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Development of a method for detecting TB-antibodies in patient serum

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Development of a Method for Detecting TB-antibodies in Patient Serum

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by

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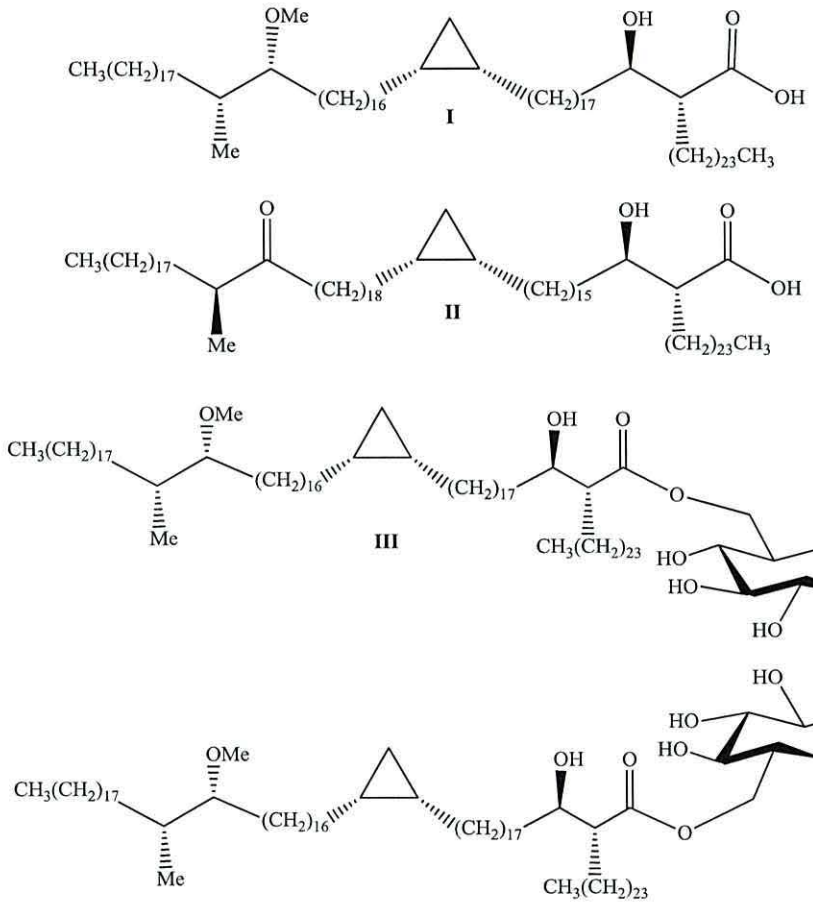
Abbreviations and Acronyms

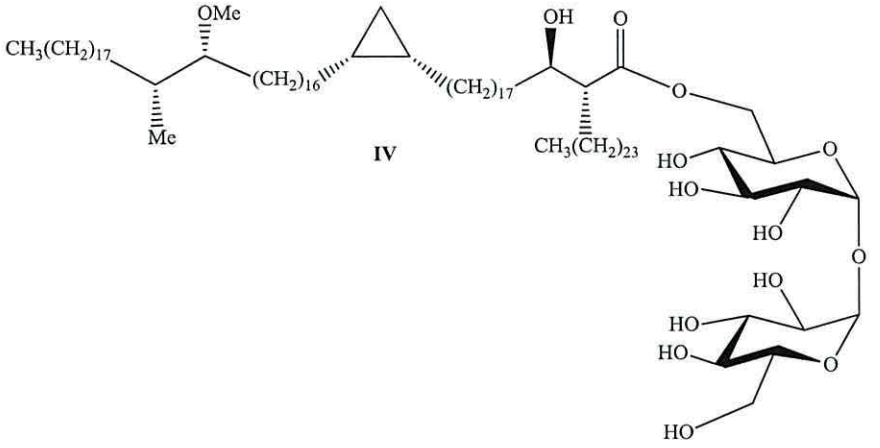
Ac	Acetyl
AIDS	Acquired Immune Deficiency Syndrome
aq.	Aqueous
br	Broad
BCG	Bacillus Calmette-Guérin
Bn	Benzyl
m-CPBA	*m*-Chloroperbenzoic acid
CID-MS	Collision-induced dissociation mass spectrometry
d	Doublet
DMAP	4-Dimethylaminopyridine
DMF	Dimethylformamide
EDCI	1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide
ELISA	Enzyme-linked immunosorbent assay
GC	Gas Chromatography
GMM	Glucose monomycolate
HIV	Human immunodeficiency virus
HMPA	Hexamethylphosphoramide
HPLC	High Performance Liquid Chromatography
IMS	Industrial methylated spirits
IR	Infra-red
J	Coupling constant
LDA	Lithium *N, N*,-diisopropylamide
m	*meta*-
m	Multiplet
MALDI	Matrix-Assisted Laser Desorption/Ionization
MALDI-TOF	Matrix Assisted Laser Desorption Ionization Time-Of-Flight
MDR-TB	Multidrug-resistance tuberculosis
MHz	MegaHertz
mol. equiv.	Molar equivalents
m.p.	Melting point
MS	Mass spectrometry
NBS	*N*-Bromosuccinimide

NMR	Nuclear magnetic resonance
Oxone	$2KHSO_5.KHSO_4.K_2SO_4$($KHSO_5$:potassium peroxomonosulfate)
OPD	*O*-phenylenediamine
PCC	Pyridinium chlorochromate
PCR	Polymerase chain reaction
Petrol	Petroleum spirit (boiling point 40 to 60 ^{o}C)
PPD	Purified Protein Derivative
PPTS	Pyridinium *p*-toluenesulfonate
q	Quartet
s	Singlet
SAM	*S*-adenosyl-*L*-methionine
sat.	Saturated
t	Triplet
TB	Tuberculosis
TBAF	Tetra-*n*-butylammonium fluoride
T-cell	T-lymphocytes
TDM	Trehalose dimycolate
THF	Tetrahydrofuran
THP	Tetrahydropyran
TLC	Thin layer chromatography
TMM	Trehalose monomycolate
TPBSH	2,4,6-Tri-isopropylbenzenesulphonyl hydrazide
TsCl	*p*-Toluenesulfonyl chloride
TST	Tuberculin Skin Test
v.br.	Very broad
UNICEF	United Nations Children's Fund (Previously United Nations International Children's Emergency Fund)
WHO	World Health Organisation
XDR-TB	Extensively drug-resistance tuberculosis

Abstract

The main objective of the work was to develop a analytical method for the diagnosis of TB, and this was achieved in three parts.The first part was involved the synthesis of two oxygenated mycolic acids, a methoxy mycolic acid **(I)** and a keto mycolic acid **(II)**. The successful synthesis of these mycolic acids led to the synthesis of the cord factors TDM **(III)**, TMM **(IV)**, TDM **(V)** and TMM **(VI)**, which were used in modified ELISA assay as new antigens to detect TB-antibodies in serum.





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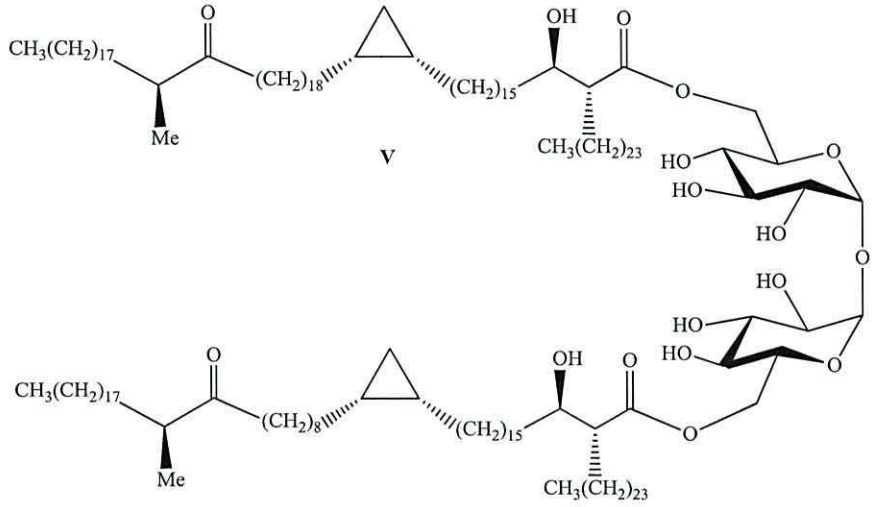

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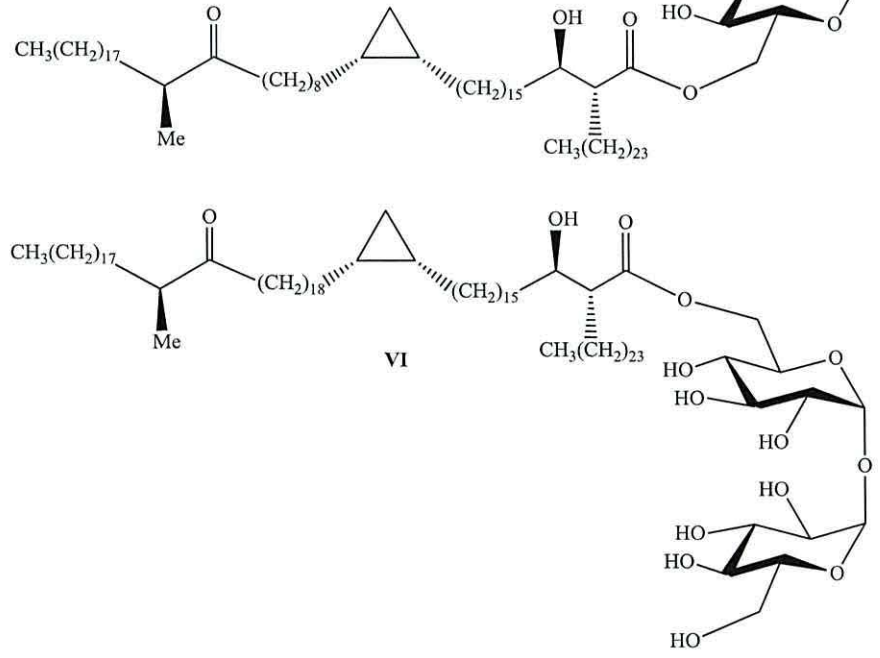
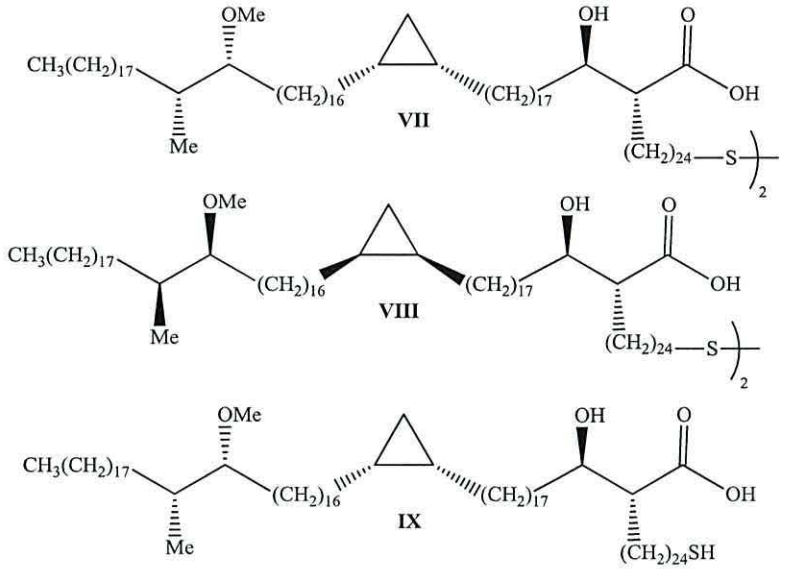


VI

The second part was involved the synthesis of thiolated derivatives **(VII)**, **(VIII)** and **(IX)** of methoxy mycolic acids. These compounds will be covalently attached to a gold surface so as to create a self assembled monolayer with antigenic properties. It is expected that this will create a stable surface for the binding of TB-antibodies in diagnosis tuberculosis tests.. This will contribute to development of a new biosensor as a rapid and accurate method for detecting TB infection.



The third part was concerned on analysing of TB positive and TB negative samples to detect TB-antibodies in patient serum using novel synthetic mycolic acids and their derivatives as antigens in modified ELISA assay, after determination of the optimisation conditions of the ELISA assay. Higher antibody binding signals were observed with cord factors. The sensitivity and selectivity for TDM **(198)** (80%, 87%), TDM **(204)** (75%, 90%) and TDM **(254)** (80%, 84%), respectively which showed good significant values in comparison with other synthetic antigens.

Chapter 1

1- Introduction

1.1 Tuberculosis

1.1.1 What Is Tuberculosis?

Tuberculosis (TB) is an ancient disease that infects humans and is caused by *Mycobacterium tuberculosis.* The term TB comes from the word *tubercle,* which was first used to describe the distinctive nodules in the lungs of TB patients by Franciscus Sylvius in 1679.[1] TB infects mainly the lungs, which are part of the pulmonary system. In addition, TB can infect different parts of the human body, such as the central nervous system, lymph nodes, kidneys, skeleton and indeed almost any organ system, when it is referred to as extra-pulmonary.[2, 3]

Annually, TB kills 1.7 million people in the world, and 9 million new cases are recorded and it is thought to have killed more people than any other microbial pathogen.[4]

Generally, TB is transmitted through the air among people, especially those who suffer from an active infection, via coughing, sneezing, talking or spitting in their environment.[5] An infectious dose of TB is very small and the inhalation of just a single bacterium in one of these transmitted droplets can cause a new infection.[6] TB can also be transmitted easily from infected animals to humans.

During the early stages of latent TB which means someone has been infected with TB infection without showing any symptoms, the diagnosis of TB is very difficult. Conversely, a patient with active TB is characterised by symptoms such as chronic coughing, fever, weight loss and haemoptysis.[7]

1.1.2 History of Tuberculosis

The earliest dating of TB in humans goes to thousands years ago. The bacterium that causes TB is believed to have co-evolved with humans, and has been carried through the rise and fall of ancient civilizations and empires. Current evidence suggests East Africa as the place of origin of *M. tuberculosis*. From its origins in East Africa, TB is believed to have been taken to all the other continents by migrating human populations. Over the preceding millennia, evidence of the disease or the reporting of it has occurred in every major civilization. TB has been noticed in Egyptian

mummies.[8] Indian texts from about 3000 years ago described TB, where it was known as the King of Diseases. TB was found in China 2300 years ago.[9,10] In the Western hemisphere, TB has been detected in Peruvian mummies from the Andean region dating back to before the arrival of Columbus and other Europeans.[11]

The disease was given many names by different scholars and commentators, at different points in time. For example, the Greek physician, Hippocrates, referred to it as 'phthisis',[12] while other literature referred to TB as 'Captain of these men of death'.[13]

The current understanding of TB began to crystallise when French physicians such as Rene Laennac described the pathology of pulmonary TB. In 1865, another French physician, the military surgeon Jean-Antoine Villemin, demonstrated the infectious nature of TB. He infected a rabbit by inoculating it with fluid from the lung cavity of a deceased TB patient.[14] In 1882, the German, Robert Koch, isolated *M. tuberculosis*.[15] Eight years later, he also purified tuberculin from *M. tuberculosis* and initially presented it as a 'treatment' for TB. Tuberculin failed dramatically as a treatment; after several years of research, it was noticed that a positive reaction to tuberculin was caused by latent TB and Koch later realized its greater potential as a diagnostic tool.

During the Industrial Revolution, TB came to be considered as a public health problem, especially when cities became overcrowded and the conditions of life in these cities, as well as health care services, were very poor. These factors led to the rapid spread of TB.[16] Throughout the 18th and 19th centuries, TB infection was the cause of more than 25% of deaths in Europe.[17]

In 1990, there was a reported increase in the deaths of patients with TB, especially compared with deaths caused by other diseases during this time such as through leprosy, malaria, AIDS and tropical diseases.[18] TB was considered as a major health problem in 1993 and the World Health Organization (WHO) declared a global health emergency.[19] The WHO determined the risk of TB and expected an increase in the number of TB infections among people, especially in the developing countries.

In 2009, there were 9.4 million new cases of TB recorded, more than at any time in history.[20] Cases were spread throughout the world, with most in Asia and Africa, and smaller proportions in the eastern Mediterranean region, the European region and the Americas (**Figure 1**). India, China, South Africa, Nigeria and Indonesia occupied the highest places in the world in terms of the total number of cases in 2009.

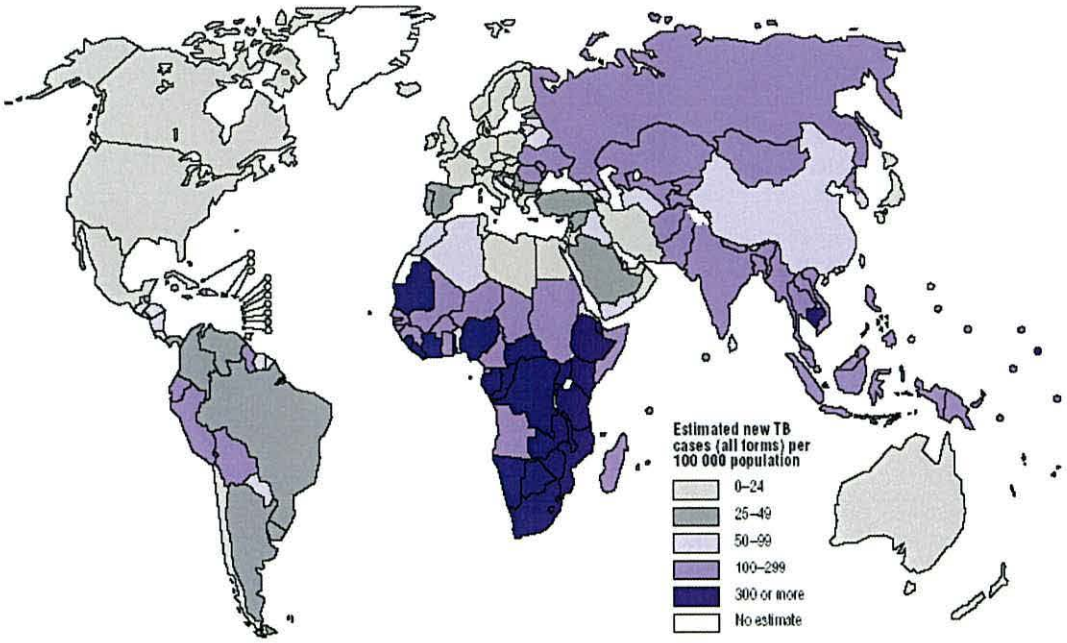


Figure 1: Estimated number of new TB cases per 100,000 population.[20]

1.1.3 TB and AIDS

A primary factor that makes people more likely to be infected with active TB is HIV/AIDS. AIDS patients are immunologically depressed and thus susceptible to being co-infected with TB, which increases the possibility of developing TB from 5 to 15% within a year.[21] This is because TB infection progresses when the bacilli can overcome the immune defence system and are able to multiply. Undoubtedly, the HIV virus is able to stimulate latent TB infection or increase TB development in patients, who are already infected with HIV.[22, 23]

A co-epidemic of AIDS and TB has caused the deaths of millions, not even sparing developed Western countries, where TB had all but disappeared. It was reported that between 1987 and 1993, TB rates increased by 15% in England and Wales. The rate was more than doubled in inner-city London.[24, 25]

The co-epidemic continues today, especially in the poorer, high HIV-burdened countries. WHO studies have reported sub-Saharan Africa and countries in South East Asia as having a high incidence of HIV/AIDS and TB.[26] TB is responsible for at least 25% of HIV deaths.[27] People living with latent TB infection and who are co-infected with HIV are 20 to 30 times more susceptible to developing an active TB infection. The Mycobacterium's incubation time is also shortened by a simultaneous HIV infection.

AIDS destroys the cellular immune system of infected persons. In particular, it targets the CD4+ helper T-cells, which are responsible for the ability of the immune system to remember past exposure to an antigen. This immunity is acquired either from an infection or an immunization vaccine. Thus, HIV/AIDS patients who had previously acquired immunity against TB are at risk of coming down with the disease because of the loss of this central component of their immune system.

1.1.4 Protection

The bacille Calmette–Guérin (BCG) was the first vaccine used against TB. It was discovered over 100 years ago by the French microbiologists, Albert Calmette and Camille Guérin. It was made from attenuated live *Mycobacterium bovis*.[28] The BCG vaccine was first used on humans in France in 1921.[29] At first, using BCG was not consistently successful–sometimes it was accepted and sometimes it was rejected. In 1930, an incident in Lübeck, Germany, temporarily reduced the usage of BCG for TB vaccinations. Infants in Lübeck were vaccinated with BCG, and 76 of them died following the vaccine and 249 other infants almost developed TB. Later, it was discovered that the BCG sample that had been given had been contaminated.[30] Since the 1940s, BCG has prevented TB infection in many people, distinguishing it as the most widely given and safest vaccine. Also, the BCG vaccine has been supported significantly by the WHO and UNICEF.[31]

Currently, the BCG vaccine is available in freeze-dried form. BCG is administered as early as possible in infancy. The immunity conferred, however, decreases by adolescence. The BCG vaccine cannot prevent TB infection in adults; therefore, it cannot be considered as an active vaccine.[32, 33]

Other TB vaccines are currently in development including MVA85A and rBCG.[34, 35] These new vaccines focus on the over-expression of TB antigens or proteins that eventually stimulate T-cell responses.[36] Hopefully, these vaccines will be more effective than BCG against TB and may protect humans and decrease the risk of TB infection in the world.

1.1.5 Treatment for Tuberculosis

TB treatment is a major problem, because it is resistant to most common antibiotics and chemotherapy agents. The first chemotherapy came in the form of the antibiotic streptomycin in 1944. Streptomycin **(1)** was first isolated by Albert Schatz and his co-

workers. It is derived from actinobacterium *Streptomyces griseus.*[37] This discovery was as dramatic as any other in the history of TB and completed the anti-TB arsenal. The use of combination therapy to treat TB has been recommended by the WHO for over half a century. Its recommended first-line treatment for drug-susceptible TB consists of isoniazid (INH) **(2)**, rifampicin (also known as rifampin; RMP) **(3)**, ethambutol (EMB) **(4)** and pyrazinamide (PZA) **(5)**.[38] This combination is taken for two months. For the remaining four months of this Directly Observed Treatment Short-course (DOTS) regimen, only isoniazid and rifampicin are taken. These drugs are used together to provide a multi-pronged sustained assault on bacteria. The aim is to kill all the bacteria infecting the host, as a single cell may give rise to drug-resistant strains. INH and EMB kill the bacteria by interfering with cell wall synthesis, while RMP, synthesised by the soil bacterium, *Amycolatopsis rifamycinica*, inhibits *M. tuberculosis* RNA polymerase.[39] PZA is able to kill bacterial cells hiding in the intracellular compartment of macrophages.

Treating TB within the context of HIV/AIDS co-infection is quite complicated. Interaction between the two drug regimens, DOTS and HAART (Highly Active Anti-retroviral Therapy), can be counter-productive.

According to the British HIV Association (BHIVA), treatment of TB in HIV patients should be delayed as much as possible. TB treatment is given precedence over HIV in patients with a $CD4^+$ count above 100.[40] The use of protease inhibitors is ill-advised during TB treatment.[41]

However, the patient must be tested to rule out the presence of active disease, in order to minimize drug resistance. Misuse of, or noncompliance with treatment can result in the development of resistant strains. This has been a major factor in increasing the incidence of multidrug-resistant TB (MDR-TB), especially in countries such as China, India and the former Soviet Union.[42] Usually, resistance is to INH and RMP.[43] Along with resistance to these two first-line drugs, some people are known to have developed extensively drug-resistant TB (XDR-TB). XDR-TB is very similar to MDR-TB, except that transmission between XDR-TB patients has a much higher mortality rate than between MDR-TB patients.

Treatment of MDR-TB and XDR-TB depends on the drugs and the extent to which there is resistance. MDR-TB treatment must extend for a minimum of 18 months and might also require invasive surgical procedures.[44]

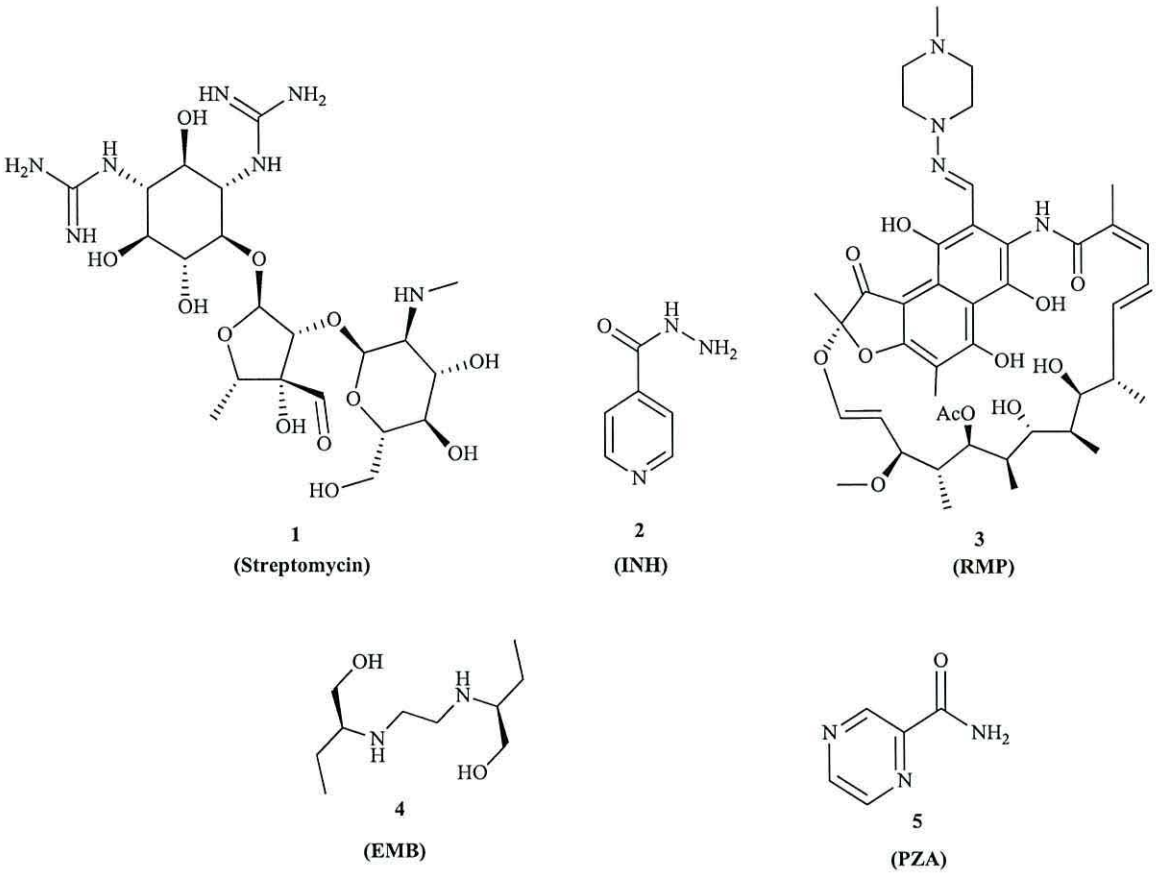


Figure 2: Molecular structures of first-line anti-TB drugs.[38]

1.2 Mycobacteria

1.2.1 Overview

The type of TB that occurs in humans is a result of infection caused by *M. tuberculosis*. It is an aerobic and small rod-shaped bacillus, 1-4 x 0.3-0.6 μm in size, which divides every 16 to 20 hours, (**Figure 3**). This rate of division is slower than that of most other bacteria.[45] It is classified as acid fast because during the staining process the bacilli are not decolourised by acids. On other hand, it can be observed by using a fluorescence microscope after addition of the Ziehl–Neelsen stain, which turns the bacilli red.[46] This organism has the ability to survive for a long time in dry conditions without any effect on its growth.

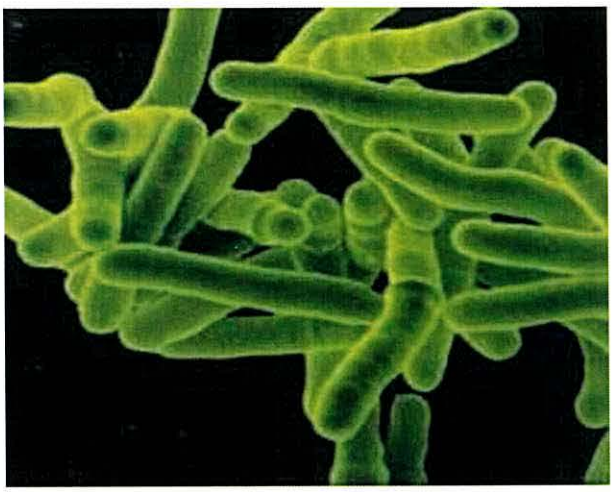


Figure 3: Scanning electron micrograph of M. tb.[47]

M. tuberculosis is responsible for causing tuberculosis in humans but there are several other mycobacteria that cause tuberculosis in different animals. The differences in these types can be determined by their shape, colour, their virulence and the temperature at which they grow. *M. bovis* infects cattle and can be transferred to humans through an infected cow's milk, raw meat or as an aerosol.[48] *Mycobacterium marinum* was isolated in 1926 from a salt water fish by J.D. Aronson,[49] and it causes tuberculosis in fish.[50] *Mycobacterium microti* causes tuberculosis disease in animals, especially voles, wood mice and shrews.[51] Recently, *Mycobacterium microti* has been found in humans in the Netherlands.[52] Another pathogenic Mycobacterium is *Mycobacterium avium,* responsible for disease in birds.[53]

There are other strains of Mycobacterium that do not develop into TB. For example *Mycobacterium fortuitum* causes diseases in soft tissues, skin and lungs.[54] In 1884, Lustgrat was the first to find and isolate *Mycobacterium smegmatis,* a type that is found in soil, water and plants which causes granulomas in soft tissues.[55,56] *Mycobacterium ulcerans* has been reported to cause skin lesions in humans and also produces the condition known as a Buruli ulcer.[57] *Mycobacterium kansasii* can be found in the environment such as in water, soil, foods and a variety of animals, and is most commonly associated with AIDS.[45] Leprosy is caused by *Mycobacterium leprae.* Recently, the spread of this disease has decreased worldwide by about 90% and more than 13 million patients have recovered. Leprosy was removed from the list of global public health problems in 2000.[58]

1.2.2 The Mycobacterial Cell Envelope

The genus Mycobacterium is characterised by a cell wall made up of very long, complex waxes called mycolic acids. Dobson *et al.* proposed a classification of these cells based on the diversity of their cell wall lipid structures.[59] The Mycobacterium has a high resistance to most available antibiotics and chemotherapeutic agents because of the composition of the bacterial cell wall.[60,61]

Generally, the cell envelope of the Mycobacterium (**Figure 4**) is mainly composed of three structural layers: the plasma membrane (the internal layer), the cell wall and the capsule (the external layer). The plasma membrane composition is typical of other types of living organisms. The capsule mainly consists of polysaccharides, proteins and lipids.[62]

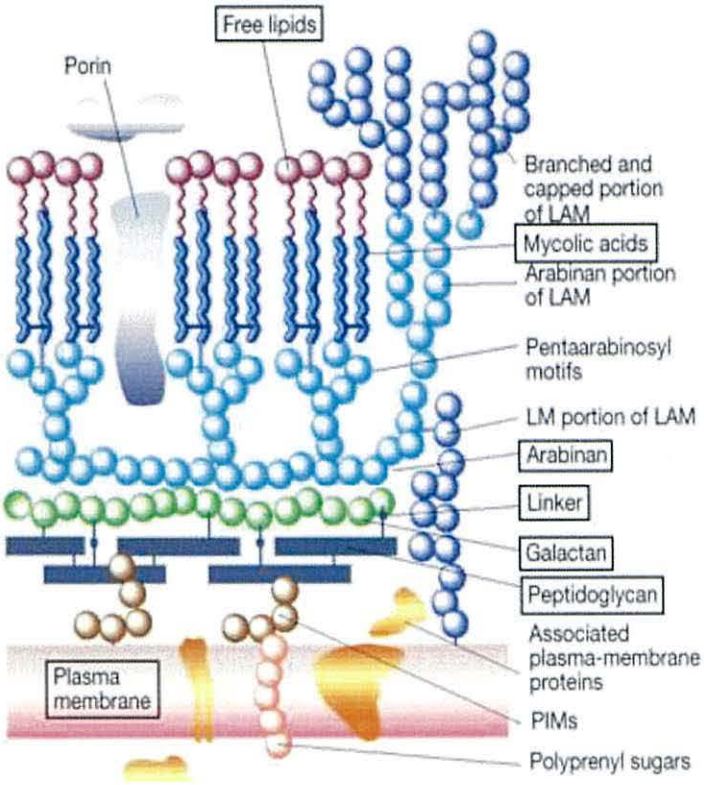


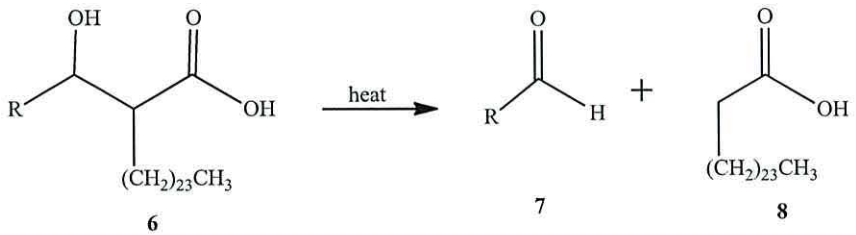
Figure 4: The cell envelope of mycobacteria.[63]

The cell wall consists of covalently bound sub-units of peptidoglycan (*N*-acetylglucosamine and *N*-acetylmuramic acid),[64] with arabinogalactan (polysaccharides) as the internal layer and a series of very long-chain fatty acids, called mycolic acids, as the external layer, which are bound to the arabinogalactan through the carboxylic acid groups. In 1938, Anderson *et al.* reported the isolation of the major lipid component (mycolic acid) of the *M. tuberculosis* cell wall, which

contributes more than 40% of the dry weight of the cell wall.[45,65] The mycolic acids are linked parallel to one another and perpendicular to the internal layer.[66,67,68] It is thought that mycolic acid has a role in the permeability of the external cell envelope of the bacteria and that it makes the mycobacterium more resistant to drugs.

1.3 Mycolic Acids

Mycolic acids are high-molecular-weight α-alkyl, β-hydroxy fatty acids found in the cell envelope of *Mycobacterium* types. They have chain lengths of between C_{60} and C_{90} and have a large α-branch (C_{20}–C_{25}).[69,70] Their name was suggested by Anderson, who described the presence of one carboxylic, one hydroxyl and one methoxy group.[65] Asselineau and Lederer confirmed the position of the hydroxyl group in relation to the carboxylic acid, and the presence of a long alkyl chain at the α-position, based on the pyrolysis of mycolic acids (**Scheme 1**).[71]



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Scheme 1: Pyrolysis of a mycolic acid.

Generally, the structure of mycobacterial mycolic acid has two parts: a main chain, called meromycolate, and mycolatemotif. The hydroxyl group and α-alkyl branch are in the (*R,R*) configuration in the structure of the mycolatemotif, which is the same in all types of mycolic acid, with there being only a small difference in the length of the chain at the α-position. The meromycolate main chain possesses two functional groups in the distal position (X) and proximal position (Y). The general structure of mycolic acids is shown in **Figure 5**.

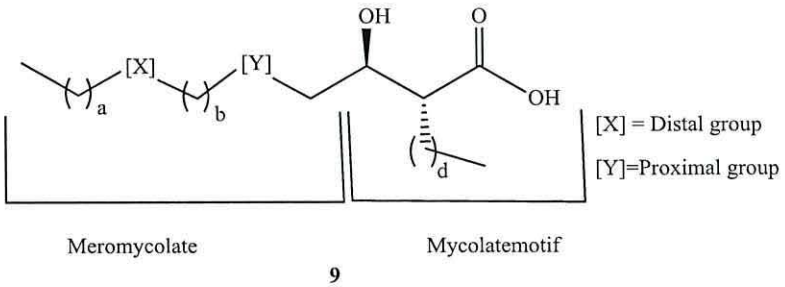

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Figure 5: The general structure of mycolic acids.

There are many types of mycolic acids, according to chain length and the presence of functional groups in the meromycolate chain. These functional groups can be cyclopropanes, double bonds or an epoxy group, methoxy group, carbonyl group or methyl group.5 Separation of mycolic acid types has been achieved by using different analytical methods, such as thin layer chromatography (TLC),[59,72,73] gas chromatography (GC)[74] and high-performance liquid chromatography (HPLC).[75,76,77]. In addition, infrared spectroscopy (IR), nuclear magnetic resonance (NMR) and mass spectrometry (MS) have been used to complete mycolic acid determination.

According to the nature of the functional groups in the meromycolate chain, Minnikin and Polgar reported three main types of mycolic acids in the cell wall of *M. tuberculosis*.[78,79,80] These three types were called α-mycolic acids, which contain two cyclopropyl rings generally in the *cis*-configuration **(10)**, methoxy **(11)** and keto **(12)** mycolic acids, which have oxygenated functional groups in distal positions (**Figure 6**). The methoxy mycolic acid is substituted in the distal position with a methoxy group while the keto mycolic acid is substituted in the distal position with a carbonyl group. Both the methoxy and keto mycolic acids have a methyl branch in the α-position with respect to the oxygenated functional groups.

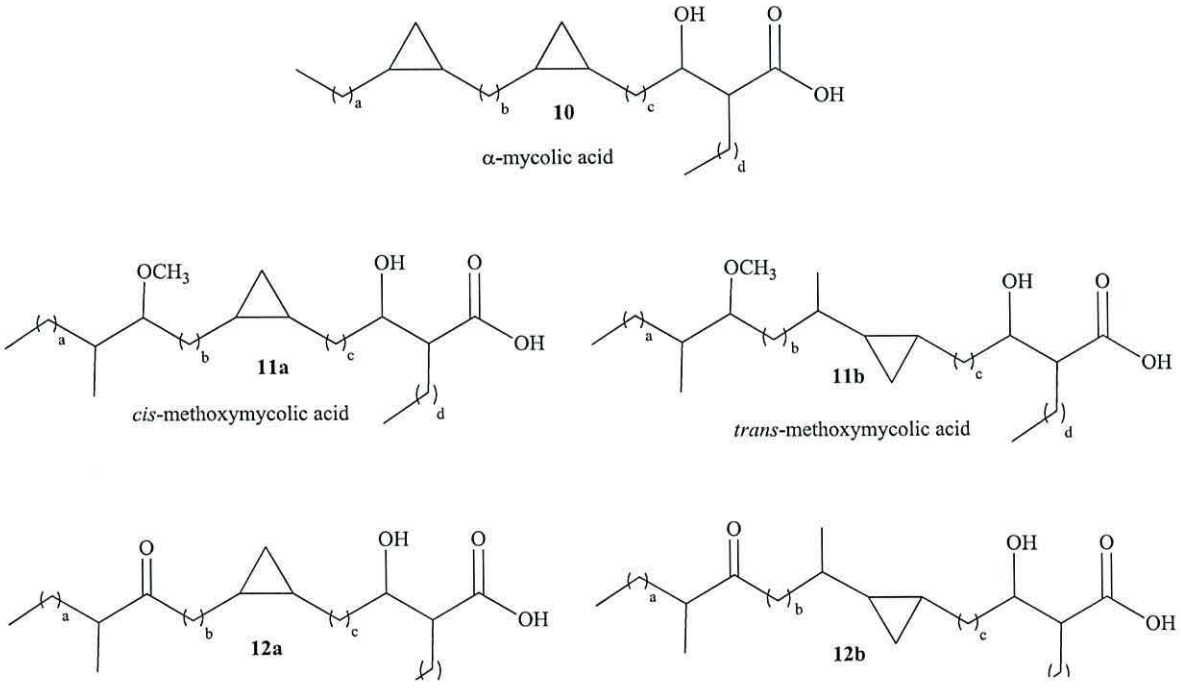


Figure 6: Schematic overview of the major types of mycolic acids in M. tuberculosis.

Other types of mycolic acids (**Figure 7**) have been found in other species of *Mycobacterium* in different sets. *M. smegmatis* contains α' and α-mycolic acids **(13)** and **(14)** with either one or two double bonds, either in the *cis* or the *trans* configuration.[81] *M. chelonae* contains α′-mycolates with a single *cis*-double bond and a small molecular size.[82,83] The epoxy-mycolic acids **(15)**, with an epoxy ring were isolated from *M. fortuitum.*[70,81] Other types of oxygenated mycolic acid exist, such as ω-carboxy-mycolic acid **(16)** which has been isolated from *Mycobacterium phlei,*[84] while ω-1-methoxy-mycolic acid **(17)** has been isolated from *Mycobacterium alvei.*[85] *M. avium* contains a different oxygenated mycolic acid, called a wax ester mycolic acid **(18)**.[86] Hydroxy-mycolic acid **(19)** is a new type of mycolic acid which has been found in small amounts in *M. bovis* BCG, *M. smegmatis* and *M. tuberculosis.*[87,88]

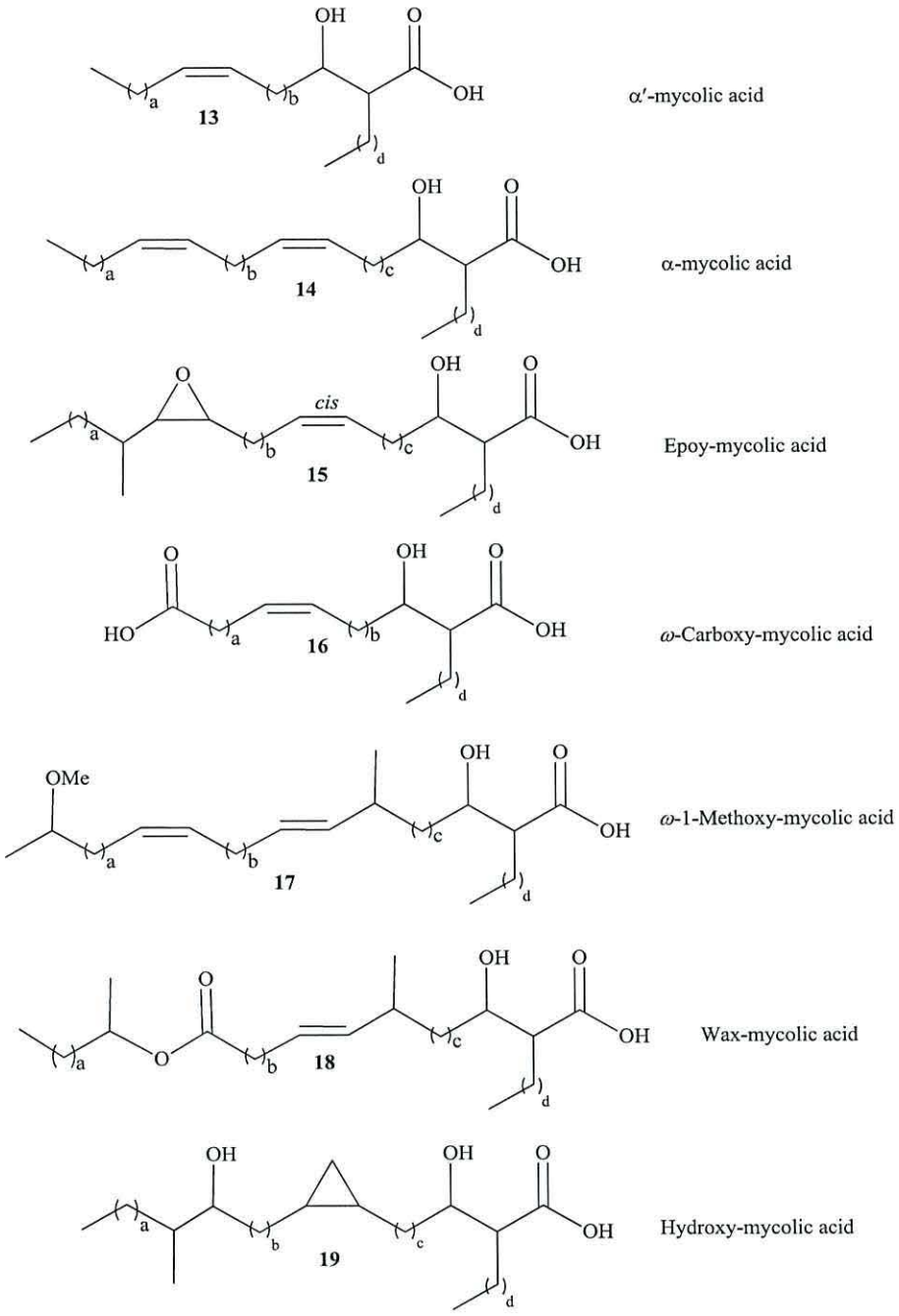
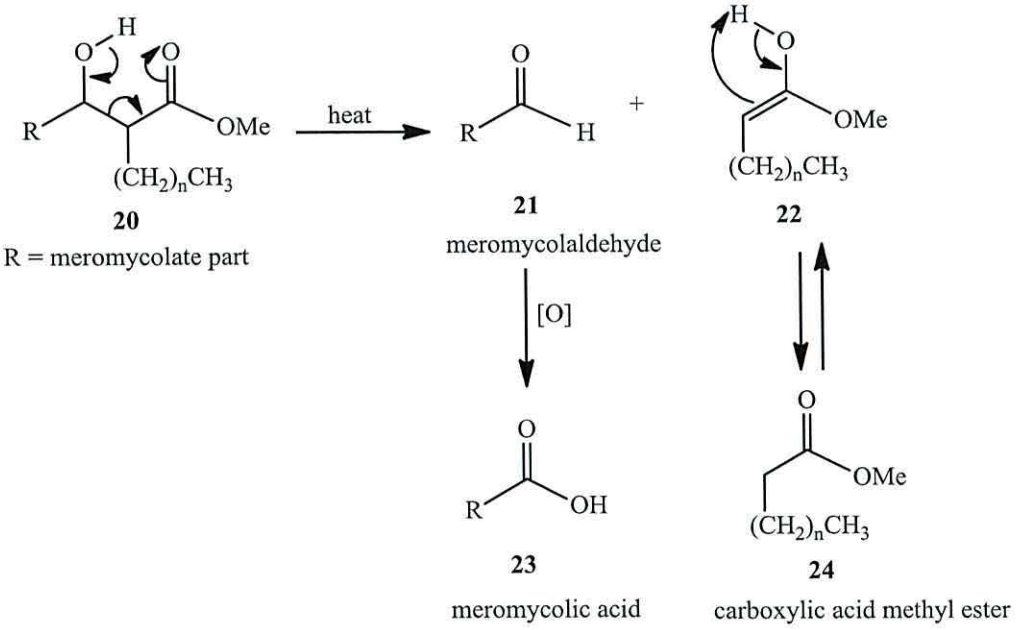


Figure 7: Schematic overview of types of mycolic acids from other mycobacteria

In all mycobacteria, there are not only different types of mycolic acids, but also different homologues for each of them. This variety has made the separation of mycolic acid and the determination of its real structure very difficult. The identification of the molecular weight of every homologue, and particularly the location of the functional groups in each mycolic acid has been particularly challenging.

Recently, MALDI-TOF spectroscopy has provided a rapid and highly sensitive analytical technique for analysis of mycolic acids and other lipids. Initially, Laval *et al.* used MALDI-TOF spectroscopy to analyse the length of the total carbon chain of the major types of mycolic acids of different mycobacteria.[89] In further studies, Watanabe *et al.* also used ^{1}H NMR and MALDI-TOF spectroscopy to determine the nature and the location of the functional group in the meromycolate chains of mycolic acids from different species of *Mycobacterium*.[90,91] Firstly, they separated the saturated, *cis* and *trans* methyl esters of the mycolic acids into different types by argentation chromatography, according to the method of Krembel and Etémadi.[92] The meromycolic acids were prepared by pyrolysis of the methyl ester, which was heated at 300 °C under a vacuum to give meromycolaldehydes and carboxylic acid methyl esters. Then, the meromycolaldehydes were oxidised to the corresponding meromycolic acids by using silver oxide, **(Scheme 2)**.



Scheme 2: Degradation of mycolic acid.

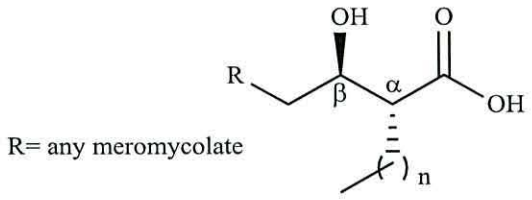
The meromycolic acids were analysed using collision-induced dissociation mass spectrometry (CID MS) and over 50 different ones were found, which were divided into α-mycolic acid, methoxy-mycolic acid and ketomycolic acid.

1.3.1 The Stereochemistry of Mycolic Acids

Although there are many studies that have been carried out to understand the stereochemistry of mycolic acids, and the determination of the stereochemistry of the functional groups and, in particular the *cis*-cyclopropane has been particularly difficult. The determination of the structure and classification of mycolic acid types has been achieved by using two-dimensional TLC, GC, HPLC, IR, NMR, MS and MALDI-TOF mass spectrometry techniques. The configuration (*R* or *S*) of the functional groups in mycolic acid cannot be distinguished by using the same techniques, therefore, only polarimetry is available to determine specific rotations and relate these to particular stereoisomers. The stereochemistry of full mycolic acids has been determined by breaking them down into smaller sub-units and comparing these with known compounds.[93]

Quémard *et al.*[87] explained the use of molecular rotations (M_D) to determine the configuration of the stereocentres in natural mycolic acids. The configuration of the complete mycolic acid was estimated by measuring the M_D from the contribution of each chiral centre present in mycolic acid and then comparing the results with the same units of synthetic mycolic acid.

The motif part has two chiral centres in the α and β positions relative to the carboxylic acid in all types of mycolic acid. The stereochemistry has been found to be an *R,R*-configuration and both centres are *anti* to each other, **(Figure 8)**.[79,81,93,94,95]



25

Figure 8: The configuration of the motif part of mycolic acid.

The configuration *(R,R)* of the hydroxyl group and the alkyl chain in the motif affects the molecular packing in the cell wall of the *Mycobacterium*. However, a hydrogen bond formed between the carboxyl group and hydroxyl group has a stabilising effect for the aligned conformation between the two long chains.[96,97,98] This configuration is essentially for the recognition of the mycolic acids by T-cells and the generation of an immune response by the host organism against TB.[99]

The study of meromycolate stereochemistry based on degradation and molecular rotation has also shown that the hydroxyl **(26)**, methoxy **(27)**, and keto **(28)** groups in the mycolic acids, and the methyl branch next to the oxygenated groups are in the *S*-configuration, (**Figure 9**). The methyl branch of the wax ester (**29**) appears in the *S*-configuration, which is derived from ketomycolic acid by an enzymatic oxidation.[88,100,101,102]

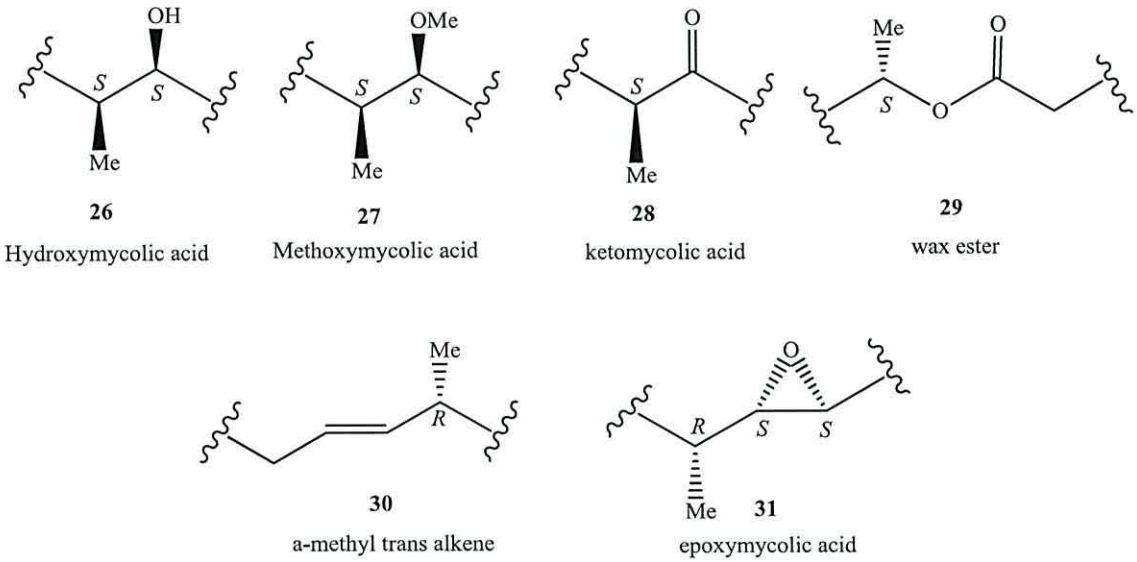


Figure 9: The stereochemistry of chiral centers of the mycolic acids.

Other studies established the stereochemistry of α-methyl-*trans*-alkene **(30)** as in the *R*-configuration.[93] The α-methyl-*trans*-unit in epoxy-mycolic acid **(31)** of *M.fortuitum* was reported to have an *R*-configuration,[93] and recently the stereochemistry of the epoxide ring was reported in *S,S*-configuration.[103]

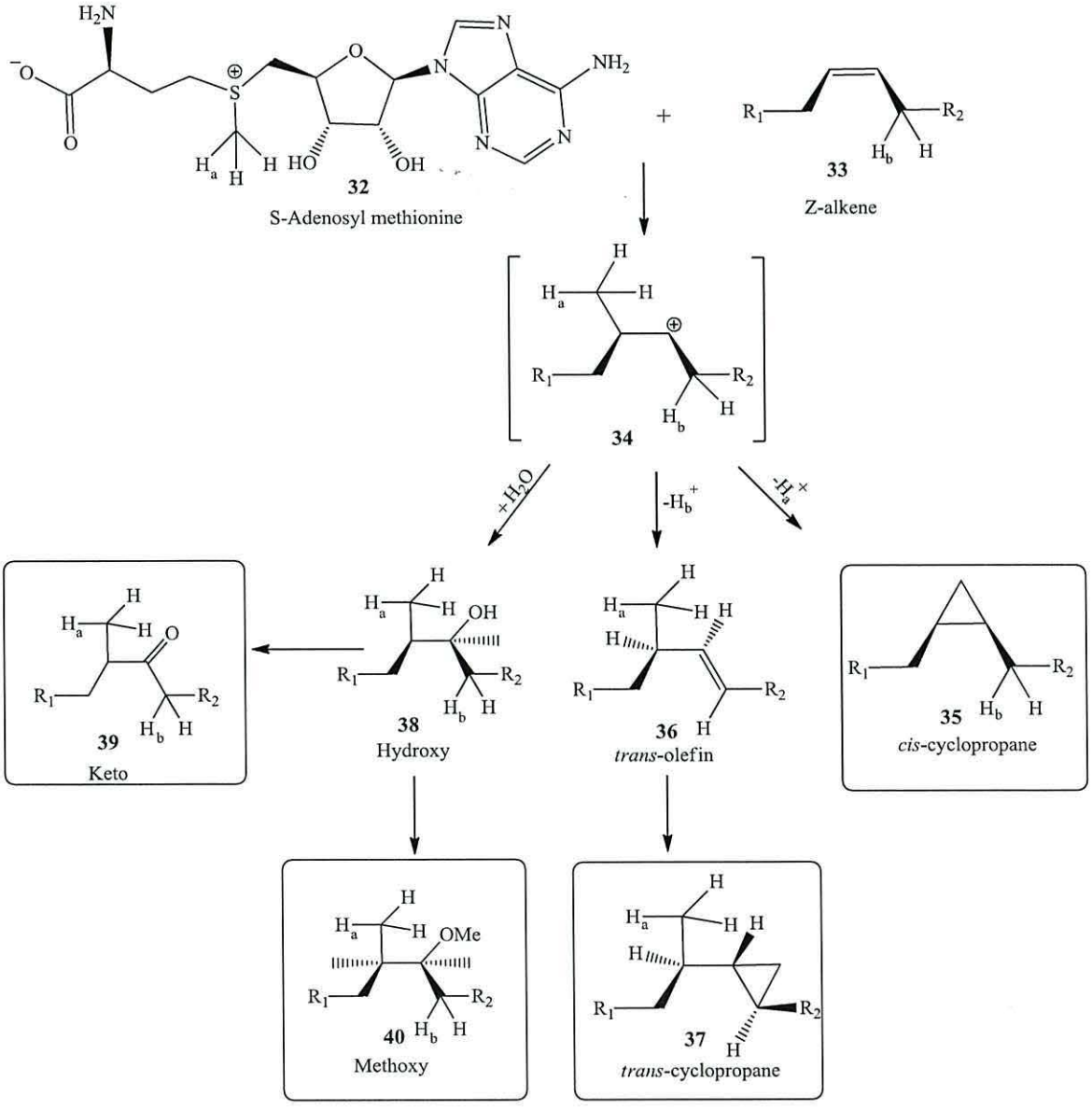
1.3.2 Biosynthesis of Mycolic Acids

Many studies have been carried out to try and understand the biosynthesis of mycolic acids due to the fact that they play an important role in the cell wall of the Mycobacterium,[69,70] and to study and identify all possible targets for drugs against TB. The biosynthesis of mycolic acid can be divided into four stages:

1. Synthesis of straight chain fatty acids up to C_{26} to provide the α-alkyl branch.
2. Synthesis of C_{18}-C_{50} fatty acid to provide the main meromycolic chain.
3. Modification of the meromycolate chain to introduce different functional groups.
4. A final condensation step to give the full mycolic acid.

Several different mechanisms have been suggested for the processes of cyclopropanation, oxygenation and methylation in the biosynthesis of mycolic acids. Studies have been conducted in which labelled methionine was incorporated in the growth of mycobacteria and it has been found that the methyl group of methionine can be directly integrated into mycolic acids. It has also been shown that the bridging methylenes of the cyclopropane rings, the carbon of the methoxy and the methyl branches adjacent to the *trans*-olefins, methoxy and keto moieties are all derived from methionine, presumably via *S*-adenosyl-*L*-methionine (SAM).[93,104,105,106]

Initially, there is transfer of a methyl group from *S*-adenosyl methionine (SAM) **(32)** to the *Z*-alkene **(33)** to give a carbocation intermediate **(34) (Scheme 3)**[70].



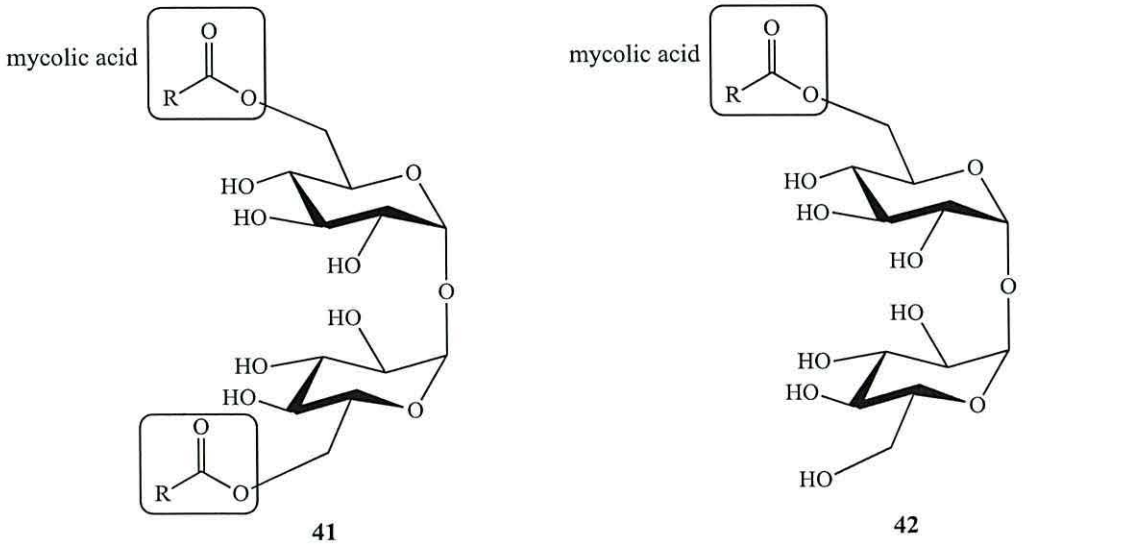
Scheme 3: The mechanism of the biosynthesis of functional groups in meromycolate.[70]

Deprotonation of the carbocation intermediate gives two different products depending on the position of the hydrogen. Deprotonation of H_a yields the *cis*-cyclopropane **(35)**, whereas deprotonation of H_b yields the *trans*-alkene **(36)**, which is converted into *trans*-cyclopropane **(37)** after a second reaction with *S*-adenosyl methionine. The hydroxy-mycolate **(38)** is formed as a result of the hydration of the carbenium ion, which is a common precursor for the biosynthesis of the oxygenated keto **(39)** and methoxy **(40)** mycolates.

1.4 Cord Factor

The cell walls of mycobacteria contain a variety of lipids in addition to mycolic acid.[70] The cord factor is mainly considered as one of these lipids. It is classified as a free lipid because it is not bond to the arabinogalactan in the cell wall. It is easy to extract and liberate with a suitable solvent, while mycolic acids bound to the arabinogalactan cannot be liberated from the cell wall without hydrolysis.

A cord factor consists of a trehalose (1α-1α'-diglycoside) esterified on the primary alcohol groups with mycolic acids. Cord factor is a complex glycolipid and much of this complexity derives from variation in the combination of mycolic acid esterified to the disaccharide. The molecular masses vary widely because the mycolic acids can be from different classes, which are further composed of homologues. Cord factor is mainly divided into two classes **(Figure 10)**: trehalose dimycolate (TDM) and trehalose monomycolate (TMM).



41 **Trehalose dimycolate (TDM)**

42 **Trehalose monomycolate (TMM)**

Figure 10: Generalised structures of cord factors (TDM, TMM).

Koch studied cord factors as early as 1884, when he showed that certain tubercle bacilli formed long strands or cords.[107]In 1950, Bloch isolated the glycolipid from the sticky substance on the surface of tubercle bacilli cells and named this cord factor.[108] He extracted four glycolipids from different types with petrol and studied the toxicity of the cord factors on mice. Noll *et al.* first purified cord factors in 1956,[109] and they could determine the structure of the cord factor and proposed it to be a 6,6'-dimycolate of α, α-D-trehalose **(43)**, **(Figure 11)**.

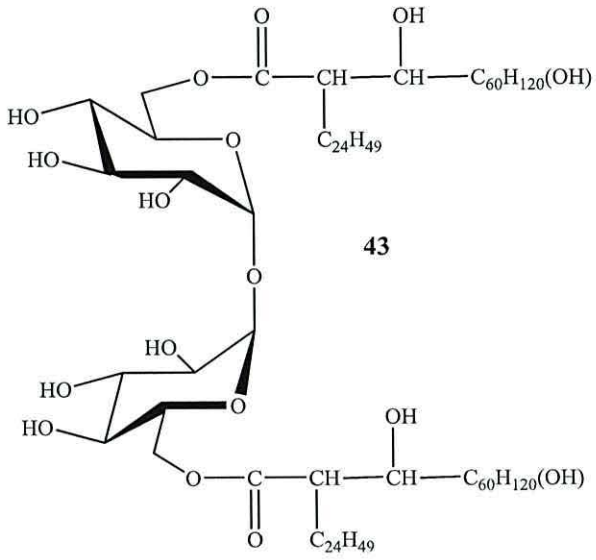


Figure 11: The structure of the 6,6-dimycolate of α,α–D-trehalose proposed by Noll et al.[109]

The glycolipid was isolated from *Corynebacterium diphtheria* by Ioneda *et al.* in 1963.[110] Puzo *et al.* isolated and identified three different compounds from *Corynebacterium matruchotii* by TLC, one of them called true coryno-cord factor and the other two were oxoacyl containing trehaloses. They used MS to determine their structures.[111] The first time the MALDI-TOF mass spectrometry technique was used to analyse TDM and TMM from nine types of Mycobacterium was in the work of Fujita *et al.*[112,113] They found extreme structural diversity in the molecules. In *M. tuberculosis* $H_{37}Rv$ and Aoyama B 60 different molecular mass ion species were found, while *M. bovis Connaught* had less than 35. *M. tuberculosis* had a higher diversity because of the presence of three types of mycolic acid (alpha, keto and methoxy). In addition, Kai *et al.*[114] used MALDI-TOF MS to determine the structure of TDM and TMM, isolated from *M. leprae* and *M. bovis* BCG.

1.5 TB Diagnosis

Diagnosing TB is complicated because the causative *M. tuberculosis* is one of several related bacilli. The inability to diagnose TB early and give correct treatment before the active assault of the disease is one of the main difficulties in controlling TB. Current diagnostic methods for TB are inadequate for treating TB in developing countries with increasing numbers of people being infected. Most tests are unable to distinguish between latent and active TB. It is also necessary to distinguish between TB and inflammatory non-tuberculosis mycobacteria (NTM), which are more prevalent in people with a compromised immune system. The best way to diagnose TB should be safe for people who are working on TB diagnosis so that other people do not become infected.

For several years, the Tuberculin Skin Test (TST) was the only diagnostic tool available for TB.[115] It was used in mass screening campaigns. In the latter decades of the 20th century, several new tests became available. However, most have a limited specificity (the percentage of true TB-negatives detected) for the tubercle bacilli. Also, the sensitivities (the percentage of true TB-positives detected) are often limited because the Mycobacterium is a slow grower, doubling only every 15 to 20 hours. The culture method from sputum or body fluid specimens is considered the gold standard for the diagnosis of TB because it has high sensitivity (80-85%) and high specificity (98%) but it takes a long time (about up to 8 weeks) to exclude active TB, and by that time, the patient may have died. The effectiveness of the culture method is very low in children.[116,117]

According to the WHO, a test for TB should have greater than 85% specificity and 85% sensitivity.[118] With the advent of the HIV/AIDS pandemic, diagnosing TB has been complicated further. Another factor, affordability, has been introduced. The advantages and disadvantages of different diagnostic classes will be discussed next.

1.5.1 Tuberculin Skin Test (TST)

The oldest TB test is the TST. It is often referred to as the Mantoux test or the Purified Protein Derivative (PPD) test. It was first discovered by Robert Koch and developed for diagnosis by Charles Mantoux. The test is based on the injection of 0.1 mL of tuberculin (PPD) under the surface of the skin of the forearm. The result will be positive when the rise of pale skin, 6 to 10 mm in diameter, is observed 48 to 72 hours

after injection, due to the human body's immune response against the bacteria.[119] The reading is dependent on a measurement of the swelling.

The TST showed great value in the first half of the 20th century, but its reliability became suspect when mass BCG vaccination was undertaken as a public programme. Individuals vaccinated against TB with BCG gave a false-positive response to the TST.[120] This is because the PPD is a mixture of proteins with antigenicity common to many members of the *Mycobacterium* genus. Additionally, HIV-positive patients with a low $CD4^+$ cell count might give a false-negative result, which results in low specificity for the test. The TST is also unable to distinguish between persons infected with non-TB Mycobacterium, such as *Mycobacterium avium,* or between those with an active infection and those with the latent form.[121] In addition, if a TST gives a false-positive result, this will lead to inappropriate treatment for the patient and is a waste of health care resources.[122] Today, the TST is mainly used in the United States, where the BCG vaccine is not given routinely to newborns.

The sensitivity of the TST also does not meet the WHO's prescribed standards. In non-HIV-infected patients, the sensitivity is about 70%. This statistic drops dramatically to as low as 30% in AIDS patients.[115] Hence, the test is ruled out in HIV/AIDS-burdened countries.

1.5.2 Radiographic Techniques

A chest X-ray of a patient with active pulmonary TB shows lesions on the lungs caused by granuloma formation. Even after full recovery, the scar tissue remains visible for several years. This means that a specialist, a roentgenologist, is often needed to accurately interpret the X-ray films.[123] Interpretation of the films is further complicated when the patient is co-infected with HIV, the modern presentation of many TB cases. Extra-pulmonary TB actually shows no lesions on the lungs and the risk of a false-negative diagnosis is greatly increased. Other tests for TB need to be carried out to confirm a negative diagnosis.[123]

X-ray diagnosis of TB is generally obsolete today. In spite of being fast, it lacks specificity and is of a low sensitivity, as only active and advanced disease can be detected. It can be relied on only in cases where there is no evidence of HIV/AIDS disease and no previous history of TB infection. The requirement for specialized equipment and experts to interpret results also limits dependence on X-rays for effective TB diagnosis. This test usually has to be run with the TST or sputum smears.

1.5.3 Interferon-γ

Interferon-γ (IFN- γ) is considered to be a new method for TB diagnosis *in vitro*. The principle of the IFN-γ assay is the stimulation of T-cells to produce IFN-γ when they re-encounter the antigen of *M. tuberculosis*.[124,125] The result of the assay depends on cell-mediated immunity by measuring IFN-γ released from T-cells in response to TB antigens. It is quicker than TST, giving a result within 24 hours. The results are not affected in people who have previously had the BCG vaccination.[126] IFN-γ results can be affected by disease severity and treatment, especially in patients with advanced disease or in those who have completed treatment. Additionally, the serum samples must be processed within 12 hours of collection and correctly stored; failure to do so will lead to a decrease in the accuracy of the test. This method detects only latent TB for certain, as active TB will suppress IFN-γ, which may have limited value for estimating infectious TB in high-endemic countries.[127,128] These factors can be affected on the sensitivity of the test.

1.5.4 The Sputum Smear

The sputum smear method relies on direct observation of mycobacteria under a light microscope. This method is significantly faster and less expensive when compared with other methods. Although often referred to as the sputum smear test, practically any body fluid, and even tissue biopsies, can be stained for direct microscopy. The stain of choice is the Ziehl–Neelsen stain, which detects acid-fast bacteria. *M. tuberculosis* is not the only acid-fast bacilli; therefore, the test cannot distinguish among the various possible mycobacterial types. In addition, to be detectable by this staining method, a minimum of 10,000 bacilli/mL sputum is required for sensitivity. The method's specificity is low and it cannot be used alone to exclude an infection.[129] Using microscopic observation directly is not valid in diagnosing children or for extra-pulmonary TB.[119,123] Additionally, as observation requires a relatively high bacterial titre, therefore, some results may be false negatives.[130] This means that early diagnosis is severely limited with this testing method and that sensitivity is low.

1.5.5 The Culture Method

The culture method is considered the gold standard for diagnosing TB. It improves the sensitivity of the microscopic testing by culturing a patient's tissue sample over several weeks. Sensitivity is increased at the expense of time.

However, the culture method requires four to eight weeks for completion because of the slow growth of *M. tuberculosis,* which may make microbiological growth more at risk of exposure to contamination.[131,132]

The presence of bacilli in the test sample is necessary when determining the sensitivity of the culture. Application of the culture method for patients with HIV and for children is difficult because of the problems associated with the production of sputum from them. Patients with HIV are more likely to die because of the risk of the growth of the Immune reconstitution inflammatory syndrome (IRIS) factor. Patients with active pulmonary TB may have the bacilli protected in the lung cavities, or it may be absent from a particular sputum sample, and thus may lead to incorrect diagnosis and the incorrect subsequent TB treatment.[133]

1.5.6 Polymerase Chain Reactions

The polymerase chain reaction (PCR) is a rapid method to diagnose diseases, identify bacteria and viruses. Amplification of DNA from the mycobacteria with polymerase chain reaction technology has introduced a powerful, highly sensitive and specific tool for TB diagnosis.[131,134] Results are ready in a few hours and require a very limited amount of genetic material for detection. This is a highly sensitive and specific method but it is relatively expensive and requires specialized equipment.[135] This method may be prone to cross contamination of genetic materials, and as a result it can give incorrect results. Furthermore, it is unable to distinguish between live or dead bacteria, therefore it is not be used with patients receiving treatment.[136]

1.5.7 Serological Tests

Serological testing for TB is not new; in fact, the TST is one such test. These tests try to detect antibodies and other immune-response factors elicited by the host in response to the presence of the mycobacteria. The antibodies are specific for the bacterial antigens, which are often polypeptides. These tests are based on using any component of the Mycobacterium cell wall as the derived antigen, but they are limited in terms of sensitivity and specificity.[137,138] Testing for TB from a serum sample is attractive as a point-of-care test because of the relative ease of drawing blood from a patient, as compared to extracting several deep-throat sputum samples. Patients with extra-pulmonary TB or children are incapable of producing quality sputum samples. In

addition, the detection of anti-TB serum factors can potentially show the presence of extra-pulmonary TB, which is currently difficult with sputum tests.

The current commercially available serological tests have variable accuracy levels.[139] In 2011, the WHO released a policy statement recommending that none of the commercially available serodiagnostic tests should be used for either pulmonary or extra-pulmonary TB.[140] This conclusion was reached after reviewing 67 pulmonary TB and 27 extra-pulmonary TB studies, in which large variability was found in the sensitivity and specificity of the tests. When compared to mycobacterial culture tests, the variability in sensitivity was 1% to 60%, while specificity varied between 53% and 99%. This was the first negative policy statement on TB issued by the global health body, and it highlights the difficulty in diagnosing the disease.

1.5.7.1 Biosensor Detection of TB

The use of biosensors to detect TB is based on an immune-assay protocol, in which the serum of TB patients contains antibodies that can be detected by binding with an antigen. Thanyani *et al.*[141] designed an Interaction Assay System (IAsys) affinity biosensor for the detection of TB infection, including HIV/AIDS co-infections. The response of this biosensor depends on the binding between the antigen (natural mycolic acid) and antibody by measuring the change in the refractive index. This work depends on the phenomenon of light exciting surface plasmons on a planar, highly refractive glass surface coated with a microscopic layer of gold and is known as Surface Plasmon Resonance (SPR). SPR is generated at a specific angle and wavelength of incident light passing through a prism to create a surface evanescent wave in the waveguide.[142] An electrochemical biosensor was used for distinguishing between TB-positive and TB-negative results, which was reported by Mathebula *et al.*[143] This measurement response is based on electrochemistry that measures the change in electroimpedance spectroscopy. This typically has a reference electrode, an active electrode and a sink electrode. Electrons generated at the active electrode due to the detection process create an electrical potential difference between the reference and active electrodes. They inserted mycolic acid into a self-assembled monolayer of *N*-(2-mercaptoethyl) octadecanamide on a gold electrode. Other biosensors have also been reported for diagnosing TB.[144,145,146] The advantages of biosensors are their simplicity and real-time and *in situ* analysis capabilities. However, there have been many

attempts to improve the sensitivity and the selectivity of these biosensors, to extend their application on large groups of people who are infected with TB.

1.5.7.2 ELISA

ELISA means enzyme-linked immunosorbent assay (ELISA). It is a simple and powerful clinical test that is often used in initial diagnoses for many common infectious diseases such as HIV/AIDS.

ELISA was developed largely as a safer immunoassay to replace radioimmunoassays. The routine use of radioactively labelled compounds posed a health risk to health workers. Enzyme-linked chromogens replaced the radioactively labelled compounds as the reporter labels. The various aspects or components of the ELISA system were developed independently by different researchers. Engvall and Perlmann, working independently, are credited for the development of the ELISA technique in the early 1970s, but their breakthrough was the result of building on the work of other researchers.[147] These researchers are credited with developing the technique to link an enzyme to an antibody.[148,149] Wide developed the immunosorbency technique, which is where the antibody or antigen is non-covalently immobilized on a hydrophobic substrate.[150]

1.5.7.2.1 Methodology

The basic principle of an enzyme-catalysed reaction being the reporter label of an antibody–antigen reaction has generally been retained. An antigen is first immobilized in the wells of a microtitre plate. A 96-well microtitre (**Figure 12**) made of plastic is often used.

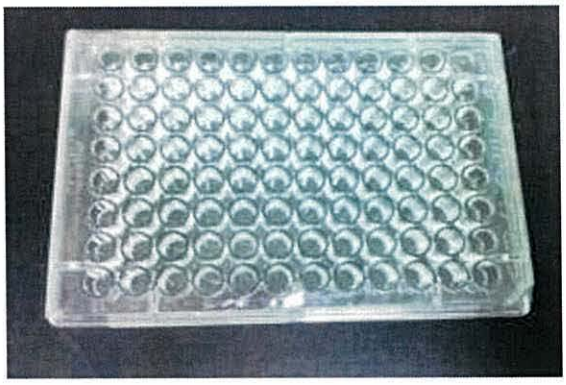


Figure 12: A 96-well microtitre plate.

Next, a protein that does not react with the antigen such as bovine serum albumin or casein is used to block the exposed plastic surfaces of the wells where no binding of the antigen has occurred. A solution of the desired antibody specific for the antigen is then applied. This may be a buffered solution of serum, whole blood, saliva or some other biological fluid. Excess unbound antibody is washed away. This step is the major action of the ELISA. Only high-affinity antibodies remain after washing and, hence, are detected by this method. The next step, however, is the major strength of ELISA. Another antibody solution (a secondary antibody) is applied to the plate. Importantly, this secondary antibody is conjugated to an enzyme. A common enzyme used is horseradish peroxidase. When the enzyme's substrate is applied to the well for a brief period, a chromogenic enzyme-catalysed reaction occurs. Wells with bound secondary antibodies will produce a coloured product. The colour intensity is read quantitatively by a spectrometer. The advantage of this colour development is that even if very little of the suspected antibody is bound initially, the signal is amplified by the enzyme–substrate reaction **(Figure 13)**.[151]

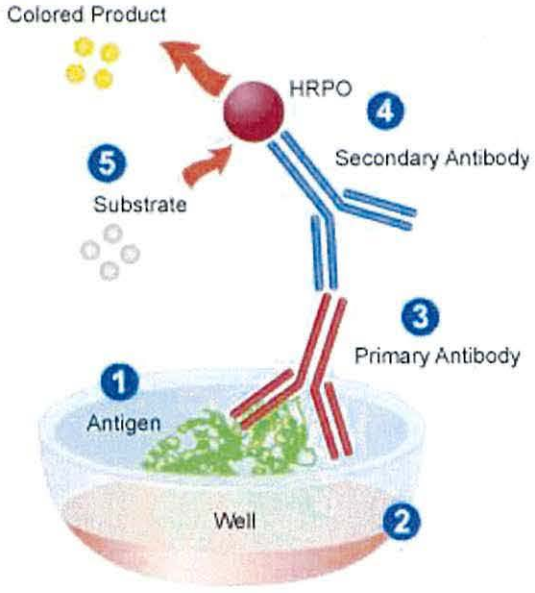


Figure 13: Classic indirect ELISA.[151]

1.6 Application of Mycolic Acid and Cord Factor in TB Diagnosis

ELISA has found acceptance in many areas of medical diagnosis. It is fast, cheap and requires minimal training to carry out. A number of mycolic acids and cord factors have been and are being tested to determine their biological activities and application to diagnosis. The glycolipid cord factor was used as an antigen in an ELISA test for

the rapid serodiagnosis of TB.[152,153] The cord factor was purified from *M. tuberculosis.* They reported excellent specificity. A study by Pan *et al.* showed that the anti-cord factor antibody IgG in the serum of TB patients has a high ability to recognise the actual mycolic acid structures in cord factor.[154] Fujiat *et al.* showed that cord factor has a high response in recognising antibodies in patient serum.[155] A later study by Schleicher *et al.* showed that running ELISA with free mycolic acid antigens to detect TB in HIV-infected patients or those not infected by the virus but exposed regularly to TB-infected persons, gave much less spectacular results.[156] They reported a serum assay level as low as 51% sensitivity and 63% specificity for *M. tuberculosis.* It was found that the presence of cross-reactive anti-cholesterol antibodies in patient's serum could be responsible for the low accuracy level in TB detection when using mycolic acid as antigen.[157]

In spite of the gloomy results reported by Schleicher *et al.,* ELISA continues to be used in TB research and is useful for the early detection of different forms of TB. The recent availability of stereochemically pure forms of synthetic mycolic acids and cord factors may offer hope for improving the sensitivity and specificity of ELISA-based TB diagnoses. The first reported use of synthetic mycolic acids as ELISA antigens was by Beukes *et al.*[158] The initial results reported by the group indicate significant disparity between the ELISA signals of the different mycolic acid subclasses. Methoxymycolic acid gave the best signal, in comparisonwith other types of mycolic acid. These results may indicate that there is room for improvement in mycolic acid-based ELISA technology with the use of specific synthetic subclasses.

1.7 Synthesis of Mycolic Acids

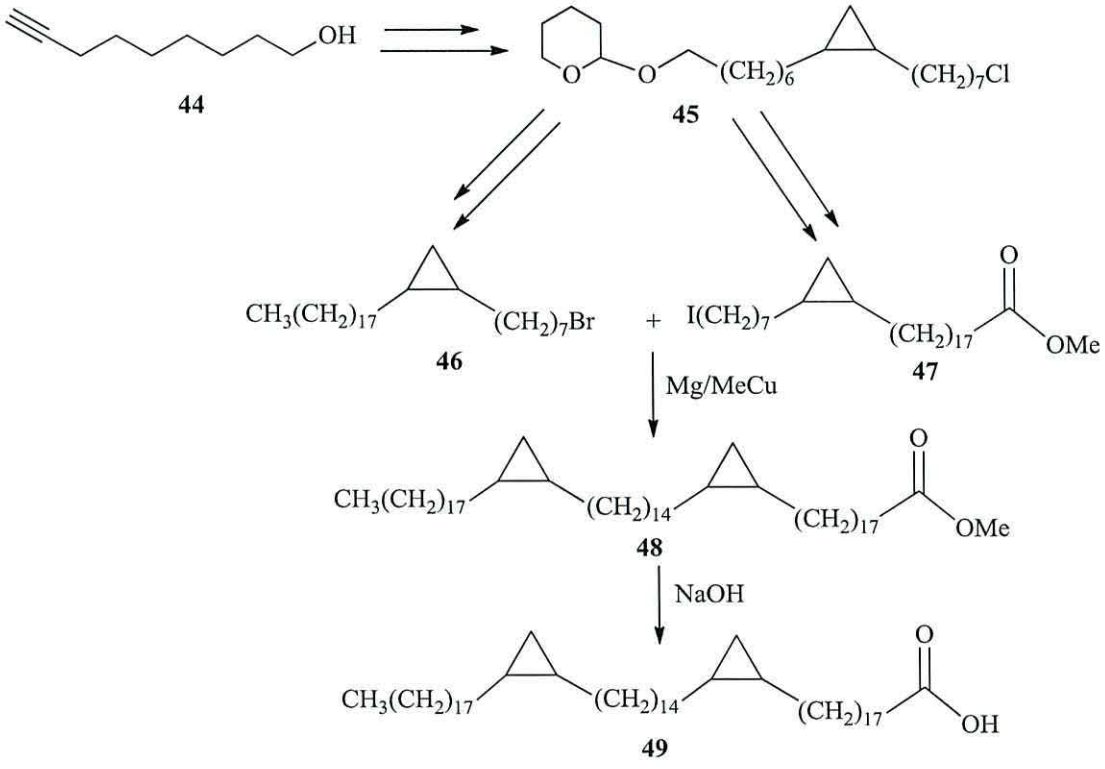
The synthesis of mycolic acid is very important in the identification of the structures of natural mycolic acid. It may help in understanding the properties and biosynthesis of natural mycolic acids. The preparation of the synthetic analogue of mycolic acids is important for research towards the development of easier, quicker and cheaper methods to diagnose and treat TB and other immune-related diseases. In ELISA assays to diagnose TB, different synthetic mycolic acids have been used as the antigens in order to select the best one, giving high sensitivity and selectivity in the assay.

The synthesis of full mycolic acid requires the preparation of two parts: the mycolatemotif part and the meromycolate part, which contains different functional

groups with different stereochemistry. The two parts are joined together in a coupling reaction to get the full mycolic acid.

1.7.1 The Synthesis of Meromycolate

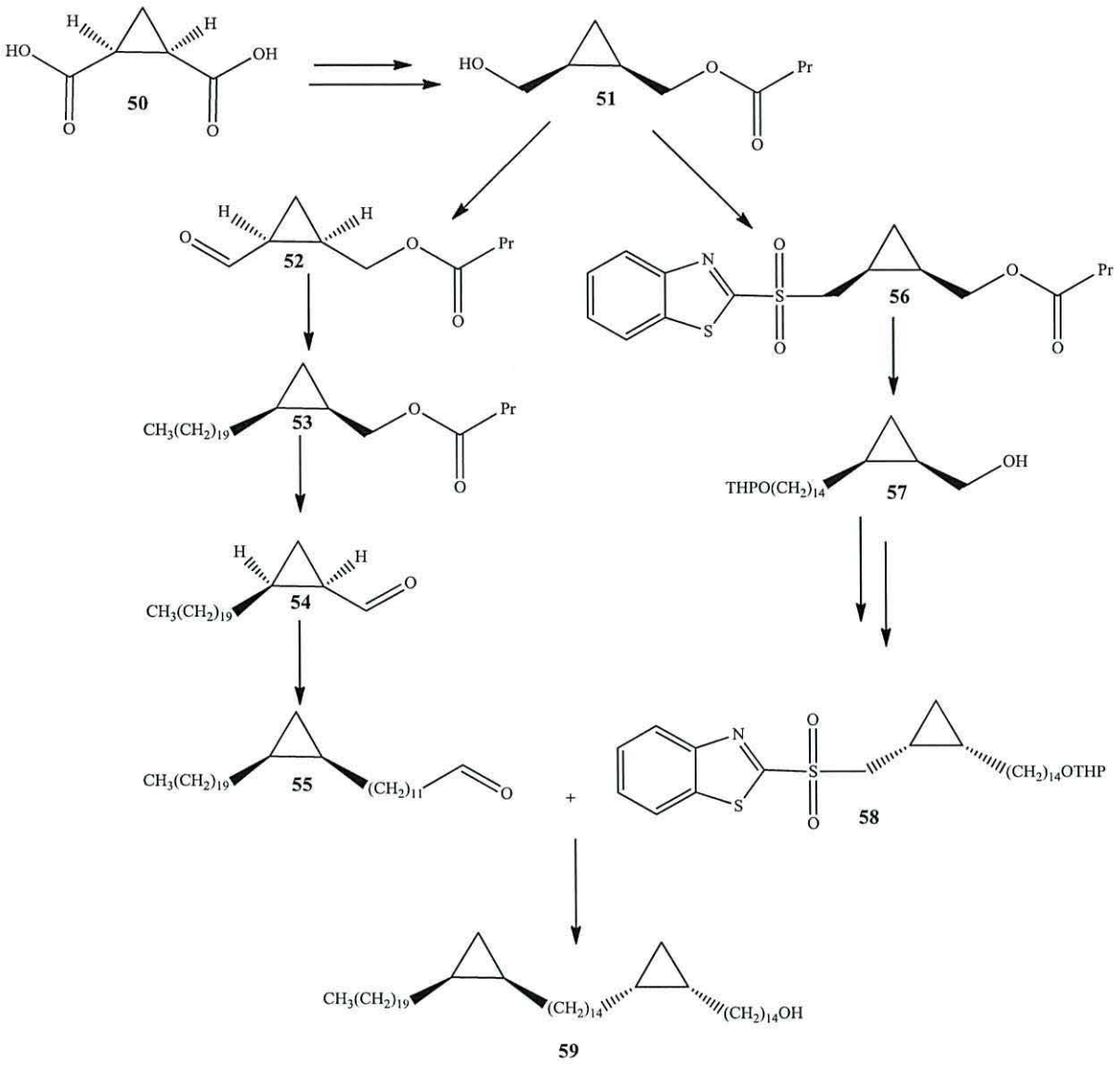
The first synthesis of α-meromycolate, which contained two *cis*-cyclopropane rings, was achieved by Gensler[159]. In this method, 1,4-cyclohexadiene was used as the starting material and after a number of steps, gave methyl meromycolate as a mixture of four stereoisomers. Later, Gensler *et al.* suggested another method for the synthesis of a meromycolic acid which was easy and short. This involved joining different fragments together to form mycolic acid.[160] The 1-hydroxy-8-nonyne **(44)** was used as a starting material and after several steps led to the formation of a compound **(45)** which was converted into two intermediates **(46)** and **(47)** in several steps. A Grignard reaction was used for the coupling of the intermediates to give **(48)**. Finally, compound **(49)** was obtained by saponification of **(48)** **(Scheme 4)**.



Scheme 4: Synthesis of an α-meromycolic acid by Gensler et al.[161]

Even thougt this route is shorter, it gave a very poor yield and does not have any control on the absolute stereochemistry of the chiral centres of cyclopropyl groups.

Recently, Al Dulayymi *et al.*[161] succeeded in a new method to prepare the first single enantiomer of α-meromycolate. This method included preparation of two parts, **(55)** and **(58)**, and joining them together. The first part **(55)** was prepared from the anhydride of cyclopropane-*cis*-1,2-dicarboxylic acid **(50)**,[162] which was converted into the protected alcohol **(51)**. The alcohol was converted into the corresponding aldehyde **(52)** and then coupled with nonadecyltriphenylphosphonium bromide and base, via a Wittig reaction, in order to make the terminal chain, followed by hydrogenation to give **(53)** **(Scheme 5)**. Compound **(53)** was reduced with lithium aluminium hydride to the corresponding alcohol and this was followed by oxidation to give the aldehyde **(54)**. The coupling reaction was done between **(54)** and a phosphonium salt using the Wittig reaction, followed by hydrogenation and oxidation to give the aldehyde **(55)**. The second part, **(58)**, was prepared from the same alcohol **(51)**, which was converted into sulfone **(56)**. A Julia coupling was done between the sulfone **(56)** and 13-tetrahydropyranyloxy-tridecanal to give the protected alcohol as an *E/Z*-mixture, followed by hydrogenation and reduction to give **(57)** which was then converted into sulfone **(58)**. Finally, a Julia reaction was carried out between the aldehyde **(55)** and the sulfone **(58)** to form an unsaturated compound, followed by deprotection and hydrogenation to give the meromycolate **(59)** as a single enantiomer.



Scheme 5: Synthesis of α-meromycolic acid by Al Dulayymi et al.[161]

The synthesis of the α-methyl-*trans*-cyclopropane functional unit is important in the synthesis of some types of meromycolate.. This unit was prepared starting from *D*-mannitol and used to prepare different meromycolates with different stereochemistry, **(Figure 14)**.[163,164]

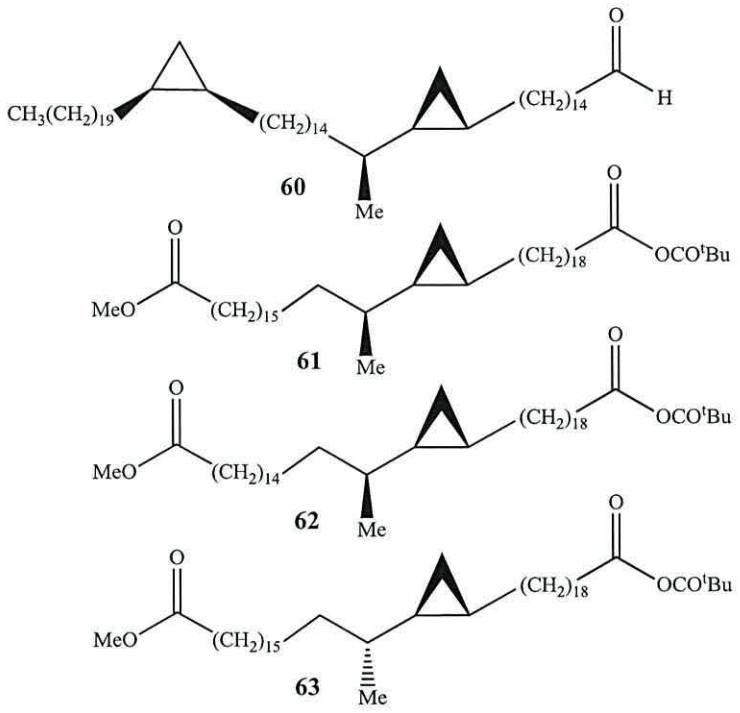
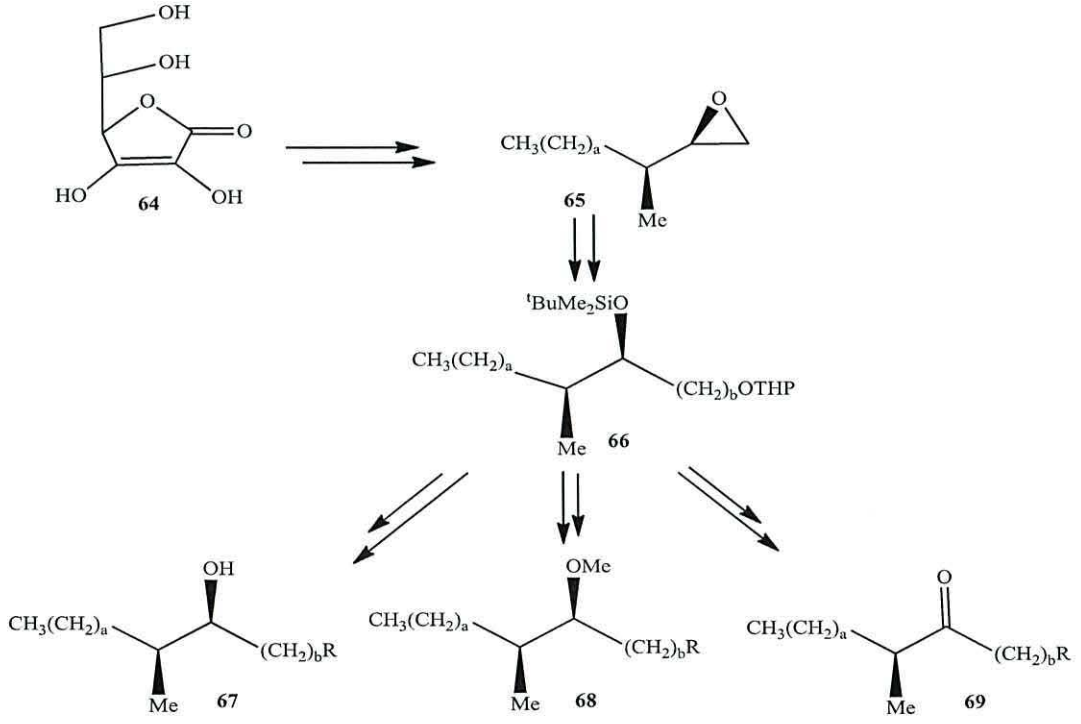


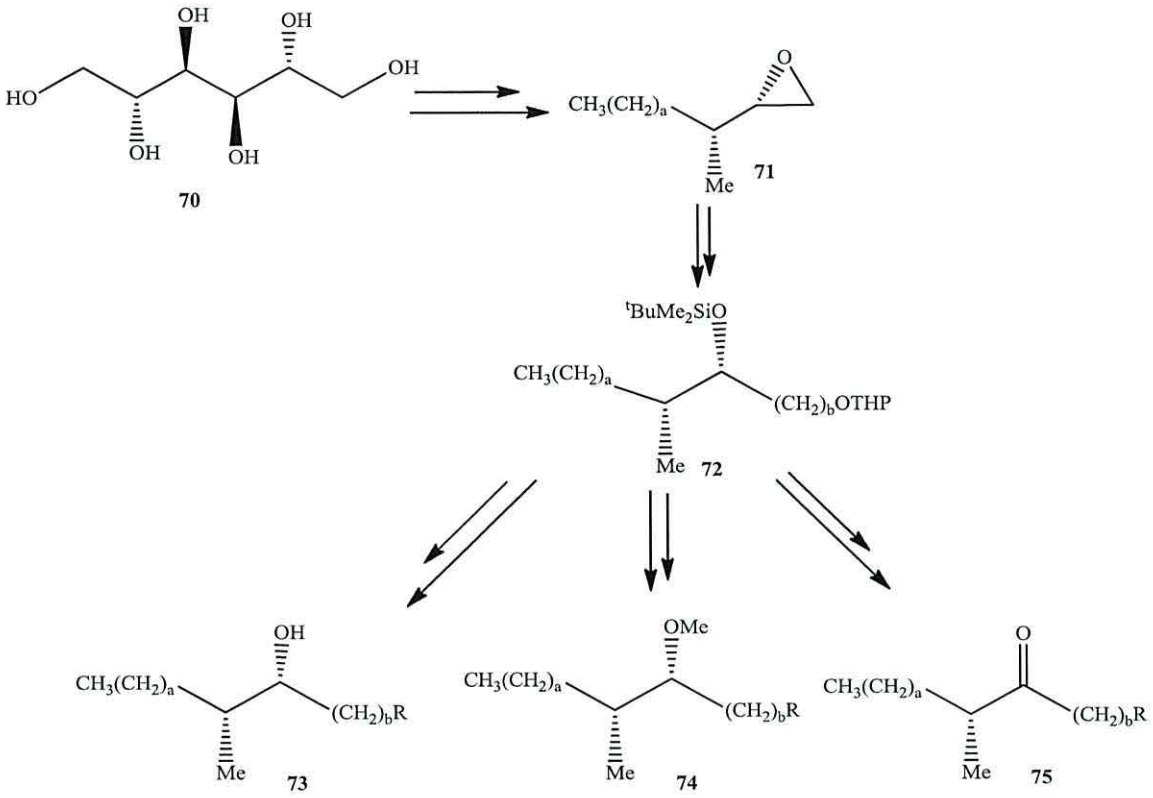
Figure 14: The different types of meromycolates containing an α-methyl-trans-cyclopropane.

