

The efficacy of coumarin derivatives against persistent mycobacterial biofilms

By

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Submitted in partial fulfilment of the requirements for the degree of

PHILOSOPHIAE DOCTOR (Medicinal Plant Sciences)

in the

Department of Plant and Soil Sciences

of the

Faculty of Natural and Agricultural Sciences

UNIVERSITY OF PRETORIA

PRETORIA



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2018

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Acknowledgements

The following individuals have played a key role during the course of this PhD study and my sincere gratitude goes out to each and every one on this list and the ones forgotten.

- My supervisor, Prof Namrita Lall, who provided me with guidance, support and motivation, I will always be truly thankful.
- My co-supervisor, Dr Anil Ojha from the University of Albany (USA) for his assistance with the experiments with regard to the mycobacterial biofilms.
- Dr Navneet Kishore and Ms Dominique Buyens, for their contributions made on the synthesis and chemistry part of this study.
- Prof Bernard Fourie, Buki Onwuegbuna, Dr Farzana Ishmail and all the staff at the NHLS, Prinshoff Campus for assistance with the antimycobacterial studies.
- Prof Lyndy McGaw and the whole team at Onderstepoort, Phytomedicine group for the assistance with the hepatoprotective assays.
- Dr Vivek Kumar, Prof Fourie Joubert, Johan Swart, Dr Andre Stander and Johan van der Westhuizen for all the assistance with the computational work.
- Ms Jeannette Brand and all the staff at the Medical Research Council for the continued support.
- Dr Chris Hamilton for the guidance and support with the enzymatic studies.
- Prof Eric Strauss and friends from the Strauss lab, for allowing me to conduct work in his lab.
- The faculty and staff from the Department of Plant and Soil Sciences.

- My family, friends and colleagues for their continued support throughout my studies.
- My dear friends Marco and Danielle for walking along this journey with me, I will always be in debt to you.
- My siblings, Hannes and Inarie, I am now officially smarter than both of you combined.
- To my wife, Annebel, all I can say is thank you, you have been my rock and thanks for being patient with me, I love you.
- My parents, Jurg and Elmarie, your love and encouragement have carried me from the start, I couldn't have asked for anything more.

I would further like to thank the following institutions for their support and funding:

- Chemistry department, University of Pretoria
- Department of Science and Technology
- Medical Research Council of South Africa
- National Research Foundation
- New York State Department of Health, Albany
- Stellenbosch University
- University of East Anglia
- University of Pretoria

Dedication

I dedicate this thesis to YOU, the reader for showing interest and taking the time to read.

Research Outputs

PhD MANUSCRIPTS

Oosthuizen CB, Gasa N, Hamilton CJ, Lall N. “Inhibition of mycothione disulphide reductase and mycobacterial biofilm by selected South African plants.” *South African Journal of Botany*. July 2018. (Accepted)

Carel B. Oosthuizen, Navneet Kishore, Vivek Kumar, Anil Ojha, Jacob Richards, Eric Strauss, Chris J. Hamilton, Namrita Lall. “Selectivity of 7-hydroxycoumarin derivatives towards mycobacterial biofilm inhibition through interactions on mycothione reductase” *Journal of Medicinal Chemistry* August 2018 (Submitted)

BOOK CHAPTERS

Oosthuizen CB, Reid A, Lall N. (2017). “Can Medicinal Plants Provide an Adjuvant for TB Patients?” *Medicinal Plants for Holistic Health and Well-Being. Elsevier* ISBN 9780128124758

Reid A, **Oosthuizen CB**, Fibrich B. D, Twilley D, Lambrechts IA, De Canha MN, Rademan S and Lall N, 2017. “Traditional medicine: The ancient roots of modern practice”. *Medicinal plants for holistic health and well-being. Elsevier* ISBN: 9780128124758

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Oosthuizen CB, Kishore N, Kumar V, Ojha A, Lall N. “Natural coumarin derivatives as inhibitors of mycobacterial biofilms” South African Association of Botanists 44th Annual Conference (SAAB). Botany for the Biodiversity Economy. Pretoria, South Africa. (9th – 12th January 2018).

Oosthuizen CB, Kishore N, Kumar V, Ojha A, Lall N. “Inhibition of Mycobacterial biofilms by natural Coumarins.” Indigenous Plant Use Forum 20th Annual Conference. Back to basics - Discovering our indigenous roots. Pretoria, South Africa. (9th – 12th June 2017). **Awarded best paper.*

Oosthuizen CB, Kishore N, Lall N. “The inhibition of Mycobacterial biofilms by natural coumarin derivatives.” The International Symposium on Methods for studying drug metabolism and transport, and African Traditional medicine. Pretoria, South Africa (22nd -25th Nov 2015).

Oosthuizen CB, Kishore N and Lall N. “The use of natural coumarin derivatives to combat Mycobacterial infections, focusing on biofilm inhibition.” IPUF (Indigenous Plant Use Forum) and SEB (Society for Economic Botany) joint international conference. Clanwilliam, South Africa. (28th June – 2nd July 2015).

FUNDING AND AWARDS

- Gauteng **Biotech Fundi Award** (2018) for an outstanding Postgraduate student.
- Award for **Best Paper** (category: young scientist) at the Indigenous Plant Use Forum Annual conference (2017)
- **Technology Innovation Agency SEED** funding for the development and commercialisation of a natural product (2017/2018)
- Recipient of the National Research Foundation **PhD Innovation Scholarship**, 2014, 2015, 2016
- **Golden Key** International Honour Society, 2015

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List of Abbreviations

7H11: Middlebrook 7H11 agar	ETH: Ethionamide
7H9: Middlebrook 7H9 broth	EtOH: Ethanol
ActinoD: Actinomycin D	FadD32: Fatty acid degradation protein D32
AI: Autoinducer	FDA: Food and Drug Administration
AIDS: Acquired immunodeficiency syndrome	GSH: Glutathiol
ATCC: American Type Culture Collection	GSSG: Glutathione disulfide (oxidized)
BF: Biofilm	Gtr: Glutathiol disulfide reductase
CADD: Computer-aided drug design	HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
CAT: catalase	HepG2: Human hepatocytes cell-line
CCl₄: tetrachloromethane	HIV: Human immunodeficiency virus
CD: Coumarin derivatives	HMBC: Heteronuclear multiple bond correlation
CFU: Colony forming units	HMQC: Heteronuclear multiple-quantum correlation
Cipro: Ciprofloxacin	IC₅₀: 50% inhibition concentration
COSY: Correlation spectroscopy	INH: Isoniazid
CPU: Computer processing unit	K_m: Michaelis constant
DEPT: Distortionless enhancement by polarization transfer	LJ: Löwenstein-Jensen
DMSO: Dimethyl sulfoxide ((CH ₃) ₂ SO)	M.tb: <i>Mycobacterium tuberculosis</i>
DNA: Deoxyribonucleic acid	MABA: Microtitre alamar blue assay
Dscore: Druggability score	MD: Molecular dynamics
DTNB: Ellman's Reagent [5,5-dithio-bis-(2-nitrobenzoic acid)]	MDR: Multiple drug-resistant
EDTA: Ethylenediaminetetraacetic acid	MDS: Molecular dynamics simulation
ELISA: Enzyme-linked immunosorbent assay	MHz: Megahertz
EMB: Ethambutol	MIC: Minimum inhibitory concentration
EMEM: Eagle's minimum essential medium	MPBA: Microtitre presto blue assay
EPS: Extracellular polysaccharides	

MS: Mass spectrometry

MSH: Mycothiol

MSSM: Mycothione or Mycothiol disulfide

Mtr: Mycothiol disulfide reductase

MU: 4-Methylumbelliferone

NADPH: Nicotinamide adenine dinucleotide phosphate

NMR: Nuclear magnetic resonance

OADC: Oleic acid, albumin, dextrose, catalase

OD: Optical density

PANTA: Polymixin B, amphotricin B, nalidixic acid, trimethoprim, azlocillin

PDB: Protein data bank

QQ: Quorum quenching

QS: Quorum sensing

RIF: Rifampicin

RMSD: Root mean square deviation

RMSF: Root mean square fluctuation

SD: Standard deviation

SI: Selectivity index

SMILES: Simplified molecular-input line-entry system

SOD: Superoxide dismutase

STR: Streptomycin

TB: Tuberculosis

TM: Traditional medicine

Txr: Thioredoxin reductase

U: Umbelliferone

U937: Human macrophage cell-line

V_{max}: Maximum velocity

WHO: World health organization

WWW: World Wide Web

XDR: Extensively drug-resistant

XP: Extra precision

XTT: 2,3-bis(2-methoxy-4-nitro-5-sulphophenyl)-5[(phenylamino)carbonyl]-2H-tetrazolium hydroxide

Summary

The efficacy of coumarin derivatives against persistent mycobacterial biofilms

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Tuberculosis remains a problematic disease to treat and eradicate and the high incidence of latent infections are of great concern. The search for new drugs as well as drug targets should always be of high importance, and nature has an important role to play in the discovery process of novel drugs through the vast selection of secondary metabolites produced by plants, marine animals and other microorganisms. Although new antibiotics continue to be developed, the onset of drug resistance is probably one of the biggest concerns for the disease prevention and treatment.

The use of different kinds of molecules that do not actively kill or inhibit the growth through bacteriostatic properties can have a pharmacological relevance in treating diseases. Quorum quenching molecules and biofilm inhibitors fall under this class of “alter-biotics”, in that they inhibit a different

process within the bacterial population that renders them less virulent and infective without actively killing the bacteria, and in the process reduce the risk of acquired and selective resistance.

In this study, coumarin derivatives were evaluated for their mycobacterial biofilm inhibitory activities. It was identified that some of these derivatives exhibit the properties discussed above, with high selectivity towards biofilm and low antibacterial activity. The possible liver toxicity was evaluated and it showed that the majority of the derivatives had *in vitro* hepatoprotective effects. Furthermore, the possible mechanism or mode of action by which the compounds exhibit their activity was evaluated on the mycobacterial enzyme, mycothione reductase. It was established that the type and position of substitution on the coumarin nucleus had a great effect on the biological and enzymatic activities. Lastly, the use of computational techniques revealed important insights into the structure-activity relationship.

This study showed the significance of a coumarin derivative as a mycobacterial biofilm inhibitor and this compound can be considered for further research for pre-clinical and clinical studies.

Abstract

Numerous strides have been made in the treatment and diagnosis of Tuberculosis (TB), but it still remains one of the deadliest infectious diseases that plague this world. The efficiency by which the causative agent, *Mycobacterium tuberculosis* infects, is due to many different evolutionary functions that the bacilli employ. It is estimated that a quarter of the world's population is infected with a form of a latent subpopulation of mycobacteria. This sustains the elevated incidences and death rates seen each year. One mechanism that the bacteria employ to overcome, or at the least tolerate, antibiotic attack and the human immune response, is the formation of a protective biofilm structure. A biofilm is an aggregation of bacteria within an enclosed extracellular polymeric matrix. Most, if not all *Mycobacterium* spp. form biofilms in nature and the clinical significance in human disease has been emphasized in the literature. There are currently no drugs or drug candidates that target biofilm formation or eradication of *M. tuberculosis*.

One group of natural secondary metabolites that exhibit a broad range of biological activities as well as the inhibition of other bacterial biofilms are the coumarins. The position and type of substitution on the coumarin nucleus directly influence the pharmacological properties of the compound. This study attempted to synthesize twelve coumarin derivatives (CD) (6 x umbelliferone **U**, and 6 x 4-methylumbelliferone **MU**) and investigated their drug-susceptibility and biofilm inhibitory properties on mycobacterial planktonic and biofilm cultures. The aim was to identify a compound that targets biofilm but not necessarily has antimycobacterial activity through bactericidal or bacteriostatic effects.

Twelve compounds (two base compounds and 12 derivatives) belonging to the coumarin class of compounds, of which two were novel (**U5** and **MU5**), were synthesized. The samples were assessed for their antimycobacterial and antibiofilm activities on two mycobacterial organisms. Derivatives **U1** and **MUA** showed moderate antimycobacterial activity against *M. smegmatis* mc² 155 with minimum inhibitory activity (MIC) values of 543 and 508 µM, respectively. Derivatives **U2**, **MU2** and **MU5** exhibited the highest selectivity towards *M. smegmatis* mc² 155 biofilm inhibition with sample **MU2**

showing a selectivity index of 64 towards biofilm inhibition when compared to its antimycobacterial activity. The structure-activity relationship indicates the importance of a methyl substitution on C-4 and an alkane with two free methyl groups on the end of the coumarin nucleus, for increased activity. These three derivatives (**U2**, **MU2** and **MU5**) were shown to be the most active ones against *M. tuberculosis* H37Rv, with a MIC's of approximately 100 μ M. The intra-biofilm activity on *M. tuberculosis* mc² 7000 revealed that derivatives **U3**, **U4** and **MUA** reduced that bacterial count within the mature biofilm by up to 90%.

Owing to TB drug-induced liver toxicity in patients receiving conventional treatment, the derivatives were assessed for their hepatoprotective activities on acetaminophen-induced toxicity in a human hepatocyte cell line, HepG2. The derivatives showed moderate to negligible antiproliferative activities when tested for their cytotoxicity. All, except three derivatives (**U2**, **MU1** and **MU5**), showed a protective effect at concentrations ranging from 100 to 200 μ M. Compounds **MU1** and **MU5** had an antagonistic effect with the acetaminophen toxicity and this data was further substantiated by these compounds showing an increased inhibition of glutathione reductase in the mechanistic study, presenting an inhibitory concentration, where 50% of the enzymatic activity was inhibited (IC₅₀), at 106.4 $\pm$ 26.2 and 253.8 $\pm$ 11.1 μ M, respectively for **MU1** and **MU5**.

Glutathione reductase (Gtr) is an enzyme that reduces glutathione to the low molecular weight glutathiol and is produced by many eukaryotic and bacterial and fungi organisms. In the case of the *Mycobacterium* spp., mycothione reductase (Mtr) takes the role of Gtr. This enzyme has not only been identified as a possible drug-target for drug development, but the encoding gene has been shown to be upregulated during biofilm formation and the enzymatic product, glutathiol, has been found to be vital for the development of the biofilm. The mechanistic study involved the assessment of the derivatives as inhibitors of the mycobacterial analog Mtr and was compared to the activity with that on the human analog, Gtr. The derivative that showed the best activity and highest selectivity towards Mtr was **MU2**, with a selectivity index of 34. This inhibitory activity correlates with the biofilm activity and one can conclude that this could be a possible mechanism by which this compound exhibits its activity.

An attempt was made to investigate the variation in activity between structurally similar coumarins (**MU1** and **MU2**). Computational chemistry was employed through molecular docking and molecular

dynamics simulations for evaluation. The only structural difference between these compounds is a double bond of the 3-methyl-2-butenyloxy moiety of **MU1** vs. the saturated 3-methylbutoxy unit of **MU2** at C-7 of the coumarin nucleus. The investigation through molecular docking revealed the most likely pose and orientation that these inhibitors/ligands will adopt in the active site of the enzyme. Further analysis with the use of molecular dynamics simulations revealed that the torsion angle, specifically at the aforementioned double bond position is important for the inhibition.

This study is the first report of coumarins as mycobacterial biofilm inhibitors. Two new coumarin derivatives have been synthesized and their activity described. Additionally, this is the first report on the inhibition of mycothione reductase by coumarin derivatives, describing their possible molecular mechanism of inhibition at an atomic level, through the use of dynamics simulations. To date, the existing conventional TB-drugs have not been reported to have inhibitory activities on mycobacterial biofilms and the mycothione reductase enzyme. Derivative **MU2** exhibited a selectivity index of 64 towards biofilm inhibition of *M. smegmatis* when compared to its antimycobacterial activity and showed significant antimycobacterial activity against *M. tuberculosis* H37Rv with an MIC value of 102 μ M. Derivative **MU2** also showed the highest selectivity towards Mtr (SI of 34) when compared to its Gtr inhibition. The preclinical and clinical assessment can be considered for future aspects to prove the efficacy of the identified CD, firstly, in an animal model followed by humans.

***T**HEY CAN SAY, IT ALL SOUNDS CRAZY. They can say, I've lost my mind. I don't care, so call me crazy. We can live in a world that we design. 'Cause every night I lie in bed, the brightest colors fill my head. A million dreams are keeping me awake. I think of what the world could be, a vision of the one I see. A million dreams is all it's gonna take. A million dreams for the world we're gonna make."*

Justin Paul & Benj Pasek

“A hard beginning maketh a good ending.”

John Heywood

1

1. General Introduction

1.1. Background and Motivation of Study

Tuberculosis remains one of the world’s deadliest diseases, infecting on average 10.4 million people per year with a mortality rate of 1.7 million according to the world health organization (WHO, 2018). The bacilli responsible for this, is the acid-fast bacterium; *Mycobacterium tuberculosis* (*M.tb*). This organism employs a range of defensive strategies to overcome the body’s immune responses and evades the attack of the current drugs (Hunter, 2016). One mechanism that has been proposed to increase the difficulty in treating the infection, and explains the persistence of latent sub-populations of bacteria, is the formation of a biofilm (Islam et al., 2012).

A biofilm is an aggregation of bacteria embedded into a controlled extra-cellular polymeric matrix (**Fig 1.1**). Within the biofilm, the residing bacilli are protected from antibiotic attack as well as evasion of the human immune response (Ojha et al., 2008). Biofilm formation is controlled by quorum sensing through the communication of small signalling molecules and polypeptides produced by the bacteria. The mechanisms by which mycobacteria form a biofilm have not been described in depth, but it has been recognized clinically to be an important pathogenic factor. Chronic and latent tuberculosis infections are strongly associated with the formation of a biofilm (Esteban and García-Coca, 2018).

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Mycothiol, the low molecular weight thiol produced by mycobacteria, is vital for the development and formation of a biofilm (Vargas et al., 2016). This molecule protects the bacteria from oxidative stresses and the detoxification of reactive oxygen species and antibiotics. The function is similar to that of glutathiol, produced by other bacteria and eukaryotic cells. The enzyme involved in the production of this molecule is mycothione reductase (Mtr), an oxidoreductase produced in the place of glutathione reductase, by Actinobacteria (Newton et al., 2002; Sareen et al., 2003). This enzyme has been proposed to be a possible drug-target for the treatment of active and latent infections of TB (Rawat and Av-Gay, 2007). There are currently no drugs available that target this specific enzyme.



Fig 1.1: Biofilm of *Mycobacterium* on solid 7H11 media (left) and a biofilm pellicle forming on the air-medium interface in liquid 7H9 broth media (Oosthuizen, 2018).

Natural products and secondary metabolites produced by plants, marine animals and other microorganisms have been a source of novel drugs for many years. Coumarin and its derivatives are phenolic compounds from the α -benzopyrone class of secondary metabolites that exhibit a broad range of biological activities (Venugopala et al., 2013). The type and position of substitutions to the coumarin nucleus have had a profound effect on the biological and pharmacological properties. In the case of mycobacterial inhibition, this has been demonstrated by the work done by Kapp et al., (2017) and Keri et al., (2015). In addition, it has been established that this group of compounds show biofilm inhibitory activities on other bacterial systems (Lee et al., 2014; Reen et al., 2018).

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Computational chemistry and techniques have developed to such an extent, that computer models can be used to study the effect of an inhibitor or ligand on a specific binding site of an enzyme. Computer-aided drug design (CADD) has played a key role in the development of new antibiotics (Jorgensen, 2004). The combination of wet-lab enzyme inhibitory experiments and computational techniques, such as molecular docking and molecular dynamics simulations can be used to elucidate the mechanism of enzyme inhibition.

This study was, therefore, undertaken to evaluate the use of coumarin derivatives (CD) as antimycobacterials and biofilm inhibitors through the inhibition of mycothione reductase as one of the possible mechanisms, and to investigate the inhibition through the use of computational techniques to identify the most potent inhibitor of *Mycobacterium* spp. biofilms.

1.2. Hypothesis

Premise 1: *Mycobacterium tuberculosis* infection remains a problem and it is known that mycobacteria spp. form persistent biofilms that render them drug-tolerant and more difficult to treat than their planktonic counterpart.

Premise 2: Coumarins are a class of secondary metabolites that contain a wide range of biological activities, including the inhibition of biofilm formation of certain bacterial species and this activity is related to the type and position of the substitution.

Hypothesis: Derivatives of coumarin have the ability and selectivity to inhibit the formation of mycobacterial biofilms as opposed to their antimycobacterial activity.

1.3. Research Questions

Tuberculosis remains one of the world's deadliest diseases. The development of resistance by *Mycobacterium tuberculosis* to many of the current drugs has increased the severity of the problem. According to the WHO, it is estimated that a quarter of the human population is infected with latent

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tuberculosis, and this high number will sustain increased numbers of active disease, new incidences and mortality rate seen on a yearly basis. *Mycobacterium*, as most bacterial species, is known to form protective biofilm structures that increase the bacilli's tolerance to drug treatment, help with the evasion of the human immune response and increase the development of acquired resistance. Although not fully understood, the biofilm growth habit explains the difficulty in treating persistent latent TB infections.

Coumarins are a class of secondary metabolites produced by plants for various functions. These α -benzopyrone compounds have been established to exhibit an enormous range of biological activities, and their pharmaceutical relevance is related to specific chemical substitutions. Coumarin and its derivatives have been found to inhibit biofilm formation of *E. coli*, as well as inhibit quorum sensing that regulates the formation of biofilm. Additionally, some derivatives have shown exceptional antimycobacterial activities, targeting the inhibition of enzymes involved in the mycolic acid synthesis, as seen in the case with diarylcoumarins inhibiting the fatty acid degradation protein D32 (FadD32).

This led to the following research questions for this study:

- Can coumarin derivatives (CD) inhibit the growth of *Mycobacterium* spp.?
- Do CD have an effect on the formation of a biofilm or on the bacterial count within a mature biofilm?
- Do these CD show any toxicity on liver cells, or possibly a hepatoprotective effect?
- Are CD able to inhibit mycothione reductase, an enzyme related to biofilm formation?
- Can computational biology techniques be used to describe the specific activity of coumarins on this enzyme, by utilizing molecular docking and molecular dynamics simulations?
- What specific substitution on the CD may lead to an increase in the biological activities?

1.4. Aims and Objectives

The main aim of this study was to investigate if CD show mycobacterial biofilm inhibition and their selectivity towards biofilm vs bactericidal and bacteriostatic activities. Furthermore, the compounds were evaluated for their hepatoprotective activity on drug-induced toxic liver cells. The mechanism was assessed by investigating the inhibition of mycothione and glutathione reductases and computational techniques were used to explore the structure-activity relationship. A graphical representation of the objectives of this study can be found in **Fig 1.2**.

The objectives for the present study were as follows:

- Synthesis, purification and characterization of the CD.
- Determination of the minimum inhibitory concentrations (MIC) of the CD on *Mycobacterium tuberculosis* and *M. smegmatis*.
- Investigation of the inhibition of mycobacterial biofilm formation by the CD.
- Evaluate the intra-biofilm activity of the CD by colony count reduction.
- Establish the cellular toxicity or antiproliferative activity on human liver cells (HepG2).
- Determine the hepatoprotective effect on drug-induced-hepatotoxic cells.
- Investigate the inhibition of CD on mycothione and glutathione reductases.
- Establish the possible poses of the active CD on the enzymes using docking studies.
- Molecular dynamic simulation studies for tracking the trajectories in an effort to describe the structure-activity relationship.

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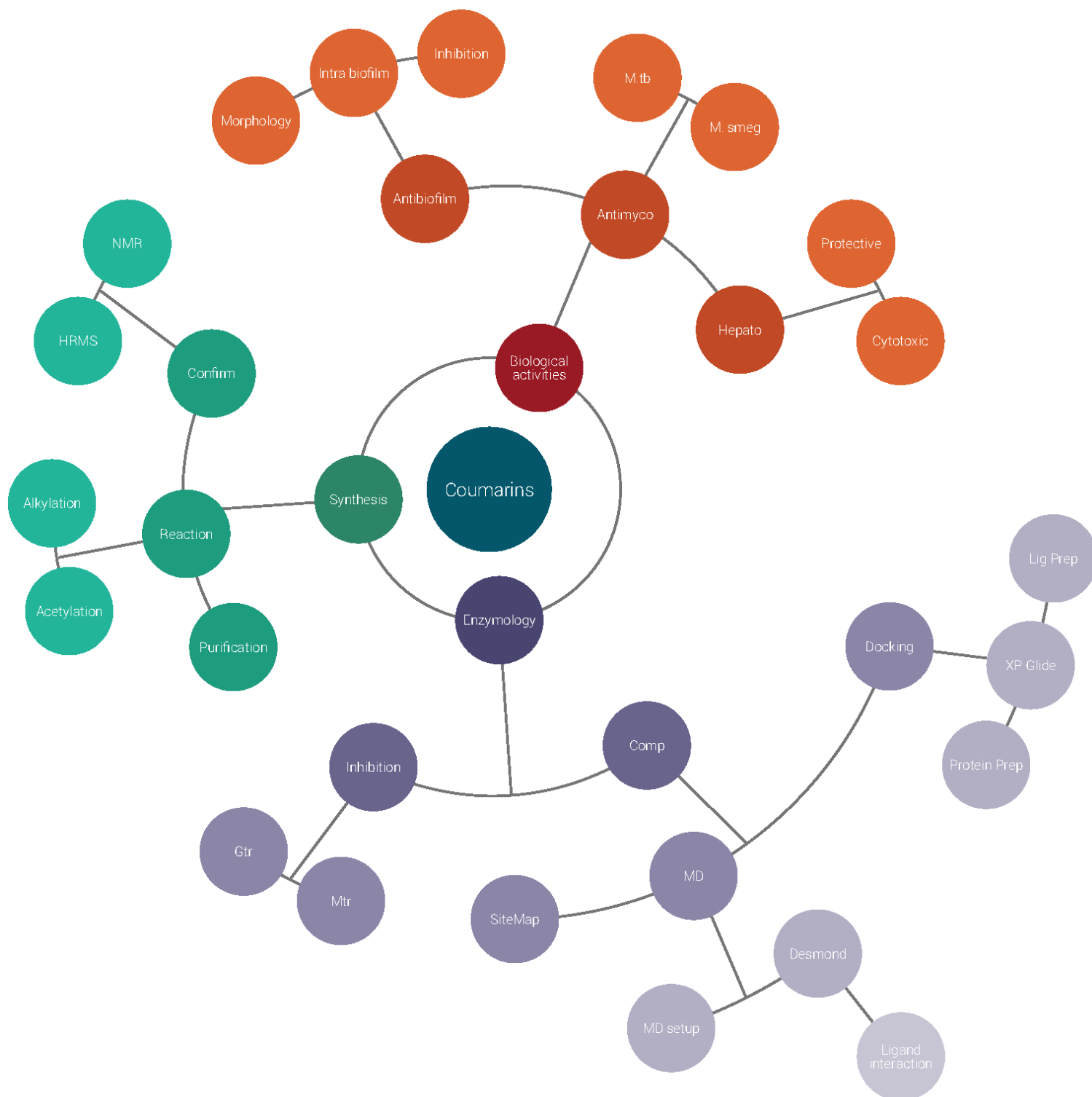


Fig 1.2: Mind map of project outline and objectives (Mindly, 2018).

1.5. Methodology

1.5.1. Synthesis of Coumarin Derivatives

Different methods of synthesis were utilized to synthesize the coumarin derivatives (CD), alkylation and acetylation as described earlier were used (Adfa et al., 2012; Devji et al., 2011; Huang et al., 2012; Khan et al., 2003, 2002). Two base compounds with a coumarin nucleus were used as the starting point of the derivatization namely; umbelliferone (**U**) (7-hydroxycoumarin) and 4-methylumbelliferone (**MU**) (7-hydroxy-4-methyl-coumarin) (**Fig 1.3**). Column chromatography was used to purify the derivatives. Nuclear magnetic resonance (400 MHz) and high-resolution mass spectrometry were used to compare with previous reports and confirm the structures (Li et al., 2010; Mishra et al., 2004).

1.5.2. Biological Assessment

1.5.2.1. Antimycobacterial Activity

Antibacterial testing of CD was conducted against *M. smegmatis*, mc² 155 and *Mycobacterium tuberculosis* H37Rv.

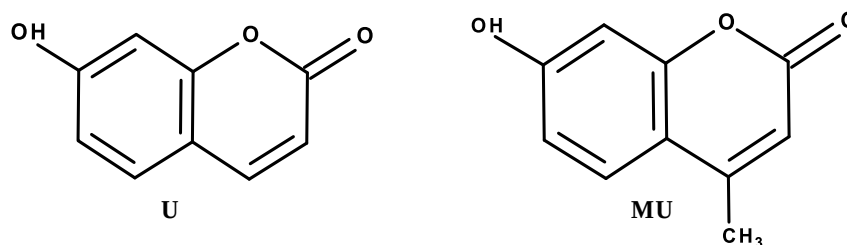


Fig 1.3: Basic starting structures of the two sets of CD, umbelliferone derivatives (labelled as **U**) and 4-methylumbelliferone derivatives (labelled as **MU**).

The microtitre alamar blue assay (MABA) and microtitre presto blue assay (MPBA) was used to evaluate the activity of the compounds against the bacteria and for the determination of the minimum inhibitory concentrations (MIC) (Franzblau et al., 1998; Lall et al., 2013).

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1.5.2.2. Antibiofilm Activity

The biofilm inhibitory activity of the derivatives was assessed by using a combination of different techniques. The non-pathogenic bacterium, *Mycobacterium smegmatis*, was used as the model organism due to its faster growth rate, *in vitro* biofilm formation capabilities and to limit the health risk associated with *M. tuberculosis* (**Fig 1.4**). The composition and structural components of mycobacterial biofilms are similar between species. The minimum biofilm formation inhibition concentration (MBFIC) was determined as the lowest concentration where no visual biofilm was formed. Crystal violet was used to quantify the amount of biofilm formed (Kulka et al., 2012; O'Toole, 2011; Peeters et al., 2008; Tram et al., 2013; Van den Driessche et al., 2014)

1.5.2.3. Biofilm Colony Morphology

Colony morphology was assessed by observing the size, shape, surface and margin of *M. smegmatis* on solid 7H11 agar discs containing a set concentration of the CD (Ealand et al., 2018).

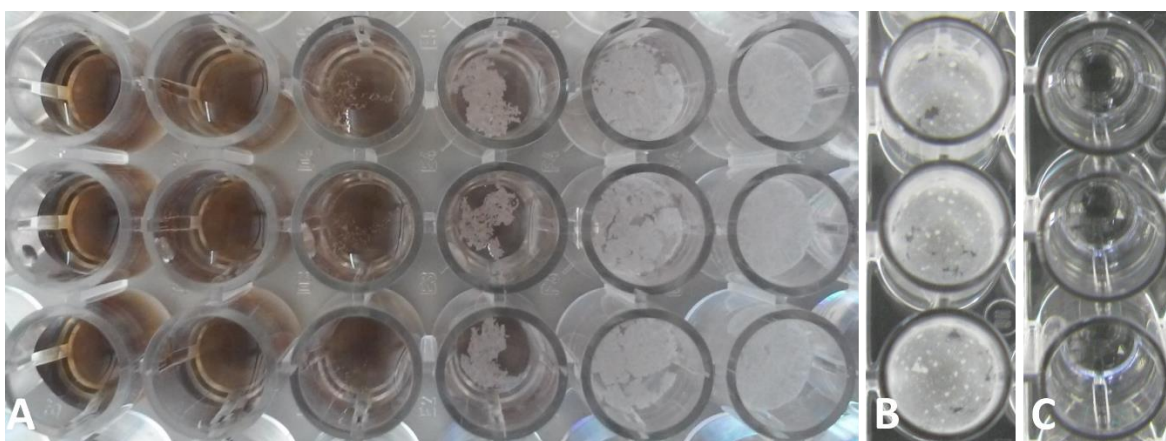


Fig 1.4: Dose-response of a plant extract on biofilm formation (A) (concentrations ranging from 1000 (left) to 31.25 (right) µg/mL), 100% biofilm formation (B), media control (C) (Oosthuizen, 2018).

1.5.2.4. Intra-biofilm Activity

The ability of the CD to reduce the bacterial count within a mature biofilm was assessed on an attenuated strain of *M. tuberculosis* H37Rv mc² 7000. Mature biofilms were treated with the CD, at a set concentration, and the biofilms were disrupted by the use of Tween80 to yield the viable bacilli from the

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enclosed structure. Bacterial colony counts were done using 7H11 agar plates, to determine the colony forming units present in the bacterial biofilm (Ojha et al., 2015).

1.5.2.5. *HepG2 Antiproliferative Activity*

The antiproliferative activity of the CD was assessed on a human liver cell line, HepG2 (ATCC HB-8065). The viability of the cells was evaluated by using presto blue, a resazurin-based salt solution that is converted from the blue resazurin to a red resorufin complex by the mitochondrial dehydrogenase enzyme in viable cells (Lall et al., 2013).

1.5.2.6. *Hepatoprotective Activity*

The first line tuberculosis drugs, although effective, have certain limitations; one of them is drug-induced-liver-toxicity related to the metabolism of the antibiotics by the liver.

The hepatoprotective activity of the CD was tested on human hepatocyte cell line HepG2 (ATCC HB-8065). Acetaminophen was used as the toxic inducer and the protective effect of the CD was evaluated by assessing the recovery of the cells after treatment by means of presto blue (Lall et al., 2016).

1.5.3. Mechanism Studies

Glutathiol is the major thiol present in almost all eukaryotes. The reduced form of glutathiol disulfide is a low molecular weight thiol, glutathiol, and it is mainly involved with the protection of cells against oxidative stress and reactive electrophiles (Newton and Fahey, 2002). Mycothiol is the bacterial analog, with the same activity in *Mycobacterium* as glutathiol in other organisms (Newton and Fahey, 2002; Rawat and Av-Gay, 2007). The enzymatic reaction of mycothione reductase that reduces the disulfide mycothione to mycothiol is depicted in **Fig 1.5**. The possible drug-target, mycothione reductase, an oxidoreductase, has been shown to be upregulated during biofilm formation of mycobacterial (Vargas et al., 2016). Additionally, this enzyme aids in bacterial tolerance towards isoniazid and ethionamide (Vilchèze et al., 2011). There are currently no drugs that act as inhibitors on this enzyme.

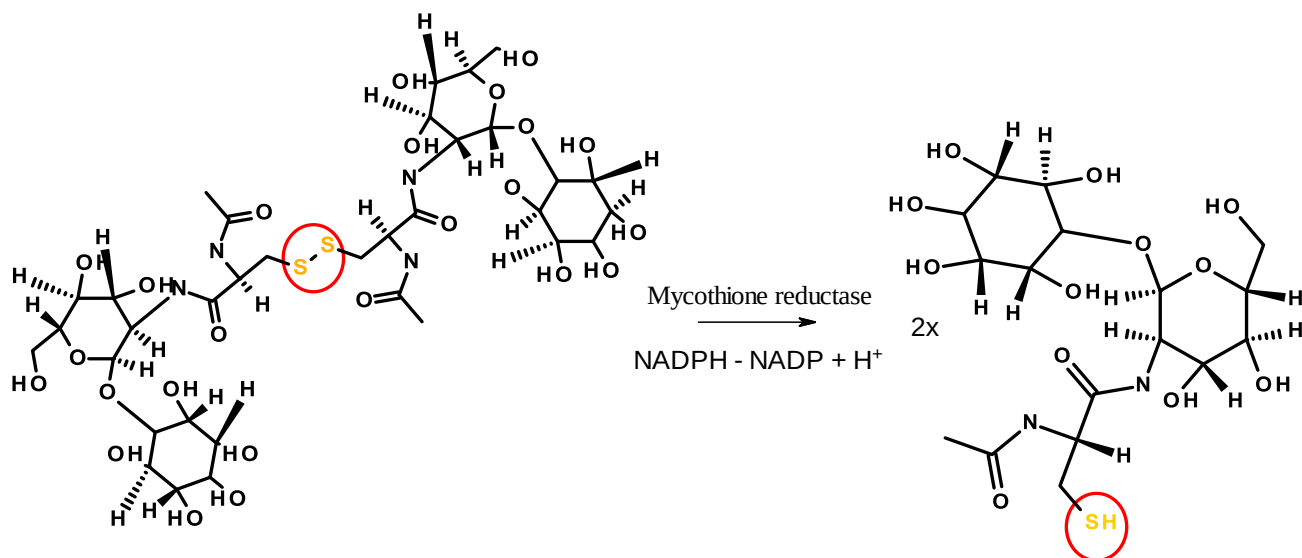


Fig 1.5: The enzymatic reaction of mycothione reductase that reduces mycothione (disulfide) to mycothiol.

Enzyme inhibition of the CD was tested on the mycobacterial enzyme and its human analog, glutathione reductase by using the assay described by Hamilton et al., (2009). Mycothione reductase was kindly donated by Dr Chris Hamilton, and/or isolated from a recombinant *E. coli* BL21 (Lamprecht et al., 2012). The initial rates were measured in the presence of saturated substrate concentrations with the CD as inhibitors at varying concentrations. The effective dose where 50% of the enzyme was inhibited due to CD, was calculated.

1.5.4. Computational Biology

1.5.4.1. Homology model

The crystal structure for mycothione reductase has not been elucidated and there are no 3-dimensional structures available in the RCSB protein data bank. The online server SWISS-MODEL (Swiss Institute of Bioinformatics, Biozentrum) was used to generate the homology model and coordinate file used for the subsequent assays. SWISS-MODEL (www.swissmodel.expasy.org) is a fully automated protein structure homology-modeling server, accessible via the ExpASY web server. (Benkert et al., 2011; Bertoni et al., 2017; Bienert et al., 2017; Guex et al., 2009; Waterhouse et al., 2018).

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1.5.4.2. *SiteMap*

For the identification and validation of possible target sites within the crystal structure of glutathione reductase (protein data bank (PDB) ID: 3SQP) and the homology model of mycothione reductase, SiteMap (Schrödinger, LLC, New York, NY, 2018) was utilized (Halgren, 2007, 2009a). The five most probable binding sites were identified and assessed for the subsequent assays.

1.5.4.3. *Molecular Docking*

Molecular docking was done to investigate the probability of the CD to bind to the enzyme as well as to determine the possible poses of the ligands within the binding site of the enzyme. The Glide (XP – extra precision) package with extensive sampling and advanced scoring functions within Schrodinger's Maestro (version 11.2.013, release 2017-2) was used for the docking study (Friesner et al., 2006, 2004; Halgren et al., 2004).

1.5.4.4. *Molecular Dynamic Simulations*

To investigate the inhibition of the derivatives/ligands on mycothione and glutathione reductases, and to examine the ligand-protein interactions, Schrodinger's Desmond Molecular Dynamics System, D. E. Shaw Research, was utilized and simulations of 50ns were run on a workstation equipped with 2x Intel Xeon CPU's and one Nvidia Tesla K40c GPU (Bowers et al., 2006). The ligand interaction analysis was done on the simulation trajectories after completion of the simulation and the data analyzed.

1.5.5. Structure-Activity Relationship

All the biological data were used to assign the activity to a specific structure and/or substitution on the structure.

1.6. Evaluation of Biological Methods

The methodology used in this study, specifically for the biofilm and enzyme inhibition assays, was established in the current study and has been published. The title of the manuscript is “*Inhibition of*

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mycothione disulphide reductase and mycobacterial biofilm by selected South African plants” and was accepted for publication in the journal, South African Journal of Botany. Twenty plants were selected based on their traditional usage against tuberculosis and related symptoms. These plants were evaluated, for their antimycobacterial, anti-biofilm and enzyme inhibitory activities. A brief summary of the paper is as follows.

The antimycobacterial activity of the ethanolic extracts from different genera and variable plant parts were evaluated using the microplate alamar blue assay. A biofilm formation inhibition assay was adapted from Mothiba et al., (2015), and optimized to determine the inhibitory activity against *Mycobacterium smegmatis* mc² 155 biofilm formation. The cytotoxic effect of the plant extracts was determined on U937 (ATCC CRL1593.2) human macrophage cells using the XTT [2, 3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide] cell proliferation kit. A DTNB-coupled glutathione/mycothiol disulfide reductase assay was used to determine the inhibitory activity against the human and bacterial reductases, respectively.

Of the 20 plant extracts tested, it was found that *Salvia africana-lutea*, *Ficus sur* and *Sphedamnocarpus pruriens* showed moderate antimycobacterial activity with minimum inhibitory concentration (MIC) values of 31.25, 62.5 and 62.5 µg/mL respectively. *S. pruriens* (Biofilm selectivity index (BF SI) = 1.00) and *S. africana-lutea* (BF SI = 0.33) showed potential biofilm formation inhibition at concentrations, 62.2 and 95.8 µg/mL respectively when compared to Ciprofloxacin (BF SI 0.32), which inhibited at a concentration of 1.9 µg/mL. Most of the plant extracts showed low to moderate cellular toxicity, *Withania somnifera* had a higher toxicity with an IC₅₀ value of 6.8 µg/mL as compared to the positive control Actinomycin D (9×10^{-4} µg/mL). *F. sur* showed the highest selectivity index of 3.36. During mechanistic studies, *S. africana-lutea* showed inhibitory activity against mycothiol disulfide reductase (Mtr) exhibiting an inhibitory concentration (IC₅₀) of 102.3 µg/mL and showed lower inhibitory activity against glutathione reductase (Gtr) with an IC₅₀ of 224.1 µg/mL. This study indicated that South African plant extracts traditionally used to treat tuberculosis-related symptoms have moderate antimycobacterial, antibiofilm and enzyme inhibitory activity with relatively low toxicity. The plant extract that showed the highest selectivity towards biofilm inhibition was *F. sur*, and further investigation into the phytochemical composition of this plant extract indicated the presence of CD (Ramde-Tiendrebeogo et al., 2012).

1.7. Structure of Thesis

The content of each chapter is as follows:

Chapter 1 General Introduction

The general background, justification of the study, the research questions, aims and objectives, the hypothesis as well as the methodology used throughout the study.

Chapter 2 Coumarins, Tuberculosis & Biofilms

The literature review on coumarins as biologically active compounds, including the pharmacological activity of coumarins specifically against the pandemic of tuberculosis, the current treatment and link between persistent *M. tuberculosis* infections and biofilms, the mechanism of formation of biofilms as well as a general discussion on inhibitors of bacterial biofilms.

Chapter 3 Selectivity of Coumarin Derivatives towards Mycobacterial Biofilm Inhibition

The synthesis and the pharmacological activities against *M. tuberculosis* and *M. smegmatis* including their biofilm inhibitory activities of the CD, the hepatoprotective vs. the hepatotoxic activities of the derivatives, and the mechanistic study on mycothione and glutathione reductases.

Chapter 4 Computational Assessment of Coumarins as Mycothione Reductase Inhibitors

The assessment of three of the CD (4-methylumbelliferone derivatives), referred to as ligands, as mycothione reductase inhibitors using computational techniques, molecular docking for the initial assessment and for the analysis of the poses and orientation of the ligands within the binding site of the enzyme, molecular dynamics simulation studies to study the interactions of the ligands with the residues of the binding site.

