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Synthesis of cord factors

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Synthesis of Cord Factors

By Max Maza Iglesias

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Contents

ACKNOWLEDGMENTS **3**

CONTENTS **4**

ABBREVIATIONS AND ACRONYMS **6**

ABSTRACT **8**

1. INTRODUCTION **10**

1.1 TUBERCULOSIS 10
1.1.1 Characteristics *10*
1.1.2 Different species *11*
1.1.3 Transmission *11*
1.1.4 Main issues: Cost and number of infected *12*
1.1.5 History of TB *12*
1.1.6 Global illness *14*
1.1.7 Cure targets *15*
1.2 IMMUNE SYSTEM MEDIATED ILLNESS 16
1.2.1 History *16*
1.2.2 People affected by allergies *17*
1.2.3 Immunity *18*
1.2.4 Immune system components *19*
1.2.5 The immune system response *20*
1.2.6 How allergies work *22*
1.2.7 Diagnosis *23*
1.2.8 Today's treatments *24*
1.2.9 The hygiene hypothesis *25*
1.2.10 Bacteria and asthma *27*
1.2.11 BCG in animal models *28*
1.2.12 Bacteria and cancer *29*
1.2.13 IFN *29*
1.2.14 Skin diseases *30*
1.3 THE MYCOBACTERIAL CELL ENVELOPE 32
1.3.1 Generalities *32*
1.3.2 Mycobacterial lipids *36*
1.4 MYCOLIC ACIDS (MAS) 37
1.5 CORD FACTORS 41
1.5.1 Trehalose dimycolates (TDM) *41*
1.5.2 History of cord factors *43*
1.5.3 Trehalose esters in other organisms *45*
1.5.4 Techniques of analysis used for cord factors *45*
1.5.5 Historical perspective of synthesis of TDM/TMM and other trehalose esters *51*
1.5.6 Model compounds for the esterification reaction with trehalose and other sugar like compounds *65*
1.5.7 Esterification reaction with enzymes *67*
1.6 GLYCEROL ESTERS 68
1.7 PATHOGENICITY AND ANTIGENIC STRUCTURE 71
1.8 PROPERTIES OF TREHALOSE DIESTER 71
1.9 IMMUNE MANIPULATIONS THAT MAY IMPROVE ALLERGIC AND LATENT TUBERCULOSIS PATIENTS 73
1.9.1 New vaccine strategies *73*
1.10 BIOLOGICAL ACTIVITY 74
1.10.1 Adjuvants *74*
1.10.2 Adjuvant activity *75*
1.10.3 Antitumor Activity *76*

1.10.4 Antibacterial Activity *77*
1.10.5 Antiparasitic Activity *77*

2. RESULTS AND DISCUSSION **79**

2.1 MODEL COUPLING REACTIONS 79
2.1.1 Preparation of trehalose dipalmitate *79*
2.1.2 Model couplings using potassium salts and iodotrehalose derivatives *81*
2.1.2.1 Preparation of potassium salts 81
2.1.2.2 Reactions of potassium salts with halogenated trehalose derivatives 83
2.1.2.3 Enzyme catalysed reactions with Novozym 435 (Candida antartica) lipase 86
2.1.2.4 Preparation of 'model' corynomycolic acids 86
2.1.2.4.1 Claisen condensation of henicosanoic acid 87
2.1.2.4.2 Claisen condensation of palmitic acid 89
2.1.3 A simple model 3-(tert-butyl-dimethylsilanyloxy)-heptadecanoic acid *90*
2.2 ESTERIFICATION OF TREHALOSE WITH MODEL COMPOUNDS 91
2.2.1 Coupling 3-hydroxyoctadecanoic acid with trehalose *91*
2.2.2 Coupling trehalose with crude mycolic acid samples *95*
2.2.3 Coupling reaction between trehalose and 3-(tert-butyldimethyl-silanyloxy)-2-eicosyltetracosanoic acid and DCC *96*
2.2.4 Coupling reactions between trehalose and acid chlorides *97*
2.2.5 Coupling reaction between protected trehalose and alpha-alkyl beta-hydroxy acids using EDCI *99*
2.2.6 Trimethylsilyl deprotection reaction of alpha-alkyl beta-hydroxy acids esterified to trehalose *101*
2.3 MYCOLIC ACID SYNTHESIS 103
2.3.1 Aim and overview *103*
2.3.2 Cyclopropane ring formation and chain extension *105*
2.3.3. The Julia reaction to couple the second cyclopropane extended chain *106*
2.3.4. Corynomycolate moiety addition *109*
2.4 CORD FACTOR SYNTHESIS 113
2.4.1 Aim and overview *113*
2.4.2 Methoxy mycolic acid protection and esterification with trehalose *113*
2.4.3 Alpha mycolic acid protection and esterification with trehalose *120*
2.5 GLYCEROL ESTER SYNTHESIS 127
2.5.1 Aim and overview *127*
2.5.2 C_{32} glycerol esters *127*
2.5.3 Alpha mycolic acid glycerol esters *129*

3. BIOLOGICAL DATA **132**

3.1 BIOLOGICAL DATA FOR ALPHA/METHOXY MYCOLIC ACIDS 132
3.2 BIOLOGICAL DATA FOR CORD FACTORS 134
3.2.1 Procedure *135*
3.2.2 Data *135*
3.3 BIOLOGICAL DATA FOR GLYCEROL ESTERS 137

4. CONCLUSIONS **140**

5. FUTURE WORK **145**

6. EXPERIMENTAL **146**

6.1 GENERAL CONSIDERATIONS 146
6.2 EXPERIMENTS 147

7. REFERENCES **232**

8. APPENDIX **249**

8.1 EXPERIMENTS 249
8.2 FIGURES 254

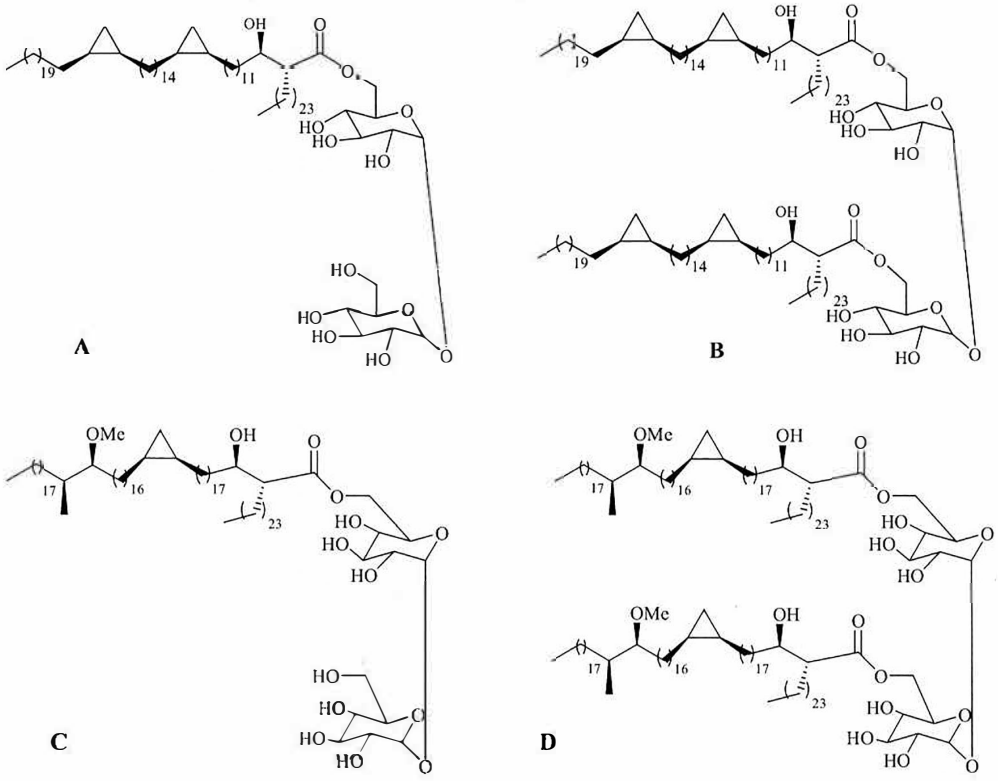
Abbreviations and acronyms

AG	Arabinogalactan
B	Broad
BCG	Bacillus Calmette-Guerin
Bn	Benzyl
BSA	N,O-bis(trimethylsilyl)acetamide
D	Doublet
DCC	Dicyclohexylcarbodiimide
DEAD	Diethyl azodicarboxylate
DIAD	Di-isopropyl azodicarboxylate
DMAP	Dimethylaminopyridine
DMF	N,N-Dimethylformamide
DMSO	Dimethyl sulfoxide
EDCl	1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride
EI	Electron Impact
EIMS	Electron Impact Mass Spectrometry
GC	Gas Chromatography
GC-MS	Gas Chromatography-Mass Spectrometry
HMPA	Hexamethyl phosphoramide
HMPT	Hexamethyl phosphorous triamide
Hz	Hertz
IFN-γ	Interferon Gamma
Ig	Immunoglobulins
IL-1	Interleukin 1
IR	Infra-red
M	Multiplet
MALDI-TOF	Matrix Assisted Laser Desorption Ionisation Time-

	Of-Flight
MDR	Multiple Drug Resistant strains
MHC	Major Histocompatibility Complex
MMG	Monomycolyl glycerol
Mmol	Millimole
mp	Melting Point
NK cells	Natural Killer cells
NMR	Nuclear Magnetic Resonance
PCC	Pyridinium Chlorochromate
PG	Peptidoglycan
PMN	Polymorphonuclear leukocytes
Q	Quartet
S	Singlet
S_N2	Nucleophilic substitution, bimolecular
t	Triplet
TB	Tuberculosis
TBAF	Tetrabutylammonium fluoride
T-cells	T-Lymphocytes
TDM	Trehalose dimycolates
TFA	Trifluoroacetic acid
THF	Tetrahydrofuran
TLC	Thin Layer Chromatography
TMM	Trehalose monomycolates
TNF-α	Tumor Necrosis Factor
Ts	Tosyl
WHO	World Health Organisation

Abstract

The aim of this thesis was to prepare different synthetic cord factors (A, B, C and D) and understand how they affect the immune system, which relates to asthma and eczema.



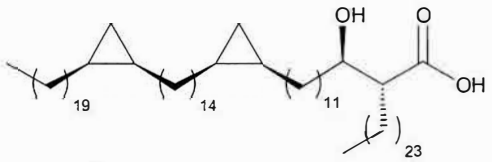
Cord factors were made by esterification of a trehalose sugar and a mycolic acid using DMAP and 4-DMAP. At this first stage the esterification produced an anhydride mycolate which was first characterised and isolated.

The next step included silyl group deprotection in two steps first using tetrabutylammonium fluoride to remove sugar silyl and then HF-pyridine complex to remove mycolic acid protection, releasing the free cord factor.

The aim of making such compounds was to be able to distinguish which specific synthetic cord factors produce noticeable beneficial effects with the minimal side effects on a variety of chemokines and cytokines. In comparison, crude natural cord factors composed of a variety of components are toxic in different ways. A synthetic route might

be able to separate inflammatory/toxic effects caused by some components and reveal beneficial effects of others.

Due the size of the molecules involved in a cord factor, a block by block synthesis was taken starting by making the mycolic acid (E, F) which were then incorporated to the cord factor



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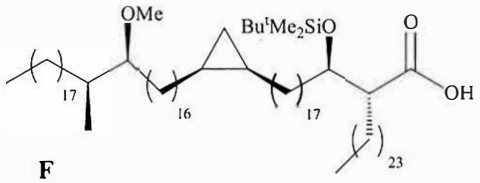
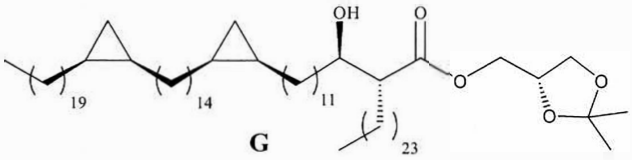


F

A different type of molecule based on a glycerol core instead of the trehalose one has been proved to be bactericidal and to have anti-cancer properties (G). For this reason the synthesis of glycerol esters was included in the thesis as a different approach to disease treatment.



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The final part of the thesis reports biological data obtained from synthetic cord factors and its implications with immune systems related illnesses.

1. Introduction

Cord factors are characteristic components of tuberculosis (TB) cells. They are potent signalling agents in the immune system which may relate to the progression of TB and of a range of other immune system disorders. Natural cord factors are very complex mixtures. This thesis sets out to synthesise individual cord factors and allow their specific effects to be determined and applied. Therefore the introduction below provides background information on TB, immune system illness, mycobacterial characteristics, mycolic acids, glycerol esters and cord factors.

1.1 Tuberculosis

1.1.1 Characteristics

Tuberculosis is a chronic or acute infection caused by bacteria belonging to the genus Mycobacterium. In tissue, these bacteria are thin rod-shapes that do not form spores. The organisms measure between 2 and 4 micrometres and are classified as 'acid fast' bacteria as they are resistant to acids or alcohol decolourization (95 % alcohol, 3 % HCl).[1] Due to that they cannot be classified as gram-positive or gram-negative.

Growth characteristics are: aerobic organisms with a growth rate which is slower than that of most bacteria. They are more resistant to chemical agents than other bacteria because of the specific hydrophobic configuration of their cell surface. In fact, dyes or antibacterial substances can be incorporated into the growth medium without interrupting growth. Dry conditions do not seem to affect these organisms either as they can survive for long periods in such an environment.[2]

1.1.2 Different species

There are over fifty types of mycobacteria and variations are due to colony shapes, colours, virulence, temperature growth and many other characteristics. The main source of TB in human beings is *Mycobacterium tuberculosis*, whereas fowl and cattle are resistant to it. This bacterium can survive several years outside the human body but it does not grow in soil or water. *Mycobacterium bovis* used to be an important source of TB in humans, coming from cattle when infected meat or milk was consumed. Hopefully, modern strict techniques, such as pasteurization or meat tests have reduced drastically this source. Alongside these two species there are many other pathogenic mycobacteria; as an example, *Mycobacterium avium* can be found in environments such as food, water, soil and animals. As its name may reveal it causes diseases in birds and may be serious in people with AIDS where between 25-90 % of HIV-infected patients developed this bacteria, due to the absence of immunosuppression. *Mycobacterium kansasii* produces pulmonary disease, especially in immune suppressed patients. *Mycobacterium scrofulaceum, Mycobacterium marinum* and *Mycobacterium ulcerans* occur mainly in water environments, infecting fish and producing human skin lesions. Another important type of bacterium, described in 1873, is *Mycobacterium leprae* which causes leprosy. This bacterium is classified as non-tuberculous, but still causes a chronic infectious disease with 5 million new cases expected to arise between 2000 and 2020, and that in 2020 there would be 1 million people affected (mainly in Asia).[3] In addition, 2-3 million people, though cured, are in need of continuous care as the result of their deformities.

1.1.3 Transmission

M. tuberculosis is air transmitted as aerosolised drops when an infected patient sneezes. The bacteria then travel in the air, enter the upper airways of the uninfected patient and are inhaled into the bronchia. They then implant in this rich oxygenation area. A small number of bacteria, such as a 100 could cause infection. Human beings are at the same time a reservoir and a transmission agent for the illness.[4] A big percentage of the infected population do not develop any symptoms as the pathogen stays in a 'latent' form. For

those who do, the infection pattern develops as follows; 2-4 % of patients develop the illness within a year. In the remaining patients, the infected cells will be surrounded by macrophages, B and T-lymphocytes and fibroblasts to form a granuloma, which seals in the infection.[5] This percentage of patients developing TB will rise to 33 % in people that are infected with HIV and the disease will develop within a maximum of 5 months.[6] HIV does, in fact, reactivate latent *M. tuberculosis* infection or facilitate TB progression in already infected patients.[7]

Tuberculosis can be divided into different types: non-pulmonary and extra-pulmonary. The first meaning the initial infection was not in the lungs and the second that the source of infection started in the lungs.[8]

1.1.4 Main issues: Cost and number of infected

Despite great advances in medical science there were more than 8.8 million new TB cases in 2005. In fact tuberculosis is the infectious disease that causes the highest number of deaths in the world.[8] The spread of cases varied, but 7.4 million were concentrated in Asia and sub-Saharan Africa. TB has caused death to 1.6 million people including almost 200,000 infected with HIV. Mortality due to infectious diseases accounts for 20 % of total deaths.[9] TB cases have been rising slowly, due to increased infection in the African, Eastern Mediterranean and South-East Asia regions. According to the WHO, total costs assigned to fight TB worldwide have risen from $ 500 million in 2002 to $ 1.25 billion in 2007.[10]

1.1.5 History of TB

Tuberculosis (also known as consumption or white plague), regardless of its origin, remained one of the leading causes of death throughout history.[11] In the Palaeolithic period people did not live in villages or in settled places but in small migrating groups. At that time TB was not epidemic but with the development of agriculture came cattle and sheep. With the number of people increasing, the disease had the opportunity to become

endemic to humans, who presumably caught it from cattle. TB has effectively killed people since early times. Historians have reported and established that mummies dating to 2000 to 4000 B.C. showed symptoms that characterised tuberculosis death.

The epidemic spread slowly as the population increased in the globe reaching a maximum in the 17th and 18th century where the illness was undoubtedly the first cause of death in the developed world. In the 19th and 20th centuries it fully spread over Asia, Africa and South America. The illness followed a pattern where the maximum peak of spread is attainted 50-70 years after exposure, declining then due probably to resistance developed by survivor hosts.[12] The fact that the body encapsulates the disease producing 'small knots' in the lungs to stop it spreading made Franciscus Sylvious call it 'tubercula'. The illness progressed until the 19th century where one-fourth of all deaths were due to Tuberculosis. At that time people did not know what caused TB but this changed in 1882 when Robert Koch described the isolation of the organism causing the disease.[8] Koch's strategy was to culture the bacillus and he developed a vaccine which would finally end in a cure. This vaccine eventually came from Calmette and Guerin after attenuating an *M. bovis* strain by serial passages for 230 times over 11 years. The attenuated strain was non-virulent against a range of animals before being tested in humans for the first time in 1921. Today a much preferred vaccination is a freeze-dried vaccine form of BCG. On average 115 million vaccines are distributed yearly in 172 countries.[13] The successful vaccination with BCG showed inconclusive results when clinical trials were performed, as the variation in protection can run on 0 to 80 %.[14]

It is now widely known that BCG often does not provide adequate protection against tuberculosis. Several trials showed that BGC cannot prevent adults from developing TB.[15] These contradictory results could be related to genetic variations in the population and exposure, attenuation of the BCG strain or even reduction of the vaccine efficiency over time.[16,14,17] Due to all these reasons new vaccine discovery and efficiency improvement are required in this area.

In the mid 20th century came the discovery of the first drug proved to be effective against TB. A variety of drugs, such as streptomycin and isoniazid came in the 1940s and 1950s. Streptomycin was isolated from *Streptomyces griseus* and was the first effective drug against TB. Isoniazid acts by inhibiting mycolic acid biosynthesis. Those novel

treatments seemed to eradicate the disease, only temporarily though as resistance to these drugs appeared. Fortunately, multi-drug treatments prevented the appearance of drug-resistance.[8] Rifampicin, a natural compound extracted from *Streptomyces mediterranei* which inhibits RNA biosynthesis came in the 1970s and gave a short term chemotherapy cure for both TB and leprosy, having many advantages compared to previous treatments such as reduced therapy time.[18] But those innovative discoveries suddenly were proven to be of temporary effectiveness as, in the mid 1980s, HIV outbreaks occurred and multidrug resistance appeared. HIV infected patients would have their T-lymphocytes destroyed by the virus which will result in a breakdown of the immune response against TB. You are 200 times more likely to develop TB if you already have HIV than if you do not. The WHO estimates that up to 50 million people worldwide are infected with multiple drug-resistant strains (MDR-TB) of TB.[10] MDR-TB are those strains resistant to isoniazid and rifampicin. Treatment for multidrug-resistant tuberculosis takes a long-time (up to two years), and is less effective, costly, and poorly tolerated.[19] As a result, there are today more cases of TB than there were in the 1950s.

To make matters worse, tuberculosis control plans took place mainly in developing countries, leaving those with low income out. This approach, typical of unbalanced societies, divided developed countries from under developed ones. This situation only worked for a period of time as in a global world, where people travel from one place to the other, there is no physical barriers for such a disease. On the positive side, efforts have been made by several governments and associations, such as the WHO, to change this. However, it must be taken into account that complete elimination of the disease will not occur due to the large number of people infected with it (33 % of global population). This will required constant and periodic surveillance of the disease.[20]

1.1.6 Global illness

Back in 1993, the WHO declared tuberculosis a global emergency illness and implemented several programs to target the disease. An estimated 2 billion people (33 % of the population worldwide) is believed to be carrying the bacillus. Globally, in 2007

there were an estimated 9.27 million cases of TB. As a comparison, there were 9.24 million cases in 2006, 8.3 million cases in 2000 and 6.6 million cases in 1990.
Although the total number of incident cases of TB is increasing, the number of cases per capita is falling slowly (1 % per year) due to population growth (Fig 1). A large majority of cases in 2007 was in Asia (55 %) and Africa (31 %).[21]

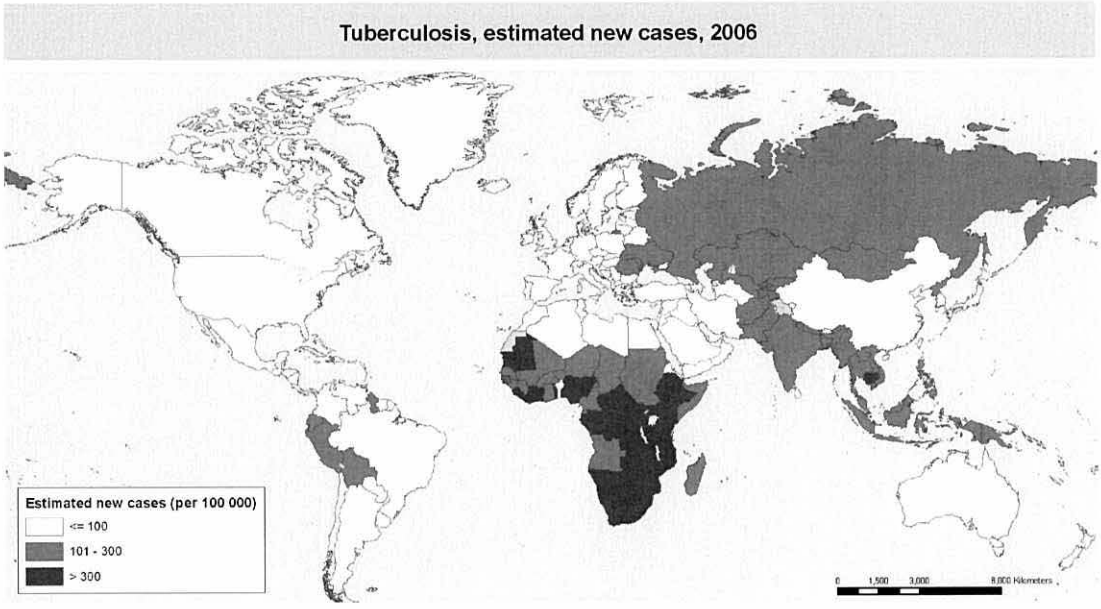


Fig 1. Estimated new cases of TB in the world. Data from WHO, 2005.[22]

Of the total number of infections for 2007, 1.4 million (14 % of total affected) were HIV-positive. The total number of deaths for 2005 was 1.7 million people.
What must also be taken into account is the social effect of TB on people's lives, such as employment, orphan children, exclusion, failure to complete treatments and deterioration of the health system.

1.1.7 Cure targets

Anti-tuberculosis compounds target the synthesis of macromolecules which are essential for bacterial survival. Potential development drugs must have the minimum impact on organisms which a Mycobacterium infects and be able to minimise resistance appearance.

1.2 Immune system mediated illness

1.2.1 History

For an average person allergies are meant to be a 21st century problem. Contradicting this, we could give several examples in antiquity which may prove that the idea of having symptoms such as bloating, itching or headaches was documented in early times.[23] In 2735 BC Chinese emperors described that some food may be not consumed by some people, such as pregnant women. Those foods included shellfish, chicken or meat that could cause skins lesions or ulcers (which may be related with today's eczema or urticaria).[24] Hippocrates described the same symptoms in his patients back in 460 BC. In the 12th century the knowledge achieved by Greek and Arabic civilisation was organised and described by the Arabic physician Moses Maimonides in 'Treatise on Asthma'. The treatments prescribed included, as today, avoiding polluted environments, control with food and drinks, everyday habits and simple remedies used against 'the disease'.[25] The term 'allergy' only came into use in 1906 and even at that time was not commonly used in the scientific community that had more important problems to deal with, such as maternal and infant high mortality rates.[26] From the 1840s to the 1890s a variety of injected treatments were performed on animals. Those treatments included egg albumin or cholera inducing vibrio but at the time the mechanism of why things happened could not be explained.[27] The term 'anaphylaxis' was described for the first time in 1912 by French doctors Richet and Portier who described it as the 'opposite to protection' when injecting plants toxins into dogs.[26] Those first discoveries opened the link to a variety of different trials and studies that would later be related to humans. All those early studies gave a therapeutic approach to diseases such as hay fever, asthma or eczema, that have been understood since the beginning of the 20th century as being allergic or immunological in nature. 'Allergic disorders' were since then understood as being created by the patient's body rather that the allergen itself.[28] In the mid 1950s histamine was found in mast cells, and during the 1960s allergic mediator IgE immunoglobulin was discovered by the Ishizaka group.[29,30]

1.2.2 People affected by allergies

Within the last two decades, asthma has unevenly proliferated to the point of being predominantly a First World problem.[31,32] In the UK, by the late 2000s, approximately 25 % of the adult population in the country suffered from an allergic disease (asthma, eczema, hay fever).[33] Statistics showed that in the UK 15 % of children suffer asthma and 10 % had eczema.[26] In terms of financial cost, the NHS is expending 1 billion pounds to treat the 3.4 million asthmatics in the UK.

If we take for example the case of the USA, 54.6 % of their citizens tested positive to one or more allergens.[34,35] Allergic diseases affect an astonishing number of Americans, as many as 40 to 50 million (Fig 2, 3).[36] These numbers reflect the cost involved which for the USA has increased from a value of 1.5 billion dollars in the 1980s to a surprisingly high 10 billion dollars in the mid 1990s.[26]

Asthma affects about 150 million people worldwide and is the most important chronic disease for children. It usually tends to be underestimated due the fact that mortality rates are lower compared with other respiratory diseases. It is predicted that the disease will increase in number of affected people if measures are not taken against it.[37]

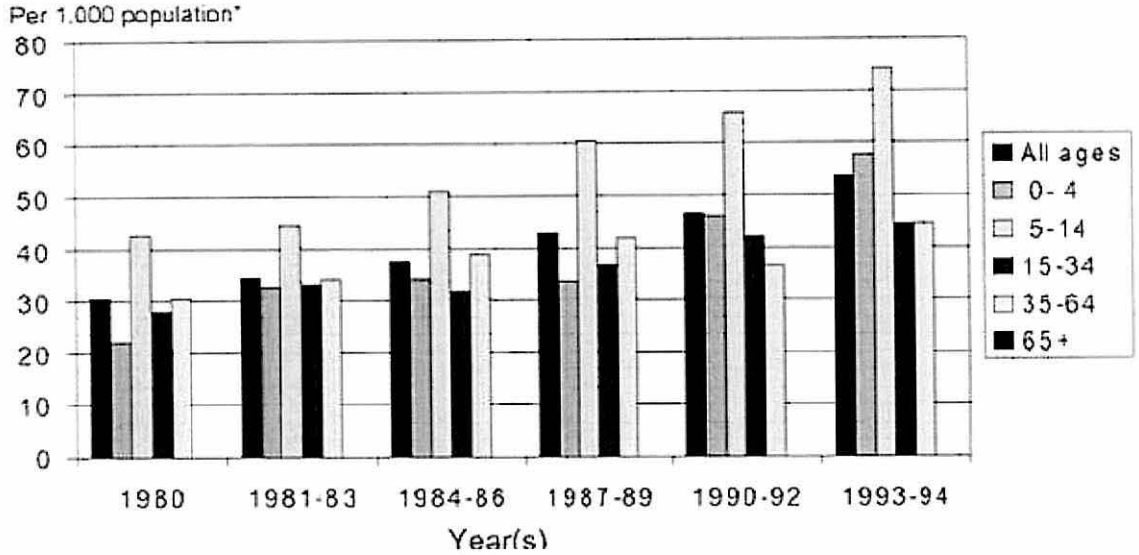


Fig 2. The variation between ages groups/time for USA population. The prevalence rates of allergic conditions have importantly risen in the world in the past decades. The graph shows differences between groups, with decreasing effect with age.[38]

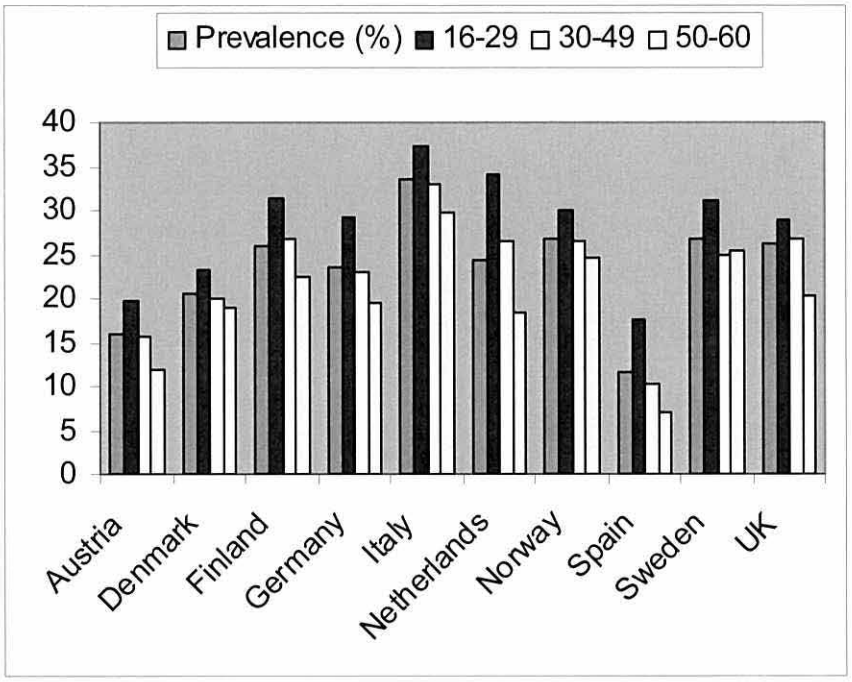


Fig 3. Distribution of respiratory allergic disorders in 10 European countries. Blue lines represent percentage of the total population affected by respiratory disorders. Purple, yellow and light blue lines represent allergic disorder age distribution pattern for the entire population ($P<0.002$). Adapted from Dalh *et al*, 2004.[33]

Eczema arguably causes a considerably greater stress for affected families than asthma or even type 1 diabetes, due to problems such as financial costs, lack of sleep and in some cases jobs lost.[39]

1.2.3 Immunity

Allergies can be described as an over reaction that your body creates against substances considered to be harmful, even if they are not. In 'normal' conditions the body prevents foreign substance attacks by defending itself, and this is the role of the immune system. Any substance capable of inducing an immune response will be considering as allergen, whatever its origin. Examples of antigens are microbes and cancerous cells that are being neutralised by immunologic cells and the antibodies that they produce.[23] The immune system consists of cellular and molecular components that work together to destroy antigens.

The development of the immune system starts early in prenatal stages and will continue as the future child confronts natural environmental conditions, filled with antigens.

1.2.4 Immune system components

The organs of the immune system are located in different parts of the body (thymus, bone marrow, spleen, lymph nodes or even the appendix) and they are called lymphoid organs because they produce lymphocytes or white cell.

The immune system has two types of cells involved in response, mononuclear and multinuclear.

The mononuclear group includes macrophages, monocytes, lymphocytes (T and B cells).

- Macrophages can destroy some allergens directly and they do alert lymphocytes to foreign invasions. They are activated by IFN-γ, and can then kill antigens and secrete IL-1 and tumour necrosis factor-α (TNF-α). These two cytokines facilitate pathogen destruction.
- Monocytes can be described as macrophage precursors. They reach infected sites and T-cells secrete cytokines (IFN-γ) that prevent macrophages from leaving the infected area.
- Lymphocytes present two types of cell:
    I. B-cells mature in the bone marrow and their primary function is developing into plasma cells that are responsible for producing and secreting antibodies (IgM, IgG, IgA, IgD and IgE).
    II. T-cells mature in the thymus and have a wide range of functions but usually detect and respond to an antigen and are stimulated by macrophages. When this happens, T-cells release a variety of lymphokines which are basically soluble polypeptides. One of those peptides, Interleukin 2 (IL-2) boosts T-cell response. On the other hand secreting polyclonal B-cells activators will boost B-cell production. These specific cells form an important barrier against virus and cancerous cell expansion in an organism. T-cells can be divided in to three main subtypes, helper, regulatory and cytotoxic.

On the other hand the multinuclear component of the immune system destroys antigens once they have been 'labelled' with antibodies. They are usually called polymorphonuclear (PMN) leukocytes, due the fact that their cytoplasm contains granules. Inflammation occurs because they produce inflammatory substances such as lysozymes, hydrogen peroxide, superoxides or prostaglandins which are toxic to foreign cells once ingested but do harm host tissue. This is the reason why inflammation processes are described as producing redness and swelling.

There are four important different types of cells: Neutrophils, Eosinophils, Basophils and Mast cells.

- Neutrophils occur in the circulation and when required can enter infected tissue and kill invasive microorganisms by phagocytosis.
- Eosinophils kill organisms which are too big to be phagocytised by releasing toxic proteins and several enzymes. They are the source of inflammatory mediators such as prostaglandins.
- Basophils release inflammatory mediators, such as histamines.
- Mast cells are an exception to the other three types as they do not occur in circulation but in different body tissues such as mucosal cells. They contain cell granules with releasing mediators that have a role in generating protective inflammatory response.

1.2.5 The immune system response

There are four main roles of the immune system. First it acts as a barrier. The second important role happens when an antigen appears in the body; this is the ablility to distinguish between 'self' cells that carry a distinctive marker molecule and cells carrying 'foreign' markers. Third, to eliminate the source of 'foreign' antigens and finally keep memory of immunological encounters.[40] When this step happens the body will launch the protective action. These markers are called Major Histocompatibility Complex (MHC) and are present in all cells in order to be recognised by the immune system. There are two classes of MHC, Class 1, the recognition portion of the molecule present in every cell and

Class 2, characteristic of immune cells. They both present a range of units that can reassemble into about 2 x 10^7 different combinations making it easy to distinguish what belongs to the body and what it does not. When the MHC of the antigen is presented to the T lymphocyte, rapid cell division happens and cytokines and interleukins are produced and released. The antigen stimulates these mediators and the B-cell is stimulated as well. B-cells are then divided into two groups. The first group will develop into Plasma cells that will produce and secrete antibodies and the other group will keep the surface cell marker which holds memory of the antigen and will rapidly respond if a second infection occurs.

These massively produce antibodies consist of two heavy and two light chains and they belong to the immunoglobulin group, which includes two categories.

Defence immunoglobulin:

- Immunoglobulin G (IgG) coats microbes and speeds antigen processing by immune cells.
- IgM: very effective bacteria killer and responding to infection.
- IgA: a high concentration is naturally occurring in body fluids, such as tears, saliva, respiratory and digestive tract.

Other immunoglobulins:

- IgD: initiates B cell response
- IgE: protects against parasites and is responsible for allergic symptoms.[23]

If the antibodies bind to antigens on bacteria or parasites, this acts as a signal for PMNs or macrophages to phagocytise and kills them. Another important function of antibodies is to initiate the "complement destruction cascade." When antibodies bind to cells or bacteria, serum proteins called complement bind to the immobilized antibodies and destroy the bacteria by creating holes in them. Antibodies can also signal natural killer cells (NK cells) and macrophages to kill viral or bacterial-infected cells. These NK cells are, among others, responsible for flushing out and eliminating all cells that become cancerous. Tumours are then the result if a system breaks down or is overwhelmed.

1.2.6 How allergies work

Sometimes the immune system goes beyond its purposes and over reacts by extremely defending the organism against substances that are not real threats. This type of reaction is commonly known as hypersensitivity. In the case of allergies, big quantities of IgE antibodies are produced. The process of being in contact with the allergen, having memorised it, and having produced IgE molecules is called sensitisation. These IgE molecules will then attach to the mast cells and, in the case of a second allergenic attack, IgE mast cells will release chemicals that will produce characteristic symptoms such as sneezing, watery eyes or itching. Some people can be sensitised and stop there without developing allergic symptoms.

Allergies can be manifested in a variety of forms and affect different organs: Respiratory, Eye allergies, Insect allergies, Food allergies, Skin allergies and Drug allergies.

Respiratory allergies can affect either the nose, nasal allergy or the bronchi, allergic asthma. Pollen, or plant seed contains a variety of allergic proteins which can be allergenic in sensitive or atopic individuals. Symptoms are expressed as rhinitis or asthma.

Some people may get affected by household dust which includes a variety of allergens such as mould or food allergens. Acarids (mites) are tiny spiders 0.3 mm long that live in dust and are responsible of respiratory allergies. They feed from dust mite and skin scales which they digest externally with enzymes. Those enzymes are highly allergenic to atopic individuals. They cause asthma, rhinitis, conjunctivitis and atopic dermatitis (eczema).

Pets such as cats, dogs, guinea-pigs, horses or birds are a source of allergy because of the secretions they produce. Saliva, skin cells, urine or in some cases excrements are an important source of allergies. Symptoms may take time to develop, as much as 2 years, and symptoms may include asthma, rhinitis, eczema and contact urticaria.[36]

Other animals, such as bees and wasps can cause allergy by stings in sensitive people. The symptoms can vary and include: generalised itching, urticaria, abdominal pain, and difficulty breathing.

Allergic rhinitis (commonly known as hay fever) causes inflammation in the nose area and produces nasal block, swelling and runny nose. It can affect sleep and concentration.

Allergic asthma produces airway narrowing (bronchial hyper-reactivity), inflammation due to allergens and excess mucus segregation.[36] Asthma can be caused by pollen, dust, animal fur, chemicals, foods or drugs. Asthma produces an important blockage of bronchi due to spasms in muscles, swelling in the bronchi due to fluid accumulation, mucus production and inflammation. All this interrupts or worsens air circulation making breathing discomforting.

Allergic conjunctivitis symptoms are itchy, watery and red eyes in conjunction with swollen lids. This is caused mainly by pollens and mould.

In the case of food allergies the immune system produces IgE against food in genetically predisposed individuals. Symptoms can vary from digestive problems (vomiting, diarrhoea), skin problems (itchy and red skin), blood circulation (blood pressure, heart rate, dizziness) and respiratory problems. Foods that may cause allergic reactions are:

- Milk: proteins present in milk may act as antigens.
- Eggs: allergies can be related to both white and yolk proteins.
- Peanuts: is a lifelong allergy compared to milk or eggs.
- Fish and shellfish: due to proteins found in their flesh. Even cooking vapours can be allergenic.
- Fruits, vegetables and nuts: even cutting some of these products may induce allergies.

1.2.7 Diagnosis

People who have the symptoms described above may need to consult an expert in allergies who will determine if the person is actively producing IgE antibodies against specific or to a broad number of allergens. Diluted extracts from specific allergens are tested on the skin by superficial injection.[36] If the patient has developed IgE against any of the tested substances, his skin will have a particular rush or redness pattern. Depending on shape, colour and size, the doctor may establish if the patient is allergic to the substance or not. Blood tests are used only in severe cases or anaphylactic profile people. They are very sensitive but not suitable for every condition.

1.2.8 Today's treatments

The mechanism behind asthma is accepted to be bronchial inflammation. Mediators such as interleukins and prostaglandins are produced by inflammatory cells. Some of those components are destructive to cell membranes. As a result, an increase in mucus production, airway narrowing, endothelium damage and finally inflammation happens.
An asthmatic episode will cause the following conditions to happen after allergen exposure:

- Uneven reduction in bronchi area (within minutes)
- Edema (within hours)
- Mucus hyper production and cell damage (within days)

These three combined factors will obstruct bronchi and in some cases, respiratory failure may occur.[23] In essence, asthma is the result of an immune response in the bronchial airways.[41]

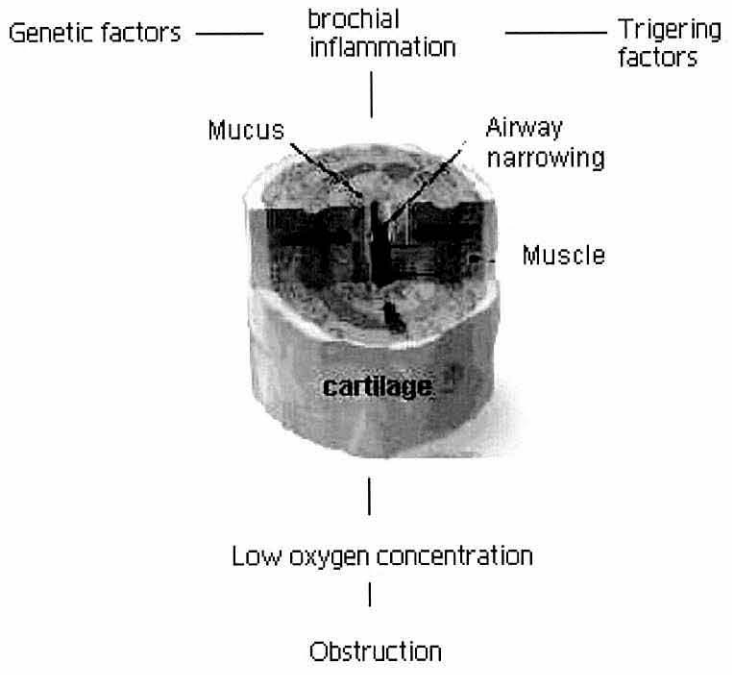


Fig 4. Acute asthma infection scheme proposed by Wolf (adaptation).[23]

The more general drugs available do not cure asthma but are only temporarily effective in de-blocking bronchi. Asthma patients are treated, in the majority of cases with a combination of inhaled corticosteroids (ICS) and a β-agonist for symptom relief.[42]
Corticosteroids are known to reduce excessive airway narrowing and can be used as an inhaler or orally. These drugs, which are bronchodilators, block inflammation and are extremely effective in relieving symptoms of asthma. When inhaled for a long time airways become less sensitive to allergens. Steroids have several side effects such as the risk of developing diabetes and decalcification. When used for longer periods, gain in weight and mental problems may occur.
Some β-agonists, such as albuterol, are effective and have a minor effect on other organs but continuous use does not add control and may be harmful. It is used as an inhaler which goes effectively into the lungs and its action lasts for up to 6 hrs.[23] Common side effects of β2-agonists depend on patient age, type of drug, preparation and dose administration but include muscle tremor, nausea, palpitations, increased blood pressure, agitation and heartburn (at higher dose).[43] Some new drugs are becoming safer and more tolerable, such as indacaterol, a novel once-daily β2-agonist bronchodilator.[44]
Almost 10 % of the total asthmatic population is considered to show symptoms persistent to such treatments and present a much more complicated issue, in terms of hospital admittance, therapy and life quality for the patients.

1.2.9 The hygiene hypothesis

In the early 1990s, a new concept was developed by Mosmann to explain the high increase in asthma and allergies.[45] According to the 'hygiene hypothesis', immune disorders occur because there are two ways in which lymphocyte T helpers can produce cytokines. A Th0 cell will mature under different factors, genetic and environmental.[46] In specific illness such as tuberculosis or viral exposure, those T helpers will mature and produce mainly interleukin-2 (IL2), interferon-γ (IFN-γ) and tumour necrosis factor (TNF), this route being known as Th1. Other specific illnesses, such as allergies will in constrast produce cytokines such as interleukin-4, interleukin-5, interleukin-9 or

interleukin-13.[47] This route is known as Th2 and will drive IgE production. It is important to emphasise that the two routes are mutually exclusive in a way that Th1 cytokine production inhibits Th2.[48]

The hygiene hypothesis says that newborns have a Th2 oriented system which after encountering the environment will develop into Th1. This mechanism is not fully understood and may include genetic and environmental factors. A very basic scheme can be seen in Fig 5.

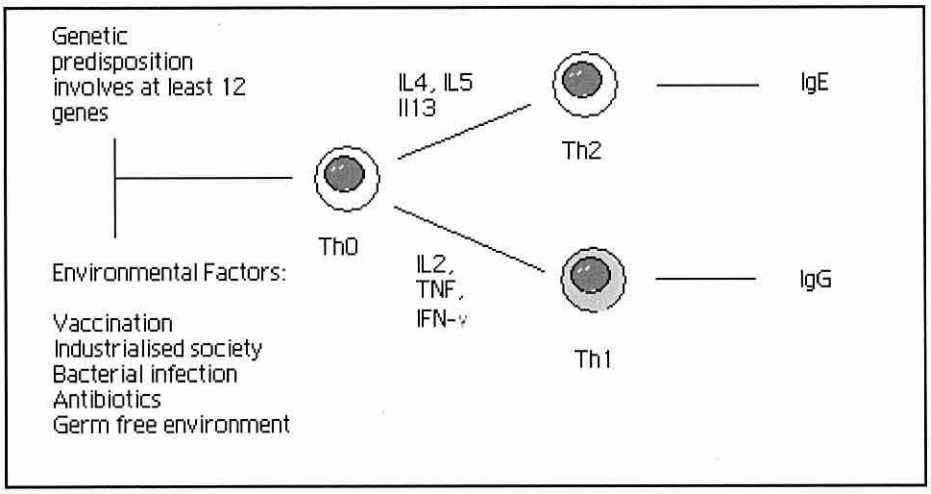


Fig 5. The influence of genetic and environmental factors will potentiate Th0 cells to mature into either the Th1 or Th2 system. Each system produces significantly different cytokines which at the end make allergic or immune responses happen.[23,47]

The hygiene hypothesis says that modern society exposes people of all ages to reduced contact with pathogens that prime Th1 immune responses. This lack of Th1 stimulation may result in reactivation of Th2 activity, actively linked with allergic conditions.

The Th1/Th2 paradigm predicts a negative association between Th1 and Th2 responses as they are mutually inhibitory.

Meanwhile, a number of epidemiological studies support the concept of the hygiene hypothesis where a variety of early life exposures to environmental factors can protect against allergic diseases. It has been observed that children under five growing up in a farm environment develop significantly less asthma and eczema symptoms through their lives.[49] Results from Gehring *et al* showed that children exposed to endotoxins early in life will have a significant reduction in the development of atopic eczema and respiratory

diseases later in life.[50,51] On the other hand, asthma impact seems higher in poor living environments of Afro-American and Hispanic populations in the USA, showing that another mechanism may be influencing Th1/Th2 connectivity. Furthermore the hygiene hypothesis ignored several Th1 immune diseases such as Crohn's disease, type-1 diabetes or multiple sclerosis. The problem here is that both Th1/Th2 immune responses seem to be widely spread which shows that the Th1/Th2 paradigm is sometimes a simple explanation for a very complex system of interactions which is far from being fully understood.[52,47] The main point is to try to understand if the timing of infection, the absence of infection, type of infection or geographical variability may cause variability in asthma cases. On the other hand, early exposure to antibiotics may affect intestinal flora and may increase allergy development.[53]

1.2.10 Bacteria and asthma

It is important to know that different bacteria may have different effects on immune response activity depending on species, immune state of the host and specific environmental exposure of the individual. *Mycobacterium tuberculosis* or respiratory viruses may increase Th1 mediated response and decrease Th2 response at the same time, this being important for the non-development of allergic symptoms. Here, we will use these bacterial infections as examples to review mechanisms of predisposition/protection to asthma.

Early studies by Ishizaka *et al* showed IgE suppression in mice models exposed to mycobacteria. IFN-γ production was increased, affecting Th1/Th2 response.[54] Some other studies confirmed those preliminary results such as a Finish study showing that *M. tuberculosis* infection in childhood prevented the development of asthma in later life and even suppressed the development of subsequent allergic conditions.[55] A variety of other studies performed in communities with higher rates of exposure to *M. tuberculosis* showed that those bacteria reduce Th2 response and consequently atopic disorders such as wheeze, asthma.[56,57]

However, not all Tb infections react in the same way, as patients with progressive TB have raised levels of IgE compared with those with the latent version of TB. The same

happens when using BCG vaccination as results are inconclusive due to differences between populations and different environment to which patients have been exposed previously.[58,59]

1.2.11 BCG in animal models

By using the BCG model as part of the hygiene hypothesis different scientist have tried to link and explain mycobacterial exposures with atopic disease prevention. When using another species such as *M. vaccae*, results were promising with animal models for lung inflammation but remained inconclusive during clinical trials.[60] Recent studies showed that a whole set of variables such as genetic variation, timing, dose, delivery route and environmental factors may play an important role in clarifying the specific process taking place in the therapeutic effect of BCG.[61] It is prohibited to deliver live BCG to humans. Therefore, several studies have shown that freeze killed BCG administered to previously ovoalbumin (OVA) sensitised mice and guinea pigs prevented allergy primarily by large IFN-γ production which boost immune response.[62] The study shows a reduction in inflammatory cell number, eosinophilia and cytokines (Fig 6). However, heat killed BCG induces macrophages to produce TNF-α and several side effects such as tissue necrosis in humans are related to that.[63]

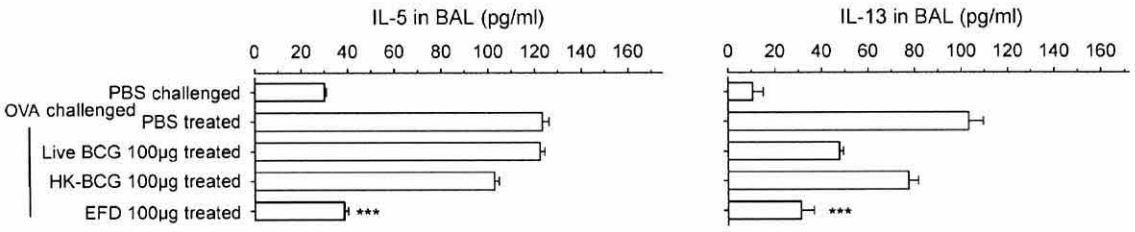


Fig 6. BCG killed by freeze-drying (EFD) shows a significant reduction in proinflamotry cytokines IL-5 and IL-13 when compared to other methods such as Heat killed-BCG (HK-BCG), Live BCG or PBS solution. BAL (bronchio-alveolar lavage).

Erb et al demonstrated that BCG immunization of children reduced drastically asthma symptoms in previously ovalbumin sensitised patients. In order to have the most

pronounced results, BCG must be administered into the lung, demonstrating the variability depending on application area.[64]

1.2.12 Bacteria and cancer

Immunotherapy for cancer started at the end of the 19th century with Coley when he developed killed streptococcal preparations (Coley's toxins) to raise immunity and fight back tumours. Coley's legacy is still in use today; as an example bladder cancer is still treated by injecting BCG to the patient. The literature on BCG use is vast, starting in 1935 when Holmgren treated cancer patients, this time with not much success.[65] Several years after this a French group demonstrated the efficacy of active immunotherapy in the control of leukæmia in man, putting BCG therapy on the map.[66]

M. vaccae immunotherapy has been shown to be a safe and effective treatment for cancers such as adenocarcinomas and melanoma while dramatically improving the patients quality of life.[67] Heat killed *M. vaccae* has been found to be a potent adjuvant fighting small cell lung cancer in Phase II studies with no increased toxicity.[68] Patients with lung melanoma showed lifestyle improvement when treated with killed *M. vaccae* in Phase III studies.[69] This capacity of inducing tumour protection comes from the Mycobacterium and other specie's ability to control interleukin-12 (IL-12). IL-12 plays a role in immunity in a way that targets both T-cells and Natural Killer (NK) cells. In fact anti-tumour activity is the response to both T and NK cells.

1.2.13 IFN

Interferons are proteins produced by animals and plants in general to fight back viral infections by interfering in virus replication. This action was first described by Lindenmann and Issac.[70]

In order for a vaccine to be efficient, T lymphocytes must secrete specific cytokines. In humans and mice the specific cytokine involved appears to be the lymphokine interferon alpha (IFN-α).[71,72] These cytokines will promote macrophage activation, induce

granulomatous response and induce antimicrobial resistance.[73] This antimycobacterial function occurs by releasing reactive nitrogen intermediates, including NO, NO_2, and HNO_2.[74] When vacuoles are formed in the macrophages, intense enzymatic activity occurs which is increased by IFN-α.[75] This is the reason why IFN- α is being used as a bacterial marker for infection.

Historically, newer interest came when in the late 1960s it was found that IFN stopped tumour growth and also stimulated immune response. Later developments in the 1970s allowed interferon purification. In the 1980s IFN-α showed good results in stopping melanoma in cell lines. Since then several clinical trials have been done on IFN-α as adjuvant but results seems to vary and the ability to destroy cancer is not supported by all researchers.[76,77] However it is effective in leukaemia or bone cancer. Results varied when treating multiple sclerosis, melanoma, renal cancer or AIDS-related sarcoma. These variations are related to patient selection, dosing or toxicity.[78]

1.2.14 Skin diseases

Eczema, or dermatitis, is a common inflammatory skin disorder characterised by itch, dry skin, and red patches. These patches may develop small blisters, known as vesicles, which can break down leading to weeping and crusting of the skin. The resultant impairment of skin barrier function leaves the skin prone to further damage from external agents such as irritants and infection. Itch leads to scratching and rubbing which over time can produce further changes such as thickening (lichenification) and fissuring.

Eczema can be present in different clinical forms, the main types being atopic eczema, irritant and allergic contact eczema, discoid eczema, venous eczema and pompholyx.

Atopic eczema typically starts in the face and later on moves to elbows and knees, particularly in adults, but can affect any part of the body. This chronic disease will continue in 10 % of children when they became adults. Atopy is the genetic predisposition to form excessive lgE antibodies and to develop one or more of a group of disorders that include asthma, hay fever, urticaria, allergies and a particular form of

eczema. The concentration of serum lgE is raised by more than five times in 80 per cent of children with eczema but its role in the disease remains uncertain.[79] Eczema is not an allergy itself, but allergies can trigger eczema. Complications of atopic eczema include chronic blepharitis, kerato-conjunctivitis, herpes simplex virus infection (eczema herpeticum) and secondary bacterial infection. Trehalose dimycolates (TDM), a sugar derivative component of mycobacterial cells walls, can act as an immunomodulator so could potentially be indicated for use in treating moderate to severe eczema. It would treat eczema by targeting the underlying disease process itself, although exactly how it works in eczema is not known. The skin's immune system of a person with eczema over-reacts when exposed to an irritant, like dust, sending chemical messages that result in an eczema flare-up. TDM blocks immune system cells from creating these chemical messages, slowing or halting the eczema by binding T cell receptors, inhibiting cytokine production and inhibiting IgE sensitivity.

More work needs to be done regarding TDM affinity to target cells. It is important to know that several factors are related to antibacterial, antitumor or antiparasitic activity such as the length of the peptide and differences in the glycosidic part. In this way research needs to target the nature, position, and length of a lipid moiety in order to improve the biological activity of these compounds.

1.3 The Mycobacterial Cell envelope

1.3.1 Generalities

The bacterial cell wall represents the ultimate separation between the environment and a very complex structure of a single cell organism. The main functions of such a separation are as follows:

- Separation from the hostile environmental conditions of the outside milieu.
- Stability, solidity and shape of the cell.
- Connection with the outside can be done in a selective way, as transportation of substances and contact with the environment can be tuned.[80]

M. tuberculosis does all these functions very effectively and many of the problems related to antibiotic resistance, chemical damage or dehydration are due to its cell wall permeability. As an example, mycobacteria are resistant to disinfectants, alkali media.[81] The cell wall is also involved in immunological host-mycobacteria reaction.[82]

The mycobacterial cell wall has a complex nature and very high lipid content. Mycobacterial cell walls contain between 30-60 % (weight) lipids, including waxes, C-mycoside glycopeptidolipids, phenol glycosides, trehalose-containing lipopoly-saccharides, sulfolipids, lipoarabinomannan, and mycolic acids.[83]
Early research aimed to define the cell wall structure of *Mycobacterium spp.* Different groups in France (Lederer) and Japan (Yamamura, Kato) started this research in the 1960s and 1970s. The cell envelope structure is today well understood thanks to Brennan, Minnikin or Daffé.[81,84,85] Electron microscopy was used to identify ultrathin sections outside the plasma membrane which was in later studies confirmed a triple layer structure.[86]

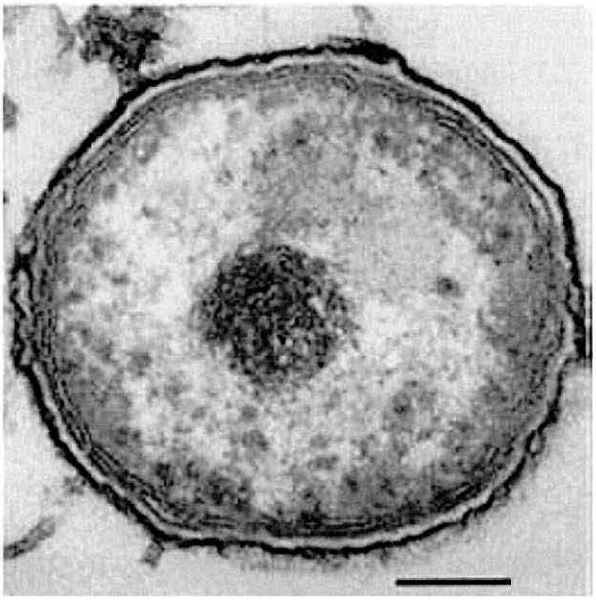
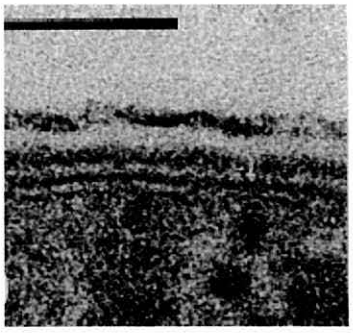


Fig 7. Thin layer of *M. szulgai*. From Rastogi *et al.*[86]

Later protocols minimise extraction, giving better quality images such as the electron microscopy of *M. thermoresistible* which shows three different layers: two electron dense layers and one middle electron transparent layer (Fig 7). The transparent layer appearance is due to the inability of water soluble stains to penetrate this region.



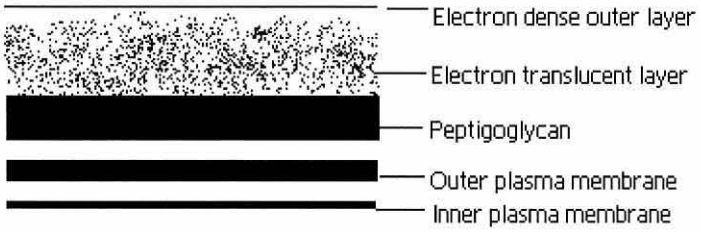


Fig 8 (left). Magnification of *M. thermoresistible* dehydrated envelope. From Paul and Beveridge.[87]
Fig 9 (right). Schematic representation of envelope profile Fig 3 (not according to scale). Adapted from Paul and Beveridge.[87]

It is difficult to know the exact inner structure of the wall but the model proposed by Minnikin in 1982 and modified thereafter is usually accepted.[83]

The cell envelope of Mycobacterium species is mainly composed by three structures:

- an internal plasma cell
- the cell wall
- an external layer mainly made of polysaccharides, proteins and lipids, called capsule.[88]

The composition of the plasma membrane remains similar to other living organism's lipid bilayer. It is composed of phospholipids derivatives from phosphatidic acid (PIMs). Other associated compounds are polyterpenes. The inner layer, overlaying the lipid bilayer is composed by PG, consisting of long polysaccharide chains that give shape and rigidity to bacteria. N-Glycolylmuramic acid is linked in *β1→4* to N-acetyl glucosamine in alternating positions and cross linked to a four chain amino acids L-alanine, D-isoglutamine, meso-diamino-pimelic acid and D-alanine. The PG is linked to AG by a phosphodiester bridge (Fig 10). The AG layer is composed of arabinose and galactose.[89]

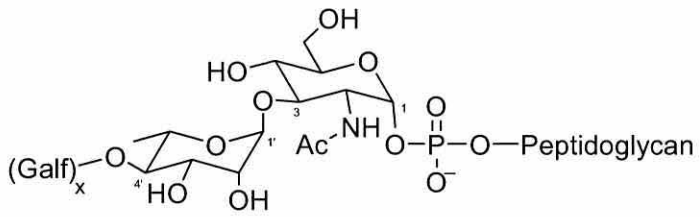


Fig 10. The galactan of AG is linked to the C-6 of some murarnyl residues of peptido-glycan via the diglycosylphosphoryl bridge, L-Rhap-(1 →3)-D-GlcNAc(1 →P).[90]

The cell wall skeleton is composed of three covalently linked substructures: peptidoglycan (PG), attached to arabinogalactan (AG) which in turn is attached to mycolic acids.[82] The junction of these three motifs is commonly known as (mAGP) mycolyl-arabinogalactan-peptidolycan complex.[91] A simple representation of these structures is shown in Fig 11. Most of these lipids assemble producing an asymmetric bilayer which is considerably thick. This particular structure reduces fluidity in the innermost part of the bilayer, gradually increasing toward the outside membrane.[81]

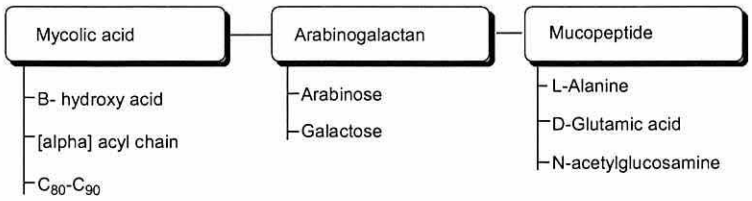


Fig 11. Structure of BCG-CWS. BCG-CWS consists of mycolic acids, arabinogalactan and mucopeptide.[92] The mycolic acids extend perpendicular to the arabinogalactan/peptidoglycan. They are long chain α-alkyl, β-hydroxy fatty acids (Fig 12).

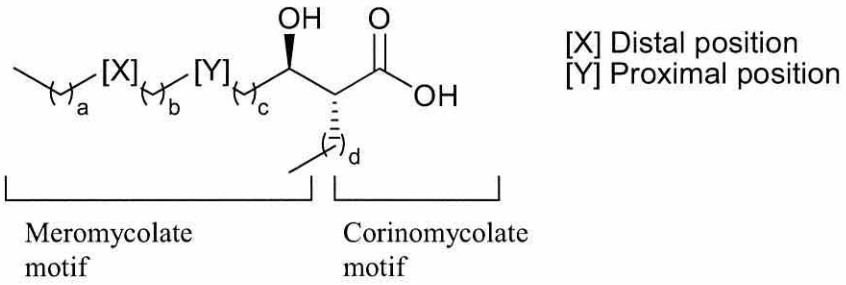


Fig 12. Basic Scheme of a mycolic acid.

They appear as bound esters of AG in the form of tetramycolylpentaarabinosyl clusters. These forms a stable 'pseudo' bilayer structure strengthened by proteins and polysaccharides.[93] The majority of the terminal hydroxy groups in the arabinofuranoside motifs are esterified by mycolic acids. The upper segment is composed of free lipids, differing in length. Interspersed are the cell wall proteins, the phosphatidylinositol mannosides (PIMs), the phthiocerol containing lipids, lipomannan (LM), and lipoarabinomannan (LAM) (See figure 13).

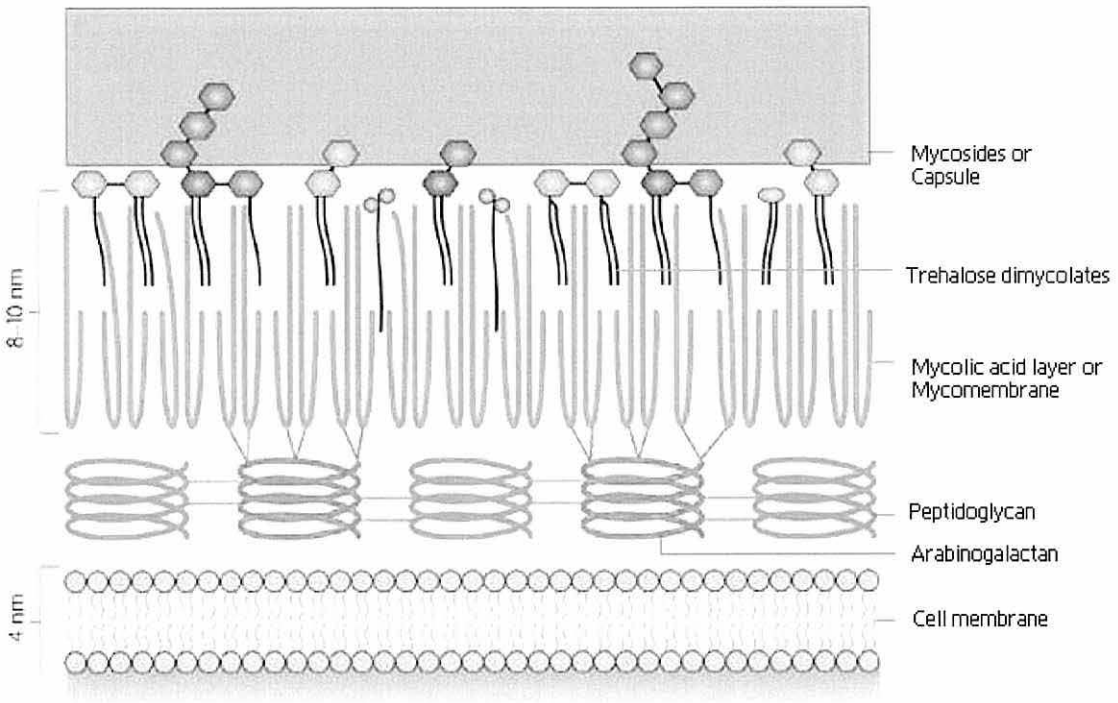


Fig 13. Representation of the cell envelope of *M. tuberculosis*. Adapted from Abdallah *et al.*[94]

The most external electron opaque layer varies in thickness and looks granular or fibrillar depending on the species.[81]

1.3.2 Mycobacterial lipids

Mycobacterial lipids have been studied for more than 70 years, due to their structural diversity and biological activities.[9] The mayor outer lipids are mycolic acids as cell wall components, glycolipids with toxic and immunological properties such as lipoarabinomannan (LAM), acylated trehalose (cord factors), mycosides and phospholipids.

A variety of lipids appears only in a related group of bacteria, such as sulfolipids which are characteristic of *M. tuberculosis* whereas others are more generally present such as LAM and cord factors. Some of those lipids are called "free'' lipids as they are easily extractable from the AG-peptidoglycan unit by using polar solvents.[83]

1.4 Mycolic acids (MAs)

Early studies targeted the composition, molecule distribution and properties of mycolic acids in *M. tuberculosis* and other related species.

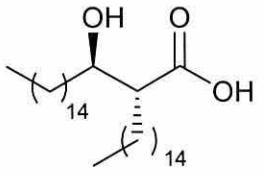
Anderson (1939) isolated for the first time mycolic acids with general formula $C_{88}H_{172}O_4$.[95,96] He described the oxygen atoms as representing a carboxyl, a hydroxy and a methoxy group. From his results mycolic acid melted at 54-56 °C and had the appearance of a white powder.[97] Later work showed that mycolic acids are a group of long-chain α-alkyl, β-hydroxy fatty acids. The general formula for a simple mycolic acid is shown in Fig 12. Mycobacterial lipids correspond up to 40 % in dry weight of the cell envelope.

In the early 1950s, Polonsky and Lederer described synthetic corynomycolate diastereoisomer separations. Many authors such as Yamura, Azuma and Lederer were involved during the 1970s and 1980s in understanding and helping to solve problems with *M. tuberculosis* as analytical techniques evolved and other close fields such as genetics and biosynthesis underwent great development.

In many ways scientific progress related to mycolic acids involved analytical chemistry development techniques. Those techniques have proved the complexity of these compounds first thought to be single structures extracted from *Mycobacterium tubercolisis* 'wax'. Nowadays the family includes over 500 related structures.[98]

The three principal categories are:

(i) The corynebacteria acids ranging from C_{28} to C_{40}, found mostly in Corynebacteria. The first to be discovered and the most common is $C_{32}H_{64}O_3$ **1** (Fig 14) found as an example in *Nocardia asteroids* esterified to trehalose.[99,100]



1

Fig 14. $C_{32}H_{64}O_3$ first found in *N. asteroids*.

A racemate of this simple natural corynomycolic was synthesised by Lederer and Pudles by a series of reactions, starting with a *Claisen* condensation.[101] In 1953 diatereosiomers of $C_{32}H_{64}O_3$ and $C_{36}H_{72}O_3$ were separated and $C_{36}H_{68}O_3$ were synthesised.[100]

(ii) The nocardiae acids ranging from C_{40} to C_{60}, produced by Nocardia and discovered by Michel *et al.*[102,103] Different species corresponding to saturated, mono-, di- and tri-unsaturated bonds have been identified.

(iii) The mycobacterial mycolic acids, ranging from C_{60} to C_{90}.[89] Some typical structures are shown in Fig 15.

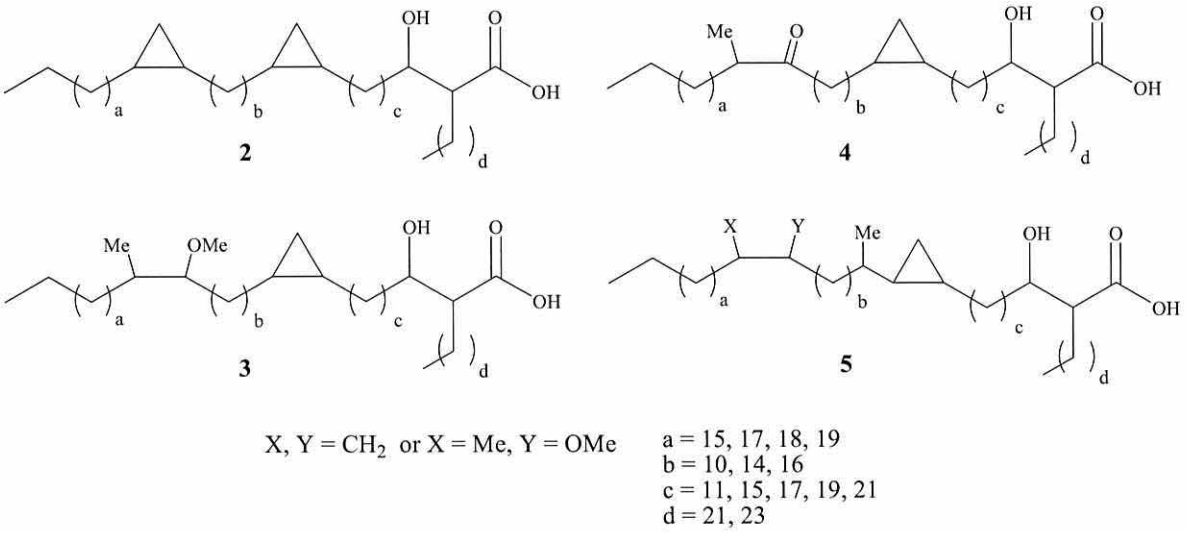


Fig 15. Structures of mycolic acids from mycobacteria. Numbers are described as follows; alpha mycolic acid **2**, methoxy-mycolic acid **3**, keto-mycolic acid **4** and **5** is a mixture of the previous ones as described.[83,104]

Each mycobacterium includes a number of the classes. Moreover, within a class different homologues with different chain lengths are found and not single molecular species.[105] The particular ratio between mycolic acids classes is usually used as a taxonomic differentiator factor.[106]

Mycolic acids are characterised by having two chiral centres at the α and β positions in the corynomycolate motif.[98] Modifications can be observed as well at two points in the mero chain, known as distal (furthest from the β-hydroxy acid) and proximal (closest to the β-hydroxy acid). Such modifications divide the mero chain into three parts.

Functional groups present within the chain influence polarity, giving three major complex structures which can be seen after TLC analysis and further purification shown in order

of increasing polarity: cyclopropyl mycolic acids (called alpha) which contains two cyclopropane rings either in *cis* or *trans* configurations (Fig 15, **2**), methoxy-mycolic acids (Fig 15, **3**) and keto-mycolic acids (Fig 15, **4**).

The most commonly seen class appears to be the α-mycolates which can either be present with two double bonds or two cyclopropane rings, followed by ketomycolates and finally methoxymycolates.[98]

Several authors have done detailed work on mycolic acid composition for the different species. Minnikin included a detailed composition of *M. bovis* BCG and after him so did other authors.[107,108]

It is thought that the biosynthesis of mycolic acids proceeds by condensation of a number of shorter-chain fatty acids. The mechanism for antituberculosis drug isoniazid suggests that the composition of lipids in the cell envelope might be somewhat altered, inhibiting mycolic acid synthesis.[109,110] Those changes may produce changes in the cell envelope and cause cell death.[111]

The fatty acid component in α-mycolic acids may be modified by unsaturated bonds, methyl side chains or cyclopropane rings. The prefix *α* has been attributed to the first acids eluted from chromatography columns. Other mycolic acids, eluted after alpha-mycolates, are named after specific group functions presence such as methoxy-, carboxy-, epoxy-, or keto-mycolic acids (Fig 15).

From 1949-60, the use of chromatographic techniques revealed the variety of mycolic acids and later on NMR spectroscopy and mass spectrometry helped establish complete mycolic acids molecular structures. The initial problem found in purification was that the high temperatures used by GC caused the molecules to break in two fragments. The problem was solved by using stabilising trimethylsilyl groups allowing then the full use of GC-MS techniques.[112] Mycolic acid biosynthesis is less known than their structure. Different models have attempted to describe their biosynthesis and a two step model is commonly accepted. The model has a chain elongation process and a mycolic acid condensation but to date it has been difficult to establish in which order the processes occur.[113]

In the early 1970s, Lanéelle and Lanéelle studied lipids extracted from the 316F strain from *M. paratuberculosis*. The mycolic acids were obtained by saponification, purified by column chromatography and identified by mass spectrometry.[114]

Early approaches included X-ray studies of mycolic acids in monolayers.[97] The difference in fluidity of the cell membrane due to mycolic acids and the effect on the X-ray diffraction data obtained from it was pointed out previously.

The α-branch lacks double bonds or any cyclopropane which may produce or increase fluidity by bifurcating the mycolic acid. Comparatively, the proximal position on the meromycolate motif can have a double bond or a cyclopropane unit which usually is *trans*. This configuration benefits mycolic acid 'packing' and decreases membrane fluidity. As we approach the distal position of the meromycolate motif, units are more often in a *cis*-configuration which increases the 'separation' of the whole structure consequently increasing membrane fluidity. This particular pattern appears to be inverted compared to Gram-positive organisms.[81]

If we take into account the β-OH group of the mycolic acid it is noticed that this group forms hydrogen bonds which stabilize the structure, benefitting 'packing'.

Mycolic acids are usually found in nature linked to sugars. In the cell wall of mycobacteria they are esterified with arabinose and with trehalose or waxes in cord factors.[6]

1.5 Cord factors

Trehalose derivatives include two classes: trehalose dimycolates and sulpholipids. Trehalose dimycolates consist of a sugar unit, the disaccharide trehalose which is esterified either at the 6 or at the 6-6' position of it primary alcohol groups with mycolic acids (Fig 16).

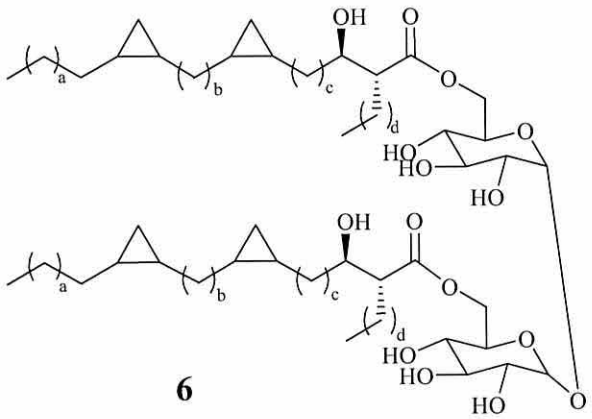


Fig 16. Model cord factor including trehalose and mycolic acid units.
This representation, although used by several author does not represent angles correctly.

1.5.1 Trehalose dimycolates (TDM)

M. tuberculosis has a variety of lipids within it cell wall.[81] From these, TDMs are present in the outer envelope of all mycobacteria and have unique properties. They appear to be the most abundant surface lipid extractable from *M. tuberculosis* comprising about 2 % of its dry weight.[83,115] This value may vary considerably between species and for some is poorly quantified.

TDM is formed by a sugar core, trehalose, which is a non-reducing disaccharide containing two glucose molecules linked by a α,α-1,1-glycosidic linkage. This disaccharide can be found as well in the cell envelope as a glycolipid component.

Trehalose monomycolate (TMM) is precursor of TDM, and is involved in providing mycolic acid to the cell wall arabinogalactan in order to form but it also appears to serve as the donor of mycolic acid residues to the cell wall arabinogalactan to form m-AGP

complex.[93] It is commonly found in bacteria, insects, invertebrates and plants where it acts as a source of energy and carbon.[116] Among its functions are preventing dehydration, stress, heat or oxidation (Fig 17).[117,118] Of the three possible existing anomers, α,β-1,1-, β,β-1,1-, and α,α-1,1-, only the last one is synthesised by organisms.

Trehalose has been regarded as a lipid preserver and stabilizer in membranes due to axial-axial linkage of the disaccharides.[119]

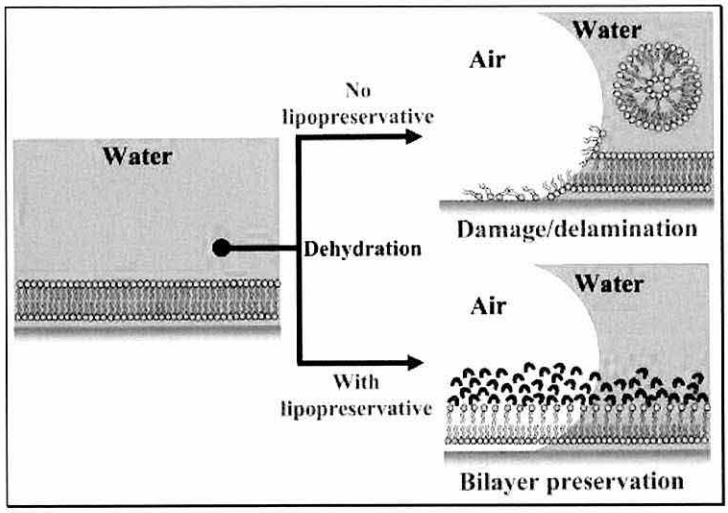


Fig 17. Dehydration of supported phospholipids membranes. Upper-Right Panel: In the absence of a lipopreservative the thin lipid film spontaneously reorganizes and delaminates from the solid support. Lower-Right Panel: The presence of a lipoprotectant suppresses damage and delamination.[119]

Trehalose coupled with glycolipids is not that common in nature, so it presence is related with membrane damage and anhydrous preservation as well with membrane functionality.[120]

Shorter chain Corynebacteria and nocardia species contain trehalose glycolipids but they differ from those found in mycobacteria.[121] Apart from being used as structural components biological functions are not known.

Mycolate synthesis needs an α-glucosyl-containing sugar to be present in the medium. The hypothesis of the coupling between the trehalose motif and the mycoloyl residue occurring outside of the cell has been proved by incorporating ^{14}C. A postulate exists that trehalose monomycolates are precursors of both trehalose dimycolate and cell wall mycolates.[122]

Wax isolation and purification was reported by Azuma and Yamamura. Biological activity and structure elucidation were detailed for the shorter mycolic acid chain ($C_{68}H_{136}O_4$).[123]

Trehalose lipids found in mycobacteria can contain different groups such as sulphate or other type of fatty acids, such as phthienoic acids.[124,125] The presence of such a variety may have a role which so far has not been elucidated.

1.5.2 History of cord factors

In 1884, Robert Koch, discovered that growing tubercle bacilli formed filaments or 'cords'. This is a toxic glycolipid discovered by Bloch when he extracted it from mycobacteria with petrol ether. The name 'cord factor' is derived from the erroneous belief that this factor was responsible for the characteristic 'serpentine cord' appearance of colonies of *M. tuberculosis.*[126]

In 1947, this characteristic serpentine cord formation in *M. tuberculosis* and the ability of avirulent species to absorb the dye natural red led to the hypothesis that the cell wall was implicated in virulence.[127] Since then, the search for the component responsible for such effects was the target, uncovering two families of trehalose based lipids: cord factors and sulfolipids (SLs).

