Fr 30. Transmission Electron Microscopy has revealed that Fr 30 affected the morphology of the cell wall in the same way as when nat is knocked out (figure 5.2.3). This might have implications on antibacterial drug entry into the bacillus.
Figure 5.2.2: Comparative inhibition of growth of *M. bovis* BCG. Fr 30 (open triangles), Fr 60 (open circles) and Fr 100 (open diamond) showed inhibitory action on the growth of *M. bovis* BCG. Fr 30 exhibited more inhibition at lower concentrations than Fr 60 and Fr 100.

Figure 5.2.3: Effect of Fr 30 on the morphology of *M. bovis* BCG cells. Transmission Electron Microscopy showed that *M. bovis* BCG wild type (*M. bovis* BCG WT) had a characteristic rugged outer cell wall membrane that is absent when *nat* was knocked out (*M. bovis* BCG natΔ). Interestingly, when *M. bovis* BCG WT was treated with Fr 30, on the 3rd day of treatment, the cells had altered phenotypic appearance as the outer cell wall membrane had disintegrated in the same way as in *M. bovis* BCG natΔ.
5.3. The isolation of Fr 30.2 from Fr 30 by HPLC

Initially, Fr 30 was subjected to HPLC using a step-wise gradient of 20%-100% acetonitrile in water on a Discovery® C-18 reversed phase column (Supelco 4.6mm X 150mm, particle size of 5µm) over a period of 20 minutes at a flow rate of 1ml/min (Table 5.3.1). A major peak of interest, Fr 30.2 (retention time = 10.5 minutes), was identified (Figure 5.3.1). This peak was collected using preparative HPLC. Two other fractions of the minor peaks present in Fr 30, Fr 30.1 (retention time = 0 to 7 minutes) and Fr 30.M (retention time = 7 to 10 minutes) were also collected.

<table>
<thead>
<tr>
<th>Time (minutes)</th>
<th>MeCN (%)</th>
<th>H2O (%)</th>
<th>F rate (ml/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.00</td>
<td>20</td>
<td>80</td>
<td>1.0</td>
</tr>
<tr>
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</tr>
<tr>
<td>20.00</td>
<td>20</td>
<td>80</td>
<td>1.0</td>
</tr>
</tbody>
</table>

Table 5.3.1: Initial HPLC method
**Figure 5.3.1:** HPLC profile of Fr 30. Fr 30.2 is a sharp major peak that eluted at 10.5 minutes. The UV wavelength of detection is 220nm.

Sub-fraction 30.1, 30.M and 30.2 were tested for inhibitory action against the activity of the MSNAT. It was found that Fr 30.2 had increased activity against the MSNAT compared to the "parent" Fr 30 and had higher activity than Fr 30.1 (Figure 5.3.2) or Fr 30.M (data not shown). When tested against the growth of *M. bovis* BCG in broth, Fr 30 and Fr 30.2 showed a similar effect, based on OD readings (Figure 5.3.3).
Figure 5.3.2: Relative activity of MSNAT when treated with fractions. Fr 30.2 (solid circles) exhibited increased inhibitory activity against the MSNAT compared to Fr 30 (open triangles) and Fr 30.1 (solid squares).

Figure 5.3.3: Effect of Fr 30.2 on growth of *M. bovis* BCG. Fr 30.2 (open squares) and Fr 30 (open triangles) had a similar effect of the growth of *M. bovis* BCG. The growth of the cultures treated with both these agents was delayed when compared to the untreated cultures (solid diamonds).
Mass Spectrometry analysis revealed that the LC-MS trace of Fr 30.2 (Figure 5.3.4) was a single major peak. However, when this major peak was specifically analyzed using an integration method, it was shown that a high molecular weight species (m/z=670.27) was present in Fr 30.2 [Figure 5.3.5]. This high molecular weight did not correspond with molecular weights of compounds that have been isolated previously from Warburgia species. We suspected that this was a dimer or perhaps two compounds that co-eluted and could not separate under the chromatographic conditions used.

Figure 5.3.4: LC-MS trace of Fr 30.2. Fr 30.2 seemed a single species.
Figure 5.3.4: MS spectrum of Fr 30.2. The high molecular weight ion m/z at 670.27 suggested a cumulative effect probably due to two co-eluting compounds. The abundant ion at 249.15 is common in the class of compounds previously isolated from Warburgia species.

5.4. Modification of the HPLC method

It is sometimes feasible to optimize the separation method such that peaks of interest are well resolved. In reversed phase, resolution can be improved by changing parameters such as: increasing run time or column length, decreasing flow rate or increasing the percentage (%) organic strength. This can be a time consuming process especially when one is dealing with complex mixtures such as in natural products. Resolution degrades during the lifetime of a column due to day-to-day fluctuations and sometimes changing a column or the sorbent could be the best decision.

In an attempt to resolve the components of Fr 30.2, a new XTerra® reversed phase C-18 column (4.6mm X 150mm, particle size of 5µm) from Waters was
used for subsequent HPLC separations and 0.1% trifluoroacetic acid (v/v) was added as an ion pairing agent to improve resolution. TFA complexes with charged biomolecules, increasing their retentivity and minimizing their interaction with the HPLC column packing. XTerra® columns combine the best properties of silica and polymers with its revolutionary Hybrid Particle Technology (www.waters.com). This results in a rugged inorganic/organic particle that can be operated at high speeds, high temperature and high pH while providing sharp, high-efficiency peaks for any compound in any mobile phase. The extra endcapping of one in every 3 silanol residues by reacting with a methyl group ensures minimal polar secondary interactions and hence improve retentivity and resolution of non-polar compounds.

The flow rate was also reduced at the region at which the peak of interest (Fr 30.2) in the initial HPLC run eluted (50%≥MeCN percentage≤62%). This region was judged from the initial HPLC chromatogram (Figure 5.3.1). The decrease in flow rate was performed in order to separate the components of Fr 30.2 that were co-eluting. The conditions of this new method are tabulated in table 5.4.1. Resolution in this new system was improved (Figure 5.4.1). A Gilson HPLC system consisting of a Gilson 205 liquid handler and a Gilson 170 diode Array detector and so was the method in table 5.4.1 was used for both analytical and preparative HPLC. The only difference was the injection volumes and amounts of material. During the analytical development of this method 20μl of a 1mg/ml
solution of extracts was injected using a while a scale up of 250 fold was possible during preparative HPLC (5mg of extracts was loaded in a 50μl volume).

**Table 5.4.1: Modified HPLC method**

<table>
<thead>
<tr>
<th>Time (minutes)</th>
<th>MeCN (%)</th>
<th>H2O (%)</th>
<th>F/rate (ml/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.00</td>
<td>40</td>
<td>60</td>
<td>1.0</td>
</tr>
<tr>
<td>5.00</td>
<td>46</td>
<td>54</td>
<td>0.7</td>
</tr>
<tr>
<td>15.00</td>
<td>62</td>
<td>38</td>
<td>0.7</td>
</tr>
<tr>
<td>16.00</td>
<td>100</td>
<td>0</td>
<td>1.0</td>
</tr>
<tr>
<td>21.00</td>
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<td>1.0</td>
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<tr>
<td>25.00</td>
<td>40</td>
<td>60</td>
<td>1.0</td>
</tr>
<tr>
<td>30.00</td>
<td>40</td>
<td>60</td>
<td>1.0</td>
</tr>
</tbody>
</table>

The two major peaks of interest that originally co-eluted in the initial HPLC chromatogram of Fr 30 as Fr 30.2 have been resolved. The improved HPLC method was adapted on a larger scale using preparative HPLC. Large amounts (5mg/run) of Fr 30 could be loaded on a C-18 reversed phase XTerra® preparative column using a 100μl injection loop. Several chromatographic runs yielded enough compound 1 for further bio-testing but insufficient amounts of compound 2.
Figure 5.4.1: The new HPLC profile of Fr 30: The new and the old HPLC profiles of Fr 30 are similar, however, Fr 30.2 has resolved into 2 distinct peaks corresponding to compound 1 (green) and 2 (blue).

