

Stereocontrolled synthesis of a thiolated mycolic acid for development of novel TB diagnostics

by

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Abbreviations

°C	degree Celsius
µm	micrometre
Å	angstrom
A	ampere
A	area of electrode surface
AC	alternating current
AIBN	azobisisobutyronitrile
AIDS	acquired immune deficiency syndrome
aq	aqueous
atm	atmosphere
Au	gold
Au—S	gold-thiolate
BCG	bacillus Calmette-Guérin
BE	binding energy
Bn	benzyl
br q	broad quartet
C	concentration
c/s	counts per second
Cp ₂ ZrCl ₂	zirconium bis(cyclopentadienyl)dichloride
D	diffusion coefficient
d	sampling depth
DC	direct current
ddd H ₂ O	double distilled deionized water
DMF	dimethyl formamide

E	electric potential
EIS	electrochemical impedance spectroscopy
ELISA	enzyme-linked immunosorbent assay
epoxy-MA	epoxy mycolic acid
EtOAc	ethyl acetate
eV	electron volts
F	Faraday's constant
FFM	friction force microscope
GC-MS	gas chromatography-mass spectrometre
h	hour
h	Planck's constant
Hex	hexane
HIV	human immunodeficiency virus
HMBC	heteronuclear multiple bond correlation
HMPA	hexamethylphosphoramide
HPLC	high performance liquid chromatography
I	current
IMS	industrial methylated spirit
IR	infra red
I_s	intensity at the surface
J	flux of ions
KE	kinetic energy
keto-MA	keto mycolic acid
KHz	kiloHertz
LDA	lithium diisopropylamide

m	multiplet
<i>M.tb</i>	<i>Mycobacterium tuberculosis</i>
MA(s)	mycolic acid(s)
MALDI-TOF	matrix-assisted laser desorption ionization-time-of-flight
MA-OH	hydroxyl mycolic acid
MARTI	mycolic acid antibodies real-time inhibition test
mCPBA	meta-chloroperoxybenzoic acid
MDR	multi-drug resistant (TB)
MEODA	N-(2-mercaptoethyl) octadecanamide
MeO-MA	methoxy mycolic acid
Mes	mesityl
MFM	magnetic force microscope
mHz	milliHertz
min	minute(s)
mM	millimolar
mol. eq.	molar equivalent
mV	millivolts
n	number of electrons
N(E)	number of electrons per second
n-BuLi	n-butyllithium
nm	nanometer
NMR	nuclear magnetic resonance
ODT	octadecanethiol
PCC	pyridinium chlorochromate
PGLs	phenolic glycolipids

ppm	parts per million
PPTS	pyridinium- <i>p</i> -toluene sulphonate
Pv	pivaloyl
r.t.	room temperature
R_{ct}	charge transfer resistance
R_f	retention factor
s	second(s)
s	singlet
SAM	self assembled monolayer
SAP	saponin
S_N2	substitution nucleophilic bimolecular
SPM	scanning probe microscopy
SPR	surface plasmon resonance
STM	scanning tunneling microscopy
t	triplet
TB	tuberculosis
TBAH	tetrabutylammonium hydroxide
TBAI	tributylammonium iodide
TBS	tertiary butyldimethylsilyl
TDM	trehalose-6,6'-dimycolate
TEM	transmission electron microscope
THF	tetrahydrofuran
THP	tetrahydropyran
TLC	thin layer chromatography
Tol	tolyl

UV	ultraviolet
ν	frequency of light
WHO	World Health Organization
x	distance from electrode surface
X_{CA}	chiral auxiliary
XDR	extensively drug resistant (TB)
XPS	x-ray photoelectron spectroscopy
Z	impedance
α -MA	alpha mycolic acid
λ	inelastic mean free path
ω	frequency
Φ	spectrometre work function

Summary

Title:

Stereocontrolled synthesis of a thiolated mycolic acid for development of novel TB diagnostics

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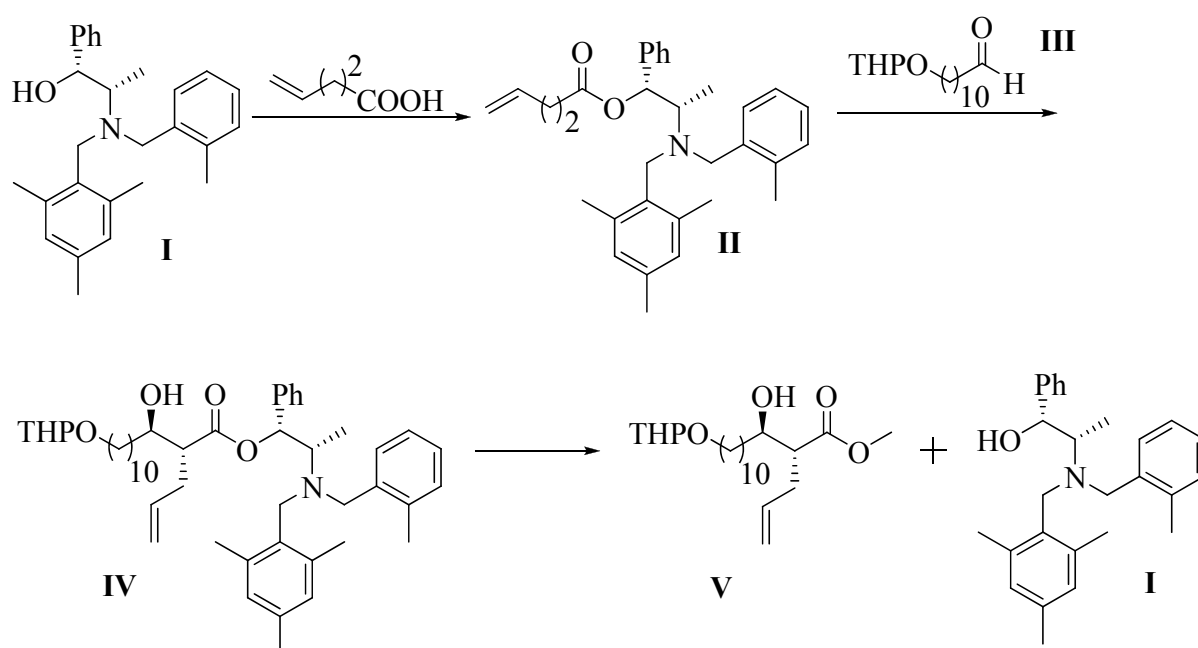
Degree:

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The current global tuberculosis epidemic has shown the need to urgently replace the aged anti-TB armamentarium. The search for a fast, accurate and reliable diagnosis of active TB has emerged as the spearhead in efforts to defeat the scourge. Traditional diagnostics that have existed for more than a century are at best inadequate against the wilier modern forms of the disease like TB-HIV/AIDS co-infection and the drug resistant TB. The development of mycolic acid based serodiagnostics has introduced a powerful tool that gives high hopes of solving the diagnostic challenges of a large percentage of TB cases. To date, these biosensor-based technologies require the immobilization of MA antigens on a gold substrate for antibody recognition. Several challenges need to be resolved before these new technologies can be rolled out as affordable and reliable diagnostic tools. This project sets out primarily to economize the synthesis of MA and develop a new and stable antigenic surface that will improve reliability of tests.

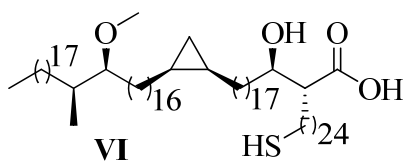
Chemical knowledge of MA has been growing over the past century but total organic synthesis of the various forms of these complex molecules have only been achieved in the past decade. This

project focused on improving a recently developed synthetic route to the mycolic motif. It successfully scaled up the synthesis by systematically troubleshooting various aspects of the synthetic protocol. This resulted in the reduction of the number of reactions by at least 6 steps compared to the current literature method [**Scheme I**]. Importantly, this method has the potential of being extended to the synthesis of much longer fragments of the mycolic acid molecule.



Scheme I

A full-length methoxy mycolic acid **VI** was functionalized with a thiol for derivatization of a solid gold substrate. The successful immobilization of the mycolic acid was confirmed by XPS, AFM, CV, and EIS, which also confirmed the stability of the new surface. This is expected to have a significant impact on the new sensor-based TB diagnostics.



1 Chapter One: Introduction

This work set out to develop lipid antigens that can provide better and more stable surfaces for sensors to be used in diagnosing tuberculosis. The introduction therefore describes the background to the disease and the methods currently used to diagnose it, the types of molecules that can be used as antigens in serodiagnosis, the nature of current and proposed diagnostics, and finally current synthetic approaches to the class of antigen chosen for development.

1.1 Tuberculosis

Tuberculosis is a facultative infection primarily associated with the lungs. It does however also affect nearly every organ system in the human body. Infection could be acute or chronic and asymptomatic. Gestation time to TB disease is often extended and most cases never really become infectious even for the entire lifespan of the carrier.¹ Co-infection with other pathogens or collapse of host immunity might trigger progression to TB. This has fuelled the recent resurgence of TB as a co-epidemic with HIV/AIDS. HIV has since the early 1990's been the single most important factor contributing to TB proliferation and TB has in turn been the major killer of AIDS patients in sub-Saharan Africa and East Asia.² To further complicate the scenario of the almost vengeful return of TB there is also the emergence of two, more dangerous forms of the disease — multidrug resistant (MDR)³ and extensively drug resistant (XDR) TB.⁴

Unlike its AIDS partner, TB is not a new disease. In fact it is one of the oldest infections of man and its neolithic existence has been confirmed.⁵ Morphological and molecular analyses present strong evidence of tuberculosis in humans from 9000 year old settlements. With such a timeline it is no surprise that TB is believed to have exerted the highest mortality of any disease on mankind.⁶

No continent has been spared the sporadic episodes of TB epidemics. Often referred to as the white plague, it is even speculated that medieval Europe infection rate was as high as 100% of the population and 25% of deaths attributed to TB.⁷ Today the World Health Organization (WHO)

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estimates that 11.1 million people have active TB disease while about 2 billion, approximately a third of the world's population, carry latent TB.²

Having been around for several millennia and survived changes in civilizations, TB has been known by several names, which portray either how the disease was perceived or euphemistically referred to, due to its horror. Probably the designations that most potently present the historical impact of the disease and its status among other diseases are 'the Captain of all these men of Death' by the English writer John Bunyan and in ancient Hindu scripts '*Rogaraj*' which translates to 'King of diseases'.⁸

The legendary and mythical dark cloud about TB began to clear in the mid 19th century when Jean-Antoine Villemin demonstrated that TB was a transmittable disease.⁹ The first strong ray of light through the cloud came with the convincing demonstration by Robert Koch in 1882 that a bacillus, *Mycobacterium tuberculosis*, was the etiological agent of TB. Other brilliant scientific breakthroughs like the development of BCG vaccine for TB prevention in 1907 and the discovery of isoniazid and other TB-specific antibiotics for chemotherapy between 1921 and 1957 terminated the spectre of the disease as an incurable affliction [**Figure 1.1**]. This confidence gradually slipped into complacency over the second half of the 20th century. Then, ironically following the jubilation over the achievement of the eradication of smallpox, TB re-emerged in a pact with AIDS, the new viral disease threat to mankind since the early 1980's.¹⁰ TB was back in the headlines, this time as the increasingly drug resistant, incurable disease that could generally not be avoided by a choice of safe sexual behaviour as was the case with AIDS. A frenzied, almost frantic search for new diagnostics and therapeutics ensued to replace the aged, inadequate diagnostics and depleted anti-TB therapeutic arsenal.

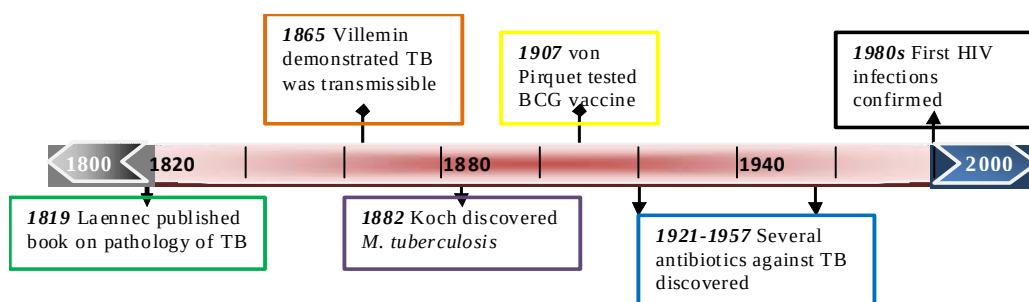


Figure 1.1: 200 years timeline of major advances in combating TB.

1.2 The Causative Organism

Mycobacterium tuberculosis (*M.tb*) belongs to a genus of the *Actinobacteria* consisting of several bacilli, some of which cause disease in humans, livestock and wild animals. The human infectious organisms are clinically classified as the *Mycobacterium tuberculosis* complex,¹¹ *Mycobacterium leprae*, and the non-tuberculous mycobacteria. A crucial point-of-note is that all the mycobacteria that primarily infect humans do not have an intermediary host, a transmission vector, or incubation reservoir from which they are transmitted to humans. Between them they infect all major organ systems of the body. These facts, coupled with the ability of a large percentage of the human population to asymptotically harbour latent *M.tb*, strongly indicate a possible co-evolutionary development.¹²

M. leprae causes Hansen's disease.¹³ This disease, better known as leprosy, has a documented history of social stigmatization dating back to the oldest civilizations of China, Egypt, and India. In Biblical times, sufferers were banished from the city walls and even had to ring a bell to warn of their approach. Its most horrid physical presentation was mutilation of limbs and facial disfiguration. Today, the WHO reports 'the diagnosis and treatment of leprosy is easy'.¹⁴ This has greatly relegated the disease to the annals of medical history although infections still occur. There are only about six countries yet to reach the WHO elimination target of 1 case per 10 000.

M. leprae was discovered by the Norwegian physician Gerhard Armauer Hansen in 1873, just under a decade before Robert Koch identified *M.tb* as the cause of tuberculosis, making this discovery the first *Mycobacterium* to be confirmed as causing a human disease. It is also the slowest replicating

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bacterium known, doubling only once in 13 days. It has never been successfully cultured *in vitro*. *In vivo* cultures in the footpads of mice and armadillos take several weeks to mature.¹⁵

Although non-tuberculous mycobacteria do not cause TB or leprosy they do cause some very discomfiting and serious infections of humans and livestock. This class of mycobacteria consists of a growing list of over a hundred species.¹⁶ Many, like *Mycobacterium ulcerans* and *Mycobacterium haemophilum*, cause disease in humans, yet they are not communicable. *Mycobacterium avium* complex is a group of non-tuberculous mycobacteria which are responsible for major opportunistic infections in HIV-immunocompromised patients.¹⁷

Mycobacterium tuberculosis complex is a group of five bacilli: *M. tuberculosis*, *M. bovis*, *M. africanum*, *M. canetti* and *M. microti*.¹¹ All cause tuberculosis in humans but, except for *M.tb*, they either have limited regional spread—as with *M. africanum*—or have rare occurrences. *M. bovis* still poses a threat to cattle, but human transmission is not a major public health challenge today, mainly due to routine pasteurization of milk.¹⁸⁻²¹ *M.tb*, therefore, represents the most dangerous mycobacterial infectious agent of disease.



Figure 1.2: *M. tuberculosis* **A.** bacilli **B.** colonies growing on solid medium.²²

M.tb is a bacillus of 2-4 μm in length and 0.2-0.5 μm in width. The binomial nomenclature was given by Lehman and Neumann in 1896.²³ During the rapid growth phase of its life cycle *M.tb* requires a constant, abundant supply of oxygen. It replicates significantly faster than *M. leprae*, yet

its 15-20 h doubling time is extremely slow compared to *Escherichia coli*'s 20 minutes division time.²⁴

Alveolar macrophages are the prime host target cells of *M.tb*.²⁵ In these macrophages *M.tb* is well adapted for long-term survival. Macrophages are the primary effector cells of the innate immune system.²⁶ They secrete cytokines and chemokines that rally other immune response cells to a site of pathogen infection. As phagocytic cells they engulf foreign detritus in tissues. They induce cellular immunity by presenting immunogenic peptides to T-cells on membrane-bound major histocompatibility complexes after intracellular digestion of the foreign body. *M.tb* resists hydrolytic digestion in these alveolar cells by inhibiting fusion of the phagosome with lysosomal vesicles.^{27,28} This arrest of phagosome maturation further enables the intracellular tubercle bacilli to evade antigen presentation on the surfaces of the host macrophages,²⁹ while keeping the nutrient supply channels open.³⁰ Mannose-capped lipoarabinomannan produced by *M.tb* inhibits the tethering cascade required for the maturation of the phagosome to a phagolysosome.³¹ This activity is a critically important factor in the latency of TB.

1.3 TB Diagnosis

Proper diagnosis of TB has become quite complicated, more so by the increased incidences of disseminated forms of the disease. This is primarily due to co-infection with HIV.³² The majority of current tests are sputum based and hence depend on the lungs being the main site of infection.³³ Even in cases where the lungs are the site of active infection the microbes may be harboured in cavities that prevent their presence in sputum samples. Serological tests using several mycobacterial cell wall-derived antigens are also available, but have shown limited success [Table 1.1].^{34,35} The tuberculin skin test, the gold standard for testing in countries like the United States and Canada where routine BCG vaccinations are not administered, is almost ineffective in the high burden countries. The skin test cannot distinguish between latent and active TB, which is another reason why it gives many false positive results in these high burden countries. ELISA tests using *M.tb* antigens have been commonly used and assessed in these countries but have given limited added value to the smear and culture tests. While much progress has been made in developing novel diagnostics for *M.tb*, most still rely on sputum and do not provide scope for the amelioration of the

challenge of diagnosing extrapulmonary TB. Extrapulmonary TB is a common manifestation of HIV co-infection and of children. Considering the high rate of TB cases in HIV-infected persons, this represents a crisis for TB management in HIV-burdened populations.¹

A recently developed diagnostic, Mycolic acid Antibody Real-Time Inhibition test (MARTI), provides a strong opportunity to adequately solve some of the major problematic areas of TB diagnosis in the era of HIV co-infection.^{36,37} This is described in more detail in **Section 1.5.1**. Because it is serum-based and detects antibodies to the mycolic acid antigens found in the *M.tb* cell wall it has the potential to detect extra-pulmonary TB more efficiently and accurately than current clinical standards. The MARTI protocol, unlike ELISA, avoids a washing process after antibody binding and is thus not restricted to detecting only high affinity antibodies. It is less invasive than procedures often applied to obtain sputum samples of desired quality for culture or smear microscopy. The aim of this work was to further develop the principles of this assay.

Table 1.1: Serological antigens for TB diagnosis.

Antigens used in serological diagnosis of TB
Mycobacterial sonicates ³⁸
Purified protein derivative ³⁹
Antigen 5 (38 kDa antigen) ⁴⁰
A60 antigen ⁴¹
45/47-kDa antigen complex ⁴²
Antigen Kp90 ⁴³
30 kDa antigen ⁴⁴
P32 antigen ⁴⁵
Cord Factor (trehalose dimycolate)/ Mycolic acids ^{37,46}
Lipoarabinomannan ⁴⁷

1.4 Mycobacterial Cell Envelope

The mycobacterial cell envelope is made up of 3 structural layers: the plasma membrane, the cell wall and the cell capsule [Figure 1.3]. While the plasma membrane chemical composition is typical of other prokaryotes, the other two layers include molecules unique to mycobacteria. These are discussed in detail below. Their 3D arrangement passively protects the cell from antibiotics and host hydrolytic enzymes. These lipids allow the bacteria to actively modulate their intracellular environment for survival, a distinctive attribute of the genus.

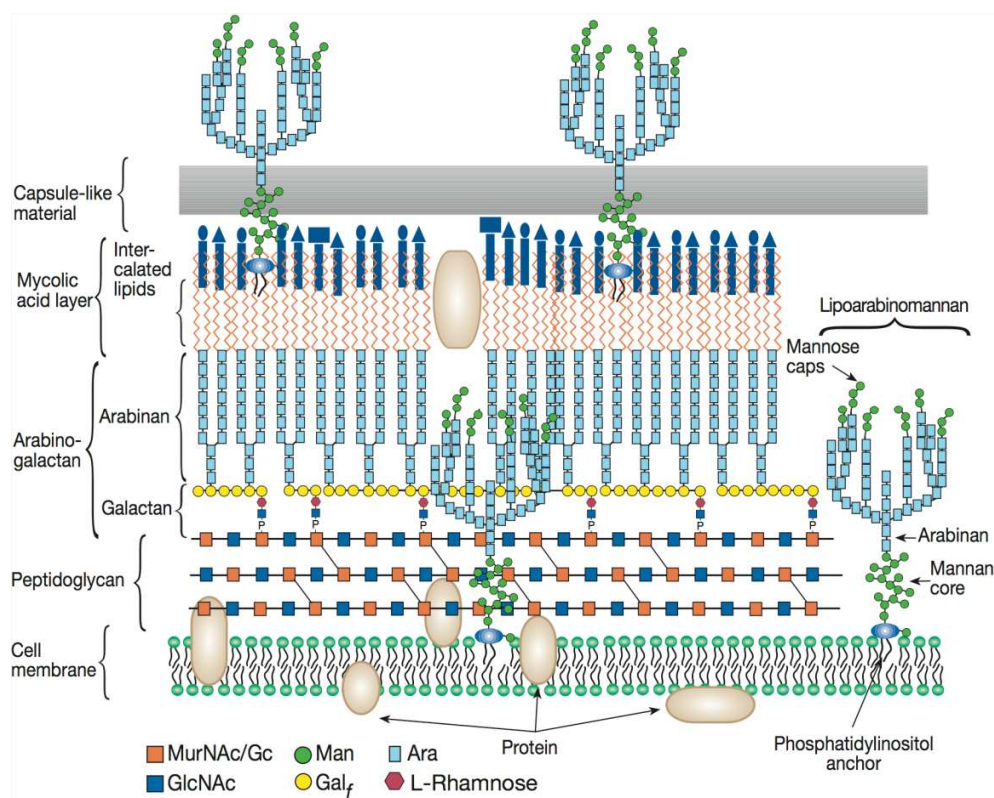


Figure 1.3: Structure of the cell wall of mycobacteria.⁴⁸

1.4.1 Major Lipids of the Mycobacterial Cell Envelope

Mycobacteria characteristically have unusual lipids in their cell envelopes. The strata of complexity go from heterogeneity in chain length to variation in functional group polarity.⁴⁹

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Early attempts at fractionating these lipids consisted of differential solvent extraction.⁵⁰ This resulted in the early classification of mycobacterial lipids into phosphatides, fats, purified wax, and soft wax. With improvement in analytical techniques, much better resolution of lipid profiles was achieved.⁵¹

The most hydrophobic mycobacterial lipids are the triacylglycerols, menaquinones, and diesters of mycocerosic acids and phthiocerols [**Table 1.2**]. Glycolipids of trehalose make up the next least polar lipids. Mycolipenic and hydroxymycolipenic acids form esters with this homo-disaccharide.⁵²⁻⁵⁴ This class of lipid appears to be peculiar to *M.tb*. The most polar lipids extracted with petroleum ether are the ‘cord factors’ and sulfoglycolipids [**Table 1.3**]. Cord factors are trehalose di-mycolates. While the trehalose dimycolates are common among mycobacterial species the sulfoglycolipids are more uniquely associated with *M.tb*.⁵⁵

Examination of the fractions of a polar extraction solvent system, chloroform-methanol-water, show that the least polar lipids are the glycopeptidolipids.⁵⁵ These lipids contain the unusual amino-acyl alcohol, alaninol, along with a few other amino acids in a short peptide. The peptide is esterified to O-methylated rhamnose and deoxytalose.⁵⁶

The antigenicity of mycolic acids (MAs) was first described in 1994.⁵⁷ It was shown that MA was presented by CD1b cells for recognition by CD4⁺CD8⁻ T-cells to elicit an immune response not dependent on the CD4⁺ T-cell targets of HIV. To begin to understand why they are able to interact with the host’s immune system in this way, one must analyze their structure and compare them to other mycobacterial cell wall lipids.

1.4.1.1 Menaquinone

Menaquinone **1** is a polyisoprenylated naphthoquinone electron carrier. It shuttles electrons between membrane-bound proteins of the bacterial electron transport chain, an analogous role to the mammalian benzoquinone, ubiquinone.⁵⁸

Menaquinone is found in pathogenic bacteria like mycobacteria, most Gram positive bacteria, and some Gram negative bacteria.⁵⁹⁻⁶¹ Mycobacterial menaquinone has nine isoprene units, while other

Table 1.2: Major non-mycolate lipids of mycobacteria.

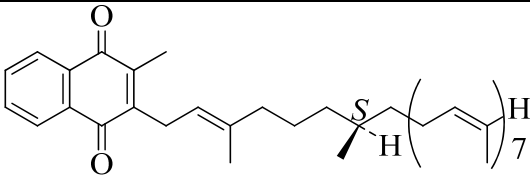
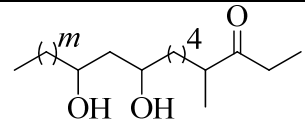
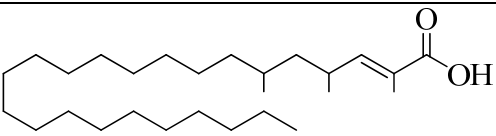
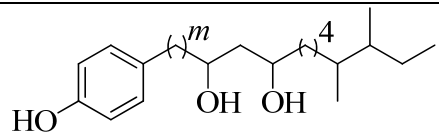
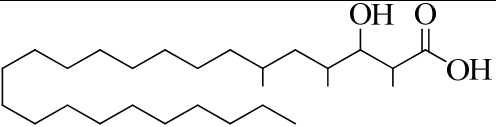
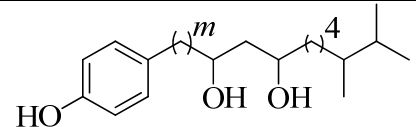
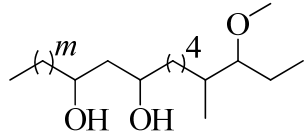
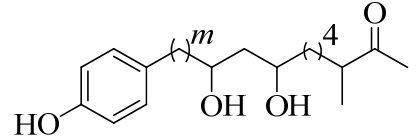
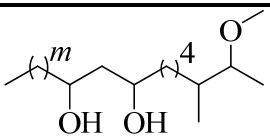
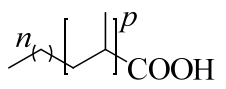
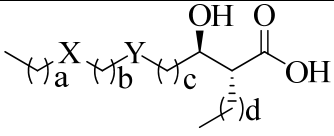
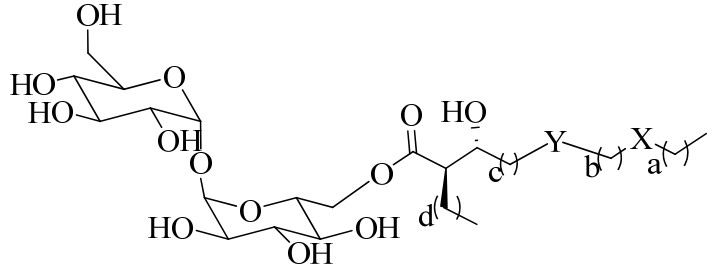
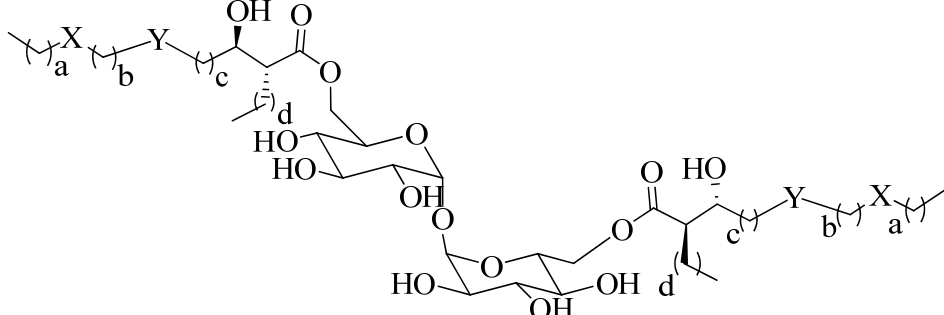
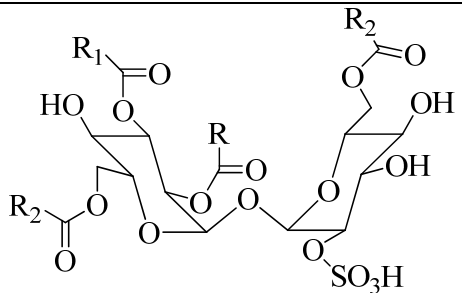
 1	 6 Phthiodiolone
 2 Mycolipenic acid	 7 Phenol phthiocerol A
 3 Mycolipanic acid	 8 Phenol phthiocerol B
 4 Phthiocerol A	 9 Phenol phthiodiolone
 5 Phthiocerol B	 10 Mycocerosic acid

Table 1.3: Mycolates and trehalose lipids.

 11 Mycolic acid
 12 Trehalose monomycolate
 13 Trehalose dimycolate
 14 Sulfolipids For SL-1: RCOO⁻ = palmitate, stearate; R₁COO⁻ = phthiocerate; R₂COO⁻ = hydroxyphthioceratanate

bacteria have 4-9 units. These mycobacterial lipids contain a stereogenic centre due to hydrogenation of one of the isoprene units [Table 1.2].⁶²

Menaquinone is an important cofactor in the human blood clotting cascade system.⁶³ It is synthesized by intestinal *E. coli* or obtained from the diet as vitamin K₂. Biosynthesis of menaquinone involves eight enzymatic steps with chorismate as the main precursor. This pathway is absent in humans and has become an attractive drug target considered for treating TB.⁶⁴ Because menaquinone is an important cofactor of the human biological system it is precluded as an antigenic modulator of the host immune system.

1.4.1.2 Mycocerosic Acids

Mycocerosates **10** are methyl-branched fatty acid salts. They may contain from 27 to 34 carbon atoms. *M.tb* has C₂₇-C₃₄ while *M. leprae* has C₃₀-C₃₄.^{65,66} The shorter mycocerosates have 22 to 26 carbons. The longer chain acids are found in esters of both phthiocerols **4-5** and phenolphthiocerols **7-8** while shorter chain mycocerosates are found esterified only to phthiocerols. The phthiocerol family **4-9** consists of long chain β -diols. These secondary alcohols form diesters with the multimethyl-branched fatty acids **10**. The L-fatty acids are called mycocerosic acids while the D-fatty acids are phthioceranic acids. The diesters of phthiocerol are respectively known as dimycocerosates and dipthioceranates.

Structurally related are the phenolic glycolipids (PGLs) which only differ in having glycosylated phenols at the ω end of the phthiocerol chain **7-9**.⁶⁷ This family of a few phthiocerol waxes is unique to slow growing pathogenic *Mycobacterium* species like *M. leprae*, *M. ulcerans*, *M. marinum*, and mycobacteria of the *M.tb* complex.⁶⁸ They have been implicated in many activities related to the pathogenicity of the mycobacteria. Particularly well studied in *M. leprae*, PGLs were found to help the bacteria enter macrophages and Schwann cells via phagocytosis, while simultaneously evading intracellular degradation. Mutant strains of *M.tb* deficient in dimycocerosate metabolism are attenuated for infection of laboratory animals.⁶⁹⁻⁷¹

Although these complex waxes have been shown to be potent virulence factors, there is limited evidence of their role in immunomodulation except in cellular immunity.⁷²⁻⁷⁴ *M.*

leprae derived PGLs were found to restrict T-lymphocyte proliferation, in contrast to PGLs derived from *M.tb* complex bacilli. Furthermore, many clinical strains of *M.tb* do not produce PGLs and no strong evidence exists for immunomodulation by dimycocerosates.⁷⁵

1.4.1.3 Sulfolipids

Mycobacterial sulfolipids **14**, also referred to as sulfoglycolipids, are multi-acylated trehaloses with a sulfate group on the sugar moiety. This makes the lipids anionic and more polar than the cord factors/trehalose-dimycolates. Five structural forms of this family of lipids have been identified in *M.tb*.⁷⁶ The principal member is the 2,3,6,6'-tetraacyl trehalose 2'-sulfate (SL-1) **14** [Table 1.3].⁷⁷ They are reported to be potent virulence factors and strong immunomodulators and are presented for T-cell recognition by CD1b molecules.⁷⁸ Khuller *et al.* reported that the antigenicity of these acidic lipids is due to the α -D-trehalose and sulfate groups.⁷⁹ This is however contrary to what was observed with the other major trehalose glycolipid, cord factor, where the antigenic epitope was demonstrated to be the mycolic acid residues.⁸⁰ Beckman *et al.* even demonstrated CD1b-linked antigenicity of non-glycosylated MA.⁵⁷

1.4.1.4 Mycolic Acids

Mycolic acids **11** are high molecular weight β -hydroxy acids with a long alkyl side branch at C₂. There is a wide spectrum of structural diversity in these waxes that has resulted in broad classifications. These include inter-genera and inter-species variations. The molecular class compositions and the distribution of homologues are different in different species, such that they form complex mixtures that are characteristic of each species.^{51,81}

MAAs are the major outer membrane lipids of *Caseobacter*, *Corynebacterium*, *Mycobacterium*, *Nocardia*, and *Rhodococcus*. Corynomycolic acids of *Corynebacterium* have 22-38 carbon atoms and nocardomycolic acids of *Nocardia* have 44-60 carbons [Table 1.4].^{82,83}

Table 1.4: Mycolic acid-containing genera.⁸³

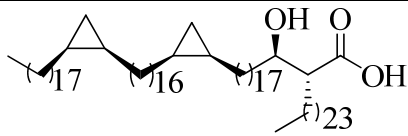
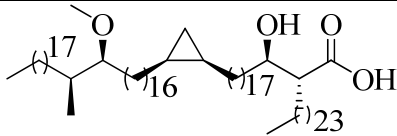
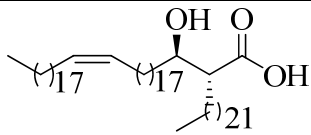
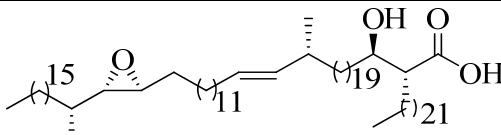
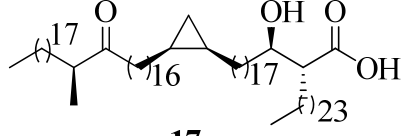
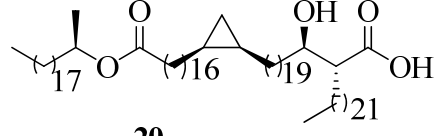
Genus	Chain length (carbon atoms)
<i>Corynebacterium</i>	22-38
<i>Rhodococcus</i>	34-52
<i>Nocardia</i>	44-60
<i>Gordonia</i>	48-66
<i>Tsukamurella</i>	67-78
<i>Mycobacterium</i>	60-90

Mycobacterial MAs are unusually large lipids. They have between 60 and 90 carbons. Unlike the MAs of other genera, mycobacterial MAs have other oxygenated functional groups along with the β -hydroxyl group. These may be a ketone, methoxy, epoxy, or even another hydroxy or carboxyl group. Adding to the complexity, these oxygenated groups occur invariably either with a cyclopropane or a double bond proximal to the β -OH. Some classes of mycolic acids totally lack any other oxygen functionality apart from the β -OH.⁸⁴ Several classes of MAs have been identified in mycobacteria based on these functional group variations [Table 1.5].⁸³

MAs are the major lipid components of mycobacterial cell walls. They occur either as free acids, glycosylated as trehalose esters or (mainly) bound to arabinogalactan. MA esterified to arabinogalactan establishes a formidable outer membrane barrier to cellular injury.⁸⁵ This biological scaffolding not only effectively resists injurious chemical penetration, it also affords a three dimensional matrix for other complex lipids and is central to many of the organism's interactions with the host.^{49,86}

Cryo-electron tomographic investigations of whole mycobacterial cells have led to revisions of models describing the cell envelope architecture.^{87,88} These studies show that the cell envelope is made up of several distinct layers with the outermost layer being a symmetrical lipid bilayer. Theoretical models of the mycobacterial outer membrane had reported a thickness of ≈ 9 nm but the experimental data reported by Hofmann *et al.* showed a slightly smaller thickness of ≈ 7 nm.

Table 1.5: Examples of mycobacterial mycolic acid from each class.

 15 α Mycolic acid	 18 Methoxy Mycolic acid
 16 α' Mycolic acid	 19 Epoxy Mycolic acid
 17 Keto Mycolic acid	 20 Wax ester Mycolic acid

After their discovery, MA classes were studied mainly for their taxonomic value. Minnikin *et al.* used TLC to demonstrate the existence of patterns that could be used for classifying the mycolata bacteria.^{49,51} HPLC, GC-MS, IR, and extensive NMR analysis have resulted in a library of over 500 chemically related compounds making up the MA family.⁸⁹ A significant breakthrough in scientific efforts of the last two decades of the 20th century is the discovery that these major cell envelope lipids of the tubercle bacillus were potent antigens in the human host.⁵⁷

MAs are classified into several different classes based on the functional group combination at the proximal (Y) and distal (X) positions of the meromycolate chain [Table 1.3]. The alpha (α) **15**, methoxy **17**, and keto **18** mycolic acids have a cyclopropane, a methyl ether, and a carbonyl respectively at the distal position along with a proximal cyclopropane [Table 1.3, Figure 1.4]. These MA classes are commonly found in *M.tb*. The cyclopropane may be *cis* or *trans* in natural mixtures. *Trans* configuration has a methyl group α to the cyclopropane. The natural ratio of the MA classes in an organism appears to be a complex

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function of the species, the stage of its life cycle, and possibly the state of its environment.^{90,91}

Alpha MAs are the major class found in *M.tb* and many other mycobacteria.^{51,91,92} They may make up about 50-70% of the total MAs present. They are the most hydrophobic class of mycobacterial MAs because they lack any intra-mero-chain oxygen functionality. Alpha MAs have either two cyclopropyl rings or two double bonds or a combination of these functional groups. Three homologous series have been characterized based on these functional groups: dicyclopropanoyl acids, monocyclopropanoyl monoenoic acids, and dienoic acids.^{49,91-93}

The cyclopropanes have been found mainly in the *cis*-configuration.⁹² Dicyclopropanoyl α -MAs are mainly made of even carbon-numbered acids. They have no methyl branches on the mero-chain. Olefinic α -MAs are mainly odd carbon-numbered chains and usually have intrachain methyl side branches alpha to the unsaturated functional group. *Trans*-configuration is observed in odd carbon-numbered monoenoic α -mycolic acids. The methyl branch is believed to be introduced during conversion from *cis* to *trans* configuration.⁴⁹

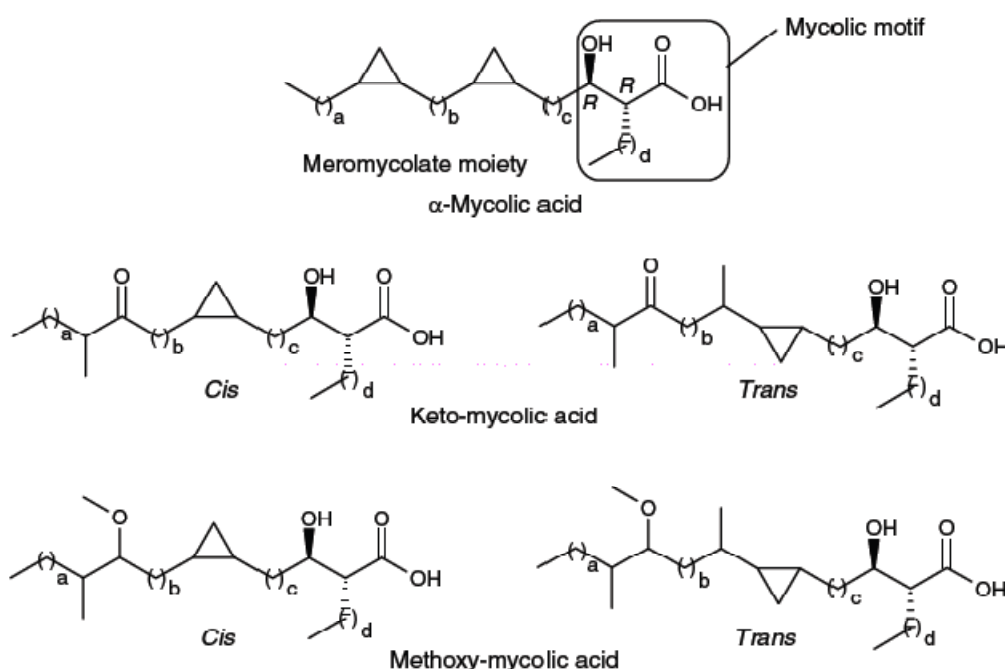


Figure 1.4: *M.tb* MA subclasses.⁹⁴

1.4.2 Biological Roles of MA Classes

MAAs have mainly been associated with maintenance of the mycobacterial cell envelope integrity due to the extreme hydrophobicity that they confer on the outer structure.^{49,87,88,95} However, evidence has been building up that the different classes play pleiotropic biological roles. The importance of oxygenated MA to the virulence of *M.tb* has been known for some time from studies on mutant strains of mycobacteria.⁹⁶⁻⁹⁸ *M. bovis* BCG Pasteur strain used for vaccination lacks the ability to synthesize MeO-MA.⁹⁶ *M.tb* strains unable to synthesize keto-MA have attenuated intracellular growth.^{97,98} MAAs containing *trans*-cyclopropanes at the proximal position in the mero-chain were reported to affect the cell wall and therefore influence the sensitivity of mycobacterial species to hydrophobic antibiotics.⁹⁶

Several interactive roles of MA with the host have also been elaborated. MAAs elicit the formation of foamy macrophages.^{99,100} These are morphological cellular changes that are normally part of the host innate response to mycobacterial pathogens like *M.tb* and *M. avium*. Foamy cells are characteristically larger in size and harbour several vacuoles in which neutral lipids and cholesterol are hoarded intracellularly.⁹⁹ The cholesterol is used as an energy source and is also implicated in the internalization of the bacillus by the phagocytic macrophage.^{101,102} Crucially, it is believed to be involved in the prevention of maturation of the phagosome.¹⁰³

When Bloch isolated cord factor from virulent *M.tb* strains he discovered that the extract was toxic to laboratory animals and also very immunogenic. He also suggested that they were responsible for the 'cord-like' morphology of the cultured bacilli.¹⁰⁴ This observation was later questioned when TDM was isolated from non-cording bacteria.¹⁰⁵ Kato demonstrated that injecting rabbits with TDMs elicited anti-cord factor antibodies which protected the laboratory mice against virulent *M.tb*.¹⁰⁶ Bekierkunst showed that cord factors induced pulmonary granuloma formation.¹⁰⁷ He further demonstrated the immunostimulatory and related anti-tumouric effects of these glycolipids.^{108,109} Several other fascinating immunological effects of TDMs have been observed but these are often a function of the animal studied, the route of administration and the nature of the TDM suspensions used.¹¹⁰

This project aims to focus on the free MAs for serodiagnosis even though the TDMs have remarkable immunological properties.⁹⁴ As discussed below [Section 1.5], TDMs possess exciting specificity as serodiagnostic tools for TB but with less impressive sensitivity.¹¹¹ Free MAs as antigens in optical and electrochemical biosensors-based diagnostics show better sensitivity and specificity.^{37,112} The free MAs show such wide structural and functional group variability among mycobacteria species that they can provide the specificity wanting in other mycobacterial antigens.

1.5 MA in TB diagnosis

Antibodies to cord factor presented an attractive choice as a surrogate marker for TB because of the latter's strong antigenicity.¹⁰⁶ They were stable on ELISA plates and gave reproducible test results.^{46,113} Two significant caveats to the anti-TDMs tests must be noted. Firstly, cord factor is a complex mixture of dimycolates. The two MA residue classes and subclasses vary. This can potentially yield inconsistent outcomes to the test. However, with the recent availability of synthetic cord factors this issue might be resolved.¹¹⁴ Secondly, the patients enlisted in the studies were not co-infected with HIV. As MAs are the epitopic determinants of cord factors¹¹⁵ it can be extrapolated that the level of sensitivity and specificity for cord factor-based ELISA might be less than reported. Free MAs have also been found to give only 57% accuracy when used in ELISA tests.¹¹⁶

In 2008 Thanyani *et al.* reported an affinity biosensor-based serological test for TB with the potential to effectively detect TB in HIV co-infected persons.³⁷ They used an immunoassay protocol in which serum antibodies to MA were pre-inhibited in high dilution MA solution and then exposed to immobilized MA for binding. The degree of antibody binding inhibition as the output signal gave much better sensitivity and specificity than ELISA to TB infection including HIV/AIDS co-infected cases. Unpublished recent results from the group gave promising results, not excluding the possibility that the test may eventually perform at a specificity and sensitivity approaching 100% on a surface plasmon resonance (SPR) biosensor. However the low throughput, degree of sophistication and price of the test make it not practical for commercial use in its current format. This test is described in more detail in the following section.

A related technology was also recently reported using electrochemical techniques.¹¹² Binding of anti-MA antibodies was detected by impedance of electron charge transfer to a gold surface with a soluble redox agent. This held out the possibility of larger sample throughput, simplified application and the possibility of using disposable printed electrodes at considerably lower cost than SPR technology. Whereas antibody detection by ELISA was possible only down to a 1 : 20 dilution, the surface plasmon resonance and impedimetric immunosensing were sensitive at dilutions as high as 1 : 2000. The electrochemical method has better potential for lower priced, large sample analysis throughput, but still has to prove its sensitivity and specificity as explained below [**Section 1.5.2**].

The chemical synthesis of MAs will greatly facilitate stable antigenic surfaces on gold-coated sensors. The ability to immobilize stereochemically defined MA molecules should improve the reliability of test results and certainly make both the SPR and EIS protocols more user-friendly.

1.5.1 The Surface Plasmon Resonance Sensor

SPR is a phenomenon of some metals that has been widely applied in photometric biosensors. Gold is the most commonly used metal for plating glass in optical biosensors because of its ability to generate surface plasmon on absorption of electromagnetic waves. When a thin coating of gold on a high refractive index glass surface is irradiated by an incident laser beam the excited metal electrons generate a detectable evanescent field. At a specific angle and wavelength of the incident light this phenomenon is dependent on the physical nature of the gold surface. Changes in the surface, such as binding of a ligand to an adsorbed receptor, results in a change in the refractive index that affects the energy whereby the incident light evokes a plasmon resonance. This is measured as the angle at which alteration of the evanescent waves on the metal surface.

The first application of SPR to MA-based TB diagnosis was reported in 2009 by Thanyani as part of a doctoral thesis.³⁶ It heralded the development of the MARTI diagnostic and showed that evanescent field biosensors were credible alternatives to ELISA for TB

serodiagnosis.³⁷ It could signal a major shift from ELISA as the technology of choice for serodiagnosis of TB.^{34,46}

A MARTI protocol sensogram typically has a series of stages which are explained in **Figure 1.5**. The TB status of a patient is determined by the separation of the last two segments of the two coloured curves.

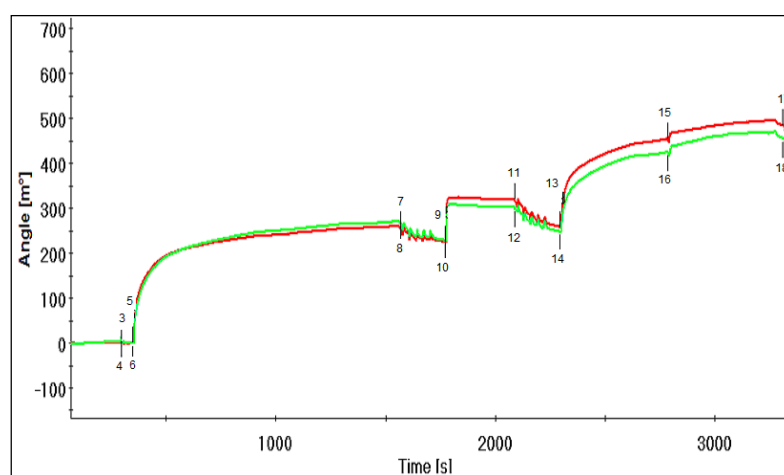


Figure 1.5: A representative sensogram of a MARTI protocol analyzing a human serum on a two-channel ESPRIT biosensor. The numbers mark the initiation of different events in the process; the red and green lines respectively represent two channels on the biosensor for the serum pre-incubated with MAs-liposomes and empty-liposomes; 3/4 correspond to the baseline; 5/6 liposome immobilisation on the sensor surface; 7/8 start of washing; 9/10 saponin blocking; 11/12 start of washing; 13/14 addition of highly diluted serum; 15/16 addition of low dilution serum; 17/18 end of experiment.³⁶

SPR has a couple of advantages over electrochemical spectroscopic techniques discussed below. It is reagentless. The sensogram is a real-time profile of the intermolecular interactions being investigated [**Figure 1.5**]. The sensitivity of SPR is higher than electrochemical methods because of its use of changes in the electromagnetic properties of light to track the progress of an experiment. Electrochemical cell experiments are affected by the physico-chemical limitations inherent in a chemical reaction. These include the electrode kinetics and diffusion rate of the redox species at the sensor surface.

The high sensitivity and real-time nature of SPR, although desirable, may also be the biggest hindrance to practical field application. Sample preparation and running an

experiment require much more instrumental sophistication and skilled knowledge than electrochemical biosensors.

1.5.2 An Electrochemical Sensor

A significant breakthrough in the development of new diagnostics against TB was recently published by Mathebula *et al.*^{112,117} The immunosensor technique applied electrochemistry, specifically electroimpedance spectroscopy, to distinguish between TB positive and TB negative patient sera.

The infrastructure of the immunosensing surface involved first creating a self-assembled monolayer of cysteamine on a gold electrode. Next, stearic acid was covalently linked to the SAM via an amide bond to create a hydrophobic chain which was used to immobilize a natural mixture of *M.tb* MA classes. The sensor surface was completed by blocking spaces in the structure with saponin [Figure 1.6].

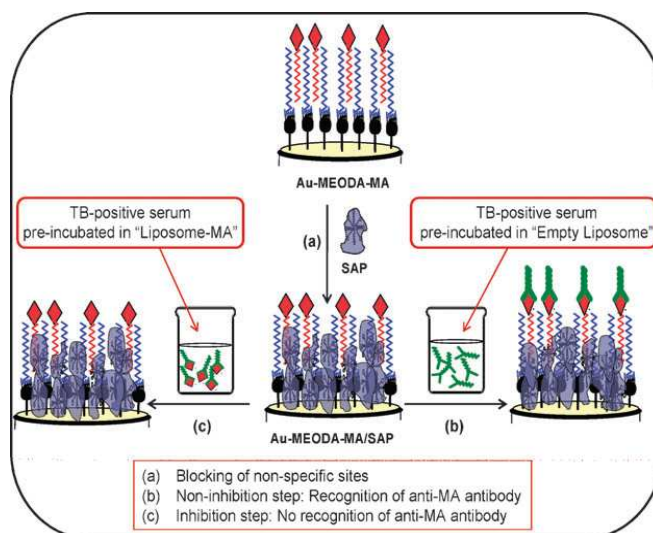


Figure 1.6: Modification of a Au-MEODA-MA electrode with a blocking agent (saponin, SAP) and subsequent interaction of the immobilized MA with anti-MA antibody in human TB-positive serum.¹¹²

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The actual diagnostic procedure closely resembles that of the wave-guide and SPR biosensor techniques.^{36,37} The patient sera were each pre-incubated with buffered saline solutions of MA-liposomes and empty liposomes respectively. The first treatment was done to remove MA-binding antibodies and inhibit binding. The second was done to provide a comparison uninhibited signal for MA binding.

The EIS results, presented as Nyquist plots, clearly distinguished between TB+ and TB- patients when the change in the charge transfer resistances (ΔR_{ct}) of the two sera was compared [Figure 1.7]. ΔR_{ct} is the difference between the charge transfer resistances of the patient serum-incubated electrode and the Au-MEODA-MA-SAP antigen surface. Some of the fundamentals of the EIS along with cyclic voltammetry will be discussed in detail later in the introduction to Chapter 4.

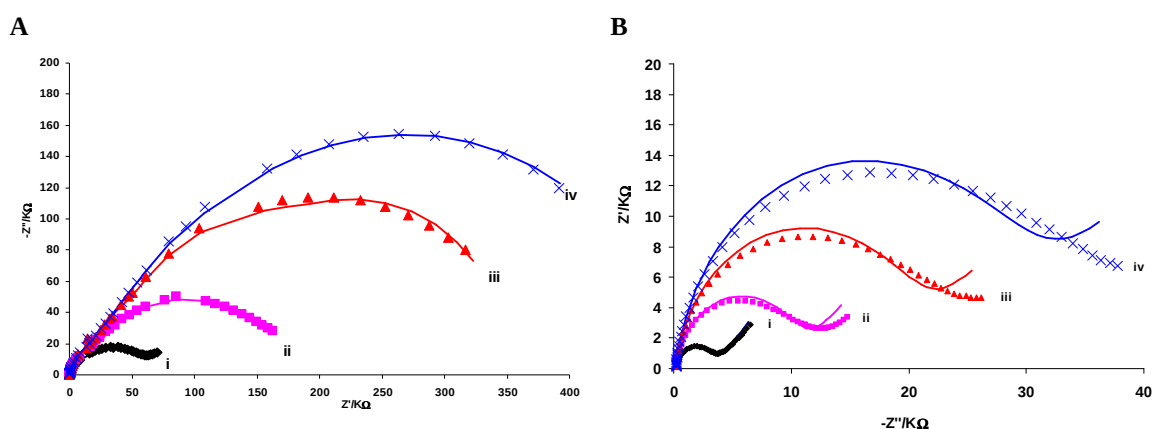


Figure 1.7: Nyquist plots of Au-MEODA-MA-SAP with **A.** HIV⁺TB⁺ and **B.** HIV⁻TB⁻ patient sera pre-incubated in empty liposomes. **i.** Au-MEODA-MA-SAP without patient serum, **ii.** 1 : 2000 (0.05% serum), **iii.** 1 : 1000 (0.10% serum), and **iv.** 1:500 (0.20% serum). The solid lines are fitted curves using equivalent circuits.¹¹²

Remarkably, detection of anti-MA antibody binding to the sensor surface was still possible even when the patient serum was diluted 1 : 2000 (0.05%). Also it confirmed that MAs without liposomes could still be recognized as antigens when used in biosensors. This was particularly relevant to the aim of the present project to create a new sensor surface by directly linking thiol-modified MA to a gold substrate in the absence of liposomes.

1.6 Gold Sensor Surfaces

Early attempts at creating hydrophobic surfaces on solid supports investigated alkyl alcohols and amines adhering to glass and platinum.^{118,119} These were physical rather than chemical interactions between the organic substrates and the inorganic supports. They were therefore unstable. Sagiv created the first stable chemisorbed surfaces when he reacted alkyl chlorosilanes with SiOH on silicon support.¹²⁰ The high reactivity of silyl chlorides excludes the use of such surfaces for biological studies.

Gold stands out among several other elements as a sensor surface for several reasons. It is chemically inert to many functional groups found in biological molecules.¹²¹ It is easy to clean. Adsorbed molecules can be removed with common laboratory reagents without risk of oxidizing the metal. The conductivity of gold has also made it useful in electrochemical sensors.

A truly remarkable property of gold, and indeed many other noble metals like silver, is its strong affinity for sulfur atoms [**Table 1.6**]. Multi-functional biological molecules do not compete with ‘soft’ sulfur because functional groups like alcohols, amines and acids are relatively ‘hard’ in comparison. Au—S bonds are formed under ambient conditions which is critical for heat labile biomolecules. This property has been widely exploited as a powerful tool in the creation of hydrophobic surfaces.

Table 1.6: Examples of modifying layers formed by chemisorption.¹²²

Molecule	Electrode material
RSH	Au, Ag, Pt, Cu, Hg
RSSR'	Au
RSR'	Au
R ₃ P	Zr
RNC	Au, Pt, Cu
RCOOH	Ag, Al ₂ O ₃
R(OArCH(NH ₂) ₂) ₂	Au
Silanes	Hydroxylated surfaces

1.6.1 Self-Assembled Monolayers of Alkanethiols on Gold

Binding of gold to sulfur can occur with thiols, disulfides, thiones, thioesters, and many other sulfur species, but the best binding is obtained with thiols [Table 1.6].¹²³ Alkanethiols spontaneously adsorb and covalently bind to the gold surface from solution to form a two-dimensional monolayer called a self-assembled monolayer (SAM).

SAMs of long alkyl chains like octadecanethiol (ODT) form quasi-crystalline arrays on gold, while shorter chains form more fluid surfaces. Long-chain alkanethiols have often been used to immobilize more complex molecules by hydrophobic interactions. Shorter chains often have a head functional group like $-\text{COOH}$, $-\text{OH}$, or $-\text{NH}_2$ that can be modified chemically for reacting with another molecule after initial monolayer formation. This benefit derives from the versatility and strength of the Au—S surface.

Deposition of thiols on the gold surface is a random spontaneous process that might require several hours or days for completion [Figure 1.8].¹²³ The lack of requirement for reagent or catalyst for this process makes it a cheap and cost effective method for creating monolayers. The affinity for gold is so strong that the thiols can displace most physically adsorbed substances from the surface thus eliminating the need for rigorously cleaned surfaces. Van der Waals interactions between the alkyl chains further strengthen the monolayer, making it resistant to washing.

