Synthesis and fluorescent labelling of model mycolic acids

By

Mr Jean Pierre KAYEMBE

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In the

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Summary

Mycolic Acids (MAs) are long chain *α*-alkyl-*β*-hydroxy fatty acids that form part of the cell wall of *Mycobacterium* species and a few other genera. They play an important role in steering the host-pathogen relationship to establish active TB disease. These compounds are recognized by antibodies and therefore show potential for use in new diagnostic techniques such as biosensor assays. Previous studies have shown that MA-methyl esters were not antigenic, while fluorescein labelled-MA maintained antigenicity. It was proposed that this was due to the presence of the carboxylic acid group on the fluorescein label that substituted for the one on MA that became esterified during conjugation. However the existence of the free carboxylic acid on fluorescein after labelling of MA was uncertain as fluorescein labelling of palmitic acid apparently produced two structural forms, the free acid and the lactone, as a mixture of tautomers. Furthermore the original biological studies were not done on characterized material and activity may have been due to the presence of some unreacted MA.

Here, the synthesis of a corynomycolic acid homologue is reported via two routes: (a) Claisen condensation followed by reduction or (b) aldol condensation. Due to the cost and poor quality of commercial “5-BromoMethylFluorescein”, this reagent had to be synthesised in the laboratory. The synthetic Corynomycolic acid homologue made in this study and a mixture of naturally occurring bovine mycolic acids obtained from Sigma-Aldrich were labelled with freshly prepared 4(5)-BMF. NMR characterization of the fluorescein labelled-MA showed the presence of a carboxylic acid group on the fluoresceinsuggesting that it is likely to maintain biological activity.

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List of Abbreviations

ACP	: Acyl carrier protein
AG	: Arabinogalactan
AG-M	: Arabinogalactan-mycolate
Ag85	: Antigen 85
AIDS	: Acquired immune deficiency syndrome
AmB	: Amphotericin B
AMP	: Adenosine mono-phosphate
aq	: Aqueous
atm	: Atmosphere
Bn	: Benzyl
Bz	: Benzoyl
BCG	: Bacillus Calmette-Guérin
4(5)-BMF	: 4(5)-bromomethyl fluorescein
BPO	: Benzoyl peroxide
CMAs	: Corynomycolic acids
DCM	: Dichloromethane
DIBPO	: Dibenzoyl peroxide
DIPT	: Diisopropyl tartrate
DMF	: Dimethyl formamide
DMSO-d_6	: Deuterated dimethyl sulfoxide
DMAP	: 4-N,N-dimethylaminopyridine
DMSO	: Dimethyl sulfoxide
ELISA	: Enzyme-linked immunosorbent assay
Epoxy-MA	: Epoxy mycolic acid
eq	: Equivalent
FA	: Fatty acids
FAM	: Fluorescein amidite (6-FAM)
FACS	: Fluorescence-activated cell sorting
FAS-I	: Fatty acid synthetase type I

FAS-II	: Fatty acid synthetase type II
FITC	: Fluorescein isothiocyanate
h	: Hour(s)
HMPA	: Hexamethylphosphoramide
HIV	: human immune deficiency virus
HPLC	: High Pressure Liquid Chromatography
IC_{50}	: Half maximal inhibitory
IFN-γ	: Interferon-gamma
INH	: Isoniazid
IR	: Infra-red
KAS	: *β*-ketoacyl synthase
Keto-MAs	: Keto-mycolic acids
LDA	: lithium diisopropylamide
Mb	: Molecular beacon
MF	: Methyl fluorescein
mRNA	: Messenger RNA
M. tb.	: *Mycobacterium tuberculosis*
MTBC	: *Mycobacterium tuberculosis* complex
α-MA	: Alpha-mycolic acid
MA(s)	: Mycolic acid(s)
MALDI-TOF	: Matrix-assisted laser desorption ionization-time-of-flight
MARTI	: Mycolic acid real-time inhibition test
MDR	: Multi-drug resistance
MHz	: Mega Hertz
mol	: Mole
Mm	: Molar mass
M. tb.	: *Mycobacterium tuberculosis*
MTBC	: *Mycobacterium tuberculosis* complex
mAGP	:Mycolyl-arabinogalactan-peptidoglycan
MIC	: Minimum inhibitory concentration
MS	: Mass spectrometry
NTM	: Non-Tuberculous Mycobacteria
NADH	: β-nicotinamide adenine dinucleotide

nm	: Nanometre
NMR	: Nuclear magnetic resonance
NBS	: *N*-Bromosuccinimide
Pks 13	: Polyketide synthase
PG	: Peptidoglycan
Ppm	: Parts per million
Pv	: Pivaloyl
R_f	: Retention factor
rt	: Room temperature
SAM	: *S*-Adenosyl-*L*-methionine
TB	: Tuberculosis
TBAH	: Tetrabutylammunium hydroxide
TDM	: Trehalose dimycolate
TML	: Thiolactomycin
TMM	: Trehalose monomycolate
THF	: Tetrahydrofuran
TLC	: Thin layer chromatography
UV	: Ultraviolet
WHO	: World health organization
XDR	: Extensive drug resistance

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Chapter 1: General introduction

1.1. Tuberculosis

Tuberculosis (TB), an infectious disease, remains a major global health problem. It was estimated in 2012, that 8.6 million people developed TB and that 1.3 million died from the TB disease and this includes 320 000 deaths among HIV-positive people (WHO, 2013). The number of TB deaths is unacceptably large given that most are preventable. It is now about 20 years since the World Health Organization (WHO) declaration of TB as a global public health emergency and major progress has been made towards the 2015 global target, which was set in the context of the Millennium Development Goals (MDGs). However, in developing countries the risk of infection and the incidence of tuberculosis in the last decades have increased due to the prevalence of HIV infection and population migration (WHO, 2013).

Mycobacterial infection of the respiratory system is the most common form of tuberculosis. Extra-pulmonary TB is rarely observed and its incidence is about 8.4%. However, in patients co-infected with HIV/TB the extra-pulmonary manifestation of TB involves almost 47.5 to 50% of patients (WHO, 2012, WHO, 2013). The risk of extra-pulmonary tuberculosis and *mycobacteremia* is augmented by immunosuppression as occurs with AIDS.HIV associated with the TB remains a major global public health challenge. The co-infection with HIV complicates both the diagnosis and treatment of TB. Rate increases in drug resistant TB, including multi-drug (MDR-TB) and extensively drug resistant TB (XDR-TB) have been observed, which are difficult cases to treat. As a consequence, there is an increased mortality due to TB. Due to the poor performance of sputum smear microscopy in diagnosis of HIV co-infected patients, new diagnostic test tools which are sensitive, specific and easy to be used in remote and resource-constrained settings are urgent. In the treatment of HIV co-infected cases, anti-tuberculosis and antiretroviral drugs need to be administered concomitantly. Some challenges with the treatment include patient compliance to chemotherapy, drug interactions, overlapping toxic effects, as well as immune reconstitution inflammatory syndrome.

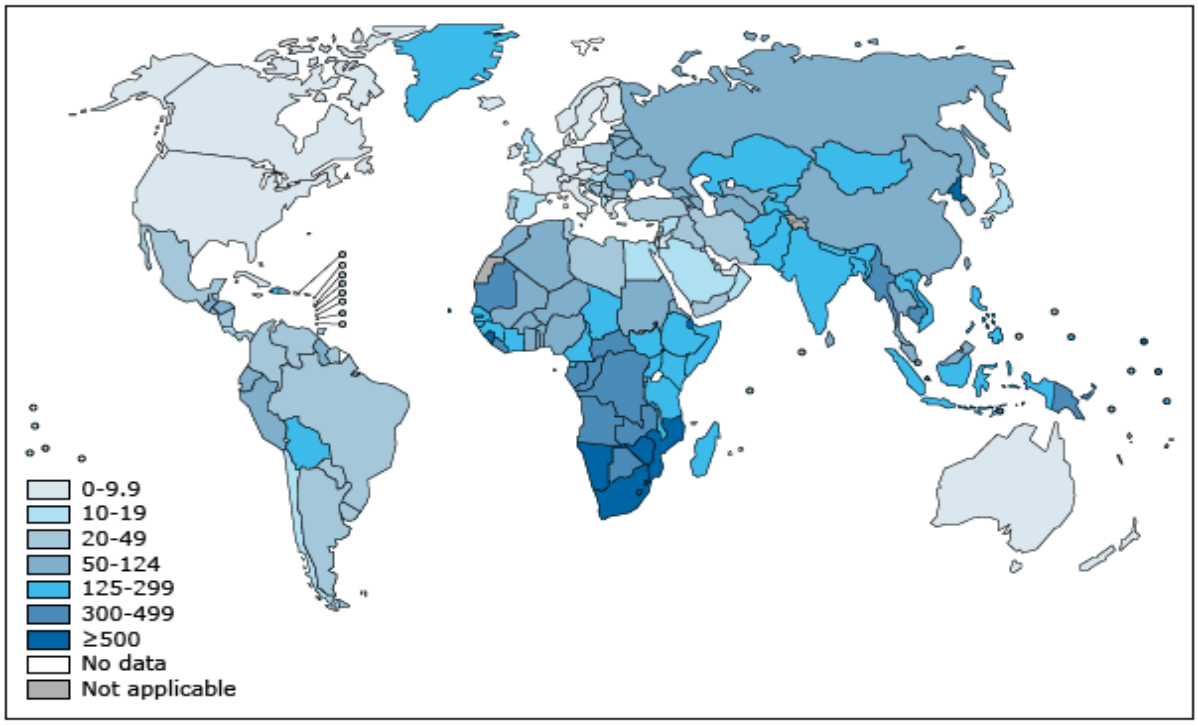


Figure 1.1: **Figure 1.1: Estimated tuberculosis incidence rates, by country in 2012 (WHO, 2013).**

The World Health Organisation recommended that tuberculosis is treated by a simultaneous combination therapy consisting of the use of more than one drug, each with different biological targets. The advantage of a combined therapy is that it boosts some important properties such as the antibacterial activity as well as the capacity to prevent drug-resistance. Generally the treatment involves an initial treatment period which may last up to 2 months and then a follow up of four to 7 months period of treatment. The four most common *anti*-TB drugs are: Ethambutol (EMB or E), Isoniazid (INH or H), pyrazinamide (PZA or Z) and rifampicin (RMP or R). The first line anti-TB drugs names have a standard three-letter and a single-letter abbreviation. Due to the high rates of resistance to streptomycin, this drug is longer considered as a first line TB drug.

Second line drugs are used to treat multi-drug resistant (MDR) TB and extensively drug-resistant (XDR) TB since there is resistance to first line drugs. These second-line drugs are are not used as first-line drugs because they may be less effective (e.g. p-aminosalicylic acid), they may have toxic side-effects (e.g. cycloserine), or may not be available in developing countries (e.g., fluoroquinolones). The WHO has recommended that MDR-TB, defined as resistance isoniazid and rifampicin, be treated for a period of 20 months with a regimen that includes second-line *anti*-TB drugs (WHO, 2013). XDR TB is resistance to any fluoroquinolone, and at least one of the

three second-line drugs such as capreomycin, kanamycin, or amikacin as well as isoniazid and rifampicin (Figure 1. 2) (WHO, 2012). Effective management of MDR- and XDR-TB is problematic. In 2006 the World Health Organization ratified a plan of 6 points to eradicate TB as a public health problem by 2050. The aim was to treat at least 50 million patients and save 14 million lives by 2015 (Stop TB Initiative, World Health Organization, 2010).

Figure 1.2: **Proportion of MDR among new TB cases latest available data, 1994-2011** (WHO, 2012)

It is estimated that two thirds of TB deaths might be prevented by starting anti-TB therapy sooner (WHO, 2007). This requires early and rapid diagnosis. Unfortunately, the quick and affordable sputum smear microscopy or the "Acid Fast Bacilli (AFB) test" has very low sensitivity and the current gold standard diagnostic for active pulmonary TB of culturing mycobacteria from sputum is too slow - taking 6 weeks to give an answer. Blood based tests fail to distinguish between active and latent TB. The current dilemma that faces researchers is the time taken to accurately diagnose TB in HIV positive patients. There is an urgent need for a fast and reliable diagnostic tool for TB, especially in high HIV incidence populations (World Health Organisation

2007). The idea of using a serum based test for antibodies to the mycobacterial cell wall lipid mycolic acids was patented (Verschoor *et al*., 2005). This method tests for the presence of biomarker *anti*-mycolic acid antibodies for diagnosing active tuberculosis (Thanyani *et al*., 2008). It is known by the abbreviation MARTI (Mycolic Acid Antibody Real Time Inhibition) assay, can differentiate well between active and latent TB and has shown potential to diagnose even extrapulmonary TB (Lemmer *et al.*, 2009). It is currently being developed for clinical trials by Verschoor and co-workers.

1.2. Mycobacterium tuberculosis

Mycobacterium tuberculosis (*M.tb.*) is a small pathogenic bacterial species that causes the disease tuberculosis (Kassim *et al*., 2004). *M. tb*. was first discovered in 1882 by Robert Koch (Robert, K., 1982). *M.tb.* is highly aerobic, i.e. it needs high levels of oxygen during the active replicative stage and hence usually attacks the lungs while also affecting other parts of the body. The bacterium is transmitted through the air when people infected with active TB people sneeze or cough (Konstantinos *et al*., 2010).

Mycobacterium tuberculosis replicates every 15–20 hours which is tremendously slow when compared to other bacteria, which typically divide in minutes. It contains an unusual cell wall which is rich in lipids (with mycolic acids the most abundant lipid, which is responsible for the resistance and is a key virulence factor (Murray *et al*., 2005).

Mycobacteria do not fit the Gram-positive group as they don't retain the crystal violet stain owing to the additional high lipid layer in its cell wall. Neither do *Mycobacteria* fit the Gram-negative group since they do not have an outer cell membrane. *Mycobacteria* have been classified as acid-fast Gram-positive bacteria because they do retain Ziehl-Neelsen stain (Kassim *et al*., 2004).

- **The Mycobacterial Cell wall structure**

The cell wall structure of *Mycobacterium tuberculosis* (Figure 1.3) needs particular attention as it is unique among prokaryotes, and it is the major virulence determinant for the bacterium. The cell wall complex contains the universal peptidoglycan but is exclusively endowed with the complex lipids. About 60% of the *mycobacterial* cell wall content is lipid consisting largely of

mycolic acids (MAs). The elevated concentration of the *Mycobacterium tuberculosis* 's cell wall lipids have been associated with characteristic properties of the bacterium such as the non-permeability to stains and dyes, the resistance to many antibiotics, the resistance to killing by acidic and/or alkaline compounds, the resistance to osmotic lysis through complement attack and the resistance to lethal oxidations within macrophages (Russell *et al*., 2010a).

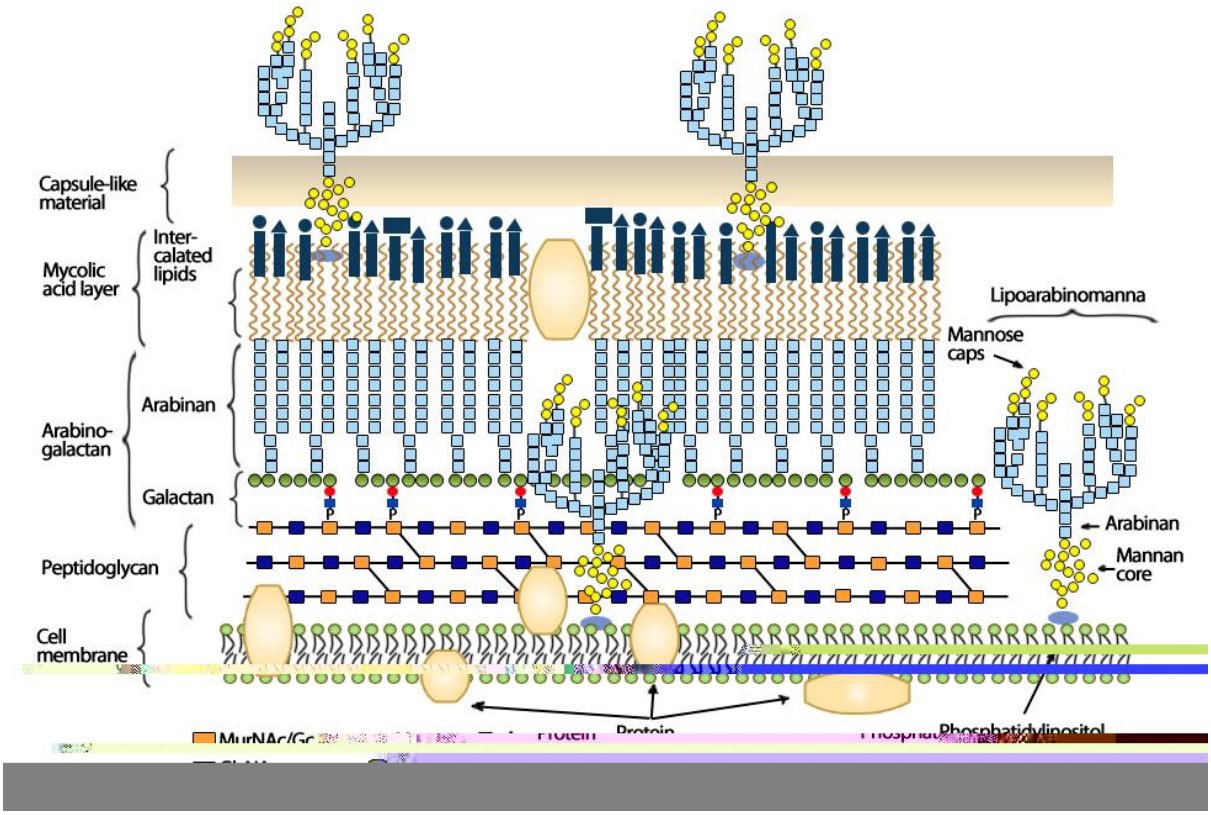


Figure 1.3: **Schematic diagram of mycobacterial cell wall** (Russell *et al*., 2010b).

The schematic diagram of the mycobacterial cell wall shows an outer lipid layer covering a layer of mycolic acids. The mycolic acids coat the arabinogalactan and peptidoglycan. The inner layers including theplasma membrane, which does not significantly differ in structure from other biological plasma membranes (Figure 1. 3). Lipoarabinomannan crosses the entire cell wall structure. The mycolic acids are linked as esters to the non-reducing ends of the arabinogalactan chains (Russell *et al*., 2010b). Mycolic acids are thought to be oriented perpendicularly to the plane of the membrane where they provide an exceptional lipid barrier which is responsible for

the physiological and disease-inducing aspects of *Mycobacterium tuberculosis* cord factor or dimycolyltrehalose (Russell *et al*., 2010b).

The three main structural layers include the plasma membrane; the cell wall and the cell capsule form the mycoylarabinogalactanpeptidoglycan (MAGP) which is the heart of the cellular membrane (Russell *et al*., 2010b). The three dimensional arrangement protects the cell wall from antibiotics and hydrolytic enzymes of the host. The diverse lipid content of the cell wall allows the bacteria to actively modulate their intracellular environment for survival, which is a special characteristic of the genus (Russell *et al.*, 2010a, b).

- **The *Mycobacterium tuberculosis* life cycle**

The life cycle of the Mycobacterium tuberculosis is divided into five different stages. The infection with *Mycobacterium tuberculosis* starts through inhalation of the bacilli in aerosolized droplets from an infected individual. The bacilli then replicate primarily in cells of the airway terminals (The first stage called “Onset:1-7 Days). If the bacterium does not get killed, it is taken up by the alveolar macrophages where it continues to replicate (Second stage or “Symbiosis”:7-21 Days). *M.tb.* carries the gene(s) which prevents the acidification of the phagosome and hence the bacteria are not destroyed. The potential contribution of mycolic acids in all of this has been discussed as being possible, but is not yet understood (Verschoor et al., 2012). Labeled mycolic acids have been applied to determine if they are able on their own to block phagosome-lysosome fusion, but the answer to that was a clear “no” (Korf et al., 2005). *M.tb.* also prevents the death of the macrophage by neutralizing reactive nitrogen intermediates (Flynn et al., 2003). The macrophages distribute the bacteria to the lungs and the lymph nodes. As soon as a cell-mediated immune response starts developing, the replication of the bacilli slows down and the bacteria are restricted to developing in granulomas. To this point, the infection is still subclinical.

Upon reinfection reactivation of the cellular immune response is weakened. Thus in approximately 5% of infected patients but in 50-100% of those co-infected with AIDS that are not under anti-TB therapy, the bacilli will continue to replicate until the macrophages burst and tubercules develop. The result is that the lungs undergo caseation and cavitation. Consequently, this releases the organism into the bronchial tree and the sputum leading to progressive pulmonary TB (with symptoms such as cough, hemoptysis and sometimes weight loss) or to

extra-pulmonary TB. This is the third stage or "Initial Caseous Necrosis"). Once the bacteria run out of macrophages reproduction slows down. The bacteria produce anoxic conditions and reduce the pH. No reproduction is observed but the bacterium may persist for long periods (Grosset, 2003).

The fourth stage consists of the interplay of tissue-damage and the macrophage activation of the immune response. Here the macrophages surround the tubercules. *M.tb* uses the inactivated macrophages to replicate causing the tubercules to grow. The tubercle can break off and spread to other parts of the lungs or into the blood supply causing "Miliary TB".

The last stage is called also "Liquefaction and cavity formation." In this stage, the tubercules liquefy allowing the bacteria to reproduce extracellularly, making the disease spread faster. The walls of the bronchi become necrotic and rupture resulting in cavity formation. (Grosset, 2003).

1.3. Mycolic acids (MA)

Mycolic acids constitute a wide family of over 500 classes. They constitute the major component of the cell wall of Actinomycetes, a distinct suprageneric taxon that encompasses the genera *Mycobacterium, Gordona, Nocardia* and *Rhodococcus* (Asselineau, 1978). MAs constitute the most abundant lipids of the *mycobacterial* cell envelope; for example, MAs constitute 34% by weight of the cell wall skeleton in *Mycobacterium microti* (McNeil, 1991, Paul, 1994).

Analysis and treatment of an extract of human *M. tb.,* by Andersen and his co-workers in 1927, (Anderson, 1927) at Chemistry Department of Yale University, furnished the hexacosanoic acid as an oil in 24% yield which has been referred to as the MA (Anderson, 1927). It was only twelve years later that its structure was elucidated by Asselineau and co-workers (Figure 1.4) (Asselineau, 1950). These authors were able to establish that mycolic acids consisted of a *β*-hydroxy-*α*-alkyl branched chain and that MA contained two major parts including the mycolic motif, which comprised of the *β*-hydroxy-*α*-alkyl branched chain, and the long alkyl chain "mero-mycolate" moiety as shown in figure 1.5 below.

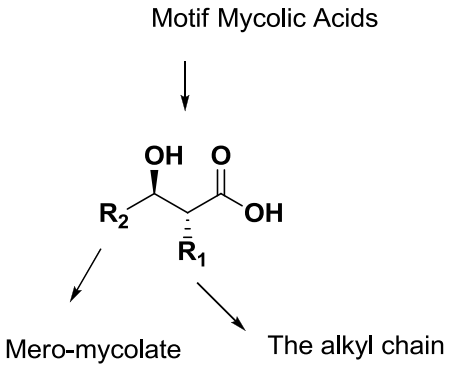
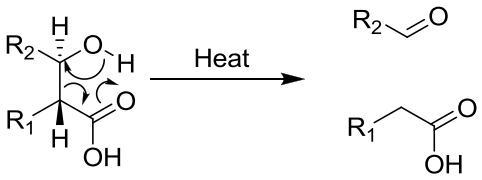


Figure 1.4**: The general structure of MA as first proposed (Asselineau, 1950).**

The mero-mycolate chain (R_2 in figure 1.4) may or may not contain other oxygenated groups, such as hydroxyl, methoxy, or keto groups. Mycobacteria are known to have MA with the longest mero-chains containing a range of functional groups while the other members of the mycolata family contain short, simple mero-chains and only *cis*-double bond functional groups (Table 1.1). The discovery of new therapeutics against tuberculosis has driven much research in this field with interesting outcomes towards a better understanding of the disease. For example, mycolic acids were the first known CD1-presented lipid antigen to stimulate a scarce sub-population of T cells that is involved in the regulation of auto-immunity (Beckman *et al*., 1994).

The mero-chain chain was found to contain more than 50 carbon atoms. Application of thermolysis at 300 °C cleaved the hydroxyl acid functionality to yield the mero-mycolaldehyde and this is shown in scheme 1.1 bellow (Asselineau, 1978)**.**

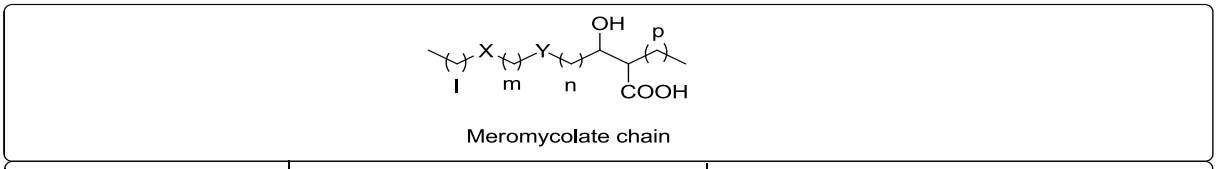


Scheme 1.1: **The structure of α-mycolic acids and the cleavage by thermolysis of hydroxyacid functionality, at 300 °C producing the meromycolaldehyde** (Asselineau, 1978).

The mycolic acids isolated from *Mycobacterium* are also named “eumycolic acids” and they have 60-90 carbon atoms, while those from other classes (such as *Corynobacterium* and *Nocardia*) are much shorter and are called “corynomycolic acids” (22-36 carbons) or “nocardomycolic acids” (44-60 carbons). A description of the various forms of mycolic acids found in *Mycobacterium* was reviewed by Minnikin and co-workers (Minnikin *et al*. 1984).

The Genus	The length of the carbon chain
Corynebacterium	22-38
Rhodococcus	34-52
Nocardia	44-60
Gordonia	48-66
Tsukamurella	67-78
Mycobacterium	60-90

Table 1.1: **The chain length of MAs in different Genera** (Palomino et al., 2007).



Meromycolate chain

Mycolate	X	Y
Type-1 Mycolate	cis / or / trans; Alpha-; CH_3, OCH_3 Methoxy-; CH_3, O Keto-	cis / or / trans; or; cis, cis, n'
Type-2 Mycolate	cis / or / trans; Alpha-; CH_3, OCH_3 Methoxy-; CH_3, O Keto-	CH_3, trans; or; CH_3, trans, cis, n'
Type-3 Mycolate	cis / or / trans; Alpha-; CH_3, OCH_3 Methoxy-; CH_3, O Keto-	cis; or; cis, cis, n'; CH_3, trans, cis, n'

Table 1.2**: The general distribution of mycolate types, the essential functional groups and the range of chain lengths of the MAs in representative *mycobacteria*** (Minnikin *et al*., 1984; McNeil, 1991)**.**

The above table illustrates some possible structures of the mycobacterial envelope especially their functional groups positions which differentiate them in different types and/or subclasses. (Table 1.2) (Minnikin *et al*., 1991). MAs usually have two intra-chain functional groups (X in the distal position and Y in the proximal position) in the main meromycolate chain (Table 1.2 and Figure 1.5), which vary in type, stereochemistry and spacing. These functional groups usually affect the packing of the long hydrocarbon mycolate chains and thus induce the fundamental physiological functions of the cell envelope. Non-oxygenated MAs are also known as *α*-mycolic acids (Figure 1. 5). α-Mas usually have cyclopropane rings in the distal and proximal positions. Both cyclopropane rings and double may be either *cis* or *trans.Trans* groups always occur with an adjacent methyl branch (Table 1. 2). The MAs from *Mycobacterium* spp. also include oxygenated functional groups such as the epoxy-, keto-, methoxy- , hydroxyl- or carboxyl groups, in the distal position, with cyclopropanes or double bonds in the proximal position. (Minnikin, 1982, Minnikin *et al*., 1982, Minnikin *et al*. 1984).

It is actually impossible to better understand the importance of MAs in cell envelope structure unless comprehensive knowledge of the composition and the nature of each one of the individual MA is established. Most previous studies have characterized the major MAs in certain species (Davidson *et al*., 1982) or recorded the general distribution of mycolate types, the essential functional groups and the range of chain lengths of the MAs in representative *mycobacteria* (Watanabe, 2001, Watanabe et al., 2002).

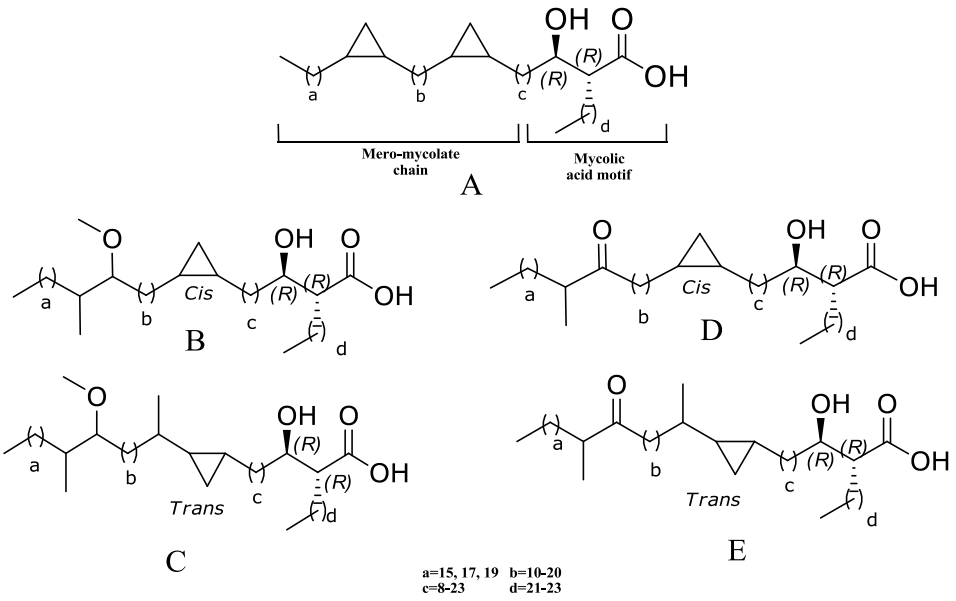


Figure 1.5: **Different structures of mycobacterial MA and mycolate classes found in *M. tb*. (A): *α*-Mycolic Acids; (B): *cis*-Methoxy-Mycolic Acids; (C): *trans*-Methoxy-Mycolic Acids; (D): *cis*-Keto-Mycolic Acids and (E): *trans*-Keto-Mycolic Acids** (Minnikin et al., 1984).

