



**Designing novel inhibitors for *Mycobacterium tuberculosis* by
targeting *inhA* and *kasA* genes using ligand-based drug
discovery**

MOJALEFA BRIAN SETLAI

Haematology Department
National Health Laboratory Service, Universitas Business Unit
Central University of Technology Free State, Bloemfontein, South Africa

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Promotor: Dr. Pravinkumar Kendrekar (Ph.D. Organic Synthesis)

Co-promotor: Dr. Lebogang F Mogongoa (DHSc. Biomedical Technology)

Co-promotor: Prof. Samson Mashele (Ph.D. Biochemistry)

Department of Health Sciences

Central University of Technology Free State, Bloemfontein, South Africa

External Co-promotor: Dr. Martin Nyaga (Ph.D. Medical Virology)

Next Generation Sequencing Unit

University of the Free State, Bloemfontein, South Africa

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DECLARATION OF INDEPENDENT WORK

I, Mojalefa Brian Setlai, declare that this research project submitted to the Central University of Technology Free State, in fulfilment of the requirements for the degree, Doctor of Health Sciences: Biomedical Technology, is my independent work that has not been submitted to any institution before, by me or any other person in fulfilment of the requirements for the attainment of any qualification.

Candidate Signature

26/09/2022

Date

VERKLARING VAN ONAFHANKLIKE WERK

Ek, Mojalefa Brian Setlai, verklaar hiermee dat die navorsingsprojek wat ter vervulling van die vereistes vir die graad Doktor in Gesondheidswetenskappe: Biomediese Tegnologie, aan die Sentraal Universiteit van Tegnologie, Vrystaat, wat deur my voorgelê word, my selfstandige werk is, en nie voorheen deur my of enige ander persoon by enige ander instelling, ter verwerwing van enige kwalifikasie voorgelê is nie.

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Abbreviations

ACP	Acyl carrier protein
AcpM	Acyl carrier protein M
AIDS	Acquired immunodeficiency syndrome
AlrA	D-alanine racemase
ART	Antiretroviral therapy
ARV	Antiretroviral
A, T, C, G	adenine, thymine, cytosine, guanine
ATP	Adenosine triphosphate
BDQ	Bedaquiline
BCG	Bacillus-Calmette Guérin
BLAST	Basic Local Alignment Search Tool
bp	Base pair
BSC II	Biohazard Safety Cabinet class II
CAN	Ceric ammonium nitrate
cDNA	Coding DNA
CFU	Colony-forming units
COSY	Correlation Spectroscopy
CYP450	Cytochrome P450
DCM	Dichloromethane
DDQ	Dichlorodicyanobenzoquinone
ddNTPs	Dideoxynucleotide triphosphates
DMSO	Dimethyl sulfoxide
dNTPs	Deoxynucleotide triphosphates
DNA	Deoxyribonucleic acid
DNA gyrase	Topoisomerase type II
DPA	Decaprenyl-phosphoryl-B-D-arabinose
DPR	Decaprenyl-phosphoryl ribose
<i>E. coli</i>	<i>Escherichia coli</i>
EEA1	Early-endosomal autoantigen-1
EMB	Ethambutol
ENR	Enoyl acyl carrier protein reductase

FAS-II	Fatty-acid synthase type II
GPK	5-fluoro-1-(glucosyl) pyrimidine-2,4(1H,3H)-dione
G6PD	Glucose-6-phosphate dehydrogenase
HIV	Human immunodeficiency virus
Hrs	Hours
HSQC	Heteronuclear Single Quantum Coherence
Hz	Hertz
IBX	Iodoxybenzoic acid
IL	Interleukin
INH	Isonicotinylhydrazine (isoniazid)
InhA	Fatty-acid enoyl-acyl carrier protein reductase
<i>inhA</i>	<i>Fatty-acid enoyl-acyl carrier protein reductase gene</i>
IQC	Internal quality control
J	Joule
JE	Jarzynski's Equality
KasA	β -ketoacyl-ACP synthase
<i>kasA</i>	<i>β-ketoacyl-ACP synthase gene</i>
MDR-TB	Multidrug resistance tuberculosis
MGIT	Mycobacteria Growth Indicator Tube
MHA	Mueller Hinton agar
MIC	Minimum inhibitory concentration
MTCYP450	<i>Mycobacterium tuberculosis</i> cytochrome P450
NALC	N-Acetyl-L-cysteine
NaOH	Sodium hydroxide
NCBI	National Center for Biotechnology Information
NGS	Next-generation sequencing
NHLS	National Health Laboratory Service
NMR	Nuclear Magnetic Resonance
OHASIS	Occupational Health and Safety Information System
PAS	Para-Aminosalicylic Acid
PDBsum	Protein Database Summaries
Pd(OH) ₂	Palladium hydroxide
PCR	Polymerase Chain Reaction

PPE	Personal protective equipment
ppm	Parts Per Million
PSi	Pound-force per square inch
PZA	Pyrazinamide
RIF	Rifampicin
RNA	Ribonucleic acid
rRNA	Ribosomal Ribonucleic acid
ROS	Reactive oxygen species
RpsA	Ribosomal protein 1
R.T	Room temperature
VTE	Venous thromboembolism
SA	South Africa
SNP	Single nucleotide polymorphism
TB	Tuberculosis
TDM	Trehalose dimycolate
TFAA	Trifluoroacetic acid anhydride
TH1	Thymus-derived helper-1
TLM	Thiolactomycin
TMM	Trehalose monomycolate
TNF	Tumor necrosis factor
USA	United States of America
XDR-TB	Extensively drug-resistant tuberculosis

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Summary

Tuberculosis (TB) affects almost one-third of the world's population and is caused by the *Mycobacterium tuberculosis* (*M. tuberculosis*) bacteria. One of the problems in treating tuberculosis is the emergence of multidrug-resistant tuberculosis (MDR-TB) and extensively drug-resistant tuberculosis (XDR-TB). The disease and the emergence of multidrug-resistant variants of *M. tuberculosis* have opened the doors for researchers to find novel drug targets. Since both fatty-acid enoyl-acyl carrier protein reductase (InhA) and β -ketoacyl-ACP synthase (KasA) enzymes are the primary targets of action for isoniazid (INH) and thiolactomycin (TLM) in *M. tuberculosis*, the aim of the study was to design novel drug candidates that target and bind to InhA and KasA enzymes responsible for the synthesis of mycolic acid of the *M. tuberculosis* cell wall from TLM derivatives and cause inhibition of the cell wall synthesis.

The research study was approved by The Environmental and Biosafety Research Ethics Committee of the University of the Free State, with Ethics approval number UFS-ESD2019/0064. The study was applied experimental research conducted in three stages to develop drug candidates for novel drugs by using the ligand-based bioinformatics tool PubChem to find the native ligand binding sites for TLM derivatives that have a strong binding capacity to the InhA and KasA proteins. We created a construct sequence for the *inhA* and *kasA* genes of *M. tuberculosis* H37Rv strain using nucleotide Basic Local Alignment Search Tool (BLAST) search on the National Center for Biotechnology Information (NCBI) and performed drug susceptibility testing on *Mycobacterium smegmatis* (*M. smegmatis*) MC2155 strain to surrogate *M. tuberculosis* H37Rv strain due to its non-pathogenic characteristic and similarity to *M. tuberculosis* in genome and structure. *M. smegmatis* strain was isolated utilizing the Becton Dickinson (BD) BBL Bactec Mycobacteria Growth Indicator Tube (MGIT) bottles and then inoculated on Mueller Hinton agar (MHA) plates infused with different concentrations of drug compounds of interest.

The *inhA* and *kasA* gene sequences were subjected to BLAST analysis at the NCBI against *M. tuberculosis* whole genome ribosomal ribonucleic acid (rRNA) sequence to identify the closest homologs. Among resulting hits, the sequences with 100%

identity homologs were selected. These strains were selected, and the *H37Rv* rRNA sequences were obtained. Phylogenetic analysis was carried out using the maximum likelihood method based on the Tamura–Nei model. All positions in the sequence containing gaps and missing data were eliminated. The phylogenetic analysis showed the branch lengths and the bacterial isolates identified in this study. Both receptors InhA and KasA were submitted to Protein Database Summaries (PDBsum) to determine their interaction, and coordinates of the interaction between the protein and the ligand noted. 5-fluoro-1-(glucosyl) pyrimidine-2,4(1H,3H)-dione (GPK) was derived from TLM and used as a ligand. After multiple runs, a cluster analysis was performed. The hydrophobic residues-binding positions for InhA and KasA proteins were acquired, and the ligands docked in the active position to determine the interactions with the highest binding affinity.

The results of *M. tuberculosis strain H37Rv* sequences with 100% identity homologs were selected from the resulting hits. The obtained sequence reads were aligned to the reference genome (CP003248) using the NCBI BLAST. There were no open gaps found within the nucleotide sequences, meaning that there were 100% identities and 0% gaps between the reference genome subject (Sbjct) and the query genes (Query) *inhA* "GeneID: 886523" (CP072765.1) with 831/831 bits, and *kasA* "GeneID: 887269" (NC_000962.3; CP072765.1; CP007027.1; CP053903.1; CP003248.2) with 1251/1251 bits.

There were seven synthesized TLM derivative compounds, GPK91, GPK92, GPK93, GPK94, GPK95, GPK96, and GPK97, but there were only two potential candidates for the novel antimycobacterial drugs, GPK91 and GPK96. The designed ligand compounds were docked with InhA PDB ID 3FNE and KasA PDB ID 4C6X imported from PDBsum. TLM was used as a reference ligand in both proteins. Our study showed that all the designed drug compounds had a good affinity toward the binding pockets of InhA and KasA. When docked with InhA, GPK91 showed the hydrogen bond interaction with GLY₉₆, ALA₁₉₈, and ILE₂₉₄ with the best docking score of -8.88 binding energy. In the study done by Sharma et al. (2015), InhA 2X23 showed a hydrogen bond interaction with VAL₆₅:HN₁ with the docking scores of -12.6 binding energy for ZINC01777652, and -12.7 for ZINC03831448.

In this study, GPK96 was docked with KasA 4C6X and showed a high affinity towards the binding pocket compared to the reference TLM, with a docking score of -7.746 binding energy. GPK96 also showed the hydrogen bond with MET₂₁₃, THR₃₁₅, THR₃₁₃, and additional Pi-Pi stacking with the PHE₄₀₄. The study by Sharma et al. (2015), KasA 4C72, showed hydrogen bond interaction with HIS₃₁₁:HE₂₁ with -10.7 binding energy for ZINC01530603 and -10.8 for ZINC01532344. In the same study by Sharma et al. (2015), all the compounds made several of interactions at the binding site with HIS₃₄₅ and GLN₁₇₁ that were similar to TLM. Similarly, in our study, there were other ligands with good affinity towards the binding positions of InhA and KasA, but none of the compounds displayed a simultaneous targeting of InhA and KasA. Previous studies have demonstrated that TLM is an inhibitor of the FAS-II condensing enzyme β -ketoacyl-ACP synthase (Slayden et al., 1996). This activity of TLM makes it a proficient reagent for designing novel drugs that could inhibit the activity of the InhA and KasA proteins. A series of articles that have been published state that, InhA is not the primary target of INH but rather that of KasA, which is the primary target of INH in *M. tuberculosis* (Mdluli et al., 1996; 1998; Barry, 1997; 2001; Slayden et al., 2000; Slayden and Barry, 2000). A study by Slayden et al., 2000, postulated that both InhA and KasA enzymes were targets for INH but that KasA was the primary target.

Mycobacterium smegmatis showed drug sensitivity on MHA plates for GPK91 and GPK96 concentrations down to 0.781 $\mu\text{g/ml}$ and 3.125 $\mu\text{g/ml}$, respectively. For GPK91 there was detectable growth with partial inhibition observed at [0.391 $\mu\text{g/ml}$] and no inhibition observed at [0.195 $\mu\text{g/ml}$] and less. Similarly, for GPK96, there was detectable growth with partial inhibition observed at [1.563 $\mu\text{g/ml}$], and no inhibition observed at [0.781 $\mu\text{g/ml}$] and less.

The minimal inhibitory concentration (MIC) of GPK91 and GPK96 for *M. smegmatis* was determined at 0.781 $\mu\text{g/ml}$ and 3.125 $\mu\text{g/ml}$, respectively (Table 4.3). The MIC is designated when a 99.9% reduction in colony-forming units (CFU) occurs at a specific compound's concentration compared to the control. The MIC results of GPK91 were lower compared to the previous results observed by Slayden et al. (2000) for TLM, and the range was between 2.5 $\mu\text{g/ml}$ – 5.0 $\mu\text{g/ml}$ on the agar plate. GPK96 MIC result was comparable with the previous results observed by Slayden et

al. (2000) for TLM. Slayden et al. (1996) also showed that TLM possesses in vivo antimycobacterial activity against the saprophytic strain *M. smegmatis* MC2155 and the virulent strain *M. tuberculosis* Erdmun, resulting in complete inhibition of growth on solid media at 75 and 25 µg/ml respectively (Slayden et al., 1996).

The results showed that GPK91 might act as an InhA protein inhibitor by targeting the GLY₉₆, ALA₁₉₈, and ILE₂₉₄ amino acid residuals that could result in the downregulation of the *inhA* gene. The results also predicted that GPK96 might act as a KasA protein inhibitor by targeting the MET₂₁₃, THR₃₁₅, THR₃₁₃, and Pi-Pi:PHE₄₀₄ amino acid residues that could result in the downregulation of the *kasA* gene. With similarity to TLM in the structure, together GPK91 and GPK96 showed selective activity on the specific binding sites of InhA and KasA proteins therefore proving to be the only two that could be developed into potential novel anti-tuberculosis drug candidates due to their high binding energy towards these amino acids. Both compounds had the highest binding energy at the binding sites of InhA and KasA proteins. The minimum inhibitory concentration values showed that GPK91 can induce inhibition of *M. smegmatis* at a lower concentration than GPK96. Further studies could determine the mechanism of action and conduct clinical trials to assess the antimicrobial efficacy and toxicity of the drug candidates to render personalized patient treatment with a high potential of improving treatment outcome and prognosis, effectively reducing drug burden that would otherwise cause adverse side effects of the current treatment.



Chapter 1

Introduction

1.1 Introduction

Tuberculosis (TB) is primarily a pulmonary disease but can also disseminate to other parts of the body like bones, kidneys, and brains, and causes miliary tuberculosis. It affects almost one-third of the world's population and is caused by *M. tuberculosis* bacterium (Appendix 1) (WHO Report, 2022). Tuberculosis is a highly contagious disease able to disarm its primary host cell, the macrophage. Once the bacteria are inhaled via droplets, they spread through person-to-person contact. Macrophages can phagocytose and kill the bacilli, but if they are not killed during that initial interaction, they can proliferate within dendritic cells and alveolar macrophages at a rapid rate, signaling the production of interleukin (IL)-1a, IL-1b, and other host proinflammatory cytokines (Fogel, 2015). Infected macrophages cluster together to form a granuloma and trigger an immune response (Cox et al., 2019). Inside the granuloma, *M. tuberculosis* organisms evade immune clearance and manifest into latent tuberculosis (Cox et al., 2019).

Within the population of tuberculosis-infected patients in South Africa alone, an estimated 7.1 million South Africans are living with human immunodeficiency virus (HIV) (Cornell et al., 2017). Approximately 10.6 million new cases of tuberculosis were reported in 2021, of which 6.7% were living with HIV (WHO Report, 2022). Over 500,000 cases were caused by multidrug-resistant tuberculosis (Becerra et al., 2018). There are an estimated 30,000 new cases of children who develop multidrug-resistant tuberculosis (MDR-TB) annually, and out of those, only 10% - 15% are diagnosed and receive appropriate treatment globally, and this leads to high morbidity and mortality (Schaaf et al., 2021). South Africa has the highest number of HIV-positive populations in the world (Cornell et al., 2017). Currently, there is no cure for HIV infection, and the presence of the virus in the body is a source of many complications and susceptibility to tuberculosis. The presence of HIV is a risk factor for developing tuberculosis because HIV within granuloma kills the clusters of differentiation 4 (CD4) T-cell lymphocytes, and that is associated with the reactivation of tuberculosis (Shankar et al., 2013; Lawn et al., 2002). Because of this, it becomes a daunting challenge to manage tuberculosis when there is possible drug-drug interference of TB drugs with antiretroviral (ARV) drugs.

The current standard of care for drug-susceptible *M. tuberculosis* infection is a two-month intensive phase of a four-drug cocktail containing rifampicin (RIF), isonicotinylhydrazine [(isoniazid) (INH)], pyrazinamide (PZA), and ethambutol (EMB). It is followed by a longer 12-week continuation phase of RIF and INH to fight residual bacteria that have entered a dormant, slow-replicating latent phase (Hoagland et al., 2016; WHO Guidelines, 2021). The anti-tuberculosis (anti-TB) therapy targets for tuberculosis treatment are the three main proteins of the causative organism, namely, fatty-acid enoyl-acyl carrier protein reductase (InhA), β -ketoacyl-ACP synthase (KasA) and a complex of an acyl carrier protein (Sharma et al., 2015). The *inhA* and *kasA* genes encode InhA and KasA enzymes that are required for the synthesis of mycolic acid. The cytokine tumor necrosis factor (TNF) is another therapeutic target for tuberculosis because of its ability to promote either cell survival, apoptosis, or necroptosis depending on the cellular context (Stutz, 2018). Kar et al. (2019) proposed that in order to disturb the essential reduction step in the mycobacterial mycolic acid biosynthesis, the entry of natural substrate could be blocked by inhibitors such as triclosan. Triclosan does not require prior activation to bind with *M. tuberculosis* as isoniazid does (Kar et al., 2019). Triclosan is a diphenyl ether derivative that targets mycobacterial enoyl acyl carrier protein reductase (ENR) at 12.5 $\mu\text{g/ml}$ against *M. tuberculosis* (Kar et al., 2019).

The multidrug-resistant tuberculosis is defined by WHO, as an infection by *M. tuberculosis* strain that is resistant to at least one of the first-line treatment drugs, rifampicin and/or isoniazid (Maharaj et al., 2016). One of the problems in treating tuberculosis is noncompliance of the patients to anti-TB treatment, and hence do not achieve the required therapeutic outcome, putting them at risk of developing drug resistance to anti-TB drugs. There are 40% and 60% mortality rates for multidrug resistance tuberculosis (MDR-TB) and extensively drug-resistant tuberculosis (XDR-TB), with an increase in the number of patients with programmatically incurable tuberculosis (Govender et al., 2018). The disease and the emergence of multidrug-resistant variants of *M. tuberculosis* have opened the doors for researchers to find novel drug targets. The need to develop new drugs to fight MDR-TB has been compounded by the emergence of XDR-TB and coinfection with HIV. Another problem faced by the current TB treatment is the adverse side effects caused by the drugs because when transported through the body via blood circulation, the majority

of the molecules do not reach their intended targets, and remain in the body. Evidence has shown that *M. tuberculosis* degrades host cell cholesterol during persistence (Pandey and Sassetti, 2008; Wiperman et al., 2014) and that the inhibition of cholesterol degradation could provide a potential route to control the growth of *M. tuberculosis* in macrophages (Van Der Ven et al., 2015). Although the current research has shed light on TB diagnosis and treatment, there is still much to be discovered to effectively decrease the incidence of tuberculosis and eradicate it. It has been demonstrated that only little modifications are allowed on the carbon-5 position to result in an active analogue reminiscent of antimycobacterial when this side chain is removed and alkylation of the oxygen on position 4. The proper management of TB requires rapid identification and treatment with high efficacy and less toxic drugs. The delay in treatment may lead to the transmission and spread of drug-resistant *M. tuberculosis* strains.

Hypothesis I

When novel drug candidates of thiolactomycin (TLM) derivatives effectively bind to the InhA and KasA enzymes of *M. tuberculosis*, they would result in the downregulation of *inhA* and *kasA* gene expression. It would render the pathogen unable to synthesize mycolic acids, thus surrendering further cell wall biosynthesis. That would subsequently cause the weakening of the cell membrane and wall structures until bacterial death occurs.

The mycolic acids of the bacterial cell wall are essential for the survival of *M. tuberculosis* within the host cell. The inhibitors of the deoxyribonucleic acid (DNA) replication of its genome will affect the antiphagocytotic properties of the microorganism itself. Thiolactomycin (TLM) is a natural inhibitor of type II fatty-acid synthase (FAS-II), and KasA is an enzyme required in the FAS-II step for the biosynthesis of mycolic acid. The time lag from the time that TLM is absorbed to the time it is activated, allows the organism the opportunity to mutate into a drug-resistant strain that gives rise to MDR-TB and later XDR-TB. It is during this activation step that the *inhA* genes mutate. The mutations occur spontaneously during DNA replication due to polymerase errors. There are no studies done yet on the downregulation of the expression of the genes responsible for the biosynthesis of the proteins responsible for the mycolic acids of the bacterial cell wall.

1.2 Aim of the Study

The aim of the study was to design drug compounds from TLM derivatives that target and bind to InhA and KasA to down-regulate the *inhA* and *kasA* gene expression that encode these enzymes responsible for the synthesis of mycolic acid of the *M. tuberculosis* cell wall.

1.3 Objectives of the Study

- Create a construct sequence for the *inhA* and *kasA* genes of *M. tuberculosis* H37Rv strain using nucleotide Basic Local Alignment Search Tool search on the National Center for Biotechnology Information.
- Synthesize methoxyl protonated aspalathin analog via C-glucosylation of pentamethoxy dihydropropane
- Synthesize racemic TLM compound and construct derivative drug compounds therefrom.
- Find the native ligand binding sites for TLM derivatives that have strong binding capacity to the InhA and KasA protein respectively, using the ligand-based bioinformatics tool PubChem.
- To determine the minimum inhibitory concentration (MIC) of GPK91 and GPK96 using *Mycobacterium smegmatis* (*M. smegmatis*) MC2155 strain as a surrogate for *M. tuberculosis* H37Rv strain.

1.4 Ethical Approval

Even though *M. smegmatis* is a non-pathogenic microorganism, if the conditions are permitting, it may still cause disease. All procedures performed in this study involved potentially infectious biological material and thus were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. The ethical approval for the research study was obtained from the Environmental and Biosafety Research Ethics Committee of the University of the Free State on 02 November 2019, with ethics number UFS-ESD2019/0064 (Appendix 2).

1.5 Rational and Motivation

Over the years, the development of antiretroviral therapy (ART) has redefined infection with human immunodeficiency virus as a manageable chronic disease. Studies have shown that HIV infection increases the risk of developing other medical complications like venous thromboembolism (VTE) (Esterly et al., 2013) and pulmonary tuberculosis. The management of HIV and tuberculosis remains a challenge due to the emergence of MDR-TB and XDR-TB (Kar et al., 2019). In a patient having a coinfection of HIV-TB, the viral replication and progression to acquired immunodeficiency syndrome (AIDS) are accelerated by TB, and in turn, the HIV infection speeds up the progression to active TB (Ticona et al., 2016). Tuberculosis is second only to HIV/AIDS in terms of high mortality rates. The drugs that inhibit the type II FAS pathway can be selectively toxic to *M. tuberculosis*, hence inhibition of the fatty acid synthesis pathway is the reasonable and logical choice to conduct research. Before the antiretroviral drugs can be excreted from the body via bile or renal filtrate, they must first be modified by the cytochrome P450 (CYP450) enzyme system in the liver because most of them are not water-soluble (Hughes et al., 2015).

Cytochrome P450 is an iron-containing hemeprotein (Gonzalez and Gelboin, 1992) of which the term “P450” is derived from the spectrophotometric peak at the wavelength of 450 nm where the maximum absorption of the enzyme when it is in the reduced state and complexed with carbon monoxide occurs. Human cytochrome Ps are located either in the inner membrane of mitochondria or in the endoplasmic reticulum of the liver cells and are primarily membrane-associated proteins (Berka et al., 2011). Many antiretroviral drugs cause drug interactions mediated via the same CYP enzymes, specifically CYP3A4, CYP2C9, and CYP2C19 (Esterly et al., 2013). Since these drugs have different effects on CYP450 liver enzymes, they may affect the management of tuberculosis in HIV-infected individuals (Jackson and Mokoena, 2017). Among antiretroviral drug classes, protease inhibitors inhibit CYP3A4, resulting in supratherapeutic toxicity, whereas non-nucleosides reverse transcriptase inhibitors generally induce CYP450 enzymes that result in the sub-therapeutic effect (Foy et al., 2014).

The rationale of the research study was to establish the mode of action of the inhibitory novel drug candidates, whereby after the identification and validation of the molecular target, a target-specific enhancement of the drug could be pursued to improve efficiency and reduce toxicity through the reduction of the length of treatment. The challenge faced by the study was the potential risk of *M. tuberculosis* developing drug resistance to the potential novel drug candidates because the pharmacokinetic pathways might have been similar to those of the drugs currently being used for tuberculosis treatment. Second-line anti-TB drugs have many adverse effects, some of which need regular monitoring assessments. Poor pharmacovigilance and poor adherence to treatment may lead to an increase in morbidity and death. Early diagnosis and appropriate treatment give the best outcome results only when patients are compliant with the treatment.

1.6 Implementation of Finding and Significant Contribution

Upon completion, the study would lead to obtaining a doctoral degree. One article has been published (section 3.10.3), and two more to follow in high-impact international peer-reviewed journals. The results of this research were disseminated to scientific and academic audiences for peer review. The decision on the total duration of treatment is informed by the severity of the disease, the extent of drug resistance, that is, to which medications are the strain resistant, the site of infection, and the response to treatment. Aligning with the government's strategy to combat tuberculosis, the study contributes to the implementation of newer policies for the management and treatment of tuberculosis. The novel drug should be able to reduce the duration of treatment, which will reduce cases of defaulting on the treatment. Currently, the biggest threat to global TB prevention and control is the increasing resistance of *M. tuberculosis* strains to the first-line anti-tuberculosis drugs. There are two main mechanisms through which drug resistance in tuberculosis occurs, primary or transmitted drug resistance, in which resistant strains are transmitted to a new host. Secondary or acquired drug resistance is the acquisition of drug resistance mutations to one or more drugs (Dookie et al., 2018). Resistance to rifampicin and isoniazid makes the treatment of MDR-TB difficult because the therapy with the available first-line and second-line drugs is less efficacious and more toxic (Ahmad et al., 2007).

The global prevention and elimination of TB is part of the WHO strategy whereby in the recent guidelines it indicated that preventative therapy should be considered in the high-risk household contacts of patients with drug-resistant TB. By developing novel drugs, the study also contributes towards improving health outcomes of long-term patient management and reducing the cost of patient care by increasing therapeutic efficacy and minimizing drug toxicity; being cost-effective by saving the laboratory and the patient unnecessary expenditures resulting from frequent patient follow-up clinic visits. With the novel drugs, treatment regimens could be better because of the improved compliance by patients and reduce the emergence of MDR-TB and XDR-TB.

Chapter 2

Literature Review

2.1 Introduction and Background

Mycobacterium tuberculosis was discovered in 1882 by a German microbiologist named Robert Koch (Fogel, 2015). There was still strong evidence at the time that tuberculosis was one of the leading causes of death in the world but the causative organism had never been seen. The name of the causative organism was derived from the tubercles that were seen in the lungs of an infected person. The presence of high surface lipid content in the cell wall of *M. tuberculosis* makes it very difficult to stain. Robert Koch employed the use of alkaline methylene blue together with Bismarck brown that stained only the tissue. The organisms appeared as bright blue, rod-shaped cells (Figure 2.1 a). Kock's method was the forerunner of the Ziehl-Nielsen stain, which is the modern-day stain for acid-fast bacteria. When stained with Auramine, a positive TB sample will show green fluorescence under the microscope (Figure 2.1 b). Apart from staining and identifying the organism, Koch also successfully isolated the organism after the development of the solid media, through culturing from various sources of human and animal origins (Madigan et al., 1997).

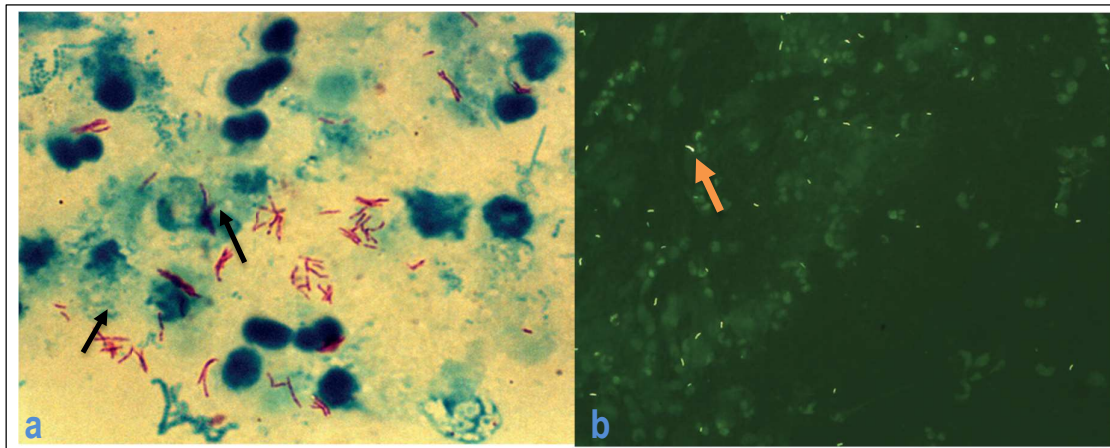


Figure 2.1: Microscopic appearance of *Mycobacterium tuberculosis* by Ziehl-Neelsen (a) and Auramine (b) stains. A positive TB sample has the presence of Gram-negative pink organisms (a), and a positive fluorescence (b).

Tuberculosis infection can be either classical pulmonary tuberculosis or extrapulmonary tuberculosis, which are tuberculosis meningitis and miliary tuberculosis. Each of these forms follows a different pathogenesis and presents with different symptoms that are associated with diverse host responses to the pathogen. HIV-infected individuals have higher cases of extrapulmonary tuberculosis reported

than immunocompetent individuals (Shankar et al., 2013; Sharma et al., 2005). Previous studies have shown that the most common site of extrapulmonary tuberculosis infection among HIV-infected individuals is the lymph nodes (Shanmuganathan et al., 2013), and it occurs most commonly in HIV patients with low CD4+ T cell counts.

Tuberculosis infection can be either latent or active. Latent tuberculosis means the patient has the infection but is asymptomatic and cannot transmit the infection to others, whereas active tuberculosis means the patient is symptomatic and capable of transmitting the infection to others (Figure 2.2). Approximately 5% of patients develop active tuberculosis within two years of exposure, and in approximately 10% of HIV-infected patients, tuberculosis develops within one year (Murray et al., 2016).

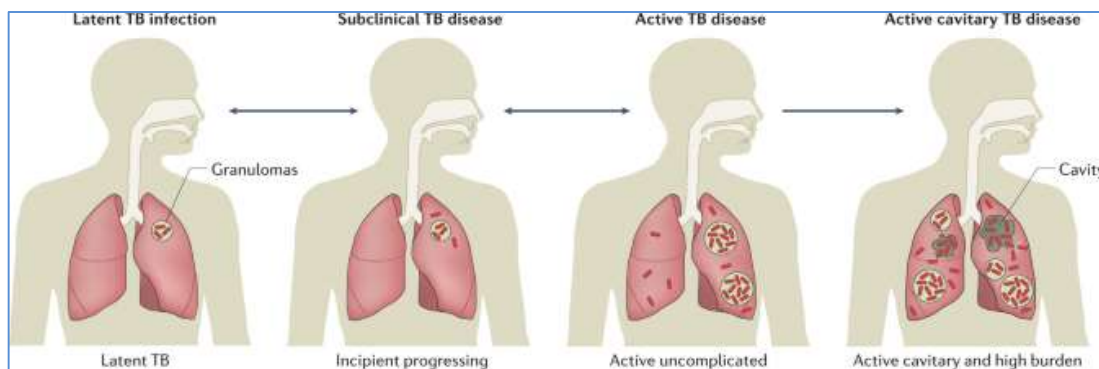


Figure 2.2: Different stages of TB infection and its progression.

The presence of HIV-tuberculosis coinfection in some patients brings about HIV-related dysfunction of the immune system, which renders the ability of the granuloma in those patients incapable of restraining the tubercle bacilli, thereby leading to the eventual multiplication and dissemination of the bacilli that result in severe pathology (Shankar et al., 2013). The increased replication of HIV within activated CD4+ T cells and macrophages at the site of *M. tuberculosis* is associated with the exacerbation of tuberculosis pathology (Imperiali et al., 2001).

Early research discovered tuberculin and Bacillus-Calmette Guérin (BCG) vaccines in 1890 and 1908, respectively (Fogel. 2015). BCG has some limitations concerning HIV-exposed individuals who can develop a disease called BCGosis (Bustamante et al., 2007). There are different types of drug-resistant tuberculosis, with the first

outbreaks of multidrug-resistant *M. tuberculosis* reported in 1990 in patients with acquired immunodeficiency syndrome. Management of MDR-tuberculosis is a challenge in South Africa (SA) because in most cases, the outcomes are suboptimal results, and these strains are untreatable because they are resistant to fluoroquinolones and at least one of the second-line drugs (Pontali et al, 2016) (Table 2.1).

Table 2.1: Definition of types of drug-resistant tuberculosis (Schaaf et al., 2021)

Type of drug-resistant TB	Definition
Mono-drug resistant TB	<i>M.tuberculosis</i> strain resistant to a single first-line anti-TB drug
Polydrug-resistant TB	<i>M.tuberculosis</i> strain resistant to two or more first-line anti-TB drugs but not both isoniazid and rifampicin
Multidrug-resistant TB (MDR-TB)	<i>M.tuberculosis</i> strain resistant to both isoniazid and rifampicin with or without resistance to other drugs
Pre-extensively drug-resistant TB	<i>M.tuberculosis</i> strain resistant to isoniazid, rifampicin plus a fluoroquinolone
Extensively drug-resistant TB (XDR-TB)	<i>M.tuberculosis</i> strain resistant to isoniazid, rifampicin, a fluoroquinolone plus either bedaquiline or linezolid (Previous definition: resistance to second-line injectable agents)

The inhibition of the main proteins of *M. tuberculosis* is the basis of the current therapy targets for tuberculosis treatment. A novel drug developed by Janssen Pharmaceuticals in the United States of America (USA) is called bedaquiline (BDQ) and belongs to the diarylquinoline group (Pontali et al., 2016). This drug is characterized by the quinolinic central heterocyclic nucleus, with alcohol and amine side chains attributing to the drug's antimycobacterial properties (Pontali et al., 2016). Bedaquiline targets bacterial energy metabolism by inhibiting its adenosine triphosphate (ATP) synthase, and it is active on drug-resistant *M. tuberculosis* isolates and drug-susceptible strains (Pontali et al., 2016). Bedaquiline was granted conditional approval in the USA (2012) and Europe (2014) for use in MDR-TB. In a randomized, placebo-controlled trial, 24 weeks of bedaquiline treatment significantly increased the culture conversion rate in patients with MDR-TB (Pym et al., 2016), but clinical resistance to BDQ has been identified. It is necessary to have a conceptual and clear understanding of tuberculosis to find a better treatment through novel and collaborative research and public health efforts.

Mycobacterium smegmatis was discovered soon after the discovery of *M. tuberculosis* by Lustgarten in 1884. It has similar characteristics to *M. tuberculosis* and more than 2000 homologous genes that they share. It is also an acid-fast bacillus with a thick cell wall that can be stained by Ziehl-Neelsen and Auramine stains. *M. smegmatis* is a non-pathogenic organism that could be used for research analysis of other pathogenic *Mycobacterium* species because it grows faster when compared to *M. tuberculosis*.

2.2 Structure and Resistance

Mycobacterium tuberculosis is a Gram-positive acid-fast bacillus that causes tuberculosis in humans. The mycobacterial cell envelope is the dominant feature of the biology of *M. tuberculosis*. The particular chemical structures and organization of the lipids between the inner and the outer membrane of the mycobacterial cell membrane account for most of its impermeable ability to biocides and for the unique staining properties that help in the diagnosis of an infected specimen (Jackson, 2014). This property is due to the presence of unique lipid components called mycolic acids found on the surface of the mycobacterial cell. The cell wall of *M. tuberculosis* contains a peptide-glycan to which it is covalently bound, and arabinose-galactose-mycolic acid polymer, a lipid-polysaccharide-peptidoglycan complex that confers the hydrophobic characteristic on the mycobacterial cell surface (Figure 2.3).

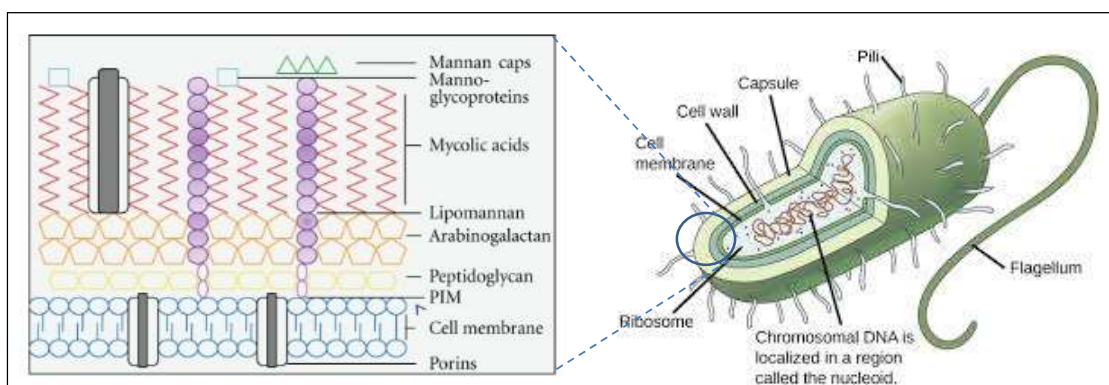


Figure 2.3: The structure of *M. tuberculosis* cell wall: a schematic representation of the major components of the cell wall and their distributions. The inner layer is composed of peptidoglycan that is covalently linked to arabinogalactan layer. The outer membrane contains mycolic acids, glycolipids like (mannose-capped) lipomannan, and mannoglycoproteins.

These unique features in the cell envelope are critical in the virulence and persistence of *M. tuberculosis* (Marrakchi et al., 2014). *M. tuberculosis* has three

main types of mycolate acids: alpha-, methoxy-, and keto-mycolic acid (Sharma et al., 2015) (Figure 2.4). Mycolic acids are the group of complexed branched-chain 2-alpha-alkyl, 3-beta-hydroxyl lipids existing in two main forms in the mycobacterial cell; either as unanchored trehalose or glycerol ester or as anchored mycolic acid, which is linked to the arabinogalactan component of the cell wall (Shaw et al., 2017).

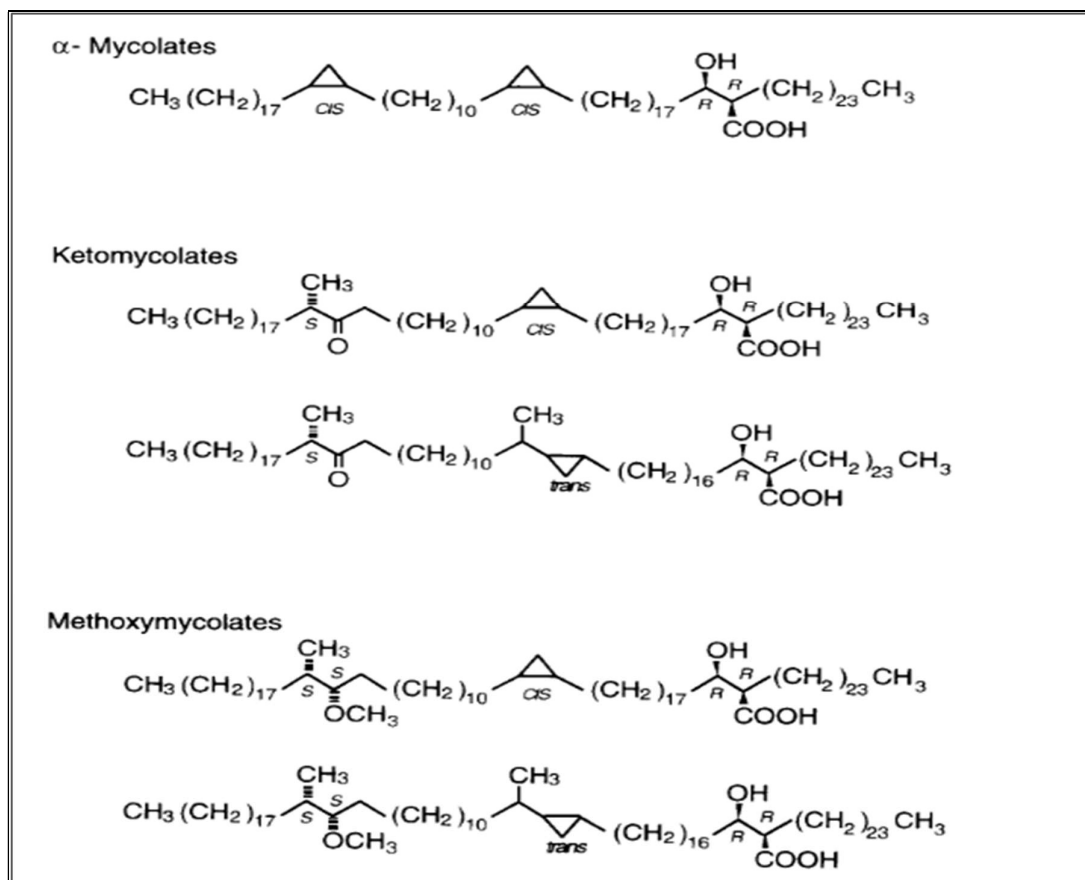


Figure 2.4: The three main mycolate acids of *M. tuberculosis*: alphamycolates, ketomycolates, and methoxymycolates

Both forms play an important role in the structural impermeability of the cell envelope, taking part in the two leaflets of the mycobacterial outer membrane called the mycomembrane (Marrakchi et al., 2014). The alpha mycolic acids of *M. tuberculosis* contain 74 – 80 carbon atoms with two double bonds of *cis*-cyclopropyl groups located in the meromycolic chain (Marrakchi et al., 2014). The ability of *M. tuberculosis* to survive through long-lasting periods in the host macrophages depends on breaking down host cell lipids as the carbon source where the nutrients are scarce inside the formed granulomas (Cox et al., 2019). The fatty acid is synthesized by *M. tuberculosis* via the type II pathway where enoyl-ACP reductase

(ENR) is a regulatory enzyme of the type II FAS pathway (Kar et al., 2019). The final step of the type II fatty acid elongation cycle is catalyzed by the enzyme ENR (Kar et al., 2019). All designed and predicted drug candidates must be tested in vitro to confirm their ability to penetrate the cell wall.

M. tuberculosis has complex lipids of long chains that form a waxy coat around the organism, which is most likely what prevents antibiotics from entering the organism (McLean et al., 2009). *M. tuberculosis* has several cytochrome P450 (MTCYP450) that metabolize fatty acids. MTCYP450 14 α -sterol demethylase (CYP51) in particular, catalyzes the biosynthesis of cholesterol, and during the catalytic cycle the substrate undergoes three successive monooxygenation reactions that result in the formation of 14-hydroxymethyl, 14-carboxaldehyde, and 14-formyl derivatives, followed by elimination of formic acid with the introduction of a 14,15 double bond (Podust et al., 2001). Various azole drugs that inhibit fungal CYP51 through heme iron coordination are also potent inhibitors of mycobacterial growth, which suggests that one or more MTCYP450s may be azole targets (McLean et al., 2009). Cytochrome P125 (CYP125) and cytochrome P142 (CYP142) are also present in *M. tuberculosis*, and CYP125 may be relatively resistant to macrophage-generated nitric oxide because earlier studies of the protein interactions of the MTCYP125 with nitric oxide have shown that its ferrous-nitric oxide complex was relatively labile (McLean et al., 2009; Ouellet et al., 2009). Various MTCYP450s that bind to econazole and other azoles include CYP121, CYP51B1, and CYP130 (McLean et al., 2009).

2.3 Pathogenesis and Immunity

Mycobacterium tuberculosis is an intracellular pathogen with the ability to establish lifelong infection in its host target (Murray et al., 2016). Tuberculosis can spread in three ways that are as a result of inoculation via cuts, the ingestion of infected food, and more commonly, through inhalation of organism particles in the air. *M. tuberculosis* can invade any organ but is more commonly acquired via inhalation of aerosolized bacilli into the respiratory tract. As alveolar macrophages and Langerhans cells in the mucosal lining of the airways pick up the organisms, they process them along transit to the regional lymph nodes to initiate the adaptive

immune responses (Shankar et al., 2013). When organisms penetrate the alveoli, they are phagocytized by alveolar macrophages, but with *M. tuberculosis*, instead of being lysed by the lysosomes, *M. tuberculosis* prevents fusion of the phagosome with them by blocking the specific bridging molecule called early-endosomal autoantigen-1 (EEA1) (Murray et al., 2016).

During primary infection, there are no significant symptoms. Granulomas or tubercles are hard masses formed around the organisms by the body's immune system, allowing tuberculosis to go into a latent stage where it lays dormant in the host tissues for years (Cox et al., 2019). Granulomas limit the replication of the organism, but *M. tuberculosis* persists within the granuloma. The phagosome fuses with other intracellular vesicles instead, permitting the tuberculosis pathogen access to nutrients and facilitating intravacuole replication in this latent state. Phagocytized microorganisms of *M. tuberculosis* survive macrophage lysis by also catalytically catabolizing the nitric oxide oxidants that are formed, mediated via MTCYP125, through protein-nitric oxide interaction that forms a labile ferrous-nitric oxide complex (McLean et al., 2009; Ouellet et al., 2009). Granuloma prevents further spread of the organism, and when a small antigenic burden is present during macrophage stimulation, the granuloma becomes small, and therefore the organism is destroyed with minimal tissue damage. However, when there is a high antigenic burden, large necrotic granulomas are formed and become encapsulated with fibrin that effectively protects the mycobacterial organisms from being killed by macrophages, and any event that distresses the integrity of the granuloma results in the reactivation of the latent tuberculosis infection (Murray et al., 2016).