Identified in the early 1950s, cord factors were described as being the lipid component of virulent bacteria which were toxic to mice and were present in filtrates from virulent bacteria cultures.[128] Later studies showed no relation between cord formation and virulence in *M. tuberculosis.*[101] Following their discovery, lipid extracts from tubercle bacilli were applied as a dermal injection to mice in order to detect toxicity and leukocyte migration.[129]

Noll *et al.* showed that the cord factor was in fact a 6,6-dimycolate of α,α–D-trehalose and although the precise structure was not known at that time they proposed an approximate formula (Fig 18, **7**).[130]

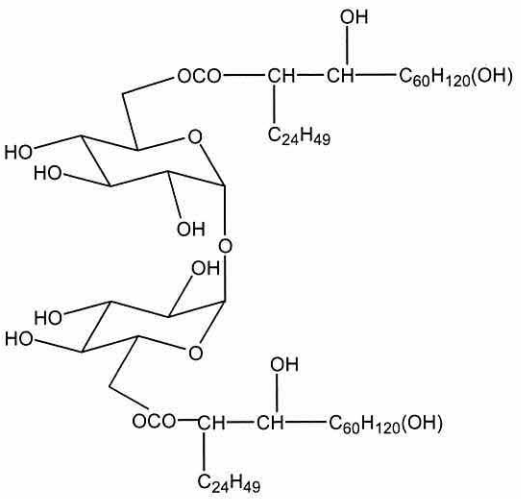


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Fig 18. Proposed structure of 6,6-dimycolate of α,α–D-trehalose **7** by Noll *et al.* The structure is presented as published in the paper.[130]

Natural diesters of trehalose have been isolated from different bacteria since the 1960s. Vilkas *et al* isolated from *M. fortuitum* an asymmetrically substituted trehalose diester where the substituents were mostly palmitic and tuberculostearic acids, which structures were determined by MS.[131,132]

In 1963 Ioneda *et al* described a glycolipid isolated from *Corynebacterium diphtheriae* and they proved that the substance (m.p 110-115 °C $[\alpha]_D$: + 64° ($CHCl_3$)) was a trehalose diester containing similar proportions of corynomycolic ($C_{32}H_{64}O_3$) and corynomycolenic acid ($C_{32}H_{62}O_3$).[133,134]

Toubiana *et al* (1975) extracted and purified dimycolates of trehalose from the strain Brevanne by C wax hydrolysis of the tubercle bacilli (Fig 19, **8**).[135]

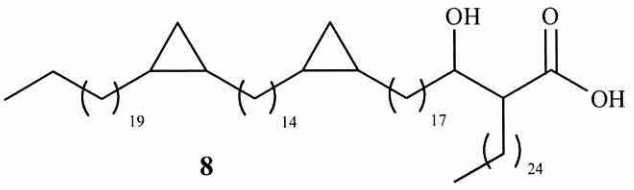

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Fig 19. Mycolates attached to trehalose from Brevanne strain.[135]

In 1976, Prome *et al* isolated mixtures of α-D-trehalose dimycolate and monomycolate from *M. phlei* as trimethylsilyl derivatives according to the polarity of the fatty acid residues.[136]

Takayama and Armstrong identified in 1977 a novel disymmetrically substituted ester, from H37Ra strain and involved in mycolic acid transfer into the cell wall.[137]

Toubiana *et al* (1979) isolated and characterised all types of mycolic acid (type α, β, γ) in a single strain of *M. tuberculosis Brevanne* and found molecular peak ion by mass spectrometry.[138]

1.5.3 Trehalose esters in other organisms

Trehalose diester can be found in mycobacteria related organisms such as *Brevibacterium thiovaginalis* or in *Arthrobacter paraffineus.* No trehalose esters have been isolated from higher organisms to date.[121]

1.5.4 Techniques of analysis used for cord factors

As described previously, TMM and TDM are based on a trehalose core and one or two mycolic acid attachments. The sugar moiety remains unchanged but not the mycolic acid which changes considerably depending on the mycobacterial species and even in the same species great variety occurs. For this reason careful analysis is required to establish this source of variety.

Classical structural identification of cord factors from *M. tuberculosis* was done by Bloch, Asselineau or Lederer. The usual separation method for cord factors is column chromatography of crude samples. Cord factor extracted from *C. diphtheriae* is in fact a mixture of corynomycolic and corynomycolenic acid esters. Because of that, TLC of mycobacterial cord factors usually shows a multiple spot. Noll and Bloch (1955) described the infra red spectra for natural cord factors with a 807 cm^{-1} peak for trehalose, 3070 cm^{-1} for the cyclopropane methylene and seven peaks at 997, 1022, 1057, 1079, 1104, 1152, 1175 cm^{-1}.[115]

Later, technique development allowed studies of protected glycolipids by mass spectrometry. The spectrum usually showed an m/e peak for both trehalose monoester and diester. The main fragmentation affects the glycosidic bond of the disaccharide. Retrospectively, mass spectrometry was used to identify structures in glycolipids mixtures such as the lower cord factor homologue **9**, **10** found in *Corynebacterium diphtheriae*. Schematic structures can be seen below.[139]

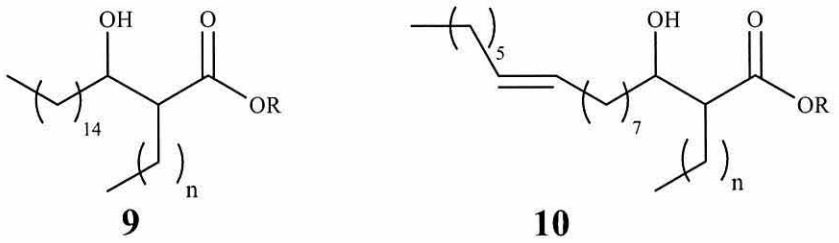


Fig 20. Saturated and unsaturated acids obtained from *C. diphtheriae*. n= 13, 11, 9 showing homologue variety.[139]

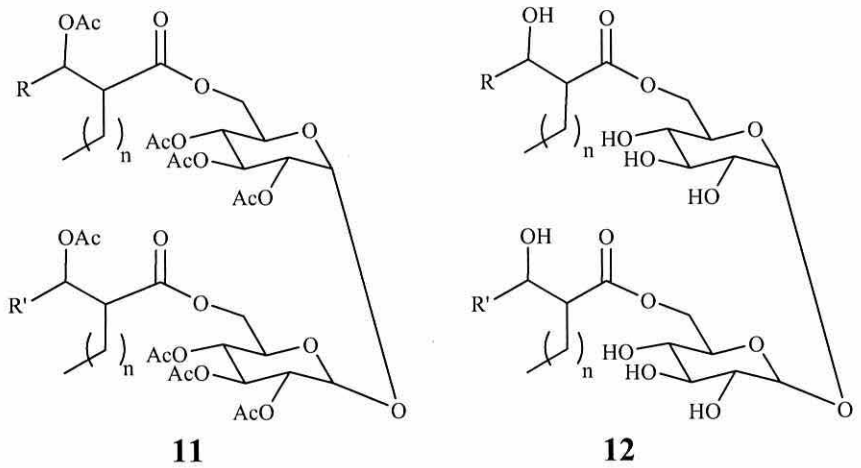


Fig 21. Peracetylated and glycolipids derivatives extracted from *C. diphtheriae*; n= 13, 11, 9; R and R' range from $C_{15}H_{29}$ to $C_{15}H_{31}$.[139]

A later paper by Adam *et al* described mass spectrometry for peracetylated derivatives of both natural and synthetic trehalose diesters with mycolic acids ranging from C_{22} to C_{90} and purified by column chromatography **11**, **12** (Fig 21). The three species targets in the experiment were *M. tuberculosis*, BCG and *M. butyricum* which produce different type of mycolic acids and consequently different cord factors.

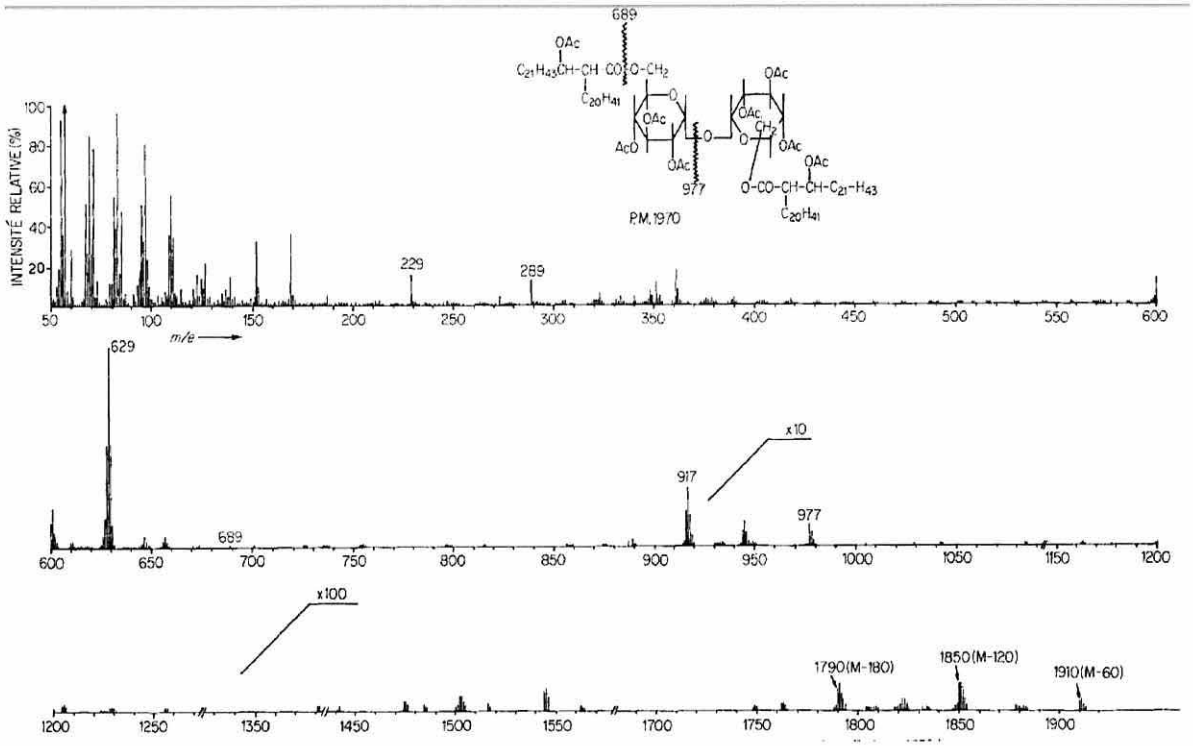


Fig 22. Mass spectrum of methylated trehalose derivative by *Adam et al.*[140] Axis show relative intensity (vertical) and m/e (horizontal). No molecular ion peak is observed but (M-60) at m/e 1910, (M-120) at m/e 1850 an oxonium ion at m/e 977 and 917 after losing 60 m.u.

The problem found here was that the molecular weight of the structures exceeded 3000 and no molecular ion peak was detected (Fig 22). Instead an oxonium ion confirmed structures as each trehalose pyranose cycle is substituted by a mycolic acid. A problem occurred as they could not differentiate between asymmetric substitution patterns in positions 6 and 6' of the sugar.[140]

Characterisation of *Corynebacterium matruchotii* by Puzo *et al* using electron impact mass spectrometry (EIMS) showed the existence of three different compounds, one called the 'true' coryno-cord factor and two 3-oxoacyl containing trehaloses. Differences between these compounds were found in the degree of unsaturation derivatives of the aliphatic chains.[141]

On the other hand, NMR is an effective tool for studying mycolic acid structure as it is for cord factors. A variety of studies used ^{13}C NMR, ^{1}H NMR and MS to elucidate the final cord factor structures.

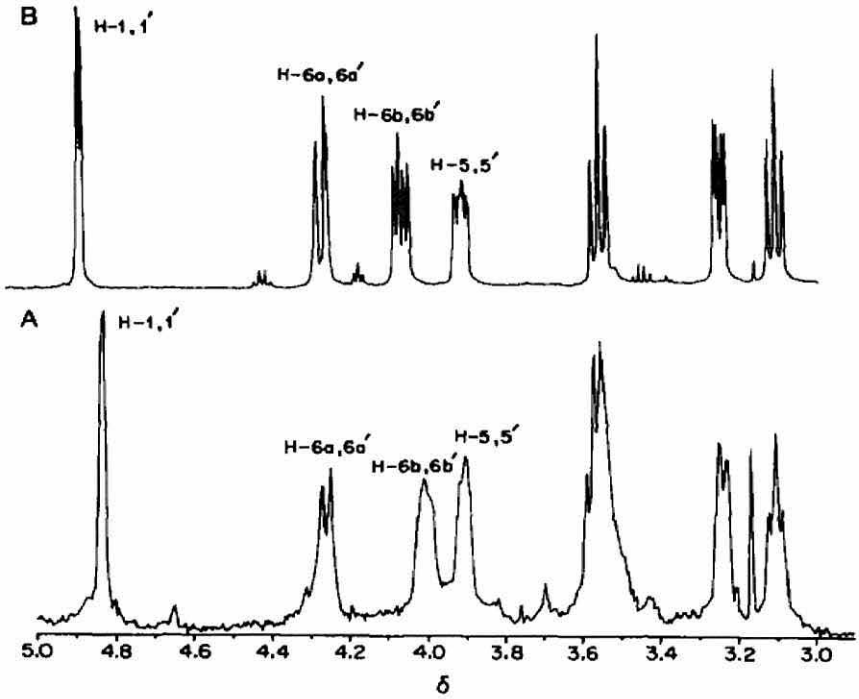


Fig 23. ^{1}H NMR segment comparison for both purified (A) trehalose dicorynomycolate and synthetic (B) samples of trehalose 6,6' dipalmitate from *Corynebacterium matruchotii*, Datta and Takyama.[142]

When comparing purified samples of natural trehalose dicorynomycolate (C_{32}) of *C. matruchotii* with synthetic trehalose dipalmitate, both ^{1}H NMR spectra looked similar as shown in the Fig 23 for the sugar region containing the CH and CH_2 signals. The ^{1}H NMR spectrum of the natural compound appears to be less simple due the fact that only one isomer is present. Determining the positions of acyl groups on trehalose using NMR gives some advantages over EIMS such as the simplicity of the method and the certainty of the results.[142]

Recently, TDM/TMM analysis using matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) for characterisation and identification has been used. This technique is characterised by giving rapid analysis with minor preparation, not only for TMMs and TDMs but also for mycolic acids. Compared to other invasive techniques, MALDI-TOF does not require any degradative process.[143]

When applied to mycolic acids, MALDI-TOF provides accurate molecular mass determination. The use of 2,5-dihydroxybenzoic acid (2,5-DHB) as matrix allows low material requirement, less than 10 pmol to establish analysis.[144] When selecting the right

conditions, such as laser intensity, number of shoots, matrix, solvent and dilution, the molecular weight detection range can vary from 700 to 3500. It must be said that laser resolution decreases dramatically when approaching these two limits. Another problem arises from the use of the matrix itself, as 2,5-DHB interferes with analysis when molecular weight falls under 700. This problem can be solved by changing the matrix for a more appropriate one, such as titanium dioxide in ethylene glycol (2 %). Comparatively TiO_2 shows a much lower interaction of the matrix with the laser during ionisation.

Mycolic acid analysis has been performed using electron impact mass spectrometry (EI/MS),[138] fast atom bombardment mass spectrometry (FAB/MS),[145] and GC/MS.[146,147] Some problems arising from those methods were: EI/MS and FAB/MS could not analyse samples whose molecular mass were higher than 1400 Da for TMM and 2600 Da for TDM. Similarly, GC/MS disadvantages were limited chain length analysis.[143]

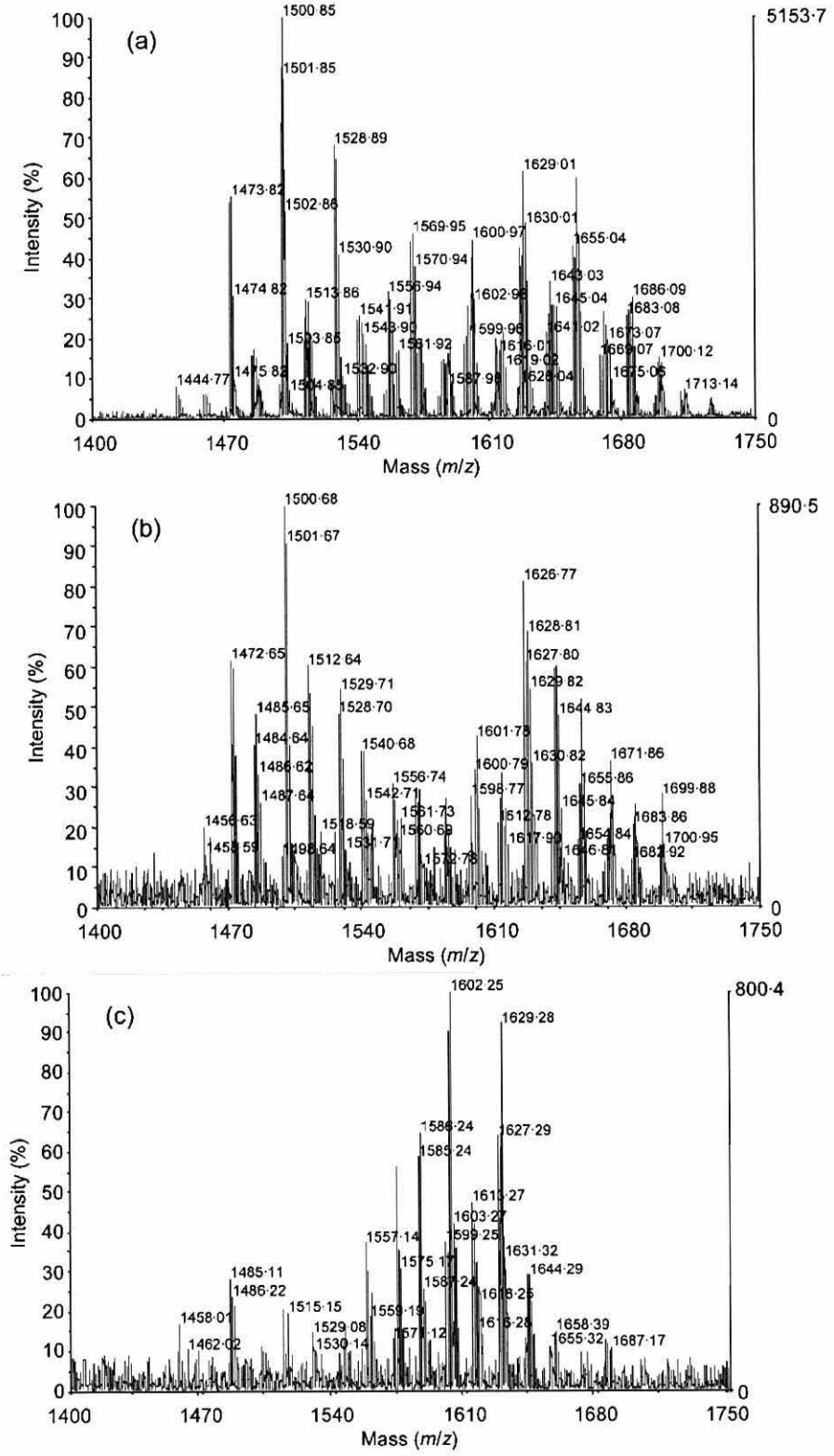


Fig 24. Accurate mass MALDI-TOF analysis. (a) shows α-mycolic acid methyl esters of TMM from *M.Tb*. (b) shows TDM from *M.Tb* extract and figure (c) shows a bound lipid of *M.Tb*.[143]

α-Mycoloyl TMM of eleven mycobacteria, including different strains of *M. tuberculosis* and *M. bovis* showed a series of even and odd number carbon ranging C_{75}-C_{85}. Strains of *M. tuberculosis* showed a MALDI biphasic distribution due the fact that α-mycoloyl TMMs have a lower mass range than methoxy- or ketomycoloyl. Abundant odd carbon (C_{77}, C_{79}, C_{81} and C_{83}) α-mycoloyl TMM showed monoenoic mycolates mass numbers compared with the even carbon having dienoic mass numbers.

These trends apply for methoxy (C_{85}, C_{87}, C_{88}, C_{89}) and ketomycoloyl (C_{87}, C_{88}) TMM.[143] Minnikin *et al* found a lack of methoxymycoloyl TMM in BCG Connaught.[107] The biphasic mass ion distribution patterns of α-mycoloyl TMM centred at C_{78} and ketomycoloyl TMM ranging from C_{82} to C_{91} corroborate these results.[143]

M. Kansasii α-mycoloyl TMMs have mainly odd-carbon-numbered monoenoic α-mycolates. In contrast, *M. bovis* has mainly even-carbon-numbered dienoic (C_{76}, C_{78}, C_{80} and C_{82}) mycolates. The same happens with other species, such as *M. phlei* and *M. smegmatis*. *M. tuberculosis* α-mycolic acids from TMM contained abundant C_{77}, C_{79}, C_{81} and C_{83} odd-carbon numbered mycolic acids, as shown in the Fig 24(a). In contrast TDM and cell-wall-bound lipids (Fig 24(b), (c)) contained abundant even-carbon-numbered dienoic α-mycolates. Mycolic acid composition of each glycolipid appears to be more variable in TMM compared to TDM. This may be consistent with TMM being a precursor of TDM or cell wall arabinose.[143]

This shows the variable number of structures that TMM and TDM have.

Mass spectrometry allows fingerprinting glycolipids from a specific strain which may give information regarding mycobacterial glycolipids synthesis.

1.5.5 Historical perspective of synthesis of TDM/TMM and other trehalose esters

The first 6,6'-trehalose diesters **17** were synthesised by mixing the 6,6'-ditosylate of trehalose **15** with the potassium salt of the acid **16** in DMF.[148,149] Problems were the low yield, difficulty in preparing the ditosylate and the formation of side products (see Fig 25).

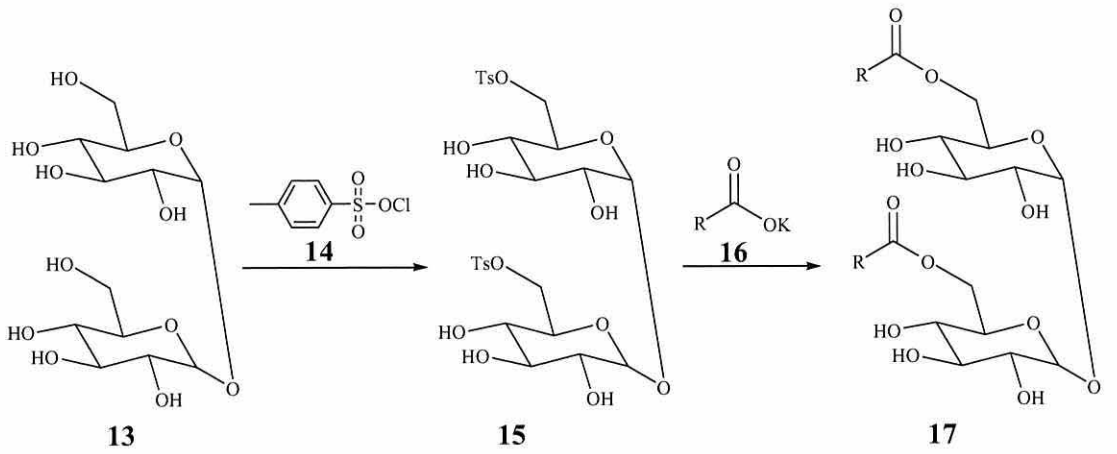


Fig 25. The synthesis of diacyl trehalose **17**. ClTs is tosyl chloride.

This method was modified by Polonsky *et al* by introducing toluene as a solvent instead of DMF and decreasing the temperature to 90 °C.[150] To increase cord factor yield the crown-ether 18-crown-6 was used as a catalyst.

The process shown in Fig 26 describes the reaction between a tosylated 6,6' protected trehalose molecule **15** with '18-crown-6' and the potassium salt of a mycolate **16** (with different chain lengths) in toluene for 4.5 hrs at 90 °C, to give the corresponding ester **17** (45 % yield, 83 % with recovery of the mycolic acid salt). The same reaction was achieved using a C_{44} and a C_{32} mycolate potassium salt.

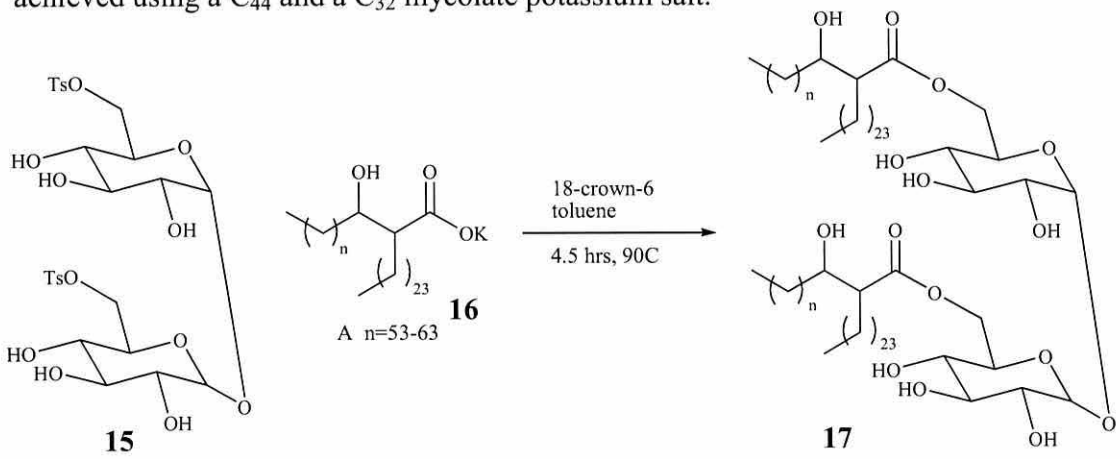


Fig 26. Synthesis of 6,6'-di-O-mycolyl-α,α-trehalose **17**.

Birch and Richardson described the advantages and simplicity of using trehalose due to the fact it has two-fold axis of symmetry which makes the two D-glucopyranose units indistinguishable chemically and physically.[151] Trehalose is then unusually symmetrical

about the central oxygen position.[152] Other properties are its being non- reducing because of the 1-glycosidic ether. Due to that, aqueous solutions of trehalose resist degradation between pH 3.5-10 at 100 °C and do not react with amines, amino acids or proteins. These and many other properties are related to its structure, symmetry, solubility or thermodynamic stability, making trehalose based compounds very attractive for clinical, pharmaceutical and immunological research.[153]

The esterification of α,α-trehalose dihydrate with methanesulphonyl chloride gave a mixture of two products, the 6-ester and 6,6'-diester, but in poor yield (8 %).[151] Modification of reaction conditions such as increasing the quantity of acid chloride, did not improve yields. Instead, bulkier substituent were tried to improve selectivity, which partially worked.

Synthetic routes to trehalose mycolates were investigated by several groups as described below. In most procedures, the ester bond is generated by the displacement, by the mycolate ion, of a leaving group (e.g., sulphonate or halide) from the C-6 atom(s) of trehalose, usually protected at its secondary position by O-(trimethysilyl) or O-benzyl groups. Methods for esterification of carboxylic acids by reaction of their salts with alkyl halides (bromides or iodides) in HMPA were described by Shaw *et al* in 1973.[154]

In 1972 Hanessian *et al* studied an efficient halogenation of carbohydrates **13** by treating them with triphenylphosphine and N-halosuccinimide (bromo, chloro and iodo), leaving by-products of succinimide and triphenylphosphine oxide which can be separated.[155] An important characteristic in halogenations using Ph_3P and N-halogenosuccinimide is the selectivity observed, where only the primary hydroxyl position reacts not the secondary one **18** (Fig 27).

When the amount of halogenating mixture was changed from two to four equivalents relative to trehalose, the major product was 6,6'-dibromo-6,6'-dideoxy-α,α-trehalose **18** in 62 % yield. On the other hand, the 6,6'-diiodo derivative could be obtained in 55 % yield when using a large excess of halogenating agent.[152]

Figure 27 shows the protecting and halogenating process which can be done either with bromine, fluorine or iodine. Deacetylation of the bromo derivatives gave 6,6'-di-bromotrehalose in 80 % yield.

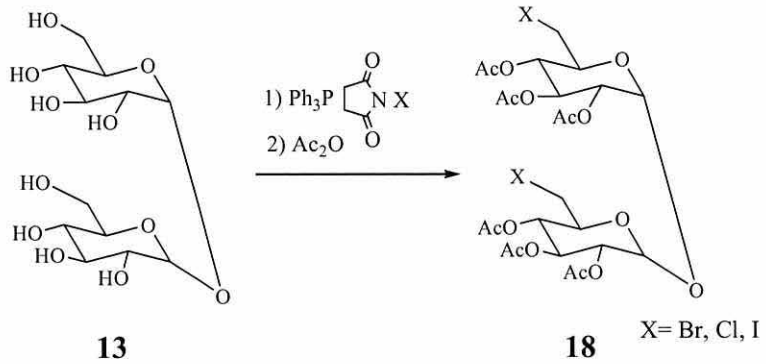


Fig 27. Halogenation and protection of anhydrous trehalose. X represent Br, Cl or I.[152]

Early synthetic methods proposed in 1956 included preparation of the mycolic acid potassium salt **20** which was then coupled to the protected trehalose 6,6'-ditosylate **19** (Fig 28).[148,156] The tosylated trehalose will behave like the corresponding halide in facilitating nucleophilic substitution (S_N2);[157] however, saponification of acetyl protecting groups in this case produced partial mycolic acid loss. Coupling conditions for the reaction were refluxing in DMF (130 °C, 3 days) with the ditosyl trehalose and 2 mol eq. of eicosyl-2 hydroxy-3 tetracosanoic. Substitution of OTs by RCOO gave **21** in good yield (80-90 %). Without protection of the trehalose secondary hydroxyl groups, the reaction proceeded with lower yields (40 %).

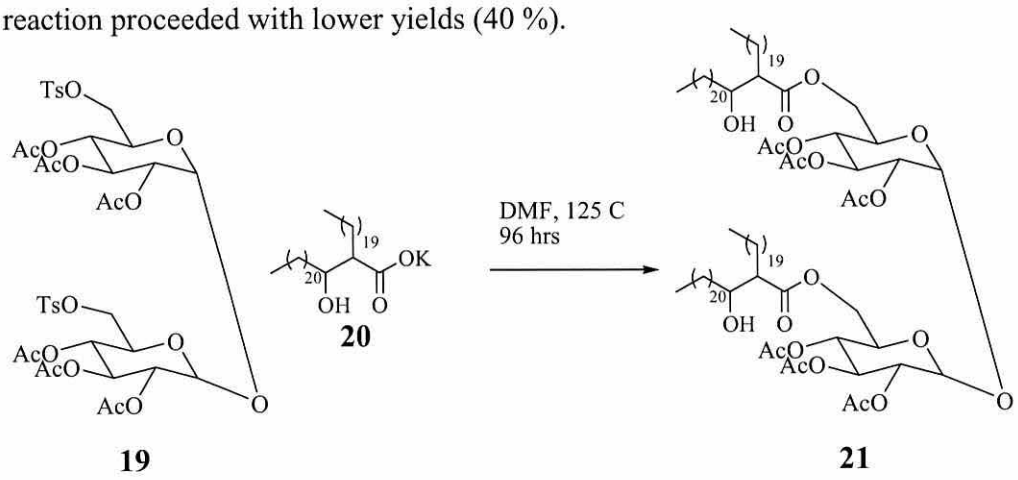


Fig 28. Reaction of the hydroxy acid potassium salt **4** with ditosyl 6,6' hexacetyl-2,3,3,2',3',4' trehalose **19**.

At the same time another method proposed was the reaction of the acid chloride corresponding to **20** (with the hydroxyl position acetylated with acetic anhydride/pyridine) coupled to anhydrous trehalose dissolved in pyridine. The problem

with the acid chloride method was the difficulty in controlling the mono-, di- and even triester produced. Yields for this reaction were really low and reaction times long (more than three weeks).[158]

Toubiana and Toubiana proposed a different approach, synthesising 6-O-acylated mono- and disaccharides by transesterification using palmitic ester **22** (Fig 29).[157]

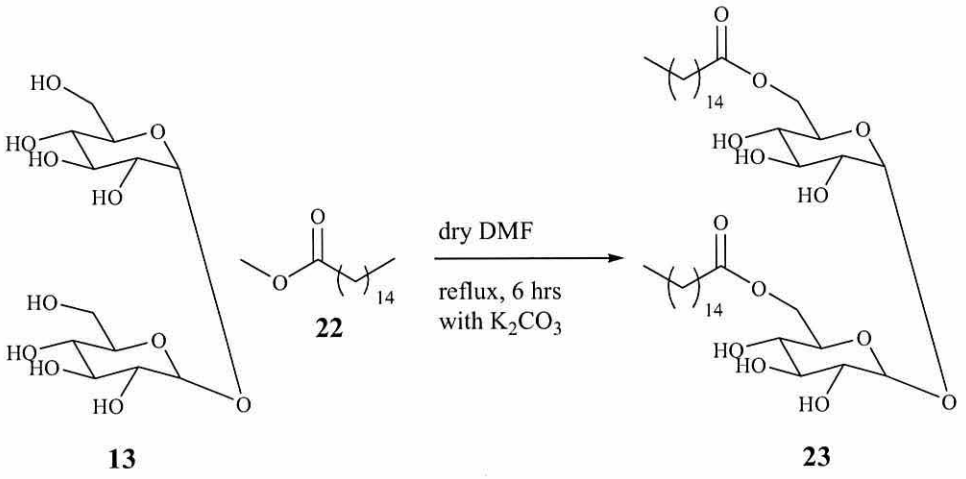


Fig 29. The synthesis of trehalose palmitate **23** using anhydrous trehalose **13**.

Methyl palmitate **22** and anhydrous trehalose **13** were dissolved in DMF to which potassium carbonate was added and the mixture was heated under reduced pressure. After 6 hrs under reflux and heat, solvent was evaporated and the residue was purified by column chromatography then precipitated in acetone and centrifuged to give the product, a variety of trehalose mono-, di-, tri- and tetra-palmitates. Products were analysed by IR, optical rotation and melting point and compared to naturally extracted samples from *M. tuberculosis*. Results showed superimposed IR data for both synthetic and natural extracts and MS of hexaacylated products with molecular ion peaks at m/e 1070 (M^+) and 527 (oxonium ion) corresponding to breakage of the glycosidic bond.[157] TLC showed a variety of esters corresponding to different degrees of substitution as well as their corresponding positional isomers. Transesterification is facilitated for primary alcohols but secondary ones may react as well under basic conditions leading to structures such as 2,2'-trehalose di-palmitate.[157] As a result yields remained low (10-20 %).

A similar method by Tocanne reacted a potassium salt of the mycolic acid with **25** (Fig 30) in hexamethylphosphoramide (HMPA).[159] Compounds **28**, **29** and **30** were prepared

by mixing compound **25** and the corresponding potassium salt (**26**, **27**, **2**) dissolved in HMPA. The resulting compounds were deprotected giving the final products **23**, **31** and **32** respectively. Compound **23** represents the TDM from palmitic acid, **31** the C_{32} and **32** the C_{80} natural TDM from human TB culture.

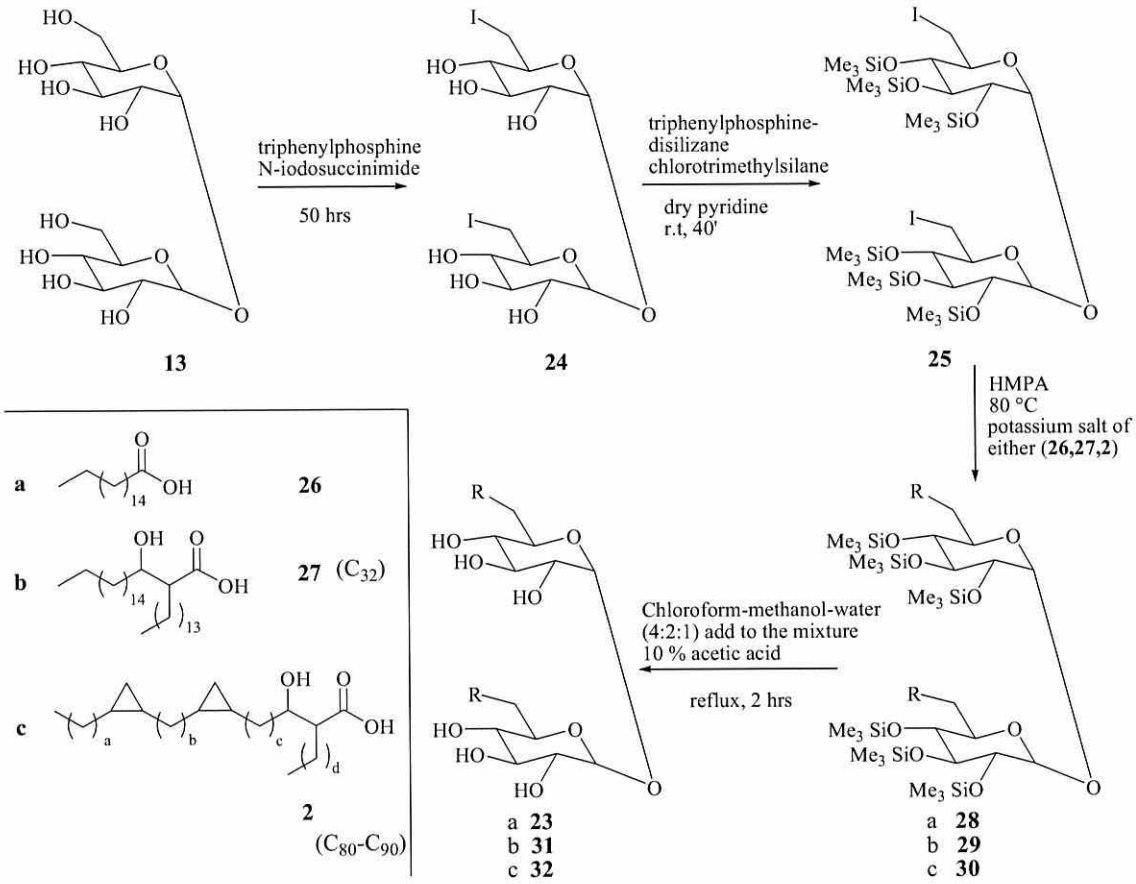


Fig 30. Synthesis of trehalose mycolate esters following the Hanessian and Lavallée method.

A longer acid chain required the reaction temperature to be increased, up to 80 °C, which seems to be the maximum tolerated without compromising the stability and reactivity as established for compound **27** (Fig 30). At higher temperatures HMPA catalyses alcohol dehydration in **27** and **2**. After reaction, protecting groups are eliminated by heating in a mixture of chloroform-methanol-water 4:2:1 (v/v) and dilute acetic acid for 2 hrs. Yields are acceptable for compounds **23**, **31** and **32** respectively (55, 65, 55 % respectively) (Fig 30).[159] Mixtures of both mono and di-substituted products were observed. For the palmitic acid potassium salts it must be taken into account that solubility in HMPA is poor, unlike mycolic acid salts.

It could be thought that during the desilylation step (Fig 30 compounds **23**, **31** and **32**) migration of ester function could happen, but due to the treatment time and low acidity this cannot happen very much. This paper represents an early cord factor synthesis due the fact that $C_{80\text{-}90}$ natural mycolic acids, **2** were attached to trehalose, although yields were not high (50 % for **32** (Fig 30)).[152]

A similar reaction using the corresponding dibromo-derivative **33** was described by Toubiana *et al.* in 1975 (Fig 31).[135] The 6(6')-bromosugar derivative **33** needed hydroxyl group protection with trimethylsilyl to avoid elimination or internal bond formation between iodine or bromine and the hydroxyl groups. Compound **34** was obtained by silylation of **33** with Tri-Sil Z (N-trimethylsilylimidazole-pyridine) at r.t for 12 hrs. The potassium/sodium salts of mycolic acids can be made and manipulated easily.

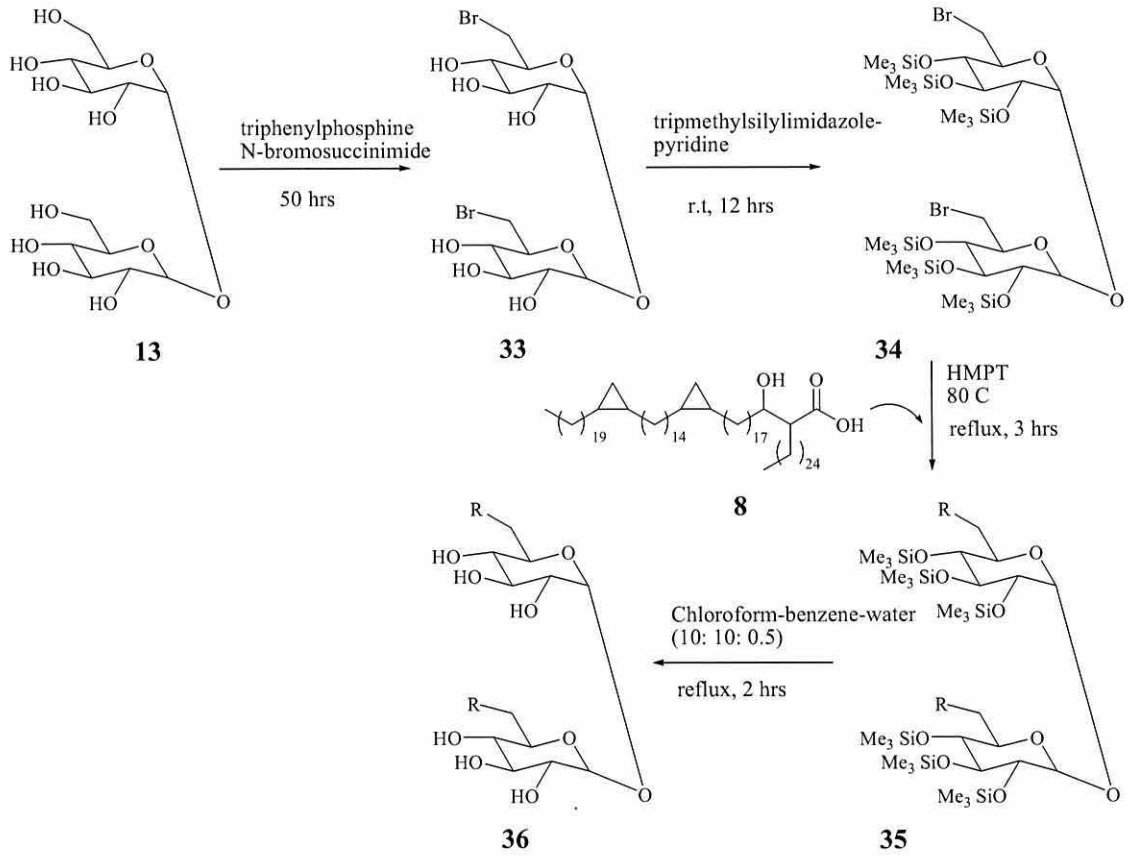
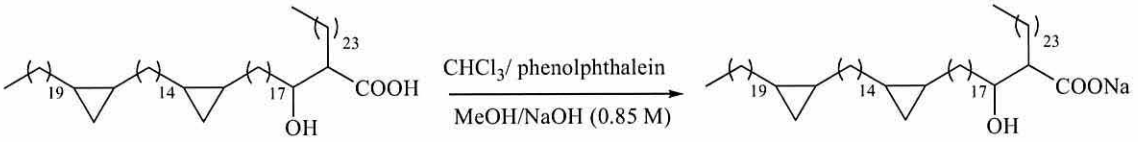


Fig 31. Synthesis of trehalose mycolates/dimycolates **36** following the Hanessian and Lavallée method.

Compound **35** was obtained by mixing the potassium salt of the natural mycolic acid **8** with **34** dissolved in HMPT.

Deprotection of **35** in a mixture of chloroform-benzene-water at reflux for 2 hrs gave the final product **36** in 33 % yield.

In this experiment, crude extracts of mycolic acid were purified by chromatography and the product was then dissolved in sodium hydroxide to get the sodium mycolates (Fig 31, **8**). An example of the mycolic acid salt formation can be seen when **8** was dissolved in chloroform, to which a drop of phenolphthalein was added. The mixture was then dissolved in MeOH/NaOH (0.85 M) and after being cooled for 12 hrs the product **37** was obtained as a white precipitate (Fig 32). The example shows a mycolic acid from *bacilli tuberculous* 'souche Brevanne' obtained after wax C saponification and purification.[135]



8 **37**

Fig 32. Mycolic acid sodium salt formation.

The S_N^2 reaction was used again, but HMPA was replaced by hexamethylphosphorus triamide (HMPT) and 6,6'-di-O-p-tolylsulfonyl-α,α-trehalose was replaced by a di-bromo protected trehalose **34** which appears to be easier to prepare and gave better yields (62 %) compared with 10 % for the tolylsulfonyl one. Once product **35** had been obtained by coupling **34** and **8,** removal of the $SiMe_3$ protection on carbons C2-C4 of the sugar freed the secondary alcohol positions. The final product **36** was obtained with 33 % yield.

The trimethylsilyl protecting method showed important advantages compared to p-tolyl sulfonyl protection. The first was to avoid preparing 6,6'-di-O-p-tolylsulfonyl-α,α-trehalose. Another is that the yield increased from 15 % to up to 62 % when using trimethylsilyl protecting instead of p-tolyl sulfonyl protection (yields are for the protected structure before coupling with the mycolate salt are shown in Fig 32).

Most of the methods reported so far had certain deficiencies (poor yields, production of multi esters, and formation of undesired by products). Because of that Yoshimoto *et al* (1982) employed **41** as a key intermediate (Fig 33) which was obtained starting from trehalose dihydrate **38** and following all step to reach compound **41**.[160]

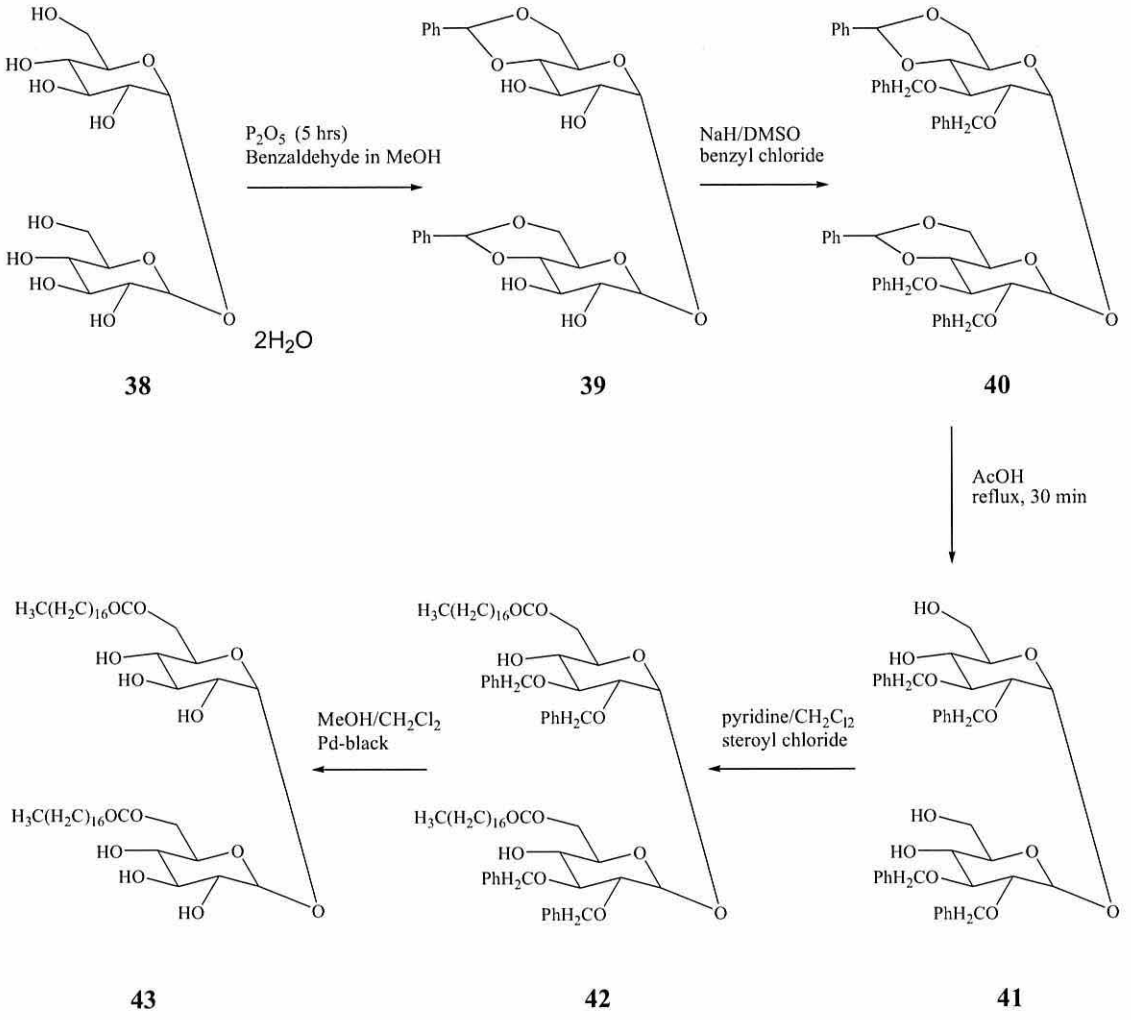


Fig 33. Esterification method proposed by Yoshimoto *et al* (1982).

Trehalose dihydrate **38** was converted into **39** using Evans[161] and Birch[162] methods using P_2O_5. Compound **39** was benzylated by first reacting NaH/DMSO under argon and stirring for 1 hr at r.t until salt formation occurred. Then addition of benzyl chloride in DMSO gave the desired compound **40**. Compound **41** was obtained by addition of AcOH to **40** and refluxing the mixture for 30 min. The esterification reaction between **41** and steroyl chloride occurred by adding pyridine and CH_2Cl_2 at 0°C and stirring at r.t overnight. Final debenzylation occurred by hydrogenolysis of **42** in MeOH/CH_2Cl_2 for 4 hrs, using Pd-black as a catalyst. This paper also introduces NMR data for the products obtained with close detail to ^{13}C shifts.

In 1984, Bottle and Jenkins reported a new, simple, mild and selective procedure for the synthesis of dipalmitate trehalose analogues, with good yield and directly for the free disaccharides using di-isopropyl azodicarboxylate (DIAD), triphenylphosphine and DMF as solvent in a Mitsunobu reaction (Fig 34).[163] Jenkins and Goren employed this reaction for the condensation of 3-O-protected mycolic acids with unsubstituted trehalose.[164] This mild reaction, first discovered in 1967 converts a hydroxyl group into a potent leaving group that is able to be displaced by a wide variety of nucleophiles.[165,166]

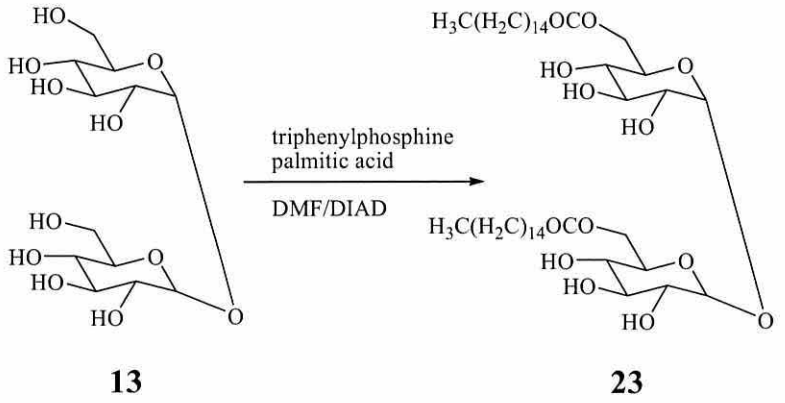


Fig 34. Esterification reaction between **1** and palmitic acid without protection of the sugar.

Yields were acceptable, with 55 % for the dipalmitate **23**.[163]

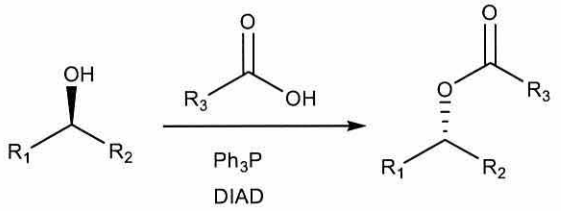


Fig 35. Mitsunobu reaction, showing alcohol stereochemistry inversion.

The mechanism of the Mitsunobu reaction (Fig 35) is fairly complex and still today is subject to debate.

Liav and Goren (1980) described a method to produce synthetic cord factor analogues and reduce deficiencies described in previous methods, such as poor selectivity, bad yield or tedious preparation (Fig 36).[167] Compound **44** was prepared according to Bredereck's method.[168] Benzylation of **44** in 2,4-dioxane gave **45** and **346** was obtained by detritylation of **45** with 80 % acetic acid.

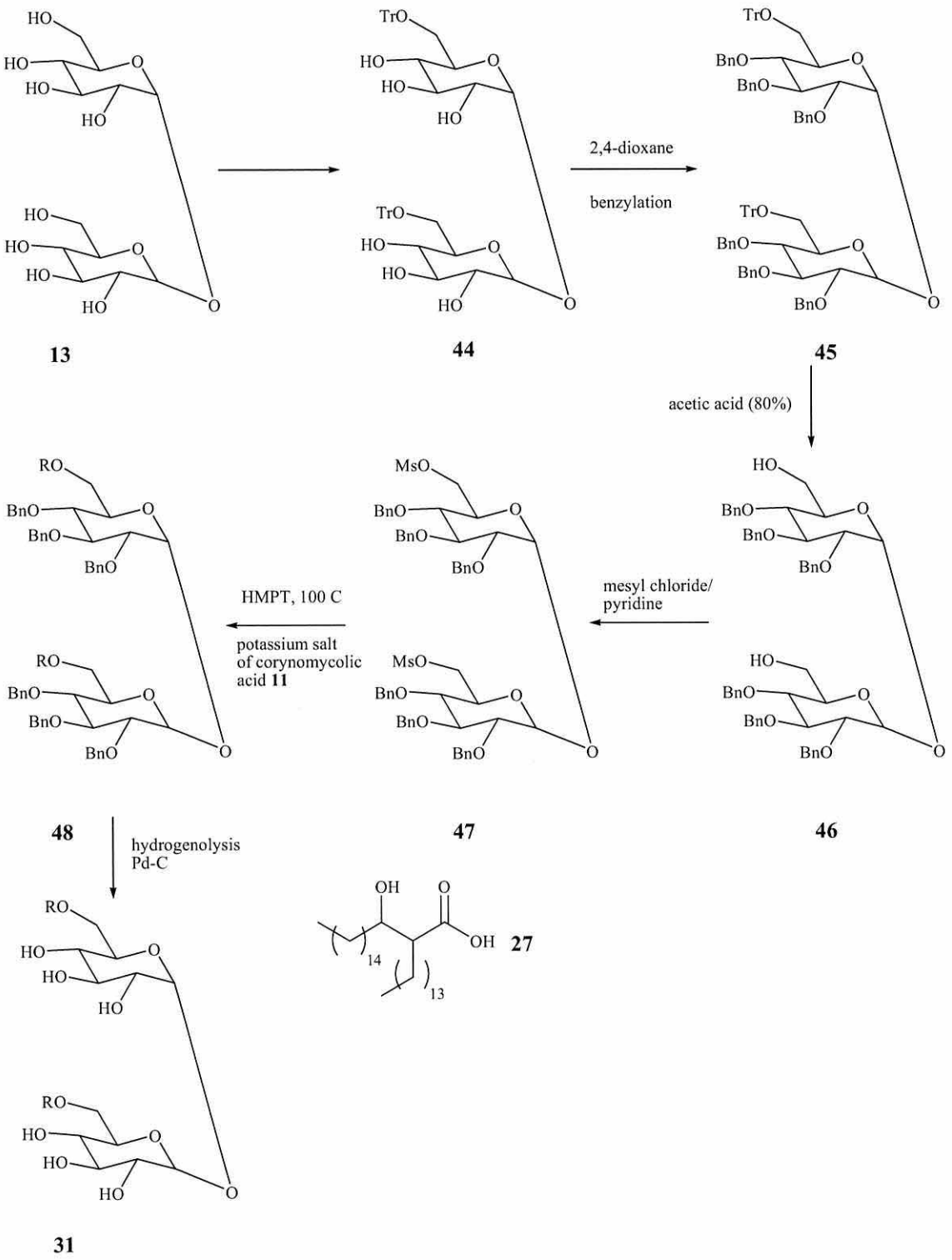


Fig 36. Synthetic path described by Liav and Goren for 6,6' trehalose analogues.

Compound **47** was then obtained by adding mesyl chloride in pyridine. Addition of the potassium salt of the corynomycolic acid **27** in HMPT as a solvent at 100 °C gave **48** which then was hydrogenolysed for 3 hrs over palladium-on-carbon to give **31**.[167] The paper indicates it is impossibile to using acylation an via acid chloride when dealing with (3-hydroxylated)mycolic acids so steps to **47** and **48** were used instead.

Numata *et al* synthesised various trehalose-6,6' diesters, such as linear C_{30}-acid **49**, α-branched β-hydroxy C_{32}-acid **27**, α-branched β-hydroxy C_{48}-acid **54,** the protected **52** (a α-branched β-hydroxy C_{32} acid with $OSiMe_3$ protection), and **53** (a α-branched β-hydroxy C_{48} acid with $OSiMe_3$ protection).[169] Figure 37 describes the steps followed to obtain compounds **31, 56** and **58**.[170] Compound **49** was hydrogenolysed with palladium black/MeOH to give **15**.

Compound **15** was silylated with chlorotrimethylsilane-hexamethyldisilazane in pyridine for 50 hrs to give **50**. Following the procedure, **57** was obtained from **50** and the potassium salt of 2-docosyl-3-hydroxyhexacosanoic acid **53** (83 %) still having silyl protection on the β-hydroxy acid. Finally compound **57** was fully deprotected under mild acidic conditions by adding $CHCl_3$/MeOH/AcH/H_2O. Compound **56** was obtained in the same way as **58**. The fact that compound **31** was esterified with a long chain acid with no hydroxyl group helps avoiding the need for $SiMe_3$ protection.

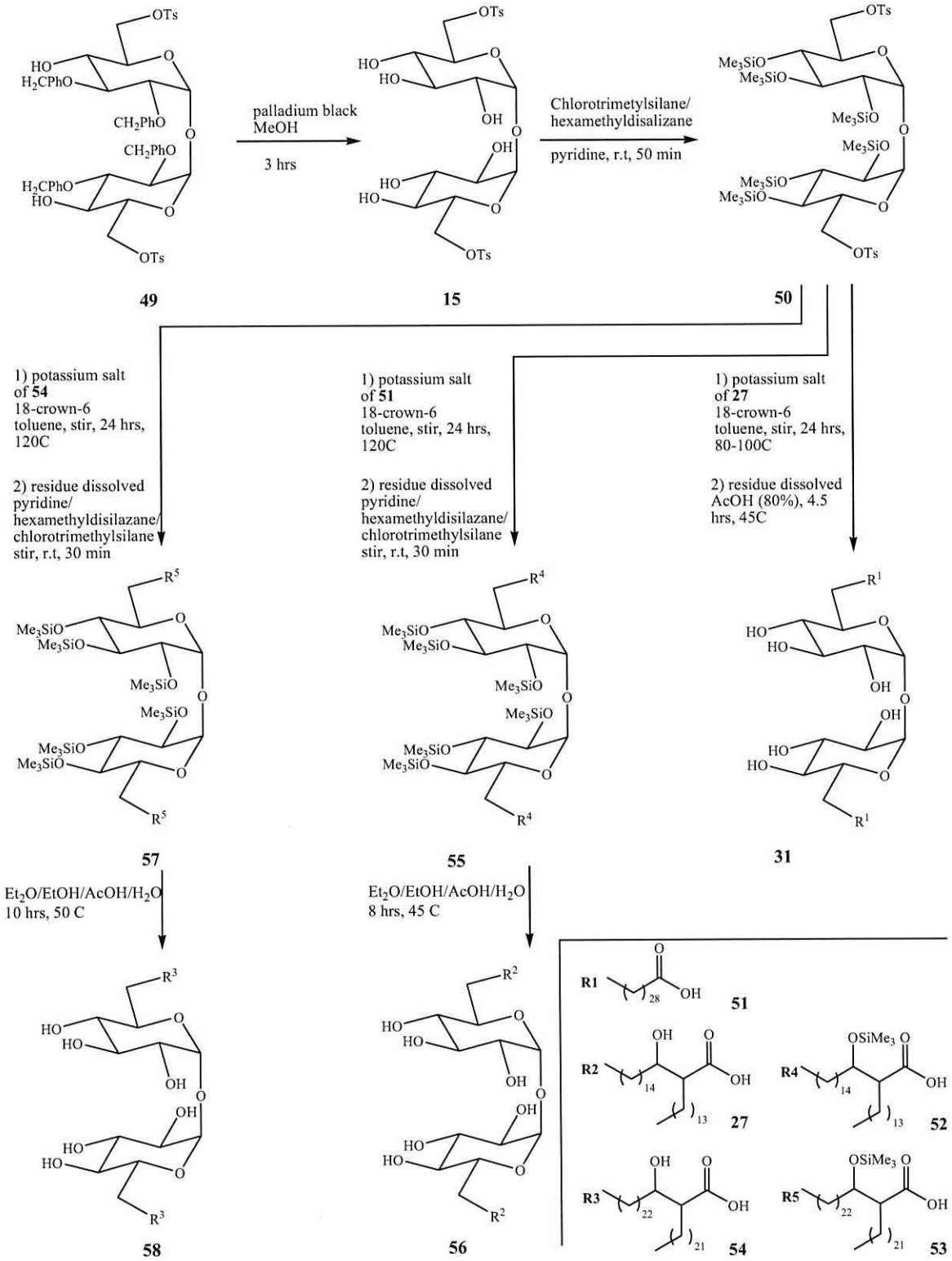


Fig 37. Synthesis using a protected trimethylsilyl trehalose proposed by Numata *et al* (1985).[169]

As methodology improved, more work was done regarding sugar protection with the aim of getting this sugar coupled to protected mycolic acids. In 1991, Datta and Takayama proposed coupling (hydroxyl-protected) acids to the partially trimethylsilylated sugar using dicyclohexylcarbodiimide (DCC) and 4-dimethylamino-pyridine 4-DMAP.[164] The partially protected trehalose 2,3,4,6,2',3',4',6'-octakis-O-(trimethyl)trehalose **59** was obtained as described by Toubiana *et al.* in 1975.[135] The method was necessary due the fact that the simplest mycolic acids, corynomycolates **27** are convenient substrates to an enzymatic reaction which has a key role in mycolic acid synthesis. This new method was aimed to avoid derivatized intermediates and the previous complicated procedures.

Groups to be used in sugar protection, more specifically within trehalose, are important as they have to be a compromise between stability during coupling reactions and easy removal when required. Due to this, the preferred protecting group for trehalose is trimethylsilyl ether. Trehalose dihydrate is fully protected by using a previously described method with pyridine/trimethylsilylchloride/hexamethyl-disilazane, giving the octakis-O-trimethylsilyl trehalose **59** (Fig 38).[135]

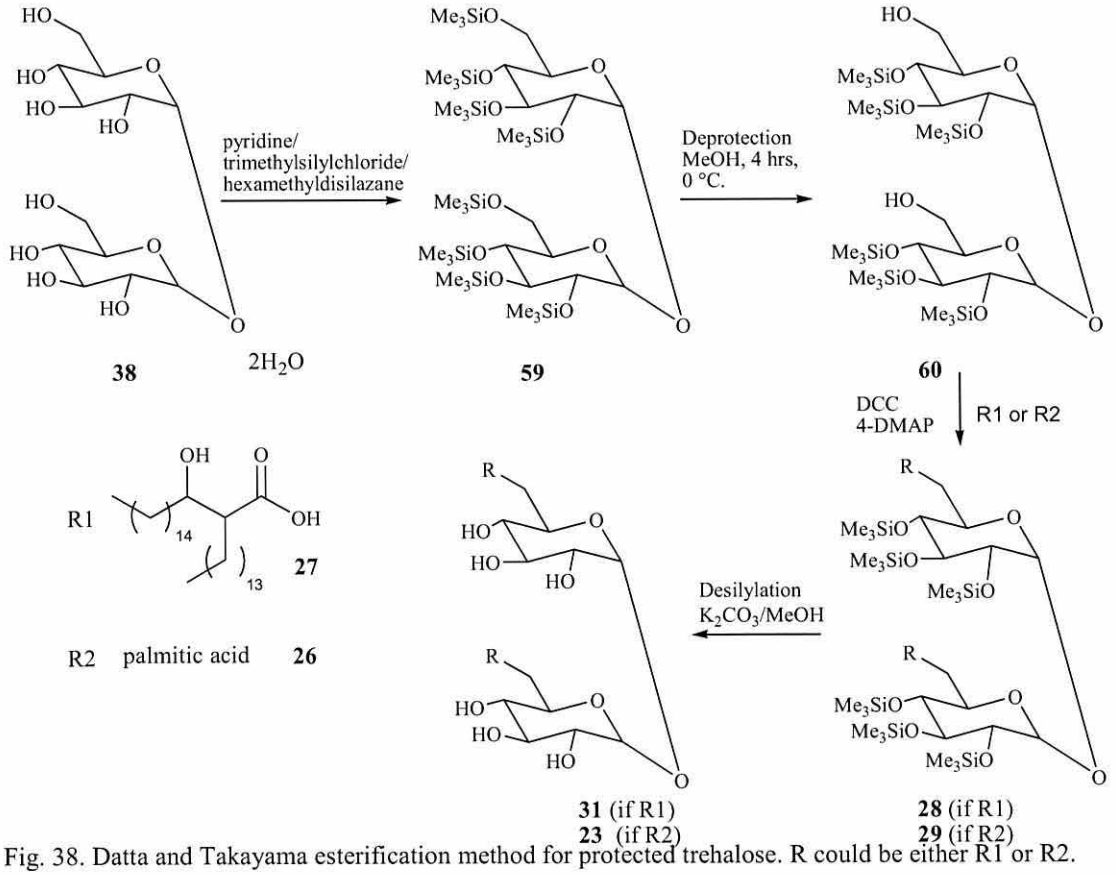


Fig. 38. Datta and Takayama esterification method for protected trehalose. R could be either R1 or R2.

Selective deprotection of the 6,6' silylated positions in **59** with methanol and potassium carbonate gave **60**.[135] The reaction of either **52** (Fig 41) or palmitic acid **26**, both in excess, with **60** (Fig 38) gives diesters **28** and **29**. The coupling takes places in the presence of dicyclohexylcarbodiimide (DCC), 4-DMAP as a catalyst and molecular sieves. The mixture was the vacuum dried for 4 hrs the stirred for 8 hrs at 60 °C using toluene as solvent. Final products **23** and **31** were obtained by gentle desilylation of **28** and **29** with methanolic potassium carbonate at r.t for 3-4 hrs.

1.5.6 Model compounds for the esterification reaction with trehalose and other sugar like compounds

Figure 36 shows that it is impossible to use the simple method of acylation via an acid chloride when dealing with 3-hydroxylated mycolic acids. The main reason for this is that the unprotected alcohol in the β-position is easily affected and reactive. Due to that it is important to prepare model compounds and test alcohol protection reactions in molecules with similar structures to mycolic acids but which are simpler and less costly make. These simpler molecules should help modelling steps regarding protection, coupling and decoupling methods which then may be applied to real mycolic acids in order to achieve reaction success and high yield.

Many authors have reported methods to make simple compounds with structures modelling real mycolates. Heathcock and Lampe used an aldol reaction of benzaldehyde with t-butylketones to get the corresponding β-hydroxy acids.[171] Figure 39 shows four ketones which were converted into lithium enolates by using LDA in THF (-78 °C) or pentane at 25 °C and then trapped with the aldehyde. Deprotonation of 'a' occurs in 20 min whereas 'd' takes more than 4 hrs.

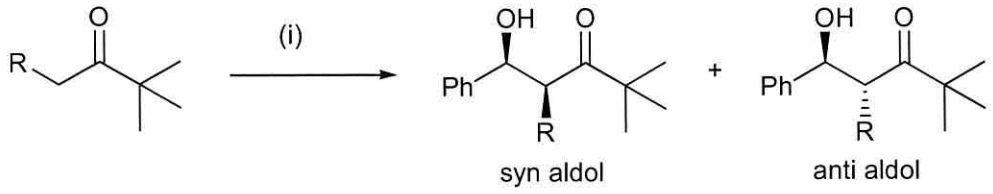


Fig 39. (i) LDA, solvent; PhCHO
a R=Me, **b** R= Et, **c** R= n-Pr, **d** R= n-Bu

This variation in the enolization corresponds to Ireland's model where an increase of the size of the R group correlated to reduced enolisation as seen in the Figure 40 below.[172]

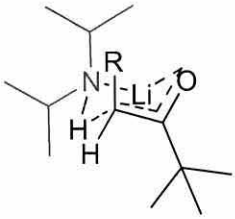


Fig 40. Effect of size of R on the enolization process.

Syn-aldols will be obtained when the reaction solvent is THF or pentane at -78 °C. On the other hand, pentane at r.t will give *anti* aldol products. The *syn:anti* ratio decreases as the chain length of R increases.

The first of set of reactions include a model compound, the C_{32} corynomycolic acid **27,** the synthesis of which is described in Fig 41. This compound had seemed a good starting point in TDM synthesis due its relatively easy synthesis. Dry methyl palmitate **22** was coupled via a Claisen condensation using NaH as base to get the keto-ester **61** after being neutralized with acetic acid. $NaBH_4$ was used to reduce the carbonyl to the hydroxyl to give stereomeric products **62**, due to the chiral centre, which were separated by chromatography. Saponification of **62** in 5 % KOH and biphasic butanol/water for 6 hrs at 120 °C followed by HCl acidification will hydrolyse the methyl ester to the corresponding carboxylic acid, giving β-hydroxy acid **27**.

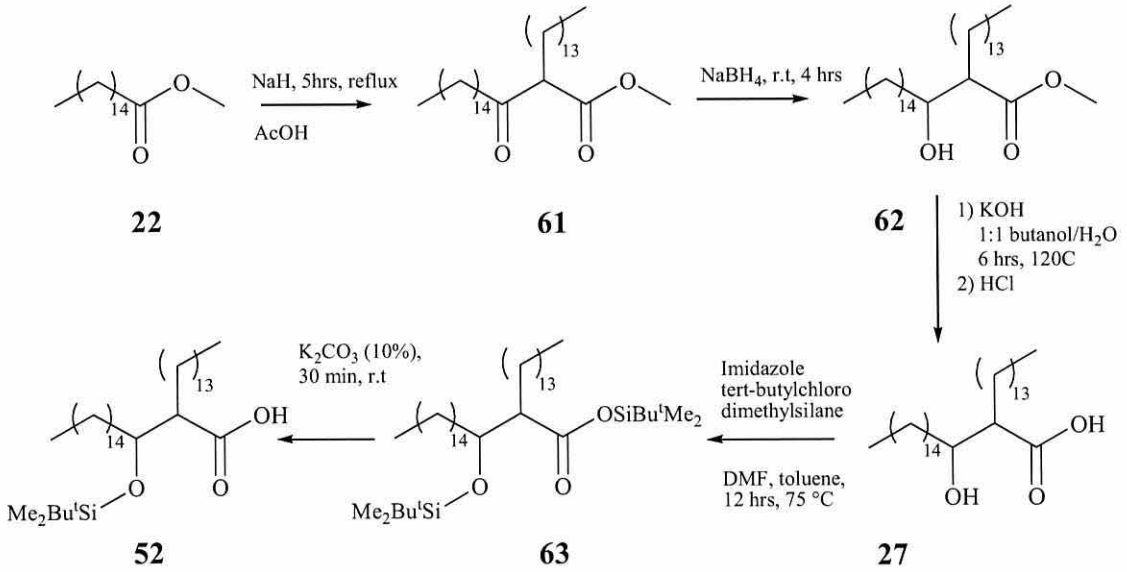


Fig 41. Synthesis of *tert*-butyldimethylsilyl *B*-hydroxy acid protected C_{32} MA proposed by Datta.

The next step was β-hydroxy acid protection to avoid unwanted couplings or self acylation. Compound **27** was dissolved in imidazole/*tert*-butylchlorodimethylsilane/DMF and dry toluene and heated at 75 °C. A variety of silylated products was obtained but only **63** was deprotected with K_2CO_3 to cleave the silyl ester groups, giving the final product **52**.[164]

1.5.7 Esterification reaction with enzymes

A different approach was followed by Gelo-Pujic *et al* using immobilised *Candida antartica* lipase (*Novozym 435*) which is thought to catalyse esterification reactions between α,α-trehalose and ethyl dodecanoate under classical heating conditions with *tert*-butyl alcohol giving yields up to 78 %. Higher rates were achieved when using microwave irradiation, and a ratio of 1:3 equivalents of the acids to the sugar. Under such conditions 80:3 of **65** to **66** was obtained.[173] (Fig 42)

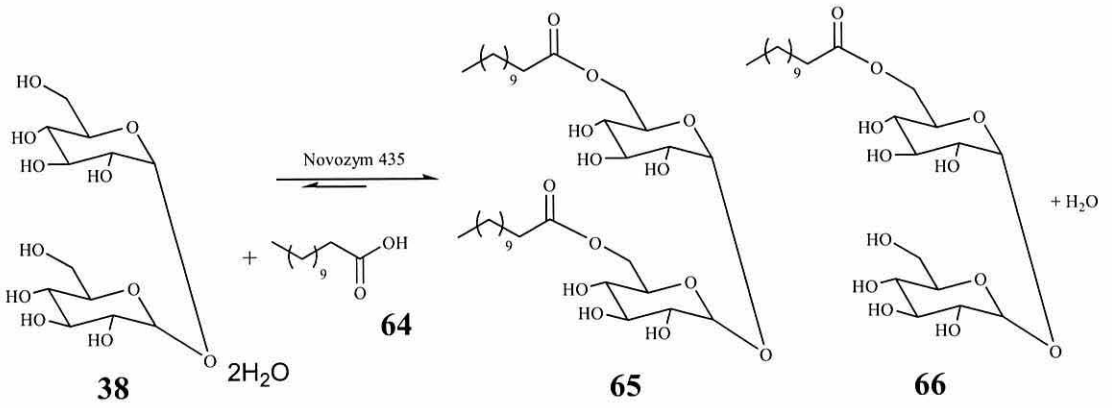


Fig 42. *Novozym 435* catalysed reaction using α,α-trehalose dihydrate 25 and dodecanoic acid.

The simplicity of the method lies in the fact that protection is not required and only trehalose and dodecanoic acid in dry media are used. *Novozym 435* was impregnated with an aqueous solution of trehalose and dried under high vacuum for 2 hrs. Dodecanoic acid, dissolved in ether was then added and the mixture was introduced into the microwave reactor for different times and power conditions. Oil bath heating reactions (80 °C) were used as a comparison method. During esterification, water is produced which has to be removed to increase the yield. This is the reason why molecular sieves (12 % v/v) or open tubes are used.[174] Microwave irradiation gave a sugar ester conversion rate of 62-95 %

depending on temperature, power or sample preparation. In comparison, oil bath conditions only achieved 48 % conversion. Yields obtained from using short fatty acids (C_8-C_{10}) were lower (85 %) than higher homologues (C_{16}-C_{18}) (95 %). In the same way yields were drastically lower when using non-commercial enzymes such as *ST389*.[173]

As seen above, esterification between sugars and mycolic acids of different chain length, appears to be a difficult process due the fact that protection of alcohol groups present in both sugar and mycolic acids is required. This protection interferes with the esterification process so the right choice of the protecting group appears to be a crucial factor to take into account. Different authors have taken a variety of routes which in some cases were not successful in terms of yields, number of steps, and complication of the method or that simply it did not work.

1.6 Glycerol esters

Once mycolic acids have been coupled successfully to trehalose to form TMM and TDM different sugars should be tried on in order to test the flexibility of the reaction. Glycerol esters have been synthesised for their different properties ranging from anticancer to antibacterial agents. These compounds, including mixtures of natural mycolic acids, have never been synthesised using a single enantiomer of mycolic acid. Therefore it would be of great interest to have such compounds to test their properties and see a possible application in medicine.

Bhowruth *et al* showed that four lipid fractions, phthiocerol dimycocerosates (PDIMs), triacylglycerols (TAGs), phenolic glycolipids (PGLs) and C_{70-90} monomycolyl glycerol (C_{70-90} MMG) induced Th1 cytokine production.[175] By incorporating lipids into cationic liposomes composed of dimethyldioctadecylammonium (DDA), a very powerful adjuvant is produced which is capable of inducing strong immune responses.[176] This important characteristic is useful for treating a variety of illnesses. The isomeric C_{32} corynomycolate esters of glycerol **68** were synthesised by the method of Datta *et al* (see Fig 43).[164]

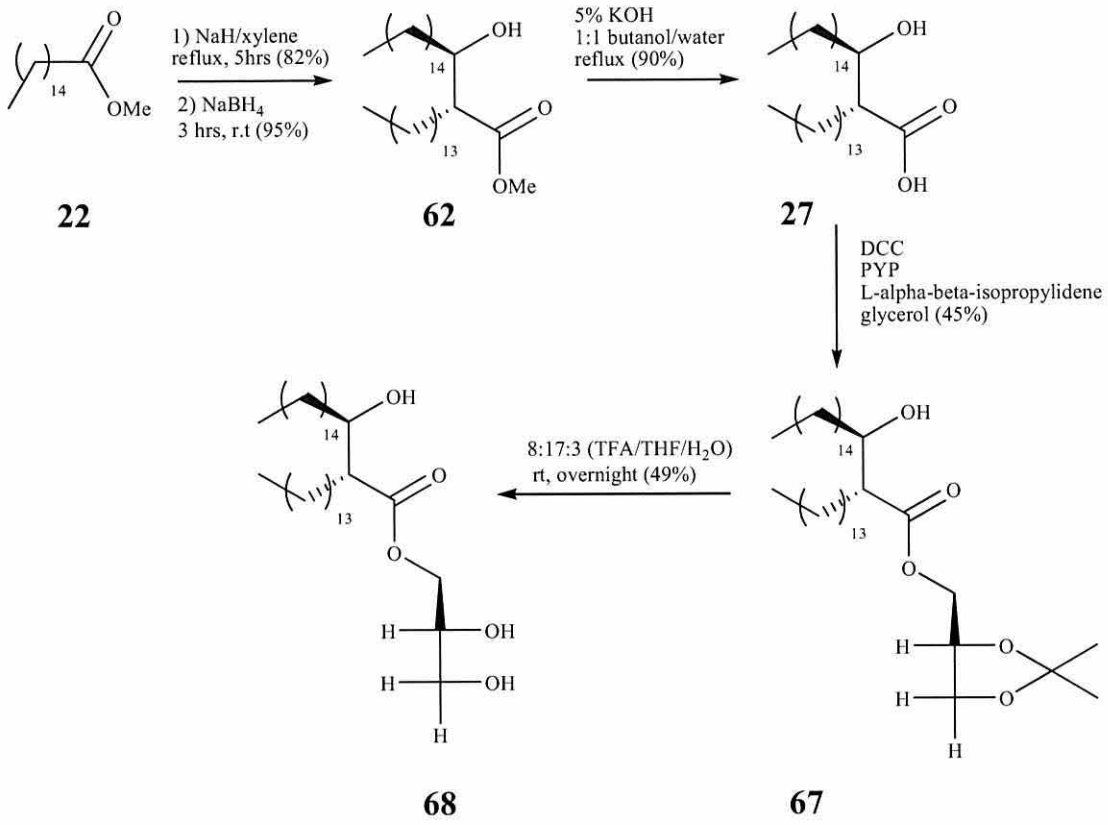
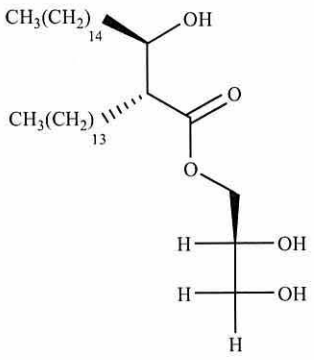


Fig 43. C_{32} corynomycolic acid glycerol ester synthesis.

A protected glycerol was coupled to the C_{32} corynomycolate silyl-protected motif using dicyclohexylcarbodiimide (DCC) and 4-pyrrolidinopyridine (PYP) (Fig 43). Trifluoroacetic acid, TFA/tetrahydrofuran and THF/water 8:17:3 was used for deprotection, giving *erythro* and *threo* diastereoisomers of glycerol coupled products **68** – **71** (Fig 44).[175]



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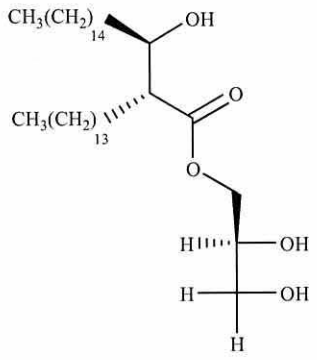

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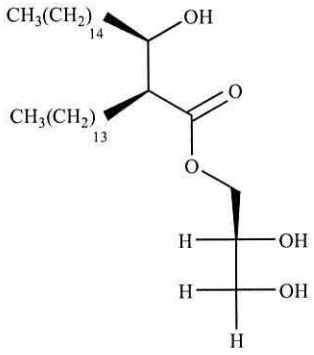

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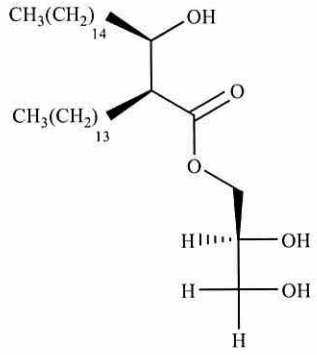

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Fig 44. Diastereoisomeic products obtained from the deprotection of coupled glycerol and C_{32} corynomycolates.

Monomycolyl glycerol (C_{70-90} MMG) increased significantly IL-12, TNF-α and IL-6 with values reaching 250 pg/ml, 3500 pg/ml and 5000 pg/ml respectively.

1.7 Pathogenicity and antigenic structure

Pathogenicity is the ability of an organism to cause disease in another organism, thus called pathogen. The susceptibility of the host and the aggressiveness of the pathogen are clearly related with the degree of pathogenicity. To quantify pathogenicity the term virulence has been introduced to describe the relative degree of damage done by a pathogen. Mycobacterial pathogens include the group of *M. tuberculosis, M. bovis, M. africanum, M. microti* and possibly *M. leprae.*

An antigen can be defined as substance that stimulates an immune response, especially the production of antibodies. Mycobacteria contain antigenic proteins and sugars in their cytoplasm (soluble) and cell wall lipid antigens (insoluble).

1.8 Properties of trehalose diester

In 1950, Bloch proved the toxicity of TDM when injected in mice.[126] At that time mainly toxic properties were studied. TDM toxicity is based on mitochondrial membrane modification which enables respiration processes to occur. In vivo and in vitro tests have proved that TDM injected in a mitochondrial suspension induced respiration inhibition, loss of respiratory control and inhibition of electron transport.[177,178] This mitochondrial attack of specific tissues will result in swelling, breakage of limiting membranes, and fragmented, swollen cristae.[101] A later discovery using electron microscopy suggested that cord factor weakened the peripheral part of the inner membrane.

Durand *et al* demonstrated that the nature of the sugar residue is a major determinant of their activity.[179] In the case of the 6,6'-bis(C_{32}-mycoloyl)-trehalose, it is the relative stereochemistry of the mycoloyl residues which modulates the lipid activity. As a consequence, comparison between different cord factors should not be made without keeping these physical parameters in mind.

In the late 1960s experiments studies showed that bacilli cord factor extract applied as intravenous injections (in mineral oil emulsions) protected mice against posterior *M. tuberculosis.*

Cord factor injection caused a response which did not differ from that caused by mycobacterial infection. This response was produced with as little as 5μg or less.

