

SYNTHESIS AND BIOLOGICAL EVALUATION OF TREHALOSE GLYCOLIPIDS

by

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Abstract

Numerous α,α -trehalose diesters have been isolated from bacteria such as *Mycobacteria* and *Corynebacteria*, and more recently from *Caenorhabditis elegans* dauer larvae. Although these glycolipids are thought to confer protection to the bacteria and larvae against harsh environmental conditions, it is the biological activities of these compounds, including anti-tumour and adjuvant activities, which have been of major interest to scientists over recent years.

In this thesis, three different aspects relating to the synthesis and testing of defined trehalose glycolipids will be presented. First, the synthesis of a variety of fatty acid trehalose diesters (TDEs) with varying lipid lengths was performed and the ability of these glycolipids to activate macrophages was studied. Two different synthetic strategies were employed to attain the TDEs of interest and it was observed that lipid lengths of more than 18 carbons were required for macrophage activation. Furthermore, the C22 fatty acid trehalose monoester (TME) and the C26 TME were also synthesised and interestingly they both showed macrophage activation abilities, with subsequent studies indicating that like TDEs, the TMEs were also ligands for mincle, a C-type lectin found on macrophages. This is the first time that TMEs have been tested for their ability to activate macrophages *via* Mincle. The cytotoxicity of these compounds and subsequent anti-tumour activity of a few selected compounds were also studied and although the TDEs and TMEs did not exhibit any significant cytotoxicity, in *in vivo* models the C10 TDE and C22 TDE both showed anti-tumour activity. This depicts that the mechanism for anti-tumour activity of these compounds is not due to cytotoxicity but due to as yet unidentified pathway.

Methodology that can be applied to the synthesis of more complex trehalose glycolipids, such as trehalose dicorynomycolates (TDCMs, isolated from *Corynebacteria*) and trehalose dimycolates (TDMs, isolated from *Mycobacteria*) was also explored. One of the key steps frequently used in the synthesis of these glycolipids is the Fráter-Seebach alkylation. To improve the efficacy of this methodology allylic iodides, rather than alkyl iodides were used for the α -alkylation of β -hydroxy esters. Our results showed that for all substrates studied, the yield of the α -alkylation was greatly improved when the allylic, rather than the alkyl halide

was used. The use of this methodology in the synthesis of trehalose monocorynomycolate (TMCM) was also investigated.

The third aspect of this thesis focuses on the use of Affinity Based Proteome Profiling (A/BPP) for elucidating the receptors that TDMs bind to upon interacting with host cell. A/BPP focuses on using small molecules which mimic the natural substrate for a particular protein and through the use of 'trap' and 'tag' groups on the molecule the identity of the protein/receptors can be determined. The synthesis of a TDM probe containing a benzophenone 'trap' group and an alkyne 'tag' group will be discussed.

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List of Abbreviations

AcOH	acetic acid	IFN	interferon
ABP	activity based proteome	IL	interleukin
AfBP	affinity based proteome	IMDM	Iscoe's Modified Dulbecco's Medium
ABPP	activity based proteome profiling	Imid.	imidazole
AfBPP	affinity based proteome profiling	IR	infrared spectroscopy
APC	antigen presenting cell	<i>J</i>	coupling constant
aq.	aqueous	LAM	lipoarabinomannan
BCG	Bacillus Calmette-Guérin	LC/MS	liquid chromatography mass spectrometry
BINAP	2,2'-bis(diphenylphosphino)- 1,1'-binaphthyl	LDA	lithium diisopropylamide
BMM	bone marrow macrophages	LM	lipomannan
BMS	borane dimethyl sulfide	LPS	lipopolysaccharide
Bn	benzyl	m	multiplet
BSA	<i>N,O</i> -bis-trimethylsilylacetamide	MCP	monocyte chemoattractant protein
Calcd.	calculated	MS	mass spectrometry
CC	click chemistry	Me	methyl
CD	cluster of differentiation	MHC	major histocompatibility complex
COSY	Correlation spectroscopy	MHz	megahertz
d	doublet	MIC	minimum inhibitory concentration
dd	doublet of doublets	min.	minutes
DC	dendritic cell	Mincle	macrophage inducible C-type lectin
DCC	<i>N,N'</i> -dicyclohexylcarbodiimide	MIP	macrophage inflammatory protein
DCM	dichloromethane	MMP	matrix metalloproteinases
DIAD	diisopropyl azodicarboxylate	Mp	melting point
DIPT	diisopropyl tartrate	<i>m/z</i>	mass to charge ratio
DMAP	4-(dimethyl)-aminopyridine	<i>M. tb</i>	<i>Mycobacterium tuberculosis</i>
DMF	<i>N,N</i> -dimethylformamide	MyD	myeloid differentiation
DMSO	dimethyl sulfoxide	NADPH	nicotinamide adenine dinucleotide phosphate
EDCI	1-ethyl-3-(3- dimethylaminopropyl) carbodiimide	NK	natural killer
eq.	equivalent	NMR	nuclear magnetic resonance
ESI	electrospray ionisation	NO	nitric oxide
Et	ethyl	NOD	nucleotide oligomerisation domain
GMCSF	granulocyte macrophage colony stimulating factor	Obsd.	observed
HepG2	human hepatocellular carcinoma	PAMP	pathogen associated molecular pattern
HDAC	histone deacetylases	PBS	phosphate-buffered saline
HMBC	heteronuclear multiple bond correlation	PCC	pyridinium chlorochromate
HMPA	hexamethylphosphoric triamide		
HRMS	high-resolution mass spectrometry		
HSQC	heteronuclear single quantum coherence		
Hz	hertz		

PDIM	phthiocerol dimycocerosate	TDM	trehalose dimycolate
PGL	phenolic glycolipid	TES	triethylsilane
ppm	parts per million	TFA	trifluoroacetic acid
PRR	pathogen recognition receptor	Tf	trifluoromethanesulfonyl
<i>p</i> TsOH	<i>para</i> -toluenesulfonic acid	THF	tetrahydrofuran
r.t .	room temperature	THP	tetrahydropyran
s	singlet	TLC	thin layer chromatography
sat.	saturated	TLR	toll-like receptor
SDS-	sodium dodecyl sulfate	TMCM	trehalose monocorynomycolate
PAGE	polyacrylamide gel electrophoresis	TME	trehalose monoester of fatty acid
SL	sulfolipid	TMS	trimethyl silyl
t	triplet	TNF	tumour necrosis factor
TAM	tumour associated macrophage	Tr	triphenylmethyl
TB	tuberculosis	VEGF	vascular endothelial growth factor
TBAF	tetrabutyl ammonium fluoride		
TBDPS	<i>tert</i> -butyldiphenylsilyl		
TBS	<i>tert</i> -butyldimethylsilyl		
TDB	trehalose dibehenate		
TDCM	trehalose dicorynomycolate		
TDE	trehalose diester of fatty acid		

1

General Introduction

1.1 Mycobacteria in the treatment of disease: A historical perspective

The use of bacteria in the treatment of disease has been a topic of much interest to the scientific community for over 100 years. In the late 1800s, William Coley was the first to illustrate that bacteria have the potential to be used in the treatment of cancer. In his seminal work, Coley injected streptococcal bacteria into a cancer patient and noted a reduction in the tumour burden.¹ Although this result was encouraging, subsequent injections with streptococcal bacteria alone sometimes resulted in fatalities. Accordingly, Coley switched to using a tumour vaccine containing *Streptococcal pyogenes* and *Serratia marcescens*. This concoction, known as ‘Coley’s toxins’, turned out to be more effective in treating malignant tumors.^{1,2}

Over the next 40 years, Coley treated more than 1000 patients, however, his work was often criticised due to poor documentation and follow up of patients, variability

in the modes of administration, and the inconsistencies in Coley's Toxin preparation. There were 13 different toxin preparations reported, of which some were more effective than others.³ Because of this, Coley's experiments were largely ignored until the late 20th century.

Science has progressed significantly since Coley's original experiments and now it is widely acknowledged that bacteria and bacterial components can be used in disease treatment and in vaccination programmes. For example, inactive and dried *Mycobacterium tuberculosis* (*M. tb*) is a major component of Freund's complete adjuvant system which has proved to be effective in disease treatments. It has shown potential to prevent juvenile-onset diabetes in mouse models⁴ and its use in Parkinson's disease treatments in rat models has also been explored.⁵ However, the use of this adjuvant in humans is forbidden due to its toxicity.⁶

Bacillus Calmette-Guérin (BCG) is the current vaccine in use for tuberculosis (TB) and is made up of attenuated live bovine tuberculosis bacillus, *Mycobacterium bovis*, which has lost its virulence in humans. BCG was first formulated by Calmette and Guérin in 1920,⁷ given to a human infant in 1921, and to date, has been administered to four billion people making it the most widely used vaccine in the world.⁸ Although the vaccine is effective against childhood tuberculosis, meningitis, and miliary tuberculosis, the efficacy of the vaccine is questionable in adult pulmonary tuberculosis where variable results have been obtained.⁹ This could be due to the inability of BCG to impart a memory T cell response which is vital for long-term immunity to TB. To address this problem, adjuvants could be incorporated into vaccines. Currently bacterial cell components known to induce T cell immunity are being tested in vaccine programmes to deduce if they boost the response of the vaccine over a long period of time.¹⁰ BCG is also used as a standard therapy in the treatment of bladder cancer.¹¹

Of particular interest to us is the potential use of mycobacterial cell wall components, from *M. tb* and related mycobacteria, in the treatment of disease. While there is much evidence to suggest that such cell wall components have important immunomodulatory effects,¹² to date, the immunomodulatory properties of distinct (homogenous) cell wall constituents has been poorly documented. Moreover, the

mechanism of action of individual mycobacterial cell wall components is not fully understood.

1.2 Overview

The objective of this thesis is to better understand the immunomodulatory properties of distinct mycobacterial cell wall components with a view that this may lead to better therapies and vaccination protocols against tuberculosis. In particular, the focus of my research relates to the effects of trehalose diesters (which are the major cell wall components of *M. tb*) on the immune response. This may be achieved *via* the synthesis of distinct trehalose diesters of differing lipid lengths using novel Fráter-Seebach alkylation methodology, and the subsequent testing of these glycolipids in a number of biological assays. In addition, modified trehalose dimycolate (TDM) containing functionalised traps (e.g. benzophenone, azides) will be prepared so that the proteins that bind to TDM can be elucidated *via* Affinity Based Proteome Profiling (A/BPP).¹³ The context of this TDM work will be discussed following a brief discussion of *M. tb* and the variety of cell wall constituents that the bacterium contains.

1.3 *Mycobacterium tuberculosis* and related mycobacteria

M. tb is a wax-coated rod-shaped bacillus discovered by Robert Koch in 1882.¹⁴ It is a slow growing, aerobic, acid-fast, non-motile, obligate intracellular pathogen that can infect several animal species, as well as humans. The bacterium divides every 15 to 20 hours which is quite slow compared to majority of other bacteria (*Escherichia coli* divides every 20 minutes).⁸ *M. tb* belongs to the Mycobacterium family in the Corynebacterinae sub-group of the Actinomycete line (Figure 1). The class Actinobacteria under phylum Actinobacteria comprises of Gram positive bacteria with high guanine and cytosine content. Although *M. tb* does not stain with Gram

staining, it is classified under this class due to lack of an outer cell membrane (as in Gram negative bacteria). The sub-order Corynebacteria contains most of the acid fast bacteria like Mycobacteriaceae, Nocardiaceae and Corynebacterineae. Bacteria classified under genus *Mycobacterium* in the Mycobacteriaceae family have a characteristic cell wall which is thicker than those found in other bacteria. In addition, the cell wall is hydrophobic, waxy and rich in mycolic acids and mycolates. The cell wall structure of *Mycobacterium* will be discussed in more detail in section 1.4.

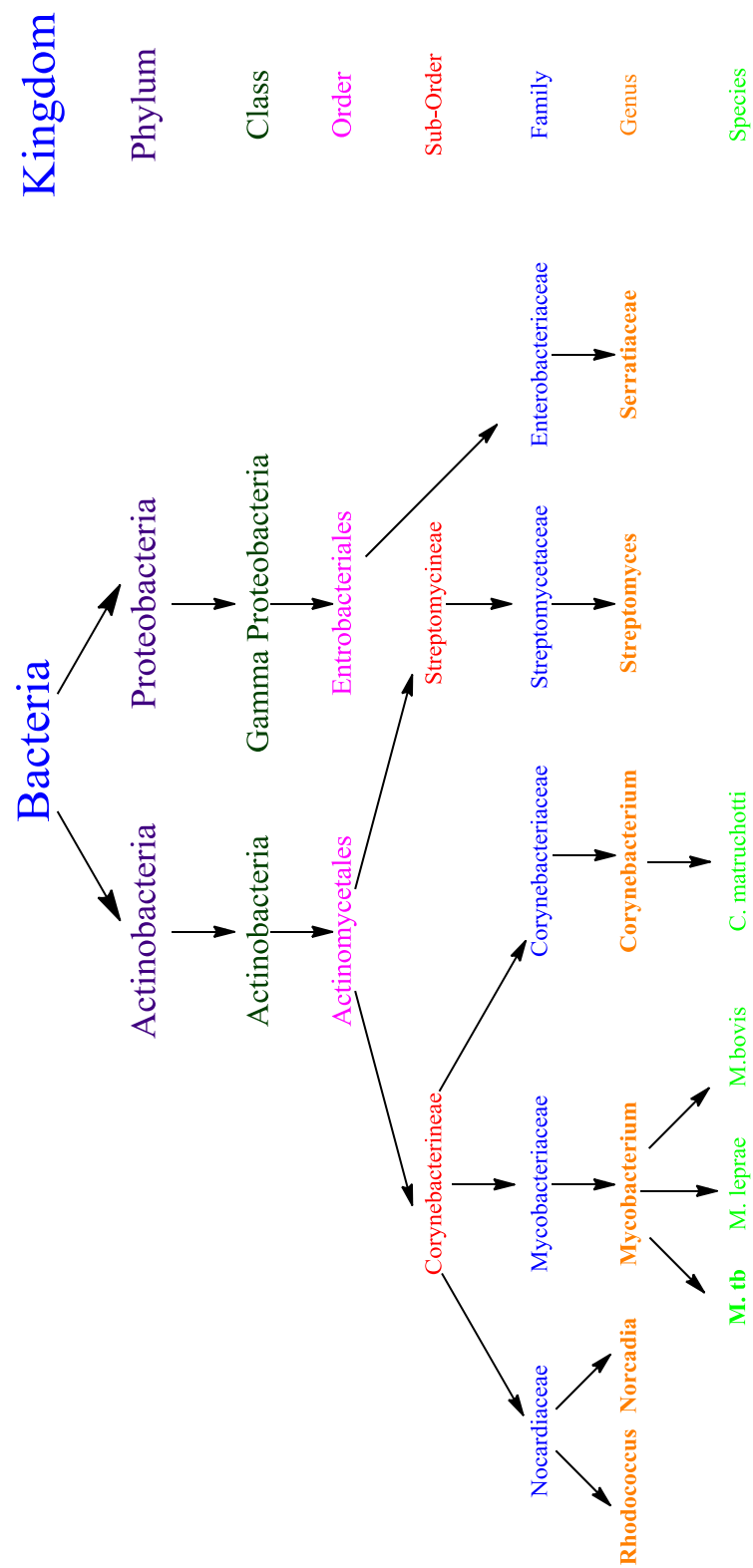


Figure 1. Taxonomic classification of bacteria.

M. tb is responsible for causing TB, a pernicious disease that affects the respiratory system (Figure 2). One third of the world's population is infected by this bacteria¹⁵ that kills about 1.7 million people every year. The occurrence of TB strongly correlates with reduced socio-economic status making TB more prominent in developing countries in Asia and Africa. *M. tb* is also an opportunistic pathogen which is quite prominent in people who are HIV positive, for whom TB is a leading cause of death. In order to treat TB, a cocktail of drugs is required along with very lengthy treatment regimes. In addition, due to the marginal efficacy of the BCG vaccine, the vaccination against *M. tb* has been discontinued in many countries, including NZ, except for those deemed to be in high risk groups. It is hoped that by understanding more about the immunomodulatory effects of the cell wall components of *M. tb*, better TB vaccines can be developed and novel drug targets identified.