- **Cholesteroid nature and the use of MA in establishing TB after infection**

MAs secreted *in vivo* and *in vitro* during the growth of *M. tb.* **(**Ojha, 2008). The different classes of Ma differ in their ability to induce foamy macrophages, attract neutrophils and in their capacity for antibody recognition (Verschoor *et al*., 2012). A study undertaken by Benadie and co-workers has demonstrated that they have been found to attract cholesterol, to bind Amphotericin B and to be recognised by monoclonal antibodies that cross-react with cholesterol (Benadie *et al*., 2008, Beukes *et al.*, 2010). It was found that this ability could be due to the presence of a range of functionalities in their structure such as but not limited to the carboxylic acid located at the end of their motif, which implies that some vital function could be hidden in their selective expression and secretion which could establish tuberculosis after infection of the host. Their cholesteroid nature may be related to how they use the host cholesterol for their persistence (Verschoor *et al*., 2012).

In studying the cholesteroid nature of mycolic acids, Beukes and co-workers (Beukes *et al*., 2010) have shown that while the simple MA-methyl esters have been proven to lose their antigenic activity, amazingly the ester formed by simple derivatization of MA with 5-Bromo-

methyl fluorescein (5-BMF) was shown to maintain 80% of its antigenic activity in ELISA/BIOSENSOR (Lemmer, 2009). It was postulated that this fact was due to the presence of the free carboxylic acid group on the fluorescein dye. However the true structure of MA-fluorescein was uncertain as labelling of palmitic acid apparently produced two structural forms and fluorescein was said to exist as a mixture of tautomers (Mukherjee *et al*., 1995).

- **Potential applications of fluorescently labelled mycolic acids**

To test the biological function of MAs in cell culture or animal studies, it is often required that the MA is fluorescently labelled. An example of how this clarified the function of MA was reported by Korf *et al*., (2005). This study established that MAs' interaction with macrophages induced a macrophage morphotype similar to the foamy macrophage derivatives observed in TB granulomas. For the determination of the specific type of phagocyte which is accountable for the in vivo phagocytosis of the liposomes, MA was derivatized with a fluorescein dye (5-BMF) before its insertion within the liposomes. This was injected into the peritoneal cavity of the mice. Results from flow cytometric analysis showed that only the F4/80-positive exudate cell population internalized the fluorescent liposomes, identifying macrophages as the exclusive cell type responsible for internalization of the MA-liposomes. These results were confirmed by the confocal microscopic analysis which showed the uptake of the MA-liposomes particles of 5-BMF-MA-positive cells and their processing into acidified vesicles (Korf et al., 2005).

Considerable structural variation exists within the *M. tb.*-MA pool.. Seppe *et. al.* studied the effect of oxygenation level and proximal cyclopropane configuration on inflammation and host immune response (Seppe *et al*., 2011). A powerful modern approach to learn how this successful human pathogen regulates inflammation and host immune responses would be the technology of molecular beacons (Chen *et al*., 2000; Chen *et al*., 1997; Tyagi *et al*, 1996; Giessendorf *et al*., 1998). This entails the microscopic assessment of the expression of immune cytokines or other marker mRNA in single cells that have been treated in a particular way. Molecular beacon-based polymerase chain reaction (PCR) assay can supply the possibility of real-time specific and quantitative detection of specific target mRNA directly in the PCR tube over a wide dynamic range of detection, all in a real-time manner (Chen *et al*., 2000). One of the most powerful aspects of molecular beacons is its specificity of detection. To link the

molecular beacon cytokine profile of a single cell to that of MA uptake, it is important to use a fluorescently labelled synthetic MA to confirm its presence in the cell under investigation. Quantitative labelling of MA with fluorescein with full structural characterisation of the conjugate will facilitate such studies.

1.4. Hypothesis

Labelling of mycobacterial-MA with bromo-methyl-fluorescein by esterification of the carboxylic acid group of MA retains a carboxylic acid functional group close to the ester bond that may sustain the natural conformational folding of MA.

1.5. The Aim of the project

The aim of this project was to synthesize and characterize a fluorescent labelled MA- ester structure using commercial 5-Bromo-methyl fluorescein (5-BMF). Since "5-BMF" is very expensive commercially, sometimes sold in a poor quality and often not readily available from the seller, the 5-BMF was prepared in the lab from basic precursors. The chemistry of the fluorescination of MA was to be done first with a simpler MA, and the technique then to be transferred to the labelling of mycobacterial MA. The sub aims were identified as follow:

- Synthesis of a model corynomycolic acid (CMA) with no controlled stereochemistry via Claisen condensation.
- Synthesis of the model corynomycolic acid (CMA) via aldol condensation methodology as an alternative route.
- Synthesis of a 4(5)-Bromo-methyl fluorescein and attaching it to the model CMA to optimise the labelling of the naturally occurred Bovine-MA.
- Purification and characterization of the newly prepared materials by NMR analysis and the confocal microscopy study of the Bovine-MA labelled with the 4(5)-BMF.

1.6. Project subdivision

Results of this research are presented and discussed in two separate chapters as follows: Synthesis of a model corynomycolic acid with no controlled stereochemistry via Claisen or alternatively aldol condensation methods to optimise the methodology for the labelling of the mycobacterial MA-BMF (chapter two) and the synthesis of a 4(5)-Bromo-methyl fluorescein and its labelling onto the MA (chapter three). The chapters four and five describe respectively the conclusion and the experiment section of this research.

Chapter 2: Synthesis of *corynomycolic* acids analogue 1

2.1. Introduction

C*orynebacteria* belong to a group of bacteria including *Mycobacteria* and *Nocardia*, which are characterized by the presence of mycolic acids in their cell wall (Barksdale *et al.*, 1977). These bacteria share the property of having an unusual cell envelope structural organization similar to Gram-negative bacteria (Barksdale *et al.*, 1977). In addition to the inner membrane, the cell envelope consists of a thick arabinogalactan-peptidoglycan polymer covalently linked to an outer lipid layer, which is mainly composed of mycolic acids and probably organized in an outer membrane like structure (Barksdale *et al*, 1977). They have a similar cell wall structure to that described for the *M. tb.* in chapter one except that CMAs are shorter than the MAs from mycobacteria and that they contain no functional groups in the mero chain. CMAs are found in the cell wall of *corynebacteria* as trehalose diesters with a general structure corresponding to a C_{18}-palmitic acid which is substituted with a C_{14}-alkyl chain in the α-position and β-hydroxy group in the (*R, R)* configuration, analogous to the mycolic motif of *Mycobacterial-MA* (Asselineau *et al.*, 1970). MAs normally vary in length from C_{60} to C_{90} in *Mycobacteria*, but their equivalents in Corynebacteria are significantly shorter in chain length (C_{22}–C_{36}) (Barry *et al.*, 1998). The corynebacterial equivalents of trehalose monocorynomycolate (TMCM) and trehalose dicorynomycolate (TDCM) are analogous to the mycobacterial trehalose esters apart from the fact that they contain the shorter corynomycolates (Tropis *et al.*, 2005).

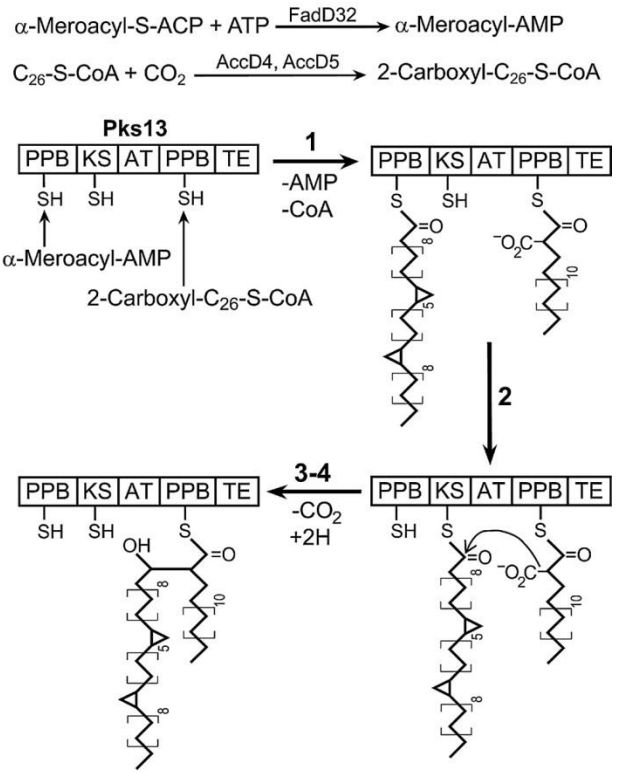
- **Biosynthesis of MA**

The biosynthesis of MAs have been extensively studied owing to their functional uniqueness, role and the finding that many drugs, including the front line TB drug isoniazid, target numerous enzymes involved in their biosynthesis (Schroeder *et al.* 2002). Even though the chain length of MAs varies between Corynebacterineae, the mycolic motif is conserved (Scheme 2.1). Most of the enzymes involved in initial steps of the MAs biosynthesis have recently been identified in *Corynebacterium glutamicum,* a species which tolerates the loss of MAs. This has allowed the characterization of viable mutants that cannot be derived in *Mycobacteria*. Studies have shown

that the polyketide synthase enzyme is required for condensation of two fatty acids to yield the 2-*alkyl, 3-ketomycolates*. Pks13 enzyme has been established to catalyze the condensation reaction (Portevin *et al.*, 2004; Bhatt *et al.*, 2007). Mature mycolates generated by this pathway are thought to be delivered to lipid carriers for transport across the plasma membrane, before being attached to trehalose to form TMM/ TMCM and TDM/TDCM and following esterification to arabinogalactan (Takayama *et al.*, 2005) (Scheme 2.1).

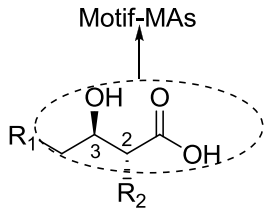
FadD32 converts the *α*-meroacyl-S-ACP derived from the FAS-II system to *α*-meroacyl-AMP. The hexacosanoyl-*S*-CoA derived from FAS-I is carboxylated by acyl-CoA carboxylases (AccD4 and AccD5) to yield 2-carboxyl-C26-S-CoA. These two products are the substrates for the condensation reaction catalyzed by Pks13. Reaction 1 is the loading step in which the two substrates are covalently attached to Pks13. Reaction 2 is the transfer of a meroacyl group from the N-terminal PPB domain to the ketosynthase condensing enzyme (KS). Reactions 3 and 4 together are the condensation step and reduction of the 3-oxo group to the secondary alcohol by an unidentified reductase to yield the mature α-mycolate. (Takayama *et al.*, 2005).

MA biosynthesis involves reduction of the β-keto ester to form the mycolic motif, followed by hydrolysis to generate the *α*-alkyl-β-hydroxy acid (Gastambide O. M., 1959). Studies conducted by Lea-Smith *et al.* (2007a) have demonstrated that NCgl2385 enzyme is actually involved in the reduction of the β-keto group following the condensation as the arabinogalactan-bound mycolic acids of the NCgl2385 deficient mutant demonstrated that all the mycolates of this mutant retain the -keto ester motif. In continuation, these studies have also speculated that "the re-expression of NCgl2385 in this mutant completely restored the synthesis of mature corynomycolates. The NCgl2385 gene is highly conserved in all sequenced genomes of members of corynebacterineae and is likely to encode the same activity in all species, including the human pathogens *M. tuberculosis, M. leprae*, and *M.ulcerans*" (Lea-Smith *et al.* 2007b)



Scheme 2.1: **Proposed mechanism of Claisen-type condensation for the synthesis of *α*-mycolic acid by Pks13 in *Mycobactrium tuberculosis*** (Takayama *et al.*, 2005).

The biosynthesis of mycolic acids from *Corynobacteria* usually follows the same pattern as for *Mycobacterial*-MA, except that there is no need for functional groups in the mero chain. It involves a simple coupling via a Claisen-type condensation which is followed by the reduction of the ketone to an alcohol to give the (*R, R*) configuration (Figure 2.1).



Corynomycolic acids

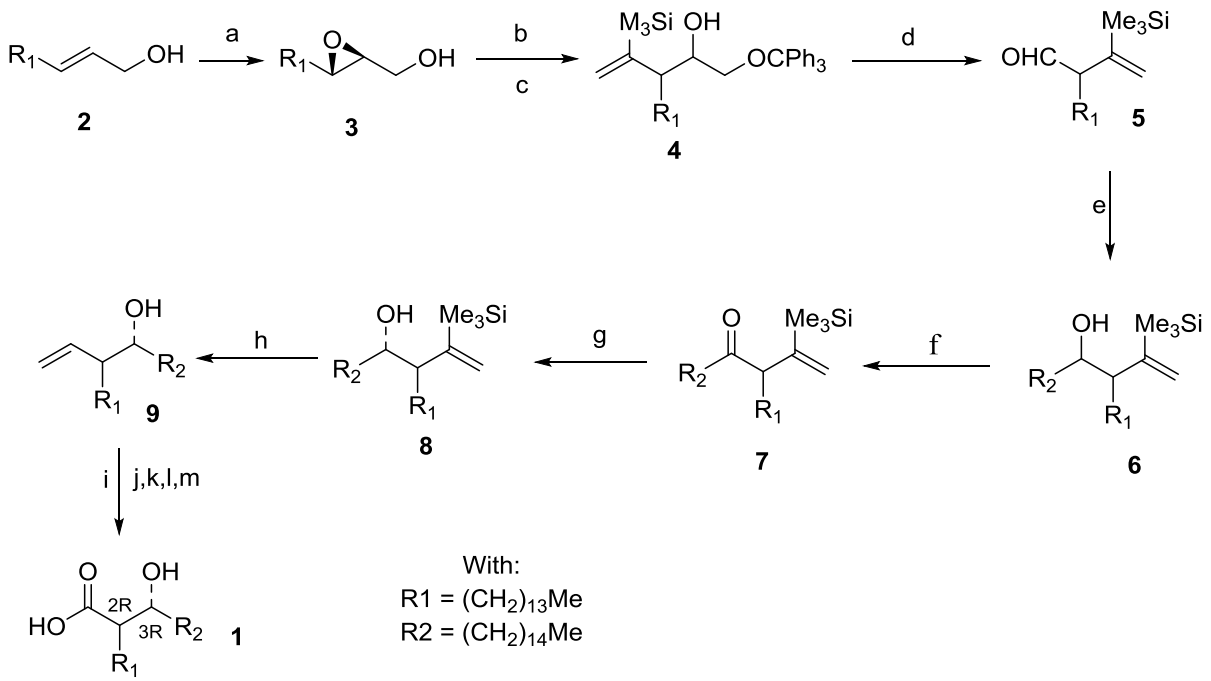
1

Figure 2.1: **General basic structure of MA in (2*R*, 3*R*) configuration as proposed by Lederer** (Lederer *et al.*1975).

- **Previous synthesis of the CMA**

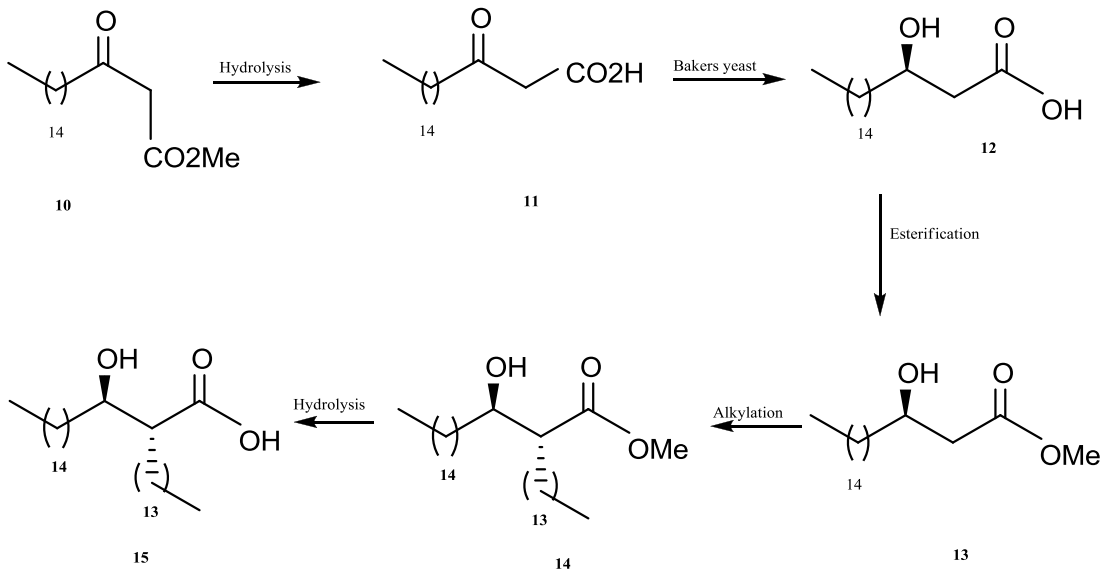
Minnikin and Polgar (1966) have established that the stereochemistry of the natural mycobacteria-MAs' is in *anti*-(*2R, 3R*) configuration**.** Therefore, optimisation of the model CMAs' preparation to get the *anti*-(2*R*, 3*R*) configuration would be the better method to generate or synthesise the motif-MA owing to the similarity of CMAs-*Corynebacteria* and *M. tb.*-MAs. Even though these acids have interesting biological properties, only a few syntheses of the racemic form of **1** have been reported where -(2*R*, 3*R*)--*anti*-stereochemistry was achieved with rather low stereo-selectivity (Kitano *et al.*, 1985). Kitano and co-workers were the first to report on the total synthesis of (+)-CMAs in a stereo and enantio-selective approach (Scheme 2.2) (Kitano *et al.*, 1985). This method therefore, constitutes a general synthetic approach for a range of optically active MAs **1**. In this approach, two alkyl chains R_1 and R_2 were introduced independently at different stages. The usefulness of this method was shown with the synthesis of the (+)-CMAs **1** which is one of the MAs produced by Corynebacteria. The shapeless asymmetric epoxidation of the alcohol **2**, yielded epoxide **3** in 77% yield (De Souza *et al*. 2008).

The protection of the hydroxyl functional group of **3** as trityl ether and subsequent regioselective epoxide ring opening, afforded the product **4**. This compound **4** was then converted into the aldehyde **5** by successive aqueous treatments as described in the scheme 2. 3. The reaction of n-pentadecylmagnesium bromide with **5** gave the product **6** exclusively in 90% yield.[4] Jones oxidation reaction of the product **6** gave the product **7** which was reduced to the product **8** in 84%. Protodesilylation of **8** gave the alcohol **9** in 86%. The conversion of the product **9** into its corresponding benzyl ether, the ozonolysis, the oxidation and lastly the hydrogenation afforded the desired product (+)-**1** in 90% (Scheme 2.2) (Kitano *et al.*, 1985).



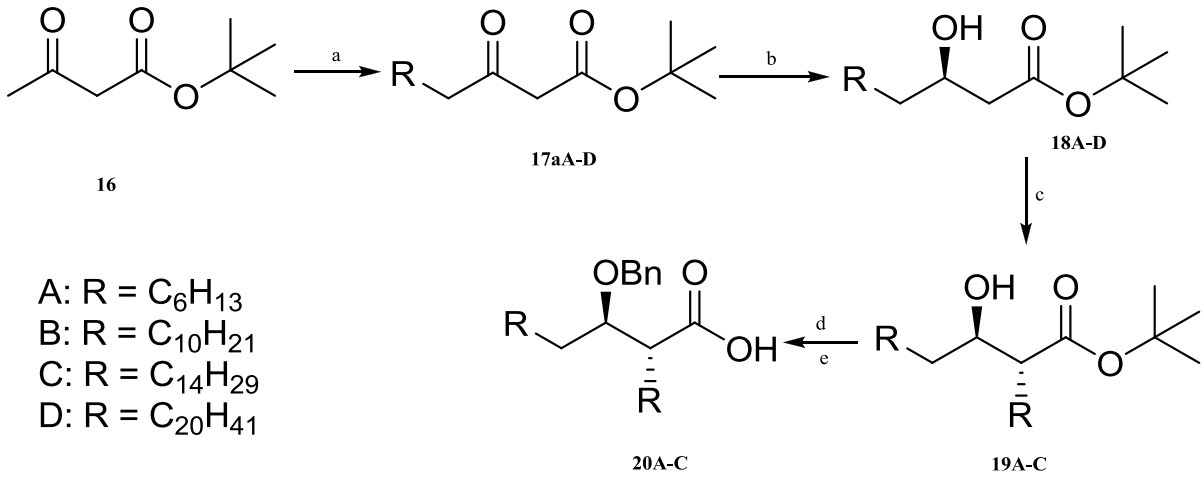
Scheme 2.2: **Preparation of the CMA via a nucleophilic addition to the *α*-alkyl-*β*-trimethylsilyl-*β, γ*-unsaturated carbonyl unit: a) *DIPT*, b) Ph_3CCl, DMAP, NEt_3, c) $C_5H_{11}BrMgSi$, d) Cl_2CHCO_2H and $NaIO_4$, e) $C_{15}H_{31}BrMg$, f) CrO_3, H_2SO_4, g) L-Selectride, h) NaH, HMPA, i) $PhCH_2Br$, KH, THF, j) O_3, k) Me_2S, l)CrO_3, H_2SO_4, and m) H_2, Pd-C** (Kitano *et al.*, 1985).

Another method developed by Utaka and co-workers to prepare an optically active (+)-(*2R, 3R*)-CMA (Scheme 2.3) was published a year later, which was based on an asymmetric reduction of the acid **11** (prepared from the hydrolysis of the ester product **10**) with a fermenting Baker's yeast (Utaka *et al.* 1987) to give the alcohol **12** and incorporation of the C_{14}-*α*-alkyl chain via a Fráter alkylation method (Fráter *et al.*, 1984, Fráter *et al.*, 1981) to yield the product **15** after hydrolysis of the methyl ester group.



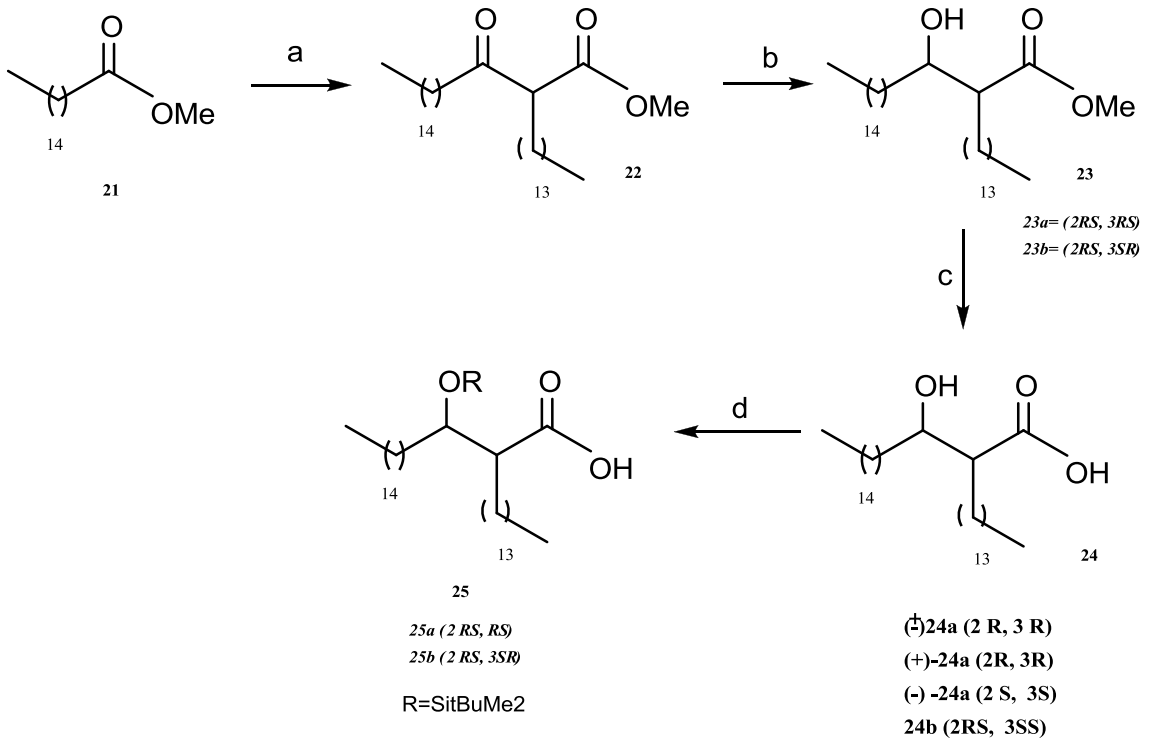
Scheme 2.3: **The asymmetric reduction of the 3-oxo-octadecanoic acid followed by the Frater alkylation method for the synthesis of CMA 1** (Utaka et al., 1987).

It was around 2007 that the work on the synthesis of CMA was completed by Nishizawa and co-workers by the insertion of the *β*-hydroxy group using a catalytic asymmetric hydrogenation with a ruthenium catalyst to yield the product **19** while the *α*-alkyl chain was introduced using Frater alkylation to produce the desired CMA **20** (Scheme 2. 4). This *α*-alkyl chain was then extended to a C_{20} carbon in an attempt to emulate the mycolic motif of the mycobacterial-MA. This method would require an alkylation with a suitable functionalised alkyl iodide before the extension of the approach to the insertion of a complex meromycolate chain for the total synthesis of the MA (Nishizawa *et al*., 2007).



Scheme 2.4: **The CMA preparation with an asymmetric catalytic hydrogenation and Frater alkylation: (a) NaH, n-BuLi, $C_{14}H_{29}I$, (b) H_2, (*R*)-BINAP-Ru, (c) LDA, $C_{14}H_{29}I$, (d) TMSCl (e) PhCHO, Et_3SiH, $TMSOT_f$** (Nishizawa *et al.*, 2007).

To synthesise racemic C_{32}-corynomycolic acid for the purpose of developing a route to the trehalose mycolates, Datta and co-workers (Datta *et al.*, 1991) turned to the procedures developed by Lederer *et al.*,1991 in which the methyl palmitate **21** was subjected to self-condensation (Claisen), and the resulting keto ester **22** which was reduced (sodium borohydride) to a mixture (proportions 2:3) of the hydroxy esters **23a** and **23b** (Scheme 2.5). After separation of the *syn*- and *anti*-diastereomers by column chromatography, the esters were saponified to yield racemic corynomycolic acid (**24a**) and its diastereomer (24**b**) respectively. For the protection of the 3-hydroxy function to prevent self-acylation during the coupling step, it seemed desirable to use a group that would be removed under the same conditions as trehalose-bound *O*-(trimethylsilyl) groups. The *O*-(trimethylsilyl)- and *O*-tetrahydropyranyl-CMA were therefore tested, but found to be unsatisfactory (Datta et al., 1991). The former was found to be unstable, and the latter was a diastereomeric mixture, which makes purification of the acylation products difficult. However, the 3-*O*-(*tert*-butyldimethylsilyl) derivative showed a satisfactory balance between stability and ease of removal. The reaction of **24a** with *tert*-butylchlorodimethylsilane initially gave a mixture of the 3-silyl ether, the silyl ether-silyl ester, and the silyl ester, but a brief treatment with potassium carbonate cleaved the silyl-ester groups, and chromatography gave the desired silyl ether plus regenerated mycolic acid that could be recycled. Similar results were obtained with the diastereomer **24b** (Datta et al., 1991).



Scheme 2.5: **General procedure for the synthesis of protected-CMA 1 by a self-condensation (Claisen) method: (a) LDA, THF, -78 °C, 2-4h, (b) $NaBH_4$, MeOH, rt, 30-45min, (c) LiOH, MeOH/water, rt, 30min, (d)** TBS (Datta *et al.*, 1991).

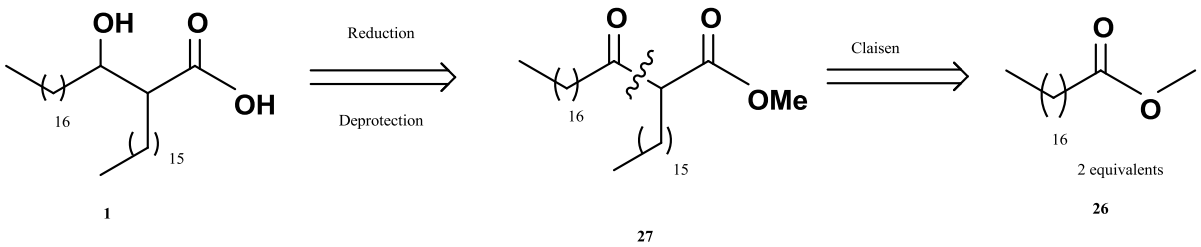
2.2. Aim

The aim of this chapter was to synthesis the model compound, CMA ester analogue to optimise the coupling reaction of a synthetic MA with 5-BMF.

2.3. Results and discussion

An improved synthetic method for the MA should be shorter and more economical, giving a more selective product with a desired specific stereochemistry such as the *α*-alkyl-*β*-hydroxy group in a *(R, R)* configuration (Minnikin *et al.*, 1966). We wished to synthesize a corynomycolic acid as a model material for the optimization of the reaction of MA with the fluorescent agent 5-BMF. We intended to use a biomimetic synthesis based on a Claisen condensation followed by reduction of the Claisen product in line with the work of Datta described above (Datta *et al.*, 1991).

The retro-synthetic analysis shows that reduction and de-protection are required to take the mycolic acid structure to the β-ketoester Claisen product. The Claisen condensation was planned to be a symmetrical self-condensation of two units of methyl stearate **26** (Scheme 2.6).

