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Mycothiol Disulfide Reductase as a Drug Target

A thesis Presented in fulfilment of the requirement of the degree of
DOCTOR OF PHILOSOPHY

Department of Clinical Laboratory Sciences
UNIVERSITY OF CAPE TOWN
In the Division of Chemical Pathology
APRIL 2010

Vuyo Bhongolethu Mavumengwana
DECLARATION

The work contained in this thesis is original, except where indicated and acknowledged. No portion of this work has been submitted for another degree at this or any other university.

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V.B. Mavumengwana

April 2010
ACKNOWLEDGEMENTS

I wish to express my gratitude to my supervisors Prof D.J. Steenkamp and Prof. D.W. Gammon for their supervision, guidance and teaching throughout this study.

I would also like to thank Dr. Leveque, Mr. Munyololo and Dr. Kinfe for their support and assistance during my stay in the chemistry department. I’m indebted to my colleagues in the Division of Chemical Pathology, Dr. Marakalala for testing my synthetic compounds against MshB and Mca and Dr. Williams for cloning mtr in pSD26. I’m grateful to Gabriel and Nick for their friendship and willingness to help whenever I needed them. Ms. Di James (Department of Molecular and Cell Biology, University of Cape Town) and Dr. Wiid (University of Stellenbosch, South Africa) are gratefully acknowledged for sequencing experiments and anti-tubercular testing of compounds respectively. I am deeply indebted to Dr. Tricia Owens (Division of Chemical Pathology, UCT) for her support and encouragement throughout my stay in the department, and to Dr. André Trollip (BiotecSA) for caring and creating a work environment that facilitated the completion of my studies. You stood by me and gave me strength when I was ready to quit. I really have no words to describe my appreciation.

Support from the National Research Foundation, and the University of Cape Town is gratefully acknowledged.
ABSTRACT

Tuberculosis, a common and deadly infectious disease is caused by the pathogen *Mycobacterium tuberculosis*. The recent emergence of multi-drug and extreme multi-drug resistant strains poses an even greater obstacle to controlling the disease, particularly in areas where efficient and effective health systems have not been properly addressed. Current treatments using first line drugs typically require extended use and strict compliance by patients, which is often not adhered to. As a result, there is an urgent need for new and highly effective drugs to combat this disease.

*Mycobacterium tuberculosis* relies on mycothiol, a low molecular mass thiol used to circumvent oxidative stress generated by activated macrophages and to deal with antibiotics such as rifamycin. Enzymes involved in the biosynthesis of mycothiol have been elucidated over the past ten years and some have been designated to be essential in the growth of *Mtb*, with mycothiol disulfide reductase (Mtr) being one. Mtr is involved in the reduction of oxidized mycothiol (MSSM) to mycothiol (MSH) upon exposure of the microbe to oxidative stress. The rational behind this study involves the use of naphthoquinones grafted onto the mycothiol template as subversive substrates against Mtr.

Initially, we had planned to clone and express Mtr in *Corynebacterium glutamicum* (*C. glutamicum*) to try to obtain enhanced expression levels of Mtr, and although the expression of the enzyme was not successful we were able to confirm that *C. glutamicum* does indeed depend on MSH as has been previously suggested but not actually proven. MSH was isolated from *C. glutamicum* with the aid of a new derivatising agent 2-bromo-acetonaphthone (BAN) and characterized using a combination of NMR and mass spectrometry. Consequently, we were also able to establish that MSSnaph (obtained during the isolation process of MSH) is an alternative and better substrate for Mtr with a $k_{m,\text{app}}$ of 23 µM, a $k_{\text{cat}}$ and $k_{\text{cat}}/k_{m}$ of 15.56 s$^{-1}$ and $8.17 \times 10^5$ respectively, as compared to the natural substrate (MSSM).
Synthesis of subversive substrates proceeded through the initial formation of 2-azido-2-deoxy-3,4,6-tri-O-acetyl-D-glucopyranosyl acetate (7). Phenyl-3,4,6-tri-O-acetyl-2-azido-1-thio-α-D-glucopyranoside (12) was generated from this and, after reduction of the azide, linked to the carboxy terminus of substituted naphthoquinones using standard N-3-(dimethylaminopropyl)-N-ethyl-carbodiimide (EDC) and Hydroxybenzotriazole (HOBt) coupling procedures. Deacetylation using the Zemplén procedure gave the target naphthoquinone derivatives 35b (34%), 36b (46%), 37b (60%), and 38b (52%).

The compounds exhibited specific activities ranging between 0.29 μmol/min/mg and 0.42 μmol/min/mg at 40 μM, and inhibition levels ranging between 4 and 14% at 40 μM when tested against Mtr. The Phenyl-2-deoxy-2-[3’-(8’’-hydroxy-3’’-methyl-1’’,4’’-dioxo-1’’,4’’-dihydronaphthalen-2’’-yl)propanamido]-1-thio-α-D-glucopyranoside (35b) was the only compound that exhibited bacteriocidal effects when tested against Mtb using the radiometric respiratory technique based on the Bactec system.

A range of thioglycosides and O-glycosides was also evaluated (at 500 μM) as possible inhibitors of Mtr and found to be of poor inhibitory nature exhibiting percentage inhibitions that ranged between 1.86-12.75% and 3.39-20.2% respectively. We also prepared INH-NAD(P) adduct mixtures based on published procedure and found for the
first time that Mtr is also inhibited by the activated isoniazid, with percentage inhibitions ranging from 16.4% to 66%. Attempts to prepare Ethionamide-NAD(P) adduct mixtures using the same procedure were however, not successful. The finding that INH-NAD(P) adducts also inhibit Mtr offers a new direction for the design of inhibitors targeted at Mtr.
**Abbreviations**

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<tr>
<td>AcCys</td>
<td>Acetyl-cystein</td>
</tr>
<tr>
<td>AcCySmB</td>
<td>Bimane derivative of N-acetylcysteine</td>
</tr>
<tr>
<td>Accq-fluor</td>
<td>6-aminoquinolyl-N-hydroxysuccinimidyl carbamate</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BAN</td>
<td>2-bromo-2’-acetonaphthone</td>
</tr>
<tr>
<td>BF3.OEt2</td>
<td>Boron triflouride etherate</td>
</tr>
<tr>
<td>Boc$_2$O</td>
<td>Di-tert-butylidicarbonate</td>
</tr>
<tr>
<td>CD$_3$OD</td>
<td>Deuterated methanol</td>
</tr>
<tr>
<td>CH$_3$CN</td>
<td>Acetonitrile</td>
</tr>
<tr>
<td>Cl$_3$CCN</td>
<td>Trichloroacetonitrile</td>
</tr>
<tr>
<td>COSY</td>
<td>Correlation spectroscopy</td>
</tr>
<tr>
<td>DCM</td>
<td>Dichloromethane</td>
</tr>
<tr>
<td>DEPT-NMR</td>
<td>Distortionless enhancement by polarization transfer</td>
</tr>
<tr>
<td>DMAP</td>
<td>4-Dimethyl aminopyridine</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA D</td>
<td>Eoxyribonucleic acid</td>
</tr>
<tr>
<td>EDC</td>
<td>N-3-(dimethylaminopropyl)-N-ethyl-carbodi mide</td>
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<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
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<td>EPO</td>
<td>Medium used for growing electroporation competent cells (described in Appl Microbiol Biotechnol, 1999, 52: 541-545)</td>
</tr>
<tr>
<td>EtOAc</td>
<td>Ethyl acetate</td>
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<tr>
<td>FAD</td>
<td>Flavin adenine dinucleotide</td>
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<tr>
<td>GlcNAc</td>
<td>N-acetylglucosamine</td>
</tr>
<tr>
<td>GlcNAc-Ins</td>
<td>1-D-myo-inosityl-2-acetamido-2-deoxy-alpha-D-glucopyranoside</td>
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<tr>
<td>GSH</td>
<td>Glutathione</td>
</tr>
<tr>
<td>HBr</td>
<td>Hydrogen bromide</td>
</tr>
<tr>
<td>HOBT</td>
<td>Hydroxybenzotriazole</td>
</tr>
<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
</tr>
<tr>
<td>Term</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------------------------------------------------------------------------</td>
</tr>
<tr>
<td>INH-NAD(P)</td>
<td>Isonicotinoylated nicotinamide adenine dinucleotides</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropyl-beta-D-thiogalactopyranoside Kcat</td>
</tr>
<tr>
<td>$k_{cat}$</td>
<td>Turn over number</td>
</tr>
<tr>
<td>$k_{cat}/k_m$</td>
<td>Overall catalytic efficiency</td>
</tr>
<tr>
<td>LB</td>
<td>Lurea broth</td>
</tr>
<tr>
<td>LBG</td>
<td>Lurea broth containing filter sterilized glucose</td>
</tr>
<tr>
<td>mBBr</td>
<td>Monobromobimane</td>
</tr>
<tr>
<td>McA</td>
<td>Mycothiol S-conjugate amidase</td>
</tr>
<tr>
<td>MDR</td>
<td>Multidrug resistance</td>
</tr>
<tr>
<td>MeOH</td>
<td>Methanol</td>
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<tr>
<td>MSH</td>
<td>Mycothiol</td>
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<tr>
<td>MshA</td>
<td>1L-my-o-inositol-1- phosphate 1--D-N-acetylglucosaminyltransferase</td>
</tr>
<tr>
<td>MshB</td>
<td>1-O-(2-acetamido-2-deoxy-D-glucopyranosyl)-D-my-o-inositol deacetylase</td>
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<td>MshC</td>
<td>ATP-dependent L-cysteine: 1-O-(2-amino-2-deoxy-D-glucopyranosyl)-D-my-o-inositol ligase</td>
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<tr>
<td>MshD</td>
<td>Mycothiol synthase</td>
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<tr>
<td>MSmB</td>
<td>Monobromobimane derivative of mycothiol</td>
</tr>
<tr>
<td>MSSM</td>
<td>Mycothiol disulfide</td>
</tr>
<tr>
<td>Mtr</td>
<td>Mycothiol disulfide reductase</td>
</tr>
<tr>
<td>NADPH</td>
<td>Reduced nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NADP⁺</td>
<td>Oxidized nicotinamide adenine dinucleotide phosphate</td>
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<tr>
<td>NADH</td>
<td>Reduced nicotinamide adenine dinucleotide</td>
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<tr>
<td>NAD⁺</td>
<td>Oxidised nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NaN₃</td>
<td>Sodium azide</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
</tr>
<tr>
<td>NQ</td>
<td>Naphthoquinone</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethylsulphonyl fluoride</td>
</tr>
<tr>
<td>RNI</td>
<td>Reactive nitrogen intermediate</td>
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</table>
ROI       Reactive oxygen intermediate
SDS-PAGE  Sodium dodecyl sulphate polyacrylamide gel electrophoresis
Tf₂O      Trifluoromethanesulfonic (triflic) anhydride
TfN₃      Triflyl azide
THF       Tetrahydrofuran
TLCK      N-p-tosyl-L-lysine-chloromethyl ketone
TMS       Trimethyl silyl
TPCK      N-p-tosyl-L-phenylalanine-chloromethyl ketone
TR        Trypanothione reductase
TS₂       Trypanothione disulfide
XDR       Extensively drug resistant tuberculosis
α-DGI     1-D-myo-inositol-2-amino-2-deoxy-alpha-D-glucopyranoside
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Chapter 1
Introduction

1.1 Origins of *Mycobacterium tuberculosis*

For the most part, mycobacteria are saprophytic organisms abundantly found in soil and untreated water, with some species being pathogens (Rook *et al*., 2007). It is therefore most likely that during the early stages of the evolution of the animal kingdom, chance mutations in mycobacteria managed to produce species capable of parasitizing animals. As a result, earlier on, it was hypothesised that there might have been a direct progression from a mycobacterial pathogen in livestock to a bacterium that would have evolved to the closely related *Mycobacterial tuberculosis*, as the domestication of animals occurred, particularly cattle. Consequently, *Mycobacterium bovis* (*M.bovis*) which results in tuberculosis (TB) like pathogenesis in cattle was seen to be the most likely precursor of *Mycobacterium tuberculosis* (*Mtb*) (Steele and Ranney, 1958).

The appeal in this hypothesis is understandable given the fact that it was formulated long before the advent of modern DNA sequencing and related techniques. Thus, recent studies have shown a marked similarity of DNA and 16S rRNA sequences among the members of the *Mtb* complex, while they show varying degrees of phenotypes, pathogenicity and host tropism (Brosch *et al*., 2002). In spite of this, closely related bacteria can be differentiated by the identification of rare synonymous single nucleotide polymorphism analysis (sSNP), which also suggests that *M.bovis* must have evolved at the same time as *Mtb* (Sreevatsan *et al*., 1997).

Although a lot of progress has been achieved with regards to *Mtb* evolutionary studies, it appears there is still open debate about the origins of tuberculosis in animals and humans and that it may be a while before the question is resolved, as is evident from Bosch *et al*., 2002, where they hypothesized that the common ancestor of the tubercle bacilli could have been a human pathogen already resembling either *Mtb* or *Mycobacterium canetti* (*M. canetti*).
Unfortunately the impact \textit{Mtb} has had on ancient populations cannot be statistically demonstrated and compared with what present societies have to contend with, where approximately one third of the world’s population, and mostly in developing countries is affected (figure 1.1) (Rook \textit{et al.}, 2007).

1.2 \textit{Mtb} pathogenesis and macrophage interaction

The transmission and perpetuation of the pathogen is both simple and ingenious and prove a daunting task to deal with therapeutically. Tuberculosis is an ailment that is almost exclusively transmitted by aerosolised droplets containing infectious \textit{Mtb} by an individual’s cough and inhaled by an uninfected person. The primary destination of the inhaled bacilli is the alveoli in the lungs, and upon reaching the oxygen exchanging sacs, they are engulfed by dendritic cells in the lung interstitium and alveolar macrophages, where they replicate (Kaufmann and McMichael, 2005).

Following the initial site of primary infection and replication the pathogen then spreads to local lymph nodes within the lung and is ultimately dispersed to remote locations in the body, hence, every so often, some patients present with tuberculous meningitis (TBM). Furthermore, \textit{Mtb} has developed the striking ability to establish and maintain latency throughout its early stages of infection, and during this period, the disease, is generally
asymptomatic, particularly in adults. Although the host’s immune response manages to control the primary infection, the bacterium is in most cases never eradicated. Individuals who are at this stage of infection usually do not have clinically apparent tuberculosis but nevertheless, harbour the pathogen that only reactivates at a later stage. As a result, infected people merely become vectors of *Mycobacterium tuberculosis* (Kaufmann and McMichael, 2005).

It has long been established that alveolar macrophages, the first line of defence in the lung, possess antimicrobial activity. When activated by cytokines (figure 1.2), the gamma interferon and tumour necrosis factor-alpha, macrophages are capable of generating a hostile environment that includes an acidic pH, reactive oxygen intermediates (ROIs), lysosomal enzymes and toxic peptides (Keane *et al*., 1997; Nathan and Hibbs, 1991). In addition, reactive nitrogen intermediates (RNIs), generated through a reaction initiated by the expression of an inducible nitric oxide synthase (iNOS also called NOS2) contribute in producing nitric oxide thereby ensuring an unsustainable environment. Moreover, RNIs have been demonstrated to be effective against virulent mycobacteria in mouse macrophages (Miller *et al*., 2004; Flynn and Chan, 2001).

However, a selection of intracellular pathogens, including mycobacteria, has evolved and devised mechanisms to reside in the macrophages while avoiding destruction. Essentially three different strategies in use have been observed so far; micro-organisms like *Leishmania spp.* and *Coxiella burnetti* reside and proliferate in the harsh environment of mature phagolysosomes. While *Listeria monocytogenes* and *Shigella spp.* avoid delivery to phagolysosomes by secreting lytic enzymes that degrade the phagosome membrane thus allowing their escape to the nutrient-rich cytoplasm, whereas, pathogens like *Mycobacterium spp.*, *Toxoplasma gondii*, *Brucella spp.*, and the promastigote form of *Leishmania spp.* are capable of inhibiting phagolysosomes biogenesis, consequently creating immature compartments lacking the means to kill and degrade pathogens (Maurin *et al*., 1992; Dermine and Desjardins, 1999; Wilson *et al*., 1980).
In theory, intracellular pathogens capable of utilizing macrophages synthesize molecules that allow them to resist the harsh environment of phagolysosomes and *Mtb* in particular, have been shown to display a number of virulent factors which have been implicated in their resistance to the sustained attack of toxic molecules produced by their host. Some of these molecules, including proteins and thiols serve a critical role in maintaining the redox status of the oxidatively active macrophage for the benefit of the pathogen (Raynaud *et al.*, 1998; Forman and Torres, 2001; Dermine and Deşjardins, 1999).

**Figure 1.2:** Schematic representation of events that take place during tuberculosis infection: *M. tuberculosis* enters the host within inhaled droplets and this, results in three possibilities: (i) Elimination of the pathogen by the host’s immune system. (ii) Infection quickly develops into tuberculosis. This is not uncommon in immuno-compromised individuals, particularly HIV infected patients who stand a chance of developing tuberculosis 800-fold times compared to immuno-competent individuals. (iii) Infection does not progress into a disease due to the containment of the bacilli in granulomas. In diseased individuals however, the *M. tuberculosis* is no longer in granulomas because caseation (necrotic degradation of bodily tissue) results in distribution and consequent transmission of *M. tuberculosis*. Upon inhalation, the mycobacteria are engulfed in alveolar macrophages and dendritic cells (DC). In draining lymph nodes, mycobacterial antigens are presented to different T cell populations (CD4+ T cells, CD8+ T cells, and CD 1 restricted T cells) by the DC’s and macrophages, which are activated by interferon gamma (IFN-γ) and tumour necrosis factor (TNF-α). Taken from: Kaufmann and McMichael (2005).
1.3 Thiols in intracellular redox reactions

1.3.1 Glutathione (GSH)

The protection of cells from reactive oxygen and nitrogen intermediates has been observed to rely on the presence of thiols, which assist in the maintenance of cellular redox potentials and protein thiol disulfide ratios. The tripeptide $\gamma$-L-glutamyl-L-cysteinylglycine (glutathione) is the most studied and best characterised thiol that has been detected in most Gram-negative bacteria and nearly all eukaryotic organisms, except those lacking mitochondria and chloroplast (Hand and Honek, 2005, Fahey, 2001).

In addition to participating in free radical and peroxide detoxification, glutathione functions in metabolism, catalysis, transport and as a storage form of cysteine moieties. It also forms conjugates with a variety of compounds such as drugs and xenobiotics and is a cofactor for a range of enzymes. Synthesis of GSH from constituent amino acids takes place intracellularly and exported from cells, where it participates in intra and interorgan transfer of cysteine moieties and in the protection of cell membranes (Meister, 1988). A comprehensive overview of GSH activities is shown in figure 1.3.
**Figure 1.3**: A comprehensive overview of GSH biochemistry. AA, amino acids; X, compounds that react with GSH to form conjugates. GSH synthesis from \(L\)-glutamate and \(L\)-cysteine is achieved by two sequential ATP dependent reactions that utilise \(\gamma\)-glutamylcysteine synthetase (1) to form the intermediate, \(\gamma\)-glutamylcysteine. This is followed by glutathione synthetase (2) that adds glycine to the C-terminal end of \(\gamma\)-glutamylcysteine to form the end product. The transfer of the \(\gamma\)-glutamyl moiety of GSH to an amino acid or peptide acceptor is catalyzed by \(\gamma\)-glutamyltranspeptidase (3), resulting in the production mercapturates. Cysteine and glycine required in GSH biosynthesis are generated by dipeptidases (4) utilizing dipeptides. \(\gamma\)-glutamylcyclotransferase (5) catalyzes the formation of 5-oxoproline which feeds onto an ATP dependent 5-oxoprolinase (6) to give glutamate. GSH conjugates to a variety of electrophilic ligands through the action of GSH-S-transferase (7) thus accomplishing its detoxification capabilities. During oxidizing conditions, GSH is transformed into the disulfide (GSSG). GSH is maintained in its reduced form by glutathione disulfide reductase which utilizes NADPH as a cofactor. (12) Enzymes 3→6 play key roles in the pathway for GSH synthesis, as they generate much required substrates (Meister, 1988; Noctor et al., 1998; Board et al., 1978; Mazelis and Creveling, 1978; Telakowski-Hopkins et al., 1986). The figure was taken from Meister (1988).
1.3.2 Mycothiol (MSH)

Although different microorganisms may employ a widespread thiol, certain intracellular pathogens depend on different antioxidant systems that display a type of chemistry that is analogous to that initially observed with glutathione. For instance, apart from GSH and ovothiol A (N\(^1\)-methyl-4-mercaptohistidine), the bis-glutathionyl conjugate of spermidine, termed Trypanothione [N\(^1\),N\(^8\)-bis(glutathionyl)spermidine] is specific to the pathogenic protozoa of the genera *Trypanosoma* and *Leishmania* (Ariyanayagam and Fairlamb, 2001). Whereas, most *Actinomycetes*, including *Mtb* exclusively synthesize 1-D-myoinositol-2-(N-acetyl-L-cysteinyl)amino-2-deoxy-α-D-glucopyranoside (Mycothiol, figure 1.4) as their major low molecular mass thiol (Spies and Steenkamp, 1994).

Mycothiol was first isolated as a symmetrical disulfide (MSSM, figure 1.4) from *Streptomyces* sp. AJ 9463 by Sakuda et al. (1994) while searching for an intermediate in allosamidin biosynthesis. Shortly thereafter, *Mycobacterium bovis* (*M. bovis*) was shown to contain the same compound as the only major low molecular mass thiol, isolated as a bimane derivative (MSmB) (figure 1.4) (Spies and Steenkamp, 1994). The same structure was later reported to be present in *Streptomyces clavuligerus* designated U17 as a previously unidentified thiol, with apparent wide distribution amongst the members of the *Actinomycetes* (Newton et al., 1995).
1.3.3 MSH Biosynthesis

Subsequent to isolating and establishing the structure of mycothiol (Sakuda et al., 1994; Spies and Steenkamp, 1994), studies were carried out to elucidate its biosynthesis (figure 1.4) using a synthetic 1D-myo-inositol-2-amino-2-deoxy-\(\alpha\)-D-glucopyranoside (GlcN-Ins) to assay extracts of *M. smegmatis* for enzyme activity. The study was able to show enzyme (ligase, MshC, Rv2130c) activity corresponding to an ATP dependent ligation of \(\text{L-cysteine} \text{ with GlcN-Ins} \) to generate Cys-GlcN-Ins and an acetyltransferase activity (MshD, Rv0819) which produced mycothiol from acetyl-CoA and Cys- GlcN-Ins (Bornemann et al., 1997).

To further elaborate on the abovementioned studies, *M. smegmatis* was confirmed to produce GlcN-Ins and Cys-GlcN-Ins as intermediates in the biosynthesis of mycothiol by directly measuring their levels in cell extracts (Anderberg et al., 1998). However, earlier studies left undefined the biochemical reactions involved in the formation of GlcN-Ins, and by comparing the steps already defined for MSH production with those for a
glycosylphosphatidylinositol (GPI) anchor production which contains as a component an isomer of GlcN-Ins with an α(1,6) linkage it was shown that 1D-myoinosityl-2-acetamido-2-deoxy-α-D-glucopyranoside (GlcNAc-Ins) is a precursor for GlcN-Ins by demonstrating a deacetylase (MshB, Rv1170) activity using GlcNAc-Ins as a substrate (Newton et al., 2000b).

Although the three genes (mshB, mshC, and mshD) involved in the biosynthesis of mycothiol had been identified, the biochemical reactions catalyzed by a putative glycosyltransferase (MshA) (Newton et al., 2003) had not been established until recently when it was shown that the production of GlcNAc-Ins is only possible when the participating substrates are UDP-N-acetylglucosamine and 1L-myoinositol-1-phosphate (1L-Ins-1P). This reaction generates UDP (uridine diphosphate) and 3-phospho-1D-myoinosityl-2-acetamido-2-deoxy-α-D-glucopyranoside (GlcNAc-Ins-P) where the latter is dephosphorylated by a postulated phosphatase, designated MshA2 to yield GlcNAc-Ins (Newton et al., 2006a). 1L-Ins-1P can also be produced from exogenous inositol, as demonstrated by Gammon et al., (2003) where they illustrated the inhibition of [3H]inositol transport into *M. smegmatis* cells. The biosynthesis is summarised in figure 1.5.
Figure 1.5: MSH biosynthesis: A summary of the events taking place in this figure is presented below.
The formation of GlcNAc-Ins proceeds through the action of a glycosyltransferase (MshA). MshA catalyzes the reaction between UDP-N-acetylglucosamine and 1L-myoinositol-1-phosphate to generate GlcNAc-Ins-P. The latter is dephosphorylated by a phosphatase (MshA2). GlcNAc-Ins is then deacetylated by MshB to yield GlcN-Ins wherein a cysteine is ligated in an ATP dependent reaction catalyzed by MshC. Mycothiol is ultimately produced by the acetylation of the cysteiny l amine of Cys-GlcN-Ins, catalyzed by an acetyltransferase (MshD). An alternative route for the production of 1L-Ins-P through the usage of exogenous inositol that is processed by a postulated inositol kinase (InoK). Although this enzyme has not yet been identified in Mtb, the ATP-dependent reaction it catalyzes in lily pollen and wheat germ using myo-inositol as a substrate (Loewus et al., 1982) is also expected to occur in Mtb (Newton et al., 2006a).

Evidence for the existence of a myo-inositol transporter in Mtb was given impetus by observing that the putative product of iolT in Bacillus subtilis exhibits considerable similarities with many bacterial sugar transporters. In B. subtilis, the iolABCDEFGHIJ and iolRS operons are known to be involved in inositol utilization and their transcription is regulated by the IolR repressor and induced by inositol (Yoshida et al., 2002).

Furthermore, this gene was shown to be the primary inositol transporter in this organism since mutants (with inactivated iolT) showed growth defects when grown on inositol as the main carbon source (Yoshida et al., 2002). Exploiting the iolT protein sequence to search for Mtb orthologs using the blastp search of the TB genome on TubercuList (http://tuberculist.epfl.ch), the only strong probable match was found to be Rv3331 that was 26% identical in a 432 residue overlap annotated as a probable sugar transport integrall membrane protein. A homolog with a 24% identity in a 384 residue overlap was also found in M. smegmatis emphasizing that Rv3331 could be the best candidate to express an inositol transport protein in Mtb (Newton et al., 2006a).
1.3.4 Mycothiol dependent detoxification

1.3.4.1 NAD/MSH-dependent formaldehyde dehydrogenase

Oxidative demethylation of amino acids or degradation of one carbon compounds like methanol, methyl halides, methane, methylated amines and sulphur compounds is usually accompanied by concomitant generation of formaldehyde (Ling and Tung, 1948). In normal cells, formaldehyde is toxic even at low concentrations due to non-specific reactivity with proteins (Fraenkel-Conrat and Mechan, 1948) and nucleic acids. To circumvent potential biochemical disturbances, glutathione producing organisms eliminate formaldehyde with the aid of a NAD/GSH-dependent formaldehyde dehydrogenase whereas Gram-positive bacteria (*Amycolatopsis methanolica* and *Rhodococcus erythropolis*) contain the factor dependent enzyme termed NAD/MySH-dependent formaldehyde dehydrogenase (Misset-Smits *et al*., 1997).

The S-hydroxymethylmycothiol formed by the spontaneous reaction between MSH and formaldehyde, is converted into a mycothiol formate ester, which is expected, by analogy with the GSH-dependent pathway to be further hydrolyzed to generate formic acid and MSH through a thiol esterase as is the case with glutathione based systems. However, since no thiol esterase activity has been detected, despite an esterase motif being present in some *Mtb* gene products an alternative path involving an aldehyde dehydrogenase that converts the mycothiol formate ester to a carbonate ester followed by hydrolysis to give MSH and CO$_2$ has been suggested. The NAD/MSH-dependent formaldehyde dehydrogenase has also been found to be highly specific for its substrate and that it cannot be used interchangeably with its GSH counterpart (Misset-Smits *et al*., 1997; Norin *et al*., 1997).

The NAD/MSH-dependent formaldehyde dehydrogenase has however, been ruled out as an essential detoxification enzyme in *Mtb* based on the fact that no thiol esterase activity had ever been detected since it is assumed that like GSH, MSH reacts spontaneously to form S-hydroxymethylmycothiol adduct that is converted to a MSH formate ester
(Misset-Smits et al., 1997). Although MSH and GlcN-Ins may not be essential for \textit{in vitro} survival of \textit{M. smegmatis}, it remains indispensable for \textit{Mtb} as confirmed by studies where the disrupted \textit{mshC} gene in \textit{Mycobacterium tuberculosis} Erdman produced no viable clones possessing either reduced levels of MSH or the \textit{mshC} product (Sareen \textit{et al}., 2003).

\textbf{1.3.4.2 Mycothiol S-Conjugate Amidase}

\textit{Mtb} uses a more general mycothiol dependent detoxification pathway that is capable of protecting against a range of electrophilic toxins such as alkylating agents, halogens and chlorine derivatives. The discovery of this pathway was based on studies that centred on the treatment of \textit{M. smegmatis} with monobromobimane (mBBr) and the cleavage of the resultant MSmB to generate GlcN-Ins and the bimane derivative of N-acetylcysteine (AcCySmB) (Newton \textit{et al}., 2000a). The bimane fluorescent label is a derivative of syn-9,10-dioxabimane: 1,5-diazabicyclo[3.3.0]octa-3,6-diene-2,8-dione that has been efficiently used to label the reactive thiol groups in haemoglobin, membrane proteins and glutathione of normal human red cells under physiological conditions (Kosower \textit{et al}., 1979).

The Mycothiol S-conjugate amidase (Mca) responsible for this cleavage was purified from \textit{M. smegmatis} and comparison of the N-terminal sequence pointed to an \textit{Mtb} ortholog Rv1082. The gene encoding this enzyme was also found to have a homolog (\textit{mshB}, Rv1170) that possesses weak Mca activity but substantial deacetylation activity with the biosynthetic precursor (Newton \textit{et al}., 2000b). The \textit{mca} gene from \textit{Mtb} was cloned and expressed in \textit{E. coli}, and found to have substrate specificity similar to that of the \textit{M. smegmatis} amidase. It showed no activity with MSH, GlcNAc-Ins and the mycothiol disulfide reductase (to be discussed in detail at a later stage) but rather a preference for the MSH moiety of the conjugates, and relatively low specificity with respect to the group attached to the sulphur moiety (Newton \textit{et al}., 2000a).
In addition to mBBr, conjugates of mycothiol with N-ethylamide (NEM), 7-diethylamino-3-(4’-maleimidyl-phenyl)-4-methylcoumarin (CPM), cerulenin, 3-(N-maleimidopropionyl)-biocytin, and rifamycin S, prepared under physiologically relevant conditions were shown to be substrates for Mca. Despite the ongoing search for natural substrates, MSmB remains the best substrate studied so far with a Km value of 95 \pm 8\mu M and a k_{cat} of 8 s^{-1} while rifamycin displayed k_{cat}/ k_m values that were 3-5% of that for MSmB (Newton et al., 2000a; Steffek et al., 2003).