When the immune response is triggered, the macrophages secrete interleukin (IL)-12 and tumor necrosis factor (TNF)- α . These two cytokines activate the chemotaxis of T-cells and natural killer cells to the infected area and induce T-cell differentiation into thymus-derived helper-1 (TH1) cells that secrete interferon-gamma (INF- γ) (Murray et al., 2016). In post-primary infection, the dormant *M. tuberculosis* organisms are reactivated because of the weakening immune system. This reactivation leads to lymphohematogenous dissemination and the onset of severe active tuberculosis marked by Ghon focus (Ghon focus is a primary lesion that develops in the lung of a nonimmune host caused by *Mycobacterium bacilli*). It

involves hilar lymph nodes and caseous necrosis, especially in the apical segment of the lungs owing to poor air-pumping mechanisms and lymphatic distribution (Shankar et al., 2013). The INF- γ activates macrophages to increase the phagosome-lysosome interaction and intracellular killing. TNF- α stimulates the production of nitric oxide that enhances alveolar macrophage apoptosis (Keane et al., 2000). In a state of a weakened immune system, patients with low levels of INF- γ or TNF- α have functional defects in the receptors of these cytokines and are at high risk for severe progressive infection (Murray et al., 2016). The patient becomes symptomatic and clinically presents with fatigue, weight loss, persistent cough with green or yellow sputum, and hemoptysis.

2.4 TB Treatment and Resistance

First-line anti-TB drugs

2.4.1 Rifampicin

Rifampicin is a derivative of rifamycin, and it was introduced as an antituberculosis agent in 1972 (Blanchard, 1996). It is one of the most effective anti-TB antibiotics, and together with isoniazid, constitutes the basis of the multidrug treatment regimen for tuberculosis. Rifampicin binds to the β -subunit of the RNA polymerase to inhibit the elongation of messenger RNA in *M. tuberculosis* (Blanchard, 1996). Most of the *M. tuberculosis* strains that are rifampicin-resistant carry mutations in the *rpoB* gene that codes for the β -subunit of the RNA polymerase. This results in a decrease in the affinity of the β -subunit of the RNA polymerase for the drug, which leads to the development of drug resistance (Telenti et al., 1993). The dormant bacilli have slow in vitro growth but active transcriptional activity even when cell division has ceased. Rifampicin and pyrazinamide have great activity against dormant *M. tuberculosis*. A high dose of rifampicin kills most non-actively replicating bacteria (Alnimr, 2015) but there is a proportion of those persisters that can resist even high doses of rifampicin in vitro (Alnimr, 2015).

In about 96% of *M. tuberculosis* isolates resistant to rifampicin, there are mutations in the so-called “hot-spot region” of 81-base pairs spanning codons 507–533 of the *rpoB* gene. This region is also known as the rifampicin resistance-determining region (Ramaswamy and Musser, 1998). In most studies, mutations in codons 516, 526,

and 531 are the most commonly associated mutations with rifampicin resistance (Caws et al., 2006). Some reports have noted the occurrence of mutations outside the hot-spot region of *rpoB* even though it is less frequent (Heep et al., 2000; Siu et al., 2011). Cross-resistance with other rifamycins can occur. In some codons, mutations have been associated with low-level resistance to rifampicin but are still susceptible to rifamycins, such as rifabutin or rifalazil (Cavusoglu et al., 2004). That is important for TB patients who receive antiretroviral therapy since rifabutin is a less effective inducer of the cytochrome P450 CYP3A oxidative enzyme (Burman and Jones, 2001) while on the other hand, monoresistance to rifampicin is quite rare, with almost all rifampicin-resistant strains resistant to other drugs also, especially to isoniazid, and this is the reason why rifampicin resistance is considered a surrogate marker for MDR-TB (Traore et al., 2000). Genome sequencing studies have uncovered the acquisition of compensatory mutations that occur when the fitness loss after one mutation, is corrected by its epistatic interaction with a second mutation at a different site in the genome. The mutation in *rpoA* and *rpoC* resulted in the loss of fitness encoding α and β' subunits of RNA polymerase, in rifampicin-resistant strains after subsequent mutations in *rpoB* corrected it (Comas et al., 2011).

2.4.2 Isoniazid

Isoniazid (INH) was discovered in 1952 to have significant bactericidal activity against *M. tuberculosis*, the causative agent of tuberculosis (Bernstein et al., 1952; Fox, 1952). After being introduced as an anti-TB agent, it was adopted as a front-line antibiotic in the treatment of tuberculosis, and together with rifampicin, they remain the basis for the treatment of the disease. Biosynthesis of some cell envelope lipids is the site of action of anti-TB drugs such as isoniazid and ethionamide (Jackson, 2014). Unlike rifampicin, isoniazid is only active against metabolically active replicating bacilli. Also known as an isonicotinic acid hydrazide, isoniazid is a pro-drug that requires activation by the catalase/peroxidase enzyme KatG, encoded by the *katG* gene in order to exert its effect (Zhang et al., 1992). Isoniazid acts by inhibiting the synthesis of mycolic acids through the NADH-dependent enoyl-acyl carrier protein (ACP)-reductase, encoded by the *inhA* gene (Rawat et al., 2003). Although simple in its structure, resistance to this drug has been associated with mutations in several genes, such as *katG*, *inhA*, *ahpC*, *kasA*, and *NDH*. The two main molecular mechanisms of isoniazid resistance are associated with gene mutations in

katG and *inhA* or its promoter region, and studies have found mutations in these two genes as the most commonly associated with isoniazid resistance (Ramaswamy et al., 2003). Among these, the most prevalent gene mutation has been identified as S315T in the *katG* gene, resulting in an isoniazid product deficient in forming the isoniazid-NAD adduct needed to exert its antimicrobial activity (Vilchèze and Jacobs, 2007). This mutation has been consistently associated with high-level resistance to isoniazid (Fenner et al., 2012) and occurs more frequently in MDR strains (Hazbón et al., 2006). The second most common mutation occurs in the promoter region of *inhA*, causing overexpression of *InhA*, or less frequently, a mutation in its active site, which decreases its affinity for the isoniazid-NAD adduct (Rozwarski et al., 1998).

The most prevalent mutation found is at position -15C/T and is more commonly associated with low-level resistance to isoniazid. Mutations in the *inhA* gene cause resistance to isoniazid and the structurally related drug ethionamide, which shares the same target (Larsen et al., 2002). A recent study found that a mutation in the *inhA* regulatory region and the *inhA* coding region, produced high-level isoniazid resistance and cross-resistance to ethionamide (Machado et al., 2013). One recent interesting finding showed that the 4R isomer of the isoniazid-NADP adduct causes inhibition of the dihydrofolate reductase (*DfrA*) in *M. tuberculosis*, suggesting that mutations in *dfrA* could possibly play a role in resistance to isoniazid (Argyrou (a) et al., 2006). Furthermore, an analysis of the proteome of isoniazid targets in *M. tuberculosis* identified that 16 other proteins, in addition to *InhA* and *DfrA*, were bound by these adducts with high affinity, which could signal that there may be other actions of isoniazid on the bacteria not yet clearly defined (Argyrou (b) et al., 2006). Two studies, however, have failed to identify any mutation in *dfrA* associated with resistance to isoniazid (Ho et al., 2009; Wang et al., 2010). In *M. tuberculosis*, *ahpC* encodes an alkyl hydroperoxidase reductase that is implicated in resistance to reactive oxygen intermediates, and it was initially proposed that mutations in the promoter region of *ahpC* could be used as proxy markers for isoniazid resistance (Rinder et al., 1998). It is now better understood that mutations in the promoter of *ahpC* are compensatory for the loss of catalase/peroxidase activity rather than the cause of isoniazid resistance (Sherman et al., 1996). Moreover, overexpression of *AhpC* does not confer resistance to isoniazid (Heym et al., 1997). Other studies have found single nucleotide polymorphisms in the other genes in isoniazid-resistant

clinical isolates of *M. tuberculosis*, including *kasA* and the *oxyR-ahpC* and *furA-katG* intergenic regions (Hazbón et al., 2006; Cardoso et al., 2007; Ando et al., 2011). However, their direct role as a cause of isoniazid resistance has not been fully demonstrated. Another study has found that a silent mutation in *mabA* conferred isoniazid resistance through upregulation of *inhA* in *M. tuberculosis* (Ando et al., 2014).

2.4.3 Thiolactomycin

TLM is an antibiotic compound that was first isolated in 1982 by Oishi and co-workers from a strain of actinomycetes belonging to the genus *Nocardia* found in a Japanese soil sample (Oishi et al., 1982). It contains thiolactone moiety in its molecule. The thiolactone ring is attached to a long hydrocarbon chain, and the ring itself contains a thiol group (-SH) and a lactone functional group (-C=O). The hydrocarbon chain is made of carbon atoms with hydrogen attached (Figure 2.5).

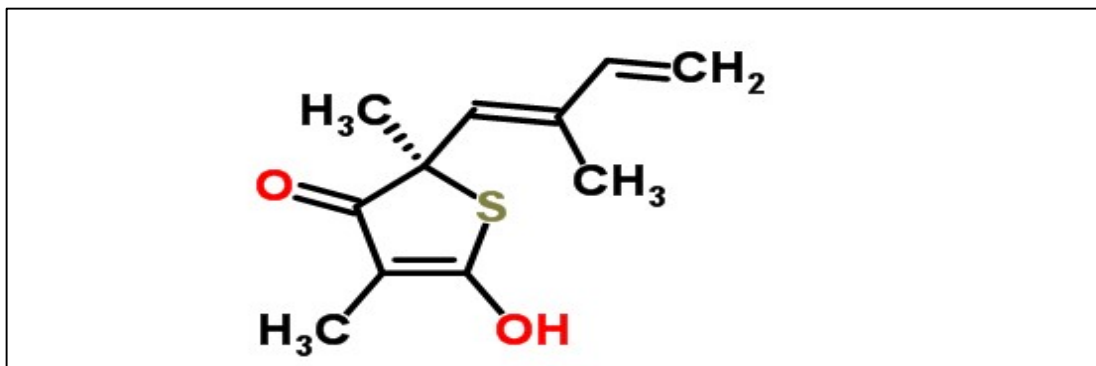


Figure 2.5: Chemical structure of thiolactomycin compound.

At the time of its discovery, TLM exhibited a broad antibacterial spectrum activity against *Salmonella*, *Serratia*, and *Bacteroides* as well as weak toxicity (Oishi et al., 1982) by the inhibition of mycolic acid synthesis (Price et al., 2001). It is a natural inhibitor of KasA that binds to this enzyme when it is in the free and acylated forms. TLM is a unique natural thiolactone molecule that reversibly inhibits FAS type II but not type I (Price et al., 2001) by targeting the beta-ketoacyl-acyl carrier protein (ACP) synthases (Luckner et al., 2009). A methyl on carbon 3 confers a selectivity of inhibition for FAS-II enzymes. It mimics malonyl-ACP in the FabB active site and forms a strong hydrogen bond with the two catalytic histidines and the unsaturated alkyl side chain (Price et al., 2001). The compound was at the origin of many structure-activity studies aimed at designing bacterial fatty acid synthase inhibitors.

The efficacy of TLM against *M. smegmatis* and *M. tuberculosis* would later provide the prospects of identifying fatty acid and mycolic acid biosynthetic genes and revealing a novel range of chemotherapeutic agents directed against *M. tuberculosis*. The advantages of new TB drugs based on this compound are their physical and pharmacokinetic properties like low molecular weight (MW 210g/mol, high water solubility, good oral absorption, and low toxicity profile in mice (de Souza, 2009).

Thiolactomycin (TLM) is a unique thiolactone that exhibits anti-mycobacterial activity by inhibiting fatty acid and mycolic acid biosynthesis (Luckner et al., 2009). It targets two β -ketoacyl-acyl-carrier protein synthases, KasA and KasB, consistent with the fact that both enzymes belong to the fatty-acid synthase type II system involved in fatty acid and mycolic acid biosynthesis (Luckner et al., 2009). Overexpression of KasA, KasB, and KasAB in *Mycobacterium bovis* BCG increased *in vivo* and *in vitro* resistance against TLM (Abrahams et al., 2016). In addition, a multidrug-resistant clinical isolate was also found to be sensitive to TLM, indicating promise in counteracting multidrug-resistant strains of *M. tuberculosis* (Abrahams et al., 2016). The design and synthesis of several TLM derivatives have led to compounds more potent both *in vitro* against fatty acid and mycolic acid biosynthesis and *in vivo* against *M. tuberculosis* (Schroeder et al., 2002). A three-dimensional structural model of KasA has been generated to improve understanding of the catalytic site of mycobacterial Kas proteins and provide a more rational approach to the design of new drugs (Kremer et al., 2000).

2.4.4 Pyrazinamide

Pyrazinamide was introduced into TB treatment in the early 1950s and constitutes now part of the standard first-line regimen to treat the disease. Pyrazinamide is an analogue of nicotinamide, and its introduction allowed reducing the length of treatment to six months. It has the characteristic of inhibiting semi-dormant bacilli residing in acidic environments like that found in TB lesions (Mitchison, 1985). Pyrazinamide is also a pro-drug that needs to be converted to its active form, pyrazinoic acid, by the enzyme pyrazinamidase/nicotinamidase coded by the *pncA* gene (Konno et al., 1967; Scorpio and Zhang, 1996). The proposed mechanism of action of pyrazinamide involves the conversion of pyrazinamide to pyrazinoic acid, which disrupts the bacterial membrane energetics-inhibiting membrane transport.

Pyrazinamide enters the bacterial cell by passive diffusion, and after conversion to pyrazinoic acid, it is excreted by a weak efflux pump. Under acid conditions, the protonated pyrazinoic acid is reabsorbed into the cell and accumulated inside due to an inefficient efflux pump, resulting in cellular damage (Zhang and Mitchison, 2003).

One study has also found that pyrazinoic acid and its n-propyl ester can inhibit the fatty acid synthase type I in replicating *M. tuberculosis* bacilli (Zimhony et al., 2000; Zimhony et al., 2007). A recent study, however, has challenged the previous model by proposing that pyrazinoic acid inhibits trans-translation, a process of ribosome-sparing in *M. tuberculosis* (Shi et al., 2011). The study was performed on pyrazinamide-resistant strains that lacked mutations in *pncA* but had mutations in *rpsA*, identifying the ribosomal protein 1 (RpsA) as the proposed target. Overexpression of RpsA conferred increased resistance to pyrazinamide, and pyrazinoic acid was confirmed to be bound to RpsA (Shi et al., 2011). Mutations in the gene *pncA* remain the most common finding in pyrazinamide-resistant strains. These mutations are scattered throughout the gene, but most occur in a 561-bp region in the open reading frame or an 82-bp region of its putative promoter (Scorpio et al., 1997; Juréen et al., 2008). Some studies have reported the occurrence of pyrazinamide-resistant strains without any mutation in *pncA*, stating that the resistance could be due to mutations in another not yet identified regulatory gene (Cheng et al., 2000). Based on that evidence, the contribution of mutations in *rpsA* to pyrazinamide resistance remains limited (Alexander et al., 2012; Simons et al., 2013; Tan et al., 2014). Pyrazinamide can be used in longer MDR-TB regimens only if susceptibility is confirmed.

2.4.4 Ethambutol

In 1966, EMB was introduced as the treatment of TB and is part of the current first-line regimen to treat the disease (Takayama et al., 1989). EMB was used with the three other combination standard first-line drugs as an alternative drug for streptomycin for TB treatment (Ahmad et al., 2007). The prevalence of resistance of *M. tuberculosis* isolates to EMB is lower than that for streptomycin (Ahmad et al., 2007). EMB is bacteriostatic against multiplying bacilli interfering with the biosynthesis of arabinogalactan in the cell wall. In *M. tuberculosis*, the genes *embCAB*, organized as an operon, code for arabinosyl transferase, which is involved

in the synthesis of arabinogalactan, producing the accumulation of the intermediate D-arabinofuranosyl-P-decaprenol (Mikusová et al., 1995). The recognized mechanism of resistance to ethambutol has been linked to mutations in the gene *embB*, with mutations at position *embB306* as the most prevalent in most of the studies performed (Telenti et al., 1997; Sreevatsan et al., 1997). Other studies have also found mutations in *embB306* in ethambutol-susceptible isolates (Ahmad et al., 2007). A study on the large number of *M. tuberculosis* isolates found that mutations in *embB306* were not necessarily associated with resistance to ethambutol but with a predisposition to develop resistance to an increasing number of drugs, and be transmitted (Hazbón et al., 2005). Allelic exchange studies have shown that individual mutations causing some amino acid substitutions produced ethambutol resistance, while other amino acid substitutions had little or no effect on ethambutol resistance (Safi et al., 2008). The same authors have recently reported that mutations in the decaprenyl-phosphoryl-B-D-arabinose (DPA) biosynthetic and utilization pathway genes, *Rv3806c* and *Rv3792*, together with mutations in *embB* and *embC* accumulate, giving rise to a range of MICs of ethambutol depending on mutation type and number (Safi et al., 2013). These findings could have an influence on the correct detection of ethambutol resistance by current molecular methods. Mutations in *embB306* cause variable degrees of ethambutol resistance and are required but are not enough to cause high-level resistance to ethambutol (Ahmad et al., 2007). There remain about 30% ethambutol-resistant strains that do not present any mutation in *embB*, stressing the need to identify other possible mechanisms of drug resistance to this drug.

2.4.5 Streptomycin

Originally isolated from the soil microorganism *Streptomyces griseus*, streptomycin was the first antibiotic to be successfully used against TB. It was identified in 1943 by Waksman and co-workers (De Souza, 2009). As soon as it was prescribed, resistance to it emerged, which was a result of being administered as monotherapy (Crofton and Mitchison, 1948). Streptomycin is an aminocyclitol glycoside active against actively growing bacilli, and its mode of action is by inhibiting the initiation of the translation in the protein synthesis (Moazed and Noller, 1987). More specifically, streptomycin acts at the level of the 30S subunit of the ribosome at the ribosomal

protein S12 and the 16S rRNA coded by the genes *rpsL* and *rrs*, respectively (Finken et al., 1993).

Consequently, mutations in *rpsL* and *rrs* are the main mechanisms of resistance to streptomycin, but account for 60%–70% of the resistance found (Gillespie, 2002). Among the mutations reported in *rpsL*, a substitution in codon 43 from lysine to arginine has been the most commonly reported. This mutation produces high-level resistance to streptomycin. In *rrs*, the most common mutations occur around nucleotides 530 and 915. There remains a significant percentage of strains resistant to streptomycin that lack mutations in either of these two genes, suggesting additional mechanisms of resistance. In the past years, it has also been reported that mutations in *gidB*, a gene encoding a conserved 7-methylguanosine methyltransferase specific for the 16S rRNA, confers low-level resistance to streptomycin (Okamoto et al., 2007; Spies et al., 2008).

Second-line anti-TB drugs

2.4.6 Fluoroquinolones

Fluoroquinolones are currently in use as second-line drugs in the treatment of MDR-TB. Both ciprofloxacin and ofloxacin are synthetic derivatives of the parent compound nalidixic acid, discovered as a by-product of the antimalarial chloroquine (Goss et al., 1965). Newer-generation quinolones such as moxifloxacin and gatifloxacin were evaluated in clinical trials and proposed as first-line antibiotics to shorten the length of TB treatment (Rustomjee et al., 2008; Palomino and Martin (a), 2013). The mode of action of fluoroquinolones is by inhibiting the topoisomerase II (DNA gyrase) and topoisomerase IV, two critical enzymes for bacterial viability. These proteins are encoded by the genes *gyrA*, *gyrB*, *parC*, and *parE*, respectively (Fàbrega et al., 2009). In *M. tuberculosis*, only type II topoisomerase (DNA gyrase) is present and, thus, is the only target of fluoroquinolone activity (Aubry et al., 2004). Type II topoisomerase is a tetramer formed by two α and β subunits, coded by *gyrA* and *gyrB*, respectively, which catalyzes the supercoiling of DNA (Takiff et al., 1994). One of the mechanisms of developing fluoroquinolone resistance in *M. tuberculosis* is by developing chromosomal mutations in the quinolone resistance-determining region of *gyrA* or *gyrB*. The most frequent mutations found are at positions 90 and 94 of

gyrA but mutations at positions 74, 88, and 91 have also been reported (Cheng et al., 2004; Sun et al., 2008). A recent systematic review of fluoroquinolone resistance-associated gyrase mutations in *M. tuberculosis* has been published (Maruri et al., 2012).

One interesting finding in *M. tuberculosis* is the presence of a natural polymorphism at position 95 in *gyrA* not related to fluoroquinolone resistance since it is also found in fluoroquinolone-susceptible strains (Musser, 1995). Another interesting finding has been about the report of the simultaneous occurrence of mutations T80A and A90G in *gyrA* led to the hypersusceptibility to several quinolones (Aubry et al., 2006). This finding could point out that the problem of fluoroquinolone resistance in *M. tuberculosis* might be more complex than initially thought. Cross-resistance is assumed to occur between fluoroquinolones, even though isolated reports have acknowledged the presence of strains resistant to gatifloxacin and moxifloxacin that were still susceptible to ofloxacin (Von Groll et al., 2008). In addition the involvement of efflux mechanisms has been suggested as a possible cause of fluoroquinolone resistance in *M. tuberculosis* (Escribano et al., 2007).

2.4.7 Kanamycin, Capreomycin, Amikacin, Viomycin

The four antibiotics have the same mechanism of action of inhibiting the protein synthesis, though kanamycin and amikacin are aminoglycosides, while capreomycin and viomycin are cyclic peptide antibiotics. All four are second-line drugs used in the management of MDR-TB. Kanamycin and amikacin inhibit protein synthesis by alteration at the level of 16S rRNA. The most common mutations found in kanamycin-resistant strains are at positions 1400 and 1401 of the *rrs* gene, conferring high-level resistance to kanamycin and amikacin. However, mutations at position 1483 have also been reported (Alangaden et al., 1998; Suzuki et al., 1998). Full cross-resistance between kanamycin and amikacin is not complete, as previously thought. A study has shown variable levels and patterns of resistance, suggesting that other mechanisms of resistance might be possible (Krüner et al., 2003). In concordance with this, low-level resistance to kanamycin has been associated with mutations in the promoter region of the *eis* gene, encoding an aminoglycoside acetyltransferase (Zaunbrecher et al., 2009). Mutations at positions –10 and –35 of the *eis* promoter led to amikacin. These mutations were found in up to 80% of clinical isolates showing

low-level resistance to kanamycin (Zaunbrecher et al., 2009; Campbell et al., 2011). Capreomycin and viomycin on the other hand, have a similar structure and bind at the same site in the ribosome, at the interface of the small and large subunits (Stanley 2010). They show full cross-resistance reported in previous studies (McClatchy et al., 1977). Mutations in the *tlyA* gene have also been associated with resistance to capreomycin and viomycin. *TlyA* is an rRNA methyltransferase specific for 2'-O-methylation of ribose in rRNA. Mutations in *tlyA* determine the absence of methylation activity (Johansen et al., 2006). Although some studies did not find this association, a recent meta-analysis evaluating the association of genetic mutations and resistance to second-line drugs has confirmed the presence of *tlyA* mutations in addition to mutations in *rrs* and *eis* (Georghiou et al., 2012).

2.4.8 Ethionamide

Ethionamide is a derivative of isonicotinic acid that is structurally similar to isoniazid. It is also a pro-drug requiring activation by a monooxygenase encoded by the *ethA* gene. It interferes with the mycolic acid synthesis by forming an adduct with NAD that inhibits the enoyl-ACP reductase enzyme. EthA is regulated by the transcriptional repressor *EthR* (Carette et al., 2012). Resistance to ethionamide occurs because of mutations in *etaA/ethA*, *ethR*, and mutations in *inhA*, which cause resistance to both isoniazid and ethionamide (DeBarber et al., 2000; Brossier et al., 2011). Moreover, studies with spontaneous isoniazid- and ethionamide-resistant mutants of *M. tuberculosis* found that they map to the *mshA* gene, encoding an enzyme essential for mycothiol biosynthesis (Vilchèze et al., 2008).

2.4.9 Para-Amino Salicylic Acid

Although it was one of the first anti-tuberculosis drugs to be used in the treatment of the disease, together with isoniazid and streptomycin, para-aminosalicylic acid (PAS) is now considered a second-line drug part of the treatment regimens for MDR-TB. Until recently, its mechanism of action was not completely defined. It has been proposed that being an analogue of para-aminobenzoic acid, it must compete with it for dihydropteroate synthase, interfering in the process of folate synthesis. A study using transposon mutagenesis identified mutations in the *thyA* gene associated with resistance to PAS that were also present in clinical isolates resistant to PAS

(Rengarajan et al., 2004). A recent study has also identified various missense mutations in *folC* encoding dihydrofolate synthase that conferred resistance to PAS in laboratory isolates of *M. tuberculosis* (Zhao et al., 2014). In a panel of 85 clinical MDR-TB isolates, mutations in *folC* were identified in five isolates resistant to PAS. Nevertheless, less than 40% of PAS-resistant strains had mutations in *thyA*, indicating that other resistance mechanisms to the drug might exist (Mathys et al., 2009).

2.4.10 Cycloserine

Cycloserine is an oral bacteriostatic second-line anti-tuberculosis drug used in MDR-TB treatment regimens. It is an analogue of D-alanine that blocks the activity of D-alanine:D-alanine ligase in order to inhibit the synthesis of peptidoglycan. Cycloserine also inhibits the D-alanine racemase (AlrA) needed to convert L-alanine to D-alanine (Zhang, 2005). Even though the actual target of cycloserine in *M. tuberculosis* is not elucidated, in previous studies on *M. smegmatis* showed that overexpression of *alrA* led to resistance to cycloserine in recombinant mutants (Càceres et al., 2008). A more recent study has also shown that a point of mutation in *cycA* that encodes a D-alanine transporter was partially responsible for resistance to cycloserine in *M. bovis* BCG (Chen et al., 2012).

2.4.11 Thioacetazone

Thioacetazone was previously used for TB treatment because of its favourable in vitro activity against *M. tuberculosis* and cost-effective. The problem with the drug is its toxicity, especially in patients co-infected with HIV. It belongs to the Group 5 drugs of the WHO, and its mode of action is the inhibition of mycolic acid synthesis (Grzegorzewicz et al., 2012). TLM is a natural inhibitor of FAS-II pathway proteins. It inhibits KasA by binding to the enzyme if it is in both the free and acylated forms (Luckner et al., 2009).

2.4.12 Macrolides

Macrolides have limited activity against *M. tuberculosis* and are hence mostly recommended for treating other mycobacterial infections. One of them is clarithromycin, which is considered part of the Group 5 drugs of the WHO. The low cell wall permeability and the expression of the *emr37* are attributing to the intrinsic resistance of *M. tuberculosis* to macrolides. The *emr37* gene encodes for a methylase enzyme at a specific site of the 23S rRNA that inhibits the binding site of the drug. Study have shown that the intrinsic resistance was inducible with sub-inhibitory concentrations of clarithromycin, leading to a four- to eight-fold increase in MIC values (Andini and Nash, 2006). In another study performed with clinical isolates of *M. tuberculosis*, the sub-inhibitory concentrations of ethambutol reversed resistance to clarithromycin, indicating that a permeability barrier was the cause of the intrinsic resistance to the macrolides (Bosne-David et al., 2000).

2.4.13 Clofazimine

Clofazimine is a riminophenazine compound reported long ago as having anti-TB activity (Barry et al., 1957). Due to the availability of other effective anti-TB drugs at the time and some other undesirable side effects like pigmentation of the skin, its use was more limited to the treatment of leprosy (Browne and Hogerzeil, 1962). It is now considered one of the Group 5 drugs of the WHO for the management of MDR-TB. Until recently, the exact mode of action of clofazimine antibiotic was not clearly understood. However, a recent study has signaled the outer membrane as the possible target of clofazimine (Cholo et al., 2012). Another study found that in *M. tuberculosis*, clofazimine is reduced by NADH dehydrogenase, subsequently after spontaneous reoxidation liberates bactericidal levels of reactive oxygen species (ROS) (Yano et al., 2011). Resistance to clofazimine has not yet completely been characterized, however, a recent study has found that in spontaneous mutants of the reference strain H37Rv, mutations in the transcriptional regulator *Rv0678* caused an upregulation of MmpL5, a multisubstrate efflux pump, which not only caused resistance to clofazimine but also to bedaquiline (Hartkoorn et al., 2014).

2.4.14 Linezolid

Linezolid is also part of the category 5 drugs of second-line anti-TB drugs. It is an oxazolidinone originally approved for clinical use for treating skin infections and nosocomial pneumonia caused by Gram-positive bacteria (Leach et al., 2011). The mode of action of linezolid is to inhibit the initial steps in the synthesis of proteins by binding to the 50S ribosomal subunit (Zhang, 2005). Resistance to linezolid in *M. tuberculosis* is still unusual, but a study analyzing 210 MDR strains found that 1.9% of strains were resistant to linezolid (Richter et al., 2007). Further analysis of in vitro selected linezolid-resistant mutants found that strains with mutations in the 23S rRNA had MICs of 16 – 32 µg/ml, while strains with MICs of 4 – 8 µg/ml or susceptible strains showed no mutations (Hillemann et al., 2008). Another study using next-generation sequencing has also found the mutation T460C in *rp1C*, encoding the 50S ribosomal L3 protein, in in-vitro-selected mutants and clinical isolates of *M. tuberculosis* resistant to linezolid (Beckert et al., 2012). Previous studies have also found evidence of the possible involvement of efflux pumps in the resistance of *M. tuberculosis* to linezolid (Escribano et al., 2007).

Next-generation anti-TB drugs

2.4.15 Bedaquiline

Bedaquiline is the first drug in a new class of agents, the diarylquinolines, to be used for TB treatment. Bedaquiline is a new antibiotic belonging to the class of diarylquinolines with specific activity against *M. tuberculosis*, which has also shown in vitro activity against other non-tuberculous mycobacteria (Huitric et al., 2007). Bedaquiline was discovered after a high-throughput evaluation of thousands of compounds using *M. smegmatis* in a whole-cell assay (Andries et al., 2005). The drug showed in vitro and in vivo activity against *M. tuberculosis* and then entered into clinical evaluation for drug-susceptible and MDR-TB (Palomino and Martin (a), 2013; Rustomjee et al., 2008; Diacon et al., 2009). Based on the results from the two phase-II clinical trials, bedaquiline received conditional approval for the treatment of MDR-TB under the tradename Sirturo. However, a “black box” warning accompanied this authorization due to the reported unexplained deaths and QT interval prolongation. Recent reviews and evaluations of this drug have been published (Chahine et al., 2014; Palomino and Martin (b), 2013). A phase III clinical trial was scheduled to begin

in 2013 but has not yet started. Bedaquiline was also evaluated in new combination regimens to reduce the length of the treatment (Diacon et al., 2012). The mode of action of bedaquiline is through the inhibition of the ATP synthase of *M. tuberculosis*, whereby it was a completely new target for action for an antimycobacterial drug (Pontali et al., 2016), inhibiting bacterial respiration. The drug is active against dormant bacilli, an invaluable characteristic of MTB infection. This mode of action was discovered by analyzing *M. tuberculosis* and *M. smegmatis* mutants' resistance to bedaquiline. By sequencing the genome of these mutants and comparing them to that of the susceptible strains, the only mutation found was in the *atpE* gene, which encodes the c part of the F₀ subunit of the ATP synthase (Andries et al., 2005). It is a complex structure that generates the ATP needed by the mycobacterial cells (Koul et al., 2007), for which bedaquiline has a favoured specificity compared to mitochondrial ATP synthase (Haagsma et al., 2009).

The most prevalent mutation in the *atpE* gene found in bedaquiline-resistant mutants is A63P, but also I66M has been found. The latter introduces a modification that interferes with the proper binding of bedaquiline to its target (Petrella et al., 2006; Segala et al., 2012). Nevertheless, in the study to further assess the mechanisms of resistance to bedaquiline in *M. tuberculosis*, only 15 out of 53 resistant mutants were found to have had mutations in *atpE*. The other 38 strains lacked mutations in *atpE* or even in the F₀ or F₁ operons, which suggested that there were still other possible mechanisms of resistance (Huitric et al., 2010).

2.4.16 Delamanid

Delamanid is a derivative of nitro-dihydro-imidazooxazole with activity against *M. tuberculosis* that inhibits the synthesis of mycolic acid (Palomino and Martin (a), 2013). Delamanid was previously shown to have very good in vitro and in vivo activity against drug-susceptible and drug-resistant *M. tuberculosis* (Matsumoto et al., 2006), as well as good early bactericidal activity comparable to that of rifampicin (Diacon et al., 2011). Delamanid has also shown its safety and efficacy in clinical evaluation for MDR-TB (Gler et al., 2012). The specific mode of action of delamanid is to inhibit the mycolic acid synthesis, but it differs from isoniazid whereby it only inhibits methoxy- and keto-mycolic acid while isoniazid also inhibits α -mycolic acid (Matsumoto et al., 2006). Delamanid also requires reductive activation by *M. tuberculosis* to exert its

activity. In experimentally generated delamanid-resistant mycobacteria, a mutation was found in the Rv3547 gene, suggesting its role in the activation of the drug (Matsumoto et al., 2006).

2.4.17 Benzothiazinones

Benzothiazinone (BTZ) has antimycobacterial activity due to the presence of a compound called 2-[2-S-methyl-1,4-dioxo-8-azaspiro[4.5]dec8-yl]-8-nitro-6-trifluoromethyl-4H-1,3-benzothiazine-4-one (BTZ043). BTZ043 had in vitro, ex vivo, and in vivo activity against *M. tuberculosis* and also active against drug-susceptible and MDR clinical isolates of *M. tuberculosis* (Pasca et al., 2010). The mode of action of BTZ043 was initially observed at the cell wall biogenesis level using transcriptome analysis. Performing further genetic analysis using in vitro generated mutants, the target of the drug was identified at the level of the gene *rv3790*, which together with *rv3791* encode proteins that catalyze the epimerization of decaprenyl-phosphoryl ribose (DPR) to decaprenyl-phosphoryl arabinose (DPA), a precursor for arabinan synthesis needed for the bacterial cell wall (Mikusová et al., 2005).

Another study has characterized the mechanism of action of BTZ043 by showing that the drug is activated in the bacteria through the reduction of an essential nitro group to a nitroso derivative, which can react with a cysteine residue in DprE1 (Trefzer et al., 2010). In studies with *M. smegmatis*, the overexpression of a nitroreductase NfnB led to the inactivation of the drug by reducing an essential nitro group to an amino group (Manina et al., 2010). The lack of nitroreductases that reduce the BTZ043 by *M. tuberculosis* is a finding that could be important for developing new BTZ analogues with improved activity. A series of piperazine-containing BTZs have been reported. The lead compound PBTZ169 has improved activity, safety, and efficacy in animal models and has shown in vitro synergy with bedaquiline, signaling it as an attractive new candidate for further clinical development (Makarov et al., 2014).

2.5 The Role of InhA and KasA

2.5.1 Fatty-acid Enoyl-acyl Carrier Protein

Fatty-acid enoyl-acyl carrier protein (InhA) is an enzyme that is encoded by the *inhA* gene of *M. tuberculosis*. It is essential for the synthesis of mycolic acid in the organism's cell wall. In the last decade, InhA has remained the prime candidate for drug development, which has intensified research development on the inhibitors against this enzyme (Shirude et al., 2013). During the biosynthesis of fatty and mycolic acids of the organism's membrane and cell wall, InhA catalyzes the NADH-dependent reduction of enoyl-ACP. The reaction starts with a direct hydride transfer from the C4 of NADH to the β -carbon of the enoyl-ACP, followed by enolate anion formation (organic anion derived from the deprotonation of carbonyl compound), which is eventually protonated to the pro-(2R) position of the α -carbon (Vögeli et al., 2018) (Figure 2.6).

2.5.2 β -Ketoacyl-ACP Synthase

β -ketoacyl-ACP synthase (KasA) is an enzyme that is encoded by the *kasA* gene of *M. tuberculosis*. There is a close relationship between KasA and InhA enzymes. Both enzymes are essential for the biosynthesis of the mycolic acids of the cell wall of *M. tuberculosis*. The initial substrates of FAS-II are medium-length keto-acyl-ACP resulting from the condensation by the *M. tuberculosis* FabH protein of the acyl-CoA products of FAS-I with malonyl-ACP. After reduction by the β -keto-acyl-ACP reductase MabA, dehydration by the (3R)-hydroxyl-dehydratases HadAB and HadBC and reduction by the enoyl-CoA reductase InhA, either the β -ketoacyl-ACP synthase KasA or KasB catalyzes the condensation of the resulting product with malonyl-ACP units, thereby initiating the next round of elongation (Jackson, 2014). Methyltransferase and other enzymes modify the meromycetyl chain and introduce double bonds, cyclopropyl, hydroxyl, methoxy, and keto functionalities. These two chains are coupled via Claisen condensation by the acyl-AMP ligase FadD32 and the polyketide synthase Pks13. Upon release from Pks13, reduction by CmrA yields mycolic acids, which are then translocated to the periplasm under the form of trehalose monomycolate (TMM) by partially elucidated translocated machinery involving the essential integral membrane transporter, MmpL3, and attached to AG or another molecule of TMM to form trehalose dimycolate (TDM), by the antigen 85

complex (Jackson, 2014) (Figure 2.6). Meromycolic acids are the direct precursors of mycolic acids synthesized by type II fatty acid synthase (FAS-II) from acyl carrier protein-bound substrates (Veyron-Churlet et al., 2010). β -ketoacyl-ACP synthase is responsible for the condensation of the elongation chain with malonyl-ACP units that form meromycolyl-ACPs, which are the direct precursors of mycolic acids (Veyron-Churlet et al., 2010). The KasA is an essential enzyme in the FAS-II pathway because it catalyzes the condensation between the growing acyl chain and the malonyl-AcpM by devising a “ping-pong” mechanism (Luckner et al., 2009). In the FAS-II pathway, short fatty acids derived from the FAS-I pathway are elongated, and Acyl-CoA and malonyl-ACP are condensed by FabH, which is a Co-A dependent β -ketoacyl-AcpM synthase. The β -ketoacyl-AcpM reductase (MabA) and trans-2-enoyl-AcpM reductase then saturate the β -ketoacyl-AcpM to form β -enoyl-AcpM via sequential reactions. The β -ketoacyl-AcpM synthase then initiates the initial rounds of elongation of the fatty acid, which is completed by KasB β -ketoacyl-AcpM synthase involved in the final steps of mycolic acid formation (Luckner et al., 2009).

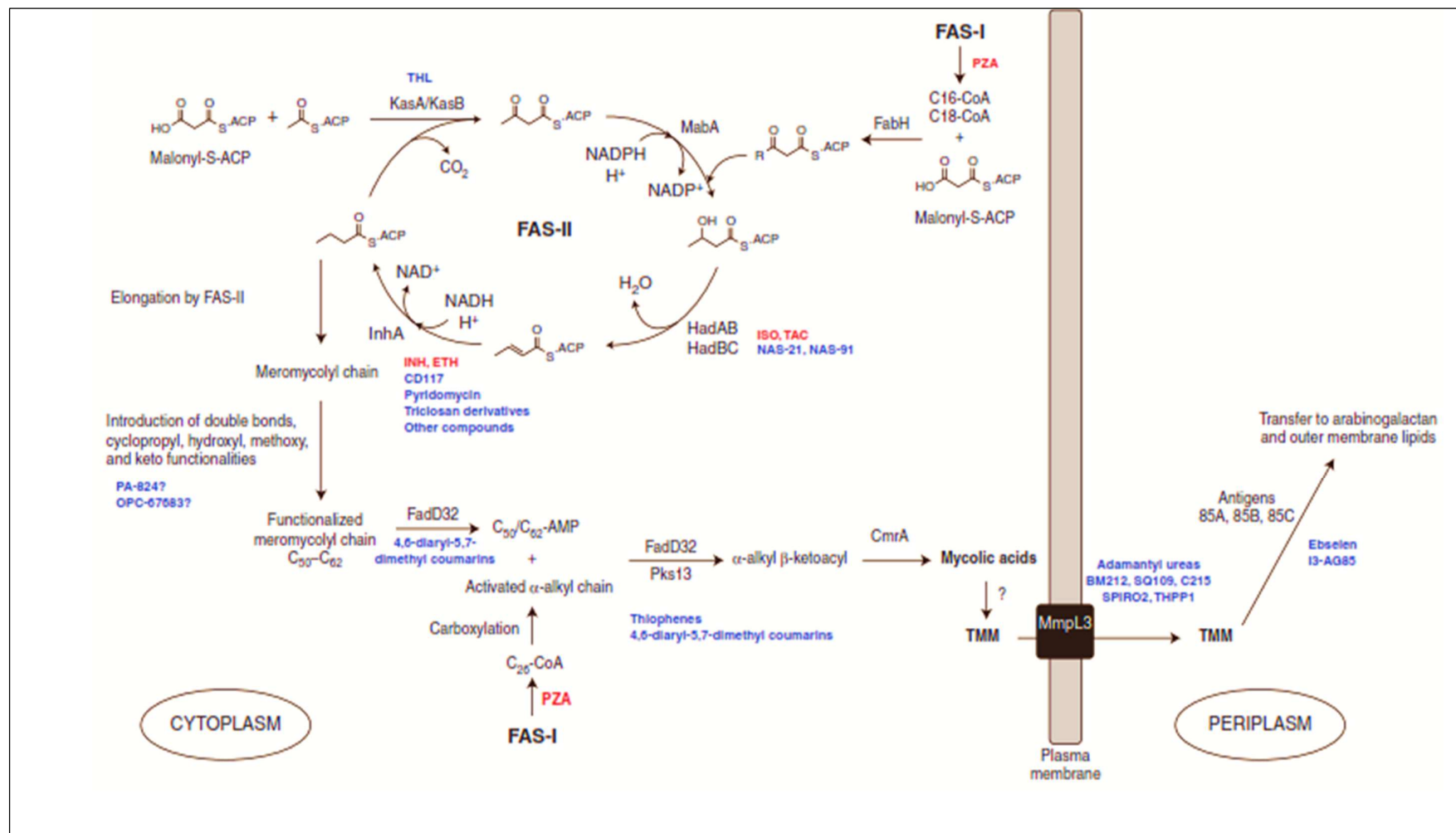


Figure 2.6: Biosynthetic pathway of mycolic acids in *M. tuberculosis* and site of action of anti-TB drugs. The saturated α -alkyl chain and the meromycolyl chain ($C_{50} - C_{62}$) are synthesized by fatty-acid synthase-I (FAS-I) and fatty-acid synthase-II (FAS-II), respectively. The site of action of *M. tuberculosis* inhibitors are indicated. Drugs presently of formerly used in the clinical treatment of TB are in red; inhibitors under development are in blue.

2.6 DNA Sequencing

2.6.1 Introduction

The hereditary information and biological properties of terrestrial life are encoded in an arranged order of nucleic acids in polynucleotide chains (Heather and Chain, 2016). DNA sequencing is the process of determining the exact order of nucleotides in the targeted region of the DNA (Venter et al., 2001). The three-dimensional structure of the DNA was solved by Watson and Crick in 1953 (Heather and Chain, 2016), however, the ability to sequence remained unsolved until Frank Sanger developed the first DNA sequencing method in 1977, where he reported his method for determining the order of nucleotides of DNA (Lu et al., 2016). All the sequence changes that may be present in a DNA fragment can be identified by this method for targeted mutation detection (Vulliamy et al., 2011). The specificity of the DNA fragment that is amplified is determined by the sequence of the primers used (Heather and Chain, 2016). A sequence of 17 – 22 base pairs is statistically likely to be unique in the human genome, and therefore, primers of this length and longer will anneal at only one specific place on a template of genomic DNA (Venter et al., 2001). This test uses DNA that has been amplified by PCR to detect gene mutations, inversions, or deletions that are associated with the presiding abnormality and may help to determine the treatment and/or prognosis of certain diseases (Vulliamy et al., 2011).

2.6.2 The Sequencing Process

With the current next-generation sequencing (NGS) technology, different strategies to prepare the sequence libraries into suitable templates, have been developed by different sequence platform vendors to detect the signal and ultimately read the DNA sequence (Buermans and den Dunnen, 2014). The PCR products derived from the DNA template are visualized by the methods of staining the amplified DNA product with a chemical dye such as ethidium bromide, which intercalates between the two strands of the duplex; labeling the PCR primers or nucleotides with fluorescent dyes before PCR amplification to allow the labels to be directly incorporated in the PCR product; and the agarose gel electrophoresis, which separates the DNA base on the size and charge (Garibyan and Avashia, 2013).