The other major peak that eluted at 4.74 minutes (Figure 5.4.1) was not included in the following testing as it corresponded to the region in the initial HPLC chromatogram of Fr 30 (Figure 5.3.1) that exhibited little activity. The purity of compounds 1 and 2 was confirmed by LC-MS (Figure 5.4.2 and Figure 5.4.3) before they were sent for structural elucidation.
Figure 5.4.2: LC-MS trace of compound 1 showed that this agent is a single species.

Figure 5.4.3: LC-MS trace of compound 2 showed that this agent is a major component when viewed under UV-VIS. However mass detection revealed a large number of interfering species in the sample.
The LC-MS analysis revealed that compound 1 was a pure single species with insignificant interference (Figure 5.4.2). On the other hand, the LC-MS trace of compound 2 under UV/VIS detection seemed to suggest a single species although the mass detector revealed that in the actual fact there were a number of impurities in the sample. Compound 2 therefore, was considered impure.

Based on OD readings, it was revealed that the activity of compound 1 was decreased compared to the activity of Fr 30.2 and Fr 30 with respect to the extension of the lag phase of growth of *M. bovis* BCG cultures in broth (Figure 5.4.5).

![Figure 5.4.5: Effect of compound 1 on the growth of *M. bovis* BCG. The growth of the cultures treated with both compound 1 and Fr 30 was delayed when compared to the untreated cultures (solid diamonds). Compound 1 (solid circles) had lesser effect on *M. bovis* BCG growth than Fr 30 (open triangles).]
5.5. Effect of Compound 1 and Fr 30 on *M. bovis* BCG growing in solid agar

In order to study the effect of compound 1 and Fr 30 on growth of *M. bovis* BCG on solid agar, the cells (200 cells/20μl) of *M. bovis* BCG were spotted on Middlebrook 7H10 agar as described by Bhakta *et al.* (2004). The organisms were allowed to grow at 37°C for 21 days. The diameters of the resulting colonies (Figure 5.4.6) were measured and the areas of each colony were calculated using the formula for finding an area of a circle (best fit), \( \pi r^2 \).

![Figure 5.4.6](image.png)

*Figure 5.4.6*: The effect of Fr 30 and Compound 1 on the growth of *M. bovis* on solid agar. An aliquot (20μl) of *M. bovis* culture, approximately 200 cells of was spotted on agar containing with different concentrations of Fr 30 or Compound 1. The colonies were allowed to grow at 37°C for 21-28 days. Compound 1 and Fr 30 inhibit growth of *M. bovis* BCG on solid agar in different potencies. There is a clear dose-dependent inhibition by Fr 30.
A dose dependent inhibition of growth of *M. bovis* BCG by Fr30 was observed (Figure 5.4.6 and Figure 5.4.7). Compound 1 showed lesser effect than Fr 30 against the growth of *M. bovis* BCG on solid agar (Figure 5.4.6 and Figure 5.4.7). The MIC for compound 1 against *M. bovis* BCG growth was found to be between 80µg/ml and 100µg/ml while that of Fr 30 was between 40µg/ml and 80µg/ml. Both Fr 30 and compound 1 had little effect on the growth of *M. bovis* BCG Δnat (figure 5.4.7), suggesting that these two agents are specific inhibitors of mycobacterial NAT.

![Figure 5.4.7: Relative inhibition of growth of wild type (●) and Δnat (❖) cultures of *M. bovis* BCG on solid agar by Fr 30 and compound 1. The areas of the resulting colonies (fig 5.4.6) were measured for the treated and for the untreated cells using the formula of an area of a circle (πr²). The areas of colonies of treated cells were expressed in relation to the area of the colonies of untreated cells. The relative areas were plotted against concentration. Compound 1 has decreased activity compared to F30 against the wild type. Both compound 1 and Fr 30 had little effect on the growth of *M. bovis* BCG Δnat.](image-url)
5.5. Prospects

A preliminary experiment similar to that described in section 5.5, carried out by a colleague at Oxford, showed that *M. tuberculosis* H37Rv was more susceptible (Compound 1: MIC 20-40μg/ml) than *M. bovis* (Compound 1: MIC 80-100μg/ml). Due to the limited amounts of compound 1 and delays in getting license to work with live *M. tuberculosis* culture in the UK, these experiments could not be carried out fully and the toxicities of these agents against mammalian cell lines have not been determined. These pending investigations are recommended in the future, once these compounds are synthesized chemically. Synergy between compounds 1 and compound 2 with respect to activities against *M. bovis* BCG and MSNAT has been suggested and will be investigated in the future.
Chapter 6

Compound 1 & 2: Structural elucidation
6.1. Introduction

Previously, from Warburgia salutaris, a series of antibacterial drimane sesquiterpenoids of a characteristic chemical skeleton, including a novel drimane sesquiterpenoid lactone, salutarisolide [3, figure 6.1.1] have been isolated (Mashimbye et al., 1999).

![Diagram](image)

Figure 6.1.1: The chemical structure of salutarisolide. Salutarisolide is an anti-bacterial sesquiterpenoid lactone from Warburgia salutaris (Mashimbye et al., 1999)

Though powerful NMR spectroscopic tools such as COSY, NOESY, HMBC and HSQC are used in the characterization of organic compounds, the identification of sesquiterpenes and diterpenes “one skeleton (of several possible) is implicitly assumed on the basis of the source and the structure of known co-occurring compounds” (Mann et al., 1994). Therefore, the NMR techniques used serve to confirm the general structure of the unknown sesquiterpene and in addition identify any other novel groups or stereochemistry.
6.2. Identification of compound 1 and 2

The identity of compound 1 (Figure 6.2.1) was determined by mass spectrometry, infrared spectrometry, specific rotation and $^1$H, $^{13}$C, HSQC, COSY, HMBC and NOESY NMR experiments and supported by data of related compounds from *Warburgia salutaris* (Mashimbye et al., 1999) and other Canellaceae plants from which sesquiterpenes have been isolated, such as *Cinnamosma fragans* Baillon (Canonica et al., 1969a & 1969b).

**Figure 6.2.1:** The chemical structure of compound 1 was determined by mass spectrometry, infrared spectrometry, specific rotation and $^1$H, $^{13}$C, HSQC, COSY, HMBC and NOESY NMR experiments and supported by data of related compounds from *Warburgia salutaris* (Mashimbye et al., 1999). (A) depicts the absolute and relative stereochemistry and (B) represents the three dimensional conformation of the molecule.
The results of the NMR experiments showing the scalar and spatial correlations between the protons of compound 1 are summarized in the following tables (6.2.1. and 6.2.2).