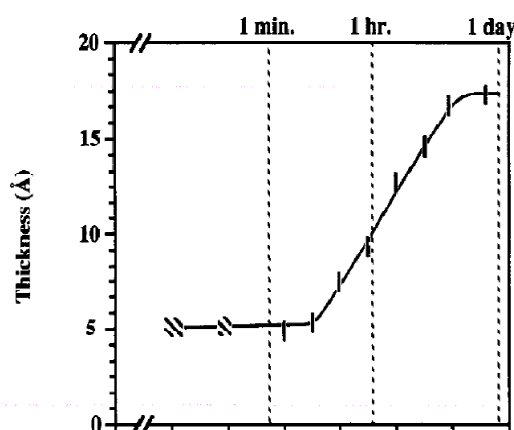


Figure 1.8: XPS measurement of thickness of organic films on polycrystalline gold after exposure to 1.4 μM solutions of $\text{HS}(\text{CH}_2)_{15}\text{CH}_3$ in ethanol.¹²³

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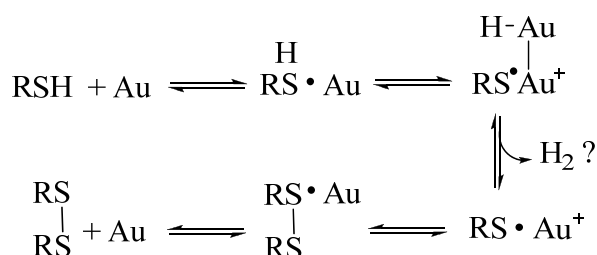
The discussed properties of SAMs on gold made them attractive for use as biological sensor films. Recently, in our group, Thanyani *et al.* used ODT SAM to immobilize mycolic acid liposomes on SPR sensor chips in the novel MARTI diagnostic test for TB.³⁶ Also, Mathebula *et al.* used cysteamine SAM on gold electrodes for their novel work on electrochemical diagnostic test for TB.¹¹² The amine head of the cysteamine SAM was reacted with stearic acid to create a hydrophobic surface for immobilizing MA liposomes.

Although many biological molecules are immobilized on gold surfaces these are often done by hydrophobic interactions to a SAM of simple thiolated organic molecules. The primary reason seems to be the difficulty in thiolating complex biological molecules without seriously disrupting their sensitive biological activities. In spite of the tenacious stability of the SAM on gold the entire system is fragile because hydrophobic interactions are several orders of magnitude weaker than covalent bonds. Further, MA-liposome systems currently used in the two new TB diagnostics of Thanyani and Mathebula and co-workers have a short shelf life, often not reliable 24 h after preparation by sonication. Stable and reliable sensor surfaces are required.

1.6.2 SAMs of Thiols versus Disulfides

Although the spontaneous oxidation of thiols to disulfides may be perceived as a nuisance, both form stable SAMs with almost identical convenience. The rates of SAM formation by dialkyl disulfides and alkanethiols are identical.¹²³ TEM and XPS characterizations do not produce any observable difference.^{123,124}

A possible mechanism for the formation of SAMs by alkane thiols and dialkyl disulfides was proposed by Biebuyck *et al.* in their 1994 publication [**Scheme 1.1**].¹²³ They came to this qualitative description of the process of SAM formation by alkanethiols and dialkyl disulfides based on observations that both species yielded epitaxial alkanethiolates, RS^-Au^+ , on gold.



Scheme 1.1: Mechanism of covalent reaction between alkanethiols and dialkyl disulfides and gold.¹²³

There are however some differences in the behavior of SAMs from the two oxidation states of the sulfur precursor. Long chain thiols and disulfides can replace SAMs formed from short chains.¹²³ Displacement of propyl thiolate SAM by hexadecanethiol was ~50 times faster than dihexadecyl disulfide.¹²³ Also the alkanethiols were better ordered than the disulfides.

1.7 Synthesis of Mycolic Acids

The major classes of mycobacterial MAs exist in subclasses which are stereochemical variants. Chain length heterogeneity also contributes to subclass diversity. These subclasses have been observed to possess significantly different, and often opposing, biological activities.^{91,92} It is extremely cumbersome to isolate stereochemically and structurally pure subclasses in enough quantities from natural mixtures for analysis.

Determination of the native stereochemistry of the natural MA target for synthesis is problematic. In some instances the stereochemistry of the natural compound is inferred from the stereochemistry of the precursors in the biochemical pathway. Even when pure compounds are obtained elucidation of absolute stereochemistry is still a challenge because of the presence of several stereogenic centres within the MA molecule. Chemical fragmentation of a MA molecule could result in alteration of the native stereochemistry. Assigning an exact absolute stereochemistry purely on the basis of physico-chemical techniques is very difficult; hence all possible stereochemical forms have to be synthesized and tested biologically. From this the native chemistry can be determined.

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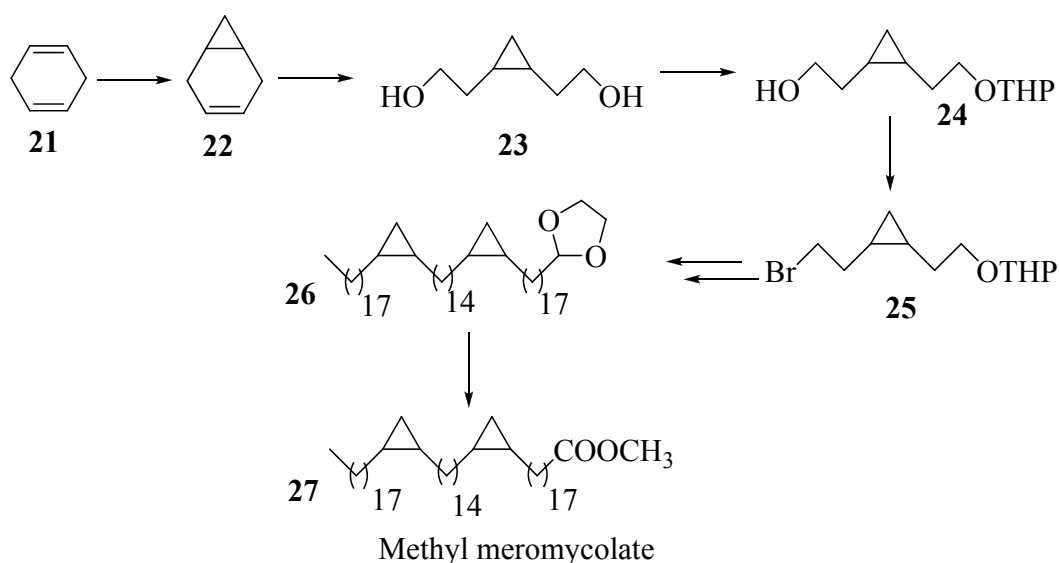
It must be noted that variations in stereochemistry and chain lengths are most profound in the merochain. Indeed, in all MAs investigated to date the absolute stereochemistry of the 2-alkyl-3-hydroxy stereocentres has been *R,R*.¹²⁵⁻¹²⁷ Only minor differences have been seen in the lengths of the 2-alkyl sidechain.¹²⁸

1.7.1 Synthesis of the Merochain

The synthesis of α -meromycolates as mixtures of diastereomers has been known for some years; more recently syntheses of a range of meromycolates as single enantiomers have been reported. The research team of Professor MS Baird at Bangor University has led in this field and some of their most significant synthetic achievements will be highlighted below.

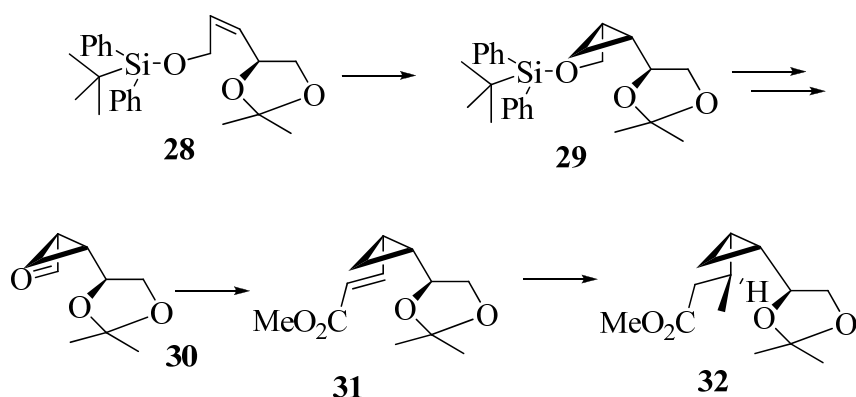
1.7.1.1 Synthesis of *cis*-cyclopropanes

Attempts to synthesize MA classes first focused on synthesis of the cyclopropane ring.¹²⁹ Gensler *et al.* synthesized the cyclopropane ring from 1,4-cyclohexadiene [**Scheme 1.2**].¹³⁰ The diene **21** was converted to norcarene **22** which was ozonolysed by a modified Weinstein and Sonnenberg process. The ozonide was reduced to the cyclopropyl diol **23**. Coupling **25** with linker chains and the distal alkyl chain yielded the methyl meromycolate **27** as a mixture of four stereoisomers.



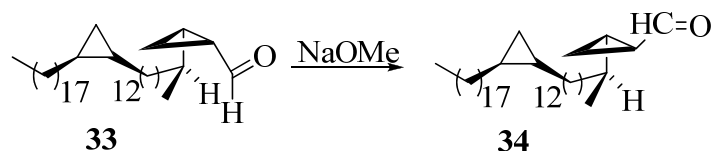
Scheme 1.2: Synthesis of methyl meromycolate **27**.¹³⁰

Coxon *et al.* successfully prepared enantiomerically pure *cis*-cyclopropane analogues from D-mannitol.¹³¹ A *cis*-alkene **28** was synthesized by Wittig chemistry. The olefin was cyclopropanated by the Simmons-Smith reaction. Stereo-control was achieved by the coordination of the zinc carbenoid intermediate to the adjacent oxygen of the isopropylidene ring, leaving the methylene group to attack from the *1re-2si* bottom face. Deprotection and oxidation of glycol **29** afforded the aldehyde **30**, which was extended to the monocyclopropyl methyl meromycolate analogue **32** by a Wittig reaction. The vinyl intermediate **31** of the monocyclopropyl chain elongation was selectively saturated by chemical hydrogenation with a diimide [Scheme 1.3].¹³²



Scheme 1.3: Stereochemical synthesis of cyclopropane derivatives.¹³²

The cyclopropyl acetal **30** was homologated to the α -vinyl ester **31**, which was methylated via Grignard chemistry. This introduced the α -methyl branch observed in MAs with a *trans*-cyclopropane. The *trans*-cyclopropane **34** was obtained by epimerization of the *cis*-cyclopropane **33** [Scheme 1.4].^{132,133} There was a 5% contamination of the *cis*-isomer.



Scheme 1.4: Epimerization of *cis*- to *trans*-cyclopropane.¹³²

With the chemistry for stereo-selective synthesis of a range of mono-cyclopropyl meromycolate analogues firmly established and a method for synthesis of the hydroxyl acid unit developed, complete synthesis of enantiomerically pure MA classes became less of a challenge.¹³⁴ By Julia-Kocienski coupling of two cyclopropyl units the first dicyclopropyl MA, α -MA, was achieved in 2005 by the Baird group.^{135,136}

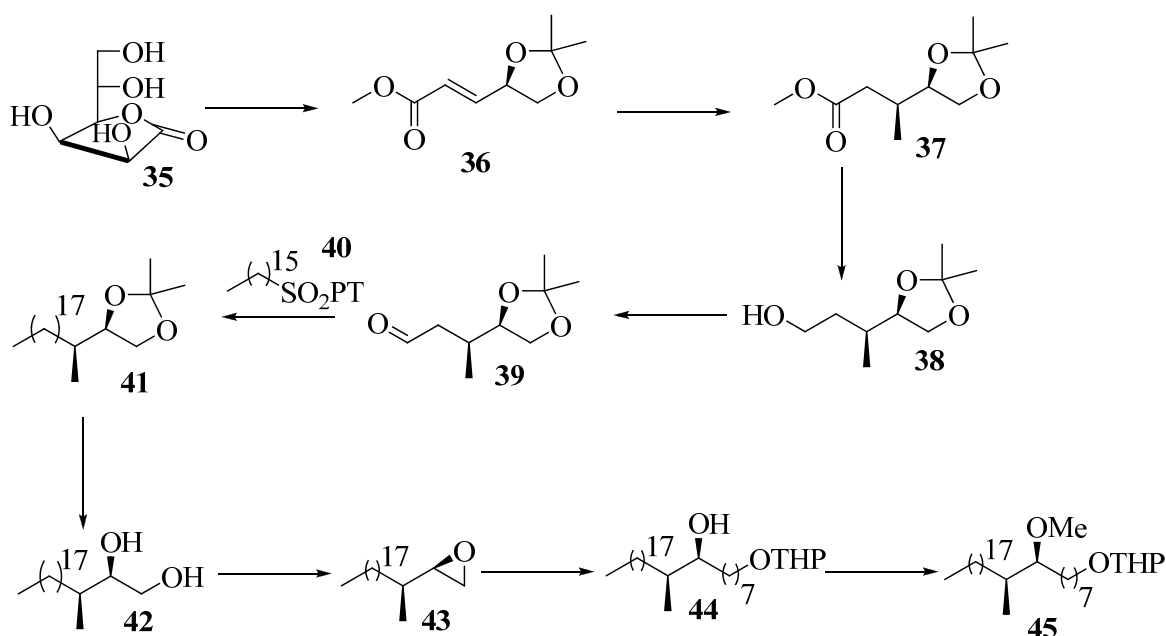
1.7.1.2 Oxygenation of Merochain

As mentioned above most mycobacteria have MAs with oxygenated functional groups on

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the mero chain. *M.tb* has a carbonyl and an ether respectively for keto-MA and methoxy-MA. *M. smegmatis* has an epoxide (epoxy-MA) and *M. avium* has an ester (wax ester).⁴⁹ These have all been successfully synthesized in enantiomerically pure subclasses.

Although not isolated as a membrane lipid, hydroxyl MA (MA-OH) has been synthesized.¹³⁷ It is an intermediate in the biosynthesis of oxygenated MA. Similar to the *in vivo* pathway, MA-OH is a precursor to the synthetic keto- and MeO-MA.^{137,138} The α -methyl OH fragment was synthesized by the opening of the α -methyl epoxide **43** to an α -methyl alcohol **44** [Scheme 1.5].



Scheme 1.5: Synthesis of the α -methyl- β -methoxy chain fragment.¹³⁸

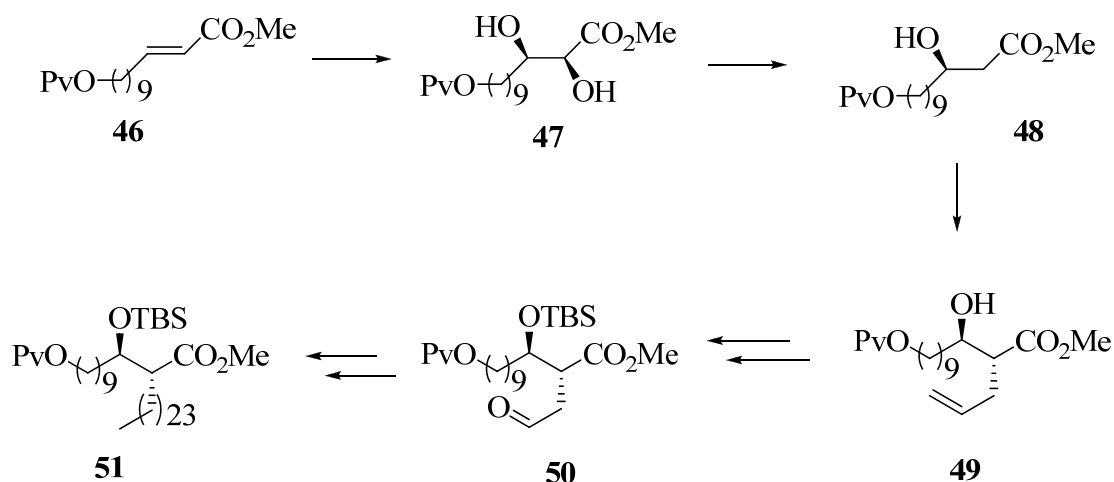
The protected hydroxyl intermediate **44** was methylated to afford the methyl-ether mero-fragment **45**. To obtain the keto-MA the hydroxyl group was oxidized with PCC. A chemical Baeyer-Villiger intra-chain oxidation was carried out to confirm that there was no epimerization of the α -methyl side group. This yielded a wax ester MA. The alcohol product of the hydrolysis of the wax ester had the same specific rotation of -3.8 as the natural reported compound.¹³⁷

Al Kremawi *et al.* recently reported the synthesis of two stereo-isomers of an epoxy-MA of *Mycobacterium fortuitum* and *M. smegmatis*.¹³⁹ Probably the most significant recent synthesis is that of trehalose monomycolate and trehalose dimycolate (cord factor).¹¹⁴

1.7.2 Synthesis of the Mycolic Motif

The successful synthesis of different classes of MA in several stereochemical permutations should assist to unravel many of the mysteries about the properties of these molecules. However, the synthetic routes are quite lengthy, challenging and expensive. Economizing the synthesis of MA will be a priority to enable its incorporation into any commercial TB diagnostic.

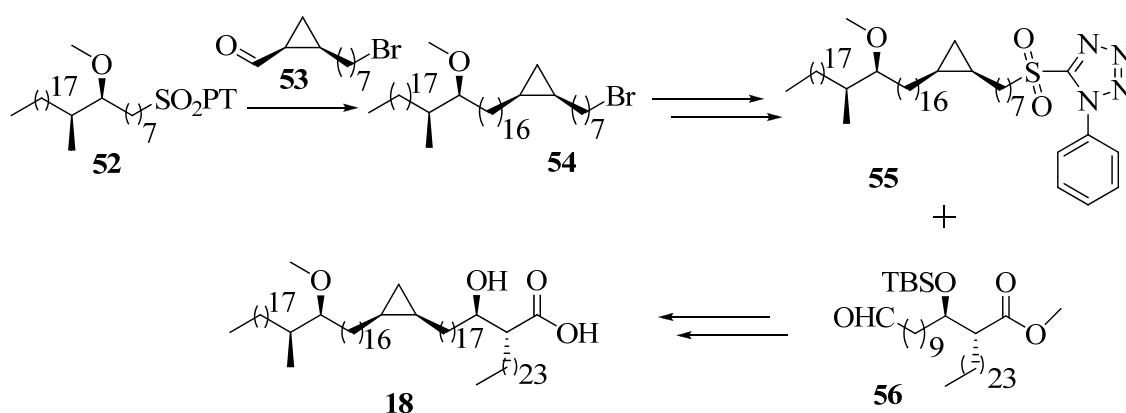
The mycolic motif is a priority for the efficient and economical synthesis of MAs due to its structural and stereochemical conservatism. The routes to its synthesis however highlight the challenges of making two stereogenic centres in an *anti*-configuration. A few approaches to the synthesis of the mycolic motif have been developed over the years but the most noteworthy is that of Toschi and Baird.^{134,140-143} The two stereogenic *2R,3R* centres were created via Sharpless hydroxylation and Frater alkylation reactions [**Scheme 1.6**]. The yield was 7% in 15 steps. The mycolic motif allyl analogue **51** could be extended to the full motif with any choice of 2-alkyl chain heterogeneity. A modified method is currently used in which the 3-OH was created by ring opening of an epoxide; this method was used in the current work and is described in detail in **Section 2.2.7**.¹⁴⁴ It is however worth stressing here that the Frater alkylation does not work directly with long chains. Finding a method to introduce the C₂₄ chain that avoids the Frater-type alkylation is a high priority. An *anti*-aldol reaction could provide the required solution. Aldol reactions have been used in the stereosynthesis of many complex biological compounds. A brief description of the chemistry of asymmetric aldol reactions is discussed in **Section 1.7.4**.



Scheme 1.6: Synthesis of mycolic motif by Toschi and Baird.¹³⁴

1.7.3 Synthesis of a Complete MA

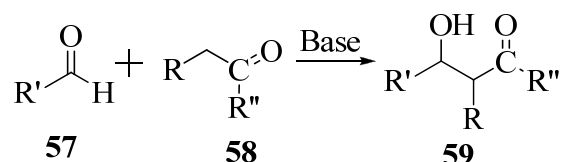
Al Dulayymi *et al.* described the total syntheses of MeO-MA enantiomers from the different methods described above. Fragment **45** was extended to sulfone **52** by standard methods and coupled to aldehyde **53** to introduce **54**. The mycolic motif aldehyde **56** was synthesized by the method of Toschi and Baird.¹³⁴ The methoxy meromycolyl sulfone **55** was coupled by a modified Julia-Kocienski reaction to **56** to yield a mixture of *E/Z* isomers. These were hydrogenated and after deprotection the MeO-MA enantiomer **18** was obtained [Scheme 1.7].



Scheme 1.7: Synthesis of MeO-MA **18**.¹³⁸

1.7.4 A New Approach to the Mycolic Motif: The Aldol Reaction

The aldol reaction is one of the most powerful carbon—carbon coupling reactions in synthetic organic chemistry [Scheme 1.8].



Scheme 1.8: A basic aldol reaction.

The ability to simultaneously create up to two stereogenic centres potentially eliminates several often challenging and expensive reaction steps. However, aldol chemistry is only possible under very specific reaction conditions and controlling these conditions is paramount.

The aldol reaction has enabled the synthesis of many complex biological molecules which were difficult to synthesize in high enantiomeric purity. Syntheses of the macrolide and ionophore antibiotics have been achieved largely through aldol condensations. For example, the 6-deoxyerythronolide B **60** of *Streptomyces erythreus* was synthesized by four successive aldol reactions in 85% stereoselectivity [Figure 1.9].¹⁴⁵

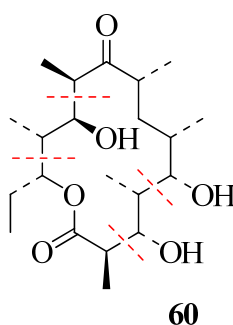


Figure 1.9: 6-Deoxyerythronolide B.¹⁴⁵

1.7.4.1 Aldol Enolate Geometry

The nucleophilic partner in an aldol reaction is an enolate intermediate formed by a base with the anionic charge stabilized by a metal cation. The configuration of the double bond has been shown to have a profound effect on the relative stereochemistry of the aldol adduct.^{146,147} *Cis*-Enolates often produce *syn*-aldols while *trans*-enolates primarily give *anti*-aldols. These stereoselectivities have mostly been observed with lithium and boron enolates under kinetic control.

With alkyl lithium amides, deprotonation of the alpha carbon to the enolate is believed to proceed via a six-membered heterocyclic transition state.¹⁴⁸ These hindered lithium amides almost exclusively form *trans*-enolates of esters. For this to occur the alpha carbon substituent will be forced to project equatorially by the sterically demanding amide substituents and the coordinating THF solvent molecules on lithium [**Figure 1.10**].¹⁴⁸

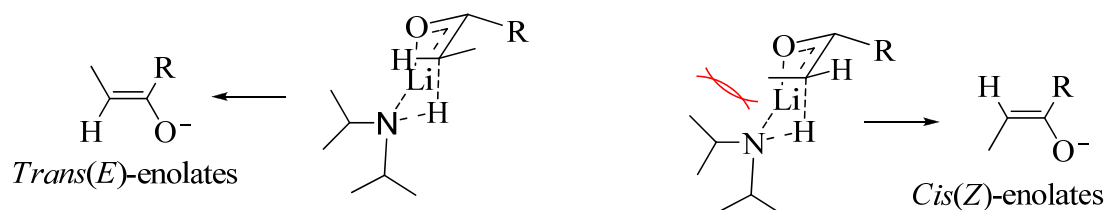


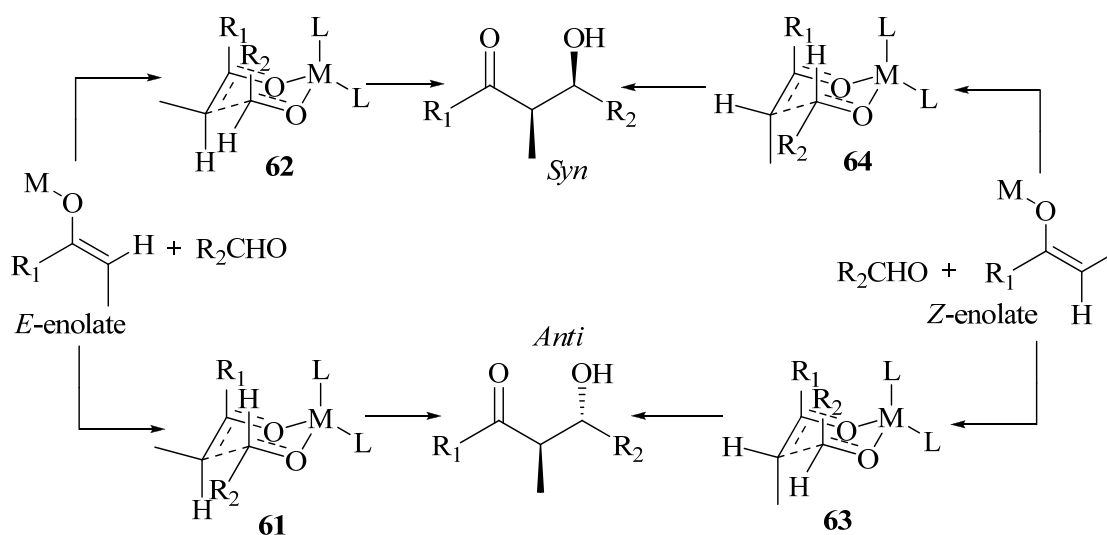
Figure 1.10: Ireland model of enolate formation.¹⁴⁸

Lithium amides are conveniently synthesized usually by treating the appropriate amine with butyl- or methyl-lithium in THF. The bulky alkyl substituents prevent the formation of amides by nucleophilic displacements on esters. Probably the biggest challenge in working with lithium enolates is the rigorously anhydrous conditions under which the reactions must be carried out.

Lithium bases are poorly selective for *cis*-enolates under kinetic control. Instead, boron is used as the coordinating metal for very good *cis*-enolate formation.¹⁴⁹ This usually gives excellent *syn*-diastereomeric selectivity.

1.7.4.2 Aldol Transition States

The most widely accepted model of the aldol transition state proposed by Zimmerman and Traxler in 1957 has a six-membered chair conformation model.¹⁵⁰ In this model the electrophilic carbonyl approaches the metal-stabilized enolate in a diastereofacial selective manner that is controlled and enhanced by coordination of the carbonyl oxygen with the metal. This results in a closed chair conformation that preferentially orientates the substituent group (R) of the carbonyl equatorially [**Scheme 1.9**].



Scheme 1.9: Zimmerman-Traxler transition states of the aldol reaction.¹⁵⁰

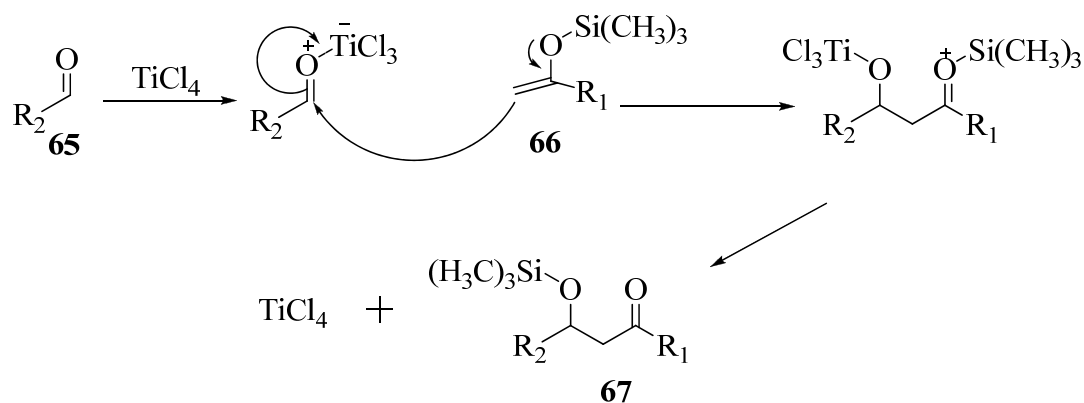
The ligating metal (M) is transferred from the anionic enolate oxygen to the carbonyl oxygen, effectively increasing the electrophilicity of the carbonyl carbon. Acidic work-up gives the alcohol.

The higher the steric demand of R₁ of the enolate the better the diastereoselection of the *syn* and *anti* products [**Scheme 1.9**]. A *syn*-pentane interaction between R₁ and R₂ in transition states **62** and **63** results in destabilization of the pericyclic chair conformations relative to **61** and **64**, respectively.¹⁵¹

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The Zimmerman-Traxler model has been good at predicting the stereochemical outcome of aldol reactions of boron or lithium metals under kinetic control. The extreme complexities of aldols with different reagents under different reaction conditions means the model has not been universally successful. A notable exception is the Mukaiyama aldol addition.¹⁵²

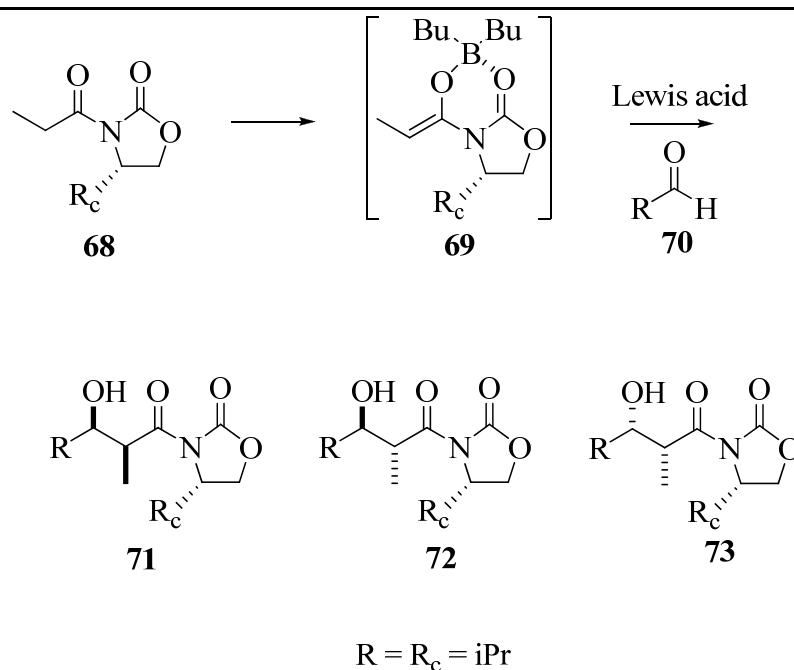
The Mukaiyama aldol reactions are believed to proceed via non-Zimmerman-Traxler open conformation transition states [**Scheme 1.10**]. These reactions are thermodynamically controlled and reversible. A silyl enol ether **66** is used as an enolate equivalent. This is a weak nucleophile and the aldol reaction is catalysed by a Lewis acid, usually a titanium IV salt such as TiCl_4 .¹⁵³



Scheme 1.10: Mukaiyama aldol reaction.

1.7.4.3 Controlling Aldol Product Stereoselection

Heathcock¹⁵¹ and Dubois¹⁵⁴ have independently shown that in cases where the metal enolate is formed before treatment with an aldehyde, kinetic diastereoselectivity is heavily influenced by the enolate geometry. In a 1991 communication Walker and Heathcock showed how slight changes in the reaction conditions of the Lewis acid used can have a disproportionate effect on stereoselectivity. Using the Evans imide **68** they demonstrated that stereoselection of a Lewis acid-catalysed aldol reaction could be affected by altering the sequence of introducing reagents [**Table 1.7**].¹⁵⁵ While aldol product **71** is formed using



Scheme 1.11: Lewis acid-mediated aldol reaction of boron enolate.¹⁵⁵

Table 1.7: Effects of reaction conditions on aldol stereoselectivity.¹⁵⁵

Reaction Sequence	Method A	Method B	Method C
Lewis Acid Used	TiCl ₄	SnCl ₄	TiCl ₄
First	Boron enolate formed	Boron enolate formed	Aldehyde pre-complexed with Lewis acid
Second	Lewis acid added in one portion to enolate	Enolate treated with aldehyde	Boron enolate added by cannula
Third	Aldehyde added over 30 min	Lewis acid added over 3-4 h	—
Total Aldol Yield (%)	77	76	71
Product ratios (71:72:73)	0:11:89	0:92:8	0:17:83

Evans base catalysed methodology, Lewis acid catalysis delivered **72** or **73** as major products depending on reaction conditions.

Another critical factor in the stereochemical outcome of *anti*-aldol reactions is the formation of a relatively tight transition state. This is often attributed in part to the chelating metal ion. Since the metal stabilizes the enolate anion and also acts as a Lewis acid for the carbonyl oxygen it is the metal-oxygen bond length that is of significance in this regard [Table 1.8]. Boron affords a tight transition state because of its relatively shorter bond length of about 1.5-1.6 Å to oxygen compared to other common metals. Lithium is often preferred because lithium enolates are much easier to work with than boron enolates.^{149,156} Under thermodynamic control other metals with relatively long bonds to oxygen also give very good *E* selections. Magnesium and zinc have been reported to produce *anti*-aldol condensation products from ketones.¹⁵⁶

Table 1.8: Metal – oxygen bond lengths.

METAL	Metal-Oxygen Bond Length (Å)
B	1.5-1.6
Li	1.96
Al	1.78-1.99
Mg	1.79
Ti	1.95-1.98
Zr	2.1-2.3

1.7.5 MA Conformational Folding

The conformational folding of MAs is known to be important for their biological activities.¹⁵⁷ Much work has been done to elucidate the exact native folding of the different MA classes.¹⁵⁸⁻¹⁶² Many of these investigations have involved molecular dynamics simulations while others were actual experiments with Langmuir monolayer films of MAs. In the latter group are the experiments of Villeneuve *et al.* which provided some novel data about the folding of the three *M. tb* MA classes.^{161,162} It was shown that the two oxygenated

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MA classes adopted a W-shaped conformation. MeO-MA melted from a 4-chain folded structure assumed at 18 °C to a more stretched out conformation at 32 °C while keto-MA maintained a 4-chain folded form over the same temperature range. The monolayer thickness of the folded keto-MA and MeO-MA were approximately 2.9 nm while the stretched-out MeO-MA was about 5.0 nm.¹⁶² Although a clear decision on the folding pattern of α -MA was not made by Villeneuve and co-workers at the time the fact that it formed a liquid film over the temperature and pressure ranges studied pointed to a stretched conformation. In 2010 they reported that α -MA does adopt a four-chain folded conformation at lower temperatures and surface pressures.¹⁶³ The extended conformation was observed at higher than 20 °C. More interestingly they showed that irrespective of the conformation adopted by α -MAs these molecules formed fluid monolayers.

Molecular dynamics computer modeling of the *in vacuo* folding patterns of the MAs remarkably showed that all three MA classes adopted W-, U-, and Z-shaped conformations when studied as single molecules.¹⁶⁰ The α -MA showed the highest frequency of folding into any of the three structures but keto-MA was more rapid in folding.

1.7.6 Functionalizing Mycolic Acids

Mycobacterial MA are antigenic molecules.⁵⁷ This implies that they have restricted three dimensional folds optimized by specific inter- and intra-molecular interactions of their functional groups. In attempting to design more stable and reproducible devices, it was thought to be important to bind MA to surfaces, such as gold surfaces, to control their presentation to antibodies. Any attempt at functionalizing these molecules must avoid disrupting the intricate native folded structural balance to maintain the biological activity. This is a challenge because of the large diversity in functional groups, chain lengths and stereochemistries.

The oxygenated functionalities of free MA are recognized in a combinatorial epitope by T-cell receptors [Figure 1.11].¹⁵⁷ The combinatorial epitope is formed when the polar functional groups are brought into close interaction as a result of the binding of the hydrophobic alkyl segments of the MA chain with CD1b. MA methyl esters were not recognized by T-cells. The extended hydrophobic alkyl chain segments resided in a pocket

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on the presenting CD1b molecule and do not seem to be involved in the recognition process.

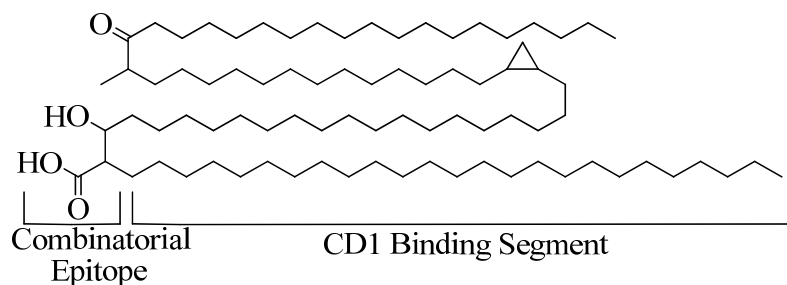
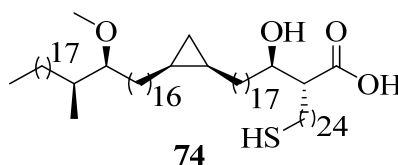


Figure 1.11: T-cell antigen receptor-binding fold of mycolic acids.¹⁵⁷

CD1b could be mimicked with a chemically inert surface like gold to which a functionalized MA is tethered covalently. The most convenient means of binding the MA to the gold surface is via a thiol on the terminal position of the CD1b binding segment (the α -chain).

1.8 Hypothesis

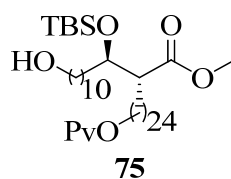
A chemically synthetic MA derivative **74** can be constructed for covalent attachment to gold on a sensor surface, with the eventual aim of applying this in a simple, rapid biosensor TB diagnostic that is not restricted to sputum sample analysis.



1.9 Aims

The aims of this project are to:

1. Synthesize a mycolic motif analogue **75** as a single stereoisomer with a functionalized α -alkyl chain that can be incorporated in the synthesis of **74**.



2. Scale up and economize an aldol reaction in a potential synthesis of the mycolic motif.
3. Synthesize a stereochemically controlled thiolated MeO-MA **74**.
4. Create a SAM of thiolated MeO-MA **74** on a gold substrate.
5. Characterize MeO-MA SAM by physical and electrochemical analytical techniques.

2 Chapter Two: Aldol Synthesis of a Mycolic Motif Analogue

2.1 Introduction

A more efficient synthesis of the mycolic motif is seen as critical in developing routes to mycolic acids that are suitable for scale up. The biosynthesis of MA involves a Claisen-type condensation to link two fragments [**Figure 2.1**]. A widely studied reaction that achieves a similar outcome is the aldol process. Use of an aldol reaction to prepare the *anti*-*R,R* configuration of the mycolic motif was explored by Driver and found to show promise.¹⁶⁴ This Chapter reports developments of that work and a comparison with the processes currently used.¹⁴⁴

2.1.1 The Claisen Reaction in the Biosynthesis of Mycolic Acids

The *M.tb* MAs' alpha alkyl side chains are often 22 or 24 carbons long. The fatty acid synthase enzyme, FAS I, synthesizes the precursors, tetracosanoyl (C₂₄) and hexacosanoyl (C₂₆), bound to the acyl carrier protein, ACP. ACP then transfers the chain to coenzyme A, where it is carboxylated at C-2. The carboxyl is later released as CO₂.¹⁶⁵

The coenzyme A derivatives of FAS I and the AMP-activated meroacyl FAS II products react in a Claisen-type condensation reaction catalyzed by polyketide synthase 13 (PKS 13).¹⁶⁵ This creates an oxo-group which is reduced to the β -OH group of the MA molecule [**Figure 2.1**]. An *in vitro* mimicry of this biological process was a major aim of this project and has been reported, including results obtained in this Chapter.¹⁶⁶

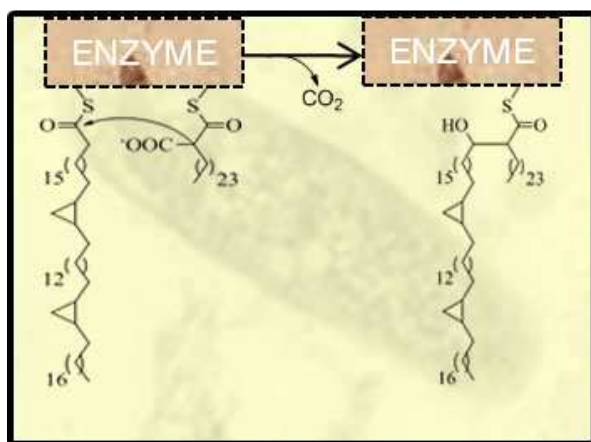


Figure 2.1: Claisen-type condensation and reduction to synthesize MA.¹⁶⁵

2.1.2 Significance for Mycolic Motif Synthesis

An *anti*-aldol reaction is obviously attractive for mycolic motif synthesis because of its powerful ability to generate the alpha and beta stereogenic centres simultaneously. To be successful it must produce a molecule with *R,R* absolute configuration at the C-2 alkyl and C-3 hydroxyl positions in high enantiomeric purity.

Walker and Heathcock used an Evans oxazolidinone chiral auxiliary **68** with an excess of the Lewis catalyst Et₂AlCl to synthesize an *anti*-aldol adduct stereoselectively in high yields [Scheme 1.11] [Table 1.7].¹⁵⁵ The aldehyde approaches the *Z*-boron enolate diastereoselectively because the *iso*-propyl substituent of the auxiliary hinders one of the faces. The ‘open’ transition state in which the aldehyde oxygen coordinates with the aluminum Lewis acid instead of the boron allows the C-2 alkyl and C-3 OH to orientate in an *anti*-relationship.¹⁵⁵ Compound **72** had the wrong configuration for the mycolic motif. This ruled out using this auxiliary for the aldol synthesis of the mycolic motif.

Kurosu and Lorca reported the use of a norephedrine-based chiral auxiliary in the synthesis of *anti*-aldol adducts.¹⁶⁷ This method has some significant advantages. It was reported to work remarkably with lithium enolates under kinetic control, hence eliminating the need for a Lewis acid catalyst to make *anti*-aldol compounds of esters. The diastereoselectivity in favor of the *R,R anti*-diastereomer was enhanced by the use of Cp₂ZrCl₂ to *trans*-metalate

the lithium enolate intermediate. It also yielded the correct *R,R* *anti*-configuration required for mycolic motif synthesis.

The norephedrine-based chiral auxiliary **76** provided very sterically congested chiral esters. The mesityl group is believed to be orientated opposite to the propionate in the least congested space around the ester. The other two aromatic groups jostle for space with the bulky mesityl and, cooperatively, with the cyclopentadienyl ligands of the zirconium metal effectively restrict access from the *re*-face of the metal enolate. The *R,R*-*anti*/*syn* selectivities of 90-95 : 10-5 of the products obtained with several different aldehydes were very impressive.¹⁶⁷

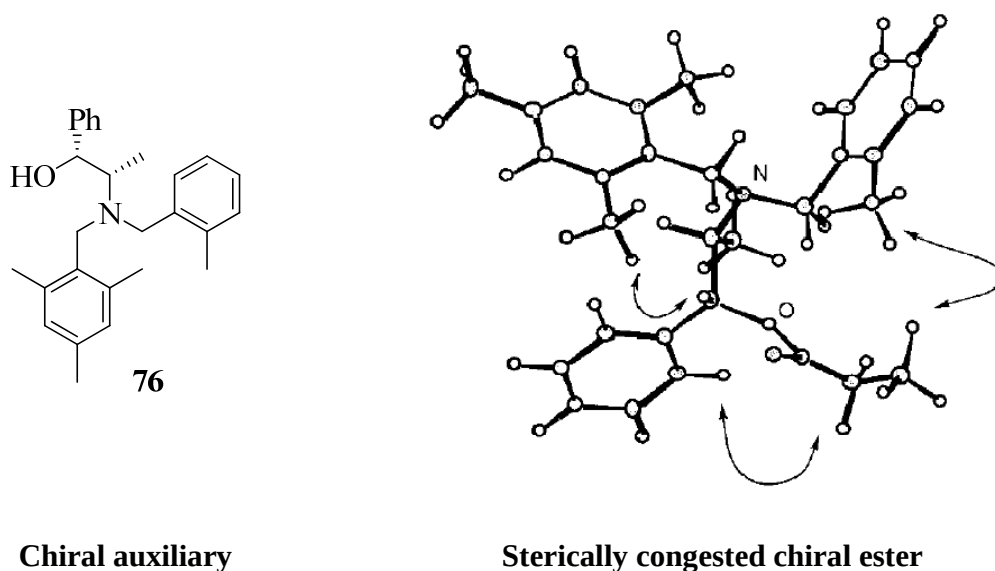


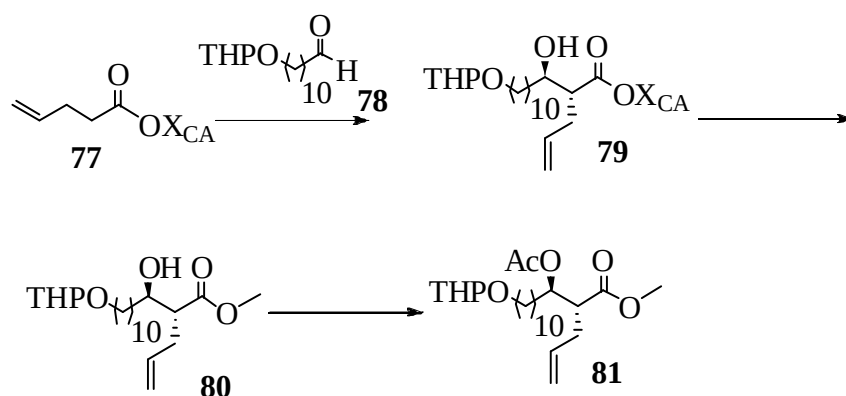
Figure 2.2: The chiral auxiliary **76** and chiral ester of Kurosu and Lorca.¹⁶⁷

This protocol of *anti*-aldol synthesis presented by Kurosu and Lorca was found attractive to apply in the synthesis of the stereogenic centres of the mycolic motif. The reagents required could be obtained relatively cheaply in bulk and are easy to work with.

2.1.3 Mycolic Motif Synthesis via an *Anti*-Aldol Reaction

Application of aldol chemistry in the synthesis of the mycolic motif was first undertaken by Driver.¹⁶⁴ After investigating several chiral esters and aldehydes the allyl ester **77** and the aldehyde **78** were found to give the best results. However, the practical application of the process was threatened by low yield and difficulty in scaling up. This provided the primary challenge for the current project.

The work of Driver established the strategy for the synthesis and proved, in principle, that the approach does produce the desired *anti*-2*R*,3*R* enantiomer in excellent enantiomeric purity [**Scheme 2.1**]. The results of her work, with significant contributions from this present project, have been published.¹⁶⁶ A detail discussion of this contribution is presented below.

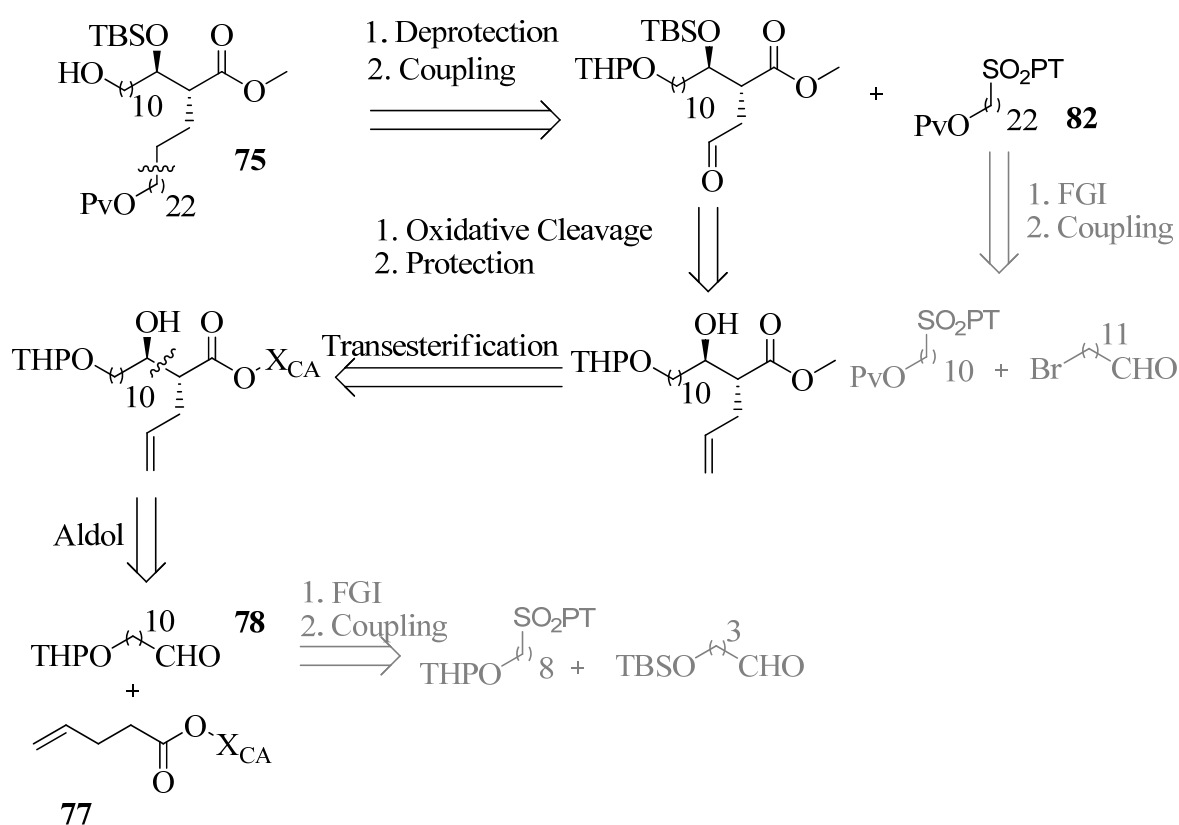


Scheme 2.1¹⁶⁴

2.2 Results and Discussion

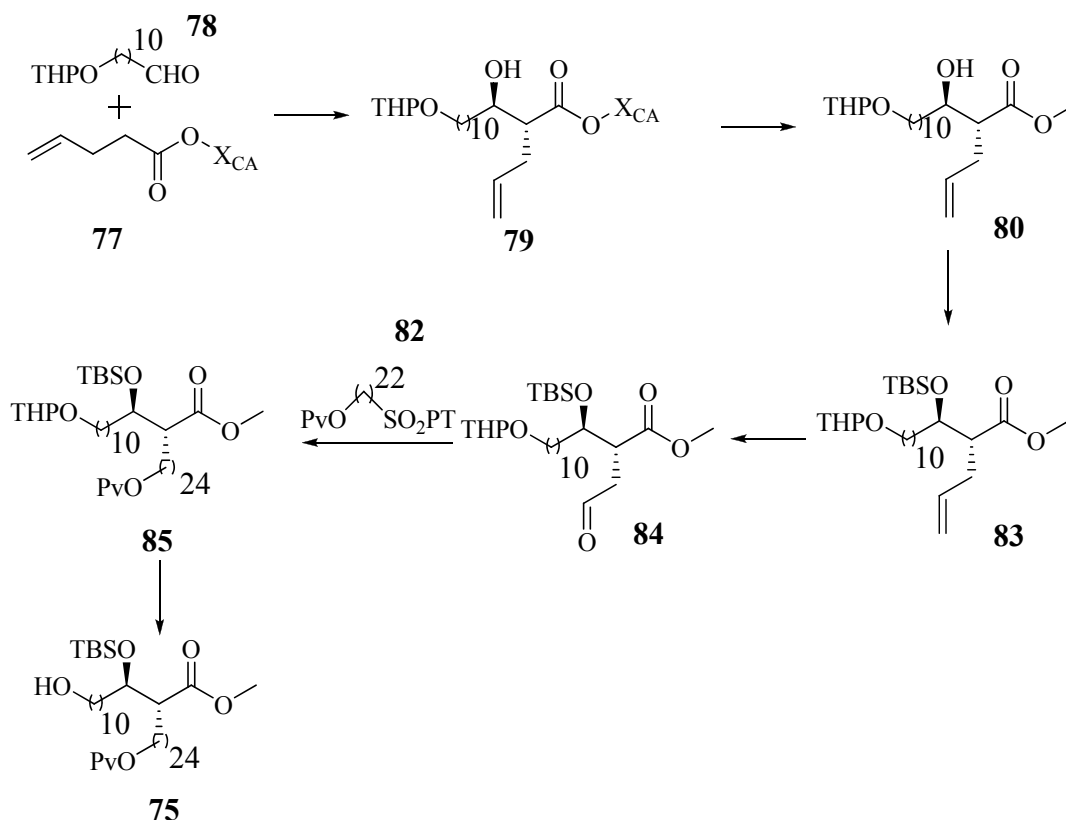
The general retrosynthetic strategy for the synthesis of the target mycolic motif analogue **75** is outlined in **Scheme 2.2** and the corresponding forward synthesis is presented in **Scheme 2.3**.

The synthesis is described in detail in the following sections, and involved a number of key processes. Two alkyl chain segments were synthesized at the onset according to standard chemistry. The carbonyl partner in the aldol reaction was a C₁₁ aldehyde **78**. This was synthesized from C₈ and C₃ diol precursors. Similarly, the C₂₂ α -alkyl chain extension **82** of the aldol allyl product **80** was synthesized from C₁₀ and C₁₂ diol precursors.



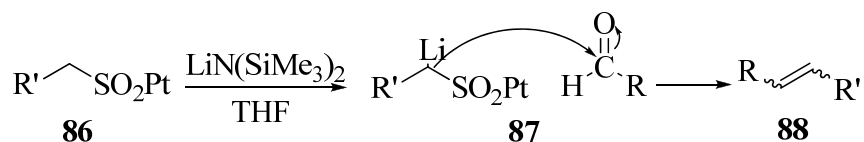
Scheme 2.2

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Scheme 2.3

A number of the steps in the sequence involved the extension of an alkyl chain. The Julia-Kocienski olefination is a popular method for the synthesis of alkenes because of its high *E/Z* selectivity and high yields under relatively mild reaction conditions [**Scheme 2.4**]. For the latter reason it was adopted for all chain extensions in this project without any attempt at stereo-control or stereo-selection because the alkenes were immediately saturated in the next reaction.

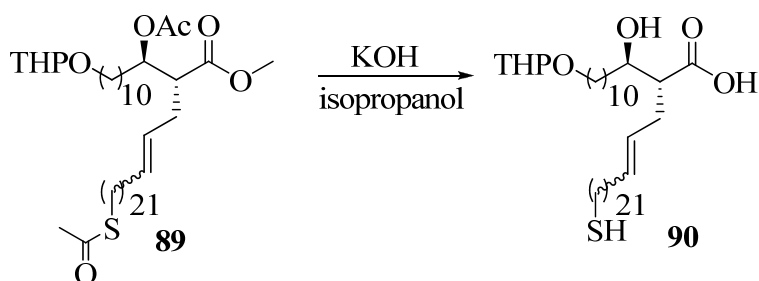


Scheme 2.4

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In this way, the α -alkyl chain of the aldol product **79** was extended to the full length C₂₄ alkyl chain, functionalized at its terminal carbon, by coupling the aldehyde product **84** of oxidative cleavage of the allyl **80** to a C₂₂ alkyl sulfone **83**.

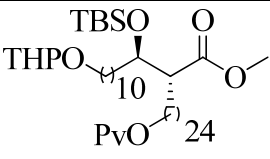
The synthesis used a number of protecting groups. In her work Driver used an acetate protecting group on the β -hydroxy group as in **81**, similar to the strategy adopted in previous mycolic motif synthesis.^{132,137} The aim of Driver was to carry out a universal deprotection of a fully-extended thiolated motif **89** in one reaction step [Scheme 2.5]. This had a limited success and a different protecting group system was adopted in this project.



Scheme 2.5¹⁶⁴

In the current work, an alternative strategy was adopted in which the four protecting groups shown in **Table 2.1** were used, to be removed in the sequence presented.

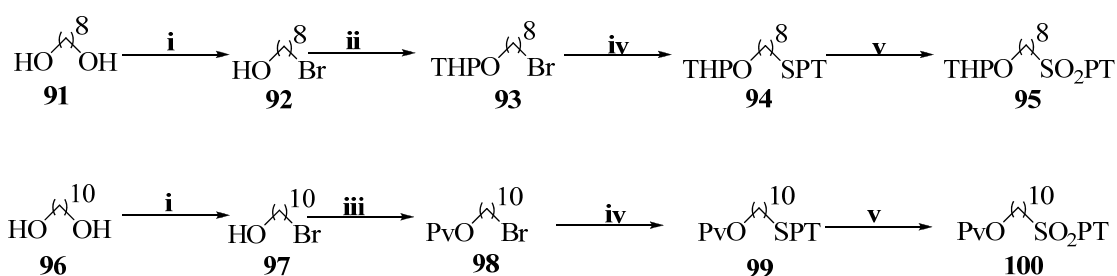
Table 2.1: Protecting group strategy for mycolic motif synthesis.

 85		
Protecting Group	Order of Cleavage	Cleavage Reagent
THP	1 st	PPTS, MeOH, EtOH
PvO	2 nd	KOH, MeOH
TBS	3 rd	HF• Pyridine, Pyridine
Me	4 th	LiOH; TBAH

The detailed synthesis is now presented.

2.2.1 Synthesis of C₈ and C₁₀ Phenyl Tetrazoyl Sulfones

1,8-Octanediol **91** was mono-brominated using HBr (48%) in high yield to afford **92** [Scheme 2.6]. The highest yield was obtained using 2.5 mol. eq. of HBr. With less HBr large amounts of diol were recovered whereas with a greater excess larger amounts of dibromide were obtained. The hydroxyl was protected with 3,4-dihydropyran under standard conditions to form the bromo-acetal **93**. The tetrahydropyran protecting group is acid labile and comes off on a silica gel column. This necessitated that the silica column was neutralized with triethylamine. The bromo group was then substituted with a 1-phenyl-1*H*-tetrazole-5-thiol to yield **94**. Oxidation of the sulfide with mCPBA afforded the sulfone **95** in 83-85% yield. A second method for oxidation of the sulfide was also used occasionally: a solution of ammonium heptamolybdate (VI) tetrahydrate in 35% hydrogen peroxide was added in small portions to a solution of sulfide **94** in industrial methylated spirit (IMS).¹³⁸ Working up this method was less cumbersome than the mCPBA method although both gave similar yields. The mCPBA route cost less.



Scheme 2.6: **i.** HBr, toluene, reflux, 48 h, 78-82%; **ii.** Dihydropyran, pyridinium-*p*-toluene sulfonate, CH₂Cl₂, r.t., overnight, 89%; **iii.** Pivaloyl chloride, Et₃N, CH₂Cl₂, r.t., 18 h, 93%; **iv.** 1-phenyl-1*H*-tetrazole-5-thiol, K₂CO₃, acetone, reflux→r.t., overnight, 82-87%; **v.** mCPBA, CH₂Cl₂, r.t., 83-85%.