The electrophiles used to generate the S-conjugates for the amidase are generally not natural toxins except for cerulenin and rifamycin S (Steffek et al., 2003). Cerulenin is a secondary metabolite that has antifungal and antibiotic properties (Hata et al., 1960) whilst rifamycin S is an antibiotic synthesised by the Gram-positive *Nocardia mediterranei* (White et al., 1974). Studies utilizing these antibiotics substantiate findings that Mca could be crucial in the detoxification of antibiotics (figure 1.6), particularly mycobacterial related antibiotics.
The detoxification pathway (figure 1.6) is based on experiments where *M. smegmatis* grown in the presence of mBBr was found to be void of cellular mycothiol by alkylation of the thiol group to form MSmB. Subsequent analysis of the medium did not show any MSmB proving that mycothiol bimane was not exported from the cells but was cleaved intracellularly by the amidase to produce GlcN-Ins and the AcCys S-conjugate (AcCys-mB) termed mercapturic acid, as is the case with the final excreted product in the mercapturic acid pathway of GSH-dependent detoxification (Newton *et al.*, 2000a).
Further studies aimed towards a better understanding of the role Mca and MSH play in xenobiotic detoxification led to the generation of a mutant of the mca gene in M. smegmatis and subsequent exposure to mBBr and the antibiotic rifamycin S. It was observed that M. smegmatis accumulated the S-conjugate of the alkylating agents, and exhibited susceptibility upon exposure to electrophilic toxins such as iodoacetamide, chlorobenzene, and oxidants such as plumbagin and menadione. Subsequent treatment of the mutant with the antituberculous antibiotic streptomycin rendered the mycobacterium even more susceptible as it led to a 10-fold decrease in the MIC compared to the parent strain M. smegmatis mc2 155 (Rawat et al., 2004). Based on these observations, it is reasonable to speculate that inhibiting Mca might sensitize M. tuberculosis to antibiotics.

1.4 Mycothiol disulfide reductase

During the process of peroxide reduction mentioned earlier, the formation of intramolecular disulfide of mycothiol (MSSM) is unavoidable and the reducing capacity of MSH is consequently compromised. It is theoretically possible that MSSM formation could undoubtedly deplete MSH pools and compromise the various MSH dependent pathways. MSH in M. smegmatis has been shown to be maintained in a highly reduced state with redox ratios that range from 200 to 1000 (Newton et al., 2005) through the action of a corresponding mycothiol disulfide reductase (Patel and Blanchard, 1999).

Mycothiol disulfide reductase (Mtr) or mycothione reductase as proposed by Patel and Blanchard, (1999) belongs to the family of dimeric flavoproteins that promote the transfer of electrons between pyridine nucleotides and disulfide/dithiol compounds, and direct catalysis via FAD and a redox active disulfide. The “thione” ending is discussed in a review by Newton and Fahey, 2002. Some of the most extensively studied enzymes in this group include lipoamide dehydrogenase, glutathione reductase, trypanothione reductase and mercuric ion reductase (Patel and Blanchard, 1999; Racker, 1965; Shames et al., 1986). Although these enzymes may be derived from different sources and exhibit strict preferences for substrates, they nonetheless show considerable similarities such as
significant amino acid homology and the ability to undergo thiol disulphide exchanges (Arscott et al., 1997; Mustacich and Powis, 2000).

The catalytic mechanism of the flavoenzymes have been thoroughly investigated and long established by spectroscopic and kinetic techniques as well as by protein crystallography, and Mtr catalysis has been shown to mirror that of other members within this family. The reaction generally begins with the flavin reduction by NADPH, the reduced flavin then reduces the cysteine disulfide in the active site and the nascent active site dithiol ultimately cleaves the disulfide of the substrate (MSSM), where active site cysteines sequentially react with MSSM to generate mixed disulfides (MSS-E). The active site thiolate then attacks ES-SM to reform the active site disulfide and liberating the second molecule of MSH. Ultimately two molecules of MSH are released and the cysteine disulfide in the active site is reformed (Krauth-Siegel et al., 1998; Patel and Blanchard, 1999; O’Donnell and Williams, 1984).

The sequence of catalytic steps outlined above for Mtr is based on the detection of various components usually expected to be part of the flavoenzyme active site. The presence of the flavin cofactor was determined by spectral characterization of oxidized Mtr that gave the spectrum eg $\lambda_{\text{max}}$ 260-265nm as observed for other flavoproteins (disulfide reductases). Reductive titration of Mtr with NADH confirmed the possible charge transfer complex of FAD and the putative thiolate of the active site. These results together with reductive alkylation and steady state kinetics experiments confirmed a kinetic mechanism that is synonymous with the catalytic steps already established for flavoenzymes in which the reduced FAD transfers reducing equivalents to the redox active disulfide to generate the stable reduced enzyme that is able to reduce MSSM via dithiol-disulfide interchanges (figure 1.7) (Patel and Blanchard, 1999).
Figure 1.7: NADPH dependent MSSM reduction. NADPH reduced FAD transferring reducing equivalents to the disulfide of the active site, which consequently reduces the disulfide of MSSM to produce 2 molecules of MSH. This figure is adapted from Patel and Blanchard, 1999.

In addition to these mechanistic studies, the bioinformatic analysis of the putative Mtr amino acid sequence also confirms this enzyme as a member of the pyridine nucleotide disulfide reductase family where it shares homology with *Crithidia fasciculata* trypanothione reductase (28%) and human glutathione reductase (31%). A Closer look at the amino acid sequence alignment (figure 1.8) further revealed the presence of the conserved redox-active cysteines (Cys-XXXX-Cys) and a nucleotide binding motif (GXGXXN) near the amino terminus for binding the ADP moiety of FAD (Patel and Blanchard, 1999).
Amino acid sequence alignment of *C. fasciculata* trypanothione reductase, human glutathione disulfide reductase and *M. tuberculosis* mycothiol disulfide reductase. Tyrosine114 and tyrosine197 involved in the substrate binding site and NADPH binding site respectively seem to be conserved in various reductases. However, Y197 in Mtr is substituted with a phenylalanine residue. The essential catalytic His-444-Glu-449 ion pair is conserved in the three reductases. The figure is adapted from Patel and Blanchard, 1999.

Homology with GSH and trypanothione disulfide reductases (figure 1.8) permitted the cloning and expression of the Mtr (Rv2855) in *M. smegmatis*. The enzyme was reported to be a 50kDa homodimer with a $k_{cat}$ for MSSM that is 400 mol per mol of FAD per minute, which is one to two orders of magnitude slower than other disulfide reductases (Patel and Blanchard, 1999). Due to the slow auto-oxidation rate of MSH as compared to that of glutathione, N-acetylcysteine or cysteine (Newton et al., 1995), it was suggested that this rate may be sufficient to maintain reduced MSH levels in mycobacteria. The Michaelis-Menten constants measured for MSH and NADPH were found to be in

**Figure 1.8.** Amino acid sequence alignment of *C. fasciculata* trypanothione reductase, human glutathione disulfide reductase and *M. tuberculosis* mycothiol disulfide reductase. Tyrosine114 and tyrosine197 involved in the substrate binding site and NADPH binding site respectively seem to be conserved in various reductases. However, Y197 in Mtr is substituted with a phenylalanine residue. The essential catalytic His-444-Glu-449 ion pair is conserved in the three reductases. The figure is adapted from Patel and Blanchard, 1999.
agreement with those of the corresponding substrates of GSH and trypanothione reductases (Patel and Blanchard, 1999).

In addition to preference for MSSM, Mtr was also shown to be active against the truncated substrate des-myoinositol mycothiol disulfide (figure 1.9), with a $k_m$ of 400 µM as compared to 70 µM for the authentic mycothiol disulfide, indicating an absolute requirement for the glucosamine moiety. It was also demonstrated that the presence of the des-myoinositol mycothiol disulfide as a mixture of $\alpha$ and $\beta$ diastereomers did not affect the activity of the enzyme since the mixture was completely converted to des-myoinositol mycothiol (Patel and Blanchard, 1998).

1.5 *Mtb* essential genes in MSH biosynthetic pathway

Analysis of *M. smegmatis* mutants disrupted in the four genes encoding the proteins needed for MSH biosynthesis have led to conclusions that mycothiol and mycothiol-dependent enzymes protect the mycobacterial cell against attack from various toxic agents and different types of stresses. *M. smegmatis* mutants deficient in MshB have been shown to produce low levels of MSH (about 20%) as compared to the parent strain (mc²155), the mutants remained viable and MSH production was never abolished. Moreover, they showed resistance to isoniazid whilst they appeared more susceptible.
when grown in the presence of rifampin. Increased MSH production was restored when
the mutants were complemented with the mshB gene confirming that the enzyme is
important for MSH production (Rawat et al., 2003; Buchmeier et al., 2003; Newton et
al., 2000a).

Contrary to MshB defective mutants, M. smegmatis clones disrupted in mshC were
unable to produce mycothiol. Moreover, they were also found to possess increased
susceptibilities to free radicals, alkylating agents and to a wide range of antibiotics such
as erythromycin, penicillin G, rifamycin, vancomycin, rifampin and azithromycin.
However, the mutants exhibited resistance to isoniazid and this is consistent with studies
involving MshB mutants. MshC has consequently been considered essential for the
growth of M. tuberculosis (Sareen et al., 2003, Rawat et al., 2002), whilst MshB is not
since its absence in M. tuberculosis and M. smegmatis does not completely eliminate
mycothiol and growth is not deterred either (Rawat et al., 2003).

To assess the viability of M. smegmatis deficient in mshA, a chemically derived mutant
and one generated by transposon mutagenesis were treated with isoniazid and compared
to the parent strain M. smegmatis mc²155. The MIC’s of isoniazid were found to be 1
µg/ml for the parent strain and more than 250 µg/ml for each of the mutants. When the
transposon mutant was complemented with the mshA gene and compared to the chemical
mutant after treatment with isoniazid, the MIC’s were found to be 2.7 µg/ml and 28
µg/ml respectively, demonstrating extensive reversion to the parental phenotype. In this
regard, the mshA gene in M. smegmatis proves to be crucial in MSH biosynthesis. The
isoniazid resistance observed corroborates previous studies in which MSH deficient
strains exhibited isoniazid resistance. It has been postulated that MSH may be directly or
indirectly involved in the activation of isoniazid (Newton et al., 2003; Koledin et al.,
2002).

The gene encoding the acetyltransferase has also been shown not to be essential in the
growth of M. tuberculosis since mshD mutants accumulate substantial levels of Cys-
GlcN-Ins, a small amount of MSH and two alternative thiols, N-formyl-Cys-GlcN-Ins
and N-succinyl-Cys-GlcN-Ins. The mutant did not exhibit any heightened sensitivity upon exposure to hydrogen peroxide, cumene hydroperoxide or tert-butyl hydroperoxide, indicating that it most probably relied on one of the alternative thiols (Newton et al., 2005).

Although Mca is not a biosynthetic enzyme, its role as a detoxifying agent remains crucial for the proliferation of \textit{Mtb} and disruption of \textit{mca} renders the \textit{M. smegmatis} mutants vulnerable to a wide range of electrophilic toxins, antituberculous antibiotic streptomycin and oxidants such as menodione and plumbagin. However, the mutant did not show any sensitivity towards hydrogen peroxide, cumene hydrogen peroxide, and nitrofurantoin, possibly due to the fact that these oxidants cannot form stable S-conjugates that may require Mca for cleavage as compared to the naphthoquinones.

There has not been a lot of transposon mutagenesis studies carried out on \textit{mtr} so as to conclusively make phenotypic characterisations of such mutants and resolve the enzyme’s indispensability. Nevertheless, findings by McAdam \textit{et al.}, 2002, that showed viability of \textit{M. tuberculosis} mutants generated by transposon mutagenesis in \textit{mtr} suggested the gene to be non essential, whereas, high density Himar-1 studies carried out by Sassetti \textit{et al.}, 2003, demonstrated that \textit{mtr} is required in \textit{M. tuberculosis} for optimal growth.

Taking into consideration the urgent need for new and effective anti-tubercular drugs, due to the persistence of the disease, the emergence of multidrug resistant (MDR) strains and more recently, extensively drug resistant (XDR) strains (Dorman and Chaisson, 2007), several enzymes involved in mycothiol biosynthesis and metabolism (table 1.1) hold promise for the development of active compounds against the mycobacterium. Moreover, according to Sacchettini \textit{et al.}, 2008, ideal TB drug targets should be required for bacterial growth and persistence and it must be feasible to temper with their activity through the use of small molecules, hence, the attraction in targeting intermediates in MSH biosynthesis.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Rv</th>
<th>Significance</th>
<th>Potential</th>
</tr>
</thead>
<tbody>
<tr>
<td>mshA</td>
<td>Rv0486</td>
<td>Essential for <em>Mtb</em> growth.</td>
<td>Drug target</td>
</tr>
<tr>
<td>mshB</td>
<td>Rv1170</td>
<td>Inactivation does not fully prevent MSH biosynthesis.</td>
<td>May hold promise if targeted in conjunction with other gene products, i.e. inhibiting the product of this gene together with Mca and one of the other essential enzymes.</td>
</tr>
<tr>
<td>mshC</td>
<td>Rv2130c</td>
<td>Essential for <em>Mtb</em> growth</td>
<td>Drug target</td>
</tr>
<tr>
<td>mshD</td>
<td>Rv0819</td>
<td>Inactivation does not fully prevent MSH biosynthesis. Production of alternative thiols by the mutant</td>
<td>Has potential as a drug target</td>
</tr>
<tr>
<td>mca</td>
<td>Rv1082</td>
<td>Mutants susceptible to electrophilic toxins and oxidants</td>
<td>Drug target</td>
</tr>
<tr>
<td><em>Mtr</em></td>
<td>Rv2855</td>
<td></td>
<td>Drug target</td>
</tr>
</tbody>
</table>
1.6 Mycothiol analogs

The genes and their respective products outlined in table 1.1 accept substrates and produce products that eventually give rise to mycothiol. It is therefore, precisely for this reason that compounds based on the GlcN-Ins moiety that bear groups on the nitrogen that resemble those of the respective transition states, are seen as potential inhibitors for any one of the enzymes involved in the biosynthesis, detoxification and reduction of mycothiol (knapp et al., 2002). However, such inhibitor studies are hampered by the scarcity of mycothiol. Although MSH can be isolated from whole cells (Spies and Steenkamp, 1994), amounts recovered are not sufficient for large scale experiments. A subsequent modification of the isolation and purification procedure developed by Steenkamp and Vogt, 2004, generates reasonable yields but poses a limitation when structural modifications are required and as such, synthetic approaches are a necessity.

Previous synthetic studies concerning the preparation of a suitably protected D-myo-inositol, its resolution and the subsequent generation of an α-glycoside were hampered by convoluted syntheses (Borneman et al., 1997; Jardine et al., 2002; Nicholas et al., 2002).

Interestingly, Mca has been shown to be active against a mycothiol-S-bimane analogue (inositol replaced with S-cyclohexyl) (figure 1.10) exhibiting specific activity of 7500 nmol min$^{-1}$ mg protein$^{-1}$ as compared to 14200 nmol min$^{-1}$ mg protein$^{-1}$ for the authentic mycothiol-S-bimane (knapp et al., 2002). In addition to the truncated des-myo-inositol disulfide (section 1.5), Mtr has recently been shown to be active against the methyl and benzyl glycoside analogs of MSSM (Stewart et al., 2007).
The enzyme activities exhibited against the mycothiol-S-bimane analogue, the MSSM analogs and the truncated *des-myo*-inositol disulfide leads to a few conclusions with regard to the inositol portion of mycothiol: that it may not always be essential for substrate recognition by certain MSH processing enzymes, that neither the inositol hydroxyls nor the glycosidic linking atom (O vs. S) plays a major role in enzyme binding. These observations also suggest that these analogs can provide a platform upon which to base inhibitor design (Stewart *et al.*, 2007; knapp *et al.*, 2002).

**Figure 1.10**: Mycothiol analogs. Structures adapted from knapp *et al.*, 2002 and Stewart *et al.*, 2007.
1.7 Mca and MshB inhibitors

In an attempt to design and optimize potent inhibitors, Nicholas et al., 2002, screened a synthetic library comprised of bromotyrosine derivatives alongside a set of related natural product inhibitors, isolated from an Australian non-verongid sponge, *Oceanapia sp.* (figure 1.11) (Nicholas et al., 2001). The IC\(_{50}\) values generated from these studies varied from 3 µM for compound 1 to 2.7 mM for compound 13 (Nicholas et al., 2002). This data, taken together with the fact that compounds 1 to 4 feature a spirocyclic isoxazoline core or a reduced bromophenyl oximinoamide functionality as is the case for compounds 5 and 6, led the authors to several conclusions regarding structure-activity relationships.

In view of the fact that compounds 1 and 6 appeared to be the most effective, whereas, compounds 3 and 4 exhibited IC\(_{50}\) values that were comparable to the synthetic set, compounds 13 and 14, it was concluded that the potency of compounds 1, 3 and 4 could not be attributed to the presence of the spirocyclic isoxazoline system since the equally inhibitory compounds 6, 13 and 14 comprise the reduced bromophenyl oximinoamide functionality. These observations suggested that the spirocyclic ring system is not necessary to achieve good inhibition (Nicholas et al., 2002), and that bromotyrosine derivatives containing a Mca specific portion could lay a foundation for a more specific and potent inhibition, as illustrated by Metaferia et al, 2007a, through the synthesis of MSH-inspired mimicking analogs that centred on the incorporation of a quinic acid template to replace the inositol ring of natural MSH (figure 1.11).

Two of the ensuing compounds (14a and 14b) in this synthesis exhibited a 45% inhibition at 50 µM. The reason for using a reduced quinic acid as a surrogate for inositol instead of the cyclohexane was that since it has some hydroxyls and is more polar than cyclohexane it would be involved in hydrogen bonding. In a different study carried out in our lab (Marakalala, M.J. 2008, PhD Thesis) using compounds 35-38 (discussed in chapters 5 and 6) it was found that the phenyl ring is a better inositol surrogate than all the other six membered rings tried so far.
Figure 1.11: Compounds 1-7 are bromotyrosine derived inhibitors, whereas, compounds 8-14 are a synthetic suit. The synthetic compounds 8-9 displayed IC$_{50}$ values in the range of 60-200 µM. Compounds 11 and 12 were poor inhibitors. Compounds 15 and 16 are MSH-inspired inhibitors. Compounds 14a and 14b incorporating a quinic acid ring as an inositol replacement. Structures adapted from Nicholas et al., 2002 and Metaferia et al., 2007a.

Given that the active sites of Mca and MshB have been shown to share 42% sequence identity (Steffek et al., 2003) based on sequence alignments, Metaferia et al., 2007b synthesised spiroisooxazoline-like analogs (figure 1.12) built on the thioglycoside
scaffold targeting the two homologous enzymes. Interestingly, the spiroisooxazoline containing derivatives proved quite potent in the dual inhibition of Mca and MshB, substantiating the notion that inhibitors bearing a portion specific to the enzyme’s active site could be very useful against significant enzymes in the MSH biosynthetic pathway.

Figure 1.12: Compounds found to inhibit MshB and Mca. The sulfonamides 16 and 18, the homophenylalanine trifluoroacetate 20 and the heterocycles 21, 22, 24 and 25 were found to inhibit both MshB and Mca. Compound 23 only showed weak inhibition towards Mca whereas, compounds 21, 22 and the N-Boc analog 19 appeared to be specific for MshB. Structures adapted from Metaferia et al, 2007b.
1.8 Naphthoquinones

The quinones (figure 1.13) are a heterogeneous group of highly reactive compounds found in several natural products and endogenous biochemicals. Over the years, they have become indispensable tools as specific metabolic inhibitors in enzyme research and as useful drugs in the control of disease (Webb, 1966; Bolton, 2006), for instance, atovaquone, a substituted 2-hydroxynaphthoquinone is used therapeutically to treat \textit{Plasmodium falciparum} malaria (Kessl \textit{et al.}, 2003). General antimicrobial activities due to these compounds had long been established using the simplest substance of this class, \(\rho\)-benzoquinone and observing its bactericidal actions against a staphylococcus isolate, \textit{Staphylococcus py. aur} (Cooper, 1913).

Even though the antimicrobial nature of quinones had long been postulated, their potent biological actions had never been correlated with specific mechanisms. Enzyme inhibition has since been shown to be possible through complexes formed between quinones and the ion of the enzyme, as illustrated in tyrosinase catalyzed oxidations (Arthur and Mclemore, 1955), and that a quinone-quinol system can be generated using xanthine oxidase and following the reduction of a quinone by monitoring its oxidation-reduction potential (Dixon, 1926). In essence, the reduction of a quinone results in the oxidation of the enzyme, which is one of a few mechanisms by which quinones can inhibit enzymes. This type of inhibition was validated by studies carried out on the inactivation of papain by selected oxidants that included a quinone. The subsequent inactivation was shown to be reversible by treating the enzyme with hydrogen sulphide and other reducing agents (Hellerman and Perkins, 1934).

Oxidation-reduction reactions of quinoid or quinol substances usually involve the formation of a semiquinone intermediate, the existence and stability of which depends mostly on the pH and nature of the quinone. In some instances, this intermediate had been a challenge to isolate as its identification tended to be colorimetric. It was also found to crystallize in cultures of \textit{Bacillus chlororaphis} without the culture medium showing the typical green colour that the intermediate is usually associated with.
Thus the semiquinone, which exists only to a slight extent, seems to be the active form in producing certain effects on metabolic processes in that it may be reduced to the hydroquinone, oxidized to the quinone, dimerize to form quinhydrone, or react with various metabolites (Michaelis and Schubert, 1937).

Since naphthoquinones can form adducts with thiols and that it is possible to discern between toxicity that can be ascribed to their redox cycling ability, which results in the formation of free radicals, as opposed to toxicity due to such addition reactions, by introducing substituents at the sites where thiols would react for example, 2,3-dimethyl naphthoquinone is used to evaluate the activity of naphthoquinones as redox cyclers and does not mop up thiols. This led to the realisation of another type of inhibition conferred by quinones involving sulfhydryl groups of enzymes.

The finding was made as a consequence of succinate dehydrogenase inhibition using a variety of quinones, including p-benzoquinone and 2-methyl naphthoquinone which were shown to decrease toxicity almost tenfold. This decrease in toxicity provided an explanation for the absence of clinical toxicity with natural vitamin K. In addition to the 2-methyl group, vitamin K contains a long side chain at the 3 position thus making any reaction with sulfhydryl groups virtually impossible (Potter and DuBois, 1943).
1.9 Subversive substrates for Trypanothione reductase

The fact that glutathione disulfide reductase was able to catalyze a NADPH dependent electron transfer to 2,4,6-trinitrobenzenesulfonate in the presence of oxygen with concomitant generation of hydrogen peroxide (Carlberg and Mannervik, 1986), set precedence for subsequent studies utilizing trypanothione reductase (TR) and suitably substituted naphthoquinone and nitrofuran derivatives. The rationale in using the latter derivatives was based on the notion that the semiquinone generated will reduce oxygen to superoxide anion radicals whilst regenerating the quinone. This would in turn subvert the physiological function of TR by increasing the levels of hydrogen peroxide and trypanothione disulfide (Henderson et al., 1988; Michaelis, 1935; Salmon-Chemin et al., 2001).
The study showed consistency with regard to observations made earlier concerning inherent enzyme inhibition by quinones. The naphthoquinone (NQ) derivatives tested against TR under aerobic conditions were not capable of inactivating the enzyme, whereas, the opposite is true when the reaction was carried out under anaerobic conditions utilising the nitrofuran derivatives, causing irreversible inactivation. Moreover, in addition to their ability to act as substrates for TR, the compounds were also found to inhibit enzymatic reduction of the physiological substrate, and subsequent exposure of *Trypanosoma cruzi* epimastigotes to these compounds resulted in trypanocidal activities (Henderson *et al*., 1988).

Continuing the development of better and efficient subversive substrates in pursuit of an effective trypanocidal compound, Salmon-Chemin *et al*., 2001, synthesised and tested a series of 2-and 3-substituted 1,4-naphthoquinone derivatives as subversive substrates targeted against *Trypanosoma cruzi* trypanothione reductase and lipoamide dehydrogenase. Three distinct series of 1,4-naphthoquinones substituted at C-2 and C-3 of the quinone moiety by alkylamino side chains of varying lengths were synthesised as potential inhibitors of *Trypanosoma cruzi* TR using menadione, plumbagin and juglone as parent molecules (figure 1.13a). NQ derivatives based on plumbagin were found to be reduced by *Trypanosoma cruzi* lipoamide dehydrogenase faster than the corresponding menadione derivatives. A derivative based on plumbagin also proved to be a potent subversive substrate and effective uncompetitive inhibitor against trypanothione disulfide and NADPH.

In addition to general inhibition, the influence of the spacer length was assessed on the inhibition of trypanothione disulfide (TS$_2$) and, interestingly, the highest antiparasitic activity within a series of bis 1,4-NQ derivatives was observed with those compounds possessing the longest spacer between the two NQ moieties (Salmon-Chemin *et al*., 2001).
Figure 1.13a: Structure of trypanothione and one of the inhibitors. Structures adapted from Salmon-Chemin et al., 2001.

1.10 Subversive substrates for Mtr

Preliminary assessment of some commercial naphthoquinones, for example, 2,6-dimethylbenzoquinone, 5-hydroxy-1,4-napthoquinone, 5,8-hydroxy-1,4-naphthoquinone and 2-methyl-1,4-napthoquinone that have previously been shown to react with various flavoproteins was undertaken and their activities verified using Mtr (Patel and Blanchard, 1999). A more involved study utilising a series of synthetic and plant derived naphthoquinone derivatives based on the 7-methyljuglone parent structure (figure 1.14) has recently been completed, and the derivatives were evaluated for antibacterial activity against Mtb, as subversive substrates against Mtr and to establish structure-activity relationship (Mahapatra et al., 2007).
Figure 1.14: 7-methyljuglone derivatives used as subversive substrates against Mtr. Mahaptra et al., 2007.

Compounds 28, 33 and 34 proved to be the most efficient exhibiting $k_m$ values that ranged between 30 and 60 $\mu$M with admirable inhibitory potential, where their MIC’s were noted to be 22, 21 and 23 $\mu$M respectively. Interestingly, even though compounds 26, 29, and 30 gave $k_m$ values in the range of 200-500 $\mu$M, their MIC values (6, 3 and 11$\mu$M) were considerably more appealing than all the compounds tested. The discrepancies in $k_m$ and MIC values of these compounds can be attributed to poor selectivities due to their nonspecific activity with other disulfide reductases which are also found in cells (Mahaptra et al., 2007), and as mentioned in section 1.8 and illustrated by Metaferia et al., 2007a, that inhibitors specific to MSH-biosynthetic enzymes could be accomplished through the design of MSH mimicking analogs. The same can also be achieved with Mtr (Mahaptra et al., 2007).
1.11 Overview of the chemistry involved in the synthesis of subversive substrates

Given the substantial homology between glutathione, trypanothione, and mycothiol disulfide reductases, and the fact that GSH and trypanothione have been shown to form disulfides upon exposure to increased levels of hydrogen peroxide, due to their respective enzyme-catalyzed reduction of quinones to semiquinone radicals (Patel and Blanchard, 1999; Salmon-Chemin et al., 2001), one of the focal points of this project also centres on the use of naphthoquinone derivatives against mycothiol disulfide reductase. As mentioned earlier (section 1.6), the conspicuous absence of mycothiol and its biosynthetic enzymes in humans provides an extremely attractive drug target against \textit{Mtb}, and Mtr was consequently chosen as an anti-tubercular target.

As illustrated by Knapp et al., 2002, and recently by Metaferia et al., 2007a through their synthesis and use of simplified thioglycoside analog active against Mca, we envisaged a similar synthetic strategy that makes use of subversive substrates built upon a thiophenol derived scaffold. During the course of this study, Stewart et al., 2007 also published the synthesis of a methyl glycoside analog of MSSM (figure 1.10) and demonstrated its activity with Mtr. Their study was also able to show that the benzyl portion of this MSSM analogue occupies the same site as inositol of the native substrate.

The substitution of the inositol portion with a phenyl group is largely based on the difficulties encountered with the synthesis and resolution of the inositol and its alpha glycosylation step (Borneman et al., 1997; Jardine et al., 2002; Nicholas et al., 2002). On the other hand, thioglycosides have been shown to be rather resistant to degradation by glycosidases and also show slower hydrolysis by aqueous mineral acid than the corresponding \textit{O}-glycosides (Knapp et al., 1996; Collins and Ferrier, 1995). Moreover, they are easy to prepare and should the need arise, they can act as good leaving groups upon complexation with a range of sulphur-specific reagents to form sulphonium cations (Collins and Ferrier, 1995).
1.12 Preparation of thioglycosides

There are several strategies available for preparing thioglycopyranosides with high stereo and regioselectivity, most of which involve treating peracetylated pyranoses with thiols such as thiophenol or thioethanol in the presence of Lewis acid such as boron trifluoride etherate (BF$_3$.Et$_2$O), to give predominantly 1,2-trans products depending on the nature of the C-2 neighbouring groups (Herzner et al., 2000). This assertion is well illustrated in the formation of the phenyl 1-thio-β-D-galactopyranoside when commercial penta-O-acetyl-D-galactose is treated with a thiophenol in the presence of BF$_3$.Et$_2$O (Janczuk et al., 2002). An alternative method for preparing alkyl and aryl 1-thioglycosides employs the use of a thiourea that is reacted with acetylated glycosyl halides to give a pseudothiouronium salt that can be hydrolyzed to the desired 1-thio-glycopyranose with aqueous potassium carbonate (Bonner and Kahn, 1950; Lindhorst, 2003).