Chapter 5 Concluding Remarks, Novel Aspects and Future Work

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“A successful book is not made of what is in it, but what is left out of it.”

Mark Twain

2

2. Coumarins, Tuberculosis & Biofilms

Abstract

The use and investigation of natural products as potential drugs have been routinely used throughout the processes of drug discovery and development. One class of natural products or secondary metabolites is the α -benzopyrone, coumarins. Coumarin and its derivatives have been studied for various pharmacological properties and have shown specificity on a range of diseases and a number of targets. *Mycobacterium tuberculosis* is the bacterium responsible for the incidence of 10.4 million new cases of tuberculosis (TB) and 1.7 million deaths each year. The treatment of TB is a lengthy process and can take from 6 months for susceptible strains to 25 months for isoniazid-monoresistant strains. One of the proposed mechanisms that the bacteria employ that protects it against, and increases its tolerance to the effects of the antibiotics is the formation of a biofilm. Mycobacterial biofilms have been studied to some degree but the processes involved in the formation and maintenance thereof have not been fully described. The use of coumarins as biofilm inhibitors has been reported throughout literature, but the research on the direct use of these compounds against mycobacterial biofilms are lacking. This chapter discusses the pharmacological activities and usage of coumarins, the pandemic of tuberculosis and mycobacterial biofilms and the mechanism involved in their formation and possible inhibition.

Keywords: Biofilm formation, Coumarin, *Mycobacterium*, Natural products, Tuberculosis, Umbelliferone

2.1. Introduction: Coumarin

In the search for novel treatments and drugs for the fight against diseases, nature has supplied a vast number of natural compounds with a wide variety of biological actions. Many organisms produce metabolites that can alter the biological activity of another organism, penicillin, produced by the fungus *Penicillium rubens*, being the most renowned example (Glynn, 2005). Natural plant products, in the form of extracts, teas, tinctures, etc. have been the treatment for human ailments since ancient times and before the development of western medicine. Natural plant based medicines still play a big part in many countries. Plants and plant metabolites have been and will always be a major source of new drugs in the process of drug discovery. Many of these metabolites have gone on to become current treatments and new candidates (Dias et al., 2012).

One class of secondary metabolites is the phenolic coumarin, first isolated in 1820 by Vogel from *Dipteryx odorata* (Aubl.) Willd., commonly known as the Tonka bean (**Fig 2.1**). Coumarins are fragrant organic compounds that fall under the α -benzopyrone class of chemical compounds and are produced and biosynthesized as depicted in **Fig 2.2** (Tandon and Rastogi, 1979; Vogel, 1820). The biosynthesis of complex coumarins in plants has not been established completely, fortunately, the biosynthesis of simple coumarins has been elucidated and falls within the phenylpropanoid pathway. Their function in plants has been linked to the inhibition of phytopathogens as in the case of phytoalexins (hydrolyzed derivatives) produced by carrots in response to fungal infections (Cowan, 1999). Due to the bitterness of some coumarins, it is understood that these compounds are strong deterrents to herbivory. Other functions include abiotic stress responses, regulation of oxidative stress and possibly the attraction of pollinators due to their volatility and sweet fragrance (Bourgaud et al., 2006).

Coumarin and coumarin-derivatives are widely distributed throughout the plant kingdom and occur naturally in several plant families and some are found to be produced by bacteria and fungi. It is reported that coumarins have been identified in more than 150 different species from 30 different families (Venugopala et al., 2013). Coumarins have been used as a fragrance in food and cosmetic products (Borges et al., 2005).

Chapter 2 Coumarins, TB & Biofilms



Fig 2.1: *Dipteryx odorata* Willd., the first plant where coumarin was isolated (Descourtilz, 1829).

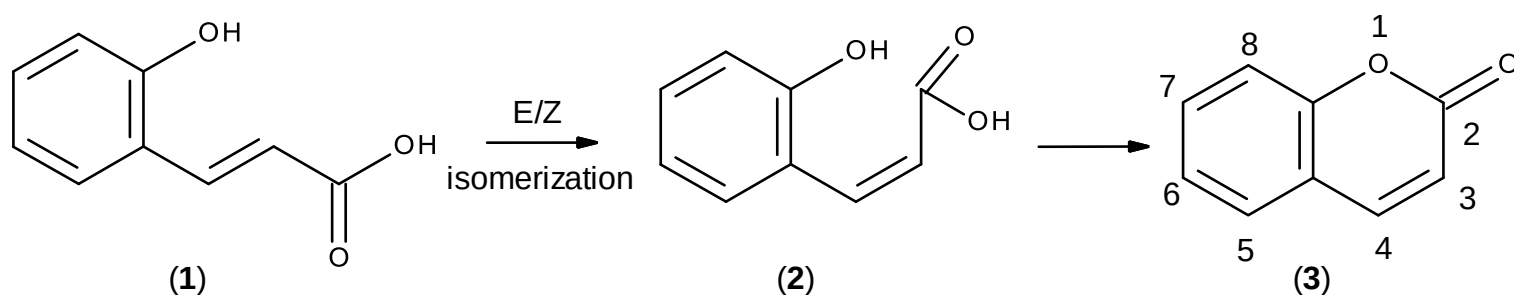


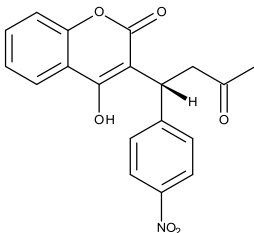
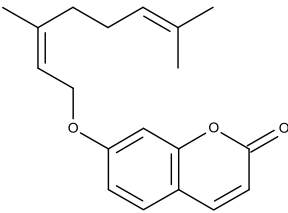
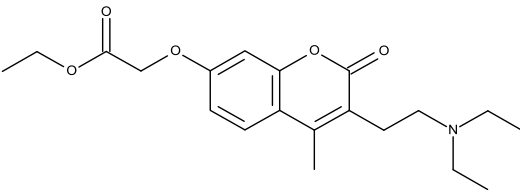
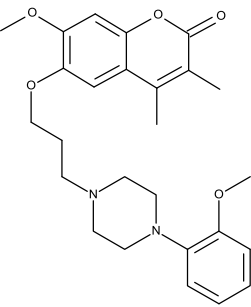
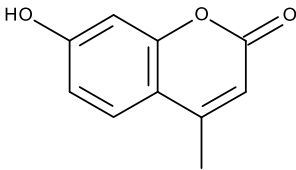
Fig 2.2: Chemical structure of coumarin (3), by the isomerization of cinnamic acid (1, 2).

In a review paper, the author stated that as of 1996, 1300 coumarins have been isolated and identified as secondary metabolites (Cowan, 1999). This number has undoubtedly increased over the last 20 years. These compounds are not only wide-spread in nature, but their biological activity is well established and pharmacologically diverse (See sections 2.1.2 and 2.1.3). Some coumarins are well recognized and reputable in the pharmaceutical market with a wide range of biological activities and mechanisms of action (Keri et al., 2015).

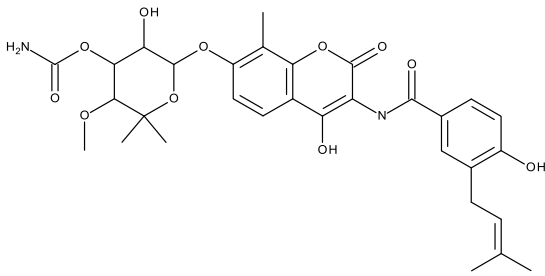
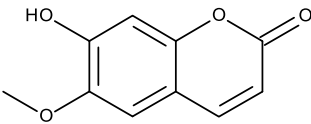
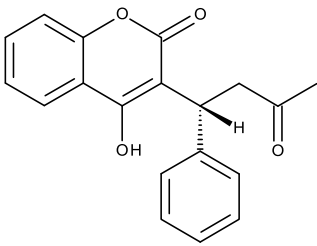
Table 2.1 lists some of these, together with their pharmaceutical indications and mechanism of action to demonstrate the varied bioactivities.

Chapter 2 Coumarins, TB & Biofilms

Table 2.1: Pharmaceutical relevant coumarins and their usage

Coumarins	Structures	Indication (mechanism of action)
Acenocoumarol		Anticoagulant (Inhibition of Vitamin-k-epoxide reductase)
Auraptene		Chemopreventative agent, degenerative diseases
Chromonar		Coronary vasodilator
Ensaculin		Dementia and amnesia (antagonistic to NMDA, agonist to 5HT _{1A})
Hymecromone (4-methylumbelliferone)		Bile therapy, choleretic and antispasmodic drugs (arylsulfate sulfotransferase)

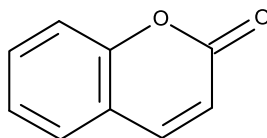
Chapter 2 Coumarins, TB & Biofilms

Coumarins	Structures	Indication (mechanism of action)
Novobiocin		Antibiotic (DNA-Gyrase)
Scopoletin		High blood pressure, bacteriostatic, anti-inflammatory
S-warfarin		Anticoagulant (Inhibition of Vitamin-k-epoxide reductase)

Coumarin and its biological and pharmaceutical properties have been reviewed in a number of inspiring papers. The early and more recent pharmacological work on coumarin have been reviewed by Egan et al., (1990) and Venugopala et al., (2013), respectively. The chemistry, toxicology and occurrence can be found in the published work of Borges et al. (2005), Lake (1999), Murray (1991), and Sarker and Nahar, (2017).

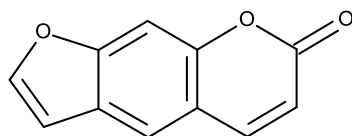
2.1.1. Classification

Coumarins can be classified into 6 main groups; 1) simple coumarins, 2) furanocoumarins, 3) dihydrofuranocoumarins 4) pyranocoumarins, 5) phenyl coumarins and 6) bi- and tricoumarins. Simple and the more complex coumarins have been isolated from various plants. The physiochemical properties, biological activity and therapeutic usage depend strongly on the type and position of substitution of simple and complex coumarin derivatives (Borges et al., 2005; Keri et al., 2015; Venugopala et al., 2013). **Fig 2.3** represents the types as well as examples of coumarins that have shown antibacterial activities.



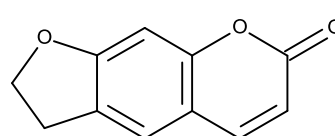
Simple coumarins

Ostruthin (Hodák et al., 1967)
Novobiocin (Chain, 1958)
Coumermycin (Gellert et al., 1976)
Umbelliferone (Chiang et al., 2010) (TB)



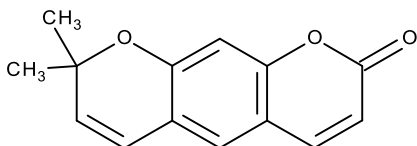
Furanocoumarins

Imperatorin (Piller, 1975)
Psoralen (Penta, 2016) (TB)
Bergapten (Chiang et al., 2010) (TB)



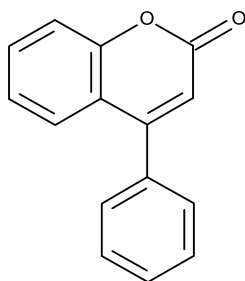
Dihydrofuranocoumarins

Anthogenol (Chakthong et al., 2012)
Felamidin (Rosselli et al., 2009) (TB)
Marmesin, (Chiang et al., 2010) (TB)



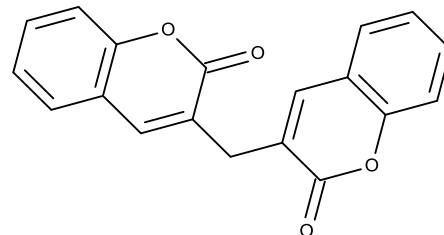
Pyranocoumarins

Grandivittin (Basile et al., 2009)
Agasyllin (Basile et al., 2009)
Aegelinol benzoate (Basile et al., 2009)
Xanthyletin (Chiang et al., 2010) (TB)



Phenylcoumarins

Mesulol (Verotta et al., 2004)



Bicoumarins

Dicoumarol (Hamdi et al., 2008)

Fig 2.3: Classification of coumarins showing examples of which some show antibacterial and some antimycobacterial activity (extracted from Venugopala et al., (2013))

The biological activities of coumarins have been well established and reported on. It has been postulated that the ability of the coumarin moiety to form stabilized interactions with several binding sites contributes towards the pharmacological versatility of coumarin derivatives (Kapp et al., 2017). The antimycobacterial and antibiofilm activities have been discussed in the following sections.

2.1.2. Coumarins and Antimycobacterial Activity

Chapter 2 Coumarins, TB & Biofilms

2.1.2.1. Isolated Natural Coumarins and Anti-TB Activity

Coumarin was first isolated from a plant and is widely distributed as a variety of active coumarin derivatives. In a study conducted by Chiang et al. (2010), the inhibitory activity of simple- and furanocoumarins, isolated from *Fatoua pilosa* Gaudich., was evaluated on the inhibition of *Mycobacterium tuberculosis* H37Rv (**Fig 2.4**). It was found that the simple coumarins umbelliferone (minimum inhibitory concentration (MIC) = 360 μ M), scopoletin (219 μ M) and phellodenol-A (291 μ M) all showed inhibitory activities approximately one magnitude lower when compared to the positive control, ethambutol (31 μ M). In the case of the furanocoumarins, psoralen and bergapten, the activities were 1184 μ M and >1156 μ M, respectively. These activities are related to coumarin derivatives substituted at C-5 and C-6.

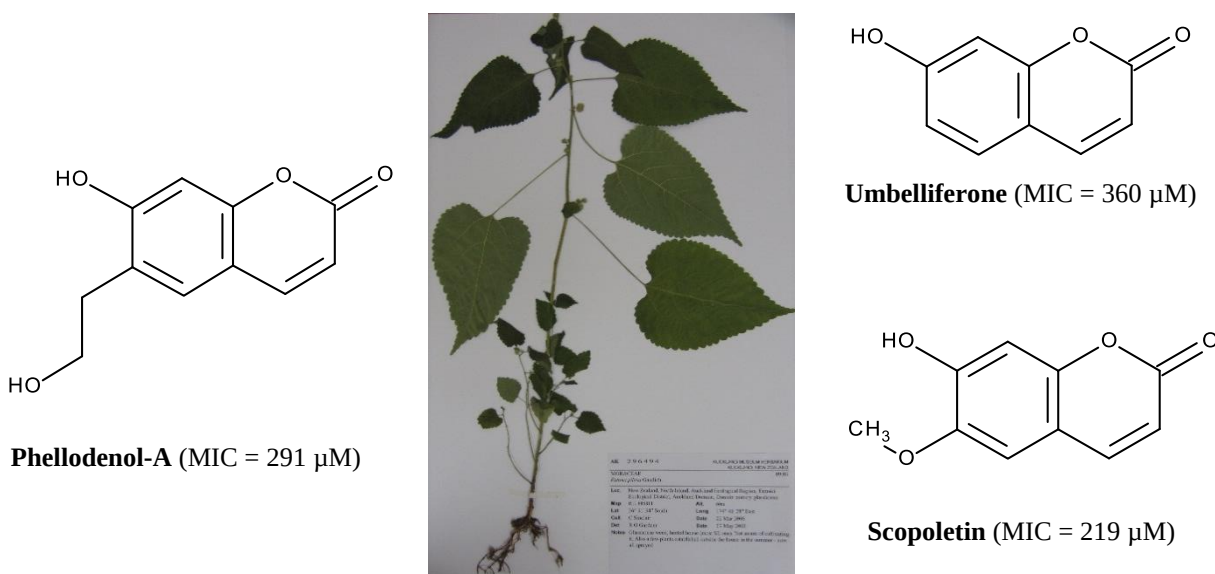
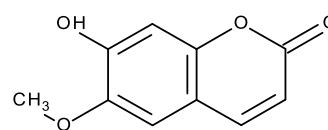


Fig 2.4: *Fatoua pilosa* Gaudich., with its isolated coumarins, umbelliferone, scopoletin and phellodenol-A exhibited antimycobacterial activity (Gardner, 2006).

Isolated coumarins from *Ferula communis* L., were evaluated for their inhibitory activity against a number of mycobacterial species. The position and type of substitution were again highlighted with an increased activity seen by the substitution at C-3 and C-4 with a benzyloxy group. The activity was reduced by a factor of eight when compared with a hydroxy group (Appendino et al., 2004). Other notable coumarins include dentatin (212 μ M) from *Clausena excavata* Burm.f., bergaptol (495 μ M)

Chapter 2 Coumarins, TB & Biofilms

from *Foeniculum vulgare* Mill (**Fig 2.5**). and the 3-phenylcoumarin, indicanine B (49 μ M) from the root bark of *Erythrina indica* Lam. (Esquivel-Ferriño et al., 2012; Nkengfack et al., 2000; Sunthitikawinsakul et al., 2003). These studies indicate that the coumarin scaffold exhibits an antimycobacterial effect and further derivatization may lead to more potent molecules.



Bergaptol (MIC = 495 μ M)

Fig 2.5: *Foeniculum vulgare* Mill., with its isolated coumarin, bergaptol exhibited antimycobacterial activity (Petruss, 2012).

There have been many coumarin derivatives reported earlier which have antibacterial activities. Novobiocin and chlorobiocin, 3-amino-4,7-dihydroxycoumarin derivatives, biosynthesized and isolated from *Streptomyces niveus* are prominent antibacterials. The mechanism of action of aminocoumarins is by the competitive inhibition of bacterial topoisomerase, and specifically DNA gyrase. This is established from the overlapping within the binding site of the sugar moiety of the aminocoumarin with the adenine ring of adenosine triphosphate (ATP). ATP is required for the activity of DNA gyrase and can therefore not bind (Heide, 2014). Another DNA gyrase inhibitor, simocyclinone D8 lacks the 7-amino moiety and the distinctive mechanism has been found to be due to the binding at two alternate binding pockets despite the structural similarities (Flatman et al., 2005).

Diarylcoumarins are natural derivatives with a very specific mechanism against *M.tb*. The researchers found that 4,6-diaryl-5,7-dimethylcoumarin derivatives were able to inhibit mycobacterial growth by the inhibition of fatty acid degradation protein D32 (FadD32), with MIC's ranging from 0.2 to 5.6 μ M. This enzyme forms part of the biosynthesis of mycolic acid which is involved in the cell-wall synthesis. The

inhibition occurs by the ability of the coumarin derivative to directly inhibit the acyl-acyl carrier activity of FadD32 (Stanley et al., 2013).

2.1.2.2. Synthetic Coumarins and Anti-TB Activity

The antimycobacterial activity of synthetic coumarin derivatives has been evaluated in a number of publications. The substitution at C-7 and the addition of a methyl group at C-4, introduce a high probability and potency for antimycobacterial activity. The researchers found that ten of these coumarins showed activity against bacteria and four were further investigated to determine the minimum inhibitory concentration. The MIC's were found to be below 30 μM and the best sample, CM15 (**Fig 2.6**) had an MIC of 8.31 μM , comparable to the positive control rifampicin at 6 μM (Kapp et al., 2017).

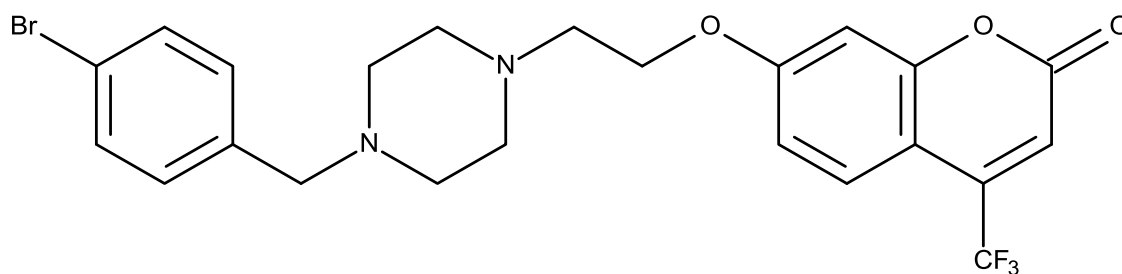


Fig 2.6: Structure of 7-substituted coumarins, CM15 showing an MIC of 8.31 μM against *M. tuberculosis* (Kapp et al., 2017).

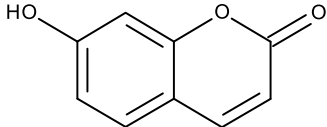
Generally, all except substitutions at C-1 and C-2 have shown antimycobacterial activities. The versatility of coumarins to bind and interact with different targets within the mycobacterial bacilli, make these compounds chemically and pharmaceutically interesting. The broad specificity and versatility of coumarins can alter the drug development process and effective use as antimycobacterial drugs, as the mechanisms are difficult and troublesome to identify. It has been established that coumarins play a role in the treatment of neurodegenerative diseases due to the inhibition of β - and γ -secretases, cholinesterases and monoamine oxidases, to name a few (de Souza et al., 2016; Skalicka-Woźniak et al., 2016). Thus, the effective establishment of selectivity and alternate pharmacological effects need to be confirmed and evaluated at an early stage of drug discovery and development.

2.1.3. Coumarins and Antibiofilm Activity

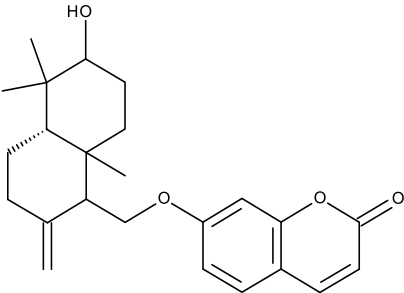
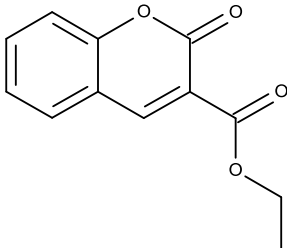
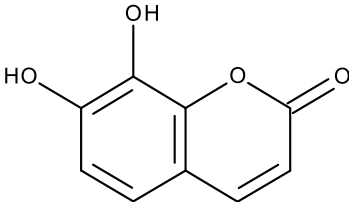
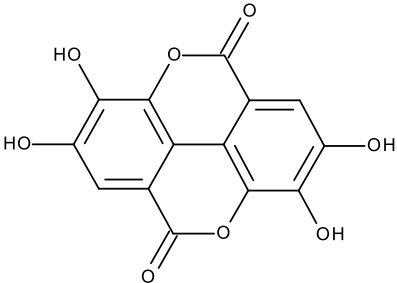
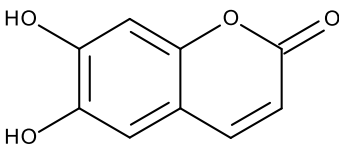
One promising mechanism of disease control has been proposed to be through the inhibition of the formation of biofilms or the disruption of a formed biofilm. Biofilms are discussed in more detail in section 2.3. Coumarin and its derivatives have been assessed to some extent for their antibiofilm activity. Most of the literature reports on biofilm inhibition of gram-negative bacteria and infections relating to chronic wounds and implant/medical device biofilms. The coumarins as biofilm inhibitors have been summarized in **Table 2.2**.

In a study by Lee et al. (2014), it was found that coumarins reduce biofilm formation at an early stage as well as the virulence of the gram-negative *E. coli*. It was established that coumarin and umbelliferone inhibited the attachment and formation of a biofilm by up to 80% at 308 μM . It was also noted that the antibiofilm activity was not related to the antibacterial activity. It was further established that coumarins downregulated the expression of genes involved in curli pili formation. Two of these genes have been identified as the *csg* gene in *E. coli* and *mtp* gene in *M. tuberculosis*. *Csg* and *mtp* mutants have shown a reduction of biofilm mass when compared to the wild-type. This indicated the importance of these genes and curli pili in the formation of biofilm (Lee et al., 2014; Ramsugit et al., 2013). The ability of coumarin to downregulate the expression of the genes involved in biofilm formation can potentially reduce persistence in latent infections.

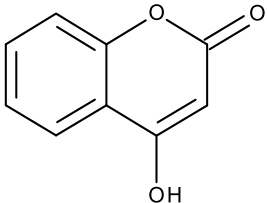
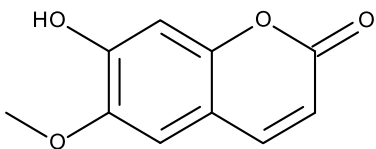
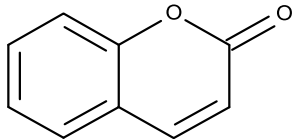
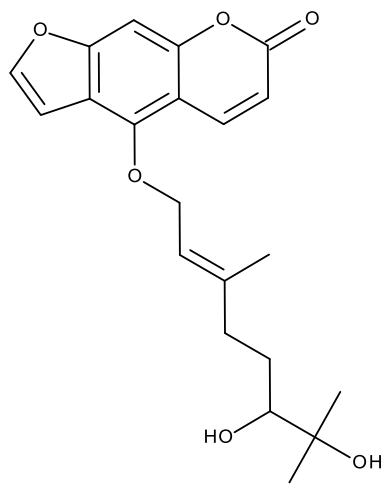
Table 2.2: Coumarin and derivatives of coumarin and their biofilm inhibitory activity on specific organisms, adapted from Reen et al., (2018).

Coumarin Compounds	Target Organisms	Concentration (μM)	Biofilm Activity ^a	Reference
 Umbelliferone	<i>E. coli</i>	308	+++	(Lee et al., 2014)

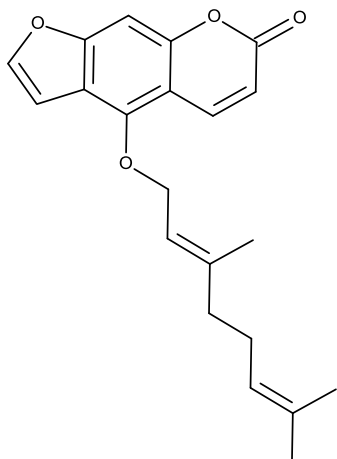
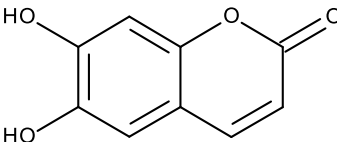
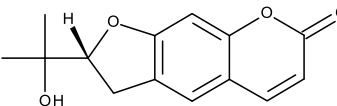
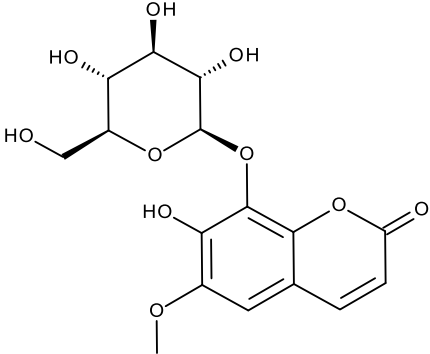
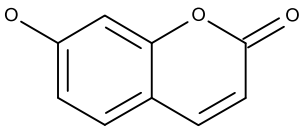
Chapter 2 Coumarins, TB & Biofilms

Coumarin Compounds	Target Organisms	Concentration (μM)	Biofilm Activity ^a	Reference
 Colladonin	<i>E. coli</i>	131	+++	(Lee et al., 2014)
 Coumarin-3-carboxylate	<i>E. coli</i>	229	++	(Lee et al., 2014)
 Dephnetin	<i>E. coli</i>	281	++	(Lee et al., 2014)
 Ellagic acid	<i>E. coli</i>	165	++	(Lee et al., 2014)
 Esculetin	<i>E. coli</i>	281	++	(Lee et al., 2014)

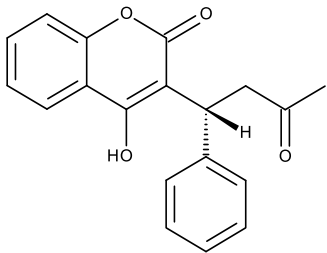
Chapter 2 Coumarins, TB & Biofilms

Coumarin Compounds	Target Organisms	Concentration (μM)	Biofilm Activity ^a	Reference
 4-Hydroxycoumarin	<i>E. coli</i>	308	++	(Lee et al., 2014)
 Scopoletin	<i>E. coli</i>	260	++	(Lee et al., 2014)
 Coumarin	<i>P. aeruginosa</i> <i>Edwardsiella tarda</i> <i>E. coli</i> <i>S. aureus</i> <i>V. anguillarum</i>	1369 1369 1369 1369 1369	++ + ++ ++ ++	(Gutiérrez-Barranquero et al., 2015)
 Dihydroxybergamottin	<i>E. coli</i> <i>S. typhimurium</i> <i>P. aeruginosa</i>	3	+++ + +	(Gutiérrez-Barranquero et al., 2015)

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Coumarin Compounds	Target Organisms	Concentration (μM)	Biofilm Activity ^a	Reference
 Bergamottin	<i>E. coli</i> <i>S. typhimurium</i> <i>P. aeruginosa</i>	3	+++ ++ ++	(Gutiérrez-Barranquero et al., 2015)
 Esculetin	<i>S. aureus</i>	719	+++	(Dürig et al., 2010a)
 Nodakenetin	<i>P. aeruginosa</i>	329	+++	(Ding et al., 2011)
 Fraxin	<i>P. aeruginosa</i>	200	++	(Ding et al., 2011)
 Umbelliferone	<i>R. solanocearum</i>	308	+++	(L. Yang et al., 2017a)

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Coumarin Compounds	Target Organisms	Concentration (μM)	Biofilm Activity ^a	Reference
 S-warfarin	<i>E. coli</i>	4995	++	(Ojima et al., 2016a)

^a Biofilm activity are represented in three classifications, + represents moderate activity, ++ represents high activity, +++ represents exceptional activity.

In another interesting study conducted it was found that autoinducer 1 (AI-1) and 2 (AI-2) were inhibited up to 95% by the furanocoumarins isolated from grapefruit juice. Autoinducers are signalling compounds used in quorum sensing, which regulate biofilm formation. The authors found that naturally occurring furanocoumarins inhibited intra-specific communication of four gram-negative bacteria; *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella typhimurium* and *Vibrio harveyi*. Autoinducer 1, acetyl homoserine lactone derivatives, are mostly used by gram-negative bacteria, however, both gram-positive and -negative utilize the boronated-diester molecule AI-2 (Girennavar et al., 2008). This indicates the possibility of coumarins as biofilm inhibitors regardless of the bacterial type, with the possibility of spanning a diverse microbial population.

2.2. Introduction: Tuberculosis

2.2.1. The Bacterium

Mycobacterium tuberculosis (*M.tb*), the etiological agent of Tuberculosis (TB) is one of the world's major epidemics and is second only to HIV/AIDS, as the biggest killer due to a single infectious organism. It is estimated that latent *Mtb* infection is present in one-quarter of the world's population (1.9×10^9 people) (**Fig 2.7**) (WHO, 2018). This staggering number of latent infections sustains the high number of new cases and deaths seen each year (Zhang, 2014). Although the death rate and incident rate are high, it is estimated that 5-10% of the people who are infected with *M.tb*, develop active TB (Ojha and Hatfull, 2012). New cases can be attributed to infection and re-infection from an active infection as

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well as activation and reactivation from a latent infection (Dye, 2006). The active disease usually occurs in an infected person when the body's resistance or immunity is low, or if there is a prolonged exposure to the bacteria that overcome the body's natural defences (Narasimhan et al., 2005). Thus, emphasis should not only be on developing treatments for active TB disease, but also latent infections.

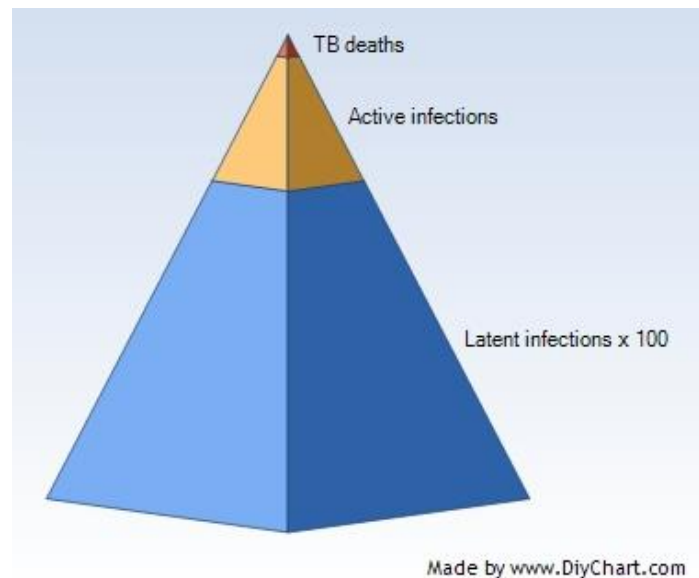


Fig 2.7: TB deaths in relation to active infections and latent infections.

In 2016 there were 10.4 million new incidences and a death rate of 1.7 million. The majority of these cases were in developing low-income countries China, India, Indonesia, Nigeria, Pakistan, Philippines and South Africa (64%). An infected patient with untreated active TB will infect on an average, 10-15 people per year. Tuberculosis is also the leading cause of death in patients with HIV/AIDS (WHO, 2018). Considering the epidemiology of TB, it is estimated that this bacterium requires a population size of a 100 people to survive. This makes TB one of the most efficient infectious diseases (Talbot and Raffa, 2015). With more than a third of the world's population living in urban areas, this creates the ideal environment and niche for the bacteria not only to thrive but also to persist in a latent form for extended periods. Fortunately, the TB death rate has declined since 1990 by 45%, but with the increase in population size, specifically in urban areas and increased incidence of multidrug and extensive drug resistance, it is still not as significant as it ought to be (Talbot and Raffa, 2015).

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South Africa is one of the countries with the highest burden of TB infections with close to 1% of the population, of approximately 56 million people, exhibiting signs of active TB. The estimated rate of latent infection is much higher than the world average, with 80% of the population infected. The treatment efficacy has increased over the last few years to about 80%, however, the number of treated cases still only remains at 50% (WHO, 2017).

As mentioned above one-quarter of the world's population is infected with *M.tb* and these infections are classified as latent bacterial infections. In latent infections, persistent bacteria can live and survive for years without causing disease (Parrish et al., 1998). Persistent bacteria are classified as a small population of bacterial cells that survive antibiotic attack and evade immunological responses. These populations can survive in adverse conditions and develop into active TB. Latent persisters are the main cause of post-treatment relapse, posing a significant problem in the treatment of tuberculosis. Although the mechanism of immunological and chemical evasion of mycobacteria is still poorly understood, evidence has indicated genes and specific pathways involved in survival of the bacteria. These include alternative energy pathways, such as the glyoxylate shunt pathway, DNA repair or protection, phosphate metabolism as well as macromolecule-degradation (Zhang, 2014). Another mechanism by which mycobacteria persist has been hypothesized to be the formation of a biofilm (Kulka et al., 2012; Ojha et al., 2008).

Tuberculosis cannot be discussed without including the development and spread of resistance in the form of multidrug-resistant (MDR) and extensively drug-resistant (XDR) TB. According to the World Health Organization (WHO), MDR-TB is defined as bacilli that are at least resistant to two of the first-line drugs; isoniazid and rifampin (TB drugs discussed in section 2.2.2). To be termed XDR-TB, in addition to the previously mentioned first-line drugs, the bacilli should be resistant to the second-line fluoroquinolones (levofloxacin or moxifloxacin) and to at least one of the three injectable second-line drugs (amikacin, capreomycin or kanamycin). Resistance has been found in all countries tested, and in 2016, 600 000 patients were diagnosed with a resistant form of tuberculosis of which 6.2 % were XDR-TB. Fortunately, the use of the two newest drugs, bedaquiline and delamanid have been made available in 89 and 54 countries, respectively, in an effort to combat the spread as well as the development of resistance (WHO, 2018).

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To overcome the problems of tuberculosis, new chemotherapeutics should possess certain abilities. Firstly, the treatment period should be shorter than the current regimen (6-9 months). Secondly, the drug target should include mechanisms that are associated with latent TB infections. This could include targeting the biofilm, either in the formation stage or in the maturation stage, or targeting enzymes that are known to be involved in persistent infections, or by altering communication by inhibiting quorum sensing.

2.2.2. The Treatment

Although effective, the current treatment of tuberculosis has some drawbacks. Firstly, patients suffering from TB, have to take a combination of drugs for as long as nine months. This could lead to non-compliance, especially in developing countries, where access to treatment remains a problem. Secondly, the current regimen is very slow in treating persistent latent infections. Thirdly, new drug candidates have been identified, but many do not pass clinical trials or have an abundance of adverse side effects (Rylance et al., 2010).

The selection process of new drug candidates is also centred around the intracellular antimycobacterial activity (Orme, 2014). *Mycobacterium tuberculosis* is considered an intracellular disease, but more evidence has indicated that bacteria can be found extracellularly on the perimeter of the granulomas (Vega-Domínguez et al., 2014). These extracellular bacteria grow in aggregates, and Kerns et al., (2014) found that a set of *M. tuberculosis* proteins, related to biofilm formation, were expressed in infected Guinea pig sera. Thus, while many candidate drugs are disregarded due to their lack of intracellular activity, some could well be able to target biofilms, and might alleviate latent infections to a certain extent. It is worthwhile mentioning that biofilms can also form intracellularly, but drug selection should not only be due to an intracellular biofilm activity. From **Table 2.3** it is clear that current drugs against TB target a wide range of mechanisms, where some of the targets relate to arabinogalactan and mycolic acid biosynthesis, which in turn can be linked to biofilm formation, although no evidence of antibiofilm activity can be found for most of the drugs.

Table 2.3: Current TB drugs and their associated targets.

TB Drug	Mechanism of action
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TB Drug	Mechanism of action
First-line TB Drugs	
Isoniazid	Mycolic acid biosynthesis - ACP reductase InhA
Rifampicin	Transcription inhibition - RNA polymerase
Pyrazinamide	Membrane transport functions
Ethambutol	Arabinogalactan biosynthesis - cell wall arabinan polymerization
Second-line TB Drugs	
Aminoglycosides	Polypeptide synthesis -30S ribosomal subunit
Fluoroquinolones	Transcription inhibition - Gyrase & Topoisomerase
Macrolides	RNA-dependent protein synthesis - 50S ribosomal subunit
Ethionamide	Mycolic acid biosynthesis - ACP reductase InhA
Capreomycin	Inhibits protein synthesis - Ribosomal structures at the 16S rRNA
Cycloserine	Peptidoglycan synthesis - d-alanine racemase
Nitroimidazoles	
PA-824	Mycolic acid biosynthesis - inhibition of the oxidation of hydroxymycolates
OPC-67683	Mycolic acid biosynthesis - synthesis of methoxy- and oxo-mycolic acids
TMC207	Synthesis of ATP - ATP synthase
SQ109	Unknown
Phenothiazines	Multifactorial - Fatty acid metabolism and membrane proteins
Oxazolidinones	
Linezolid	Protein synthesis - Ribosomal 50S subunit
PNU-100480	Protein synthesis - Ribosomal 50S subunit
AZD5847	Protein synthesis - Ribosomal 50S subunit
Benzothiazinones	synthesis of cell-wall arabinans - decaprenyl-phosphoryl- β -D-ribose 2'-epimerase

The fight against tuberculosis is an ongoing battle. Many different approaches can be taken for the identification and selection of new treatments. Biofilms and latent infections are one of many possible targets one may consider. It is important to consider other factors, other than antibiotic activity when discovering and developing new drugs. These include the determination of the cytotoxic and hepatoprotective effect of the candidate. For a drug to be efficient and effective it should have high activity and selectivity towards the bacteria and not cause acute or chronic toxic effects in the host. Many of the current drugs are associated with hepatotoxicity, due to the long exposure required for eradication, and the toxic metabolites that are produced during metabolism. New treatments should not aid in this hepatotoxic effect, but rather protect the liver against drug-induced toxicity.

Conventional antibiotics for TB have a cell growth inhibiting effect, either bacteriostatic or bactericidal, by disrupting cell walls, inhibiting DNA replication and the inhibition of target enzymes, to name a few

(Lohrasbi et al., 2018). These antibiotics usually culminate in the death of the bacteria but consequently select the resistant subpopulation. Rather than focusing on therapeutics that target cell death, much like these conventional antibiotics, an alternative approach would be to target functions essential for latent infection and immune evasion, such as alternative energy production or biofilm formation through quorum sensing. This approach will not only increase the possible drug targets, but it may reduce the risk of drug resistance by exerting less selective pressure (Clatworthy et al., 2007; Lee et al., 2014).

2.2.3. Persisters and Biofilms

Persisters were discovered more than 70 years ago and are defined as a small population of quiescent bacterial cells that survive an antibiotic attack or other stresses in the form of immunological responses. The formation of a biofilm is related to, and may trigger, drug tolerance and resistance as well as an inflammatory response, resulting in a persistent infection (Chen and Wen, 2011). Additionally, latent infections are also caused by persistent bacteria. They are responsible for post-treatment relapses, thus posing a significant challenge for the treatment of tuberculosis and other diseases (Zhang, 2014). The following section relates to biofilms and the formation of mycobacterial biofilms, which attempts to explain why persisters are difficult to treat.

2.3. Introduction: Biofilms

A biofilm can be defined as a sessile microbial structure, characterized by the attachment of cells to a surface or each other, embedded in a matrix of extracellular polysaccharides, DNA and protein and that demonstrates an altered phenotype with respect to growth, gene expression and protein production (Donlan and Costerton, 2002). The structure of the biofilm can differ significantly between different bacteria, ranging between a single-cell layer to a community of cells embedded in a thick polymeric matrix with an intricate channel network to provide the community with environmental nutrients and water (Costerton et al., 1995; Kulka et al., 2012; Richards and Ojha, 2014; Shirtliff et al., 2002; Sousa et al., 2015; Xiang et al., 2014).

Biofilm formation occurs in distinct stages as depicted in **Fig 2.8**. It often begins with the attachment of individual planktonic cells to a biotic or abiotic surface. However, substratum attachment can also occur

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at a later stage after intercellular aggregation of planktonic cells and growth of aggregates (Yang et al., 2017). After the initial growth of surface-attached aggregates, the genetic program for the synthesis of extracellular matrix is induced (Kulka et al., 2012). In some species, matrix synthesis appears to be controlled through quorum sensing. Quorum sensing is a specific process that the bacteria employ in response to the bacterial density via small signalling molecules called autoinducers. This allows for inter as well as intra-species communication (Cady et al., 2012; Joo and Otto, 2012). The biofilm matrix consists of proteins, lipids and in the case of mycobacteria, free mycolic acid as well. Maturation of biofilms is associated with the acquisition of a new phenotype, including extreme tolerance to antibiotics (Ojha and Hatfull, 2012). Lastly, dispersal of bacterial cells from matured biofilms and initiation of biofilm formation at new locations completes the cycle (Hall-Stoodley and Stoodley, 2005; Xiang et al., 2014)

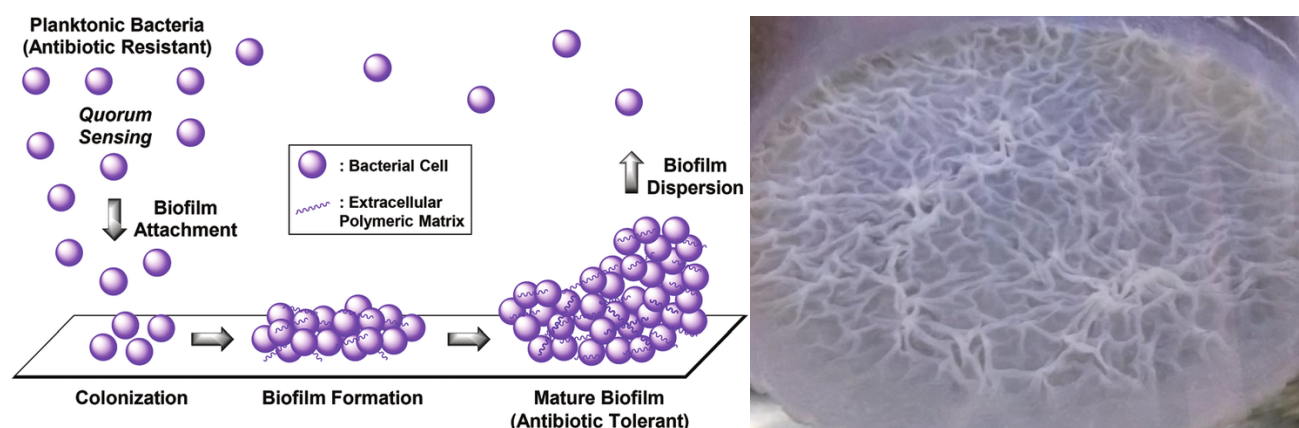


Fig 2.8: Stages of biofilm formation (left) (Basak et al., 2017), a mature biofilm of *Mycobacterium smegmatis* growing on the air-medium interface (right).