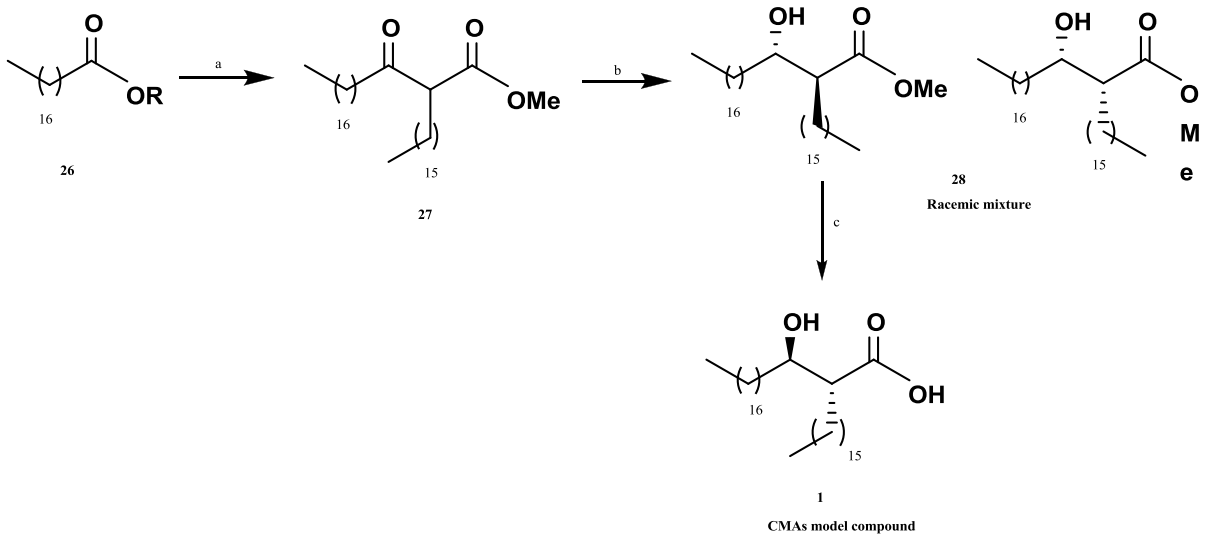


1 **27** **26**

Scheme 2.6: **The retrosynthetic analysis for the preparation of corynomycolic acid analogue 1**

- **Synthesis of the CMA 1 via the Claisen condensation route**

For the synthesis of corynomycolic acid **1** (Scheme 2.7), a combination of different methods including the one developed by Datta *et al.,* was used (Datta et al., 1991). The methyl stearate **26** was prepared by Fisher esterification of stearic acid in 95% yield. Analysis of the ^{1}H NMR spectrum of the product **26** showed the broad singlet at 3.63 ppm, integrating for 3H, which indicated the presence of the methyl ester. The broad peaks between 1.26-1.64 ppm correspond to the methylene groups integrating for 28H. The triplet at 2.34 ppm integrating for two protons corresponds to the two protons of the methylene group adjacent to the carbonyl carbon group of the ester. The triplet at 0.88 ppm integrating for 3H corresponds to the 3H of the terminal methyl group.



Scheme 2.7: **Procedure for the synthesis of the corynomycolic acids 1: (a) LDA, THF, -78 °C, 2-4 h (63%). (b) $NaBH_4$, MeOH, rt, 30-45 min (92%). (c) LiOH, MeOH/water, rt, 30 min.**

The product **26** was self-condensed via Claisen condensation route (Scheme 2.7) in the presence of NaH (60% dispersion in hexane) as the base. Initially this gave poor yields of an "acid" as a result of the hydrolysis of the methyl ester **26** by the NaOH which forms when NaH decomposes. To assist in monitoring the progress of the Claisen reaction by TLC, we synthesised compound 27 via an aldol reaction, followed by oxidation of the β-hydroxyketone with Pyridinium chlorochromate (PCC). Then Claisen condensation was repeated using THF instead of the Xylene while modifying the temperature to optimize this reaction. This resulted in an improvement of the product **27** since it gave 63% yield of the desired product. It was then decided to replace NaH by lithium diisopropyl amide (LDA) and use it as the base. This showed excellent results as the Claisen product was obtained in 96% yield.

Formation of **27** was confirmed by the ^{1}H NMR (300 MHz, $CDCl_3$) and ^{13}C NMR (75 MHz, $CDCl_3$) analysis (Figure 2.2 and 2.3) by the presence of some characteristic peaks such as the triplet *α*-proton peak at 3.41 ppm integrating for one proton. This proton is particulary downfield because it is adjacent to two carbonyl carbons, and it resonates as a triplet due to the two protons of the methylene group. The methylene group in turn can be observed at 1.82 ppm (doublet of triplets). The singlet peak integrating for 3H at 3.68 ppm corresponds to the methyl group of the ester which has been shifted slightly downfield when compared to the spectrum of starting material **26**. Another characteristic peak which confirmed the product formation is the presence of two overlapping triplets at 0.79 ppm integrating for six protons which correspond to

six protons of the two terminal methyl groups of the α- and β-chains. On the other hand, the ^{13}C NMR confirmed the ^{1}H NMR by the presence of a peak at 51.9 ppm which corresponds to the carbon of the methyl ester. The peak situated at 170.8 ppm corresponds to the carbonyl carbon of the ester while the ketone carbonyl is situated at 206.1 ppm. The carbon bearing the α-proton is located at 58.9 ppm while the two terminal carbons resonate at 14.3 ppm (figure 2.3).

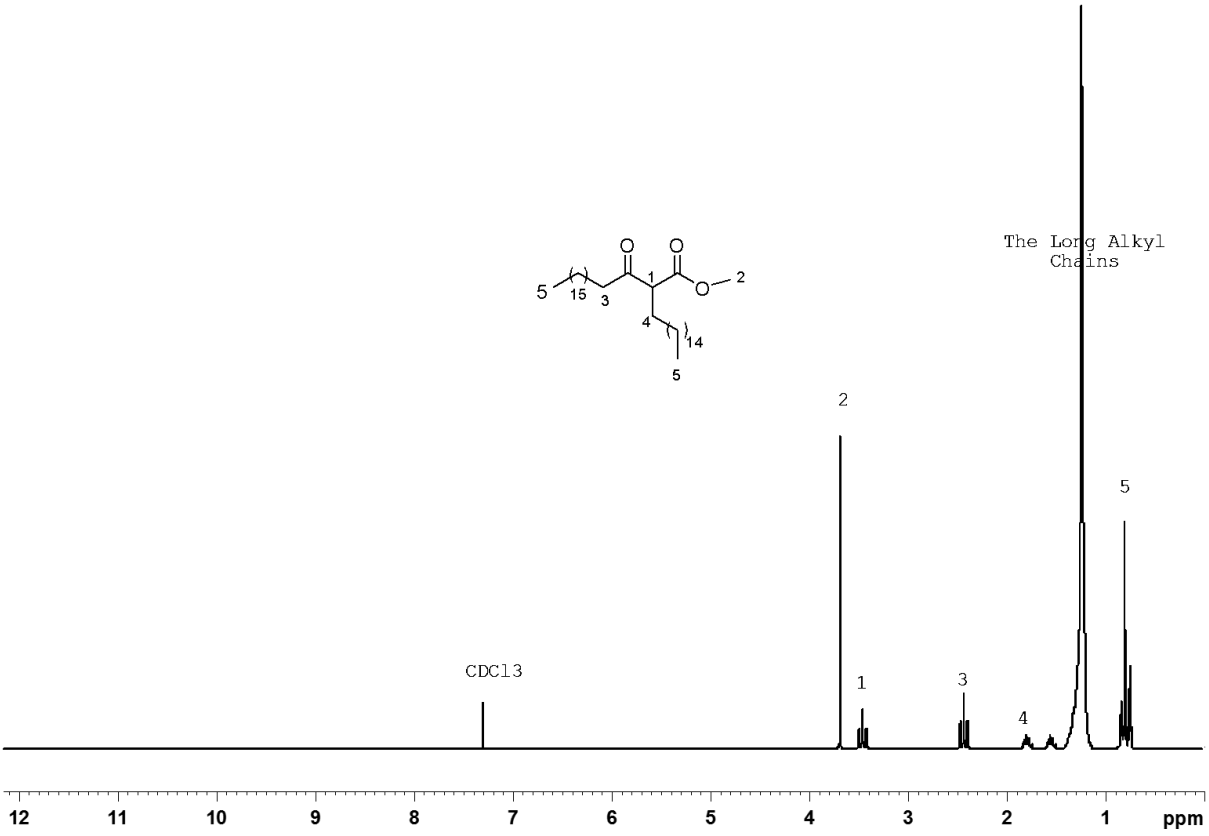


Figure 2.2: **The ^{1}H NMR spectrum of compound 27 (300 MHz, $CDCl_3$)**

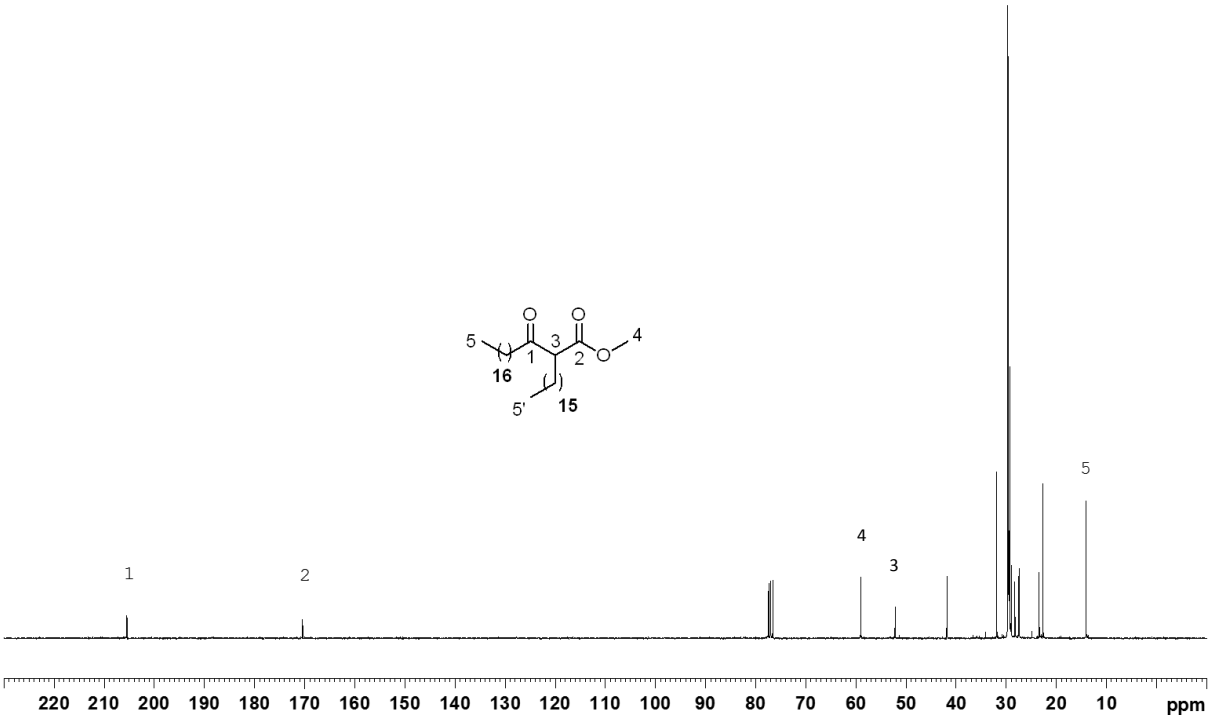


Figure 2.3: The ^{13}C NMR spectrum of compound 27 (75 MHz, $CDCl_3$)

The reduction of the product **27** using sodium borohydride gave the β-hydroxy esters **28** in 63% yield as a 56:44 (*anti-syn*) mixture of diastereomers. These diastereomers were quantitatively separated by repeated column chromatography to give pure *anti*- and *syn*-products. Thus the *anti*-product was obtained in 35% yield. This was confirmed after analysing the ^{1}H NMR spectra which showed the presence of a new doublet of triplets peak of the *β*-proton which appeared at 3.84 ppm, integrating for 1H and confirming the conversion or the reduction of the ketone of the product **28** to an alcohol group. Another doublet of triplet at 2.39 integrating for one proton corresponds to the *α*-proton (Figure 2.4). These characteristic peaks were confirmed by the ^{13}C NMR.The peak at 175.2 ppm corresponds to the carbonyl carbon adjacent to the methyl ester which is in accord with the literature. The peak at 59.2 and 52.0 ppm corresponds respectively to the carbon bearing the *α*-proton as well as the carbon of the methyl ester (Figure 2.5).

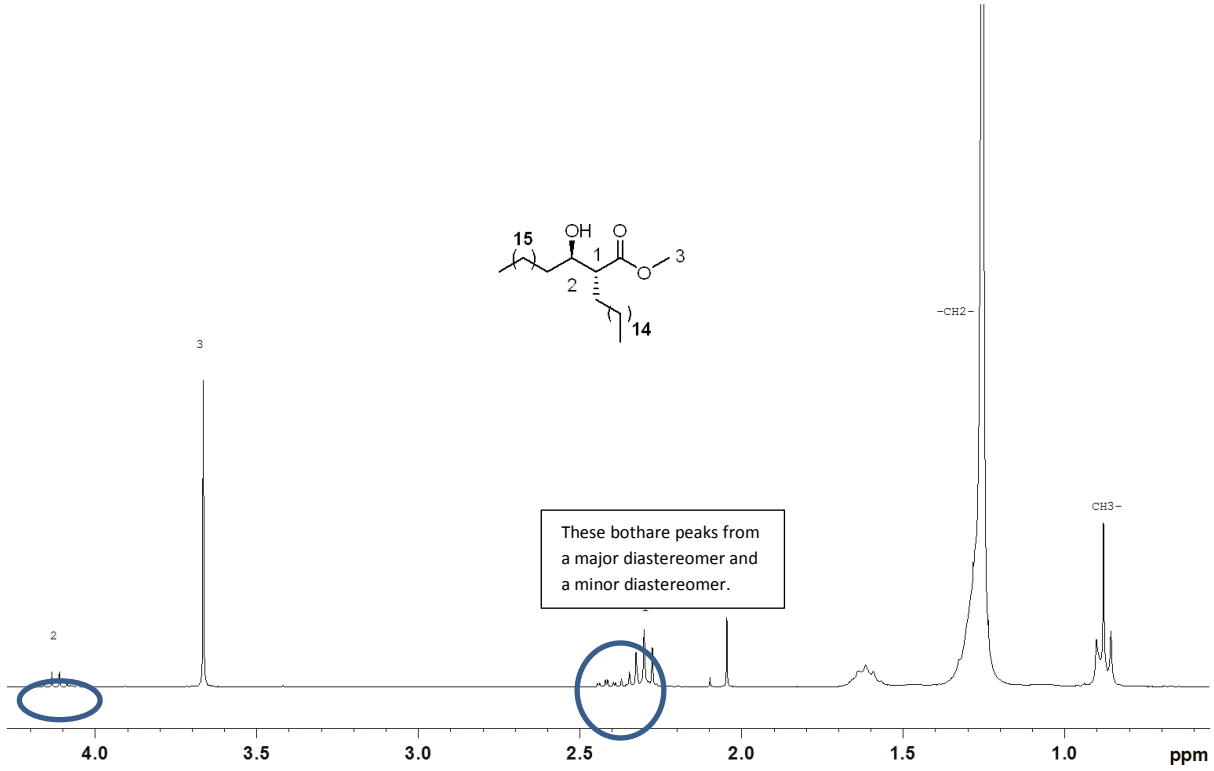


Figure 2.4: **The ^{1}H NMR spectrum of compound 28 (300 MHz, $CDCl_3$)**

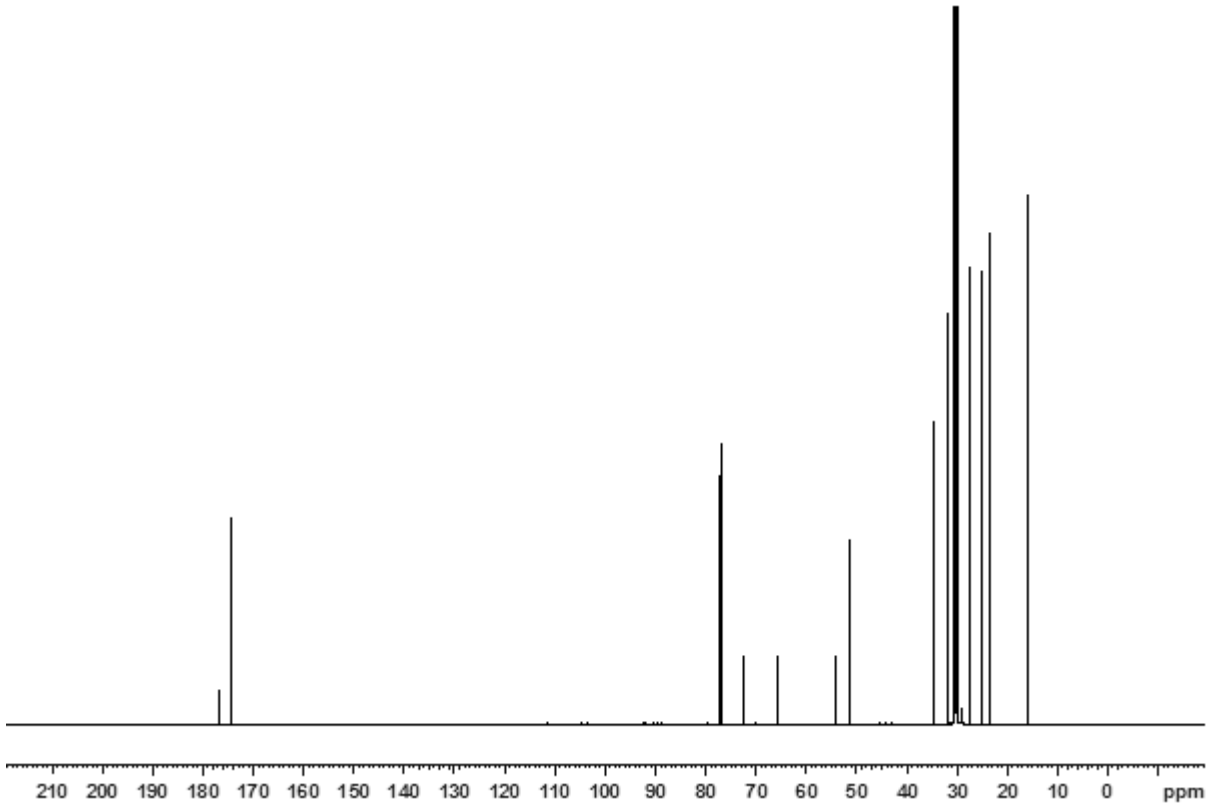


Figure 2.5: **The ^{13}C NMR of the compound 28 (75 MHz, $CDCl_3$).**

After testing several bases (e.g. LiOH, NaOH, Bu_4NOH) at several different concentrations for the hydrolysis of the methyl ester of the *anti*-product **28**, the best result was obtained when a 20% aqueous solution of tetrabutylammonium hydroxide (TBAH) was used to yield racemic corynomycolic acid **1** as a yellowish solid in 96% yield. The ^{1}H and ^{13}C NMR spectral analysis of **1** (Figure 2.6) showed that the methyl ester singlet (3.68 ppm) was no longer present. The signals at 2.29 ppm and 4.15 ppm correspond to the *α*-proton (^{13}C NMR, 75 MHz, $CDCl_3$: 54.2 ppm) and the *β*-protons (^{13}C NMR, 75 MHz, $CDCl_3$: 71.4 ppm) respectively while the peak at 0.79 ppm corresponds to the two terminal methyl groups of the alpha and mero chains which integrates for 6H. The peak at 3.31 ppm is related to the hydroxy group from the reduction of the ketone functional group. The ^{13}C NMR (75 MHz, $CDCl_3$) spectral analysis confirmed the loss of the methyl ester signal (52.3 ppm) and the carboxylic acid carbonyl signal resonated at 176.5 ppm.

Thus the optimised Claisen route to a racemic *anti*-CMA **1** was completed in 4 steps with an overall yield of 31% with little diastereoselectivity (racemic mixture 56:44 *anti: syn*).

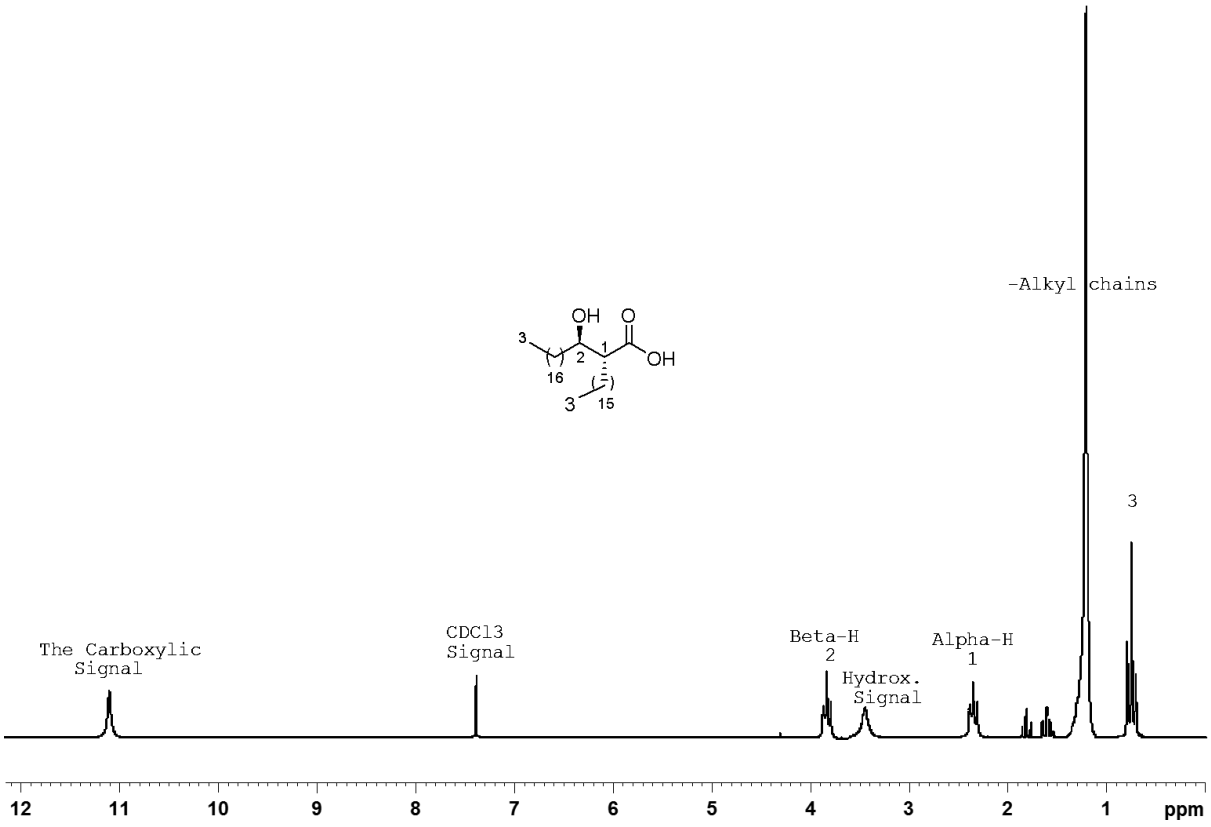
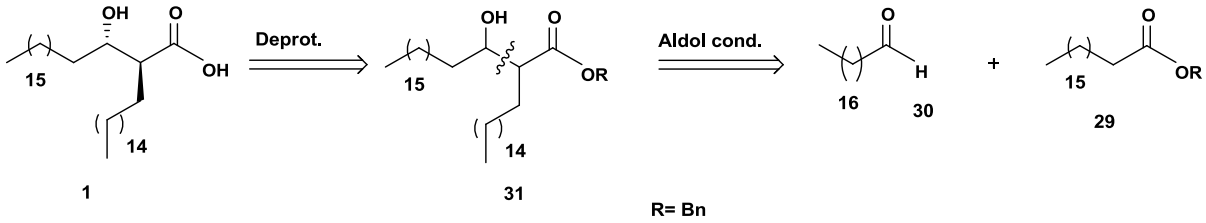


Figure 2.6: **The ^{1}H NMR (300 MHz, $CDCl_3$) Spectrum for CMA 1**

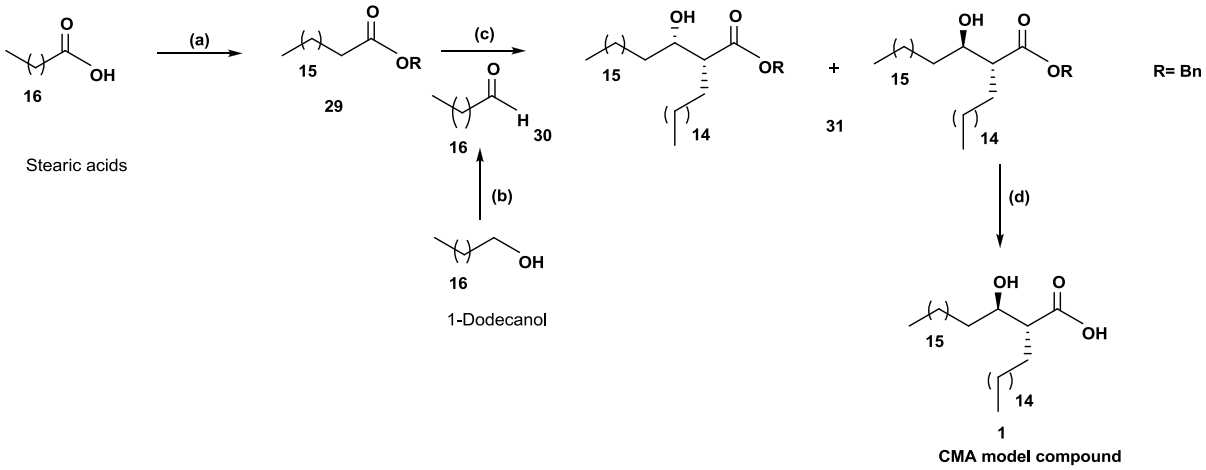
- **Synthesis of the CMA 1 via the aldol condensation route**

The aldol route to corynomycolic acids is an attractive alternative to the Claisen route, since the *α*- and *β*-stereo centres are created simultaneously providing a simpler synthetic approach as given in scheme 2.8. Having found that the Mycolic acid methyl ester was rather resistant to ester hydrolysis and prone to retro-aldol condensation under basic conditions, in considering the protection strategy for the aldol route, we decided to use the benzyl ester as a protecting group for the carboxylic acid with a view to using hydrogenation for deprotection.



Scheme 2.8: **Retrosynthesis analysis for the aldol condensation reaction.**

Benzyl stearate **29** was therefore prepared in 99% yield from the reaction of the stearic acid and benzyl bromide in the presence of $NaHCO_3$ as the base in DMF/1,4-Dioxane (1:1) (Scheme 2.9). The benzyl stearate formation was confirmed by the presence of the broad singlet peak at 5.20 ppm integrating for 2H. The peaks between 7.29 ppm and 7.47 ppm integrating for 5H are indicative of the benzene ring. Others peaks were also observed such as the broad peak between 1.26 ppm and 1.64 ppm integrating for 30H. A methylene group adjacent to the carbonyl carbon of the benzyl ester group is indicated at 2.42 ppm.



Scheme 2.9: **Proposed synthetic procedure for the CMA 1 via the aldol route: (a) $NaHCO_3$, BnBr, DMF/Dioxane, (b) PCC, DCM, 2 h, 35 °C. (c) LDA, -78 °C-rt, 30, THF, 2-4 h, (d) $Pd(OH)_2/C$, H_2, EtOAc, 45 min.**

The aldol reaction is one of the most powerful and selective carbon-carbon bond forming reactions in synthetic organic chemistry and has been used to prepare numerous series of natural product derivatives. For the aldol reaction the aldehyde **30** was freshly prepared in 99% yield from the oxidation of 1-octadecanol with 2.5 equivalents Pyridinium chlorochromate (PCC) within two hours. ^{1}H NMR analysis showed the characteristic triplet peak at 9.75 ppm which corresponds to the single aldehyde proton in accordance with the literature. The broad peak between 1.29 ppm and 1.57 ppm which integrates for 28H corresponds to the methylene groups (Figure 2.7).