**Table 6.2.1:** $^1$H, HSQC and HMBC data of Compound 1, in MeOH-d$_4$

<table>
<thead>
<tr>
<th>Proton</th>
<th>COSY</th>
<th>NOESY</th>
</tr>
</thead>
<tbody>
<tr>
<td>1$\alpha$</td>
<td>1$\beta$, 2$\beta$, 2$\alpha$</td>
<td>H-2$\alpha$, H-3$\alpha$</td>
</tr>
<tr>
<td>1$\beta$</td>
<td>1$\alpha$, 2$\alpha$</td>
<td>H-11, CH$_3$-13</td>
</tr>
<tr>
<td>2$\alpha$</td>
<td>1$\alpha$, 2$\beta$, 3$\beta$</td>
<td>H-1$\alpha$, H-3$\alpha$</td>
</tr>
<tr>
<td>2$\beta$</td>
<td>1$\alpha$, 2$\alpha$, 3$\alpha$</td>
<td>H-3$\beta$, CH$_3$-13, CH$_3$-14</td>
</tr>
<tr>
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<td>2$\beta$, 3$\beta$</td>
<td>H-1$\alpha$, CH$_3$-15</td>
</tr>
<tr>
<td>3$\beta$</td>
<td>2$\alpha$, 2$\beta$, 3$\alpha$</td>
<td>H-2$\beta$, CH$_3$-14</td>
</tr>
<tr>
<td>4</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>-</td>
<td>H-6</td>
</tr>
<tr>
<td>6</td>
<td>H-5, H-7</td>
<td>H-5, H-7, CH$_3$-15</td>
</tr>
<tr>
<td>7</td>
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</tr>
<tr>
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</tr>
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<td>12</td>
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<td>-</td>
</tr>
<tr>
<td>13</td>
<td>-</td>
<td>H-1$\beta$, H-2$\beta$, CH$_3$-14</td>
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</tr>
<tr>
<td>17</td>
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</table>
Table 6.2.2: Scalar and Spatial Couplings of the protons of Compound 1

<table>
<thead>
<tr>
<th>Proton</th>
<th>$\delta_H$ (ppm)</th>
<th>$J$ (Hz)</th>
<th>HSQC</th>
<th>HMBC</th>
</tr>
</thead>
<tbody>
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<td>1α</td>
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<td>m</td>
<td>31.9</td>
<td>t</td>
</tr>
<tr>
<td>1β</td>
<td>1.35</td>
<td>m</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2α</td>
<td>1.55</td>
<td>m</td>
<td>17.9</td>
<td>t</td>
</tr>
<tr>
<td>2β</td>
<td>1.73</td>
<td>m</td>
<td>-</td>
<td>C-4, C-10</td>
</tr>
<tr>
<td>3α</td>
<td>1.32</td>
<td>d (2.5)</td>
<td>44.7</td>
<td>t</td>
</tr>
<tr>
<td>3β</td>
<td>1.45</td>
<td>d (12.5)</td>
<td>-</td>
<td>C-4</td>
</tr>
<tr>
<td>4</td>
<td>-</td>
<td>-</td>
<td>33.6</td>
<td>s</td>
</tr>
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<td>5</td>
<td>2.13</td>
<td>d (4.0)</td>
<td>45.6</td>
<td>d</td>
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<td>C-4, 14-CH₃, 13-CH₃</td>
</tr>
<tr>
<td>6</td>
<td>5.79</td>
<td>t (4.5)</td>
<td>66.6</td>
<td>d</td>
</tr>
<tr>
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<td></td>
<td></td>
<td></td>
<td>C-5, C-7, C-8, C-10, C-16</td>
</tr>
<tr>
<td>7</td>
<td>6.69</td>
<td>d (2.5)</td>
<td>133.7</td>
<td>d</td>
</tr>
<tr>
<td>8</td>
<td>-</td>
<td>-</td>
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<td>s</td>
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<td>-</td>
<td>39.0</td>
<td>s</td>
</tr>
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<td>11</td>
<td>5.87</td>
<td>brs</td>
<td>99.4</td>
<td>d</td>
</tr>
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<td>12</td>
<td>-</td>
<td>-</td>
<td>168.0</td>
<td>s</td>
</tr>
<tr>
<td>13</td>
<td>1.21</td>
<td>s</td>
<td>18.7</td>
<td>q</td>
</tr>
<tr>
<td>14</td>
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<tr>
<td>15</td>
<td>1.04</td>
<td>s</td>
<td>32.4</td>
<td>q</td>
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<td>170.7</td>
<td>s</td>
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<td></td>
<td></td>
<td>C-16</td>
</tr>
</tbody>
</table>
The HRCIMS of compound 1 showed a molecular ion at m/z 342.1914 [M+NH₄]⁺ together with a suggested isotopic composition of C₁₇H₂₄O₆, calculated for 342.1916. In general, the proton NMR spectra of compound 1 showed resonances due to three tertiary methyl groups, a strong acetoxy peak, one olefinic proton and no aldehyde signals.

The identity of compound 2 (Figure 6.2.2) was determined by mass spectrometry and ¹H NMR experiments coupled with the assumed biogenetic relationship to compound 1.

![Figure 6.2.2: The chemical structure of compound 2. Determined mass spectrometry and ¹H NMR experiments coupled with the assumed biogenetic relationship to compound 1.](image)

The HRCIMS of compound 2 showed a molecular ion at m/z 309.1700 [M+H]⁺ together with a suggested isotopic composition of C₁₇H₂₄O₅, calculated for 309.1701. The NMR spectrum of compound 2 was similar to that of compound 1 except for the presence of the two characteristic aldehyde peaks at δH 9.48 and δH 9.76.
6.2.1. Interpretation of NMR data

The construction of the skeleton of compound 1 starts with the proton NMR resonance at $\delta_{\text{H}}$ 5.79 (1H, t, $J_1 = J_2 = 4.5$Hz). The coupling constant of this triplet due to the C-6 proton (H-6) is similar to that reported by Canonica et al. (1969b) for H-6 of the sesquiterpene lactone, cinnamosmolide [4, figure 6.2.1.1, C-6 proton resonance at $\delta_{\text{H}}$ 5.80, t, $J_1 = J_2 = 4.3$Hz]. Theoretically, this proton at C-6 should resonate as a double doublet giving rise to 4 peaks as in the case of bumarivolide [5, figure 6.2.1.1] (Canonica et al., 1969a) due to its splitting by the two neighbouring protons at C-5 and C-7. However, in practice the two centre peaks do not resolve, hence we report a triplet.

![Chemical structures of compounds 4 and 5](image)

**Figure 6.2.1.1:** Sesquiterpene lactones. The chemical structures of sesquiterpene lactones from *Cinnamosma fragans*. Cinnamosmolide (4) and bumarivolide (5) (Canonica et al., 1969).
In the COSY spectrum, H-6 correlated with the vicinal protons at δ_H 6.69 (1H, d, J = 2.5Hz) and at δ_H 2.13 (1H, d, J = 4.0Hz) assigned as H-7 and H-5 respectively. The HMBC spectrum showed that H-6 correlated with C-5 and C-7, together with C-10 and C-16. The deshielding of C-16 is typical of a carbonyl carbon, resonating at δ_C 170.7. Furthermore, the OAc protons δ_H 2.10 (3H, s) correlated with C-16; hence the OAc group is attached at C-6. HSQC showed that the OAc protons were attached to the carbon at δ_C 20.4 (C-17).

The special correlations of H-6 with H-5 and H-7 as shown in the NOESY spectrum meant H-5 and H-6 have an α-orientation. The resonance of H-7 is typical of an olefinic proton conjugated to a carbonyl group. When isolated, the olefinic protons should resonate between δ_H 4.00 and δ_H 5.00. The IR spectrum of compound 1 has confirmed the presence of a lactone carbonyl group (1746 cm⁻¹) [Harwood and Claridge, 1997]. The presence of this lactone carbonyl group together with the UV absorption maximum at 213 suggested an α,β-unsaturated-γ-lactone (Canonica et al., 1969a). If one considers the general structure of known sesquiterpenoid lactones and the resonance of the olefinic proton (H-7); then the lactone carbonyl group is at position 12, resonating at δ_C 168.0 (C-12).