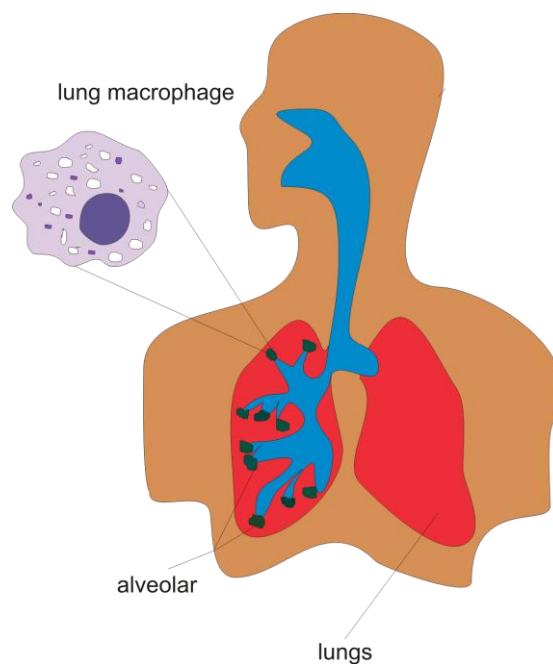


Figure 2. Respiratory system.

1.4 Mycobacterial cell wall components

The ability of *M. tb* to remain in a dormant state in the host organism and evade destruction from drug treatment, chemical injury and the host immune system has been attributed to the outer cell wall structure of *M. tb* that has been deemed crucial in the survival of the bacteria.¹⁶ Made up of various types of lipids, the cell wall structure of *M. tb* is highly complex and contains several key features (Figure 3).^{17,18} Closest to the plasma membrane is the peptidoglycan layer comprising of a highly cross-linked polymer of amino acids, like alanine and glutamic acid, and amino sugars. Protruding from the peptidoglycan layer are arabinogalactan saccharides which contain linear galactan and branched arabinose motifs. The arabinose chains are then linked to mycolic acids which are primarily present in two forms in the cell wall: covalently bonded to arabinogalactan, and as esters of trehalose giving rise to trehalose dimycolate (TDM), the most abundant glycolipid present in the cell wall of *M. tb*. The cell wall also contains phthiocerol dimycocerosate (PDIM) and phenolic glycolipids (PGL) (not shown), along with lipoarabinomannan (LAM) which consists of a phosphoryl-inositol-mannose (PIM) anchor and a polysaccharide backbone. The individual cell wall glycoconjugates are discussed in more details as follows.

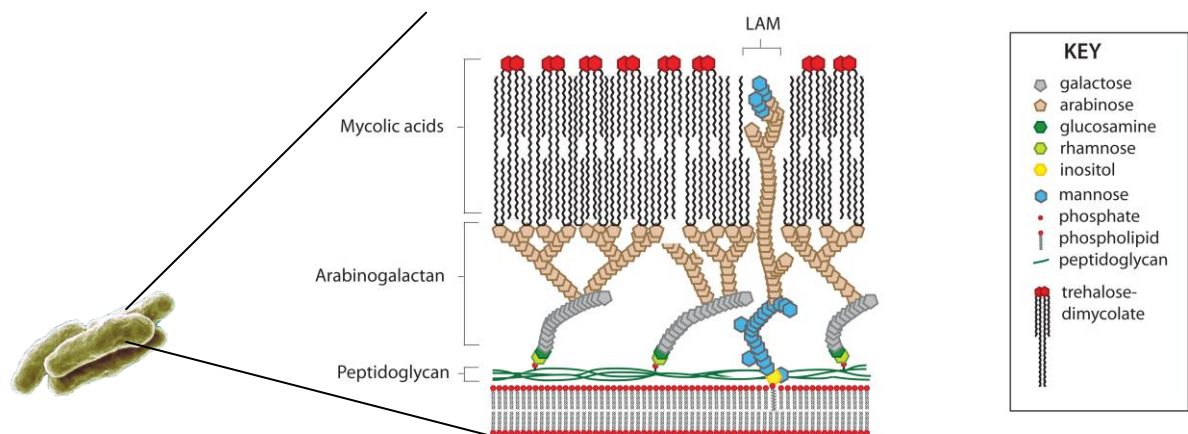


Figure 3. Cartoon of *M. tb* cell wall.

1.4.1 Phosphoryl-inositol-mannosides (PIMs)

M. tb contains several phosphoryl-inositol-mannosides (PIMs) structures (**1**, Figure 4) including PIM₁ (where the inositol residue of PI [phosphatidyl-*myo*-inositol] is mannosylated at the C-2 position), PIM₂ (where PIM₁ is further mannosylated at C-6 of inositol).¹⁹ Further α -1,6 mannosylation of PIM₂ gives rise to PIM₃ and PIM₄ the common precursor of PIM₅ and PIM₆ (made by further α -1,2 mannosylation of PIM₄). In addition, the core PI unit is found as a unique entity on its own. The synthesis of naturally occurring PIMs and analogues has been undertaken and many elegant syntheses have been reported. (For a recent review see ref.²⁰)

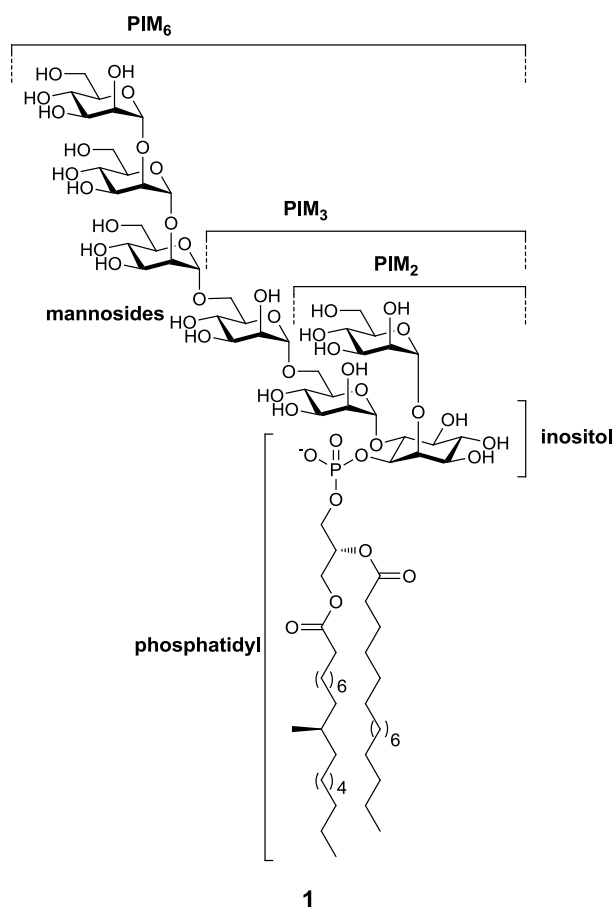


Figure 4. Structure of PIMs (**1**).

PIMs exhibit both immunosuppressive and adjuvant activities. The ability of PIMs to induce cell mediated immunity, particularly, a Th1 type immune response illustrates

their potential to be used as adjuvants in sub-unit vaccines.^{21,22} In a study by Painter and co-workers, a synthetic analogue of PIM₆ was shown to induce DC-mediated suppression of human CD8⁺ T cell proliferation, which can enhance the survival of *M. tb* when resident in the host cell.²³ PIM₆ has also been found to interact with DC-SIGN, a receptor on dendritic cells. DC-SIGN has also been shown to be regulated following host infection with *M. tb*, thus suggesting that PIMs could potentially be involved in the virulence and pathogenesis of *M. tb*.²⁴

1.4.2 Lipoarabinomannan (LAMs)

PIMs are common precursors for more complex mycobacterial cell wall components like lipomannan (LM, **2**, Figure 5), lipoarabinomannan (LAM, **3**) and mannan capped lipoarabinomannan (ManLAM). PIM₄ is the key biosynthetic intermediate whereby α -1,6 linked mannose to the PIM₄ backbone leads to mature branched lipomannan, which undergoes subsequent arabinosylation to form lipoarabinomannan and further mannosylation of LAM leads to ManLAM.²⁵ The use of LAM as a diagnostic tool for tuberculosis was investigated by Sada *et al.*, whereby they noted the presence of LAM in the serum of most patients with pulmonary tuberculosis, indicating that the detection of LAM is an accurate diagnostic tool for pulmonary tuberculosis.²⁶ In another study, purified LM, LAM and PIM₁ from a cell wall fraction of *M. bovis* BCG Tokyo-172 strain were examined for their ability to induce differentiation of human peripheral blood naive CD4 T cells into Th1 and Th2 cells.²⁷ The results showed that LAM/LM enhanced T cell differentiation under both Th1 and Th2 conditions, since both Th1 [e.g. interleukin (IL)-2, tumour necrosis factor (TNF)- α , interferon (IFN)- γ] and Th2 (e.g. IL-4, IL-5) cytokines were observed, indicating that LAM/LM possess a potent modulating activity in human systems and could thus be used in vaccine development for both Th1 and Th2 diseases.

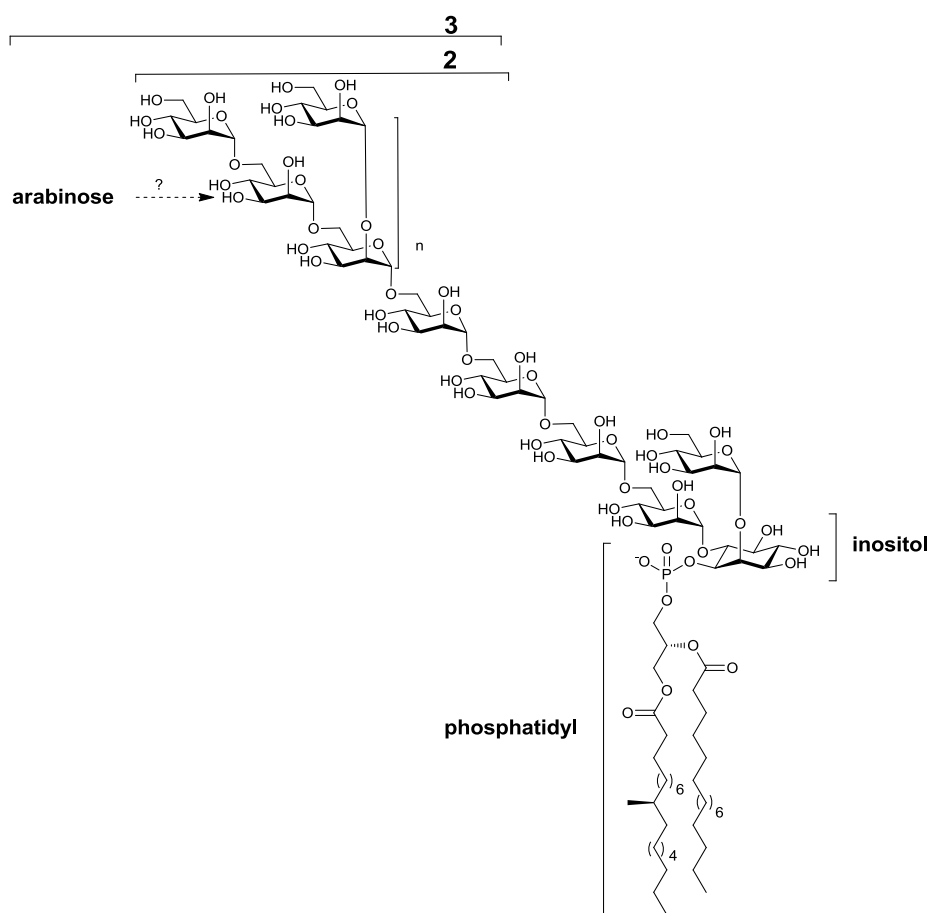


Figure 5. LM (2) and LAM (3) structures.

1.4.3 Phthiocerol dimycocerosate (PDIMs)

Phthiocerol dimycocerosate (PDIM) (4, Figure 6) and its derivatives consist of a long chain β -diol (phthiocerol), which occurs naturally as diesters of polymethyl branched fatty acids (mycocerosic acid), and have been found in all pathogenic *Mycobacteria* except *M. gastri*.²⁸ The methylated branched asymmetric centres can be of L or D configuration depending on the strain of mycobacterium. For instance, in *M. marinum*, the configuration of the asymmetric centres is L, whereas in *M. leprae* and *M. tb* they are of the D configuration.²⁹

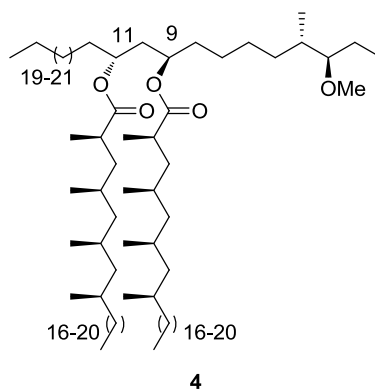


Figure 6: PDIM (**4**) from *M. tb*.

The ester groups present at carbons 9 and 11 in PDIMs can have either an *anti* or *syn* relationship, again depending on the mycobacterial strain. For PDIMs isolated from *M. tb*, *M. leprae*, *M. bovis* and *M. kansasii* the esters are *anti* to each other (**5**), whereas those obtained from *M. marinum* (**6**) and *M. ulcerans* (**7**) display a *syn* relationship (Figure 7).³⁰ PDIMs have also been found to be involved in the pathogenesis of *M. tb*, as illustrated by mutant strains of *M. tb* without PDIMs in the cell wall which exhibit higher cell permeability, thus making them more vulnerable to detergents and the host immune response.³¹

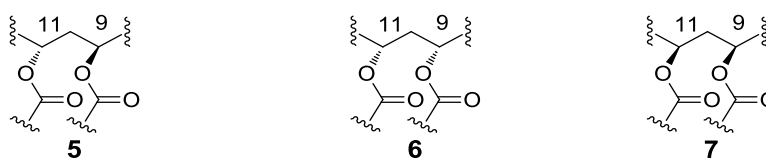


Figure 7: Stereochemistry of esters in PDIM from various mycobacterial species.

1.4.4 Phenolic glycolipids (PGLs)

Structurally related to PDIMs are phenolic glycolipids (PGLs) where the lipid core is ω -terminated by an aromatic nucleus, which in turn is glycosylated by different saccharide units leading to a variety of phenolic glycolipids. The structures of PGLs from different mycobacterial species differ in the sugar moiety attached to the

aromatic nucleus.³⁰ PGL (8) from *M. leprae* and PGL (9) from *M. tb* each contain trisaccharide sugar units (Figure 8). Similarly, PGL from *M. bovis* and *M. marinum* both have a single rhamnose sugar that only differs in its methylation pattern. PGLs from *M. tb* have been shown to inhibit the innate immune response in the host organism and thus play an important role in the virulence of TB.³²

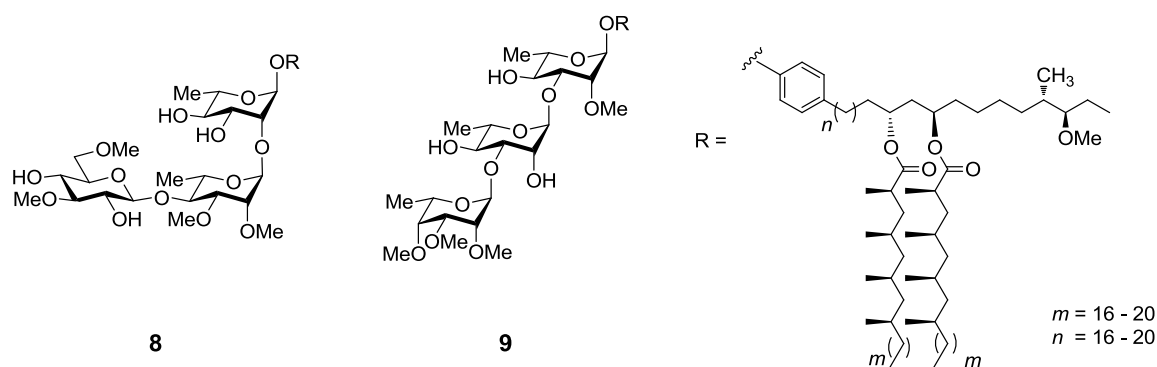


Figure 8: PGL from *M. leprae* and *M. tb*.