Al Dulayymi *et al.*[165] prepared an oxygenated unit of the meromycolate part in an *S,S*-configuration from *L*-ascorbic acid as the starting material **(64)**. The procedure included multiple-steps to give the key intermediate **(66)**, which it is possible to convert into hydroxyl **(67)**, methoxy **(68)** and keto **(69)** groups **(Scheme 6)**.



Scheme 6: Schematic overview ofthe synthesis of S,S-oxygenated mycolic acid

The same work described the synthesis of the oxygenated unit of the meromycolate part in an *R,R*-configuration from *D*-mannitol **(70)** in the same way as above **(Scheme 7)**.[165]



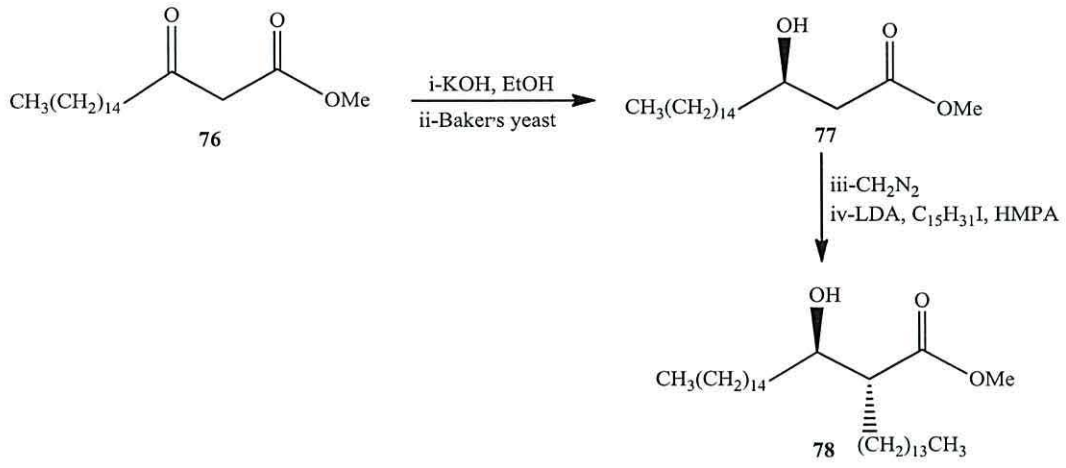
Scheme 7: Schematic overview of the synthesis of R,R-oxygenated mycolic acid

1.7.2 Synthesis of Mycolatemotif

The mycolatemotif part is the same unit in all mycolic acids, and contains a β-hydroxyl group and a α-alkyl group in an *R,R*-configuration which are both *anti* to each other.

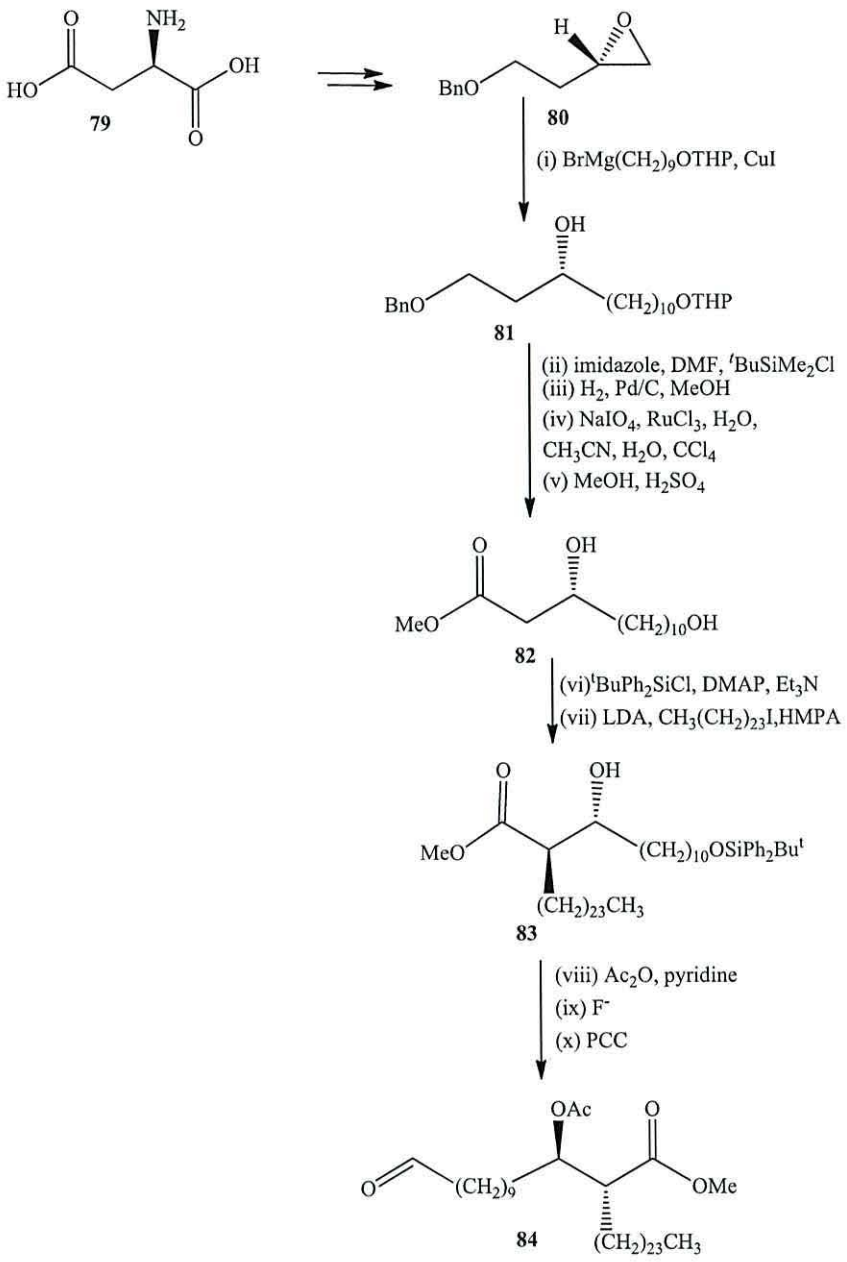
The first mycolatemotif synthesis was reported by Lederer *et al.* and the method was based on the condensation between two fatty acid molecules.[166] However; the method gave the product as a mixture of diastereoisomers. Further studies were carried out to prepare a motif part by using the Claisen method, but still the product was a mixture of diastereomers.[167,168]

Utaka *et al.*[169] described a method to insert a hydroxyl group at the β-position by selective reduction of methyl-3-oxo-octadecanoate **(76)** with baker's yeast **(Scheme 8)**. The insertion of the alkyl chain at the α-position with correct configuration was achieved by using a Fräter reaction to give the desired compound **(78)**.[170]



Scheme 8: The preparation of mycolatemotif according to the method byUtaka et al.[169]

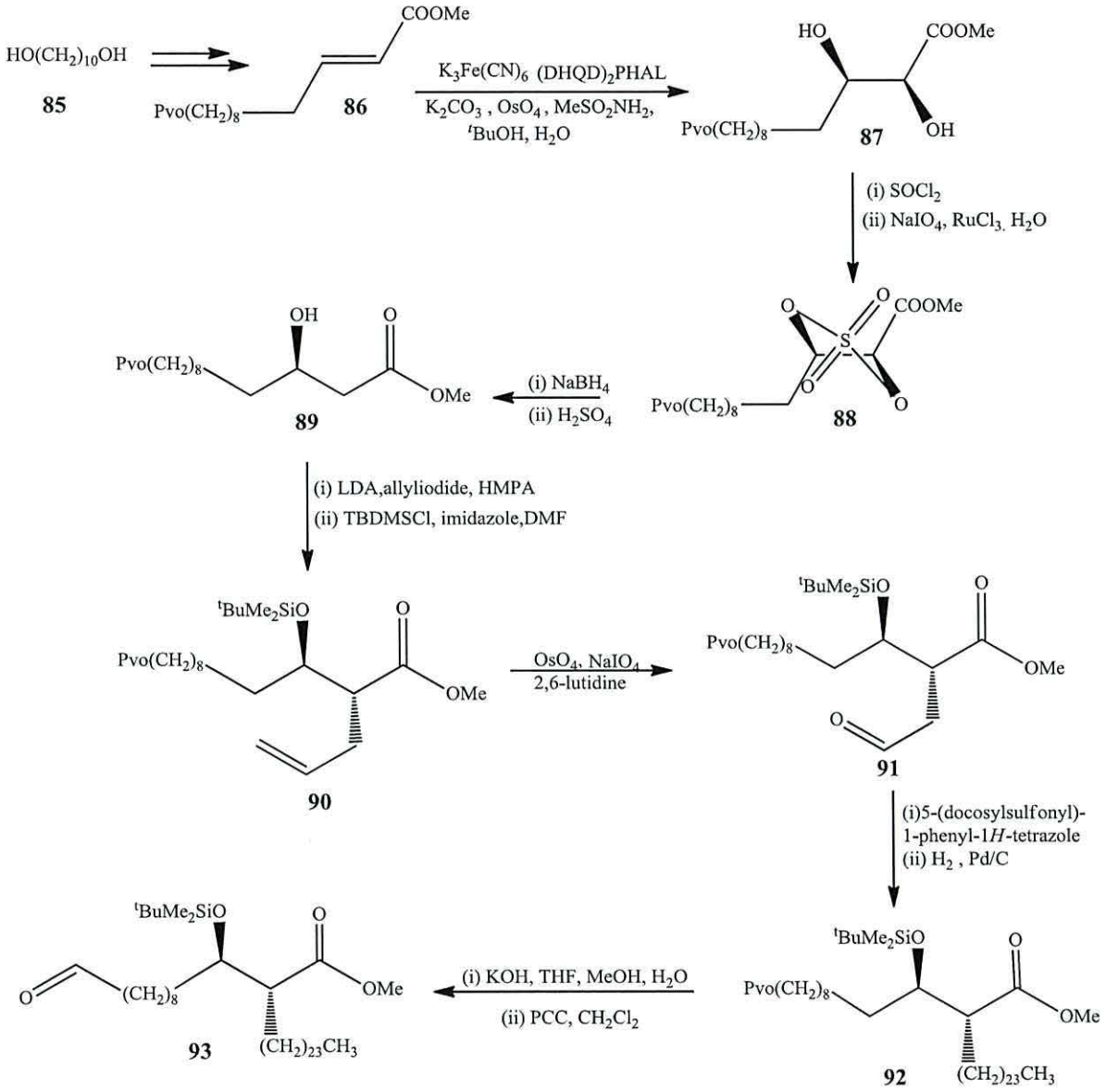
Al Dulayymi *et al.*[171] reported a different method to prepare the mycolatemotif from *R*-aspartic acid **(79)**, which was converted into an epoxide after several steps. The intermediate **(80)** was ring opened with a Grignard reagent followed by several steps, which led to the formation the diol **(81)**. The allyl chain at the α-position was introduced by performing the Fräter reaction upon compound **(82)**, after the primary hydroxy group had been protected. The secondary hydroxy was protected with an acetate to give **(83)**. Deprotection of the primary alcohol was followed by oxidation to the corresponding aldehyde **(84)**. Thus, the mycolatemotif was then ready to couple with any sulfone meromycolate to give full mycolic acid **(Scheme 9)**.



Scheme 9: Synthesis of the mycolatemotif part of R-aspartic acid.

Koza.[172] prepared the mycolatemotif part by using the same method as above, but they used *L*-aspartic acid as the starting material instead of *R*-aspartic acid because *L*-aspartic acid is cheaper than *R*-aspartic acid. Toschi *et al.*[173] improved the method to prepare the mycolatemotif. They used 1,10-decanediol **(85)** as a simple starting material to get an α,β-unsaturated ester **(86)** as an intermediate. The ester was converted into diol ester **(87)** using a Sharpless dihydroxylation. After that, the diol was converted to the sulfate **(88)**, which was reduced and hydrolysed to give a (3*R*)-3-hydroxy ester **(89)**. The Fräter reaction was used to introduce the allyl chain at the α-

position to give the alkene **(90)**. Protection of the secondary alcohol was carried out prior to conversion of the alkene to aldehyde **(91)**. The alkene was oxidised to an aldehyde and coupled with sulfone in a Julia reaction, followed by hydrogenation to give **(92)**. The motif **(93)** was obtained by deprotection of the compound **(92)** and oxidation with PCC in dichloromethane, **(Scheme 10)**.

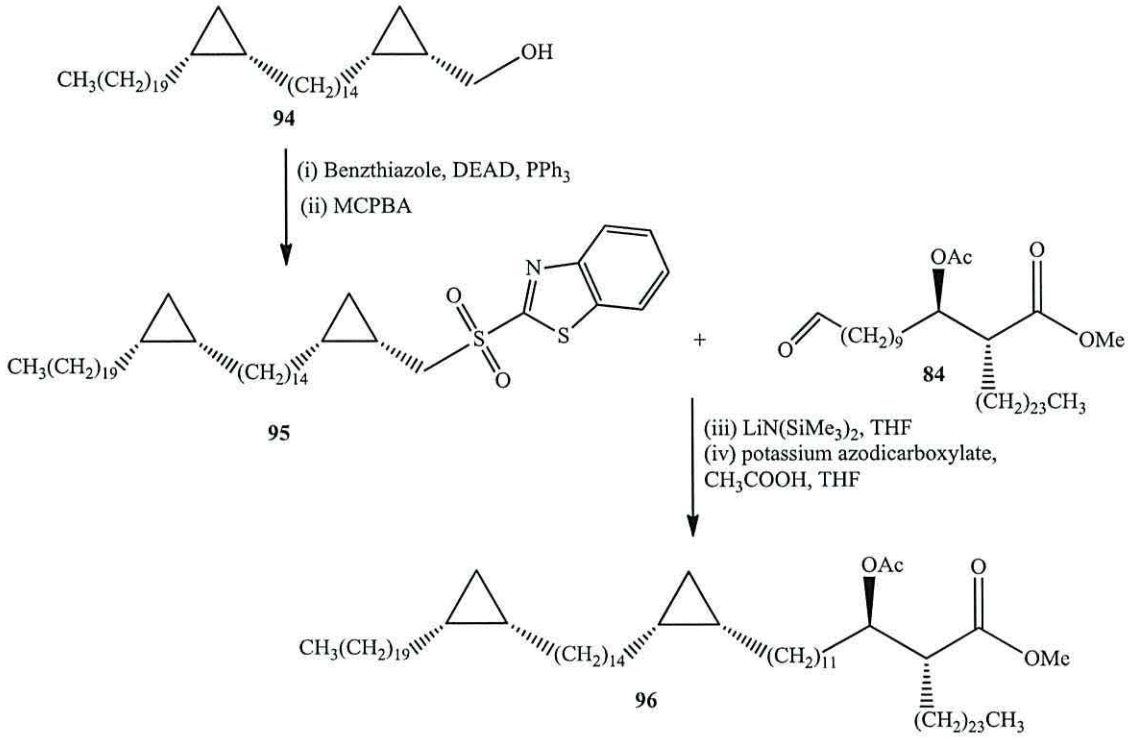


Scheme 10: The improved method of synthesis of the mycolatemotif.

1.7.3 Synthesis of Full Mycolic Acid

The first synthesis of the pure enantiomer of α-mycolic acid, which can be found as a major mycolic acid in *M. tuberculosis,* was achieved by Al Dulaymmi *et al.*[174] This

method included the preparation of the alcohol meromycolate **(94)** using the same methods as for the meromycolate **(59)**, as explained previously. The meromycolate was converted into sulfone **(95)**. A Julia coupling reaction was achieved between the sulfone meromycolate **(95)** and the aldehyde **(84)** **(see Scheme 9)**, followed by hydrogenation to give protected mycolic acid **(96)** **(Scheme 11)**.



Scheme 11: Synthesis of protected α-mycolic acid of M. tuberculosis.

Recently, the free α-mycolic acid **(97)** with the same stereochemistry as the protected α-mycolic acid **(96)** was achieved by Muzael *et al.*[175]

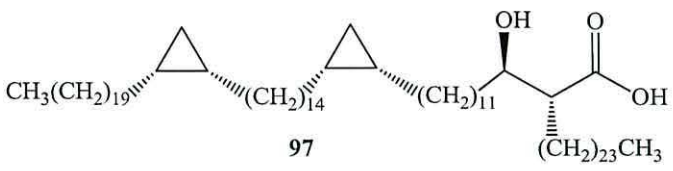


Figure 15: Synthesis of free α-mycolic acid of M. tuberculosis.

Additionally, the syntheses of three α-mycolic acids with different stereochemistry were reported.[176,177,178]

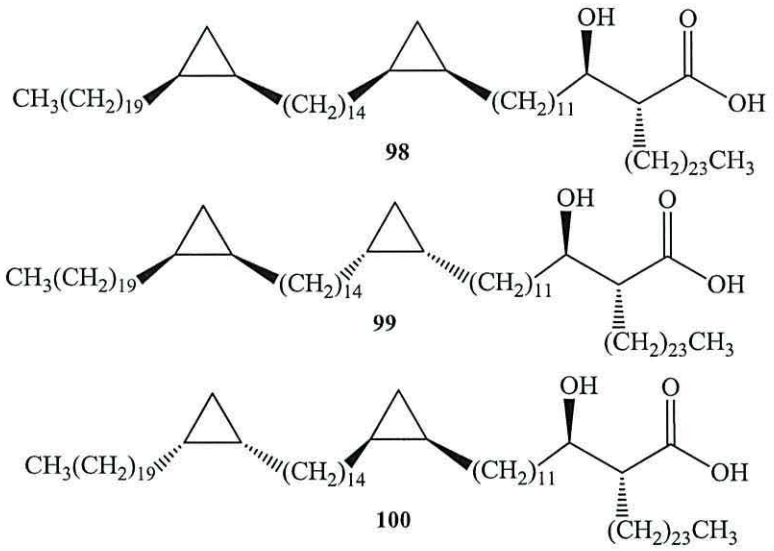


Figure 16: Synthesis of α-mycolic acids with different stereochemistry.

Subsequently, methoxymycolic acids from *M. tuberculosis* were prepared with different stereochemistries. [165,179]

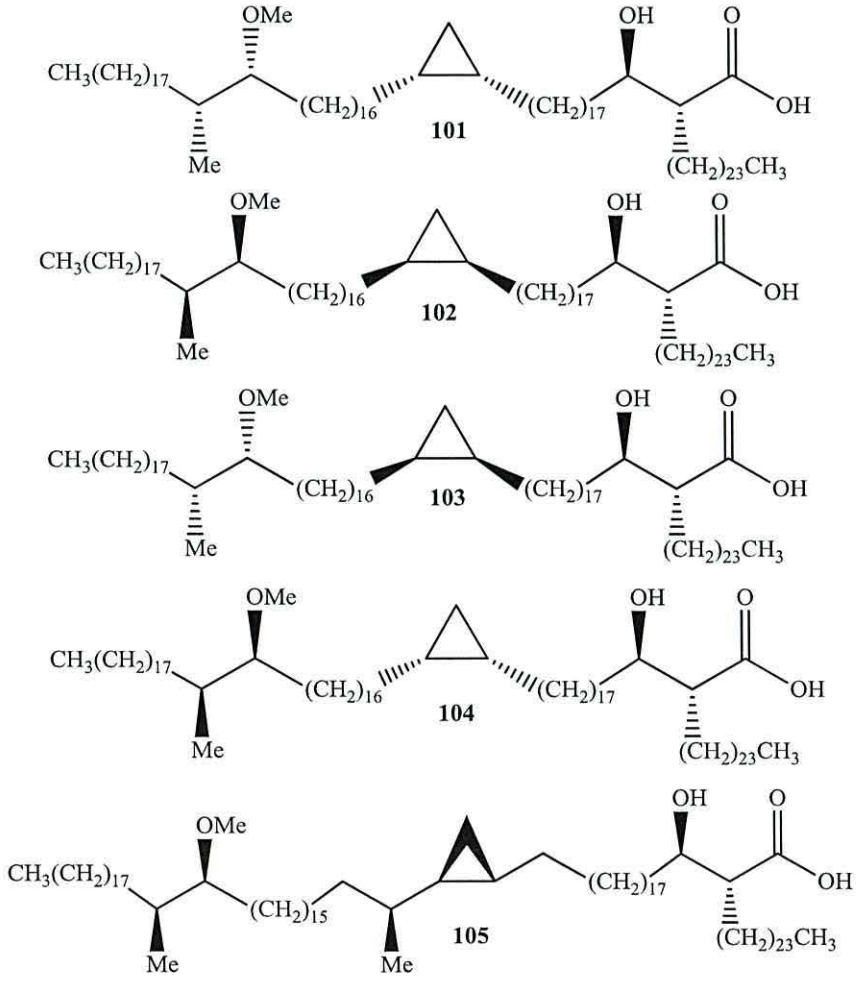


Figure 17: Synthesis of methoxy-mycolic acids with different stereochemistry.

Baird *et al.* have contributed significantly to the field of synthesis of complete mycolic acids, where they established a number of methods to give enantiomerically pure mycolic acids. Recently, the Baird group prepared other types of mycolic acids in other mycobacteria. For example, they synthesised epoxy-mycolic acids, **(106)** and **(107)**. They also reported that the structure of **(106)** was the same natural epoxy-mycolic acid in *M. smegmatis*.[180] Furthermore, the complete free *cis*-alkene mycolic acid **(108)** from *M. smegmatis* was synthesised by Muzael *et al.*[181] **(Figure 18)**.

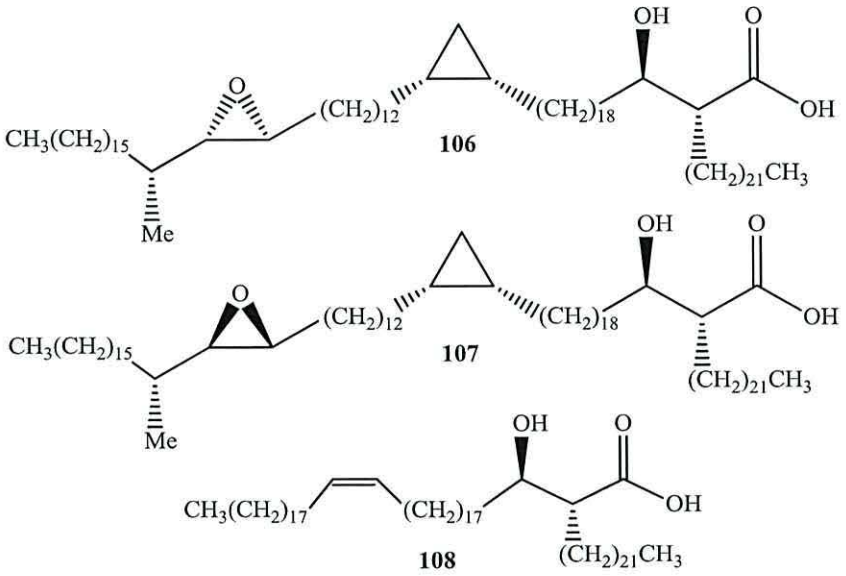
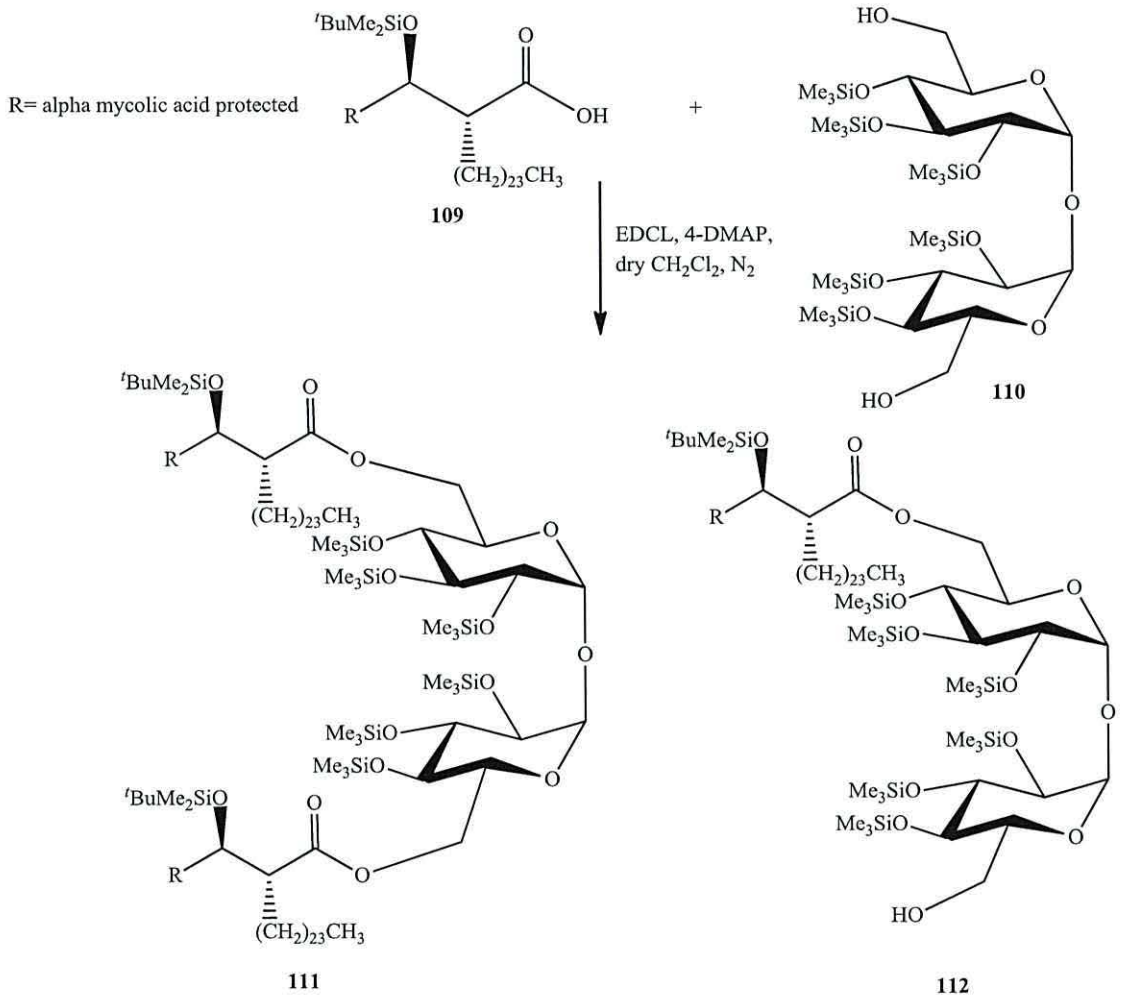


Figure 18: Synthesis of different types of mycolic acids from other mycobacteria

1.8. The Synthesis of Cord Factor

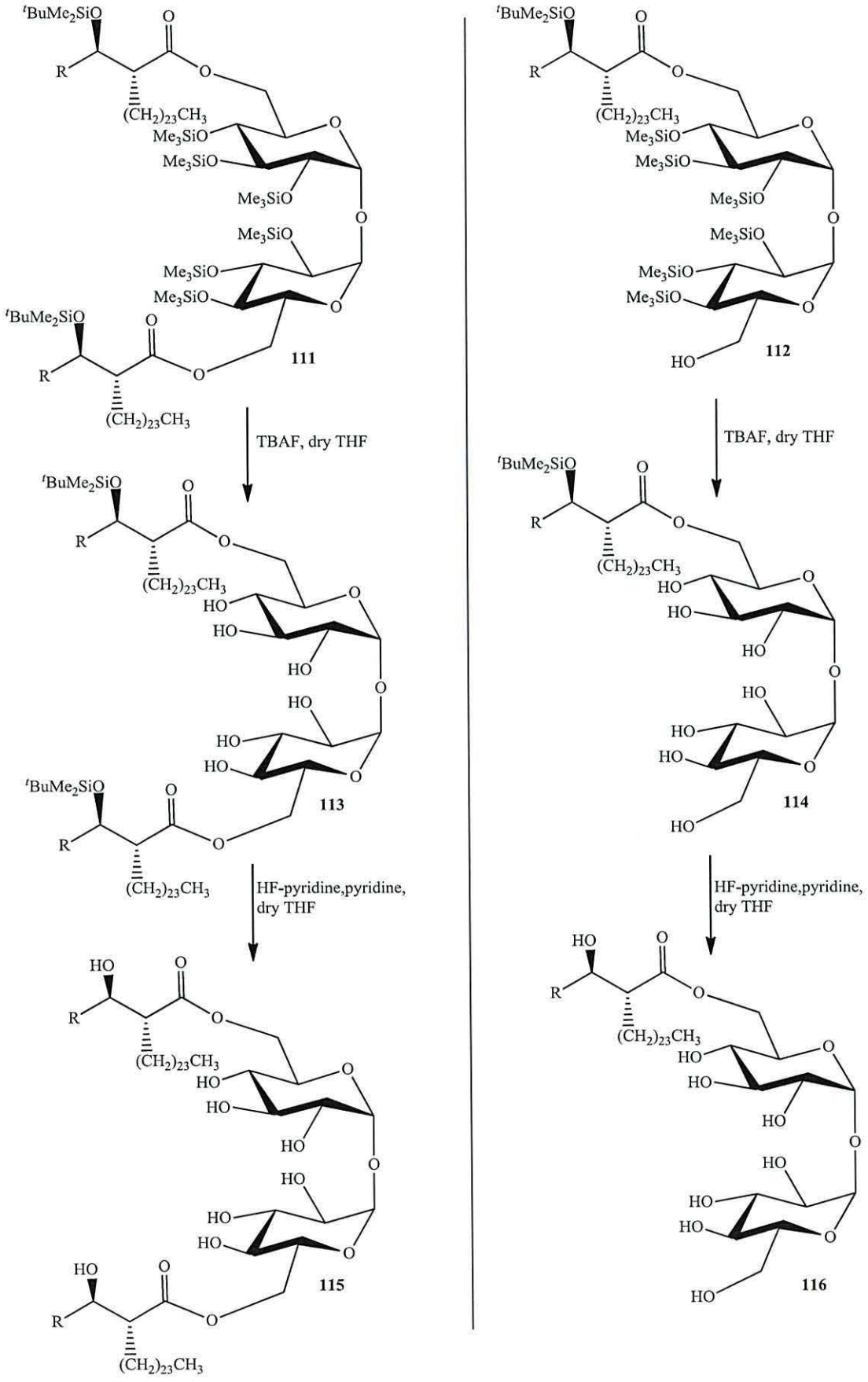
There have been many attempts to synthesise cord factors and their model structures before knowing their true structures. The early studies were focused on preparing a model of the cord factor. Palmitic acid, behenic acid and corynomycolic acid were used instead of mycolic acid in the preparation of cord factor.[182,183,184] Tocanne [185] used natural α-mycolic acid and a model of mycolic acid to prepare cord factor. Initially, the secondary hydroxyl groups of trehalose were protected by trimethylsilyl, while the primary hydroxyl was replaced with iodine as a good leaving group. The esterification was carried out between the protected trehalose sugar and a potassium salt of natural mycolic acid, followed by removing the protecting groups to give TDM. The same method to prepare different TDMs but using the salt of another natural mycolic acid and its model was completed by Toubiana *et al.*[186]

Recently, Baird *et al.*[176,177] prepared the first cord factors (TDM and TMM) of synthetic α-mycolic acid. The synthetic α-mycolic acid was protected at the β-hydroxyl group with a silyl group **(109)**. The secondary alcohols of trehalose were protected with trimethylsilyl groups **(110)**. The esterification was achieved between the protected mycolic acid and protected trehalose after 6 days at room temperature using EDCI and DMAP as coupling reagents. The esterification reaction gave two products, protected TDM **(111)** and protected TMM **(112)**, which were separated by column chromatography **(Scheme 12)**.



Scheme 12: Esterification of protected mycolic acid with protected trehalose.

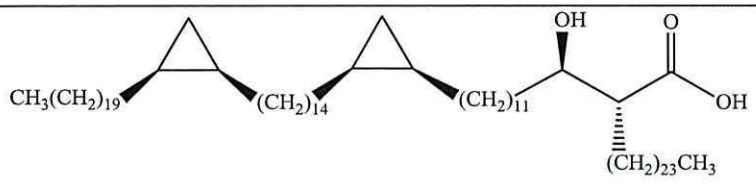
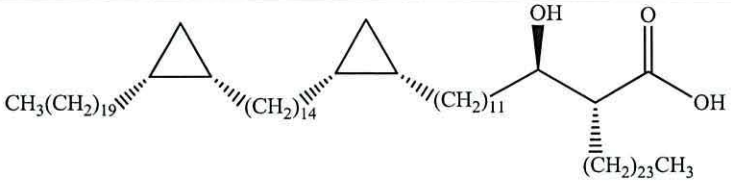
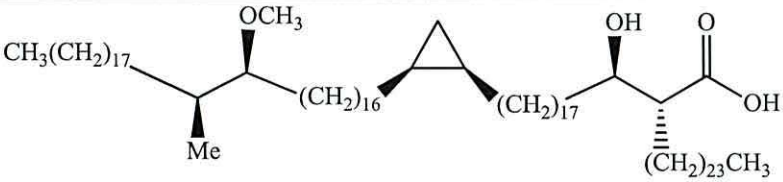
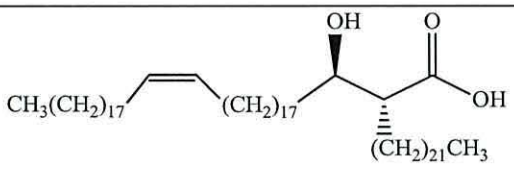
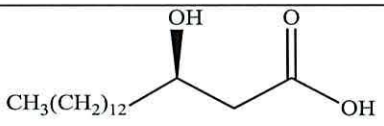
Deprotection was carried out in two steps; first deprotection of the trimethylsilyl groups in trehalose was achieved with TBAF to give silyl-protected TDM **(113)** and silyl-protected TMM **(114)**. Secondly, deprotection of the silyl group present upon the mycolic acid was achieved with HF.pyridine to give a pure TDM **(115)** and TMM **(116)**. The structures of the products were confirmed by MALDI-TOF MS and NMR spectroscopy **(Scheme 13)**.



Scheme 13: Deprotection of trimethyl silyl groups and the silyl group in TDM and TMM.

Following the method above, different cord factors and a model cord factor were prepared (**Table 1**).

Table 1: The synthesis of mycolic acids as starting material of different cord factors

	(TDM,TMM)[176,177]
	(TDM,TMM)[175]
	(TDM,TMM)[177]
	(TDM)[175]
	(TDM)[178]

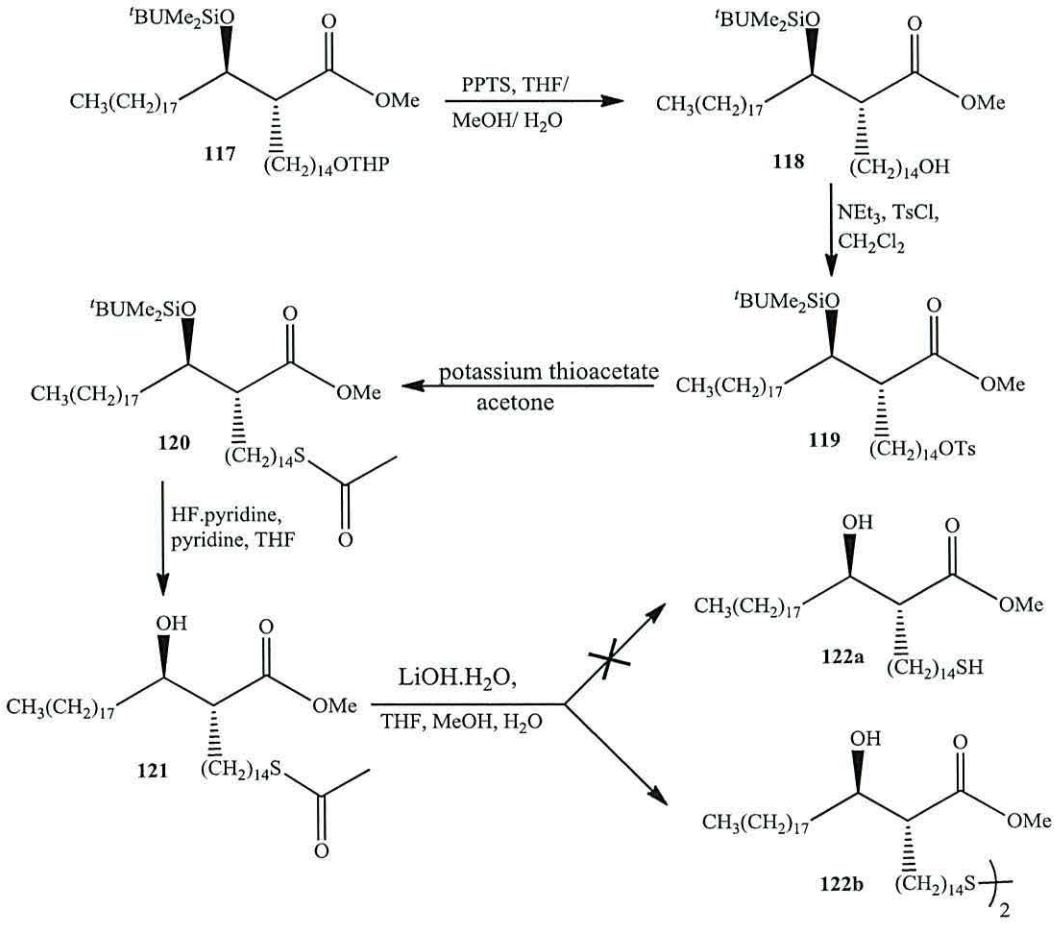
1.9 Thiolated Mycolic Acid

Free mycolic acid has been used as an antigen to detect TB, as described previously. Generally, most serodiagnostic assays for detecting TB have shown results below the levels recommended by the WHO, including sensitivity and specificity requirements. Currently, there are attempts to improve the TB assay by synthesising mycolic acid with a thiol group, which will be attached to a gold surface.

Initially, Nuzzo *et al.* described a study of self-assembled monolayers of alkanethiols and alkyl disulfides on gold.[187] Following this, the self-assembly of alkanethiols on a gold surface has become a useful device for surface modification. The advantage of the thiol group is its high specific affinity for gold, which allows for the covalent attachment of sulphur to the gold in the presence of other functional groups on the alkanethiols.[188,189,190] Conversely, the gold is relatively inert to the commonly

occurring functional groups in biological systems, that is, –OH, –COOH, $–NH_2$, –CO etc. In addition, the gold is more sensitive than other metals as a sensor surface. It has high conductivity and high sensitivity to light, which makes it useful in electrochemical and optical sensors. Additionally, the adsorbed molecules on the gold surface are easy to remove with laboratory reagents without the risk of oxidation.

Recently, Huws completed the first synthesis of a simple thiolated mycolic acid.[191] The motif part **(114)** with a short α-alkyl protected chain was used as a model because the mycolatemotif part is similar in all mycolic acids Furthermore, the synthesis of full mycolic acid is an extremely long and difficult process. The strategy of this work included the hydrolysis of a protecting group at the terminus of the α-chain to give primary alcohol **(118)** (**Scheme 14**). The next step was conversion of the hydroxyl group into a tosylate **(119)**, followed by a thioacetylation to give **(120)**. The silyl group was removed by using HF.pyridine complex and pyridine in dry THF. The final step was hydrolysis of the methyl ester and thioacetate by using lithium hydroxide, but this in fact gave disulfide **(122a)** instead of the thiol **(122b)**.



Scheme 14: Synthesis of of simple a thiolated mycolic acid

Chapter 2

2. Results and Discussion

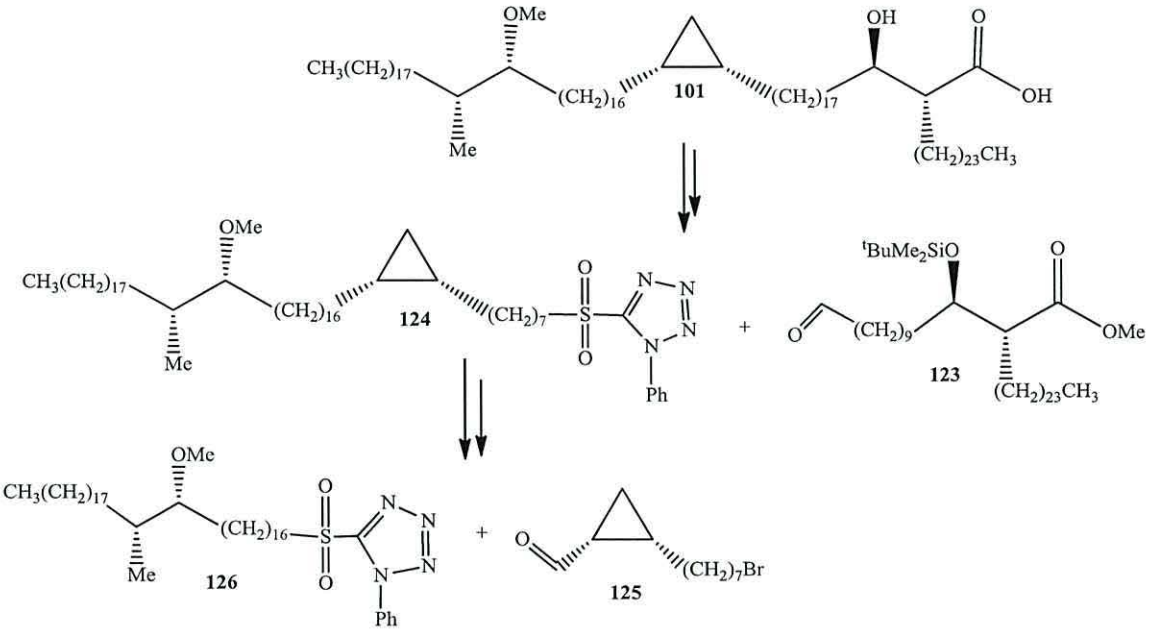
2.1 The aims of this chapter:

- To prepare a stereoisomer of a methoxymycolic acid matching the chain length of the main isomer from *Mycobacterium tuberculosis*.
- To prepare a *cis* ketomycolic acid of *M. tuberculosis* with the same the absolute stereochemistry as the natural compound.
- To prepare TDM and TMM for both mycolic acids.
- To use them as antigens in developing a method for TB diagnosis.

2.2 Synthesis of a methoxymycolic acid

The synthesis of the full methoxymycolic acid **(101)** containing a cyclopropane ring in (*S,R*)-configuration in the proximal position, and methoxy and methyl groups in the (*R,R*)-configuration in the distal position was achieved according to the work which was previously carried out by Al-Dulayymi *et al.*[165] It would be used to couple with trehalose to prepare cord factors (TDM and TMM), in order to use them as antigens in ELISA tests to check their ability to detect TB-antibodies, and to determine if the stereochemistry of the mycolic acid in the cord factor has any effect on its biological activities (This will be described in Chapter four).

For ease of synthesis, the full methoxymycolic acid can be divided into mycolatemotif part and meromycolate part. Sequentially, the meromycolate part can be split into a *R,R*-α-methyl-methoxy unit and a *S,R-cis*-cyclopropane unit **(Scheme 15)**.

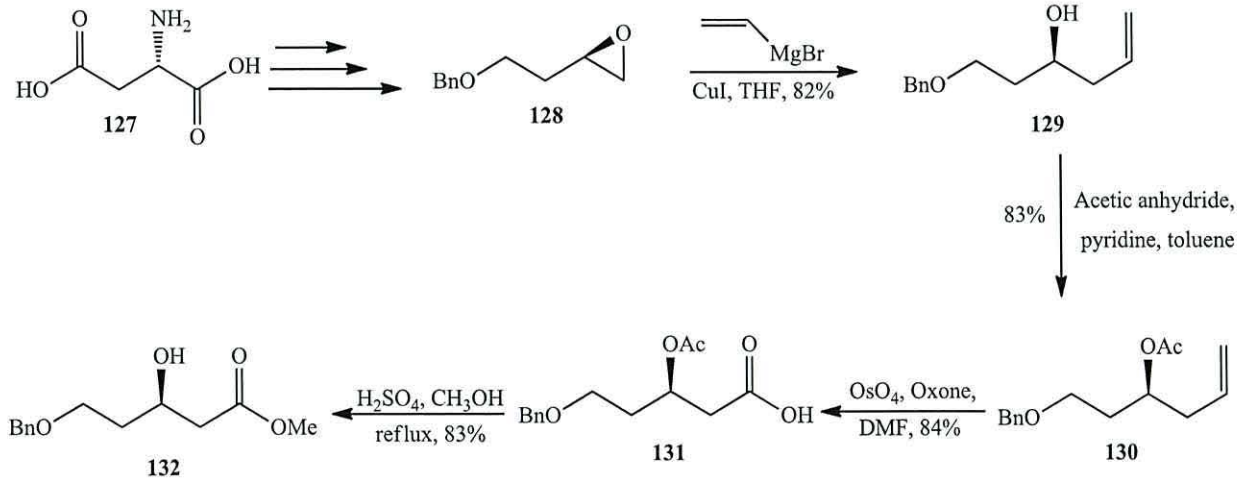


Scheme 15: Retrosynthesis of the full methoxymycolic acid

2.2.1 Synthesis of mycolatemotif part

2.2 1.1 Synthesis of β-hydroxy ester (132)

L-Aspartic acid **(127)** was used as the starting material for the preparation of the main part of mycolatemotif **(132) (Scheme 16)**. This work was done according to the literature methods and the resulting proton and carbon NMR spectra for **(132)** were similar to those in the literature[172,192]

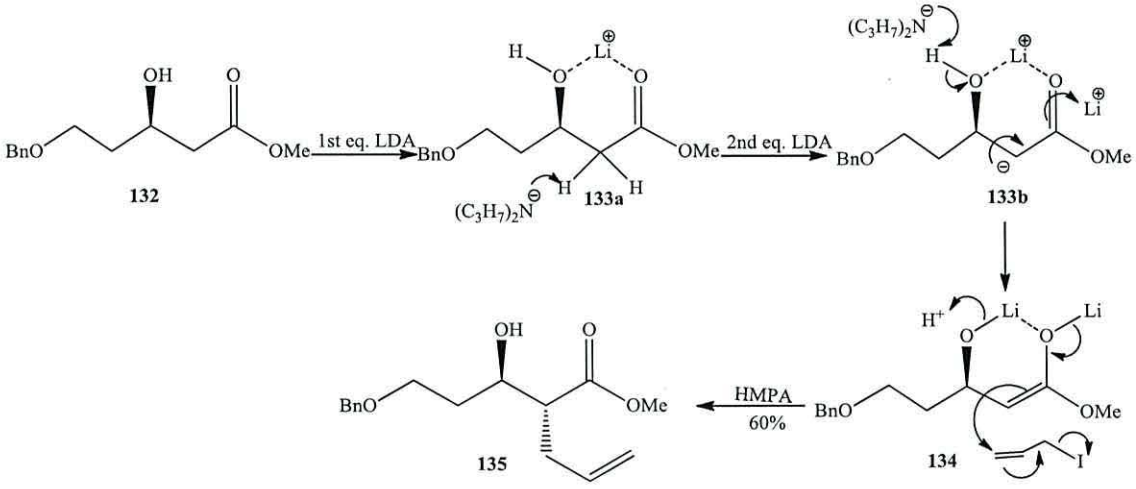


Scheme 16: Synthesis of the ester (132)

2.2.1.2 The addition of α-alkyl chain

The Fräter allylation[193,194] has been reported to be highly diastereoselective and as such was considered most appropriate for a stereo-controlled insertion of the alkyl chain at the α-position of the β-hydroxy ester **(132)**. The allyl chain was first introduced at the α-position to give the α-alkyl-β-hydroxy fragment in the (*R,R*)-configuration, because the insertion of the full alkyl group in one step had always led to variable results and poor yields.[173]

The first step of this reaction is the generation of LDA (2 mol eq.) *in situ* by reacting diisopropylamine with MeLi at -78 °C, followed by the addition of the β-hydroxy ester **(132)** at - 62 °C and stirring for 2 h at this temperature to ensure the generation of a stable chelated enolate intermediate **(134)**. The mixture was allowed to stir for 2 h between -60 °C to -10 °C. It was then cooled to – 62 °C, and a mixture of allyl iodide and HMPA in dry THF was added. The allyl chain was attached from the bottom because of the steric effect on the top of the six membered ring of the intermediate **(134)** which gave the *anti*-alkylated product, (2*R*,3*R*)-2-allyl-3-hydroxy ester **(135)** **(Scheme 17)**.[195]



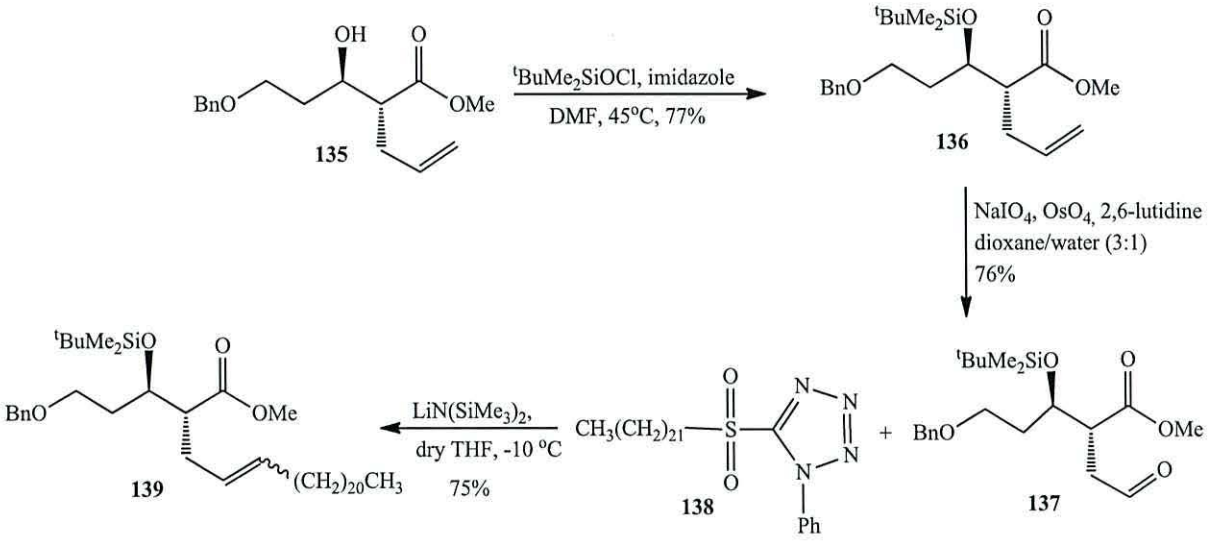
Scheme 17: The insertion of the α-allyl chain

The resulting compound **(135)** gave the same proton and carbon NMR spectra as in the literature.[172]

2.2.1.3 The extension of allyl chain

The allyl chain was extended to the required long alkyl chain by a standard procedure involving cleavage to an aldehyde, chain extension by a Julia-Kocienski reaction, and then hydrogenation.

The β-hydroxy-group of the compound **(135)** was protected to give the compound **(136)** **(Scheme 18)** before the oxidation of alkene into the aldehyde. A *tert*-butyldimethylsilylether was chosen as the protecting group because it has been reported to be stable during the next reaction steps.[196] The alkene group of the compound **(136)** was oxidised to the aldehyde **(137)**, using OsO_4 and $NaIO_4$ in the presence of 2,6-lutidine, which improves the yield and suppresses any side reactions.[197] A modified Julia-Kocienski olefination of the resultant aldehyde **(137)** and 22 carbon sulfone **(138)** resulted in the alkenes **(139)**.

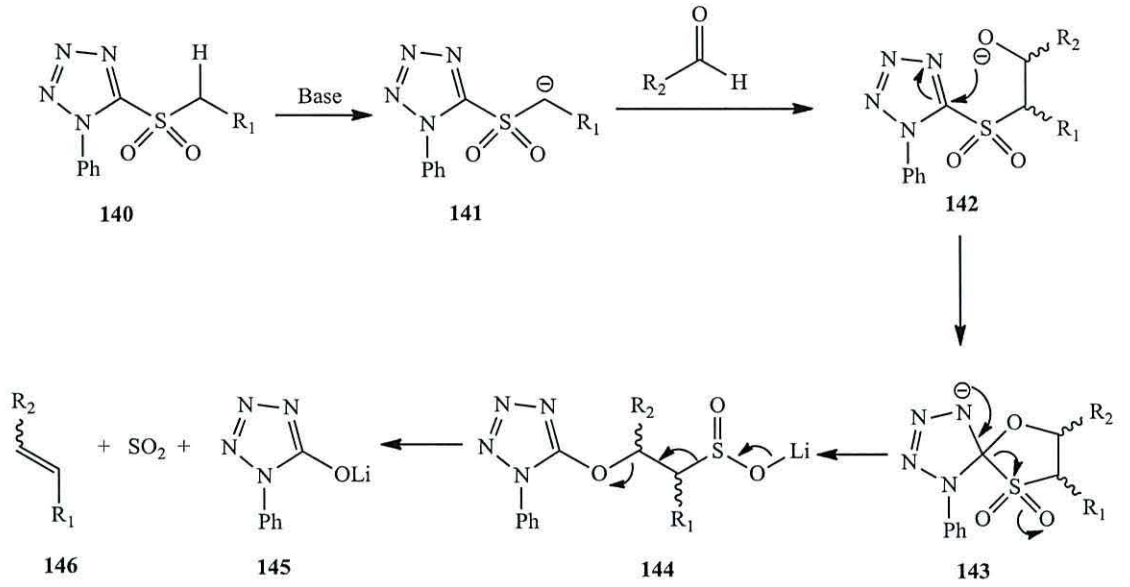


Scheme 18: The chain extension

Because the chain extension is used in novel chain extensions later in this work, it is described in some detail below.

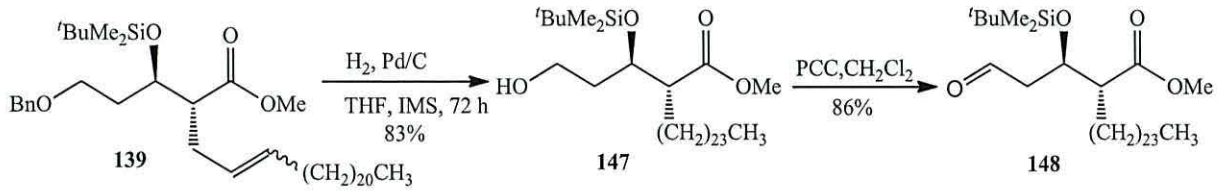
Julia *et al.* first found this reaction which involved using a phenylsulfone and an aldehyde.[198] Later, the reaction was modified by Kocienski and Lythgoe, and became called the modified Julia-Kocienski reaction.'[199,200,201] The mechanism of this reaction involves formation of the β-alkoxysulfone **(142)** as an intermediate, as a result of reacting the metallated sulfone **(141)** with the aldehyde. The β-alkoxysulfone **(142)** is unstable and is converted into an intermediate **(143)** by a Smiles rearrangement that

leads to the transfer of the heterocycle from sulfur to oxygen. Finally, the sulfur dioxide and lithium 1-phenyl-1-*H*-tetrazolene **(145)** is eliminated from **(144)** to give the alkene **(146)** as a mixture of *(E/Z)*-isomers **(Scheme 19)**.



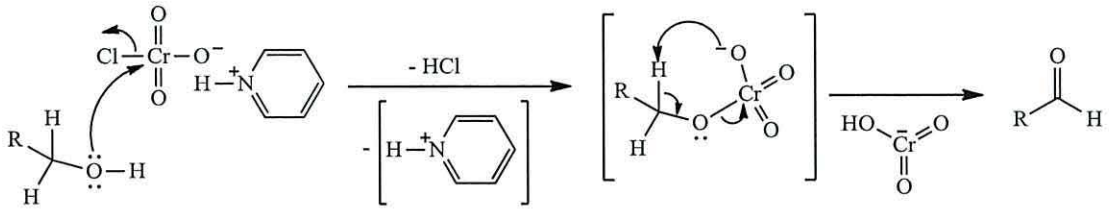
Scheme 19: Mechanism of the modified Julia reaction

Hydrogenation and debenzylation of the unsaturated compound **(139)** was carried out with hydrogen gas using a palladium on charcoal catalyst to give the primary alcohol **(147)**, which was then oxidised with pyridinium chlorochromate (PCC) to give the required aldehyde **(148)** to extend the motif from the side with any chain length required **(Scheme 20)**.



Scheme 20: Hydrogenation (139) and oxidation of the primary alcohol (147)

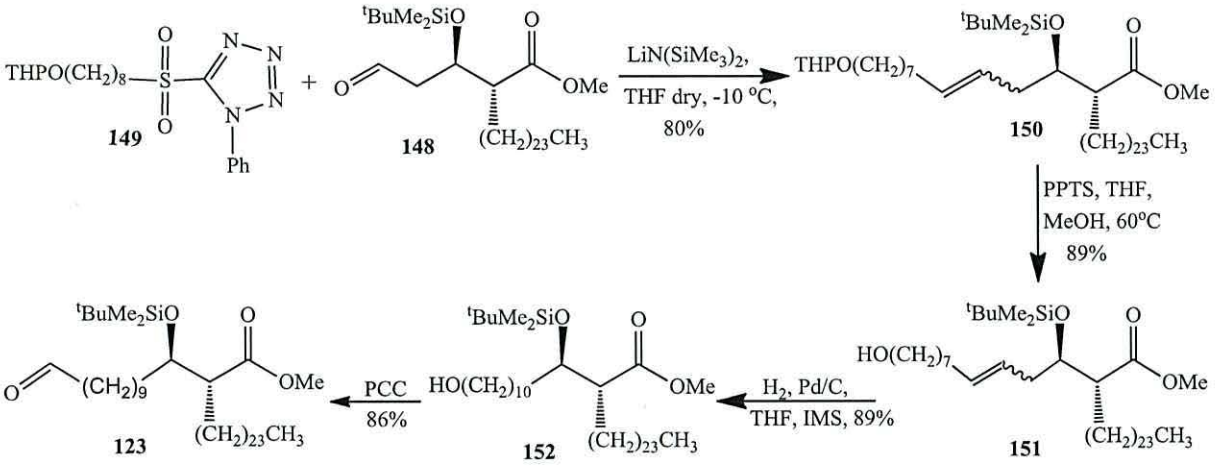
The aldehyde is unstable and can undergo further oxidation to carboxylic acid, thus it was used for the next step immediately after purification. The detailed spectroscopic data for this compound were matching with those given in the literature.[172] The general mechanism of this oxidation reaction is belived to be as follows **(Scheme 21)**.



Scheme 21: The mechanism of oxidation by PCC

2.2.1.4 Extension of the mycolatemotif chain

In order to obtain the correct chain length between mycolatemotif part and meromycolate part, the aldehyde **(148)** was coupled with 1-phenyl-5-((8-((tetrahydro-2*H*-pyran-2-yl)oxy) octyl)-sulfonyl)-1*H*-tetrazole **(149)** in a modified Julia reaction to give alkene **(150)** as a mixture of isomers **(Scheme 22)**. The crude product was purified by column chromatography on silica gel neutralized with a few drops of triethylamine because the THP-group is acid labile and comes off on silica gel. The next step before hydrogenation was deprotection of the tetrahydropyranyloxy (THP) by using pyridinium-*p*-toluene sulfonate as a mild acid which is a better reagent than the *p*-toluene sulfonic acid monohydrate and gave a good yield of unsaturated alcohol **(151)**. The hydrogenation of the double bond was carried out by using hydrogen gas in the presence of Pd on carbon (10%) to give saturated compound **(152)**. The success of the hydrogenation was confirmed by the proton and the carbon NMR spectra which showed the disappearance of all signals for the protons of the double bond. Subsequently, the alcohol was oxidised to the aldehyde **(123)**, ready to couple with the meromycolate. The resulting aldehyde showed comparable spectroscopic data to those in the literature.[179]



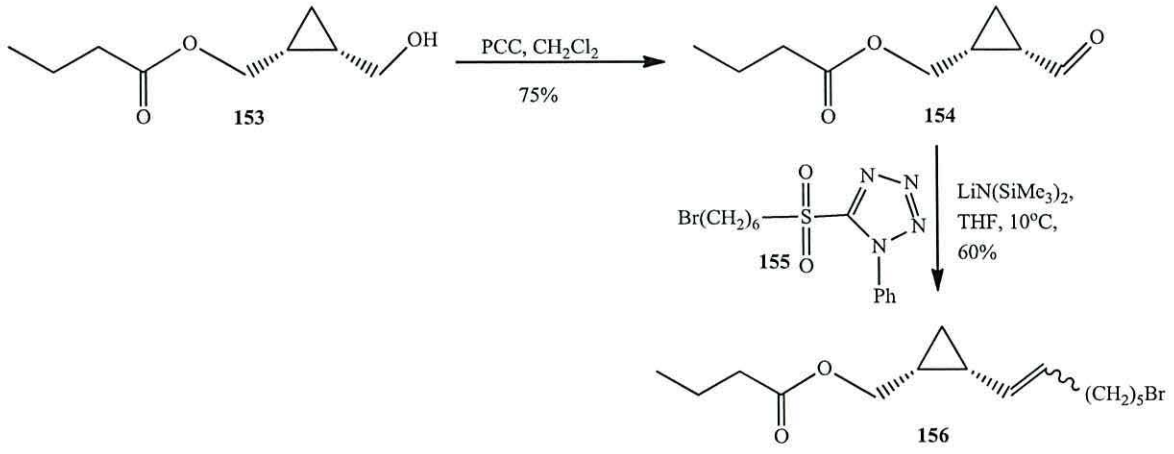
Scheme 22: Synthesis of full mycolatemotif part (123) of methoxymycolic acid

2.2.2 Synthesis of the meromycolate part

The general way to prepare the meromycolate part required for the methoxy mycolic acid **(101)** included coupling the methoxy unit **(125)** with the *cis*-cyclopropane unit **(126)** in a Julia reaction.

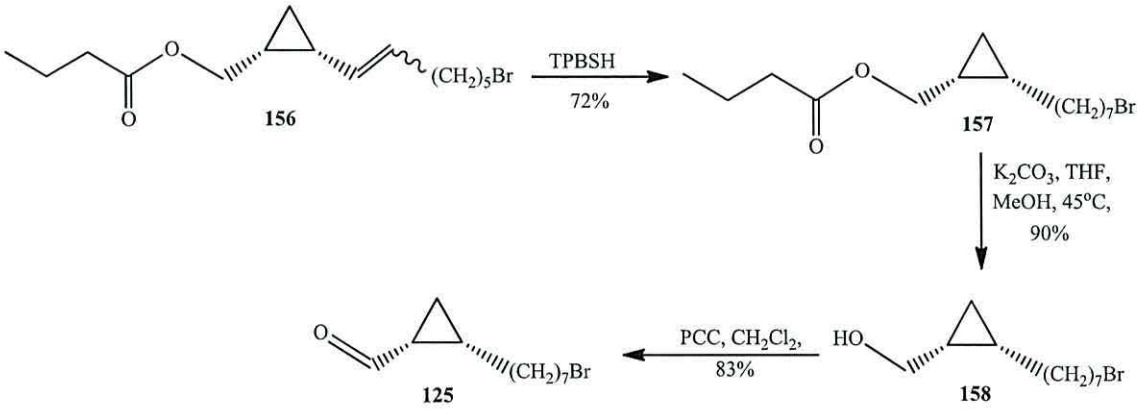
2.2.2.1 Synthesis of the proximal *cis*-cyclopropane unit (125)

In order to chain extend the cyclopropane unit, firstly the hydroxy-cyclopropyl monoester **(153)** was oxidised to the corresponding aldehyde **(154)**. A typical modified Julia-Kocienski olefination was performed between the resulting aldehyde and the sulfone **(155)**, using lithium *bis* (trimethylsilyl)amide as the base, to give the olefin **(156)** as a mixture of (*E/Z*) isomers **(Scheme 23)**.



Scheme 23: Chain extension of the compound (153)

It is known that hydrogenation of a double bound in the presence of the cyclopropane ring with palladium on carbon and hydrogen gas can lead to ring opening.[202,203] For that reason, the hydrogenation was achieved by using di-imide (N_2H_2) as a milder hydrogenation agent. This can reduce the double bond while maintaining the cyclopropane ring. In this work 2,4,6-triisopropyl-benzenesulfonyl hydrazide (TPBSH) was used to generate di-imide. The alkene **(156)** was dissolved in THF and TPBSH was added and heated for three hours, followed by the addition of another mole equivalent of TPBSH and stirring under the same conditions for 24 h **(Scheme 24)**. The proton NMR spectrum of the product **(157)** showed no signals for the alkene protons. The product ester **(157)** was deprotected to give the (1*R*,2*S*)-2-(7-bromoheptyl)cyclopropyl)methanol **(158)**, by using anhydrous potassium carbonate in THF and methanol at 45 °C. The resulting alcohol was then oxidised to the aldehyde **(125)**. The NMR spectra of this compound were found to be similar to the given in the literature.[165]

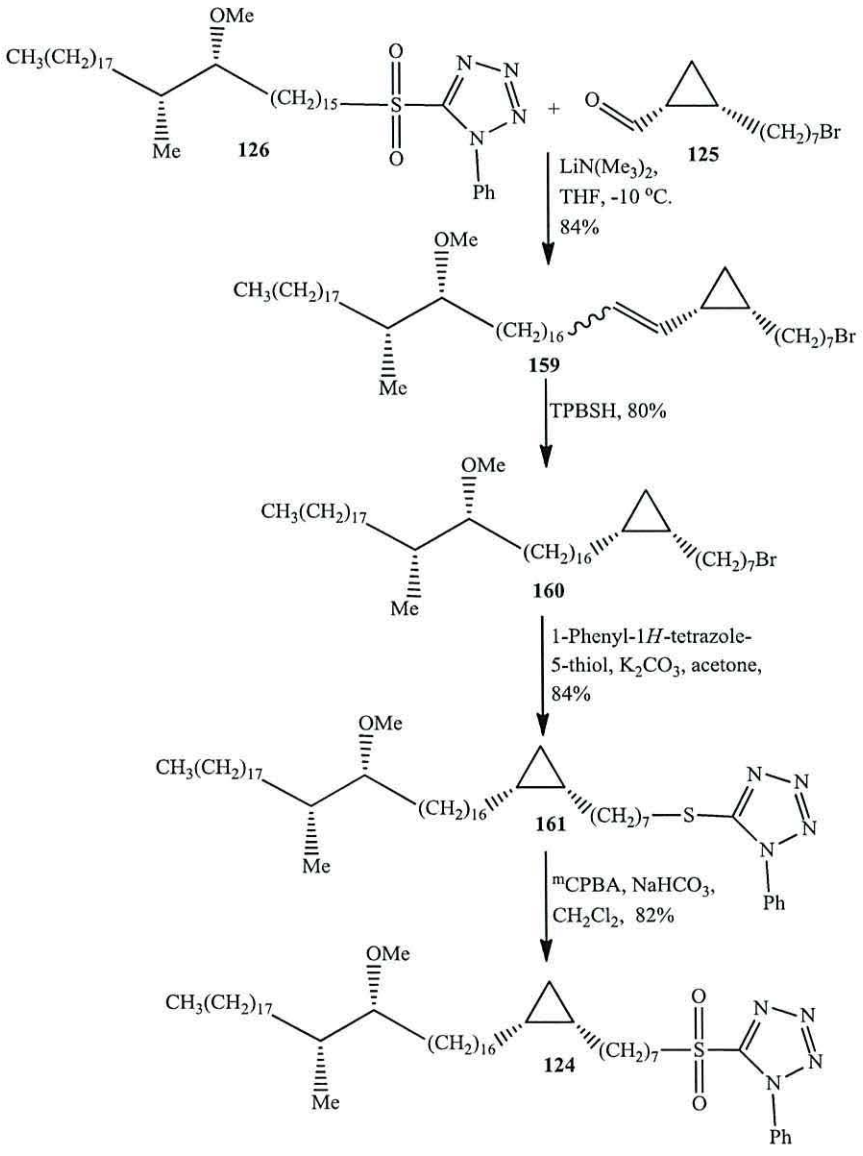


Scheme 24: Synthesis of *cis*-cyclopropane unit (125)[165]

2.2.2.2 Synthesis of the full meromycolate (124)

The aldehyde **(125)** was coupled with the methoxy sulfone unit **(126)** (It was made by Dr. J. R. Al Dulayymi and Evan Roberts) in the presence of the base to give the alkene **(159)** as an *(E/Z)*-mixture. Hydrogenation of this mixture was undertaken using TPBSH to give the compound **(160)**. The bromo-compound was reacted with 1-phenyl-*H*-tetrazole-5-thiol in the presence of K_2CO_3 and acetone to give the sulfide **(161)**. Subsequently, this was oxidised to the corrsponding sulfone **(124)**, using *m*-chloroperbenzoic acid in dichloromethane with sodium bicarbonate **(Scheme 25)**. The

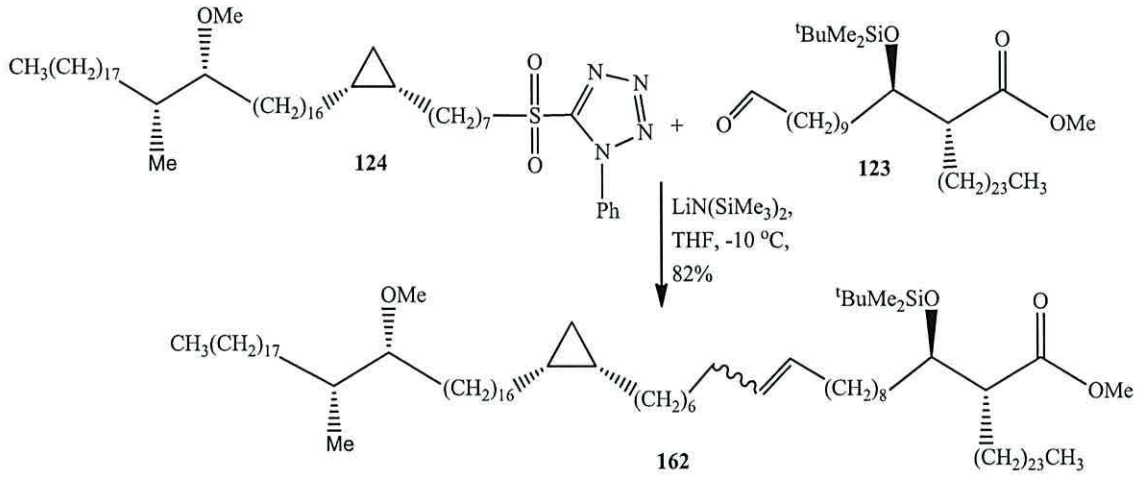
spectroscopic data obtained for the sulfane **(161)** and the sulfone **(124)** were similar. However, the proton NMR for the sulfane showed a multiplet between δ 7.58-7.54 representing five aromatic protons, and a triplet at δ 3.40 *(J* 7.25 Hz) for the two protons adjacent to the sulfur atom. Due to the presence of oxygen atoms in the sulfone, the five aromatic protons did not appear together, rather there were two sets of multiplets between δ 7.71-7.69 for two protons and δ 7.63-7.61 for three protons. Also, a characteristic peak for the two protons next to sulfur was shifted further down field to δ 3.74 (*J* 8.2 Hz) because of the deshielding effect of oxygen. The detailed spectroscopic data of this compound were commensurate with those given in the literature.[165]



Scheme 25: Synthesis of the meromycolate intermediate (124)

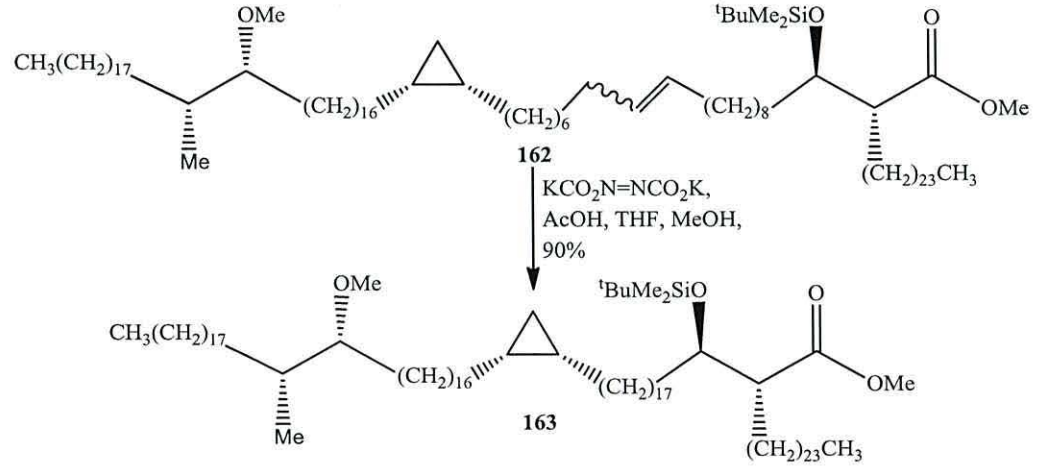
2.2.3 Final coupling to form the full methoxymycolic acid

The modified Julia reaction, as discussed before, was used to achieve the coupling between meromycolate sulfone **(124)** and mycolatemotif aldehyde **(123).** This gave the alkene product **(162)** as an (*E/Z*) mixture **(Scheme 26)**.



Scheme 26: The final coupling to form the methoxymycolic acid

Following this, hydrogenation of the double bond in the alkene **(162)** was achieved using dipotassium azodicarboxylate as the diimide source. The alkene **(162)** was dissolved in THF and methanol, then an excess of dipotassium azodicarboxlate was added at 0 °C under nitrogen. A solution of glacial acetic acid in THF was added dropwise over 18 h **(Scheme 27)**. The hydrogenation was repeated twice in order to make sure that the saturation of the alkene was complete.



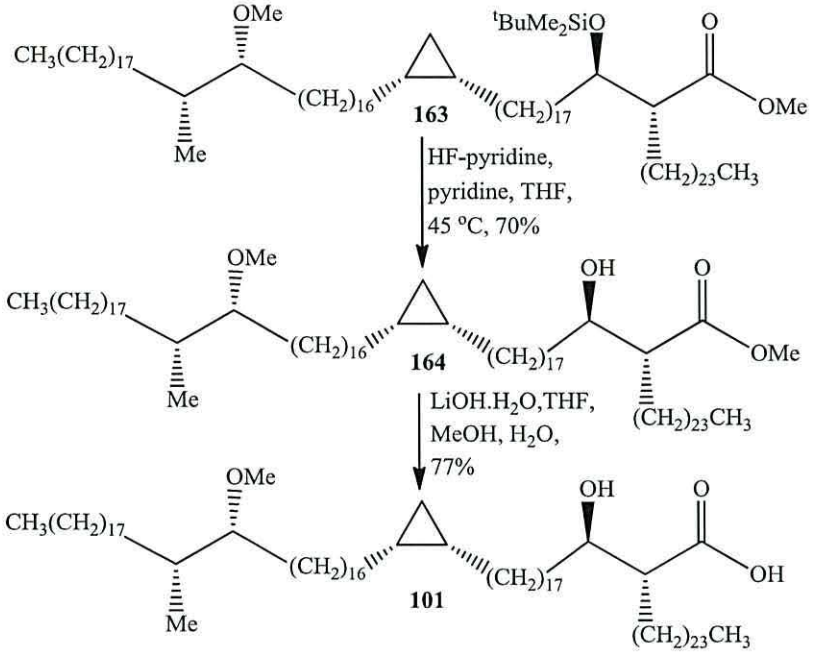
Scheme 27: Hydrogenation with dipotasium azodicarboxylate

The proton NMR spectrum of compound **(163)** showed no signal for the alkene protons. The proton next to the silyl group appeared as a multiplet at δ 3.93-3.89 and the two methoxy groups showed singlets at δ 3.66 and 3.34. The proton next to the methoxy group appeared as a multiplet between δ 2.97-2.94 and the α-proton of the motif part appeared as a doublet of doublets of doublets at δ 2.53 (*J* 4.1, 6.9, 10.4 Hz). The protons of the silyl protecting group appeared as a singlet at δ 0.86 for *tert*-butyl group and two singlets at δ 0.05 and 0.02 for the methyl groups. The cyclopropane ring protons appeared as a multiplet between δ 0.65-0.62, a triplet of doublets at δ 0.57 (*J* 4.1, 8.2 Hz) and a broad quartet at δ -0.32 (*J* 5.35 Hz).

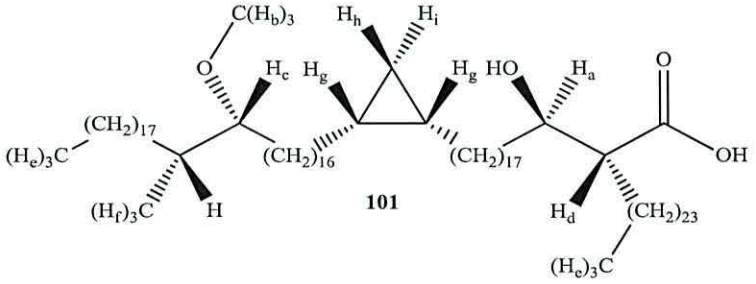
2.2.4 Deprotection and Hydrolysis

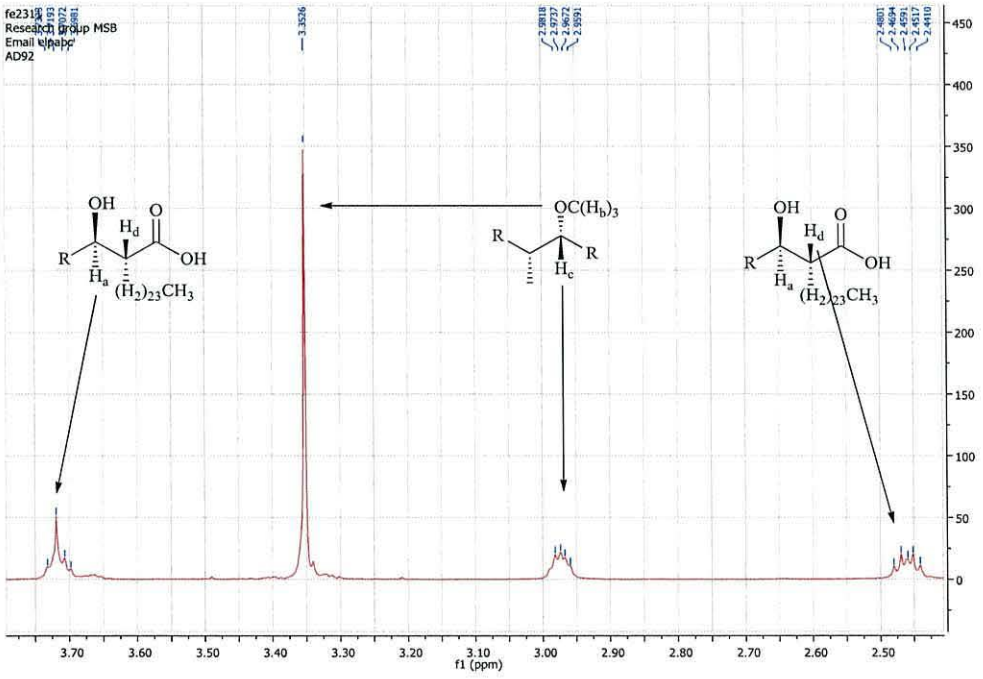
The *tert*-butyldimethylsilyl group of the compound **(163)** was removed using HF.pyridine and pyridine in dry THF at 45 °C for 18 h, giving the ester **(164) (Scheme 28)**. This was carried out in a clean dry polyethylene vial and required careful handling of the reagents. A less sensitive method, which involves the use of an acidic medium, was not an option due to the reactivity of the cyclopropane ring.[204] After work-up, the proton NMR spectrum of compound **(164)** showed the loss of the signals for the silyl group at δ 0.86 for *tert*-butyl and δ 0.05 and 0.02 for the two methyl groups. The carbon NMR spectrum showed no signals corresponding to the *tert*-butyldimethylsilyl group.

The final step to obtain free methoxymycolic acid involved the hydrolysis of the methyl ester group by using lithium hydroxide in a mixture of THF, methanol and water at 45 °C for 18 h. The successful formation of the free methoxymycolic acid **(101)** was confirmed by proton NMR which showed the disappearance of the singlet at δ 3.71 for the methyl ester. The MALDI MS of free methoxymycolic acid gave the correct $(M + Na)^+$ at 1276.55. In addition, the proton NMR **(Figure 19)** included a multiplet between δ 3.73-3.69 for the H_a next to the β-hydroxyl group and a singlet at δ 3.35 for $C(H_b)_3$. Also, proton H_c next to the methoxy group appeared as multiplet between δ 2.98-2.95, while H_d at the α-position appeared as a multiplet between δ 2.47-2.44. The two terminal methyl groups $C(H_e)_3$ showed a triplet at δ 0.88(*J* 6.9 Hz) while the methyl group $C(H_f)_3$ next to the methoxy group showed a doublet at δ 0.85 (*J* 6.6 Hz). The four protons of the *cis*-cyclopropane appeared as a multiplet at δ 0.68-0.64 for H_g, a doublet of triplets at δ 0.56 (*J* 4.05, 8.15 Hz) for H_h and a quartet at δ -0.32 (*J* 5.05 Hz) for H_i.

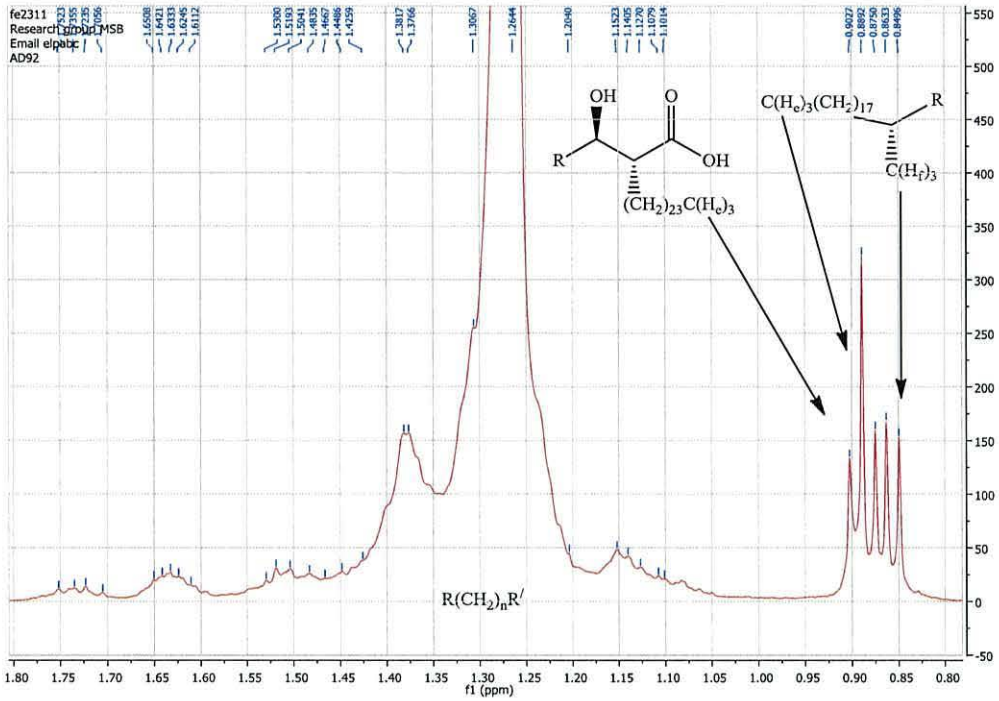


Scheme 28: Deprotection and hydrolysis to obtain the free methoxy mycolic acid

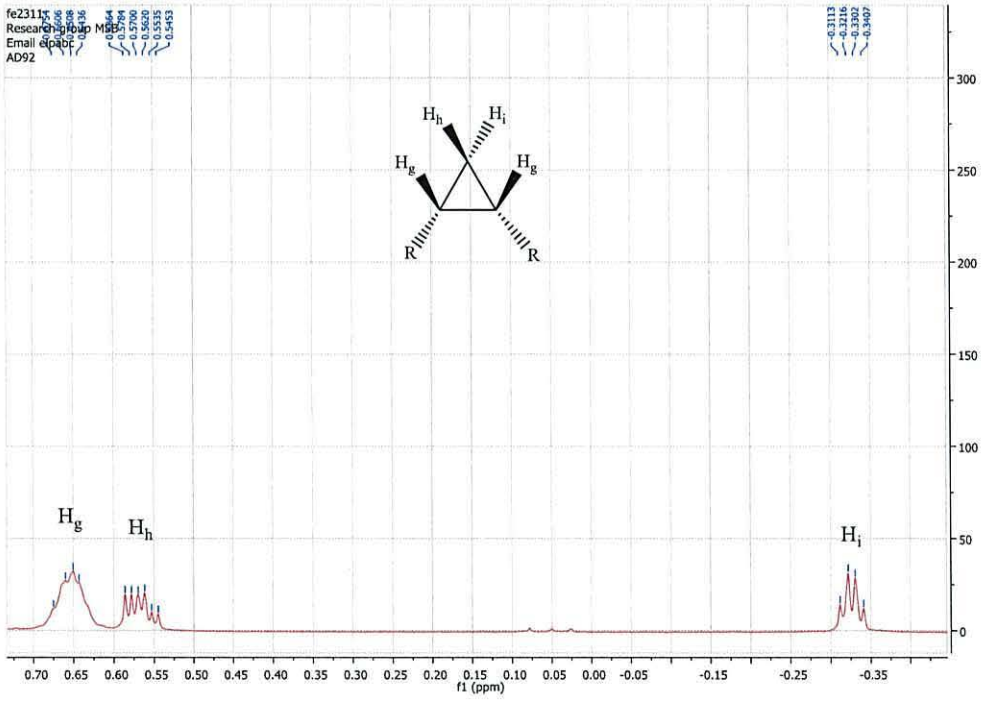




A



B



C

Figure 19: ^{1}H NMR expansions of free methoxy mycolic acid (101)

2.3 Synthesis of ketomycolic acid (165)

Methoxymycolic acids and ketomycolic acids are found in the cell wall of *Mycobacterium. tuberculosis* as the major oxygenated mycolic acids. It was decided to synthesise the ketomycolic acid **(165)** and its derivatives (TDM and TMM). It is believed that the ketomycolic acid **(165)** **(Figure 20)** has the same stereochemistry as the natural ketomycolic acid, because it is thought that the cyclopropane configuration in the ketomycolic acid **(165)** has the same stereochemistry in the natural ketomycolic acid.[205] Also, the methyl branch next to carbonyl group in the natural ketomycolic acid was proved to exist in the *S*-configuration.[87]

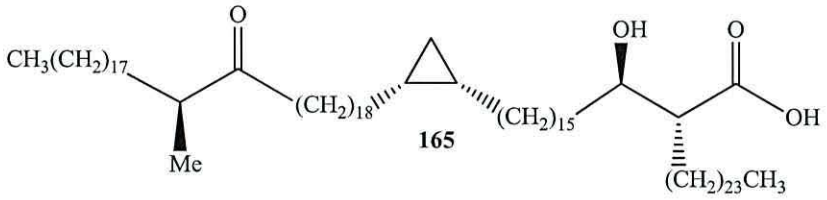
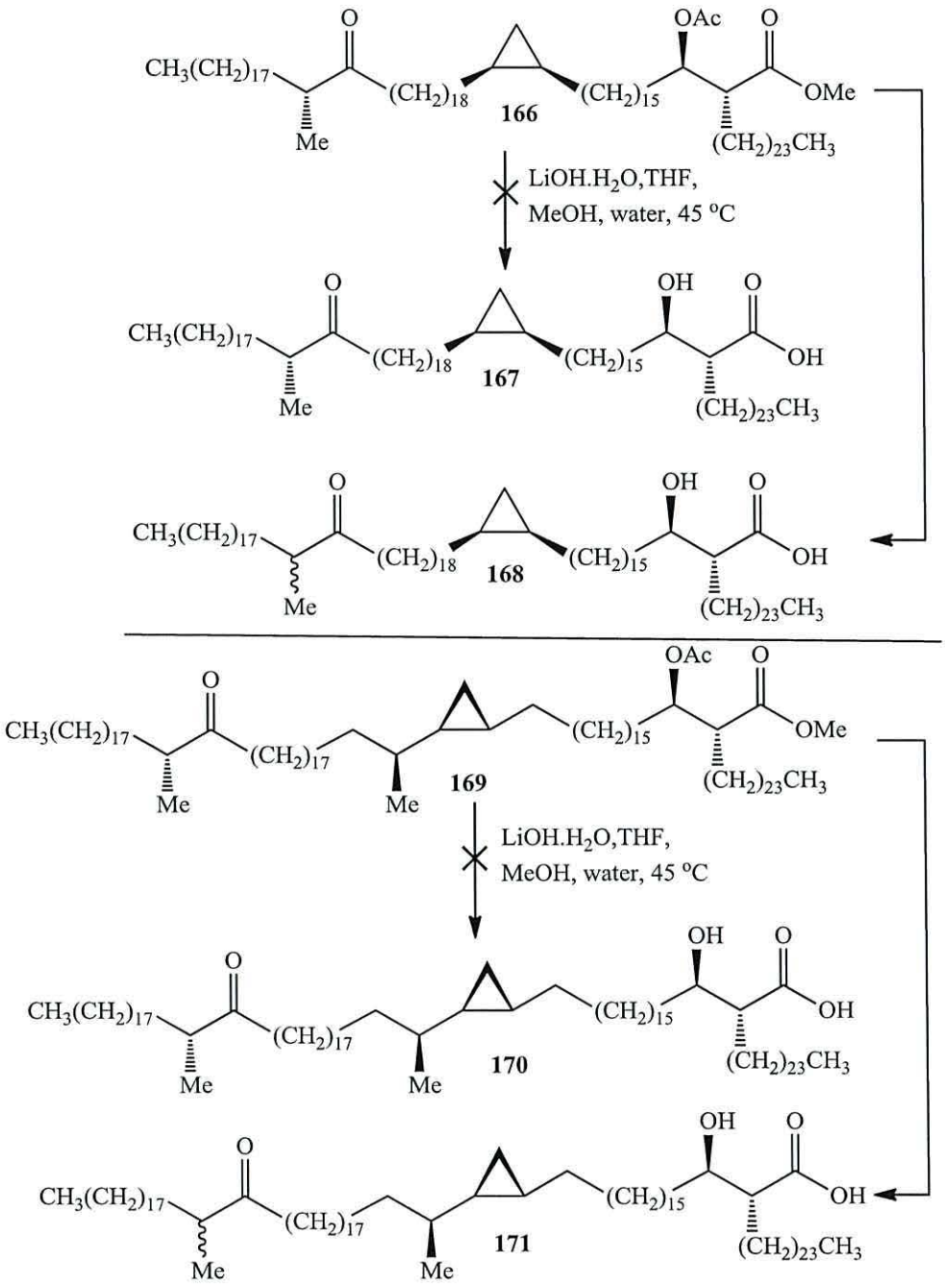


Figure 20: The target of a ketomycolic acid of Mycobacterium tuberculosis

2.3.1 Previous synthesis of ketomycolic acids

In early attempts, Koza[172] tried to prepare the free *cis*-ketomycolic acid **(167)** and the free *trans*-ketomycolic acid **(170)**. Koza reported that the final step to deprotect the methyl ester and acetyl protecting groups of **(166)** and **(169)** using lithium hydroxide monohydrate caused the epimerisation of the methyl group adjacent to the carbonyl, to give **(168)** and **(171)** instead of the free ketomycolic acids **(167)** and **(170)** **(Scheme 29)**.



Scheme 29: Base hydrolysis of the protected ketomycolic acids (166) and (169)

Later, the free *cis*-ketomycolic acid **(172)**[206] and the free *trans*-ketomycolic acid **(173)**[207] **(Figure 21)** were prepared without epimerisation. This was achieved by using different protecting groups for the secondary hydroxyl groups in both the meromycolate and mycolatemotif and making sure the oxidation of the alcohol of the meromycolate chain to a carbonyl group was carried out under a condition not causing epimerisation. Eventually complete deprotection was carried out under acidic conditions.