Dosage can potentiate cellular response in a way in which two injections of the cord factor (10μg each) are much more effective than a 20μg single one. The emulsion administration (oil in water or water in oil) can make a difference in response, as well as the animal used for the experiment (mouse versus guinea pig).[180]

Kato (1971) remarked that synthetic analogues possessing cord factor-like toxicity inhibited the mitochondrial oxidative phosphorylation as does cord factor and in some case even the dipalmitate (C_{44}) was found to be active.[177,181] Moreover, the synthetic analogues are non-toxic and less granulomagenic making them perfect candidates for veterinary and clinical applications. In fact neither the toxicity nor the granulomagenicity of cord factor is required for expression of antitumor activity.

A body weight loss will follow α,α-TDM injection and finally the mice die. However β,β-TDM and α,β-TDM caused only a transient loss of body weight in the first days following the injection after which mice weight recovered and no mice died.

These results point out clearly that the α,α-anomer of the trehalose moiety is essential for the manifestation of the lethal toxicity of TDM.[182]

Trehalose is unusual in having a symmetrical disposition of α-D-glucosyl groups about the central oxygen atom; this feature and the α-D-linkage have a strong bearing on its behaviour toward enzymes.[152] 6,6'-Diesters of α,α-trehalose have also been extensively studied by different groups in the past.[135,159] Goren et al remarked on the link between the carbohydrate core and the lipid substituent in relation to the biological activities of TDM, depending on the amphipathic properties conferred by a polar core surrounded by large lipophilic groups.[101]

1.9 Immune manipulations that may improve allergic and latent tuberculosis patients

More than 2 billion people have latent tuberculosis, so it is important to find effective methods of preventing infection. Different methods may be of great importance in reducing infection:

- By boosting Th1 cell immunity we strengthen protective immunity.
- Th2 cell immunity inhibition will potentially increase immune response.
- Increased antigenic response to lipid metabolism may help in recognising antigens during TB latency.[183]

1.9.1 New vaccine strategies

Many new attenuated/killed mycobacterial vaccines are in current development. A common strategy is to use killed mycobacteria (*M. vaccae*) to induce immune response that will effectively interact with *M. tuberculosis* antigens which may lead to immune system boost.[183] These new vaccines focus in over-expressing TB antigens or proteins which finally stimulate T cell response.[184] A Phase III study is taking place in Tanzania, where killed *M. vaccae* is given to HIV patients previously treated with BCG to boost their immunity.[185] This approach might help to develop a new vaccine more effective than BCG in helping protecting against TB.

1.10 Biological activity

1.10.1 Adjuvants

In recent years modern biology techniques have improved purification and vaccine synthesis. Vaccines have the general problem of being poorly immunogenic.[186] That is the reason why researchers need to find compounds that will boost immune response. For more than 60 years many adjuvants have been added to vaccine formulation in order to target infectious and cancerous diseases effectively by augmenting immunity.[187] Adjuvant, a Latin word which originally meant 'to help' has been used for any material capable of accelerate, prolong or increase humoral or cellular immunity response towards an antigen.[175,187]

Bloch discovered Cord factors in the 1950s, as a toxic component of mycobacterial cell walls,[129] the chemical structure of which was later discovered and described as trehalose-6,6′-dimycolate (TDM).[130] TDMs are not exclusive to mycobacteria as they appear to be present in nocardia and corynebacteria groups.

Mycobacteria have long been known to exert a number of immunomodulatory effects and have been used extensively as a source of adjuvant preparations. Despite that, Th2-aluminum-containing compounds, such as hydroxides, are the only adjuvants approved for human use worldwide.[188] The problem of these is not being active with all immunogens, such as allergy.[189] The development of new adjuvants to replace the existing one is required.

The best known adjuvant is Freund's complete adjuvant consisting of a paraffin oil emulsion and heat-killed mycobacteria.[190] Similarly, purified components of mycobacteria have been shown to have immunostimulatory activity. Wax D (a complex of peptidoglycan, arabinogalactan, and mycolic acids) was found to possess strong adjuvant activity,[191] and trehalose 6,6′-dimycolate administered as an o/w emulsion has been included in various adjuvant formulations.[75,186] These bacterial fractions have been used as adjuvants to fight immunological disorders, cancer or even infections.[192]

In the 1970s was established that the more immune-active component in mycobacteria and related groups was the cell wall by producing adjuvant arthritis in rats.[193] Clinical trials with oil treated kill BCG-cell walls were carried out in patients with gastric cancer. The two year study concluded that immunotherapy with BCG-CWS prolonged the survival of patients who underwent operations for gastric cancer compared with the control group.[194] A twenty two years clinical trial showed that cancer patients treated with BCG-CWS had longer survival rates.[195]

Z-100, is a lipid extracted from *M. tuberculosis* which have antitumor activity and so is used in Japan clinically.[196] It seems possible to extract lipids which will then be used for their immunostimulatory properties but getting single molecules for therapeutic use may be too complicated for the simple reason that it is very expensive and time consuming. That is why a synthetic route may be of great interest for future adjuvant formulation.[188]

In the 1970s, Ellouz *et al* showed that the minimal subunit of bacterial cell wall capable of inducing adjuvant activity was the disaccharide tetrapeptide N-acetyl glucosaminyl-N-acetylmuramyl-L-alanyl-D-isoglutaminyl-meso-diaminopimelyl-D-alanine (muramyl dipeptide, MDP). This peptidoglycan can in fact replace whole killed mycobacteria and still induce adjuvancy. Simplifications of that muramyl dipeptide structure and synthetic similar compounds were found to have adjuvant activity.[197] A variety of those derivatives have been proved to be host stimulating against bacterial, fungal and viral infections as well as cancer induced in laboratory animals.[198,199]

In recent years, the mechanism behind adjuvant activity, including Toll receptor signalling, has started to be more understood, confirming the importance of mycobacterial constituents acting as adjuvants.[188]

1.10.2 Adjuvant activity

Adjuvants are agents which modify the effect of other agents while having few if any direct effects when given by themselves. In this sense, they are very roughly analogous with chemical catalysts. Trehalose dimycolates have emerged as a principal factor in contributing to antitumor activity of a variety of substances in themselves much less

active (or even inactive) in the absence of α,α-trehalose 6,6'-dimycolate (or closely related analogs).[200]

In 1977, a study by Saito *et al* confirmed that cord factor was an adjuvant in mice and rats by enhancing antibody production and causing delayed hypersensibility.[201,202] The Cord factor itself is not antigenic, but it stimulates antibodies when injected in swine serum as a "schlepper" or carrier protein.[203]

When the functions of RES (Straight endoplasmic reticulum) were suppressed, tolerance was facilitated. The importance is that, cord factor, is partly responsible for RES function activation. Sharma *et al* in 1985 used trehalose-6,6'-dimycolate (TDM) in aqueous solution as an adjuvant for immunizing rabbits with a E. histolytica antigen. Intravenous inoculations of antigen plus TDM were found to induce protective immunity, both in vivo and in vitro.[204]

From the work of Kato and Asselineau on an in vitro model (mitochondrial phosphorylation), it seems that the D-gluco configuration is the most active.[205]

In 1976, Saito *et al* responded to two important questions about cord factors; the first is whether impurities in cord factor preparation might contribute to its adjuvant activity. Second, adjuvant activity of a given mycobacterial component may be expressed in some animal species but not in others.[201] A 1 μg cord factor injection produced antibody response while 5 μg of wax D did not. About specificity it has to be said that cord factor may induce antibody production in one species and not in others.

1.10.3 Antitumor Activity

Trehalose dimycolates C_{180} and C_{76} produced regression of a fibrosarcoma after injection; all cured animals were also resistant to a second inoculation of the same tumor.[181]

In 1974, Bekierkunst *et al* induced skin tumour and transplanted to guinea pigs in order to be treated for tumour regression. Delipidated and deproteinized cell wall (BGC) from *M. tuberculosis* was introduced in mineral oil (1.25 %) and applied to the animals which caused regression in 33 % of the cases.[206] This percentage increased when to 83 % when TDM was combined with BCG. The fact of changing mineral oil into peanut oil produced

a significant effect in carcinoma regression with 36 % and 80 % reduction when BGC and BGC+TDM were applied respectively. The antitumor activity depends on the oil concentration and the type of emulsion.[181] Tumour growth in the granulomatous lesion was strongly inhibited, whereas on the opposite site it developed quite normally (Fig 45).

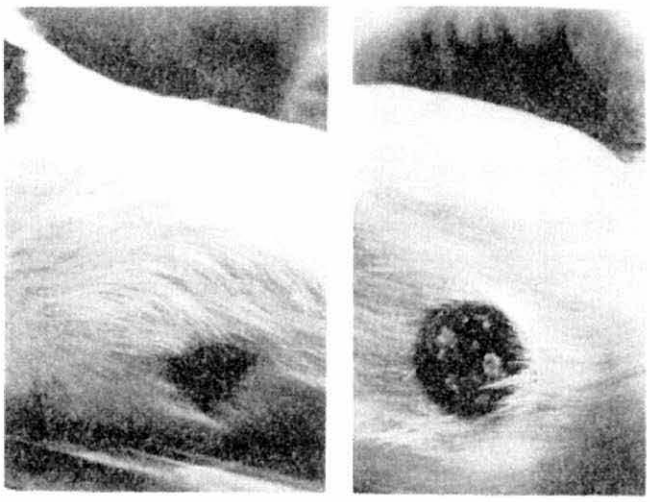


Fig 45. Skin tumours in guinea pig after 26 days line 10 tumour treatment (left) and at the opposite side untreated (right).

1.10.4 Antibacterial Activity

TDM and synthetic trehalose diesters proved to be active against infection of mice by different bacterial species, such as *Salmonella typhi, S. typhimurium, Klebsiella pneumoniae* and *Listeria monocytogenes.*[207,208] This activity remained even when oil emulsion were substituted by water.

1.10.5 Antiparasitic Activity

Clark showed that the antiparasitic effect of TDM of *Babesia microti* in mice was still protective after 7 weeks of receiving TDM injection. Similarly, Olds *et al* results showed that infection with *Schistosoma mamoni* in mice still gave a 32 % protection after infection, a percentage that rises to 71 % after a 200 μg trehalose dimycolate injection.[181] Due to these important characteristics, a US patent claimed that the use of sugar ester and fatty acids containing α- and β-hydroxycarboxylic acids, α- and β-ketocarboxylic acids

may have antibacterial, antifungal, and antiviral properties making them useful as topical bronchial dilators and dermatological agents.[209]

2. Results and discussion

The overall aim of this project was to prepare complete cord factors by coupling synthetic mycolic acids to trehalose. In order to do this, three steps were required which will be described in the following section: firstly to show that the coupling of model acids to trehalose could be achieved; secondly, to synthesise a mycolic acid representative of those present in *M.tuberculosis* and thirdly to couple this to trehalose.

2.1 Model coupling reactions

2.1.1 Preparation of trehalose dipalmitate

Direct reaction of the primary alcohols of trehalose to form esters is complicated by the formation of mixtures of protected/unprotected products at the other alcohol groups.

Trehalose was therefore firstly protected as its octatrimethylsilyl ether **59** (Fig 46). The protecting groups were then removed selectively from the primary alcohol groups to give **60** in a one pot reaction as described by Johnson.[210]

The use of trimethylsilyl groups for protecting alcohols has been proved to be inexpensive and straightforward even for tertiary and sterically hindered secondary alcohols.[211] N,*O*-bis(trimethylsilyl)acetamide (BSA) and tetrabutylammonium fluoride (TBAF) in an aprotic solvent specially DMF afforded the corresponding silyl ether **59** from **13** which was not isolated as the reaction occurred in one pot. The use of an aprotic solvent changes the solvation of anions compared to protic solvents, increasing reactivity but in this case was not in fact necessary. For less reactive alcohols more equivalents of reagent and higher temperature are required for them to be protected. This is not the case for the per-O-silylation of trehalose dihydrate which requires as little as 0.05 mol eq of TBAF.[210]

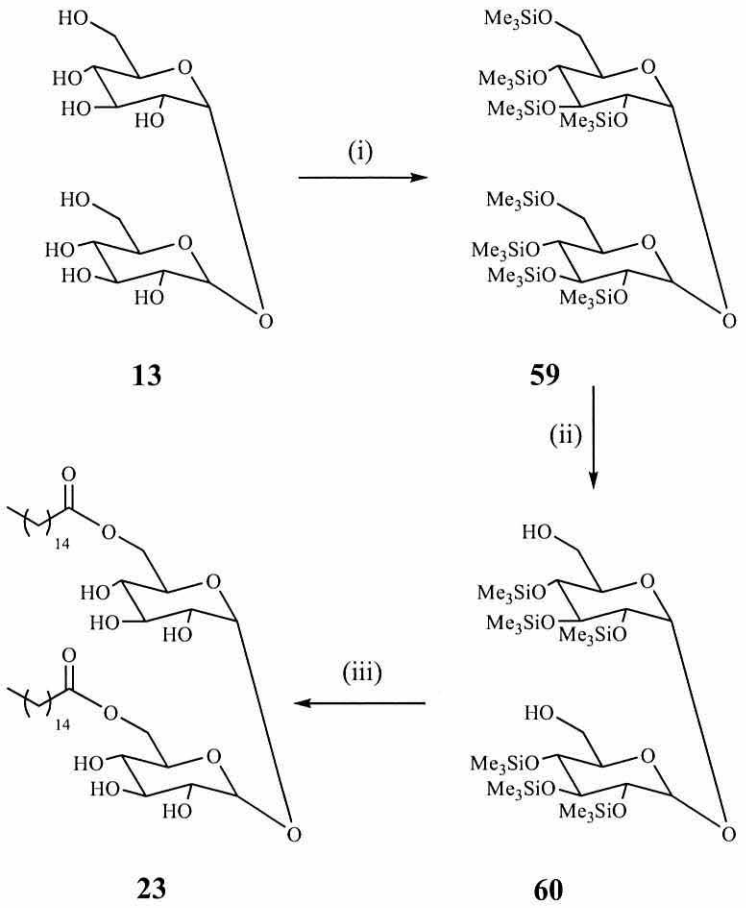


Fig 46. (i) (BSA), (TBAF 0.24 mmol), DMF, 30 min, r.t, 2-propanol; (ii) K_2CO_3/MeOH, 2 hrs, 0 °C (71 %); (iii) hexadecanoyl chloride; toluene/pyridine, 3 hrs, 80 °C; 1:10 MeOH/H_2O , 2 hrs, reflux (54 %).

Silylation of all the alcohol groups occurs in the first step and the second step involves the methanolysis of the trimethylsilyl ethers at the 6,6'-position with potassium carbonate in methanol to release the primary alcohols.

Hexadecanoyl chloride **68** used in the above reactions was either bought or synthesised. The reaction occurred by adding hexadecanoic acid, oxalyl chloride and DMF as a catalyst. These react to give an iminium intermediate.[212] Once the iminium ion is formed it will interact with the hexadecanoic acid and regenerate DMF.

Yields for the esterification and deprotection were acceptable with 54 % and 79 % when using commercial hexadecanoyl chloride and freshly synthesised **68** respectively. The spectroscopic data correspond reasonably to those found in the literature.

Although this method works well with a simple long chain acid, acyl chloride derivatives for esterification have not been used extensively in preparing TDM/TMM from mycolic

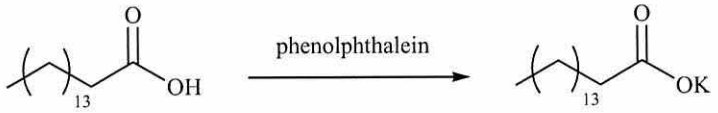
acids due the fact that the β-hydroxy group present in the mycolic acid often needs to be protected. The acyl chloride appears as be too aggressive so this method of coupling will probably remain marginal and restricted to simple acids.

An older method proposed by Toubiana *et al* was carried out in two steps; first the fully protected **59** was prepared, followed by deprotection. This method used trimethylsilyl chloride, hexamethyldisilazane and pyridine allowing separation of the fully silylated protected trehalose which was then deprotected in the same way as in Johnson's method.[135] Final yields for this method were 90 %.

2.1.2 Model couplings using potassium salts and iodotrehalose derivatives

2.1.2.1 Preparation of potassium salts

Model potassium salts of acids were made in order to try to couple them to a protected diiodotrehalose. The first of these model compounds was simple to make, as it only involved the use of hexadecanoic acid and a base using phenolphthalein as an indicator, to give potassium hexadecanoate **72** (Fig 47).



26 **72**

Fig 47. Potassium salt of hexadecanoic acid. $CHCl_3$/phenolphthalein, KOH (0.85M in MeOH) (90%).

The second product was potassium (*R*)-5-benzyloxy-3-hydroxypentanoate **75**. (*R*)-5-Benzyloxy-3-hydroxy-pentanoic acid methyl ester **73** (provided by G.Koza) was hydrolysed to the free acid **74** (Fig 48).

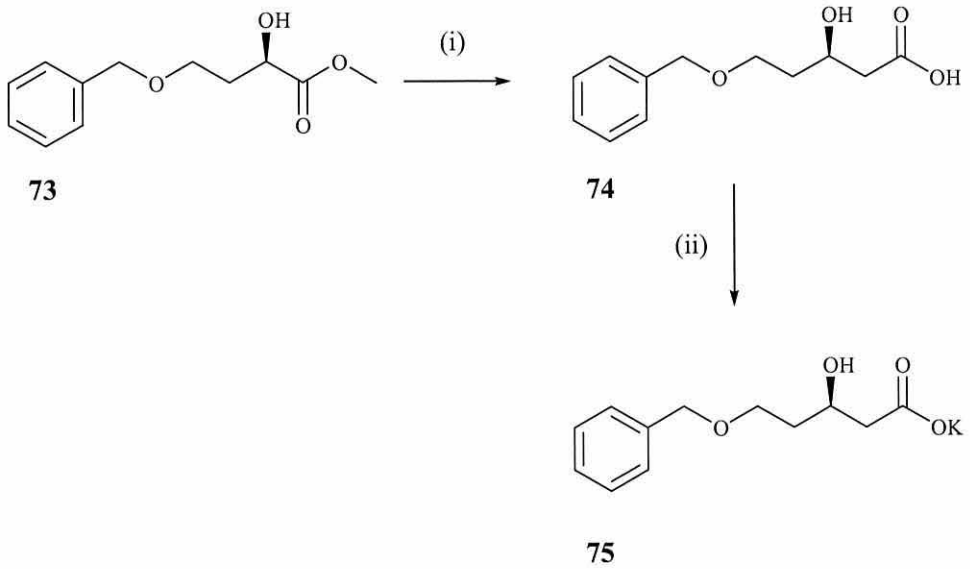


Fig 48. (i) NaOH hydrolysis, 12 hrs, r.t: HCl (79%); (ii) $CHCl_3$/phenolphthalein, KOH (0.85M in MeOH) (85%).

Protection of the free β-hydroxy acid **74** was attempted with acetic anhydride but a mixture of products were obtained, (*R*)-3-acetoxy-5-benzyloxypentanoic acid **76,** (*E*)-5-benzyloxy-pent-2-enoic acid **77** and the eliminated protecting group as methyl acetate **78** (see Fig 48 iii); see appendix). It was then decided to prepare the corresponding potassium salt to couple to trehalose.

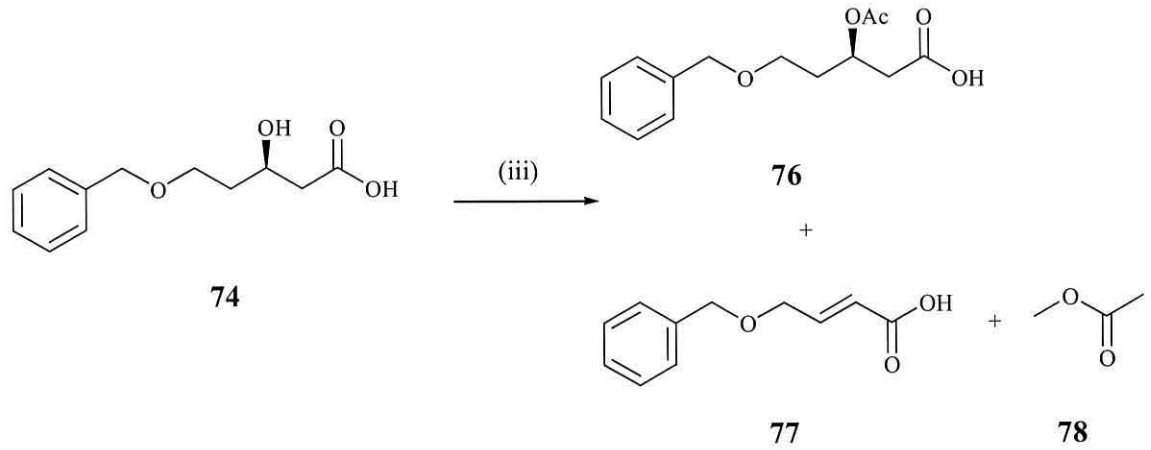


Fig 48. (iii) Ac_2O/pyridine, dry toluene, 10 hrs, r.t; HCl.

2.1.2.2 Reactions of potassium salts with halogenated trehalose derivatives

The first step of this reaction consisted in the halogenation of anhydrous trehalose **13** following the Hanessian and Lavalée method to give **24,** which could then be protected *in situ.*[155] This method is capable of halogenating carbohydrates and nucleosides by using triphenylphosphine-N-halosuccinimide. Major advantages are the selective halogenation in this case of primary alcohol positions 6 and 6' of trehalose and compatibility with protecting groups within the sugar.

Once the diiodo product **24** had been formed, two different routes were taken in order to protect the secondary alcohol positions. The first route, introduced by Tocanne in 1975 involved using hexamethyldisilazane and chlorotrimethylsilane in anhydrous pyridine to obtain the corresponding crystalline 6,6'-diiodo trimethylsilyl protected trehalose **25** in 30 % yield, compared to 60 % in the literature (Fig 49).[159]

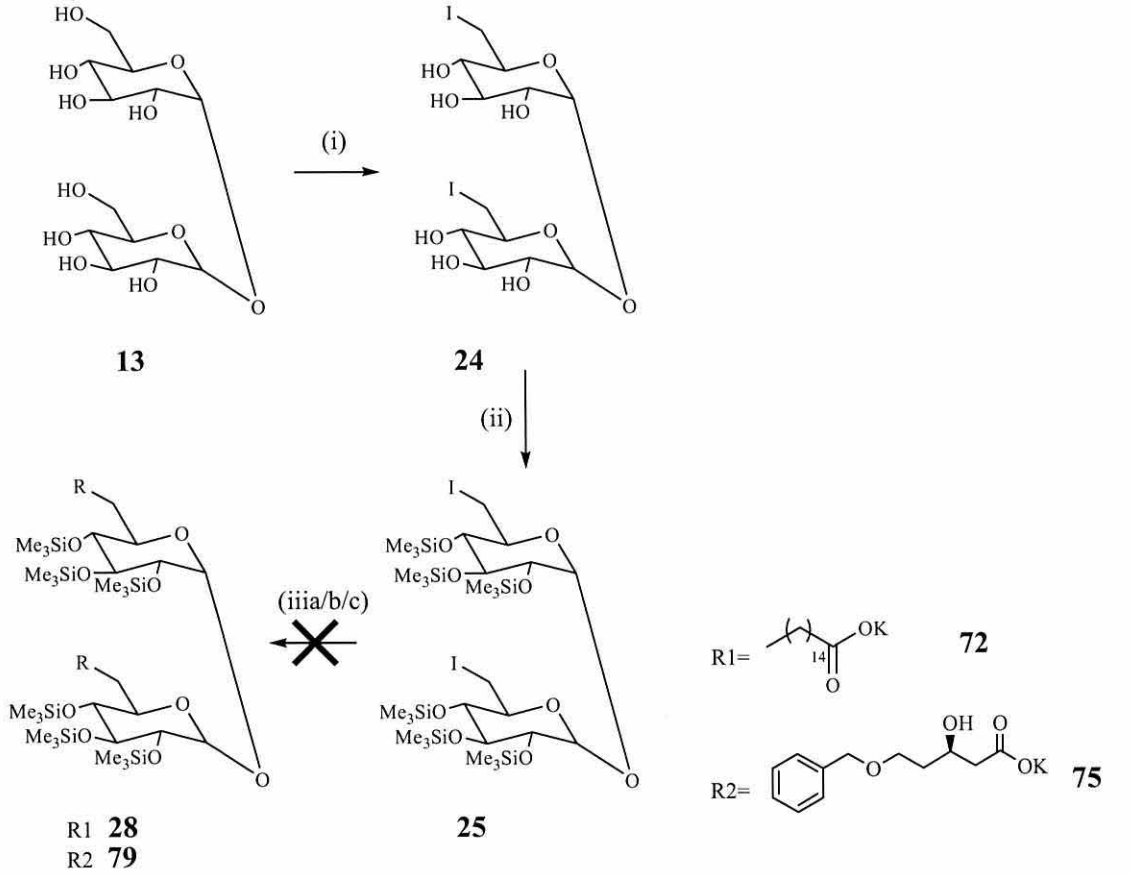


Fig 49. (i) PPh_3/DMF, N-iodosuccinimide, 50 hrs, 50 °C; (ii) hexamethyldisilazane, chlorotrimethylsilane, anhydrous pyridine, 40 min, r.t, N_2 (30 %); (iiia) potassium pentadecanoate, HMPA, 10 hrs, 80 °C; (iiib) potassium pentadecanoate, 1,3-dimethyl-2-imidazolidinone (DMI), 48 hrs, 90 °C ; (iiic) potassium (R)-5-benzyloxy-3-hydroxypentanoate, HMPA, 20 hrs, 80 °C.

The spectroscopic data for **25** corresponded to those in the literature; $[\alpha]^{23}_{D}$ = + 96 whereas Lit.[159] $[\alpha]^{20}_{D}$ = + 96. ^{1}H NMR shows on the literature 3x18H as 3 singlets resonating at δ 0.15, 0.3 and 0.35 compared to δ 0.15, 0.16 and 0.21. Signals for the protons in the sugar core appeared in both cases at δ 4.9 for the acetal proton continuing with the remaining 6 until δ 3.3.

Compound **25** was then coupled to potassium pentadecanoate **72** using HMPA by the Toubiana *et al* method,[135] promoting a S_N2 reaction as described on page 55. Instead of using the 6(6') bromo sugar derivatives **33** (as described on Fig 31), it was decided to use the more reactive di-iodide and the sodium salt of the mycolic acid **8** was replaced by the corresponding more reactive potassium salt. Such changes should result in an improved yield compared to the literature values found (30 %). The first reaction attempted to couple the palmitate salt **72** with the silyl protected sugar **25** but after 10 hrs at 80 °C, no products were observed in the IR (ester band). The literature suggested that this method was more convenient for mycolic acids as the shorter the acid chain is, the more insoluble salt becomes. The reaction was tried with a different coupling agent, DMI (1,3-dimethyl-2-imidazolidinone) which was stirred at 80 °C for 24 hrs, but once again no signs of product were detected. It was thought that a model compound containing just a single alkyl chain might react differently to a more complex mycolic acid, so a second model compound, closer to the mycolic acid in structure, was tried instead. Potassium (*R*)-5-benzyloxy-3-hydroxypentanoate **75** was reacted with **25** using HMPA (reaction iiic Fig 49); no product was obtained. In this case DMI conditions were not tried, due to the small quantity left of reagent **75** and the fact that the bulky trimethylsilyl groups might be responsible of hiding the reaction site and preventing coupling.

A different sugar protection was required in order to avoid hindered reaction sites. The second route followed the Hanessian and Lavalée paper and involved the *in situ* acetylation of 6,6' diiodotrehalose **24** to give **80** (Fig 50). In this case literature yields for **80** matched experimental ones (55 % and 50 % respectively).[152]

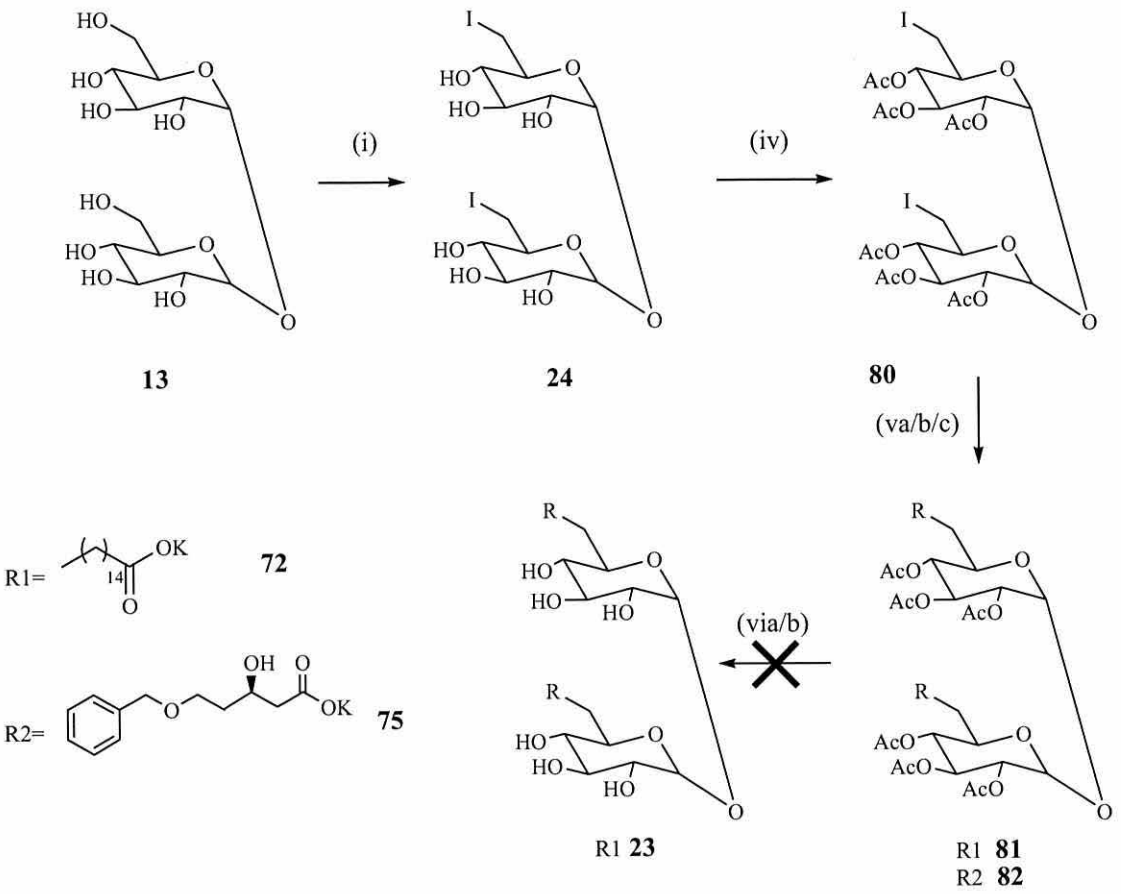


Fig 50. (i) PPh_3/DMF, N-iodosuccinimide, 50 hrs, 50 °C; (iv) Ac_2O/pyridine, 24 hrs, r.t (50 %); (va) potassium pentadecanoate, HMPA, 10 hrs, 80 °C (60 %); (vb) potassium pentadecanoate, 1,3-dimethyl-2-imidazolidinone (DMI), 48 hrs, 90 °C (66 %); (vc) potassium (*R*)-5-benzyloxy-3-hydroxypentanoate, HMPA, 10 hrs, 80 °C (68 %); (via) THF/MeOH/H_2O, K_2CO_3, 12 hrs, r.t then 12 hrs, 40 °C; (vib) THF, diethylmethylamine/H_2O, 20 hrs, r.t.

Esterification of the acetylated sugar was attempted using the same method as for the trimethylsilyl protected sugar, using HMPA and DMI. When using the potassium salt **72**, yields for the coupled product **81** were 60 % when using HMPA and 66 % with DMI. On the other hand, potassium (*R*)-5-benzyloxy-3-hydroxypentanoate **75** gave a 68 % of **82** when using HMPA. DMI was not used as a solvent when coupling **80** and **75** to give **82** due to the small quantities available and to the fact that the compound had already been obtained by the other route. On the other hand, compound **81** was successfully obtained using both solvents in similar yields.

Deprotection of the protected trehalose **81** using THF/MeOH/H_2O in the presence of K_2CO_3 was tried following the Polonsky *et al* method.[148] NMR data showed palmitic acid as a main product, which meant cleavage of the ester bond happened somehow. A

different approach was tried using the Kunz and März method involving a gentle hydrolysis of **81** with diethylmethylamine/water.[213] The method gave only the starting material **81** and did not deprotect the acetyl on the trehalose core. NMR data of products correspond to those found for starting materials. Because the final deprotection was not successful even with this model compound, an alternative approach had to be examined.

2.1.2.3 Enzyme catalysed reactions with Novozym 435 (Candida antartica) lipase

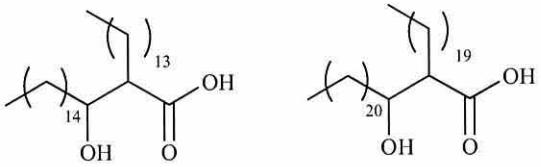
A method for protecting 6,6'-positions of trehalose directly with simple acids introduced by Gelo-Pujit *et al* which has been described in the introduction was next examined to determine whether it would also work with mycolic acids. This method was primarily used to introduce acyl groups regioselectively into sugars without the need for protection,[173] promoted by microwave irradiation in a solvent free reaction catalysed by Novozym 435. Reaction times usually varied depending on chain length, acceptor and receptor structure but ranged from 1-22 hrs. The literature values for trehalose coupling with dodecanoic acid at 110 °C under oil bath conditions without microwave irradiation record a 78 % conversion. It was these thermal conditions that were used here.

Reaction of unprotected trehalose dihydrate **38** impregnated with *Candida antartica* lipase with palmitic acid, pentadecanoic acid or docosanoic acid under the conditions described gave only starting material but no products **23**, **157** or **158**.

2.1.2.4 Preparation of 'model' corynomycolic acids

Better model compounds were needed in order to accomplish trehalose esterification reactions and in the longer term full mycolic acid-trehalose coupling. A paper by Datta and Takayama described corynomycolic acid preparation starting with methyl palmitate **22** and ending in corynomycolic acid and achieving good yields using a Claisen condensation.[164] It is important to say the resulting products **83** and **27** were a mixture of racemic *threo* and *erythro* diastereoisomers (Fig 51). The preparation of a single isomer

with the correct stereochemistry appears to be of crucial importance in relation to mycolic acid stereochemistry, structure and toxicity but it was not a problem to be solved at this stage.



27 **83**

Fig 51. Final structures for 27 and 83 obtained from palmitic acid (C_{16}) and henicosanoic acid (C_{22}).

2.1.2.4.1 Claisen condensation of henicosanoic acid

Henicosanoic acid, like corynomycolic acids is much simpler than a full mycolic acid and thus a convenient model compound that can be obtained easily and used in a latter stage for esterification with sugar. The first step in the reaction was methylation of henicosanoic acid (see appendix) followed by a self condensation (Claisen). Self condensation promoted by a base (NaH) afforded ketoester **86** (89 %) (Fig 52).[164] Claisen condensation allows different chain length choice delineating a flexible approach to future substituent groups.[214] As expected, NaH is preferred over sodium ethoxide, being a stronger base leading to higher yields and, following this reasoning, sodium ethoxide was not used for the condensation. A second method describes a Ti-Claisen condensation of henicosanoic acid methyl ester. The literature identifies the advantages of Ti-Claisen condensation as high yields, mild conditions and short time reactions, when compared to previous NaH conditions.[215,216] However, results showed that yields for compound **86** were 40 % when using $TiCl_4$ whereas NaH achieved 89 %.

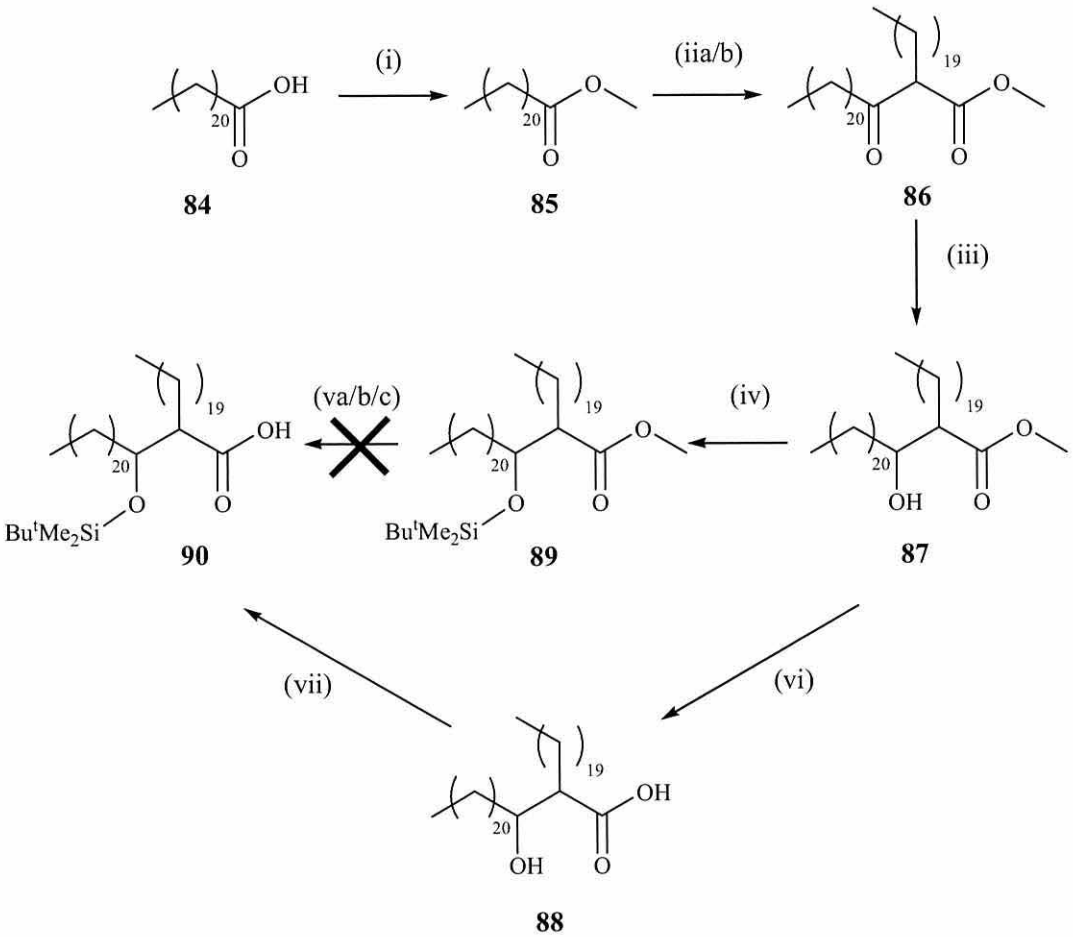


Fig 52. (i) MeOH/H_2SO_4, 4hrs, 73 °C (69 %); (iia) NaH/xylene, 12 hrs, 145 °C (89 %); (iib) $TiCl_4$/toluene, Bu_3N/toluene, 0-5 °C (40 %); (iii) $CHCl_3$/MeOH, $MgSO_4$, 12 hrs, r.t; $NaBH_4$, 72 hrs, 22 °C (48 %); (iv) imidazole, *tert*-butylchlorodimethylsilane/dry toluene, 40 hrs, 75 °C (85 %); (va) MeOH, KOH, 48 hrs, 75 °C, AcOH; (vb) THF, MeOH, H_2O, LiOH, 12 hrs, r.t, $KHSO_4$; (vc) dioxane, LiOH, AcOH; (vi) KOH, Butanol/H_2O, 8 hrs, 105 °C; HCl (92 %); (vii) imidazole, *tert*-butylchlorodimethylsilane/dry toluene, 48 hrs, 75 °C; K_2CO_3/H_2O, 24 hrs, r.t; $KHSO_4$ (58 %).

Compound **86** was reduced to the keto-ester **87** with $NaBH_4$ to give a mixture of two diastereoisomers which were not separated. At this stage, the synthesis did not target individual stereoisomers, but simply a mixture of model isomers. From this stage two main routes were taken. The first one included *tert*-butyldimethylsilyl protection of the β-hydroxy acid **89** which was achieved with a good overall yield (85 %). Deprotection of the ester was not successful when tried by three different methods including MeOH and KOH; THF and LiOH or dioxane, LiOH and AcOH. A second route was taken starting from the previously synthesised keto-ester **87** which was saponified to yield **88** (92 %).

From here β-hydroxy acid protection again with a *tert*-butyldimethylsilyl group gave the final product **90** with 58 % yield.

2.1.2.4.2 Claisen condensation of palmitic acid

An identical procedure was carried out for condensing hexadecanoic acid methyl ester **22** using Claisen self-condensation (Fig 53). Condensation with $TiCl_4/Bu_3N$ was preferred to NaH for convenience, although yields were found to be slightly lower (50 %). Once the keto-ester **62** was obtained from **61** by $NaBH_4$ reduction (75 %), it was saponified to the β-hydroxy acid **27** (69 %). This was first fully protection with *tert*-butyldimethylsilyl groups to give **63** (66 %) and then selective deprotection of acid protecting group gave **52** (83 %), keeping the required β-hydroxy protection in order to achieve a trehalose esterification reaction. A final step was obtaining the corresponding acid chloride **91** (which will be used later to make a glycerol ester).

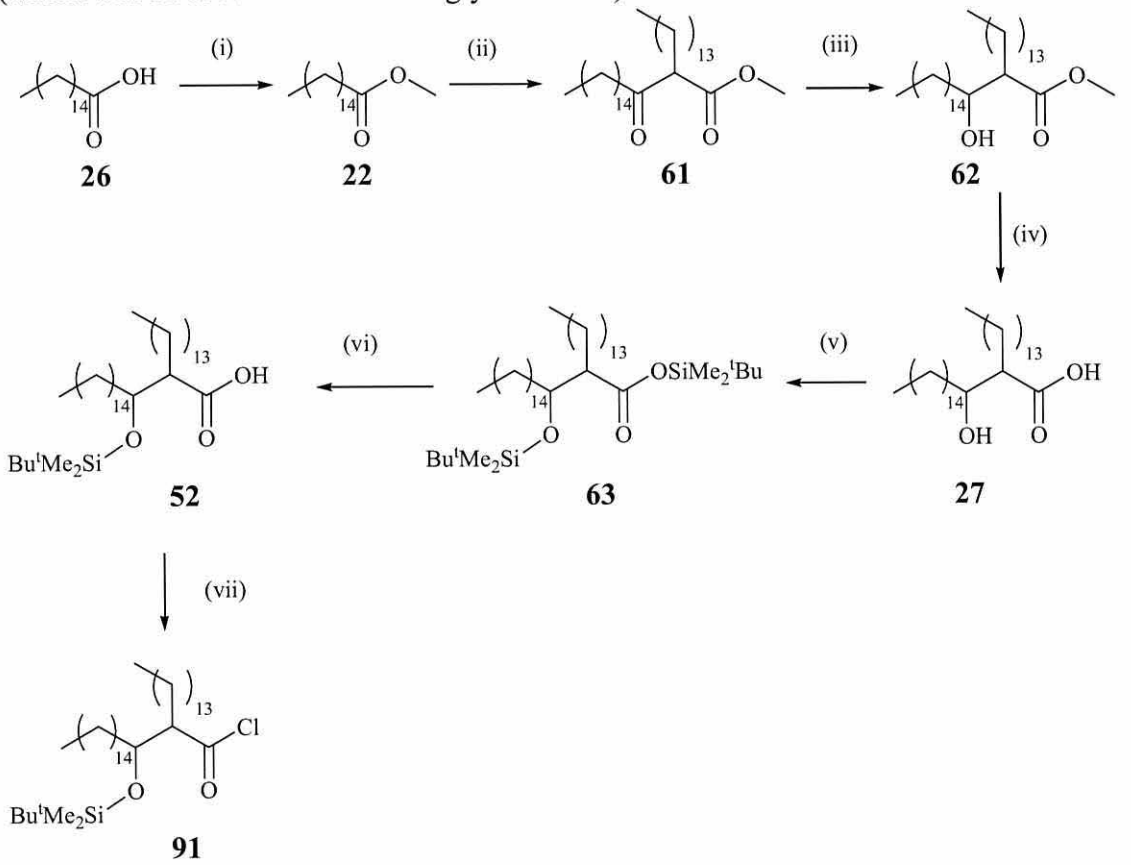


Fig 53. (i) $MeOH/H_2SO_4$, 4hrs, 73 °C (69 %); (ii) $TiCl_4$/toluene, Bu_3N/toluene, 0-5 °C (50 %); (iii) $CHCl_3$/MeOH, $MgSO_4$, 12 hrs, r.t; $NaBH_4$, 72 hrs, 22 °C (75 %); (iv) KOH, Butanol/H_2O, 6 hrs, 120 °C; HCl (69 %); (v) imidazole, *tert*-butylchlorodimethylsilane/dry toluene, 14 hrs, 75 °C (66 %); (vi) $MeOH/THF/K_2CO_3/H_2O$, 24 hrs, r.t; $KHSO_4$ (83 %); (vii) oxalyl chloride, dry toluene/THF.

2.1.3 A simple model 3-(*tert*-butyl-dimethylsilanyloxy)-heptadecanoic acid

Another model compound was synthesised, in this case a simple β-hydroxy acid protected heptadecanoic acid **96**. This compound has been used in the litterature for a variety of coupling reactions.[217] The reaction started by generating the lithium enolate from the ester **78**. This was acylated with hexadecanoyl chloride **92** to give the ketoesters **93**. Reduction with $NaBH_4$ and saponification with KOH led to the target model **95**. Finally β-hydroxy silylation gave compound **96** with a 47 % yield (Fig 54). Reactions including compounds **93** to **96** were based on the Datta and Takayama method, which was used to synthesise **52** and **90**.[164]

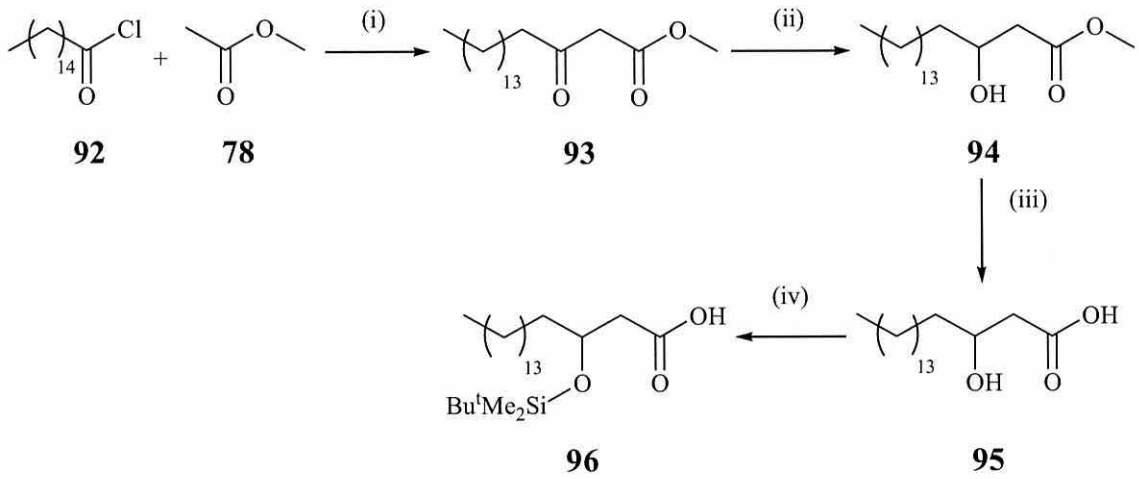


Fig 54. (i) Methyllithium, anhydrous diisopropylamine (-40 °C)/THF, N_2, 1 hr, r.t; Methyl acetate (-78 °C), 10 min, -78 °C; hexadecanoyl chloride (-78 °C)/THF, 48 hrs, r.t (50 %); (ii) MeOH, $NaBH_4$, 16 hrs, r.t (76 %); (iii) KOH, butanol/H_2O; HCl (83 %); (iv) imidazole, *tert*-butylchlorodimethylsilane/dry DMF/dry toluene, 12 hrs, 75 °C; ethyl triethylamine, K_2CO_3/CH_3Cl, 30 min, r.t; H_2SO_4 (47 %).

These model corynomycolic acid, **90**, **52** and **96** have been synthesised successfully and will be tested in the next section when coupling trehalose sugars with those model compounds using esterification on either the 6 or the 6,6' position of the trehalose core. Such reactions are a crucial step towards understanding esterification coupling conditions in order to apply it to mycolic acids.

2.2 Esterification of trehalose with model compounds

Firstly, palmitic acid was activated with N-hydroxysuccinimide to give **97** (51 %) that then was reacted with 1-decanol in a model reaction.[218] Results showed no product formation under such conditions (Fig 55) (see appendix for reactions).

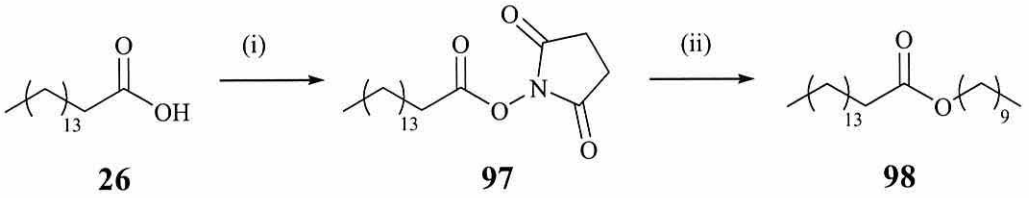


Fig 55. (i) N-hydroxysuccinimide, dicyclohexylcarbodiimide (DCC), 4-pyrrolidinopyridine, 1 hr, r.t; AcOH, 2 hrs, r.t (51 %); (ii) 1-decanol, dry toluene, 14 hrs, r.t; 8 hrs, 50 °C.

2.2.1 Coupling 3-hydroxyoctadecanoic acid with trehalose

It was then decided to move directly to trehalose to which different reaction conditions and acids would be applied. Figure 56 shows a series of reaction involving esterification of unprotected trehalose. A paper by Bottle and Jenkins[163] suggested a method with no protection for any of the alcohols on the sugar core. The inconvenience of this method is that it had only been done for very simple short chain acids. When tried, this method did give product **23** in very low yield (< 20 %) when coupling anhydrous trehalose to palmitic acid **26**.

MALDI was used for the mass analysis of cord factors as they don't ionise well using ESI, but ESI can still be used for simpler acid structures coupled to trehalose, such as **23**. NMR data and ESI (Fig 57 appendix) confirmed the structures. The ESI data showed $[M+Na]^+$: 841.5592 when $C_{44}H_{82}O_{13}Na$ requires: 841.5647.

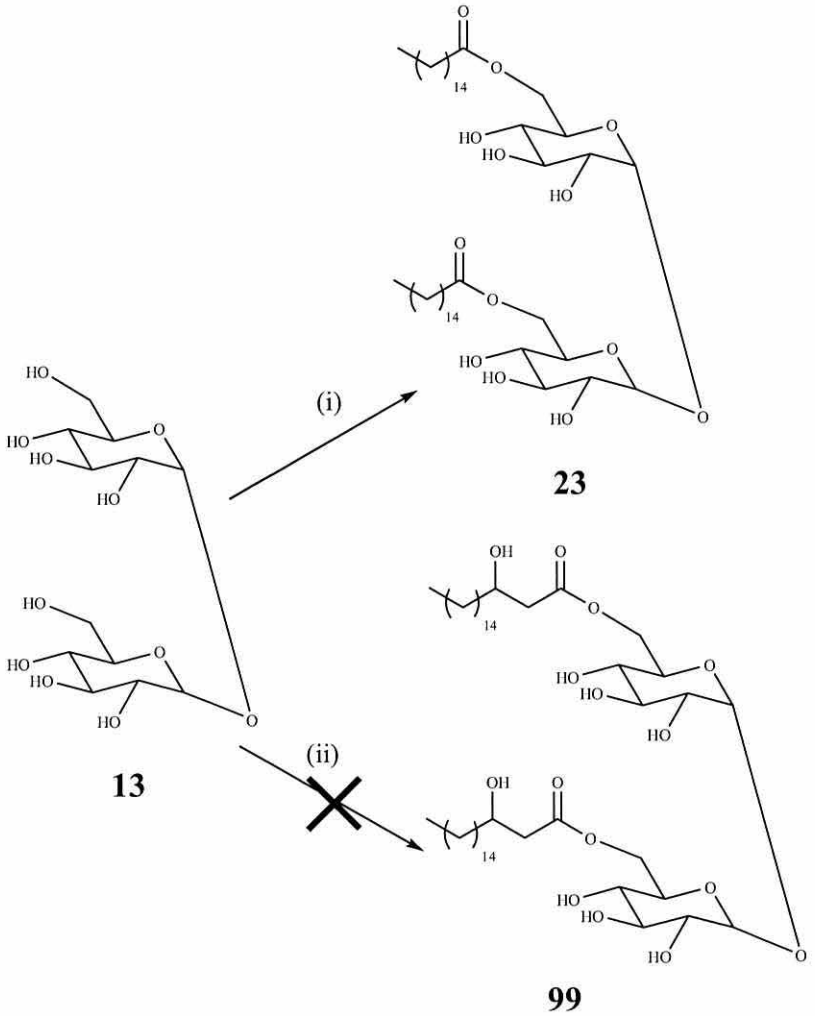


Fig 56. Unprotected trehalose coupling reactions. (i) hexadecanoic acid, PPh_3/dry DMF, N_2, 0 °C, DIAD, 36 hrs, r.t (20 %); (ii) 3-hydroxyoctadecanoic acid **73**, PPh_3/dry DMF, DIAD, 36 hrs, 30 °C.

In contrast, when the reaction was repeated with the β-hydroxy acid **95**, no product was obtained even though reaction time and temperature were increased.

The next set of reactions that were carried out to try to couple a variety of acids to the protected trehalose **60**. Once again the same reagents were used for DIAD coupling following Bottle and Jenkins method, trying to couple protected trehalose **60** with palmitic acid.[163] After 24 hrs stirring at r.t, TLC showed a new spot above both starting materials, so the reaction was worked up. The first column fraction was identified as being palmitic acid by ^{1}H NMR. The second fraction's ^{1}H NMR included PPh_3 signals in the aromatic region downfield, followed by DIAD signals at δ 4.85 and 1.18. Signals at δ 3.5 - 4 showed the trehalose core protons and MALDI-TOF confirmed no product

formation. ^{1}H NMR integral ratios between trimethylsilyl groups and acyl groups for the required product **28** should have been 18:2 respectively, which was not the case (Fig 58).

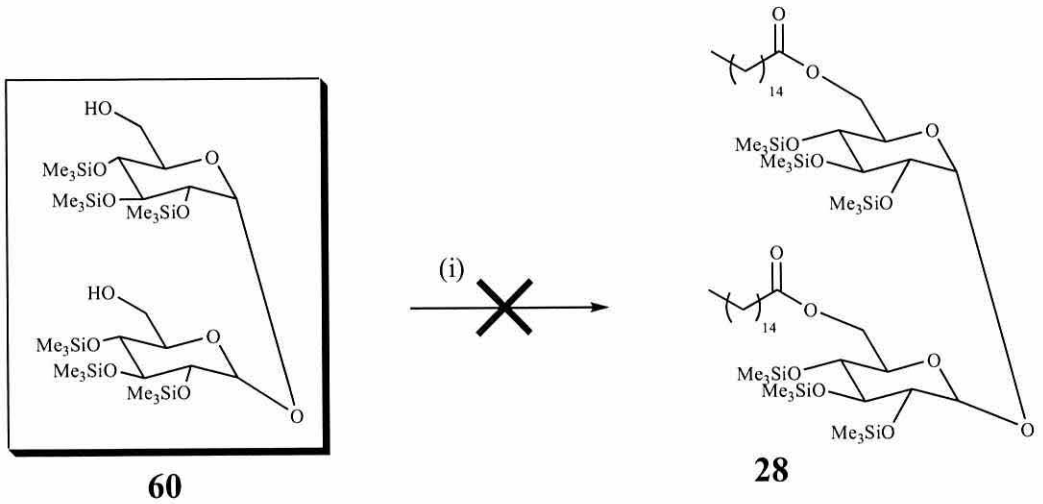


Fig 58. (i) Hexadecanoic acid, PPh_3/dry DMF, 0 °C, N_2, DIAD, 24 hrs, r.t.

The second reaction (Fig 59 (ii)) shows the coupling of unprotected 3-hydroxy-octadecanoic acid **95** with protected trehalose **60**. Care was taken not to allow the reaction temperature to rise, so instead the reaction time was increased to 72 hrs. TLC after a column showed **100** in the first fraction and 3-hydroxy-octadecanoic **95** in the second (Fig 60).

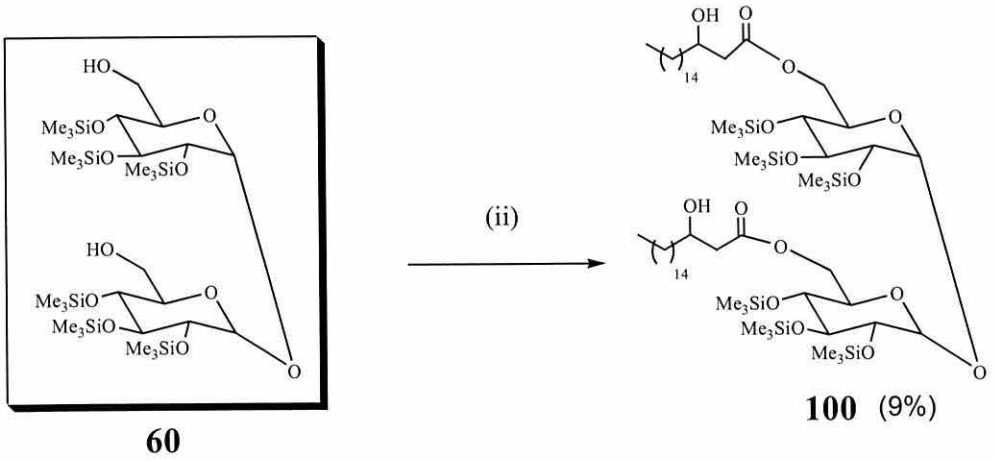


Fig 59. (ii) 3-hydroxy-octadecanoic acid **73**, DCC, 4-dimethylaminopyridine (DMAP), molecular sieves, 4 hrs, r.t; dry toluene (0 °C), 72 hrs, 30 °C.

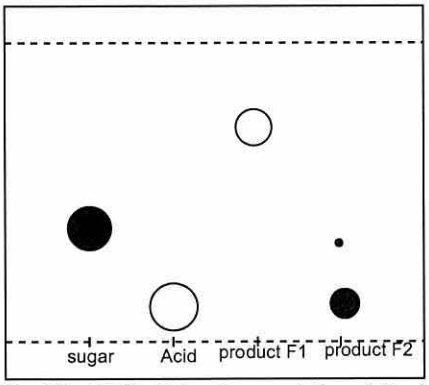


Fig 60. TLC plate, hexane/ether 1:2 showing column chromatography fractions F1 (**100**) and F2 (**95**) compared to starting materials.

Confirmation of the formation of **100** was achieved by proton ^{1}H NMR (Fig 61 appendix). A downfield signal at δ 4.90 included 2 protons attached to the C_1-$C_{1'}$ carbons of the sugar core. The remaining 12 protons on C_2-C_6 appeared in the range from δ 3.5 - 4.5. Protons corresponding to the *β*-hydroxy acid came around δ 3.85 as a multiplet. The two protons in the α-position appeared up field, below δ 3.5. The trimethylsilyl protecting groups on the sugar appeared as a total of six singlets ranging from δ 0.17 - 0.13 and including nine protons each. The ^{13}C NMR spectrum showed a carbonyl at δ 167, C_1 at δ 94 and the remaining sugar carbons in the region of δ 73 - 61 where the *β*-hydroxy carbon could be seen. The CH_2 chain ranged from δ 37 - 22 and the CH_3 came up-field around δ 15. Trimethylsilyl carbons signal were at the highest field at δ 0.9. Mass spectrometry of the products confirmed the structures to be those of mono- and di-substituted trehalose protected sugars with $[M+Na]^+$: 1080.66, $[M+K]^+$: 1095.62 and $[M+Na]^+$: 1363.93, $[M+K]^+$: 1379.28 respectively (Fig 62-63 appendix). These molecular ions including sodium and potassium were formed due to the presence of small amounts of Na^+ and K^+ ions in the samples and the matrix used in MALDI-TOF technique.

Although MALDI did show peaks for both mono- and di-substituted products, this was not clear in the NMR. This may be due to the fact that only a small amount of monosubtituted product was formed.

2.2.2 Coupling trehalose with crude mycolic acid samples

As this simple 3-hydroxy-octadecanoic acid **95** did couple with trehalose giving as a product **100**, the same reaction was tried using a natural mycolic acid to corroborate the results from the model (reaction (iii) in Fig 64).

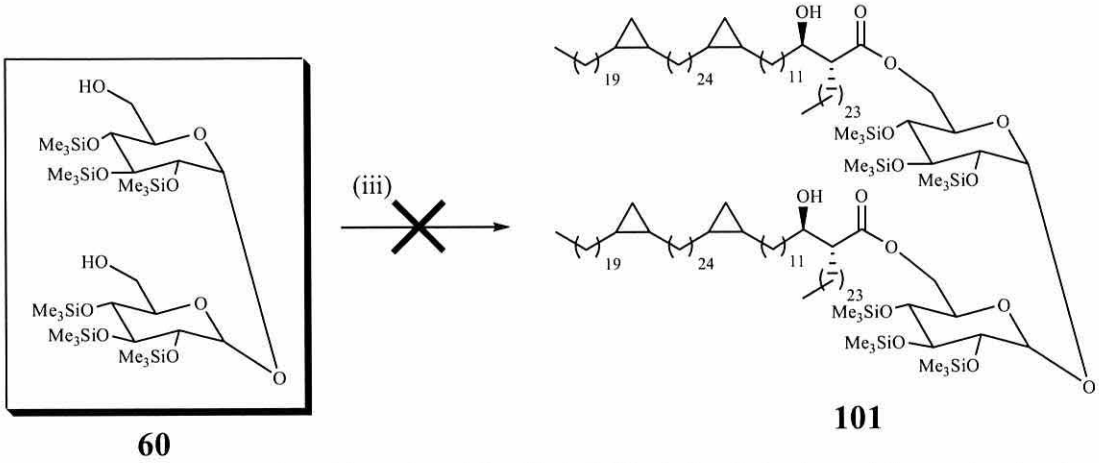


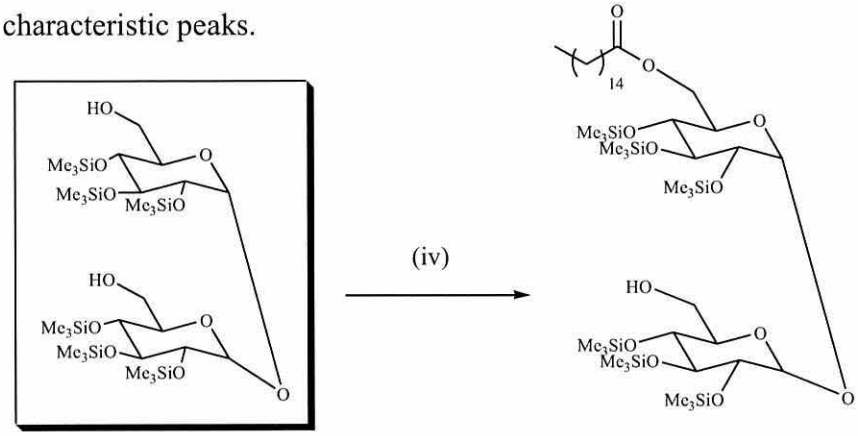
Fig 64. (iii) natural mycolic acid (Prof. D. Minnikin), DCC, 4-dimethylaminopyridine, molecular sieves, N_2, 4 hrs, r.t; dry toluene (0 °C), 24 hrs, 30 °C then 24 hrs, 70 °C.

The NMR of the natural mycolic acid used for the coupling can be seen in Fig 65 of the appendix. The ^{1}H NMR shows a downfield signal at δ 3.53 representing the 3 H in the methyl ester position, a 1 proton signal corresponding to the proton next to the *β*-hydroxy, followed by a δ 2.43, 1 proton signal due to an α-proton. Characteristic CH_2 signals appeared ranging from δ 1.3 - 1.0 and a CH_3 at δ 0.88. Finally signals at δ 0.19 and δ -0.33 corresponded to the cyclopropane protons.

The coupling was tried once again using same reagents as for the previous reaction but changing the temperature, which started at 30 °C but was increased to 70 °C. ^{1}H NMR showed signals for both protected sugar cores ranging from δ 3.4 - 4.5 and mycolic acids cyclopropane rings at δ -0.3 but no trace of esterification by changes on the H_6-$H_{6'}$ protons on the sugar that should have shifted downfield. The MALDI-TOF mass spectrum confirmed no product formation but showed sugar protected starting material. The fact that the sample provided was a methyl ester was probably responsible for the lack of reaction success.

2.2.3 Coupling reaction between trehalose and 3-(*tert*-butyldimethyl-silanyloxy)-2-eicosyltetracosanoic acid and DCC

Reactions (ii)-(iv) (Fig 59, 64, 66) used DCC and DMAP as reagents. In this case the coupling was first tried using hexadecanoic acid and increasing reaction temperature to 70 °C. TLC showed new spots after 28 hrs which increased at 60 hrs. After work up and a column, the first fraction corresponded to palmitic acid. The second fraction showed product **28b** with characteristic signals in the ^{1}H NMR with two protons in the H_6 position shifting from their initial position to δ 4.32, 4.0 and showing both sugar and acid characteristic peaks.

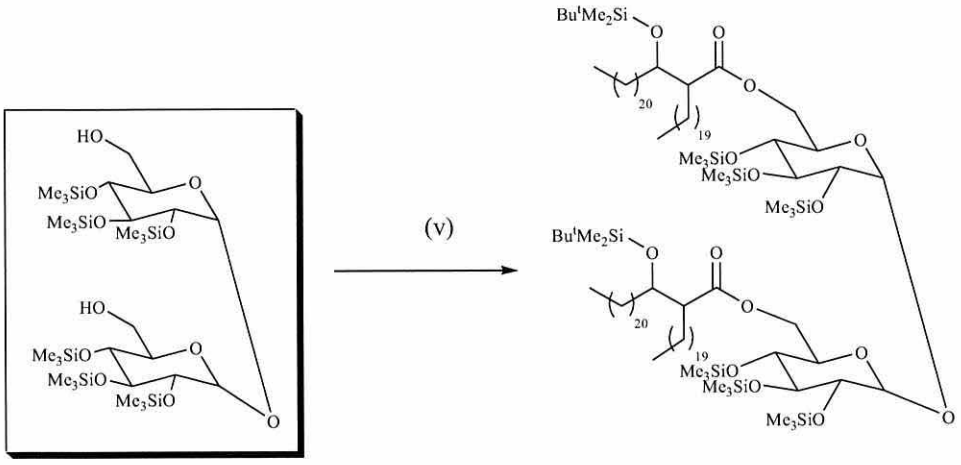


60 **28b** (43%)

Fig 66. (iv) hexadecanoic acid, DCC, DMAP, molecular sieves, N_2, 4 hrs, r.t; dry toluene (0 °C), 24 hrs, 30 °C then 24 hrs, 70 °C (43 % TMM).

The NMR spectrum showed a single palmitic acid attached to trehalose (Fig 67 appendix). MALDI-TOF confirmed this as peaks were seen at $[M+Na]^+$: 1037.07; $C_{46}H_{100}O_{12}Si_6Na$ requires: 1036.80 (Fig 68 appendix). The yield for this reaction was 43 % but did not include any di-substituted product, due the fact that this was lost during column purification.

In reaction (v) (Fig 69), the same reagents were used, but hexadecanoic acid was replaced by **90** for the esterification, a molecule much more similar to a mycolic acid. Reaction conditions were changed from 24 hrs at 30 °C then 24 hrs at 70 °C to 12 hrs at 22 °C then 5 days at 55 °C and the product **102** was obtained in 40 % yield.



60 **102**(40%)

Fig 69. (v) 3-(*tert*-Butyl-dimethyl-silanyloxy)-2-icosyltetracosanoic acid **90**, DCC, DMAP, molecular sieves, N_2, 3 hrs; dry toluene (0 °C), 12 hrs, 22 °C then 5 days, 55 °C.

The ^{1}H NMR spectrum showed trace signals downfield between δ 7.1 - 7.3. A signal at δ 4.91 with an integral value of two corresponded to H_1-$H_{1'}$ of the sugar. The remaining H_2-H_6 protons were distributed around δ 3.9 - 3.2. This area included as well a signal at δ 3.8 for the proton in the β-hydroxy acid protected position. The trimethylsilyl protecting group of the β-hydroxy acid came at δ 0.93 (15 protons) followed by a signal at δ 0.88 for the terminal methyl's (6 protons). Finally, signals at δ 0.17 (27H) and 0.13 (27H) corresponded to the trimethyl protecting groups on the sugar alcohols. NMR data suggested the presence of both mono and di-substituted sugar esters as confirmed by MALDI-TOF analysis (Fig 70 appendix).

2.2.4 Coupling reactions between trehalose and acid chlorides

The use of DCC and DMAP with protected trehalose **60** and either palmitic acid, 3-hydroxy-octadecanoic acid **95**, 3-(*tert*-Butyl-dimethyl-silanyloxy)-2-icosyltetracosanoic acid **90** did give the corresponding esters. However, yields were generally low (43 %, 9 % and 40 % respectively) so a method which might increase the yield of the coupling was required. The first reaction examined involved an acid chloride coupling, using docosanoyl chloride and pyridine on a protected trehalose (Fig 71 (i)).[135,158] The reaction time was short and after column chromatography a first fraction was collected which corresponded to docosanoic acid (0.15 g, 34%). The second fraction contained the

product which was shown to be **103**, derived by coupling followed by loss of the protecting groups, by NMR and mass analysis (Fig 72-73 appendix). The mono-substituted product has a mass of $[M+Na]^+$ <700 and so it could not be detected by MALDI as the matrix interferes producing 'noise'.

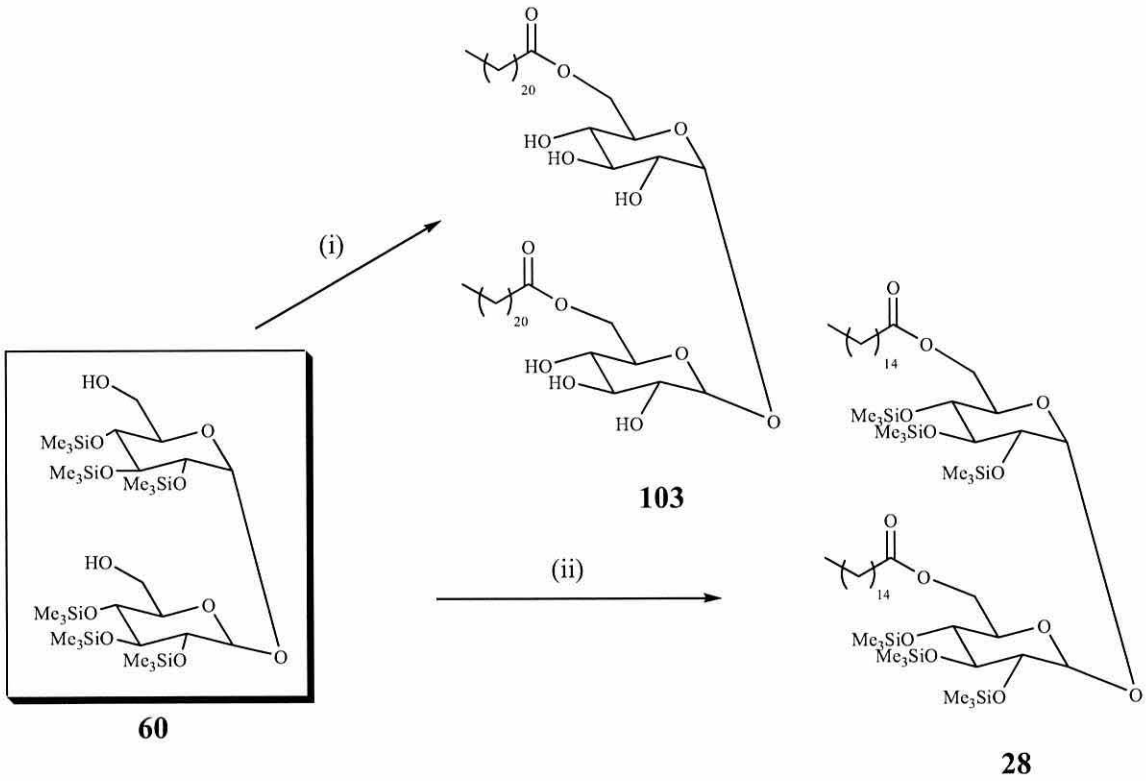


Fig 71. (i) dry pyridine/dry toluene, docosanoyl chloride/anhydrous toluene, 2.5 hrs, 80 °C (68 %); (ii) dry toluene/dry pyridine, hexadecanoyl chloride/anhydrous toluene, 12 hrs, 80 °C (85 %).

A shorter chain acid chloride, hexadecanoyl chloride was then used, under the same reaction conditions as before, keeping the same temperature, 80 °C but changing the reaction time from 2.5 to 12 hrs as in Fig 71 (ii). In this case, $MeOH/CHCl_3$ was not as in the previous case used in the reaction to promote OSiMe deprotection, in order to characterise the protected intermediate compound **28**. The $^1H/^{13}C$ NMR spectra for **28** were extremely clean with sharp clear peaks (Fig 74 appendix).

The intermediate **28** was then treated with trifluoroacetic acid/THF/H_2O for deprotection but the starting material product **60** was obtained; this may have been produced by breakage of the ester bond between sugar and the acid.

2.2.5 Coupling reaction between protected trehalose and alpha-alkyl beta-hydroxy acids using EDCI

A different coupling method involving EDCI and DMAP was tried to compare the yield and speed of reaction. The best method in terms of efficiency, speed of reaction and simplicity would finally be chosen to couple full mycolic acids to trehalose and other sugar like compounds. Instead of using DCC as coupling agent, EDCI was used (Fig 75, (i)) as described by Nishizawa *et al.*[219]

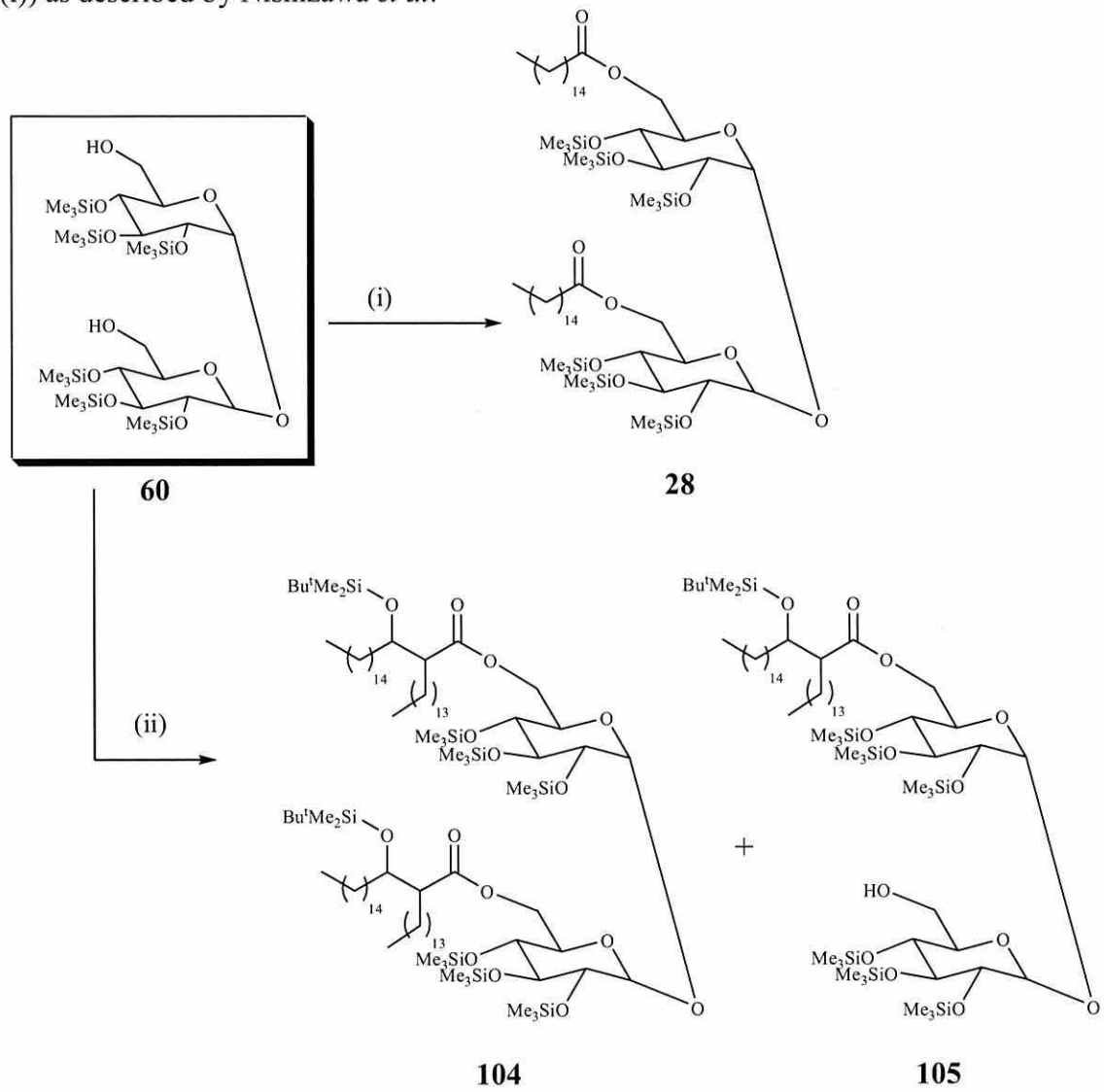


Fig 75. (i) hexadecanoic acid, EDCI, DMAP, molecular sieves, CH_2Cl_2, 56 hrs, 20 °C, N_2 (55 %); (ii) 3-(*tert*-Butyl-dimethyl-silanyloxy)-2-tetradecyl-octadecanoic acid **52**, DMAP, EDCI, molecular sieves, CH_2Cl_2, 60 hrs, r.t, N_2 **105** (40 %), **104** (25 %).

The reaction used DMAP as a catalyst to couple **60** to hexadecanoic acid, giving **28** as product (Fig 76-77 appendix) (Fig 78 shows the TLC of the products). The yield was 55 % compared to 85 % when using pyridine and hexadecanoyl chloride.

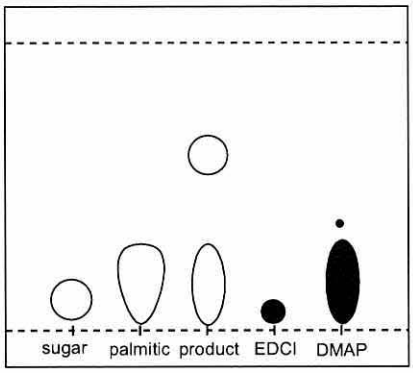


Fig 78. TLC for **28**. Top product includes di-substituted palmitate **28**. On the other hand, mono-substituted product comes in the same are as the starting material/reagents. Solvent used chloroform.

In the next step, the coupling of **60** to a protected alpha-alkyl beta-hydroxy acid **52** was examined. The reaction of **52** and **60** with DMAP/EDCI over 60 hrs gave a mixture of both di- and mono-substituted products, isolating 25 % (**105**) and 40 % (**104**) respectively (Fig 75, (ii)). The order of addition of reagents was important and was as follows; first the acid was added, followed by the protected sugar, DMAP, EDCI and finally molecular sieves. In order to get both mono- and di- substituted products, a minimum of 2.5 mol eq. of the acid compared to the sugar had to be used in the reaction.

The first fraction expected on column chromatography was the di-substituted trehalose **104**. Although NMR data confirmed that coupling had occurred, mass spectrometry gave the mono-substituted trehalose mass. This could be related to bad ionization of the di-substituted product. It must be taken into account that carbon NMR requires a bigger sample quantity than proton NMR in order to give good resolution and signal to noise ratio which is not always possible due the quantity of samples produced (Figure 79-80 appendix for ^{1}H and ^{13}C NMR). The ^{1}H NMR spectrum for **104** showed H1-H1' signals in the sugar resonating at δ 4.86 - 4.83 with an integral of 2H. The H6-H6' protons come next in the NMR at δ 4.67 - 4.53 (again 2H). The remaining sugar core signals, a total of 10 hydrogens could be seen from δ 4.01 - 3.49. The hydrogens in the beta position in the acid came next at δ 3.39, one for each acid. The next signal corresponded to the alpha

position two protons resonating at δ 2.56 and the CH_2 within the acid chain. On the other hand the ^{13}C NMR spectrum showed the most downfield signal at δ 178 corresponding to the carboxylic acid, followed by sugar core carbon signals ranging from δ 80 - 64. The next two signals corresponded to the beta position carbon to the acid and the alpha one, resonating at δ 64 and 50 respectively. The methyl carbon signals close to the silicon were at -4.39 and -4.64.

The second fraction corresponded to the mono-substituted trehalose **105** as confirmed by MALDI MS (the mass found was $[M+Na]^+ = 1390.22$ compared to required 1391.49) and NMR. The ^1H NMR spectrum showed the same pattern of signals as **104** but the sugar signals were doubled due to lack of symmetry. The ^{13}C NMR spectrum showed the same pattern with the carboxylic acid resonating at δ 177, the sugar core carbons showing signals from δ 94 - 72. In this case the split pattern for the sugar carbons was not observed completely, probably due to overlap. The remaining signals showed the same pattern observed in **104**. Although signals were satisfactory, they were a little bit broad; this may be due to a dilute/weak sample or the high molecular weight of the compound. (see Fig 81 appendix for ^1H NMR).

2.2.6 Trimethylsilyl deprotection reaction of alpha-alkyl beta-hydroxy acids esterified to trehalose

A further step was achieved with the deprotection of the silyl groups for both sugar and acid in compounds **104** and **105**. The reaction involved a two step deprotection starting with the removal of the trimethylsilyl groups on the sugar with TBAF (1.5 mol .eq) to get **106** (Fig 82 appendix). The second step involved deprotection of the *tert*-butyldimethylsilyl group on the *β*-hydroxy position using TBAF (1.5 mol.eq.) and trifluoroacetic acid (2 mol.eq.). The problem when using TBAF was that it is difficult to remove tetrabutylammonium oxide which runs at the same point as the product on TLC and on a column. Deprotection of **104** did not occur fully as the ^1H NMR spectrum showed a signal at δ 0.07 corresponding to dimethylsilyl protons on the β-hydroxy acid. It was then thought that a simpler method would be treating **104** with TBAF/TFA to fully

deprotect in one go (Fig 83). Once again the reaction was unsuccessful as protecting group still remained.