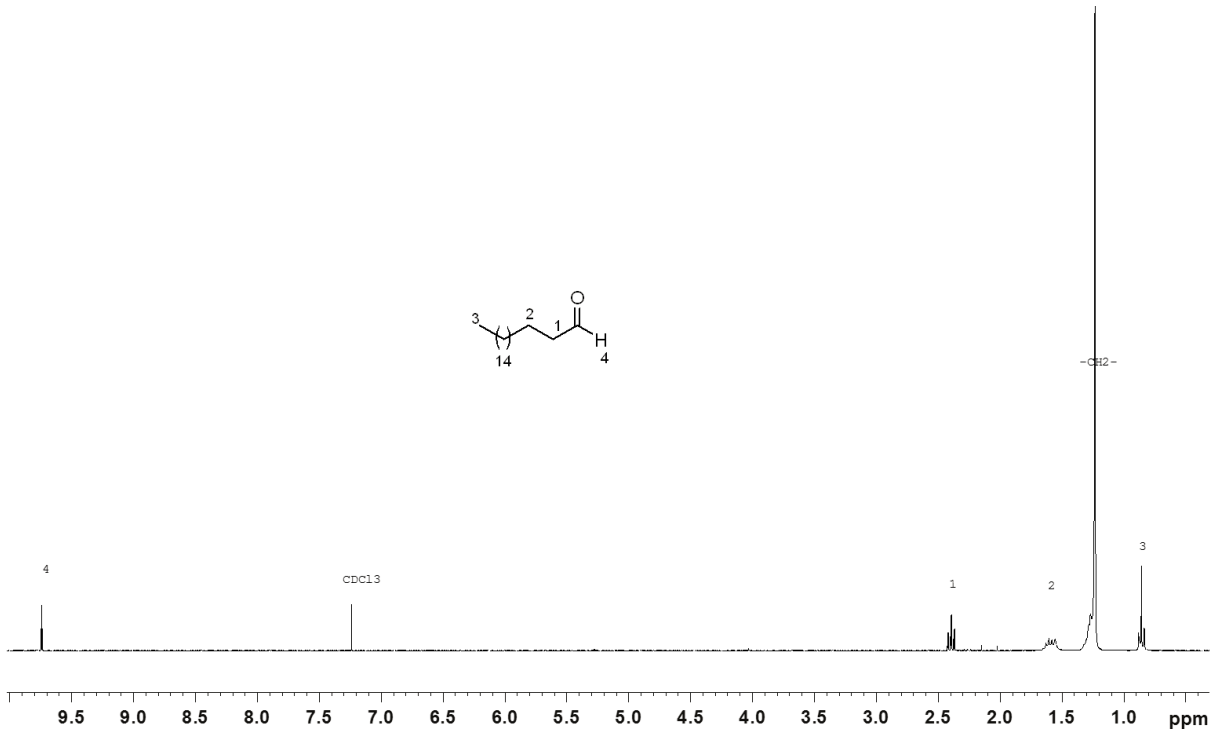


Figure 2.7: **The ^{1}H NMR spectra of the aldehyde product 30 (300 MHz, $CDCl_3$).**

Aldehyde **30** was condensed with benzyl stearate **29** in an aldol reaction with freshly prepared LDA in THF at -78 °C as described in scheme 2.9. This gave the product **31** in excellent yield (94%). The diastereomers were readily separated by flash column chromatography eluting in hexane: ethyl acetate: ether (6:2:2) to give separate *anti*-**31** (50%) and *syn*-**31** (50%) products. ^{1}H NMR spectrum of the *anti*-product **31** (figure 2.8) showed characteristic peaks such as the two doublet of triplets signals at 2.39 ppm and 3.76 ppm each integrating for one proton which corresponds to the α- and the *β*-protons respectively. The peak at 0.88 ppm integrating for 6H corresponds to two triplets of the two terminal methyl groups from both *α*- and *β*-chains. The analysis of the ^{13}C NMR confirmed the above mentioned results. The peaks at 52.1 ppm and 71.6 ppm correspond to the *α*- and *β*-carbon atoms respectively. The ester carbonyl carbon is situated at 174.6 ppm.

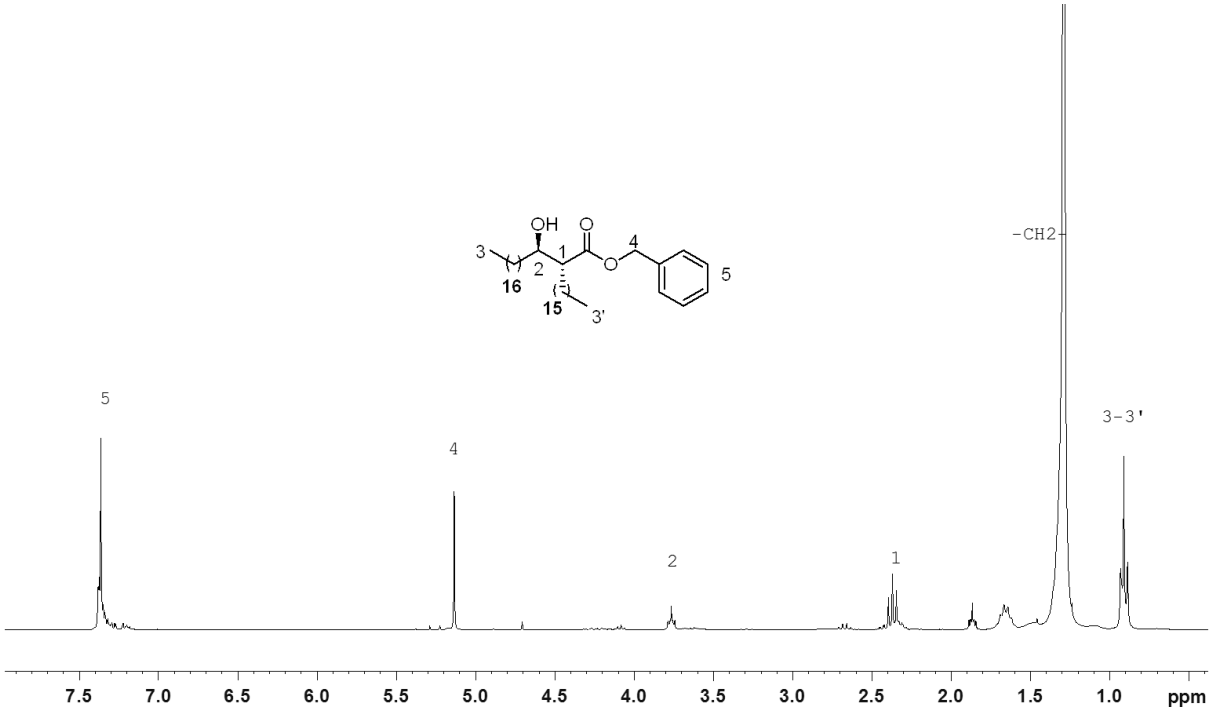


Figure 2.8: **The ^{1}H NMR (300 MHz, $CDCl_3$) of the *anti*-aldol product 31.**

The deprotection of the benzyl ester of **31** to the carboxylic acid **1** was first achieved by hydrolysis in the presence of tetrabutylammonium hydroxide (20%) which gave a yield of 66%. Lower concentrations of TBAH gave reduced yields. Secondly, the benzyl ester was removed by hydrogenation with Palladium hydroxide on carbon ($Pd(OH)_2/C$) catalyst. Different results were recorded: when 0.5 equivalents of the $Pd(OH)_2/C$ catalyst was used followed by the hydrogenation , the conversion was achieved in 99%, within 48 minutes. It was investigated that 0.1 and 0.01 equivalents of the catalyst gave respectively 95 and 90% of conversion within 1.20 h and 2.50 h respectively. Decreasing the number of equivalent of the catalyst caused an increased time necessary for the reaction to reach completion and consequently decreased the final yield of the acids. Hydrogenation of the benzyl ester group gave a higher yield with a shorter reaction time and low temperature when compared to the TBAH approach. Both reactions afforded the CMA product **1** with a ^{1}H and ^{13}C NMR spectra identical to those obtained for CMA **1** prepared via hydrolysis of the methyl ester in the Claisen route. The NMR spectra clearly showed the absence of the characteristic benzyl peaks. Thus the optimised aldol route to a racemic *anti*-CMA **1** was completed in 3 steps in the longest linear sequence with an overall yield of 44-46% with no diastereoselectivity (1:1 *anti: syn*).

2.4. Conclusion

The Claisen product **27** exists as a pair of enantiomers containing just one stereocentre. Attempts to control the stereochemistry at this centre would be futile as the proton between two carbonyl groups is highly acidic and would racemise. However, after the condensation step it might be possible to conduct a selective reduction of the ketone to an alcohol using the method of Nishizawa and co-workers (2007) that relies on a ruthenium catalysed asymmetric hydrogenation to form the (*R, R*) configuration of the mycolic motif if the *anti*-product is more stable than the *syn*-product. However, explicit control of stereochemistry at the *α*-carbon would not be possible.

The low yield (63%) and lack of diastereoselectivity in the reduction step as shown in the scheme 2.7 constitutes a major challenge of the Claisen route. In addition, multiple purifications by column chromatography are needed to get a quantitative separation of the *syn*- and the *anti*-products **28**. Separation of the benzyl ester was achieved much more easily by column chromatography.

The choice of the aldol route as an alternative route to the synthesis of the CMAs **1** was based in the fact that, in contrast to the claisen route, the aldol route allows the insertion of both *α*- and *β*-stereo-centres simultaneously in one step but with higher overall yield (Scheme 2.9). There is strong evidence in the literature that shows that the aldol route can be made enantio- and diastereo-selective by use of a chiral auxiliary to give the desired *α*-alkyl-*β*-hydroxy product in the required (*R, R*) configuration (Driver, 2009; Driver *et al*., 2010).

Chapter 3: Synthesis of the Fluorescent dye: 4(5)-BMF

3.1. Introduction

- **Discovery and first applications of Fluorescein dye**

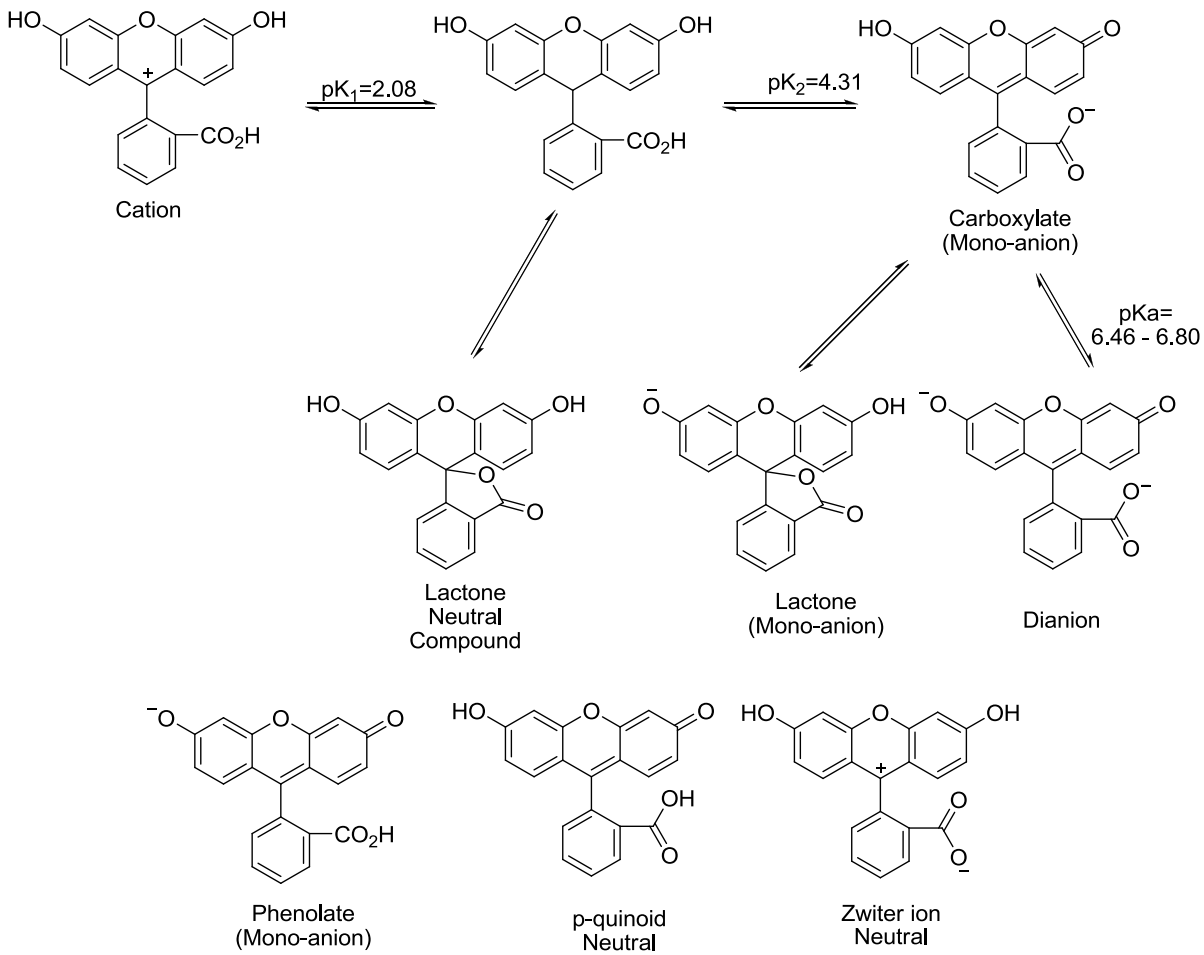
Von Baeyer Adolf, a German chemist, was awarded a Nobel Prize in chemistry in 1905 for his work on organic dyes and hydroaromatic compounds (De Meijere, 2005). He first prepared the 3',6'-dihydroxyspiro[isobenzofuran-1(3H),9'-(9H)xanthen]-3-one, which is called "fluorescein". The name comes from "fluo" and resorcin, deriving from resorcinol that was reacted with phthalic anhydride (Von Baeyer, 1878).

- **The properties of fluorescein**

Fluorescein is a synthetic organic compound available as a dark orange/red powder soluble in water and alcohol (Schubert *et al*., 1995a, Schubert *et al*., 1995b, Valeur, 2011). Fluorescein belongs to the xanthene fluorophores which also include rhodamine. It is widely used as a fluorescent tracer for many applications. Fluorescein is a fluorophore commonly used in microscopy, as the gain medium in a laser dye, in forensics and serology to detect latent blood stains, and in dye tracing. Fluorescein has an absorption maximum at 494 nm and emission maximum of 521 nm (in water). Fluorescein also has an isosbestic point (equal absorption for all pH values) at 460 nm. Fluorescein is known as a colour additive (D&C Yellow no. 7) (Schubert *et al*., 1995a, Schubert *et al*., 1995b, Valeur, 2011). The disodium salt form of fluorescein is known as uranine or D&C Yellow no. 8. More concentrated solutions of fluorescein can even appear red.

The fluorescence property of fluorescein is mostly based on the formation of a conjugated system in the xanthene moiety (Kotásková, 2012, Fráter, 1981, Fráter*et al*., 1984). At physiological conditions of pH (between 6 – 8) the fluorescein exists in equilibrium between the "closed" lactone containing two phenol functional groups and the "open" quinoid form

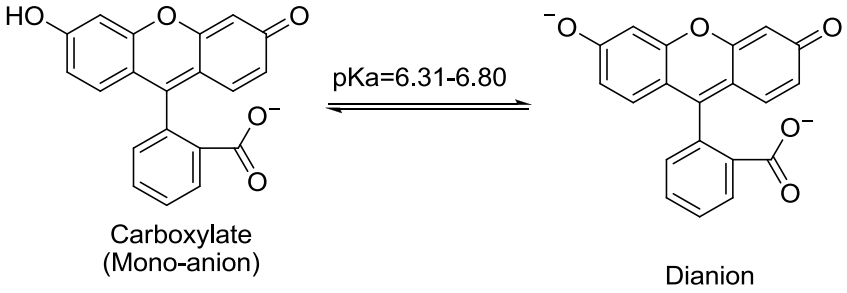
containing one phenol group. This results in various ionization equilibria as shown in the scheme 3.1, making for a band of excitation wavelengths that depends on pH (Sun *et al*., 1997; Slyusareva *et al*., 2009). This probably explains the pH dependent absorption and emission wavelength maxima for fluorescein over the pH range of 5 to 9 (Gee *et al*., 1997). It has been found that under acidic pH ranging between 2 to 4; generally the fluorescein moiety exists in a "closed" lactone form that is not fluorescent. Under a milder acidic pH ranging between 5 and 7 a significant part of the fluorescein remains in the protonated, non-fluorescent form and in the less fluorescent mono-anionic form. It has been found that under physiological conditions with a pH of around 7.4, the fluorescein is primarily a highly hydrophilic and fluorescent dianion. Details are given in the scheme 3.1 below (Sjöback *et al*., 1995, Sjöback *et al*., 1998).



Scheme 3.1: **The pH dependence of fluorescein equilibrium** (Adapted from Sjöback *et al*., 1995, Sjöback *et al*., 1998).

Most of the biologically relevant molecular forms exist as mono-anion and dianion at approximately neutral pH. These two forms are the most principal ground-state species which

interchange due to the phenolic pKa values ranging between 6.31 and 6.80 as illustrated in the scheme 3.2. Their corresponding spectral properties in aqueous solution have been found to be as follows: For the Mono-anion: Maximal absorption wavelength (λmax) = 453, 472 nm, ε = 29 000-32 600 $M^{-1}.cm^{-1}$, Φ = 0.36-0.37, and for the Dianion: λmax = 490 nm, ε = 76 900-87 600 $M^{-1}.cm^{-1}$ and Φ = 0.92-0.95 (Haugland et *al*., 2005).



Scheme 3.2: **The Mono-anionic and dianionic forms of fluorescein** (Kotásková, 2012)

Generally the mono-anionic form has been found to have a lower absorptivity and the absorption maxima are blue-shifted relative to the dianionic form. The fluorescence quantum yield of the mono-anion is also significantly lower than that for the dianion. Thus, the dianionic form has a very strong visible light absorbance and potent fluorescent emission. The structure of fluorescein needs to be further modified in such a way to overcome the pH sensitivity. For example researchers have found that 2',7'-dichlorofluorescein is less sensitive to pH dependence (pKa: 4.6) than fluorescein itself (pKa = 6.4)(Lavis *et al*, 2007; Mchedlov-Petrossyan *et al*., 1992, Leonhadt *et al*., 1971).

- **Fluorescein derivatives**

Different derivatives of the fluorescein dyes have been prepared for covalent attachment to compounds such as proteins (antibodies), nucleotides, oligonucleotides, drugs, hormones, lipids, peptides and other biomolecules(Kotásková, 2012). The major derivatives are 5-bromomethyl fluorescein (5-BMF), fluorescein isothiocyanate (FITC), and fluorescein amidite (6-FAM) phosphoramidite. These reagents possess high molar absorptivity (128,832 M^{-1} cm^{-1}) and quantum yield (0.92) (which corresponds to the ratio between the number of emitted photons at a given wavelength usually in the UV-visible region and the number of absorbed photons) in

alkaline media. The excitation and emission maxima of 5-BMF are at 490 nm and 520 nm respectively. 5-BMF has been shown to be a useful reagent for achieving low concentration detection limits in biopharmaceutical analysis. 5-BMF is one of several fluorescent reagents used for the derivatization of carboxylic acids (Toyoo`ka, 2002; Tortoli *et al.*, 2002; Butler *et al.*, 2001; Tortoli, 1996). FITC has been found reactive towards most primary amine groups of biological molecules such as intracellular proteins, forming a thiourea linkage. Several phosphoramidite reagents which contain a protected fluorescein such as but not limited to 6-FAM phosphoramidite (Figure 3.1) have been widely used in the synthesis of fluorescein-labelled oligonucleotides. Thiol reactive fluorescein maleimides and 5-iodoacetamides (5-IAAF) have been also used (Haugland *et al*. 2005; Slyusareva e*t al.*, 2009; Gee *et al.*, 1996). Fluorescein azide derivatives have been used in the CuI-catalyzed Huisgen 1,3-dipolar cycloaddition reaction with terminal alkyne labelled biomolecules to give the 1,4-regioisomers of 1,2,3-triazole in the "click reaction" (Rostovtsev *et al.*, 2002; Kolb *et al.*, 2001).

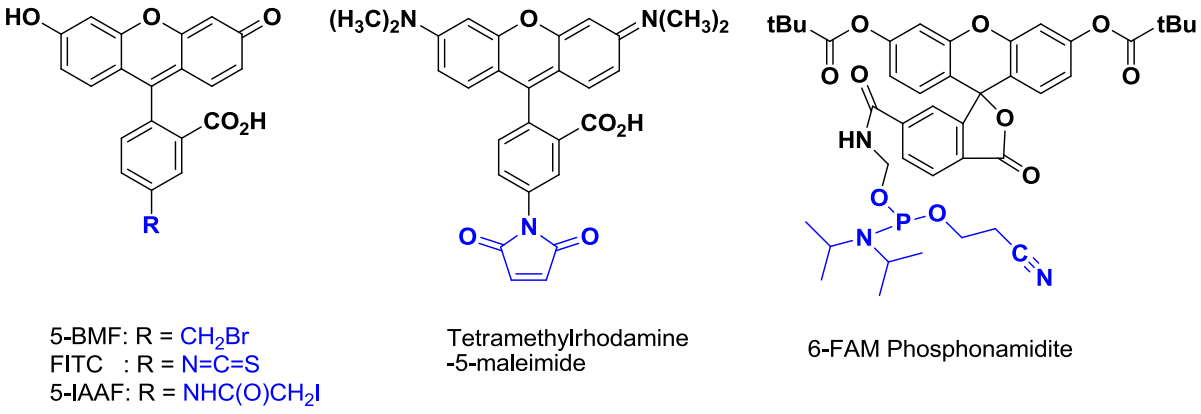


Figure 3.1: **Structures of some fluorescein dyes and derivatives containing different reactive groups** (Gee *et al.*, 1996).

It has been found that iodoacetamide and maleimide derivatives are very difficult to prepare in very pure form. As a consequence, the commercially available batches generally contain different variable mixtures of 5- and 6- isomers (Haugland *et al.* 2005). To the list of the above mentioned fluorescein derivatives, we also include 5-BMF. This is one of the reasons we set out to prepare our own dye for this project. The commercially available 5-BMF is in the form of "sum of tautomers" (Sigma Aldrich) which complicates the derivatization process as well as the NMR spectrum analysis of the resulting product. In this project the bromomethyl reagent was the choice for the derivatisation of the carboxylic acid functional group of the CMAs **1.**

- **Some other applications of fluorescein labelling**

Antibodies and so many other compounds such as enzyme substrates, nucleotides, and drug-markers have been fluoresceinated for a number of applications. For example in 1941 Albert Coons for the first time directly conjugated a fluorescein label to an antibody for its visualization and proved that the antibody could still retain the antigen binding activity (Coons *et al*., 1941). This discovery made the fluorescent antibodies technique one of the most valuable reagents for the study of some basic issues in cell biology, immunology, and neurobiology as well as in clinical medicine. Nowadays huge numbers of different fluorescein conjugated biological compounds with distinct excitation and emission spectra have been prepared and can be used in microscopy techniques or in Fluorescence-activated cell sorting (FACS) (Herzenberg *et al*., 1972)

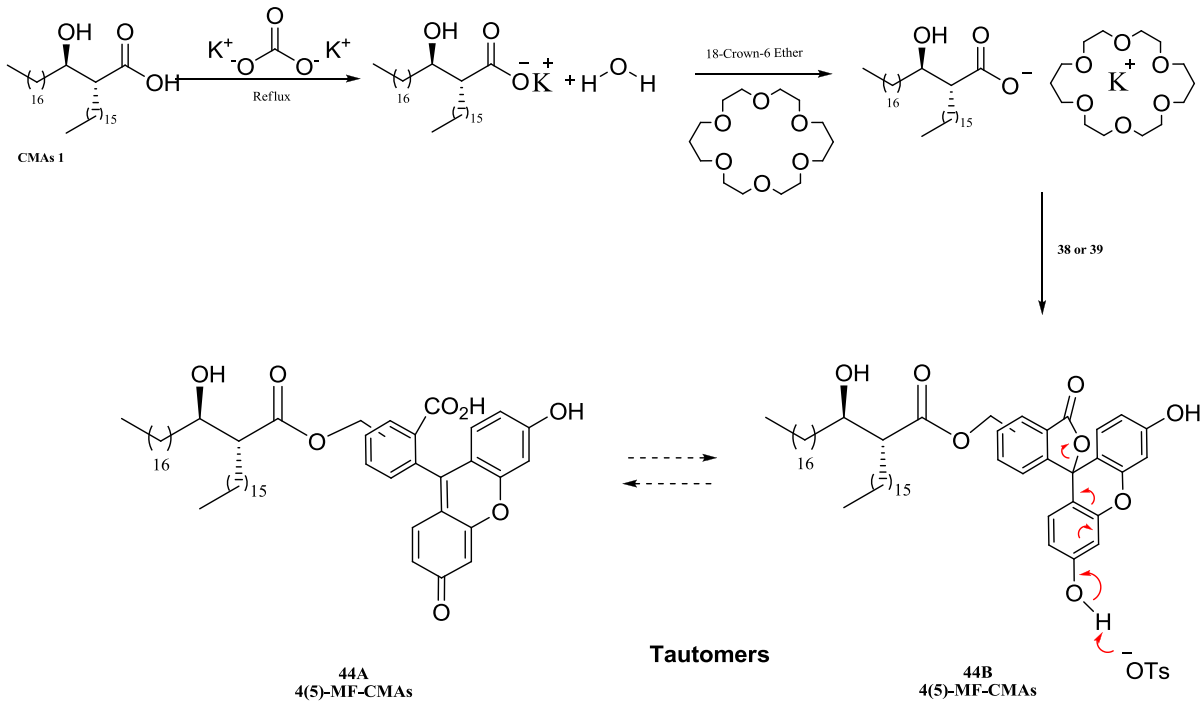
- **Why label mycolic acids with a fluorescent dye?**

MA's role has been largely discussed in the first and second chapter. The analysis of the lipid fractions of the *M. tb,* has broadly elicited the better understanding of its composition. However it was very difficult to purify the wax fraction previously called "unsaponifiable wax" (Stodola *et al*., 1938). This "unsaponifiable wax" (later called "Mycolic acid" following its isolation from the original H37 strain of *M. tb.*), was clarified as an "alcohol-insoluble" and high molecular weight hydroxy acid (Stodola *et al*., 1938; Kanetsuna, 1968; Lechevalier *et al*., 1971).

A study conducted by Korf and co-workers in 2005 has established how the MA interaction with macrophages induced a macrophage morphotype that is similar to the foamy macrophage derivatives seen in TB granulomas (Korf *et al*., 2005). In order to get around the *M. tb.*-MAs' poor solubility in water, they were inserted into liposomes (sized between 0.1–10 µm using flow cytometry), which were used as vehicles for their subsequent administration into the mice. For the determination of the specific type of phagocyte which is accountable for the *in vivo* phagocytosis of the liposomes, MA was first derivatized with a fluorescein dye (5-BMF) before its insertion within the liposomes and injection into the peritoneal cavity of the mice. About 2 hours after administration of MA-liposomes particules the peritoneal exudate cells were isolated and then stained for the macrophage specific marker F4/80. Results from flow cytometric analysis showed that only the F4/80-positive exudate cell population internalized the fluorescent liposomes, identifying macrophages as the exclusive cell type responsible for internalization of the MA-liposomes. Confocal microscopic analysis confirmed the uptake of the

MA-liposomes containing 5-BMF-MA and their processing into acidified vesicles (Korf *et al.*, 2005).

Another reason why MAs should be labelled is that, as indicated in their name and/or structures, they contain a free carboxylic acid functional group in their motifs. Long chain aliphatic carboxylic acids are notoriously difficult to separate and detect in chromatography, or to characterise them by techniques such as infrared spectroscopy or mass spectrometry (Tortoli *et al.*, 2002; Tortoli *et al.*, 1996; Crawford, 1994) . Usually, at least methyl esterification is needed to enable separation of MAs on any suitable kind of chromatography (Watanabe *et al*. 2001, Pan *et al*. 1999). To study function, such methylated, separated MAs then need to be hydrolyzed to the free carboxylic acid, as it was shown that MAs lose their functional activity upon esterification (Lemmer *et al.*, 2009, Beukes *et al.*, 2010). For sensitivity of detection of MAs among many other aliphatic carboxylic acids in a sample, esterification to fluorophores may provide a solution. The application of chemoluminescence detection may be restricted and radio-immunological detection is rather time-consuming and laborious (Tortoli *et al.*, 2002; Tortoli *et al.*, 1996; Crawford, 1994). Therefore to improve sensitivity, carboxylic acids may be derivatized with suitable fluorophore reagents. Fluorescent detection has the potential for analysis in the low picomolar (pM) range (Azuma, 1975; Barry III, 1996). Several fluorescent dyes have been prepared for the analysis of carboxylic acids by HPLC, (Tortoli, 2002; Tortoli, 1996; Crawford, 1994). Among them, 4-Bromomethyl-7-methoxycoumarin (Br-MMC) and its analogues have been used to derivatize aliphatic mono-carboxylic acids and dicarboxylic acids (Wolf, 1992; Tortoli, 2002; Tortoli, 1996; Crawford, 1994). The reaction mechanism involved in the ester formation is simple and short (Scheme 3.3).



Scheme 3.3: **The derivatisation procedure adapted from Toyoo`ka, et al** (Toyoo`ka *et al*., 2002)**.**

The reaction of Bromo-methyl-type reagents with carboxylic acids is generally performed by refluxing at 60 °C for 30–60 minutes for mono-carboxylic acids in aprotic solvents in the presence of a base (K_2CO_3) and a catalyst (18-crown-6 ether) which acts as phase transfer agent (Toyoo`ka, 2002). However, due to the high cost and unreliable purity of the commercial "5-BMF", it became necessary to synthesise and purify it in our laboratory.

- **The fluorescein derivatization effect on MA antigenicity**

According to an investigation conducted by Lemmer and co-workers (Lemmer *et al*. 2009), simple MA-methyl-ester mixtures were not recognized by antibodies, while a mixture of free MAs was recognized by both TB positive and TB negative patient sera. The antibody recognition was stronger in TB positive sera. However when MA was esterified with a fluorescein label, as described in chapter 1, the antibody binding signal was only slightly affected. These authors speculated that this could possibly be due to the presence of a free carboxyl group on the fluorescein remaining after conjugation, which substitutes one of the MA that has been liganded

in the derivatization reaction to form the ester (Lemmer *et al*. 2009). The presence of this carboxylic acid group was hypothesized by Lemmer *et al.* to be necessary to sustain a MA conformation by internal hydrogen bonding that could support antigenicity (recognition by antibody).

In order to support the claim of Lemmer and co-workers that it is the carboxylic acid moiety in the fluorescein label that allows labelled mycolic acids to retain their antigenic properties, it was our aim to synthesize a fluorescein labelled MA using 4(5)-BMF and to characterize the structure fully by NMR spectroscopic analysis. It is worth noting that all original biological studies so far available in the literature on fluoresceinated mycolic acids were not done on characterized materials.

3.2. Aims

1. To synthesis the Bromo-methyl fluorescein dye;
2. To optimize the labelling reaction using the model CMA **1**;
3. To label a natural mycobacterial-MA mixture with the 4(5)-BMF and to determine whether it maintains biological properties;
4. Characterize the newly prepared 4(5)-BMF, CMAs-4(5)-MF and the natural mycobacterial-MAs-4(5)-MF materials by NMR analysis.

3.3. Results and discussions

The commercially available so-called "5-BMF" is supplied in the form of a "sum of tautomers" (Sigma Aldrich). The structure provided is of the lactone tautomer, but there is no indication of alternative tautomer structures and no ratio of the tautomers is given. Furthermore at a price of R 6,990.20 per 50 mg sample, it was very disappointing that the batch was 4 years old as evidenced from the ^{1}H NMR spectrum that was supplied on request, the quality was poor and several derivatisation reactions were unsuccessful.