In addition to their stability, thioglycosides are so versatile in that they can be converted into a variety of other common glycosyl donors upon exposure to an appropriate thiophilic promoter (figure 1.15). It has also been shown that acetylated thioglycosides can be deacetylated without degradation (see reviews by Davis, 2000; Demchenko, 2003), which is another attractive feature, since acyl protecting groups were employed in the synthesis of the target subversive substrates.
Figure 1.15: Generation of a β-D-thioglycopyranoside from penta-O-acetyl-D-glucose, with subsequent activation by a wide variety of promoters to different glycosyl donors such as the bromide, imidate, fluoride, and the alcohol. This Figure is adapted from Lindhorst, 2003.

1.13 N-protection of amino sugars

One of the requirements for the target subversive substrates was the stringent stereoselective synthesis of the α-thioglycoside, as dictated by the α-GlcN-Ins portion of mycothiol, and as a result, a choice of a suitable C-2 non-participatory protective group had to be carefully selected since glucosamine hydrochloride was chosen as the foundation on which to initiate the synthesis. Even though there is a wide variety of groups that can be utilized for the N-protection of amino sugars (figure 1.16), most were
deemed undesirable for our purposes since they tend to influence the formation of 1,2-trans glycosides.

**Figure 1.16:** A selection of N-protecting groups. These groups tend to influence the formation of 1,2-trans glycosides during glycosylation steps. The phthalimido (Pht) and the N-tetrachlorophthalimido (TCP) groups are introduced by treating the free glucosamine with phthalic anhydride and N-tetrachlorophthalic anhydride respectively. Whilst TCP groups can be cleaved under mild conditions using ethylenediamine, the removal of the Pht group can be a mission that requires rather harsh conditions such as methanol-hydrazine-hydrate at 100°C (Debenham et al., 1995; Jain et al., 1997). Protection of an amino sugar with a dithiasuccinoyl (Dts) group is achieved by treating glucosamine hydrochloride with bis(ethoxythiocarbonyl)sulphide in ethanol or S-carbomethyl O-ethylidithiocarbonate in methanol. Cleavage of the Dts group is attained by using thiols such as dithiothreitol or other reducing agents (Barany and Merrifield, 1977). The introduction of Allyloxy carbonyl amides as protecting agents is achieved by treating the amino sugar with allyloxycarbonyl chloride and triethylamine in THF. This figure was adapted from Lindhorst, 2003.

In instances where C-2 protecting groups are participatory as in acetylated glucopyranosides, the glycosylation reaction normally proceeds through the formation of an oxocarbonium ion intermediate that when attacked by a nucleophile (e.g. -OH), leads to the formation of an orthoester which is eventually isomerised to the β-glycoside (e.g. the abovementioned phenyl 1-thio-β-D-galactopyranoside) (figure 1.17). By comparison, N-acetylated amino sugars tend to form stable 1,2-oxazoline derivatives which may be used as glycosyl donors with unavoidable generation of the 1,2-trans glycosides (Wittmann and Lennartz, 2002).
To effect the desired 1,2-cis thioglycosides used in this thesis, azides were selected as suitable amino protecting groups due to their non-participatory nature, less steric hindrances and their lack of hydrogen or carbon nuclei that complicate nuclear magnetic resonance (nmr) spectra (Nyffeler et al., 2002). Moreover, azides have been shown to be resistant to many reaction conditions and can be easily reduced to the corresponding primary amine using either the Staudinger reaction or hydrogen over palladium-on-charcoal at atmospheric pressure (Nyffeler et al., 2002 Lange et al., 1998).
In general, there are various methods available for the preparation of azide derivatives, the earliest being the one developed by Lemieux and Ratcliffe, 1979, where sodium azide and ceric ammonium nitrate (CAN) are used to generate the azido radical that adds to glycals to afford 2-azido-2-deoxyglycosylnitrates. However, this method was established utilising glycals and generates epimeric mixtures of 2-azido-2-deoxy-1-\(\text{O}\)-nitropyranoses, the ratio of which is dependent on the structure of the glycal starting material.

This is best illustrated by the azidonitration of the galactal in figure 1.18 where the 2-equatorial product is highly favoured leading to 2-azido-deoxygalactose nitrate (36) whereas, the azidonitration of the glucal (37) affords mixtures of 2-azido-2-deoxy-D-gluco and D-manno derivatives (38 and 39). The regioselectivity observed for these transformations has been established to be influenced by the steric hindrance of the top face of the D-galactal derivative as opposed to that of the D-glucal derivative (Lemieux and Ratcliffe, 1979; Paulsen, 1984, Banoub et al., 1992).

The fact that steric hindrances at the top face of the galactal are responsible for the observed regioselectivity was also confirmed in studies carried out by Seeberger et al., 2000, where a series of C-4 and C-6 conformationally constrained glucals was shown to selectively furnish C-2 azido-deoxy-D-glucoses over their corresponding D-manno isomers when subjected to azidonitration, as shown in table 1.2.

![Figure 1.18: Regioselective azidonitration of D-galactal and D-glucal derivatives.](image-url)
Table 1.2: Regioselective synthesis of C-2 azido-deoxy-D-glucoses using glucals constrained at C-4 and C-6 hydroxyl groups. Seeberger et al., 2000.

<table>
<thead>
<tr>
<th>R</th>
<th>α-β-gluco</th>
<th>α-β-manno</th>
<th>Yield (%)</th>
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<tbody>
<tr>
<td>Ac</td>
<td>8</td>
<td>1</td>
<td>71</td>
</tr>
<tr>
<td>Piv</td>
<td>5,5</td>
<td>1</td>
<td>56</td>
</tr>
<tr>
<td>Bn</td>
<td>14</td>
<td>1</td>
<td>59</td>
</tr>
<tr>
<td>TBDMS</td>
<td>12</td>
<td>1</td>
<td>58</td>
</tr>
<tr>
<td>TIPS</td>
<td>20</td>
<td>1</td>
<td>71</td>
</tr>
<tr>
<td>Ac</td>
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<td>1</td>
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</tr>
<tr>
<td>TIPS</td>
<td>11</td>
<td>1</td>
<td>63</td>
</tr>
<tr>
<td>Ac</td>
<td>3</td>
<td>1</td>
<td>75</td>
</tr>
</tbody>
</table>

The study also found that while the reaction of the acetylated D-glucal furnished the desired C-2 azide substituted D-glucose with selectivity over the D-mannose derivative, it was in fact the introduction of large protecting groups at C-3 that provided additional bulkiness and resulted in increased yields as well as excellent selectivities, as evident in the 14:1 and 20:1 ratios of the desired 2-azido-2-deoxy-D-glucose over the manno derivative when benzyl and TIPS protecting groups were used respectively (a). Surprisingly, the use of the sterically more demanding pivaloyl group exhibited lower selectivities (a). Selectivities were slightly lower in cases where benzylidene acetals (b) were used as opposed to isopropylidene (1), and increased yields were observed when di-tert-butylsilane (c) protecting groups were used but exhibited low selectivities when acetates were fixed at C-3.
The synthesis of 2-azido-2-deoxy-3,4,6-tri-O-acetyl-d-glucopyranosyl acetate was consequently adapted from Martin-Lomas et al., 2000; Cavender and Shiner Jr, 1972, where the azido moiety is introduced to d-glucosamine via a triflyl azide (TiN₃) that is generated in a reaction between sodium azide (NaN₃) and trifluoromethanesulfonic anhydride (triflic anhydride) to afford the desired 2-azido-2-deoxy glucopyranoside. The latter is acetylated and ultimately converted into a thioglycoside using benzenenethiol (figure 1.19).

![Figure 1.19: Generation of Phenyl 3,4,6-Tri-O-acetyl-2-azido-2-deoxy-1-thio-d-glucopyranoside from d-glucosamine (40). The latter is treated with sodium methoxide and DMAP in methanol and then treated with a freshly prepared solution of TiN₃ and stirred for 18 hrs at room temperature. Methanol is removed under reduced pressure and the resulting residue is dissolved in pyridine and acetic anhydride is added at 0°C. The mixture is stirred between 0 and 4°C for 10 hours. Flash chromatography gives (41) and treatment thereof with thiophenol in the presence of a Lewis acid (BF₃,Et₂O) generates (42). Crystallization of (42) in absolute ethanol gives the α-isomer.](image)

### 1.15 Aims and objectives

The over-expression of *Mtb* recombinant proteins in *Escherichia coli* (E. coli) remains a challenge and in some instances poor protein yields are often observed even when mycobacterial genes are placed behind strong *E. coli* promoters. It is suspected that these difficulties may be due to differences in the GC contents of the two microorganisms where *Mtb* has been shown to have a GC content of about 70% as opposed to the 50% observed in *E. coli* (Matsuo et al., 1988; Lakey et al., 2000).

This prompted us to (1) attempt the cloning and expression of the gene encoding mycothiol disulfide reductase in *Corynebacterium glutamicum* (*C. glutamicum*) utilising pEC-XK99E, an *E. coli-C. glutamicum* shuttle expression vector developed by Kichner
and Tauch, 2003. This would be carried out in an attempt to enhance the amount of mycothiol disulfide reductase produced.

(2) To synthesise subversive substrates based on a 1-O-(2-Amino-2-deoxy-α-D-glucopyranosyl)-D-myoinositol (GlcN-Ins) scaffold.

(3) To carry out enzyme kinetic studies using the synthesised subversive substrates.

(4) Evaluation of the subversive substrates against *Mtb*. 
Chapter 2
Materials and Methods

2.1 Molecular biology methods for cloning the mtr gene

*Corynebacterium glutamicum* and the pEC-XK99E *E. coli*-C. glutamicum shuttle expression vector were a kind gift from Dr. Andreas Tauch of the Center for Biotechnology (CeBiTec) at the Universität Bielefeld, Germany, while the *E. coli*-mycobacteria shuttle vector pSD26 was a donation from Dr W. R. Jacobs (Department of Microbiology and Immunology, Albert Einstein College of Medicine) to Professor D.J. Steenkamp. *Mtb* DNA was obtained from Professor van Helden, Division of Medical Biochemistry, University of Stellenbosch, South Africa. One Shot™ TOP10 chemically competent *E. coli* cells and the Zero Blunt™ PCR cloning kit were purchased from Invitrogen. Restriction enzymes (Sac I, EcoR I, Kpn I and Xba I along with their buffers) and agarose were obtained either from Fermentas or Roche. Pfu DNA polymerase, IPTG T4 DNA ligase and the 1 kb DNA ladder were purchased from either Promega or Roche. The antibiotics, kanamycin and hygromycin were obtained from Calbiochem and Fluka respectively. The MinElute™ Gel Extraction and QiAprep™ Spin Miniprep kits were from Qiagen, whilst the Wizard® SV Gel and PCR Clean-Up system was purchased from Promega. The NUCLEOBOND AX® for large scale purification of plasmids was obtained from Macherey-Nagel. Tryptone, yeast extract and agar were purchased either from Difco, Biolab, Fluka or Oxoid whilst, Middlebrook 7H9 Broth was exclusively obtained from Difco and Tryptic soy broth from Merck. Tween® 80, acetonamide, ferric ammonium citrate and acrylamide, Acetonitrile E chromasolv®, TFA, Acetonitrile-d₆, β-NADPH, 4,4-dithiopyridine, and 2-bromo-2’-acetonaphthone were obtained from Sigma-Aldrich. N,N’-methylenebisacrylamide was purchased from Fluka. Ammonium persulfate, sodium dodecyl sulphate and TEMED were purchased from BDH. Isoniazid and Ethionamide were purchased from Sigma.
2.1.1 Introduction of a His\textsubscript{6} tag sequence into the vector pEC-XK99E

pEC-XK99E (6 µg) was digested with 20 U of the restriction enzyme SacI in the presence of a Sure/Cut restriction buffer A, diluted 10X upon addition to the mixture and incubated at 37 °C for 16 hours (as recommended by the supplier). The enzyme digest mixture was heated at 70 °C for 10 minutes to denature the enzyme and 20 U of the restriction enzyme EcoR1 were added and the reaction mixture incubated at 37 °C for a further 16 hours (the sequential digestion was done because of the close proximity of the restriction sites). The result of the digest was analysed on a 0.8% agarose gel, incorporating ethidium bromide and the DNA was visualised using a Fluor Chem 5500 Imager. The band corresponding to the SacI-EcoR1 restricted plasmid was extracted from the agarose gel using the Wizard\textsuperscript{®} SV Gel and PCR Clean-Up system or the MinElute\textsuperscript{™} Gel extraction kit, as per manufacturer’s instructions. This procedure was used in order to remove the piece that had been excised so it is not ligated back in when ligating the His\textsubscript{6} tag sequence in the gene (section 2.1.2).

2.1.2 Preparation of the His\textsubscript{6} tag sequence

Two oligonucleotide sequences (designated oligo 1 and the reverse sequence, oligo 2) were synthesized to generate SacI and EcoR1 recognition sites upon ligation into the pEC-XK99EE, oligo 1: 5’-ATGAAT TCA GCC ACC ACC ACC ACC ACA GCA GCG GCC TGG CGC GCG GCA GCG AGC T-3’ and oligo 2: 3’-GTCG GTG GTG GTG GTG GTG TCG TCG CCG GAC CAC GGC GCG CCG TCG C-5’. The His\textsubscript{6} tag and the thrombin are highlighted in red and green respectively. The progression of the envisaged process is shown below, starting with the cloning region of pEC-XK99E.

```
<table>
<thead>
<tr>
<th>EcoR1</th>
<th>SacI</th>
</tr>
</thead>
<tbody>
<tr>
<td>5’-ATGG</td>
<td>AA TTC GAG CT</td>
</tr>
<tr>
<td></td>
<td>Sequential digestion</td>
</tr>
<tr>
<td>5’-ATGG</td>
<td>C GTG ACC CGG GGA TCC TCT AGA GTC GAC CTG CAG GCA TG C AAG CTT-3’</td>
</tr>
</tbody>
</table>
```
Ligation of the His\textsubscript{6} tag sequence into the cut vector would then reform EcoR1 and Sac1 restriction sites (figure shown in chapter 3).

A nucleotide sequence coding for a His\textsubscript{6} tag was prepared by mixing 9.8 µg of each of the two complimentary oligonucleotides in 100 µl of nuclease free water. The mixture was heated to 95 °C for 5 minutes and allowed to cool to facilitate annealing of the oligonucleotides, the result being a sticky ended fragment (as shown below, figure 2.1). A 2% agarose gel was used to analyse the result of the annealed sequences.

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5'-AATTCAGCCACCACCCACACCACACAGGCCTGGTGCCGGGCGGCAGCGAGCT-3’
3’-GTCGCTGGTGGTGGTGGTGGTGTCGCCGGACCAAGGCCCGCCGTCG-5’

Figure 2.1: Expected annealed oligonucleotides

2.1.3 Generation of the pEC-XK99E-his

The ligation of the His\textsubscript{6} tag sequence to the pEC-XK99E vector (linearized in 2.1.1) was performed by mixing 5ng of the His\textsubscript{6} tag sequence with 50 ng of the vector using 10X ligation buffer (1.2 µl) and 2 U of the ligase. The volume of the ligation mixture was made up to 12 µl with nuclease free water. The ligation reaction was incubated overnight at 4 °C. The reaction was stopped by incubating the ligation mixture at 70 °C for 10 minutes. Confirmation of the ligation is described in 2.1.5.

2.1.4 Transformation with One Shot\textsuperscript{®} TOP10 competent cells

Top10 competent cells (50 µl vial) were thawed on ice and the ligation reaction (2 µl) was added to the cells and incubated on ice for 30 minutes. This was followed by incubation at 42 °C for 45 seconds and immediately incubated on ice for 2 minutes. A pre-warmed (37 °C) SOC medium (250 µl) was then added and the transformation reaction incubated at 37°C for 1 hour at 225 rpm in an orbital shaker. The vials with the transformed cells were then placed on ice and increasing volumes (typically, 10-100 µl) aseptically plated onto LB plates (10 g/l tryptone, 5g/l yeast extract, 5g/l NaCl and15g/l agar, pH 7.5)
supplemented with 50 µg/mL kanamycin. The plates were incubated overnight at 37 °C. Untransformed Top10 competent cells were used as control.

2.1.5 Confirmation of the pEC-XK99E-his

A total of 7 colonies from the LB plates prepared in section 2.1.4 were inoculated into LB media supplemented with 50 µg/mL kanamycin and grown overnight at 37 °C. The cells were harvested and the plasmids extracted using a QiAprep® Spin Miniprep kit. The resulting plasmids were cleaved with EcoRV and BamHI and increasing volumes of the digest mixture were analysed on a 2% agarose gel. The sample that gave rise to a 685 bp band was used to transform Top10 competent cells (vector diagram and its characteristics are shown and discussed in chapter 3). Single colonies resulting from LB agar plates supplemented with kanamycin were grown in LB media and a large scale plasmid extraction using NUCLEOBOND AX® was carried out. The modified plasmids were then submitted for sequencing to confirm the presence of the His6 tag (discussed in detail in chapter 3)

2.1.6 Amplification of the mtr gene

The mtr gene was amplified by PCR using 5’-GGTACCCCCCTACAAATGGAAACG-3’ and 5’-TCTAGAGTCACGCAGGCCAG-3’ as the forward and the reverse primers respectively. The primers were synthesised to generate kpn1 and Xba1 recognition sites (underlined). The amplification reaction consisted of 76 ng of the template (Mtb DNA), 400 ng of each primer in 10X pfu buffer, 0.2 mM of each dNTP, 6 U of pfu DNA polymerase, and the volume made up to 100 µl with nuclease-free water. PCR was carried out using an Eppendorf master cycler gradient with the following temperature profile:

denaturation 98 °C for 5 minutes
denaturation 98 °C for 1 minute, 35 cycles
annealing 65 °C for 1 minute, 35 cycles
extension 72 °C for 2 minutes, 35 cycles
final extension 72 °C for 10 minutes
The PCR reaction (5 µl) was analysed on a 1.5% agarose gel

2.1.7 Extraction of the mtr gene from agarose

The agarose gel from section 2.1.6 was re-ran using 20 µl of the PCR reaction mixture and the band corresponding to the mtr gene was excised and purified using the MinElute™ Gel extraction kit.

2.1.8 Ligation into pCR®-Blunt

The ligation of the blunt ended mtr gene into the pCR®-Blunt vector was performed by mixing linearized blunt pCR®-Blunt vector (25 ng) with the blunt ended mtr gene (75 ng) in 10X ligation buffer. T4 DNA ligase (4 U) was added to facilitate ligation and the reaction volume was made up to 20 µl with nuclease-free water. The ligation reaction was incubated overnight at 4 °C.

2.1.9 Transformation of Top 10 competent cells with pCR®-Blunt-mtr

Transformation was essentially carried out as described in section 2.1.4. Twenty colonies were selected for growth in LB complemented with kanamycin and subsequent plasmid extraction was performed using the QiAprep® Spin Miniprep kit.

2.1.10 Restriction endonuclease digestion of the pCR®-Blunt-mtr

pCR®-Blunt-mtr was digested overnight with 10 U of each of the restriction enzymes (fastDigest®kpnI and fastDigest®XbaI) in the presence of 10X fastDigest® buffer. Agarose gel (0.8%) electrophoresis was used to analyse the result of the digest and the band corresponding to the mtr gene was excised and purified using the Wizard® SV Gel and PCR Clean-Up system.
2.1.11 Ligation of the \textit{mtr} gene into pEC-XK99E-his to generate pEC-XK99E-his-\textit{mtr}

The pEC-XK99E-his vector (50 ng) prepared in section 2.1.3 was mixed with the \textit{mtr} DNA (45 ng) in 10X ligation buffer. T4 DNA ligase (2 U) was added and the volume of the reaction mixture made up to 14 µl with nuclease-free water. The ligation reaction was then incubated at 4°C for 16 hours.

2.1.12 Transformation of Top 10 competent cells with pEC-XK99E-his-\textit{mtr}

The ligation mixture (2 µl) was subsequently used to transform Top 10 competent cells following the procedure described before. The transformation mixture was aseptically plated in increasing volumes (50 µl, 100 µl, and 150 µl) onto LB agar plates supplemented with kanamycin (50 µg/mL). Also included were the positive and negative controls (competent cells transformed with pEC-XK99E-his and competent cells only). Two colonies from each plate were inoculated into LB medium supplemented with kanamycin (50 µg/mL) and incubated overnight at 37°C.

2.1.13 Confirmation of the presence of the \textit{mtr} gene

Plasmid (pEC-XK99E-his-\textit{mtr}) extraction was achieved through the use of QiAprep® Spin Miniprep kits and subjected to a restriction digest using \textit{kpn1} and \textit{Xba1} (10 U each) to confirm the presence of the insert (\textit{mtr} gene). The digest was analysed by electrophoresis on an agarose gel (0.8%). The colony that contained the correct recombinant was inoculated into 50 mL of LB medium and after an overnight growth of cells at 37 °C; a large scale plasmid extraction was carried out and submitted for sequencing.
2.1.14 pEC-XK99E-his\textendash{}mtr electroporation into \textit{C. glutamicum}

2.1.14.1 Preparation of the EPO medium and LBG plates

The method for growing electroporation-competent \textit{C. glutamicum} cells was slightly modified from van der Rest \textit{et al.}, 1999. Isoniazid (400 mg), glycine (2.5 g), and Tween 80 (0.1 mL) were dissolved in water (20 mL), filter sterilized and added into autoclaved LB medium to make up the EPO medium. Petri dishes containing LBG were poured as a mixture containing tryptone (5 g/l), NaCl (5 g/l), yeast extract (2.5 g/l), agar (15 g/l). Glucose (2\%) was added as a filter sterilized solution immediately before pouring the plates.

2.1.14.2 Preparation of competent \textit{C. glutamicum} cells

A freshly grown colony of \textit{C. glutamicum} was selected from the Petri dishes, inoculated into LB medium (5 mL) and cultivated for 18 hours at 37 °C. The EPO medium (50 mL) prepared in section 2.1.14.1 was inoculated with the overnight culture and incubated at 37 °C to an optical density (OD) of 0.25 at 600 nm. Upon reaching this OD, the temperature was lowered to 18 °C and the incubation continued for 28 hours, until the OD was 1.

The culture was chilled on ice for 20 minutes and the cells harvested by centrifugation at 10 000 g. The cells were washed four times with ice-cold 10\% glycerol (50 mL) and resuspended in 10\% glycerol (0.5 mL). Aliquots (100 µl) were stored frozen at -80°\textdegree C. For electroporation the cells were thawed on ice and 3 µl of the pEC-XK99E-his\textendash{}mtr (section 2.1.11) was added.

The mixture was transferred to an electroporation cuvette, and the electroporation parameters set at 25 µF, 600 Ω and 2.5 kV. LBG (1 mL) was added immediately after electroporation, the cells were transferred into an Eppendorf tube and incubated for 6 minutes at 46 °C This was followed by incubation at 30 °C for 1 hour, after which the
cells were plated on agar containing LBG supplemented with kanamycin (25 µg/mL). LBG plates were incubated at 30 °C for 2 days.

2.1.14.3 Confirmation of the presence of pEC-XK99E-his-mtr in *C. glutamicum* cells

Two colonies were inoculated into sterile distilled water (5 µl), and into this, 400 ng of the forward and the reverse primer (used in section 2.1.6) in 10X pfu buffer, 0.2 mM of each dNTP, and pfu (6 U) DNA polymerase were added. The volume was made up to 100 µl with sterile distilled water. PCR was carried out as described in section 2.1.6. The results of the colony PCR were analyzed on agarose gel (1.5 %). *C. glutamicum* colonies harbouring pEC-XK99E-his-mtr were grown in LB medium, glycerol stocks were prepared and stored at -80 °C. Large scale plasmid isolation was also carried out for DNA sequencing experiments.

2.1.15 Induction of the mtr gene expression with IPTG

Glycerol stocks of *C. glutamicum* wild type (control) and *C. glutamicum* containing pEC-XK99E-his-mtr and *C. glutamicum* (wild type) were used to inoculate LBG media supplemented with kanamycin (25 µg/mL) and incubated at 30 °C until an OD$_{600nm}$ of 1 was reached. The culture flasks were then allowed to equilibrate to 25 °C, at which point IPTG (1mM) was added for induction of the mtr gene. Expression was attempted at this temperature in an orbital shaker at 225 rpm.

A 4 mL aliquot was taken immediately and thereafter at 1 hour intervals up to 4 hours. The cell pellets of the aliquots were collected by centrifugation at 10000xg for 10 minutes. The cells were resuspended in 50 mM Hepes buffer (300 µl) pH 7.6 containing EDTA (0.1 mM), protease inhibitors (120 µl), PMSF, TLCK, TPCK, and sonicated (using a Branson sonifier 250) for 2 minutes at 30 second intervals on ice at the following settings: output level 2.5 at 20% duty cycle and the temperature maintained at 4 °C. The sonicated mixture was centrifuged at 10000xg for 10 minutes at 4 °C. The pellets were
resuspended in 50 mM Hepes buffer (120 µl) and an equal volume 2X SDS-PAGE solubilisation buffer (0.125 M Tris/HCl, 20% (v/v) glycerol, 4% (w/v) SDS, 10% 2-mercaptoethanol, and 0.2 mg bromophenol blue) was added. The corresponding supernatants were treated the same. The samples were mixed by vortexing and heated at 70 °C for 3 minutes and each sample (12 µl) analyzed by SDS-PAGE using a 10% polyacrylamide gel.

### 2.1.16 Cloning of the mtr gene without the His₆ tag to generate pEC-XK99E-mtr

pEC-XK99E (1.4 µg) was sequentially digested with 10 U of each of the restriction enzymes kpn1 and Xba1 in the presence of kpn1 buffer at 37 °C for 16 hours. The enzyme digestion mixture was heated at 65 °C for 15 minutes to stop the reaction and the result of the digestion was analysed on a 0.8% agarose gel, incorporating ethidium bromide. A FluorChem 5500 Imager was used to visualise the agarose gel. The band corresponding to the kpn1-Xba1 restricted plasmid was extracted from the agarose gel using the MinElute™ Gel extraction kit, as per manufacturer’s instructions.

The mtr gene was amplified as previously described (section 2.1.6) using the same set of primers and PCR conditions. Blunt ligation was carried out as in section 2.1.8, except that 300 ng of blunt mtr DNA was used. Transformation of the cells with pCR®-Blunt-mtr was also carried out as described before except that chemically competent E. coli DH5α cells were used instead of Top 10 cells. Transformation into DH5α competent cells was carried out as described in section 2.1.4 except that in addition to being supplemented with kanamycin, LB agar plates used to obtain colonies contained IPTG (1mM) since DH5α instead of TOP10 cells was used, and in such cases IPTG is required for the expression of the ccdB gene (discussed in chapter 3). Colonies were inoculated into LB media, grown overnight and the QiAprep® Spin Miniprep kit used to extract plasmids containing the blunt mtr gene.

Restriction endonuclease digestion of pCR®-Blunt-mtr and the excision and purification of the mtr gene was essentially carried out as in section 2.1.10. Ligation of the mtr gene
into pEC-XK99E to generate pEC-XK99E-mtr was based on the procedure used in section 2.1.11. Transformation of *E. coli* DH5α cells with pEC-XK99E-mtr was performed as previously described in section 2.1.12. Plasmid extraction from colonies grown overnight in LB media containing kanamycin was achieved through the use of QiAprep® Spin Miniprep kit and a restriction digest utilizing kpn1 and Xba1 performed to confirm the presence of the mtr gene, results of which were analysed on an agarose gel (0.8%) stained with ethidium bromide.

Electroporation of pEC-XK99E-mtr into electro-competent *C. glutamicum* cells was performed as described in section 2.1.14.2 and upon plating on LGB agar plates supplemented with kanamycin, colony PCR was carried out as a rapid means of verifying the presence of the mtr gene within *C. glutamicum*.

### 2.1.17 Attempted induction of the mtr gene with IPTG

Induction was carried out as described in section 2.1.15 and *C. glutamicum*, electroporated with pEC-XK99E, was used as a control.

### 2.1.18 Preparation of mycothiol disulfide (MSSM)

#### 2.1.18.1 *M. smegmatis* culture

*M. smegmatis* was cultured as described in Bornemann *et al.*, 1997 and harvested in the late log phase of growth.
2.1.18.2 Mycothiol isolation from *M. smegmatis*

Mycothiol was isolated from *M. smegmatis* essentially as described by Steenkamp and Vogt, 2004. Packed cells (114g wet weight) were resuspended in 200 mL of a 1:1 mixture (v/v) of acetonitrile and perchloric acid (0.75 M) containing EDTA (2 mM). The cells were disrupted by sonication at 4 °C at the following settings: output control of 8, duty cycle of 30, and timer set at 10 minutes.

The mixture was clarified by centrifugation at 10000 g for 10 minutes. The supernatant was adjusted to pH 4.8 by addition of solid K$_2$CO$_3$ and the resulting precipitate (potassium perchlorate) removed by centrifugation. The mycobacterial extract contained 440 μmoles thiols and was then added dropwise with stirring to the reagent, 2-S-(2’-thiopyridyl)-6-hydroxynaphthylisulfide (880 μmoles), prepared beforehand as described by Steenkamp and Vogt, 2004. The reaction was incubated overnight at room temperature and the formation of S-2-(mycothioly)-6-hydroxynaphthylisulfide was monitored using a Luna 5μ C-18 analytical HPLC column (250 x 4.6mm) under the optimised conditions in table 2.1. The UV detector was set at 230nm and the flow rate at 0.8 mL/min.

Thiols were generally estimated by adding 20 μl of a sample to 970 μl of 25 mM K$_2$HPO$_4$ buffer, pH 8.0, followed by the addition of 10 μl of 20 mM 4,4-dithiodipyridyl (dTdP). The absorbance was measured at 325 nm.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>0</th>
<th>5</th>
<th>35</th>
<th>40</th>
<th>45</th>
</tr>
</thead>
<tbody>
<tr>
<td>TFA (0.1%) A</td>
<td>95%</td>
<td>95%</td>
<td>10%</td>
<td>10%</td>
<td>95%</td>
</tr>
<tr>
<td>CH$_3$CN B</td>
<td>5%</td>
<td>5%</td>
<td>90%</td>
<td>90%</td>
<td>5%</td>
</tr>
</tbody>
</table>

The mixture was then diluted five fold with water and passed through SepPak C18 cartridges. The cartridges were washed with water and the S-2(mycothioly)-6-
hydroxynaphthyldisulfide was eluted using 50% acetonitrile/water (v/v). The acetonitrile was removed by passing air, and the solution subsequently chromatographed isocratically using 25% buffer A (CH$_3$CN) and 75% buffer B (0.1% TFA) on a Vydac C18 preparative column.