By growing in a biofilm matrix the microbes enjoy a number of advantages over the single planktonic bacterial cells. Firstly, a biofilm enables the bacteria to overcome several removal strategies. These strategies include resistance/tolerance to antimicrobial agents, shear stress, evading immune phagocytic clearance, as well as the protection against free radicals and protease defences. Secondly, the poly matrix biofilm has the ability to capture and concentrate nutrients. Thirdly, this growth habit has the potential for dispersion through detachment for the repopulation and colonization of new sites after the termination of drug treatments (Boyd and Chakrabarty, 1994).

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Not all bacterial biofilms are the same, the structure, chemistry and physiology are dependent on the residing microorganism, but all biofilms form an extracellular matrix which contributes significantly towards the organization of the community (Branda et al., 2005). Not only does a biofilm form a niche for bacteria to grow and persist, it is recognized as an important contributor to drug tolerance as well as acquiring resistance through horizontal gene transfer within the biofilm (Hall-Stoodley and Stoodley, 2005; Tram et al., 2013).

The resistance/tolerance of the biofilm to antibiotics has been attributed to different factors; These include the low metabolic levels of bacteria in biofilms and the drastically reduced rate of cell division (Brown et al., 1988). Biofilms have been shown to possess the ability to act as diffusion barriers and limit the penetration of some antibiotics. It is important to understand the mechanisms involved in the initial attachment, development and maturation of the biofilm to elucidate the impact of the structure and identify new drug targets to overcome the problem of persistence and resistance in biofilms (Shirtiliff et al., 2002).

2.3.1. Mycobacterial Biofilms

Persistence of many infectious diseases is associated with the ability of the bacteria to grow in an organized community, referred to as a biofilm (Ojha and Hatfull, 2012; Zhang, 2014). Several *Mycobacterium* species including *M. smegmatis* and *M. tuberculosis* have been identified to grow *in vitro* in spontaneous biofilm structures that contain persistent drug-tolerant and drug-resistant bacilli (Ojha and Hatfull, 2012). Although the occurrence of an *in vivo* mycobacterial biofilm remains controversial, some studies have found the presence of biofilm-like structures.

As is the case with a study performed by Lenaerts et al., (2007), where aggregates of mycobacteria microcolonies were found to be present in the acellular rim surrounding the primary granuloma. Another study identified *M. tuberculosis* proteins in the serum of infected Guinea pigs which are associated with biofilm growth and not expressed when cells are cultured in their planktonic state (Kerns et al., 2014). In another study it was established that *M. tuberculosis* encodes pilin-like proteins which are not only expressed *in vivo* but also bind strongly to the eukaryotic extracellular matrix, supporting the idea of attachment in the initial formation of a biofilm (Alteri et al., 2007). Histopathological studies of infected

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human lung tissue have described the growth of *M. tuberculosis* in large multicellular clusters, although it is unclear if the structure represents the genetically programmed growth pattern of a persistent biofilm (Canetti et al., 1972). This strongly suggests the presence of an *in vivo* biofilm.

Furthermore, biofilms explain the efficient ability of *M. tuberculosis* to evade host defence and antibacterial attack during latent infections, and it maintains an environment for persisters to reside within. Bacon et al. (2014), investigated persisters by nutrient depletion and found that the persistent sub-population was surrounded by an extracellular matrix, resembling a biofilm-like structure. Additionally, they identified genes (oxidoreductases, Acyl-CoA dehydrogenase) that were up-regulated within the non-replicating persisters that were also present within the aggregates in the granuloma.

A question that arises when discussing mycobacterial biofilms is whether *M. tuberculosis* can form drug-tolerant biofilms *in vivo*. If so, does the biofilm growth contribute to the persistence of the bacteria? It has been well documented that mycobacterial species form pellicle biofilms in detergent-free media on the air-medium interface (Ojha et al., 2008). Biofilms have formed spontaneously *in vitro* and a method was developed to disperse the bacteria in a homogeneous suspension by the addition of a detergent to the liquid media, without affecting the virulence properties of the pathogen (Dubos and Middelbrook, 1948). This method is still widely used today to test new therapeutic agents, but many researchers believe that it has several pitfalls in the interpretation of biological activities and characteristics of the bacilli grown in such conditions. The addition of a detergent can have a profound impact on the functional properties of the bacterial envelope, interacting with bacterial surface lipids and altering the permeability of small molecules. As in the case where mycobacteria grown in media containing ‘Tween80’ as a detergent have shown significant sensitivity to antibiotics compared to bacteria grown without Tween80 (Islam et al., 2012). Another problem identified with planktonic cultures is that the bacilli are homogeneously exposed to nutrients and oxygen, this is not the case in biofilms, where there are heterogeneous microenvironments with a gradient of nutrients and oxygen from the outer layers to the inner core of the biofilm (Islam et al., 2012).

2.3.2. Mechanism of Biofilm Formation

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The question then arises as to how the bacteria regulate biofilm formation and maturation. Many scientists believe that this mechanism is very precisely regulated via quorum sensing (Carter et al., 2003; Hentzer et al., 2002; Kulka et al., 2012; Proal, 2008; Shirliff et al., 2002). Quorum sensing is a cell density-dependent regulatory mechanism utilized by many microorganisms, including gram-positive and negative bacteria and also fungi. It is a cell to cell communication method mediated by small signalling molecules to regulate a variety of physiological functions, including virulence and biofilm formation (Bassler, 1999; Hentzer et al., 2002). By inhibiting quorum sensing, termed quorum quenching (QQ), a QQ molecule can inhibit the communication and not target a mechanism that will culminate in the bactericidal effect of the known drugs. Thus, by inhibiting communication the bacteria cannot regulate mechanisms based on cell density. This will lead to the interrupted activation of virulence, which is also regulated by quorum sensing, as well as biofilm formation (Bassler, 1999).

Many genes have been identified to be up-regulated at different stages during the development of a biofilm. Their specific role and how they interconnect are not clearly understood, but some of these genes and gene products include; isocitrate lyase, thioredoxin-like protein, mycothione reductase, other oxidoreductases and DNA gyrase, to name a few (Islam et al., 2012; Vargas et al., 2016). Mycothiol, the low molecular weight thiol produced by the enzymatic reaction of mycothione reductase has been shown to be vital for the survival during stress and biofilm formation of *Mycobacterium smegmatis* (Vargas et al., 2016). Some of the genes that are induced during biofilm formation, development and maturation are listed in **Table 2.4**.

Table 2.4: Genes induced during the development of *Mycobacterium smegmatis* biofilms and their associated *M. tuberculosis* homologs (partial list from (Islam et al., 2012)).

Locus tag	Annotation	<i>M. tuberculosis</i> homologs
MSMEG_0313	6-Phosphogluconate dehydratase	Rv0189c
MSMEG_3297	Adenylatecyclase	Rv1900c
MSMEG_6487	Amidohydrolase family superfamily	NA ^a
MSMEG_0782	Aminotransferase, class III	Rv2598
MSMEG_3194	Biotin synthase	Rv1589

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Locus tag	Annotation	<i>M. tuberculosis</i> homologs
MSMEG_0457	DNA gyrase subunit B, putative	Rv0005
MSMEG_1336	Flavohemoprotein, putative	Rv3571
MSMEG_0020	FxuD protein	Rv0265c
MSMEG_5680	Glyoxalase family protein	Rv0887c
MSMEG_2945	Holliday junction DNA helicase RuvB	Rv2592c
MSMEG_4477	Hydrolase, α - β hydrolase fold family	Rv1900c
MSMEG_0911	Isocitrate lyase	Rv0467
MSMEG_2740	LexA repressor	Rv2720
MSMEG_5556	Major facilitator family transporter	NA
MSMEG_0225	Membrane protein, MmpL family	Rv2339
MSMEG_1268	MutT-nudix family protein	Rv1593c
MSMEG_1742	Oxidoreductase	Rv3554
MSMEG_6030	P450 heme-thiolate protein, putative	Rv1777
MSMEG_4512	Polyketide synthase, putative	Rv2381c
MSMEG_2601	Protocatechuate 3,4-dioxygenase b subunit	NA
MSMEG_0504	Ribokinase	Rv2436
MSMEG_5310	SAM-dependent methyltransferases	Rv2622
MSMEG_1515	Sensor histidine kinase	NA
MSMEG_5004	Ser-Thr protein phosphatase family	Rv1277
MSMEG_2380	Sugar transporter family protein	Rv2994
MSMEG_6618	Superfamily II DNA and RNA helicases	Rv2092c
MSMEG_5308	Surface antigen, putative	Rv1057
MSMEG_2268	Thioredoxin-like protein	NA
MSMEG_4057	Transcriptional regulator, GntR family	Rv0165
MSMEG_4038	Vanillin dehydrogenase	NA
MSMEG_6540	Virulence factor	Rv0589

^aNot available

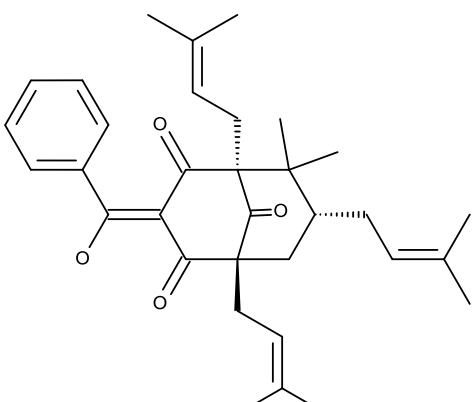
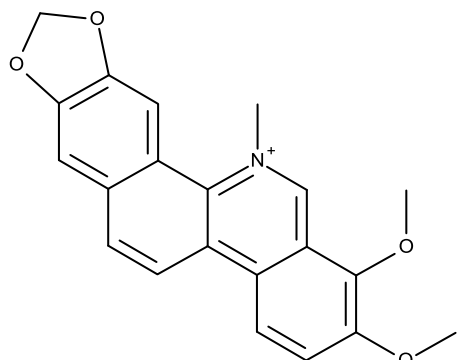
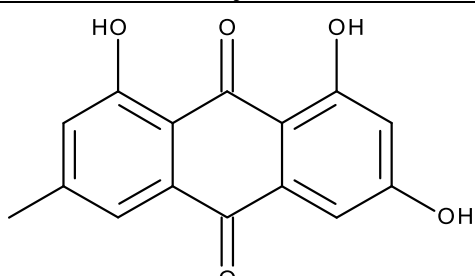
2.3.3. Inhibitors of Biofilm Formation

To a great extent, research has gone into the use of acetyl homoserine lactone (AHL) derivatives as quorum sensing inhibitors or quorum quenching molecules and in the process inhibits the formation of biofilms. These AHL signalling compounds are only used by gram-negative bacteria, while gram-positive bacteria use autoinducer 2 and polypeptides as signalling molecules (Bassler, 1999). The use of other natural and synthetic molecules to target different biofilm mechanisms have been evaluated

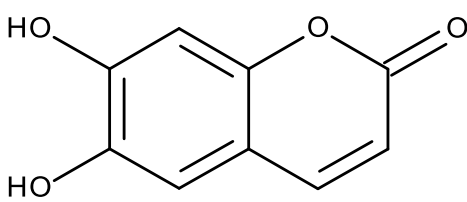
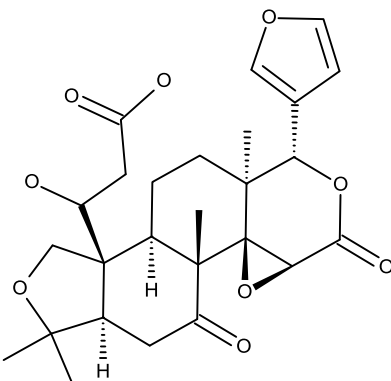
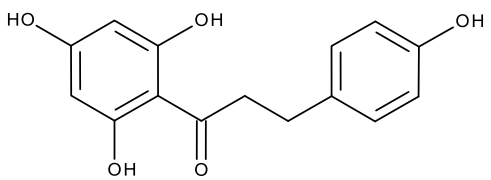
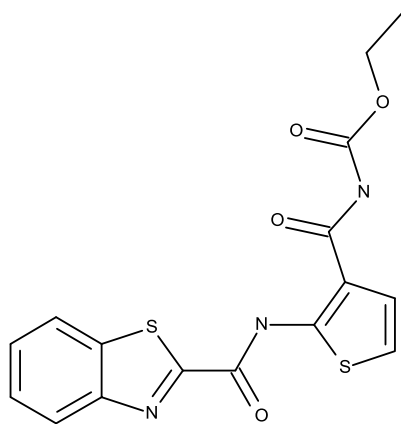
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throughout the literature and additional information can be found in the work of Geske et al., (2005), Grandclément et al., (2016), Musk Jr. and Hergenrother, (2006) and Rabin et al., (2015). These compounds have been identified to act against biofilm formation, all targeting different mechanisms. Some of these are summarized in **Table 2.5**. Coumarin and its derivatives that have shown inhibition of biofilms can be found in section 2.1.3.

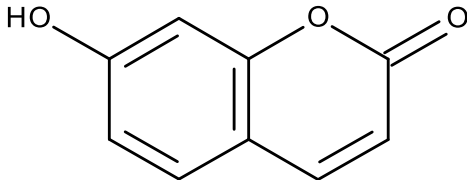
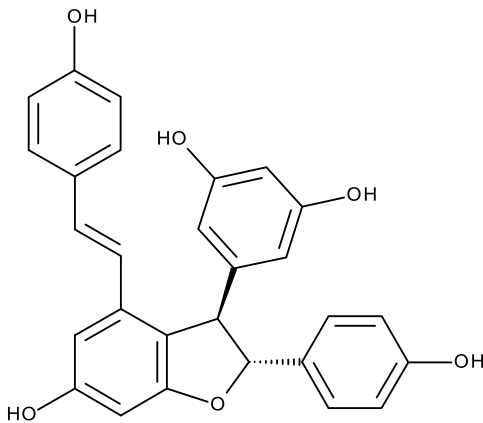
Table 2.5: A few natural and synthetic compounds that show antibiofilm activity.

Chemical Compounds	Activity	Organisms	References
 7-Epiclusianone	497 μ M	<i>S. mutans</i>	(Murata et al., 2010)
 Chelerythrine	8.6 μ M	<i>S. epidermidis</i>	(Artini et al., 2012)
 Emodin	20 μ M	<i>P. aeruginosa</i> and <i>S. maltophilia</i>	(Ding et al., 2011)

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Chemical Compounds	Activity	Organisms	References
 Esculetin	200 μ M	<i>P. aeruginosa</i>	(Zeng et al., 2008)
 Isolimonic acid	19.7 μ M	<i>E. coli</i>	(Vikram et al., 2012)
 Phloretin	182 μ M	<i>E. coli</i>	(Lee et al., 2011)
 TCA1	0.1 μ M	<i>M. smegmatis</i>	(Wang et al., 2013)

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Chemical Compounds	Activity	Organisms	References
 Umbelliferone	308 μ M	<i>E. coli</i>	(Lee et al., 2014)
 ε-Viniferin	110 μ M 22 μ M	<i>P. aeruginosa</i> <i>E. coli</i>	(Cho et al., 2013)

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“Research is to see what everybody else has seen, and to think what nobody else has thought.”

Albert Szent-Gyorgyi

3

3. Selectivity of Coumarin Derivatives towards Mycobacterial Biofilm Inhibition

** The editorial style of the Journal of Medicinal Chemistry (Impact Factor: 6.2) has been followed for this chapter.*

Abstract

Tuberculosis (TB), the second deadliest infectious disease, remains a problem, especially in underdeveloped countries. It is estimated that one-quarter of the world's population is infected with an asymptomatic latent infection with a 10% chance for the development of active TB disease. Together with the development of drug-resistant strains of TB, new strategies for the treatment of *Mycobacterium tuberculosis* infections are vital. One proposed mechanism that renders this disease so difficult to treat, is the ability of the bacteria to grow in complex biofilms. Within this extracellular matrix, composed of polysaccharides, proteins and mycolic acids, the residing bacteria are protected from antibiotic attack and the human immune responses. Additionally, it renders the population more tolerant to the TB drugs and there is a higher probability for the development of acquired resistance through horizontal gene transfer. Biofilms should, therefore, be considered as a probable target for drug discovery and development. One group of natural products, produced by a number of different plant

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species and other organisms is coumarins. This biologically diverse class of compounds has shown biofilm inhibition in other bacterial species. This study aimed to investigate the antibiofilm activity of coumarin derivatives (CD), using a set of umbelliferone (**U**) and 4-methylumbelliferone (**MU**) derivatives, and comparing their antimycobacterial activity to determine the selectivity. Furthermore, the hepatoprotective effect of the derivatives was screened against acetaminophen-induced toxicity in human hepatic cells (HepG2). Lastly, in an attempt to investigate the mechanism of biofilm inhibition, the derivatives were screened for their mycothione reductase inhibition, an enzyme important throughout bacterial growth and biofilm formation. One of the derivatives, **MU2** showed exceptional selectivity towards biofilm inhibition of *M. smegmatis*, with a selectivity of 64 followed by **MU5** and **U2** with selectivities of 32 and 15 respectively. The best antimycobacterial activity was observed by the 3-methylbutyloxy substitution at C-7 of samples **U2** and **MU2** showing minimum inhibitory concentrations (MIC) of 108 μM and 102 μM on *M. tuberculosis*. Some of the derivatives had the ability to intercalate into a mature biofilm and reduce the bacterial count by up to 90%. During the hepatoprotective study, it was observed that most of the compounds and especially the **U** derivatives showed a protective effect against the toxicity induced by acetaminophen with sample **U3** showing the highest protection of 40%. The enzymatic inhibitory assay correlated with the biofilm inhibition with sample **MU2** showing a 50% inhibitory effect at a concentration of $29.1 \pm 11.9 \mu\text{M}$. This is the first report of coumarin derivatives as inhibitors of mycobacterial biofilm as well as their selectivity towards biofilm inhibition. Furthermore, it indicates the *in vitro* safety of these derivatives as well as describes a possible mechanism to explain the biological activity.

Keywords: 4-Methylumbelliferone, Antibiofilm, Antimycobacterial, Coumarin, Mycobacterium, Mycothione reductase, Tuberculosis, Umbelliferone

3.1. Introduction

Over the last few decades, and with the onset of resistant strains of bacteria, the treatment of infectious diseases has become a greater challenge. The persistence and antibiotic tolerance of *Mycobacterium tuberculosis* make this bacterium extremely effective at inducing infections and causing disease. The first and second-line tuberculosis (TB) drugs are accompanied by a range of different side effects, which inevitably influences the compliance of patients with the scheduled treatment regimen, usually for a duration of 6-9 months. The most common side-effect is TB-drug induced hepatotoxicity that leads to an interruption of the treatment and contributes to non-compliance (Singla et al., 2010). Current TB antibiotics display their mechanism of action as a growth-dependent bacteriostatic and/or bactericidal effect and very few of these focus on the persistent, non-replicating, hypoxic communities of bacteria within a biofilm (Islam et al., 2012).

It is well established that *Mycobacterium* species form complex biofilms in nature. Moreover, evidence-based results indicate the existence of an *in vivo* biofilm composed of non-replicating, drug-tolerant persister cells within a heterogeneous extracellular matrix contributing to a high level of tolerance towards antibiotic therapy. A biofilm structure has been identified in the acellular rim of a granuloma in a study done on guinea pigs (Lenaerts et al., 2007). The existence of a biofilm and the accompanying difficulty in treating and eradicating the structure and enclosed bacteria focusses on the possible mode of persistence in latent infections. The mechanisms and processes involved in the formation of biofilms are therefore an ideal source for new drug targets. In broader terms, this leads to two classes of biofilm-related drugs; inhibition of biofilm formation and disruption of biofilm structure to expose the enclosed bacterium to an appropriate drug.

In the search for novel treatments and drugs for the fight against diseases, nature has supplied us with a vast number of natural compounds with a wide variety of biological actions. One of these classes of natural compounds is coumarins, first isolated in 1820 from *Dipteryx odorata* commonly known as the Tonka bean. Coumarins are fragrant organic compounds that fall under the α -benzopyrone class of chemical compounds (Tandon and Rastogi, 1979). Umbelliferone, a 7-hydroxycoumarin derivative is the most abundant coumarin found in nature. It has been reported earlier that coumarins reduce biofilm

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formation as well as the virulence of *Escherichia coli*, *Edwardsiella tarda*, *Pseudomonas aeruginosa*, *Ralstonia solanacearum*, *Staphylococcus aureus*, *Salmonella typhimurium*, *Vibrio anguillarum* (Ding et al., 2011; Dürig et al., 2010b; Gutiérrez-Barranquero et al., 2015; Lee et al., 2014; Ojima et al., 2016b; L. Yang et al., 2017b). It was established that the coumarin compounds inhibited the attachment and formation of *E. coli* biofilm by up to 80%. It was noted that the antibiofilm activity was not related to the antibacterial activity indicating a mechanism that does not have a bactericidal effect (Lee et al., 2014).

Many genes have been identified to be up-regulated at different stages during the development of a biofilm. Their specific role and how they interconnect is, however, not clearly understood. These genes and gene products include; isocitrate lyase, thioredoxin-like protein, mycothione reductase, other oxidoreductases and DNA gyrase, to name a few (Islam et al., 2012; Vargas et al., 2016). Mycothiol, the low molecular weight thiol produced by the enzymatic reaction of mycothione reductase (Mtr) has been shown to be vital for the survival during stress of *Mycobacterium smegmatis* biofilm formation (Vargas et al., 2016). It has been proposed that by using synthetic libraries and drugs that capture the chemical diversity obtained from synthesis with the physiochemical properties of natural products, a potent inhibitor can be obtained with greater bioavailability (Brown and Wright, 2016).

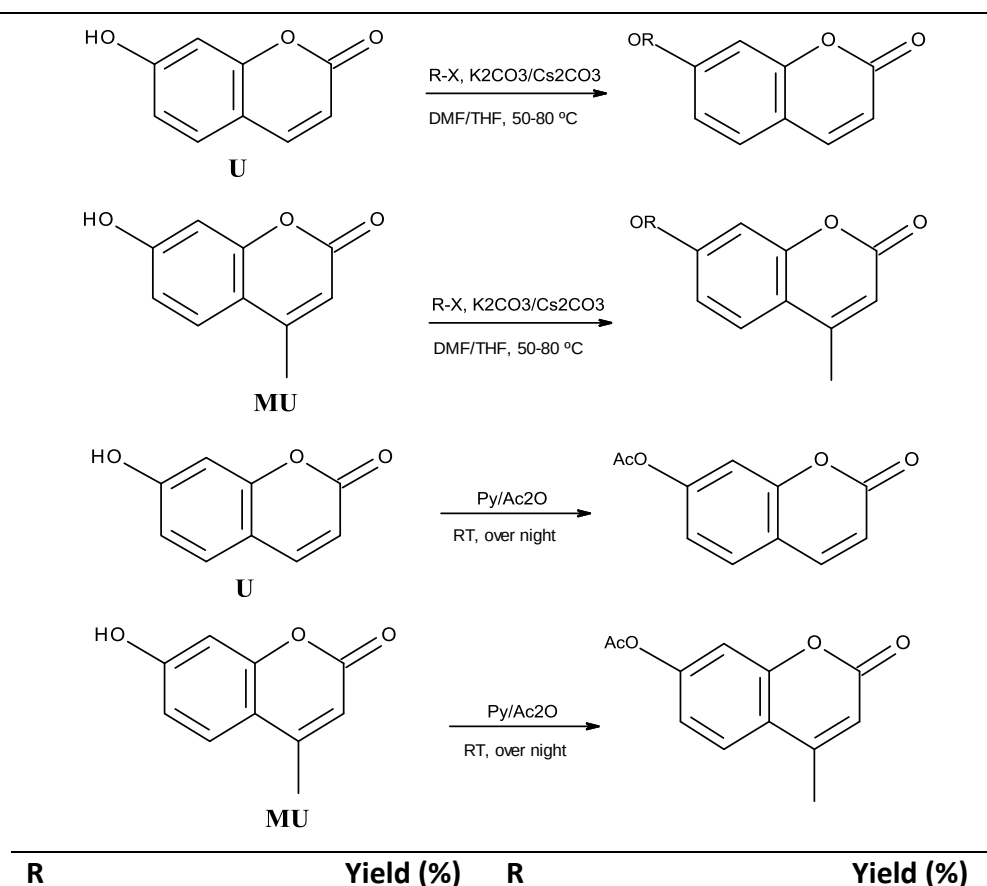
The aim of the present study was to investigate the possibility of coumarin derivatives (CD) for their mycobacterial biofilm inhibitory activities by using *M. smegmatis* mc² 155 and an attenuated biosafety level 2 strain of *M. tuberculosis* H37Rv mc² 7000 (Ojha et al., 2008). This was achieved by investigating the selectivity of these derivatives towards the inhibition of biofilm formation as compared to planktonic growth inhibitory activity. Different bacterial assays were conducted to determine the selectivity. These included planktonic growth inhibition assays, intra-biofilm antibacterial activity and biofilm formation inhibition. Additionally, the hepatoprotective activity of the coumarin derivatives was evaluated using human liver hepatocytes (HepG2). Lastly, the inhibition of mycothione reductase was evaluated to investigate the possible mechanism of action of the CD.

3.2. Results and Discussion

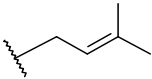
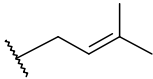
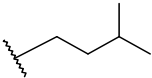
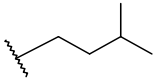
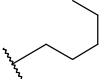
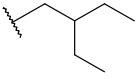
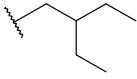
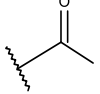
3.2.1. Chemistry

The CD were synthesized according to the methods previously described (Adfa et al., 2012; Huang et al., 2012; Khan et al., 2003). The two commercially available base compounds used were umbelliferone (7-hydroxycoumarin, labelled as **U**) and 4-methylumbelliferone (7-hydroxy-4-methylcoumarin, labelled as **MU**). Six of each umbelliferone and 4-methylumbelliferone derivatives were synthesized and summarized in **Table 3.1**. Two novel compounds, **U5** and **MU5** were synthesized. All the derivatives dried to form white or pale-white crystals. The final products were obtained in good to high yields (52 - 99%). The base compounds, as well as the synthesized derivatives, were characterized by ^1H , ^{13}C NMR, GCMS and HRMS and compared with published data.

Table 3.1: Scheme for the synthesis of 7-hydroxycoumarin and related derivatives.



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R		Yield (%)	R		Yield (%)
U1		95	MU1		93
U2		98	MU2		84
U3		65	MU3		68
U4		53	MU4		52
U5		89	MU5		99
UA*		98	MUA*		99

*Pyridine instead of K₂CO₃ as a catalyst

3.2.2. Antimycobacterial vs. Antibiofilm Activity

The antimycobacterial activity of the 14 coumarin derivatives (two base compounds and 12 derivatives) was evaluated on two species of mycobacteria; *Mycobacterium tuberculosis* H37Rv and *Mycobacterium smegmatis* mc² 155 for their inhibitory and antibiofilm activities (an attenuated biosafety level 2 strain was used for the biofilm work, called *M. tuberculosis* H37Rv mc² 7000). *M. smegmatis* does not cause tuberculosis infection in humans but was selected for the study as it is the model mycobacterial biofilm organism and it forms biofilms at a much faster rate than *M. tuberculosis*. The results are summarized in **Table 3.2**. The derivatives showed higher activity on *M. tuberculosis* when compared to *M. smegmatis*. This can be explained by the metabolic processes within the organisms as well as the growth rate, where the doubling time of *M. smegmatis* is six times higher than the slow-growing *M. tuberculosis* (Klann et al., 1998).

The **MU** samples had on average higher antimycobacterial activity when compared to the **U** samples, indicating that the substitution of a 4-methyl (-CH₃) group increased the activity. A similar effect was observed by Appendino et al., (2004), where the substitution at C-4 increased the activity. The CD, **U1** and **MUA** showed moderate inhibition of *M. smegmatis* exhibiting MIC values of 543 and 508 µM,

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respectively. Three of the derivatives had a moderate antimycobacterial activity with MIC values ranging between 102 μ M and 108 μ M against *M. tuberculosis* (**U2**, **MU2**, **MU5**). Comparing the structures of these compounds, all three have two free methyl groups at the C-7 substituent. The second best activity was observed by **U1** and **MU1**, with MIC values of 217 μ M and 205 μ M, both having two free allylic methyl groups. The acetoxy substitution (**U5**, **MU5**) had comparable activity when compared to the base compounds **U** and **MU**. The C-7 substitution involving a pentyloxy (**U3**, **MU3**) or a cyclohexyloxy (**U4**, **MU4**) moiety had a reduced effect and should not be considered for antimycobacterial activity. In this study, the antimycobacterial activity of the tested coumarin derivatives was comparable to previous reports, where MIC values of 153 μ M and 247 μ M were observed for dentatin and bergaptol, respectively (Esquivel-Ferriño et al., 2012; Sunthitikawinsakul et al., 2003).

Although some of the derivatives showed activity, it is not comparable to the positive controls which showed activities below 2 μ M. The contradictory result can be seen in the observation of the antibiofilm activity, where the derivatives had a higher tendency to inhibit the formation of biofilm. This can be observed in the activities of the controls and samples for their respective activities in **Fig 3.1** and **Fig 3.2**. The biofilm inhibitory activity was obtained by visually observing the formation of a biofilm. The minimum biofilm formation inhibitory concentration (MBFIC) is described as the lowest concentration where no biofilm formed on the air-medium interface. Four of the coumarin derivatives showed an MBFIC lower than 70 μ M with **MU2** showing the best activity of 32 μ M. Collectively, samples **MU5**, **U1** and **U2** followed by **MU1** had the second and third best activity of $\pm$ 68 μ M and 128 μ M, respectively.

Table 3.2: The bacterial growth and biofilm inhibitory activities of coumarin derivatives against *Mycobacterium* spp. and their selectivity for biofilm inhibition.

Compound	<i>M. smeg</i> mc ² 155					<i>M.tb</i> H37Rv	<i>M.tb</i> mc ² 7000 ^d
	MIC ^a (μ M)	MBFIC ^b (μ M)	Biofilm IC ₅₀ ^c (μ M)	$\pm$ SD	Selective Index	MIC ^a (μ M)	Intra-Biofilm (% reduction)
U	3084	1542	366*	91	2	463	-
U1	543	68	5	7	8	217	-
U2	1076	67	1	0	16	108	0
U3	2153	1076	371*	28	2	431	93

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Compound	<i>M. smeg</i> mc ² 155					<i>M.tb</i> H37Rv	<i>M.tb</i> mc ² 7000 ^d
	MIC ^a (μ M)	MBFIC ^b (μ M)	Biofilm IC ₅₀ ^c (μ M)	$\pm$ SD	Selective Index	MIC ^a (μ M)	Intra-Biofilm (% reduction)
U4	2047	512	97*	25	4	409	93
U5	2153	1076	104*	34	2	431	-
UA	2449	2449	174*	40	1	367	-
MU	2838	1419	242*	59	2	426	0
MU1	2047	128	3	2	16	205	81
MU2	2030	32	1	0	64	102	80
MU3	1015	508	53	13	2	406	64
MU4	968	484	62	22	2	387	73
MU5	2030	63	2	1	32	102	-
MUA	508	508	49	4	1	305	93
Ciprofloxacin^e	2	1	1	0	2	-	-
Isoniazid^f	-	-	-	-	-	1	-
Rifampin^f	-	-	-	-	-	0	100
Streptomycin^f	-	-	-	-	-	1	-

^a Minimum inhibitory concentration, ^b Minimum biofilm formation inhibitory concentration, ^c Inhibitory concentration where 50% of biofilm formation was inhibited (Data is represented as mean IC₅₀ $\pm$ SD, One-way ANOVA * = p-value < 0.05 when compared to the positive control), ^d *Mycobacterium tuberculosis* strain that forms biofilms *in vitro*, ^e Positive control for *M. smegmatis*, ^f positive control for *M. tuberculosis*

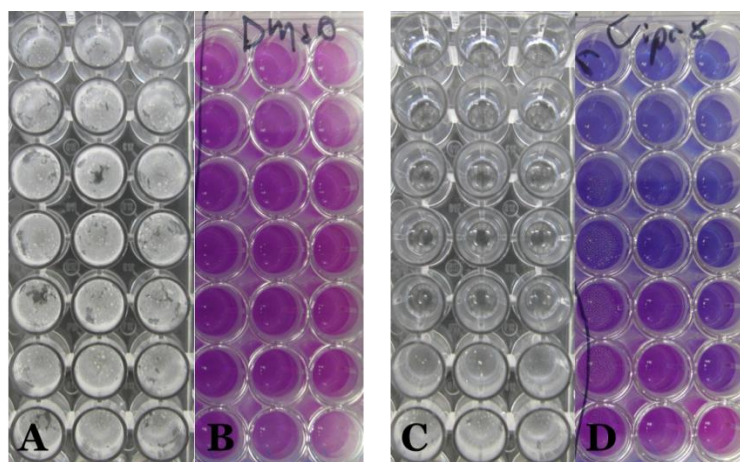


Fig 3.1: Antimycobacterial activity vs. antibiofilm activity on *M. smegmatis* indicating the controls and non-selectivity of ciprofloxacin towards biofilm inhibition. (A) Biofilm control (DMSO 5%). (B) Bacterial control (DMSO 5%). (C) Antibiofilm activity of ciprofloxacin (positive control) with an MBFIC of 1 μ M. (D) Antimycobacterial activity of ciprofloxacin with an MIC of 2 μ M indicating a selective index of 2.

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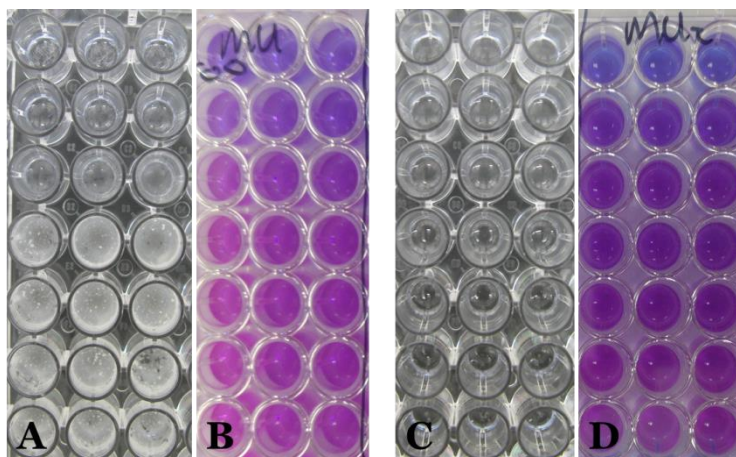


Fig 3.2: Antimycobacterial activity vs. antibiofilm activity on *M. smegmatis* indicating the selectivity of **MU2** towards biofilm inhibition. (A) Antibiofilm activity of **MU** with an MBFIC of 1419 μM . (B) Antimycobacterial activity of **MU** with an MIC of $> 2838 \mu\text{M}$. (C) Antibiofilm activity of **MU2** with an MBFIC of 32 μM . (D) Antimycobacterial activity of **MU2** with an MIC of 2030 μM indicating a selective index of 64.

The concentration where 50 % of the biofilm was inhibited (IC_{50}), was calculated for each derivative. Untreated biofilms were considered as 0% inhibited and biofilm-free media was considered as 100% biofilm inhibition. The best activities were observed for **MU2**, **U2**, **MUA**, **MU1** and **U1** with IC_{50} 's lower than 5 μM . These samples showed biofilm inhibition as high as 96% even at the lowest tested concentration when compared to untreated biofilms. The sigmoidal dose-response curves are depicted in **Fig 3.3** and **Fig 3.4**. The position and type of substitution had an effect and all the results indicate to an increased activity when coumarin is substituted with a methyl group at C-4 and two free methyl groups at the end of the moiety at C-7.

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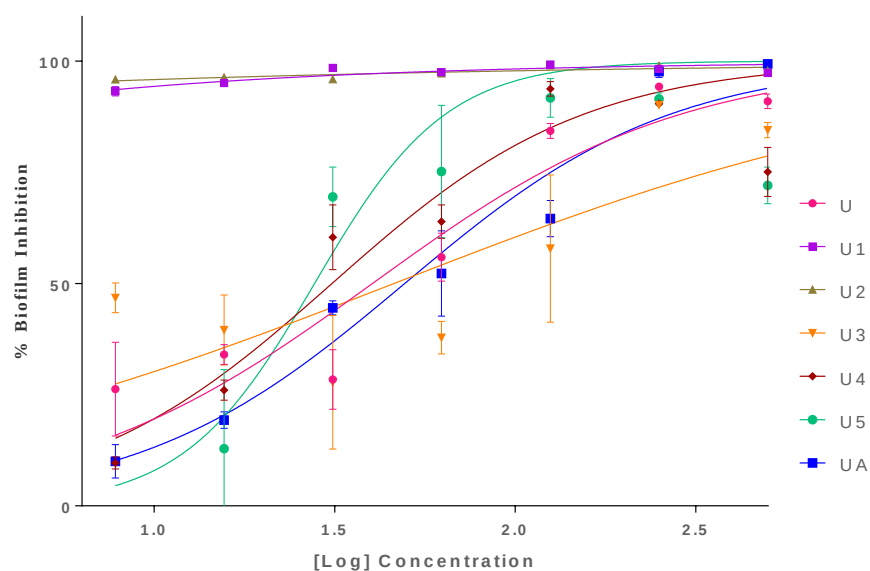


Fig 3.3: Dose-response curves of coumarin derivatives (U samples) on biofilm inhibition. Untreated biofilm = 0% inhibited, biofilm-free media = 100% inhibited. Data are represented as means $\pm$ SEM, non-linear regression curve using a four-parameter logistic equation.

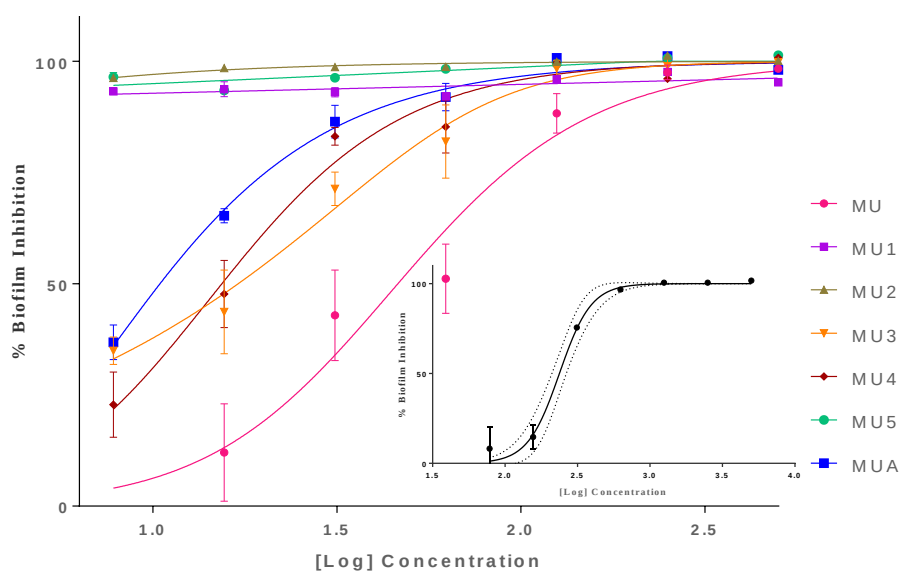


Fig 3.4: Dose-response curves of coumarin derivatives (MU samples) on biofilm inhibition and ciprofloxacin as a positive control (bottom right corner). Untreated biofilm = 0% inhibited, biofilm-free media = 100% inhibited. Data are represented as means $\pm$ SEM, non-linear regression curve using a four-parameter logistic equation.

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The selectivity index (SI) is a representation of the antimycobacterial vs. the antibiofilm activity of the coumarin derivatives. The SI values were calculated by dividing the MIC with the MBFIC and can be seen in **Fig 3.5**. Sample **MU2** showed the highest selectivity of 64. This was followed by **MU5** with an SI of 32. A trend can be observed with the addition of a (3-methylbut-2-en-1-yl)oxy moiety as in the case of **U1** and **MU1** and the addition of a 3-methylbutyloxy in the case of **U2** and **MU2**. The change from a 2-butenyloxy (alkene) to a butyloxy (alkane) substitution increased the activity in both sets of compounds. The C-4 substitution, together with the addition of a 3-methylbutyloxy on C-7 had a profound effect increasing the antimycobacterial activity on *M. tuberculosis* from 463 to 102 μ M (4-fold increase) and the antibiofilm activity on *M. smegmatis* from 1542 to 32 μ M (48-fold increase), this may be due to the possible interactions (hydrogen bonding and electrostatic) of the compound within the respective binding site of a target enzyme.