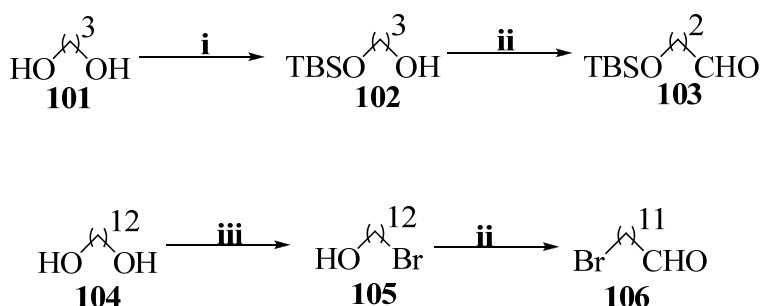
1,10-Decanediol **96** was similarly brominated, protected and sulfonated [Scheme 2.6] but with a different protecting group. A base-sensitive trimethylacetate protecting group on the alcohol **98** would be carried through the synthesis to allow discrimination in the deprotection of the primary alcohols of the mycolic motif **85**. This was efficiently introduced by reaction of the bromo-alcohol **97** with pivaloyl chloride and triethylamine.

All the reactions to synthesize the protected sulfones **95** and **100** gave favorable yields. Yields ranged between 80 and 85% even for multi-gram scaled up reactions. The reaction conditions were mild and product purifications were uncomplicated.

In order to proceed with the synthesis, the two sulfones **95** and **100** next needed to be chain extended to give the aldehyde **78** and the sulfone **82** respectively. C₃ and C₁₂ aldehyde linkers **103** and **106** were required.

2.2.2 Syntheses of C₃ and C₁₂ Aldehydes

Aldehydes are often the electrophiles of the Julia reactions [Scheme 2.4]. The sulfone makes the proton on the α-carbon highly acidic and hence easily abstracted by strong bases to give the nucleophilic anion for the Julia coupling.



Scheme 2.7: **i.** NaH, TBSCl, THF, r.t., 54%; **ii.** PCC, CH₂Cl₂, r.t., 2 h, 78-89%; **iii.** HBr, toluene, reflux, 48 h, 76%.

1,3-Propanediol **101** was monosilylated with TBSCl to give the protected alcohol **102** in moderate yield. This was oxidized to the aldehyde **103** with PCC. This aldehyde was a bit of a challenge to concentrate as it is quite volatile and this affected the yield. Hexane/ether eluant was used in column purification of the product and exposure to reduced pressure was carefully controlled using only a cold water bath. On average, moderate yields of about 78% were obtained due to loss during the concentration processes and some solvent remained. This unstable aldehyde was used immediately as described in the next section and was not fully characterised.

To avoid the use of such a volatile C₃ intermediate another chain combination of C₅ and C₆ might be considered for future C₁₁ synthesis. If another MA homologue is targeted for synthesis or the linker chain of the synthetic meromycolyl moiety is of a different length it may be possible to purchase a diol of suitable length so that the formation of precursors and Julia coupling will be completely avoided.

The 1,12-dodecanediol **104** was brominated as described for compounds **91** and **96**. The bromoalcohol **105** was oxidized with PCC to the aldehyde **106** with 89% yield. Again, the aldehyde was used immediately.

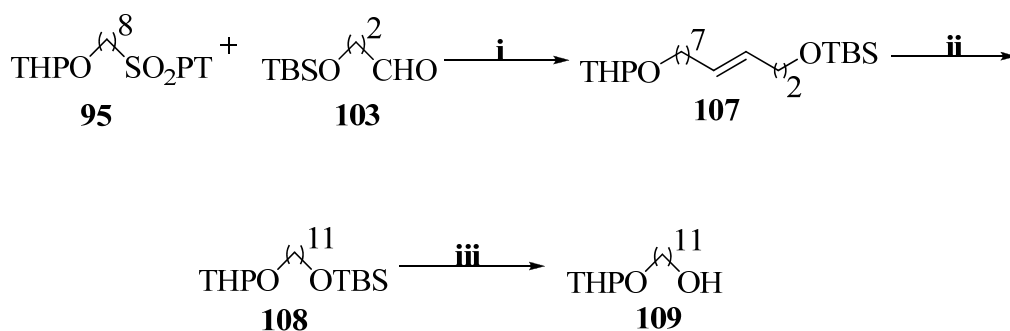
2.2.3 Modified Julia-Kocienski Coupling of Alkyl Chains¹⁶⁸⁻¹⁷⁰

The C₈ and C₁₀ sulfones (**95**, **100**) were coupled to the C₃ and C₁₂ aldehydes (**103**, **106**), respectively, in modified Julia-Kocienski reactions using lithium bis(trimethylsilyl)amide

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as base to give alkenes **107** and **110**. To prevent undesirable polymerization of the sulfones, the base was added to a stirred solution of the sulfone and aldehyde at 0 °C. The temperature was then allowed to reach room temperature. Although the reaction of the aldehyde with the sulfone was reported to be fast, some starting material was observed after an hour and it was decided to leave the reaction overnight. There was then very little aldehyde observed on the TLC. The *E/Z* alkene products were easily purified by standard work-up followed by silica column chromatography eluting with hexane : ethyl acetate in a 10 : 1 ratio. The coupling of C₁₀ and C₁₂ fragments gave a much better yield (84-90%) than the C₈ to C₃ coupling (77-80%). This might be due to the volatility of the C₃ aldehyde which prevented full evaporation of the solvent and meant less aldehyde would be in the reaction solution than anticipated. The proton and carbon NMR spectra of these products were each complicated due to the presence of *E/Z*-diastereoisomers, but the alkene groups could be clearly seen in the region $\delta = 4-5$ ppm.

After hydrogenation of **107** using 10% palladium on carbon at a pressure of 2.4 atmospheres overnight the alkene signals completely disappeared. The structure of the pure product was confirmed by proton and carbon NMR spectroscopy.



Scheme 2.8: **i.** LiN(SiMe₃)₂, THF, r.t., overnight, 77-80%; **ii.** 10% Pd/C, hexane/ ethyl acetate, H₂, overnight, 100%; **iii.** Pyridinium-*p*-toluene sulfonate, MeOH/ EtOH/ H₂O, r.t., 8 h, 63%.

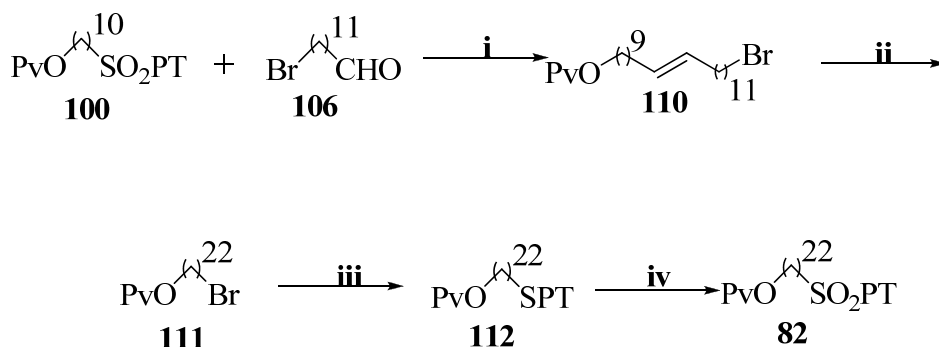
In the case of **108**, the ¹H NMR signals of the (CH₃)₂Si group of the TBS and OCHO of the THP protecting groups at 0.02 ppm and 4.55-4.56 ppm respectively indicated that the diprotected C₁₁ chain **108** had been obtained. That all double bonds had been saturated was confirmed by the absence of any signal in the alkene region. The carbon NMR signals for

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the $(\text{CH}_3)_2\text{Si}$ at -5.3 ppm and the acetal carbon (OCHO) of the THP at 98.8 ppm confirmed the formation of the desired product; no alkene signals were observed.

The diprotected compound **108** was desilylated by stirring in a MeOH/ EtOH solution with a catalytic amount of PPTS (0.1 mol. eq.) for 8 h. This reaction had to be carefully monitored to limit the simultaneous cleavage of the THP group. A moderate yield of 55% of **109** was obtained and remaining starting material **108** was recovered and the reaction repeated. With the repetition the total yield of **109** was 63%. TLC showed a faster running spot just above the THP-alcohol **109** which was suspected to be the mono-deprotected alcohol in which the THP, rather than the TBS, had been cleaved. The proton NMR spectrum of **109** confirmed the presence of the desired alcohol **109** by the disappearance of the $(\text{CH}_3)_2\text{Si}$ group signal but the retention of the acetal (OCHO) multiplet at 4.52-4.54 ppm. The silyl protecting group's carbons were absent in the ^{13}C NMR spectrum.

In the same way, the bromide **110** was hydrogenated as before at 2.4 atm over palladium 10% on carbon to give **111**. It was however observed that hydrogenation at 1 atm led rapidly to debromination if left for more than 2 h.



Scheme 2.9: **i.** $\text{LiN}(\text{SiMe}_3)_2$, THF, r.t., overnight, 84-90%; **ii.** 10% Pd/ C, hexane/ ethyl acetate, H_2 , overnight, 100%; **iii.** 1-phenyl-1H-tetrazole-5-thiol, K_2CO_3 , acetone, reflux $\rightarrow$ r.t., overnight, 82%; **iv.** mCPBA, CH_2Cl_2 , r.t., 85%.

The structure of the C_{22} chain **111** was again confirmed by NMR analysis. The CH_2Br at 3.38 ppm with a coupling constant for the C_1 and C_2 protons of 6.9 Hz and the CH_2OPv at 4.02 ppm were indicators of the desired compound. Furthermore, the sharp singlet of $(\text{CH}_3)_3\text{CCO}$ of the trimethylacetate protecting group at 1.17 ppm integrating for 9H

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supported the structure of the compound. That all alkenes were fully hydrogenated was confirmed as above by the absence of any double bond signals in both the ^1H and ^{13}C NMR spectra. The most significant diagnostic ^{13}C NMR peak was the carbonyl carbon at 178.5 ppm.

The protected C_{22} bromo-alkyl chain **111** was converted into a sulfonyl phenyltetrazole as described above for **93** and **98**. The white solid obtained, **82** (m.p. = 57 °C), was easily purified on a silica column eluting with warm hexane/ethyl acetate solution. The oxidation of the sulfide **112** to the sulfone **82** was supported by the shift in R_f from 0.47 to 0.44 with hexane/ ethyl acetate (8 : 2). The most significant ^1H NMR signal shift was of the sulfide methylene downfield from 3.35 ppm to 3.71 ppm in the sulfone.

2.2.4 The Synthesis of the Aldol Chiral Ester **77**

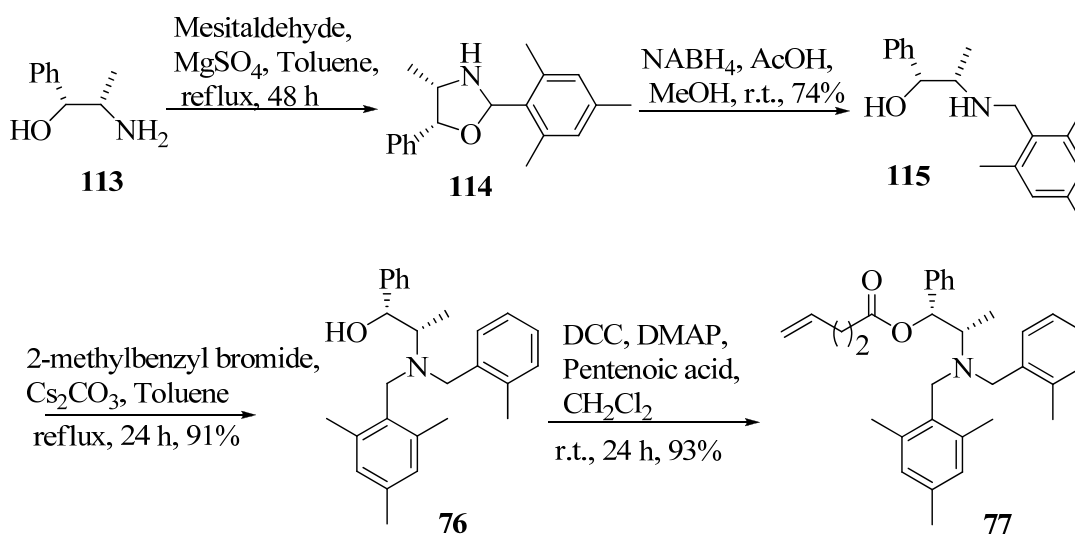
The amine **115** was synthesized from (-)-norephedrine **113** and mesitaldehyde in refluxing toluene in 74% yield [**Scheme 2.10**]. Anhydrous MgSO_4 was used *in situ* to remove water to drive the equilibrium towards the unstable oxazolidine intermediate **114**. Reduction of **114** with NaBH_4 in the presence of acetic acid afforded the amine **115**. *N*-benzylation of the amine **115** by refluxing with 2-methylbenzyl bromide in the presence of caesium carbonate yielded the chiral auxiliary **76**. The ^1H and ^{13}C NMR spectra of this correlated well with the data reported by Kurosu and Lorca.¹⁶⁷

Esterification of the alcohol **76** with pentenoic acid was achieved in 24 h with a 93% yield using DCC and DMAP under standard conditions [**Scheme 2.10**]. The allyl methylene and methine proton signals of **76** were observed as multiplets at 4.84-4.98 ppm and 5.62-5.75 ppm, respectively. The single proton of the secondary alcohol carbon gave a significant peak shift from 4.74 ppm in the alcohol **76** to 5.96 ppm in the ester **77**. The ^{13}C NMR spectrum also confirmed the structure of the desired ester by the appearance of the quaternary carbon signal at 172.2 ppm.

Chiral ester **77** and its precursor intermediates **115**, **76** were readily purified by column chromatography. The viscous chiral auxiliary **76** and ester **77** were stable under storage at

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room temperature for prolonged periods. The chiral auxiliary moiety will be represented as X_{CA} in the esters of the aldol reaction and products described in the subsequent sections.

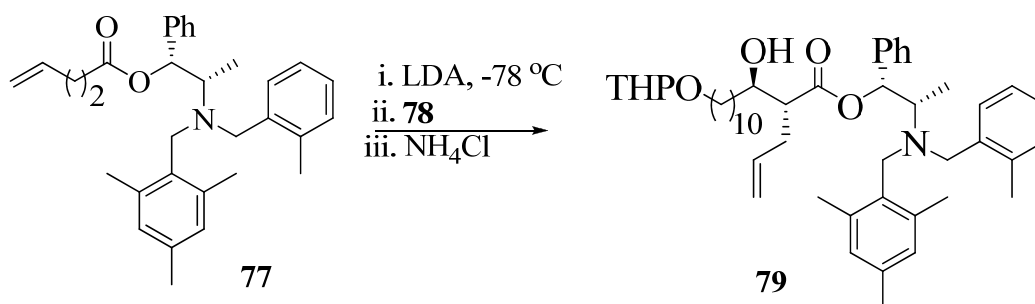


Scheme 2.10

2.2.5 The Aldol Reaction to Synthesize 2*R*,3*R* Stereogenic Centres

The *anti*-aldol reaction was initially performed as described by Driver with C_{11} aldehyde **78** and chiral ester **77**.¹⁶⁴ Commercial LDA (7.0 mol. eq.) was added to a stirred solution of Cp_2ZrCl_2 (0.3 mol. eq.) in THF -78 °C under N_2 . The chiral ester **77** (1.0 mol. eq.) was added over about 1 h in THF and stirred for 2.5 h after which additional Cp_2ZrCl_2 (3.0 mol. eq.) was added over 1 h. The reaction was stirred for 45 min after which the aldehyde **78** (1.1 mol. eq.), dissolved in THF, was added over 45 min. After 2.5-3 h, the mixture was allowed to reach about -10 °C, and then quenched with 1 M HCl.

The *anti*- and *syn*- diastereomers were obtained with R_f 0.38 and 0.25 in hexane : ether (7 : 3), respectively. Unreacted aldehyde **78** was also observed, with an R_f between those of the products. The products were readily separated from each other by flash column chromatography, but central fractions contained some aldehyde. The yield of the *anti*-aldol product **79** was 29%.

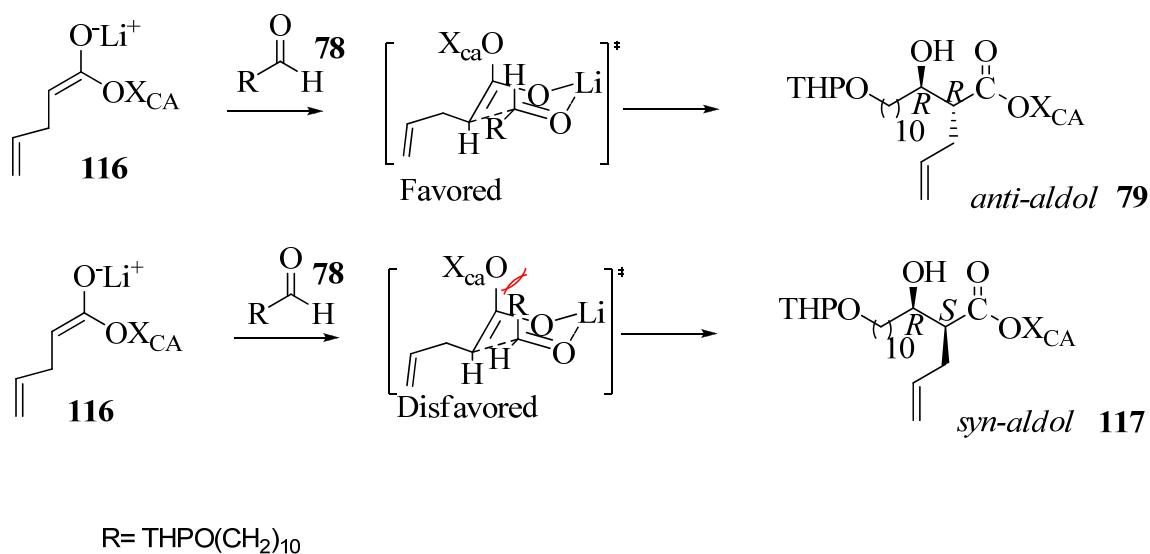


Scheme 2.11

In this initial procedure, the enolate **116** was made by slowly adding the chiral ester **77** to a stirred solution of LDA in THF at -78 °C. A dry ice-in-acetone bath was used to achieve this temperature although it was suspected that the temperature within the reaction vessel was not constant during the reaction period and this was not directly monitored at this stage. Enolate formation and stability is very temperature sensitive and this must be maintained in a tight range.¹⁶⁷

Addition of the electrophilic aldehyde **78** to the Li⁺-stabilized enolates **116** yielded the aldol adduct **79**. The mechanism of this process is believed to proceed via a six-membered chair conformation transition state proposed by Zimmerman and Traxler in 1957.¹⁵⁰ The possibility of the aldehyde extending its chain in two orientations for proper coordination with the Li⁺ counterion means that *E* and *Z* configurations of enolates can have favored and disfavored states [**Scheme 2.12**]. The enolate itself does not have a choice of orientations, hence the favored state in both cases will be with the aldehyde H axial and *syn* to the chiral auxiliary.

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Scheme 2.12

The ¹H and ¹³C NMR spectra of **79** were identical to those reported by Driver who had used Mosher ester derivatization of the methylated aldol product **80**, obtained by transesterification, to confirm that the β-OH was in the *R* configuration.¹⁶⁴ The α_D of +3.3 for the Me-aldol **80** closely matched the value of +3.1 reported in the literature by Toschi and Baird.¹³⁴ The ¹³C NMR shifts of the vinyl vicinal carbons were identical to the published data [Table 2.2].¹³⁴

Table 2.2: ¹³C NMR shifts of the vinyl vicinal carbons.



Carbon	80	<i>epi</i> - 80	<i>anti</i> - 49
H <u>C</u> =CH ₂	134.9	135.6	134.9
HC= <u>C</u> H ₂	117.1	116.6	117.1

The methine carbon of the allyl group in the aldol product **79** gave a signal at 134.9 ppm, while the vinyl methylene had a signal at 117.1 ppm. Although the β-carbon proton signal

could not be defined due to the presence of the THP signal in the same region (3.78-3.88 ppm) of the spectrum, the α -carbon proton multiplet was easily resolved between 2.48-2.56 ppm.

Driver's investigation of the stereochemical nature of the aldol products demonstrated that the major diastereomeric product was the desired *anti*-2*R*,3*R* compound.¹⁶⁴ It did not however rule out the presence of the *anti*-2*S*,3*S* enantiomer as a minor component or what the stereochemical make-up of the minor *syn*-diastereomer was. This project confirmed the enantiomeric purity of both the products by ¹⁹F NMR analysis of the Mosher's ester derivatives.

Mosher's derivatization is often used to determine the absolute configuration of secondary alcohols.¹⁷¹ α -Methoxy- α -(trifluoromethyl)phenylacetic acid (MTPA), as either the *R*- or *S*-enantiomer, is converted to the acid chloride and reacted with the alcohol to obtain the respective esters as two diastereomers. The changes in the chemical shifts of as many of the protons of the *R*- and *S*-esters as possible are determined from the ¹H NMR spectra. Using a model described by Ohtani *et al.* the absolute configuration of the stereogenic centre can be assigned.¹⁷² Another useful aspect of the Mosher's ester analysis, and important to this project, is that the fluorinated Mosher's esters can be analysed by ¹⁹F NMR to conveniently determine the enantiomeric purity of the compound.

Using this, the *S*-Mosher's ester of both diastereomeric products of the aldol reaction were analysed. It must be pointed out that the methylated products of transesterification of the aldol diastereomers were used. The model described by Mosher and modified by Ohtani *et al.* cannot be applied to a sterically hindered alcohol^{171,172} For this reason the chiral auxiliary had to be cleaved before Mosher's ester derivatization. The transesterification method will be described in detail in **Section 2.2.6**.

The ¹⁹F NMR of the Mosher's ester of **80** gave a single peak for the *anti*-aldol product at -71 ppm confirming a greater than 98% enantiomeric excess. The methylated *syn*-diastereomers showed two strong peaks indicative of a mixture of two *syn*-aldol products **117** and **118** in a 2 : 1 ratio in the *syn*-aldol diastereomers [**Figure 2.3**, **Figure 2.4**]. This conclusion was further supported by the duplication of the alkene peaks in the ¹³C spectrum of the *syn*-diastereomer prior to transesterification.



Figure 2.3: Aldol syn-enantiomers.

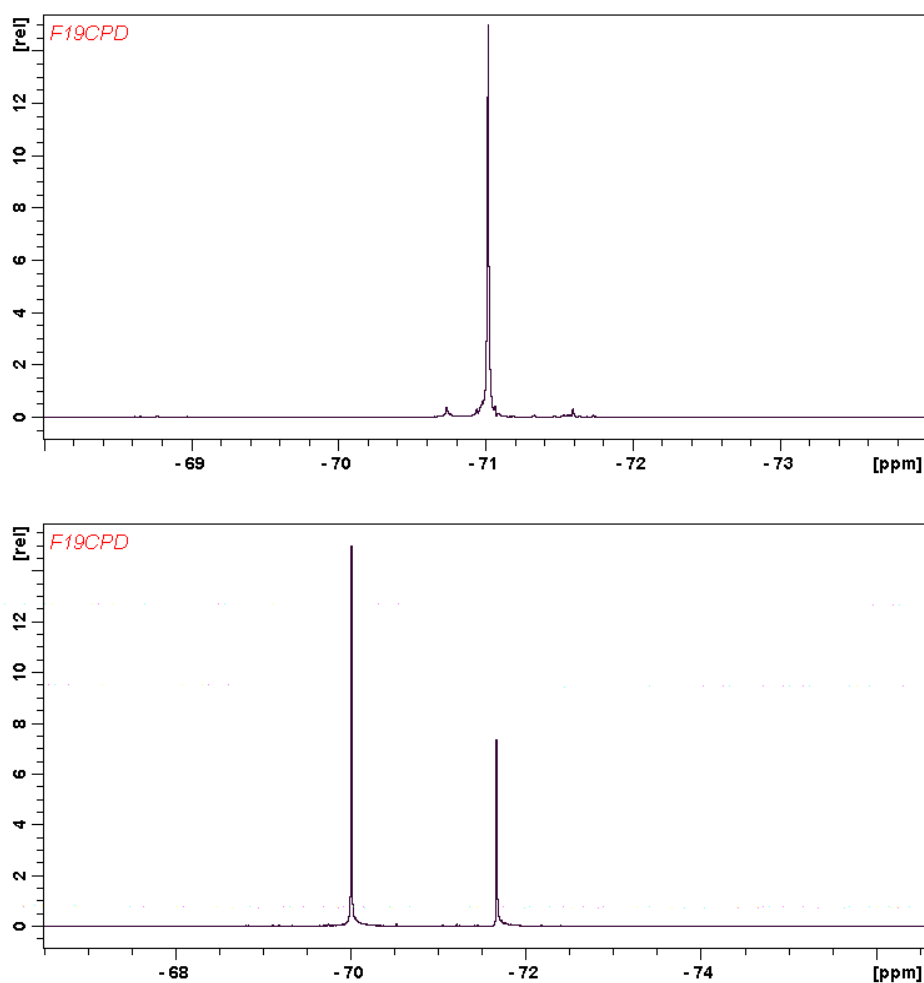


Figure 2.4: ^{19}F NMR spectra of: **A.** *Anti*-aldol product. **B.** *Syn*-aldol products.

In preliminary work by Driver the best yield from chiral ester **77** was 35% for the *anti*-aldol product **79** on 200 mg of the ester and 25% when the scale was doubled.¹⁶⁴ No product was obtained when the reaction was attempted on a 1.0 g scale. The low yield in this process

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was even more disappointing given the excellent yields reported for other reactions with the same chiral auxiliary **76**.¹⁶⁷ It had been predicted that the steric hindrance created by the bulky auxiliary would almost completely exclude one diastereomer and result in good yields of the *anti*-compound **79**, yet the *syn*-isomers made up as much as 40% of the total aldol products. In order to try to overcome this problem, it was decided to carry out a meticulous control of the experimental conditions. To do this several aspects of the experimental process were carefully investigated. These included:

1. Control of temperature,
2. Investigation of LDA integrity,
3. Investigation of co-catalyst,
4. Changing the limiting reagent,
5. Purity of reactants,
6. Equivalents of LDA used.

2.2.5.1 Temperature control

For kinetic control, the temperature in the reaction had to be maintained at -78 °C with minimal variation. Using a dry ice/ acetone cooling bath the temperature of reaction solution, monitored by an internal temperature probe, rose by as much as 10 °C. This problem was overcome by using an electronic cooling bath with which the reaction temperature range was successfully restricted to between -75.4 °C and -80.5 °C and an average of -78.4 °C over the reaction period.

It was observed that the temperature did not fluctuate significantly when reagents were added slowly such that two drops of a reagent from a syringe were not added in quick succession. This particularly applied to the addition of the solution of chiral ester **77** in THF. Provided two drops were not added too close together, the yield of the reaction remained similar to what was obtained before the acquisition of the electronic cooling bath system.

2.2.5.2 The Nature of the Base

Next, doubt was cast on the integrity of the commercially supplied LDA. Attempts were not made to titrate the commercial base but instead it was decided to make a fresh preparation at the time of use from re-distilled diisopropylamine and n-butyl lithium. This was titrated against diphenylacetic acid and the exact concentration was determined. The new base was used as described by Kurosu and Lorca on 250 mg of chiral ester **77**.¹⁶⁷ This, coupled with the electronic cooling bath, gave a significantly better result. The desired *anti*-product **79** yield was 45% with 80 : 20 selectivity against the *syn*-diastereomer. This was more encouraging, and reproducible, but still not comparable to the literature values.

A threefold scaling up of the process using both freshly prepared LDA and good temperature control gave mixed results. In the first experiment, the only difference from the reaction above was that the addition times were doubled rather than trebled. This experiment completely failed. It was concluded that the failure might be attributable to the addition times; however, later experiments also indicated that the dryness of the substrates might have been an issue. The second experiment used the same molar equivalents of reagents but half the THF solvent in preparing the LDA. This gave 25% yield of **77**.

The process was further scaled up to 1 g using fresh chiral ester **77** and ester recovered from previous reactions in separate experiments. In both cases the aldehyde was used in 1.1 mol. eq. excess of the ester **77**. The concentration of the LDA prepared was also doubled to 0.96 M but still used as a 7 mol. eq. of the chiral ester **77**. The yields of **79** in both cases were similar, approximately 45%.

2.2.5.3 The Nature of the Catalyst

The cyclopentadienyl zirconium dichloride, Cp_2ZrCl_2 , was introduced as a co-catalyst to transmetalate the lithium enolate. According to Kurosu and Lorca this improved the diastereoselectivity of the process.¹⁶⁷ In her work, Driver reported that only Cp_2ZrCl_2 from a particular manufacturer worked in the aldol process.¹⁶⁴ This was investigated and found not to be so. Three experiments to investigate this were setup. One experiment used Cp_2ZrCl_2 from Fluka (the manufacturer of the Cp_2ZrCl_2 used by Driver); a second

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experiment used Cp_2ZrCl_2 from Merck, while a third experiment used no Cp_2ZrCl_2 . These experiments were conducted simultaneously and all reagents, except the Cp_2ZrCl_2 , were aliquoted from the same stock. These three experiments were conducted using the original Driver conditions but with improved temperature control and freshly made LDA. All three experiments gave comparable yields with similar diastereomeric selectivity. The Fluka Cp_2ZrCl_2 experiment gave 25% of **77**; the Merck Cp_2ZrCl_2 experiment gave 30% while the experiment without Cp_2ZrCl_2 gave 29%. The Merck Cp_2ZrCl_2 gave slightly the better yield probably because it was obtained from a freshly opened bottle while the Fluka Cp_2ZrCl_2 had been in use for about two years. Both zirconium experiments gave a 4 to 1 selectivity for the *anti*-aldol product while the third experiment gave a 1 to 1 selectivity. The rather poor yields of these experiments were probably due to the difficulty, even inefficiency, of running three aldol experiments simultaneously.

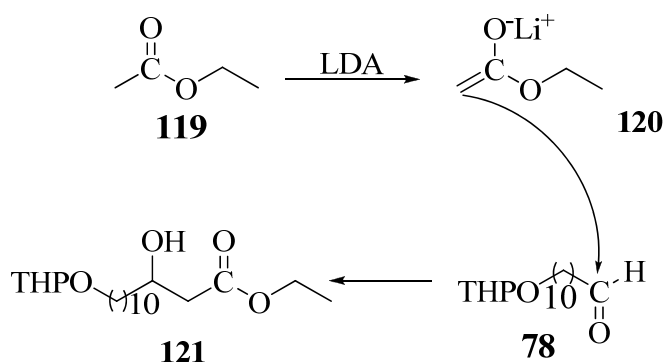
Running the aldol reaction without Cp_2ZrCl_2 was deemed crucial to improving the yield obtained from the aldehyde **78**. Particularly, the use of 1 M HCl to work up the reaction when Cp_2ZrCl_2 was used could have also resulted in significant cleavage of the THP protecting group of **79**. Such a harsh work up might also induce dehydration to an alkene.

The reaction without the Cp_2ZrCl_2 was repeated and the diastereoselectivity improved to 70% of the *anti*-diastereomer. The yield of **79** was 12%. This lack of control over the product yield, in spite of rigorous attempts to control the reaction conditions, elicited investigation of the degree of purity of the reactants. At this stage 0.5 M HCl was still used to work up the reaction. It was however confirmed that in the present process the marginal effect of using Cp_2ZrCl_2 was no greater than a 10% improvement in selection. Although the diastereoselectivity changed from 80 : 20 to about 70 : 30 for the *anti* : *syn* selection in the absence of Cp_2ZrCl_2 , removing it halved the reaction time. Also there was an opportunity to improve the overall product yield by using a weaker acid to work up the reaction and maintaining protecting group integrity. Moreover, the non-catalysed reaction provided enhanced quantities of the *syn*-isomer, should this be required.

2.2.5.4 The Effect of the Reactants' Purity

The state of the reactants was next examined. It was ensured that both reactants were of the

highest purity. The ester **77** was placed under high vacuum repeatedly for several days prior to use to remove all residual solvents, especially ethyl acetate. With an abstractable α proton the ethyl ethanoate ester **119** could react with the LDA to form a lithium enolate **120** [Scheme 2.13]. This would have the effect of depleting the available base for chiral enolate formation and possibly encourage other disruptive side reactions.



Scheme 2.13

The aldehyde **78**, prepared just before use, was purified by column chromatography using hexane and ether. Excess solvent was removed by high vacuum pressure at room temperature and gentle blowing with a stream of nitrogen gas.

The yield was 34% of the *anti*-aldol product **79** in a 7 : 3 diastereoselectivity against the *syn*-diastereomer.

2.2.5.5 Effect of Changing the Limiting Reagent

The aldol process described by Driver used the aldehyde **78** as the limiting reagent and up till this stage in the present process this had also generally been the case. Although the chiral ester **77** was the more costly reagent using it in excess was preferred in order to minimize the complication posed to column chromatography purification of the aldol products. The aldehyde **78** had a R_f between the two aldol diastereomers. However, with the improvements described above the aldol reaction was attempted with the aldehyde **78** in

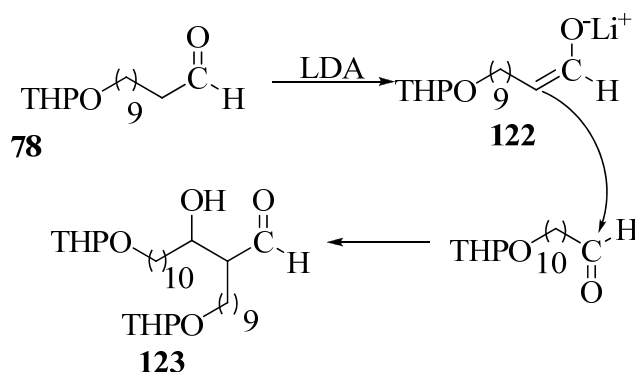
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a 1.1 mol. eq. to the chiral ester **77**. The yield was 40% of the *anti*-aldol product **79**. Significantly it must be pointed out that the column chromatography was much easier with less contamination of the middle fractions with the aldehyde. It is also worth noting that less complication with the column purification process was noticed when the reagents were subjected to rigorous purification before use [Section 2.2.5.4].

2.2.5.6 Reduction of LDA Molar Equivalents

The initial process described by Driver required about 7 mol. eq. of LDA. With the removal of residual ethyl acetate solvent and other improvements already described in the preceding sections above the amount of LDA used was reduced to 2.5 mol. eq. The yield was 42%.

Fewer impurities were observed on the TLC of the crude product. It was proposed that excess base might have catalysed dimerization of the aldehyde **78** [Scheme 2.14]. This would also decrease the experimental yield.



Scheme 2.14

2.2.5.7 The Optimised Procedure and Scale-up

When all the improvements above had been determined, an optimized procedure could be

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identified. This was: To a stirred solution of freshly prepared LDA (2.5 mol. eq.) in THF at -78 °C is added a solution of chiral ester **77** (1.0 mol. eq.) in THF (2 ml) slowly over 1 h under nitrogen. The solution is stirred for 2 h when aldehyde **78** (1.1 mol. eq.) in THF (2 ml) is added slowly over 45 min. Stirring is continued for another 2.5 h. The reaction is worked up by allowing the temperature of the solution to rise to between -10 °C and 0 °C and adding NH₄Cl (30 ml). The organic layer is separated and the aqueous layer extracted thrice with diethyl ether (3 x 100 ml).

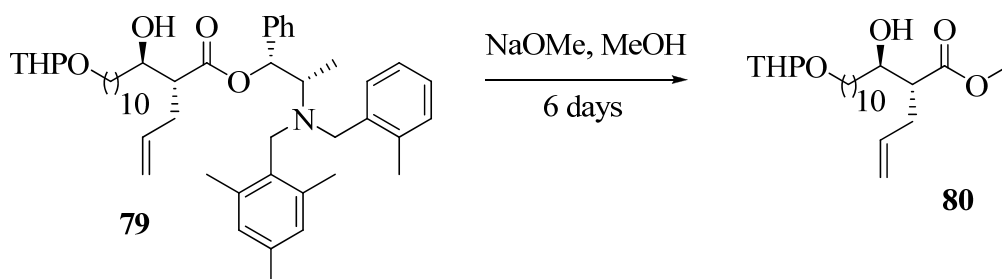
Using this procedure, except that 1.0 mol. eq. of the aldehyde was used, the aldol reaction was attempted on a multi-gram scale. A 4 g reaction of the ester **77** gave a yield of 40% and the diastereoselectivity was approximately 70 : 30, *anti* : *syn*. When compared to previous attempts to scale up which had either completely failed or given very low yields, this was a major success. Importantly, the zirconocene co-catalyst was not used.

All the precautions used resulted in a reproducible experimental yield constantly between 40-45% for the *anti*-aldol product **79** and diastereoselectivity of about 70 : 30.

Separating the diastereomers was not problematic. While the *anti*-product **79** had a *R_f* of 0.38 the *syn*-product was 0.25 with a hexane/ether (7 : 3) solvent system. During the multi-gram scale up the aldehyde was once again used as the limiting reagent to further improve the purity of the product; this reduced the amount of unreacted aldehyde. Flash column chromatography provided the *anti*-product **79** in high purity. No wastage of expensive reagent was incurred because the crucial chiral ester **77**, used in 1.1 mol. eq. relative to the aldehyde, could be recovered and re-used.

2.2.6 Transesterification of the *Syn*- and *Anti*-Aldol Products

Transesterification of the aldol product **79** with NaOMe in methanol was used to simultaneously hydrolyze the chiral auxiliary **76** and protect the carboxylic group to give methyl ester **80**.



Scheme 2.15

Base hydrolysis was chosen to prevent cleavage of the acid labile THP group. A major disadvantage of the one-pot base-catalysed transesterification process is epimerization. To minimize epimerization at the α -carbon, the reaction was done in a refrigerator at $-4\text{ }^{\circ}\text{C}$.¹⁶⁴ The lower temperature meant the reaction was slow and several equivalents of NaOMe were added at 48 h intervals for six days to ensure maximum conversion of the aldol product **79**. The yield of **80** was a moderate 65% with quantitative recovery of the chiral auxiliary alcohol **76**.

Formation of the methylated product was easily detected by the loss of UV activity and lower R_f (0.29, Hex : EtOAc (10 : 3) compared to 0.38 for the *anti*-aldol product **79**) on TLC. ^1H NMR gave a spectrum which was identical to that reported and showed the disappearance of the signals in the aromatic ring region for **79** and concomitant appearance of a sharp $-\text{CH}_3$ singlet at 3.68 ppm for the methyl ester.

The rate and extent of epimerization of the *anti*-aldol product **79** during base hydrolysis was compared to the *syn*-diastereomers [Figure 2.5]. Transesterification of the *syn*-diastereomers was complete in 2 days compared to the 6 days for the *anti*-product. It was not clear the reason or mechanism for this difference. It was also observed from TLC spots intensities that greater epimerization occurred in the *syn*-aldol products than the *anti*. As reported above the major *anti*-aldol methyl ester gave only a single signal in the ^{19}F NMR and its absolute configuration was confirmed by Driver to be $2R,3R$. The *syn*-product was a mixture of two *syn*-enantiomers [Figure 2.3].

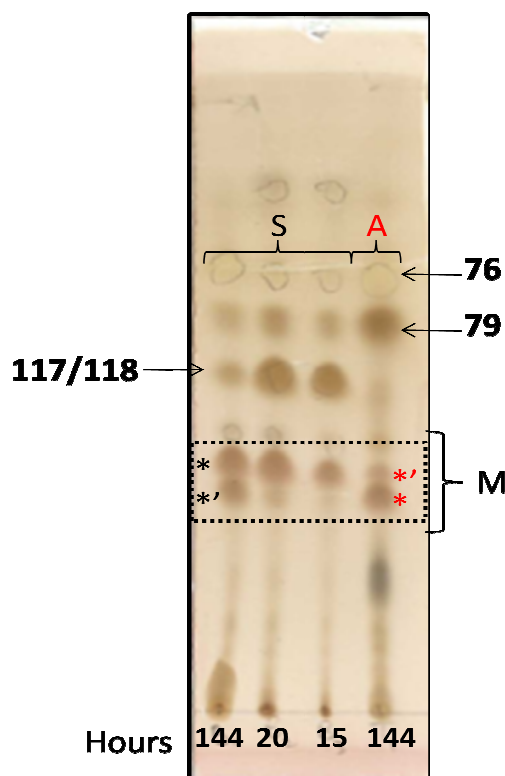


Figure 2.5: TLC of crude reaction samples of six-day transesterification experiments of *anti*- and *syn*-aldol products. **A** = *anti*-aldol product **79**, **S** = *syn*-aldol products (**117** and **118**), **M** = methylated aldol products, * = methylated *anti*-aldol product **80**, *' = epimer of methylated *anti*-aldol product, * = methylated *syn*-aldol products, *' = epimers of methylated *syn*-aldol products. (**76** = recovered chiral auxiliary; Solvent system = Hex : EtOAc, 10 : 1; TLC development stain = anisaldehyde).

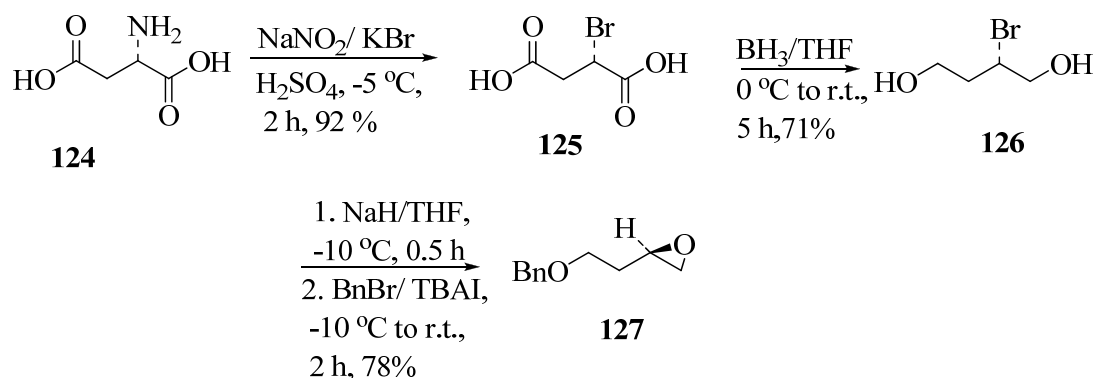
Since it was initially difficult to obtain the mycolic motif via aldol synthesis on a sufficient scale for other aspects of this project an established and published method was used to synthesize the required quantity of **75**.¹⁴⁴ This also offered a good opportunity to compare the new aldol method to the published method in the synthesis of a common mycolic motif analogue **75**.

2.2.7 Synthesis of Mycolic Motif via a Frater Reaction

The oxirane **127** was obtained in 3 steps from (*S*)-aspartic acid **124** [Scheme 2.16].¹⁷³ Treatment of the amino acid with sodium nitrite and potassium bromide in sulfuric acid gave (*S*)-bromosuccinic acid **125** in 92% yield. This unusual reaction proceeding with

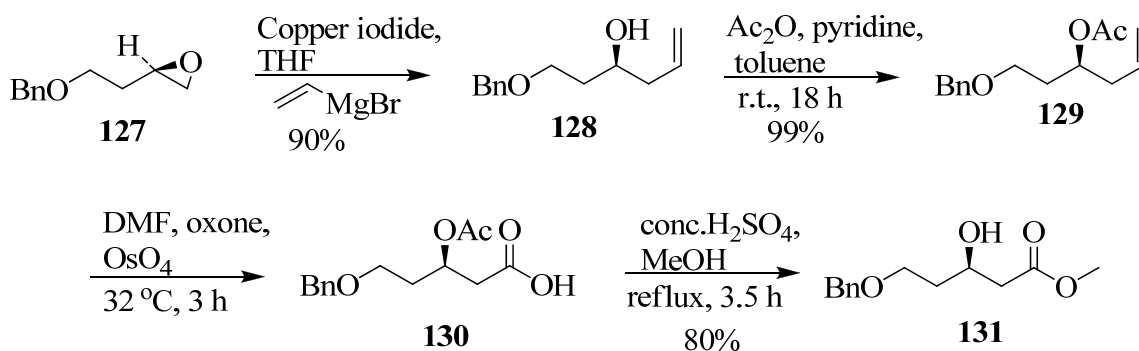
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overall retention of stereochemistry actually involves an intramolecular substitution of the derived diazo-species by the acid with inversion, followed by intermolecular opening of the derived α -lactone by bromide ion, with a second inversion. The bromo-diacid **125** was reduced to the diol **126** with diborane in THF at room temperature. This polar product **126** is difficult to purify on silica column. Using a petroleum ether/ ethyl acetate solvent system (1 : 2) the column took several hours to complete, resulting in a lower yield than anticipated. Epoxidation and protection of the diol **126** was achieved in a one-pot reaction with sodium hydride and benzylbromide in THF to yield the (*R*)-(2-benzyloxyethyl)oxirane **127**.



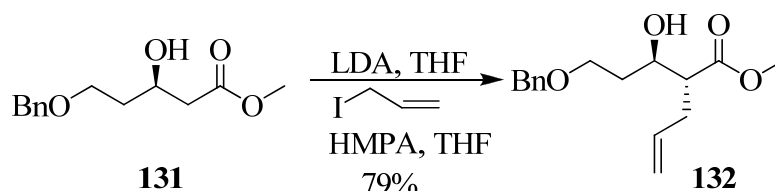
Scheme 2.16

The *R*-3-hydroxy ester **131** for Frater alkylation was introduced in 4 steps from the oxirane **127** [Scheme 2.17]. The epoxide ring was opened with vinylmagnesium bromide under an inert atmosphere to afford the enol **128**. The secondary alcohol was protected with acetate and the alkane oxidized to a carboxylic acid **130**. Deprotection of 3-hydroxy group with concentrated H_2SO_4 and methylation of the acid yielded the *R*-3-hydroxy methyl ester **131** in good yields. The aromatic multiplet at 7.28-7.37 ppm, integrating for five protons, the $-\text{OCH}_3$ singlet at 3.71 ppm, and the single β -proton at 3.66 ppm confirmed that a structure identical to the literature compound was obtained.¹⁴⁴ The quarternary carbonyl carbon was observed at 172.8 ppm.



Scheme 2.17

Allylation of ester **131** was achieved by the Frater method as described by Toschi and Baird [Scheme 2.18].^{134,174} LDA was synthesized *in situ* and the ester **131** added slowly at -78 °C. Allyl iodide (1.5 mol. eq.) was added at -62 °C. The nucleophilic kinetic enolate attacks the allylic halide via an S_N2 mechanism to yield the product **132**. The very reactive allylic halide encouraged high yields for the process which were typically between 70 and 80%.^{134,144}



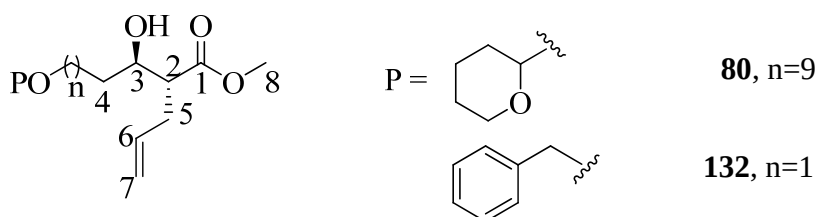
Scheme 2.18

The allylation process, like the aldol, proceeds via a cyclic transition state.¹⁷⁵ The Li⁺ strongly chelates the two oxygen anions in a rigid chair conformation. This facilitates stereoselection. HMPA was mixed with the allyl iodide in THF. HMPA enhances the reactivity of the enolate by strongly coordinating with the lithium. This effectively traps the enolate and probably limits undesirable side reactions.¹⁴⁸ HMPA was not used in the aldol as it reverses the kinetic *E* : *Z* selectivity in the formation of the lithium enolate. The basic HMPA-THF solvent system might also facilitate a retro-aldol reaction.¹⁷⁶

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The allyl methyl ester **132** was obtained in 79% yield as a yellow oil. NMR characterization data were identical to the published results.¹⁴⁴ The NMR of the aldol methyl ester **80** and the Frater methyl ester **132** were also quite similar with the major functional groups chemical shifts almost identical [Table 2.3, Table 2.4].

Table 2.3: Comparison of ¹H NMR chemical shifts of allyl methyl esters from aldol (**80**) and Frater (**132**) syntheses. (Proton numbers correspond to carbon numbers in structure.)



Proton (¹ H)	Aldol 80 (δ_H (400 MHz))	Frater 132 (δ_H (500 MHz))
	ppm	ppm
H₂	2.48-2.56	2.50-2.57
H₃	3.78-3.88	3.90-3.97
H₄	1.77-1.85	1.70-1.85
H₅	2.33-2.47	2.28-2.40
H₆	5.68-5.82	5.65-5.75
H₇	4.98-5.10	4.95-5.10
H₈	3.68	3.68

Table 2.4: Comparison of ^{13}C NMR chemical shifts of allyl methyl esters from aldol (**80**) and Frater (**132**) syntheses.

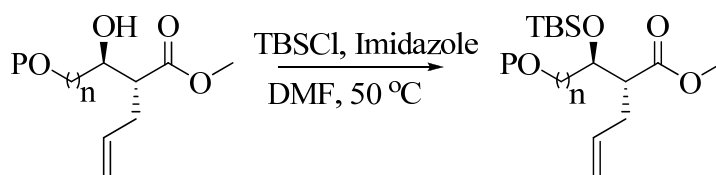
Carbon (^{13}C)	Aldol (δ_{H} (100 MHz))	Frater (δ_{H} (125 MHz))
	ppm	Ppm
C ₁	175.2	174.8
C ₂	50.5	51.1
C ₃	71.8	70.9
C ₄	34.3	34.6
C ₅	33.8	33.3
C ₆	134.9	135.0
C ₇	117.2	117.1
C ₈	51.6	51.6

2.2.8 Extension of the α -Chain

Having prepared the substituted allyl mycolate motifs **80** and **130**, the next step required the extension of the allyl chain to the full length alkyl chain.

2.2.8.1 Protection of β -OH Group

The β -OH group of the methyl esters **81** and **131** were protected with TBS under the same reaction conditions [Scheme 2.19].



P = THP, n=10	80	83	Yield = 90%
P = Bn, n=2	132	133	Yield = 90%

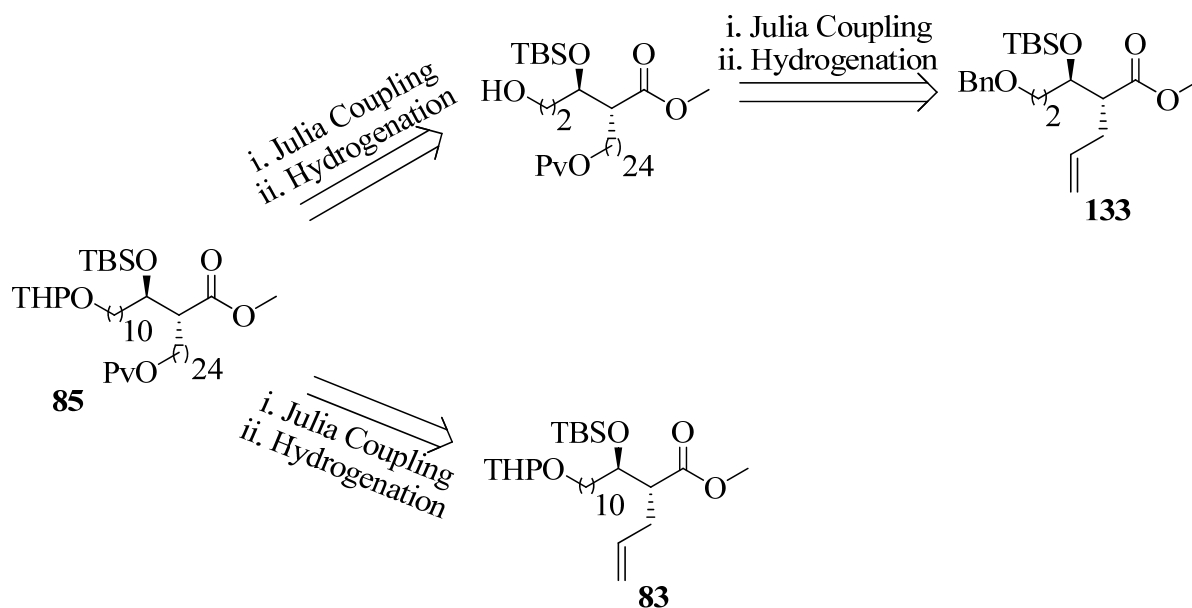
Scheme 2.19

Protection of the Frater product **132** was faster and completed in 18 h while the aldol product **80** was only completely protected at 48 h. This significant difference in time could not be explained except that the longer alkyl chain of the aldol product might fold over to encourage interaction between the oxygenated protected end and the –OH group. This would hinder access to the –OH.

The structures of the TBS-protected aldol and Frater products (**83**, **133**) were confirmed by ^1H and ^{13}C NMR analysis. The two singlets at 0.00 ppm and 0.85 ppm integrating for 6H's and 9H's respectively were diagnostic of the successful protection of the alcohol.

2.2.8.2 Chain Extension

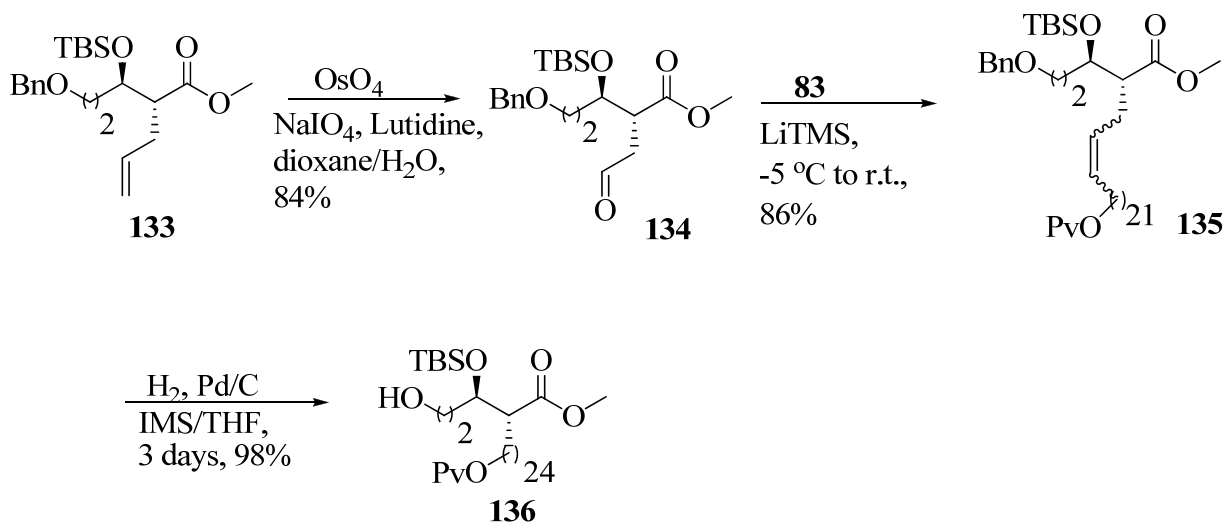
The protected Frater product **133** required two coupling reactions to extend the alkyl chains to the required lengths for preparation of the complete mycolic acid while the protected aldol product **83** needed only one as shown retrosynthetically in **Scheme 2.21**.



Scheme 2.21

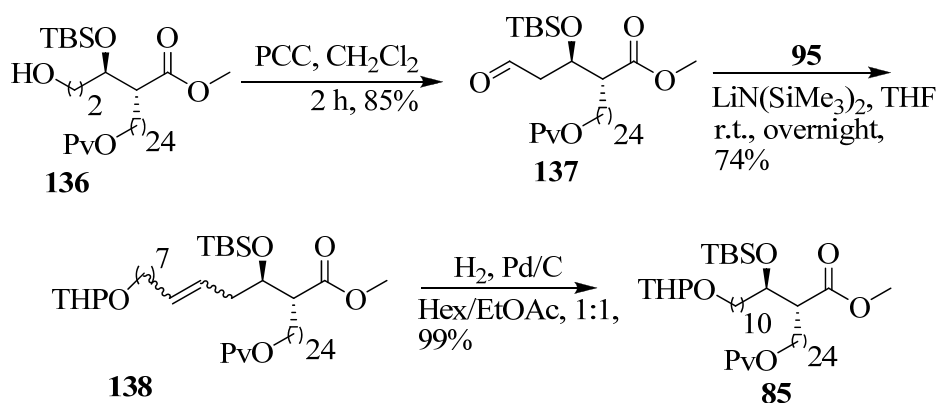
Oxidative cleavage of the allyl methyl ester **133** with osmium tetroxide and sodium metaperiodate conveniently introduced the aldehyde **134** in approximately 84% yield [Scheme 2.22]. This was not characterized but was immediately coupled to the C₂₂ sulfone **82** prepared earlier, using a modified Julia-Kocienski reaction to give isomeric *E/Z*-mixtures of alkenes **135**. Hydrogenation on palladium 10% on carbon at 1.0 atm H₂ pressure was continued for 3 days to effect cleavage of the benzyl protecting group and give **136** in 98% yield.

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Scheme 2.22

PCC oxidation of the alcohol **136** gave the aldehyde **137** which was coupled to the $\text{C}_8\text{SO}_2\text{PT}$ **95** by a second Julia-Kocienski reaction [Scheme 2.23]. Overnight catalytic hydrogenation of **138** at 2.4 atm of hydrogen for 18 h yielded the protected motif analogue **85** at the convergent point of both synthetic routes.