1.4.5 Trehalose dimycolates (TDMs)

As alluded to before, trehalose dimycolates (TDMs, 10, Figure 9) are the most abundant glycolipids found on the cell wall of *M. tb* and were first discovered in the bacterium in the early 1950s when it was observed that virulent strains of *M. tb* expressed a material named “cord factor” which was essential for the pathogenesis of the bacteria.³³ It was not until 1956, that “cord factors” were characterised as TDMs and since then a vast number of studies have looked at the structural requirements and biological activities of this class of compounds.^{12,34,35}

Amongst the different mycobacterial species, the variability of these glycolipids is apparent both in terms of the type and the amount of individual TDMs present.³⁴ TDMs are characterised by the presence of a mycolic acid (11, Figure 9) and a trehalose moiety, with the mycolic acid consisting of at least two chiral centres, α and β to the carboxylate, both of *R* configuration.^{36,37} The lipid chain α to the carboxylate is known as the α branch, while the other portion of the mycolic acid is termed the meromycolate. Among different TDMs, the α branch of the mycolic acid varies only in the number of carbons present. The meromycolate region, however, is

more variable with different functionalities present.³⁸ Mycolic acids are generally divided into two groups - the α -mycolates, which have no oxygenated functional groups, and the oxygenated mycolates. The most common mycolic acids are the α -mycolates in which *cis*-cyclopropanes are present in the meromycolate branch. The oxygenated mycolates include methoxy mycolates, keto mycolates and epoxy mycolates.¹⁸ The biological activity and synthesis of TDMs and related trehalose 6,6'-diesters will be discussed in more detail later (Section 1.5.3).

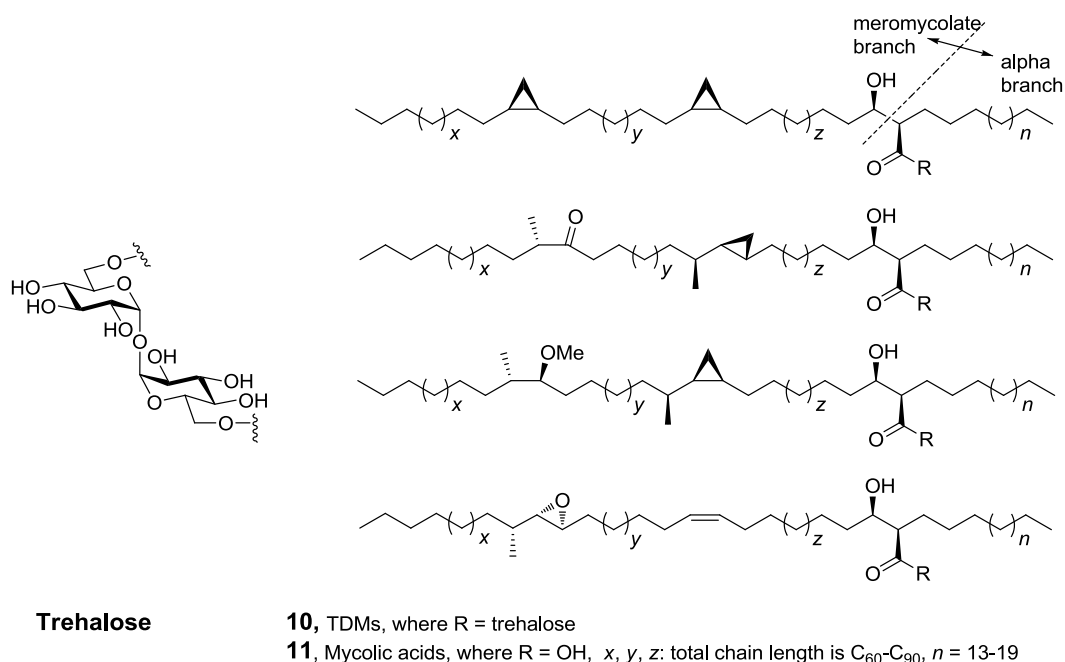


Figure 9. TDMs (**10**) and mycolic acids (**11**).

1.4.6 Other Trehalose Glycolipids

A second class of trehalose glycolipids found in virulent strains of mycobacteria are termed sulfolipids (*e.g.* SL-1, **12**, Figure 10), to account for the presence of sulfate esters.³⁹ Although first isolated in 1959, the structure of these sulfolipids was not elucidated until 1970 when Goren *et al.* characterised them as 2,3,6,6'-tetraesters of trehalose-2'-sulfate.⁴⁰ It was not until 2004 that diacyltrehalose sulfates (**13**), the biosynthetic precursors for SL-1, were isolated from *M. tb* independently by Domenech *et al.*⁴¹ and Gilleron *et al.*⁴²

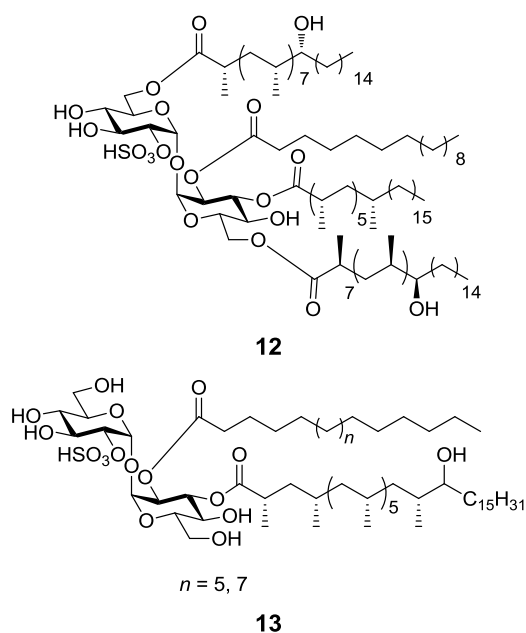


Figure 10. Sulfolipids.

1.5 Trehalose 6,6'- diesters

1.5.1 Overview

In addition to TDMs, sulfolipids, and diacyl trehalose sulfates found in mycobacteria, a number of other trehalose diesters have been observed in other bacterial species, and recently in *Caenorhabditis elegans*. Notably, these include the trehalose diesters of fatty acids (TDEs) (including maradolipids) and the trehalose dicorynomycolates (TDCMs). Collectively, these can be defined as trehalose 6,6'-diesters, whereby two esters are present on the 6 and 6'-positions of the trehalose moiety, as opposed to the 2,3-trehalose diesters (as in the case of diacyl trehalose sulfates) or the 2,3,6,6'-tetraesters (SL-1). The focus of this thesis concerns the synthesis and biological activity of trehalose 6,6'-diesters, which will be discussed in more detail below.

1.5.2 Trehalose diesters of fatty acids (TDEs)

1.5.2a Introduction

Trehalose diesters of fatty acids (TDEs) (**14**, Figure 11) are the simplest glycolipids found in the trehalose 6,6'-diester series and can be differentiated by lipid length and the branching of the fatty acids. Historically, linear TDEs have been studied for their anti-tumor^{43–47} and anti-bacterial activities,^{48,49,50} however, it is only in recent years that the presence of these compounds has been detected in the animal kingdom.⁵¹ The isolation of the palmitic acid diester of trehalose (**14**, $n = 14$), from *Mycobacterium fortuitum* was also reported in the 1960s.⁵² Branched TDEs form an integral part of the diacyl trehalose isolated from dauer larvae of *C. elegans* (commonly known as maradolipids, **15**), while linear TDEs and cyclopropanated trehalose diesters are also found in maradolipid extracts, albeit in smaller quantities.⁵¹

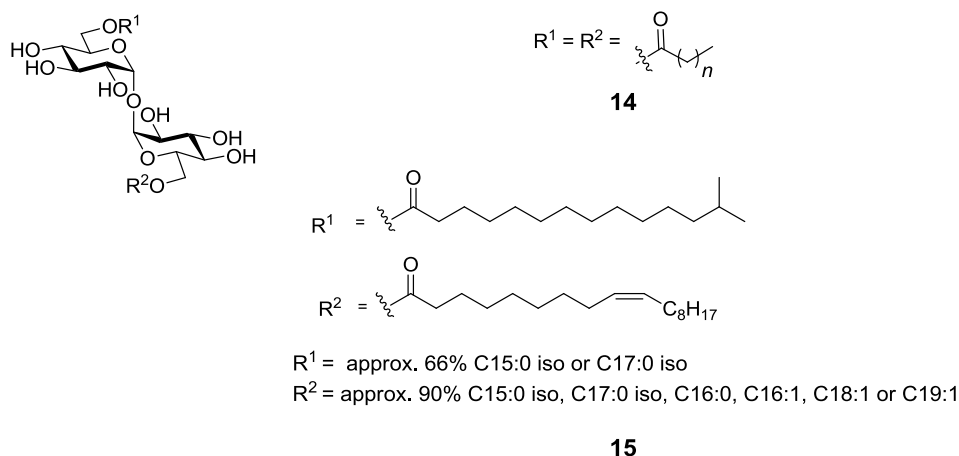


Figure 11. TDEs (**14**) and maradolipids (**15**).

Of the TDEs, perhaps the most noteworthy family member is the C22 homologue, trehalose dibehenate (TDB), which has recently been shown to bind to and activate macrophages in a similar way to its more complex counterpart, TDM. TDB binds to Macrophage Inducible C-type Lectin (mincle, Figure 12), a calcium dependent receptor that possesses a carbohydrate identifying domain in its extracellular

region,^{53,54} and activates macrophages through a FcRgamma-Syk-Card9-dependant pathway. These studies have important implications as they provide insight into the mechanism of action of TDEs, which in turn will aid in the development of better therapeutics for cells of the myeloid lineage. Toll-like receptor (TLR)-2 and dectin-1 are examples of other surface membrane receptors found on immune cells which recognise certain bacteria, fungi and exogenous substances.

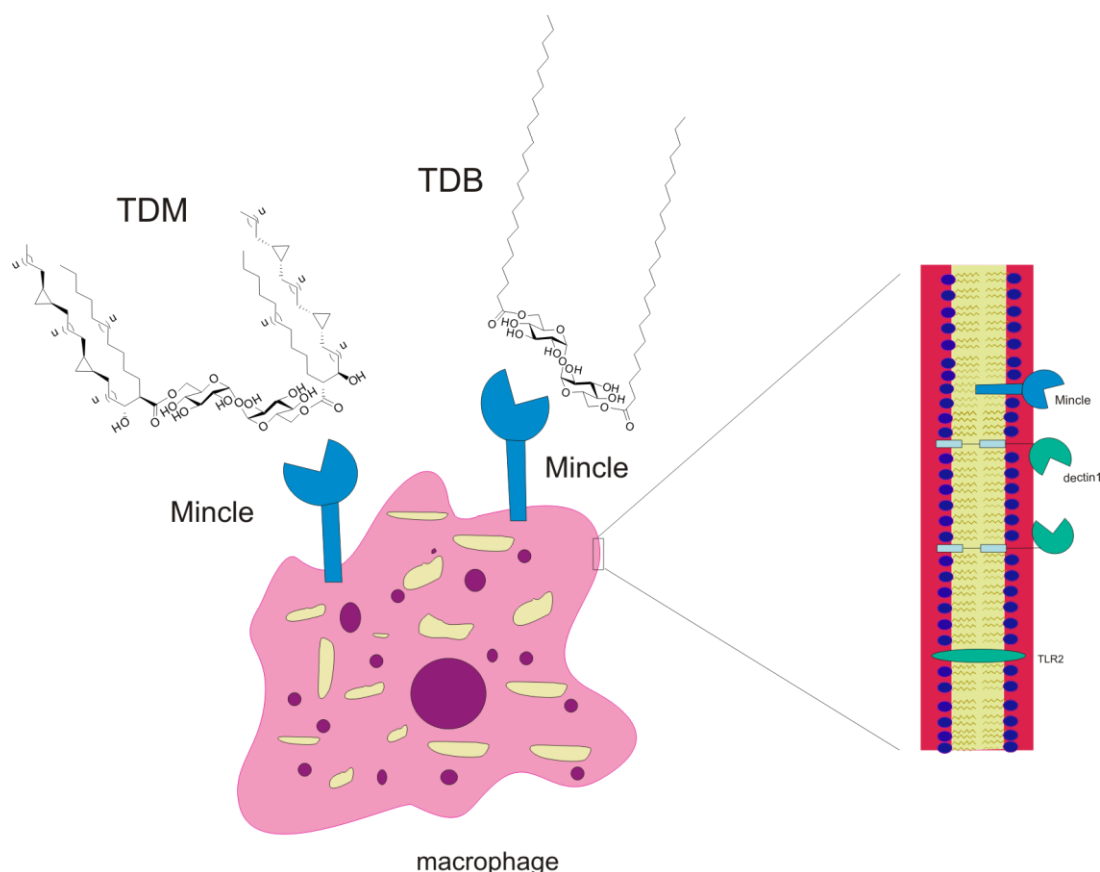


Figure 12. Cartoon of Mincle interacting with TDM and TDB.

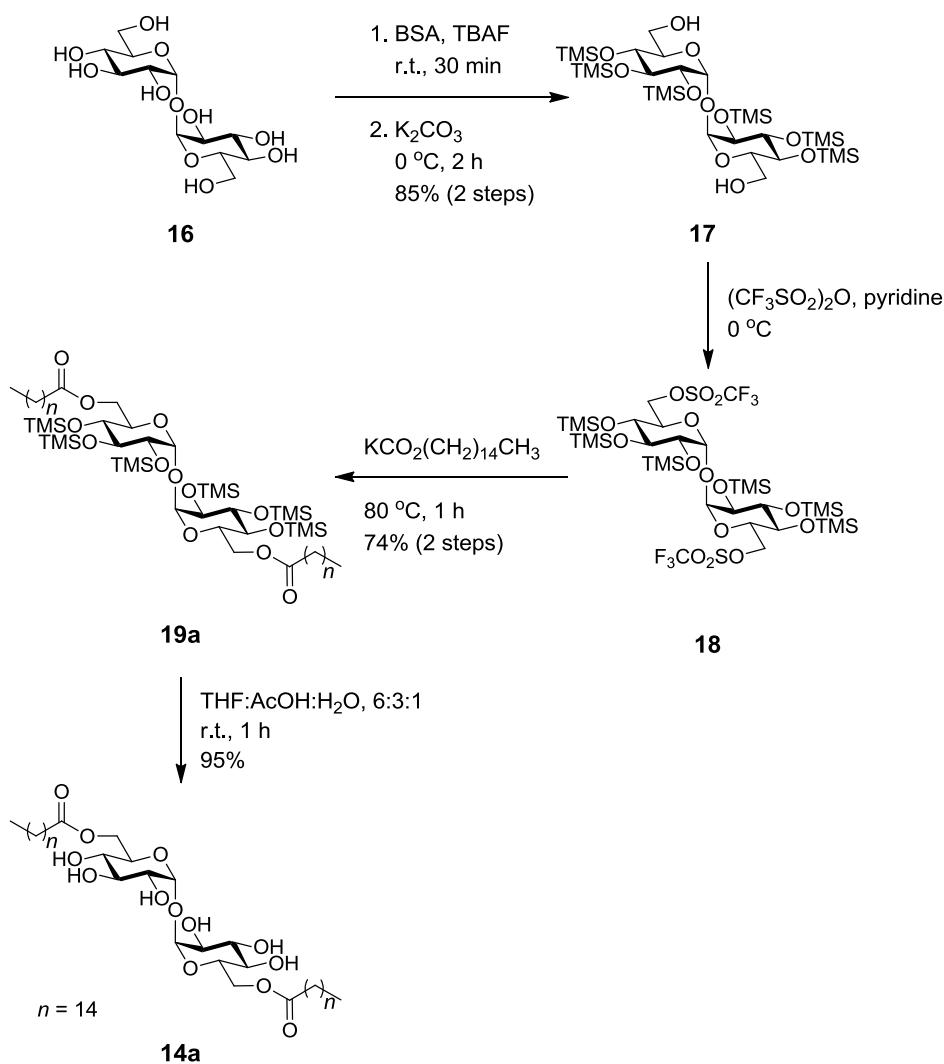
1.5.2b Linear TDE syntheses

Various synthetic methodologies for TDE syntheses have been developed over time to gain access to these intriguing diesters. One of the first synthetic methods reported was the potassium carbonate mediated trans-esterification of fatty acid methyl esters with trehalose.⁵⁵ This reaction, however, was not selective with various other mono, di- and tri-esters also being formed. The synthesis of TDEs has also been reported

whereby 6,6'-ditosylate trehalose was subjected to esterification conditions with the potassium salt of palmitic acid,⁵⁶ based on methodology developed previously.⁵⁷ This synthetic strategy posed a few difficulties including the formation of 3,6-anhydro-trehalose products and other by-products that were difficult to separate from the desired TDEs.⁵⁸ Toccane⁵⁹ and Toubiana *et al.*⁶⁰ independently proposed the use of a 6,6'-dihalo-6,6'-dideoxy trehalose derivative for the synthesis of trehalose diesters to eliminate these side reactions. The esterification reaction could also be improved by using 2,2',3,3',4,4'-hexa-*O*-benzyl-trehalose as an intermediate.⁵⁸