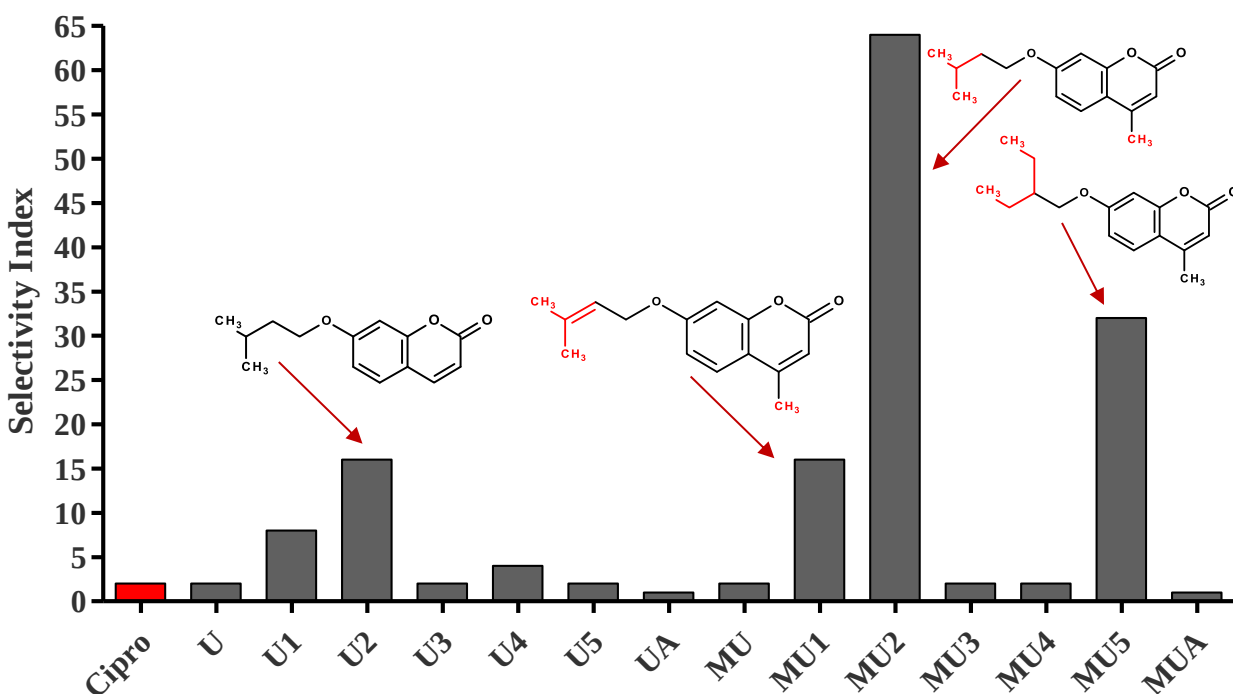


Fig 3.5: Selectivity of coumarin derivatives for biofilm inhibition ($SI = MIC/MBFIC$), indicating the structural changes between **U2**, **MU1** and **MU2**. Positive control: ciprofloxacin (red). Data are represented as absolute SI-values.

The colony morphology of *M. smegmatis* mc² 155 was evaluated to determine the inhibition of a biofilm on solid 7H11 media. The formation of the biofilm pellicles on the solid media is depicted in **Table 3.3**.

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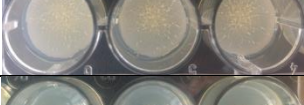
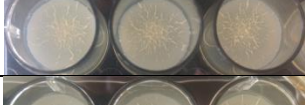
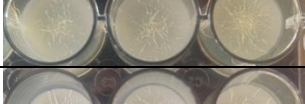
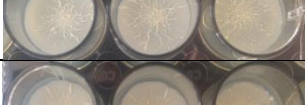
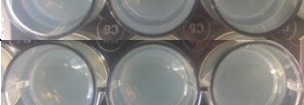
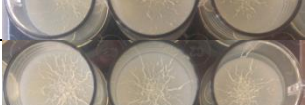
Two test concentrations were selected at 100 and 1000 μM . The formation of biofilm was ranked on a scale of 0 to 10, with 0 showing complete inhibition of biofilm formation and 10 indicating no inhibition. The positive control, ciprofloxacin was tested at 3 and 30 μM and showed complete inhibition of colony growth and biofilm formation. A similar observation as seen in the antibacterial and antibiofilm activity was made, where the **MU** samples showed higher activity when compared to the **U** samples. Derivatives **U1** and **U2** were the only compounds that showed complete inhibition at 1000 μM . Their activity at 100 μM is just as notable, where none of the other derivatives had any activity. Sample **U5** had a reverse effect and did not show an improved activity when compared to the base compound **U**.

Sample **MU1** at 1000 μM had an effect on the perimeter and shape of the colony and led to a marginal reduction of biofilm. Although it did not inhibit the growth there was no formation of pellicle structures to indicate a biofilm phenotype. The only derivative that showed complete inhibition of biofilm formation at a concentration of 100 μM was **MU2**.

Some of the derivatives showed a high reduction in the bacterial load within the biofilm structure with **MUA** showing a reduction of 96% of colony forming units per millilitre (CFU/mL) when compared to untreated biofilm. **Fig 3.6** shows the reduction of colony forming units by one of the derivatives when compared to the control. There is no direct correlation between the antimycobacterial activity and intra-biofilm reduction of bacterial count. The compounds that showed inhibitory activity were not inevitably the best intra-biofilm samples, as seen in the case of **U3** and **U4** which showed a low antimycobacterial activity with MIC values of 431 μM and 409 μM , respectively, but had the highest reduction in the bacterial count during the intra-biofilm assay. This cannot clearly be explained and more work needs to be done to fully understand and describe the observation and possible mechanisms that underlie the reduction in bacterial counts associated with these compounds. The observation can either be due to the intercalation and absorption of the coumarins into the biofilm having a bacteriostatic or bactericidal effect on the residing bacilli or due to a disruption of the structural components of the mature biofilm.

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Table 3.3: Colony morphology of *M. smegmatis* on (72 h) 7H11 solid agar media after the treatment of coumarin derivatives.

Compound	1000 μ M	Rating	100 μ M	Rating
U		7		10
U1		0		2
U2		0		1
U3		6		10
U4		1		10
U5		9		10
UA		3		10
MU		1		10
MU1		4		8
MU2		0		0
MU3		0		10
MU4		0		10
MU5		0		1
MUA		0		10
Cipro (30 & 3 μ M)		0		0
Media control			Bacterial Control	
Controls		0		10

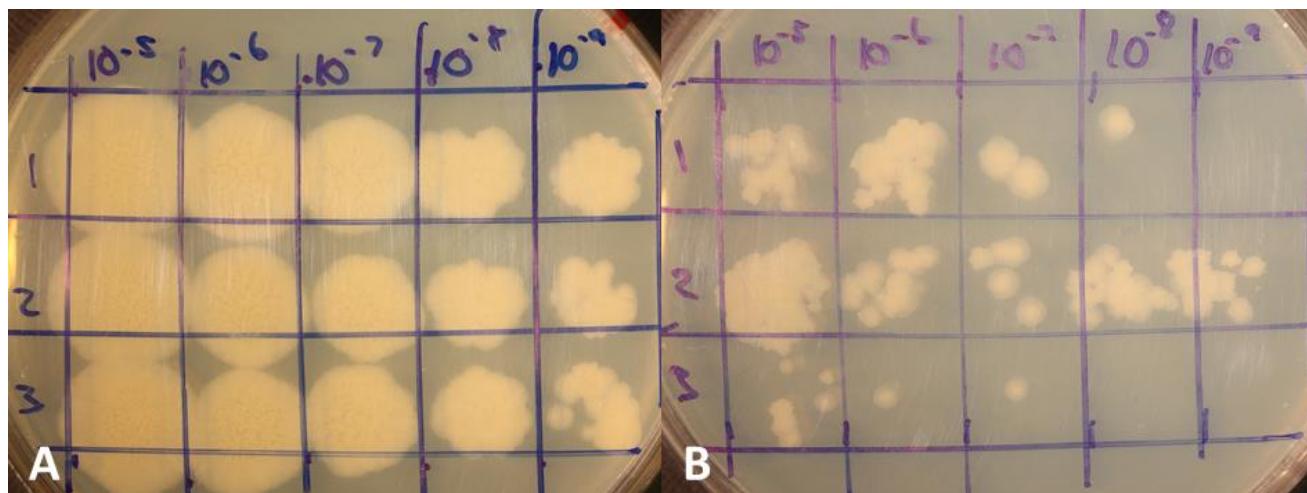


Fig 3.6: The intra-biofilm activity of sample **MUA** on *M. tuberculosis* mc² 7000 (B), compared to the solvent control.

Throughout this study, it was evident that these 7-hydroxycoumarin derivatives showed above all an inhibitory effect on the formation of a biofilm. The substitution of a C-4 methyl moiety increased the activity and relates to previous reports indicating an increased biological activity of coumarins substituted on this position and C-5 (Keri et al., 2015). The latter showed enhanced anti-TB activity, although the substitution on the fourth position may relate more to biofilm inhibition as opposed to anti-TB activity. The structural difference between **MU2** and **MU5** is two methyl moieties replaced by two ethyl moieties, both showing exceptional biofilm inhibitory activities. The third best inhibitor is **MU1** which has a double bond in the butenyl substitution. This indicates the importance of bond rotations and angles at this position. It signifies that a free rotation around the 2,3 bond of the isopentyloxy moiety can possibly have an increase in activity. It is worth mentioning that the mechanisms by which these compounds confer their biological activities might be different, and this can be observed in the hepatoprotective study.

3.2.3. Hepatoprotection vs Hepatotoxicity

During the early stages of drug discovery, many different compounds and scaffolds are discarded as possible drugs due to the toxicity associated with these compounds (Lohrasbi et al., 2018). Drug-induced liver toxicity is one of the most prominent side effects of the current TB drugs (Jong et al., 2013; Sia and Wieland, 2011). Therefore, the coumarin derivatives were assessed not only for their

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possible hepatotoxic properties but also for their protective effect on drug-induced hepatotoxicity using acetaminophen. In **Fig 3.7** the HepG2 cells are depicted together with the cells treated with acetaminophen and **MU5** showing a cytotoxic effect.

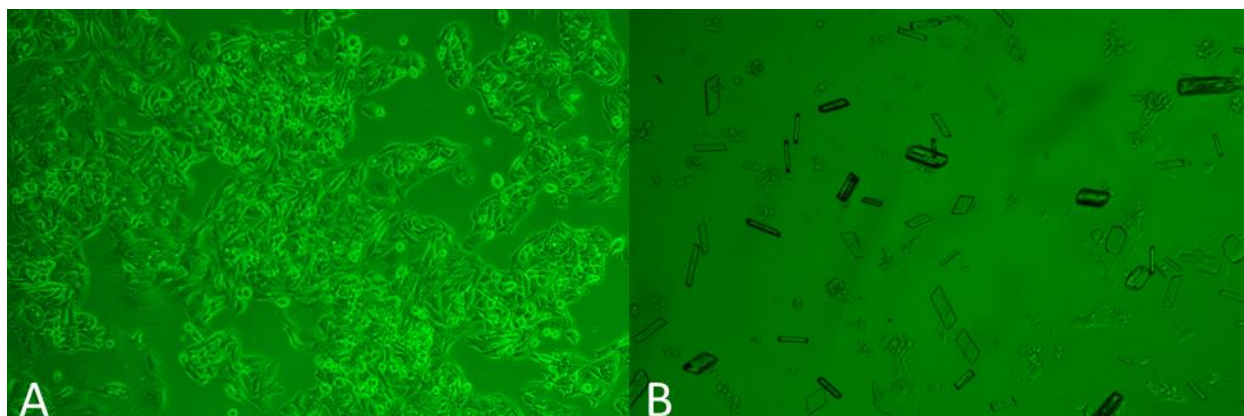


Fig 3.7: Human liver cell line HepG2 treated with acetaminophen. Cell control with no toxin (A), cells treated with 20 mM acetaminophen and **MU5** at 200 μM , showing some crystallization and an antagonistic effect (B).

The cellular toxicity of the derivatives was evaluated to determine the inhibitory concentration where 50% of the cells were inhibited and to identify the concentration range to be used for the hepatoprotective assay. The toxicity values are summarized in **Table 3.4** and the sigmoidal dose-response curve for acetaminophen can be found in **Fig 3.8**. Five of the derivatives had IC_{50} values lower than the highest concentration tested. Moderate toxicity was observed for compound **U2** (IC_{50} of 200 μM), and low toxicity for compounds **U5** (859 μM), **MU1** (1113 μM), **MU2** (682 μM) and **MU4** (761 μM). From the toxicity values, it was decided to test the hepatoprotective effect of the derivatives at two set concentrations of 200 (50 $\mu\text{g/mL}$) and 100 μM (25 $\mu\text{g/mL}$). The IC_{50} for acetaminophen was calculated to determine the concentration needed to induce a toxic effect.

Table 3.4: The cellular toxicity of coumarin derivatives against the human liver cell line HepG2 expressed as 50% inhibitory concentrations.

Compounds	HepG2 $\text{IC}_{50}^{\text{a}}$ (μM)	$\pm\text{SD}^{\text{b}}$
U	- ^e	-
U1	-	-
U2	200*	27

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Compounds	HepG2 IC ₅₀ ^a (μM)	±SD ^b
U3	-	-
U4	-	-
U5	849*	54
UA	-	-
MU	-	-
MU1	1113*	30
MU2	682*	29
MU3	-	-
MU4	761*	25
MU5	-	-
MUA	-	-
Acetaminophen ^c	11.4*	1.5
Actinomycin D ^d	2.4 x 10 ⁻²	1.6 x 10 ⁻³

^a Concentration where 50% of the cells were inhibited, ^b Standard deviation, ^c Toxic inducer, ^d positive control for cellular toxicity, ^e No activity was observed at the tested concentrations. Data are expressed as mean IC₅₀'s ± SEM. One-way ANOVA p<0.05. Post-hoc Tuckey multiple comparison test, * p-value<0.001, when compared to the positive control.

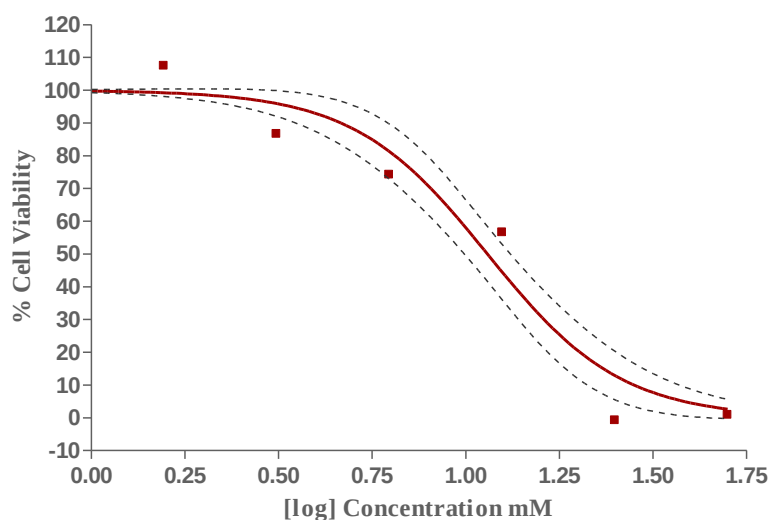


Fig 3.8: Dose-response of acetaminophen on HepG2 cells with an IC₅₀ of 11 mM

Twenty millimolar of acetaminophen was used to induce toxicity on HepG2 cells. Most of the coumarin derivatives showed a protective effect at a concentration of 200 μM, with compound **U3** showing 40% protection (**Fig 3.9**). Compounds **U2** and **MU1** showed no observable protective effect at 200 μM but had a protective effect at 100 μM, indicating a possible interaction at higher concentrations, with these

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samples having an additive effect. The only compound that showed an antagonistic effect was **MU5** (Fig 3.7) indicating that this sample increased the toxicity of acetaminophen at the tested concentrations.

In a previous study by Atmaca et al., (2011), the hepatoprotective effect of coumarin derivatives was evaluated on CCl₄-induced toxicity in male Sprague-Dawley rats. Similarly, it was found that 4-methylumbelliferone (**MU**) and the coumarin nucleus showed no significant protective effect. It was however observed, that 6,7-dihydroxycoumarin and 6,7-dimethoxycoumarin had a protective effect. Pre-treatment with these compounds reduced the hepatotoxic markers superoxide dismutase (SOD) and catalase (CAT) (Atmaca et al., 2011).

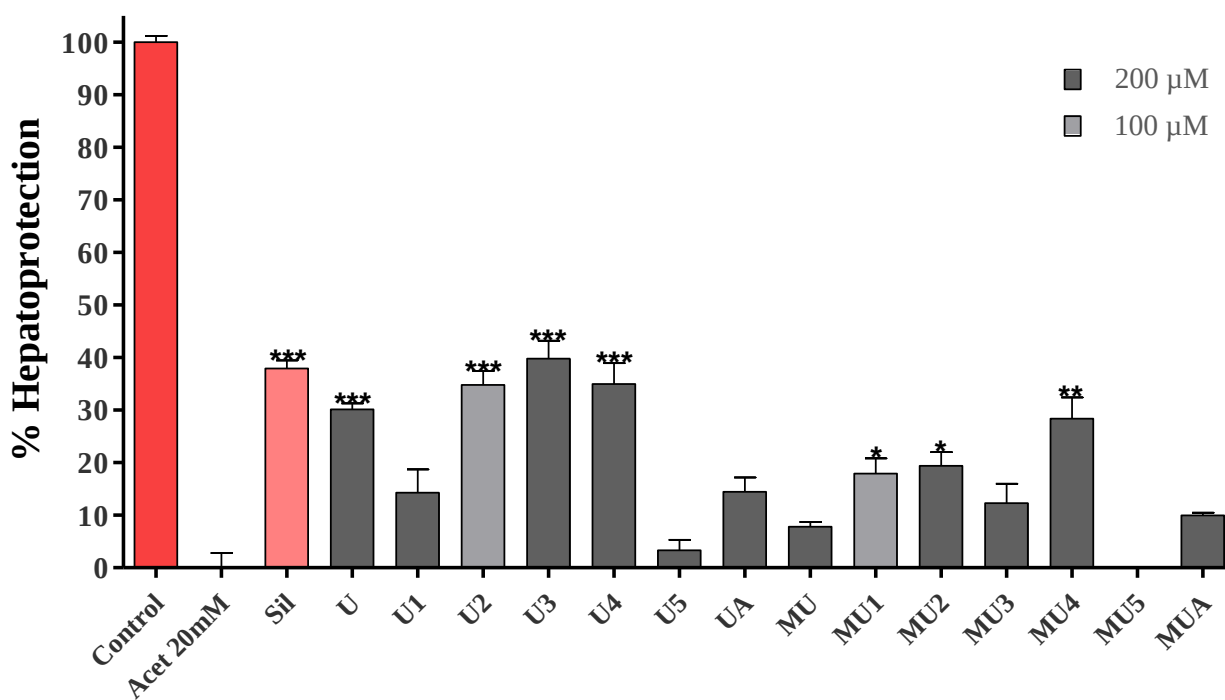


Fig 3.9: Protection effect of coumarins on acetaminophen treated (20mM) HepG2 cells at $\frac{1}{4}IC_{50}$. Untreated cells (red), positive control silymarin (pink). Data are represented as means % protection $\pm$ SEM. One-way ANOVA $p < 0.05$. Post-hoc Tuckey multiple comparison test, * p -value < 0.05 , ** p -value < 0.01 , *** p -value < 0.001 when compared to acetaminophen.

In another study, coumarin glucosides isolated from *Hydrangea paniculata* with the glucoside substitution on position 7, showed a protective effect against DL-galactosamine-induced toxicity on the human hepatic cells (HL-7702 cells) (Shi et al., 2014). Both these studies, together with the findings from the present study, suggest that a hydroxy or other substitution at C-7 can improve the protective

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effect of the coumarin nucleus and that a methyl substitution at C-4 might be detrimental if not combined with other substitutions. The 4-methyl substitution together with a substitution containing two free ethyl groups on position 7 (**MU5**) could potentially be hepatotoxic or have a detrimental drug-drug interaction.

3.2.4. Mycothione and Glutathione Reductase Inhibition

As part of the mechanistic study, the inhibitory effect of the coumarin derivatives was assessed on mycothione reductase (Mtr) and using the human analog, glutathione reductase (Gtr) as the control in an attempt to identify the selectivity towards the bacterial analog. The concentration where 50% of the enzyme was inhibited was calculated followed by a calculation of the inhibition constant K_i . The selectivity towards Mtr was calculated by dividing the IC_{50} on Gtr with the IC_{50} value on Mtr. The values are summarized in **Table 3.5**. None of the umbelliferone derivatives (**U** samples) showed inhibition, at the tested concentration, on Mtr and Gtr indicating the importance of the 4-methyl substitution on C-4. Samples **MU4** and **MU3**, with its cyclohexyloxy and pentyloxy substitutions at C-7, exhibited no inhibition on both enzymes. The acetoxo substitution (**MUA**) had a negligible activity on Gtr with an IC_{50} value of 994.3 μ M. A substitution with 3-methylbut-2-en-1-yloxy (**MU1**) at C-7 had a detrimental effect and showed selectivity towards Gtr. The base compound, 4-methylumbelliferone (**MU**), and the 2-ethylbutyloxy substitution (**MU5**) showed better inhibition of the bacterial enzyme when compared to the human enzyme with a selectivity indices of 6 and 3, respectively. When comparing the glutathione reductase inhibition values with the hepatoprotective activity, sample **MU1** and **MU5** showed the lowest IC_{50} values. These two compounds showed an antagonistic effect on the acetaminophen-treated cells. Glutathiol is important in the detoxification of and stress management in cellular redox homeostasis (Couto et al., 2016). In a classic study done by Mitchell and colleagues, in 1973, the authors indicated that the addition of acetaminophen had a reduction in cellular glutathiol and increased hepatic necrosis. A combination in the depletion of glutathiol and the inhibition of glutathione reductase can then partly explain the antagonistic effect seen with samples **MU1** and **MU5**.

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Table 3.5: The enzyme inhibitory activity of coumarin derivatives against the human analog glutathione and mycobacterial analog mycothione reductases. The K_i -values were calculated according to Cer et al., (2009).

Sample	Glutathione reductase			Mycothione reductase			SI^d Gtr/Mtr
	IC_{50}^a (μM)	SD^b	K_i^c (μM)	IC_{50} (μM)	SD	K_i (μM)	
U Samples (U – UA)	>1000	-	-	>200	-	-	-
MU	778.2	11.3	404.7	136.3	15.5	135.8	6
MUA	994.3	29.9	517.1	>200	-	-	-
MU1	106.4	26.2	55.3	244.2	13.7	243.3	-2
MU2	>1000	-	-	29.1	11.9	28.9	34
MU3	>1000	-	-	>200	-	-	-
MU4	>1000	-	-	>200	-	-	-
MU5	253.8	11.1	131.9	78.2	13.2	73.2	3

^a Inhibitory concentration where 50% of the enzyme was inhibited, ^b Standard deviation, ^c Inhibition constant, ^d Selective index by dividing the IC_{50} of Gtr with Mtr

Sample **MU2** showed no inhibition of Gtr but had the best activity against Mtr with an IC_{50} of 29.1 μM and a selectivity of 34 towards Mtr inhibition. This activity can be related to the whole-cell bacterial assays where **MU2** showed an MIC of 108 μM on *M. tuberculosis* and a selectivity of 64 towards *M. smegmatis* biofilm inhibition. These activities can partially explain the mechanism by which this compound inhibits the formation of a mycobacterial biofilm.

3.3. Experimental Section

3.3.1. General

All reagents and chemicals were purchased from Sigma-Aldrich and of a commercial grade and used directly without any additional purification. Acetylation and alkylation derivatisation reactions were carried out in oven-dried glassware. Reaction mixtures were washed with distilled- H_2O , dried using sodium sulphate and purified by silica gel 60 column chromatography (**Fig 3.10**). Thin layer chromatography was performed on aluminum-backed silica gel (60 F₂₅₄) plates and long and shortwave UV chamber. The NMR spectra were recorded on a 400 MHz Bruker AvanceIII NMR spectrometer equipped with a 5 mm BBO probe and mass (MS) fragmentation patterns were recorded Bruker maXis impact quadrupole-time-of-flight mass spectrometer. The purified compounds were stored at 4°C

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throughout the duration of the study. Dimethyl sulfoxide (DMSO) was used to dissolve all the test samples for the various biological assays.



Fig 3.10: Procedures during synthesis, from left to right; washing of reaction mixture with H₂O to remove salts, separating funnel to collect the organic layer containing the synthesized derivatives, drying of the collected organic layer using sodium sulfate, column chromatography to purify the synthesized derivative.

General Procedure for the Synthesis of Acyl, Alkyl and Aryl derivatives; 2-oxo-2H-chromen-7-yl acetate (7-acetoxycoumarin, UA) and 7-acetoxy-4-methylcoumarin (MUA). A mixture of 7-hydroxycoumarin (U - umbelliferone) and/or 7-hydroxyl-4-methylcoumarin (MU - 4-methylumbelliferone) (6 mmol) in dimethyl formamide (DMF) or tetrahydrofuran (THF) with alkyl, aryl halide or acetic acid (6 mmol) and calcium, caesium or potassium carbonate (5 mmol) in a round bottom flask was stirred at various temperatures ranging from 45-80 °C for 16-24 h. The reaction mixture was dried under vacuum for the removal of DMF/THF, and extracted with dichloromethane (DMC), and washed with H₂O for the removal of the salts. The organic layer was collected and dried with sodium sulfate for 45 min and roto-evaporated to dryness. The collected residue was subjected to column chromatography on silica gel with DCM to yield the product. The method for the synthesis of the derivatives was previously described by Adfa et al. (2012), Devji et al. (2011), Huang et al. (2012) and Khan et al. (2002, 2003).

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7-(3-Methylbut-2-en-1-yloxy)coumarin (U1). Pale white crystals. 99% yield. MP 103 °C. ^1H NMR (400 MHz, CDCl_3) δ 1.81 (d, J = 15.4 Hz, 3H), 4.59 (d, J = 6.8 Hz, 2H), 5.49 (tt, J = 6.8, 1.4 Hz, 1H), 6.26 (d, J = 9.5 Hz, 1H), 6.84 (d, J = 2.4 Hz, 1H), 6.87 (dd, J = 8.6, 2.4 Hz, 1H), 7.38 (d, J = 8.6 Hz, 1H), 7.66 (d, J = 9.5 Hz, 1H). ^{13}C NMR (101 MHz, CDCl_3) δ 162.11, 161.33, 155.85, 143.48, 139.35, 128.70, 118.59, 113.23, 112.95, 112.42, 101.53, 65.42, 25.85, 18.31. MS (ESI) m/z : Calcd for $\text{C}_{14}\text{H}_{14}\text{O}_3$ ($\text{M}+\text{H}$) $^+$ 230.094294, found 230.094825

7-(Isopentyloxy)coumarin (U2). White crystals. 91% yield. MP 77 – 78 °C. ^1H NMR (400 MHz, CDCl_3) δ 0.99 (d, J = 6.6 Hz, 6H), 1.73 (q, J = 6.7 Hz, 2H), 1.90 – 1.81 (m, 1H), 4.06 (t, J = 6.6 Hz, 2H), 6.26 (d, J = 9.5 Hz, 1H), 6.83 (d, J = 2.3 Hz, 1H), 6.85 (dd, J = 8.5, 2.3 Hz, 1H), 7.38 (d, J = 8.5 Hz, 1H), 7.66 (d, J = 9.5 Hz, 1H). ^{13}C NMR (101 MHz, CDCl_3) δ 162.40, 161.32, 155.92, 143.47, 128.69, 113.01, 112.91, 112.36, 101.30, 67.05, 37.62, 25.01, 22.54. MS (ESI) m/z : Calcd for $\text{C}_{14}\text{H}_{16}\text{O}_3$ ($\text{M}+\text{H}$) $^+$ 232.109944, found 232.110425

7-(Pentyloxy)coumarin (U3). Pale white crystals. 99% yield. MP 40 °C. ^1H NMR (600 MHz, CD_3OD) δ 0.87 (3H, t, J = 7.0 Hz), 1.20-1.45 (4H, 1.38 (tt, J = 7.5, 7.0 Hz), 1.29 (h, J = 7.0 Hz)), 1.81 (2H, quint, J = 7.4 Hz), 4.25 (2H, t, J = 7.4 Hz), 6.44 (1H, d, J = 9.5 Hz), 6.52 (1H, dd, J = 8.3, 1.5 Hz), 6.66 (1H, dd, J = 1.5, 0.4 Hz), 7.98 (1H, dd, J = 8.3, 0.4 Hz), 8.10 (1H, d, J = 9.5 Hz). ^{13}C NMR (101 MHz, CD_3OD) δ 161.9, 128.4, 68.2, 112.8, 28.3, 28.7, 143.4, 101.5, 14.0, 113.5, 113.2, 161.4, 155.7, 22.4. MS (ESI) m/z : Calcd for $\text{C}_{14}\text{H}_{16}\text{O}_3$ ($\text{M}+\text{H}$) $^+$ 232.109944, found 232.15

7-(Cyclohexyloxy)coumarin (U4). Pale white crystals. 99% yield. MP 93 – 94 °C. ^1H NMR (600 MHz, CD_3OD) δ 1.39 (1H, dq, J = 12.7, 2.8 Hz), 1.36-1.74 (9H, 1.65 (dtd, J = 13.2, 10.3, 2.8 Hz), 1.46 (dtd, J = 12.7, 10.3, 2.8 Hz), 1.55 (dq, J = 12.7, 2.8 Hz), 1.51 (dtd, J = 12.7, 10.3, 2.8 Hz), 1.65 (dq, J = 13.2, 2.8 Hz)), 4.80 (1H, tt, J = 10.3, 2.8 Hz), 6.44 (1H, d, J = 9.5 Hz), 6.54 (1H, dd, J = 8.3, 1.5 Hz), 6.67 (1H, dd, J = 1.5, 0.4 Hz), 7.98 (1H, dd, J = 8.3, 0.4 Hz), 8.10 (1H, d, J = 9.5 Hz). ^{13}C NMR (101 MHz, CD_3OD) δ 23.4, 23.4, 114.6, 99.6, 113.5, 143.4, 161.4, 112.8, 155.7, 31.6, 31.6, 72.5, 128.4, 162.5, 25.3. MS (ESI) m/z : Calcd for $\text{C}_{15}\text{H}_{16}\text{O}_3$ ($\text{M}+\text{H}$) $^+$ 244.109944, found 244

7-(2-Ethylbutyloxy)coumarin (U5). White crystals. 99% yield. ^1H NMR (600 MHz, CD_3OD) δ 0.83 (6H, t, J = 7.1 Hz), 1.29-1.44 (4H, 1.36 (qd, J = 7.1, 6.7 Hz), 1.36 (qd, J = 7.1, 6.7 Hz)), 1.63 (1H, sept, J

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= 6.7 Hz), 4.26 (2H, d, J = 6.7 Hz), 6.44 (1H, d, J = 9.5 Hz), 6.53 (1H, dd, J = 8.3, 1.5 Hz), 6.66 (1H, dd, J = 1.5, 0.4 Hz), 7.98 (1H, dd, J = 8.3, 0.4 Hz), 8.10 (1H, d, J = 9.5 Hz). ^{13}C NMR (101 MHz, CD_3OD) δ 161.4, 112.8, 71.2, 143.4, 161.7, 40.6, 113.5, 23.5, 23.5, 11.1, 11.1, 128.4, 113.2, 101.5, 155.7. MS (ESI) m/z : Calcd for $\text{C}_{15}\text{H}_{18}\text{O}_3$ ($\text{M}+\text{H}$) $^+$ 246.125594, found 246.126425

7-Acetoxycoumarin (UA). Pale white crystals. 60% yield. ^1H NMR (400 MHz, CDCl_3) δ 2.37 (s, 3H), 6.42 (d, J = 9.6 Hz, 1H), 7.08 (dd, J = 8.4, 2.2 Hz, 1H), 7.14 (d, J = 2.2 Hz, 1H), 7.51 (d, J = 8.4 Hz, 1H), 7.72 (d, J = 9.6 Hz, 1H). ^{13}C NMR (101 MHz, CDCl_3) δ 168.75, 160.39, 154.66, 153.14, 142.88, 128.58, 118.43, 116.68, 116.09, 110.47, 21.14. MS (ESI) m/z : Calcd for $\text{C}_{11}\text{H}_8\text{O}_4$ ($\text{M}+\text{H}$) $^+$ 204.042259, found 204.043025

4-Methyl-7-(3-methylbut-2-en-1-yloxy)coumarin (MU1). White crystals. 99% yield. ^1H NMR (400 MHz, CDCl_3) δ 1.81 (d, J = 16.0 Hz, 3H), 2.41 (s, 1H), 4.59 (d, J = 6.8 Hz, 2H), 5.49 (tt, J = 6.8, 1.4 Hz, 1H), 6.15 (s, 0H), 6.84 (d, J = 2.5 Hz, 1H), 6.88 (dd, J = 8.8, 2.5 Hz, 1H), 7.51 (d, J = 8.8 Hz, 1H). ^{13}C NMR (101 MHz, CDCl_3) δ 161.91, 161.41, 155.23, 152.63, 139.28, 125.47, 118.66, 113.46, 112.91, 111.83, 101.54, 65.37, 25.85, 18.70, 18.31. MS (ESI) m/z : Calcd for $\text{C}_{15}\text{H}_{16}\text{O}_3$ ($\text{M}+\text{H}$) $^+$ 244.109960, found 244.110525

4-Methyl-7-(3-methylbutyloxy)coumarin (MU2). White crystals. 99% yield. ^1H NMR (400 MHz, CDCl_3) δ 1.00 (d, J = 6.6 Hz, 5H), 1.73 (q, J = 6.7 Hz, 2H), 1.87 (dt, J = 13.4, 6.7 Hz, 1H), 2.42 (s, 2H), 4.07 (t, J = 6.6 Hz, 2H), 6.15 (s, 0H), 6.83 (d, J = 2.4 Hz, 1H), 6.87 (dd, J = 8.7, 2.5 Hz, 1H), 7.51 (d, J = 8.8 Hz, 1H). ^{13}C NMR (101 MHz, CDCl_3) δ 162.21, 161.42, 155.30, 152.61, 125.46, 113.40, 112.71, 111.81, 101.31, 67.00, 37.63, 25.01, 22.55, 18.70. MS (ESI) m/z : Calcd for $\text{C}_{15}\text{H}_{18}\text{O}_3$ ($\text{M}+\text{H}$) $^+$ 246.125594, found 246.125925

4-Methyl-7-(pentyloxy)coumarin (MU3). White fluffy crystals. 99% yield. MP 135 – 137 °C ^1H NMR (600 MHz, CD_3OD) δ 0.87 (3H, t, J = 7.0 Hz), 1.20-1.45 (4H, 1.38 (tt, J = 7.5, 7.0 Hz), 1.29 (h, J = 7.0 Hz)), 1.82 (2H, quint, J = 7.4 Hz), 2.42 (3H, s), 4.25 (2H, t, J = 7.4 Hz), 5.86 (1H, s), 6.54 (1H, dd, J = 8.3, 1.5 Hz), 6.67 (1H, dd, J = 1.5, 0.4 Hz), 8.09 (1H, dd, J = 8.3, 0.4 Hz). ^{13}C NMR (101 MHz, CD_3OD) δ 28.3, 108.4, 68.2, 151.4, 19.1, 152.5, 128.2, 14.0, 114.6, 161.0, 22.4, 161.9, 28.7, 99.6, 111.4. MS (ESI) m/z : Calcd for $\text{C}_{15}\text{H}_{18}\text{O}_3$ ($\text{M}+\text{H}$) $^+$ 246.125594, found 246

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7-(Cyclohexyloxy)-4-methylcoumarin (MU4). White crystals. 99% yield. ^1H NMR (600 MHz, CD_3OD) δ 1.32-1.74 (10H, 1.65 (dtd, $J = 13.2, 10.3, 2.8$ Hz), 1.46 (dtt, $J = 12.7, 10.3, 2.8$ Hz), 1.39 (dq, $J = 12.7, 2.8$ Hz), 1.55 (dq, $J = 12.7, 2.8$ Hz), 1.51 (dtt, $J = 12.7, 10.3, 2.8$ Hz), 1.65 (dq, $J = 13.2, 2.8$ Hz)), 2.42 (3H, s), 4.80 (1H, tt, $J = 10.3, 2.8$ Hz), 5.86 (1H, s), 6.55 (1H, dd, $J = 8.4, 1.5$ Hz), 6.67 (1H, dd, $J = 1.5, 0.4$ Hz), 8.09 (1H, dd, $J = 8.4, 0.4$ Hz). ^{13}C NMR (101 MHz, CD_3OD) δ 23.4, 23.4, 111.4, 161.0, 99.6, 19.1, 72.5, 114.6, 152.5, 151.4, 128.2, 162.5, 31.6, 31.6, 108.4. MS (ESI) m/z : Calcd for $\text{C}_{16}\text{H}_{18}\text{O}_3$ ($\text{M}+\text{H}$) $^+$ 258.125594, found 260

7-(2-ethylbutyloxy)-4-methylcoumarin (MU5). Pale white semicrystal. 99% yield. MP 86°C. ^1H NMR (400 MHz, CDCl_3) δ 0.94 (t, $J = 7.5$ Hz, 6H), 1.42 – 1.37 (m, 4H), 1.70 (p, $J = 6.3$ Hz, 1H), 2.39 (s, 3H), 3.90 (d, $J = 5.8$ Hz, 2H), 6.12 (s, 1H), 6.81 (d, $J = 2.4$ Hz, 1H), 6.86 (dd, $J = 8.8, 2.5$ Hz, 1H), 7.48 (d, $J = 8.8$ Hz, 1H). ^{13}C NMR (101 MHz, CDCl_3) δ 162.47, 161.44, 155.30, 152.62, 125.41, 113.35, 112.74, 111.76, 101.32, 70.69, 40.68, 23.30, 18.71, 11.12. MS (ESI) m/z : Calcd for $\text{C}_{16}\text{H}_{20}\text{O}_3$ ($\text{M}+\text{H}$) $^+$ 260.141245, found 260.141325

7-Acetoxy-4-methylcoumarin (MUA). White crystals. 99% yield. ^1H NMR (400 MHz, CDCl_3) δ 2.27 (s, 3H), 2.37 (s, 3H), 6.20 (s, 1H), 7.01 (dd, $J = 8.6, 2.3$ Hz, 1H), 7.05 (d, $J = 2.2$ Hz, 1H), 7.54 (d, $J = 8.6$ Hz, 1H). ^{13}C NMR (101 MHz, CDCl_3) δ 168.77, 160.51, 154.15, 153.03, 151.94, 125.40, 118.10, 114.51, 110.47, 25.36, 21.13, 18.74. MS (ESI) m/z : Calcd for $\text{C}_{12}\text{H}_{10}\text{O}_4$ ($\text{M}+\text{H}$) $^+$ 218.057909, found 218.058325

* The ^1H and ^{13}C NMR spectra as well as the MS data can be found in the appendix (3, 4, 5 and 6) for U2, MU2, U5, and MU5, all the data for the derivatives have been uploaded in a data repository, links are available in the appendix section.

3.3.2. Antimycobacterial Activity - Planktonic Cell Suspension

The microtitre alamar blue assay (Franzblau et al., 1998) with the presto blue modifications (Lall et al., 2013) was used to assess the antimycobacterial activity of the derivatives on *M. smegmatis* mc² 155 and *M. tuberculosis* H37Rv. Briefly, the derivatives were tested at eight concentrations ranging from 50 to 4000 μM (7.5 - 1000 $\mu\text{g/mL}$) in 7H9 broth supplemented with 2% glycerol 0.2% Tween 80.

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Ciprofloxacin tested at a concentration range of 0.02 to 30 μM was used as the positive control. A bacterial, media and solvent control (DMSO 5%) were included. One hundred microliters of a prepared inoculum of 1.5×10^6 CFU/mL of *M. smegmatis* mc² 155 culture was added to yield a final assay volume of 200 μl . The plates were sealed and incubated for 24 h. Presto blue or alamar blue at a ratio of 10 μl per 100 μl were added and incubated for another 1-2 hours. The MIC values were visually determined as the lowest concentration where no colour change could be observed. All the derivatives were tested in triplicates in at least three independent assays.

Antimycobacterial activity against *M. tuberculosis* was done as described above with the following modifications. Briefly: the outer wells of a 96-well plate contained 200 μl of sterile distilled water to compensate for evaporation. The growth media used was 7H9 broth (Sigma Aldrich), supplemented with 10% OADC and 2% PANTA. The samples were tested at six concentrations (120 – 4000 μM). Isoniazid, at test concentrations ranging from 0.5 to 18 μM was used as the positive control. A solvent (2.5% DMSO), bacteria and medium controls were added. One hundred microliters of the *M. tuberculosis* inoculum (1.5×10^6 CFU/mL) was added to all the test wells to produce a final assay volume of 200 μl . The plates were incubated at 37°C for 5-7 days. After incubation, 20 μl of presto blue was added to all the wells. The minimum inhibitory concentration (MIC) was defined as the lowest concentration where no colour change from blue to pink could be observed.

* The microtitre plates indicating the antimycobacterial activity can be found in Appendix 1.

3.3.3. Biofilm Formation Inhibition Assay

One hundred microliters of a 1.5×10^6 CFU/mL *M. smegmatis* mc² 155 inoculum was added into the wells of a polystyrene 96-well microplate (7H9 broth media supplemented with 2% glycerol and no Tween 80). Serially diluted samples (100 μl) ranging from 30 to 4000 μM (7.8 to 1000 $\mu\text{g/mL}$) were added to yield a final assay volume of 200 μl . Ciprofloxacin (0.02 - 30 μM) was used as the positive control. A solvent control, untreated bacteria and media controls were included. The plates were incubated at 37°C for 2-4 days under stationary conditions which induce biofilm formation. The lowest concentration where no visible biofilm formation could be observed was considered the minimum biofilm formation inhibitory concentration (MBFIC) (Cady et al., 2012; Ishida et al., 2011).

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The biofilm inhibition activity of the derivatives was quantitatively analysed by utilizing 0.01% crystal violet, to determine the 50% biofilm inhibition concentrations (IC₅₀). Briefly: after the visual analysis, the plates were dried in a desiccating oven to remove all the liquid media. The dried biofilms were fixed to the plates by the addition of 200 µl of methanol. The plates were washed three times with sterile distilled water to remove planktonic cells and any broth residue. The washed plates were left to air dry and 200 µl of a 0.01% crystal violet solution was added to each well as a biofilm biomass indicator. The plates were left at room temperature on an orbital shaker (170 rpm) for 15 min followed by three wash steps as mentioned above. The bound crystal violet was extracted by using 200 µl ethanol and transferred to a new microtitre plate. The amount of crystal violet was quantified by measuring the absorbance at OD600 nm using a Biotek ELISA plate reader. GraphPad Prism 4.0 software was used to calculate the IC₅₀ by means of a four-parameter logistic equation.

* The microtitre plates indicating the antibiofilm activity can be found in Appendix 2. The data underlying the IC₅₀ calculations are uploaded to the data repository, links provided in the appendix section.

3.3.4. Biofilm Morphology

Colony morphology was assessed by observing the size and shape of *M. smegmatis* mc² 155 on solid 7H11 agar discs containing a set concentration of the coumarin derivatives (Ealand et al., 2018). Briefly, fresh 7H11 media was prepared according to the manufacturer's instructions with the omission of OADC. When the media cooled to 50°C, the coumarin derivatives dissolved in DMSO, were added to 2 mL of the prepared media and plated into a sterile 24-well microtiter plate in triplicate. The plates were left for the content to solidify. The compounds were tested at two set concentrations of 100 and 1000 µM. The bacterial inoculum was prepared as described above and 10 µl of a 1.5x10⁶ CFU/mL *M. smegmatis* inoculum was added to the middle of each prepared plate on the 24-well plate, sealed with parafilm and incubated for 72 hours at 37°C. The formation of the colonies was observed and the size and shape recorded on a scale of 0 to 10, where 0 indicated no biofilm (media control) formation and 10 indicated an uninterrupted biofilm (bacterial control).