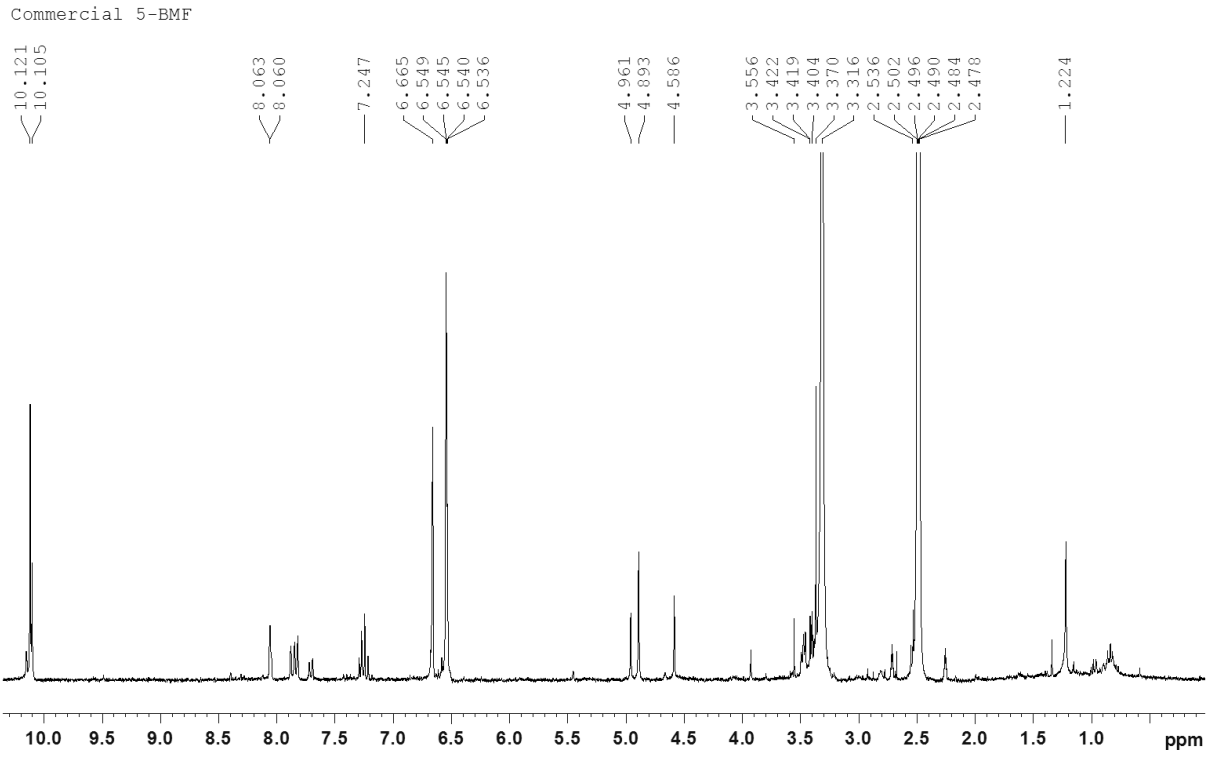


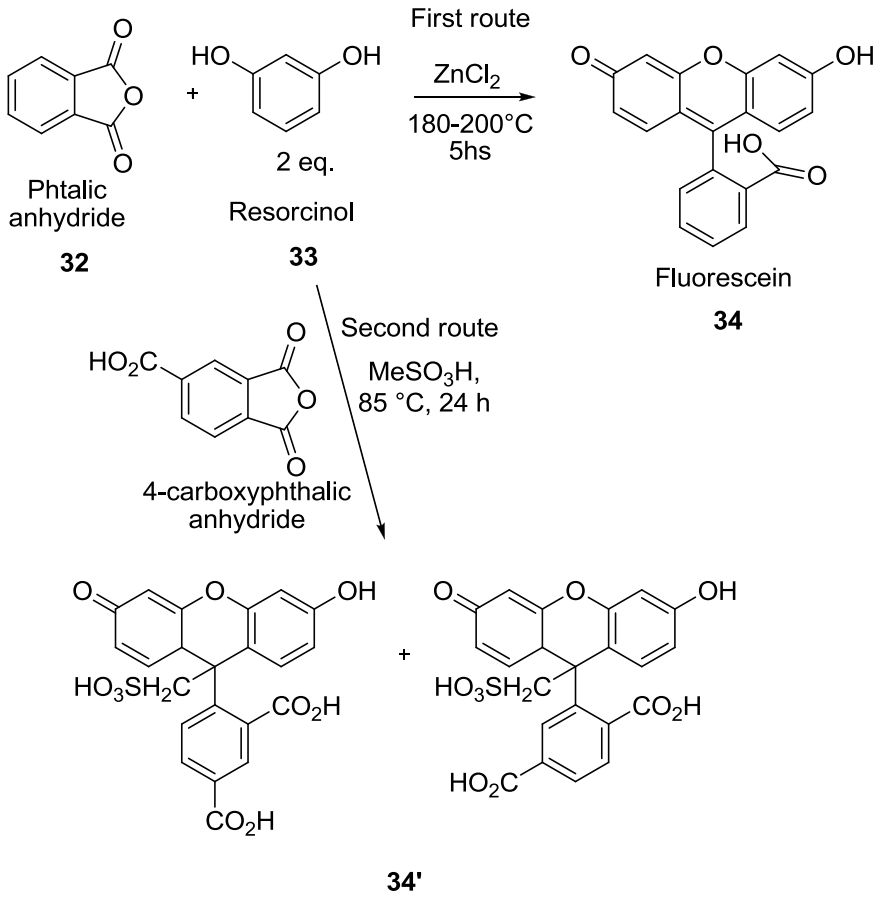
Figure 3.2: **The ^{1}H NMR spectrum analysis of the first commercially available "5-BMF" sample**.

For example in both NMR spectra analyses of the commercially available samples as described in the figures 3.1, all the peaks between 0 ppm and 2.49 ppm as well as between 2.60 ppm and 4.10 ppm correspond to impurities in the compound although the purity was claimed to be greater than 98%. Additionally, the carboxylic signal integrated to 3H rather than 1H.

Therefore we set out to synthesise, purify and characterize a fresh dye (Schemes 3.4-3.9) for labelling our synthesized CMAs.

3.3.1. Previous synthesis of the BMF

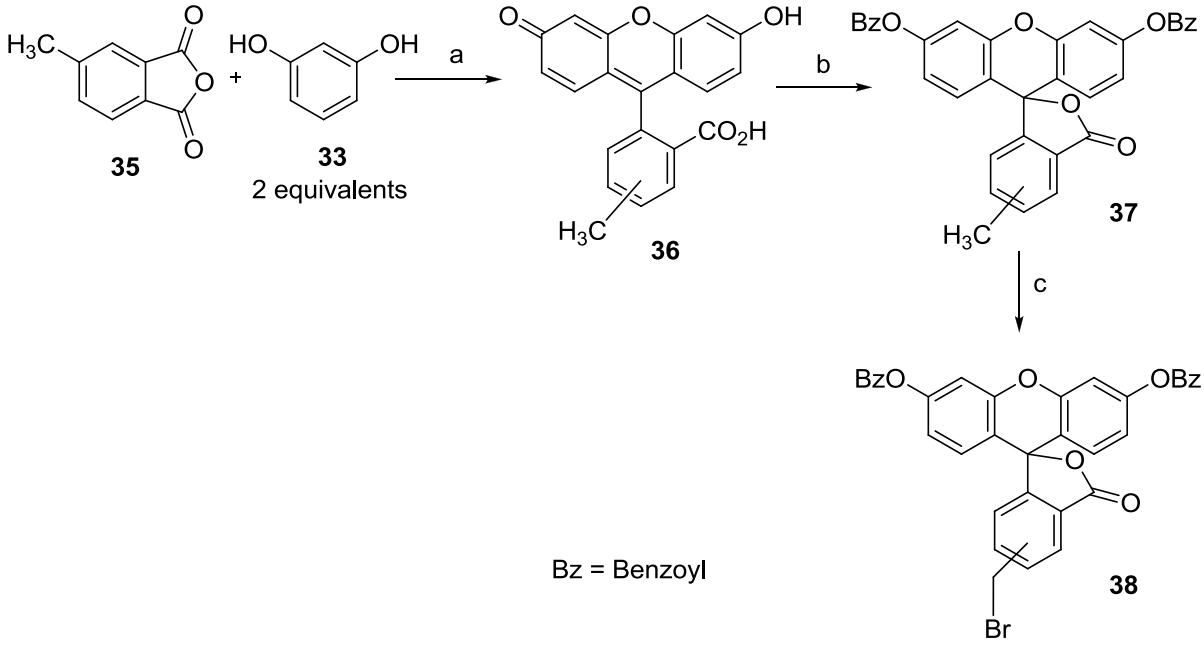
Fluorescein was first synthesized by Adolf von Baeyer in 1871 (De Meijere, 2005, Von Baeyer, 1878) from phthalic anhydride and resorcinol in the presence of zinc chloride via the Friedel-Crafts reaction (Kotásková, 2012). A second method to prepare a fluorescein derivative used methanesulfonic acid as a Brønsted acid catalyst. This route has a high yield under milder conditions (Scheme 3.4).



Scheme 3.4: **Synthetic reaction of the fluorescein dye 34** (Ueno *et al.*, 2004, Holletz *et al.*, 1993).

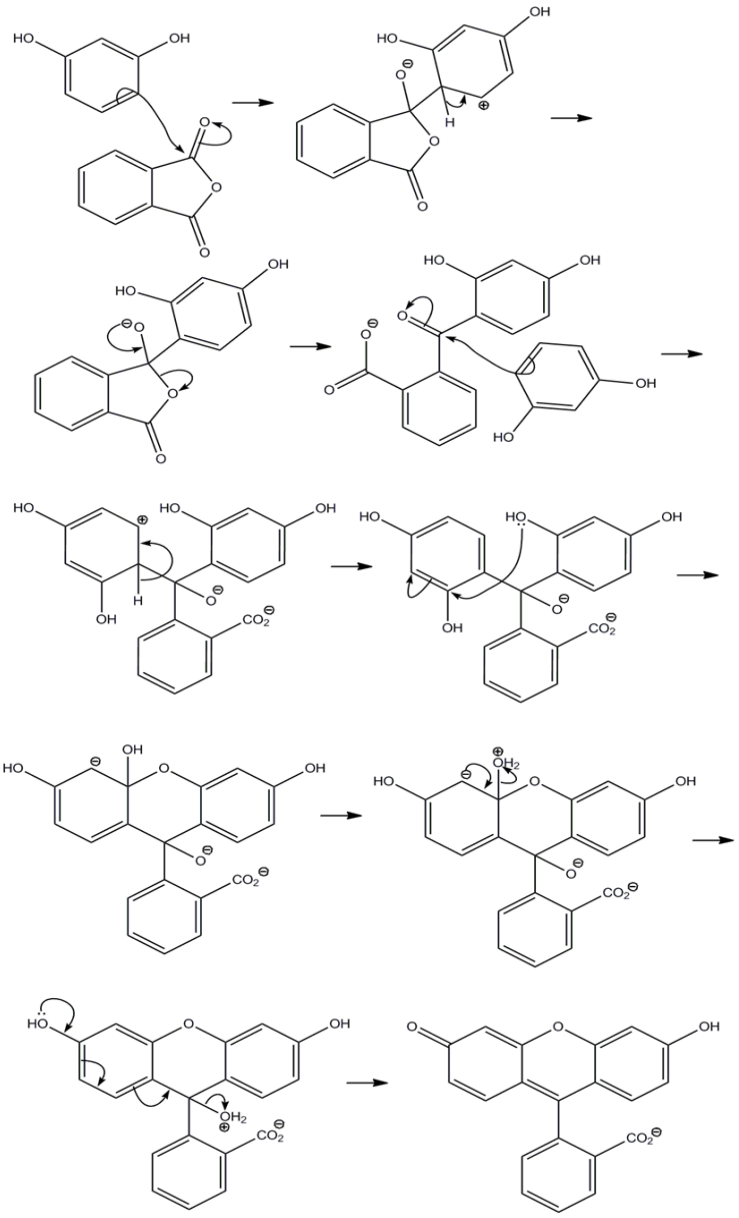
The method of von Baeyer refined by Gattermann and Weinandy (Gattermann*et al.*, 2002) was extrapolated by Torsten Holletz and co-workers (Holletz*et al.*, 1993) for the synthesis of 4(5)-BMF, used as its 3', 6'-dibenzoyl derivative **38** to label nucleosides. Dibenzoyl protection raised the alkali resistance and thermal stability of the label. These authors showed that the

photophysical fluorescence properties were poorer than for unmodified fluorescein (Schubert *et al*., 1995a, Schubert*et al*., 1995b).



Scheme 3.5**: Synthesis of 3',6'-dibenzoyl-4(5)-(bromomethyl)fluorescein 38: a) $ZnCl_2$, 170-180 °C, 5h, 20-61%; b) C_6H_5COCl, C_5H_5N, 3.15 h, 0 °C-rt, 85 °C; c) NBS, $(PhC(O)_2)$, CCl_4, 80 °C, 97%** (Holletz *et al*., 1993).

Using this approach, 4-methylphthalic anhydride **35** was condensed with resorcinol **33** in the presence of anhydrous zinc chloride anhydrous (Scheme 3.5) to yield the desired dyes without additional purification in yields of 20% (**34)** and 61% (**36)** respectively. This condensation reaction involves the ether bond formation which is initiated by the protonation of one of the carbonyl oxygen's from phthalic anhydride by a catalytic amount of sulphuric acid. The overall process is two sequential Friedel–Crafts type reactions that occur in the same reaction flask without having to isolate the product of the first reaction as shown in scheme 3.6. Reliant on the type and orientation of the substituents present on the phenol, either a triarylmethane or fluorescein derivative-type dye is produced as the final result (attack at the para- and ortho-position simultaneously (Scheme 3.6 and 3.7) (McCullagh *et al*., 2007). The synthesis of methylfluorescein **36** gave a mixture of isomeric 4- and 5-methylfluoresceins in a 1:1 ratio which were distinguished by their ^{1}H-NMR spectra (Holletz *et al*., 1993).

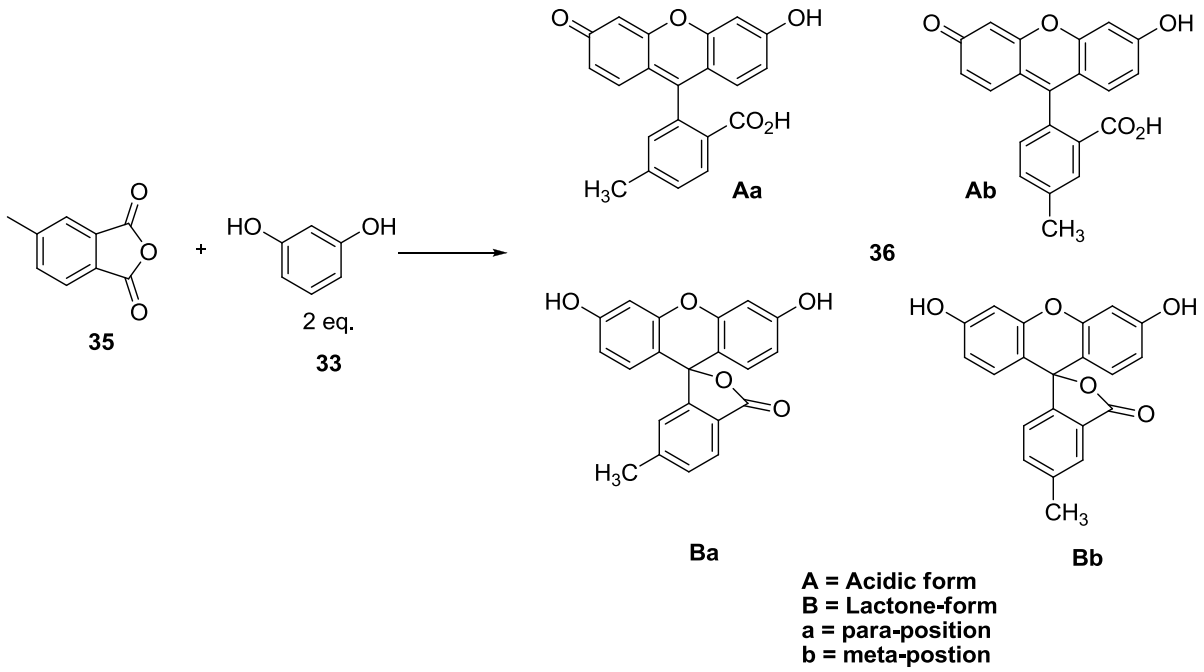


Scheme 3.6: **Proposed reaction mechanism for the formation of the fluorescein dye.**

The phenolic hydroxy groups of the dye **36** were protected as the dibenzoyl derivative **37** in 85% yields by reacting it (**36**) with benzoyl chloride in anhydrous pyridine. Selective side-chain bromination with *N*-bromosuccinimide (NBS) yielded the 3‘, 6’-dibenzoyl-protected dye **38** with the 4(5)-bromomethyl function in almost 100% yield (scheme 3.5) (Holletz *et al.*, 1993).

- **Synthesis of 4(5)-BMF**

To prepare the fluorescein dye (5-BMF) **39AB**, the same procedure as developed by Holletz and co-workers (Scheme 3.5) was followed as the aim was to get a pure material that will be characterized to confirm its exact structure. Unless the structural isomers **36**-**38** could be separated, this synthesis would yield a mixture of 4- and 5-bromomethylfluorescein. 4-Methyl phthalic anhydride **35** was fused with resorcinol **33** in the presence of a fine powdered anhydrous $ZnCl_2$ as the catalyst. A catalytic amount of sulphuric acid was added and the mixture fused to yield a pure orange solid product **36** consistent with reported values from the literature in 90% yields which needed no further purification (Scheme 3.7).



Scheme 3.7: **The synthetic route for the product 36Aab: Reaction conditions: $ZnCl_2$, 170-180 °C, 5 h.**

One remarkable difference from the literature was that the ^{1}H and ^{13}C NMR spectral analysis revealed the presence of four products. These corresponded to the expected "*para-*" (**36Aa** and **36Ba)** and the "*meta–*" (**36Ab** and **36Bb)** structural isomers respectively each in an acidic form **36A** and the lactone-form **36B**. After successive column chromatography, we were able only to separate and isolate the tautomers **36A** and **36B** in a 1:1 ratio. Unfortunately the 4- and 5-methyl structural isomers ("a" and "b") were inseparable.

The ^{1}H and ^{13}C NMR spectral analysis clearly distinguished between the acid and lactone forms of **36.** The spectra for the lactone, which has a plane of symmetry, had fewer signals than for the acid tautomer. The ^{1}H NMR spectrum of **36A** (Figure 3.3) showed a carboxylic acid proton signal at 10.80 ppm integrating to one which was notably absent from the ^{1}H NMR spectrum of **36B** (Figure 3.4). The hydroxyl signal in the lactone form integrated to two (symmetrical) while in the acid it integrated to one. Due to the presence of the 4- and 5-methyl structural isomers in a 1:1 ratio in both samples, many peaks were doubled complicating the analysis. The acid form (**36A**) revealed one carbonyl carbon peak at 186.5 corresponding to the ketone in both structural isomers and two acid carbonyl carbons in the ^{13}C NMR spectrum (75 MHz, $CDCl_3$) at 170.0 and 155.3 ppm for **36Aa** and **36Ab** respectively. In contrast the lactone tautomers **36Bab** revealed only one carbonyl carbon peak at 166.06 ppm. The *para*- and *meta*- methyl singlet peaks resonated at 2.33 and 2.36 ppm in **36Aab** and at 2.72 ppm (2 peaks) in **36Bab**.

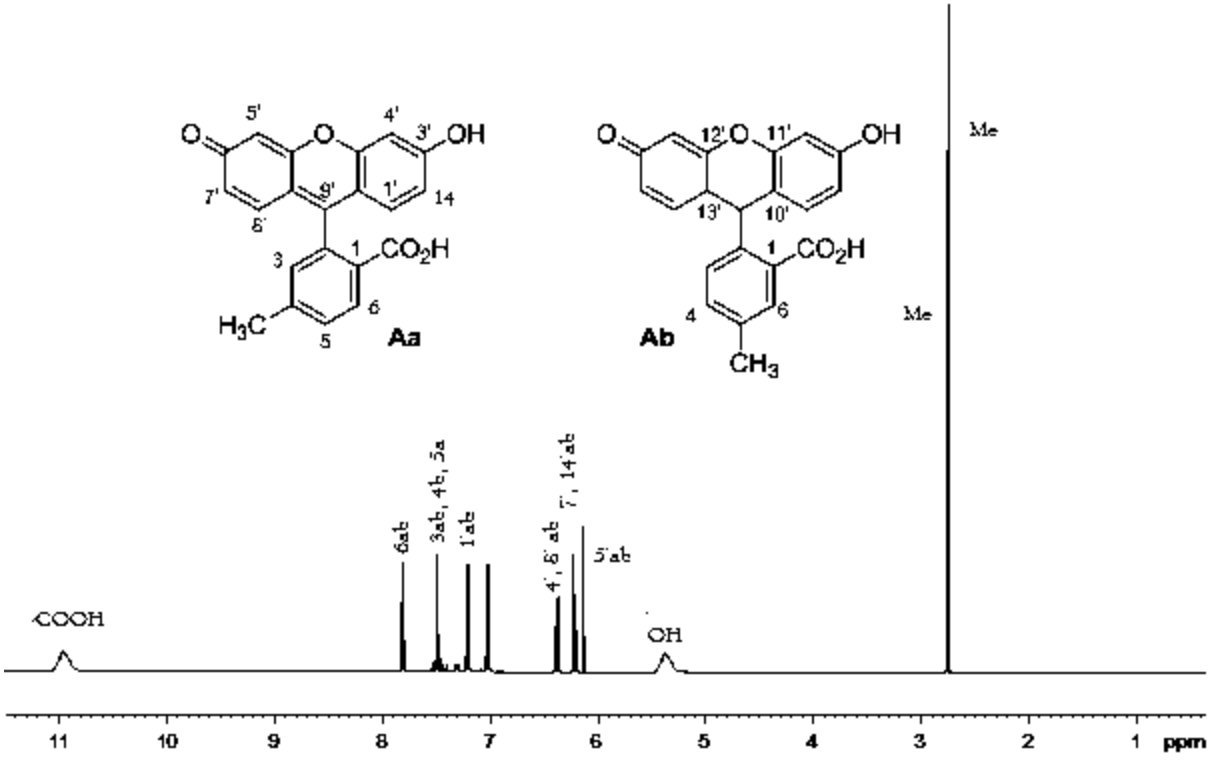


Figure 3.3: **The ^{1}H NMR spectrum of the product 36Aab (300 MHz, $CDCl_3$).**

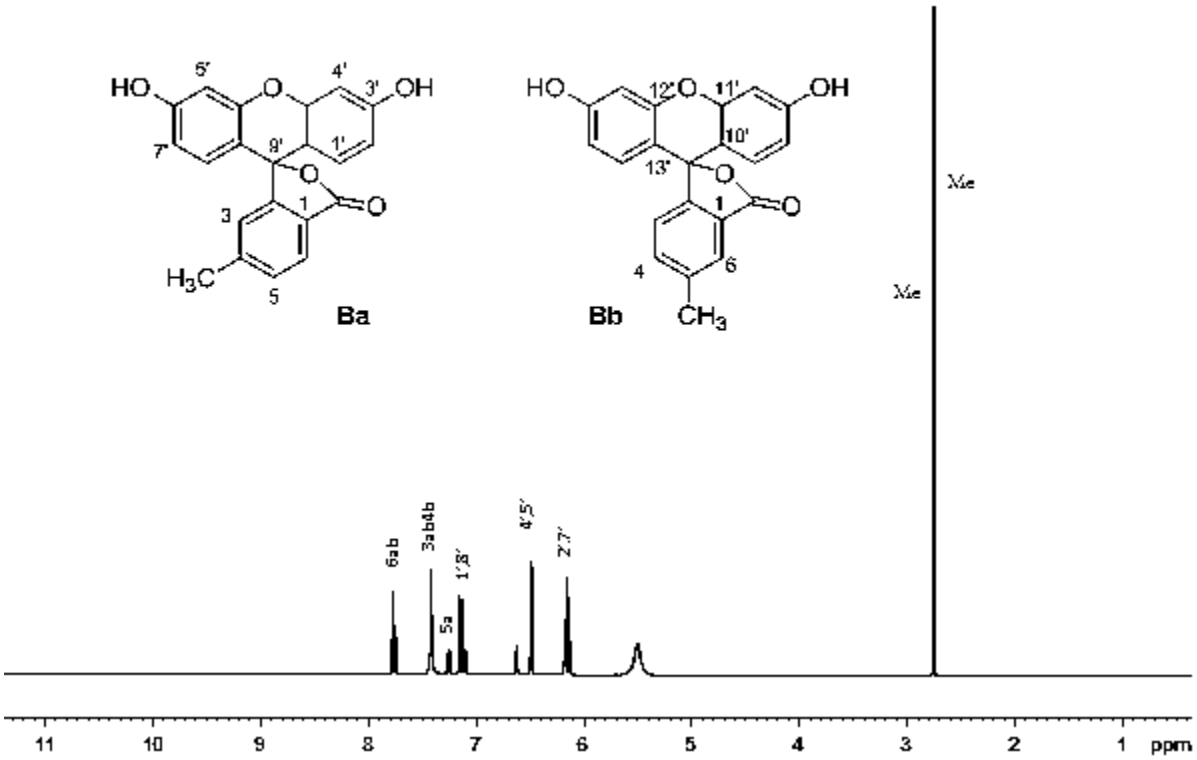


Figure 3.4: **The ^{1}H NMR spectra of the product 36Bab (300 MHz, $CDCl_3$)**

To assist in the interpretation of the ^{1}H and ^{13}C NMR spectra of the 4(5)-methyl mixtures, the spectra of each structural isomer was predicted using ChemBioDraw Ultra and ChemSketch software. Combined results are presented in tables 3.1 and 3.2 below for the ^{1}H and ^{13}C NMR spectral analysis respectively.

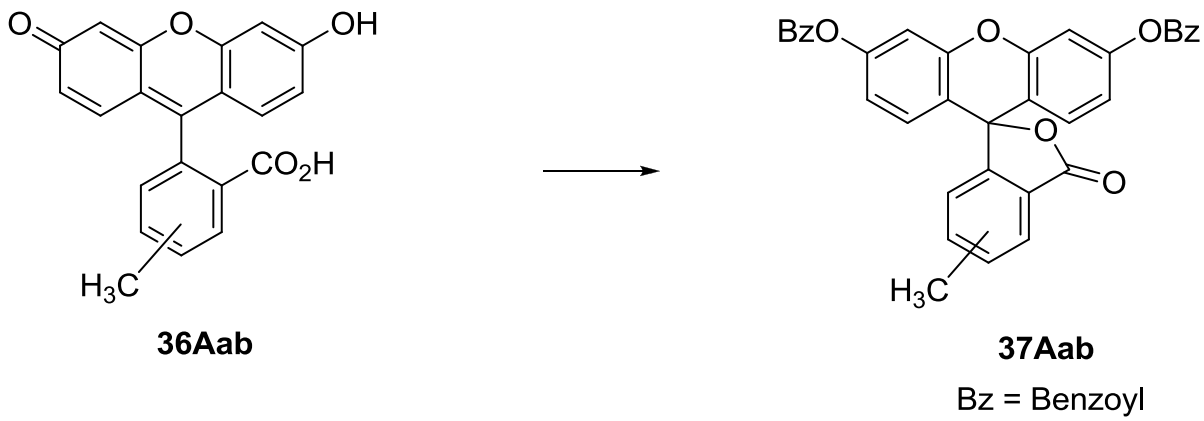
H #	36Aa		36Ab		36Ba		36Bb	
	Predicted	Actual	Predicted	Actual	Predicted	Actual	Predicted	Actual
COOH	11.00	10.80	11.00	10.80	-	-	-	-
OH	5.35	5.28	5.35	5.28	5.35	5.40	5.35	5.40
Me	2.34	2.33	2.34	2.36	2.34	2.72	2.34	2.72
3	7.30	7.12	7.47	7.43	7.34	7.39	7.35	7.39
4	-	-	7.52	7.50	-	-	7.37	7.70
5	7.32	7.39	-	-	7.27	7.29	-	-
6	7.78	7.81	7.87	7.81	7.86	7.91	7.69	7.72
1'	7.04	7.00	7.04	7.10	7.15	7.14	7.15	7.14
2'	6.21	6.20	6.21	6.20	6.40	6.40	6.40	6.40
4'	6.45	6.43	6.45	6.43	6.62	6.60	6.62	6.60
5'	6.11	6.09	6.11	6.10	6.62	6.60	6.62	6.60
7'	6.25	6.20	6.25	6.40	6.40	6.40	6.40	6.40
8'	6.46	6.43	6.46	6.50	7.15	7.14	7.15	7.14

Table 3.1: **Comparison between the predicted and actual ^{1}H NMR spectra for products 36Aab and 36Bab**

C #	36Aa		36Ab		36Ba		36Bb	
	Predicted	Actual	Predicted	Actual	Predicted	Actual	Predicted	Actual
Me	21.7	20.8	21.3	19.6	21.6	20.6	21.3	18
CO_2H	167.6	170	167.6	168.9	169.5	166.1	169.5	166.1
1	126	122.7	128.9	131.7	124.2	124.2	127.1	125.1
2	139.7	137.6	136.8	136.7	151.6	153.3	148.7	153.3
3	126.5	124.9	126.2	124.6	130	131	128	128.1
4	143.5	137.6	134.1	133	143.2	142.1	133.8	134.2
5	128.1	128.5	137.5	137.3	126.4	124.5	135.8	137.3
6	130.1	132.3	130.4	132.3	130.3	133.3	128.7	129.9
1'	128.5	131	128.5	131	129.3	129.9	129.3	129.9
2'	110	112	110	112	109.5	111.4	109.5	111.4
3'	158.4	156.4	158.4	156.4	155.7	158.1	155.7	158.1
4'	100.8	99	100.8	99	105.7	106.2	105.7	106.2
5'	95.3	96.6	95.3	96.6	105.7	106.2	105.7	106.2
6'	185.7	186.5	185.7	186.5	155.7	158.1	155.7	158.1
7'	115.3	118.7	115.3	118.7	109.5	111.4	109.5	111.4
8'	147.9	150.3	147.9	150.3	129.3	129.9	129.3	129.9
9'	134.3	134	134.3	134	85.1		84.8	
10'	108.4	110.9	108.4	110.9	107.7		107.7	
11'	155.1	155.3	155.1	155.3	152.4	153.3	152.4	153.3
12'	156.9	155.3	156.9	155.3	152.4	153.3	152.4	153.3
13'	128.5	131	128.5	131	107.7		107.7	
Peak not assigned						119.9		119.9

Table 3.2: **Comparison between the predicted and actual ^{13}C NMR spectra for product 36Aab and 36Bab.**

The acid tautomers **36Aab** were protected with the dibenzoyl chloride in anhydrous pyridine to afford product **37** as a mixture of *para* (**37a**) and *meta*-positions (**37b**) in 97% yield (Scheme 3.9).