In 1983, Bottle and Jenkins reported on a new method for the synthesis of TDEs *via* the reaction of trehalose with fatty acids in the presence of diisopropyl azodicarboxylate (DIAD) and triphenyl phosphine under exceptionally mild conditions.⁶¹ In their seminal work, unprotected trehalose and palmitic acid were subjected to the Mitsunobo reaction⁶² to yield 6,6'-trehalose dipalmitate in 59% yield.⁶¹ Later, Jenkins and Goren extended this methodology for the synthesis of more complex TDMs.⁶³

In the continual search for the optimum conditions for the synthesis of TDEs, the use of 2,2',3,3',4,4'-hexa-*O*-trimethylsilyl trehalose as an intermediate for the synthesis of dipalmitate trehalose was reported.⁶⁴ This intermediate was appealing due to the ease in which labile trimethylsilyl (TMS) protecting groups could be removed after the esterification reaction. In the initial studies by Toubiana *et al.*, hexa-*O*-trimethylsilyl trehalose was synthesised in 2-steps from commercially available α,α -trehalose, while in the later work by Johnson, an elegant 2-step, one pot synthesis of hexa-trimethylsilyl trehalose was developed,⁶⁵ and later used for the synthesis of trehalose dipalmitate **14a** (Scheme 1).⁶⁶ Here, α,α -trehalose (**16**) was persilylated using *N,O*-bis(trimethylsilyl)acetamide (BSA) and tetra-butylammonium fluoride (TBAF) and the more labile primary TMS ethers were removed *via* the addition of potassium carbonate to the same reaction vessel to give **17** in good (85%) yield. Diol **17** was then converted to triflate **18** and condensed with the potassium salt of the acid to give the fully protected diacyl trehalose **19a**, which was deprotected under acidic conditions to provide trehalose dipalmitate **14a** in good overall yield.



Scheme 1. Trehalose dipalmitate synthesis.⁶⁶

1.5.2c Biological Activity of linear TDEs

The anti-tumour activity of TDEs has been studied for many years and though it is difficult to deduce with certainty which factors are responsible for good anti-tumour activity due to the different ways in which the assays were performed, several general conclusions can be drawn. Lipopolysaccharide (LPS) has been shown to enhance the anti-tumour activity of the linear TDMs in *in vivo* tumour assays,^{44–47} and the co-presence of an oil vehicle also appears to enhance the response.^{67–70} The importance of the acyl chain length was seen in an *in vivo* tumour assay, where an

acyl chain of more than 10 carbons was essential to induce anti-tumour activity in an Ehrlich ascites carcinoma model.⁴³ The most common tumour model employed in these studies was the rat hepatoma cell line.

The anti-bacterial activity of linear TDEs has also been studied by several groups and varying results obtained depending on the bacterial strain used. When tested against *Klebsiella pneumoniae* and *Listeria monocytogenes* in mice, the C22 diester as well as the C16 diester did not confer any protection.⁴⁸ However, when tested on *Schistosoma mansoni*, the C16 diester conferred protection, whereas no protection was seen with the C22 diester.⁴⁹ Similarly, the C16 diester and monoester were seen to confer protection against *Salmonella typhi* and *Salmonella typhimurium*.⁵⁰ In all studies, the glycolipids were formulated in oil and administered to the mice as an emulsion. In the case of *Schistosoma mansoni* and *Salmonella* challenges, the mice were pre-treated with the TDEs before being challenged with the bacteria, whereas, in the *K. pneumoniae* and *L. monocytogenes* studies, the mice were first challenged with the infection and then the TDEs were introduced. C16 and C22 diesters of trehalose have also shown potential to be used in the serodiagnosis of tuberculosis.⁷¹

The singularly most studied glycolipid in the TDE family is the C22 diester of trehalose, TDB. In addition to the aforementioned anti-tumour and anti-bacterial studies, TDB has been combined with dimethyldioctadecylammonium bromide (DDA), a cationic surfactant to produce a liposome adjuvant system which induces a strong cell-mediated and antibody response,⁷² and thus could be an effective adjuvant in *M. tb* subunit vaccines.⁷³ Indeed, such cationic liposomes are now in Phase I clinical trials for their use during TB vaccination.⁷⁴

1.5.2d Maradolipid Syntheses

Maradolipids are diacyltrehalose glycolipids containing linear, branched and cyclopropanated fatty acid chains, observed in the dauer larvae of *C. elegans*.⁵¹ The lipid composition and the relative abundances of the lipids of the dauer larvae were analysed using extensive NMR spectroscopy and mass spectrometric analysis and it

was revealed that the major TDE was maradolipid (**15**, Figure 13) which is an acylated trehalose containing a branched fatty acid moiety at position 6 and either a mono-unsaturated or saturated linear fatty acid at the 6' position. MS/MS analysis revealed that 40% of the fatty acid moieties were 15:0 and 17:0 fatty acid (FA) (Figure 14) and about 60% of the maradolipids were found to have a branched fatty acid chain. The presence of cyclopropanated lipids was also found on the maradolipids.⁵¹ As the first diacyl trehalose glycolipid found in the animal kingdom, the discovery of maradolipids represents a first step in understanding stress related lipid composition changes in cells.

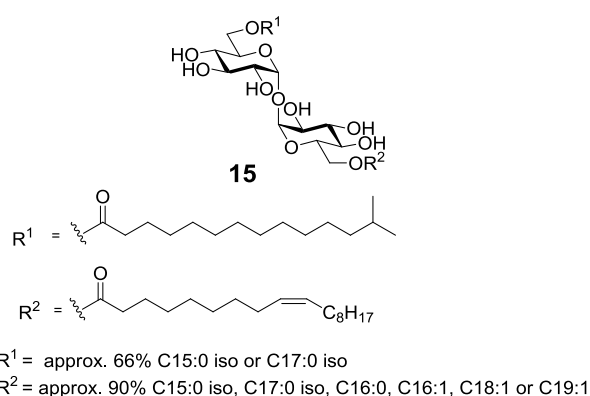


Figure 13. Representative maradolipid.

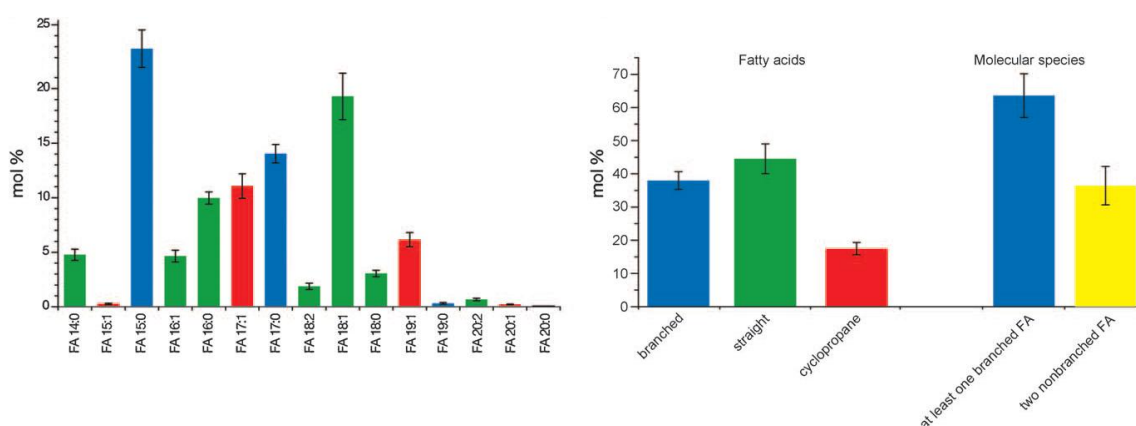
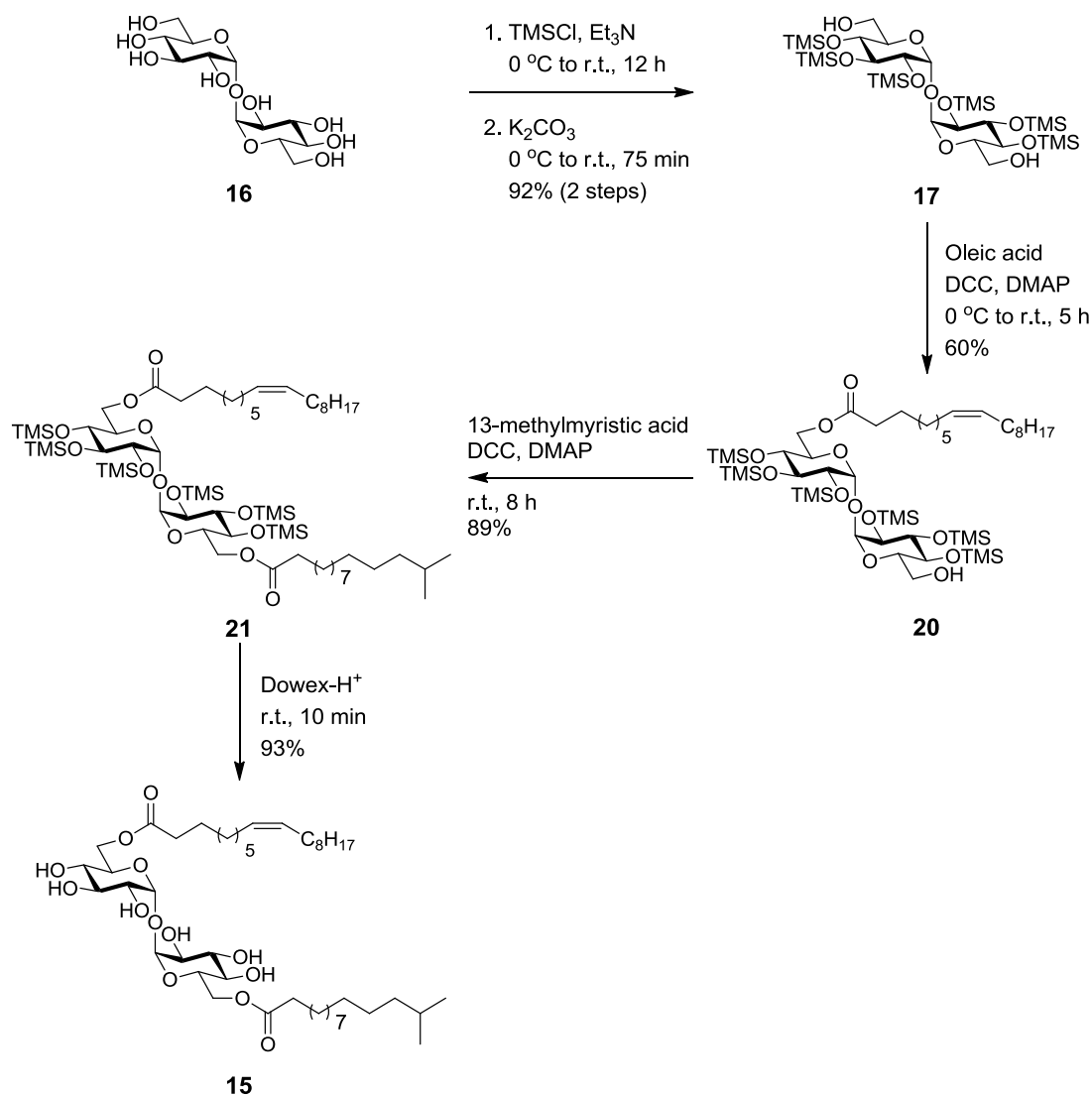


Figure 14. Lipid composition of maradolipids.⁵¹

Two syntheses of the major maradolipids from *C. elegans* were reported simultaneously in 2011 by the groups of Sarpe and Kulkarni⁷⁵ (Scheme 2) and Knölker and colleagues.⁷⁶ In the former synthesis, α,α -trehalose (**16**) was transformed into diol **17** using similar methodology as that developed by Johnson,⁶⁵ and the diol was then subjected to esterification with oleic acid (1 equiv.) in the presence of *N,N'*-dicyclohexylcarbodiimide (DCC) and 4-dimethylaminopyridine (DMAP) to give mono-ester **20**. A further esterification with 13-methylmyristic acid using the same coupling conditions then gave the fully protected diester **21**, which was deprotected in the presence of acidic dowex to yield maradolipid **15** in 46% yield (five steps). To date, there have been no biological activities reported on the maradolipids aside from those saturated derivatives discussed in section 1.5.2c above.



Scheme 2. Maradolipid synthesis.⁷⁵

1.5.3. Trehalose Dicorynomycolate (TDCM)

1.5.3a Introduction

Trehalose dicorynomycolates (TDCMs) are found in *Corynebacteria* and consist of a trehalose carbohydrate group esterified at the 6 and 6' position with corynomycolic acid. The major TDCM (**22**, Figure 15) contains a C32 corynomycolic acid, however, TDCMs with shorter acid chains (C24–C30) have also been found in *Corynebacteria*. TDCMs were first isolated in 1963,⁷⁷ and later found to be a mixture

of three components: dicorynomycolate, monocorynomycolate-mono(dehydrocorynomycolate) and the bis(dehydrocorynomycolate)^{78,79} which were separable once converted to their trimethylsilyl derivatives.⁷⁸ In more recent years, the isolation, and characterisation of TDCM (**22**) and mono-corynomycolate from *Corynebacterium matruchotti* using ¹H NMR spectroscopy has been reported by Datta and Takayama.⁸⁰ The stereochemistry of the two chiral centres in TDCMs lipid chain have been shown to be *R,R*.³⁶

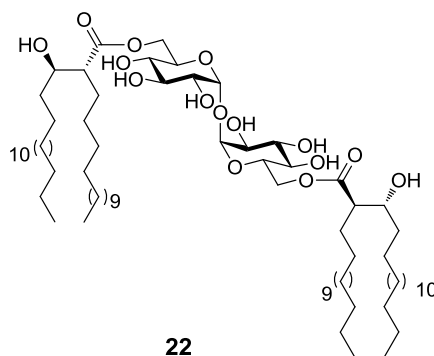


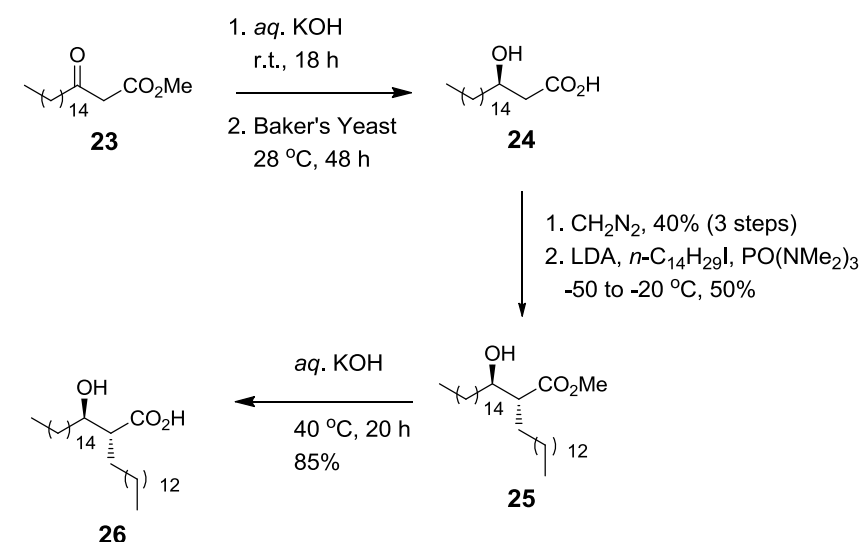
Figure 15. Trehalose dicorynomycolate (**22**).