During DNA sequencing, the DNA chain is lengthened by DNA polymerase using deoxynucleotide triphosphates (dNTPs) as in the PCR, but a small proportion of labeled dideoxynucleotide triphosphates (ddNTPs) are included in the reaction mixture of the PCR to prevent any further extension of the chain (Vulliamy et al., 2011). The 3' hydroxyl group that is essential for the extension of DNA chains is absent from the ddNTPs and therefore cannot bond with the 5' phosphate of the next dNTP (Heather and Chain, 2016). Each of the ddNTPs is labeled with a different fluorochrome colour, which serves to identify the base (Buermans and den Dunnen, 2014), and so that the products of the sequencing reaction will consist of single-stranded DNA fragment that differs in size by one base pair (bp) (Vulliamy et al., 2011). The base pairs are identified by each of the four fluorescently labeled dNTP nucleotides (A, C, T, G), and the sequence is read by following the fluorescent signal per extension step for each cluster (Buermans and den Dunnen, 2014).

2.6.3 Application of DNA Sequencing

Sequencing has been evaluated for drug resistance surveillance. Apart from being used for prenatal testing for detecting trisomies (Buermans and den Dunnen, 2014) and drug resistance prediction, whole genome sequencing allows for differentiation of clinical isolates into major *M. tuberculosis* lineages and sub-lineages. A recent study showed that certain specific lineages are essentially resistant to certain anti-TB agents and, in the same way, acquire mutations at a higher rate and have been associated with MDR-TB resurgence (Telenti et al., 1993).

Giving a significantly too high number of reads that map to a particular chromosome is how trisomies reveal their presence in the chromosome (Buermans and den Dunnen, 2014). The genome sequences that may be associated with a disease or are predictive of response to medication, are identified by the personal genome sequencing. Thus, it is an important milestone to be capable of making critical correlations and connecting personalized genomes to personalized medicine (Wheeler et al., 2008).

2.7 Molecular Docking

Molecular docking is a method currently used in drug discovery and medicinal chemistry, providing information into molecular recognition through the analysis of the conformation and orientation of molecules into a binding site of a larger molecular target (Klauda, 2021). A docking method is a software tool that uses the graph-based signatures of a protein structural environment to assess the structural ramifications on drug binding affinity. The computer-generated 3D structure of small ligands is positioned into a receptor structure in different orientations and positions (Klauda, 2021). It is achieved by reducing the degree of freedom whereby the complexity of the two molecules binding together is reduced, allowing for an accurate estimate of molecule-molecule binding affinity (Klauda, 2021). Molecular docking is a tool used to predict the binding affinity of drugs by targeting specific proteins on a gene. The presence of single nucleotide polymorphism (SNP) in the gene protein can destabilize the effects of protein-drug binding affinity. The docking methods have been used as starting points for more extensive molecular dynamics simulations of ligands binding to the androgen receptor or initial conformations to calculate the smallest amount of mechanics of the catalytic cycle of nogalamycin monooxygenase essential for the biosynthesis of tetracycline antibiotics, bioluminescence of nanoluciferase-furimamide complex, and substrates in glutathione S-transferases (Klauda, 2021). Docking methods also allow for more accurate predictions of ligand poses and binding free energies (Klauda, 2021).

Molecular docking uses ligand-based bioinformatics, a principle based on the binding of an ion to an atom through coordinated bonding. Ligands are molecules or ions with a lone pair of electrons that attach themselves to ions with high positive charge density (Evangelista et al., 2019). It is not easy to determine the accurate binding affinity of a ligand to a receptor in computer-aided drug design. The most important quantity classification of the binding affinity between the receptor and the ligand is the binding free energy because it is directly related to the inhibition constant that can be measured experimentally using kinetic enzyme assay (Ho et al., 2020). Molecular docking is used in computer-aided drug design to select potential compounds from a large database because of its high speed, but it is not sufficiently accurate (Ho et al., 2020). Ho et al (2020) combined Jarzynski's Equality (JE) and the Hummer-Szabo formalism to demonstrate that the nonequilibrium binding free

energy is a good descriptor for binding affinity. It is, however, unclear if it is valid for other systems because the conclusion was obtained for only one data set, which included 23 ligands in complex with beta-lactamase protein.

Traditional docking methods, the lock-and-key methods, suffer from limitations of semi-flexible treatment of targets and ligands (Figure 2.7). There was the development of a different docking method, the induced-fit model, which incorporates protein-ligand flexibility where the receptor and ligands have to change their conformation to fit into each other (Fan et al., 2019) (Figure 2.7). Accurate binding pose predictions and more rational indications of protein binding interactions with the ligands are a result of flexible protein-ligand receptors. However, the traditional lock-and-key methods still give a more realistic representation of the behavior of protein in a solution. They can perform simultaneous exploration of binding modes and conformations that reveal unstable bound complexes and help identify new or filter out physically unreliable docking solutions (Fan et al., 2019).

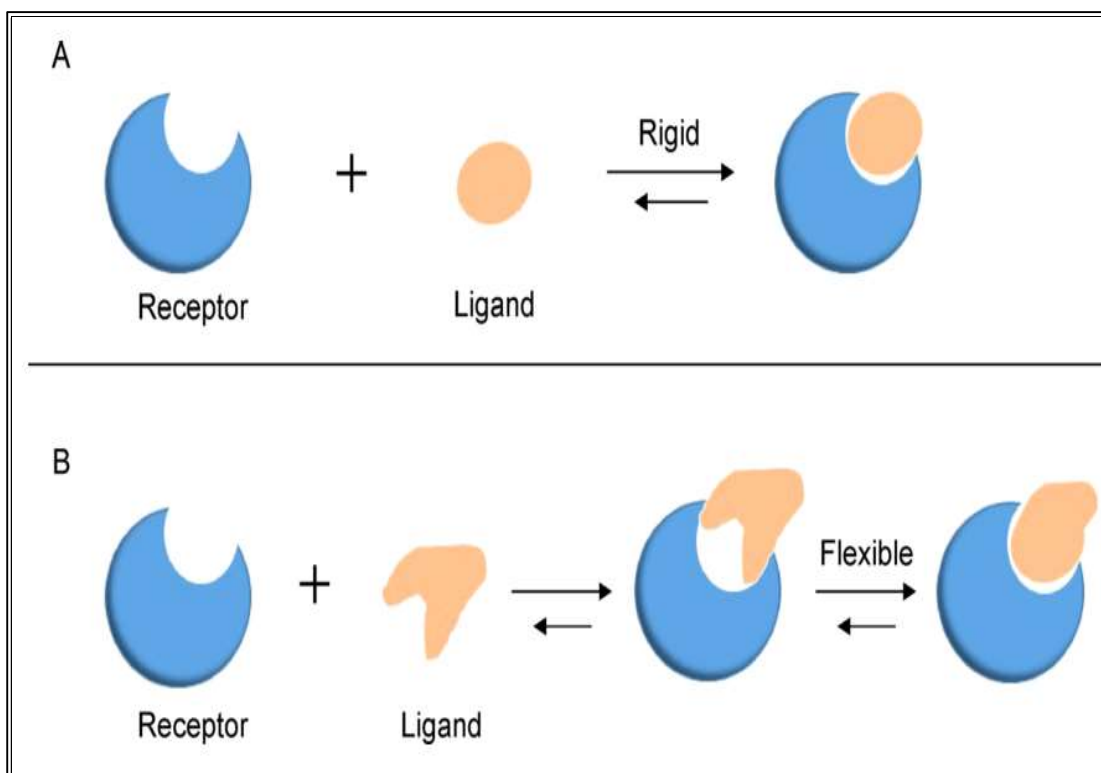


Figure 2.7: Docking methods for protein-ligand interaction. (A) Lock-and-key. (B) Induced fit method.

A 5-fluoro-1-(glucosyl) pyrimidine-2,4(1H,3H)-dione (GPK) is a chemical compound that can be employed as a ligand in drug discovery. It consists of a pyrimidine ring with a glucosyl group attached to carbon 1, a fluorine atom at carbon 5, and keto groups at positions 2 and 4 of the pyrimidine ring (Figure 2.8 a). The compound is a derivative of uracil, a naturally occurring pyrimidine found in nucleic acids. The addition of glucosyl group allows the compound to be used for research in drug development due to its potential biological activity when reacting with amino acids. Amino acids are essential building blocks of proteins and play an important role in protein synthesis. The basic structure of an amino acid consists of an amino group and a carboxylic acid group attached to a carbon. Each amino acid differs from another amino acid based on the composition of its R group (Figure 2.8 b) (Appendix 3). There are three main chemical properties that amino acids can be categorized based on their side chains: charge, hydrophobicity, and polarity. Few amino acids are referred to as special cases because their chemical property and role in specific processes are unique only to them. These properties affect amino acid's structure and the interaction with other compounds (Mustatea et al., 2019).

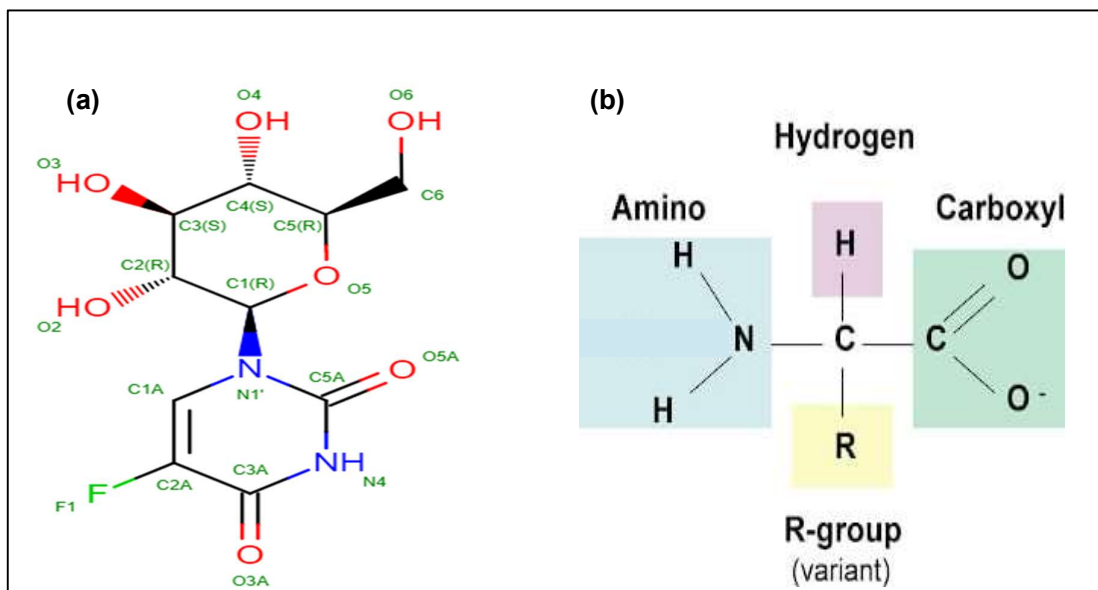


Figure 2.8: Chemical structures of GPK (a) ($C_{10}H_{13}FN_2O_7$) and amino acid (b) ($R-CH(NH_2)-COOH$).

2.8 Glucosylation

Glycoside is a molecule that contains a sugar moiety attached to another moiety through a glycosidic bond *via* its anomeric (C-1') carbon. The sugar moiety is known as glycone, and another moiety is the aglycone part of the glycoside. Glycosides can be linked *via* oxygen- (an O-glycoside), nitrogen- (α -glycosylamine), sulfur- (α -thioglycoside), or carbon- (α -C-glycoside) glycosidic bonds. Glycosides play essential biological functions, and as polymers, they store energy in plants, which serve as food sources for animals and humans (Isono, 1988). Oligomeric glycosides on mammalian cell surfaces play an essential role in the immune system. The aglycone moiety usually renders the glycoside more water-soluble. Thus, glycosylation makes metabolite toxins and other unwanted secondary metabolites in mammals water-soluble for excretion by the kidneys (Wang et al., 1998; Ayan et al., 2009).

Many secondary metabolites in plants are glycosides (Cirak and Radusiene, 2019). Some of these are toxins that protect the plant against herbivores. Plethoras of other essential activities in mammalian and human biology have been demonstrated (Isono, 1988; Giri and Singh, 2020). Flavonoids accumulate in plants as O-glycosylated derivatives, but several species, including the major cereal crops, predominantly synthesize flavone C-glycosides. These are stable for hydrolysis and biologically active in both plants and mammals. Activities ascribed to these plant secondary metabolites include them functioning as antioxidants (Fouda et al., 2021; Ramarathnam et al., 1989), insect-feeding attractants (Xiao et al., 2015), antimicrobial agents (Dinda et al., 2006), promoters of mycorrhizal symbioses (Akiyama et al., 2002) and UV-protective pigments (Middleton and Teramura, 1993).

3.1 Introduction

The cell wall structure of *M. tuberculosis* consists of mycolic acids, mainly facilitated by the enzymes InhA and KasA for their synthesis. A drug of interest, (R)-(+)-thiolactomycin with Lot number 22-THT-134-2, was purchased from Toronto Research Chemicals. All the procedures in the research were performed according to the written in-house standard operating procedures. These procedures included NCBI nucleotide BLAST, synthesis of racemic TLM, target screening, organism inoculation, and the determination of minimum inhibitory concentration.

The study was conducted in three stages; first, it was the nucleotide BLAST for gene (*inhA* and *kasA*) sequence. Secondly, it was the synthesis and screening of the compound molecules targeted against these proteins using docking study employing ligand-based bioinformatics. Thirdly, it was the determination of minimum inhibitory concentration. In this study, we used *M. smegmatis* as the surrogate because it is a non-pathogenic organism similar to *M. tuberculosis* in genome and structure and a perfect model to predict the outcome of *M. tuberculosis*. Working with *M. smegmatis* only required a biosafety level 1 laboratory because it was easy to work with.

3.2 Background

Tuberculosis is a contagious disease that affects one-third of the world's population, and while there is a new development in tuberculosis treatment, there are new cases of MDR-TB and XDR-TB that emerge. The World Health Organization has underlined the "End TB Strategy" in its priority action by emphasizing the need for new anti-TB drugs through intensified research and innovation (Pontali et al., 2016). The ability of the *M. tuberculosis* strain to go into a latent state inside the host cells, as well as to mutate into a multi/extremely-drug resistant strain, is a call for concern to researchers in this field. The hardest hit people are the people who have a low socio-economic background, live in dense and populated communities, and are HIV infected. Drug resistance may worsen further as there is still a high number of TB patients who default on their TB treatment and do not complete their treatment successfully. There is a critical need to develop a new novel drug to improve current regimens.

3.3 Study Design

- The study was applied experimental research to develop novel drugs that target and bind to InhA and KasA proteins.
- By creating a construct sequence for the *inhA* and *kasA* genes of the *M. tuberculosis* H37Rv strain using nucleotide BLAST search on the NCBI, we could find the native ligand binding sites for TLM derivatives that have the strong binding capacity to InhA and KasA proteins, respectively, using ligand-based bioinformatics tool PubChem.
- Isolated the *M. smegmatis* strain utilizing the Becton Dickinson (BD) BBL Bactec Mycobacteria Growth Indicator Tube (MGIT) bottles and then inoculated on Mueller Hinton agar (MHA) plates for determination of the MIC.

3.4 Sample Collection and Preparation

A medium consisting of 8.5 g/l of disodium phosphate, 1.0 g/l of ammonium chloride, 0.5 g/l of sodium chloride, 3.0 g/l of potassium dihydrogen phosphate, 14.2 mg/l of calcium chloride, 0.5 g/l of magnesium sulfate heptahydrate and 0.15 g/l of potassium chloride, was used to isolate the *M. smegmatis* strain. The medium was further supplemented with 10.0 ml of trace element solution that consisted of 4.0 mg/l of manganese sulfate, 0.4 mg/l of copper sulfate, 4.0 mg/l of zinc sulfate heptahydrate, 1.0 mg/l of potassium iodide, 2.0 mg/l of iron chloride hexahydrate, 5.0 mg/l of boric acid and 1.2 mg/l sodium permanganate per liter of medium. We used the standard sterile culturing techniques to culture and isolate the *M. smegmatis* strain and cultured it on the MGIT, a liquid media used to isolate *M. tuberculosis*.

The *inhA* and *kasA* gene sequences were subjected to BLAST analysis at NCBI against *M. tuberculosis*'s whole genome ribosomal RNA sequence to identify the closest homologs. Among the resulting hits, sequences with 100% identity homologs were selected. These strains were selected, and the H37Rv rRNA sequences were retrieved. Phylogenetic analysis was carried out using the maximum likelihood method based on the Tamura–Nei model. All positions in the sequence containing gaps and missing data were eliminated. The phylogenetic analysis showed the branch lengths, and the bacterial isolates identified in this study. Drug susceptibility testing was performed using MHA plates infused with different concentrations of

drugs of interest under supranational proficiency testing according to World Health Organization standards (World Health Organization 2009).

3.5 Environmental and Safety Precautions

The Laboratory Safety training was completed on a program called Occupational Health and Safety Information System (OHASIS). Universal safety precautions, aseptic technique, and Good Laboratory Practice guidelines to avoid contamination were observed for these procedures. All the work on the *M. smegmatis* isolate was performed in a Biohazard Safety Cabinet class II (BSC II) at the Microbiology laboratory at Universitas National Health Laboratory Service (NHLS), in Bloemfontein (Appendix 4). The laboratory health and safety regulations were observed throughout the research process, which included working in a biohazard safety cabinet and wearing personal protective equipment (PPE), i.e., protective eyewear, gloves, N95 mask, and a lab coat. The daily decontamination of all work surfaces was carried out when work was completed. The UV light was switched off before commencing work, and the working light was switched on. Fresh disinfectant (Aglusept IDP 700, 2%) and 1% sodium hypochlorite were made up daily and a bottle of 70% alcohol was available. The inside of the cabinet was wiped with 2% aglusept and 1% sodium hypochlorite, followed by 70% alcohol. A small sharps container and an autoclavable plastic bag for all solid (non-sharp) waste were placed in the cabinet. A 50 ml discard tube containing approximately 10 ml of neat 5% sodium hypochlorite was placed in the cabinet for discarding the liquid waste. A clean paper towel was laid down inside the cabinet to cover the workspace. Made sure that the paper towel was sufficiently soaked with disinfectant and that a bottle of diluted aglusept (disinfectant) was available in the cabinet to disinfect in the event of a spillage. Washed hands with recommended hand-wash soap when finished with the experiment for the day. Eating, drinking, and smoking are not allowed inside the laboratory. Biohazard signs are displayed at the lab entrance. In addition, an indemnity form was signed where it was required or mandatory.

3.6 Environmental and Resource Capacity

Environmental and resource capacity was crucial for the delivery of the outcome of the research study. It is the ability to execute core functions while linked to the achievement of the objectives of the study. During the experimental phase of the study, the researcher considered the reduction of environmental stress and minimized the human risk when working with the *M. smegmatis* MC2155 strain, by making available the proper disposing materials, personal protective equipment, and decontamination materials. The NHLS granted permission to conduct the MIC experiment at the Microbiology laboratory in Bloemfontein (Appendix 5).

The laboratory is equipped with required analyzers to carry out research studies and boasts some of the latest technologies that are conducive to conducting a good research study. Despite the availability of some of the latest technologies, there was a barrier that prevented the production of more solid reports. A lack of a level 3 biosafety cabinet mitigated the use of the *M. smegmatis* MC2155 strain instead of the *M. tuberculosis* H37Rv strain. There is an existing bilateral agreement that allows collaboration between the two universities. Permission from the head of the Next Generation Sequencing laboratory has been granted. The application for funding from the CUT Faculty Research and Innovation Committee was approved. The NHLS also funded the research. The facility operates 24 hours even though the core operational times are from 08:00 am to 05:00 pm, Monday to Friday. Access to the laboratory was granted to continue the experiments outside the core operational times.

3.7 Sample and Data Analysis

3.7.1 Gene Sequencing

Phylogenetic analysis was carried out using the maximum likelihood method based on the Tamura–Nei model. It is used to study the changing relationships between organisms or genes by constructing interactions and showing the ancestral relationships and divergence graphs among different species or genetic sequences. The Tamura-Nei (1993) model adjusts for multiple hits by considering variations in substitution rates among nucleotides and uneven distribution of nucleotide frequencies. The model discerns the transitional substitution rate, which involves

purines, from transversional substitution rates, which involve pyrimidines. Additionally, it posits uniform substitution rates across sites, Q is the proportion of transversional differences, and g_A , g_C , g_G , and g_T , are the respective frequencies of A, C, G, and T (Tamura and Nei, 1993).

3.7.2 Binding Site Identification

The bioinformatics tool, Protein Database Summaries (PDBsum), and ZINCPharmer are the online servers used to predict the binding positions of the developed novel drugs. The servers provide a detailed quantitative characterization of the interior cavities and surface pockets of proteins, which are prominent concave regions of proteins that are frequently associated with binding events. PDBsum database was used to find the native ligand-binding site for the given receptor. The InhA and KasA were downloaded and checked in PDBsum for the ligplot. Ligands bind to specific residues in a protein structure. The amino acid residue of the pocket of the protein that interacts with the ligand can be generated by a ligplot. The ligplot generates binding site information based on previously deposited ligand-protein interactions. It demonstrates the exact binding amino acid of the receptor with the ligand and shows the bond between them. Both receptors InhA and KasA were submitted to PDBsum to identify the interaction of these protein genes, and the coordinates of the interaction between protein and ligand were noted down. GPK, 5-fluoro-1-(beta-D-glucosyl) pyrimidine-2,4(1H,3H)-dione, (GPK-91, 92, 93, 94, 95, 96 and 97) were synthesized and used as ligands.

3.7.3 Docking Process

After the binding sites were identified for InhA and KasA proteins, ligands were docked at those positions to determine the interactions with the highest binding affinity. The first step of docking was structure determination on the protein molecules for receptor and ligand, obtained from ZINCPharmer that are 3FNE and 4C6X for InhA and KasA respectively. The chemical structure of the TLM drug was obtained from the PubChem compound database and was used as a ligand in this study. The second step was to use the LigPrep module to prepare the receptor-ligand structure, which generated accurate conformation of the compounds. The third step was a computational screen of the drug compounds to distinguish all the chemical and

structural properties that the compounds could have, that could affect the computational results. The last step was the prediction evaluation step in which after multiple runs, a cluster analysis was performed. The three-dimensional structure of the GPK ligands and the target protein were brought closer together to optimize the conformation and orientation. A search for an algorithm to find the possible binding modes was applied to predict the binding modes. The stable binding modes were obtained and their affinity scored. Scoring was obtained according to the protein-ligand binding energy.

3.8 Confidentiality

There were no human participants in this study; therefore, no informed consent was required. However, the results of this study will only be made available to the greater public after they have been presented and accepted by peer review.

3.9 Materials and Methods

3.9.1 Reagents

1. (R)-(+)-Thiolactomycin 25mg
2. 2.9% Sodium citrate
3. 4% Sodium hydroxide (NaOH)
4. 70% alcohol
5. BD Bactec Supplement kit
6. N-Acetyl-L-cysteine powder (NALC)
7. Phosphate buffer pH of 6.8
8. Sodium Hypochlorite
9. 2,3,4,-tri-O-benzyl acetate
10. Dichloromethane [(DCM) (CH_2Cl_2)]
11. Trifluoroacetic acid anhydride (TFAA)
12. Acetic acid (CH_3COOH)
13. Trifluoromethanesulfonic acid trimethylsilylester (TMSOTf)
14. Boron trifluoride estherate (BF_3OEt_3)
15. 50% sodium hydroxide (NaOH)
16. Acetonitrile (CH_3CN)
17. Methyl α -propionylpropionate

18. Pyridium bromide perbromide
19. Thioacetic acid
20. 3-ethoxy-2-methyl-2-propenal
21. Methylene triphenylphosphorane

3.9.2 Equipment

1. 37°C Incubator
2. Appropriate mycobacterial disinfectant (Aglusept)
3. BD Bactec MGIT instrument
4. BD Bactec MIGT tubes
5. Mueller Hinton agars
6. Centrifuge with an aerosol-tight rotor
7. Class II Biosafety Cabinet with UV light
8. Disposable powder free gloves
9. Falcon Tubes 50 ml
10. Finn tip pipettes 1000 µl
11. Glass pasture pipettes
12. Glass Test tubes 10x75
13. Nuclease free water/Sterile deionised Water
14. Paper towel
15. Pipette 10 µl and filter tips
16. Pipette 200 µl
17. Sterile glass pipettes
18. Timer
19. Vortex mixer
20. Waste disposable containers

3.10 Methods

3.10.1 Inoculation of the MGIT Tubes

3.10.1.1 *Intended Use*

The BD MGIT tube is used for the detection and recovery of mycobacteria. The tube contains 7 ml of modified Middlebrook 7H9 broth base supplemented with growth supplements for the rapid growth of mycobacteria. Albumin acts as a protective agent

by binding free fatty acids that may be toxic to *Mycobacterium* species. Dextrose is an energy source. Catalase destroys toxic peroxides that may be present in the medium. BD MGIT PANTA antibiotic mixture reduces contamination when supplemented before inoculation with the clinical specimens.

3.10.1.2 Principle of the Method

A fluorescent compound is embedded in silicone on the bottom of 16 X 100 mm round bottom tubes. The fluorescent compound is sensitive to oxygen dissolved in the broth. Initially, the large amount of dissolved oxygen quenches the emissions from the compound, and little fluorescence can be detected. Later, actively respiring microorganisms consume the oxygen and allow the fluorescence to be detected. Tubes entered into the BD Bactec MGIT instrument are continuously incubated at 37°C and monitored every 60 minutes for increasing fluorescence. Analysis of the fluorescence is used to determine if the tube is instrument-positive, i.e., the test sample contains a viable organism.

3.10.1.3 Procedure

A lyophilized vial of BD BBL MGIT PANTA antibiotic mixture was reconstituted with 15 ml of BD Bactec MGIT Growth Supplement. The MGIT tube was labeled with a unique number. Prior to specimen inoculation, the cap was removed and aseptically added 0.8 ml of Growth Supplement / BD BBL MGIT PANTA antibiotic mixture.

A 0.5 ml of the concentrated specimen suspension prepared was added to the MGIT tube and 0.1 ml onto the mycobacterium agar plate. The tube was recapped tightly and then mixed well. The tube was entered into the instrument and automatically tested for the duration of the 42-day testing protocol.

3.10.2 Screening the Targets for the InhA and KasA Proteins

3.10.2.1 Principle of the Test

Mycobacterium tuberculosis is known to synthesize alpha-, methoxy-, and ketomycolic acids. The InhA and KasA are the enzymes commonly involved in the synthesis of mycolic acid. The genes that encode these proteins are *inhA* and *kasA* respectively. A molecular interaction of the ligand binding sites of the complex chemical, TLM, for a given receptor on the InhA and KasA protein molecules, will

give an informative representation of the strength of the interaction including hydrogen bonds, hydrophobic interactions, and atom accessibilities. Nuclear Magnetic Resonance (NMR) techniques were used for the synthesis and characterization of benzyl-methoxy-protected aspalathin analog via C-glucosylation of pentamethoxy dihydropropane for drug synthesis of racemic TLM. NMR uses the magnetic field principle where a sample is exposed to radiofrequency radiation, the nuclei absorb energy and go into a state of nuclear spin. When the radiofrequency is turned off, the nuclei revert to their original state, releasing radiofrequency signals that are detected and analyzed to generate an NMR spectrum (Mlynarik, 2017).

3.10.2.2 Procedure

The chemical formula of TLM was imported from PubChem in the SDF file format. The docking studies were performed using Schrodinger Maestro 2018. The designed ligands were docked with InhA 3FNE and KasA 4C6CX, which were imported from the gene data bank. They were combined into a single molecule file in the Discovery Studio and then were submitted to the PharmaGist server to generate pharmacophores that use a ligand-based pharmacophore detection (http://bioinfo3d.cs.tau.ac.il/pharma/in_ex.html). Pharm Gist server functions by aligning a set of drug-like molecules that can bind to receptors and pharmacophore detection of ligands. The molecule was set as the first input molecule while submitting to the PharmaGist server, and the minimum number of pharmacophore features to “5”. The complex file of TLM was taken as a pivot molecule with five features, and the result was calculated each time. The pharmacophore alignment obtained from the uploaded complexes that showed the highest score was chosen for further analysis. These pharmacophores were then uploaded to the ZINCPharmer server (<http://zincpharmer.csb.pitt.edu/>). This server is free pharmacophore search software for screening the purchasable subset of the ZINC database.

The pharmacophores were analyzed using different subsets of the ZINC database like the Zinc Drug Database, Zinc in Man, and Zinc Drug Database (Metabolites). Finally, the ligands file for the uploaded pharmacophores was downloaded and the binding sites were identified by selecting the ligplot co-ordinates where the ligand shows interaction. The results were analyzed to select the appropriate binding site for our protein-ligand dockings. PDBsum database was used to find the native ligand binding site for the target receptor (<http://www.ebi.ac.uk/thornton-srv/databases/pdbsum/>).

The InhA and KasA proteins were downloaded and checked in PDBsum for the ligplot. Using the ligplot coordinates where the ligand can bind was estimated and noted down as X, Y, and Z axis, and the mean sum was calculated for the prediction of the active sites or the binding positions.

3.10.3 Synthesis and characterization of benzyl-methoxy-protected aspalathin analog via C-glucosylation of pentamethoxy dihydropropane for drug synthesis of racemic TLM

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Novel Synthesis of benzyl-Methoxyl Protected Aspalathin Analog *via* C-Glucosylation of Pentamethoxy Dihydropropane

Pravin Kendrekar ^{1*}, Mojalefa Setlai ¹, Sunil Tekale ², Rajita Ingle ², Chandrashekhar V. Kulkarni ³, Rajendra Pawar ^{2,*}

¹ Unit for Drug Discovery Research (UDDR), Department of Health Sciences, Central University of Technology, Free State (CUT), Private Bag X20539, Bloemfontein 9300, South Africa

² Department of Chemistry, Deogiri College, Aurangabad, 431005, Maharashtra, India

³ Centre for Smart Materials School of Natural Sciences, University of Central Lancashire, Preston PR12HE United Kingdom

* Correspondence: kkpravin@gmail.com (P.K.); rppawar@yahoo.com (R.P.);

Scopus Author ID 7003738785

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Abstract: The first report on a novel and efficient synthesis of benzyl-methoxy protected aspalathin derivative has been described via C-glucosylation of pentamethoxy dihydropropane. The synthesized compound was characterized by ¹H, ¹³C NMR, COSY, and HSQC techniques.

Keywords: aspalathin; glucolysation; glycoside.

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1. Introduction

Glycoside is a molecule that contains a sugar moiety attached to another moiety through a glycosidic bond *via* its anomeric (C-1') carbon. The sugar moiety is known as glycone and another moiety as the aglycone part of the glycoside. Glycosides can be linked *via* oxygen- (an *O*-glycoside), nitrogen- (a glycosylamine), sulfur- (a thioglycoside), or carbon- (a *C*-glycoside) glycosidic bond. Glycosides show important biological functions. As polymers, they are an important store of energy in plants that serve as food sources for animals and humans. Oligomeric glycosides on mammalian cell surfaces play an important role in the immune system. The glycone moiety usually renders the glycoside more water-soluble. Thus, glycosylation makes metabolite toxins and other unwanted secondary metabolites in mammals water-soluble for excretion by the kidneys. Many secondary metabolites in plants are glycosides. Some of these are toxins that protect the plant against herbivores. Plethoras of other activities in mammalian and human biology has been demonstrated [1-8]. Flavonoids normally accumulate in plants as *O*-glycosylated derivatives. However, several species, including major cereal crops, predominantly synthesize flavone *C*-glycosides. These are stable to hydrolysis and are biologically active both in plants and mammals. Activities ascribed to these plant secondary metabolites include them functioning as antioxidants [9-10], insect feeding attractants [11], antimicrobial agents [12], promoters of mycorrhizal symbioses [13], and UV-protective pigments [14].

2. Materials and Methods

Aspalathin (1a) (Figure 1) (3'- β -D-glucopyranosyl-2', 3, 4, 4', 6'-pentahydroxydihydrochalcone) is a dihydrochalcone C-glucopyranoside. It was first characterized by Koeppen [15] and co-workers in 1965. Notably is the β -stereochemistry at the anomeric carbon. It occurs exclusively in leaves of *Aspalathus linearis* (rooibos) where it is the major component. It has recently received considerable interest due to its plasma sugar lowering properties [16-17]. Nothofagen (1b) (3'- β -D-glucopyranosyl-2', 4, 4', 6'-tetrahydroxydihydrochalcone) differs from aspalathin in the absence of the 3-hydroxy on the A-ring. It occurs in a much lower concentration in rooibos.

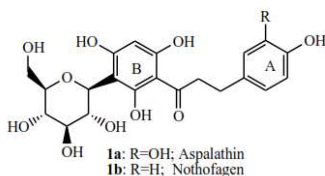
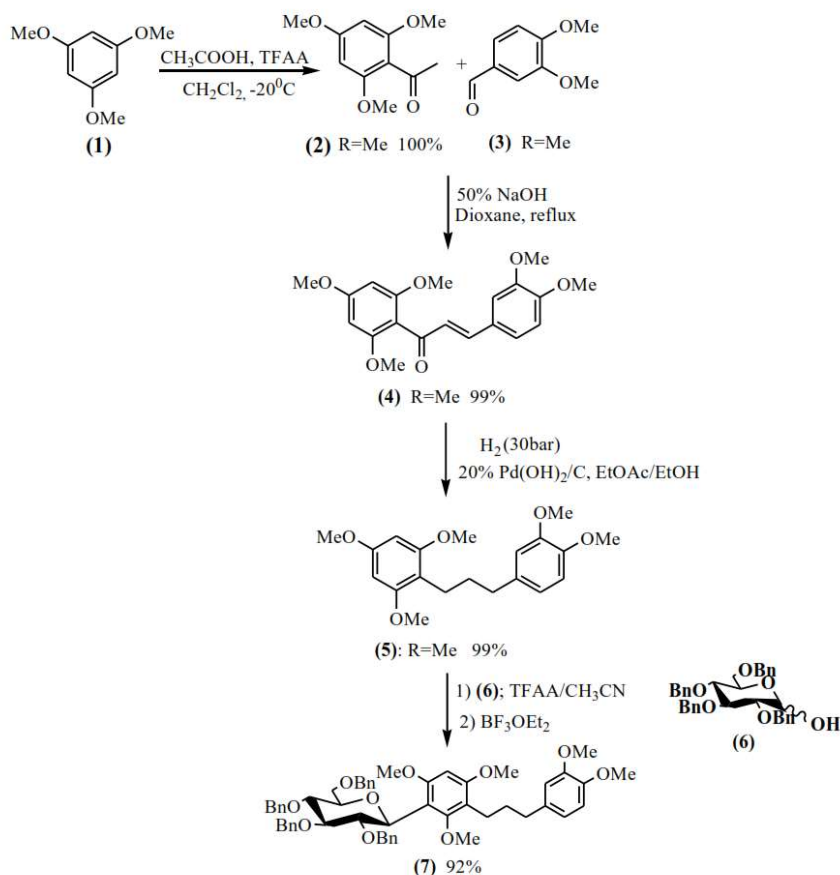


Figure 1. Structures of aspalathin (1a) and nothifagen (1b).



Scheme 1. Synthesis of diarylpropane (5) and C-diarylpropane-glycoside (7).

The isolation of naturally occurring C-aryl glycosides with important pharmacological properties [18-23] has prompted synthetic methods that are also relevant to the synthesis of

aspalathin and analogs. The C-glycosidic bond confers stability to O-glycosides to both enzymatic and chemical hydrolysis and, thus, probably contributes to enhanced bioavailability. However, it is much more difficult to form a carbon-carbon bond than an ether bond, and C-glycoside synthesis has remained a challenge. The regio- and stereoselective requirements of the C-aryl linkage are the additional complications.

In continuation of our previous work on the synthesis of some bioactive heterocyclic compounds [24-27], in the present work, we report synthesis and characterization of benzyl-methoxyl protected aspalathin analog via C-glucosylation of pentamethoxy dihydropyranone (Scheme 1).

Table 1. High-pressure hydrogenation of fully OMe protected chalcone.

Substrate	Hydrogen Pressure (Bar/PSi)	10% Pd/C (Equivalent)	Solvents (EtOAc/MeOH (Ratio))	Time (h)	Yield (%)
4	30/435	(0.1)	(50:50)	24	(100)
4	R.T. Using H ₂ , Balloon	(0.2)	EtOAc/H ₂ O/Dil, HCl	Overnight	99%

Table 2. Synthesis of (7) via C-glycosylation of diarylpropane (5).

Entry	Sugar donor	Catalyst (equivalents)		Solvent	Temperature (°C)	Time (h)	Yield (%)
1	6	BF ₃ OEt ₂ (2)		DCM	0	14	14
2	Sugar-1 [#]	TMSOTf (2)		DCM	-10	8	23
3	Sugar-2 [#]	BF ₃ OEt ₂ (2)		DCM	-20	6	42
4	Sugar-3 [#]	SnCl ₄ (2)		DCM	0	7	30
5	6	TFAA* (3)	BF ₃ OEt ₂ (2)	DCM	-12	6	59
6	6	TFAA* (3)	BF ₃ OEt ₂ (2)	CH ₃ CN	-12	3	92

* Preactivation with TFAA was essential for the coupling reaction to take place.

[#]Sugar-1: 2, 3, 4, 6-tetra-O-benzylglucosyl acetate, Sugar-2: 2, 3, 4, 6-tetra-O-benzylglucosyl acetamide, Sugar-3: 2, 3, 4, 6-tetra-O-benzyl-glycopyranosyl fluoride.

C-aryl glycosylation has been reviewed by Palmacci [28] and Seeberger [29]. The substituents may influence glycosidic bond formation's regioselectivity on the aromatic ring and the reaction conditions such as the temperature and pressure employed. C-aryl glycosidic bond formation's stereoselectivity was influenced by the structure of carbohydrate moiety, e.g., neighboring group effects, anomeric effects, and synthetic conditions such as the choice of catalysts and solvents.

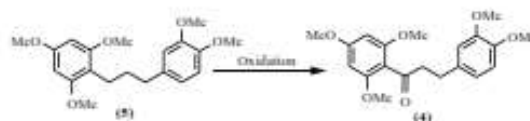
3. Results and Discussion

The methoxy-protected chalcone (4) was obtained in quantitative yields. High-pressure catalytic hydrogenative reduction of (4) gave the corresponding 1, 3-diarylpropane (5) quantitatively (Scheme 1) (Table 1). Salient in the NMR of (5) is the following: The absence of the two chalcone proton resonances in the ¹H NMR spectrum [H_α (d, 7.25 ppm, J = 15.5 Hz) and H_β (d, 6.88 ppm, J = 15.5 Hz) for corresponding chalcone, and the absence of the carbonyl resonance (δ = 196) in the ¹³C NMR; The three propane CH₂ groups are represented in the ¹H NMR at 2.58 ppm (two overlapping benzylic CH₂ groups that integrate four hydrogen atoms) and 1.77 ppm (a multiplet that integrates two hydrogen atoms). They correspond to two cross-peaks at 35.5 and 31.1 ppm in the HSQC; The 1,3-diarylpropane-C-glycoside (7) was subsequently obtained in almost quantitative isolated yield (92%, Entry 6, Table 2) from (6) via the trifluoroacetic anhydride (TFAA) method described under approach 1 (preactivation of the anomeric OH with TFAA) (Scheme 1); The glycosyl fluoride and BF₃OEt₂ gave (7) in a yield of only 42% (Entry 3, Table 2). The C-diarylpropane-glycoside (7) was characterized by the following observations:

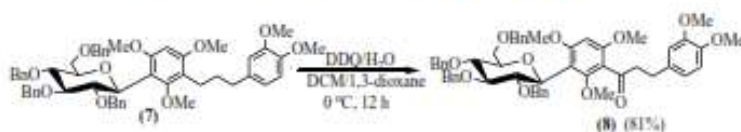
- It has complex ^1H and ^{13}C NMR spectra at room temperature due to the expected rotational isomerism about the glycosidic carbon-carbon bond. Heating of the sample to 140°C in $\text{DMSO}-d_6$ was required for NMR elucidation.
- The anomeric proton resonates at 4.73 ppm in the ^1H and its corresponding C at 74.2 ppm in the ^{13}C NMR spectra. These correspond with a carbon-carbon and not carbon-oxygen bond.
The $J = 9.8$ Hz coupling constant agrees with the required β -stereochemistry on the anomeric carbon.

An edited two-dimensional HSQC experiment; (CH and CH_3 cross-peaks having a different color from CH_2 cross-peaks) allows facile differentiation between the benzylic protons (CH_2) of the benzyl protection groups and the anomeric sugar proton (CH), all of which resonate in the range of δ 4.00-5.20 ppm.

We thus proved our hypothesis that the carbonyl group in chalcone or the phloroacetophenone moiety prevents C-glycosylation. Initial efforts to regenerate the carbonyl group *via* oxidation of (7) with Dess-Martin reagent, IBX (*o*-iodoxybenzoic acid), CAN (ceric ammonium nitrate), pyridine-dichromate, $\text{Na}_2\text{Cr}_2\text{O}_7$, and DDQ (dichlorodicyanoquinone) under anhydrous conditions failed. Upon further literature search and model reactions (Table 3) with the diarylpropane (5), we realized that the presence of water as a source of oxygen is essential for this oxidation. Upon treatment of (5) with DDQ in the presence of H_2O (1.5 equivalent), the corresponding dihydrochalcone (4) was obtained quantitatively at room temperature (Scheme 2). Compared to other oxidative conditions, it is mild with water as the oxygen source.



Scheme 2. Benzylic oxidation of the diarylpropane (5).



Scheme 3. Optimized synthesis of methyl-benzyl-protected aspalathin (8) *via* benzylic oxidation of (7).

Table 3. Oxidation condition for the synthesis of (5) at room temperature.

Entry	Oxidant	Solvents (2:1)	Time (h)	Yield (%)
1	IBX	Acetone*	14	0
2	Des-Martin	DCM*	8	0
3	CAN	DCM*	6	0
4	Pyridine-dichromate	DCM*	7	0
5	$\text{Na}_2\text{Cr}_2\text{O}_7$	DCM*	6	0
6	DDQ	DCM/Dioxane*	3	0
7	IBX	DCM*	14	14
8	Des-Martin	DCM*	8	23
9	CAN	DCM*	6	33
10	pyridine-dichromate	DCM*	7	30
11	$\text{Na}_2\text{Cr}_2\text{O}_7$	DCM*	6	59
12	DDQ	DCM/Dioxane*	3	92

* Anhydrous solvents were used in reactions

* 1.5 equivalent of H_2O was present in the reaction solvents

Table 4. Optimized oxidation conditions for the synthesis of *C*-dihydrochalcone glycoside (7).

Entry	Oxidant (Eq)	Solvents (2:1)	Temp. (°C)	Time (h)	Yield %
1	DDQ (4)	DCM/Dioxane ^a	r.t.	2	44
2	DDQ (4)	DCM/Dioxane ^a	0	12	81

* 1.5 equivalent of H₂O was present in the reaction solvents

The same treatment of (7) with DDQ at room temperature under the conditions optimized for (5) yielded the expected dihydrochalcone-*C*-glycoside (8) in a 44% yield. Upon lowering the reaction temperature to 0 °C, the reaction proceeded slower but produced a higher yield (81%) (Scheme 3, Table 4). We attributed this to the partial removal of the aliphatic benzyl protection groups on the sugar moiety with DDQ at room temperature. The NMR of (8) also required elevated temperature (140 °C) to remove rotational isomerism and simplify interpretation. Notable are the following: The carbonyl resonance at δ 198.4 in the ¹³C NMR spectrum; Two multiplets at δ 3.05 and 2.93 in the ¹H NMR. These CH₂ resonances correlate with the carbon resonances at δ 38.8 and 19.7 ppm, respectively, in an edited HSQC experiment. The two multiplets also cross-couple to each other in the COSY spectrum. They represent the dihydrochalcone's CH₂-CH₂ moiety; the anomeric proton of the sugar moiety resonates as a doublet at δ 4.73 ppm that correlates with the anomeric carbon at δ 73.6 ppm in the edited HSQC. The *J* = 9.8 Hz coupling constant indicates β -stereochemistry for the *C*-glycosidic bond; The four benzylic CH₂ resonances in the δ 4.55 to 4.90 ppm range of the ¹H NMR spectrum indicate that the four benzyl groups on the sugar moiety remained intact (stable to the oxidation conditions); The same treatment of (7) with DDQ at room temperature under the conditions optimized for (5) yielded the expected dihydrochalcone-*C*-glycoside (8) in a 44% yield. Upon lowering the reaction temperature to 0 °C, the reaction proceeded slower but produced a higher yield (81%) (Scheme 3, Table 4). We attributed this to the partial removal of the aliphatic benzyl protection groups on the sugar moiety with DDQ at room temperature.

4. Conclusions

Thus the present protocol represents the first synthesis of methyl-benzyl-protected aspalathin analog (8). However, attempts to deprotect the analog (8) using BBr₃ could not lead to the successful synthesis of aspalathin. Efforts for demethylation by using the excess of BBr₃ lead to a breakdown of sugar moieties and decomposition.

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Conflicts of Interest

The authors declare no conflict of interest.

References

1. Isono, K. Nucleoside antibiotics: Structure, biological activity, and biosynthesis. *The Journal of Antibiotics* **1988**, *41*, 1711-1739, <https://doi.org/10.7164/antibiotics.41.1711>.
2. Cirak, C.; Radusiene, J. Factors affecting the variation of bioactive compounds in *Hypericum* species. *Biologia Futura* **2019**, *70*, 198-209, <https://doi.org/10.1556/019.70.2019.25>.