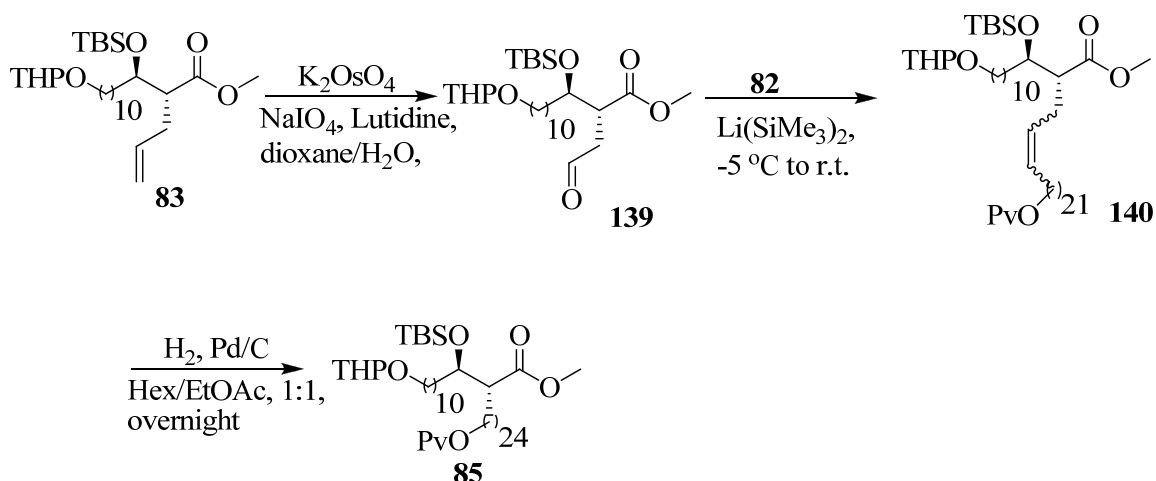
Scheme 2.23

^1H NMR analysis of **85** confirmed the presence of diagnostic signals for the four protecting groups. The six dimethylsilyl protons were observed as a singlet at 0.02 ppm while the nine

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tertiary butyl protons gave a singlet at 0.84 ppm. The THP acetal triplet of the pyran ring at 4.55 ppm along with the multiplet of the protons of the α -methylene at 3.68-3.73 ppm confirmed the presence of the THP protecting group. The nine protons of the trimethylacetate group were observed as a singlet at 1.24 ppm and the α -methylene triplet appeared at 4.02 ppm. The methyl ester singlet was seen at 3.63 ppm.

In the same way the aldol-derived intermediate **83** was chain extended by a modified Julia-Kocienski reaction using lithium bis(trimethylsilyl)amide as base. However, because of the high toxicity of the pure osmium tetroxide (OsO_4), the much less toxic potassium salt (K_2OsO_4) was used instead for oxidative cleavage of the allyl **83**. The *E/Z* alkene mixture **140** was hydrogenated overnight at 2.4 atm of hydrogen using palladium 10% on carbon as catalyst. This gave **85** in an overall yield of 67% from **83**.

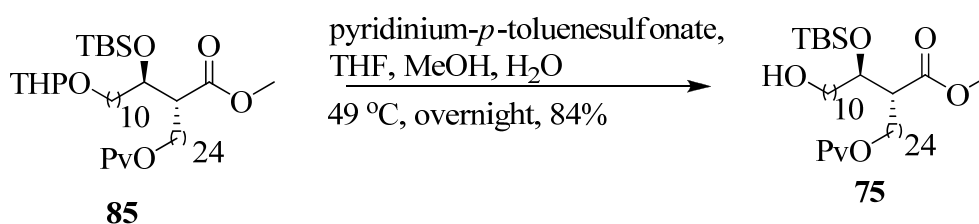


Scheme 2.24

Once again the product **85** could be fully characterized by proton and carbon NMR and gave the required accurate mass. The NMR spectra of **85** prepared by the two different routes were identical to each other.

2.2.9 Cleavage of THP Protecting Group

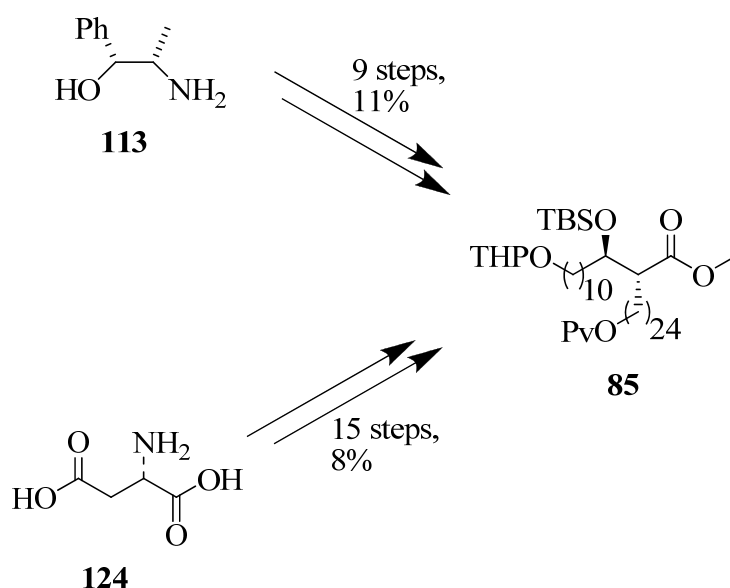
The THP protecting group was cleaved with pyridinium-*p*-toluene sulfonate in a MeOH/ THF/ H₂O solvent system first at 60 °C for 2 h and then at room temperature overnight. The primary alcohol **75** was obtained in 84% yield. The cleavage of the protecting group was confirmed by the disappearance of THP signals in the NMR spectra.



Scheme 2.25

2.3 Conclusions

It required significantly fewer reaction steps to arrive at the common product **85** via the aldol synthetic route than Frater alkylation route. The total yield for the aldol route is 11% in 9 steps while the Frater route gave a yield of 8% in 15 steps [Scheme 2.26]. Because the chiral auxiliary can be recovered it is possible to reduce the aldol route by a further 2 steps.



Scheme 2.26

Epimerization of the *anti*-aldol product during hydrolysis of the chiral auxiliary taints the attractiveness of the shorter aldol route. This might necessitate the introduction of additional steps involving reduction of the ester **79** to the alcohol, reoxidation to the acid and methylation to obtain **80**. No easily observable enantio-impurity was detected in the Frater product **132** but the length of the synthetic process and harsh conditions of some of the reactions are significant.

Several of the reagents used in the Frater route are either toxic or notoriously difficult to handle. HMPA is a powerful carcinogen, which makes it difficult to use on a large scale. Special disposal systems must be incorporated into the cost of the process. The Frater alkylation reaction, although highly enantioselective and successful, is very sensitive to the specific reaction condition. The linearity of the reaction sequence leading to the Frater starting material **131** means failure of the coupling step when carried out by an unskilled experimenter will lead to an expensive loss of material with little chance of recovery of precious starting compounds.

Although the aldol is also quite sensitive to the reaction conditions the aldol starting ester **77** is fully recoverable and cleaved chiral auxiliary **76** can be re-used. The reagents used are

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much less toxic and more easily handled. Development of the aldol process is still in its infancy. It has only been possible to scale up current process so as to produce about 1 g of methyl ester **83** from a single reaction; in contrast, the Frater process has been more extensively developed and can produce 25-50 g of intermediate. Given that the intermediates must be taken through a number of steps to produce a full mycolic acid, efficient further scale up of the aldol process is a high priority. A further advantage for the aldol reaction would be the possibility of directly coupling longer chains in place of the allyl chain of **77** which will bring the process closer to actual mimicry of the *in vivo* MA synthesis.¹⁷⁷ This would further reduce the steps involved in the synthesis of the motif and the MA.

This project has successfully scaled up the aldol synthesis of mycolic motif allyl analogue **79**. It has also identified several factors that bedeviled the synthesis initially. The possibility now exists for the aldol coupling of longer fragments of the motif.

3 Chapter Three: Synthesis of a Thiolated Methoxy Mycolic Acid

3.1 Introduction

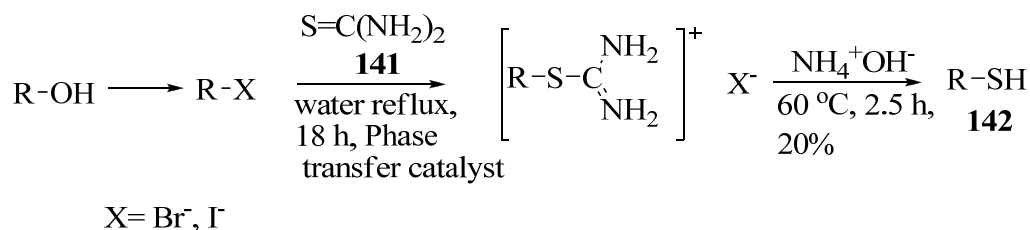
3.1.1 Synthesis of Thiolates

Many biological molecules have hydroxyl groups which can be conveniently converted into thiols via a more reactive intermediate. The hydroxyl group itself is a poor leaving group with a pK_a of 15 and relatively low reactivity [Table 3.1].

Table 3.1: Reactivity and pK_a of common leaving groups.

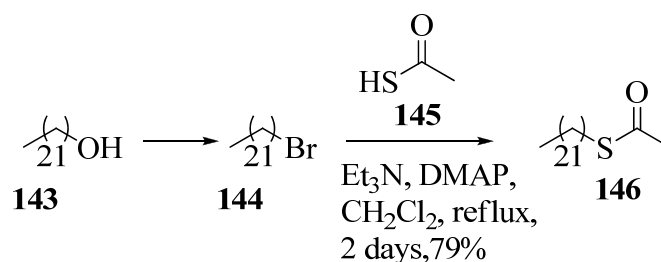
Leaving group A^-	HO^- , H_2N^- , RO^-	F^-	Cl^-	Br^-	I^-	$TosO^-$
Relative reactivity	$\ll 1$	1	200	10, 000	30, 000	60, 000
pK_a of HA	> 15	3.45	-7.0	-8.0	-9.0	-2.5

Primary alcohols can easily be converted into a range of more powerful leaving groups, like bromide. Nucleophilic substitution of the halide by sulfur can afford the thiol.¹⁷⁸ One method to achieve this uses thiourea **141** to thiolate *n*-alkyl halides. The bromo-thiuronium salt intermediate is then hydrolyzed to the free alkyl thiol [Scheme 3.1]. Using this method Speziale reported a 61-78% recovery of the starting alkyl halides and 20% of actual product.¹⁷⁹ NMR analysis of the crude product showed it to be contaminated with 4-19% of the dialkyl disulfide.



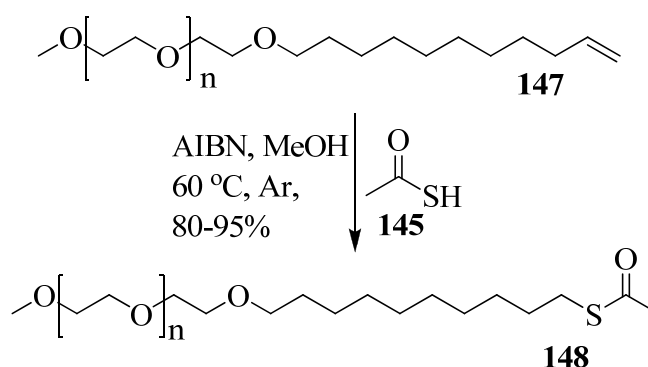
Scheme 3.1¹⁷⁹

Another process for thiolation involves refluxing an alkyl bromide **143** with 3 mol. eq. of thioacetic acid **145** over three days [Scheme 3.2]. This method was attempted in Pretoria using model compounds and gave a better yield than the thiourea method.¹⁶⁴



Scheme 3.2

A terminal alkene **147** can also be thiolated with thioacetic acid **145** [Scheme 3.3].¹⁸⁰ Azobisisobutyronitrile (AIBN) generates a radical of the thiol under reflux that attacks the alkene in an *anti*-Markovnikov process.¹⁸¹ Although this method gives high yields of 80-95%, terminal alkenes are not always easily obtained commercially. The maximum yield was obtained at 1.5 mol. eq. of AIBN, 12 mol. eq. of thioacetic acid, and refluxing at 60 °C under argon for 72 h.^{181,182}



Scheme 3.3¹⁸⁰

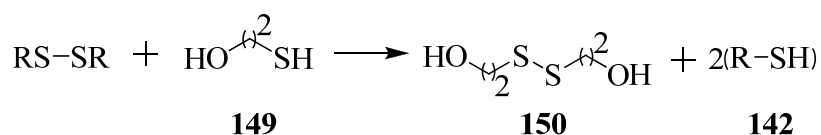
The conditions described above highlight the challenge of thiolating organic compounds even with reactive halide substituents. Tosylates are much more reactive than the halides [Table 3.1] and thiolation via a tosylate was attempted in this project.

3.1.2 The Risk of Dimerization of Thiols

Thiols very readily and spontaneously oxidize in air and dimerize to disulfides. The most effective means to prevent this is to keep the thiols protected until all chemical processes are accomplished. It has been reported that base hydrolysis of thioesters aid disulfide formation more than acid hydrolysis.¹⁸¹ It is therefore important to ensure that deprotected thiols are stored under inert conditions and are used as early as possible.

3.1.3 Reduction of Disulfides

The disulfide bond has bond dissociation energy of 60 kcal/ mol, which makes it a relatively strong and stable bond. It plays an important role in the tertiary folding of polypeptides and its reduction is a hurdle that must be surmounted in the sequencing of proteins. The traditional reagents used by biochemists to cleave cystine, the predominant disulfide in proteins, are mercaptoethanol **149** and dithiothreitol [Scheme 3.4].

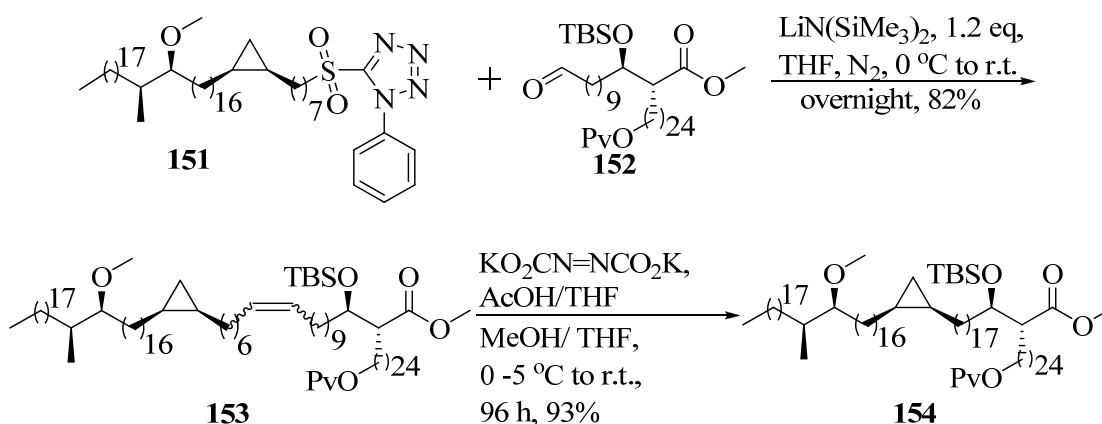


Scheme 3.4

Disulfide bonds are actually susceptible to cleavage by a wider range of polar reagents, which may be either nucleophilic or electrophilic.¹⁸³ In organic chemistry nucleophilic reagents like sodium borohydride are used to reduce the disulfide to thiols. The alkali metals can also be used as electrophiles to produce the corresponding metal thiolates which are further protonated by a strong acid.

3.2 Results and Discussion

The meromycolyl sulfone **151** was generously provided by Dr Juma Al'Dulayymi.* Aldehyde **152** was prepared by PCC oxidation of alcohol **75** with an 80% yield. Sulfone **151** was coupled to the motif aldehyde **152** by a modified Julia-Kocienski reaction [Scheme 3.5]. It was observed that warming the THF solution of the sulfone slightly prior to cooling to 0 °C improved the yield. The yield was 82%.



Scheme 3.5

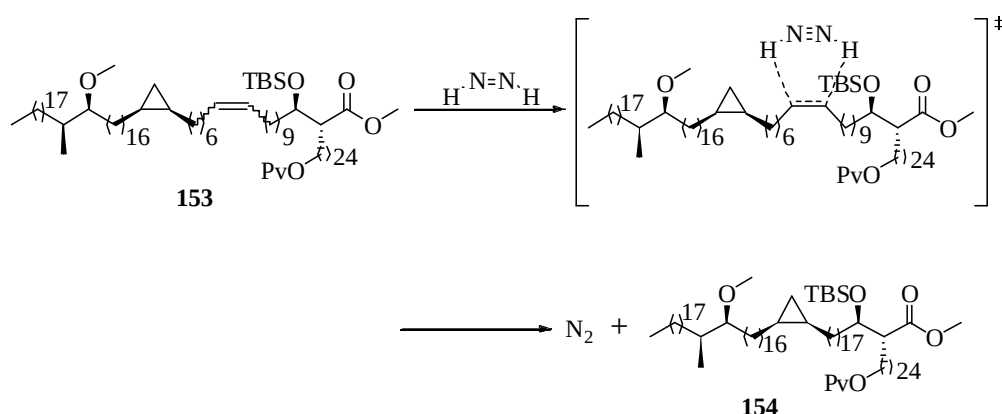
* Dr Al Dulayymi provided 500 mg of sulfone **151** for the synthesis of the thiolated MA.

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The presence of the alkenes **153** was confirmed by ^1H and ^{13}C NMR analysis. The characteristic multiplet of the *E/Z*-isomers at double bond was observed at 5.31-5.85 ppm. The unsaturated vicinal carbons appeared at 130.3 ppm for the diastereomers. One of the cyclopropane protons was observed at -0.36 ppm as a broad quartet while the methoxy protons appeared as a singlet at 3.32 ppm. The methyl ester singlet appeared at 3.63 ppm. At 4.02 ppm the protons of the PvO α carbon appeared as a triplet [**Figure 3.1**]. The characteristic hydroxyl acid α -carbon proton was observed as a multiplet at 2.47-2.53 ppm while the β -carbon proton was observed as a multiplet at 3.86-3.90 ppm.

Catalytic hydrogenation of the newly formed double bond is precluded for conversion of the alkene to alkane because rapid hydrogenation of the cyclopropane can occur under these conditions.¹⁸⁴ Chemical hydrogenation via diimide generated *in situ* is commonly used.¹³¹ This process is much slower.

A slurry of the potassium salt of the azodicarboxylate was formed with a solution of the *E/Z* alkenes **153** at a temperature between 0 °C and 5 °C [**Scheme 3.6**]. Since free azodicarboxylic acid decomposes rapidly at room temperature, acetic acid in THF was added slowly over 8 h to the saturated solution. Hydrogenation occurs via a transient diimide formed by decarboxylation of the diacid [**Scheme 3.6**]. The *cis*-isomer is less stable than the *trans* and is believed to carry out a *syn* addition of two hydrogen atoms to the alkene.



Scheme 3.6

Figure 3.1: ^1H NMR spectrum of *E/Z* MeO-MA alkenes **153** (400 MHz, CDCl_3).

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After 24 h ^1H NMR showed the presence of a substantial amount of alkene [Figure 3.2]. The reaction was repeated several times and the final yield of the alkane **154** was 93%.

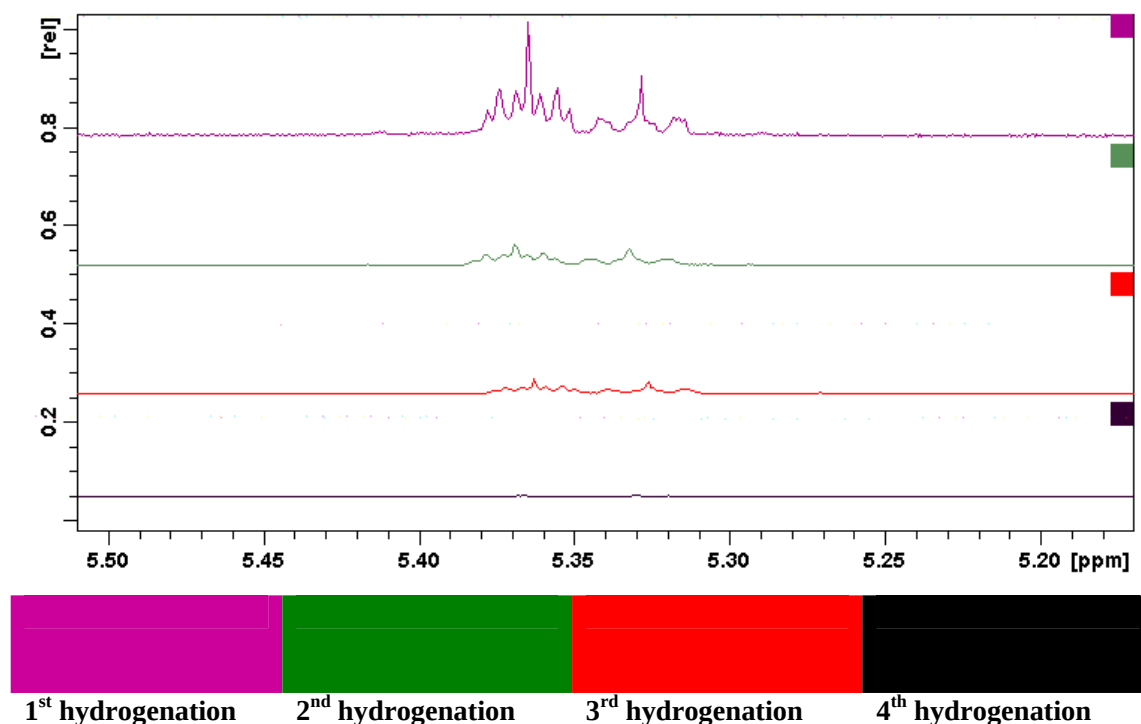
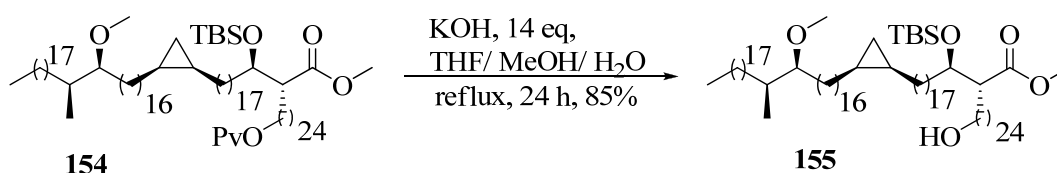


Figure 3.2: Comparison of ^1H NMR spectra of alkene **153** hydrogenation (400 MHz, CDCl_3).

3.2.1 Deprotection of α -alkyl chain

The above coupling successfully produced the skeleton of a complete substituted mycolic acid, but to produce the free acid, several protecting groups needed to be removed and the primary alcohol of **155** converted into a thiol.

The first protecting group to be cleaved was the base-labile trimethyl acetate of the C_{24} -chain [Scheme 3.7]. The hydrolysis was readily achieved with 14 mol. eq. of KOH at reflux.



Scheme 3.7

^1H NMR confirmed the success of the reaction, and the fact that the rest of the molecule remained intact, by the disappearance of the singlet integrating for the 9H's of the pivalate at 1.17 ppm. Also significant is the shift of the $\text{CH}_2\text{—OPv}$ triplet upfield from 4.02 ppm to 3.62 ppm for $\text{CH}_2\text{—OH}$. ^{13}C NMR spectrum lost the signals at 178.7 ppm for the carbonyl carbon C—COO and C—COO at 38.7 ppm. The remaining characteristic signals of the mycolic acid were still present.

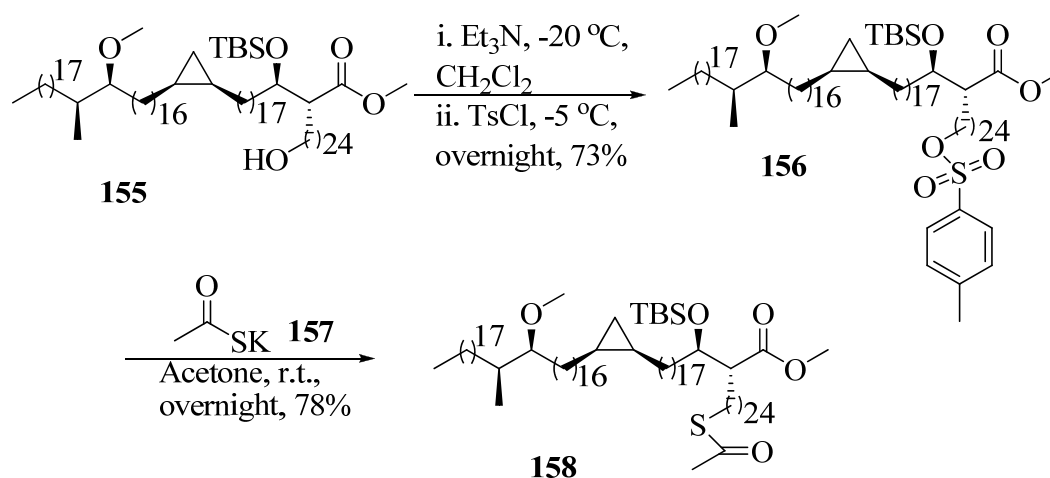
Deprotection of the C_{24} hydroxyl provided a uniquely significant molecule with a wide potential. Its versatility could be likened to the allyl motif synthesized by Toschi and Baird.¹³⁴ The functionalized MA molecule **155** can be attached via its α -alkyl chain to appropriate substrates and possibly nanoparticles for therapeutic applications like drug delivery. The first application is in the design of a new antigenic surface on a gold sensor substrate for improving TB serodiagnosis.^{37,112} This means conversion of the hydroxyl group to a thiol.

The hydroxyl group is one of the most convenient and commonly used starting functional groups in synthetic organic chemistry. Although it is a poor leaving group for substitution reactions it is easily replaced by the more reactive halides [Table 3.1]. Further substitution of the halides by other functional groups can then be achieved. In this project halogenation was avoided in the route to thiolation. Driver did brominate the distal terminal of the α -alkyl chain of a mycolic motif analogue before thioacetylation.¹⁶⁴ The strategy was successful but took a few days. Tosylation, followed by thioacetylation, was more convenient and rapid [Scheme 3.8].^a

^a Method developed by Enlli Huws of Bangor University on model mycolic acid compounds.

3.2.2 Tosylation and Thioacetylation

Tosylation is one of the most convenient methods of activating the rather stable hydroxyl group. The sulfonyl group increases significantly the electrophilicity of the alkyl C₂₄ [Scheme 3.8]. The relative reactivity of the tosylate is six times that of bromide [Table 3.1]. Because of this the tosylate was used as soon as possible.



Scheme 3.8

Tosylation of the alcohol **155** was done overnight in the refrigerator at about $-5\text{ }^\circ\text{C}$ with a 73% yield. The product **156** was easily observed running at $R_f = 0.63$ (Hex : EtOAc, 8:2) on TLC by its UV visibility. A quick ^1H NMR spectrum clearly showed a pair of doublets at 7.3 ppm and 7.8 ppm for the phenyl ring protons of the tosylate. The methylene protons adjacent to the oxygen of the C₂₄ chain also shifted from 3.62 ppm for the alcohol **155** to the higher chemical shift of 4.09 ppm for **156**.

The high reactivity of the tosylate functional group allowed a facile introduction of a MA thioacetate **158** under ambient conditions by a $\text{S}_\text{N}2$ reaction in 78% yield. An excess of potassium thioacetate **157** was required [Scheme 3.8].

The product **158** was readily purified by flash column chromatography, eluting just after unreacted tosylate **156**. Proton NMR spectrum analysis showed the three acetyl protons as a

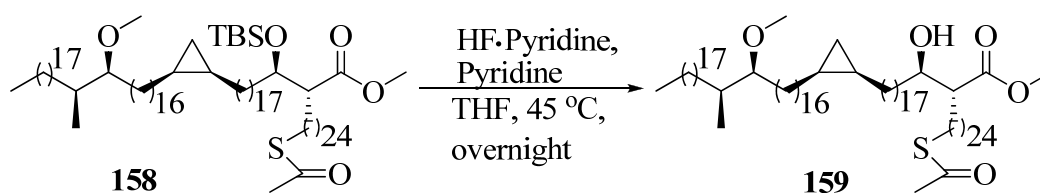
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singlet at 2.30 ppm. The triplet signal of the α methylene protons adjacent to the thioacetate had shifted to 2.84 ppm from the 4.09 ppm of the tosylate **156**. Other signals confirmed that the entire molecule was intact and all the protecting groups were still in place. The characteristic α and β carbon protons of the hydroxyl acid segment were observed as multiplets at 2.48-2.53 and 3.86-3.90 ppm, respectively. The chemical shift of the three protons of the methyl ester was observed as a singlet at 3.63 ppm while the methoxy ether singlet remained at 3.32 ppm. The dimethyl and tertiary butyl substituents of the TBS protecting group were observed as usual as the highest field signals, apart from the cyclopropane proton. One of the cyclopropane methylene protons was characteristically observed at -0.35 ppm as a broad quartet while another was observed at 0.50-0.57 ppm as a broad double triplet. The carbonyl carbon of the acetyl thioester was observed at 175.1. MALDI-TOF analysis gave the accurate mass.

The ester **158** was stable especially since all the reactive functional groups were stably protected. The first step in obtaining the final fully deprotected target compound **74** was the cleavage of the TBS protecting group. This was followed by hydrolysis of the acetyl and methyl esters in a single pot reaction. These reactions are described in the remaining sections of this chapter.

3.2.3 Deprotection of β -OH

Tertiary butyldimethylsilyl ether of the β -OH was cleaved efficiently with HF•pyridine. The mildly acidic cleavage was complete within 12 h at 45 °C [**Scheme 3.9**].



Scheme 3.9

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NMR analysis confirmed that the silyl protecting group had come off and the acetyl and methyl esters were still in place. The singlet for the six protons of the two silyl methyl groups at 0.02 ppm disappeared along with the singlet for the nine protons of the tertiary butyl group at 0.84 ppm. The ^{13}C NMR spectrum accounted for all the resonances anticipated for the different functional groups, but the carbonyl carbon of the thioacetate was difficult to identify in the baseline noise. This peak was clearly identified by HMBC analysis that gave a cross peak signal which coupled the acetyl protons to the carbonyl carbon at 195.9 ppm [Figure 3.3].

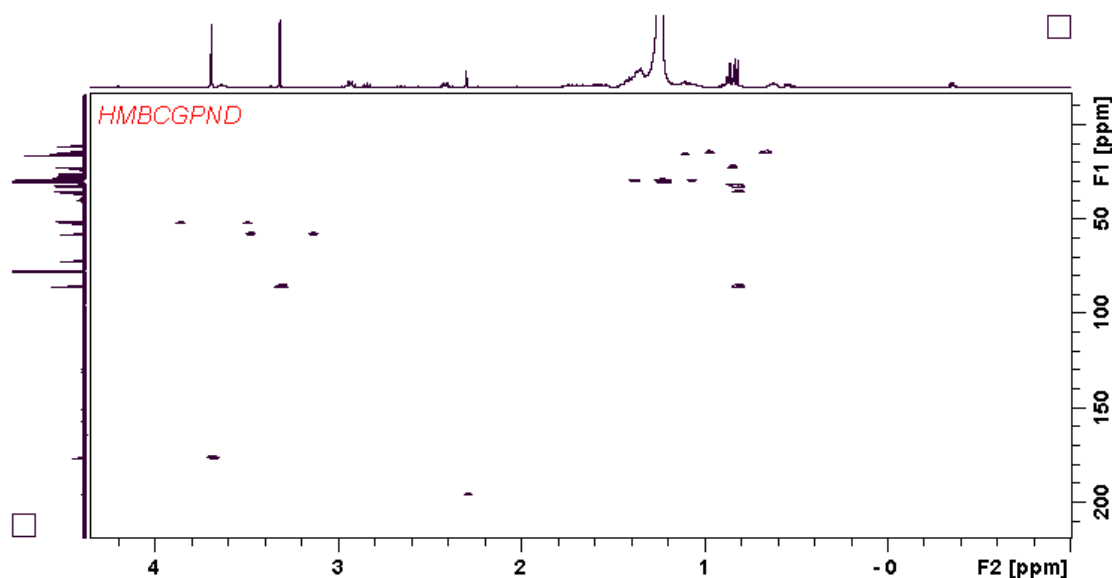


Figure 3.3: HMBC NMR spectrum of **159** (400 MHz, CDCl_3 , contour view).

3.2.4 Deprotection of Carboxyl and Thiol Functional Groups

3.2.4.1 Deprotection Reactions

Hydrolysis of the methyl and thioacetyl esters was first attempted with LiOH (4 mol. eq.) in a THF/ H_2O / MeOH (1 : 0.1 : 0.1) solvent system at 45 °C for 18-24 h. LiOH has been the base-of-choice to demethylate the carboxyl group of MAs.^{114,132,133,135-139,144} The reaction was however of limited success as the completely deprotected product **74** was obtained along with some methylated compound. The yield was quite poor and ^1H NMR indicated

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the presence of some impurity. Only 2.0 mg of partially purified product **74** was obtained from 13 mg of starting material **159**. The hydrolysis of the two esters was confirmed by the disappearance of the methyl and acetyl singlets at about 3.70 ppm and 2.31 ppm, respectively, [Figure 3.4]. The methoxy singlet was clearly visible at 3.35 ppm while the diagnostic cyclopropane quartet was almost lost in the baseline at -0.36 ppm. Although other characteristic peaks were obscured by the presence of much residual diethyl ether and dichloromethane it is clear that a MA compound is present and the disappearance of the esters' signals show that it must be the deprotected version of the starting compound **159**. Apart from the residual solvents seen in the NMR spectrum the major contaminants were the singlets seen at 6.55 ppm and 0.05 ppm. No further characterization was carried out as a repeat of the reaction also gave a very poor yield of less than 20% and another method of hydrolysis was investigated.

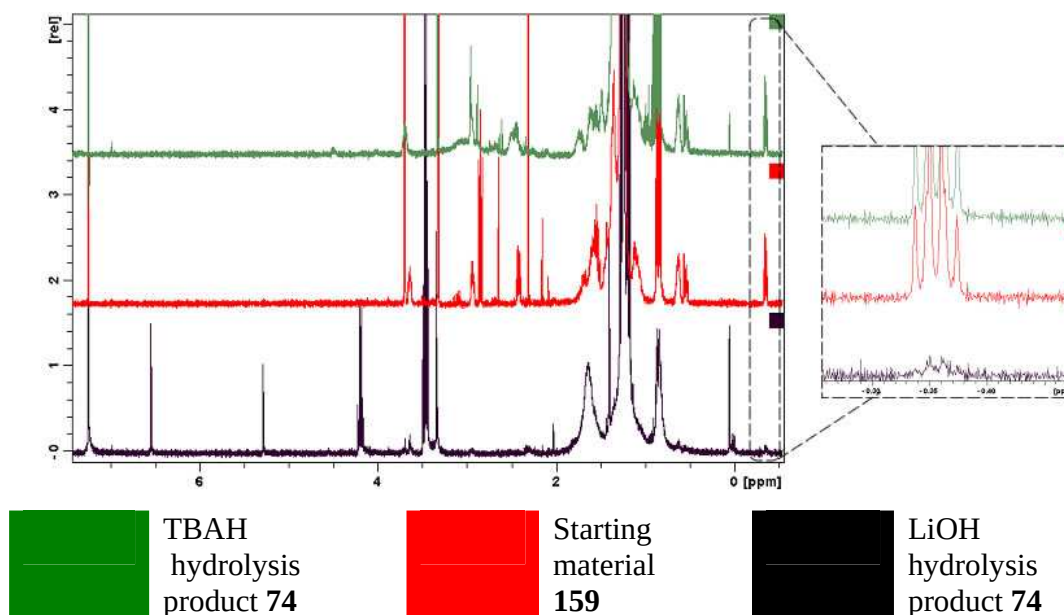


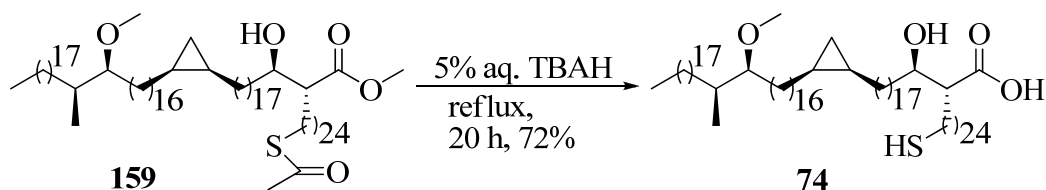
Figure 3.4: ^1H NMR spectra comparison of hydrolysis products of **159**, (400 MHz, CDCl_3).

The ester **159** was refluxed in a 5% aqueous solution of tetrabutylammonium hydroxide (TBAH) overnight [Scheme 3.9].* The first attempt of this reaction for 24 h gave a totally deprotected product **74** with recovery of unreacted starting material **159**. The yield was

* Enlli Huws of Bangor University had developed this method on model thiolated MA compounds.

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63%; remarkably better than the previous attempts. The reaction was repeated for a second time for 18 h and a lower yield of 41% was obtained with almost no starting material recovered. A third repeat of the reaction for 20 h and using double distilled deionized water (ddd H₂O) to prepare the aqueous TBAH solution resulted in 72% yield of the target compound **74** with TLC showing no starting material remaining. Also, there was almost no other product seen in the TLC.



Scheme 3.9

The TBAH reaction was interesting in that, although MAs have a limited selection of solvents in which they are soluble, the reaction was done by suspending the MA **159** in the aqueous solution. According to the method worked out by Dr Al' Dulayymi of Bangor University the solution must be brought to a boil at 100 °C for the MA to fully dissolve in the solution and the maximum yield to be obtained. It was noticed that here at the University of Pretoria water will only boil at about 95 °C. We therefore attempted to raise the boiling temperature of the 5% aqueous TBAH solution by adding NaCl. It required a concentration of about 10% NaCl to get the temperature to 100 °C. This level of salinity resulted in almost no product formation and evaporation of the water even with a reflux condenser attached. When attempted with the ddd H₂O the reaction went with a high yield.

Although the three attempts reported above were carried out for different durations with significantly different yields it is probably the extraction methods of the crude product after acidification to pH 1 and the column purification methods that had the greater influence on the final yields. In the first two attempts the crude product was extracted with warm diethyl ether only while the third experiment was further extracted with hot hexane. Given that MAs generally melt around 60 – 65 °C, which is approximately the boiling temperature of hexane, the latter method would certainly have effected a better extraction of the product.

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In the purification of the products of the first two reactions a flash column eluting with hexane : ethyl acetate (8 : 2) only was used while the product of the third reaction was purified with a solvent system consisting of first hexane : diethyl ether (10 : 1) followed by chloroform : methanol (10 : 1). In hexane : ethyl acetate (8 : 2) the R_f of **74** is 0.20 while in chloroform : methanol (10 : 1) the R_f is 0.56. This meant that changing to a more polar solvent system encouraged better elution of the relatively polar thiol **74**.

The target thiol **74** was fully characterized. ^1H and ^{13}C NMR spectra analysis confirmed the successful deprotections. The acetyl and methyl singlets at 2.30 ppm and 3.69 ppm, respectively, were absent. This resulted in the shift of the CH_2S protons to higher field at 2.48 ppm from 2.84 ppm. The characteristic shifts of the other functional groups of the MeO-MA structure confirmed the integrity of **74**. The α and β protons were observed as multiplets at 2.41-2.48 ppm and 3.65-3.72 ppm. The methoxy singlet remained at 3.32 ppm while a characteristic cyclopropane proton was seen as a broad apparent quartet at -0.35 ppm. The other cyclopropane protons were observed at 0.50-0.57 ppm and 0.58-0.66 ppm. ^{13}C NMR spectrum confirmed the ^1H NMR structural analysis. The free carboxyl group was seen at 177.2, shifting slightly downfield after deprotection. The methoxy ether carbons were observed at 57.7 ppm and 85.6 ppm while the β -OH carbon was at 72.1 ppm. The methyl ester carbon at 51.5 ppm had disappeared.

Thiols readily form disulfides as discussed in **Section 3.2.4.2** below. It has even been observed that the TBAH hydrolysis reaction also yields an alternative compound so far suspected to be the disulfide **160** as the major product.* From the experimental data obtained in this project it is believed with high degree of certainty that compound **74** is the target thiol. Firstly, all the ^1H NMR integrals of the diagnostic peaks gave the expected number of protons for a thiol compound with structure **74**. The CH_2S triplet of 159 shifted from 2.84 ppm to the main chain α carbon proton multiplet region (2.41-2.48 ppm) where it was not clearly discernible. However, integration of this region showed that it represented three protons. This multiplet region was also partially obscured by a broad peak which was suspected to be that of the SH. A D_2O exchange experiment resulted in the disappearance of this broad signal, implying that it belonged to the SH. In a personal communication with Professor Baird the suspected disulfide compound did not give a CH_2S signal at around 2.5

* Personal communication with Professor Mark Baird and his research group.

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ppm but instead the signal was observed further downfield between 2.6 and 2.7 ppm. Also, a comparison of the R_f of the thiol **74** obtained in this project and the suspected disulfide obtained by Professor Baird's group in Bangor showed a large difference.* The thiol **74**, as reported above, had a R_f of about 0.20 while the disulfide was 0.76 using a hexane : ethyl acetate (8 : 2) solvent system. As is obvious from the structures of the thiol **74** and the disulfide **160** the former will be more polar and should run lower on a normal silica TLC plate with the solvent system used. A final observation made was a difference in solubility in the two compounds. While **74** was readily soluble in the halogenated solvents and warm hexane, like other non-thiolated MAs, **160** was insoluble in these solvents. Solvation was only achieved after heating the suspension and **160** rapidly precipitated on cooling.

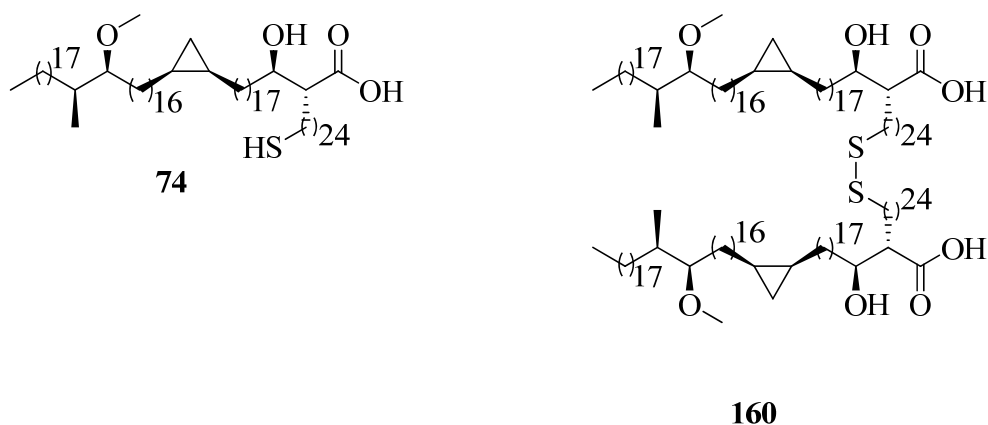


Figure 3.5: MA thiol **74** and MA disulfide **160**.

Several MALDI-TOF experiments were run on **74** and the suspected disulfide. These did not produce any accurate mass ion data. Methyl ester derivatives of these thiolated compounds also failed to give any accurate data. A mass spectrometer analysis would have given a conclusive distinction of the products obtained from the TBAH hydrolysis. The

* The alternative product of the TBAH hydrolysis, suspected to be compound **160**, was not directly synthesized in this project but was obtained by Professor Baird's research group using TBAH as described here to deprotect **159**. A 2 mg sample of **160** was provided to us in Pretoria to conduct a comparative R_f study in order to determine whether the compounds were indeed different. It was on this sample that the physical properties of **160** reported here were made. The ^1H chemical shift data were obtained from personal communications with Professor Baird.

only explanation so far is that the thiolated molecules might be fragmenting during the MS experiments.

3.2.4.2 Stability of Product

Thiols are notorious for their short shelf life under ambient conditions. They slowly oxidize to disulfides. MA thiol **74** prepared in the first TBAH reaction above was stored under argon at about -8 °C. After 6 weeks it was observed by TLC that much of the thiol had ‘decomposed’ to what was suspected to be the disulfide. This new product had a R_f of 0.84 compared to 0.20 for the thiol **74** in hexane : ethyl acetate (8 : 2). After purification the recovered thiol **74** was less than 15% of the original amount.

For the purpose of immobilization on a gold substrate oxidation to the disulfide is of little concern except for possible solvent solubility issues. The thiol **74** is insoluble in the alcoholic solvents methanol and ethanol, has limited solubility in hexane which increases at elevated temperatures but dissolves well in the ethereal and halogenated solvents even at ordinary room temperatures. However, the huge MA-S-S-MA **160** is insoluble in almost all of these solvents at normal room temperatures. Thus, it is advised that deprotection to the thiol should be carried out as close to the time of use as possible. The current observations indicate that dimerization might occur even under inert atmosphere after a prolonged period; hence long term storage under an inert gas does not eliminate this problem.

3.3 Conclusion

Tosylation of the MA alcohol **155** and thiolation with potassium thioacetate was a simple and high yielding thiolation process. The selection of protecting groups also allowed for a relatively easy deprotection process. The thioester and methyl ester are both base labile and this meant that only two deprotection steps were required to obtain **74** from the fully protected **158**. Complications with spontaneous oxidation of the free thiols show that final deprotection should only be carried out when the compound is required. This effectively argues against a strategy of introducing the SH directly rather than through a thioacetate.

4 Chapter Four: Characterizing and Interrogating Alkanethiol SAM on Gold Substrate

4.1 Introduction

The thiolated MeO-MA **74** synthesized in the last chapter was immobilized on a solid gold substrate from a chloroform solution. The self-assembled monolayer (SAM) created is intended to be used as a stable antigenic surface to improve the biosensor-based TB diagnostic technologies developed at the University of Pretoria.^{36,112,117} In this chapter, the thiolated MeO-MA SAM (referred to as MASH SAM below) was characterized by various analytical techniques to investigate its integrity and stability.

Several techniques are available to characterize the chemical and physical architecture of thin films formed on gold substrate. The choice of technique is heavily influenced by the chemical properties of the immobilized molecules. Many of the techniques have been reviewed extensively¹²² but those that will be applied in this research project are discussed below. These analytical methods were applied by Mathebula *et al.* to characterize their new MA-based TB diagnostic.^{112,117}

The first technique discussed is X-ray photoelectron spectroscopy (XPS). It was used primarily to confirm that the MASH was covalently bound to the gold substrate. Next, the atomic force microscope was used to graphically visualize the SAM topography. Two electrochemical techniques, cyclic voltammetry and electrochemical impedance spectroscopy, were used to interrogate the electrical properties of the SAM. These last two techniques are particularly significant because they were used by Mathebula *et al.* to determine the TB status of serum samples.^{112,117}

4.1.1 X-ray Photoelectron Spectroscopy

X-ray photoelectron spectroscopy is an analytical technique used to determine the chemical

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composition of surfaces. Developed in 1967 by Kai Siegbahn at the University of Uppsala, Sweden,¹⁸⁵ it uses a beam of X-rays to eject electrons from atoms in the top 1 to 10 nm of the material. A detector measures the kinetic energy and number of electrons released.

The XPS spectral lines are identified by the shell from which the electron is ejected. The core or inner electrons have binding energies characteristic of the particular element. This is in contrast to the near-Fermi level or valence electrons which move randomly in space and carry little specific binding information.

An incident X-ray photon ejects a photoelectron with kinetic energy:

$$\mathbf{KE} = \mathbf{h\nu} - \mathbf{BE} - \mathbf{\Phi} \quad \text{Equation 4.1}$$

where **KE** is electron kinetic energy, **h** is Planck's constant, **v** is frequency of light, **BE** is electron binding energy, and **Φ** is the spectrometer work function. The monovalent cation emits another electron, an Auger electron, to become divalent. To do this an Auger electron from an outer shell will fall to the inner shell, which is a lower energy level, to replace the ejected photoelectron [Figure 4.1] while another Auger electron will be emitted to conserve energy released in this process according to the equation:

$$\mathbf{KE} = \mathbf{E(K)} - \mathbf{E(L_1)} - \mathbf{E(L_2)} \quad \text{Equation 4.2}$$

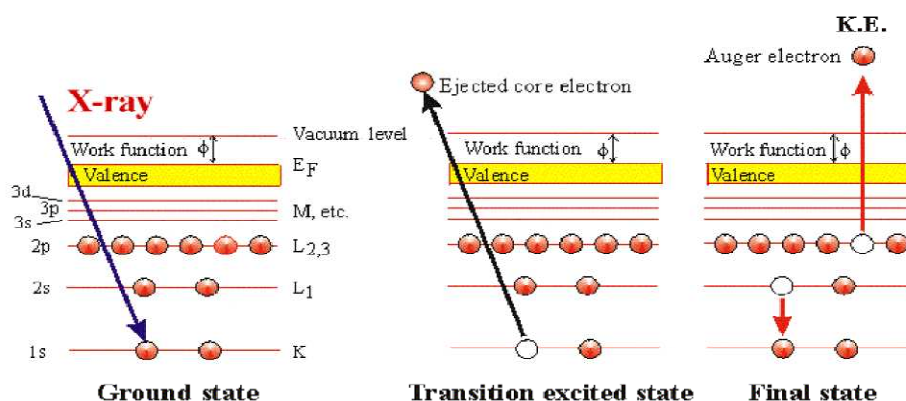


Figure 4.1: Schematic diagram of the basic principle of XPS.¹⁸⁶

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The observed XPS signal is a combination of both the photoelectron and Auger kinetic energies. While the photoelectron energy is observed as a strong sharp line the Auger energy signal is broader and less prominent. The energies are typically recorded as eV with binding energy increasing from right to left and kinetic energy increasing from left to right on the abscissa.

Unlike other spectroscopic methods XPS requires only that the incident photon be of sufficient energy to eject an electron; it does not have to be in resonance with the transition energy. Also, the kinetic energy of the ejected electrons is measured, not absorbance or transmittance of photons.

Accurate analysis is aided by using different vacuum pump systems even up to ultra high vacuum conditions. The sample is also bombarded with argon ions to remove any environmental contaminants.

4.1.2 Atomic Force Microscopy

Atomic force microscopy (AFM) is a relatively modern surface analytical technique. Unlike the older STM (Scanning Tunneling Microscopy), which requires the sample to be conductive, AFM measures attractive or repulsive forces. These forces often include Van der Waals, magnetic, electrostatic, capillary, ionic or frictional forces, measured as interactions between a sharp probing tip attached to a cantilever and the sample surface [Figure 4.2].¹⁸⁷ Deflections of the cantilever are digitized to create an image of the surface. The z-movement is a function of the lateral x, y-movement of the lever. Quantitative data about roughness and height of sample surface are obtained.¹⁸⁷

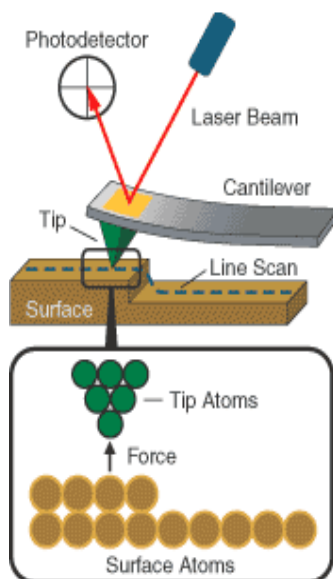


Figure 4.2: Schematic diagram of basic working principle of AFM.¹⁸⁸

When run in non-contact mode the tip-to-sample separations can be between 10-100 nm, while in contact mode the tip is only several angstroms away from the sample. Non-contact mode provides information about surface topography, distributions of charges, magnetic domain wall structure or liquid film distribution. Contact mode images can give images of atomic resolution. The imaging processes are non-destructive.

AFM is often used in combination with other scanning probe microscopy (SPM). The most successful is AFM and STM. This allows, respectively, for both non-conductive and conductive samples to be analyzed. Other combinations include AFM/ MFM (Magnetic Force Microscope) and AFM/ FFM (Friction Force Microscope).

Samples do not require any pre-analysis preparations. Unlike electron microscopes it can analyze samples in ambient air and liquid, avoiding expensive vacuum pumps. This is crucial for studying live biological samples. Usually though, the microscope is housed in an acoustically designed box which minimizes the effects of environmental noise.

AFM is well applied in analysis of organic samples like Langmuir-Blodgett films. It is used in the study of unfolding of proteins, imaging biomolecules, antibody-antigen binding interactions, *etc.*

4.1.3 Cyclic Voltammetry

Cyclic voltammetry is often used to investigate the electrochemical properties of a substance. An electric potential applied to a working electrode is ramped linearly over a time period and when a pre-set potential is reached the ramping, or scan rate, is reversed. Often, three electrodes are involved: the reference, working, and auxiliary electrodes.

The working electrode usually is made of glassy carbon, platinum, silver, or gold. An insulating and inert material encapsulates the electrode such that only a disk of about 2 mm diameter is exposed at the end which is dipped into the electrolyte [Figure 4.3].



Gold sensor surface

Figure 4.3: A working electrode with a gold sensor surface.

The reference electrode has a stable and known electrode potential. It is used as a half cell to determine the potential of the working electrode. The counter electrode is made of any convenient conductive chemically inert material like platinum wire which will not react with the bulk electrolyte solution. It is very useful when charge-sensitive biological materials like proteins or cellular matter is being investigated.

The Faradaic current conditions of CV depend on the kinetics of electron transfer and the rate at which a redox species diffuses to the surface of the working electrode. Although capacitive current is present it is relatively small compared to Faradaic current from electron transfer.

$[\text{Fe}(\text{CN})_6]^{3-} / [\text{Fe}(\text{CN})_6]^{4-}$ is usually used as a redox couple in many CV experiments. The kinetics of electron transfer for this redox species is fast; hence the concentrations at the electrode surface can be represented as:

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$$E = E^0 - 0.0592 \log \left(\frac{[Fe(CN)_6]^{-4}}{[Fe(CN)_6]^{-3}} \right)$$

Equation 4.3

where E is the applied potential and E^0 is the formal electrode potential. This is the Nernst equation. From this relationship, it is obvious that the concentration of the oxidized species, $[Fe(CN)_6]^{3-}$, will decrease as the negative potential is ramped linearly to a more negative value. Assuming that the electron transfer rate is very rapid, the measured current i is directly proportional to the diffusion rate, or flux, J of the oxidized species to the electrode surface:

$$i = kJ$$

Equation 4.4

where k is a constant represented by $n \times F \times A$ where n = number of electrons, F = Faraday's constant, A = area of the electrode surface. Therefore **Equation 4.4** can be written as:

$$I = nFAJ$$

Equation 4.5

Fick's Law of Diffusion is represented by:

$$J = -D \left(\frac{dC}{dx} \right)_{x=0} \cong D \frac{(C^* - C_{x=0})}{\Delta x}$$

Equation 4.6

where D is the diffusion coefficient of the species, x is the distance from the electrode surface, $(dC/dx)_{x=0}$ is the concentration gradient at the surface, C^* is the concentration of the oxidized species in the bulk solution, and $C_{x=0}$ is its concentration at the surface. It predicts that the greater the concentration gradient, the greater the flux J and hence the greater the cathodic current.

The uniform bulk concentration, C^* , changes such that the oxidized species at the electrode surface are depleted as the electrode is ramped with a higher negative potential. This

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creates a concentration gradient that induces an increased flux to the surface until the concentration of the oxidized species is zero. The concentration gradient will weaken, which means current, being proportional to flux J , will decrease. When this cathodic process is reversed in CV, an anodic current takes over and a mirror profile is observed for the reduced species. This reversible cyclic ramping is generically represented by the cyclic voltammogram shown in **Figure 4.4**.

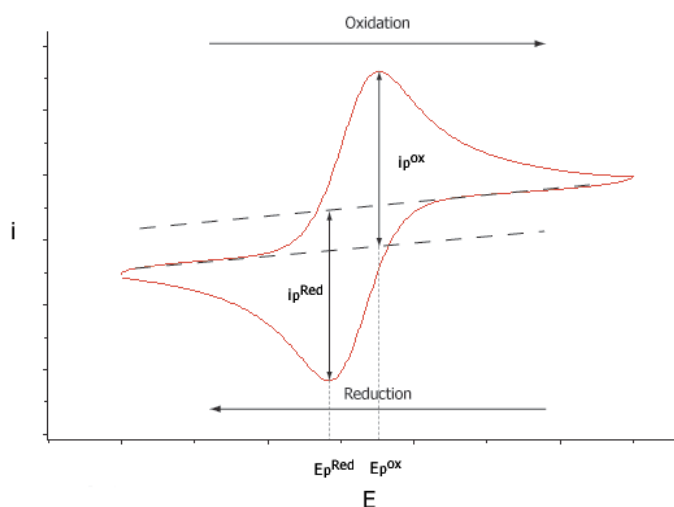


Figure 4.4: A typical cyclic voltammogram for a reversible single electron transfer. (i_p = peak current)¹⁸⁹

It must be noted that a perfectly Nernstian system is an ideal scenario which is never achieved, because electron transfer between an electrode and a redox species is much slower than assumed above. An approximation is possible by slowing the ramping. This allows the current flow rate to be fast compared to the diffusion rate.

Modification of the bare gold surface could increase electron transfer resistance measured by cyclic voltammetry and electrochemical impedance spectroscopy.

4.1.4 Electrochemical Impedance Spectroscopy

Electrochemical impedance spectroscopy (EIS) is a valuable electrochemical method used to experimentally characterize the dielectric properties of a substance. It measures the electrical impedance of a sample as a function of frequency. Impedance is the alternating current (AC) equivalent of the direct current (DC) electrochemical cell resistance. Mathematically, impedance (Z) is related to potential (E) and current (I) by Ohm's Law:

$$Z = E/I$$

Equation 4.7

As described for cyclic voltammetry above, the flow of electrons in a DC electrochemical cell is limited by the electrode kinetics, the nature of the electrochemical reactions occurring in the cell and the flux of redox species from the electrodes. Similarly, resistors, capacitors, and inductors hinder the flow of current through an AC circuit. The applied potential of EIS is very small compared to CV. The voltage probe signal is usually between 1-10 mV which is low enough to prevent perturbation of the sample as in CV. This is crucial to investigating biological molecules like MA and the protein antibodies that will be used for diagnostic purposes.^{112,117}

EIS data are often represented either as a Nyquist plot (also referred to as a Cole-Cole plot) or the Bode plot [Figure 4.5]. The Bode plot will not be used to analyse the EIS data of the MASH SAM in this project.