Fractions containing S-2(mycothiolyl)-6-hydroxynaphthyldisulfide were lyophilised overnight and the resultant dry material dissolved in 3 mL of 25 mM sodium phosphate buffer (pH 8.7). The S-2(mycothiolyl)-6-hydroxynaphthyldisulfide mixture was then reduced with 25mg/mL NaBH$_4$ (350 µl) and separated on an HPLC Vydac 218TP1022 reversed phase preparative column. The separation was achieved using a method which starts with 100% buffer A (0.1% TFA) until 10 minutes, followed by the gradient from 0% to 50% B (100% (CH$_3$CN) until 55 min and then back to 100% A at 60 minutes. The MSH fractions were pooled, the pH converted to 8 with triethylamine and thereafter treated with diamide (12 µmol) for 15 minutes. The MSSM generated was separated from the excess diamide on a preparative Vydac C18 column. The acetonitrile was removed by passing air and the samples stored at -20 °C.

2.1.19 Mycothiol isolation and confirmation of the presence of mycothiol in *C. glutamicum*

*C. glutamicum* packed cells (74g) were resuspended in 222 mL of a 1:1 mixture (v/v) of acetonitrile and perchloric acid (0.375 M) containing EDTA (2 mM). The cells were disrupted by sonication at the following settings: output control of 8, duty cycle of 30, and timer set at 10 minutes. The resulting homogenous suspension was clarified by centrifugation at 10000 g for 10 minutes. The supernatant was adjusted to pH 4.8 by addition of solid K$_2$CO$_3$ and the resulting potassium perchlorate precipitate removed by centrifugation at 10000 g for 10 minutes. The pH of the *C. glutamicum* thiol extract (116 µmoles) was then adjusted to 7.6 and treated with 2-bromo-2’-acetonaphthone (BAN) (131 µmoles) and left to react overnight at room temperature.
The formation of mycothiol-2’-acetonaphthone (MSH-BAN) was monitored by HPLC using a Phenomenex Phenyl-Hexyl column (250 x 4.6 mm) under the optimised conditions in table 2.2. The UV detector was set at 248nm and the flow rate at 0.8 mL/min. Collected fractions were verified to be BAN derivatives by monitoring absorbance at 248, 300 and 350 nm using the OceanOptics USB2000 spectrophotometer.

Table 2.2: HPLC conditions for monitoring the formation of MSH-BAN

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>0</th>
<th>5</th>
<th>30</th>
<th>33</th>
<th>35</th>
<th>37</th>
</tr>
</thead>
<tbody>
<tr>
<td>TFA (0.1%) A</td>
<td>90%</td>
<td>75%</td>
<td>65%</td>
<td>10%</td>
<td>10%</td>
<td>90%</td>
</tr>
<tr>
<td>CH(_3)CN B</td>
<td>10%</td>
<td>25%</td>
<td>35%</td>
<td>90%</td>
<td>90%</td>
<td>10%</td>
</tr>
</tbody>
</table>

The MSH-BAN extract was diluted with water to decrease the acetonitrile content to 15% and loaded onto a column packed with a Vydac C18 resin. The column was initially washed with 2 bed volumes of water until the colour of the eluent changed from yellow to clear. The column was then eluted with 50% acetonitrile and the fractions confirmed to be BAN derivatives by checking absorbance at 248, 300 and 350 nm. Fractions showing a BAN spectrum and absorbing at 248 nm were pooled and purified by HPLC on a preparative Phenomenex Phenyl Hexyl column (21.8 x 250 mm) using conditions in table 2.3 at a flow rate of 4 mL/min.

Table 2.3: HPLC conditions for the separation of MSH-BAN from excess BAN

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>0</th>
<th>5</th>
<th>30</th>
<th>33</th>
<th>35</th>
<th>37</th>
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<tbody>
<tr>
<td>TFA (0.1%) A</td>
<td>90%</td>
<td>72%</td>
<td>65%</td>
<td>65%</td>
<td>10%</td>
<td>10%</td>
<td>90%</td>
</tr>
<tr>
<td>CH(_3)CN B</td>
<td>10%</td>
<td>28%</td>
<td>35%</td>
<td>35%</td>
<td>90%</td>
<td>90%</td>
<td>10%</td>
</tr>
</tbody>
</table>

Fractions eluting at 15 minutes that exhibited a BAN spectrum were pooled and the acetonitrile removed by bubbling air for 16 hours. The solution was subsequently lyophilised, dissolved in acetonitrile-d\(_6\) and submitted for \(^1\)H, \(^{13}\)C, 2-D NMR (COSY and HSQC) and electrospray ionisation mass spectrometry.
2.1.20 Assays for activity

Samples taken during induction of the mtr gene with IPTG (sections 2.1.15 and 2.1.17) were assayed for enzyme activity using a continuous spectrophotometric assay at 30 °C. The absorbance change at 340 nm was recorded by means of an OceanOptics USB2000 spectrophotometer, and the standard assay consisted of Tris/HCl (44.5 mM) pH 7.6, 152 µM NADPH, and MSSM (7.5 µM). Protein concentration was determined by the BioRad protein micro-assay procedure according to the manufacturer’s instruction, using bovine serum albumin as standard.

2.1.21 Induction of the mtr gene with acetamide (pSD26-mtr)

Three different media Issabelle (Issabelle, et al., 2003), Middlebrook, tryptone and yeast extract enriched Middlebrook supplemented with hygromycin (100 µg/mL) were prepared and inoculated with pSD26-mtr glycerol stocks (prepared in our lab by Dr M.J. Williams). Expression of the mtr was induced at 37 °C (OD₆₀₀ = 1) by adding acetamide (0.2%), the liquid cultures were allowed to incubate for 16 hours at this temperature.

2.1.22 Sonication and Assays for activity

Sonication of M. smegmatis cells and electrophoresis (10% SDS PAGE gel) of the soluble fraction were performed as previously described for C. glutamicum in section 2.1.15 except that the 50 mM Hepes buffer pH 7.6 containing EDTA (0.1 mM) used for re-suspension of the cells contained a protease inhibitor cocktail (Roche). Assays for activity were carried out essentially as described in section 2.1.20 except that 37.8 µM 2-S-(mycothiolyl)-6-hydroxynaphthyl disulfide (MSSnaph) was used as a substrate.
2.1.23  Acetamide concentration dependant induction

Induction of the mtr gene at 37 °C with acetamide (0.1%, 0.2%, and 0.3%, in Issabelle, LB and Middlebrook media) was carried out as in section 2.1.21. Aliquots (5 mL) taken at 15, 30 and 45 hrs were centrifuged at 10000xg for 10 minutes, pellets were resuspended in 50 mM Hepes buffer pH 7.6 containing EDTA (0.1 mM) and a protease inhibitor cocktail (complete protease inhibitor cocktail, Roche). The cell suspensions were sonicated on ice and aliquots analyzed by SDS-PAGE (10 %) as in section 2.1.15.

2.1.24  Acetamide concentration dependant induction at 20 °C

Induction and sampling was carried out as in section 2.1.15, except that acetamide and M.smegamatis (as an expression host) were used instead of IPTG and C. glutamicum. SDS-PAGE analysis was performed as described in section 2.1.15.

2.2  MSSnaph as an alternative substrate for Mtr and its kinetic parameters

The ability of Mtr to utilise MSSnaph as a substrate was determined as in section 2.1.20, except that MSSnaph (10-55 µM) was used instead of MSSM.

2.2.1  Reduction of 35-38 (Discussed in chapters 5 and 6) by Mtr

The capability of Mtr to reduce 35-38 was determined by monitoring the oxidation of NADPH at 340 nm. The reaction mixture contained, in a final volume of 1 mL: 50 mM Tris/HCl (pH 7.6), NADPH (0.11 µmoles), and 35-38 (40 µM) at 30 °C. The reaction was started by adding Mtr (48 nM). Stock solutions of 35-38 were prepared in DMSO.

2.2.3  Evaluation of 35-38 as substrates or inhibitors of Mtr

Compounds 35-38 (40 µM) were incubated at 30 °C with Mtr (6 nM) and NADPH (0.11 µmoles) in 50 µmoles Tris/HCl (pH 7.6) in a total volume of 1 mL and the reaction
initiated by adding MSSnap (24.9 µM). The reaction was followed by monitoring the oxidation of NADPH at 340 nm.

2.2.4 Anti-mycobacterial activities of 35-38

In vitro anti-mycobacterial activities of 35-38 were assayed by the BACTEC system at the University of Stellenbosch, South Africa, following a procedure by Heifts and Good, 1994.

2.2.5 Evaluation of O-linked glycosides and bi-cyclic thioglycosides as inhibitors for Mtr

Evaluation of O-linked glycosides and bi-cyclic thioglycosides as Mtr inhibitors was carried out as described in 2.2.3, except that SPh 2 to SPh 7 and Th 1 to Th 7 (structures shown in chapter 6) were used instead of 35-38. Both sets of compounds were evaluated at 500 uM.

2.2.6 Preparation of INH-NAD and INH-NADP adducts

The synthesis of INH adducts was carried out as described by Ducasse-Cabanot et al., 2004). Briefly, 2mM INH, 2mM NAD⁺ and 4mM Mn⁺⁺⁺-PyrPh in 100 mM sodium phosphate buffer, pH7, were incubated for 20 min at room temperature to generate INH-NAD. The INH-NADP adduct was prepared in exactly the same way except that NAD⁺ was replaced with NADP⁺.

2.2.7 Purification of INH-NAD adduct mixture

The pool of INH-NAD(P) adducts was separated from the contaminants by chromatography on Sep-Pak reversed phase C18 cartridges. The cartridge was equilibrated with an aqueous solution of ammonium acetate (75 mM) after an extensive wash with CH₃CN. After the reaction mixture was loaded onto the Sep-Pak C18 cartridges, the elution of the contaminants by ammonium acetate (75 mM) was monitored.
by measurement of the OD at 260 nm. The elution of the adduct mixture by water was monitored by measuring the OD at 326 nm.

### 2.2.8 Preparation of Mn\textsuperscript{III}-PyrPh

Mn\textsuperscript{III}-PyrPh was prepared as essentially described by Nguyen \textit{et al.}, 2001. Briefly, An aqueous solution of sodium pyrophosphate (200 mM) containing Mn\textsuperscript{III}(OAc)\textsubscript{3} (5mM) was acidified to pH 4.5 with phosphoric acid and stirred for 24 hrs at room temperature to produce red complex [Mn\textsuperscript{II}(H\textsubscript{2}P\textsubscript{2}O\textsubscript{7})\textsubscript{3}]Na\textsubscript{3}.

### 2.2.9 Mtr inhibition by INH-NAD(P) adduct mixture

Inhibition of Mtr was carried out by incubating various concentrations of the INH-NAD(P) adduct mixture (2 µM-10 µM) with MSSnaph (24.9 µM) and NADH (0.105 µmoles) in 50 mM Tris/HCl (pH 7.6) at 30 °C. The total reaction volume was 1 mL and the reaction was initiated by adding Mtr (6 nM). MSSnaph was usually dissolved in 50% acetonitrile since it is not sufficiently soluble in water. The concentration of Mtr used in all the inhibition studies was measured in terms of its FAD content (at an absorbance of 450 nm using a molar extinction coefficient of 11300 M\textsuperscript{-1}.cm\textsuperscript{-1}).

### 2.2.10 Generation of MSSM from MSSnaph

MSSnaph (4 mg) was dissolved in 40 % acetonitrile (4 mL), TEA was then added to raise the pH to 8. Acetonitrile was then removed by passing a stream of nitrogen and dithiothreitol (DTT) (0.003 M) added. The solution was then acidified to pH 3 with TFA and purified on HPLC using a Phenomenex Phenyl-Hexyl preparative column (21.8 X 250mm). The pH of the recovered fractions was then raised to 8 and the fractions left overnight to oxidise. For quantification purposes, each fraction (20 µl) was treated with sodium borohydride (0.35 M) and incubated at 30 °C for 30 minutes after which the pH was lowered to 5 with 1 M acetic acid. Then dTdT (0.4 mM) was added to the 20 µl of the borohydride reduced fractions and the absorbance measured at 325 nm. To estimate the amount of MSSM formed, the concentration of MSH obtained was divided by 2.
Chapter 3

Results and Discussion

Cloning of the mtr gene and attempted expression

3.1 Cloning of the mtr gene in pEC-XK99E

The aim of this section was to clone the Mtb mtr gene into pEC-XK99E, an E.coli- C. glutamicum shuttle expression vector that is based on the medium copy number plasmid pGA1 and on a P_{trc} promoter (Kirchner and Tauch, 2003). C. glutamicum is a Gram-positive soil bacterium that is extensively used in the fermentative production of amino acids such as L-valine, L-isoleucine, L-threonine, and L-glutamic acid. As such, the biotechnological importance displayed by this organism has led to significant improvements in its bioprocess as well as its molecular biology, which also make it an attractive vehicle with which to attempt protein expression (Hermann, 2003).

The rationale behind the utilisation of C. glutamicum as an expression system was based on the fact that Mtb genes need relevant transcriptional and translational machinery required to produce target proteins, and difficulties in over-expressing Mtb genes in well characterized host cells such as E.coli has led to the use of bacteria that are phylogenetically related to mycobacteria (Lakey et al., 2000). In addition, this expression system was utilised to see whether enhanced expression of Mtr could be obtained in C. glutamicum, since expression of Mtr using M. smegmatis and a heat shock promoter resulted in a recovery of only 3mg Mtr per liter of culture medium (Patel and Blanchard, 1999).

Since pEC-XK99E is not His\textsubscript{6}tagged, the first step towards the cloning of the mtr gene was to modify the vector by inserting a His\textsubscript{6}tag to precede the N-terminus of the protein.
3.1.2 pEC-XK99E-his generation

The hybridized oligomers (with SacI and EcoR1 restriction sites) coding for the His<sub>6</sub>-tag were cloned into pEC-XK99E (figure 3) digested with SacI and EcoR1. The ligation mixture was used to transform TOP10 competent cells and the positive clones were identified by restriction endonuclease digestion.

Figure 3.0: pEC-XK99E developed for genetic engineering in *C. glutamicum* is based on the medium copy number pGA1, *lacI*<sup>q</sup> gene and on a P<sub>trc</sub> promoter. The vector map shows a multiple cloning site for cloning of PCR products and it also carries the kanamycin resistance gene *aph(3')-Ila* as a selectable marker. This figure was taken from Kirchnier and Tauch, 2003.

It was therefore, decided that since the restriction sites were close together it would be better to carry out sequential digestion. In view of the fact that SacI cannot be heat inactivated, as opposed to EcoR1, SacI was used first and the digestion carried out for 16
hours. The digestion mixture was then incubated at 75 °C for 30 min to inactivate EcoR1 and left to cool for 30 min. An aliquot (2µl) was put aside for agarose gel analysis, EcoR1 was then added to the remainder of the mixture and incubated at 37 °C for a further 16 hrs, after which an aliquot was taken for analysis by agarose gel electrophoresis.

Because the EcoR1 and Sac1 restriction sites were in close proximity it was not possible to ascertain whether both cleavage events or only a single one had occurred on the basis of a shift in mobility as determined by agarose gel electrophoresis. The gel bands were therefore excised, the DNA extracted using the Wizard® SV gel and PCR clean up system and ligation of the His6-tag insert was continued.

The ligation reaction typically used an excess of the insert in a 1:3 vector:insert ratio, so as to increase the success of the ligation. The ligation reaction was used to transform TOP10 competent cells and the transformed cells were grown for 1 hour in SOC medium and were then plated on LB plates supplemented with kanamycin.

3.1.3 Plasmid DNA preparations

Individual colonies were selected and grown overnight at 37 °C in LB liquid medium supplemented with kanamycin, and the plasmids isolated using a QiAprep® Spin Miniprep kit. The presence of the recombinant pEC-XK99E-his was confirmed by digesting the vector with EcoRV and BamH1; as a control pEC-XK99E was digested with the same enzymes.

The rationale behind this was that digestion of pEC-XK99E without the insert with these two restriction enzymes would result in excision of a 627 bp fragment while the His6-tagged plasmid would give rise to a fragment that is 36bp larger, i.e. 663 bp when analysed on an agarose gel. It was therefore, decided to run increasing volumes on a 2% agarose gel. The bands observed suggested that the cloning was successful and a large scale plasmid isolation was carried out using a NUCLEOBOND AX® kit and submitted for sequencing. Although the original pEC-XK99E was not sequenced, figure 3.1 shows
part of the sequencing data of the modified region and contrasted with the original portion of the vector map of the cloning region as published in Pubmed (shown below figure 3.1). The complete original pEC-Xk99E sequence is shown in appendix 1B, which shows the promoter region to be between the TATA box and the ATG, where the A of ATG is actually residue 7016 at the end of the vector. It is this ATG that was planned to be used for the initiation codon rather than the one at the start of the mtr gene sequence.

Figure 3.1: Sequencing data showing the cloning of the His6tag sequence (bases 20-38) and the thrombin region (bases 47-64) regions into pEC-XK99E. The plasmid is henceforth called pEC-KX99E-his.

cctgtataat gatgagcggat aacaatttca cacaggaac cc acc atg gaattgagc tccgtataat gtgagcggat aacaatttca cacaggaac cc acc atg gaattgagc gac tgagcggat aacaatttca cacaggaac cc acc atg gaattgc

tcggtacccg gggatctct agagtcgacc tgcaggcatg caagcttcgg

5'-ATG G AAT TC A GCC ACC ACC ACC ACC ACA GCA GCG GCC TGG CGC

EcoR1 Histag Thrombin
Sac1 Kpn1 Xba1

GCG GCA GCG AGC TC GGT ACC CGG GGA TCC TCT AGA GTC GAC CTG

CAG GCA TG C AAG CTT-3’

Figure 3.1.1: Modified cloning region of pEC-XK99E. The region beyond the Sac1 restriction site was not tampered with and maintained as in figure 3 (multiple cloning site). The His6tag is shown in red, whereas the thrombin cleavage site is depicted in green.
3.1.4 Amplification of the mtr gene

The PCR reaction (section 2.1.6) utilised *pfu* which generates a blunt ended PCR product, and upon running an agarose gel, it was discovered that the amplification process was not as specific as it was intended as the gel showed two more bands. The band corresponding to the *mtr* gene was therefore excised and the DNA extracted and purified using the MinElute™ Gel Extraction and QiAprep® Spin Miniprep kit. The amplified *mtr* gene is shown in figure 3.2.

![Amplified mtr gene analysed on a 0.8% agarose gel. Lane 1, 1Kb ladder; lane 2 PCR reaction. DNA was visualised by ethidium bromide.](image)

3.1.5 Ligation into PCR blunt

The ligation of the blunt *mtr* gene fragment into pCR-Blunt was performed according to the manufacturer’s instructions and as described in section 2.1.8. A useful feature of pCR®-Blunt is the presence of the lethal gene *ccdB* that promotes the ease and direct selection of positive recombinants. Expression of the lacZα-ccdB gene fusion protein is...
toxic to the cells, as a result, upon transformation, cells that contain a pCR\textsuperscript{\textregistered}-Blunt with a functional \textit{ccdB} gene are not viable whereas, successful ligation of a blunt pcr product disrupts the \textit{ccdB} gene and consequent expression of the lacZ\textalpha-\textit{ccdB} gene fusion protein (Invitrogen manual catalogue numbers K2700, K2700-40).

### 3.1.6 Transformation of TOP10 competent cells with PCRBlunt-\textit{mtr}

The transformed cells were grown at 37 \textdegree C for 1 hour in SOC medium and plated on LB plates supplemented with kanamycin. Twenty colonies were inoculated into 50 mL of LB medium containing kanamycin and cultured for 16 hrs subsequent to plasmid isolation, the pCR\textsuperscript{\textregistered}-Blunt containing the \textit{mtr} gene pcr product was cleaved with kpn1 and Xba1 for 16 hrs. All the colonies processed contained the plasmid. The digestion mixture was run on an agarose gel (0.8%). The band corresponding to the \textit{mtr} gene (figure 3.3) was excised and the DNA extracted and purified.

![Figure 3.3 Restriction digests of PCRBlunt-\textit{mtr} and pEC-XK99E-his. Lane 1, 1kb ladder, lane 2: pEC-XK99E-his cut with Kpn1 and Xba1, lane 3: uncut pEC-XK99E-his, lane 4: Incompletely cut pEC-XK99E, lane 5: PCRBlunt-\textit{mtr} cut with Kpn1 and Xba1. Analysis was carried out on a 0.8% agarose visualised by ethidium bromide staining.](image-url)
3.1.7 Ligation of the mtr gene into pEC-XK99E-his

The mtr gene with kpn1 and Xba1 restriction ends was ligated, using a 3:1 insert:vector ratio, into pEC-XK99E-his that had been digested with the same enzymes at 4°C for 16 hrs.

3.1.8 Transformation of TOP10 competent cells

TOP10 competent cells were transformed with the ligation mixture (section 3.3.6) and increasing volumes of the transformation reaction plated on LB plates supplemented with kanamycin. Two colonies from each plate were inoculated into LB media containing kanamycin and plasmids isolated. Restriction digestion was carried out using kpn1 and Xba1 for 3 hrs followed by analysis on an agarose gel. DNA isolation was carried out from a total of 8 colonies (figure 3.4.1). Lanes 5, 9, 10, 11, & 12 were sent for sequencing and only 9 gave the desired result. Sequencing results confirmed the presence of the mtr gene through a blast search from the NCBI database and are shown in figures 3.4.2 and 3.4.2.1.

![Restriction digests of plasmid DNA extracts. Analysis was carried out on a 0.8% agarose visualised by ethidium bromide staining. Lane 7 was excluded.](image)
Features in this part of subject sequence:

**NADPH-dependent mycothiol reductase mtr gene**

Score = 1127 bits (610), Expect = 0.0
Identities = 610/610 (100%), Gaps = 0/610 (0%)
Strand=Plus/Minus

Figure 3.4.2: Blast search of the mtr gene sequence performed in the NCBI database indicated that the experimental nucleotide sequence had 100% similarity with *M. tuberculosis* H37Rv mycothiol disulfide reductase. The result with the forward primer starts with the start codon in the 10th position. Sequencing experiments were carried out at the University of Cape Town, Molecular and Cell Biology department.
Features in this part of subject sequence:

NADPH-dependent mycothiol reductase mtr

Score = 1382 bits (748), Expect = 0.0
Identities = 748/748 (100%), Gaps = 0/748 (0%)
Strand = Plus/Plus

Figure 3.4.2.1: Blast search of the mtr gene sequence performed in the NCBI database indicated that the nucleotide sequence had 100% similarity with M. tuberculosis H37Rv mycothiol disulfide reductase. The result with the reverse primer is for the non-coding strand and starts with the stop codon at 5’. Sequencing experiments were carried out at the University of Cape Town, Molecular and Cell Biology department.
3.1.9 *C. glutamicum* electroporation with pEC-XK99E-his-*mtr*

The colony that gave rise to PEC-XK99E-*mtr*-his (lane 9, figure 3.4) was grown in LB medium, a large scale plasmid isolation carried out and electroporated into competent *C. glutamicum* cells. After electroporation and heat shock, the transformation mixture was plated on LBG agar plates containing kanamycin and incubated at 30 °C for 2 days. As a rapid verification, colony PCR was carried out to confirm positive recombinants (figure 3.5). Although the control of WT *C. glutamicum* was not run, the evidence that the vector contained the *mtr* gene rested mainly on lane 4.

![Figure 3.5](image-url)

Figure 3.5: Agarose gel (0.8%) visualised by Ethidium bromide showing the result of a colony PCR carried out as a rapid confirmation of the pEC-XK99E-*mtr*-his in *C. glutamicum*. Lane 1, 1kb ladder, lane 2, TOP 10 cells transformed with pEC-XK99E, lane 3, TOP 10 cells transformed with pEC-XK99E-his, lane 4, TOP 10 cells transformed with pEC-XK99E-his-*mtr*, and lane 5, *C. glutamicum* transformed with pEC-XK99E-*mtr*-his.
3.1.10 Induction of pEC-XK99E-mtr-his

*C. glutamicum* electroporated with pEC-XK99E-mtr-his was grown at 30 °C in LB medium (500 mL) containing kanamycin until OD$_{600}$ was 1. IPTG (1mM) was then added and the flasks transferred to 25 °C. SDS PAGE (figure 3.6) analysis of the samples did not show a band corresponding to Mtr (50 kDa band), this is despite the fact that the sequencing results indicated the *mtr* gene to be in-frame and that the plasmid is meant to ensure tight control of protein expression, since the insert is placed under the regulation of a strong *E.coli* promoter (P$_{trc}$).

Activity assays using MSSM as a substrate and *C. glutamicum* crude extracts also suggested that there was no expression of Mtr in active form. This is surprising since *C. glutamicum* is known to produce its own MSH and Mtr.

![Figure 3.6: Attempted time course induction of the mtr gene in C. glutamicum. Lane 1, molecular weight markers, lanes 2 and 3 (pellet and its supernatant) uninduced. Lanes 4-11 pellets and their respective supernatants taken at times 1hr-4hrs.](image)
Attempting induction at 18 °C using IPTG concentrations ranging from 0.8 mM to 2 mM resulted in more or less the same result as in figure 3.8. It has been noted however, that certain C. glutamicum strains possess very strong restriction systems that efficiently degrade foreign DNA or DNA with a foreign methylation pattern (Liebl et al., 1989).

It was also suspected that the His6-tag could be interfering with the folding of the protein, and it was consequently undertaken to clone and express the mtr gene without the His6-tag. A fresh PCR product of the mtr gene was prepared and cloned into the pCR®-Blunt as described previously.

3.1.11 Generation of pEC-XK99E-mtr

The pCR®-Blunt containing the mtr gene PCR product was cleaved with Kpn1 and Xba1 for 16 hrs (figure 3.7), the band corresponding to the mtr gene excised and the DNA extracted and purified.

![Figure 3.7: Restriction digests of plasmid DNA extracts. Lane 1, 1 kb ladder, lanes 2, 3, 5, and 6 DNA extracts digested with Kpn1 and Xba1. DNA was analysed on a 0.8% agarose gel, and was visualised using ethidium bromide staining.](image-url)
The purified mtr gene was then ligated into pEC-XK99E previously cleaved with Kpn1 and Xba1. Transformed TOP10 cells were grown at 37°C for 1 hour in SOC medium and plated on LB plates supplemented with kanamycin (30 µg/mL). Plasmid isolation and restriction digests were carried out using the above mentioned enzymes to confirm the presence of the insert (figure 3.8). Figures 3.9, 3.10, 3.11 show the nucleotide and protein alignments of the clone and published mtr, sequencing experiments were carried out at the University of Cape Town, Molecular and Cell Biology department (as previously described).

Figure 3.8: Restriction digests of plasmid DNA extracts to confirm if the mtr gene was successfully cloned into pEC-XK99E. Lane 1, 1 kb ladder, lanes 2-9 DNA extracts digested with Kpn1 and Xba1. DNA was analysed on a 0.8% agarose gel, and was visualised by ethidium bromide staining.
Figure 3.9: Nucleotide sequence alignment of the published mtr and mtr cloned into pEC-XK99E, dashes (residues 640-662) indicate a gap (region not covered by the primers during sequencing experiments). The first 30 nucleotides on the clone that do not correspond to \textit{Mtb}-mycothiol disulfide reductase belong to the vector.
Figure 3.10 Continuation.
Figure 3.11: Protein alignment of the cloned Mtr and published Mtb Mtr. BioEdit was used to convert the nucleotide sequence into a protein sequence. Dashes (residues 214-221) indicate a gap (region not covered by the primers during sequencing experiments)
3.1.12 *C. glutamicum* electroporation with pEC-XK99E-*mtr*

Electroporation of competent *C. glutamicum* was carried out as previously described in 2.1.14.2

3.1.13 Induction of pEC-XK99E-*mtr*

Induction at 18 and 25 °C was attempted as before using IPTG (1mM). The SDS PAGE result was the same as in section 3.1.10, figure 3.6. The promoter region was suspected to have been disrupted or undergone some form of mutation during electroporation, as a result, large scale plasmid isolation was carried out in preparation for sequencing experiments, which showed the promoter region to be still intact.

Moreover, at the time, no background activity could be detected when testing the *C. glutamicum* crude extract, as *C. glutamicum* electroporated with pEC-XK99E was used as a control. Since MSH is the major thiol found in actinomycetes, it was speculated that perhaps *C. glutamicum* uses a different thiol as opposed to the MSH it had been thought to utilise. We therefore, proceeded to isolate the *C. glutamicum* thiol for structural comparisons with the published MSH structure (discussed in chapter 4).

3.1.14 Expression of the *mtr* gene in *M. smegmatis* (pSD26-*mtr*)

Since the expression of *mtr* gene in *C. glutamicum* was not successful, expression in *M. smegmatis* was attempted using pSD26-*mtr* recombinants prepared in our lab. pSD26 (Daugelat *et al.*, 2003), is an *M. smegmatis* vector with an inducible acetamidase promoter, and allows expression of foreign genes by translational fusion with a C-terminal his-tag. This expression vector is basically a modification of a pSD24 vector that is derived from a promoterless episomal *E.coli- mycobacteria* shuttle expression vector, pMV206-Hygro (Patel and Blanchard 2001 cross reference), the difference between pSD26 and the pSD24 vector being the C-terminus his-tag in the former, as shown in figure 3.12.
Figure 3.12: Map of pSD24 *E.coli-mycobacteria* shuttle plasmid containing the inducible acetamidase promoter. pSD24 is derived from a promoterless pMV206-Hygro plasmid, the pSD26 is a derivative of pSD24. Partial sequences are shown below. The plasmid map and the partial sequences were taken from Daugelat *et al.*, 2003.

**BamHI PvuII**

\[
\begin{align*}
\text{pSD24} & \quad \text{atg ccc gag gta gtt ttt atc cat} \text{ gga tcc age tgc aga} \quad \text{atg ccc gag gta gtt ttt atc cat} \\
& \quad \text{MP E V V F I H G S S C R} \quad \text{acetamidase}
\end{align*}
\]

**BamHI EcoRV**

\[
\begin{align*}
\text{pSD26} & \quad \text{atg ccc gag gta gtt ttt atc cat} \text{ gga tcc gat atc cac cac cac cac cac tga} \quad \text{atg ccc gag gta gtt ttt atc cat} \\
& \quad \text{MP E V V F I H G S S I H H H H H H *} \quad \text{acetamidase}
\end{align*}
\]

Since the acetamidase promoter is tightly regulated (Daugelat *et al.*, 2003) and *M. smegmatis* is phylogenetically closer to *Mtb*, we expected the expression of *mtr* gene to proceed without difficulties. However, as can be seen in figure 3.13, no significant change in the band intensities could be seen, with the exception of Issabelle medium.
(lane 7), where there was a slight increase in the band intensity, but not conclusive enough for us to proceed.