<https://nanobioletters.com/>

3. Wang, M.; Li, J.; Rangarajan, M.; Shao, Y.; LaVoie, E.J.; Huang, T.-C.; Ho, C.-T. Antioxidative phenolic compounds from *Salvia officinalis*. *J. Agric. Food Chem.* **1998**, *46*, 4869-4873, <https://doi.org/10.1021/jf980614b>.
4. Ayan, A.K.; Radušienė, J.; Çirak, C.; Janulis, V. Secondary metabolites of *Hypericum scabrum* and *Hypericum hupleuroides*. *Pharmaceutical Biology* **2009**, *4*, 847-853, <https://doi.org/10.1080/13880200902942436>.
5. Jain, R.; Beyer, H.G.; Standish, R. *Proceedings of the National Academy of Sciences, India, Section A: Physical Sciences*. Volume 77, **2007**; pp. 99-100.
6. Jung, H.-A.; Su, B.-N.; Keller, W.J.; Mehta, R.G.; Kinghorn, A.D. Antioxidant xanthenes from the pericarp of *Garcinia angostana* (Mangosteen). *Journal of Agriculture and Food Chemistry* **2006**, *54*, 2077-2082, <https://doi.org/10.1021/jf052649z>.
7. Subramoniam, A. *Plants with anti-diabetes mellitus properties*. Boca Raton: CRC Press, **2016**; <https://doi.org/10.1201/9781315371481>.
8. Giri, P.; Singh, I. Development and evaluation of muco adhesive tablets of cinnarizine using carboxymethylated guar gum by compression coating technique. *Biointerface Research in Applied Chemistry* **2020**, *10*, 6076-6081, <https://doi.org/10.33263/BRIAC105.63656376>.
9. Fouda, A.E.-A.S.; El-Maksoud, S.A.A.; El-Habab, A.T.; Ibrahim, A.R. Synthesis and characterization of new ethoxylated carbohydrate based surfactants for corrosion inhibition of low cost steel in aqueous solutions. *Biointerface Research in Applied Chemistry* **2021**, *11*, 9382-9404, <https://doi.org/10.33263/BRIAC112.93829404>.
10. Ramarathnam, N.; Osawa, T.; Namiki, M.; Kawakishi, S. Chemical studies on novel rice hull antioxidants. 2. Identification of isovitexin, a C-glycosyl flavonoid. *J. Agri. Food. Chem.* **1989**, *37*, 316-319, <https://doi.org/10.1021/jf00086a009>.
11. Xiao, J.; Capanoglu, E.; Jassbi, A.R.; Miron, A. Dietary phytochemicals: Nutrition and health advance on the flavonoid C-glycosides and health benefits. *Dietary Phytochemicals: Nutrition and Health* **2015**, s29-s45, <https://doi.org/10.1080/10408398.2015.1067595>.
12. Dinda, B.; Bhattacharya, A.; Takayanagi, H.; Harigaya, Y. Antimicrobial C-Glucoside from aerial parts of *Diospyros nigra*. *Chem. Pharm. Bull.* **2006**, *54*, 679-681, <https://doi.org/10.1248/cpb.54.679>.
13. Akiyama, K.; Matsumoto, H.; Hayashi, H. Isolation and identification of a phosphate deficiency-induced C-glycosyl flavonoid that stimulates Arbuscular Mycorrhiza formation in melon roots. *Molecular Plant-Microbe Interactions* **2002**, *15*, 334-340, <https://doi.org/10.1094/MPMI.2002.15.4.334>.
14. Middleton, E.M.; Teramura, A.H. The role of flavonol glycosides and carotenoids in protecting soybean from ultraviolet-B damage. *Plant Physiology* **1993**, *103*, 741-752, <https://doi.org/10.1104/pp.103.3.741>.
15. Pérez Gutierrez, R. M.; García Campoy, A.H.; Paredes Carrera, S.P.; Muñoz Ramirez, A.; Mota Flores, J.M.; Flores Valle, S.O. 3'-O-β-D-glucopyranosyl-α,4,2',4',6'-pentahydroxy-dihydrochalcone, from Bark of *Eysenhardtia polystachya* Prevents Diabetic Nephropathy via Inhibiting Protein Glycation in STZ-Nicotinamide Induced Diabetic Mice. *Molecules* **2019**, *24*, <https://doi.org/10.3390/molecules24071214>.
16. Kawano, A.; Nakamura, H.; Hata, S.-I.; Minakawa, M.; Miura, Y.; Yagasaki, K. Hypoglycemic effect of aspalathin, a rooibos tea component from *Aspalathus linearis*, in type 2 diabetic model db/db mice. *Phytomedicine : International Journal of Phytotherapy And Phytopharmacology* **2009**, *16*, 437-443, <https://doi.org/10.1016/j.phymed.2008.11.009>.
17. Plante, O.J.; Palmacci, E.R.; Andrade, R.B.; Seeberger, P.H. Oligosaccharide synthesis with glycosyl phosphate and dithiophosphate triesters as glycosylating agents. *Journal of the American Chemical Society* **2001**, *123*, 9545-9554, <https://doi.org/10.1021/ja016227r>.
18. Kitamura, K.; Ando, Y.; Matsumoto, T.; Suzuki, K. Total synthesis of aryl C-glycoside natural products: Strategies and tactics. *Chemical Reviews* **2018**, *118*, 1495-1598, <https://doi.org/10.1021/acs.chemrev.7b00380>.
19. Ben, A.; Yamauchi, T.; Matsumoto, T.; Suzuki, K. Sc(OTf)₃ as efficient catalyst for aryl C-glycoside synthesis. *Chem Inform* **2004**, *35*, 225-230, <https://doi.org/10.1002/chin.200423210>.
20. Li, Y.; Mo, H.; Lian, G.; Yu, B. Revisit of the phenol O-glycosylation with glycosyl imidates, BF₃·OEt₂ is a better catalyst than TMSOTf. *Carbohydrate Research* **2012**, *363*, 14-22, <https://doi.org/10.1016/j.carres.2012.09.025>.
21. Yamauchi, T.; Watanabe, Y.; Suzuki, K.; Matsumoto, T. Facile One-pot synthesis of resorcinol bis-C-glycosides possessing two identical- sugar moieties. *Synthesis* **2006**, *2006*, 2818-2824, <https://doi.org/10.1055/s-2006-942526>.
22. Alexei, V. *Handbook of Chemical Glycosylation: Advances in Stereoselectivity and Therapeutic Relevance*. **2008**, Wiley-VCH.
23. Kaliappan, K.P.; Subrahmanyam, A.V. A New versatile strategy for C-aryl glycosides. *Organic Letters* **2007**, *9*, 1121-1124, <https://doi.org/10.1021/ol701159>.
24. Gore, V.A.; Tekale, S.U.; Bhale, S.P.; Rajani, D.P.; Domb, A.J.; Pawar, R.P. Synthesis and biological evaluation of novel thiazole hydrazines as antimicrobial and antimalarial agents. *Letters in Applied NanoBioScience* **2021**, *10*, 1846-1855, <https://doi.org/10.33263/LIANBS101.18461855>.

25. Kaminwar, N.S.; Tekale, S.U.; Chidrawar, A.B.; Kótai, L.; Pawar, R.P. Eco-friendly synthesis of 1,4-dihydropyrano-[2,3-c]pyrazoles using copper nanoparticles grafted on carbon microsphere as a heterogeneous catalyst. *Letters in Applied NanoBioScience* **2020**, *9*, 1521-1528, <https://doi.org/10.33263/LIANBS94.15211528>.
26. Bhale, S.; Gore, V.; Tekale, S.; Pawar, R. Synthesis, characterization and antimicrobial activity of Ni(II), Zn(II), and Cd(II) complexes of 3/4-bromobenzoic acid (phenyl-pyridine-2-yl-methylene)-hydrazide ligand. *Letters in Applied NanoBioScience* **2020**, *9*, 1529-1537, <https://doi.org/10.33263/LIANBS94.15291537>.
27. Kendrekar, P.; Mashele, S.; Tekale, S.; Pawar, R. Synthesis of some novel and potent anti-plasmodial aminoalkyl chalcone derivatives. *Biointerface Research in Applied Chemistry* **2020**, *10*, 6076-6081, <https://doi.org/10.33263/BRIAC105.60766081>.
28. Palmacci, E.R.; Seeberger, P.H. Synthesis of C-aryl and C-alkyl glycosides using glycosyl phosphates. *Organic Letters* **2001**, *3*, 1547-1550, <https://doi.org/10.1021/ol0158462>.
29. Nakajima, N.; Abe, R.; Yonemitsu, O. 3-Methoxybenzyl(3-MPM) and 3,5-dimethoxybenzyl(3,5-DMPM) protecting groups for the hydroxy functionless readily removable than 4-methoxybenzyl(MPM) and 3,4-dimethoxybenzyl (DMPM) protecting groups by DDQ oxidation. *Chemical and Pharmaceutical Bulletin* **1988**, *36*, 4244-4247, <https://doi.org/10.1248/cpb.36.4244>.

3.10.4 Synthesis of racemic thiolactomycin

A convenient five-step procedure for the chemical synthesis by Wang and Salvino (1984) was used to synthesize TLM drug compounds. The synthetic strategy involved bromination of methyl α -propionylpropionate (compound 1) with pyridium bromide perbromide (compound 2) in acetic acid followed by treatment of compound 2 with thioacetic acid to yield the thioacetate (compound 3). The essential step involved the addition of 3-ethoxy-2-methyl-2-propenal to the thiolactone (compound 4), followed by mild acid hydrolysis and subsequent treatment with methylene triphenylphosphorane to afford racemic TLM (compound 6) (Figure 3.1).

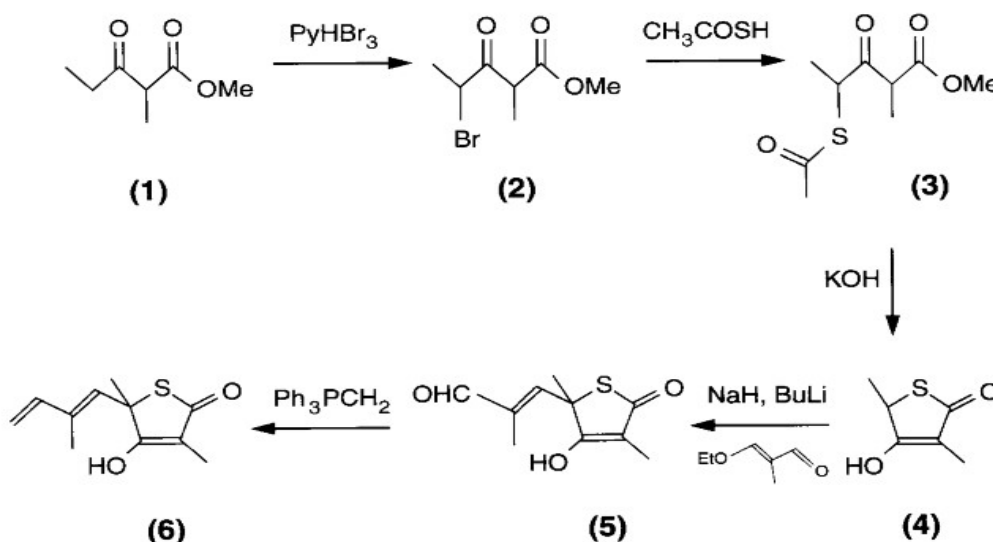


Figure 3.1: Synthesis procedure of racemic thiolactomycin

3.10.5 Minimal Inhibitory Concentration

3.10.5.1 Intended use

This method determines the absolute minimum concentration that will cause bacterial growth inhibition. It employs the use of the agar-disc elution method.

3.10.5.2 Principle of the test

An antimicrobial agent of different concentrations was placed on the agar plate inoculated with the organism of interest. Beef extract and acid hydrolysate of casein provide nitrogen, vitamins, carbon, amino acids, sulphur, and nutrients to facilitate organism growth. Starch absorbed any toxic metabolites produced and served as a source of energy. The plates were incubated and observed for the presence of

inhibition around the antibiotic area. The lowest concentration of the antimicrobial agent where there was evidence of growth inhibition was considered the minimal inhibitory concentration of the drug.

3.10.5.3 Procedure

We determined the susceptibility of *M. smegmatis* to various concentrations of TLM derivatives of interest on MHA plate containing beef extract and acid hydrolysate of casein.

Eight MHA plates with lot number J202153 were received (Appendix 6). The internal quality control (IQC) for bacteriological media was carried out before proceeding with the MIC testing. One plate was inoculated with *Enterococcus faecalis* for positive control to check for growth, while another was a negative control for checking sterility and contamination. Both plates were incubated at 37°C and were examined every 24 hours for three days. They both passed the IQC test to validate the rest of the plates from that batch lot to be used for testing (Appendix 7). The remaining six MHA plates were used for the experiment, and each plate was divided into six sections (Figure 4.20). On each medium plate, there were positive and negative controls as well as the numbers corresponding to the drugs and their concentration (Table 4.3).

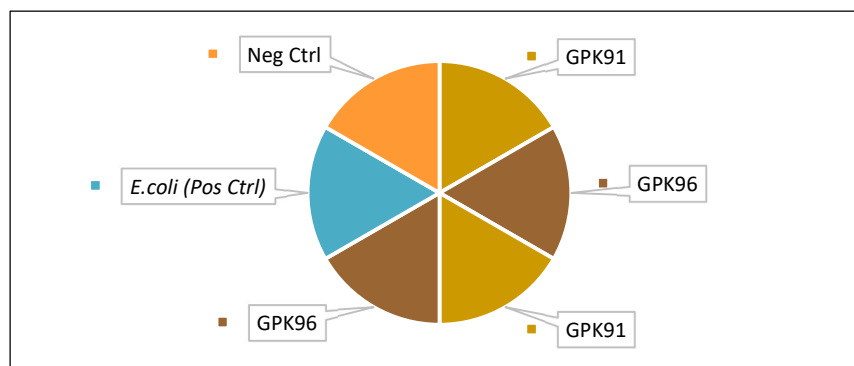


Figure 3.2: Diagram representation of how the Mueller Hinton agar plates were divided.

M. smegmatis was inoculated across 4/6 sections on MHA plates that contained 2.0 g/l of beef extract, 17.5 g/l acid hydrolysate of casein, and 1.5 g/l starch. *Escherichia coli* (*E. coli*) AATC25922 strain (Positive control) was inoculated across 1/6 sections of the MHA plates, and the sixth section was not inoculated (negative control). MHA_1 to MHA_5 were treated with different concentrations of GPK91 and GPK96 inoculated with *M. smegmatis* on areas 1–4, 5–8, 9–12, 13–16, and 17–20. MHA-6

was treated with TLM [25.0 µg/ml], a reference antimicrobial, on area 21 and also inoculated with *M. smegmatis*. *E. coli* and uninoculated areas served as positive and negative controls on all MHA plates, each on the demarcated areas of the plates as illustrated in Figure 3.2.

We separately diluted 25 µg/ml of our compounds in 100 µl of phosphate-buffered saline to make the working compound solutions. From the working solutions, serial titrations were prepared starting from a concentration of 25.0 µg/ml down to 0.049 µg/ml (Appendix 8). A 1.0 µl of different concentrations of the antimicrobial drug compounds were placed at the center of the sections inoculated with *M. smegmatis* and were allowed to set on the media.

Once the drops were set, the plates were incubated for up to 72 hrs. at 37°C. The MHA plates were examined for organism growth as well as the presence of any inhibition every 24 hours. The results were recorded in Table 4.3.

4.1 Introduction

The *M. tuberculosis* genome has been fully sequenced and this provided an opportunity to explore the database to identify the *inhA* and *kasA* DNA polymerase gene sequences within the *M. tuberculosis* genome. NCBI was used to perform BLAST searches on the gene database and carry out the phylogenetic analysis on the genes of interest of a specific microorganism, the *M. tuberculosis* H37Rv strain (www.ncbi.nlm.nih.gov). We used a Nucleic acid sequence massager to remove FASTA comments, HTML mark-up, line feeds, numbers, and blank spaces before submitting the sequence for BLAST (biomodel.uah.es/en/lab/cybertory/analysis/massager.htm). Since both InhA and KasA enzymes are the primary targets of action for INH and TLM in *M. tuberculosis*, the study aimed to design TML derivatives novel drug compounds that target InhA and KasA proteins. TLM specifically inhibits the mycobacterial acyl carrier protein-dependent type II fatty acid synthase (FAS-II) (Slayden et al., 1996). The efficacy of TLM against *M. smegmatis* and *M. tuberculosis* provides the prospects of identifying fatty acid and mycolic acid biosynthetic genes and revealing a novel range of chemotherapeutic agents directed against *M. tuberculosis*.

With the isolation of naturally occurring C-aryl glycosides with important pharmacological properties, synthetic methods that are also relevant to the synthesis of aspalathin and analogs have been prompted. The C-glycosidic bond confers stability compared to O-glycosides to both enzymatic and chemical hydrolysis and thus contributes towards enhanced bioavailability. It is, however, much more difficult to form a carbon-carbon bond than an ether bond, and C-glycoside synthesis has remained a challenge. The regio- and stereoselective requirements of the C-aryl linkage are additional complications.

4.2 Results of NCBI Nucleotide Sequence

4.2.1 Introduction

DNA sequencing is the process of determining the exact order of nucleotides in the targeted region of the DNA. The proteins of interest were InhA and KasA, therefore we obtained the nucleotide sequences of *M. tuberculosis H37Rv inhA* and *kasA* genes from the *M. tuberculosis* whole genome ribosomal ribonucleic acid (rRNA) sequence (Accession: CP003248) that would be used to identify the sequences with closest homologs.

4.2.2 *Mycobacterium tuberculosis* H37Rv, complete genome sequence

LOCUS CP003248 4411709 bp DNA circular BCT 01-OCT-2014
 DEFINITION *Mycobacterium tuberculosis* H37Rv, complete genome.
ACCESSION: CP003248 VERSION: CP003248.2
 DBLINK BioProject: [PRJNA37301](#) BioSample: [SAMN03081436](#)

4.2.3 *Mycobacterium tuberculosis* strain H37Rv *kasA* gene sequence

/db_xref="GeneID: [887269](#)" CDS=1..1251

ORIGIN

```

1  GTGAGTCAGC CTTCCACCGC TAATGGCGGT TTCCCCAGCG TTGTGGTGAC CGCCGTCACA
61  GCGACGACGT CGATCTCGCC GGACATCGAG AGCACGTGGA AGGGTCTGTT GGCCGGCGAG
121 AGCGGCATCC ACGCACTCGA AGACGAGTTC GTCACCAAGT GGGATCTAGC GGTCAAGATC
181 GGCGGTACAC TCAAGGATCC GGTTCGACAGC CACATGGGCC GACTCGACAT GCGACGCATG
241 TCGTACGTCC AGCGGATGGG CAAGTTGCTG GGCGGACAGC TATGGGAGTC CGCCGGCAGC
301 CCGGAGGTCG ATCCAGACCG GTTCGCCGTT GTTGTCGGCA CCGGTCTAGG TGGAGCCGAG
361 AGGATTGTCG AGAGCTACGA CCTGATGAAT GCGGGCGGCC CCCGGAAGGT GTCCCCGCTG
421 GCCGTTGAGA TGATCATGCC CAACGGTGCC GCGGCGGTGA TCGGTCTGCA GCTTGGGGCC
481 CGCGCCGGGG TGATGACCCC GGTGTCGGCC TGTTCGTCGG GCTCGGAAGC GATCGCCAC
541 GCGTGGCGTC AGATCGTGAT GGGCGACGCC GACGTCGCCG TCTGCGGCGG TGTCGAAGGA
601 CCCATCGAGG CGCTGCCCAT CGCGGCGTTC TCCATGATGC GGGCCATGTC GACCCGCAAC
661 GACGAGCCTG AGCGGGCCTC CCGGCCGTTT GACAAGGACC GCGACGGCTT TGTGTTTCGGC
721 GAGGCCGGTG CGCTGATGCT CATCGAGACG GAGGAGCACG CCAAAGCCCG TGGCGCCAAG
781 CCGTTGGCCC GATTGCTGGG TGCCGGTATC ACCTCGGACG CCTTTCATAT GGTGGCGCCC
841 GCGGCCGATG GTGTTCTGTC CGGTAGGGCG ATGACTCGCT CGCTGGAGCT GGCCGGGTTG
901 TCGCCGGCGG ACATCGACCA CGTCAACGCG CACGGCACGG CGACGCCTAT CGGCGACGCC
961 GCGGAGGCCA ACGCCATCCG CGTCGCCGGT TGTGATCAGG CCGCGGTGTA CGCGCCGAAG
1021 TCTGCGCTGG GCCACTCGAT CGGCGCGGTC GGTGCGCTCG AGTCGGTGCT CACGGTGCTG
1081 ACGCTGCGCG ACGGCGTCAT CCCGCCGACC CTGAACACG AGACACCCGA TCCGAGATC
1141 GACCTTGACG TCGTCGCCGG CGAACCGCGC TATGGCGATT ACCGCTACGC AGTCAACAAC
1201 TCGTTCGGGT TCGGCGGCCA CAATGTGGCG CTGCCTTCG GCGTTACTG A

```

4.2.4 *Mycobacterium tuberculosis* strain H37Rv *inhA* gene sequence

Gene symbol: *inhA* **Gene ID:** 886523

Gene description: NADH-dependent enoyl-[ACP] reductase

Locus tag: Rv1484 [CDS](#): 22..831

ORIGIN

```

1  TGACACAACA CAAGGACGCA CATGACAGGA CTGCTGGACG GCAAACGGAT TCTGGTTAGC
61  GGAATCATCA CCGACTCGTC GATCGCGTTT CACATCGCAC GGGTAGCCCA GGAGCAGGGC
121  GCCCAGCTGG TGCTCACC GGTTTCGACCGG CTGCGGCTGA TTCAGCGCAT CACCGACCGG
181  CTGCCGGCAA AGGCCCGCT GCTCGAACTC GACGTGCAAA ACGAGGAGCA CCTGGCCAGC
241  TTGGCCGGCC GGGTGACCGA GGCGATCGGG GCGGGCAACA AGCTCGACGG GGTGGTGCAT
301  TCGATTGGGT TCATGCCGCA GACCGGGATG GGCATCAACC CGTTCTTCGA CGCGCCCTAC
361  GCGGATGTGT CCAAGGGCAT CCACATCTCG GCGTATTTCG ATGCTTCGAT GGCCAAGGCG
421  CTGCTGCCGA TCATGAACCC CGGAGGTTCC ATCGTCGGCA TGGACTTCGA CCCGAGCCGG
481  GCGATGCCGG CCTACAACTG GATGACGGTC GCCAAGAGCG CGTTGGAGTC GGTCAACAGG
541  TTCGTGGCGC GCGAGGCCGG CAAGTACGGT GTGCGTTCGA ATCTCGTTGC CGCAGGCCCT
601  ATCCGGACGC TGGCGATGAG TGCATCGTC GCGGTGCGC TCGGCGAGGA GGCCGGCGCC
661  CAGATCCAGC TGCTCGAGGA GGGCTGGGAT CAGCGCGCTC CGATCGGCTG GAACATGAAG
721  GATGCGACGC CGGTCGCCAA GACGGTGTGC GCGCTGCTGT CTGACTGGCT GCCGGCGACC
781  ACGGGTGACA TCATCTACGC CGACGGCGGC GCGCACACCC AATTGCTCTA GA

```

4.3 Results of NCBI Nucleotide BLAST

4.3.1 Introduction

A nucleotide BLAST search was used to create a construct sequence for the *inhA* and *kasA* genes of the *M. tuberculosis* H37Rv strain on the NCBI. The *inhA* and *kasA* gene sequences were subjected to BLAST analysis against *M. tuberculosis*'s whole genome ribosomal ribonucleic acid (rRNA) sequence to identify the closest homologs. Among the resulting hits, the sequences with 100% identity homologs were selected. These strains were selected, and the H37Rv rRNA sequences were obtained. The acquired sequence reads were aligned to the reference genome (CP003248).

Your search is limited to records that include: *Mycobacterium tuberculosis* H37Rv (taxid:83332)

•	
Job Title	NC_000962: <i>Mycobacterium tuberculosis</i> H37Rv,...
RID	0MRPWF4Z013 Download All
Program	BLASTN Help Citation
Query ID	NC_000962.3
Description	<i>Mycobacterium tuberculosis</i> H37Rv, complete genome
Query Length	1251
Other reports	Distance tree of results MSA viewer Help
Organism	only top 20 will appear
○	5 sequences selected

4.3.2 *Mycobacterium tuberculosis* strain H37Rv_CG chromosome, complete genome Phylogenetic analysis for *kasA* gene

Sequence ID: [CP072765.1](#) Length: 4415999 Number of Matches: 1
Range 1: 2521046 to 2522296 [GenBankGraphics](#)

Score	Expect	Identities	Gaps	Strand
2311 bits(1251)	0.0	1251/1251(100%)	0/1251(0%)	Plus/Plus
Query 1	GTGAGTCAGCCTTCCACCGCTAATGGCGGTTTCCCCAGCGTTGTGGTGACCGCCGTCACA	60		
Sbjct 2521046	GTGAGTCAGCCTTCCACCGCTAATGGCGGTTTCCCCAGCGTTGTGGTGACCGCCGTCACA	2521105		
Query 61	GCGACGACGTCGATCTCGCCGGACATCGAGAGCACGTGGAAGGGTCTGTTGGCCGGCGAG	120		
Sbjct 2521106	GCGACGACGTCGATCTCGCCGGACATCGAGAGCACGTGGAAGGGTCTGTTGGCCGGCGAG	2521165		
Query 121	AGCGGCATCCACGCACTCGAAGACGAGTTCGTCACCAAGTGGGATCTAGCGGTCAAGATC	180		
Sbjct 2521166	AGCGGCATCCACGCACTCGAAGACGAGTTCGTCACCAAGTGGGATCTAGCGGTCAAGATC	2521225		
Query 181	GGCGGTCACCTCAAGGATCCGGTCGACAGCCACATGGGCCGACTCGACATGCGACGCATG	240		
Sbjct 2521226	GGCGGTCACCTCAAGGATCCGGTCGACAGCCACATGGGCCGACTCGACATGCGACGCATG	2521285		
Query 241	TCGTACGTCCAGCGGATGGGCAAGTTGCTGGGCGGACAGCTATGGGAGTCCGCCGGCAGC	300		
Sbjct 2521286	TCGTACGTCCAGCGGATGGGCAAGTTGCTGGGCGGACAGCTATGGGAGTCCGCCGGCAGC	2521345		
Query 301	CCGGAGGTCGATCCAGACCGGTTCCGCCGTTGTTGTCGGCACCGGTCTAGGTGGAGCCGAG	360		
Sbjct 2521346	CCGGAGGTCGATCCAGACCGGTTCCGCCGTTGTTGTCGGCACCGGTCTAGGTGGAGCCGAG	2521405		
Query 361	AGGATTGTCGAGAGCTACGACCTGATGAATGCGGGCGGCCCGGAAGGTGTCCCGCTG	420		
Sbjct 2521406	AGGATTGTCGAGAGCTACGACCTGATGAATGCGGGCGGCCCGGAAGGTGTCCCGCTG	2521465		
Query 421	GCCGTTTCAGATGATCATGCCAACGGTGCCGCGGCGGTGATCGGTCTGCAGCTTGGGGCC	480		
Sbjct 2521466	GCCGTTTCAGATGATCATGCCAACGGTGCCGCGGCGGTGATCGGTCTGCAGCTTGGGGCC	2521525		
Query 481	CGCGCCGGGGTGATGACCCCGGTGTCGGCCTGTTTCGTCGGGCTCGGAAGCGATCGCCAC	540		
Sbjct 2521526	CGCGCCGGGGTGATGACCCCGGTGTCGGCCTGTTTCGTCGGGCTCGGAAGCGATCGCCAC	2521585		
Query 541	GCGTGGCGTCAGATCGTGATGGGCGACGCCGACGTCGCGTCTGCGGCGGTGTGCAAGGA	600		
Sbjct 2521586	GCGTGGCGTCAGATCGTGATGGGCGACGCCGACGTCGCGTCTGCGGCGGTGTGCAAGGA	2521645		
Query 601	CCCATCGAGGCGCTGCCCATCGCGGCGTTCTCCATGATGCGGGCCATGTCGACCCGCAAC	660		
Sbjct 2521646	CCCATCGAGGCGCTGCCCATCGCGGCGTTCTCCATGATGCGGGCCATGTCGACCCGCAAC	2521705		
Query 661	GACGAGCCTGAGCGGGCCTCCCGGCCGTTTCGACAAGGACCGCGACGGCTTTGTGTTCCGGC	720		
Sbjct 2521706	GACGAGCCTGAGCGGGCCTCCCGGCCGTTTCGACAAGGACCGCGACGGCTTTGTGTTCCGGC	2521765		
Query 721	GAGGCCGGTGCGCTGATGCTCATCGAGACGGAGGAGCACGCCAAAGCCCGTGGCGCCAAG	780		



Sbjct	2521766	GAGGCCGGTGCGCTGATGCTCATCGAGACGGAGGAGCACGCCAAAGCCCGTGGCGCCAAG	2521825
Query	781	CCGTTGGCCCGATTGCTGGGTGCCGGTATCACCTCGGACGCCTTTCATATGGTGGCGCCC	840
Sbjct	2521826	CCGTTGGCCCGATTGCTGGGTGCCGGTATCACCTCGGACGCCTTTCATATGGTGGCGCCC	2521885
Query	841	GCGGCCGATGGTGTTCGTGCCGGTAGGGCGATGACTCGCTCGCTGGAGCTGGCCGGGTTG	900
Sbjct	2521886	GCGGCCGATGGTGTTCGTGCCGGTAGGGCGATGACTCGCTCGCTGGAGCTGGCCGGGTTG	2521945
Query	901	TCGCCGGCGGACATCGACCACGTCAACGCGCACGGCACGGCGACGCCTATCGGCGACGCC	960
Sbjct	2521946	TCGCCGGCGGACATCGACCACGTCAACGCGCACGGCACGGCGACGCCTATCGGCGACGCC	2522005
Query	961	GCGGAGGCCAACGCCATCCGCGTCGCCGGTTGTGATCAGGCCGCGGTGTACGCGCCGAAG	1020
Sbjct	2522006	GCGGAGGCCAACGCCATCCGCGTCGCCGGTTGTGATCAGGCCGCGGTGTACGCGCCGAAG	2522065
Query	1021	TCTGCGCTGGGCCACTCGATCGGCGCGGTTCGGTGCCTCGAGTCGGTGCTCACGGTGCTG	1080
Sbjct	2522066	TCTGCGCTGGGCCACTCGATCGGCGCGGTTCGGTGCCTCGAGTCGGTGCTCACGGTGCTG	2522125
Query	1081	ACGCTGCGCGACGGCGTCATCCCGCCGACCCTGAACTACGAGACACCCGATCCCGAGATC	1140
Sbjct	2522126	ACGCTGCGCGACGGCGTCATCCCGCCGACCCTGAACTACGAGACACCCGATCCCGAGATC	2522185
Query	1141	GACCTTGACGTCGTCGCCGGCGAACC GCGCTATGGCGATTACCGCTACGCAGTCAACAAC	1200
Sbjct	2522186	GACCTTGACGTCGTCGCCGGCGAACC GCGCTATGGCGATTACCGCTACGCAGTCAACAAC	2522245
Query	1201	TCGTTTCGGGTTTCGGCGGCCACAATGTGGCGCTTGCCTTCGGGCGTTACTGA	1251
Sbjct	2522246	TCGTTTCGGGTTTCGGCGGCCACAATGTGGCGCTTGCCTTCGGGCGTTACTGA	2522296

4.3.3 *Mycobacterium tuberculosis* H37RvSiena, complete genome Phylogenetic analysis for *kasA* gene

Sequence ID: [CP007027.1](#) Length: 4410911 Number of Matches: 1
Range 1: 1 to 1251 [GenBankGraphics](#)

Score	Expect	Identities	Gaps	Strand
2311 bits (1251)	0.0	1251/1251(100%)	0/1251(0%)	Plus/Plus
Query	1	TTGACCGATGACCCCGGTTTCAAGGCTTCAACACAGTGTGGAACGCGGTCTCGTCTCCGAACTT		60
Sbjct	1	TTGACCGATGACCCCGGTTTCAAGGCTTCAACACAGTGTGGAACGCGGTCTCGTCTCCGAACTT		60
Query	61	AACGGCGACCCCTAAGGTTGACGACGGACCCAGCAGTGTGCTAATCTCAGCGCTCCGCTG		120
Sbjct	61	AACGGCGACCCCTAAGGTTGACGACGGACCCAGCAGTGTGCTAATCTCAGCGCTCCGCTG		120
Query	121	ACCCCTCAGCAAAGGGCTTGGCTCAATCTCGTCCAGCCATTGACCATCGTCGAGGGGTTT		180
Sbjct	121	ACCCCTCAGCAAAGGGCTTGGCTCAATCTCGTCCAGCCATTGACCATCGTCGAGGGGTTT		180
Query	181	GCTCTGTTATCCGTGCCGAGCAGCTTTGTCCAAAACGAAATCGAGCGCCATCTGCGGGCC		240
Sbjct	181	GCTCTGTTATCCGTGCCGAGCAGCTTTGTCCAAAACGAAATCGAGCGCCATCTGCGGGCC		240
Query	241	CCGATTACCGACGCTCTCAGCCGCCGACTCGGACATCAGATCCAACCTCGGGGTCCGCATC		300
Sbjct	241	CCGATTACCGACGCTCTCAGCCGCCGACTCGGACATCAGATCCAACCTCGGGGTCCGCATC		300
Query	301	GCTCCGCCGGCGACCGACGAAGCCGACGACACTACCGTGCCGCCTTCCGAAAATCCTGCT		360
Sbjct	301	GCTCCGCCGGCGACCGACGAAGCCGACGACACTACCGTGCCGCCTTCCGAAAATCCTGCT		360
Query	361	ACCACATCGCCAGACACCACAACCGACAACGACGAGATTGATGACAGCGCTGCGGCACGG		420
Sbjct	361	ACCACATCGCCAGACACCACAACCGACAACGACGAGATTGATGACAGCGCTGCGGCACGG		420
Query	421	GGCGATAACCAGCACAGTTGGCCAAGTTACTTCACCGAGCGCCCGCACAATACCGATTCC		480
Sbjct	421	GGCGATAACCAGCACAGTTGGCCAAGTTACTTCACCGAGCGCCCGCACAATACCGATTCC		480
Query	481	GCTACCGCTGGCGTAACCAGCCTTAACCGTCGCTACACCTTTGATACGTTTCGTTATCGGC		540
Sbjct	481	GCTACCGCTGGCGTAACCAGCCTTAACCGTCGCTACACCTTTGATACGTTTCGTTATCGGC		540
Query	541	GCCTCCAACCGGTTTCGCGCACGCCGCCGCTTGGCGATCGCAGAAGCACCCGCCGCGCT		600
Sbjct	541	GCCTCCAACCGGTTTCGCGCACGCCGCCGCTTGGCGATCGCAGAAGCACCCGCCGCGCT		600
Query	601	TACAACCCCTGTTTATCTGGGGCGAGTCCGGTCTCGGCAAGACACACCTGCTACACGCG		660
Sbjct	601	TACAACCCCTGTTTATCTGGGGCGAGTCCGGTCTCGGCAAGACACACCTGCTACACGCG		660
Query	661	GCAGGCAACTATGCCCAACGGTTGTTCCCGGGAATGCGGGTCAAATATGTCTCCACCGAG		720
Sbjct	661	GCAGGCAACTATGCCCAACGGTTGTTCCCGGGAATGCGGGTCAAATATGTCTCCACCGAG		720
Query	721	GAATTACCAACGACTTCATTAACCTCGCTCCGCGATGACCGCAAGGTCGCATTCAAACGC		780
Sbjct	721	GAATTACCAACGACTTCATTAACCTCGCTCCGCGATGACCGCAAGGTCGCATTCAAACGC		780
Query	781	AGCTACCGCGACGTAGACGTGCTGTTGGTCGACGACATCCAATTTCATTGAAGGCAAAGAG		840
Sbjct	781	AGCTACCGCGACGTAGACGTGCTGTTGGTCGACGACATCCAATTTCATTGAAGGCAAAGAG		840

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Query 841 GGTATTCAAGAGGAGTTCTTCCACACCTTCAACACCTTGCACAATGCCAACAAGCAAATC 900
          |||
Sbjct 841 GGTATTCAAGAGGAGTTCTTCCACACCTTCAACACCTTGCACAATGCCAACAAGCAAATC 900

Query 901 GTCATCTCATCTGACCGCCACCCAAGCAGCTCGCCACCCTCGAGGACCGGCTGAGAACC 960
          |||
Sbjct 901 GTCATCTCATCTGACCGCCACCCAAGCAGCTCGCCACCCTCGAGGACCGGCTGAGAACC 960

Query 961 CGCTTTGAGTGGGGGCTGATCACTGACGTACAACCACCCGAGCTGGAGACCCGCATCGCC 1020
          |||
Sbjct 961 CGCTTTGAGTGGGGGCTGATCACTGACGTACAACCACCCGAGCTGGAGACCCGCATCGCC 1020

Query 1021 ATCTTGCGCAAGAAAGCACAGATGGAACGGCTCGCGGTCCCCGACGATGTCCTCGAACTC 1080
          |||
Sbjct 1021 ATCTTGCGCAAGAAAGCACAGATGGAACGGCTCGCGGTCCCCGACGATGTCCTCGAACTC 1080

Query 1081 ATCGCCAGCAGTATCGAACGCAATATCCGTGAACTCGAGGGCGCGCTGATCCGGGTCACC 1140
          |||
Sbjct 1081 ATCGCCAGCAGTATCGAACGCAATATCCGTGAACTCGAGGGCGCGCTGATCCGGGTCACC 1140

Query 1141 GCGTTCGCCTCATTGAACAAAACACCAATCGACAAAGCGCTGGCCGAGATTGTGCTTCGC 1200
          |||
Sbjct 1141 GCGTTCGCCTCATTGAACAAAACACCAATCGACAAAGCGCTGGCCGAGATTGTGCTTCGC 1200

Query 1201 GATCTGATCGCCGACGCCAACACCATGCAAATCAGCGCGGCGACGATCATG 1251
          |||
Sbjct 1201 GATCTGATCGCCGACGCCAACACCATGCAAATCAGCGCGGCGACGATCATG 1251

```

4.3.4 *Mycobacterium tuberculosis* strain H37Rv_IC1 chromosome Phylogenetic analysis for *kasA* gene

Sequence ID: [CP053903.1](#) Length: 4411530 Number of Matches: 1

Range 1: 2517975 to 2519225 [GenBankGraphics](#)

Score	Expect	Identities	Gaps	Strand
2311 bits(1251)	0.0	1251/1251(100%)	0/1251(0%)	Plus/Plus
Query 1	GTGAGTCAGCCTTCCACCGCTAATGGCGGTTTCCCCAGCGTTGTGGTGACCGCCGTCACA	60		
Sbjct 2517975	GTGAGTCAGCCTTCCACCGCTAATGGCGGTTTCCCCAGCGTTGTGGTGACCGCCGTCACA	2518034		
Query 61	GCGACGACGTCGATCTCGCCGGACATCGAGAGCACGTGGAAGGGTCTGTTGGCCGGCGAG	120		
Sbjct 2518035	GCGACGACGTCGATCTCGCCGGACATCGAGAGCACGTGGAAGGGTCTGTTGGCCGGCGAG	2518094		
Query 121	AGCGGCATCCACGCACTCGAAGACGAGTTTCGTACCAAGTGGGATCTAGCGGTCAAGATC	180		
Sbjct 2518095	AGCGGCATCCACGCACTCGAAGACGAGTTTCGTACCAAGTGGGATCTAGCGGTCAAGATC	2518154		
Query 181	GGCGGTACCTCAAGGATCCGGTCGACAGCCACATGGGCCGACTCGACATGCGACGCATG	240		
Sbjct 2518155	GGCGGTACCTCAAGGATCCGGTCGACAGCCACATGGGCCGACTCGACATGCGACGCATG	2518214		
Query 241	TCGTACGTCCAGCGGATGGGCAAGTTGCTGGGCGGACAGCTATGGGAGTCCGCCGGCAGC	300		
Sbjct 2518215	TCGTACGTCCAGCGGATGGGCAAGTTGCTGGGCGGACAGCTATGGGAGTCCGCCGGCAGC	2518274		
Query 301	CCGGAGGTTCGATCCAGACCGGTTCCCGGTTGTTGTCGGCACCGGTCTAGGTGGAGCCGAG	360		
Sbjct 2518275	CCGGAGGTTCGATCCAGACCGGTTCCCGGTTGTTGTCGGCACCGGTCTAGGTGGAGCCGAG	2518334		
Query 361	AGGATTGTCGAGAGCTACGACCTGATGAATGCGGGCGGCCCGGAAGGTGTCCCGCTG	420		
Sbjct 2518335	AGGATTGTCGAGAGCTACGACCTGATGAATGCGGGCGGCCCGGAAGGTGTCCCGCTG	2518394		



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4.3.5 *Mycobacterium tuberculosis* H37Rv, complete genome Phylogenetic analysis for *kasA* gene

Sequence ID: [CP003248.2](#) Length: 4411709 Number of Matches: 1

Range 1: 1 to 1251 [GenBankGraphics](#)

Score	Expect	Identities	Gaps	Strand
2311 bits (1251)	0.0	1251/1251(100%)	0/1251(0%)	Plus/Plus
Query 1	TTGACCGATGACCCCGGTTTCAGGCTTCACCACAGTGTGGAACGCGGTCGTCTCCGAAC	60		
Sbjct 1	TTGACCGATGACCCCGGTTTCAGGCTTCACCACAGTGTGGAACGCGGTCGTCTCCGAAC	60		
Query 61	AACGGCGACCCCTAAGGTTGACGACGGACCCAGCAGTGATGCTAATCTCAGCGCTCCGCTG	120		
Sbjct 61	AACGGCGACCCCTAAGGTTGACGACGGACCCAGCAGTGATGCTAATCTCAGCGCTCCGCTG	120		
Query 121	ACCCCTCAGCAAAGGGCTTGGCTCAATCTCGTCCAGCCATTGACCATCGTCGAGGGGTTT	180		
Sbjct 121	ACCCCTCAGCAAAGGGCTTGGCTCAATCTCGTCCAGCCATTGACCATCGTCGAGGGGTTT	180		
Query 181	GCTCTGTTATCCGTGCCGAGCAGCTTTGTCCAAAACGAAATCGAGCGCCATCTGCGGGCC	240		
Sbjct 181	GCTCTGTTATCCGTGCCGAGCAGCTTTGTCCAAAACGAAATCGAGCGCCATCTGCGGGCC	240		
Query 241	CCGATTACCGACGCTCTCAGCCGCCGACTCGGACATCAGATCCAACTCGGGGTCCGCATC	300		
Sbjct 241	CCGATTACCGACGCTCTCAGCCGCCGACTCGGACATCAGATCCAACTCGGGGTCCGCATC	300		
Query 301	GCTCCGCCGGCGACCGACGAAGCCGACGACACTACCGTGCCGCCTTCGGAATACTCTGCT	360		
Sbjct 301	GCTCCGCCGGCGACCGACGAAGCCGACGACACTACCGTGCCGCCTTCGGAATACTCTGCT	360		
Query 361	ACCACATCGCCAGACACCACAACCGACAACGACGAGATTGATGACAGCGCTGCGGCACGG	420		
Sbjct 361	ACCACATCGCCAGACACCACAACCGACAACGACGAGATTGATGACAGCGCTGCGGCACGG	420		
Query 421	GGCGATAACCAGCAGAGTTGGCCAAGTTACTTCACCGAGCGCCGCAATACCGATTCC	480		
Sbjct 421	GGCGATAACCAGCAGAGTTGGCCAAGTTACTTCACCGAGCGCCGCAATACCGATTCC	480		
Query 481	GCTACCGCTGGCGTAACCAGCCTTAACCGTCGCTACACCTTGATACGTTTCGTTATCGGC	540		
Sbjct 481	GCTACCGCTGGCGTAACCAGCCTTAACCGTCGCTACACCTTGATACGTTTCGTTATCGGC	540		
Query 541	GCCTCCAACCGGTTTCGCGCACGCCGCCGCTTGGCGATCGCAGAAGCACCAGCCGCGCT	600		
Sbjct 541	GCCTCCAACCGGTTTCGCGCACGCCGCCGCTTGGCGATCGCAGAAGCACCAGCCGCGCT	600		
Query 601	TACAACCCCTGTTTCATCTGGGGCGAGTCCGGTCTCGGCAAGACACACCTGCTACACGG	660		
Sbjct 601	TACAACCCCTGTTTCATCTGGGGCGAGTCCGGTCTCGGCAAGACACACCTGCTACACGG	660		
Query 661	GCAGGCAACTATGCCCAACGGTTGTTCCCGGAATGCGGGTCAAATATGTCTCCACCGAG	720		
Sbjct 661	GCAGGCAACTATGCCCAACGGTTGTTCCCGGAATGCGGGTCAAATATGTCTCCACCGAG	720		
Query 721	GAATTACCAACGACTTCATTAACCTCGCTCCGCGATGACCGCAAGGTCGCATTCAAACGC	780		
Sbjct 721	GAATTACCAACGACTTCATTAACCTCGCTCCGCGATGACCGCAAGGTCGCATTCAAACGC	780		
Query 781	AGCTACCGCGACGTAGACGTGCTGTTGGTCGACGACATCCAATTCATTGAAGGCAAAGAG	840		
Sbjct 781	AGCTACCGCGACGTAGACGTGCTGTTGGTCGACGACATCCAATTCATTGAAGGCAAAGAG	840		
Query 841	GGTATTCAAGAGGAGTTCTTCCACACCTTCAACACCTTGACAATGCCAACAAGCAAATC	900		
Sbjct 841	GGTATTCAAGAGGAGTTCTTCCACACCTTCAACACCTTGACAATGCCAACAAGCAAATC	900		

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Query 901 GTCATCTCATCTGACCGCCACCCAAGCAGCTCGCCACCCTCGAGGACCGGCTGAGAACC 960
          |||||||
Sbjct 901 GTCATCTCATCTGACCGCCACCCAAGCAGCTCGCCACCCTCGAGGACCGGCTGAGAACC 960

Query 961 CGCTTTGAGTGGGGGCTGATCACTGACGTACAACCACCCGAGCTGGAGACCCGCATCGCC 1020
          |||||||
Sbjct 961 CGCTTTGAGTGGGGGCTGATCACTGACGTACAACCACCCGAGCTGGAGACCCGCATCGCC 1020

Query 1021 ATCTTGCGCAAGAAAGCACAGATGGAACGGCTCGCGGTCCCCGACGATGTCCTCGAACTC 1080
          |||||||
Sbjct 1021 ATCTTGCGCAAGAAAGCACAGATGGAACGGCTCGCGGTCCCCGACGATGTCCTCGAACTC 1080

Query 1081 ATCGCCAGCAGTATCGAACGCAATATCCGTGAACTCGAGGGCGCGCTGATCCGGGTCACC 1140
          |||||||
Sbjct 1081 ATCGCCAGCAGTATCGAACGCAATATCCGTGAACTCGAGGGCGCGCTGATCCGGGTCACC 1140

Query 1141 GCGTTCGCCTCATTGAACAAAACACCAATCGACAAAGCGCTGGCCGAGATTGTGCTTCGC 1200
          |||||||
Sbjct 1141 GCGTTCGCCTCATTGAACAAAACACCAATCGACAAAGCGCTGGCCGAGATTGTGCTTCGC 1200

Query 1201 GATCTGATCGCCGACGCCAACACCATGCAAATCAGCGCGGCGACGATCATG 1251
          |||||||
Sbjct 1201 GATCTGATCGCCGACGCCAACACCATGCAAATCAGCGCGGCGACGATCATG 1251

```

4.3.6 *Mycobacterium tuberculosis* strain H37Rv _CG chromosome, complete genome Phylogenetic analysis for *inhA* gene

Sequence ID: [CP072765.1](#) Length: 4415999 Number of Matches: 1

Range 1: 1675752 to 1676583 [GenBank](#)

Score	Expect	Identities	Gaps	Strand
1537 bits(832)	0.0	832/832(100%)	0/832(0%)	Plus/Plus
Query 1	TGACACAACACAAGGACGCACATGACAGGACTGCTGGACGGCAAACGGATTCTGGTTAGC	60		
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Sbjct 1675872	GCCCAGCTGGTGCTACCGGGTTCGACCGGCTGCGGCTGATTACAGCGCATCACCGACCGG	1675931		
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Sbjct 1675932	CTGCCGGCAAAGGCCCGCTGCTCGAACTCGACGTGCAAAACGAGGAGCACCTGGCCAGC	1675991		
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Sbjct 1675992	TTGGCCGGCCGGGTGACCGAGGCGATCGGGGCGGGCAACAAGCTCGACGGGTGGTG CAT	1676051		
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Sbjct 1676052	TCGATTGGGTTTCATGCCGCAGACCGGGATGGGCATCAACCCGTTCTTCGACGCGCCCTAC	1676111		
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Sbjct 1676352 ATCCGGACGCTGGCGATGAGTGCGATCGTCGGCGGTGCGCTCGGCGAGGAGGCCGCGGCC 1676411

Query 661      CAGATCCAGCTGCTCGAGGAGGGCTGGGATCAGCGCGCTCCGATCGGCTGGAACATGAAG 720
               |||
Sbjct 1676412 CAGATCCAGCTGCTCGAGGAGGGCTGGGATCAGCGCGCTCCGATCGGCTGGAACATGAAG 1676471

Query 721      GATGCGACGCCGGTCGCCAAGACGGTGTGCGCGCTGCTGTCTGACTGGCTGCCGGCGGACC 780
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Sbjct 1676472 GATGCGACGCCGGTCGCCAAGACGGTGTGCGCGCTGCTGTCTGACTGGCTGCCGGCGGACC 1676531

Query 781      ACGGGTGACATCATCTACGCCGACGGCGGCGCGCACACCCAATTGCTCTAGA 832
               |||
Sbjct 1676532 ACGGGTGACATCATCTACGCCGACGGCGGCGCGCACACCCAATTGCTCTAGA 1676583

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After aligning the sequence reads to the reference genome (CP003248), there were no open gaps found within the nucleotide sequences, which meant that there were 100% identities and 0% gaps between the reference genome subject (Sbjct) and the query genes (Query) *InhA* (CP072765.1) with 831/831 bits, and *kasA* (NC_000962.3; CP072765.1; CP007027.1; CP053903.1; CP003248.2) with 1251/1251 bits.