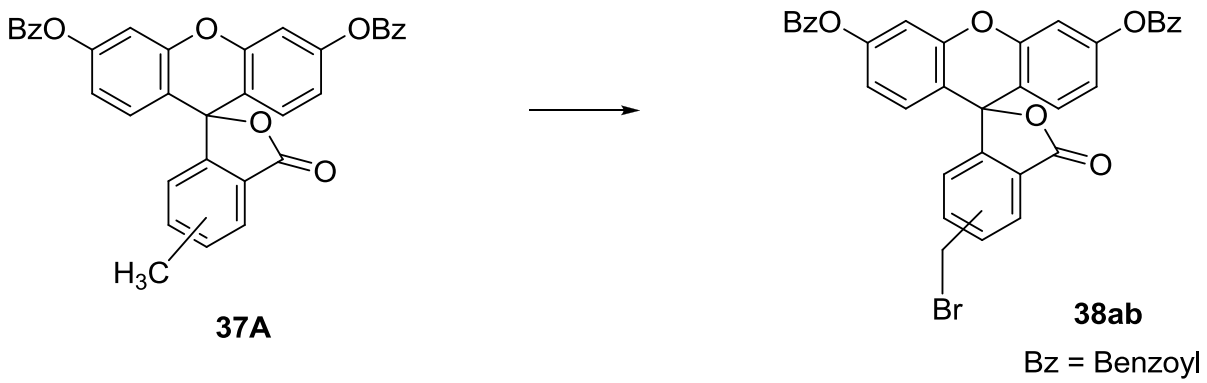
36Aab **37Aab**

Bz = Benzoyl

Scheme 3.8: **The proposed synthetic route for the product 37ab. Reaction conditions: C_6H_5COCl, anhydrous pyridine, 3.25 h, 0 °C - rt.**

Once again, it was impossible to separate the 4(5)-methyl structural isomers **37a** and **37b**. The presence of these products was confirmed by the ^{1}H and ^{13}C NMR spectral analysis. The spectra were similar to those for **36Bab** but the ^{1}H NMR spectrum of dibenzoate **37** showed additional multiplets between 7.00 ppm and 8.23 ppm corresponding to additional aromatic protons from the benzylic groups. The ^{13}C NMR spectrum also showed an additional ester carbonyl peak at 166.4 ppm.

The product **37** was converted into the product **38** by a free radical bromination reaction in the presence of the *N*-bromosuccinimide (NBS) in tetra-chloromethane (CCl_4) and of dibenzoyl peroxide (Scheme 3.9). After purification by column chromatography the bromomethyl product was obtained as a light yellow foam solid in 99% yield. It was necessary to recrystallize the NBS prior to use. This was achieved by dissolving it rapidly in boiling water followed by the filtration step which gave very pure white crystals after drying over calcium chloride ($CaCl_2$).



Scheme 3.9: **The free radical bromination reaction to prepare the product 38ab. Reaction conditions: NBS, $(Ph(CO)_2$, CCl_4, 80 °C, 6 h**.

The formation of product **38Aab** was confirmed by the NMR spectral analysis (Figure 3.5), which showed similarity with the previous spectra but was distinguished by the loss of the methyl peaks and the appearance of two singlet peaks at 4.75 ppm integrating for 2H which corresponds to the methylene group between the terminal bromine atom and the toluene group for both *para* and *meta* isomers. This result was confirmed by the ^{13}C NMR spectral analysis which showed two new singlets at 33.7 ppm which corresponds to this above mentioned methylene group.

The ^{1}H NMR spectral analysis (300MHz, $CDCl_3$) of the product **38Aab** is shown in the figure 3.5 below.

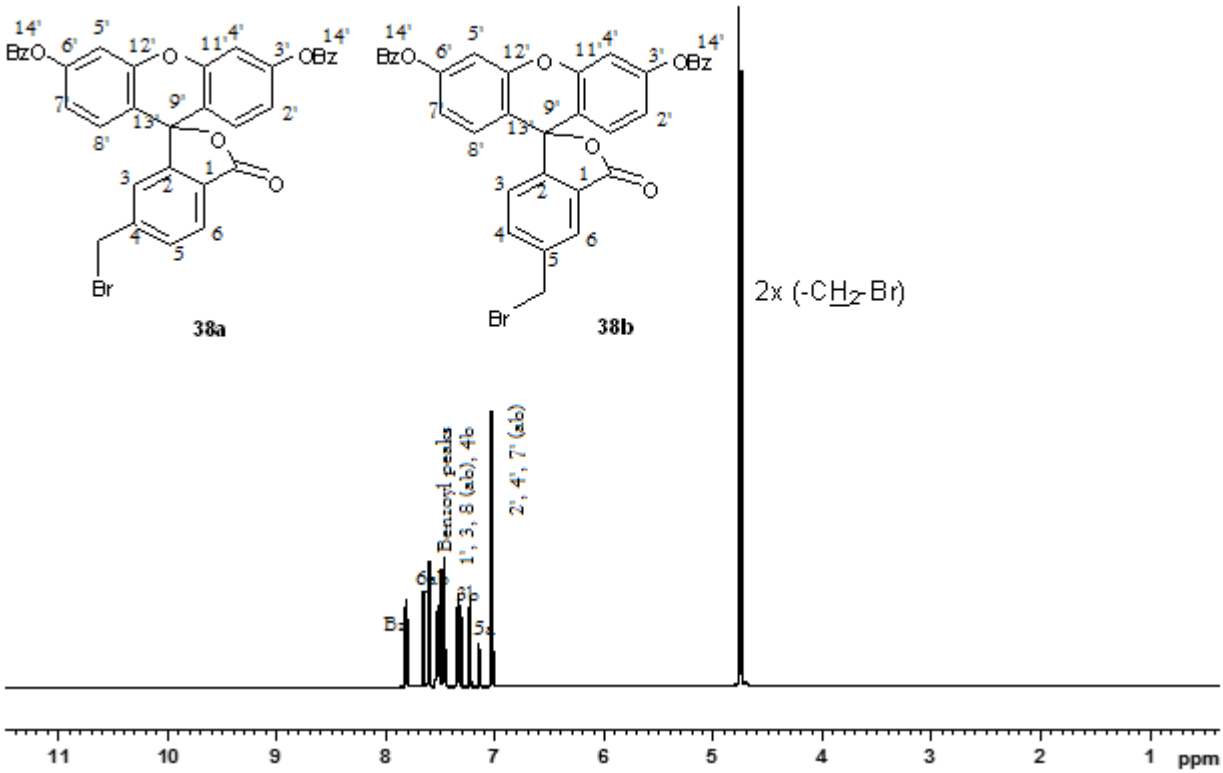
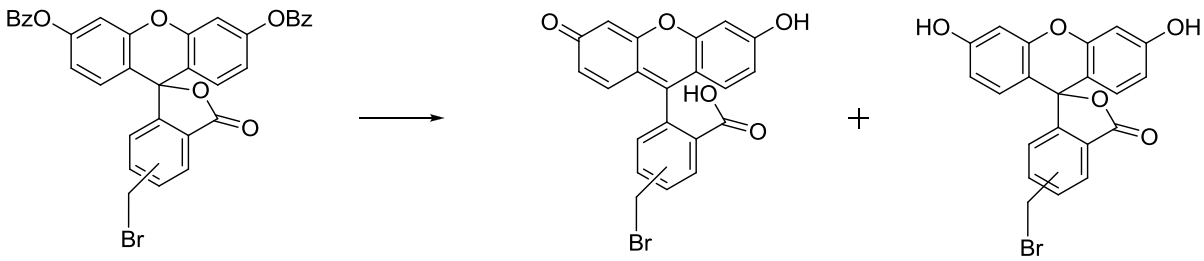


Figure 3.5: **The ^{1}H NMR spectrum of the product 38ab (300 MHz, $CDCl_3$)**

Holletz and co-workers did not report the removal of the benzoate protecting groups before their labeling procedure. Based on our previous experience described in the second chapter we chose to remove the benzoate groups by hydrogenation as this method gave better final yield than the hydrolysis using the TBAH base. Hydrogenation of the product **38ab** in the presence of $Pd(OH)_2/C$ in ethyl acetate at room temperature gave 99% yield of a crude mixture of four isomers. Flash column chromatography gave a green-yellow solid 4(5)-(bromomethyl)-2-(6-hydroxy-3-oxo-*3H*-xanthen-9-yl)benzoic acid **(39Aab)** containing a free carboxylic acid functional group, as the major product (90%) and the 4(5)-(bromomethyl)-3',6'-dihydroxy-*3H*-spiro[isobenzofuran-1,9'-xanthen]-3-one (**39Bab**) as a minor product in 10% yield (Scheme 3.10). Each of these products occurred as a mixture of two isomers with the bromomethyl groups in *para*- and *meta*-positions respectively.



38ab **39Aab** **39Bab**

Scheme 3.10: **Synthesis of the 4(5)-BMF product 39Aab: Reaction conditions: $Pd(OH)_2/C$, H_2, EtOAc, rt, 45 minutes.**

The synthesis of this compound was one of the key aims of this project as described in the first chapter. The two isomeric products **39A** and **39B** were confirmed by the NMR analysis. The presence of the mixture was demonstrated by the presence of the two closer peaks at 4.33 ppm and 4.49 ppm (^{13}C NMR, 75 MHz, $CDCl_3$: 32.98 ppm for both signals) which integrated to two protons each. These two peaks correspond to the methylene groups attached to the bromine atom from each of the two isomers respectively. The presence of the acidic isomer was confirmed by the presence of the peak at 10.99 ppm. The ^{13}C NMR (75 MHz, $CDCl_3$) also confirmed this result by the presence of the peak at 186.23 ppm which corresponds to the ketone group on the acidic isomer. The peak at 165.4 ppm corresponds to the acid carbonyl carbon while the one at 168.8 ppm corresponds to the lactone carbonyl carbon. A comparison of the ^{1}H NMR spectrum of the 5-BMF obtained from Sigma Aldrich with that of our freshly synthesised, purified and characterised isomeric products **39Aab** showed that the "5-BMF" sample obtained from Sigma Aldrich was impure and contained little 5-BMF.

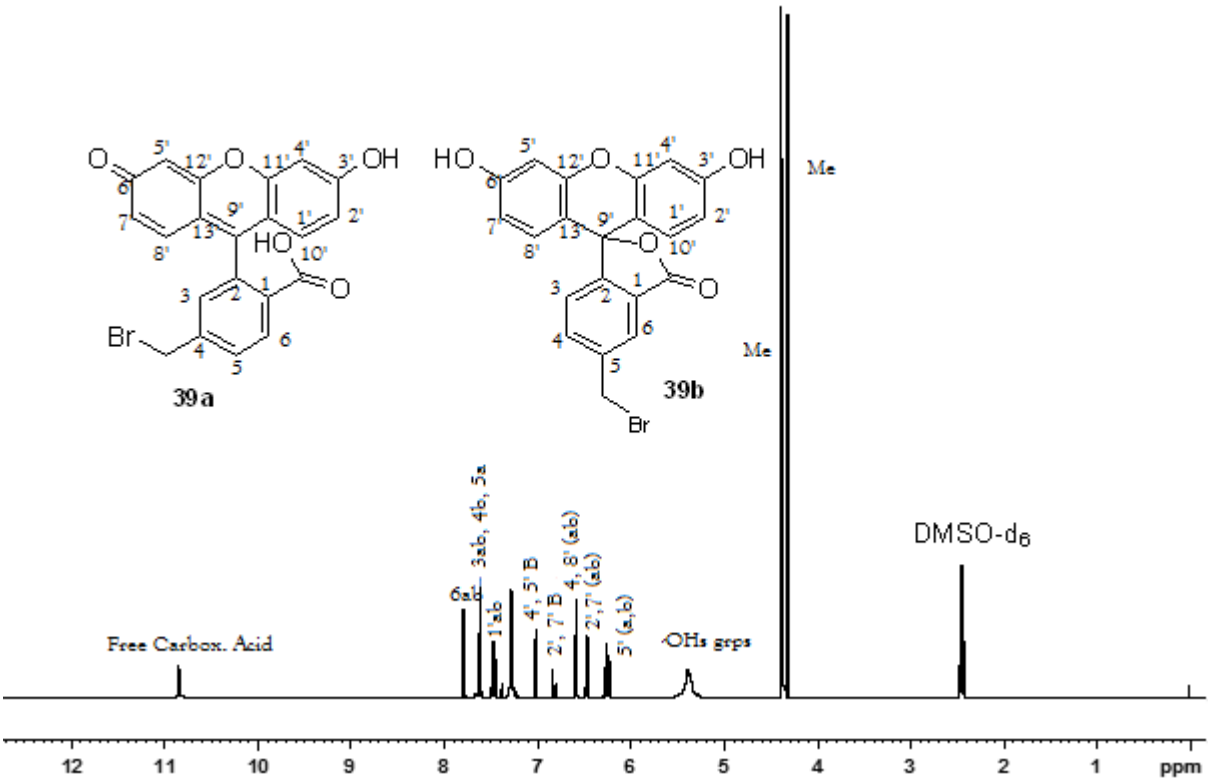


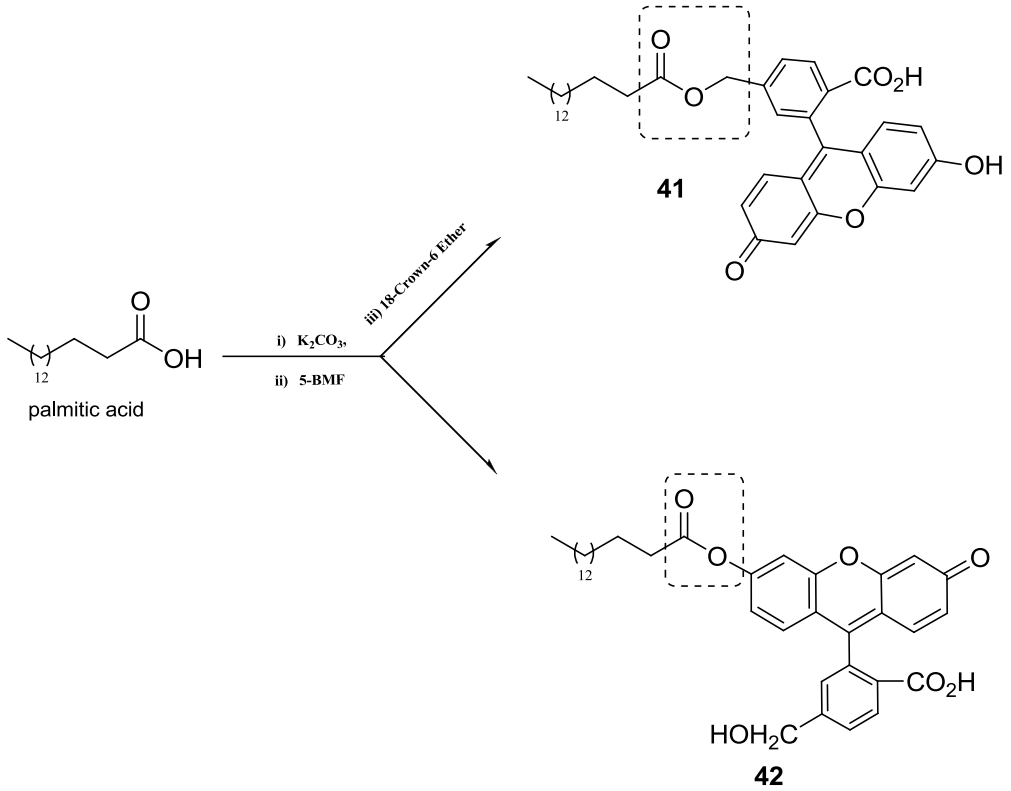
Figure 3. 6: **The ^{1}H NMR spectrum of the product 39ab (300 MHz, DMSO).**

- **Labelling of the different MAs**

For the labelling and/or derivatization process of our different acids, the method described by Toyoo`kaand co-workers (Scheme 3.12), (Toyoo`ka*et al*., 2002). Amazingly when we tried this method without the crown-ether catalyst, the product was obtained in just 1-3% lower yields than the catalytic method. Therefore the second method would be much more cost effective than combining the catalyst and the base in one reaction since at least one reagent can be avoided.

Labelling of corynomycolic acid **1** with the acid form of 4(5)-BMF (**39A**) gave quantitative yields (98%) of two pairs of products **44A** as the major product and **44B** as the minor product in 90:10 ratios. These acid and lactone forms could be easily separated by flash chromatography. No

other products were observed. In a paper published by Mukherjee and co-workers on the labelling of palmitic acid with "5-BMF" under these same reaction conditions, they observed the formation of two products in HPLC analysis (Mukherjee *et al.*, 1995). One product was attributed to the fluorescein palmitate ester in the acid form (**41**) and the other was proposed to be the palmitate ester linked via a fluorescein phenolic OH group (**42**) (Scheme 3.11). These researchers reported that if one of the peaks were isolated and re-injected in the absence of the catalyst, isomerization occurred and once again the same two peaks were observed. No attempt was made to characterize the product structures by NMR analysis (Mukherjee, *et al.*, 1995). Our results suggest that it is likely that they were observing the acid and lactone tautomers rather than a phenolic ester product. Having separated the acid and lactone forms, we did not observe any tautomerism on standing in DMSO-d_6 in an NMR tube for a few weeks, stored in the freezer for months and on exposure to sunlight

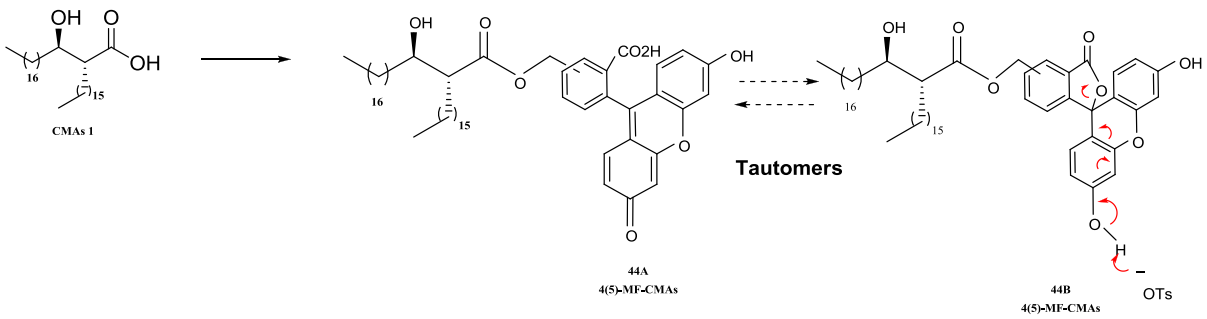


Scheme 3.11: **The proposed fluorescein derivatives of palmitic acid** (Mukherjee*et al.*, 1995).

To avoid any side reaction or any by-product formation as proposed by Mukherjee and co-workers (Mukherjee*et al.*, 1995) and maximise therefore the yield of our desired products or

TM, it was suggested that we label MAs with the protected dye **38ab** and subsequently remove the benzoate groups.

The product **38ab** was successfully attached onto our freshly synthesized *anti*-corynomycolic acids **1 to** give the solid product **40** in 99.8% yield and the commercially available natural mycobacterial-MAs mixture to give the solid product **43** in 94% yield by simple esterification in the presence potassium carbonate (K_2CO_3) as the base, refluxing in a mixture of dioxane: DMF as the solvent system. About 1.01 molar equivalents of the fluorescein were used per equivalent of the respective acids to avoid excess of the unreacted fluorescein and/or the acids after the esterification reaction which would need extensive removal steps. The same procedure as developed by **Toyoo**`ka **and co-workers (2002)** was used for the whole labelling process (Scheme 3.12).



Scheme 3.12: **Thegeneral derivatisation procedure of MAs (CMAs 1) adapted from Toyoo`ka, *et al* (2002): K_2CO_3, 4(5)-BMF, DMF: 1,4-Dioxane, Reflux, 24 h.**

The formation of the ester **40** was confirmed by the presence of the singlet at 5.32 ppm (^{13}C NMR; 75 MHz; DMSO-d_6: 67.3 ppm) which integrates for two protons. When looking closer, two peaks are observed at the same position due to the *para*- and *meta*-positions of the $–CH_2Br$ group on the fluorescein dye. The multiplet between 6.88 ppm and 8.30 ppm correspond to phenyl groups from the protected fluorescein dye. The other signals have been discussed previously. The disappearance of the signal between 3.00 ppm and 3.50 ppm is due to the fact that all the OH groups are protected. The only doublet of triplets at 3.61 ppm and 2.39 ppm both integrating for 1H correspond to the β- and α-proton respectively. The peak at 2.50 ppm corresponds to the DMSO solvent. The triplet at 0.87 ppm, which integrates to 6H corresponds to the two terminal methyl groups of the CMAs.

The benzoate groups were removed by hydrogenation using the same method developed for the hydrogenation of protected 4(5)-BMF **38** to **39**. Hydrogenation gave the solid yellowish CMAs-4(5)-MF **44** in 98% yield and solid mycobacterial-MAs-4(5)-MF mixture product mixture **45** in 95% yield after flash chromatography. The total 98% yield of the product **44** contained about 99.9% of the acid form (**44Aab**) and some lactone form (**44Bab**) in about 0.1% that was separated by the column chromatography eluting in acetonitrile:methanol:hexane (3:1:1). No further separation attempt on the mycobacterial-MAs **45** was made since it was a complex mixture of all three MA subclasses.

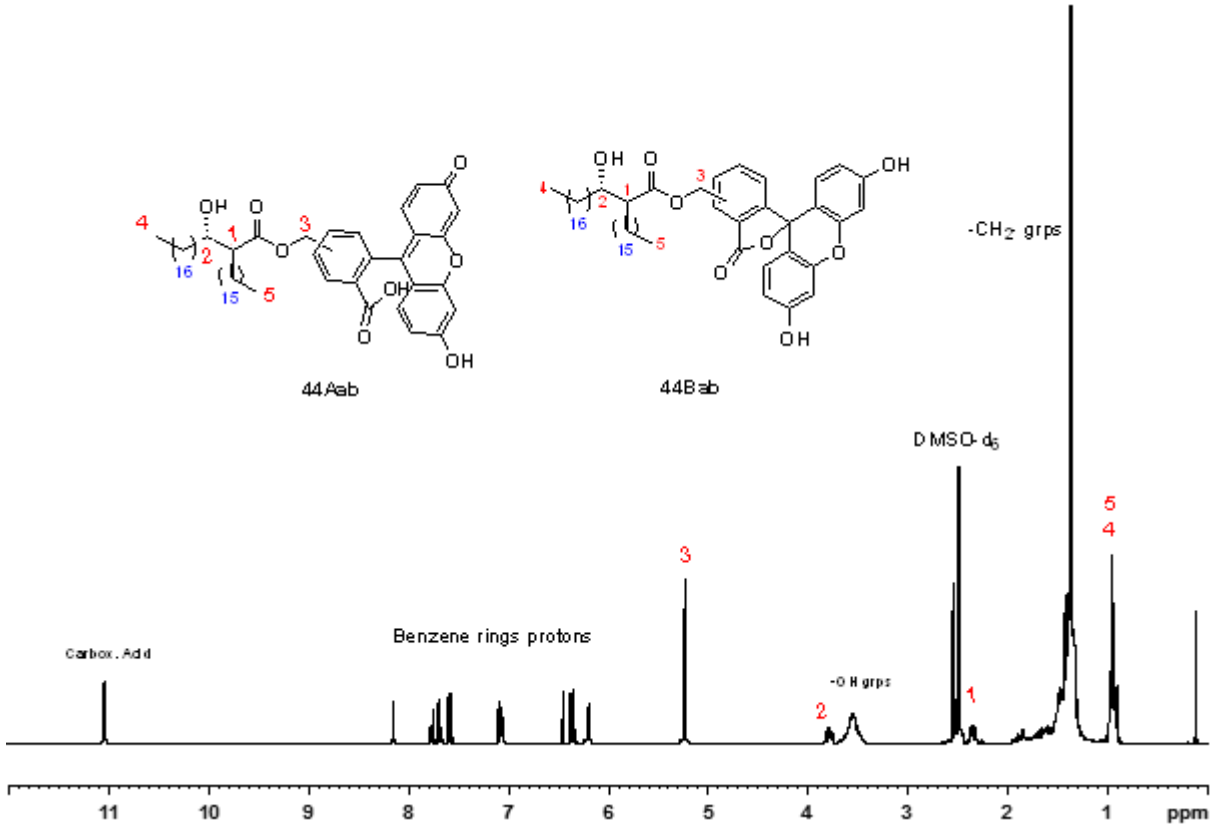


Figure 3. 7: The ^{1}H NMR spectra (300MHz, DMSO-d_6) analysis of the fluorescently labelled-CMAs 44.

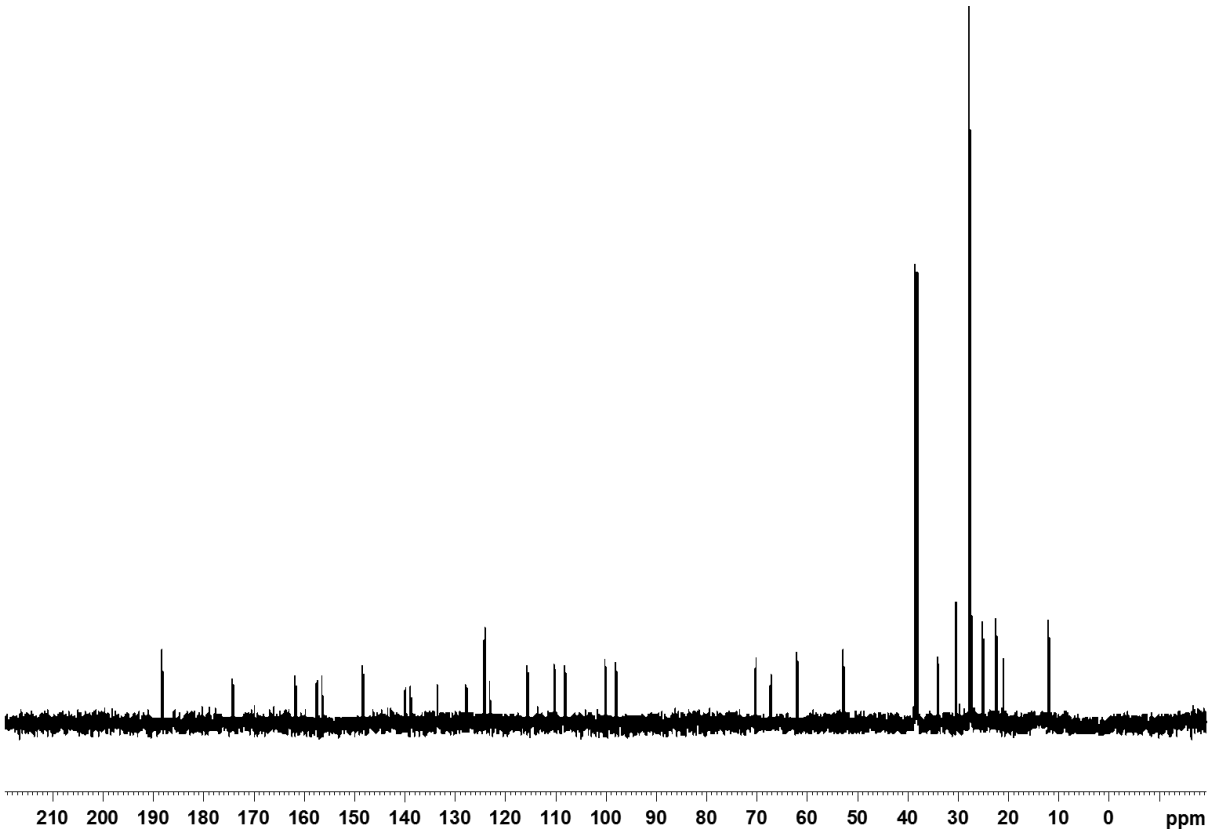


Figure 3. 8: The ^{13}C NMR Spectra (75 MHz, DMSO-d_6) analysis of the fluorescently labelled-CMAs 44.

The formation of the mycolic acid methyl fluorescein esters **44Aab** (CMAs-4(5)-MF) (Scheme 3.12) and **45** (Mycobacterial-MAs-4(5)-MF) was confirmed by NMR analysis as shown in the figures 3.7, 3.8, 3.9 and 3.10 respectively. As expected the products were collected as a mixture of the *para*- and *meta*-structural isomers, thus the use of "4(5)-MF" nomenclature. The NMR spectra of the products **44A** and **44B** clearly indicated that the dominant tautomer was the acid tautomer even though these products were obtained by hydrogenation of the lactone tautomer. A mechanism for the formation of tautomers is given in Scheme 3.3.