A starter culture containing pSD26-mtr was used to inoculate LB, Middlebrook and Issabelle and superbroth media (100 mL) supplemented with hygromycin and grown for 16 hrs. Small samples were then transferred to separate flasks and cultured separately as control (uninduced). When OD$_{600}$ had reached 1, acetamide (0.2%) was added (induction flasks) and the cultures incubated at this temperature for 16 hrs. Figure 3.9 shows an SDS PAGE of the attempted induction. Media components are shown in appendix 1A.

### 3.1.15 Induction of PSD26-mtr

According to experiments performed by Parish et al., 1997, which demonstrated that certain growth media contribute to the regulation of the acetamidase gene, we were encouraged to also explore the use of different media in an attempt to resolve the expression of the mtr gene. As such, four different media (LB, superbroth, Middlebrook and Issabelle medium) were used to determine whether the expression of the acetamidase gene can be enhanced and consequently express the mtr gene.

Unfortunately, there was no difference in the band intensities with all the growth media used (except for an elevation of a band between 43 kDa and 66 kDa in the presence of acetamide, the inducer) (figure 3.13), activity assays were thus carried out, and instead of utilising the natural substrate, MSSM as before, MSSnaph was used as an alternative substrate (discussed in chapter 4) as it was found to be versatile and showed better activity than MSSM. Activity assays showed expression in *M. smegmatis* grown in Issabelle to be promising as compared to *M. smegmatis* grown in LB media (compared in section 3.1.16). In addition longer SDS PAGE gels were performed to effect better resolution (figures 3.14, 3.15, 3.16).
Figure 3.13: Attempted time course induction of the \textit{mtr} gene in \textit{C. glutamicum}. Lane 1, molecular weight markers, lanes 2 and 3, \textit{M. smegmatis} transformed with pSD26-\textit{mtr} grown in LB (uninduced and induced respectively), Lanes 4 and 5, \textit{M. smegmatis} transformed with pSD26-\textit{mtr} grown in Middlebrook (uninduced and induced respectively), lanes 6 and 7, growth in Issabelle medium (uninduced and induced), lanes 8 and 9, growth in superbroth (uninduced and induced). No Mtr bands corresponding to the expected 50 kDa could be conclusively identified. Although the band below 67 KDa (lane 3) was suspected of being Mtr, activity assays carried out in section 3.1.16 excluded LB as growth media supportive of expressing the \textit{mtr} gene.

3.1.16 Activity assays

Cells were grown (in LB, Middlebrook, Superbroth and Issabelle media respectively) and induced with acetamide as described in 3.1.14; this was followed by sonication and separation of cell debris from the cytosolic contents. The latter was dialysed overnight in 1 mM Tris-HCl (pH7.6) and activity assays conducted using dialysed extracts.

Activity results (from crude extracts) suggested that only cells grown in Issabelle and to a lesser extent LB media, showed potential for the induction of the \textit{mtr} gene. Cells grown in Issabelle medium gave activities of 115 \(\mu\)mol/min/mg as opposed to 62.7 \(\mu\)mol/min/mg noted for those grown in LB media, while uninduced cells grown in Issabelle medium gave activities of 48.2 \(\mu\)mol/min/mg. As a result, it was decided to optimise the induction of the \textit{mtr} gene in Issabelle medium, in conjunction with the use of other modified media.
3.1.17 Expression of the mtr gene in *M. smegmatis*, using different media at 20 °C

Since expression in Issabelle medium seemed promising, it was decided to attempt expression in Issabelle and in rich media, Middlebrook (containing tryptone and yeast extract) media only using three different concentrations of acetamide (0.1%, 0.2%, and 0.3%), and sampling after 15 hours. Induction was carried out at 20 °C, using different media at 20 °C (figure 3.14). The figure below showed mycothiol disulfide reductase to be expressed as inclusion bodies, as a result, variables such as temperature and the inducer were further investigated in an attempt to optimize the expression of the target protein in a soluble fraction.

![Figure 3.14: Attempted concentration dependent induction of the mtr gene in M. smegmatis. Lanes 2-4, pellet fractions of cells transformed with pSD26-mtr grown and induced (0.1%, 0.2% and 0.3% acetamide respectively) in Issabelle medium. Lanes 6-8, pellet fractions of cells transformed with pSD26-mtr grown and induced (0.1%, 0.2% and 0.3% acetamide respectively) in enriched Middlebrook media. Lane 10, molecular weight markers. Lanes 12-14, cytosolic fractions of cells transformed with pSD26-mtr grown and induced in Issabelle medium. Lanes 16-18, cytosolic fractions of cells transformed with pSD26-mtr grown and induced in enriched Middlebrook medium. Lane 19: marker lane: 10. The composition of the media used is described in appendix 1A](image-url)
Since the temperature used for induction was significantly lower and judging from the SDS PAGE in figure 3.10 it seemed as if in addition to the acetamide, there was also autoinduction by some components in the complex growth media used, as the protein still expressed as inclusion bodies in just 15 hrs. The phenomenon of autoinduction has been described by Studier, 2005, and Grossman et al., 1998, who observed that cultures growing in certain complex media tend to induce significant amounts of target protein upon approach to elevated cell growth in the absence of the inducer.

However, in this case, there seemed to be this sort of induction even before the saturation point is reached. This prompted us to grow cultures at 20 °C in the absence of acetamide to verify if autoinduction described by Studier was the issue here.

**3.1.18 Expression of Mtr in *M. smegmatis*, using different media at 20 °C in the absence of the inducer (acetamide).**

A starter culture containing pSD26-*mtr* was used to inoculate Middlebrook and Issabelle media supplemented with hygromycin. Sampling was done at 15 hrs. Figure 3.15 shows an SDS PAGE of the attempted induction. Once more, the Mtr was found to be concentrated in the insoluble fraction from cells grown in Issabelle medium in particular, and to a lesser extent in Middlebrook and the LB media.
The SDS PAGE above shows samples grown at 20 °C without acetamide for 15 hrs. The fact that the enzyme goes into inclusion bodies leads to speculations that there may be certain components in the media responsible for the observed induction as noted by Studier and others. It was therefore, decided to omit the glycerol in the Middlebrook and carry out the expression in the absence of the inducer at 20 °C.
3.1.19 Expression of the mtr gene in *M. smegmatis*, using Issabelle and Middlebrook media at 17 °C in the absence of the inducer.

SDS PAGE analysis of Issabelle and Middlebrook cultures incubated at 17 °C in the absence of the inducer also showed the expressed Mtr to be in inclusion bodies (figure 3.16).

Figure 3.16: Lanes 1 and 3, soluble fractions of cells transformed with pSD26-*mtr* grown in Issabelle and Middlebrook media without acetamide. Lane 5, molecular weight markers. Lanes 7 and 9 pellet fractions of cells transformed with pSD26-*mtr* grown in Issabelle (lane 7), Middlebrook (lane 9).

Due to unsuccessful attempts at expressing Mtr using pEC-XK99E and pSD26, pMV261 was obtained from the Dept of Medical Microbiology at UCT and was used for the cloning and expression of Mtr. Briefly, the gene encoding *M. tuberculosis* mtr was expressed in *M. smegmatis* as described by Patel and Blanchard, with minor modifications: The forward primer was 5’-CCC CTA CAA GTT TAA ACG TAC GAC-3’. To lower the level of constitutive expression of Rv2855 the transformed cells were cultured at 30 °C and expression was induced by raising the temperature to 42 °C. Under these conditions a ~160-fold increase in the level of mtr specific activity was achieved as compared to *M. smegmatis* lacking the pMV261 plasmid with Rv2855 insert.
3.1.20 Conclusion

The unsuccessful expression of Mtr in *C. glutamicum* could not be explained, and more studies in the feasibility of using pEC-XK99E to clone and express *Mtb* proteins are required. One of a few published works dealing with expression of *Mtb* related proteins in *C. glutamicum*, which laid precedent to the adaptation of expressing the *mtr* gene (in this study) was carried out by Engels *et al.*, 2004. These authors successfully managed to express a *clgR* gene (a transcriptional activator involved in the regulation and expression of the *clpP1P2* operon) in *C. glutamicum*, using a different expression vector (pXMJ19). It is therefore possible to express foreign genes in *C. glutamicum* and perhaps the only difference lies in the expression vectors.

Experiments involving the use of pSD26 as an expression vector suggested that the induction and subsequent expression of Mtr in *M. smegmatis* was influenced by certain components in the Issabelle medium since there was still expression of the enzyme (as seen by the intense band at 50 kDa, figure 3.10) even with the inducer absent. Middlebrook medium on the other hand seemed to lack components capable of inducing the enzyme, as can be seen when comparing figures 3.14 and 3.15, the common factor between the two being the absence of acetamide in the latter. Whilst Issabelle medium contains yeast extract, peptone, NaH$_2$PO$_4$.H$_2$O, and Ferric ammonium citrate; Middlebrook is much more complex in its composition and it’s not clear whether the expression of the *mtr* gene observed with Issabelle (despite being insoluble) has to do with its simplicity and why it was unsuccessful with LB media which contains both peptone and the yeast extract.

The only successful Mtr expression system so far is the one carried out by Patel and Blanchard, 1999, that utilises the pMV261 vector incorporating a heat shock promoter. Stover *et al.*, 1991, successfully utilised pMV261 as an expression vector for some *Mtb* proteins and managed to show that the regulatory sequences of the BCG *hsp60* and *hsp70* genes were capable of driving the expression of foreign antigen genes in BCG. Eckstein *et al.*, 1998, have also demonstrated the generation of recombinant gene products in *M.*
*smegmatis* when foreign genes are cloned under the control of an *hsp60* promoter using the abovementioned pMV261 (Stover *et al*., 1991). It is therefore plausible that certain *Mtb* genes express better if placed under the control of a heat shock promoter.
Chapter 4
Results and Discussion

Isolation and characterization of mycothiol from *C. glutamicum* and evaluation of MSSnaph as an alternative substrate for Mtr

4.1 Substrate preparation for activity assays

MSSM was prepared as described by Steenkamp and Vogt, 2004, briefly, S-2-(mycothiolyl)-6-hydroxynaphthylisulfide (MSSnaph) (figure 4.1) was generated from a reaction between *M. smegmatis* crude extract and 2-S-(2'-thiopyridyl)-6-hydroxynaphthylisulfide. MSSnaph was purified by HPLC followed by reduction with NaBH₄, the resultant MSH was then oxidised with diamide to generate MSSM. While MSSM was essentially needed for Mtr assays, we had anticipated to evaluate MSSnaph as a possible and alternative substrate for Mtr (discussed in section 4.3). Most importantly, the major thrust of this chapter centred on the characterisation of a *C. glutamicum* thiol and its comparison with authentic MSH.

![Figure 4.1: Structure of MSSnaph](image)

The inability to detect MSSM reduction using crude *C. glutamicum* extracts (induced, uninduced and the wild type) prompted us to structurally verify the major low molecular mass thiol in *C. Glutamicum*. 
4.2 Isolation of *C. glutamicum* thiol

4.2.1 Activity assays using *C. glutamicum* thiol

Initially a crude *C. glutamicum* thiol extract was injected on HPLC and the fraction that eluted at 21 minutes was oxidised with diamide. Since this is the normal retention time for authentic MSH, based on conditions outlined in section 2.1.19, it was reasoned that the thiol from *C. glutamicum* should have more or less the same retention time.

Activity assays were once again carried out using the oxidized sample (*C. glutamicum* isolated thiol) instead of MSSM as substrate and still no activity was detected. A small scale thiol extraction was then carried out, derivatised with 2-bromo-acetonaphthone (BAN) and incubated at 37 °C with mycothiol s-conjugate amidase (Mca). MSH-BAN (MSH previously isolated from *M. smegmatis* treated with BAN) was used as a control to assess the extent of Mca cleaved MSH-BAN from *C. glutamicum*. The reaction samples were then spotted and the TLC performed with butanol: acetic acid: water/2:1:1, as a mobile phase. The plates were air-dried and the spots detected under UV light, to assess the extent of cleavage by Mca.

The Mca activity observed gave preliminary indications that the thiol could be similar to mycothiol, however, this was not conclusive enough due to incomplete conversion of the starting material, MSH-BAN (Rf = 0.015). In fact, the spot corresponding to the product, α-DGI (Rf = 0.65) on the TLC was very faint, even after long incubation periods. As a result, a large scale thiol extraction was carried out in preparation for structural studies.

4.2.2 *C. glutamicum* thiol isolation and structural elucidation

*C. glutamicum* thiol extract was derivatised with BAN at pH 8 and purified on a Vydac C18 low pressure column using acetonitrile (50%). The extracts were spectrophotometrically monitored at 248nm, the absorbance maximum of BAN (molar extinction coefficient, 34000 M⁻¹.cm⁻¹) (figure 4.2). These fractions were pooled and purified by HPLC on a preparative phenyl-hexyl column, lyophilised and
submitted for 600MHz NMR analysis at the Central Analytical Facility at the University of Stellenbosch. A small scale comparative elution was carried out using *M. smegmatis* extract derivatised with BAN (figure 4.3, panels A and B). The thiol extracts from *C. glutamicum* showed the same retention time (21 min) as the *M. smegmatis* extract, both of which corresponded to MSH-BAN. At this stage, it was becoming even more evident that *C. glutamicum* contained MSH. Nevertheless, it was decided to continue and carry out a full NMR analysis of the compound isolated as the BAN derivative so as to conclusively substantiate the *C. glutamicum* thiol as MSH.

![Absorption spectrum of BAN](image)

*Figure 4.2: The absorption spectrum of BAN in 50% acetonitrile with an absorbance peak at 248 nm*
Figure 4.3: Analytical HPLC traces showing *C. glutamicum* thiol extract (Panel A) and that of *M. smegmatis* (Panel B). Both extracts had the same retention time as MSH-BAN. HPLC was carried out in accordance with the gradient conditions in table 4 below, using a phenyl-hexyl column at a flow rate of 0.8 ml/min and at a wavelength of 248 nm.

Table 4: HPLC conditions for monitoring the formation of *C. glutamicum*-BAN thiol (Panel A) and *M. smegmatis*-BAN thiol (Panel B)

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>0</th>
<th>5</th>
<th>30</th>
<th>33</th>
<th>35</th>
<th>37</th>
</tr>
</thead>
<tbody>
<tr>
<td>TFA (0.1%) A</td>
<td>90%</td>
<td>75%</td>
<td>65%</td>
<td>10%</td>
<td>10%</td>
<td>90%</td>
</tr>
<tr>
<td>CH$_3$CN B</td>
<td>10%</td>
<td>25%</td>
<td>35%</td>
<td>90%</td>
<td>90%</td>
<td>10%</td>
</tr>
</tbody>
</table>
Analysis of the $^1$H-NMR spectrum (figure 4.4) showed consistency with published data for MSH (Spies and Steenkamp, 1994), with the exception of the downfield region where the BAN moiety substituted the bimane used to derivatise the original isolate. Expansions on figure 4.4 confirmed the diagnostic features (figure 4.5) of the glucosamine, inositol and the acetyl-cysteine moieties of MSH-BAN from C. glutamicum to be comparable to published MSH data.

Figure 4.4: 1H-NMR spectrum of BAN derivatised mycothiol isolated from C. glutamicum.

Figure 4.5: Structure of MSH derivatised with BAN isolated from C. glutamicum.
The stereochemistry at C-1, C-2’ and C-2 was confirmed by a doublet, a triplet and a doublet of doublets in the 600MHz $^1$H-NMR spectrum shown above and expanded in figure 4.6 below at $\delta$ 4.979, $\delta$ 4.04, and $\delta$ 3.816 respectively, with coupling constants of 3.66 Hz, 2.81 Hz for H-1 and H-2’ respectively and integrating for one proton each whilst H-2 had a J value of 10.5 Hz, 3.66 Hz also integrating for one proton. The expanded spectrum below also showed the $\alpha$-proton with a chemical shift of 4.495 ppm and appearing as a doublet of doublets with a coupling constant of 8.91 Hz, 5.13 Hz, also integrating for one proton. H-3 and H-4 appeared at 3.694 ppm (dd, $J$= 10.74 Hz, 8.79 Hz) and 3.31 ppm (t, $J$= 9.32) respectively. H-3 could be easily identified from expanding the region between 3.8 to 3.6 ppm (figure 4.7).

Figure 4.6: $^1$H NMR expanded region showing the anomeric ($\delta$ 4.979), H-2’ ($\delta$ 4.04), H-2 ($\delta$ 3.816), H-3 ($\delta$ 3.694) protons and the $\alpha$ proton ($\delta$ 4.495), all integrating for one proton each. The peak at 4.2 ppm is a solvent peak. (The appearance of a minor doublet at $\delta$ 5 ppm indicates the presence of a minor glycoside which has not been identified.)
Figure 4.7: $^1$H NMR expanded region showing H-2 ($\delta$ 3.816), H-3 ($\delta$ 3.694), H-5 and H-6$_{a\&b}$ protons in detail.

H-5, H-6a and H-6b were also identified from figure 4.7 above where H-5 and H-6a appeared as multiplets between 3.76 and 3.715 ppm, whilst H-6b also appeared as a multiplet between 3.67 and 3.64 ppm. This is further substantiated by the integration of four protons in figure 4.6 (3.76-3.64 ppm), which includes H-3.

The remaining signals corresponding to the inositol portion were detected at 3.417 ppm for H-1’ (dd, $J = 10.01 \text{ Hz}, 2.93 \text{ Hz}$), 3.35 ppm for H-3’ (dd, $J = 9.89 \text{ Hz}, 2.81 \text{ Hz}$), 3.476 ppm for H-4’ (dd, $J = 9.5 \text{ Hz}, 9.11 \text{ Hz}$), 3.135 ppm for H-5’ (t, $3.52 \text{ Hz}$) whilst H-6’ appeared as a multiplet (figures 4.6 and 4.7) between 3.63-3.58 ppm and integrating for one proton. Figure 4.8 shows the $^1$H NMR spectrum for the remainder of the inositol protons mentioned above as well as H-$\beta_\text{A&B}$ protons of acetyl cysteine moiety, where H-$\beta_\text{A}$ appeared at 2.99 ppm (dd, $J = 14.04 \text{ Hz}, 5.13 \text{ Hz}$) and H-$\beta_\text{B}$ at 2.795 ppm (dd, $J = 14.04 \text{ Hz}, 8.79 \text{ Hz}$).
Figure 4.8: $^1$H NMR spectrum showing chemical shifts and integrations for H-1’ (3.417 ppm), H-3’ (3.35 ppm), H-4’ (3.476 ppm), H-5’ (3.135 ppm), H-βA (2.99 ppm), and H-βB (2.795 ppm).

The N-acetyl-methyl group of the thiol appeared as a singlet at 1.901 ppm integrating for three protons.

Figure 4.9: $^1$H NMR spectrum showing chemical shift for the –CH$_3$ group (1.901 ppm).
The assignment of the BAN portion of the molecule revealed a total of seven protons as expected (figure 4.10), where H-6 and H-7 (BAN portion) appeared as triplets integrating for one proton each. Since H-5 and H-8 are equivalent, they appeared at around 8.74 ppm integrating for two protons, whilst H-2 and 4 appeared as doublets at around 8.02 and 7.92 ppm respectively.

Figure 4.10: Downfield region of the $^1$H-NMR spectrum showing the BAN protons. However, the CH2 signal could not be detected due to the protons being in enol-keto tautomerism and, therefore, exchangeable with the solvent deuterium. The interconversion of the two forms involves the movement of a proton and the shifting of bonding electrons where the carbonyl group is in rapid equilibrium with an enol tautomer.
Table 4.1 below summarizes published mycothiol proton assignments in comparison with mycothiol isolated from *C. glutamicum*.

**Table 4.1: Spectral data of published MSH and MSH isolated from *C. glutamicum***

<table>
<thead>
<tr>
<th>Published MSH data (Spies &amp; Steenkamp, 1994)</th>
<th>MSH data from the <em>C. glutamicum</em> isolate</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Compound</strong></td>
<td><strong>Proton</strong></td>
</tr>
<tr>
<td>Glucosamine</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>6&lt;sub&gt;A&lt;/sub&gt;</td>
</tr>
<tr>
<td></td>
<td>6&lt;sub&gt;B&lt;/sub&gt;</td>
</tr>
<tr>
<td>Inositol</td>
<td>1’</td>
</tr>
<tr>
<td></td>
<td>2’</td>
</tr>
<tr>
<td></td>
<td>3’</td>
</tr>
<tr>
<td></td>
<td>4’</td>
</tr>
<tr>
<td></td>
<td>5’</td>
</tr>
<tr>
<td></td>
<td>6’</td>
</tr>
</tbody>
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Continuation of table 4.1

<table>
<thead>
<tr>
<th></th>
<th>$\alpha$</th>
<th></th>
<th></th>
<th>$\alpha$</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>$N$-Acetylcysteine</td>
<td>4.548 (dd, 8.4, 5.2)</td>
<td>$N$-Acetylcysteine</td>
<td>4.495 (dd, 8.91, 5.13)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\beta_A$</td>
<td>3.123 (dd, 13.9, 5.2)</td>
<td>$\beta_A$</td>
<td>2.99 (14.04, 5.13)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\beta_B$</td>
<td>2.939 (dd, 13.9, 8.4)</td>
<td>$\beta_B$</td>
<td>2.795 (14.04, 8.79)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$-\text{CH}_3$</td>
<td>2.017 (s)</td>
<td>$-\text{CH}_3$</td>
<td>1.901 (s)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
In addition to the $^1$H NMR data shown above, mass spectral data also confirmed the isolate as MSH-BAN as shown in figure 4.11 below. Mass spectroscopy was carried out at the Central Analytical Facility, University of Stellenbosch, South Africa, on a Waters API Q-TOF Ultima.

Figure 4.11: Mass spectrometric analysis of MSH-BAN. The analysis worked best in the ESI negative mode. Panel A shows the ESI negative spectrum, the 653 signal is the M-1 ion. The m/z 451 (panel B) is a fragment ion as was proven with the MSMS spectrum of m/z 653.
It is interesting to note that soon after we had confirmed mycothiol to be the major low molecular mass thiol in *C. glutamicum*, Feng *et al.*, 2006, published similar data where they had isolated mycothiol from the same organism and showed that maleylpyruvate isomerase from this bacterium uses it as a cofactor. In addition, they demonstrated that mycothiol functions as an essential growth factor for *C. glutamicum* when gentisate and 3-hydroxybenzoate were supplied as carbon sources. However, this is the first time that mycothiol from *C. glutamicum* has been characterised using NMR and mass spectroscopy and compared with the original published data. In addition, this study was able to show BAN as an alternative derivatising agent as compared to monobromo-bimane. Over and above being photosensitive, bimanes have a tendency to be carcinogenic.

**4.3 MSSnaph as an alternative substrate for Mtr**

During the isolation of MSH from *M. smegmatis* using the procedure described in materials and methods and briefly examined in the opening section of this chapter (section 4.1 and figure 4.1), MSSnaph is formed as a precursor to MSH, and whilst MSH can be easily oxidised to MSSM it was of interest to investigate MSSnaph as a possible substrate for Mtr. In addition, MSSnaph can be easily converted to MSSM should the need arise (as described in section 2.2.10).

The double reciprocal plot (figure 4.12) generated in the exploration of MSSnaph as a substrate for Mtr resulted in kinetic parameters which suggested that MSSnaph was a better substrate for the reductase as compared to the natural substrate, where the $k_{m, app}$ was found to be 23 µM, whilst $k_{cat}$ and $k_{cat}/k_{m}$ were calculated to be 15.56 s$^{-1}$ and 8.17 x 10$^5$ respectively. This was an improvement to the kinetic parameters observed for MSSM with a higher $k_{m, app}$ (73 µM) and a lower $k_{cat}/k_{m}$ (1.64 x 10$^5$). Studies in our lab have also shown MSSnaph to be versatile as it can also be used to assay Mca activity, eliminating the need to reduce it to generate MSH and then derivatising with monobromobimane.
Since Mtr inhibition studies are severely restricted by the scarcity of MSH and MSSM, which are difficult to prepare in sufficient quantities via chemical (Jardine et al., 2002 and Nicholas et al., 2002) or whole cell synthesis (Steenkamp and Vogt, 2004). Hamilton et al., 2009, recently synthesized the mixed disulfide, (figure 4.13a) to establish its suitability as an Mtr substrate.

Interestingly, the authors found the Km value of 43 to be approximately 1.5-fold lower than that of the symmetrical disulfide, 44, (figure 4.13b discussed in section 1.6), and only twice as large as that of the native substrate MSSM. In terms of overall substrate efficiency, the kcat/Km value for 43 was found to be nearly 5-fold greater than that of 44 and 70% of that observed for MSSM. Whilst, our study finds the Km of MSSnaph to be 11.6 and 19 fold lower than 43 and 44 respectively.
4.4 Conclusion

Isolation of MSH from *C. glutamicum* was largely based on the fact that no reductase activity could be observed when investigating Mtr expression (chapter 3), and as explained before, this consequently led to speculations that perhaps this bacterium makes use of a different substrate.

The successful isolation of mycothiol and its characterisation reinforced the notion that indeed all actinomycetes depend on this low molecular mass thiol for regulating their redox chemistry, and offers *C. glutamicum* as an alternative for the study of mycothiol dependant enzymes pathways and their isolation. Moreover, given the resurgence of *diphtheriae* infections in recent years, particularly within the eastern European regions (Dover et al., 2004) targeting MSH dependant pathways may lead to the development of new drugs against *C. diphtheriae*.

The demonstration that MSSnaph is a better substrate for Mtr than MSSM which has a significantly higher $k_m$ than MSSnaph leads to speculations that the active site of Mtr is perhaps capable of accommodating aromatic $\alpha$-DGI derivatives, which may be a valuable feature in the design of drugs targeting Mtr. However, this remains to be established until the crystal structure of the reductase is solved.

The fact that both Mtr and Mca can concurrently utilise MSSnaph as an alternative substrate means that it is possible to design drugs based on the $\alpha$-DGI scaffold that can target both enzymes. Yet again, this would be greatly facilitated once both crystal structures are solved and compare
Chapter 5
Synthesis of compounds 35-38

The goal of this work was to prepare compounds of the general type I shown below, where the core 2-deoxy-2-amino-glucose unit has a suitable aglycone \( R^2 \) and is tethered to plumbagin via its amino group.

![Chemical Structure](image)

**Synthesis of suitable glycoside of 2-deoxy-2-amino-\( \beta\)-D-glucose**

The first goal of the synthetic work was to prepare a suitably protected glycoside of glucosamine, having a free amine for later coupling to the substituted naphthoquinone. The initial focus was on the synthesis of the protected pseudodisaccharide 5 (scheme 1), via glycosylation of D-2,3,4,5,6-penta-\( O\)-acetyl-myoinositol 2 with the protected (2',4'-dinitrophenylamino)-2-deoxy-\( \alpha\)-D-glucosyl bromide 4. The resulting glycoside could then be selectively deprotected to form the corresponding 2-deoxy-2-amino glucoside, for subsequent amide coupling.

The selectively deprotected D-2,3,4,5,6-penta-\( O\)-acetyl-\( \beta\)-myo-inositol 2 and glucosyl bromide 4 were prepared from precursors 1 and 3 respectively (Scheme 1) according to a literature procedure (Jardine et al., 2002). The attempted Koenings-Knorr coupling of 2 and 4 to afford the desired \( \alpha\)-linked glycoside 5 was, however, not successful, and use of a different glycosyl donor, the trichloroacetimidate (9, Scheme 2), also did not give rise
to the desired glycoside, but instead gave a major product tentatively identified as the unexpected TMS glucoside 10 (scheme 3).

Scheme 1: Reagents and conditions: (i) H₂/Pd, EtOAc, RT, 3 hrs; (ii) HBr (30% in acetic acid), CH₂Cl₂, RT, 8 hrs; (iii) AgOTf, CH₂Cl₂, 2,4,6-lutidine, RT, 30 hrs.

Scheme 2: Reagents and conditions: (i) NaOMe, MeOH, TfN₃, RT, 18 hrs, then Ac₂O, pyridine, RT, 10 hrs. (ii) THF, piperidine, RT, 16 hrs. (iii) CH₂Cl₂, Cl₃CCN, K₂CO₃, RT.

However, the glycosylation of inositol 2 using the imidate 9 also proved unsuccessful despite attempts to optimize reaction conditions including increasing the number of equivalents of 2, raising the temperature, ensuring anhydrous solvents, addition of powdered molecular sieves (4Å) and use of TMSOTf as a promoter. In all cases, TLC revealed the conversion of the starting materials into a new product, but after separation by column chromatography the major (least polar) product was tentatively identified as the unexpected TMS glucoside 10 (scheme 3).
Scheme 3: Reagents and conditions: CH$_2$Cl$_2$, TMSOTf, 4Å molecular sieves, 0°$^\circ$C to RT, 30 hrs.

These failed attempts prompted a further change in focus towards producing thioglycosides as analogues for the inositol glycosides. This was justified, as mentioned earlier, from the observation that the phenylthioglycoside analogue of the mshB substrate had been shown to be a competitive substrate, and that thioglycosides have been found to be more resistant towards glycosidases than the corresponding O-glycosides.

The 2-azido-$\alpha$-phenylthioglycoside 12 was identified as a key precursor for generating the amine required for subsequent coupling to naphthoquinone-bearing carboxylic acids and was prepared as shown in Scheme 4. Glucosamine hydrochloride (6) was treated with triflic azide followed by acetylation with acetic anhydride to give the acetylated 2-azidoglucose 7 in 50% yield. Treatment of this with thiophenol in the presence of BF$_3$·Et$_2$O gave an inseparable mixture (3:1 $\alpha/\beta$) of phenyl glycosides 11. However, crystallization of the product mixture from absolute ethanol gave the pure $\alpha$-isomer 12 in 65% yield (scheme 4). Confirmation of the $\alpha$-stereochromy was obtained from appearance in the $^1$H NMR spectrum of a doublet for H-1 at $\delta$ 5.61 ppm, with coupling constant ($J_{1,2}$) of 5.6Hz consistent with an axial-equatorial coupling, whilst the diagnostic signals for the phenyl group were also observed in the region 7.51-7.29 ppm integrating for five protons.