4.4 Results of synthesis and characterization of benzyl-methoxy-protected aspalathin analog via C-glucosylation of pentamethoxy dihydropropane for drug synthesis of racemic TLM

These results are available in the published article in section 3.10.3.

4.4.1 Nuclear Magnetic Resonance (NMR) data for benzyl-methoxyl protected aspalathin analogue via C-glucosylation of pentamethoxydihydropropane

4.4.1.1 Introduction

¹H NMR spectrum provides details about the chemical environment of different hydrogen atoms in a molecule. The detail includes the number of chemically distinguished hydrogen environments shown by the number of peaks, the chemical shift (δ) of these peaks, and the coupling patterns between adjacent hydrogen atoms, which provide information about the connectivity of atoms within the molecule. The

chemical shift is measured in parts per million (ppm) and depends on the electronic environment of the hydrogen atoms (Lambert et al., 2019). The bonding strength between neighboring hydrogen atoms is called coupling constant (J) and is measured in Hertz (Hz). Multiplicity is the splitting pattern of each peak, which indicates the number of adjacent hydrogen atoms where isolated hydrogen atoms are called singlets (s), one neighboring hydrogen atom is doublets (d), two neighboring hydrogen atoms are triplets (t), the naming continues like that according to the number of the neighboring hydrogen atoms (Mlynarik, 2017).

^{13}C NMR gives information on a carbon connectivity and structural features of the organic molecule. The resulting spectrum shows peaks corresponding to the different types of carbon atoms in a molecule, providing details about the number of distinct carbon environments and the chemical environments surrounding each carbon atom (Lambert et al., 2019).

Heteronuclear Single Quantum Coherence (HSQC) is a two-dimensional NMR used to correlate proton and carbon chemical shifts in a molecule. It gives details about the bonding between hydrogen and carbon atoms in a molecule (Lambert et al., 2019).

Correlation Spectroscopy (COSY) is also a two-dimensional NMR that gives details about the correlation between hydrogen atoms in a molecule by showing cross-peaks between coupled protons (Lambert et al., 2019).

4.4.1.2 3, 4, 2', 4', 6'-pentamethoxy-chalcone (4)

Notable in the NMR of (4) were as follows:

^1H NMR (600 MHz, CDCl_3 , Me_4Si): 7.25 (H- α , d, $J=15.5$ Hz), 7.09 (H-6, dd, $J = 2.2$; 8.5 Hz), 7.06 (H-2, d, $J= 2.2$ Hz), 6.88 (H- β , d, $J = 15.5$ Hz), 6.84 (H-5, d, $J = 8.5$ Hz), 6.17 (H-3 & H-5, s, $2\times\text{H}$), 4.90~5.02 (ArOCH_3 , s, $15\times\text{H}$) (Figure 4.1).

COSY NMR (CDCl_3): Cross-peaks between H- α and H- β , H-6 and H-5 (Figure 4.2)

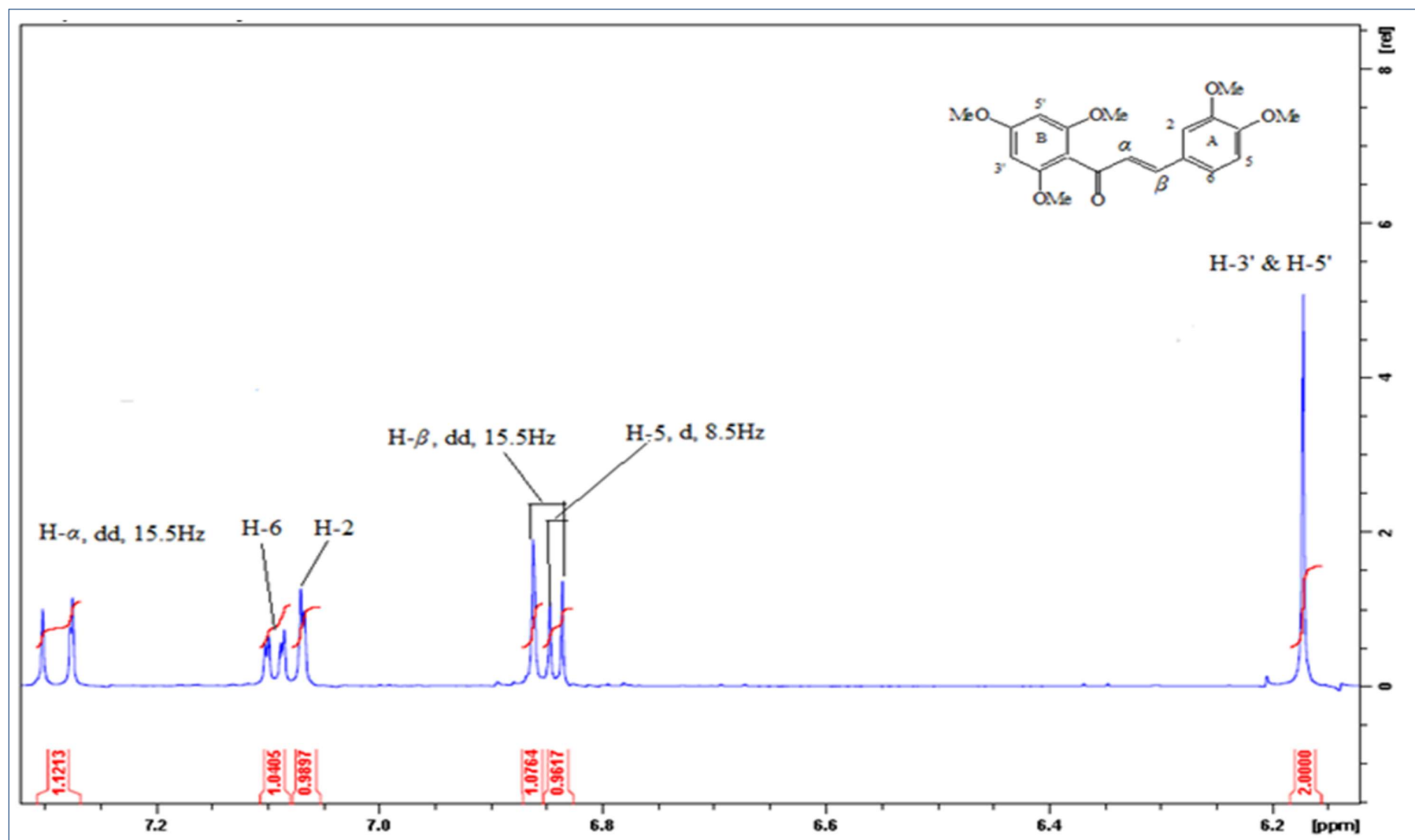


Figure 4.1: ^1H NMR spectrum of 3, 4, 2', 4', 6'-pentamethoxy-chalcone (4)

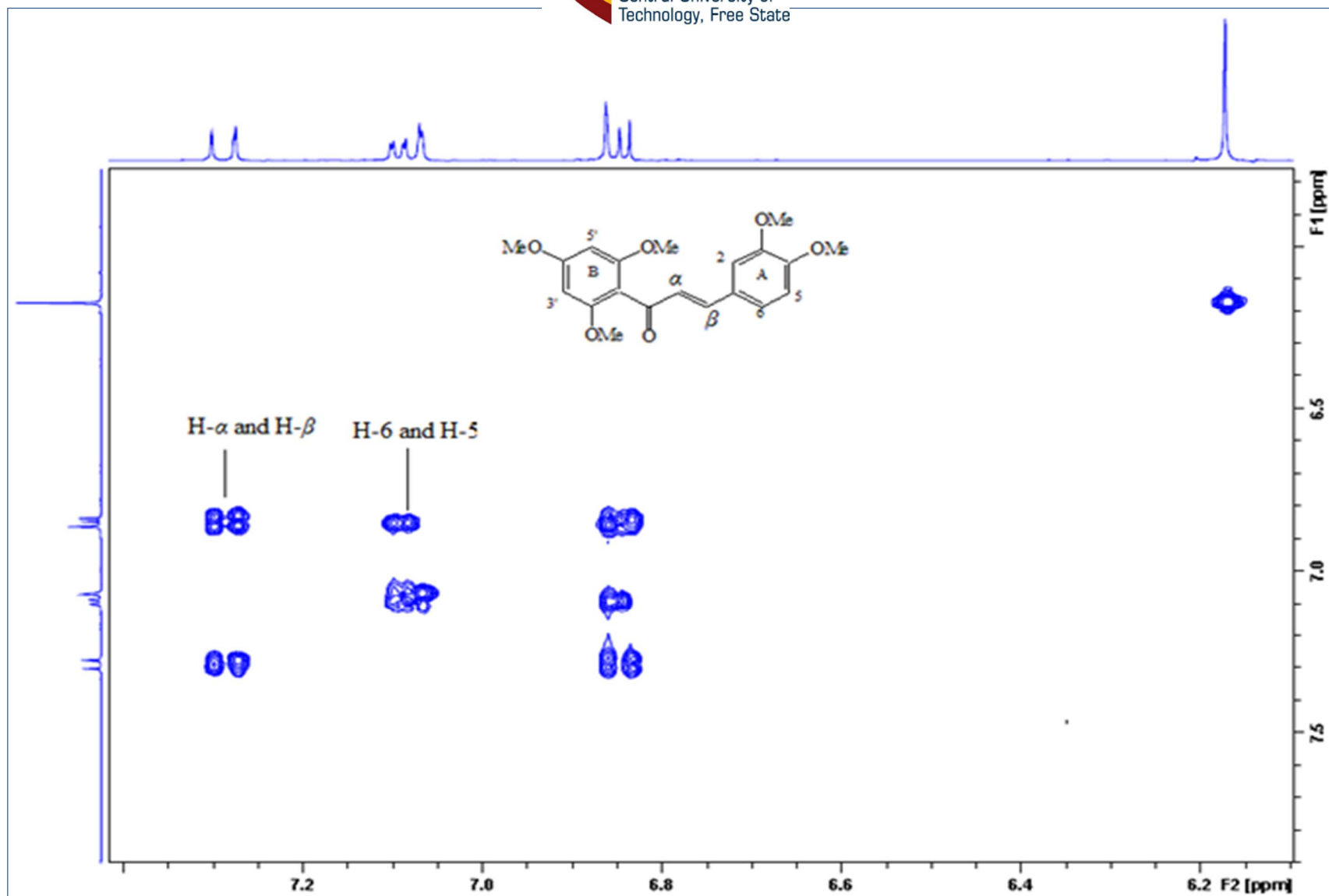


Figure 4.2: COSY NMR spectrum of 3, 4, 2', 4', 6'-pentamethoxy-chalcone (4).

4.4.1.3 3, 4, 2', 4', 6'-pentahydroxy-dihydrochalcone (5)

Notable in the NMR of (5) were as follows:

^1H NMR: (600 MHz, CDCl_3 , Me_4Si ; Plate 27): 6.74~6.80 (H-2'' & H5'' & H-6'', m, 3×H), 6.21 (H-3' & H-5', s, 2×H), 3.88 (ArOCH_3 , 3×H), 3.86 (ArOCH_3 , 3×H), 3.82 (ArOCH_3 , 3×H), 3.80 (ArOCH_3 , 6×H), 2.58 (H-1 & H-3, m, 4×H), 1.77 (H-2, m, 2×H) (Figure 4.3)

^{13}C NMR: (150 MHz, CDCl_3 , Me_4Si): 158.7~159.3 (aromatic O-C, 5×C), 120.28~148.7 (aromatic C, 7×C), 111.8, 111.5, 111.2 (C-2'' & C5'' & C-6'', 3×C), 90.6 (C-3' & C-5', 2×C), 55.9, 55.8, 55.6, 55.3, 55.2 (ArOCH_3 , 5×C), 35.5 (C-1 & C-3, 2×C), 31.1 (C-2) (Figure 4.4).

HSQC (CDCl_3): Cross-peaks between H-2'' and C-2''; H5'' and C5''; H-6'' and C-6''; H-3' & H-5' and C-3' & C-5'; H-1 and C-1; H-2 and C-2; H-3 and C-3 (Figure 4.5).

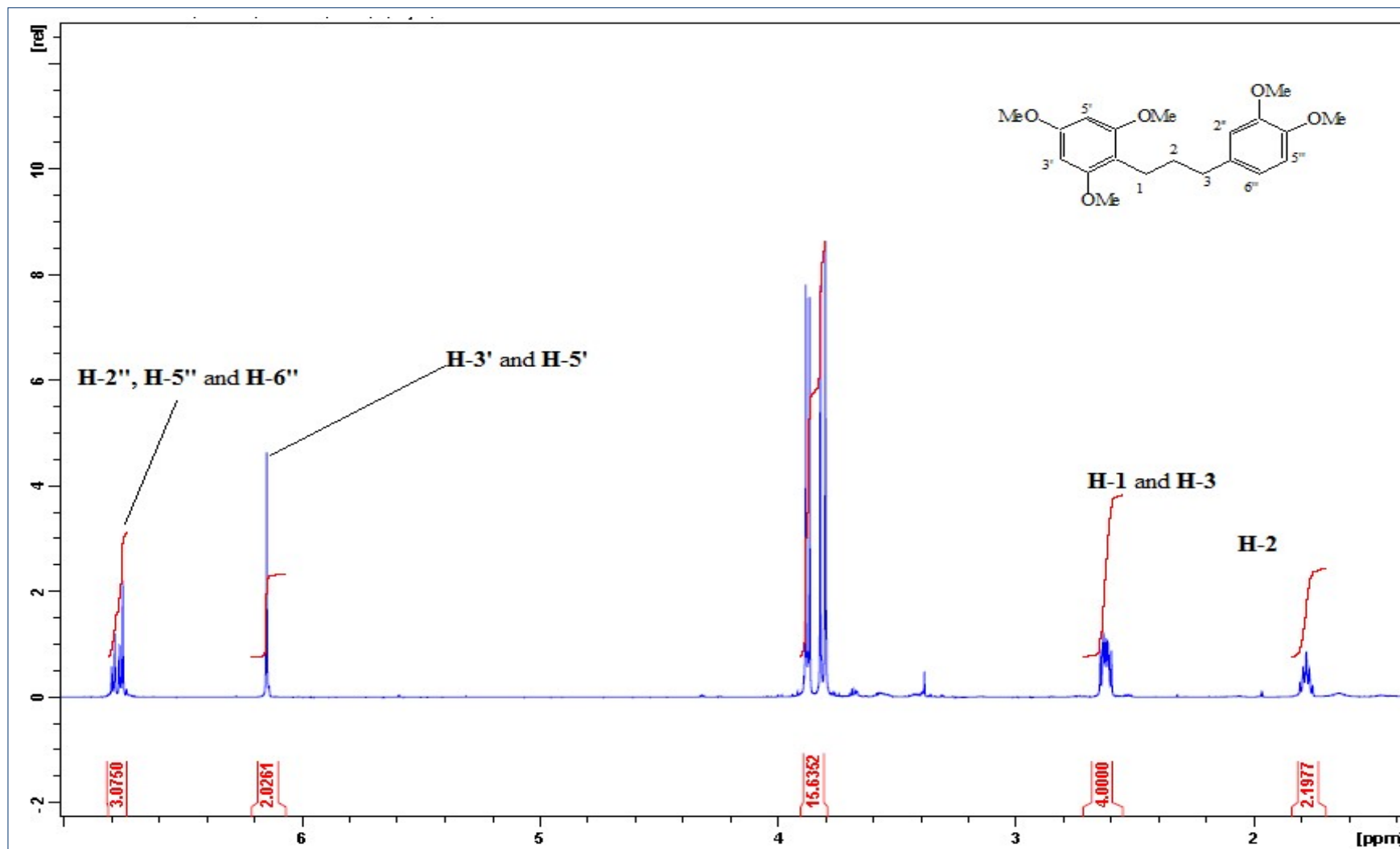


Figure 4.3: ^1H NMR spectrum of 3, 4, 2', 4', 6'-pentahydroxy-dihydrochalcone (5)

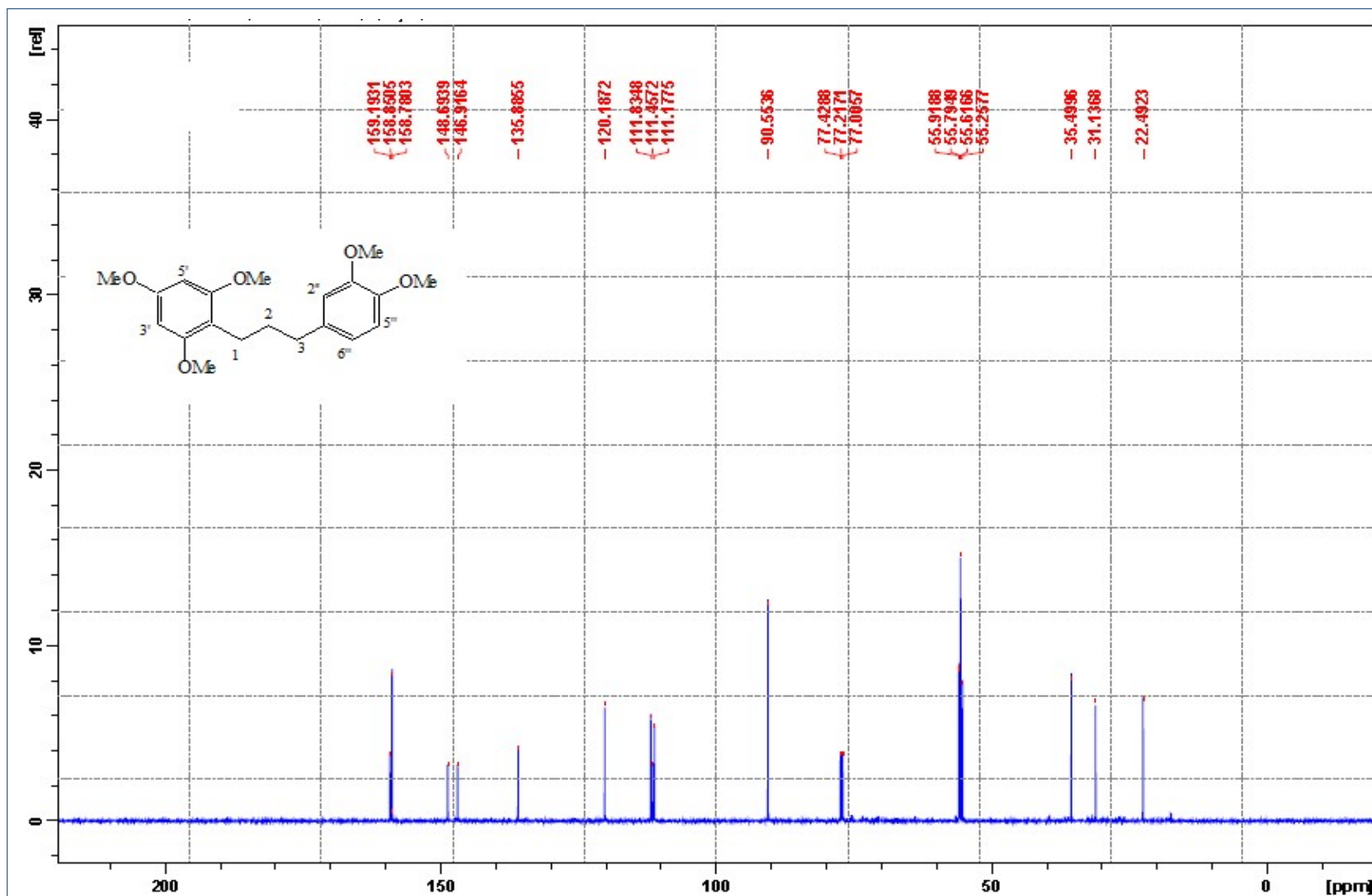


Figure 4.4: ^{13}C NMR spectrum of 3, 4, 2', 4', 6'-pentahydroxy-dihydrochalcone (5)

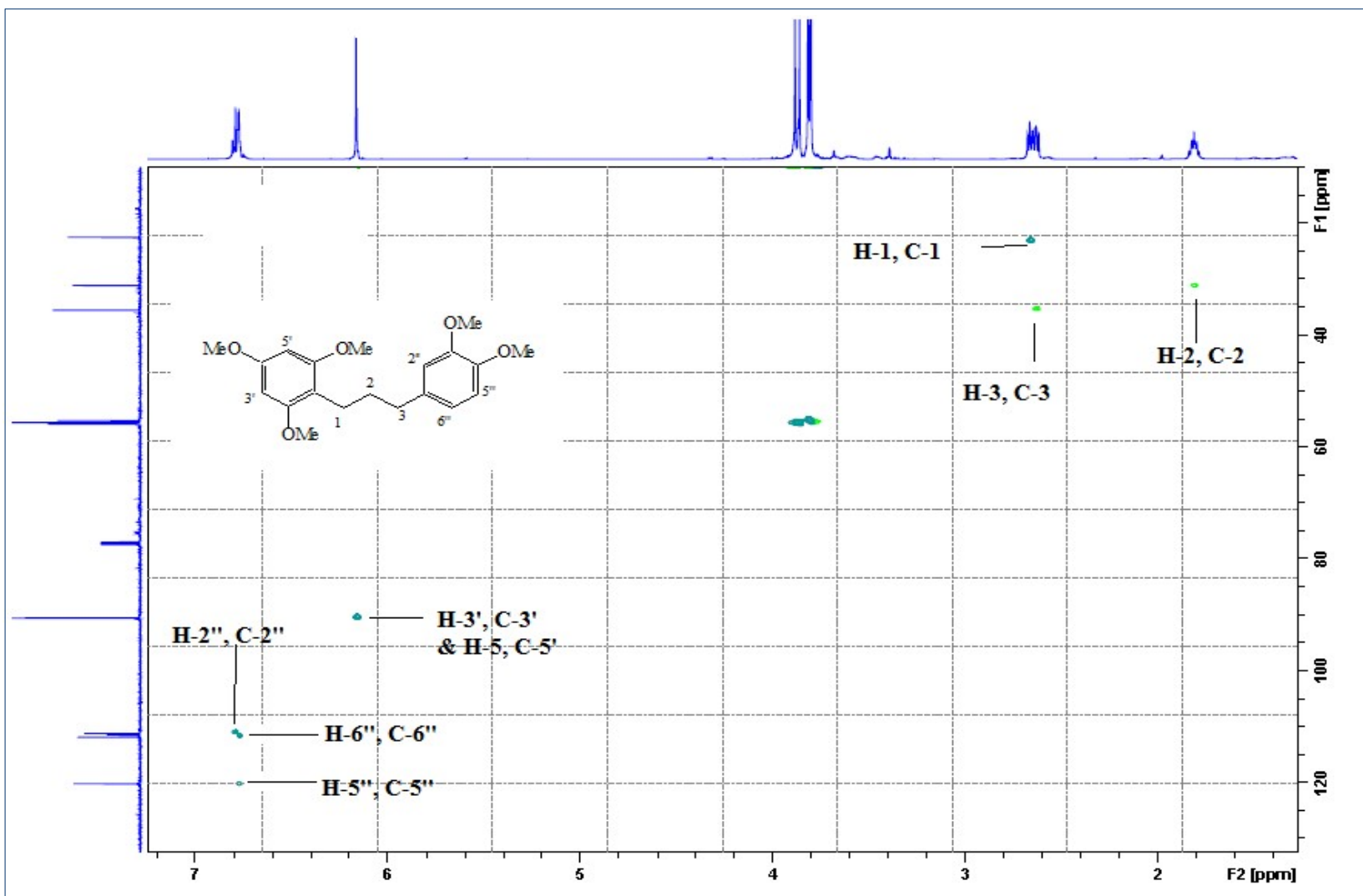


Figure 4.5: HSQC NMR spectrum of 3, 4, 2', 4', 6'-pentahydroxy-dihydrochalcone (5)

4.4.1.4 3'',4'',2',4',6'-pentamethoxy-3'-C-(2''', 3''', 4''', 6'''-tetra-O-benzyl- β -D-glucopyranosyl)-1,3-diarylpropane (7)

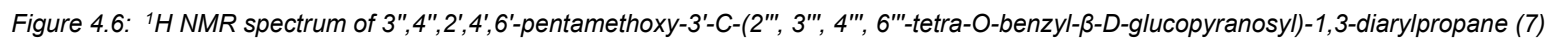
Notable in the NMR of (7) were as follows:

^1H NMR: (600 MHz, DMSO- d_6 , Me $_4$ Si; 140 °C): 7.10~7.40 (Ar-H, 20×H), 6.85 (H-6'', dd, J = 2.2; 8.5 Hz), 6.77 (H-2'', d, J = 2.2 Hz), 6.65 (H-5'', d, J = 8.5 Hz), 6.48 (H-5', s), 4.73 (H-1''', d, J = 9.8 Hz), 4.01~4.95 (benzylic H, 8×H), 4.37 (H-2''', t, J = 9.8 Hz), 3.50~3.80 (ArOCH $_3$, 15×H), 3.69 (H-3''', d, J = 9.8 Hz), 3.63 (H-6''', m, 2×H), 3.60 (H-4''', t, J = 9.8 Hz), 3.53 (H-5''', m), 2.63 (H-1, m), 2.57 (H-3, t, J = 7.5 Hz), 1.80 (H-2, m) (Figure 4.6).

^{13}C NMR: (150 MHz, DMSO- d_6 , Me $_4$ Si; 140 °C): 159.8 (C-2' & C-4' & C-6'), 150.2 (C-3''), 148.5 (C-4''), 114.3~139.6 (Ar-C, 24×C), 121.2 (C-5''), 115.0 (C-2''), 114.7 (C-6''), 95.5 (C-5'), 87.3 (C-3'''), 80.6 (C-2'''), 79.5 (C-4'''), 79.4 (C-5'''), 74.2 (C-1'''), 70.0~80.1 (benzylic OCH $_2$, 8×C), 62.7 (C-6'''), 56.5~57.2 (ArOCH $_3$, 5×C), 35.4 (C-3), 31.5 (C-2), 23.4 (C-1) (Figure 4.7).

HSQC NMR: (DMSO- d_6 , 140 °C): Cross-peaks between H-5'' (6.65 ppm) and C-5'' (121.2 ppm); H-2'' (6.77 ppm) and C-2'' (115.0 ppm); H-6'' (6.85 ppm) and C-6'' (114.7 ppm); H-5' (6.48 ppm) and C-5' (95.5 ppm); H-1''' (4.73 ppm) and C-1''' (74.2 ppm); H-2''' (4.37 ppm) and C-2''' (80.6 ppm); H-3''' (3.69 ppm) and C-3''' (87.3 ppm); H-4''' (3.60 ppm) and C-4''' (79.5 ppm); H-5''' (3.53 ppm) and C-5''' (79.4 ppm); H-6''' (3.63 ppm) and C-6''' (62.7 ppm); H-1 (2.63 ppm) and C-1 (23.4 ppm); H-3 (2.57 ppm) and C-3 (35.4); H-2 (1.80 ppm) and C-2 (31.5 ppm); O-benzylic carbons and protons; OMe carbons and protons (Figure 4.8)

COSY NMR (DMSO- d_6 , 140 °C): Cross-peaks between H-1''' and H-2''; H-2''' and H-3''; H-4''' and H-5''; H-5''' and H-6''; H-2 and H-1 & H-3 (Figure 4.9).



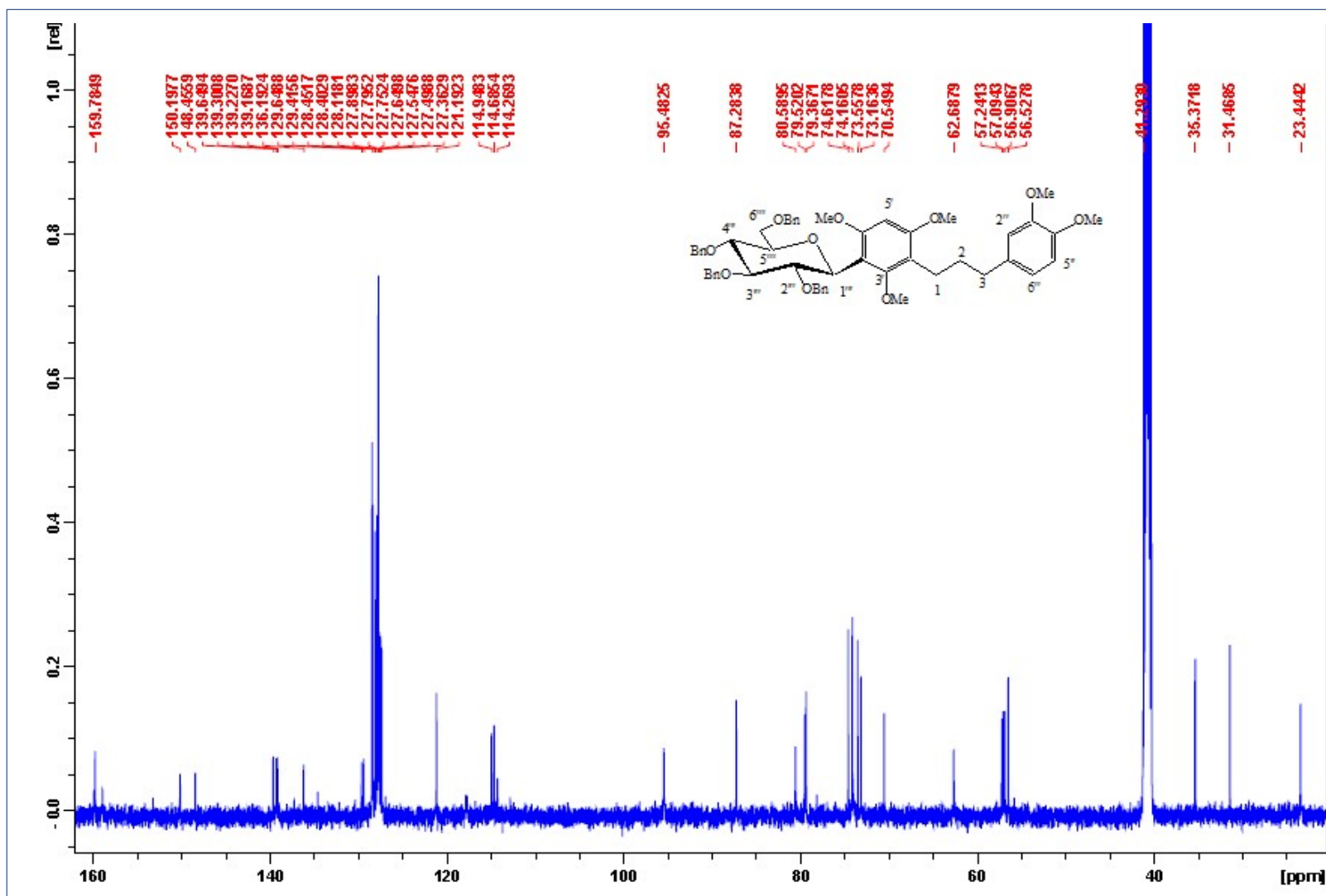


Figure 4.7: ^{13}C NMR spectrum of 3',4'',2',4',6'-pentamethoxy-3'-C-(2'', 3'', 4'', 6''-tetra-O-benzyl- β -D-glucopyranosyl)-1,3-diarylpropane (7)

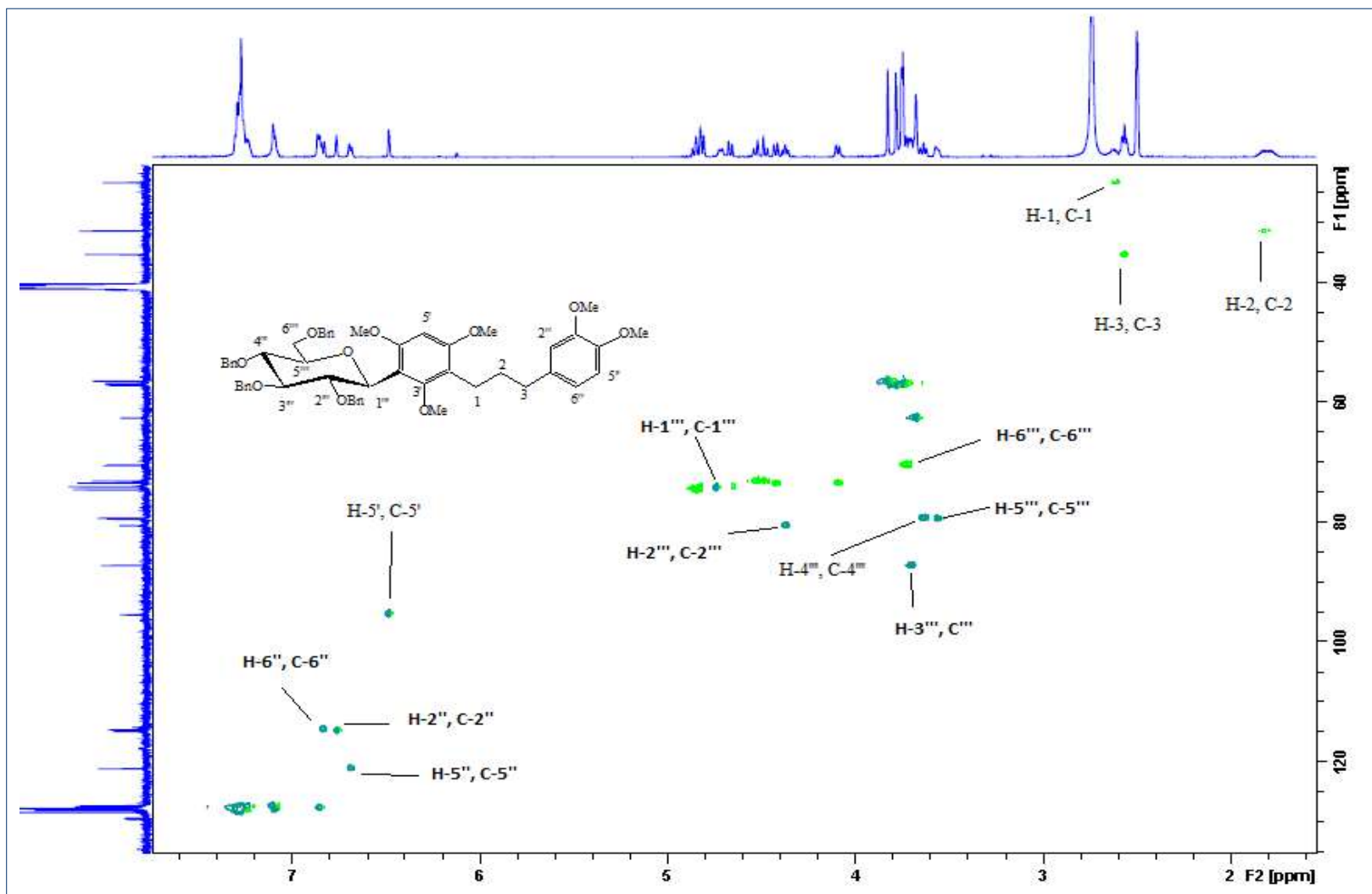


Figure 4.8: HSQC NMR spectrum of 3'',4'',2',4',6'-pentamethoxy-3'-C-(2'', 3'', 4'', 6'')-tetra-O-benzyl-β-D-glucopyranosyl)-1,3-diarylpropane (7)

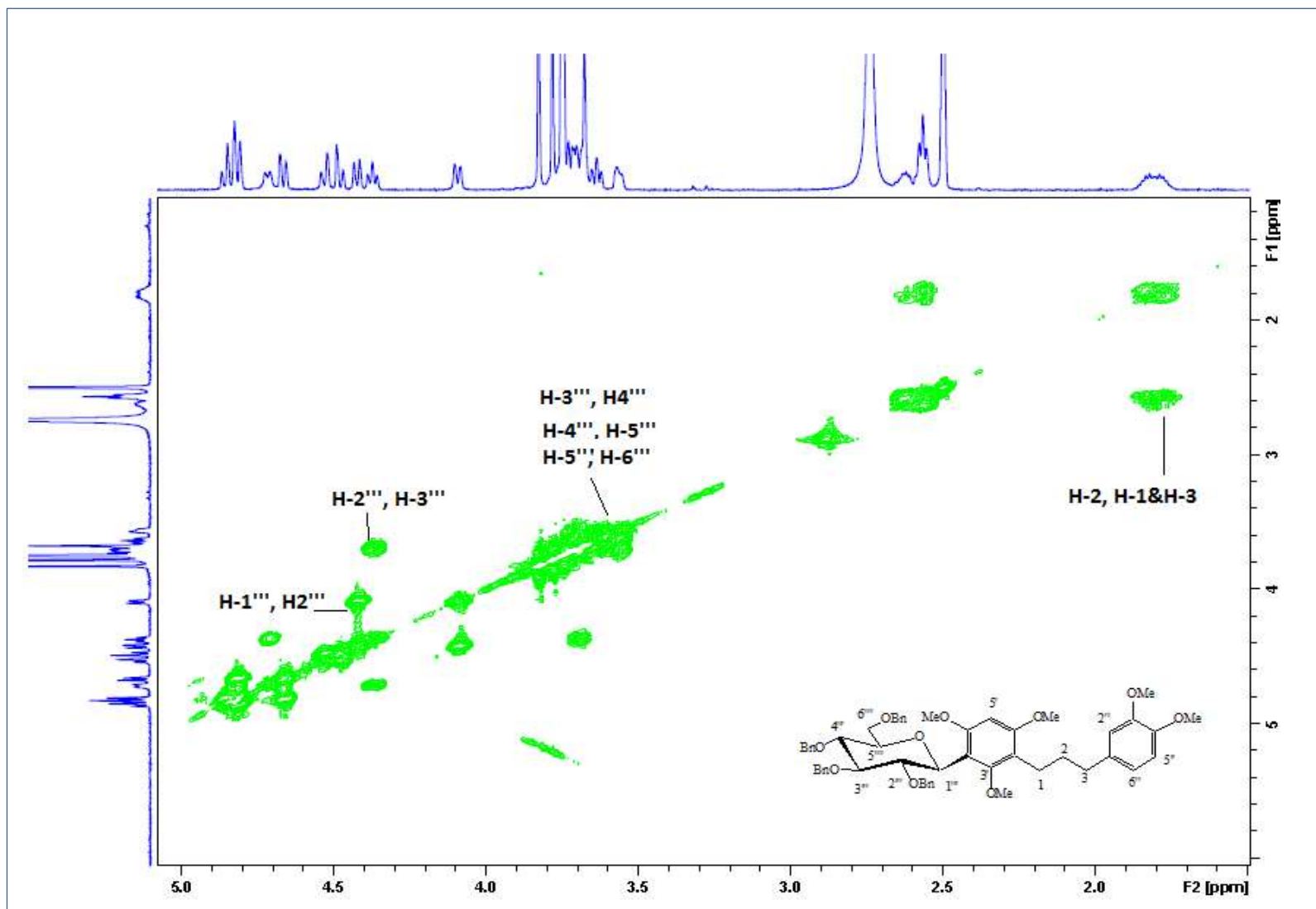


Figure 4.9: COSY NMR spectrum of 3'',4'',2',4',6'-pentamethoxy-3'-C-(2''', 3''', 4''', 6'''-tetra-O-benzyl- β -D-glucopyranosyl)-1,3-diarylpropane (7).

4.4.1.5 3,4,2',4',6'-pentamethoxy-3'-C-(2'',3'',4'',6''-tetra-O-benzyl- β -D-glucopyranosyl)-dihydrochalcone (8)

Notable in the NMR of (8) were as follows:

^1H NMR: (600 MHz, DMSO- d_6 , Me $_4$ Si; 140 °C): 6.82-7.60 (aromatic Hs, 20×H), 6.52 (H-5', s), 4.55-4.90 (benzylicHs, 8×H), 4.73 (H-1'', d, J = 9.8 Hz), 4.38 (H-2'', t, J = 9.8 Hz), 3.75~3.95 (ArOCH $_3$, 15×H), 3.76 (H-6'', m, 2×H), 3.72 (H-3'', d, J = 9.8 Hz), 3.63 (H-4'', t, J = 9.8 Hz) δ = 3.60 (H-5'', m), 3.05 (H- α), 2.93 (H- β) (Figures 4.10 & 4.11).

^{13}C NMR: (150 MHz, DMSO- d_6 , Me $_4$ Si; 140 °C): 198.4 (CO-C), 159.7, 154.3, 149.9, 139.7, 139.3 (O-aromatic, 5× C), 113.0~139.3 (aromatic, 31×C), 95.5 (C-3' & C-5'), 87.3 (C-3''), 80.6 (C-2''), 79.5 (C-4''), 79.4 (C-5''), 73.6 (C-1''), 74.6, 74.2, 73.2, 70.6 (benzylic OCH $_2$, 4×C), 62.9 (C-6''), 56.5~57.3 (ArOCH $_3$, 5×C), 38.8 (C- α), 19.7 (C- β) (Figure 4.12)

HSQC NMR: (DMSO- d_6 , 140 °C): Cross-peaks between H-5' (6.52 ppm) and C-5' (95.5 ppm); H-1''(4.73 ppm) and C-1'' (73.6 ppm); H-2'' (4.38 ppm) and C-2'' (80.6 ppm); H-3'' (3.72 ppm) and C-3'' (87.3 ppm); H-4'' (3.63 ppm) and C-4'' (79.5 ppm); H-5'' (3.60 ppm) and C-5'' (79.4 ppm); H-6'' (3.76 ppm) and C-6'' (62.9 ppm); H- α (3.05 ppm) and C- α (38.8 ppm); H- β (2.93 ppm) and C- β (19.7 ppm); O-benzylic carbons and protons; OMe carbons and protons (Figure 4.13).