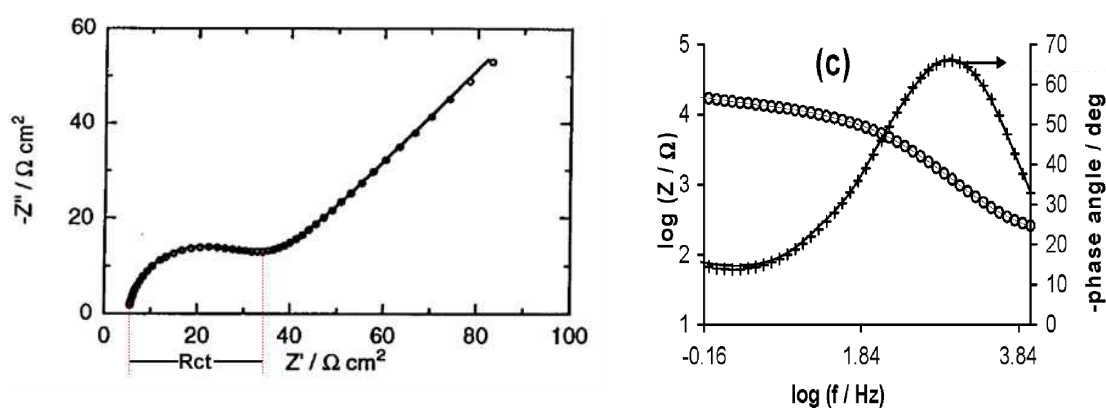


Figure 4.5: A. Simple Nyquist plot. B. Simple Bode plot.¹¹²

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The Nyquist plot presents an imaginary impedance, Z'' , against the real impedance, Z' , at each excitation frequency such that the frequency range decreases from left to right. Characteristically, Nyquist plots exhibit semicircular plots at high frequencies and straight lines at low frequencies.

The most significant data derived from the Nyquist plot is the charge transfer resistance R_{ct} [Figure 4.5A]. It is a quantitative measure of the resistance (impedance) to charge transfer between an electrode and the redox couple. This parameter was used by Mathebula *et al.* in their innovative diagnostic EIS-based test to differentiate between TB-positive and TB-negative patient sera.^{112,117} This test was described in details in Section 1.5.2.

The Bode plot presents the impedance $|Z|$ as a function of frequency, ω [Figure 4.5B]. It offers a visual image of how impedance changes with respect to frequency.

4.2 Results and Discussion

4.2.1 Fabrication of SAM on Gold Substrates

To investigate the physico-chemical properties of a thiolated MA on a solid gold substrate MASH was immobilized on SPR and voltammetry electrode gold disks [Figure 4.6]. The SPR gold disk was used in X-ray photoelectron spectroscopy (XPS) and atomic force microscopy (AFM) analyses while the gold electrode was used in electrochemical studies.



SPR gold disk



Gold disk electrode

Figure 4.6: Gold substrates on which thiolated MA was immobilized.

4.2.2 X-ray Photoelectron Spectroscopy

XPS analysis of the surface of SPR gold disks fabricated with thiolated MeO-MA **74** (MASH) SAM was performed with an ALK α instrument at a photoelectron take-off angle of 45°. **Figure 4.7** shows a survey spectrum of the MASH-Au surface with the peaks referenced to the Au $_{4f7/2}$ signal shift at 84.1 eV. A well resolved peak of the C(1s) photoelectron binding energy appears at 285.1 eV. This confirms the presence of sp³ carbon species in the molecule immobilized on the gold surface. In **Figure 4.8** computer-generated peak fits of the different carbon species are presented in a multiplex spectrum. The aliphatic carbons, –C–C–, gave a characteristic peak at 284.8 eV. The oxygenated carbon species appear at higher energies. The probable bonds for the signals at 289.0 eV, 287.9 eV, and 286.45 eV are O–C=O, –C=O, and C–O, respectively. The C–O could be either an ether or an alcohol; the former is suspected in this case because the MASH has a methyl ether.

Although these observations indicate the presence of the MA on the gold surface they did not provide information of covalent immobilization of the acid on the gold substrate. Binding energy of the sulfur to gold as indicated by the S(2p) signal at approximately 162.5 eV was conspicuously absent. According to Biebuyck *et al.* the S(2p) photoelectron signal of the gold-thiolate bond appears at 162.5 eV.¹²³ A probable explanation for this is that the signal might have been attenuated such that it is lost in the background noise.

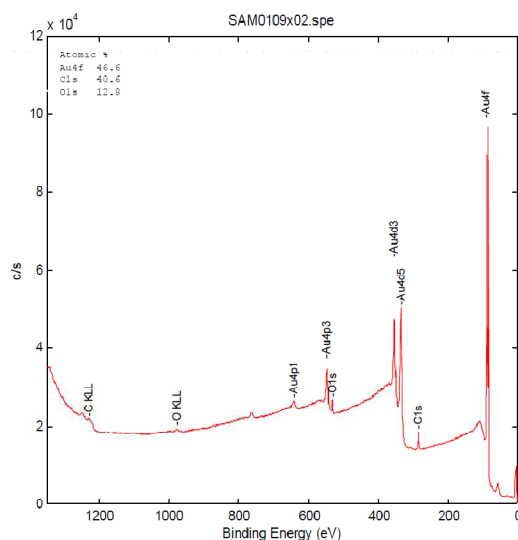
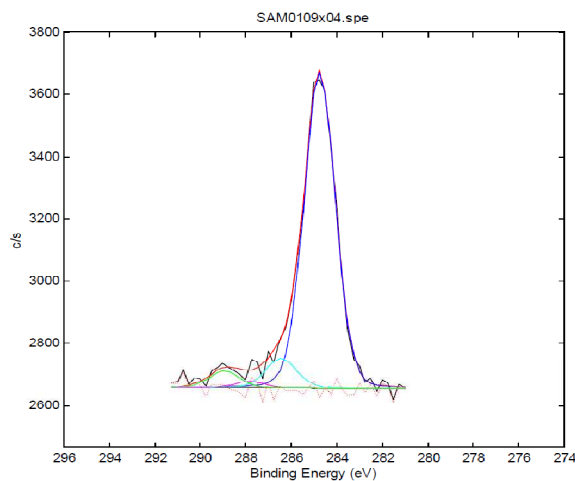


Figure 4.7: XPS survey spectrum of MASH-Au. (c/s = electron counts per second).



Binding Energy (eV)	Probable Bond
284.75	C—C
286.45	C—O—C
287.85	C=O
288.95	O—C=O

Figure 4.8: Multiplex XPS spectrum for expanded C(1s) region of MASH-Au. (Table cell colour match bond peak colour in the graph. c/s = electron counts per second)

Binding, or kinetic, energy intensity is attenuated when an abstracted photoelectron has to take a long path from deep in the surface layer before escaping. XPS is most effective for the detection of the top 2-3 atomic monolayers on a substrate. Collisions with electrons of

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atoms of upper layers dissipate the energy of the inner layer photoelectron such that the energy detected by the electron analyzer is non-characteristic of the element. It instead contributes to the noise of the spectrum. This phenomenon is explained by Beer-Lambert's rule [$I_s = I_0 e^{-d/\lambda}$ Equation 4.8] where λ is the inelastic mean free path of an electron in a solid, I_0 is the initial energy intensity of the photoelectron at sampling depth, d , and I_s is the intensity at the surface. The deeper in the layer the photoelectron originates the lower will be the observed I_s [Figure 4.9].

$$I_s = I_0 e^{-d/\lambda}$$

Equation 4.8

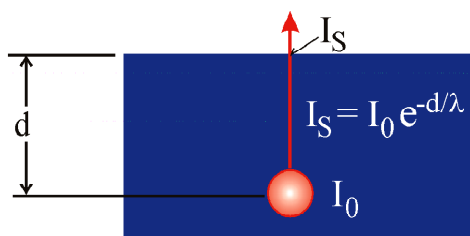


Figure 4.9: Attenuation of photoelectron energy described by Beer-Lambert equation.¹⁸⁶

Mathebula *et al.* reported 'broadening' of the S(2p) signal when an extra layer of 18 carbons of stearic acid is coupled to cysteamine. They explained this loss of signal by suggesting that SAM structural rearrangement might have occurred during the coupling process.¹¹² In the publication by Bain *et al.* the S(2p) signal was also not easily detectable for C₈-C₁₀ alkanethiols [Figure 4.10].¹⁹⁰

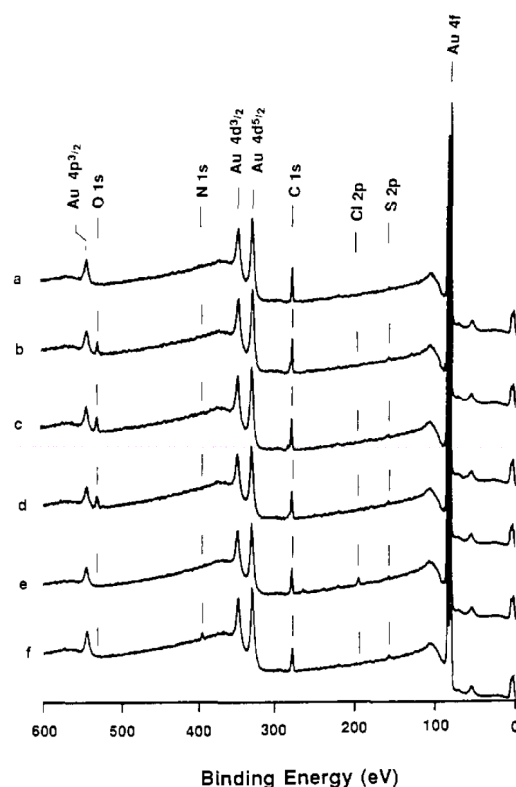


Figure 4.10: XPS survey spectra of thiol monolayers on gold. (a) $\text{HS}(\text{CH}_2)_{10}\text{CH}_3$, (b) $\text{HS}(\text{CH}_2)_{10}\text{CH}_2\text{OH}$, (c) $\text{HS}(\text{CH}_2)_{10}\text{CO}_2\text{H}$, (d) $\text{HS}(\text{CH}_2)_{10}\text{CO}_2\text{CH}_3$, (e) $\text{HS}(\text{CH}_2)_{10}\text{CH}_2\text{Cl}$ and (f) $\text{HS}(\text{CH}_2)\text{CN}$.¹⁹⁰

The experiment was repeated with a MgK_α X-ray XPS instrument at a takeoff angle of 45° . MgK_α give better signal resolution than AlK_α X-rays. The putative $\text{S}(2p)$ signal of the gold-thiolate bond was observed at 162.3 eV [Figure 4.11]. This confirmed that the MASH was indeed covalently bound to the gold surface.

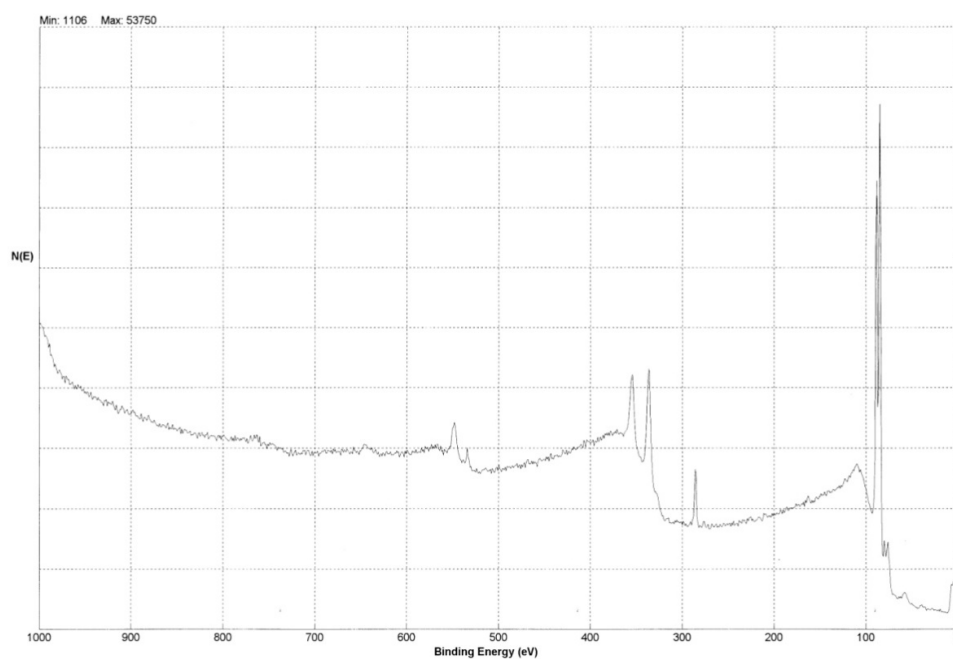


Figure 4.11: MgK α XPS survey spectrum of MASH-Au surface. ($N(E)$ = number of electrons per second)

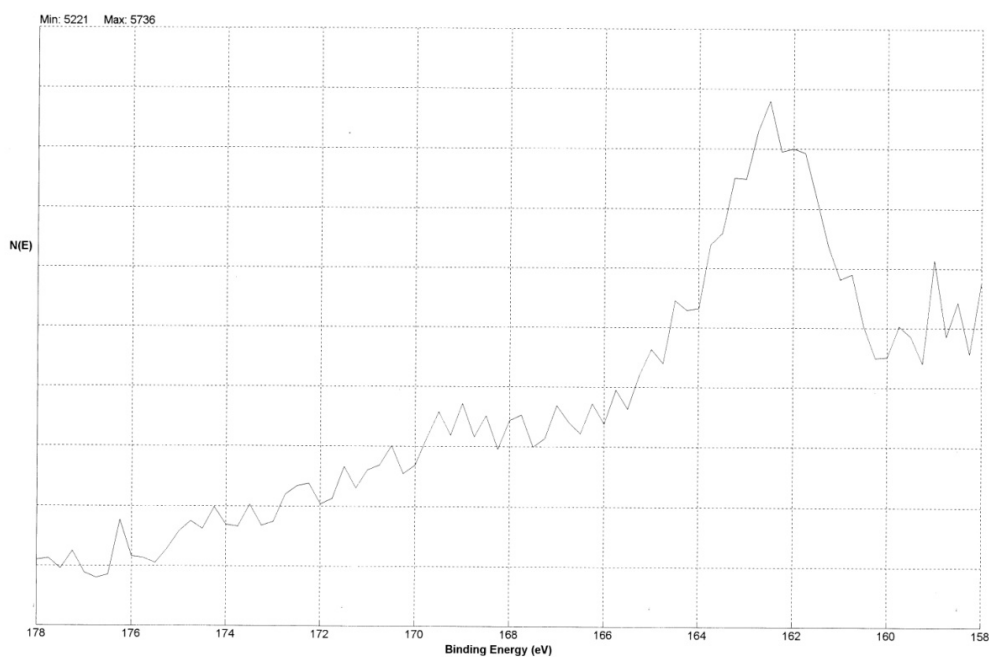


Figure 4.12: Multiplex spectrum of thiolate S(2p) signal of MASH-Au. ($N(E)$ = number of electrons per second)

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Attenuation of the Au signal observed in the bare gold however provided further evidence of the presence of an overlayer on the substrate [**Figure 4.13**]. The Au_{4f} spectrum has a doublet structure due to the presence of $Au_{4f7/2}$ and $Au_{4f5/2}$ peaks. The peak signals appeared at 84.1 eV and 88 eV, respectively. The $Au_{4f7/2}$ of bare gold was weakened from 48575 c/s to 39978 c/s.

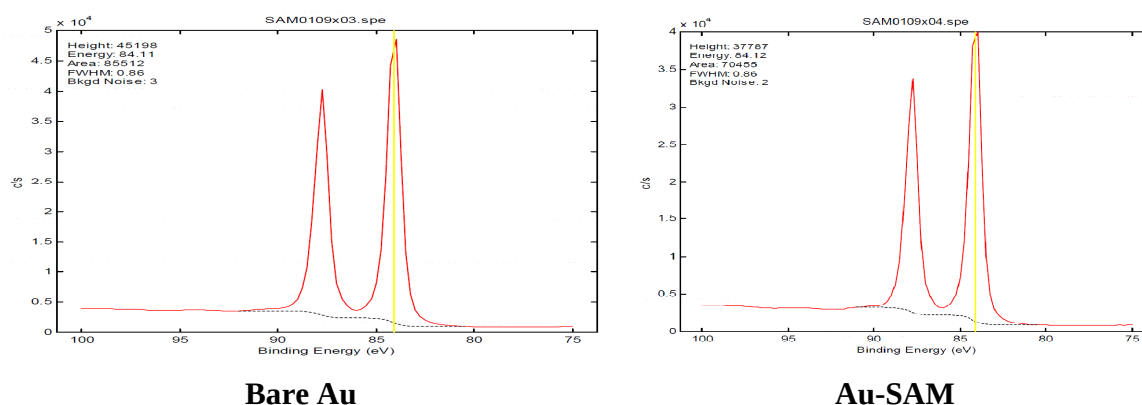


Figure 4.13: XPS spectra of Au_{4f} doublets of bare gold and Au-SAM.

4.2.3 Atomic Force Microscopy

The topography of the MASH SAM on a SPR gold sensor disk was investigated by tapping mode AFM [**Figure 4.14**]. The bare gold surface height was determined to be 6 nm. To obtain maximum surface coverage the gold disk was incubated for a week in a 1 mM chloroform solution of the MASH. The MASH overlayer mean height was measured to be 3-4 nm, **A** [**Figure 4.15**]. The maximum height was 6.75 nm, **B**. The trough observed at **C** is an area of etched gold. These are visible as deep streaks in **Figure 4.14**.



Figure 4.14: AFM phase image of SAM of MASH.

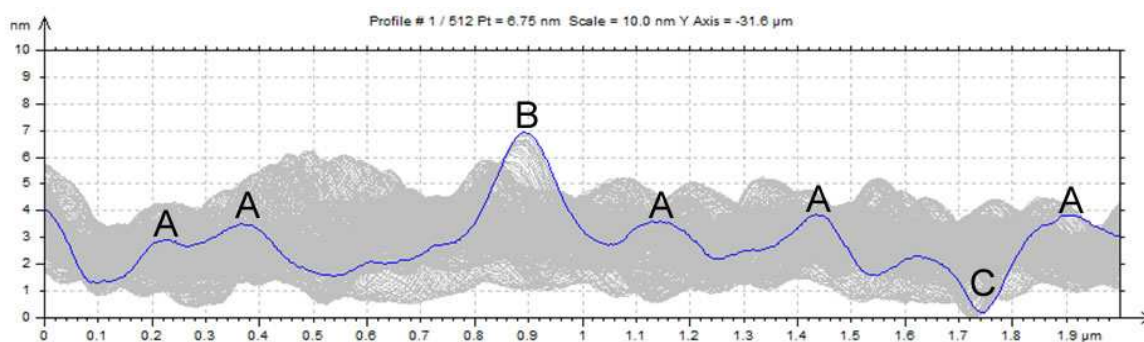


Figure 4.15: Cross-section profile of MASH overlayer.

The data acquired correlated well with the report of Mathebula *et al.*¹¹² They reported that the height of the Au-cysteamine surface was increased by 3.4 nm after immobilization of MA. This falls in the average height range of 3-4 nm observed for the directly immobilized MASH. The difference in the measured heights might be due to the partial insertion into the cysteamine monolayer of the MA rather than the interfacial interaction with the gold surface in a directly immobilized MASH. It may further be proposed that the maximum height of 6.75 nm is due to multiple layers of MASH interacting hydrophobically via Van der Waal's attraction.

It is difficult to postulate what the folding pattern of the molecules is on the gold substrate. From the works of Hasegawa, Villeneuve, and Prinsloo it would be expected that the MeO-MA will adopt a multiple folding conformation to maximize interactions between the hydrophobic chain segments of the molecule. It must be quickly emphasized, however, that the present results cannot be directly related either to the works of Hasegawa or Prinsloo.¹⁵⁸⁻¹⁶⁰ Those experiments were either done with heterogeneous mixtures of natural MAs on non-gold surfaces or in virtual experiments. This highlights the uniqueness of the current observations and data and further investigations will be required to obtain more insight.

4.2.4 Cyclic Voltammetry

Cyclic voltammetry (CV) is one of two electrochemical techniques used to probe the MASH SAM. Electroimpedance spectroscopy (EIS), the other technique, will be discussed later. These methods have gained wide acceptance in recent literature for characterization of monolayers deposited onto solid substrates and, in the case of EIS, as powerful immunosensors.¹⁹¹ In the experimental results discussed below the MASH SAM was investigated by these two methods. A SAM of octadecanethiol (ODT), one of the most commonly used n-alkanethiols, is also studied as a comparative standard to help understand the electrochemical behavior of MASH SAM on gold. A third SAM system consisting of a mixed monolayer of MASH and ODT was also studied to explore the ability of the MASH SAM to accommodate long chain thiols. This might prove to be crucial for the antigenicity of the MASH-Au system as the mimicry of biological membranes require a mixed monolayer system for biological activity.¹⁹²

The bare gold was cleaned before every experiment. The cleaning process is a rigorous protocol that involves physical, chemical, and electrochemical cleaning. Details of this protocol are given in **Section 6.2.2** below. The underivatized gold electrode was then incubated in either an ethanolic solution of ODT or a chloroform solution of MASH.

CV is most useful for tracking changes occurring on the electrode as successive fabrication steps modify the gold surface. **Figure 4.16** shows an overlay plot of the cyclic

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voltammograms of the gold electrode before and after it was modified with ODT, MASH, and MASH/ODT mixed monolayers. The redox probe $[\text{Fe}(\text{CN})_6]^{3-}/[\text{Fe}(\text{CN})_6]^{4-}$ reveals a reversible process in all the experiments.

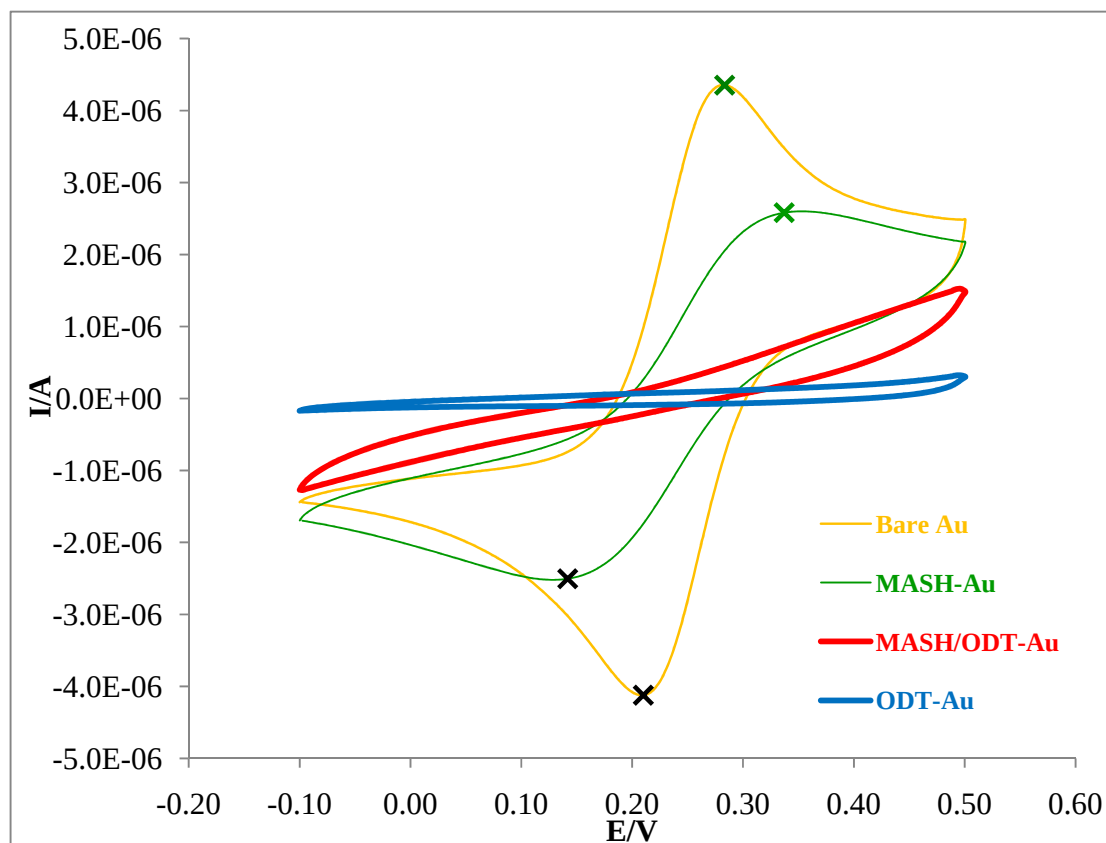


Figure 4.16: Overlay plot of CV voltammograms of bare gold and SAM-fabricated gold electrodes. (x = cathodic peak potential, x = anodic peak potential)

Alkanethiols are known to form quasi-crystalline SAMs relatively rapidly and their electrochemical behaviors are well studied.¹⁹² A fully formed alkanethiolate SAM lacks pinholes and there is almost total resistance to charge transfer between the underlying gold and the redox probe solution. ODT was used as a standard to which the MASH SAM could be compared.

When the bare gold was incubated in a 10 mM ethanolic solution of ODT for 12 h and investigated with CV the voltammogram assumed an almost linear shape. A 24 h

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incubation period would have resulted in a horizontal linear plot representative of a total lack of charge transfer between the gold and the redox probe.¹⁹³ A similar incubation process of 0.5 mM chloroform solution of MASH was also carried out but for 24 h. The longer incubation period for the MASH was chosen because a lower concentration was used and because it was anticipated that the longer MASH chains would not readily pack into a neat array as the ODT due to their inclination to adopt folded conformations. The CV plot of MASH-Au system gave a profile indicative of the formation of a SAM. Evidence of resistance to electron transfer caused by SAM fabrication of the gold surface is seen in the change in the peak-to-peak separation of electric potential (ΔE_p). The bare gold had a separation of the minimum and maximum peak electrochemical potentials of 73 mV while the MASH-modified surface had a separation of 222 mV [**Figure 4.16**]. The ΔE_p of the bare gold is very close to the theoretical value of 59 mV for a single electron transfer reaction process. This confirms the cleanliness of the bare gold surface prior to immobilization of MASH.

The sigmoidal shape of the plot supports the hypothesis that the MASH will not form an ordered SAM like ODT. Instead, there are areas of structural defects and exposed gold. These electro-active sites are responsible for charge transfer and are known as pinholes.¹⁹⁴ The lack of discernable peak potentials in the ODT-Au electrode means a severe limitation on charge transfer between the gold and the redox probe solution. This is an indication that the ODT SAM system almost lacked any pinholes.

Pinholes are sites of coverage defect on the gold surface that allow for the electrode to be in contact with the redox solution. When these sites are surrounded and insulated by the inert alkanethiol, microelectrodes are formed. If it is assumed that the MASH adopt any of the multiple folded conformations predicted by Prinsloo then it is easy to understand why pinholes occur in the SAMs of these molecules.¹⁶⁰ A folded MASH will create a region on the gold surface that disallows another MASH to bind. The need to maximize Van der Waals interactions between the hydrophobic chain segments leads to aggregation of the MASH molecules. Evidence of this is observed in the AFM cross-sectional profile of the SAM [**Figure 4.15**]. These aggregates appear as mounds giving an undulating topography.

The ΔE_p is significant for determining the extent of separation of pinhole sites. An increase in ΔE_p represents an increase in the distance between pinholes. With a homogeneous

distribution of pinholes in a monolayer there is an inverse relationship between the number of pinholes and the distance between them.^{194,195} The fewer the pinhole sites the larger the distance between them. It can thus be argued that as the number of pinholes tends towards infinity so does the distance between them approach zero. Hence, a bare gold surface can be seen as an infinite number of contiguous pinholes or micro-electrodes.

In the data presented above it was noted that ΔE_p increased significantly when the bare gold was incubated in MASH. The sigmoidal shape of the CV suggested the presence of pinholes and when the bare gold was fabricated with ODT, ΔE_p was much higher still. The ability of the MASH pinhole system to accommodate the straight-chain ODT was then investigated.

The MASH-derivatized gold electrode was incubated in a 10 mM ethanolic solution of ODT for 12 h. The time period was chosen to allow for comparison with the ODT SAM prepared earlier. **Figure 4.16** shows a significant change in the CV. The ΔE_p was greater than 0.40 V. This suggested that the number of pinhole spaces had reduced. The shorter chain ODT might have, as expected, inserted themselves into the pinhole spaces of the MASH SAM.¹⁹⁶

This result also presents other interesting facts about the MASH-Au system. Firstly, the ODT was unable to displace the MASH thiolate from the gold surface. This provides strong support for a covalent link between MASH and gold suggested by the XPS data presented above. Long chain alkanethiols are able to displace non-covalently bound molecules from the gold surface.¹⁹⁰ They are able to do this efficiently because of the very strong affinity of thiols for gold. If the ODT had displaced the MASH the CV would have more closely resembled the ODT-Au profile [**Figure 4.16**]. Secondly, the ability of the ODT to bind to the MASH-derivatized gold system is significant for the practical application of the new surface mimicking a biological membrane for TB diagnosis. Often, immobilized biological molecules require a co-molecule to be biologically active.¹⁹² The data presented here show that this can be done without dislodging the bound MAs.

Concurrent with the increase in ΔE_p , in an inverse relationship, is the change in the peak current densities. They decreased in the order bare gold > MASH-Au > mixed monolayer-Au > ODT-Au. When the MASH-Au was mixed with ODT the current was approximately

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halved [Figure 4.16]. This further indicates that the ODT had effectively occupied the spaces left in the MASH-Au monolayer that were acting as microelectrodes through which electrons could be transferred between the underlying gold and the redox pair solution.¹⁹⁷

In the CV experiments presented above the ramping potential was 25 mV and it was repeated for 20 cycles. The MASH SAM remained stable during the ramping process. It maintained its stability even when the ramping potential was doubled to 50 mV for 20 cycles. The CV profile maintained its shape [Figure 4.17]. This confirms that the MASH-Au surface possessed electrical stability.

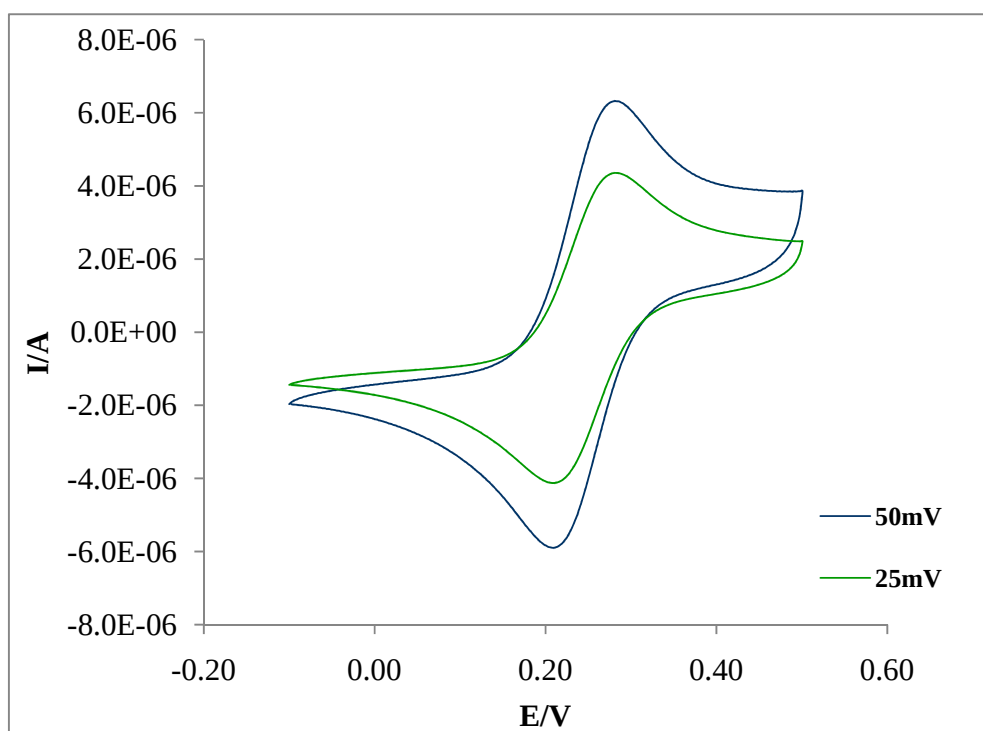


Figure 4.17: CV of MASH-Au at 25 mV and 50 mV.

4.2.5 Electrochemical Impedance Spectroscopy

EIS was also used to characterize the MASH-Au system relative to a bare gold and ODT-Au electrodes and determine their permittivity to single-electron transfer between the gold and the $[\text{Fe}(\text{CN})_6]^{3-}/[\text{Fe}(\text{CN})_6]^{4-}$ redox probe. Often ran with CV during electrochemical

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experiments it provides corroborative data of the electron transfer kinetics and more details about the actual electrochemical reactions occurring at the surface-solution interface.

Resistance to electron transfer is reported as impedance, Z , in the alternating current EIS technique. In the Nyquist plots of the bare and SAM-derivatized gold electrode systems investigated here, the real impedance (Z') is compared to an imaginary ($-Z''$) impedance. An alternating perturbing potential of 5 mV was applied between 10 mHz and 10 kHz at the formal potential of $E_{1/2} = 209$ mV.

Figure 4.18 shows the Nyquist plots of the four different electrode systems studied. The bare gold has a linear profile at approximately 45° angle to the horizontal axis, a characteristic property of an unimpeded transfer of charge between the gold and the redox probe solution. This also shows planar diffusion across the naked gold surface. Fabrication of the gold surface resulted in the evolution of the plot to characteristic semicircular shapes at high frequencies [**Figure 4.18**]. This is indicative of resistance of the charge transfer process.

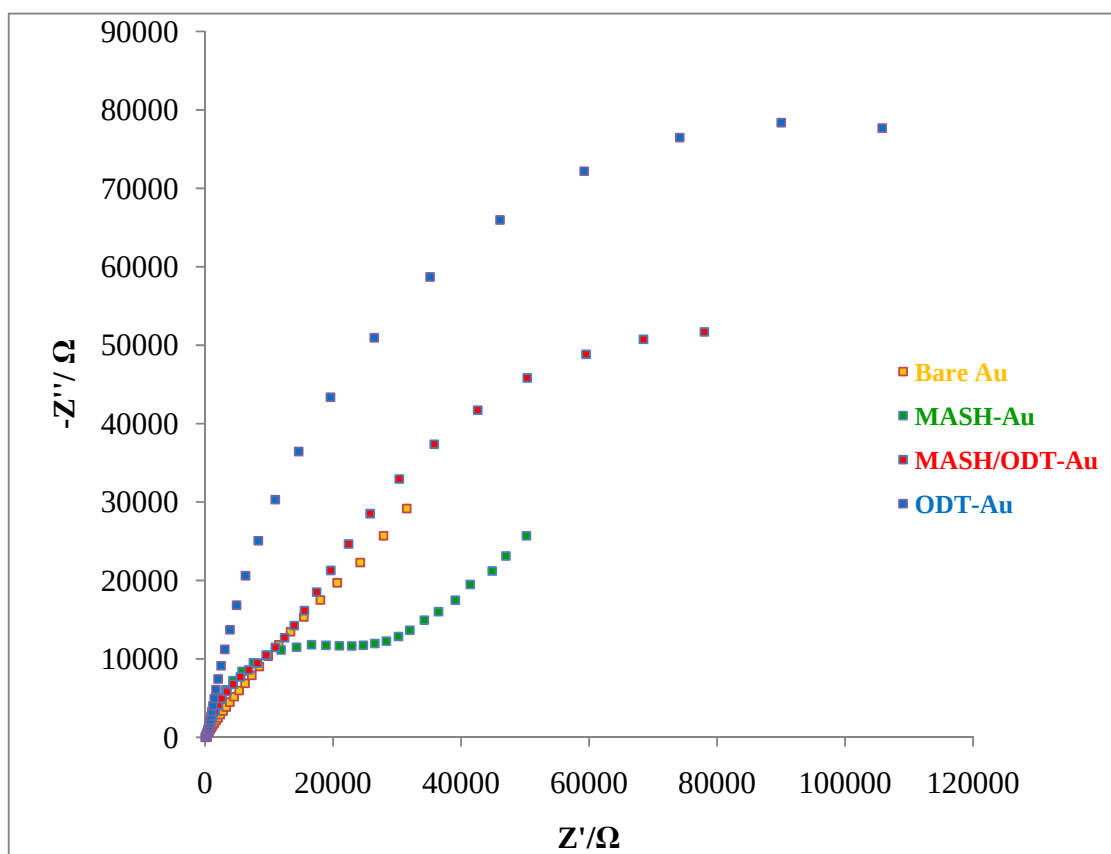


Figure 4.18: Overlay plot of EIS voltammograms of bare gold and SAM-fabricated gold electrodes.

The increased resistance to electron transfer is attributable to the formation of SAM on the gold surface. The MASH SAM plot followed a semicircular path at high frequency (the leftmost segment of plot) which then turns linear at lower frequencies (the rightmost segment of plot).*

Compared to the ODT-fabricated surface the MASH-Au surface allows significantly higher charge transfer. This confirms that while the ODT SAM possesses a neat packing of the linear alkanethiols which almost totally prevents interaction between the $[\text{Fe}(\text{CN})_6]^{3-}/[\text{Fe}(\text{CN})_6]^{4-}$ and the gold, the MASH SAM is irregular in topography. There are areas of easy electron transfer between the gold electrode and redox probe. These areas, described as pinholes in the discussion above, allowed for ODT to bind directly to the gold after immobilization of the MASH. The conclusion that ODT was unable to dislodge the bound

* AC current is applied at a frequency range starting from 10 KHz to 10 mHz.

MASH is supported by the charge transfer resistance of the MASH/ODT mixed SAM being between that of the ODT and MASH SAM systems.

4.2.5.1 Circuit Fit and Charge Transfer Resistance of the MASH-Au

A significant advantage of an EIS experiment is that it provides an opportunity to determine the electrical properties of a SAM system by comparing it to a model electronic circuit consisting of resistors and capacitors [Figure 4.19]. This is done by fitting the Nyquist plot to an appropriate circuit that may consist of different electrical elements. The MASH SAM was fitted to an equivalent circuit consisting of $R_1(Q_1[R_2W_1])$, i.e., two resistors (R), a constant phase element capacitor (Q), and a Warburg impedance (W).

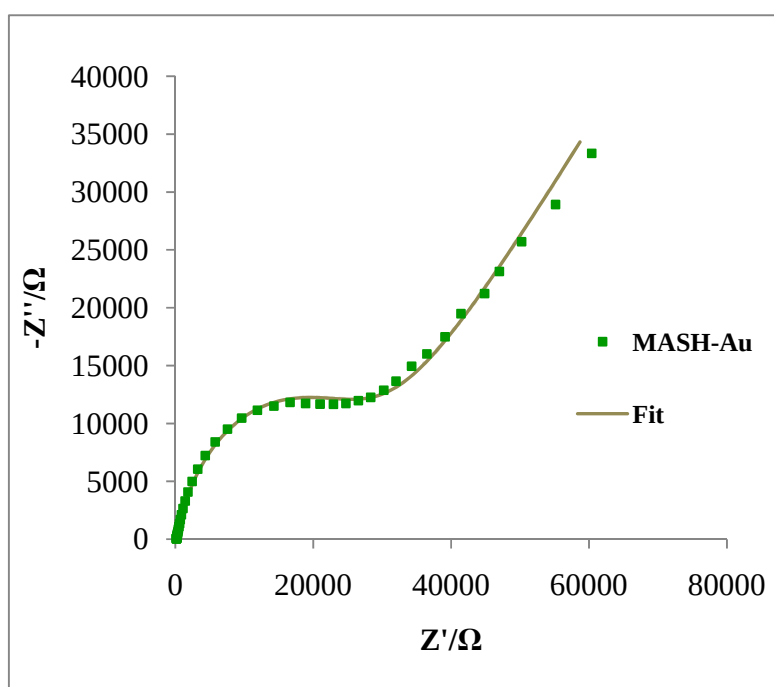


Figure 4.19: Equivalent electronic circuit fit plot of MASH-Au SAM.

The most important quantity calculated from the Nyquist plot of a fitted circuit is the charge transfer resistance, R_{ct} . It is a measure of the resistance to electron transfer provided

by the SAM. The R_{ct} of the MASH SAM system was calculated to be about 27.5 K Ω . The R_{ct} was used by Mathebula *et al.* to differentiate between TB⁺ and TB⁻ patient sera.^{112,117} It is an important value used in the quantitative characterization of SAMs by EIS.

It was not possible to calculate the exact R_{ct} values for the ODT and MASH/ODT SAMs because these systems still impeded electron transfer within the frequency range of 10 mHz to 10 KHz used in this project. It is however obvious from **Figure 4.18** that these values were significantly higher than that of the MASH SAM.

4.2.6 Effect of Purity on SAM Formation

The strong affinity of thiols for gold would imply that the presence of non-thiol compounds in a thiol solution will not interfere with SAM formation. Some of the experiments described above were carried out with the partially purified MA thiol **74** obtained by LiOH hydrolysis in **Section 3.2.4** and whose ¹H NMR is presented in **Figure 3.4**.^{*} To eliminate doubts about the validity of the data obtained the electrochemical experiments were repeated on a pure MA thiol **74** sample which had been fully characterized by NMR spectroscopy.

The electrochemical data of the partially purified and pure samples were very similar [**Figure 4.20**]. It must first be noted that cleaning of the gold electrode creates a surface that might vary between experiments. Hence, every experiment is always preceded by electrochemical scanning of the bare gold on which a thiol is going to be immobilized. This provides a reference to which any derivatization can be compared. In **Figure 4.20** it is obvious the two bare gold surfaces offered different resistances to charge transfer. The bare gold surface on which the pure MASH was immobilized appears to offer less resistance to charge transfer than the gold surface used in immobilizing the partially purified MASH.

^{*} The impurities have been discussed in **Section 3.2.4.1** but the sample of **74** used for SAM formation did not have the solvents which were observed in the NMR spectrum because it was blown with nitrogen while on a heating block at 85 °C for 30 minutes before use.

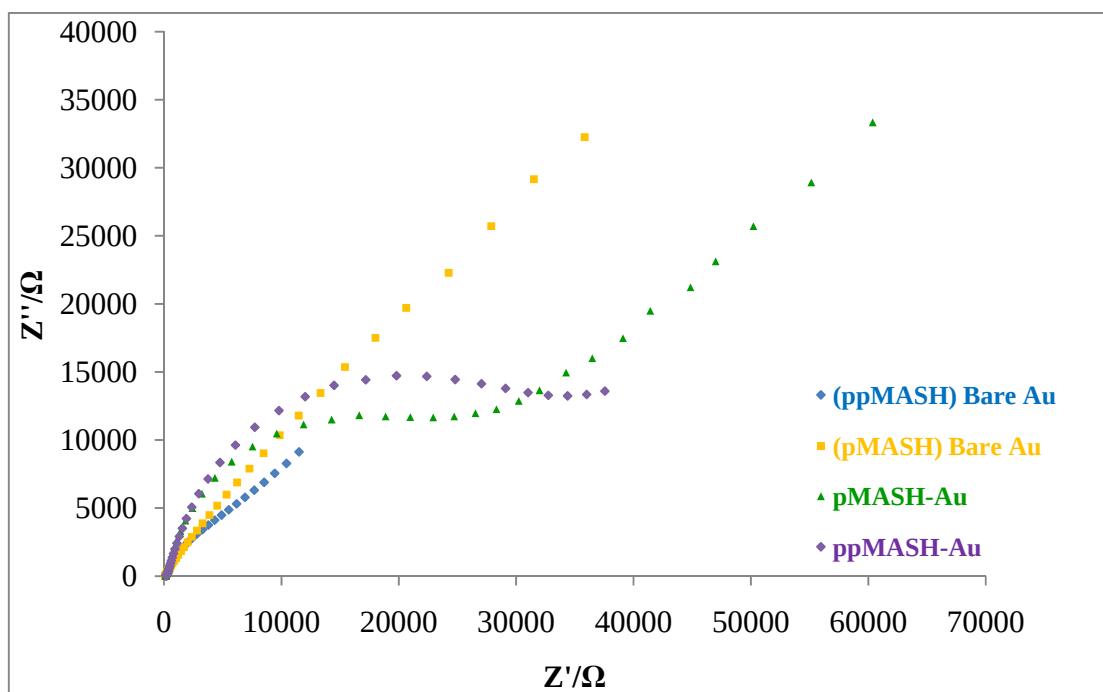
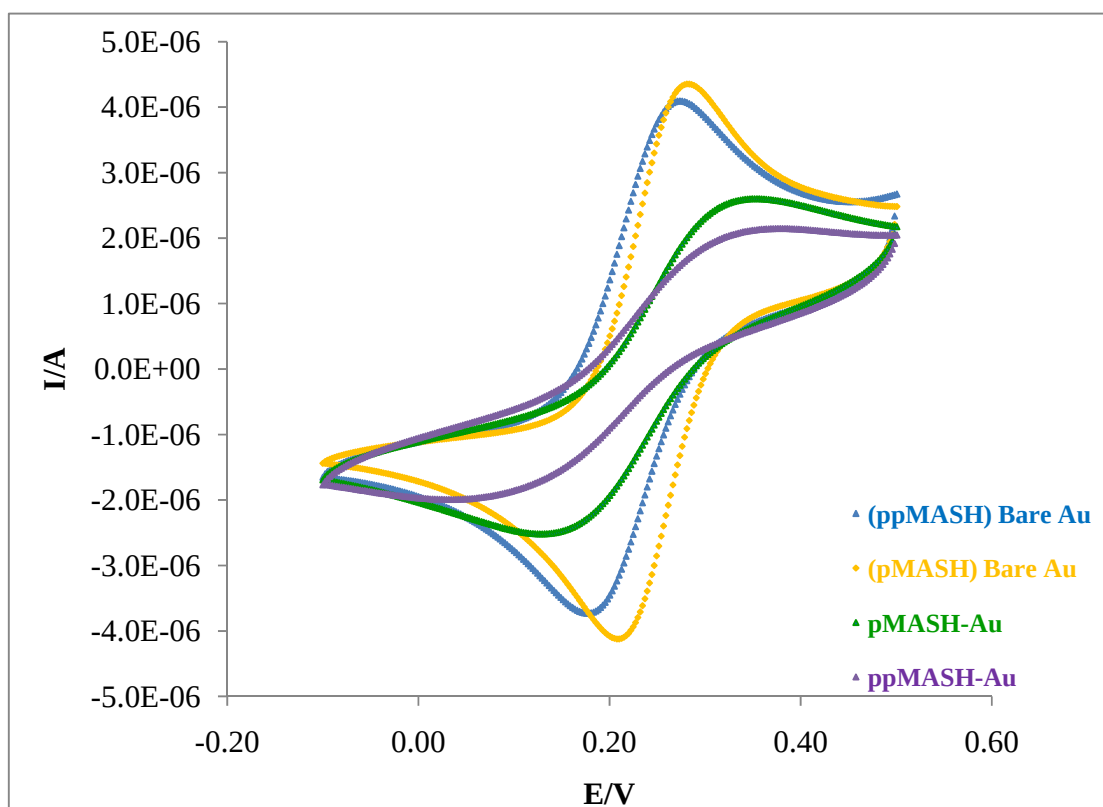


Figure 4.20: Comparative **A.** CV and **B.** EIS overlay plots of SAMs formed from pure and partially purified MASH samples.

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The pure MASH SAM thus appears to resist electron transfer slightly less than the partially purified MASH SAM. These observations are clearly evident in both the CV and EIS plots.

It is also observed from the EIS Nyquist plot that the pure MASH SAM exhibited significant diffusion as evident by the linear segment of its plot. This means that the topography was irregular. The partially purified MASH SAM did not show diffusion; a signal that immobilized molecules were relatively orderly packed. A couple of explanations can be proposed to explain the differences in packing:

1. Differences in the ambient temperature during immobilization experiments. The pure MASH SAM was created at about 25 °C while the partially purified MASH SAM was created at about 15 °C.
2. Concentration of thiol. The concentration of the pure MASH solution in which the gold electrode was incubated was 0.5 mM while the partially purified MASH was estimated to be 1 mM.
3. Incubation time. The gold electrode was incubated in the pure MASH solution for 24 h while the partially purified MASH incubation time was 25 h.

These factors might have contributed to the observed differences in the two SAM systems. However, it is clear that the impurities observed in the NMR spectrum did not inhibit thiolate formation and hence binding of the ppMASH to gold.

4.3 Conclusion

The successful covalent binding of MASH to gold was confirmed by XPS and the topography of the MASH SAM visualized by AFM. The height of the SAM was determined to be between 3-4 nm, which implies that the MASH molecules adopted a folded conformation in the SAMs. Electrochemical investigation of the MASH SAM showed that the surface was stable under electrical perturbation. Compared to the linear

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ODT the MASH SAM was irregularly packed. This allowed for relatively easy transfer of electrons between the underlying gold surface and the $[\text{Fe}(\text{CN})_6]^{3-}/[\text{Fe}(\text{CN})_6]^{4-}$ redox probe.

5 Chapter Five: Conclusion

Tuberculosis continues to be a leading public health challenge even though it has been around for millennia. It has in part been able to achieve such a phenomenal survival by having periods of relative dormancy between episodes of destructive epidemics.

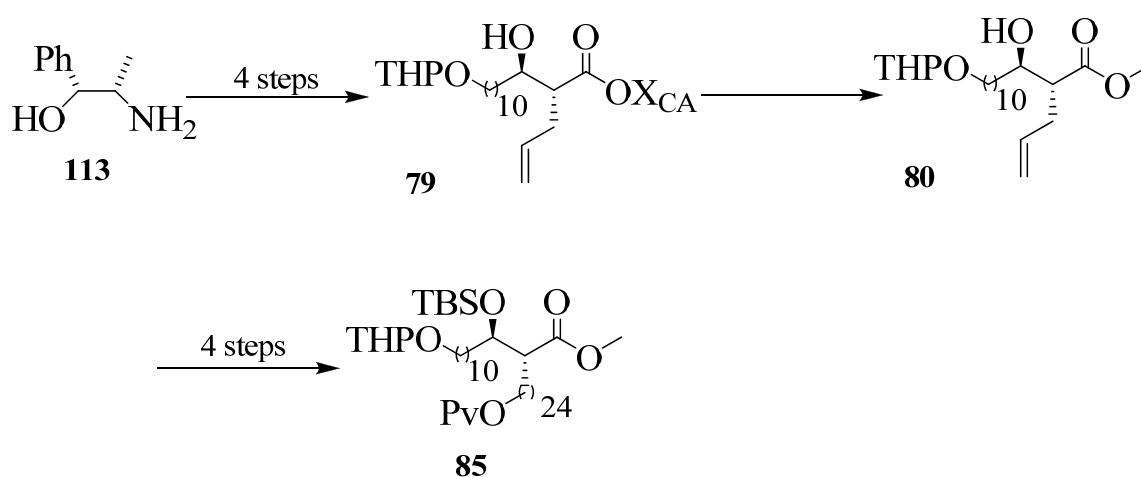
Proper diagnosis of TB still remains a major challenge in the battle against the disease. The WHO gold standard for diagnosing TB, *M.tb* culture, and other sputum-based tests have become less reliable since the advent of the HIV/AIDS pandemic. Most serological assays have so far failed to provide appropriate sensitivity and specificity required to herald a true departure from sputum as a test specimen. One recently developed technology has so far proven to be a strong candidate to achieve this.

The gold sensor disk-based tests using SPR biosensor and EIS immunosensor offer a renewed opportunity to properly address the need for a new testing technology in the age of TB-AIDS co-infections. However, to be marketable these tests still require improvements in their current states. Making the antigenic substrate, MA, more synthetically facile and economically affordable, and creating more stable antigenic sensor surfaces to increase the throughput and reproducibility of tests were the central aims of this project.

The possibility of using an *anti*-aldol reaction to synthesize the two stereogenic centres of the mycolic motif fragment of MA was previously investigated by Driver. She showed that the synthesis was feasible, but only succeeded on a very limited scale. This project worked systematically through the Driver synthetic protocol and successfully optimized several aspects of the procedure which culminated in the scaling up of the synthesis to multi-gram level. With an electronically regulated cooling bath system the temperature within the aldol reaction flask was kept at approximately -78 °C for the entire duration of the reaction. Secondly, it was shown that fresh preparation of the LDA base not only yielded higher quantities of the *anti*-aldol product **79** but also allowed for successful scaling up of the reaction and could be used in a more reasonable ratio.

Conclusion

An established route to synthesis of a 2*R*,3*R* α -alkyl β -hydroxyl mycolic motif was also carried out primarily to obtain enough intermediate compound **75** but also to compare the new aldol synthetic route to the current published method. The allyl products of both the aldol and Frater routes were conveniently protected and chain extended to the common intermediate **85**. The shorter aldol route achieved this in 9 steps and overall yield of 11%, starting from norephedrine **113** [Scheme 5.1]. The same compound **85** was prepared via the Frater route in 15 steps and an overall yield of 8%, starting from aspartic acid.



Scheme 5.1

The Frater route has been used successfully to provide the allyl motif intermediate on a scale greater than 20 g. However, the toxicity of some of the reagents, for example HMPA, pose a challenge to regular large scale synthesis. On the contrary the aldol was only attempted on a 4 g scale with approximately 1 g of allyl methyl ester **80** obtained after transesterification of the aldol product. The cleaved chiral auxiliary was re-usable and if this fact is taken into consideration the number of steps required to make **85** is further reduced by 2 steps. In addition, recovered unreacted chiral ester was successfully re-used.

A possible further extension of the aldol route is in the synthesis of longer chain fragments. The Frater alkylation was not successful with longer chains.

Conclusion

In 1981 Heathcock *et al.* successfully synthesized a methylated corynomycolic acid analogue in a racemic mixture.¹⁵⁶ They used achiral aryl hindered esters of palmitic acid and palmitaldehyde in an asymmetric aldol reaction. A chiral auxiliary could give enantiomeric selectivity but with the low temperatures used in the synthesis of the enolates the major constraint to applying the aldol reaction to synthesis of full-length MA chains is efficiently dissolving the chains. This is most important for synthesizing the kinetic *trans*-enolate. According to Kurosu and Lorca the enolate decomposes at above -50 °C.¹⁶⁷ Whether this applies to enolates of longer chains will have to be investigated. One solution to this might be to first stir the chiral ester at an intermediate temperature between -78 °C and -50 °C for several hours before the base is added. Heathcock *et al.* stirred the enolate solution for over 10 h.¹⁵⁶

In an application of the aldol procedure to the synthesis of MA the α -alkyl side chain is introduced as the chiral ester. For functionalized α -alkyl chains the problem of solubility might be ameliorated by using adequately polar protecting functional groups whereas for the non-functionalized α -alkyl chains it might only be possible to use short allyl chiral esters, as in this project. The allyl can then be oxidatively cleaved for chain extensions.

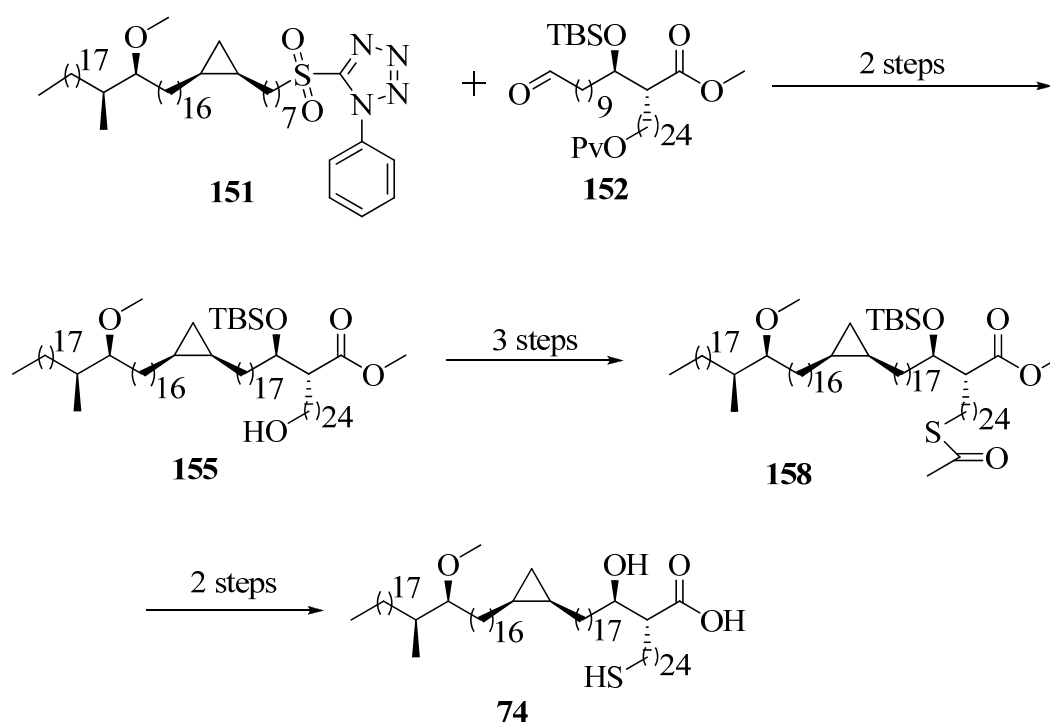
To develop the aldol route for synthesis of MA it will be important to investigate other bases and chiral auxiliaries. LDA was used throughout this project and the kinetic enolate formed by this base requires very low temperatures like the -78 °C used here. Other bases with different counterion might work well at higher temperatures. This will facilitate the application of the aldol process to the longer chains.

Apart from extending the aldol reaction to longer chains it would be pressing in the immediate future to attempt the aldol protocol as optimized in this project on a scale comparable to that achieved with the Frater alkylation method. This will provide confidence in the aldol synthesis.

Norephedrine has recently been placed on an international list of restricted compounds because of its use in preparing illicit drugs and obtaining it has become more difficult. This eliminates one of the advantages of using it – its ready commercial availability for bulk purchase. This may necessitate the search for alternative chiral auxiliaries.