The 2-azido-thioglucoside 12 was then reduced with H$_2$ over Pd/C in ethanol to generate phenyl-3,4,6-tri-$O$-acetyl-2-amino-2-deoxy-1-thio-$\alpha$-$D$-glucopyranoside 13. $^1$H NMR spectroscopy confirmed the reduction, with the presence of the -NH$_2$ protons evident.
from the signal at 1.50 ppm with all the other signals conserved as for phenyl 3,4,6-tri-\(O\)-acetyl-2-azido-2-deoxy-1-thio-\(\alpha\)-D-glucopyranoside 12.

\[
\begin{align*}
\text{6} & \rightarrow \text{7} \\
\text{7} & \rightarrow \text{11} \\
\text{11} & \rightarrow \text{13} \\
\text{13} & \rightarrow \text{12}
\end{align*}
\]

\text{Scheme 4: Reagents and conditions: (i) NaOMe, MeOH, TfN, RT, 18hrs, then Ac}_2\text{O, pyridine, RT, 10hrs; (ii) PhSH, BF}_3\text{,Et}_2\text{O; (iii) crystallize from EtOH; (iv) H}_2\text{,Pd/C, EtOH}}

First attempt at developing coupling strategy to incorporate tethered naphthoquinone

With 2-amino-phenylthioglucoside 13 in hand attention was turned to methods for coupling this to the naphthoquinone unit via suitable spacers. Our initial attempts to derivatize plumbagin (see below) involved inserting a side chain terminated by an amine, in the hope that a simple strategy for coupling two amines could be developed via suitable derivatization of one amine. Initially, oxalyl chloride was tried as a coupling agent (scheme 5), in the hope that intermediate acid chloride 14 could be isolated, but although starting material was consumed in this reaction, TLC analysis of the reaction mixture did not show formation of a single product, suggesting that 14 may not be stable.

To ascertain if 14 had indeed formed, a one pot reaction was attempted where, upon the disappearance of the starting material, aniline was added to try to trap the acid chloride.
Although several products were isolated from this experiment, they did not show the spectroscopic features of the expected product, with the anomeric proton signal absent and the integration of signals for the acetoxy groups indicating less than the expected nine protons. The $^{13}$C NMR spectra of the three isolated products also did not reveal the expected two amide carbonyls.

Scheme 5: Reaction and conditions: CH$_2$Cl$_2$, pyridine, oxalyl chloride, 0 °C, 30 mins.

In an alternative approach, treatment of 13 with chloroacetyl chloride in CHCl$_3$ gave phenyl-3,4,6-tri-O-acetyl-2-chloroacetamido-2-deoxy-1-thio-α-D-glucopyranoside (15) in excellent yield (scheme 6). TLC showed complete conversion of the starting material and no side products, such that 15 could be recovered after washing with 1M HCl and water, and used without further purification.

The $^1$H NMR spectrum of 15 confirmed the presence of a chloromethylene group from the two-proton singlet at 3.98 ppm whilst the $^{13}$C NMR spectrum exhibited the corresponding chloroacetyl carbon signal at 42 ppm, with a DEPT experiment confirming that this was a methylene carbon.

Scheme 6: Reagents and conditions: 2,4,6-lutidine, chloroacetyl chloride, CHCl$_3$, 0 °C, 2hrs.
Preparation of first set of naphthoquinone derivatives

Taking into account the objective of the study to synthesize naphthoquinone derivates that would be more potent and specific to the \textit{Mtb} reductase, and the fact that the catalytic residues of the enzyme’s active site have not yet been characterised, it was decided to incorporate alkyl chains of variable length as spacers between plumbagin and thioglucoside 13 for the purposes of probing the active site. The synthesis of plumbagin derivatives was carried out following the approach of Salmon-Chemin \textit{et al}, 2001, where Boc-protected amino acids where attached to plumbagin using an oxidative decarboxylation.

The Boc-protected amino acids 17\textit{a-d} were prepared from the corresponding amino-acids by reported methods. The spectroscopic data for these was consistent with that reported by Salmon-Chemin \textit{et al}, 2001, with the diagnostic signals for the tert-butyloxycarbonyl group in $^1$H NMR spectra appearing at 1.36 ppm and integrating for nine protons as expected, and the secondary amine appearing as a triplet that integrates for one proton. The $^{13}$C NMR spectrum revealed the presence of methyl groups from the single peak at 28.94 ppm.

The butyloxycarbonyl-amino acids 17\textit{a-d} were then reacted with 5-hydroxy-2-methyl-1,4-naphthoquinone 16 (plumbagin) to give alkylated naphthoquinones 18\textit{a-d} (scheme 7) in low to moderate yields. In these reactions, it was observed that 16 was never completely consumed, while reaction times longer than 10 hrs generated side products that complicated the purification process. Reactions were therefore not carried out for longer than 8 hrs. In trying to improve the yield, the number of equivalents of ammonium persulfate added was varied whilst maintaining silver(I) in catalytic amounts, and in all attempts the average yield obtained was 58\% with a small amount of unreacted plumbagin recovered after purification.
Scheme 7: Reagents and conditions: 30% CH₃CN, (NH₄)₂S₂O₄, AgNO₃, 7.5hrs, 70 °C. 18a-d were recovered in yields of 34%, 31%, 32%, and 31% respectively.

Spectroscopic values of 18a-d were consistent with those reported in literature. The ¹H NMR signals for the aromatic protons in the quinone moiety appeared as expected between 7.6 ppm and 7.2 ppm, with the hydroxyl group signal appearing at around 12 ppm and integrating for one proton. The diagnostic signal for the Boc-protected amino acids appeared at around 1.4 ppm, integrating for nine protons. The conjugated 1,4-dicarbonyl unit was evident from two characteristic signals in the ¹³C NMR spectrum at 190 and 184 ppm respectively.

Oxidative decarboxylation of butyloxy carbonyl-amino acids was also attempted with 5-hydroxy-1,4-naphthoquinone (juglone), an analogue of plumbagin which is not substituted at C-2 or C-3 of the quinonoid ring. In this case both mono- and di-substitution is possible, and this was evident from TLC of the reaction products which showed two overlapping spots. Attempts to separate the two products were unsuccessful and NMR analysis of the crude sample was complicated by the presence of a further side product in proximity to the two products of interest. It was therefore decided to only proceed with the plumbagin derivatives.

Acid hydrolysis of the butyloxy carbonyl-amino acids gave the amines 19 – 22 in yields around 60% (scheme 8). The absence of the tert-butyloxy carbonyl group was evident from both ¹H and ¹³C NMR spectra, which also confirmed the presence of the primary amine whose protons appear at 1.6 ppm as a broad singlet.
Scheme 8: Reagents and conditions: CH$_2$Cl$_2$, TFA, 0 °C, 1hr. 19-22 were recovered in yields of 30%, 27%, 29%, and 28% respectively.

**Attempted coupling of naphthoquinonylalkylamines 19 - 22 to alkyl chloride 15**

Having successfully generated 3-substituted quinones 19, 20, 21, and 22, the next step involved coupling them to the chloroacetylated aminoglucoside 15, described earlier. Refluxing 21 or 22 with 15 in CH$_3$CN afforded 23 or 24 (schemes 9) in poor yields and low levels of purity. The reactions did not go to completion even after 2 days, whilst column purification was complicated by the co-elution of the side products associated with the target compound. Relatively pure samples were eventually obtained by preparative TLC so that the structures of the products could be confirmed by NMR spectroscopy. The presence of the proton signals of the quinone and phenyl moieties was evident from the signals between 7.6 and 7.2 ppm integrating for 8 protons. The hydroxyl signal appeared at 12.05 ppm, which was consistent with previous naphthoquinone spectra. In addition, the diagnostic signal for the anomeric proton of the glucopyranosyl moiety was detected at 5.82 ppm, with an axial-equatorial coupling constant ($J_{1,2}$) of 5.64 Hz confirming the $\alpha$-configuration, whilst the signals for the acetoxy groups appeared at around 2 ppm, integrating for 12 protons as expected. However, despite the detection of these diagnostic signals, the NMR revealed signals for contaminants which were difficult to remove.

The naphthoquinone derivatives 19 and 20 (scheme 10) were however not successfully converted to the desired products, with a substantial amount of unreacted 15 recovered.
together with unidentified minor products. In an attempt to drive the reactions to completion reagents and reaction conditions were varied without success.

Scheme 9: Reagents and conditions: (i) CH$_3$CN, NaHCO$_3$, 80 °C, 48hrs.

Scheme 10: Reagents and conditions: (i) CH$_3$CN, NaHCO$_3$, 80 °C, 48hrs
Alternative strategy: use of naphthoquinonyl carboxylic acids

The mixed success of the coupling approach described above prompted consideration of a different strategy, illustrated in scheme 11. This involved formation of naphthoquinone-terminated carboxylic acids II via oxidative decarboxylation of diacids with plumbagin, and coupling of these to amine 13 using well-established coupling reagents HOBT and EDC. In this method the carbodiimide EDC reacts with a carboxyl group to form a highly reactive O-acylisourea intermediate that readily reacts with amines. However, since the O-acylisourea is inclined to contribute to racemisation, hydroxybenzotriazole, HOBT, is normally used as a reactive nucleophile to intercept the O-acylisourea and form a further reactive intermediate (Montalbetti and Falque, 2005).

Scheme 11: Envisaged formation of naphthoquinonyl carboxylic acids II and coupling via amide bonds to amine 13.

A series of naphthoquinonyl carboxylic acids 27 – 30 was therefore prepared, from plumbagin (16) and dicarboxylic acids succinic, glutaric, adipic, and pimelic acid, using the oxidative decarboxylation procedure employed for the preparation of 18.
The spectroscopic data for 27, 28, 29, and 30 was consistent with that for 19, 20, 21, and 22 prepared earlier, where the signal for the 5-hydroxyl group appeared at 12.09 ppm in the $^1$H NMR spectrum, and the $^{13}$C NMR signal for the carboxyl group was detected at 173.64 ppm. The signals for the three protons of the phenyl appeared in the $^1$H NMR spectrum between 7.7 and 7.26 ppm. All of these diagnostic signals, together with those for the alkyl methylene groups in the $^1$H NMR and $^{13}$C NMR spectra confirmed the assigned structures.

With compounds 27-30 in hand, we then proceeded to form peptide bonds with 13 using HOBT and EDC. The amides 31, 32, 33, and 34 were obtained in reasonable yields (39%, 56.6%, 42.6%, and 50% respectively) from the reaction of 13 and each of 27-30 in a mixture of $\text{CH}_2\text{Cl}_2$/THF in the presence of EDC and HOBT. The reactions did not go to completion and there was difficulty in obtaining better yields even when reaction times were prolonged. Interestingly, improved yields were obtained when the number of molar equivalents of HOBT was reduced from 1.1 to 0.5 eq. This observation is consistent with data published by Nozaki, 1999, where it was noted that 0.02-0.1 HOBT molar equivalents were sufficient in enhancing chemical yields of tripeptides. Unfortunately, due to insufficient starting material the reactions could not be repeated, in order to attempt to optimize yields for this step.
The $^1$H NMR spectra for 31-34 showed signals for the anomeric proton as a doublet at 5.72 ppm with an axial-equatorial coupling constant of 5.2 Hz confirming the alpha configuration of the thioglycoside. The presence of the naphthoquinone portion was confirmed by the appearance of the signal for the 5-hydroxyl group at 12.0 ppm, whilst the three aromatic protons together with those from the aromatic portion of the phenylthioglycoside appeared between 7.59 and 7.26 ppm, integrating for 8 protons. The acetoxy groups were accounted for by the signals appearing at 2.05, 2.04, and 2.03 ppm, and integrating for nine protons, whilst the $^{13}$C NMR revealed the carbonyls of the acetoxy groups and the naphthoquinone between 205.4 and 180 ppm, with the carbonyl of the peptide bond appearing at 173.6 ppm.

Treatment of acetylated derivatives 31-34 in methanol with catalytic NaOMe and subsequent neutralisation with Dowex-50WX-200 resin (H$^+$ form) gave the target compounds 35-38 in 34%, 46%, 60%, and 52% yields respectively (scheme 12). The $^1$H NMR and $^{13}$C NMR spectroscopic data was consistent with that observed for 31-34, except that the signals corresponding to the acetoxy groups were absent, indicating the formation of the respective triols. The presence of unprotected triols was further
confirmed by \( ^1\)H NMR spectra of D\(_2\)O-washed samples, which made it possible to identify exchangeable protons and assign the final structures.

Scheme 12: Reagents and conditions: (i) PhSH, BF\(_3\)Et\(_2\)O, 55 °C, 12 h, followed by crystallization from EtOH. (ii) H\(_2\), Pd/C, EtOH, rt, 5 h. (iii) HO\(_2\)(CH\(_2\))\(_n\)CO\(_2\)H, 30% aq CH\(_3\)CN, AgNO\(_3\), NH\(_4\)(S\(_2\)O\(_8\))\(_2\), 70 °C, 8 h. (iv) EDC, HOBt, CH\(_2\)Cl\(_2\)/THF, rt, 18 h. (v) cat. NaOMe, MeOH, Dowex-50WX-200 resin, rt, 1 h. Plumbagin and diacids were commercial.

**Experimental**

**General procedures**

All solvents used were freshly distilled. Dichloromethane was distilled over phosphorus pentoxide, while lutidine and tetrahydrofuran were distilled over sodium wire under nitrogen. Other solvents were purified using established procedures (Perrin, Armarego, 1963). Commercially available reagents were used without purification unless otherwise
stated. All reactions were performed under an inert atmosphere of nitrogen in flame dried glassware.

Reactions were monitored by thin-layer chromatography (TLC) carried out on Merck F254 silica plates and products visualised under UV light and by wetting the plate with a solution of anisaldehyde and sulphuric acid in ethanol, followed by heating. Column chromatography was performed on glass columns loaded with Merck silica gel 60 (70-230 mesh) eluting with mixtures of light petroleum and ethyl acetate.

Nuclear magnetic resonance spectra (\(^1\)H and \(^{13}\)C) were recorded on either a Varian Mercury (300 MHz) or Varian Unity (400 MHz) with CDCl\(_3\) as the solvent and TMS (\(\delta = 0\) ppm) as the internal standard. All chemical shifts are reported in ppm. Melting points were determined using a Reichert-Jung Thermovar hot plate microscope.

**Materials**

5-hydroxy-2-methyl-1,4-naphthoquinone, 5-hydroxy-1,4-naphthoquinone, 2-hydroxy-1,4-naphthoquinone, adipic acid, 6-amino-hexanoic acid, \(\beta\)-alanine, 4-aminobutyric acid, 5-amino-hexanoic acid, 5-amino-pentanoic acid, glutaric acid, acetic anhydride, trifluoromethanesulfonic acid, acetic anhydride, Di-tert-butyl dicarbonate, acetonitrile-d\(_3\), DMSO-d\(_6\), acetone-d\(_6\), CD\(_3\)OD, D\(_2\)O, glucosamine hydrochloride, pyridine, silver nitrate, trifluoroacetic acid, thiophenol, hydroxybenzotriazole hydrate, N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride, chloroacetyl chloride, oxalyl chloride, Lawesson’s reagent, cyclohexene and pimelic acid were purchased either from SigmaAldrich or Fluka. Lutidine, sodium azide, and ZnCl\(_2\) were obtained from BDH. They were all used as received. The rest of the chemicals were analytical reagent grade.

2-azido-2-deoxy-3,4,6-tri-O-acetyl-\(\alpha\),\(\beta\)-D-glucopyranosyl acetate (Martin-Lomas *et al.*, 2000) (7)
To a round bottomed flask equipped with a nitrogen inlet containing NaN$_3$ (0.17 mol, 3.6g) water (32 mL) was added followed by CH$_2$Cl$_2$ (30 mL). The emulsion was stirred and cooled at 0 °C, dry Tf$_2$O (0.0354 mol, 6mL) added via syringe and the reaction mixture was stirred at 0 °C for 2 hrs, then at 25 °C for 15 min. The organic layer was then extracted with CH$_2$Cl$_2$ (2x10 mL) and the combined organic layers were neutralised with a saturated solution of NaHCO$_3$, washed with water, and dried over MgSO$_4$ and filtered to recover the triflic azide (TfN$_3$).

In a separate 3 necked flask equipped with a nitrogen inlet, D-glucosamine hydrochloride (0.0167 mol, 3.6g) was dissolved in anhydrous methanol (100 mL), and NaOMe (32 mL of 0.5 M solution in MeOH) was added and the reaction stirred for 30 min. DMAP (2g) was then added and the reaction mixture was diluted further with additional MeOH (40 mL). The freshly prepared solution of TfN$_3$ was added to this reaction mixture via syringe and the reaction left to stir at room temperature for 18 hrs. The solvent was then removed in vacuo to give a dark yellow oil which was then acetylated by dissolving in anhydrous pyridine (100 mL) at 0-4 °C and adding anhydrous acetic anhydride (100 mL), then stirring for 10 hrs at room temperature. On completion, the reaction mixture was co-evaporated with toluene (4x100 mL) to give a brown sticky residue. Chromatography of the crude product on silica (EtOAc/petroleum ether 40:60) gave the desired product (7) as a mixture of α and β anomers as colourless oil (3g, 50%). $^1$H NMR (CDCl$_3$): δ 6.27 (d, 1H, $J = 3.66$ Hz, H-1α), 5.52 (d, $J = 8.57$ Hz, H-1β), 5.01 (m, 3H), 5.42 (t, 1H, $J = 8.0$, 5.14 Hz), 5.1-4.8 (m 3H), 4.29-4.23 (m 2H), 4.12-4.0 (m, 4H), 3.79-3.75 (m, 1H), 3.65-3.61 (m, 2H), 2.16 (s, 6H), 2.065 (s, 3H), 2.06 (s, 3H), 2.04 (6H), 2.01 (s, 3H), 1.98 (3H).

$^{13}$C NMR (CDCl$_3$): δ 170.5, 170, 169.7, 169.6, 169.5, 168.5, 168.4, 92.6, 90, 72.8, 72.8, 70.8, 69.8, 68, 67.9, 62.6, 61.5, 61.4, 60.4, 20.9, 20.8, 20.6, 20.6, 20.5
Phenyl 3,4,6-tri-O-acetyl-2-azido-2-deoxy-1-thio-α-D-glucopyranoside (12)

\[
\begin{align*}
&\text{BF}_3\cdot\text{OEt}_2 (2.6 \text{ mL, } 18.1 \text{ mmol}) \text{ was added to a solution of } 7 (1.5 \text{ g, } 4.0 \text{ mmol}) \text{ and } \\
thiophenol (0.862 \text{ mL, } 8.0 \text{ mmol}) \text{ in } \text{CH}_2\text{Cl}_2 (30 \text{ mL}) \text{ at } 0 ^\circ \text{C. The reaction mixture was} \\
stirred at 55 ^\circ \text{C for 12 hrs. On completion, the reaction mixture was dil}\\nted with \text{CH}_2\text{Cl}_2, \text{washed with brine and the organic layer separated. The aqueous layer was}\\nextracted with \text{CH}_2\text{Cl}_2. \text{The combined organic layers were dried over MgSO}_4 \text{ and concentrated under}\\nreduced pressure. Silica gel chromatography (EtOAc/} \\
\text{petroleum ether 10: 90) of the} \\
crude mixture afforded 3 as an α/β mixture (1.03g, 61%). \text{The α-anomer was obtained as}\\nwhite crystals by crystallization from absolute ethanol: mp 93 - 97 ^\circ \text{C.} \\
^1\text{H-NMR} (400 \text{ MHz}) 7.51-7.47 (m, 2H, Ar-H), 7.34-7.29 (m, 3H, Ar-H), 5.61 (d, 1H, H-1, J=5.6Hz), \\
5.31 (dd, 1H, H-3, J=9.2Hz, J=10.5Hz), 5.01 (dd, 1H, H-4, J=9.2Hz, J=10.3Hz), 4.56 \\
(ddd, 1H, H-5, J=2.3Hz, J=5.1Hz, J=10.3Hz), 4.26 (dd, 1H, H-6a, J=5.1Hz, J=12.4Hz), \\
4.04 (dd, 1H, H-6b, J=6.1Hz, J=11.1Hz), 2.07 (s, 3H, CH_3), 2.02 (s, 3H, CH_3), 1.99 (s, \\
3H, CH_3). ^13\text{C-NMR} (\text{CDCl}_3) \delta 170.4, 169.7, 132.4, 132.2, 129.2, 128.0, 86.5 (C-1), 76.6, \\
72.0, 68.7, 68.5, 61.9, 61.5, 20.6, 20.5. \text{Anal. calcd. for } C_{18}H_{21}N_3O_7S: \text{ C 51.06, H 5.19, N} \\
9.92; \text{found: C 51.64, H 5.11, N 9.94, S 7.23.}
\end{align*}
\]

Phenyl-3,4,6-tri-O-acetyl-2-amino-2-deoxy-1-thio-α-D-glucopyranoside (14)

\[
\begin{align*}
&\text{Phenyl-3,4,6-tri-O-acetyl-2-amino-2-deoxy-1-thio-α-D-glucopyranoside (14)} \\
&\text{BF}_3\cdot\text{OEt}_2 (2.6 \text{ mL, } 18.1 \text{ mmol}) \text{ was added to a solution of } 7 (1.5 \text{ g, } 4.0 \text{ mmol}) \text{ and} \\
thiophenol (0.862 \text{ mL, } 8.0 \text{ mmol}) \text{ in } \text{CH}_2\text{Cl}_2 (30 \text{ mL}) \text{ at } 0 ^\circ \text{C. The reaction mixture was} \\
stirred at 55 ^\circ \text{C for 12 hrs. On completion, the reaction mixture was dil}\\nted with \text{CH}_2\text{Cl}_2, \text{washed with brine and the organic layer separated. The aqueous layer was}\\nextracted with \text{CH}_2\text{Cl}_2. \text{The combined organic layers were dried over MgSO}_4 \text{ and concentrated under}\\nreduced pressure. Silica gel chromatography (EtOAc/} \\
\text{petroleum ether 10: 90) of the} \\
crude mixture afforded 3 as an α/β mixture (1.03g, 61%). \text{The α-anomer was obtained as}\\nwhite crystals by crystallization from absolute ethanol: mp 93 - 97 ^\circ \text{C.} \\
^1\text{H-NMR} (400 \text{ MHz}) 7.51-7.47 (m, 2H, Ar-H), 7.34-7.29 (m, 3H, Ar-H), 5.61 (d, 1H, H-1, J=5.6Hz), \\
5.31 (dd, 1H, H-3, J=9.2Hz, J=10.5Hz), 5.01 (dd, 1H, H-4, J=9.2Hz, J=10.3Hz), 4.56 \\
(ddd, 1H, H-5, J=2.3Hz, J=5.1Hz, J=10.3Hz), 4.26 (dd, 1H, H-6a, J=5.1Hz, J=12.4Hz), \\
4.04 (dd, 1H, H-6b, J=6.1Hz, J=11.1Hz), 2.07 (s, 3H, CH_3), 2.02 (s, 3H, CH_3), 1.99 (s, \\
3H, CH_3). ^13\text{C-NMR} (\text{CDCl}_3) \delta 170.4, 169.7, 132.4, 132.2, 129.2, 128.0, 86.5 (C-1), 76.6, \\
72.0, 68.7, 68.5, 61.9, 61.5, 20.6, 20.5. \text{Anal. calcd. for } C_{18}H_{21}N_3O_7S: \text{ C 51.06, H 5.19, N} \\
9.92; \text{found: C 51.64, H 5.11, N 9.94, S 7.23.}
\end{align*}
\]

Phenyl-3,4,6-tri-O-acetyl-2-amino-2-deoxy-1-thio-α-D-glucopyranoside (14)
A suspension of 12 (0.844 g, 2.0 mmol) and palladium on carbon (0.42 g) in ethanol (48 mL) under hydrogen (1 atm) was stirred for 5 hrs at room temperature. The reaction mixture was then filtered through celite and further purified by silica column chromatography (EtOAc/petroleum ether 60:40) to yield 14 (0.739 g, 93%) as a white solid, m.p. 72-74°C. $^1$H NMR (CDCl$_3$): δ 7.425-7.23 (m, 5H, Phenyl), 5.56 (d, 1H, $J = 5.2$ Hz, H-1), 5.05 (dd, 2H, $J = 2.2$, 10.25 Hz, H-4, H-6a), 4.6 (m, 1H, H-5), 4.32 (dd, 1H, $J = 5.12$, 12.29 Hz, H-2), 4.07 (dd, 1H, $J = 2.3$, 12.25 Hz, H-3), 3.32 (dd, 1H, $J = 5.19$, 10.22 Hz, H-6b), 2.1 (s, 3H), 2.04 (s, 6H). $^{13}$C NMR (CDCl$_3$): δ 170.70, 170.57, 169.78, 133.50, 131.88, 129.13, 127.74, 91.23, 74.72, 69.00, 62.30, 55.15, 20.68

Phenyl-3,4,6-tri-O-acetyl-2-chloroacetamido-2-deoxy-1-thio-α-D-glucopyranoside (16)

To a solution of 14 (1.723 mmol, 0.684 g) and 2.6 lutidine (2.585 mmol, 0.301 mL) in chloroform (7 mL), chloroacetyl chloride (1.723 mmol, 0.137 mL) was added at -10°C. The reaction mixture was allowed to stir between -10°C and 0°C for 1 hr. On completion, the mixture was diluted with chloroform and washed twice with HCl (1 M). The organic layer was separated and the aqueous layer extracted with chloroform. The combined organic layers were then washed with ice cold NaHCO$_3$, then with water, dried over MgSO$_4$ and evaporated under reduced pressure to yield 16 (0.9 g, 75%) as off-white crystals. m.p. 112-114°C. $^1$H-NMR (400 MHz) 7.44-7.42 (m, 2H, Ar-H), 7.31-7.26 (m, 3H, Ar-H), 6.91 (d, 1H, NH, $J=8.5$ Hz), 5.65 (d, 1H, H-1, $J=5.4$Hz), 5.17 (td, 2H, H-3&4, $J=9.3$Hz, $J=20.7$Hz), 4.55-4.48 (m, 2H, H-2&5), 4.28 (dd, 1H, H-6a, $J=4.9$Hz &12.3Hz), 4.08 (dd, 1H, H-6b, $J=2.3$Hz, $J=12.4$Hz), 3.98 (d, 2H, COCH$_2$-, $J=1.3$Hz), 2.04,
2.03, 2.02 (s, 9H, 3x-COCH₃). $^{13}$C-NMR (CDCl₃) δ 171.2, 170.5, 169.3, 166.2, 132.7, 131.8, 129.2, 128.0, 87(C-1), 70.9, 69.1, 68.08, 62.2, 61.9, 53.08, 42.2, 20.61

**General procedure for the preparation for (N-tert-Butyloxycarbonyl) aminoalkyl acids**

To a solution of aminoalkyl acid (11.2 mmol, 1 g) in dioxane/H₂O (30 mL) was added 1 M NaOH (10 mL). Upon cooling to 0°C, Boc₂O (10.5 mmol, 2.3 g) was added and after stirring at room temperature for 16 hrs, the solvent was removed and the residue was taken up in EtOAc. The organic layer was washed with 5% citric acid (100 mL), dried over MgSO₄, filtered and the solvent evaporated to give the N-tert-Butyloxycarbonyl)-amino acid. as a white solid.

**(N-tert-Butyloxycarbonyl)-β-alanine** (Salmon-Chemin et al., 2001) (17a)

Solid (1.36g, 69%). White solid; $^1$H NMR (DMSO-d₆) δ 11.93 (bs, 1H, COOH, exchanged with D₂O), 6.72 (t, 1H, 5.06Hz, NH), 3.11 (t, 2H, J=7.01Hz, CH₂-NH), 2.34 (t, 2H, J=7Hz, CH₂-COOH), 1.34 (s, 9H, Boc). $^{13}$C NMR (DMSO-d₆) δ 172.2, 154.3, 76.7, 35, 33.2, 29.1.

**4-(N-tert-Butyloxycarbonyl)-aminobutanoic acid** (17b)
Solid (0.73g, 93%). $^1$HNMR (DMSO-d$_6$) $\delta$ 12.01 (bs, 1H, COOH, exchanged with D$_2$O), 6.75 (t, 1H, J=5.07Hz, NH), 2.90 (q, 2H, J=6.21Hz, CH$_2$-NH), 2.17 (t, 2H, J=7.44Hz, CH$_2$-COOH), 1.58 (qt, 2H, J=7.3Hz, CH$_2$), 1.37 (s, 9H, Boc). $^{13}$C NMR (DMSO-d$_6$) $\delta$ 174.8, 156.3, 78.1, 60.4, 31.7, 28.9, 25.6.

5-(N-tert-Butyloxycarbonyl)-aminopentanoic acid (17c)

Solid (1.32g, 71%). $^1$HNMR (DMSO-d$_6$) $\delta$ 11.91 (bs, 1H, COOH) 6.72 (t, 1H, 5.06Hz, NH), 2.90 (q, 2H, J=6.68Hz, CH$_2$-NH), 2.19 (t, 2H, J=7.21Hz, CH$_2$-COOH), 1.52-1.33 (m, 13H, (CH$_2$)$_2$, Boc). $^{13}$C NMR (DMSO-d$_6$) $\delta$ 174.2, 155.5, 77.24, 39.7, 28.9, 24.5.

6-(N-tert-Butyloxycarbonyl)-aminohexanoic acid (17d)

Solid (1.4g, 79.5%). $^1$HNMR (DMSO-d$_6$) $\delta$ 11.94 (bs, 1H, COOH), 6.69 (t, 1H, J=5.15Hz, NH), 2.87 (q, 2H, J=6.88Hz, CH$_2$-NH), 2.16 (t, J=7.37Hz, CH$_2$-COOH), 1.47 (qt, 2H, J=7.25Hz, CH$_2$), 1.35-1.14 (m, 13H, (CH$_2$)$_2$, Boc); $^{13}$C NMR (DMSO-d$_6$) $\delta$ 175, 156.3, 78, 60.4, 34.3, 28.9, 26.5, 24.9.

General procedure for the preparation of N-Boc aminoalkyl derivatives

A solution of 5-hydroxy-2-methyl-1,4-naphthoquinone (3.2 mmol, 0.6 g) and Boc amino acid (9.66 mmol, 1.8 g) in 30% CH$_3$CN (26 mL) was stirred at 65 °C for 1 hr, after which AgNO$_3$ (0.96 mmol, 0.163 g) was added. A solution of ammonium persulphate (4.152
mmol, 0.947 g) in 30% CH$_3$CN (6.6 mL) was then added drop wise over a period of 3.5 hrs. The mixture was maintained between 65 and 70 °C for a further 4 hrs. On cooling, the mixture was diluted with EtOAc, then the organic layer was separated, washed with water, dried over MgSO$_4$, and the solvent removed under reduced pressure. Silica gel chromatography (EtOAc/petroleum ether 10:90), of the crude extract afforded 18a (34%) (light orange solid), 18b (47%) (light orange solid), 18c (40%) (deep yellow solid), and 18d (31%) (yellow solid).