COSY NMR: (DMSO- d_6 , 140 °C): Cross-peaks between H-1'' and H-2''; H-2'' and H-3''; H-4'' and H-5''; H-5'' and H-6''; H- α and H- β (Figure 4.14).

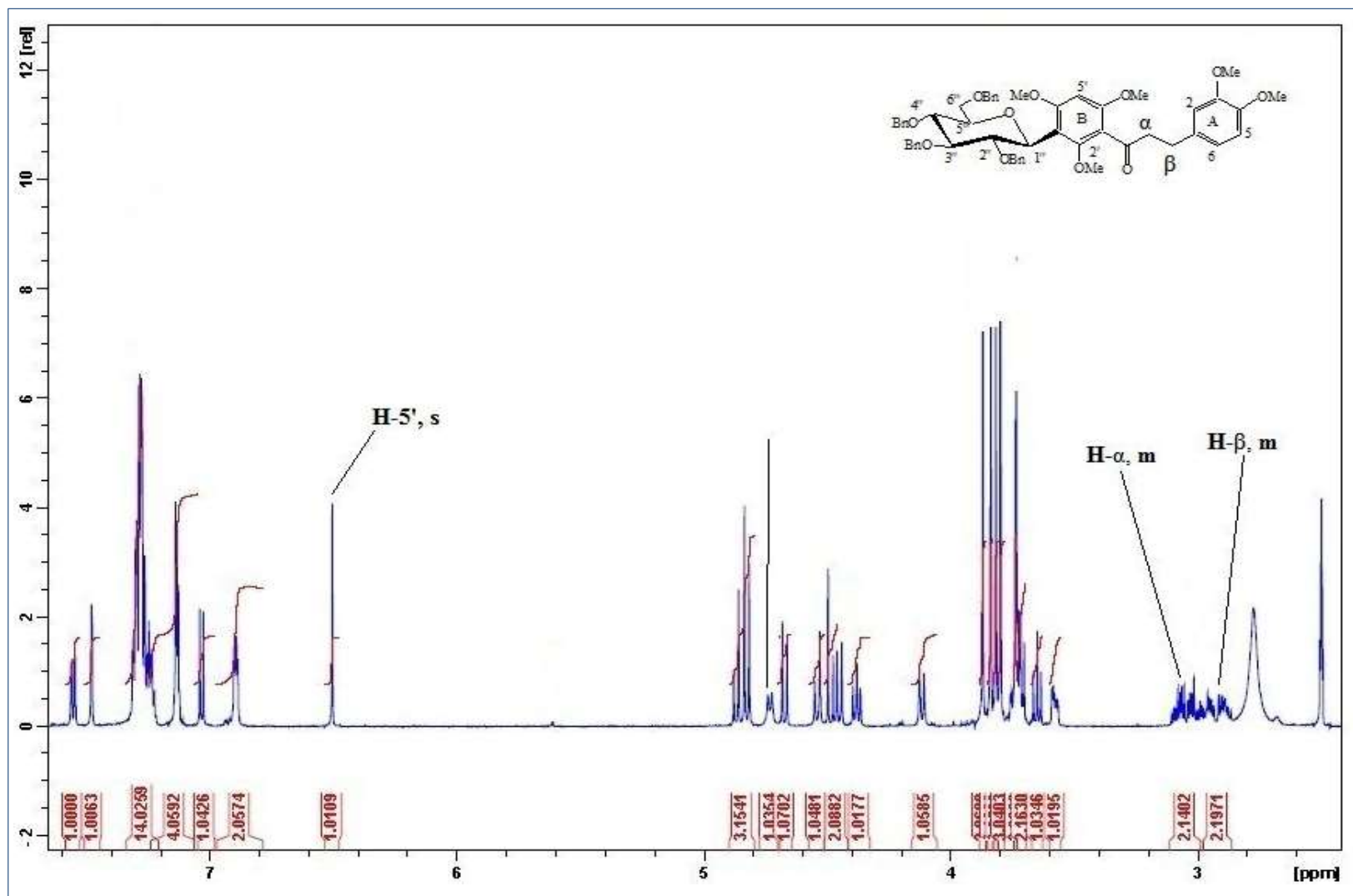


Figure 4.10: ¹H NMR spectrum of 3,4,2',4',6'-pentamethoxy-3'-C-(2'',3'',4'',6'')-tetra-O-benzyl-β-D-glucopyranosyl)-dihydrochalcone (8)

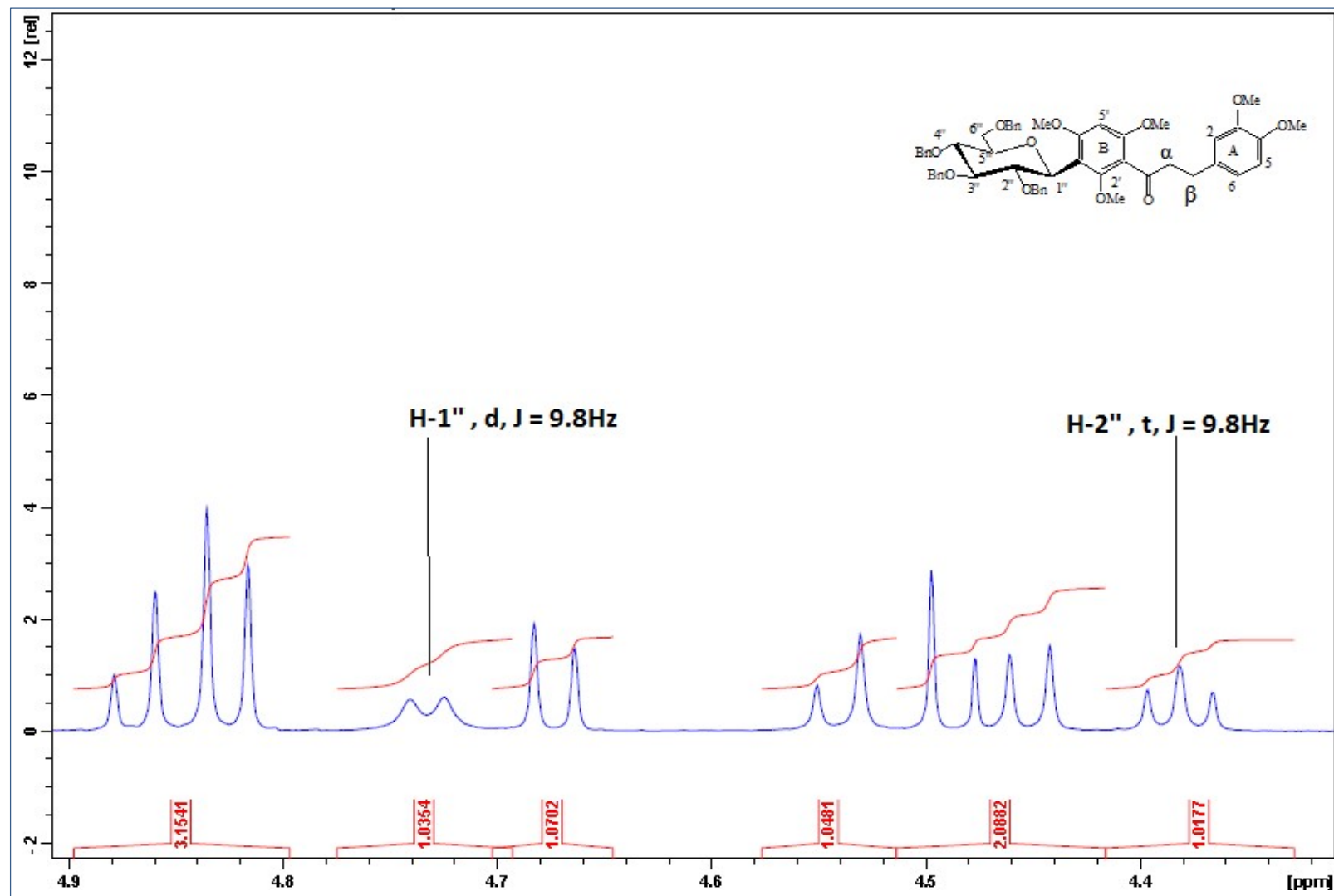


Figure 4.11: ^1H NMR spectrum of 3,4,2',4',6'-pentamethoxy-3'-C-(2'',3'',4'',6'')-tetra-O-benzyl- β -D-glucopyranosyl)-dihydrochalcone (8) H-1, H-2 J

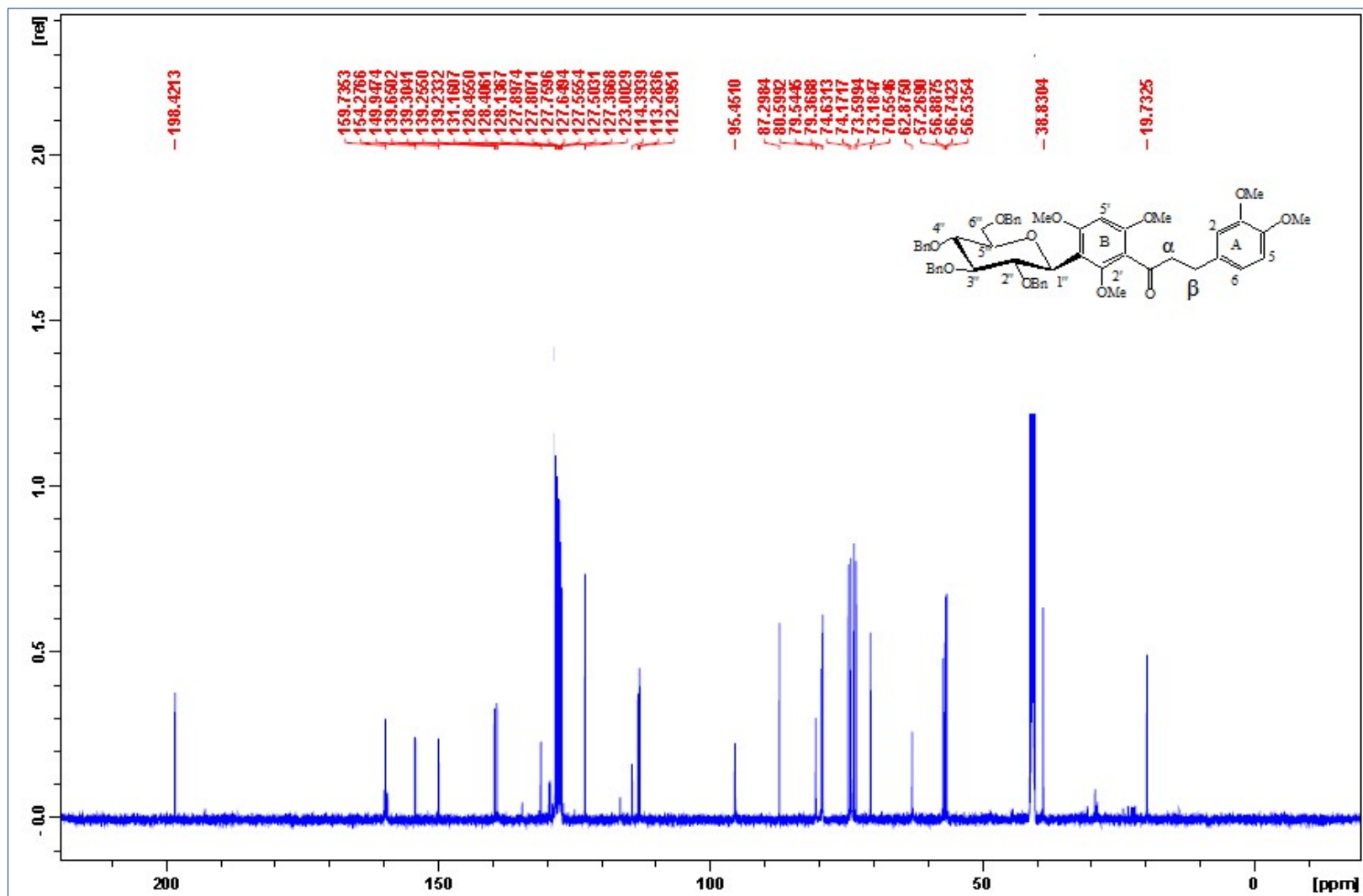


Figure 4.12.: ^{13}C NMR spectrum of 3,4,2',4',6'-pentamethoxy-3'-C-(2'',3'',4'',6'')-tetra-O-benzyl-β-D-glucopyranosyl)-dihydrochalcone (8)

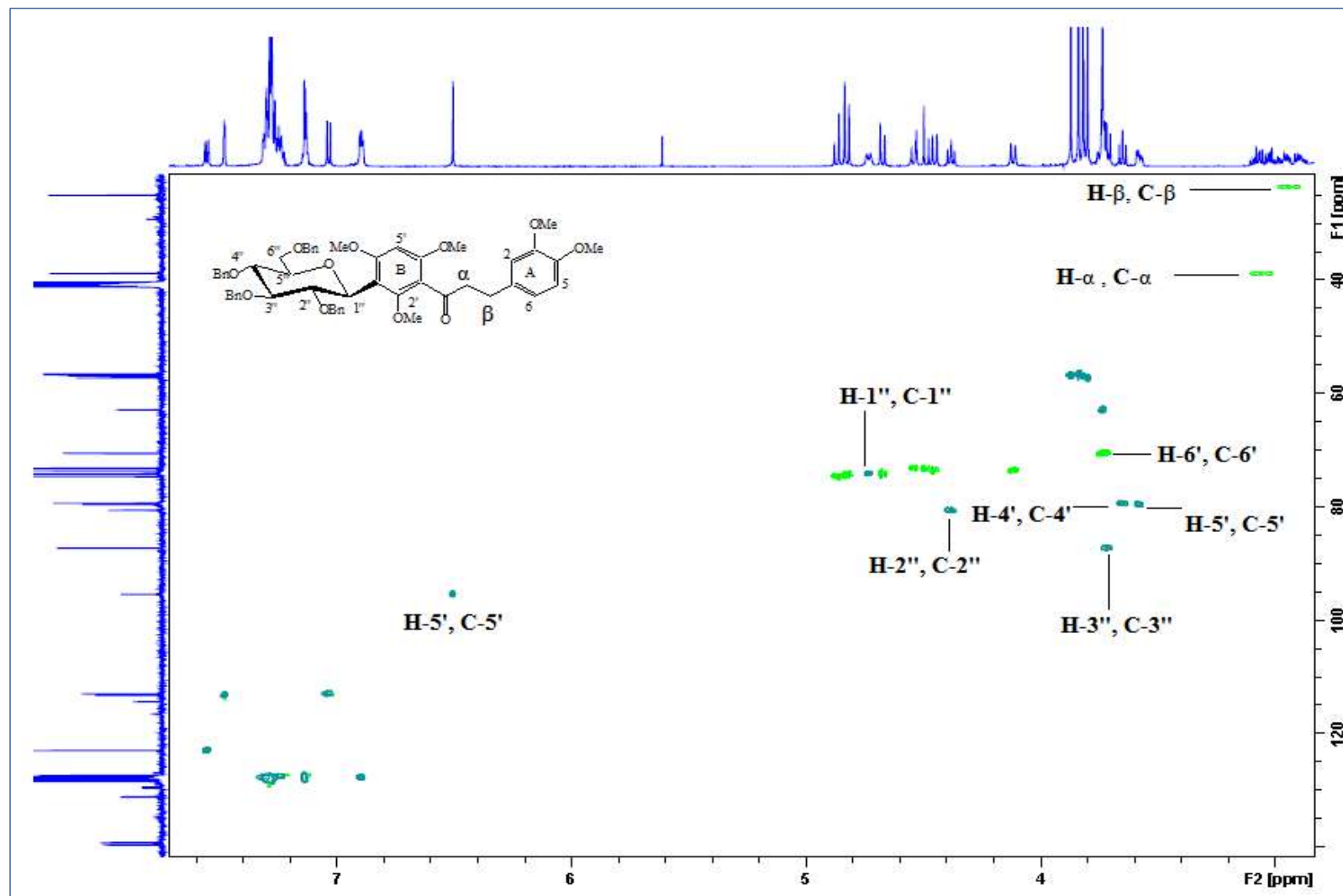


Figure 4.13: HSQC NMR spectrum of 3,4,2',4',6'-pentamethoxy-3'-C-(2'',3'',4'',6''-tetra-O-benzyl-β-D-glucopyranosyl)-dihydrochalcone (8)

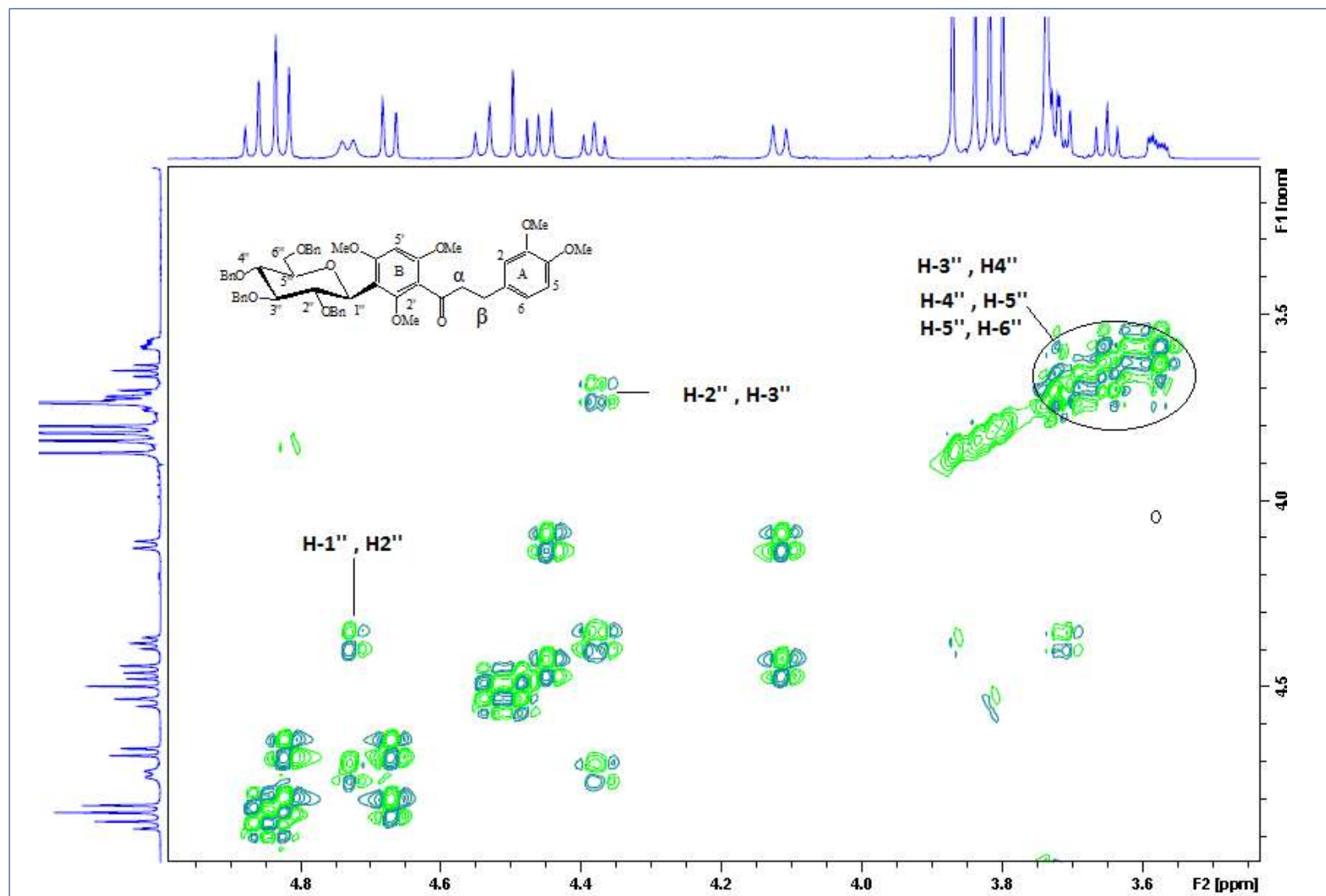


Figure 4.14: COSY NMR spectrum of 8 3,4,2',4',6'-pentamethoxy-3'-C-(2'',3'',4'',6''-tetra-O-benzyl- β -D-glucopyranosyl)-dihydrochalcone (8)

4.5 Results of Synthesis of Racemic Thiolactomycin

Seven TLM drug compounds (Figure 4.15) were synthesized by a convenient five-step procedure for chemical synthesis by Wang and Salvino⁵ method. Only GPK91 and GPK96 showed highest binding capacity at the binding sites of InhA and KasA therefore selected for potential drug candidates for antimicrobial drugs.

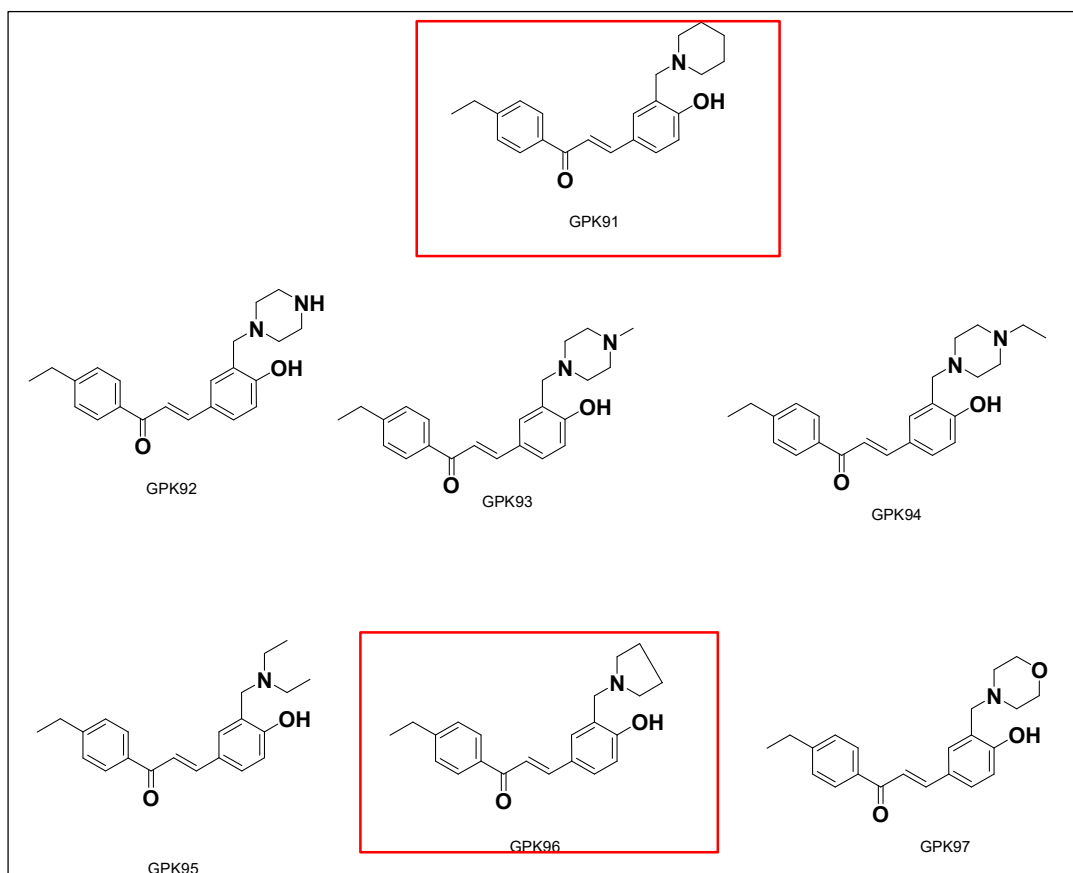


Figure 4.15: Designed Thiolactomycin ligand compounds

4.6 Docking Results

4.6.1 Introduction

Docking involves predicting the favoured alignment of one molecule relative to another when they come together to create a stable complex. This technique is vital in structural molecular biology and computer-assisted drug design. The objective of ligand-protein docking is to anticipate the primary binding configuration of a ligand with a protein of known three-dimensional structure.

4.6.2 Results of thiolactomycin derivative compounds docking with InhA

The reference ligand, TLM, formed a hydrogen bond interaction with LYS₁₆₅ and TYR₁₅₈ when docked with the InhA protein (Table 4.1; Figure 4.16). The results suggested that all the designed ligands had a high affinity toward the binding pocket of InhA. GPK91 showed the hydrogen bond interaction with GLY₉₆, ALA₁₉₈, and ILE₂₉₄, had the best docking score of -8.88 binding energy. The other ligands also had better docking scores than the reference compound, with the scores as follows: GPK94 -8.767, GPK93 -8.335, GPK95 -8.162, GPK92 -7.763, GPK97 -7.709, and GPK96 -7.469 (Figure 4.17). The reference ligand TLM had a low affinity towards the receptor, with a docking score of -7.021.

Table 4.1. The results of TLM compounds interaction binding energy at the binding site of InhA

Protein	Compound	Binding energy	Interacting amino acid
InhA 3FNE	GPK91	-8.880	GLY ₉₆ ; ALA ₁₉₈ ; ILE ₂₉₄
	GPK94	-8.767	
	GPK93	-8.335	
	GPK95	-8.162	
	GPK92	-7.763	
	GPK97	-7.709	
	GPK96	-7.496	
	TLM	-7.021	LYS ₁₆₅ ; TYR ₁₅₈

During interaction with InhA 3FNE, TLM formed a covalent bond with LYS₁₆₅. The racemic TLM ligand was oriented in such a way that thioester carbonyl oxygen is also bound to ILE₁₂₂ and TYR₁₅₈ at the terminal carboxyl group (Figure 4.16). This allowed

the electrons to move away from the carboxyl group of the thiolactone ring of the TLM as we bent thiolactone with the binding site.

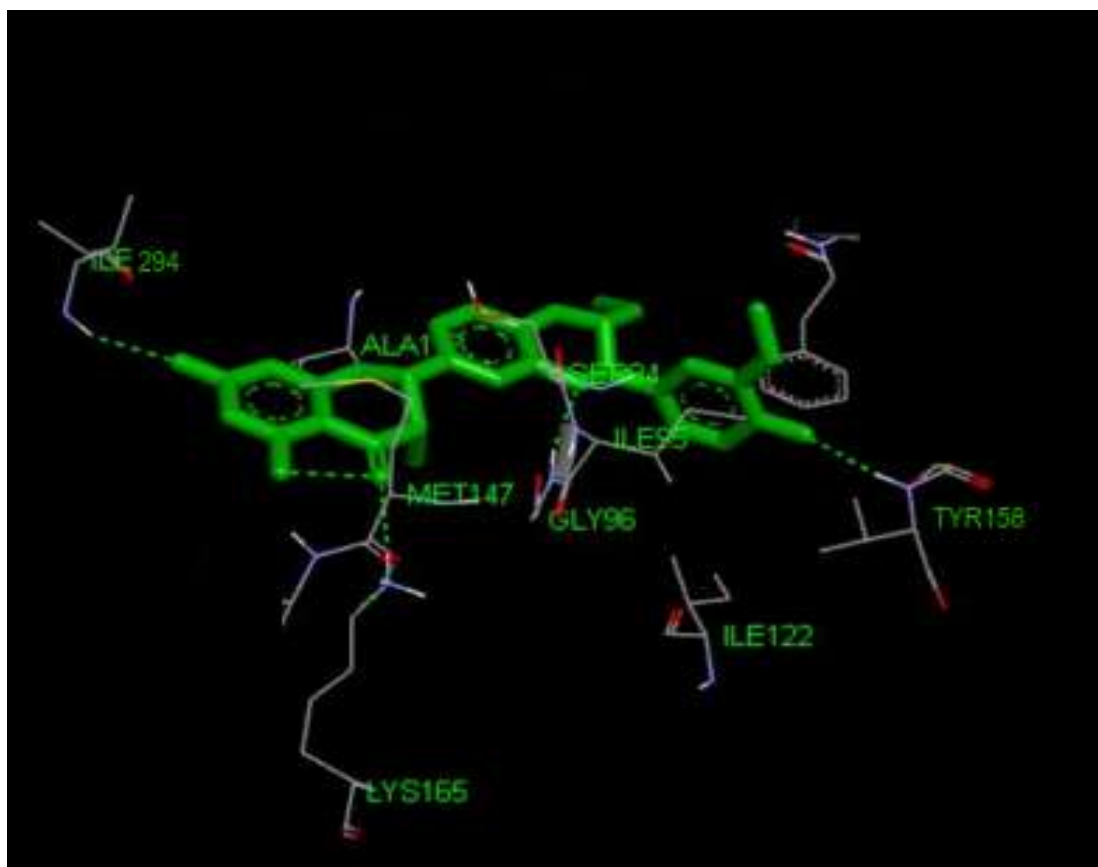
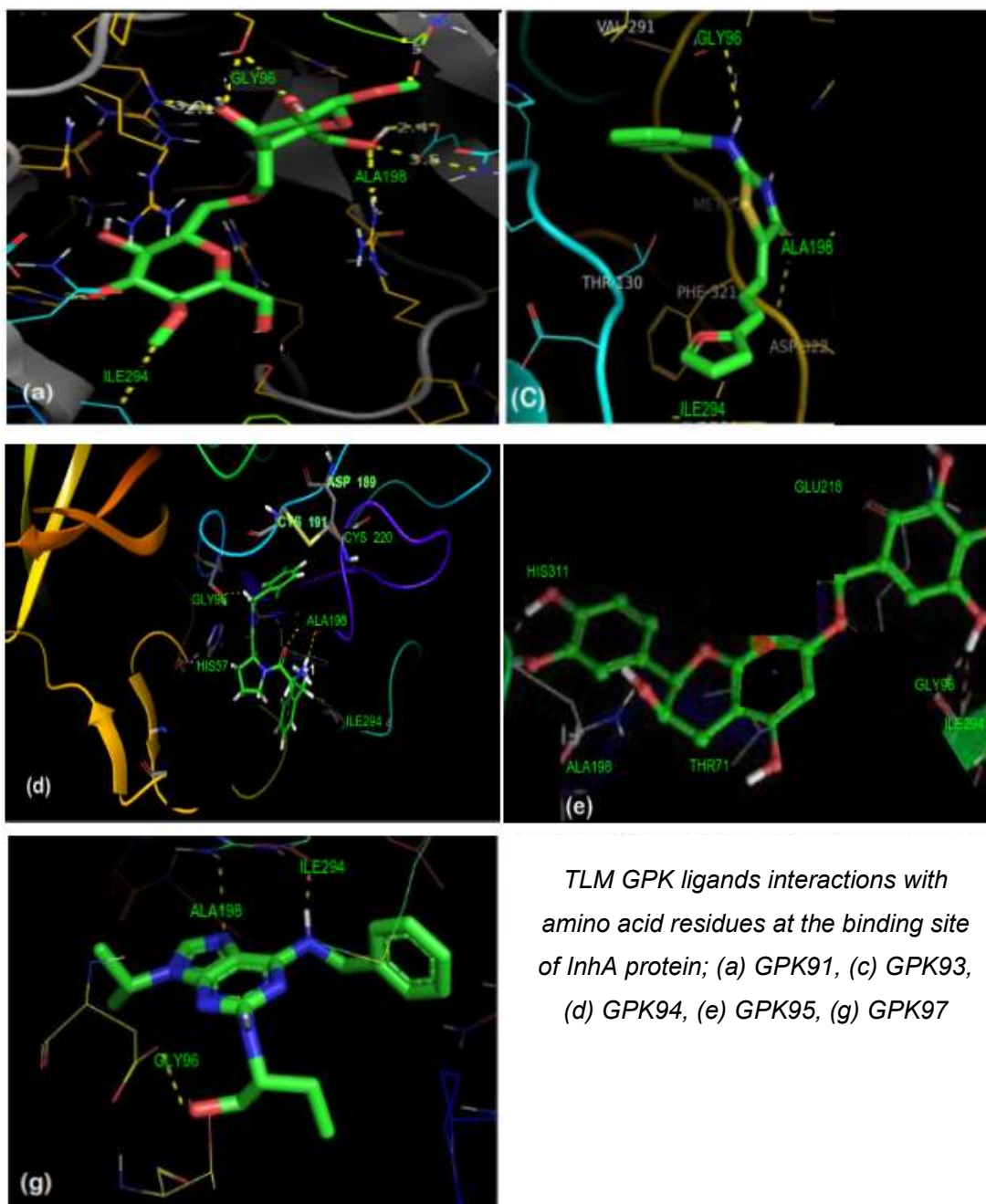


Figure 4.16: TLM reference ligand interactions with amino acid residues at the binding site of InhA.



TLM GPK ligands interactions with amino acid residues at the binding site of InhA protein; (a) GPK91, (c) GPK93, (d) GPK94, (e) GPK95, (g) GPK97

Figure 4.17: The docking interactions of TLM GPK ligands at the binding site of InhA.

4.6.3 Results of thiolactomycin derivative compounds docking with KasA

The reference ligand, TLM, formed a hydrogen bond interaction with the HIS₃₁₁ and HIS₃₄₅ when docked with the KasA protein (Table 4.2; Figure 4.18). The docking results (Figure 4.19) showed that the designed ligand GPK96 had a high affinity towards the binding pocket of KasA compared to the reference TLM, with a docking score of -7.746 binding energy. The GPK96 also showed the hydrogen bond with MET₂₁₃, THR₃₁₅, THR₃₁₃, and additional Pi-Pi stacking with the PHE₄₀₄. Ligand GPK93, showed a comparable docking score on HIS₃₁₁ and HIS₃₄₅ with the reference TLM, with docking scores of -7.149 and -7.256, respectively. GPK93 also interacted with the PHE₄₀₄ and MET₃₄₅. Other ligands also had good affinity towards the binding pocket of KasA with the docking scores as follows: GPK95 -7.092, GPK91 -7.045, GPK97 -6.849, GPK94 -6.815, GPK92 and -6.559 (Figure 4.19).

Table 4.2. The results of TLM compound interactions binding energy at the binding site of KasA

Protein	Compound	Binding energy	Ligand amino acid interaction
KasA 4C6X	135403829 (TLM)	-7.256	HIS ₃₁₁ ; HIS ₃₄₅
	GPK96	-7.746	MET ₂₁₃ ;THR ₃₁₅ ; THR ₃₁₃
			Pi-Pi:PHE ₄₀₄
	GPK93	-7.149	PHE ₄₀₄ ; MET ₃₄₅ ; HIS ₃₁₁ ; HIS ₃₄₅
	GPK95	-7.092	
	GPK91	-7.045	
	GPK97	-6.849	
	GPK94	-6.815	
	GPK92	-6.559	

During interaction with KasA 4C6X, the TLM ligand formed a covalent bond with HIS₃₁₁ and HIS₃₄₅ (Figure 4.18). Again, the TLM was oriented accordingly to allow the electrons to move away from the carboxyl group of the thiolactone ring of the TLM as we bent it within the binding site.

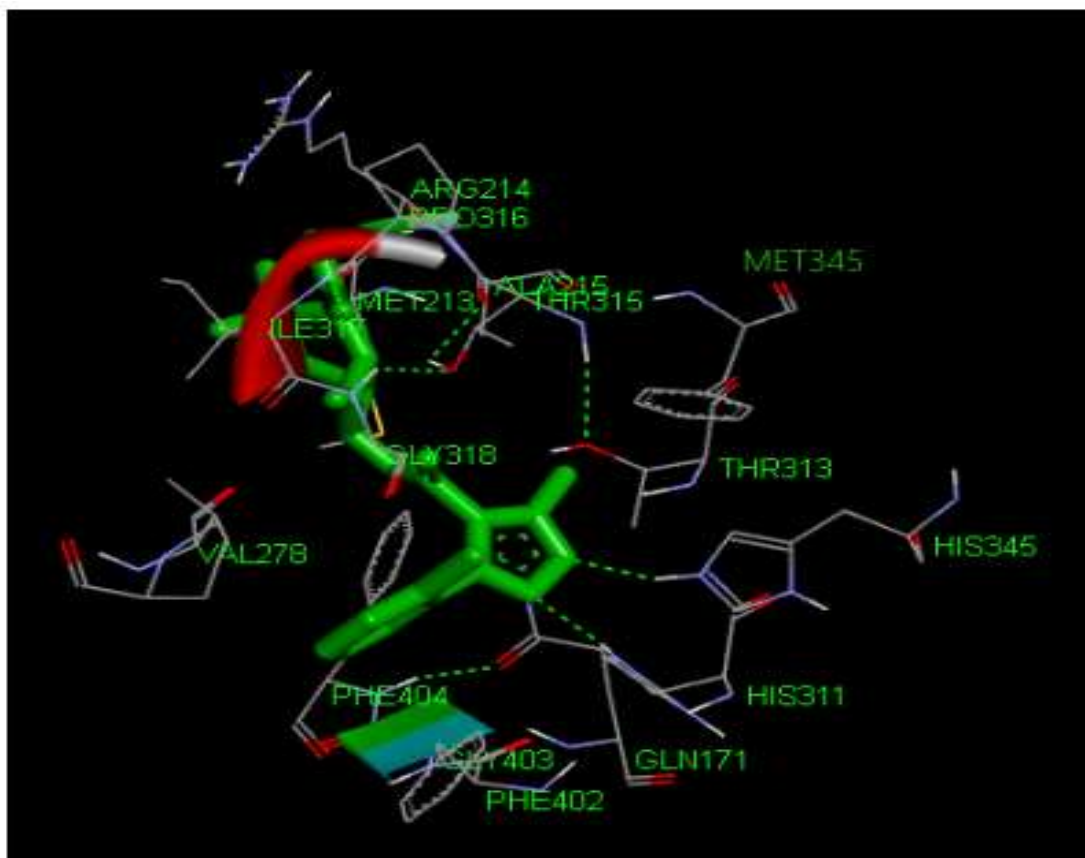


Figure 4.18: TLM reference ligand interactions with amino acid residues at the binding site of KasA.

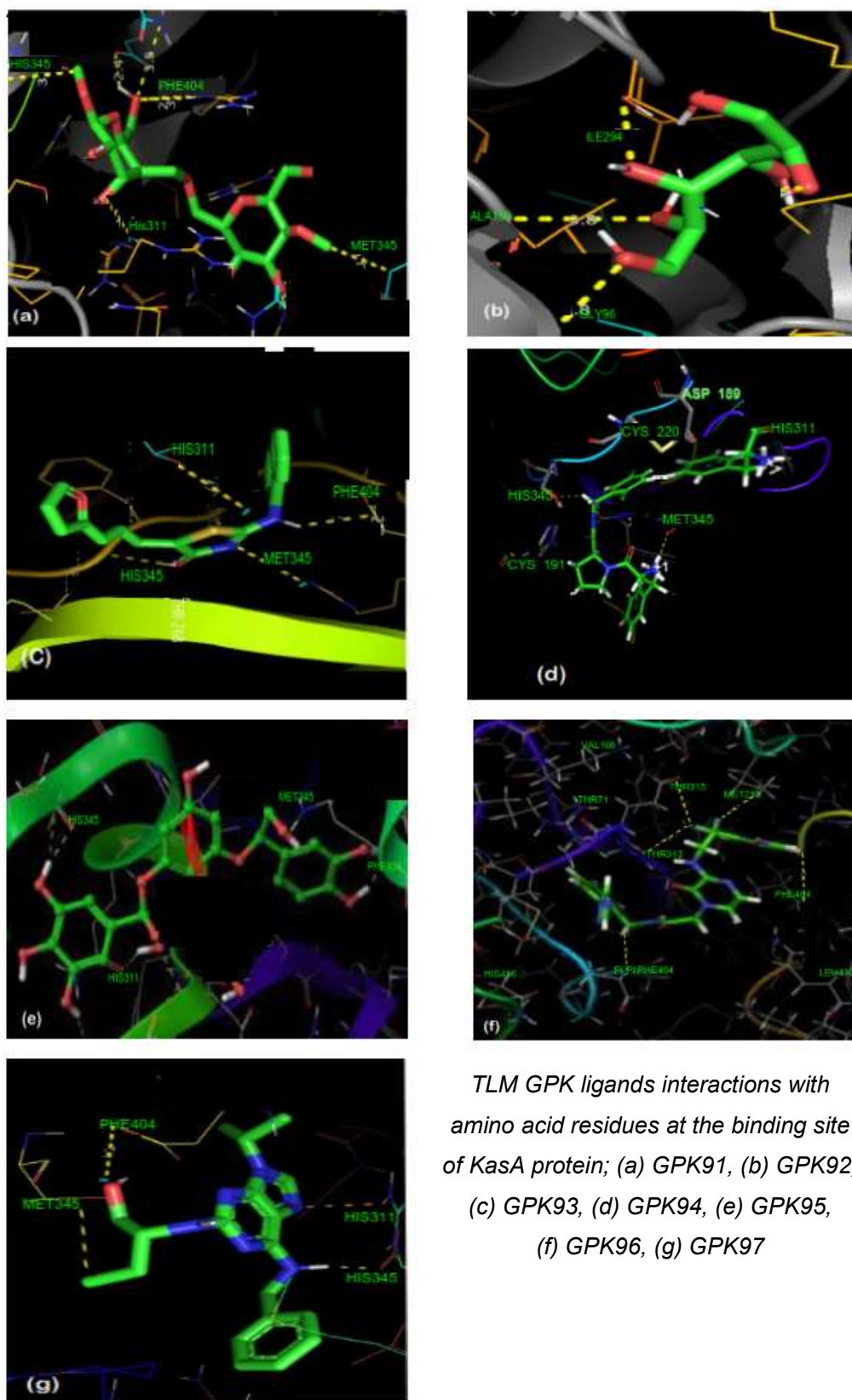


Figure 4.19: The docking interactions of TLM GPK ligands at the binding site of KsaA.

4.7 Minimum Inhibitory Concentration Results

The results of inhibitory concentrations were validated by the positive and negative controls on each MHA plate, all of which were from the same lot number, J212053. Growth was observed in all areas inoculated with the *E. coli* ATCC25922 strain (positive control), while no growth was observed in all the uninoculated areas (negative control). GPK91 showed inhibition (no growth) down to [0.781 µg/ml], while GPK96 showed inhibition down to [3.125 µg/ml]. TLM [25.0 µg/ml] was used as a reference antimicrobial drug (Table 4.3).

Table 4.3. Inhibitory results of the novel drug compounds GPK91 and GPK96 for *M. smegmatis* MC2155

Compounds	# on the MHA plate	Serial Concentrations of GPK91 (µg/ml)	Inhibition / Positive growth observed	Compounds	# on the MHA plate	Serial Concentrations of GPK96 (µg/ml)	Inhibition / Positive growth observed
GPK91	1	25.0	Inhibition present	GPK96	2	25.0	Inhibition present
GPK91	3	12.5	Inhibition present	GPK96	4	12.5	Inhibition present
GPK91	5	6.25	Inhibition present	GPK96	6	6.25	Inhibition present
GPK91	7	3.125	Inhibition present	GPK96	8	3.125	Inhibition present
GPK91	9	1.563	Inhibition present	GPK96	10	1.563	Partial inhibition
GPK91	11	0.781	Inhibition present	GPK96	12	0.781	No inhibition
GPK91	13	0.391	Partial inhibition	GPK96	14	0.391	No inhibition
GPK91	15	0.195	No inhibition	GPK96	16	0.195	No inhibition
GPK91	17	0.098	No inhibition	GPK96	18	0.098	No inhibition
GPK91	19	0.049	No inhibition	GPK96	20	0.049	No inhibition
TLM	21	25.0	Inhibition present				
Negative Control (un-inoculated area)			No growth observed	Positive control (<i>E. coli</i> ATCC25922)			Positive growth

Figure 4.20 illustrates the sensitivity results of serial concentrations that ranged from 25.0 µg/ml down to 0.049 µg/ml. GPK91 concentrations were denoted as odd numbers up to position 19, and GPK96 concentrations as even numbers. The symbols below illustrate the sensitivity results on the MHA plates when treated with different concentrations of GPK91 and GPK96 compounds.

+Ctrl = Positive control * = Positive control;

Neg Ctrl = Negative control

 = Complete inhibition / No growth

 = Partial inhibition

  = No inhibition / Full growth

MHA_1 to MHA_5 were treated with different concentrations of GPK91 and GPK96. MHA_6 showed complete inhibition on area 21 treated with TLM [25.0 µg/ml], a known antimicrobial that served as a reference antimicrobial, while *E. coli* and uninoculated areas, served as positive and negative controls on all MHA plates.

M. smegmatis was inoculated on areas 1–4, 5–8, 9–12, 13–16, and 17–20. GPK91 showed inhibition (no growth) down to [0.781 µg/ml], while GPK96 showed inhibition down to [3.125 µg/ml].