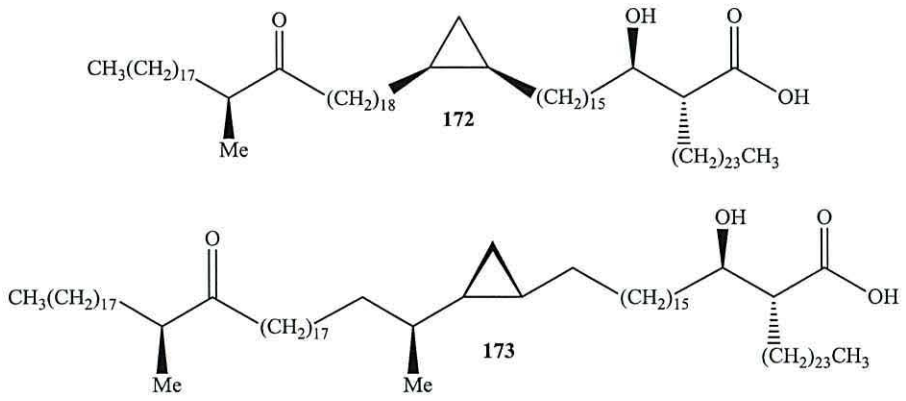
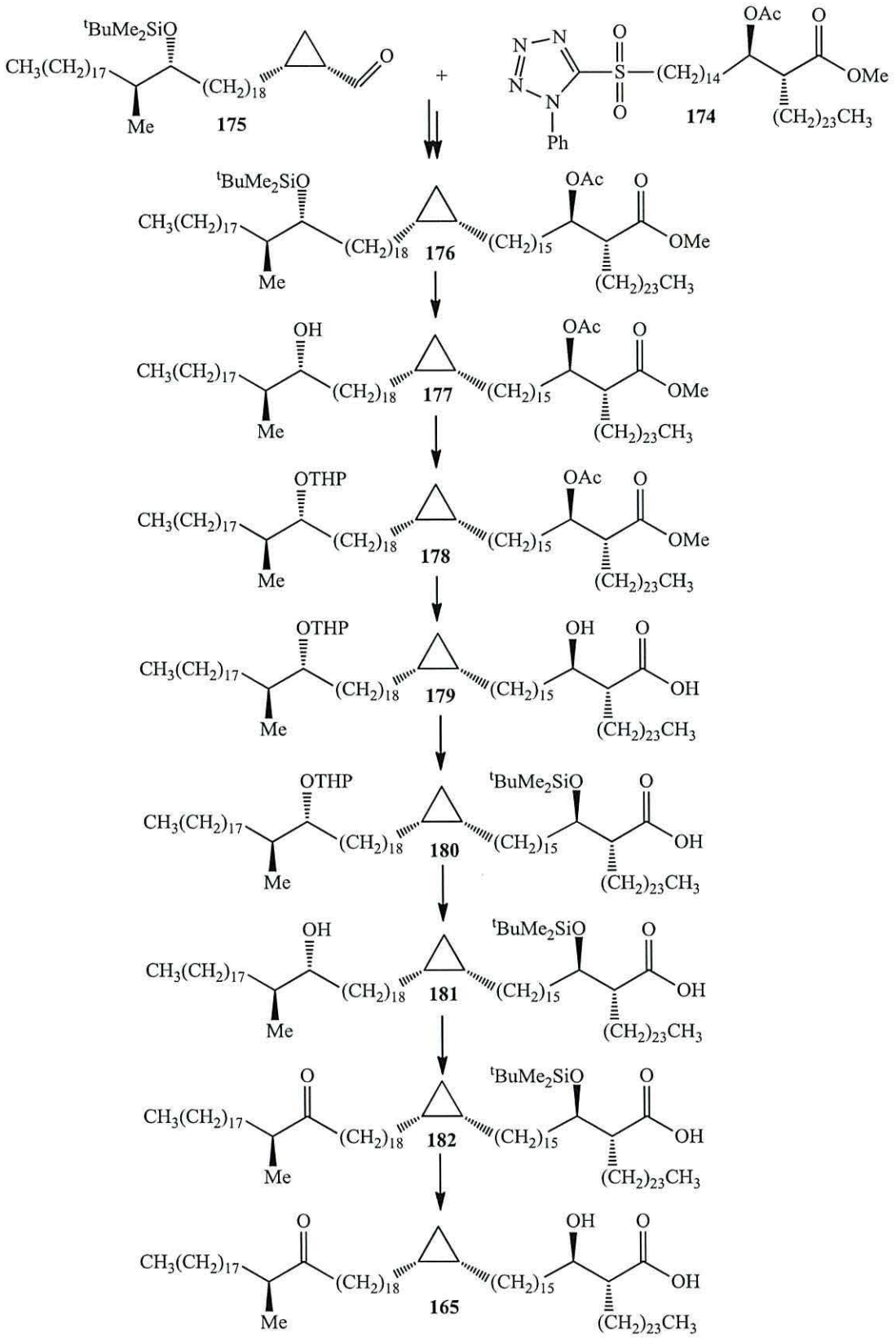


Figure 21: The synthesis of free keto mycolic acids with different stereochemistry

2.3.2 Synthesis of a new ketomycolic acid containing a *cis*-cyclopropane (165)

In this study a new free ketomycolic acid with correct stereochemistry was prepared by adapting the method in the literature.[206,207] This involved the coupling of the meromycolate part **(175)** with mycolatemotif part **(174)**, which had different protecting groups on them. This was followed by replacing the silyl protecting group with a THP group, which is resistant to basic media, and is cheap and easy to remove.[196] The methyl ester and acetyl groups of **(178)** were deprotected in basic media. The subsequent steps included the reprotection of the hydroxyl group in the motif with a silyl group, deprotection of the THP-group and oxidation of the resulting hydroxyl to give the ketone **(182)**. Finally, the silyl group was removed with HF-pyridine complex to give the free ketomycolic acid **(165) (Scheme 30).**

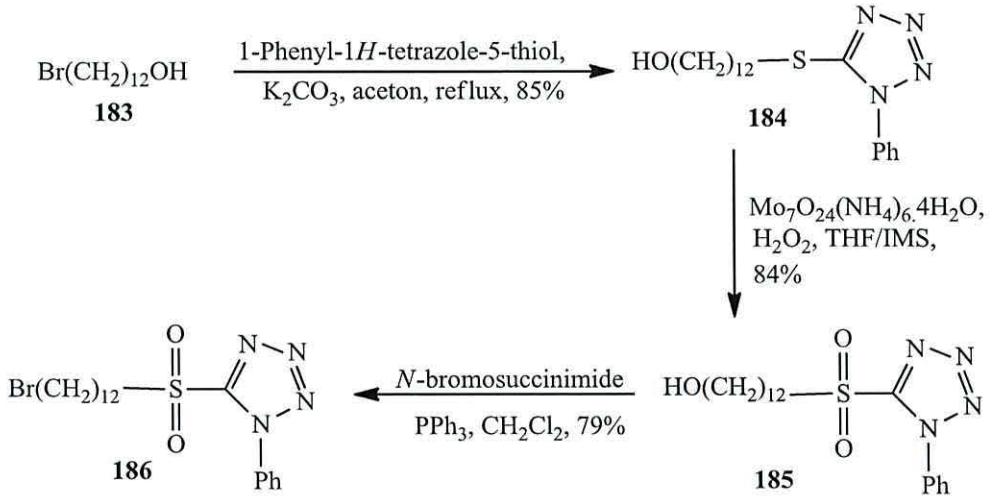


Scheme 30: The approach to the synthesis of a new ketomycolic acid (165)

2.3.3 Synthesis of mycolatemotif part of ketomycolic acid (174)

2.3.3.1 Synthesis of bromo-sulfone (186)

Twelve carbon atoms were needed to be added to the side chains of the mycolatemotif aldehyde **(148)** (as prepared earlier in Section **2.2.1.3**) to give the correct chain length between the meromycolate and the mycolatemotif. The bromo-sulfone **(186)** was prepared from 12-bromododecan-1-ol **(183)** which was reacted with 1-phenyl-1*H*-tetrazole-5-thiol and potassium carbonate to give the sulfide **(184)**. The sulfide was oxidised by using ammonium molybdate (VI) tetrahydrate and hydrogen peroxide to give the sulfone **(185)**. Finally, the sulfone was converted into bromo-compound **(186)** by reacting with *N*-bromosuccinimide and triphenylphosphine in dichloromethane **(Scheme 31)**. The detailed spectroscopic data of the compound **(186)** were as reported in the literature.[180]

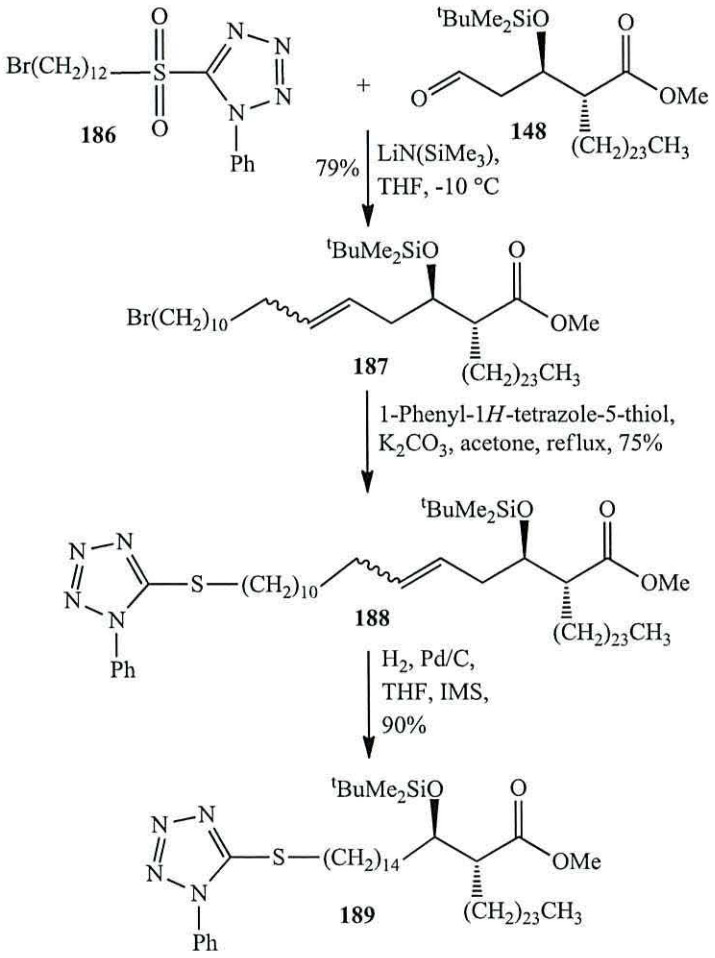


Scheme 31: Synthesis of the bromo-sulfone (186)

2.3.3.2 The coupling reaction

A Julia reaction was carried out between the aldehyde **(148)** and the sulfone **(186)** in the presence of the base in dry THF and gave the alkene **(187)** as an (*E/Z*) mixture **(Scheme 32)**. The unsaturated bromoester **(187)** was converted into sulfide **(188)**, using 1-phenyl-1*H*-tetrazol-5-thiol and potassium carbonate in acetone. The double bond of the resulting sulfide **(188)** was hydrogenated by using hydrogen gas with Pd on carbon (10%) as discussed earlier. The proton NMR spectrum of the saturated sulfide **(189)** showed no signals belonging to the alkene protons which confirmed the

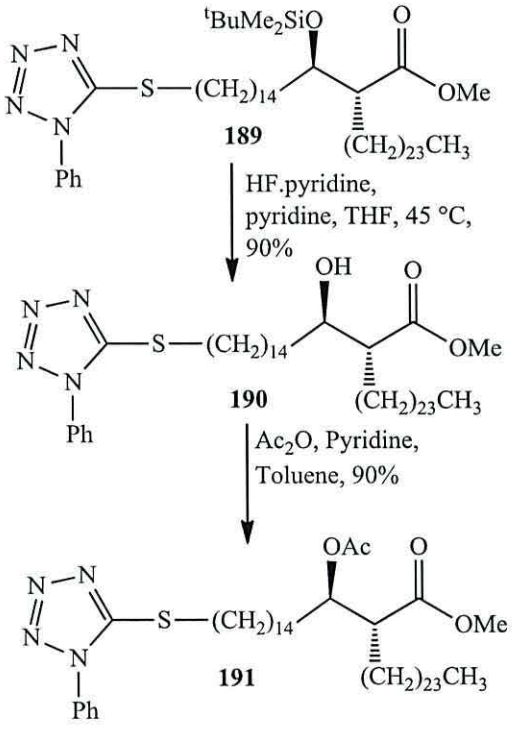
success of the hydrogenation. The five protons of the phenyl group showed a multiplet between δ 7.60-7.53. The proton at the β-position contiguous to the silyl protected alcohol, showed a multiplet between δ 3.92-3.88, while the proton at the α-position showed a doublet of doublets of doublets at δ 2.52 (*J* 3.0, 5.88, 10.85 Hz). The methoxy group appeared as a singlet at δ 3.65 and the two protons next to sulphur gave a triplet at δ 3.40 (*J* 5.88 Hz). The silyl group showed a singlet at δ 0.86 integrating to the nine protons of the *tert*-butyl and two singlets at δ 0.04 and 0.02 with an integration of three protons each for the two methyl groups. Also, the carbon NMR showed no signals belonging to alkene carbons, while it showed four signals at δ 133.79, 130.10, 128.23 and 123.87 for the phenyl group. The signal of the tetrazole carbon appeared at δ 154.45.



Scheme 32: Synthesis of compound (189)

2.3.3.3 Desilylation and acetylation

It was necessary to replace the silyl protecting group of mycolatemotif **(189)** with an acetyl group because the hydroxyl on the meromycolate was also protected with the same silyl group. The removal of the silyl group was carried out using HF.pyridine complex in dry THF at 45 °C **(Scheme 33)**. Compound **(190)** was characterised by the proton NMR spectrum which showed the disappearance of the singlet at δ 0.86 belonging to the *tert*-butyl group and also the loss of the two singlets at δ 0.04 and 0.02 for the two silyl methyls. The proton at the β- position appeared as a multiplet between δ 3.69-3.63, which was shifted slightly to high field because of the removal of the silyl group. The carbon NMR spectrum confirmed the absence of the signals at δ -4.45 and -4.96 for the two methyl carbons next to silicon. The IR spectrum showed very a broad peak at 3486 cm^{-1} for the hydroxyl group.



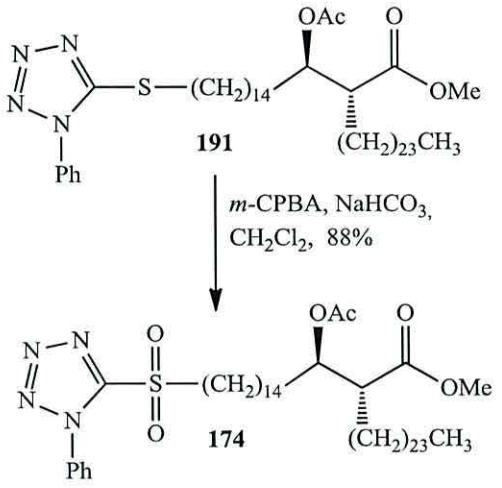
Scheme 33: Exchange of the protecting group of mycolatemotif part (189)

Then the secondary hydroxyl group of mycolatemotif **(190)** was protected with an acetyl group using acetic anhydride and anhydrous pyridine in dry toluene. The product **(191)** was confirmed by the proton NMR spectrum which showed the proton next to the acetoxy group as a multiplet between δ 5.08-5.05 which was shifted downfield because the acetate group made it more acidic. The methyl ester and methyl

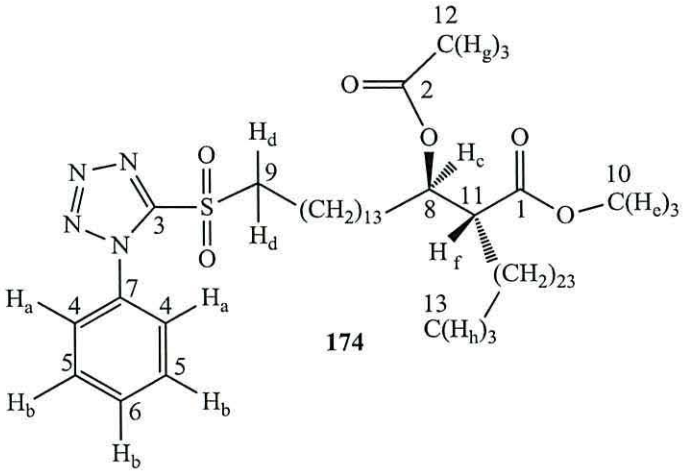
of the acetyl group appeared as singlets at δ 3.66 and 2.20 respectively. The methylene group adjacent to sulfur appeared as a triplet at δ 3.38 (*J* 5.88 Hz). The carbon NMR spectrum gave two signals at δ 170.42 and 173.56 for the acetyl carbonyl and ester carbonyl respectively. The IR spectrum showed the disappearance of any signal belonging to the hydroxyl group.

2.3.3.4. Oxidation of the sulfide (191)

The final step of the synthesis of mycolatemotif part **(174)** involved the oxidation of the sulfide (**191**) to the sulfone, using *m*-chloroperbenzoic acid in dichloromethane with sodium bicarbonate **(Scheme 34)**. The structure of the motif sulfone **(174)** was confirmed by the proton and the carbon NMR spectra as shown in **Table 2.**



Scheme 34: Synthesis of mycolatemotif sulfone part (174) of the keto-mycolic acid

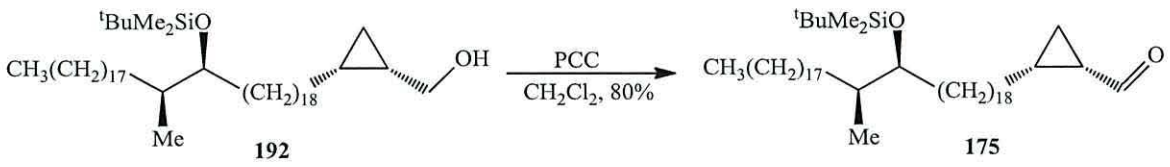


H_x	δ	Multiplicity	Integration	*J* (Hz)	C_x	δ
H_a	7.71-7.69	m	2	-	C_1	173.63
H_b	7.63-7.61	m	3	-	C_2	170.45
H_c	5.10-5.07	m	1		C_3	153.53
H_d	3.73	t	2	6.4	C_4	133.06
H_e	3.68	s	3	-	C_5	131.40
H_f	2.62	ddd	1	3.44,5.48,8.68	C_6	129.74
H_g	2.03	s	3	-	C_7	125.18
H_h	0.88	t	3	5.44	C_8	73.85
-	-	-	-	-	C_9	55.89
-	-	-	-	-	C_{10}	51.57
-	-	-	-	-	C_{11}	49.97
-	-	-	-	-	C_{12}	20.98
-	-	-	-	-	C_{13}	14.09

Table 2: ^1H and ^{13}C NMR analysis of the compound (174)

2.3.4 Final coupling to form ketomycolic acid (165)

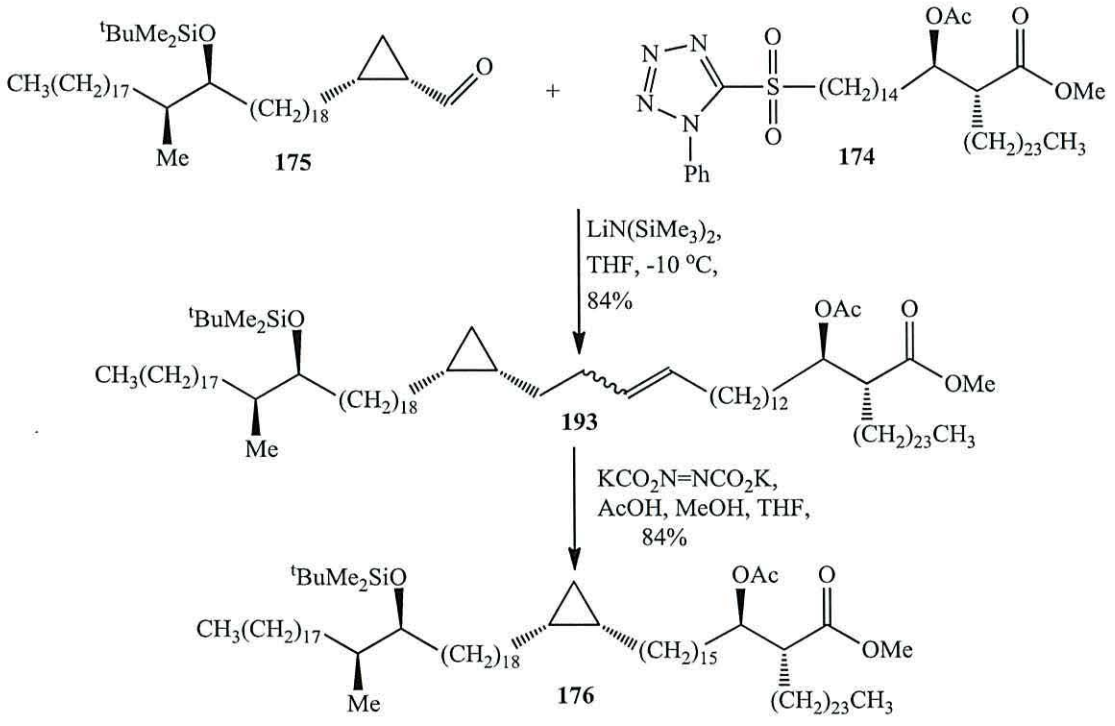
Initially, the alcohol meromycolate **(192)** (kindly provided by Dr. J. R. Al Dulayymi) was oxidised into the corresponding aldehyde **(175)**, using PCC in dichloromethane **(Scheme 35)**. The proton NMR spectrum of the aldehyde product showed a doublet at δ 9.36 (*J* 4.44 Hz) for the aldehyde proton. The carbon NMR spectrum of the aldehyde showed a signal at δ 201.75 for the carbonyl carbon, while the IR spectrum showed a peak at 1709 cm^{-1} for the carbonyl group.



Scheme 35: Oxidation of the keto meromycolate

Following this, the sulfone **(174)** was coupled with the meromycolate aldehyde **(175)** to give the alkene **(193)** as an (*E/Z*) mixture **(Scheme 36)**. The hydrogenation of the

alkene was carried out by using dipotassium azodicarboxylate as a described before. The success of the hydrogenation was confirmed by the proton NMR of compound **(176)** which showed no signals in the alkene region. The β-proton adjacent to the acetoxy group gave a doublet of triplets at δ 5.09 (*J* 4.1, 8.15 Hz) while the proton adjacent to the silyl group appeared as a multiplet between δ 3.51-3.48. The methoxy group showed a singlet at δ 3.68 and the methyl of the acetyl group gave a singlet at δ 2.03. The α-proton showed a doublet of doublets of doublets at δ 2.62 (*J* 4.1, 6.95, 10.75 Hz). The cyclopropane ring give a multiplet at δ 0.66-0.63 for two protons; one proton showed a doublet of triplets at δ 0.56 (*J* 4.1, 7.85 Hz) and one proton gave a broad quartet at δ – 0.32 (*J* 5.35 Hz). The carbon NMR spectrum showed the two carbonyl carbons at δ 173.65 and 170.34, with the absence of all signals belonging to the alkene carbons.

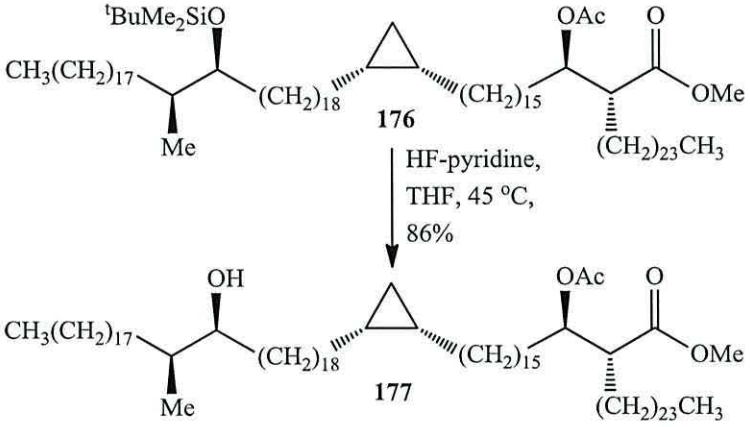


Scheme 36: The final coupling to form keto-mycolic acid

2.3.5 Deprotection of the silyl group in meromycolate

The silyl protecting group was removed from compound **(176)** by using HF.pyridine complex and pyridine in dry THF **(Scheme 37)**. The proton NMR spectrum for the resulting compound **(177)** revealed a doublet of triplets at δ 5.09 (*J* 3.75, 7.85 Hz) for the proton next to the acetoxy group and the proton at the α-position appeared as a

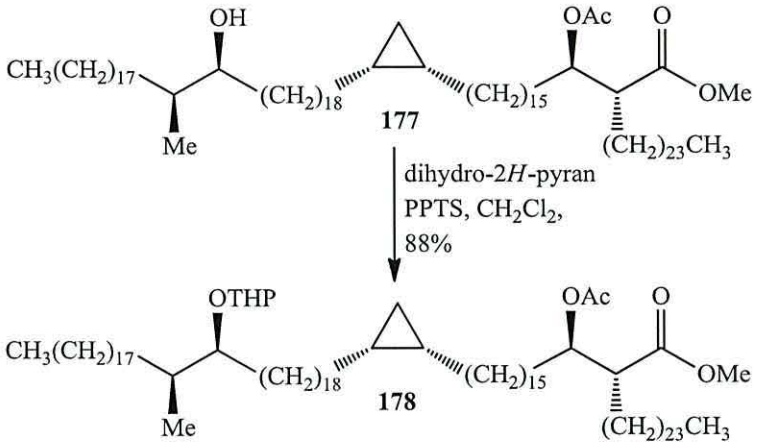
doublet of doublets of doublets at δ 2.62 (*J* 4.4, 6.95, 10.7 Hz). The proton next to the resulting hydroxyl group appeared as a multiplet between δ 3.50-3.49, with the absence of all signals belonging to the silyl group. The carbon NMR spectrum indicated the loss of the signals at δ -4.18 and -4.42 for the two methyl carbons next to silicon. The IR spectrum showed a broad peak at 3555 cm^{-1} for the hydroxyl stretch.



Scheme 37: Deprotection of the silyl group of (176)

2.3.6 Re-protection of the secondary hydroxyl group in meromycolate

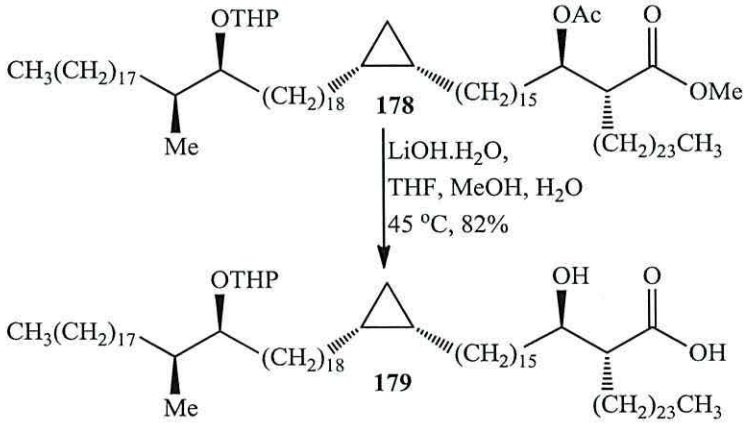
The secondary hydroxyl group of meromycolate was re-protected with a THP protecting group. Compound **(177)** was treated with freshly distilled dihydro-2*H*-pyran in the presence of pyridine *p*-toluene sulfonate in dry dichloromethane **(Scheme 38)**. The proton NMR spectrum identified the formation of the THP protected compound **(178)** which gave a broad triplet at δ 4.65 (*J* 3.15 Hz), another broad triplet at δ 4.62 (*J* 2.2 Hz) for the acetal proton, a multiplet between δ 3.94-3.89, and another multiplet between δ 3.48-3.42 for the protons next to the oxygen of the THP group along with the proton adjacent to the THP group. The IR spectrum did not show the peak at 3555 cm^{-1} for the hydroxyl group.



Scheme 38: Protection the secondary alcohol with a THP group

2.3.7 Hydrolysis of the acetyl and methyl ester protecting groups

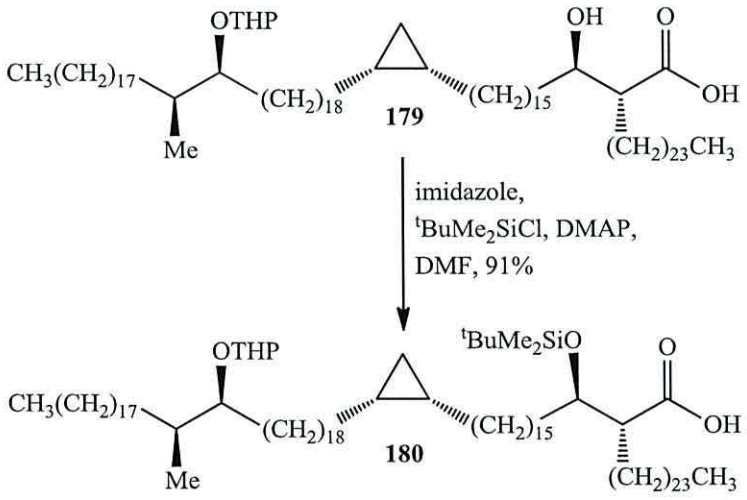
The hydrolysis of the acetyl and methyl ester groups of the above compound **(178)** was carried out by using lithium hydroxide monohydrate in a mixture of THF, MeOH and H_2O **(Scheme 39)**. The proton NMR spectrum of the resulting compound **(179)** showed a multiplet between δ 3.72-3.70 for the proton next to the β-hydroxyl group, shifted to high field because of the removal of the acetyl group. The singlet at δ 3.68 for the methyl ester and the singlet at δ 2.02 for methyl of the acetyl group had also disappeared. The IR spectrum showed a broad peak at 3435 cm^{-1} for the new hydroxyl group which showed the success of the hydrolysis.



Scheme 39: Hydrolysis of the acetyl and methyl ester protecting groups

2.3.8 Re-protection of the β-hydroxyl group

The β-hydroxyl group in compound **(179)** was re-protected with *tert*-butyl-dimethylsilyl. This protection was carried out by using *tert*-butyldimethylsilyl-chloride and imidazole in dry DMF at 70 °C which led to protection of both the acid and the β-hydroxyl group. Therefore, the crude product was treated with potassium carbonate in a mixture of THF, CH_3OH and water at 45 °C overnight, in order to deprotect the silyl on the carboxylic acid **(Scheme 40)**. The proton NMR spectrum of the silyl protected compound **(180)** showed a singlet at δ 0.92 for the protons of the *tert*-butyl and two singlets at δ 0.14 and 0.13 for the dimethyl groups. The proton next to the silyl protected hydroxyl group appeared as a multiplet between δ 3.85-3.82, which was shifted slightly downfield in comparison with the signal for the unprotected hydroxyl group. The carbon NMR spectrum confirmed the presence of the silyl group which gave two signals at δ - 4.26 and -4.99 for the two methyl carbons next to silicon.

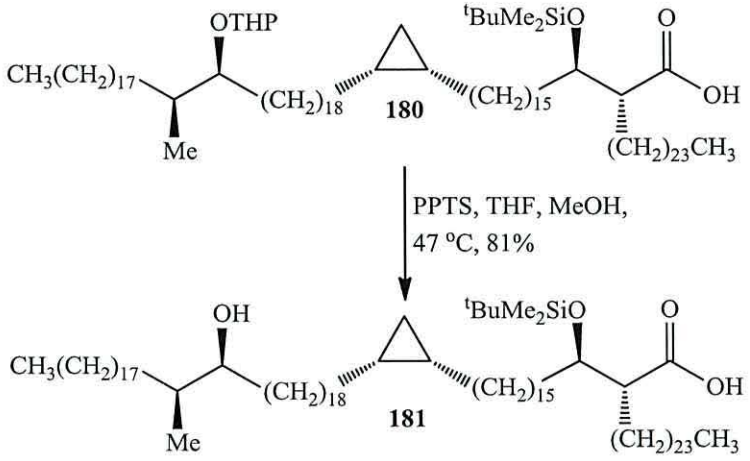


Scheme 40: Re-protection of β-hydroxyl with a silyl protecting group

2.3.9 Deprotection of THP-group

The THP protecting group was removed from **(180)** to give a secondary hydroxyl group, with the silyl group on the motif part not being affected. The THP was removed by stirring a solution of compound **(180)** in a mixture of THF, MeOH and H_2O with pyridinium-*p*-toluenesulfonate **(Scheme 41)**. The proton NMR spectrum of the product **(181)** clearly showed the disappearance of the characteristic signals for the tetrahydropyranyl group. The proton next to the hydroxyl group in the meromycolate

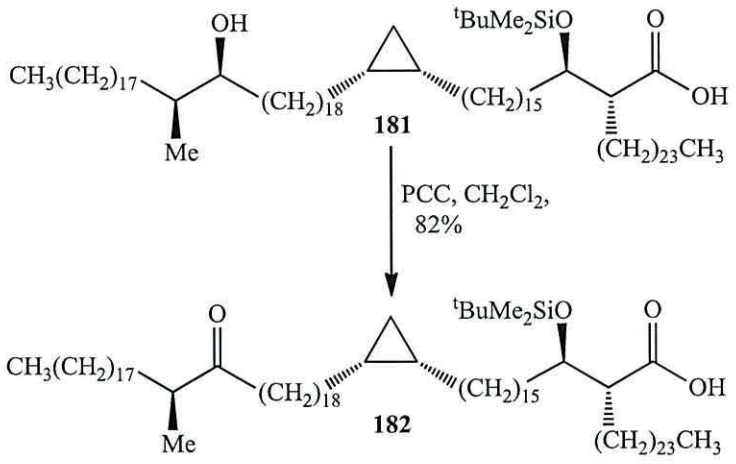
appeared as a broad pentuplet at δ 3.50 (*J* 3.8 Hz). The IR spectrum showed a broad peak at 3410 cm^{-1} corresponding to the hydroxyl group.



Scheme 41: Deprotection of the THP protecting group

2.3.10 Oxidation of the secondary hydroxyl group

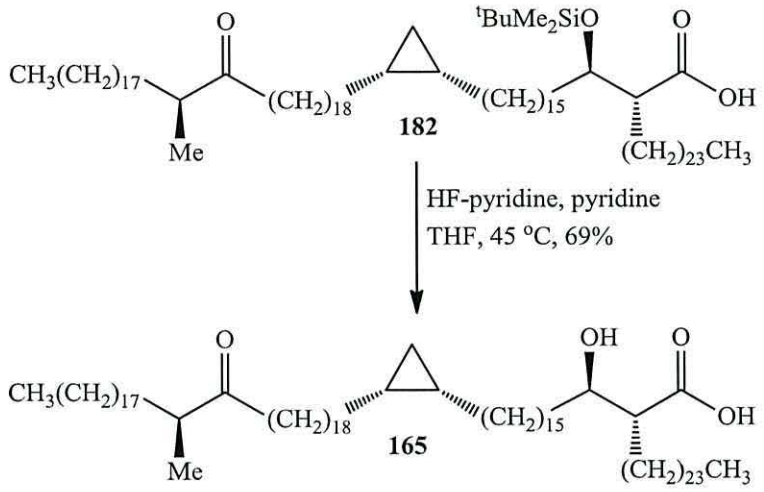
The secondary hydroxyl group of the compound **(181)** was converted into the corresponding carbonyl group by using same method as described before with PCC in dichloromethane **(Scheme 42)**. The proton NMR spectrum of the protected ketomycolic acid **(182)** showed a multiplet between δ 3.85-3.82 for the β-proton. The α-proton and the proton next to the new carbonyl group appeared as a multiplet between δ 2.54-2.48 while the two protons next to the carbonyl adjacent to the long chain appeared as a doublet of triplets at δ 2.41 (*J* 2.0, 7.25 Hz). The methyl group next to the carbonyl appeared as a doublet at δ 1.05 (*J* 6.6 Hz), while the terminal methyl groups showed a triplet at δ 0.89 (*J* 6.95 Hz). The protons of the *t*-butyl of the silyl group appeared as a singlet at δ 0.92 and the protons of the two methyl groups appeared as two singlets at δ 0.14 and δ 0.13. The carbon NMR spectrum gave signals at δ 215.16 and 175.93 for the keto and ester functional groups, respectively. Compound **(182)** was divided into two portions; one for the next step to prepare the full free keto mycolic acid and another to prepare the TDM and TMM.



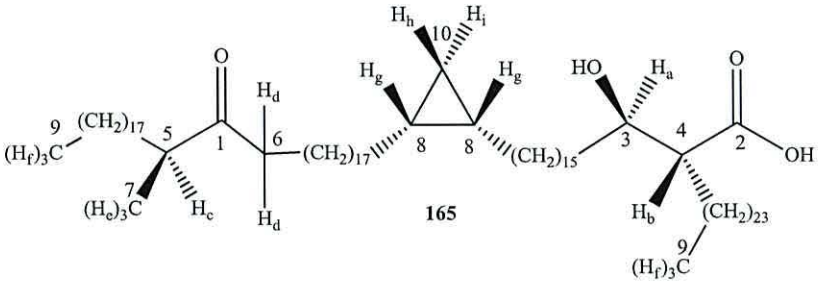
Scheme 42: Oxidation of the secondary alcohol of compound (181)

2.3.11 Deprotection of the silyl group

The *tert*-butyldimethylsilyl group of compound **(182)** was removed by treating with HF-pyridine, giving the free ketomycolic acid **(165) (Scheme 43)**. The proton and carbon NMR spectra showed the absence of all signals corresponding to the silyl group, as is shown in **Table 3** and **Figure. 22**. The MALDI MS of free ketomycolic acid showed the correct mass ion at $(M + Na)^+$: 1260.32. The specific rotation was $[\alpha]_D^{21} = +9.14$ (*c* 0.71, $CHCl_3$). The specific rotation can be converted to a molecular rotation, M_D which is found to be +113 by using the equation: $M_D = [\alpha]_D \times$ (Mol. Wt./100). According to the research of Quémard *et al.*,[87] the predicted molecular rotation M_D of **(165)** can be calculated by adding the rotations of the fragments to each other. The M_D of the *R, R*-β-hydroxy-α-alkyl carboxylic acid fragment was determined to be M_D +40, and that of the *S*-methyl keto fragment to be M_D +44, while the *cis*-cyclopropane has a contribution of approximately 0, therefore, the predicted M_D of **(165)** is +84. This indicated that the free mycolic acid had been obtained without epimerization of the α-methyl next to the carbonyl group.

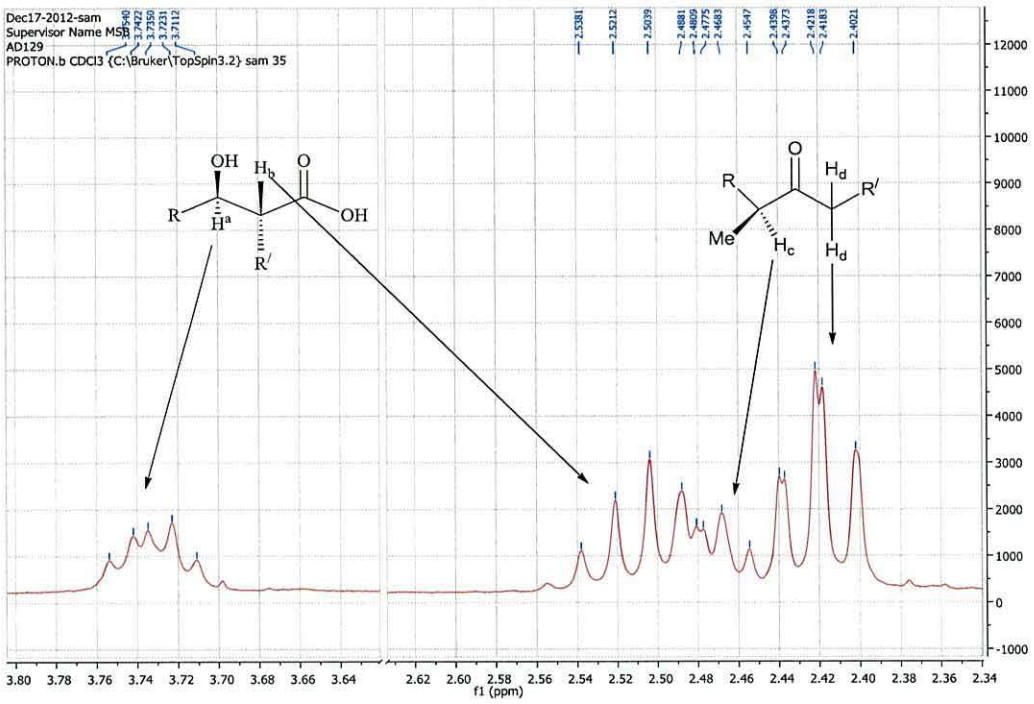


Scheme 43: Synthesis of the free keto-mycolic acid

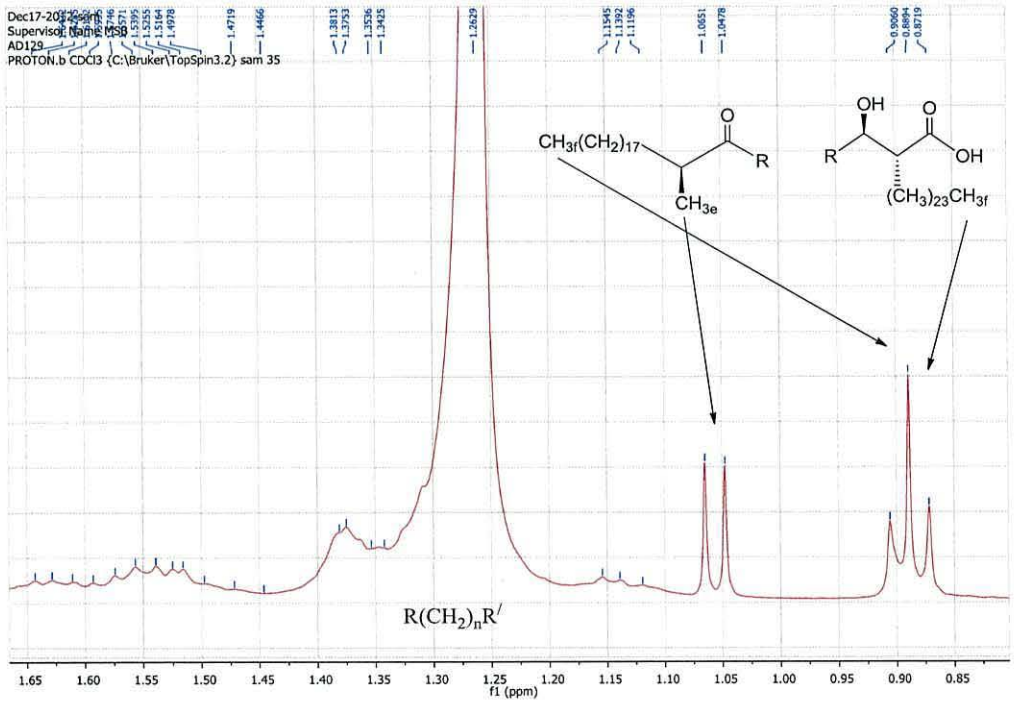


H_x	δ	Multiplicity	Integration	*J* (Hz)	C_x	δ
H_a	3.75-3.71	m	1	-	C_1	215.43
H_b	2.5	q	1	5.6	C_2	178.42
H_c	2.48-2.45	m	1	-	C_3	72.11
H_d	2.42	dt	2	1.04, 5.7	C_4	50.69
$R(CH_2)_nR$	1.74-1.11	br. m	146	-	C_5	46.33
H_e	1.05	d	3	5.5	C_6	41.14
H_f	0.88	t	6	5.4	C_7	16.35
H_g	0.66-0.63	m	2	-	C_8	15.76
H_h	0.56	dt	1	3.08, 6.5	C_9	14.10
H_i	-0.32	br.q	1	4.12	C_{10}	10.89

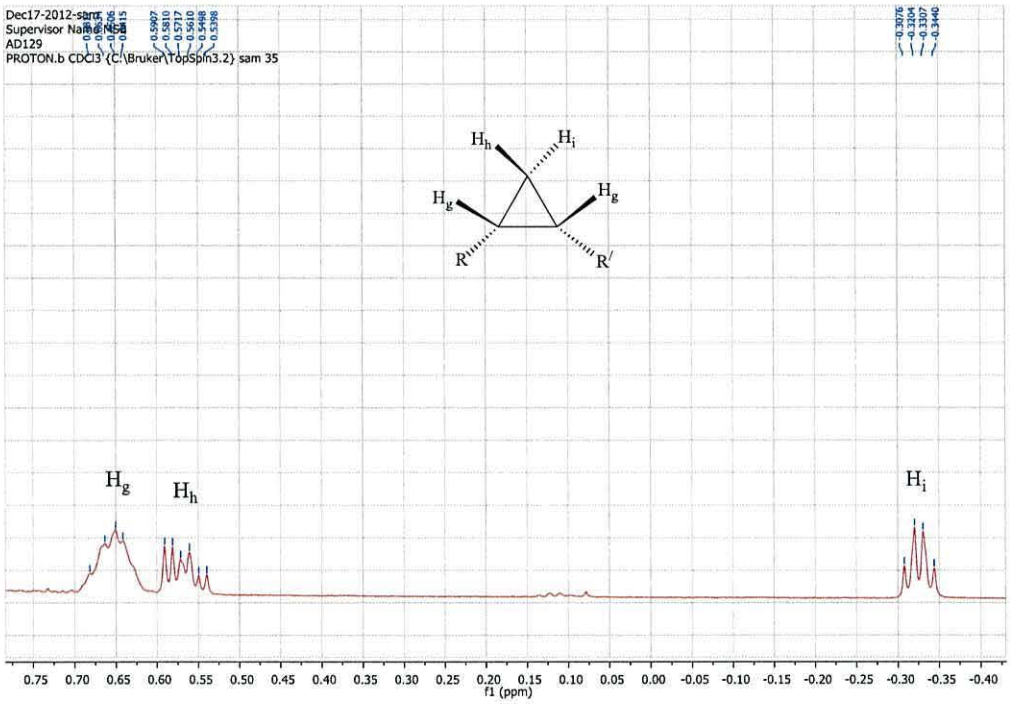
Table 3: ^{1}H and ^{13}C NMR analysis of the free keto mycolic acid (165)


Dec17-2012-sam
Supervisor Name MSB
AD129
PROTON.b CDCl3 {C:\Bruker\TopSpin3.2} sam 35
OH
H_b
O
R
H^a
OH
R/
O
H_d
R
R/
Me
H_c
H_d
f1 (ppm)

A


Dec17-2012-sam
Supervisor Name MSB
AD129
PROTON.b CDCl3 {C:\Bruker\TopSpin3.2} sam 35
$CH_{3f}(CH_2)_{17}$
O
R
CH_{3e}
OH
O
R
OH
$(CH_3)_{23}CH_{3f}$
$R(CH_2)_nR'$
f1 (ppm)

B



C

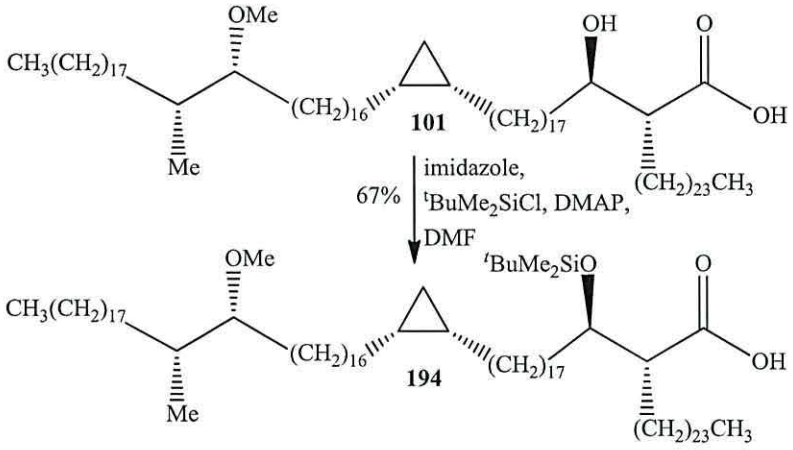
Figure 22: ^{1}H NMR expansion of free keto mycolic acid (165)

2.4 Synthesis of trehalose esters of mycolic acid (cord factors)

The synthesis of new cord factors of mycolic acids **(101)** and **(165)** was carried out in this study according to the same procedure as described in the literature.[176] This involved the esterification of the protected mycolic acid with protected trehalose. After that, the protecting groups on both trehalose and mycolic acid were removed to give free cord factor.

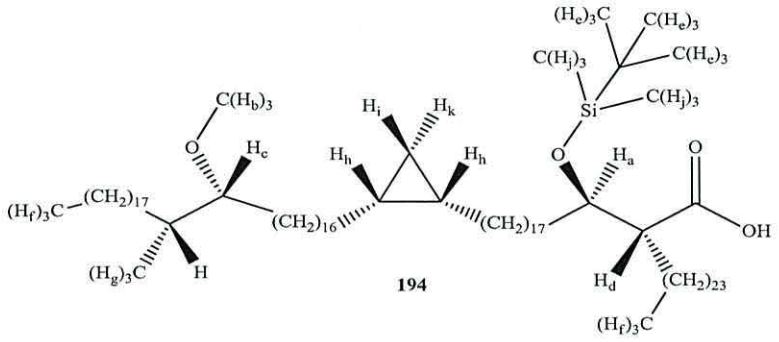
2.4.1 Protection of the secondary hydroxyl group of methoxymycolic acid

The secondary hydroxyl group at the β-position of methoxy mycolic acid **(101)** was protected before the esterification to avoid alcohol reactions. The *tert*-butyldimethylsilyl ether group was chosen to protect the β-hydroxyl group. The same procedure used in Section **2.3.8** was repeated to protect the β-hydroxyl group of **(101)** **(Scheme 44)**.



Scheme 44: Protected mycolic acid (101)

The product was identified by the proton NMR spectrum which gave a singlet at 0.91 for the *tert*-butyl and two singlets at δ 0.12 and 0.11 for the protons of the two methyl groups of the silyl group **(Table 4)**.

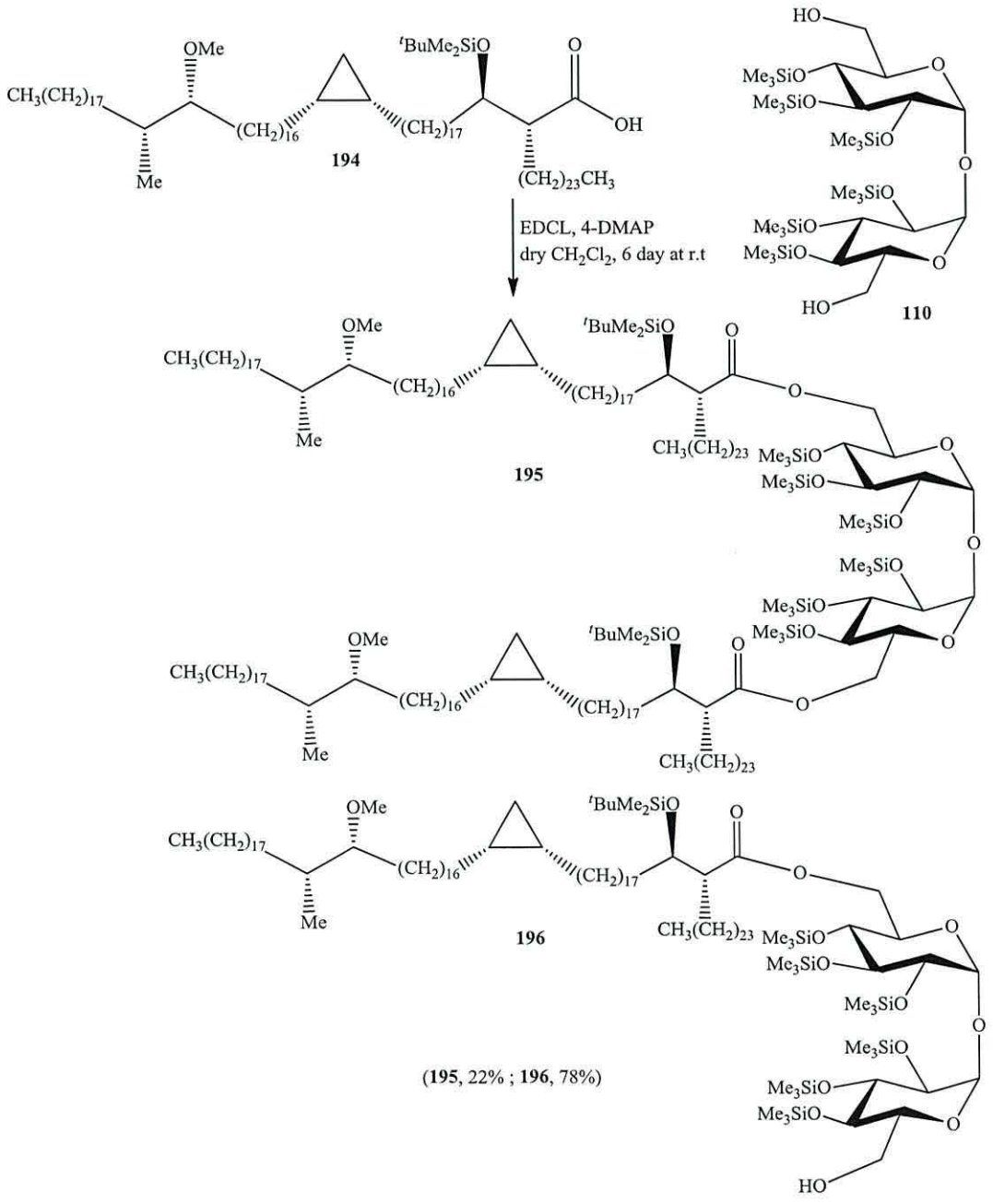


H_x	δ	Multiplicity	Integration	J (H_z)
H_a	3.87-3.84	m	1	-
H_b	3.34	s	3	-
H_c	2.96	pent.	1	5.05
H_d	2.53	ddd	1	3.8, 5.8, 9.45
H_e	0.91	s	9	-
H_f	0.89	t	6	6.9
H_g	0.86	d	3	6.6
H_h	0.67-.63	m	2	-
H_i	0.56	dt	1	4.1, 8.2
H_j	0.12, 0.11	s	2 x 3H	-
H_k	-0.32	br.q	1	5.05

Table 4: ^1H NMR analysis of the protected methoxy mycolic acid (194)

2.4.2 Coupling of protected methoxymycolic acid with trehalose.

The esterification reaction was carried out between protected methoxymycolic acid **(194)** and protected trehalose **(110)** in dry dichloromethane in the presence of EDCL and DMAP as catalysts which have been reported to improve the yield.[208] The reaction mixture was stirred at room temperature for 6 days, at which point TLC showed that no starting material was left. The solvent was evaporated and the residue was purified by column chromatography. The first fraction was TDM **(195)** and the second fraction was TMM **(196) (Scheme 45)**.



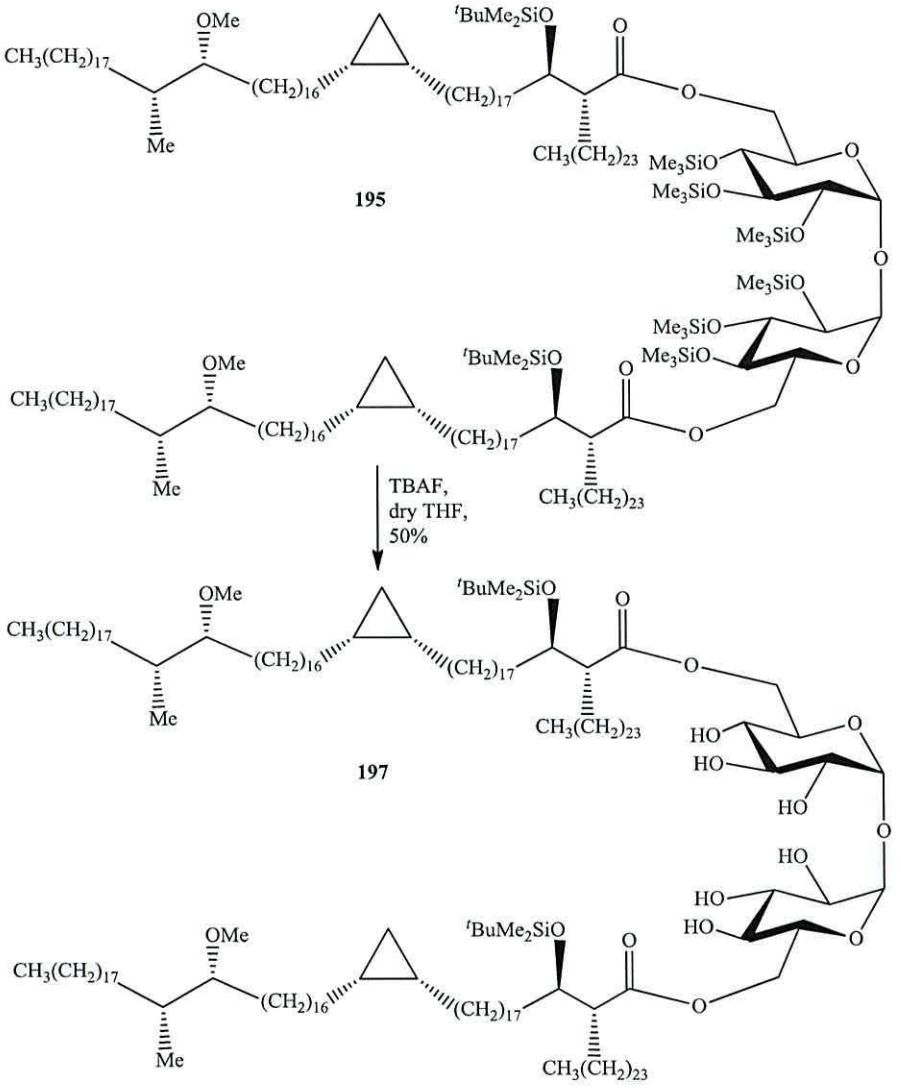
Scheme 45: Esterification of protected trehalose with methoxy mycolic acid (194)

The proton NMR spectrum of TDM **(195)** showed a doublet at δ 4.86 (*J* 2.85 Hz) for the hemiacetal protons. The remaining signals for the sugar protons and the β-hydroxyl protons resonated at δ 4.37, 4.04-3.99, 3.96-3.88, 3.52 and 3.39. The protons of the methoxy groups showed a singlet at δ 3.34 and the protons adjacent to the methoxy groups showed a broad pentuplet at δ 2.96 (*J* 4.1 Hz). The α-protons of the mycolic acid appeared as a doublet of doublets of doublets at δ 2.55 (*J* 3.6, 4.7, 10.1 Hz). The terminal methyl groups gave a triplet at δ 0.89 (*J* 6.9 Hz) and the *tert*-butyl groups showed a singlet at δ 0.88 with an integration of eighteen protons. The trimethylsilyl protecting groups on the sugar appeared as singlets at δ 0.16, 0.148 and 0.14 with an integration of eighteen protons for each. The twelve protons of dimethyl groups bounded to silicon atom appeared as a singlet at δ 0.064. The protons of the cyclopropane ring showed a multiplet at δ 0.66-0.63 for four protons, a doublet of triplets at δ 0.56 (*J* 4.1, 8.2 Hz) for two protons, and a broad quartet at δ -0.31 (*J* 5.05 Hz) for the other two protons. The carbon NMR spectrum showed a signal at δ 173.8 for the carbonyl carbon. The anomeric carbon signal appeared at δ 94.86 and the remainder of the sugar carbons between δ 73.56-70.76. The methyl carbon signals of the protecting silyl groups of the sugar appeared at δ 1.18, 1.10 and 0.16. Additionally, the carbons of the dimethyl silyl groups of the mycolic acid appeared at δ -4.50 and -4.63. The MALDI MS showed the correct mass ion at $(M + Na)^+$: 3496.75. The proton NMR spectrum of the TMM **(196)** was more complicated than the TDM because of the loss of the symmetry. The hemiacetal protons appeared as doublets at δ 4.91 (*J* 3.15 Hz) and δ 4.84 (*J* 2.85 Hz) with an integration one proton for each. The remaining of the sugar protons resonated between δ 4.91-3.39. The signals of the methoxy mycolic acid protons were similar to TDM **(195)**, but with half the number of protons. The MALDI MS showed the correct mass ion at $(M + Na)^+$: 2146.75.

2.4. 3 Deprotection of TDM (195)

The trimethylsilyl and *t*-butyldimethylsilyl protecting groups of TDM **(195)** were deprotected in two steps. The first step used TBAF in dry THF at room temperature for one hour to deprotect the trimethylsilyl groups on the sugar molecules **(Scheme 46)**. The success of this step in forming compound **(197)** was confirmed by the proton NMR spectrum which showed the absence of the signals belonging to the trimethylsilyl protecting groups at δ 0.16, 0.148 and 0.140. Also, the carbon NMR

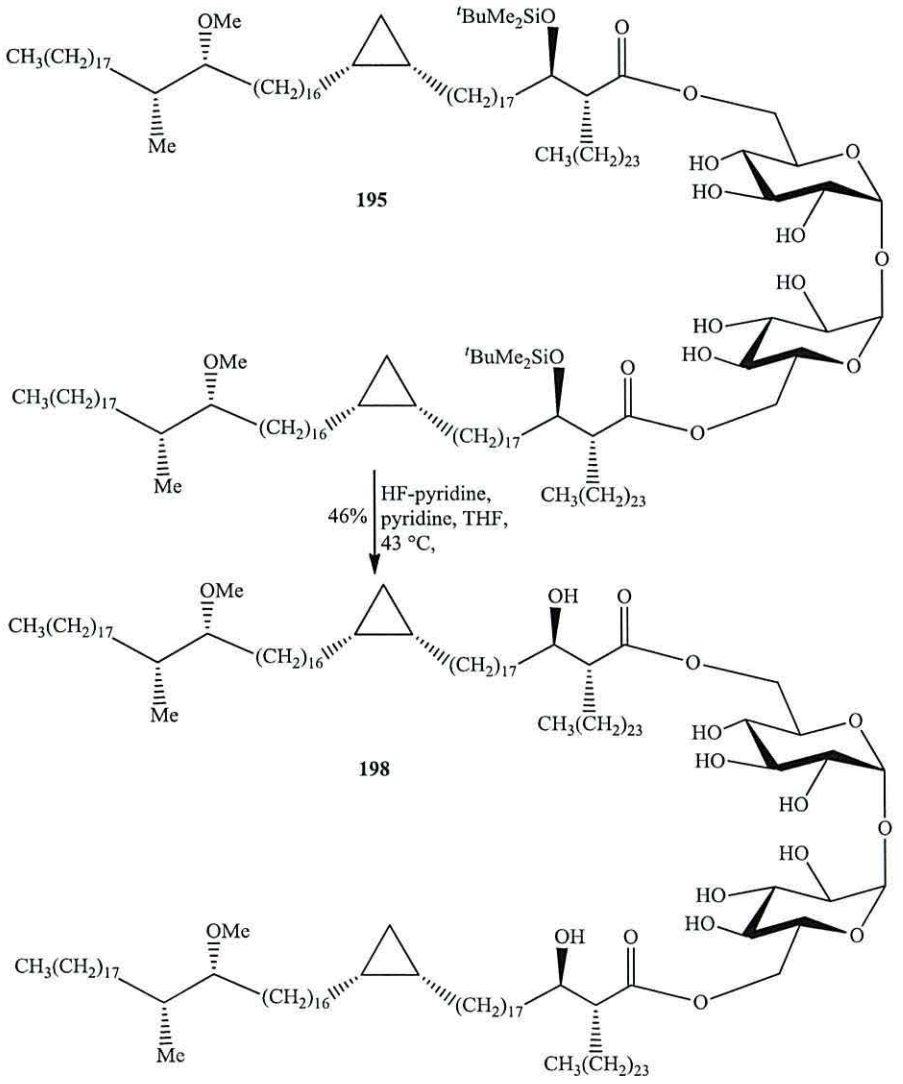
spectrum showed no signals at δ 1.18, 1.10 and 0.16 corresponding to the carbons of the trimethylsilyl groups.



Scheme 46: Deprotection of the trehalose moiety (195)

The second step was to remove the *t*-butyl silyl ether protecting group at the β-position of the mycolic acid using HF.pyridine complex and pyridine in dry THF at 45 °C overnight **(Scheme 47)**. The structure of free TDM **(198)** was established by the proton NMR spectrum which showed no signals for the *tert*-butyldimethylsilyl group. The hemiacetal protons showed a doublet at δ 4.98 (*J* 3.15 Hz). The rest of the sugar protons resonated at δ 4.74, 4.29, 3.75, 3.5, 3.37 and 3.19 with an integration of two protons for each. The protons adjacent to β-hydroxyl of the mycolic acids showed a broad triplet at δ 3.89 (*J* 7.9 Hz) and the methoxy groups of mycolic acids gave a

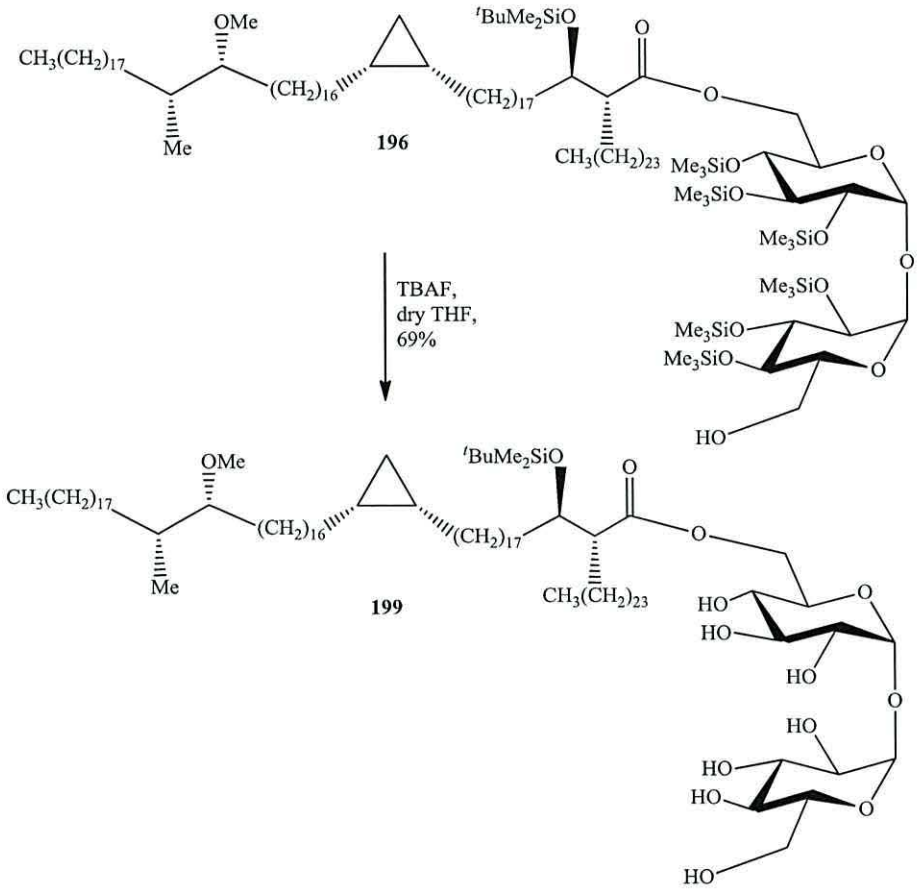
singlet at δ 3.31. A broad quartet signal appeared at δ 2.95 (*J* 5.1 Hz) for the protons adjacent to the methoxy group and a multiplet occurred between δ 2.39-2.28 for the protons in the α-position next to the alkyl chain of mycolic acid. The terminal methyl groups gave a triplet at δ 0.85 (*J* 6.6 Hz) with an integration of twelve protons, while the methyl adjacent to the methoxy group gave a doublet at δ 0.82 (*J* 6.95 Hz) with an integration of six protons. The protons of the cyclopropane ring gave a multiplet at δ 0.64-0.58 for four protons, a doublet of triplets at δ 0.53 (*J* 4.1, 8.15 Hz) for two protons and a broad quartet at δ -0.35 (*J* 5.05 Hz) for two protons. Also, the carbon NMR spectrum showed no signals belonging to the *tert*-butyldimethylsilyl group. The MALDI MS showed the correct mass ion at $(M + Na)^+$: 2838.01.



Scheme 47: Synthesis of free TDM of methoxymycolic acid (198)

2.3. 4 Deprotection of TMM (196)

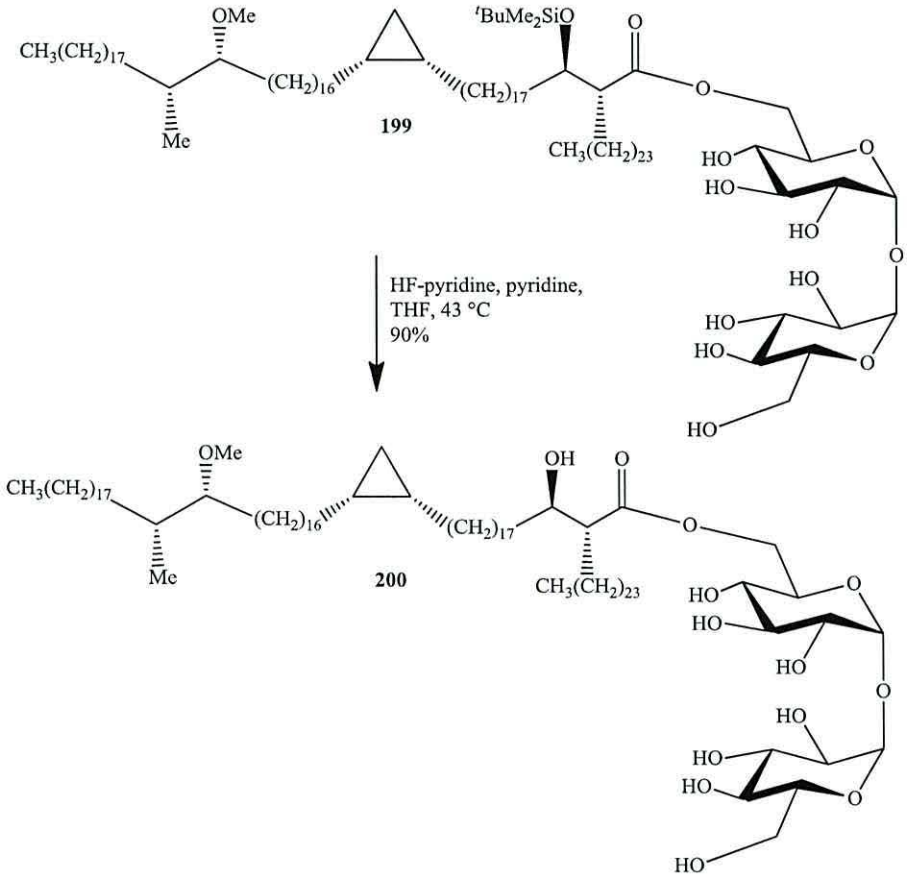
The method which was applied for the deprotection of TDM **(195)** was repeated to obtain free TMM. The protecting groups of the sugar were removed by stirring compound **(196)** with TBAF in dry THF at room temperature for one hour **(Scheme 48)**. The proton NMR spectrum of the resulting compound **(199)** showed the loss of the signals at δ 0.17, 0.16, 0.155, 0.150, 0.148 and 0.122, and the carbon NMR spectrum showed the absence of the signals at δ 1.05, 1.01, 0.92, 0.84, 0.17 and 0.045, which correspond to the carbons of the trimethylsilyl groups.



Scheme 48: Deprotection of (196)

Subsequently, the *tert*-butyldimethylsilyl group on the β-position of compound **(199)** was removed by using HF.pyridine complex and pyridine in dry THF **(Scheme 49)**. The structure of the free TMM **(200)** was identified by the proton NMR spectrum. It showed no signals for the *tert*-butyl at δ 0.83, or for the protons of the two methyl groups bonded to silicon at δ 0.02 and 0.00. The hemiacetal protons appeared as a

doublets at δ 5.07 (*J* 4.0 Hz) and 5.00 (*J* 3.45 Hz). The rest of the sugar protons resonated between δ 4.68 and 3.2. The signals of methoxymycolic acid protons were similar to free TDM with half the number of protons. The carbon NMR spectrum confirmed the formation of free TMM which showed the loss of the signals at δ -4.74 and -5.11 for the carbons of the two methyl groups bonded to silicon. The MALDI MS of free TMM **(200)** showed the correct mass ion at $(M + Na)^+$: 1600.65.

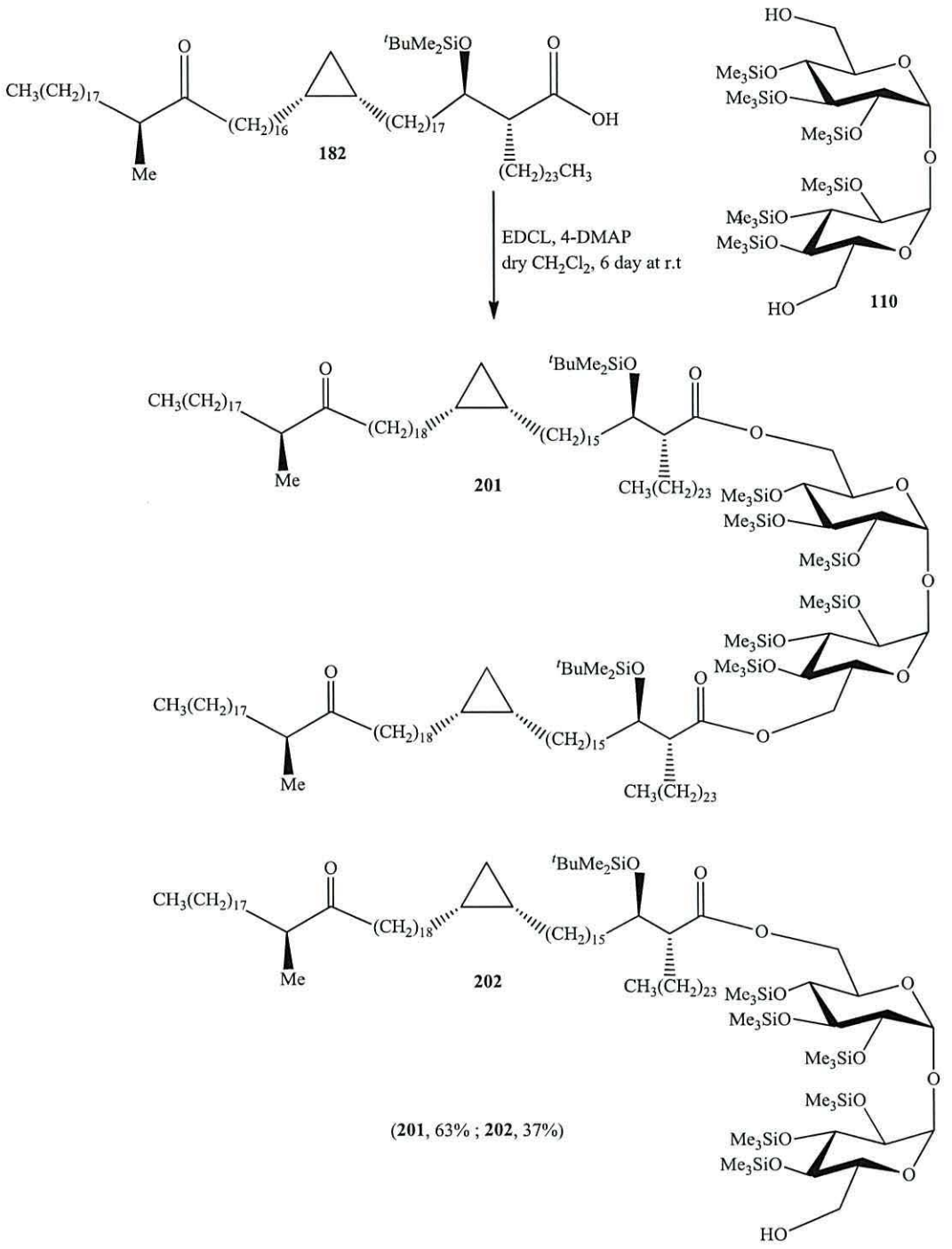


Scheme 49: Synthesis of free TMM of methoxymycolic acid (200)

2.3.5 Synthesis of cord factors of ketomycolic acid

The same method employed to prepare TDM **(198)** and TMM **(200)** was adopted to prepare the TDM and TMM of *cis*-ketomycolic acid **(165)**. The protected ketomycolic acid **(182)** was reacted with protected trehalose **(110)** in dry dichloromethane in the presence of EDCI and DMAP. After work up, the product was separated into two fractions by column chromatography **(Scheme 50)**. The first fraction was TDM **(201)** which was confirmed by the proton NMR spectrum. The hemiacetal protons showed a

doublet at δ 4.85 (*J* 2.86 Hz). The remaining sugar protons resonated between δ 4.37-3.38, including the two protons at the β-position of the motif part. The proton at the α-position relative to the carboxyl group and the proton α to the ketone appeared as a multiplet between δ 2.56-2.48 with an integration of four protons. The protons next to the carbonyl adjacent to the long chain of mycolic acid appeared as a triplet at δ 2.41 (*J* 7.4 Hz) with an integration of four. The protons of the methyl next to the carbonyl appeared as a doublet at δ 1.05 (*J* 6.95 Hz) with an integration of six protons. The terminal methyl groups and the protons of *tert*-butyl of the silyl protecting group of the mycolic acid appeared as a multiplet between δ 0.9-0.88. The protons of the trimethyl silyl protecting groups upon the sugar appeared as singlets at δ 0.16, 0.148 and 0.14. The dimethylsilyl ether of mycolic acid appeared as a singlet at δ 0.064. The cyclopropane ring protons showed a multiplet at δ 0.65-0.63 for four protons, a doublet of triplets at δ 0.57 (*J* 3.52, 7.52 Hz) for two protons and a broad quartet at δ -0.31 (*J* 4.76 Hz) for two protons. The carbon NMR spectrum showed two signals at δ 215.19 and 173.84 for the carbonyl groups of the ketone and carboxylic acid respectively. The anomeric carbon signal appeared at δ 94.80 and the rest of the sugar carbons between δ 73.52-70.70. The methyl carbon signals of the protecting silyl groups of the sugar appeared at δ 1.08, 0.93 and 0.14. As well, the carbons of the dimethylsilyl groups of the mycolic acid appeared at δ -4.53 and -4.66. The MALDI MS showed the correct mass ion at $(M + Na)^+$: 3467.31. The second fraction was protected TMM **(202)**. The proton NMR spectrum showed the hemiacetal protons as doublets at δ 4.92 (*J* 2.46 Hz) and 4.85 (*J* 2.40 Hz) with an integration of one proton for each. The rest of the protons of the sugar resonated between δ 4.35-3.42. The signals of the keto-mycolic acid protons were similar to TDM, with half the number of protons. The MALDI MS showed the correct mass ion at $(M + Na)^+$: 2131.96.

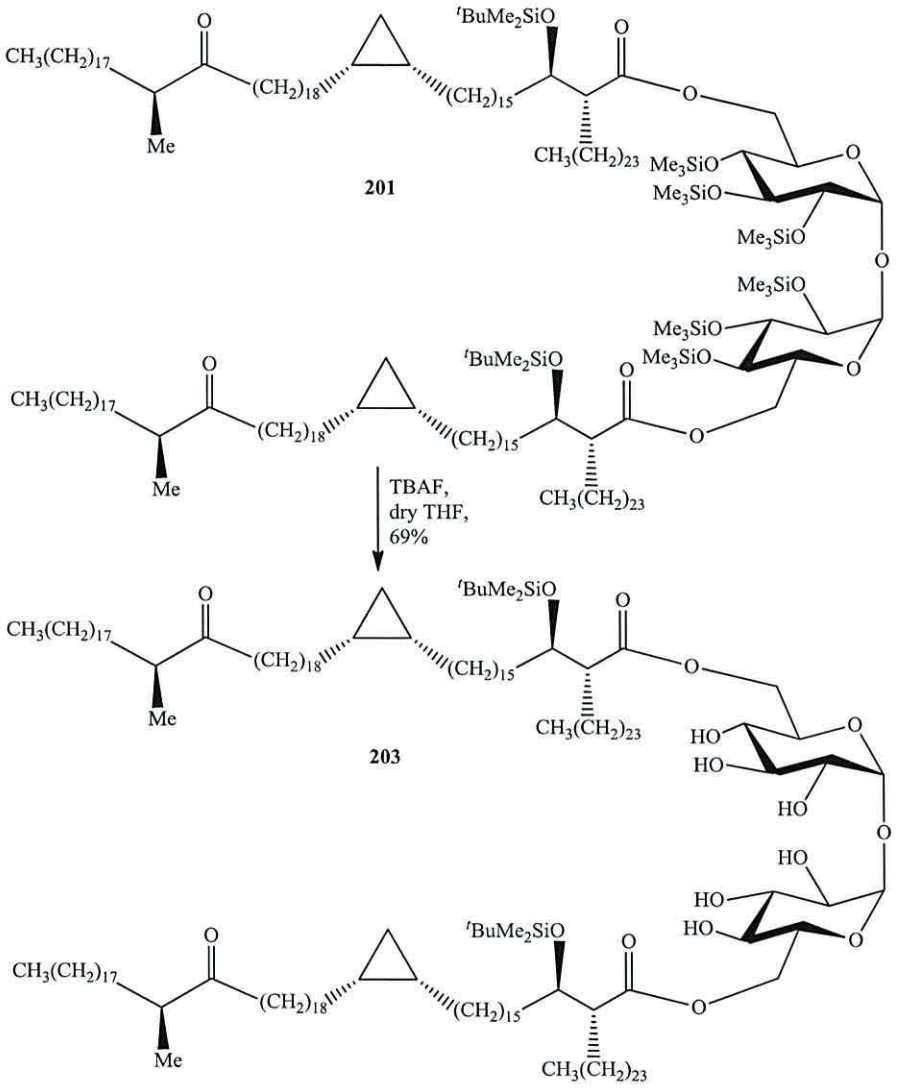


Scheme 50: Esterification of protected keto mycolic acid with protected trehalose

2.3. 6 Deprotection of TDM (201)

The trimethylsilyl protecting groups of the sugar of compound **(201)** were removed by using TBAF in dry THF to yield **(203) (Scheme 51)**. These conditions of deprotection did not cause any epimerization of the methyl group next to the carbonyl in the mycolic acid moiety. This was proved by the Baird research group when they applied the same conditions on similar model.[209] The formation of compound **(203)** was

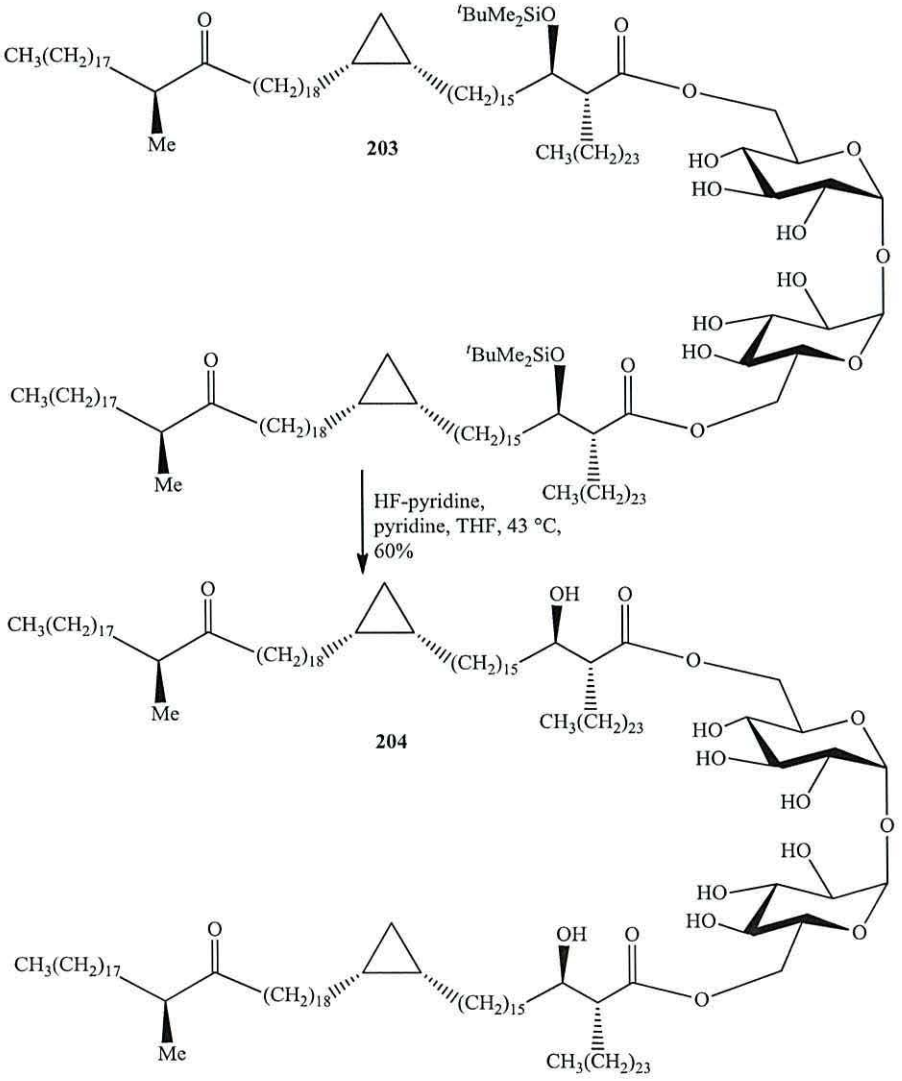
confirmed by the proton NMR spectrum, which showed the loss of the signals at δ 0.16, 0.148 and 0.14. Also, the carbon NMR spectrum showed no signals at δ 1.08, 0.93, and 0.14 corresponding to the carbons of the trimethylsilyl groups.



Scheme 51: Deprotection of the trimethylsilyl protecting groups of compound (201)

Following this, the *tert*-butyldimethylsilyl protecting group of the mycolic acid was removed by using HF.pyridine complex and pyridine to obtain a free TDM **(204) (Scheme 52)**. The proton NMR spectrums of free TDM **(204)** gave a doublet at δ 4.98 (*J* 3.04 Hz) for the hemiacetal protons. The rest of the protons of the sugar protons resonated between δ 4.67 and 3.19. The protons at the β-hydroxyl position of the mycolic acid showed a broad triplet at δ 3.93 (*J* 11.4 Hz). The proton at the α-position

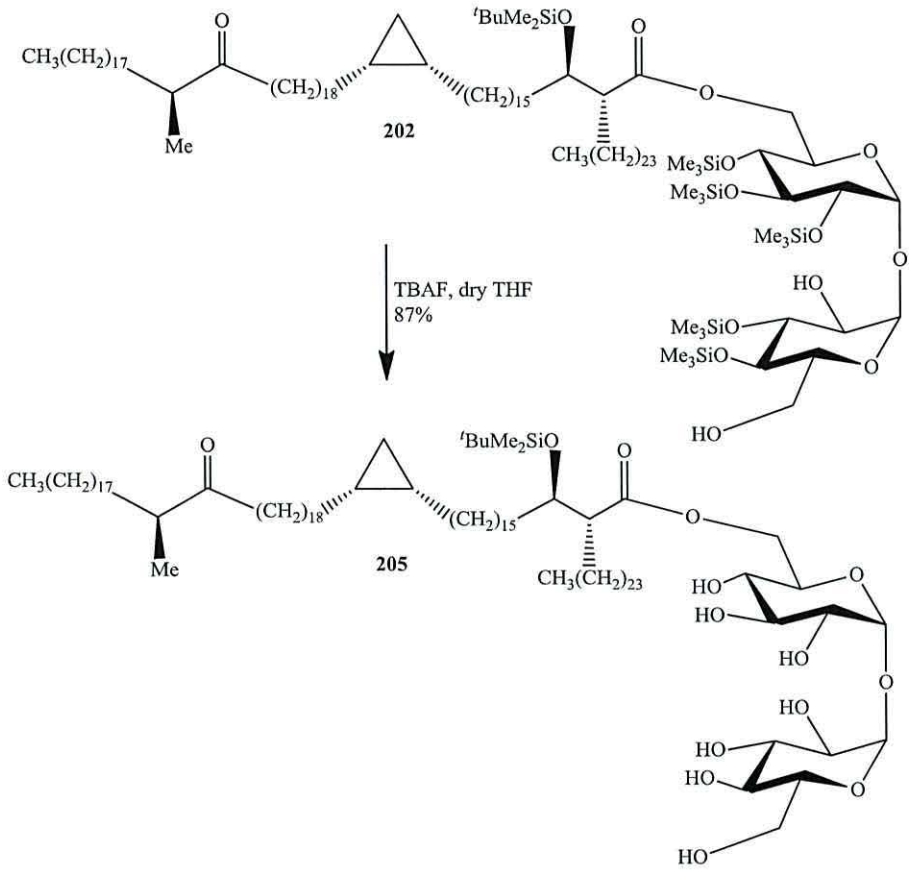
to the alkyl chain of the motif, with the proton at α-ketone of mycolic acid showed a quartet at δ 2.47 (*J* 6.6 Hz) with an integration of four protons. A triplet at δ 2.38 (*J* 7.36 Hz) corresponded to the protons next to the carbonyl adjacent to the long chain of the mycolic acid. The methyl group adjacent to the ketone appeared as a doublet at δ 1.00 (6.96 Hz) with an integration of six protons while the terminal methyl groups showed a triplet at δ 0.83 (6.48 Hz) with an integration of twelve protons. The cyclopropane ring gave a multiplet at δ 0.6 for four protons, a doublet of triplets at δ 0.53 (*J* 3.76, 8.4 Hz) for two protons and a broad quartet at δ -0.37(*J* 5.16 Hz) for two protons. The signals belonging to the protons of the silyl group were not present. The carbon NMR spectrum confirmed the removal of the silyl group with the loss of the signals at δ -4.56 and -4.97 for the carbons of the methyl groups on silicon. The MALDI MS showed the correct mass ion at $(M + Na)^+$: 2804.65



Scheme 52: Synthesis of free TDM of keto-mycolic acid (204)

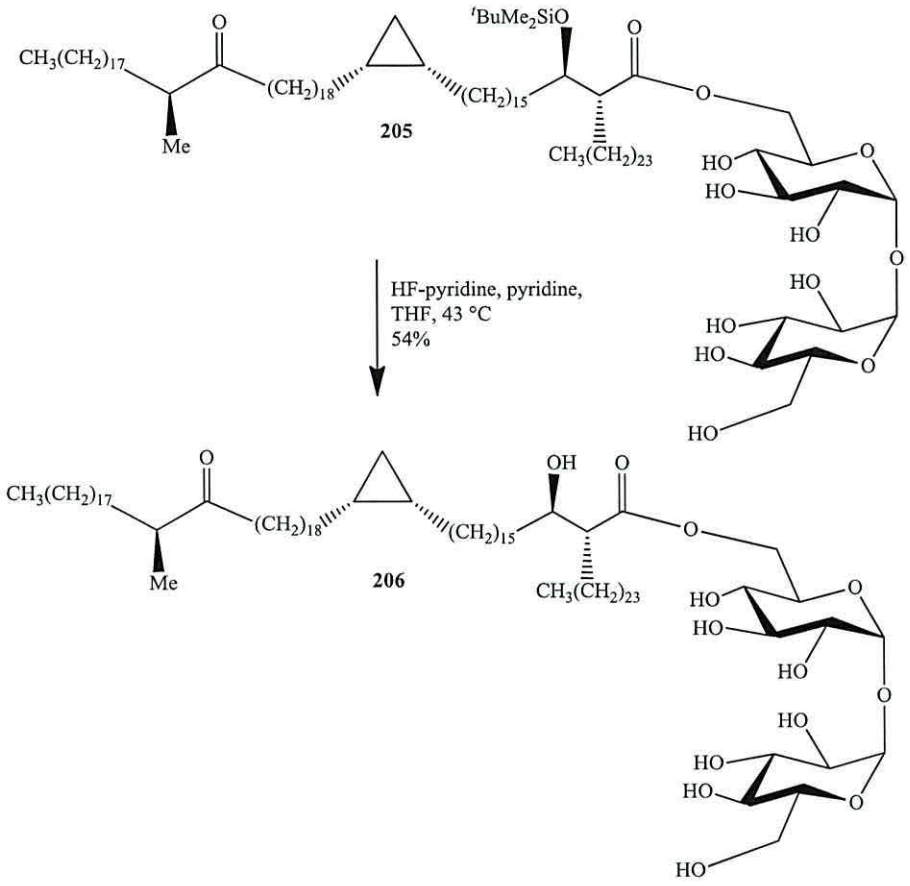
2.3.7 Deprotection of TMM (202)

In this reaction, the trimethylsilyl groups of the sugar of TMM **(202)** were first removed by using TBAF in dry THF, **(Scheme 53)**. The proton NMR spectrum of the resulting compound **(205)** showed the absence of signals at δ 0.17, 0.158, 0.152, 0.147, 0.141 and 0.12, and the carbon NMR spectrum the absence of signals at δ 1.04, 0.99, 0.92, 0.84, 0.16, and 0.033 corresponding to the trimethylsilyl groups.



Scheme 53: Deprotection of the trimethylsilyl groups of compound (202)

The *tert*-butyldimethylsilyl group was then removed using HF.pyridine and pyridine **(Scheme 54)**. The stucture of free TMM **(206)** was confirmed by the proton NMR spectrum which showed similar peaks to **(206)**, but lacking the *tert*-butyl dimethylsilyl signals on the β-hydroxy acid. Also, the carbon NMR spectrum showed no signals for the carbons of the silyl group which gave evidence for the success of the deportection. The MALDI MS showed the correct mass ion at $(M + Na)^+$: 1585.81.



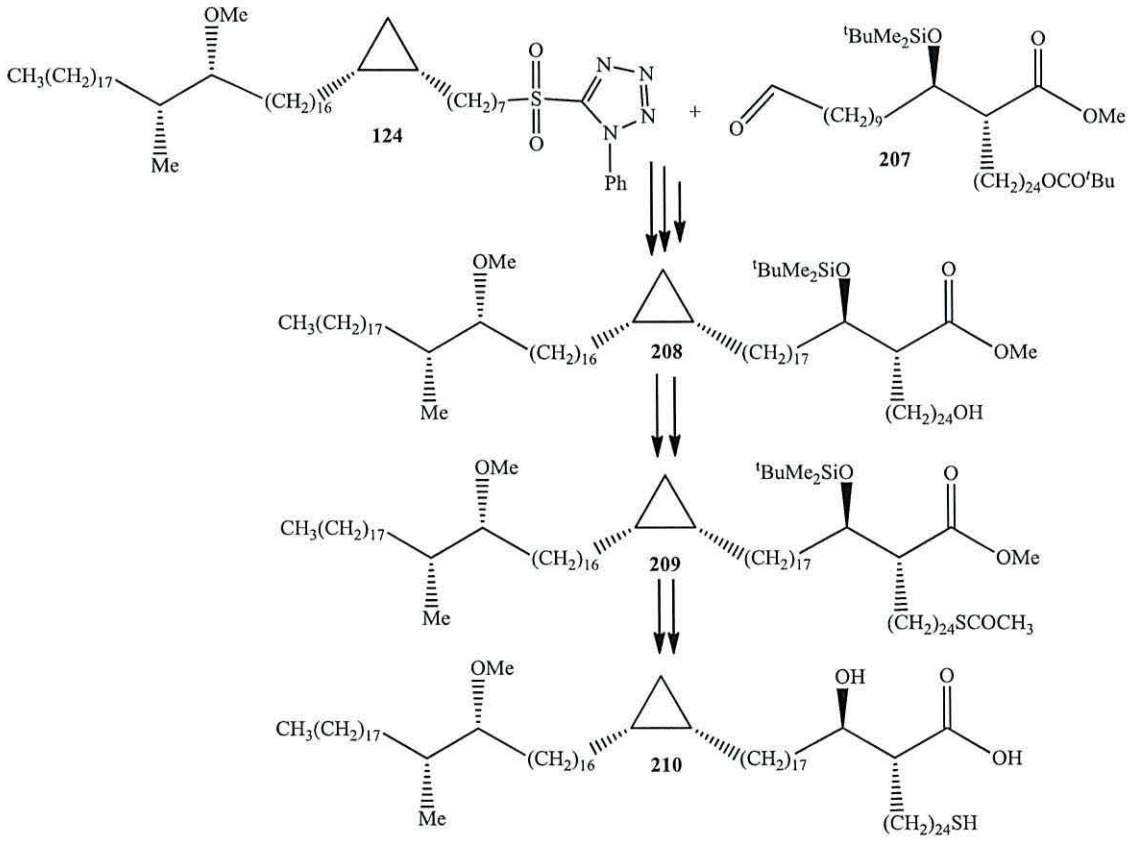
Scheme 54: ***Synthesis of free TMM of ketomycolic acid (206)***

Chapter 3

3. Synthesis of a thiolated mycolic acid

This chapter of the thesis concerns the synthesis of a novel methoxymycolic acid with a thiol group at the terminal position of the α-alkyl chain. This position for thiolation was chosen in order to minimise conformation change around the β-hydroxy acid and its interaction with the long functional mero-chain. The thiol group will be allowed to bind with gold and to develop second generation sensors for detecting TB-antibodies.