To overcome this problem, HF-pyridine complex was used to fully deprotect **106**. The method involved adding the complex at 10 °C and stirring for 2.5 hrs. Neutralization with sodium bicarbonate, extraction and purification did give the starting material **106** as confirmed by NMR.

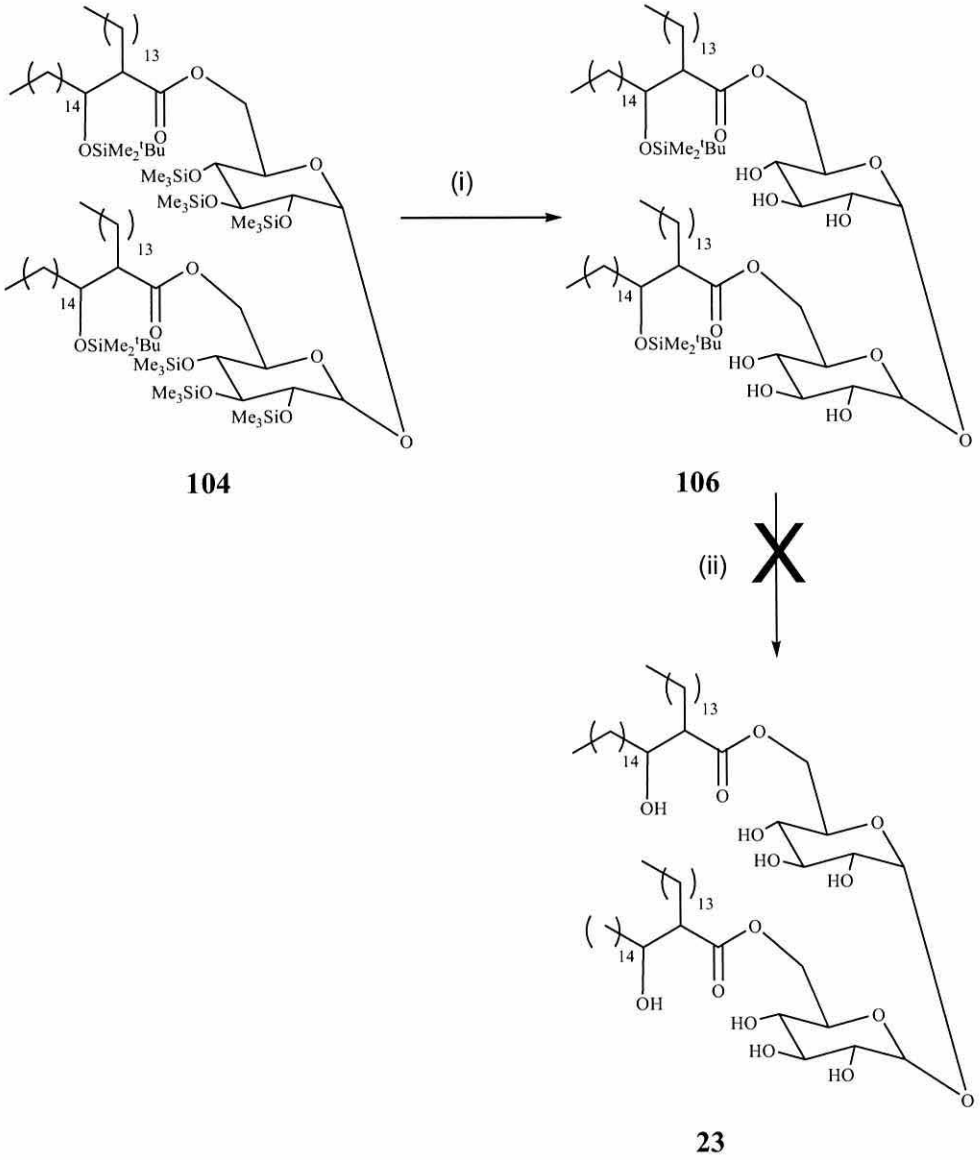


Fig 83. Deprotection of **104**. (i) dry THF, TBAF, 90 min, r.t. (ii) pyridine, THF, HF-pyridine complex as 70 % hydrogen fluoride at 10 °C, 2.5 hrs, r.t.

2.3 Mycolic acid synthesis

2.3.1 Aim and overview

The molecule targeted in this section was the α-mycolic acid **107** (see Fig 84) which is one of the major components of *M. tuberculosis*. On the other hand **108** had already been synthesised by Al Dulayymi *et al.*[220]

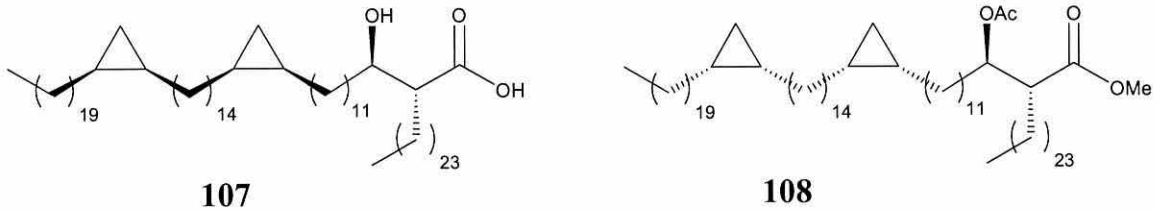


Fig 84. The target mycolic acid **107** and the previously synthesised diastereoisomer **108**.

As described in the introduction tuberculosis is responsible for million people deaths every year. The fact that there are not many drugs available for treating the disease makes things worse. Mycolic acids are responsible for antibiotic resistance due the fact that the cell wall structure containing them acts as an impermeable membrane or barrier to outside compounds. By changing the composition of these mycolic acids, membrane permeability changes and this will have an influence in resistance to antibiotics.

Mycolic acids are complex mixtures which vary from one species to another.[221] It is of great importance to know the different configurations and structures of such compounds and over the years several efforts have been made in this area. As an example, Minnikin and Polgar reported that mycolic acids containing two *cis*-cyclopropanes were the major components of *M. tuberculosis.*[222] Naturally isolated α-mycolates were detailed and described in previous papers.[223] It was decided to synthesise **107** as the stereochemistry of the *cis*-cyclopropane in alpha-mycolic acids remains unclear. Several mycolic acids have been prepared in recent years,[224,225] and in this section mycolic acid **107** will be compared with the diastereomeric di-*cis*-cyclopropane **108** having the opposite absolute stereochemistry at each cyclopropane, synthesised by Al Dulayymi *et al.*[220] The final aim

of this thesis was to obtain a full mycolic acid, in this case an alpha mycolic acid to be used in TDM/TMM synthesis.

The synthetic steps followed in the synthesis of **107** are similar to those followed in the diastereomeric mycolic acid **108** using 'building blocks' for the preparation of the corynomycolate moiety and in some parts of the meromycolate moiety.

The preparation of mycolic acids has been achieved in the past following methods reported in the literature.[226] The retrosynthesis shows some key intermediates (Fig 85): cyclopropane ring formation and chain extension to give **113**; chain extension on the other side of the cyclopropane to give **116**; Julia reaction to couple a second cyclopropane extended chain to give **121** and finally addition of the corynomycolate **125** moiety to give **127**.

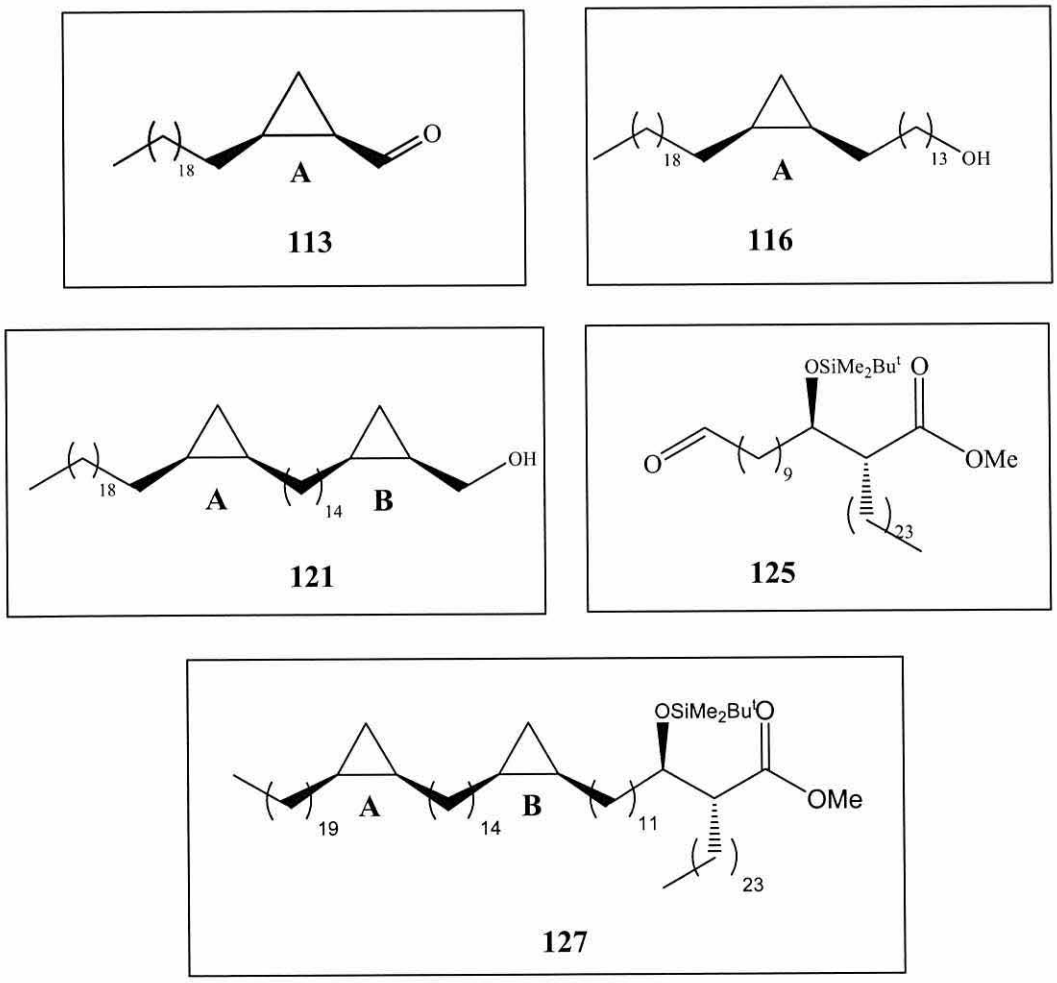
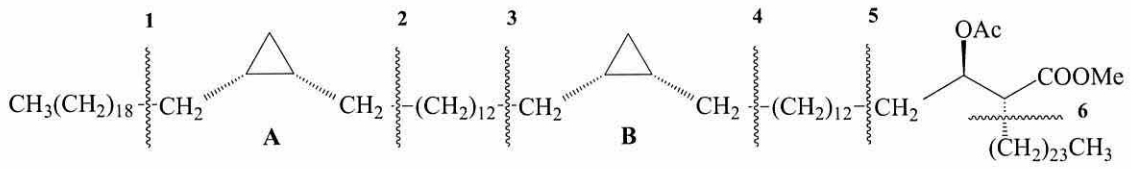


Fig 85. Building blocks involved in alpha mycolic acid **107** syntheses.

The order in which experiments will be shown follows a chronological sequence where specific problems will be highlighted.



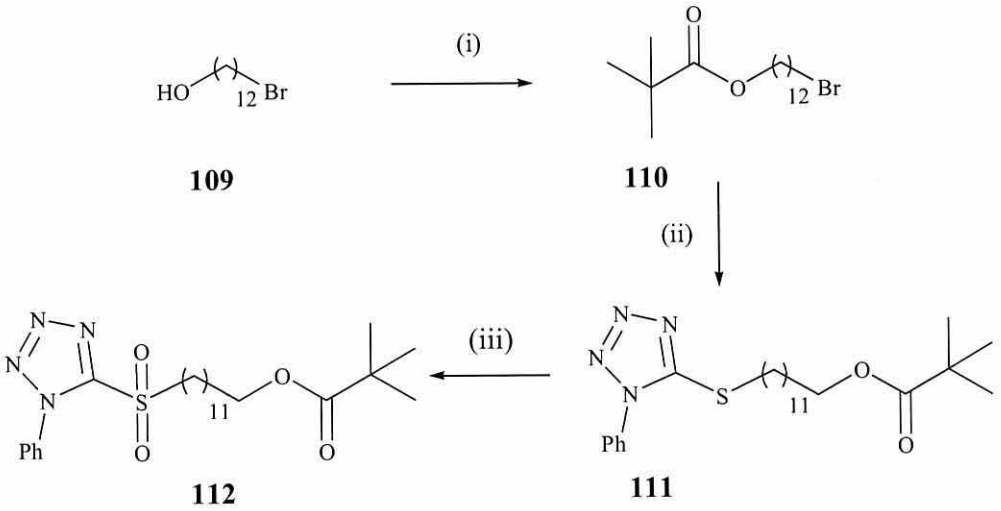
108

Fig 86. Carbon-carbon attachment of synthesised units is shown for Al Dulayymi's model for diasteromer **108**.[220] Numbers show attachment points and the letter are to differentiate between cyclopropane rings.

Although the synthetic methods used varied in some cases depending on the absolute stereochemistry for each cyclopropane, the bonds were created in positions as in Al Dulayymi's synthesis as shown in Fig 86.

2.3.2 Cyclopropane ring formation and chain extension

Figures 87, 88 and 90 show the chain extension on both sides of the cyclopropane ring to produce **116**. Bromoalcohol **109** was obtained with 44 % yield from HBr and 1,12-dodecanediol. Protection of **109** with trimethylacetyl chloride gave **110** (89 %).



109 **110** **111** **112**

Fig 87. Chain extension from the cyclopropane ring. (i) trimethylacetyl chloride, $CHCl_2$, pyridine, 4-DMAP, r.t, 18 hrs; (ii) 1-phenyl-1H-tetrazole-5-thiol, KCO_3 in acetone, 18 hrs, r.t; (iii) ammonium molybdate (VI) tetrahydrate, H_2O_2, THF, IMS, 2 hrs, r.t.

Conversion of the primary bromide **110** into the corresponding sulfide **111** and then sulfone **112** by routine procedures was achieved in 86 and 73 % yield respectively.

2.3.3. The Julia reaction to couple the second cyclopropane extended chain

The newly synthesised (1*R*,2*S*)-aldehyde **114** (supplied by S.Heitmuller) was obtained by the standard method from the corresponding alcohol using PCC in 86 % yield (Fig 88). The method as described by Al Dulayymi *et al,*[220] includes the coupling of a nona-decyltriphenylphosphonium salt to (1*S*,2*R*)-butyryloxymethyl-2-formylcyclopropane using butyllithium in THF, leading to the ester (Fig 86) which was reduced to the alcohol and the double bond saturated with hydrazine hydrate, sodium periodate, copper sulphate and acetic acid.

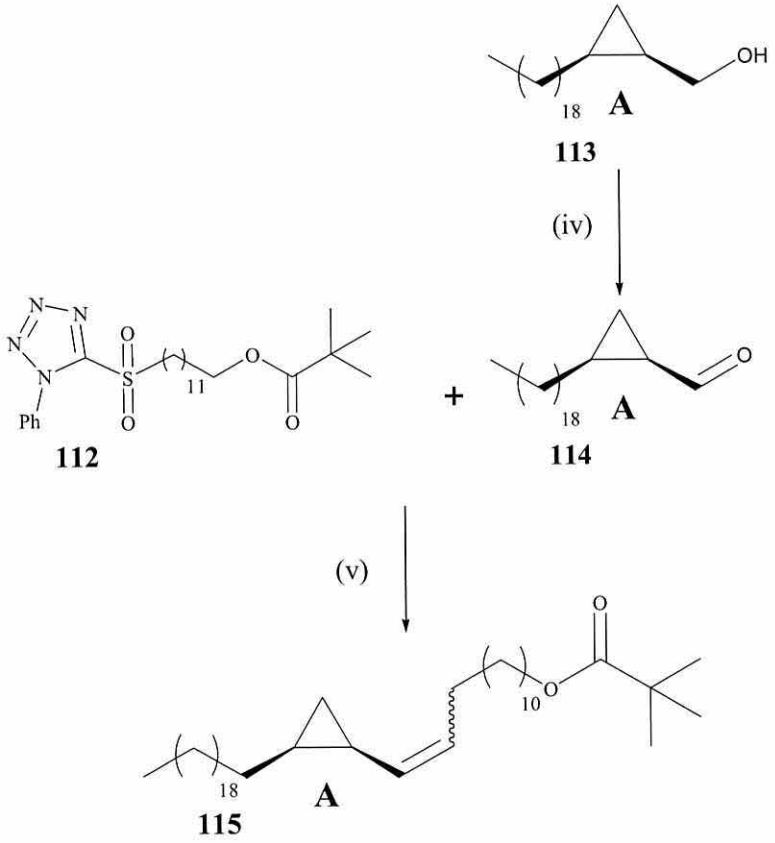


Fig 88. (iv) PCC in CH_2Cl_2, 2 hrs, r.t; (v) Lithium bis(trimethylsilyl)amide, THF, 3 hr, r.t.

Finally oxidation of the alcohol led to **119** (see Fig 89 appendix). Using the same reaction, Al Dulayymi *et al* obtained yields up to 90 % for the (1*S*,2*R*)-aldehyde **119** and the specific rotation was was -3.9 (c = 1.22, $CHCl_3$) whereas it was +3.76 (c = 1.1, $CHCl_3$) for **114**. The ^{1}H and ^{13}C NMR spectra were the same as those reported for the enantiomer except that the latter were broader possibly due the fact they were obtained on a 250 MHz spectrometer.[220]

A Julia reaction of **114** with sulfone **112** amend **115**; alcohol deprotection with $LiAlH_4$ giving **116** and double bond hydrogenation with sodium (meta)periodate generated **117** in 85 % yield (Fig 90). Conversion of **117** into **118** was achieved using PCC in 84 % yield, and the aldehyde gave a specific rotation of +1.59.

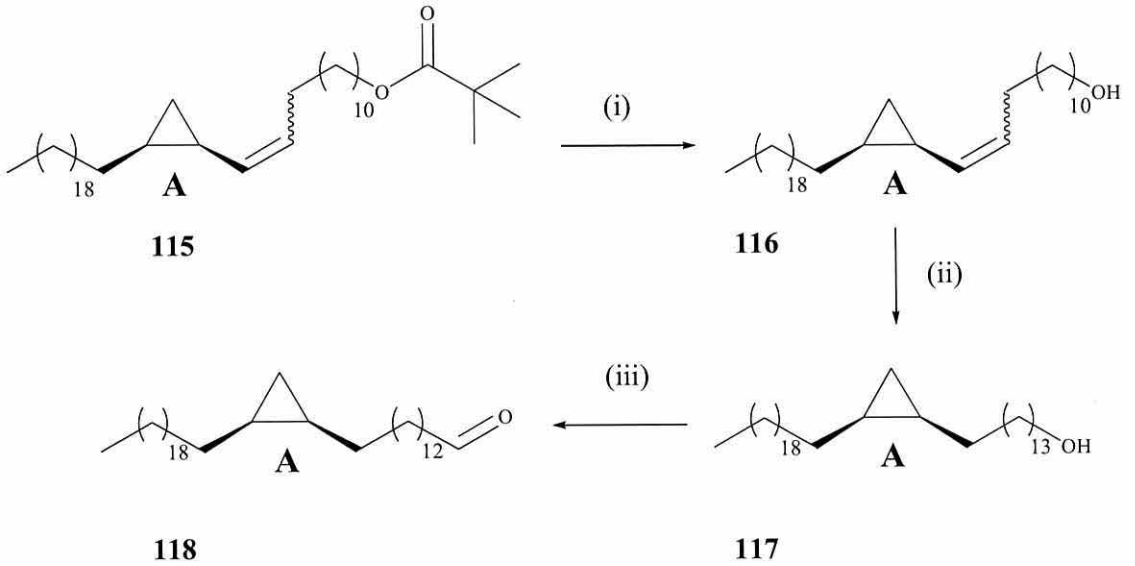


Fig 90. Final chain extension on the cyclopropane ring. (i) $LiAlH_4$, THF; (ii) Sodium (meta) periodate, isopropyl alcohol, acetic acid, copper sulphate, hydrazine hydrate, 2 hrs, r.t; (iii) PCC in CH_2Cl_2, 2 hrs, r.t.

The enantiomer **120** had been obtained in 93 % yield with a specific rotation of -1.69.[220] The ^{1}H NMR spectrum for compound **118** agreed with those of the enantiomer (Fig 91)[220] except that the signal for the proton next to the aldehyde that should be seen around 9.72 ppm was not present in **118**; however the carbon signal at 202.0 was seen.

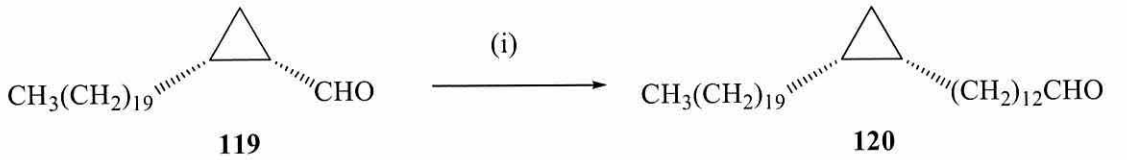


Fig 91. Homologation of 88a, (i) $MeO_2(CH_2)11PPh_3I$, MeONa, THF, DMF (70 %); $LiAlH_4$, THF (92 %); N_2H_4, $NaIO_4$, AcOH, $CuSO_4$, *i*-PrOH (85 %); PCC, CH_2C_{l2} (93 %).

A second Julia reaction of **118** with ((1*R*,2*S*)-2-((1-phenyl-1H-tetrazol-5-ylsulfonyl)methyl)cyclopropyl)methyl butyrate gave **122**, followed by alcohol deprotection with $LiAlH_4$ giving **123**; double bond hydrogenation with sodium (meta) periodate generated **124** in 63 % yield, whereas the yield reported for the enantiomer was 89 % (Fig 92 and Fig 93 appendix for **124**).[220] Melting point values were 72-74 °C for **124** whereas the value for its diastereisomer ((1*S*,2*R*)-2-(14-((1*S*,2*R*)-2 icosylcyclopropyl)tetradecyl)cyclopropyl)methanol was 74-76 °C. Rotation values were +8.33° for **124** compared to -2.04 ° for its diastereoisomer. The ^{1}H NMR results for **124** were performed on a 500 MHz machine compared to the 250 MHz which had been used for its diastereoisomer. Compound **124** showed two downfield signals for the two protons close to the alcohol resonating at δ 3.66 and 3.55 respectively.

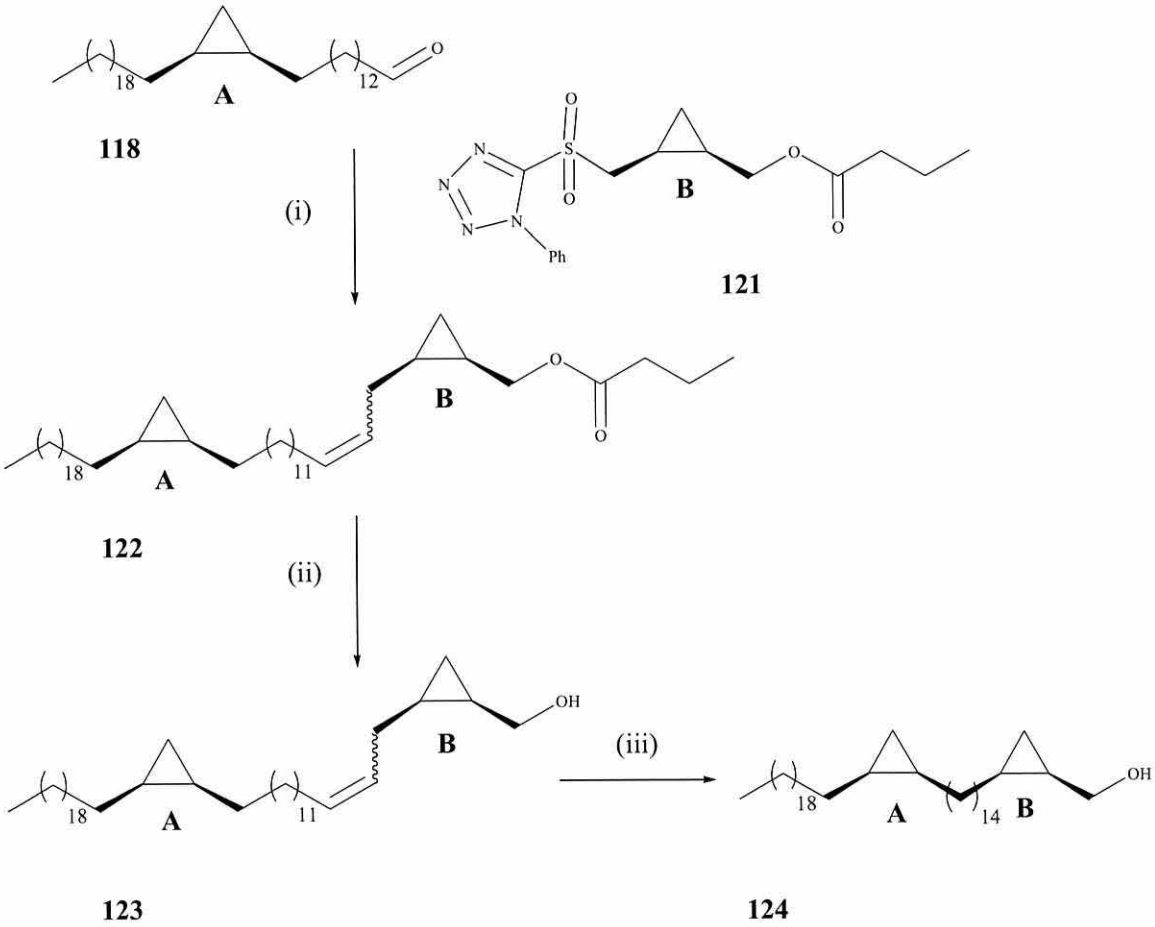


Fig 92. The second cyclopropane ring addition. (i) Lithium bis(trimethylsilyl)amide, THF; ((1*R*,2*S*)-2-((1-phenyl-1H-tetrazol-5-ylsulfonyl)methyl)cyclopropyl)methyl butyrate, 5 hrs, r.t; (ii) $LiAlH_4$, THF, reflux, 3 hrs; (iii) Sodium (meta) periodate, isopropyl alcohol, acetic acid, copper sulphate, hydrazine hydrate, 2 hrs, r.t.

The next signal was the CH_2 in the chain resonating at δ 1.56 - 1.20. Signals at δ 1.17 integrating to 4H corresponded to the protons close to the cyclopropane ring on both sides of the ring opposite to the alcohol position. Signals corresponding to the cyclopropane came next resonating at δ 0.8 (2H), 0.70 (1H), 0.66 (2H), 0.57 (1H), -0.03 (1H) and -0.32 (1H) making a total of 8H for both rings.

Signals in the ^{13}C NMR spectrum were not shown in Al Dulayymi paper for ((1*S*,2*R*)-2-(14-((1*S*,2*R*)-2 icosylcyclopropyl)tetradecyl)cyclopropyl)methanol;[220] in the case of **124**, signals were as follows, δ 64 for the carbon close to the alcohol followed by δ 32 corresponding to the carbons attached to the cyclopropanes. The CH_2 signals ranged from δ 28 - 22 whereas the cyclopropane rings showed signals from δ 15 - 9. The IR spectra for both diastereoisomers showed characteristic signals for the alcohol at 3400 and 3375 respectively.

2.3.4. Corynomycolate moiety addition

Compound **124** was transformed into the corresponding sulfide **125** and from there to the sulfone **126** (Fig 94). The yields for those two steps were 93 % and 85 % respectively. Protection of the alcohol position in **124** was achieved by using 1-phenyl-1H-tetrazole-5-thiol, whereas in Al Dulayymi's method, 2-mercaptobenzothiazole was used. The modified aldehyde **128** was also prepared using a more efficient method, giving a 97 % yield using a silyl protecting group which was easier to work with and to remove.[227] In Al Dulayymi's[220] route to the isomer, an OAc protection on the alcohol giving ((1*R*, 2*R*)-1-acetoxy-11-oxoundecyl) hexacosanoic acid methyl ester in 95 % yield was used. A later paper by Al Dulayymi *et at* [226] described the synthesis of **128** in 84 % yield (Fig 95 appendix). Fig 94 shows the last step in the synthesis of **128**. Mass spectrometry showed a $[M+Na]^+$ = 731.63 compared to the 731.63 expected. On the other hand rotation values were similar with -4.50 compared to -4.48 in Al Dulayymi's case. ^1H NMR and ^{13}C NMR data coincided with those found in the paper.

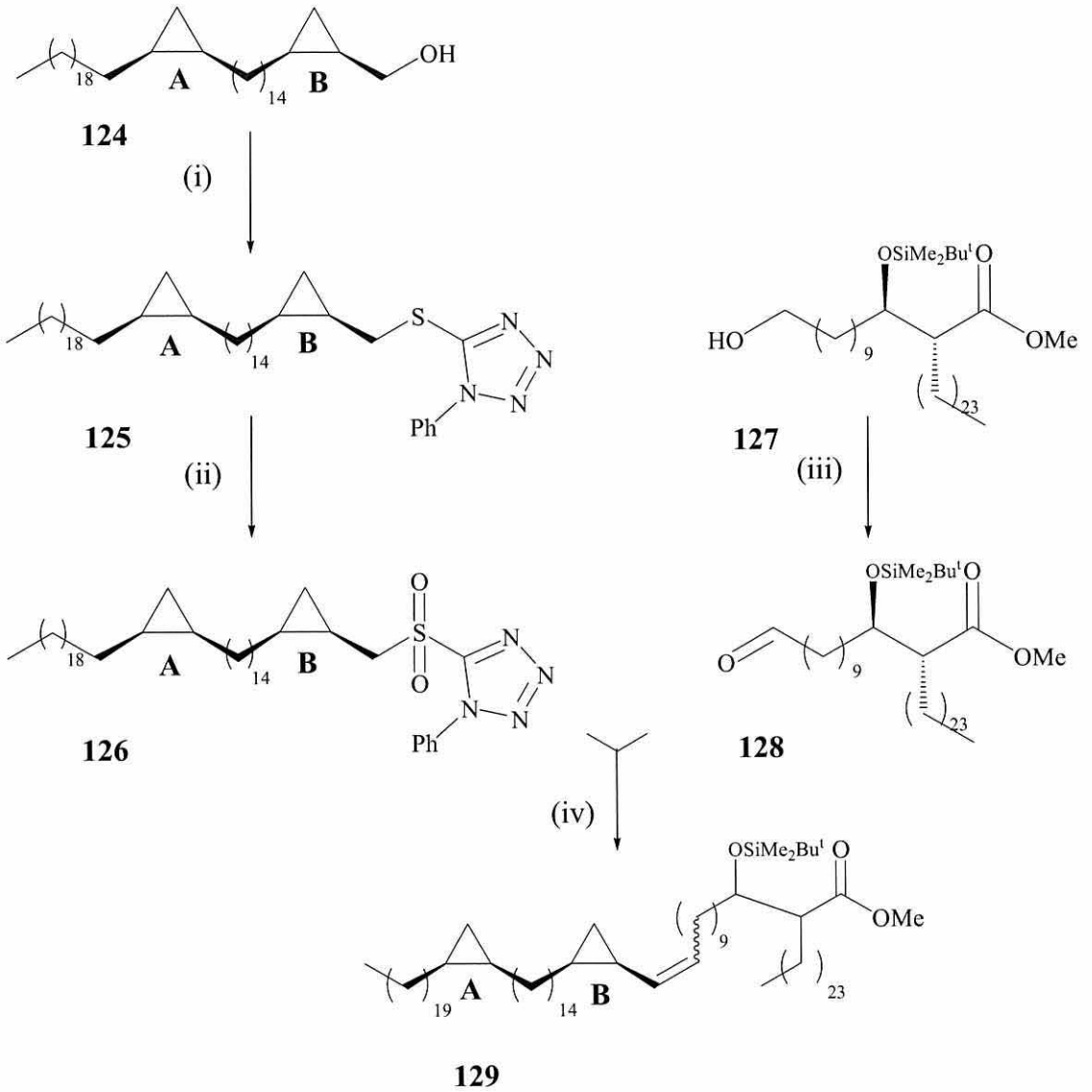


Fig 94. (i) Diethyl azodicarboxylate, THF, triphenylphosphine, 1-phenyl-1H-tetrazole-5-thiol in THF, 45 min, r.t; (ii) CH_2Cl_2, $NaHCO_3$ at r.t; meta-chloroperbenzoic acid 70 % in CH_2Cl_2 previously dried with $MgSO_4$; (iii) PCC in CH_2Cl_2, stir 3hrs, r.t; (iv) lithium bis(trimethylsilyl) amide, THF.

Reaction of the aldehyde **128** with the sulfone **126** in the presence of lithium bis(trimethylsilyl)amide gave **129**. Saturation of the double bond and removal of the silyl group led to **130** and **131** respectively (Fig 96). Compound **131** still showed a signal for three hydrogens at δ 3.71 corresponding to the OMe but had lost the signals for the silyl group on the *β*-hydroxy acid corresponding to the two methyl units and the *tert*-butyl. The ^{13}C NMR signals corresponding to these groups at δ -4.37 and -4.93 had also disappeared.

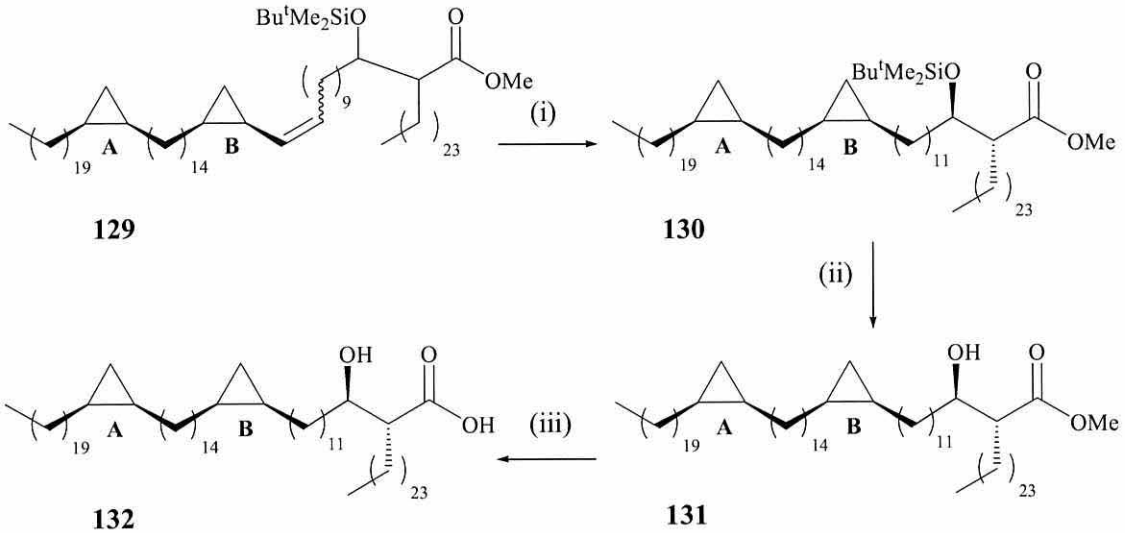


Fig 96. (i) THF/MeOH, acetic acid in THF dropwise, stir 9 hrs, r.t; (ii) anhydrous pyridine in THF, stir r.t; HF-pyridine complex, stir 17 hrs, 45 °C; (iii) THF/H_2O/MeOH, add LiOH, stir 12 hrs, r.t.

Removal of the methyl group in **131** (that was hydrolysed to the acid) was done under standard method using LiOH to obtain the final alpha mycolic acid **132** with 93 % yield in this final step. The ^{1}H NMR spectrum of **132** showed the characteristic peaks for the proton on the carbon having the β-hydroxy-acid resonating at δ 3.71; the α carbon resonated at δ 2.44 and both cyclopropane rings including protons at δ 0.64 corresponding to 4H, δ 0.57 for 2H and δ -0.32 for another 2 H. These figures matched it diasteroisomer but peaks for the latter were less sharp due the fact that they were recorded on a 250 MHz machine. It must be taken into account that Al Dulayymi's compound is acetyl protected and has a methyl protection in the carboxylic acid making direct comparisons difficult. In the ^{13}C NMR spectrum for **132**, the carboxylic acid signal was at δ 182, followed upfield by the carbon on the beta hydroxy acid position resonating at δ 72. The alpha carbon resonated at δ 51. The carbons adjacent to the cyclopropanes showed signals between δ 35 - 34 whereas the long chain CH_2 carbons ranged from δ 29 - 22. Signals for the cyclopropane rings came between δ 14 - 10. IR showed the alcohol peak at 3427 and carbonyl at 1467 cm^{-1}. MALDI MS gave an $[M+Na]^+$ = 1160.59 while the expected value was 1161.06.

Fig 97 shows a TLC plate including the alpha mycolic acid synthesised comparing it with it natural homologue as well as with other mycolic acids.

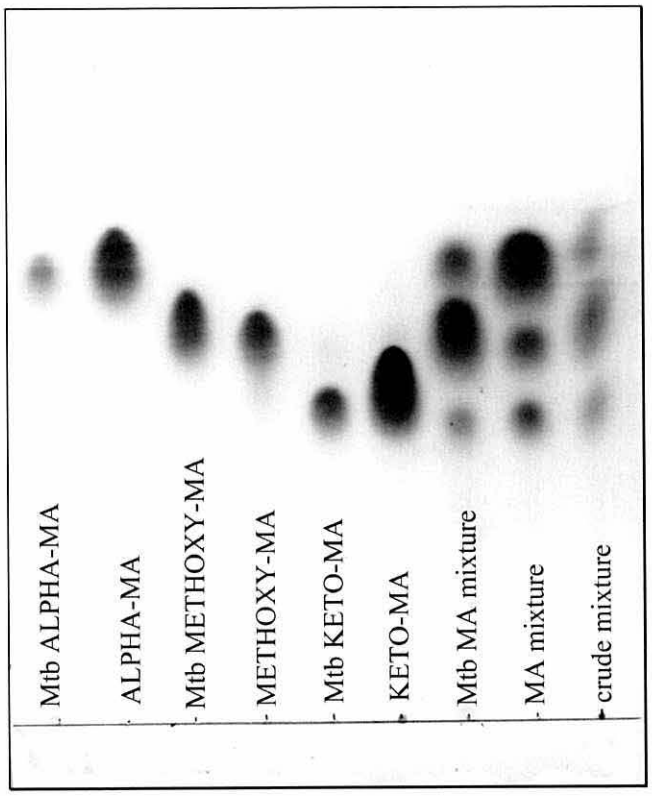


Fig 97. TLC plate including final alpha mycolic acid **132**. From left to right. Mtb alpha-MA (natural alpha mycolic acid separated by purification), alpha-MA correspond to **132**, Mtb methoxy-MA (natural methoxy mycolic acid separated by purification), methoxy-MA correspond to **133**, Mtb Keto-MA (natural Keto mycolic acid separated by purification), Keto-MA correspond to synthetic compound. The last two samples represent a MA mixture for the three synthetic compounds whereas crude mixture includes a mixture of the three naturally purified samples. Samples were run in apolar solvent for 12 hrs in a long glass TLC plate (20 cm long) to allow sample separation.

2.4 Cord factor synthesis

2.4.1 Aim and overview

Mycobacteria contain long chain β-hydroxy acids (MAs) the synthesis of one of which has been described in the previous section. Such compounds are present in the membrane free as MAs or esterified to trehalose, forming trehalose-6,6'-dimycolates (TDMs cord factors) and trehalose monomycolates (TMMs).The aim of this section was to synthesise a complete TDM based on a synthetic mycolic acid.

2.4.2 Methoxy mycolic acid protection and esterification with trehalose

All mycolic acids must be protected in their *β* hydroxy acid position to avoid alcohol reactions. The most commonly used group for this protection is the *tert*-butyldimethyl-silyl ether due to simplicity of removal and because it is is an easy compound to work with.

The methoxy mycolic acid used to couple to trehalose was provided by Dr. Al Dulayymi **133** and was converted to **134** following a standard silylation method as described in Fig 98.

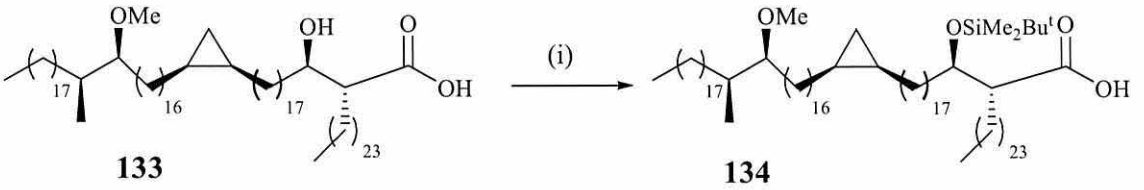


Fig 98 (i) Imidazole, (*R*)-2-{(*R*)-1-hydroxy-18-[(1*R*,2*S*)-2-((17*S*,18*S*)-17-methoxy-18-methylhexatria-contyl)-cyclopropyl]-octadecyl}hexacosanoic acid **133**, dry DMF, dry toluene; *tert*-butyldimethylsilylchloride, 4-dimethylaminopyridine, 70 °C, 24 hrs and r.t, 18 hrs.

The ^{1}H NMR spectrum showed 3H resonating at δ 0.15 and 3H resonating at δ 0.13 which corresponded to the methyl close to the silicon atom in the protecting *tert*-butyldimethyl silyl group. The *tert*-butyl protons, 9 in total appeared at δ 0.89.

Characteristic signals in the methoxy mycolic acid were also present, such as OMe at δ 3.36 and the proton on the β-hydroxy acid resonating at δ 3.85. Once again, the cyclopropane ring signals were found at δ 0.63, δ 0.57 and δ -0.33 with two, one and one protons respectively. Signals in the ^{13}C spectrum were consistent with structure **134**, including δ 177.29 corresponding to the carboxylic acid, the methoxy carbon at δ 85.47 and δ 73.56 for the β-hydroxy carbon.

The protecting group carbons next to silicon resonated at δ -4.31 and 4.92 and the three carbons in the cyclopropane ring within δ 10-15.

A variety of methods were used in Section 2.2 to esterify acids to trehalose, each of them presenting some advantages and disadvantages. The majority of the methods required alcohol protection at sites not involved in the coupling, such as the 3,4,5 positions in the sugar core of trehalose. Protecting groups on the sugar were chosen depending on the simplicity of reaction and easy removal, and bulkier structural compounds were avoided due the fact that they could hinder reaction sites, in this case the 6,6'-positions on the sugar core. The results and discussion section describes all the protecting groups used for both acid and sugar reactants.

After modelling the reaction using a variety of model acids such as **68, 72, 75, 90, 52, 91** or **96** in coupling to protected trehalose, the reaction giving the best results was found to be a modified version of the Datta and Takayama procedure.[164] The protecting group finally used on the sugar in cord factor preparation was trimethylsilyl, which shows a balance between stability and easy removal which was not the case for example with AcO-groups. Model reactions including trehalose ester compounds were not successful in deprotecting AcO-groups in the sugar as shown for compounds **81** and **82**.

Esterification of trehalose anhydride **60** and **134** was accomplished using dicyclohexacarbodiimide (DCC) as an activating agent and 4-dimethylaminopyridine (4-DMAP) as catalyst.[228] The reaction was carried out under dry conditions under a nitrogen atmosphere and the mixture was stirred for 6 days at r.t as temperatures above 45 °C produce ester bond destruction. The Datta and Takayama method was described in the literature for simple short chain corynomycolates and reaction times were 6-8 hrs. It was found that, in the case of **134**, the reaction takes place forming an intermediate compound

shown in the TLC from days 1-4. This compound was characterised as **135** corresponding to the anhydride coupling of two compound **134** (Fig 99).

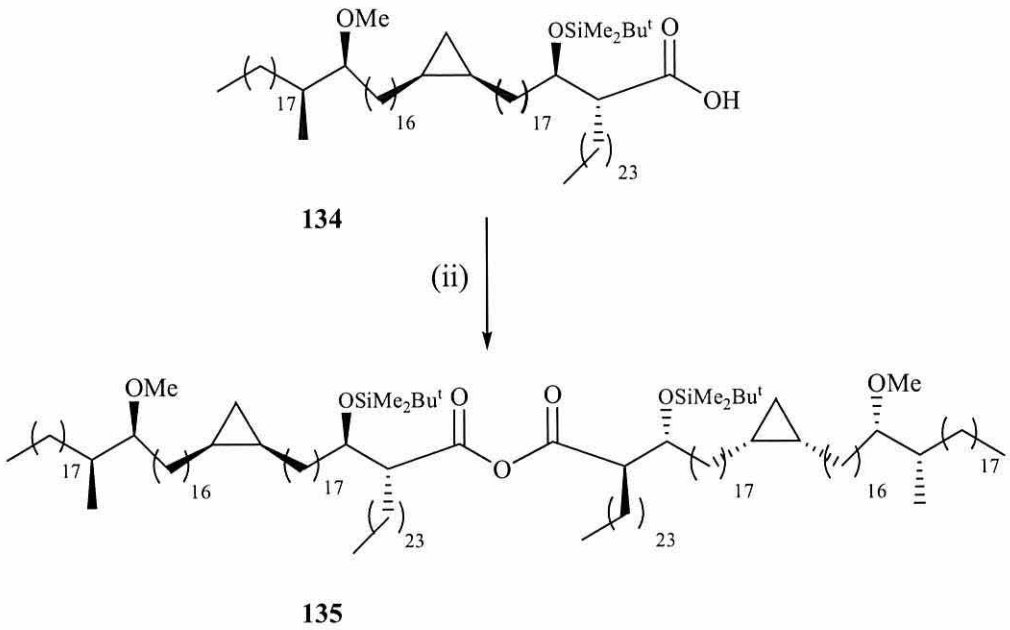


Fig 99. Anhydride formation, (ii) EDCI, 4-DMAP, 47, dry CH_2Cl_2, 6 days r.t.

MALDI MS results showed $[M+Na]^+$ = 2742.10 compared with the required 2742.05. The starting material **134** had only a mass of 1390 whereas the two other products, **136** and **137** had masses of 3498 and 2146.

The ^1H NMR spectrum showed two singlets for the methyl protons close to the silicon atom in the protecting dimethyl silyl, going from 6H in compound **134** to 12H resonating at δ 0.07 in **135**. The *tert*-butyl protons, 9 in total for **134** were doubled for **135,** resonating at δ 0.90. The same happened to the characteristic signals in the methoxy mycolic acid that were still present, such as OMe at δ 3.35 and the 2H (one for each chain) in the β-hydroxy acid resonating at δ 3.95. In fact all signals were reduced in number and doubled in relative size due to symmetry. Once again, the cyclopropane ring signals were found at δ 0.67, δ 0.57 and δ -0.33 integrating for four, two and two protons respectively. The ^{13}C NMR signals followed a similar pattern with δ 169.47 corresponding to the carbon of the anhydride; the methoxy carbon resonated at δ 85.44 and the β-hydroxy carbon at δ 72.72. Figure 100 shows a TLC plate representation for a

crude sample before column for **136** and **137**. The sample shows anhydride **135** at the top, followed by **136**, **137** and **134** respectively.

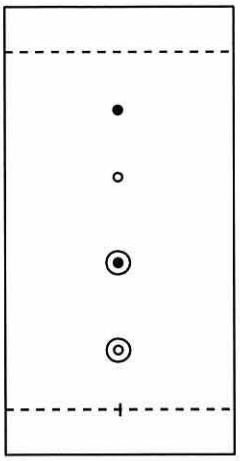


Fig 100. TLC for crude TDM/TMM sample of **136/137**. Petrol/ethyl acetate 20:1. From the top to the bottom; solid dot (anhydride), hollow bold (TDM), hollow (TMM) and hollow double (acid).

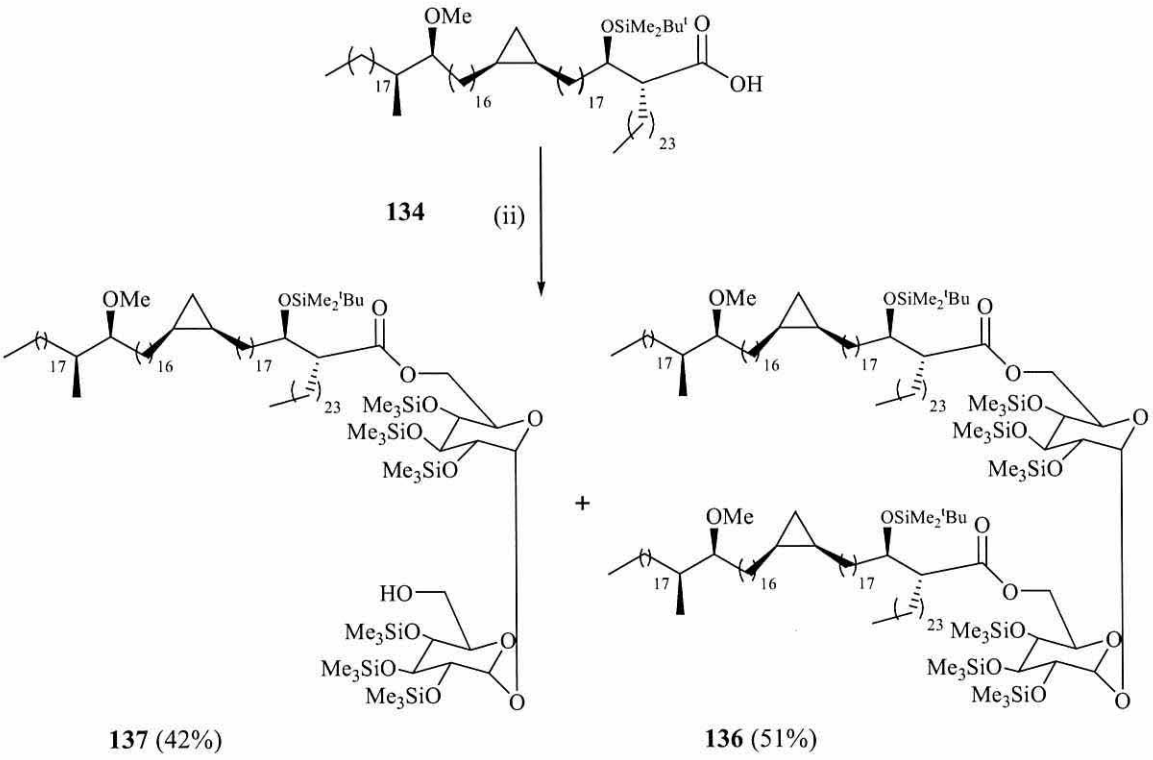


Fig 101. Esterification reaction, (ii) EDCI, 4-DMAP, 47, dry CH_2Cl_2, 6 days r.t.

The reaction began to form the products **136** and **137** from days 4 – 6 (Fig 101). TDM **136** was the first product obtained in 51 % yield (Datta and Takayama obtained 69 % for a TDM **28** involving a corynomycolic acid) and its structure was confirmed by MALDI mass spectrometry and NMR. The ^{1}H NMR spectrum for TDM **136** showed signals for the silyl protection for both alcohol positions in the methoxy acids resonating at δ 3.35 and at δ 0.88 for the *tert*-butyl protons, 18 in total. Characteristic signals in the methoxy mycolic acid were still present, such as OMe at δ 3.35 and the proton adjacent to the β-hydroxy group resonating at δ 3.96. Cyclopropane ring protons gave similar signals to those observed for **134**. The 6 sugar core signals appeared at δ 4.85, for H1 close to the hemiacetal, δ 4.37 for H6 and δ 4.04, 3.94, 3.90, 3.52 and 3.38 for the remaining protons. In comparison, signals in **60** were at δ 4.91 for H1, δ 3.89 for H6, and δ 3.85, 3.73, 3.67, 3.48 and 3.42. The signals for H6 shifted downfield after esterification. In contrast the H1 protons had moved upfield. Silyl protecting groups for the alcohols in the sugar core were found at δ 0.16, 0.14, and 0.12 integrating to 18H each.

The second fraction obtained after the column was the TMM **137** in 42 % yield, with both MALDI and NMR results confirming the structure. The signals for the sugar protons ranged from δ 4.91 to δ 3.39. In this case, the signals were not symmetrical and the 14H expected for the sugar core of trehalose were distributed mainly in a one signal to one proton basis. The methyl signals for the protecting groups on the sugar were found split into six different groups (one for each Me_3 situated in C2-C3-C4) ranging from δ 0.172 - 0.124 and corresponding in each case to 9H and to a total of 54 hydrogens. Once again the spectrum reflects the non-symmetrical pattern in the TMM structure leading to much more complicated signals. The methoxy mycolic acid showed signals at δ 3.85 for the β-hydroxy acid and δ 3.34 for the OMe. Signals for the remaining groups did not change. The ^{13}C NMR spectrum showed the same number of signals due to asymmetry but clearly had δ 174.1 for the ester group, δ 94.52 and 94.39 for C1 in the sugar cores, and δ -4.45 and -4.68 for the two methyl groups next to the silicon in the $OSiMe^{2}Bu^{t}$.

The next step in the sequence corresponded to sugar deprotection of both products obtained in the previous step under separate reactions. Compounds **136** and **137** were each deprotected using TBAF and dry THF under dry conditions and a nitrogen

atmosphere to give **138** (78 %) and **139** (80 %) respectively (Fig 102). In comparison, Datta and Takayama reported a 69 % yield when deprotecting **28** and obtaining **31**.

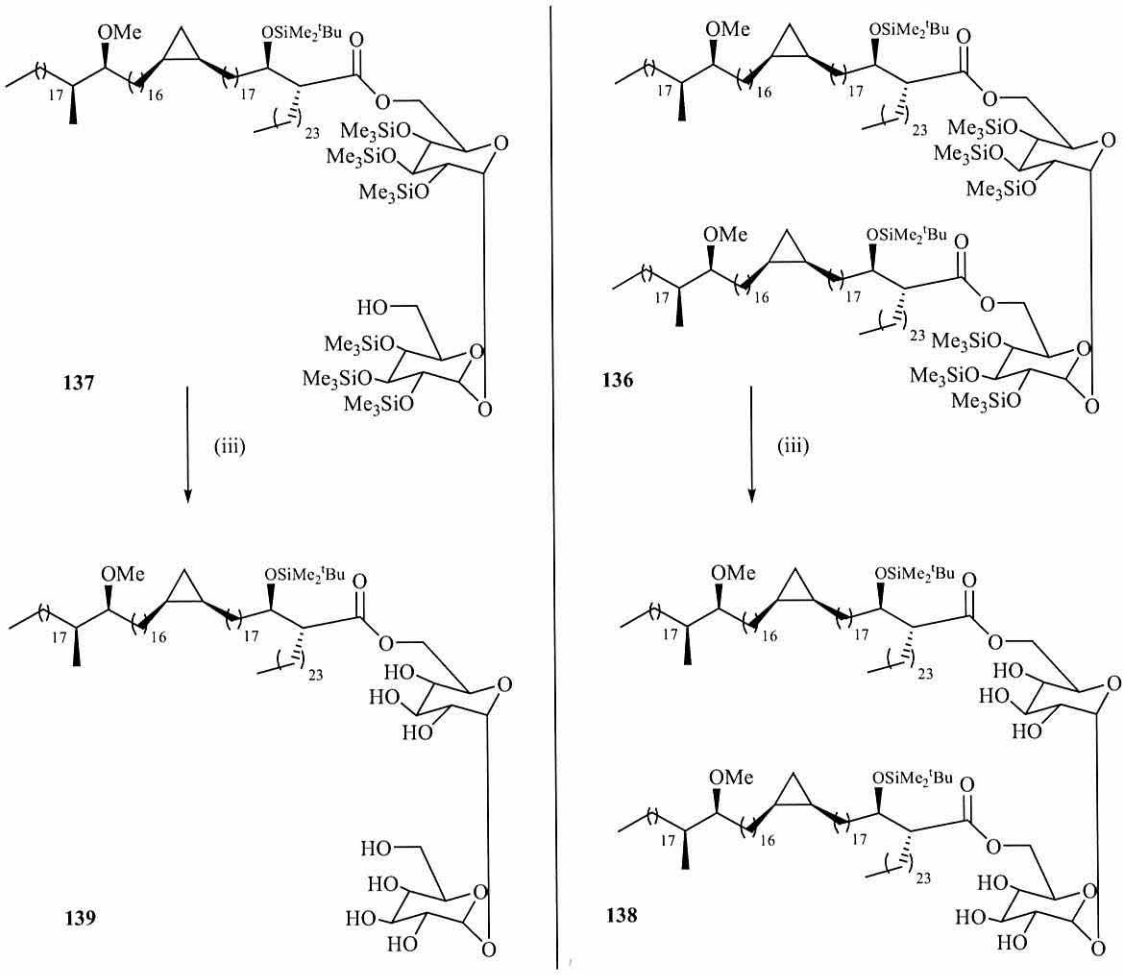


Fig 102. TDM/TMM suagr deprotection step, (iii) TBAF, dry THF.

Compound **138** has lost the signals for the 54 protons corresponding to the 18 methyl groups previously resonating at δ 0.16 - 0.14. In Fig 103 (appendix) can be seen the a MALDI-MS showing the theoretical value for $[M+Na]^+$ for compound **138** at 3065.9 and the observed value of 3065.8. On the other hand, compound **139** had lost the protons previously resonating at δ 0.172 - 0.124.

The last step was to remove the $SiMe_2Bu^t$ protection from the alcohol in the β-position of the mycolic acid, leading to compounds **140** (72 %) and **141** (78 %) (Fig 104).

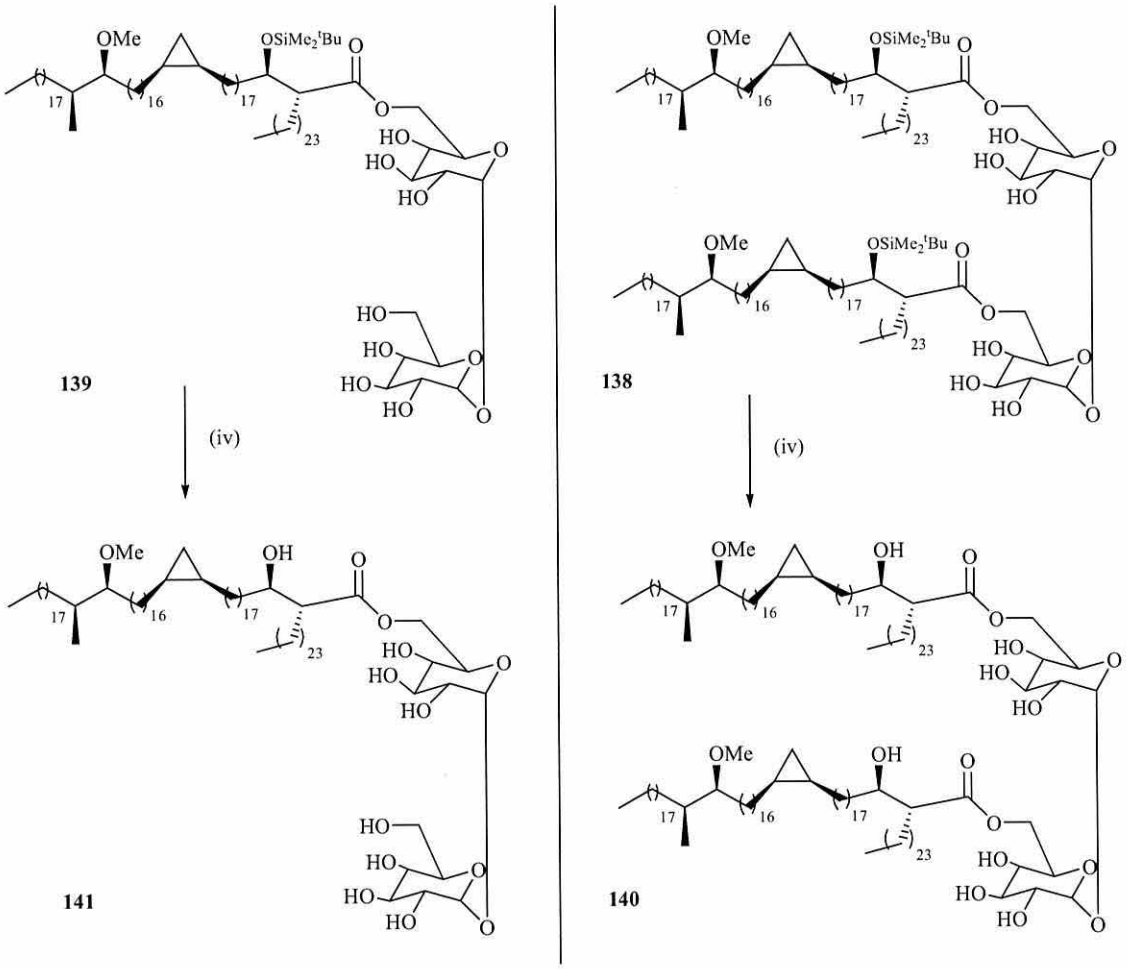


Fig 104. TDM/TMM beta alcohol deprotection step, (iv) pyridine in dry THF, Hydrogen fluoride-pyridine complex, stirred at 43 °C, 17 hrs.

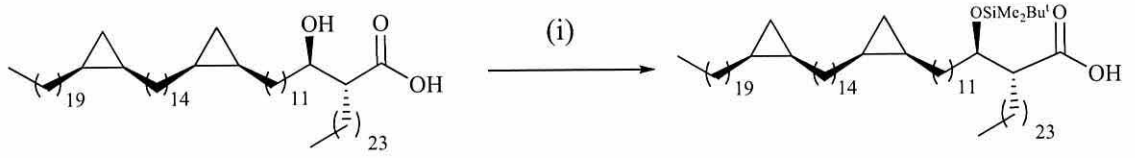
A MALDI MS for compound **140** is shown in Fig 104 (appendix), with $[M+Na]^+$ = 2837.660 in good agreement with the required 2837.744. The ^{1}H NMR spectrum can be seen in Fig 105 (appendix), showing signals for protons in the trehalose core ranging from δ 4.93 to 3.42 and integrating to 12H. The mycolic acid dowfield signals were at δ 3.53 for the protons in the beta position (2H in total) and the OMe signals in the two units resonated at δ 3.25 (6H). A signal at δ 2.35 corresponded to the proton in the alpha position adjacent to the alkyl chain. On the other hand signals upfield corresponding to the cyclopropane ring at δ 0.60, δ 0.47 and δ -0.43 integrated to four, two and two protons respectively. Signals in the ^{13}C NMR spectrum showed the carboxylic acid at δ 175, followed by the sugar core signals between δ 94 and δ 79. The IR spectrum showed a carbonyl band at 1722 cm^{-1} and an alcohol at 3362 cm^{-1}. The ^{1}H NMR for **141** can be

seen in Fig 106 of the appendix, showing signals for protons in the trehalose core ranging from δ 5.07 to 3.48, this time as two sets of signals for the protons in the two different sugar core, being different for H1 and H1', H2 and H2' and others. The three protons corresponding to the OMe resonated at δ 3.32 - 3.27 and the cyclopropane ring gave signals at δ 0.63, δ 0.51 and δ -0.38 corresponding to two, one and one proton respectively. The ^{13}C NMR signals were split into a more complicated pattern due the lack of symmetry but a carbonyl signal was still present at δ 175, whereas twice the number of signals for the sugar core carbons were seen compared to compound **141,** ranging from δ 94 to 64. IR signals were the same as for **141,** with carbonyl and alcohol bands at 3362 and 1722 cm^{-1} respectively. The MALDI MS results for **141** showed $[M+Na]^+ = 2837.66$ compared to the required 2837.74.

In this reaction HF-pyridine complex, pyridine and dry THF was used under nitrogen conditions to remove silyl protection in the beta position of the mycolic acid. This deprotection method is not the standard used by Datta and Takayama, with methanolic K_2CO_3 giving compound **31** with 61 % yield.

2.4.3 Alpha mycolic acid protection and esterification with trehalose

The alpha mycolic acid **132** was esterified with trehalose in order to obtain the corresponding TDM/TMM, following the same procedure as described for the methoxy mycolic acid above (Fig 107). The β-hydroxy acid protection was again necessary, so compound **132** was silylated with imidazole, TBDMS-Cl and 4-DMAP to give **142**.



132 **142**

Fig 107. Alpha cord factor synthesis. (i) imidazole, *tert*-butyldimethylsilylchloride, 4-DMAP, 70 °C, 24 hrs, then r.t 18 hr.

This had the correct molecular weight from the mass spectrum. The ^1H NMR results showed 9 new protons resonating at δ 0.91 and corresponding to the *tert*-butyl group

protons. The methyl groups close to the silicon resonated at δ 0.12 and 0.10. On the other hand the ^{13}C spectrum showed the signals for these last two methyl groups at δ -4.29 and -4.93.

Esterification of **142** and **60** under the same conditions as above (DCC, 4-DMAP) gave products **143**, **144** and **145** (Fig 108). The alpha mycolic acid anhydride **143** was reused to generate more **144** and **145,** increasing the yield reaction to a total of 33 % for compound **144** and 49 % for **145**.

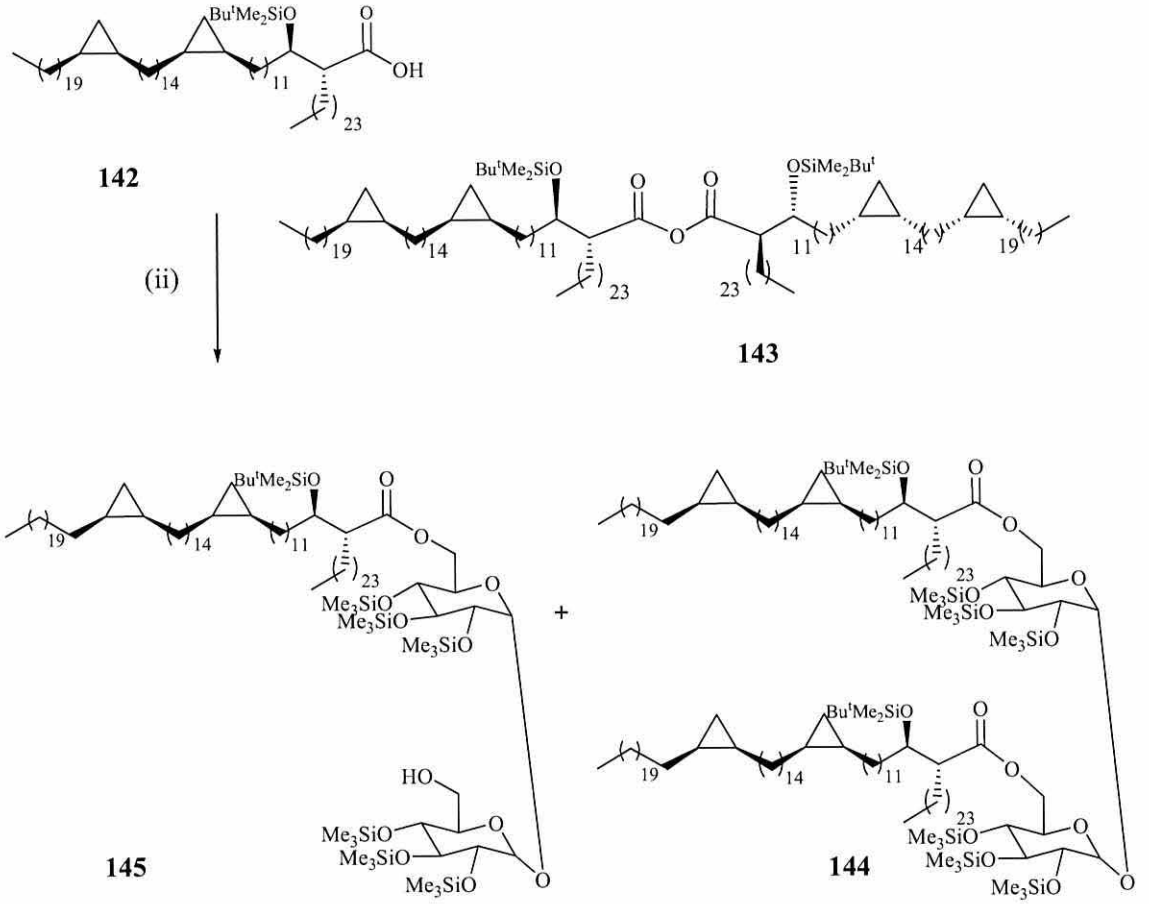


Fig 108. Alpha cord factor synthesis (ii) 3-ethylcarbodiimidehydrochloride (EDCI), 4-DMAP, molecular sieves 4A, dry CH_2Cl_2, r.t, N_2, 6 days.

In this case yields are considerably lower than those achieved for compounds **136** and **137** when using the methoxy mycolic acid. Anhydride **143** showed the same NMR as **132** but duplicating the proton signals due to the symmetry. The MALDI MS results showed $[M+Na]^+$ = 2509.55 compared with the required 2509.64. The starting material **142** had only a mass of 1275 whereas the two other products, **144** and **145** had masses of 3266

and 2032 respectively. The ^{1}H NMR spectrum for **143** showed the 18 *tert*-butyl protons resonating at δ 0.90. In the same way the characteristic signals in the alpha mycolic acid were still present, such as a two hydrogen signal (one for each side) for the β-hydroxy acid resonating at δ 3.85. Once again, the cyclopropane ring signals were found at δ 0.66, δ 0.57 and δ -0.32 now corresponding to eight, four and four protons respectively. Signals corresponding to the four methyl groups attached to the silicon were found upfield at δ 0.07. The ^{13}C NMR signals followed these results, with δ 178.37 corresponding to the carbonyl carbon and δ 73.63 for the β-hydroxy carbon. Figure 100 shows a TLC plate representation of a crude sample before columning for **144** and **145**. The sample shows anhydride **143** at the top, followed by **144**, **145** and **142** respectively. By TLC, both anhydrides (methoxy and alpha) ran fastest and clearly separated from the remaining products in the TLC, making isolation easier.

The ^{1}H NMR spectrum for TDM **144** showed the seven signals corresponding to the core sugar protons starting with the H1 resonance at δ 4.86, H6 at δ 4.37 and the rest of the signals at δ 4.04, 3.99, 3.9, 3.53 and 3.38. The β-hydroxy protected position showed a proton at δ 3.84. The next signal, δ 2.56 corresponded to the proton in the α-position in the alpha mycolic acid. The terminal methyl groups appeared at 0.89 (12 H), whereas the methyls of the alcohol protecting groups in the sugar appeared at δ 0.166, 0.15 and 0.14 corresponding to 18 H in each case. The cyclopropane ring signals appeared at δ 0.65, δ 0.57 and δ -0.32 corresponding to 8, 4 and 4 protons respectively. The ^{13}C NMR spectrum showed the ester carbonyl at δ 173 whereas C1 signals for the sugar core were around δ 94 - 95. The rest of the sugar core signals ranged between δ 73 - 70. The infra-red spectrum showed the characteristic signals for the ester at 1743 cm^{-1}. The molecular mass results from MALDI found $[M+Na]^{+}$ = 3266.83 compared to 3266.93 expected.

TMM **145** showed a more complex signal pattern due to lack of symmetry in the molecule. The sugar core protons in the NMR started at δ 4.91 and 4.84 for the H1 close to the hemiacetal and δ 4.35 for H6 close to the ester. The remaining sugar signals, mostly accounting for 1H, were at δ 4.08, 3.99, 3.95, 3.91, 3.85, 3.66, 3.48, 3.43 and 3.39. Signals resonating at δ 3.74 and 2.55 corresponded to the proton in the β-hydroxy acid carbon and the α-carbon respectively. The signals the terminal methyls were δ 0.89 (6H) and 0.88 (9H) corresponding to the *tert*-butyl group protecting the alcohol position.

Cyclopropane ring signals showed δ 0.67 - 64, 0.57 and -0.32 corresponding to 4, 2 and 2 protons respectively. On the other hand methyls of the protecting groups on the sugar alcohol positions appeared at δ 0.174, 0.164, 0.159, 0.155, 0.153, and 0.127 integrating to 9H each and to a total of 54H. Signals in the ^{13}C NMR spectrum reflected once again the lack of symmetry. The ester carbon appeared at δ 174, followed by the two carbons close to the hemiacetal at δ 94.52 and 94.40. The rest of the signals ranged within δ 73.44 - 70. The CH_2 signals were in the region of δ 30 - 20 and finally the methyl groups close to the silicon in the protecting alcohol position in the mycolic acid were seen at δ -4.48 and -4.68. The mass ion found for **145** by MALDI MS was $[M+Na]^+ = 2032.68$, compared to a required value of 2032.66 (Fig 109 appendix). In Fig 109 (appendix) can be seen the $[M+K]^+$ peak characteristic in MALDI samples using a matrix which is rich in such alkali metal.

TBAF was used in the next reaction in order to remove the silyl protection from the sugar core of **144** and **145** (Fig 110). This reagent proved to be difficult to remove after the reaction. It was observed that NMR peaks were broad and not sharp enough to assign when only $CDCl_3$ was used as solvent after deprotection of **144** and **145**. The same occurred with **138** and **139**. This problem was discussed in previous synthetic papers and is probably due to molecular aggregation. In some cases, even after D_2O exchange the signals were still broad and indistinct, so the answer to the problem was to add di-($^2H_3$)methyl sulfoxide and a drop of trifluoroacetic acid.[164] By doing so, OH signals were suppressed leaving clear peaks for the anomeric protons. The problem found with this method was the acidity produced and possible sample loss. In the case of TDM/TMM, D_2O addition to $CDCl_3$ improved signal resolution.

Compounds **146** and **147** were obtained in 53 % and 90 % yields respectively. In comparison, Datta and Takayama got a 69 % yield when deprotecting protected dipalmitate trehalose **28** and obtaining fully deprotected **31**.

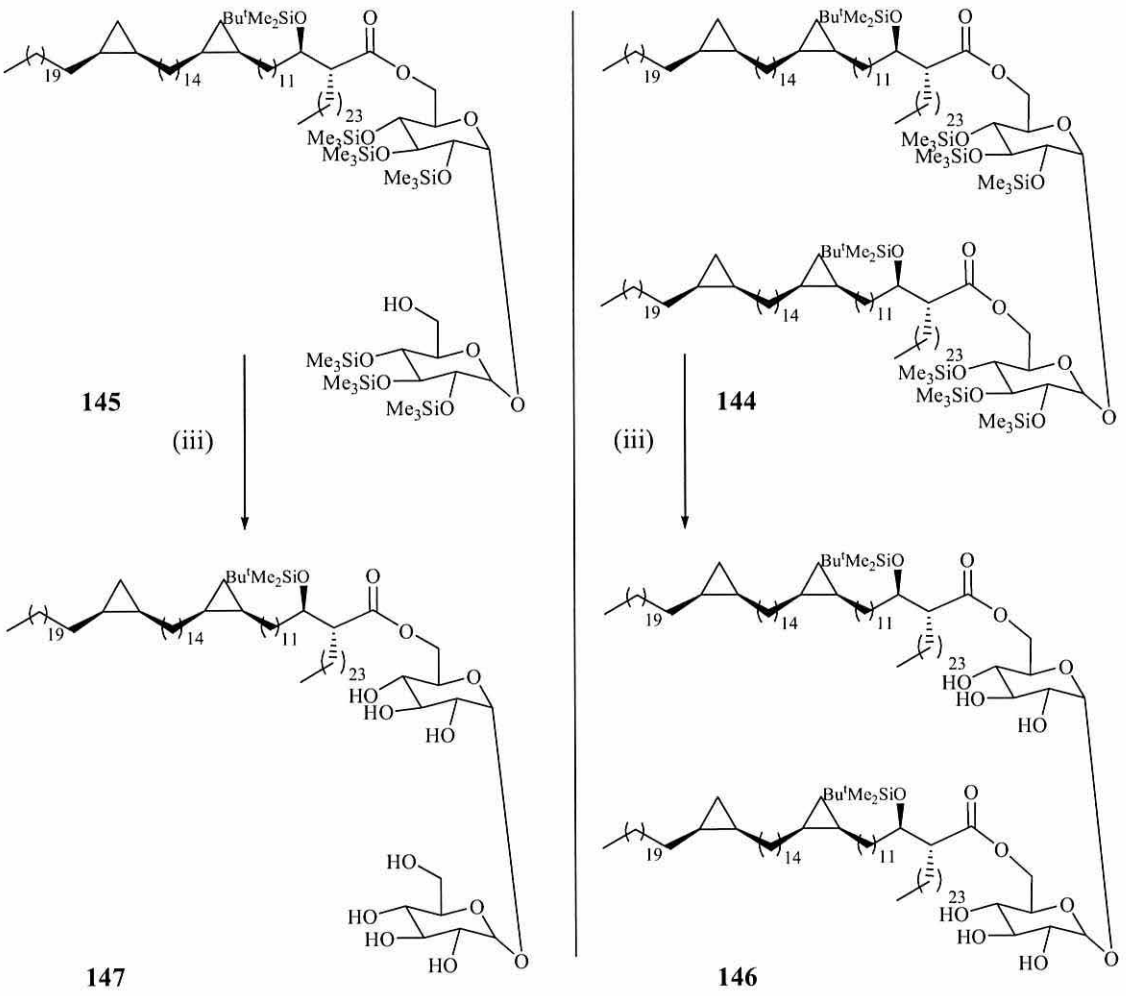


Fig 110. (iii) TBAF, dry THF, N_2. stir 5 °C, r.t then 1h.

Compound **146** gave a mass ion $[M+Na]^+$ = 2833.90, compared to the expected value of 2833.87. The ^1H NMR spectrum showed similar peak patterns and shifts to **144,** but lacking the methyl signals for the protecting groups on the sugar alcohols. Compound **147** gave similar values to **145** for the ^1H and ^{13}C NMR spectra but lacking methyl protection on the sugar. In this case the NMR results were sometimes less sharp compared to the previous two samples of **144** and **145**. MALDI MS for **147** gave $[M+Na]^+$ = 1599.30 compared to 1599.60 required.

The next step in the reaction was removal of the silyl protection from the β-hydroxy acid in the mycolic acid with HF-pyridine complex (Fig 111). In this case to increase sample purity after it had been columned, cold methanol was added to the fully deprotected samples. This produced a precipitate that then was centrifuged, giving product **149**. This

procedure was not necessary with sample **148** as purity was adequate. Yields after this reaction and the centrifugation were 22 % for **149** and 54 % for **148**.

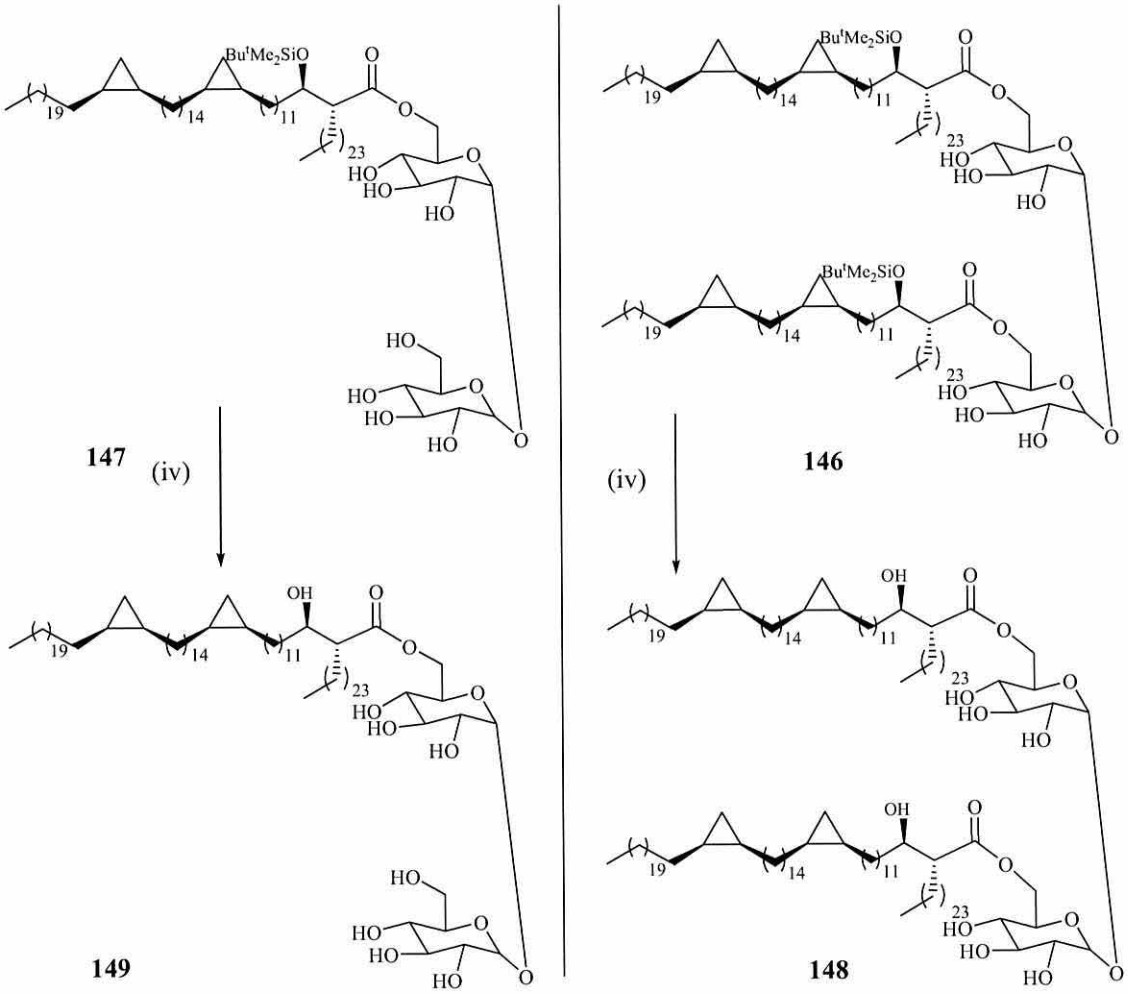


Fig 111. TDM/TMM beta alcohol deprotection step, (iv) pyridine in dry THF, Hydrogen fluoride-pyridine complex, stirred at 43 °C, 17 hrs.