3.3.5. Intra-biofilm Activity

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Mature biofilms of the attenuated strain, *M. tuberculosis* H37Rv^{mc²7000} were cultured in 6-well polystyrene plates by inoculating 6 mL of Sautons Medium (without Tween-80) with 60 µl of planktonic cultures. The plates were wrapped in parafilm and incubated at 37°C for 5 weeks without shaking in humidified conditions. The test samples were dissolved in DMSO at appropriate concentrations and 10 µl of the sample was injected through the biofilm into the media to yield a test concentration of 300 µM (100 µg/mL). A drug-free control, solvent control, as well as a positive control (rifampicin at 364 µM (50 µg/mL)), were included in the assay. After 7 days the biofilms and cells were harvested and gently agitated at 4°C for 24 h with 0.1% Tween 80 and 1 mm glass beads. Cell viabilities were determined by plating 10 µl of serial dilutions onto fresh 7H11 agar plates followed by a colony count.

3.3.6. Hepatoprotection vs Antiproliferative Activity

The cytotoxic effects of the coumarin samples were assessed on HepG2 (ATCC HB-8065), cells, maintained in Eagle's minimum essential medium (EMEM) by using the method described previously (Lall et al., 2013). Briefly, HepG2 cells were plated in the inner wells of a 96-well plate at a cell density of 100 000 cells/mL. The samples were tested at varying concentration ranging from 3 to 1000 µM (0.78 to 200 µg/mL). A positive control, actinomycin D and solvent control, DMSO (0.04%) were included in the assay. The outer wells contained 200 µl of sterile distilled water to compensate for evaporation. After the incubation of 72 hours (37°C, 5% CO₂), 20 µl of presto blue (Thermofisher) reagent was added and incubated for a further 1-2 hours. The absorbance of the resorufin colour complex was quantified spectrophotometrically by measuring the absorbance at a primary wavelength of 570 nm with a reference wavelength of 600 nm on a microplate reader (Bio-Tek, PowerWave XS). The 50% inhibitory concentration was statistically calculated by utilizing a four parameter logistic regression. An ANOVA analysis with a Tukey post-hoc test was run for the statistical significance analysis. The IC₅₀ values were used to determine the appropriate concentration for the hepatoprotective assay.

The samples were tested for their hepatoprotective activity at ½ and ¼ IC₅₀ (**Fig 3.11**). The cells were plated as stated above and incubated overnight for the attachment of the cells. Together with 50 µl of a 20 mM acetaminophen solution, dissolved in the media, 50 µl of the prepared sample was added. A toxic inducer control (acetaminophen only), positive control (silymarin) and an untreated cell control was included and the plates were incubated for 18 hours. The viability of the cells was assessed by

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measuring the absorbance (Abs) at 570 nm (600 nm reference). Percentage hepatoprotection was calculated by the following equation:

$$\% \text{ Hepatoprotection} = \frac{(\text{Abs Sample} - \text{Abs Toxic inducer})}{(\text{Abs Untreated cells} - \text{Abs Toxic inducer})} \times 100$$

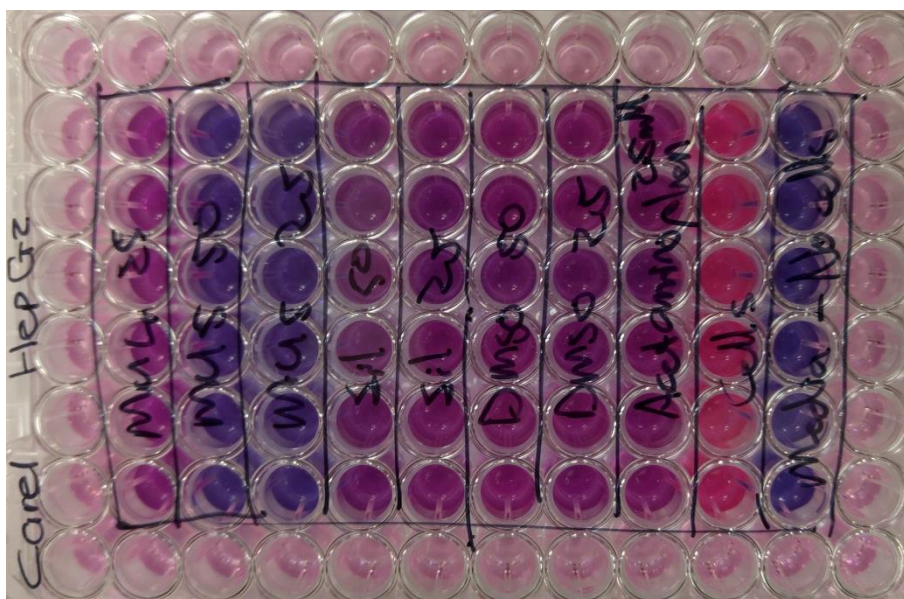


Fig 3.11: Hepatoprotective assay showing some of the CD and controls, indicating the toxicity of **MU5**.

* The data underlying the IC_{50} calculations are uploaded to the data repository, links provided in the appendix section.

3.3.7. Enzyme Inhibition Assay

The DTNB coupled mycothiol (Mtr) and glutathione reductase (Gtr) assays were carried out as described by Hamilton et al (2009) to determine the inhibitory effect (IC_{50}) of the compounds. Briefly, the assay was carried out with Gtr/Mtr at 32°C in a 96-well microtitre plate with an assay volume of 200 μ l (0.1 mM EDTA, 100 μ M Ellman's reagent (DTNB, [5,5'-dithio-bis-(2-nitrobenzoic acid)]), 50 mM Hepes (pH 7.6), 70 μ M NADPH and substrate (60 μ M)) Ten microliters of the samples were added at a concentration ranging from 3 – 1000 μ M. The enzyme, coenzyme and compounds were pre-incubated for 5 min before initiating the reaction through the addition of the substrate. Initial velocities

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were recorded by measuring the increase in absorbance at 405 nm due to the formation of TNB (5'-mercapto-2-nitrobenzoic acid). Rates were measured from the linear regression of the progress curve. The inhibition values were calculated by a 4-parameter logistic equation to determine the IC₅₀'s.

3.3.8. Pan Assay Interference assessment

Theoretical screening of the compounds for their interference was done on the PAINS-remover of false positives as well as ZINC 15 (Baell and Holloway, 2010; Sterling and Irwin, 2015). All compounds passed the filter and cannot be considered as false positives.

3.4. Conclusion

Based on the present study, the results indicate that umbelliferone and 4-methylumbelliferone derivatives have the potential to act as mycobacterial biofilm inhibitors, especially if the substitution contains free methyl groups on an alkane chain. Furthermore, the 4-methylumbelliferone derivatives showed inhibition of mycothione reductase. The toxicity assessment of the derivatives indicated that these compounds, at least from an *in vitro* point of view are relatively safe and may possibly have a protective effect in drug-induced liver toxicity. Lastly, the emphasis should be placed on the ability of these compounds towards biofilm inhibition with low to weak antimycobacterial activity.

The biological and pharmacological properties of coumarins and the mechanisms by which they exhibit their activities are and will remain a difficult process to elucidate. In the field of drug discovery and development, emphasis can be placed on the development of an “alter-biotic” with clinical relevance. An “alter-biotic” in this case is defined as a substance or chemical compound that does not confer its inhibitory effect through bactericidal or bacteriostatic activities, but rather through a different process such as targeting biofilm formation and in this manner potentially reduce the risk of development of resistance.

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“True knowledge exists in knowing that you know nothing.”

Socrates

4

4. Computational Assessment of Coumarins as Mycothione Reductase Inhibitors

** The editorial style of the Journal of Medicinal Chemistry (Impact Factor: 6.2) has been followed for this chapter.*

Abstract

Computational biology and -chemistry contain a range of tools that can be employed to study the interactions between a protein and ligand or inhibitor. The inhibitory activities of three 4-methylumbelliferone derivatives were evaluated on two enzyme systems, glutathione and mycothione reductases. The latter has been proposed as a probable and important target for tuberculosis treatment. The selected derivatives of coumarin were assessed for their fit and pose within the predicted active site through the use of molecular docking. All the selected derivatives showed successful docking with both enzymes. Molecular dynamics (MD) simulations were performed for 50 ns and the interaction analysis between the ligands and proteins were evaluated. The MD simulations revealed the importance of a rotatable bond within the structure of ligand **MU2**.

Keywords: 4-Methylumbelliferone, Coumarin, Docking, Molecular dynamics, Mycothione reductase, Tuberculosis

4.1. Introduction

The development of technology and computational methods available to study chemistry and biochemical systems have made computational biology one of the optimal tools to use in the realm of drug-discovery, drug-development and lead optimization. Computer-aided drug design (CADD) has played a key role in a number of drugs that have gone from design, through clinical trials and ultimately commercial use (Bajorath, 2015; Talele et al., 2010). Computational chemistry uses computer science to solve chemical or biochemical problems (Jensen, 2007).

Molecular docking is an *in silico* computational technique to predict binding affinities and poses that ligands or inhibitors can assume within a binding pocket or surface of a protein. Binding affinity is the attractive force between a ligand and protein and it is influenced by non-covalent interactions such as hydrogen bonds, van der Waals interactions and electrostatic interactions (Friesner et al., 2006). The two main outputs of a docking study are the poses and scoring. The pose is the orientation of a specific ligand within an active site and the scoring involves the actual fit that is calculated from energy functions. The predictive capabilities of this technique still have some drawbacks and do not always correlate with the biological activities seen in conventional wet-lab inhibitory and enzymology studies. Ligand and protein flexibility is usually underestimated (Basciu et al., 2017). There are a number of different force fields and software to perform a docking study, each of which has their own methodologies with pros and cons (Yuriev and Ramsland, 2013).

Molecular dynamics simulations (MDS) use force field calculations with a set of parameters (bond torsion angles, length and dihedral angles) to study the movement of atoms and molecules. The simulation is set up to run for a set period of time to give insight into the evolution of a protein-ligand complex through the recording of trajectories. It is roughly based on Newton's second law of motion with the equation:

$$Force = Mass \times Acceleration$$

This technique is another prediction model to study the interactions between a ligand and the residues within an active site by observing the movement of atoms.

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Coumarins are a class of small molecules with a broad range of biological activities. It has previously been established that 4-methylumbelliferone derivatives show inhibition of mycothione reductase, an important enzyme of *Mycobacterium tuberculosis* (Chapter 3). This enzyme has been identified as a probable target for drug development, not only for the treatment of active tuberculosis infection but also latent infections (Hernick, 2013; Newton et al., 2008).

This study attempted to investigate and predict the interactions and molecular mechanisms of the ligands, in order to explain their enzyme inhibitory activities, and to describe the effect of different types of substitutions or additions to the coumarin nucleus on the observed biological activity.

4.2. Results and Discussion

4.2.1. Selection of Ligands Based on the Biological Activity

In the previous chapter, it was shown that some of the coumarin derivatives exhibited inhibitory activities on mycothione (Mtr) and glutathione reductases (Gtr). Seven 4-methylumbelliferone derivatives (**MU-MUA**) were selected for molecular docking and three were further studied using MDS. Sample **MU2** was selected based on the inhibitory activity on Mtr ($IC_{50} = 29.1 \mu M$) and **MU1** was selected based on Gtr activity ($IC_{50} = 106.4 \mu M$). The base compound, sample **MU**, was included in the experiment as a “non-active” control. The 3-dimensional structures of the selected ligands together with their biological activities are depicted in (Fig 4.1).

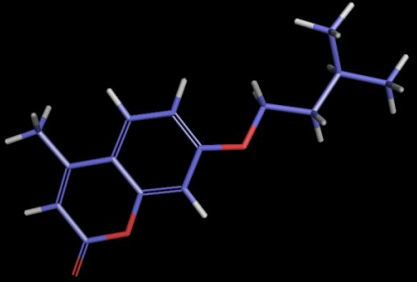
MU 4-Methyl-7-hydroxy-coumarin		MU1 4-Methyl-7-(3-methyl-2-butenyloxy)-coumarin		MU2 4-Methyl-7-(3-methylbutoxy)-coumarin	
					
Gtr IC₅₀	136.3		106.4		>1000
Mtr IC₅₀	778.2		244.2		29.07

Fig 4.1: 3-Dimensional structures of **MU**, **MU1** and **MU2** ligands with their reported enzyme inhibitory activities (μM).

4.2.2. Binding Site Prediction

The crystal structure of glutathione reductase (PDB: 3SQP) has been co-crystallized with an inhibitor, pyocyanin bound to the active site (Kasozi et al., 2011). For the effective assessment of an inhibitor, it is vital to evaluate the possible binding sites, and this is even more important with coumarin molecules due to their broad specificity and biological targets (Venugopala et al., 2013). SiteMap from the Schrodinger (2017) suite was employed to predict the possible binding pocket. The 3d mol2 files of the derivatives were additionally submitted on the SwissModel blind docking server in an attempt to identify the most favourable binding site (Grosdidier et al., 2011). Both analyses identified the same site as the ideal binding pocket for coumarin structures and incidentally the same site as the crystal-bound ligand, pyocyanin. **Table 4.1** presents the results obtained from the SiteMap analysis. Site 1 showed the largest size and volume, and this is caused by the quaternary structure of the enzyme. Both Mtr and Gtr are dimers and the two subunits intersect where site one was predicted. This site showed the highest drugability score (Dscore) and hydrophobic potential energy. The Dscore is useful for the identification and differentiation between “undruggable” and “druggable” ligands (Halgren, 2009b).

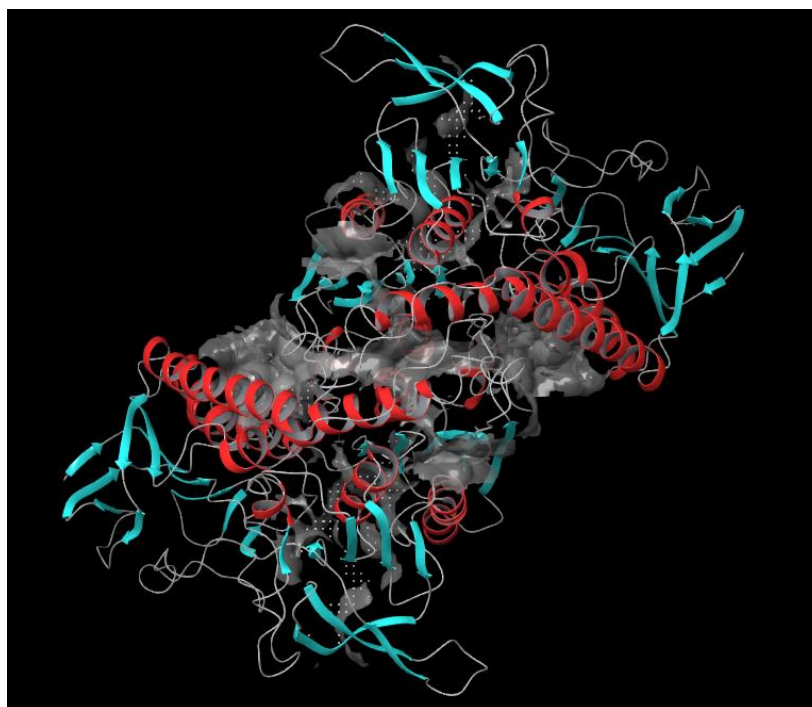


Fig 4.2: Visual representation of the five identified binding sites on glutathione reductase (PDB: 3SQP)

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Table 4.1: Analysis of possible binding sites of mycothione and glutathione reductases using Sitemap.

Site #	size	Dscore	volume	exposure	enclosure	contact	phobic	philic	balance	don/acc
Site 1	606.00	1.08	1512.63	0.48	0.80	1.05	1.14	1.03	1.11	0.85
Site 2	130.00	1.05	426.01	0.55	0.76	0.94	0.73	1.05	0.69	1.01
Site 3	134.00	0.97	406.11	0.56	0.75	0.96	0.40	1.29	0.31	1.41
Site 4	174.00	1.03	629.75	0.72	0.68	0.82	0.42	0.96	0.44	0.58
Site 5	184.00	1.03	674.34	0.74	0.64	0.77	0.34	0.92	0.37	0.80

The confirmation of site 1 as the most preferred binding site for the coumarin derivatives were selected for the subsequent assays and a receptor grid file was prepared with a 6 Å box around the centroid of the site. It is worth emphasizing that this site is still a prediction although most likely correct. For a definitive confirmation, the ligand needs to be co-crystallized with the enzyme to determine the 3D conformation. This can be included for further studies, especially on mycothione reductase, as the crystal structure for this important target has not been elucidated at the time of this study.

4.2.3. Docking Analysis and Pose Assessment

The main usage of the molecular docking study was for the determination of the binding modes, poses and fit of the coumarin derivatives within the defined binding site. All seven derivatives were successfully docked using Glide XP, with scores ranging from -6.1 to -8.81. A lower value indicates a higher approximation of binding free energies. Although some correlations are made between docking scores and biological activities, Glide and similar docking programs (GOLD, AutoDock, SwissDock) are intended for database enrichment and pose analysis and ranking rather than for prediction of binding affinities. Errors between the docking score and experimental values are common and can be quite large (4 kcal/mol). This is attributed to a number of factors including rigid receptor structures and inaccuracies in the scoring function. Fortunately, these issues are worked on continuously by other researchers and groups and will improve as time goes on.

In addition to the Glide docking score, a Glide Emodel score was also calculated (last column in **Table 4.2**). The Emodel incorporates the contributions of the electrostatic and van der Waals interaction to a greater extent, which proves to be more relevant when testing conformers such as the coumarin derivatives (Halgren et al., 2004). From the table, the glide Emodel seems to correlate better with the biological activities observed in the inhibition assays. The reference ligand had the lowest score, which

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is expected and indicates a successful docking. This was followed by **MU2**, **MU3** and **MU1** respectively. These values correlate to some extent with the biological activities, but not fully. This observation may have countless explanations. As mentioned above, it could be due to the computational functions or underestimation of the rigidity of the binding site residues. What is noteworthy about the result is a successful docking and pose analysis, indicating a positive binding.

Table 4.2: Docking scores and contributing factors of 4-methylumbelliferone (**MU**) ligands docked in active site 1 of glutathione reductase. Ref is the co-crystallized ligand; pyocyanin. Conditional formatting was employed to visually indicate the values.

Ligand*	Glide XP score	Rotatable bonds	Glide evdw ^a	Glide ecoul ^b	Glide energy	Glide Emodel
MU4	-8.81	2	-31.08	-2.28	-33.36	-41.75
MU3	-8.36	5	-27.26	-3.82	-31.08	-46.60
MU5	-8.01	4	-28.84	-2.97	-31.81	-43.61
MUA	-7.81	2	-27.58	-4.34	-31.92	-44.65
MU1	-7.63	3	-32.97	-1.11	-34.08	-46.16
Ref	-7.52	0	-34.86	-1.51	-36.36	-52.06
MU2	-7.17	4	-33.18	-0.82	-33.99	-48.22
MU	-6.86	1	-27.74	-6.52	-34.26	-45.19

* The structure files (SMILES) for the compounds can be found under the appendix, ^a Van der Waals energy, ^b coulomb energy.

In the case of mycothione reductase, the docking scores related very well to the biological activity with a good correlation between the Glide XP score and the Emodel (**Table 4.3**). All the derivatives showed better predictive binding free energies when compared to the reference ligand. Discrepancies between docking score and biological activity can be expected to a greater extent with Mtr due to the variations of the homology model to the un-elucidated crystal structure.

It is interesting to observe the changes in docking scores of sample **MU2**, where it showed the second to worst score on Gtr but the second to best score on Mtr. This correlates with the enzyme inhibition values that exhibited the same trend. The question then arises as to why **MU1** showed the best docking score on Mtr. This can be explained by the similarities in the structure in combination with the ligand rigidity during the docking study. The only structural difference between **MU1** and **MU2** is a double bond of the 3-methylbut-2-en-1-yloxy moiety of **MU1** vs. the single bond in 3-methylbutyloxy unit of **MU2**. The rotation around this single bond can have a tremendous effect on the activity which is not examined to

Chapter 4 Computational Assessment

its full extent with only a docking study. The use of a time-dependent molecular dynamics simulation might provide a better explanation for the biological activities of these two ligands and will be evaluated in the following section.

Table 4.3: Docking scores and contributing factors of 4-methylumbelliferone (**MU**) ligands docked in active site 1 of mycothione reductase homology model. Ref is the co-crystallized ligand; pyocyanin. Conditional formatting was employed to visually indicate the values.

Ligand*	Docking score	Rotatable bonds	Glide evdw ^a	Glide Ecoul ^b	Glide energy	Glide emodel
MU1	-7.26	3	-33.21	-2.69	-35.90	-47.08
MU2	-6.25	4	-32.68	-2.43	-35.11	-47.25
MU5	-6.09	4	-33.80	-2.47	-36.27	-46.76
MU4	-5.98	2	-31.80	-1.65	-33.44	-43.72
MU	-5.71	1	-25.76	-6.58	-32.33	-40.76
MUA	-5.64	2	-30.89	-2.19	-33.08	-43.21
MU3	-5.33	5	-31.58	-2.70	-34.28	-45.03
Ref	-4.95	0	-21.53	-7.20	-28.73	-33.61

* The structure files (SMILES) for the compounds can be found under the appendix, ^a Van der Waals energy, ^b coulomb energy.

Figures 4.3 and 4.4 illustrates the binding poses of **MU1** and **MU2** in the active site of mycothione reductase. It indicates the importance of the residue GLN 415 for the hydrogen bonding for both ligands and TYR 414 for the π - π stacking interaction between the residue and ligand **MU2**

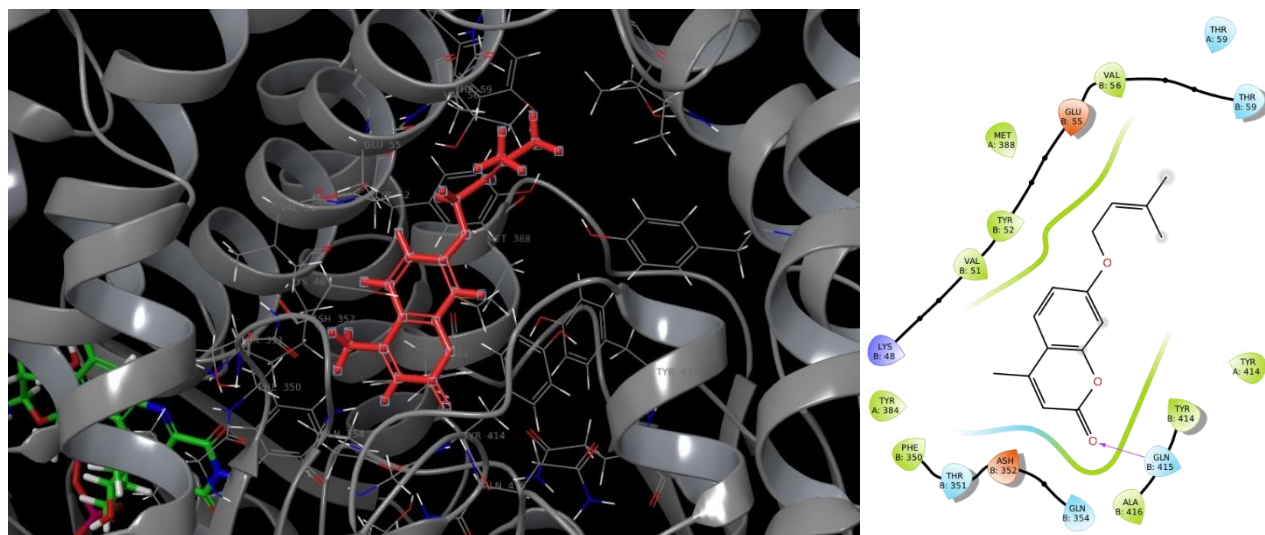


Fig 4.3: Ligand **MU1** in the binding pocket (site 1) of mycothione reductase (left), the two-dimensional ligand interaction diagram indicating the pose as well as the different interactions (right).

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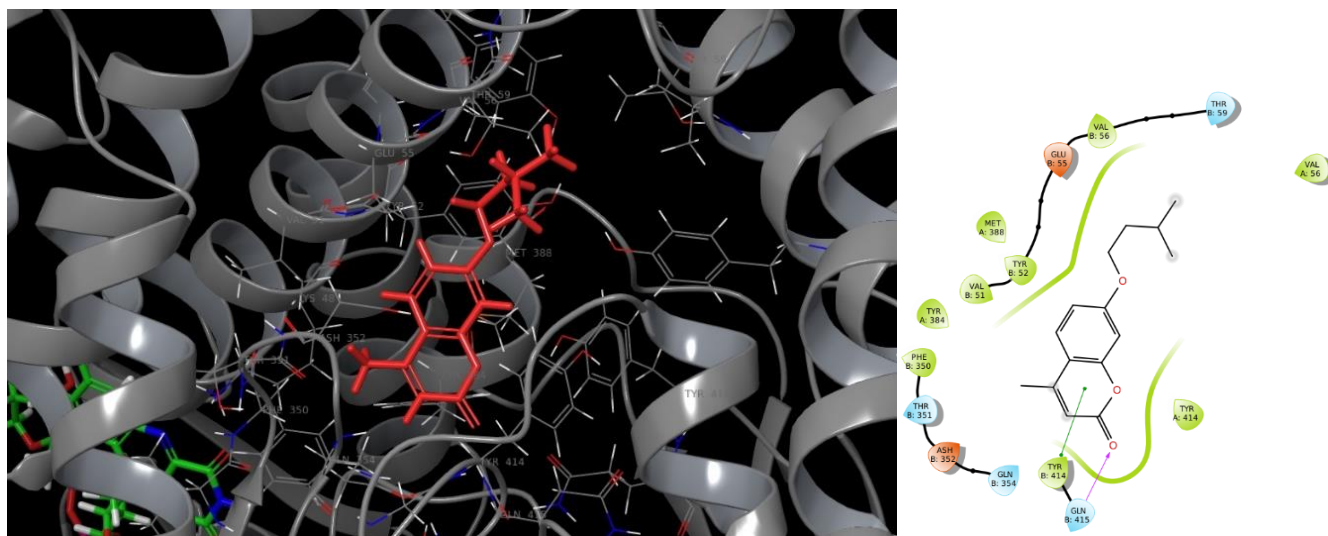


Fig 4.4: Ligand **MU2** in the binding pocket (site 1) of mycothione reductase (left), the two-dimensional ligand interaction diagram indicating the pose as well as the different interactions (right).

4.2.4. Molecular Dynamics and Ligand Interaction Analysis

The root mean square deviation (RMSD) was calculated for each simulation (three ligands on both enzymes) for the whole duration and depicted for each enzyme in **Fig 4.5** (Gtr) and **Fig 4.6** (Mtr). Frame one of the trajectory ($t = 0$) was selected as the reference point. The RMSD gives insight into the conformational changes of the enzyme through the duration of the simulation. Values between 1 Å and 3 Å are considered to be within normal range. The RMSD of Gtr and all three ligands stabilized at an early stage. There were no noticeable structural changes in the 50 ns of the production run and the RMSD averaged out at about 1.3 Å. Although not a significant deviation, the simulation between Gtr and ligand **MU1** showed, on average, higher values of deviation indicating a slightly more advanced conformational change when compared to the base compound/ligand **MU**. The simulation that showed the lowest deviation and subsequently the least conformational changes were the Gtr-**MU2** complex.

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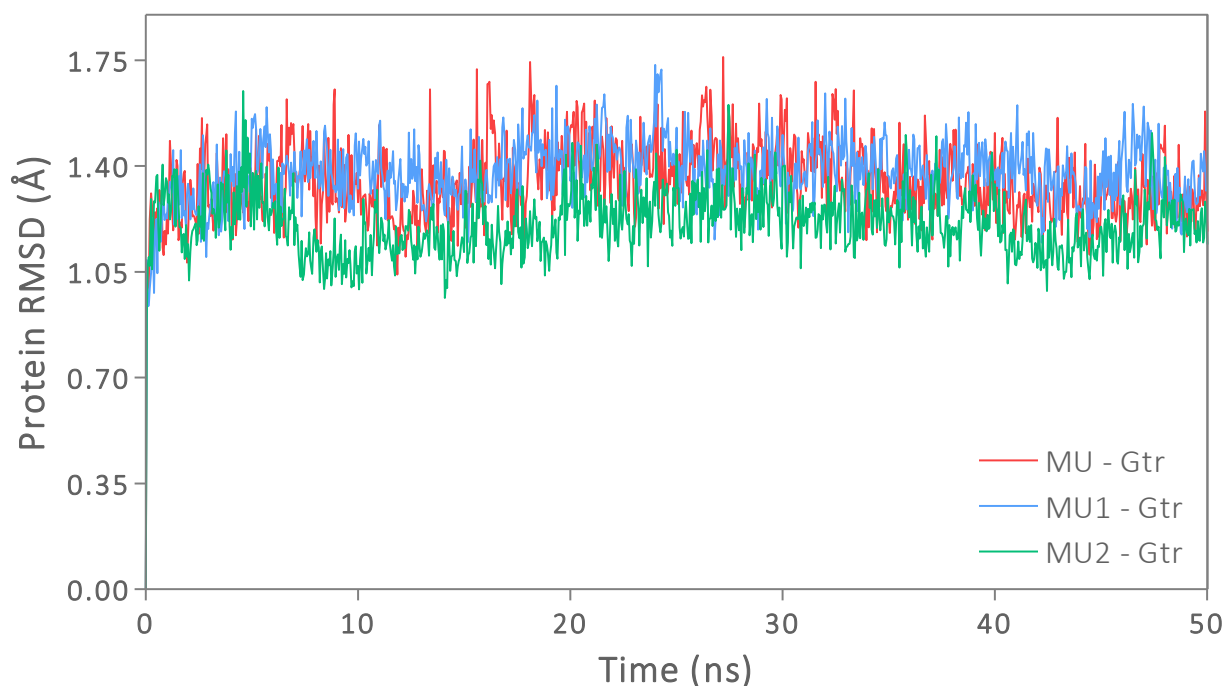


Fig 4.5: Root mean square deviation (RMSD) of glutathione reductase complexed with 4-methylumbelliferone derivatives (**MU**, **MU1**, **MU2**) over a simulation period of 50 ns.

The deviations on the simulations of Mtr fluctuated considerably more, averaging at an RMSD of 3 Å, and were much greater than the RMSD's seen with the Gtr simulations. This can either be explained by an increased affinity or more likely, due to the homology model. By using a homology model, one is expected to observe greater deviation during the simulations due to the fact that the model is a predictive model, and not based on the exact coordinates of a crystal structure.

By investigating the trends of the deviation between all three complexes an interesting observation can be made on the conformational changes that the enzyme undergoes. From 30 ns onwards the fluctuations for **MU** (red) and **MU2** (green) are increasing marginally while the RMSD for **MU1** (blue) stays relatively constant. By comparing the total number of contacts from **Fig 4.10**, it is evident that for **MU**, the number of contacts is decreasing in the same time span (30 – 50 ns). This can potentially indicate a diffusion of the ligand away from the binding site, correlating with the experimental data, where **MU** did not show inhibition of Mtr. The MU simulations were run as the “non-active” ligand and this observation supports to some degree the hypothesis.

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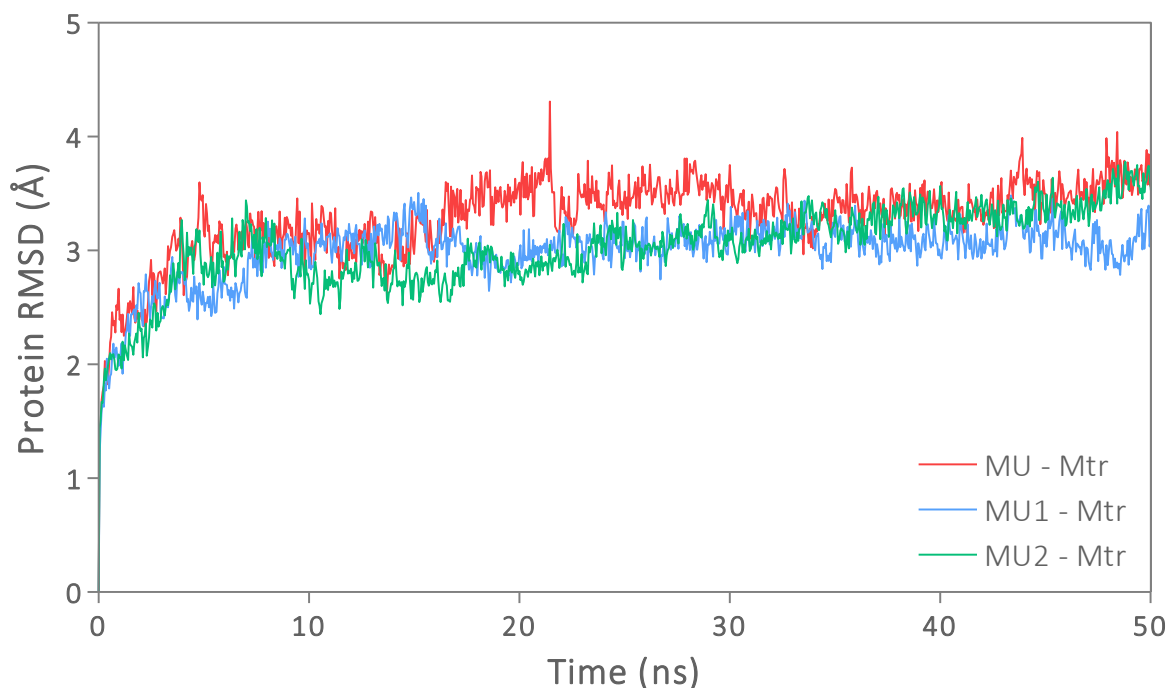


Fig 4.6: Root mean square deviation (RMSD) of mycothione reductase complexed with 4-methylumbelliferone derivatives (**MU**, **MU1**, **MU2**) over a simulation period of 50 ns.

The explanation used for the fluctuation of Mtr-**MU** complex cannot be used for the Mtr-**MU2** complex, which showed a similar trend in the fluctuation of the enzyme. In this case, the number of total contacts has not decreased. The integration diagram from **Fig 4.11** shows a constant interaction throughout the simulation with residue GLN 415 of chain B. The interactions with other residues such as VAL 51 (chain B) and TYR 414 (chain A) are shown to increase during the later stages of the simulation. Ligand **MU2**, in relation to the other two ligands, showed a higher number of interactions with residues from chain A.

Figures **4.7** and **4.8** represent the root mean square fluctuation against each residue or part of the enzyme (**MU2**-Gtr/Mtr complexes). The graph is divided into three sections based on the secondary structures of the enzyme; β -strands, α -helices and loops. The highest fluctuations were seen on the loop regions of the enzyme, this is expected, as these residues are not in a conformed secondary structure. The green lines indicate the residues that showed interactions or contacts with the ligand and it can be concluded that most of the interactions formed contacts with residues that form part of the α -helices.

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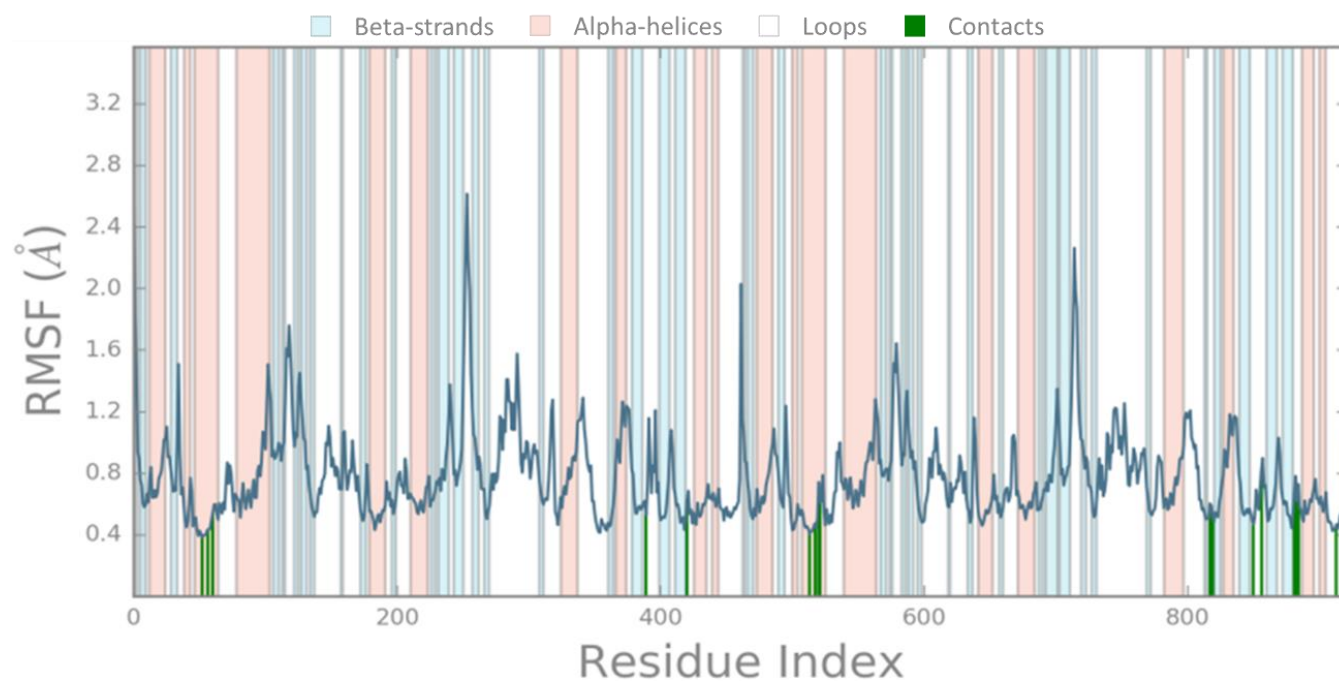


Fig 4.7: Root mean squared fluctuation of the α -carbons of glutathione reductase in complex with sample **MU2**.

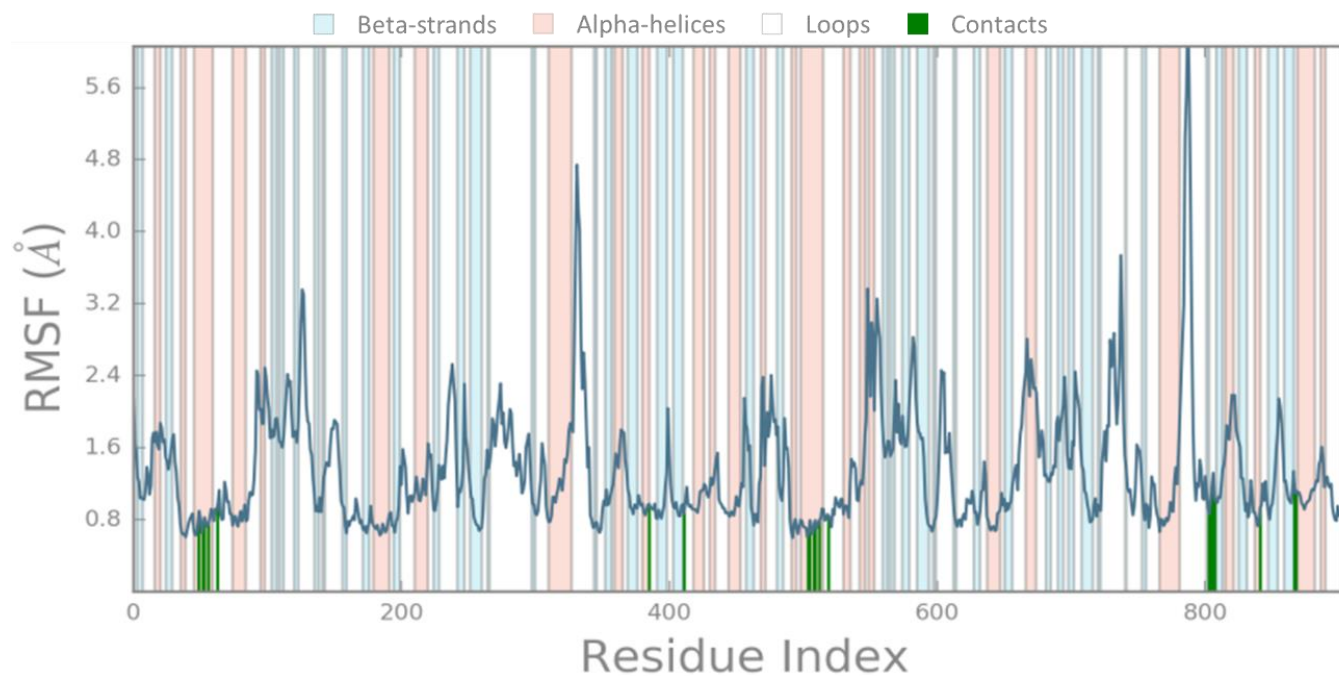


Fig 4.8: Root mean squared fluctuation of the α -carbons of mycothione reductase in complex with sample **MU2**.