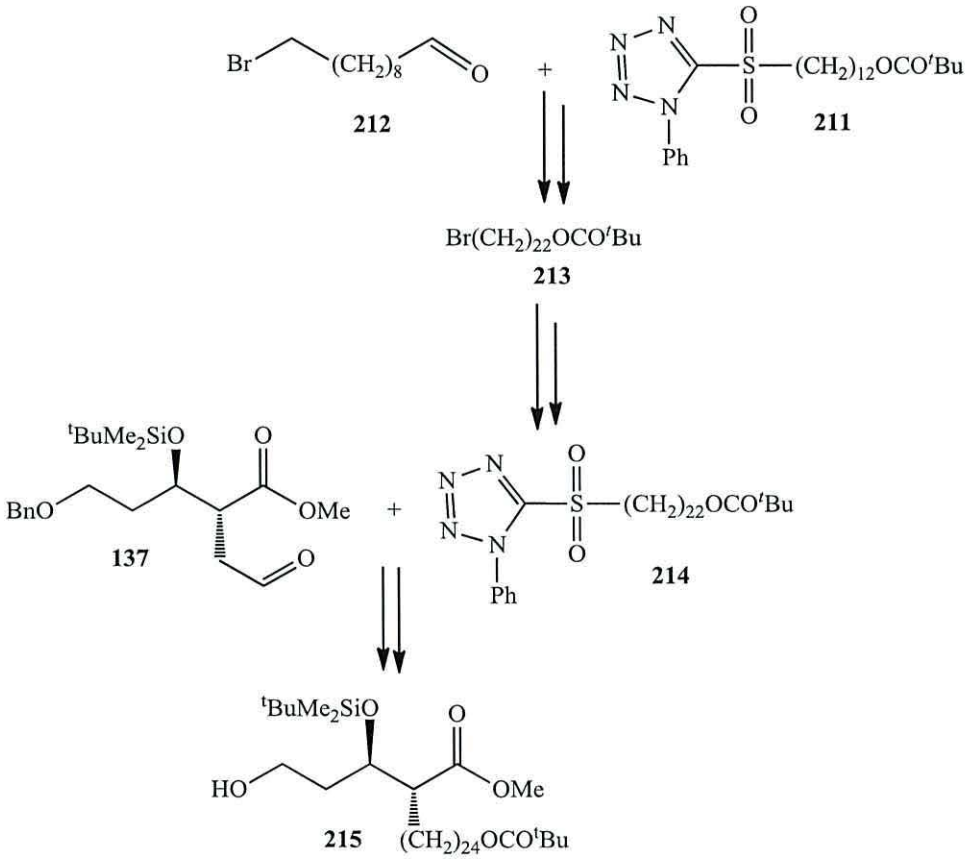
The synthesis of target compound **(210)** was carried out by coupling the meromycolate **(124)** with the mycolatemotifmotif protected at the end of the α alkyl chain **(207)**, followed by hydrogenation and deprotection of the protecting group at the α alkyl chain terminus. The resulting hydroxyl group **(208)** was converted into tosylate which was then converted into a thioacetate **(29)**. Eventually, the remaining protecting groups were removed to give free thiol compound **(210) (Scheme 55)**.



Scheme 55: The synthesis steps for the preparation of thiolated methoxy mycolic acid (210)

3.1 Synthesis of the mycolatemotif part with a protected α-alkyl chain

The α-alkyl chain generally contains 22 or 24 methylene carbon atoms with a terminal methyl group in the full mycolic acid types. In this work, the α-alkyl chain of the motif **(137)** (preparation described previously in Section **2.2.1.3**) was extended with the C_{22} protected sulfone reagent **(214)**, bearing pivalate protection at its terminus. **(Scheme 56)**. The pivalate group was selected to be different to the other protecting groups, as it can be removed selectively and easy using a strong base. Epimerisation in the α-alkyl chain is not an issue due to steric hindrance. Also this group is better than the tetrahydropyran (THP) group because it gives one signal in proton NMR spectrum.



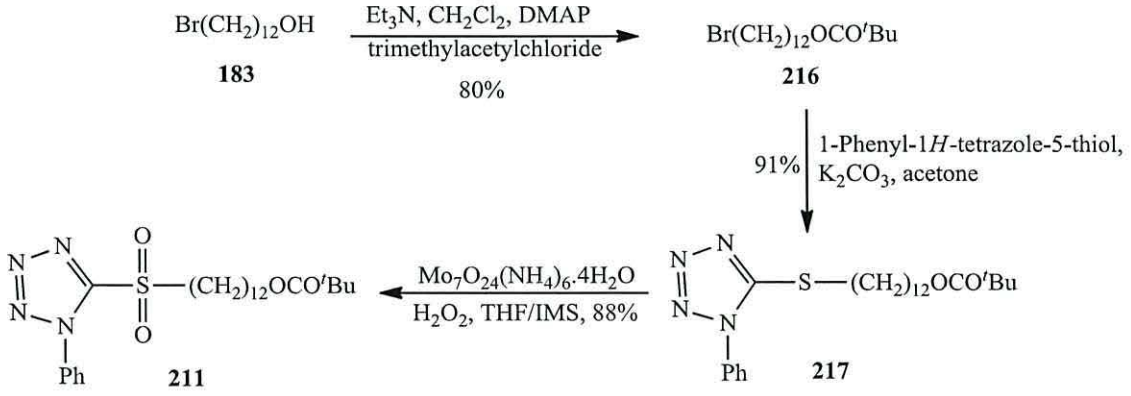
Scheme ***56: The approach to synthesise the mycolatemotif part with protected alkyl chain***

3.1.1 Extension of the α-allyl chain

2,2-Dimethylpropanoic acid-22-(1-phenyl-*1H*-tetrazole-5-ylsulfonyl)docosyl ester **(214)** was selected to complete the chain extension at the α-position of the mycolatemotif. This sulfone is not commercially available, therefore, it was prepared in the manner given below.

3.1.1.a Synthesis of the protected sulfone (211)

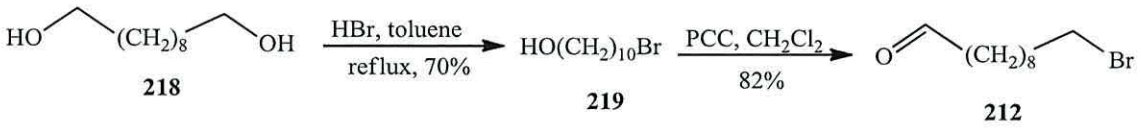
The hydroxyl group of 12-bromododecan-1-ol **(183)** was protected with the pivalate group by using trimethylacetyl chloride in the presence of pyridine and DMAP as catalysts **(Scheme 57)**. The formation of compound **(216)** was confirmed by the proton NMR spectrum which gave a triplet at δ 4.03 (*J* 6.6 Hz) for the protons next to the oxygen, and a singlet at δ 1.18 for the nine protons of the *tert*-butyl group. In addition, the two protons next to bromine appeared as a triplet at δ 3.39 (*J* 7.0 Hz). The carbon NMR spectrum showed a signal at δ 178.54 for the carbonyl carbon and a signal at δ 38.66 for the quaternary carbon of the *tert*-butyl group. The IR spectrum showed a peak at 1729 cm^{-1} for the carbonyl group. The bromo ester **(216)** was then converted into the sulfide **(217)**, by treating it with 1-phenyl-1*H*- tetrazole-5-thiol and potassium carbonate. The proton NMR spectrum of the sulfide showed a multiplet between δ 7.55-7.49 for the five protons of the phenyl group. A triplet appeared at δ 3.90 (*J* 6.6 Hz) for the two protons next to the oxygen atom and another triplet at δ 3.56 (*J* 7.2 Hz) for the two protons next to the sulfur atom. Four signals appeared between δ 133.8-125.0 in the carbon NMR spectrum for the aromatic carbons. Subsequently, the sulfide was converted into the sulfone **(211)** using hydrogen peroxide and ammonium molybdate (VI) tetrahydrate. The proton NMR spectrum of the resulting sulfone showed a multiplet between δ 7.70-7.68 for two aromatic protons and another multiplet between δ 7.61-7.59 for three aromatic protons. Also, the two protons next to the sulfur atom of the sulfone appeared as a triplet at δ 3.73 (*J* 7.9 Hz), which was further downfield in comparison to the position of its counterpart in the spectrum of the sulfide.



Scheme 57: Synthesis of the protected sulfone (211)

3.1.1.b Synthesis of the aldehyde (212)

1,10-Decanediol **(218)** was monobrominated with hydrobromic acid in toluene to give 10-bromodecan-1-ol **(219)**.[210, 211] The hydroxyl group of compound **(219)** was then oxidised to the corresponding aldehyde **(212)**, using PCC in dichloromethane **(Scheme 58)**.

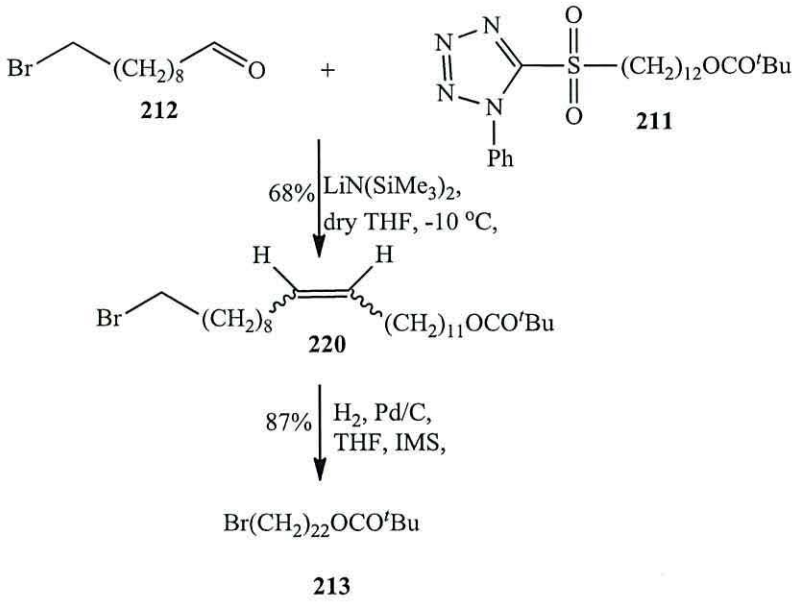


Scheme 58: Synthesis of the aldehyde (213)

The proton NMR spectrum showed a singlet at δ 9.75 for the aldehyde proton and a triplet at δ 3.39 (*J* 6.95 Hz) for the two protons adjacent to bromine. The carbon NMR spectrum gave evidence for the formation of the aldehyde, by showing a signal at δ 202.79 for carbonyl carbon.

3.1.1.c The coupling of (211) with (212)

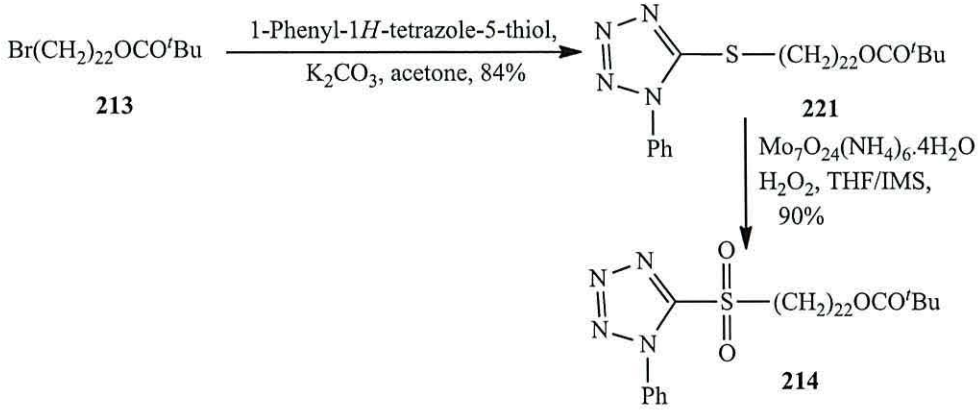
The aldehyde **(212)** and sulfone **(211)** were reacted by the Julia-Kocienski olefination reaction in dry THF with lithium *bis*(trimethylsilyl)amide to give the alkene **(220)** as an (*E/Z*) mixture **(Scheme 59)**. The product alkene **(220)** was hydrogenated with Pd on carbon (10 %) catalyst under hydrogen atmosphere. It has been reported, that the timing of the hydrogenation of a substrate bearing bromine substitution is crucial, given that the reductive removal of the bromine atom is a possibility under these conditions.[212] Therefore, the hydrogenation was carried out for one hour to give the saturated compound **(213)**. The proton NMR spectrum showed no signals corresponding to the alkene protons. The two protons next to oxygen showed a triplet at δ 4.05 (*J* 6.65 Hz) and the two protons next to bromine showed a triplet at δ 3.41 (*J* 6.95 Hz).



Scheme 59: Synthesis of the bromo ester (213)

3.1.1.d Synthesis of the protected C22 sulfone

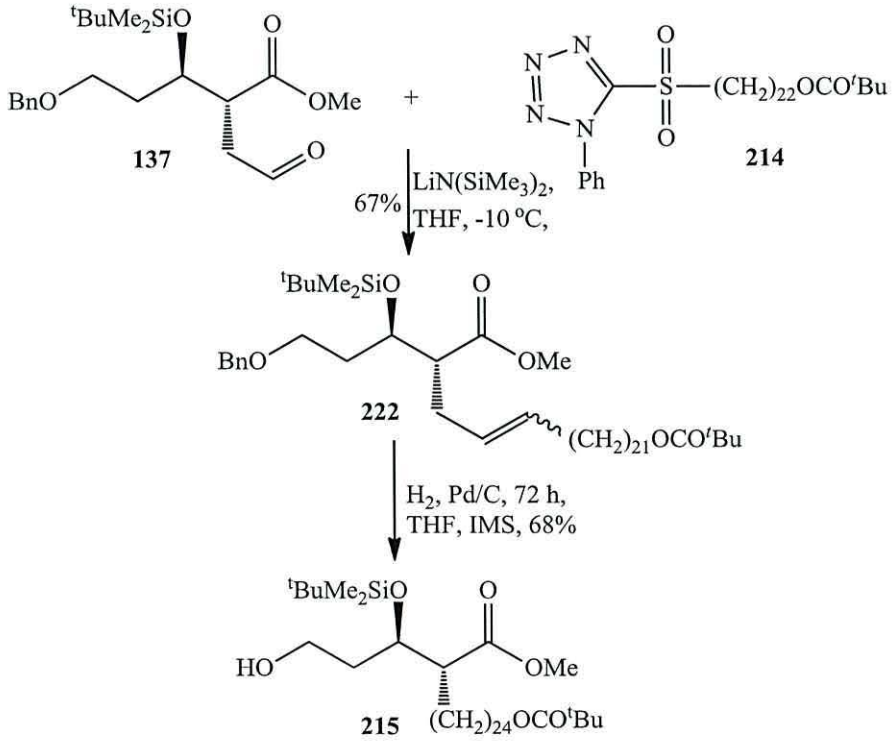
In order to prepare the sulfone **(214)**, the bromo ester **(213)** was reacted with 1-phenyl-1*H*-tetrazole-5-thiol, using potassium carbonate to yield the sulfide **(221)**. Subsequently, the sulfide **(222)** was oxidised with hydrogen peroxide to give the corresponding sulfone **(214)** **(Scheme 60)**. The proton NMR of the sulfone showed the five protons of the phenyl group as two sets of multiplets occurring between δ 7.61-7.60 for two protons and δ 7.59-7.58 for three protons, whereas the proton NMR spectrum of the sulfide showed one multiplet between δ 7.55-7.49. A triplet appeared at δ 4.04 (*J* 6.6 Hz) for the two protons next to the oxygen and another triplet at δ 3.73 (*J* 7.4 Hz) for the two protons next to the sulfonyl group.



Scheme 60: Synthesis of the protected C_{22} sulfone

3.1.2 Julia-Kocienski reaction between the aldehyde (137) and sulfone (214)

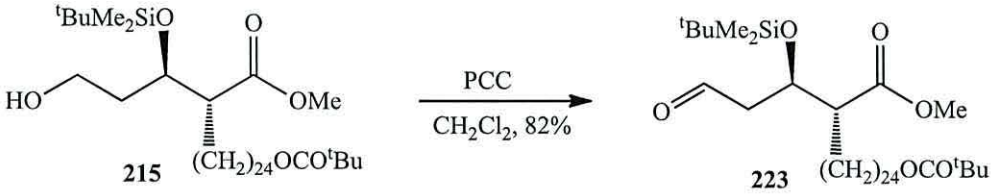
This reaction involved the coupling of the aldehyde **(137)** and sulfone **(214)** using lithium *bis*(trimethylsilyl)amide as a base in dry THF and gave the alkene product **(222)** as an (*Z/E*) mixture. The simultaneous hydrogenation of the alkene and de-benzylation of the primary alcohol **(222)** was carried out in IMS/THF, using Pd on carbon (10 %) as the catalyst under hydrogen atmosphere to give **(215) (Scheme 61)**. The hydrogenation was continued for three days in order to ensure complete alkene saturation and de-benzylation. The proton NMR spectrum of the resulting compound **(215)** showed no signals belonging to either aromatic or alkene protons during their region. There was a triplet signal at δ 4.04 (*J* 6.5 Hz) for the two protons adjacent to the oxygen of the ester. Two protons next to the hydroxyl group appeared as a multiplet between δ 3.78-3.70. The carbon NMR spectrum did not show any signals for either aromatic or alkene carbons. There were signals at δ 64.47 for the carbon next to ester group and δ 59.54 for the carbon next to hydroxyl group. Also, the two carbonyl groups showed signals at δ 178.6 and δ 174.6 respectively. The IR spectrum showed a broad peak at 3521 cm^{-1} corresponding to the hydroxyl group. All of these data confirmed that the hydrogenation had been successful.



Scheme 61: Synthesis of the protected mycolatemotif (215)

3.1.3 Oxidation of the alcohol (215)

In order to complete the construction of the meromycolate chain, the primary alcohol **(215)** was oxidised to give aldehyde **(223)** using PCC **(Scheme 62)**. The proton NMR spectrum of the aldehyde product revealed a singlet at δ 9.82 for the aldehyde proton and the carbon NMR spectrum showed a signal at δ 201.27 for the aldehydic carbonyl and two signals at δ 178.64 and δ 174.0 respectively, corresponding to the two ester carbonyls. Later, this aldehyde motif was extended with two different chain lengths to prepare different types of thiolated mycolic acids.

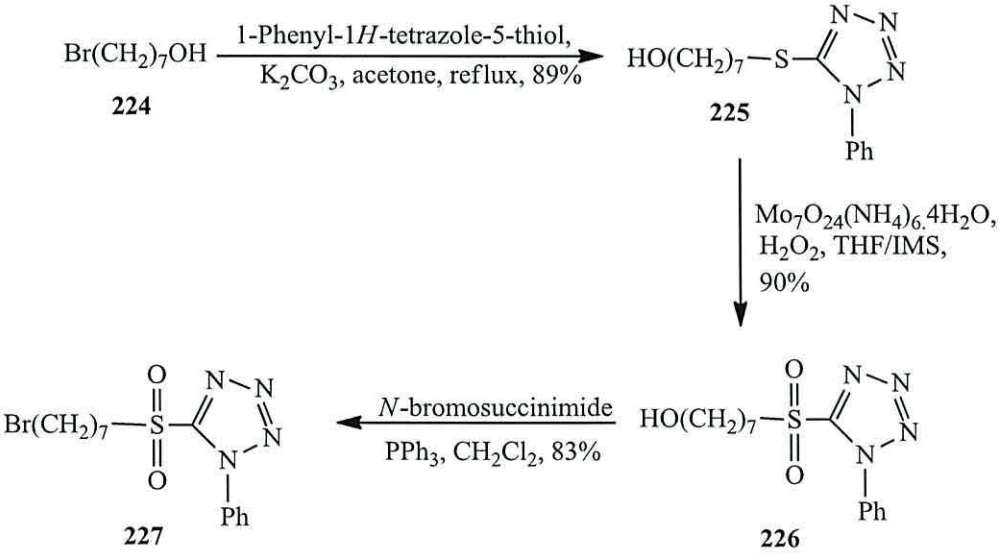


Scheme 62: Oxidation of the alcohol (215)

3.1.4 The chain extension to prepare the sulfone (230)

3.14.a Preparation of C7 sulfone (227)

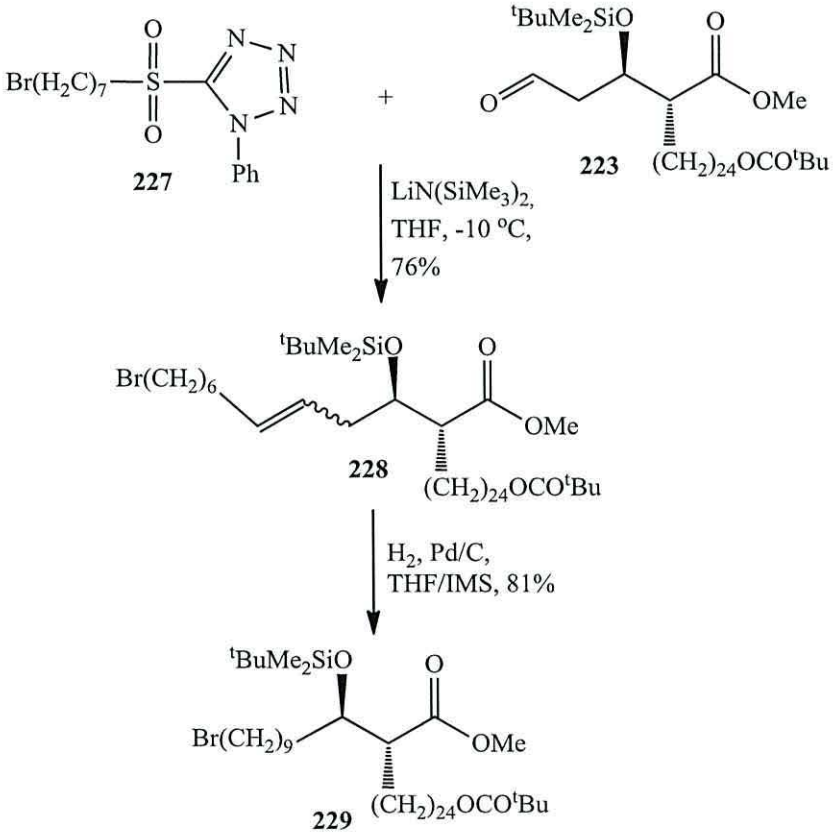
The sulfone **(227)** was prepared from 7-bromo-1-ol **(224)** as the starting material **(Scheme 63)**, employing the procedure given previously in Section **2.3.3.1**



Scheme 63: Synthesis of the sulfone (227)

3.1.4.b Julia-Kocienski coupling of the aldehyde (223) and the sulfone (227)

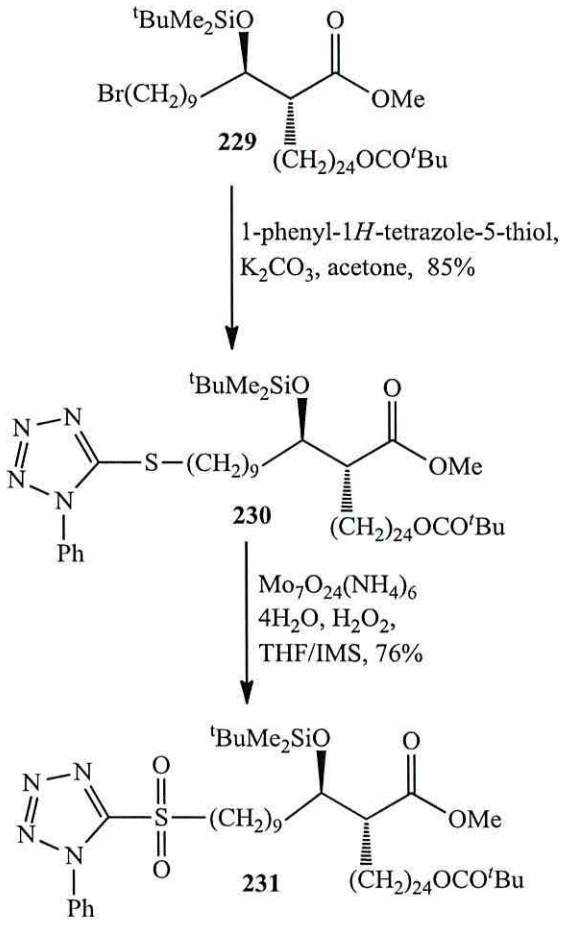
A Julia-Kocienski reaction was carried out between the aldehyde **(223)** and the sulfone **(227)**, using lithium *bis*(trimethylsilyl) amide as the base in dry THF, and gave the alkene **(228)** as an (*E/Z*) mixture **(Scheme 64)**, which was followed by hydrogenation of the alkene mixture **(228)** employing palladium catalyst under an atmosphere of hydrogen gas and for one hour to give **(229)**. The proton NMR spectrum of the saturated compound **(229)** showed no signals for the olefinic protons and the proton next to the silyl group appeared as a multiplet between δ 3.91-3.88. There are two triplet signals, occurring at δ 4.03 (*J* 6.6 Hz) and δ 3.39 (*J* 6.9 Hz), which correspond to the methylene groups next to oxygen and bromine respectively. The α-position proton showed as a doublet of doublets of doublets at δ 2.51 (*J* 3.8, 7.25, 11.05 Hz). The carbon NMR spectrum showed signals at δ 178.56 and 175.05 for the two respective carbonyl groups.



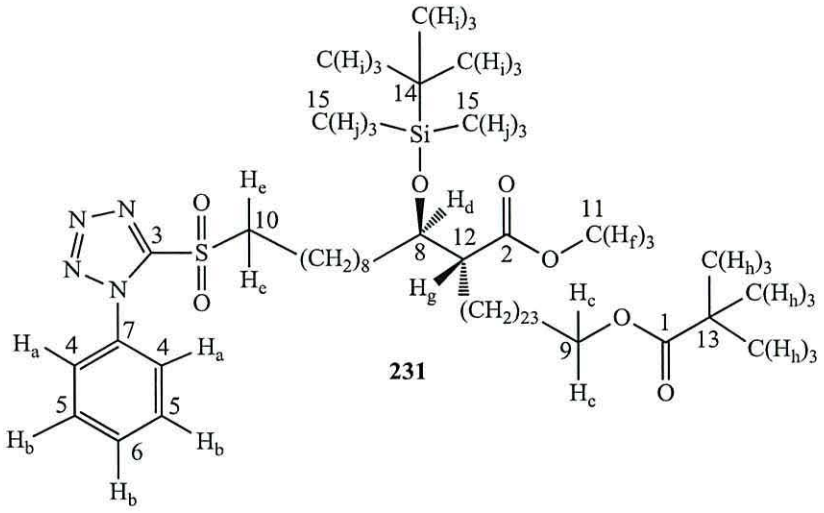
Scheme 64: Julia-Kocienski between aldehyde (223) and sulfone (227)

3.1.4.c Conversion into the sulfone (231)

As discussed before, the bromo ester compound **(229)** was reacted with 1-phenyl-1*H*-tetrazole-5-thiol to prepare the sulfide **(230)**, which was then oxidised with ammonium molybdate (VI) tetrahydrate and hydrogen peroxide to give the corresponding sulfone **(231)** (**Scheme 65**). The proton and carbon NMR data of the sulfone **(231)** are shown in **Table 5.** (Note that, this mycolatemotif sulfone was given to E. F. Huws (a previous member of Professor Baird's group) who required it for the preparation of thiolated *trans*-methoxymycolic acid)[191,213]



Scheme 65: Synthesis of the sulfone (231)



231

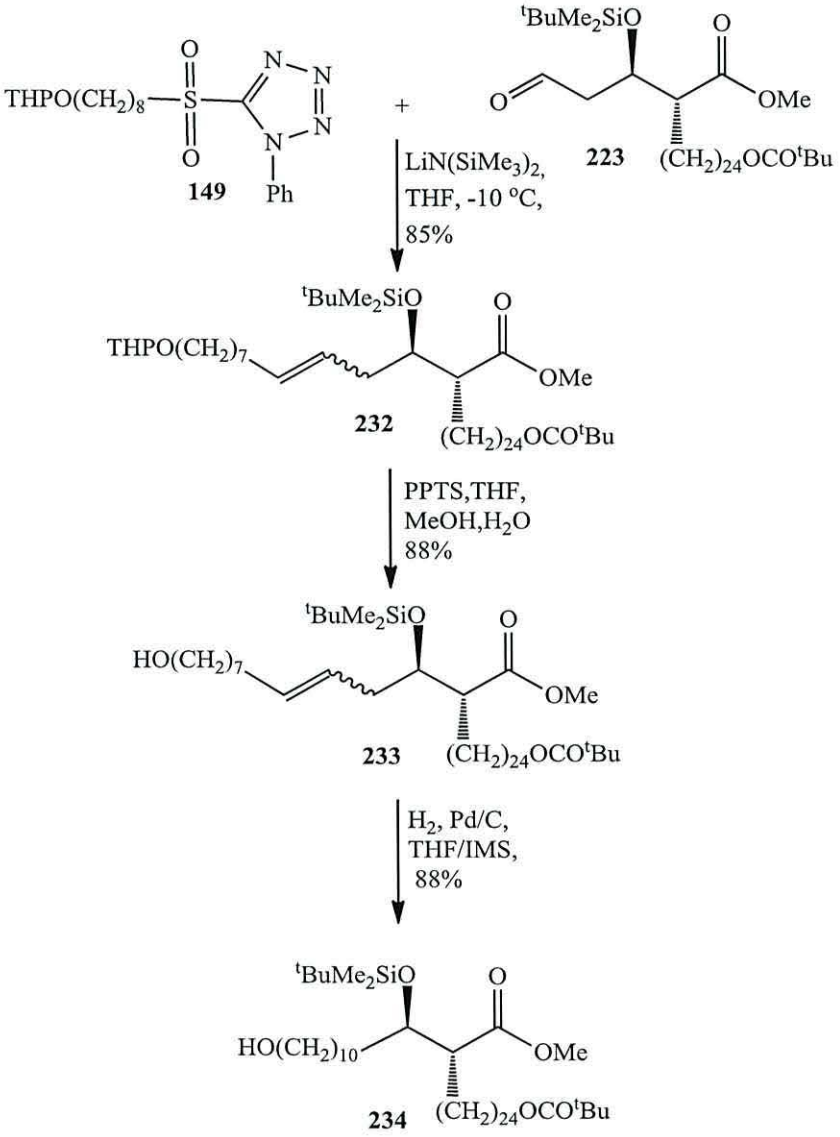
H_x	δ	Multiplicity	Integration	*J* (Hz)	C_x	δ
H_a	7.71-7.69	m	2	-	C_1	178.66
H_b	7.62-7.60	m	3	-	C_2	175.08
H_c	4.04	t	2	6.6	C_3	153.48
H_d	3.92-3.88	m	1	-	C_4	133.03
H_e	3.73	t	2	7.85	C_5	131.43
H_f	3.65	s	3	-	C_6	129.70
H_g	2.52	ddd	1	3.45,6.9,10.7	C_7	125.04
H_h	1.19	s	9	-	C_8	73.15
H_i	0.86	s	9	-	C_9	64.46
H_j	0.04, 0.02	s	2x3H	-	C_{10}	55.99
-	-	-	-	-	C_{11}	51.58
-	-	-	-	-	C_{12}	51.23
-	-	-	-	-	C_{13}	38.71
-	-	-	-	-	C_{14}	3361
-	-	-	-	-	C_{15}	-4.37,-4.93

Table 5: ^{1}H and ^{13}C NMR data for the sulfone (231)

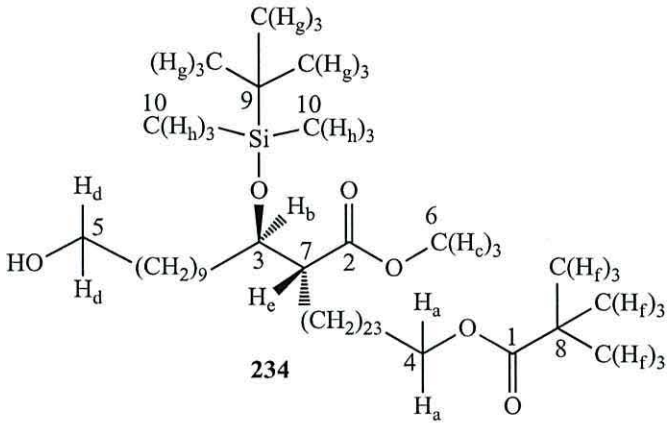
3.1.5 The side chain extension to prepare the alcohol motif (234).

In order to prepare the alcohol motif **(234)**, the aldehyde **(223)** was coupled with the sulfone **(149)**, using lithium *bis*(trimethylsilyl)amide in dry THF to give the alkene **(232)** as an *(E/Z)* mixture. The tetrahydropyran protecting group was removed by

using pyridinium-*p*-toluene sulfonate as described before to give the primary alcohol **(233)**. The saturation of the double bond in the compound **(233)** was achieved by catalytic hydrogenation as described before **(Scheme 66)**. The proton and carbon NMR data analysis of saturated compound **(234)** are shown in **Table 6**. (Note that, some of the mycolatemotif **(234)** was given to M. M. Sirhan (a Bangor PhD student) who required it for preparation of thiolated α-mycolic acid).[213,214]



Scheme 66: Synthesis of the alcohol (234)



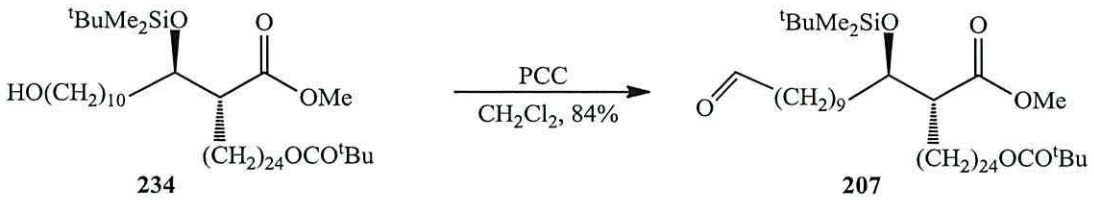
234

H_x	δ	Multiplicity	Integration	*J* (Hz)	C_x	δ
H_a	4.03	T	2	7.3	C_1	178.62
H_b	3.90-3.87	m	1	-	C_2	175.10
H_c	3.64	S	3	-	C_3	73.16
H_d	3.62	T	2	6.5	C_4	64.43
H_e	2.51	ddd	1	3.75,7.25,10.7	C_5	62.97
H_f	1.18	S	9	-	C_6	51.53
H_g	0.85	S	9	-	C_7	51.18
H_h	0.03, 0.01	S	2x3H	-	C_8	38.68
-	-	-	-	-	C_9	32.76
-	-	-	-	-	C_{10}	-4.40,-4.96

Table 6: ^{1}H-and ^{13}C-NMR data analysis of the compound (234)

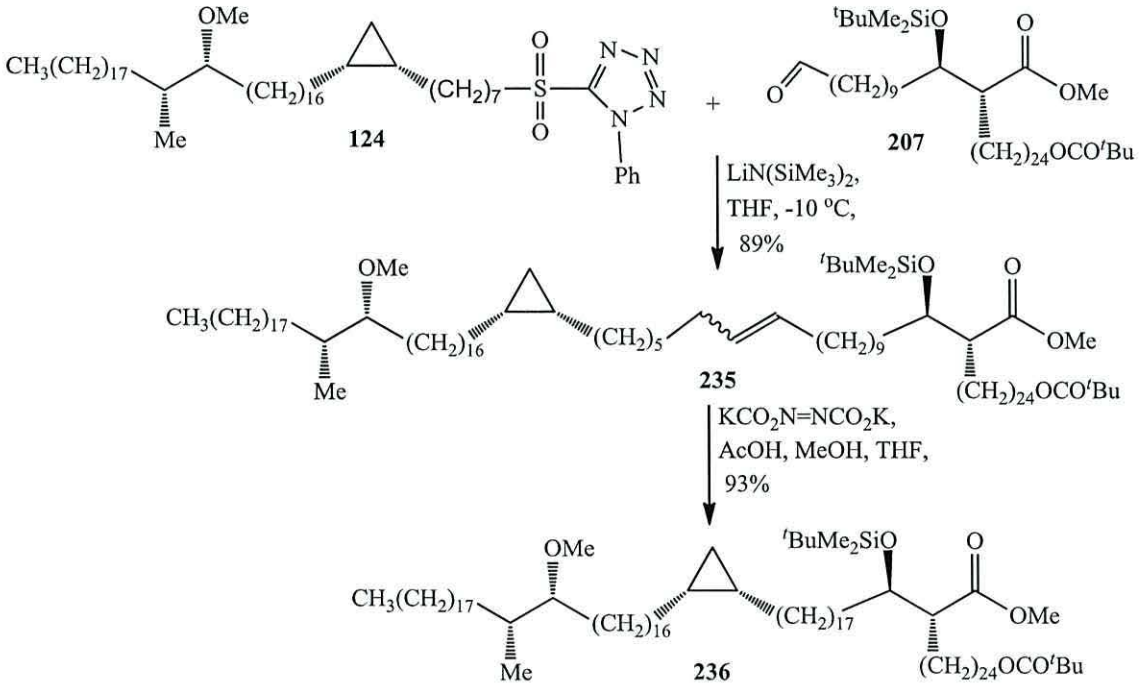
3.2 The coupling to form the fully protected *R,R*-methoxymycolic acid (237)

Prior to the coupling reaction, the alcohol **(234)** was oxidised using PCC in dichloromethane to give the corresponding aldehyde **(207) (Scheme 67)**. The proton NMR spectrum of the aldehyde **(207)** gave a triplet at δ 9.77 (*J* 3.75 Hz) for the aldehydic proton. The carbon NMR spectrum showed a signal at δ 202.80 for the aldehyde carbonyl, with two signals at δ 178.62 and 175.06 respectively, corresponding to the ester carbonyl groups.



Scheme 67: Oxidation of the motif (234)

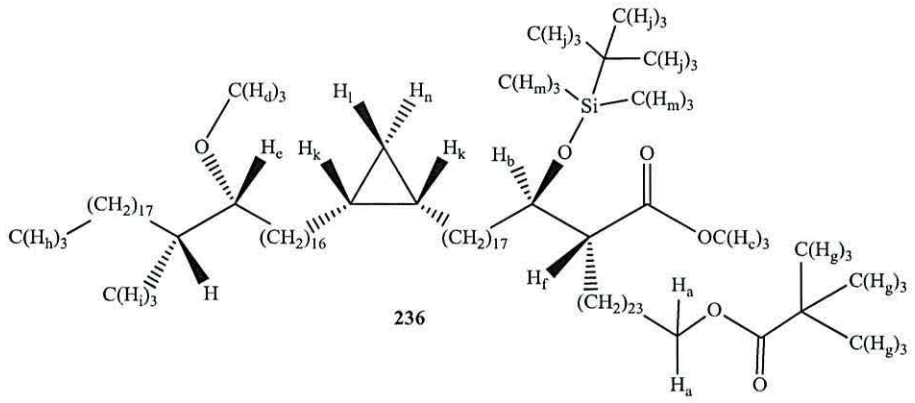
Subsequently, the aldehyde **(207)** was reacted with the meromycolate sulfone **(124)** (prepared earlier as described in Section **2.2.2.2**) via a Julia-Kocienski reaction to give the alkene **(235)** as an (*E/Z*) mixture. The resulting alkene **(235)** was hydrogenated with dipotassium azodicarboxylate **(Scheme 68)**.



Scheme 68: The final coupling of the thiolated R,R-methoxymycolic acid

The proton NMR spectrum of the compound **(236)** showed no olefinic signals. In addition, the spectrum included a triplet at δ 4.05 (*J* 6.65 Hz) for the two protons next to the oxygen of the pivalate protecting group. The β-proton appeared as a multiplet between δ 3.95-3.89, the α-proton showed as a doublet of doublets of doublets at δ 2.53 (*J* 3.8, 7.25, 11.05 Hz) and the proton next to the methoxy showed as a multiplet between δ 2.96-2.94. The methyl ester was seen as a singlet at δ 3.66, whilst the

methoxy appeared as a singlet at δ 3.34. The *tert*-butyl of the pivalate protecting group appeared as a singlet at δ 1.20, whilst the silyl protecting group appeared as three singlets at δ 0.87, 0.02 and 0.04 for the *tert*-butyl and the two methyl groups respectively. The protons of the cyclopropane ring showed as a multiplet between δ 0.66-0.63, doublet of triplets at δ 0.56 (*J* 4.1, 8.2 Hz) and a broad quartet at δ -0.32 (*J* 4.75 Hz) **(Figure 23)**. The specific rotation was $[\alpha]_D^{22} = +2.75$ (*c* 0.87 , $CHCl_3$).



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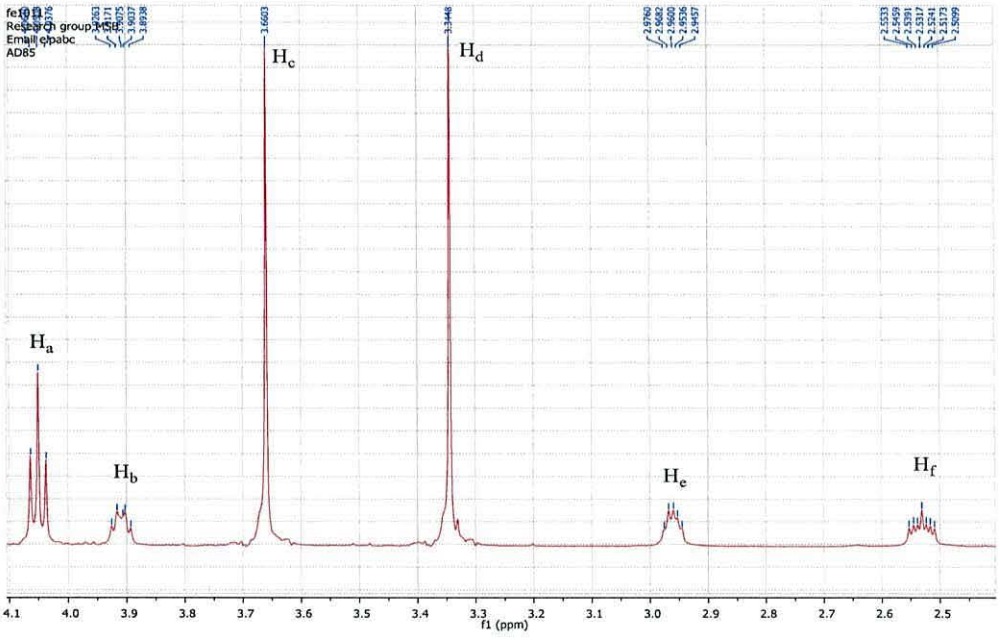

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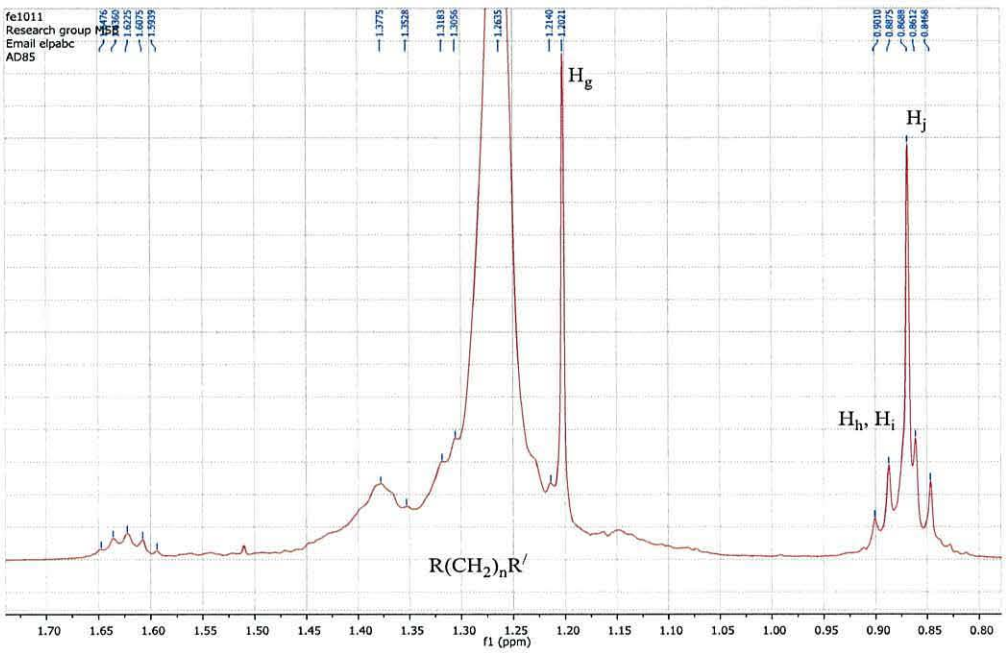

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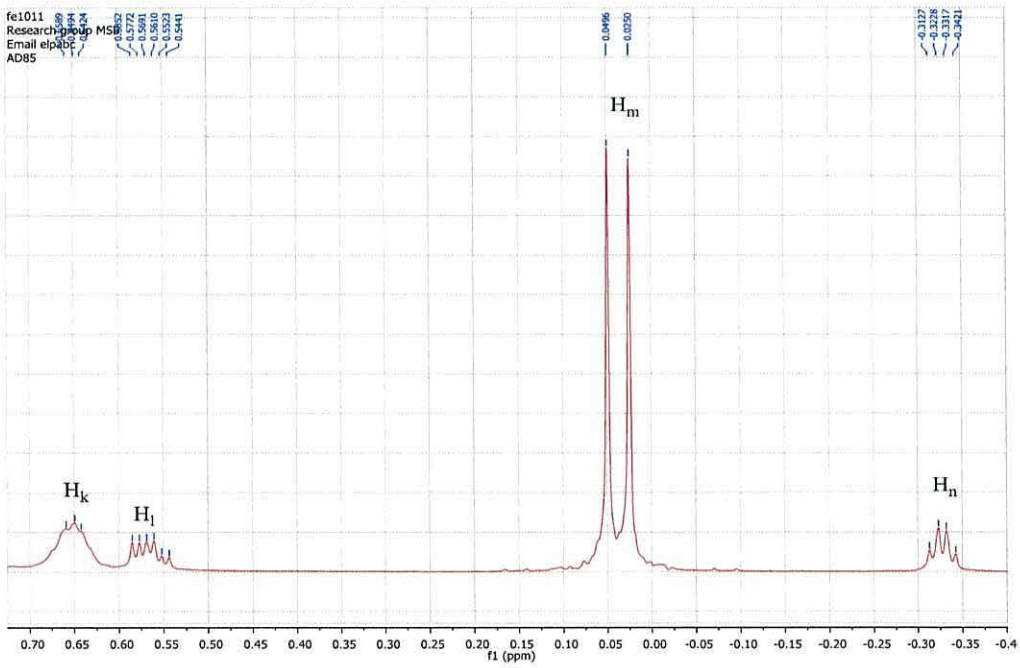


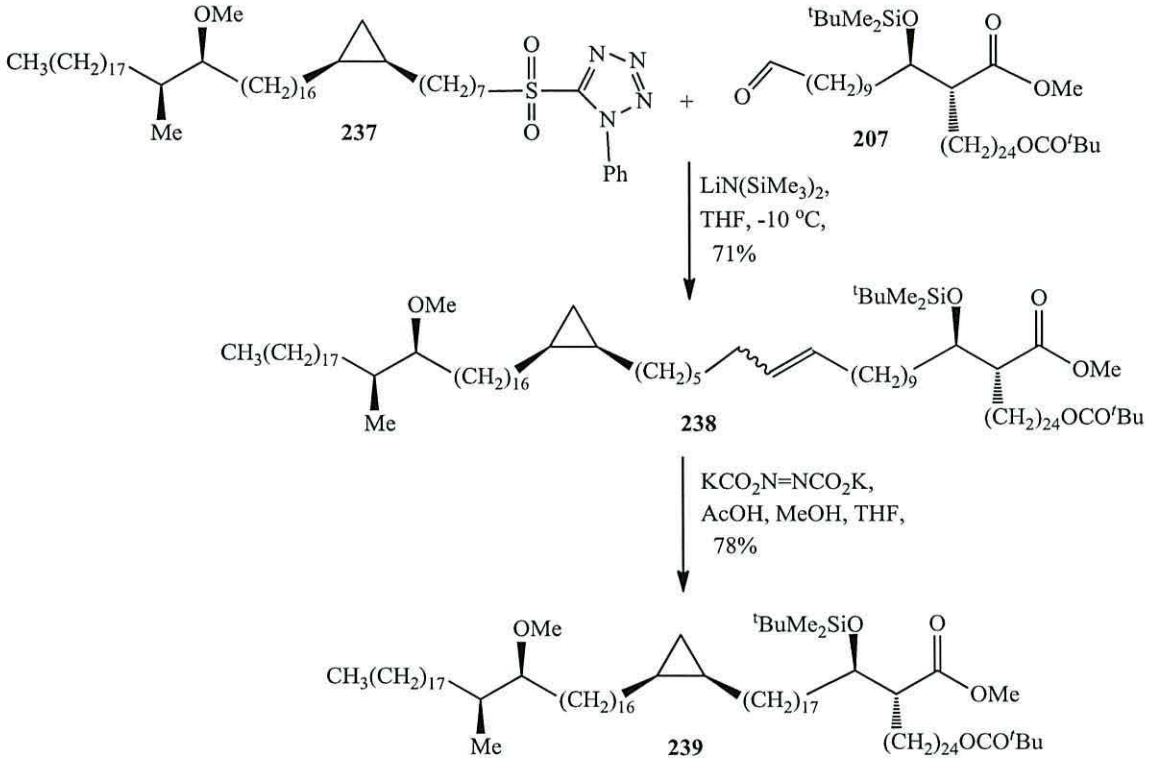
C

Figure 23: Expansion of the ^{1}H NMR spectrum of the compound (236)

3.3 Synthesis of thiolated *S,S*- methoxymycolic acid

In order to study the effect of the absolute stereochemistry of the mycolic acid on its use in diagnostics, a second diastereoisomer was prepared by coupling the mycolatemotif part **(207)** with the meromycolate **(237)** (kindly provided by Dr. J. R. Al Dulayymi). The resulting alkene **(238)** was hydrogenated as before to give the

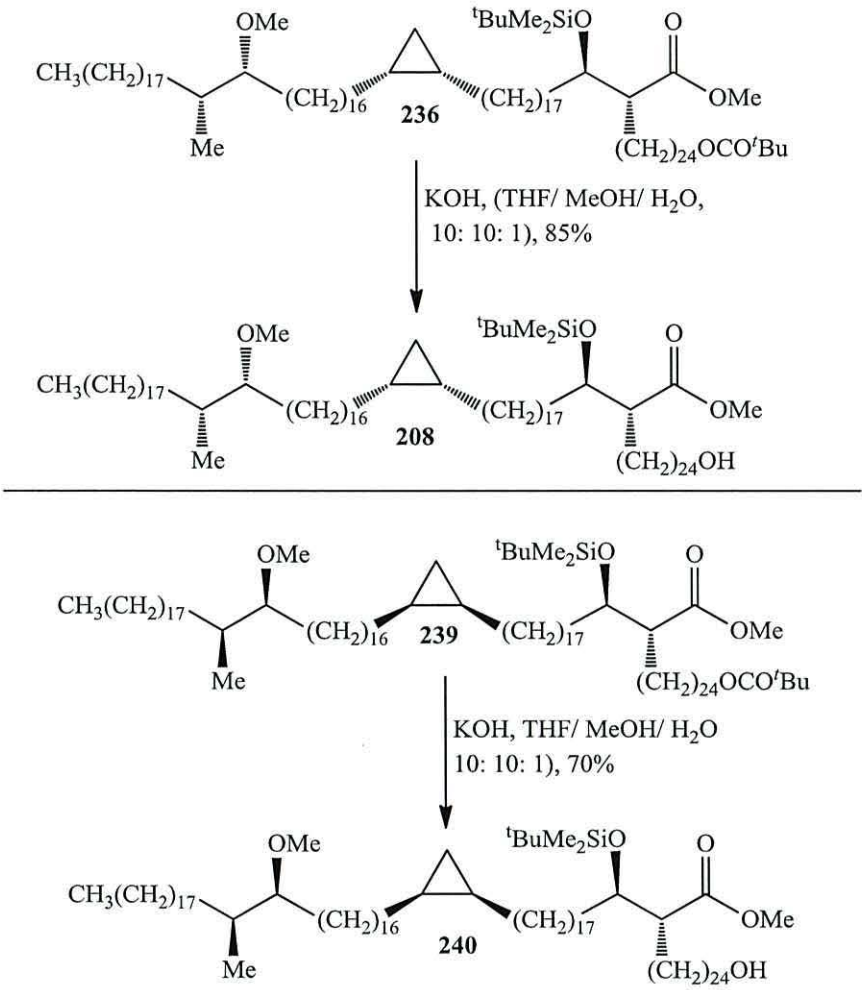
saturated compound **(239)** **(Scheme 69)**. All spectroscopic data of the compound **(239)** were similar to those of the compound **(236)**, and showed opposite specific rotation $[\alpha]_D^{23}$ = -5.60 (*c* 1.07 g, $CHCl_3$).



Scheme 69: Coupling reaction to prepare the thiolated S,S-methoxymycolic acid

3.4 Deprotection of α-alkyl chain

The pivalate protecting group situated at the α-alkyl chain terminial of **(236)** and **(239)** was removed by using potassium hydroxide in a mixture of THF, MeOH and water (10: 10: 1) to give the corresponding primary alcohols **(Scheme 70)**. The hydrolytic removal of the protecting group was confirmed through the disappearance of the singlet for *tert*-butyl of the pivalate group at δ 1.20 in the proton NMR spectrum for **(208)**. The triplet signal for the two protons next to the newly liberated hydroxyl group was shifted highfield from δ 4.05 (*J* 6.65 Hz) to δ 3.64 (*J* 6.9 Hz). The carbon NMR spectrum showed the disappearance of the signals at δ 178.61 for the carbonyl carbon, and at δ 38.71 for the quaternary carbon of the pivalate group. IR spectrum showed a broad peak at 3450 cm^{-1} for the hydroxyl group. The proton and carbon spectra of the compound **(240)** were similar to those of the compound **(208)**.

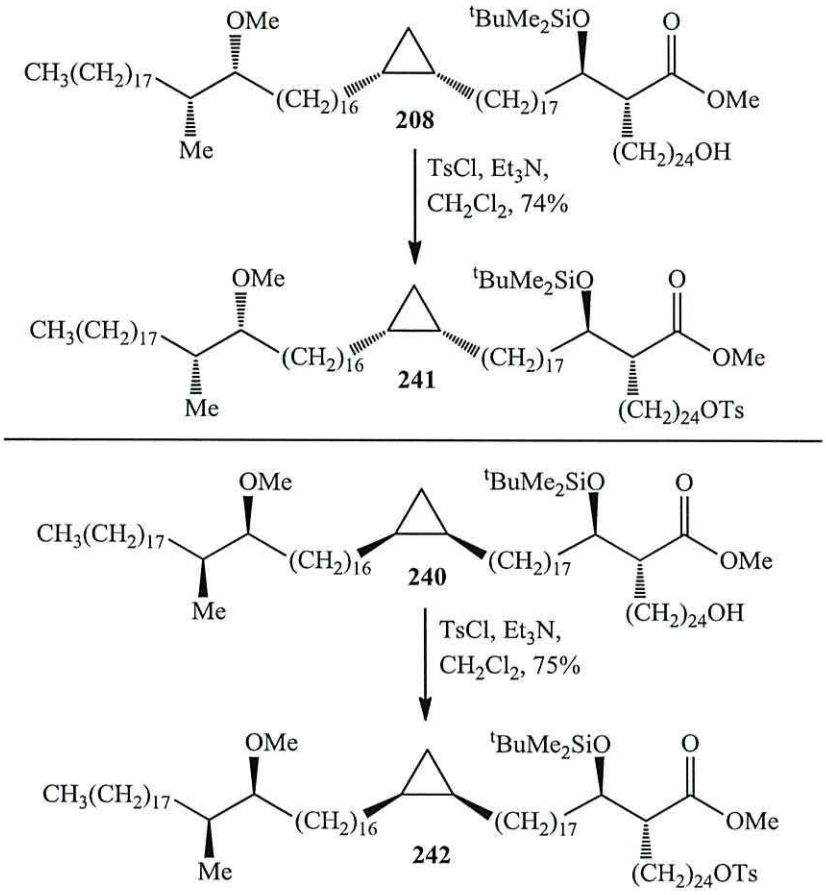


Scheme 70: Removal of the pivalate ester group from (236) and (239)

3.5 Tosylation of the hydroxyl group of α-alkyl chain

Compounds **(208)** and **(240)** were converted into their tosylates **(241)** and **(242)** through reaction with *para*-toluene sulfonyl chloride and triethylamine in dry dichloromethane. **(Scheme 71)**. After purification, the formation of the tosylate **(241)** was proved by the proton NMR spectrum which gave two doublets at δ 7.80 (*J* 8.2 Hz) and 7.35 (*J* 8.0 Hz) for the phenyl ring protons of the tosylate. A triplet signal appeared at δ 4.02 (*J* 7.4 Hz) for the two protons next to the oxygen of the alkyl chain and was shifted downfield in comparison to the alcohol starting material **(208)**. The three protons for the methyl group at the *para*-position of phenyl ring appeared as a singlet at δ 2.45. The carbon NMR gave four signals at δ 144.55, 133.36, 129.76 and 127.88 belonging to the aromatic carbons. The carbon of the ester carbonyl appeared at

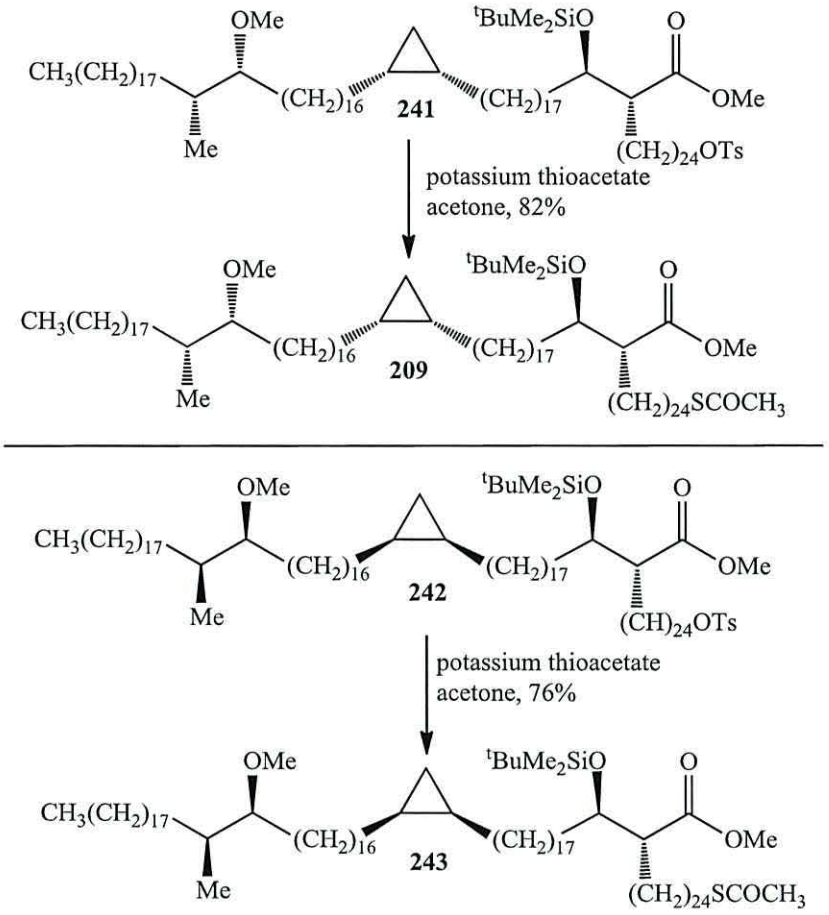
δ 175.10. The proton and carbon NMR spectra of the compound **(242)** were similar to those of its diastereoisomer **(241)**.



Scheme 71: Tosylation of the hydroxyl groups of α-chain (208) and (240)

3.6 Formation of the thioacetate

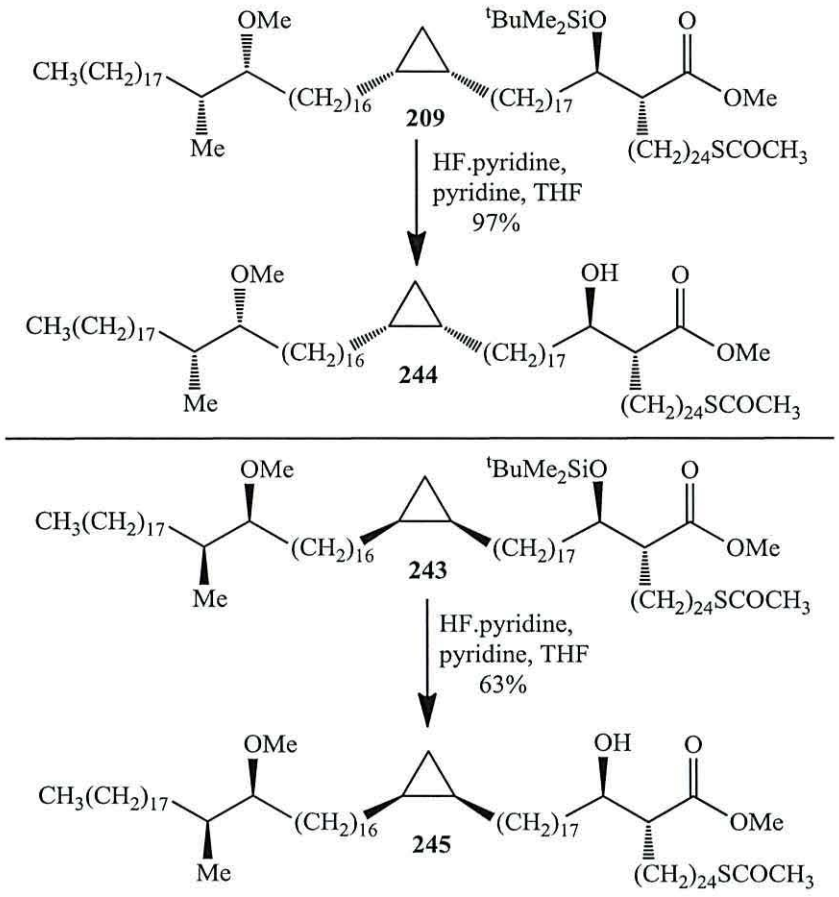
Compounds **(241)** and **(242)** were converted into their corresponding thioacetates **(209)** and **(243)** through reaction with potassium thioacetate in acetone **(Scheme 72)**. The proton NMR spectrum of the thioacetate **(209)**, showed a singlet at δ 2.32 for the three protons of the acetyl group. The triplet signal appeared at δ 2.86 (*J* 7.5 Hz) for the two protons next to the thioacetate, which was shifted highfield in comparison to the position of its counterpart in the NMR spectrum of the tosylate starting material. The carbon NMR spectrum gave a peak at δ 195.98 for the carbonyl of the thioacetate. The compound **(243)** gave the same proton and carbon NMR spectra for the compound **(209)**.



Scheme 72: Synthesis of the thioacetate compounds (209) and (243)

3.7 Desilylation

The silyl group was removed from compounds **(209)** and **(243)** using HF-pyridine complex and pyridine in dry THF to give the secondary alcohols **(244)** and **(245)** respectively **(Scheme 73)**. The removal of the silyl group from the compound **(244)** was verified by the proton NMR spectrum which showed the loss of the singlet at δ 0.86 for the the *tert*-butyl group, and the two singlets at δ 0.04 and 0.02 for the two methyl groups. The signals corresponding to the remaining protons were seen as before **(Figure 24)**. The carbon NMR spectrum did not show any signals for the silyl group. The mass spectrum of **(244)** showed the correct mass ion at $(M + Na)^+$: 1364.2809. The compound **(245)** showed similar spectroscopic data to the compound **(244)**.



Scheme 73: Deprotection of the silyl group in (209) and (243)

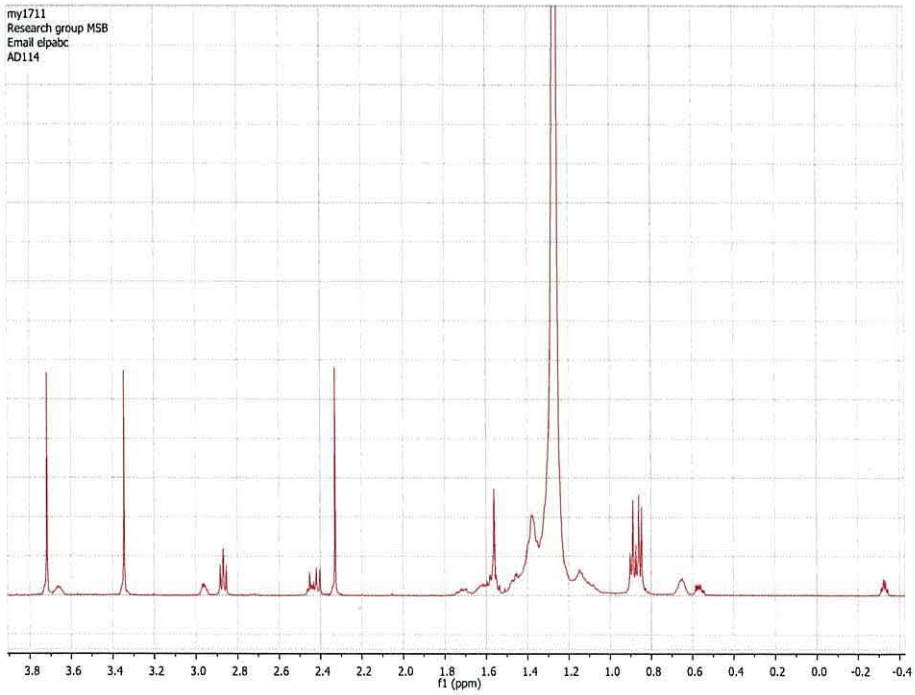
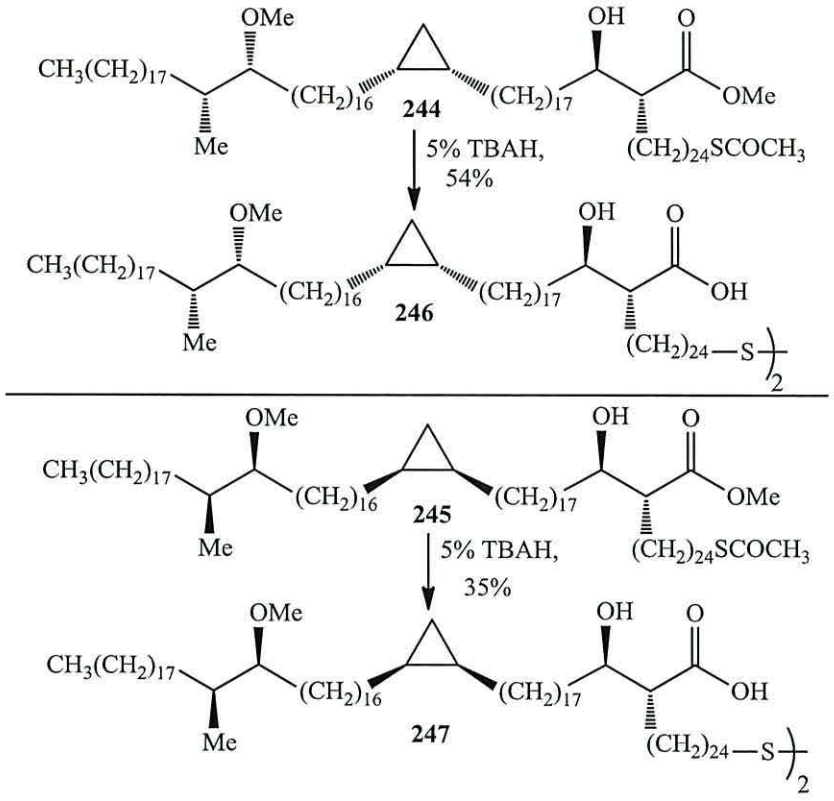


Figure 24: ^{1}H NMR spectrum of the compound (244)

3.8 The hydrolysis of the methyl ester and thioacetyl

The final step involved the simultaneous hydrolysis of the methyl ester and thioacetyl protecting groups of the motif. This involved heating the compound **(244)** or **(245)** in a 5% aqueous solution of tetrabutylammonium hydroxide at a temperature 100 °C overnight **(Scheme 74)**. After workup and purification, the proton NMR spectrum of product **(246)** or **(247)** showed the removal of all signals associated with methyl ester and thioacetyl groups. Mainly fragments of the spectrum for the final product **(247)** represented in **(Figure 25)**. The β and α protons appeared as multiplets between δ3.73-3.69 and 2.48-2.44 respectively. The methoxy group was observed as a singlet at δ 3.35 while the protons adjacent to the methoxy group gave a multiplet between δ 2.98-2.95. A triplet signal appeared at δ 2.69 (*J* 7.25 Hz) for the protons next to the sulfur. This gave a hint that the disulfide had been obtained instead of free thiol. The remaining protons were seen as before. However, the molecular mass spectrum could not be obtained for this product. The formation of a disulfide instead a thiol is not considered to be a problem because a disulfide can be bonded to gold in the same way as a thiol.[215] These compounds **(246)** and **(247)** have been bound to gold nanoparticles and used in a novel biosensor to diagnose TB by Dr. M. Pitts.[216] These compounds showed reasonable results. The compound **(246)** showed a sensitivity of 82% and specificity of 76%, while the compound **(247)** showed values of 80% and 70% respectively.



Scheme 70: Removal of methyl ester and thioacetyl groups

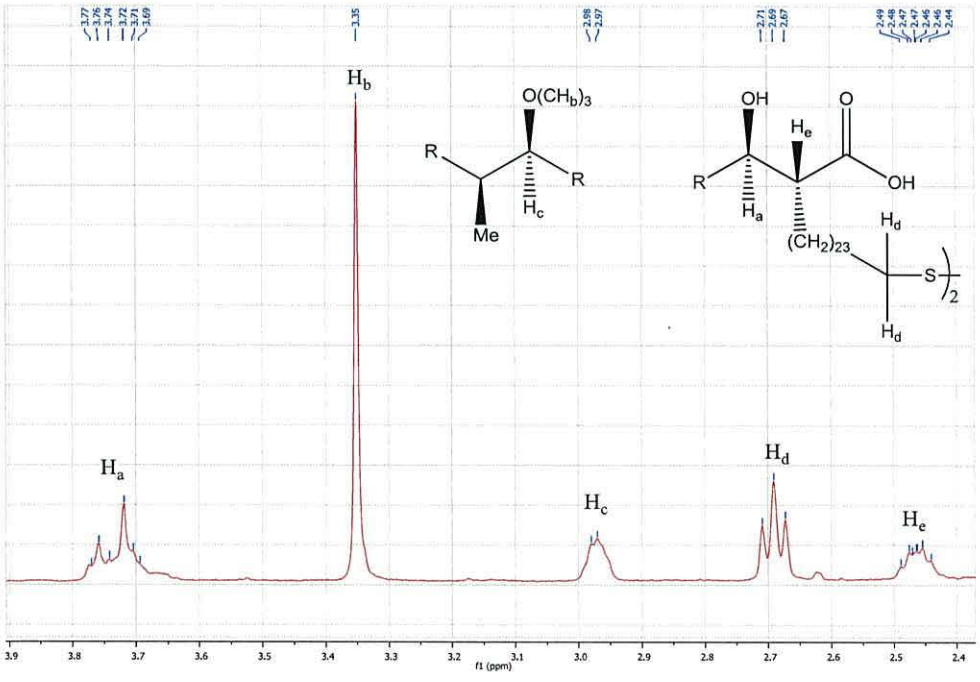


Figure 25: selected signals ^{1}H NMR spectrum of disulfide (247)

3.9 The verification of the formation of the disulfide (246)

In order to confirm the structure of the disulfide, the acid **(246)** was esterified with diazomethane and the alcohol was re-protected as an acetate. The disulfide was then reductively cleaved to give the thiol **(250)** using *DL*-dithiothreitol.

3.9.a Protection of hydroxyl group of carboxylic acid

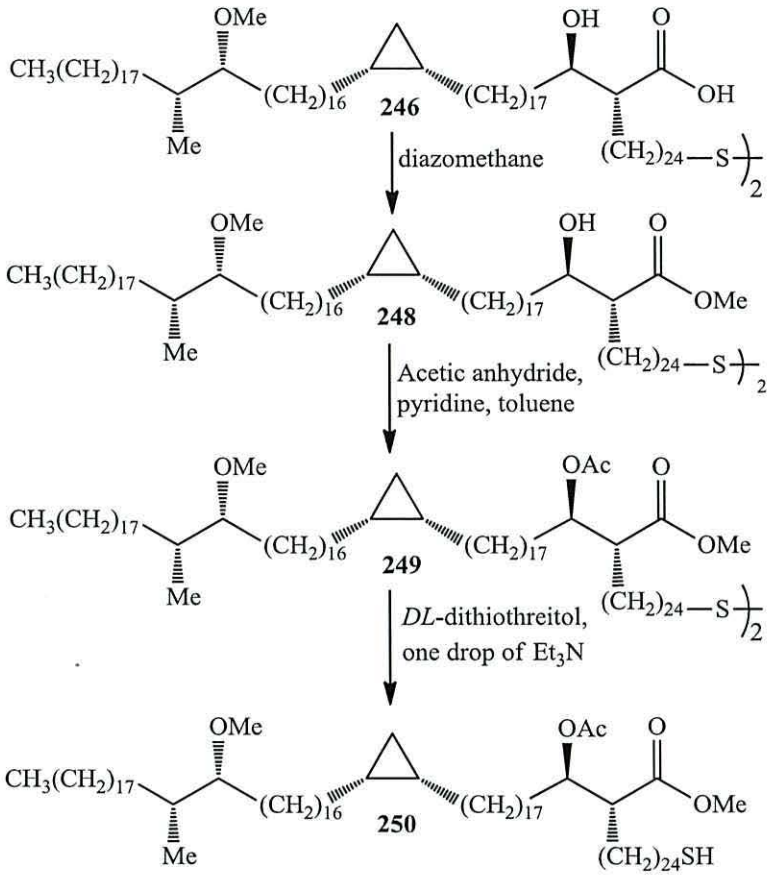
An excess of diazomethane in ether was added to the putative disulfide **(246)** and stirred for 30 minutes. The solvent was evaporated to give the ester **(248) (Scheme 75)**. The proton NMR spectrum of the compound **(248)** showed a singlet at δ 3.70 for the methyl ester group, and another singlet at δ 3.34 for the methoxy group, which confirmed that the esterification had proceeded.

3.9.b Protection of the β-hydroxyl group

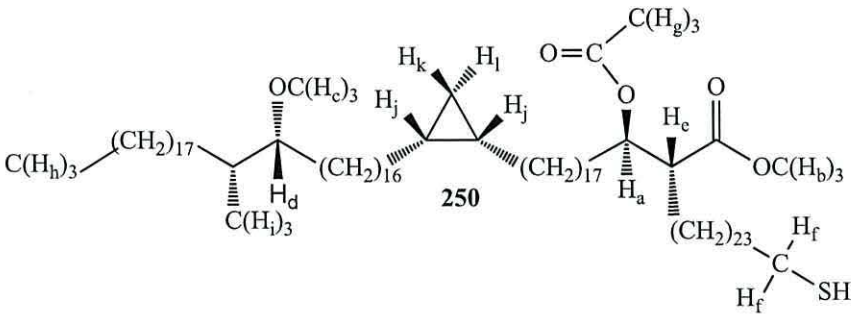
The secondary alcohol of the motif **(248)** was re-protected with the acetyl group. The protection of the secondary hydroxyl was achieved by using acetic anhydride and anhydrous pyridine in dry toluene **(Scheme 75)**. The proton NMR spectrum for the compound **(249)** showed the proton next to the acetoxy group to be a multiplet at δ 5.10-5.06, which was shifted downfield relative to its position in the unprotected species **(248)**. The methyl protons of the acetyl group appeared as a singlet at δ 2.03. The carbon NMR spectrum showed two signals at δ 173.67 and 170.37 corresponding to the two carbonyl groups of the methyl ester and acetyl respectively.

3.9.c The cleavage of the disulfide bond

This step involved the cleavage of the disulfide bond of **(249)** by adding *DL*-dithiothreitol to a stirred solution of compound **(249)** in chloroform followed by the addition of one drop of triethylamine under a nitrogen atmosphere.[217] The flask was covered with aluminium foil and left stirring for 48 hours **(Scheme 75)**. After work up, the proton NMR spectrum of the compound **(250)** showed a quartet at δ 2.53 (*J* 7.52 Hz) for the two protons next to sulfur. In addition, it was found that the carbon signal for the methylene group adjacent to sulfur had shifted from δ 39.5 in the disulfide to 24.66 in the thiol **(Table 7)** and **(Figure 26)**. This is typical for the carbon adjacent to sulfur in sulphides.[218,219,220] MALDI MS analysis gave the correct mass ion for the free-thiol protected mycolic acid **(250)** at $(M + Na)^+$: 1364.10.

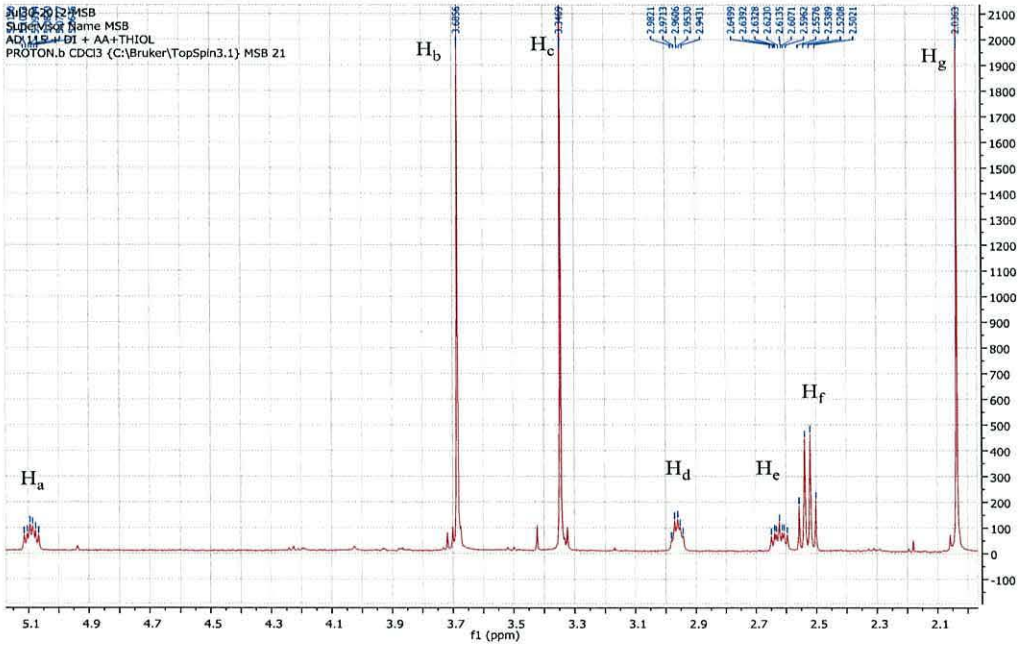


Scheme 75: Cleavage of the disulfide (246) to give thiol (250) after protection of the hydroxyl groups

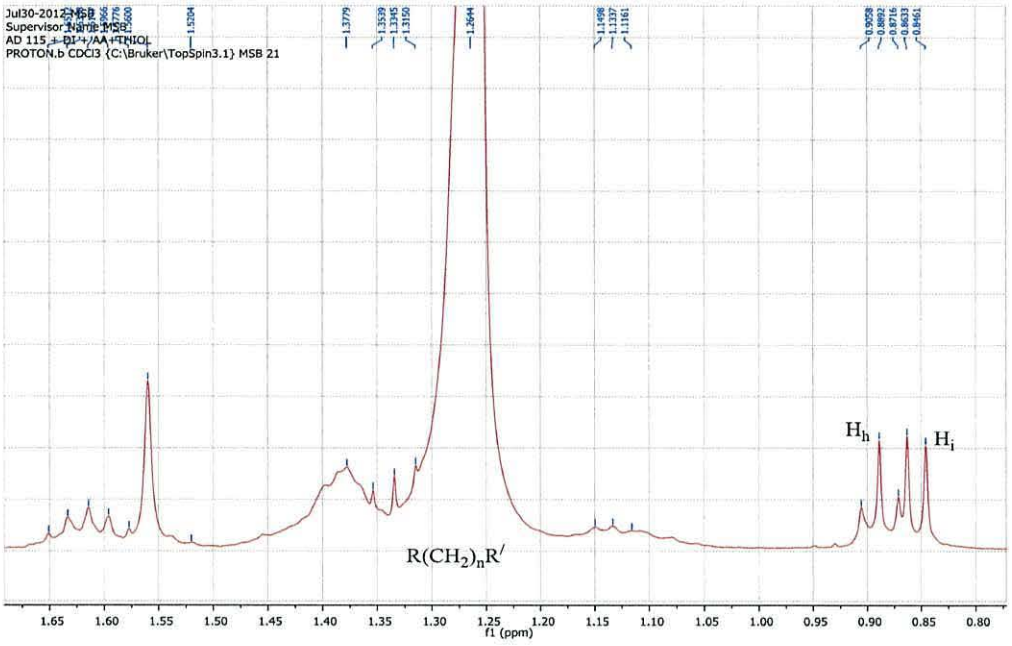


H_x	δ	Multiplicity	Integration	J (H_z)
H_a	5.13-5.06	m	1	-
H_b	3.68	s	3	-
H_c	3.34	s	3	-
H_d	2.97-2.94	m	1	-
H_e	2.62	ddd	1	4.36, 6.88, 10.88
H_f	2.53	q	2	7.25
H_g	2.03	s	3	-
H_h	0.88	t	3	6.52
H_i	0.81	d	3	7.4
H_j	0.68-0.64	m	2	-
H_k	0.57	dt	1	3.76, 8.04
H_l	-.032	br. q	1	5.16

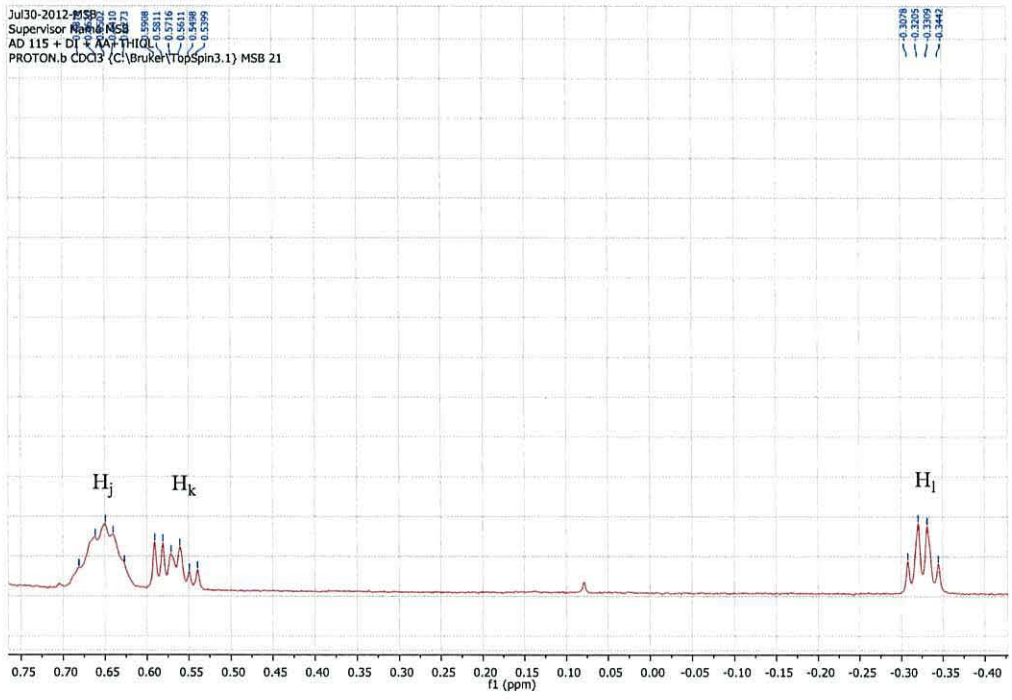
Table 7: ^{1}H NMR data analysis of the compound (250)



A



B



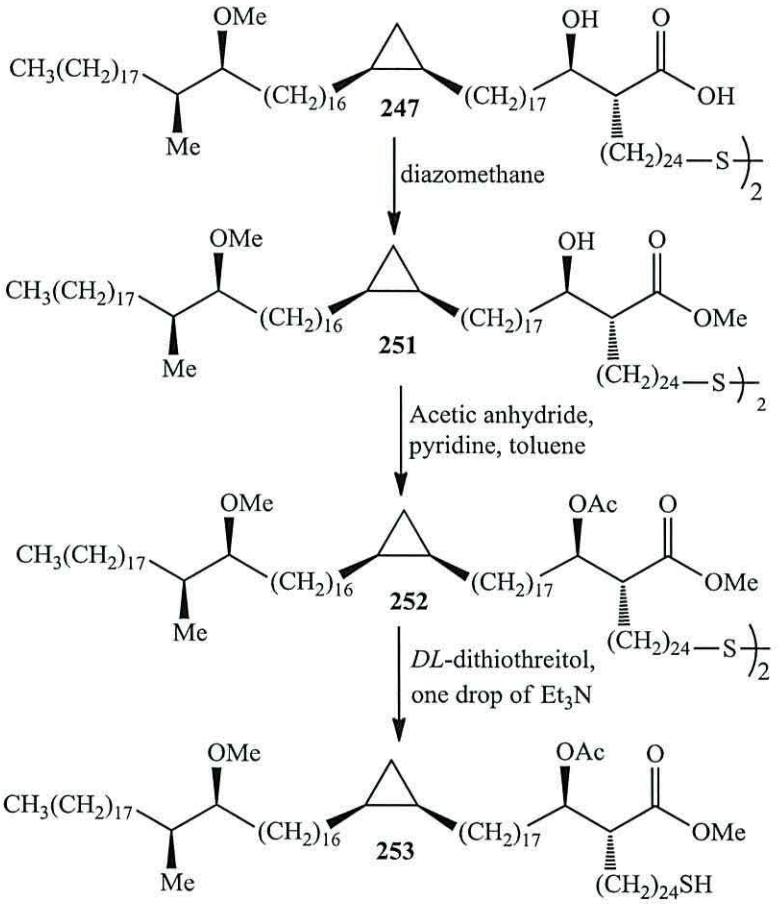
C

Figure 26: *^{1}H NMR* expansion of the protected thiol methoxy mycolic acid (250)

3.10 The verification of disulfide (247) formation

The structure of the disulfide **(247)** was confirmed through the application of that method discussed previously for the diastereoisomer **(246)**. Firstly, the terminal carboxy groups and β-hydroxyl groups were protected with diazomethane and acetic

anhydride respectively, which was followed by the cleavage of the disulfide bond using the *DL*-dithiothreitol reagent **(Scheme 76)**. The final compound **(253)** and the intermediates **(251)** and **(252)** were found to give identical spectroscopic data to those compounds in **(Scheme 75)**.

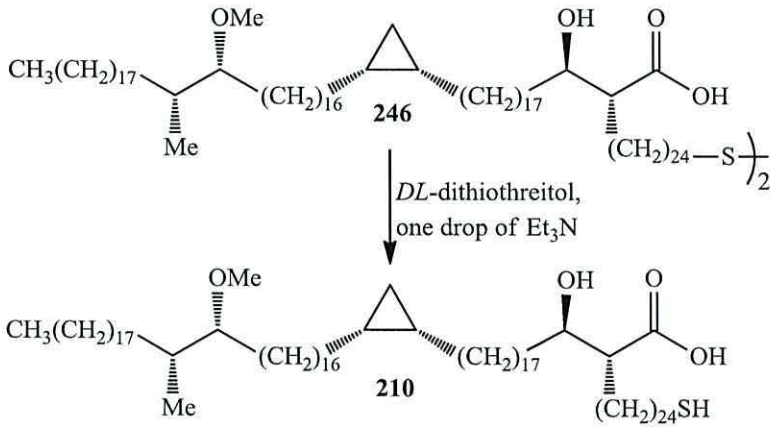


Scheme 76: Cleavage of the disulfide (247) to thiol (253) after the protection of the carbonyl and hydroxyl groups

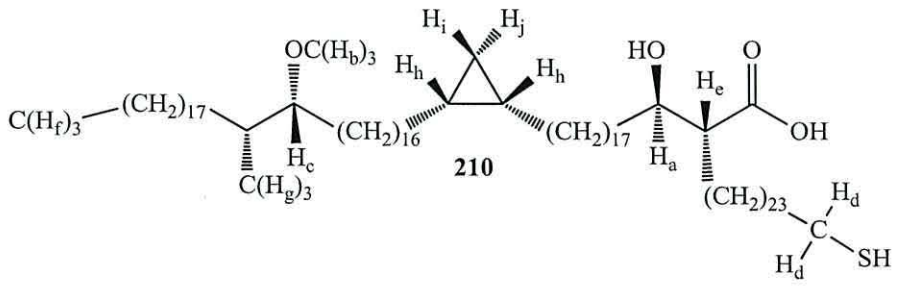
3.11 One step to cleave the disulfide (246)

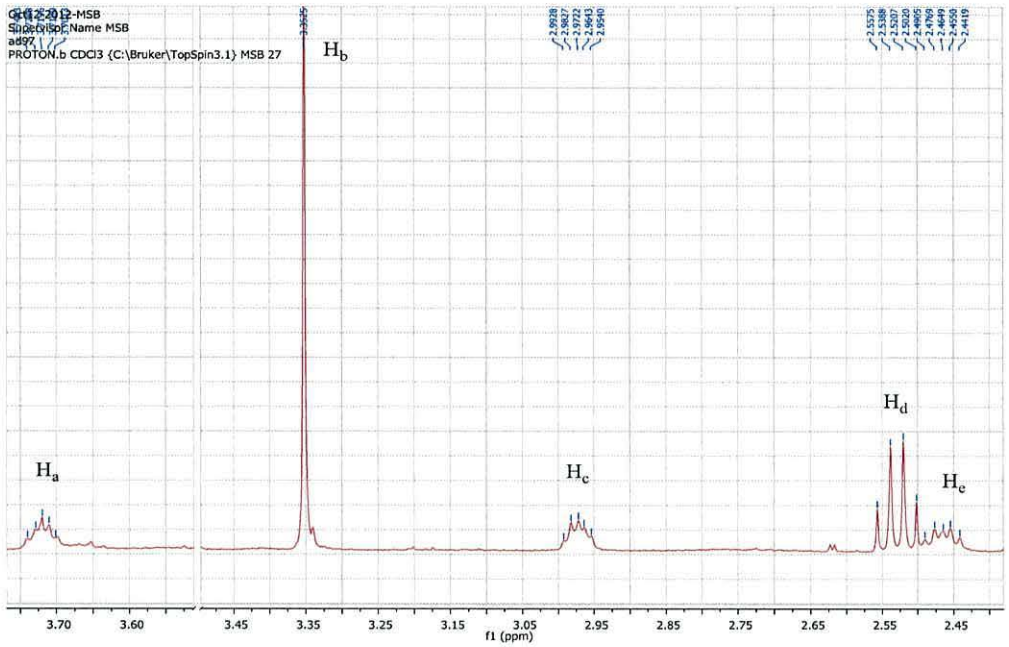
In this method, the free thiol **(210)** was obtained through the cleavage of the disulfide bond of **(246)** without protection of the alcohol and acid groups. This method was applied only on the disulfide **(246)** because only a very small quantity of the disulfide **(247)** was available. The disulfide **(246)** was dissolved in chloroform, and following the addition of *DL*-dithiothreitol and one drop of triethylamine, the flask was covered with aluminium foil and left for 48 hours at room temperature **(Scheme 77)**. After work up, the formation of the desired free thiol methoxy mycolic acid **(210)** was

confirmed by both proton and carbon NMR spectroscopy and MALDI MS. The proton NMR spectrum **(Figure 27)** gave a multiplet between δ 3.74-3.69 for the β-proton of the motif part. The methoxy group appeared as a singlet at 3.35 and the proton next to the methoxy was seen as a multiplet between δ 2.99-2.95. The two protons next to the sulfur were observed as a quartet at δ 2.52 (*J* 7.52 Hz) which gave evidence for the formation of the free thiol. When the sample was shaken with D_2O, the quartet became a triplet (*J* 7.16 Hz). The proton at the α-position appeared as a multiplet between δ 2.49-2.44. The terminal methyl group appeared as a triplet at δ 0.88 (*J* 6.52 Hz) while, the methyl substituent at the C_{19} branch gave a doublet at δ 0.85 (*J* 6.92 Hz). The four *cis*-cyclopropane protons appeared as a multiplet at δ 0.68-0.64 for $2H_h$, a doublet of triplets at δ 0.56 (*J* 3.76, 7.52 Hz) for H_i and a broad quartet at δ -0.32 (*J* 5.05 Hz) for H_j. The MALDI MS showed the correct mass ion at $(M + Na)^+$: 1308.78.