HSQC showed that H-5, H-6 and H-7 were attached to carbons at δ_C 45.6 (C-5), δ_C 56.6 (C-6) and δ_C 133.7 (C-7) respectively. The other olefinic carbon (C-8) is also deshielded and resonated at δ_C 134.1, confirmed by an HMBC correlation with H-6. In the NOESY spectrum, H-6 showed a strong correlation with the
methyl protons at δH 1.04 (3H, s), meaning that they are less than 5Å apart and hence it was assigned as CH3-15α, which must be attached to C-4, at δC 33.6. The protons of CH3-15α were further coupled to C-4, C-5 and C-14 (assigned below) as shown by HMBC. CH3-15α correlated with the proton multiplet at δH 1.32 (1H, m) [NOESY], thus identifying H-3α. In the COSY spectrum H-3α is in turn coupled to a proton at δH 1.45 (1H, d, J = 12.5Hz) assigned as H-3β as a coupling constant of this magnitude is usually geminal, seen between the axial and the equatorial protons attached to the same proton in a closed cyclohexane ring (Harwood and Claridge, 1997).

HSQC showed that H-3α and H-3β were linked to the same carbon at δC 44.7 (C-3). A COSY coupling of H-3α with the proton at δH 1.73 (1H, m), identified H-2β. HSQC showed that H-2β and the multiplet at δH 1.55 (1H, m) were attached to the same carbon at δC 17.9 (C-2), hence this multiplet was assigned as H-2α. H-2β and H-3β are both coupled to the methyl at δH 1.20 (1H, s), designated as CH3-14β. The two C-2 protons correlated to the proton at δH 2.05 (1H, s), assigned as H-1α. H-1α is deshielded due to the through space inductive effect of the OH group at C-9. C-9 is more deshielded than C-10 due to the inductive effect of the tertiary OH group immediately attached to it and hence its resonance is at δC 75.8 while the one for C-10 is at δC 39.0. H-1α is coupled to the proton multiplet at δH 1.35 (1H, m), assigned as H-1β. HSQC showed that H-1α and H-1β are attached to the same carbon at δC 31.9 (C-1).
NOESY showed that H-1β is in close proximity to the H-11 proton at δH 5.87 (1H, s), and the methyl at δH 1.19 (1H, s), CH3-13β. H-11 and C-11 (δC 99.4) are very deshielded, due to the combined inductive effects of the adjacent hydroxyl and acyloxy groups. HSQC showed that the three methyl groups, CH3-13β, CH3-14β and CH3-15α are attached to the carbons at δC 18.7, δC 24.0 and δC 32.4 respectively.

6.3. Anti-tubercular Sesquiterpenes

Sesquiterpene lactones of the germacranoide, guaianolide and eudesmanolide type have been tested against Mycobacterium tuberculosis with minimum inhibitory concentrations (MIC) ranging between less than 128μg/ml and 2μg/ml. It has been found that if they posses an α-methylene-γ-lactone ring on their structure together with another alkylating group such as an epoxide or a conjugated carbonyl group, then they possess MICs of 64μg/ml and less (Cantrell et al., 2001). The same review article by Cantrell et al. (2001) reveals that the sesquiterpenoid lactone that exhibited the highest activity (MIC=2μg/ml) against Mycobacterium tuberculosis was the lipophilic dehydrocostuslactone (6, figure 6.3.1). The two new sesquiterpenoids with potential antitubercular activity, (1S,4S,5R,10R)-1-hydroxy-6-isobutyryloxy-10H-9-oxofuraneremophilane (7), and 1α-hydroxy-6β-(2,5-methylbutyryloxy)-10αH-9-oxofuraneremophilane (8) have been isolated from the n-hexane and dichloromethane extracts of the roots of Senecio chionophilus (Gu et al., 2004) and are illustrated in figure 6.3.1.
Figure 6.3.1: Structures of anti-tubercular sesquiterpenoids. dehydrocostus lactone (6), (1S,4S,5R,10R)-1-hydroxy-6-isobutyryloxy-10α,9-oxofuranooeremophilane (7), and 1α-hydroxy-6β-(2,4-methylbutyryloxy)-10α,9-oxofuranooeremophilane (8).

6.4. Other Sesquiterpenes

Sesquiterpenoids have a wide range of activities ranging from anti-bacterial, anti-cancer, anti-HIV and anti-fungal. Sesquiterpenoids with antibacterial and antifungal properties have been isolated from Warburgia species. These sesquiterpenoids include warburganal, polygodial (Kubo and Himejima, 1992), muzigadial (Rabe and van Staden, 2000), cinnamodial (Brooks and Draffan, 1969) and cinnamosmolide (Canonica et al., 1969b). Drimane sesquiterpenoids have been isolated from the bark Cistus creticus ssp. creticus (Hatzellis et al., 2004), from the bark of Taxus yunnanensis (Nguyen et al., 2003) and from the leaves of Warburgia stuhlmannii (Manguro et al., 2003). Derivatives of polygodial have been isolated from the bark of Drimys winterii Forst (Rodriguez et al.,
Several sesquiterpenes from *Stylotella aurantium* have been patented as anti-tumor agents (Fraga, 2002). Sesquiterpenes have also been found in the fungus, *Botrytis cinerea* (Collado *et al*., 1996) and possibly in algae (Elias *et al*., 1997).

### 6.5. Biosynthetic Pathway of Sesquiterpenoids

The "biogenetic isoprene rule" of Ruzicka (Mann *et al*., 1994) states that each member of a terpenoid sub-type, be it monoterpoid or sesquiterpenoid, is derived from a single compound that is unique to that group. Basically sesquiterpenoids are made from the enzyme-mediated cyclization, functionalization and sometimes rearrangements of 2E,6E-farnesyl pyrophosphate (FPP), a 15 carbon membered compound (Bohlmann *et al*., 1998). FPP itself is formed in the cytoplasm or endoplasmic reticulum from mevalonic acid. Mevalonic acid is in turn formed via a sequence of steps including the Claisen ester condensation of two molecules of acetyl-S-CoA by the acetyl-S-CoA thiolase followed by the synthesis of hydroxymethylglutaryl-S-CoA (HMG-S-CoA) by the HMG-S-CoA synthase and the reduction of HMG-S-CoA by HMG-S-CoA reductase (Mann, 1994). Mevalonic acid is pyrophosphorylated to produce MVA-5-pyrophosphate and this compound upon decarboxylation forms isopentenyl pyrophosphate (IPP), the first 5-carbon unit of terpenoid synthesis. Isomerization enables another five-carbon unit, 3,3- dimethylallyl pyrophosphate (DMAP). DMAP is ionized to form a highly electrophilic ion pair, which undergoes
nucleophilic attack by IPP to form geranyl pyrophosphate. Geranyl pyrophosphate condenses with IPP to form farnesyl pyrophosphate, a precursor of most sesquiterpenoids. A schematic representation of the mevalonic pathway of terpenoid and sterol synthesis is shown in figure 6.5.1.

![Diagram of the mevalonic acid biosynthetic pathway.](image)

**Figure 6.5.1**: The Mevalonic acid biosynthetic pathway. Biosynthetic pathway of sterols, carotenoids and sesquiterpenoids from mevalon acid (MVA).

There has been considerable interest in the cloning and expression of genes encoding terpenoid synthases (cyclases) and those encoding the HMG-SCoA reductases. Genes of three HMG-SCoA reductases from *Capsicum annuum* have been characterized (Ha et al., 2003). The 5-Epi-Aristolochene synthase from *Nicotiana tabacum* has been cloned, expressed and shown to convert FPP into a sesquiterpenoid known as 5-Epi-Aristolochene, a precursor of an antifungal
phytoalexin capsidiol (Back et al., 1994). The 3D structure of this enzyme has been elucidated from x-ray data and shows a hydrophobic pocket at the active site where an analog of FPP, farnesyl hydroxyphosphate (FHP) binds.

Ongoing research on the expression of the terpene synthases has suggested that Saccharomyces cerevisiae or yeast could be a better expression system than E.coli. The best yields of the sesquiterpenoid epi-cedrol by genetically modified epi-cedrol synthase and HMG-SCoA reductase system in yeast cells was 370μg/L, a figure six times more than the yields obtained in E.coli for other sesquiterpenoids (Jackson et al., 2003). However, these yields are still not practical for industrial purposes. More work still needs to be done because this is a very interesting field with respect to production of essential terpenoids on an industrial scale by fermentation for cosmetic, pharmaceutical and agricultural purposes.