1.5.3b TDCM Syntheses

Most earlier syntheses of TDCMs were reported by Liav *et al.* whereby the synthesis of this glycolipid along with analogues of TDCM with different carbohydrate groups (*e.g.* dimannosides, digalactosides), and the synthesis of mono-esterified compounds were undertaken in order to correlate the structure to the biological activities of these compounds.^{81–86} In these earlier reactions, the racemic corynomycolic acid was synthesised using Lederer and Polonsky's method⁸⁷ which involved the self condensation of methyl palmitate, subsequent reduction of the keto ester using sodium borohydride (NaBH₄), and then hydrolysis of the methyl ester in 10% potassium hydroxide (KOH) to give the crystalline potassium corynomycolate as the product.

It was not until 1985, that the synthesis of optically pure corynomycolic acid was reported by Sato and co-workers. Here, corynomycolic acid was synthesised in nine

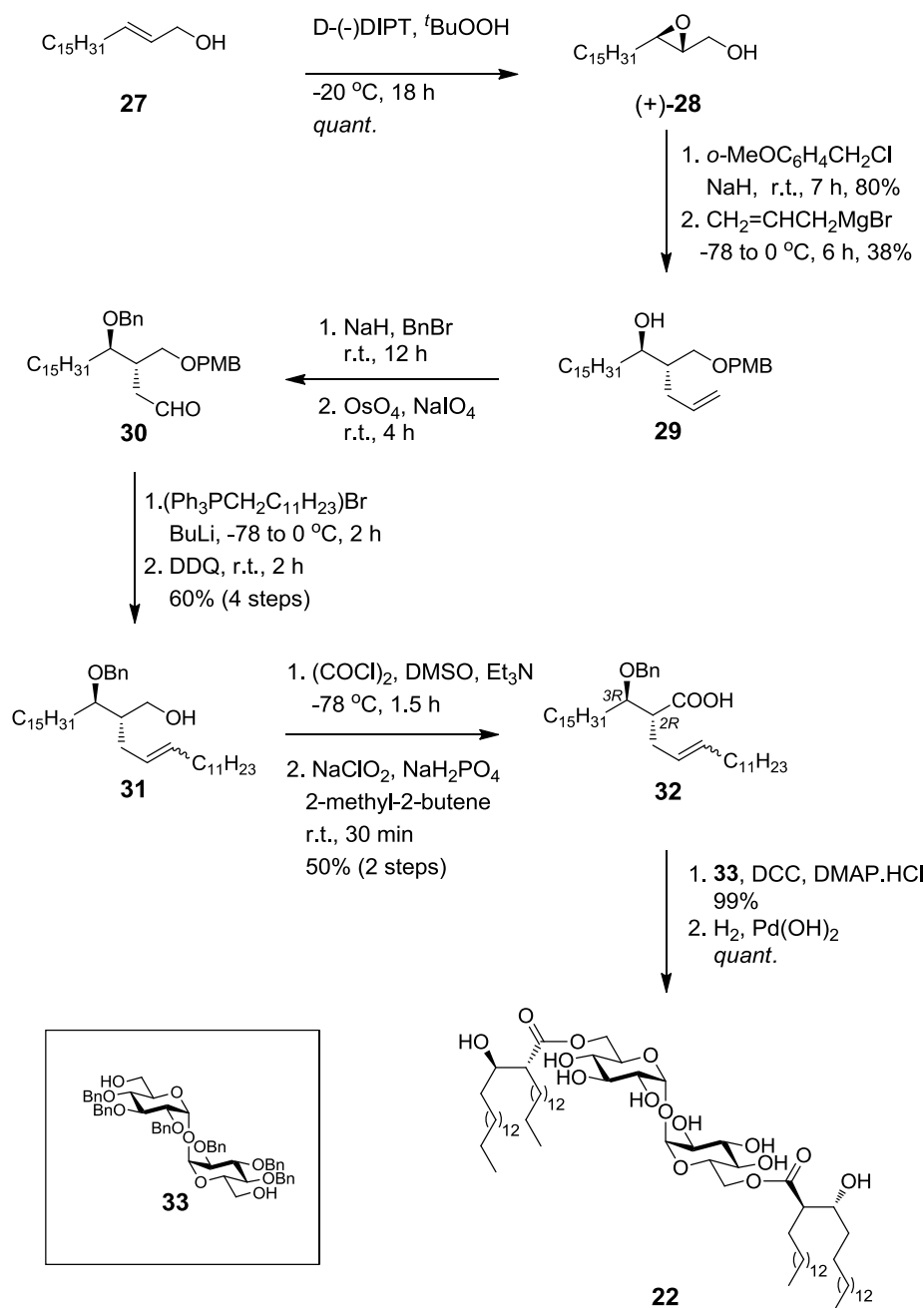
steps starting from *n*-tetradecyl iodide.⁸⁸ Later, Ukata reported a more efficient six step synthesis of (+)-corynomycolic acid from 3-oxo-octadecanoic acid using an asymmetric reduction mediated by fermenting baker's yeast (Scheme 3).⁸⁹ In their synthesis, methyl 3-oxo-octadecanoate (**23**) prepared from methyl acetoacetate and *n*-tetradecyl iodide in 75% yield, was hydrolysed and subsequently subjected to enzymatic reduction to form hydroxy acid **24**. The acid was then methylated using diazomethane and subjected to alkylation *via* treatment with lithium diisopropylamide (LDA) and *n*-tetradecyl iodide in the presence of hexamethylphosphoric triamide (HMPA) to form methyl corynomycolate **25**. Finally, hydrolysis of the methyl ester led to the single enantiomer of corynomycolic acid (**26**) in 13% overall yield.



Scheme 3. Corynomycolic acid synthesis.⁸⁹

The first total synthesis of naturally occurring TDCM, the 6,6'-trehalose diester of (2*R*,3*R*)-corynomycolic acid, was reported by Nishizawa *et al.* in 1994 (Scheme 4).⁹⁰ The required stereochemistry at the 2- and 3-positions was installed using a Katsuki-Sharpley catalytic asymmetric epoxidation⁹¹ of 2-octadecenol (**27**) using D-(-)-diisopropyl tartrate (DIPT) in CH₂Cl₂ to give epoxide (+)-**28** in quantitative yield. Protection of the free hydroxyl in **28** as the *p*-methoxybenzyl (PMB) ether, followed by epoxide ring opening with allylmagnesium bromide resulted in the formation of

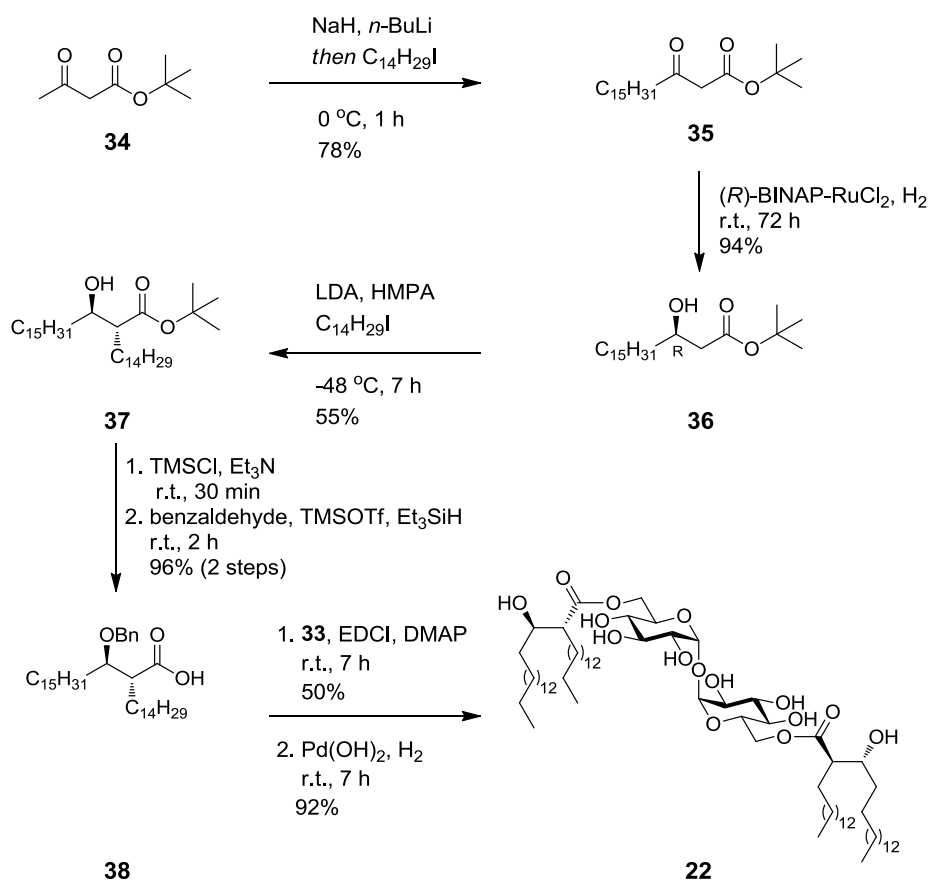
alcohol **29** in combination with the undesired regioisomeric ring-opened product in a 38:62 ratio. Benzylation of the alcohol and cleavage of the double bond by Lemieux-Johnson oxidation⁹² provided aldehyde **30**, which was then subjected to a Wittig reaction with the ylide derived from triphenyldodecylphosphonium bromide, followed by removal of the PMB protective group to give **31** as a mixture of *E* and *Z* isomers. Subsequent Swern⁹³ and NaClO₂ oxidations of **31** resulted in the protected corynomycolic acid **32**, ready for coupling with the selectively protected trehalose **33**. Three other stereoisomers of **32**, the (2*R*,3*S*)-, (2*S*,3*R*)-, and (2*S*,3*S*)-isomers, were also prepared in analogous fashion. Coupling of the corynomycolates with hexa-benzyl trehalose derivative **33**, followed by global deprotection by Pd-catalysed hydrogenation, with concomitant reduction of the double bond, provided the target TDCM **22** in excellent yield.



Scheme 4. Synthesis of TDCM.⁹⁰

Nishizawa *et al.* also reported on a more efficient route to corynomycolic acids that makes use of Noyori's 2,2'-bis(diphenylphosphino)-1,1'-binaphthyl (BINAP)-Ru-catalysed asymmetric reduction,⁹⁴ in the dynamic kinetic resolution of α -palmityl-butyrolactone.⁹⁵ This reduction was later used in the synthesis of corynomycolic acid analogues, whereby a *tert*-butyl acylacetate was stereoselectively reduced (Scheme

5).⁹⁶ In order to form the natural (2*R*,3*R*)-TDCM isomer **22**, the dianion of *tert*-butyl acetoacetate (**34**) was generated with NaH and *n*-BuLi, and subsequently treated with *n*-tetradecyl iodide to form the γ -alkylated product **35**. Stereoselective reduction of the ketone in **35** using (*R*)-BINAP-RuCl₂ and hydrogen then gave (*R*)-hydroxy ester **36** in an excellent yield of 94% and more than 98% ee. Subsequent Fráter-Seebach alkylation^{97,98,99} of **36** with tetradecyl iodide introduced the second stereocentre, again with excellent selectivity (>99% de, **37**). Next, reductive etherification and ester cleavage led to the benzyl protected corynomycolic acid **38**, which was then coupled to the hexa-benzyl protected trehalose derivative **33**. Again global deprotection using catalytic hydrogenation led to the target TDCM **22** in a highly efficient seven step synthesis. In addition, further stereoisomers and branched chain TDCM analogues were produced using the same strategy, but using (*S*)-BINAP-RuCl₂ for the stereoselective reduction, and a variety of alkylating reagents in the γ -alkylation of the acetoacetate and Fráter-Seebach alkylation.



Scheme 5. TDCM synthesis *via* Noyori's asymmetric reduction.⁹⁵

1.5.3c TDCM Biological Activity

Over the years, TDCMs isolated from the cell wall of *Corynebacterium* have been shown to have a variety of biological activities, including acting as agonists for macrophage activation during *in vivo* studies.¹⁰⁰ In combination with monophosphoryl lipid A, TDCMs form an integral part of the Ribi Adjuvant system which has shown to be effective in *Leshmania*¹⁰¹ and *M. tb* vaccine formulations.¹⁰² The tumour regression property of synthetic racemic TDCMs has also been reported whereby the TDCMs were injected intralesionally as an oil emulsion in mice with fibrosarcoma.¹⁰⁰

The first testing on homogenous TDCMs, was carried out in 1996 when four TDCMs synthesised by Nishizawa *et al.* were tested individually for their biological activity.¹⁰³ In the study, the immunoadjuvant activity of the TDCMs was analysed, by considering the cytolytic activity of peritoneal macrophages obtained from mice treated peritoneally with TDCM, to Lewis lung carcinoma cells. The results indicated that all four diastereomeric TDCMs had adjuvant activities, however, the natural (2*R*,3*R*) and its enantiomer (2*S*, 3*S*) were more potent than the 2*R*,3*S* and the 2*S*,3*R* isomers in terms of stimulating the cytolytic activity of the macrophages. Subsequent *in vivo* studies showed that all four TDCMs were effective in prophylactically, as well as therapeutically, inhibiting lung metathesis produced by the highly metastatic tumour cells, colon 26-M3.1 carcinoma and B16-BL6 melanoma. The compounds were injected intravenously into mice as oil emulsions and again the 2*R*,3*R*-TDCM and 2*S*,3*S*-TDCM were shown to be most effective at inhibiting metathesis. The anti-metastatic effects of TDCMs were seen to be much higher than that of TDMs. Furthermore, it was shown that the lethal toxicity of TDMs, apparent in mice, was not exhibited by the TDCMs thereby making TDCMs better candidates for future biological studies.¹⁰⁴

The effect of TDCMs on the production of IL-6 in *in vivo* murine models has also been explored.⁹⁶ TDCMs induced a more potent immune response than the mono-esters; the natural TDCM with 2*R*,3*R* stereochemistry and a C14 lipid on the fatty acid residue was the most active (based on IL-6 accumulation in mice serum). This is

an important observation and provides further evidence for the potential use of natural TDCM in the treatment of cancer and other diseases.