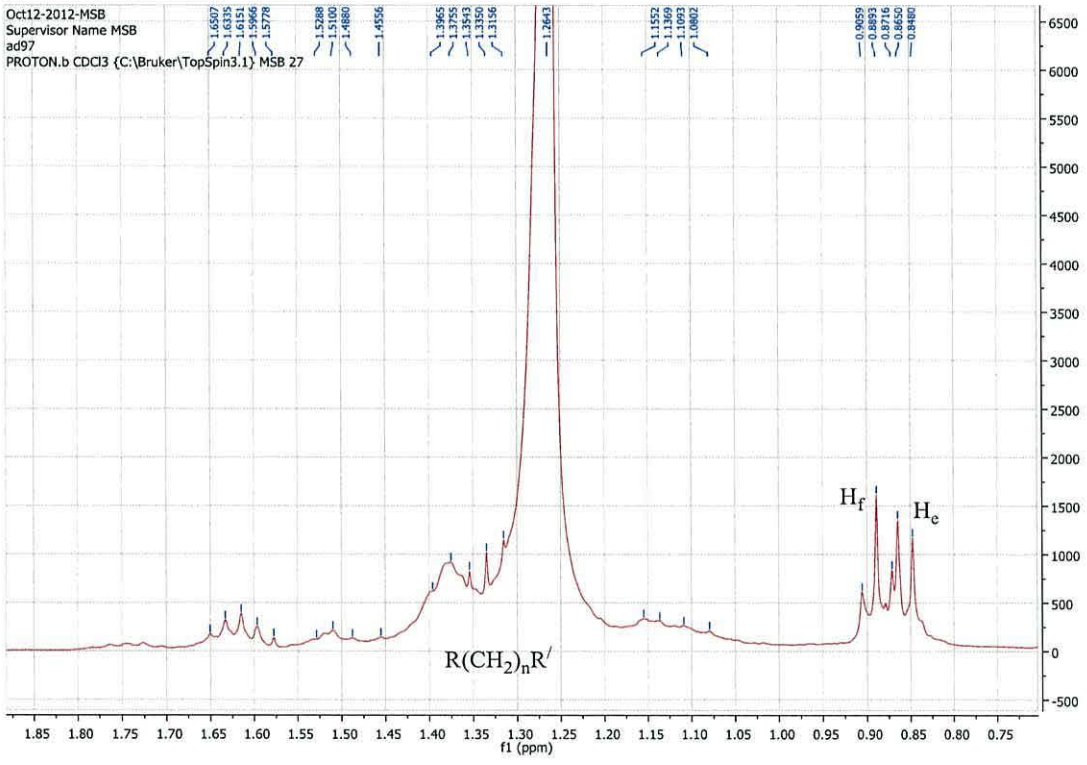


Scheme 77: One step cleavage of the disulfide (246) to thiol (210)

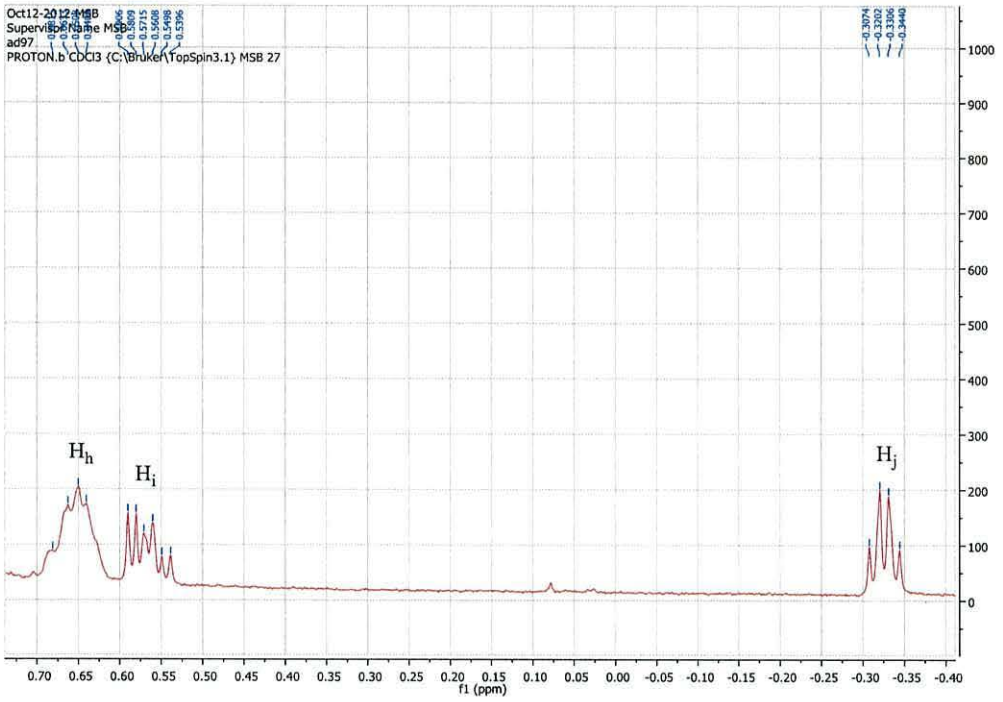



PROTON.b CDCl3 {C:\Bruker\TopSpin3.1} MSB 27
H_a
H_b
H_c
H_d
H_e
f1 (ppm)

A


Oct12-2012-MSB
Supervisor Name MSB
ad97
PROTON.b CDCl3 {C:\Bruker\TopSpin3.1} MSB 27
H_f
H_e
$R(CH_2)_nR'$
f1 (ppm)

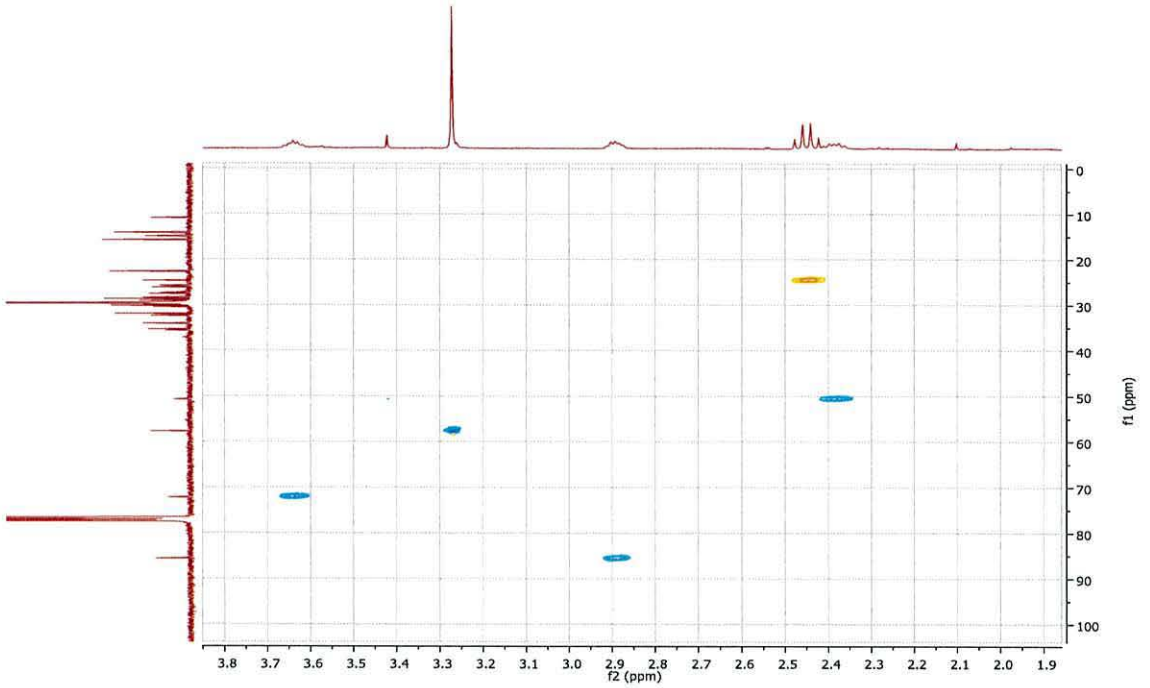
B



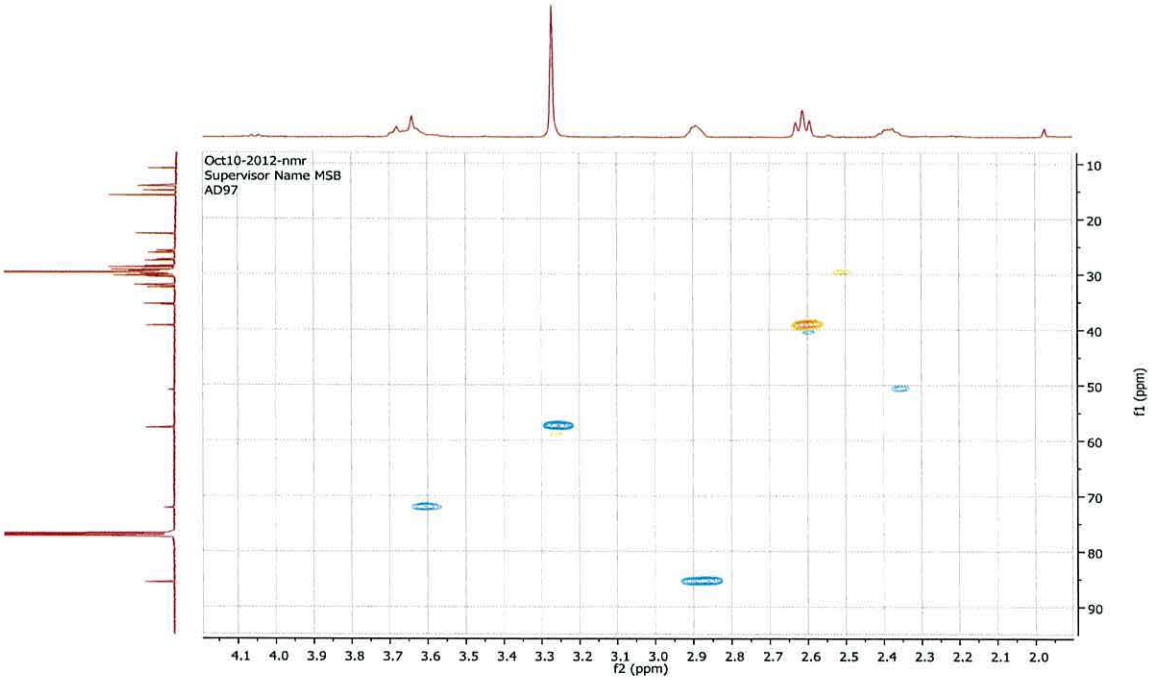
C

Figure 27: Expansion of the proton NMR spectrum of the free thiol methoxy (210)

The signal corresponding to the methylene carbon next to the thiol group was identified by a 2D proton carbon correlation NMR analysis (HSQC) which showed a signal at δ 24.65, which in the disulfide appeared at δ 39.09 **(A, B, Figure 28).** This provided another means of discriminating between the thiol and the disulfide.



A



B

Figure 28: Proton carbon correlation spectrum for thiol (A) and disulfide (B)

Chapter 4

4. Diagnosis of Tuberculosis

4.1 ELISA assay

The use of the ELISA assay as a tool for diagnosing TB is attractive for a number of reasons. The tool is relatively simple, cheap and quick to administer (see page 25). However, the results drawn from the ELISA assay largely depend on the degree of antibodies detected in the TB patient's blood sample using recombinant TB antigens. It has been established that the results of the ELISA method in diagnosing TB infection depend on the quality of the antigen used. Mycolic acids and their derivatives are mycobacterium cell wall antigens, which have attracted great interest as surrogate markers for tuberculosis serodiagnosis.[154] They are the dominant lipids in the bacterium cell wall.

The present chapter presents a discussion of results obtained from an investigation of several new synthetic mycolic acids and their derivatives as antigens in ELISA experiments. TB diagnosis is dependent on the measurement of absorbance. A TB-positive sample is distinguished by a significantly high absorbance, while a low-absorbance sample is indicative of negative TB status. Thus, the challenge in this work is to select the most suitable antigen, which will produce reliable results in TB diagnosis. This process will largely depend on the sensitivity and specificity of the antigen used. Sensitivity is the proportion of positive samples correctly identified by the antigen to the total TB-positive sera, while specificity is the proportion of negative samples correctly identified by the antigen to the total TB-negative sera.[221] Both values are expressed as percentages and are determined on the basis of the cut-off value of the absorbance to distinguish a negative result from a positive result for the test antigen.

The study was conducted with three serum sample groups: WHO serum samples of known and unknown TB status and Bangor samples. These WHO samples were collected from many sources which included tropical regions of Asia, Africa and the Americas. The WHO sample was divided into two groups. The first group consisted of 99 known serum samples of both TB positive and TB negative sera while the second group consisted of 249 unknown sera. (Provided by WHO). The Bangor samples were obtained from the north-western region of Wales in the United Kingdom. This region

was chosen because the UK is not a TB endemic country. (Kindly provided by Dr. Prysor Williams, Bangor University)

Initially, experiments were carried out with the first group of samples from the WHO in order to optimise the conditions of the ELISA assay. Later, experiments were conducted with the remaining samples drawn from the WHO and Bangor. The method in the literature[140] was modified and optimized to detect anti-TB antibodies so as to obtain good results from the ELISA assay. All this was done with a view to distinguishing TB infection from non-infected serum.

4.2 Optimisation of the ELISA assay

4.2.1 The wavelength selection

First, the reaction between the secondary antibody, o-phenylenediamine dihydrochloride (OPD) and hydrogen peroxide was investigated in order to determine the wavelength that provided the maximum absorbance. The secondary antibody was diluted to various dilutions in casein/phosphate buffered saline (PBS) and placed in a cuvette (100 μL). The OPD substrate (containing 0.8 mg/mL H_2O_2 and 1 mg/mL OPD in citrate buffer) was then added to the cuvette (900 μL), and the UV-visible spectrum of the solution was measured from 300 – 800 nm for 5 minutes after the addition of the OPD substrate. The wavelength 492 nm was discovered to be the best wavelength. This was then used to run all the ELISA assay experiments **(Figure 29)**.

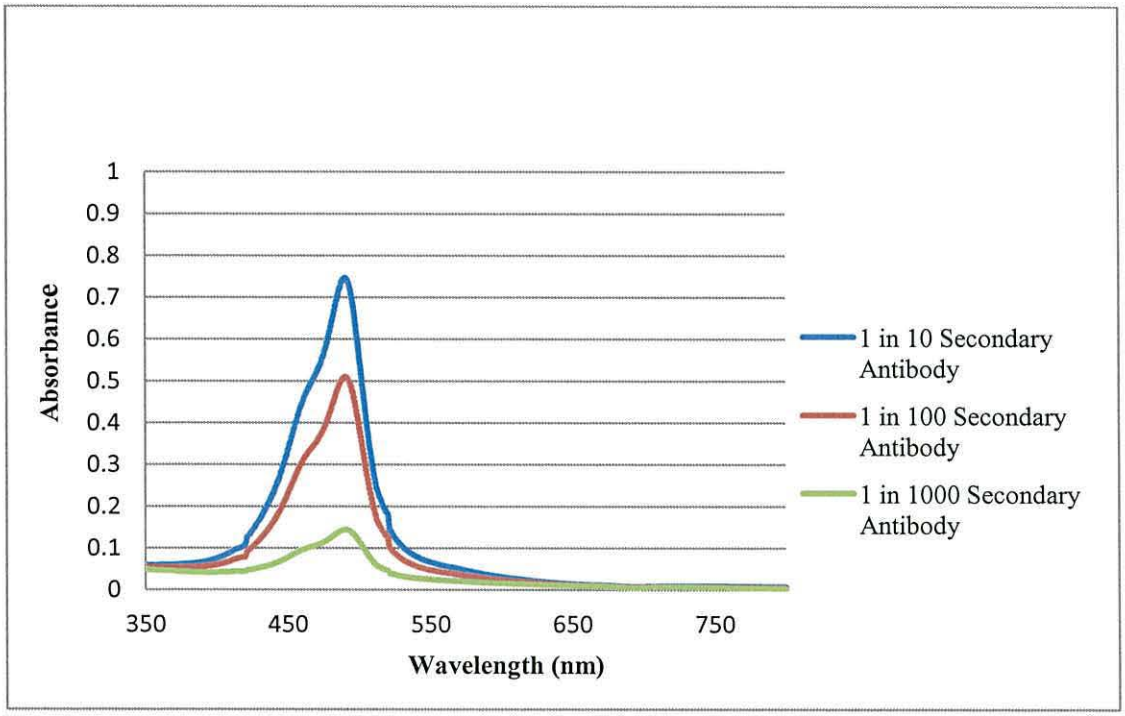


Figure 29: Change in absorbance with change in secondary antibody concentration

4.2.2 Selection of the best serum concentration

Serum concentration may affect ELISA results. Thus, in the ELISA assay, different concentrations of serum from the first group of samples were diluted with casein in (PBS) buffer to obtain concentrations of 1:20 to 1:160 against natural TDM as an antigen **(Figure 30)**. TDM or TMM cord factors were preferred over free mycolic acids because they gave a high level of response to TB antibodies, as mentioned before (see page 26).

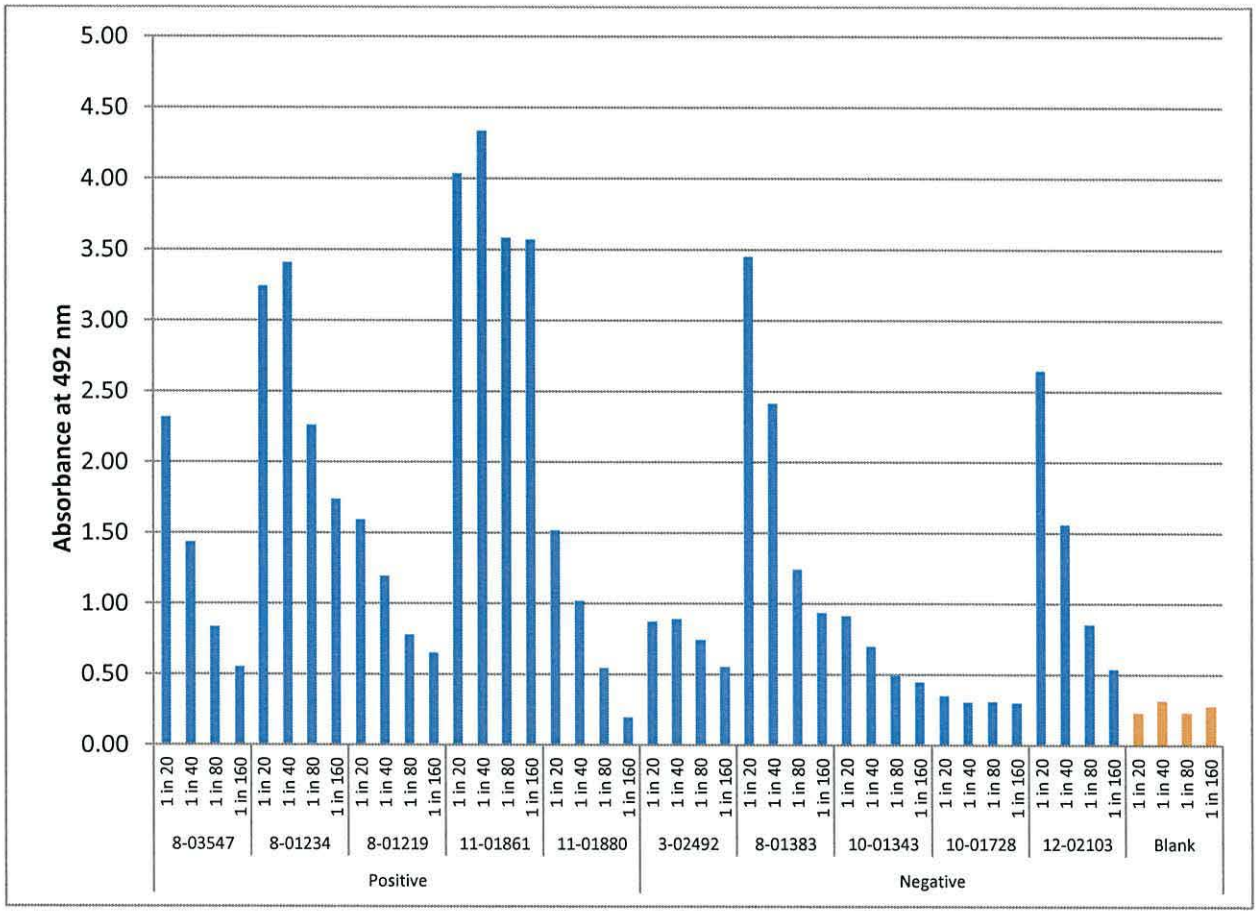


Figure 30: The effect of serum concentration on ELISA results with natural TDM as an antigen

Figure 30 above shows the effect of serum dilution on the absorbance and its effect on the ELISA assay results. It shows that the absorbance decreased with increased dilution of the serum. A dilution of 1:20 gave a high absorbance in comparison with other concentrations. Therefore, this was used during subsequent experiments

4.2.3 Selecting the Secondary Antibody Conjugate

An antibody or immunoglobulin (Ig) is a glycoprotein found in plasma and extracellular fluids. The human body has the ability to produce antibodies from specialized white blood cells called B lymphocytes as an immune response to an antigenic target, such as a bacterium or virus. Moreover, antibodies have the ability to bind to antigens with a high affinity and specificity. These antibodies are called primary antibodies, which are naturally unconjugated.[222,223] There are five main types of antibodies: IgG, IgM, IgA, IgD, and IgE. Of these, IgA, IgG and IgM are more prominent in serum, with IgG being the most abundant found in humans and animals. Each type of antibodies contains a light chain and a heavy chain, with the heavy chain being specific to each type of antibody.[156] Another type of antibody, called a secondary antibody, is created by injecting an animal, such as a goat, donkey, sheep, chicken or rabbit, with whole or fragment of IgG, IgM, IgA, IgD, or IgE drawn from the various species (e.g., a mouse) that produce primary antibodies. The secondary antibody has the ability to bind to the primary antibody.[224] By using the secondary antibody, it is possible to detect the primary antibody as an antigen. The secondary antibody is conjugated to an enzyme that catalyzes a simple chemical reaction that results in a colour, which can be measured by using spectrophotometer. As a result, secondary antibodies become useful in detecting many diseases.

Whole molecule IgG (WM) and fragmented IgG (Fc), as secondary antibodies, were used with various cord factors, which used antigens such as TDM of methoxy mycolic acid **(198)**, TDM of α-mycolic acid **(254)**[175] and TDM of *trans*-ketomycolic acid **(255)**[209] **(Figure 31)**. These antigens were tested with a randomly selected group of 10 positive and 20 negative sera from the first group of WHO samples. An experiment to determine the more appropriate secondary antibody to use and the results are presented in Figure **32**.

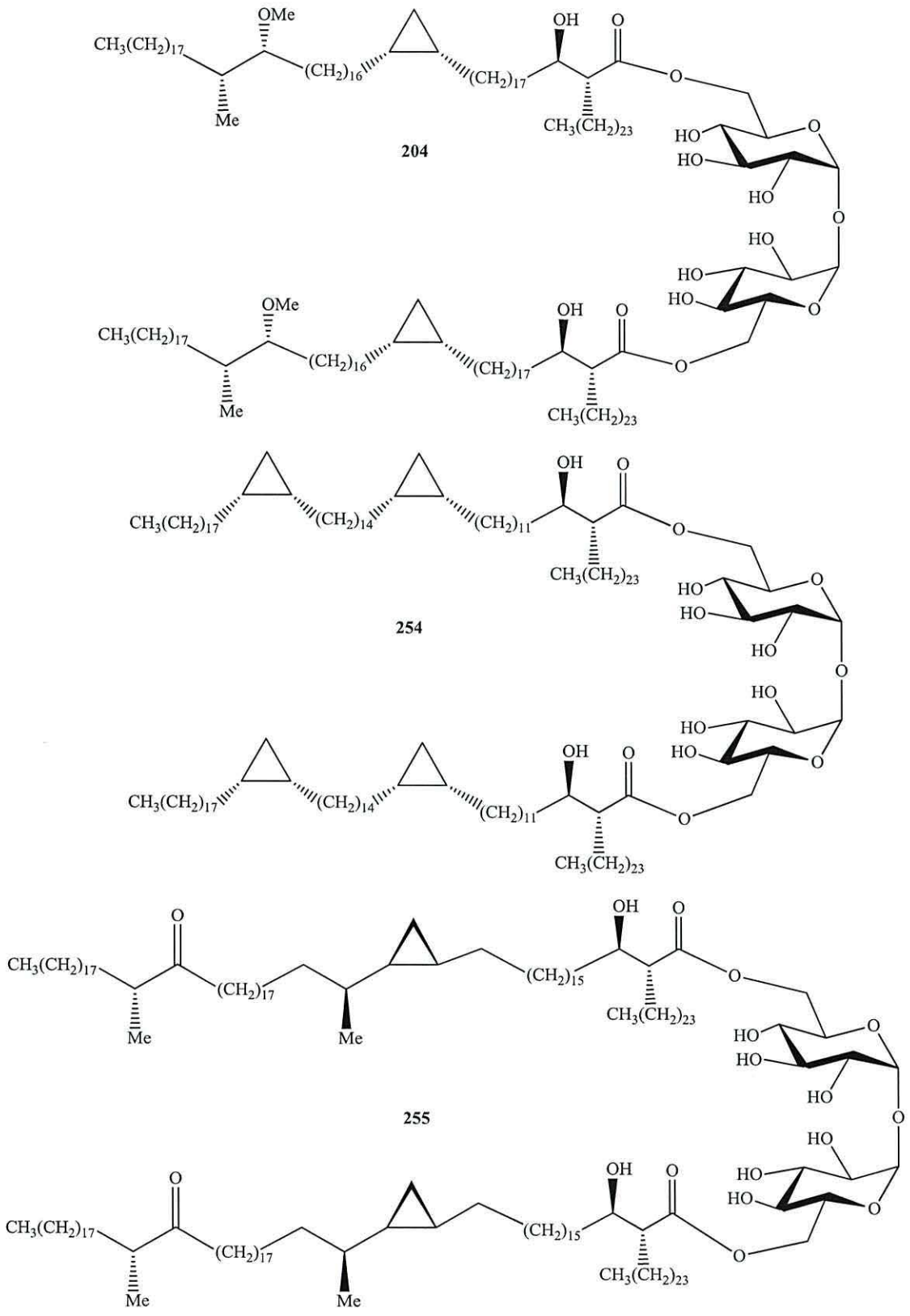


Figure 31: Structure of the cord factors (198), (254) and (255)

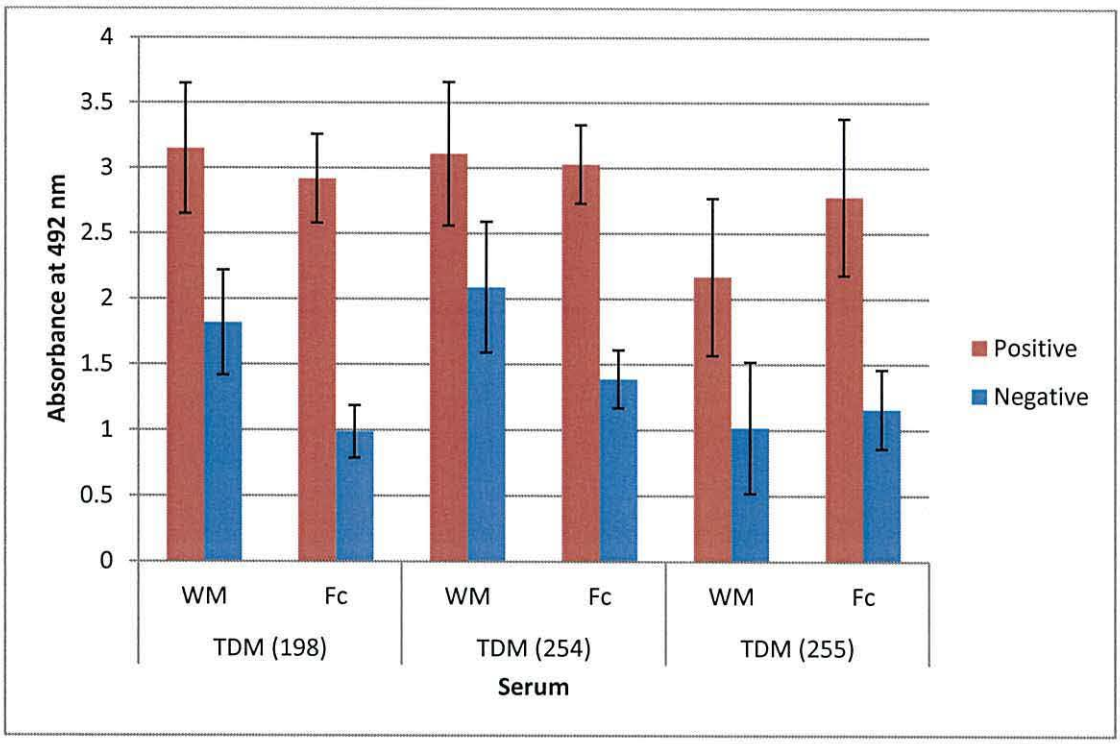


Figure 32: The effect of a change in the secondary antibody on the ELISA results of the serum samples from the first group

Figure 32 shows that there is a significant difference in average absorbance between the positive and negative sera when IgG (WM) and IgG (Fc) are used as secondary antibodies in an ELISA assay. A better distinction was observed between the two serum types when using IgG (Fc) as the secondary antibody than when using IgG (WM). The sensitivity and specificity values for these antigens are illustrated in **Table 8.**

Table 8: The data analysis of ELISA results when using IgG (WM) and IgG (Fc) as secondary antibodies with (199), (255) and (256) as antigens

Antigen	Average absorbance positive	Average absorbance negative	Sensitivity (%)	Specificity (%)
TDM (198) with IgG (WM)	3.15	2.14	90	50
TDM (198) with IgG (Fc)	3.04	1.10	80	85
TDM (254) with IgG (WM)	3.11	2.39	100	40
TDM (254) with IgG (Fc)	2.80	1.28	80	85
TDM (255) with IgG (WM)	2.17	1.01	50	90
TDM (255) with IgG (Fc)	2.39	1.29	60	85

In conclusion, these results revealed that the use of the IgG(Fc) secondary antibody seemed to be much better than the use of the IgG(WM) secondary antibody when distinguishing between TB+ and TB- samples. This result may be because the IgG(Fc) has specific detection (binding) only for the IgG antibody type, while the IgG (WM) has the ability to detect (bind) to any type of antibody (with preference for IgG). The results showed that TDM **(198)** and TDM **(254)** were good antigens when using the IgG (Fc) secondary antibody, which gave the best sensitivity and specificity values. Thus, TDM **(198)** and TDM **(254)** appeared to be the most likely candidates for use in detecting TB infection.

4.3 Analysis of first-group sera with TDM (198) and TDM (254)

In the previous experiments, TDM **(198)** and TDM **(254)** showed good results in terms of sensitivity and specificity in the ELISA assay when applied as antigens to selected specimens of the first-group sera of the WHO samples. It was decided to test TDM **(198)** and TDM **(254)** as antigens against the full list of the first-group sera to estimate the accuracy of the ELISA assay with these antigens. This was done in order to determine how many true positive and true negative samples would be detected during the assay. The results of these experiments using TDM **(198)** and TDM **(254)** as antigens showed a higher absorbance response with positive sera than with negative sera **(Figure 33)**.

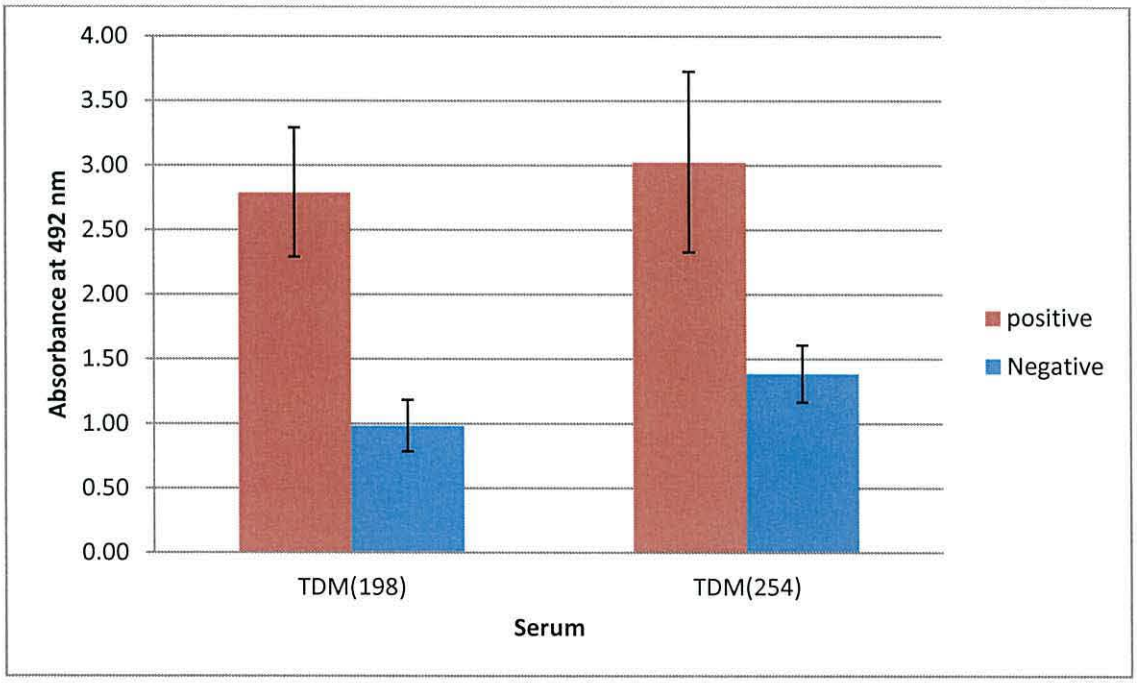


Figure 33: The average absorbance of the serum samples (+) and (-) from the first group against TDM (198) and TDM (254)

The results for the absorbance values of the first group of WHO samples against TDM **(198)** and TDM **(254)** as antigens are summarised in **Table 9**. The results for TDM **(198)** showed five false negatives out of 26 known positive samples. This showed the sensitivity of TDM **(198)** to equal 81%. Also, there were seven false positive samples out of 73 known negative samples, which showed the specificity of TDM **(198)** to be 90%. The results for TDM **(254)** included five false negatives and 17 false positives. It showed a sensitivity and specificity of 81% and 77% respectively, for the same set of TB serum samples. The sensitivity and specificity values of TDM **(198)** and TDM **(254)** were calculated by using a cut-off absorbance value of 2. The cut-off was chosen in order to achieve the best sensitivity and specificity values. On other hand, if the cut-off was reduced to detect all the positive samples (100% sensitivity), the specifities were very low (53% and 56%) for TDM **(198)** and TDM **(254)** respectively, which were considered unacceptable.

Table 9: Absorbance values of first group sera (unblind) using synthetic antigens TDM (198) and TDM (254). Red background represents TB+ sample, blue TB-, as diagnosed by WHO; pink boxes for results are TB+ using ELISA assay with a cut-off of 2

Serum	TDM(198)	TDM(254)	Serum	TDM(198)	TDM(254)
BA 1	1.35	1.23	CO 7	2.07	2.21
BA 2	4.00	3.11	CO 8	1.00	1.68
BA 3	4.27	3.08	CO 9	0.63	0.65
BA 4	2.02	2.11	CO 10	0.43	0.71
BA 5	2.13	3.35	CO 11	0.40	0.50
KE 1	3.25	3.21	CO 12	0.22	0.21
KE 2	1.19	1.73	CO 13	1.12	1.51
KE 3	4.10	3.19	CO 14	1.14	2.93
KE 4	3.70	2.83	CO 15	1.92	3.14
KE 5	4.39	4.12	CO 16	0.79	0.37
BA 19	3.52	3.57	CO 17	0.76	1.14
BA 20	3.23	3.14	CO 18	0.58	0.47
BA 21	3.36	2.99	KE 6	0.55	0.95
PE 3	2.42	3.00	KE 7	0.45	0.29
PE 4	3.49	3.60	PE 1	1.18	2.24
SA 6	3.14	3.14	PE 2	0.72	1.45
KE 8	3.43	3.70	SA 7	0.55	1.43
KE 9	0.76	1.15	SA 8	0.27	0.49
GA 1	3.27	4.28	CO 19	0.54	0.93
GA 2	3.28	3.13	CO 20	0.40	0.79
SP 1	2.24	3.15	CO 21	0.91	1.79

VI 1	4.11	4.03	CO 22	1.09	3.16
VI 2	1.47	1.76	CO 23	0.38	0.99
VI 3	2.91	3.75	CO 24	1.12	1.72
GA 3	1.26	1.98	CO 25	0.25	0.42
GA 4	3.58	4.38	CO 26	0.99	2.34
BA 6	1.25	2.79	CO 27	0.35	0.55
BA 7	2.28	2.99	CO 28	0.37	0.52
BA 8	3.20	3.38	CO 29	0.48	1.10
SA 1	0.71	1.20	GA 5	1.23	1.15
SA 2	1.19	1.31	GA 6	1.34	1.44
SA 3	0.43	1.05	GA 7	1.15	1.38
SA 4	0.67	0.30	GA 8	3.82	4.08
SA 5	2.45	0.82	GA 9	1.20	2.43
BA 9	0.69	1.67	GA 10	1.19	2.78
BA 10	0.47	1.09	GA 11	1.73	2.96
BA 11	0.87	0.96	GA 12	1.99	4.19
BA 12	0.87	1.60	BR 1	0.41	0.98
BA 13	0.98	0.91	CA 1	4.09	4.07
BA 14	1.60	1.87	CA 2	0.15	0.39
BA 15	1.19	0.82	CA 3	0.18	0.32
BA 16	0.44	0.65	CA 4	0.28	0.54
BA 17	0.26	0.59	CA 5	0.74	1.27
BA 18	0.73	0.28	SP 2	0.23	0.32
CO 1	0.46	0.54	SP 3	4.31	4.40
CO 2	1.32	0.83	SP 4	0.23	0.64
CO 3	1.80	2.73	SP 5	0.38	0.58
CO 4	0.66	0.96	SP 6	0.25	0.45
CO 5		0.25	SP 7	0.29	0.54
CO 6	0.26	0.40			

To obtain a more detailed explanation of the results for TDM **(198)** and TDM **(254)** as synthetic antigens, further analysis of the data was carried out using the Minnitab 16 software programme. The individual values were plotted as dot plots so as to clearly differentiate between positive sera (1) and negative sera (0) for TDM **(198)** and TDM **(254)** **(Figure 34)**. The cut-off absorbance was set at 2. The dot plot showed the accumulation of most of the positive sera (1) above the cut-off absorbance value of 2, with few false negatives. Conversely, most negative sera (0) were found under or at the cut-off absorbance value of 2, with few false positives. From the results obtained above, it was shown that TDM **(198)** and TDM **(254)** acted as good antigens in terms of having the ability to bind to TB antibodies. It was therefore decided to use them to detect TB antibodies in the blind sera group **(see Section 4.13)**. Both had the same

sensitivity value, although TDM **(198)** had a higher specificity. For this reason, TDM **(198)** was considered a better antigen than TDM **(254)**.

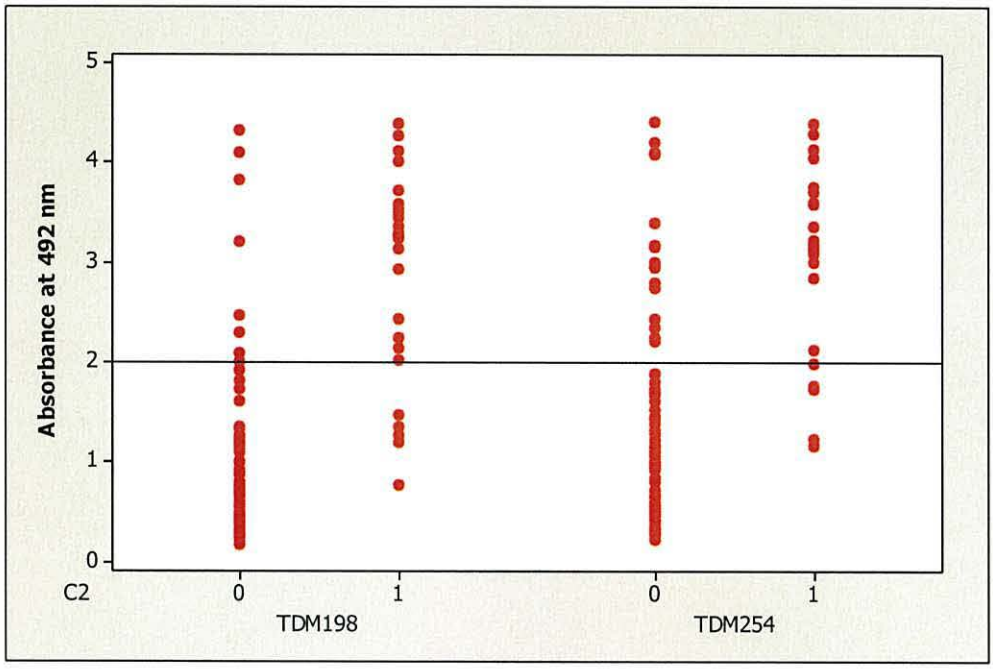


Figure 34: Individual value plots of TDM (198) and TDM (254) as antigens to full first-group sera. (TB- = 0, TB+ = 1)

4.4 A comparison between TDM (198) and TDM (256)

The compound **(256)**[177] is a synthetic methoxy TDM that differs from **(199)** only in the stereochemistry of the mycolic acid **(Figure 35)**.

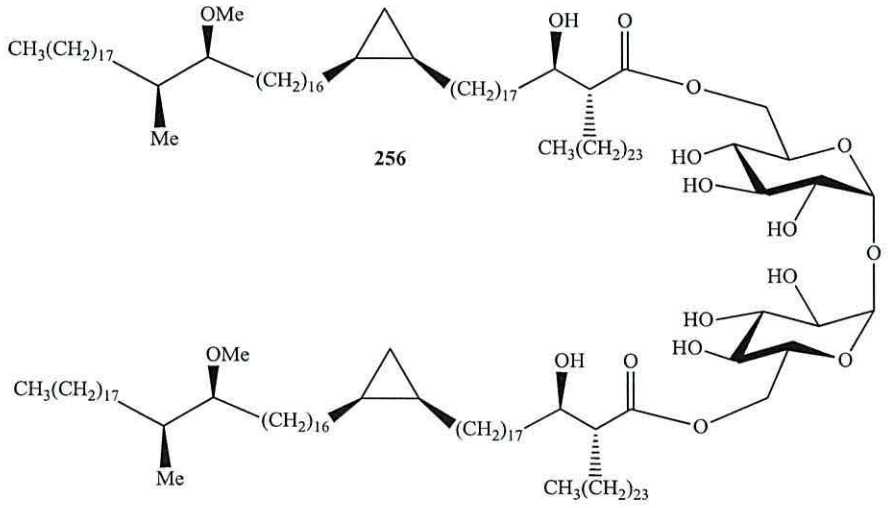


Figure 35: Structure of TDM (256)

ELISA experiments were carried out to investigate the effect of stereochemistry on the antigenicity of cord factors **(256)** and **(198)**. TDM **(256)** was tested with the secondary antibody IgG (Fc) and a first-group serum from the WHO with a dilution of 1:20. The absorbance was measured at 492 nm **(Figure 36)**. The analysis drawn from this experiment found a significant difference in response between positive serum and negative serum.

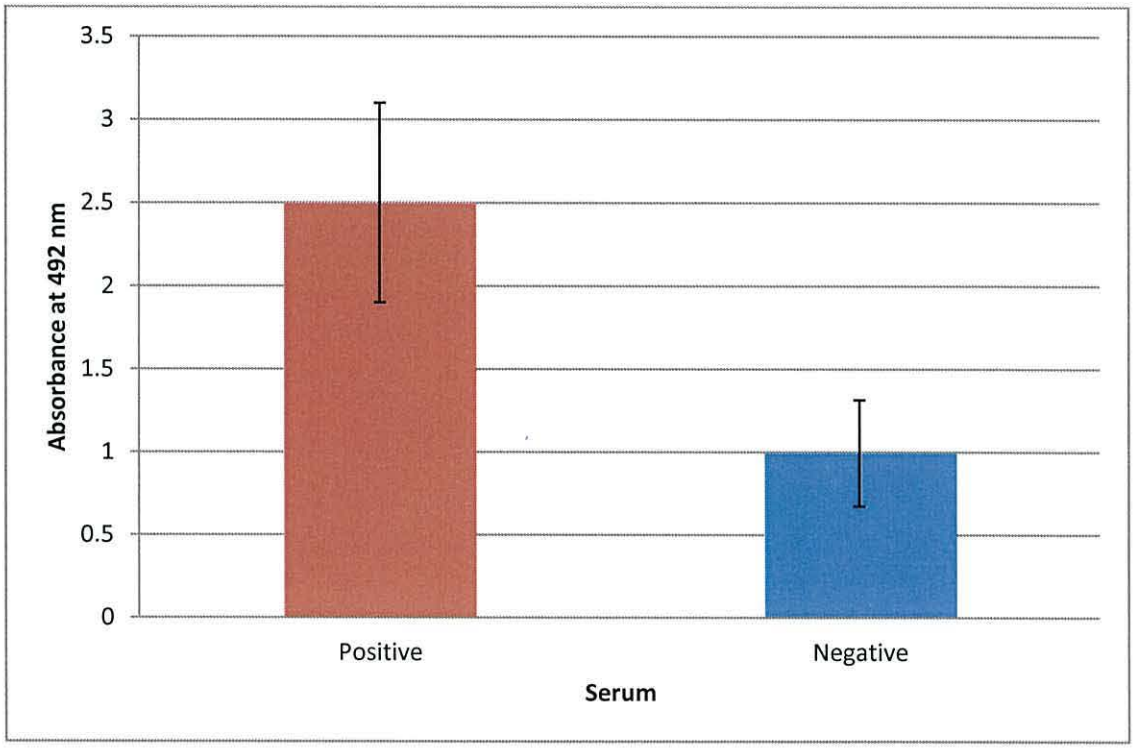


Figure36: The average absorbance of the serum samples (+) and (-) from first group against TDM (256)

The results obtained with the full list of the first group using this antigen can be seen in **Table 10.** There were seven false negatives and eight false positives from the total number of the first group of WHO samples. This made the sensitivity and specificity of this antigen equal to 73% and 89% respectively. These values were calculated by taking a cut-off absorbance value of 2. These values were lower than the values of TDM **(199)**. When looking at the individual samples the 4 positive samples (BA 1, KE 9, VI 2 and GA 3) that gave negative responses with this antigen also gave low values with the previous antigen TDM **(198)**. This is not the case with the other 3 positive samples (BA 4, KE 4 and PE 3) that gave negative responses with TDM **(256)** which were identified correctly with the previous antigens TDM **(198)**. This difference indicated that stereochemistry has an effect on the antigenic properties of mycolic acids.

Table 10: Absorbance values of first group sera (unblind) using synthetic antigen TDM (256). Red background represents TB+ sample, blue TB-, as diagnosed by WHO; pink boxes for results are TB+ using ELISA assay with a cut-off of 2

Serum(+)	TDM(257)	Serum(-)	TDM(257)	Serum(-)	TDM(257)	Serum(-)	TDM(257)
BA 1	0.61	BA 6	0.64	CO 9	1.57	CO 29	0.30
BA 2	3.14	BA 7	1.13	CO 10	0.56	GA 5	0.46
BA 3	2.79	BA 8	1.28	CO 11	0.36	GA 6	2.20
BA 4	1.40	SA 1	2.14	CO 12	0.54	GA 7	1.42
BA 5	2.65	SA 2	1.04	CO 13	0.26	GA 8	1.52
KE 1	2.61	SA 3	1.15	CO 14	1.59	GA 9	1.79
KE 2	2.35	SA 4	0.67	CO 15	2.04	GA 10	1.29
KE 3	2.55	SA 5	0.59	CO 16	2.87	GA 11	2.05
KE 4	1.84	BA 9	0.70	CO 17	0.41	GA 12	1.92
KE 5	2.78	BA 10	0.69	CO 18	0.76	BR 1	1.82
BA 19	3.16	BA 11	0.69	KE 6	0.50	CA 1	0.66
BA 20	2.78	BA 12	0.49	KE 7	1.39	CA 2	2.94
BA 21	3.77	BA 13	0.81	PE 1	0.29	CA 3	0.42
PE 3	1.43	BA 14	0.36	PE 2	1.19	CA 4	0.36
PE 4	3.12	BA 15	1.71	SA 7	0.38	CA 5	0.41
SA 6	4.46	BA 16	0.80	SA 8	0.64	SP 2	1.58
KE 8	3.67	BA 17	1.30	CO 19	0.24	SP 3	0.44
KE 9	1.23	BA 18	0.63	CO 20	0.40	SP 4	4.18
GA 1	2.98	CO 1	0.26	CO 21	0.28	SP 5	0.47
GA 2	3.51	CO 2	0.47	CO 22	0.77	SP 6	0.47
SP 1	3.30	CO 3	1.52	CO 23	1.27	SP 7	0.51
VI 1	2.94	CO 4	2.14	CO 24	0.36		
VI 2	0.88	CO 5	0.88	CO 25	0.90		
VI 3	3.25	CO 6	0.18	CO 26	0.17		
GA 3	0.64	CO 7	0.35	CO 27	1.48		
GA 4	3.51	CO 8	1.43	CO 28	0.51		

4.5 A comparison of TDM (198) and TMM (200)

TMM **(200)** consists of the same methoxy mycolic acid in TDM **(198)** the only difference being that while TMM has one copy of the methoxy mycolic acid moiety, TDM has two **(Figure 37).**

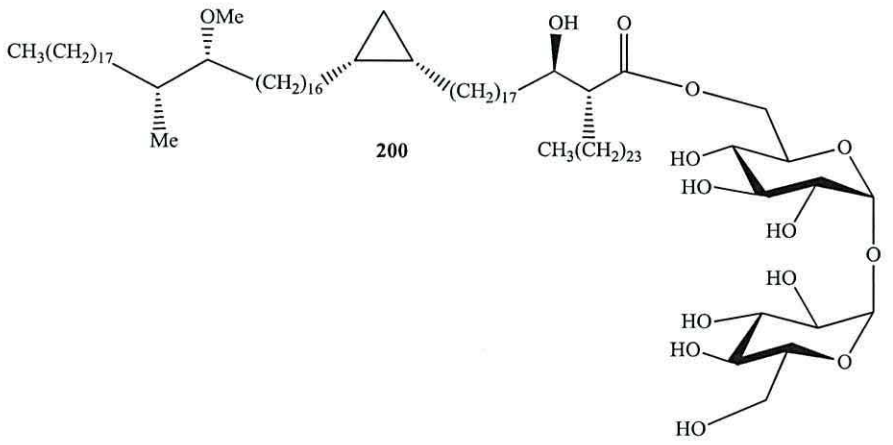


Figure 37: Structure of TMM (200)

An ELISA investigation to determine the significance of multiple mycolic acid copies in terms of antigenicity was carried out. The experiment was conducted under the same assay conditions as previously used with TDM **(198)**. As presented in **Figure 38**, TMM **(200)** clearly differentiated between TB^+ and TB^- sera.

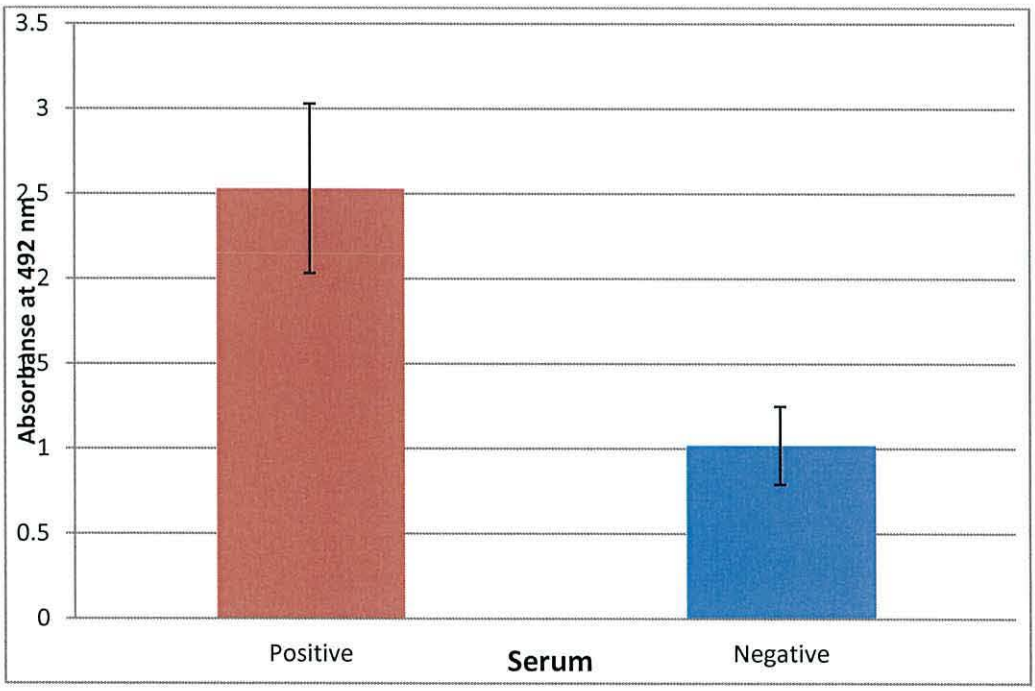


Figure38: The average absorbance of the serum samples (+) and (-) from first group against TMM (200)

A full list of the absorbance values obtained from TMM **(200)** is summarised in **Table 11**. The results showed nine false negative from true positive sera and eight false positives from the true negative sera. The sensitivity and specificity of TMM **(200)** were 67% and 88% respectively, by taking a cut-off absorbance value of 2. If the cut-

off was reduced to 1, the sensitivity which became to 85%, also the specificity was reduced to 67%. These results indicate that there is a clear difference between the antigenicity of TDM **(198)** and that of TMM **(200)**. This difference may be as a result of the presence of two molecules of mycolic acid in TDM, which showed a greater ability to bind with antibodies in the serum. This raises the possibility that anti-mycolic acid antibodies recognize the antigen more as a cluster rather than as individual molecules.

Table 11: Absorbance values of first group sera (unblind) using synthetic antigen TMM (200). Red background represents TB+ sample, blue TB-, as diagnosed by WHO; pink boxes for results are TB+ using ELISA assay with a cut-off of 2

Serum(+)	TMM(201)	Serum(-)	TMM(201)	Serum(-)	TMM(201)	Serum(-)	TMM(201)
BA 1	1.77	BA 6	1.08	CO 9	0.48	CO 29	0.54
BA 2	3.78	BA 7	1.50	CO 10	0.26	GA 5	0.93
BA 3	3.62	BA 8	1.91	CO 11	0.42	GA 6	0.80
BA 4	2.02	SA 1	1.24	CO 12	0.30	GA 7	0.87
BA 5	2.70	SA 2	1.21	CO 13	0.86	GA 8	2.28
KE 1	1.33	SA 3	0.55	CO 14	1.24	GA 9	1.39
KE 2	1.09	SA 4	0.42	CO 15	1.31	GA 10	1.07
KE 3	4.39	SA 5	3.67	CO 16	0.61	GA 11	0.60
KE 4	3.22	BA 9	0.64	CO 17	0.79	GA 12	1.06
KE 5	4.39	BA 10	0.59	CO 18	0.55	BR 1	2.38
BA 19	3.11	BA 11	1.12	KE 6	0.49	CA 1	3.55
BA 20	3.35	BA 12	0.87	KE 7	0.54	CA 2	0.14
BA 21	3.01	BA 13	0.53	PE 1	0.30	CA 3	0.13
PE 3	1.54	BA 14	1.00	PE 2	0.43	CA 4	0.32
PE 4	2.37	BA 15	1.15	SA 7	0.77	CA 5	1.08
SA 6	3.24	BA 16	0.46	SA 8	0.27	SP 2	0.29
KE 8	0.99	BA 17	0.40	CO 19	0.34	SP 3	3.16
KE 9	0.45	BA 18	0.65	CO 20	0.28	SP 4	0.30
GA 1	3.23	CO 1	0.47	CO 21	3.21	SP 5	0.41
GA 2	2.84	CO 2	1.59	CO 22	1.45	SP 6	0.48
SP 1	0.22	CO 3	2.53	CO 23	0.28	SP 7	0.61
VI 1	3.60	CO 4	0.96	CO 24	0.72		
VI 2	1.17	CO 5		CO 25	0.26		
VI 3	3.39	CO 6	0.41	CO 26	1.40		
GA 3	0.94	CO 7	3.59	CO 27	0.23		
GA 4	3.68	CO 8	0.78	CO 28	0.28		

4.6 Study of TDM (204) and TMM (206) as antigens

After the synthesis of the cord factor TDM **(204)** and TMM **(206)** of *cis*-keto mycolic acid **(Figure 39)** by the author, it was decided to test them as antigens for use in TB diagnosis.

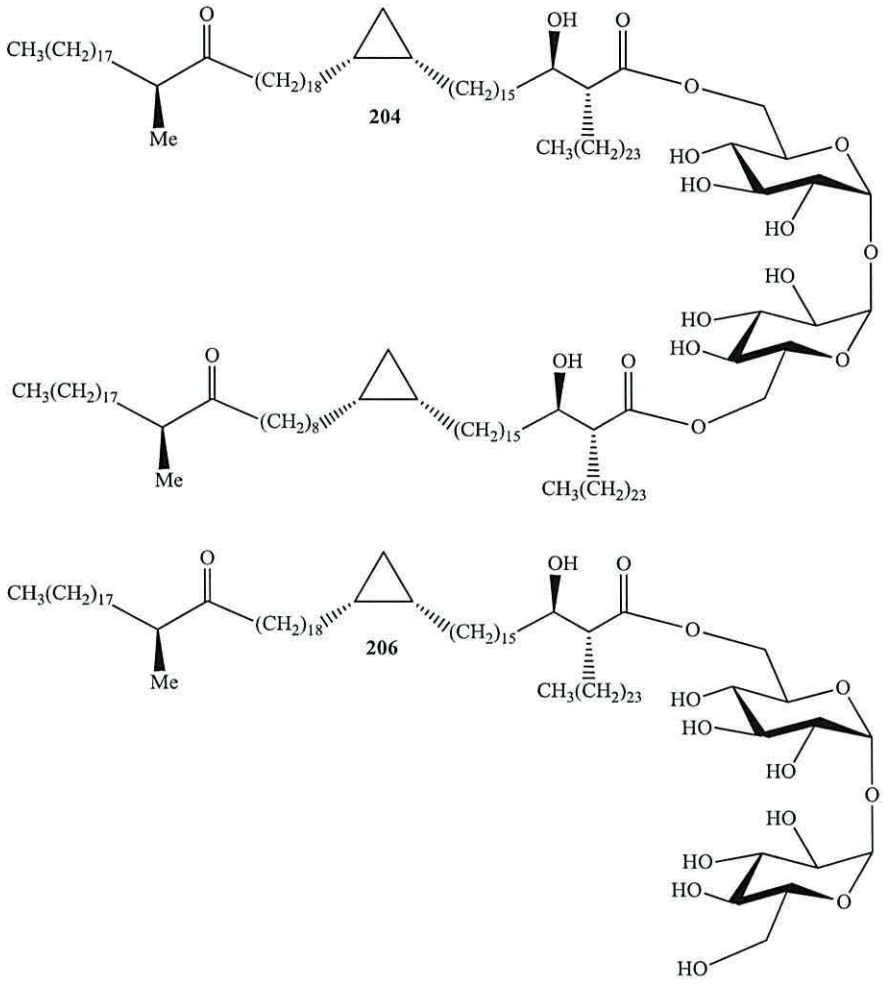


Figure 39: Structure TDM (204) and TMM (206) of ketomycolic acid

The initial testing of TDM **(204)** and TMM **(206)** was performed with a selected group, including 10 positive and 20 negative sera from the first group of WHO samples, in order to determine their ability to detect TB infection. They were tested via ELISA assay under the same wavelength (492 nm) and serum dilution (20:1) conditions, and IgG (Fc) was used for detection. The results of this experiment can be seen in **Figure 40**.

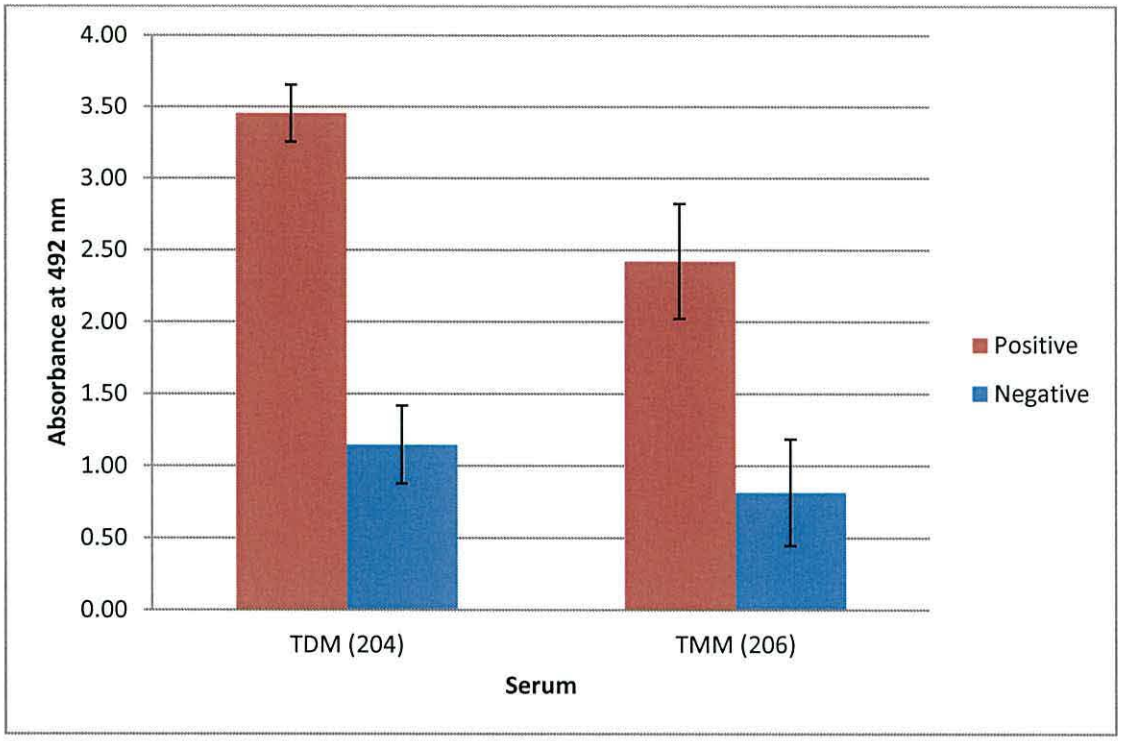


Figure 40: The average absorbance of some serum samples (+) and (-) from the first group against TDM (204) and TMM (206)

The results for TDM **(204)** and TMM **(206)** produced a clear distinction between (TB^+) and (TB^-). TDM **(204)** gave average absorbance values of 3.41 for the positive sera and 1.12 for the TB-negative samples. The sensitivity and specificity of TDM **(204)** were 100% and 90% respectively. From these data, it is clear that TDM **(204)** has a high level of ability to distinguish between TB+ and TB- patient serum. TMM **(206)** gave lower average absorbance values than TDM **(204)**: 1.12 and 0.72 for positive and negative sera respectively. TMM **(206)** has a higher degree of specificity (90%), but the sensitivity was low (70%). Also, this result suggests that TB antibodies bind more readily to TDM **(204)** than to TMM **(206)**. Thus, the results for TMM **(206)** can be rejected, and it cannot be accepted as a good antigen. This is because it was unable to detect the TB antibodies correctly.

4.7 Analysis of first-group sera against TDM (204)

From the results of the preliminary testing of TDM **(204)**, which showed good sensitivity and specificity values, it was decided to verify its action with all of the samples from the first group. An ELISA assay was carried out under the same conditions as were used in the above experiment. The full data are presented in **Table 12**. The correct positive and negative sera were calculated by using a cut-off

absorbance value of 2. The results of this experiment showed two false negatives out of 26 true positive sera, and six false positives out of 73 true negative sera. As a result, the sensitivity and specificity of TDM **(204)** were 92% and 84% respectively. In order to detect all positive sera (100% sensitivity) the cut-off was reduced to 1.46, but the specificity was reduced to 67%. Thus, the cut-off was set to be 2 with this antigen, which gave a good sensitivity and a good specificity value.

Table 12: Absorbance values of first group sera (unblind) using synthetic antigens TDM (204).Red background represents TB+ sample, blue TB-, as diagnosed by WHO; pink boxes for results are TB+ using ELISA assay with a cut-off of 2

Serum(+)	TDM(205)	Serum(+)	TDM(205)	Serum(+)	TDM(205)	Serum(+)	TDM(205)
BA 1	1.50	BA 6	1.66	CO 9	0.61	CO 29	1.05
BA 2	4.18	BA 7	0.77	CO 10	0.48	GA 5	0.67
BA 3	3.45	BA 8	2.50	CO 11	0.50	GA 6	0.59
BA 4	2.32	SA 1	1.11	CO 12	0.21	GA 7	0.29
BA 5	3.30	SA 2	0.77	CO 13	1.24	GA 8	2.97
KE 1	3.17	SA 3	0.64	CO 14	2.61	GA 9	0.89
KE 2	2.06	SA 4	0.32	CO 15		GA 10	1.02
KE 3	2.65	SA 5	0.34	CO 16	0.35	GA 11	1.57
KE 4	3.51	BA 9	0.83	CO 17	1.54	GA 12	2.13
KE 5	3.86	BA 10	0.70	CO 18	0.51	BR 1	0.06
BA 19	3.57	BA 11	1.54	KE 6	0.63	CA 1	3.63
BA 20	3.25	BA 12		KE 7		CA 2	0.17
BA 21	3.19	BA 13	1.04	PE 1	1.70	CA 3	0.16
PE 3	3.09	BA 14	1.69	PE 2	0.47	CA 4	0.33
PE 4	4.17	BA 15	0.63	SA 7	1.40	CA 5	0.58
SA 6	3.90	BA 16	0.50	SA 8	0.31	SP 2	0.15
KE 8	3.66	BA 17		CO 19	1.06	SP 3	3.46
KE 9	3.00	BA 18	0.30	CO 20	0.71	SP 4	0.28
GA 1	2.99	CO 1	0.48	CO 21	2.24	SP 5	0.28
GA 2	3.30	CO 2	0.57	CO 22	4.40	SP 6	0.28
SP 1	3.75	CO 3	2.35	CO 23	0.64	SP 7	0.64
VI 1	3.72	CO 4	0.70	CO 24	1.81		
VI 2	1.47	CO 5	0.14	CO 25	0.30		
VI 3	3.00	CO 6	0.26	CO 26	0.10		
GA 3	2.95	CO 7	2.87	CO 27	0.65		
GA 4	3.66	CO 8	1.06	CO 28	0.69		

The data were further analysed using an individual value plots graph **(Figure 41)**. Most of the positive sera (1) were found to be above the cut-off absorbance value of 2, while most of the negative sera (0) were clustered below the cut-off value. Two false positives and ten false negatives were obtained from the experiment. These results

showed that TDM **(204)** has a higher accuracy than the other synthetic cord factors. This indicated that the functional groups in cord factor mycolic acids had a significant effect on the result of the ELISA assay. Hence, TDM **(204)** represented the best of our synthetic cord factor antigens for the detection of TB infection in human serum via ELISA.

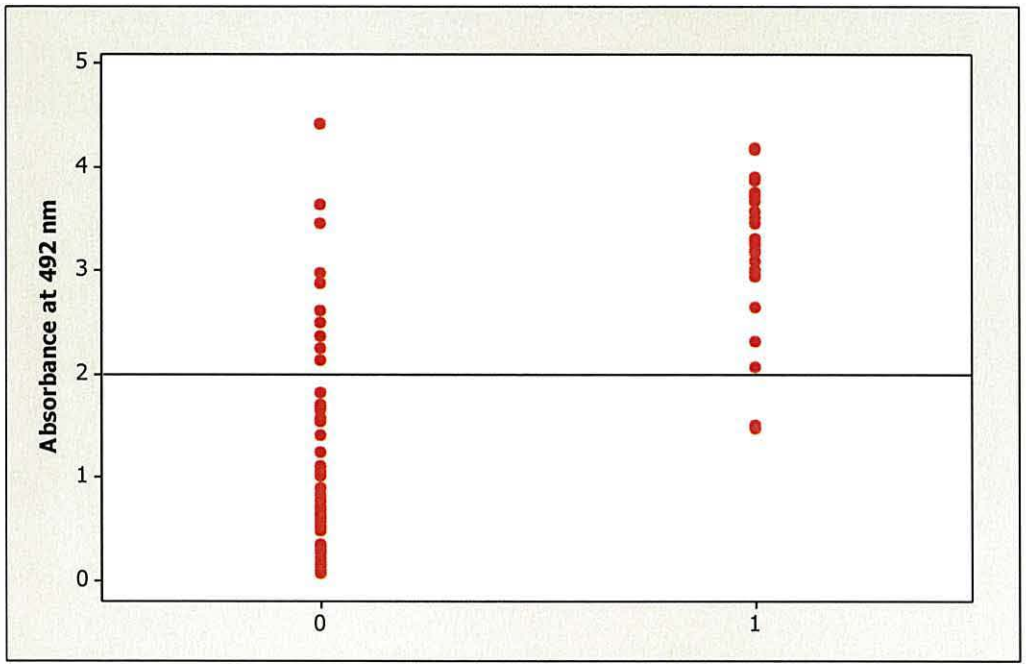
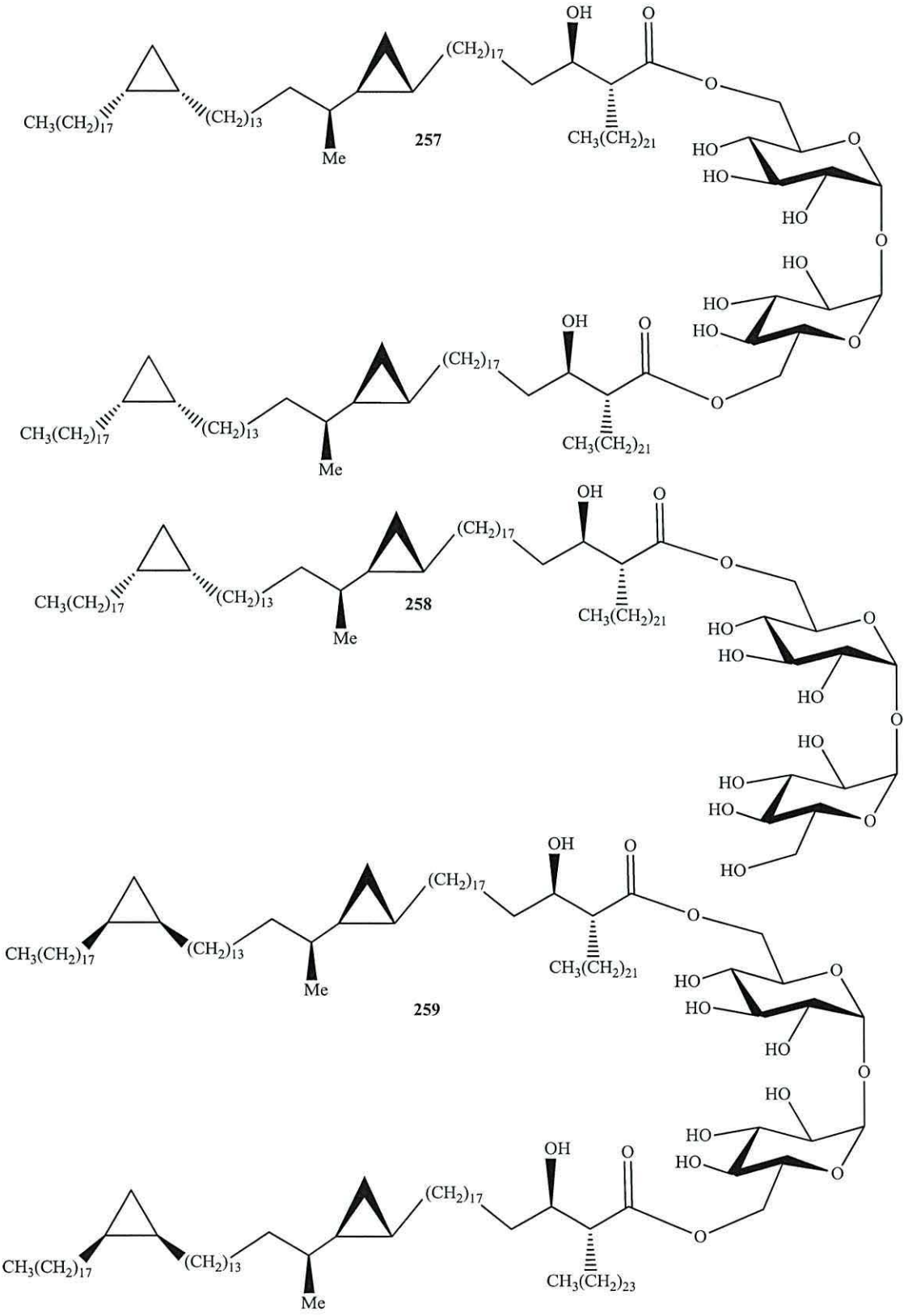
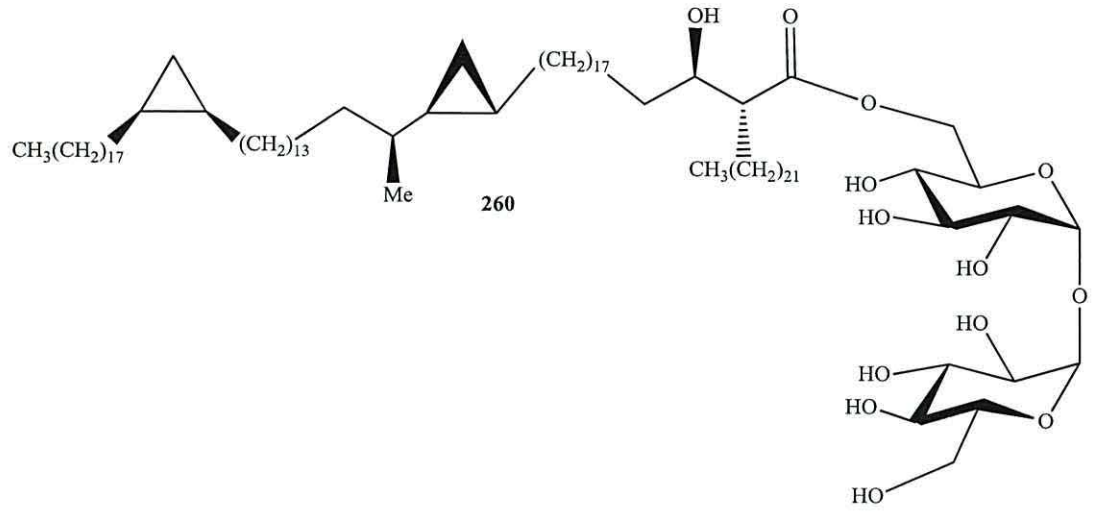


Figure 41: Individual value plots of TDM (204) as an antigen. (TB- = 0, TB+ = 1)

4.8 The study of cord factors in other Mycobacterium species as antigens

M. kansasii and *M. avium* complex are species of non *Mycobacterium tuberculosis* commonly found in the human physical environment. Often, *M. kansasii* contains *trans* methyl α-mycolic acid as a major type of mycolic acid.[90,91] M. M. Sirhin (a Bangor PhD student) prepared different cord factors of *trans* methyl α-mycolic acid such as TDM **(257)**, TMM **(258)** TDM **(259)**, and TMM **(260)** which differ in terms of the stereochemistry of the mycolic acid **(Figure 42)**.[214]


OH
O
(CH2)17
CH3(CH2)17
(CH2)13
Me
CH3(CH2)21
HO
O
257
258
259



260

Figure 42: Synthetic cord factors from other mycobactria

These compounds were tested with some selected positive sera and negative sera samples from the first group. The emergent results showed a clear signal against TB+ sera for TDM **(257)** and TMM **(258)**. In contrast, TDM **(259)** and TMM **(260)** produced only a low signal **(Figure 43)**. This may be due to the stereochemistry of the mycolic acid. The sensitivity values of these compounds were low. The specificity values were good **(Table 13)**. This suggests that these compounds did not have the ability to properly bind to the TB antibodies in the patient serum correctly. These compounds may have the ability to detect special antibodies related to *M. kansasii* and *M. avium* complex. Therefore, it was also decided to exclude these compounds as antigens for the diagnosis of TB.

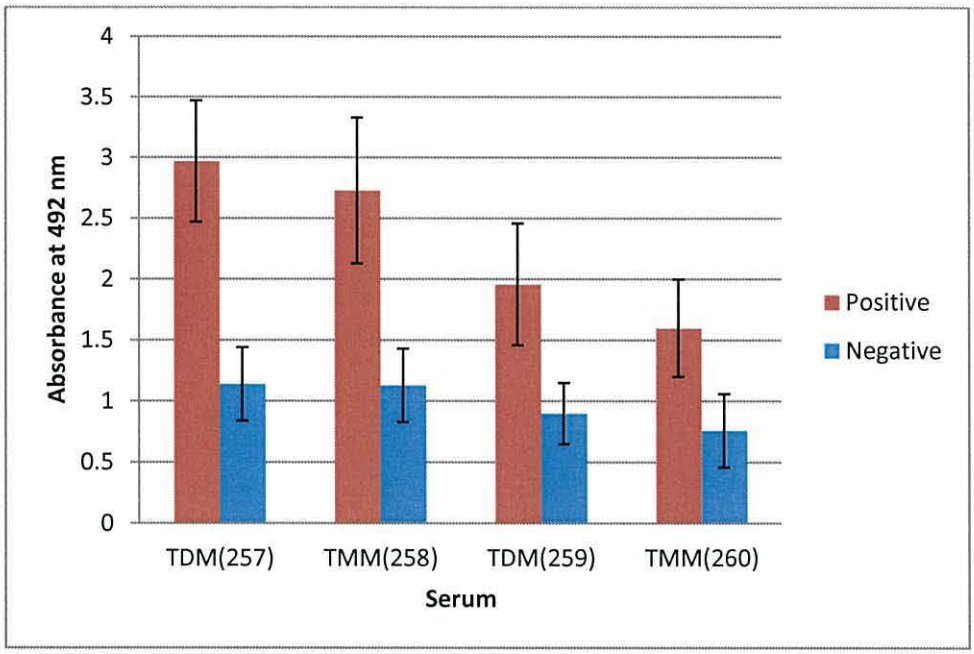


Figure 43: The average absorbance of the serum samples (+) and (-) from the first group against TDM (257), TMM (258), TDM (259) and TMM (260)

Table 13: The data analysis of the ELISA results when using TDM (257), TMM (258), TDM (259) and TMM (260) as antigens

various antigens	Average absorbance positive	Average absorbance negative	Sensitivity (%)	Specificity (%)
TDM (257)	2.8	1.22	68	82
TMM (258)	2.73	1.23	68	82
TDM (259)	1.96	0.9	54	85
TMM (260)	1.6	0.76	31	85

4.9 Study of glucose monomycolate as antigens.

In addition to cord factors, there are other derivatives of mycolic acid, such as glucose monomycolate (GMM).[225] The structure of GMM is simple; it is a free glucose that is esterified at the 6-position to a mycolic acid. Some types of these compounds, such as GMM **(261)** for *trans*-α-mycolic acid, GMM **(262)** for methoxy mycolic acid and GMM for epimerised *trans*-ketomycolic acid **(263)** **(Figure 44)**, were prepared by M. M. Saheb (a Bangor PhD student).[226]

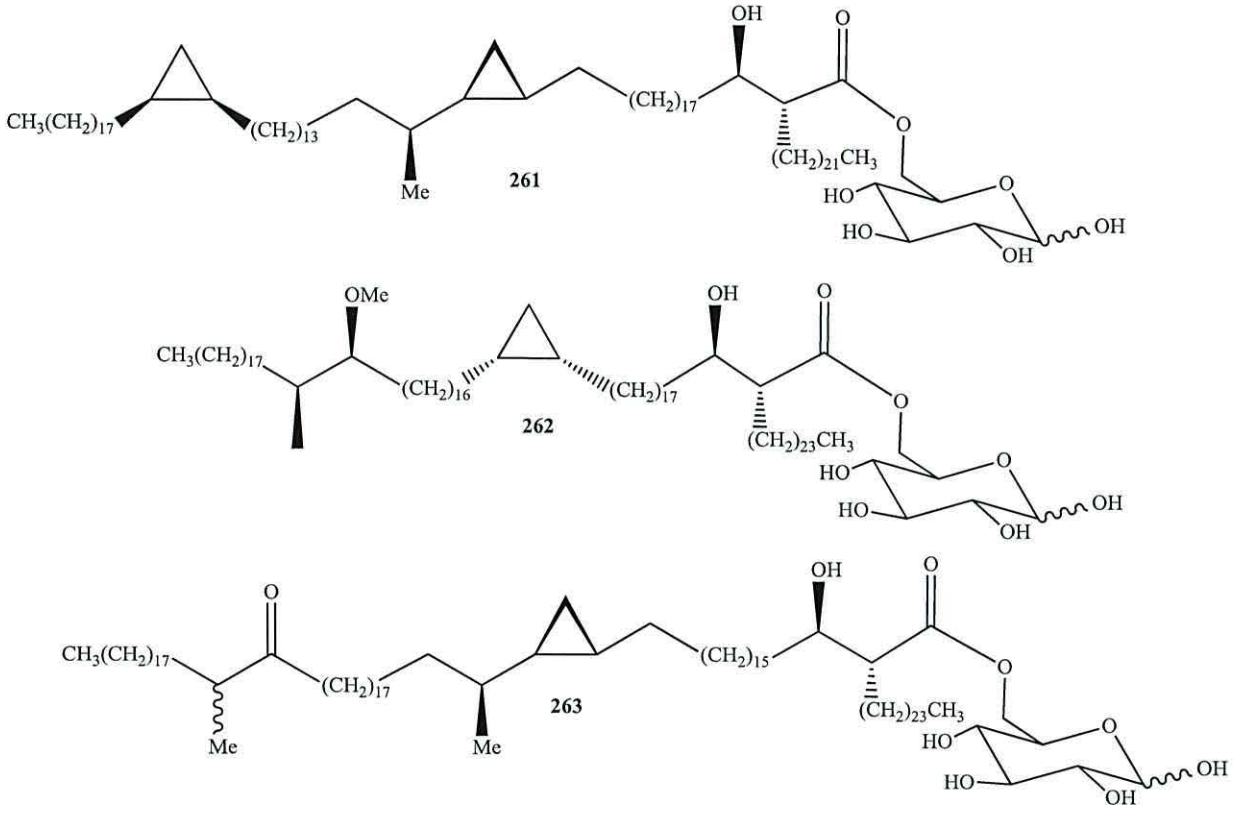


Figure 44: structure of synthtic glucose monomycolate types (GMM)

It was decided to estimate the ability of these compounds to detect TB-antibodies in human serum. They were tested with selected sera (positive and negative) from the first group. The testing of these compounds was conducted employing the same conditions as were used for the testing of TDM **(198)**. **Figure 45** presents the results of the testing of these compounds. These results clearly indicate that there are positive sera (+), but they produce low absorbance in comparison with the response of the TDM cord factors. Evidently, there is a small, but significant, difference between

positive and negative sera, especially with the compounds GMM **(262)** and GMM **(263)**. In addition, the findings showed that all types of glucose monomycolate indicated low sensitivity and specificity. These results imply that glucose monomycolate types may not be very useful in the serodiagnosis of TB infection

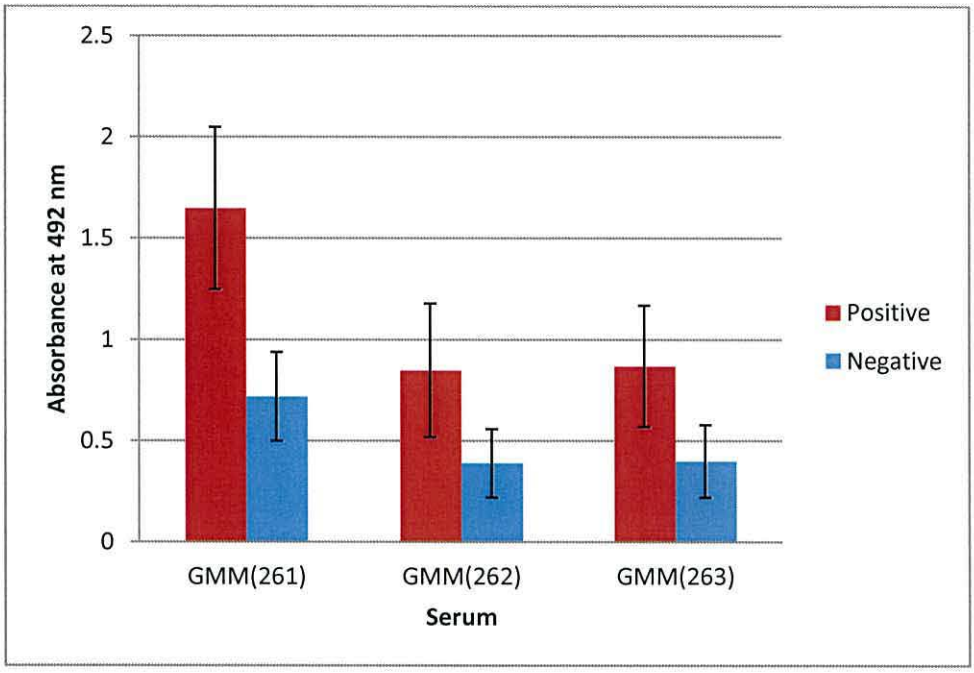


Figure 45: The average absorbance of the serum samples (+) and (-) from group 1 against various types of glucose monomycolate

4.10 Study of methyl arabinosyl mycolate as antigen

Methyl arabinosyl mycolate consists of mycolic acid and one or more of the methyl arabinosyl sugars that can be found in the cell walls of mycobacteria.[227] M. O. Mohammed (a Bangor PhD student) synthesised different types of mono methyl arabinosyl mycolate, such as methyl arabinosyl for methoxymycolic acids **(264, 265, 266)** methyl arabinosyl for epimerised *trans* and *cis*-ketomycolic acids **(267, 268)** and methyl arabinosyl for α-mycolic acid **(269)** **(Figure 46)**.[228]

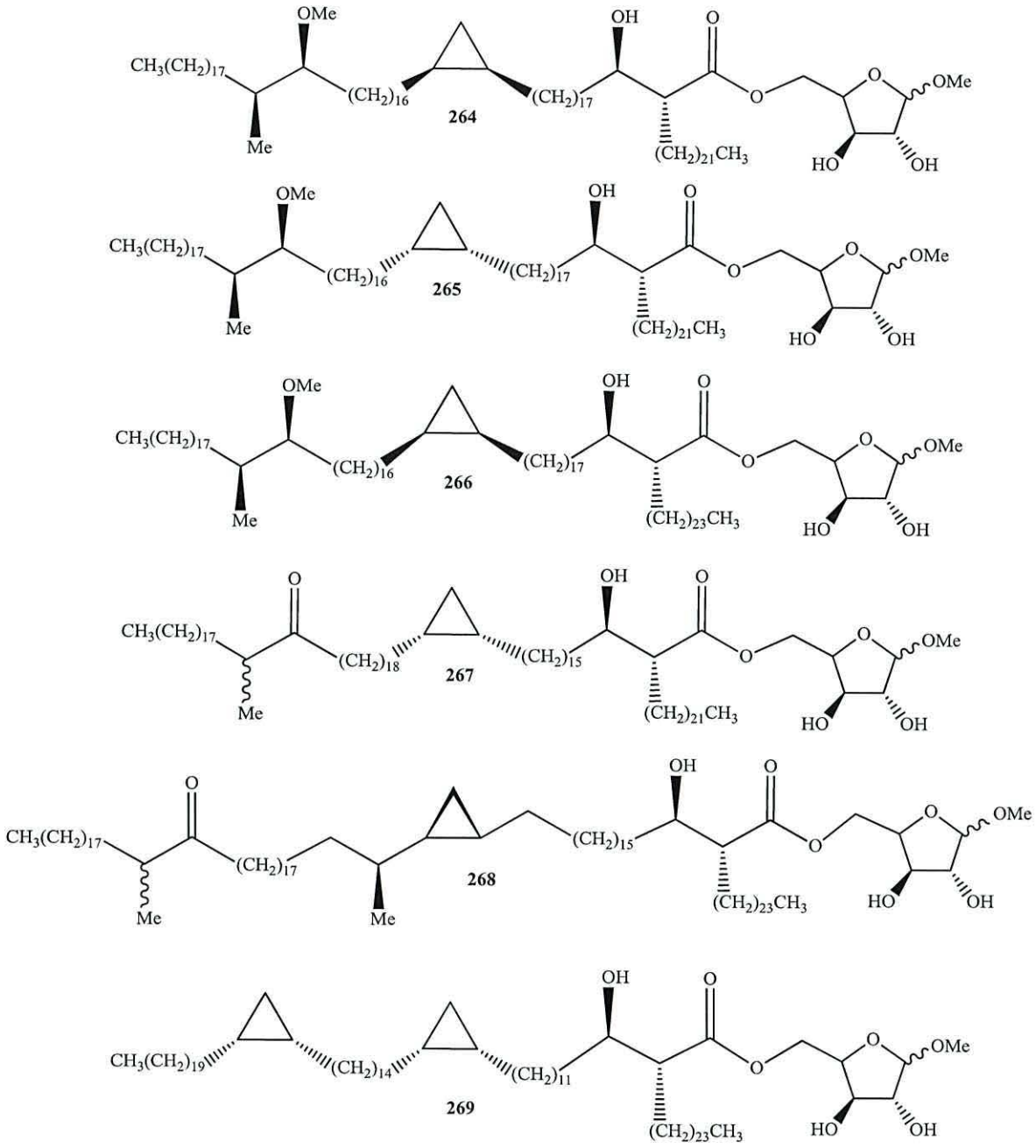


Figure 46: structure of synthetic methyl arabinosyl monomycolate types

These compounds were used in an ELISA assay with some sera (positive and negative) of the first group in order to investigate their abilities as antigens capable of binding to TB-antibodies in sera. The test experiments were carried out under the same serum dilution, secondary antibody and wavelength conditions as before. The results obtained from these antigens are illustrated in **Figure 47**. As shown in **Figure 47**, the methyl arabinosyl mycolates did not give significantly different ELISA profiles for TB^+ and

TB^- sera. The sensitivities and specificities were variable and very low. Thus, these molecules can be considered unsuitable as antigens for TB diagnosis.

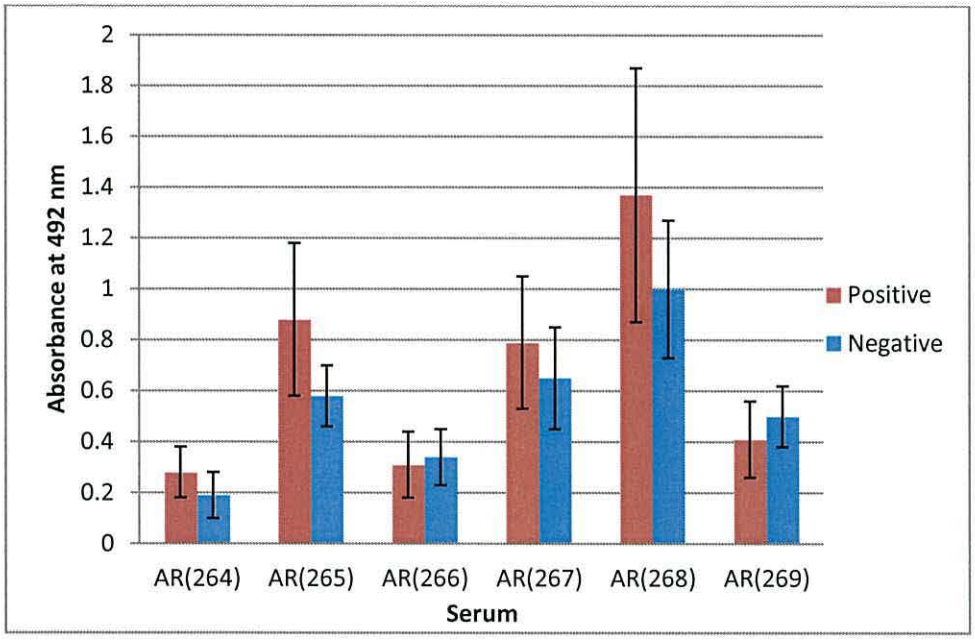


Figure 47: The average absorbance of the serum samples (+) and (-) from group 1 against different types of methyl arabinosyls mycolate

4.11 The study of free mycolic acid as an antigen

The synthetic free mycolic acids were also investigated as antigens for distinguishing between TB^+ and TB^- sera. The synthesis of free mycolic acid is a shorter process than that of cord factor, and Baird's group has synthesised many types of free mycolic acids from various types of Mycobacteria. Therefore, in this ELISA assay, several types of synthetic free mycolic acids were used that are different in terms of stereochemistry, such as α-mycolic acids (**(98)**, **(99)** and **(100)**),[175,176,177,178] epoxy mycolic acids (**(106)** and **(107)**)[180] and ketomycolic acid **(165)** **(Figure 48)**.

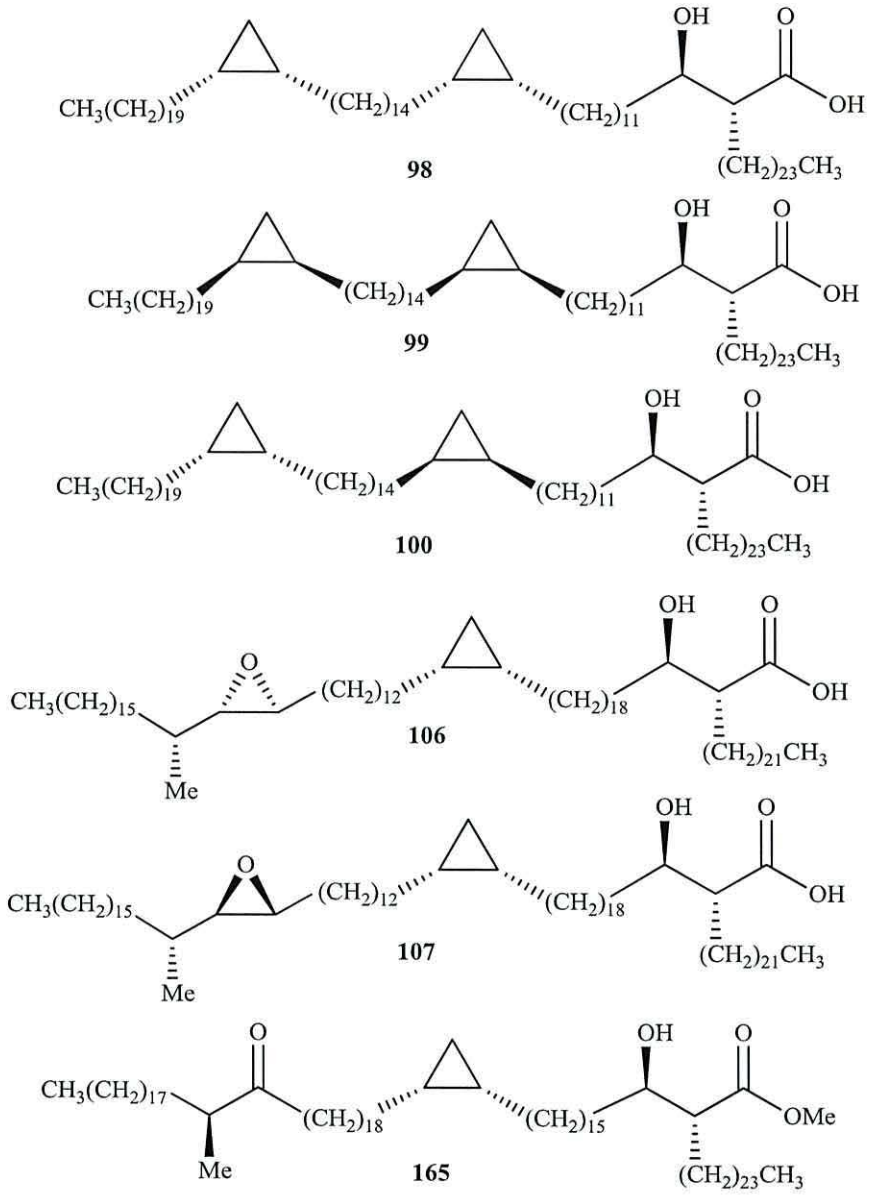


Figure 48: Structure of synthetic mycolic acids as antigens

Most of these molecules generated low absorbance in ELISA assays, and it was difficult to differentiate between TB^+ and TB^- sera **(Figure 49)**. The resulting absorbance values are much lower than those obtained by using cord factors for the same serum sample. These results suggest that as an antigen, free mycolic acid may not be very useful in detecting TB antibodies in serum.