6.6. Discussion

Sesquiterpenoid lactones, though they have been shown to possess antitubercular properties, have not been tested against the NAT enzyme and there is no structure activity data available at present. These sesquiterpenoid lactones may well be inhibitors of human NATs and tests need to be carried out on compound 1 and 2 to determine their effect on the human NATs. Sesquiterpenoid lactones that are specific and selective inhibitors of the bacterial NATs are most likely to be useful.
Chapter 7

Research Summary and Conclusions
7.1. Research Summary

Tuberculosis is prevalent in Sub-Saharan Africa and is the leading cause of mortality in HIV infected individuals. Globally, 1.7 million deaths were due to tuberculosis in 2003; the latest death statistics for South Africa indicate that there is an increase in deaths caused by tuberculosis. The emergence of resistance to existing drugs and the increasing Human Immunodeficiency Virus infection, especially in Sub-Saharan Africa interferes with current tuberculosis control measures. Novel drugs as much as novel target proteins are sought in order to control tuberculosis that does not respond to current cures. Natural compounds provide such diversity of chemical entities that can be developed into anti-tubercular drugs.

In an effort to identify potential anti-tubercular natural compounds, an initial screen of several South African medicinal plants that were identified during a field trip in November of 2000 was conducted. In that screen and in subsequent investigations it has been shown that a crude dichloromethane extract of *Warburgia salutaris* bark exhibited anti-mycobacterial action at a concentration of 50μg/ml but not against *Escherichia coli*, a gram-negative bacterium even at 100μg/ml. *Warburgia salutaris* is a commonly used medicinal plant in South Africa such that this “tree of the year 1996” is endangered. The bark of *Warburgia salutaris* is used as an expectorant for coughs, and as a natural antibiotic to treat chest infections productive of purulent sputum, venereal diseases and constipation. It is also used to treat cancer, rheumatism and stomach ulcers. It is applied to cuts on temples for
headaches and has been used as an aphrodisiac. Leaves of *Cannabis sativa* L. mixed with powdered bark of *Warburgia salutaris* are smoked for dry coughs.

A novel target of mycobacteria, the arylamine N-acetyltransferase (NAT) is crucial for mycobacterial survival. Independent genetic studies of the NAT in *Mycobacterium bovis* BCG have shown that knocking out the *nat* interferes with the integrity of the bacterial cell wall, slows the growth of the organisms, increases the susceptibility of the bacilli to antibacterials and increases intracellular killing of the bacilli inside the macrophages. Identifying natural compounds that would induce a similar phenotype in *M. bovis* BCG and ultimately in *Mycobacterium tuberculosis* would have implications for anti-tubercular intervention. The crude extract inhibited the activity of the *Mycobacterium smegmatis* arylamine N-acetyltransferase (IC50=22μg/ml). Transmission Electron Microscopy showed that a sub-fraction (Fr 30) of the crude extract from which two active purified agents were isolated affected *M. bovis* BCG cell wall integrity in a manner similar to that which was observed in the genetic *nat* knockout strain of *M. bovis* BCG (*M. bovis* BCG Δnat). In addition, quantitative evidence has shown clearly that treatment of *M. bovis* BCG with crude extract, its sub-fractions and the purified agents induced a phenotype similar to that of *M. bovis* BCG Δnat. These findings have implications for antibiotic entry into the tubercle bacillus and intracellular killing of the bacilli by macrophages.
A Bioassay-guided fractionation process using a combination of Solid Phase Extractions, analytical and preparative High Performance Liquid Chromatography has been used to isolate and purify agents that simultaneously inhibit the growth of *M. bovis* BCG and the activity of *M. smegmatis* arylamine N-acetyltransferase.

The two purified anti-mycobacterial agents are: a novel sesquiterpenoid lactone compound 1 (C_{17}H_{24}O_{6}) and a known drimane sesquiterpenoid dialdehyde, compound 2 (C_{17}H_{24}O_{5}) isolated for the first time from *Warburgia salutaris* which affected growth of *M. bovis* BCG at concentrations of 50μg/ml. Compound 1 specifically inhibited the arylamine N-acetyltransferase in *M. bovis* BCG. Chemical synthesis of these natural compounds would be a favorable source as yields obtained from the plant material were low, 0.015% for compound 1 and 0.002% for compound 2. All the activities mentioned above would need to be verified once these agents have been synthesized chemically and their toxicities should be determined before any further development takes place.

7.2. Research Prospects

*Warburgia salutaris* was identified in May 2005 at the Centurion Lake Declaration as one of the medicinal plants that need to be researched. *Warburgia* species and Callenaceae family have yielded several antibacterial sesquiterpenoids. Sesquiterpenoids from *Warburgia salutaris* have never been investigated for anti-tubercular action. Yet several anti-tubercular
sesquiterpenoid lactones from other natural products have been reported in the literature. This creates a new resource for the identification and development of novel anti-tubercular agents from Warburgia species.

The synthetic route of compound 2 has been reported in the literature. Though it is a lengthy and complex method, it can be modified and used in obtaining these agents in large amounts. Biosynthesis of sesquiterpenoids in genetically modified organisms has been reported and is another area that needs investigation. Sesquiterpenoids that target the arylamine N-acetyltransferase of mycobacteria could be crystallized with the enzyme and better inhibitors modeled in order to develop better and more selective anti-tubercular agents.

Sesquiterpenoids have been reported to possess other activities such as anticancer, antifungal antibacterial and anti-plasmodial. They are components of aromatic oils and have been used in perfumery and as spices. There is potential for the development of innovative and commercial agents from these agents such as drugs, perfumes and spices.

**7.3. Conclusions**

This investigation of *Warburgia salutaris* revealed a novel drimane sesquiterpenoid lactone and a known drimane sesquiterpenoid dialdehyde, cinnamodial. Both these natural anti-tubercular compounds are reported for the first time from *Warburgia salutaris*. 
The specific activity of this novel drimane sesquiterpenoid lactone against the arylamine N-acetyltransferase, an enzyme involved in cell wall mycolic acid biosynthesis in *M. bovis* BCG has implications for susceptibility of tubercle bacillus to killing by antibacterial drugs and by macrophages in the host.
Chapter 8

Materials and Methods
All organic solvents used for plant extractions were purchased either from Scharlau, Merk, or Fisher Scientific and were of highest purity (HPLC grade). General chemicals were purchased from sigma unless stated otherwise.

8.1. Preparation of Middlebrook 7H9 (M7H9) Broth
2.35g of 7H9 broth powder (Difco) was dissolved in 450ml of double distilled water containing 1ml of glycerol (0.2% v/v, Sigma) and 0.25g of Tween 80 (0.05% wt/v, Sigma) in a 500ml glass bottle. The bottle was shaken well to thoroughly mix the contents. Then the resulting solution was autoclaved at 121-124°C for 10-12 minutes.

8.2. Preparation of Middlebrook 7H10 (M7H10) Agar
9.5g of the powder (Difco) was dissolved in 450ml of double distilled water containing 2.5ml of glycerol (0.5% v/v, Sigma) in a 500ml glass bottle. The contents were mixed thoroughly and heated in a microwave with frequent agitation until it started to boil. Once the contents were completely dissolved, the solution was autoclaved at 121-124°C for 10-12 minutes.