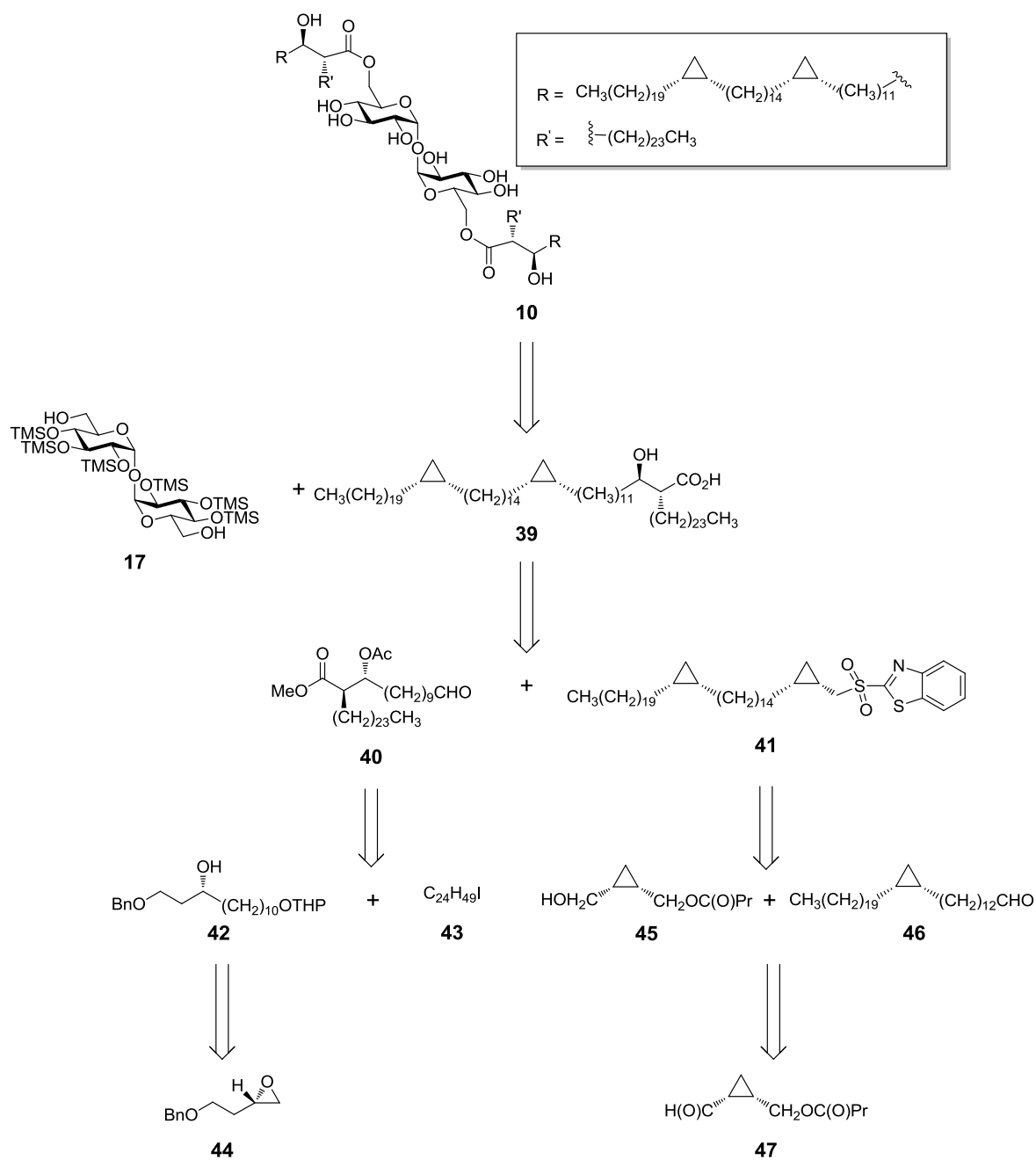
1.5.4 TDM- Reported Synthesis and Biological Activity

1.5.4a Introduction

TDMs are the most complex form of 6,6'-diesters isolated from mycobacterial species. For isolation and characterisation of TDMs see section 1.4.5. The complexity of TDMs has presented many challenges during their synthesis, though much elegant work has been achieved in this field, particularly by Baird and co-workers whose initial work focussed on the synthesis of various mycolic acids,^{105–113} and more recently extended to the synthesis of individual TDMs.¹¹⁴

1.5.4b TDM synthesis

One of the first mycolic acids prepared by Baird and co-workers was α -mycolate (**39**, Scheme 6)¹⁰⁶ which they later transformed into α -TDM **10**.¹¹⁴ The key steps in the synthesis included esterification between mycolic acid **39** and TMS trehalose **17** (obtained from α,α -trehalose following Johnson's procedure),⁶⁵ with mycolic acid **39** obtained from Julia reaction¹¹⁵ between aldehyde **40** and cyclopropane building block **41**. Aldehyde **40**, in turn, was synthesised *via* Fráter-Seebach alkylation between THP-ether **42** and tetracosanoyl iodide (**43**). THP-ether **42** was accessed from epoxide **44**, itself obtained from aspartic acid.¹¹⁶ The cyclopropyl building block **41** was prepared *via* Julia reaction between cyclopropyl building blocks **45** and **46**, whereby both intermediates were synthesised from the same common precursor **47**.¹¹⁷



Scheme 6. Retrosynthesis of α -TDM **10**.

1.5.4c TDM Biological activity

Although TDMs have been shown to play a role in the pathogenesis of *M. tb* by conferring protection to the bacteria against harsh environmental conditions,^{17,18,118,119} it is the biological activity of TDMs, which have been of much interest over the past years. TDMs exhibit anti-tumour activity,^{120,121} adjuvant activity,⁷³ anti-bacterial properties,^{122,123} and have the ability to induce granuloma formation^{124–127} and angiogenesis.¹²⁸

TDMs exhibit anti-tumour activity, through tumour prevention, tumour regression and immunisation. A number of studies have been conducted to illustrate the effect of TDM on various tumour cells. In a study by Yarkoni *et.al.*, prior injection of TDMs in mice was shown to suppress the growth of Ehrlich Ascites tumour cells.¹²⁹ The tumour regression property of TDMs was demonstrated in a study where the growth of an ascetic rat hepatoma was suppressed in a mouse model following injection of TDM as an emulsion in peanut oil.¹³⁰ In another study, administration of TDMs as liposomes led to activation of macrophages, subsequent nitric oxide (NO) production, and the development of anti-tumour activity against synergenic P77 fibrohistiocyoma tumour cells.¹²⁰

TDMs exhibit adjuvant activity, as seen by Bekierkunst *et al.*,¹³¹ where it was found that pre-injection of TDMs followed by injection of antigen into the same footpad of mice induced a high antibody production, whereas injection of antigen alone into contralateral footpads resulted in a lower antibody production. A number of studies have also shown induction of IL-4, IL-6 and IL-10 production after exposure of mice cells to TDMs, indicating that TDM could potentially enhance antibody production.³⁶ In another study, it was found that TDMs caused a significant increase in IFN- γ production, which was accompanied by the expansion of natural killer (NK) cells and upregulation of MHC class II on macrophages.¹³² The increased proportion of Th1 cells evident after 3 weeks suggest that TDMs could be potentially used as the adjuvant component of Th1 vaccines (e.g. cancer vaccines).

TDMs have also been shown to produce granulomas similar to those formed during mycobacterial infection.¹³³ The purpose of granulomas is to contain the mycobacterium in distinct foci preventing the spread of infection and making

destruction of the bacterium by cell-mediated immunity easier. However, it also serves to protect the bacterium from the host organism's natural defence system and allow the bacterium to multiply inside host cells.

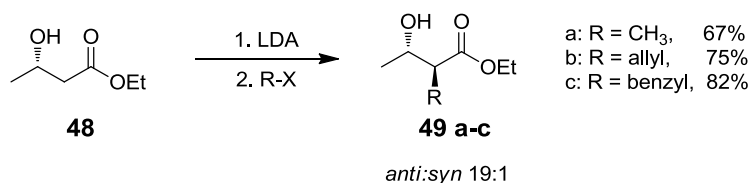
Inflammatory granulomas are usually associated with angiogenesis, and several studies have been conducted to determine the effects that TDMs have on granuloma formation and angiogenesis.^{128,134,135} The production of vascular endothelial growth factor (VEGF) and IL-8, which are crucial for angiogenesis, were initiated in the presence of TDMs in macrophages indicating that TDMs play a major role in angiogenesis by inducing the production of cytokines required for this process.

Cord factors have also been shown to stimulate non-specific resistance against bacterial infections and parasites. Mice injected with TDMs were protected against *Salmonella typhi* strain Ty2 or *Salmonella typhi* strain SR11.¹³⁶ Similar studies found that TDMs induce non-specific response against *K. pneumoniae* and *L. monocytogenes*.¹²³

1.6 Fráter-Seebach alkylation in TDCM and TDM syntheses

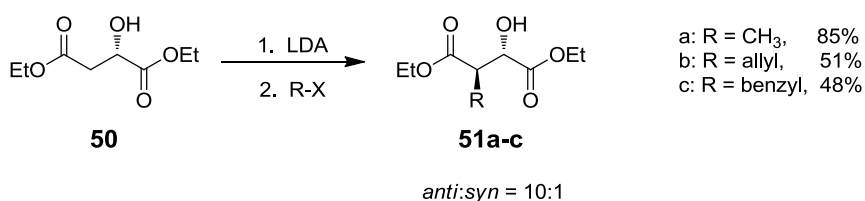
1.6.1 The Fráter-Seebach alkylation

As highlighted previously in 1.5.3b and 1.5.4b one of the key steps in TDCM and TDM synthesis is the Fráter-Seebach alkylation. Defined as a highly efficient means of diastereoselectively introducing α -substituents to chiral β -hydroxy-esters, this methodology was proposed by Fráter in 1979. He reported that by utilising LDA as a base, ethyl (*S*)-3-hydroxybutyrate (**48**) could be alkylated with methyl, allyl, butyl and benzyl bromide to give the corresponding (2*S*, 3*S*)-alkylated products **49a-c** in good yield and excellent diastereoselectivity (Scheme 7).^{98,99} Similar methodology was also used for the alkylation of ethyl (*R*)-3-hydroxyvalerate using propyl iodide as the electrophile.¹³⁷



Scheme 7. Fráter's alkylation.^{98,99}

At approximately the same time as Fráter, Seebach also performed pioneering studies in this field, as illustrated by the alkylation of diethyl malate **50** with methyl, allyl and benzyl halides to yield the alkylated product **51a-c** (Scheme 8).⁹⁷ Again, excellent diastereoselectivities, in favour of the *anti*-isomer, and good yields were reported for the α -alkylations. To recognise the contributions by both research groups to this field, the α -alkylation of β -hydroxy-esters is thus referred to as the Fráter-Seebach alkylation.

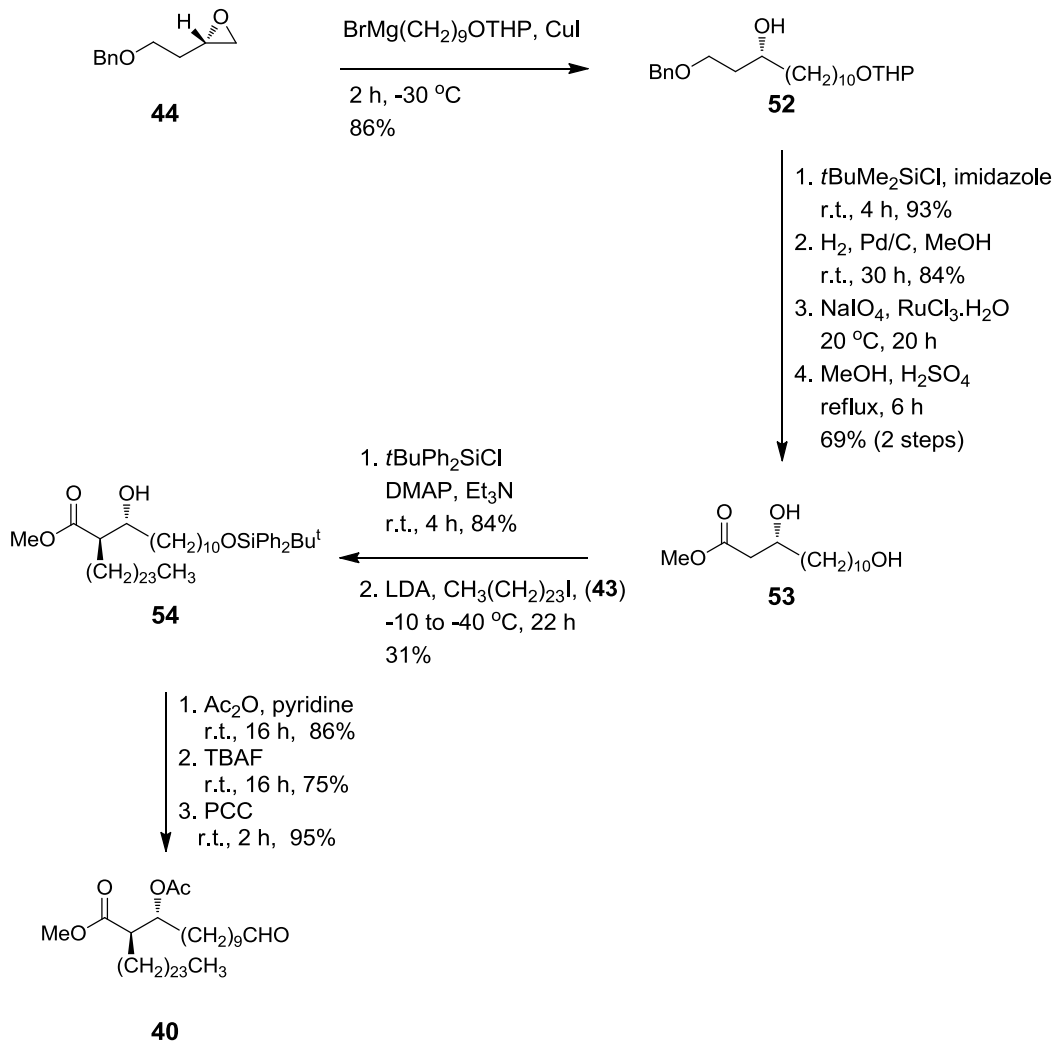


Scheme 8. Seebach's alkylation.⁹⁷

Over the years, the Fráter-Seebach alkylation has formed a crucial step in the preparation of a variety of biologically important compounds including natural products^{138,139,140} and derivatives thereof,^{141,142,143} enzyme inhibitors,¹⁴⁴ and other key chiral building blocks.¹⁴⁵ The methodology has also been modified by the addition of HMPA to the reaction vessel so as to enhance the efficacy of the reaction. Despite the versatility of the Fráter-Seebach alkylation in organic synthesis, the extension of this methodology to longer chain alkyl halides, however, has been met with mixed success.^{96,105,106}

1.6.2 Scope and limitations of the Fráter-Seebach alkylation in the synthesis of trehalose glycolipids

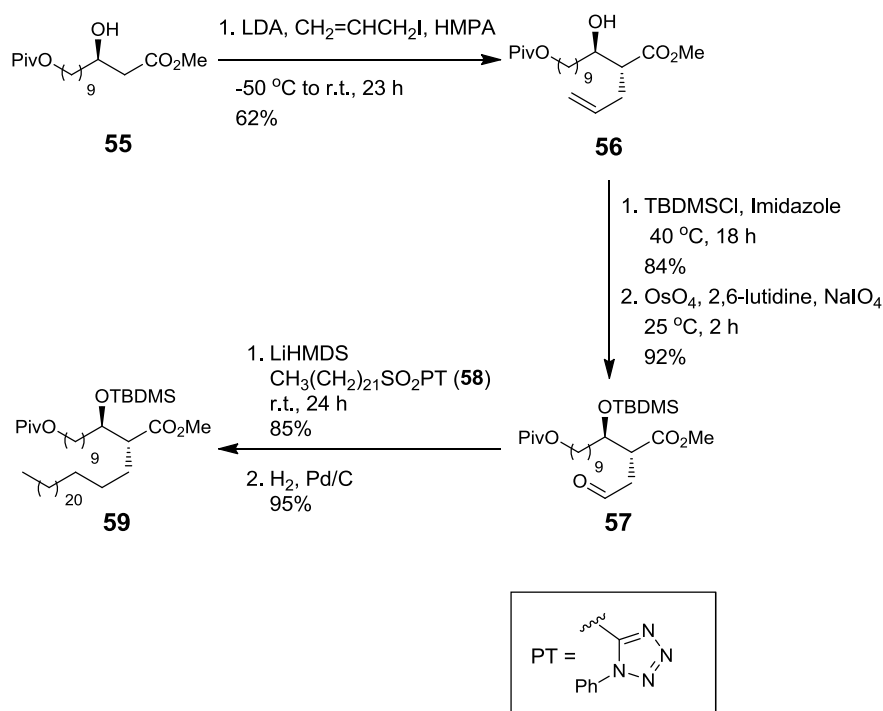
During their initial syntheses of mycolic acids *en route* to the preparation of TDMs, Baird and co-workers used the Fráter-Seebach alkylation for the introduction of the α -chain of the mycolic acid.^{105,106} The synthesis of the α -alkyl- β -hydroxy fragment started with a Grignard type ring-opening of epoxide **44** to produce alcohol **52** as a single enantiomer (Scheme 9). Alcohol **52** was then transformed into diol **53** in four steps, after which selective protection of the primary hydroxyl in **53** as the *tert*-butyldiphenylsilyl (TBDPS) ether, followed by Fráter-Seebach alkylation using tetracosanyl iodide **43** furnished hydroxy-ester **54**, albeit in modest (31%) yield. Protection of the secondary alcohol in **54** as an acetate, followed by deprotection of the primary hydroxyl, and subsequent oxidation then yielded aldehyde **40**, the core fragment of mycolic acid.