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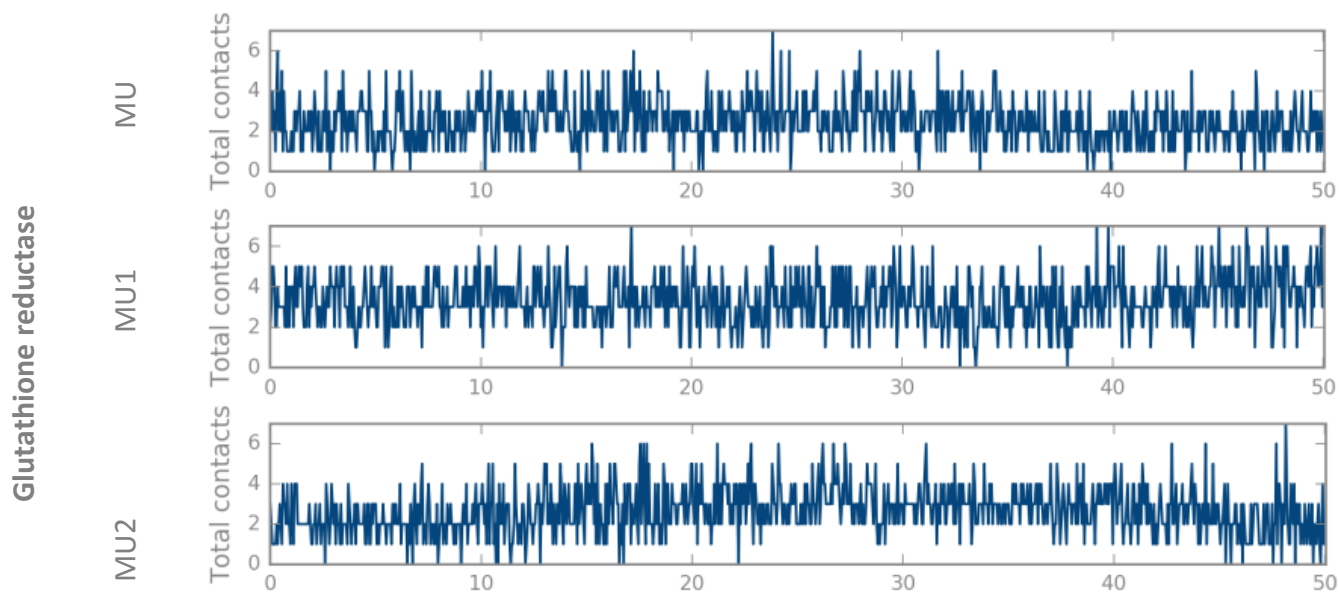


Fig 4.9: Number of contacts (H-bonds, Hydrophobic, Ionic, Water bridges) between the ligands and glutathione reductase

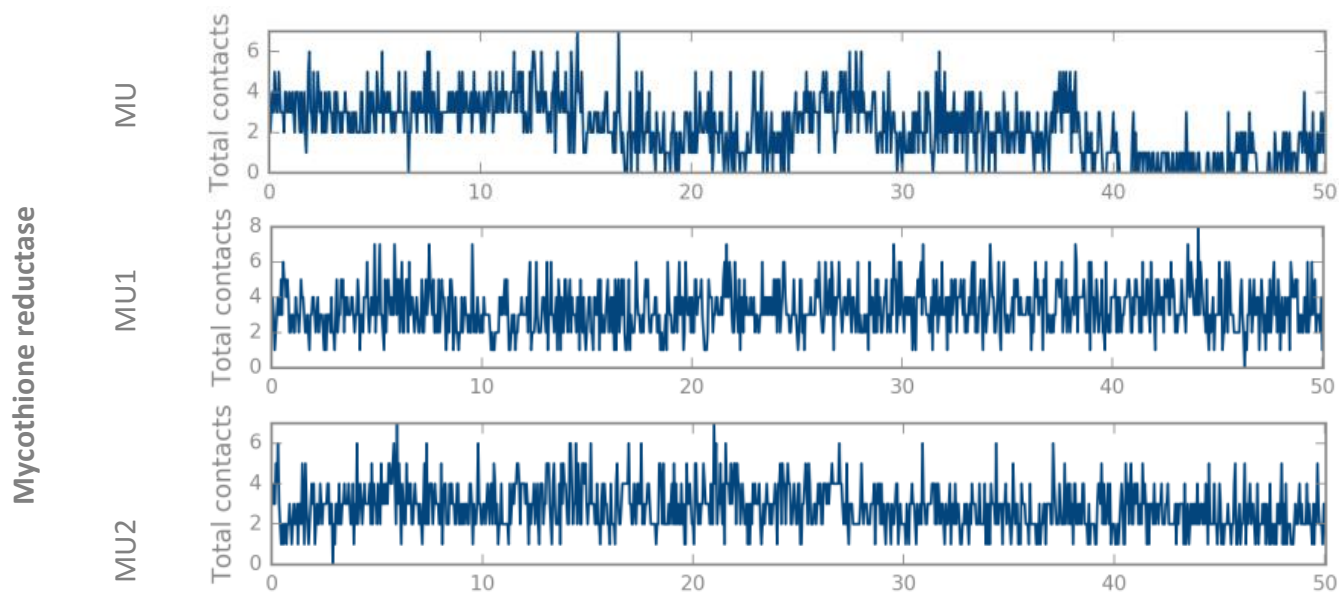


Fig 4.10: Number of contacts (H-bonds, Hydrophobic, Ionic, Water bridges) between the ligands and mycothione reductase

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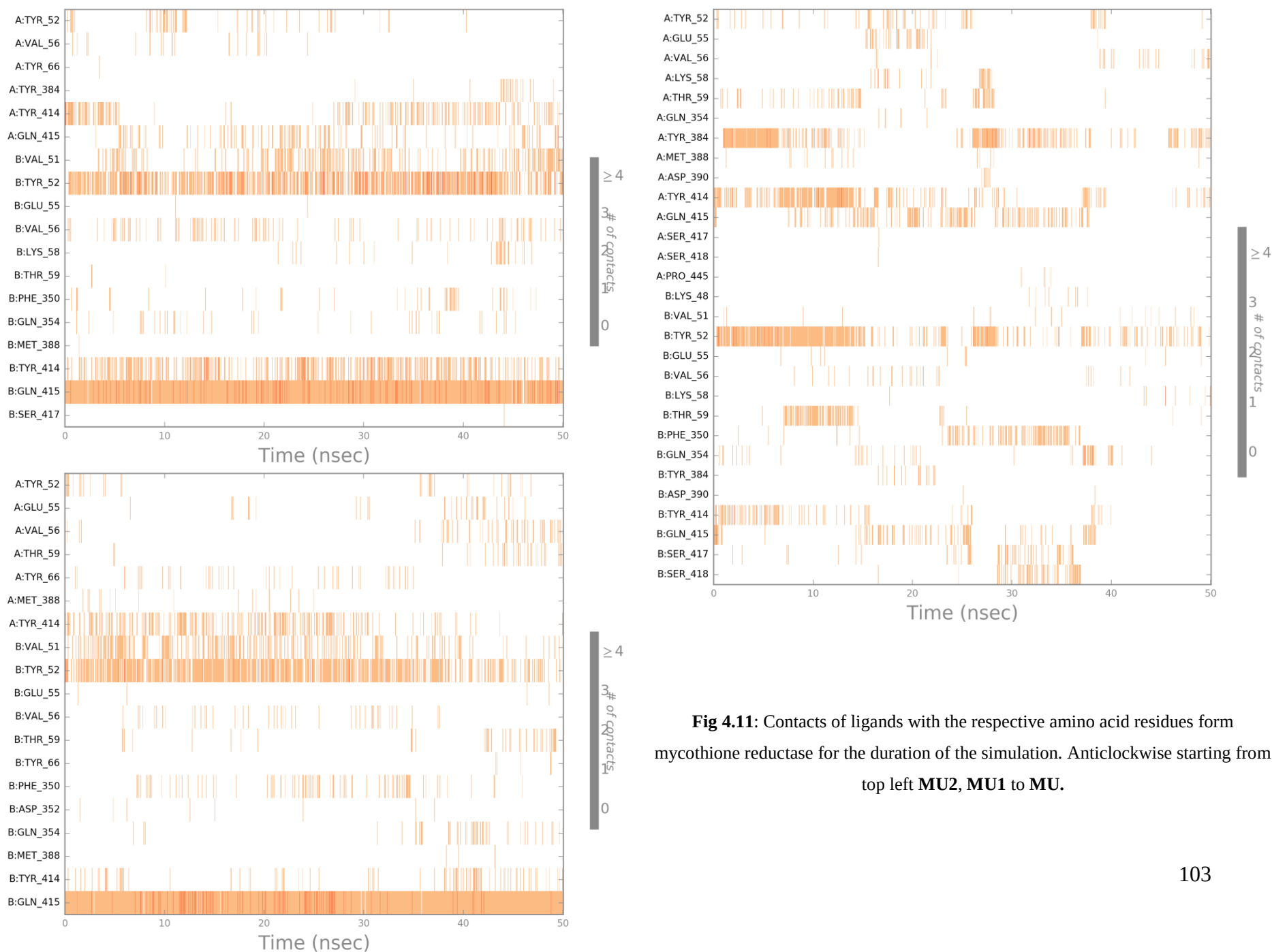


Fig 4.11: Contacts of ligands with the respective amino acid residues form mycothione reductase for the duration of the simulation. Anticlockwise starting from top left MU2, MU1 to MU.

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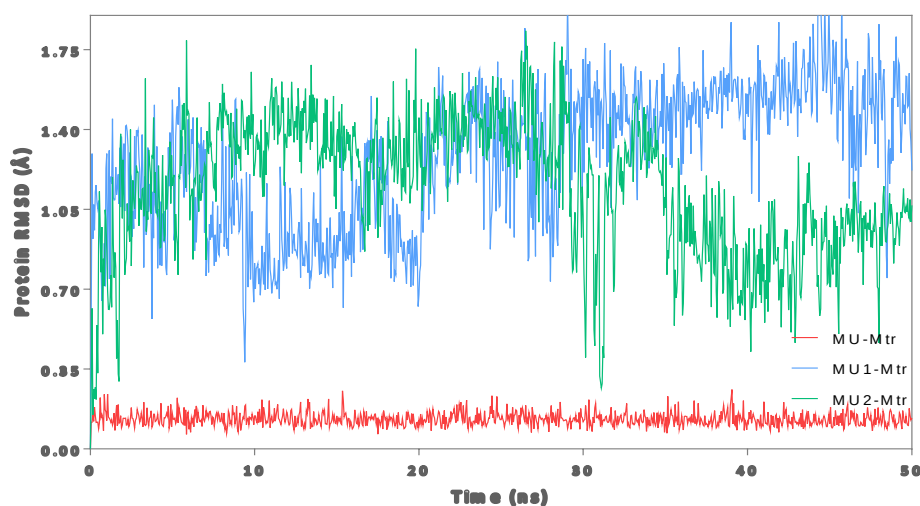


Fig 4.12: Root mean square deviation (RMSD) of 4-methylumbelliferone derivatives (**MU**, **MU1**, **MU2**) with reference to the ligand initial position over a simulation period of 50 ns.

The root mean square deviation of the ligands during the simulations are depicted in **Fig 4.12**. This indicates the changes that the ligand undergoes. It is evident that **MU** showed no conformational changes during the course of the 50 ns simulation. Ligands **MU1** and **MU2** showed much greater deviations, which are indicative of the interactions between the ligand and active site residues.

The ligand torsion plots summarized in **Fig 4.13** and **Fig 4.14** indicates the conformational evolution of every rotatable bond within each ligand. The rotatable bonds are colour-coded to the corresponding radial plots (left) and bar plots (right). The radial plots indicate the position of the torsion angles throughout the whole simulation. The centre point of the radial plot represents the beginning of the simulation and the time evolution radiates outwards. The bar plots indicate the probability density of the torsion angles summarized from the radial plots.

Within the complex between Gtr-**MU2**, the last rotatable bond had the probability to have two torsion angles, at 80° and 180°. When comparing this to the same bond in the Mtr-**MU2** complex, the simulation indicated a third torsion angle at -80° which was not observed in the Gtr simulation. This observation, together with the increased interaction between the residues of chain A, can partly explain the increase in activity and selectivity towards Mtr.

* The raw data, full analysis with all the graphs of the ligand interaction analysis can be found under in the data repository, links are available in the appendix section.

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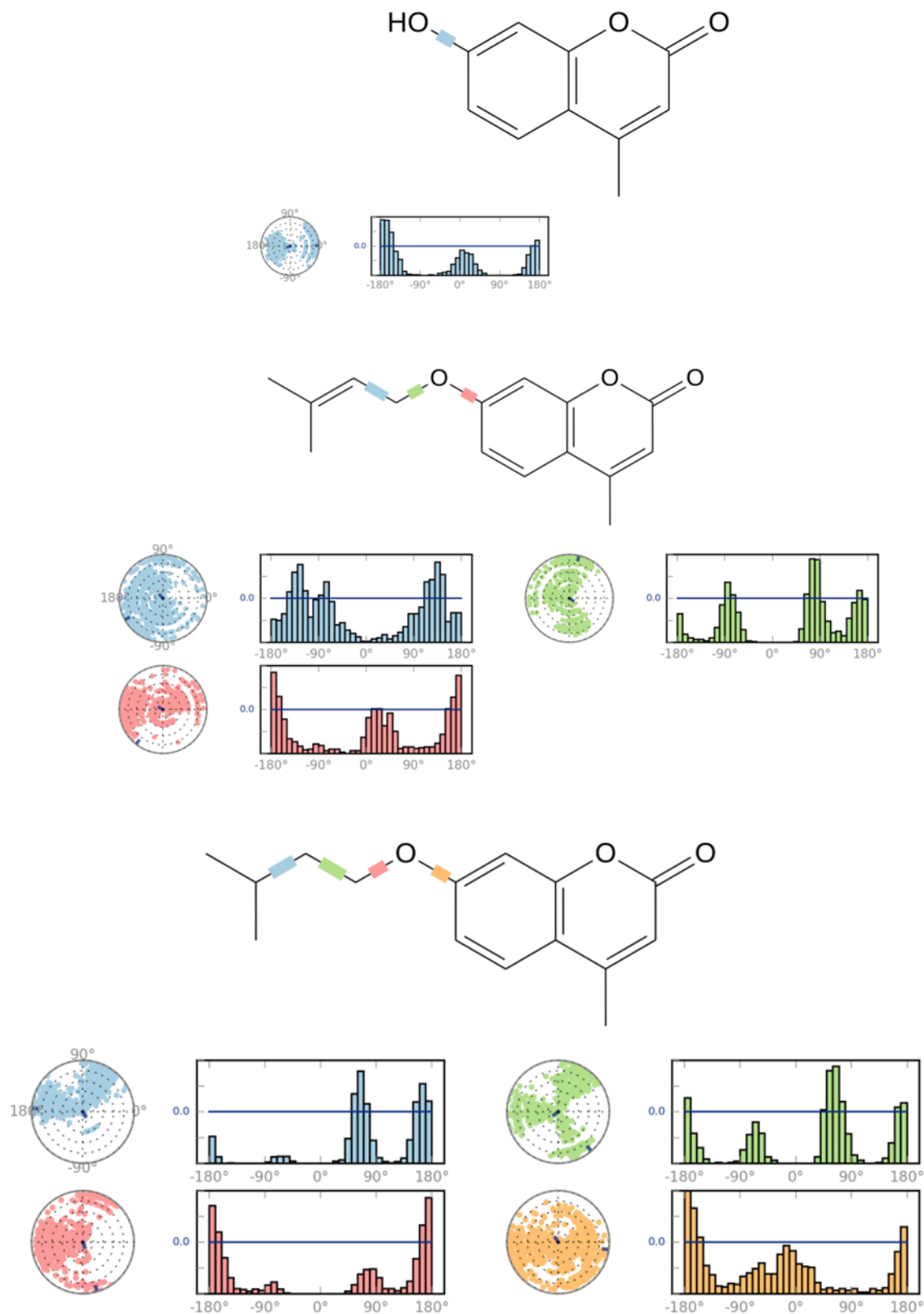


Fig 4.13: Ligand torsion profiles of MU, MU1 and MU2 on glutathione reductase.

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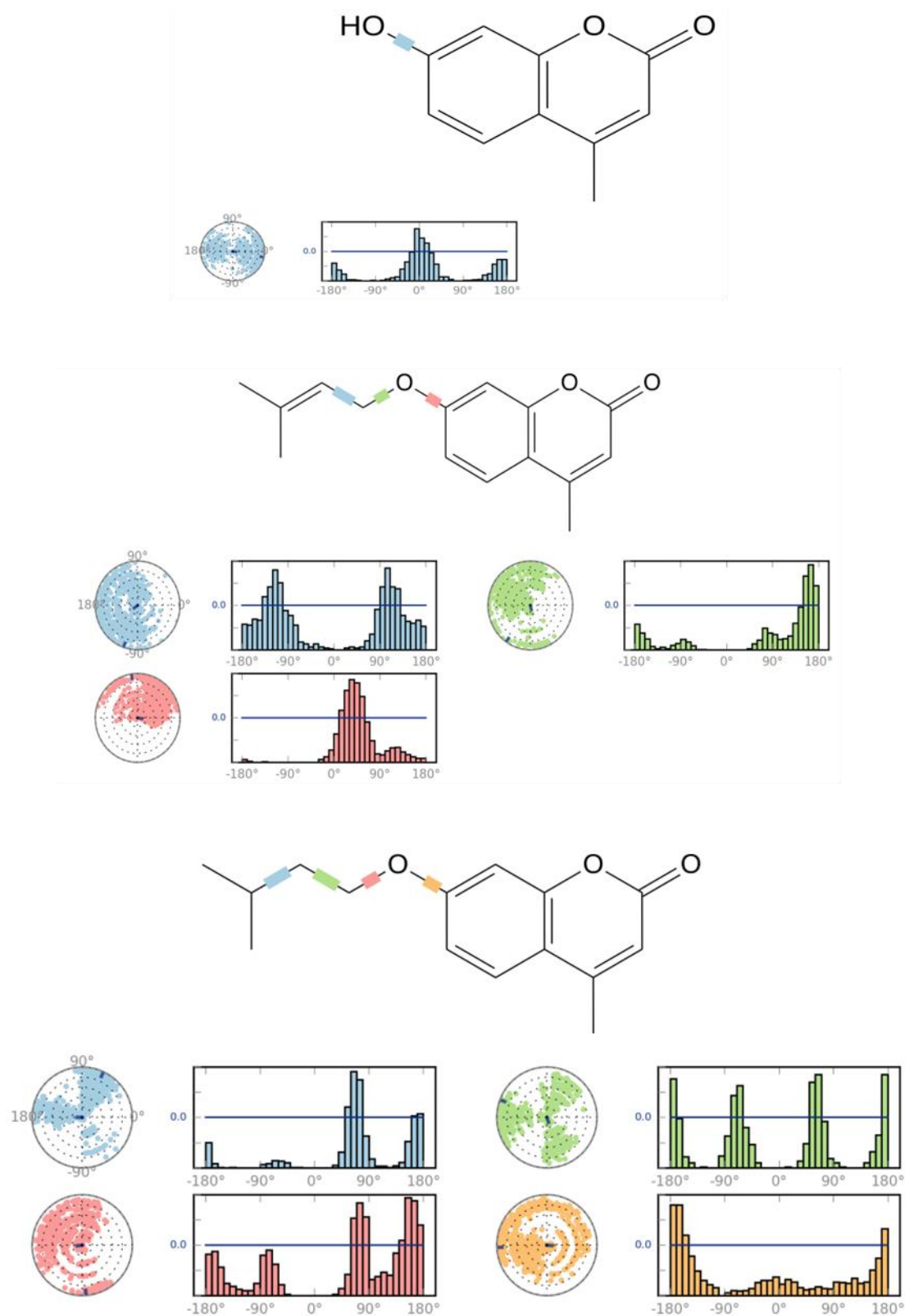


Fig 4.14: Ligand torsion profiles of MU, MU1 and MU2 on mycothione reductase.

4.3. Experimental Section

4.3.1. Structure selection and protein preparation

For the computational analysis of glutathione reductase a crystal structure of the enzyme was selected (PDB ID: 3SQP). The enzyme is co-crystallized with pyocyanin in the active site and two flavin-adenine dinucleotides (FAD). The complex was pre-processed using the protein preparation wizard for the assignment of bond orders, addition of hydrogens and completion of missing side chains and loops, if necessary. The hydrogen bonds were optimized using PROPKA at a pH of 7.0 followed by the minimization of the complex using the OPLS3 force field (Harder et al., 2016). For the assessment of other possible binding sites, the co-crystal structure of pyocyanin was removed.

* The SMILES strings for the derivatives have been uploaded to the data repository, links provided in the appendix section.

4.3.2. SiteMap

For the identification and validation of possible target sites within the crystal structure of glutathione reductase and the homology model of mycothione reductase, SiteMap (Schrödinger, LLC, New York, NY, 2018) was utilized (Halgren, 2007, 2009a). SiteMap uses algorithms to predict binding sites based on size, functionality, and extent of solvent exposure. The program was run with the default settings selected and to identify the five highest scoring binding pockets.

4.3.3. Molecular Docking - Glide

The compounds were prepared for molecular docking on mycothiol and glutathione reductases by using LigPrep. The three selected coumarin ligands (**MU**, **MU1** and **MU2**) were drawn and Epik was used to generate possible states at a pH of 7.0 and the OPLS3 force field was used for the minimization. Docking analysis was performed using Glide. Receptor grid files were prepared for each identified site using the receptor grid generation tool at a 6Å region around the centre. Docking of the prepared 3D structures of the ligands and reference compound, pyocyanin were performed using the XP function.

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Furthermore, all molecules were evaluated for possible molecular interactions with active site residues. The protein-ligand complexes, interaction analysis and their amino acid positions with bond distances were calculated and examined. The highest scoring poses were selected and transferred to the coordinate files of glutathione and mycothione reductases for the molecular dynamics simulation.

* The full docking results for the derivatives have been uploaded to the data repository, links provided in the appendix section.

4.3.4. Molecular Dynamic Simulation Setup

The docked complexes of three coumarin derivatives were selected for molecular dynamic simulations (MDS) on mycothiol and glutathione reductase. The same protein files were used as generated in the docking study. Desmond from Schrodinger was used to run the production of the MDS. Briefly, the complexes were pre-processed using the protein preparation wizard for assignment of bond orders and the addition of hydrogens. The hydrogen bonds were optimized using PROPKA at a pH of 7.0 followed by the minimization of the complex using the OPLS3 force field (Harder et al., 2016). System builder was used to solvate the model with the predefined TIP3P parameters in an orthorhombic periodic boundary box with 10 Å buffering distance. Sodium or chloride ions were added to neutralize the system and placed at a random distance at least 10 Å away from the ligand. A relevant salt concentration of 0.15 M of Na⁺/Cl⁻ were added to mimic a biological system. The simulation was set to run for 50 ns with a recording interval of 50 ps. The NPT ensemble class was selected with a constant temperature and pressure of 300 K and 1.01 bar respectively. The simulation was run using GPU acceleration on a workstation equipped with a Tesla K40c GPU with the following stages; stage 1 - task. Stage 2 - simulate, Brownian Dynamics NVT, T = 10 K, small time steps, and restraints on solute heavy atoms, 100ps. Stage 3 - simulate, NVT, T = 10 K, small time steps, and restraints on solute heavy atoms, 12ps. Stage 4 - simulate, NPT, T = 10 K, and restraints on solute heavy atoms, 12ps. Stage 5 - solvate pocket. Stage 6 - simulate, NPT and restraints on solute heavy atoms, 12ps. Stage 7 - simulate, NPT and no restraints, 24ps. Stage 8 - simulate.

4.3.5. Simulation Analysis

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Simulation quality analysis was used to calculate the total energy of the system in kcal/mol. The simulation interaction diagram panel was used to calculate the protein-ligand root mean square deviation (RMSD) to measure the distance or displacement of a selection of atoms throughout the simulation based on the reference structure ($t = 0$). The RMSD of the protein was calculated using the following equation:

$$RMSD = \sqrt{\frac{1}{N} \sum_{i=1}^N (r'_i(t_x)) - r_i(t_{ref}))^2}$$

The root mean square fluctuation (RMSF) was calculated to analyse the changes in the protein chain using the following equation:

$$RMSF = \sqrt{\frac{1}{T} \sum_{T=1}^T < (r'_i(t)) - r_i(t_{ref}))^2 >}$$

Protein-ligand contacts, including hydrogen and ionic bonds, hydrophobic interactions and water bridges were analysed together with the ligand torsion profiles, to establish the conformational changes the rotatable bonds, within the ligand, undergoes throughout the course of the simulation.

* The ligand interaction analysis data for the simulations have been uploaded to the data repository, links provided in the appendix section.

4.4. Conclusion

The use of the molecular docking in this instance gave some indication to the possible binding of a ligand to the enzyme to form a complex. Molecular dynamics simulations revealed the interactions in much greater detail and were able to differentiate the actives from the non-actives as examined experimentally. Specific coumarin derivatives can, therefore, be considered as mycothione reductase

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inhibitors, but the activity is dependent on the type and position of the substitution. This remains a predictive model and experimental validation is required to confirm the observations.

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“It is the mark of an educated mind to be able to entertain a thought without accepting it.”

Aristotle

5

5. Concluding Remarks, Novel Aspects and Future Work

5.1. Concluding Remarks

- Coumarins exhibit a broad range of activities and it has been proven that they act as inhibitors of mycobacterial biofilm formation. The derivatives were moderately active against *M. smegmatis* but had selectivity towards the inhibition of biofilm formation. During the *M. tuberculosis* assays, it was found to be more active and the derivatives were able to reduce the bacterial load within a mature biofilm. This is the first report of **umbelliferone** and **4-methylumbelliferone** derivatives as mycobacterial biofilm inhibitors.
- The *in vitro* cellular toxicity and antiproliferative activities of these derivatives were found to be low. With the lowest activity being that of **U2** with an IC_{50} value of $200 \pm 27 \mu M$ on HepG2 cells. The coumarins did show a hepatoprotective effect on the toxicity induced by acetaminophen on hepatocytes, and caution should be taken with the use of **7-acetoxy-4-methylcoumarin** which showed an antagonistic effect.
- Mycothione reductase inhibition assays revealed some insight into the possible mode of action with the best biofilm inhibitor showing the highest enzyme inhibitory properties. This is the first

Chapter 5 Conclusion and Final Remarks

report of coumarins acting as Mtr inhibitors. More experimentation should be done on the enzyme kinetics to improve the structure-activity relationship.

- This study identified the most potent derivative and attempts to explain the molecular mechanisms associated with the activity. Compound **4-methyl-7-(3-methylbutyloxy)coumarin (MU2)** had the best overall activity and selectivity, selecting biofilm inhibition over antimycobacterial activity with a selective index of 64 and inhibiting the mycobacterial Mtr over the humans analog Gtr with a selectivity of 32. Furthermore, the **3-methylbutyloxy** substitution showed higher activities compared to the **3-methyl-but-2-en-1-yloxy** which is attributed to the rotation around the double/single bond.
- The position and type of substitution to the coumarin have a major influence on the biological activities. The library of possible substitutions is broad and the activities observed can be improved by a different type of substitution.
- Computational biology can be used in the field of medicinal plant sciences to explain in more detail the experimental observations and should be considered in other projects within this field.

The hypothesis was that “Derivatives of coumarin have the ability and selectivity to inhibit the formation of mycobacterial biofilms as opposed to their antimycobacterial activity”, there is not enough evidence to support the null hypothesis.

5.2. Future Recommendations

As is the case with any research project, this study has its limitations, and extended work can be conducted to confirm the observations made independently. The following steps can be considered after the proof of concept and *in vitro* screening as future recommendations:

- Pharmacokinetic assessment through absorption, distribution, metabolism and excretion experiments. A computational analysis can be considered for the initial screening by employing Quantitative structure-activity models to determine the pharmacokinetic profiles. QuikProp is

Chapter 5 Conclusion and Final Remarks

one such analysis tool to assign descriptors to the derivatives and based on models can predict the ADME profiles. Wet-lab experiments, such as cytochrome inhibition or induction assays can shed light on the metabolism.

- Investigation into other possible mechanisms of action, including quorum sensing inhibition, autoinducer disruption and genes regulation involved with curli pili formation.
- Possible investigation into microbial transformation.
- Exploring the effect of nanoparticlization on the activity and delivery of the CD
- The crystal structure of mycothione reductase should be elucidated and co-crystallized with the ligands to confirm the binding site and pose.
- This study was an *in vitro* screening project. *In vivo* assessment on an appropriate animal model can be considered too.

“I am a brain, Watson. The rest of me is a mere appendix.”

Arthur Conan Doyle

Appendices

This section contains selected additional data as appendices, data for this PhD has been uploaded to a data repository and made available, the published links for the various aspects can be found below:

Data:

- Oosthuizen, Carel Basson (2018), “Confirmation of coumarin derivatives through **NMR** and **HRMS**”, Mendeley Data, v1. <http://dx.doi.org/10.17632/hsrdkj66y6.1>
- Oosthuizen, Carel Basson (2018), “**Biofilm Inhibition** of coumarin derivatives (IC₅₀)”, Mendeley Data, v1. <http://dx.doi.org/10.17632/rjxx4zn6m.1>
- Oosthuizen, Carel Basson (2018), “**Cellular toxicity** of coumarins on HepG2 cells”, Mendeley Data, v1. <http://dx.doi.org/10.17632/f885dypc7w.1>
- Oosthuizen, Carel Basson (2018), “**SMILES** of coumarin derivatives as mycobacterial biofilm inhibitors”, Mendeley Data, v1. <http://dx.doi.org/10.17632/nncpkv2nj9.1>
- Oosthuizen, Carel Basson (2018), “**Docking results** of coumarin derivatives on glutathione and mycothione reductases”, Mendeley Data, v1. <http://dx.doi.org/10.17632/3th33fs5vv.1>
- Oosthuizen, Carel Basson (2018), “Molecular dynamics simulation, **ligand interaction analysis** of three coumarin derivatives on glutathione and mycothione reductase”, Mendeley Data, v1. <http://dx.doi.org/10.17632/s9jjjsycrm.1>

Appendices:

APPENDIX 1: Antimycobacterial activities of derivatives on *M. smegmatis*

APPENDIX 2: Antibiofilm activities of derivatives on *M. smegmatis*

APPENDIX 3: NMR and MS confirmation of **MU5**

APPENDIX 4: NMR and MS confirmation of **U5**

APPENDIX 5: NMR and MS confirmation of **U2**

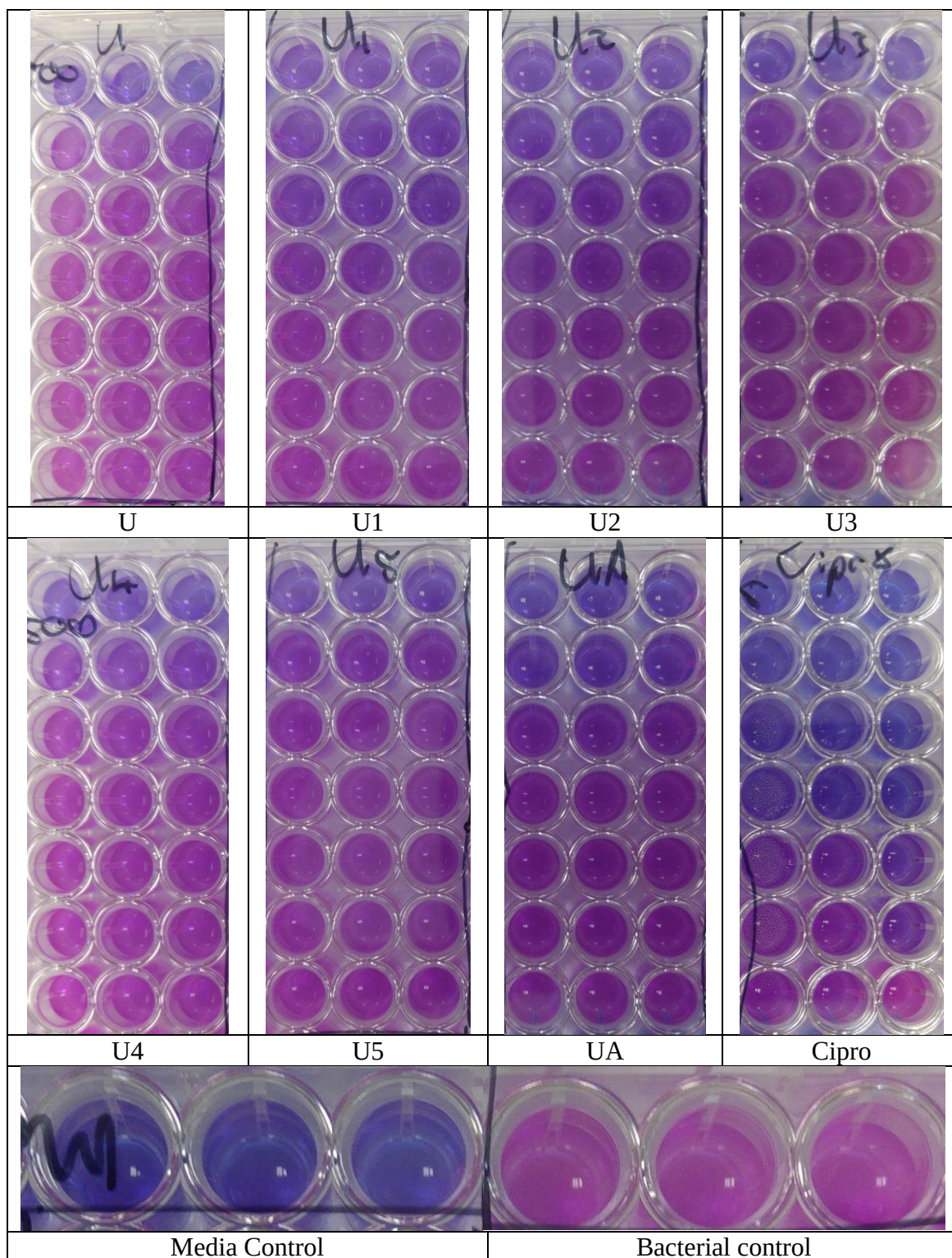
APPENDIX 6: NMR and MS confirmation of **MU2**

Appendix 1

This appendix contains a set of the antimycobacterial assay plates, indicating the inhibitory activity of the coumarins derivatives against *Mycobacterium*. A blue color indicated no bacterial growth or inhibition a pink color indicated bacterial growth.

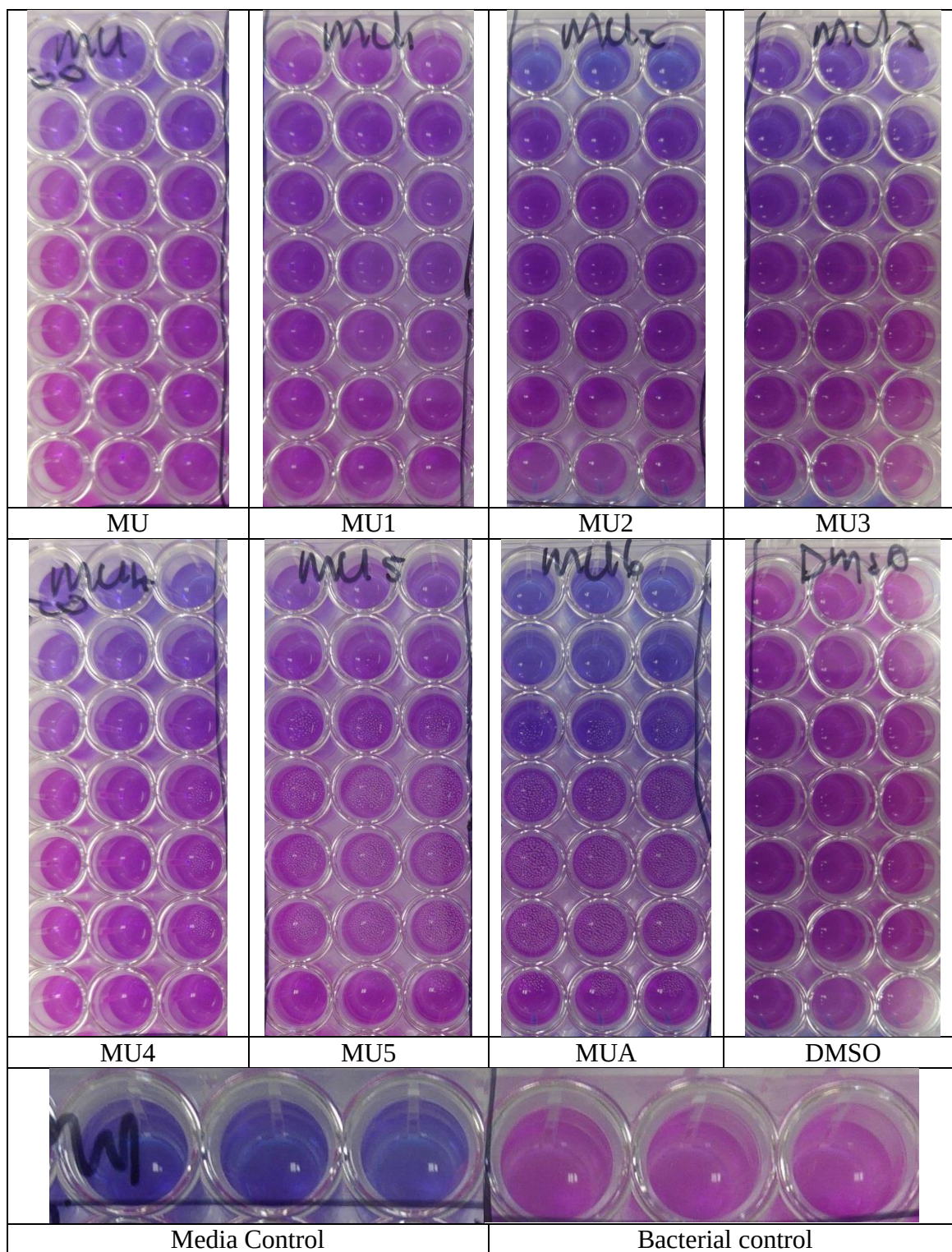
Appendices

APPENDIX 1: Antimycobacterial activities of U Samples on *M. smegmatis*



Appendices

APPENDIX 1: Antimycobacterial activities of MU Samples on *M. smegmatis*

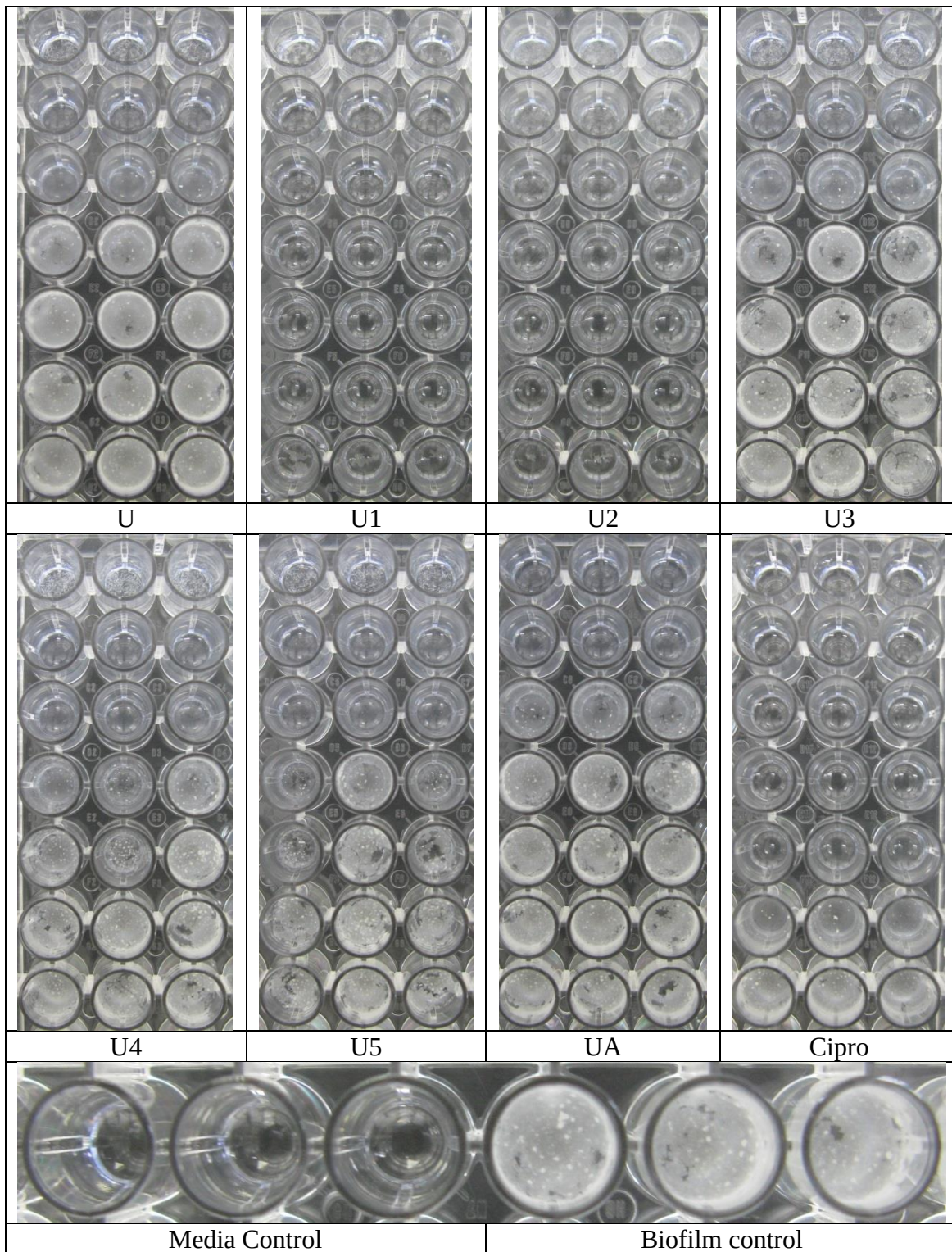


Appendix 2

This appendix contains a set of the antibiofilm assay plates, indicating the inhibitory activity of the coumarins derivatives against the formation of *Mycobacterium* biofilms.