The formation of product **44Aab** was confirmed by the appearance of the signal 11.01 ppm which is associated with the free carboxylic acid on the fluorescein dye. The peak at 3.50 ppm corresponds to the phenol group on the dye. The singlet at 6.15 ppm integrating for 1H corresponds to the methine group adjacent to the carbonyl carbon. The doublet at 6.36 ppm integrating for 1H corresponds to the other methine group also adjacent to the same carbonyl carbon. This proton couples with the other singlet proton whose signal is observed at 7.60 ppm. The singlet integrating to 2H at 5.21 ppm corresponds to the methylene group adjacent to the ester group. All the other signals have been discussed in detail previously.

In the same manner, the labelling of the naturally occurring mycobacterial-MA was confirmed by the ^{1}H and ^{13}C NMR spectra analysis (Figure 3.9 and 3.10). The formation of the ester **45** was confirmed by the presence of a singlet integrating to 2H at 5.14 ppm (^{13}C NMR (75 MHz, DMSO-d_6): 67.96 ppm). The peak at 11.01 ppm is associated with the free carboxylic acid group on the labelled material. The doublet of triplets at 2.29 and at 3.55 ppm which both integrate to 1H correspond to α-and β-protons of the MAs. The singlet at 3.28 ppm integrating for 3H adjacent to the –OH group at 3.10 ppm is related to the methoxy-group of the MA mixture. The multiplet between 0.15 and 0.49 ppm integrating to 8H are most likely associated with the two possible cyclopropane rings in the mero-chain (^{13}C NMR, 75 MHz, DMSO-d_6: 33.9-36.8 ppm). Other peaks are similar to the spectrum of the product **44** as discussed above.

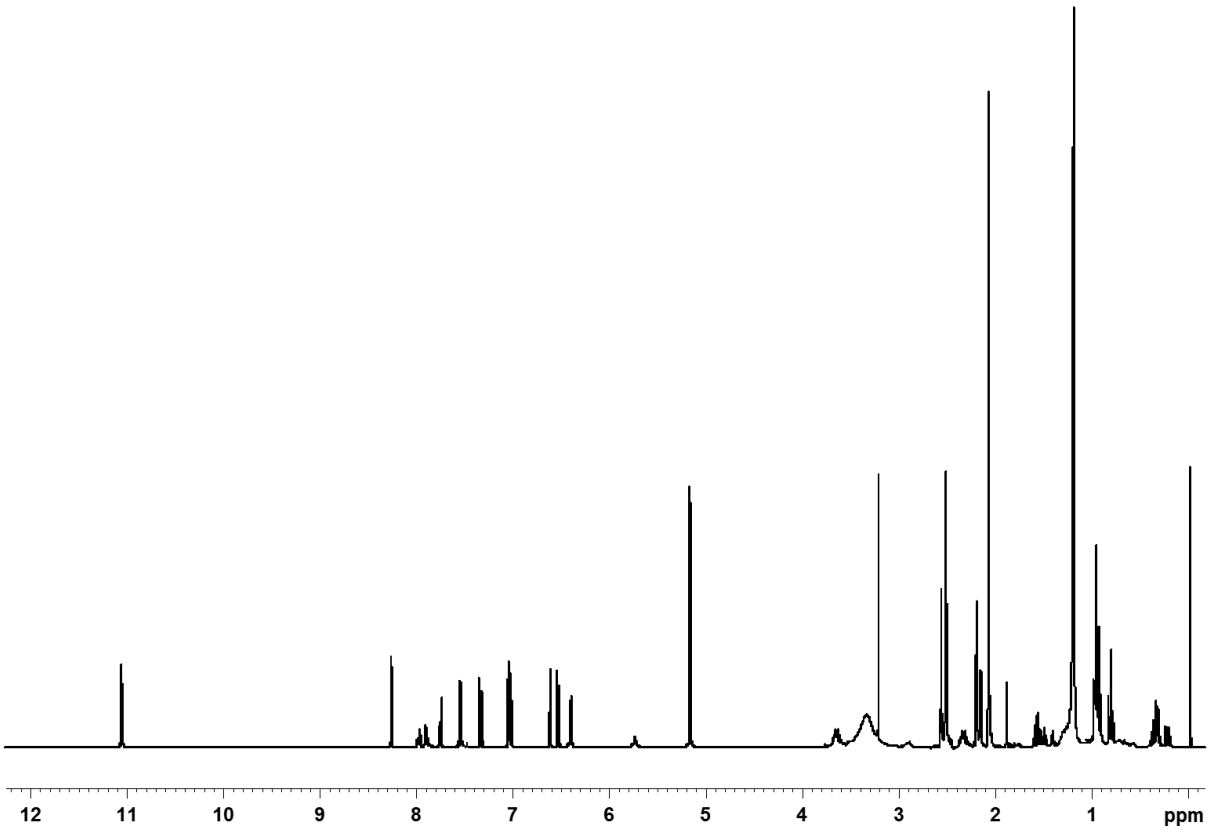


Figure 3. 9: The ^{1}H NMR spectrum of the mycobacterial-MAs-MF 45

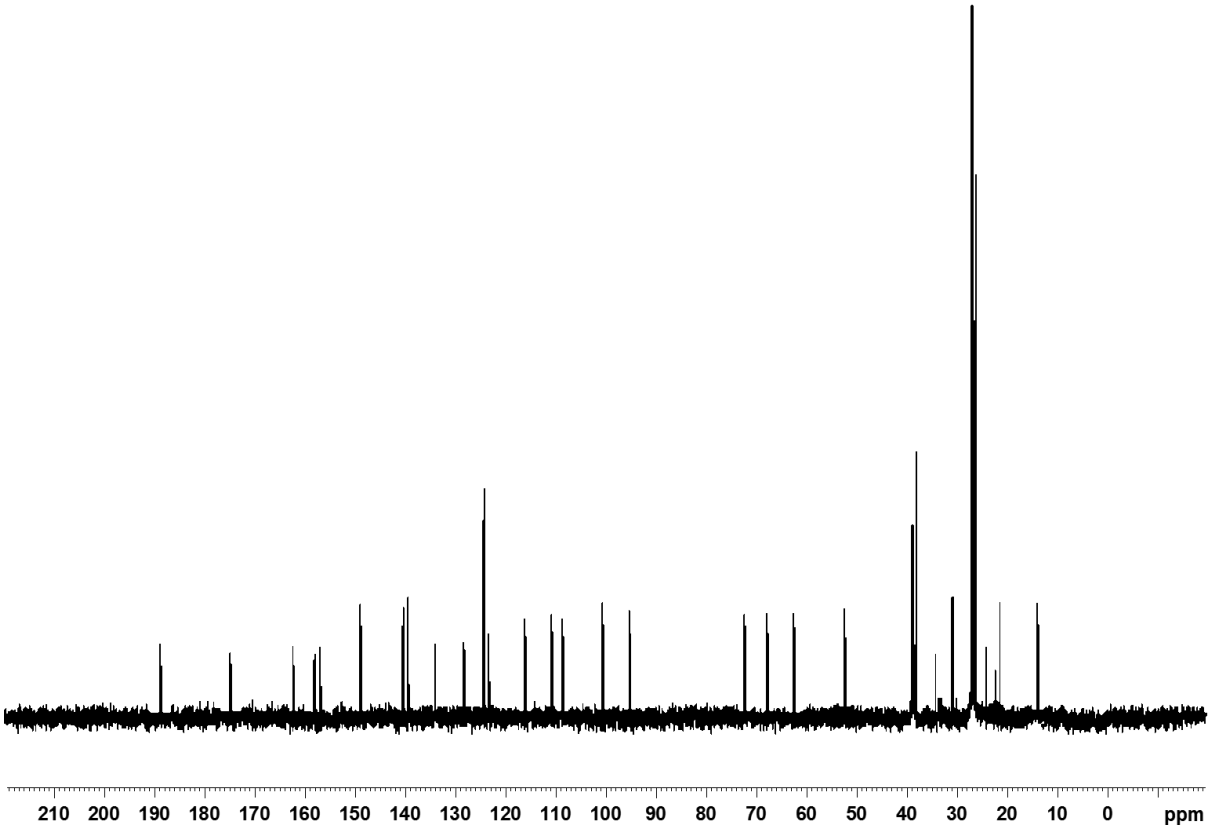


Figure 3. 10: **The ^{13}C NMR Spectrum (75 MHz, DMSO-d_6) for the Mycobacterial-MAs 45.**

3.4. Conclusion

The aim of this chapter was to label mycolic acids with fluorescein and determine the structure of the labelled compounds using NMR analysis. This was achieved firstly by synthesis and purification of the 4(5)-BMF according to the literature (Wolf, 1992; Tortoli, 2002; Tortoli, 1996; Crawford, 1994). This product was prepared in the acid tautomeric form as a mixture of 4(5)-bromomethyl structural isomers **39Aab** in 78% yield over 4 steps. Both CMAs and Mycobacterial-MAs were labelled with **39Aab** by the method developed by Toyoo`ka and co-workers and with **38ab,** which had both phenol groups protected. The latter product was then hydrogenated to give the same product in two steps with comparable yield (Toyoo`ka *et al*., 2002). Both methods only gave one major product – the acid tautomer. All the newly prepared materials such as the 4(5)-BMF **39Aab**, CMAs-4(5)-MF **44Aab** and the natural mycobacterial-MAs-4(5)-MF **45** were purified by flash column chromatography. Their structures were characterized and/or confirmed by NMR analysis. There was no evidence of any esterification of

the phenolic OH's of the fluorescein and there was little evidence for the formation of the lactone tautomer. This means that Mycobacterial-MAs-4(5)-MF material could maintain its biological properties by providing a free carboxylic acid close the ester bond, in order to maintain a functional conformation by internal hydrogen bonding as hypothesized by Lemmer *et al*. (2009). This illustrated the importance of understanding how pH dependent tautomerism affects the labelling process and the analysis of the labelled materials.

This chapter demonstrated that the fluorescein labelled mycolic acids favour the acid tautomer. This supports the hypothesis of Lemmer that replacement of the acid functional group of MA with another could prevent loss of biological activity by esterification (Lemmer *et al*. 2009).

Chapter 4: Conclusion and Future Work

Mycolic acids (MAs) have been shown to play an important role in the virulence of *Mycobacterium tuberculosis* (*M. tb.*). In host-pathogen interactions, they have been found to re-programme murine macrophages and to modulate their inflammatory activity (Korf *et al*., 2005, Korf *et al*., 2006). Antibodies to MAs in patient serum can be used as surrogate markers of active TB via a biosensor technique (Schleicher *et al*., 2002, Lemmer *et al*., 2009). Using this method and ELISA analysis the cross-reactivity of *anti*-MA antibodies with cholesterol was discovered. This may have strong implications for how the latent TB bacilli can feed off cholesterol for months on end (Verschoor *et al.,* 2012).

Considerable structural variation exists within the *M. tb.*-MA pool. Seppe *et. al.* have studied the effect of the oxygenation level and proximal cyclopropane configuration on anti-inflamatory activity (Seppe *et al*., 2011). The importance of MA in TB warrants structure function relationship studies to learn how subtle structural aspects, such as functional groups, chain lengths, and glycosylations, in MA may influence biological activity. This can be achieved by stereo-controlled chemical synthesis of MA types, the first of which was reported in 2005 (Al Dulayymi *et al*., 2005). Since then, a large number of different MA types have been generated by the University of Bangor (eg. Al Dulayymi *et al*., 2005; Al Dulayymi *et al*., 2006a; Al Dulayymi *et al*., 2006b, 2007). An important step forward now would be the economical production of such types of MA on a larger scale. One approach may be to investigate the possibility of using the *anti*-aldol methodology to insert both stereocentres and to couple full length *α*- and mero chains to get a pure *anti*-diastereomer of the *α*-MA motif with desired *(R,R)*-configuration in high yield with fewer steps (Driver *et al.*, 2010; Kurosu, 2001). This may be achieved by using a chiral ester which can be predicted to yield a the (*R,R*)-diastereomer enantioselectively as well as the *syn*-products that may readily be separated by flash column chromatography (Driver *et al.*, 2010; Kurosu, 2001).

The fluorescein dye is probably the most common fluorescent probe today. Its very high molar absorptivity at the wavelength of the argon laser (488 nm), large fluorescence quantum yield and high photostability makes it a very useful and sensitive fluorescent label. The emission spectrum of fluorescein overlaps extensively with the absorption spectrum of tetramethyl rhodamine, which is a related strongly fluorescent dye, making this pair very suitable for energy

transfer experiments to determine distances within and between labeled macromolecules (Murchie *et al*., 1989).

Fluorescein in aqueous solution occurs in cationic, neutral, anionic and dianionic forms (Zanker, 1958) making its absorption and fluorescence properties strongly pH dependent. The protolytic constants relating the concentrations of the protolytic forms have been difficult to determine, because their spectra overlap substantially and the different pK_a values are quite close. With the recent advent of powerful chemometric methods for spectral analysis, complicated spectroscopic mixtures can today be analyzed with confidence (Kubista *et al*., 1993).

The pathway of MA processing in a cell entails the acidification of vesicles, which favours the acidic form of the labelled MA-MF as it progresses through the cell. Our findings reported in Chapter 3 concluded that the protonated free carboxylic acid form is prevalent under neutral conditions. This corroborated the observation made by Korf *et al*. (2005), who showed that the fluorescence of the labelled MA was not quenched in the acidified phagosomes. The structural characterization of the fluorescein labelled MA that identified the acidic form as the dominant tautomer supports the assertion made by Lemmer that the carboxylic acid moiety enables the labelled MA to maintain biological function (Lemmer *et. al.* 2009).

The chemistry of quantitative labelling of MA developed in this study may be used in future to detect and quantify MA from a mixture of organic acids in cell wall extracts of different mycobacterial species. This could possibly increase the determination of purity of MA isolated from extracts, which, in the past, proved challenging (Goodrum *et al*., 2002). It will also be possible to use fluorescently labelled MAs in conjuction with molecular beacon technology. Detection of the labelled MAs in cells will confirm their presence in the cell and link the molecular beacon cytokine profile to MA activation. This has been made possible after the fulfilment of the aim of this project which was to label MA with fluorescein with full structural characterisation of the conjugate.

Chapter 5: Experimental section

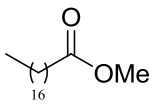
5.1. Introduction

All chemicals reagents were obtained from Sigma Aldrich, Ltd, or Merk. Glasswares were dried overnight in an oven at 120 °C. Some of the solvents used in this project were dried and/or distilled under nitrogen gas before use. THF was always dried over Na/benzophenone and distilled freshly under nitrogen prior to use. To visualise the TLC plates (Kieselgel 60 F254 from Merk), a solution of phosphomolybdic acid (12 g) in ethanol (250 ml) was used. Flash column chromatography was done under air pressure using silica gel 60 from Merk (0.040-0.063 mm). Eluents mixtures are quoted as volume: volume ratios. ^{1}H, ^{13}C NMR spectra were recorded on Bruker 300 MHz spectrometers in $CDCl_3$ and DMSO-d_6. All the chemical shifts were recorded relative to the resonance of the residual protons in the $CDCl_3$ (7.25 ppm) or DMSO-d_6 (2.50 ppm). IR spectra were recorded on a Perking. Organic solvents were dried over anhydrous magnesium sulphate. Reactions that required heating were carried out using an oil bath while cooling to -78 °C was achieved by using acetone-dry ice cold baths.

5.2. Experiments for Chapter 2

5.2.1. Preparation of CMA 1 via the Claisen Route

Synthesis of Methyl stearate 26

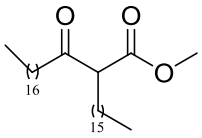


The Stearic acid (10.1 g; 0.037 moles; 1 eq.) was dissolved in methanol (100 ml) in a 250 mL round bottomed flask equipped with a refluxing condenser and a magnetic stirrer bar, under nitrogen gas. Thionyl chloride (5.40 ml, 0.074 moles, 2 eq) was added drop wise and themixture was allowed to reflux for 2h. After that, it was allowed to cool to room temperature for 45

minutes. Aqueous $NaHCO_3$ (10 ml) and ethyl acetate (20 ml) were added to the reaction mixture and it was stirred for 30min. The product was extracted with ethyl acetate 3 times and the organic layers were combined, dried over $MgSO_4$ and concentrated to yield an off-white solid which was purified by flash chromatography (4:1 hexane: ethyl acetate) to yield the methyl stearate **26** as an off-white solid (10.9691 g, 99%): **Molecular formula: $C_{19}H_{38}O_2$; Mw: 298.50 g/mol**, **R_f: 0.62** (4:1 hexane: ethyl acetate); **^{1}H NMR: δ_H (300 MHz, $CDCl_3$)**: 3.63 (3H, s, -$CO_2C\underline{H}_3$), 2.34 (2H, t, *J*= 7.4, -$C\underline{H}_2$-CO_2Me), 1.55 (2H, tt, *J*=7.7, J=7.4), 1.25 (2H, tt, *J*=7.7, *J*=7.0), 1.23-1.27 (m, 26H, -$C\underline{H}_2$-), 0.88 ppm (3H, t, *J* 7.0, $C\underline{H}_3$-CH_2-).**^{13}C NMR (75 MHz, $CDCl_3$)**: 14.1, 22.7-31.9, 33.6, 51.9 173.1 (**C=O**).

Synthesis of the methyl 2-hexadecyl-3-oxoicosanoate 27

(a) with NaH as the base



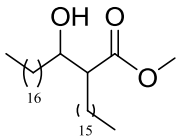
Ester **26** (0.100 g; 0.335 mmol; 2 eq.) was dissolved in freshly distilled dried THF (5 ml) in a 20 mL round bottomed flask equipped with a magnetic stirrer bar. NaH (0.010 g; 0.435 mmol, 1.3 eq) from NaH 60% dispersion (0.017 g) washed in dried hexane was added and the mixture was allowed to stir at 65 °C for 5h. The reaction was neutralized with acetic acid, and the product extracted 3 times with ethyl acetate. The product was purified by flash column chromatography eluting in 4:1 hexane: ethyl acetate, the solvent was evaporated to yield keto ester **27** (0.059 g, 63%) as an off-white solid. **Molecular formula: $C_{37}H_{72}O_3$; Mw: 564.96 g/mol, R_f: 0.64** (4:1 hexane: ethyl acetate); **^{1}H NMR: δ_H (300 MHz, $CDCl_3$)**: 3.41 (1H, t, *J*= 7.3,-C(O)-C$\underline{H}$(CH_2-)-CO_2-Me), 3.68 (3H, s, -$CO_2C\underline{H}_3$), 1.73 (2H, td, *J*=7.6, J=7.3), 2.46 (2H, t, J=7.4), 1.28 (2H, tt, *J*=7.5, *J*=7.1),), 1.22-1.27 (m, 56H, 28x-$C\underline{H}_2$-), 0.79 (6H, t, *J*= 7.0, 2x$C\underline{H}_3$-CH_2-). **^{13}C NMR (75 MHz, $CDCl_3$)**: 14.3, 20.3, 25-3, 51.9, 58.9, 170.8 and 206.1.

(b) with LDA as the base

Diisopropylamine (4 ml) and n-BuLi (16 ml, 1.6 M in hexane) were added to freshly distilled, dry THF (25 ml) at -78 °C under nitrogen. The reaction mixture was stirred for 15 min and warmed to room temperature with stirring for 45 min. Then the methyl stearate **26** (1.90 g, 6.4 mmol, 2 eq) was dissolved in THF (10ml) and then added to the reaction mixture slowly over a period of 1 h. The reaction mixture was allowed to stir for 2 h until the reaction was completed. It was then quenched with HCl aqueous (10ml, 1M). The product was extracted 3 times with DCM; the combined organic layers were dried over $MgSO_4$ and concentrated. Purification by flash chromatography gave solid product **27** (1.735 g, 96%).

Synthesis of methyl 2-hexadecyl-3-hydroxyicosanoate 28

(a**) with $LiBH_4$**

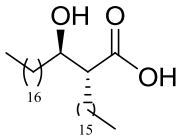


Keto ester **27** (0.75 g, 132 x 10^{-5} mol; 1 eq.), and $LiBH_4$ (0.043 g; 199 x 10^{-5} mol; 1.5 eq) were dissolved in freshly distilled THF (25ml) and the mixture was refluxed for almost 40 minutes. Methanol (6 ml) was added drop-wise and the mixture was refluxed for an extra 2 h at 60 °C under N_2 gas. The reaction mixture was quenched with HCl aqueous (8 ml, 2M) stirred for 1 h, then diluted with water (25 ml) and saturated aqueous $NaHCO_3$ (10 ml) while cooling to room temperature. The organic layer was washed 3 times with diethyl ether, combined, concentrated and dried over $MgSO_4$ to afford product **28** (0.739 g, 98,8%) after flash chromatography (4:1; hexane: EtOAc). The *anti*-diastereomer was separated from the *syn*-diastereomer by repeated column chromatography (4:1; hexane: EtOAc) to give racemic *anti*-**28** in 50% yield (0.369 g) and *syn*-**28** in 50% (0.369 g) **Molecular formula: $C_{37}H_{74}O_3$; Mw: 566.98 g/mol, R_f: 0.64** (4:1; hexane: EtOAc); **^{1}H NMR: δ_H (300 MHz, $CDCl_3$)**: 3.84 (1H, dt, *J* = 7.41, *J* = 7.50, -C<u>H</u>(OH)-CH-), 3.62 (3H, s, -O-C<u>H</u>$_3$), 2.39 (1H, dt, *J*= 3.4 , *J*=7.5, -CH(OH)-C<u>H</u>-), 1.72 (2H, td, *J*=7.5, J=7.5), 1.54 (2H, td, *J*=7.3, *J*=6.2), 1.28 (2H, tt, *J*=7.5, *J*=7.1), 1.37 (2H, tt, *J*=7.3, *J*=7.1), 1.25 (2H, tt, *J*=7.1, *J*=6.5), 1.3 (2H, tt, *J*=7.1, *J*=6.6), 1.22 (2H, tt, *J*=6.5, J=6.5), 1.20 (2H, tt, *J*=6.6, J=6.5), 1.23-1.28 (m, 46, -C<u>H</u>$_2$-), 0.79 ppm (6H, t, *J*=7.0, 2x C<u>H</u>$_3$-CH$_2$-). **^{13}C NMR (75 MHz, $CDCl_3$)**: 14.1, 22.4-35.0, 50.8, 52.4, 71.5, 174.6.

(b) With $NaBH_4$

The same procedure reported above was followed except that $NaBH_4$ (0.103 g, 297 x 10^{-5} mol, 1.5 eq) and product **27** (1.120 g, 198 x 10^{-5} mol, 1 eq.) were used. Product **28** was obtained in 81% yield (0.91 g) this afforded 56 % (0.51 g) *anti*-product and 44 % (0.40 g) *syn*-product after flash chromatography eluting from 4:1; hexane: EtOAc.

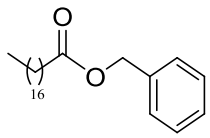
Synthesis of 2-hexadecyl-3-hydroxyicosanoic acid (CMAs) 1



Anti-methyl ester **28** (1.50 g, 2.65 mmol, 1 eq) was dissolved in dichloromethane (DCM) (5 ml). Tetrabutylammonium hydroxide aqueous (3 ml, 20% aq.; 11.39 mmol, 4.3 eq) was added to the reaction mixture. The reaction mixture was allowed to reflux for 20h at 97 °C and then cooled to room temperature. The reaction was quenched with HCl aqueous (1ml, 1M) and diethyl ether (10 ml) and the product were extracted with diethyl ether followed by hexane (3x30ml). The organic layers were combined, concentrated, dried over $MgSO_4$ and the crude product was purified via flash chromatography (hexane: ethyl acetate 9:1 then chloroform: methanol 9:1) to yield the final product **1** (1.4g, 96%). **Molecular formula: $C_{36}H_{72}O_3$; Mw: 552.95 g/mol**; **R_f: 0.31** (hexane: ethyl acetate 9:1 then chloroform: methanol 9:1), **^{1}H NMR: δ_H (300 MHz, $CDCl_3$)**: 2.39 ppm (1H, td, *J*=7.3, *J*=4.2, -CH(OH)-C<u>H</u>-), 3.68 ppm (1H, td, *J*=6.2, *J*=4.2, -C<u>H</u>(OH)-CH-), 1.52 (2H, td, *J*=7.5, *J*=7.3, -CH_2-C<u>H_2</u>-CH(OH)-), 1.53 (2H, td, *J*=7.3, *J*=6.1, -CH_2-C<u>H_2</u>-CH-), 1.28 (2H, tt, J=6.5), 1.23-1.27 (m, 42H, -C<u>H_2</u>-), 0.79 (6H, t, *J*=7.0, C<u>H_3</u>-CH_2-).**^{13}C NMR (75 MHz, $CDCl_3$)**: 14.2 (**<u>C</u>H_3-C-**), 21.9 (**CH_3-<u>C</u>H_2-**), 26.3 (**-C(OH)-CH(<u>C</u>H_2-)-CO_2H**), 28.2, 28.6, 31.1, 32.4, 36.8 (**-CH_2-<u>C</u>H_2-C(OH)-**), 54.2 (**α-C**), 71.4 (**β-C**), and 176.5 (**-<u>C</u>O_2-H**).

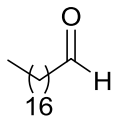
5.2.2. Preparation of the CMA 1 via the Aldol Route

Synthesis of the Benzyl stearate 29



To a solution of stearic acid (5.00 g, 0.017 moles, 1 eq.) and benzyl bromide (2.3 ml, 3.31 g, 0.019 mol, 1.1 eq.) dissolved in 30 ml DMF/1,4-Dioxane (1:1), was added $NaHCO_3$ (2.067g, 0.024 moles, 1.4 eq.) at room temperature. The reaction mixture was then refluxed with stirring at 90 °C for 24 h. The reaction was allowed to cool to room temperature and diluted with EtOAc and washed with saturated aqueous NaCl solution and water. The organic layer was collected, combined, concentrated, dried over $MgSO_4$ and the solvent evaporated under vacuum to produce crude product that was purified by flash chromatography (hexane: EtOAc 4:1) to yield the benzyl carboxylate **29** (6.53 g, 99%) as an off-white solid. **Molecular formula: $C_{25}H_{42}O_2$; Mw: 374.59 g/mol; R_f: 0.71** (hexane: EtOAc 4:1) ; **^{1}H NMR: δ_H (300 MHz, $CDCl_3$)**: 5.20 ppm (2H, s, -O-C$\underline{H}_2$-Ph), 2.24 (2H, t, *J*=7.4, -C$\underline{H}_2$-CO_2-), 1.52 (2H, tt, *J*=7.6, *J*=7.4,), 7.29 (1H, m, -C$\underline{H}$-), 7.38 (1H, m, , -C$\underline{H}$-), 7.47 (1H, m, -C$\underline{H}$-) 7.35 (1H, m, -C$\underline{H}$-), 7.33 (1H, tt, *J*=7.7, *J*=1.6), 1.23-1.64 (m, 32H, 16x -C$\underline{H}_2$-), 0.88 (3H, t, *J*=7.0, C$\underline{H}_3$-CH_2-). **^{13}C NMR (75 MHz, $CDCl_3$)**: 14.1, 21.4, 26.0, 28.8-32.7, 34.2, 67.9, 126.5, 128.2, 128.6, 129.9, 138.0, and 174.6.

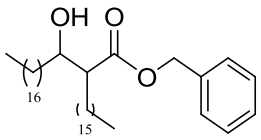
Synthesis of the stearaldehyde 30



1-octadecanol ($C_{18}H_{37}OH$, 10.00g, 0.0369 mol, 1 eq) was dissolved in dichloromethane DCM (150 ml). Pyridinium chlorochromate (PCC) (9.562g, 0.044 mol, 1.2 eq) was added and the reaction mixture was allowed to stir for 2 h at 35 °C. The reaction mixture was quenched with 50 ml hexane: ethyl acetate (8:2) and stirred for an extra 20min. The reaction mixture was filtered through a bed of celite and the solvent evaporated. The product was purified by a flash column chromatography eluting in diethyl ether: hexane: ether 1:1:8 to yield a white solid **30** (9.807 g, 99%). **Molecular formula: $C_{18}H_{36}O$; Mw: 268.47 g/mol; R_f: 0.55** (Diethyl ether: hexane: ether 1:1:8) ; **^{1}H NMR: δ_H (300 MHz, $CDCl_3$)**: 9.75 (1H, t, J=6.9, -C$\underline{H}$(O)-), 2.50 (2H, td, *J*=7.4, *J*=6.90, -CH_2-C$\underline{H}_2$-C(O)H), 1.51 (2H, tt, *J*=7.6, *J*=7.5), 1.28 (2H, tt, *J*=7.6, *J*=7.0), 1.25- 1.30 (m, 26H, -C$\underline{H}_2$-

)(3H, t, *J*=7.0, C<u>H</u>$_3$-CH$_2$-); **^{13}C NMR (75 MHz, CDCl$_3$)**: 14.1 (<u>C</u>H$_3$-C-); 22.4 (CH$_3$-<u>C</u>-C-); 27.9-30.0; 32.0; 44.8 (<u>C</u>-CO) and 204.0 **(**-C-<u>C</u>=O).

Synthesis of benzyl 2-hexadecyl-3-hydroxyicosanoate 31



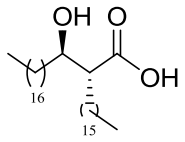
To distilled THF (50.00 ml) under N_2, at -78 $^{\circ}$C were added di-isopropylamine (4ml) and n-BuLi (16ml, 1.6 M in hexane) and the reaction mixture was allowed to stir at that temperature for 15 minumtes before being allowed to warm to ambient temperature; stirring was continued at that point for a further 45 min.