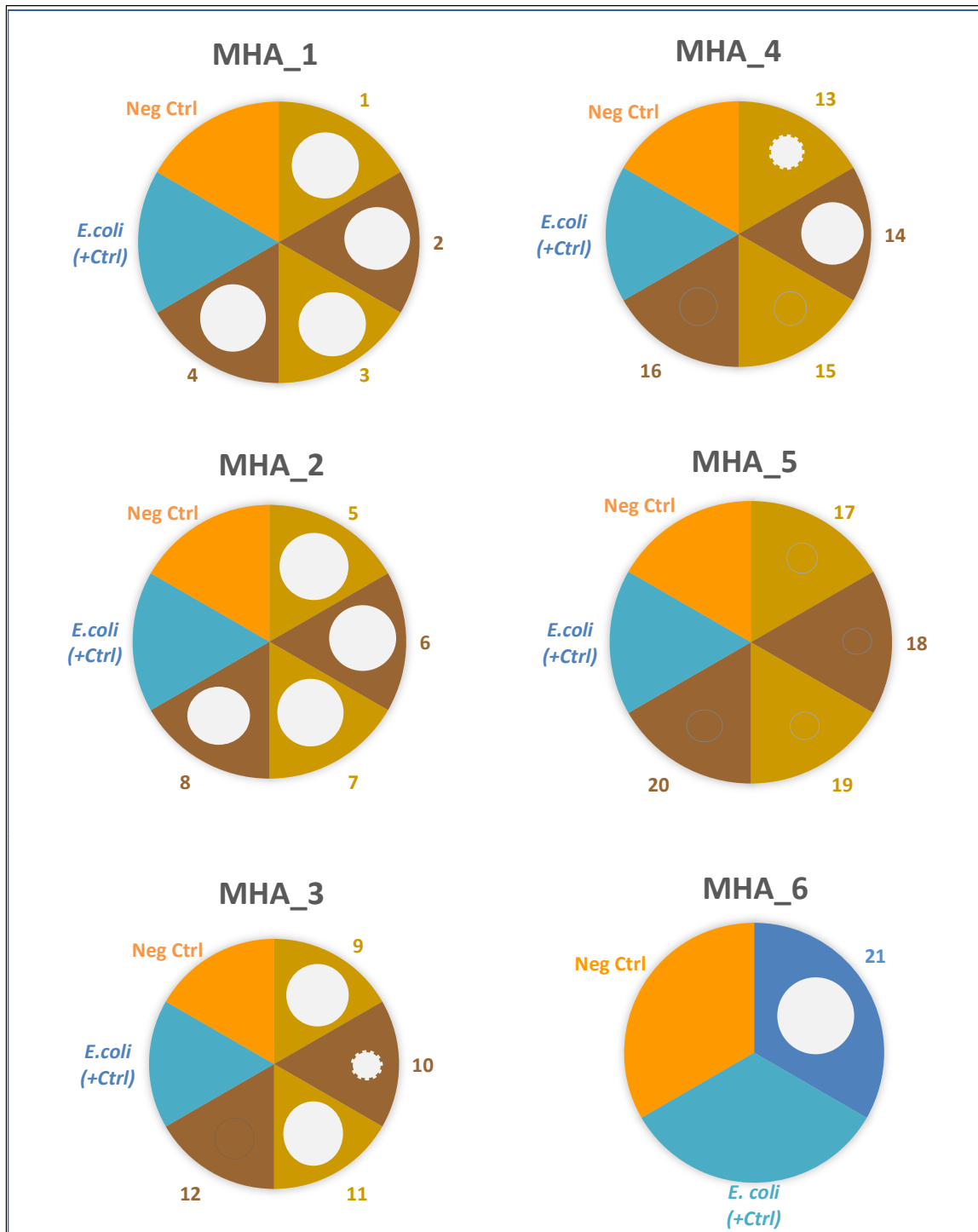


Figure 4.20: Results of serial concentrations of GPK91 and GPK96 illustrated on MHA_1 to 5 inoculated with *M. smegmatis* on areas 1-4, 5-8, 9-12, 13-16 and 17-20. MHA_6 showed complete inhibition on area 21 treated with TLM [25.0 $\mu\text{g/ml}$], with *E. coli* and uninoculated areas serving as positive and negative controls on all MHA.

Nucleotide sequences

The NCBI BLAST was used to identify the closest homolog in order to exclude any transposon inserts in the *M. tuberculosis* H37Rv strain genome and perform phylogenetic analysis for the selection of the gene sequences that were used for the docking studies. The *M. tuberculosis* H37Rv strain sequences with 100% identity homologs were selected from the resulting hits.

Nuclear Magnetic Resonance (NMR) data for benzyl-methoxyl protected aspalathin analogue via C-glucosylation of pentamethoxy-dihydropropane

The methoxy-protected chalcone (4) was obtained in quantitative yields. High-pressure catalytic hydrogenative reduction of (4) gave the corresponding 1,3-diarylpropane. On the NMR spectra of (5), the absence of the two chalcone proton resonances in the ^1H NMR spectrum [H_α (d, 7.25 ppm, $J = 15.5$ Hz) and H_β (d, 6.88 ppm, $J = 15.5$ Hz) for corresponding chalcone, and the absence of the carbonyl resonance ($\delta = 196$) in the ^{13}C NMR. The three propane CH_2 groups are represented in the ^1H NMR at 2.58 ppm (two overlapping benzylic CH_2 groups that integrate four hydrogen atoms) and 1.77 ppm (a multiplet that integrates two hydrogen atoms). They correspond to the two crosspeaks at 35.5 and 31.1 ppm in the HSQC spectrum.

The 1,3-diarylpropane-C-glycoside (7) was subsequently obtained from (6) via the Trifluoroacetic anhydride (TFAA) method. The C-diarylpropane-glycoside (7) has complex ^1H and ^{13}C NMR spectra at room temperature due to the expected rotational isomerism about the glycosidic carbon-carbon bond. Heating of the sample to 140°C in DMSO-d_6 was required for NMR elucidation. The anomeric proton resonates at 4.73 ppm in the ^1H and its corresponding C at 74.2 ppm in the ^{13}C NMR spectra. These correspond with a carbon-carbon and not a carbon-oxygen bond. The $J = 9.8$ Hz coupling constant agrees with the required β -stereochemistry on the anomeric carbon. An edited two-dimensional HSQC experiment (CH and CH_3 cross-peaks having a different color from CH_2 cross-peaks), allows facile differentiation between the benzylic protons (CH_2) of the benzyl protection groups and the anomeric sugar proton (CH), all of which resonate in the range of δ 4.00 – 5.20 ppm.

The NMR of (8) also required elevated temperature (140°C) to remove rotational isomerism and simplify interpretation. Notable were the following: the carbonyl resonance at δ 198.4 in the ^{13}C NMR spectrum. There were two multiplets at δ 3.05 and 2.93 in the ^1H NMR. These CH_2 resonances correlate with the carbon resonances at δ 38.8 and 19.7 ppm, respectively, in an edited HSQC experiment. The two multiplets also cross-couple each other in the COSY spectrum. They represent the $\text{CH}_2\text{-CH}_2$ moiety of the dihydrochalcone. The anomeric proton of the sugar moiety resonates as a doublet at δ 4.73 ppm, which correlates with the anomeric carbon at δ 73.6 ppm in the edited HSQC. The $J = 9.8$ Hz coupling constant indicates β -stereochemistry for the C-glycosidic bond. The four benzylic CH_2 resonances in the δ 4.55 to 4.90 ppm range of the ^1H NMR spectrum indicate that the four benzyl groups on the sugar moiety remained intact.

Docking results

Seven TLM derivative compounds, GPK91, GPK92, GPK93, GPK94, GPK95, GPK96, and GPK97, were synthesized, but only GPK91 and GPK96 were identified as potential candidates for novel antimycobacterial drugs. The designed ligand compounds were docked with InhA PDB ID 3FNE and KasA PDB ID 4C6X imported from the PDBsum bank (Figure 4.17 & 4.19). TLM was used as a reference ligand in both protein enzymes. Our study showed that all the designed drug compounds had a high affinity toward the binding pockets of InhA and KasA. When docked with InhA, GPK91 showed the hydrogen bond interaction with GLY₉₆, ALA₁₉₈, and ILE₂₉₄ with the best dock score of -8.88 binding energy. In the study done by Sharma et al. (2015), InhA 2X23 showed hydrogen bond interaction with VAL₆₅:HN₁ with the docking score of -12.6 binding energy for ZINC01777652, and -12.7 for ZINC03831448.

The study also showed that when we docked GPK96 with KasA 4C6X, it demonstrated a high affinity towards the hydrophobic side chain of the amino acids compared to the reference TLM, with docking score of -7.746 binding energy. The GPK96 also showed the hydrogen bond with MET₂₁₃, THR₃₁₅, THR₃₁₃ and additional Pi-Pi stacking with the PHE₄₀₄.

In the hydrogen bond interactions of GPK91 with ALA₁₉₈ and ILE₂₉₄ within InhA, and GPK96 with MET₂₉₃, THR₃₁₅, THR₃₁₃, and PHE₄₀₄ within KasA, all the amino acids had a hydrophobic side chain that made them unable to dissolve in water. The hydrogen bond is essential for the stability of the structure of the molecule, as hydrophobic interactions facilitate the folding process, and the hydrogen bonds support the final structure. These amino acids play a role in stabilizing the protein structures. Most enzymes have one active site per molecule, but the hydrophobic side chain amino acids do not have those functional groups in their molecules, with them, is referred to as the binding site, which is the position within the protein structure. Alanine forms long structures that are flexible and stretch resistant, while isoleucine plays a role in the synthesis of the tertiary structure of a protein due to its branched structured methyl group to make the protein stronger. During protein synthesis, methionine is an essential starting amino acid in most protein sequences. Threonine plays a role in forming hydrogen bonds within the protein structures, thereby forming a more stable structure. Phenylalanine has the highest hydrophobic property due to its benzene ring side chain, making it the main contributor to structure's inability to dissolve in water.

These properties of toughness, insolubility and resistance to changes in pH and temperature are the main features of the mycolic cell wall structure of *M. tuberculosis*. The probable clinical relevance of GPK91 and GPK96 is that when they show affinity towards these amino acids, they can dissociate the organism's hydrogen bonds and form hydrogen with them. That can affect the different properties of the proteins resulting in an unstable and weakened cell wall structure. Alone, GPK91 can only affect the protein structural strength inside InhA protein. GPK96 can induce its inhibitory action on the protein structural strength, hydrophobic property, and the initial phase of the protein synthesis, but only inside KasA protein. However, a combination of the two inhibitors can achieve effective results. While GPK91 can induce its inhibitory action on the two amino acids, alanine and isoleucine, which are the major role players in the protein's strength, GPK96 can induce its inhibitory action on the three amino acids, threonine, phenylalanine, and methionine, which are the major role players in the protein's strength, hydrophobic property, and protein synthesis initial phase, respectively. In the study by Sharma et al. (2015), KasA 4C72 showed hydrogen bond interaction with HIS₃₁₁:HE₂₁ within the FabB active site, with

-10.7 binding energy for ZINC01530603, and -10.8 for ZINC01532344. In the same study by Sharma et al. (2015), all the compounds made several interactions at the binding sites with HIS₃₄₅ and GLN₁₇₁ similar to TLM. Similarly, in our study, there were other ligands that had a high affinity towards the binding pocket of InhA and KasA, but none of the compounds displayed a simultaneous targeting of InhA and KasA. Previous studies have demonstrated that TLM is an inhibitor of the FAS-II condensing enzyme β -ketoacyl-ACP synthase (Slayden et al., 1996). This activity of TLM makes it a useful reagent for designing novel drugs that could inhibit the activity of the InhA and KasA proteins. A series of articles have been published stating that InhA is not the primary target of INH rather that KasA, which encodes the primary target of INH in *M. tuberculosis* (Mdluli et al., 1996; 1998); (Barry, 1997; 2001); (Slayden et al., 2000); (Slayden and Barry, 2000). Slayden et al. (2000) postulated that both InhA and KasA enzymes encode targets on INH, but that KasA encodes the primary target.

Minimum inhibitory concentration

M. smegmatis showed drug sensitivity on MHA plates for GPK91 and GPK96 concentrations down to 0.781 $\mu\text{g/ml}$ and 3.125 $\mu\text{g/ml}$, respectively. For GPK91 there was detectable growth with partial inhibition observed at [0.391 $\mu\text{g/ml}$] and no inhibition observed at [0.195 $\mu\text{g/ml}$] and less. Similarly, for GPK96, there was detectable growth with partial inhibition observed at [1.563 $\mu\text{g/ml}$] and no inhibition observed at [0.781 $\mu\text{g/ml}$] and less. The minimal inhibitory concentration of GPK91 and GPK96 for *M. smegmatis* was determined at 0.781 $\mu\text{g/ml}$ and 3.125 $\mu\text{g/ml}$, respectively (Table 4.3). The MIC is designated when a 99.9% reduction in CFU occurs at a specific compound concentration compared to the control. Our MIC results for the GPK91 compound were lower than the previous results observed by Slayden et al., (2000) for TLM on *M. tuberculosis* and *M. smegmatis*, with the range of between 2.5 $\mu\text{g/ml}$ – 5.0 $\mu\text{g/ml}$ on the agar plate. GPK96 compound result was within consensus with the results observed by Slayden et al., (2000) for TLM. Slayden et al. (1996) also showed that TLM possesses in vivo antimycobacterial activity against the saprophytic strain *M. smegmatis* MC2155 and the virulent strain *M. tuberculosis* Erdmun, resulting in complete inhibition of growth on solid media at 75 and 25 $\mu\text{g/ml}$ respectively. (Slayden et al., 1996). A study by Oishi and co-workers (1982) observed MIC of 62.52 $\mu\text{g/ml}$ against *M. tuberculosis* (Oishi et al., 1982).

Study limitations and challenges

1. *Health risk*: Lack of level 3 biosafety cabinet led to *M. tuberculosis* being surrogated with *M. smegmatis* due to potentially high levels of health risks.
2. *The measure of uncertainty*: Because the *M. smegmatis inhA* gene has a promoter immediately upstream of its *inhA* gene, whereas the *M. tuberculosis inhA* gene is in an operon with *mabA* (Banerjee et al., 1994; 1998), there was an uncertainty of measurement when the outcome results obtained through *M. smegmatis* were applied on *M. tuberculosis*.
3. *Logistical problems*: The research study peaked at the time when Covid-19 was declared a global pandemic. Part of the study was to include isoniazid but we ended up working only with thiolactomycin. Our collaboration with the Indian Institute of Technology was negatively impacted by the travel restrictions across the globe and the daily tragedies that were ravaging India.
4. *Training*: Due to the limited personnel available to offer training during the pandemic, I was only afforded training on ligand-based bioinformatics PDBsum and ZINCpharm tools. As a result, I did not get the opportunity to use AutoDock Vina or QikProp in my study.

Peer reviews

Congress participation; Paper presented at “Free State Department of Health Provincial Research Day” held at Bloemfontein on 07 – 08 November 2019 (Appendix 9).

Publication; Kendrekar P, Setlai M, Tekale S, Ingle R, Kulkarni CV, Pawar R. Novel Synthesis of benzyl-Methoxyl Protected Aspalathin Analog via C-Glucosylation of Pentamethoxy Dihydropropane. *Letters in Applied NanoBioScience* 2021; 10 (3): pp. 2382 – 2388. ISSN 2284-6808

Setlai MB, Mogongoa LF, Mashele S (*Submitted for publication*). Determining the minimum inhibitory concentration of the novel drug candidates of *Mycobacterium tuberculosis*, GPK91 and GPK96 that target fatty-acid enoyl-acyl carrier protein reductase and β -ketoacyl-acyl carrier protein synthase.

With the help and suggestions of the bioinformatics tool, PubChem, we were able to synthesize novel drug compounds that target InhA and KasA proteins that could potentially down-regulate *inhA* and *kasA* genes of the *M. tuberculosis* organism. Molecular docking was investigated and the study showed that, with similarity to TLM in the structure, both GPK91 and GPK96 had the highest binding energy and showed high affinity towards amino acid hydrophobic residues of InhA and KasA proteins. The study showed that GPK91 could be an inhibitor of the InhA protein by targeting the GLY₉₆, ALA₁₉₈, and ILE₂₉₄ amino acid residues resulting in the downregulation of the *inhA* gene. The results also predicted that GPK96 could be an inhibitor of the KasA protein by targeting the MET₂₁₃, THR₃₁₅, THR₃₁₃, and Pi-Pi:PHE₄₀₄ amino acid residues resulting in the downregulation of the *kasA* gene. However, the minimum inhibitory concentrations were different. The study showed that GPK91 was able to induce inhibition of *M. smegmatis* at a lower concentration than GPK96. Just as the current TB treatment requires the combination of different regimens to achieve more effective outcome results from the treatment, the recommendation would be for the two inhibitors used as a combination due to their inhibitory effect on the different properties of the proteins. Due to its inhibitory action at low MIC, GPK91 could prove to be fast-acting, and therefore, considered for the initiation of treatment, while GPK96 due to its broader inhibitory action, can be considered for the step-up treatment towards longer TB management. The results were supportive of our hypothesis, therefore it was concluded that both GPK91 and GPK96 compounds have good potential as novel anti-TB drugs and be considered for antimycobacterial drug prescreening.

Potential impact and applications of the findings of this research

The greatest challenge to global TB prevention and control is the increasing resistance of *M. tuberculosis* strains to the first-line anti-tuberculosis drugs. The two pre-clinical screening drug candidates could offer an alternative treatment option for patients who do not respond to the conventional treatment. The current TB treatment duration varies from 9 months to 18 months. The drug candidates could turn out to be more efficient with less duration of treatment, which could reduce cases of defaulting on the treatment, and compliance by patients will reduce the emergence

of MDR-TB and XDR-TB. Treatment of MDR-TB poses a challenge because the therapy with the available first-line and second-line drugs is less efficacious and more toxic (Ahmad et al., 2007). The two potential drug candidates being derivatives of thiolactomycin could present less toxicity compared to rifampicin. In a high-risk household where there is contact with MDR-TB patients, the drug candidates could be considered for preventative therapy that could render a personalized patient treatment that has a high potential of improved treatment outcome and prognosis. The drug candidates could also be considered for pregnant women and breast-feeding mothers. The outcome of the research could contribute towards improving health outcomes of long-term patient management by reducing patient care costs through the improvement of therapeutic efficacy, reduction of drug toxicity, and reduction of laboratory testing if the novel drug candidates were offered as an alternative treatment option.

Potential future research prospects from these research outcomes

- Conduct a similar study using isoniazid to construct derivatives for drug compounds. We would follow the same protocol as current research. AutoDock Vina would be used for molecular docking.
- To determine the minimum bactericidal concentration assay against *M. smegmatis*. This would require the bacterial cultures to be suspended in phosphate-buffered saline, make serial dilutions thereof, and inoculated on MHA plates. After an incubation period at 37°C, the CFU would be counted.
- Even though thiolactomycin has low toxicity, its clinical use has been limited due to poor pharmacokinetic properties. It would be clinically significant to research pharmacokinetic and physiochemical properties (absorption, distribution, metabolism, and excretion) of the GPK91 and GPK96 using the QikProp tool.
- Research *inhA* and *kasA* gene mutations and polymorphism as a possible cause of drug resistance using the Next Generation Sequence.

Chapter 7

References

- Abrahams KA, Chung Cw, Ghidelli-Disse S, Rullas J, Rebollo-López MJ, Gurcha SS, Cox JAG, Mendoza A, Jiménez-Navarro E, Martínez-Martínez MS, Neu M, Shillings A, Homes P, Argyrou A, Casanueva R, Loman NJ, Moynihan PJ, Lelièvre J, Selenski C, Axtman M, Kremer L, Bantscheff M, Angulo-Barturen I, Izquierdo MC, Cammack NC, Drewes G, Ballell L, Barros D, Besra GS, Bates RH. Identifications of KasA as the cellular target of an anti-tubercular scaffold. *Nature Communication* 2016; 7 (12581): 1 – 13. Doi.org/10.1038/ncomms12581.
- Ahmad S, Jaber AA, Mokaddas E. Frequency of embB codon 306 mutations in ethambutol-susceptible and -resistant clinical *Mycobacterium tuberculosis* isolates in Kuwait. *Tuberculosis* 2007; 87 (2): 123 – 129.
- Akiyama K, Matsuoka H, Hayashi H. Isolation and identification of a phosphate deficiency-induced C-glycosyl flavonoid that stimulates Arbuscular Mycorrhiza formation in melon roots. *Molecular PlantMicrobe Interactions*® 2002; 15: 334 – 340.
- Alangaden GJ, Kreiswirth BN, Aouad A, Khetarpal M, Igno FR, Moghazeh SL, Manavathu EK, Lerner SA. Mechanism of resistance to amikacin and kanamycin in *Mycobacterium tuberculosis*. *Antimicrobial Agents and Chemotherapy* 1998; 42 (5): 1295 – 1297.
- Alexander DC, Ma JH, Guthrie JL, Blair J, Chedore P, Jamieson FB. Gene sequencing for routine verification of pyrazinamide resistance in *Mycobacterium tuberculosis*: A role for pncA but not rpsA. *Journal of Clinical Microbiology* 2012; 50 (11): 3726 – 3728.
- Alnimr AM. Dormancy models for *Mycobacterium tuberculosis*: A minireview. *Brazilian Journal of Microbiology* 2015; 46(3), 641 – 647.
- Andini N, Nash KA. Intrinsic macrolide resistance of the *Mycobacterium tuberculosis* complex is inducible. *Antimicrobial Agents and Chemotherapy* 2006; 50 (7): 2560 – 2562.
- Ando H, Kitao T, Miyoshi-Akiyama T, Kato S, Mori T, Kirikae T. Downregulation of katG expression is associated with isoniazid resistance in *Mycobacterium tuberculosis*. *Molecular Microbiology* 2011; 79 (6): 1615 – 1628.
- Ando H, Miyoshi-Akiyama T, Watanabe S, Kirikae T. A silent mutation in mabA confers isoniazid resistance on *Mycobacterium tuberculosis*. *Molecular Microbiology* 2014; 91 (3): 538 – 547.

Andries K, Verhasselt P, Guillemont J, Göhlmann HW, Neefs JM, Winkler H, van Gestel J, Timmerman P, Zhu M, Lee E, Williams P, de Chaffoy D, Huitric E, Hoffner S, Cambau E, Truffot-Pernot C, Lounis N, Jarlier V. A diarylquinoline drug active on the ATP synthase of *Mycobacterium tuberculosis*. *Science* 2005; 307 (5707): 223 – 227.

Argyrou A, Vetting MW, Aladegbami B, Blanchard JS. *Mycobacterium tuberculosis* dihydrofolate reductase is a target for isoniazid. *Nature Structural and Molecular Biology* 2006; 13 (5): 408 – 413. (a)

Argyrou A, Jin L, Siconilfi-Baez L, Angeletti RH, Blanchard JS. Proteome-wide profiling of isoniazid targets in *Mycobacterium tuberculosis*. *Biochemistry* 2006; 45 (47): 13947 – 13953. (b)

Aubry A, Pan XS, Fisher LM, Jarlier V, Cambau E. *Mycobacterium tuberculosis* DNA gyrase: Interaction with quinolones and correlation with antimycobacterial drug activity. *Antimicrobial Agents and Chemotherapy* 2004; 48 (4): 1281 – 1288.

Aubry A, Veziris N, Cambau E, Truffot-Pernot C, Jarlier V, Fisher LM. Novel gyrase mutations in quinolone-resistant and -hypersusceptible clinical isolates of *Mycobacterium tuberculosis*: Functional analysis of mutant enzymes. *Antimicrobial Agents and Chemotherapy* 2005; 50 (1): 104 – 112.

Ayan AK, Radušienė J, Çirak C, Janulis V. Secondary metabolites of *Hypericum scabrum* and *Hypericum bupleuroides*. *Pharmaceutical Biology* 2009; 4: 847 – 853.

Banerjee A, Dubnau E, Quemard A, Balasubramanian V, Um KS, Wilson T, Collins D, de Lisle G, Jacobs Jr. WR. *inhA*, a Gene Encoding a Target for Isoniazid and Ethionamide in *Mycobacterium tuberculosis*. *Science* 1994; 263: 227 – 230.

Banerjee A, Sugantino M, Sacchettini JC, Jacobs Jr. WR. The *mabA* gene from the *inhA* operon of *Mycobacterium tuberculosis* encodes a 3-ketoacyl reductase that fails to confer isoniazid resistance. *Microbiology* 1998; 144: 2697 – 2707.

Barry 3rd CE. New horizons in the treatment of tuberculosis. *Biochemical Pharmacology* 1997; 54 (11): 1165 – 1172.

Barry 3rd CE. Interpreting cell wall 'virulence factors' of *Mycobacterium tuberculosis*. *TRENDS in Microbiology* 2001; 9 (5): 237 – 241.

Barry VC, Belton JG, Conalty ML, Denneny JM, Edward DW, O'Sullivan JF, Twomey D, Winder F. A new series of phenazines (rimino-compounds) with high antituberculosis activity. *Nature* 1957; 179 (4568): 1013 – 1015.

Becerra MC, Huang CC, Lecca L, Bayona J, Contreras C, Calderon R, Yataco R, Galea J, Zhang Z, Atwood S, Cohen T, Mitnick CD, Farmer P, Murray M. Resistance at no cost: The transmissibility and potential for disease progression of drug-resistant *M. tuberculosis*. *bioRxiv* 2018: 1 – 21.

Beckert P, Hillemann D, Kohl TA, Kalinowski J, Richter E, Niemann S, Feuerriegel S. rplC T460C identified as a dominant mutation in linezolid-resistant *Mycobacterium tuberculosis* strains. *Antimicrobial Agents and Chemotherapy* 2012; 56 (5): 2743 – 2745.

Berka K, Hendrychová T, Anzenbacher P, Otyepka M. Membrane position of ibuprofen agrees with suggested access path entrance to cytochrome P450 2C9 active site. *The Journal of Physical Chemistry A* 2011; 115 (41): 11248 – 11255.

Bernstein J, Lott WA, Steinberg BA, Yale HL. Chemotherapy of Experimental Tuberculosis: V. Isonicotinic acid hydrazide (nydrazid) and related compounds. *The American Review of Tuberculosis* 1952; 65 (4): 357 – 364.

Blanchard JS. Molecular mechanisms of drug resistance in *Mycobacterium tuberculosis*. *Annual Review of Biochemistry* 1996; 65 (1): 215 – 239.

Bosne-David S, Barros V, Verde SC, Portugal C, David HL. Intrinsic resistance of *Mycobacterium tuberculosis* to clarithromycin is effectively reversed by subinhibitory concentrations of cell wall inhibitors. *Journal of Antimicrobial Chemotherapy* 2000; 46 (3): 391 – 395.

Brossier F, Veziris N, Truffot-Pernot C, Jarlier V, Sougakoff W. Molecular investigation of resistance to the antituberculous drug ethionamide in multidrug-resistant clinical isolates of *Mycobacterium tuberculosis*. *Antimicrobial Agents and Chemotherapy* 2011; 55 (1): 355 – 560.

Browne SG, Hogerzeil L.M. “B 663” in the treatment of leprosy. Preliminary report of a pilot trial. *Leprosy Review* 1962; 33 (1): 6 – 10.

Burman WJ, Jones BE. Treatment of HIV-related tuberculosis in the era of effective antiretroviral therapy. *American Journal of Respiratory and Critical Care Medicine* 2001; 164 (1): 7 – 12.

Bustamante J, Aksu G, Vogt G, de Beaucoudrey L, Genel F, Chapgier A, Filipe-Santos O, Feinberg J, Emile J.F, Kutukculer N, Casanova J.L. BCG-osis and tuberculosis in a child with chronic granulomatous disease. *Journal of Allergy and Clinical Immunology* 2007; 120 (1): 32 – 38

Cáceres NE, Harris NB, Wellehan JF, Feng Z, Kapur V, Barletta RG. Overexpression of the D-alanine racemase gene confers resistance to D-cycloserine in *Mycobacterium smegmatis*. *Journal of Bacteriology* 1997; 179 (16): 5046 – 5055.

Campbell PJ, Morlock GP, Sikes RD, Dalton TL, Metchock B, Starks AM, Hooks DP, Cowan LS, Plikaytis BB, Posey JE. Molecular detection of mutations associated with first- and second-line drug resistance compared with conventional drug susceptibility testing of *Mycobacterium tuberculosis*. *Antimicrobial Agents and Chemotherapy* 2011; 55 (5): 2032 – 2041.

Cardoso RF, Cardoso MA, Leite CQ, Sato DN, Mamizuka EM, Hirata RD, de Mello FF, Hirata MH. Characterization of *ndh* gene of isoniazid resistant and susceptible *Mycobacterium tuberculosis* isolates from Brazil. *Mem. Inst. Oswaldo Cruz, Rio de Janeiro* 2007; 102 (1): 59 – 61.

Carette X, Blondiaux N, Willery E, Hoos S, Lecat-Guillet N, Lens Z, Wohlkönig A, Wintjens R, Soror SH, Frénois F. Structural activation of the transcriptional repressor EthR from *Mycobacterium tuberculosis* by single amino acid change mimicking natural and synthetic ligands. *Nucleic Acids Research* 2012; 40 (7): 3018 – 3030.

Cavusoglu C, Karaca-Derici Y, Bilgic A. In-vitro activity of rifabutin against rifampicin-resistant *Mycobacterium tuberculosis* isolates with known *rpoB* mutations. *Clinical Microbiology Infections* 2004; 10 (7): 662 – 665.

Caws M, Duy PM, Tho DQ, Lan NT, Hoa DV, Farrar J. Mutations prevalent among rifampin and isoniazid-resistant *Mycobacterium tuberculosis* isolates from a hospital in Vietnam. *Journal of Clinical Microbiology* 2006; 44 (7): 2333 – 2337.

Chahine EB, Karaoui LR, Mansour H. Bedaquiline: A novel diarylquinoline for multidrug-resistant tuberculosis. *Annals of Pharmacotherapy* 2014; 48 (1): 107 – 115.

Cheng SJ, Thibert L, Sanchez T, Heifets L, Zhang Y. *pncA* mutations as a major mechanism of pyrazinamide resistance in *Mycobacterium tuberculosis*: Spread of a monoresistant strain in Quebec, QC, Canada. *Antimicrobial Agents and Chemotherapy* 2000; 44 (3): 528 – 532.

Cheng AF, Yew WW, Chan EW, Chin ML, Hui MM, Chan RC. Multiplex PCR amplicon conformation analysis for rapid detection of *gyrA* mutations in fluoroquinolone-resistant *Mycobacterium tuberculosis* clinical isolates. *Antimicrobial Agents and Chemotherapy* 2004; 48 (2): 596 – 601.

Chen JM, Uplekar S, Gordon SV, Cole ST. A point mutation in *cycA* partially contributes to the D-cycloserine resistance trait of *Mycobacterium bovis* BCG vaccine strains. *PLoS One* 2012; 7 (8): e43467.

Cholo MC, Steel HC, Fourie PB, Germishuizen WA, Anderson R. Clofazimine: Current status and future prospects. *Journal of Antimicrobial Chemotherapy* 2012; 67 (2), 290 – 298.

- Cirak C, Radusiene J. Factors affecting the variation of bioactive compounds in *Hypericum* species. *Biologia Futura* 2019; 70: 198 – 209.
- Comas I, Borrell S, Roetzer A, Rose G, Malla B, Kato-Maeda M, Galagan J, Niemann S, Gagneux S. Whole-genome sequencing of rifampicin-resistant *Mycobacterium tuberculosis* strains identifies compensatory mutations in RNA polymerase genes. *Nature Genetics* 2011; 44 (1): 106 – 110.
- Cornell M, Johnson LF, Wood R, Tanser F, Fox MP, Prozesky H, Schomaker M, Egger M, Davies MA, Boule A. Twelve-year mortality in adults initiating antiretroviral therapy in South Africa. *Journal of the International AIDS Society* 2017; 20(1): 1– 10.
- Cox JAG, Taylor RC, Brown AK, Attoe S, Besra GS, Fütterer K. Crystal structure of *Mycobacterium tuberculosis* FadB2 implicated in mycobacterial β -oxidation. *Acta Crystallographica* 2019; D75: 101 – 108.
- Crofton J, Mitchison DA. Streptomycin resistance in pulmonary tuberculosis. *British Medical Journal* 1948; 2 (4588): 1009 – 1015.
- DeBarber AE, Mdluli K, Bosman M, Bekker LG, Barry 3rd CE. Ethionamide activation and sensitivity in multidrug-resistant *Mycobacterium tuberculosis*. *Proceedings of the National Academy of Science of USA* 2000; 97 (17): 9677 – 9782.
- De Souza M V N. Promising candidates in clinical trials against multidrug-resistant tuberculosis (MDR-TB) based on natural products. *Fitoterapia* 2009; 80 (8): 453 – 460. doi:10.1016/j.fitote.2009.07.010.
- Diacon AH, Pym A, Grobusch M, Patientia R, Rustomjee R, Page-Shipp L, Pistorius C, Krause R, Bogoshi M, Churchyard G, Venter A, Allen J, Palomino JC, De Marez T, van Heeswijk RPG, Lounis N, Meyvisch P, Verbeeck J, Parys W, de Beule K, Andries K, Mc Neeley DF. The diarylquinoline TMC207 for multidrug-resistant tuberculosis. *New England Journal of Medicine* 2009; 360 (23): 2397 – 2405.
- Diacon AH, Dawson R, Hanekom M, Narunsky K, Venter A, Hittel NL, Geiter J, Wells CD, Paccaly AJ, Donald PR. Early bactericidal activity of delamanid (OPC-67683) in smear-positive pulmonary tuberculosis patients. *The International Journal of Tuberculosis and Lung Disease* 2011; 15 (7): 949 – 954.
- Diacon AH, Dawson R, von Groote-Bidlingmaier F, Symons G, Venter A, Donald PR, van Niekerk C, Everitt D, Winter H, Becker P, Mendel CM, Spigelman MK. 14-day bactericidal activity of PA-824, bedaquiline, pyrazinamide, and moxifloxacin combinations: A randomised trial. *The Lancet* 2012; 380: 986 – 993.
- Dinda B, Bhattacharya A, Takayanagi H, Harigaya Y. Antimicrobial C-glucoside from aerial parts of *Diospyros nigra*. *Chem. Pharm. Bull.* 2006; 54: 679 – 681.

Dookie N, Rambaran S, Padayatchi N, Mahomed S, Naidoo K. Evolution of drug resistance in *Mycobacterium tuberculosis*: a review on the molecular determinants of resistance and implications for personalized care. *Journal of Antimicrobial Chemotherapy* 2018; 73 (5): 1138 – 1151.

Escribano I, Rodríguez JC, Llorca B, García-Pachon E, Ruiz M, Royo G. Importance of the efflux pump systems in the resistance of *Mycobacterium tuberculosis* to fluoroquinolones and linezolid. *Chemotherapy* 2007; 53 (6): 397 – 401.

Evangelista W, Ellingson SR, Smith JC, Baudry JY. Ensemble Docking in Drug Discovery: How Many Protein Configurations from Molecular Dynamics Simulations Are Needed to Reproduce Known Ligand Binding? *The Journal of Physical Chemistry B* 2019. [acs.jpcb.8b11491](https://doi.org/10.1021/acs.jpcb.8b11491)–. doi:10.1021/acs.jpcb.8b11491.

Fàbrega A, Madurga S, Giralt E, Vila J. Mechanism of action of and resistance to quinolones. *Microbial Biotechnology* 2009; 2 (1): 40 – 61.

Fenner L, Egger M, Bodmer T, Altpeter E, Zwahlen M, Jaton K, Pfyffer GE, Borrell S, Dubuis O, Bruderer T, Siegrist HH, Furrer H, Calmy A, Fehr J, Stalder JM, Ninet B, Böttger EC, Gagneux S. Effect of mutation and genetic background on drug resistance in *Mycobacterium tuberculosis*. *Antimicrobial Agents and Chemotherapy* 2012; 56 (6): 3047 – 3053.

Finken M, Kirschner P, Meier A, Wrede A, Böttger EC. Molecular basis of streptomycin resistance in *Mycobacterium tuberculosis*: Alterations of the ribosomal protein S12 gene and point mutations within a functional 16S ribosomal RNA pseudoknot. *Molecular Microbiology* 1993; 9 (6): 1239 – 1246.

Fogel N. Tuberculosis: A disease without boundaries. *Tuberculosis* 2015; 95 (5): 527 – 531.

Fouda AE-AS, El-Maksoud SAA, El-Habab AT, Ibrahim AR. Synthesis and characterization of new ethoxylated carbohydrate based surfactants for corrosion inhibition of low cost steel in aqueous solutions. *Biointerface Research in Applied Chemistry* 2021; 11 (2): 9382 – 9404.

Garibyan L, Avashia N. Polymerase Chain Reaction. *Journal of Investigative Dermatology* 2013; 133 (3): 1 – 4.

Georgiou SB, Magana M, Garfein RS, Catanzaro DG, Catanzaro A, Rodwell TC. Evaluation of genetic mutations associated with *Mycobacterium tuberculosis* resistance to amikacin, kanamycin and capreomycin: a systematic review. *PLoS One* 2012; 7 (3): e33275.

Gillespie SH. Evolution of drug resistance in *Mycobacterium tuberculosis*: Clinical and molecular perspective. *Antimicrobial Agents and Chemotherapy* 2002; 46 (2): 267 – 274.

Giri P, Singh I. Development and evaluation of muco adhesive tablets of cinnarizine using carboxymethylated guar gum by compression coating technique. *Biointerface Research in Applied Chemistry* 2020; 10: 6076 – 6081.

Gler MT, Skripconoka V, Sanchez-Garavito E, Xiao H, Cabrera-Rivero JL, Vargas-Vasquez DE, Gao M, Awad M, Park SK, Shim TS, Suh TS, Danilovits MD, Ogata H, Kurve A, Chang J, Suzuki K, Tupasi T, Koh WJ, Seaworth B, Geiter LJ, Wells CD. Delamanid for multidrug-resistant pulmonary tuberculosis. *New England Journal of Medicine* 2012; 366 (23): 2151 – 2160.

Gonzalez FJ, Gelboin HV. Human cytochromes P450: evolution and cDNA-directed expression. *Environmental Health Perspectives* 1992; 98: 81 – 85.

Goss WA, Deitz WH, Cook TM. Mechanism of action of nalidixic acid on *Escherichia coli*. II. Inhibition of deoxyribonucleic acid synthesis. *Journal of Bacteriology* 1965; 89 (4): 1068 – 1074.

Govender T, Naidoo S, Padayatchi N, Gwyther L. Palliative care for drug-resistant tuberculosis: An urgent call to action. *South African Medical Journal* 2018; 108 (5): 360.

Grzegorzewicz AE, Korduláková J, Jones V, Born SE, Belardinelli JM, Vaquié A, Gundi VA, Madacki J, Slama N, Laval F, Vaubourgeix J, Crew RM, Gicquel G, Daffé M, Morbidoni HR, Brennan PJ, Quémard A, McNeil MR, Jackson MA common mechanism of inhibition of the *Mycobacterium tuberculosis* mycolic acid biosynthetic pathway by isoxyl and thiacetazone. *Journal of Biological Chemistry* 2012; 287 (46): 38434 – 38441.

Haagsma AC, Abdillahi-Ibrahim R, Wagner MJ, Krab K, Vergauwen K, Guillemont J, Andries K, Lill H, Koul A, Bald D. Selectivity of TMC207 towards mycobacterial ATP synthase compared with that towards the eukaryotic homologue. *Antimicrobial Agents and Chemotherapy* 2009; 53 (3): 1290 – 1292.

Hartkoorn RC, Uplekar S, Cole ST. Cross-resistance between clofazimine and bedaquiline through upregulation of MmpL5 in *Mycobacterium tuberculosis*. *Antimicrobial Agents and Chemotherapy* 2014; 58 (5): 2979 – 2981.

Hazbón MH, Bobadilla del Valle M, Guerrero MI, Varma-Basil M, Filliol I, Cavatore M, Colangeli R, Safi H, Billman-Jacobe H, Lavender C, Fyfe J, Garcia-Garcia L, Davidow A, Brimacombe M, Leon CI, Porras T, Bose M, Chaves F, Eisenach KD, Sifuentes-Osornio J, de Leon AP, Cave MD, Alland D. Role of embB codon 306 mutations in *Mycobacterium tuberculosis* revisited: A novel association with broad drug resistance and IS6110 clustering rather than ethambutol resistance. *Antimicrobial Agents and Chemotherapy* 2005; 49 (9): 3794 – 3802.

Hazbón MH, Brimacombe M, del Valle, MB, Cavatore M, Guerrero MI, Varma-Basil M, Billman-Jacobe H, Lavender C, Fyfe J, García-García L, León CI, Bose M, Chaves F, Murray M, Eisenach KD, Sifuentes-Osornio J, Cave MD, de León AP, Alland D. Population genetics study of isoniazid resistance mutations and evolution of multidrug-resistant *Mycobacterium tuberculosis*. *Antimicrobial Agents and Chemotherapy* 2006; 50 (8): 2640 – 2649.

Heather JM, Chain B. The sequence of sequencers: The history of sequencing DNA. *Genomics* 2016; 107 (1): 1 – 8.

Heep M, Rieger U, Beck D, Lehn N. Mutations in the beginning of the *rpoB* gene can induce resistance to rifamycins in both *Helicobacter pylori* and *Mycobacterium tuberculosis*. *Antimicrobial Agents and Chemotherapy* 2000; 44 (4): 1075 – 1077.

Heym B, Stavropoulos E, Honoré N, Domenech P, Saint-Joanis B, Wilson TM, Collins DM, Colston MJ, Cole ST. Effects of overexpression of the alkyl hydroperoxide reductase AhpC on the virulence and isoniazid resistance of *Mycobacterium tuberculosis*. *Infection and Immunity* 1997; 65 (4): 1395 – 1401.

Hillemann D, Rüscher-Gerdes S, Richter E. In vitro-selected linezolid-resistant *Mycobacterium tuberculosis* mutants. *Antimicrobial Agents and Chemotherapy* 2008; 52 (2): 800 – 801.

Ho K, Truong DT, Li MS. How good is the Jarzynski's Equality for Computer-Aided Drug Design? *The Journal of Physical Chemistry B* 2020, *acs.jpcc.0c02009*–. doi:10.1021/acs.jpcc.0c02009

Ho YM, Sun YJ, Wong SY, Lee AS. Contribution of *dfrA* and *inhA* mutations to the detection of isoniazid-resistant *Mycobacterium tuberculosis* isolates. *Antimicrobial Agents and Chemotherapy* 2009; 53 (9): 4010 – 4012.

Hoagland DT, Liu J, Lee RB, Lee R. New agents for the treatment of drug-resistant *Mycobacterium tuberculosis*. *Advanced Drug Delivery Reviews* 2016; 102: 55 – 72.

Hughes CA, Tseng A, Cooper R. Managing drug interactions in HIV-infected adults with comorbid illness. *Canadian Medical Association Journal* 2015; 187 (1): 36 – 43.

Huitric E, Verhasselt P, Andries K, Hoffner SE. In vitro antimycobacterial spectrum of a diarylquinoline ATP synthase inhibitor. *Antimicrobial Agents and Chemotherapy* 2007; 51 (11): 4202 – 4204.

Huitric E, Verhasselt P, Koul A, Andries K, Hoffner S, Andersson DI. Rates and mechanisms of resistance development in *Mycobacterium tuberculosis* to a novel diarylquinoline ATP synthase inhibitor. *Antimicrobial Agents and Chemotherapy* 2009; 54 (3): 1022 – 1028. doi:10.1128/AAC.01611-09

Imperiali FG, Zaninoni A, La Maestra L, Tarsia P, Blasi F & Barcellini W. Increased *Mycobacterium tuberculosis* growth in HIV-1-infected human macrophages: role of tumor necrosis factor- α . *Clinical and Experimental Immunology* 2001; 123 (3): 435 – 442.

Isono K. Nucleoside antibiotics: Structure, biological activity, and biosynthesis. *The Journal of Antibiotics* 1988; 41: 1711 – 1739.

Jackson M. The Mycobacterial Cell Envelope – Lipids. *Cold Spring Harbor Perspect Med* 2014; 4: a021105.

Johansen SK, Maus CE, Plikaytis BB, Douthwaite S. Capreomycin binds across the ribosomal subunit interface using tlyA-encoded 2'-O-methylations in 16S and 23S rRNAs. *Molecular Cell* 2006; 23 (2): 173 – 182.

Juréen P, Werngren J, Toro JC, Hoffner S. Pyrazinamide resistance and pncA gene mutations in *Mycobacterium tuberculosis*. *Antimicrobial Agents and Chemotherapy* 2008; 52 (5): 1852 – 1854.

Kar SS, Bhat VG, Shenoy VP, Bairy I, Shenoy GG. Design, synthesis, and evaluation of novel diphenyl ether derivatives against drug- susceptible and drug- resistant strains of *Mycobacterium tuberculosis*. *Chemical Biology and Drug Design* 2019; 93 (1): 60 – 66.

Keane J, Remold HG, Kornfeld H. Virulent *Mycobacterium tuberculosis* strains evade apoptosis of infected alveolar macrophages. *The Journal of Immunology* 2000; 164 (4): 2016 – 2020.

Klauda JB. Virtual Issue on Docking. *The Journal of Physical Chemistry B* 2021; 125(21): 5455 – 5457.

Konno K, Feldmann FM, McDermott W. Pyrazinamide susceptibility and amidase activity of tubercle bacilli. *American Review of Respiratory Disease* 1967; 95 (3): 461 – 469.

Koul A, Dendouga N, Vergauwen K, Molenberghs B, Vranckx L, Willebrords R, Ristic Z, Lill H, Dorange I, Guillemont J, Bald D, Andries K. Diarylquinolines target subunit c of mycobacterial ATP synthase. *Nature Chemical Biology* 2007; 3 (6): 323 – 324.