MALDI MS results matched the predicted structure but the ^{1}H NMR spectrum was still broad. This was first noticed when the silyl groups had been removed in the previous step (compounds **146** and **147**), but in this case increasing relaxation time did not improve results. It was decided to increase NMR temperature to the maximum possible without compromising compound stability, in this case 40 °C. Even though results were better than before there were still not as sharp as those obtained with the methoxy TDM and TMM (**140** and **141**).

In the ^{1}H NMR spectrum, sample **148** showed similar chemical shifts to **146** but lacking the *tert*-butyl dimethylsilyl signals on the β-hydroxy acid. The fourteen hydrogens for the sugar core were present ranging from δ 4.98 to δ 3.21. The proton on the carbon containing the β-hydroxy group resonated at δ 3.64 and that on the α-position in the mycolic acid at δ 2.40. The cyclopropane ring hydrogens appeared at δ 0.62, 0.52 and -0.36 integrating for 4, 2 and 2 protons respectively. The ^{13}C NMR spectrum showed the carboxylic acid signal at δ 175.40 followed by the sugar carbons ranging from δ 94 - 69. MALDI MS gave $[M+Na]^+$ = 2605.38 and the predicted value was 2605.40 (Fig 112 appendix). The IR spectrum showed characteristic signals for the carbonyl and the alcohol at 3391 and 1730 cm^{-1} respectively.

Sample **149** showed signals for the 14 hydrogens of the sugar core resonating at δ 5.11 and 5.07 for the protons close to the hemiacetal, δ 4.62 and 4.13 for the protons in H5, H6 positions, and δ 4.06, 3.93, 3.58, 3.53, 3.39 and 3.36 for the remaining ones. Cyclopropane ring signals appeared δ 0.62, 0.56 and -0.34 corresponding to 4, 2 and 2 protons respectively. At this stage there was no signal in the carbon or the proton NMR for the silyl protecting group. The ^{13}C NMR spectrum showed the carboxylic acid signal at δ 175.45 followed by the sugar carbons ranging from δ 94 - 64. As explained before, due to the lack of symmetry the number of signals was higher. The MALDI MS showed $[M+Na]^+$ = 1485.14 against a required value of 1485.31. The IR spectrum showed bands for the alcohol at 3356 cm^{-1} and for the carbonyl at 1728 cm^{-1}.

A final comparison can be made between a crude cord factor sample bought in the Aldrich catalogue (10-06-08) and **148** to see the variety of signals observed in the crude mixture, including different length chains of both TDM and TMM (Fig 113-114 appendix).

2.5 Glycerol ester synthesis

2.5.1 Aim and overview

Simpler structures compared to TMM and TDM have been proved to be effective in increasing immune system responses. Some of these compounds, such as C_{32} MMG (monomycoloyl glycerol) act as Th1 adjuvants, and their activity in immunising animals such as mice is similar to TDB (trelalose behenate).[175]

These synthesised compounds can be added to a culture plate with different concentrations of the antigen and after incubation assessment for different immune system responses such as IFN-c and IL-6, IL-5, IL-10 can be run.

2.5.2 C_{32} glycerol esters

Bhowruth *et al* tested the adjuvant activity of a C_{32} MMG analogue.[175] The synthesis of this compound was done as a starting point to develop the methodology used for TDM/TMM coupling for a different range of compounds which may also be important from a therapeutical point of view.

This synthesis started from compound **91**, which was coupled to ((*S*)-2,2-dimethyl-[1,3]dioxolan-4-yl)-methanol using 4-DMAP as catalyst (Fig 115). The product **150** was obtained in 53 % yield. Rotation values were not taken as compound **91** was not optically pure. An ^{1}H NMR spectrum showed a downfield signal for the single proton in the ring at δ 4.34 followed by the two protons close to the ester at δ 4.15. The next two signals corresponded to the remaining two protons in the ring resonated at δ 4.06 and 3.84. The proton in the β-hydroxy acid carbon showed a signal at δ 3.77 whereas the proton for the α-carbon of the C_{32} chain appeared at δ 2.55. The methyls in the *tert*-butyl protecting group appeared at δ 0.93. The terminal methyls appeared at δ 0.90 and the two methyl protecting groups in the alcohol of the glycerol came at δ 1.43 and 1.37. Methyl groups close to the silicon appeared at δ 0.04 and 0.14. The ^{13}C spectrum confirmed the values

obtained in the proton NMR, showing a downfield signal at δ 174 corresponding to the carboxylic acid carbon. The carbon in the alpha position to the carboxylic acid showed a signal of δ 64 whereas the one in the beta position gave a signal of δ 73. The silyl protecting group showed methyl groups adjacent to silicon at δ -4.29 and δ -4.53 whereas *tert*-butyl carbons showed signals at δ 25 for the primary and δ 18 for the quaternary one.

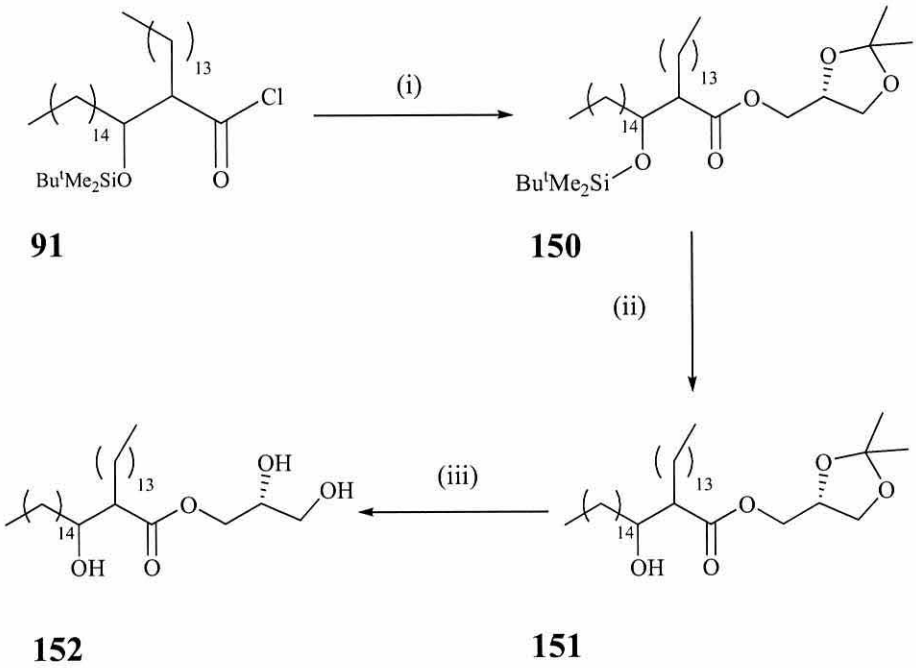


Fig 115. Glycerol ester reaction with C_{32} corynomycolate mycolic acid. (i) ((*S*)-2,2-Dimethyl-[1,3]dioxolan-4-yl)-methanol (0.1 g, 0.76 mmol, supplied by Dr.Al-Dulayymi), THF, 4-DMAP, r.t, 3 hrs; (ii) Dry THF, HF-pyridine complex, 45 °C, 12 hrs under N_2; (iii) THF, MeOH/water, DOWEX ion exchange resin, r.t 24 hrs.

Deprotection of **150** was achieved using dry THF and HF-pyridine complex to give **151** in 61 % yield. The ^{1}H NMR spectrum showed the loss of the protecting group on the acid as the *tert*-butyl group previously appearing at δ 0.93 and the two methyl groups close to the silicon at δ 0.04 and 0.14 had each disappeared. The ^{13}C NMR spectrum also showed no signals corresponding to the silyl protecting group.

Final deprotection of the alcohol groups was done using DOWEX ion exchange resin. In the first instance, the reaction did not happen at r.t even when left for a long time (48 hrs). It was decided then to increase the temperature, but only to 45 °C so as not to compromise stability of the ester bond. After 48 hrs, the reaction gave product **152** in 54 % yield. The reaction gave some starting material **150**. Compound **152** showed a similar

NMR spectrum to **150** but signals at δ 1.43 and 1.37 (each 3H) had been lost. The MALDI MS showed the product mass ion $[M+Na]^+$ = 593.63 compared to the calculated 593.51. Results for m.p were 70-72 °C compared to 72-74 °C literature values.[229]

2.5.3 Alpha mycolic acid glycerol esters

The same procedure used for the model C_{32} glycerol ester was used when coupling longer chain mycolic acids to glycerol.

The first step in this reaction involved ((*S*)-2,2-dimethyl-[1,3]dioxolan-4-yl)-methanol, pyridine and p-toluenesulfonyl chloride. The reaction gave **154** in 69 % yield (Fig 116).

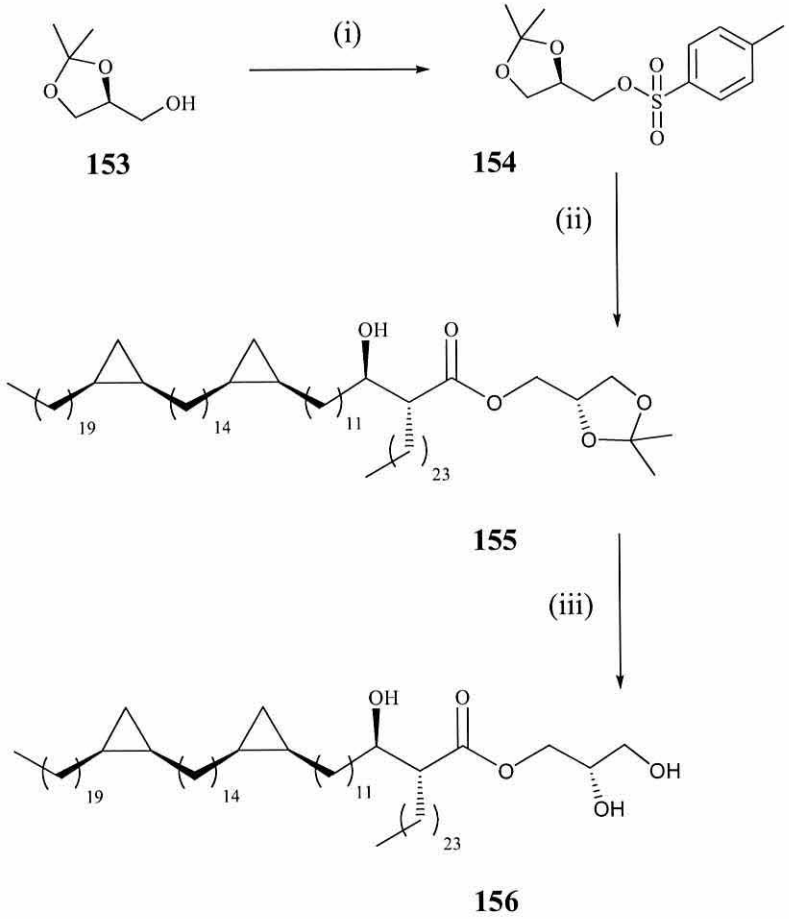


Fig 116. Alpha mycolic acid glycerol ester synthesis. (i) ((*S*)-2,2-Dimethyl-[1,3]dioxolan-4-yl)-methanol, pyridine, p-toluenesulfonyl chloride, left in fridge 16 hrs; (ii) Compound **132**, Dry DMF/THF, $CsHCO_3$, 48 hrs, 70 °C; (iii) MeOH/water, DOWEX ion exchange resin, 80 hrs, 45 °C.

Proton NMR of **154** confirmed the structure, showing the aromatic ring signals at δ 7.74 and δ 7.30 integrating to 2H each. A signal for the single proton in the acetal ring at δ 4.25 was followed by the two protons close to the ester at δ 3.98 and 3.95. The next two signals corresponded to the remaining two protons in the acetal ring resonating at δ 3.91 and 3.70. The methyl group on the aromatic ring resonated at δ 2.40 and the two methyl's of the acetal were found at δ 1.27 and 1.24. The ^{13}C NMR results showed aromatic signals for the carbon close to the sulfone at δ 144, the para position at δ 132 (1C), the meta position at δ 129 (2C) and finally the ortho position at δ 127 (2C). Signals at δ 72.66, 69.38 and 65.73 corresponded to the three carbons in the glycerol unit whereas those at δ 26.30 and 24.84 corresponded to the methyls of the protecting group.

The coupling method for the C_{32} model compound used the acid chloride to couple to glycerol. In this case, it was decided to try a different approach so p-toluenesulfonyl was used instead, being a better leaving group. Compound **155** was obtained by coupling unprotected alpha mycolic acid **132** with glycerol protected **154** using dry DMF/THF and $CsHCO_3$. The product was obtained with 76 % yield compared with 61 % attained for **151** using the model compound C_{32}. The MALDI MS mass ion at $[M+Na]^+$ = 1275.32 corresponded to structure requirements, with a calculated value of 1275.21 (Fig 117 appendix). The ^1H NMR spectrum showed a downfield signal for the single proton in the acetal ring at δ 4.39 followed by the two protons close to the ester at δ 4.21. The remaining two protons in the acetal ring resonated at δ 4.05 and 3.77. The proton on the β-hydroxy acid carbon showed a signal at δ 3.71, whereas the proton on the α-carbon of the alpha mycolic acid showed a value of δ 2.50. Signals at δ 1.44 and 1.34 corresponded to the methyl groups in the protecting acetal ring. Signals for the cyclopropane ring were at δ 0.66, 0.57 and -0.32 integrating to 4, 2 and 2 protons respectively. The ^{13}C NMR spectrum showed a downfield signal at δ 172 for the carboxylic acid, followed by the carbon located between the two oxygen positions resonating at δ 109. The next three signals correspond to the carbons in the glycerol at δ 73.39, 72.66 and 66.30. The carbon in the alpha position to the carboxylic acid appeared at δ 51.

The final step involved removing the alcohol protection and was done as described in the previous method using DOWEX ion exchange resin. Some problems occurred when deprotecting **152,** so the temperature and reaction times were increased considerably. In

this case, **156** was not obtained as the deprotection failed even on stirring for more than 3 days at 45 °C. The reaction was repeated using more ion exchange resin and increasing the reaction time to 100 hrs, keeping the temperature the same for stability reasons. The deprotection failed and, due the small amount of material used (0.025 g) over several trials, the compound was finally lost when columned.

3. Biological data

3.1 Biological data for alpha/methoxy mycolic acids

Natural mycolic acids can be used for the serodiagnosis for TB run in an ELISA plate assay though the selectivity is not high enough for application. Patients with HIV and TB still have high levels of antibodies to mycolic acids, making this method attractive in coinfected patients, but the use of natural mixtures of mycolic acids was still not adequate for serodiagnosis. It has been reported that cholesterol may interact with mycolic acids and give a low accuracy when testing patients for TB.[230] Due to that it is important to know the different characteristics of mycolic acids and identify the one or the ones that interact with cholesterol. In contrast, natural cord factors, containing complex mixtures of isomers, give a higher selectivity and sensitivity. For this the reason it is of interest to examine how single synthetic TDM/TMM molecules may be applied in diagnosis. This testing remains to be done.

Having an early detection method for TB means not spending time in quarantine and being able to receive anti-TB therapy in more quickly, leading to a non-infectious patient within days.

A more sensitive diagnosis can be made by using synthetic enantiomers of a single MA instead of the complex natural mixtures. A specific synthetic MA antigen could give more reliable data and giving better distinction between TB positive and TB negative patient sera.

The first synthetic enantiomer controlled synthesis was done by Al Dulayymi *et al* [220] and synthesis of **132** is the natural continuation of this work. The purpose of making such compounds is to study the antigenic activity of natural and synthetic MA mixtures using TB positive and TB negative serum samples with ELISA. Such studies would reveal the role of functional groups within the different structures as well as the cross reactivity with cholesterol.

In work by collaborators in Pretoria, a range of alpha, methoxy and keto-MA samples were coated onto ELISa plates in PBS. Five TB positive and five TB negative patient sera

were selected in each case. The plates were coated with the different MA at 50 μl.[231] This work proves that free mycolic acid but not methyl ester is recognized by antibodies. Fig 118 gives the results for antibody response against synthetic and natural MA. Generally, synthetic methoxy have the best recognition, followed by hydroxy, keto and finally alpha. Although the detailed structures will not be presented here, synthetic **132** correspond to number 14 in Fig 118 whereas its diastereisomer (*R*)-2-((*R*)-1-Hydroxy-12-{(1*R*,2*S*)-2-[14-((1*R*,2*S*)-2-icosyl-cyclopropyl)-tetradecyl]-cyclopropyl}-dodecyl)-hexacosanoic acid is number 13.

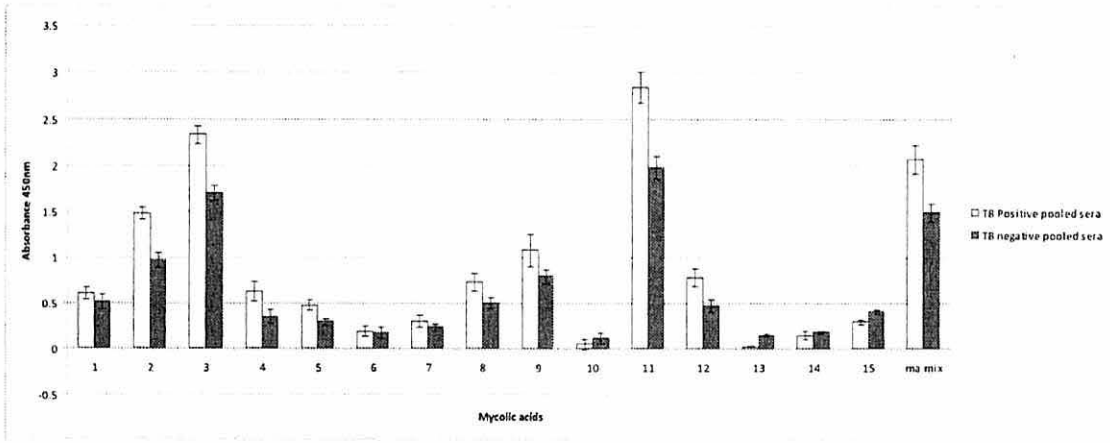


Fig 118. ELISA signals of antibody binding to both TB positive and TB negative sera for synthetic (1-14), natural alpha (15) and natural mixture MA (ma mix). Methoxy MA: 1-4, 11, Hydroxy MA: 5,7-9, Keto MA: 6,10,12, alpha MA (13-15). Error bars indicate deviation.

If we compare results within alpha mycolic acids number 13 (*cis*-enantiomer) and 14 (corresponding to **132**) against the natural alpha MA (15) and the natural MA mix it can be seen that both natural and synthetic alpha MA failed to distinguish between TB negative and TB positive patient sera (Fig 119).[231]

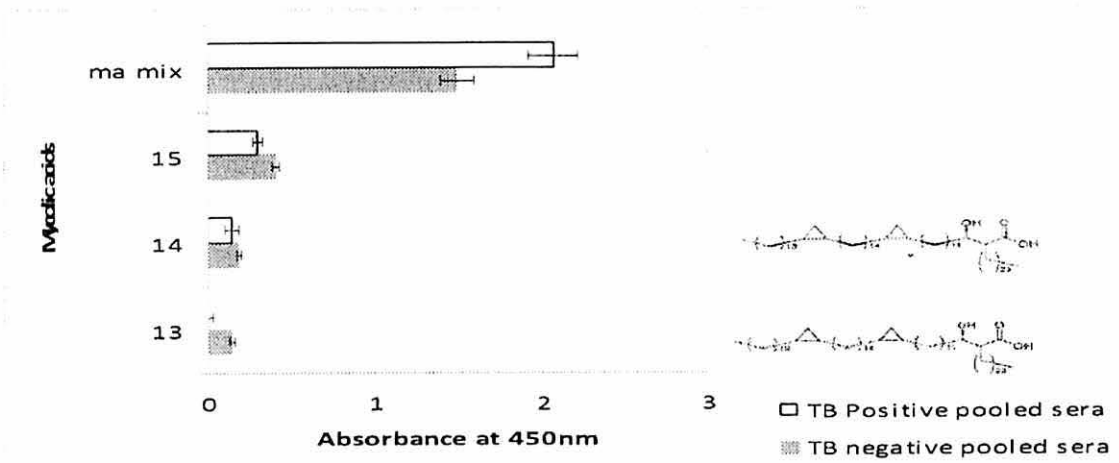


Fig 119. ELISA antibody binding for TB positive and TB negative sera. Ma mix is a natural mixture of MA. *Sample 15* natural isolated alpha. *Sample 14* synthetic trans- alpha (**132**). *Sample 13* synthetic cis-alpha.[231]

3.2 Biological data for cord factors

It is important to know that natural TDM analysis is fairly complex as many combinations of MAs can be bonded to trehalose, increasing the possible number of structures.

TDM show a variety of immune related effects as previously described in the introduction. They are able to stimulate the immune system so it will produce a range of chemokines (MCP-1, IL-8) and cytokines (IFN-γ, TNF-α, IL-12, IL-10, IL-4).[232] Vascularization can be achieved through neutrophils and macrophages. Intraperitoneal TDM treatment prior to virus infection inhibits viral growth and this relates to IFN production.

3.2.1 Procedure

Purified Cord Factor was used to stimulate mouse RAW 264.7 cells. The selected cord factor sample was suspended at a concentration of 10μg/well of each compound in isopropanol and sonicated in a bath sonicator for 5 min. The suspension was incubated at 60 °C for 10 min. and sonication repeated. The resulting solution was layered onto tissue culture in 24-well cultures cell plates at the indicated concentrations and incubated at 37 °C in order to ensure complete evaporation of the solvent. Control wells were layered with solvent without Cord Factor and incubated at 37 °C. To this layer of Cord Factor, RAW 264.7 cells incubated at 37 °C for 20 hours before activation. TNF-α and MCP-1 production were measured in the supernatant and analysed using a Mouse Inflammation Cytometric Bead Array kit (BD) on a FACScalibur. The activity was compared to that of a commercial sample of M. tuberculosis TDM (Sigma). Asterisks indicate significance of the differences in mean as compared to the Mtb-TDM group, determined by Turkey's multiple comparison test (**$p < 0.01$, ***$p < 0.001$).

3.2.2 Data

The data show a clear increase in chemokine MCP-1 production compared with the control. Commercial TDM samples obtained from Sigma were proved to induce similar levels of chemokine to both methoxy samples (TMM and TDM containing mycolic acid **134**) but results were significantly higher when dealing with TDM containing the alpha mycolic acid **132** (Fig 120).

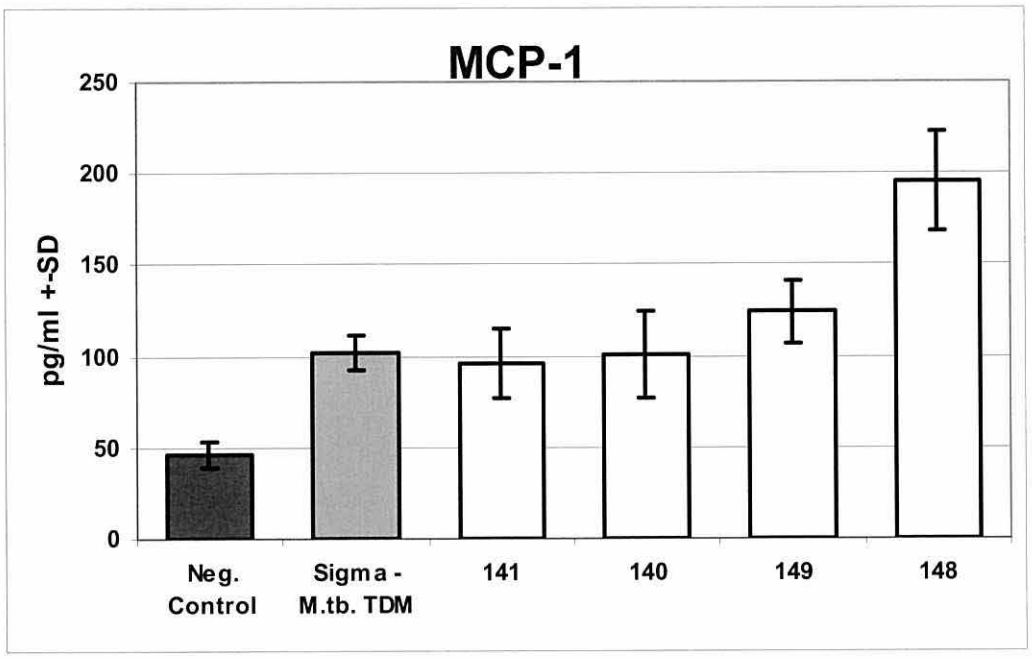


Fig 120. MCP-1 assays for commercial TDM (Mtb-TDM) compared to synthetic TMMs (**141**, **149**) and TDMs (**140**, **148**). Placebo sample (**), sample **148** (***).[104]

Those results confirm that changing the detailed structure of the mycolic acids on the cord factor may lead to a significant change in the immune response. If we compare the placebo with sample **148,** MCP-1 activation seems to be four times higher for the latter. Figure 121 below shows data corresponding to TNF-alpha cytokine activity after mouse cell exposure to different cord factor samples. The placebo or control showed low TNF-alpha activation whereas the commercial TDM samples obtained from Sigma tripled those activation levels. Taking into account that commercial samples are composed of a very diverse range of individual cord factor components no relationship could be defined to relate cause and effect. Comparatively, synthetic samples of single mycolic acids, both TDM and TMM were able to distinguish between structure type and effect caused. Methoxy TMM sample **141** showed similar activation to the mixture but significantly higher than the placebo. The methoxy TDM **140** produced higher TNF-alpha activation than the placebo but lower than the mixture. Comparatively, compound **140** had a higher ability to induce this cytokine production than **141** did.

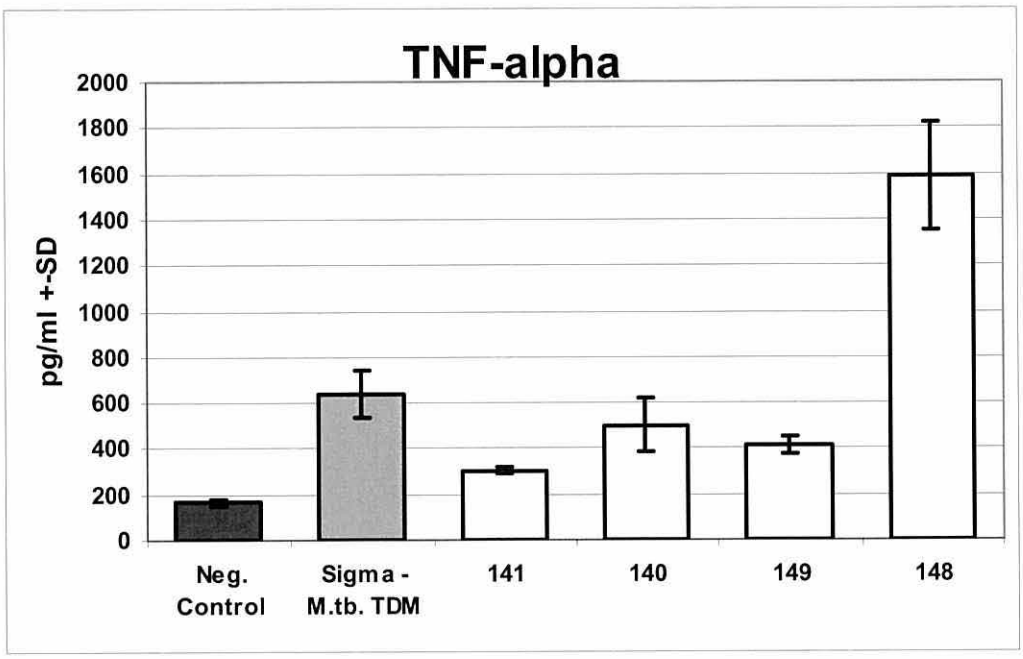


Fig 121. TNF-α assays for commercial TDM (Mtb-TDM) compare to synthetic TMMs (**141**, **149**) and TDMs (**140**, **148**). Placebo sample (***), sample **141** (**), sample **148** (***).[104]

Compound **149** (synthetic alpha-TMM) induced higher TNF-alpha production than the placebo but lower than the natural mixture. Compound **148** induced a much higher cytokine production than both the placebo and the mixture of natural TDMs. Results were up to eight times higher than the placebo and up to three times the values obtained using the commercial TDM mixture.

In this specific case both TMMs samples induced lower levels of cytokine production than their corresponding TDMs for the same quantity of material.

3.3 Biological data for glycerol esters

Mycobacteria have been known as modulators of the immune systems and as source of adjuvant preparations. In fact, *M. bovis* BCG vaccine is nothing more than an attenuated version of *M. bovis*. Vaccines lacks antigenicity or being able to induce immune system

protection and this is the role of the adjuvants; just to potentiate or prolong the specific action of a vaccine.

Many mycobacteria component are proven to be strong adjuvants. As an example the lipid extract of *M. bovis* BCG Copenhagen was separated into its components showing four lipids fractions. One of those fractions was a C_{70-90} MMG (monomycolyl glycerol). This component induced high levels of IL-12 (250 pico gram/ml) and TNF-α (3500 pico gram/ml).[175]

Bhowruth *et al* decided to synthesise and test immune activity of simplified version compounds. The targeted molecule was C_{32} MMG. After synthesis compounds were added to capture ELISA kits and a variety of cytokines and chemokines were tested. Different *erythro* and *threo* enantiomers for the C_{32} were synthesis and results showed that only one of the diastereoisomers would induce high levels of both IFN-γ and IL-6.

A non separated mixture of this C_{32} was prepared (compound **68**) as control but it was not tested to the moment.

Fig 122 show the results for different compounds Isolated of lipids from the immunostimulatory apolar fraction of *M. bovis* BCG and their ability to induce DC (dendritic cells) activation. Such activity was characterized by two cytokines, IL-6 and TNF-α. Results clearly show an important activity in small molecular weight compounds such as C_{32}.

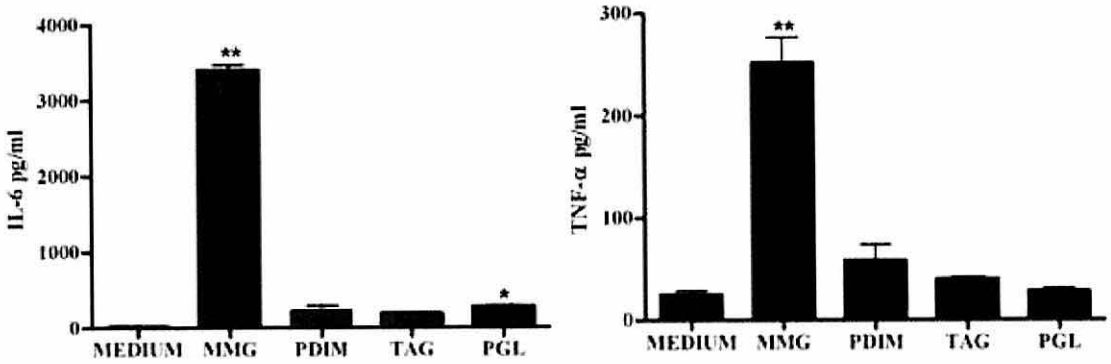


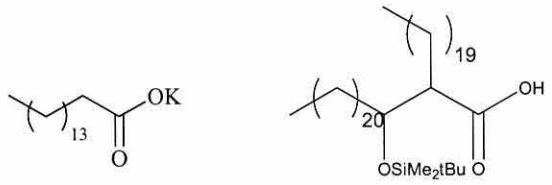
Fig 122. Culture supernatants obtained following treatment with MMG (C_{32}), PDIM (Phthiocerol dimycocerosate), PGL (Glycosylphenol PDIM), or TAG (triacylglycerol) (10 μg/ml) were analyzed by ELISA for the presence of the cytokines IL-6 and TNF-α.[229]

On the other hand taking into account results from the alpha TDM section described above, it was decided to extend alpha mycolic acid to a range of different cores not limited to trehalose. Glycerol seemed to be a good choice and an alpha mycolic acid glycerol ester was successfully synthesized **155**. The compound was successfully characterized but no final deprotection was neither achieved nor biological assays.

4. Conclusions

The aim of this thesis was to synthesise for the first time pure TDM/TMM and to test these synthetic compounds for immune response activity using a variety of chemokines and cytokines.

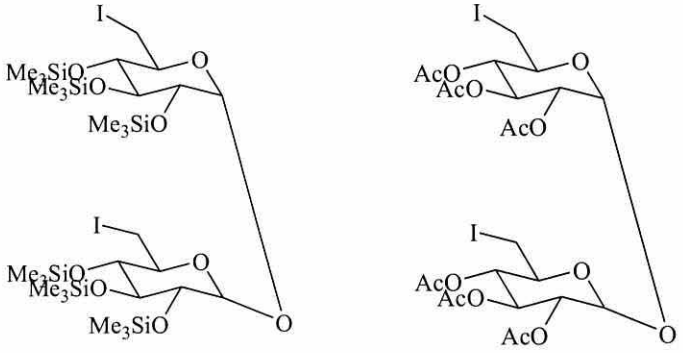
In order to achieve esterification reaction between the trehalose sugar and the corresponding mycolic acid, model compounds were made in the first instance due to the long process required to make a mycolic acid. The first reactions to couple an acid to trehalose were relatively simple, using an acid chloride of either palmitic or behenic acid and couple it to a protected sugar. Results were as expected but this method could not be applied to β-hydroxy acids so it was not used further. In this regard the second model compounds synthesised were **72** and the potassium salts of **90** (Fig 123).



72 **90**

Fig 123. Structures for compounds **72** and **90**.

These compounds were then coupled to trehalose protected with acetate groups (**80**), or trimethylsilyl groups (**25**), using HMPA as promoter. Reaction using acetate protection did give the required product but then deprotection seemed impossible to achieve.



25 **80**

Fig 124. Structures for acetate and trimethylsilyl protected trehalose.

In comparison, using trimethylsilyl protection did not give the ester coupling in the first place. Enzymatic reactions using Novozym 435 lipase did not give target product **23** but only starting material **38**.

A more advanced model approach was to include compounds having a β-hydroxy acid, such as **95** which could be then protected (Fig 125).

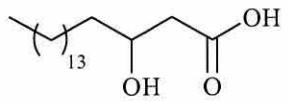
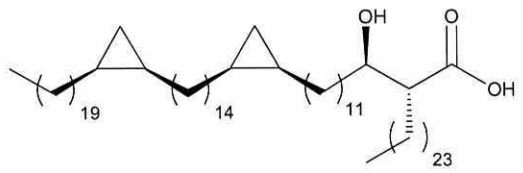


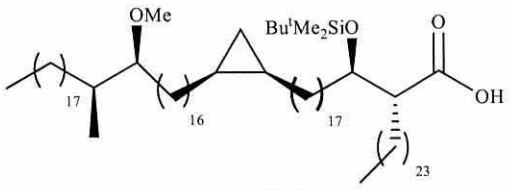
Fig 125. Structure for **95**.

A step forward was taken by synthesising structurally similar compounds to mycolic acids to couple to trehalose. Such compounds were simply obtained from condensation of methyl esters (**85** or **61**) into their corresponding products **86** or **62**. These compounds then had their alcohol groups protected before esterification with trehalose was attempted. Model acid compounds were then used in a variety of reactions to try the coupling with trehalose. Yields for those reactions were variable and their effectiveness depended on the coupling reagent used, the acid structure and the protection used for trehalose.

The alpha mycolic acid **132** was then synthesised (Fig 126). This synthesis had two main purposes.



132



134

Fig 126. Structure for alpha mycolic acid **132** and **134**.

First this mycolic acid had not been prepared previously and would be tested by Professor Verschoor's group in Pretoria (South Africa) in relation to TB, to determine which of the mycolic acids enantiomers is most effective in serodiagnosis with infected patients and in Ghent (Belgium) by Professor J.Grooten to test its effects on the immune system. Results showed that both *cis* and *trans* synthetic MA were able to distinguish between TB

positive and TB negative patient sera but only in a limited way. In fact, alpha MAs were the least antigenic of the four groups tested, including keto, hydroxy and methoxy MAs. From those methoxy seemed to be the most antigenic. Secondly, this mycolic acid could then be coupled to trehalose in order to synthesise new cord factors, both TDM/TMM, single enantiomers of which would be tested in Belgium for human immune response against a variety of chemokines and cytokines.

The route taken when making mycolic acid **132** followed a series of known steps for some of the 'building blocks' or different part of this mycolic acid with some other variation in standard methods. Overall yields were satisfactory and the final compound was obtained in quantity to be used as part of cord factors and even glycerol esters.

The final cord factors synthesis was achieved by coupling two major single mycolic acids with trehalose; the methoxy mycolic acid **134** (supplied by Dr. Al-Dulayymi) and the one synthesised during this project (**132**). From the model reactions used to esterify acids with trehalose, the one that appeared most suitable to use with cord factors was the use of EDCI, 4-DMAP using CH_2Cl_2. This reaction did not work at first and several modifications were made leading to the final reaction conditions, including extending the reaction time to 6 days and keeping the temperature under 40 °C. An intermediate anhydride of the mycolic acids was identified in these reactions, which could be recycled. All reactions required protection of the alcohol groups in both sugar and mycolic acids which were then deprotected giving clean TMM/TDM products. Methoxy mycolic acid coupling finally gave compounds TMM **141** (78 %) and TDM **140** (72 %) (Fig 127) whereas alpha-mycolic acid coupling gave compounds TMM **149** (22 %) and TDM **148** (54 %) (Fig 128).

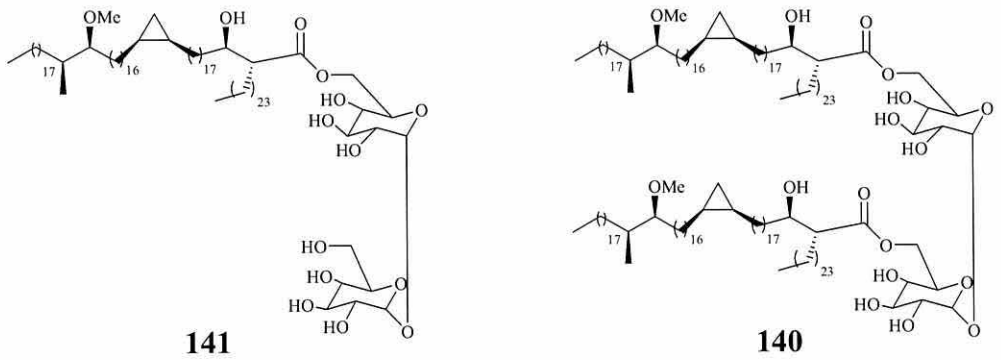


Fig 127. Final methoxy cord factor structures (TMM and TDM).

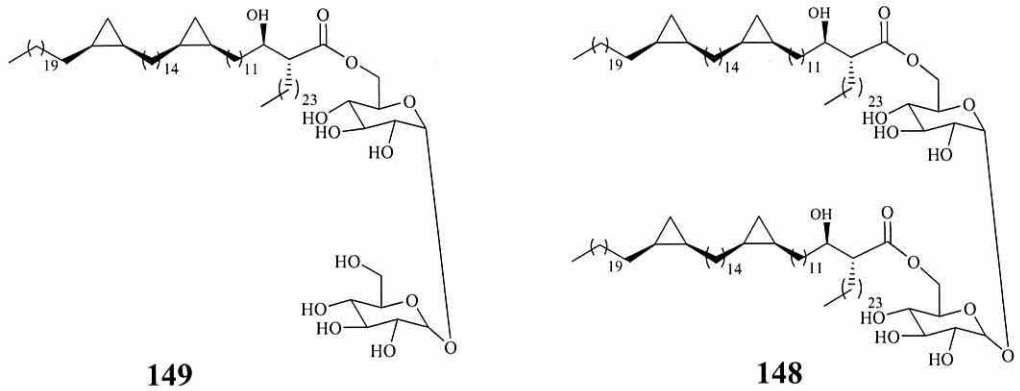


Fig 128. Final alpha cord factor structures (TMM and TDM).

The four cord factors were then tested for immune activity in mouse cells for one chemokine (MCP-1) and one cytokine (TNF-γ). Results for those assays can be seen in the biological assay section. General trends showed stimulation of raw cells leading to either chemokine or cytokine production in levels which were higher for all compounds than the placebo. When testing MCP-1 activity compound **148** produced extremely high activity whereas the other three cord factors did give results similar to the natural mixture. The same pattern was repeated when testing for TNF-α but in this case compounds **141** and **149** showed significantly lower activity than the natural mixture. Once again compound **148** exhibited high values of TNF-alpha activation. These results give a clear idea of how important such single synthetic compounds can be for therapeutical and pharmaceutical use.

Another part of the work involved synthesising glycerol esters. Model compounds such as **152** were prepared in the first instance in good yield. Later work was done including coupling the mycolic acid **132** (Fig 126) to give a final product **155**, which unfortunately could not be fully deprotected (Fig 129).

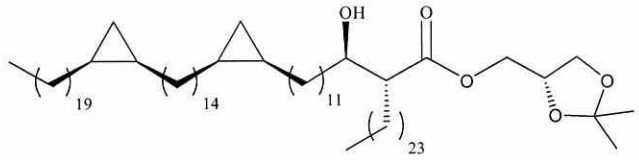


Fig 129. Structure for unprotected **155**.

Once again the importance of synthesising these compounds for the first time opens the way to understanding their properties and applications not only in immune mediated illnesses but also in other areas such as cancer and viral inhibition.

5. Future work

This section aim is to clarify further work which need to be done or could be done to further this work.

It must be said that mycolic acid synthesis was a challenging and new area even a few years ago. Research is progressively moving from synthesis towards getting mycolic acids with specific activity or discrimination by TB antibodies with leading to sensors for TB in the presence of HIV-AIDS. In this regard interactions between cholesterol and mycolic acids could be responsible for current low accuracy when diagnosing TB patients.

On the other hand the adjuvant activity of cord factors is of major importance as, until the present work, no single enantiomers have been synthesised so no effects of single structures over a diverse range of chemokines-cytokines could be drawn. Further synthetic compounds must be prepared and tested in order to have a full picture on how this family of compounds interacts with human body and immune system. Their activity is not only restricted to immunity but also with cancer and viral infections due the fact that these compounds are potentially immune system busters.

A further step can be taken when including two different single enantiomers of mycolic acids to trehalose or even other sugar like structures to see if immune effect/activity is increased. This area of work has an incredible potential which may be responsible for giving cure or improving millions of people lives in a near future.

6. Experimental

6.1 General considerations

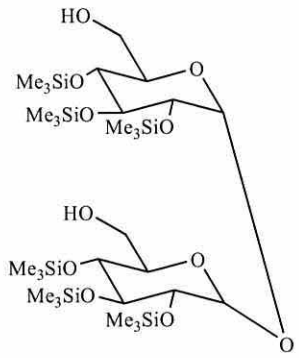
All chemicals were purchased from Aldrich Chemical Co. Ltd, Lancaster Synthesis Ltd, or Avocado Chemical Co. Ltd. THF was distilled over sodium and benzophenone under nitrogen, while dichloromethane was distilled over calcium hydride. Petrol refers to the fraction bp 40–60 °C. Organic solutions were dried over anhydrous magnesium sulfate and solvents were removed at 14 mmHg; residual traces of solvent were finally removed at 0.1 mmHg. All glassware used in anhydrous reactions was dried for not less than 5 h in a 250 °C oven.

Column chromatography was conducted under medium pressure using silica gel (BDH, particle size 33–70 mm); TLC was carried out on pre-coated Kieselgel 60 F254 (Art. 5554; Merck) plates. Optical rotations were measured as solutions in chloroform of known concentration using a Polar 2001 automatic polarimeter. Melting points were measured using a Gallenkamp melting point apparatus. Infra-red spectra were recorded as KBr discs (solids) or thin films on NaCl windows or using a Perkin Elmer 1600 series FT-IR spectrometer. NMR spectra were recorded either on a Bruker AC 250 spectrometer with 5 mm Dual probe or on a Bruker Advance 500 spectrometer with 5 mm BBO probe as solutions in deuterated chloroform ($CDCl_3$) if not differently indicated. Chemical shifts for $^{1}H/^{13}C$ are quoted in δ relative to chloroform (δ 7.27 ppm), and $CDCl_3$ (δ 77.0 ppm). Mass spectra were obtained using a Bruker MicroTOF time of flight mass spectrometer with an ESI source. Matrix assisted laser desorption ionisation (MALDI) were obtained using a Bruker daltonics *Reflex IV*. A laboratory book was filled in including chemical safety information following COSHH regulations.

6.2 Experiments

Experiment 1:

[6-(6-Hydroxymethyl-3,4,5-tris-trimethylsilanyloxytetrahydropyran-2-yloxy)-3,4,5-tris-trimethylsilanyloxytetrahydropyran-2-yl]methanol 60



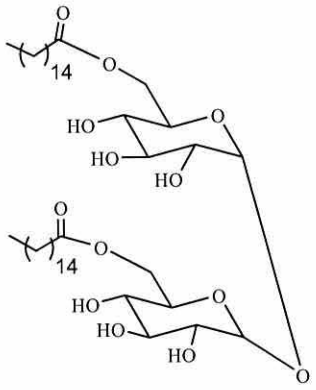
60

D-(+)-Trehalose dihydrate **38** (1.5 g, 4 mmol) was dissolved in dry DMF (5 ml) at 15 °C under N_2 and stirred. To this was added N-O-bis(trimethylsilyl)acetamide (BSA) (8.46 ml, 34.2 mmol) followed by tetrabutylammonium fluoride (TBAF) (0.063 g, 0.24 mmol, 1.0M in THF) dropwise over 10 min. The reaction mixture was vigorously stirred at r.t for 30 min and 2-propanol (1.5 ml) was slowly added. The mixture was diluted with dry MeOH (90 ml), cooled to 0 °C and treated dropwise over 20 min with a solution of K_2CO_3 (0.55 g, 3.96 mmol) in dry MeOH (90 ml). After stirring for 2 hrs at 0 °C, the solution was neutralized with acetic acid (0.6 ml) and the MeOH was removed *in vacuo* at 35 °C. The resulting thin oil was diluted with diethyl ether (150 ml), brine (150 ml) and the organic layer was separated and the water layer re-extracted with diethyl ether (2 x 150 ml). The ethereal layers were dried ($MgSO_4$) and evaporated to give a crude product which was purified by column chromatography eluting with ethyl acetate/petroleum ether 1:2 to give the pure [6-(6-hydroxymethyl-3,4,5-tris-trimethylsilanyloxytetrahydropyran-2-yloxy)-5-methyl-3,4,5-tris-trimethylsilanyloxytetrahydropyran-2-yl]-methanol **60** (2.2 g, 71 %); mp (115-116 °C); $[\alpha]_D^{27}$: + 105 °, (c = 1.4, $CHCl_3$) (Lit.[135] mp 115-118 °C); $[\alpha]_D^{20}$: + 100 °); δ_H (500 MHz, $CDCl_3$): 4.91 (2H, d, *J* 3.15 Hz), 3.89 (2H, t, *J* 9.15 Hz), 3.85 (2H, dt, *J* 2.8, 6.6 Hz), 3.73

(2H, dd, J 2.5, 11.6 Hz), 3.67 (2H, dd, J 3.45, 11.6 Hz), 3.48 (2H, t, J 9.1 Hz), 3.42 (2H, dd, J 3.15, 9.45 Hz), 1.81 (2H, br, s), 0.16 (18H, s), 0.14 (18H, s), 0.12 (18H, s); δ_C (500 MHz, $CDCl_3$): 171.2, 94.6, 73.3, 72.9, 72.7, 71.3, 61.6, 60.4, 21.0, 14.2, 1.3, 1.0, 0.8, 0.1.

Experiment 2:

Hexadecanoic acid (2*R*,3*S*,4*S*,5*R*,6*R*)-6-((2*R*,3*R*,4*S*,5*S*,6*R*)-6-hexadecanoyloxymethyl-3,4,5-trihydroxytetrahydropyran-2-yloxy)-3,4,5-trihydroxytetrahydropyran-2-ylmethyl ester 23



23

(a) Hexadecanoyl chloride (0.43 g, 1.56 mmol) dissolved in anhydrous toluene (4 ml) was added to a stirred solution of [6-(6-hydroxymethyl-3,4,5-tris-trimethylsilanyloxytetrahydropyran-2-yloxy)-3,4,5-tris-trimethylsilanyloxytetrahydro-pyran-2-yl]-methanol **60** (0.54 g, 0.69 mmol) dissolved in anhydrous toluene (10 ml) and dry pyridine (1 ml). The solution was heated (80 °C) for 3 hrs. The solvent was then eliminated under vacuum and the remaining solution dissolved in methanol/water (1:10, 6.6 ml) and refluxed for 2 hrs. Once the reaction was complete by TLC solvents were eliminated under vacuum and the crude product was purified by column chromatography eluting with chloroform/methanol 85:15 to give hexadecanoic acid (2*R*,3*S*,4*S*,5*R*,6*R*)-6-((2*R*,3*R*,4*S*,5*S*,6*R*)-6-hexadecanoyloxymethyl-3,4,5-trihydroxy-tetrahydropyran-2-yloxy)-3,4,5-trihydroxytetrahydropyran-2-ylmethyl ester **23** (0.31 g, 54 %); m.p 155-158 °C, lit. 154-158 °C; $[\alpha]^{23}_{D}$ = + 86.5 (c = 1.1, $CHCl_3$), $[\alpha]^{23}_{D}$ = + 80; {Found $[M+Na]^+$: 841.5600; $C_{44}H_{82}O_{13}Na$ requires: 841.5647}; which showed δ_H (500 MHz, $CDCl_3$ + few drops of CD_3OD): 4.92 (2H, d, J 3.5 Hz), 4.13 (2H, d, *J* 2.2 Hz), 4.09 (2H, dd, *J* 5.05, 12 Hz), 3.80-3.77 (2H, ddd, *J* 2.5, 5, 10.5 Hz), 3.59 (2H, t, *J* 9.15 Hz), 3.34 (2H, dd, *J* 3.8, 9.8 Hz), 3.19-3.15 (6H, m, including a triplet resonated at δ 9.5 Hz), 2.16 (4H, t, *J* 7.9 Hz), 1.43 (4H, q, *J* 7.9 Hz), 1.15-1.07 (44H, m), 0.69 (6H, t, *J* 7 Hz); δc: 174.3, 93.3, 73.0, 71.3, 69.7, 62.9, 33.8, 31.5, 29.3, 29.3, 29.2, 29.1, 29.0, 28.9, 28.8, 24.5, 22.3, 13.6.

(b) Hexadecanoyl chloride **68** (0.5 g, 1.8 mmol, 2.5 mol eq.) (prepared as in Appendix 1) dissolved in anhydrous toluene (4 ml) was added to a stirred solution of **60** (0.54 g, 0.69 mmol) dissolved in anhydrous toluene (10 ml) and dry pyridine (1 ml). The solution was heated at 80 °C for 3 hrs. The solvent was then eliminated under vacuum and the remaining solution dissolved in methanol/water (1:10, 6.6 ml) and refluxed for 2 hrs. Once the reaction was complete by TLC solvents were eliminated under vacuum and the crude product was purified by column chromatography eluting with chloroform/methanol 85:15 to give **23** (0.45 g, 79 %), m.p 156-158 °C, $[\alpha]^{23}_{D} = + 84.5$ (c = 1.3, $CHCl_3$), which showed the same NMR as above.

(c) To a stirred solution of **81** (prepared below) (0.1 g, 0.096 mmol) in THF (16.5 ml), MeOH (5.5 ml) and H_2O (1.1 ml) was added K_2CO_3 (0.17 g, 1.23 mmol, 10 mol eq.).[156] The solution was stirred at r.t for 12 hrs. TLC was checked but no reaction. The temperature was increased to 40 °C and continued left stirring for 12 extra hrs. The solution was then quenched with water (25 ml) and extracted with $CHCl_3$ (3x 25 ml). The combined organic layers were dried and evaporated to give palmitic acid as the main product, mp: 52-53 °C; lit. 52-54 °C, which showed an identical NMR to an authentic sample.

(d) Ester **81** (0.2 g, 0.187 mmol) was dissolved in THF (5 ml) and treated with diethyl methylamine/water 1:1 until pH=9 then stirred for 20 hrs at r.t.[213] The solution was then quenched with water (25 ml) and extracted with $CHCl_3$ (3x 25 ml). The combined organic layers were dried, evaporated to give **81**, $[\alpha]^{24}_{D} = + 131.81$ (c = 1.1, $CHCl_3$), which showed an identical NMR to the starting material.

(e) Anhydrous trehalose **13** (0.5 g, 1.46 mmol), triphenylphosphine (1.04 g, 3.94 mmol) and hexadecanoic acid (0.97 g, 3.79 mmol) were dissolved in dry DMF (9 ml) and cooled to 0 °C under N_2. Di-isopropyl azodicarboxylate (DIAD) (0.826 g, 4.1 mmol) was added dropwise to the cooled, stirred solution over 15 min. The mixture was stirred at r.t for 36 hrs. TLC was checked to show reaction ending. The mixture was then evaporated (bath temperature <50 °C). The solid obtained was evaporated to dryness (<50 °C) then dissolved in chloroform (50 ml), filtered and columned in 79:11:8:2 chloroform/MeOH/acetic acid/H_2O to give **23** (0.23 g, 20 %); m.p 154-158 °C, {Found

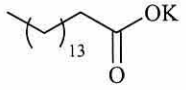
$[M+Na]^+$: 841.86; $C_{44}H_{82}O_{13}Na$ requires: 842.12} which showed the same NMR data as above

(f) *Candida antarctica* lipase (Novozym 435) (2 g) was impregnated with an aqueous solution of D-(+)-trehalose dihydrate **38** (1.12 g, 2.96 mmol) dissolved in water (2 ml). The sample was the dried for 13 hrs in a desiccator under vacuum (5mm Hg). Palmitic acid (2.28 g, 8.9 mmol, 3 mol.eq.) and *tert*-butyl alcohol (20 ml) were added to the mixture that was heated for 14 hrs. After cooling to r.t, ether (100 ml) was added and the mixture was stirred for 10 min before being filtered to remove excess pentadecanoic acid.[173] The solid was the dissolved in methanol (20 ml), stirred for 20 min and filtered again. The sample was then evaporated and the crude product was purified by column chromatography eluting with chloroform/methanol 85:15 to give **38** which showed same ^1H and ^{13}C NMR as the starting material.

Attempted **(g)** hexadecanoic acid 6-(6-hexadecanoyloxy-methyl-3,4,5-tris-trimethylsilanyloxytetra-hydropyran-2-yloxy)-3,4,5-tris-trimethylsilanyloxy-tetrahydropyran-2-ylmethyl ester **28** (0.2 g, 0.16 mmol) were dissolved in trifluoroacetic acid/THF/H_2O (8:17:33, 20 ml) and kept at r.t until TLC showed hydrolysis completion (2 hrs). The resulting mixture was evaporated under vaccum and purified by column chromatography eluting with 85:15 chloroform/MeOH showing starting material.

Experiment 3:

Potassium pentadecanoate 72

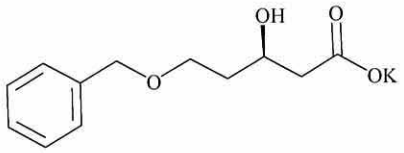


72

Pentadecanoic acid (1 g, 3.89 mmol) was dissolved in $CHCl_3$ (25 ml) to which 5 drops of phenolphthalein were added. The change in colour from transparent to pink was followed by the addition of 2.5 ml of a 0.85M KOH solution (0.95 g KOH in 20 ml MeOH). The solution was kept refrigerated for 10 hrs and a precipitate was formed. The precipitate was washed with ether (20 ml), dried to give **72** (1.04 g, 90 %).[135]

Experiment 4:

Potassium (*R*)-5-benzyloxy-3-hydroxypentanoate 75

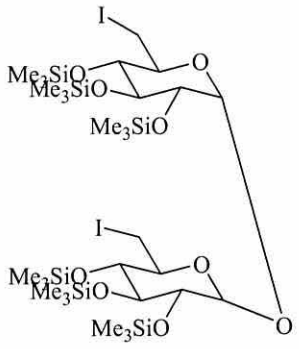


75

Compound (*R*)-5-benzyloxy-3-hydroxy-pentanoic acid **76** (0.2 g, 0.89 mmol) was dissolved in $CHCl_3$ (4 ml) to which 2 drops of phenolphthalein were added. The change in colour from transparent to pink was followed by the addition of a 0.85M KOH solution (0.47 g KOH in 10 ml MeOH, 2 ml).[135] The solution was kept refrigerated for 10 hrs and a precipitate was formed. The precipitate was washed with ether (10 ml) and dried to give the product potassium (*R*)-5-benzyloxy-3-hydroxypentanoate **75** (0.2 g, 85 %) which was used directly in experiment 11.

Experiment 5:

6-Desoxy-6-iodo-2,3,4-tri-O-trimethylsilyl-α-D-glucopyranosyl)-6-desoxy-6-iodo-2,3,4-tri-O-trimethylsilyl-α-D-glucopyranoside 25

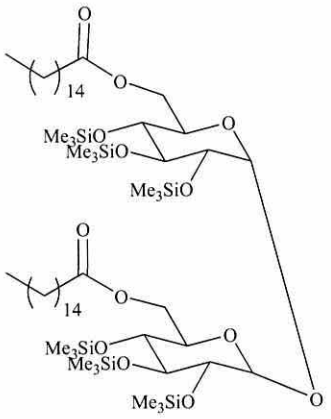


25

To a cooled solution of anhydrous D-(+)-trehalose **13** (5 g, 14.61 mmol) and powdered triphenylphosphine (15.34 g, 58.48 mmol) in DMF (50 ml) was added N-iodosuccinimide (13.16 g, 58.49 mmol) slowly with stirring. It was then heated for 50 hrs at 50 °C. TLC was checked to show complete reaction.[152] The solvent was evaporated under high vacuum and methanol (100 ml) was added. The solution was evaporated in the presence of toluene (150 ml) and $CHCl_3$ (100 ml) was added twice and evaporated from the resulting oil. Water (400 ml) was added to this oil and it was extracted with $CHCl_3$ (4x150 ml). The combined organic layers were washed with water (400 ml), dried and evaporated to give a thick oil (10.84 g) of compound **24** that was silylated by stirring with hexamethyldisilazane (50 g, 0.31 mol) and chlorotrimethylsilane (25 g, 0.23 mol) in anhydrous pyridine (100 ml) at r.t for 40 min under N_2.[159] Ether (150ml) was then added to the resulting mixture was washed with cold water (3x 150 ml). The combined organic layers were dried and evaporated. The crude product was purified by column chromatography eluting with petrol/ether 10:0.5 to give 6-desoxy-6-iodo-2,3,4-tri-O-trimethylsilyl-α-D-glucopyranosyl)-6-desoxy-6-iodo-2,3,4-tri-O-trimethylsilyl-α-D-glucopyranoside **25** (4.05g, 30 %), $[\alpha]^{23}_D = +96$ (c = 2, $CHCl_3$); (Lit.[159] $[\alpha]^{20}_D = +96$ (c = 2.4, $CHCl_3$)); δ_H (500 MHz, $CDCl_3$): 4.99 (2H, d, *J* 3.15 Hz), 3.95 (2H, t, *J* 9.1 Hz), 3.49-3.47 (3H, m), 3.45 (2H, t, *J* 3.4 Hz), 3.43 (1H, d, *J* 3.15 Hz), 3.30 (2H, t, *J* 8.2 Hz), 3.27 (2H, t, *J* 5.35 Hz), 0.21 (18H, s), 0.16 (18H, s), 0.15 (18H, s), δc: 94.5, 76.1, 72.9, 72.7, 70.6, 10.3, 1.3, 1.1, 0.4.

Experiment 6:

Hexadecanoic acid 6-(6-hexadecanoyloxy-methyl-3,4,5-tris-trimethylsilanyloxytetra-hydropyran-2-yloxy)-3,4,5-tris-trimethylsilanyloxy-tetrahydropyran-2-ylmethyl ester 28



28

Attempted (**a**) Compound **25** (0.10 g, 0.100 mmol) and **72** (0.1 g, 3.5 mol eq) were dissolved in HMPA (5 ml) and the mixture was stirred at 80 ° C for 20 hrs. The reaction was followed by TLC and no changes were observed so the time was increased to 40 hrs then to 50 hrs. After that the reaction was taken up in ether (60 ml). The organic layer was washed with water to eliminate the remaining HMPA then dried over $MgSO_4$ and evaporated under high vacuum. TLC in $CHCl_2$/ethyl acetate 4:2 did not show product formation and NMR confirmed starting material and HMPA for both fractions collected. The water layer used in the previous step for the extraction was re-extracted with chloroform (3x 25 ml) and the combined organic layers were washed with water (100 ml). The resulting product was evaporated under high vacuum and crude NMR showed HMPA.

Attempted (**b**) Compound **25** (0.2 g, 0.20 mmol) and **72** (0.2 g, 2.5 mol eq) were dissolved in DMI (7 ml) and the mixture was stirred at 90 ° C for 40 hrs. The reaction was followed by TLC and no changes were observed so the time was increased to 50 hrs. After that the reaction was taken up in ether (100 ml). The organic layer was washed with water to eliminate the remaining HMPA then dried over $MgSO_4$ and evaporated under

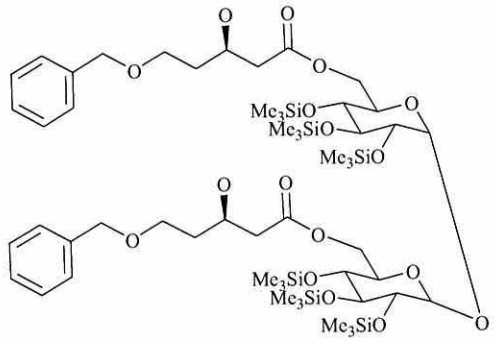
high vacuum. TLC in petrol/ether 10:0.5 did not show product formation and NMR confirmed starting material and HMPA for both fraction collected.

(**c**) Compound **60** (0.3 g, 0.38 mmol, 1 mol .eq) was dissolved in dry toluene (5 ml) and dry pyridine (0.5 ml). To this mixture commercial palmitoyl chloride (0.27 g, 0.99 mmol, 2.5 mol e.q) in anhydrous toluene (2 ml) was added. The solution was heated overnight for 12 hrs at 80 °C. The reaction was cooled down to r.t and solvents were evaporated then water was added (100 ml) and it was extracted with petrol/ether (1:1, 3x 100 ml).[135] The organic layer were collected, dried and evaporated to give a crude product which was purified by column chromatography eluting with petrol/ether 10:1 to give **28** (0.40 g, 85 %) showing δ_H (500 MHz, $CDCl_3$): 4.928 (1H, s), 4.922 (1H, s), 4.29-4.27 (2H, dd, *J* 2.2, 12 Hz), 4.07-4.04 (2H, dd, *J* 2.4, 11.65 Hz), 4.02-3.99 (2H, m) 3.92-3.89 (2H, dt, *J* 5.05, 9 Hz), 3.50-3.46 (2H, t, *J* 8.8 Hz), 3.45-3.42 (2H, dd, *J* 3.15, 9.45 Hz), 2.38-2.29 (4H, m), 1.62-1.59 (4H, m), 1.34-1.22 (48, m), 0.89-0.86 (6H, t, *J* 7 Hz), 0.16 (18H, s), 0.14 (18H, s), 0.13 (18H, s); δc: 173.7, 94.3, 73.4, 72.6, 71.9, 70.7, 63.2, 34.1, 31.9, 29.7, 29.6, 29.6, 29.4, 29.3, 29.3, 29.1, 24.8, 22.6, 14.1, 1.0, 0.9, 0.2.

(d) A mixture of palmitic acid (0.26 g, 1 mmol, 4 mol eq), trehalose 47 (0.4 g, 0.52 mmol, 2 mol eq), EDCI (0.29 g, 1.5 mmol, 5.8 mol eq), DMAP (0.031 g, 0.26 mmol, 1 mol eq) and grounded/dry molecular sieves in dry CH_2Cl_2 (4 ml) were stirred for 8 hrs at r.t. TLC in chloroform was checked and after that the reaction was left stirring for 48 hrs and TLC was checked again to shown reaction ending. The mixture was then filtrated in a sinter funnel and concentrated under vaccum to give a crude product which was then purified by column chromatography eluting with chloroform to give **28** (0.31 g, 55 %) wich showed thee same analysis as above.

Experiment 7:

Attempted preparation of (*R*)-5-Benzyloxy-3-hydroxy-pentanoic acid 6-[6-((*R*)-5-benzyloxy-3-hydroxy-pentanoyloxymethyl)-3,4,5-tris-trimethylsilanyloxytetrahydro-pyran-2-yloxy]-3,4,5-tris-trimethylsilanyloxy-tetrahydro-pyran-2-ylmethyl ester 79

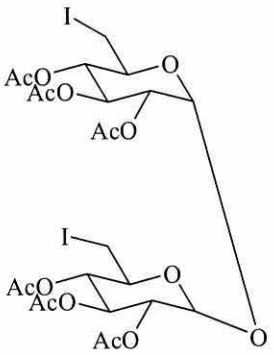


79

6-Desoxy-6-iodo-2,3,4-tri-O-trimethylsilyl-α-D-glucopyranosyl)-6-desoxy-6-iodo-2,3,4-tri-O-trimethylsilyl-α-D-glucopyranoside **25** (0.113 g, 0.114 mmol) and potassium (*R*)-5-benzyloxy-3-hydroxypentanoate **75** (0.1 g, 0.38 mmol) were dissolved in hexamethylphosphoramide (5 ml) and the mixture was stirred at 80 °C for 19 hrs. The reaction was followed by the formation of the ester band by IR. After condensation, ether (60 ml) was added. The organic layer was washed with water (2x 60 ml). The combined organic layers were dried and evaporated to give a crude product which was purified by column chromatography eluting with first with 9:1 CH_2Cl_2/MeOH to give HMPA, but no compounds **25** or **75** were found. Crude NMR did show starting material but no product formation.

Experiment 8

Acetic acid 4,5-diacetoxy-2-iodomethyl-6-(3,4,5-triacetoxy-6-iodomethyltetrahydropyran-2-yloxy)-tetrahydropyran-3-yl ester 80

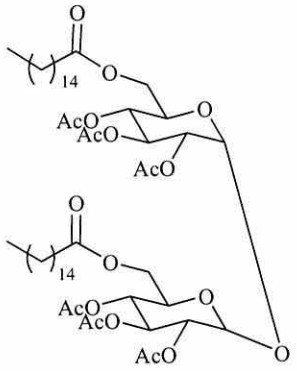


80

D-(+)-Trehalose dehydrate **38** (0.5 g, 1.33 mmol) was dried under high vacuum at 130 °C for 4 hrs to give anhydrous D-(+)-trehalose **13** (0.47 g, 1.37 mol). To a cooled and homogeneous solution of **13** (0.47 g, 1.37 mmol) and powdered triphenylphosphine (1.44 g, 5.5 mmol) in DMF (50 ml) was added N-iodosuccinimide (1.24 g, 5.5 mmol) slowly and with stirring. It was then heated for 50 hrs at 50 °C. The TLC was checked to show complete reaction. The solvent was evaporated under vacuum and methanol (20 ml) was added. The solution was evaporated in the presence of toluene (20 ml) and $CHCl_3$ (20 ml) was added twice and evaporated from the resulting oil. To this oil water (100 ml) was added and it was extracted with $CHCl_3$ (4x 50 ml). The combined organic layers were washed with water (60 ml), dried and evaporated to give a thick oil that was acetylated by reaction with acetic anhydride (5 ml) in pyridine (20 ml). After being stirring for 24 hrs at r.t, the solution was poured into iced water (250 ml), and left to settle for 2 hrs when the crystalline crude product was filtered, dried and evaporated. The crude product was purified by column chromatography eluting with 4:1 petroleum ether/ethyl acetate to give 4,5-diacetoxy-2-iodomethyl-6-(3,4,5-triacetoxy-6-iodomethyl-tetrahydro-pyran-2-yloxy)-tetrahydro-pyran-3-yl ester **80** (0.55 g, 50 %), m.p (193-195 °C), (Lit.[152] (191-193 °C)); δ_H (500 MHz, $CDCl_3$): 5.48 (1H, t, *J* 9.75 Hz), 5.42 (1H, d, *J* 3.8 Hz), 5.21 (1H, dd, *J* 3.75. 10.1 Hz), 4.9 (1H, t, *J* 9.75 Hz), 3.97-3.93 (1H, dt, *J* 2.55, 9.8 Hz), 3.24 (1H, dd, *J* 2.55, 11 Hz), 3.07 (1H, dd, *J* 9.15, 11 Hz), 2.15 (3H, s), 2.08 (3H, s), 2.03 (3H, s); δc: 169.9, 169.6, 169.4, 91.8, 72.3, 69.9, 69.7, 69.3, 30.9, 21.2, 20.7, 20.6, 2.4.

Experiment 9:

Hexadecanoic acid 3,4,5-triacetoxy-6-(3,4,5-triacetoxy-6-hexadecanoyloxymethyl-tetrahydropyran-2-yloxy)-tetrahydropyran-2-ylmethyl ester 81



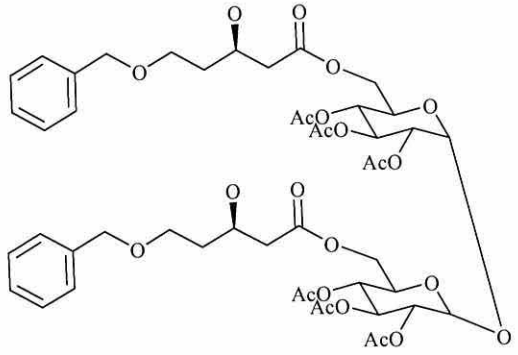
81

(a) Acetic acid 4,5-diacetoxy-2-iodomethyl-6-(3,4,5-triacetoxy-6-iodomethyl-tetrahydro-pyran-2-yloxy)-tetrahydro-pyran-3-yl ester **80** (1.11 g, 1.364 mmol) and potassium pentadecanoate **72** (1 g, 3.39 mmol) were dissolved in hexamethylphosphoramide (15 ml) and the mixture was stirred at 80 °C for 10 hrs. The reaction was followed by the formation of the ester band in the IR spectrum. After condensation, ether (200 ml) was added. The organic layer was washed with water (2x 200 ml). The combined organic layers were dried and evaporated to give a crude product which was purified by column chromatography eluting with first with petroleum ether/ethyl acetate 10:1 increasing to a final 1:1 to give the product hexadecanoic acid 3,4,5-triacetoxy-6-(3,4,5-triacetoxy-6-hexadecanoyloxymethyl-tetrahydro-pyran-2-yloxy)-tetrahydropyran-2-ylmethyl ester **81** (0.85 g, 60 %) as a white solid, Found $[M+Na]^+$: 1094.58; $C_{56}H_{94}O_{19}Na$ requires: 1094.34, $[\alpha]^{24}_D = +131.8$ (c = 1.1, $CHCl_3$)[156], which showed δ_H (500 MHz, $CDCl_3$): 5.49 (2H, t, *J* 9.45 Hz), 5.28 (2H, d, *J* 4.1 Hz), 5.07-5.02 (4H, m, including a dd at δ 5.04, *J* 1.3, 7.9 Hz), 4.24 (2H, dd, *J* 5.35, 12.3 Hz), 4.07-4.04 (2H, m), 4.02-4.00 (2H, dd, *J* 2.2 , 11.95 Hz), 2.34-2.30 (4H, m), 2.08 (6H, s), 2.05 (6H, s), 2.03 (6H, s), 1.63-1.57 (4H, m), 1.25 (48H,s), 0.88 (6H, t, *J* 7.25 Hz), δc: 173.4, 169.9, 169.5, 169.4, 92.3, 70.1, 69.8, 68.5, 68.2, 61.5, 33.8, 31.9, 29.7, 29.6, 29.5, 29.3, 29.2, 29.1, 24.73, 24.7, 22.7, 20.6, 20.6, 20.5, 14.1.

(b) Compounds **80** (1.11 g, 1.364 mmol) and **72** (1 g, 3.39 mmol) were dissolved in 1,3-dimethyl-2-imidazolidinone (DMI) (16 ml) and the mixture was stirred at 90 °C for 48 hrs. The reaction was followed by the formation of the ester band in the IR spectrum. After condensation, ether (200 ml) was added. The organic layer was washed with water (2x 200 ml). The combined organic layers were dried, evaporated to give a crude product which was purified by column chromatography eluting with $CHCl_2$/ethyl acetate 2:1 to give the product hexadecanoic acid 3,4,5-triacetoxy-6-(3,4,5-triacetoxy-6-hexadecanoyloxymethyl-tetrahydro-pyran-2-yloxy)-tetrahydro-pyran-2-ylmethyl ester **81** (0.95 g, 66 %) as a white solid (Found $[M+Na]^+$: 1094.5300; $C_{56}H_{94}O_{19}Na$ requires: 1094.3443), $[\alpha]^{24}_D = +141.81$ (c = 1.3, $CHCl_3$), which showed identical ^1H and ^{13}C NMR spectra to the product in (a).

Experiment 10:

(*R*)-5-Benzyloxy-3-hydroxypentanoic acid 3,4,5-triacetoxy-6-(3,4,5-triacetoxy-6-hexadecanoyloxymethyltetrahydropyran-2-yloxy)-tetrahydropyran-2-ylmethyl ester 82

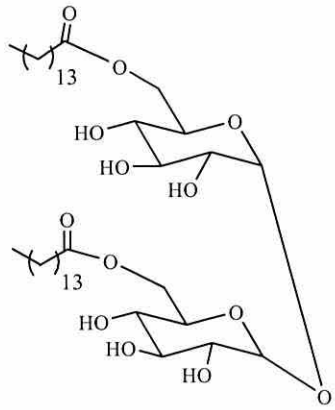


82

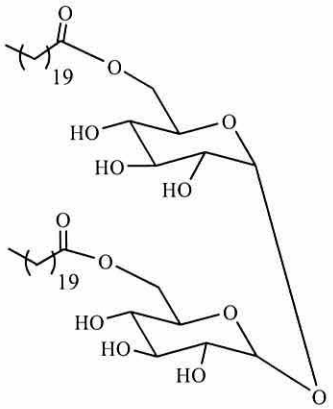
Acetic acid 4,5-diacetoxy-2-iodomethyl-6-(3,4,5-triacetoxy-6-iodomethyl-tetrahydro-pyran-2-yloxy)-tetrahydro-pyran-3-yl ester **80** (0.12 g, 0.15 mmol) and potassium (R)-5-benzyloxy-3-hydroxypentanoate **75** (0.1 g, 0.38 mmol) were dissolved in hexamethylphosphoramide (5 ml) and the mixture was stirred at 80 °C for 10 hrs. The reaction was followed by the formation of the ester band by IR. After condensation, ether (200 ml) was added. The organic layer was washed with water (2x 200 ml). The combined organic layers were dried and evaporated to give a crude product which was purified by column chromatography eluting with first with CH_2Cl_2/ethyl acetate 4:2 increasing to a final 1:1 to give (*R*)-5-benzyloxy-3-hydroxy-pentanoic acid 3,4,5-triacetoxy-6-(3,4,5-triacetoxy-6-hexadecanoyloxymethyl-tetrahydro-pyran-2-yloxy)-tetrahydro-pyran-2-ylmethyl ester **82** (0.105 g, 68 %) as a white solid, (Found $[M+Na]^+$: 1029.41; $C_{48}H_{62}O_{23}Na$ requires: 1029.99), $[\alpha]^{24}_D$ = + 92.6 (c = 2.9, $CHCl_3$), which showed δ_H (500 MHz, $CDCl_3$): 7.34-7.33 (10H, m), 5.49 (2H, dd, J 9.45, 10.1 Hz), 5.29 (2H, d, J 3.8 Hz), 5.03-4.99 (4H, m, including a dd at δ 5.01, *J* 3.8, 10.4 Hz), 4.52 (4H, s), 4.27-4.20 (4H, m, including a dd at δ 4.22, *J* 6.3, 12.6 Hz), 4.06-4.04 (4H, dd, *J* 2.5 , 9.75 Hz), 3.72-3.63 (4H, m), 2.52-2.51 (4H, m), 2.08 (6H, s), 2.05 (6H, s), 2.03 (6H, s), 1.82-1.78 (4H, m), δc: 171.7, 169.8, 169.7, 169.6, 138.0, 128.4, 127.7, 127.6, 91.9, 73.2, 69.9, 69.8, 68.6, 67.9, 67.8, 66.9, 61.8, 41.6, 36.0, 20.6, 20.5, 20.5.

Experiment 11:

Attempted preparation of pentadecanoic acid 3,4,5-trihydroxy-6-(3,4,5-trihydroxy-6-pentadecanoyloxymethyltetrahydropyran-2-yloxy)-tetrahydropyran-2-ylmethyl ester 157 and henicosanoic acid 6-(6-henicosanoyloxymethyl-3,4,5-trihydroxy-tetrahydropyran-2-yloxy)-3,4,5-trihydroxytetrahydropyran-2-ylmethyl ester 158



157



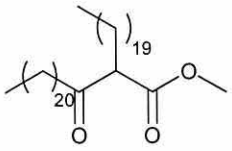
158

Candida antarctica lipase (Novozym 435) (1 g) was impregnated with an aqueous solution of D-(+)-trehalose dihydrate **38** (0.56 g, 1.5 mmol) dissolved in water (0.5-1 ml). The sample was dried for 13 hrs in a desiccator under vacuum (5mm Hg). Pentadecanoic acid (1.15 g, 4.5 mmol, 3 mol.eq.) and *tert*-butyl alcohol (30 ml) were added to the mixture that was heated for 14 hrs. After cooling to r.t, ether (30 ml) was added and the mixture was stirred for 10 min before being filtered to remove excess pentadecanoic acid.[173] The solid was the dissolved in methanol (20 ml), stirred for 20 min and filtered again. The sample was then evaporated and the crude product was purified by column chromatography eluting with chloroform/methanol 85:15 to give **38** which showed same ^{1}H and ^{13}C NMR as the starting material.

(b) Anhydrous trehalose **13** (0.5 g, 1.46 mmol) and docosanoic acid (1 g, 2.93 mmol) were dissolved in 2-methyl-2-propanol (15 ml) to which *Candida antarctica* lipase (Novozym 435) (0.2 g) and molecular sieves 12 % (w/w) as 10 % (w/w) were added. The mixture was then heated at 60 °C for 72 hrs at 600 rpm.[174] TLC showed no product, so the mixture was left for a further 120 hrs. After that TLC showed only starting material.

Experiment 12:

2-Icosyl-3-oxo-tetracosanoic acid methyl ester 86



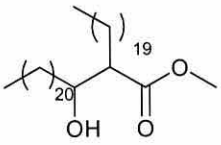
86

(a) Compound **85** (see appendix) (7.0 g, 19.7 mmol) was dried under high vacuum for 1 hr to eliminate moisture and then dissolved in dry xylene (40 ml). NaH (1.46 g, 60 % dispersion, 1.5 mol.eq.) was washed in hexane (15 ml) and added to the previous solution which was heated for 12 hrs under reflux at 145 °C.[100,164] The mixture was neutralized with acetic acid (3 ml) until pH 6. Then it was extracted with chloroform (3x 60 ml). After evaporation of the solvents, the keto-ester **86** was obtained as a yellowish solid, (12 g, 89 %); mp (65-66 °C), δ_H (500 MHz, $CDCl_3$): 3.72 (3H, s), 3.43 (1H, t, *J* 7.25 Hz), 2.6-2.4 (2H, m), 1.83 (2H, m), 1.58-1.55 (2H, m), 1.31-1.26 (78H, m), 0.90-0.87 (6H, t, *J* 7 Hz); δ_C: 205.5, 170.5, 59.0, 52.2, 41.9, 31.9, 29.7, 29.6, 29.5, 29.5, 29.3, 29.3, 29.0, 28.3, 27.5, 23.4, 22.7, 14.1.

(b) $TiCl_4$ (15.8 g, 0.083 mol, 1.5 mol .eq) in toluene (50 ml) was added to a stirred solution of **85** (20 g, 0.056 mol, 1 mol .eq) and Bu_3N (18.82 g, 0.10 mol, 1.8 mol .eq) in toluene (120 ml) at 0-5 °C.[216] After stirring at the same temperature for 1 hr, the mixture was quenched with water (150 ml) and extracted with ether (3x 100 ml). The organic phases were collected together, dried and evaporated to give a crude product which was purified by column chromatography eluting with petrol/ether 10:1 to give **86** (14.5 g, 40 %); which showed identical ^1H and ^{13}C NMR spectra to the product in **(a).**

Experiment 13:

3-Hydroxy-2-icosyl-tetracosanoic acid methyl ester 87

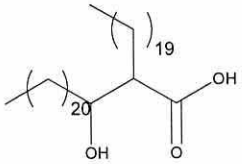


87

Compound **86** (8 g, 11.81 mmol) was dissolved in chloroform/methanol 1:1 (150 ml) and $MgSO_4$ (3 g) was added to the mixture that was left stirring for 12 hrs at r.t. As the sample was not completely soluble it was slightly warmed (40 °C) and filtered through a sinter funnel. Powdered $NaBH_4$ (0.67 g, 17.71 mmol, 1 mol .eq) was slowly added over several minutes in small portions. The mixture was left stirring for 24 hrs at r.t. TLC was checked still showed some starting material so extra $NaBH_4$ (0.5g, 13.21 mmol, 1 mol .eq) was added and the mixture left stirring for a further 48 hrs at 22 °C.[100,164] The reaction was worked up by adding water (40 ml) and extracted it with chloroform (3x 50 ml). The organic layers were dried, evaporated to give a crude product that was purified by column chromatography eluting with chloroform to give **87** (4 g, 48 %); mp (73-74 °C), δ_H (500 MHz, $CDCl_3$): 3.79-3.71 (1H, m), 3.71 (3H, s), 2.46-2.43 (1H, m), 1.58 (2H, m), 1.47-1.42 (2H, m), 1.30-1.24 (75H, m), 0.90-0.87 (6H, t, *J* 7 Hz); δ_C: 176.1, 72.1, 51.6, 51.0, 41.0, 34.3, 31.9, 29.7, 29.6, 29.5, 29.4, 29.3, 27.9, 26.9, 25.9, 22.7, 14.1.