*N-tert-Butyloxycarbonyl-2-(8-hydroxy-3-methyl-1,4-dioxo-1,4-dihydronaphthalen-2-yl)ethylamine* (Salmon-Chemin *et al.*, 2001) (18a).

\[
\begin{align*}
\text{N-tert-Butyloxycarbonyl-2-(8-hydroxy-3-methyl-1,4-dioxo-1,4-dihydronaphthalen-2-yl)ethylamine} \\
\text{(Salmon-Chemin *et al.*, 2001) (18a).}
\end{align*}
\]

$^{1}$H-NMR (CDCl$_3$) $\delta$ (400 MHz) 12.09 (OH, 1H), 7.69 (dd, 1H, H-6, J=7.5Hz, J=8.4Hz), 7.57 (dd, 1H, H-7, J=1.1Hz, J=7.5Hz), 7.25 (dd, 1H, H-5, J=1.1Hz, J=8.4Hz), 4.73 (bs, 1H, NH-CO), 3.33 (q, 2H, J=6.6 Hz, CH$_2$-NH), 2.87 (t, 2H, J=6.82 Hz, CH$_2$), 2.23 (s, 3H, CH$_3$), 1.39 (s, 9H, Boc). $^{13}$C-NMR (CDCl$_3$) $\delta$ 190.1, 184.3, 161.3, 155.1, 146.4, 143.8, 136, 132.2, 123.8, 119, 115, 79.5, 39.5, 28.3, 27.4, 13.1.

*\text{N-tert-Butyloxycarbonyl-2-(8-hydroxy-3-methyl-1,4-dioxo-1,4-dihydronaphthalen-2-yl)propylamine} (18b).*
\[ \text{CH}_2\text{NH} \]

\[ \text{OH} \]

$^1$H-NMR (CDCl$_3$) $\delta$ (400 MHz) 12.13 (s, 1H, OH), 7.69 (dd, 1H, H-6, J=7.5Hz, J=8.4Hz), 7.57 (dd, 1H, H-7, J=1.1Hz, J=7.48Hz), 7.25 (dd, 1H, H-5, J=1.1Hz, J=8.4Hz), 4.74 (bs, 1H, NH-CO), 3.18 (q, 2H, J=6.53 Hz, CH$_2$-NH), 2.66 (m, 2H, CH$_2$), 2.18 (s, 3H, CH$_3$), 1.68 (qt, 2H, J=7.53 Hz, CH$_2$-CH$_2$-NH), 1.44 (s, 9H, Boc). $^{13}$C-NMR (CDCl$_3$) $\delta$ 190.2, 184.3, 161.3, 155.9, 146.3, 145.1, 136, 132.2, 123.8, 118.9, 114.9, 79.3, 40.3, 29.7, 23.6, 12.8.

\[ \text{N-tert-Butyloxycarbonyl-2-(8-hydroxy-3-methyl-1,4-dioxo-1,4-dihydronephthalen-2-yl)butylamine (18c).} \]

$^1$H-NMR (CDCl$_3$) $\delta$ (400 MHz) 12.12 (s, 1H, OH), 7.69 (dd, 1H, H-6, J=7.5Hz, J=8.4Hz), 7.57 (dd, 1H, H-7, J=1.1Hz, J=7.48Hz), 7.25 (dd, 1H, H-5, J=1.1Hz, J=8.4Hz), 4.56 (bs, 1H, NH-CO), 3.17 (q, 2H, J=6 Hz, CH$_2$-NH), 2.79 (t, 2H, 7.0 Hz, CH$_2$), 2.17 (s, 3H, CH$_3$), 1.64-1.42 (m, 13H, CH$_2$-CH$_2$-NH, CH$_2$-(CH$_2$)$_2$-NH, Boc). $^{13}$C-NMR (CDCl$_3$) $\delta$ 190, 183, 160, 156, 174.3, 143, 135.2, 130, 125.3, 118.4, 115, 78, 40.2, 28, 26.2, 13.4.

\[ \text{N-tert-Butyloxycarbonyl-2-(8-hydroxy-3-methyl-1,4-dioxo-1,4-dihydronephthalen-2-yl)pentylamine (18d).} \]
1H-NMR (CDCl₃) δ (400 MHz) 12.1 (s, 1H, OH), 7.69 (dd, 1H, H-6, J=7.5Hz, J=8.4Hz), 7.57 (dd, 1H, H-7, J=1.1Hz, J=7.48Hz), 7.25 (dd, 1H, H-5, J=1.1Hz, J=8.4Hz), 4.53 (bs, 1H, NH-CO), 3.12 (q, 2H, J=6.5Hz, CH₂-NH), 2.6 (t, 2H, J=7.1 Hz, CH₂), 2.16 (s, CH₃), 1.56-1.43 (m, 15H, CH₂-CH₂-NH, CH₂-(CH₂)₂-NH, CH₂-(CH₂)₃-NH, Boc). 13C-NMR (CDCl₃) δ 190, 184.5, 161.2, 156, 146.9, 144.6, 135.9, 132.2, 123.7, 118.8, 114.9, 77.4, 40.4, 29.9, 28.4, 27, 26.3, 12.7.

**General procedure for the preparation of napthoquinonyl carboxylic acids**

A similar procedure to the one outlined for 18a-d was followed except that 5-hydroxy-2-methyl-1,4-naphthoquinone (2.66 mmol, 0.5 g) and an appropriate diacid (7.98 mmol, 0.942 g) were used. Preparative thin layer chromatography (MeOH/DCM 2.5:97.5) gave 27 as a dark orange solid (32%), 28 as an orange solid (40%), 29 as an orange solid (40%), and 30 as a red solid (50%).

**3-(8-hydroxy-3-methyl-1,4-dioxo-1,4-dihydronaphthalen-2-yl)propanoic acid (27)**

1H-NMR (Acetone-d₆) δ (300 MHz) 12.09 (OH, 1H), 7.70 (dd, 1H, H-6, J=7.5Hz, J=8.4Hz), 7.57 (dd, 1H, H-7, J=1.1Hz, J=7.5Hz), 7.26 (dd, 1H, H-5, J=1.2Hz, J=8.4Hz), 2.96 (t, J=8.2, 2H, CH₂-COOH), 2.58 (t, 2H, CH₂-CH₂- COOH, J=7.9Hz), 2.22 (s, 3H, CH₃); 13C-NMR (Acetone-d₆) δ 190.4, 184, 173, 162, 145.7, 145, 136.4, 133, 123.5, 118.6, 114.9, 31.9, 22.1, 12.
3-(8-hydroxy-3-methyl-1,4-dioxo-1,4-dihyronaphthalen-2-yl)butanoic acid (28)

![Chemical structure](image)

$^1$H-NMR (Acetone-d$_6$) $\delta$ (400 MHz) ppm 12.09 (OH, 1H), 7.69 (dd, 1H, H-6, $J=7.5$Hz, $J=8.4$Hz), 7.57 (dd, 1H, H-7, $J=1.1$Hz, $J=7.5$Hz), 7.25 (dd, 1H, H-5, $J=1.1$Hz, $J=8.4$Hz), 2.74 (t, $J=7.9$, 2H, $CH_2$-COOH), 2.44 (t, $J=7.2$, 2H, $CH_2$-($CH_2$)$_2$- COOH), 2.21 (s, 3H, $CH_3$), 1.84 (m, 2H, $CH_2$-($CH_2$)$_2$- COOH). $^{13}$C-NMR 190.4, 184, 173.4, 161, 146, 145, 136, 132, 123, 118, 115, 32.9, 25.5, 23.3, 12.

3-(8-hydroxy-3-methyl-1,4-dioxo-1,4-dihyronaphthalen-2-yl)pentanoic acid (29)

![Chemical structure](image)

$^1$H-NMR (Acetone-d$_6$) $\delta$ (400 MHz) ppm 12.1 (OH, 1H), 7.69 (dd, 1H, H-6, $J=7.5$Hz, $J=8.4$Hz), 7.56 (dd, 1H, H-7, $J=1.1$Hz, $J=7.5$Hz), 7.25 (dd, 1H, H-5, $J=1.1$Hz, $J=8.4$Hz), 2.69 (t, $J=7.8$, 2H, $CH_2$-($CH_2$)$_3$- COOH), 2.36 (t, $J=7.3$, 2H, $CH_2$-COOH), 2.2 (s, 3H, $CH_3$), 1.73 (td, 2H, $CH_2$-($CH_2$)$_2$- COOH, $J=7.1$Hz, $J=14.5$Hz), 1.60 (m, 2H, $CH_2$-($CH_2$)$_2$- COOH); $^{13}$C-NMR 190.4, 184, 174, 162, 146.5, 144.7, 136, 133, 124, 119, 115, 33, 25.9, 25, 11.9.

3-(8-hydroxy-3-methyl-1,4-dioxo-1,4-dihyronaphthalen-2-yl)hexanoic acid (30)
\[ ^1H\text{-NMR (Acetone-d}_6) \delta (400 MHz) \text{ ppm 12.1 (OH, 1H), 7.69 (dd, 1H, H-6, J=7.5Hz, J=8.4Hz), 7.56 (dd, 1H, H-7, J=1.1Hz, J=7.5Hz), 7.25 (dd, 1H, H-5, J=1.1Hz, J=8.4Hz), 2.67 (t, J=7.9, 2H, CH}_2\text{-COOH), 2.31 (t, J=7.3, 2H, CH}_2\text{-}(CH}_2)_4\text{-COOH), 2.2 (s, 3H, CH}_3\text{), 1.67 (td, 2H, CH}_2\text{-CH}_2\text{-COOH, J=7.2Hz, J=14.6Hz), 1.54 (m, 4H, (CH}_2)_2\text{-}(CH}_2)_2\text{-COOH);} \]
\[ ^13C\text{-NMR 190.2, 184.4, 173.8, 161, 146.6, 145, 136.3, 132.3, 12.43, 118.4, 114.9, 33.3, 25.9, 24.3, 11.9.} \]

**General procedure for coupling reactions:**

A mixture of phenyl-2-amino-1-thioglycoside 14 (0.378 mmol, 0.15 g), and each of carboxylic acids 27, 28, 29, and 30 (0.090 g, 0.34 mmol), EDC (0.0797 g, 0.416 mmol), and HOBt (0.0562 g, 0.416 mmol) were dissolved in a mixture of CH\(_2\)Cl\(_2\) (5 mL) and THF (10 mL) under nitrogen. After stirring for 18 hrs at room temperature, the solvents were evaporated. The residue was washed with 5% NaOH (5 mL), 0.5 M HCl (2.5 mL), and then dried under vacuum. Preparative TLC (MeOH/DCM 2.5:97.5) was performed to yield 31 as an orange solid (57%), mp 163-165°C; 32 as a yellow solid (84%), mp 157-158°C; 33 as a red solid (64%), mp 127-130 °C; and 34 as a red solid (78%), mp 55-58 °C.

**Phenyl-3,4,6-tri-O-acetyl-2-deoxy-2-[3'-(8''-hydroxy-3''-methyl-1'',4''-dioxo-1'',4''-dihydronaphthalen-2''-yl)propanamido]-1-thio-α-D-glucopyranoside (31)**
1H-NMR (CDCl$_3$) δ (300 MHz) 12.0 (s, 1H, OH), 7.59-7.52 (m, 2H, Ar-H), 7.42-7.39 (m, 2H, Ar-H), 7.31-7.26 (m, 5H, Ar-H), 6.00(d, 1H, NH, J=8.5 Hz), 5.72 (d, 1H, H-1, J=5.2Hz), 5.18-5.14 (m, 2H, H-3&4), 4.61- 4.47 (m, 2H, H-2&5), 4.27 (dd, 1H, H-6a, J=5.0Hz, J=12.4Hz), 4.07(dd, 1H, H-6b, J=2.3Hz, J=12.3Hz), 2.93 (dd, J=6.6Hz, J=8.5Hz, 2H, CH$_2$-CONH), 2.41 (dd, 2H, CH$_2$-CH$_2$- CONH, J=6.6Hz, J=8.5Hz), 2.2 (3H, CH$_3$), 2.05, 2.04, 2.03 (s, 9H, 3x-COC$_3$H$_7$).

Phenyl-3,4,6-tri-O-acetyl-2-deoxy-2-[3'-(8''-hydroxy-3''-methyl-1'',4''-dioxo-1'',4''-dihydronaphthalen-2''-yl)butanamido]-1-thio-α-D-glucopyranoside (32)
Phenyl-3,4,6-tri-O-acetyl-2-deoxy-2-[3'-(8''-hydroxy-3''-methyl-1'',4''-dioxo-1'',4''-dihydronaphthalen-2''-yl)pentanamido]-1-thio-α-D-glucopyranoside (33)

**1H-NMR** (CDCl$_3$) δ (300 MHz) 12.0 (s, 1H, OH), 7.6-7.56 (m, 2H, Ar-H), 7.44-7.41 (m, 2H, Ar-H), 7.30-7.20 (m, 5H, Ar-H), 5.9 (d, 1H, NH, J=8.5 Hz), 5.76 (d, 1H, H-1, J=5.4Hz), 5.17-5.13 (m, 2H, H-3&4), 4.65- 4.48 (m, 2H, H-2&5), 4.28 (dd, 1H, H-6a, J=4.9Hz, J=12.4Hz), 4.09 (dd, 1H, H-6b, J=2.3Hz, J=12.3Hz), 2.63 (dt, 1H, J=1.5Hz, J=7.1Hz, CH$_2$-CONH), 2.25 (dt, 1H, J=2.3Hz, J=7.2Hz, -CH$_2$-(CH$_2$)$_3$- CONH), 2.15 (3H, CH$_3$), 2.05, 2.04, 2.03 (s, 9H, 3x-COCH$_3$), 1.7-1.6 (m, 2H, CH$_2$-CH$_2$- CONH), 1.5-1.4 (m, 2H, CH$_2$-(CH$_2$)$_2$- CONH). **13C-NMR** 190, 184, 172, 171.6, 170.5, 169.2, 161.2, 146, 144.8, 136.8, 135.9, 132.8, 132.1, 131.4, 129.2, 127.8, 123.8, 118.8, 114.9, 87.6(C-1), 71.3, 68.9, 68.1, 62, 52.7, 36.1, 27.9, 25.9, 25.5, 20.7, 20.66, 20.6, 12.7. Anal. calcd. for C$_{33}$H$_{35}$NO$_{11}$S: C 60.63; H 5.4; N 2.14; S 4.91; found: C 59.78; H 5.75; N 1.71; S 4.0. [M-H]$^+$ calcd for C$_{33}$H$_{35}$NO$_{11}$S: 652.1852; found 652.1981.

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Phenyl-3,4,6-tri-O-acetyl-2-deoxy-2-[3′-(8′-hydroxy-3′-methyl-1′,4′-dioxo-1′,4′-dihydronaphthalen-2′-yl)hexanamido]-1-thio-α-D-glucopyranoside (34)

\[
\begin{align*}
\text{H-NMR (CDCl}_3\text{) } & \delta \text{ (300 MHz)} \quad 12.0 \text{ (s, 1H, OH)}, \quad 7.6-7.56 \text{ (m, 2H, Ar-H)}, \quad 7.44-7.41 \text{ (m, 2H, Ar-H)}, \quad 7.30-7.20 \text{ (m, 5H, Ar-H)}, \quad 5.9 \text{ (d, 1H, NH, J=8.5 Hz)}, \quad 5.76 \text{ (d, 1H, H-1, J=5.4Hz)}, \quad 5.17-5.13 \text{ (m, 2H, H-3&4)}, \quad 4.65-4.48 \text{ (m, 2H, H-2&5)}, \quad 4.28 \text{ (dd, 1H, H-6a, J=4.9Hz, J=12.4Hz)}, \quad 4.09 \text{ (dd, 1H, H-6b, J=2.3Hz, J=12.3Hz)}, \quad 2.6-2.5 \text{ (m, 2H, CH}_2\text{CONH)}, \quad 2.2-2.18 \text{ (m, 2H, CH}_2\text{-(CH}_2\text{)}_4\text{CONH)}, \quad 2.2 \text{ (3H, CH}_3\text{)}, \quad 2.058, 2.056, 2.04 \text{ (s, 9H, 3x-COC}_3\text{H}_3\text{)}, \quad 1.6-1.5 \text{ (m, 2H, CH}_2\text{CH}_2\text{CONH)}, \quad 1.5-1.4 \text{ (m, 4H, (CH}_2\text{)}_2\text{CH}_2\text{CONH)}.
\end{align*}
\]

\[\text{^{13}C-NMR} \quad 190, 184, 172, 171.6, 170.5, 169.2, 161.2, 146.4, 144.8, 136.8, 135.9, 132.8, 132.1, 131.4, 129.2, 127.8, 123.8, 118.8, 114.9, 87.6(C-1), 71.3, 68.9, 68.1, 62, 52.7, 36.1, 27.9, 25.9, 25.5, 20.7, 20.66, 20.6, 12.7. \text{Anal. calcd for C}_{35}\text{H}_{39}\text{NO}_{11}\text{S: C 61.66; H 5.77; N 2.05; S 4.7; found: C 63.9; H 6.14; N 1.6; S 3.47. [M-H]}^+ \text{calcd for C}_{35}\text{H}_{39}\text{NO}_{11}\text{S: 680.2165; found 679.5982.}
\]

**General procedure for deacetylation:**

A portion of 0.2 M NaOMe in MeOH was added to a stirred suspension of acetylated glycoside (31-34 respectively) in MeOH, at room temperature. When TLC showed complete conversion to a single product with lower polarity (typically after 1h) Dowex-50WX-200(H\(^+\)) ion exchange resin was added to the reaction mixture until a neutral pH was reached. The dowex resin was then removed by filtration and the clear filtrate concentrated *in vacuo* to give deacetylated product: 35 as a red/brown solid (18mg, 34%), mp 238-241 °C; 36 as a yellow/brown solid (25mg, 46%), mp 196-199 °C; 37 as a
brown solid (32mg, 60%), mp 170-173°C; and 38 as a brown/orange solid (30mg, 52%), mp 157-159 °C.

Phenyl-2-deoxy-2-[3'-(8''-hydroxy-3''-methyl-1'',4''-dioxo-1'',4''-
dihydronaphthalen-2''-yl)propanamido]-1-thio-α-D-glucopyranoside (35)

\[
\text{OH} \quad \text{O} \\
\text{HO} \quad \text{SPh} \\
\text{NH} \\
\text{OH}
\]

\[\begin{align*}
1^H-NMR \ (DMSO-d_6) & \delta \ (400 \ MHz) 12.0 \ (s, \ H, \ OH), 8.09 \ (d, \ 1H, \ NH, \ J=6.5Hz), 7.69 \ (t, \\
& 1H, H-7, J=8.0Hz), \ 7.5-7.4 \ (m, \ 1H, H-5,1H), \ 7.38-7.35(2H, m, Ar-H), 7.31-7.24(4H, m, \\
& Ar-H), \ 5.60 \ (d, \ 1H, H-1, J=5.1Hz), 5.12 \ (d, \ 1H, OH, J=5.6Hz), \ 4.85 \ (d, \ 1H, OH, \\
& J=5.6Hz), \ 4.51 \ (t, \ 1H, OH, J=6.0Hz), \ 3.87-3.80(2H, m, H-2&3), \ 3.58-3.54 \ (2H, m, H-
\end{align*}
\]

\[\begin{align*}
& 5& \text{a}), \ 3.46-3.40(1H, m, H-6b), \ 3.3-3.2 \ (1H, m, H-4), \ 2.8-2.7 \ (m, \ 2H, CH_2-CNH), \\
& 2.38-2.34 \ (m, \ 2H, CH_2-CH_2-CNH), \ 2.1 \ (3H, CH_3). \ ^{13}C-NMR \ 183.8, 172, 171.9, 159.7, \\
& 145.1, 136.2, 134.2, 131.6, 130.9, 129.08, 129.03, 128.9, 127, 123.3, 118.4, 114.5, 87(C-
\end{align*}
\]

\[\begin{align*}
& 1), \ 73.8, 70.37, 70.30, 70, 60, 54.5, 54.4, 33.52, 33.48, 22.2, 12.4. \ [M+H] \ calcd \ for \\
& C_{26}H_{27}NO_8S: \ 514.1536; \ found \ 514.1544.
\end{align*}
\]
Phenyl-2-deoxy-2-[3'-(8''-hydroxy-3''-methyl-1'',4''-dioxo-1'',4''-dihydronaphthalen-2''-yl)butanamido]-1-thio-α-D-glucopyranoside (36)

\[ \text{Phenyl-2-deoxy-2-[3'-(8''-hydroxy-3''-methyl-1'',4''-dioxo-1'',4''-dihydronaphthalen-2''-yl)butanamido]-1-thio-α-D-glucopyranoside (36)} \]

\[ \begin{align*}
\text{O} & \quad \text{OH} \\
\text{HO} & \quad \text{SPh} \\
\text{NH} & \quad \text{OH} \\
\text{O} & \quad \text{OH} \\
\text{O} & \quad \text{O} \\
\end{align*} \]

\[ ^1\text{H-NMR (DMSO-d}_6\text{)} \delta (300 \text{ MHz}) \]

\[ \begin{align*}
12.0 & (s, 1\text{H, OH}), \\
7.86 & (d, 1\text{H, } \text{NH}, J=6.6\text{Hz}), \\
7.69 & (d, 1\text{H, } \text{J}=7.6\text{Hz}, J=8.3\text{Hz}), \\
7.51 & (d, 1\text{H, } \text{J}=1.1\text{Hz}, J=7.5\text{Hz}), \\
7.40 & (m, 2\text{H, Ar-H}), \\
7.29 & (m, 4\text{H, Ar-H}), \\
5.64 & (d, 1\text{H, } \text{OH}, J=5.1\text{Hz}), \\
5.01 & (d, 1\text{H, } \text{OH}, J=5.6\text{Hz}), \\
4.76 & (d, 1\text{H, } \text{OH}, J=5.6\text{Hz}), \\
3.9 & (t, 1\text{H, } \text{OH}, J=5.6\text{Hz}), \\
3.6 & (m, 2\text{H, CH}_2\text{-CONH}), \\
3.49 & (m, 1\text{H, } \text{H}-6_a), \\
3.29 & (m, 1\text{H, } \text{H}-6_b), \\
2.6 & (m, 2\text{H, CH}_2\text{-CONH}), \\
1.7 & (s, 3\text{H, CH}_3), \\
1.2 & (m, 2\text{H, CH}_2\text{-CONH}).
\end{align*} \]

\[ ^{13}\text{C-NMR} \]

\[ 189, 184, 172, 160, 146, 145, 136, 135, 132, 131, 129, 127, 123, 118, 88(\text{C-1}), 74, 71, 70, 61, 55, 34, 28, 25, 24, 12. \]

\[ [\text{M-H}]^+ \text{ calcd for C}_{27}\text{H}_{29}\text{NO}_8\text{S} 526.1536; \text{ found 526.1526.} \]

Phenyl-2-deoxy-2-[3'-(8''-hydroxy-3''-methyl-1'',4''-dioxo-1'',4''-dihydronaphthalen-2''-yl)pentanamido]-1-thio-α-D-glucopyranoside (37)

\[ \begin{align*}
\text{O} & \quad \text{OH} \\
\text{HO} & \quad \text{SPh} \\
\text{NH} & \quad \text{OH} \\
\end{align*} \]
H-NMR (DMSO-d<sub>6</sub>) δ (300 MHz) 12.0 (s, 1H, OH), 7.86 (d, 1H, NH, J=6.5Hz), 7.69 (dd, 1H, J=7.6Hz, J=8.3Hz), 7.51 (d, 1H, J=7.4Hz), 7.40-7.3(m, 2H, Ar-H), 7.26-7.16(m, 4H, Ar-H), 5.6 (d, 1H, J=5.1Hz), 5. (d, 1H, OH, J=5.5Hz), 4.76(d, 1H, OH, J=5.5Hz), 4.39 (t, 1H, OH, J=5.8Hz), 3.9-3.8(m, 2H, H-2&3), 3.6-3.5(m, 2H, H-5&6<sub>a</sub>), 3.5-3.4(m, 1H, H-6<sub>b</sub>), 3.3-3.2(m, 1H, H-4), 2.6-2.5(2H, m, CH<sub>2</sub>-CONH), 2.2-2.1(2H, m, CH<sub>2</sub>-CONH), 2.0(3H, s, CH<sub>3</sub>), 1.63-1.57(2H, m, CH<sub>2</sub>-CH<sub>2</sub>-CONH), 1.5-1.4(2H, m, CH<sub>2</sub>-CONH). 13<sup>C</sup>-NMR 189, 184, 173, 160, 146, 144, 136, 135, 132, 131, 129, 128, 127, 123, 118, 114, 87(C-1), 74, 70.4, 70.1, 61, 55, 35, 27, 26, 25,12. [M-H]<sup>+</sup> calcd for C<sub>28</sub>H<sub>31</sub>NO<sub>8</sub>S: 540.1692; found 540.1685.

Phenyl-2-deoxy-2-[3'-(8''-hydroxy-3''-methyl-1'',4' '-dioxo-1'',4''-dihydronaphthalen-2''-yl)hexanamido]-1-thio-α-d-glucopyranoside (38)

H-NMR (DMSO-d<sub>6</sub>) δ (300 MHz) 12.0 (s, 1H, OH), 7.86 (d, 1H, NH, J=6.5Hz), 7.7 (t, 1H, J=8.3Hz), 7.51 (d, 1H, J=7.5Hz), 7.4(td, 2H, J=1.2 & 8.2, Ar-H), 7.3-7.2(m, 4H, Ar-H), 5.6 (d, 1H, J=4.8Hz), 5.1 (d, 1H, OH, J=5.7Hz), 4.8(d, 1H, OH, J=5.8Hz), 4.5 (t, 1H, J=6.1Hz), 3.9-3.8(m, 2H, H-2&3), 3.6-3.5(m, 2H, H-5&6<sub>a</sub>), 3.5-3.4(m, 1H, H-6<sub>b</sub>), 3.3-3.2(m, 1H, H-4), 2.6-2.5(2H, m, CH<sub>2</sub>-CONH), 2.2-2.1(2H, m, CH<sub>2</sub>-CONH), 2.0(3H, s, CH<sub>3</sub>), 1.63-1.57(2H, m, CH<sub>2</sub>-CH<sub>2</sub>-CONH), 1.5-1.4(4H, m, (CH<sub>2</sub>)<sub>2</sub>-CONH). 13<sup>C</sup>-NMR 189.7, 184, 173.5, 160, 146.8, 144.5, 136.6, 135, 132, 131.5, 129, 127, 123.6, 118, 114, 87(C-1), 74, 70.8, 70.6, 60.7, 55, 35, 29, 27.9, 26, 25,12.6. [M-H]<sup>+</sup> calcd for C<sub>29</sub>H<sub>33</sub>NO<sub>8</sub>S: 554.1849; found 554.1843.
Chapter 6
Results and Discussion
Evaluation of subversive substrates (35-38), thioglycosides, O-glycosides and INH-NAD(P) adducts against Mtr

6.1 Effects of INH-NAD(P) adducts on crucial Mtb proteins: Overview

Isoniazid (INH) and ethionamide (ETH) are essential drugs in the treatment of tuberculosis, even though ETH is generally considered a second line drug for TB. The use of ethionamide has significantly increased over the years due to the appearance of cases of MDR and XDR (Argyrou, et al., 2006b and Wang et al., 2007).

INH and ETH are pro-drugs that require activation by KatG, a catalase peroxidase (Johnsson et al., 1995 and Zhang et al., 1992), and ethA, a flavin monooxygenase (Wang et al., 2007) respectively to form adducts with NAD\(^+\) resulting in concomitant inhibition of InhA, an NADH-dependant enoyl-acyl carrier protein reductase of the fatty acid biosynthesis type II system (Rozwarski et al., 1998; Vilcheze et al., 2006; Lei et al., 2000 and Marrakchi, et al., 2000). The formation of these adducts proceeds through the oxidative activation of INH by KatG to generate an isonicotinoyl radical that reacts non-enzymatically with NAD(P) to generate a mixture of INH-NAD(P) isomers (figure 6.1) (Nguyen et al., 2001). Over the last few years, it has also become evident that INH resistance is predominantly due to mutations in the KatG and inhA genes.

In addition to the NADH-dependant enoyl-ACP reductase, INH-NAD(P) adducts have also been shown to inhibit the \textit{M. tuberculosis} NADPH-dependant dihydrofolate reductase (DHFR) with nanomolar affinity (Argyrou et al., 2006a). A proteome wide profiling of \textit{Mtb} using both the INH-NAD and INH-NADP adducts identified 16 other proteins, most of which were predicted to be pyridine nucleotide-dependant dehydrogenases/reductases, to bind to these adducts with high affinity (Argyrou, et al., 2006b).
6.2 Percentage inhibition of Mtr by INH-NAD(P) adduct mixture

INH-NAD and INH-NADP adduct mixtures were prepared and purified using Sep-pak C-18 cartridges as described by Ducasse-Cabanot et al., 2004. Briefly, 2mM INH, 2mM NAD$^+$ and 4mM Mn$^{III}$-PyrPh in 100 mM sodium phosphate buffer, pH7, were incubated for 20 min at room temperature to generate INH-NAD. The INH-NADP adduct was prepared in exactly the same way except that NAD$^+$ was replaced with NADP$^+$. Unfortunately, the preparation of ethionamide adducts (ETH-NAD(P)) using the same procedure was not successful, as no product peak formed at 326 nm even after 1 hr of incubation, whilst INH-NAD(P) adducts formed after 20 mins as reported.

Although ETH is structurally similar to INH and although the primary target for both drugs is the mycolic acid biosynthetic pathway (Takayama et al., 1972; Winder et al., 1971), it has been demonstrated that while INH requires activation by KatG to form INH-NAD(P), activation of ETH involves ethA, which encodes a flavin monooxygenase that catalyzes the Baeyer-Villiger reaction (Fraaijie et al., 2004). To date, in-vitro activation...
of ETH has not been possible by either chemical or enzymatic methods, the only viable method being a cell based approach devised by (Wang et al., 2007).

Even though Mtr has been shown to have a 13-fold preference for NADPH over NADH based on V/K values (Patel and Blanchard, 1999), preliminary percentage inhibitions of Mtr by INH-NAD(P) adducts were carried out using NADH due to the fact that the $k_m$ value for the binding of NADPH by Mtr is only $7 \mu M$, at which concentration the absorbance at 340nm will be only 0.0435. This would have made it difficult to obtain accurate results.