Scheme 9. Fráter-Seebach alkylation in mycolic acid synthesis.^{105,106}

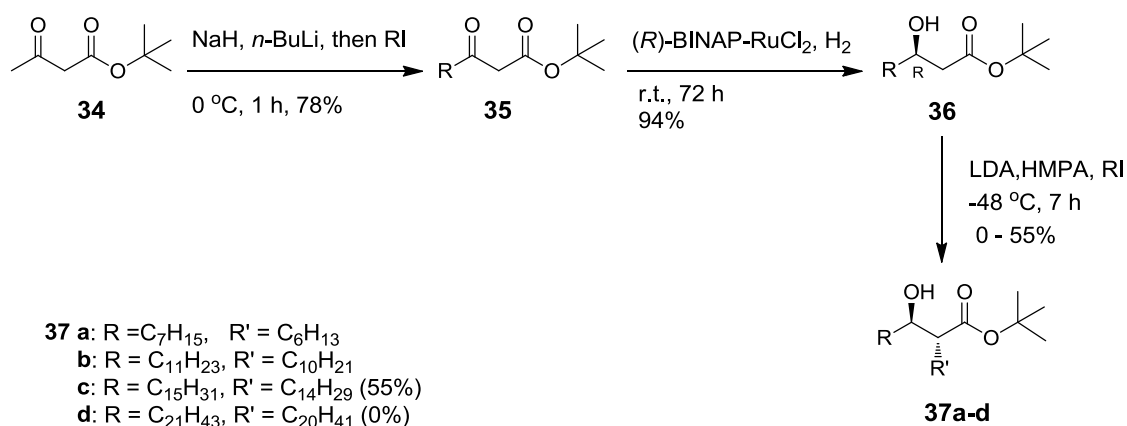
To circumvent the low yield of α -alkylation with tetracosyl iodide, Baird and co-workers later developed a chain-elongation strategy¹¹² in order to install the required lipid at the α -position (Scheme 10). Here, hydroxy ester **55** was subjected to the Fráter-Seebach reaction with allyl iodide in the presence of HMPA to yield the α -alkylated ester **56** in an improved 62% yield. The α -alkylated ester **56** was further derivatised to oxo-ester **57** via protection of the hydroxyl as the TBDMS ether followed by oxidation of the alkene to the corresponding aldehyde. Elongation of the α -position in **57** was achieved via Julia olefination with tetrazole **58**, followed by subsequent removal of the TBDMS group and hydrogenation of the double bond to yield hydroxy ester **59**. Although this methodology led to an increased yield for the

Fráter-Seebach reaction, additional steps were required to generate the desired alkyl length at the α -position.



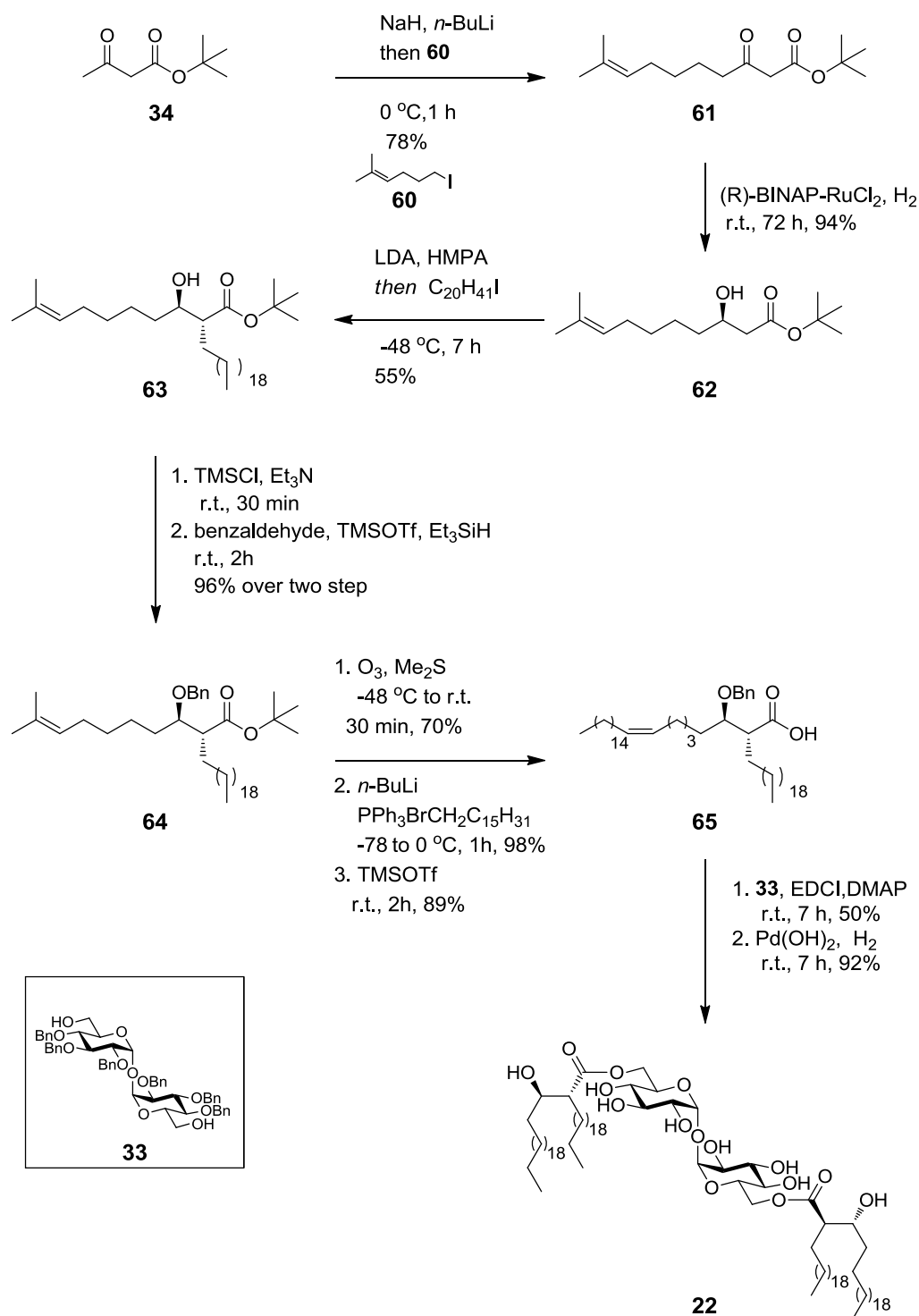
Scheme 10. Chain-elongation methodology in mycolic acid synthesis.¹¹²

In their synthesis of TDCM and analogues, Nishizawa *et al.* noted similar difficulties when performing a Fráter-Seebach alkylation with long chain alkyl (eicosanyl) iodide.⁹⁶ Although the synthetic strategy was successful for the synthesis of natural TDCM and analogues with shorter corynomycolic chains (*e.g.* **37a-c**) (Scheme 5), TDCM analogues with the C₂₀ alkyl chain (*e.g.* **37d**) could not be synthesised *via* a Fráter-Seebach alkylation of tetracosanoyl β -hydroxy functionalised ester **36** with eicosanyl iodide (Scheme 11).



Scheme 11. Fráter-Seebach alkylation in corynomycolic acid and analogues syntheses.⁹⁶

To accomplish the synthesis of the C20 TDCM analogue, **22**, the authors thus resorted to a different strategy involving the γ -alkylation of *tert*-butyl acetoacetate (**34**) with bis-homoprenyl iodide **60** to form ester **61** (Scheme 12). Stereoselective reduction of the ketone in **61** using (*R*)-BINAP-RuCl₂ and hydrogen gave hydroxy ester **62**, which was subjected to a Fráter-Seebach alkylation using eicosanyl iodide to form product **63** in 55% yield. Here, the presence of a shorter lipid on ester **62** was thought to facilitate the Fráter-Seebach alkylation by increasing the solubility of the substrate. Reductive etherification of **63** then afforded benzyl ether **64**, which was subjected to ozonolysis, followed by Wittig reaction and subsequent treatment with TMSOTf, to liberate mycolic acid **65**. Finally, acid **65** was coupled to the hexa-benzyl protected trehalose derivative **33**, followed by hydrogenation of the benzyl groups and reduction of the double bond to yield the desired C20 TDCM **22**.



Scheme 12. Alternate route for C20 TDCM analogue synthesis.⁹⁶

1.7 Trehalose diester probes to study trehalose immunology

1.7.1 Introduction

To date, aside from Mincle, there are no other known receptors for TDMs (and associated linear analogues such as TDB). One way to determine potential proteins that trehalose diesters bind to and interact with, is through the development of trehalose-diester probes, for use in affinity based proteome profiling (A/BPP). This, in turn, will aid in our understanding of the mechanism of action of this intriguing class of bio-molecules.

1.7.2 Affinity Based Proteome Profiling (A/BPP)

1.7.2a Overview

Proteomic research focuses on the study of proteins, their functions, and subsequent interactions with other bio-molecules in complex biological systems. Instead of studying individual proteins separately, proteomic research allows for the study of distinct proteins expressed at the same time by an organism or cell type. Traditionally, proteins were separated using techniques such as 1D/2D gel-electrophoresis and isotope coded affinity tagging (ICAT), followed by determination of the relative abundance.¹⁴⁶ An alternate strategy that is now commonly employed is activity or affinity based proteome profiling (ABPP or A/BPP). ABPP and A/BPP use site-directed chemical probes to elucidate the functional state of enzymes in complex biological systems. They use relatively small organic molecules to label specific enzymes at their active site (and not their inactive precursor or inhibitor-bound forms).¹⁴⁶ These methods are better since they can detect important post-translational forms of protein regulation like those mediated by protein-protein and/or protein-small molecule interactions.¹⁴⁷ To date, probes have been developed for a number of enzyme classes, and these have provided a means of

elucidating enzyme functions, and understanding enzyme activity in specific physiological and pathological processes.

1.7.2b Basic Technology of A/BP Probes

To ensure selective binding of activity and affinity based proteome (ABP and A/BP) probes to proteins and prevent cross reactivity with other proteins in the proteome, most probes consist of a combination of binding and reactive groups that target conserved mechanistic or structural features in a protein's active site. The probe contains three major elements: the recognition group, the reactive group, and the tag. The recognition group directs the ABP probe and A/BP probe to the target protein and is designed in a way to resemble the natural substrate of the target protein. Closely attached to the recognition group is the reactive group ('trap') that reacts with amino acids in the proteins active site and thus forms a bond between the probe and protein. The recognition and reactive group are linked most often *via* a spacer unit to a tag which allows for visualisation and/or purification of the bound protein (Figure 16).¹⁴⁸

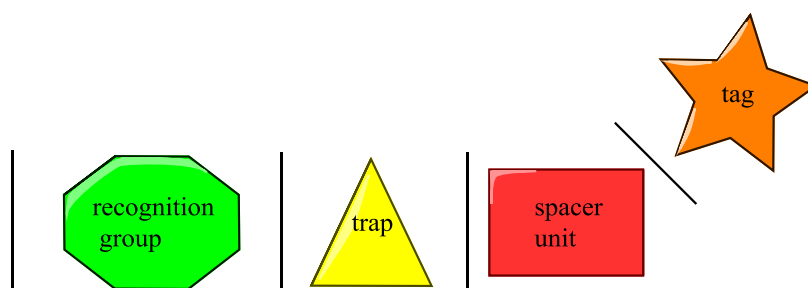


Figure 16. Representative cartoon of Activity/Affinity Based Proteome Probe.

1.7.2c A/BPP vs ABPP

The difference between ABPP and A/BPP arises from the different reactive groups employed by the two profiling methods.¹³ In ABP probes, electrophilic traps are present which are suited for proteins that contain a nucleophilic amino acid side chain residue (*e.g.* serine, cysteine, threonine) in their active site such as: cysteine proteases, serine hydrolases, and proteasome subunits. ABP probes thus involve a directed approach in which individual probes are designed to target specific proteins (Figure 17A). In contrast, A/BP probes employ the use of photoreactive trap groups and are commonly used for proteins not relying on nucleophilic residues in their active site, for example, metalloproteases and histone deacetylases. Most photoreactive groups are used to label proximal residues in the proteins active site following irradiation with light (Figure 17B). The use of A/BP probes offer a non-directed way of profiling proteins from several different classes, and in many cases are commonly used to profile proteins not accessible by ABP probes.

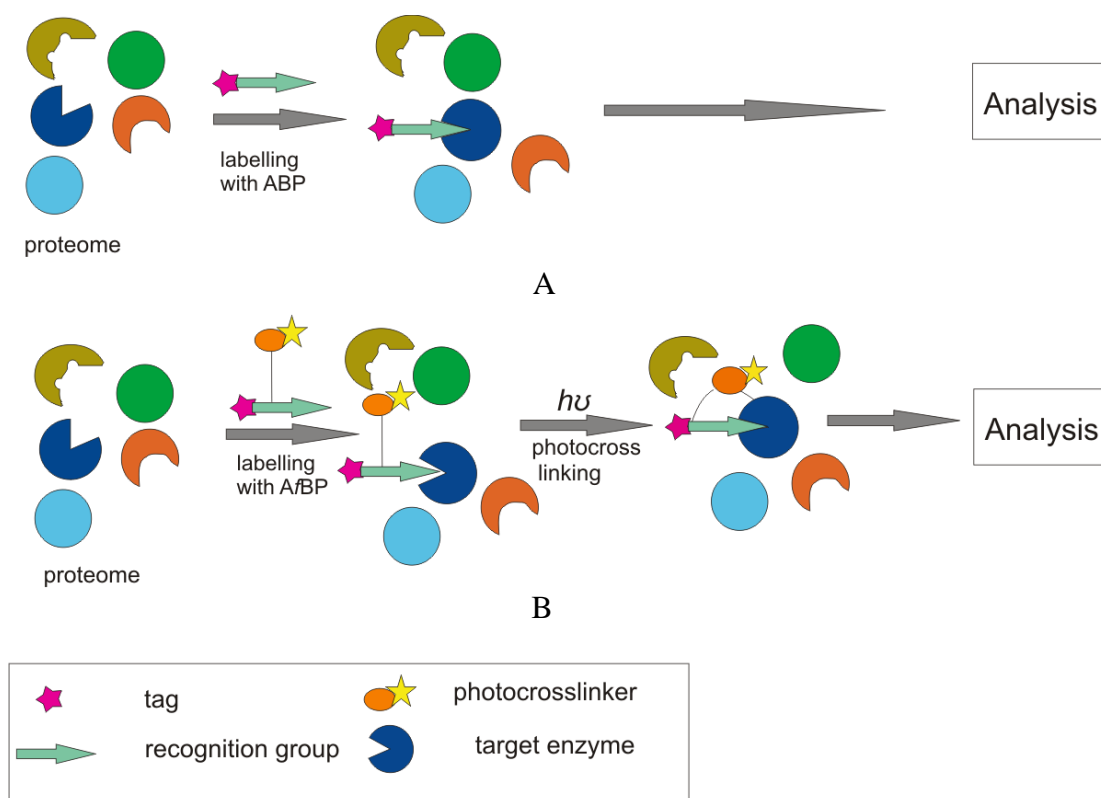


Figure 17. ABPP (A) vs A/BPP (B).

1.7.2d Tag Groups

The tag group on a probe is used to facilitate the characterisation of the target protein. Some tags that are commonly used include fluorophores [(e.g. rhodamine, **66**)], biotin (**67**) and latent analytical handles like azides and alkynes (**68**) (Figure 18).^{149,150}

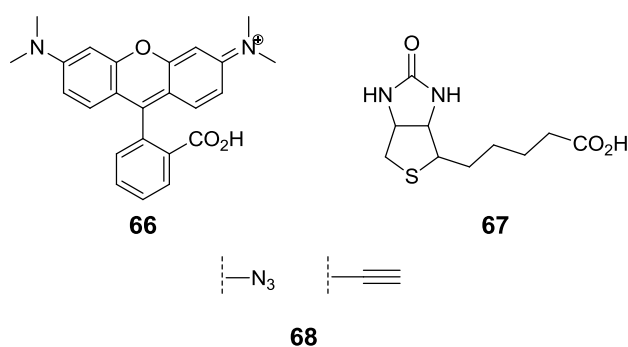


Figure 18. Common reporter tags used in ABP and A/BP probes.