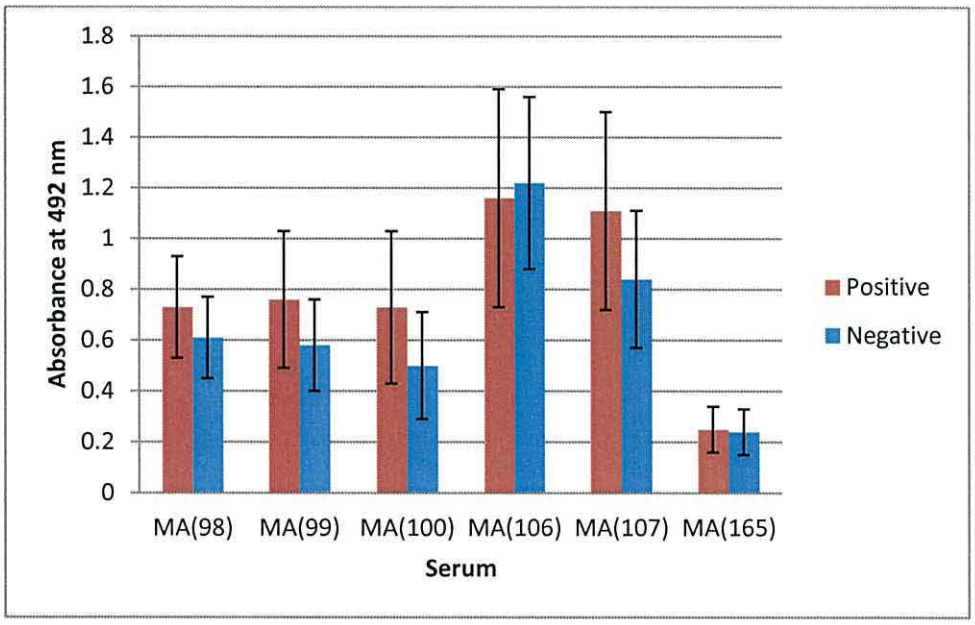


Figure 49: The average absorbance of the serum samples (+) and (-) from group 1 against various types of free mycolic acids

4.12 The study of Bangor samples

It was decided to analyse Bangor samples, which are believed to be uninfected, by using TDM **(198)**, TDM **(204)** and TDM **(254)** as good synthetic antigens. Bangor samples were collected from people who lived in different area (Bangor Urban (BU), Bangor Rural (BR), Bangor Framers (BF) and Bangor Abattoir (BA)). These experiments were carried out under the same conditions described above, in order to confirm the accuracy of these antigens with different sera. **Figure 50** shows the low absorbance with all sera group, which indicated that they were TB negative sera, compared to the average absorbance of the TDM **(198)**, TDM **(204)** and TDM **(254)** respectively for the TB+ samples of the WHO, and for the TB- samples. This further confirmed that these antigens could be recognized by anti-mycolic acid antibodies present in TB positive sera.

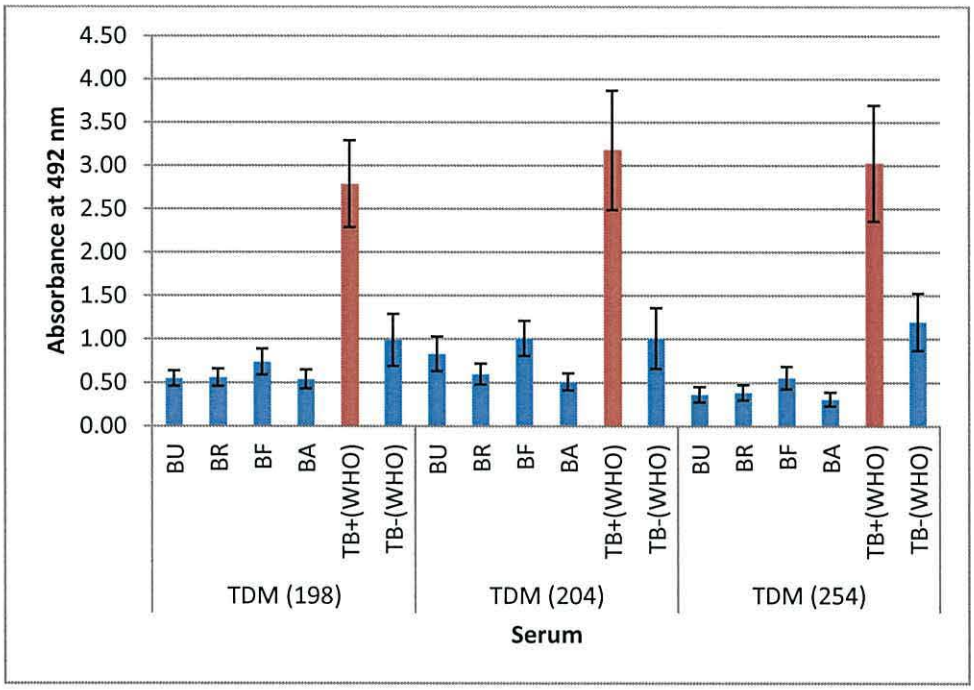


Figure 50: The Response of TDM (198), TDM (204) and TDM (254) as antigens against different sera groups (Bangor samples)

4.13 Analysis of TDM (198), TDM (204) and TDM (254) with blind sera

From the preceding experiments, it was shown that TDM cord factor is better than other synthetic mycolic acid antigens. TDM **(198)**, TDM **(204)** and TDM **(254)** showed high absorbances for positive sera as compared with those for negative sera. Also, these compounds had good sensitivity and specificity values. It was decided to use these compounds as antigens during ELISA with blind sera in order to verify the sensitivity and specificity values of these antigens in a larger serum sample group. The experiments were carried out under the same conditions as were used before. All of these serum samples were tested blindly, and the following analysis was performed after the unblinding of the samples. The results of these experiments are presented in **Figure 51** and **Table 14.** As shown in **Figure 51**, there is a clear distinction between positive sera (TB^+) and negative sera (TB^-). The results show TDM **(198)** to have 15 false negatives and 22 false positives. There were 19 false negatives and 17 false positives for TDM **(204)**, and there were 15 false negatives and 27 false positives for TDM **(254)** out of the total positive and negative sera samples. The sensitivity and specificity values for the synthetic antigens used are shown in **Table 15**.

Table 14: Absorbance values of second group sera (blind) using synthetic antigens TDM (198), TDM (204) and TDM (254). Red background represents TB+ sample, blue TB-, as diagnosed by WHO; pink boxes for results are TB+ using ELISA assay with a cut-off of 2

Serum	TDM(199)	TDM(205)	TDM(255)	Serum	TDM(199)	TDM(205)	TDM(255)
BA 22	3.31	3.47	3.29	CO 83	0.30	0.20	0.31
BA 23	0.82	1.24	1.18	CO 84	3.05	2.34	2.92
BA 24	3.05	3.00	2.89	GA 13	0.49	0.39	0.71
BA 25	2.04	2.58	2.23	GA 14	0.80	0.71	1.01
BA 26	3.78	3.77	3.67	GA 15	4.42	4.06	4.41
BA 27	4.03	4.05	3.96	GA 16	0.90	0.64	0.92
BA 28	3.12	3.60	3.61	GA 17	1.06	1.25	1.61
BA 29	3.11	3.34	3.36	GA 18	0.67	0.60	0.97
BA 30	3.99	3.93	4.06	GA 19	0.34	0.21	0.37
BA 31	3.78	4.25	4.09	GA 21	1.66	1.54	1.56
BA 32	1.05	1.21	1.83	GA 23	0.46	0.36	0.59
BA 33	2.00	1.99	2.85	GA 29	1.33	1.17	2.07
BA 34	3.82	4.39	4.09	GA 30	0.97	0.51	0.87
PE 6	1.30	3.36	2.25	GA 31	0.80	0.55	0.90
PE 7	3.56	4.15	3.89	GA 32	2.66	2.53	2.79
PE 8	3.50	4.31	3.44	GA 33	0.52	0.33	0.59
PE 9	3.20	1.26	1.52	GA 34	3.23	1.66	3.40
PE 10	3.43	4.40	4.39	GA 35	0.53	0.19	0.58
PE 11	0.31	0.30	0.49	GA 36	1.67	0.62	1.60
PE 12	3.68	4.15	3.57	GA 37	1.32	0.55	1.00
PE 13	0.77	0.48	0.55	GA 38	0.54	0.30	0.61
PE 14	3.65	4.40	4.38	GA 39	0.48	0.31	0.63
PE 15	0.85	1.16	1.54	GA 40	0.58	0.36	0.62
PE 16	3.68	3.56	3.84	GA 41	0.74	0.45	0.98
PE 17	3.10	3.80	3.55	GA 42	2.49	1.29	2.55
SA 12	3.64	3.45	3.73	GA 43	0.62	0.52	0.72
SA 13	2.85	2.27	2.83	GA 44	1.25	0.46	1.15
SA 14	4.37	3.92	4.38	GA 45	0.54	0.39	0.63
SA 15	4.00	2.30	1.61	GA 46	0.30	0.25	0.37
SA 16	4.22	3.80	4.40	GA 47	1.24	1.32	1.58
SA 17	0.48	0.34	0.57	GA 48	0.73	0.81	0.93
SA 18	4.04	1.42	4.06	GA 49	0.32	0.27	0.35
SA 19	2.24	2.12	2.90	GA 50	0.50	0.41	0.59
SA 20	2.14	1.44	2.15	GA 51	0.33	0.23	0.49
SA 22	3.65	2.07	1.70	GA 52	0.54	0.50	0.73
SA 23	1.26	1.04	1.36	GA 53	3.32	2.34	3.03
SA 24	3.77	4.08	4.25	GA 54	1.26	0.74	1.09
CO 39	3.05	3.25	3.49	GA 55	0.65	0.32	0.62
CO 44	3.26	3.37	3.28	GA 56	0.70	0.55	0.86
CO 45	3.21	3.35	3.53	GA 57	1.34	0.92	1.65
CO 51	2.05	1.87	2.13	GA 58	0.55	0.49	0.99
CO 53	3.47	3.74	3.82	GA 59	1.46	2.37	2.17
CO 55	4.07	4.27	4.12	GA 60	2.00	1.66	2.86
CO 56	0.86	0.49	1.05	GA 61	0.88	0.54	1.24
CO 58	3.67	4.03	4.01	GA 62	1.15	0.87	1.52
CO 85	3.06	4.08	4.10	GA 63	2.79	2.11	3.09
KE 10	3.13	3.34	4.24	GA 64	0.63	0.39	1.23
KE 11	2.94	3.22	3.90	GA 65	0.90	0.65	1.22
KE 12	3.88	3.78	3.66	GA 66	1.27	1.39	1.84

KE 13	3.60	3.49	3.04	GA 67	0.42	0.41	0.64
KE 14	2.99	3.12	3.39	GA 68	1.43	1.11	2.29
KE 15	3.30	3.02	3.09	GA 69	1.34	1.16	2.20
KE 16	3.99	2.55	3.93	GA 70	0.77	0.63	1.18
KE 17	2.57	1.41	2.69	GA 71	2.13	1.64	3.14
GA 20	4.42	4.41	4.00	GA 72	0.61	0.48	0.74
GA 22	3.71	3.92	4.40	GA 73	3.81	4.32	3.94
GA 24	2.86	3.31	4.00	BR 2	2.76	3.09	3.88
GA 25	4.03	4.22	3.50	BR 3	2.48	2.87	3.29
GA 26	3.00	3.55	4.04	BR 4	1.03	0.90	1.27
GA 27	2.47	3.88	3.86	BR 5	0.71	0.63	1.13
GA 28	1.15	1.31	1.45	CA 6	1.72	2.03	3.27
SP 10	3.01	3.00	2.98	CA 7	0.32	0.23	0.26
SP 13	4.27	3.80	3.41	CA 8	0.63	0.53	0.70
SP 40	0.59	0.55	0.69	CA 9	0.35	0.24	0.35
VI 4	3.72	4.10	3.98	CA 10	2.30	2.48	2.85
VI 5	3.35	4.25	3.63	CA 11	0.32	0.14	0.29
VI 10	4.04	4.38	4.40	CA 12	1.09	0.87	1.00
VI 11	4.00	4.00	4.00	CA 13	0.29	0.24	0.34
VI 12	3.40	4.27	3.93	CA 14	0.34	0.25	0.36
VI 13	1.29	1.56	2.39	CA 15	0.44	0.37	0.45
VI 14	0.71	0.69	1.20	CA 16	0.26	0.27	0.37
VI 15	1.94	3.50	4.26	CA 17	0.33	0.32	0.50
VI 16	3.76	4.00	4.09	CA 18	0.25	0.23	0.30
VI 17	0.54	0.45	0.67	CA 19	0.38	0.40	0.65
VI 18	2.94	3.43	3.57	CA 20	3.01	3.13	4.26
PE 18	1.82	1.86	2.21	CA 21	2.28	2.26	3.75
SA 9	0.64	0.38	0.75	CA 22	0.58	0.51	1.34
SA 10	0.79	0.63	0.66	CA 23	0.61	0.71	0.99
SA 11	0.55	0.43	0.72	CA 24	0.25	0.22	0.27
SA 21	1.14	0.60	0.93	CA 25	0.37	0.23	0.42
CO 30	0.83	1.01	0.87	CA 26	0.28	0.32	0.37
CO 31	0.81	0.67	0.76	CA 27	0.53	0.64	0.51
CO 32	4.00	4.07	4.38	CA 28	0.28	0.15	0.35
CO 33	0.53	0.49	0.67	CA 29	0.43	1.30	0.96
CO 34	0.35	0.21	0.36	CA 30	0.23	0.14	0.33
CO 35	1.36	1.30	1.99	CA 31	3.59	3.41	3.85
CO 36	0.31	0.20	0.34	CA 32	0.73	0.57	0.79
CO 37	0.37	0.28	0.49	CA 33	0.77	0.66	0.73
CO 38	0.98	0.77	1.30	CA 34	0.45	0.65	0.53
CO 40	0.50	0.27	0.35	SP 8	0.56	0.42	0.70
CO 41	1.27	0.51	1.30	SP 9	0.70	0.43	0.83
CO 42	0.57	0.42	0.83	SP 11	0.59	0.53	0.75
CO 43	3.20	3.31	3.74	SP 12	0.15	0.13	0.21
CO 46	0.60	0.46	0.78	SP 14	0.68	0.44	0.84
CO 47	1.50	1.01	1.48	SP 15	0.95	0.85	1.06
CO 48	0.38	0.19	0.29	SP 16	0.81	0.43	0.97
CO 49	0.57	0.20	0.37	SP 17	0.61	0.47	0.92
CO 50	0.28	0.14	0.27	SP 18	0.70	0.48	0.94
CO 52	0.57	0.30	0.28	SP 19	0.42	0.41	0.42
CO 54	0.42	0.21	0.33	SP 20	0.34	0.34	0.38
CO 57	1.43	0.45	0.76	SP 21	0.61	0.51	0.83
CO 59	0.21	0.15	0.26	SP 22	1.30	0.99	1.62
CO 60	0.40	0.39	0.53	SP 23	0.33	0.30	0.36
CO 61	0.24	0.23	0.31	SP 24	0.30	0.25	0.38

CO 62	0.48	0.28	0.47	SP 25	0.37	0.38	0.56
CO 63	0.52	0.49	0.68	SP 26	2.45	1.72	2.82
CO 64	0.46	0.38	0.62	SP 27	1.57	1.06	1.78
CO 65	0.20	0.21	0.23	SP 28	0.29	0.21	0.37
CO 66	0.88	0.52	0.19	SP 29	0.51	0.51	0.80
CO 67	0.35	0.27	0.95	SP 30	0.50	0.61	0.65
CO 68	0.59	0.48	0.80	SP 31	1.58	1.18	2.08
CO 69	4.40	4.07	4.18	SP 32	0.46	0.39	0.59
CO 70	0.56	0.62	0.78	SP 33	0.74	0.59	0.93
CO 71	0.27	0.27	0.40	SP 34	0.71	0.68	0.86
CO 72	0.87	0.58	1.00	SP 35	0.73	0.56	1.14
CO 73	1.77	0.93	1.77	SP 36	0.57	0.47	1.04
CO 74	1.23	0.37	0.63	SP 37	0.40	0.31	0.52
CO 75	0.35	0.37	0.49	SP 38	0.75	0.77	1.02
CO 76	1.25	0.89	1.46	SP 39	0.47	0.35	0.52
CO 77	0.23	0.22	0.27	VI 6	3.16	2.64	4.26
CO 78	0.92	0.78	1.09	VI 7	1.15	1.00	1.99
CO 79	1.95	1.01	1.78	VI 8	0.62	0.63	0.90
CO 80	0.70	0.61	1.04	VI 9	1.50	1.52	2.13
CO 81	0.92	0.53	0.88	VI 19	2.77	3.09	3.73
CO 82	0.21	0.17	0.20				

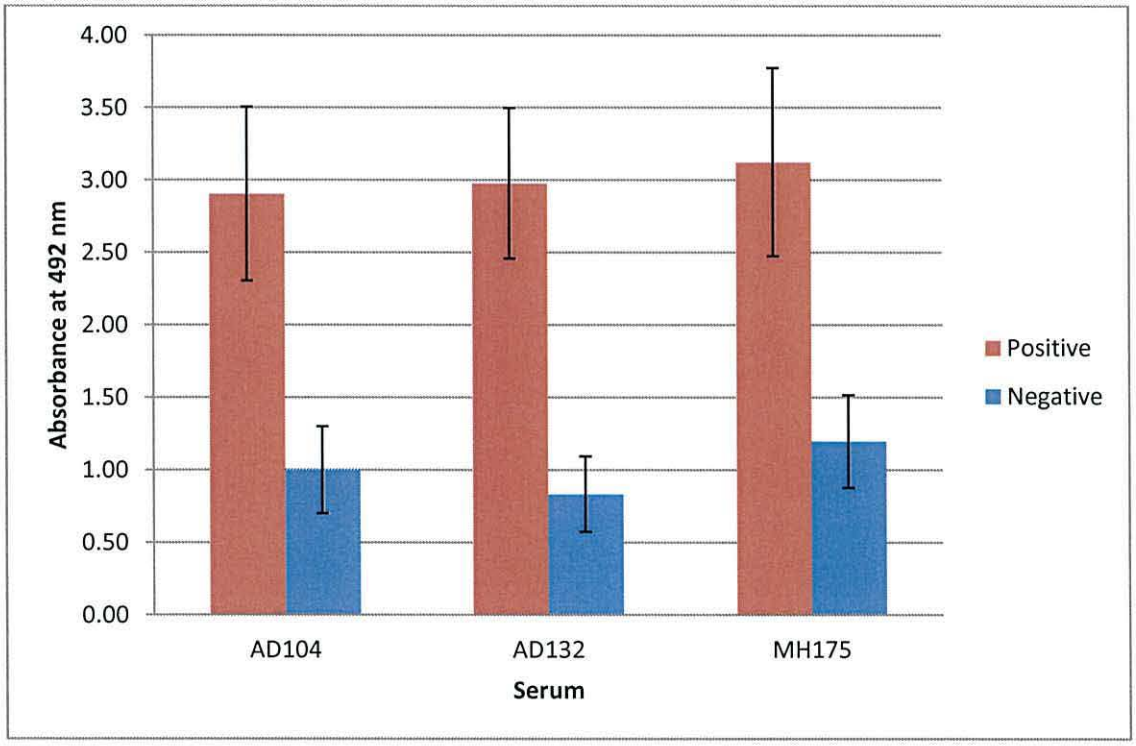


Figure 51: ***The average absorbance of the serum samples (+) and (-) from blind sera against TDM (198), TDM (204) and TDM (254)***

Table 15: The data analysis of ELISA assay using TDM (198), TDM (204) and TDM (254) as antigens against 249 sera samples

antigens	Average absorbance positive	Average absorbance negative	Sensitivity (%)	Specificity (%)
TDM **(198)**	2.90	1.00	80	87
TDM **(204)**	2.98	0.83	75	90
TDM **(254)**	3.13	1.20	80	84

In order to perform additional analysis on the results, an individual value plots graph was used to confirm the results. It showed that most positive sera (1) were found above the cut-off value, which was equal to the number two, with a few false negatives. On other hand, most negative sera (0) were found under or at the number two, with few false positives for the three antigens and some differences among them **(Figure 52)**. Finally, TDM **(198)**, TDM **(204)** and TDM **(254)** showed good results, suggesting that they could be used as antigens for the diagnosis of TB

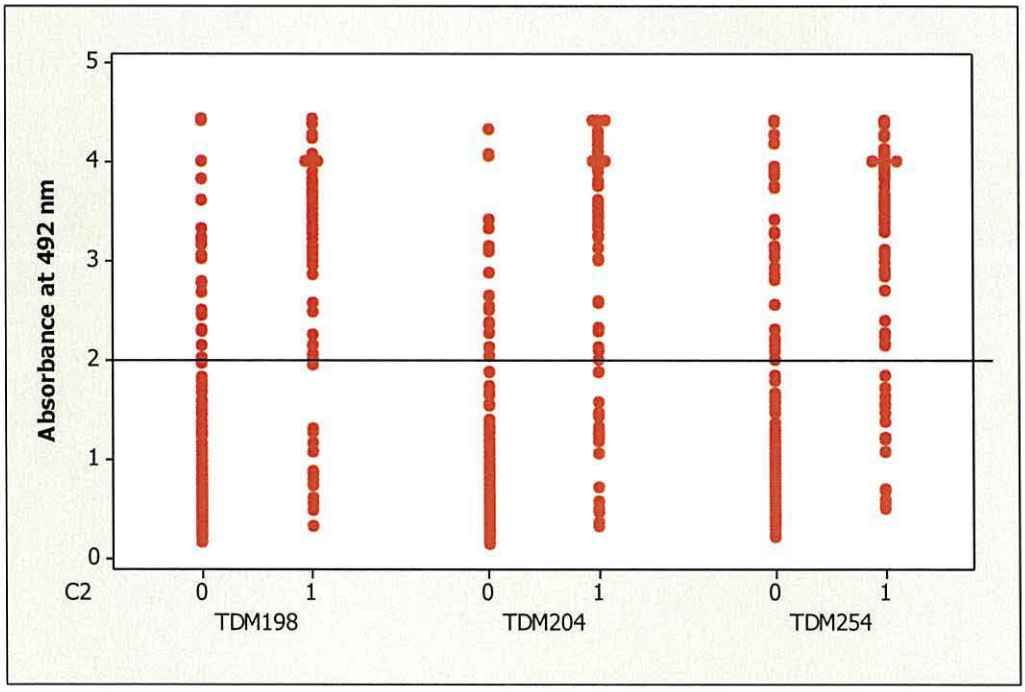


Figure 52: Individual value plots of TDM (198), TDM (204) and TDM (254) as antigens to blind sera

4.15 ELISA Experiment

4.15.1 Reagents

1. Phosphate buffered saline (PBS).

The stock solution of 20x PBS was prepared by dissolving NaCl (160.0 g), KCl (4 g), KH_2PO_4 (4.0 g) and Na_2HPO_4 (23.0 g) in 900 mL of double distilled de-ionised water ($dddH_2O$). The solution was then made up to a final volume of 1 L using $dddH_2O$ and filtered through a 0.22 μL membrane filter.

2. Casein/PBS buffer (0.5%).

20x PBS stock solutions (100 mL) was added to 1.4 L of double distilled de-ionised water ($dddH_2O$) in 2 L beaker, and casein (carbohydrates and fatty acid free) (10.0 g) was added. This solution was stirred for two hours at 37 °C and then stored overnight at 4 °C. The next day the pH was adjusted to 7.4 with sodium hydroxide NaOH (1.0 M) and the volume made up to 2 L with ddd H_2O.

3. Secondary antibody (Goat anti-human IgG peroxidise conjugate).

Secondary antibody (10 μL) was added to 10 mL of 0.5% casein/PBS. This solution was prepared 5 minutes before use.

4. Citrate Buffer (0.1 M).

Citric acid solution (450 mL, 0.1 M) was added to tri-sodium citrate solution (450 mL, 0.1 M) until a pH of 4.5.

5. O-phenylenediamine dihydrochloride (OPD) substrate.

OPD (10.0 mg) and H_2O_2 (8.0 mg) was added to citrate buffer (0.1 M, 10 mL). The substrate was prepared 5 minutes prior to use.

6. Natural Mixture TDM

Trehalose-6,6-dimycolate extracted from *M. Tuberculosis* was purchased from Aldrich.

7. Synthetic antigens.

Synthetic antigens were dissolved in hexane to give an antigen solution of concentration 62.5 μg/mL.

4.15.2 ELISA Procedure

The synthetic antigen solution (50 µl) was added to each well of a 96 well microtiter plate, and the solvent was left to evaporate at room temperature and stored overnight in a plastic bag. Plate was blocked with (0.5%) casein in PBS buffer (400 µL/well), and incubated at 25 °C for 30 min. The buffer was removed and any excess buffer was flicked out until the plates were dry. The serum sample (50 µL) was diluted with casein/PBS (1:20) and was added to each well and incubated at 37 °C for 2 hours. The plates were washed three times with casein/PBS buffer (with an automatic washer). The plates were then flicked out and dried very well. The goat anti-human IgG-peroxidase (50 µL) was added to each well, and incubated at 37 °C for 30 min. The plates were then washed three times with casein/PBS and then flicked out and dried very well. The OPD substrate solution (50 µL) was added to each well and the plates were again incubated at 37 °C for 30 min. After that, the reaction was terminated by adding H_2SO_4 (50 µL, 2.5 M) to each well. The absorbance was measured by using UV-visible spectrophotometer at 492 nm.

All values quoted are average values for four readings. In instances where error bars are shown these represent the standard deviation of the values used. The cut-off values were chosen by conditional formatting in Exel software programme.

Chapter 5

5. Conclusions

The aim of the first part of this study was the synthesis of two oxygenated mycolic acids: the methoxy mycolic acid **(101)** and ketomycolic acid **(165)**. These mycolic acids have been reported as important components of the *M. tuberculosis* cell wall.

The methoxy mycolic acid was prepared by coupling a methoxy meromycolate fragment **(124)** to a mycolic motif aldehyde **(123)** via a Julia coupling reaction. This coupling gave very good yield of alkenes. Hydrogenation of the alkenes was carried out using di-imide as a mild hydrogenation system to avoid the hydrogenolysis of the cyclopropyl ring. The silyl protecting group of secondary alcohol on the β-carbon was removed by using HF.pyridine while the methyl ester group was hydrolysed with lithium hydroxide to give free methoxy mycolic acid **(101)**.

On the other hand, the synthesis of ketomycolic acid **(165)** was achieved by coupling the motif part **(174)** with the meromycolate part **(175)** which had different protecting groups on them. After the hydrogenation of the alkenes, a series of steps involving protection and de-protection were carried out. The final reaction step was a deprotection in an acid media to avoid epimerisation of the methyl substituent α to the ketone group of the mycolic acid.

The completion of the synthesis of these mycolic acids gave the starting materials for the preparation of four new cord factors as derivatives of mycolic acids **(101)** and **(165)**. The first step in the synthesis of the cord factors was the protection of the secondary hydroxyl groups in both the mycolic acid and the trehalose sugar with two different protecting groups to allow selective deprotections later in the synthesis. These reactions were followed by an esterification reaction between the mycolic acids and trehalose. These resulted in two protected cord factors (TDM and TMM). The two products were separated by column chromatography and the protecting groups were cleaved in two steps. The first step was the deprotection of the trehalose sugar with TBAF, while the second step was the deprotection of the mycolic acids with HF. pyridine complex to obtain the free cord factors **(198)**, **(200)**, **(204)** and **(206)**.

The second aim of this study was the synthesis of mycolic acids with thiol groups which could eventually be bound to gold nanoparticles and their activity against tuberculosis antibodies tested. The position of the thiol group within the mycolic acid

molecule required careful consideration. The thiol group was introduced at the end of the α-alkyl chain because it is believed that introducing it at this position would not affect the antigenicity of the molecule. This synthesis was carried out by a Julia coupling reaction between the motif protected at the terminus of its α alkyl chain **(232)** and the meromycolate **(124)** used previously to prepare the free methoxy mycolic acid **(101)**. This process yielded an alkene which was hydrogenated as described previously, followed by deprotection of the α alkyl chain. The resulting primary hydroxyl group was converted into a tosylate group as a good leaving group, subsequent to its conversion into a thioacetate. The silyl protecting group was removed using HF.pyridine. The final deprotection of the methyl ester and thioacetate groups in a single reaction, gave the disulfide **(246)** instead of the desired free thiol. This pointed to the sensitivity of the deprotection conditions. The formation of the disulfide was proved by NMR spectroscopy which showed a triplet for the the methylene group next to sulfur at δ 2.69 in the proton NMR spectrum, and a signal for the same methylene carbon at δ 39.09 in the carbon NMR spectrum. However, an accurate mass spectrum could not be obtained for this product. The difficulty in obtaining a free thiol monomer has a redundant effect on the eventual linking to gold substrate because both thiols and disulfides have almost identical binding properties to the metal. A second diastereoisomer was prepared by using the same procedure to determine the effect of the stereochemistry of the mycolic acid on its use in TB diagnosis. Also, the final deprotection gave the disulfide **(247)**, which showed similar spectroscopic data for **(246)**, and did not provide a molecular ion.

The characterization of the formation the disulfide **(246)** or **(247)** was carried out by esterification of the acid with diazomethane and the alcohol was reprotected as an acetate. The disulfide was then reductively cleaved to give the thiol **(250)** or **(253)** using *DL*-dithiothreitol. The resulting compounds **(250)** and **(253)** gave the expected molecular ion in MALDI MS, and the methylene group next to sulfur in both them, appeared as a quartet at δ 2.53 (*J* 7.52). In addition, the carbon NMR spectrum showed a signal at δ 24.66 for the methylene next to sulfur.

In the same way, the disulfide **(246)** was split to the free thiol **(210)** by using *DL*-dithiothreitol without protection of the hydroxyl groups. The proton NMR spectrum of the thiol **(210)** again showed a quartet at δ 2.52 (*J* 7.52 Hz) for the methylene protons alpha to the sulfur atom. The methylene carbon adjacent to sulfur appeared at δ 24.65 in the carbon NMR spectrum. This gave the expected molecular ion in MALDI MS.

The goal of the synthesis of mycolic acid molecules is to use them in a rapid, accurate and inexpensive method for TB diagnosis. ELISA assay was selected to investigate the potential of the synthesized molecules for the diagnosis of TB disease. Initially, ELISA assay conditions were optimised by choosing the best wavelength for absorbance, serum dilution and the type of the secondary antibody which gave a clear distinction between TB+ and TB-. The different types of synthetic mycolic acids and their derivatives were analysed to select the best synthetic antigen which will give a high difference between TB^+ and TB^- sera, and a good accuracy (sensitivity and specificity). The ELISA result using different types of free mycolic acids showed low sensitivity, since the TB positive and negative absorbances appeared similar. The cord factors gave high response to antibodies in sera of TB infected persons. However, the test results of TDM **(198)**, TDM **(204)** and TDM **(254)** as synthetic antigens showed a significant difference between the average absorbance recorded for the TB positive sera and the TB negative sera. These antigens gave good values of selectivity and specificity when used as antigens to analyse different sera in modified ELISA method, comporison with free mycolic acids and their derivatives as synthetic antigens. The sensitivity and selectivity for TDM **(198),** TDM **(204)** and TDM **(254)** were ((80%, 87%), (75%, 90%) and (80%, 84%)), respectively. This gave an indication as to which antigens would be best to use in the further analysis of the diagnosis of TB using different method. Also, this study set toward for preparing a rapid method for detecting TB-antibodies, in order to improve the diagnosis of TB which will help to control on the spread of TB disease. Accurate detection of early TB infection could lead to patients being treated more efficiently and more quickly, leading to fewer deaths from the disease, as well as a reduction in it's spreading.

Chapter 6

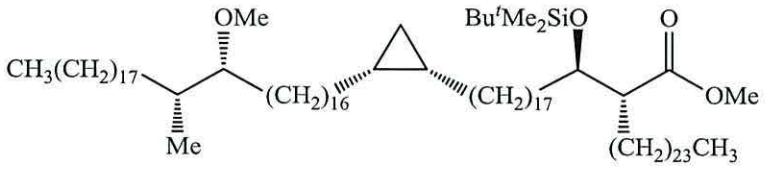
6. Experimental

6.1 General considerations

Starting materials and reagents were purchased from Alfa Aesar, Acros, Lancaster or Sigma-Aldrich. However, solvents such as THF and diethyl ether were dried over sodium wire and benzophenone under nitrogen, whereas dichloromethane, diisopropylamine and HMPA were dried over calcium hydride. Anhydrous magnesium sulfate was used to dry organic solutions. Petrol used was of boiling point 40-60 °C. Reactions carried out under inert conditions were subjected to a slow stream of nitrogen using a nitrogen balloon. Reactions carried out at low temperatures were cooled using a bath of methylated spirit and liquid nitrogen. Silica gel (Merck 7736) and silica gel plates(Merck 7736) used for column chromatography and thin layer chromatography were obtained from Aldrich; separated components were detected using variously UV light, I_2 and phosphomolybdic acid solution in IMS followed by charring. Melting points were measured using a Gallenkamp melting point apparatus. Optical rotations $[\alpha]_D^T$ were measured as solutions in chloroform of known concentration using a POLAR 2001 optical activity polarimeter. Infrared spectra were recorded on Perkin Elmer 1600 F.T.I.R. spectrometer as liquid films or KBr discs (solids). A Bruker Advance 500 NMR spectrometer was used to record both ^1H and ^{13}C NMR spectra in $CDCl_3$ unless otherwise stated. Chemical shifts for ^1H and ^{13}C were at δ 7.27 ppm and δ 77.0 ppm respectively. Data were reported as follows: chemical shift, integration, multiplicity (br, broad; s, singlet; d, doublet; t, triplet; q, quartet; pent, pentet; sext, sextet; m, multiple), coupling constant. Mass spectra samples were run on a Bruker Microtof by direct infusion and matrix-assisted laser desorption/ionisation mass spectra were run on a Bruker Reflex IV.

6.2 Experiments

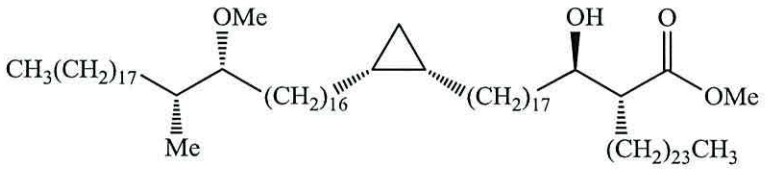
Experiment 1: (*R*)-2-{(*R*)-1-(*tert*-Butyldimethylsiloxy)-18-[(1*S*,2*R*)-2-(17*R*,18*R*)-17-methoxy-18-methylhexatricontyl)cylopropyl]octadecyl}hexacosanoic acid methyl ester (163)



Lithium bis(trimethylsilyl)amide (3.33 mL, 3.53 mmol, 1.06 M) was added dropwise to a stirring solution of methyl (2*R*)-2-((*R*)-1-(*tert*-butyldimethylsiloxy)-11-oxoundecyl)hexacosanoate **(123)** (1.45 g, 2.04 mmol) and 5-{7-[(1*S*,2*R*)-2-((17*R*,18*R*)-17-methoxy-18-methylhexatriacontenyl)cyclopropyl]heptylsulfonyl}-1-phenyl-*1H*-tetrazole **(124)** (2.11 g, 2.35 mmol) in dry THF (50 mL) at -10 °C under nitrogen. The reaction turned bright yellow and was left to reach room temperature and stirred for 1 hour under nitrogen. TLC was taken to show no starting material. The reaction was quenched by adding sat. aq. NH_4Cl. The product was extracted with petrol / ethyl acetate (10:1) (3 x 100 mL), dried over $MgSO_4$, filtered and the solvent evaporated. The crude product was purified by column chromatography over silica gel, eluting solvent with petrol/ethyl acetate (20:1) to give a colourless oil, ((*E/Z*)(*R*)-2-{(*R*)-1-(*tert*-butyldimethylsiloxy)-18-[(1*S*,2*R*)-2-((17*R*,18*R*)-17-methoxy-18-methylhexatricon-tyl)-cyclopropyl]octadecyl}hexacosanoic acid methyl ester **(162)** (2.25 g, 82%) as a mixture. Hydrogenation was carried out with dipotassium azodicarboxylate (2 g, 10.3 mmol), which was added to a stirred solution of the above alkenes **(162)** (2.1 g, 1.55 mmol) in THF (30 mL) and methanol (7 mL) at 5 °C. A solution of glacial acetic acid (5 mL) in THF (5 mL) was added dropwise over a period of two days. Further portions dipotassium azodicarboxylate (1.5 g) and glacial acetic acid (2 mL) were added and the mixture was stirred overnight. This mixture was added slowly to a sat.aq. $NaHCO_3$ and the product was extracted with petrol/ethyl acetate (1:1, 3 × 80 mL) and the combined organic layers were washed with water (50 mL) dried and evaporated. The crude product was purified by column chromatography eluting with petrol/ethyl acetate (15:1) to give (*R*)-2-{(*R*)-1-(*tert*-butyldimethylsiloxy)-18-[(1*S*,2R)-2-((17*R*,18*R*)-17-methyoxy-18-methylhexatricontyl)cyclopropyl]octadecyl}hexacosano-

ic acid methyl ester **(163)** as a colourless oil (1.8 g, 90%), $[\alpha]_D^{22}$ = +2.60 (*c* 0.96, $CHCl_3$) {Found $(M + Na)^+$:1404.3833, $C_{92}H_{184}O_4SiNa$ requires: 1404.3862}, which showed δ_H (500MHz, $CDCl_3$): 3.93-3.89 (1H, m), 3.66 (3H, s), 3.34 (3H, s), 2.97-2.94 (1H, m), 2.53 (1H, ddd, *J* 4.1, 6.9, 10.4 Hz),1.64-1.47 (6H, m), 142-1.13 (144H, m), 0.90-0.84 (15H, m, including a singlet at δ 0.86), 0.65-0.62 (2H, m), 0.57 (1H, dt, *J* 4.1, 8.2 Hz), 0.05 (3H, s), 0.02 (3H, s), -0.32 (1H, br.q, *J* 5.35 Hz); δc (126MHz, $CDCl_3$): 175.10, 85.45, 73.24, 57.69, 51.58, 51.17, 35.37, 33.70, 32.41, 31.94, 30.51, 30.24, 29.99, 29.95, 29.84, 29.72, 29.68, 29.59, 29.45, 29.38, 28.74, 27.83, 27.59, 26.29, 25.11, 22.78, 21.18, 15.89, 15.02, 10.90, -4.29, -5.06; ν_{max}: 2920, 2851, 1741, 1464, 1361,1254, 1162, 836 cm^{-1}.

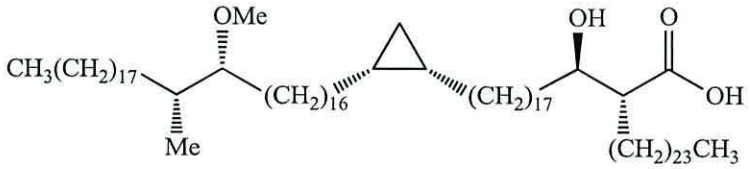
Experiment 2: (*R*)-2-{(*R*)-1-Hydroxy-18-[(1*S*,2*R*)-2-((17*R*,18*R*)-17-methoxy-18-methylhexatricontyl)cyclopropyl]octadecyl}hexacosanoic acid methyl ester (164)



(*R*)-2-{(*R*)-1-(*tert*-Butyldimethylsiloxy)-18-[(1*S*,2*R*)-2-((17*R*,18*R*)-17-methoxy-18-methylhexatricontyl)cylopropyl]octadecyl}hexacosanoic acid methyl ester **(163)** (1.8 g, 1.30 mmol) was dissolved in dry THF (25 mL) in a dry polyethylene vial under nitrogen at 0 °C. Pyridine (0.2 mL) and hydrogen fluoride-pyridine complex (1.5 mL) were added and the mixture was stirred at 45 °C for 18 hours. When TLC showed no starting material was left, the mixture was added slowly to sat. aq. $NaHCO_3$ (20 mL). The product was extracted with petrol/ethyl acetate (5:1, 3 × 50 mL) and the combined organic extracts were dried, filtered and evaporated. The crude product was purified by column chromatography eluting with petrol/ethyl acetate (10:1) to give (*R*)-2-{(*R*)-1-hydroxy-18-[(1*S*,2*R*)-2-((17*R*,18*R*)-17-methoxy-18-methyheexatricontyl)cylopropyl] octadecyl}hexacosanoic acid methyl ester **(164)** as a white solid (1.15 g, 70%), $[\alpha]_D^{22}$ = +1.12 (*c* 0.90, $CHCl_3$), m.p. 60-62 °C {Found $(M + Na)^+$:1290.2987, $C_{86}H_{170}O_4Na$ requires: 1290.2997}, which showed δ_H (500MHz, $CDCl_3$): 3.71 (3H, s), 3.69-3.64 (1H, m), 3.34 (3H, s), 2.96-2.94 (1H, br.pent., *J* 4.6 Hz), 2.46-2.42 (1H, m), 1.74-1.68 (2H, m), 1.64-1.53 (4H, m), 1.48-1.12 (142H, m), 0.88 (6H, t, *J* 6.3 Hz), 0.86 (3H, d, *J*

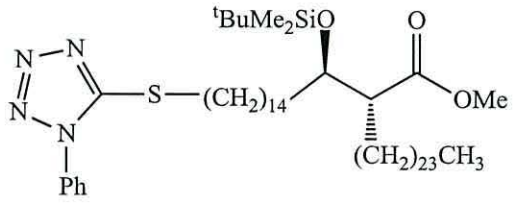
6.65 Hz), 0.68-0.64 (2H, m), 0.56 (1H, dt, *J* 4.1, 8.2 Hz), -0.32 (1H, br.q, *J* 5.05 Hz); δ_C (126MHz, $CDCl_3$): 176.19, 85.44, 72.29, 57.68, 51.45, 50.96, 35.70, 35.37, 32.40, 31.93, 30.51, 30.22, 29.98, 29.94, 29.70, 29.63, 29.61, 29.58, 29.50,29.42, 29.36, 28.72, 27.58, 27.42, 26.17, 25.73, 22.68, 22.59, 20.43, 19.42, 15.77, 14.87, 14.09, 10.91; ν_{max}: 3512, 2917, 2848, 1713, 1464, 1376, 1195, 1167, 1098, 720 cm^{-1}.

Experiment 3: (*R*)-2-{(*R*)-1-Hydroxy-18-[(1*S*,2*R*)-2-((17*R*,18*R*)-17-methoxy-18-methylhexatricontyl)cyclopropyl]octadecyl}hexacosanoic acid (101)



Lithium hydroxide monohydrate (0.57 g, 13.61 mmol) was added to a stirred solution of (*R*)-2-{(*R*)-1-hydroxy-18-[(1*S*,2*R*)-2-((17*R*,18*R*)-17-methyoxy-18-methylhexatricontyl)cyclopropyl]octadecyl}hexacosanoic acid methyl ester **(164)** (1.15 g, 0.90 mmol) in THF (15 mL), methanol (2 mL) and water (1 mL) at room temperature. The mixture was stirred at 45 ˚C for 24 hours. When TLC showed no starting material was left, the reaction was acidified with 5% HCl and the product was extracted with petrol/ethyl acetate (5:1) (3 × 50 mL), dried and evaporated to give a white solid. The crude product was purified by column chromatography eluting with petrol/ethyl acetate (7:2) gave (*R*)-2-{(*R*)-1-hydroxy-18-(1*S*,2*R*)-2-[(17*R*,18*R*)-17-methoxy-18-methylhexatricontylcyclopropyl]octadecyl}hexacosanoic acid **(101)** (0.86 g, 77%) as a white solid, $[\alpha]_D^{25}$ = +6.22 (*c* 0.98, $CHCl_3$), m.p. 58-60 ˚C {MALDI Found (M + Na)$^+$:1276.55, $C_{85}H_{168}O_4Na$ requires: 1276.28}, which showed δ_H (500MHz, $CDCl_3$): 3.73-3.69 (1H, m), 3.35 (3H, s), 2.98-2.95 (1H, m), 2.48-2.44 (1H, m), 1.75-1.70 (1H, m), 1.65-1.61 (2H, m), 1.53-1.10 (146H, m), 0.88 (6H, t, *J* 6.95 Hz), 0.85 (3H, d, *J* 6.65 Hz), 0.68-0.64 (2H, m), 0.56 (1H, dt, *J* 4.05, 8.15 Hz), -0.32 (1H, br.q, *J* 5.05 Hz); δ_C (126MHz, $CDCl_3$): 178.62, 85.56, 72.12, 57.67, 50.76, 35.54, 35.36, 32.38, 31.92, 30.49, 30.22, 29.98, 29.93, 29.70, 29.61, 29.59, 29.52, 29.42, 29.36, 28.72, 27.56, 27.33, 26.16, 25.73, 22.68, 15.78, 14.88, 14.10, 10.91; ν_{max}: 3282, 2917, 2850, 1708, 1471, 1377, 1095, 717 cm^{-1}.

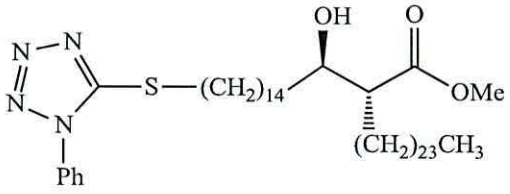
Experiment 4: (*R*)-2-[(*R*)-1-((*tert*-Butyldimethylsilyloxy)-15-(5-phenyl-5*H*-tetrazol-1-ylsulfanyl)pentadecyl]hexacosanoic acid methyl ester (189)



The procedure used in experiment **1** was repeated in order to couple (*R*)-2-[(*R*)-1-(*tert*-butyldimethylsilanyloxy)-3-oxopropyl]-hexacosanoic acid methyl ester **(148)** (2.9 g, 4.85 mmol) and 5-((12-bromododecyl)sulfonyl)-1-phenyl-1*H*-tetrazole **(186)** (2.9 g, 6.33 mmol) using lithium bis(trimethylsilyl)amide (8.93 mL, 9.46 mmol) in dry THF (80 mL). The crude product was purified by column chromatography eluting with petrol/ethyl acetate (20:1) to give a colourless oil, (*R*)-2-[(*E*/*Z*)-(*R*)-15-bromo-1-((*tert*-butyldimethylsilyl)oxy)pentadec-3enyl]hexacosanoic acid methyl ester **(187)** (3.2 g, 79%), as a mixture. 1-Phenyl-1*H*-tetrazole-5-thiol (0.75 g, 4.24 mmol) was added to a stirred solution of the above alkenes **(187)** (3.2 g, 3.86 mmol) and anhydrous potassium carbonate (1.17 g, 8.46 mmol) in acetone (100 mL) at room temperature. The mixture was vigorously stirred for 18 hours. TLC analysis indicated that the reaction was complete. Water (150 mL) was added and the product was extracted with dichloromethane (1 × 200 mL, 2 × 100 mL). The combined organic phases were washed with brine (2 × 200 mL), dried and evaporated. The crude product was purified by column chromatography eluting petrol/ethyl acetate (20:1) to give a colourless oil of (*R*)-2-[(*E*/*Z*)-(*R*)-15-(5-phenyl-5*H*-tetrazol-1-ylsulfanyl)-1-((*tert*-butyldimethylsilyl -oxy)-pentadec-3-enyl]hexacosanoic acid methyl ester **(188)** (2.7 g, 75%), as a mixture. Hydrogenation was carried out with palladium (10% on carbon, 0.5 g) which was added to a stirred solution of the above unsaturated sulfide **(188)** (2.7 g, mmol) in THF (30mL) and IMS (30 mL), for one hour. The crude product was purified by column chromatography eluting with petrol/ethyl acetate (15:1) to give a colourless oil, (*R*)-2-[(*R*)-1-((*tert*-butyldimethylsilyloxy)-15-((5-phenyl-5*H*-tetrazol-lylsulfanyl) pentadecyl]hexacosanoic acid methyl ester **(189)** (2.42, 90%), $[\alpha]_D^{26}$= -5.6 (*c* 1.1, $CHCl_3$) {Found $(M + Na)^+$: 949.7385, $C_{55}H_{102}N_4O_3SSiNa$ requires: 949.7334}, which showed δ_H (500MHz, $CDCl_3$): 7.60-7.53 (5H, m), 3.92-3.88 (1H,m), 3.65 (3H,s), 3.40

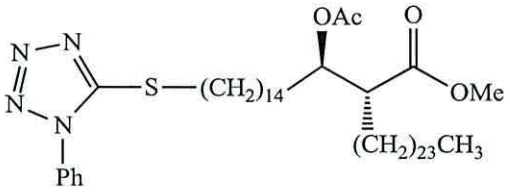
(2H, t, *J* 5.88 Hz), 2.52 (1H, ddd, *J* 3.0, 5.8, 10.85 Hz), 1.83 (2H, pent., *J* 5.88 Hz), 1.45-1.11 (70H, br.m), 0.88 (3H, t, *J* 5.44 Hz), 0.86 (9H, s), 0.04 (3H, s), 0.02 (3H, s); δ_C (126MHz, $CDCl_3$): 175.12, 154.54, 133.72, 130.04, 129.72, 123.83, 73.11, 51.54, 51.22, 33.64, 33.35, 31.93, 29.84, 29.72, 29.66, 29.46, 29.10, 28.85, 28.23, 27.84, 27.56, 25.73, 23.64, 22.73, 17.97, 14.14, -4.45, -4.96; ν_{max}: 2932, 2858, 1741, 1602, 1506, 1466, 1387, 1253, 1169, 1077, 838, 765 cm^{-1}.

Experiment 5: (*R*)-2-[(*R*)-1-Hydroxy-15-((5-phenyl-5*H*-tetrazol-1-ylsulfanyl) pentadecyl]hexacosanoic acid methyl ester (190)



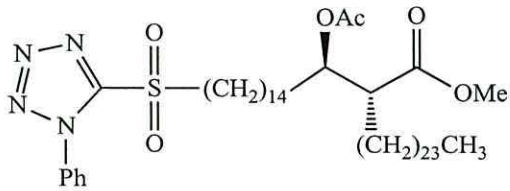
The procedure used in experiment **2** was repeated using (*R*)-2-[(*R*)-1-((*tert*-butyldimethylsilyloxy)-15-((5-phenyl-5*H*-tetrazol-1-ylsulfanyl)pentadecyl)hexacosano -ic acid methyl ester **(189)** (2.4 g, 2.58 mmol), pyridine (1.2 mL) and HF.pyridine (2.5 mL) in dry THF (15 mL). The product was purified by column chromatography eluting with petrol/ethyl acetate (10:1) to give a white solid, (*R*)-2-[(*R*)-1-hydroxy-15-((5-phenyl-5*H*-tetrazol-1-ylsulfanyl)pentadecyl]hexacosanoic acid methyl ester **(190)** (1.9 g, 90%), $[\alpha]_D^{26}$ = + 3.8 (*c* 1.12, $CHCl_3$), m.p.: 62-64 °C {Found $(M + Na)^+$: 835.6456, $C_{49}H_{88}N_4O_3SNa$ requires: 835.6469}, which showed δ_H (500MHz, $CDCl_3$): 7.60-7.53 (5H, m), 3.71 (3H, s), 3.69-3.63 (1H, m), 3.40 (2H, t, *J* 6.25 Hz), 2.50-2.47 (1H, m), 1.80 (2H, pent., *J* 6.30 Hz), 1.75-1.68 (1H, m), 1.62-1.55 (1H,m), 1.51-1.38 (6H, m), 1.36-1.10 (63 H, br.m), 0.88 (3H, t, *J* 5.84 Hz); δ_C (126MHz, $CDCl_3$): 176.26, 154.56, 133.73, 130.05, 129.76, 123.84, 72.35, 51.49, 50.93, 35.74, 33.38, 31.93, 29.74, 29.68, 29,56, 29.53, 29.48, 29.34, 29.10, 29.06, 28.63, 27.44, 25.73, 22.74, 14.14; ν_{max}: 3486, 2915, 1716, 1596, 1498, 1459, 1375, 1291, 1280, 1240, 1189, 1132, 1077, 836 cm^{-1}.

Experiment 6: (*R*)-2-[(*R*)-1-Acetoxy-15-((5-phenyl-5*H*-tetrazol-1-ylsulfanyl) pentadecyl]hexacosanoic acid methyl ester (191)



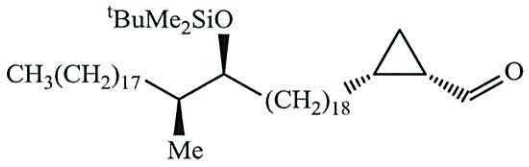
A mixture of acetic acid anhydride (7 mL) and anhydrous pyridine (7 mL) was added to stirred solution of the (*R*)-2-[(*R*)-1-hydroxy-15-((5-phenyl-5*H*-tetrazol-1-ylsulfanyl) -pentadecyl]hexacosanoic acid methyl ester **(190)** (1.9 g, 2.33 mmol) in dry toluene (25 mL) at room temperature for 18 hours, diluted with toluene (10 mL), and then the solvent was evaporated to give a solid. The crude product was purified by column chromatography eluting with petrol/ethyl acetate (10:1) to give a white solid, (*R*)-2-[(*R*)-1-acetoxy-15-((5-phenyl-5*H*-tetrazol-1-ylsulfanyl)pentadecyl]hexacosanoic acid methyl ester **(191)** (1.8 g, 90%), $[\alpha]_D^{26} = +6.4$ (*c* 1.0, $CHCl_3$), m.p.: 51-53 °C {Found $(M + Na)^+$: 877.6535, $C_{51}H_{90}N_4O_4SNa$ requires: 877.6575}, which showed δ_H (500MHz, $CDCl_3$): 7.58-7.53 (5H, m), 5.08-5.05 (1H, m), 3.66 (3H, s), 3.38 (2H, t, *J* 5.88 Hz), 2.60 (1H, ddd, *J* 3.4, 5.48, 8.6 Hz), 2.02 (3H, s), 1.80 (2H, pent., *J* 5.92 Hz), 1.55-1.48 (2H, m), 1.42 (2H, br.pent., *J* 6.22 Hz), 1.31-1.12 (66H, m), 0.88 (3H, t *J* 5.80 Hz); δ_C (126MHz, $CDCl_3$): 173.56, 170.42, 154.75, 133.75, 130.10, 129.83, 123.87, 73.86, 51.52, 49.96, 33.36, 32.00, 31.74, 29.78, 29.68, 29.54, 29.46, 29.10, 28.68, 28.21, 27.44, 25.14, 22.78, 21.13, 14.14; ν_{max}: 2922, 2852, 1743, 1602, 1503, 1468, 1238,1074, 838 cm^{-1}.

Experiment 7: (*R*)-2-[(*R*)-1-Acetoxy-15-((5-phenyl-5*H*-tetrazol-1-ylsulfonyl)-pentadecyl]hexacosanoic acid ester (174)



m-Chloroperbenzoic acid (1.56 g, 8.98 mmol) in dichloromethane (10 mL) was added at 0 °C to a stirred solution of (*R*)-2-((*R*)-1-acetoxy-15-((5-phenyl-5*H*-tetrazol-1-ylsulfanyl)pentadecyl)hexacosanoate **(191)** (1.8 g, 2.10 mmol) and $NaHCO_3$ (0.8 g, 9.52 mmol) in dichloromethane (25 mL) and stirred at room temperature for 20 hours. The mixture was quenched by addition of a sat.aq. $NaHCO_3$ (30 mL) and extracted with dichloromethane (1 × 100, 3 × 30). The combined organic layers were washed with water (100 mL), dried and evaporated. The crude product was purified by column chromatography eluting with petrol/ethyl acetate (5:1) to give a white solid, (*R*)-2-[(*R*)-1-acetoxy-15-(5-phenyl-5*H*-tetrazol-1-ylsulfonyl)pentadecyl]hexacosanoic acid methyl ester **(174)** (1.65 g, 88%), $[\alpha]_D^{25} = +9.2$ (*c* 0.95, $CHCl_3$), m.p.: 51-53 °C {Found $(M + Na)^+$: 909.6456, $C_{51}H_{90}N_4O_6SNa$ requires: 909.6473}, which showed δ_H (500MHz, $CDCl_3$): 7.71-7.69 (2H, m), 7.63-7.61 (3H, m), 5.10-5.07 (1H, m), 3.73 (2H, t, *J* 6.4 Hz), 3.68 (3H, s), 2.62 (1H, ddd, *J* 3.44, 5.48, 8.68 Hz), 2.03 (3H, s), 1.96 (2H, br.pent., 6.04 Hz), 1.69-1.47 (6H, m), 1.40-1.11 (64H, br.m), 0.88 (3H, t, *J* 5.44 Hz); δ_C (126MHz, $CDCl_3$): 173.63, 170.45, 153.53, 133.06, 131.40, 129.74, 125.18, 73.85, 55.89, 51.57, 49.97, 31.98, 31.76, 29.75, 29.69, 29.58, 29.51, 29.48, 29.35, 29.26, 29.12, 28.87, 28.24, 28.11, 27.49, 25.04, 22.73, 21.10, 20.98, 14.09; ν_{max}: 2928, 2853, 1736, 1467, 1375, 1595, 1498, 1375, 12245, 1167, 836 cm^{-1}.

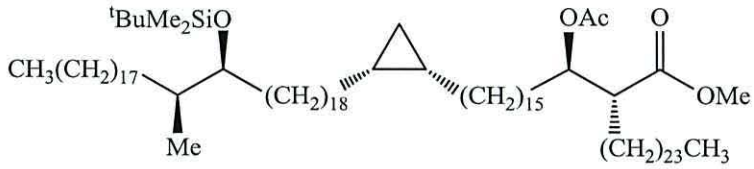
Experiment 8: (1*S*,2*R*)-2-[(19*S*,20*S*)-19-(*tert*-Butyldimethylsilanyloxy)-20-methyl-octatriacontyl]cyclopropanecarbaldehyde (175)



(1*S*,2*R*)-2-[(19*S*,20*S*)-19-(*tert*-Butyldimethylsilanyloxy)-20-methyloctatriacontyl]cyclo-propyl}methanol **(192)** (1.5 g, 1.73 mmol) in dichloromethane (10 mL) was added in portions to a stirred solution of PCC (1.08 g, 936.55 mmol) in dichloromethane (30 mL) at room temperature. The mixture was stirring vigorously for 2 hours, when TLC showed no starting material was left. It was poured in petrol/ethyl acetate (10:1, 100 mL), filtered through a pad of silica and the solvent evaporated. The crude product was purified by chromatography eluting with petrol/ethyl acetate (10:1) to give (1*S*,2*R*)-2-

[(19*S*,20*S*)-19-(*tert*-butyldimethylsilanyloxy)-20-methyloctatriacontyl]cyclopropaneca -rbaldehyde **(175)** as colourless oil (1.2 g, 80%), $[\alpha]_D^{23}$ = -10.72 (*c* 1.02, $CHCl_3$) {Found $(M + Na)^+$: 769.7223, $C_{49}H_{98}O_2SiNa$ requires: 769.7211}, which showed δ_H (500MHz , $CDCl_3$): 9.35 (1H, d, *J* 5.55), 3.51-3.48 (1H, m), 1.88-1.84 (1H, m), 1.63-1.58 (2H, m), 1.51-1.17 (71H, m), 1.08-1.01 (1H, m), 0.90-0.86 (12H, m, including a singlet at δ 0.88), 0.80 (3H, d, *J* 6.75 Hz), 0.03 (3H, s), 0.02 (3H, s); δ_C (126MHz, $CDCl_3$): 201.75, 75.88, 37.73, 33.55, 32.50, 31.92, 30.00, 29.98, 29.89, 29.70, 29.65, 29.60, 29.55, 29.36, 29.26, 28.88, 28.22, 27.80, 27.71, 25.96, 25.92, 24.78, 22.68, 22.32, 18.18, 14.73, 14.41, 14.10, -4.19, -4.43; ν_{max}: 2924, 2853, 1709, 1464, 1252, 1056, 835, 773 cm^{-1}.

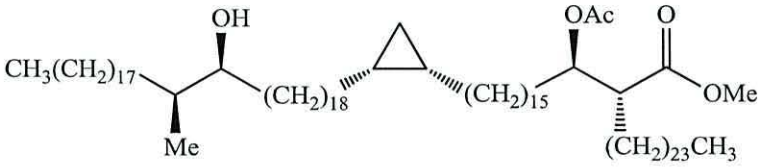
Experiment 9: (*R*)-2-((*R*)-1-Acetoxy-16-{(1*S*,2*R*)-2-[(19*S*,20*S*)-19-(*tert*-butyldimethylsilanyloxy)-20-methyloctatriacontyl]cyclopropyl}hexdecyl)hexacosanoic acid methyl ester (176)



The procedure used in Experiment **1** was repeated in order to couple (1*S*,2*R*)-2-[(19*S*,20*S*)-19-(*tert*-butyldimethylsilanyloxy)-20-methyloctatriacontyl]cyclopropane-carbaldehyde **(175)** (1.2 g, 1.60 mmol) and (*R*)-2-[(*R*)-1-acetoxy-15-(5-phenyl-5*H*-tetrazole-1-sulfonyl)pentadecyl]hexacosnoic acid methyl ester **(174)** (1.63 g, 1.84 mmol) using lithium bis(trimethylsilyl)amide (2.61 mL, 2.77 mmol, 1.06 M) in dry THF (25 mL) at -10 °C. The crude prodect was purified by column chromatography eluting with petrol/ethyl acetate (20:1) to give a colourless oil, (*E/Z*)(*R*)-2-((*R*)-1-acetoxy-16-{(1*S*,2*R*)-2-[(19*S*,20*S*)-19-(*tert*butyldimethylsilanyloxy)-20-methyloctatria -contyl]cyclopropyl}hexadec-15-enyl)hexacosanoic acid methyl ester **(193)** (1.9 g, 84%) as a mixture. Hydrogenation was carried out using dipotassium azodicarboxylate (2.0 g, 10.31 mmol) which was added to a stirred solution of the above alkenes **(193)** (1.9 g, 1.34 mmol) in THF (25 mL) and methanol (5 mL) and a solution of glacial acetic acid (5 mL) and THF (5 mL) was added as before. The crude product was purified by column chromatography eluting with petrol/ethyl acetate (20:1) to give a

white simi-solid, (*R*)-2-((*R*)-1-acetoxy-16-{(1*S*,2*R*)-2-[(19*S*,20*S*)-19-(*tert*-butyldimeth -ylsilanyloxy)-20-methyloctatriacontyl]cyclopropyl}hexadecyl)hexacosanoic acid methyl ester **(176)** (1.6, 84%), $[\alpha]_D^{24}$= - 3.9 (*c* 0.86, $CHCl_3$) {Found $(M + Na)^+$: 1433.3826, $C_{93}H_{184}O_5SiNa$ requires: 1432.3805}, which showed δ_H (500MHz, $CDCl_3$): 5.09 (1H, br.dt, *J*, 4.1, 8.15 Hz), 3.68 (3H, s), 3.51-3.48 (1H, m), 2.62 (1H, ddd, *J* 4.1, 6.95, 10.75 Hz), 2.03 (3H, s), 1.62-1.50 (4H, m), 1.41-1.07 (143 H, m), 0.90-0.87 (15H, m, including a singlet at 0.88), 0.81 (3H, d, *J* 6.65 Hz), 0.66-0.63 (2H, m), 0.56 (1H, dt, *J* 4.1, 7.85 Hz), 0.03 (3H, s), 0.02 (3H, s), -0.32 (1H, br.q, *J* 5.35 Hz); δ_C (126MHz, $CDCl_3$): 173.65, 170.34, 75.88, 74.11, 51.52, 49.59, 37.73, 33.56, 32.51, 31.92, 31.73, 30.22, 30.00, 29.88, 29.70, 29.56, 29.47, 29.39, 29.35, 28.72, 28.12, 27.71, 27.47, 25.96, 25.92, 24.99, 22.62, 21.01, 18.18, 15.77, 14.41, 14.10, 10.91, -4.18, -4.42; ν_{max}: 2919, 2851, 1747, 1464, 1370, 1236, 1164, 1022, 835, 773, 720 cm^{-1}.

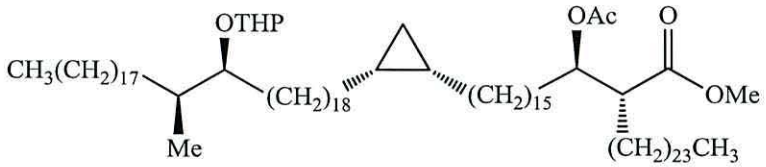
Experiment 10 : (*R*)-2-((*R*)-1-Acetoxy-16-{(1*S*,2*R*)-2-[(19*S*,20*S*)-19-hydroxy-20-methyloctatriacontyl]cyclopropyl}hexadecyl)hexacosanoic acid methyl ester (177)



The procedure used in Experiment **2** was repeated using (*R*)-2-((*R*)-1-acetoxy-16-{(1*S*,2*R*)-2-[(19*S*,20*S*)-19-(*tert*-butyldimethylsilanyloxy)-20-methyloctatriacontyl]cycl -opropyl}octadecyl)hexacosanoic acid methyl ester **(176)** (1.6 g, 1.12 mmol) pyridine (0.3 mL) and HF.pyridine (1.8 mL) in dry THF (25 mL). The product was purified by column chromatography eluting with petrol/ethyl acetate (20:1) to give a white semi-solid, (*R*)-2-((*R*)-1-acetoxy-16-{(1*S*,2*R*)-2-[(19*S*,20*S*)-19-hydroxy-20-methyloctatriaco -ntyl]cyclopropyl}hexadecyl)hexacosanoic acid methyl ester **(177)** (1.25 g, 86%), $[\alpha]_D^{25}$= - 4.5 (*c* 0.6, $CHCl_3$) {Found $(M + Na)^+$: 1318.2918, $C_{87}H_{170}O_4Na$ requires: 1318.2940}, which showed δ_H (500MHz, $CDCl_3$): 5.09 (1H, dt, *J* 3.75, 7.85 Hz), 3.68 (3H, s), 3.50-3.49 (1H, m), 2.62 (1H, ddd, *J* 4.4, 6.95, 10.7 Hz), 2.03 (3H, s), 1.57-1.51 (4H, m), 1.49-1.13 (144H, m, v.br), 0.88 (6H, t, *J* 6.95 Hz), 0.85 (3H, d, *J* 7.0 Hz), 0.66-0.64 (2H, m), 0.57 (1H, br.dt, *J* 3.75, 7.85 Hz), -0.32 (1H, br.q, *J* 5.35 Hz); δ_C (126MHz, $CDCl_3$): 173.65, 170.34, 75.78, 75.22, 74.11, 60.38, 56.50, 51.52, 49.60,

41.70, 41.36, 38.18, 36.08, 34.51, 34.12, 33.73, 33.38, 31.93, 31.73, 31.58, 31.41, 30.23, 30.11, 29.96, 29.57, 29.47, 29.44, 29.40, 29.36, 29.19, 29.06, 28.89, 28.73, 28.12, 27.67, 27.47, 27.42, 26.29, 26.08, 25.83, 24.99, 22.69, 22.61, 22.33, 21.01, 20.44, 19.43, 18.75, 15.78, 14.31, 14.19, 14.10, 14.04, 13.58, 11.41, 10.92 ; ν_{max}: 3555, 2923, 2853, 1747, 1465, 1371, 1235, 1021, 730 cm^{-1}.

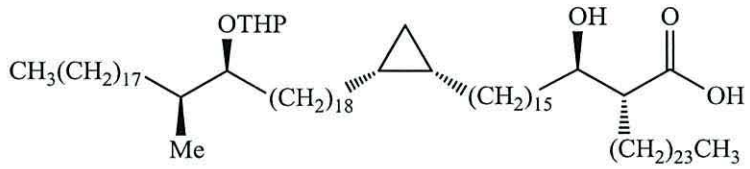
Experiment 11: (*R*)-2-((*R*)-1-Acetoxy-16-{(1*S*,2*R*)-2-[(19*S*,20*S*)-19-(tetrahydro pyran-2-yloxy)octatriacontyl]cyclopropyl}hexadecyl)hexacosanoic acid methyl ester (178)



Pyridinium p-toluene sulfonate (0.121 g, 0.482 mmol) in dry dicholoromethane (1 mL) was added with stirring to (*R*)-2-((*R*)-1-acetoxy-16-((1*S*,2*R*)-2-[(19*S*,20*S*)-19-hydroxy-20-methyloctatriacontyl]cyclopropyl)octadecyl)hexacosanoic acid methyl ester **(177)** (1.25 g, 0.964 mmol) and freshly distilled dihydro-2*H*-pyran (1 mL, 11.09 mmol) in dry dichloromethane (25 mL) at room temperature under nitrogen. After 3 hours, the reaction was quenched with a sat. aq. $NaHCO_3$ (15 mL), extracted with dichloromethane (3 × 50 mL) and the combined organic layers were dried and evaporated. The crude product was purified by column chromatography eluting with petrol/ethyl acetate (20:1) to give a white semi-solid, (*R*)-2-((*R*)-1-acetoxy-16-{(1*S*,2*R*)-2-[(19*S*,20*S*)-19-(tetrahydropyran-2-yloxy)octatriacontyl]cyclopropyl}hexad -ecyl)hexacosanoic acid methyl ester as a mixture of diastereoisomers **(178)** (1.17 g, 88%), {Found $(M + Na)^+$: 1402.3439, $C_{92}H_{178}O_6Na$ requires: 1402.3466}, which showed δ_H (500MHz, $CDCl_3$): 5.08-5.07 (2H, m), 4.65 (1H, br.t, *J* 3.15 Hz), 4.62 (1H, br.t, *J* 2.2 Hz), 3.94-3.89 (2H, m), 3.68 (6H, s), 3.48-3.42 (4H, m), 2.62 (2H, ddd, *J* 4.1, 6.95, 10.75 Hz), 2.03 (6H, s), 1.86-1.80 (2H, m), 1.77-1.06 (304H, m), 0.88 (12H, t, *J* 6.95 Hz), 0.84 (6H, d, *J* 6.6 Hz), 0.67-0.64 (4H, m), 0.56 (2H, br.dt, *J* 4.1, 7.85 Hz), -0.32 (2H, br.q, *J* 5.35 Hz); δ_C (126MHz, $CDCl_3$): 173.65, 170.34, 98.50, 97.84, 81.43, 80.96, 74.11, 62.77, 62.44, 51.52, 49.60, 36.45, 35.11, 32.49, 32.03, 31.93, 31.73, 31.42, 31.29, 31.23, 30.23, 30.00, 29.94, 29.90, 29.70, 29.57, 29.47, 29.44,

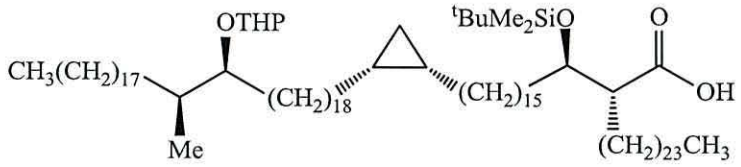
29.40, 29.36, 28.73, 28.12, 27.60, 27.55, 27.47, 26.13, 25.74, 25.67, 25.61, 24.99, 22.69, 21.01, 20.09, 19.82, 15.78, 15.18, 14.95, 14.11, 10.92; ν_{max}: 2921, 2852, 1746, 1468, 1373, 1237, 1200, 1167, 1077, 1023, 869, 720 cm^{-1}.

Experiment 12: (*R*)-2-((*R*)-1-Hydroxy-16-{(1*S*,2*R*)-2-[(19*S*,20*S*)-19-(tetrahydropy -ran-2-yloxy)octatriacontyl]cyclopropyl}hexadecyl)hexacosanoic acid (179)



The procedure used in Experiment **3** was repeated in order to hydrolyse (*R*)-2-((*R*)-1-acetoxy-16-{(1*S*,2*R*)-2-[(19*S*,20*S*)-19-(tetrahydropyran-2-yloxy)octatriacontyl]cyclopr -opyl}hexadecyl)hexacosanoic acid methyl ester **(178)** (1.15 g, 8.337 mmol) using lithium hydroxide monohydrate (0.56 g, 133.39 mmol) in THF (20 mL), methanol (1.2 mL) and water (1.5 mL). The crude product was purified by column chromatography eluting with petrol/ethyl acetate (10:1) to give a white semi solid (*R*)-2-((*R*)-1-hydroxy-16-{(1*S*,2*R*)-2-[(19*S*,20*S*)-19-(tetrahydropyran-2-yloxy)octatriacontyl]cyclopr -opyl}hexadecyl)hexacosanoic acid **(179)** as a mixture of diastereoisomers (0.9 g, 82%), {Found $(M + Na)^+$: 1346.3270, $C_{89}H_{174}O_5Na$ requires: 1346.3319}, which showed δ_H (500 MHz, $CDCl_3$): 4.67 (1H, br.t, *J* 3.15 Hz), 4.63 (1H, br.t, *J* 2.5 Hz), 3.95-3.89 (2H, m), 3.72-3.70 (2H, m), 3.50-3.44 (4H, m), 2.45 (2H, br.pent, *J* 5.35 Hz), 1.85-1.80 (4H, m), 1.74-1.01 (306H, m), 0.88 (12H, t, *J* 6.95 Hz), 0.84 (6H, d, *J* 6.6 Hz), 0.67-0.63 (4H, m), 0.56 (2H, br.dt, *J* 4.1, 8.2 Hz), -0.32 (2H, br.q, *J* 5.35 Hz); δ_C (126MHz, $CDCl_3$): 175.01, 98.49, 97.77, 81.52, 80.99, 72.16, 62.74, 62.38, 50.61, 36.43, 35.58, 35.11, 32.50, 32.02, 31.92, 31.41, 31.26, 31.20, 30.22, 29.99, 29.94, 29.90, 29.61, 29.58, 29.51, 29.42, 29.36, 28.72, 27.59, 27.54, 27.33, 26.12, 25.73, 25.66, 25.58, 22.68, 20.02, 19.75, 15.78, 15.17, 14.94, 14.10, 10.91; ν_{max}: 3435, 2918, 2850, 1683, 1470, 1378, 1131, 1024, 719 cm^{-1}.

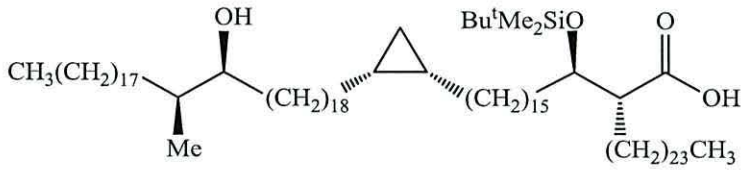
Experiment 13: (*R*)-2-((*R*)-1-(*tert*-Butyldimethylsilanyloxy)-16-{(1*S*,2*R*)-2-[(19*S*,20*S*)-19-(tetrahydropyran-2-yloxy)octatriacontyl]cyclo-propyl}hexadecyl) hexacosanoic acid (180)



Imidazole (0.52 g, 7.68 mmol) was added with stirring to (*R*)-2-((*R*)-1-hydroxy-16-{(1*S*,2*R*)-2-[(19*S*,20*S*)-19-(tetrahydropyran-2-yloxy)octatriacontyl]cyclopropyl}hexad -ecyl)hexacosanoic acid **(179)** (0.9 g, 0.6684 mmol) in dry DMF (6 mL) and dry toluene (5 mL) at room temperature followed by the addition of tertbutyldimethylsilyl chloride (1.142 g, 7.627 mmol) and 4-dimethylaminopyridine (0.05 g, 0.41 mmol). The reaction mixture was stirred at 70 °C for 18 hours at room temperature. When TLC showed no starting material was left, the solvent was removed under high vacuum and the residue was diluted with petrol/ethyl acetate (10:1) (50 mL) and sat. aq. $NaHCO_3$ (10 mL). The organic layer was separated, and the aqueous layer was re-extracted with petrol/ethyl acetate (3 × 20 mL). The combined organic layers were washed with water, dried and evaporated. The residue was dissolved in THF (15 mL), water (2 mL) and methanol (2 mL); to this was added a potassium carbonate (0.3 g). The reaction mixture was stirred at 45 °C for 6 hours, when TLC showed no starting material was left. The mixture was diluted with petrol/ethyl acetate (10:1, 20 mL) and water (2 mL), then acidified with potassium hydrogen sulfate topH 2. The organic layer was separated and the aqueous layer was re-extracted with petrol/ethyl acetate (2 × 20 mL). The combined organic layers were washed with water, dried and evaporated to give a residue, which was purified by column chromatography eluting with petrol/ethyl acetate (20:1) to give a white semi-solid, (*R*)-2-((*R*)-1-(*tert*-butyldimethylsilanyloxy)-16-{(1*S*,2*R*)-2-[(19*S*,20*S*)-19-(tetrahydropyran-2-yloxy) octatriacontyl]cyclopropyl}he -xadecyl)hexacosanoic acid **(180)** as a mixture of diastereoisomers (0.9 g, 91 %) {Found $(M + Na)^+$: 1460.4015, $C_{95}H_{188}O_5SiNa$ requires: 1460.4064}, which showed δ_H (500MHz, $CDCl_3$): 4.66 (1H, br.t, *J* 3.15 HZ), 4.62 (1H, br.t, *J* 2.5 Hz), 3.93-3.89 (2H, m), 3.85-3.82 (2H, m), 3.49-3.42 (4H, m), 2.53 (2H, ddd, *J*, 3.15, 5.65, 8.8 Hz), 1.85-1.80 (4H, m), 1.72-1.13 (304H, m), 0.92

(18H, s), 0.88 (12H, t, *J*, 6.95 Hz), 0.84 (6H, d, *J*, 6.6 Hz), 0.66-0.64 (4H, m), 0.56 (2H, br.dt, *J*, 4.1, 7.85 Hz), 0.14 (6H, s), 0.13 (6H, s), -0.32 (2H, br.q, *J*, 5.35 Hz); δ_C (126MHz, $CDCl_3$): 175.17, 98.49, 97.82, 81.43, 80.95, 73.69, 62.75, 62.42, 50.13, 36.43, 35.68, 35.10, 32.48, 32.02, 31.92, 31.42, 31.27, 31.21, 30.22, 29.98, 29.94, 29.89, 29.62, 29.59, 29.54, 29.46, 29.42, 29.39, 29.35, 28.72, 27.59, 27.54, 27.43, 26.11, 25.72, 25.68, 25.64, 25.59, 25.07, 22.67, 22.59, 20.06, 19.80, 18.11, 17.92, 15.77, 15.16, 14.94, 14.09, 10.91, -4.26, -4.88; δc: 175.17, 98.49, 97.82, 81.43, 80.95, 73.69, 62.75, 62.42, 50.13, 36.43, 35.68, 35.10, 32.48, 32.02, 31.92, 31.42, 31.27, 31.21, 30.22, 29.98, 29.94, 29.89, 29.62, 29.59, 29.54, 29.46, 29.42, 29.39, 29.35, 28.72, 27.59, 27.54, 27.43, 26.11, 25.72, 25.68, 25.64, 25.59, 25.07, 22.67, 22.59, 20.06, 19.90, 18.11, 17.92, 15.77,15.16, 14.94, 14.09, 10.91, -4.26, -4.99; ν_{max}: 2924, 2853, 1708, 1465, 1254, 1077, 836, 775, 720 cm^{-1}.

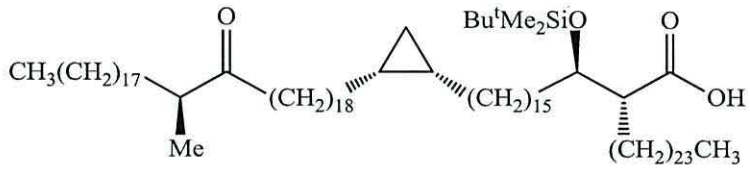
Experiment 14: (*R*)-2-{(*R*)-1-(*tert*-Butyldimethylsilanyloxy)-16-[(1*S*,2*R*)-2-[(19*S*, 20*S*)-19-hydroxy-20-methyloctatriacontyl)cyclopropyl]-hexadecyl} hexacosanoic acid (181)



Pyridinium-p-toluenesulfonate (300 mg, 1.2 mmol) was added to *R*)-2-((*R*)-1-(*tert*-butyldimethylsilanyloxy)-16-{(1*S*,2*R*)-2-[(19*S*,20*S*)-19-(tetrahydropyran-2-yloxy)octat -riacontyl]cyclopropyl}hexadecyl)hexacosanoic acid **(180)** (0.9 g, 0.62 mmol) in THF (10 mL), MeOH (1 mL) and stirred at 47 °C for 7 hours. Sat. aq. sodium bicarbonate (0.5 mL) was added and the product was extracted with petrol/ethyl acetate (3 × 25 mL, 1:1). The combined organic layers were dried and evaporated. The crude product was purified by column chromatography eluting with petrol/ethyl acetate (10:1) to give a white semi solid, (*R*)-2-{(*R*)-1-(*tert*-butyldimethylsilanyloxy)-16-[(1*S*,2*R*)-2-[(19*S*,20*S*)-19-hydroxy-20-methyloctatriacontyl)cyclopropyl]hexadecyl }hexacosanoic acid **(181)** (0.7 g, 81%), $[\alpha]_D^{21}$ = - 3.16 (*c* 0.79, $CHCl_3$) {Found $(M + Na)^+$: 1376.3523, $C_{90}H_{180}O_4SiNa$ requires: 1376.3543}, which showed δ_H (500MHz, $CDCl_3$): 3.84 (1H, br.pent, *J* 5.05 Hz), 3.50 (1H, br.pent, *J* 3.8 Hz), 2.53 (1H, ddd, *J* 3.15, 5.35, 9.15 Hz),

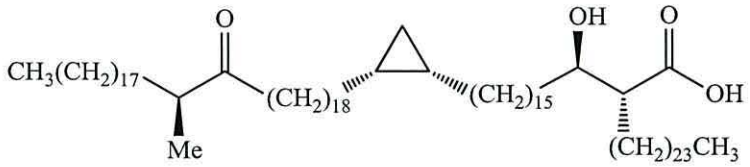
1.69-1.60 (1H, m), 1.58-1.53 (2H, m), 1.43-1.13 (146H, m), 0.92 (9H, s), 0.88 (9H, br.q, *J* 6.95 Hz), 0.66-0.64 (2H, m), 0.56 (1H, dt, *J* 4.05, 8.15 Hz), 0.14 (3H, s), 0.129 (3H, s), -0.32 (1H, br.q, *J* 5.05 Hz); δ_C (126MHz, $CDCl_3$): 175.47, 75.25, 73.68, 50.23, 38.16, 35.58, 34.50, 33.37, 31.92, 30.22, 29.96, 29.70, 29.66, 29.47, 29.43, 29.40, 29.36, 28.72, 27.45, 27.41, 26.27, 25.73, 25.01, 22.68, 17.93, 15.78, 14.10, 13.57, 10.9, -4.26, -4.88; ν_{max}: 3410, 2919, 2851, 1708, 1467, 1361, 1253, 1103, 907, 835, 775, 721 cm^{-1}.

Experiment 15: (*R*)-2-{(*R*)-1-(*tert*-Butyldimethylsilanyloxy)-16-[(1*S*,2*R*)-2-((*S*)-20-methyl-19-oxo-20-octatriacontyl)cyclopropyl]hexadecyl}-hexacosanoic acid (182)



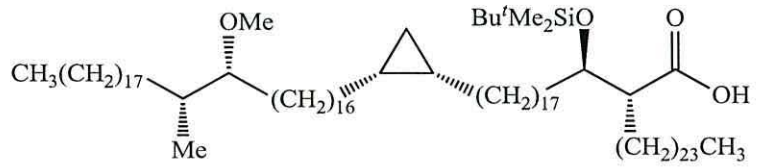
The procedure used in Experiment **8** was repeated in order to oxidise (*R*)-2-{(*R*)-1-(*tert*-butyldimethylsilanyloxy)-16-[(1*S*,2*R*)-2-[(19*S*,20*S*)-19-hydroxy-20-methyloctatri -acontyl)cyclopropyl]hexadecyl}hexacosanoic acid **(181)** (0.7 g, 0.513 mmol) using PCC (0.38 g, 1.76 mmol) in dichloromethane (25 mL). The crude product was purified by chromatography eluting with petrol/ethyl acetate (20:1) to give (*R*)-2-{(*R*)-1-(*tert*-butyldimethylsilanyloxy)-16-[(1*S*,2*R*)-2-((*S*)-20-methyl-19-oxo-20-octatriacontyl)cycl -opropyl]hexadecyl}hexacosanoic acid **(182)** as a white semi-solid (0.57 g, 82%), $[\alpha]_D^{23}$ = +7.04 (*c* 0.71, $CHCl_3$) {Found $(M + Na)^+$: 1374.3350, $C_{90}H_{178}O_4SiNa$ requires: 1374.3367}, which showed δ_H (500MHz, $CDCl_3$): 3.85-3.82 (1H, m), 2.54-2.48 (2H, m), 2.41 (2H, dt, *J* 2.0, 7.25 Hz), 171-160 (2H, m), 1.58-1.13 (143H, m), 1.05 (3H, d, *J* 6.6 Hz), 0.92 (9H, s), 0.89 (6H, t, *J* 6.95 Hz), 0.67-0.64 (2H, m), 0.56 (1H, br.dt, *J* 4.1, 8.2 Hz), 0.14 (3H, s), 0.13 (3H, s), -0.32 (1H, br.q, *J* 5.0 Hz); δ_C (126MHz, $CDCl_3$): 215.16, 175.93, 73.64, 50.39, 46.32, 41.70, 41.35, 41.13, 35.38, 34.12, 33.04, 31.92, 30.22, 29.70, 29.60, 29.55, 29.50, 29.49, 29.46, 29.40, 29.35, 29.34, 29.05, 28.72, 27.66, 27.46, 27.32, 26.08, 25.82, 25.73, 24.85, 23.72, 22.67, 22.59, 20.42, 17.93, 16.35, 15.77, 14.29, 14.09, 10.91, -4.27, -4.90; ν_{max}: 2919, 2851, 1708, 1467, 1361, 1253, 1075, 908, 836, 775, 735 cm^{-1}.

Experiment 16: (*R*)-2-{(*R*)-1-Hydroxy-16-[(1*S*,2*R*)-2-((*S*)-20-methyl-19-oxo-20-octatriacontyl)cyclopropyl]hexadecyl}hexacosanoic acid (165)



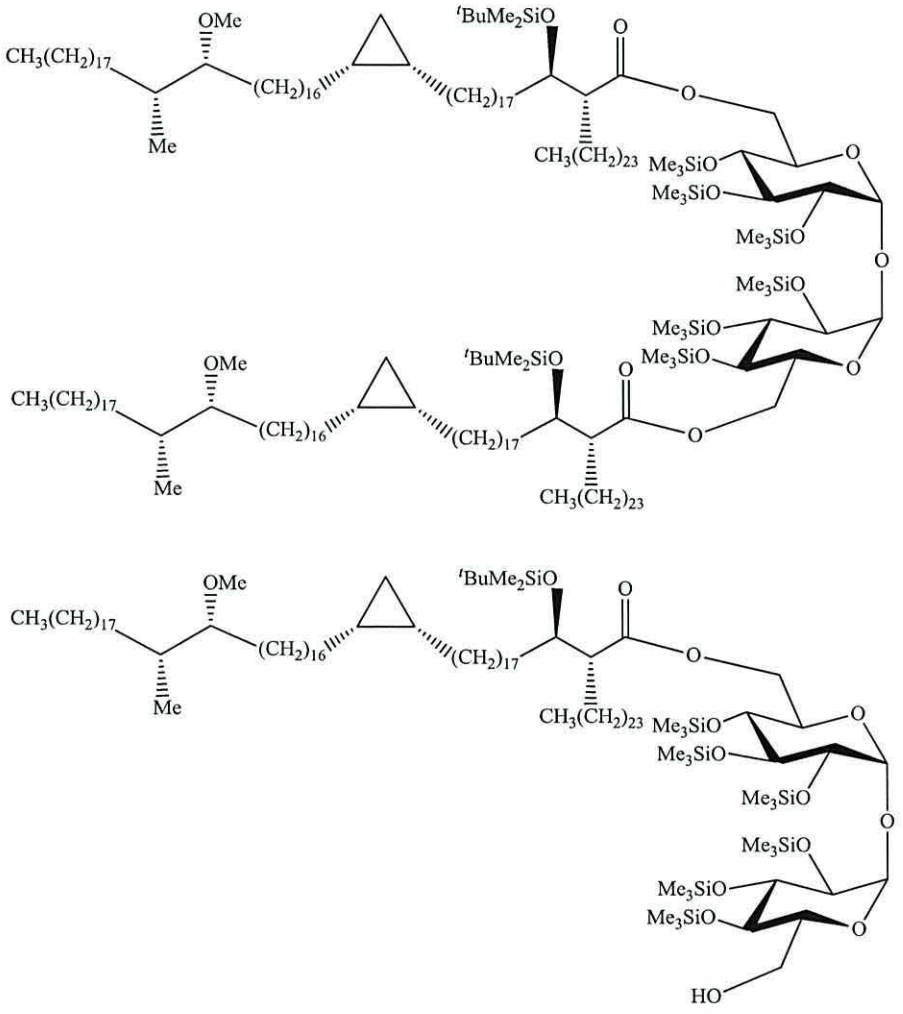
The procedure used in Experiment **2** was repeated using (*R*)-2-{(*R*)-1-(*tert*-butyldimethylsilanyloxy)-16-[(1*S*,2*R*)-2-((*S*)-20-methyl-19-oxo-20-octatriacontyl)cycl -opropyl]hexadecyl}hexacosanoic acid **(182)** (0.12 g, 0.088 mmol), pyridine (0.1 mL) and HF.pyridine (0.6 mL) in dry THF (8 mL) The crude product was purified by column chromatography eluting with petrol/ethyl acetate (10:1) to give (*R*)-2-{(*R*)-1-hydroxy-16-[(1*S*,2*R*)-2-((*S*)-20-methyl-19-oxo-20-octatriacontyl)cyclopropyl]hexadec -yl}hexacosanoic acid **(165)** as a white solid (75 mg, 69%), m.p 65-67 °C, $[\alpha]_D^{21} = +$ 9.14 (*c* 0.71, $CHCl_3$) {MALDI Found $(M + Na)^+$: 1260.32, $C_{84}H_{164}O_4Na$ requires: 1260.25}, which showed δ_H (500MHz, $CDCl_3$): 3.75-3.71 (1H, m), 2.50 (1H, br.q, *J* 5.6 Hz), 2.48-2.45 (1H, m), 2.42 (2H, dt, *J* 1.04, 5.7 Hz), 1.74-1.11 (146H, m), 1.05 (3H, d, *J* 5.5 Hz), 0.88 (6H, t, *J* 5.4 Hz), 0.66-0.63 (2H, m), 0.56 (1H, dt, *J* 3.08, 6.5 Hz), -0.32 (1H, br.q, *J* 4.12 Hz); δ_C (126MHz, $CDCl_3$): 215.43, 178.42, 72.11, 50.69, 46.33, 41.14, 35.54, 33.03, 31.92, 30.91, 30.21, 29.69, 29.65, 29.59, 29.58, 29.50, 29.46, 29.41, 29.35, 29.32, 28.71, 27.32, 25.72, 23.72, 22.68, 16.35, 15.76, 14.10, 10.89; ν_{max}: 3470, 2922, 2852, 1725, 1709, 1470, 1377, 717 cm^{-1}.

Experiment 17: (*R*)-2-{(*R*)-1-(*tert*-Butyldimethylsilanloxy)-18-[(1*S*,2*R*)-2-((17*R*, 18*R*)-17-methoxy-18-methylhexatriacontyl)cyclopropyl]octadecyl}hexacosanoic acid (194)



Imidazole (0.4 g, 5.89 mmol) was added to a stirred solution of (*R*)-2-{(*R*)-1-hydroxy-18-(1*S*,2*R*)-2-[(17*R*,18*R*)-17-methyoxy-18-methylhexatricontyl)cylopropyl]octadecyl }hexacosanoic acid **(101)** (0.66 g, 0.52 mmol) in dry DMF (3 mL) and dry toluene (4 mL) at room temperature, followed by the addition of *tert*-butyldimethylsilylchloride (0.9 g, 6 mmol) and 4-dimethylaminopyridine (0.068 g, 0.56 mmol). The reaction mixture was stirred at 70 °C for 24 hours. When TLC showed no starting material was left, the solvent was removed under high vacuum and the residue was diluted with petrol/ethyl acetate (10:1) (50 mL) and water (20 mL). The organic layer was separated and the aqueous layer was re-extracted with petrol/ethyl acetate (2 × 30 mL). The combined organic layers were washed with water, dried and evaporated to give a colourless oil. The residue was dissolved in THF (15 mL), water (2 mL) and methanol (2 mL) and to this was added potassium carbonate (0.3 g). The reaction mixture was stirred at 45 °C for 18 hours, then TLC showed no starting material was left. The mixture was diluted with petrol/ethyl acetate (10:1, 20 mL) and water (2 mL) then acidified with potassium hydrogen sulfate to pH 2. The organic layer was separated and the aqueous layer was re-extracted with petrol/ethyl acetate (2 × 20 mL). The combined organic layers were washed with water, dried and evaporated to give a residue, which was purified by column chromatography eluting with petrol/ethyl acetate (10:1) to give (*R*)-2-{(*R*)-1-(*tert*-butyldimethylsilanloxy)-18-[(1*S*,2*R*)-2-((17*R*,18*R*)-17-methoxy-18-methylhexatriacontyl)cyclopropyl]octadecyl}hexacosanoic acid **(194)** as a colourless oil (0.48 g, 67 %), $[\alpha]_D^{23} = +5.62$ (*c* 0.80, $CHCl_3$) {Found $(M + Na)^+$: 1390.3708, $C_{91}H_{182}O_4SiNa$ requires: 1390.3705}, which showed δ_H (500MHz, $CDCl_3$): 3.87-3.84 (1H,m), 3.34 (3H, s), 2.96 (1H, pent, *J* 5.05 Hz), 2.53 (1H, ddd, *J*, 3.8, 5.8, 9.45 Hz), 1.73-1.10 (148H, m), 0.91 (9H, s), 0.89 (6H, t, *J* 6.9 Hz), 0.86 (3H, d, *J* 6.6 Hz) 0.67-0.63 (2H, m), 0.56 (1H, dt, *J* 4.1, 8.2 Hz), 0.12 (3H, s), 0.11 (3H, s), -0.32 (1H, br.q, *J* 5.05 Hz); δ_C (126MHz, $CDCl_3$): 176.47, 85.47, 73.36, 57.68, 50.48, 41.35, 36.08, 35.30, 33.72, 32.40, 32.06, 31.92, 31.58, 30.50, 30.38, 30.22, 29.98, 29.94, 29.56, 29.49, 29.46, 29.41, 29.36, 29.05, 28.88, 28.72, 27.66, 27.57, 27.49, 26.16, 25.74, 24.81, 22.68, 22.60, 20.43, 19.42, 18.74, 17.94, 15.77, 14.88, 14.29, 14.09, 10.91, -4.28, -4.90; ν_{max}: 3450, 2921, 2851, 1709, 1465, 1362, 1254, 1099, 836 cm^{-1}.