Kremer L, Douglas JD, Baulard AR, Morehouse C, Guy MR, Alland D, Dover LG, Lakey JH, Jacobs, Jr. WR, Brennas PJ, Minnikin DE, Besra GS. Thiolactomycin and Related Analogues as Novel Anti-mycobacterial Agents Targeting KasA and KasB Condensing Enzymes in *Mycobacterium tuberculosis*. *Journal of Biological Chemistry* 2000; 275 (22): 16857 – 16864. doi.10.1074/jbc.M000569200.

Krüüner A, Jureen P, Levina K, Ghebremichael S, Hoffner S. Discordant resistance to kanamycin and amikacin in drug-resistant *Mycobacterium tuberculosis*. *Antimicrobial Agents and Chemotherapy* 2003; 47 (9): 2971 – 2973.

Lambert JB, Mazzola EP, Ridge CD. (2019). *Nuclear Magnetic Resonance Spectroscopy: An Introduction to Principles, Applications and Experimental Methods*, 2nd edition, Wiley Publishers, New Jersey USA, Chichester UK. 1 – 50.

Larsen MH, Vilchèze C, Kremer L, Besra GS, Parsons L, Salfinger M, Heifets L, Hazbon MH, Alland D, Sacchetti JC, Jacobs WR Jr. Overexpression of *inhA*, but not *kasA*, confers resistance to isoniazid and ethionamide in *Mycobacterium smegmatis*, *M. bovis* BCG and *M. tuberculosis*. *Molecular Microbiology* 2002; 46: 453 – 466.

Lawn SD, Butera ST, Shinnick T. Tuberculosis unleashed: the impact of human immunodeficiency virus infection on the host granulomatous response to *Mycobacterium tuberculosis*. *Microbes and Infection* 2002; 4 (6): 635 – 646.

Leach KL, Brickner SJ, Noe MC, Miller PF. Linezolid, the first oxazolidinone antibacterial agent. *Annals of the New York Academy of Science* 2011; 1222 (1): 49 – 54.

Lu H, Giordano F, Ning Z. Oxford Nanopore MinION sequencing and genome assembly. *Genomics, Proteomics and Bioinformatics* 2016; 14 (5): 265 – 279.

Luckner SR, Machutta CA, Tonge PJ, Kisker C. Crystal structures of *Mycobacterium tuberculosis* KasA show mode of action within cell wall biosynthesis and its inhibition by thiolactomycin. *Structure* 2009; 17 (7): 1004 – 1013.

Machado D, Perdigão J, Ramos J, Couto I, Portugal I, Ritter C, Boettger EC, Viveiros M. High-level resistance to isoniazid and ethionamide in multidrug-resistant *Mycobacterium tuberculosis* of the Lisboa family is associated with *inhA* double mutations. *Journal of Antimicrobial Chemotherapy* 2013; 68 (8): 1728 – 1732.

Madigan MT, Martinko JM, Parker J. (1997). In: Brock Biology of Microorganisms 8th ed. London, Sydney, Toronto, Mexico. New Delhi, Tokyo, Singapore, Rio de Janeiro: Prentice-Hall. 23 – 29; 938 – 940.

Maharaj J, Ross A, Maharaj NR, Campbell L. Multidrug-resistant tuberculosis in KwaZulu-Natal, South Africa: An overview of patients' reported knowledge and attitudes. *African Journal of Primary Health Care & Family Medicine* 2016; 8(1):1 – 6.

Makarov V, Lechartier B, Zhang M, Neres J, van der Sar AM, Raadsen SA, Hartkoorn RC, Ryabova OB, Vocat A, Decosterd LA, Widmer N, Buclin T, Andries K, Pojer F, Dyson P, Cole ST. Towards a new combination therapy for tuberculosis with next generation benzothiazinones. *EMBO Molecular Medicine* 2014; 6 (3): 372 – 383.

Manina G, Bellinzoni M, Pasca MR, Neres J, Milano A, Ribeiro AL, Buroni S, Skovierová H, Dianišková P, Mikušová K, Marák J, Makarov V, Giganti D, Haouz A, Lucarelli AP, Degiacomi G, Piazza A, Chiarelli LR, DE Rossi E, Salina E, Cole ST, Alzari PM, Riccardi G. Biological and structural characterization of the *Mycobacterium smegmatis* nitroreductase NfnB, and its role in benzothiazinone resistance. *Molecular Microbiology* 2010; 77 (5): 1172 – 1185.

Marrakchi H, Lanéelle, MA, Daffé M. Mycolic Acids: Structures, Biosynthesis, and Beyond. *Chemistry and Biology* 2014; 21(1): 67 – 85.

Martinelli LKB, Rotta M, Villela AD, Rodrigues Junior VS, Abbadi BL, Trindade RV, Petersen GO, Danesi GM, Nery LR, Pauli I, Campos MM, Bonan CD, de Souza ON, Basso LA, Santos DS. Functional, thermodynamics, structural and biological studies of in silico-identified inhibitors of *Mycobacterium tuberculosis* enoyl-ACP (CoA) reductase enzyme. *Scientific Reports* 2017; 7 (1): 46696 | doi: 10.1038/srep46696.

Maruri F, Sterling TR, Kaiga AW, Blackman A, van der Heijden YF, Mayer C, Cambau E, Aubry A. A systematic review of gyrase mutations associated with fluoroquinolone-resistant *Mycobacterium tuberculosis* and a proposed gyrase numbering system. *Journal of Antimicrobial Chemotherapy* 2012; 67 (4): 819 – 831.

Mathys V, Wintjens R, Lefevre P, Bertout J, Singhal A, Kiass M, Kurepina, N, Wang XM, Mathema B, Baulard A, Kreiswirth BN, Bifani P. Molecular genetics of para-aminosalicylic acid resistance in clinical isolates and spontaneous mutants of *Mycobacterium tuberculosis*. *Antimicrobial Agents and Chemotherapy* 2009; 53 (5): 2100 – 2109.

Matsumoto M, Hashizume H, Tomishige T, Kawasaki M, Tsubouchi H, Sasaki H, Shimokawa Y, Komatsu M. OPC-67683, a nitro-dihydro-imidazooxazole derivative with promising action against tuberculosis in vitro and in mice. *PLoS Medicine*. 2006; 3 (11): 2131 – 2144.

- McClatchy JK, Kanes W, Davidson PT, Moulding TS. Cross-resistance in *M. tuberculosis* to kanamycin, capreomycin and viomycin. *Tubercle* 1977; 58 (1): 29 – 34.
- McLean KJ, Lafite P, Levy C, Cheesman MR, Mast N, Pikuleva IA, Leys D, Munro AW. The Structure of *Mycobacterium tuberculosis* CYP125: Molecular basis for cholesterol binding in a P450 needed for host infection. *Journal of Biological Chemistry* 2009; 284 (51): 35524 – 35533.
- Mdluli K, Sherman DR, Hickey MJ, Kreiswirth BN, Morris S, Stover CK, Barry 3rd CE. Biochemical and genetic data suggest that *InhA* is not the primary target for activated isoniazid in *Mycobacterium tuberculosis*. *The Journal of Infectious Diseases* 1996; 174 (5): 1085 – 1090.
- Mdluli K, Slayden RA, Zhu Y, Ramaswamy S, Pan X, Mead D, Crane DD, Musser JM, Barry 3rd CE. Inhibition of a *Mycobacterium tuberculosis* β -ketoacyl ACP Synthase by isoniazid. *Science* 1998; 280(5369): 1607 – 1610.
- Middleton EM, Teramura AH. The role of flavonol glycosides and carotenoids in protecting soybean from ultraviolet-B damage. *Plant Physiology* 1993; 103: 741 – 752.
- Mikusová K, Slayden RA, Besra GS, Brennan PJ. Biogenesis of the mycobacterial cell wall and the site of action of ethambutol. *Antimicrobial Agents and Chemotherapy* 1995; 39 (11): 2484 – 2489.
- Mikusová K, Huang H, Yagi T, Holsters M, Vereecke D, D'Haeze W, Scherman MS, Brennan PJ, McNeil MR, Crick DC. Decaprenylphosphoryl arabinofuranose, the donor of the D-arabinofuranosyl residues of mycobacterial arabinan, is formed via a two-step epimerization of decaprenylphosphoryl ribose. *Journal of Bacteriology* 2005; 187 (23): 8020 – 8025.
- Mitchison DA. The action of antituberculosis drugs in short-course chemotherapy. *Tubercle* 1985; 66 (3): 219 – 225.
- Mlynarik V. Introduction to nuclear magnetic resonance. *Analytical Biochemistry* 2017 (529): 4 – 9.
- Moazed D, Noller HF. Interaction of antibiotics with functional sites in 16S ribosomal RNA. *Nature* 1987; 327 (6121): 389 – 394.
- Murray PR, Rosenthal KS, Pfaller MA (2016). *Mycobacterium* and related acid-fast bacteria. In: *Medical Microbiology, 8th edition*, Canada, Elsevier Inc.: 218 – 225.
- Musser JM. Antimicrobial agent resistance in mycobacteria: Molecular genetic insights. *Clinical Microbiology Reviews* 1995; 8 (4): 496 – 514.

Mustatea G, Ungureanu EL, Iorga E. Protein acidic hydrolysis for amino acids analysis in food - Progress over time: A short review. *Journal of Hygienic Engineering and Design* 2019: 81 – 87.

National Center for Biotechnology Information nucleotide BLAST
(<http://www.ncbi.nlm.nih.gov>) [Accessed: 12 March 2022].

Nucleic acid sequence massager
(biomodel.uah.es/en/lab/cybertory/analysis/massager.htm)
[Accessed: 12 March 2022].

Oishi H, Noto T, Sasaki H, Suzuki K, Hayashi T, Okazaki H, Ando K, Sawada M. Thiolactomycin, a new antibiotic i. Taxonomy of the producing organism, fermentation and biological properties. *Journal of Antibiotics* 1982;35 (4): 391 – 395.

Okamoto S, Tamaru A, Nakajima C, Nishimura K, Tanaka Y, Tokuyama S, Suzuki Y, Ochi K. Loss of a conserved 7-methylguanosine modification in 16S rRNA confers low-level streptomycin resistance in bacteria. *Molecular Microbiology* 2007; 63 (4): 1096 –1106.

Ouellet H, Lang J, Couture M, de Montellano PRO. Reaction of *Mycobacterium tuberculosis* Cytochrome P450 Enzymes with Nitric Oxide. *Biochemistry* 2009; 48 (5): 863 – 872.

Palomino JC, Martin A. Tuberculosis clinical trial update and the current anti-tuberculosis drug portfolio. *Current Medicinal Chemistry* 2013; 20: 3785 – 3796. (a)

Palomino JC, Martin A. TMC207 becomes bedaquiline, a new anti-TB drug. *Future Microbiology* 2013; 8 (9): 1071 – 1080. (b)

Palomino JC, Martin A. Drug Resistance Mechanisms in *Mycobacterium tuberculosis*. *Antibiotics* 2014; 3 (3): 317 – 340.

Pasca MR, Degiacomi G, Ribeiro AL, Zara F, De Mori P, Heym B, Mirrione M, Brerra R, Pagani L, Pucillo L, Troupioti P, Makarov V, Cole ST, Riccardi G. Clinical isolates of *Mycobacterium tuberculosis* in four European hospitals are uniformly susceptible to benzothiazinones. *Antimicrobial Agents and Chemotherapy* 2010; 54 (4): 1616 – 1618.

PDBSum Database (<http://www.ebi.ac.uk/thornton-srv/databases/pdbsum/>)
[Accessed: 28 May 2021].

Petrella S, Cambau E, Chauffour A, Andries K, Jarlier V, Sougakoff W. Genetic basis for natural and acquired resistance to the diarylquinoline R207910 in mycobacteria. *Antimicrobial Agents and Chemotherapy* 2006; 50 (8): 2853 – 2856.

Podust LM, Poulos TL, Waterman MR. Crystal structure of cytochrome P450 14 α -sterol demethylase (CYP51) from *Mycobacterium tuberculosis* in complex with azole inhibitors. *Proceedings of the National Academy of Science of USA* 2001; 98 (6): 3068 – 3073.

Pontali E, Sotgiu G, D'Ambrosio L, Centis R, Migliori GB. Bedaquiline and multidrug-resistant tuberculosis: a systematic and critical analysis of the evidence. *European Respiratory Journal* 2016; 47 (2): 394 – 402.

Price AC, Choi K-H, Heath RJ, Li Z, White SW, Rock CO. Inhibition of beta -Ketoacyl-Acyl Carrier Protein Synthases by Thiolactomycin and Cerulenin. Structure and mechanism. *Journal of Biological Chemistry* 2001; 276 (9): 6551 – 6559.

Pym AS, Diacon AH, Tang SJ, Conradie F, Danilovits M, Chuchottaworn C, Vasilyeva I, Andries K, Bakare N, De Marez T, Haxaire-Theeuwes M, Lounis N, Meyvisch P, Van Baelen B, van Heeswijk RPG, Dannemann B on behalf of the TMC207-C209 Study Group. Bedaquiline in the treatment of multidrug- and extensively drug resistant tuberculosis. *European Respiratory Journal* 2016; 47 (2): 564 – 574.

Ramarathnam N, Osawa T, Namiki M, Kawakishi S. Chemical studies on novel rice hull antioxidants. 2. Identification of isovitexin, a C-glycosyl flavonoid. *Journal of Agricultural and Food Chemistry* 1989; 37: 316 – 319.

Ramaswamy S, Musser JM. Molecular genetic basis of antimicrobial agent resistance in *Mycobacterium tuberculosis*: 1998 update. *The International Journal of Tuberculosis and Lung Disease* 1998; 79 (1): 3 – 29.

Ramaswamy SV, Reich R, Dou SJ, Jasperse L, Pan X, Wanger A, Quitugua T, Graviss EA. Single nucleotide polymorphisms in genes associated with isoniazid resistance in *Mycobacterium tuberculosis*. *Antimicrobial Agents and Chemotherapy* 2003; 47 (4): 1241–1250.

Rawat R, Whitty A, Tonge PJ. The isoniazid-NAD adduct is a slow, tight-binding inhibitor of InhA, the *Mycobacterium tuberculosis* enoyl reductase: Adduct affinity and drug resistance. *Proceedings of the National Academy of Science of USA* 2003; 100 (24): 13881 – 13886.

Rengarajan J, Sasseti CM, Naroditskaya V, Sloutsky A, Bloom BR, Rubin EJ. The folate pathway is a target for resistance to the drug para-aminosalicylic acid (PAS) in mycobacteria. *Molecular Microbiology* 2004; 53 (1): 275 – 282.

Richter E, Rüscher-Gerdes S, Hillemann D. First linezolid-resistant clinical isolates of *Mycobacterium tuberculosis*. *Antimicrobial Agents and Chemotherapy* 2007; 51 (4): 1534 – 1536.

Rinder H, Thomschke A, Rüscher-Gerdes S, Bretzel G, Feldmann K, Rifai M, Löscher T. Significance of *ahpC* promoter mutations for the prediction of isoniazid resistance in *Mycobacterium tuberculosis*. *European Journal of Clinical Microbiology and Infectious Disease* 1998; 17 (7): 508 – 511.

Rozwarski DA, Grant GA, Barton DH, Jacobs WR Jr., Sacchettini JC. Modification of the NADH of the isoniazid target (InhA) from *Mycobacterium tuberculosis*. *Science* 1998; 279 (5347): 98 – 102.

Rustomjee R, Lienhardt C, Kanyok T, Davies GR, Levin J, Mthiyane T, Reddy C, Sturm AW, Sirgel FA, Allen J, Coleman DJ, Fourie B, Mitchison DA and the Gatifloxacin for TB (OFLOTUB) study team. A Phase II study of the sterilising activities of ofloxacin, gatifloxacin and moxifloxacin in pulmonary tuberculosis. *The International Journal of Tuberculosis and Lung Disease* 2008; 12 (2): 128 – 138.

Rustomjee R, Diacon AH, Allen J, Venter A, Reddy C, Patientia RF, Mthiyane TCP, De Marez T, van Heeswijk R, Kerstens R, Koul A, De Beule K, Donald PR, McNeeley DF. Early bactericidal activity and pharmacokinetics of the diarylquinoline TMC207 in treatment of pulmonary tuberculosis. *Antimicrobial Agents and Chemotherapy* 2008; 52 (8): 2831 – 2835.

Safi H, Sayers B, Hazbón MH, Alland D. Transfer of *embB* codon 306 mutations into clinical *Mycobacterium tuberculosis* strains alters susceptibility to ethambutol, isoniazid, and rifampin. *Antimicrobial Agents and Chemotherapy* 2008; 52 (6): 2027 – 2034.

Safi H, Lingaraju S, Amin A, Kim S, Jones M, Holmes M, McNeil M, Peterson SN, Chatterjee D, Fleischmann R, Alland D. Evolution of high-level ethambutol-resistant tuberculosis through interacting mutations in decaprenylphosphoryl- β -D-arabinose biosynthetic and utilization pathway genes. *Nature Genetics* 2013; 45 (10): 1190 – 1197.

Schaaf H, Mabena F, Lee C. Guide to Patient Health; HIV, TB and Mental Health newsletter for healthcare professionals across the continent. *Medical Focus* 2021; 3 (4): 1 – 16.

Schroeder EK, de Souza ON, Santos DS, Blanchard JS, Basso LA. Drugs that inhibit mycolic acid biosynthesis in *Mycobacterium tuberculosis*. *Current Pharmaceutical Biotechnology* 2002; 3 (3): 197 – 225. doi.org/10.2174/1389201023378328.

Scorpio A, Zhang Y. Mutations in *pncA*, a gene encoding pyrazinamidase / nicotinamidase, cause resistance to the antituberculous drug pyrazinamide in tubercle bacillus. *Nature Medicine* 1996; 2 (6): 662 – 667.

Scorpio A, Lindholm-Levy P, Heifets L, Gilman R, Siddiqi S, Cynamon M, Zhang Y. Characterization of *pncA* mutations in pyrazinamide-resistant *Mycobacterium tuberculosis*. *Antimicrobial Agents and Chemotherapy* 1997; 41 (3): 540 – 543.

Segala E, Sougakoff W, Nevejans-Chauffour A, Jarlier V, Petrella S. New mutations in the mycobacterial ATP synthase: New insights into the binding of the diarylquinoline TMC207 to the ATP synthase C-ring structure. *Antimicrobial Agents and Chemotherapy* 2012; 56 (5): 2326 – 2634.

Shankar EM, Vignesh R, Ellegård R, Barathan M, Chong YK, Bador MK, Rukumani DV, Sabet NS, Kamarulzaman A, Velu V, Larsson M. HIV-*Mycobacterium tuberculosis* co-infection: A 'danger-couple model' of disease pathogenesis. *Pathogens and Disease* 2013; 70 (2): 110 – 118.

Shanmuganathan A, Srinivasan R, Thilagavathy G, Sathishkumar D, Sidduraj C, James B. Determination of sites involved, HIV co-infection and utility of diagnostic modalities in EPTB. *Journal of Clinical and Diagnostic Research* 2013; 7 (8): 1644 – 1646.

Sharma P, Chaudhary S, Pardeshi T, Sood N, Sheikh S, Deshmukh K. Designing Novel Inhibitors for Tuberculosis (Tb) by Targeting InhA and KasA Using Ligand Based Drug Design. *Journal of Innovations in Pharmaceuticals and Biological Sciences* 2015; 2 (2): 159 – 168.

Sharma SK, Mohan A, Kadiravan T. HIV-TB co-infection; epidemiology, diagnosis and management. *Indian Journal of Medical Research* 2005; 121: 550 – 567.

Shaw DJ, Robb K, Vetter BV, Tong M, Molle V, Hunt NT, Hoskisson PA. Disruption of key NADH binding pocket residues of the *Mycobacterium tuberculosis* InhA affects DD-CoA binding ability. *Scientific Reports* 2017; 7 (1): 1 – 7.

Sherman DR, Mdluli K, Hickey MJ, Arain TM, Morris SL, Barry 3rd CE, Stover CK. Compensatory *ahpC* gene expression in isoniazid-resistant *Mycobacterium tuberculosis*. *Science* 1996; 272 (5268): 1641 – 1643.

Shi W, Zhang X, Jiang X, Yuan H, Lee JS, Barry CE, Wang H, Zhang W, Zhang Y. Pyrazinamide inhibits trans-translation in *Mycobacterium tuberculosis*. *Science* 2011; 333 (6049): 1630 – 1632.

Shirude PS, Ramachandran S, Hosagrahara V. Challenges and opportunities in tuberculosis drug discovery: an industry perspective. *Future Medicinal Chemistry* 2013; 5 (5): 499 – 501.

Simons SO, Mulder A, van Ingen J, Boeree MJ, van Soolingen, D. Role of *rpsA* gene sequencing in diagnosis of pyrazinamide resistance. *Journal of Clinical Microbiology* 2013; 51 (1): 382 – 382.

Siu GK, Zhang Y, Lau TC, Lau RW, Ho PL, Yew WW, Tsui SK, Cheng VC, Yuen KY, Yam WC. Mutations outside the rifampicin resistance-determining region associated with rifampicin resistance in *Mycobacterium tuberculosis*. *Journal of Antimicrobial Chemotherapy* 2011; 66 (4): 730 – 733.

Slayden RA, Lee RE, Armour JW, Cooper AM, Orme IM, Brennan PJ, Besra GS. Antimycobacterial action of thiolactomycin: an inhibitor of fatty acid and mycolic acid synthesis. *Antimicrobial Agents and Chemotherapy* 1996; 40 (12): 2813 – 2819.

Slayden RA, Barry 3rd CE. The genetics and biochemistry of isoniazid resistance in *Mycobacterium tuberculosis*. *Microbes and Infection* 2000; 2 (6): 659 – 669.

Slayden RA, Lee RE, Barry 3rd CE. Isoniazid affects multiple components of the type II fatty acid synthase system of *Mycobacterium tuberculosis*. *Molecular Microbiology* 2000; 38(3): 514 – 525.

Spies FS, da Silva PE, Ribeiro MO, Rossetti ML, Zaha A. Identification of mutations related to streptomycin resistance in clinical isolates of *Mycobacterium tuberculosis* and possible involvement of efflux mechanism. *Antimicrobial Agents and Chemotherapy* 2008; 52 (8): 2947 – 2949.

Sreevatsan S, Stockbauer KE, Pan X, Kreiswirth BN, Moghazeh SL, Jacobs WR Jr., Telenti A, Musser JM. Ethambutol resistance in *Mycobacterium tuberculosis*: Critical role of embB mutations. *Antimicrobial Agents and Chemotherapy* 1997; 41 (8): 1677 – 1681.

Stanley RE, Blaha G, Grodzicki RL, Strickler MD, Steitz TA. The structures of the anti-tuberculosis antibiotics viomycin and capreomycin bound to the 70S ribosome. *Nature Structural and Molecular Biology* 2010; 17 (3): 289 – 293.

Stutz MD. 2018. Dissecting the role of TNF signaling in *Mycobacterium tuberculosis* disease pathogenesis to identify novel therapeutic targets. University of Melbourne Library. Minerva access.

Sun Z, Zhang J, Zhang X, Wang S, Zhang Y, Li C. Comparison of gyrA gene mutations between laboratory-selected ofloxacin-resistant *Mycobacterium tuberculosis* strains and clinical isolates. *International Journal of Antimicrobial Agents* 2008; 31 (2): 115 – 121.

Suzuki Y, Katsukawa C, Tamaru A, Abe C, Makino M, Mizuguchi Y, Taniguchi H. Detection of kanamycin-resistant *Mycobacterium tuberculosis* by identifying mutations in the 16S rRNA gene. *Journal of Clinical Microbiology* 1998; 36 (5): 1220 – 1225.

Takayama K, Kilburn JO. Inhibition of synthesis of arabinogalactan by ethambutol in *Mycobacterium smegmatis*. *Antimicrobial Agents and Chemotherapy* 1989; 33 (9): 1493 – 1499.

Takiff HE, Salazar L, Guerrero C, Philipp W, Huang WM, Kreiswirth B, Cole ST, Jacobs WR Jr, Telenti A. Cloning and nucleotide sequence of *Mycobacterium tuberculosis* gyrA and gyrB genes and detection of quinolone resistance mutations. *Antimicrobial Agents and Chemotherapy* 1994; 38 (4): 773 – 780.

Tamura K, Nei M. Model Selection in the estimation of the number of nucleotide substitutions. *Letter to the Editor* 1993: 154 – 157.

Tan Y, Hu Z, Zhang T, Cai X, Kuang H, Liu Y, Chen J, Yang F, Zhang K, Tan S, Zhao Y. Role of pncA and rpsA gene sequencing in detection of pyrazinamide resistance in *Mycobacterium tuberculosis* isolates from southern China. *Journal of Clinical Microbiology* 2014; 52 (1): 291 – 297.

Telenti A, Imboden P, Marchesi F, Lowrie D, Cole S, Colston MJ, Matter L, Schopfer K, Bodmer T. Detection of rifampicin-resistance mutations in *Mycobacterium tuberculosis*. *The Lancet* 1993; 341 (8846): 647 – 550.

Telenti A, Philipp WJ, Sreevatsan S, Bernasconi C, Stockbauer KE, Wieles B, Musser JM, Jacobs WR Jr. The emb operon, a gene cluster of *Mycobacterium tuberculosis* involved in resistance to ethambutol. *Nature Medicine* 1997; 3 (5): 567 – 570.

Ticona E, Huaroto L, Kirwan DE, Chumpitaz M, Munayco CV, Maguiña M, Tovar MA, Evans CA, Escombe R, Gilman RH. Impact of Infection Control Measures to Control an Outbreak of Multidrug-Resistant Tuberculosis in a Human Immunodeficiency Virus Ward, Peru. *American Journal of Tropical Medicine and Hygiene* 2016; 95(6): 1247 – 1256.

Traore H, Fissette K, Bastian I, Devleeschouwer M, Portaels F. Detection of rifampicin resistance in *Mycobacterium tuberculosis* isolates from diverse countries by a commercial line probe assay as an initial indicator of multidrug resistance. *The International Journal of Tuberculosis and Lung Disease* 2000; 4 (5): 481 – 484.

Trefzer C, Rengifo-Gonzalez M, Hinner MJ, Schneider P, Makarov V, Cole ST, Johnsson K. Benzothiazinones: Prodrugs that covalently modify the decaprenylphosphoryl- β -D-ribose 2'-epimerase DprE1 of *Mycobacterium tuberculosis*. *Journal of the American Chemical Society* 2010; 132 (39): 13663 – 13665.

Venter GJ, Adams MD, Myers EW, Li PW, Mural RJ, Sutton GG, Smith HO, Yandell M, Evans CA, Holt RA, et al. The Sequence of the Human Genome. *Science* 2001; 291 (5507): 1304 – 1351.

Veyron-Churlet R, Zanella-Cléon I, Cohen-Gonsaud M, Molle V, Kremer L. Phosphorylation of the *Mycobacterium tuberculosis* β -Ketoacyl-Acyl Carrier Protein Reductase MabA Regulates Mycolic Acid Biosynthesis. *Journal of Biological Chemistry* 2010; 285 (17): 12714 – 12725.

Vilchèze C, Jacobs WR Jr. The mechanism of isoniazid killing: Clarity through the scope of genetics. *Annual Review of Microbiology* 2007; 61 (1): 35 – 50.

Vilchèze C, Av-Gay Y, Attarian R, Liu Z, Hazbón MH, Colangeli R, Chen B, Liu W, Alland D, Sacchettini JC, Jacobs WR Jr. Mycothiol biosynthesis is essential for ethionamide susceptibility in *Mycobacterium tuberculosis*. *Molecular Microbiology* 2008; 69 (5): 1316 – 1329.

Vögeli B, Rosenthal RG, Stoffel GMM, Wagner T, Kiefer P, Cortina NS, Shima S, Erb TJ. InhA, the enoyl-thioester reductase from *Mycobacterium tuberculosis* forms a covalent adduct during catalysis. *Journal of Biological Chemistry* 2018; 293 (44): 17200 – 17207.

Von Groll A, Martin A, Jureen P, Hoffner S, Vandamme P, Portaels F, Palomino JC, da Silva PA. Fluoroquinolone resistance in *Mycobacterium tuberculosis* and mutations in gyrA and gyrB. *Antimicrobial Agents and Chemotherapy* 2009; 53 (10): 4498 – 4500.

Vulliamy T, Kaeda J, Foroni L, Bain BJ (2011). Molecular and cytogenetic analysis. In: Dacie JV, Lewis M, *Practical Haematology*, 11th edition, Elsevier Churchill Livingstone: 139 – 145.

Wang CLJ, Salvino JM. Total synthesis of ($\pm$) thiolactomycin. *Tetrahedron Letters* 1984; 25 (46): 5243 – 5246.

Wang F, Jain P, Gulten G, Liu Z, Feng Y, Ganesula K, Motiwala AS, Loerger TR, Alland D, Vilchèze C, Jacobs WR Jr., Sacchettini JC. *Mycobacterium tuberculosis* dihydrofolate reductase is not a target relevant to the antitubercular activity of isoniazid. *Antimicrobial Agents and Chemotherapy* 2010; 54 (9): 3776 – 3782.

Wang M, Li J, Rangarajan M, Shao Y, LaVoie EJ, Huang TC, Ho CT. Antioxidative phenolic compounds from Sage (*Salvia officinalis*). *Journal of Agricultural and Food Chemistry* 1998; 46: 4869 – 4873.

World Health Organization. Guidelines on treatment of tuberculosis. Module 4: (2021) Geneva, Switzerland. (<https://www.who.int/publications-detail-redirect/9789240048126>) [Accessed: 19 June 2022].

World Health Organization. Global Tuberculosis Report 2022. Geneva, Switzerland.

Xiao J, Capanoglu E, Jassbi AR, Miron A. Dietary phytochemicals: Nutrition and health advance on the flavonoid C-glycosides and health benefits. *Dietary Phytochemicals: Nutrition and Health 2015*: s29 – s45.

Yano T, Kassovska-Bratinova S, Teh JS, Winkler J, Sullivan K, Isaacs A, Schechter NM, Rubin H. Reduction of clofazimine by mycobacterial type 2 NADH:Quinone oxidoreductase. *Journal of Biological Chemistry 2011*; 286 (12): 10276 – 10287.

Zaunbrecher MA, Sikes RD Jr., Metchock B, Shinnick TM, Posey JE. Overexpression of the chromosomally encoded aminoglycoside acetyltransferase eis confers kanamycin resistance in *Mycobacterium tuberculosis*. *Proceedings of the National Academy of Sciences of the USA 2009*; 106 (47): 20004 – 20009.

Zhang Y, Heym B, Allen B, Young D, Cole S. The catalase-peroxidase gene and isoniazid resistance of *Mycobacterium tuberculosis*. *Nature 1992*; 358 (6387): 591 – 593.

Zhang Y, Mitchison D. The curious characteristics of pyrazinamide: A review. *The International Journal of Tuberculosis and Lung Disease 2003*; 7 (1): 6 – 21.

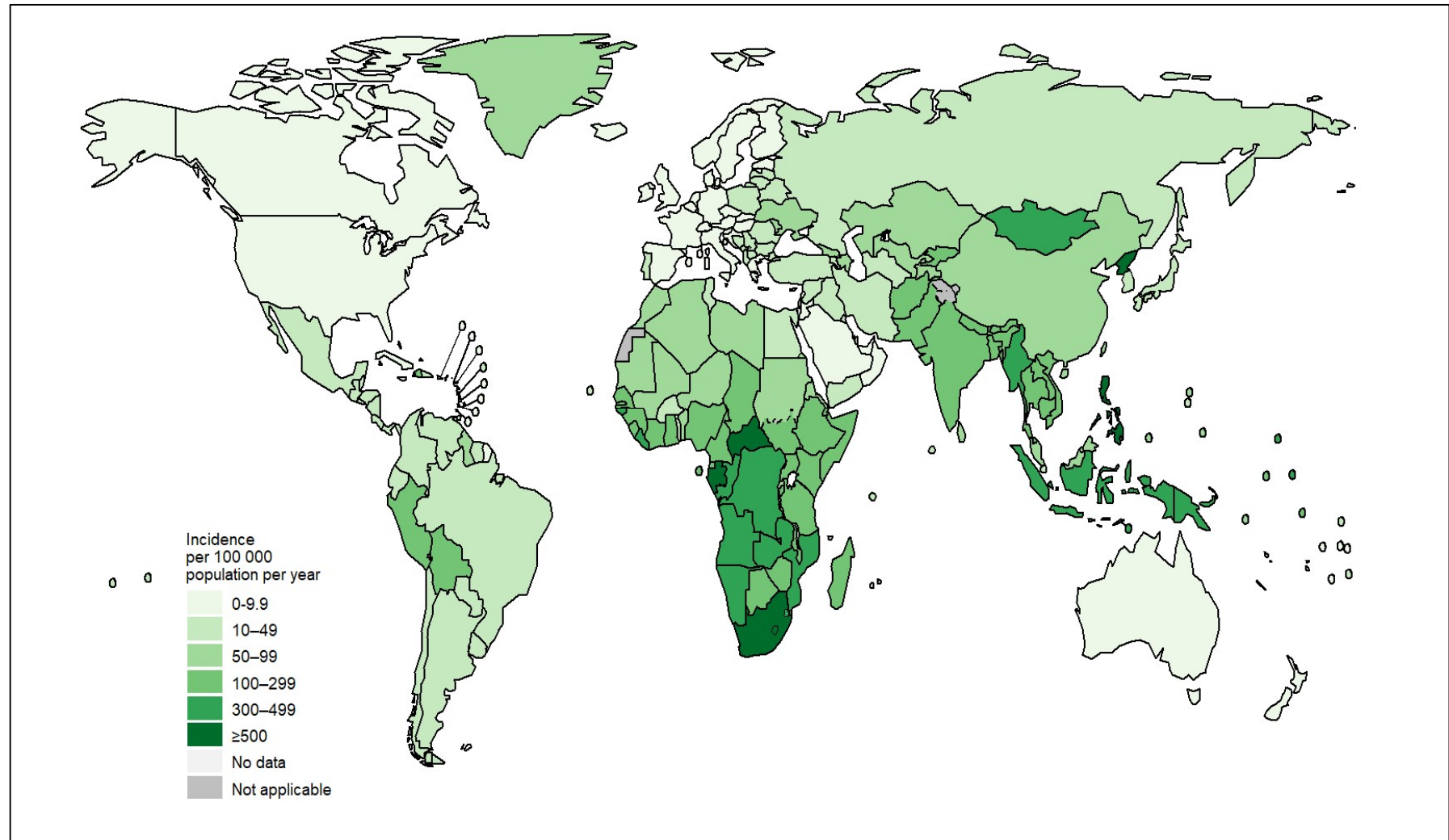
Zhang Y. The magic bullets and tuberculosis drug targets. *Annual Review of Pharmacology and Toxicology 2005*; 45 (1): 529 – 564.

Zhao F, Wang XD, Erber LN, Luo M, Guo AZ, Yang SS, Gu J, Turman BJ, Gao YR, Li DF, Cui ZQ, Zhang ZP, Bi LJ, Baughn AD, Zhang XE, Deng JY. Binding pocket alterations in dihydrofolate synthase confer resistance to para-aminosalicylic acid in clinical isolates of *Mycobacterium tuberculosis*. *Antimicrobial Agents and Chemotherapy 2014*; 58 (3): 1479 – 1487.

Zimhony O, Cox JS, Welch JT, Vilchèze C, Jacobs WR Jr. Pyrazinamide inhibits the eukaryotic-like fatty acid synthetase I (FAS-I) of *Mycobacterium tuberculosis*. *Nature Medicine 2000*; 6 (9): 1043 – 1047.

Zimhony O, Vilchèze C, Arai M, Welch JT, Jacobs WR Jr. Pyrazinoic acid and its n-propyl ester inhibit fatty acid synthase type I in replicating tubercle bacilli. *Antimicrobial Agents and Chemotherapy 2007*; 51 (2): 752 – 754.

Appendix 1: Estimated 2021 global distribution of TB cases according to WHO



Appendix 2: Letter of Ethics approval



Environmental & Biosafety Research Ethics Committee

02-Nov-2019

Dear Mr Mojalefa Setlai

Project Title: Designing novel inhibitors for Mycobacterium tuberculosis by targeting InhA and KasA using ligand-based drug discovery

Department: CUT - Central University of Technology

APPLICATION APPROVED

This letter confirms that this research proposal was given ethical clearance by the Biosafety & Environmental Research Ethics Committee of the University of the Free State.

Your ethical clearance number, to be used in all correspondence is: UFS-ESD2019/0064

Please note the following:

1. This ethical clearance is valid for one year from the issuance of this letter.
2. If the research takes longer than one year to complete, please submit a Continuation Report to the Ethics Committee before ethical clearance expires.
3. If any changes are made during the research process (including a change in investigators), please inform the Ethics Committee by submitting an Amendment.
4. When the research is concluded, please submit a Final Report to the Ethics Committee.

Thank you for your application and we wish you well in all of your research endeavours.

Yours Sincerely



Prof. RR (Robert) Bragg

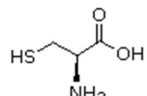
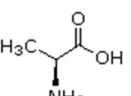
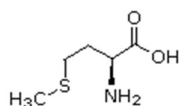
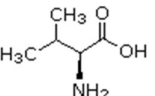
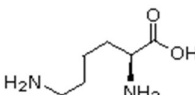
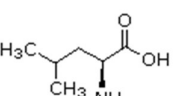
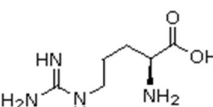
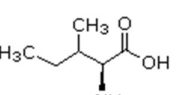
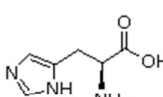
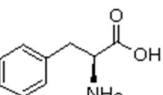
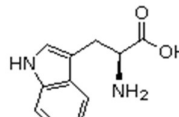
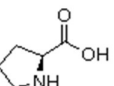
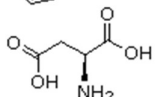
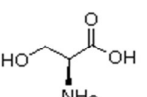
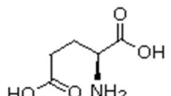
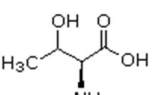
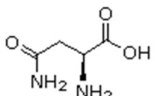
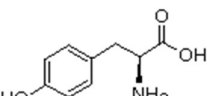
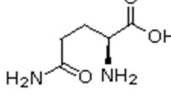
Chairperson: Biosafety & Environmental Research Ethics Committee

University of the Free State

Directorate: Research Development
T: +27 (0)51 401 9398 | +27 (0)51 401 2075 | E: smitham@ufs.ac.za
Johannes Brill Building, Room 106D, First Floor
205 Nelson Mandela Drive | Park West, Bloemfontein 9301 | South Africa
P.O. Box 339 | Bloemfontein 9300 | South Africa | www.ufs.ac.za



Appendix 3: List of amino acids

Name	Formula	Abbreviations	Name	Formula	Abbreviations
Glycine		Gly G	Cysteine		Cys C
Alanine		Ala A	Methionine		Met M
Valine		Val V	Lysine		Lys K
Leucine		Leu L	Arginine		Arg R
Isoleucine		Ile I	Histidine		His H
Phenylalanine		Phe F	Tryptophan		Trp W
Proline		Pro P	Aspartic Acid		Asp D
Serine		Ser S	Glutamic Acid		Glu E
Threonine		Thr T	Asparagine		Asn N
Tyrosine		Tyr Y	Glutamine		Gln Q

Appendix 4: Biohazard Safety Cabinet class II ISO Certificate



WORLD CLASS. WORLDWIDE.
International distributors worldwide in more than 100 countries
Escoc and/or its officers are members of CETA/IST/SEFA/ABSA



Escoc Micro Plc Ltd
Cert No. 307589



Escoc Micro Plc Ltd
Cert No. 021323/1

Model: AC2-451 Serial Number: 2009-41911

This document certifies that the Airstream Class II Biohazard Safety Cabinet bearing the markings above has been individually factory balanced after production in accordance with our published specifications for exhaust / inflow air volume.

Before shipment, the cabinet was individually factory-tested and found to be in compliance with the following international standards and norms:

For biohazard safety cabinetry construction
EN12469:2000 (Class II) requirements

For HEPA / ULPA filter performance
EN 1822 "High Efficiency Air Filters (HEPA and ULPA)"
IEST-RP-CC001.3 "Recommended Practice for HEPA and ULPA Filters"

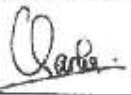
For cabinet cleanliness and product protection
Class 3 as per ISO 14644 · Class 1 / M1.5 as per US Federal Standard 209E
Class C as per British Standard BS 5295 · Class 1 as per German Standard VDI 2083
Class 3 as per Japanese Standard JIS B9920 · Class 0.035 as per Australian Standard AS 1386
Class 40 as per French Standard AFNOR X44001
Class M100 as per Korean Standard Z7030.1

Independent type certification
Airstream Class II BSC (AC2-3E1, AC2-4E1, and AC2-6E1) has been certified by HPA – Health Protection Agency (UK)
for compliance with EN12469:2000 (Class II) requirements

Testing has been performed in accordance with established international procedures, using the latest instruments, properly calibrated. For more details on methodologies and results obtained, please refer to the report that accompanies this certificate.

Date of Testing: 2nd September 2009

Test Department Supervisor



Charles Sitindaon

Test Engineer



Name: Rommel Saragih

Escoc Micro Plc. Ltd. • 21 Changi South Street 1 • Singapore 486777
Tel: +65 6542 0933 • Fax: +65 6542 9920 • mail@escocglobal.com • www.escocglobal.com
Singapore • Bangkok • Phnom Penh • Port Moresby • Port of Spain • Pretoria • Tokyo • Kuala Lumpur • Manila • Beijing • Shanghai • Guangzhou • Hong Kong • Melbourne • Sydney • Perth

Appendix 5: Permission letter from NHLS



Practice No. 5200296

Office of the Business Manager
UNIVERSITAS ACADEMIC LABORATORIES
PO BOX 339 (G3)
C/O: CHEMICAL PATHOLOGY
1ST FLOOR
BLOCK C
FACULTY OF HEALTH SCIENCES
UNIVERSITY OF FREE STATE
BLOEMFONTEIN
9301

Date: 08 October 2019

Request: Mr. Mojalefa Setlai

Project Name: "Designing novel inhibitors for Mycobacterium tuberculosis by targeting inhA and kasA genes using ligand-based drug discovery."

Dear Mr. Setlai,

Your request for use of laboratory facilities / data is hereby granted under following conditions:

- 1) That University Ethical Committee approval is obtained.
- 2) All laboratory data remain confidential to the patient and doctor (anonymity is maintained).
- 3) This office must be notified before any publication of any results / findings are made.
- 4) NHLS is recognised in all publications.
- 5) That a successful K-project application be made and relevant NHLS project cost centre be created to utilise testing at NHLS as per your protocol.

May your project be successful

Regards,



Mr. Pakiso Letanta
Acting Business Manager



Appendix 6: Proof of delivery of MHA plates

Page 1 of 1

NHLS DELIVERY/ PICKING NOTE

Ship: Rietfontein Diagnostica Media
From: Pro A
1 Modderfontein Road
2000
GP

Ship: UNI MICRO E
To: UNI - Microbiology
UNI MICRO E
NHLS MICROBIOLOGY
Second Floor, Block C, R
Medical Faculty, Francois
Bloomfontein

Date: 26-10-2020
Time: 08:19:25
Page: 1

Customer No: int2311

Customer PO:

Req No: 1996924
Order No: 405788

Requestor: JASON.JASPER
Delivery Note: 33317197

Line	Code	Description	UOM	Locator Number	Lot Number	Expiry Date	Req Qty	Pick Qty	Shipped Qty	BackOrder Qty
1	BMFA0365	Mueller Hinton agar + sheep blood	EA		3212053	23-12-20	8	8	8	0

Comments: drp order

Picked By: Name: Aphelele Signature: [Signature]

Date: 26/10/20 Time: 08:19

Despatched By: Name: Anderson Signature: [Signature]

Date: 26/10/20 Time: 08:19

Received By: Name: MB SETUAI Signature: [Signature]

Date: 27/10/20 Time: 08:45

Comments:

Appendix 7: Internal quality control for bacteriological media



Records of Internal Quality Control for Bacteriological Media upon arrival from DMP Stores F0209

Date: 27-10-2020 Delivery Note: 33317197 Order number: 405788 Lot numbers/Expiry – refer to attached

Delivery Note: Order number:

Delivery Note: Order number:

Delivery Note: Order number:

Item checked	LOT	Visual check	Sterility check following incubation		Control organism Planted	Results		Sign	Date
		Date/Sign	Passed/Failed	Date/Sign		Expected	Obtained		
<i>MHA plates</i>	<i>J22033</i>	<i>27/10/20 AB</i>	<i>Pass</i>	<i>28/10/20 AB</i>	<i>E. faecalis</i>	<i>+ Growth</i>	<i>✓</i>	<i>AB</i>	<i>30/10/2020</i>

Appendix 8: Calculation of serial concentrations

Concentrations Calculation			
GPK91	GPK96	V1	C2
# on the plate			
1	2	100.000	25.000
3	4	50.000	12.500
5	6	25.000	6.250
7	8	12.500	3.125
9	10	6.250	1.563
11	12	3.125	0.781
13	14	1.563	0.391
15	16	0.781	0.195
17	18	0.391	0.098
19	20	0.195	0.049

C1 = Initial concentration = 25.0 µg/ml

C2 = Final concentration

V1 = Initial volume

V2 = Final volume = 100 µl

Formula: $C2 = (C1 \cdot V2) / V2$

Appendix 9: Congress participation: Paper presentation