8.3. Preparation of a new batch of the crude CH₂Cl₂ extract
The bark of *Warburgia salutaris* that Dr. Crouch collected from the Silver Glen Nursery was cut into small pieces (ca. 0.5mm) then air-dried at room temperature in a ventilated room for 7 days. Once dried the pieces of bark were ground into a fine powder using a blender in order to facilitate an
exhaustive extraction. The dried powdered material (1.3kg) was exhaustively extracted in methanol followed by another exhaustive extraction in dichloromethane. The resulting dichloromethane extract was concentrated using a rotary evaporator (Buchi) at a low temperature of 40°C and then dried under inert nitrogen gas and finally in a desiccator. Once completely dry the dichloromethane extract of the authenticated bark (15g) was kept in a sealed bottle in a dark cupboard at room temperature until use. All preparations for subsequent screens of this natural product were done using this new batch.

8.4. Preparation of M. tuberculosis H37Rv cultures

Technologists at the Groote Schuur Hospital TB diagnostic laboratory performed this experiment, in my presence using the Bactec 460® method, which is a gold standard for M. tuberculosis diagnostic purposes. Four Bactec 460® vials were prepared; each contained 3800μl of broth that had radiolabelled 14C-palmitate as a source of carbon for growing M. tuberculosis H37Rv. M. tuberculosis H37Rv was inoculated into these vials and allowed to grow at 37°C for several days, the released radiolabelled carbon dioxide (14CO2) would be measured using the Bactec 460® instrument as a growth index between the range of zero to the highest detection limit of 999. Usually the organisms will grow very slowly for a few hours (about 36hrs) then start growing exponentially (Log phase culture); then after about 168 hours they will stop growing exponentially, the growth index approaching 999 (Stationary phase culture).

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In each of the 4 Bactec 460® vials, *M. tuberculosis* H37Rv (100µl) culture that had grown to the maximum growth index of 999 was added by injection using a sterilized syringe.

**8.5. Preparation of crude extract for Bactec® method**

As we knew that a concentration of 500µg/ml of crude bark extract exhibited anti-mycobacterial action, we decided to reduce the concentration for subsequent screens to 200µg/ml. The crude bark extract (1.6mg) from the Silver Glen batch was dissolved in 200µl of 100% acetonitrile in a glass vial and was submitted for testing using the Bactec 460® method. Half of the crude extract solution (100µl) was injected in one of the Bactec 460® vials. In each of the 3 remaining vials, an aliquot (100µl) of either one of the following: 100% acetonitrile (a solvent control), TB dilution buffer (untreated cells) or isoniazid (a positive control) to the final concentration of 0.05 µg/ml [MIC of isoniazid for *M. tuberculosis* H37Rv]. The vials were incubated at 37°C for several days and readings were taken each day at the exact time using the Bactec 460® instrument. A plot of growth index versus time (hours) was prepared in order to graphically determine the effect of the crude dichloromethane bark extract of *Warburgia salutaris* on the growth of *M. tuberculosis* H37RV.
8.6. Preparation of *M. bovis* BCG for growth curves

An *M. bovis* BCG Pasteur culture was prepared from a glycerol (25%) stock, which was stored frozen in a cryo-vial at -70°C. One milliliter (1ml) of this stock was transferred into a 100ml tissue culture treated roller bottle (Corning®), containing 100ml of Middlebrook® 7H9 broth supplemented with 10ml of aseptically added ADC (Albumin, Dextrose, Catalase; Difco) enrichment (M7H9ADC). The bottle was incubated at 37°C for about 5-6 days with constant mixing until the cells reached the mid log phase of growth (OD = 1). Fresh M7H9ADC broth (99ml) was added into four tissue culture treated roller bottles, then 1ml of the mid log phase culture was added in each of the bottles to create a 1% of the mid log phase culture.

8.7. Preparation of crude extract for growth curves

The crude bark extract was prepared to the stock solution of 10mg/ml in 100% acetonitrile. An aliquot (100μl) of 100% acetonitrile or M7H9ADC broth was added in either of two bottles. In the other 2 bottles, an aliquot of 100μl or 20μl of the 10mg/ml stock solution of crude bark extract were added to yield the final concentrations of 100μg/ml and 20μg/ml respectively. The bottles were incubated at 37°C for 7 days. Optical density readings were taken every 12 hours from each bottle in duplicate using a spectrophotometer (Eppendorf, BioPhotometer). A plot of Growth (in terms of OD) versus time (Hours) was prepared in order to determine whether the crude extract affects *M. bovis* BCG growth.
8.8. Ziehl – Neelsen hot staining procedure

The organisms (20μl) were spotted on microscope slides in duplicate and were air-dried and fixed on them by heating the slide moderately in an oven at 100-110°C for 15 minutes. The slides were flooded with carbol fuchsir red dye to cover the slide for 5 minutes. The stain was discarded and they were rinsed with double distilled water until the water that runs off was colorless. They were tilted on a tissue paper, to dry, in between each of the steps. The second time they were flooded with acid decolorizing solution for about 10 to 15 seconds. The solution was washed off completely with double distilled water. Lastly, a counter stained with Malachite green solution was performed for 1 minute. Then, the slides were washed with double distilled water and dried. The organisms were visualized under oil using a light microscope (Olympus BH2) under 1000 magnitudes of magnification.

8.8.1. Preparation of stained cells for photography

Dako® Glycergel mounting medium was liquidified by immersing the reagent bottle in a 50°C water bath for 5 minutes. Then the liquid was spotted on top of the fixed organisms on the microscope slide. A cover slip was carefully put on top making sure the mounting medium was spread out over the whole smear. The slide was left for 24 hours to glue. Pictures were then taken using a digital camera (Nikon).
8.9. *Warburgia salutaris* extract vs *E. coli*

An overnight culture of *E. coli* JM109 was prepared from glycerol stocks in fresh LB broth. One milliliter (1ml) of the overnight culture (OD=1.9) was transferred into 100ml of LB broth into 3 conical flasks. In each of these flasks 100µl of either LB broth, 100% acetonitrile or extract (10mg/ml stock) was added. The flasks were then incubated at 37°C with constant shaking. OD readings were taken every 30 minutes for 7 hours. A plot of growth versus time (hours) was prepared to graphically determine the effect of the crude extract on the growth of *M. bovis* BCG.

8.10. Protein expression and purification

8.10.1. Expression of MSNAT

The gene for the open reading frame of *Mycobacterium smegmatis* N-acetyltransferase containing an N-terminal hexa-histidine tag (Hexa-his-tag) was cloned in a pET28b vector which was transformed into *E.coli* BL21(DE3)pLysS cells for expression, as described (Payton *et al.*, 1999). Glycerol stocks were prepared and kept at -70°C until use; cells were transferred from the glycerol stock into 100ml of LB media containing 34µg/ml of Chloramphenicol and 30µg/ml of Kanamycin and incubated for 16 hours at 37°C with shaking. The resulting culture was inoculated into 3 flasks containing in total a litre of LB media containing 1.0M Sorbitol, 2.5mM Betaine, 34µg/ml of Chloramphenicol and 30µg/ml of Kanamycin. The flasks were incubated at 37°C with constant shaking until the bacteria had grown to an optical density of 0.4-0.6 at 600nm. The temperature was dropped to 27°C and 30 minutes
later at this temperature; cells were induced by the addition of isopropyl β-D-thiogalactopyranoside (0.25mM) in each flask. Cultures were allowed to grow at this temperature for another 16 hours with constant shaking.

8.10.2. Harvesting of cells

Cells were centrifuged at a speed of 5000g for 20min at 4°C. The supernatant was discarded and the pellets were resuspended in 50ml of 20mM Tris.HCl pH 8.0, 300mM NaCl pH 8.0 and 1mM Pefabloc SC (Pentapharm). The cells were then frozen at -70°C to facilitate the subsequent lysis. Frozen cells were thawed at 37°C then sonicated on wet ice using twenty bursts of 45 seconds on, 30 seconds off as described previously (Sinclair et al., 2000). Then, 1ml of a 10mg/ml solution of lysozyme was added to the final concentration of 0.5mg/ml. Lysed cells were centrifuged at a speed of 12000g for 20 minutes at 4°C. The resulting whole lysate was used for the subsequent enzyme purification process, while the pellets were discarded.