Experiment 18: 6,6'-bis-O-(*R*)-2-{(*R*)-1-(*tert*-Butyldimethylsilanyloxy)-18-[(1*S*,2*R*)-2-((17*R*,18*R*)-17-methoxy-18-methylhexatriacontyl)cyclopropyl]octadec -yl}hexacosanoic-2,3,4,2',3',4',-hexakis-O-(trimethylsiyl)α,α'-trehalose (195) and 6-O-(*R*)-2-{(*R*)-1-(*tert*-butyldimethylsilanyloxy)-18-[(1*S*,2*R*)-2-((17*R*,18*R*)-17-methoxy-18-methylhexatriacontyl)cyclopropyl]octadecyl}hexacosanoic-2,3,4, 2',3',4',-hexakis-O-(trimethylsilyl)-α,α'-trehalose (196)

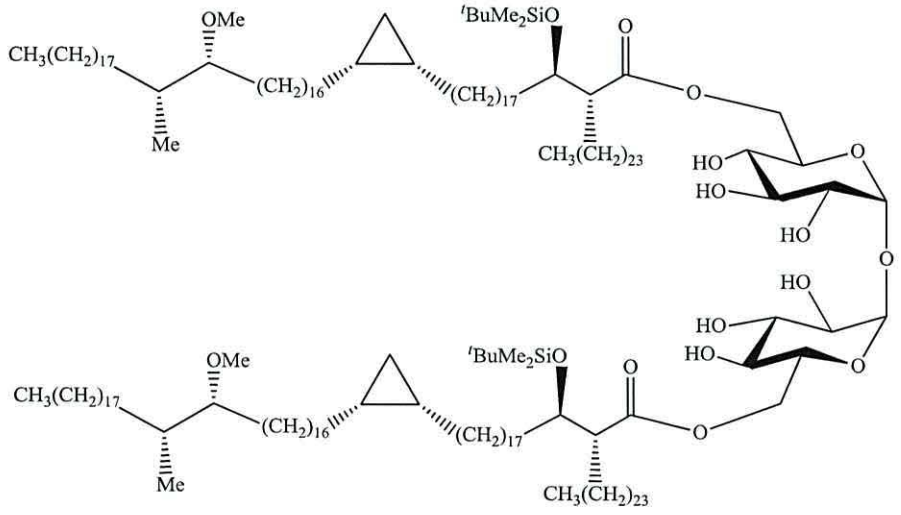


1-(3-Dimethylaminopropyl)-3-ethylcarbodimidehydrochloride (EDCL) (257 mg, 1.34 mmol) and 4-dimethylaminopyridine (143 mg, 1.17 mmol) were added to a stirred solution of (*R*)-2-{(*R*)-1-(*tert*-butyldimethylsilanloxy)-18-[(1*S*,2*R*)-2-((17*R*,18*R*)-17-methoxy-18-methylhexatriacontyl)cyclopropyl]octadecyl}hexacosanoic acid **(194)** (481 mg, 0.35 mmol), 2,3,4,2',3',4'-hexaakis-O-(trimethylsilyl)-α,α'-trehalose **(110)** (130 mg, 0.167 mmol) and powdered 4 ˚A molecular sieve in dry dichloromethane (3 mL) at room temperature under nitrogen. The reaction mixture was stirred for 6 days at

room temperature when TLC showed no starting material was left. The reaction mixture was diluted with dichloromethane and filtered. The filtrate was evaporated under reduced pressure to give a residue, which was purified by column chromatography eluting with petrol/ethyl acetate (20:1) to give the first fraction 6,6'-bis-O-(*R*)-2-{(*R*)-1-(*tert*-butyldimethylsilanyloxy)-18-[(1*S*,2*R*)-2-((17*R*,18*R*)-17-methoxy-18-methylhexatriacontyl)cyclopropyl]octadecyl}hexacosanoic-2,3,4,2',3',4'-hexakis-O-(trimethylsilyl)-α,α'-trehalose **(195)** (0.14 g, 22%) as a colourless thick oil, $[\alpha]_D^{23}$ = + 24.48 (*c* 0.98, $CHCl_3$) {MALDI Found (M + Na)$^+$: 3496.75, $C_{212}H_{430}O_{17}Si_8Na$ requires: 3496.08}, which showed δ_H (500MHz, $CDCl_3$): 4.86 (2H, d, *J* 2.85 Hz), 4.37 (2H, br.d, *J* 10.10 Hz), 4.04-3.99 (4H, m), 3.96-3.88 (4H, m), 3.52 (2H, t, *J* 9.15 Hz), 3.39 (2H, dd, *J* 3.15, 9.45 Hz), 3.34 (6H, s), 2.96 (2H, br.pent, *J* 4.1 Hz), 2.55 (2H, ddd, *J* 3.6, 4.7, 10.1 Hz), 1.67-1.61 (4H, m), 1.46-1.10 (290H, m), 0.89 (12H, t, *J* 6.9 Hz), 0.88 (18H, s), 0.85 (6H, d, *J* 6.9 Hz) 0.66-0.63 (4H, m), 0.56 (2H, dt, *J* 4.1, 8.2 Hz), 0.16 (18H, s), 0.148 (18H, s), 0.140 (18H, s), 0.064 (12H, s), -0.31 (2H, br.q, *J* 5.05 Hz); δ_C (126MHz, $CDCl_3$): 173.79, 94.86, 85.45, 73.56, 73.43, 73.24, 72.85, 71.84, 70.76, 62.38, 57.69, 51.87, 35.38, 33.47, 32.40, 31.94, 30.52, 30. 24, 29.99, 29.95, 29.86, 29.71, 29.61, 29.59, 29.52, 29.44, 29.37, 28.74, 28.13, 27.83, 27.59, 27.51, 26.24, 26.17, 25.84, 25.20, 22.69, 18.03, 15.78, 14.88, 14.11, 10.94, 1.18, 1.10, 0.16, -4.50, -4.63; ν_{max}: 2924, 2853, 1742, 1465, 1251, 1099, 1077, 872, 841 cm^{-1}; and the second fraction was 6-O-(*R*)-2-{(*R*)-1-(*tert*-butyldimethylsilanyloxy)-18-[(1*S*,2*R*)-2-((17*R*,18*R*)-17-methoxy-18-methylhexatriacontyl)-cyclopropyl]octadecyl}hexacosanoic-2,3,4,2',3',4',-hexakis-O-(trimethylsilyl)-α,α'-trehalose **(196)** (0.3 g, 78%), $[\alpha]_D^{23}$ = + 45.83 (*c* 0.96, $CHCl_3$) {MALDI Found (M + Na)$^+$: 2146.75, $C_{121}H_{250}O_{14}Si_7Na$ requires: 2146.71}, which showed δ_H (500MHz, $CDCl_3$): 4.91 (1H, d, *J* 3.15 Hz), 4.84 (1H, d, *J* 2.85 Hz), 4.35 (1H, dd, *J* 4.1, 11.90 Hz), 4.08 (1H, dd, *J* 4.1, 12.00 Hz), 3.98 (1H, br.q, *J* 5.25 Hz), 3.94-3.83 (4H, m), 3.72-3.66 (2H, m), 3.48 (2H, dt, *J* 6.6, 8.85 Hz), 3.44 (1H, dd, *J* 3.15, 9.45 Hz), 3.39 (1H, dd, *J* 2.85, 9.15 Hz), 3.34 (3H, s), 2.96-2.94 (1H, m), 2.55 (1H, ddd, *J*, 3.65, 5.75, 10.41 Hz), 1.73-1.70 (1H, m), 1.63-1.59 (2H, m), 1.49-0.96 (148H, m), 0.90-0.81 (15H, m, including a singlet at δ 0.86), 0.65 (2H, br.m), 0.56 (1H, dt, *J* 3.75, 8.15 Hz), 0.17 (9H, s), 0.16 (9H, s), 0.155 (9H, s), 0.150 (9H, s), 0.148 (9H, s), 0.122 (9H, s), 0.060 (3H, s), 0.056 (3H, s), -0.32 (1H, br.q, *J*, 4.75); δ_C (126MHz, $CDCl_3$): 174.05, 94.53, 94.41, 85.44, 73.44, 73.37, 72.89, 72.83, 72.78, 72.00, 71.44, 70.75, 62.45,

61.68, 57.70, 51.84, 35.36, 33.43, 32.39, 31.92, 30.50, 30.22, 29.98, 29.94, 29.82, 29.70, 29.54, 29.36, 28.72, 28.11, 27.57, 26.38, 26.16, 25.82, 24.88, 22.68, 18.02, 15.77, 14.88, 14.10, 10.92, 1.05, 1.01, 0.92, 0.84, 0.17, 0.045, -4.48, -4.67; ν_{max}: 2924, 2854, 1743, 1464, 1251, 1165, 1077, 843 cm^{-1}.

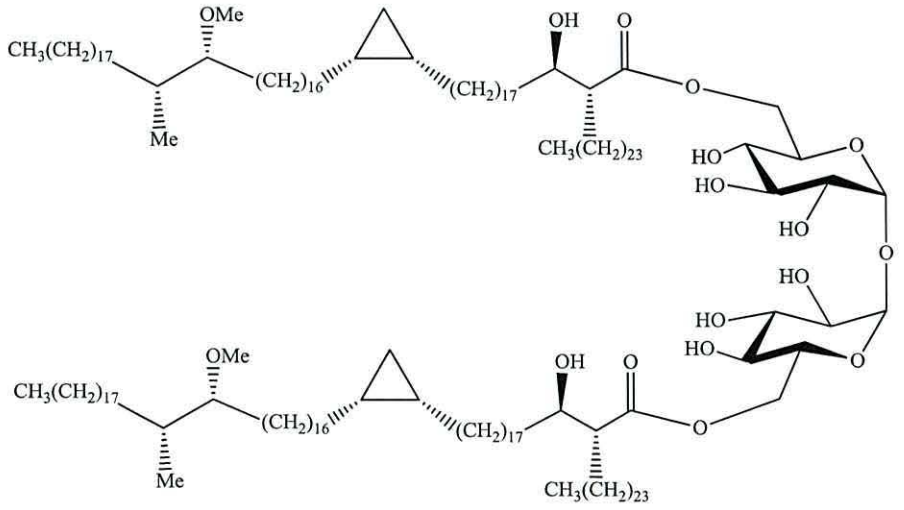
Experiment 19: 6,6'-bis-O-(*R*)-2-{(*R*)-1-(*tert*-Butyldimethylsilanyloxy)-18-[(1*S*,2*R*)-2-((17*R*,18*R*)-17-methoxy-18-methylhexatriacontyl)cyclopropyl]octadec -yl}hexacosanoic-α,α'-trehalose (197)



Tetrabutylammonium fluoride (0.32 mL, 0.32 mmol, 1 M) was added to a stirred solution of 6,6'-bis-O-(*R*)-2-{(*R*)-1-(*tert*-butyldimethylsilanyloxy)-18-[(1*S*,2*R*)-2-((17*R*,18*R*)-17-methoxy-18-methylhexatriacontyl)cyclopropyl]octadecyl}hexacosanoic -2,3,4,2',3',4'-hexakis-O-(trimethylsiyl)-α,α'-trehalose **(195)** (0.14 g, 0.04 mmol) in dry THF(6 mL) at 5 °C under nitrogen. The reaction mixture was allowed to reach room temperature and stirred for one hour, When TLC showed no starting material was left. The reaction was cooled to 5 °C and quenched with sat. aq. sodium bicarbonate (3 mL) then diluted with cold $CHCl_3$ (50 mL). The organic layer was separated and the aqueous layer was re-extracted with $CHCl_3$ (2 × 50 mL). The combined organic layers were washed with brine solution (50 mL), dried and evaporated to give a residue, which was purified by column chromatography eluting with $CHCl_3$/MeOH (10:1) to give 6,6'-bis-O-(*R*)-2-{(*R*)-1-(*tert*-butyldimethyl-silanyloxy)-18-[(1*S*,2*R*)-2-((17*R*,18*R*) -17-methoxy-18-methylhexatriacontyl)cyclopropyl]octadecyl}hexacosanoic-α,α'-trehal -ose **(197)** (0.06 g, 50%), $[\alpha]_D^{23}$ = +38.47 (*c* 0.92, $CHCl_3$) {MALDI Found $(M + Na)^+$:

3065.95, $C_{194}H_{382}O_{17}Si_2Na$ requires: 3065.85}, which showed δ_H (500MHz, $CDCl_3$ + few drops of CD_3OD): 5.03 (2H, d, *J* 3.15 Hz), 4.28 (2H,br.d, *J* 8.2 Hz), 4.20 (2H, br.d, *J* 10.7 Hz), 3.92 (2H, br.d, *J* 9.15 Hz), 3.86 (2H, d *J* 5.05 Hz), 3.79 (2H, t, *J* 9.45 Hz), 3.44 (2H, d, *J* 9.75 Hz), 3.32-3.27 (8H, m, including a singlet at δ 3.29), 2.93 (2H, d, *J* 3.75 Hz), 2.53 (2H, ddd, *J* 3.6, 6.95, 10.70 Hz),1.56-1.05 (300H, m), 0.83 (12H, t, *J* 7.25 Hz), 0.82 (18H, s), 0.79 (6H, d, *J* 7.25 Hz), 0.62-0.56 (4H, br.m), 0.50 (2H, dt, *J* 4.1, 8.15 Hz), -0.007 (6H, s), -0.02 (6H, s), -0.32 (2H, br.q, *J* 5.30 Hz); δ_C (126MHz, $CDCl_3$): 175.05, 94.18, 93.57, 85.52, 73.17, 73.01, 71.64, 70.24, 69.88, 62.81, 57.52, 51.61, 35.25, 33.51, 32.26, 31.78, 30.37, 30.09, 29.82, 29.76, 29.69, 29.57, 29.55, 29.33, 29.22, 28.59, 27.66, 27.38, 26.85, 25.97, 25.74, 25.63, 24.18, 22.54, 17.82, 15.64, 14.66, 13.91, 10.76, -4.64, -5.02; ν_{max}: 3368, 2920, 2851, 1732, 1466, 1377, 1254, 1100, 1049, 836, 760, 720 cm^{-1}.

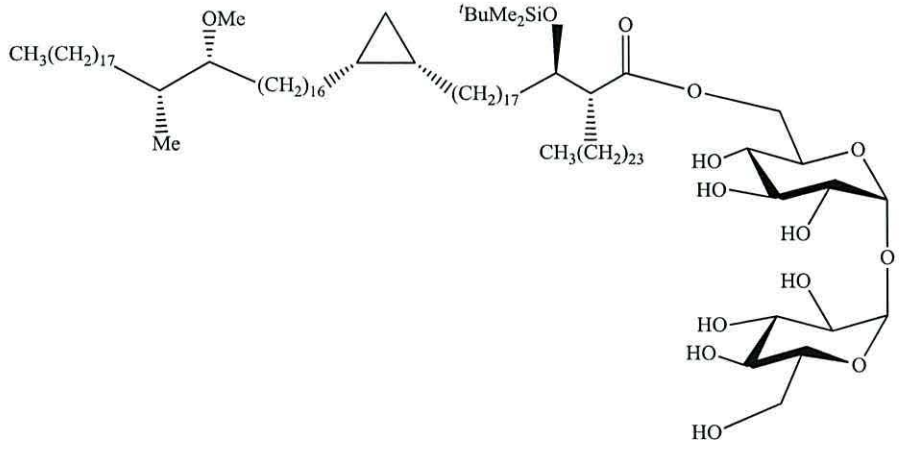
Experiment 20: 6,6'-bis-O-(*R*)-2-{(*R*)-1-Hydroxy-18-[(1*S*,2*R*)-2-((17*R*,18*R*)-17-methoxy-18-methylhexatriacontyl)cyclopropyl]octadecyl}hexacosanoic-α,α'-trehalose (198)



The procedure used in Experiment **2** was repeated using 6,6'-bis-O-(*R*)-2-{(*R*)-1-(*tert*-butyldimethylsilanyloxy)-18-[(1*S*,2*R*)-2-((17*R*,18*R*)-17-methoxy-18-methylhexatriaco-ntyl)cyclopropyl]octadecyl}hexacosanoic-α,α'-trehalose **(197)** (50 mg, 0.016 mmol), pyridine (0.1 mL) and HF.pyridine (0.2) in dry THF (5 mL). The crude product was purified by chromatography column eluting with $CHCl_3$/MeOH (10:1) to give 6,6'-bis-O-(*R*)-2-{(*R*)-1-hydroxy-18-[(1*S*,2*R*)-2-((17*R*,18*R*)-17-methoxy-18-methylhexatriacon

-tyl)cyclopropyl]octadecyl}hexacosanoic-α,α'-trehalose **(198)** (20 mg, 46%) as syrup, $[\alpha]_D^{23}$ = +20.83 (*c* 0.96, $CHCl_3$) {MALDI Found $(M + Na)^+$: 2838.01, $C_{182}H_{354}O_{17}Na$ requires: 2837.74}, which showed δ_H (500MHz, $CDCl_3$ + few drops of CD_3OD): 4.98 (2H, d, *J* 3.15 Hz), 4.74 (2H, br.d, *J* 10.40 Hz), 4.29 (2H, br.t, *J* 9.15 Hz), 3.89 (2H, br.t, *J* 7.9 Hz), 3.75 (2H, t, *J* 9.8 Hz), 3.5 (2H, dd, *J* 2.8, 9.45 Hz), 3.37 (2H, d, *J* 2.0 Hz), 3.31 (6H, s), 3.19 (2H, br.t, *J* 9.8 Hz), 2.95 (2H, br.q, *J* 5.1 Hz), 2.39-2.28 (2H, m), 1.58-1.07 (302H, m), 0.85 (12H, t, *J* 6.6 Hz), 0.82 (6H, d, *J* 6.95 Hz), 0.64-0.58 (4H, m), 0.53 (2H, dt, *J* 4.1, 8.15 Hz), -0.35 (2H, br.q, *J* 5.05); δ_C (126MHz, $CDCl_3$): 175.34, 94.51, 85.51, 72.51, 71.20, 71.00, 69.81, 64.01, 57.46, 52.32, 35.17, 34.60, 32.19, 31.73, 30.30, 30,04, 30,03, 29.76, 29.69, 29.50, 29.36, 29.26, 29.17, 28.53, 27.32, 27.13, 25.89, 25.66, 25.09, 22.49, 15.58, 14.59, 13.85, 10.69; ν_{max}: 3389, 2918, 2851, 1731, 1467, 1377, 1149, 1100, 992, 836, 720 cm^{-1}.

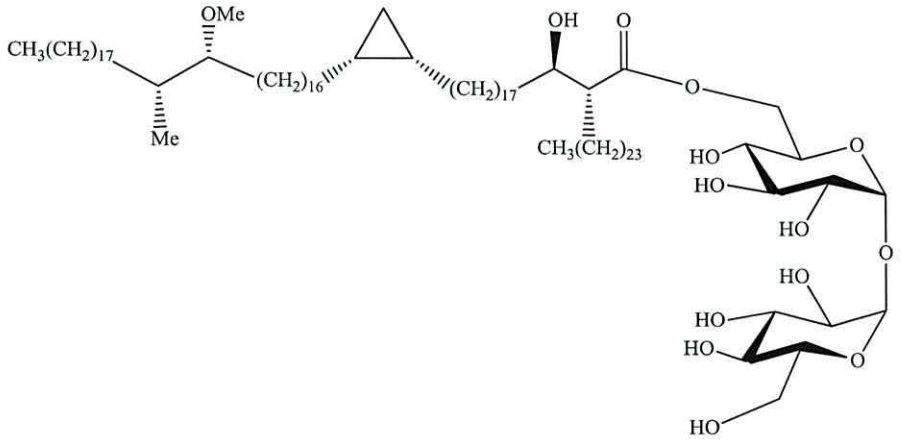
Experiment 21: 6-O-(*R*)-2-{(*R*)-1-(*tert*-Butyldimethylsilanyloxy)-18-[(1*S*,2*R*)-2-((17*R*,18*R*)-17-methoxy-18-methylhexatriacontyl)cyclopropyl]-octadecyl}hexacos-anoic-α,α'-trehalose (199)



The procedure used in Experiment **19** was repeated using 6-O-(*R*)-2-{(*R*)-1-(*tert*-butyldimethylsilanyloxy)-18-[(1*S*,2*R*)-2-((17*R*,18*R*)-17-methoxy-18-methylhexatriaco-ntyl)cyclopropyl]octadecyl}hexacosanoic-2,3,4,2',3',4',-hexakis-O-(trimethylsiyl)-α,α'-trehalose **(196)** (0.3 g, 0.14 mmol) and tetrabutylammonium fluoride (1.12 mL, 1.129 mmol, 1M) in dry THF (6 mL). The crude product was purified by column chromatography eluting with $CHCl_3$/MeOH (10:1) to give 6-O-(*R*)-2-{(*R*)-1-(*tert*-butyldimethylsilanyloxy)-18-[(1*S*,2*R*)-2-((17*R*,18*R*)-17-methoxy-18-methylhexatriaco

-ntyl)cyclopropyl]octadecyl}hexacosanoic-α,α'-trehalose **(199)** (0.16 g, 69%), $[\alpha]_D^{23}$ = +30.56 (*c* 0.88, $CHCl_3$), {MALDI Found $(M + K)^+$: 1730.45, $C_{103}H_{202}O_{14}SiK$ requires: 1730.45}, which showed δ_H (500MHz, $CDCl_3$ + few drops of CD_3OD): 5.05 (2H, br.s), 4.32-4.24 (2H, m), 3.92-3.89 (3H, m), 3.81 (2H, br.d, *J* 9.5 Hz), 3.72-3.68 (2H, m), 3.52 (2H, br.m), 3.37-3.34 (2H, m), 3.31 (3H, s), 2.95-2.92 (1H, m), 2.52 (1H, br.m), 1.61-1.07 (154H, m), 0.86-0.79 (18H, m, including a singlet at δ 0.83), 0.62-0.61 (2H, br.m), 0.53 (1H, dt, *J* 3.8, 7.9 Hz), 0.02 (3H, s), 0.00 (3H, s), -0.35 (1H, br.q, *J* 5.35 Hz); δ_C (126MHz, $CDCl_3$): 175.03, 93.52, 93.46, 85.51, 73.12, 72.98, 72.67, 72.09, 71.55, 70.65, 70.19, 69.87, 67.76, 62.66, 61.86, 60.39, 57.42, 51.61, 35.23, 33.46, 32.25, 31.71, 30.34, 30.01, 29.73, 29.67, 29.61, 29.53, 29.48, 29.44, 29.21, 29.14, 28.52, 27.56, 27.30, 26.79, 25.87, 25.63, 25.53, 25.33, 24.14, 22.45, 17.75, 15.58, 14.56, 13.83, 13.79, 10.68, -4.74, -5.11; ν_{max}: 3369, 2919, 2851, 1723, 1465, 1100, 1048, 994, 836, 760 cm^{-1}.

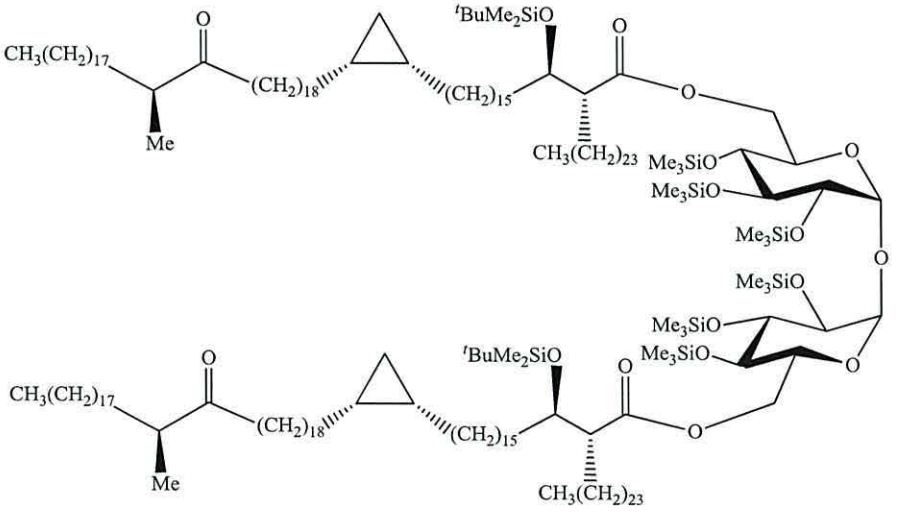
Experiment 22: 6-O-(*R*)-2-{(*R*)-1-hydroxy-18-[(1*S*,2*R*)-2-((17*R*,18*R*)-17-methoxy-18-methylhexatriacontyl)cyclopropyl]octadecyl}hexacosanoic-α,α'-trehalose (200)

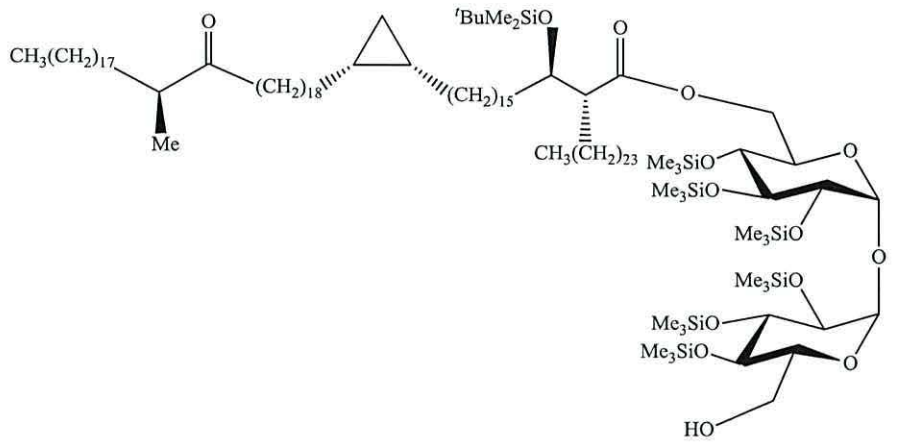


The procedure in Experiment **2** was repeated using 6-O-(R)-2-{(R)-1-(*tert*-butyldimethylsilanyloxy)-18-[(1S,2R)-2-((17R,18R)-17-methoxy-18-methylhexatriaco -ntyl)-cyclopropyl]octadecyl}hexacosanoic-α,α'-trehalose **(199)** (0.12 g, 0.070 mmol), pyridine (0.1 mL) and HF.pyridine (0.2) in dry THF (5 mL). The product was purified by chromatography eluting with $CHCl_3$/MeOH (10:1) to give 6-O-(*R*)-2-{(*R*)-1-hydroxy-18-[(1*S*,2*R*)-2-((17*R*,18*R*)-17-methoxy-18-methylhexatriacontyl) cyclopropyl]octadecyl}hexacosanoic-α,α'-trehalose **(200)** (0.1 g, 90%) as a syrup, $[\alpha]_D^{23}$ = +35.11

(*c* 0.9, $CHCl_3$) {MALDI Found $(M + Na)^+$: 1600.65, $C_{97}H_{188}O_{14}Na$ requires: 1600.39}, which showed δ_H (500MHz, $CDCl_3$ + few drops of CD_3OD); 5.07 (1H, d, *J* 4.0 Hz), 5.00 (1H, d, *J* 3.45 Hz), 4.68 (1H, br.d, *J* 11.65 Hz), 4.23 (1H, br.t, *J* 8.2 Hz), 3.97-3.77 (4H, m), 3.64-3.54 (4H, m), 3.47 (1H, d, *J* 10.4 Hz), 3.31-3.25 (4H, m, including a singlet at 3.30), 3.20 (1H, br.t, *J* 9.1 Hz), 2.94-2.93 (1H, m), 2.42-2.36 (1H, m), 1.57-1.55 (2H, m), 1.34-1.04 (153H, m), 0.84 (6H, t, *J* 6.6 Hz), 0.81 (3H, d, *J* 6.6 Hz), 0.62-0.58 (2H, m), 0.52 (1H, dt, *J* 3.75, 8.5 Hz), -0.36 (1H, br.q, *J* 5.05); δ_C (126MHz, $CDCl_3$): 175.45, 93.90, 85.51, 72.54, 72.28, 71.35, 70.74, 70.12, 63.87, 62.00, 57.60, 52.41, 35.30, 34.63, 32.33, 31.85, 30.78, 30.44, 30.18, 30.15, 30.07, 30.04, 29.89, 29.85, 29.75, 29.71, 29.65, 29.62, 29.42, 29.28, 28.67, 27.47, 27.20, 26.06, 25.12, 22.60, 15.69, 14.75, 14.00, 13.43, 10.84; ν_{max}: 3429, 2919, 2850, 1643, 1466, 1184, 1106 cm^{-1}.

Experiment 23: 6,6-bis-O-(*R*)-2-{(*R*)-1-(*tert*-Butyldimethylsilanyloxy)-16-[(1*S*,2R)-2-((*S*)-20-methyl-19-oxo-20-octatriacontyl)cyclopropyl]hexadecyl}hexacosanoic-2,3,4,2',3',4',-hexakis-O-(trimethylsiyl)-α,α'-trehalose (201) and 6-O-(*R*)-2-{(*R*)-1-(*tert*-butyldimethylsilanyloxy)-16-[(1*S*,2*R*)-2-((*S*)-20-methyl-19-oxo-20-octatriacontyl)-cyclopropyl]hexadecyl}hexacosanoic-2,3,4,2',3',4',-hexakis-O-(trimethylsiyl)-α,α'-trehalose (202)

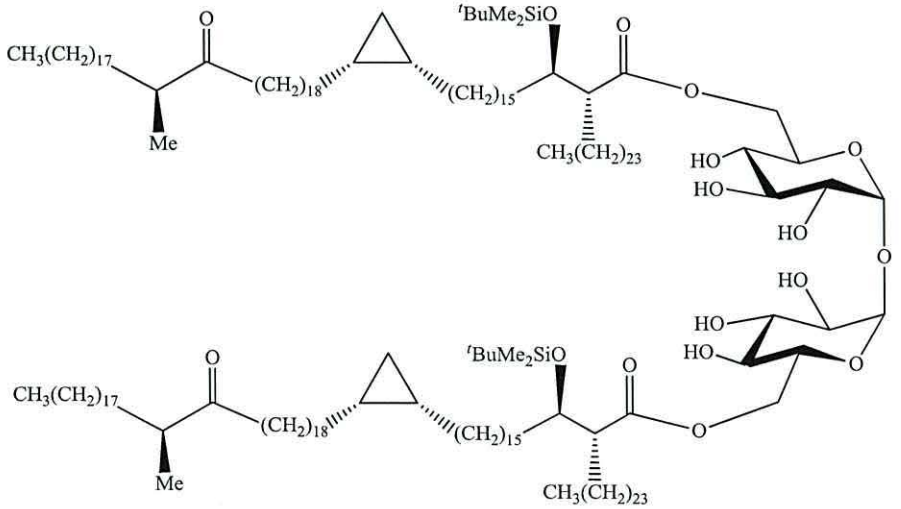




The procedure used in Experiment **18** was repeated using of (*R*)-2-{(*R*)-1-(*tert*-butyldimethylsilanyloxy)-16-[(1*S*,2*R*)-2-((*S*)-20-methyl-19-oxo-20-octatriacontyl) cyclopropyl]hexadecyl}hexacosanoic acid **(165)** (400 mg, 0.296 mmol), 2,3,4,2',3',4'-hexaakis-O-(trimethylsilyl)-α,α'-trehalose **(110)** (110 mg, 0.141 mmol), 1-(3-dimethylaminopropyl)-3-ethylcarbodimidehydrochloride (EDCL) (217 mg, 1.13 mmol), 4-dimethylaminopyridine (121 mg, 0.993 mmol) and powdered 4 ˚A molecular sieve in dry dichoromethane (3 mL). The crude product was purified by column chromatography on silica gel eluting with petrol/ethyl acetate (20:1) to give the first fraction 6,6-bis-O-(*R*)-2-{(*R*)-1-(*tert*-butyldimethylsilanyloxy)-16-[(1*S*,2*R*)-2-((*S*)-20-methyl-19-oxo-20-octatriacontyl)cyclopropyl]hexadecyl}hexacosanoic-2,3,4,2',3',4',-hexakis-O-(trimeth -ylsiyl)-α,α'-trehalose **(201)** (0.30 g, 63%) as a colourless thick oil, $[\alpha]_D^{23} = +35.58$ (*c* 0.77, $CHCl_3$) {MALDI Found $(M + Na)^+$: 3467.31, $C_{210}H_{422}O_{17}Si_8Na$ requires: 3467.26}, which showed δ_H (400MHz, $CDCl_3$): 4.85 (2H, d, *J* 2.86 Hz), 4.37 (2H, br.d, *J* 10.4 Hz), 4.01 (4H, br.t, *J* 11.0 Hz), 3.98-3.88 (4H, m), 3.53 (2H, t, *J* 8.76 Hz), 3.38 (2H, br.d, *J* 9.28 Hz), 2.56-2.48 (4H, m), 2.41(4H, t, *J* 7.4 Hz), 1.64-1.15 (288H, m), 1.05 (6H, d, *J* 6.95 Hz), 0.9-0.88 (30H, m, including singlet at δ 0.88), 0.65-0.63 (4H, br.m), 0.57 (2H, dt, *J* 3.52, 7.52 Hz), 0.16 (18H, s), 0.148 (18H, s), 0.140 (18H, s), 0.064 (12H, s), -0.31 (2H, br.q, *J* 4.76 Hz); δ_C (100MHz, $CDCl_3$): 215.19, 173.84, 94.80, 73.52, 73.39, 72.78, 71.78, 70.70, 62.37, 51.83, 46.31, 41.14, 33.42, 33.03, 31.92, 30.23, 29.84, 29.79, 29.72, 29.70, 29.65, 29.59, 29.51, 29.49, 29.46, 29.35, 29.32, 28.72, 28.11, 27.32, 25.91, 25.82, 25.14, 23.70, 22.68, 18.00, 16.36, 15.75, 14.11, 10.91, 1.08, 0.93, 0.14, -4.53, -4.66; ν_{max}: 2919, 2850, 1743, 1700, 1470, 1251, 1164, 1111, 1077, 872, 820 cm^{-1}. The second fraction was 6-O-(*R*)-2-{(*R*)-1-(*tert*-butyldimethylsilanyloxy)-16-[(1*S*,2*R*)-2-((*S*)-20-methyl-19-oxo-20-octatriacontyl)cyclopropyl]hexadecyl}hexacosanoic-2,3,4,2',3',4',-hexakis-O-(trime

-thylsiyl) -α,α'-trehalose **(202)** (0.11 g, 37%); as a colourless thik oil, $[\alpha]_D^{23} = +46.12$ (*c* 0.80, $CHCl_3$) {MALDI Found $(M + Na)^+$: 2131.96, $C_{120}H_{246}O_{14}Si_7Na$ requires: 2131.69}, which showed δ_H (400MHz, $CDCl_3$): 4.92 (1H, d, *J* 2.64 Hz), 4.85 (1H, d, *J* 2.40 Hz), 4.35 (1H, br.d, *J* 11.40 Hz), 4.07 (1H, dd, *J* 3.76, 11.80 Hz), 3.99 (1H, br.d, *J* 9.16 Hz), 3.95-3.88 (3H, m), 3.84 (1H, br.d, *J* 9.40 Hz), 3.70-3.65 (2H, m), 3.48 (2H, q, *J* 8.88 Hz), 3.42 (2H, dt, *J* 2.88, 9.52 Hz), 2.57-2.48 (2H, m), 2.41 (2H, t, *J* 7.4 Hz), 1.71 (2H, t, *J* 6.88 Hz), 1.63-1.14 (143H, m), 1.05 (3H, d, *J* 6.80 Hz), 0.88 (6H, t, *J* 8.8 Hz), 0.87 (9H, s), 0.67-0.62 (2H, m), 0.57 (1H, dt, *J* 3.76, 8.4 Hz), 0.17 (9H, s), 0.158 (9H, s), 0.152 (9H, s), 0.147 (9H, s), 0.141 (9H, s), 0.12 (9H, s), 0.06 (3H, s), 0.02 (3H, s), -0.31 (1H, br.q, *J* 5.04 Hz); δ_C (100MHz, $CDCl_3$): 215.23, 174.09, 94.48, 94.36, 73.41, 73.33, 72.85, 72.78, 72.73, 71.96, 71.38, 70.70, 62.43, 61.65, 56.08, 51.80, 46.31, 41.13, 33.39, 33.03, 31.91, 30.22, 29.81, 29.77, 29.71, 29.69, 29.59, 29.53, 29.49, 29.46, 29.35, 29.32, 28.71, 28.09, 27.31, 25.80, 24.82, 23.70, 22.68, 18.00, 16.36, 15.75, 14.11, 10.90, 1.04, 0.99, 0.92, 0.84, 0.16, 0.033, -4.50, -4.70; ν_{max}: 2924, 2853,1742, 1715, 1464, 1251, 1165, 1076, 1007, 898, 843 cm^{-1}.

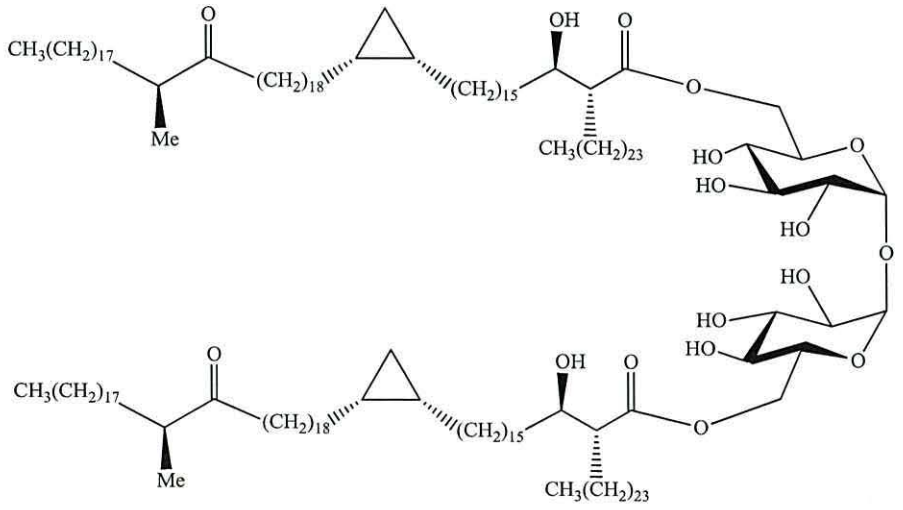
Experiment 24: 6,6-bis-O-(*R*)-2-{(*R*)-1-(*tert*-Butyldimethylsilanyloxy)-16-[(1*S*,2*R*) -2-((*S*)-20-methyl-19-oxo-20-octatriacontyl)cyclopropyl]hexadecyl}hexacosanoic α,α'-trehalose (203)



The procedure used in Experiment **19** was repeated using 6,6-bis-O-(*R*)-2-{(*R*)-1-(*tert*-butyldimethylsilanyloxy)-16-[(1*S*,2*R*)-2-((*S*)-20-methyl-19-oxo-20-octatriacontyl) cyclopropyl]hexadecyl}hexacosanoic-2,3,4,2',3',4',-hexakis-O-(trimethylsiyl)-α,α'-

trehalose **(201)** (0.3 g, 0.087 mmol) and tetrabutylammonium fluoride (0.26 mL, 0.26 mmol, 1 M) in dry THF (6 mL). The crude product was purified by column chromatography eluting with $CHCl_3$/MeOH (10:1) to give 6,6-bis-O-(*R*)-2-{(*R*)-1-(*tert*-butyldimethylsilanyloxy)-16-[(1*S*,2*R*)-2-((*S*)-20-methyl-19-oxo-20-octatriacontyl)cyclopropyl]hexadecyl}hexacosanoic-α,α'-trehalose **(203)** (0.180 g, 69%), $[\alpha]_D^{22} = +$ 29.33 (*c* 0.74, $CHCl_3$) {MALDI Found (M + Na)$^+$: 3033.92, $C_{192}H_{374}O_{17}Si_2Na$ requires: 3033.79}, which showed δ_H (400MHz, $CDCl_3$ + few drops of CD_3OD): 5.04 (2H, d, *J* 3.52 Hz), 4.32 (2H, br.d, *J* 11.92 Hz), 4.19 (2H, br.d, *J* 11.32 Hz), 3.87 (4H, br.t, *J* 11.44 Hz), 3.72 (2H, t, *J* 9.28 Hz), 3.44 (2H, dd, *J* 4.2, 9.64 Hz), 3.29 (2H, t, *J* 9.80 Hz), 2.52-2.44 (4H, m), 2.37 (4H, t, *J* 7.52 Hz), 1.56-1.07 (294H, m), 1.00 (6H, d, *J* 6.88 Hz), 0.83 (12H, t, *J* 6.28 Hz), 0.81 (18H, s), 0.67-0.63 (4H, m), 0.52 (2H, dt, *J* 3.64, 7.64 Hz), 0.0019(6H, s), -0.019 (6H, s), -0.37 (2H, br.q, *J* 5.24 Hz); δ_C (100MHz, $CDCl_3$): 216.02, 175.22, 93.43, 73.23, 72.95, 71.68, 70.23, 70.01, 62.88, 51.60, 46.27, 41.10, 33.60, 32.96, 31.85, 30.81, 30.17, 30.15, 29.76, 29.69, 29.62, 29.59, 29.52, 29.43, 29.41, 29.28, 29.24, 28.65, 27.72, 27.24, 25.79, 25.69, 23.64, 22.61, 17.89, 16.25, 15.68, 14.01, 10.83, -4.56, -4.97; ν_{max}: 3411, 2919, 2850, 1734, 1714, 1467, 1254, 1076, 1050, 991, 939, 835, 720 cm^{-1}.

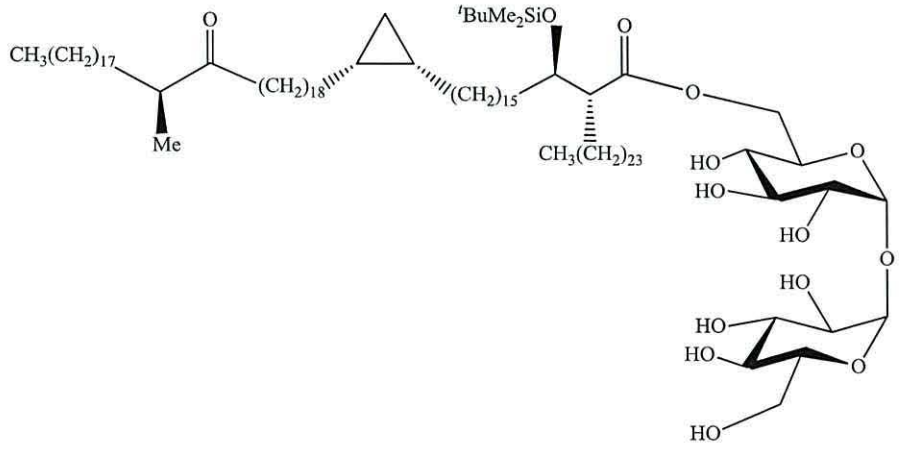
Experiment 25: 6,6-bis-O-(*R*)-2-{(*R*)-1-(Hydroxy)-16-[(1*S*,2*R*)-2-((*S*)-20-methyl-19-oxo-20-octatriacontyl)cyclopropyl]hexadecyl}hexacosanoic-α,α'-trehalose (204)



The procedure used in Experiment **2** was repeated using 6,6-bis-O-(*R*)-2-{(*R*)-1-(*tert*-butyldimethylsilanyloxy)-16-[(1*S*,2*R*)-2-((*S*)-20-methyl-19-oxo-20-octatriacontyl)cycl

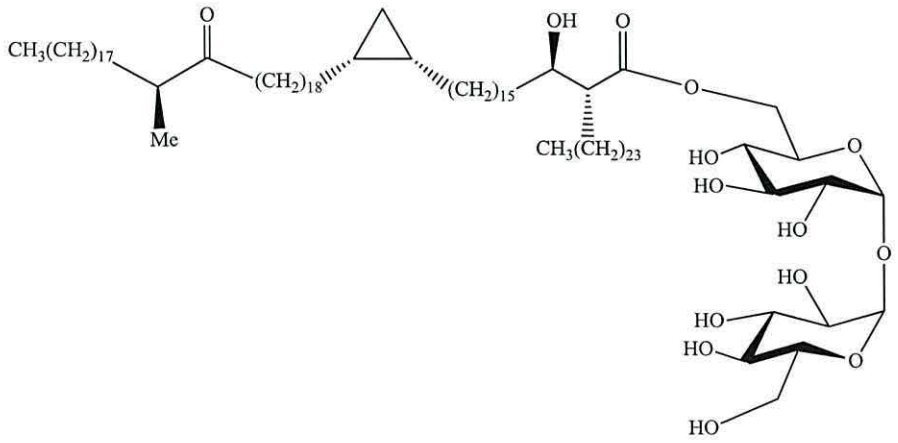
-opropyl]hexadecyl}hexacosanoic-α,α'-trehalose **(203)** (180 mg, 0.059 mmol), pyridine and HF.pyridine (0.2 mL) in dry THF (15 mL). The crude product was purified by chromatography eluting with $CHCl_3$/MeOH (10:1) to give 6,6-bis-O-(*R*)-2-{(R)-1-(hydroxy)-16-[(1*S*,2*R*)-2-((*S*)-20-methyl-19-oxo-20-octatriacontyl)cyclopropyl] hexadecyl}hexacosanoic-α,α'-trehalose **(204)** (100 mg, 60%) as a syrup, $[\alpha]_D^{23}$ = + 34.62 (*c* 0.76, $CHCl_3$) {MALDI Found $(M + Na)^+$: 2804.65, $C_{180}H_{346}O_{17}Na$ requires: 2804.62}, which showed δ_H (400MHz, $CDCl_3$ + few drops of CD_3OD): 4.98 (2H, d, *J* 3.04 Hz), 4.67 (2H, br.d, *J* 11.12 Hz), 4.23 (2H, br.d, *J* 8.32 Hz), 3.93 (2H, br.t, *J* 11.4 Hz), 3.72 (2H, br.t, *J* 9.32 Hz), 3.63 (2H, m), 3.48 (2H, dd, *J* 3.25, 9.8 Hz), 3.19 (2H, br.t, *J* 9.4 Hz), 2.47 (4H, q, *J* 6.6 Hz), 2.38 (4H, t, *J* 7.36 Hz), 1.63-1.50 (4H, m), 1.40-1.08 (292H, m), 1.00 (6H, d, *J* 6.96 Hz), 0.83 (12H, t, *J* 6.48 Hz), 0.60 (4H, m), 0.53 (2H, dt, *J* 3.76, 8.4 Hz), -0.37 (2H, br.q, *J* 5.16 Hz); δ_C (100MHz, $CDCl_3$): 215.87, 175.47, 95.13, 72.56, 72.37, 71.48, 71.09, 69.85, 64.63, 52.15, 46.26, 41.09, 34.64, 32.95, 31.83, 30.14, 29.50, 29.42, 29.36, 28.64, 27.22, 25.68, 25.07, 23.62, 22.59, 16.23, 15.67, 13.99, 10.81; ν_{max}: 3436, 2920, 2850, 1733, 1714, 1494, 1467, 1147, 1107, 1050, 907, 824, 734 cm^{-1}.

Experiment 26: 6-O-(*R*)-2-{(*R*)-1-(*tert*-butyldimethylsilanyloxy)-16-[(1*S*,2*R*)-2-((*S*)-20-methyl-19-oxo-20-octatriacontyl)cyclopropyl]hexadecyl}hexacosanoic-α,α'-trehalose (205)



The procedure used in Experiment **19** was repeated using 6-O-(*R*)-2-{(*R*)-1-(*tert*-butyldimethylsilanyloxy)-16-[(1*S*,2*R*)-2-((S)-20-methyl-19-oxo-20-octatriacontyl)-cyclopropyl]hexadecyl}hexacosanoic-2,3,4,2',3',4',-hexakis-O-(trimethylsiyl)-α,α'-trehalose **(202)** (0.11 g, 0.052 mmol) and tetrabutylammonium fluoride (0.165 mL, 0.165 mmol, 1M) in dry THF(8 mL). The product was purified by column chromatography on silica gel eluting with $CHCl_3$/MeOH (10:1) to give 6-O-(*R*)-2-{(*R*)-1-(*tert*-butyldimethylsilanyloxy)-16-[(1*S*,2*R*)-2-((*S*)-20-methyl-19-oxo-20-octatriacontyl)cyclopropyl]hexadecyl}hexacosanoic-α,α'-trehalose **(205)** (80 mg, 87%), $[\alpha]_D^{23}$= + 30.52 (*c* 0.67, $CHCl_3$) {MALDI Found $(M + Na)^+$: 1698.93, $C_{102}H_{198}O_{14}SiNa$ requires: 1698.44}, which showed δ_H (400MHz, $CDCl_3$ + few drops of CD_3OD): 5.00 (2H, d, *J* 2.24 Hz), 4.26-4.18 (2H, m), 3.89-3.87 (1H, m), 3.86 (2H, br.d, *J* 4.28 Hz), 3.83 (2H, br.d, *J* 4.64 Hz), 3.61 (1H, dd, *J* 4.88, 11.04 Hz), 3.44-3.39 (3H, m), 3.37-3.25 (2H, m), 2.47-2.41 (2H, m), 2.34 (2H, t, *J* 7.16 Hz), 1.47-1.05 (151H, m), 0.95 (3H, d, *J* 6.92 Hz), 0.78 (6H, t, *J* 6.56 Hz), 0.76 (9H, s), 0.56-0.57 (2H, m), 0.48 (1H, dt, *J* 3.46, 8.2 Hz), -0.04 (3H, s), -0.06 (3H, s), -0.41 (1H, br.q, *J* 4.88 Hz); δ_C (100MHz, $CDCl_3$): 216.34, 175.05, 93.52, 93.45, 73.08, 72.06, 71.48, 70.57, 70.15, 69.81, 61.81, 51.54, 46.16, 41.01, 33.36, 32.85, 31.73, 29.55, 29.45, 29.28, 29.20, 29.13, 28.52, 26.80, 25.49, 23.50, 22.48, 17.75, 15.56, 13.85, 10.67, -4.75, -5.15; ν_{max}: 3436, 2920, 2851, 1735, 1714, 1493, 1452, 1050, 990, 824 cm^{-1}.

Experiment 27: 6-O-(*R*)-2-{(*R*)-1-hydroxy-16-[(1*S*,2*R*)-2-((*S*)-20-methyl-19-oxo-20-octatriacontyl)cyclopropyl]hexadecyl}hexacosanoic-α,α'-trehalose (206)



The procedure in Experiment **2** was repeated using 6-O-(*R*)-2-{(*R*)-1-(*tert*-butyldimethylsilanyloxy)-16-[(1*S*,2*R*)-2-((*S*)-20-methyl-19-oxo-20-octatriacontyl) cyclopropyl]hexadecyl}hexacosanoic-α,α'-trehalose **(205)** (80 mg, 0.040 mmol), pyridine (0.1 mL) and HF.pyridine (0.2 mL) in dry THF (12 mL). The crude product was purified by chromatography eluting with $CHCl_3$/MeOH (10:1) to 6-O-(*R*)-2-{(*R*)-1-hydroxy-16-[(1*S*,2*R*)-2-((*S*)-20-methyl-19-oxo-20-octatriacontyl)cyclopropyl]hexad -ecyl}hexacosanoic-α,α'-trehalose **(206)** (40 mg, 54%) as syrup, $[\alpha]_D^{23}$ = + 43.25 (*c* 0.73, $CHCl_3$) {MALDI Found $(M + Na)^+$: 1585.81, $C_{96}H_{184}O_{14}Na$ requires: 1585.36}, which showed δ_H (400 MHz, $CDCl_3$ + few drops of CD_3OD): 5.08 (1H, d, *J* 2.88 Hz), 5.01 (1H, d, *J* 3.28 Hz), 4.67 (1H, br.t, *J* 10.56 Hz), 4.23 (1H, br.t, *J* 8.92 Hz), 3.96 (1H, br.d, *J* 10.92 Hz), 3.91 (1H, br.d, *J* 10.04 Hz), 3.87 (1H, d, *J* 4.88 Hz), 3.83-3.76 (2H, m), 3.63-3.58 (2H, m), 3.55 (1H, dd, *J* 3.60, 9.80 Hz), 3.47 (1H, dd, *J* 3.64, 10.28 Hz), 3.26 (1H, br.t, *J* 9.40 Hz), 3.20 (1H, br.t, *J* 9.52 Hz), 2.47 (2H, q, *J* 6.64 Hz), 2.38 (2H, t, *J* 7.4 Hz), 1.57-1.51 (4H, m), 1.33-1.08 (148H, m), 1.01 (3H, d, *J* 7.0 Hz), 0.84 (6H, t, *J* 6.64 Hz), 0.61-0.60 (2H, m), 0.52 (1H, dt, *J* 3.64, 7.8 Hz), -0.36 (1H, br.q, *J* 4.76 Hz); δ_C (100MHz, $CDCl_3$): 216.31, 175.47, 94.38, 72.55, 72.36, 71.35, 71.14, 70.97, 70.87, 70.04, 64.15, 62.19, 52.29, 41.08, 34.61, 32.94, 31.83, 30.15, 29.70, 29.66, 29.63, 29.60, 29.54, 29.49, 29.41, 29.35, 29.26, 28.63, 27.21, 25.05, 23.61, 22.58, 16.21, 15.66, 13.98, 10.80; ν_{max}: 3435, 2918, 2850, 1735,1714, 1494, 1452, 1105, 1049, 992, 824 cm^{-1}.

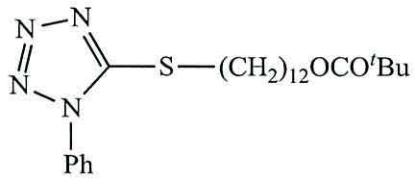
Experiment 28: 2,2-Dimethylpropionic acid 12-bromododecyl ester (216)

$Br(CH_2)_{12}OCO^tBu$

Trimethylacetyl chloride (22.19 mL, 180.79 mmol) in dichloromethane (80 mL) was added to a stirred solution of 12-bromododecan-1-ol **(183)** (40 g, 150.85 mmol), triethylamine (31.30 mL, 226.28 mmol) and 4-dimethylaminopyridine (0.74 g, 5.92 mmol) in dichloromethane (200 mL) over a period of 15 min at 0°C under nitrogen. The reaction mixture was stirred 18 hours then quenched with dilute hydrochloric acid (100 mL). The aqueous layer was re-extracted with dichloromethane (2 × 50 mL). The combined organic layers were washed with brine, dried and evaporated to give a crude product. The crude product purified by column chromatography eluting with

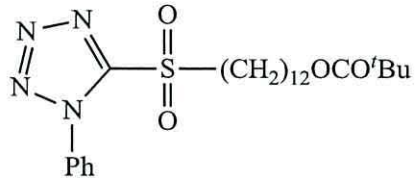
petrol/ethyl acetate (1:1), to give colouress oil, 2,2-dimethylpropionic acid 12-bromododecyl ester **(216)** (42 g, 80%) {Found $(M + Na)^+$: 371.1552, $C_{17}H_{33}O_2BrNa$ requires: 371.1561}, which showed δ_H (500MHz, $CDCl_3$) : 4.03 (2H, t, *J* 6.65 Hz), 3.39 (2H, t, *J* 7.0 Hz), 1.83 (2H, pent, *J* 7.0 Hz), 1.60 (2H, pent, *J* 6.6 Hz), 1.41 (2H, pent, *J* 7.0 Hz), 1.30-1.22 (14H, m), 1.18 (9H, s); δ_C (126MHz, $CDCl_3$): 178.54, 64.37, 38.66, 33.90, 32.78, 29.42, 29.36, 29.15, 28.99, 28.69, 28.55, 27.97, 27.14, 26.44; ν_{max}: 2928, 2855, 1729, 1480, 1461, 1284, 1158, 1042, 1005, 852 cm^{-1}.

Experiment 29: 2,2-Dimethylpropionic acid 12-(1-phenyl-1*H*-tetrazol-5-ylsulfonyl)dodecyl ester (217)



1-Phenyl-*1H*-tetrazole-5-thiol (23.57 g, 132 mmol), 2, 2-dimethylpropionic acid 12-bromo-dodecyl ester **(216)** (42 g, 0.12mol) and anhydrous potassium carbonate (36.44 g, 260 mmol) in acetone (250 mL) were mixed at room temperature. The mixture was vigorously stirred for 18 hours when TLC analysis indicated that the reaction wase complete. Water (1 L) was added to the mixture and the product was extracted with dichloromethane (1 × 200 mL, 2 × 100 mL). The combined organic layers were washed with brine (2 × 200 mL), dried and evaporated. The crude product was purified by column chromatography eluting with petrol/ethyl acetate (5:1) to give a colorless oil, 2,2-dimethylpropionic acid 12-(1-phenyl-1*H*-tetrazol-5-ylsulfanyl)-dodecyl ester **(217)** (49 g, 91%) {Found $(M + Na)^+$: 469.2615, $C_{24}H_{38}O_2N_4SNa$ requires: 469.2613}, which showed δ_H (500MHz, $CDCl_3$): 7.55-7.49 (5H, m), 3.90 (2H, t, *J* 6.6 Hz), 3.56 (2H, t, *J* 7.2 Hz), 1.83 (2H, pent, *J* 7.0 Hz), 1.63 (2H, pent, *J* 7.0 Hz), 1.46-142 (2H, m), 1.32-1.26 (14H, m), 1.20 (9H, s); δ_C (126MHz, $CDCl_3$): 178.60, 154.50, 133.81, 131.08, 129.70, 125.09, 63.12, 56.09, 32.69, 29.48, 29.40, 29.33, 29.08, 28.76, 28.10, 25.69; ν_{max}: 2927, 2854, 1726, 1500, 1462, 1387, 1284, 1159, 761 cm^{-1}.

Experiment 30: 2,2-Dimethylpropionic acid 12-((1-phenyl-1*H*-tetrazol-5-ylsulfon -yl)dodecyl ester (211)



A solution of ammonium molybdate (VI) tetrahydrate (67.8 g, 54.8 mmol) in 35% H_2O_2 (100 mL), prepared and cooled in an ice bath was added to a stirred solution of 2,2-dimethylpropionic acid 12-(1-phenyl-1*H*-tetrazol-5-ylsulfanyl)-dodecyl ester **(217)** (49 g, 109.7m mol) in THF (150) and IMS (300 mL) at 10 °C. The reaction mixture was stirred at room temperature for 2 hours. A further solution of ammonium molybdate (VI) tetrahydrate (33.88 g, 27.0 mmol) in 35% (50 mL) was added and the mixture was stirred at room temperature for 18 hours. The mixture was poured into water (1.2 L) and extracted with dichloromethane (1 × 250 mL, 3 × 150 mL). The combined organic phases were washed with water (500 mL), dried and the solvent was evaporated. The crude product was purified by column chromatography eluting with petrol/ethyl acetate (5:1,then 1:1) to give a yellow oil, 2,2-dimethylpropionic acid 12-(1-phenyl-1*H*-tetrazol-5-ylsulfonyl)dodecyl ester **(211)** (46 g, 88 %) {Found $(M + Na)^+$: 501.2526, $C_{24}H_{38}O_4N_4SNa$ requires: 501.2511},which showed δ_H (500 MHz, $CDCl_3$): 7.70-7.68 (2H, m), 7.61-7.59 (3H,m), 4.04 (2H, t, *J* 6.6 Hz), 3.73 (2H, t, *J* 7.9 Hz), 1.95 (2H, pent, *J* 7.5 Hz), 1.61 (2H, pent, *J* 6.65 Hz), 1.49 (2H, pent, *J* 7.0 Hz), 1.34-123 (14H, m), 1.19 (9H, s); δ_C (126MHz, $CDCl_3$): 178.59, 153.41, 133.04, 131.08, 129.66, 125.06, 64.40, 55.99, 38.70, 29.48, 29.40, 29.32, 29.05, 28.76, 28.10, 25.81, 21.90; ν_{max}: 2930, 2857, 1725, 1498, 1480, 1342, 1285, 1154, 735 cm^{-1}.

Experiment 31: 10-bromodecanal (212)

$Br(CH_2)_9CHO$

The proceduer used in Experiment **8** was repeated in order to to oxidise 10-bromodecan-1-ol **(219)** (11.0 g, 464.0 mmol) using PCC (22.0 g, 102 mmol) in dichloromethane (300 mL). The crude product was purified by column

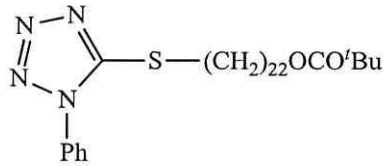
chromatography eluting with petrol/ethyl acetate (10:1) to give a colorless oil of 10-bromodecanal **(212)** (9 g, 82%), which showed δ_H (500 MHz, $CDCl_3$): 9.75 (1H, s), 3.39 (2H, t, *J* 6.95 Hz), 2.43-2.39 (2H, m), 1.84 (2H, pent, *J* 6.9 Hz), 1.62 (2H, pent, *J* 7.25 Hz), 1.30-1.23 (10H, br. m); δ_C (126MHz, $CDCl_3$): 202.79, 64.56, 43.82, 33.90, 33.66, 32.76, 29.26, 28.05, 25.82; ν_{max}: 2925, 2854, 1706, 1412, 1230, 1102, 1035, 721 cm^{-1}.

Experiment 32: 2,2-Dimethylpropionic acid (22-bromo)docosyl ester (213)

$Br(CH_2)_{22}OCO^tBu$

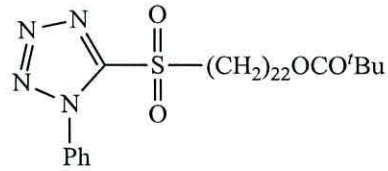
The procedure used in Experiment **1** was repeated in order to couple 10-bromodecanal **(212)** (9.0 g, 38.2 mol) and 2,2-dimethylpropionic acid 12-(1-phenyl-1*H*-tetrazol-5-ylsulfonyl)dodecyl ester **(211)** (21.98 g, 45.9 mmol) using lithium bis(trimethylsilyl) amide (70 mL, 74.6 mmol) in dry THF (200 mL). The product was purified by column chromatography eluting with petrol/ethyl acetate (20:1) to give (*E/Z*)-2,2-dimethylpropionic acid 22-bromodocos-12-enyl ester **(220)** (12.60 g, 68%) as a mixture. Palladium on (10% on carbon, 1.0 g) was added to stirred solution of alkenes (12.60 g, 0.025 mmol) in IMS/THF (1:1) under hydrogen. Hydrogenation was carried out for 1 h, then the mixture was filtered off through a bed of celite and the solvent was evaporated. The crude product was purified by column chromatography eluting with petrol/ethyl acetate (10:1) to give a white solid, 2,2-dimethylpropionic acid (22-bromo)docosyl ester **(213)** (11 g, 87%), m.p. 37-38 °C, {Found $(M + Na)^+$: 511.3125, $C_{27}H_{53}BrO_2Na$ requires: 511.3121} which showed δ_H (500MHz, $CDCl_3$): 4.05 (2H, t, *J* 6.65 Hz), 3.41 (2H, t, *J* 6.95 Hz), 1.86 (2H, pent, *J* 6.65 Hz), 1.61 (2H, *J* 6.6 Hz), 1.45-1.26 (36H, br. m), 1.20 (9H, s); δ_C (126MHz, $CDCl_3$): 178.67, 67.89, 64.45, 58.27, 33.93, 32.79, 29.62, 29.57, 29.55, 29.50, 29.47, 29.45, 29.37, 29.16, 28.70, 28.55, 28.12, 27.13, 25.84, 25.53, 18.26; ν_{max}: 2914, 2328, 1719, 1476, 1286, 1159, 1034, 889 cm^{-1}.

Experiment 33: 2,2-Dimethylpropionic acid 22-((1-phenyl-1*H*-tetrazol-5-ylsulfanyl)docosyl ester (221)



The procedure used in Experiment **29** was repeated using 2,2-dimethylpropionic acid (22-bromo)docosyl ester **(213)** (11 g, 22.4 mmol), 1-phenyl-1*H*-tetrazole-5-thiol (4.40 g, 24.6 mmol) and anhydrous potassium carbotonate (6.81 g, 49.3 mmol) in acetone (250 mL). The crude product was purified by column chromatography eluting with petrol/ethylacetate (10:1) to give a semi-solid 2,2-dimethylpropionic acid 22-(1-phenyl-1*H*-tetrazol-5-ylsulfanyl)docosyl ester **(221)** (13.0 g, 84 %) {Found $(M + H)^+$: 587.4344, $C_{34}H_{59}O_2N_4S$ requires: 587.4353}, which showed δ_H (500MHz, $CDCl_3$): 7.58-7.51 (5H, m), 4.03 (2H, t, *J* 6.65 Hz), 3.38 (2H, t, *J* 7.55 Hz), 1.84 (2H, pent, *J* 6.5 Hz), 1.60 (2H, pent, *J* 6.3 Hz), 1.44-1.39 (2H, m), 1.33-1.21 (34H, m), 1.18 (9H, s); δ_C (126MHz, $CDCl_3$): 178.56, 154.44, 133.72, 129.97, 129.69, 123.78, 67.89, 64.39, 53.36, 38.65, 33.31, 30.84, 29.50, 29.48, 29.45, 29.37, 29.16, 29.02, 28.58, 28.55, 27.13, 25.84, 25.55; ν_{max}: 2925, 2853, 1728, 1597, 1500, 1462, 1397, 1283, 1156 cm-^{1}.

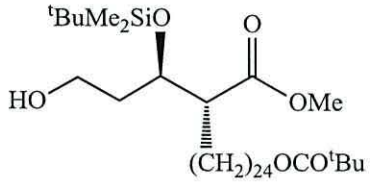
Experiment 34: 2,2-Dimethylpropionic acid 22-(1-phenyl-1*H*-tetrazol-5-ylsulfonyl)docosyl ester (214)



The procedure used in Experiment **30** was repeated using 2,2-dimethylpropionic acid 22-((1-phenyl-1*H*-tetrazol-5-ylsulfanyl)docosyl ester **(221)** (13.00 g, 22.18 mmol), ammonium molybdate (VI) tetrahydrate (13.70 g, 11.09 mmol) in ice cold H_2O_2 (35% w/w, 50 mL), THF (50) and IMS (100 mL), and further solution of ammonium molybdate (VI) tetrahydrate (6.85 g, 5.554 mmol) in ice cold H_2O_2 (35% w/w, 25 mL)

to give a crude product. This was purified by column chromatography eluting petrol/ethylacetate (5:1, then 1:1) to give a white solid, 2,2-dimethylpropionic acid 22-(1-phenyl-1*H*-tetrazol-5-ylsulfonyl)docosyl ester **(214)** (12.4 g, 90%), m.p. 41-42°C {Found $(M + Na)^+$: 641.4071, $C_{34}H_{58}O_2SNa$ requires: 641.4076}, which showed δ_H (500MHz, $CDCl_3$); 7.61-7.60 (2H, m), 7.59-7.58 (3 H, m), 4.04 (2H, t, *J* 6.65 Hz), 3.73 (2H, t, *J* 7.4 Hz), 1.95-1.92 (2H, m), 1.61 (2H, pent, *J* 6.95 Hz),1.50 (2H, pent, *J* 6.65 Hz), 1.37-1.22 (34H, m), 1.19 (9H, s); δ_C (126MHz, $CDCl_3$): 178.60, 153.47, 133.03, 131.40, 129.66, 125.04, 64.42, 60.34, 55.98, 38.68, 29.66, 29.60, 29.53, 29.47, 29.42, 29.18, 29.15, 28.85, 28.57, 28.10, 27.16, 25.87, 21.90, 20.99; ν_{max}: 2918, 2850, 1725, 1617, 1497, 1473, 1342, 1285, 1155, 824 cm^{-1}.

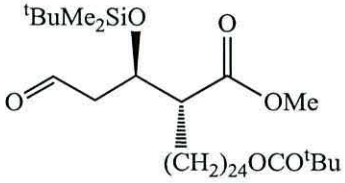
Experiment 35: (*R*)-2-[(*R*)-1-((*tert*-Butyldimethylsilyloxy)-3-hydroxypropyl]-26-(2,2-dimethylpropionyloxy)hexacosanoic acid methyl ester (215)



The procedure used in Experiment **1** was repeated in order to couple (2*R*,3*R*)-5-bezyloxy-3-(*tert*-butyldimethylsilanyloxy)-2-(oxoethyl)pentanoic acid methyl ester **(137)** (3.7 g, 9.38 mmol) and 2,2-dimethylpropionic acid 22-((1-phenyl-1*H*-tetrazol-5-ylsulfonyl)docosyl ester **(214)** using lithium bis(trimethylsilyl)amide (14.6mL,15.48 mmol) in dry THF (100 mL). The crude product was purified by column chromatography eluting with petrol/ethyl acetate (20:1), to give a colourless oil, (*E/Z*)(*R*)-2-[(*R*)-3-(benzyloxy)-1-((*tert*-butyldimethylsilyloxy)propyl]-26-(2,2-dimethy -lpropionyloxy)hexacos-4-enoic acid methyl ester **(222)** as a mixture (5.0 g, 67%). The hydrogenation was carried out with palladium on carbon (10%, 1.0 g) which was stirred with a solution of the above alkenes (5 g, 6.35 mmol) in THF (50 mL) and IMS (50 mL) under hydrogen for 3 days. The crude product was purified by column chromatography eluting with petrol/ethyl acetate (5:1) to give a white solid, (*R*)-2-[(*R*)-1-(*tert*-butyldimethylsilyloxy)-3-hydroxypropyl]-26-(2,2-dimethylpropionloxy)hexaco -sanoic acid methyl ester **(215)** (3 g, 68%), m.p. 37-39 °C, $[\alpha]_D^{23}$ = -8.89 (*c* 1.54,

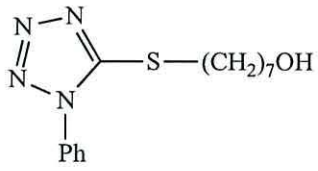
$CHCl_3$) {Found $(M + Na)^+$: 721.5739, $C_{41}H_{82}O_6SiNa$ requires: 721.5773}, which showed δ_H (500 MHz, $CDCl_3$): 4.04 (2H, t, *J* 6.5 Hz), 3.81-3.70 (1H,m), 3.78-3.70 (2H, m), 3.67 (3H, s), 2.64 (1H, ddd, *J* 3.75, 6.9, 10.7 Hz), 1.83-1.73 (2H, m), 1.64-1.58 (4H, m), 1.29-1.18 (52H, m, including a singlet at δ 1.20), 0.88 (9H, s), 0.11 (3H, s), 0.07 (3H, s); δ_C (126MHz, $CDCl_3$): 178.66, 174.66, 72.06, 64.47, 59.54, 51.61, 51.42, 38.72, 35.21, 29.70, 29.63, 29.55, 29.51, 29.43, 29.22, 28.61, 27.86, 27.20,25.90, 25.70, 22.60, 22.33, 21.03, 17.86, 14.19, -4.51, -4.96; ν_{max}: 3521, 2925, 2854, 1731, 1463, 1285, 1255, 1163, 1092,837 cm^{-1}.

Experiment 36: (*R*)-2-[(*R*)-1-((*tert*-Butyldimethylsilyloxy)-3-oxopropyl]-26-(2,2-dimethylpropionyloxy)hexacosanoic acid methyl ester (223)



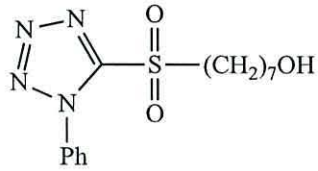
The procedure used in Experiment **8** was repeated to oxidise (*R*)-2-[(*R*)-1-(*tert*-butyldimethylsilyloxy)-3-hydroxypropyl]-26-(2,2-dimethylpropionyloxy)hexacosanoic acid methyl ester **(215)** (3 gm, 4.46 mmol) using PCC (2.40 g, 11.15 mmol) in dichloromethane (130 mL). The crude product was purified by column chromatography eluting with petrol/ethyl acetate (10:1) to give a colourless oil, (*R*)-2-[(*R*)-1-(*tert*-butyldimethylsilyloxy)-3-oxopropyl]-26-(2,2-dimethylpropionloxy)hexaco -sanoic acid methyl ester **(223)** (2.46 g, 82.2%), $[\alpha]_D^{19}$= - 4.16 (*c* 0.96, $CHCl_3$) {Found $(M + Na)^+$: 719.5565, $C_{41}H_{80}O_6SiNa$ requires: 719.5616}, which showed δ_H (500MHz, $CDCl_3$): 9.82 (1H, br.s), 4.44 - 4.41 (1H, m), 4.04 (2H, t, *J* 6.5 Hz), 3.68 (3 H, s), 2.67-2.57 (3H, m), 1.61 (2H, pent, *J* 6.5 Hz), 1.25-1.19 (53 H, m, including a singlet at δ 1.19), 0.85 (9H, s), 0.074 (3 H, s) 0.072 (3H, s); δ_C (126MHz, $CDCl_3$): 201.27, 178.64, 174.00, 68.80, 64.45, 52.25, 51.52, 48.07, 38.71, 31.57, 29.69, 29.61, 29.55, 29.50, 29.38, 29.21, 29.04, 28.60, 27.74, 27.19, 27.02, 25.90, 25.61, 22.63, 17.86, 14.10, -4.64, -4.91; ν_{max}: 2927, 2856, 1731, 1463, 1364, 1285, 1162, 1005, 837, 777 cm^{-1}.

Experiment 37: 7-((1-Phenyl-1*H*-tetrazol-5-ylsulfanyl)heptanol (225)



The procedure used in Experiment **29** was repeated using 7-bromoheptan-1-ol **(224)** (10.5 g, 53.84 mmol), 1-phenyl-1*H*-tetrazole-5-thiol (10.55 g, 59.23 mmol) and anhydrous potassium carbotonate (16.30 g, 118.46 mmol) in acetone (250 mL). The crude product was purified by column chromatography eluting petrol/ethyl acetate (5:1) to give a colourless oil of 7-((1-phenyl-1*H*-tetrazol-5- ylsulfanyl)heptanol **(225)** (14 g, 89%) {Found $(M + Na)^+$: 315.1256, $C_{14}H_{20}ON_4SNa$ requires: 315.1255}, which showed δ_H (500MHz, $CDCl_3$): 7.61-7.52 (5H, m), 3.6 (2H, t, *J* 6.6 Hz), 3.4 (2H, t, *J* 7.6 Hz), 1.82 (2H, pent, *J* 7.6 Hz), 1.5 (1H, s), 1.42 (2H, pent, *J* 6.6 Hz), 1.29-1.21 (6H, m); δ_C (126MHz, $CDCl_3$): 153.52, 133.71, 130.00, 129.72, 123.81, 62.93, 33.37, 32.72, 29.51, 29.35, 28.93, 28.61, 25.70; ν_{max}: 3401, 2929, 2857, 1461 cm^{-1}.

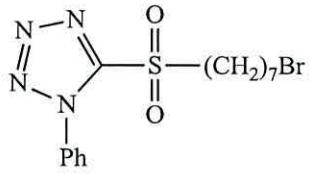
Experiment 38: 7-((1-Phenyl-1*H*-tetrazol-5-yl)sulfonyl)heptanol (226)



The procedure used in Experiment **30** was repeated using 7-((1-phenyl-1*H*-tetrazol-5-ylsulfanyl)heptanol **(225)** (14 g, 47.90 mmol), ammonium molybdate (VI) tetrahydrate (29.60 g, 23.95 mmol) in ice cold H_2O_2 (35% w/w, 50 mL), THF (100 mL) and IMS (150 mL), and further solution of ammonium molybdate (VI) tetrahydrate (14.80 g, 11.95 mmol) in ice cold H_2O_2 (35% w/w, 25 mL) to give a crude product. This was purified by column chromatography eluting petrol/ethyl acetate (5:1, then (1:1) to give a yellow oil of 7-((1-phenyl-1*H*-tetrazol-5-yl)sulfonyl)heptanol **(226)** (14 g, 90%) {Found $(M + Na)^+$: 347.1172, $C_{14}H_{20}O_3N_4SNa$ requires: 347.1153}, which showed δ_H (500MHz, $CDCl_3$): 7.69-7.66 (2H, m), 7.62-7.54 (3H, m), 3.72 (2H, t, *J* 6.95 Hz), 3.61 (2H, t, *J* 6.6 Hz), 1.86 (2H, pent, *J* 6.95 Hz), 1.62 (1H, s), 1.54 (2H, pent, *J* 6.95 Hz),

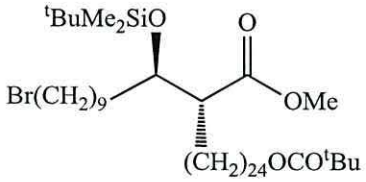
1.39-1.21 (6H, m); δ_C (126MHz, $CDCl_3$): 153.49, 133.04, 131.48, 129.73, 125.08, 62.76, 55.95, 32.71, 29.56, 28.75, 28.07, 25.84; ν_{max}: 3401, 2929, 2857, 1596, 1463, 1338,1152,764 cm^{-1}.

Experiment 39: 5-((7-Bromoheptyl)sulfonyl)-1-phenyl-1*H*-tetrazole (227)



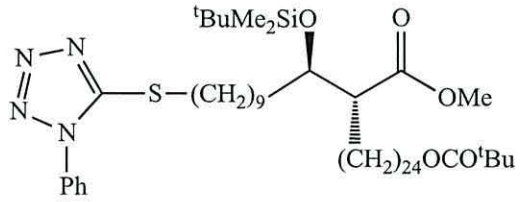
N-Bromosuccinimide (9.99 g, 56.13 mmol) was added portionwise to a stirred solution of 7-((1-phenyl-1*H*-tetrazol-5-yl)sulfonyl)heptanol **(226)** (14.0 g, 43.18 mmol) and triphenylphosphine (14.72 g, 54.34 mmol) in dichloromethane (300 mL) at 0 °C over 10 min. The mixture was allowed to reach room temperature and stirred at room temperature for 75 min, then sat. aq. sodium metabisulfate (60 mL) was added and organic layer was separated. The aqueous layer was re-extracted with dichloromethane (2 x 200 mL) and the combined organic layers were dried and evaporated to give a residue which was treated with petrol/ethyl acetate (5:1, 300 mL) and stirred for 30 min. The triphenylphosphonium oxide was filtered and washed well with a mixture of petrol/ethylacetate (5:1, 100 mL) then the solvent was evaporated. The crude product was purified by column chromatography eluting with petrol/ethyl acetate (5:1) to give a colourless oil, 5-((7-bromoheptyl)sulfonyl)-1-phenyl-1*H*-tetrazole **(227)** (14 g, 83%),{Found $(M + Na)^+$: 409.0311, $C_{14}H_{19}O_2N_4SNaBr$ requires: 409.0309} which showed δ_H (500MHz, $CDCl_3$): 7.69-7.67 (2H, m), 7.64-7.59 (3H, m), 3.74 (2H, t, *J* 7.95 Hz), 3.40 (2H, t, *J* 6.95 Hz), 1.96 (2H, *J* 7.9 Hz), 1.86 (2H, *J* 6.65), 1.56-1.39 (6H, m); δ_C (126MHz, $CDCl_3$): 153.42, 132.99, 131.44, 129.69, 125.02, 55.86, 33.59, 32.44, 28.02, 27.91, 27.65, 21.88; ν_{max}: 2934, 2859, 1739, 1596, 1497, 1466, 1341, 1152, 764 cm^{-1}.

Experiment 40: (*R*)-2-[(*R*)-10-Bromo-1-(*tert*-butyldimethylsilyloxy)decyl]-26-(2,2-dimethylpropionyloxy)hexacosanoic acid methyl ester (229)



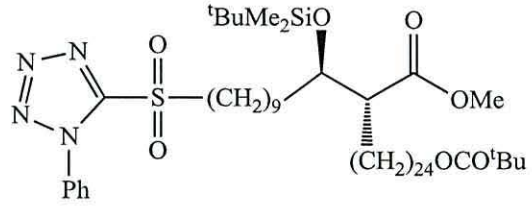
The procedure used in Experiment **1** was repeated in order to couple (*R*)-2-[(*R*)-1-(*tert*-butyldimethylsilyloxy)-3-oxopropyl]-26-(2,2-dimethyl-propionyloxy)hexacosanoic acid **(223)** (2.46 g, 3.67 mmol) and 5-((7-bromoheptyl)sulfonyl)-1-phenyl-1*H*-tetrazole **(227)** (1.84 g, 4.77 mmol) using lithium bis(trimethylsilyl)amide (5.85 mL, 4.77 mmol) in dry THF (100 mL). The crude prodect was purified by column chromatography eluting solvent with petrol/ethyl acetate (20:1) to give a colourless oil, (*R*)-2-[(*E*/*Z*)-(*R*)-10-bromo-1-(*tert*-butyldimethylsilyloxy)dec-3-enyl)-26-(2,2-dimethyl -propionyloxy] hexacosanoic acid methyl ester, **(228)** (2.30 g, 76%) as a mixture. Hydrogenation was carried out with palladium (10% on carbon, 0.3 g) which was added to a stirred solution of the above alkenes (2.30 g, 2.68 mmol) in THF (20 mL) and IMS (40 mL) under hydrogen for one hour. The crude product was purified by column chromatography eluting with petrol/ethyl acetate (10:1) to give a colourless oil, (*R*)-2-[(*R*)-10-bromo-1-((*tert*-butyldimethylsilyloxy)-decyl]-26-(2,2-dimethylpropi -onyloxy) hexacosanoic acid methyl ester **(229)** (2.0 g, 81%), $[\alpha]_D^{22} = -17.56$ (*c* 1.4, CH_3Cl) {Found $(M + Na)^+$: 881.5904, $C_{48}H_{95}O_5SiBrNa$ requires: 881.6024}, which showed δ_H (500MHz, $CDCl_3$); 4.03 (2H, t, *J* 6.6 Hz), 3.91-3.88 (1H, m), 3.64 (3H, s), 3.39 (2H, t, *J* 6.9 Hz), 2.51 (1H, ddd, *J* 3.8, 7.25, 11.05 Hz), 1.84 (2H, pent, *J* 6.95 Hz), 1.60 (2H, pent, *J* 6.6 Hz), 1.30-1.14 (67H, m, including singlet at δ 1.18), 0.86 (9H, s), 0.03 (3H, s), 0.02 (3H,s); δ_C (126MHz, $CDCl_3$): 178.56, 175.05, 73.14, 64.41, 60.32, 51.52, 51.17, 41.31, 38.67, 36.03, 33.60, 32.79, 31.87, 29.78, 29.68, 29.55, 29.49, 29.31, 29.19, 29.01, 28.69, 28.58, 28.13, 27.79, 27.44, 27.16, 25.87, 25.71, 23.64, 22.64, 20.98, 17.92, 14.16, -4.40, -4.97; ν_{max}: 2923, 2853, 1732, 1463, 1366, 1284, 1254, 1159, 1070, 836, 775 cm^{-1}.

Experiment 41: (*R*)-2-((*R*)-1-(*tert*-Butyldimethylsilyloxy)-10-((1-phenyl-1*H*-tetrazol-5-yl)thio)decyl)-26-(2,2-dimethylpropionyloxy)hexacosanoic acid methyl ester (230)



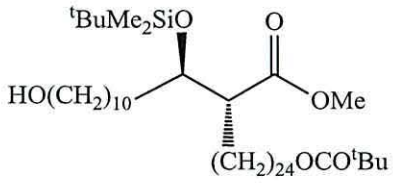
The procedure used in Experiment **29** was repeated using (*R*)-2-[(*R*)-10-bromo-1-(*tert*-butyldimethylsilyloxy)decyl)-26-(2,2-dimethylpropionyloxy)hexacosanoic acid methyl ester **(229)** (1.8 g, 2.093 mmol), 1-phenyl-1*H*-tetrazole-5-thiol (0.41 g, 2.29 mmol) and anhydrous potassium carbotonate (0.63 g, 4.60 mmol) in acetone (30 mL) and THF (15 mL). The crude product was purified by column chromatography eluting with petrol/ethyl acetate (5:1) to give a colourless oil, (*R*)-2-((*R*)-1-(*tert*-butyldimethylsilylo -xy)-10-((1-phenyl-1*H*-tetrazol-5-ylsulfanyl)decyl)-26-(2,2-dimethylpropionyloxy)hex -acosanoic acid methyl ester **(230)** (1.70 g, 85%), $[\alpha]_D^{22}$= -3.84 (*c* 1.24, CH_3Cl) {Found $(M + Na)^+$: 979.7046, $C_{55}H_{100}O_5SiN_4SNa$ requires: 979.7076}, which showed δ_H (500MHz, $CDCl_3$): 7.58-7.53 (5H, m), 4.04 (2H, t, *J* 6.65 Hz), 3.91-3.88 (1H, m), 3.65 (3H, s), 3.39 (2H, t, *J* 7.5 Hz), 2.52 (1H, ddd, *J* 3.8, 7.25, 10.75 Hz), 1.82 (2H, pent, *J* 7.6 Hz), 1.60 (2H, pent, *J* 6.9 Hz), 1.35-1.13 (67H, m, including a singlet at δ 1.19), 0.86 (9H, s), 0.04 (3H, s), 0.02 (3H, s); δ_C (126MHz, $CDCl_3$): 178.65, 175.10, 154.49, 133.75, 130.03, 129.78, 123.83, 73.16, 64.45, 51.57, 51.22, 38.71, 33.62, 33.34, 29.77, 29.69, 29.57, 29.46, 29.36, 29.20, 29.07, 28.99, 28.64, 27.83, 27.44, 27.19, 25.89, 25.74, 23.71, 22.63, 17.95, 14.09, 11.41, -4.38, -4.93; ν_{max}: 2928, 2854, 1731, 1500, 1463, 1284, 1250, 1160, 1073, 836, 775 cm^{-1}.

Experiment 42: (*R*)-2-((*R*)-1-(*tert*-butyldimethylsilyloxy)-10-((1-phenyl-1*H*-tetrazol-5-yl)sulfonyl)decyl)-26-(2,2-dimethylpropionyloxy)hexacosanoic acid methyl ester (231)



The procedure used in Experiment **30** was repeated using methyl (*R*)-2-((*R*)-1-(*tert*-butyldimethylsilyloxy)-10-((1-phenyl-1*H*-tetrazol-5-ylsulfanyl)decyl)-26-(2,2-dimethylpropionyloxy) hexacosanoic acid methyl ester **(230)** (1.63 g, 1.70 mmol), ammonium molybdate (VI) tetrahydrate (1.05 g, 0.85 mmol) in ice cold H_2O_2 (35% w/w, 10 mL), THF (15) and IMS (20), and further solution of ammonium molybdate (VI) tetrahydrate (0.52 g, 0.42 mmol) in ice cold H_2O_2 (35% w/w, 7 mL) to give a crude product. This was purified by column chromatography eluting with petrol/ethyl acetate (5:1) then (1:1) to give a colourless oil, methyl (*R*)-2-((*R*)-1-(*tert*-butyldimethylsilyloxy)-10-((1-phenyl-1*H*-tetrazol-5-ylsulfonyl)decyl)-26-(2,2-dimethylpropionyloxy)hexacosanoic acid methyl ester **(231)** (1.28 g, 76%), $[\alpha]_D^{23}$ = -7.64 (*c* 0.89, CH_3Cl) {Found $(M + Na)^+$: 1011.6958, $C_{55}H_{100}O_7SiN_4SNa$ requires: 1011.6974}, which showed δ_H (500MHz, $CDCl_3$): 7.71-7.69 (2H, m), 7.62-7.60 (3H, m), 4.04 (2H, t, *J* 6.6 Hz), 3.92-3.88 (1H, m), 3.73 (2H, t, *J* 7.85 Hz), 3.65 (3H, s), 2.52 (1H, ddd, *J* 3.45, 6.9, 10.7 Hz), 1.95 (2H, pent, *J* 7.6 Hz), 1.61 (2H, pent, *J* 6.9 Hz), 1.53-1.15 (67H, m, including a singlet at δ 1.19), 0.86 (9H,s), 0.04 (3H, s), 0.02 (3H, s); δ_C (126MHz, $CDCl_3$): 178.66, 175.08, 153.48,133.03, 131.43, 129.70, 125.04, 73.15, 64.46, 55.99, 51.58, 51.23, 38.71, 33.61, 29.55, 29.44, 29.39, 29.21, 28.87, 28.59, 28.13, 27.84, 27.43, 27.19, 25.89, 25.74, 23.73, 21.94, 17. 96, -4.37, -4.93; ν_{max}: 2925, 2853, 1731, 1463, 1344, 1284, 1254, 1154, 1099, 1074, 836, 775 cm^{-1}.

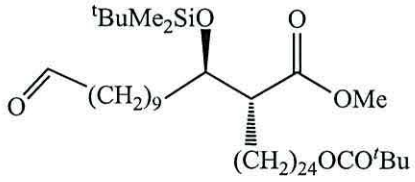
Experiment 43: (*R*)-2-((*R*)-1-(*tert*-Butyldimethyylsilyoxy)-11-hydroxyundecyl)-26-(2,2-dimethylpropionyloxy)hexacosanoic acid methyl ester (234)



The procedure used in Experiment **1** was repeated to couple (*R*)-2-((*R*)-1-(*tert*-butyldimethylsilyloxy)-3-oxopropyl)-26-(2,2-dimethylpropionyloxy)hexacosanoic acid methyl ester **(223)** (3.14 g, 4.5 mmol) and 1-phenyl-5[(8-(tetrahydropyran-2-yloxy)octyl)sulfonyl]-1*H*-tetrazole **(149)** (2.47, 5.8 mmol) using lithium bis (trimethylsilyl)amide (7.33 mL, 8.4 mmol) in dry THF (80 mL). The crude product was purified by column chromatography eluting with petrol/ethyl acetate (20:1) to give a colourless oil of (*R*)-methyl-2-((*E/Z*)-1-(*tert*-butyl-dimethylsilyloxy)-11-((tetrahydro-2*H*-pyran-2-yl)oxy)undec-3-enyl)-26-(2,2-dimethylpropionyloxy)hexacosanoic acid methyl ester **(232)** (3.4 g, 85%) as a mixture. Pyridnium-*p*-toluene sulfonate (0.47 g, 1.90 mmol) was added to stirred solution of the above alkenes **(232)** (3.4 g, 3.80 mmol) in MeOH (30 mL) and THF (70 mL) at 50 °C for 3 hours. The solvent was evaporated and the residue was treated with sat. aq. $NaHCO_3$ (30 mL) and petrol/ethyl acetate (10:1, 70 mL). The aqueous layer was separated and the aqueous layer was re-extracted with petrol/ethyl acetate (10:1) (3 × 50 mL). The combined organic layers were dried and evaporated. The crude product was purified by chromatography eluting with petrol/ethyl acetate (15:1 then 5:1) to give (R)-methyl-2-((*E/Z*)-1-((*tert*-butyldimethylsilyloxy)-11-hydroxyundec-3-enyl)-26-(2,2-dimethylpropionyloxy) hexa -cosanoic acid methyl ester as a colourless oil **(233)** (2.7 g, 88 %) as a mixture. The hydrogenation was carried out with palladium on (10% carbon, 0.5 g) which was added to a stirred solution of the above alkenes (2.7 g, 3.33 mmol) in THF (20 mL) and IMS (40mL) under hydrogen for 1 hour. The crude product was purified by column chromatography eluting with petrol/ethyl acetate (5:1) to give a semi-solid, methyl (*R*)-2-((*R*)-1-(*tert*-butyldimethyylsilyoxy)-11-hydroxyundecyl)-26-(2,2-dimeth -ylpropionyloxy)hexacosanoic acid methyl ester **(234)** (2.4 g, 88%), $[\alpha]_D^{22}$ = -3.69 (*c* 1.19, $CHCl_3$) {Found $(M + Na)^+$: 833.7025, $C_{49}H_{98}O_6SiNa$ requires: 833.7030}, which showed δ_H (500MHz, $CDCl_3$): 4.03 (2H, t, *J* 7.3 Hz), 3.90-3.87 (1H, m), 3.64 (3H, s),

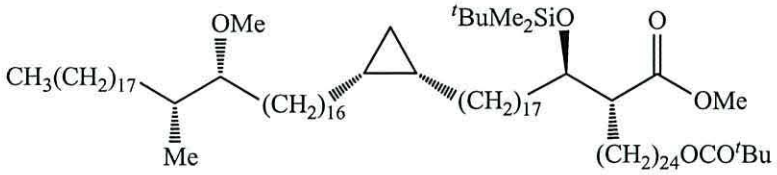
3.62 (2H, t, *J* 6.5 Hz), 2.51 (1H, ddd, *J* 3.75, 7.25, 10.7 Hz), 1.61-1.50 (6H, m), 1.44-1.18 (68H, m, including a singlet at δ 1.18), 0.85 (9H, s), 0.03 (3H, s), 0.01 (3H, s); δ_C (126MHz, $CDCl_3$): 178.62, 175.10, 73.16, 64.43, 62.97, 51.53, 51.18, 38.68,33.62, 32.76, 29.77, 29.67, 29.61, 29.53, 29.48, 29.39, 29.18,28.57, 27.79,27.16, 25.87, 25.79, 25.71, 23.67, 20.98, 17.93, 14.15, -4.40, -4.96; ν_{max}: 3344, 2927, 2854, 1732, 1655, 1546, 1463, 1284, 1253, 1157, 1034, 836, 775 cm^{-1}.

Experiment 44: (*R*)-2-[(*R*)-1-*tert*-Butyldimethylsilanyloxy)-11-oxopropyl]-26-(2,2-dimethylpropionyloxy)hexacosanoic acid methyl ester (207)



The procedure used in Experiment **8** was repeated to oxdise (*R*)-2-((*R*)-1-(*tert*-butyl-dimethylsilanyloxy)-11-hydroxy-propyl)-26-(2,2-dimethylpropionyloxy)hexacosanoic acid methyl ester **(234)** (1.2 g, 1.47 mmol) using PCC (0.79 g, 3.69 mmol) in dichloromethane (50 mL). The crude product was purified by chromatography eluting with petrol/ethyl acetate (10:1) to give a colourless oil, (*R*)-2-[(*R*)-1-*tert*-butyl-dimethylsilanyloxy)-11-oxopropyl]-26-(2,2-dimethylpropionyloxy)hexacosanoic acid methyl ester **(207)** (1 g, 84 %) {Found $(M + Na)^+$:831.6858, $C_{49}H_{96}O_6SiNa$ requires: 831.6874}, $[\alpha]_D^{20}$ = -3.98 (*c* 0.94, $CHCl_3$), which showed δ_H (500MHz, $CDCl_3$): 9.77 (1H, t, *J* 3.75 Hz), 4.03 (2H, t, *J* 5.05 Hz), 3.92-3.89 (1H, m), 3.66 (3H, s), 2.54-2.50 (1H, m), 2.44-2.40 (2H, m), 1.62 (2H, pent, *J* 6.9 Hz), 1.43-1.14 (69H, m, including s at δ 1.20), 0.86 (9H, s), 0.04 (3H, s), 0.02 (3H, s); δ_C (126MHz, $CDCl_3$): 202.80, 178.62, 175.06, 73.21, 64.45, 51.61, 51.20, 43.90, 38.71, 33.67, 29.79, 29.70, 29.64, 29.58, 29.55, 29.51, 29.48, 29.44, 29.34, 29.31, 29.22, 29.16, 28.61, 27.84, 27.45, 27.20, 25.90, 23.78, 22.08, 17.97, -4.37, -4.91; ν_{max}: 2924, 2852, 1734, 1709, 1607, 1494, 1402, 1107, 1050, 824 cm^{-1}.