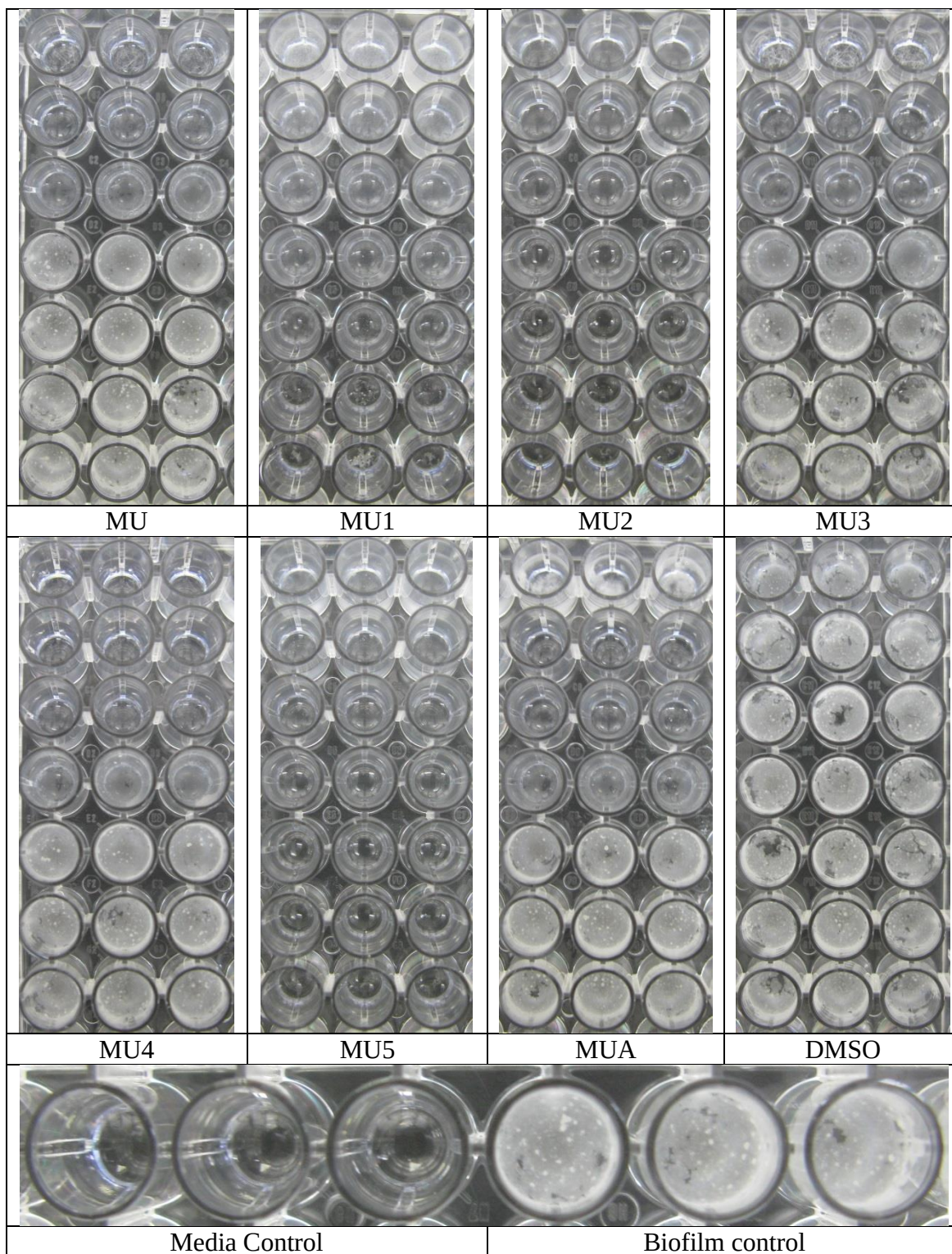
Appendices

APPENDIX 2: Antibiofilm activities of U Samples on *M. smegmatis*



Appendices

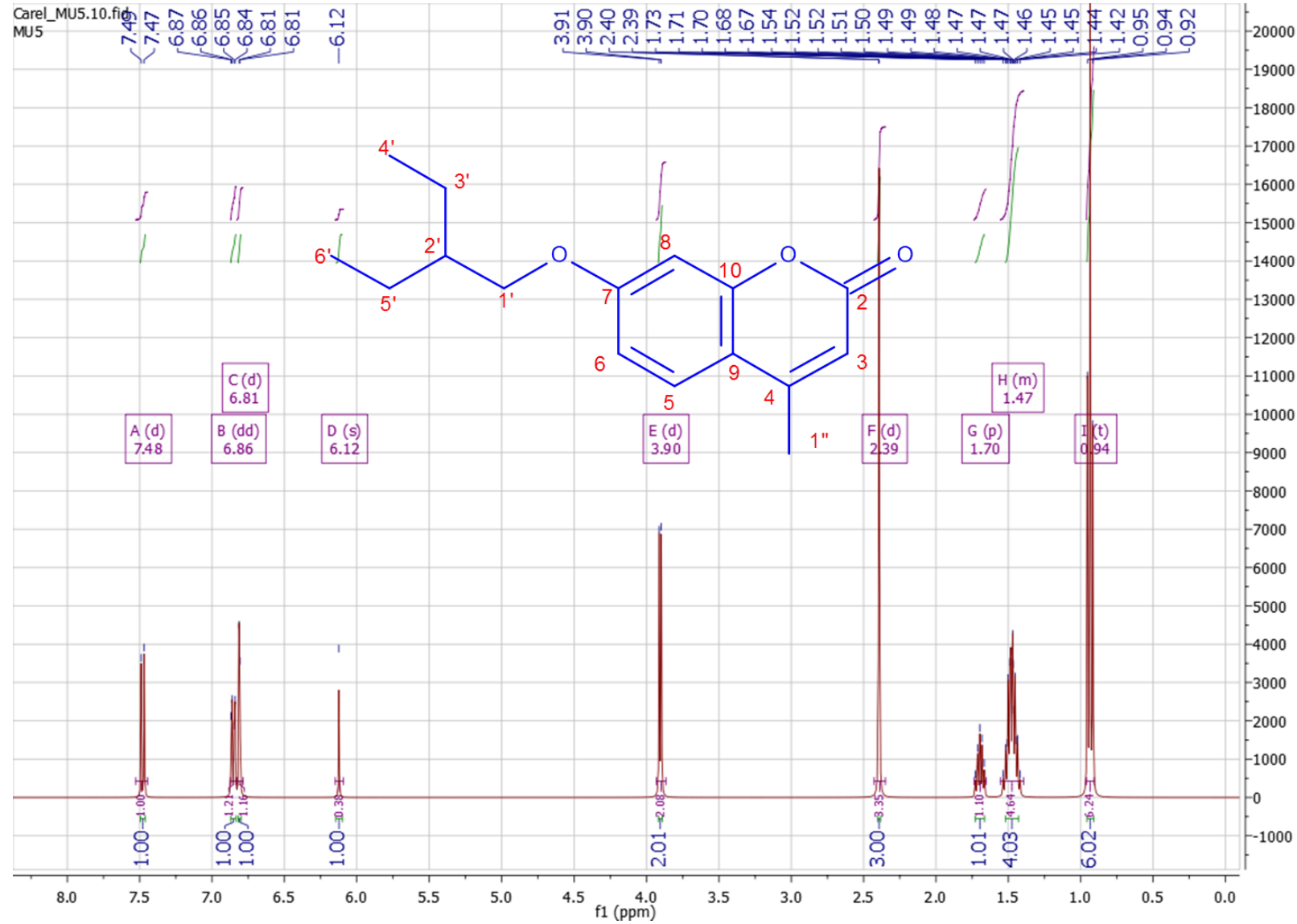
APPENDIX 2: Antibiofilm activities of MU Samples on *M. smegmatis*



Appendix 3

This appendix contains the ^1H , ^{13}C , 2D-NMR, mass spectra and fragmentation pattern of derivative MU5.

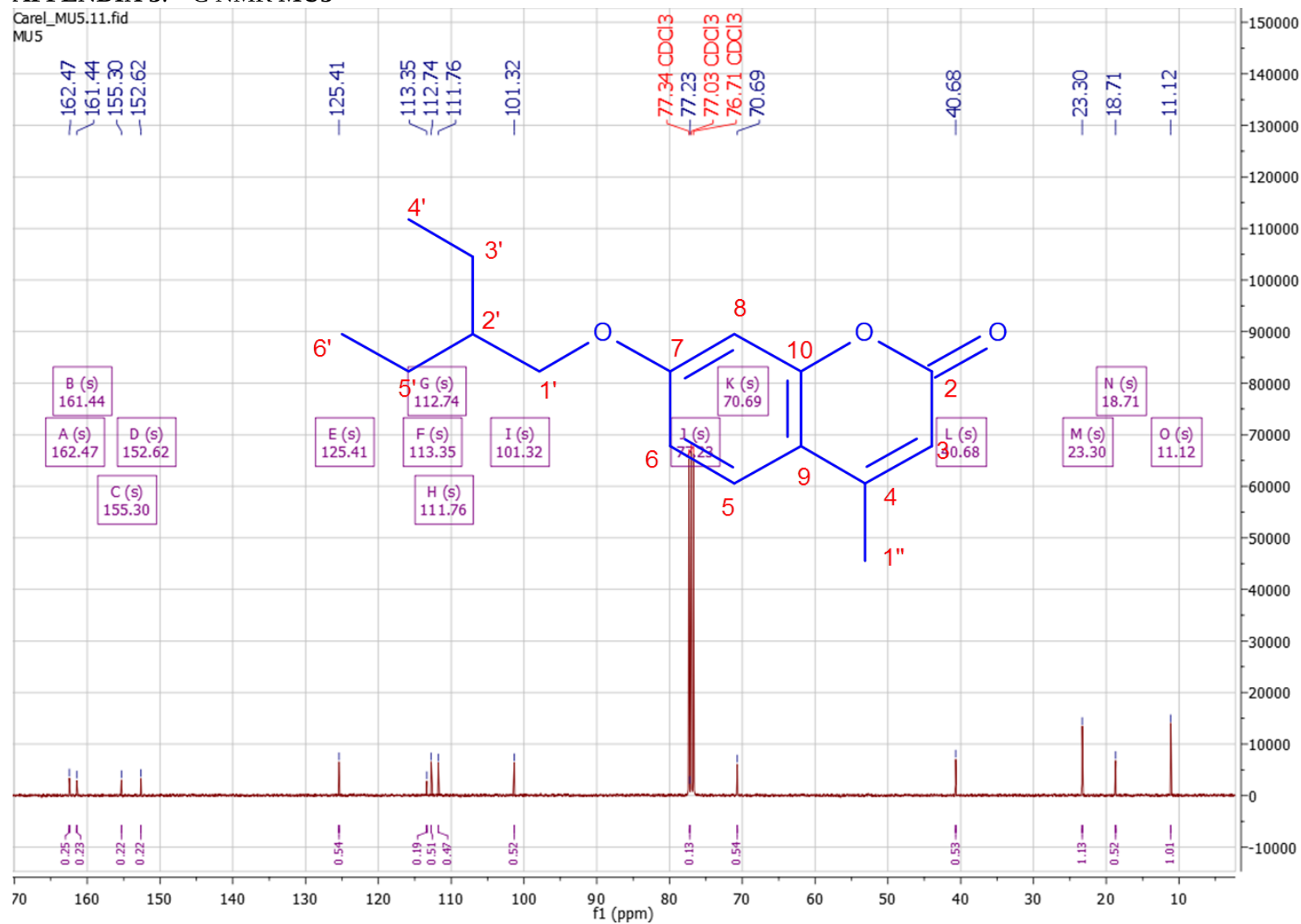
APPENDIX 3: ^1H NMR MU5



Appendices

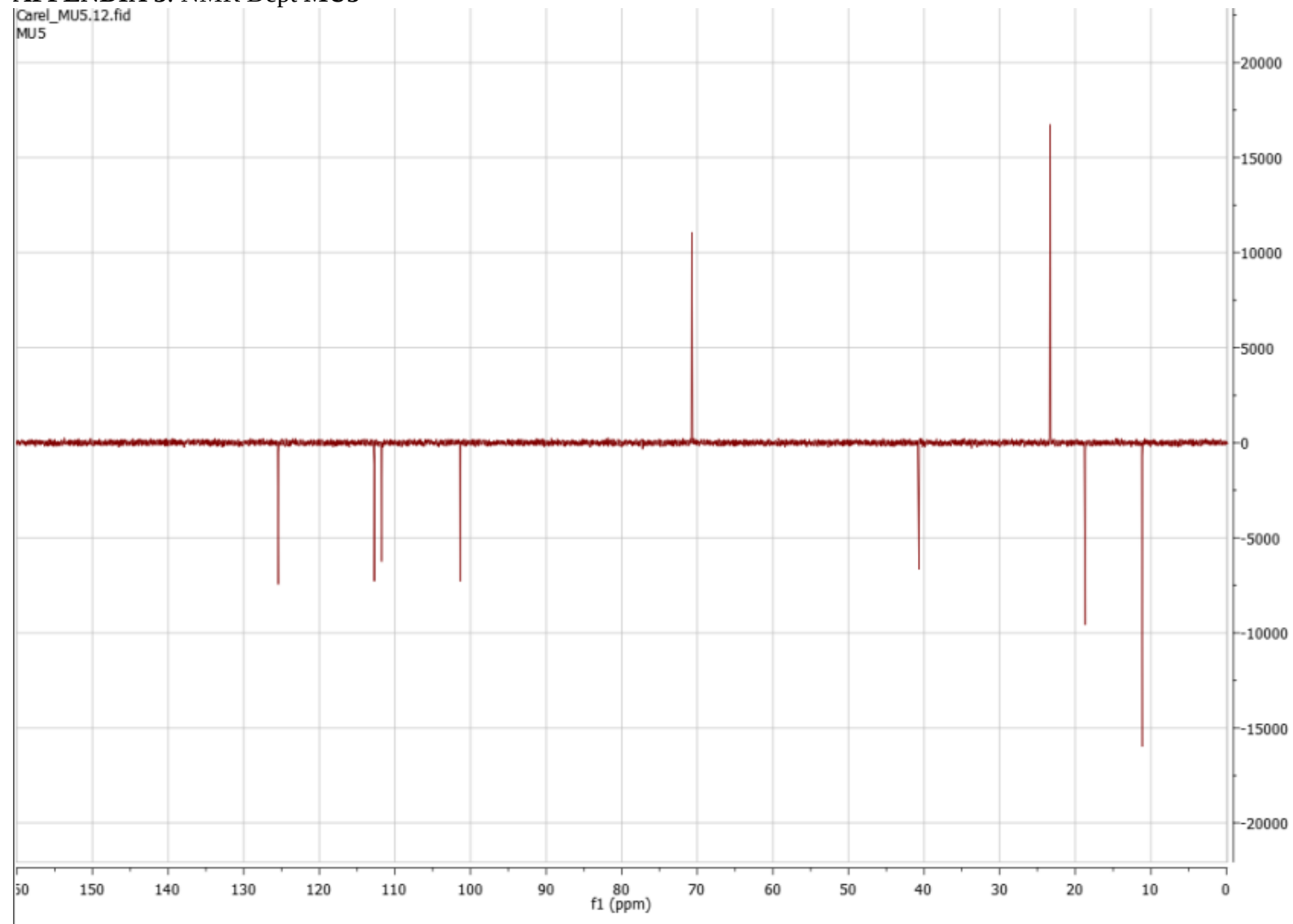
APPENDIX 3: ^{13}C NMR MU5

Carel_MU5.11.fid
MU5



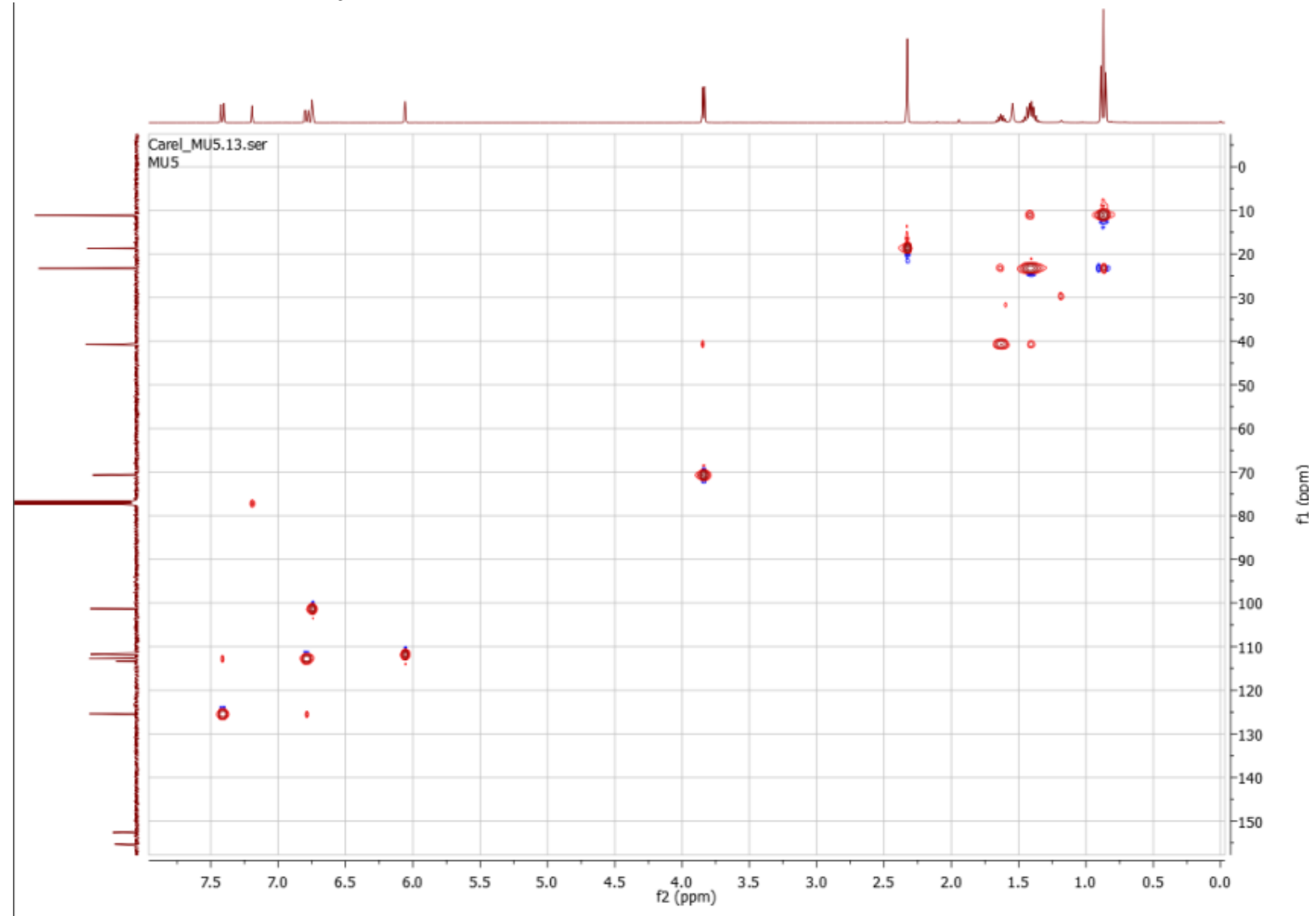
Appendices

APPENDIX 3: NMR Dept MU5



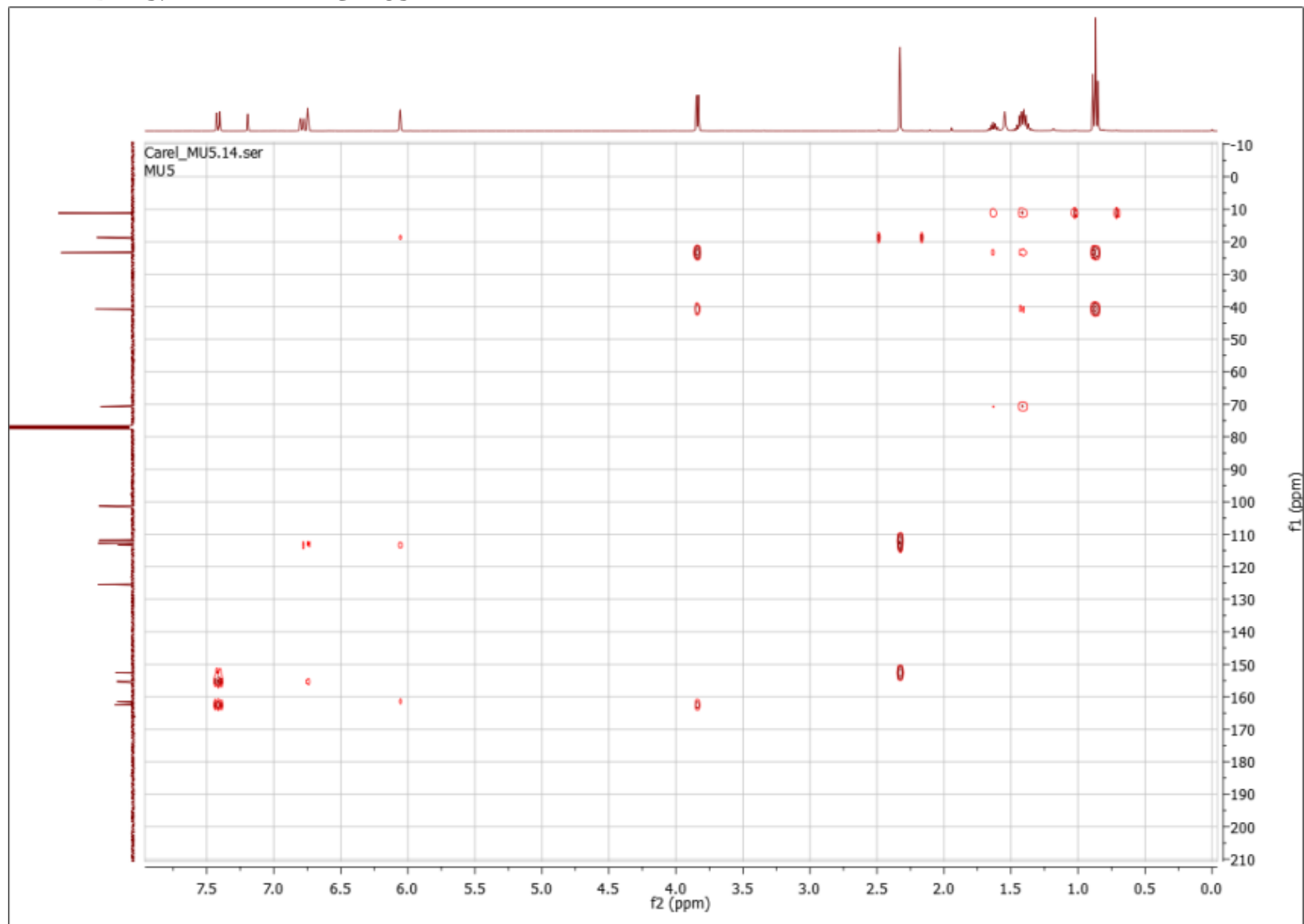
Appendices

APPENDIX 3: 2D NMR HMQC MU5

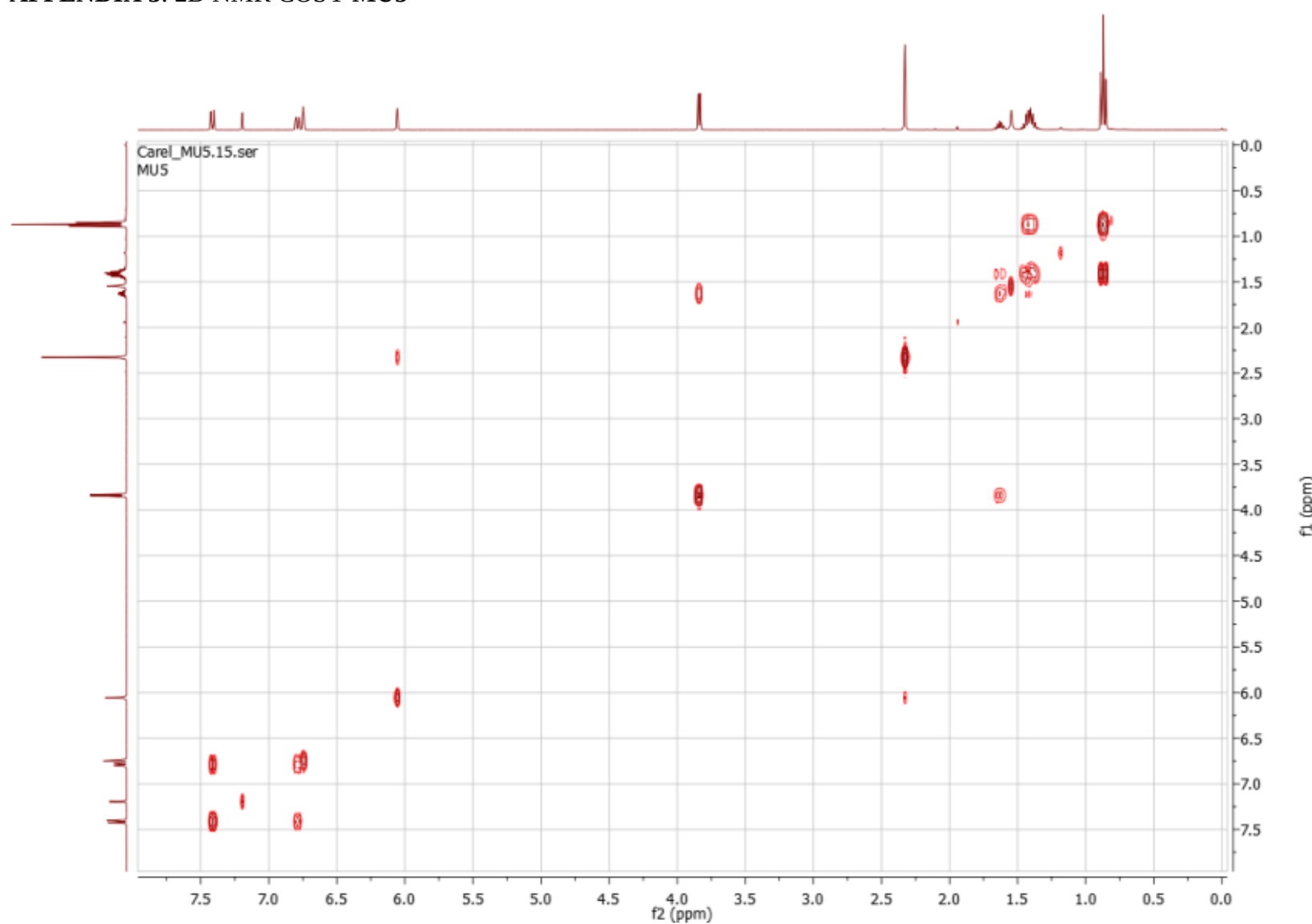


Appendices

APPENDIX 3: 2D NMR HMBC MU5

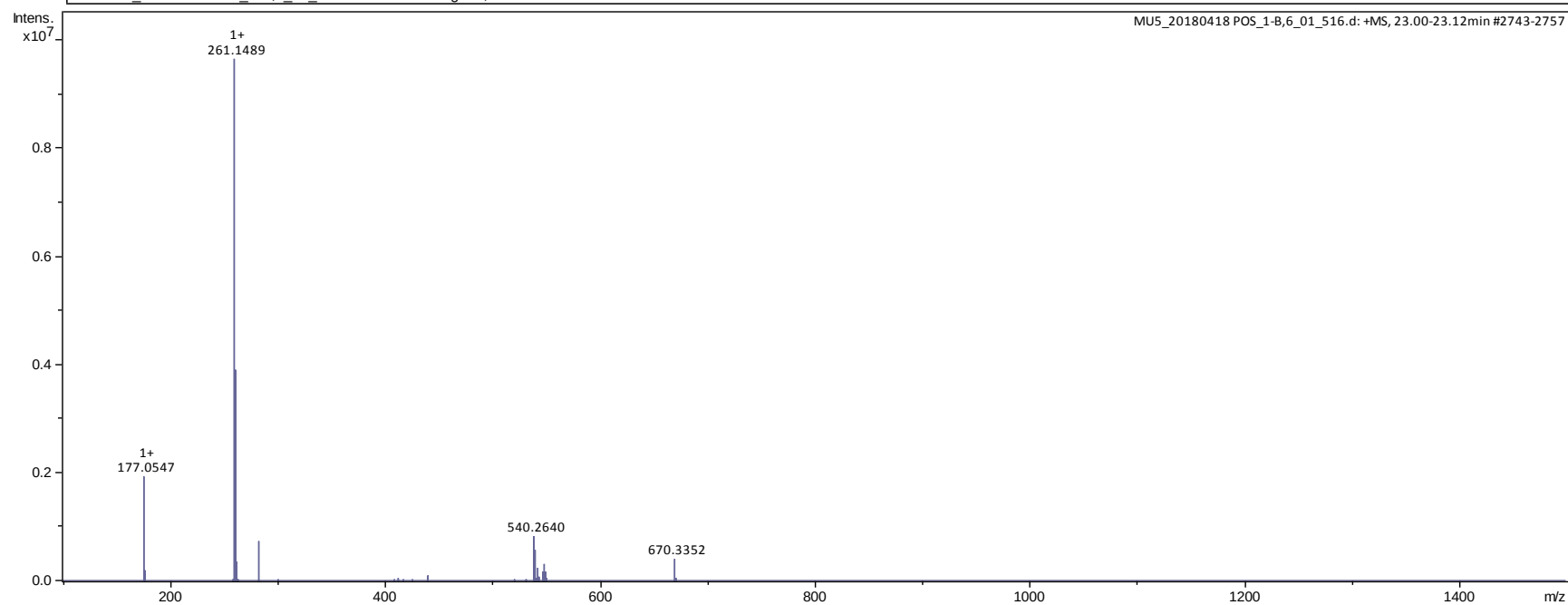
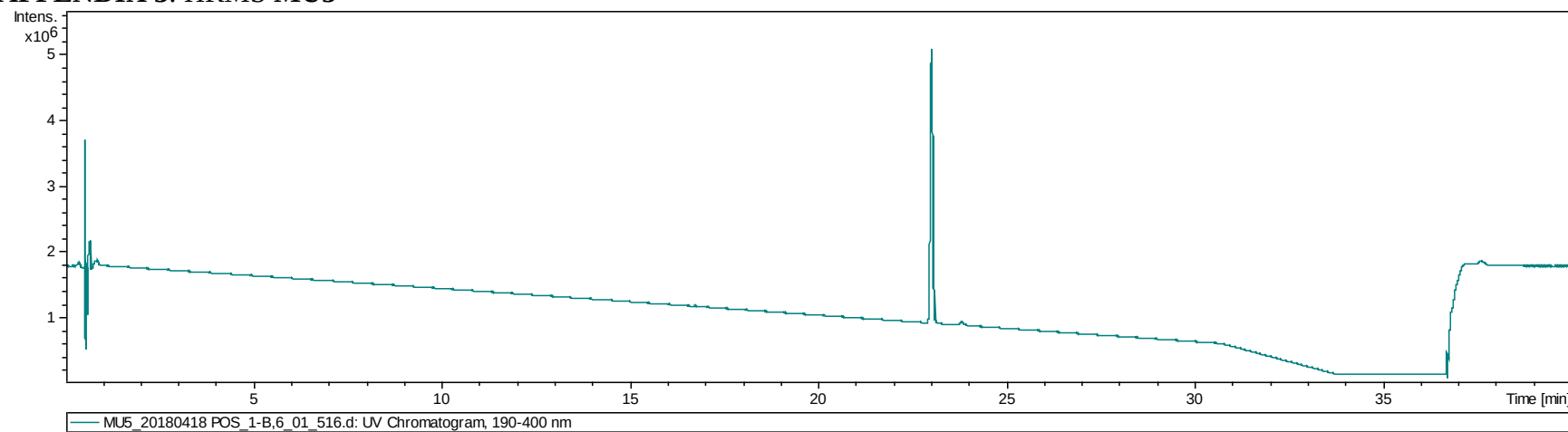


APPENDIX 3: 2D NMR COSY MU5



Appendices

APPENDIX 3: HRMS MU5

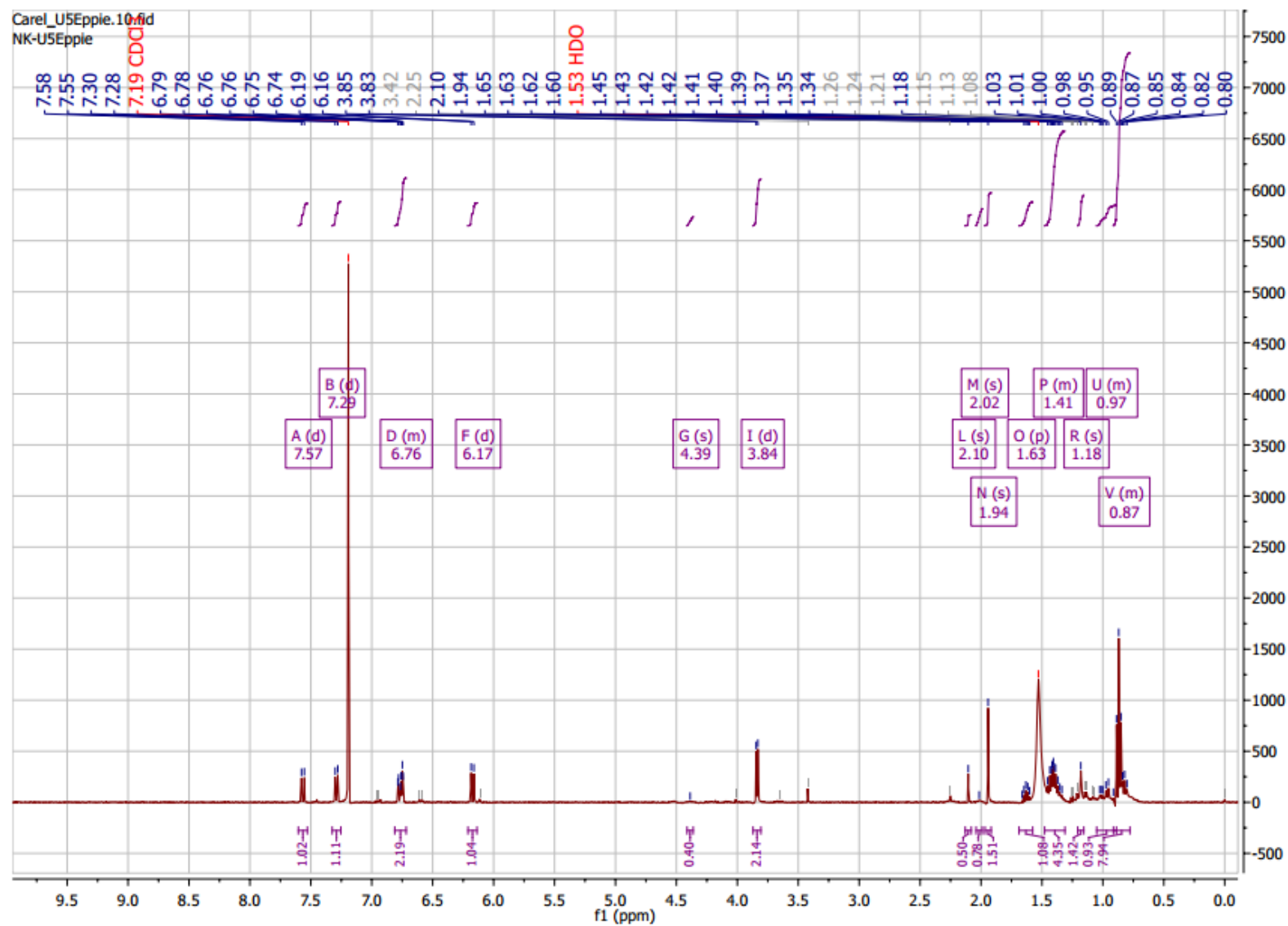


Appendix 4

This appendix contains the ^1H , ^{13}C , 2D-NMR, mass spectra and fragmentation pattern of derivative **U5**.