To a solution of **29** (5.00 g, 0.013 mol, 1 eq) dissolved in THF (15 ml) was added freshly prepared LDA (4.42 ml, 0.033 mol, 2 eq) slowly over a period of 1 h. The reaction mixture was allowed to stir for 2 h. Freshly prepared aldehyde **30** (4.49 g, 0.016 mol, 0.9 eq) dissolved in THF (5 ml) was also added to the reaction mixture over 1 h and the reaction was allowed to stir for another 2 h. The reaction was quenched with aqueous HCl (30 ml, 2M). The product extracted 3 times with DCM The organic layer was combined, dried over $MgSO_4$ and concentrated and the solvent was evaporated to give a solid that was purified by column chromatography to afford the product **31** in 94% yield (8.059 g). **Molecular formula: $C_{43}H_{78}O_3$; Mw: 643.08 g/mol; R_f: 0.64** (Hexane: etOAc: diethyl ether 6:2:2; v/v/v); **^{1}H NMR: δ_H (300 MHz, CDCl$_3$)**: 2.39 (1H, td, *J*=7.5, *J*=4.2, α-H-), 3.76 (1H, td, *J*=6.2, *J*=4.2, β-H-), 5.15 (2H, s), 1.73 (2H, td, *J*=7.5, *J*=7.5**,** -C<u>H</u>$_2$-α-H-), 1.55 (2H, td, *J*=7.3, *J*=6.2, -C<u>H</u>$_2$-β-H-), 1.28 (2H, tt, *J*=7.5, *J*=7.1), 7.38-7.46 (4H, m, -C<u>H</u>-), 1.45 (2H, tt, *J*=7.3, *J*=7.1), 1.26 (4H, tt, *J*=7.1, *J*=6.5, 2x -C<u>H</u>$_2$-), , 1.26 (2H, tt, *J*=7.1, J=6.5), 1.25 (2H, tt, *J*=6.5, *J*=6.5, -C<u>H</u>$_2$-), 7.34 (1H, tdd, *J*=7.5, *J*=1.1, *J*=1.2), 1.23 (2H, tt, *J*=6.5, *J*=6.5), 1.23-1.24 (m, 45H, -C<u>H</u>$_2$-), 0.88 (6H, t, J=7.0,2 x C<u>H</u>$_3$-CH$_2$-). **^{13}C NMR** (75 MHz, $CDCl_3$): 14.1, 21.9-29.6, 30.0-36.1, 65.5, 72.1, 125.4-136.1 and 74.9.

Synthesis of methyl 2-hexadecyl-3-hydroxyicosanoate 28 via an Aldol reaction

The same experiment described above was repeated but this time using the methyl ester **26** (1.90 g; 6.37 mmol, 1 eq) and C_{18}-aldehyde **30** (1.709 g; 6.37 mmol; 1 eq) to give 97% of the product **28** (3.50 g, 0.617 mmol) after column chromatography (hexane: diethyl ether 8:2).

Synthesis 2-hexadecyl-3-hydroxyicosanoic acid 1



- ***TBAH-Hydrolysis***

This reaction was done first using TBAH in the same procedure as described above for the hydrolysis of methyl ester **29.** Benzyl ester **31** (4.028 g**;** 6.264 mmol; 1 eq) was refluxed and/or reacted with 20% aqueous TBAH (40 ml) to give CMA **1** in 66% (2.286 g) of final off-yellow pure product **1** after column chromatography eluting with hexane: EtOAc (4:1).

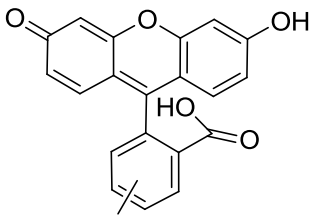
- ***Hydrogenation of the product 31 to give the CMAs 1***

To a degassed solution of benzyl ester **31** (3.90 g, 6.064 mmol, 1 eq) dissolved in ethyl acetate (50 ml), was added 0.85 mg (6.06 x10^{-5} mmol, 0.01 eq) of $Pd(OH)_2/C$ and the reaction mixture evacuated and flushed with H_2 gas several times using a balloon while stirring vigorous until TLC indicated a complete conversion to a single product. In our case, the reaction times were 48 minutes when 0.5 equivalents were used; 1h20 minutes when 0.1 equivalents of Pd/C were used and 2h50 minutes for this current one, using 0.01 equivalents of Pd/C catalyst. The suspension was filtered through a bed of silica and celite and washed with EtOAc. The product was concentrated under vacuum and purified on silica gel eluted with hexane: ethyl acetate; 8:2; v/v or Petroleum ether to yield CMA **1** (3.01 g, 90%) with the same values as described previously.

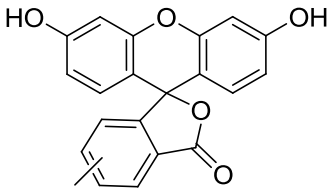
5.3. Experiments for Chapter 3

5.3.1. Synthesis of the fluorescein dye 39

Synthesis of 2-(6-hydroxy-3-oxo-*3H*-xanthen-9-yl)-4(5)-methylbenzoic acid 36A and 3', 6'-dihydroxy-4(5)-methyl-*3H*-spiro[isobenzofuran-1,9'-xanthen]-3-one 36B



36A

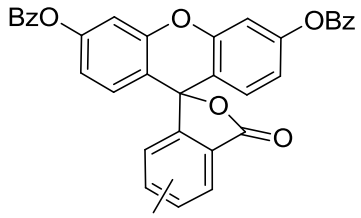


36B

4-Methyl phthalic anhydride **35** (2.00g, 0.012 mol, 1 eq) was fused at 180-200 °C with anhydrous zinc chloride (0.2 g, 0.172 mol, 0.14 eq) as the catalyst, and then resorcinol **33** (2.716 g, 0.024 mol, 2 eq) and a few drops of concentrated sulphuric acid was added and the mixture heated for 5 hours. The cooled melt was dissolved in aqueous sodium hydroxide (10 ml, 1M) and filtered. The solid dye was obtained by neutralizing the solution with diluted hydrochloric acid (1M). The solid was collected, washed with water, air-dried, then dried in vacuum at 60 °C, over P_4O_{10} to give 3',6'-dihydroxy-4-methyl-*3H*-spiro[isobenzofuran-1,9'-xanthen]-3-one in 90% yield (3.83 g) as an orange solid **36** containing a mixture of **36Aab** and **36Bab** which were separated by flash column chromatography eluting in acetonitrile:hexane:methanol (3:1:1) **Molecular formula: $C_{21}H_{14}O_5$; Mw: 346.33 g/mol; m.p. 225°C; R_f: 0.38** (acetonitril: hexane: methanol (3:1:1)). ^{1}H NMR: δ_H (300 MHz, DMSO-d_6): (**36Aa**): 10.80 (**-CO_2**), 5.28, (-OH), 2.33 (3H, s, Ph-C$\underline{H}_3$), 6.50 (1H, d, *J*=9.5,), 7.81 (1H, d, *J*=9.5), 6.50 (2H, dd, *J*=1.5, *J*=0.7), 7.91 (1H, dd, *J*=8.1, *J*=1.0), 7.713 (1H, dd, *J*=7.8, *J*=0.4), 6.40 (1H, dd, *J*=8.1, *J*=1.5), 7.00 (1H, dd, *J*=1.4, *J*=0.4), 7.43 (1H, dd, *J*=7.8, *J*=1.4). **(36Ab)**: 5.28 (-OH), 2.36 (3H, s, Ph-C$\underline{H}_3$), 6.50 (1H, d, *J*=9.5), 7.50 (1H, d, *J*=9.5), 6.50 (1H, dd, *J*=1.5, *J*=0.7), 7.39 (1H, dd, *J*=8.1, *J*=0.7), 7.12 (1H, dd, *J*=7.8, *J*=1.5), 6.20 (1H, dd, *J*=8.1, *J*=1.5), 7.12 (1H, dd, *J*=8.1, J=7.8), 7.10 (1H, dd, *J*=8.1, *J*=1.5). **^{13}C NMR** (75 MHz, DMSO-d_6): (**36Aab**): 20.7, 96.5, 98.9, 110.8, 118.6, 124.8, 128.4,131.0, 131.6, 132.2, 136.6, 137.3, 150.2, 168.8, 170.0, 186.5, 156.4, 155.3, 186.5, 19.5, 112.0, 134.0, 124.6, 137.6, 122.7, 133.0, 96.5, 118.6, 150.2, 98.9, 110.8 and 131.0. (**36Bab**): 153.2, 166.0, 20.5, 128.1, 142.1, 125.1, 124.5,

134.2, 133.3, 106.2, 111.3, 129.8, 106.2, 111.3, 129.8, 153.2, 166.0, 17.9, 119.8, 158.1, 124.2, 137.3, 131.0, 133.3, 106.2, 111.3, 129.8, 106.2, 111.3, 129.8. (**36Bab**: Tables 3.1 and 3.2).

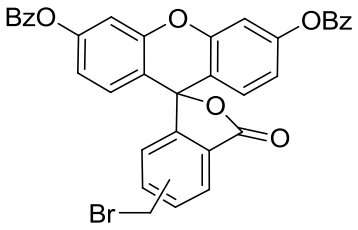
Synthesis of 4(5)-methyl-3-oxo-*3H*-spiro[isobenzofuran-1,9'-xanthene]-3',6'-diyl dibenzoate 37ab



37ab

4(5)-Methyl fluorescein **36Aab** (1.70 g, 4.9 mmol, 1eq) was dissolved in 20 ml of anhydrous pyridine. The solution was cooled to 0 °C and benzoyl chloride (1.25 ml, 10.7 mmol, 2.2 eq) diluted with pyridine (20 ml) was added with stirring. After stirring for 3 hours at room temperature, the solution was again cooled to 0 °C and water (4 ml) was added. The solution was stirred for another 15 min and subsequently concentrated under reduced pressure. The resultant oil was extracted three times with toluene (50 ml) and twice with chloroform (50 ml). The residue was dissolved in chloroform (50 ml) and the solution washed three times with a saturated aqueous $NaHCO_3$ (50 ml), solution and water (50 ml). The organic layer was dried with anhydrous Na_2S0_4 and the solvent evaporated under reduced pressure. The crude product was purified by column chromatography eluting in acetonitrile:hexane: methanol (3:1:1) and the solvent evaporated to yield **37ab** (2.635 g, 97%) as a white solid product. **Molecular formula**: **$C_{35}H_{22}O_7$**; **Mw: 554.54 g/mol; m.p. 226°C; R_f: 0.51** (acetonitrile:hexane:methanol (3:1:1)), **^{1}H NMR:** δ_H (300 MHz, DMSO-d_6): (**37a**): 7.74 (1H, dd, *J*=2.4, *J*=1.3), 7.68 (3H, m, -C<u>H</u>-), 7.24 (1H, dd, *J*=9.5, *J*=1.3), 7.20 (1H, dd, *J*=4.4, *J*=2.2), 6.87 (1H, dd, *J*=8.1, *J*=2.2), 7.21 (1H, dd, *J*=4.5, *J*=2.2), 6.87 (1H, dd, *J*=8.1, *J*=2.2), 8.04 (m, 5H, -C<u>H</u>-) 7.46 (m, 4H, -C<u>H</u>-), 7.60 (2H, tt, 2 x *J*=7.5, *J*=1.5). (**37b**): 7.26 (1H, dd, *J*=11.5, *J*=1.1), 7.41 (1H, dd, *J*=10.5, *J*=1.1), 7.68 (2H, m, -C<u>H</u>-), 7.54 (1H, dd, *J*=11.5, *J*=10.5, -C<u>H</u>-), 7.21 (2H, dd, *J*=4.5, *J*=2.2 and *J*=4.4, *J*= 2.2, -C<u>H</u>-), 6.87 (2H, dd, 2x *J*=8.1, *J*=2.2, -C<u>H</u>-), , 8.04 (4H, m, -C<u>H</u>-), 7.46 (4H, m, -C<u>H</u>-), 7.60 (2H, 2x tt, *J*=7.5, *J*=1.5, -C<u>H</u>-). **^{13}C NMR (75 MHz, DMSO-d_6)**: **37ab**: 18.6, 21.4, 84.5, 110.5, 116.0, 120.4, 124.5, 127.0-128.7, 129.9-139.0, 147.7-148.9, and 165.0-166.4.

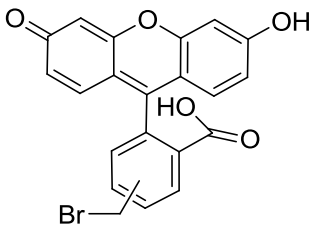
Synthesis of 4(5)-(bromomethyl)-3-oxo-*3H*-spiro[isobenzofuran-1,9'-xanthene]-3',6'-diyl dibenzoate 38ab



38ab

N-bromosuccinimide (5 g) was dissolved rapidly in boiling water (50 ml) and filtered through a filter paper into a flask which was immersed in an ice cold bath and left for 2 hours. The crystals were filtered, washed thoroughly with ice-cold water (18 ml), dried over $CaCl_2$ and the solvent evaporated. A mixture of purified NBS (0.401 g, 2.254 mmol, 1.25 eq) and 3',6'-dibenzoyl-4(5)-methyl fluorescein **37ab** (1.00 g, 1.803 mmol, 1 eq) dissolved in tetrachloro-methane was stirred and heated to 80 °C. Dibenzoyl peroxide (0.054 g, 0.225 mmol, 0.125 eq) was added and the solution refluxed for 6 hours. The solid was extracted with acetonitrile (3x 20 ml) and the solution concentrated under reduced pressure and chromatographed on silica gel eluting in acetonitrile:hexane:methanol (3:1:1) to afford 99% yield of product **38ab** (1.13 g) containing a mixture of **38ab** as a light yellow foam. **Molecular formula: $C_{35}H_{21}O_7$; Mw: 633.44 g/mol; R_f: 0.56** (acetonitrile:hexane:methanol (3:1:1)); **m.p. 115 °C**; **^{1}H NMR**: **δ_H (300 MHz, DMSO-d_6)**: (**38a**): 4.75 (2H, s, -O-C$\underline{H}_2$-Ph), 7.86 (1H, dd, *J*=1.2, *J*=0.5, -C$\underline{H}$-), 7.13 (1H, dd, *J*=10.4, *J*=1.2, -C$\underline{H}$-), 7.48 (1H, dd, *J*=10.4, *J*=0.5, -C$\underline{H}$-), 7.30 (1H, dd, *J*=8.0, *J*=3.7, -C$\underline{H}$-), 7.36 (1H, dd, *J*=10.3, *J*=4.2, -C$\underline{H}$-), 7.20 (1H, dd, *J*=3.7, *J*=2.2, -C$\underline{H}$-), 7.06 (1H, dd, *J*=8.1, *J*=2.2, -C$\underline{H}$-), 6.56 (1H, d, *J*=10.3, -C$\underline{H}$-), 7.40-7.47 and 7.62-7.78 (7H, m, -C$\underline{H}$-), 7.60 (2H, tt, 2x *J*=7.5, *J*=1.4, -C$\underline{H}$-). (**38b**): 4.75 (2H, s), 7.34 (1H, dd, *J*=11.5, *J*=1.3, -C$\underline{H}$-), 7.55 (1H, dd, *J*=11.5, *J*=11.5, -C$\underline{H}$-), 7.41 (1H, dd, *J*=11.5, *J*=1.3, -C$\underline{H}$-), 3.27 (1H, dd, *J*=9.8, *J*=4.1, -C$\underline{H}$-), 7.06 (1H, dd, *J*=8.1, *J*=2.2, -C$\underline{H}$-), 7.16 (1H, d, *J*=10.3, -C$\underline{H}$-), 7.35-7.78 (8H, m, -C$\underline{H}$-), 7.60 (1H, tt, *J*=7.49, *J*=1.5), 7.60 (1H, tt, *J*=7.5, *J*=1.5). **^{13}C NMR (75 MHz, DMSO-d_6)**: **38ab:** 30.7**,** 33.7, 39.5, 85.9, 111.0-116.5, 123.6, 124.9, 126.8-128.7, 129.0-139.7, 147.2-152.0, 152.6, 165.3, and 168.2.

Synthesis of 4(5)-(bromomethyl)-2-(6-hydroxy-3-oxo-*3H*-xanthen-9-yl) benzoic acid 39Aab



38ab

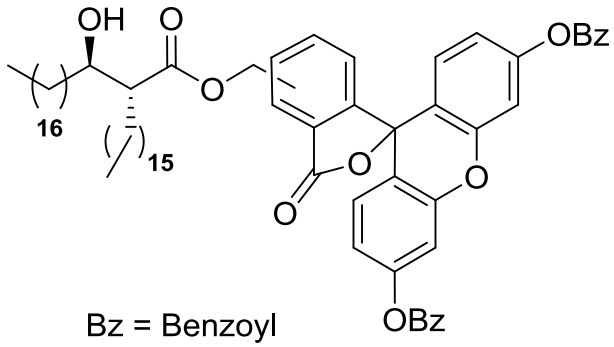
To prepare the product **39A**, the benzoate groups were removed by hydrogenation using the same procedure as developed for the removal of the benzyl group to give CMA **1**. To a degassed solution of dibenzoate **38** (1.12 g, 1.768 mmol, 1 eq) dissolved in ethyl acetate (20 ml), was added of $Pd(OH)_2/C$ (2.48 mg, 0.0177 mmol, 0.01 eq) and the reaction mixture evacuated and flushed with H_2 gas in a balloon several times with vigorous stirring until TLC indicated a complete conversion to a single product. After 2.9 h, the suspension was filtered through a bed of silica and celite with EtOAc. The product was concentrated in vacuum and purified on silica gel (hexane:EtOAc, 4:1) to afford 99% yield (0.744 g) of a mixture of **39A** and **39B.** These two products were successfully separated by flash chromatography eluting in hexane: EtOAc (4:1) **39A** (0.669 g or 90%) and **39B** (0.074 g or 10%). **Molecular formula**: **$C_{21}H_{13}BrO_5$**; **Mw: 425.22 g/mol; R_f: 0.24**; **^{1}H NMR: δ_H (300 MHz, DMSO-d_6)**: (**39Aa**): 10.99, (**-C(O)OH**) 4.33 (2H, s),Phenyl rings -CH-: 7.57 (1H, dd, *J*=1.4, *J*=0.5), 7.63 (1H, dd, *J*=7.8, *J*=1.4), 7.73 (1H, dd, *J*=7.8, *J*=0.5), 7.80 (1H, d, *J*=9.5), 7.76 (1H, dd, *J*=8.1, *J*=0.7), 6.12-6.56 (3H, m, -CH-). (**39Ab**): 4.49 (2H, s) 7.38 (1H, dd, *J*=7.9, *J*=1.5), 7.76 (1H, dd, *J*=7.9, *J*=7.8), 7.70 (1H, dd, *J*=7.8, *J*=1.5), 7.80 (1H, d, *J*=9.5), 7.70 (1H, dd, *J*=8.3, *J*=1.0), 6.12-6.57 (4H, m, -CH-). Some peaks for (**39Ba**): 7.87 (1H, dd, *J*=1.2, *J*=0.7), 7.13 (1H, dd, *J*=9.4, *J*=1.2), 7.64 (1H, dd, *J*=9.4, *J*=1.0), 7.41 (1H, dd, *J*=7.9, *J*=3.2), 7.40 (1H, dd, *J*=7.9, *J*=3.1), 6.74 (1H, dd, *J*=3.2, *J*=2.5), 6.69 (1H, dd, *J*=7.9, *J*=2.5), 6.74 (1H, dd, *J*=3.1, *J*=2.5), 6.69 (1H, dd, *J*=7.9, *J*=2.5) and (**39Bb**): 7.345 (1H, dd, *J*=11.4, *J*=1.1), 7.56 (1H, dd, *J*=11.5, *J*=11.5), 7.41 (1H, dd, *J*=11.5, *J*=1.2), 7.41 (1H, dd, *J*=7.9, *J*=3.2), 7.41 (1H, dd, *J*=7.9, *J*=3.1), 6.74 (1H, dd, *J*=3.2, *J*=2.5, 6.69 (1H, dd, *J*=7.9, *J*=2.5), 6.74 (1H, dd, *J*=3.1, *J*=2.5), 6.69 (1H, dd, *J*=7.9, *J*=2.5). **^{13}C NMR (300 MHz, DMSO-d_6)**: **39ab**: 30.9, 33.6, 96.5, 99.8, 107.2, 110.3-115.1, 124.2, 127.6, 128.7, 134.0., 137.8, 139.2, 140.3, 146.9, 155.0-158.6, 168.8, and 186.2.

5.3.2. Labeling of MAs

A General Procedure used for all mycolic acids

To a solution of the acid (CMAs **1** or commercial natural mycobacterial-MA) (1 eq) and 4(5)-(bromomethyl)-3-oxo-*3H*-spiro[isobenzofuran-1,9'-xanthene]-3',6'-diyl dibenzoate (**38ab**) and/or 4(5)-bromomethyl)-2-(6-hydroxy-3-oxo-*3H*-xanthen-9-yl) benzoic acid (**39Aab**) (1.01 eq) in DMF: 1,4-Dioxane (1:1, 10 ml) was added potassium bicarbonate (1.4 eq) and 18-crown-6 (optional), 1.4 eq at room temperature. The reaction mixture was refluxed at 90 °C for 24 hours then cooled to room temperature when TLC showed no starting materials. It was then diluted with EtOAc (10 ml) and washed with saturated sodium chloride (10 ml) and water (3x 10 ml). The organic layers were dried over $MgSO_4$ and the solvent evaporated under vacuum. The crude product was purified via flash column chromatography.

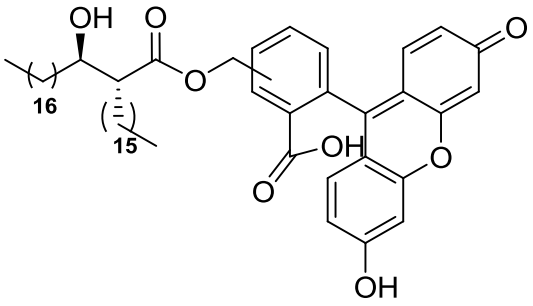
Synthesis of 4(5)-(((3R)-2-hexadecyl-3-hydroxyicosanoyloxy) methyl)-3-oxo-*3H*-spiro(isobenzofuran-1,9'-xanthene)-3',6'-diyl dibenzoate 40



To label the **CMAs 1**, the general procedure described above was used. A mixture of CMAs **1** (0.10 g, 0.18 mmol, 1 eq) and **38ab** (0.115 g, 0.182 mmol, 1.01 eq) were reacted in the same conditions to yield 99.8 % (0.201 g) of yellowish solid **40**. **Molecular formula**: $C_{72}H_{94}O_{10}$; **Mw: 1119.51 g/mol; R_f: 61**; **^{1}H NMR**: **δ_H (300 MHz, DMSO-d_6)**: 2.39 (2H, dt, *J*=7.5, *J*=4.2, α-H) 3.61 (1H, td, *J*=6.2, *J*=4.2, β-H), 8.27 (1H, dd, *J*=1.3, *J*=0.5), 7.64 (1H, dd, *J*=9.0, J=0.5), 7.68 (1H, dd, *J*=8.1, *J*=4.4), 7.68 (1H, dd, *J*=8.19, *J*=4.4), 7.47 (1H, dd, *J*=9.0, *J*=1.2), 5.32 (2H, s), 7.21 (1H, dd, *J*=4.4, *J*=2.2), 6.87 (2H, 2x dd, *J*=8.1, *J*=2.2, -C<u>H</u>-), 7.21 (1H, dd, *J*=4.4, *J*=2.2, -C<u>H</u>-), 1.73 (2H, td, *J*=7.5, *J*=7.5, -C<u>H$_2$</u>-), 1.55 (2H, td, *J*=7.3, *J*=6.2, -C<u>H$_2$</u>-), 1.28 (2H, tt, *J*=7.5, *J*=7.1, -C<u>H$_2$</u>-), 8.04 (4H, m, -C<u>H</u>-), 1.41 (2H, tt, *J*=7.3, *J*=7.1, -C<u>H$_2$</u>-), 1.25 (2H, tt, *J*=7.1, *J*=6.5, -C<u>H$_2$</u>-), 7.46 (4H, m, -C<u>H</u>-), 1.25

(2H, tt, *J*=7.1, *J*=6.5), 1.22 (2H, tt, *J*=6.5, J=6.5), 7.60 (1H, tt, *J*=7.5, *J*=1.4), 7.60 (1H, tt, *J*=7.5, *J*=1.5), 1.24 (2H, tt, *J*=6.5, *J*=6.5), 1.23-1.27 (48H, m, -C$\underline{H}_2$-), 0.87 (6H, t, *J*=7.0) and 2.50 (DMSO peak). **^{13}C NMR (75 MHz, DMSO-d_6)**: 14.1, 22.5, 24.9, 29.1-29.9, 30, 0, 30.5, 36.0, 52.7, 66.7, 70.6 (**-C(OH)-**), 94.9, 99.8, 108.8, 110.3, 127.3, 128.5, 1304.3, 134.8, 138.4, 140.0, 155.9, 156.4, 167.6, 174.1, and 185.6.

Synthesis of the 4(5)-((2-hexadecyl-3-hydroxyhenicosanoyloxy) methyl)-2-(6-hydroxy-3-oxo-*3H*-xanthen-9-yl) benzoic acid 44



To prepare the product **44**, the general procedure as described for the synthesis of the product **39** was used. To a degassed solution of dibenzoate **40** (0.200 g, 0.178 mmol, 1 eq) dissolved in ethyl acetate (100 ml), was added of $Pd(OH)_2$ (0.249 mg, 178 x 10^{-5} mmol, 0.01 eq) and the reaction mixture evacuated and flushed with H_2 gas in a balloon several times with vigorous stirring until TLC indicated a complete conversion to a single product. After 2.9 h, the suspension was filtered through a bed of silica and celite with EtOAc. The product was concentrated under vacuum and purified on silica gel (hexane: EtOAc, 4:1) to yield 98% (0.16 g) of a mixture of **44A** and **44B.** These two products were successfully separated via flash column chromatography eluting in hexane: EtOAc (4:1) **44Aa** (0.144 g or 90%) and **44Bab** (0.016 g or 10%). **Molecular formula**: **$C_{21}H_{13}BrO_5$**; **Mw: 897.27 g/mol; R_f: 0.24** (hexane:EtOAc, 4:1); **^{1}H NMR (300 MHz, DMSO-d_6)**: 6.51-7.80 (1H, d, *J*=9.5, -C$\underline{H}$-), 5.14 (2H, s, -C$\underline{H}_2$-), 2.68 (1H, td, *J*=7.5, *J*=4.2, α-H), 3.83 (1H, td, *J*=6.2, *J*=4.2, β-H), 1.73 (2H, td, *J*=7.5, *J*=7.5, -C$\underline{H}_2$-), 1.55 (2H, td, *J*=7.3, *J*=6.2, -C$\underline{H}_2$-), 1.23-1.28 (58H, m, CH_3-C$\underline{H}_2$-), 0.88 (6H, t, *J*=7.0, *J*=7.0). For **44Ab**: some characteristics peaks are such as but not limited to 6.57 (1H, d, *J*=9.5), 7.80 (1H, d, *J*=9.5), 6.68 (1H, dd, *J*=1.5, *J*=0.7), 7.70 (1H, dd, *J*=8.4, *J*=0.7), 7.71 (1H, dd, *J*=7.8, *J*=1.4), 6.51 (1H, dd, *J*=8.4, *J*=1.5), 7.70 (1H, dd, *J*=8.0, *J*=7.8), 7.54 (1H, dd, *J*=8.0, *J*=1.4), 5.20 (2H, s), 2.68 (1H, td, *J*=7.5, *J*=4.2, α-H), 3.83 (1H, td, *J*=6.2, J=4.2), 1.74 (2H, td, *J*=7.5, *J*=7.5), 1.52 (2H, td, *J*=7.32, *J*=6.2), 1.28 (2H, tt, *J*=7.5, *J*=7.1), 1.41 (2H,

tt, *J*=7.32, 0.88 (6H, t, *J*=7.0, *J*=7.0).**^{13}C NMR (300 MHz, DMSO-d$_6$)**: 14.1, 22.7, 23.8, 29.0-32.1, 35.3, 52.2, 68.0, 70.8, 93.8, 101.6, 106.1, 110.3-116.1, 128.8-134.7, 139.1, 140.1, 156.7, 156.9, 168.0, 174.3, and 184.6.

Synthesis of natural mycobacterial- MA/ 4(5)-MF conjugate (45)

To label the commercially available sample of naturally occurring mycobacterial-MAs the general procedure described above was used. A mixture of mycobacterial-MAs (1 mg, 8 x 10^{-7} mmol, approx 1 eq) and **39** (3.465 mg, 8.1 x 10^{-7} mmol, 1.01 eq) were reacted under the same conditions to yield about 95 % (1.211 mg) of a yellowish solid after flash column chromatography eluting in chloroform:methanol:hexane (8:1:1). **Mw: 1594.22 g/mol**; **^{1}H NMR (300MH$_Z$, DMSO-d$_6$)**: 11.01, (-C(O)-OH) 2.29 (1H, td, *J*=5.5, *J*=2.0, α-H), 3.55 (1H, td, *J*=5.4, *J*=2.0, β-H), 3.28 (s, -OC$\underline{H}_3$), 0.15-0.49 (m, 8H, cyclopropane rings) 6.35-8.23 (m, 11H, phenyl rings), 0.87 (6H, t, *J*=7.0, *J*=7.0, C$\underline{H}_3$-CH$_2$-), 3.10 (-OH group), **^{13}C NMR (75 MHz, DMSO-d$_6$)**: 10.66, (-$\underline{C}$H$_2$- first cyclopropane ring), 11.23 (-$\underline{C}$H$_2$- second cyclopropane ring), 14.2 (two terminal methyl groups, $\underline{C}$H$_3$-CH$_2$-), 17.99-18.66 (2x-CH$_2$-$\underline{C}$(CH$_2$)-$\underline{C}$-CH$_2$-), 21.02, 22.95, 24.25, 25.01, 30.04, 33.99, 34.36, 39.98. **^{13}C (DMSO-d$_6$):** 52.0 (-C-$\underline{C}$H-CO$_2$-, α-C), 56.8 (-O$\underline{C}$H$_3$, methoxy-group), 61.0, 67.9, 72.1 (-$\underline{C}$H(OH)-) β-C), 95.3, 101.7, 108.2, 110.0, 116.1, 122.5, 124.3-129.9 (Alkyl chains -$\underline{C}$H$_2$-), 133.2, 137.9, 139.9, 148.6, 156.5, 158.0, 162.0, 174.6 (-$\underline{C}$O$_2$-C, and 188.9 (carbonyl carbon, -CH-$\underline{C}$=O-CH-).

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