Experiment 14:

3-Hydroxy-2-icosyltetracosanoic acid 88

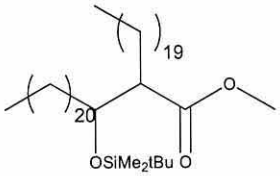


88

Compound **87** (0.7 g, 1.03 mmol) was dissolved in 5 % KOH (0.75 g in 15 ml) in biphasic butanol-water 1:1 (30 ml) and then it was stirred for 8 hrs at 105 °C. The mixture was cooled to r.t and acidified with HCl until the water layer pH was 1-1.5.[100] It was then extracted with chloroform (3x 70ml). The combined organic layers were dried, evaporated and the product was then crystallized from ethanol (100 ml) to give **88** (0.64 g, 92 %); δ_H (500 MHz, $CDCl_3$): 3.75-3.71 (1H, m), 2.51-2.45 (1H, m), 1.54-1.44 (2H, m), 1.42-1.37 (2H, m), 1.30-1.20 (74H, m), 0.90-0.87 (6H, s); δ_C: 177.3, 71.7, 54.2, 31.9, 30.0, 29.7, 29.6, 29.6, 29.3, 22.7, 14.1.

Experiment 15:

3-(*tert*-Butyl-dimethyl-silanyl)-2-icosyltetracosanoic acid methyl ester 89

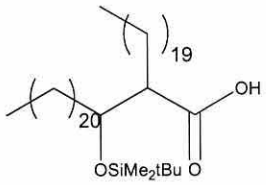


89

A solution of imidazole (1.61 g, 23.6 mmol, 8 mol.eq) and *tert*-butylchlorodimethylsilane (1.33 g, 8.9 mmol, 3 mol .eq) in dry DMF (10ml) was added to **87** (2 g, 2.95 mmol) in dry toluene (14 ml). The mixture was heated for 40 hrs at 75 °C. TLC petrol/ether 10:1 was checked to show reaction ending. After that solvents were removed under vacuum and more toluene was added and evaporated to eliminate DMF.[100,164] The crude sample was extracted with petrol/ether (1:1, 3x 40 ml) and water (40 ml). The organic layers were collected, dried and evaporated off. The crude product was then purified by column chromatography eluting with petrol/ether 10:0.5 to give **89** as a yellow solid (2 g, 85 %); δ_H (500 MHz, $CDCl_3$): 3.84-3.81 (1H, q, *J* 2.85 Hz), 3.66 (3H, s), 2.53-2.46 (1H, m), 1.57-1.53 (1H, m), 1.44-1.42 (1H, m), 1.30-1.20 (70H, m), 0.90-0.83 (6H, m), 0.05 (3H, s), 0.04 (3H, s); δ_C: 175.2, 73.4, 51.5, 34.8, 31.9, 29.8, 29.7, 29.6, 29.6, 29.5, 29.4, 28.4, 27.9, 25.8, 25.8, 24.7, 22.7, 22.6, 18.0, 14.1, -4.3, -4.5.

Experiment 16:

3-(*tert*-Butyl-dimethyl-silanyloxy)-2-icosyltetracosanoic acid 90



90

(**a**) A solution of imidazole (0.41 g, 6.02 mmol) and *tert*-butylchlorodimethylsilane (0.34 g, 2.25 mmol) in dry DMF (5 ml) was added to **88** (0.5 g, 0.75 mmol) in toluene (75 ml). The mixture was heated at 75 °C for 48 hrs then extracted with hexane (3x 50 ml). The hexane layers were passed through a thin column of neutral silica to which ethyl triethylamine was added. The column was prewashed with ether and then washed with petrol (100 ml). The product was dissolved in methanol (10 ml) and THF (3 ml), then treated with a solution of K_2CO_3 (10 %) in water (10 ml). After stirring for 24 hrs at r.t, it was concentrated under *vacuo* to a quarter of the volume and diluted with brine (10 ml).[164] The mixture was cooled to 0 °C, the pH adjusted to 4-5 with aq. $KHSO_4$ (1M) and solution. The mixture was then extracted with ether (3x 20 ml). The organic layers were collected, dried and evaporated to give a crude product which was purified by column chromatography eluting with chloroform to give syrupy **90** (0.2 g, 58 %); δ_H (500 MHz, $CDCl_3$): 3.83-3.81 (1H, m), 2.49-2.46 (1H, m), 1.56 (9H, s), 1.33-1.22 (78H, m), 0.90-0.87 (6H, m), 0.05 (3H, s), 0.04 (3H, s); δ_C: 175.2, 73.4, 51.5, 40.8, 34.8, 31.9, 29.7, 29.6, 29.4, 29.3, 25.8, 24.7, 22.7, 18.0, 14.1, -4.3, -4.5.

(b) To a stirred solution of **89** (0.17 g, 0.21 mmol) in MeOH (5 ml) under N_2 was added KOH aq (0.7 g in 2.5 ml water, 5N) dropwise. The mixture was stirred under reflux at 75 °C for 48 hrs.[164] It was then cooled and the solvents evaporated under vacuum. The residue was dissolved in water (20 ml) extracted with ether (20 ml). The water layer was acidified to pH 3-5 by the addition of AcOH. The organic layer was then washed with water (20 ml), dried and evaporated to give a crude product which showed an identical NMR to the starting material **89**.

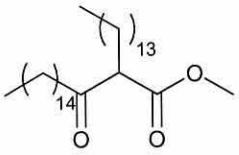
(**c**) Compound **89** (0.2 g, 0.252 mmol) in THF (10 ml), MeOH (1.5 ml), water (1 ml) was stirred at r.t. To this was added LiOH (0.16 g, 3.8 mmol, 15 mol.eq) and it was stirred at

r.t for 12 hrs. The mixture was then dissolved in petrol/ether 1:1 (40 ml) and aq. $KHSO_4$ (at pH=1) was added until the water reached pH 1-2. The organic phase was extracted, washed with water (20 ml), dried and evaporated to give a crude material which showed an identical NMR to the starting material **89**.

(**d**) To a stirred solution of **89** (0.2 g, 0.242 mmol) in dioxane (15 ml) at 0 °C was added LiOH (0.036 g, 1.5 mmol, 5.8 mol .eq) in water (5 ml), dropwise over 10 min under N_2. The solution was stirred until it reached r.t, then cooled to 0 °C again and AcOH was added dropwise until pH=4. The mixture was then extracted with dichloromethane (3x 20ml). The combined organic layers were dried, evaporated to give a crude product which showed an identical NMR to the starting material **89**.

Experiment 17:

3-Oxo-2-tetradecyloctadecanoic acid methyl ester 61

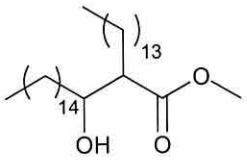


61

$TiCl_4$ (21.05 g, 0.111 mol, 1.5 mol.eq.) in toluene (50 ml) was added to a stirred solution of **22** (20 g, 0.074 mol, 1 mol .eq) and Bu_3N (24.7 g, 0.133 mol, 1.8 mol .eq) in toluene (120ml) at 0-5 °C.[164,216] After stirring at the same temperature for 1 hr, the mixture was quenched with water (150 ml) and extracted with ether (3x 100 ml). The organic phases were collected together, dried and evaporated to give a crude product which was purified by column chromatography eluting with petrol/ether 10:1 to give solid **61** (19 g, 50 %); m.p (52-54 °C), (Lit.[233] (50-51 °C)); δ_H (500 MHz, $CDCl_3$): 3.72 (3H, s), 3.43 (1H, t, *J* 7 Hz), 2.55-2.44 (2H, m), 1.86-1.80 (2H, m), 1.60-1.55 (4H, m), 1.31-1.26 (46H, m), 0.90-0.87 (6H, t, *J* 7 Hz); δ_C: 205.5, 170.5, 59.0, 52.2, 41.9, 31.9, 29.7, 29.6, 29.6, 29.5, 29.4, 29.3, 29.0, 28.3, 27.5, 23.5, 22.7, 14.1.

Experiment 18:

3-Hydroxy-2-tetradecyloctadecanoic acid methyl ester 62

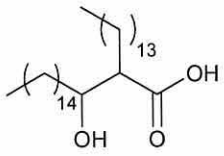


62

Compound **61** (10 g, 19.65 mmol) was dissolved in chloroform/methanol (1:1, 150 ml) and $MgSO_4$ (3 g) was added to the mixture and stirred for 12 hrs at r.t. Powdered $NaBH_4$ (2 g, 52.86 mmol, 2.7 mol .eq) was slowly added over several minutes in small portions. This gave a crude product that was purified by column chromatography was left stirring for 24 hrs at r.t. TLC was checked and the reaction was worked up by adding water (90 ml) and extracted it with chloroform (3x 70 ml).[100,164] The organic layers were dried, evaporated chloroform to give solid **62** (7.5 g, 75 %); m.p (62-64 °C), (Lit.[234] (61-62 °C)); δ_H (500 MHz, $CDCl_3$): 3.79-3.75 (1H, m), 3.71 (3H, s), 2.46-2.43 (1H, m), 1.72-1.68 (2H, m), 1.47-1.42 (2H, m), 1.30-1.24 (50H, m), 0.90-0.87 (6H, t, *J* 7 Hz); δ_C: 176.1, 72.1, 51.6, 51.0, 31.9, 29.7, 29.6, 29.6, 29.5, 29.4, 29.3, 27.8, 26.9, 25.9, 22.7, 14.1

Experiment 19:

3-Hydroxy-2-tetradecyloctadecanoic acid 27

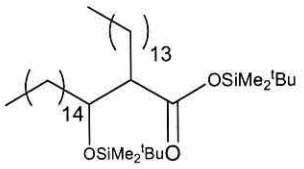


27

Compound **40** (7.2 g, 14 mmol) was saponified in refluxing 5 % KOH in biphasic butanol/water (1:1, 150 ml) for 6 hrs at 120 °C. The mixture was then acidified with HCl until water pH 1-2 and it was extracted with chloroform (2x 120 ml). The organic layers were dried, evaporated and crystallised overnight from ethanol (150 ml).[100,164] After filtration over a sinter funnel solid **27** (4.83 g, 69 %) was obtained which showed; m.p (73-75 °C), (Lit.[235] (72-75 °C)); δ_H (500 MHz, $CDCl_3$): 3.88-3.85 (1H, m), 2.50-2.46 (1H, m), 1.58-1.55 (2H, m), 1.42-1.37 (2H, m), 1.35-1.25 (52H, m), 0.90-0.87 (6H, t, *J* 6.8 Hz); δ_C: 180.2, 62.8, 54.8, 34.8, 31.9, 29.7, 29.4, 22.7, 14.1, 13.8.

Experiment 20:

(2*RS*,3*RS*)-3-(*tert*-Butyldimethylsilyloxy)-2-tetradecyloctadecanoic acid 63

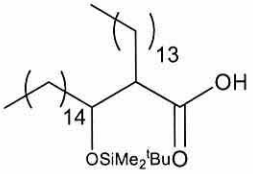


63

A solution of imidazole (4.97 g, 73 mmol, 8 mol .eq) and *tert*-butylchlorodimethylsilane (4.07 g, 27 mmol, 3 mol .eq) in dry DMF (50 ml) was added to **27** (4.5 g, 9 mmol, 1 mol .eq) in dry toluene (75 ml). The mixture was heated for 14 hrs at 75 °C and then cooled to r.t. The solution was extracted with hexane (3x 50 ml). The combined hexane layers were passed through a column of silica prewashed with ether (100 ml) to which a few drops of triethylamine were added. The column was the washed with petrol (150 ml). Elutes were pooled and evaporated to dryness. The crude product obtained this way was purified by column chromatography eluting with petrol/ether 10:1 to obtain solid **63** (4.80 g, 66 %); δ_H (500 MHz, $CDCl_3$): 3.81-3.79 (1H, m), 2.5-2.43 (1H, m), 1.62-1.56 (2H, m), 1. 52-1.47 (2H, m), 1.34-1.22 (48H, m), 0.96-94 (9H, s), 0.90-0.89 (9H, s), 0.90-0.87 (6H, t, *J* 7 Hz), 0.06-0.05 (6H, s), 0.05-0.02 (6H, s); δ_C: 175.1, 73.4, 53.2, 35.3, 31.9, 29.9, 29.7, 29.7, 29.6, 29.5, 29.5, 29.5, 29.4, 27.7, 25.9, 25.9, 25.7, 25.5, 25.5, 24.2, 22.7, 18.1, 17.5, 14.1, -2.9, -4.2, -4.5, -4.9, -4.9.

Experiment 21:

3-(*tert*-Butyl-dimethylsilanyloxy)-2-tetradecyl-octadecanoic acid 52

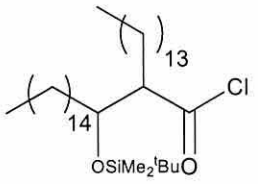


52

Compound **63** (2.26 g, 3.31 mmol) was dissolved in MeOH/THF (10:3, 52 ml) and treated with 10 % aq.K_2CO_3 (12 ml). The mixture was stirred for 14 hrs at r.t. It was then concentrated to one fourth of the volume, brine (40 ml) was added and it was cooled down to 0 °C. The pH was then corrected with an aqueous solution of $KHSO_4$ until the water layer reached pH 4-5.[164,236] The resulting solution was extracted with ether (3x 20 ml). The combined organic layers were collected, dried and evaporated to give a crude product which was purified by column chromatography eluting with chloroform to give the syrupy **52** (1.6 g, 83 %); δ_H (500 MHz, $CDCl_3$): 3.95-3.85 (1H, m), 2.50-2.45 (1H, m), 1.72-1.65 (2H, m), 1.55-1.45 (2H, m), 1.35-1.20 (51H, m), 0.90 (9H, s), 0.90-0.87 (6H, t, *J* 7 Hz), 0.09-0.08 (6H, s) δ_C: 178.7, 73.4, 50.9, 31.9, 29.7, 29.7, 29.6, 29.6, 29.5, 29.5, 29.4, 29.4, 29.4, 27.9, 27.6, 27.4, 25.8, 25.2, 22.7, 18.0, 18.0, 14.1, -4.3, -4.4, -4.6, -4.9.

Experiment 22:

3-(*tert*-Butyl-dimethyl-silanyloxy)-2-tetradecyl-octadecanoyl chloride 91

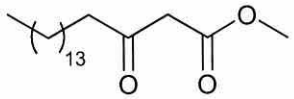


91

Compound **52** (0.3 g, 0.49 mmol) was dissolved in dry toluene (2.5 ml), oxalyl chloride (86 μl) and dry THF (5μl). The mixture was then stirred for 12 hrs at r.t under N_2. NMR was checked to see if the reaction was finished and then solvents were carefully evaporated, then dry ether (2 ml) was added and evaporated so any remaining oxalyl chloride will be eliminated. Compound **91** was used without any further purification in the next step. (δ_H (250 MHz, $CDCl_3$): 4.17-4.05 (1H, br, q, *J* 5.2 Hz), 2.92-2.87 (1H, m), 1.70-1.20 (54H, m), 0.90 (9H, s), 0.89-0.87 (6H, t, *J* 7 Hz), 0.09-0.08 (6H, s)).

Experiment 23:

3-Oxo-octadecanoic acid methyl ester 93

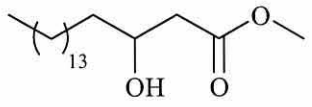


93

Methyllithium (2.35 ml, 18.32 mmol) was added dropwise at -40 °C to a solution of anhydrous diisopropylamine (3.1 ml, 22 mmol) in dry THF (20 ml) under N_2. The mixture was stirred for 1 hr at r.t. Subsequently, it was cooled to -78 °C and a solution of methyl acetate (1.05 ml, 13.2 mmol) in dry THF (6 ml) was added at a rate that allowed the temperature to remain below -65 °C, then the mixture was stirred at -78 °C for 10 min. Finally a solution of hexadecanoyl chloride (2 g, 7.3 mmol) in dry THF (6 ml) was added dropwise at -78 °C. The reaction was stirred at r.t for 48 hrs then quenched with diluted hydrochloric acid (10 %) until pH 5.[237] The aqueous layer was extracted with dichloromethane (3x 50 ml) and the combined organic layers were dried and the solvent evaporated to give a crude product that was purified by column chromatography eluting with petrol/ether 2:1 to give **93** (1.2 g, 50 %) as an oil; m.p (46-48 °C), (Lit.[238] (46.2-48 °C)); δ_H (500 MHz, $CDCl_3$): 3.97-3.92 (2H, m), 2.26-2.23 (2H, t, *J* 7.9 Hz), 2.14 (3H, s), 1.62-1.55 (2H, m), 1.27-1.2 (24H, m) ; 0.85-0.83 (3H, t, *J* 7.25 Hz); δc: 206.8, 172.2, 65.8, 48.2, 45.5, 35.4, 31.9, 30.8, 29.6, 29.6, 29.5, 29.4, 29.3, 29.3, 25.4, 22.6, 21.0, 20.6, 14.0.

Experiment 24:

3-Hydroxy-octadecanoic acid methyl ester 94

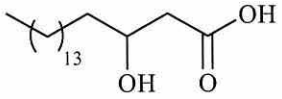


94

Compound **93** (1 g, 3.2 mmol) was dissolved in MeOH (20 ml) and the solution was cooled to 0 °C and treated with sodium borohydride (0.2 g, 5.16 mmol). The mixture was stirred for 16 hrs at r.t, and then quenched with water (40 ml).[239] The mixture was then extracted with ether (3x 40 ml) and the combined organic layers were dried and concentrated to give a crude product which was purified by column chromatography eluting with petrol/ether 1:1 to give **94** (0.76 g, 76 %), m.p (52-54 °C), (Lit.[240] (51-52 °C)) which showed δ_H (500 MHz, $CDCl_3$): 4.02-3.99 (1H, m), 3.72 (3H, s), 2.85-2.80 (1H, br, s), 2.54-2.50 (1H, dd, *J* 2.85, 16.4 Hz), 2.44-2.39 (1H, dd, *J* 8.8, 16.4 Hz), 1.45-1.42 (2H, m), 1.30-1.22 (26H, m), 0.90-0.87 (3H, t, *J* 7 Hz); δc: 173.5, 68.0, 51.7, 41.1, 36.5, 31.9, 29.7, 29.6, 29.6, 29.5, 29.5, 29.3, 25.5, 22.7, 14.1.

Experiment 25:

3-Hydroxy-octadecanoic acid 95

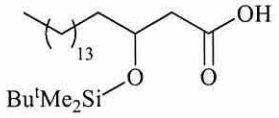


95

Compound **94** (0.5 g, 1.6 mmol) was hydrolyzed in refluxing 5 % KOH (25 ml) in biphasic butanol/water (1:1, 30 ml) for 6 hrs at 120 °C. TLC showed complete reaction. The mixture was then acidified with HCl (6 ml) until the pH turned acidic (1.5-2) and then it was extracted with chloroform (3x 30 ml).[164] The combined organic layers were dried and evaporated to give a crude product which was purified by column chromatography eluting with chloroform/methanol 5:1 to give **95** (0.4 g, 83 %); m.p (87-89 °C), (Lit.[217] (88-90 °C)); δ_H (250 MHz, $CDCl_3$): 4.10-3.99 (1H, m), 2.65-2.40 (2H, m), 1.60-1.15 (28H, m), 0.90-0.87 (3H, t, *J* 7 Hz) δc: 177.3, 67.4, 41.1, 37.2, 31.9, 29.9, 29.7, 29.7, 29.6, 29.6, 29.6, 29.6, 29.6, 29.3, 29.3, 22.7, 14.1.

Experiment 26:

3-(*tert*-Butyl-dimethylsilanyloxy)-octadecanoic acid 96

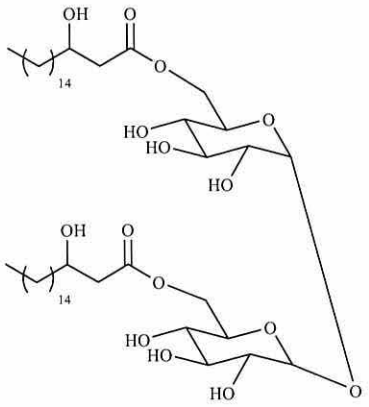


96

A solution of imidazole (0.73 g, 10.67 mmol) and *tert*-butylchlorodimethylsilane (0.6 g, 4 mmol) in dry DMF (5 ml) was added to **95** (0.4 g, 1.33 mmol) in dry toluene (7 ml). The mixture was heated at 75 °C for 12 hrs. TLC was checked to show the reaction ending. The mixture was then extracted with hexane (3x 25ml). The organic layers were then evaporated and passed through a silica column to which ethyl triethylamine was added. The column was pre-washed with ether (100 ml), washed with petrol (100 ml) and eluants were collected and evaporated. The mixture was then dissolved in chloroform (25 ml) and stirred with K_2CO_3 aq (10 %, 12 ml) at r.t for 30 min to cleave the acid protecting groups. It was then acidified with H_2SO_4 (2.5M) until pH 2.[164] The product was extracted with chloroform (3x 30 ml), dried and evaporated to give a crude product which was purified by column chromatography eluting with petrol/ether 2:1 to give solid **96** (0.45 g, 47 %); δ_H (500 MHz, $CDCl_3$): 4.12-4.07 (1H, pent, *J* 6.3 Hz), 2.52-2.46 (1H, dd, *J* 6, 14.8 Hz), 2.44-2.40 (1H, dd, *J* 6.35, 15 Hz), 1.50-1.46 (2H, m), 1.32-1.25 (26H, m), 0.94 (9H, s), 0.90-0.87 (3H, t, *J* 7 Hz), 0.07 (3H, s), 0.06 (3H, s) δc: 172.1, 69.5, 44.5, 37.5, 31.9, 29.7, 29.7, 29.6, 29.4, 25.7, 25.5, 25.1, 22.7, 18.0, 17.5, 14.1, -4.5, -4.6.

Experiment 27:

Attempted preparation of 3-Hydroxy-octadecanoic acid 3,4,5-trihydroxy-6-[3,4,5-trihydroxy-6-(3-hydroxy-octadecanoyloxymethyl)-tetrahydropyran-2-yloxy]-tetrahydropyran-2-ylmethyl ester 99

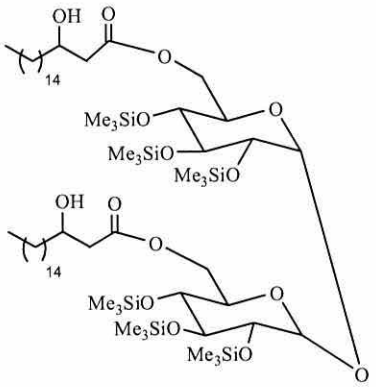


99

Anhydrous trehalose **13** (0.1g, 0.29 mmol), triphenylphosphine (0.2g, 0.79 mmol) and 3-hydroxy-octadecanoic acid **95** (0.23g, 0.25 mmol) were dissolved in dry DMF (5 ml) and cooled to 0 °C. DIAD (0.17g, 0.81 mmol) was added dropwise to a cooled and stirred solution over 15 min. The mixture was then stirred over 36 hrs at 30 °C. TLC was checked to show reaction ending. The resulting mixture was then evaporated to dryness under high vacuum (temp < 50 °C). The residue was taken up in chloroform, filtrated and purified by column chromatography with Chloroform/MeOH/acetic acid/H_2O 79:11:8:2. TLC and NMR showed staring material and no product formation.

Experiment 28:

3-Hydroxy-octadecanoic acid 6-[6-(3-hydroxy-octadecanoyloxymethyl)-3,4,5-tris-trimethylsilanyloxytetrahydropyran-2-yloxy]-3,4,5-tris-trimethylsilanyloxytetra-hydropyran-2-ylmethyl ester 100



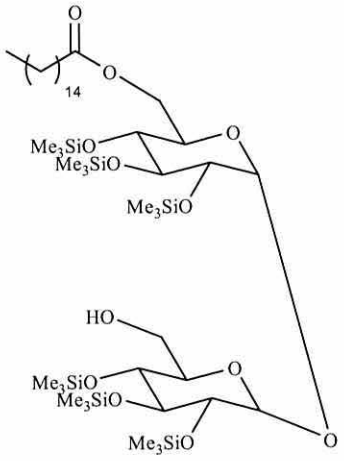
100

Compound **95** (0.2 g, 0.66 mmol), **60** (0.17 g, 0.22 mmol), DCC (0.136 g, 0.66 mmol), 4-dimethylaminopyridine (20 mg) and 4Å molecular sieves (1.5 g) were put together, ground and placed under N_2 and then a vacuum was applied.[241] This procedure was done twice in two different flasks to apply two different times to the experiment. Both mixtures were stirred for 4 hrs. After that, dry toluene (10 ml) was added at 0 °C under N_2. One mixture was left stirring for 50 hrs at 30 °C and the other was left for 72 hrs.[164] TLC was checked at 50 hrs so the first reaction was worked up by evaporating the solvent and purifying the resulting solid by column chromatography eluting with hexane/ether 1:1 to give solid **100** (0.025 g, 9 %); which showed δ_H (500 MHz, $CDCl_3$ + few drops of CD_3OD): 4.92-4.90 (2H, d, *J* 3.5 Hz), 4.36-4.32 (1H, ddd, *J* 2.5, 5.7, 12 Hz), 4.14-4.10 (1H, dd, *J* 4.1, 12 Hz), 4.04-3.97 (2H, m), 3.93-3.87 (2H, m), 3.86-3.82 (1H, m), 3.74-3.65 (2H, dt, *J* 3.5, 9.45 Hz), 3.52-3.47 (3H, t, *J* 7 Hz), 3.46-3.42 (3H, m), 3.00 (1H, br, s), 2.60-2.40 (3H, m), 1.31-1.26 (57H, m), 0.89-0.87 (6H, t, *J* 7.25 Hz), 0.17 (9H, s), 0.16 (9H, s), 0.157 (9H, s), 0.152 (9H, s), 0.14 (9H, s), 0.13 (9H, s); δc: 168.5, 94.4, 73.3, 72.8, 71.4, 67.9, 65.8, 61.6, 36.5, 31.9, 29.7, 29.6, 29.6, 29.6, 29.5, 29.3, 25.5, 22.7, 15.3, 14.1, 1.0, 1.0, 0.9, 0.8, 0.1, 0.1.

After 72 hrs, the second reaction was worked up the same way as the previous one and columned to give solid **100** (0.025 g, 9 %), {Found $[M+Na]^+$: 1363.93; $C_{66}H_{138}O_{15}Si_6Na$ requires: 1363.3215}; showed same ^1H and ^{13}C NMR spectra as one worked up 72 hrs before.

Experiment 29:

[Hexanoic acid 6-(6-Hydroxymethyl-3,4,5-tris-trimethylsilanyloxytetrahydropyran-2-yloxy)-3,4,5-tris-trimethylsilanyloxytetrahydropyran-2-yl]-methyl ester 28b



28b

(**a**) Hexadecanoic acid **26** (0.4 g, 1.56 mmol), **60** (0.4 g, 0.52 mmol), DCC (0.32 g, 1.56 mmol), DMAP (15 mg) and dry molecular sieves (1 g) were put together and finely ground. The mixture was put under N_2, stirred and high vacuum was applied for 4 hrs. After that dry toluene (14 ml) was added at 0 °C and the mixture was stirred for 60 hrs under N_2. TLC was checked to show reaction ending.[164] Solids were removed by filtration and the filtrate was evaporated to dryness. The solid was then dissolved in chloroform (10 ml) and the mixture was purified by column chromatography eluting with chloroform to give solid **28b** (0.22 g, 43 %),[164]{Found $[M+Na]^+$: 1036.65; $C_{46}H_{100}O_{12}Si_6Na$ requires: 1036.79 (TMM)}; which showed δ_H (500 MHz, $CDCl_3$): 4.92 (1H, s), 4.91 (1H, s), 4.31-4.29 (1H, dd, *J* 2.2, 12 Hz), 4.08-4.05 (1H, dd, *J* 4.75, 11.65 Hz), 4.02-4.00 (1H, m), 3.93-3.88 (2H, dt, *J* 5.05, 9 Hz), 3.87-3.83 (1H, m), 3.73-3.70 (1H, dd, *J* 2.55, 11.65 Hz), 3.70-3.65 (1H, dd, *J* 3.8, 12 Hz), 3.50-3.42 (4H, m), 2.38-2.33 (2H, t, *J* 7.5 Hz), 1.34-1.22 (27, m), 0.88 (3H, t, *J* 7.2 Hz), 0.17 (9H, s), 0.16 (9H, s), 0.15 (9H, s), 0.14 (18H, s), 0.13 (9H, s); δc: 173.7, 94.5, 94.4, 73.3, 72.9, 72.7, 72.6, 71.9, 71.4, 70.7, 63.3, 62.3, 34.1, 31.9, 29.7, 29.6, 29.4, 29.35, 29.31, 24.8, 22.6, 14.1, 1.0, 1.0, 0.9, 0.8, 0.1, 0.1.

Attempted (b) Compound **60** (0.3 g, 0.39 mmol), triphenylphosphine (0.27 g, 1.04 mmol) and hexadecanoic acid (0.258 g, 1 mmol) were dissolved in dry DMF (7 ml) and

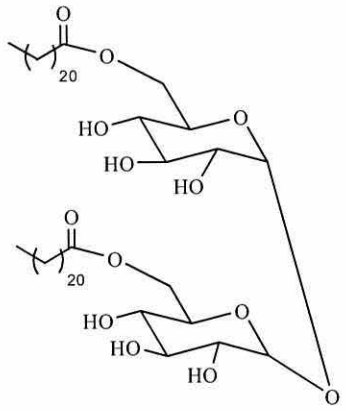
cooled to 0 °C under N_2. Di-isopropyl azodicarboxylate (DIAD) (0.22 g, 1.1 mmol) was added dropwise to the cooled, stirred solution over 15 min. The mixture was stirred at r.t for 24 hrs. TLC was checked to show reaction ending.[163] The mixture was then evaporated (bath temperature <50 °C). The solid obtained was dissolved in ethyl acetate (30 ml), and then washed with water (3x 30 ml). The combined organic layers were dried and evaporated. The crude product was then purified by column chromatography eluting with chloroform to give staring material **60** by ^{1}H NMR and ^{13}C NMR; {Found $[M+Na]^+$: 798.58, 1028.27; $C_{62}H_{130}O_{13}Si_6Na$ requires: 1275.21, $C_{46}H_{100}O_{12}Si_6Na$ requires: 1036.79}.

(c) Compound **60** (0.3 g, 0.38 mmol, 1 mol .eq) was dissolved in dry toluene (5 ml) and dry pyridine (0.5 ml). To this mixture commercial palmitoyl chloride (0.27 g, 0.99 mmol, 2.5 mol e.q) in anhydrous toluene (2 ml) was added. The solution was heated overnight for 12 hrs at 80 °C. The reaction was cooled down to r.t and solvents were evaporated then water was added (100 ml) and it was extracted with petrol/ether (1:1, 3x 100 ml).[135] The organic layer were collected, dried and evaporated to give a crude product which was purified by column chromatography eluting with petrol/ether 10:1 to give **28b** (0.10 g, 26 %) showing the same ^{13}C and ^{1}H NMR as above.

(d) Palmitic acid **26** (0.26 g, 1.01 mmol, 4 mol .eq), **60** (0.4 g, 0.51 mmol, 2 mol .eq), EDCI (0.29 g, 1.51 mmol, 6 mol .eq), DMAP (0.031 g, 0.25 mmol, 11 mol .eq) and dried/finely powdered molecular sieves (0.3 g) were dissolved in dry CH_2Cl_2 (4 ml) and stirred for 56 hrs at 20 °C. TLC was checked to show reaction end. The mixture was then filtered in a sinter funnel, dried and evaporated to dryness.[219] The crude sample was purified by column chromatography eluting with chloroform to give the product **28b** (0.30 g, 55 %) showing the same ^{13}C and ^{1}H NMR as above.

Experiment 30:

Docosanoic acid 6-(6-docosanoyloxymethyl-3,4,5-trihydroxytetrahydropyran-2-yloxy)-3,4,5-trihydroxytetrahydropyran-2-ylmethyl ester 103

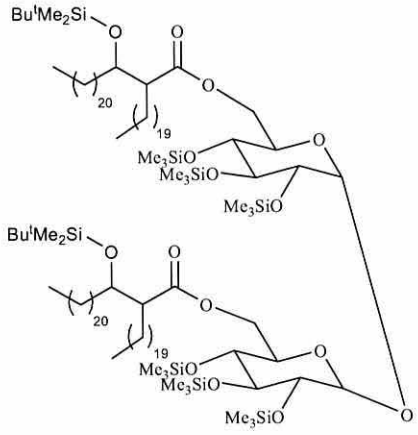


103

Compound **60** (0.5 g, 0.645 mmol) was dissolved in dry toluene (10 ml) and dry pyridine (1 ml). To this **159** (0.46 g, 1.29 mmol, 2 mol .eq) dissolved in anhydrous toluene (4 ml) was added. The solution was stirred and heated at 80 °C for 2.5 hrs. TLC in chloroform showed complete. The solvent was eliminated under vacuum and the crude product dissolved in MeOH/H_2O (1:10, 6.6 ml) and refluxed for 2 hrs. TLC in chloroform showed complete reaction. The solvents were eliminated under vacuum and the crude product was purified by column chromatography eluting with chloroform/MeOH 0.85:0.15 to give the final product **103** (0.43 g, 68 %); {Found $[M+Na]^+$: 1010.01; $C_{56}H_{106}O_{13}Na$ requires: 1010.44}; which showed δ_H (500 MHz, $CDCl_3$ + few drops of CD_3OD): 5.05 (2H, d, *J* 3.8 Hz), 4.28-4.22 (4H, m), 3.94-3.90 (2H, dt, *J* 3.15, 9.8 Hz), 3.84-3.80 (2H, t, *J* 9.15 Hz), 3.50-3.47 (2H, dd, *J* 3.75, 9.75 Hz), 3.32-3.28 (2H, t, *J* 9.75 Hz), 2.30-2.27 (4H, t, *J* 7.55 Hz), 1.58-1.52 (4H, m), 1.28-1.20 (78H, m), 0.84-0.81 (6H, t, *J* 7 Hz); δc: 174.5, 93.4, 73.0, 71.5, 71.4, 70.4, 70.1, 69.9, 34.0, 31.8, 29.6, 29.6, 29.5, 29.4, 29.2, 28.2, 29.0, 24.7, 22.5, 13.9.

Experiment 31:

3-(*tert*-Butyl-dimethyl-silanyloxy)-2-icosyl-tetracosanoic acid[6-(6-Hydroxymethyl-3,4,5-tris-trimethylsilanyloxytetrahydropyran-2-yloxy)-3,4,5-tris-trimethylsilanyl-oxytetrahydropyran-2-yl]-methyl ester 102

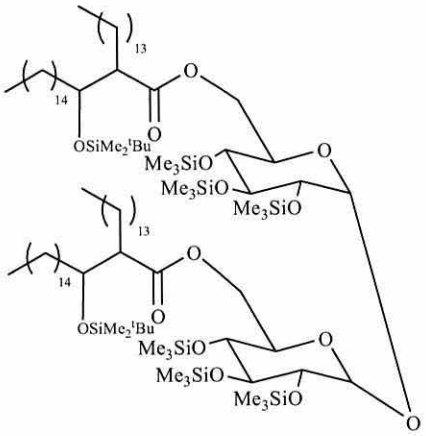


102

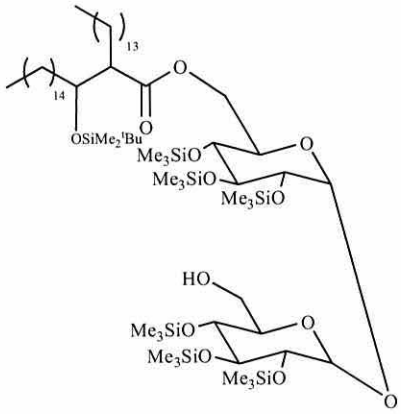
Compound **90** (0.06 g, 0.077 mmol, 2.4 mol .eq), **60** (0.025 g, 0.032 mmol, 1 mol .eq), 1,3-dicyclohexylcarbodiimide (0.016 g, 0.077 g, 2.4 mol .eq) and DMAP (5 mg) were put together and vacuum dried in a round bottled flask for 3 hrs. Dry toluene (5 ml) was then added under N_2 and the mixture was stirred for 12 hrs at 22 °C, then at 55 °C for 5 days until TLC showed no starting material left. The crude product was filtered through a sinter funnel to which THF was added (100 ml), then evaporated and purified by column chromatography eluting with petrol/ether 19:1 to give **102** (0.03 g, 40 %), {Found $[M+Na]^+$: 1557.96; $C_{800}H_{170}O_{13}Si_7Na$ requires: 1558.09}; {Found $[M+Na]^+$: 2318.65; $C_{130}H_{270}O_{15}Si_8Na$ requires: 2318.84}; δ_H (500 MHz, $CDCl_3$): 4.92 (1H, s), 4.91 (1H, s), 3.92-3.85 (4H, m, including a triplet resonated at δ 3.90, *J* 9 Hz), 3.73-3.68 (2H, m), 3.51-3.47 (2H, m, including a triplet resonated at δ 3.49, *J* 9 Hz), 3.44-3.41 (2H, dd, *J* 3.15, 9.15 Hz), 2.22-2.18 (2H, m), 1.70-1.68 (4H, m), 1.35-1.15 (152H, m), 0.93 (18H, s), 0.90-0.87 (12H, t, *J* 7 Hz), 0.17 (18H, s), 0.15 (18H, s), 0.13 (18H, s), 0.09 (3H, s), 0.07 (3H, s), 0.06 (3H, s), 0.05 (3H, s); δc: 172.3, 94.6, 73.3, 73.0, 72.7, 71.3, 61.6, 52.4, 39.0, 31.9, 29.9, 29.7, 29.5, 29.5, 29.3, 26.0, 25.9, 25.9, 25.7, 25.6, 21.4, 14.1, 1.0, 1.0, 0.9, -4.3, -4.4.

Experiment 32:

3-(*tert*-Butyl-dimethyl-silanyloxy)-2-tetradecyl-octadecanoic acid 6-(6-hydroxymethyl-3,4,5-tris-trimethylsilanyloxytetrahydropyran-2-yloxy)-3,4,5-tris-trimethylsilanyloxytetrahydropyran-2-ylmethyl ester 104 and 3-(*tert*-Butyl-dimethyl-silanyloxy)-2-tetradecyl-octadecanoic acid 6-{6-[3-(*tert*-butyl-dimethyl-silanyloxy)-2-tetradecyl-octadecanoylmethyl]-3,4,5-tris-trimethylsilanyloxy-tetrahydro-pyran-2-yloxy)-3,4,5-tris-trimethylsilanyloxytetrahydropyran-2-ylmethyl ester 105



104



105

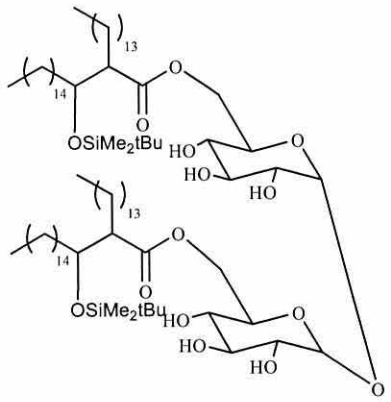
To **152** (0.2 g, 0.32 mmol, 2 mol .eq) was added **60** (0.127 g, 0.164 mmol, 1 mol .eq), DMAP (0.1 g, 0.82 mmol, 5 mol .eq), EDCI (0.188 g, 0.98 mmol, 6 mol .eq) and finally dried molecular sieves (0.2 g). To this dry dichloromethane (4 ml) was added and the mixture was stirred for 60 hrs at r.t under N_2. TLC was checked to show reaction ending. The reaction was worked up by adding water (10 ml) and ether (10 ml). The resulting mixture was extracted with ether (2x 10 ml) and the combined organic layers were dried and evaporated to give a crude product which was purified by column chromatography eluting with petrol/ether 20:1 to give a first fraction of oily **104** (0.061 g, 25 %);[164] {Found $[M+Na]^+$: 1546.55; $C_{106}H_{222}O_{15}Si_8Na$ requires: 1984.6039}, δ_H (500 MHz, $CDCl_3$): 4.86-4.85 (1H, d, *J* 3.15 Hz), 4.84-4.83 (1H, d, *J* 3.15 Hz), 4.67-4.65 (1H, br, dd, *J* 2.2, 11.8 Hz), 4.53-4.50 (1H, m), 4.01-3.93 (4H, m), 3.89-3.86 (4H, m), 3.56-3.49 (2H, m), 3.39-3.33 (2H, dt, *J* 2.5, 9.45 Hz), 2.56-2.49 (2H, m), 1.61 (2H, m), 1.48-1.47 (4H,

m), 1.31-1.24 (98H, m), 0.90 (18H, s), 0.89-0.86 (12H, t, *J* 7.25 Hz), 0.17 (18H, s), 0.15 (18H, s), 0.13 (18H, s), 0.07 (3H, s), 0.06 (3H, s), 0.05 (3H, s), 0.04 (3H, s); δ_C: 178.7, 75.5, 75.1, 73.4, 65.9, 64.9, 50.9, 44.0, 43.8, 35.7, 34.7, 34.0, 33.5, 31.9, 29.7, 29.7, 29.6, 29.6, 29.6, 29.5. 29.4, 29.4, 28.6, 27.9, 27.4, 26.4, 25.8, 25.8, 25.1, 24.4, 22.7, 18, 1, -4.4, -4.6

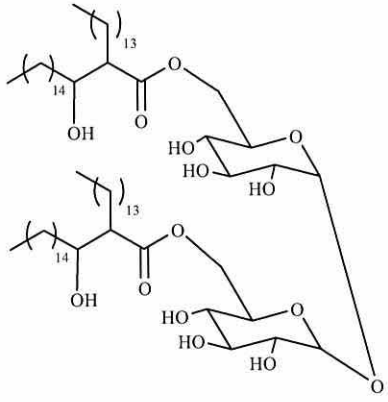
This was followed by oily **105** (0.084, 40 %), {Found $[M+Na]^+$: 1390.22; $C_{68}H_{146}O_{13}Si_7Na$ requires: 1391.49}, δ_H (500 MHz, $CDCl_3$): 4.94 (1H, d, *J* 3.15 Hz), 4.90 (1H, d, *J* 3.15 Hz), 4.63-4.60 (1H, dd, *J* 2.2, 11.7 Hz), 4.48-4.45 (1H, dd *J* 2.2, 11.9 Hz), 4.01-3.93 (2H, m), 3.90-3.83 (4H, m), 3.56-3.46 (2H, m), 3.39-3.33 (2H, m), 2.56-2.49 (2H, m), 1.61 (2H, m), 1.48-1.47 (4H, m), 1.31-1.24 (49H, m), 0.90 (9H, s), 0.89-0.86 (6H, t, *J* 7.25 Hz), 0.17 (18H, s), 0.15 (18H, s), 0.13 (18H, s), 0.05 (3H, s), 0.04 (3H, s), δ_C: 177.0, 94.5, 75.3, 73.5, 72.9, 72.8, 50.9, 31.9, 29.7, 29.7, 29.6, 29.5, 29.4, 29.4, 27.8, 25.9, 25.8, 22.7, 18.0, 14.1, 1.1, 1.0, 1.0, 0.8, 0.2, 0.1, 0.0, -4.3, -4.4, -4.5, -4.9.

Experiment 33:

Attempted preparation of 3-Hydroxy-2-tetradecyl-octadecanoic acid 3,4,5-trihydroxy-6-[3,4,5-trihydroxy-6-(3-hydroxy-2-tetradecyloctadecanoyloxymethyl)-tetrahydropyran-2-yloxy]-tetrahydropyran-2-ylmethyl ester 31



106



31

(a) Compound **104** (0.28 g, 0.14 mmol) in dry THF (3 ml) was dissolved in TBAF (1.74 ml, 1.5 mol .eq). The reaction mixture was stirred for 90 minutes at r.t. TLC $CHCl_3$/MeOH 85:15 was checked to show complete reaction. The sample was then evaporated and then extracted with $CHCl_3/H_2O$. Purification by column chromatography eluting with $CHCl_3$/MeOH 85:15 gave the resulting solid **106** (0.08, 36 %), mp (147-148 °C); (Lit.[164] 150-151 °C); which showed δ_H (500 MHz, $CDCl_3$): 5.09 (2H, d, *J* 3.15 Hz), 4.67-4.65 (1H, br, dd, *J* 2.2, 11.8 Hz), 4.40-4.36 (2H, m), 4.30-4.23 (2H, m), 3.89-3.86 (4H, m), 3.56-3.49 (2H, m), 3.39-3.33 (2H, dt, *J* 2.5, 9.45 Hz), 2.56-2.49 (2H, m), 1.61 (2H, m), 1.48-1.47 (4H, m), 1.31-1.24 (98H, m), 0.90 (18H, s), 0.89-0.86 (12H, t, *J* 7.25 Hz), 0.17 (18H, s), 0.15 (18H, s), 0.13 (18H, s), 0.07 (3H, s), 0.06 (3H, s), 0.05 (3H, s), 0.04 (3H, s); δ_C: 178.7, 75.5, 75.1, 73.4, 65.9, 64.9, 50.9, 44.0, 43.8, 35.7, 34.7, 34.0, 33.5, 31.9, 29.7, 29.7, 29.6, 29.6, 29.6, 29.5, 29.4, 29.4, 28.6, 27.9, 27.4, 26.4, 25.8, 25.8, 25.1, 24.4, 22.7, 18, 1, -4.4, -4.6.

This was taken to the next step without further purifications. Compound **106** (0.053 g, 0.034 mmol) was dissolved in TBAF (100 µl, 1.5 mol .eq) and trifluoroacetic acid (TFA) (10 µl, 2 mol.eq.). The mixture was then stirred for 2 hrs at r.t. The reaction was worked

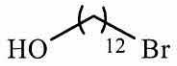
up by extracting it with $CHCl_3$/MeOH (30 ml), dried and evaporated to give starting material **106** (0.032, 60 %).

(b) A dry polyethylene vial with acid proof rubber septum was charged with **106** (0.05 g, 0.03 mmol) and pyridine (50 µl) in dry THF (0.7 ml) and stirred at r.t under argon. To it it was added HF-pyridine complex as 70 % hydrogen fluoride (100 µl) at 10 °C. The mixture was stirred for 2.5 hrs at r.t and then worked up by pouring slowly into sat. aq sodium bicarbonate until no more CO_2 was released. The product was extracted with $CHCl_3$ (3x 15 ml) then the combined organic layers were dried and evaporated to give a residue which was purified by chromatography eluting with $CHCl_3$/MeOH 85:15 to give starting material **106** (0.035, 70 %).

(c) Compound **106** (0.06 g, 0.03 mmol) was stirred in a round bottomed flask to which TBAF (0.37 ml, 1.5 mol .eq) and TFA (37 µl, 12 mol .eq) in THF (1 ml) were added. The mixture was stirred for 1.5 hrs at r.t and then it was extracted with $CHCl_3$/MeOH (15 ml), dried, evaporated and purified by column chromatography eluting with $CHCl_3$/MeOH 85:15 to give starting material **106** (0.025, 42 %).

Experiment 34:

12-Bromododecan-1-ol 109



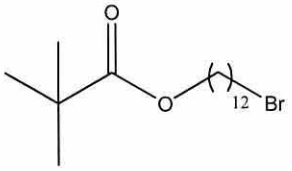
109

HBr (48 %, 10 ml) was added to a stirred solution of 1, 12-dodecanediol (7.15 g, 35.3 mmol) in toluene (80 ml). The mixture was refluxed for 18 hrs. To check the end of the reaction a sample of the reaction mixture was taken (0.5 ml); toluene was evaporated then added to a saturated $NaHCO_3$ solution and extracted with dichloromethane (10 ml). The organic layer was dried and evaporated, and then GC was checked to show complete reaction.

The rest of the mixture was cooled to r.t and the toluene was removed by simple distillation at atmospheric pressure. Sat.aq. $NaHCO_3$ (60 ml) was added and the mixture extracted with dichloromethane (3 x 50 ml).[242] The combined organic layers were dried and evaporated; the product was purified by column chromatography on silica eluting with petrol/ ether 5:1, then 1:1 to give **109** (4 g, 44 %) as a white solid; δ_H (500 MHz, $CDCl_3$): 4.78 (1H, s), 3.64 (2H, t, *J* 6.6 Hz), 3.41 (2H, t, *J* 6.95 Hz), 1.86 (2H, pent, *J* 6.95 Hz), 1.57 (2H, pent, *J* 6.95 Hz), 1.28 (16H, m); δ_C: 63.3, 34.3, 33.1, 33.0, 29.8, 29.7, 29.7, 29,6, 29.0, 28.4, 26.0, 25.9; ν_{max}: 3326, 2921, 2850, 1465, 1053, 1028, 645, 476, 455, 444, 430, 417, 411 cm^{-1}.

Experiment 35:

12-Bromododecyl pivalate 110

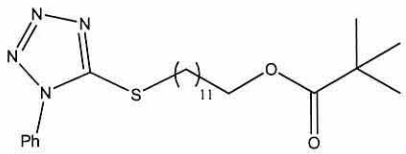


110

A solution of trimethylacetyl chloride (4.91 g, 40.75 mmol, 1.2 mol eq.) in dichloromethane (18 ml) was added to a stirred solution of **109** (9.0 g, 34 mmol), pyridine (5.37 ml, 68 mmol, 2 mol eq.) and 4-dimethylaminopyridine (0.16 g, 1.4 mmol, 0.04 mol eq.) in dichloromethane (60 ml) over 15 minutes at 5 °C. The mixture was stirred at r.t for 18 hrs. Dil. hydrochloric acid (100 ml, 5 %) was added and the organic layer separated and washed with dil. hydrochloric acid (1 x 70 ml) and then with brine (2 x 130 ml), dried and evaporated. The crude product was dissolved in petrol (100 ml) and filtered through a pad of silica with petrol (30 ml).[243] The silica pad was then washed with petrol/ether (1:1, 100 ml) and the solvent was evaporated to give **110** as colourless oil (10.6 g, 89 %); δ_H (500 MHz, $CDCl_3$): 4.04 (2H, t, *J* 6.6 Hz), 3.40 (2H, t, *J* 6.6 Hz), 1.84 (2H, pent, *J* 7 Hz), 1.60 (2H, pent, *J* 6.9 Hz), 1.41 (2H, pent, *J* 6.9 Hz), 1.27 (14H, m), 1.19 (9H, s); δ_C: 178.6, 64.4, 38.7, 34.0, 32.8, 29.4, 29.3, 29.2, 28.7, 28.6, 28.1, 27.2, 25.8; ν_{max}: 2938, 2855, 1728, 1480, 1284, 1157, 456, 450, 443, 437, 405 cm^{-1}.

Experiment 36:

12-(1-Phenyl-1H-tetrazol-5-ylthio)dodecyl pivalate 111

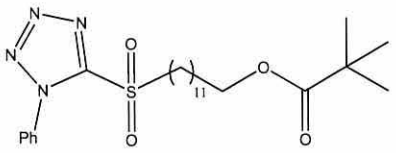


111

Compound **110** (10 g, 28.65 mmol) was added to a stirred solution of 1-phenyl-1H-tetrazole-5-thiol (5.57 g, 31.25 mmol, 1.10 mol eq.) and anhydrous potassium carbonate (8.81 g, 63.74 mmol, 2.2 mol eq.) in acetone (160 ml). The mixture was stirred for 18 hrs at r.t. TLC in petrol/ether 1:1 showed reaction completion, so the mixture was added to water (1 L) and extracted with dichloromethane (1 x 200 ml, 2 x 50 ml). The combined organic layers were washed with brine (2 x 200 ml), dried and the solvent evaporated to give a colourless oil, **111** (11 g, 86 %) {Found $[M+H]^+$: 447.6582; $C_{24}H_{39}N_4O_2S$ requires: 447.6578}; δ_H (500 MHz, $CDCl_3$): 7.58 (5H, m), 4.04 (2H, t, *J* 6.6 Hz), 3.40 (2H, t, *J* 7.55 Hz), 1.82 (2H, pent, *J* 7.25 Hz), 1.62 (2H, pent, *J* 6.95 Hz), 1.44 (2H, pent, *J* 7.6 Hz), 1.27 (14H, m), 1.19 (9H, s); δ_C : 178.6, 154.5, 133.7, 129.7, 123.8, 64.4, 38.7, 33.3, 29.4, 29.3, 29.1, 29.0, 28.9, 28.6, 28.5, 27.2, 25.9; ν_{max}: 2927, 2855, 2361, 1727, 1598, 1500, 1480, 1462, 1387, 1284, 1242, 1158, 1074, 1052, 1015, 761, 694, 551, 461, 435, 425, 418 cm^{-1}.

Experiment 37:

12-(1-Phenyl-1H-tetrazol-5-ylsulfonyl)dodecyl pivalate 112

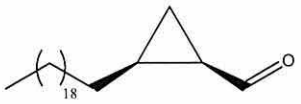


112

A solution of ammonium molybdate (VI) tetrahydrate (14.35 g, 11.61 mmol) in ice cold H_2O_2 35% (w/w, 40 ml) was added to a stirred solution of **111** (11 g, 24.6 mmol, 2.12 mol eq.) in THF (140 ml) and IMS (280 ml) at 10 °C and then stirred at r.t for 2 hrs. A further solution of ammonium molybdate (VI) tetrahydrate (7.5 g, 60.55 mmol) in ice cold H_2O_2 35 % (w/w, 20 ml) was added and the mixture that was then stirred for a further 18 hrs at r.t. The mixture was poured into water (1.2 L) and extracted with dichloromethane (1 x 200 ml, 3 x 40 ml). The combined organic layers were washed with water (1 x 500 ml), dried and the solvent evaporated to give a white solid which was purified by column chromatography on silica eluting with petrol/ether 3:1 to give **112** (8.55 g, 73 %) {Found $[M+H]^+$: 449.6574; $C_{24}H_{39}N_4O_4S$ requires: 449.6564}; δ_H (500 MHz, $CDCl_3$): 7.67 (2H, m), 7.58 (3H, m), 4.03 (2H, t, *J* 6.6 Hz), 3.71 (2H, distorted t, *J* 7.85 Hz), 1.93 (2H, m), 1.60 (2H, m), 1.48 (2H, m), 1.31 (2H, m), 1.26 (12, m), 1.18 (9H, s); δ_C: 178.5, 153.4, 133.0, 131.3, 129.6, 125.0, 64.3, 55.9, 38.6, 29.3, 29.3, 29.3, 29.1, 29.0, 28.8, 28.5, 28.0, 27.1, 25.8, 21.8; ν_{max}: 2927, 2855, 1726, 1498, 1462, 1342, 1286, 1154, 763, 689, 629, 519, 468, 458, 445, 420, 408 cm^{-1}.

Experiment 38:

(1*R*,2*S*)-2-Eicosylcyclopropanecarbaldehyde 114



114

((1*R*,2*S*)-2-Eicosylcyclopropyl)methanol[220] **113** (6.0 g, 17 mmol) in dichloromethane (80 ml) was added to a stirred suspension of pyridinium chlorochromate (9.2 g, 42 mmol, 2.5 mol eq.) in dichloromethane (200 ml) at r.t. The mixture was stirred for 2 hrs at r.t and when TLC showed no starting material was left, the reaction mixture was diluted with ether/petrol (2:1, 300 ml), then filtered through a pad of silica and celite, washed well with ether (2x 100 ml) and evaporated to give a white solid which was purified by column chromatography on silica eluting with petrol/ether 2:1 to give **114** (5.1 g, 86 %); $[\alpha]_D^{28}$: +3.76 °, (c = 1.1, $CHCl_3$), δ_H (500 MHz, $CDCl_3$): 9.37 (1H, d, *J* 5.4 Hz), 1.87 (1H, m), 1.60 (1H, m), 1.50 (1H, m), 1.27 (38H, m), 1.19 (1H, m), 0.88 (3H, t, *J* 6.9 Hz); δ_C: 201.8, 31.9, 30.0, 29.7, 29.6, 29.6, 29.5, 29.3, 29.3, 29.2, 28.2, 27.8, 26.9, 24.8, 23.0, 22.7, 17.7, 14.7, 14.3, 14.1; ν_{max}: 2921, 2851, 1700, 1465, 1215, 758, 470, 457, 441 cm^{-1}.

Experiment 39:

13-((1*R*,2*S*)-2-Eicosylcyclopropyl)tridecan-1-ol 117

117

(a) 13-((1*R*,2*S*)-2-Eicosylcyclopropyl)tridec-12-enyl pivalate 115

Lithium bis(trimethylsilyl)amide (25 ml, 27.25 mmol, 1.06 M, 1.5 mol eq. to aldehyde) was added dropwise to a stirred solution of **114** (5 g, 13.94 mmol) and **112** (8.6 g, 18 mmol, 1.3 mol eq.) in dry THF (40 ml) under nitrogen at – 10 °C. The mixture was allowed to reach r.t and then stirred for 3 hrs. When TLC showed no starting material was left, the mixture was quenched with a sat.aq. ammonium chloride (25 ml) and ether (75 ml). The organic layer was separated and the organic re-extracted with ether (2x 75 ml). The combined organic layers were dried and evaporated to give a solid which was purified by column chromatography on silica eluting with petroleum ether/diethyl ether 10:0.6 to give **115** (7.28 g, 85 %)ref which was used for the next step.

(b) 13-((1*R*,2*S*)-2-Eicosylcyclopropyl)tridec-12-en-1-ol 116

$LiAlH_4$ (0.2 g) was added to stirred THF (100 ml) at 0 °C under nitrogen to check THF dryness. Then further $LiAlH_4$ (1 g, 18.1 mmol, 1.5 mol eq.) was added. A solution of **115** (7.28 g, 12.07 mmol) in dry THF (20 ml) was added dropwise by syringe at 0 °C and the mixture was refluxed for 1 hr. When TLC showed no starting material was left, the mixture was cooled down to 0 °C and quenched with sat.aq. sodium sulphate decahydrate (20 ml) which was added dropwise with stirring at r.t until it became a solid (30 min). THF (40 ml) was added and the mixture was filtered through a pad of silica, dried and the solvent evaporated to give **116** (5.38 g, 86 %), which was used for the next step without further purification.

(c) 13-((1*R*,2*S*)-2-Eicosylcyclopropyl)tridecan-1-ol 117

Sodium (meta) periodate (37.25 g, 174 mmol) in hot water (90 ml) was added over 70 minutes at 70-80 °C, to a stirred solution of **116** (5.38 g, 10.4 mmol) in isopropyl alcohol (250 ml), acetic acid (2.5 ml), sat.aq. copper sulphate (2.5 ml) and hydrazine hydrate (0.78 mol, 25 ml). The mixture was stirred for 2 hrs until it reached r.t, then diluted with

water (400 ml) and petrol/ether (5:1, 500 ml). Due to the low solubility of the product, the mixture was warmed (40 °C) to allow separation. The aqueous layer was re-extracted with warm petrol/ether (5:1, 3x 150 ml). The combined organic layers were dried and evaporated, to give a solid, which was recrystallized from petrol/ether 1:1 to give **117** (4.6 g, 85 %); $[\alpha]_D^{32}$: -0.33 °, (c = 2.03, $CHCl_3$),[220] δ_H (500 MHz, $CDCl_3$): 3.65 (2H, t, *J* 6.6 Hz), 1.58 (4H, pent, *J* 6.65 Hz), 1.26 (58H, m), 0.89 (3H, t, *J* 6.6 Hz), 0.65 (2H, m), 0.56 (1H, m), -0.32 (1H, q, *J* 5.35 Hz); δ_C: 63.1, 32.8, 32.0, 30.2, 29.7, 29.6, 29.6, 29.4, 29.3, 28.7, 25.7, 22.7, 15.8, 14.1, 10.9; ν_{max}: 3383, 2917, 2848, 1465, 1064, 723, 449, 426, 417, 412 cm^{-1}

Experiment 40:

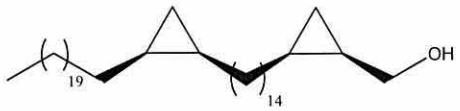
13-((1*R*,2*S*)-2-Eicosylcyclopropyl)tridecanal 118

118

Compound **117** (4.5 g, 8.6 mmol) in dichloromethane (80 ml) was added to a stirred suspension of pyridinium chlorochromate (4.66 g, 22 mmol, 2.5 mol eq.) in dichloromethane (60 ml) in portions at r.t. The mixture was stirred for 2 hrs and when TLC showed no starting material left, the mixture was diluted with petrol/ether 2:1 (300 ml) and filtered through a pad of celite on silica, then well wash with warm ether (400 ml) and the filtrate was evaporated to give a residue which was purified by column chromatography on silica eluting with petrol/ether 2:1 to give **118** (3.6 g, 84 %), m.p 61-64 °C, lit. 64-66 °C,[220] $[\alpha]_D^{25}$: +1.59 °, (c = 1.2, $CHCl_3$) δ_H (500 MHz, $CDCl_3$): 3.65 (2H, t, *J* 6.65 Hz), 1.56 (2H, q, *J* 6.6 Hz), 1.38 (6H, m), 1.26 (54H, s), 1.15 (2H, m), 0.89 (3H, t, *J* 7.25 Hz), 0.66 (2H, m), 0.57 (1H, dt, *J* 3.8, 8.2 Hz), -0.32 (1H, q, *J* 5.4 Hz); δ_C: 202.0, 63.1, 32.8, 31.9, 30.2, 29.7, 29.7, 29.7, 29.6, 29.4, 29.4, 28.7, 25.7, 22.7, 15.8; ν_{max}: 2917, 2847, 1464, 462 cm^{-1}.

Experiment 41:

((1*R*,2*S*)-2-(14-((1*R*,2*S*)-2 icosylcyclopropyl)tetradecyl)cyclopropyl)methanol 124



124

(a) ((1*R*,2*S*)-2-(15-((1*R*,2*S*)-2-icosylcyclopropyl)pentadec-2-enyl)cyclopropyl)methyl butyrate 122

Lithium bis(trimethylsilyl)amide (12.8 ml, 13 mmol, 1.06 M, 1.5 mol eq. to **118**) was added dropwise to a stirred solution of **118** (3.6 g, 6.9 mmol) and ((1*R*,2*S*)-2-((1-phenyl-1H-tetrazol-5-ylsulfonyl)methyl)cyclopropyl)methyl butyrate (3.3 g, 7.53 mmol, 1.3 mol eq. supplied by Dr. Al-Dulayymi) in dry THF (30 ml) under nitrogen at -10 to -4 °C. The solution mixture was allowed to reach r.t and stirred for 5 hrs. When TLC showed no starting material was left, the mixture was quenched with a sat.aq. ammonium chloride (50 ml) and petrol/ether (1:1, 100 ml). The organic layer was separated and the organic re-extracted with petrol/ether (1:1, 2x 75 ml). The combined organic layers were dried and evaporated to give a solid which was purified by column chromatography on silica eluting with petrol/ether 10:1 to give **122** (3 g, 65 %).

(b)((1*R*,2*S*)-2-(15-((1*R*,2*S*)-2-Eicosylcyclopropyl)pentadec-2-enyl)cyclopropyl)methanol 123

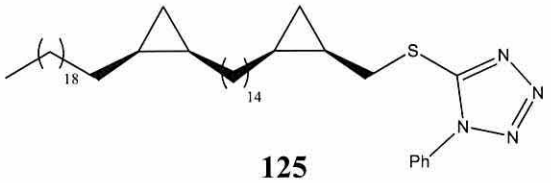
$LiAlH_4$ (0.2 g) was added to stirred THF (100 ml) at 0 °C under nitrogen to check THF dryness. Then further $LiAlH_4$ (1 g, 26 mmol) was added. A solution of **122** (3 g, 4.47 mmol, 1 mol eq.) in dry THF (20 ml) was added dropwise by syringe at 0 °C and the mixture was refluxed for 3 hrs. When TLC showed no starting material was left, the mixture was cooled to 0 °C and quenched with a sat.aq. sodium sulphate decahydrate (20 ml) which was added dropwise and stirred at r.t until it became solid (30 minutes). THF (40 ml) was added and the mixture was filtered through a pad of silica, dried and the solvent evaporated to give **123** (2.4 g, 89 %) which was used for the next step without further purification.

(c) ((1*R*,2S)-2-(14-((1R,2S)-2 icosylcyclopropyl)tetradecyl)cyclopropyl)methanol 124

Sodium (meta) periodate (20.33 g, 95 mmol) in hot water (50 ml) was added over 70 min at 70-80 °C, to a stirred solution of **123** (2.9 g, 4.8 mmol) in isopropyl alcohol (250 ml), acetic acid (1.5 ml), sat.aq. copper sulphate (1.5 ml) and hydrazine hydrate (20 ml). The mixture was stirred for 2 hrs until it reached r.t, then diluted with water (100 ml) and petrol/ether (5:1, 400 ml). Due to the low solubility of the product, the mixture was warmed up (40 °C) to allowed separation. The aqueous layer was re-extracted with warm petrol/ether (5:1, 3x 100 ml). The combined organic layers were dried and evaporated, to give a solid which was purified by column chromatography on silica eluting with petrol/ether 5:1 to give a white solid **124** (1.8 g, 63 %); {Found $[M+H]^+$: 590.0980; $C_{41}H_{81}O$ requires: 590.0978}; m.p 72-74 °C, lit. 74-76 °C; $[\alpha]_D^{28}$: + 8.33 °, (c = 0.86, $CHCl_3$), δ_H (500 MHz, $CDCl_3$): 3.66 (1H, dd, *J* , 7.25, 11.35 Hz), 3.59 (1H, dd, *J* 7.9, 11 Hz), 1.56 (4H, br, s), 1.45-1.20 (61H, br, m), 1.17-1.05 (4H, m), 0.9-0.84 (5H, including a triplet resonated at δ 0.88 with *J* 6.95 Hz), 0.70 (1H, dt, *J* 4.4, 8.2 Hz), 0.66 (2H, m), 0.57 (1H, dt, *J* 4.1, 8.5 Hz), -0.03 (1H, q, *J* 5.35 Hz), -0.32 (1H, q, *J* 5.35 Hz); δ_C (500 MHz, $CDCl_3$): 63.4, 31.9, 30.2, 30.2, 29.7, 29.6, 29.6, 29.3, 28.7, 28.6, 22.7, 18.2, 16.2, 15.8, 14.1, 10.9, 9.5; ν_{max}: 3375, 2852, 1771, 1464, 1370, 1170, 1064, 1037, 964, 932, 823 cm^{-1}.

Experiment 42:

5-(((1*R*,2*S*)-2-(14-((1*R*,2*S*)-2-Eicosylcyclopropyl)tetradecyl)cyclopropyl)methylthio)-1-phenyl-1H-tetrazole 125

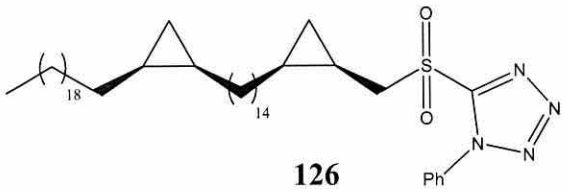


125

Diethyl azodicarboxylate (0.82 g, 4 mmol, 1.4 mol eq.) in THF (5 ml) was added to a stirred solution of **124** (1.7 g, 2.9 mmol), triphenylphosphine (1.14 g, 4.3 mmol, 1.5 mol eq.) and 1-phenyl-1H-tetrazole-5-thiol (0.82 g, 4.6 mmol, 1.6 mol eq.) in THF (50 ml) at 0-4 °C. The mixture was evaporated to a small volume to which petrol/ether (1:1, 100 ml) was added and then was stirred at r.t for 45 min. TLC was checked to showed no starting material was left, then the mixture was evaporated and the triphenylphosphine oxide formed during reaction was separated out as a white solid by filtration. The filtrate was dried and evaporated to give a solid which was purified by column chromatography on silica eluting with petrol/ether 10:2 to give **125** (2 g, 93 %), $[\alpha]_D^{32}$: + 3.15 °, (c = 1.3, $CHCl_3$); δ_H (500 MHz, $CDCl_3$): 7.60 (2H, m), 7.58 (3H, m), 3.49 (2H, d, *J* 7.9 Hz), 1.56 (3H, br s), 1.49 (1H, m), 1.38-1.13 (65H, br m), 0.95 (1H, m), 0.88 (3H, t, *J* 7.25 Hz), 0.83 (1H, dt, *J* 5.05, 8.5 Hz), 0.66 (2H, m), 0.56 (1H, dt, *J* 4.1 Hz, *J* 8.2 Hz), 0.08 (1H, q, *J* 5.35 Hz), -0.32 (1H, q, *J* 5.35 Hz); δ_C: 154.6, 133.8, 132.0, 130.0, 129.7, 128.5, 128.4, 123.8, 35.0, 31.9, 30.2, 30.0, 29.7, 29.6, 29.5, 28.7, 28.4, 22.7, 17.9, 15.8, 14.6, 14.1, 12.5, 10.9; ν_{max}: 3068, 2988, 2916, 2849, 1599, 1502, 1469, 1381, 1234, 1016, 824, 754, 694, 542, 458, 451, 435 cm^{-1}.

Experiment 43:

5-(((1*R*,2*S*)-2-(14-((1*R*,2*S*)-2-Eicosylcyclopropyl)tetradecyl)cyclopropyl)methyl-sulfonyl)-1-phenyl-1H-tetrazole 126

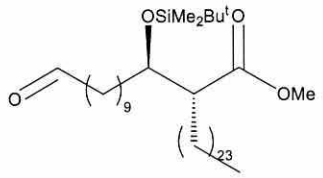


126

Compound **125** (2 g, 2.7 mmol) was dissolved in CH_2Cl_2 (100 ml) and $NaHCO_3$ (1.03 g, 12 mmol, 4.5 eq) was added at r.t. A solution of meta-chloroperbenzoic acid 70 % (1.67 g, 9.67 mmol, 2.5 eq) in CH_2Cl_2 (50 ml) previously dried with $MgSO_4$ and cooled to 5 °C was added to the previous mixture. It was then stirred for 48 hrs at r.t and when TLC petrol/ether 10:2 showed no starting material left, the mixture was quenched by adding 80 ml of NaOH aq (5 %, 4 g, 0.1 mol). Extra CH_2Cl_2 (30 ml) was added and the solution was stirred for 1 hr. It was then extracted with warm CH_2Cl_2 (1 x 150 ml, 2 x 100 ml), the combined organic layers were dried, evaporated to give a crude product which was crystallized from acetone/methanol 1:1 to give the product **126** (1.77 g, 85 %); {Found $[M+H]^+$: 782.2980; $C_{48}H_{85}N_4O_2S$ requires: 782.2976}; $[\alpha]_D^{20}$: - 18.38 °, (c = 1.5, $CHCl_3$); δ_H (500 MHz, $CDCl_3$): 7.58-7.71 (5H, m), 3.98 (1H, dd, *J* 5.35, 14.5 Hz), 3.57 (1H, dd, *J* 9.15, 14.5 Hz), 1.57 (2H, br, s), 1.5- 1.1 (65H, m), 0.95 (1H, m), 0.90-0.86 (4H, including a triplet resonated at δ 0.89 with *J* 6.95 Hz), 0.66 (2H, m), 0.56 (1H, dt, *J* 4.1, 8.2 Hz), 0.25 (1H, q, *J* 5.7 Hz), -0.32 (1H, q, *J* 5.35 Hz); δ_C: 153.7, 133.1, 131.4, 129.6, 125.2, 57.1, 31.9, 30.2, 29.6, 29.6, 29.4, 29.3, 29.1, 28.7, 22.7, 15.9, 15.8, 14.1, 11.3, 10.9, 8.0; ν_{max}: 2990, 2916, 2849, 1498, 1470, 1340, 1152, 761, 718, 687 cm^{-1}.

Experiment 44:

(*R*)-2-[(*R*)-1-(*tert*-Butyl-dimethylsilanyloxy)-11-oxoundecyl]hexacosanoic acid methyl ester 128

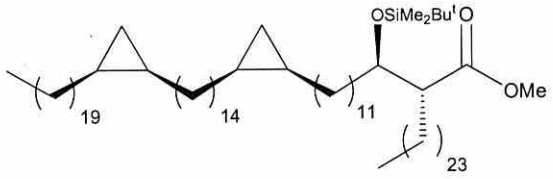


128

(*R*)-2-[(*R*)-1-(*tert*-Butyl-dimethyl-silanyloxy)-11-hydroxy-undecyl]-hexacosanoic acid methyl ester **127** (1.88 g, 2.64 mmol, provided by Dr. Al-Dulayymi) was dissolved in 25 ml CH_2Cl_2 and added to a solution of PCC (1.71 g, 7.93 mmol, 3 eq) dissolved in CH_2Cl_2 (75 ml).[226] The mixture was stirred for 3 hrs at r.t. TLC petrol/ether 5:1 was checked and the mixture was diluted with ether (100 ml), then filtered through a bed of silica, evaporated under 40 °C and purified by column chromatography eluted with petrol/ether 5:1 to give the product **128** as an oil (1.87 g, 97 %); {Found $[M+Na]^+$: 731.63; $C_{44}H_{88}O_4SiNa$ requires: 731.63}; $[\alpha]_D^{28}$: -4.50 °, (c =1.40, $CHCl_3$), which showed δ_H (500 MHz, $CDCl_3$): 9.76 (1H, br, t, 1.8 Hz), 3.90 (1H, m), 3.65 (3H, s), 2.52 (2H, m), 2.43-2.40 (2H, dt, *J* 1.55, 7.25 Hz), 2.34 (2H, t, *J* 7.6 Hz), 1.45-1.25 (60H, m), 0.86 (3H, t, *J* 7 Hz), 0.85 (9H, s), 0.04 (3H, s), 0.02 (3H, s); δ_C: 202.9, 175.1, 73.2, 51.6, 51.2, 43.9, 33.9, 31.9, 29.8, 29.7, 29.6, 29.5, 29.4, 29.3, 29.3, 29.2, 29.1, 27.8, 27.5, 25.7, 24.7, 23.7, 22.7, 22.0, 17.9, 14.1, -4.4, -4.9; ν_{max}/cm^{-1}: 2924, 2855, 1740, 1460.

Experiment 45:

(*R*)-2-((*R*)-1-(*tert*-Butyldimethylsilanyloxy)-12-{(1*R*,2*S*)-2-[14-((1*R*,2*S*)-2-eicosyl-cyclopropyl)-tetradecyl]-cyclopropyl}-dodecyl)-hexacosanoic acid methyl ester 130



130

(a) (*R*)-2-((*E*)-(*R*)-1-(*tert*-Butyldimethylsilanyloxy)-12-{(1*R*,2*S*)-2-[14-((1*R*,2*S*)-2-eicosyl-cyclopropyl)-tetradecyl]-cyclopropyl}-dodec-11-enyl)-hexacosanoic acid methyl ester 129

Compound **126** (1.77 g, 2.27 mmol) was dissolved in dry THF (20 ml) and a solution of **128** (1.73 g, 2.49 mmol, 1.1 mol eq.) in dry THF (20 ml) was added at r.t. This solution was cooled to -12 °C and lithium bis(trimethylsilyl) amide (2.78 ml, 2.94 mmol, 1.3 mol eq., 1.06 M) was added between -12/-4 °C. The solution was allowed to reach r.t and then stirred for 2 hrs. TLC showed complete reaction. Petrol/ether (10:1, 100 ml) and sat. aq. NH_4Cl (50 ml) were added. The organic layer was separated and the water layer was re-extracted with petrol/ether (10:1, 2x 100 ml). The combined organic layers were dried and the solvent was evaporated. The crude product was purified by column chromatography eluting with petrol/ether 20:1 to give the product **129** (2.5 g, 87 %) as an *E/Z*-mixture.

(b) Potassium azodicarboxylate

Azodicarbonamide (15 g, 0.13 mol) was added in small portions to a stirred solution of potassium hydroxide (29.7 g, 0.53 mol, 4.13 mol eq) in distilled water (30 ml at 0-5 °C. After addition was completed the reaction was left stirring for a further 1.5 hrs, when the solution turned thick. A thick slurry of di-potassium salt forms towards the end of the reaction. The precipitate was filtered through a sinter funnel and washed with cold methanol (100 ml). The precipitate was the dissolved in water (40 ml) at 18 °C and the solution was filtered into cold IMS (60 ml) to give a yellow precipitate. The precipitate was filtered through a sinter funnel which was washed with cold methanol (20 ml) and

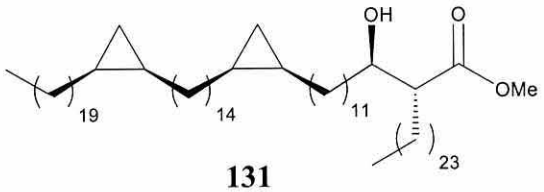
cold petroleum ether (50 ml). The dry solid **potassium azodicarboxilate** (6.10 g, 49 %) was transferred into a cold flask under nitrogen and kept in the freezer.

(c) (*R*)-2-((*R*)-1-(*tert*-Butyl-dimethyl-silanyloxy)-12-{(1*R*,2*S*)-2-[14-((1*R*,2*S*)-2-eicosyl-cyclopropyl)-tetradecyl]-cyclopropyl}-dodecyl)-hexacosanoic acid methyl ester

Compound **129** (2.4 g, 1.9 mmol) was added to a stirred solution of THF (20 ml) and methanol (25 ml) and then cooled to 0 °C. Potasium azodicarboxilate (3 g, 0.015 mol) was added, then acetic acid (5 ml) in THF (5 ml) was added dropwise at a rate of 1 ml/15 min. The reaction turned bright yellow and was left stirring for 9 hrs at r.t. The addition procedure was repeated as described above with further compound potassium azodicarboxilate, acetic acid and the solution was stirred for a further 9 hrs. The mixture was then quenched by adding it in small portions to a saturated aqueous solution of $NaHCO_3$ (50 ml). After extraction, the crude product was purified by column chromatography eluting with petrol/ether 20:1 to give **130** (2.07 g, 86 %); $[\alpha]_D^{28}$: +3.79 °, (c = 1.40, $CHCl_3$), δ_H (500 MHz, $CDCl_3$): 3.92 (1H, m), 3.66 (3H, s), 2.54 (1H, m), 1.38-1.15 (118H, m), 0.9-0.83 (31H, m, including a 9H, singlet resonated at δ 0.87 and a 6H, triplet resonated at δ 0.86 with *J* 6.7 Hz), 0.65 (4H, m), 0.58 (2H, dt, *J* 3.75, 7.85 Hz), 0.05 (3H, s), 0.03 (3H, s), -0.32 (2H, q, *J* 4.75 Hz); δ_C (500 MHz, $CDCl_3$): 175.1, 73.7, 73.2, 51.6, 51.2, 41.4, 36.1, 33.7, 33.7, 32.0, 30.2, 29.9, 29.7, 29.7, 29.6, 29.6, 29.5, 29.4, 28.9, 28.7, 27.8, 27.7, 27.5, 25.8, 23.7, 22.7, 22.6, 20.5, 19.4, 18.0, 15.8, 14.1, 10.9, -4.4, -4.9.

Experiment 46:

(*R*)-2-((*R*)-1-Hydroxy-12-{(1*R*,2*S*)-2-[14-((1*R*,2*S*)-2-icosyl-cyclopropyl)-tetradecyl]-cyclopropyl}-dodecyl)-hexacosanoic acid methyl ester 131

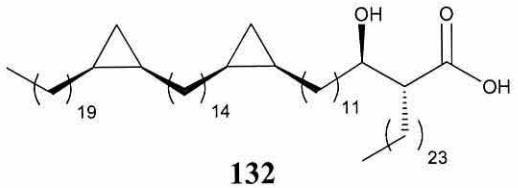


131

A dry polyethylene vial equipped with an acid proof rubber septum was charged with **130** (1.9 g, 1.53 mmol), anhydrous pyridine (2 ml) in dry THF (25 ml) and stirred at r.t under argon. To it was added hydrogen fluoride-pyridine complex as ~70% hydrogen fluoride (3 ml) at 5 °C. The mixture was then stirred at 45 °C for 17 hrs. When TLC showed no starting material was left, the mixture was diluted with petrol/ether (10:1, 100 ml) and neutralized by pouring it slowly into sat. aq. sodium bicarbonate until no more CO_2 was liberated. The product was extracted with warm petrol/ether (10:1, 2x 100 ml), and then the combined organic layers were washed with brine (100 ml). The organic layer was dried, evaporated to give a residue which was purified by chromatography eluting with petrol/ether 10:1 to give **131** (1 g, 60 %). The reaction was repeated with the unreacted starting material left, giving an overall (1.32 g, 76 %), {Found $[M+Na]^+$: 1174.99; $C_{79}H_{154}O_3Na$ requires: 1175.08} $[\alpha]_D^{32}$: + 4.29 ° (c = 0.94, $CHCl_3$); δ_H (500 MHz, $CDCl_3$): 3.71 (3H, s), 3.66 (1H, m), 2.44 (1H, dt, *J* 5.35, 9.45 Hz), 1.72-1.14 (134H, m), 0.88 (6H, t, *J* 6.95 Hz), 0.66 (4H, m), 0.57 (2H, dt, *J* 4.1, 8.55 Hz), -0.32 (2H, q, *J* 4.75 Hz); δ_C: 176.2, 72,3, 51,5, 50.9, 35.7, 31.9, 30.2, 29.7, 29.7, 29.6, 29.6, 29.6, 29.5, 29.5, 29.4, 29.4, 28.7, 27.4, 25.7, 22.7, 15.8, 14.1, 10.9. ν_{max}: 3400, 3016, 2905, 2858, 1463, 1199, 669 cm^{-1}.

Experiment 47:

(*R*)-2-((*R*)-1-Hydroxy-12-{(1*R*,2*S*)-2-[14-((1*R*,2*S*)-2-icosyl-cyclopropyl)-tetradecyl]-cyclopropyl}-dodecyl)-hexacosanoic acid 132

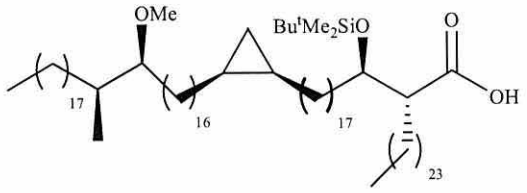


132

Compound **131** (1 g, 0.87 mmol) in THF (15 ml), water (1 ml), MeOH (1 ml) was stirred and LiOH (1.10 g, 26.21 mmol, 30 mol eq.) was added. The mixture was stirred for 12 hrs at r.t. When TLC showed no starting material was left, the mixture was dissolved in warmed petrol/ether (5:2, 100 ml) and an aqueous solution of 5 % HCl was added until water pH 1-2. The organic phase was then re-extracted (2x 100 ml) and the combined organic layers were dried, evaporated to give a crude product which was purified by column chromatography on silica gel eluting with petrol/ether 5:2 to give **132** (93 %), {Found $[M+Na]^+$: 1160.59; $C_{78}H_{152}O_3Na$ requires: 1161.06}; $[\alpha]_D^{24}$: + 2.01 °, (c = 1.2, $CHCl_3$) which showed δ_H (500 MHz, $CDCl_3$): 3.71 (1H, m), 2.44 (1H, dt, *J* 5.05, 8.8 Hz), 1.74-1.14 (134H, m), 0.88 (6H, t, *J* 6.95 Hz), 0.65 (4H, m), 0.57 (2H, dt, *J* 4.1, 8.55 Hz), -0.32 (2H, q, *J* 5.05 Hz); δ_C: 182.0, 72.2, 50.8, 35.5, 31.9, 30.2, 29.7, 29.7, 29.6, 29.5, 29.4, 29.4, 28.7, 27.3, 25.7, 22.7, 15.8, 14.1, 10.9; ν_{max}: 3427, 3019, 2916, 2848, 1467, 1215, 759, 669 cm^{-1}.