Conclusion

The second part of this project successfully synthesized a MA functionalized at the ω carbon of the α -alkyl side chain. A mycolic motif aldehyde **152** synthesized in the first part of this project was successfully coupled by modified Julia-Kocienski coupling to a methoxy meromycolyl sulfone **151** [Scheme 5.2]. After saturation of the double bond to the MA alkane a series of deprotections and functional group transformations afforded the thiolated MA with a total yield of 28% in 7 steps from the Julia coupling of the two MA fragments.



Scheme 5.2

The thiolated MA was immobilized on both SPR gold disks and a bulk gold electrode from a chloroform solution. This was a major aim of this project for the improvement of sensor-based TB diagnosis. The stability and properties of the new surface were investigated by several analytical methods: XPS, AFM, CV, and EIS.

XPS spectra confirmed that immobilization was through a gold-thiolate bond. The S(2p) signal at 162.5 eV correlated excellently with what was reported in the literature for a gold-thiolate bond. Supporting evidence for an overlayer on the gold surface was obtained from

Conclusion

attenuation of the 4f gold signals between the bare and SAM-modified gold surfaces. That it was MA immobilized on the surface was corroborated by the detection of the photoelectrons of the oxygenated functional groups HO-C=O, -C-OCH₃, and C-OH.

A visual image of the MA-SH SAM was obtained by AFM. This gave a MA monolayer of about 3-4 nm thickness. This compared well with the report of Villeneuve *et al.*¹⁶²

The electrochemical characterization experiments showed that the MASH SAM was stable when subjected to an electrical potential. It revealed that the packing achieved by the MASH molecules was not uniform but possessed areas of disorder or deformity known as pinholes. The pinhole sites allowed for single electron transfer between the underlying gold substrate and the [FeCN₆]³⁻/ [FeCN₆]⁴⁻ redox probe. This was initially indicated by the ΔE_p of the bare gold and the MASH-derivatized gold surface. Corroboration of this data was obtained from the resistance to charge transfer (R_{ct}) calculated from the Nyquist plots of the EIS experiments.

The strong affinity of gold for sulfur, particularly thiol, makes the functionalization of MAs with -SH convenient for immobilization of the molecules on a solid substrate. This use of a thiolate-gold bond has a couple of important attractions. Firstly, the very strong attraction between gold and the thiol means that binding will occur between MASH and gold even in a relatively impure solution. Secondly, the orientation of the molecule can be reliably predicted because the MASH will be linked to the gold surface only via the -SH, not any other functional group. This is important for antibody binding. On the contrary, other forms of solid substrates like silver and carbon have affinity for other functional groups present on the MASH.

The electrochemical experiments, particularly EIS, were significant because one of the MARTI test technologies expected to be enhanced by this project is based on EIS. Increasing the overlayer, probably by MA-antibody binding, is expected to result in an increase in the observed R_{ct} . The covalent linkage of the MA antigen directly to the gold substrate is expected to increase the stability of the antigenic surface. It is also anticipated that this achievement will result in disposable screen-printed electrodes which will improve test throughput and user-friendliness.

Conclusion

Similarly, it is also expected that the successful immobilization of MA on a solid gold substrate will bring the SPR-MARTI test technology closer to market readiness. It will reduce the sample preparation time, hence it will increase the throughput and reproducibility of the test.

The primary focus in this project has been on thiolating a MeO-MA because of its significance to improving the MARTI tests for TB diagnosis. However, a couple of key intermediates in the synthetic process also have significant roles beyond TB diagnosis. Mycolic acids are finding application in many other areas of medical research. The use of MAs as drug delivery vessels, adjuvants, and even therapeutic agents are actively being researched. These roles for this molecule with its limited functionality will likely require introduction of additional functionality. This has been achieved here and quite early on in the synthetic process. The functionalized mycolic motif **85** can be selectively deprotected and the primary alcohols converted to other functional groups. Also, the hydroxylated MA **155** which was converted to a thiol could also be converted to MA with new functional groups. One possibility is to convert it to an azide which could be linked to nanoparticles via click chemistry for drug delivery. Another interesting application is to introduce appropriately labeled functionality at the α alkyl terminus to track *in vivo* localization of the MA molecule in any of the exciting applications being investigated.

6 Chapter Six: Experimental

6.1 Experimental for Chapters Two and Three

General

All chemicals were purchased from reputable chemical manufacturers or approved distributors. Glassware was washed and dried in an oven at 90 °C before use. For experiments requiring rigorously dry conditions all non-melting equipment was further dried at 120 °C for a minimum of 12 h before use. Solvents used in such reactions were appropriately dried also for a minimum of 12 h and where necessary distilled immediately before use. THF for the aldol and Julia coupling reactions was dried on sodium chips and benzophenone and distilled under nitrogen. Dichloromethane was dried over calcium hydride (CaH₂).

The organic fractions extracted from the aqueous phases of a reaction solution were dried with anhydrous magnesium sulfate, MgSO₄, and concentrated *in vacuo*. Residual solvent was removed via a high vacuum pump.

Column chromatography was done with Merck silica gel 60 (0.063-0.200 mm) and flash chromatography was done with Fluka silica 60 (0.040-0.063 mm). Thin layer chromatography was done on Merck silica gel 60 F₂₅₄ aluminum sheets. TLC plates were developed with ethanolic solution of phosphomolybdic acid^α and charred with an electric heat gun. Aldol products were visualized with anisaldehyde.^β

The ¹H and ¹³C nuclear magnetic resonance (NMR) spectra were recorded on Bruker Advance DRX-500 or Bruker AM400 spectrometers. Chemical shifts (δ) are reported in

^α Phosphomolybdic acid (6 g) was dissolved in ethanol (125 ml).

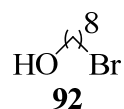
^β To a stirring solution of anisaldehyde (6 g) in ethanol (250 ml) was added H₂SO₄ (2.5 ml) dropwise.

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parts per million (ppm) downfield with deuteriated chloroform (CDCl_3) as a solvent and an internal reference.

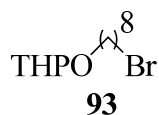
Infra-red spectra were recorded on a Perkin Elmer Spectrum RXI FT-IR system. Absorbance bands are reported in wavenumbers (cm^{-1}). Melting points were determined on a Reichert hot stage microscope. Optical rotations were determined at the indicated ambient temperature (T) using a Perkin Elmer Model 431 polarimeter at the sodium D line (589 nm) and reported as α_D^T . Mass spectra were recorded on a Bruker Microtof ESI instrument.

8-Bromooctan-1-ol (92)



A 250 ml round bottom flask was charged with 1,8-octanediol **91** (15 g, 102.6 mmol) and toluene (100 ml) added. Hydrobromic acid (13.9 ml, 256.5 mmol) was added slowly and the solution refluxed at about 110 °C. The reaction was quenched at 48 h and the crude extract purified by silica gel chromatography eluting with hexane : ethyl acetate (8 : 2) to yield the pure title compound **92** (16.8 g, 78%) as a yellow oil, consistent with reported values.¹⁹⁸ R_f = 0.15 (hexane : ethyl acetate, 8 : 2); $\nu_{\text{max}}/\text{cm}^{-1}$: 3406 (OH), 2932, 1734, 1265, 734 (C—Br); δ_H (400 MHz, CDCl_3) 1.27-1.32 (8H, m, CH_2), 1.48-1.54 (2H, m, CH_2), 1.77-1.84 (2H, m, CH_2), 3.36 (2H, t, J = 6.8 Hz, CH_2Br), 3.57 (2H, t, J = 6.6 Hz, CH_2OH); δ_C (100 MHz, CDCl_3) 25.5, 28.0, 28.6, 29.1, 32.5, 32.7, 33.9, 63.0.

2-(8-Bromo-octyloxy)-tetrahydropyran (93)

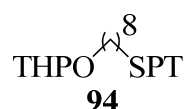


To a stirred solution of the alcohol **92** (11 g, 52.6 mmol) in dichloromethane (150 ml) was added 3,4-dihydropyran (10.5 ml, 115.6 mmol) and pyridinium-*p*-toluene sulfonate (1.3 g, 5.3 mmol) and the reaction allowed to run under nitrogen overnight. It was quenched

Experimentals

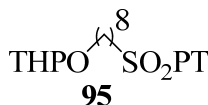
according to the literature to yield the crude extract which was purified by silica gel chromatography eluting with hexane : ethyl acetate (8 : 2) to yield the pure title compound **93** (27.5 g, 89%) as a colourless oil, consistent with literature.¹⁶⁴ R_f = 0.55 (hexane : ethyl acetate, 8 : 2); $\nu_{\max}/\text{cm}^{-1}$: 2929, 1021, 904, 726 (C—Br); δ_H (400 MHz, CDCl_3) 1.28-1.35 (8H, m, CH_2), 1.37-1.43 (2H, m, CH_2), 1.47-1.60 (4H, m, CH_2), 1.66-1.72 (2H, m, CH_2), 1.78-1.86 (2H, m, CH_2), 3.33-3.36 (2H, m, CH_2), 3.38 (2H, t, J = 6.8 Hz, CH_2Br), 3.70 (1H, dt, J = 9.6, 6.7 Hz, OCH_2), 3.82-3.87 (1H, m, CH_2), 4.54 (1H, t, J = 2.9 Hz, OCHO); δ_C (100 MHz, CDCl_3) 19.7, 25.5, 26.1, 28.1, 28.7, 29.2, 29.7, 30.8, 32.8, 34.0, 62.4, 67.6, 98.9.

1-Phenyl-5-[8-(tetrahydropyran-2-yloxy)-octylsulfanyl]-1H-tetrazole (**94**)



To a stirred solution of the acetal **93** (13.6 g, 46.4 mmol) in acetone (150 ml) was added anhydrous potassium carbonate (9.6 g, 69.6 mmol) and 1-phenyl-1H-tetrazole-5-thiol (8.3 g, 46.4 mmol). The reaction was refluxed overnight after which it was concentrated, and then diluted with water (100 ml). The crude product was extracted with dichloromethane (3 x 150 ml), dried on MgSO_4 , concentrated *in vacuo* and purified by silica gel chromatography eluting with hexane : ethyl acetate (10 : 1 and 8 : 2) to yield the title compound **94** (19.6 g, 87%) as a yellow oil consistent with reported values.¹⁶⁴ R_f = 0.30 (hexane : ethyl acetate, 8 : 2); $\nu_{\max}/\text{cm}^{-1}$: 2925, 1497, 1033, 902, 759; δ_H (400 MHz, CDCl_3) 1.19-1.34 (8H, m, CH_2), 1.37-1.43 (2H, m, CH_2), 1.47-1.60 (4H, m, CH_2), 1.66-1.72 (2H, m, CH_2), 1.78-1.86 (2H, m, CH_2), 3.30 (2H, distorted t, J = 6.8 Hz, CH_2S), 3.56 (2H, t, J = 6.8 Hz, OCH_2), 3.64-3.67 (2H, m, CH_2OTHP), 3.75-3.82 (1H, m, CH_2OTHP), 4.49 (1H, t, J = 3.5 Hz, OCHO), 7.48-7.54 (5H, m, ArCH); δ_C (100 MHz, CDCl_3) 19.5, 25.3, 26.0, 28.4, 28.8, 28.9, 29.1, 29.5, 30.6, 33.2, 62.2, 67.4, 98.6, 123.6, 129.6, 129.6, 129.9, 129.9, 133.6, 154.3.

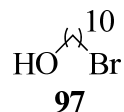
1-Phenyl-5-[8-(tetrahydropyran-2-yloxy)-octane-1-sulfonyl]-1H-tetrazole (95)



To a stirred solution of acetal **94** (6.2 g, 14.7 mmol) in dichloromethane (100 ml) was added 3-chloroperoxybenzoic acid (mCPBA) (8.9 g, 51.4 mmol) slowly at 0 °C. The reaction was stirred at room temperature for 48 h. It was worked up by quenching with Na₂S₂O₇ (14 g) and stirred for 1 h. Both the organic and aqueous phases were washed with excess sat. aq. NaHCO₃ solution (3 x 100 ml) and extracted with dichloromethane (3 x 200 ml). The organic phases were pooled, dried on MgSO₄, and concentrated *in vacuo* to yield the crude extract which was purified by silica gel chromatography eluting with hexane : ethyl acetate (9 : 1) to yield the title compound **95** (5.5 g, 83%) as a yellow oil consistent with reported values.¹⁶⁴ **R_f** = 0.25 (hexane : ethyl acetate, 8 : 2); **v_{max}/cm⁻¹**: 2929, 1498, 1024, 906, 760; **δ_H** (500 MHz, CDCl₃) 1.19-1.34 (8H, m, CH₂), 1.37-1.43 (2H, m, CH₂), 1.47-1.60 (4H, m, CH₂), 1.66-1.72 (2H, m, CH₂), 1.78-1.86 (2H, m, CH₂), 3.34 (2H, dt, *J* = 9.6, 6.5 Hz, CH₂OTHP), 3.59 (2H, t, *J* = 6.6 Hz, CH₂SO₂PT), 3.68-3.71 (1H, m, CH₂OTHP) 3.81-3.84 (1H, m, CH₂OTHP), 4.54 (1H, t, *J* = 3.5 Hz, OCHO), 7.54-7.60 (3H, m, CH), 7.64-7.66 (2H, m, CH); **δ_C** (125.7 MHz, CDCl₃) 19.6, 21.9, 25.4, 26.0, 28.0, 28.8, 28.9, 29.6, 30.7, 55.9, 62.3, 67.4, 98.8, 125.0, 129.6, 129.6, 131.4, 131.4, 133.0, 153.4.

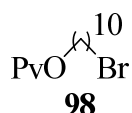
A solution of ammonium heptamolybdate (VI) tetrahydrate (61.2 g, 0.05 mmol) in H₂O₂ (150 ml, 35% w/w), prepared and cooled in an ice bath, was added to a stirred solution of acetal **94** (43 g, 0.11 mmol) in IMS (500 ml) at 12 °C and stirred at 15-20 °C for 2 h. A further solution of ammonium heptamolybdate (VI) tetrahydrate (61.2 g, 0.05 mmol) in H₂O₂ (150 ml, 35% w/w) was added and the mixture was stirred at room temperature for 18 h. The mixture was poured into water (3 L) and extracted with dichloromethane (3 x 200 ml). The combined organic phases were washed with water (2 x 300 ml), dried over MgSO₄ and concentrated *in vacuo*. The crude product was purified by silica gel chromatography eluting with petrol / ether (10:1) to give the title compound **95** as a yellow oil (37.7 g, 81%).

10-Bromodecan-1-ol (**97**)



A 250 ml round bottom flask was charged with 1,10-decanediol **96** (10 g, 68.4 mmol) and toluene (50 ml) added. Hydrobromic acid (9.3 ml, 171.0 mmol) was added slowly. The reaction was refluxed at about 110 °C. The reaction was quenched with sat. aq. NaHCO₃ (30 ml) at 48 h and the crude extract purified by silica gel chromatography eluting with hexane : ethyl acetate (8 : 2) to yield the pure title compound **97** (11.7 g, 82%) as a yellow oil, consistent with reported values.¹⁹⁸ R_f = 0.27 (hexane : ethyl acetate, 8 : 2); $\nu_{\max}/\text{cm}^{-1}$: 3610 (OH), 3406, 2929, 1265, 644 (C—Br); δ_H (400 MHz, CDCl₃) 1.27-1.41 (12H, m, CH₂), 1.51-1.56 (2H, m, CH₂), 1.79-1.86 (2H, m, CH₂), 3.38 (2H, t, J = 6.9 Hz, CH₂Br), 3.62 (2H, t, J = 6.6 Hz, CH₂OH); δ_C (100 MHz, CDCl₃) 25.9, 28.7, 29.0, 29.3 (2 peaks), 29.4, 32.8, 32.8, 34.0, 63.0.

2,2-Dimethylpropionic acid 10-bromodecyl ester (**98**)

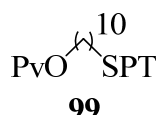


Triethylamine (14.9 g, 147.0 mmol) and pivaloyl chloride (10.2 g, 84.0 mmol) were added to a stirred solution of the alcohol **97** (10.0 g, 42.0 mmol) in dry dichloromethane (200 ml) at 0 °C under nitrogen. After 18 h the reaction was quenched by adding water (100 ml) and a few drops of 1 M HCl and the crude product was extracted with dichloromethane (3 x 100 ml). The crude product was dried over MgSO₄, concentrated *in vacuo* and purified by silica gel chromatography eluting with hexane : ethyl acetate (7 : 3) to yield the pure title compound **98** (12.4 g, 93%) as a colourless oil consistent with reported values.¹³⁸ R_f = 0.63 (hexane : ethyl acetate, 8 : 2); $\nu_{\max}/\text{cm}^{-1}$: 2932, 1807, 1722 (COO), 1265, 644 (C—Br); δ_H (400 MHz, CDCl₃) 1.17 (9H, s, CH₃), 1.23-1.32 (12H, m, CH₂), 1.56-1.63 (2H, m, CH₂), 1.79-1.86 (2H, m, CH₂), 3.38 (2H, t, J = 6.9 Hz, CH₂Br), 4.02 (2H, t, J = 6.6 Hz, CH₂OPv);

Experimentals

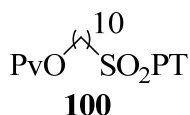
δ_C (100 MHz, $CDCl_3$) 25.9, 27.2 (3 peaks), 28.1, 28.6, 28.7, 29.1, 29.3, 29.4, 32.8, 34.0, 38.7, 64.4, 178.6.

2,2-Dimethylpropionic acid 10-(1-Phenyl-1*H*-tetrazole-5-ylsulfanyl)-decanyl ester (**99**)



To a stirred solution of ester **98** (14.0 g, 43.6 mmol) in acetone (250 ml) was added anhydrous potassium carbonate (8.90 g, 64.4 mmol) and 1-phenyl-1*H*-tetrazole-5-thiol (8.0 g, 44.9 mmol). The reaction was refluxed for 2 h and then stirred overnight at room temperature after which the solvent was evaporated. Water (100 ml) was added to the concentrated crude product. The crude product was extracted with dichloromethane (3 x 150 ml), dried over $MgSO_4$, concentrated *in vacuo* and purified by silica gel chromatography eluting with hexane : ethyl acetate (9 : 1) to yield the title compound **99** (14.9 g, 82%) as a colourless oil consistent with reported values.¹³⁸ R_f = 0.44 (hexane : ethyl acetate, 8 : 2); ν_{max}/cm^{-1} : 2930, 1715 (COO), 1163, 902, 727; δ_H (400 MHz, $CDCl_3$) 1.17 (9H, s, CH_3), 1.23-1.32 (12H, m, CH_2), 1.55-1.62 (2H, m, CH_2), 1.75-1.83 (2H, m, CH_2), 3.37 (2H, t, J = 7.4 Hz, CH_2S), 4.02 (2H, t, J = 6.6 Hz, CH_2OPv), 7.51-7.58 (5H, m, ArCH); δ_C (100 MHz, $CDCl_3$) 25.6, 27.2 (3 peaks), 28.6, 28.6, 29.0, 29.1 (2 peaks), 29.3, 29.4, 33.3, 38.7, 64.4, 123.8, 129.7 (2 peaks), 130.0 (2 peaks), 154.5, 178.6.

2,2-Dimethylpropionic acid 10-(1-Phenyl-1*H*-tetrazole-5-sulfonyl)-decanyl ester (**100**)

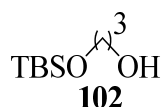


To a stirred solution of the ester **99** (16.0 g, 38.3 mmol) in dichloromethane (200 ml) was added 3-chloroperoxybenzoic acid (mCPBA) (21.3 g, 123.3 mmol) slowly at 0 °C. The reaction was stirred at room temperature for 72 h. It was quenched with excess sat. aq. $Na_2S_2O_7$ (~28 g) and stirred for 1 h. Both the organic and aqueous phases were washed

Experimentals

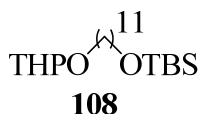
with NaHCO₃ solution (3 x 100 ml) and extracted with dichloromethane (3 x 150 ml). The organic phases were pooled, dried over MgSO₄, and concentrated to yield the crude extract which was purified by silica gel chromatography eluting with hexane : ethyl acetate (9 : 1) to yield the title compound **100** (14.6 g, 85%) as a colourless oil consistent with reported values.¹³⁸ R_f = 0.40 (hexane : ethyl acetate, 8 : 2); $\nu_{\max}/\text{cm}^{-1}$: 2930, 1715 (COO), 1163, 902, 727; δ_H (500 MHz, CDCl₃) 1.17 (9H, s, CH₃), 1.27 (12H, m, CH₂), 1.55-1.64 (2H, m, CH₂), 1.90-1.96 (2H, m, CH₂), 3.71 (2H, t, J = 8.0 Hz, CH₂SO₂), 4.02 (2H, t, J = 6.6 Hz, CH₂OPv), 7.57-7.62 (3H, m, ArCH), 7.66-7.68 (2H, m, ArCH); δ_C (125.7 MHz, CDCl₃) 21.9, 25.8, 27.2 (3 peaks), 28.1, 28.6, 28.8, 29.1, 29.1, 29.3, 38.7, 56.0, 64.4, 125.0, 129.7, 129.7, 131.4, 131.4, 153.5, 178.6.

3-(tert-Butyldimethylsilyloxy)propan-1-ol (**102**)



Sodium hydride (1.2 g, 51.2 mmol) was suspended in distilled THF (40 ml). 1,3-Propanediol **101** (10 g, 131.4 mmol) was added dropwise to the solution and stirred for 1.5 h. tert-Butyldimethylsilyl chloride was added slowly at 0 °C and the reaction was stirred for a further 1.5 h. The reaction was worked up according to the literature and the crude extract purified by flash silica column chromatography eluting with hexane : ethyl acetate (9 : 1) to yield the title compound **102** (14.7 g, 54%) as a colourless oil consistent with reported values.¹⁹⁹ R_f = 0.24 (hexane : ethyl acetate, 8 : 2); $\nu_{\max}/\text{cm}^{-1}$: 3337 (OH), 2926, 1252, 1063; δ_H (500 MHz, CDCl₃) 0.05 (6H, s, (CH₃)₂), 0.87 (9H, s, (CH₃)₃), 1.75 (2H, p, J = 5.6 Hz, CH₂), 2.35 (1H, s, OH), 3.77 (2H, t, J = 5.6 Hz, CH₂OH), 3.81 (2H, t, J = 5.5, CH₂OSi); δ_C (125.7 MHz, CDCl₃) -5.5 (two peaks), 25.8 (three peaks), 34.2, 59.7, 62.4, 62.9.

tert-Butyldimethyl(11-(tetrahydro-2H-pyran-2-yloxy)undecyloxy)silane (108)



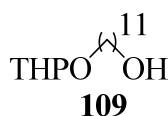
To a stirred solution of the alcohol **102** (10.0 g, 52.5 mmol) in dichloromethane (100 ml) was added pyridinium chlorochromate (PCC) (22.6 g, 105.1 mmol). The reaction was stirred for 2.5 h at which time TLC showed the reaction was complete and diethyl ether (150 ml) was added to the reaction solution. It was filtered through a bed of celite to yield a solution of the crude product which was concentrated *in vacuo* and purified by silica gel chromatography eluting with hexane : diethyl ether (9 : 1) to give the aldehyde 3-(tert-butyldimethylsilyloxy)propanal **103** (7.7 g, 78%) as a colourless volatile oil. The solvent was carefully evaporated at 14 mmHg using a cold water bath; if the temperature was increased the yield of aldehyde was considerably reduced. It was used immediately for the next reaction without confirmation of structure by NMR spectroscopy but it showed one spot on TLC with a higher R_f (0.62, hexane : ethyl acetate, 8 : 2) than the starting alcohol.

To a stirred solution of sulfone **95** (2.7 g, 5.8 mmol) in THF (30 ml) was added the aldehyde **103** (1.0 g, 5.3 mmol) at 0 °C under nitrogen. Lithium bis(trimethylsilyl)amide (7.6 ml, 7.6 mmol, 1 M in THF) was added dropwise by syringe. The cooling bath was removed and the reaction was stirred at room temperature overnight and worked up according to the literature by adding excess sat. aq. NH_4Cl (20 ml) to the solution and extracting with ethyl acetate (3 x 50 ml) to yield a crude extract. This was dried over MgSO_4 , concentrated *in vacuo* and then purified by silica gel column chromatography eluting with hexane : ethyl acetate (10 : 1) to yield an *E/Z* mixture of alkenes **107** (1.63 g, 77%). δ_{H} (500 MHz, CDCl_3) 0.02 (6H, s, $\text{Si}(\text{CH}_3)_2$), 0.87 (9H, s, $(\text{CH}_3)_3$, diastereomer at 0.89), 1.19-1.36 (14H, m, CH_2), 1.45-1.62 (6H, m, CH_2), 1.64-1.74 (2H, m, CH_2 , diastereomer at 1.75-1.85), 1.90-2.08 (4H, m, $(\text{CH}_2\text{CH}=\text{CHCH}_2)$), 3.33-3.39 (1H, dt, $J = 9.5, 6.6$, CH_2OTHP), 3.45-3.50 (1H, m, CH_2OTHP), 3.57 (2H, dt, $J = 6.6$ Hz, CH_2OSi), 3.67-3.72 (1H, dt, $J = 9.5, 6.6$ Hz, CH_2OTHP), 3.83-3.88 (1H, m, CH_2OTHP), 4.55 (1H, t, $J = 3.3$ Hz, OCHO), 5.30-5.75 (2H, m, $\text{CH}=\text{CH}$); δ_{C} (125.7 MHz, CDCl_3) -5.4 (two peaks), 25.5, 25.9 (three peaks), 26.2, 29.4, 29.5 (two peaks), 29.6, 29.8, 30.8 (two peaks), 32.9, 62.3, 63.1, 63.3, 67.7, 98.8, 114.6, 116.7.

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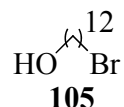
The mixture of *E/Z* alkenes **107** was dissolved in hexane : ethyl acetate (100 ml, 1 : 1) and saturated with hydrogen gas overnight at 2.4 atm using palladium 10% on carbon (160 mg, 10%) to yield the title compound **108** (1.6 g, 98%) as a yellow oil consistent with reported values.¹⁶⁴ R_f = 0.64 (hexane : ethyl acetate, 8 : 2); $\nu_{\max}/\text{cm}^{-1}$: 2923, 1461, 1096, 1021, 831, 772; δ_H (500 MHz, CDCl_3) 0.02 (6H, s, $\text{Si}(\text{CH}_3)_2$), 0.87 (9H, s, $(\text{CH}_3)_3$), 1.19-1.33 (14H, m, CH_2), 1.45-1.60 (8H, m, CH_2), 1.75-1.85 (2H, m, CH_2), 3.33-3.38 (1H, dt, J = 9.5, 6.6, CH_2OTHP), 3.45-3.50 (1H, m, CH_2OTHP), 3.57 (2H, t, J = 6.6 Hz, CH_2OSi) 3.67-3.72 (1H, dt, J = 9.5, 6.6 Hz, CH_2OTHP), 3.83-3.88 (1H, m, CH_2OTHP), 4.52 (1H, t, J = 3.3 Hz, OCHO); δ_C (125.7 MHz, CDCl_3) -5.3 (two peaks), 25.5 (three peaks), 25.8, 26.0 (two peaks), 29.4, 29.5 (two peaks), 29.6 (three peaks), 29.8, 30.8, 32.9, 62.3, 63.1, 63.3, 67.7, 98.8.

11-(Tetrahydro-2H-pyran-2-yloxy) undecan-1-ol (**109**)



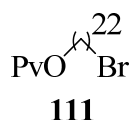
To a stirred solution of the diprotected compound **108** (1.2 g, 3.11 mmol) in a solvent mixture of MeOH/ EtOH/ H_2O (70 ml, 30 : 4 : 1) was added pyridinium-*p*-toluene sulfonate (0.008 g, 0.031 mmol) and the reaction was stirred for 8 h at room temperature. To quench, the solvent was evaporated *in vacuo* at 14 mmHg and the crude product diluted with water (40 ml) and extracted with dichloromethane (3 x 50 ml), dried over MgSO_4 , filtered, concentrated and purified by flash silica chromatography eluting with hexane : ethyl acetate (9 : 1, then 7 : 3) to yield the title compound **109** (0.53 g, 63%) as a colourless oil consistent with reported values.¹⁶⁴ R_f = 0.19 (hexane : ethyl acetate, 8 : 2); $\nu_{\max}/\text{cm}^{-1}$: 3337, 2926, 1252, 1063, 832; δ_H (400 MHz, CDCl_3) 1.23 (14H, s, CH_2), 1.42-1.55 (8H, m, CH_2), 1.63-1.69 (8H, m, CH_2), 1.75-1.81 (2H, m, CH_2), 3.30-3.36 (1H, dt, J = 9.5, 6.6, CH_2OTHP), 3.42-3.47 (1H, m, CH_2OTHP), 3.57 (2H, t, J = 6.6 Hz, CH_2OH) 3.65-3.71 (1H, dt, J = 9.5, 6.6 Hz, CH_2OTHP), 3.79-3.84 (1H, m, CH_2), 4.52-4.54 (1H, t, J = 3.3 Hz, OCHO); δ_C (125.7 MHz, CDCl_3) 19.6, 25.4, 25.7, 26.2, 29.3, 29.4 (three peaks), 29.5, 29.7, 30.7, 32.7, 62.2, 62.9, 67.6, 98.8.

12-Bromo-dodecan-1-ol (**105**)



A 250 ml flask was charged with 1,12-dodecandiol **104** (10 g, 49.5 mmol) and toluene (100 ml) was added. To the suspension was added hydrobromic acid (9.36 ml, 115.7 mmol) slowly and the reaction refluxed for 48 h. The reaction was quenched with sat. aq. NaHCO₃ (30 ml) and the crude extract purified by silica gel chromatography eluting with hexane : ethyl acetate, initially at 9 : 1 and then 8 : 2, to yield the title compound **105** (9.32 g, 76%) as a white solid consistent with reported values.¹⁹⁸ R_f = 0.27 (hexane : ethyl acetate, 8 : 2); m.p. = 27-31 °C; $\nu_{\max}/\text{cm}^{-1}$: 3267 (OH), 2916, 1461, 1029, 650 (C—Br); δ_H (400 MHz, CDCl₃) 1.24-1.42 (16H, m, CH₂), 1.51-1.58 (2H, m, CH₂), 1.79-1.87 (2H, m, CH₂), 3.38 (2H, t, J = 6.9 Hz, CH₂Br), 3.62 (2H, t, J = 6.7 Hz, CH₂OH); δ_C (100 MHz, CDCl₃) 25.7, 28.2, 28.7, 29.4, 29.4, 29.5, 29.5, 29.6, 32.8, 32.8, 34.0, 63.1.

22-Bromodocosyl pivalate (**111**)



To a stirred solution of the alcohol **105** (2.5 g, 9.3 mmol) in dichloromethane (250 ml) was added PCC (5.0 g, 23.3 mmol). The reaction was stirred for 2 h at which time TLC showed the reaction was complete and diethyl ether (400 ml) was added to the reaction solution. It was filtered through a bed of celite to yield a solution of the crude product which was concentrated *in vacuo* and purified by silica gel chromatography eluting with hexane : ethyl acetate (9 : 1) to give the aldehyde **106** (2.2 g, 89%) as a yellow oil. R_f = 0.51 (hexane : ethyl acetate, 8 : 2).

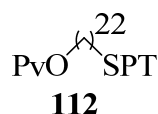
To a stirred solution of sulfone **100** (20.1 g, 42.0 mmol) in THF (100 ml) was added the freshly prepared aldehyde **106** (9.0 g, 38.0 mmol) and lithium bis(trimethylsilyl)amide (51.5 ml, 55.0 mmol, 1 M in THF) at 0 °C under nitrogen. The reaction was stirred at room temperature overnight and worked up by adding sat. aq. NH₄Cl (50 ml) and extracting with

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ethyl acetate (3 x 150 ml) to yield the crude extract. The crude extract was dried over MgSO_4 , concentrated *in vacuo*, and purified by silica gel chromatography eluting with hexane : ethyl acetate (10 : 1) to yield an *E/Z*-mixture of alkenes **110** (15.5 g, 84%) as a colourless oil. δ_{H} (400 MHz, CDCl_3) 1.17 (9H, s, CH_3), 1.22-1.36 (30H, m, CH_2), 1.57-1.62 (2H, m, CH_2), 1.80-1.86 (2H, m, CH_2), 1.90-2.02 (4H, m, $(\text{CH}_2\text{CH}=\text{CHCH}_2)$), 3.38 (2H, t, $J = 6.9$ Hz, CH_2Br), 4.02 (2H, t, $J = 6.7$ Hz, CH_2OPv), 5.32-5.37 (2H, m, $\text{CH}=\text{CH}$); δ_{C} (125.7 MHz, CDCl_3) 25.9, 27.2 (3 peaks), 28.1, 28.6, 28.7, 29.0, 29.1 (2 peaks), 29.2 (2 peaks), 29.3, 29.4 (3 peaks), 29.5 (2 peaks), 29.6, 29.7, 32.6, 32.8, 33.9, 38.7, 64.4, 129.8 (2 peaks), 130.3 (2 peaks), 178.5.

The mixture of *E/Z* alkenes **110** was dissolved in hexane : ethyl acetate (100 ml, 1 : 1) and saturated with hydrogen gas overnight at 2.4 atm using palladium 10% on carbon to yield the title compound **111** as a white solid (15.3 g, 99%). $R_f = 0.62$ (hexane : ethyl acetate, 8 : 2); **m.p.** 40-43 °C; $\nu_{\text{max}}/\text{cm}^{-1}$: 2916, 1717 (COO), 1159, 719, 653 (C—Br); δ_{H} (400 MHz, CDCl_3) 1.17 (9H, s, CH_3), 1.22-1.34 (36H, m, CH_2), 1.57-1.62 (2H, m, CH_2), 1.80-1.86 (2H, m, CH_2), 3.38 (2H, t, $J = 6.9$ Hz, CH_2Br), 4.02 (2H, t, $J = 6.7$ Hz, CH_2OPv); δ_{C} (125.7 MHz, CDCl_3) 25.9, 27.2 (3 peaks), 28.2, 28.6, 28.8, 29.2, 29.4, 29.5 (2 peaks), 29.6, 29.7 (many peaks), 32.8, 34.1, 38.7, 64.5, 178.5; **MS** {Found $(\text{M} + \text{Na})^+$: 511.3126, $\text{C}_{27}\text{H}_{53}\text{BrNaO}_2$ requires: 511.3121}.

22-(1-Phenyl-1H-tetrazol-5-ylthio)-docosyl pivalate (**112**)

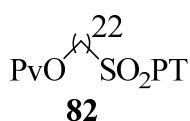


To a stirred solution of the ester **111** (5.0 g, 10.2 mmol) in acetone (150 ml) was added anhydrous potassium carbonate (4.2 g, 15.3 mmol) and 1-phenyl-1H-tetrazole-5-thiol (1.8 g, 10.2 mmol). The reaction was refluxed overnight after which it was concentrated *in vacuo*, diluted with water (100 ml) and extracted with dichloromethane (3 x 150 ml) according to literature and the crude extract purified by silica gel chromatography eluting with hexane : ethyl acetate (9 : 1) to yield the title compound **112** (4.9 g, 82%) as a white solid. $R_f = 0.47$ (hexane : ethyl acetate, 8 : 2); **m.p.** 55-57 °C; $\nu_{\text{max}}/\text{cm}^{-1}$: 2917, 1722

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(COO), 1499, 1159, 758; δ_{H} (400 MHz, CDCl_3) 1.21 (9H, s, $(\text{CH}_3)_3$), 1.36-1.43 (36H, m, CH_2), 1.54-1.61 (2H, m, CH_2), 1.74-1.81 (2H, m, CH_2), 3.35 (2H, t, $J = 7.28$ Hz, CH_2S), 4.00 (2H, t, $J = 6.8$ Hz, CH_2OPv), 7.47-7.56 (5H, m, ArCH); δ_{C} (100 MHz, CDCl_3) 25.8, 27.1 (3 peaks), 28.5, 28.6, 29.0 (2 peaks), 29.2, 29.4, 29.5 (2 peaks), 29.6 (9 peaks), 33.3, 34.1, 38.7, 64.4, 123.8, 129.7 (2 peaks), 130.0 (2 peaks), 154.4, 178.5; **MS** {Found (M + Na) $^+$: 609.4201, $\text{C}_{34}\text{H}_{58}\text{N}_4\text{NaO}_2\text{S}$ requires: 609.4173}.

22-(1-Phenyl-1H-tetrazol-5-ylsulfonyl)docosyl pivalate (**82**)



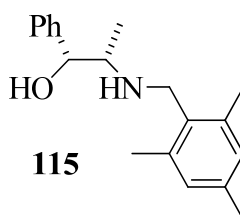
To a stirred solution of ester **112** (4.9 g, 8.4 mmol) in dichloromethane (100 ml) was added 3-chloroperoxybenzoic acid (mCPBA) (5.1 g, 29.3 mmol) slowly at 0 °C. The reaction was stirred at room temperature for 72 h. It was worked up by quenching with $\text{Na}_2\text{S}_2\text{O}_7$ (~9 g) and stirred for 1 h. Both the organic and aqueous phases were washed with NaHCO_3 (100 ml) and extracted with dichloromethane (3 x 150 ml). The organic phases were pooled, dried over MgSO_4 , and concentrated to yield the crude extract which was purified by silica gel column chromatography eluting with hexane : ethyl acetate (9 : 1) to yield the title compound **82** (14.6 g, 85%) as a white solid. $R_f = 0.44$ (hexane : ethyl acetate, 8 : 2); **m.p.** 55-57 °C; $\nu_{\text{max}}/\text{cm}^{-1}$: 2914, 1724 (COO), 1344, 1156, 629; δ_{H} (500 MHz, CDCl_3) 1.17 (9H, s, $(\text{CH}_3)_3$), 1.23-1.33 (34H, m, CH_2), 1.43-1.51 (2H, m, CH_2), 1.56-1.63 (2H, m, CH_2), 1.89-1.94 (2H, m, CH_2), 3.71 (2H, t, $J = 8.0$ Hz, CH_2SO_2), 4.02 (2H, t, $J = 6.6$ Hz, CH_2OPv), 7.56-7.62 (3H, m, ArCH), 7.66-7.70 (2H, m, ArCH); δ_{C} (125.7 MHz, CDCl_3) 21.9, 25.9, 27.2 (3 peaks), 28.1, 28.6, 28.9, 29.2 (2 peaks), 29.5, 29.6 (2 peaks), 29.7 (8 peaks), 38.7, 56.0, 64.5, 125.1, 129.7 (2 peaks), 131.4 (2 peaks), 133.1, 153.5, 178.6; **MS** {Found (M + Na) $^+$: 641.4071, $\text{C}_{34}\text{H}_{58}\text{N}_4\text{NaO}_4\text{S}$ requires: 641.4071}.

A solution of ammonium heptamolybdate (VI) tetrahydrate (1.9 g, 1.5 mmol) in H_2O_2 (20 ml, 35% w/w), prepared and cooled in an ice bath, was added to a stirred solution of ester **112** (2 g, 3.41mmol) in THF (10 ml) and in IMS (100 ml) at 12 °C and stirred at 15-20 °C for 1 h. A further solution of ammonium heptamolybdate (VI) tetrahydrate (1.9 g, 1.5

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mmol) in H_2O_2 (20 ml, 35% w/w) was added and the mixture was stirred at room temperature for 66 h. The mixture was poured into water (0.5 L) and extracted with dichloromethane (100 ml, and 2 x 50 ml). The combined organic phases were washed with water (2 x 400 ml), dried over MgSO_4 and concentrated *in vacuo*. The crude product was purified by column chromatography eluting with hexane : ethyl acetate (10:1) to give the title compound **82** as a white solid (1.69 g, 80%).

(1*R*,2*S*)-1-Phenyl-2-(2,4,6-trimethylbenzylamino]-propan-1-ol (**115**)

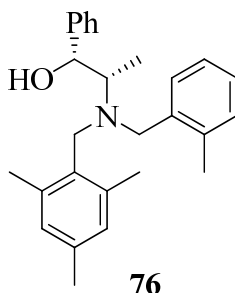


A round bottom flask was charged with (1*R*,2*S*) (-)-norephedrine **113** (10 g, 22.5 mmol) and toluene (100 ml) added. To the stirred solution was added 2,4,6-trimethylbenzaldehyde (14.7 g, 99 mmol) and anhydrous MgSO_4 (12.9 g). The reaction was refluxed under nitrogen overnight. It was worked up according to the literature and the crude product used immediately in the next reaction.¹⁶⁷

To a stirred solution of the crude **114** (27.8 g, 99 mmol) in MeOH (150 ml) and AcOH (35 ml) was added NaBH_4 (3.4 g, 198 mmol) in small portions at 0 °C. The reaction was stirred overnight at room temperature and worked up according to the literature.¹⁶⁷ The crude product was purified by column chromatography eluting with hexane : ethyl acetate (10 : 1) to yield the title compound **115** (20.8 g, 74%) as a viscous colourless oil consistent with reported values.¹⁶⁷ R_f = 0.40 (hexane : ethyl acetate, 8 : 2); $\nu_{\text{max}}/\text{cm}^{-1}$: 3562, 3427, 2845, 1619, 1578; δ_{H} (500 MHz, CDCl_3) 0.90 (3H, d, J = 6.5 Hz, CH_3), 2.26 (3H, s, Mes-*p*- CH_3), 2.36 (6H, s, Mes-*o*- CH_3), 3.06-3.08 (1H, m, NCHCH_3), 3.86 (2H, s, NCH_2), 4.86 (1H, d, J = 3.9 Hz, CHOH), 6.86 (2H, s, ArMesCH), 7.24-7.26 (5H, m, ArCH); δ_{C} (125.7 MHz, CDCl_3) 14.4, 19.4 (two peaks), 20.8, 45.4, 59.3, 72.9, 126.0 (two peaks), 127.0, 128.0 (two peaks), 129.1 (two peaks), 132.6, 136.9 (three peaks), 141.2.

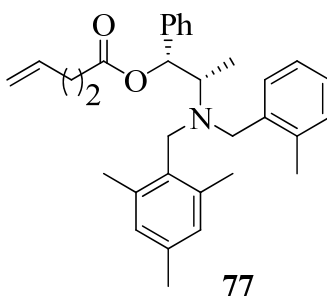
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(1*R*,2*S*)-2-((2-Methylbenzyl)-(2,4,6-trimethylbenzyl)amino)-1-phenylpropan-1-ol (**76**)



To a stirred solution of chiral amine **115** (6.9 g, 24.43 mmol) in toluene (80 ml) and acetonitrile (50 ml) was added Cs_2CO_3 (14.0 g, 42.97 mmol) and 1-bromomethyl-2-methylbenzene (4.52 g, 24.43 mmol). The reaction mixture was refluxed at 110 °C for 30 h. To quench it was diluted and extracted according to the literature to yield the crude product. The crude product was purified by silica column chromatography eluting with hexane : ethyl acetate (9 : 1) to yield the title compound **76** (8.6 g, 91%) as a colourless viscous oil consistent with reported values.¹⁶⁷ $R_f = 0.50$ (hexane : ethyl acetate, 8 : 2); $\alpha_D^{20} +26.2$ ($c = 0.01$ in CHCl_3) (lit $[\alpha]_D = +28.9$ ($c = 0.2$ in CHCl_3)); $\nu_{\text{max}}/\text{cm}^{-1}$: 3551, 2968, 1735, 1265, 738; δ_H (500 MHz, CDCl_3) 1.15 (3H, d, $J = 6.7$ Hz, CHCH_3), 2.08 (3H, s, CH_3 -*p*-Mes), 2.17 (6H, s, CH_3 -*o*-Mes), 2.97-3.00 (1H, m, NCHCH_3), 3.40 (2H, s, NCH_2), 3.44 (2H, s, NCH_2), 3.60 (3H, t, $J = 7.2$ Hz, NCH_2), 4.74 (1H, d, $J = 4.7$ Hz, CHOH), 6.69 (2H, s, ArMesCH), 6.93-7.18 (9H, m, ArCH); δ_C (125.7 MHz, CDCl_3) 8.4, 19.3, 20.2 (three peaks), 20.9, 48.3, 51.6, 58.3, 76.1, 125.4, 126.5 (two peaks), 126.8, 127.1, 128.1 (three peaks), 129.1, 130.2, 130.6, 132.1, 136.3, 137.3, 137.6, 138.4, 143.5.

(1*R*,2*S*)-2-((2-Methylbenzyl)(2,4,6-trimethylbenzyl)amino)-1-phenylpropyl pent-4-enoate (**77**)



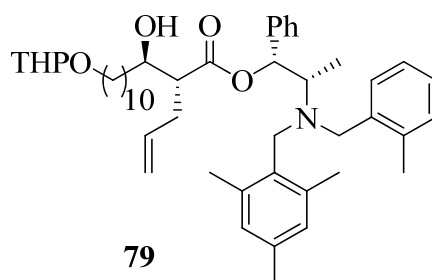
Experimentals

To a stirred solution of chiral alcohol **76** (3.5 g, 9.03 mmol) in dichloromethane (80 ml) was added DCC (3.2 g, 27.1 mmol), DMAP (1.98 g, 22.6 mmol) and pentenoic acid (1.14 g, 13.5 mmol). The reaction was stirred under nitrogen at room temperature for 35 h when the crude product was extracted according to the literature. It was purified by flash silica column chromatography eluting with hexane : ethyl acetate (9 : 1) to yield the title compound **77** (3.95 g, 93%) as a colourless viscous oil consistent with reported values.¹⁶⁴

R_f = 0.63 (hexane : ethyl acetate, 8 : 2); α_D^{20} = -0.11 (c = 0.01 in CHCl_3); $\nu_{\text{max}}/\text{cm}^{-1}$: 2975, 2850, 1720, 1628, 1614; δ_{H} (500 MHz, CDCl_3) 1.17 (3H, d, J = 8.1 Hz, NCHCH_3), 2.02 (3H, s, CH_3 - p -Mes), 2.16 (6H, s, CH_3 - o -Mes), 2.21 (3H, s, CH_3 -tol), 2.24-2.36 (4H, m, $(\text{CH}_2)_2\text{CO}$), 3.08-3.15 (1H, m, NCHCH_3), 3.42-3.50 (2H, m, NCH_2), 3.64 (2H, d, J = 12.6 Hz, NCH_2), 4.84-4.98 (2H, m, $\text{HC}=\text{CH}_2$), 5.62-5.75 (1H, m, $\text{HC}=\text{CH}_2$), 5.96 (1H, d, J = 6.3 Hz, CHOCO), 6.67 (2H, s, ArMesCH), 6.78-6.84 (2H, m, CH Ar-tol), 6.95-7.14 (7H, m, ArCH); δ_{C} (125.7 MHz, CDCl_3) 9.3, 19.3, 20.2 (two peaks), 20.9, 28.7, 33.9, 47.4, 51.3, 56.4, 76.7, 76.9, 115.6, 125.4, 126.9, 127.1 (two peaks), 127.4, 128.0 (two peaks), 129.0 (two peaks), 130.2, 130.7, 131.7, 136.3, 136.6, 137.0, 137.4, 138.5, 139.7, 172.2.

The Aldol Reaction:

(2R,3R)-((1R,2S)-2-((2-methylbenzyl)(2,4,6-trimethylbenzyl)-amino)-1-phenylpropyl) 2-allyl-3-hydroxy-13-(tetrahydro-2H-pyran-2-yloxy)tri-decanoate (79**)**



(a) Original Method Described by Driver¹⁶⁴

To a stirred solution of the alcohol **109** (100 mg, 0.37 mmol) in dichloromethane (50 ml) was added PCC (160 mg, 0.74 mmol). The reaction was stirred for 2 h at which time TLC showed the reaction was complete and diethyl ether (100 ml) was added to the reaction solution. It was filtered through a bed of celite to yield a solution of the crude product

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which was concentrated *in vacuo* and purified by silica gel chromatography eluting with hexane : ethyl acetate (9 : 1) to give the aldehyde **78** (60 mg, 60%) as a colourless oil. R_f = 0.51 (hexane : ethyl acetate, 8 : 2).

To a stirred solution of Cp_2ZrCl_2 (49 mg, 0.126 mmol) in THF (4 ml) was added LDA (1.6 ml, 1.8 M) at -78°C under nitrogen. The solution was stirred for 0.5 h after which the chiral ester **77** (0.2 g, 0.42 mmol), dissolved in THF (4 ml), was also added slowly over 1 h. The solution was stirred for 2 h after which more Cp_2ZrCl_2 (378 mg, 0.97 mmol), dissolved in THF (5 ml), was added slowly over 1.5 h. The reaction was stirred for 0.5 h after which the aldehyde **78** (60 mg, 0.21 mmol), dissolved in THF (3.5 ml), was added slowly over another 45 min. After stirring for 2.5 h the reaction was worked up with HCl (10 ml, 1.0 M). The mixture was stirred for 10 min and a thick white precipitate formed. The organic layer was extracted with diethyl ether (3 x 50 ml). The organic phases were pooled, a few drops of Et_3N added, dried over MgSO_4 , filtered and concentrated *in vacuo*. The crude product was purified by column chromatography eluting with hexane : ethyl acetate (10 : 1) to yield the title compound **79** (45.2 mg, 29%) as a yellow oil which gave identical NMR signals to those reported.¹⁶⁴

The first compound off the column was unreacted chiral ester **77** which was followed by the *anti*-aldol product **79**. Next, unreacted aldehyde **78** eluted very closely after the product **79** such that the latter fractions of **79** were often contaminated with **78**. The *syn*-diastereomers **116** and **117** were obtained as a mixture of enantiomers which eluted after the aldehyde **78**. The early fractions of the *syn*-diastereomers were also often contaminated with unreacted **78**.

Several repeats of the above experimental procedure were conducted except that 0.5 M HCl was used in the work ups. These gave identical products with the yield of **79** in the range 20 to 30%.

(b) Effect of Temperature Control

The experimental process described above was repeated with an internal temperature probe to monitor temperature fluctuations during the reaction period. A dry ice-acetone bath was used to achieve -78°C . The addition of reagents was in very small portions such that a 2 ml THF solution of any of the reagents would be added over a 1 h to 1.5 h. Later an electronic

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cooling bath with a thermostat was used to achieve and maintain $-78\text{ }^{\circ}\text{C}$. The yield of **79** was still in the same range of 20 to 30%.

(c) Effect of Freshly Synthesized LDA

Fresh LDA was synthesized according to the method reported by Kurosu and Lorca for large-scale aldol synthesis.¹⁶⁴ To a stirred solution of diisopropylamine (3 ml) in THF (50 ml) was added n-butyllithium (n-BuLi) (12.25 ml, 1.6 M) at $0\text{ }^{\circ}\text{C}$. The solution was stirred under nitrogen for 15 min. The temperature reduced to $-78\text{ }^{\circ}\text{C}$ for 30 min and 1.96 ml was used as described in the aldol procedure above. TLC showed no product yield and the chiral ester **77** was recovered by column chromatography.

Synthesis of the LDA was repeated with less THF (35 ml). To this stirred solvent was added diisopropylamine (5 ml) and n-BuLi (20 ml, 1.6 M) at $-78\text{ }^{\circ}\text{C}$. The solution was stirred for 30 min and then warmed up to room temperature for 30 min. A concentration of 0.5 M was determined by titrating against diphenylacetic acid (1 mmol, 0.212 g). The freshly synthesized LDA (6 ml, 3.2 mmol) was used in an aldol reaction as described above. The *anti*-aldol product **79** yield was 42%.

The aldol process described above was scaled up to 750 mg of **77** using freshly synthesized LDA (0.5 M). The yield of **79** was not consistent with repeats of the experiment. One experiment gave absolutely no yield while the second gave 25%. In the first experiment the quantities of the reagents and solvent used in the preparation of the LDA were just a threefold multiplication of those used in the successful reaction described above with the freshly prepared LDA. This was corrected in the second experiment when less solvent was used [Table 6.1]. From each preparation 18 ml of LDA was drawn with syringe for the aldol syntheses.

Table 6.1: LDA preparation for scaling up of aldol reaction.

Reagents	Experiment I	Experiment II
Diisopropylamine	15 ml	15 ml
n-Butyllithium	20 ml	20 ml
THF	105 ml	53 ml

Using the higher concentration LDA the aldol process of Driver described above was scaled up to 1.1 g of the chiral ester **77** using freshly prepared LDA (0.96 M). The yield was 45%. This reaction was repeated with 1.0 g of unreacted chiral ester **77** recovered from previous aldol reactions. These were re-purified by column chromatography after the initial column recovery. Although the purified chiral ester **77** had a yellow colour, unlike the colourless freshly synthesized **77**, the NMR of both were identical and showed no contaminant. The yield of **79** was similar.

(d) Effect of Zirconocene

Three simultaneous aldol experiments were setup to investigate the effect of the catalyst zirconocene, Cp_2ZrCl_2 . Experiment I and II were run with chiral ester **77** (300 mg, 0.64 mmol), aldehyde **78** (160 mg, 0.59 mmol), LDA (0.5 M) and Cp_2ZrCl_2 as described in the process above according to the method of Driver.¹⁶⁴ The two experiments differ in that Experiment I used Cp_2ZrCl_2 from Fluka manufacturer while Experiment II used Cp_2ZrCl_2 from Merck manufacturer. Experiment III did not use any Cp_2ZrCl_2 . Chiral ester **77** (300 mg, 0.64 mmol) was added slowly to a stirred LDA solution (6 ml, 0.5 M) at -78°C . The solution was stirred for 2 h when aldehyde **78** (160 mg, 0.59 mmol) dissolved in THF (2 ml) was added slowly. The solution was stirred for another 2.5 h when it was worked up with HCl (20 ml, 0.5 M) and the crude product extracted with diethyl ether (3 x 50 ml). The crude products of the three experiments were purified by column chromatography separately. Experiment I (with Fluka Cp_2ZrCl_2) gave a yield of **79** of 25%, Experiment II (with Merck Cp_2ZrCl_2) gave a yield of **79** of 30%, while Experiment III (no Cp_2ZrCl_2 used) gave a yield of **79** of 29.0%. The NMR spectra were identical to those reported by Driver.¹⁶⁴

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The non-catalysed reaction was repeated with the chiral ester **77** (463.6 mg, 0.98 mmol), aldehyde **78** (133 mg, 0.49 mmol) and LDA (0.5 M) according to the process described above. The yield was 12% with a diastereoselectivity of 70 : 30 for the *anti*-product **79**.

(e) Effect of Reactants' Purity

All reactants we obtained from the purest column chromatography fractions. The chiral ester **77** was repeatedly put under high vacuum in a warm waterbath for several hours and sealed under nitrogen prior to use. The aldehyde **78** was also put under high vacuum for an hour and blown with a gentle stream of nitrogen just before use. Also, because the aldehyde could only be subjected to high vacuum pressure at room temperature for a short period before use ethyl acetate was replaced with ether in the column purification process along with hexane. The THF was distilled for several days under nitrogen before use. With these controls the aldol was repeated with the chiral ester **77** (97.5 mg, 0.206 mmol), 1.1 mol. eq. of the aldehyde **78** and freshly prepared LDA (7.0 mol. eq., 0.5 M). The yield was 34% of **79** in 70% diastereoselectivity.

(f) Reduction of LDA Molar Equivalentents and Scaling Up of Reaction

To a stirred solution of the alcohol **109** (2.5 g, 9.2 mmol) in dichloromethane (250 ml) was added PCC (4.0 g, 18.4 mmol). The reaction was stirred for 2 h at which time TLC showed the reaction was complete and diethyl ether/ hexane (400 ml : 40 ml) was added to the reaction solution. It was filtered through a bed of celite to yield a solution of the crude product which was concentrated *in vacuo* and purified by silica gel chromatography eluting with hexane : ethyl acetate (9 : 1) to give the aldehyde **78** (2.2 g, 88%) as a yellow oil. R_f = 0.47 (hexane : ethyl acetate, 8 : 2).

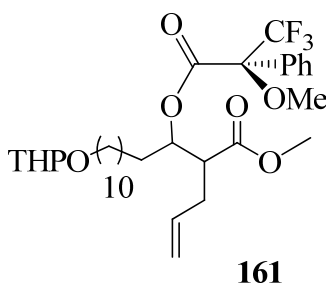
To distilled THF (35 ml) was added diisopropylamine (5.0 ml, 21.5 mmol) at -78 °C under nitrogen. n-BuLi (20.0 ml, 29.1 mmol, 1.6 M) was added to the stirred solution and the reaction stirred for 30 min. The solution was allowed to warm to room temperature. To the freshly prepared LDA (43 ml, 21.5 mmol) was added the chiral ester **77** (4.08 g, 8.6 mmol) dissolved in THF (4 ml) at -78 °C slowly over 1 h. The reaction was stirred for 2 h. The aldehyde **78** (2.1 g, 7.8 mmol) dissolved in THF (3 ml) was added slowly to the stirring solution over 45 min and the reaction stirred for 2.5 h. The reaction was worked up by warming the solution to room temperature and adding sat. aq. NH_4Cl (10 ml). The solution

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was extracted with diethyl ether (3 x 80 ml) and the organic fractions pooled, dried over MgSO_4 , and concentrated *in vacuo*. The crude product was purified by flash column chromatography eluting with hexane : diethyl ether (10 : 1) to give the title compound **79** (2.40 g, 42%) as a yellow oil. R_f = 0.38 (hexane : diethyl ether, 7 : 3); α_D^{20} = -4.5 (c = 0.01 in CHCl_3); $\nu_{\text{max}}/\text{cm}^{-1}$: 3515, 3036, 2910, 2801, 1698; δ_H (400 MHz, CDCl_3) 1.75-1.20 (27H, m, CH_2), 1.77-1.85 (2H, m, CH_2), 2.02 (6H, s, CH_3 -*o*-Mes), 2.07 (3H, s, CH_3 -*p*-Mes), 2.24 (3H, s, CH_3 -tol), 2.27-2.40 (2H, m, CH_2) 2.43-2.52 (1H, m, CCHCOO), 3.29-3.33 (1H, m, NCHCH_3), 3.38-3.42 (1H, m, CH_2OTHP), 3.49-3.54 (3H, m, NCH_2Mes , NCH_2Tol , CH_2OTHP), 3.65-3.80 (4H, m, NCH_2Mes , NCH_2Tol , CH_2OTHP , CHOH), 3.85-3.91 (1H, m, CH_2OTHP), 4.59 (1H, t, J = 3.3 Hz, OCHO), 4.78-4.90 (2H, m, $\text{H}_2\text{C}=\text{C}$), 5.44-5.57 (1H, m, $\text{C}=\text{CH}$), 6.00 (1H, d, J = 8.0 Hz, CHOCO), 6.73 (2H, s, ArMesCH), 6.90 (2H, d, J = 7.0 Hz, CH Ar-tol), 7.02-7.24 (7H, m, ArCH); δ_C (100 MHz, CDCl_3) 9.9, 14.1, 19.2, 19.6, 20.0, 20.7, 22.6, 25.5 (two peaks), 25.7, 26.2 (two peaks), 29.4 (two peaks), 29.5, 29.7, 30.7 (three peaks), 33.5, 35.4, 47.3, 50.4, 50.9, 55.5, 62.2 (two peaks), 67.6 (two peaks), 71.5, 98.8 (two peaks), 117.1, 125.2, 126.8, 127.8, 128.9 (two peaks), 130.0 (two peaks), 131.3, 134.4, 136.2, 136.7, 137.4, 138.5, 139.1, 174.2.