8.10.3. Purification of recombinant MSNAT

The soluble whole lysate was loaded onto 6mls of Ni-NTA agarose (Qiagen), which has been thoroughly washed with double distilled water. The mixture was incubated at 4°C for 2 hours rotating at 15 revolutions per minute to allow the necessary binding of proteins on the resin. The agarose was pelleted by centrifugation at a speed of 3000g for 5min at 4°C. The supernatant consisting of unbound material was removed and then after, the protein was eluted by washing with 2 X 25ml volumes of increasing concentrations of imidazole
(1mM, 10mM, 50mM and 250mM) in 20mM Tris.HCl pH 8.0, 300mM NaCl pH 8.0.

8.10.4. Detection of protein using SDS-PAGE

An aliquot (20μl) of fractions of each of the whole lysate, unbound material and each of the 4 imidazole eluates were added to 20μl of 5 times concentrated Sample Preparation Buffer (SPB) or Denaturation Buffer (DB). These mixtures, including a sample of BioRad® molecular weight markers that was already prepared in Sample Preparation Buffer, were heated to 95°C for 10 minutes to denature the proteins. Each of the denatured samples (10μl) was loaded into an individual well of the stacking gel. The gel was run at 100mV until the bromphenol blue dye reached the bottom of the separating gel. This separating gel was then stained with Comassie blue for 5 minutes at room temperature. It was then destained with a solution containing 0.5% Methanol and 0.75% Glacial acetic acid for several days. The gel was used to determine the purity of the NAT enzyme.

8.10.5. Dialysis

The purified recombinant protein contained in the 50mM imidazole wash was dialysed against 100 volumes (5L) of dialysis buffer (20mM Tris.HCl pH 8.0, 1mM EDTA pH 8.0 and 1mM DTT) at 4°C for 16-18 hours. DTT (dithiothreitol; Cleland's reagent) reduce disulfide bonds quantitatively and maintain monothiols in the reduced state (Cleland, 1964).
8.10.6. Cleavage of the His-Tag

There are 20 extra amino acids on the N-terminus of the recombinant MSNAT protein due to the histidine tag (Sandy et al., 2002). To cleave the hexa-his-tag from the purified recombinant protein, a protease called thrombin was used. Five (5) units of thrombin per mg of protein were added. The cleavage was allowed to take place at 4°C overnight. The thrombin reaction was inactivated by adding to the 50mls of purified recombinant protein solution, 1ml (1M) of pefabloc to final concentration of 20mM.

The protein was dialysed against 100 volumes (5L) of dialysis buffer (20mM Tris.HCl pH 8.0, 1mM EDTA pH 8.0 and 1mM DTT) at 4°C for 16-18 hours. The protein solution was then concentrated using an Amicon-10® concentrator by centrifugation at a speed of 3000g for to-15 minutes at 4°C until the final concentration was 6mg/ml. The concentrated protein solution was kept at 4°C and was used within 30 days. To confirm that cleavage had taken place, SDS-PAGE was carried out as outlined in section 4.3.1.4 above. Figure 4.3.1.6.1 illustrates the difference in the amino acid sequences for the MSNAT protein when the histidine tag is present or removed.

8.10.7. Determination of MSNAT activity

8.10.7.1. The CoA Standard Curve

CoA, a product in the NAT reaction, is commercially available and has been used to detect acetyl-CoA hydrolysis in the presence of arylamine substrates and inhibitors (Brooke et al., 2003a). To plot a standard curve, different
concentrations of CoA ranging from 0-500\,\mu M were prepared in 20mM Tris.HCl pH 8.0, using a 96-well plate format. A blank, containing 125\,\mu l 20\,mM Tris.HCl pH 8.0; a control, where 100\,\mu l 20\,mM Tris.HCl pH 8.0 was added and tests, with 100\,\mu l different concentrations of CoA were prepared. Then 25\,\mu l of 5mM DTNB in 6.4M Guanidine-HCl, 0.1M Tris.HCl pH 7.3, was added in the control and test wells before the spectrophotometric measurements were done on the plate reader at a wavelength of 405\,nm (Anthos 2020); all experiments were done in triplicate. A plot of OD versus CoA concentration was prepared (Figure 4.3.2.1.1). This plot gives a direct way of determining CoA concentration in an unknown sample at any given OD within the OD range of the standard curve. For the purposes of the subsequent enzyme activity assays, the CoA standard curve was specifically used to determine the amount of CoA produced by MSNAT per one OD unit.

8.10.7.2. Dilution of DTT concentration

Brooke et al. (2003a) has reported that when a solution of CoA (20mM Tris–HCl, pH 8.0, 100 \,\mu L) is treated with DTNB solution (6.4M guanidine–HCl, 0.1M Tris–HCl, pH 7.3, 25 \,\mu L) in a flat-base polystyrene 250 \,\mu L 96-well plate, which has a liquid depth in the centre (or path length) of 3.9mm, the TNB has a molar extinction coefficient (\varepsilon) of 8.5 \,mmol\(^{-1}\) \,dm\(^3\) \,cm\(^{-1}\) at \lambda_{\text{max}} of 405 \,nm. DTT, the reducing agent that stabilizes the enzyme interferes with the MSNAT assay. When DTNB is added to DTT (1mM) under the buffer conditions above, it releases two equivalents of TNB with a molar absorptivity or extinction
coefficient of 13.6 mmol$^{-1}$dm$^{-3}$cm$^{-1}$ at a wavelength of 405 nm. According to the Beer-Lambert Law, absorbance or the optical density of a solution at a given wavelength is proportional to the extinction coefficient. If the extinction coefficient is large then so will the absorbance be.

Under the assay conditions used, the molar absorptivity due to the reaction of DTT and DTNB is larger than that resulting from the reaction of free CoA and DTNB. This is a problem as the assay is exclusively meant to determine the amount of free CoA by measuring TNB. If any other chemical species contributes to the formation of TNB, the optical density observed at 405 nm due to the reaction of DTNB and free CoA will be higher than it actually is as a result of the additional absorptivity due to DTT. Therefore, the stock solution of MSNAT and hence DTT concentration, was diluted as a result in 20mM Tris.HCL pH 8.0, before each assay, 6-fold from 6mg/ml to 1mg/ml, then 10-fold to 0.1mg/ml; affording a final DTT concentration of 16μM. In each well of the 96-well plate, DTT was further diluted 10-fold to 1.6μM. Using what we know about the extinction coefficients relating to DTT and free CoA, it is clear the contribution of DTT in absorbance terms will only be 0.04%, which is negligible.

### 8.10.7.3. Assaying for MSNAT activity

A volume of 50μl of a 1mM isoniazid solution was added to wells in a 96-well format and then 10μl of the pure freshly diluted (in 20mM Tris.HCL, 300mM NaCl pH 8.0) recombinant enzyme [0.1mg/ml; 1μg per well] was added. The
plate was shaken to mix the reagents of the reaction. Both the 96-well plate and the AcCoA solution (1mM) were pre-incubated at 37°C for 5 minutes to thermally equilibrate them at the temperature of the reaction. Then 40µl of this 1mM acetyl-CoA solution was added at different time points, to start the reaction at 37°C. Each time point was done in triplicate. Then, 25µl of a 5mM stock solution of DTNB (Dissolved in 6.4M Guanidine, 0.1M Tris.HCl pH 7.3) was added to quench the reaction. TNB production was measured at 405nm using a plate reader (Anthos 2020). The data was used to calculate the specific activity of the enzyme using equation 4.3 shown below. The values for specific activity were plotted against substrate concentration while the values of product produced were plotted against time.

\[
\text{Specific Activity} = \frac{([\text{OD/min}] \cdot \text{CoA/OD}) \cdot \text{Volume of assay}}{[\text{mg of Protein}]}
\]

where \( \text{OD} = \) optical Density

\[\text{units} = \text{nmoles/min/mg Protein}\]

\[\ldots4.3\]

**8.10.8. Effect of *Warburgia salutaris* on MSNAT activity**

The dried dichloromethane extract of the bark of *Warburgia salutaris* (0.1g) was dissolved in 1ml HPLC grade acetonitrile to make a stock solution of 100mg/ml. A 10-fold dilution was prepared in 20mM Tris.HCl pH 8.0, and then sonicated for 5 minutes. Three 2-fold dilutions of the 10mg/ml stock were prepared in 20mM Tris.HCl pH 8.0 containing 10 % HPLC grade acetonitrile.
Each concentration was sonicated for 5 minutes before the inhibition assay was done.