Experiment 48:

(*R*)-2-{(*R*)-1-(*tert*-Butyl-dimethylsilanyloxy)-18-[(1*R*,2*S*)-2-((17*S*,18*S*)-17-methoxy-18-methyl-hexatriacontyl)cyclopropyl]octadecyl}hexacosanoic acid 134



134

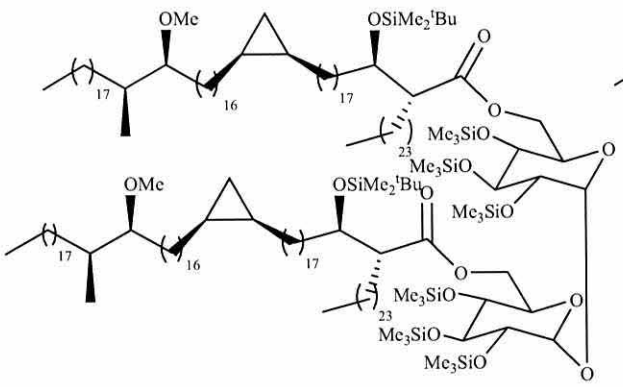
Imidazole (0.38 g, 5.6 mmol) was added to a stirred solution of (*R*)-2-{(*R*)-1-hydroxy-18-[(1*R*,2*S*)-2-((17*S*,18*S*)-17-methoxy-18-methyl-hexatriacontyl)-cyclopropyl]-octadecyl}-hexacosanoic acid **133** (0.7 g, 0.56 mmol, supplied by Dr. Al-Dulayymi) in dry DMF (3 ml) and dry toluene (4 ml) at r.t followed by the addition of *tert*-butyldimethylsilylchloride (0.84 g, 5.6 mmol) and 4-dimethylaminopyridine (0.068 g, 0.56 mmol). The reaction mixture was stirred at 70 °C for 24 hrs and at r.t for another 18 hrs. When TLC showed that no starting material was left, the solvent was removed under high vacuum and the residue was diluted with petrol/ ethyl acetate (10:1, 50 ml) and water (20 ml). The organic layer was separated and the aqueous layer was re-extracted with petrol/ethyl acetate (10:1, 2 x 30 ml). The combined organic layers were washed with water, dried and evaporated to give a colourless oil residue. The residue was dissolved in THF (15 ml), water (2 ml), and methanol (2 ml), to this was added potassium carbonate (0.30 g). The reaction mixture was stirred at 45 °C for 18 hrs, and then TLC showed no starting material was left. The mixture was diluted with petrol/ethyl acetate (10:1, 20 ml) and water (2 ml) then acidified with potassium hydrogen sulphate to pH 2. The organic layer was separated and the aqueous layer was re-extracted with petrol/ethyl acetate (2 x 20 ml). The combined organic layers were washed with water, dried and evaporated to give a residue, which was purified by column chromatography on silica eluting with petrol/ethyl acetate 10:1 to give **134** as colourless oil (0.61 g, 80 %). $[\alpha]^{24}_{D}$ = - 1.03 ° (c = 1.46, $CHCl_3$), {Found $[M+Na]^+$: 1390.3687; $C_{91}H_{182}O_4SiNa$ requires: 1390.3700}, which showed δ_H (500 MHz, $CDCl_3$): 3.85-3.8 (1H, ddd, *J* 3.15, 5, 7.55 Hz), 3.36 (3H, s), 2.96 (1H, br, pent, *J* 4.1 Hz), 2.53 (1H, ddd, *J* 3.15, 5.7, 9.15 Hz), 1.75-1.05 (148H, m), 0.93 (9H, s), 0.89 (6H, t, *J* 6.6 Hz), 0.85 (3H, d, *J* 6.9 Hz), 0.67-0.63 (2H, m),

0.57 (1H, dt, *J* 4.1, 7.9 Hz), 0.15 (3H, s), 0.13 (3H, s), -0.33 (1H, br, q, *J* 5.35 Hz); δ_C: 177.3, 85.5, 73.6, 57.7, 50.7, 35.3, 35.0, 32.4, 31.9, 30.5, 30.2, 30.0, 29.9, 29.7 (very broad), 29.7, 29.56 29.5, 29.5, 29.4, 29.4, 29.0, 28.7, 27.6, 27.53, 26.16, 25.74, 24.63, 22.69, 17.95, 15.78, 14.88, 14.1, 10.92, -4.31, -4.92; υ_{max}: 3500-2500 (very broad, OH for the carboxylic group), 2922, 2852, 1708, 1465, 1361, 1293, 1253, 1099, 1005, 939, 836 cm^{-1}.

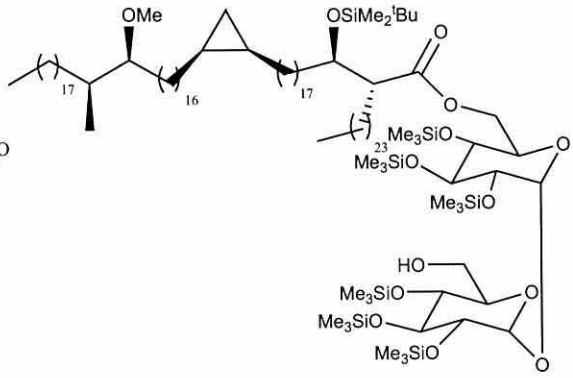
Experiment 49:

6,6'-Bis-O-(*R*)-2-{(*R*)-1-(*tert*-Butyl-dimethyl-silanyloxy)-18-[(1*R*,2*S*)-2-((17*S*,18*S*)-17-methoxy-18-methylhexatriacontyl)-cyclopropyl]-octadecyl}-hexacosanoic-2,3,4, 2',3',4',-hexakis-O-(trimethylsilyl)-α,α'-trehalose 136

6-O-(*R*)-2-{(*R*)-1-(*tert*-Butyl-dimethyl-silanyloxy)-18-[(1*R*,2*S*)-2-((17*S*,18*S*)-17-methoxy-18-methylhexatriacontyl)-cyclopropyl]-octadecyl}-hexacosanoic - 2,3,4,2',3',4'-hexakis-O-(trimethylsilyl)-α,α'-trehalose 137

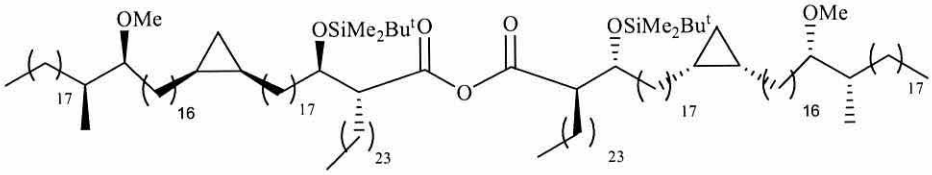


136



137

1-(3-Dimethylaminopropyl)-3-ethylcarbodimidehydrochloride (EDCI) (197.93 mg, 1.03 mmol) and 4-dimethylaminopyridine (110.4 mg, 0.903 mmol) were added to a stirred solution of **134** (441 mg, 0.323 mmol), **60** (100 mg, 0.13 mmol) and powdered 4 A° molecular sieves in dry dichloromethane (3.5 ml) at r.t under nitrogen. The mixture was stirred for 6 days at r.t until TLC showed no starting material was left. The mixture was then diluted with dichloromethane (5 ml) and filtered. The filtrate was evaporated under reduced pressure to give a residue, which was purified by column chromatography on silica eluting with petrol/ ethyl acetate 20:1 to give, in order:



135

(a) (*R*)-2-((*R*)-1-(*tert*-Butyldimethylsilyloxy)-18-((1*R*,2*S*)-2-((17*S*,18*S*)-17-methoxy-18-methylhexatriacontyl)cyclopropyl)octadecyl)hexacosanoic anhydride 135 (0.132

g, 20 %); {Found $[M+Na]^+$: 2742.10; $C_{182}H_{362}O_7Si_2Na$ requires: 2742.05}, which showed δ_H (500 MHz, $CDCl_3$): 3.98-3.95 (2H, q, *J* 6, 10.4 Hz), 3.35 (6H, s), 3.00-2.95 (2H, pent, *J* 4.1 Hz), 2.58-2.54 (2H, m), 1.75-1.05 (292H, m), 0.90 (18H, s), 0.89 (12H, t, *J* 6.6 Hz), 0.85 (6H, d, *J* 6.9 Hz), 0.67-0.63 (4H, m), 0.57 (4H, dt, *J* 4.1, 7.9 Hz), 0.07 (12H, s), -0.33 (2H, br, q, *J* 5.35 Hz); δ_C: 169.5, 85.4, 72.7, 57.7, 52.8, 35.3, 33.7, 32.4, 31.9, 30.5, 30.2, 30.0, 29.9, 29.7 (very broad), 29.5, 29.4, 29.4, 28.7, 27.9, 27.5, 26.6, 26.2, 25.9, 25.8, 24.7, 22.7, 18.0, 15.8, 14.9, 14.1, 10.9, -4.4, -4.7; υ_{max}: 2962, 2853, 1816, 1748, 1465, 1361, 1255, 1099, 908, 836, 776, 735 cm^{-1}.

This resulting anhydride (0.130 g) was coupled again with **134** (441 mg, 0.323 mmol), and **60** as described above to regenerate the acid and obtain the coupling; giving two fractions corresponding to di and mono-substituted products.

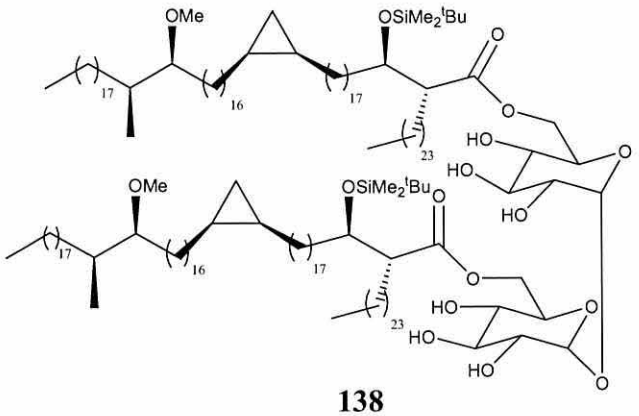
(b) 6,6'-bis-O-(*R*)-2-{(*R*)-1-(tert-butyl-dimethyl-silanyloxy)-18-[(1*R*,2*S*)-2-((17*S*,18*S*)-17-methoxy-18-methyl-hexatriacontyl)-cyclopropyl]-octadecyl}-hexacosanoic -2,3,4, 2',3',4',-hexakis-O-(trimethylsilyl)-α,α'-trehalose 136 (0.23 g, 51.28 %) as a colourless thick oil; $[\alpha]^{24}_D$ = + 19.57 (c = 1.08, $CHCl_3$), {Found $[M+Na]^+$: 3498.72; $C_{212}H_{430}O_{17}Si_8Na$ requires: 3499.36}; which showed δ_H (500 MHz, $CDCl_3$): 4.85 (2H, d, *J* 2.85 Hz), 4.37 (2H, br, d, *J* 9.75 Hz), 4.04-3.98 (2H, m), 3.96 (2H, br, pent, *J* 4.1 Hz), 3.94 (2H, br, q, *J* 5.22 Hz), 3.9 (2H, t, *J* 9.0 Hz), 3.52 (2H, t, *J* 9.0 Hz), 3.38 (2H, dd, *J* 2.8, 9 Hz), 3.35 (6H, s), 2.55 (2H, ddd, *J* 3.5, 4.75, 10.1 Hz), 1.67-1.6 (4H, m), 1.56-1.05 (290H, m), 0.89 (12H, t, *J* 7 Hz), 0.88 (18H, s), 0.85 (6H, d, *J* 7 Hz), 0.65 (4H, br, m), 0.57 (2H, dt, *J* 3.8, 8 Hz), 0.16 (18H, s), 0.145 (18H, s), 0.138 (18H, s), 0.062 (12H, s), -0.32 (2H, br, q, *J* 5.05 Hz); δ_C: 173.8, 94.9, 85.5, 73.5, 73.4, 72.8, 71.8, 70.7, 62.4, 57.7, 51.9, 35.4, 33.5, 32.4, 31.9, 30.5, 30.2, 30.0, 29.9, 29.8, 29.7 (very broad), 29.7, 29.5, 29.4, 28.7, 28.1, 27.6, 26.2, 25.8, 25.2, 22.7, 18.0, 15.8, 14.9, 14.1, 10.9, 1.1, 0.9, 0.2, -4.5, -4.6; υ_{max}: 2924, 2853, 1743, 1464.9, 1251.6, 1163, 1099, 872, 839 cm^{-1}.

(c) 6-O-(*R*)-2-{(*R*)-1-(*tert*-Butyl-dimethyl-silanyloxy)-18-[(1*R*,2*S*)-2-((17*S*,18*S*)-17-methoxy-18-methyl-hexatriacontyl)-cyclopropyl]-octadecyl}-hexacosanoic -2,3,4,2',3',4'-hexakis-O-(trimethylsilyl)-α,α'-trehalose 137 (0.114 g, 41.73 %); $[\alpha]^{24}_D$ = + 42.48 (c = 1.13, $CHCl_3$)}, {Found $[M+Na]^+$: 2146.7125; $C_{121}H_{250}O_{14}Si_7Na$ requires: 2146.7128}; which showed δ_H (500 MHz, $CDCl_3$): 4.91 (1H, d, *J* 3.15 Hz), 4.84 (1H, d, *J* 2.85 Hz), 4.35 (1H, dd, *J* 2.2, 11.7 Hz), 4.08 (1H, dd, *J* 4.1, 11.7 Hz), 3.99 (1H, br, qd, *J*

2.2, 11.7 Hz), 3.95 (1H, br, m), 3.91 (2H, dt, *J* 6.6, 9.0 Hz) 3.85 (1H, br, td, *J* 3.45, 9.45 Hz), 3.73-3.65 (2H, m), 3.48 (2H, dt, *J* 6.3, 9.0 Hz), 3.43 (1H, dd, *J* 3.15, 9.45 Hz), 3.39 (1H, dd, *J* 2.85, 9.15 Hz), 3.34 (3H, s), 2.96 (1H, br, pent, *J* 4.4 Hz), 2.55 (1H, ddd, *J* 3.5, 5.7, 10.4 Hz), 1.71 (1H, dd, *J* 5.1, 7.6 Hz), 1.64-1.58 (2H, m), 1.51-1.06 (148H, m), 0.89 (3H, t, *J* 7 Hz), 0.88 (9H, s), 0.86 (3H, d, *J* 7 Hz), 0.67-0.64 (2H, m), 0.57 (1H, dt, *J* 4.1, 8.2 Hz), 0.172 (9H, s), 0.161 (9H, s), 0.156 (9H, s), 0.151 (9H, s), 0.15 (9H, s), 0.124 (9H, s), 0.061 (3H, s), 0.057 (3H, s), -0.32 (1H, br, q, *J* 5.4 Hz); δ_C: 174.1, 94.5, 94.4, 85.4, 73.4, 73.4, 72.9, 72.8, 72.8, 72.0, 71.4, 70.7, 62.4, 61.7, 57.7, 51.8, 35.4, 33.4, 32.4, 31.9, 30.5, 30.2, 30.0, 29.9, 29.9, 29.8, 29.7, 29.7 (very broad), 29.5, 29.4, 28.7, 28.1, 27.6, 26.4, 26.2, 25.8, 24.8, 22.7, 18.0, 15.8, 14.9, 14.1, 10.8, 1.1, 1.0, 1.0, 0.9, 0.8, 0.2, 0.0, -4.4, -4.7; υ_{max}: 2924, 2853, 1742, 1465, 1251, 1215, 1100, 965 cm^{-1}.

Experiment 50:

6,6'-Bis-O-(*R*)-2-{(*R*)-1-(*tert*-Butyldimethylsilanyloxy)-18-[(1*R*,2*S*)-2-((17*S*,18*S*)-17-methoxy-18-methylhexatriacontyl)cyclopropyl]octadecyl}-hexacosanoic-α,α'-trehalose 138



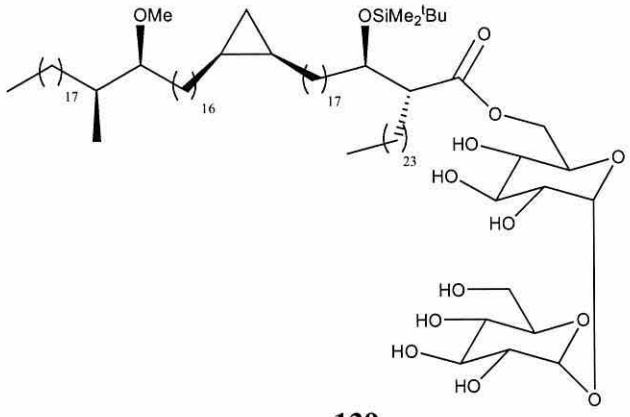
138

Tetrabutylammonium fluoride (0.336 ml, 0.336 mmol, 1M) was added to a stirred solution of **136** (0.15 g, 0.043 mmol) in dry THF (6 ml) at 5 °C under nitrogen atmosphere. The mixture was allowed to reach r.t and then it was stirred for 1 hr, until TLC showed no starting material was left. The reaction was cooled to 5 °C and quenched with sat.aq. sodium bicarbonate (3 ml) then diluted with cold $CHCl_3$ (50 ml). The organic layer was separated and the aqueous layer was re-extracted with $CHCl_3$ (2 x 50 ml). The combined organic layer were washed with brine (50 ml), dried and evaporated to give a residue, which was purified by column chromatography on silica eluting with $CHCl_3$/MeOH 10:1 to give **138** (0.102 g, 78 %) as a colourless thick oil, $[\alpha]^{26}_D = +10.79$ (c = 0.091, $CHCl_3$), {Found $[M+Na]^+$: 3065.80; $C_{194}H_{382}O_{17}Si_2Na$ requires: 3065.95}; which showed δ_H (500 MHz, $CDCl_3$ + few drops of CD_3OD): 5.05 (2H, d, *J* 3.5 Hz), 4.32 (2H, br, dd, *J* 4.4, 12.3 Hz), 4.2 (2H, br, d, *J* 10.75 Hz), 3.91 (2H, br, d, *J* 8.2 Hz), 3.87 (2H, br, q, *J* 5.65 Hz), 3.76 (2H, br, t, *J* 9.45 Hz), 3.45 (2H, dd, *J* 3.8, 9.8 Hz), 3.31-3.28 (8H, including a singlet for the methoxy groups resonated at δ 3.3), 2.93 (2H, br, pent, *J* 4.31 Hz), 2.52 (2H, ddd, *J* 3.8, 6.35, 10.4 Hz), 1.6-1.01 (300H, m), 0.84 (12H, t, *J* 7 Hz), 0.82 (18H, s), 0.81 (6H, d, *J* 7 Hz), 0.63-0.56 (4H, m), 0.52 (2H, dt, *J* 4.05, 7.85 Hz), 0.004 (6H, s), -0.02 (6H, s), -0.38 (2H, br, q, *J* 5.35 Hz); δ_C: 175.6, 93.4, 85.4, 73.3, 72.8, 71.8, 70.4, 70.3, 62.8, 57.7, 51.5, 35.2, 33.7, 32.4, 31.9, 30.5, 30.4, 30.3, 30.2, 30.1, 30.0, 29.9, 29.8, 29.7, 29.7, 29.6, 29.5, 29.5, 29.4, 29.4, 29.3, 28.7, 27.8, 27.6, 27.1, 26.2, 25.9,

25.8, 24.3, 22.7, 18.0, 15.8, 14.9, 14.1, 10.9, -4.4, -4.7; υ_{max}: 3406, 2925, 1737, 1465, 1373, 1254, 1100, 1078, 992, 939, 836, 775, 720, 665 cm^{-1}.

Experiment 51:

6-O-(*R*)-2-{(*R*)-1-(*tert*-Butyl-dimethyl-silanyloxy)-18-[(1*R*,2*S*)-2-((17*S*,18*S*)-17-methoxy-18-methyl-hexatriacontyl)-cyclopropyl]-octadecyl}-hexacosanoic-α,α'-trehalose 139

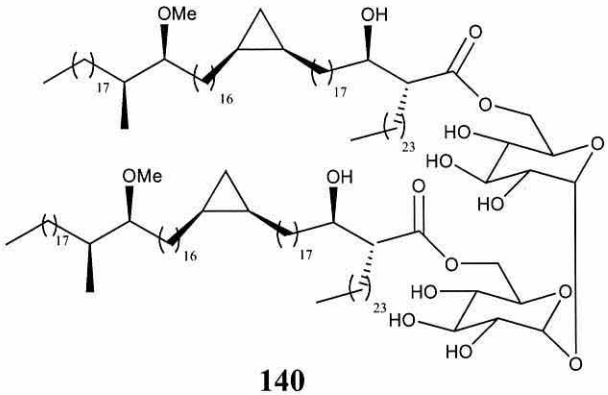


139

Tetrabutylammonium fluoride (0.558 ml, 0.558 mmol, 1M) was added to a stirred solution of **137** (0.14 g, 0.066 mmol) in dry THF (7 ml) at 5 °C under nitrogen. The mixture was allowed to reach r.t, then stirred for 1 hr until TLC showed no starting material. The mixture was worked up as above and purified by column chromatography on silica eluting with $CHCl_3$/MeOH 8.5:1.5 to give **139** (0.09 g, 80 %) as a colourless syrup, {$[\alpha]^{26}_D$ = + 28.38 (c = 0.97, $CHCl_3$)} {[Found M+Na]$^+$: 1714.47; $C_{103}H_{202}O_{14}SiNa$ requires: 1714.47} which showed δ_H (500 MHz, $CDCl_3$ + few drops of CD_3OD): 5.05 (2H, d, *J* 3.15 Hz), 4.29-4.22 (2H, m), 3.92 (1H, br, d, *J* 9.45 Hz), 3.87 (3H, br, t, *J* 9.0 Hz), 3.78 (2H, br, m), 3.66 (1H, br, d, *J* 7 Hz), 3.49 (2H, br, d, *J* 9.45 Hz), 3.34 (2H, br, d, *J* 8.5 Hz), 3.3 (3H, s), 2.93 (1H, br, pent, *J* 4.4 Hz), 2.5 (1H, m), 1.6-1.03 (154, m), 0.83 (6H, t, *J* 6.65 Hz), 0.81 (9H, s), 0.8 (3H, d, *J* 7 Hz), 0.61-0.56 (2H, m), 0.51 (1H, dt, *J* 3.8, 8.2 Hz), -0.003 (3H, s), -0.03 (3H, s), -0.38 (1H, br, q, *J* 5.05 Hz); δ_C (500 MHz, $CDCl_3$): 175.1, 93.5, 93.4, 85.5, 73.2 (broad), 72.9, 72.6, 72.1, 71.6, 70.7, 70.2, 69.9, 62.7, 62.0, 57.5, 51.6, 35.2, 33.5, 29.6, 29.6, 29.6, 29.5, 29.5, 29.2, 28.6, 27.6, 27.4, 26.9, 26.0, 25.6, 24.2, 22.6, 17.8, 15.6, 14.7, 13.9, 10.8, -4.6, -5.0; υ_{max}: 3363, 2923, 2853, 1733, 1464, 1100, 836, 760 cm^{1}.

Experiment 52:

6,6'-Bis-O-(*R*)-2-{(*R*)-1-hydroxy-18-[(1*R*,2*S*)-2-((17*S*,18*S*)-17-methoxy-18-methyl-hexatriacontyl)-cyclopropyl]-octadecyl}-hexacosanoic-α,α'-trehalose 140

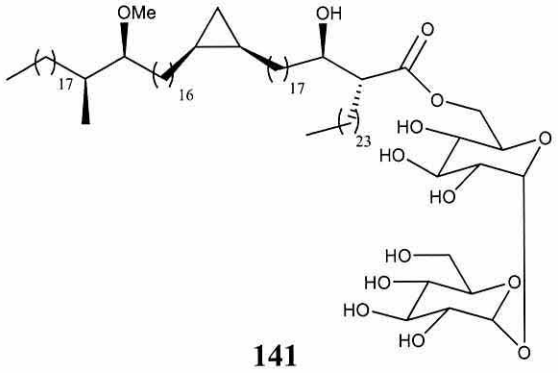


140

A dry polyethylene vial equipped with a rubber septum was charged with **138** (0.090 g, 0.029 mmol) and pyridine (100 μl) in dry THF (5 ml) was stirred at r.t under argon. Hydrogen fluoride-pyridine complex as ~70% hydrogen fluoride (0.422 ml) was then added. The mixture was stirred at 43 °C for 17 hrs, until TLC showed no starting material was left, then it was neutralized by pouring it slowly into sat. aq. sodium bicarbonate until no more CO_2 was liberated. The product was extracted with chloroform (3 x 50 ml), then the combined organic layers were dried and evaporated to give a residue which was purified by column chromatography eluting with $CHCl_3$/MeOH 10:1 to give **140** (0.06 g, 72.3 %) as a syrup, {$[\alpha]^{26}_D$ = + 31.43, (c = 0.84, $CHCl_3$)} {Found $[M+Na]^+$: 2837.6600; $C_{182}H_{354}O_{17}Na$ requires: 2837.7441} which showed δ_H (500 MHz, $CDCl_3$ + few drops of CD_3OD): 4.93 (2H, d, *J* 3.15 Hz), 4.56 (2H, br, d, *J* 11.05 Hz), 4.15 (2H, br, t, *J* 9.15 Hz), 3.93 (2H, br, q, *J* 7.25 Hz), 3.71 (2H, t, *J* 9.5 Hz), 3.58 (2H, br, m), 3.42 (2H, dd, *J* 3.2, 9.75 Hz), 3.25 (6H, s), 3.17 (2H, t, *J* 9.45 Hz), 2.89 (2H, br, pent, *J* 4.4 Hz), 2.35 (2H, m), 1.57-0.98 (302H, m), 0.78 (12H, t, *J* 7 Hz), 0.76 (6H, d, *J* 7 Hz), 0.6-0.52 (4H, m), 0.47 (2H, dt, *J* 4.1, 8.2 Hz), -0.43 (2H, br, q, *J* 5.05 Hz); δ_C: 175.4, 94.7, 85.5, 72.4, 71.1, 71.1, 69.7, 64.1, 57.4, 52.2, 35.2, 34.6, 32.2, 31.7, 30.3, 30.0, 30.0, 29.7, 29.2, 29.6, 29.5, 29.5, 29.5, 29.4, 29.3, 29.2, 29.1, 28.5, 27.3, 27.1, 25.9, 25.1, 22.5, 15.6, 14.6, 13.8, 10.7; υ_{max}: 3362, 2920, 2851, 1722, 1467, 1100, 720 cm^{-1}.

Experiment 53:

6-O-(*R*)-2-{(*R*)-1-Hydroxy-18-[(1*R*,2*S*)-2-((17*S*,18*S*)-17-methoxy-18-methyl-hexatriacontyl)-cyclopropyl]-octadecyl}-hexacosanoic-α,α'-trehalose 141

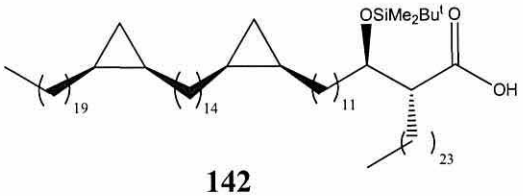


141

A dry polyethylene vial equipped with a rubber septum was charged with **139** (0.060 g, 0.035 mmol) and pyridine (0.1 ml) in dry THF (5 ml) and stirred at r.t under nitrogen. Hydrogen fluoride-pyridine complex as ~70% hydrogen fluoride (0.506 ml) was then added. The mixture was stirred at 43 °C for 17 hrs, until TLC showed no starting material was left, then it was worked up as above to give a residue which was purified by column chromatography eluting with $CHCl_3$/MeOH 10:1 then 1:1 to give **141** (0.043 g, 78 %) as a syrup, $[\alpha]^{26}_D$ = + 47.75, (c = 0.49, $CHCl_3$)} {Found $[M+Na]^+$: 1601.39; $C_{97}H_{188}O_{14}Na$ requires: 1601.39} which showed δ_H (500 MHz, $CDCl_3$ + few drops of CD_3OD): 5.07 (1H, br, s), 5.02 (1H, br, s), 4.62 (1H, br, d, *J* 10.7 Hz), 4.17 (1H, br, s), 3.99 (1H, br, m), 3.87-3.81 (4H, m), 3.64-3.61 (2H, m), 3.54 (1H, br, d, *J* 9.5 Hz), 3.48 (1H, br, d, *J* 9.75 Hz), 3.32-3.27 (4H, including a singlet (OMe) resonated at δ 3.30), 3.23 (1H, br, t, *J* 9.45 Hz), 2.93 (1H, m), 2.36 (1H, m), 1.65-1.50 (2H, m), 1.45-1.02 (153H, m), 0.83 (6H, t, *J* 7 Hz), 0.8 (3H, d, *J* 7 Hz), 0.63-0.55 (2H, m), 0.51 (1H, dt, *J* 3.75, 7.85 Hz), -0.38 (1H, br, q, *J* 5.1 Hz); δ_C: 175.5, 94.4, 85.5, 72.5 (broad), 72.4, 72.3, 71.4, 71.2, 71.0, 70.9, 70.0, 64.2, 62.1, 57.6, 52.3, 35.3, 34.6, 32.3, 31.8, 30.4, 30.1, 29.8, 29.8, 29.7, 29.6, 29.6, 29.5, 29.3, 29.2, 28.6, 27.4, 27.2, 26.0, 25.1, 22.6, 15.6, 14.7, 13.9, 10.8; υ_{max}: 3349, 2919, 2850, 1719, 1467, 992 cm^{-1}.

Experiment 54:

(*R*)-2-((*R*)-1-(*tert*-Butyldimethylsilanyloxy)-12-{(1*R*,2*S*)-2-[14-((1*R*,2*S*)-2-eicosyl-cyclopropyl)-tetradecyl]-cyclopropyl}-dodecyl)-hexacosanoic acid 142



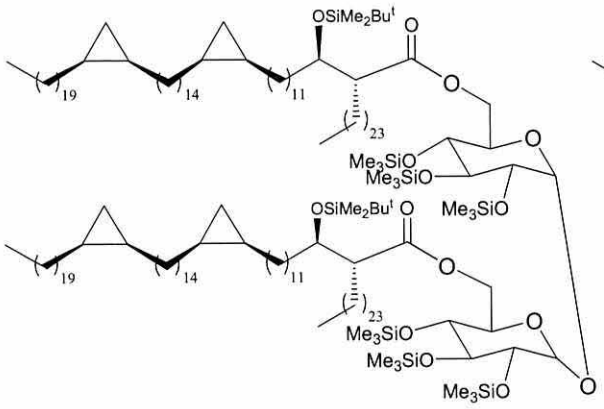
142

Imidazole (0.117 g, 1.72 mmol) was added to a stirred solution of **132** (0.39 g, 0.34 mmol) in anhydrous DMF (3 ml) and dry toluene (4 ml) at r.t followed by *tert*-butyl-dimethylsilyl chloride (0.517 g, 3.4 mmol) and 4-dimethylaminopyridine (0.042 g, 0.34 mmol). The mixture was stirred at 70 °C for 24 hrs and at r.t for another 18 hrs. The solvent was removed under high vacuum and the residue was diluted with petroleum ether/ ethyl acetate (10:2, 50 ml) and water (20 ml). The organic layer was separated and the aqueous layer was re-extracted with petroleum ether/ethyl acetate (10:2, 2 x 30 ml). The combined organic layers were washed with water, dried and evaporated to give a colourless oil. The residue was dissolved in THF (11 ml), water (1.4 ml), and methanol (1.4 ml), to this was added potassium carbonate (0.20 g). The mixture was stirred at 45 °C for 18 hrs, when TLC showed no starting material. The sample was evaporated to 1/4 of the volume and diluted with petroleum/diethyl ether (5:1, 30 ml) then acidified with potassium hydrogen sulphate to pH 2. The organic layer was separated and the aqueous layer was re-extracted with petrol/ethyl acetate (2 x 20 ml). The combined organic layers were washed with water, dried and evaporated to give a residue, which was purified by column chromatography on silica eluting with petroleum ether/ethyl acetate 10:1 to give **142** (0.28 g, 68 %), $[\alpha]^{30}_{D}$ = + 4.012 (c = 1.13 g, $CHCl_3$), {Found $[M+Na]^+$: 1275.3345; $C_{84}H_{166}O_3Si_1Na$ requires: 1275.3243} which showed δ_H (500 MHz, $CDCl_3$) : 3.85-3.88 (1H, br, q, *J* 5.35 Hz) , 2.53 (1H, br, pent, *J* 4.7 Hz), 1.75-1.10 (134H, m), 0.91 (9H, s), 0.89 (6H, t, *J* 7 Hz), 0.66-0.64 (4H, m), 0.57 (2H, dt, *J* 4.1, 8.15 Hz), 0.12 (3H, s), 0.10 (3H, s), -0.32 (2H, br, q, *J* 5.35 Hz); δ_C: 177.3, 73.6, 50.6, 35.1, 31.9, 30.2, 29.7, 29.6, 29.5, 29.5, 29.4, 29.1, 28.7, 27.5, 25.7, 24.6, 22.7, 17.9, 15.8, 14.1, 10.9, -4.3, -4.9; υ_{max}: 3500-2500 (very broad, OH for the carboxylic group), 2919, 2850, 1707, 1466, 1361, 1254, 1215, 1075, 939, 836, 761, 669, 420 cm^{-1}.

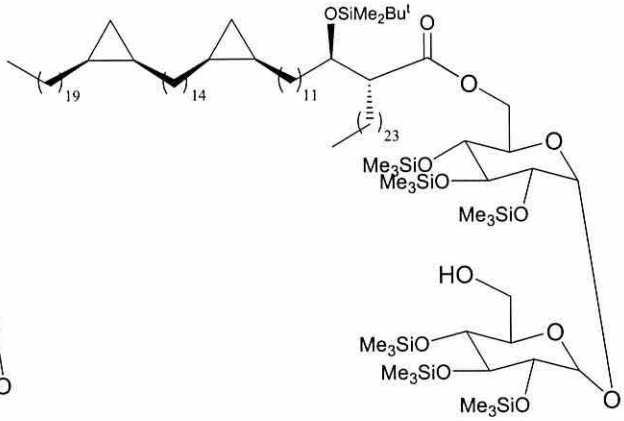
Experiment 55:

6,6'-Bis-O-(*R*)-2-((*R*)-1-(*tert*-Butyl-dimethyl-silanyloxy)-12-{(1*R*,2*S*)-2-[14-((1*R*,2*S*)-2-eicosylcyclopropyl)-tetradecyl]-cyclopropyl}-dodecyl)-hexacosanoic-2,3,4,2',3',4',-hexakis-O-(trimethylsilyl)-α,α'-trehalose 144

6-O-(*R*)-2-((*R*)-1-(*tert*-Butyl-dimethyl-silanyloxy)-12-{(1*R*,2*S*)-2-[14-((1*R*,2*S*)-2-eicosylcyclopropyl)-tetradecyl]-cyclopropyl}-dodecyl)-hexacosanoic-2,3,4,2',3',4',-hexakis-O-(trimethylsilyl)-α,α'-trehalose 145

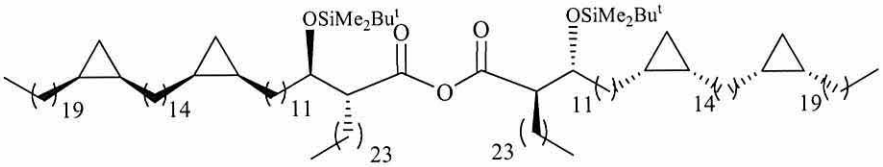


144



145

1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDCI) (125 mg, 0.65 mmol) and 4-dimethylaminopyridine (70 mg, 0.57 mmol) were added to a stirred solution of **142** (256 mg, 0.204 mmol), **60** (63 mg, 0.082 mmol) and powdered 4 A° molecular sieves in dry dichloromethane (3 ml) at r.t under nitrogen. The mixture was stirred for 6 days at r.t when TLC showed no starting material was left then diluted with dichloromethane (5 ml) and filtered. The filtrate was evaporated under reduced pressure to give a residue, which was purified by column chromatography on silica eluting with petroleum ether/ethyl acetate 20:1 to give a first fraction (**(a)** (*R*)-2-((*R*)-1-(*tert*-butyl-dimethylsilanyloxy)-12-{(1*R*,2*S*)-2-[14-((1*R*,2*S*)-2-eicosylcyclopropyl)tetradecyl]-cyclopropyl}-dodecyl)-hexacosanoic anhydride **143** (0.148 g), a second fraction **(b)** compound **144** (0.040 g, 15 %) as a colourless thick oil and a third fraction **(c)** compound **145** (0.114 g, 70 %).



143

(a) Compound **143** (0.148 g); {Found $[M+Na]^+$: 2509.55; $C_{168}H_{330}O_5Si_2Na$ requires: 2509.64} showed δ_H (500 MHz, $CDCl_3$): 3.85-3.88 (2H, br, q, *J* 5.35 Hz) , 2.62-2.52 (2H, m), 1.50-1.10 (268H, m), 0.90 (18H, s), 0.91-0.85 (12H, t, *J* 7 Hz), 0.66-0.64 (8H, m), 0.57 (4H, dt, *J* 4.1, 8.55 Hz), 0.07 (12H, s), -0.32 (4H, br, q, *J* 5.35 Hz); δ_C: 178.4, 73.6, 50.8, 35.1, 31.9, 30.3, 30.2, 29.7, 29.7, 29.5, 29.5, 29.4, 28.7, 26.0, 25.9, 25.9, 25.8, 22.7, 18.0, 15.8, 14.1, 10.9, 1.0, -4.4, -4.5.

The sample was dried and evaporated. The same procedure as described above was repeated to give a first fraction (anhydride **143**, 0.0045 g), a second fraction **(a1)** compound **144** (0.11 g, 57 %) and a third fraction **(a2)** compound **145** (0.0254 g, 21 %).

(b+a1) Compound **144** (0.15 g, 33 %), a colourless thick oil, $[\alpha]^{28}_D$ = +23.08 (c = 1.32 g, $CHCl_3$), {Found $[M+Na]^+$: 3266.83; $C_{198}H_{398}O_{15}Si_8Na$ requires: 3266.93} showed δ_H (500 MHz, $CDCl_3$): 4.86 (2H, d, *J* 2.8 Hz), 4.37 (2H, br, d, *J* 10.4 Hz), 4.04-3.99 (2H, m), 3.94 (2H, br, q, *J* 5.35 Hz), 3.9 (2H, t, *J* 9.1 Hz), 3.53 (2H, t, *J* 8.9 Hz), 3.38 (2H, dd, *J* 2.8, 9.1 Hz), 3.32 (2H, m), 2.56 (2H, ddd, *J* 3.5, 4.75, 10.1 Hz), 1.55-1.05 (268H, m), 0.89 (12H, t, *J* 7 Hz), 0.88 (18H, s), 0.65 (8H, br, m), 0.57 (4H, dt, *J* 4.1, 8.2 Hz), 0.166 (18H, s), 0.15 (18H, s), 0.14 (18H, s), 0.067 (12H, s), -0.32 (4H, br, q, *J* 5.35 Hz); δ_C: 173.8, 130.5, 94.8, 73.6, 73.4, 72.8, 71.8, 70.7, 62.4, 51.9, 33.5, 31.9, 30.2, 30.0, 29.8, 29.7, 29.5, 29.5, 29.5, 29.4, 28.7, 28.1, 26.2, 26.0, 25.9, 25.9, 25.2, 22.7, 18.0, 15.8, 14.1, 10.9, 1.1, 0.9, 0.2, -4.5, -4.6; υ_{max}: 2922, 2852, 1743, 1466.7, 1252, 1077, 838 cm^{-1}.

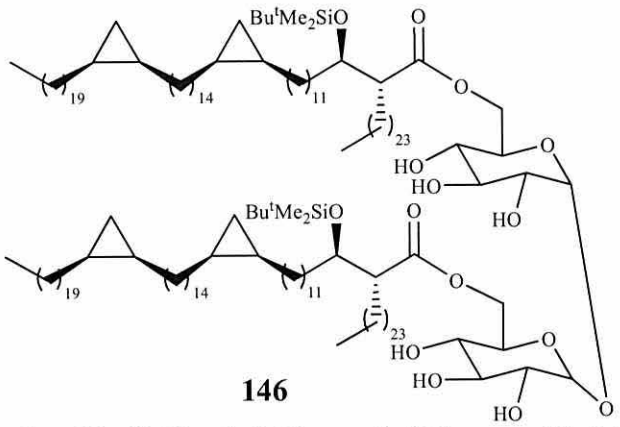
(c+a2) Compound **145** (0.139 g, 49 %), {$[\alpha]^{28}_D$ = +33.54 (c = 0.96 g, $CHCl_3$)}, {Found $[M+Na]^+$: 2032.67; $C_{114}H_{234}O_{13}Si_7Na$ requires: 2032.66} showed δ_H (500 MHz, $CDCl_3$): 4.91 (1H, d, *J* 2.85 Hz), 4.84 (1H, d, *J* 3.15 Hz), 4.35 (1H, dd, *J* 2.2, 11.65 Hz), 4.08 (1H, dd, *J* 4.1, 12 Hz), 3.99 (1H, br, dq, *J* 2.5, 9.5 Hz), 3.95 (1H, m), 3.91 (2H, dt, *J* 6.65, 9.15 Hz) 3.85 (1H, dt, *J* 3.45, 9.45 Hz), 3.74-3.66 (2H, m), 3.48 (2H, dt, *J* 6, 9.15 Hz), 3.43 (1H, dd, *J* 3.15, 9.15 Hz), 3.39 (1H, dd, *J* 3.15, 9.15 Hz), 2.55 (1H, ddd, *J* 3.5, 5.4, 9.15 Hz), 1.72 (1H, dd, *J* 5.1, 7.6 Hz), 1.64-1.60 (2H, m), 1.44-1.08 (132H, m), 0.89 (6H, t, *J* 7

Hz), 0.88 (9H, s), 0.67-0.64 (4H, m), 0.57 (2H, dt, *J* 4.1, 8.2 Hz), 0.174 (9H, s), 0.164 (9H, s), 0.159 (9H, s), 0.155 (9H, s), 0.153 (9H, s), 0.127 (9H, s), 0.065 (3H, s), 0.061 (3H, s), -0.32 (2H, q, *J* 5 Hz); δ_C: 174.1, 94.5, 94.4, 73.4, 73.4, 73.4, 72.9, 72.8, 72.8, 72.0, 71.4, 70.7, 62.5, 61.7, 51.8, 41.4, 33.4, 31.9, 30.2, 29.8, 29.7, 29.6, 29.4, 28.7, 28.1, 26.4, 25.9, 25.8, 24.9, 22.7, 19.4, 18.4, 18.0, 15.8, 14.1, 10.9, 1.1, 1.0, 0.9, 0.8, 0.2, 0.0, -4.5, -4.7; υ_{max}: 2924, 2853, 1742, 1465, 1251, 1165, 1110, 1076, 1006, 898, 873, 842, 748 cm^{-1}.

Experiment 56:

6,6'-Bis-O-(*R*)-2-((*R*)-1-(*tert*-Butyldimethylsilanyloxy)-12-{(1*R*,2*S*)-2-[14-((1*R*,2*S*)-2-eicosyl-cyclopropyl)-tetradecyl]-cyclopropyl}-dodecyl)-hexacosanoic-α,α'-trehalose

146

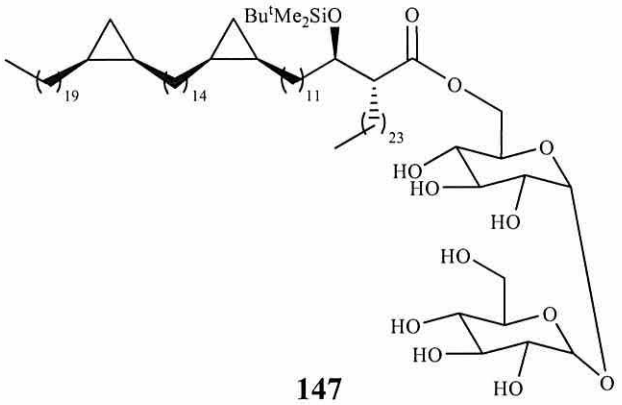


146

Tetrabutylammonium fluoride (0.27 ml, 0.27 mmol, 1M) was added to a stirred solution of **144** (0.11 g, 0.035 mmol) in dry THF (7 ml) at 5 °C under nitrogen. The mixture was allowed to reach r.t and stirred for 1 hr when TLC showed no starting material. The reaction was cooled to 5 °C and quenched with sat.aq. sodium bicarbonate (3 ml) then diluted with cold $CHCl_3$ (50 ml). The organic layer was separated and the aqueous layer was re-extracted with $CHCl_3$ (2 x 50 ml). The combined organic layers were washed with brine (50 ml), dried and evaporated to give a residue, which was purified by column chromatography on silica eluting with $CHCl_3$/MeOH 85:15 to give **146** (0.051 g, 53 %) as a colourless thick oil, $[\alpha]^{26}_D$ = + 15.02 ° (c = 3.2 g, $CHCl_3$), {Found $[M+Na]^+$: 2833.90; $C_{180}H_{350}O_{15}Si_2Na$ requires: 2833.8628} which showed δ_H (500 MHz, $CDCl_3$ + few drops of CD_3OD): 5.05 (2H, d, *J* 3.15 Hz), 4.29 (2H, br, dd, *J* 4.4, 12.3 Hz), 4.2 (2H, br, d, *J* 10.75 Hz), 3.91 (2H, br, d, *J* 9.2 Hz), 3.87 (2H, br, q, *J* 5.4 Hz), 3.80 (2H, br, t, *J* 9.45 Hz), 3.45 (2H, dd, *J* 3.5, 9.8 Hz), 3.32 (2H, m), 2.93 (2H, br, dt, *J* 4.31 Hz), 2.52 (2H, ddd, *J* 3.8, 6.35, 10.4 Hz), 1.6-1.01 (300H, m), 0.83 (12H, t, *J* 7.25 Hz), 0.82 (18H, s), 0.63-0.56 (8H, m), 0.52 (4H, dt, *J* 4.1, 8.2 Hz), 0.02 (6H, s), -0.02 (6H, s), -0.38 (4H, br, q, *J* 5.35 Hz); δ_C: 175.1, 93.4, 85.4, 73.3, 72.8, 71.7, 70.4, 70.3, 62.8, 57.7, 51.5, 35.2, 33.7, 32.4, 31.9, 30.5, 30.4, 30.3, 30.2, 30.1, 30.0, 29.9, 29.8, 29.7, 29.7, 29.6, 29.5, 29.5, 29.4, 29.4, 29.3, 28.7, 27.8, 27.6, 27.1, 26.2, 25.9, 25.8, 24.3, 22.7, 18.0, 15.8, 14.8, 14.1, 10.9, -4.4, -4.7; υ_{max}: 3384, 2920, 2851, 2360, 1739, 1469, 1253, 1077, 837, 775, 721 cm^{-1}.

Experiment 57:

6-O-(*R*)-2-((*R*)-1-(*tert*-Butyldimethylsilanyloxy)-12-{(1*R*,2*S*)-2-[14-((1*R*,2*S*)-2-eicosyl-cyclopropyl)-tetradecyl]-cyclopropyl}-dodecyl)-hexacosanoic-α,α'-trehalose 147

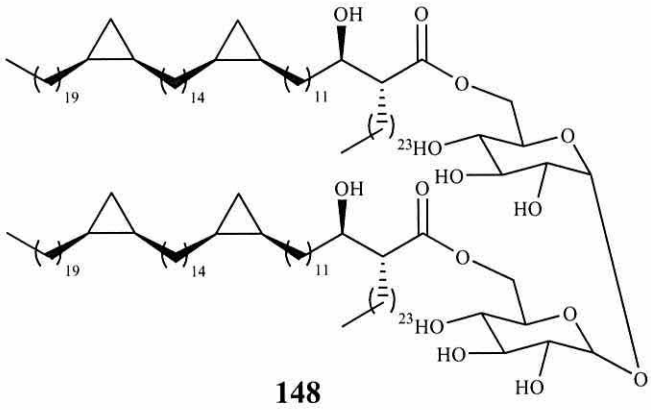


147

Tetrabutylammonium fluoride (0.538 ml, 0.538 mmol, 1M) was added to a stirred solution of **145** (0.139 g, 0.0691 mmol) in dry THF (7 ml) at 5 °C under nitrogen. The mixture was allowed to reach r.t and stirred for 1 hr, when TLC showed no starting material was left. It was worked up as described above and purified by column chromatography on silica eluting with $CHCl_3$/MeOH 8.5:1.5 to give **147** (0.098 g, 90 %) as a colourless syrup, {$[\alpha]^{26}_D$ = + 11.94 (c = 2.95, $CHCl_3$)}, {[Found M+Na]$^+$: 1599.30; $C_{96}H_{186}O_{13}SiNa$ requires: 1599.60} which showed δ_H: (500 MHz, $CDCl_3$ + few drops of CD_3OD): 5.03 (2H, d, *J* 2.2 Hz), 4.22 (1H, br, s), 4.00 (1H, br, dt, *J* 2.85, 9.4 Hz), 3.92 (1H, br, d, *J* 9.7 Hz), 3.85 (1H, m), 3.73-3.77 (3H, m), 3.65 (1H, m), 3.41 (4H, m), 2.45 (1H, m), 1.17-1.30 (143H, m), 0.79 (15H, m, including a triplet resonated at δ 0.80, *J* 7.25 Hz), 0.56 (4H, m), 0.47 (2H, dt, *J* 4.1, 8.5 Hz), -0.04 (3H, s), -0.06 (3H, s), -0.42 (2H, q, *J* 5.3 Hz); δ_C: 93.4, 73.0, 72.7, 72.6, 72.3, 72.1, 70.1, 62.8, 52.1, 49.2, 49.0, 31.7, 30.0, 29.6, 29.5, 29.5, 29.2, 28.5, 25.7, 25.0, 23.9, 22.5, 19.6, 15.6, 13.9, 13.5, 10.7, -4.7, -4.9, υ_{max}: 3384.8, 2922.5, 2852.2, 1731.3, 1466.7, 1381.7, 991.9, 482.6 cm^{-1}.

Experiment 58:

6,6'-Bis-O-(*R*)-2-((*R*)-1-hydroxy-12-{(1*R*,2*S*)-2-[14-((1*R*,2*S*)-2-eicosyl-cyclopropyl)-tetradecyl]-cyclopropyl}-dodecyl)-hexacosanoic-α,α'-trehalose 148

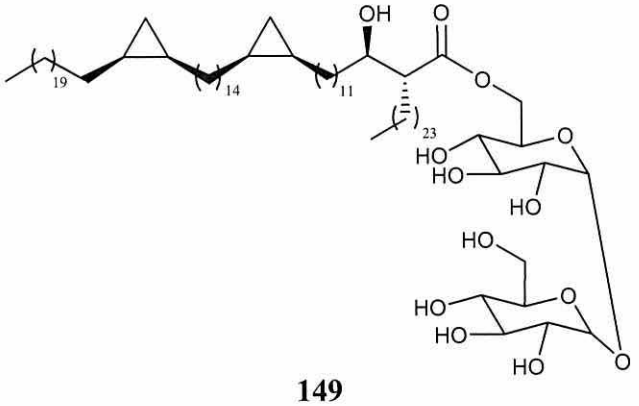


148

A dry polyethylene vial equipped with an acid proof rubber septum was charged with **146** (0.05 g, 0.018 mmol) and pyridine (100 μl) in dry THF (4.5 ml) and stirred at r.t under argon. To it was added hydrogen fluoride-pyridine complex as ~70% hydrogen fluoride (0.253 ml) at 5 °C. The mixture was stirred at 43 °C for 17 hrs, when TLC showed no starting material was left, then neutralized by pouring slowly into sat.aq. sodium bicarbonate until no more CO_2 was liberated. The product was extracted with chloroform (3 x 50 ml), then the combined organic layers were dried, evaporated to give a residue which was purified by chromatography eluting with $CHCl_3$/MeOH 10:1 to give **148** (0.024 g, 54 %) as a syrup, {$[\alpha]^{27}_D$ = + 27.91, (c = 2.2, $CHCl_3$)} {Found $[M+Na]^+$: 2605.32; $C_{168}H_{322}O_{15}Na$ requires: 2605.40} which showed δ_H (500 MHz, $CDCl_3$ + few drops of CD_3OD): 4.98 (2H, br, d, *J* 3.75 Hz), 4.6 (2H, br, d, *J* 11.35 Hz), 4.16 (2H, br, t, *J* 9.45 Hz), 4.00 (2H, br, dd, *J* 7.6, 11.65 Hz), 3.73 (2H, br, t, *J* 9.45), 3.64-3.6 (2H, m), 3.46 (2H, br, dd, *J* 3.45, 9.75 Hz), 3.21 (2H, br, t, *J* 9.75 Hz), 2.39-2.34 (2H, br, m), 1.38-1.05 (290H, m), 0.83 (6H, t, *J* 6.65 Hz), 0.62-0.57 (8H, m), 0.52 (4H, dt, *J* 4.1, 8.2 Hz), -0.36-0.39 (4H, br, q, *J* 5.1 Hz); δ_C: 175.4, 94.9, 72.5, 72.5, 71.3, 71.2, 69.8, 64.4, 52.2, 34.7, 31.8, 30.1, 30.1, 29.7, 29.6, 29.6, 29.4, 29.3, 29.2, 28.6, 27.2, 25.1, 22.5, 15.6, 13.9, 10.8, 0.8; υ_{max}: 3391, 2918, 2850, 1730, 1467, 1260, 1020, 800, 464 cm^{-1}.

Experiment 59:

6-O-(*R*)-2-((*R*)-1-Hydroxy-12-{(1*R*,2*S*)-2-[14-((1*R*,2*S*)-2-eicosyl-cyclopropyl)-tetradecyl]-cyclopropyl}-dodecyl)-hexacosanoic-α,α'-trehalose 149



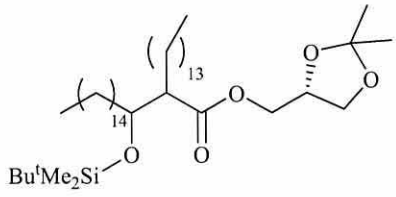
149

A dry polyethylene vial equipped with a rubber septum was charged with **147** (0.083 g, 0.053 mmol) and pyridine (0.1 ml) in dry tetrahydrofuran (5 ml) and stirred at r.t under nitrogen. To it was added hydrogen fluoride-pyridine complex as ~70% hydrogen fluoride (0.75 ml). The mixture was stirred at 43 °C for 17 hrs, when TLC showed no starting material, then neutralized by pouring slowly into sat. aq. sodium bicarbonate until no more CO_2 was liberated. The product was extracted with chloroform (3 x 50 ml), then the combined organic layers were dried, evaporated to give a residue which was purified by chromatography eluting with $CHCl_3$/MeOH 10:1 then 1:1 to give crude **149** (0.024 g, 32 %) as a syrup. The sample was then purified by dissolving it in chloroform to which cold methanol was added, after which a precipitate was formed. It was then centrifuged for 10 minutes at 8000 rev/min and the product **149** was obtained as a white solid (0.017 g, 22 %), {$[\alpha]^{26}_D$ = + 41.53, (c = 1.83, $CHCl_3$)} {Found $[M+Na]^+$: 1485.14; $C_{90}H_{172}O_{13}Na$ requires: 1485.31} which showed δ_H (500 MHz, $CDCl_3$ + few drops of CD_3OD): 5.11 (1H, br, s), 5.07 (1H, br, s), 4.62 (1H, br, d, *J* 8.2 Hz), 4.13 (1H, br, m), 4.06 (1H, br, m), 3.92-3.83 (5H, m), 3.58 (1H, br, d, *J* 7.5 Hz), 3.53 (1H, br, d, *J* 8.85 Hz), 3.39 (1H, m), 3.36 (1H, t, *J* 6.5 Hz), 2.69 (1H, br, s), 2.41-2.39 (1H, br, m), 1.72 (1H, m), 1.61 (3H, m), 1.50-1.11 (143H, m), 0.87-0.84 (6H, t, *J* 7 Hz), 0.62 (4H, m), 0.56-0.51 (2H, dt, *J* 4.1, 8.2 Hz), -0.34-0.37 (2H, q, *J* 5 Hz); δ_C: 175.4, 94.1, 72.6, 72.5, 72.3, 71.4, 70.9, 70.1, 64.0, 62.1, 58.8, 52.1, 31.8, 30.2, 30.2, 30.1, 29.8, 29.6, 29.6, 29.5,

29.4, 29.3, 28.7, 28.6, 23.8, 22.6, 19.6, 15.7, 15.7, 14.0, 13.5, 10.9, 10.8; υ_{max}: 3356, 2919, 2850, 1728, 1468, 1148, 1106, 992, 721, 427 cm^{-1}.

Experiment 60:

3-(*tert*-Butyldimethylsilanyloxy)-2-tetradecyloctadecanoic acid (*R*)-2,2-dimethyl[1,3]dioxolan-4-ylmethyl ester 150

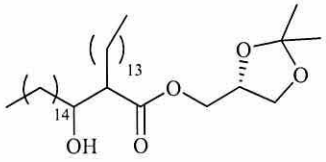


150

((*S*)-2,2-Dimethyl-[1,3]dioxolan-4-yl)-methanol (0.1 g, 0.76 mmol, supplied by Dr.Al-Dulayymi) was dissolved in THF (3 ml) and 4-DMAP (0.01 g, 0.08 mmol) was added. The reaction was stirred and cooled to 4 °C after which compound **91** (0.28 g, 0.44 mmol) in THF (0.7 ml) was added slowly. As the temperature increased a white precipitate formed, after whic it was stirred for 3 hrs at r.t. The mixture was then quenched with water (3 ml). Any alcohol excess was removed under high vacuum and the sample was then washed with water (10 ml) and extracted with petroleum ether/ethyl acetate (3x 10 ml). The combined organic layers were then washed with brine (2x 10 ml), then dried, evaporated to give a crude product that was purified by column chromatography eluting with petroleum ether/diethyl ether 5:1 to give **150** (0.17 g, 53 %) δ_H (500 MHz, $CDCl_3$): 4.34-4.28 (1H, pent, *J* 6 Hz), 4.15-4.06 (3H, m), 3.84-3.81 (1H, br, q, *J* 5.7 Hz), 3.77-3.72 (1H, br, q, *J* 9 Hz), 2.55-2.45 (1H, m), 1.60-1.56 (2H, m), 1.43 (3H, s), 1.37 (3H, s), 1.29-1.26 (52H, m), 0.93 (9H, s), 0.90-0.86 (6H, t, *J* 7 Hz), 0.14 (3H, s), 0.04 (3H, s); δc: 174.4, 73.5, 73.3, 73.3, 66.6, 66.6, 64.3, 34.9, 31.9, 29.8, 29.7, 29.6, 29.6, 29.4, 29.3, 28.6, 28.5, 25.8, 25.8, 25.7, 25.4, 24.6, 24.6, 22.7, 18.0, 14.1, -4.3, -4.5; υ_{max}: 2925.2, 2854.5, 1738.5, 1467.7, 1254.3, 836.5 cm^{-1}.

Experiment 61:

3-Hydroxy-2-tetradecyloctadecanoic acid (*R*)-2,2-dimethyl-[1,3]dioxolan-4-ylmethyl ester 151

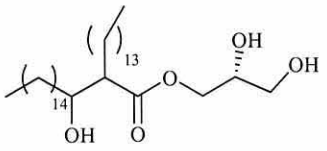


151

Compound **150** (0.2 g, 0.26 mmol) was dissolved in dry THF (15 ml) with dry pyridine (0.3 ml). The mixture was stirred and under N_2 and HF-pyridine complex (0.8 ml) was added slowly. The resulting mixture was stirred at 45 °C for 12 hrs under N_2. TLC was checked to show reaction was complete and then the mixture was added to aq. $NaHCO_3$ (40 ml) until no more CO_2 was released. The solution was then extracted with petroleum ether/diethyl ether 1:1 (3x 25ml).[175] The resulting organic layers were purified by column chromatography eluting with petroleum ether/diethyl ether 20:1 to give the product **151** (0.1 g, 61 %) as an oil which showed δ_H (500 MHz, $CDCl_3$): 4.34-4.28 (1H, pent, *J* 6 Hz), 4.22-4.12 (2H, m), 4.09-4.06 (1H, m), 3.83-3.79 (1H, br, q, *J* 5.7 Hz), 3.77-3.72 (1H, br, q, *J* 9 Hz), 2.55-2.45 (1H, m), 1.60-1.56 (2H, m), 1.43 (3H, s), 1.37 (3H, s), 1.29-1.26 (52H, m), 0.90-0.86 (6H, t, *J* 7 Hz); δc: 175.0, 109.9, 73.4, 72.2, 66.2, 64.7, 51.1, 31.9, 29.7, 29.6, 29.6, 29.5, 29.4, 29.3, 27.8, 26.7, 26.0, 25.2, 25.2, 22.7, 14.1.

Experiment 62:

3-Hydroxy-2-tetradecyl-octadecanoic acid (*R*)-2,3-dihydroxy-propyl ester 152

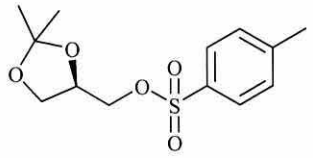


152

Compound **151** (0.1 g, 0.16 mmol) were dissolved in warm THF (2 ml), to which MeOH/water (9:1, 3 ml) was added followed by DOWEX ion exchange resin (110 mg). The mixture was left stirred for 48 hrs at 45 °C. Another 200 mg of ion exchange resin were added and the mixture was stirred for a further 24 hrs. Solvents were evaporated off, then petrol/ethyl acetate (1:1, 10 ml) was added and it was again evaporated off. More petrol/ethyl acetate was added (10 ml) and the mixture was warmed and filtered.[229] The crude product was then evaporated off and the purified by column chromatography eluting with petroleum ether/ethyl acetate 5:2 to give **152** (0.05 g, 54 %), m.p 70-72 °C, lit. 72-74 °C; which showed {Found $[M+Na]^+$: 593.63; $C_{35}H_{170}O_5Na$ requires: 593.51} as an oil which showed δ_H (500 MHz, $CDCl_3$): 4.37-4.25 (1H, pent, *J* 6 Hz), 4.19-4.12 (2H, m), 4.09-4.06 (1H, m), 3.84-3.79 (1H, br, q, *J* 5.7 Hz), 3.77-3.64 (1H, br, q, *J* 9 Hz), 2.54-2.47 (1H, m), 1.72-1.65 (2H, m), 1.29-1.23 (52H, m), 0.89-0.87 (6H, t, *J* 7 Hz); δc: 175.3, 71.9, 71.0, 67.7, 66.5, 51.8, 31.9, 29.7, 29.6, 29.6, 29.5, 29.5, 29.5, 29.4, 29.3, 27.9, 26.1, 22.7, 14.1.

Experiment 63:

Toluene-4-sulfonic acid (*R*)-2,2-dimethyl-[1,3]dioxolan-4-ylmethyl ester 154

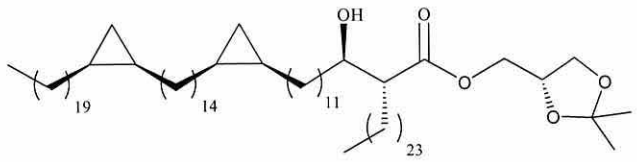


154

To an ice-cooled solution of ((*S*)-2,2-Dimethyl-[1,3]dioxolan-4-yl)-methanol (1 g, 7.5 mmol, supplied by Dr.Al-Dulayymi) in pyridine (6 ml) p - toluenesulfonyl chloride (1.43 g, 7.5 mmol) was added in small portions. The sample was then left standing in a refrigerator for 16 hrs, then diluted with ether (6 ml), washed with 1N HCl until the aqueous wash was acidic, and then with sat.aq. $NaHCO_3$ (2x 10 ml).[244] The ether layer was dried and concentrated to give **154** (1.5 g, 69 %)[245], which was used without further purification δ_H (500 MHz, $CDCl_3$): 7.74-7.72 (2H, dd, *J* 1.9, 6.65 Hz), 7.30-7.29 (2H, d, *J* 7.9 Hz), 4.25-4.19 (1H, pent, *J* 6 Hz), 3.98-3.95 (2H, dd, *J* 6.3, 9 Hz), 3.95-3.91 (1H, dd, *J* 5.65, 10 Hz), 3.70-3.67 (1H, dd, *J* 5.05, 9 Hz), 2.40 (3H, s), 1.27 (3H, s), 1.24 (3H, s); δc: 144.8, 132.4, 129.7, 127.7, 109.7, 72.7, 69.4, 65.7, 27.3, 26.3, 24.8.

Experiment 64:

(*R*)-2-((*R*)-1-Hydroxy-12-{(1*R*,2*S*)-2-[14-((1*R*,2*S*)-2-eicosyl-cyclopropyl)-tetradecyl]-cyclopropyl}-dodecyl)-hexacosanoic acid (*S*)-2,2-dimethyl-[1,3]dioxolan-4-ylmethyl ester 155



155

To a mixture of **154** (0.077 g, 0.27 mmol) and **132** (0.05 g, 0.22 mmol) in dry DMF-dry THF (1:5, 10 ml) was added $CsHCO_3$ (0.21 g, 1.1 mmol). The sample was then stirred for 48 hrs at 70 °C, then the suspension was diluted with petroleum ether/diethyl ether (1:1, 20 ml).[246] The organic layer were washed with brine (2x 20 ml), dried and evaporated. The crude residue obtained this way was purified by column chromatography eluting with chloroform-methanol 1:1 to give **155** (0.025 g, 76 %), $[\alpha]^{24}_{D}$ = + 7.46 (c = 0.63 g, $CHCl_3$), {Found $[M+Na]^+$: 1275.32; $C_{84}H_{162}O_5Na$ requires: 1275.21} which showed δ_H (500 MHz, $CDCl_3$): 4.39-4.31 (1H, pent, *J* 6 Hz), 4.21-4.17 (2H, m), 4.10-4.05 (1H, dd, *J* 6.3, 8.2 Hz), 3.77-3.74 (1H, dd, *J* 6, 8.5 Hz), 3.71-3.65 (1H, m), 2.50-2.45 (1H, m), 1.72-1.68 (2H, m), 1.60-1.55 (4H, m), 1.44 (3H, s), 1.34 (3H, s), 1.30-1.14 (129H, m), 0.88 (6H, t, *J* 6.95 Hz), 0.66-0.65 (4H, m), 0.57 (2H, br, dt, *J* 4.0, 8.7 Hz), -0.32 (2H, dd, *J* 5.35, 9.45 Hz); δc: 172.4, 109.8, 73.4, 72.7, 66.3, 51.3, 31.9, 30.2, 29.7, 29.3, 28.7, 26.7, 25.3, 22.7, 15.8, 14.1, 10.9, υ_{max}: 3468, 289, 2918, 2850, 2360, 1732, 1468, 1371, 1215, 1168, 1058, 834, 759, 541 cm^{-1}.

Experiment 65:

Attempted hydrolysis of 155

Compound **155** (0.025 g, 0.02 mmol) were dissolved in warm THF (1 ml), to which MeOH/water (9:1, 1.5 ml) was added followed by DOWEX ion exchange resin (10 mg). The mixture was left stirring for 80 hrs at 45 °C.[229] Solvents were evaporated off, then petrol/ethyl acetate (1:1, 5 ml) was added and it was evaporated off. More petrol/ethyl acetate was added (5 ml) and the mixture was warmed and filtered. The crude product was then evaporated off and the purified by column chromatography eluting with petroleum ether/ethyl acetate 5:2 to give **155**. The reaction was repeated using an excess of ion exchange resin (110 mg) and leaving the reaction stirring for 100 hrs. NMR showed once again starting material.

7. References

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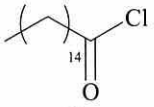
8. Appendix

8.1 Experiments

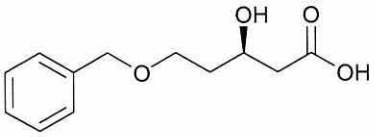
Hexadecanoyl chloride 68



68

To a solution of palmitic acid **26** (2 g, 7.8 mmol) and DMF (4 drop) in anhydrous dichloromethane (56 ml) at 0 °C under a nitrogen atmosphere was added oxalyl chloride (2.04 ml, 23.4 mmol) as a solution in anhydrous dichloromethane (56 ml). The solution was then stirred for 2 hours at 0 °C then allowed to attain ambient temperature and stirred for a further 2 hours. The volatiles were then removed *in vacuo* to give crude **68**, which was used without further purification; δ_H (250 MHz, $CDCl_3$): 2.89 (2H, t, *J* 7.3 Hz), 1.71 (2H, t, *J* 7 Hz), 1.26 (24H, s), 0.89 (3H, t, *J* 6.7 Hz).[247]

(*R*)-5-Benzyloxy-3-hydroxy-pentanoic acid 74

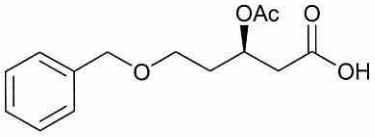


74

(*R*)-5-Benzyloxy-3-hydroxypentanoic acid methyl ester **73** (0.5 g, 2.1 mmol) (provided by G. Koza) was hydrolysed with refluxing NaOH (2M, 0.8 g), for 12 hrs at r.t. Reaction completion was checked by TLC ethyl acetate/petroleum ether 2:1. Then the methanol was evaporated off and ether (20 ml) was added. The solution was stirred vigorously and then diluted with diluted HCl (8 %, 7 ml) until pH reached 1. At that point the mixture was extracted with ether (100 ml). The water layer was re extracted with ether (2 x 100

ml). The combined organic layer were collected, dried and evaporated to give (*R*)-5-benzyloxy-3-hydroxypentanoic acid **74** (0.37 g, 79 %), which showed δ_H (500 MHz, $CDCl_3$): 7.37-7.28 (5H, m), 4.53 (2H, s), 4.30-4.25 (1H, m), 3.74-3.64 (2H, m), 2.56 (2H, d, 6.7 Hz), 1.87-1.78 (2H, m); δc: 176.4, 137.8, 128.5, 128.4, 127.8, 127.7, 73.3, 68.1, 67.2, 41.2, 35.7.

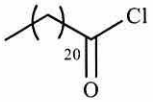
Attempted preparation of (*R*)-3-Acetoxy-5-benzyloxy-pentanoic acid 76



76

A mixture of acetic anhydride (10 ml), and anhydrous pyridine (10 ml) was added to a stirred solution of **74** (*R*)-5-Benzyloxy-3-hydroxy-pentanoic acid (0.37 g, 1.615 mmol) in dry toluene (20 ml) at r.t. The mixture was stirred for 10 hrs at r.t under N_2. When finished, the mixture was diluted with toluene (10 ml) and the solvent was evaporated. Diluted HCl (5 %, 50 ml) in water was added, then washed with CH_2Cl_2 (2x 50 ml), then extracted, dried and evaporated. The resultant product was purified by column chromatography eluting with ethyl acetate/petrol 2:1 to give a mixture of (*R*)-3-acetoxy-5-benzyloxy-pentanoic acid **76**, (*E*)-5-Benzyloxypent-2-enoic acid and the eliminating product, acetic acid methyl ester; δ_H (500 MHz, $CDCl_3$): 7.35-7.31 (5H, m), 7.1-7.2 (1H, m), 5.93-5.87 (1H, m), 5.37 (0.5H, quintet, *J* 6.3 Hz), 4.52 (1H, s), 4.47 (1H, s), 3.61-3.59 (2H, br, dt, *J* 6.3, 11.3 Hz), 3.54-3.46 (2H, br, dt, *J* 6.9, 14.3 Hz), 2.82-2.75 (1H, dd, J 7.55, 18 Hz), 2.57-2.52 (1H, br, dd, *J* 6.6, 13.25 Hz), 2.09 (3H, s); δ_C: 171.3, 166.4, 150.8, 138.0, 128.4, 128.4, 128.4, 128.3, 127.7, 127.7, 127.6, 127.6, 121.7, 73.0, 68.0, 68.0, 65.8, 33.8, 33.7, 32.8, 21.0, 20.7, 15.1.

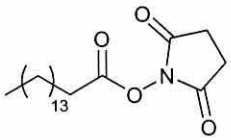
Docosanoyl chloride 159



159

To a solution of docosanoic acid **84** (2 g, 5.87 mmol) and DMF (4 drop) in anhydrous dichloromethane (56 ml) at 0 °C under a nitrogen atmosphere was added oxalyl chloride (2.04 ml, 23.4 mmol) as a solution in anhydrous dichloromethane (56 ml).[248,249] The solution was then stirred for 2 hrs at 0 °C then allowed to attain ambient temperature and stirred for a further 2 hours. The volatiles were then removed in vacuum to give crude docosanoyl chloride **159** (2 g, 94 %),[249] which was used without further purification, δ_H (500 MHz, $CDCl_3$): 2.90-2.87 (2H, t, *J* 7.5 Hz), 1.73-1.70 (2H, q, *J* 7.5 Hz), 1.35-1.33 (2H, m), 1.30-1.26 (34H, s), 0.90-0.87 (3H, t, *J* 7 Hz); δ_C: 173.9, 47.1, 31.9, 29.7, 29.7, 29.6, 29.6, 29.5, 29.4, 29.3, 29.0, 28.4, 25.0, 22.7, 14.1.

Hexadecanoic acid 2,5-dioxopyrrolidin-1-yl ester 97

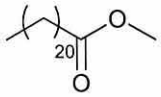


97

Commercial palmitic acid **26** (3 g, 0.012 mol, 1 mol .eq) and N-hydroxysuccinimide (1.35 g, 0.012 mol, 1 mol .eq) were suspended in dry ethyl acetate (50 ml). A solution of dicyclohexylcarbodiimide (DCC) (2.72 g, 0.013 mol, 1.1 mol .eq) and 4-pyrrolidinopyridine (0.18 g, 1.2 mmol, 0.1 mol .eq) in dry ethyl acetate (50 ml) was added. To increase solubility dry THF was added (10 ml). The clear solution was stirred at r.t for 1 hrs, after what it became whitish. Glacial acetic acid (1 ml) was added to the stirring mixture which was stirred for a further 2 hrs. The unwanted dicyclohexylurea was filtered off and washed with ethyl acetate (50 ml). The filtrate was allowed to stand at 4 °C for 12 hrs and dicyclohexylurea was filtered off. The filtrate was washed with water and brine (3x 50 ml) and evaporated to dryness.[218] The crude product was then purified by crystallisation from ethanol to give solid **88** (2.2 g, 51 %); δ_H (500 MHz, $CDCl_3$): 2.84

(4H, br, s), 2.62-2.59 (2H, t, *J* 7.25 Hz), 1.77-1.72 (2H, m), 1.41-1.37 (2H, m), 1.35-1.24 (22H, m), 0.90-0.87 (3H, t, *J* 6.6 Hz); δ_C: 169.2, 168.7, 31.9, 30.9, 29.6, 29.6, 29.5, 29.5, 29.3, 29.0, 28.7, 25.5, 24.5, 22.6, 18.3, 14.0.

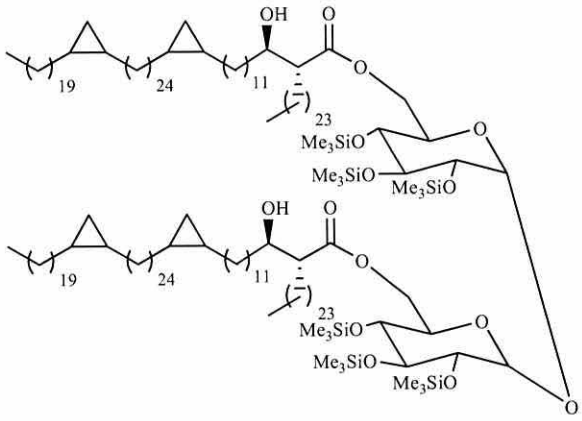
Henicosanoic acid methyl ester 85



85

Henicosanoic acid **84** (20 g, 58.72 mmol) was dissolved in MeOH (200 ml) and conc. H_2SO_4 (4 ml). The mixture was refluxed for 4 hrs at 73 °C.[250] The solvents were evaporated and was washed with saturated Sodium bicarbonate solution (100 ml) and extracted with CH_2Cl_2 (3x 150 ml). The organic phases were collected, evaporated and dried. The white crude product was then dissolved in petroleum ether (200 ml) and recrystallized over 48 hrs to give **85** (14.2 g, 69 %), m.p (47-50 °C),[251] lit 48-50 °C; δ_H (500 MHz, $CDCl_3$): 3.67 (3H, s), 2.32-2.29 (2H, t, *J* 7.25 Hz), 1.65-1.59 (2H, q, *J* 7.25 Hz), 1.30-1.26 (36H, m), 0.90-0.87 (3H, t, *J* 7 Hz); δ_C: 174.3, 51.4, 34.1, 31.9, 29.7, 29.6, 29.4, 29.4, 29.2, 29.1, 25.0, 22.7, 14.1.

Attempted preparation of (2*S*,2'*S*,175S)-(6,6'-oxybis(3,4,5-tris(trimethylsilyloxy)-tetrahydro-2H-pyran-6,2-diyl))bis(methylene) bis(2-((*S*)-1-hydroxy-12-(2-(24-(2-icosylcyclopropyl)tetracosyl)cyclopropyl)dodecyl)hexacosanoate) 101



101

Natural mycolic acids from a mycobacteria extract (0.03 g, 0.024 mmol) (supplied by Prof Minikin), **60** (0.01 g, 0.013 mmol), DCC (0.005 g, 0.024 mmol), 4-dimethylaminopyridine (10 mg) and 4Å molecular sieves (0.5 g) were put together, ground and placed in a round bottled flask to which N_2 and vacuum were applied. The mixture was stirred for 4 hrs. After that, dry toluene (10 ml) was added at 0 °C under N_2. The mixture left stirred for 24 hrs at 30 °C. TLC was checked and the temperature as increased to 70 °C for 24 hrs. After that it was worked up by evaporating the solvent and purifying the resulting solid by column chromatography eluting with hexane/ether 1:1 to give **60**; {Found $[M+Na]^+$: 798.58; $C_{186}H_{370}O_{15}Si_6Na$ requires: 3035.66} with the same ^1H and ^{13}C NMR.

8.2 Figures

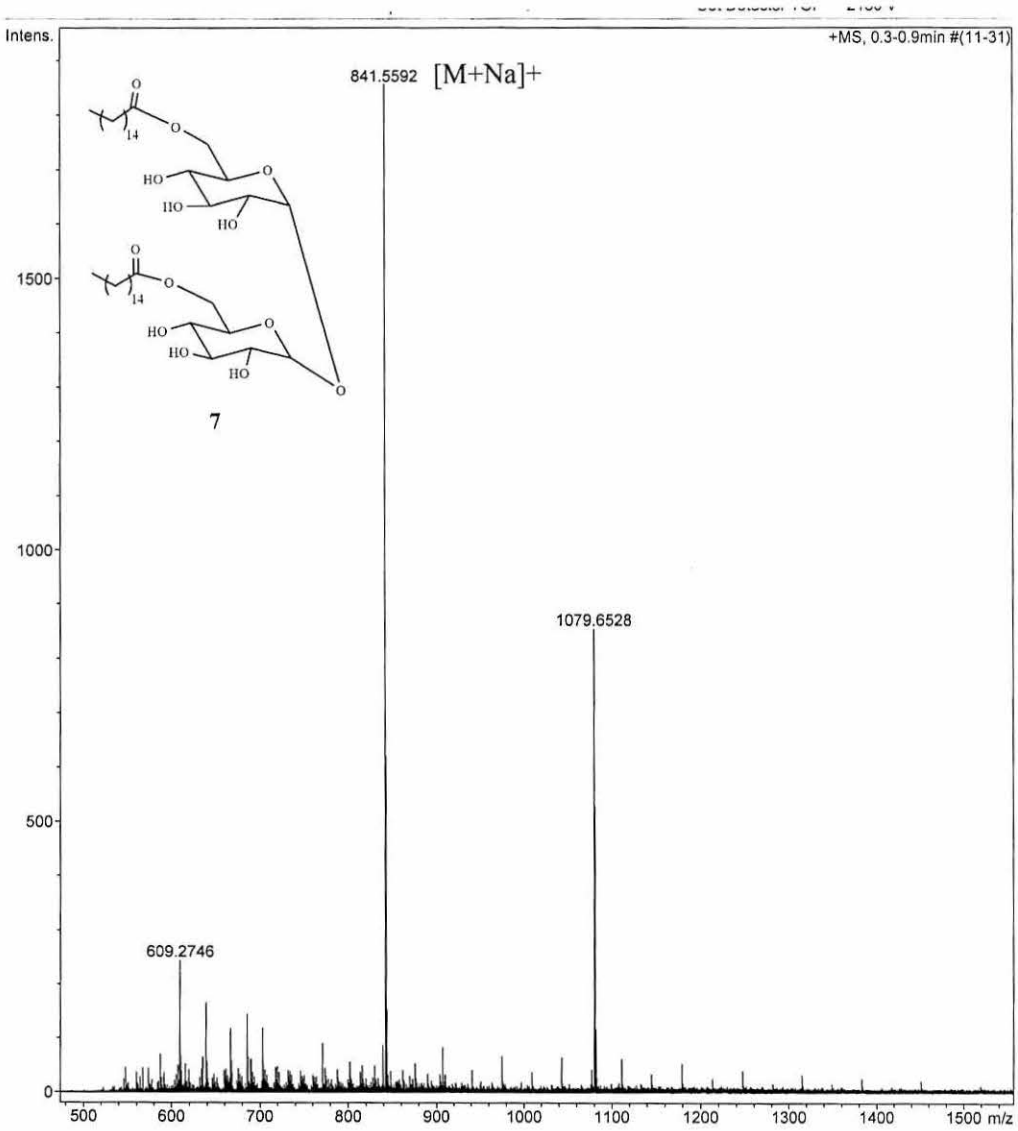


Fig 57. ESI micro TOF MS for compound **7**.