The *syn*-diastereomers were found at R_f = 0.25. The significant NMR shifts were of the alkene protons: δ_H 4.96-5.11 (2H, m, $\text{H}_2\text{C}=\text{C}$), 5.66-5.80 (1H, m, $\text{C}=\text{CH}$), δ_C 117.2, 135.0.

Mosher Ester Derivatization of *Syn*-Aldol Products.



The *syn*-aldol product was first methylated by transesterification before derivatization with *S*- MTPA.

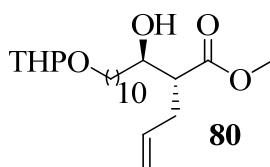
Experimentals

To a stirred solution of *syn*-aldol product (100 mg, 0.135 mmol) in anhydrous MeOH (2 ml) was added freshly prepared NaOMe (0.18 g Na in 1.5 ml MeOH) and the reaction stirred in the refrigerator (-7 °C). At 24 h more NaOMe (0.1 g Na in 1.5 ml MeOH) was added. The reaction was quenched at 48 h with sat. aq. NH₄Cl (20 ml). The methanol was evaporated *in vacuo* and the crude product diluted with water (30 ml) and extracted with CH₂Cl₂ (2 x 50 ml). The organic fractions were pooled, dried over MgSO₄, filtered and concentrated *in vacuo*. The crude product was purified by flash chromatography eluting with hexane : ethyl acetate (10 : 1) to yield the methylated *syn*-products (25 mg, 48%) as a yellow oil.

To a stirred solution of *S*-MTPA (14 mg, 0.06 mmol) in dry hexane (3 ml) and DMF (10 µl) was added oxalyl chloride (25.4 mg, 20 µl, 0.2 mmol). The solution was stirred for 1 h and decanted into a clean flask and the reaction flask was washed with hexane (2 x 10 ml). The hexane fractions were pooled and concentrated *in vacuo*. The acid chloride was then dissolved in dichloromethane (2 ml). Et₃N (20 µl), followed by DMAP (1 mg), was added to the stirred solution. Finally, the *syn*-isomers of **80** (10 mg) were added and the reaction stirred for 2.5 h. To quench, dichloromethane (4 ml) was added and washed with HCl (2 ml, 0.5 M). The organic layer was separated and the aqueous layer extracted with dichloromethane (2 x 20 ml). The combined organic phases was washed with NaHCO₃ (10 ml) and the aqueous layer extracted twice with dichloromethane (2 x 20 ml). The organic extracts were pooled, dried over MgSO₄, and concentrated *in vacuo*. ¹⁹F NMR of the crude was run.

The same procedure was repeated for methylated *anti*-aldol product **80**.

(2R,3R)-methyl 2-allyl-3-hydroxy-13-(tetrahydro-2H-pyran-2-yloxy)tridecanoate (**80**)

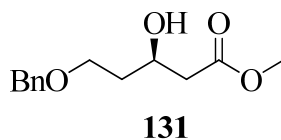


To a stirred solution of *anti*-aldol product **79** (490 mg, 0.27 mmol) in anhydrous MeOH (5 ml) was added freshly prepared NaOMe (0.5 g Na in 5 ml MeOH) and the reaction stirred

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in the refrigerator (-7 °C). At 48 h more NaOMe (0.1 g Na in 1.5 ml MeOH) was added and this was repeated at 72 h. The reaction was quenched at 96 h with sat. aq. NH₄Cl (20 ml). The methanol was evaporated *in vacuo* and the crude product diluted with water (50 ml) and extracted with CH₂Cl₂ (3 x 100 ml). The organic fractions were pooled, dried over MgSO₄, filtered and concentrated *in vacuo*. The crude product was purified by flash chromatography eluting with hexane : ethyl acetate (10 : 1) to yield the title compound **80** (165 mg, 65%) as a yellow oil. **R_f** = 0.29 (hexane : ethyl acetate, 8 : 2); α_D^{20} = +3.3 (c = 1.1 in CHCl₃); $\nu_{\max}/\text{cm}^{-1}$: 3421, 2923, 1438, 1260, 1022; δ_H (400 MHz, CDCl₃) 1.25-1.57 (25H, m), 1.77-1.85 (2H, m, CH₂), 2.33-2.47 (2H, m, CH₂), 2.48-2.56 (1H, m, CCHCOO), 3.33-3.38 (1H, m, CH₂OTHP), 3.45-3.51 (1H, m, CH₂OTHP), 3.68 (3H, s, OCH₃), 3.63-3.73 (1H, m, CH₂OTHP), 3.78-3.88 (2H, m, CH₂OTHP, CHOH), 4.55 (1H, s, OCHO), 4.98-5.10 (2H, m, H₂C=C), 5.68-5.82 (1H, m, C=CH); δ_C (100 MHz, CDCl₃) 19.7, 25.5, 25.7, 25.8, 26.2, 29.4, 29.5, 29.7, 30.8, 31.5, 32.8, 33.8, 34.3, 50.5, 51.6, 62.3, 67.7, 71.8, 98.8, 117.2, 134.9, 175.2.

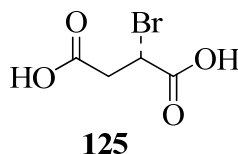
Synthesis of (R)-Methyl 5-(benzyloxy)-3-hydroxypentanoate (**131**) for Frater Alkylation



The synthesis of **131** described below was done at Bangor University under the close mentorship of Dr Al Dulayymi during a six weeks visit to the laboratory of Professor Baird. Because it is an established method, and very limited available time, detail characterization of the products were not done. In nearly all the cases an almost identical reaction method to the published literature was followed.^{144,173}

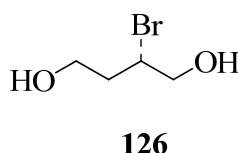
a. Synthesis of Oxirane (127)

(S)-2-Bromosuccinic acid (125)



A 3-necked 2 L round-bottom flask equipped with a mechanical stirrer and an electronic thermometer was charged with L-aspartic acid **123** (50.0 g, 0.38 mol) and KBr (201.1 g, 1.69 mol). H₂SO₄ (1 L, 2.5 M) was added and the solution stirred below 0 °C in an ice-bath. A solution of NaNO₂ (46.7 g, 0.68 mol) was added slowly over 1.5 h while keeping the temperature of the reaction solution below 0 °C. The resulting dark brown solution was stirred for 2 h below 0 °C and extracted with ethyl acetate (500 ml). The organic layer was separated and the aqueous layer further extracted with ethyl acetate (3 x 500 ml). The organic phases were pooled, dried over MgSO₄, filtered and concentrated *in vacuo* to yield the product **125** (69.0 g, 92%) as a white solid.

(S)-2-Bromobutane-1,4-diol (126)

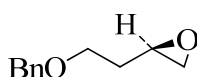


S-Bromosuccinic acid **125** (52.4 g, 0.27 mol) was suspended in dry THF (400 ml) in a 3-necked 2 L round-bottom flask equipped with a mechanical stirrer and an electronic thermometer. The mixture was cooled to 0 °C in an ice-bath and boranetetrahydrofuran (0.8 L, 0.8 mol) was added slowly via a syringe over 1 h. After the addition the reaction was stirred for 4 h at room temperature although the temperature was still carefully monitored. The reaction was quenched by adding THF/ H₂O (100 ml, 1:1) and K₂CO₃ (160 g). The mixture was stirred and then filtered through a sintered glass funnel under vacuum. The solid residue was washed with ethyl acetate (3 x 100 ml). The organic filtrate and washes were combined and concentrated to a mixture of an oil and borate salts. The oil was re-

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dissolved in diethyl ether (2 x 200 ml) and the borate salts filtered off. The filtrate was dried over MgSO_4 , filtered, and concentrated *in vacuo* to yield the crude product which was purified by column chromatography eluting with petrol : ethyl acetate (1 : 2) to yield the diol **126** (32.0 g, 71%) as a colourless oil.

(*R*)-2-(2-(benzyloxy)ethyl)oxirane (**127**)

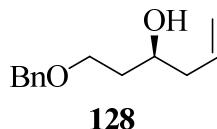


127

NaH (23.3 g, 60% dispersion, 0.97 mol) was washed with petrol (3 x 40 ml) and then suspended in dry THF (200 ml). The suspension was chilled in a methanol-ice bath to -10°C and bromodiol **126** (32 g, 0.19 mol) in dry THF (32 ml) was added over 5 min via a syringe. After 25 min benzylbromide (14.1 ml, 20.4 g, 0.21 mol) and tributylammonium iodide (4.0 g, 11.0 mmol) were added. The mixture was stirred at -10°C for 5 min then the cold bath was removed and the reaction proceeded for 2 h at room temperature. Saturated aqueous NH_4Cl (100 ml) along with ethyl acetate (20 ml) was added and the organic layer separated. The aqueous layer was extracted with ethyl acetate (3 x 200 ml) and the organic extracts combined, washed with H_2O and brine (2 x 200 ml), dried over MgSO_4 , filtered and concentrated *in vacuo*. The crude product was purified by silica gel chromatography eluting with petrol : ethyl acetate (4 : 1) to yield the oxirane **125** (27.5 g, 81%) as a colourless oil.

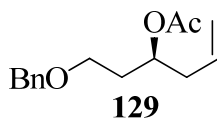
b. Synthesis of Hydroxyl Methyl Ester (131)

(S)-1-Benzyloxy-hex-5-en-3-ol (128)



Copper bromide (3.5 g, 24.1 mmol) was dissolved in dry THF (300 ml) at room temperature under nitrogen and cooled to $-75\text{ }^{\circ}\text{C}$. Vinylmagnesium bromide (23.2 mg, 23.6 ml, 176.7 mmol, 1 M in THF) was added between -75 to $-50\text{ }^{\circ}\text{C}$ and the mixture was stirred $-50\text{ }^{\circ}\text{C}$ to $-40\text{ }^{\circ}\text{C}$ for 30 min. The solution was re-cooled to $-75\text{ }^{\circ}\text{C}$ and a solution of the oxirane **127** (12.0 g, 67.4 mmol) in dry THF (100 ml) was added between -75 to $-40\text{ }^{\circ}\text{C}$ and the reaction was stirred at -40 to $-30\text{ }^{\circ}\text{C}$ for 1 h then at $-20\text{ }^{\circ}\text{C}$ for 15 min. Saturated aqueous NH_4Cl (400 ml) was added and the crude product extracted with ethyl acetate (3 x 300 ml) and the combined organic layers were washed with water, dried over MgSO_4 and the solvent was evaporated *in vacuo*. The crude product was purified by silica gel chromatography eluting with petrol : ethyl acetate (2:1) to give the title compound **128** (12.6 g, 90%) as a colourless oil. $\nu_{\text{max}}/\text{cm}^{-1}$: 3425, 3069, 2919, 1863, 1206; δ_{H} (500 MHz, CDCl_3): 1.73-1.82 (2H, m, CH_2), 2.25-2.28 (2H, m, CH_2), 3.66-3.91 (1H, m, CH_2OH), 3.73 (1H, dt, $J = 9.5, 5.4\text{ Hz}$, CH), 3.95-4.11 (1H, m, CH_2), 4.54 (2H, s, $\text{Ph-CH}_2\text{-O}$), 5.10-5.14 (2H, m, CH_2), 5.85 (1H, m, CH), 7.28-7.38 (5H, m, Ar-CH); δ_{C} (125.7 MHz, CDCl_3): 35.9, 41.9, 69.0, 70.4, 73.3, 117.6, 127.9, 128.3, 128.5, 134.9, 138.0.

Acetic acid (S)-1-(2-benzyloxy-ethyl)-but-3-enyl ester (129)

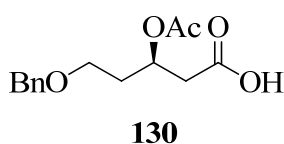


Acetic anhydride (50 ml) and then anhydrous pyridine (50 ml) were added to a stirred solution of the alkenol **128** (12.5 g, 60.6 mmol) in dry toluene (100 ml) at room temperature and the mixture was stirred for 18 h after which it was diluted with toluene (100 ml) and the solvent was evaporated. The crude product was purified by silica gel

Experimentals

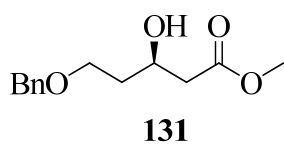
chromatography eluting with petrol : diethyl ether (6:1) to give the title compound **129** (14.8 g, 99%) as a colourless oil. $\nu_{\text{max}}/\text{cm}^{-1}$: 3066, 2923, 1737, 1496, 1241; δ_{H} (500 MHz, CDCl_3): 1.82-1.94 (2H, m, CH_2), 2.02 (3H, s, CH_3CO), 2.30-2.40 (2H, m, CH_2), 3.46-3.58 (2H, m, CH_2), 4.50 (2H, s, $\text{Ph-CH}_2\text{-O}$), 5.09-5.18 (2H, m, CH_2), 5.72-5.85 (1H, m, CH), 7.27-7.40 (5H, m, Ar-CH); δ_{C} (125.7 MHz, CDCl_3): 21.2, 33.7, 38.9, 66.6, 70.8, 73.1, 117.9, 127.6, 127.7, 128.2, 133.5, 138.3, 170.6.

(R)-3-Acetoxy-5-benzyloxy-pentanoic acid (**130**)



The allyl **129** (14.8 g, 59.6 mmol) was dissolved in dry DMF (400 ml) and oxone (146.5 g, 287.1 mmol) then OsO_4 , 2.5% in 2-methyl-2-propanol, (7.5 ml, 0.60 mmol) were added at 10 °C. The temperature of the solution was allowed to reach 32 °C and stirred for 3 h. The mixture was diluted with water (3.0 L) and extracted with ethyl acetate (1 x 500 ml, 2 x 250 ml). The combined organic layers were washed with water (700 ml), dried over MgSO_4 , and the solvent was evaporated *in vacuo*. The crude product was not purified but used directly in the next reaction.

(R)-5-Benzyloxy-3-hydroxy-pentanoic acid methyl ester (**131**)



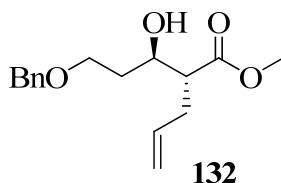
To a stirred solution of the crude acid **130** (11.0 g, 41.3 mmol) in methanol (200 ml) was added conc. H_2SO_4 (70 drops) and refluxed for 3.5 h. When TLC showed the reaction was complete the methanol was evaporated and the crude mixture dissolved with ethyl acetate (250 ml) and sat. aq. NaHCO_3 (200 ml) was added. The organic layer was separated and the aqueous layer extracted with ethyl acetate (2 x 150 ml). The combined organic layers were

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dried over MgSO_4 and the solvent evaporated *in vacuo*. The crude product was purified by column chromatography eluting with petrol : ethyl acetate (3 : 2) to give the title compound **131** (7.9 g, 80%) as a colourless oil. $\nu_{\text{max}}/\text{cm}^{-1}$: 3467, 3031, 2951, 1496, 1168; δ_{H} (500 MHz, CDCl_3): 1.78-1.88 (2H, m, CH_2), 2.53 (2H, d, $J = 6.3$ Hz CH_2), 3.66 (1H, t, $J = 6.9$ Hz, CH_2), 3.71 (3H, s, COOCH_3), 3.72 (1H, m, CH_2OH), 4.54 (2H, s, $\text{Ph-CH}_2\text{-O}$), 7.38-7.40 (5H, m, Ar-CH); δ_{C} (125.7 MHz, CDCl_3): 36.0, 41.4, 51.7, 67.1, 68.0, 73.3, 127.6 (two peaks), 127.7 (two peaks), 128.5, 138.0, 172.8.

The Frater Alkylation.

(*R*)-2-((*R*)-3-Benzyloxy-1-hydroxy-propyl)-pent-4-enoic acid methyl ester (**132**)

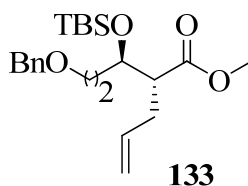


To a stirred solution of diisopropylamine (1.54 g, 57.7 mmol) in dry THF (80 ml) was added methyllithium (51.9 ml, 77.9 mmol, 1.5 M) under nitrogen at -78 °C. The reaction mixture was allowed to warm up to room temperature. After 30 min it was re-cooled to -61 °C and methyl ester **131** (6 g, 25.1 mmol) in dry THF (40 ml) was added drop-wise. The reaction mixture was allowed to slowly warm to -45 °C over 1 h, then to -20 °C for 40 min and finally maintained between -20 °C and -10 °C for 20 min. The solution was again cooled to -62 °C when a mixture of allyl iodide (6.30 ml, 37.6 mmol) and HMPA (8.8 ml, 50.2 mmol) in dry THF (20 ml) was added drop-wise. The mixture was stirred at -45 °C for 1 h, -45 °C to -20 °C for 30 min and then -20 °C for 30 min. When TLC showed little starting material left sat. aq. NH_4Cl (50 ml) was added and the product extracted with petrol : ethyl acetate (1 : 1, 3 x 100 ml). The combined organic fractions was dried over MgSO_4 , and concentrated *in vacuo* to give the crude product which was purified by flash column chromatography eluting with petrol : ethyl acetate (4 : 1) to give the title compound **132** (5.6 g, 79%) as a pale yellow oil. $R_f = 0.21$ (petrol : ethyl acetate, 8 : 2); $\nu_{\text{max}}/\text{cm}^{-1}$: 3452, 2952, 1436, 1270, 1122; δ_{H} (500 MHz, CDCl_3) 1.70-1.85 (2H, m, CH_2), 2.30-2.45 (2H, m, CH_2), 2.50-2.55 (1H, m, CH-alkyl), 3.59-3.69 (1H, m, CH_2), 3.69 (3H, s, CH_3), 3.94 (1H, m, HCOH), 4.45 (2H, s, CH_2), 4.95-5.10 (2H, m, CH_2), 5.65-5.70 (1H, m, CH), 7.20–7.38

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(5H, m, ArCH); δ_C (125.7 MHz, $CDCl_3$) 33.3, 34.6, 51.1, 51.6, 68.3, 70.9, 73.3, 117.1, 127.7 (two peaks), 128.5, 135.0, 137.9, 174.8.

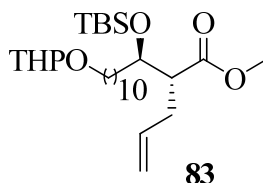
(*R*)-2-[(*R*)-3-Benzyloxy-1-(*tert*-butyl-dimethyl-silanyloxy)-propyl]-pent-4-enoic acid methyl ester (**133**)



To a stirred solution of the allyl methyl ester **132** (5.5 g, 19.7 mmol) in dry DMF (100 ml) was added imidazole (3.6 g, 52.3 mmol). The mixture was stirred for 30 min at room temperature and then cooled to 0 °C when *tert*-butyldimethylchlorosilane (3.85 g, 25.5 mmol) was added. The solution was stirred at 49 °C for 18 h, quenched with water (50 ml) and extracted with dichloromethane (3 x 80 ml). The organic fractions were pooled, dried over $MgSO_4$, and concentrated *in vacuo*. The crude product was purified by flash column chromatography eluting with petrol : ethyl acetate (8 : 2) to give the title compound **133** (7.0 g, 90%) as a colourless oil. R_f = 0.32 (petrol : ethyl acetate, 8 : 2); $[\alpha]_D^{20}$ -8.0 (c = 0.01 in $CHCl_3$); ν_{max}/cm^{-1} : 3032, 2954, 1739, 1437, 1254; δ_H (500 MHz, $CDCl_3$) 0.05 (6H, s, $Si(CH_3)_2$), 0.85 (9H, s, $SiC(CH_3)_3$), 1.75-1.81 (2H, m, CH_2), 2.25-2.38 (2H, m, CH_2), 2.60-2.65 (1H, m, CH-alkyl), 3.44-3.55 (1H, m, CH_2), 3.60 (3H, s, CH_3), 4.05-4.10 (1H, m, HCO_2Si), 4.45 (2H, s, CH_2), 4.90-5.02 (2H, m, CH_2), 5.65-5.71 (1H, m, CH), 7.20-7.35 (5H, m, ArCH); δ_C (125.7 MHz, $CDCl_3$) -4.9, -4.4, 18.0, 25.7 (three peaks), 31.3, 33.7, 51.3, 51.7, 66.3, 70.2, 72.9, 116.4, 127.5 (two peaks), 127.6, 128.3 (two peaks), 135.9, 138.5, 173.7.

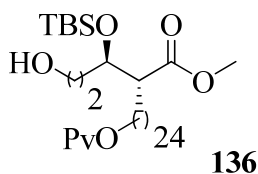
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(2*R*,3*R*)-methyl 2-allyl-3-(tert-butyldimethylsilyloxy)-13-(tetrahydro-2*H*-pyran-2-yloxy)tridecanoate (**83**)



To a stirred solution of the allyl methyl ester **80** (0.10 g, 0.26 mmol) in DMF (10 ml) was added imidazole (0.044 g, 0.65 mmol). The mixture was stirred for 30 min and tert-butyldimethylchlorosilane (0.051 g, 0.34 mmol), dissolved in DMF (2 ml), was added at 0 °C. The reaction was stirred at 49 °C under nitrogen for 2 days. It was quenched with water (10 ml) and extracted with dichloromethane (3 x 50 ml). The organic fractions were pooled, dried over MgSO₄, filtered and concentrated *in vacuo*. The crude product was purified by flash column chromatography eluting with hexane : ethyl acetate (10 : 1) to yield the title compound **83** (117 mg, 90%) as a yellow oil. R_f = 0.41 (hexane : ethyl acetate, 8 : 2); $[\alpha]_D^{20}$ +9.1 (c = 0.01 in CHCl₃); $\nu_{\max}/\text{cm}^{-1}$: 3052, 2946, 1684, 1403, 1076; δ_H (400 MHz, CDCl₃) - 0.0002 (6H, s, Si(CH₃)₂), 0.85 (9H, s, SiC(CH₃)₃), 1.22-1.54 (25H, m, CH₂), 2.33-2.47 (2H, m, CH₂), 2.48-2.56 (2H, m, CH₂), 3.30-3.37 (1H, m, CH₂OTHP), 3.40-3.50 (1H, m, CH₂OTHP), 3.66 (3H, s, OCH₃), 3.61-3.71 (2H, m, CH₂OTHP, HCOSi), 3.76-3.87 (1H, m, CH₂OTHP), 4.54 (1H, t, J = 3.3 Hz, OCHO), 4.92-5.10 (2H, m, H₂C=C), 5.65-5.80 (1H, m, C=CH); δ_C (100 MHz, CDCl₃) -5.2 (two peaks), 18.4, 19.7, 25.5 (two peaks), 25.8, 26.0 (three peaks), 26.3, 29.5 (two peaks), 29.8, 30.8, 31.5, 32.9, 33.9, 34.3, 50.5, 51.6, 62.4, 67.8, 71.8, 98.9, 117.2, 134.9, 175.4; **MS** {Found ($M + Na$)⁺: 521.3629, C₂₈H₅₄NaO₅Si requires: 521.3633}.

(*R*)-methyl 2-((*R*)-1-(tert-butyldimethylsilyloxy)-3-hydroxypropyl)-26-(pivaloyloxy)hexacosanoate (**136**)

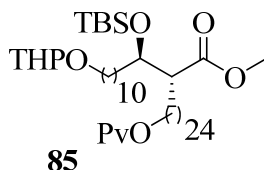


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To a stirred solution of allyl ester (**133**) (6.0 g, 15.0 mmol) in 1,4-dioxane-water (180 ml, 3 : 1) was added 2,6-lutidine (3.55 ml, 31.0 mmol), OsO₄, 2.5% in 2-methyl-2-propanol, (3.39 ml, 0.3 mmol), and NaIO₄ (12.8 g, 60 mmol). The reaction was stirred for 2 h when it was diluted with water (300 ml) and extracted with dichloromethane (3 x 200 ml). The organic fractions were pooled, dried over MgSO₄, and concentrated *in vacuo*. The crude product was purified by flash column chromatography eluting with petrol : ethyl acetate (2 : 1) to yield the aldehyde **134** (5.1 g, 84%), as a colourless oil.

To a stirred solution of sulfone (**83**) (9.5 g, 15.4 mmol) in dry THF (50 ml) was added the freshly prepared aldehyde (**134**) (5.1 g, 12.8 mmol) and lithium bis(trimethylsilyl)amide (18.2 ml, 20.0 mmol, 1.06 M in THF) at 0 °C under nitrogen. The reaction was stirred at room temperature for 2 h and quenched with sat. aq. NH₄Cl (30 ml). The crude product was extracted with ethyl acetate (3 x 200 ml) and the organic fractions pooled, dried and concentrated *in vacuo*. The crude product was purified by flash column chromatography eluting with hexane : ethyl acetate (10 : 1) to yield a mixture of *E/Z* alkenes **135** (8.7 g, 86%). The *E/Z* mixture of alkenes was dissolved in MeOH : THF (70 ml, 5 : 2) and palladium 10% on carbon (0.9 g) was added. The solution was stirred under 1 atm hydrogen for 3 days after which it was filtered on a bed of celite. The crude product was concentrated *in vacuo* and purified by column chromatography eluting with petrol : ethyl acetate (2 : 1) to give the title compound **136** (8.5 g, 98%) as a white, waxy solid. *R*_f = 0.40 (hexane : ethyl acetate, 10 : 1); α_D^{20} -7.3 (c = 1.1 in CHCl₃); *m.p.* 33-35 °C; $\nu_{\max}/\text{cm}^{-1}$: 3452, 2927, 2853, 1741, 1440; δ_H (500 MHz, CDCl₃) 0.02 (6H, s, Si(CH₃)₂), 0.80 (9H, s, SiC(CH₃)₃), 1.11 (9H, s, C(CH₃)₃), 1.12-1.30 (34H, m, CH₂), 1.42-1.61 (14H, m, CH₂), 2.49-2.58 (1H, m, CH-alkyl), 3.37 (2H, t, *J* = 6.6 Hz, CH₂OH), 3.55 (3H, s, OCH₃), 3.59-3.71 (1H, m, HCOSi), 3.95 (2H, t, *J* = 6.7 Hz, CH₂OPv); δ_C (125.7 MHz, CDCl₃) -4.4 (two peaks), 25.8 (three peaks), 27.2 (two peaks), 27.5, 27.8, 28.6, 29.2, 29.4, 29.5 (two peaks), 29.6 (two peaks), 29.7 (15 peaks), 29.8, 32.8, 33.7, 51.2, 51.6, 63.1, 64.5, 73.2, 175.1, 178.7; *MS* {Found (M + Na)⁺: 721.5756, C₄₁H₈₂NaO₆Si requires: 721.5773}.

(2R)-methyl 2-((1R)-1-(ethyldimethylsilyloxy)-11-(tetrahydro-2H-pyran-2-yloxy)undecyl)-26-(pivaloyloxy)hexacosanoate (85**)**



The title compound **85** was obtained by chain extensions of **136** and **83**. Both gave identical proton and carbon NMR spectra.

a. Chain extension of the Frater alkylation-derived compound 136.

To a stirred solution of the alcohol **136** (0.25 g, 0.36 mmol) in dichloromethane (50 ml) was added pyridinium chlorochromate (0.15 g, 0.72 mmol). The reaction was stirred for 2.5 h at which time TLC showed the reaction was complete and diethyl ether/ hexane (100 ml : 20 ml) was added to the reaction solution. It was filtered through a bed of celite to yield a solution of the crude product which was concentrated *in vacuo* and purified by flash column chromatography eluting with hexane : ethyl acetate (9 : 1) to give the aldehyde **137** (0.21 g, 85%) as a colourless oil. R_f = 0.49 (hexane : ethyl acetate, 8 : 2).

To a stirred solution of sulfone **95** (0.14 g, 0.32 mmol) in dry THF (20 ml) was added the freshly prepared aldehyde **137** (0.20 g, 0.29 mmol) and lithium bis(trimethylsilyl) amide (0.4 ml, 0.41 mmol, 1.0 M in THF) at 0 °C under nitrogen. The solution was stirred at room temperature overnight and quenched with sat. aq. NH_4Cl (10 ml). The crude product was extracted with ethyl acetate (100 ml, 50 ml x 2) and the organic fractions pooled, dried over MgSO_4 , and concentrated *in vacuo*. It was purified by flash column chromatography eluting with hexane : ethyl acetate (10 : 1) to yield a mixture of *E/Z* alkenes **138** (0.21 g, 74%). The *E/Z* mixture of alkenes **138** was dissolved in hexane : ethyl acetate (40 ml, 1 : 1) and palladium 10% on carbon (20 mg) was added. The solution was stirred under hydrogen (2.4 atm) overnight after which it was filtered on a bed of celite to yield the title compound **85** (0.21 g, 99%) as a colourless oil. R_f = 0.54 (hexane : ethyl acetate, 10 : 1); α_D^{20} -6.5 (c = 0.01 in CHCl_3); $\nu_{\text{max}}/\text{cm}^{-1}$: 3036, 2974, 2899, 1436, 1052; δ_H (400 MHz, CDCl_3) 0.02 (6H,

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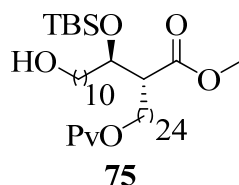
s, Si(CH₃)₂), 0.84 (9H, s, SiC(CH₃)₃), 1.17-1.25 (58H, m, CH₂; 9H, s, C(CH₃)₃), 1.47-1.63 (4H, m, CH₂), 1.66-1.73 (1H, m, CH₂), 1.75-1.85 (4H, m, CH₂), 2.46-2.53 (2H, m, CH₂), 3.33-3.39 (1H, m, CH₂OTHP), 3.45-3.50 (1H, m, CH₂OTHP), 3.63 (3H, s, OCH₃), 3.67-3.73 (2H, m, CH₂OTHP, HCOSi), 4.02 (2H, t, *J* = 6.6 Hz, CH₂OPv), 4.55 (1H, t, *J* = 3.4 Hz, OCHO); δ_C (100 MHz, CDCl₃) -4.4 (two peaks), 19.5, 25.4, 25.8 (three peaks), 27.2 (two peaks), 27.5, 27.8, 28.6, 29.4 (two peaks), 29.5 (two peaks), 29.6 (two peaks), 29.7 (24 peaks), 30.5, 32.8, 33.7, 51.2, 51.6, 62.4, 67.1, 64.5, 73.2, 98.9, 175.1, 178.7; **MS** {Found (M + Na)⁺: 917.7592, C₅₄H₁₀₆NaO₇Si requires: 917.7600}.

b. Chain extension of the Aldol-derived compound **83**.

To a stirred solution of allyl ester **83** (100 mg, 0.20 mmol) in 1,4-dioxane-water (24 ml, 3 : 1) was added 2,6-lutidine (47 ml, 0.40 mmol), K₂OsO₄ (1.20 mg, 0.004 mmol), and NaIO₄ (171.10 mg, 0.80 mmol). The reaction was stirred for 2 h when it was diluted with water (20 ml) and extracted with dichloromethane (3 x 200 ml). The organic fractions were pooled, dried over MgSO₄, and concentrated *in vacuo*. The crude product was purified by flash column chromatography eluting with hexane : ethyl acetate (9 : 1) to yield the aldehyde **139** (73 mg, 73%) as a colourless oil. *R*_f = 0.45 (hexane : ethyl acetate, 8 : 2).

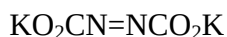
To a stirred solution of sulfone **82** (108 mg, 0.175 mmol) in dry THF (15 ml) was added the freshly prepared aldehyde **139** (73 mg, 0.146 mmol) and lithium bis(trimethylsilyl)amide (0.23 ml, 0.23 mmol, 1.0 M in THF) at 0 °C under nitrogen. The reaction was stirred at room temperature overnight and quenched with sat. aq. NH₄Cl (10 ml). The crude product was extracted with ethyl acetate (3 x 50 ml) and the organic fractions pooled, dried over MgSO₄ and concentrated *in vacuo*. The crude product was purified by flash column chromatography eluting with hexane : ethyl acetate (10 : 1) to yield a mixture of *E/Z* alkenes **140** (101 mg, 69%). The mixture of *E/Z* alkenes **140** was dissolved in hexane : ethyl acetate (100 ml, 1 : 1) and saturated with hydrogen gas overnight at 2.4 atm using palladium 10% on carbon (10 mg, 10%) after which it was filtered on a bed of celite. The solution was concentrated *in vacuo* to give the title compound **85** (99 mg, 98%) as a colourless oil.

(R)-methyl 2-((R)-1-(ethyldimethylsilyloxy)-11-hydroxyundecyl)-26-(pivaloyloxy)hexacosanoate (75)



To a stirred solution of methyl ester **85** (0.2 g, 0.2 mmol) in MeOH/THF/H₂O (12.2 ml, 1 : 5: 0.1) was added pyridinium-*p*-toluene sulfonate (2.5 mg, 0.01 mmol) and stirred at 60 °C for 2 h. The solution was cooled to room temperature and allowed to run overnight. The solution was diluted with water (50 ml) to quench and extracted with CH₂Cl₂ (3 x 50 ml). The combined organic fractions was dried over MgSO₄, concentrated *in vacuo*, and the crude product purified by flash column chromatography eluting with hexane : ethyl acetate (8 : 2) to yield the title compound **75** (139 mg, 84%) as a colourless oil. **R_f** = 0.33 (hexane : ethyl acetate, 8 : 2); α_D^{20} -4.8 (c = 0.01 in CHCl₃); $\nu_{\max}/\text{cm}^{-1}$: 3478, 2917, 2850, 1730, 1156; δ_H (400 MHz, CDCl₃) 0.08 (6H, s, Si(CH₃)₂), 0.89 (9H, s, SiC(CH₃)₃), 1.17 (9H, s, C(CH₃)₃), 1.21-1.29 (50H, m, CH₂), 1.40-1.84 (14H, m, CH₂), 2.39-2.42 (1H, m, CH-alkyl), 3.37 (2H, t, *J* = 6.6 Hz, CH₂OH), 3.69 (3H, s, OCH₃), 3.86-3.90 (1H, m, HCO₂Si), 4.02 (2H, t, *J* = 6.7 Hz, CH₂OPv); δ_C (100 MHz, CDCl₃) -4.4 (two peaks), 25.8 (three peaks), 27.2 (two peaks), 27.5, 27.8, 28.6, 29.2, 29.4, 29.5 (two peaks), 29.6 (two peaks), 29.7 (23 peaks), 29.8, 32.8, 33.7, 51.2, 51.6, 63.1, 64.5, 73.2, 175.1, 178.7; **MS** {Found (M + Na)⁺: 833.7064, C₄₉H₉₈NaO₆Si requires: 833.7025}.

Synthesis of Dipotassium Azodicarboxylate

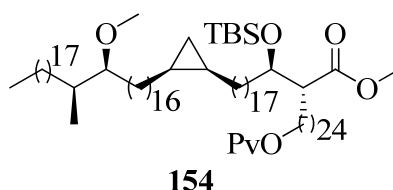


Azodicarbonamide (7.5 g, 64.61 mmol) was added in small portions to a stirred solution of KOH (15 g, 267.33 mmol) in distilled water (15 ml) at 0 – 5 °C and maintaining the temperature below 5 °C by means of a MeOH/ ice bath. After addition was complete the reaction was stirred for a further 1.5 h. A thick, yellow slurry of the dipotassium salt forms towards the reaction. The precipitate was filtered through a sintered-glass funnel under

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suction and washed with cold MeOH (50 ml). The precipitate was re-dissolved in water (40 ml) at 18 °C and filtered into cold IMS (60 ml) to give a yellow precipitate. The precipitate was filtered through a sintered-glass funnel and washed with cold MeOH (20 ml) and cold petrol (50 ml) to yield dipotassium azodicarboxylate (10.1 mg, 80%) as a yellow solid. It was transferred into an empty cold flask under nitrogen in the freezer until it was used because it decomposes almost instantaneously at room temperature.

(R)-methyl 2-((R)-1-(tert-butyldimethylsilyloxy)-18-((1R,2S)-2-((17S,18S)-17-methoxy-18-methylhexatriacontyl)cyclopropyl)octadecyl)-26-(pivaloyloxy)hexacosanoate (**154**)



To a stirred solution of the alcohol **75** (0.30 g, 0.33 mmol) in dichloromethane (50 ml) was added pyridinium chlorochromate (0.14 g, 0.65 mmol). The reaction was stirred for 2.5 h. It was filtered on a bed of celite to yield the crude product which was purified by flash column chromatography eluting with hexane : ethyl acetate (9 : 1) to give the aldehyde **152** (0.24 g, 80%) as a colourless oil. R_f = 0.56 (hexane : ethyl acetate, 8 : 2).

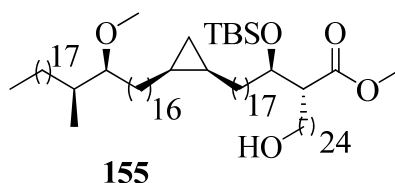
To a stirred solution of the methoxy meromycolyl sulfone **151** (0.10 g, 0.11 mmol) in dry THF (20 ml) was added the freshly prepared mycolic motif aldehyde **152** (0.12 g, 0.13 mmol) and lithium bis(trimethylsilyl)amide (0.15 ml, 0.15 mmol, 1.0 M in THF) at 0 °C under nitrogen. The reaction was stirred at room temperature overnight and quenched with sat. aq. NH_4Cl (10 ml). The crude product was extracted with warm dichloromethane (3 x 50 ml) and the organic fractions pooled, dried over MgSO_4 , and concentrated *in vacuo*. It was purified by flash column chromatography eluting with hexane : ethyl acetate (10 : 1) to yield the mixture of *E/Z* alkenes **153** (0.14 g, 82%) as a colourless oil. R_f = 0.78 (hexane : ethyl acetate, 8 : 2).

The mixture of *E/Z* alkenes **153** (0.215 g, 0.165 mmol) was stirred in THF (10 ml) and methanol (5 ml), cooled to 5 °C and excess of dipotassium azodicarboxylate was added.

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Acetic acid (3 ml) in methanol (2 ml) was added drop-wise to the reaction mixture over 8 h. The reaction was stirred for 18 h and then poured into ammonium chloride (20 ml). The reaction solution was extracted with warm diethyl ether (3 x 50 ml) and the combined organic layers was dried over MgSO_4 , and concentrated *in vacuo*. The hydrogenation reaction procedure was repeated until the NMR spectra showed no remaining alkenes. The crude product was purified by flash column chromatography eluting with hexane : ethyl acetate (8 : 2) to give the title compound **154** (0.13 g, 93%) as a viscous colourless oil. R_f = 0.78 (hexane : ethyl acetate, 8 : 2); α_D^{20} -4.1 (c = 0.01 in CHCl_3); $\nu_{\text{max}}/\text{cm}^{-1}$: 2921, 2952, 1740, 1469, 1156; δ_H (400 MHz, CDCl_3) -0.36 (1H, br q, J = 5.2 Hz, $\underline{\text{HCH}}$ -cyclopropane), 0.05 (6H, s, $(\text{CH}_3)_2\text{Si}$), 0.51-0.57 (1H, br dt, J = 3.8, 7.3 Hz, HCH -cyclopropane), 0.59-0.66 (2H, m, 2 x CH-cyclopropane), 0.82–0.86 (15H, including s for $(\text{CH}_3)_3\text{CSi}$, t, J = 6.8 Hz, for CH_3 , d, J = 6.8 Hz, for CHCH_3), 1.17 (9H, s, $(\text{CH}_3)_3\text{CCOO}$), 1.18-1.44 (146H, m, CH_2), 1.50-1.64 (1H, m, CH_2), 2.47-2.53 (1H, m, CHCOO), 2.91-2.95 (1H, m, CHCOCH_3), 3.32 (3H, s, COCH_3), 3.63 (3H, s, COOCH_3), 3.86-3.90 (1H, m, HCOSi), 4.02 (2H, t, J = 6.6 Hz, CH_2OPv); δ_C (100 MHz, CDCl_3) -4.9, -4.4, 10.9, 14.1, 14.9, 15.8, 22.7, 23.7, 25.8, 25.9, 26.2, 27.2, 27.6, 27.8, 28.6, 28.7, 29.2, 29.3, 29.4, 29.4, 29.5, 29.6, 29.7, 29.8, 29.9, 30.0, 30.2, 30.3, 30.5, 31.9, 32.4, 33.7, 35.3, 51.2, 57.7, 64.5, 73.2, 85.4, 175.1, 178.7; **MS** {Found ($M + \text{Na}$) $^+$: 1505.4419, $\text{C}_{97}\text{H}_{193}\text{NaO}_6\text{Si}$ requires: 1505.4459}.

(R)-methyl-2-((R)-1-(tert-butyldimethylsilyloxy)-18-((1R,2S)-2-((17S,18S)-17-methoxy-18-methylhexatriacontyl)cyclopropyl)octadecyl)-26-hydroxyhexacosanoate (155)

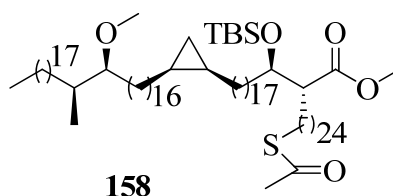


To a stirred solution of ester **154** (0.06 g, 0.04 mmol) in THF/ MeOH/ H_2O (12.2 ml, 5 : 1 : 0.1) was added KOH (31.4 mg, 0.56 mmol) and the reaction refluxed for 24 h. The solution was diluted with water (20 ml) to quench and extracted with warm diethyl ether (3 x 50 ml). The organic fractions were pooled, dried over MgSO_4 , and concentrated *in vacuo*. The crude product was purified by flash column chromatography eluting with hexane : ethyl

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acetate (8 : 2) to yield the title compound **155** (0.048 g, 85%) as a viscous colourless oil. R_f = 0.49 (hexane : ethyl acetate, 8 : 2); α_D^{20} -2.0 (c = 0.007 in CHCl_3); $\nu_{\text{max}}/\text{cm}^{-1}$: 3511, 2921, 2952, 1740, 1469, 1156; δ_H (400 MHz, CDCl_3) -0.36 (1H, br q, J = 5.2 Hz, HCH-cyclopropane), -0.003 (3H, s, $(\text{CH}_3)_2\text{Si}$), -0.02 (3H, s, $(\text{CH}_3)_2\text{Si}$), 0.52-0.57 (1H, br dt, J = 3.8, 7.3 Hz, HCH-cyclopropane), 0.61-0.65 (2H, m, 2 x CH-cyclopropane), 0.80–0.88 (15H, including s for $(\text{CH}_3)_3\text{CSi}$, t, J = 6.8 Hz, for CH_3 , d, J = 6.8 Hz, for CHCH_3), 1.03-1.44 (173H, m, CH_2), 1.51-1.65 (1H, m, CH_2), 2.49-2.56 (1H, m, CHCOO), 2.91-2.95 (1H, m, CHCOCH_3), 3.31 (3H, s, COCH_3), 3.62 (2H, t, J = 6.6 Hz, CH_2OH), 3.63 (3H, s, COOCH_3), 3.86-3.90 (1H, m, HCOSi); δ_C (100 MHz, CDCl_3) -4.9, -4.4, 10.9, 14.1, 14.9, 15.8, 22.7, 23.7, 25.8, 25.9, 26.2, 27.2, 27.6, 27.8, 28.6, 28.7, 29.2, 29.3, 29.4, 29.4, 29.5, 29.6, 29.7, 29.8, 29.9, 30.0, 30.2, 30.5, 31.9, 32.4, 33.7, 35.3, 51.2, 51.6, 57.7, 63.1, 73.2, 85.4, 175.1; **MS** {Found $(M + \text{Na})^+$: 1421.3773, $\text{C}_{92}\text{H}_{184}\text{NaO}_5\text{Si}$ requires: 1420.3805}.

(R)-methyl 26-(acetylthio)-2-((R)-1-(tert-butyldimethylsilyloxy)-18-((1R,2S)-2-((17S,18S)-17-methoxy-18-methylhexatriacontyl)cyclopropyl)octadecyl)hexacosanoate (158)



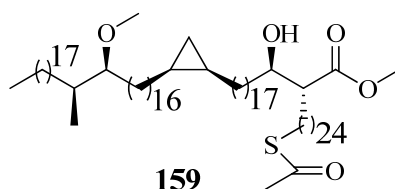
To a stirred solution of alcohol **155** (0.043 g, 0.029 mmol) in dry CH_2Cl_2 (10 ml) was added triethylamine (1 ml). The solution was cooled to -20 °C and stirred for 30 min under nitrogen. Toluene sulfonylchloride (7 mg, 35 mmol) was added and the reaction stirred in the refrigerator overnight. The solvent was evaporated and the crude product purified by flash column chromatography eluting with hexane : ethyl acetate (10 : 1) to yield the tosylate **156** (34 mg, 73%) as a viscous colourless oil. R_f = 0.63 (hexane : ethyl acetate, 8 : 2).

To a stirred solution of the tosylate **156** (0.05 g, 0.03 mmol) in acetone (10 ml) was added potassium thioacetate **157** (0.026 g, 0.23 mmol) and the reaction was stirred overnight at room temperature. The solvent was then evaporated and the crude product purified by flash

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column chromatography eluting with hexane : ethyl acetate (10 : 1) to yield the title compound **158** (36 mg, 82%) as a colourless oil. $R_f = 0.70$ (hexane : ethyl acetate, 8 : 2); $\alpha_D^{20} -5.2$ ($c = 0.009$ in CHCl_3); $\nu_{\text{max}}/\text{cm}^{-1}$: 2943, 2967, 1710, 1509, 1150; δ_H (400 MHz, CDCl_3) -0.35 (1H, br q, $J = 5.2$ Hz, $\underline{\text{HCH}}$ -cyclopropane), -0.004 (3H, s, $(\text{CH}_3)_2\text{Si}$), -0.02 (3H, s, $(\text{CH}_3)_2\text{Si}$), 0.50-0.57 (1H, br dt, $J = 3.8, 7.3$ Hz, HCH -cyclopropane), 0.60-0.67 (2H, m, 2 x CH-cyclopropane), 0.82–0.88 (15H, including s for $(\text{CH}_3)_3\text{CSi}$, t, $J = 6.8$ Hz, for CH_3 , d, $J = 6.8$ Hz, for CHCH_3), 1.00-1.49 (173H, m, CH_2), 1.49-1.64 (1H, m, CH_2), 2.30 (3H, s, CH_3CO), 2.48-2.53 (1H, m, CHCOO), 2.84 (2H, t, $J = 7.4$ Hz, CH_2S), 2.91-2.95 (1H, m, $\underline{\text{CHCOCH}_3}$), 3.32 (3H, s, COCH_3), 3.63 (3H, s, COOCH_3), 3.86-3.90 (1H, m, HCOSi); δ_C (100 MHz, CDCl_3) -4.9, -4.4, 10.9, 14.1, 14.9, 15.8, 22.7, 23.7, 25.8, 26.2, 27.5, 27.6, 27.8, 28.7, 28.8, 29.1, 29.2, 29.4, 29.5, 29.6, 29.7, 29.8, 29.9, 30.0, 30.2, 30.5, 31.9, 32.4, 33.7, 35.3, 51.2, 51.6, 57.7, 65.8, 66.1, 73.2, 85.4, 175.1; **MS** {Found ($M + \text{Na}$) $^+$: 1479.3765, $\text{C}_{94}\text{H}_{186}\text{NaO}_5\text{SSi}$ requires: 1478.3682}.

(R)-methyl-26-(acetylthio)-2-((R)-1-hydroxy-18-((1R,2S)-2-((17S,18S)-17-methoxy-18-methylhexatriacontyl)cyclopropyl)octadecyl)hexacosanoate (**159**)

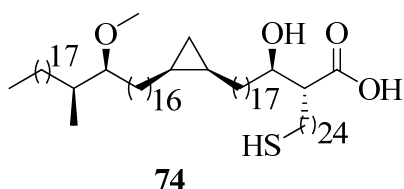


The MA thioacetate **158** (30 mg, 0.02 mmol) was dissolved in dry THF (4 ml) in a dry polyethylene vial under nitrogen at 0 °C. Pyridine (0.2 ml) and HF•Pyridine (0.7 ml) were added and the mixture stirred at 45 °C overnight. To quench the mixture was added slowly to a sat. aq. solution of NaHCO_3 (20 ml) until evolution of CO_2 stopped. The crude compound was extracted with warm diethyl ether (3 x 30 ml), dried over MgSO_4 , and concentrated *in vacuo*. The crude product was purified by flash column chromatography eluting with hexane : ethyl acetate (10 : 1) to give the title compound **159** (19 mg, 71%) as a white solid. $R_f = 0.56$ (hexane : ethyl acetate, 8 : 2); $\alpha_D^{20} -5.3$ ($c = 0.009$ in CHCl_3); **m.p.** 58-60 °C; $\nu_{\text{max}}/\text{cm}^{-1}$: 3411, 2915, 2848, 1466, 1167; δ_H (400 MHz, CDCl_3) -0.35 (1H, br q, $J = 5.2$ Hz, $\underline{\text{HCH}}$ -cyclopropane), 0.50-0.57 (1H, br dt, $J = 3.8, 7.3$ Hz, HCH -cyclopropane),

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0.58-0.66 (2H, m, 2 x CH-cyclopropane), 0.82 (1H, d, $J = 6.9$ Hz, CHCH_3), 0.86 (6H, t, $J = 7.0$ Hz, 2 x CH_3), 1.00-1.49 (173H, m, CH_2), 1.49-1.64 (1H, m, CH_2), 2.30 (3H, s, CH_3CO), 2.38-2.44 (1H, m, CHCOO), 2.84 (2H, t, $J = 7.4$ Hz, CH_2S), 2.91-2.96 (1H, m, CHCOCH_3), 3.32 (3H, s, COCH_3), 3.60-3.66 (1H, m, HCO), 3.69 (3H, s, COOCH_3); δ_{C} (100 MHz, CDCl_3) 10.9, 14.1, 14.9, 15.7, 22.7, 25.7, 26.2, 27.4, 27.6, 28.5, 28.7, 28.8, 29.0, 29.1, 29.2, 29.3, 29.4, 29.5, 29.6, 29.7, 29.9, 30.0, 30.2, 30.5, 30.6, 31.9, 32.4, 35.3, 35.7, 38.9, 39.4, 50.9, 51.5, 57.7, 72.3, 85.4, 176.2, 195.9; **MS** {Found ($M + \text{Na}$)⁺: 1364.2819, $\text{C}_{88}\text{H}_{172}\text{NaO}_5\text{S}$ requires: 1364.2818}.

(R)-2-((R)-1-hydroxy-18-((1R,2S)-2-((17S,18S)-17-methoxy-18-methylhexatriacontyl)cyclopropyl)octadecyl)-26-mercaptohexacosanoic acid (**74**)



a. Deprotection with lithium hydroxide

To a stirred solution of the alcohol **159** (13 mg, 0.01 mmol) in THF/water/methanol (4.8 ml, 4.0 : 0.4 : 0.4) was added LiOH (0.93 mg, 0.039 mmol) in one portion. The solution was stirred overnight at 45 °C. TLC showed formation of products which indicated that partially deprotected product was obtained while some starting material remained. To quench the solution was cooled to room temperature and diluted with hexane : ether (10 ml, 1 : 1). The pH was lowered to 3 by dropwise addition of HCl (0.1 M) and the crude product extracted with warm hexane : ether (1 : 1, 5 x 20 ml). The organic extracts were pooled, dried over MgSO_4 , and concentrated *in vacuo*. The crude product was purified by flash column chromatography eluting with hexane : ether (5 : 2) to yield a partially purified product **74** (2 mg).

The reaction was repeated on the same scale during which it was left to run for 24 h. A partially deprotected product was again obtained along with the fully deprotected compound **74**. Because LiOH could not completely cleave the methyl and acetyl protecting

groups and the poor yields obtained, hydrolysis with tetrabutylammonium hydroxide (TBAH) was attempted.

b. Deprotection with tetrabutylammonium hydroxide

Hydrolysis with a 5% (v/v) aqueous TBAH solution was attempted a few times for different reaction times, with slightly different extraction techniques and column purification solvents. Different yields were obtained. The full procedure described below is for the best yielding protocol.

In the first reaction attempt the MA methyl thioester **159** (15 mg, 0.01 mmol) was dissolved in TBAH (2 ml, 5%, v/v) and refluxed overnight for 24 h. The solution was cooled to room temperature and the reaction worked up by acidifying to pH 1 with 1.0 M HCl and extracted with warm diethyl ether (3 x 20 ml). The combined organic layers were pooled, dried over MgSO₄, and the solvent evaporated *in vacuo*. The crude product was purified by flash column chromatography eluting with hexane : ethyl acetate (8 : 2) to yield the title compound **74** (8.0 mg, 63%) as a white solid.

The reaction was repeated with 4.1 mg of the ester **159** refluxing in TBAH (2 ml, 5%) for 18 h. The reaction work up, extraction, and purification of the product were identical to the first attempt but the yield was 41%.

The third reaction attempt gave the best yield and is as follows:

MA thioester **159** (11.8 mg, 0.009 mmol) was suspended in aqueous TBAH* (2 ml, 5%, v/v) and refluxed at ~97 °C overnight for 20 h. The solution was cooled to room temperature and the reaction worked up by acidifying to pH 1 with 1.0 M HCl and extracted with warm diethyl ether (2 x 30 ml) followed by hot hexane (3 x 30 ml). The combined organic layers were dried over MgSO₄, and the solvent evaporated. The crude product was purified by flash column chromatography eluting first with warm hexane : diethyl ether (10 : 1) then warm chloroform : methanol (10 : 1) to yield the title compound **74** (8.3 mg, 73%) as a white solid. $R_f = 0.19$ (hexane : ethyl acetate, 8 : 2); $\alpha_D^{20} -3.5$ (c =

* TBAH was prepared in ddd H₂O.

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0.008 in CHCl_3); **m.p.** 59-61 °C; $\nu_{\text{max}}/\text{cm}^{-1}$: 3411, 2916, 2847, 2339, 1681, 1465, 1092, 760; δ_{H} (400 MHz, CDCl_3) -0.35 (1H, br q, $J = 5.2$ Hz, HCH-cyclopropane), 0.50-0.57 (1H, br dt, $J = 3.8, 7.3$ Hz, HCH-cyclopropane), 0.58-0.66 (2H, m, 2 x CH-cyclopropane), 0.83 (1H, d, $J = 6.9$ Hz, CHCH_3), 0.86 (6H, t, $J = 7.0$ Hz, 2 x CH_3), 1.00-1.49 (173H, m, CH_2), 1.49-1.66 (1H, m, CH_2), 1.67-1.80 (1H, m, CH_2), 2.41-2.48 (3H, m, CHCOOH , t, $J = 7.4$ Hz, CH_2SH), 2.91-2.96 (1H, m, CHCOCH_3), 3.32 (3H, s, COCH_3), 3.65-3.72 (1H, m, HCOH); δ_{C} (100 MHz, CDCl_3) 10.9, 14.1, 14.9, 15.8, 22.7, 25.7, 26.1, 27.3, 27.6, 28.7, 29.0, 29.1, 29.3, 29.4, 29.5, 29.6, 29.7, 29.9, 30.0, 30.2, 30.5, 31.9, 32.3, 35.3, 35.6, 38.9, 50.5, 57.7, 72.1, 85.6, 177.2.

6.2 Experimental for Chapter Four

General

All reagents were of analytical grade. Potassium hexacyanoferrate (II) was purchased from B. Jones Ltd., South Africa, while potassium hexacyanoferric (III) was purchased from Bio-Zone Chemicals, South Africa. All other reagents used were obtained from reputable suppliers. SPR gold disks and polycrystalline gold electrode (BAS, $r = 0.8$ mm) were obtained from Metrohm, South Africa.

All solvents were purged of air by bubbling nitrogen gas prior to each characterization experiment. Incubation of SPR gold disks and electrodes were done at room temperatures. Except for the X-ray photoelectron spectroscopy (XPS) experiments which were done at ultra-high vacuum all characterization experiments were done at 1 atm. Sonification was done with an Ultrasonic cleaner, Optima Scientific CC, Model: DC150H.

XPS spectra were obtained on two instruments: a Physical Electronics model Quantum 2000 with Al $K\alpha$ radiation at 1486 eV and Physical Electronics model 5400 spectrometer system with monochromatic Mg $K\alpha$ radiation at 1253.6 eV. The take-off angle was 45°.

Atomic force microscopy (AFM) images were obtained on an AFM 5100 System of Agilent Technologies in AC mode. The images were taken in air at room temperature at a scan rate of 0.9-1.0 lines s^{-1} .

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The electrochemical experiments, cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS), were done on an Autolab Potentiostat PGSTAT 302 from Eco Chemie, Utrecht, Netherlands. Data was acquired and processed with General Purpose Electrochemical Systems (GPES) and Frequency Response Analyser (FRA) software version 4.9.