All assays were carried out in triplicate. In different 96-well plate wells, 10μl of different concentrations of the crude extract were added. Then 40μl of different concentrations of isoniazid were added followed by 10μl of the pure recombinant enzyme (0.1mg/ml; 1μg per well). Once the plate had been shaken to mix the contents, the pre-incubation step was done at 37°C for 5 minutes. To start the reaction, 40μl of a 1mM Acetyl-CoA solution was added at different time points at 37°C. Each time point was done in triplicate. Then, 25μl of a 5mM stock solution of DTNB (Dissolved in 6.4M Guanidine, 0.1M Tris.HCl pH 7.3) was added to quench the reaction. TNB production was measured at 405nm using a plate reader (Anthos 2020). The specific activities were calculated as in equation 4.3. A plot of specific activity versus substrate concentration with or without the extract was prepared to see the effect of the extract of Warburgia salutaris on the activity of the MSNAT.

8.11. Solid Phase Extractions

A C-18 reversed phase column from isolute® can take 200mg of material for each run. The 10 g silica bed embedded in a column of a maximum volume of 70mls could be loaded with a maximum of 50mls of solution. Endcapping of the silica matrix ensures reduced secondary polar and ionic interactions silica surface thereby facilitating the retention of non-polar compounds.
8.11.1. Preparation of crude extract for SPE

The crude dichloromethane extract of *Warburgia salutaris* bark (2g) was dissolved in 100ml of 100% acetonitrile. The solution was filtered through a Watman® 0.45μm filter paper to exclude material that did not dissolve, which comprised of inactive waxy material. Double distilled water was added into the 100ml of filtered crude extract solution. The resulting milky solution (50ml) was loaded into a pre-conditioned column.

8.11.2. Column conditioning

The silica was washed with two volumes of 50ml each of acetonitrile, then two similar volumes of double distilled water and then 2 similar volumes of 20% acetonitrile in water before 50ml of the crude extract solution was loaded.

8.11.3. Elution

The loaded material of crude extract was allowed to wash through the silica bed under vacuum at a flow rate of 1ml/minute until all the 50ml of solution had washed through before the bound material was eluted with increasing concentrations of acetonitrile. The unbound material was air dried and kept but not investigated further as it showed no anti-mycobacterial activity in the initial screens. The bound material was eluted with 2 volumes of 50ml each of 30% HPLC grade acetonitrile under vacuum at a flow rate of 1ml/minute, followed by 3 volumes of 50ml each of 60% HPLC grade acetonitrile and lastly with 3 volumes of 50ml each of 100% HPLC grade acetonitrile. The column was washed with acetone and then re-conditions as in 8.11.2, for the
subsequent fractionations. The acetone fraction showed no anti-mycobacterial activity in the initial screens, hence it was not tested further.

There are 4 fractions that were generated, Fr 20 (unbound), Fr 30 (eluted with 30% acetonitrile), Fr 60 (eluted with 60% acetonitrile) and Fr100 (eluted with 100% acetonitrile). These fractions were concentrated using a rotary evaporator at a temperature of 40°C, air-dried and finally dried to completion in a dessicator. SPE was very efficient as almost 96% recovery of the material was obtained (Table 8.11.3.1). The acetone wash also constitutes the 4% of the unaccounted material. Some material binds permanently to the column bed thereby decreasing its efficiency and hence each column was only used at least 5 times then discarded.

| Table 8.11.3.1: Yields of fractions from 2g of the crude extract (by SPE) |
|--------------------|---------|----------|---------|----------|-------------|
| %Yields            | Fr 20   | Fr 30    | Fr 60   | Fr 100   | Filtrate    | Total       |
|                    | 10.77   | 15.2     | 42.83   | 12.75    | 14.5        | 96          |

8.12. Comparative M. bovis BCG growth inhibition

An M. bovis BCG culture was grown at 37°C for several days until it reached an OD of 0.5 at 600nm. An aliquot of this culture (80µl) was added into different wells of the 96-well plate. The fractions from SPE (20µl) of different concentrations were added in different wells containing the organisms. The final concentrations of the fractions per well ranged from 62.5µg/ml to 500µg/ml. Before the 96-well plates were incubated at 37°C for 7 days, they
were sealed with a polypropylene-based sealing film and were covered in parafilm and aluminum foil to prevent evaporation. Growth of *M. bovis* BCG in the presence and absence of the different concentrations of fractions was determined by measuring OD at 595nm using a plate reader (Anthos 2020). The OD readings were plotted against fraction concentration.

### 8.13. Optical rotation

Optical rotations were recorded on a Perkin-Elmer 241 polarimeter, using a path length of 10cm. The observed rotation, $\alpha$, was found to be $-0.945$ degrees, suggesting a levorotary rotation. The corresponding specific rotation was $\left[\alpha\right]_d^{20} = -308.10^{-1}\text{deg cm}^{-1}\text{g}^{-1}$ $(c = 0.306, \text{acetonitrile})$.

### 8.1.4. Infra Red Spectrometry

The infra-red spectrum of compound 1 was recorded as KBr disc using a Bruker Tensor 27 FT-IR spectrophotometer. Selected peaks are reported in cm$^{-1}$. 

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Appendix

NMR, IR, and MS spectra
A1.1a – A1.8 Compound 1

List of spectra:

- $^1$H NMR
- $^{13}$C NMR
- COSY
- NOESY
- HSQC
- HMBC
- IR
Figure A1.1a: $^1$H NMR data of compound 1 in MeOH-d₄, 500MHz
Figure A1.1b: Expansion of $^1$H NMR spectrum of compound 1 in MeOH-\textit{d}_4, 500MHz
Figure A1.2a: $^{13}$C NMR spectrum of compound 1 in MeOH-d$_4$, 250MHz
Figure A1.2b: $^{13}$C NMR spectrum of compound 1 showing the carbons assignments
Figure A1.3a: COSY spectrum of compound 1 MeOH-d$_4$, 500MHz
**Figure A1.3b:** An expansion of the COSY spectrum between 1.20-2.16ppm
Figure A1.4b: An expansion of the NOESY spectrum of compound 1.
Figure A1.5a: HSQC spectrum of compound 1 MeOH-d₄, 500MHz
Figure A1.5b: An expansion of the HSQC spectrum between 1-2.5ppm.
Figure A1.6a: HMBC spectrum of compound 1 MeOH-d₄, 500MHz
Figure A1.6b: Expansion of HMBC spectrum of compound 1
Figure A1.7: HRMS spectrum of compound 1
Figure A1.8: IR spectrum of compound 1
A2.1 – A2.2 Compound 2

List of spectra:

- $^1H$ NMR
- HRMS
Figure A2.1: $^1$H NMR spectrum of compound 2 in MeCN-d$_4$, 500MHz
Figure A2.2: HRMS spectrum of compound 2