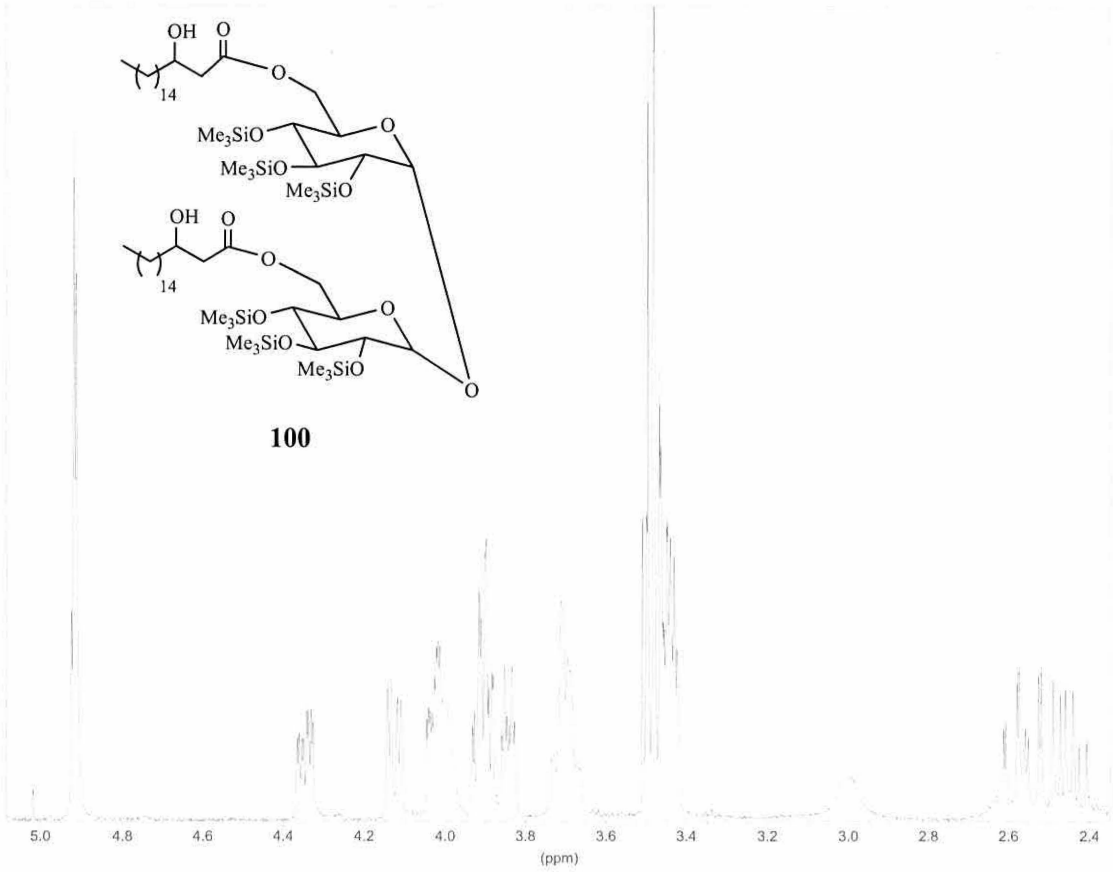


Fig 61. ^{1}H NMR expansion for **100** showing sugar core protons ranging from δ 2.2-4.9.

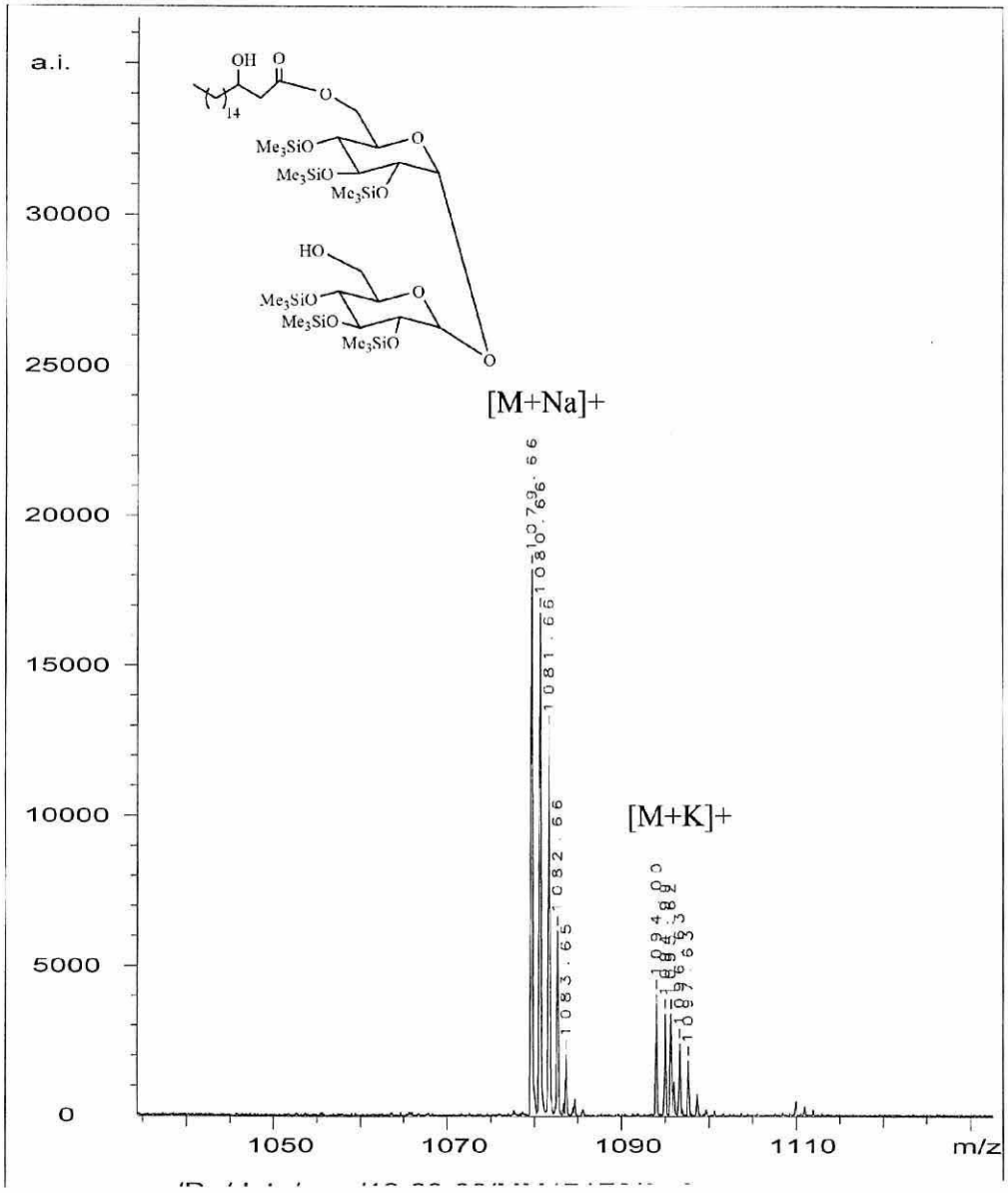


Fig 62. MALDI for the TMM corresponding to **100** that was not characterised due to the small sample amount obtained with **100**.

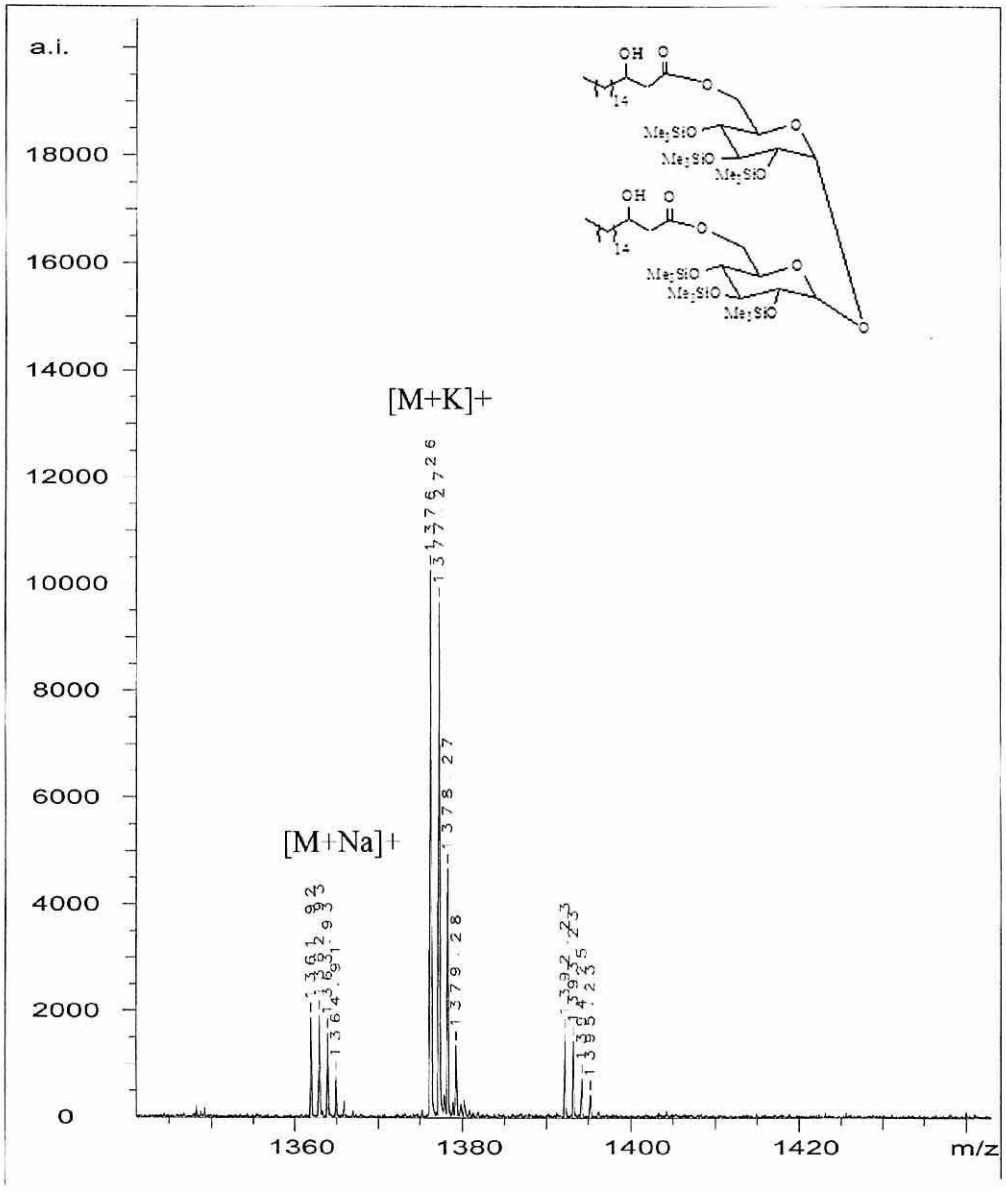


Fig 63. MALDI for compound **100**.

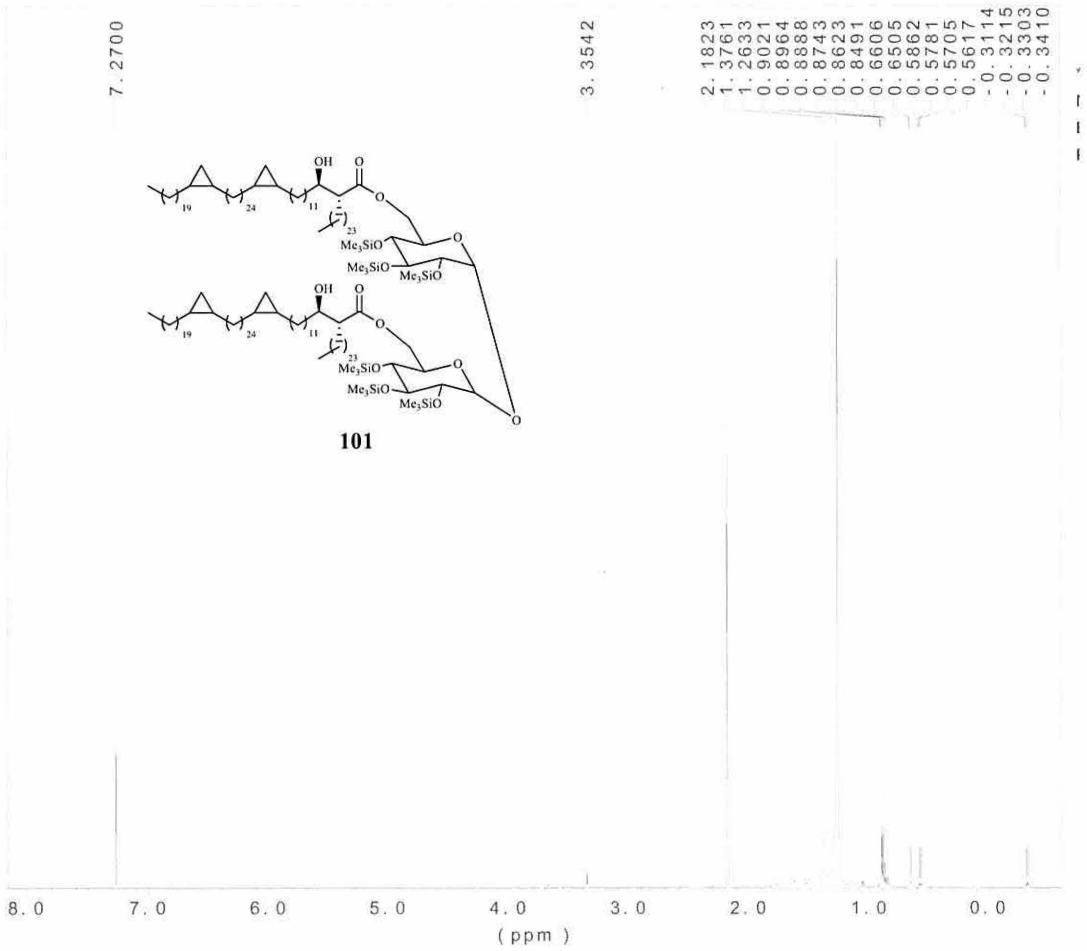


Fig 65. Natural product ^{1}H NMR of mycolic acid natural product supplied by Prof. D. Minnikin.

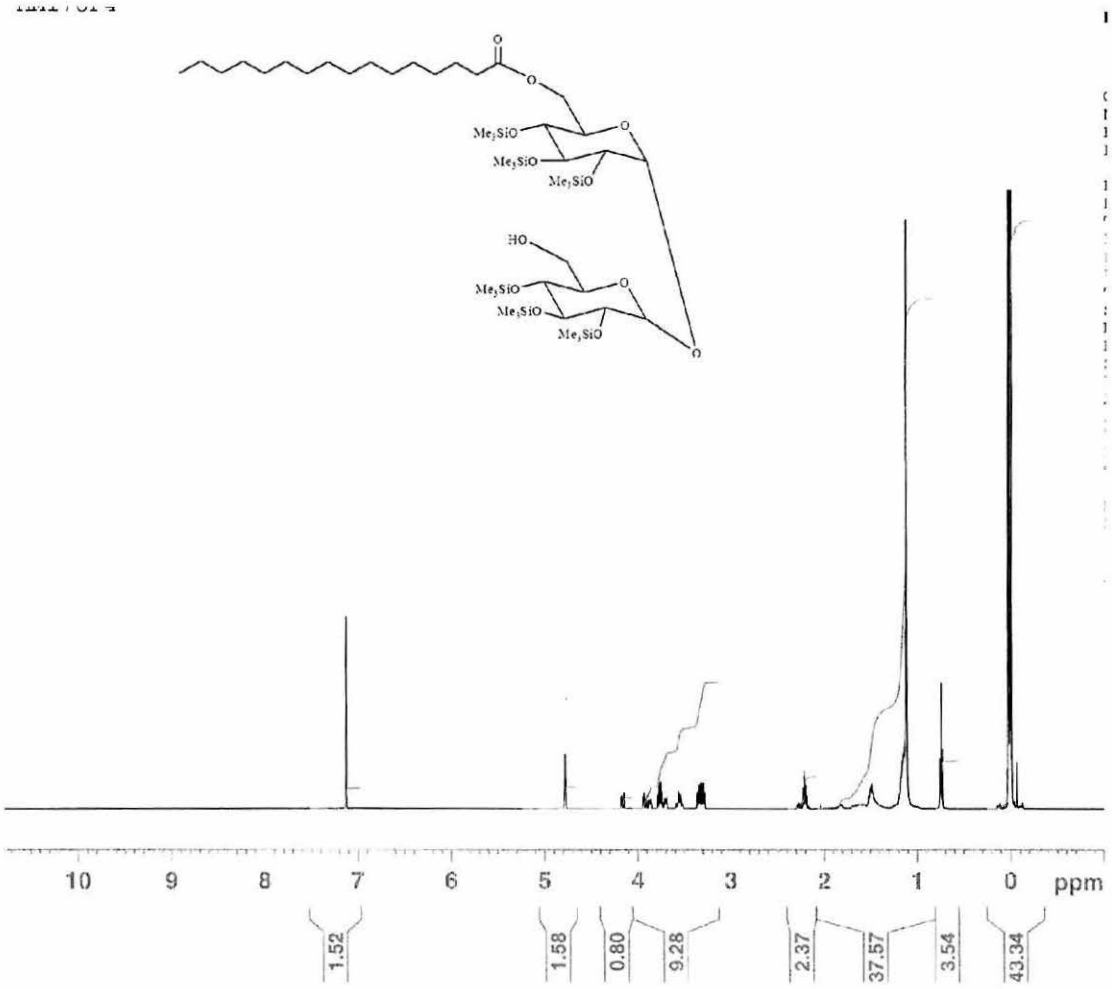


Fig 67. ^{1}H HNMR for compound **28b**.

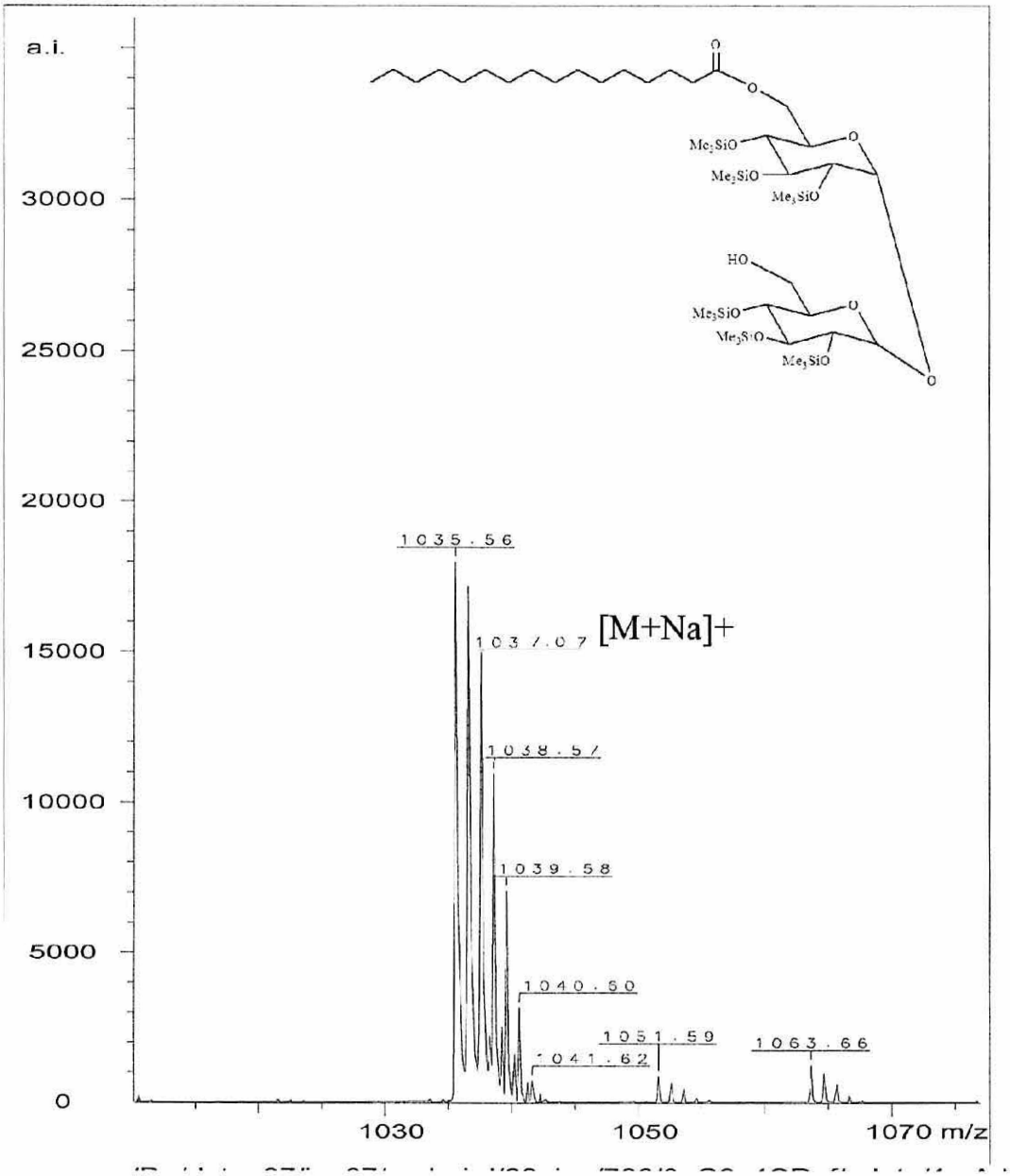


Fig 68. MALDI for compound **28b**.

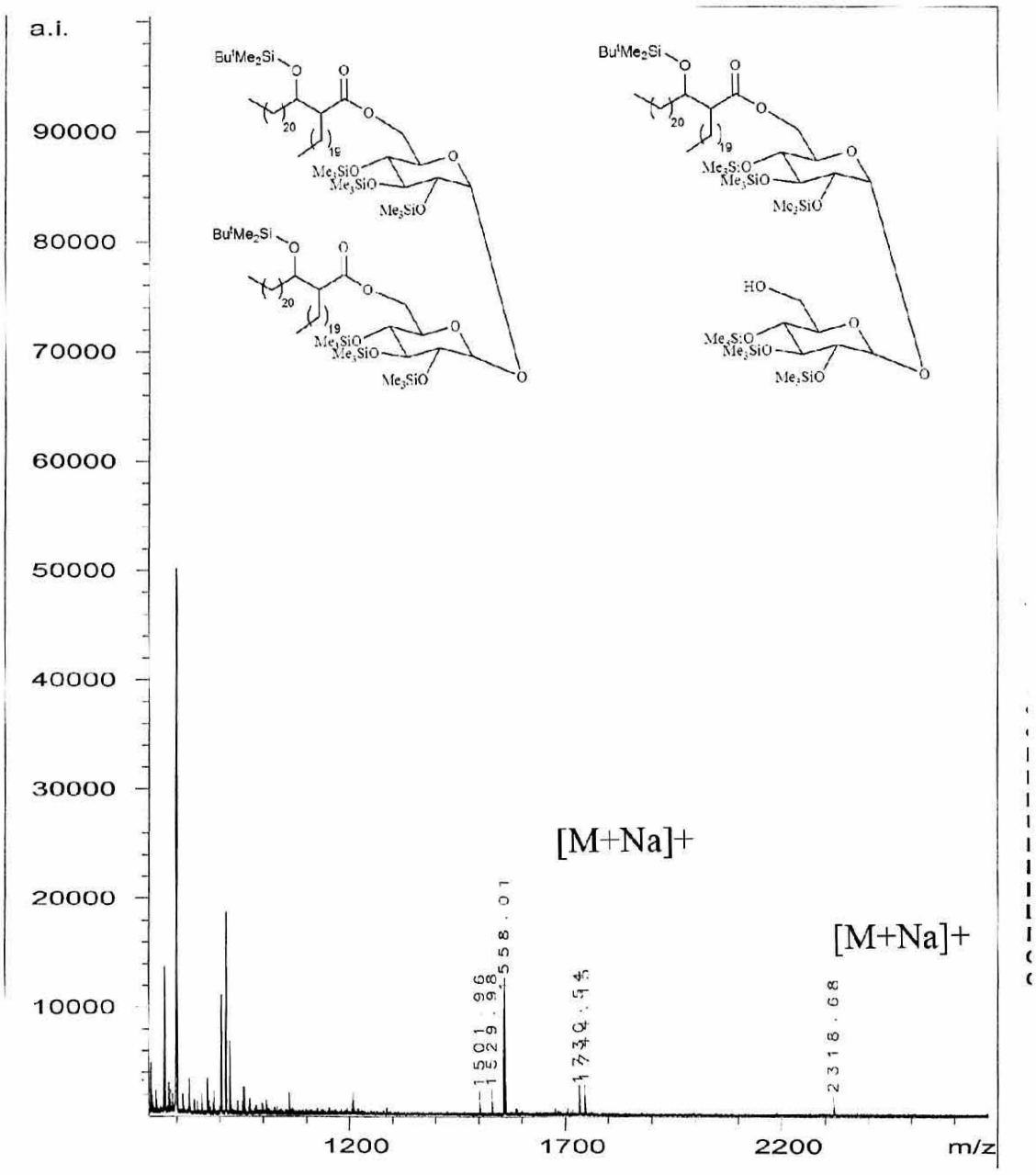


Fig 70. MALDI-TOF for compounds **102** including both mono- and di-substituted products for esterification after 6 days reaction.

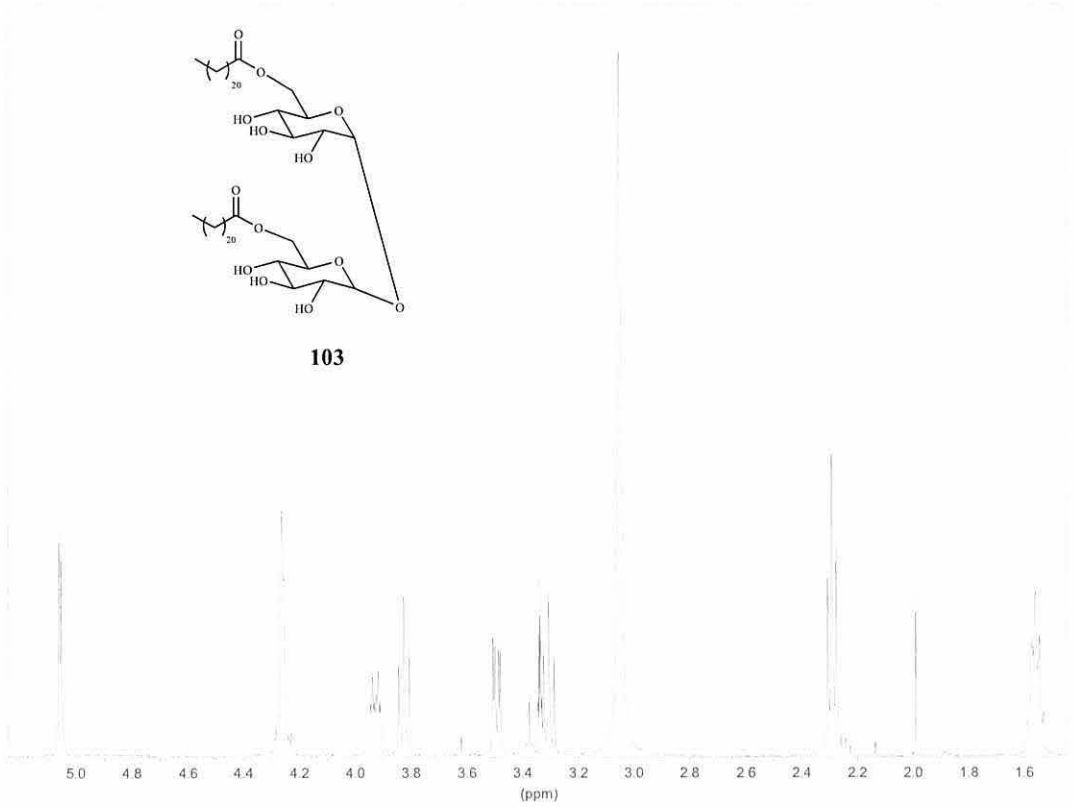


Fig 72. ^{1}H NMR for **103**.

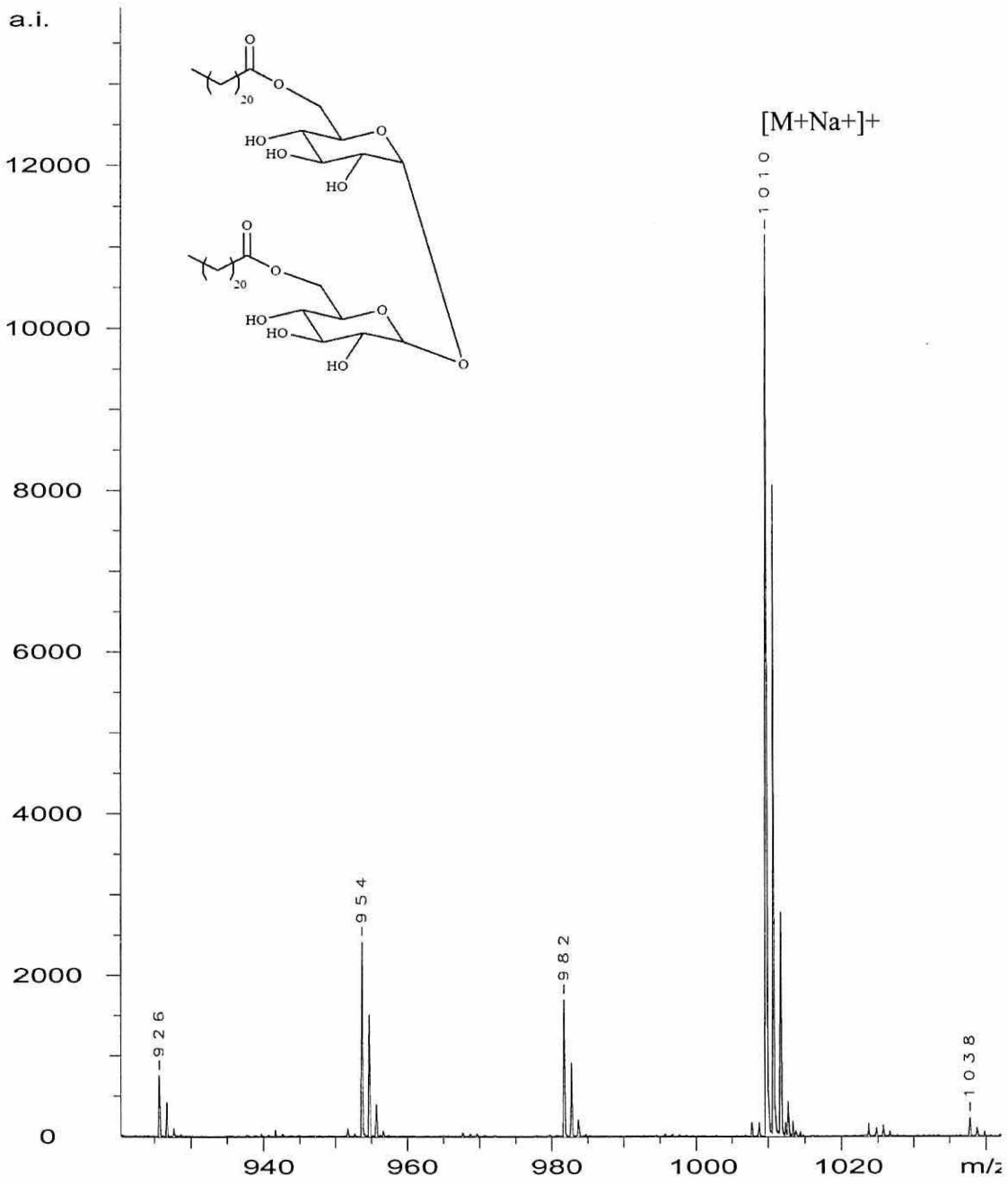


Fig 73. MALDI MS showing molecular ion for di-substituted product **103**, Found $[M+Na]^+$: 1010.01; $C_{56}H_{106}O_{13}Na$ requires: 1010.44.

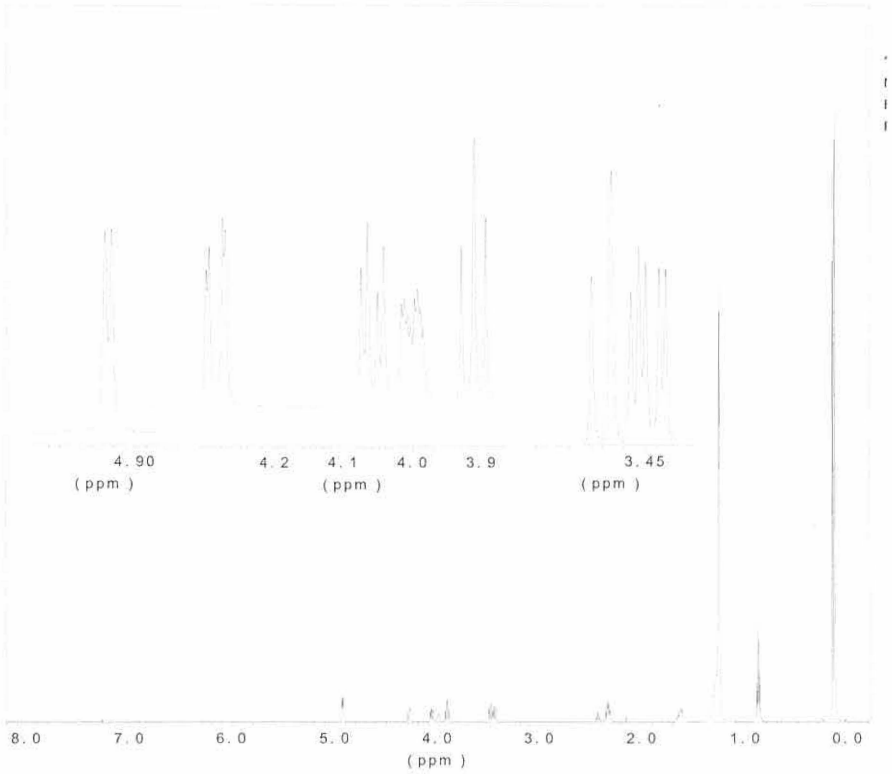


Fig 74. ^{1}H NMR corresponding to **28**, product for the acid chloride esterification.

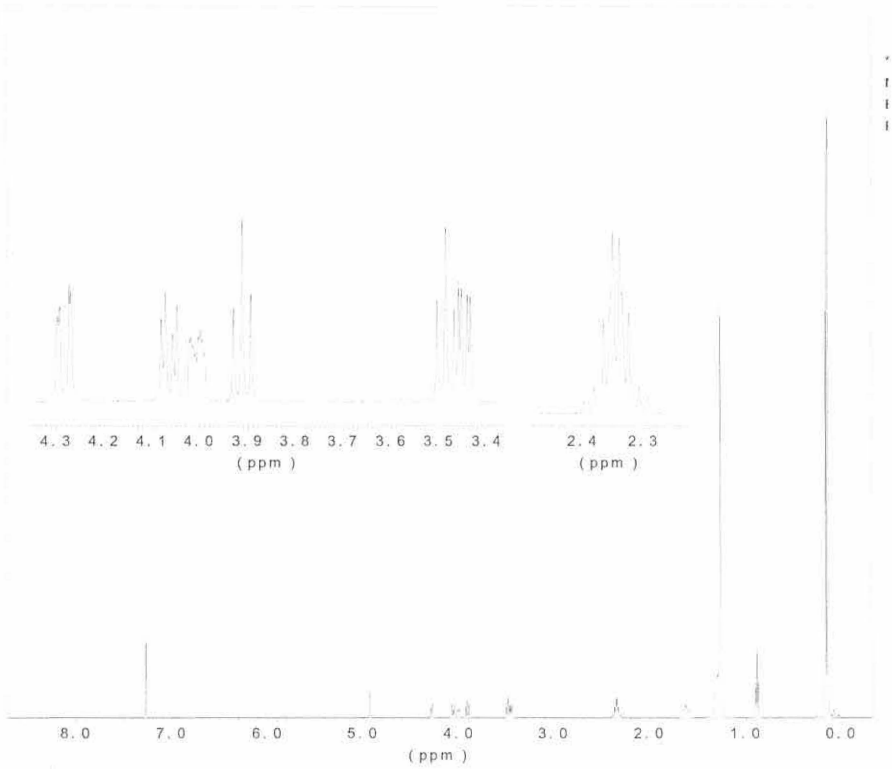


Fig 76. ^{1}H NMR for **13**, product for EDCI, DMAP reaction.

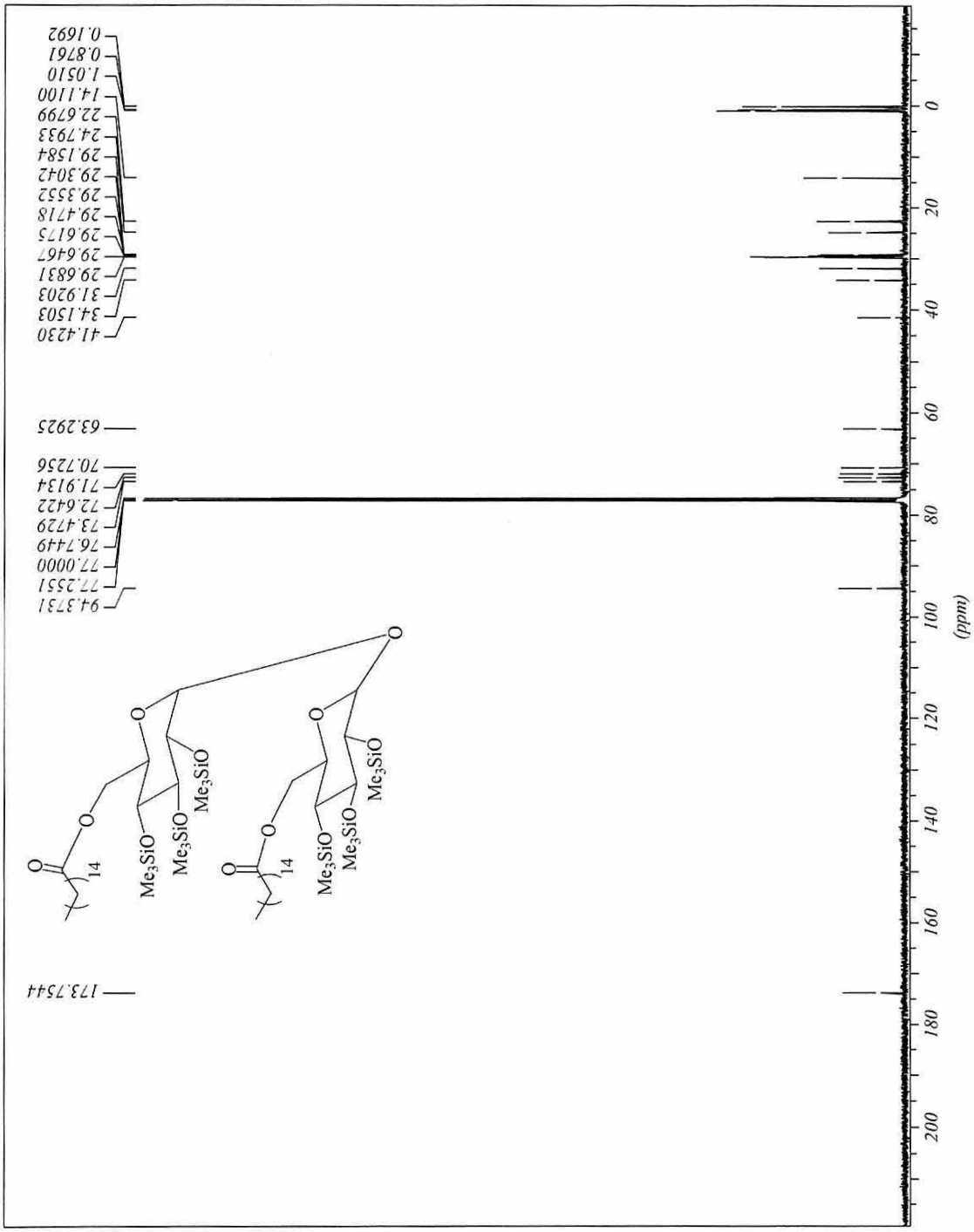


Fig 77. ^{13}C for **28** obtained following the EDCI, DMAP method.

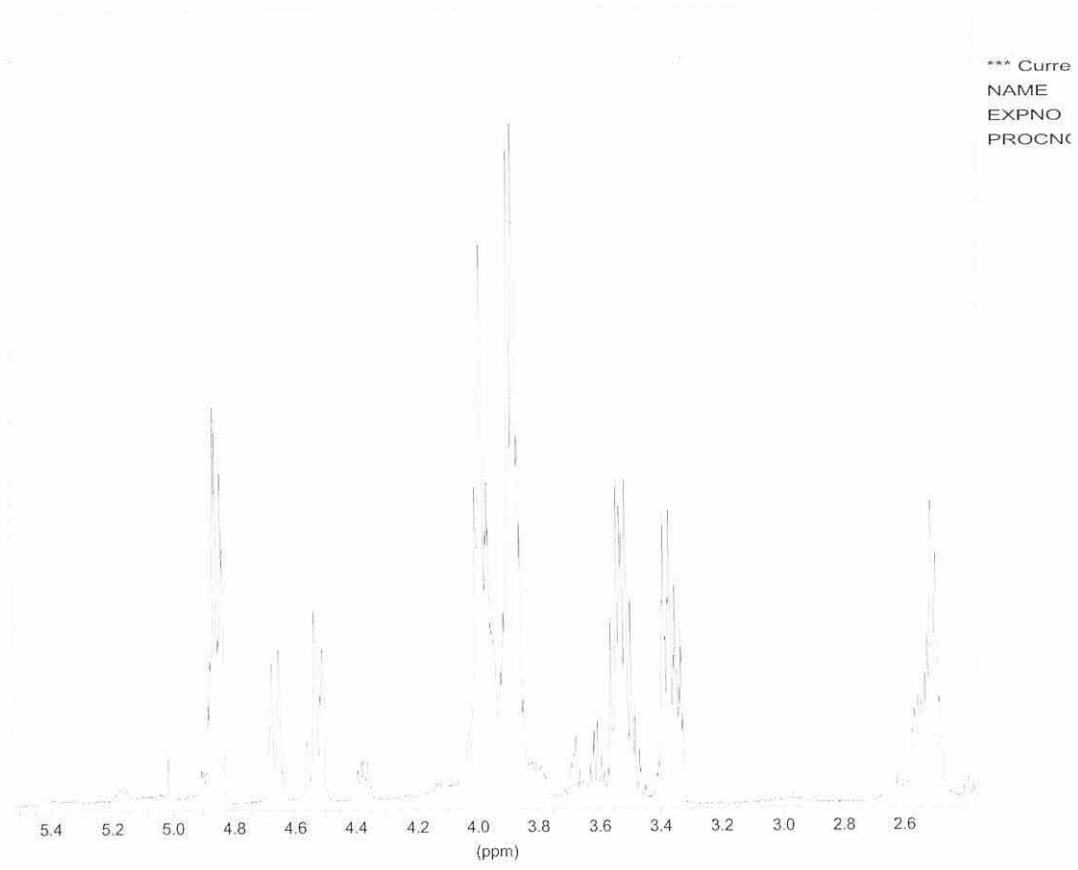


Fig 79. ^{1}H NMR for **104** showing the sugar region on the trehalose core.

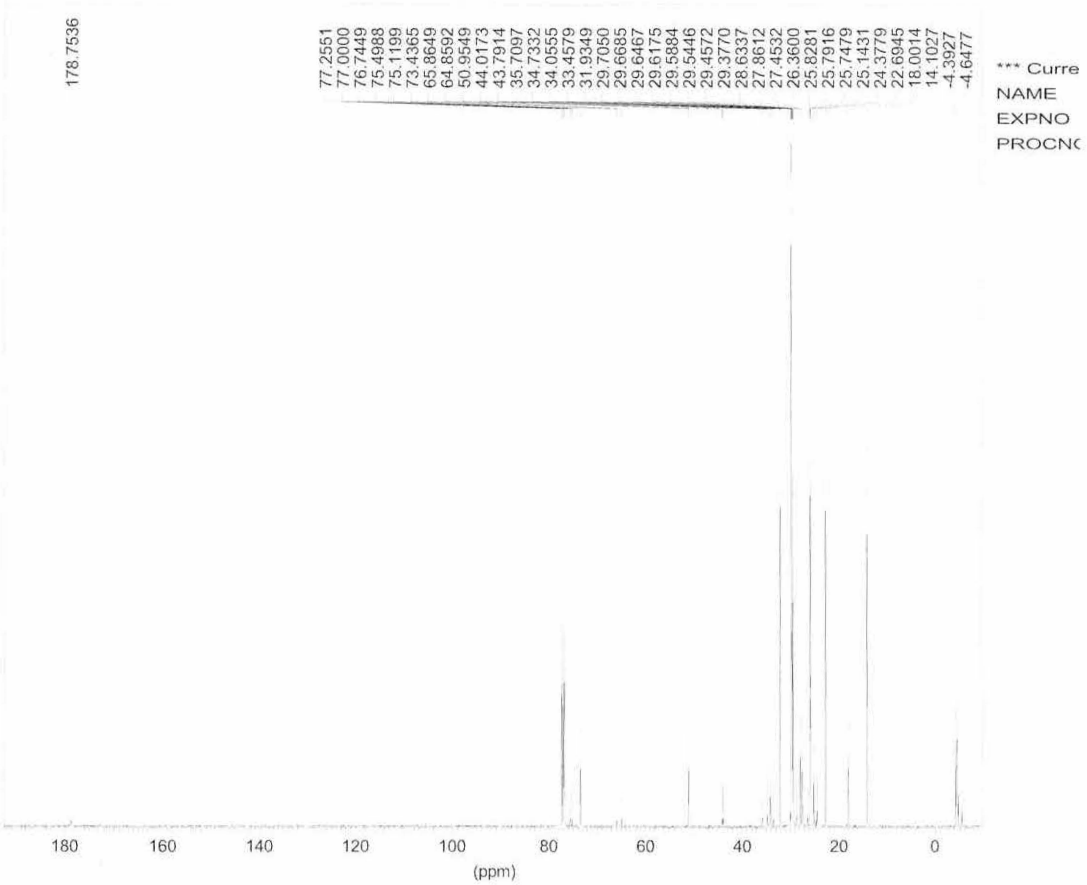


Fig 80. ^{13}C NMR for **104** showing the full spectrum.

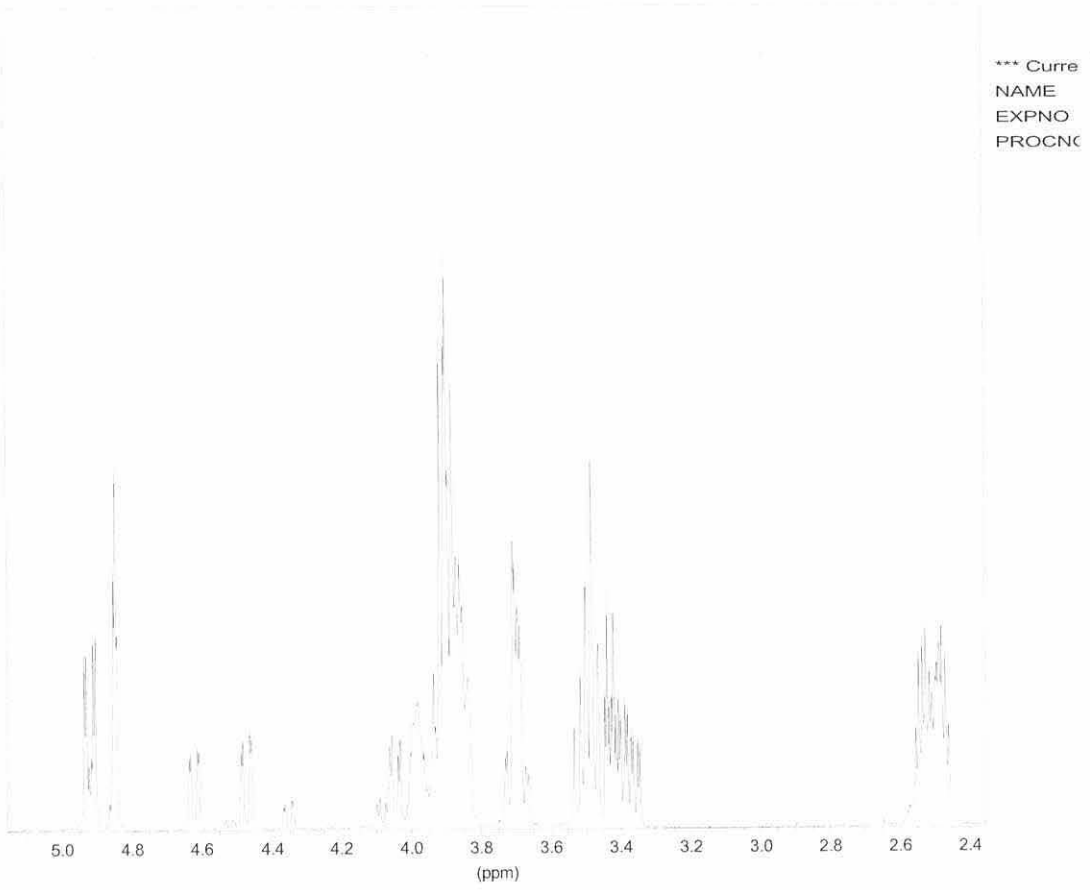


Fig 81. ^{1}H NMR for **105** showing the sugar region on the trehalose core.

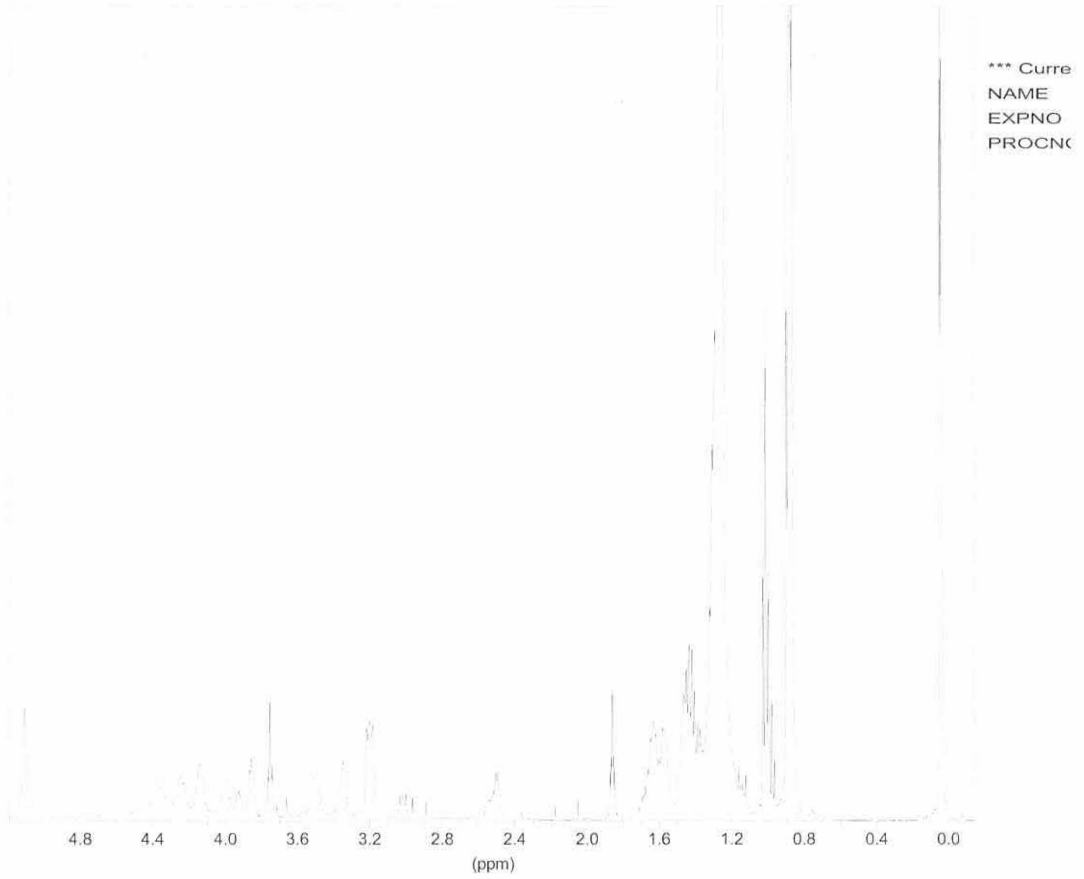


Fig 82. ^{1}H NMR for **106** showing sugar deprotected and hydroxy acid sylil protection.

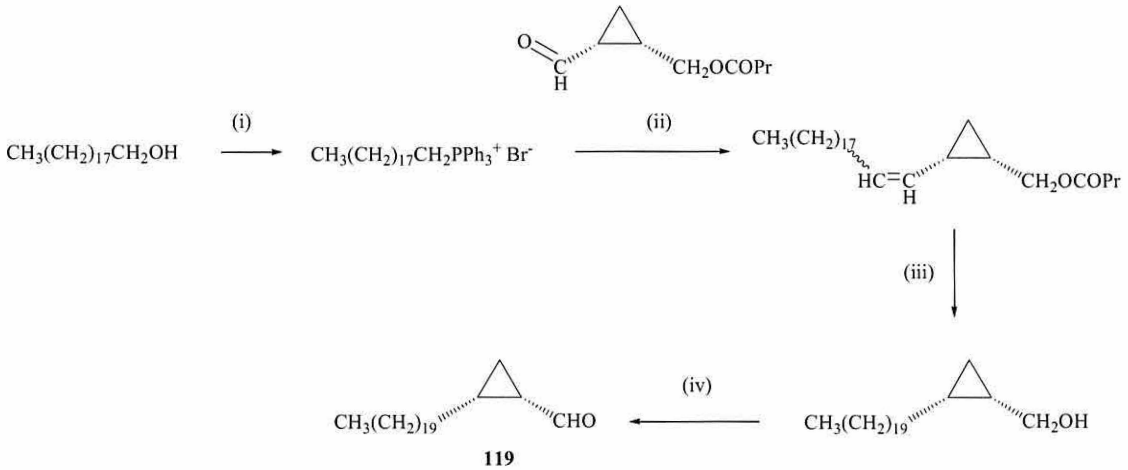


Fig 89. Cis single enantiomers formation following Al Dulyymi's reaction scheme.(i) aq. HBr, Bu4N$^+$ Br$^-$ (99%), PPh_3, toluene (85%); (ii) reaction of aldehyde with the nonadecyltriphenylphosphonium salt in THF, BuLi (81%, 6:1 Z/E), (iii) $LiAlH_4$, THF (95%), N_2H_4, $NaIO_4$, AcOH, $CuSO_4$, *i*-PrOH (80%), (iv) PCC, CH_2Cl_2 (90%).

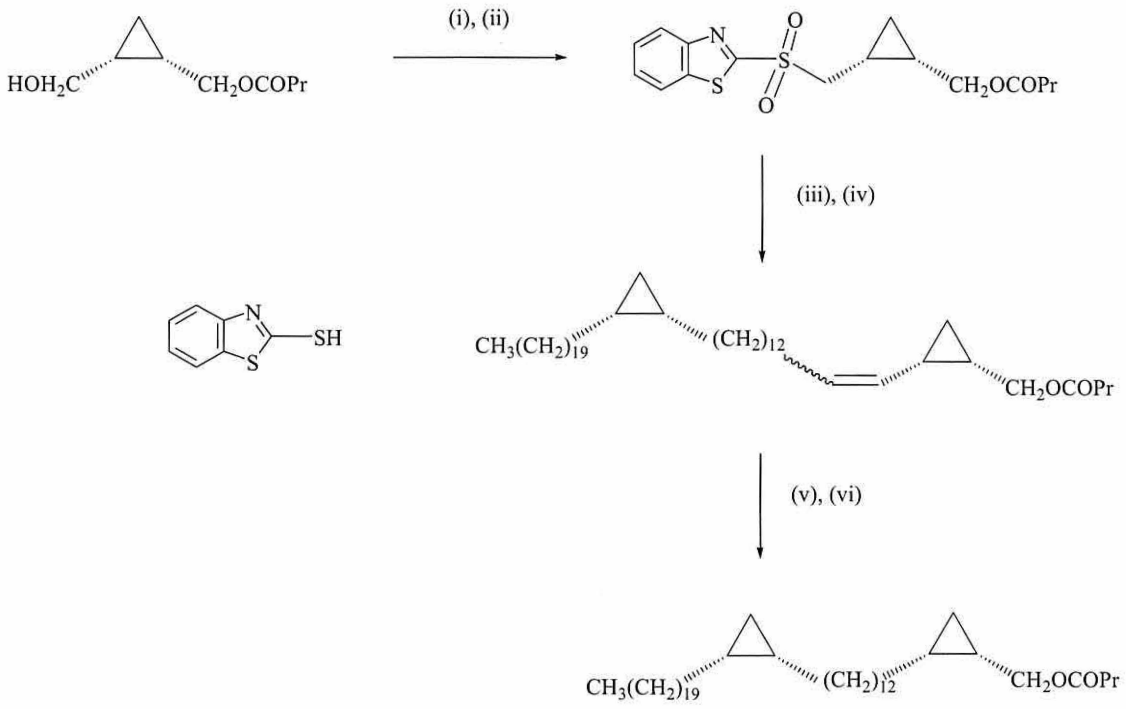


Fig 93. (i) Thiazole, PPh_3, DEAD; (ii) H_2O_2, CH_2Cl_2; (iii) LiHMDS; (iv) compound **120**; (v) $LiAlH_4$; (vi) NH_2NH_2, $NaIO_4$, $CuSO_4$, AcOH, i-PrOH (77%).

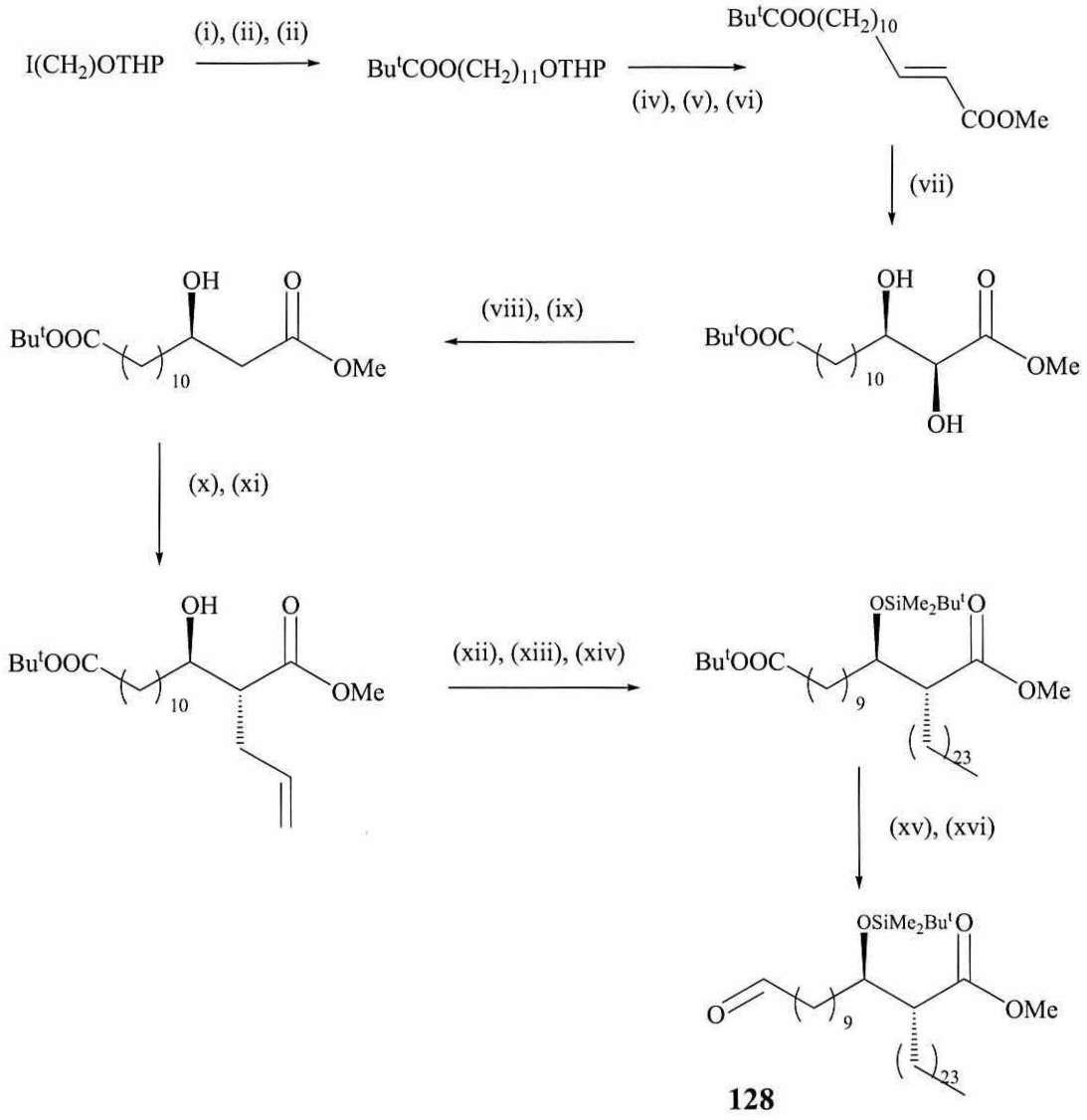


Fig 95. Showing synthesis of **128** after Al Dulyymi *et al.*[226]

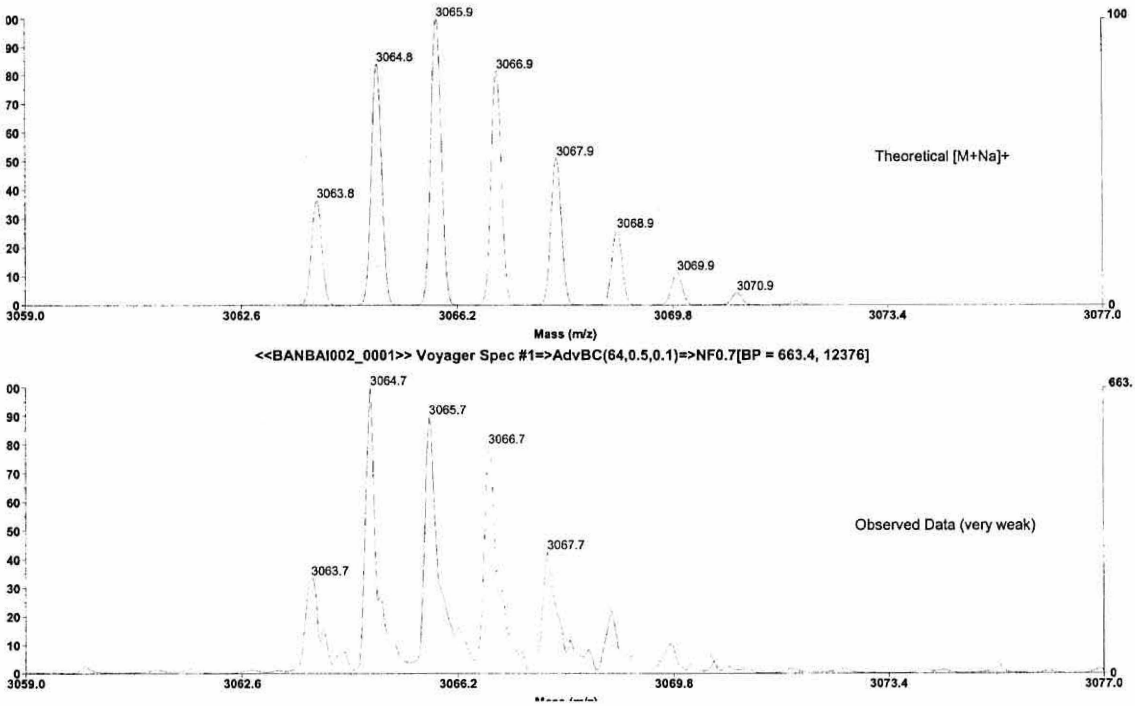


Fig 103. MALDI for compound **138** showing a theoretical prediction and the observed data.

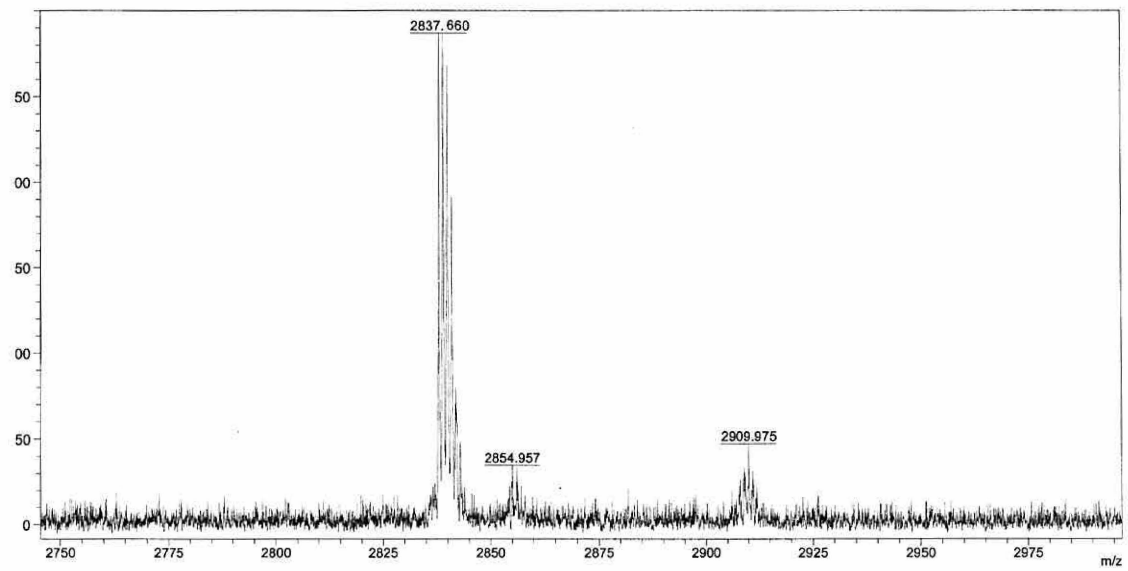


Fig 104. MALDI for compound methoxy TDM **140** showing molecular ion peak.

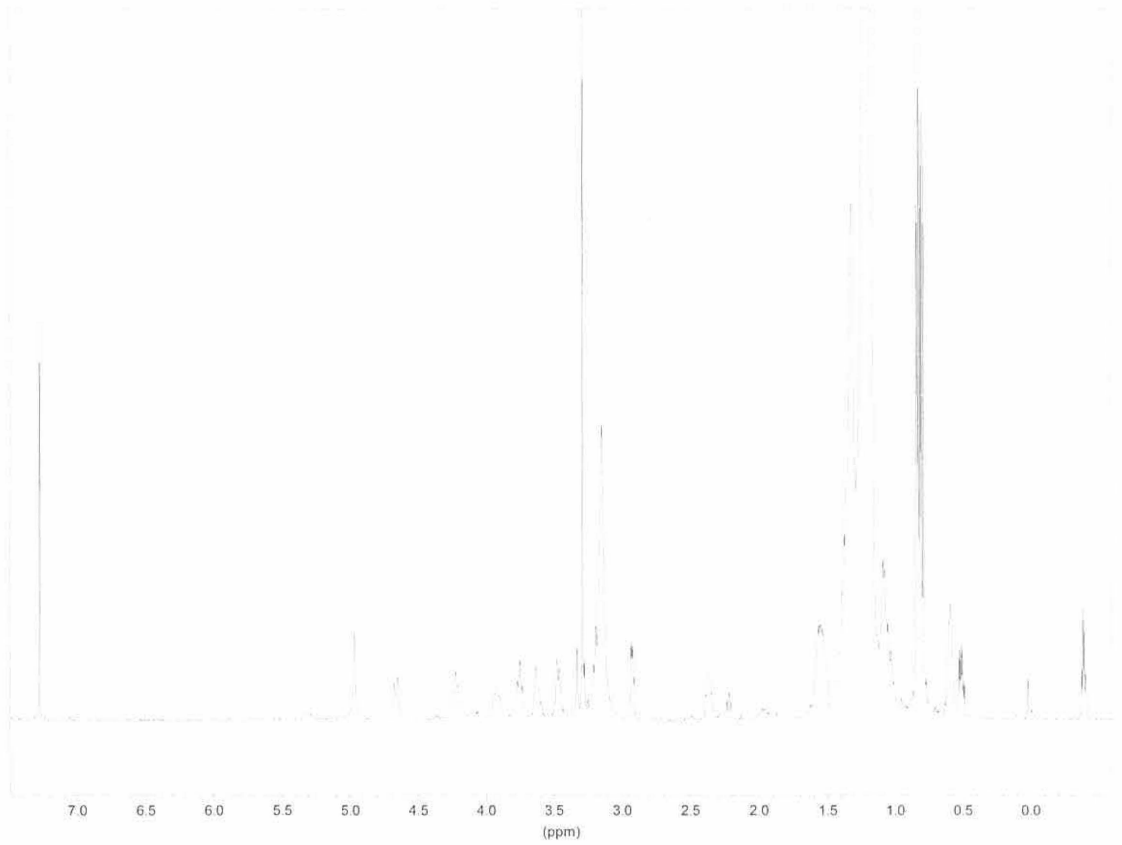


Fig 105. Compound **140** ^{1}H NMR showing the sugar and the mycolic acid region.

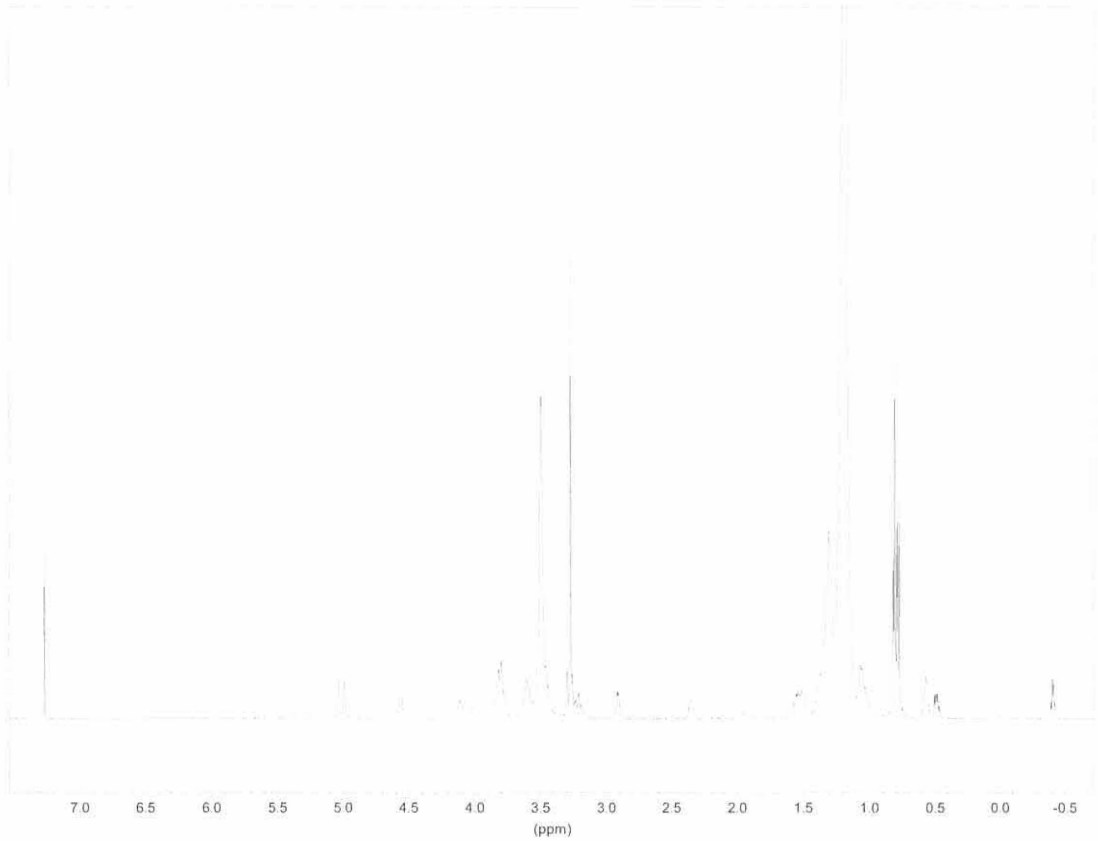


Fig 106. Compound **141** ^{1}H NMR showing the sugar and the mycolic acid region.

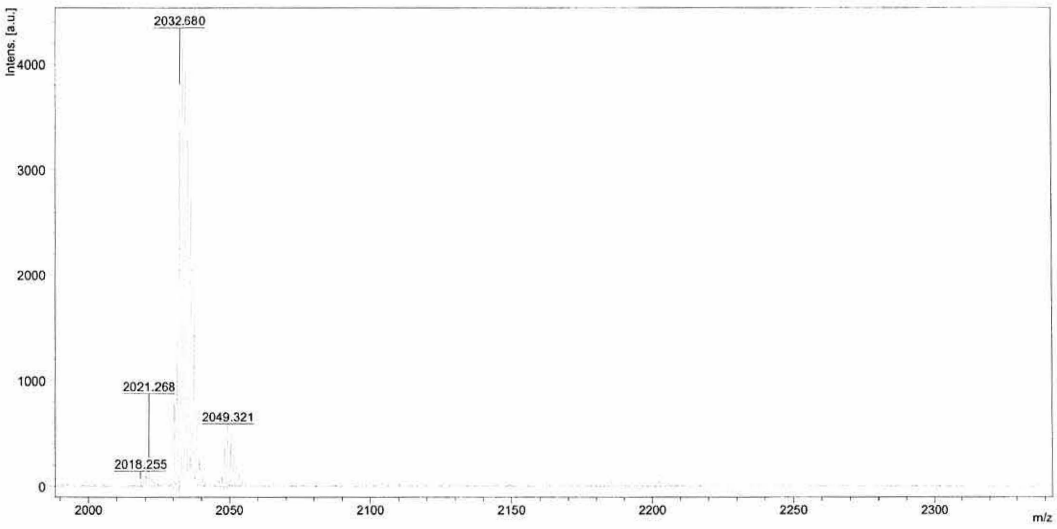


Fig 109. MALDI for **145** showing molecular peak ion.

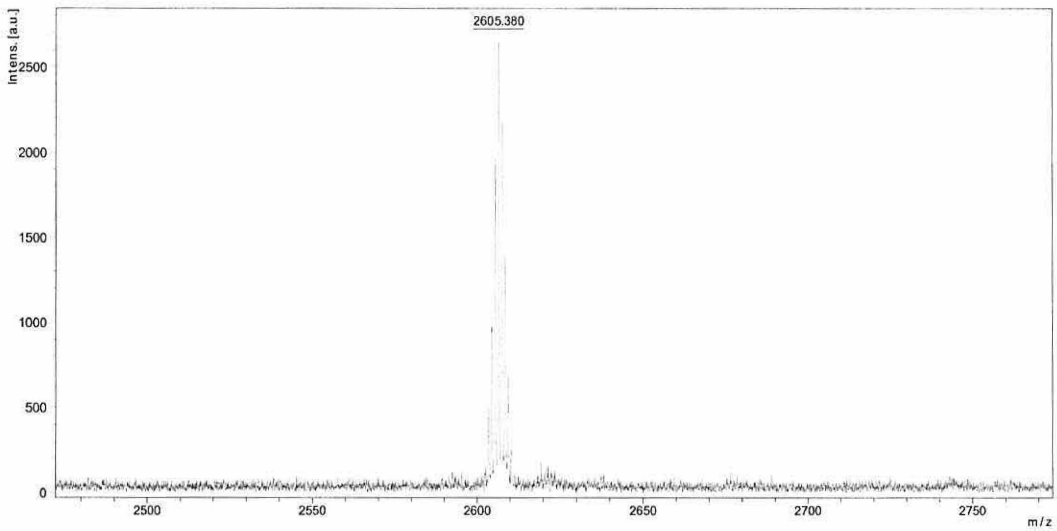


Fig 112. MALDI for **148** showing molecular peak ion.

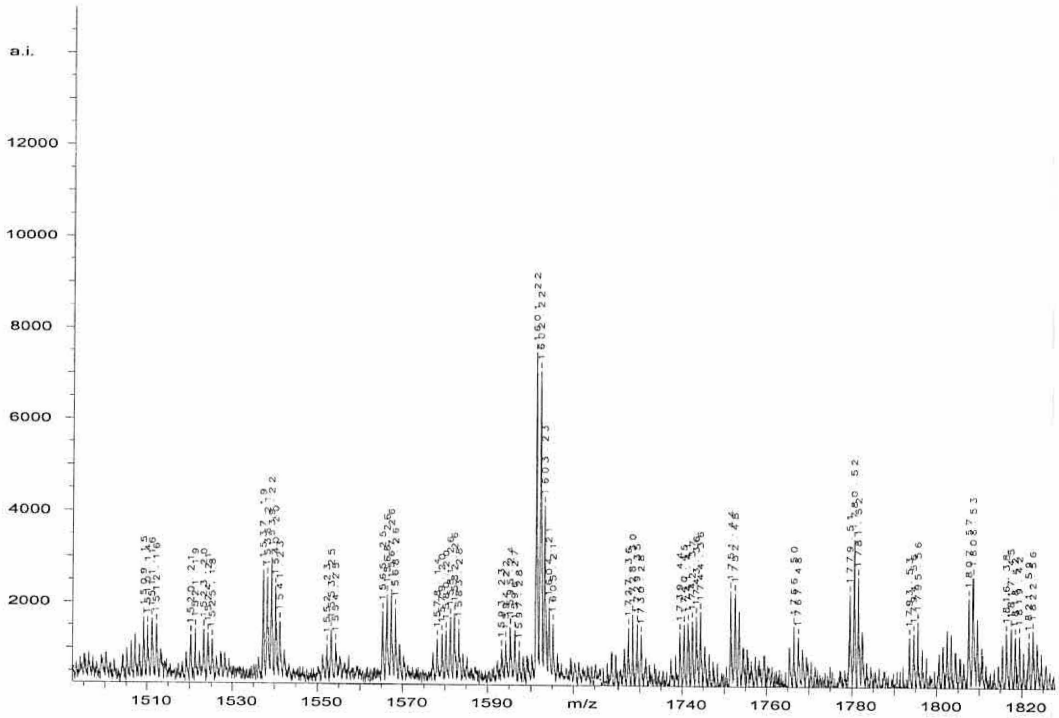


Fig 113. MALDI for crude cord factor obtained from the Sigma catalogue showing molecular peak ion for each compound (region 1510-1820 m/z).

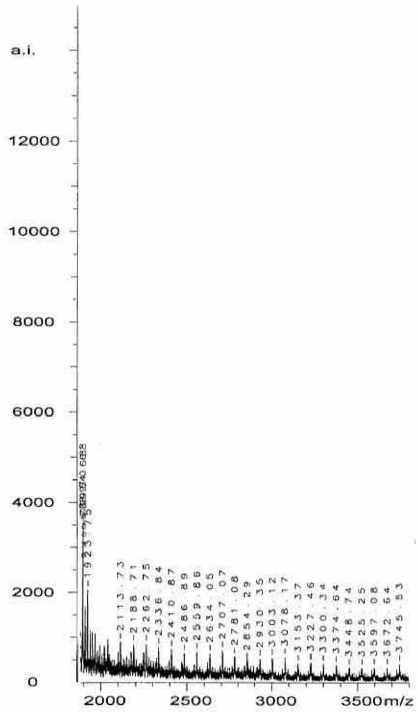


Fig 114. MALDI for crude cord factor obtained from the Sigma catalogue showing molecular peak ion for each compound (region 1900-3900 m/z).

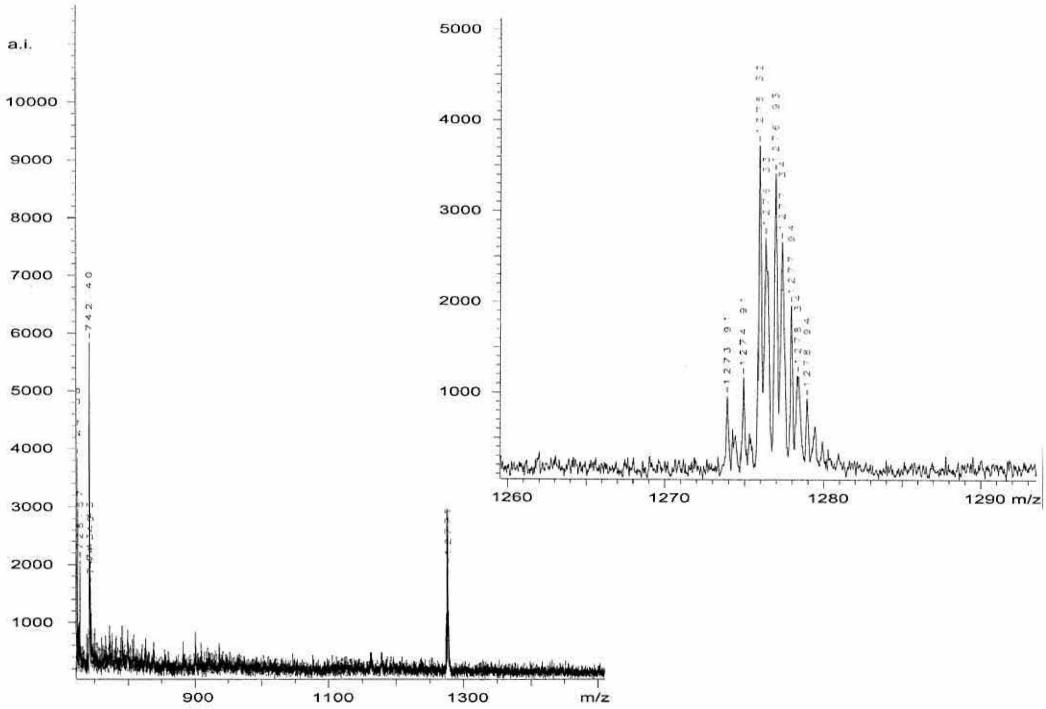


Fig 117. MALDI for compound **155**. The sample includes a zoom on the right hand side showing the molecular peak ion region with closer detail.