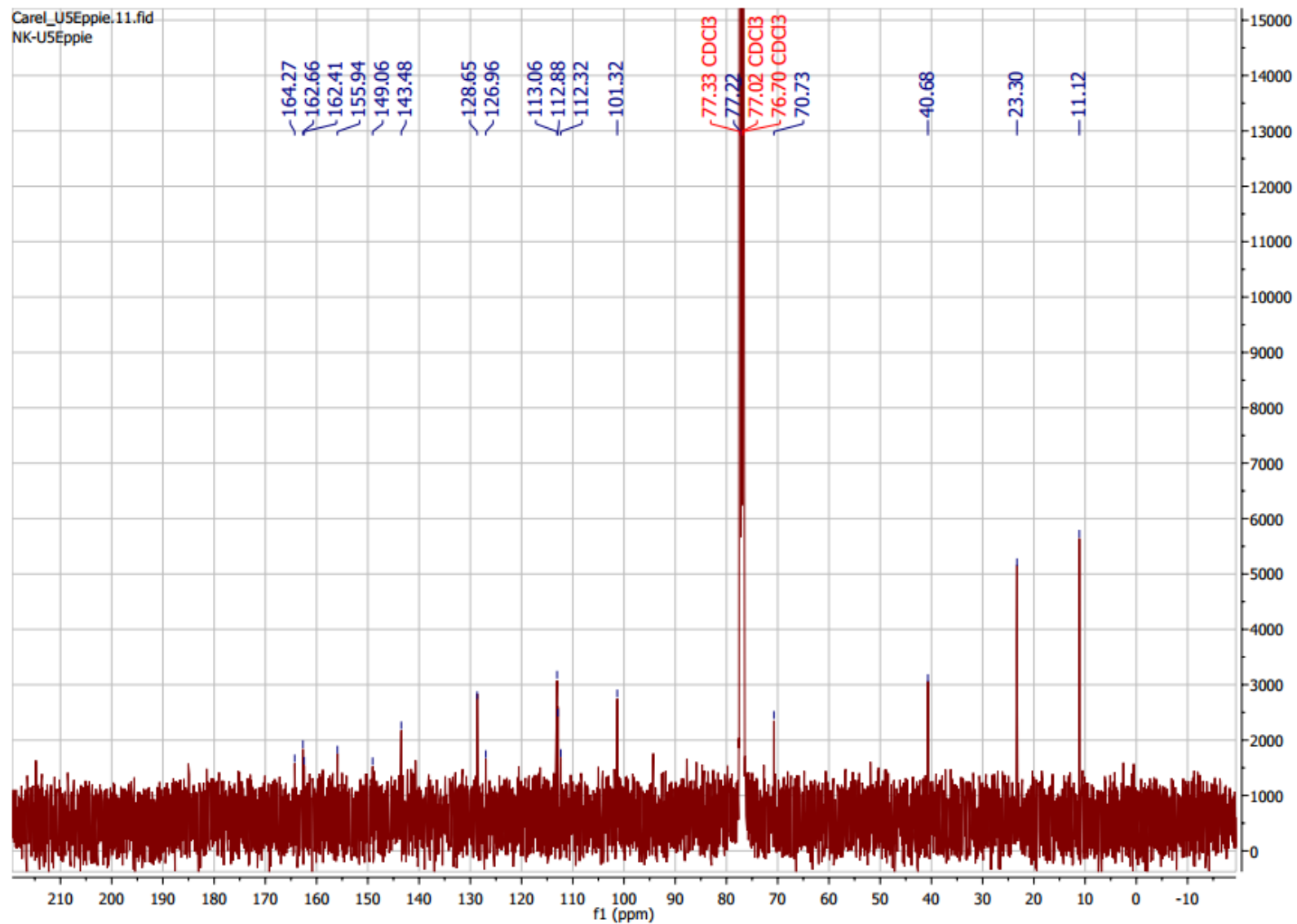
Appendices

APPENDIX 4: ^1H NMR U5



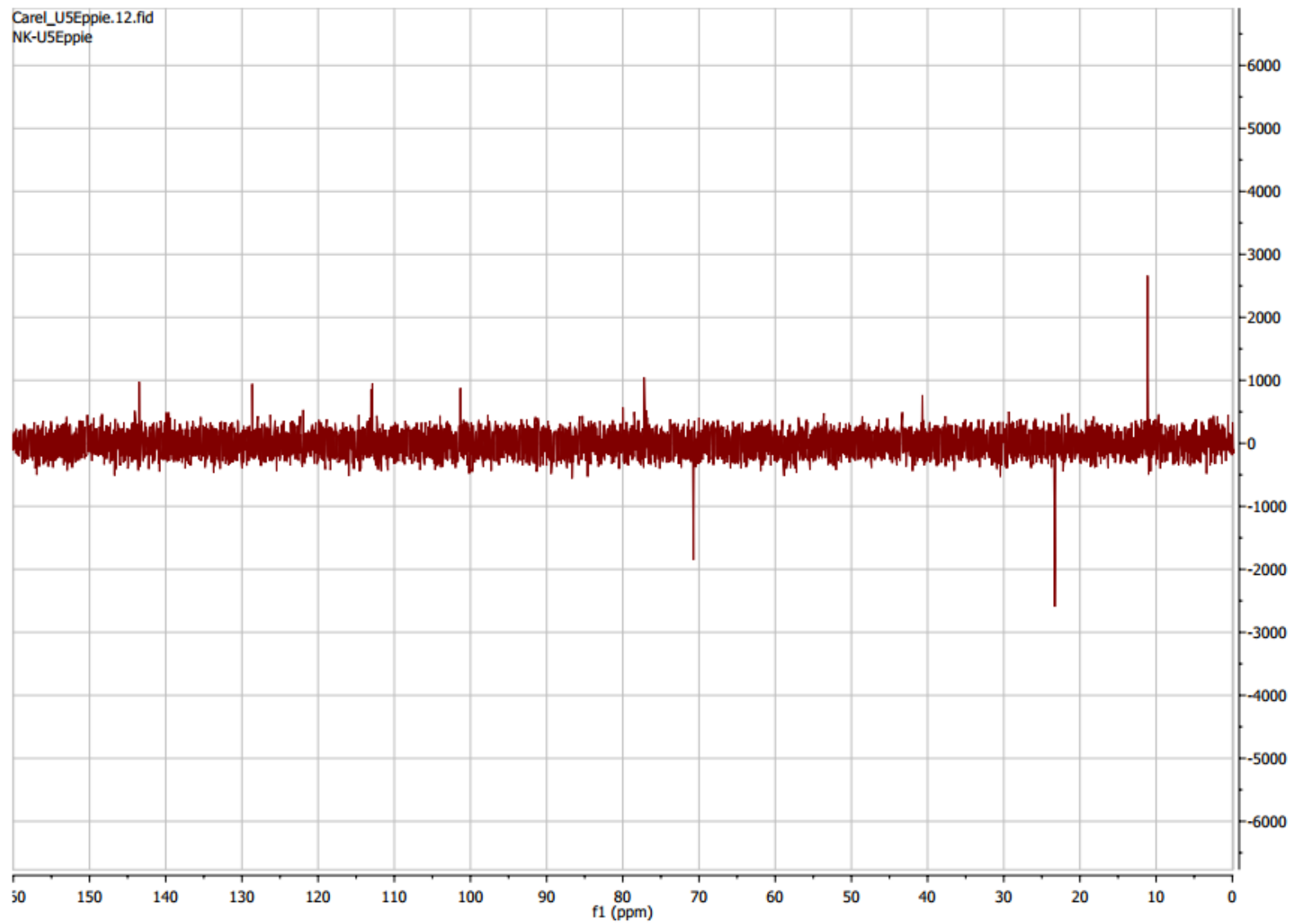
Appendices

APPENDIX 4: ^{13}C NMR U5



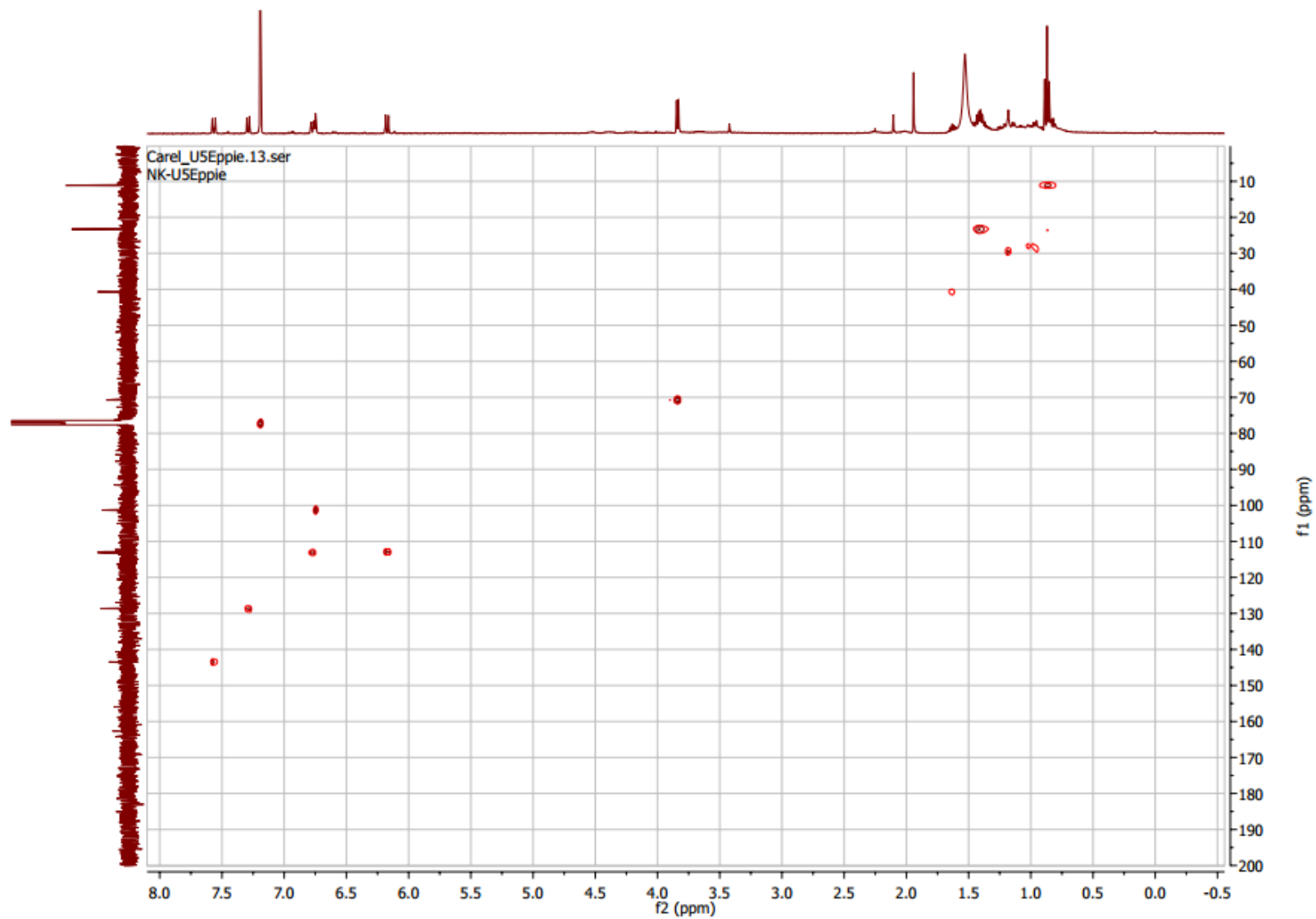
Appendices

APPENDIX 4: NMR Dept U5



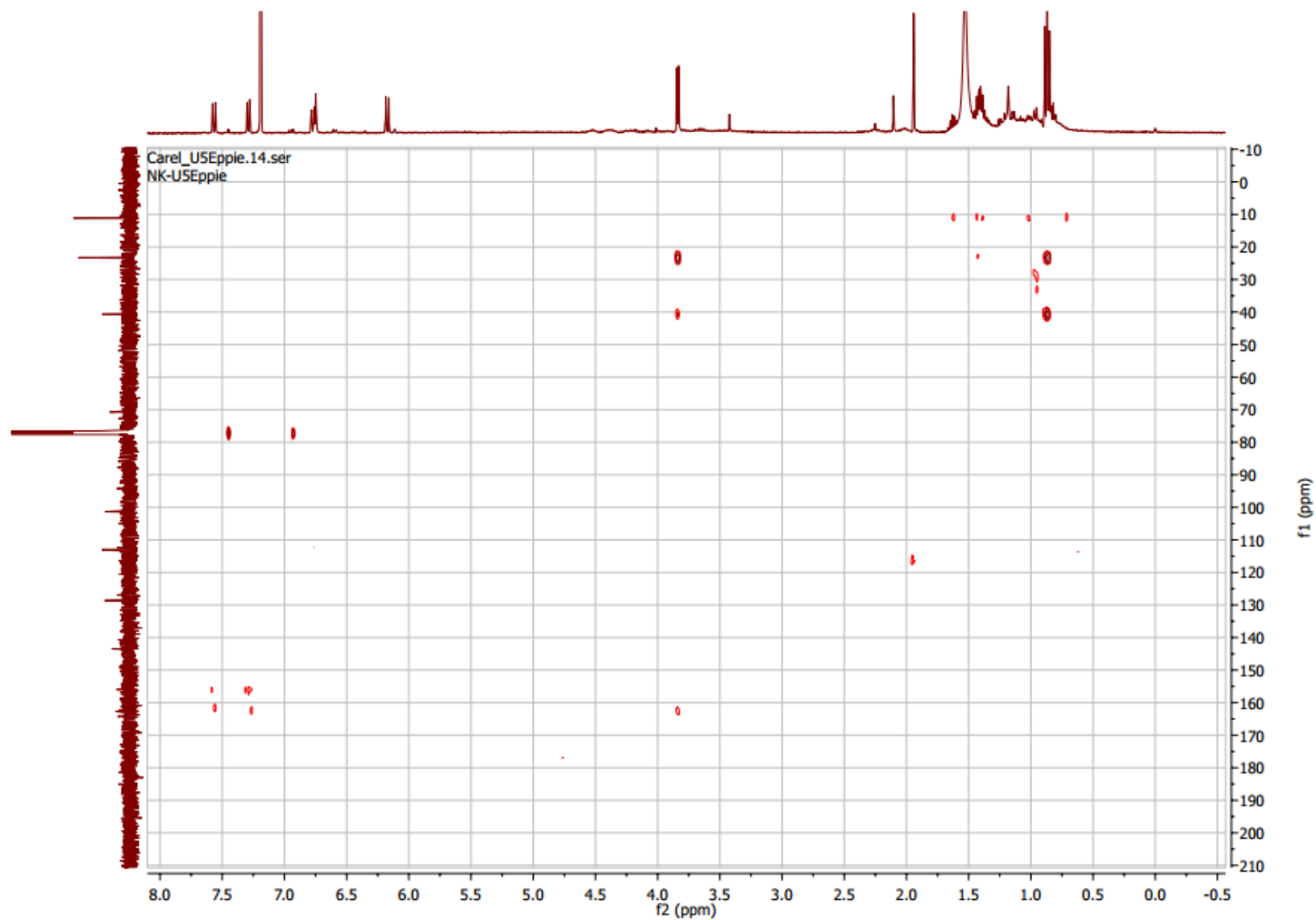
Appendices

APPENDIX 4: 2D NMR HMQC U5



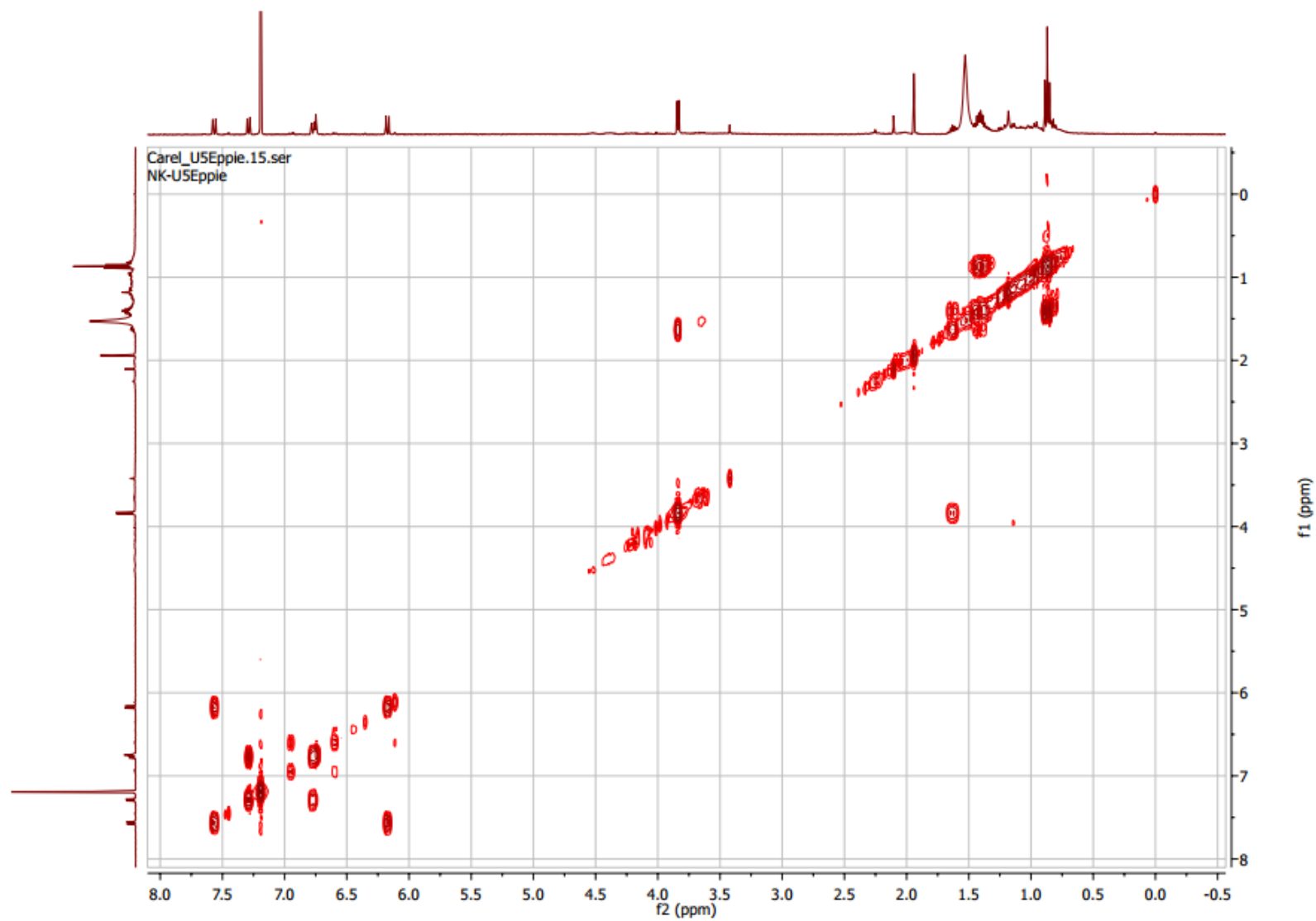
Appendices

APPENDIX 4: 2D NMR HMBC U5



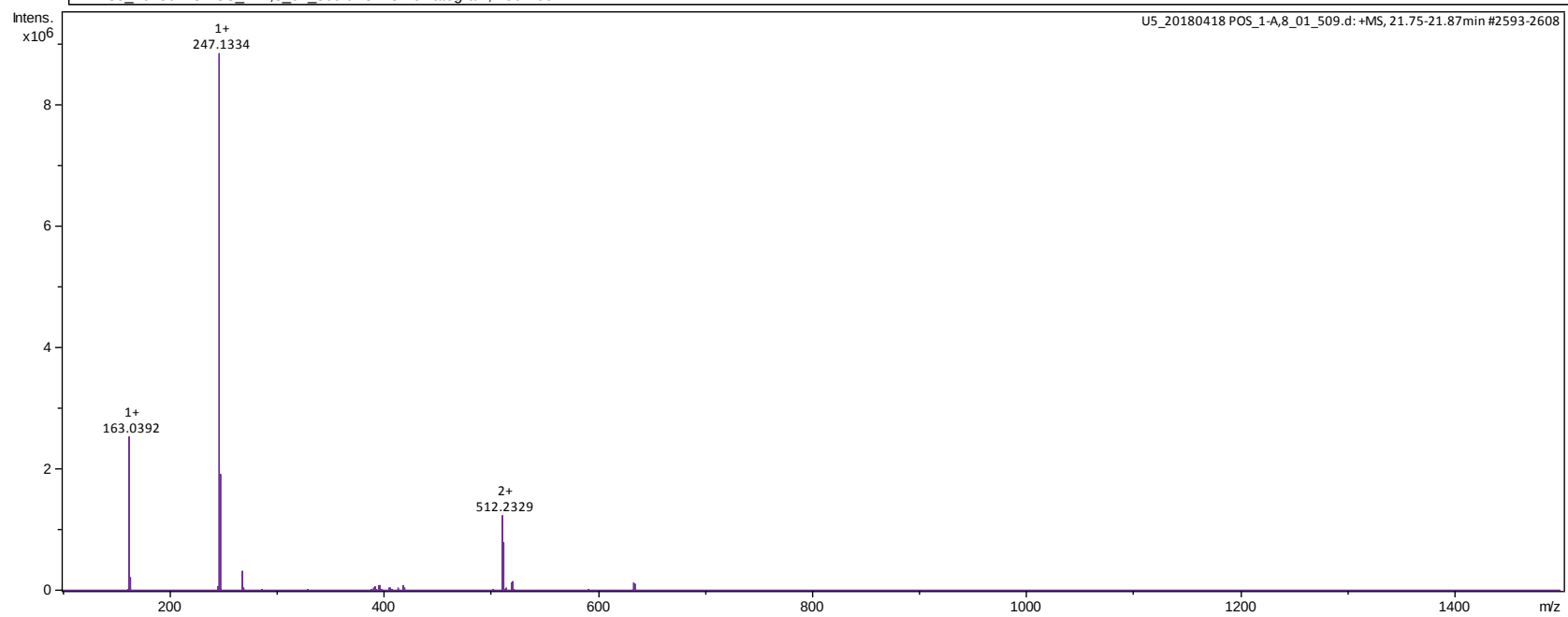
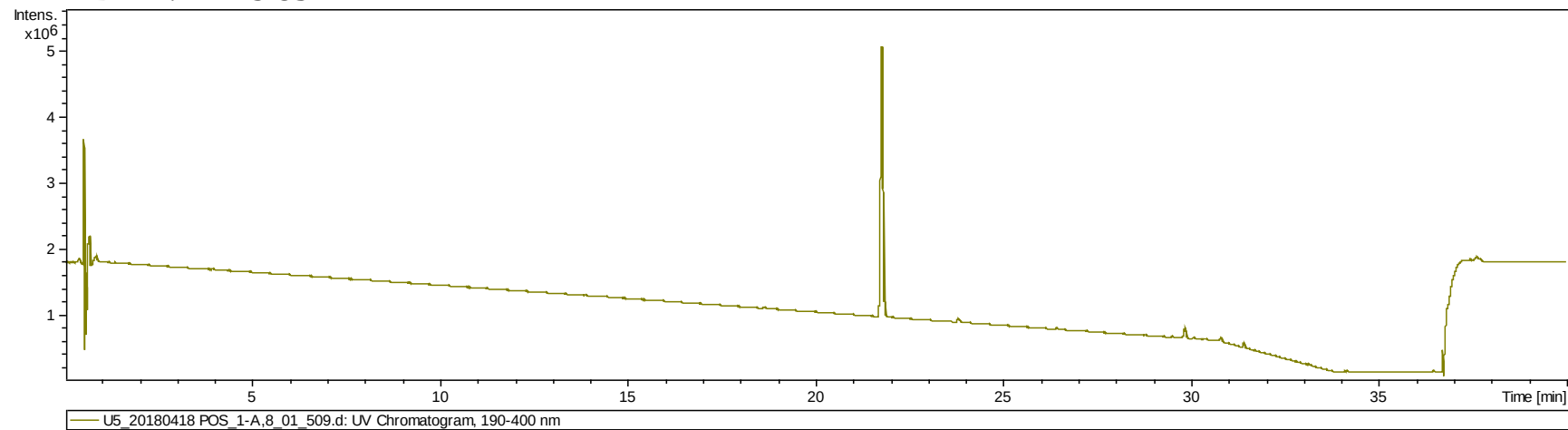
Appendices

APPENDIX 4: 2D NMR COSY U5



Appendices

APPENDIX 4: HRMS U5



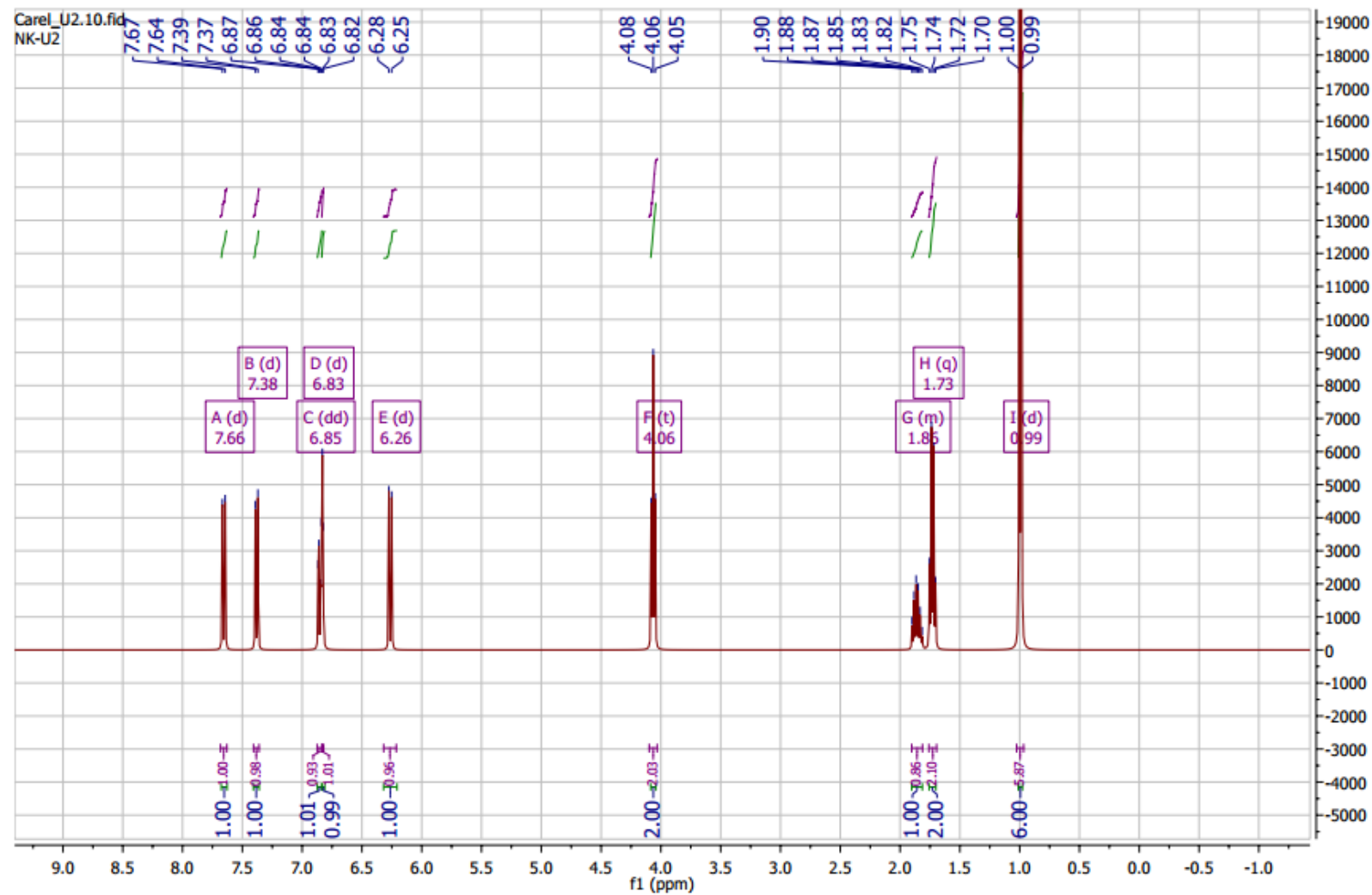
Appendix 5

This appendix contains the ^1H , ^{13}C , mass spectra and fragmentation pattern of derivative **U2**.

Appendices

APPENDIX 5: ^1H NMR U2

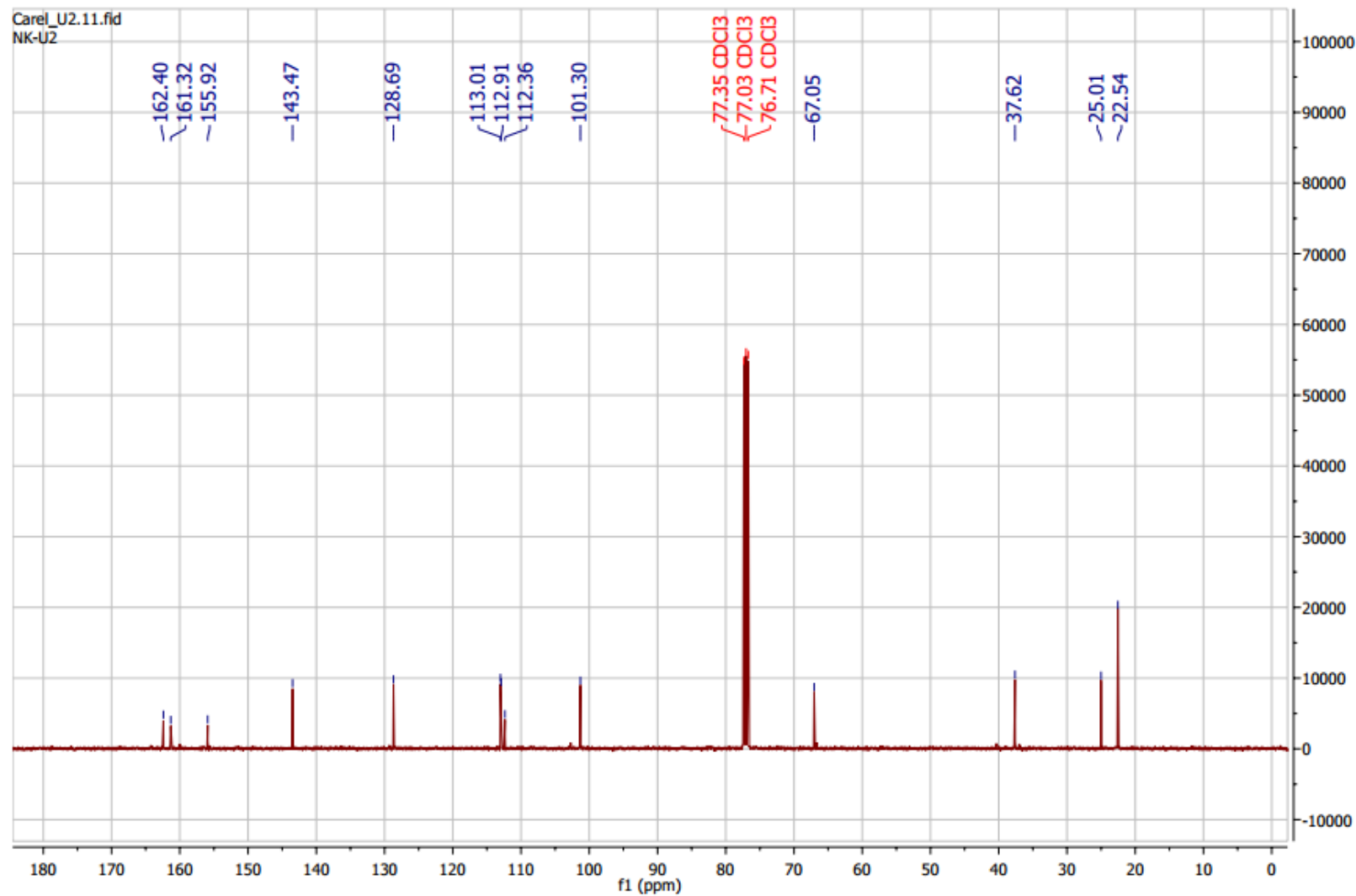
^1H NMR (400 MHz, Chloroform- d) δ 7.66 (d, J = 9.5 Hz, 1H), 7.38 (d, J = 8.5 Hz, 1H), 6.85 (dd, J = 8.5, 2.3 Hz, 1H), 6.83 (d, J = 2.3 Hz, 1H), 6.26 (d, J = 9.5 Hz, 1H), 4.06 (t, J = 6.6 Hz, 2H), 1.90 – 1.81 (m, 1H), 1.73 (q, J = 6.7 Hz, 2H), 0.99 (d, J = 6.6 Hz, 6H).



Appendices

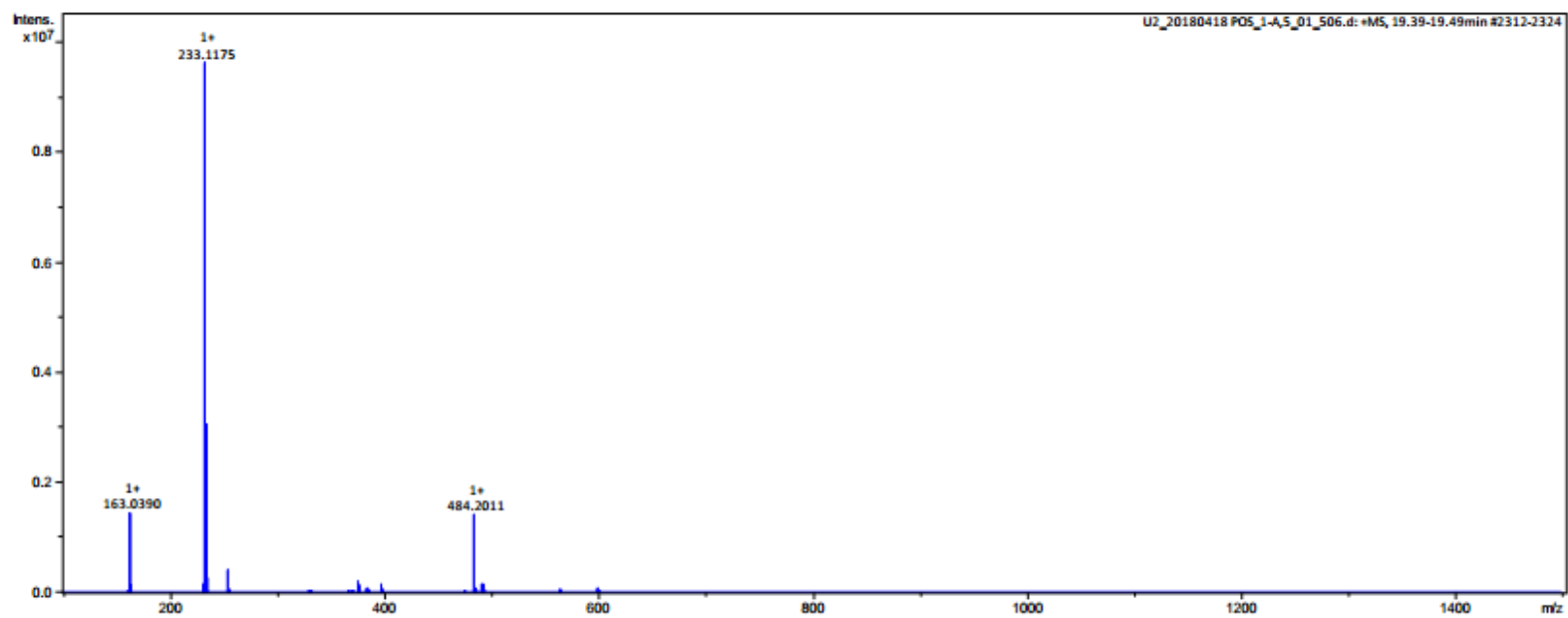
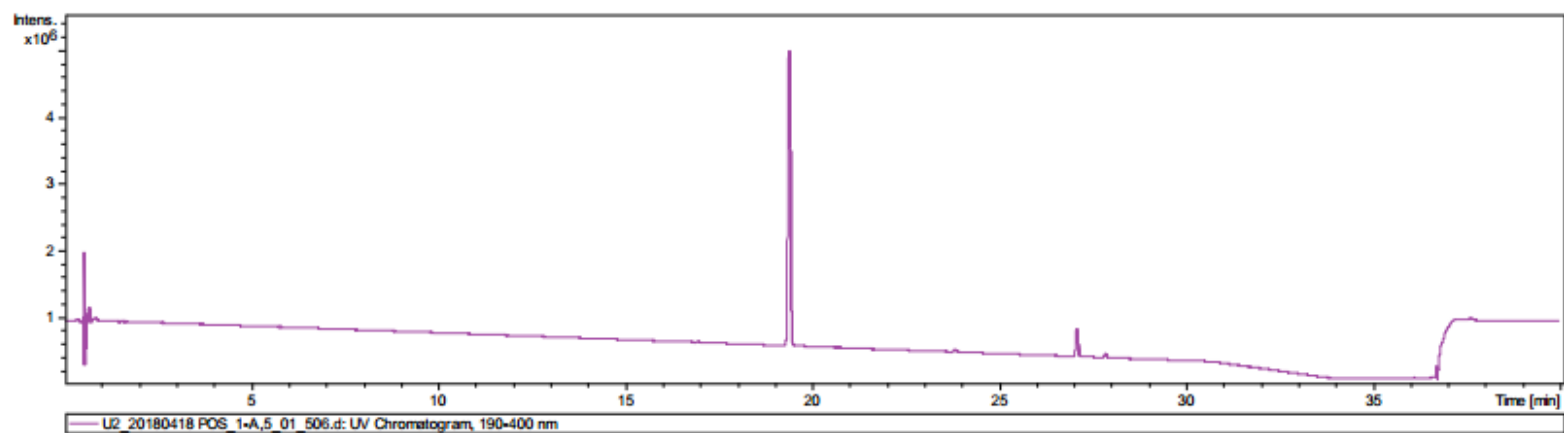
APPENDIX 5: ^{13}C NMR U2

^{13}C NMR (101 MHz, CDCl_3) δ 162.40, 161.32, 155.92, 143.47, 128.69, 113.01, 112.91, 112.36, 101.30, 67.05, 37.62, 25.01, 22.54.



Appendices

APPENDIX 5: HRMS U2



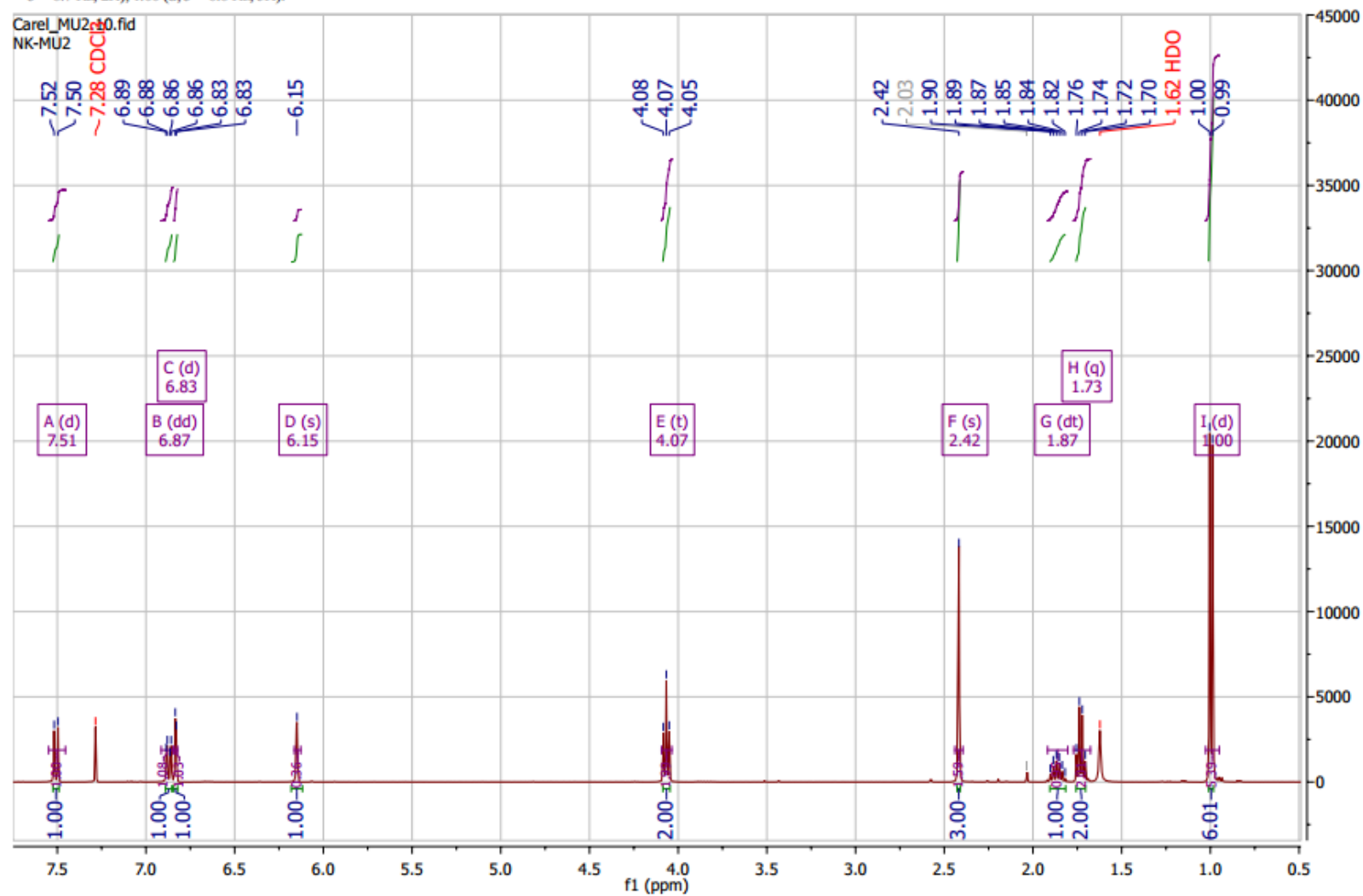
Appendix 6

This appendix contains the ^1H , ^{13}C , mass spectra and fragmentation pattern of derivative **MU2**.

Appendices

APPENDIX 6: ^1H NMR MU2

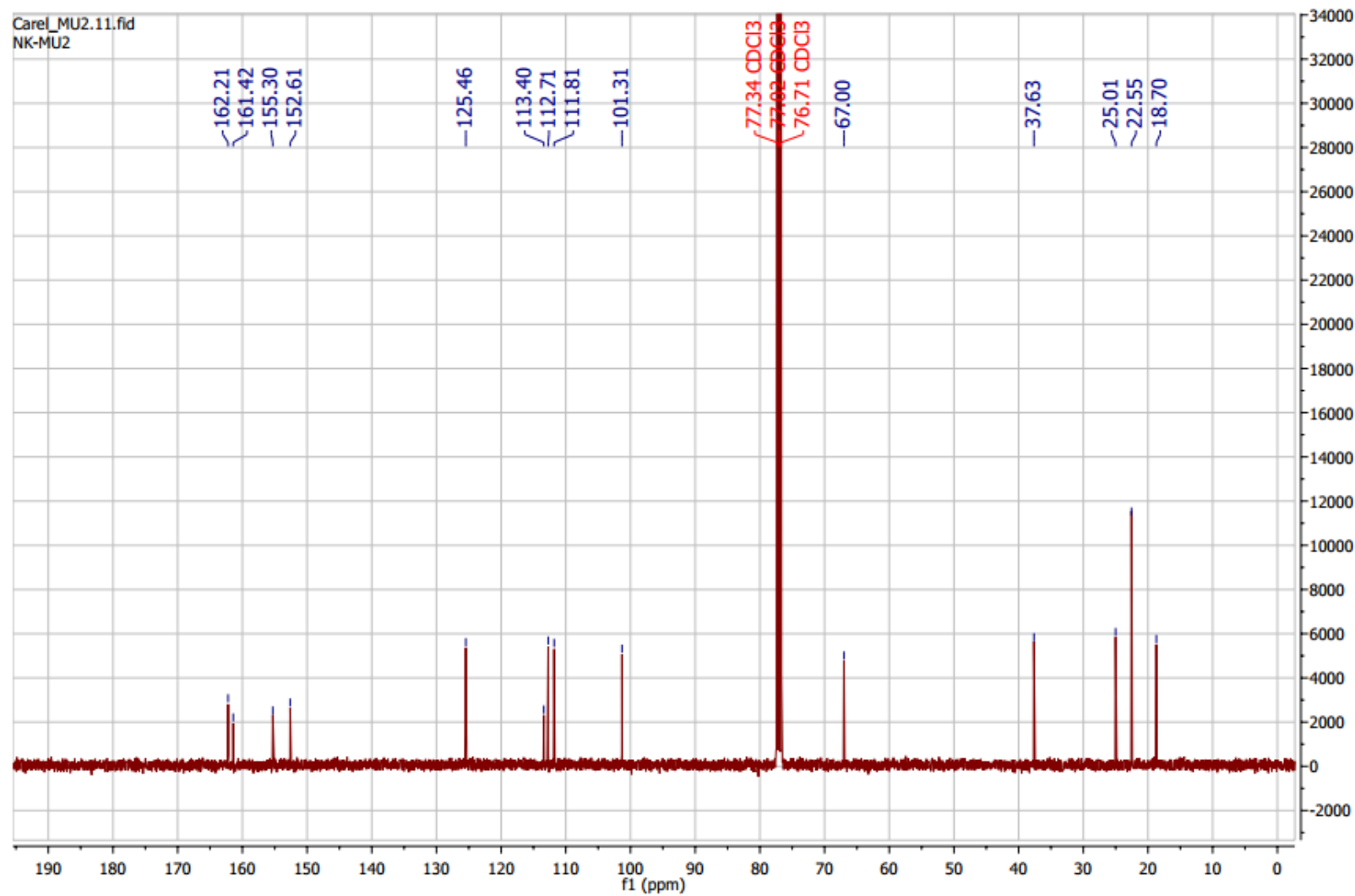
^1H NMR (400 MHz, Chloroform- d) δ 7.51 (d, J = 8.8 Hz, 1H), 6.87 (dd, J = 8.7, 2.5 Hz, 1H), 6.83 (d, J = 2.4 Hz, 1H), 6.15 (s, 0H), 4.07 (t, J = 6.6 Hz, 2H), 2.42 (s, 2H), 1.87 (dt, J = 13.4, 6.7 Hz, 1H), 1.73 (q, J = 6.7 Hz, 2H), 1.00 (d, J = 6.6 Hz, 5H).



Appendices

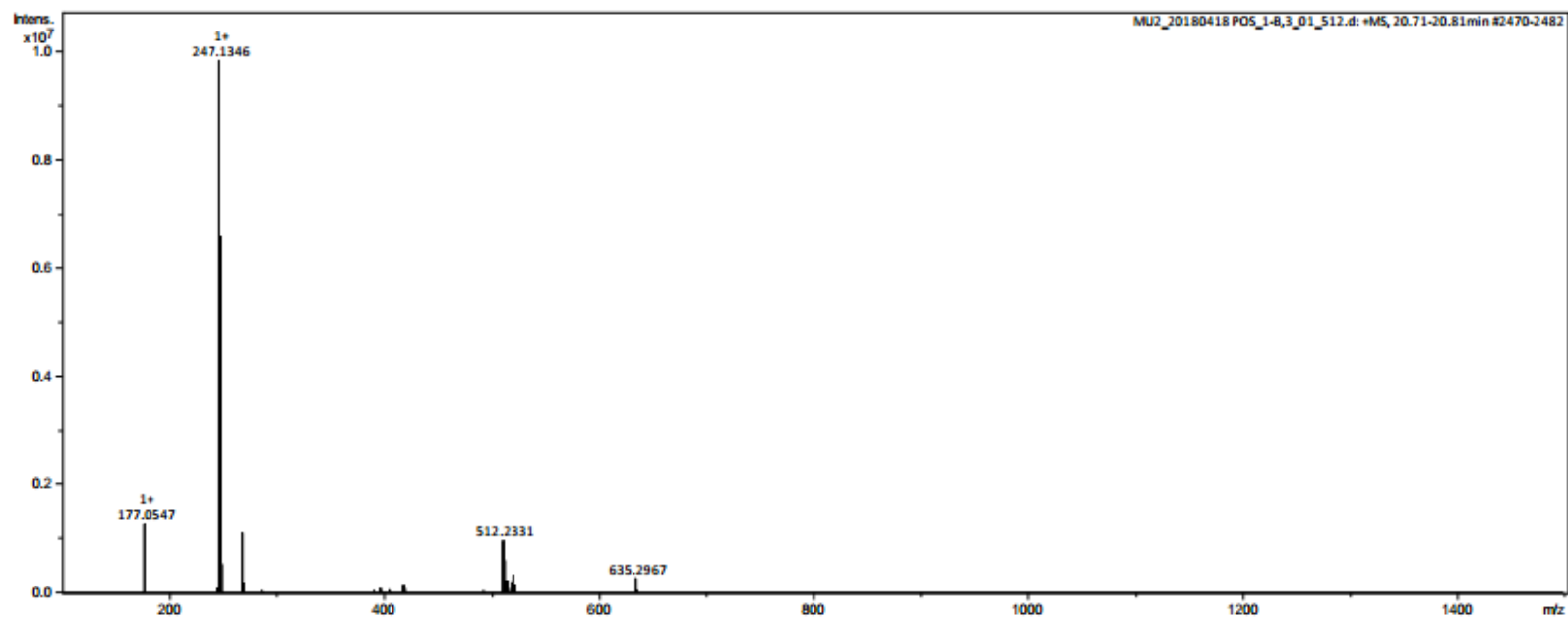
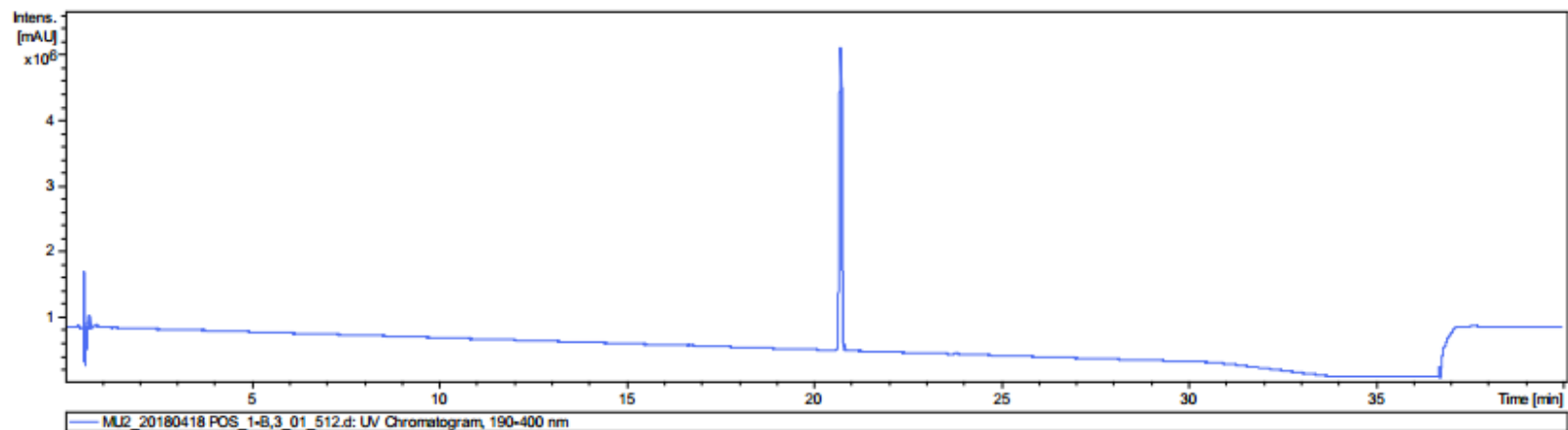
APPENDIX 6: ^{13}C NMR MU2

^{13}C NMR (101 MHz, CDCl_3) δ 162.21, 161.42, 155.30, 152.61, 125.46, 113.40, 112.71, 111.81, 101.31, 77.34, 77.02, 76.71, 67.00, 37.63, 25.01, 22.55, 18.70.



Appendices

APPENDIX 6: HRMS MU2



Fil 4:13

“Omnia possum in eo qui me confortat.”

*** FINIS ***