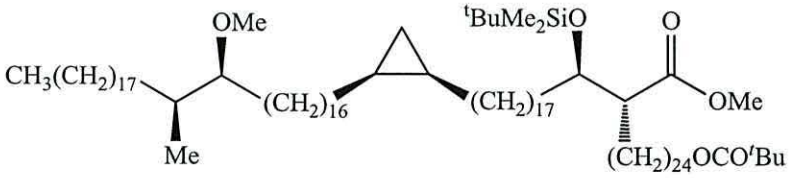
Experiment 45: Methyl (*R*)-2-((*R*)-1-(*tert*-butyldimethylsiloxy)-18-(1*S*,2*R*)-2-(17*R*, 18*R*)-17-methoxy-18-methylhexatriacontyl)cyclopropyl)octadecyl)-26-(2,2-dimeth -ylpropionyloxy)hexacosanoate(236)



The procedure used in Experiment **1** was repeated in order to couple (*R*)-2-[(*R*)-1-*tert*-butyldimethylsilanyloxy)-11-oxopropyl]-26-(2,2-dimethylpropionyloxy)hexacosanoic acid methyl ester **(207)** (1.0 g, 1.33 mmol) and 5-{7-[(1*S*,2*R*)-2-((17*R*,18*R*)-17-methoxy-18-methylhexatriacontenyl)cyclopropyl]heptylsulfonyl}-1-phenyl-1*H*-tetraz -ole **(124)** (1.37 g, 1.53 mmol) using lithium bis(trimethylsilyl)amide (2.17 mL, 2.30 mmol, 1.06 M) in dry THF (50 mL). The crude prodect was purified by column chromatography eluting with petrol/ethyl acetate (20:1) to give a colourless oil, methyl (*E/Z*)-2-((*R*)-1-(*tert*-butyldimethylsiloxy)-18-(1*S*,2*R*)-2-(17*R*,18*R*)-17-methoxy-18-hex -atriacontyl)cyclopropyl)octadec-11-enyl)-26-(2,2-dimethyl-propionyloxy)hexacosano -ate **(235)** (1.6 g, 89%) as a mixture. Hydrogenation was carried out with dipotassium azodicarboxylate (2.0 g, 10.31 mmol) which added to a stirred solution of the above alkenes (1.6 g, 1.10 mmol) in THF (30 mL) and methanol (7 mL) and a solution of glacial acetic acid (5 mL) in THF (5 mL) was added as before. The crude product was purified by column chromatography eluting with petrol/ethyl acetate (15:1) to give methyl (*R*)-2-((*R*)-1-(*tert*-butyldimethylsiloxy)-18-(1*S*,2*R*)-2-(17*R*,18*R*)-17-methoxy-18-methylhexatriacontyl)cyclopropyl)octadecyl)-26-(2,2-dimethylpropionyloxy)hexac -osanoate **(236)** as a thick colourless oil (1.5 g, 93%), $[\alpha]_D^{22} = +2.75$ (*c* 0.87, $CHCl_3$) {Found $(M + Na)^+$: 1504.4309, $C_{97}H_{192}O_6SiNa$ requires: 1504.4580}, which showed δ_H (500MHz, $CDCl_3$): 4.05 (2H, t, *J* 6.65 Hz), 3.95-3.89 (1H, m), 3.66 (3H, s), 3.34 (3H, s), 2.96-2.94 (1H, m), 2.53 (1H, ddd, *J* 3.8, 7.25, 11.05 Hz), 1.64-1.59 (4H, m), 1.37-1.22 (143H, m), 1.20 (9H, s), 0.90-0.84 (15H, m, including a singlet at δ 0.87), 0.66-0.63 (2H, m), 0.56 (1H, br.dt, *J* 4.1, 8.2 Hz), 0.04 (3H, s), 0.02 (3H, s), -0.32 (1H, br.q, *J* 4.75 Hz); δ_C (126MHz, $CDCl_3$): 178.61, 175.10, 85.44, 73.23, 64.45, 57.69, 51.58, 51.18, 38.71, 35.36, 33.69, 32.39, 31.92, 30.50, 30.22, 29.98, 29.94, 29.82, 29.70, 29.58, 29.52, 29.44, 29.36, 29.23, 28.72, 28.62, 27.83, 27.57, 2749, 27.20,

26.16, 25.91, 25.76, 23.72, 22.68, 17.97, 15.77, 14.88,14.10, 10.91, -4.37, -4.93; ν_{max}: 2924, 2853, 1733, 1465,1157, 1099, 836, 775 cm^{-1}.

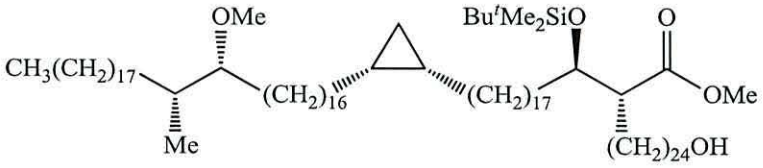
Experiment 46: Methyl (*R*)-2-((*R*)-1-(*tert*-Butyldimethylsiloxy)-18-(1*R*,2*S*)-2-(17*S*,18*S*)-17-methoxy-18-methylhexatriacontyl)cyclopropyl)octadecyl)-26-(2,2-dimethylpropionyloxy)hexacosanoate(239)



The procedure used in Experiment **1** was repeated in order to couple (*R*)-2-[(*R*)-1-*tert*-butyldimethylsilanyloxy)-11-oxopropyl]-26-(2,2-dimethylpropionyloxy)hexacosanoic acid methyl ester **(207)** (0.97 g, 1.19 mmol) and 5-{7-[(1*R*,2*S*)-2-((17*S*,18*S*)-17-methoxy-18-methylhexatriacontenyl)cyclopropyl]heptylsulfonyl}-1-phenyl-1*H*-tetrazo-le **(237)** (1.23 g, 1.37 mmol) using lithium bis(trimethylsilyl)amide (2.17 mL, 2.30 mmol, 1.06 M) in dry THF (50 mL). The crude product was purified by column chromatography eluting with petrol/ethyl acetate (20:1) to give a colourless oil, methyl (*E*/*Z*) (*R*)-2-((*R*)-1-(*tert*-butyldimethylsiloxy)-18-(1*R*,2*S*)-2-(17*S*,18*S*)-17-methoxy-18-methylhexatriacontyl)cyclopropyl)octadec-11-enyl)-26-(2,2-dimethyl-propionyloxy) hexacosanoate **(238)** (1.2 g, 71%) as a mixture. Hydrogenation was carried out with dipotassium azodicarboxylate (2.0 g, 10.31 mmol) which was added to a stirred solution of the above alkenes (1.2 g, 0.81 mmol) in THF (30 mL) and methanol (7 mL) followed by a solution of glacial acetic acid (3 mL) in THF (3 mL). The crude product was purified by column chromatography eluting with ethyl acetate (15:1) to give methyl-(*R*)-2-((*R*)-1-(*tert*-butyldimethylsiloxy)-18-(1*R*,2*S*)-2-(17*S*,18*S*)-17-methoxy-18-methylhexatriacontyl)cyclopropyl)octadecyl)-26-(2,2-dimethyl-propionyloxy)hexa-cosanoate as a thick colourless oil, **(239)** (0.94 g, 78%), $[\alpha]_D^{23}$ = -5.60 (*c* 1.07 g, CH_3Cl) {Found $(M + Na)^+$: 1504.4639, $C_{97}H_{192}O_6SiNa$ requires: 1504.4580}, which showed δ_H (500MHz, $CDCl_3$): 4.06 (2H, t, *J* 6.6 Hz), 3.92-3.89 (1H, m), 3.66 (3H, s), 3.34 (3H, s), 2.97-2.94 (1H,m), 2.53 (1H, ddd, *J* 3.8, 7.25, 11.00 Hz), 1.64-1.53 (4H, m), 1.37-1.23 (143H, m), 1.20 (9H, s), 0.90-0.82 (15H, m, including a singlet at δ 0.86), 0.66-0.64 (2H, br. m), 0.56 (1H, dt, *J* 3.75, 7.9 Hz), 0.04 (3H, s), 0.02 (3H, m), -

0.32 (1H, br.q, *J*, 5.35 Hz); δ_C (126MHz, $CDCl_3$): 178.25, 175.12, 85.43, 73.22, 64.45, 57.70, 51.57, 51.19, 38.71, 35.34, 33.68, 32.37, 31.92, 30.49, 30.22, 29.97, 29.82, 29.66, 29.58, 29.52, 29.44, 29.35, 29.23, 28.88, 28.61, 27.83, 27.56, 27.19, 26.15, 25.75, 23.70, 22.68, 17.97, 15.77, 14.87, 14.10, 10.91, -4.37, -4.93; ν_{max}: 2923, 2853, 1732, 1464, 1156, 1099, 836 cm^{-1}.

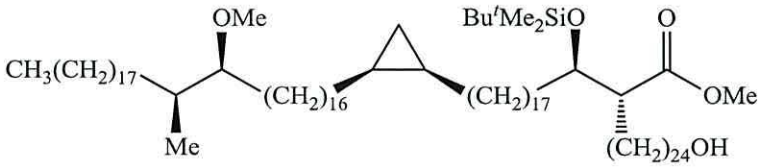
Experiment 47: Methyl (*R*)-2-((*R*)-1-(*tert*-butyldimethylsiloxy)-18-(1*S*,2*R*)-2-(17*R*,18*R*)-17-methoxy-18-methylhexatriacontyl)cyclopropropyl)octadecyl)-26-hydroxyhexacosanoate(208)



Methyl (*R*)-2-((*R*)-1-(*tert*-butyldimethylsiloxy)-18-(1*S*,2*R*)-2-(17*R*,18*R*)-17-methoxy-18-methylhexatriacontyl)cyclopropyl)octadecyl)-26-(2,2-dimethyl-propionyloxy)hexa -cosanoate **(236)** (1.5 g, 1.02 mmol) in THF (3 mL) was added to a stirred solution of potassium hydroxide (0.88 g, 15.51 mmol) in a mixture of THF (15 mL), methanol (15 mL) and water (1.5 mL). The mixture was heated under reflux at 70 °C and monitored by TLC. After 3 hours, TLC showed no starting material was left then the reaction was quenched with water (10 mL) and extracted with petrol/ethyl acetate (10:1) (3 × 25 mL). The combined organic extracts were dried, filtered and evaporated. The crude prodect was purified by column chromatography eluting with petrol/ethyl acetate (10:1) to give methyl (*R*)-2-((*R*)-1-(*tert*-butyldimethylsiloxy)-18-(1*S*,2*R*)-2-(17*R*,18*R*)-17-methoxy-18-methylhexatriacontyl)cyclopropyl)octadecyl)-26-hydroxyhexacosanoa -te **(208)** (0.85 g, 60%), as a thick colourless oil; $[\alpha]_D^{22} = +4.14$ (*c* 0.94, $CHCl_3$) {Found $(M + Na)^+$: 1420.3663, $C_{92}H_{184}O_5SiNa$ requires: 1420.3805}, which showed δ_H (500MHz, $CDCl_3$): 3.92-3.89 (1H, m), 3.66 (3H, s), 3.64 (2H, t, *J* 6.9 Hz), 3.34 (3H, s), 2.97-2.94 (1H, m), 2.53 (1H, ddd, *J* 3.45, 6.95, 10.7 Hz), 1.57 (2H, pent, *J* 6.6 Hz), 1.39-1.15 (146H,m), 0.9-0.84 (15H, m including a singlet at δ 0.86), 0.67-0.64 (2H, m), 0.57 (1H, dt, *J* 4.1, 8.2 Hz), 0.05 (3H, s), 0.02 (3H, s) -0.32 (1H, br.q, *J* 5.05 Hz); δ_C (126MHz, $CDCl_3$): 175.12, 85.45, 73.23, 63.10, 57.71, 51.59, 51.19, 35.36, 33.70, 32.83, 32.40, 31.92, 30.51, 30.22, 29.98, 29.93, 29.82, 29.60, 29.43, 29.35, 28.72,

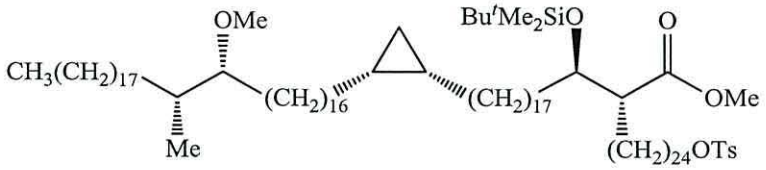
27.83, 27.57, 27.49, 26.17, 25.76, 23.73, 22.68, 17.97, 15.78, 14.80, 14.10, 10.91, -4.37, -4.92; ν_{max}: 3450, 2923, 2853, 1741, 1464, 1361, 1254, 1099, 836, 720 cm^{-1}.

Experiment 48: Methyl (*R*)-2-((*R*)-1-(*tert*-butyldimethylsiloxy)-18-(1*R*,2*S*)-2-(17*S*,18*S*)-17-methoxy-18-methylhexatriacontyl)cyclopropropyl)octadecyl)-26-hydroxyhexacosanoate (240)



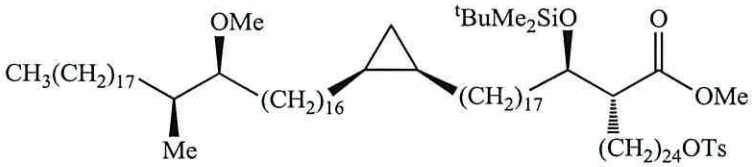
The procedure used in Experiment **47** was repeated to hydrolyse methyl (*R*)-2-((*R*)-1-(tert-butyldimethylsiloxy)-18-(1*R*,2*S*)-2-(17*S*,18*S*)-17-methoxy-18-methylhexa -triacontyl)cyclopropyl)octadecyl)-26-(2,2-dimethylpropionyloxy)hexacosanoate **(239)** (0.94 g, 0.63 mmol) using potassium hydroxide (0.53 g, 9.45 mmol) in a mixture of THF (15 mL), methanol (15 mL) and water (1.5 mL). The crude product purified by column chromatography eluting with petrol/ethyl acetate (10:1) gave methyl (*R*)-2-((*R*)-1-(tert-butyldimethylsiloxy)-18-(1*R*,2*S*)-2-(17*S*,18*S*)-17-methoxy-18-methylhexa -triacontyl)cyclopropyl)octadecyl)-26-hydroxyhexacosanoate **(240)** (0.62 g, 70%), as a thick colourless oil, $[\alpha]_D^{23}$ = -4.62 (*c* 0.67, $CHCl_3$) {Found $(M + Na)^+$: 1420.3700, $C_{92}H_{184}O_5SiNa$ requires: 1420.3805}, which showed δ_H (500MHz, $CDCl_3$): 3.92-3.89 (1H, m), 3.66 (3H, s), 3.64 (2H, t, *J* 6.95 Hz), 2.97-2.94 (1H, m), 2.55 (1H, ddd, *J* 3.8, 7.25, 11.00 Hz), 1.59-1.54 (8H, m), 1.37-1.14 (140H, m), 0.90-0.84 (18H, m, including a singlet at δ 0.86), 0.64 (2H, br. m), 0.56 (1H, dt, *J* 4.1, 8.15 Hz), 0.04 (3H, s), 0.02 (3H, s), -0.32 (1H, br.q, *J*, 5.00 Hz); δ_C (126MHz, $CDCl_3$): 175.14, 85.44, 73.22, 63.10, 57.70, 51.57, 51.21, 35.33, 33.67, 32.82, 32.37, 31.92, 30.49, 30.22, 29.97, 29.93, 29.82, 29.60, 29.43, 29.35, 28.72, 27.83, 27.56, 27.49, 26.15, 25.75, 23.71, 22.66, 17.94, 15.75, 14.88, 14.11, 10.91, -4.37, -4.93; ν_{max}: 3371, 2921, 2852, 1741, 1466, 1097, 836 cm^{-1}.

Experiment 49: Methyl (*R*)-2-((*R*)-1-(*tert*-butyldimethylsiloxy)-18-(1*S*,2*R*)-2-(17*R*,18*R*)-17-methoxy-18-methylhexatriacontyl)cyclopropyl)octadecyl)-26 (tosyloxy)hexacosanoate (241)



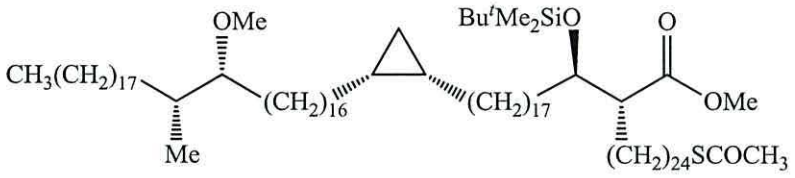
Toluene sulfonylchloride (0.143 g, 0.754 mmol) was added to a stirred solution of methyl (*R*)-2-((*R*)-1-(*tert*-butyldimethylsiloxy)-18-(1*S*,2*R*)-2-(17*R*,18*R*)-17-methoxy-18-methylhexatriacontyl)cyclopropyl)octadecyl)-26-hydroxyhexacosanoate **(208)** (0.81 g, 0.58 mmol) and triethylamine (3 mL) in dry dichloromethane (25 mL) at -20 °C under nitrogen. The solution was kept in the refrigerator overnight. When TLC showed no starting material was left, the solvent was evaporated. The crude product was purified by column chromatography eluting with petrol/ethyl acetate (10:1) to give methyl (*R*)-2-((*R*)-1-(*tert*-butyldimethylsiloxy)-18-(1*S*,2*R*)-2-(17*R*,18*R*)-17-methoxy-18-methylhexatriacontyl)cyclopropyl)octadecyl)-26-(tosyloxy)hexacosanoate **(241)** (0.66 g, 74%), as a colourless oil, $[\alpha]_D^{22}$ = +15.17 (*c* 0.87, $CHCl_3$) {Found $(M + Na)^+$: 1574.3817, $C_{99}H_{190}O_7SSiNa$ requires: 1574.3894}, which showed δ_H (500 MHz, $CDCl_3$): 7.80 (2H, d, *J*, 8.2 Hz), 7.35 (2H, d, *J* 8.00 Hz), 4.02 (2H, t, *J* 6.30 Hz), 3.92-3.89 (1H, m), 3.66 (3H, s), 3.34 (3H, s), 2.96-2.94 (1H, m), 2.53 (1H, ddd, *J* 3.8, 7.25, 10.9 Hz), 2.45 (3H, s), 1.63 (4H, pent, *J* 6.3 Hz), 1.57-1.53 (2H, m), 1.41-1.19 (141H, m), 0.90-0.83 (15H, m, including a singlet at δ 0.88), 0.68-0.66 (2H, m), 0.56 (1H, dt, *J* 4.1, 8.2 Hz), 0.05 (3H, s), 0.02 (3H, s), -0.32 (1H, br.q, *J* 5.05 Hz); δ_C (126MHz, $CDCl_3$): 175.10, 144.55, 133.36, 129.76, 127.88, 85.44, 73.23, 70.68, 57.71, 51.59, 51.19, 35.36, 33.69, 32.39, 31.92, 30.50, 30.22, 29.98, 29.94, 29.82, 29.71, 29.70, 29.58, 29.50, 29.45, 29.39, 29.36, 28.93, 28.83, 28.72, 27.83, 27.57, 27.49, 26.17, 25.76, 25.33, 23.73, 22.68, 21.60, 19.42, 17.97, 14.88, 14.10, 10.91, -4.37, -4.92; ν_{max}: 2923, 2852, 1740, 1464, 1253, 1099,836, 720 cm^{-1}.

Experiment 50: Methyl (*R*)-2-((*R*)-1-(*tert*-butyldimethylsiloxy)-18-(1*R*,2*S*)-2-(17*S*,18*S*)-17-methoxy-18-methylhexatriacontyl)cyclopropyl)octadecyl)-26-(tosyl oxy)hexacosanoate (242)



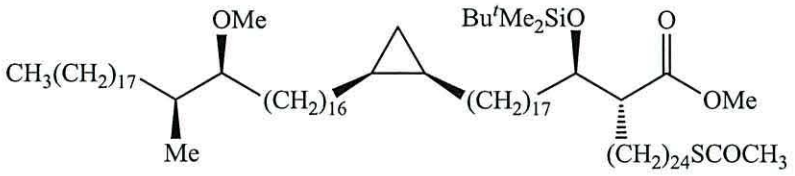
The procedure used in Experiment **49** was repeated using methyl (R)-2-((*R*)-1-(*tert*-butyldimethylsiloxy)-18-(1*R*,2*S*)-2-(17*S*,18*S*)-17-methoxy-18-methylhexatriacon -tyl)cyclopropyl)octadecyl)-26-hydroxyhexacosanoate **(240)** (0.61 g, 0.43 mmol), toluene sulfonylchloride (0.108 g, 0.56 mmol) and triethylamine (3 mL) in dry dichloromethane (25 mL). The crude product was purified by column chromatography petrol/ethyl acetate (10:1) gave methyl (R)-2-((R)-1-(*tert*-butyldimethylsiloxy)-18-(1R,2S)-2-(17S,18S)-17-methoxy-18-methylhexatriacontyl)cyclopropyl)octadecyl)-26 -(tosyloxy)hexacosanoate **(242)** (0.5 g, 75%), as a colourless oil, $[\alpha]_D^{20} = -7.22$ (*c* 0.83, $CHCl_3$) {Found $(M + Na)^+$: 1574.3777, $C_{99}H_{190}O_7SiSNa$ requires: 1574.3894}, which showed δ_H (500MHz, $CDCl_3$): 7.79 (2H, d, *J* 8.2 Hz), 7.34 (2H, d, *J* 7.9 Hz), 4.02 (2H, t, *J* 6.3 Hz), 3.92-3.89 (1H, m), 3.65 (3H, s), 3.34 (3H, s), 2.97-2.94 (1H, m), 2.53 (1H, ddd, *J* 3.8, 7.25, 11.05 Hz), 2.45 (3H, s), 1.64-1.60 (2H, m), 1.42-1.11 (145H, m), 0.89-0.82 (15H, m, including a singlet at δ 0.86), 0.65-0.64 (2H, br. m), 0.56 (1H, dt, *J* 3.4, 8.12 Hz), 0.04 (3H, s), 0.02 (3H, s), -0.32 (1H, br.q, *J* 5.35 Hz); δ_C (126MHz, $CDCl_3$): 175.12, 144.54, 133.31, 129.76, 127.87, 85.43, 73.21, 70.66, 57.69, 51.57, 51.19, 35.33, 33.66, 32.37, 31.92, 30.47, 30.21, 29.96, 29.93, 29.81, 29.71, 29.69, 29.60, 29.58, 29.49, 29.44, 29.38, 29.35, 28.91, 28.80, 28.71, 27.82, 27.56, 27.48, 26.14, 25.74, 25.31, 23.69, 22.67, 21.60, 19.40, 17.96, 14.87, 14.18, 10.90, -4.38, -4.94; ν_{max}: 2922, 2853, 1740, 1465, 1369, 1178, 1098, 836 cm^{-1}.

Experiment 51: Methyl (*R*)-2-methyl-26-(acetylthio)-2-((*R*)-1-(*tert*-butyldimethylsiloxy)-18-(1*S*,2*R*)-2-(17*R*,18*R*)-17-methoxy-18-methylhexatriacontyl)cyclopropyl)octadecyl)hexacosanoate (209)



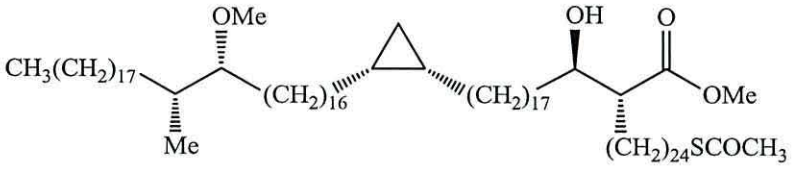
Potassium thioacetate (0.2 g, 1.78 mmol) was added to a stirred solution of methyl (*R*)-2-((*R*)-1-(*tert*-butyldimethylsiloxy)-18-(1*S*,2*R*)-2-(17*R*,18*R*)-17-methoxy-18-methylhe-xatriacontyl)cyclopropyl)octadecyl)-26-(tosyloxy)hexacosanoate **(241)** (0.66 g, 0.42 mmol) dissolved in dry THF (5 mL) and acetone (15 mL) at room temperature. The reaction mixture was stirred at room temperature for 16 hours. When TLC showed no strating material was left, the solvent was evaporated. The crude product was purified by column chromatography eluting with petrol/ethyl acetate (20:1) to give methyl (*R*)-26-(acetylthio)-2-((*R*)-1-(*tert*-butyldimethylsiloxy)-18-(1*S*,2*R*)-2-(17*R*,18*R*)-17-metho-xy-18-methylhexatriacontyl)cyclopropyl)octadecyl)-hexacosanoate **(209)** (0.51 g, 82%) as a pale yellow thick oil, $[\alpha]_D^{22} = +5.28$ (*c* 0.87, $CHCl_3$), {Found $(M + Na)^+$: 1478.3565, $C_{94}H_{186}O_5SSiNa$ requires: 1478.3682}, which showed δ_H (500 MHz, $CDCl_3$): 3.91 (1H, br.q, *J* 5.0 Hz), 3.66 (3H, s), 3.34 (3H, s), 2.96-2.94 (1H, m), 2.86 (2H, t, *J* 7.5 Hz), 2.53 (1H, ddd, *J* 3.8, 7.25, 10.70 Hz), 2.32 (3H, s), 1.61-1.51 (6H, m), 1.42-1.14 (141H, m), 0.89-0.83 (15H, m, including a singlet at δ 0.87), 0.67-0.64 (2H, m), 0.56 (1H, dt, *J* 3.75, 8.2 Hz), 0.04 (3H, s), 0.02 (3H, s), -0.32 (1H, br.q, *J*, 5.05 Hz); δ_C (126MHz, $CDCl_3$): 195.98, 175.11, 85.45, 73.24, 57.71, 51.59, 51.19, 35.36, 33.70, 32.40, 31.94, 30.61, 30.61, 30.51, 30.23, 29.99, 29.95, 29.84, 29.72, 29.71, 29.59, 29.51, 29.49, 29.46, 29.37, 29.17, 29.13, 29.06, 28.84, 27.85, 27.57, 26.19, 25.79, 25.35, 23.75, 22.71, 19.47, 17.81, 14.91, 14.11, 10.92, -4.38, -4.93; ν_{max}: 2923, 2853, 1740, 1465, 1099, 836, 720 cm^{-1}.

Experiment 52: Methyl (*R*)-26-(acetylthio)-2-((*R*)-1-(*tert*-butyldimethylsiloxy)- 18-(1*R*,2*S*)-2-(17*S*,18*S*)-17-methoxy-18-methylhexatriacontyl)cyclopropyl)octadecyl) hexacosanoate (243)



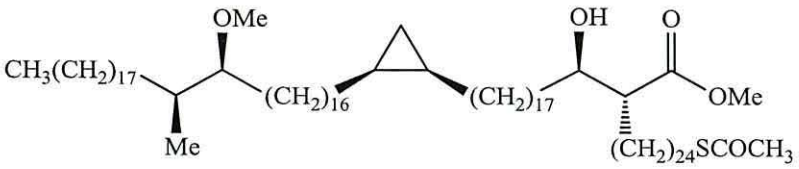
The procedure used in Experiment **51** was repeated using methyl (*R*)-2-((*R*)-1-(*tert*-butyldimethylsiloxy)-18-(1*R*,2*S*)-2-(17*S*,18*S*)-17-methoxy-18-methylhexatriacontyl)-cyclopropyl)octadecyl)-26-(tosyloxy)hexacosanoate **(242)** (0.5 g, 0.32 mmol) and potassium thioacetate (0.147 g, 1.29 mmol) in dry THF (5 mL) and acetone (15 mL). The crude product was purified by column chromatography eluting with petrol/ethyl acetate (20:1) to give methyl (*R*)-26-(acetylthio)-2-((*R*)-1-(*tert*-butyldimethylsiloxy)-18-(1*R*,2*S*)-2-(17*S*,18*S*)-17-methoxy-18-methylhexatriacontyl)cyclopropyl)octadecyl) hexacosanoate **(243)** (0.35 g, 76%) as a pale yellow thick oil, $[\alpha]_D^{20} = -5.83$ (*c* 1.37, $CHCl_3$) {Found $(M + K)^+$: 1478.3724, $C_{94}H_{186}O_4SSiK$ requires: 1478.3473}, which showed δ_H (500MHz, $CDCl_3$): 3.92-3.89 (1H, m), 3.66 (3H, s), 3.34 (3H, s), 2.97-2.94 (1H, m), 2.86 (2H, t, *J* 7.25 Hz), 2.53 (1H, ddd, *J* 3.8, 7.25, 10.75 Hz), 2.32 (3H, s), 1.64-1.53 (6H, m), 1.37-1.08 (141H, m), 0.90-0.84 (15H, m, including a singlet at δ 0.86), 0.65-0.64 (2H, br. m), 0.58-0.54 (1H, m), 0.04 (3H,s), 0.02 (3H, s), -0.32 (1H, br.q, *J* 5.00 Hz); δ_C (126MHz, $CDCl_3$): 196.05, 175.14, 85.44, 73.22, 57.71, 51.57, 51.21, 35.33, 33.67, 32.37, 31.92, 30.62, 30.48, 30.22, 29.98, 29.93, 29.82, 29.71, 29.69, 29.58, 29.49, 29.47, 29.36, 29.12, 28.82, 27.83, 27.57, 26.16, 25.76, 23.69, 22.68, 17.97, 15.77, 14.88, 14.11, 10.91, -4.37, -4.93; ν_{max}: 2924, 2853, 1740, 1697, 1465, 1360, 1254, 1099, 836 cm^{-1}.

Experiment 53: Methyl (*R*)-26-(acetylthio)-2-((*R*)-1-hydroxy-18-(1*S*, 2*R*)-2-(17*R*, 18*R*)-17-methoxy-18-methylhexatriacontyl)cyclopropyl)octadecyl)hexacosanote (244)



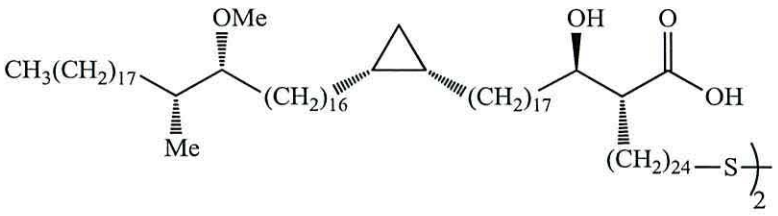
The procedure used in Experiment **2** was repeated using methyl (*R*)- 26-(acetylthio)-2-((*R*)-1-(*tert*-butyldimethylsiloxy)-18-(1*S*,2*R*)-2-(17*R*,18*R*)-17-methoxy-18-methylhexa -triacontyl)cyclopropyl)octadecyl)hexacosanoate **(209)** (0.45 g, 0.30 mmol), pyridine (0.2 mL) and HF.pyridine (0.8 mL) in dry THF (12 mL). The crude product was purified by column chromatography eluting with petrol/ethyl acetate (10:1) gave methyl (*R*)-26-(acetylthio)-2-((*R*)-1-hydroxy-18-(1*S*,2*R*)-2-(17*R*,18*R*)-17-methoxy-18-methylhex -atriacontyl)cyclopropyl)octadecyl)hexacosa-noate **(244)** (0.4 g, 97%) as a white solid, m.p. 40-42 °C, $[\alpha]_D^{23}$ = + 6.34 (*c* 0.82, $CHCl_3$) {Found $(M + Na)^+$: 1364.2809, $C_{88}H_{172}O_5SNa$ requires: 1364.2818}, which showed δ_H (500MHz, $CDCl_3$): 3.71 (3H, s), 3.68-3.64 (1H, m), 3.34 (3H, s), 2.96-2.94 (1H, m), 2.86 (2H, t, *J* 7.6 Hz), 2.46-2.42 (1H, m), 2.32 (3H, s), 1.73-1.69 (2H, m), 1.63-1.53 (4H, m), 1.46-1,14 (142H, m), 0.88 (3H, t, *J* 5.65 Hz), 0.85 (3H, d, *J* 6.9 Hz), 0.66-0.65 (2H, br.m), 0.56 (1H, dt, *J* 4.1, 8.55 Hz), -0.32 (1H, br.q, *J* 5.00 Hz); δ_C (126MHz, $CDCl_3$): 195.96, 176.18, 85.43, 72.29, 57.68, 51.46, 50.95, 35.69, 35.35, 32.38, 31.92, 30.59, 30.49, 30.21, 29.97, 29.93, 29.69, 29.63, 29.60, 29.57, 29.54, 29.49, 29.47, 29.42, 29.35, 29.15, 29.11, 28.82, 28.72, 27.56, 27.42, 26.15, 25.72, 22.67, 15.77, 14.87, 14.09, 10.91; ν_{max}: 3518, 2920, 2850, 1709, 1694, 1466, 1165, 1098, 720 cm^{-1}.

Experiment 54: Methyl (*R*)-26-(acetylthio)-2-((*R*)-1-hydroxy-18-(1*R*,2*S*)-2-(17*S*,18*S*)-17-methoxy-18-methylhexatriacontyl)cyclopropyl)octadecyl)hexacosan -ote (245)



The procedure used in Experiment **2** was repeated using (*R*)-methyl-26-(acetylthio)-2-((*R*)-1-(*tert*-butyldimethylsiloxy)-18-(1*R*,2*S*)-2-(17*S*,18*S*)-17-methoxy-18-methylhexa -tria-contyl)cyclopropyl)octadecyl)hexacosanoate **(243)** (0.35 g, 0.24 mmol), pyridine (0.2 mL) and HF.pyridine (0.8 mL) in dry THF (12 mL). The crude product was purified by column chromatography eluting with petrol/ethyl acetate (10:1) to give methyl (*R*)-26-(acetylthio)-2-((*R*)-1-hydroxy-18-(1*R*,2*S*)-2-(17*S*,18*S*)-17-methoxy-18-methylhexa -triacontyl)cyclopropyl)octadecyl)hexacosanoate **(245)** (0.2 g, 63%) as white solid, m.p. 43-45°C, $[\alpha]_D^{20}$ = - 6.02 (*c* 0.88, $CHCl_3$) {Found $(M + Na)^+$: 1364.2836, $C_{88}H_{172}O_5SNa$ requires: 1364.2818}, which showed δ_H (500MHz, $CDCl_3$): 3.70 (3H, s), 3.67-3.65 (1H, m), 3.34 (3H, s), 2.97-2.94 (1H, m), 2.87 (2H, t, *J* 7.55 Hz), 2.45-2.40 (1H, m), 2.32 (3H, s), 1.74-1.69 (2H, m), 1.58-1.13 (146H, m), 0.90-0.84 (6H, including t, *J* 6.65 Hz and d, *J* 6.95 Hz), 0.66-0.65 (2H, br.m), 0.56 (1H, dt, *J* 3.82, 7.76 Hz), -0.32 (1H, br.q, *J* 5.05 Hz); δ_C (126MHz, $CDCl_3$): 196.23, 176.22, 85.44, 72.30, 57.71, 51.49, 50.94, 35.70, 35.34, 32.37, 31.92, 30.62, 30.49, 30.22, 29.98, 29.93, 29.69, 29.60, 29.57, 29.49, 29.42, 29.35, 29.16, 29.11, 28.82, 28.72, 27.57, 27.42, 26.16, 25.72, 22.68, 15.77, 14.88, 14.11, 10.91; ν_{max}: 3285, 2917, 2850, 1691, 1470, 1167, 1104, 720 cm^{-1}.

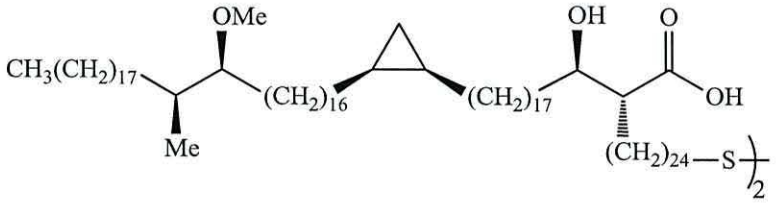
Experiment 55: (*R*,*R*,*R*,*S*,*R*,2*R*,2'*R*)-26,26'-Disulfanediylbis(2-((*R*)-1-hydroxy-18-((1*S*,2*R*)-2-((17*R*,18*R*)-17-methoxy-18-methylhexatriacontyl)cyclopropyl) octadecyl) hexacosanoic acid) (246)



Methyl (*R*)-26-(acetylthio)-2-((*R*)-1-hydroxy-18-(1*S*,2*R*)-2-(17*R*,18*R*)-17-methoxy-18-methylhexatriacontyl)cyclopropyl)octadecyl)hexacosanoate **(244)** (0.3 g, 0.22 mmol) was suspended in 5% aq. tetrabutylammonium hydroxide (20 mL) and heated to 100 °C overnight. The mixture was cooled to room temperature and acidified to pH 1 with 1M HCl and extracted with petrol/ethyl acetate (1:1, 3 × 30 mL). The combined organic layers were dried, filtered and evaporated. The product was purified by column

chromatography eluting with chloroform/methanol (10:1) to give (*R*,*R*,*R*,*S*,*R*,2*R*,2'*R*)-26,26'-disulfanediylbis(2-((*R*)-1-hydroxy-18-((1*S*,2*R*)-2-((17*R*,18 *R*)-17-methoxy-18-methylhexatriacontyl)cyclopropyl)octadecyl)hexacosanoic acid) **(246)** (150 mg, 54 %) as a white solid, $[\alpha]_D^{23}$ = +3.76 (*c* 0.85, $CHCl_3$), m.p. 61-63 °C; δ_H (500MHz, $CDCl_3$): 3.61-3.5 (2H, m), 3.29 (6H, s), 2.94-2.91 (2H, m), 2.63 (4H, t, *J* 7.25 Hz), 2.35-2.31 (2H, m), 1.62 (4H, pent, *J* 6.9 Hz), 1.42-1.05 (294H, m), 0.81 (6H, t, *J* 6.65 Hz), 0.79 (6H, d, *J* 6.60 Hz), 0.63-058 (4H, br.m), 0.51 (2H, dt, *J* 4.1, 8.5 Hz), -0.37 (2H, br.q, *J* 5.05); δ_C (126MHz, $CDCl_3$): 177.76, 85.55, 71.96, 57.53, 50.94, 39.09, 35.25, 32.27, 31.78, 30.37, 30.08, 29.82, 29.76, 29.55, 29.39, 29.38, 29.33, 29.21, 29.10, 28.59, 28.39, 27.38, 27.27, 25.96, 25.58, 22.54, 15.64, 14.66, 13.91, 10.75; ν_{max}: 3280, 2917, 2849, 1714, 1470, 1377, 1100, 719 cm^{-1}.

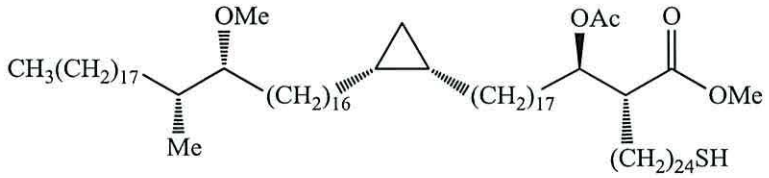
Experiment 56: (*R*,*R*,*R*,*S*,*R*,2*R*,2'*R*)-26,26'-Disulfanediylbis(2-((*R*)-1-hydroxy-18-((1*R*,2*S*)-2-((17*S*,18*S*)-17-methoxy-18-methylhexatriacontyl)cyclopropyl) octadecyl) hexacosanoic acid (247)



The procedure used in Experiment **55** was repeated using methyl (*R*)-26-(acetylthio)-2-((*R*)-1-hydroxy-18-(1*R*,2*S*)-2-(17*S*,18S)-17-methoxy-18-methylhexatri - acontyl)cyclopropyl)octadecyl)hexacosanoate **(245)** (0.1 g, 0.074 mmol) and 5% aqueous solution of TBAH (20 mL). The crude product was purified by column chromatography eluting with chloroform/methanol (10:1) to give a white solid (*R*,*R*,*R*,*S*,*R*,2*R*,2'*R*)-26,26'-disulfanediylbis(2-((*R*)-1-hydroxy-18-((1*R*,2*S*)-2-((17*S*,18*S*)-17-methoxy-18-methylhexatriacontyl)cyclopropyl)octadecyl)hexacosanoic acid **(247)** (25 mg, 26 %), m.p. 60-62 °C, $[\alpha]_D^{23}$ = -2.48 (*c* 1.37, CH_3Cl), δ_H (500MHz, $CDCl_3$): 3.73-3.69 (2H, m), 3.35 (6H, s), 2.98-2.97 (2H, m), 2.69 (4H, t, *J* 7.25 Hz), 2.48-2.44 (2H, m), 1.69-1.15 (298H, m), 0.90-0.84 (12H, including t, *J* 6.65 Hz and d, *J* 6.95 Hz), 0.64-0.65 (4H, br.m), 0.58-0.56 (2H, m), -0.32 (2H, br.q, *J* 4.7 Hz); δ_C (126MHz, $CDCl_3$): 179.77, 85.57, 72.14, 71.95, 57.67, 50.85, 39.28, 35.51, 35.33,

32.35, 31.92, 30.47, 30.22, 29.98, 29.93, 29.72, 29.70, 29.60, 29.53, 29.50, 29.44, 29.36, 29.22, 28.72, 28.50, 27.56, 27.34, 26.15, 25.73, 22.69, 15.77, 14.88, 14.11, 10.91; ν_{max}: 3278, 2921, 2851, 1708, 1465, 1376, 1098, 719 cm^{-1}.

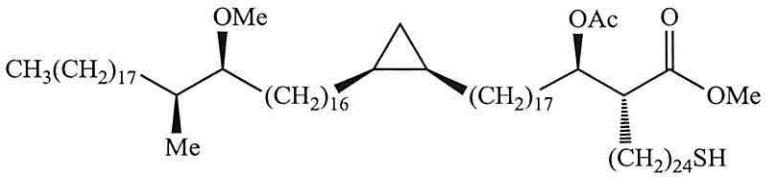
Experiment 57: Methyl (*R*)-2-((*R*)-1-acetoxy-18-((1*S*,2*R*)-2-((17*R*,18*R*)-17-methoxy-18-methylhexatriacontyl)cyclopropyl)octadecyl)-26-mercaptohexacosan –oate (250)



Excess diazomethane in ether was added to (*R*,*R*,*R*,*S*,*R*,2*R*,2'*R*)-26,26'-disulfanediylbis(2-((*R*)-1-hydroxy-18-((1*S*,2*R*)-2-((17*R*,18*R*)-17-methoxy-18-methyl-hexatriacontyl)cyclopropyl)octadecyl)hexacosanoic acid) **(246)** (7 mg) and stirred for 30 min. The solvent was evaporated to give dimethyl (*R*,*R*,*R*,*S*,*R*,2*R*,2'*R*)- 26,26'-disulfanediylbis(2-((*R*)-1-hydroxy-18-((1*S*,2*R*)-2-((17*R*,18*R*)-17-methoxy-18-methylh -exatriacontyl)cyclopropyl)octadecyl)hexacosanoate **(248)**, which showed δ_H (400MHz, $CDCl_3$): 3.70 (6H, s), 3.67-3.64 (2H, m), 3.34 (6H, s), 2.97-2.95 (2H, m), 2.68 (4H, t, *J* 7.28 Hz), 2.46-2.41 (2H, m), 1.72-1.13 (296H, m), 0.88 (6H, t, *J* 6.56 Hz), 0.85 (6H, d, *J* 6.88 Hz), 0.68-0.65 (4H, m), 0.57 (2H, dt, *J* 3.76, 7.92 Hz), -0.32 (2H, br.q, *J* 4.76 Hz); δ_C (100MHz, $CDCl_3$): 176.25, 85.44, 72.30, 57.70, 51.51, 50.92, 39.20, 35.70, 35.31, 32.35, 31.92, 30.47, 30.22, 29.97, 29.93, 29.69, 29.61, 29.57, 29.52, 29.43, 29.36, 29.25, 29.22, 28.71, 28.54, 27.56, 27.42, 26.15, 25.73, 22.69, 18.43, 15.76, 15.26, 14.88, 14.11, 10.90. The crude product was used for next step without purification. Acetic anhydride (0.3 mL) and pyridine (0.3 mL) were added to a stirred solution of dimethyl (*R*,*R*,*R*,*S*,*R*,2*R*,2'*R*)- 26,26'-disulfanediylbis(2-((*R*)-1-hydroxy-18-((1*S*,2*R*)-2-((17*R*,18*R*)-17-methoxy-18-methylhexatriacontyl)cyclopropyl) octadecyl)-hexacosanoate) **(248)** in toluene (0.3 mL). The reaction mixture was stirred for 18 hours then the solvent was evaporated under reduced pressure to give dimethyl (*R*,*R*,*R*,*S*,*R*,2*R*,2'*R*)-26,26'-disulfanediylbis(2-((*R*)-1-acetoxy-18-((1*S*,2*R*)-2-((17*R*,18*R*) -17-methoxy-18-methylhexatriacontyl)cyclopropyl)octadecyl)hexacosanoate **(249)**, which showed δ_H (400MHz, $CDCl_3$): 5.10-5.06 (2H, m), 3.68 (6H, s), 3.34 (6H, s),

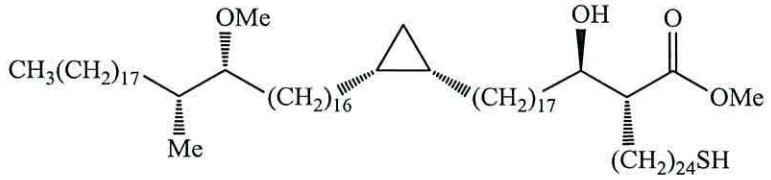
2.97-2.96 (2H,m), 2.68 (4H, t, *J* 7.28 Hz), 2.62 (2H, ddd, *J* 4.3, 7.0, 10.64 Hz), 2.03 (6H, s), 1.71-1.13 (294H, m), 0.88 (6H, t, *J* 6.40 Hz), 0.85 (6H, d, *J* 6.88 Hz), 0.68-0.66 (4H, m), 0.57 (2H, dt, *J* 3.92, 7.92 Hz), -0.32 (2H, br.q, *J* 4.92 Hz); δ_C (100MHz, $CDCl_3$): 173.67, 170.37, 85.46, 74.11, 57.70, 51.54, 49.59, 39.21, 35.32, 32.36, 31.92, 31.72, 30.59, 30.11, 29.98, 29.94, 29.65, 29.52, 29.40, 29.22, 29.10, 29.02, 28.65, 28.47, 28.11, 27.57, 27.47, 26.15, 24.98, 22.68, 21.01, 20.55, 15.77, 14.88, 14.11, 10.91. The product was used for next step without purification. *DL*-Dithiothreitol (100 mg) was added to a stirred solution of dimethyl (*R*,*R*,*R*,*S*,*R*,2*R*,2'*R*)-26,26'-disulfanediylbis(2-((*R*)-1-acetoxy-18-((1*S*,2*R*)-2-((17*R*,18*R*)-17-methoxy-18-methylhe -xatriacontyl)cyclo -propyl)octadecyl)hexacosa-noate **(249)** in chloroform (1 mL) followed by the addition of one drop of triethylamine under nitrogen. The flask was covered with aluminium foil. The reaction mixture was stirred for 48 hours at room temperature. The solvent was evaporated and the crude product was purified by column chromatography eluting with petrol/ethyl acetate (10:1) to give methyl (*R*)-2-((*R*)-1-acetoxy-18-((1*S*,2*R*)-2-((17*R*,18*R*)-17-methoxy-18-methylhexatriacontyl)cyclop -ropyl)octadecyl)-26-mercapto-hexacosanoate **(250)** (3.5 mg) {MALDI Found (M + Na)$^+$: 1364.10, $C_{88}H_{172}O_5SNa$ requires: 1364.29}, which showed δ_H (400MHz, $CDCl_3$): 5.13-5.06 (1H, m), 3.68 (3H, s), 3.34 (3H, s), 2.97-2.94 (1H, m), 2.62 (1H, ddd, *J* 4.36, 6.88, 10.88 Hz), 2.53(2H, q, *J* 7.52 Hz), 2.03 (3H, s), 1.17-1.13 (148H, m), 0.83 (3H, t, *J* 6.52 Hz), 0.81 (3H, d, *J* 7.4 Hz), 0.68-0.64 (2H, m), 0.57 (1H, dt, *J* 3.76, 8.04 Hz), -.032 (1H, br.q, *J* 5.16 Hz); δ_C (100MHz, $CDCl_3$): 173.67, 170.37, 85.43, 74.10, 57.71, 51.55, 49.58, 35.29, 34.063, 32.34, 31.92, 31.71, 30.45, 30.22, 29.98, 29.94, 29.70, 29.60, 29.57, 29.52, 29.47, 29.44, 29.40, 29.36, 29.08, 28.72, 28.38, 28.11, 27.57, 27.47, 26.16, 24.97, 24.66, 22.69, 22.34, 21.04, 15.76, 14.88, 14.12, 10.90; ν_{max}: 2921, 2851, 1746, 1609, 1493, 1452, 824 cm^{-1}.

Experiment 58: Methyl (*R*)-2-((*R*)-1-acetoxy-18-((1*R*,2*S*)-2-((17*S*,18*S*)-17-methoxy-18-methylhexatriacontyl)cyclopropyl)octadecyl)-26-mercaptohexacosan –oate (253)



Excess diazomethane in ether was added to (*R*,*R*,*R*,*S*,*R*,2*R*,2'*R*)-26,26'-disulfanediylbis(2-((*R*)-1-hydroxy-18-((1*R*,2*S*)-2-((17*S*,18*S*)-17-methoxy-18-methylhexatriacontyl)cyclopropyl)octadecyl)hexacosanoic acid **(247)** (8 mg) and stirred for 30 min. The solvent was evaporated to give dimethyl (*R*,*R*,*R*,*S*,*R*,2*R*,2'*R*)- 26,26'-disulfanediylbis(2-((*R*)-1-hydroxy-18-((1*R*,2*S*)-2-((17*S*,18*S*)-17-methoxy-18-methylhexatriacontyl)cyclopropyl)octadecyl)hexacosanoate) **(251)**, which showed an identical spectrum to that compound **(248)**. The crude product was used for next step without purification. Acetic anhydride (0.3 mL) and pyridine (0.3 mL) were added to a stirred solution of **(251)** in toluene (0.3 mL). The reaction mixture was stirred for 18 hours then the solvent was evaporated under reduced pressure to give dimethyl (*R*,*R*,*R*,*S*,*R*,2*R*,2'*R*)-26,26'-disulfanediylbis(2-((*R*)-1-acetoxy-18-((1*R*,2*S*)-2-((17*S*,18*S*)-17-methoxy-18-methylhexatriacontyl)cyclopropyl)octadecyl)hexacosanoate) **(252)**, which showed an identical spectrum to that compound **(249)**. *DL*-Dithiothreitol (100 mg) was added to a stirred solution of **(252)** in chloroform (1 mL) followed by the addition of one drop of triethylamine under nitrogen. The flask was covered with aluminium foil. The reaction mixture was stirred for 48 hours at room temperature. The solvent was evaporated and the crude product was purified by column chromatography eluting with petrol/ethyl acetate (10:1) to give methyl (*R*)-2-((*R*)-1-acetoxy-18-((1*R*,2*S*)-2-((17*S*,18*S*)-17-methoxy-18-methylhexatriacontyl)cyclopropyl) octadecyl)-26-mercaptohexacosanoate **(253)** (3 mg) {MALDI Found $(M + Na)^+$: 1364.83, $C_{88}H_{172}O_5SNa$ requires: 1364.29}, which showed identical spectrum to that compound **(250)**.

Experiment 59: (*R*)-2-((*R*)-1-hydroxy-18-((1*S*,2*R*)-2-((17*R*,18*R*)-17-methoxy-18-methylhexatriacontyl)cyclopropyl)octadecyl)-26-mercaptohexacosanoic acid (210)



DL-Dithiothreitol (150 mg) was added to a stirred solution of (*R*,*R*,*R*,*S*,*R*,2*R*,2'*R*)-26,26'-disulfanediylbis(2-((*R*)-1-hydroxy-18-((1*S*,2*R*)-2-((17*R*,18*R*)-17-methoxy-18-methylhexatriacontyl)cyclopropyl)octadecyl)hexacosanoic acid **(246)** (10 mg) in

chloroform (1.5 mL) followed by the addition of one drop of triethylamine under nitrogen. The flask was covered with aluminium foil and the reaction mixture was stirred for 48 hours at room temperature. The reaction was quenched with 5 drops of dil. HCl (5%) and water (5 mL). The product was extracted with $CHCl_3$ (3 x 10 mL) and the combined organic layers were washed with brine solution, dried and evaporated to give a residue which was purified by column chromatography eluting with chloroform/methanol (10:1) to give (*R*)-2-((*R*)-1-hydroxy-18-((1*S*,2*R*)-2-((17*R*,18*R*)-17-methoxy-18-methylhexatriacontyl)cyclopropyl)octadecyl)-26-mercapto-hexacosanoic acid **(210)** (6.5 mg), which showed, {MALDI Found $(M + Na)^+$: 1308.78, $C_{85}H_{168}O_4SNa$ requires: 1308.26}, which showed δ_H (400MHz, $CDCl_3$): 3.74-3.69 (1H, m), 3.35 (3H, s), 2.99-2.95 (1H, m), 2.52 (2H, q, *J* 7.52 Hz), 2.49-2.44 (1H, m), 1.74-1.11 (150H, m), 0.88 (3H, t, *J* 6.52 Hz), 0.85 (3H, d, *J* 6.92 Hz), 0.68-0.64 (2H, m), 0.56 (1H, dt, *J* 3.76, 7.52 Hz), -0.32 (1H, br.q, *J* 5.05 Hz); δ_C (100MHz, $CDCl_3$): 178.00, 85.55, 72.14, 57.67, 50.65, 35.58, 35.33, 34.05, 32.35, 31.92, 30.47, 30.21, 29.97, 29.93, 29.70, 29.59, 29.51, 29.43, 29.35, 29.07, 28.71, 28.38, 27.56, 27.33, 26.15, 25.73, 24.65, 22.68, 15.77, 14.89, 14.11, 10.90. When the sample was shaken with D_2O, the quartet at 2.52 becomes a triplet with *J* 7.16 Hz.

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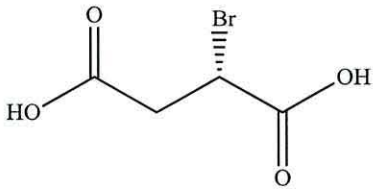
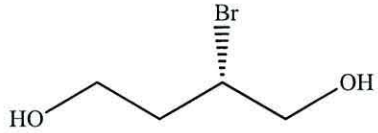
Appendices

Appendix 1: (*S*)-(-)-Bromosuccinic acid [192]



L-Aspartic acid **(127)** (50.03 g, 380 mmol) and potassium bromide (201.06 g, 1690 mmol) in H_2SO_4 (2.5 M, 1L) was cooled to -5 °C and solution of sodium nitrite (46.68 g, 0.680 mol) in water (90 mL) was added slowly without allowing the temperature to exceed 0 °C. The resulting dark brown mixture was stirred for 2 hours at -5 °C after whicht the product was extracted with ethyl acetate (4 × 500 mL). The combine organic extracts were dried and the solvent was evaporated to give a white powder of (*S*)-(-)-bromosccinic acid (65 g, 87%), which showed δ_H (500MHz, $CDCl_3$), δ_C (126MHz, $CDCl_3$), ν_{max} identical to the literature.[192]

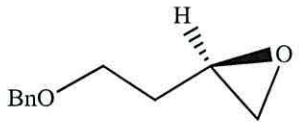
Appendix 2: (*S*)-2-Bromo-1, 4-butanediol [192]



Boranetetrahydrofuran (800 mL, 1M, 0.8 mol) was slowly added to a solution of (*S*)-(-)-bromosuccinic acid (52.46 g, 260 mmol) in dry THF (400 mL) at 0 °C under nitrogen over a period of 1 hour. After the addition was completed the cooling bath was removed and the reaction mixture was stirred for 4 hours. The reaction was quenched by slowly addition of THF/H_2O (100 mL 1:1) at 10 °C was followed by the addition of anhydrous K_2CO_3 (160 g). The mixture was stirred and then filtered through sinter glass funnel under high vacuum and the solid residue was washed with ethyl acetate (3 × 100 mL). The combined washed filtrates were concentrated to a mixture of an oil and borate salt. The oil was re-dissolved in ethyl acetate (2 × 200 mL), filtered to

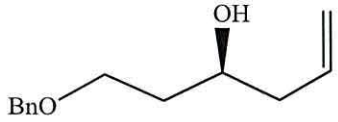
remove any borate salts present and then dried and the solvent was evaporated. The crude product was purified by column chromatography eluting with petrol/ethyl acetate (1:1) to give an oil of (*S*)-2-bromo-1,4-butanediol (40 g, 88%), which showed δ_H (500MHz, $CDCl_3$), δ_C (126MHz, $CDCl_3$), ν_{max} identical to the literature.[192]

Appendix 3 :(*R*)-(2-Benzyloxyethyl)oxiran) (128)[192]



Sodium hydride (13.5 g, 60% dispersion in oil, 337 mmol) was washed with petrol (3 x 40 mL) and suspended in dry THF (200 mL). (*S*)-2-Bromo-1,4-butanediol (18.65 g, 0.11 mol) in dry THF (20 mL) was added over a 5 min period at -10 °C. The mixture was stirred for 25 min before adding benzylbromide (14.12 mL, 20.35 g, 0.119 mol) followed by tetra-butyl ammonium iodide (4.03 g, 11 mmol). The reaction mixture was stirred at -10 °C for a further 5 min before removing the cooling bath and allowing it to warm to room temperature. The mixture was stirred at room temperature for 2 hours before cooling to -10 °C and quenched with sat. aq. ammonium chloride (100 mL). The aqueous layer was extracted with ethyl acetate (3 × 200 mL), dried and the solvent evaporated. The crude product was purified by column chromatography eluting with petrol/ethyl acetate (5:1) to give an oil of (*R*)-(2-benzyloxyethyl)oxiran **(128)** (16 g, 81%), which showed δ_H (500MHz, $CDCl_3$), δ_C (126MHz, $CDCl_3$), ν_{max} identical to the literature.[192]

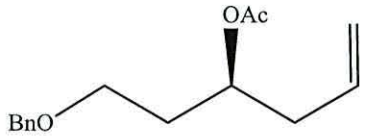
Appendix 4: (*S*)-1-Benzyloxy-hex-5-en-3-ol (129)[172]



Copper iodide (5.76 g, 30.31 mmol) was dissolved in dry THF (300 mL) at room temperature under nitrogen and cooled to -75 °C. Vinyl magnesium bromide (194 mL, 194 mmol, 1M in THF) was added between -75 °C to -50 °C and the mixture was

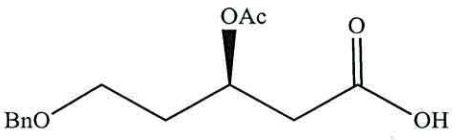
stirred at -50 to -40 °C for 30 min, then re-cooled to -75 °C and a solution of (*R*)-(2-benzyloxyethyl)oxirane **(128)** (20 g,112 mmol) in dry THF (100 mL) was added between -75 °C -40 °C and the reaction was stirred at -40 °C to -30 °C for 1 hour then at -20 °C for 15 min. Sat. aq. ammonium chloride (400 mL) was added and extracted with ethyl acetate (3 × 300 mL) and the combined organic layers were washed with water, dried and the solvent was evaporated. The crude product was purified by column chromatography eluting with petrol/ethyl acetate (5:1) to give a colourless oil (*S*)-1-bezyloxy-hex-5-en-3-ol **(129)** (19 g, 82%), which showed δ_H (500MHz, $CDCl_3$), δ_C (126MHz, $CDCl_3$), ν_{max} identical to the literature.[172]

Appendix 5: Acetic acid (*S*)-1-(2-benzyloxy-ethyl)-but-3-enyl ester (130) [172]



Acetic anhydride (80 mL) and pyridine (80 mL) were added to a stirred solution of (*S*)-1-benzyloxy-hex-5-en-3-ol **(129)** (45 g, 218 mmol) in dry toluene (200 mL) at room temperature and the mixture was stirred at room temperature for 18 hours under nitrogen. After that the solvent was evaporated. The crude product was purified by column chromatography eluting with petrol/ethyl acetate (5:1) to give a colorless oil, acetic acid (*S*)-1-(2-benzyloxy-ethyl)-but-3-enyl ester **(130)** (45 g, 83%), which showed δ_H (500MHz, $CDCl_3$), δ_C (126MHz, $CDCl_3$), ν_{max} identical to the literature.[172]

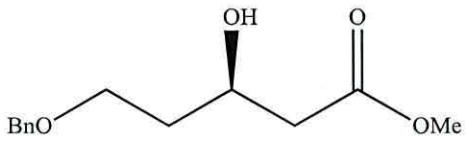
Appendix 6: (*R*)-3-Acetoxy-5-benzyloxy-pentanoic acid (131) [172]



Acetic acid (*S*)-1-(2-benzyloxy-ethyl)-but-3-enyl ester **(130)** (10 g, 40.20 mmol) was dissolved in dry DMF (150 mL) and oxone (98.85 g, 160.70 mmol) then OsO_4 2.5% in 2-methyl-2-propanol (5.04 mL, 0.40 mmol) were added at 10 °C. The mixture's

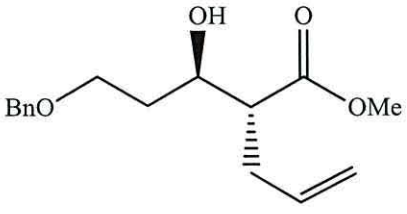
temperature was allowed to reach 34 °C and it was stirred for 5 hours. The mixture was diluted with water (3 L) and extracted with ethyl acetate (1 × 500 mL, 2 × 250 mL). The combined organic layers were washed with water (700 mL), dried and the solvent was evaporated. The crude product was purified by column chromatography eluting with petrol/ethyl acetate (1:2) to give (*R*)-3-acetoxy-5-benzyloxy-pentanoic acid **(131)** (8.5 g, 84%), which showed δ_H (500MHz, $CDCl_3$), δ_C (126MHz, $CDCl_3$), ν_{max} identical to the literature.[172]

Appendix 7 :(*R*)-5-Benzyloxy-3-hydroxy-pentanoic acid methyl ester (132) [172]



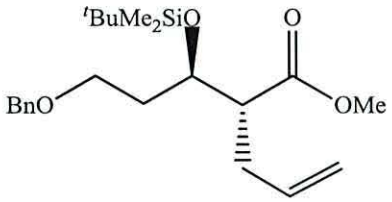
Conc. H_2SO_4 (70 drops) was added to a stirred solution of (*R*)-3-acetoxy-5-benzyloxy-pentanoic acid **(131)** (10 g, 39.9 mmol) in methanol (150 mL) at room temperature. The mixture was refluxed for 3 hours. TLC showed no starting material was left. The methanol was evaporated and ethyl acetate (250 mL) and sat. aq. $NaHCO_3$ (200 mL) were added. The organic layer was separated and the aq. layer was re-extracted with ethyl acetate (2 × 150 mL). The combined organic layers were dried and the solvent was evaporated. The crude product was purified by column chromatograph eluting with petrol/ethyl acetate (5:2) to give a colorless oil, (*R*)-5-benzyloxy-3-hydroxy-pentanoic acid methyl ester **(132)** (8 g, 83%), which showed δ_H (500MHz, $CDCl_3$), δ_C (126MHz, $CDCl_3$), ν_{max} identical to the literature.[172]

Appendix 8 :(*R*)-2-((*R*)-3-Benzyloxy-1-hydroxy-propyl)-pent-4-enoic acid methyl ester (135)[172]



Diisopropylamine (4.864 g, 48 mmol) was dissolved in dry THF (50 mL) and cooled to -60°C. MeLi (72 mL, 108 mmol, 1.5M) was added and stirred to +16 °C for 20 min. then re-cooled to -60 °C and (*R*) -5-benzyloxy-3-hydroxy-pentanoic acid methyl ester **(132)** (5 g, 20.9 mmol) in dry THF (10 mL) was added and the mixture was stirred at -45 °C for 1 h, -20 °C for 40 min. and then at -20 °C to -10 °C for 20 min. It was re-cooled to - 62 °C and allyl iodide (2.87 mL, 31.11 mmol) in dry THF (15 mL) and HMPA (7.27 mL, 41.8 mmol) were added and the mixture was stirred at - 45 °C for 1 hour, - 45 °C to - 20 °C for 30 min and then -20 °C to -10°C for 30 min. Sat.aq.NH_4Cl (70 mL) was added and the product was extracted with ethyl acetate (3 × 100 mL). The combined organic layers were dried and evaporated. The crude product was purified by column chromatograph eluting with petrol/ethyl acetate (2:1) to give a colorless oil, (*R*)-2-((*R*)-3-benzyloxy-1-hydroxy-propyl)-pent-4-enoic acid methyl ester **(135)** (3.5 g, 60%), which showed δ_H (500MHz, $CDCl_3$), δ_C (126MHz, $CDCl_3$), ν_{max} identical to the literature.[172]

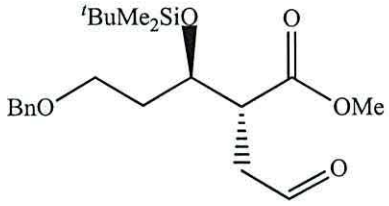
Appendix 9: (*R*)-2-[(*R*)-3-Benzyloxy-1-(*tert*-butyl-dimethyl-silanyloxy)-propyl]-pent-4-enoic acid methyl ester (136)[172]



Imidazole (6.85 g, 0.10075mol) was added to a stirred solution of (R)-2-((R)-3-benzyloxy-1-hydroxy-propyl)-pent-4-enoic acid methyl ester **(135)** (11.25 g, 40.3 mmol) in dry DMF (100 mL) at room temperature and stirred for 30 min. the mixture was then cooled to 0°C and *tert*-butyldimethylchlorosilane (7.8 g, 52.39 mmol) in dry DMF (10 mL) was added. The cooling bath was removed and the reaction mixture was stirred at 45 °C for 20 hours. Then TLC was showed no starting material was left and DMF was removed by flash distillation under high vacuum. Water (500 mL) was added and the product was extracted with dichloromethane (3 × 200 mL). The combined organic layers were washed with water (200 mL), dried and evaporated. The crude product was purified by column chromatography eluting with petrol/ethyl

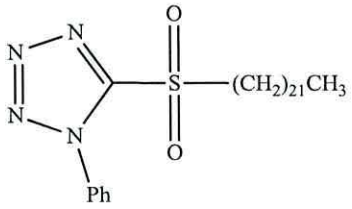
acetate (5:1) to give a colorless oil, (*R*)-2-[(*R*)-3-benzyloxy-1-(*tert*-butyl-dimethyl-silanyloxy)-propyl]-pent-4-enoic acid methyl ester **(136)** (12.23 g, 77%), which showed δ_H (500MHz, $CDCl_3$), δ_C (126MHz, $CDCl_3$), ν_{max} identical to the literature.[172]

Appendix 10: (2*R*,3*R*)-5-Benzyloxy-3-(*tert*-butyl-dimethyl-silanyloxy)-2-(oxo-ethyl) pentanoic acid methyl ester (137)[172]



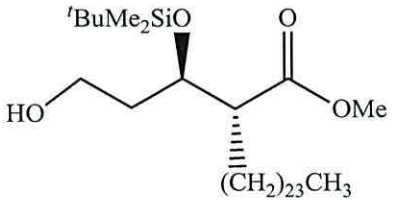
2,6-Lutidine (2.96 mL, 25.42 mmol), OsO_4 2.5 % in 2-methyl-2-propanol (2.87 mL, 0.22 mol), and $NaIO_4$ (10.87 g, 50.84 mmol) were added to a stirred solution of (*R*)-2-[(*R*)-3-benzyloxy-1-(*tert*-butyl-dimethyl-silanyloxy)-propyl]-pent-4-enoic acid meth -yl ester **(136)** (5.0 g, 12.71 mmol) in 1,4-dioxane water (3:1, 200 mL) at room temperature. The reaction was stirred at room temperature 2 hours, when TLC showed complete reaction. Water (300 mL) and dichloromethane (300 mL) were added and the organic layers separated. The water layer was re-extracted with dichloromethane (2 × 100 mL) and the combined organic layers were washed with brine (200 mL) and dried. The solvent was evaporated and the crude product was purified by column chromatography eluting with petrol/ethyl acetate (10:3) to give a colorless oil, (2*R*,3*R*)-5-benzyloxy-3-(*tert*–butyldimethylsilanyloxy)-2-(2-oxo-ethyl)-pentanoic acid methyl ester **(137)** (3.80 g, 76%); which showed δ_H (500MHz, $CDCl_3$), δ_C (126MHz, $CDCl_3$), ν_{max} identical to the literature.[172]

Appendix 11: 5-(docosane-1-sulfonyl)-1-phenyl-1*H*-tetrazole (138)[172]



1-Phenyl-1*H*-tetrazole-5-thiol (13.45 g, 75.48 mmol), 1-bromodocosane (28 g, 71.88 mmol) and anhydrous potassium carbonate (14.90 g, 107.83 mmol) were mixed together in acetone (600 mL). The mixture was stirred vigorously under reflux at 60 °C for 15 hours, when TLC showed no starting material was left. The inorganic salts were filtered off and washed well with acetone. The solvent was evaporated to a small bulk and dissolved in dichloromethane (200 mL) and water (300 mL). The organic layer was separated and the aqueous layer was re-extracted with dichloromethane (2 × 50 mL). The combined organic phases were washed with water (300 mL), dried and the solvent was evaporated. The crude product was recrystallised from acetone (100 mL) and methanol (200 mL) to give a white solid, 5-docosylsulfanyl-1-phenyl-1*H*-tetrazole (31.73 g, 90%). *m*-Chloroperbenzoic acid (36.95 g, 214.16 mmol) in dichloromethane (100 mL) was added to a solution of 5-docosylsulfanyl-1-phenyl-1*H*-tetrazole (31.73 g, 65.18 mmol) in dichloromethane (200 mL) and sodium bicarbonate (24.64 g, 293.31 mmol) at 0 °C. The reaction mixture was stirred at room temperature for 22 hours, the reaction was quenched with solution of sodium hydroxide 5% (200 mL) and stirred vigorously for 2 h. The organic layer was separated and the aqueous layer was re-extracted with dichloromethane (2 × 200 mL). The combined organic layers were dried and evaporated to give a white solid which was recrystallised from acetone/methanol (200 mL 1:1) to give a white solid, 5-(docosan-1-sulfonyl)-1-phenyl-1*H*-tetrazole **(138)** (30 g, 91 %), which showed δ_H (500MHz, $CDCl_3$), δ_C (126MHz, $CDCl_3$), ν_{max} identical to the literature.[172]

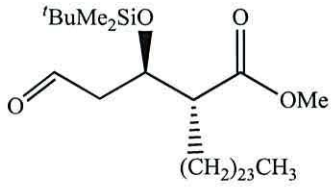
Appendix 12: (*R*)-2-[(*R*)-1-(*tert*-Butyldimethylsilanyloxy)-3-hdroxy-propyl]-hexacosanic acid methyl ester (147)[172]



The procedure used in experiment **1** was repeated in order to couple (2*R*,3*R*)-5-benzyloxy-3-(*tert*-butyldimethylsilanyloxy)-2-(2-oxo-ethyl)-pentanoic acid methyl ester **(137)** (3.80 g, 9.6 mmol) and 5-(docosane-1-sulfonyl)-1-phenyl-1*H*-tetrazole

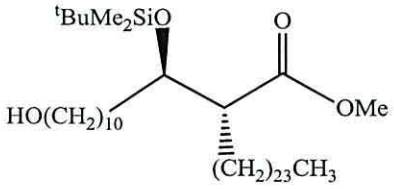
(138) (6.5 g, 12.53 mmol) using lithium bis(trimethylsilyl)amide (17.73 mL, 18.78 mmol,) in dry THF(100 mL). The crude product was purified by column chromatography eluting with petrol/ethylacetate (20:1) to give a colourless oil, (*E/Z*)-(*R*)-2-[(*R*)-3-benzyloxy-1-(*tert*-butyl-dimethylsilanyloxy)-propyl]-hexacos-4-enoic acid methyl ester **(139)** (4.80 g, 73%) as a mixture. Palladium (10% on carbon, 1.0 g) was added to a stirred solution of the above alkenes **(139)** in THF (30 mL) and IMS (30 mL) under hydrogen. Hydrogenation was carried out for 3 days. The solution was filtered over a bed of celite and the solvent was evaporated. The crude product was purified by column chromatography eluting with petrol/ethyl acetate (5:1), to give a white solid, (*R*)-2-[(*R*)-1-(*tert*-butyl-dimethylsilanyloxy)-3-hydroxy-propyl]hexacosan -oic acid methyl ester **(147)** (4 g, 93%), which showed δ_H (500MHz, $CDCl_3$), δ_C (126MHz, $CDCl_3$), ν_{max} identical to the literature.[172]

Appendix 13: (*R*)-2-[(*R*)-1-(*tert*-butyldimethylsilanyloxy)-3-oxo-propyl]hexacosan -oic acid methyl ester (148)[172]



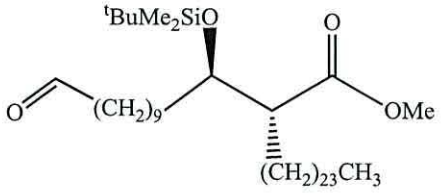
The procedure used in experiment **8** was repeated in order to to oxidise (*R*)-2-[(*R*)-1-(*tert*-butyldimethylsilanyloxy)-3-hydroxypropyl]-hexacosanoic acid methyl ester **(147)** (4.0 g, 6.67 mmol) using pyridinium chlorochromate (3.6 g, 16.69 mmol) in dichloromethane (200 mL). The crude product was purified by column chromatography eluting with petrol/ethyl acetate (10:1) to give colorless oil, (*R*)-2-[(*R*)-1-(*tert*-butyldimethylsilanyloxy)-3-oxo-propyl]hexacosanoic acid methyl ester **(148)** (3.1 gm, 78%), which showed δ_H (500MHz, $CDCl_3$), δ_C (126MHz, $CDCl_3$), ν_{max} identical to the literature[172]

Appendix 14: (*R*)-2-[(*R*)-1-(*tert*-butyldimethylsiloxy)-11-hydroxyundecyl]-hexacosanoic acid methyl ester (152)[179]



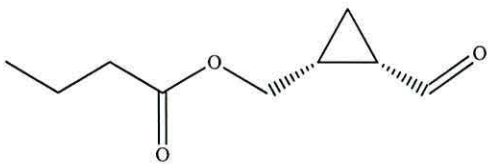
The procedure used in Experiment **1** was repeated in order to couple (*R*)-2-[(*R*)-1-(*tert*-butyl-dimethyl-silanyloxy)-3-oxo-propy]-hexacosanoic acid methyl ester **(148)** (3.10 g, 5.36 mmol) and 1-phenyl-5[(8-(tetrahydropyran-2-yloxy)octyl)sulfonyl]-1*H*-tetrazole **(149)** (2.94 g, 6.97 mmol) using lithium bis(trimethylsilyl)amide (9.4 mL, 10.46 mmol) in dry THF (80 mL). The crude prodect was purified by column chromatography eluting with petrol/ethyl acetate (20:1) to give a colourless oil, (*R*)-methyl-2-((*E*/*Z*)(*R*)-1-((*tert*-butyldimethylsilyl)-oxy)-11-((tetrahydro-2*H*-pyran-2-yl) oxy)undec-3-en-1-yl)hexacosanoate **(150)** (3.38 g, 80%) as a mixture. Pyridanium-*p*-toluene sulfonate (0.52 g, 2.09 mmol) was added to a stirred solution of the above alkenes **(150)** (3.38 g, 4.19 mmol) in THF (50 mL) and MeOH (30 mL) at 60 °C for 3 h, when TLC showed that the reaction was complete. The solvent was evaporated and the residue was dissolved with sat. aq. $NaHCO_3$ (30 mL) and CH_2Cl_2 (100 mL). The organic layer was separated and the aqueous layer was re-extracted (3 × 50 mL) with CH_2Cl_2, dried and evaporated. The crude product was purified by column chromatography with eluting petrol/ethyl acetate (15:1) to give a colourless oil, (*R*)-methyl-2-((*E*/*Z*)(*R*)-1-((*tert*-butyldimethylsilyl)oxy)-11-hydroxyun-dec-3-en-1-yl)hex - acosanoate **(151)** (2.68 g, 89 %) as a mixture. Palladium (10% on carbon, 0.5 g)) was added to a stirred solution of the above alkenes in THF (20 mL) and IMS (20 mL) under hydrogen. Hydrogention was carried for one hour, then the mixture was filtered over a bed of celite and the solvent was evaporated. The crude product was purified by column chromatography eluting with petrol/ethylacetate (5:1) to give a colourless oil, (*R*)-2-[(*R*)-1-(*tert*-butyldimethylsiloxy)-11-hydroxyundecyl] hexacosanoic acid methyl ester **(152)** (2.4 g, 89%), which showed δ_H (500MHz, $CDCl_3$), δ_C (126MHz, $CDCl_3$), ν_{max} identical to the literature.[179]

Appendix 15: (*R*)-2-[(*R*)-1-(*tert*-Butyldimethylsiloxy)-11-oxoundecyl]-hexacosanoic acid methyl ester (123)[179]



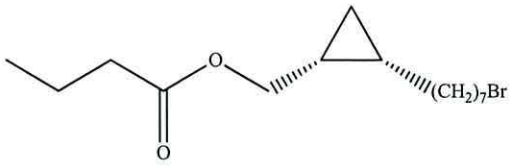
The procedure used in Experiment **8** was repeated in order to to oxidise (*R*)-2-[(*R*)-1-(*tert*-butyldimethylsiloxy)-11-hydroxyundecyl]hexacosanoic acid methyl ester **(152)** (1.7 g, 2.39 mmol) using pyridinium chlorochromate (1.54 g, 7.18 mmol) in dichloromethane (50 mL). The crude product was evaporated and purified by chromatography eluting with petrol/ethyl acetate (10:1) to give a colourless oil, (*R*)-2-[(*R*)-1-(*tert*-butyldimethylsiloxy)-11-oxoundecyl]hexacosanoic acid methyl ester **(123)** (1.45, 86%), which showed δ_H (500MHz, $CDCl_3$), δ_C (126MHz, $CDCl_3$), ν_{max} identical to the literature.[179]

Appendix 16: (1*S*,2*R*)-1-Butyryloxymethyl-2-formyl-cyclopropane (154)[162]



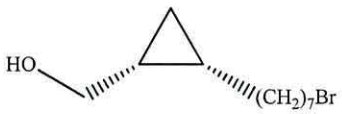
The procedure used in Experiment **8** was repeated in order to to oxidise (1*S*,2*R*)-2-hydroxymethyl-cyclopropyl-methylbutyrate **(153)** (7g, 40.69mmol) using pyridinium chlorochromate (19.30 g, 89.53 mmol) in dichloromethane (150 mL). The crude product was evaporated and purified by chromatography eluting with petrol/ethyl acetate (5:2) to give colourless oil (1*S*,2*R*)-1-butyryloxymethyl-2-formyl-cyclopropane **(154)** (5.2 g, 75%), which showed δ_H (500MHz, $CDCl_3$), δ_C (126MHz, $CDCl_3$), ν_{max} identical to the literature.[162]

Appendix 17: ((1*R*,2*S*)-2-(7-Bromoheptyl)cyclopropyl butyrate.(157) [165]



The procedure used in Experiment **1** was repeated in order to couple (1*S*,2*R*)-1-butyryloxymethyl-2-formyl-cyclopropane **(154)** (5 g, 29.41 mmol) and 5-((6-bromohexyl)sulfonyl)-1-phenyl-1*H*-tetrazole **(155)** (10.97 g, 35.29 mmol) using lithium bis (trimethylsilyl) amide (41.62 mL, 44.11 mmol, 1.06 M) in dry THF(100 mL). The crude prodect was purified by column chromatography eluting with petrol/ethyl acetate (20:1) to give a thick oil of butyric acid (1*S*,2*R*)-2-(*E*/*Z*)-bromohept-1-enyl)cyclopropyl methyl ester **(156)** (5.5 g, 60%) as a mixture. 2,4,6-Triisopropylbenzene sullfonylhydrazide (13.97 g, 46.88 mmol) was added to a stirred solution of butyric acid (1*S*,2*R*)-2-(*E*/*Z*)-bromohept-1-enyl)cyclopropyl methyl ester (5.5 g, 17.35 mmol) in tetrahydrofuran (150 mL) at room temperature. The mixture was stirred at 50 °C for 3 hours, followed by the addition of another mole equivalent of the hydrazide and stirring under the same conditions for 24 hours. It was diluted with petrol/ether (1:1,100 mL) and washed with aq sodium hydroxide (2%, 200 mL). The aqueous layer was re-extracted with petrol/ether (1:1, 2x100 mL), and the combined organic layers were washed with water, dried and evaporated. The crude product was purified by column chromotography eluting with petrol/ethyl acetate (10:1) to give ((1R,2S)-2-(7-bromoheptyl)cyclopropyl butyrate **(157)** as yellow oil (4 g, 72%), which showed δ_H (500MHz, $CDCl_3$), δ_C (125MHz, $CDCl_3$), ν_{max} identical to the literature.[165]

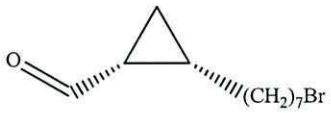
Appendix 18: [(1*R*,2*S*)-2-(7-Bromoheptyl)cyclopropyl] methanol (158) [165]



Anhydrous potassium carbonate (4.84 g, 35.10 mmol) was added to a stirred solution of butyric acid (1*S*, *R*)-2-(7-bromoheptyl) cyclopropyl methyl ester **(157)** (4 g, 12.53

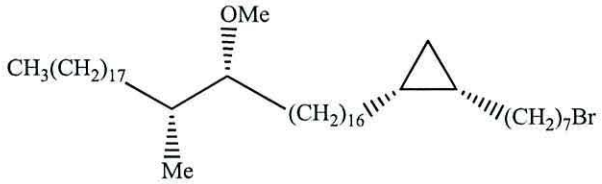
mmol) in methanol (30 mL) and THF (20 mL) at room temperature. After 2 hours at 45 °C, it was diluted with water (200 mL) and ether (100 mL). The aqueous layer was re-extracted with ether (2×50 mL). The combined organic layers were washed with brine, dried and evaporated. The product was purified by column chromatography eluting with petrol/ethyl acetate (5:1) to give [(1*S*,*R*)-2-(7-bromoheptyl) cyclopropyl]methanol **(158)** as a colourless oil (2.8 g, 90%), which showed δ_H (500MHz, $CDCl_3$), δ_C (126MHz, $CDCl_3$), ν_{max} identical to the literature.[165]

Appendix 19: (1*R*,2*S*)-2-(7-Bromoheptyl)cyclopropane carbaldehyde (125) [165]



The procedure used in Experiment **8** was repeated in order to to oxidise [(1*S*,*R*)-2-(7-bromoheptyl)cyclopropyl]methanol **(158)** (2.8 g, 11.24 mmol) using pyridinium chlorochromate (6.06 g, 28.11 mmol) in dichloromethane (75 mL). The product was purified by chromatography with petrol/ethyl acetate (10:1) to give colourless oil (1*R*,2*S*)-2-(7-bromoheptyl)cyclopropane carbaldehyde **(125)**, which showed δ_H (500MHz, $CDCl_3$), δ_C (126MHz, $CDCl_3$), ν_{max} identical to the literature.[165]

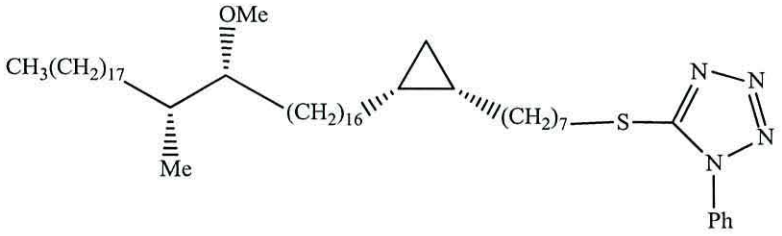
Appendix 20: (1*S*,2*R*)-1-(7-Bromoheptyl)-2-(17*R*,18*R*)-17-methoxy-18-methyl hexatriacontenyl)cyclopropan (160)[165]



The procedure used in Experiment **1** was repeated in order to couple (1*R*,2*S*)-2-(7-bromoheptyl)cyclopropane carbaldehyde **(125)** (2 g, 8.09 mmol) and 5-((16*R*,17*R*)-16-methoxy-17-methylpentriacontane-1-sulfonyl)-1-phenyl-1*H*-tetrazole **(126)** (6.93 g, 9.31 mmol) using lithium bis (trimethylsilyl) amide (13.17 mL, 13.96 mmol, 1.06 M) in dry THF (80 mL). The crude prodect was purified by column chromatography

eluting with petrol/ethyl acetate (20:1) to give a colourless oil of (1*R*,2*S*)-1-(7-bromoheptyl)-2-(*Z/E*)-(17*R*,18*R*)-17-methoxy-18-methylhexatriacont-1-enyl)cyclopro -pane **(159)** (5.25 g, 84%) as a mixture. Hydrogenation was carried out by using 2,4,6-triisopropylbenzene sulfonylhydrazide (5.14 g, 17.14 mmol) which was added to a stirred solution of the above alkenes (5.25 g, 6.85 mmol) in THF (150 mL) at room temperature. The mixture was stirred at 50 °C for 3 hours, followed by the addition of another mole equivalent of the 2,4,6-triisopropylbenzene sulfonylhydrazide and stirring under the same conditions for 24 hours. It was diluted with petrol/ether (1:1,100 mL) and washed with aq. sodium hydroxide (2%, 200 mL). The organic layer was separated and the aqueous layer was re-extracted with petrol/ ethyl acetate (1:1, 2 × 100 mL). The combined organic layers were washed with water, dried and evaporated. The crude prodect was purified by column chromatography eluting with petrol/ethyl acetate (20:1) to give a viscous colourless oil, (1*S*,2*R*)-1-(7-bromoheptyl)-2-(17*R*,18*R*)-17-methoxy-18-methylhexatriacontenyl) cyclopropan **(160)** (4.2 g, 80%), which showed δ_H (500MHz, $CDCl_3$), δ_C (126MHz, $CDCl_3$), ν_{max} identical to the literature.[165]

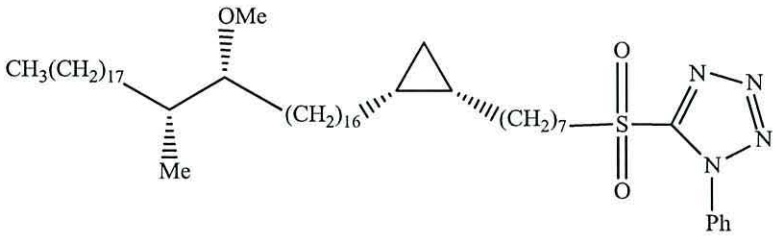
Appendix 21: 5-{7-[(1*S*,2*R*)-2-((17*R*,18*R*)-17-Methoxy-18-methyl-hexatriacont-enyl) cyclo-propyl]heptylsulfanyl}-1-phenyl-1*H*-tetrazole (161)[165]



The procedure used in Experiment **29** was repeated using, (1*S*,2*R*)-1-(7-bromoheptyl)-2-(17*R*,18*R*)-17-methoxy-18-methylhexatriacontenyl)cycl opropane **(160)** (4.2 g, 5.46 mmol), 1-Phenly-1*H*-tetrazol-5-thiol (1.2 g, 6.73 mmol) and potassium carbonate (2.96 g, 23.27 mmol) in acetone (100 mL). The crude product was purified by column chromatography eluting with petrol/ethyl acetate (20:1) to give as a simi-solid, 5-{7-[(1*S*,2*R*)-2-((17*R*,18*R*)-17-methoxy-18-methylhexatriacontenyl)cyclopropyl]heptylsul

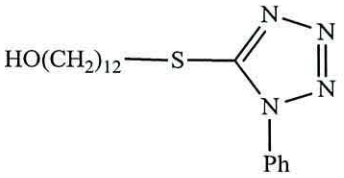
-fanyl}-1-phenyl-1*H*-tetrazole **(161)** (4.0 g, 84%), which showed δ_H (500MHz, $CDCl_3$), δ_C (126MHz, $CDCl_3$), ν_{max} identical to the literature.[165]

Appendix 22: 5-{7-[(1*S*,2*R*)-2-((17*R*,18*R*)-17-Methoxy-18-methylhexatriacontenyl) cyclopropyl]heptylsulfonyl}-1-phenyl-1*H*-tetrazole (124) [165]



The procedure used in Experiment **7** was repeated using, 5-{7-[(1*S*,2*R*)-2-((17*R*,18*R*)-17-methoxy-18-methylhexatriacontenyl)cyclopropyl]heptylsulfanyl}-1-phenyl-1*H*-tetrazole **(161)** (4.0 g, 4.62 mmol), *m*-chloroperbenzoic acid (2.98 g, 17.3 mmol) and sodium bicarbonate (1.83 g, 21.84 mmol) in dichloromethane (50 mL). The crude product was purified by by column chromatography eluting with petrol/ethyl acetate (5:1) to give 5-{7-[(1*S*,2*R*)-2-((17*R*,18*R*)-17-methoxy-18-methyl-hexatriacontenyl)cyclopropyl] heptylsulfonyl}-1-phenyl-1*H*-tetrazole **(124)** as a white solid (3.4 g, 82%), which showed δ_H (500MHz, $CDCl_3$), δ_C (126MHz, $CDCl_3$), ν_{max} identical to the literature.[165]

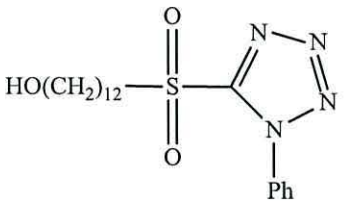
Appendix 23: 12-(1-Phenyl-1*H*-tetrazol-5-ylsulfanyl)-dodecan-1-ol (184)[180]



The procedure used in Experiment **29** was repeated using, 12-bromododecan-1-ol **(183)** (30 g, 113 mmol), 1-phenyl-1*H*-tetrazole-5-thiol (22.17 g, 118 mmol), and anhydrous potassium carbonate (34.22 g, 248 mmol) in acetone (250 mL).The crude product was re-crystallized from acetone (50 mL) and diluted with methanol (100 mL) to give a white solid of 12-(1-phenyl-1*H*-tetrazol-5-ylsulfanyl)-dodecan-1-ol **(184)** (35

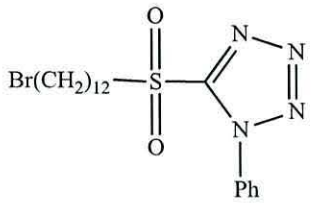
g, 85 %), which showed δ_H (500MHz, $CDCl_3$), δ_C (126MHz, $CDCl_3$), ν_{max} identical to the literature.[180]

Appendix 24: 12-(1-Phenyl-1*H*-tetrazol-5-sulfonyl)-dodecan-1-ol (185) [180]



The procedure used in Experiment **30** was repeated using, 12-(1-phenyl-1*H*-tetrazol-5-ylsulfanyl)-dodecan-1-ol **(184)** (35 g, 95.98 mmol), ammonium molybdate (VI) tetrahydrate (59.71 g, 48.30 mmol) in ice cold H_2O_2 (35% w/w, 100 mL), THF (200) and IMS (300 mL), and further solution of ammonium molybdate (VI) tetrahydrate (22.09 g, 17.8 mmol) in ice cold H_2O_2 (35% w/w, 50 mL) to give a crude product. This was purified by crystallization from acetone/methanol (1:2) to give a white solid of 12-(1-phenyl-1*H*-tetrazol-5-sulfonyl)-dodecan-1-ol **(185)** (32 g, 84 %), which showed δ_H (500MHz, $CDCl_3$), δ_C (126MHz, $CDCl_3$), ν_{max} identical to the literature.[180]

Appendix 25: 5-(12-Bromododecane-1-sulfonyl)-1-phenyl-1*H*-tetrazole (186)[180]



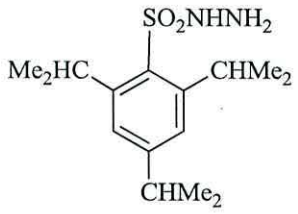
The procedure used in Experiment **39** was repeated using, 12-(1-phenyl-1*H*-tetrazol-5-sulfonyl)-dodecan-1-ol **(185)** (32 g, 81.21 mmol), N-bromosuccinimide (18.8 g, 105.62 mmol) and triphenylphosphine (27.68 g, 105 mmol) in dichloromethane (350 mL). The crude product was purified by column chromatography eluting with petrol/ethyl acetate (5:1) to give 5-(12-Bromododecane-1-sulfonyl)-1-phenyl-1*H*-tetrazole **(186)** (29.5 g, 79%), which showed δ_H (500MHz, $CDCl_3$), δ_C (126MHz, $CDCl_3$), ν_{max} identical to the literature.[180]

Experiment 26: 10-Bromodecan-1-ol (219)[172]

$Br(CH_2)_{10}OH$

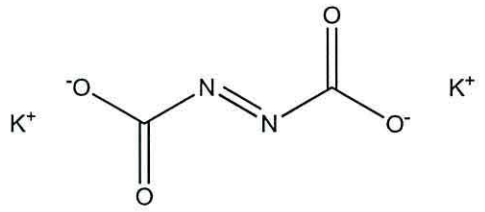
Hydrobromic acid (50 mL, 0.45 mol, 48%) was added to a stirred solution of 1, 10-decanediol **(218)** (50 g, 280 mmol) in toluene (400 mL). The mixture was refluxed for 20 hours. The mixture was then cooled to room temperature, the organic layer was separated and the solvent evaporated. The brown oil residue was dissolved in dichloromethane (600 mL) and washed with sat. aq. sodium bicarbonate (300 mL). The aqueous layer was re-extracted with dichloromethane (3 x 150 mL). The combined organic layers were dried and evaporated. The crude product was purified by column chromatography eluting with petrol/ethyl acetate (5:2) to give colourless oil, 10-bromodecan-1-ol **(219)** (48 g, 70%), which showed δ_H (500MHz, $CDCl_3$), δ_C (126MHz, $CDCl_3$), ν_{max} identical to the literature.[172]

Appendix 27: 2,4,6-Triisopropyl-benzenesulfonyl hydrazide[172]



2,4,6-Triisopropyl-benzenesulphonyl chloride (24.3 g, 80.2 mmol) was dissolved in THF (60 mL) and cooled to -10 °C. Hydrazine hydrate (8.2 g, 163 mmol) was slowly added by maintaining the solution in the temperature range -4 °C to -10 °C with constant stirring. The mixture was kept in the temperature range for a further 2 hours. Water (3 mL) was added to dissolve the solid. The mixture separated into two phases; the aqueous phase was separated and the organic phase washed with brine (2 x 20 mL). The organic phase was dried at 0 °C for 30 min., filtered and washed with diethyl ether. The solvent was evaporated at 10 °C to a white solid, 2,4,6-triisopropyl-benzenesulphonyl hydrazide (21.5 g, 90 %), which was stored at -18 °C.

Appendix 28: Di-potassium azodicarboxylate [172]



Azodicarbonamide (7.5 g, 64 mmol) was slowly added in small portions to a vigorously stirred solution of KOH (15 g, 260 mmol) in de-ionised water (15 mL) at 0 ^{0}C on a salted ice-water bath, maintaining the temperature below 5 °C. The bright yellow solution was stirred at 0–5 °C for a further 45 min., during which time a thick bright yellow precipitate of di-potassium salt formed. The precipitate was filtered into a sintered funnel and washed with ice-cold methanol (60 mL). The yellow precipitate was dissolved in water (40 mL) on the sintered glass funnel at 18 °C. The yellow solution was sucked through the sinter by vacuum into pre-cooled (- 20 °C) methylated spirit (60 mL) giving a yellow precipitate. The yellow precipitate was again filtered through a sinter funnel and washed with cold (- 20 °C) methanol (50 mL), followed by cold (- 20 °C) petrol (50 mL). The solid was dried by vacuum and powdered with a spatula before being transferred under nitrogen into a pre-cooled round bottomed flask. The flask was sealed and stored at -18 °C.