6.2.1 Preparation of Solutions

a. Preparation of Phosphate-Buffered Saline Containing Sodium Azide and EDTA (PBS/AE, pH 7.4)

Phosphate buffered saline (PBS) azide EDTA buffer (PBS/AE) with the following: NaCl (8.0 g), KCl (0.2 g), KH_2PO_4 (0.2 g) and Na_2HPO_4 (1.05 g) dissolved in ddd H_2O (1.0 L) with 1 mM EDTA and sodium azide 0.025% (m/v). The solution was adjusted to pH 7.4.

b. Preparation of Ferricyanide/Ferrocyanide Redox Probe Solution (1.0 mM)

In PBS/AE buffer (250 ml) was dissolved $\text{K}_3[\text{Fe}(\text{CN})_6]$ (82.3 mg, 0.25 mmol), $\text{K}_4[\text{Fe}(\text{CN})_6]$ (105.6 mg, 0.25 mmol), and KCl (1.86 g, 25 mmol). The solution was prepared a few days before use and purged of air by bubbling an inert gas through it for at least 30 min immediately before every electrochemistry experiment.

6.2.2 Cleaning of Gold Substrates

SPR disks were cleaned prior to an experiment by spraying with absolute ethanol. Next, the disks were dipped into boiling isopropanol for 10 s and then blown with a gentle stream of nitrogen gas. The disks were similarly treated after incubation in a thiol solution.

The gold electrode was cleaned according to the following protocol in the exact sequence:

1. Firstly, the electrode was polished on an aqueous slurry of alumina and then polished to mirror finish on Buehler felt paper.

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2. It was rinsed with a lot of distilled water followed by absolute ethanol and then subjected to an ultrasonic vibration in absolute ethanol for about 1 h to remove residual alumina particles that might have been trapped on the surface.
3. The electrode was then rinsed with excess distilled water and then absolute ethanol.
4. Next it was incubated in a hot piranha solution* for about 2 min followed by a copious rinse with ddd H₂O followed by ethanol.
5. Finally the electrode was cleaned with 0.5 M H₂SO₄ at a scan rate of 100 mV/s and a potential window of -0.2 V and -1.2 V measuring 10 scan cycles. A reproducible scan was obtained.

6.2.3 Preparation of SPR Gold Disks for XPS and AFM Characterizations

SPR gold disk was incubated in a dry chloroform solution of MASH (5 ml, 1 mM) in a sealed tube for 24 h. On removal from the solution the disk was washed by dipping in boiling isopropanol and immediately placed in a clean polytop. After blowing with a gentle stream of nitrogen the polytop was capped and sealed with parafilm. No further preparation was made and the sample was submitted for analysis.

A control disk was similarly prepared but incubation was done in dry chloroform (5 ml) only.

6.2.4 Investigation of Bare Gold Electrode Surface by CV and EIS

CV experiments were run in duplicates in a solution of K₃[Fe(CN)₆]/ K₄[Fe(CN)₆] (40 ml, 1mM) containing KCl (1 M) at a scan rate of 25 mV/s and a potential window of -0.1 V to 0.5 V. The experiments were also repeated at a scan rate of 50 mV/s.

EIS experiments were run in the same redox solution at a formal potential of 209 mV, which is equal to the E_{1/2} of the electrode.

* 30% H₂O₂ : conc H₂SO₄, 1 : 3 (v/v)

6.2.5 Fabrication of Gold Electrode

The gold electrode was fabricated with ODT, MASH, and MASH/ODT. Two different MASH samples were used; both samples chemically identical but differing only in their purity. The experimental conditions for these two samples were also identical except that the purer sample was run at an ambient temperature of about 15 °C while the other sample was run at about 25 °C.

a. Fabrication of Bare Gold Electrode with ODT

The clean and characterized gold electrode was dipped into an ethanolic solution of ODT (5 ml, 10 mM) and incubated at room temperature for 12 h. At the end of this period the electrode was removed, rinsed successively in excess quantities of absolute ethanol and ddd H₂O. This was repeated thrice and the gold surface blown with nitrogen. CV and EIS characterizations were done immediately as described above for the bare gold.

b. Fabrication of Bare Gold Electrode with MASH

The clean and characterized gold electrode was dipped into a dry chloroform solution of MASH (2 ml, 0.5 mM) and incubated at room temperature for 24 h. At the end of this period the electrode was removed, rinsed successively in excess chloroform, sonified in chloroform for 1 min, rinsed again in excess quantities of chloroform, absolute ethanol and finally ddd H₂O. This was done to remove any non-covalently bound MASH. The gold surface was then blown gently with nitrogen. CV and EIS characterizations were done immediately as described above for the bare gold.

c. Mixed Monolayer Fabrication

The MASH-fabricated electrode above was rinsed in excess ddd H₂O after the CV and EIS experiments and re-incubated in an ethanolic solution of ODT (5 ml, 10 mM) for 12 h. At the end of this period the electrode was treated and characterized as described in (a) above.

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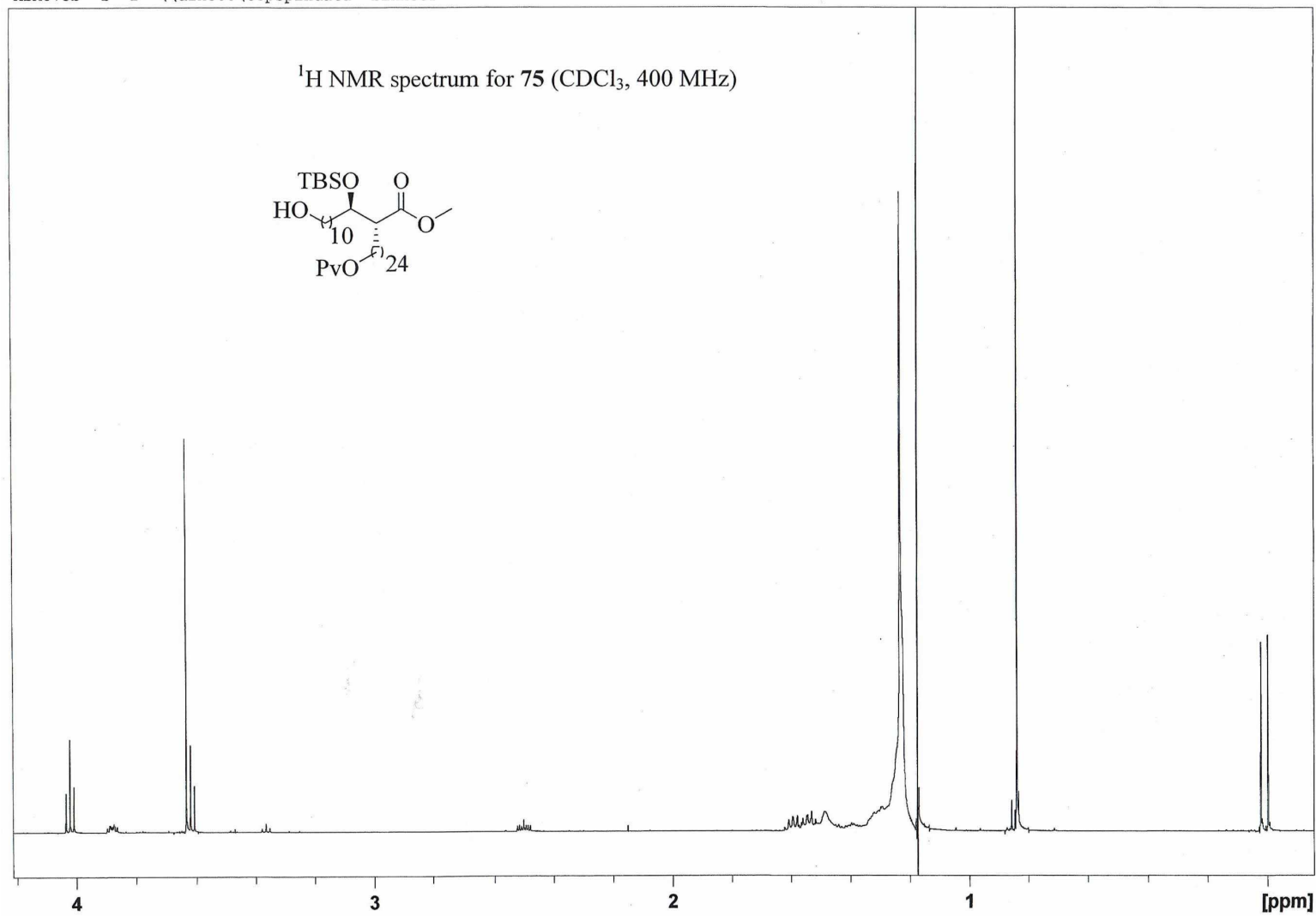
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Appendix

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Appendix

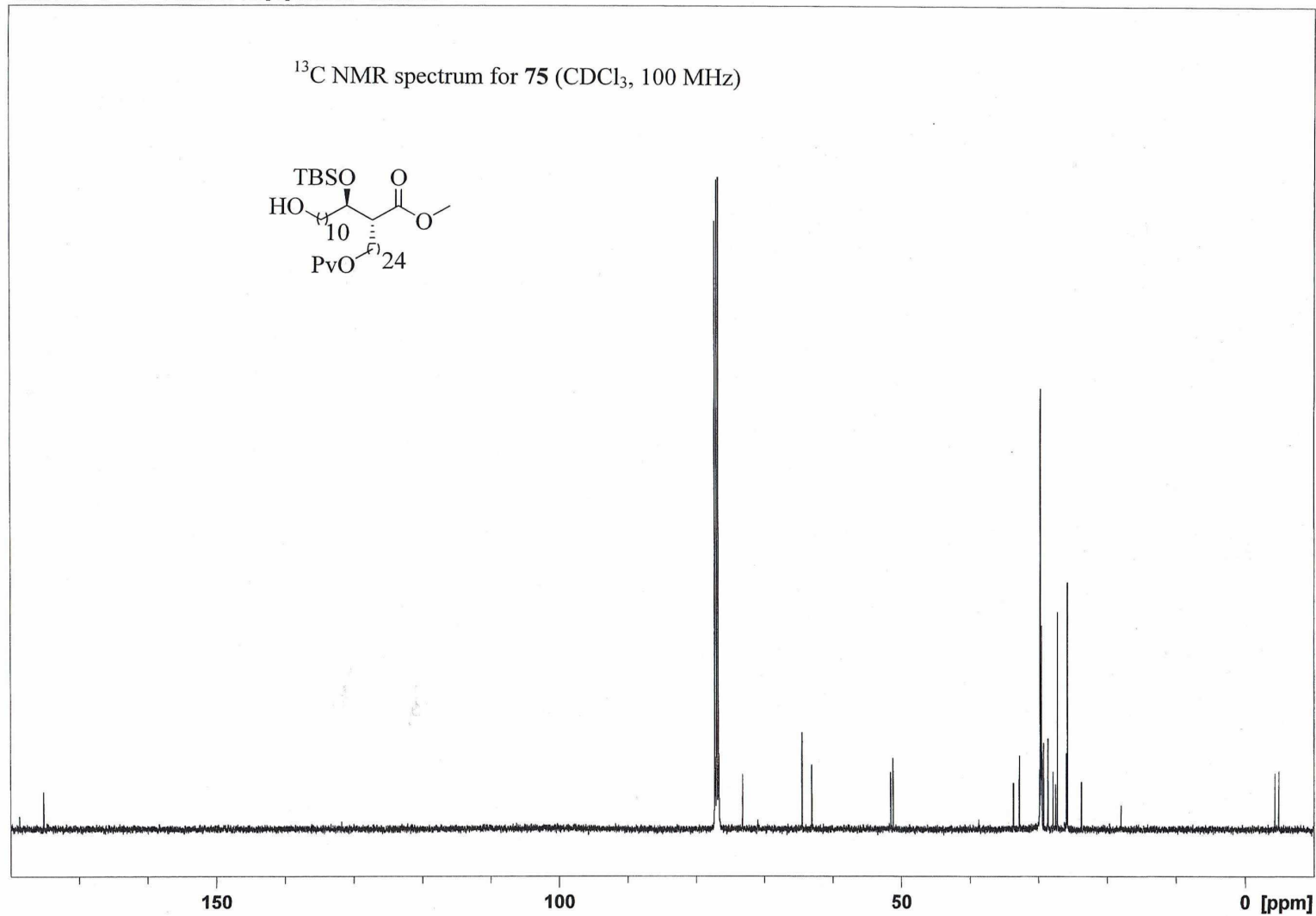
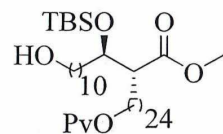
MBA073b 1 1 \\arx300\topspindata binneUP



Appendix

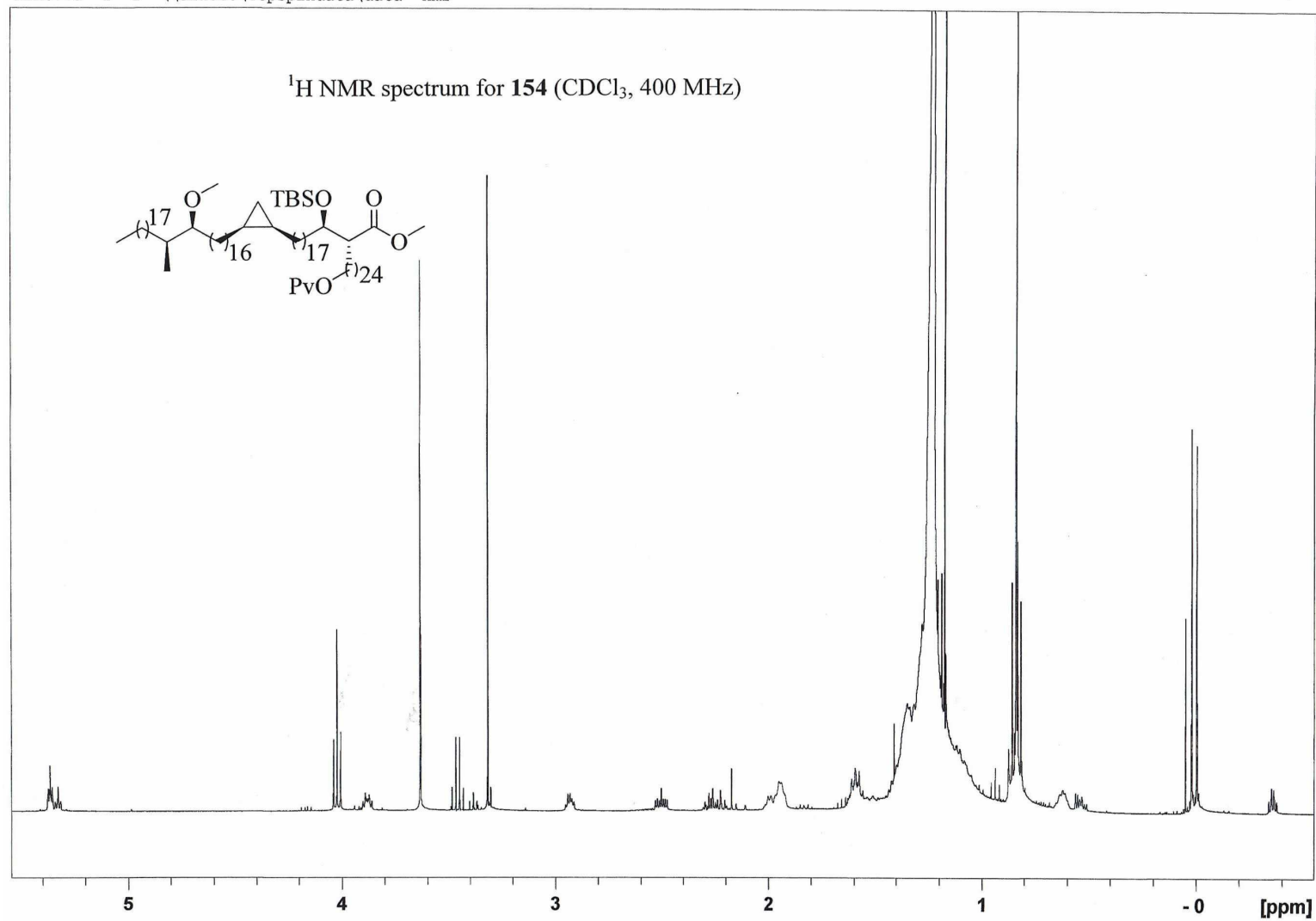
MBA073b 2 1 \\arx300\topspindata binneUP

^{13}C NMR spectrum for **75** (CDCl_3 , 100 MHz)



Appendix

MBA0770 1 1 \\arx300\topspindata\data nmr

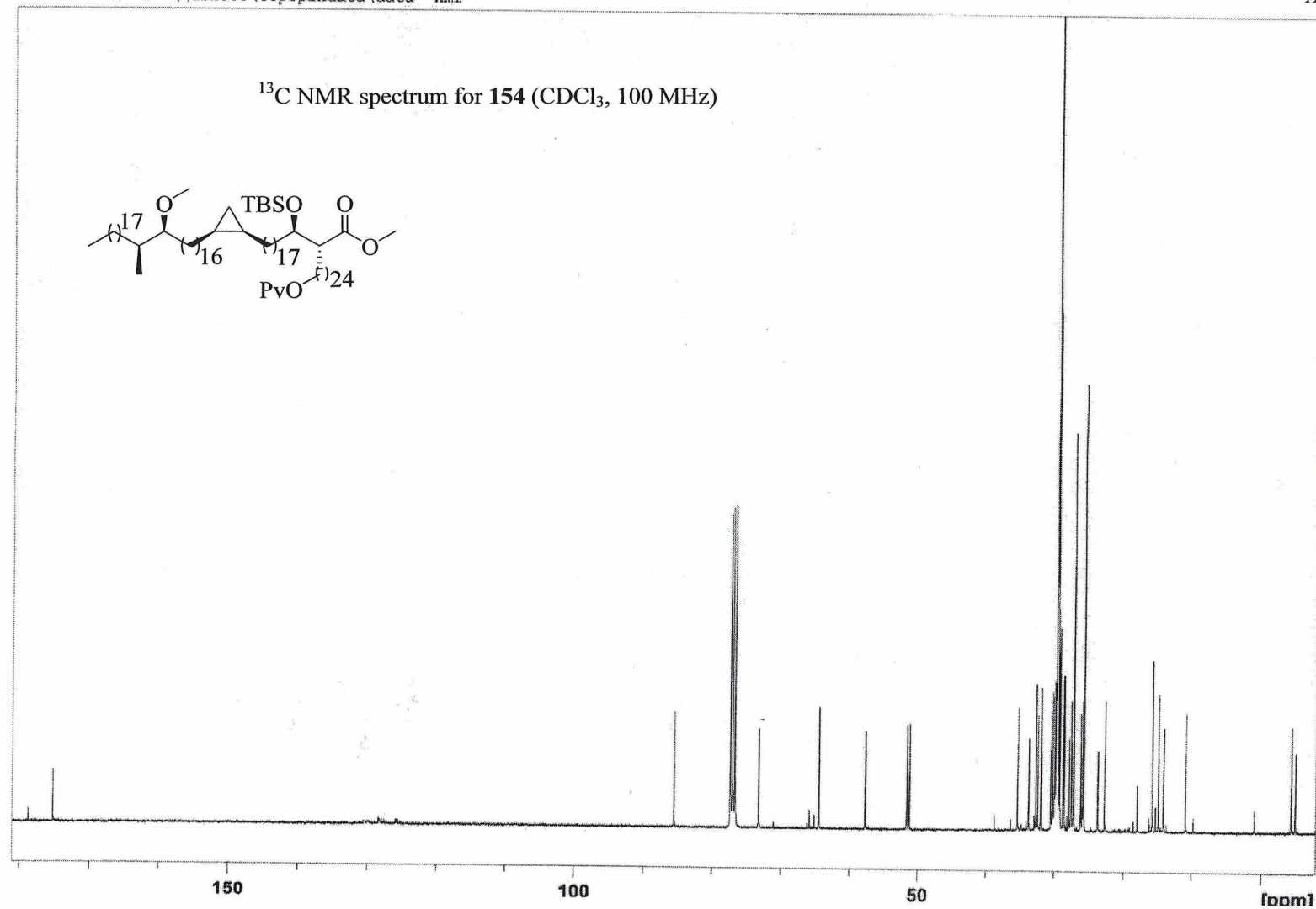
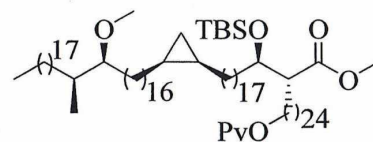


Appendix

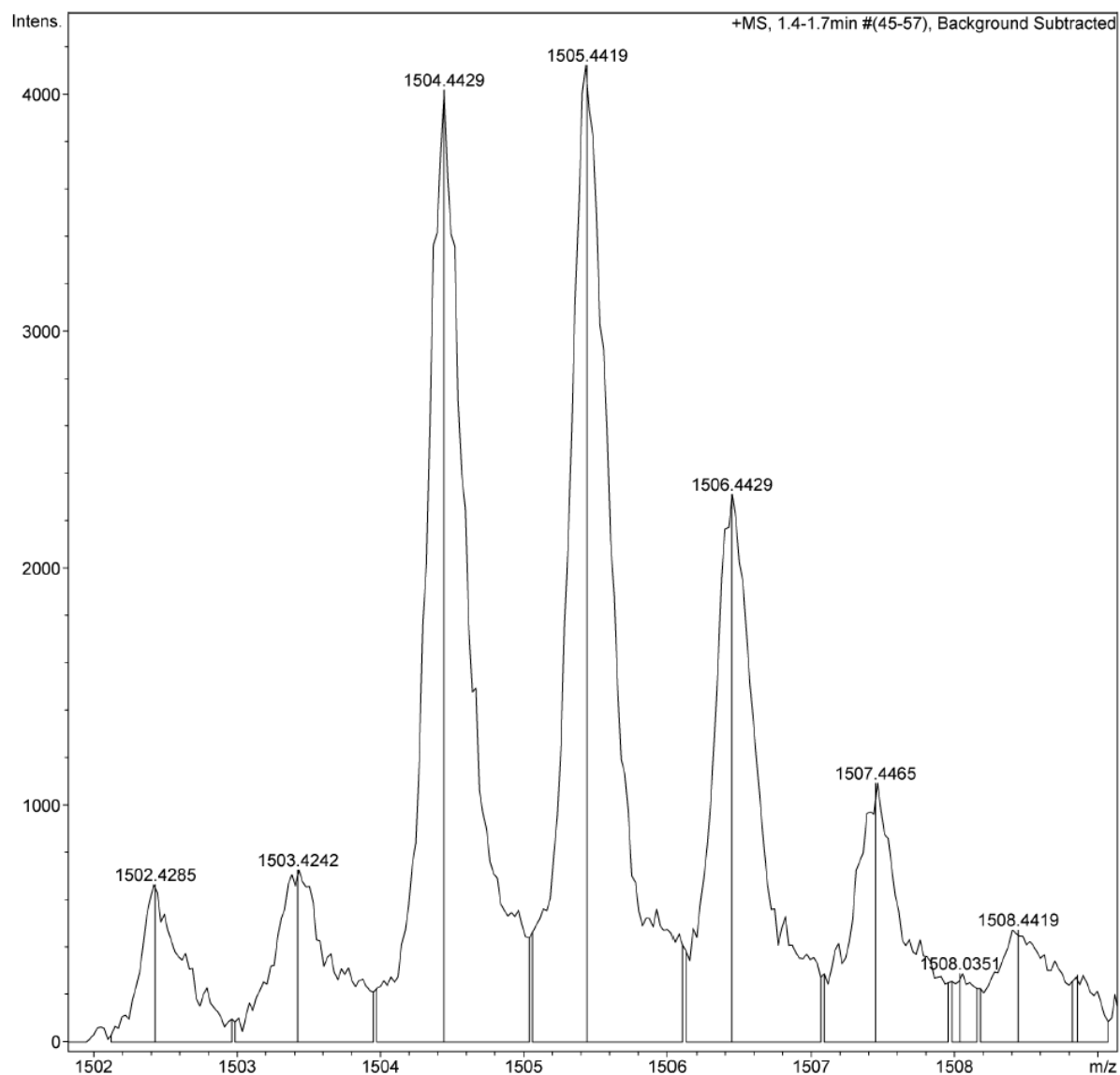
MBA0770 2 1 \\arx300\topspindata\data nmr

Ap

^{13}C NMR spectrum for **154** (CDCl_3 , 100 MHz)



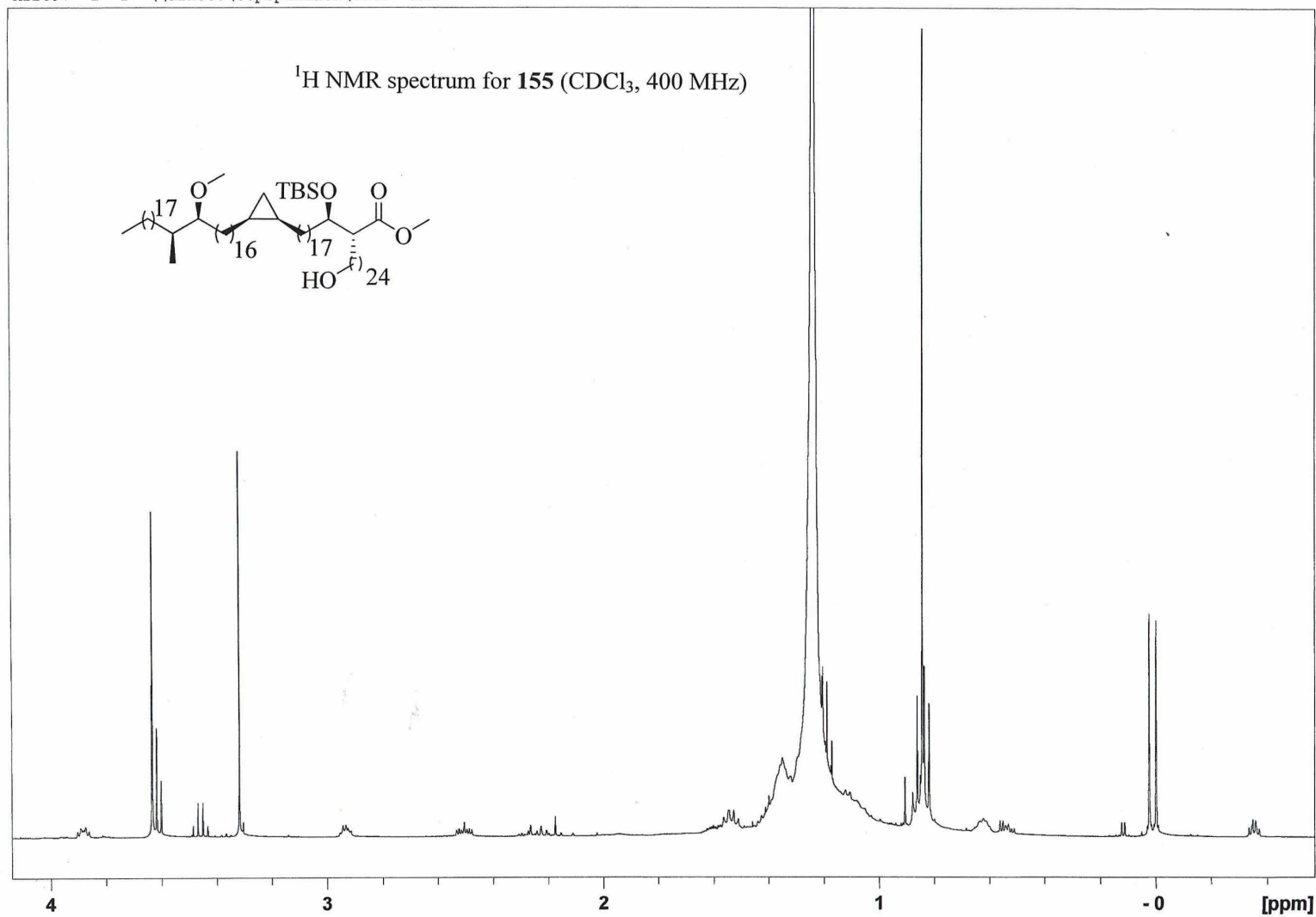
Appendix



MALDI-TOF spectrum of **154**

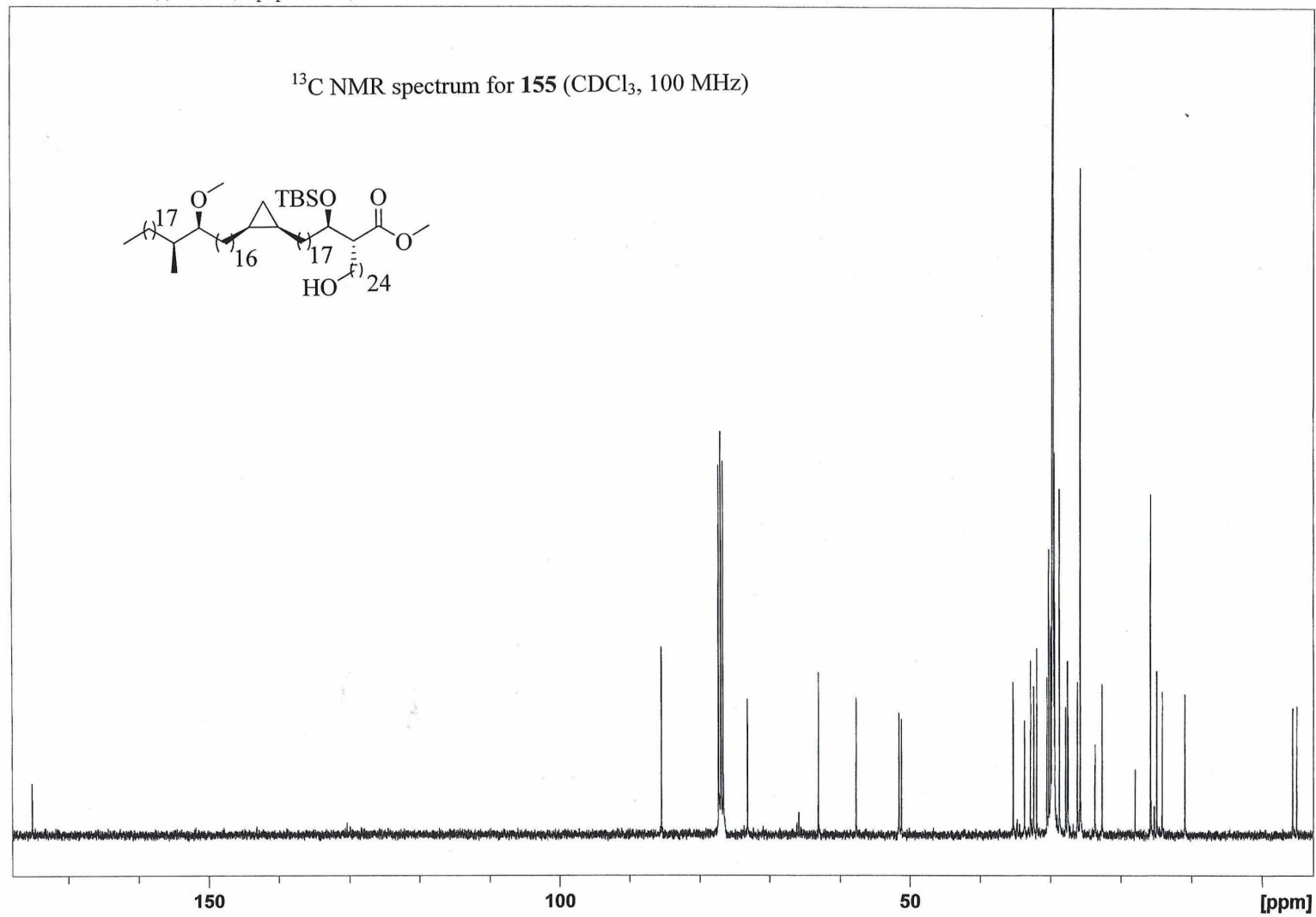
Appendix

MBB097 1 1 \\arx300\topspindata\data nmr



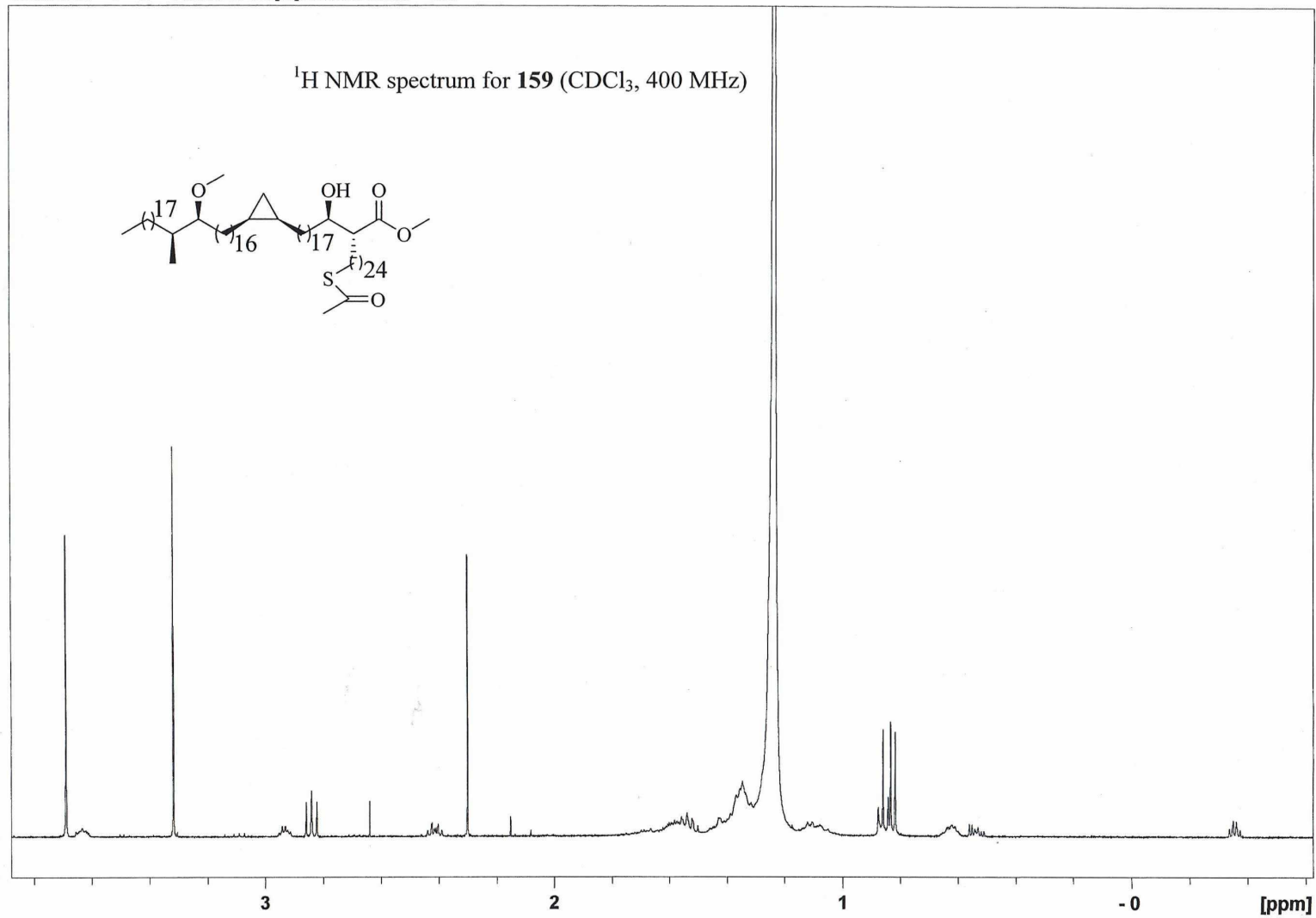
Appendix

MBB097 2 1 \\arx300\topspindata\data nmr



Appendix

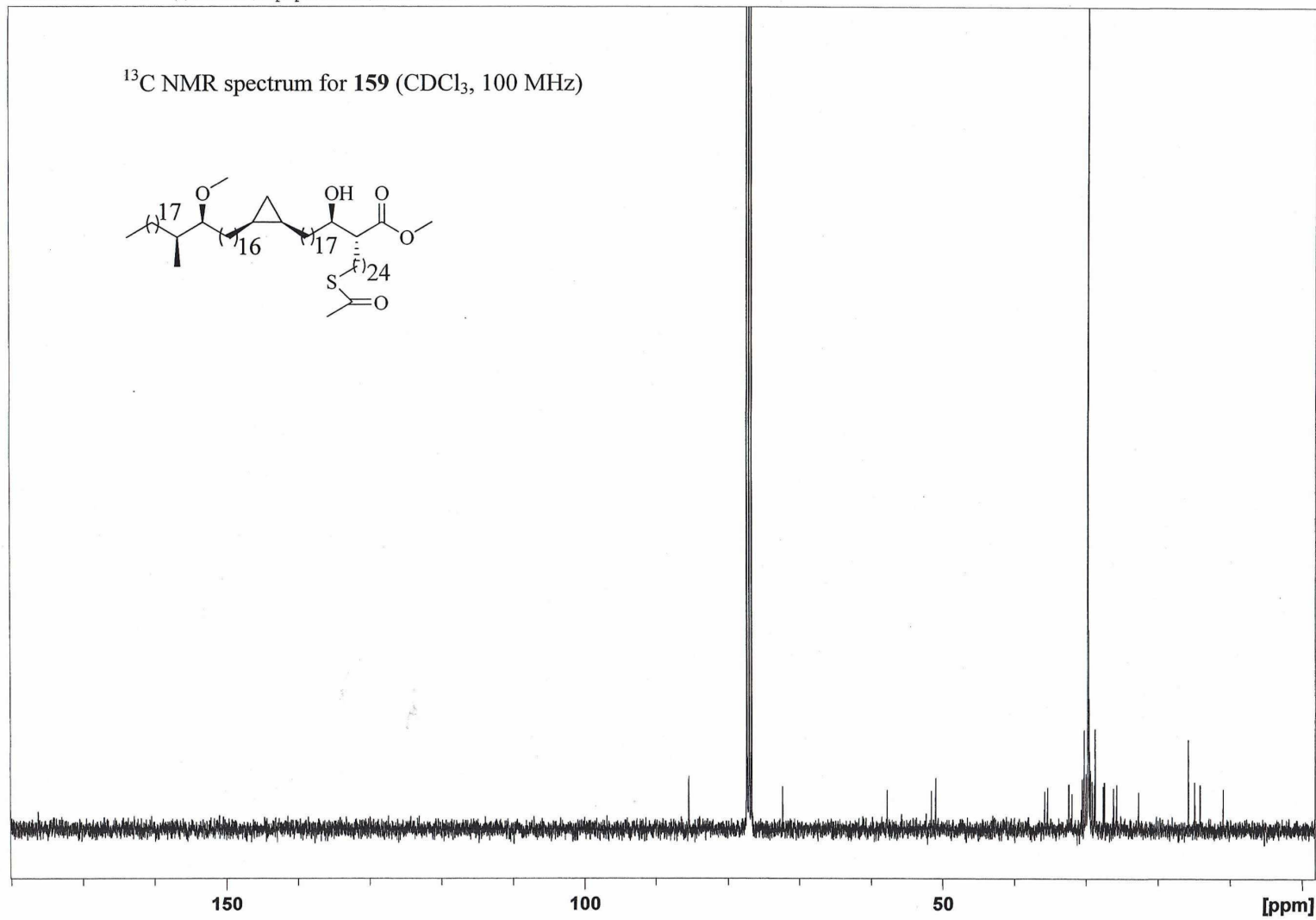
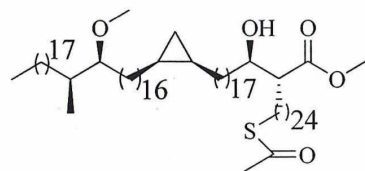
MBUK2011 1 1 \\arx300\topspindata\data nmr



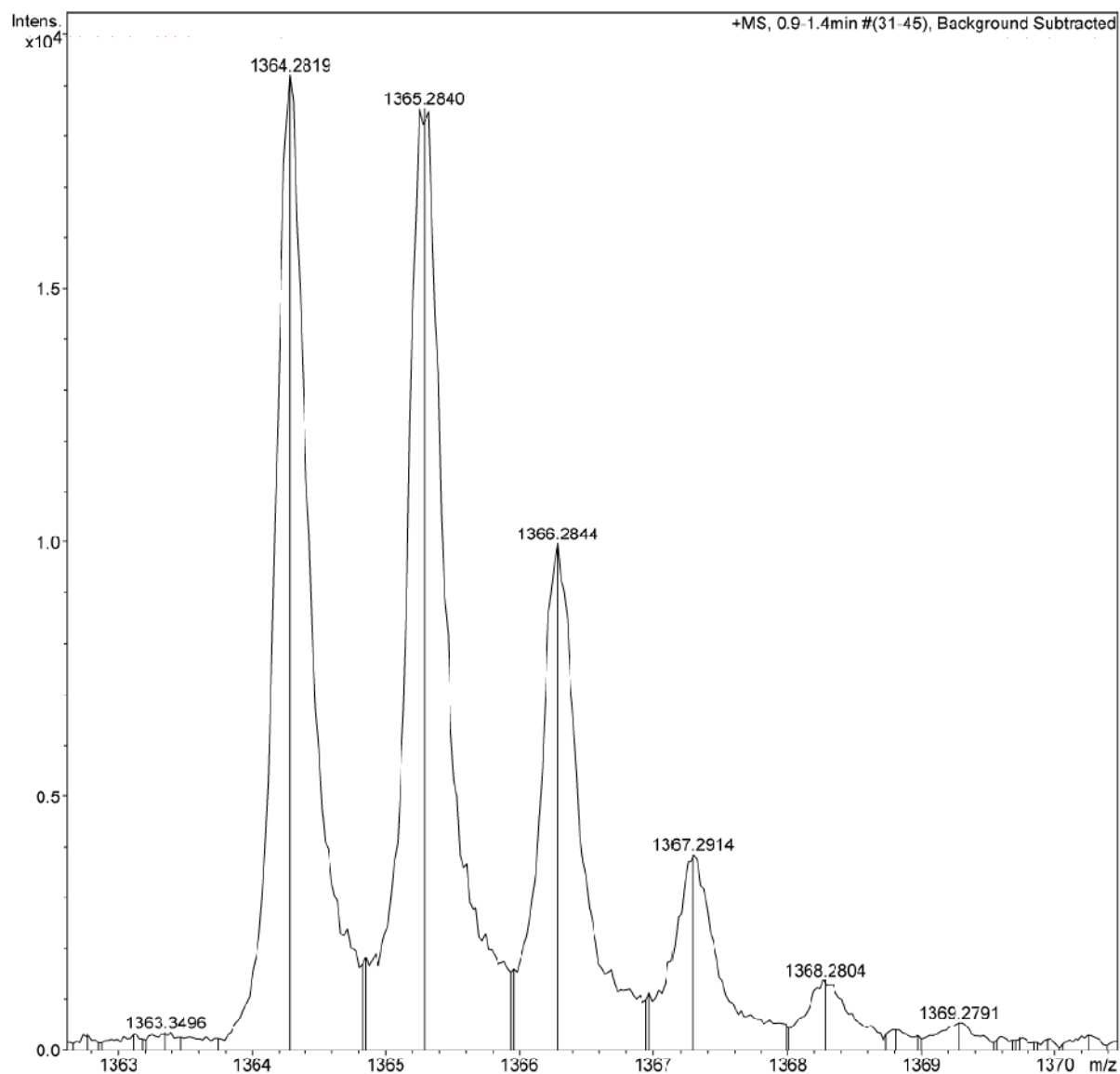
Appendix

MBUK2011 2 1 \\arx300\topspindata\data nmr

^{13}C NMR spectrum for **159** (CDCl_3 , 100 MHz)



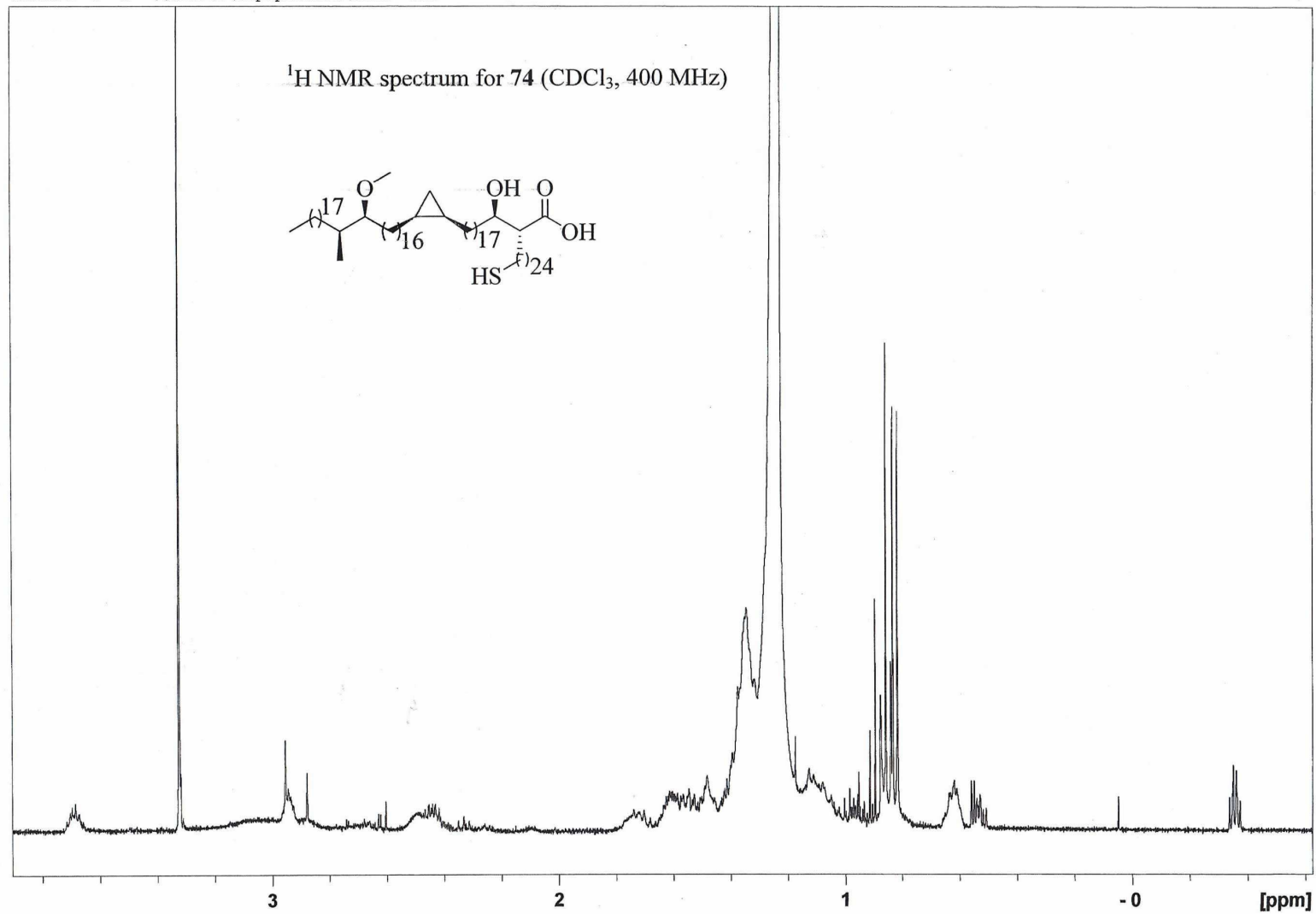
Appendix



MALDI-TOF spectrum of **159**

Appendix

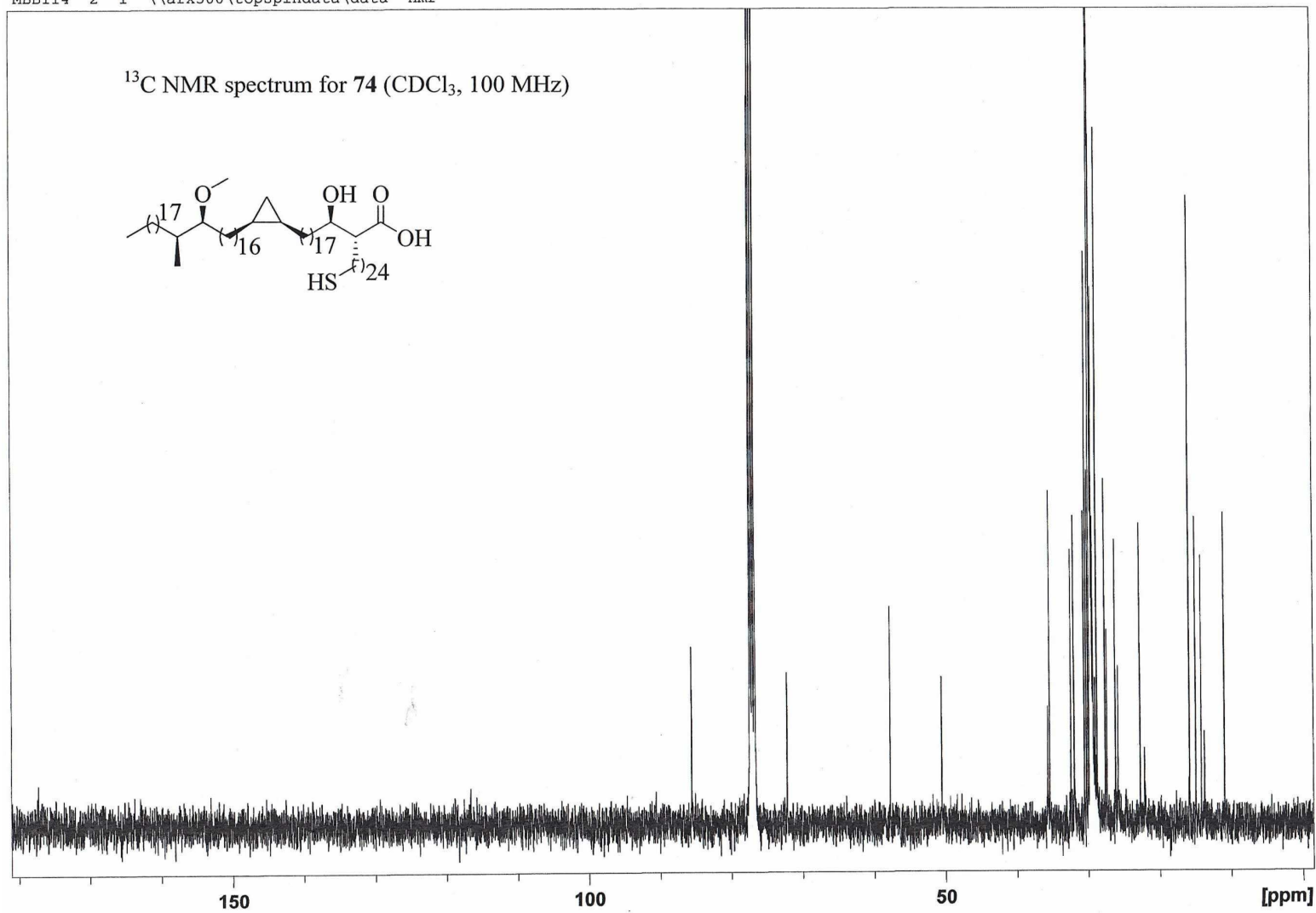
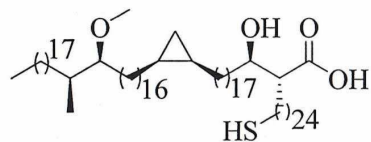
MBB114 1 1 \\arx300\topspindata\data nmr



Appendix

MBB114 2 1 \\arx300\topspindata\data nmr

^{13}C NMR spectrum for **74** (CDCl_3 , 100 MHz)



Conference Presentations and Publication

Some of the results of this project have been presented at two international conferences and published in one peer-reviewed organic chemistry journal. The titles of the conference abstracts are listed below while a copy of the publication is attached.

1. **MRC 40th Anniversary TB Colloquim, 2009:** *‘Towards an Optimal Stereocontrolled Chemical Synthesis of Mycolic Acid Antigens of Mycobacterium tuberculosis.’* (Poster presentation)
2. **11th Frank Warren Organic Chemistry International Conference, 2010:** *‘Synthesis of a functionalized Mycolic Acid for TB research’* (Oral and poster presentations)



A biomimetic approach to the synthesis of a mycolic acid motif

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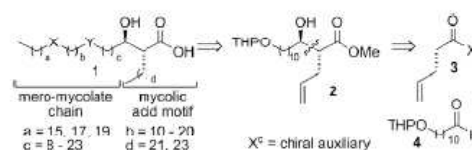
ABSTRACT

A new method for the stereoselective synthesis of the (*R,R*)- β -hydroxy- α -alkyl fatty acid fragment of mycolic acids, via an asymmetric *anti*-aldol reaction is reported. The 'mycolic acid motif' fragment was prepared in three steps and >98% ee.

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Mycolic acids **1** (MAs) are located in the protective wax coat of the cell wall of *Mycobacterium tuberculosis* and other mycobacteria.¹ MAs consist of two main parts, a mycolic acid motif with a 22 or 24 carbon α -alkyl chain and a β -hydroxy group, and a mero-mycolate chain with variable functional groups.² The mycolic acid motif, common to MAs from all mycobacteria, usually has an *R,R* configuration.³ These complex waxes are recognized by antibodies⁴ and therefore show potential for use in TB therapy and new diagnostic techniques such as biosensor assays.⁵ Furthermore, they have been shown to re-programme the macrophages to promote a tolerogenic response in experimental asthma and are being explored for their potential in the prevention and treatment of asthma.⁶

Natural mycobacterial MAs are present as complex mixtures containing different functionalities X and Y, and a range of homologues with different chain lengths.^{1,2} To fully understand the biological role of MAs there is a need for the synthesis of individual acids with known stereochemistry. In the biosynthesis of mycolic acids, the α and β carbons are joined in a Claisen-type condensation to give a β -keto product which is selectively reduced.⁷ In published syntheses of the mycolic acid motif, two stereocentres are inserted consecutively in three to five steps.^{8,9} We now report a more biomimetic approach (Scheme 1) whereby an auxiliary-mediated *anti*-aldol reaction gives the α -alkyl- β -hydroxy product **2** in the required *R,R* configuration in one step. We have adapted the

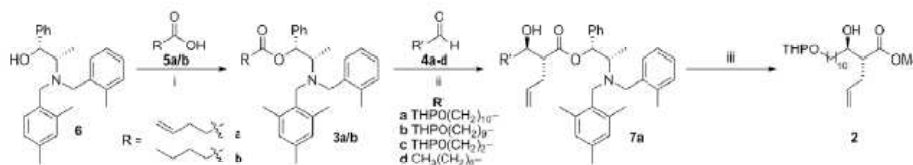


Scheme 1. Mycolic acid structure^{1,2} and retrosynthetic analysis.

method developed by Kurosu and Lorca,¹⁰ to couple saturated carboxylic acids with short chain aldehydes for the synthesis of **2**, containing a terminal alkene. This can be used to extend the α -alkyl chain in the mycolic acid motif to its full length of 22 or 24 carbons as described by Toschi and Baird,⁹ and can be extended to the full MA by reaction at the THPO-group.¹¹

The norephedrine-based auxiliary **6** was attached to both the required unsaturated acid **5a** and a saturated acid **5b** to give chiral esters **3a** and **3b** (Scheme 2). The corresponding zirconium enolates were prepared by treatment with LDA and transmetalation with a zirconium complex (Cp₂ZrCl₂). Coupling of these enolates to a variety of aldehydes **4a–d** showed that chain length and functionality in the aldehyde had little effect on the outcome of the reaction, but the introduction of the terminal alkene in the enolate led to lower yields (30–45% for **3a** with **4a–d** vs 50% for **3b** with **4b/d**). Using freshly prepared LDA, strictly anhydrous conditions, a constant temperature of –78 °C (monitored by an internal probe) and dropwise addition of the reagents, the *anti*-aldol product **7a** was obtained from **3a** and **4a** on a 1 g scale in 45% yield with high

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Scheme 2. Synthesis of chiral esters **3a** and **3b** and subsequent *anti*-aldol reaction with various aldehydes. Reagents and conditions: (i) EDCI, DMAP, CH_2Cl_2 (**3a** = 89%, **3b** = 91%); (ii) LDA, Cp_2ZrCl_2 , THF, -78°C ; (iii) Na(s), MeOH (70%).

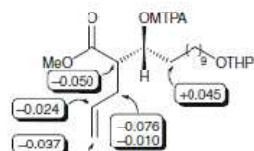


Figure 1. MTPA ester of compound **2** ($\Delta\delta_{\text{H}}$ values in ppm).

Table 1

Comparison of the ^{13}C NMR alkene signals (in ppm) of diastereomers **2** with the literature compound **8**¹¹

Carbon	2	<i>epi</i> - 2	<i>anti</i> - 8
$\text{H}_2\text{C}=\text{CH}_2$	134.9	135.6	134.9
$\text{HC}=\text{CH}_2$	117.1	116.6	117.1

diastereoselectivity (>98%) (determined as described below) after separation from the two *syn*-diastereomers by flash chromatography.¹² The chiral auxiliary was cleaved from aldol product **7a** by transesterification with sodium methoxide, rather than reductively,¹⁰ to yield the methyl ester **2** (70%) (Scheme 2). The configuration of the hydroxy group in **2** was determined using Mosher's method, by conversion into the (*R*)- and (*S*)-MTPA esters.¹³ The $\Delta\delta_{\text{H}}$ values for the protons in the left and right segments (Fig. 1), respectively, indicated an *R* configuration.

On standing in CDCl_3 at room temperature for a week, the solution of **2** partly epimerized at the α position giving a mixture of *anti*- and *syn*-diastereomers. A comparison of the ^{13}C NMR data for the two diastereomers with those published for mycolic acid motif **8**¹¹ demonstrated that the relative stereochemistry of **2** was *anti* (Table 1).

This synthesis of the mycolic acid motif methyl ester **2** represents a novel approach to a key intermediate that has been used to prepare mycolic acids.¹¹ The use of *anti*-aldol methodology reduces the number of steps required from six,¹¹ to two. Prior to the removal of the auxiliary, the diastereomeric products are readily separated giving the desired *R,R*-product in >98% ee. The chiral auxiliary can be recovered and recycled, increasing the atom econ-

omy of the process. By applying the method directly to the aldol reaction of mero-mycolate aldehydes and long chain acids, it is hoped that more efficient syntheses of a range of homologues of natural mycolic acids can be achieved.

Acknowledgements

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1015/j.tetlet.2009.12.105.

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