Inhibition experiments were thus performed as described in section 2.2.9, where increasing concentrations of INH-NAD and INH-NADP adduct mixtures were incubated with MSSnaph, NADH for 1 minute at 30 $^\circ$C and the reaction initiated by the addition of Mtr. The percentage inhibitions shown in table 6.1 increase with increasing adduct concentration and show more than 50% inhibition because MSSNaph and MSSM are not saturating (MSSnaph and MSSM concentrations were kept constant at 25 $\mu M$), moreover, the amount of data presented is rather limited.

Control experiments showed that INH, NAD$^+$ or Mn$^{III}$-PyrPh alone (concentrations of up to 1mM) had no effect on Mtr activity. When MSSnaph was substituted with MSSM, a similar trend was observed where the highest INH-NAD(P) adduct concentration used (10 uM) resulted in 66% inhibition, comparable to that obtained with 10 $\mu M$ INH-NAD(P) when MSSnaph was used as a substrate.
Table 6.1: Percentage inhibitions of Mtr by INH-NAD(P) adducts, MSSnaph and MSSM were kept constant at 25 µM.

<table>
<thead>
<tr>
<th>MSSnaph used as a substrate</th>
<th>[INH-NAD]</th>
<th>Percentage inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2 uM</td>
<td>29.5 %</td>
</tr>
<tr>
<td></td>
<td>4 uM</td>
<td>35.4 %</td>
</tr>
<tr>
<td></td>
<td>6 uM</td>
<td>54.5 %</td>
</tr>
<tr>
<td></td>
<td>10 uM</td>
<td>60.9 %</td>
</tr>
<tr>
<td>MSSnaph used as a substrate</td>
<td>[INH-NADP]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2 uM</td>
<td>16.4 %</td>
</tr>
<tr>
<td></td>
<td>4 uM</td>
<td>37.7 %</td>
</tr>
<tr>
<td></td>
<td>6 uM</td>
<td>55.5 %</td>
</tr>
<tr>
<td></td>
<td>10 uM</td>
<td>65.5 %</td>
</tr>
<tr>
<td>MSSM used as a substrate</td>
<td>[INH-NADP]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2 uM</td>
<td>16.8 %</td>
</tr>
<tr>
<td></td>
<td>4 uM</td>
<td>26 %</td>
</tr>
<tr>
<td></td>
<td>6 uM</td>
<td>53 %</td>
</tr>
<tr>
<td></td>
<td>10 uM</td>
<td>66 %</td>
</tr>
</tbody>
</table>
6.3 Inhibition of Mtr by INH-NAD(P) adduct mixture

The classic double reciprocal plots of Mtr inhibition by INH-NAD(P) adducts showed variable inhibition (figures 6.2, 6.3, 6.4), where inhibition was initially mostly slope effects, approximating competitive inhibition at low inhibitor concentrations and then slowly changing over into uncompetitive inhibition, where the uncompetitive part seemed to suggest that INH-NAD(P) could also bind to the reduced enzyme in such a way that it cannot be reversed by NADH.

Moreover, the adducts only showed incomplete inhibition when either substrate (NADH or MSSnaph) was varied at five fixed levels of INH-NAD(P). Figure 6.5, shows the degree of incomplete inhibition of Mtr by INH-NADP at substrate saturation. Incidentally, Blanchard and Patel, 1999, also found that the reduction of Mtr by NADPH was only partial (reducing only one FAD in the dimer).

Whilst the inhibition observed on double reciprocal plots with INH-NADP starts with slope effects, because INH-NADP competes with NADH for the same binding site, another type appears which could be binding of inhibitor to the reduced enzyme resulting in no further slope effects. Moreover, the fact that inhibition appears to be incomplete suggests probable half sites reactivity. The study is still ongoing to fully clarify the mechanism of inhibition of Mtr by INH-NAD(P) adducts.
Figure 6.2: Representative plot of $1/V$ vs $1/[\text{NADH}]$. Assays contained 24 nM Mtr, 25 µM MSSnaph, variable NADH (19, 28, 38, 47, and 67 µM) at five fixed levels (0, 3.5, 7, 10.5, and 14 µM) of INH-NAD. Data points represent the average of duplicate determinations.

Figure 6.2.1: Slope vs [INH-NAD]. Re-plot of figure 6.2
Figure 6.3: Representative plot of $1/V$ vs $1/[\text{MSSnaph}]$. Assays contained 24 nM Mtr, 105 µM NADH, variable MSSnaph (8, 10, 20, 30, and 40 µM) at five fixed levels (0, 3.5, 7, 10.5, and 14 µM) of INH-NAD. Data points represent the average of duplicate determinations.

Figure 6.3.1: Slope vs [INH-NAD]. Re-plot of figure 6.3
Figure 6.4: Representative plot of 1/V vs 1/[NADH]. Assays contained 24 nM Mtr, 25 µM MSSnaph, variable NADH (19, 28, 38, 47, and 67 µM) at five fixed levels (0, 3.5, 7, 10.5, and 14 µM) of INH-NADP. Data points represent the average of duplicate determinations.

Figure 6.4.1: Slope vs [INH-NADP]. Re-plot of figure 6.4. The data of the inhibitor experiments showed that slopes change dramatically for INH-NADP up to about 2 micromolar INH-NADP and then less so, while intercepts change. This also seems to differ quantitatively for INH-NAD. Inhibition of Mtr by INH-NADP is thus not a continuous process but a step wise one signified by a jump at 3.5 µM.
Although data points (figure 6.4) were all collected from 3.5 micromolar upwards, the experiment has been repeated several times, but at submicromolar concentrations of INH-NADP, where the inhibition was found to be truly competitive (unpublished observations, this laboratory).

Figure 6.5: Represents the rate of the enzymatic reaction as a function of INH-NADP at different fixed levels of the variable substrate (NADH). The dots are experimental data (intercepts) and the curve represents the theoretical values of the parameter (y) from Equation 1 (shown below) fitted to these experimental points. The fact that hyperbolic decay fits the data quite well suggests that a maximal degree of inhibition exists.

The equation used to generate figure 6.5 is the one for a three parameter hyperbolic decay, equation 1:

\[ y = y_0 + \frac{ab}{b + x} \]

Then the \( V_{\text{max}} \) values fit the predicted curve of y’s, where \( y_0 = 0.08914 \pm 0.01605 \), \( a = 0.1307 \pm 0.01557 \) and \( b = 7.485 \pm 2.196 \). So when INH-NADP is very large \( y = y_0 \) and when there is no INH-NADP \( y = y_0 + a \) which equals \( (0.08914 \pm 0.01605) \) + \( (0.1307 \pm 0.01557) \).
Given the relatively large standard errors on the parameters, at infinite INH-NADP the inhibition at $V_{\text{max}}$ is about 60%, which means that the $K_i$ for the uncompetitive part of the inhibition could be as high as 7.485 µM. Equation 1 can also be rearranged to show that:

$$y - y_o = \frac{a K_i}{1 + \frac{I}{K_i}} = \frac{a}{1 + \frac{I}{K_i}}$$

where $a$ is the extent of the inhibition and $b$ is $K_i$.

### 6.3.1 Conclusion (INH-NAD(P) adducts)

An interesting observation made in this study is that while Mtr is inhibited by both INH-NAD and INH-NADP, most of the other enzymes (e.g. dihydrofolate reductase and the enoyl-ACP-reductase) are specifically inhibited by one of the two adducts (as discussed in section 6.1). The effects of these INH-NAD(P) complexes on Mtr are thus likely to be additive. Moreover, INH will likely generate both INH-NADH and INH-NADP, both of which will inhibit Mtr and that the inhibition constants that can be estimate fall probably below 1 µM for the competitive part of inhibition by the entire INH-NADP mixture and at about 7.5 µM for the uncompetitive part of the inhibition, again for the mixture. However, is not yet known what the effect of mixtures of INH-NADP and INH-NAD will be on Mtr. While insights with regard to the mechanism of inhibition of the mycobacterial fatty acid biosynthesis pathway (FAS II), the enoyl-ACP and the dihydrofolate reductases by INH have been provided by a number of studies as discussed in section 6.1, it was only recently that Argyrou, et al., 2006b managed to identify a number of additional reductases in mycobacteria that can bind adducts formed between INH and NAD(P). However, Mtr was not amongst the newly identified targets.

In this study, the use of INH-NAD(P) adducts against Mtr indicated that the enzyme was not completely inhibited (noted in section 6.3) as a result, the inhibition observed was mainly of interest from a mechanistic point of view and also in practical terms, because it means complete inhibition by the adducts in the low micromolar range is not attainable. Despite this, the study was able to demonstrate Mtr as a possible target for INH, however, more in depth studies (e.g. crystallography) are required to explain the exact interaction of Mtr with activated INH.
One of the problems frequently encountered with the preparation of the INH-NAD(P) adducts was that although they were quick and easy to prepare, they seemed to be unstable immediately after the Sep-pak C18 step (given by the decline of absorbance at 326 nm). Moreover, after storage overnight a broad absorbance peak at 4-500 nm develops and it was also found that even when stored at -80 °C the mixture was no longer inhibitory. As a result, it became extremely important to collect the kinetic data as quickly as possible and certainly within a day.

A possible explanation for the observed decline in absorbance was attributed to the fact that perhaps an equilibrium between the different isomeric forms had not yet been established, or that the adduct itself undergoes changes that go beyond that of isomeric equilibrium. Because of this, it is not certain whether the different phases of Mtr inhibition can be ascribed to interactions with different isomers in the mixture, whereas, with DHFR and InhA, the inhibition was observed to be very tight and selective for one isomer. Also, because the inhibition was classically competitive, although bisubstrate competitive, it was possible to obtain reliable $K_i$’s, while in our case, a mixture of different inhibition types was observed which made it difficult to assign $K_i$’s to the different stages.

6.4 Evaluation of 35-38

6.4.1 Effect of 35-38 against MshB

Given the fact that naphthoquinones can readily accept electrons from flavoproteins, we envisaged that naphthoquinones linked to $\alpha$-DGI or, as in our case, a thiophenol moiety, would offer specificity towards Mtr whilst discriminating against the host’s glutathione reductase, and with the successful synthesis of 35-38 (discussed in chapter 5), we proceeded to the next phase which involved their biological evaluation.
Compounds 35-38 were tested (Marakalala, M.J., 2008) for inhibition of MshB utilising a fluorescence-detected HPLC assay in which acetyl-α-DGI was used as substrate. Inhibition of MshB was obtained by measuring the extent of acetyl-α-DGI cleavage in the presence of each of 35-38. Compound 38 was found to be the most active against MshB, inhibiting the enzyme by 94.8% at 500 µM, whilst 37 and 36 exhibited about 81% inhibition, with moderate levels displayed by 35 (57.4%).

Inhibition of MshB seemed to be directly correlated to the length of the alkyl chain linking the quinone and the glucosamine moiety, as the deacetylase activity decreased when 35 was present and was virtually absent with 38. Since the compounds were limited to a five-carbon spacer, the use of longer spacers would be of much interest in future studies. The $K_i$ (µM) values for 35, 37 and 38 were found to be $162.7 \pm 15$, $93.9 \pm 11$, and $16.8 \pm 1.9$ respectively. Comparing the activity of these compounds to the reported $K_m$ of about $340 \pm 80$ µM (Newton et al., 2006b) for the cleavage of GlcNAc-Ins by MshB it becomes apparent that 35, 37, and 38 are the most potent inhibitors of MshB yet described.

### 6.4.2 Effect of 35-38 against Mca

MshB is not essential for growth or synthesis of MSH in $Mtb$, since Mca exhibits low levels of the deacetylase activity that allows for the formation of MSH in the absence of MshB. The compounds were therefore, also tested as Mca inhibitors, but were found to be less potent inhibitors of this enzyme than of MshB.

The crystal structures of the two enzymes with 35-38 co-crystallised may reveal interesting information with regard to interactions between the enzyme’s active site and the inhibitors. However, in light of the difficulties frequently experienced in obtaining suitable crystals of proteins, perhaps 38, which exhibited the highest inhibition with both enzymes will have to be utilised for crystal and subsequent docking studies. Indeed, the inhibition of Mca by 35-38 is an interesting and useful foundation on which alternative
and more potent inhibitors can be designed to add onto those that have already shown significant inhibitory properties, as described in chapter 1.

Rawat et al., 2004, suggested that MSH can form stable S-conjugates (figure 6.6) when exposed to naphthoquinones, and as such, these conjugates may be suitable substrates for Mca. An interesting observation made with 35-38 is that they inhibited both MshB and Mca, confirming that substituted (on carbon 3) naphthoquinones may be a suitable basis for the design of compounds targeted at Mca.

Figure 6.6: Hypothetical formation of mycothiol S-conjugate when mycothiol is exposed to plumbagin. Cleavage by Mca results in the generation of α-DGI with AcCys-5-hydroxy-2-methyl-1,4-naphthoquinone being disposed out of the cell. However, with the use of compounds 35-38 the C-3 position is already occupied preventing any further substitution.
**6.4.3 Effect of 35-38 against Mtr**

The use of naphthoquinones as subversive substrates for reductases is well documented and previous studies have successfully demonstrated the utility of quinones against trypanothione reductases of pathogens such as those of *Trypanosoma congolense* (Cenas *et al.*, 1994) and *Trypanosoma cruzi* (Salmon-Chemin *et al.*, 2001). And recently, towards the completion of the synthesis of subversive substrates used in this study Mahapatra *et al.*, 2007, reported on the use of 7-methyljuglone derivatives as subversive substrates for Mtr and their activity against *Mtb*.

In this study plumbagin derivatives linked to a glucosamine through variable chain lengths were assessed as alternative substrates (with a view of using them as subversive substrates) and inhibitors of Mtr. Investigation of 35-38 as alternative substrates was limited to 40 µM since they were predominantly hydrophobic and poorly soluble above this concentration, and their specific activities were compared to that of plumbagin. They were thus evaluated and compared at the same concentration. The hydrophobicity of the compounds became evident when evaluating them in spectrophotometric assays, and this made the generation of catalytic constants difficult and unreliable.

**6.4.4 Specific activities of 35-38**

Since naphthoquinones are readily reduced by reductases (discussed in chapter 1) and since compounds 35-38 are conjugates of plumbagin and phenyl-2-amino-1-thioglycoside it was of interest to observe how they would compare with unconjugated plumbagin when reduced by Mtr. The ability of Mtr to reduce 35-38 was assayed as described in materials and methods. High specific activity was found with 37 (0.42 µmol/min/mg) followed by 36 (0.37 µmol/min/mg), 38 (0.32 µmol/min/mg) and 35 (0.29 µmol/min/mg) with the least activity, whilst plumbagin demonstrated a specific activity of 0.32 µmol/min/mg, which was comparable to that observed for 38 (figure 6.7).
Since the compounds showed comparable activities with plumbagin, further quinone reductase activity utilising cytochrome c was not attempted. Coupling semiquinone formation to the reduction of cytochrome c, is usually used to measure the amount of one electron reduction of the naphthoquinones by reductases.

The results do not show any direct correlation between the specific activity observed and the spacer used. Compound 35 employs a 2-carbon spacer as opposed to the 5-carbon spacer found in 38, and both of these compounds have more or less similar specific activities as plumbagin. Compounds 36 and 37 with 3 and 4-carbon spacers respectively proved to be slightly better substrates as compared to plumbagin.

![Figure 6.7: Comparative specific activities of plumbagin, 35, 36, 37, and 38. The capability of Mtr to reduce plumbagin, 35-38 was determined by monitoring the oxidation of NADPH at 340 nm. The reaction mixture contained, in a final volume of 1 mL: 50 mM Tris/HCl (pH 7.6), NADPH (0.11 µmoles), and 35-38 (each compound measured at 40 µM) at 30 °C. The reaction was started by adding Mtr (48 nM). Stock solutions of 35-38 were prepared in DMSO. Data points represent the average of duplicate determinations.](image)

6.4.5 35-38 as competing substrates or inhibitors of Mtr

The biological activities of 35-38 (tested at a concentration of 40 µM) as competing substrates or inhibitors of Mtr were evaluated according to the procedure described in section 2.2.3. As far as the percentage inhibition was concerned, the compounds were
not found to be promising inhibitors (table 6.2) with the highest inhibition displayed by 38 at 14%, as a result no further exploration was attempted. The poor activities displayed by these compounds (which lack the N-acetyl group of the N-acetyl moiety as found in MSH) seem to support the observation made by Newton et al., 2005, in which the activity of Mtr was found to be more substantial with the natural substrate than with desacetylmycothiol or N-succinylated mycothiol (succ-Cys-GlcN-Ins). This indicates that the presence of the N-acetyl group of the N-acetyl moiety of mycothiol seems to be crucial for the activity of mycothiol analogues as substrates for Mtr, features that are absent on compounds 35-38.

Table 6.2 Compounds 35-38 and their percentage inhibitions against Mtr. The compounds were evaluated at a concentration of 40 µM.
Despite the minimal inhibition shown in figure 6.7, the relationship between the kind of spacer used and inhibition displayed appears to be related. Compound 35 with a 2-carbon spacer showed 0% inhibition as opposed to 38 (utilising a 5-carbon spacer) showing 14% inhibition. It would be interesting to explore the effects of these compounds when concentrations above 40 µM are used. Studies looking at substituting the phenyl group with inositol are in progress (using a fused bicyclic thioglycoside) in a bid to encourage solubility, and consequent evaluation at high concentrations.

6.4.6 Anti-mycobacterial activities of 35-38

Anti-mycobacterial activities of the compounds toward *Mtb* are shown in figure 6.8. Bactericidal activity was shown only by 35, whilst 36 and 37 resulted in growth indexes of 87, 38 did not show significant inhibitory properties after 4 days, a trend almost similar to that observed for the control.

Figure 6.8: Anti-mycobacterial activities of 35-38 on *Mtb* were assessed using the radiometric respiratory technique based on the Bactec system. The assay was carried out by Dr Wiid, Department of Medical Biochemistry at the University of Stellenbosch, South Africa.

The trend observed in figure 6.8 is in direct contrast to that observed in figure 6.7 and table 6.2, where 35 was shown to exhibit low specific activity and no enzyme inhibition.
but elevated antimycobacterial properties as compared to 36 and 37. It is possible that 35 interacts with other metabolic pathways not considered in this study or that perhaps metabolic products of 35 are more active than 35. This remains an intriguing observation worth investigating further. The lack of correlation between antibacterial activity and kinetic parameters has also been observed in a previous study involving the activity of 7-methyljuglone derivatives against Mtr (Mahapatra et al., 2007).

Mahapatra et al., also postulated that the reason their methyljuglone derivatives lacked specificity between antitubercular activity and subversive substrate efficiency with Mtr could be ascribed to a possible interaction with other flavoproteins such as lipoamide dehydrogenase and thioredoxin. It is also probable that 35, which showed more or less the same specific activity as plumbagin, had ambiguous interactions with other flavoproteins, hence the observed diminished growth index of Mtb after 4 days (figure 6.8).

### 6.5 Thioglycosides as Mtr inhibitors

The necessity to assess whether thioglycosides can act as possible Mtr inhibitors is derived from established observations where aminoglycosides have been shown to act as broad spectrum antimicrobial agents that are widely used in the treatment of a range of Gram-positive and negative bacterial infections (Miller et al., 1995; Wright, 1995). And since an aminoglycoside based pseudodisaccharide (α-DGI) is the foundation of MSH and subsequent MSSM, it was thought necessary to test a variety of these compounds so as to find a lead compound that might be useful in the establishment of a potent Mtr inhibitor. The range of thioglycosides evaluated in inhibiting Mtr is shown in table 6.3.
Table 6.3: Structures of the thioglycosides used and their percentage inhibition of Mtr. The thioglycosides used in this study were obtained from Prof. Spencer Knapp, Rutgers University chemistry department. The compounds were evaluated at a concentration of 500 µM.

<table>
<thead>
<tr>
<th>R₁</th>
<th>R₂</th>
<th>Percentage inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>SPh2</td>
<td><img src="image" alt="Structure" /></td>
<td>1.86%</td>
</tr>
<tr>
<td>SPh3</td>
<td><img src="image" alt="Structure" /></td>
<td>0%</td>
</tr>
<tr>
<td>SPh4</td>
<td><img src="image" alt="Structure" /></td>
<td>5.25%</td>
</tr>
<tr>
<td>SPh5</td>
<td><img src="image" alt="Structure" /></td>
<td>8.75%</td>
</tr>
<tr>
<td>SPh6</td>
<td><img src="image" alt="Structure" /></td>
<td>17.1%</td>
</tr>
<tr>
<td>SPh7</td>
<td><img src="image" alt="Structure" /></td>
<td>12.75%</td>
</tr>
</tbody>
</table>

The maximum inhibition achieved with this set of thioglycosides was 17% (SPh6).
6.6 *O*-glycosides as Mtr inhibitors

As a comparative measure a series of *O*-glycosides was also tested as inhibitors of Mtr and is shown in table 6.4.

Table 6.4: Structures and percentage inhibitions of Mtr by *O*-glycosides. The *O*-glycosides were obtained from Prof. D.G Gammon, Chemistry department, University of Cape Town, South Africa. The compounds were evaluated at a concentration of 500 µM.

<table>
<thead>
<tr>
<th>R</th>
<th>Percentage inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>(β-anomer of Th2) Th1</td>
<td>0%</td>
</tr>
<tr>
<td>Th2</td>
<td>0%</td>
</tr>
<tr>
<td>Th3</td>
<td>0%</td>
</tr>
<tr>
<td>Th4</td>
<td>3.39%</td>
</tr>
<tr>
<td>Th5</td>
<td>3.65%</td>
</tr>
<tr>
<td>Th6</td>
<td>12%</td>
</tr>
<tr>
<td>Th7</td>
<td>20.2%</td>
</tr>
</tbody>
</table>
As observed for the thioglycosides tested in section 6.5, the maximum inhibition achieved with this range of O-glycosides was 20% (Th7), followed by Th6 with 12% whereas, Th1-Th3 showed 0% inhibition. In general, inhibition observed with both O-glycosides and thioglycosides including 35-38 was extremely poor and raises questions over the use of a thiophenol or cyclohexane as a substitute for inositol in designing Mtr inhibitors. Efforts aimed at solving the crystal structure of Mtr are currently underway, and until such time studies pertaining to structure-activity relationships will be valuable in establishing a framework for the design of potent inhibitors for this enzyme.

6.7 Conclusion

The consistent Mtb infections and increasing prevalence of drug resistant strains is of great concern; hence the search for new anti-mycobacterials as outlined in this chapter and by numerous other studies. Whilst our efforts were focused on the development of a class of compounds (35-38) targeted against Mtr, it was their observed activity against MshB and Mca that proved valuable. As this showed them as lead compounds to which further modifications such as improving their solubility, incorporating an N-acetyl group (mentioned in section 6.4.5) may lead to the development of a dual inhibitor for the two enzymes (MshB and Mtr).

Evaluation of O-glycosides (Th1-7) and thioglycosides (SPh2-7) was motivated by studies carried out by Patel and Blanchard, 1998, and Stewart et al., 2008 where the O-benzyl, O-methyl and des-myoinositol-glycoside derivatives of MSSM have previously been shown to be recognised as alternative substrates by Mtr. The Km values for these substrates were found to be 3-6 fold greater than for MSSM, which demonstrated a relatively modest compromise in substrate recognition for quite significant changes to the aglycone moiety. For all the compounds tested as Mtr inhibitors, the weak inhibition is more likely attributed to the collective effects of modification of the aglycone as well as modifications to the glucosamine N-substituent.
**General Conclusions**

Although Mtr from *Mycobacterium tuberculosis* had been cloned, expressed in *Mycobacterium smegmatis* and purified (Patel and Blanchard, 1999), this study attempted to explore alternative expression systems in a quest to recover increased enzyme yields since the original expression resulted in modest recoveries as discussed in chapter 3. Unfortunately, the two expression systems tried were not successful. However, whilst exploring the use of *C. glutamicum*, MSH was isolated using an alternative and inexpensive derivatizing agent (2-bromoacetonaphthone, BAN), purified, characterised and confirmed to be a low molecular mass thiol exclusively used by *C. glutamicum* as had been expected. Furthermore, MSSnaph was shown to be an alternative substrate for *M. tuberculosis* Mtr with a $K_{m,app}$ value of 23 µM which is 3-5 times lower than values previously reported for the reduction of MSSM.

As discussed in section 1.6, the suitability of a few different aglycons instead of inositol in mycothiol were explored in an attempt to shed light on the substrate specificity of Mtr, and the benzyl group was found to be the most suitable (Stewart et al., 2008). In addition, an earlier study by Knapp *et al.*, 2002 showed that Mca was active against a mycothiol-S-bimane analogue. These findings provided the impetus for a successful design of a set of compounds (35-38) targeted at Mtr in which plumbagin was tethered via 2 to 5 methylene carbons and an amide linkage to phenyl-2-deoxy-2-amino-1-thio-α-D-glucopyranoside. Although the compounds did not show promise as potential subversive substrates as had been initially envisaged, they were however, found to be the most potent inhibitors of MshB, and also displayed some activity against Mca (Gammon et al., 2010). In spite of the compound’s poor activity against Mtr, only compound 35 showed bactericidal activity. An interesting and unexpected finding made in this study is that Mtr is also inhibited (although incomplete) by both INH-NADP and INH-NAD adducts. Although the adducts were easy to prepare, they were nonetheless unstable, most probably due to the re-oxidation of the nicotinamide ring over time to produce a more conjugated molecule as being the decomposition product. The study is still in progress and more in...
depth studies (e.g. crystallography) will be required to explain the exact interaction of Mtr with activated INH.
Appendix 1A: Composition of growth media used in chapter 3.

Middlebrook

4.7g Middlebrook in 800 mL dH2O
50 mL glycerol
0.5g Tween 80
Made up to 1L with dH2O and autoclaved

LB media

10g Tryptone
5g Yeast extract
5g NaCl
Dissolved in 800 mL, pH adjusted to 7.5, made up to 1L with dH2O and autoclaved

Superbroth

35g Tryptone
20 Yeast extract
5g NaCl
Dissolved in 800 mL, pH adjusted to 7.5, made up to 1L with dH2O and autoclaved.

Issabelle media

40g Glucose
15g Yeast extract
15g Peptone
1.75g NaH2PO4.H2O
0.04g Ferric ammonium citrate
Dissolved in 900 mL, pH adjusted to 7.5 and autoclaved
N.B. Glucose was filter sterilised

**Appendix 1B: pEC-XK99E sequence. The multiple cloning site is underlined.**

LOCUS AY219683 7018 bp DNA circular SYN 05-SEP-2003
DEFINITION Shuttle expression vector pEC-XK99E, complete sequence.
ACCESSION AY219683
VERSION AY219683.1 GI:29164935
KEYWORDS.
SOURCE Shuttle expression vector pEC-XK99E
ORGANISM Shuttle expression vector pEC-XK99E
other sequences; artificial sequences; vectors.
REFERENCE 1 (bases 1 to 7018)
AUTHORS Kirchner,O. and Tauch,A.
TITLE Tools for genetic engineering in the amino acid-producing bacterium Corynebacterium glutamicum
PUBMED 12948646
REFERENCE 2 (bases 1 to 7018)
AUTHORS Kirchner,O. and Tauch,A.
TITLE Direct Submission

JOURNAL Submitted (15-JAN-2003) Department of Genetics, University of Bielefeld, Universitaetsstrasse 25, Bielefeld D-33615, Germany

FEATURES Location/Qualifiers

source 1..7018

/organism="Shuttle expression vector pEC-XK99E"

/mol_type="genomic DNA"

/db_xref="taxon:224638"

/note="Escherichia coli-Corynebacterium glutamicum shuttle vector"

terminator 263..306

/note="terminator T1"

terminator 438..465

/note="terminator T2"

gene complement(705..920)

/gene="per"

CDS complement(705..920)

/gene="per"

/note="positive effector of replication"

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CDS complement(1563..3026)
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Appendix 1C: Mycothiol disulfide reductase protein sequence
[Mycobacterium tuberculosis H37Rv]

LOCUS YP_177910 459 aa linear BCT 29-
MAR-2010
DEFINITION mycothione reductase [Mycobacterium tuberculosis H37Rv].
ACCESSION YP_177910
VERSION YP_177910.1 GI:57117030
DBLINK Project:224
DBSOURCE REFSEQ: accession NC_000962.2
KEYWORDS complete genome.
SOURCE Mycobacterium tuberculosis H37Rv
ORGANISM Mycobacterium tuberculosis H37Rv
Bacteria; Actinobacteria; Actinobacteridae;
Actinomycetales;
Corynebacterineae; Mycobacteriaceae; Mycobacterium;

Mycobacterium tuberculosis complex.

REFERENCE 1

AUTHORS Camus, J.C., Pryor, M.J., Medigue, C. and Cole, S.T.

TITLE Re-annotation of the genome sequence of Mycobacterium tuberculosis H37Rv

JOURNAL Microbiology (Reading, Engl.) 148 (PT 10), 2967-2973 (2002)

PUBMED 12368430

REFERENCE 2 (residues 1 to 459)


TITLE Deciphering the biology of Mycobacterium tuberculosis from the complete genome sequence

JOURNAL Nature 393 (6685), 537-544 (1998)

PUBMED 9634230

REMARK Erratum:[Nature 1998 Nov 12;396(6707):190]

REFERENCE 3 (residues 1 to 459)

CONSRTM NCBI Genome Project

TITLE Direct Submission

JOURNAL Submitted (13-SEP-2001) National Center for Biotechnology Information, NIH, Bethesda, MD 20894, USA

COMMENT REVIEWED REFSEQ: This record has been curated by NCBI staff. The reference sequence was derived from CAE55526.

Method: conceptual translation.
FEATURES Location/Qualifiers

source 1..459

/organism="Mycobacterium tuberculosis H37Rv"

/strain="H37Rv"

/db_xref="taxon:83332"

Protein 1..459

/product="mycothione reductase"

/EC_number="1.8.1.7"

/function="INVOLVED IN REDUCTION OF MYCOTHIOL."

/calculated_mol_wt=49815

Region 3..459

/region_name="PRK07846"

/note="mycothione/glutathione reductase; Reviewed; PRK07846"

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Region 174..252

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/note="Rossmann-fold NAD(P)(+)-binding proteins; cl09931"

/db_xref="CDD:164178"

Region 345..454

/region_name="Pyr_redox_dim"

/note="Pyridine nucleotide-disulphide oxidoreductase, dimerization domain; pfam02852"

/db_xref="CDD:145812"

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361 tenqavakgl disvkiqdyg dvaygwamed tsqivklite rgsgrllgah imgyqassli
421 qqliqamsfg ltaemargq ywiupalpev venallglr
References