The use of azides and alkynes to visualise protein targets post-labelling overcomes the problem of the bulkiness associated with fluorophores which can influence the uptake of the probe into the cells. In this approach (known as a two step labelling process), the ABP or A/BP probes tag is replaced by a ligation handle, which can be connected to the reporter group after the protein is captured. The most common ligation reactions used are Huisgen 2,3-dipolar cycloaddition or ‘click’ reactions. These can be copper catalysed (**I**)¹⁵¹ or strain-promoted (**II**) (Figure 19).¹⁵² Recently a new reagent has been reported by Boons and co-workers to promote a strain-promoted click reaction after irradiation with light.¹⁵³ This has been termed the ‘photoclick’ reaction (**III**).

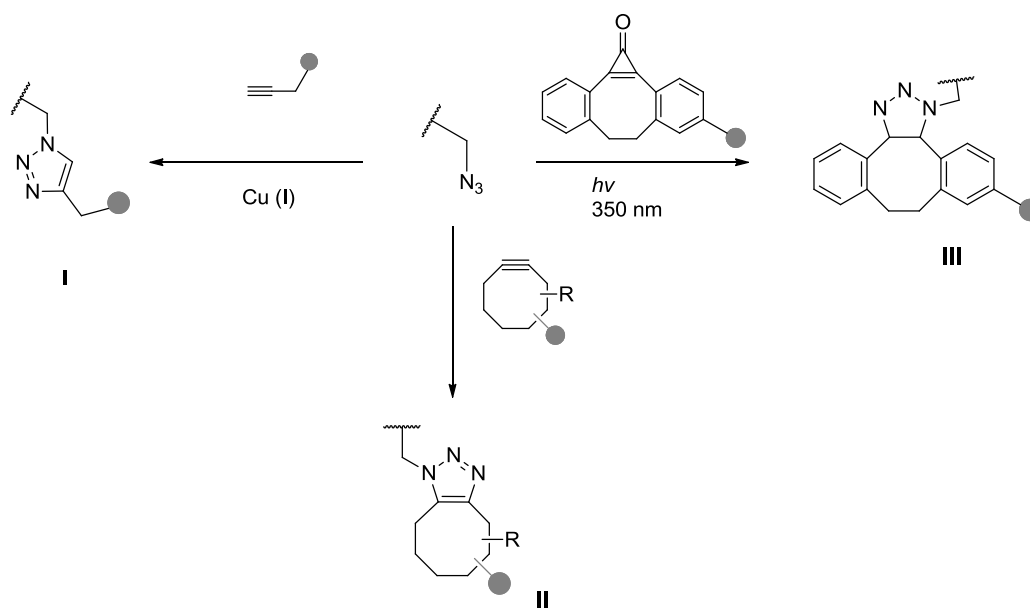


Figure 19. Ligation methods for azide and alkyne coupling.

1.7.2e Reactive Groups

To be used in AfBP probes, a photoreactive group needs to meet certain criteria. First, they need to be activated only upon irradiation with light of a specific wavelength which will not damage biological systems ($\lambda_{\text{act}} > 300 \text{ nm}$).¹⁵⁴ Second, the generated reactive species should have a shorter life span than the lifetime of the studied protein-substrate complex in order to limit non-specific labelling. It is also important that the activated species reacts with any chemical entity in close proximity to form a stabile covalent adduct. Finally, the photoreactive group should be small in order to minimise its influence on the binding mode and activity of the AfBP probe towards the protein.

The use of photoreactive groups was first reported by Westheimer and co-workers in 1962 whereby they used a diazoacetyl group to deactivate chymotrypsin via photolysis.¹⁵⁵ Over the years, many photoreactive groups have been developed but only few meet the requirements set out above. The common trap systems used today for probes are benzophenones (**69**, first reported in 1973),¹⁵⁶ aryl azides (**70**)¹⁵⁷ and diazirines (**71**) (Figure 20).¹⁵⁸

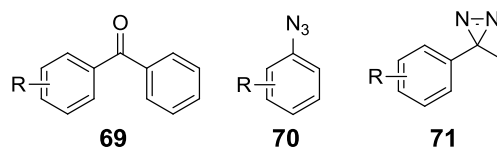
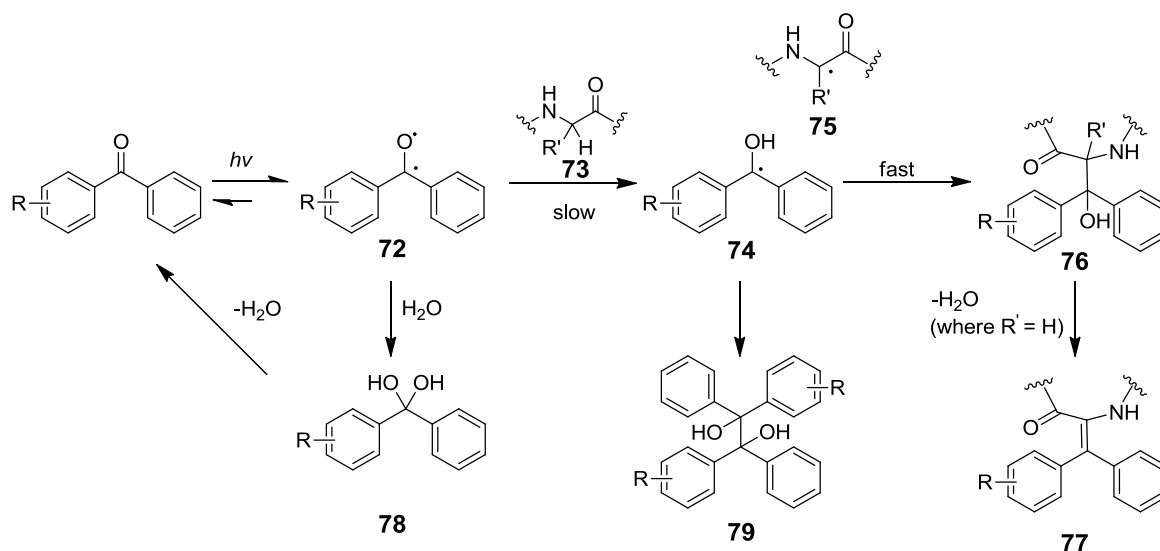


Figure 20. Examples of common trap systems for AfBP probes.

A major advantage of using benzophenones and diazirines, as opposed to aryl azides, is that they can be excited at wavelengths of 350-360 nm, and therefore no significant damage to the biological system occurs. Aryl azides have their maximum absorption wavelength below 300 nm; however this could be improved by using an electron withdrawing substituent (*e.g.* nitro, hydroxyl, and acyl groups) on the phenyl group. The major advantage of using aryl azides, is their small size which allows for easy incorporation into natural biological compounds without too much alteration of the original structure.

Unlike diazirines and aryl azides, most benzophenone building blocks are commercially available and in recent years, many examples of AfBP probes containing benzophenone have been reported.^{159,160,161} Although, the benzophenone group might seem like an ideal photo-crosslinking reagent, it has drawbacks such as being relatively bulky, which may influence the interaction between the enzyme and substrate and lead to non-specific labelling. Benzophenones also need prolonged time for irradiation (>30 minutes) to result in reasonable cross-linking. The mechanism of cross-linking of the benzophenones to proteins can be explained as depicted in Scheme 13.¹⁶² After irradiation of benzophenone, the triplet diradical **72** is formed which abstracts a hydrogen atom from the α -centre of a nearby amino acid **73** to form a ketyl radical (**74**) and an amino acid alkyl radical (**75**). These radical species then rapidly combine to form the desired cross-linked species, benzhydrol **76**. In the presence of a glycine amino acid residue ($R' = H$), there is the possibility of water eliminating from benzhydrol (**76**) resulting in olefin **77**, detection of which can be used to elucidate the presence of glycine in the active site of the protein. If diradical **72** reacts with water, the corresponding hydrate **78** forms, which rapidly loses water to form the starting benzophenone. One possible side reaction that can

take place is homodimerisation of the ketyl **74** to form benzopinacol **79**, however due to the relative large difference between hydrogen abstraction and recombination to form the benzhydrol **76**, only a small amount of this by-product is formed.



Scheme 13. Chemistry of benzophenone after photolysis.

1.7.2f Applications and Synthesis of A/BP probes

Over the years, many aryl azide based A/BP probes have been developed which can target various classes of enzymes and enhance our understanding of the mechanistic and functional states of these enzymes. In one such study, Dive and co-workers synthesised A/BP biotinylated probe **80** and ABP biotinylated probe **81** to study matrix metalloproteinases (MMPs) from tumour extracts (Figure 21).¹⁶³

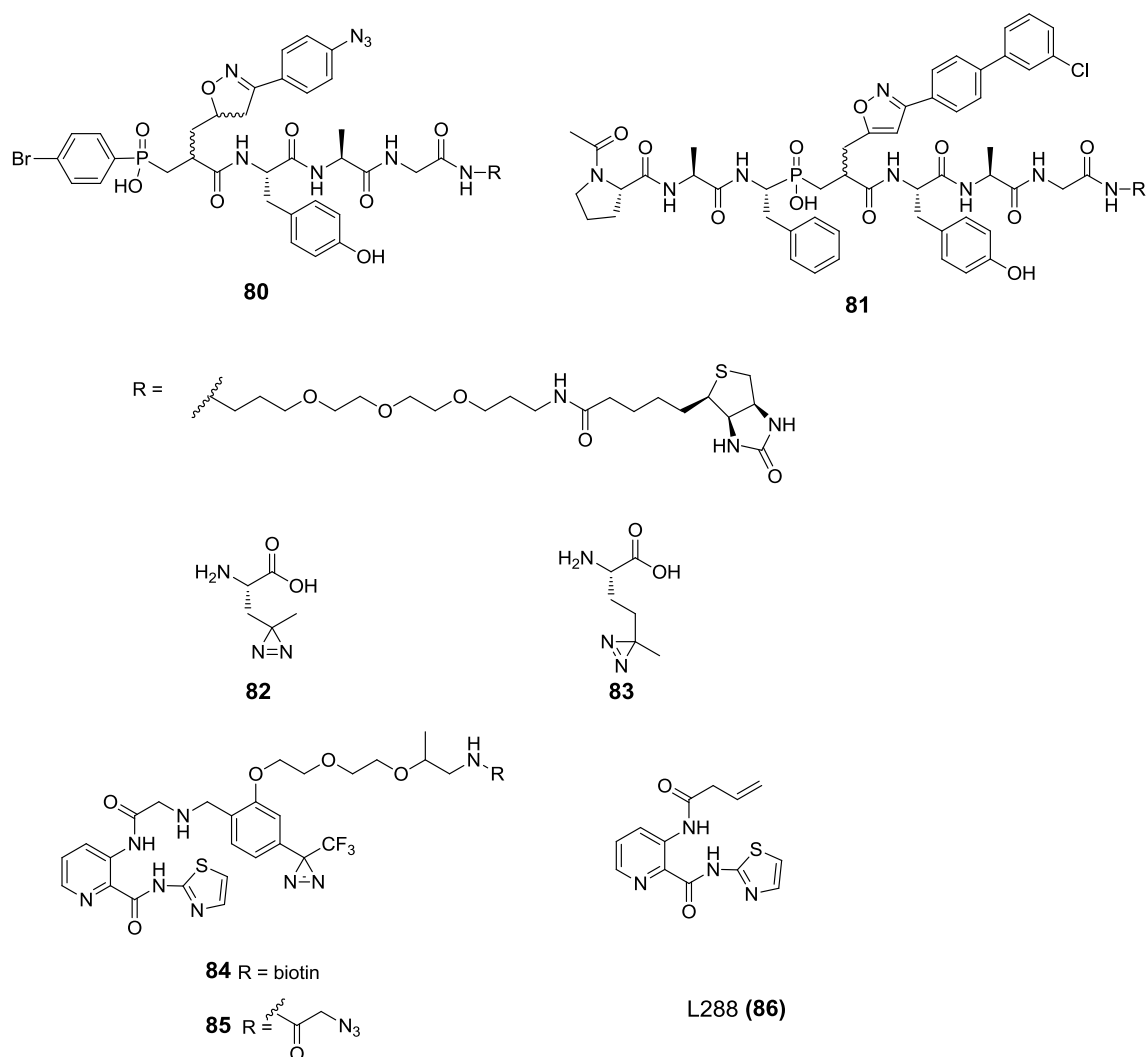


Figure 21. Aryl azide and diazirines based A/BPs.

There are also a number of interesting A/BP probes containing diazirines as ‘traps’, and these include photo-leucine (**82**) and photo-methionine (**83**), synthesised in 2005 by Thiele and co-workers, which were used as photoactivatable amino acids to study protein-protein interactions in living cells.¹⁶⁴ Here, it was shown that both photoactivatable amino acids could be readily incorporated into proteins, and protein-protein interactions not identified previously were detected using these traps. Qui *et al.* reported on the synthesis of L288–probes **84** and **85**, and analysed them for their ability to label methionine aminopeptidases (MetAPs).¹⁶⁵ L288 (**86**) is a potent inhibitor of type I MetAP, an enzyme that is a potential target for new anti-bacterial

and anti-fungal therapeutics. Both ABPs **84** and **85**, were able to bind MetAP, with probe **85** being more selective for MetAP protein labelling.

There are many examples of AfBP probes containing benzophenone groups reported in literature. To study histone deacetylases (HDACs), a key regulator of gene expression that assembles into larger protein complexes, Salisbury and Cravatt reported the synthesis of SAHA-Bryne **87** (Figure 22).¹⁵⁹ This probe contains the important structural elements of HDAC inhibitor suberoylanilide hydroxamic acid [SAHA, (**88**)] as well as benzophenone and alkyne moieties for targeting the HDACs. SAHA-Bryne **87** demonstrated good HDAC binding specificity and could also be used to measure HDAC content in different forms of cancer. Results indicated that SAHA was quite selective for HDACs and thus could be a valuable tool for profiling both the activity of HDAC proteins, and the binding proteins that regulate their function, particularly in cancer.¹⁵⁹ Benzophenone based ABPs have also been used for targeting metalloproteases. For example, GM600 (**89**) a matrix metalloprotease inhibitor, was converted to photoaffinity probe **90** by incorporating a benzophenone building block and a fluorophore.¹⁶⁰ This probe could then be used to covalently label and visualise several active MMPs in complex proteomes.

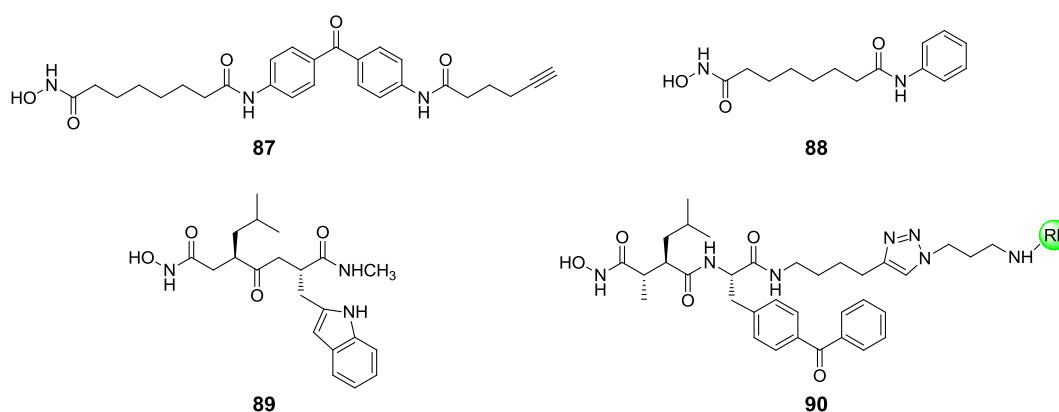


Figure 22. Benzophenone based AfBP probes.

1.7.2g Click chemistry based A/BP probes

In recent years, the use of click chemistry (CC) in proteomic research has significantly increased. Earlier designs of A/BP probes used CC as a mode of introducing the linker unit. Due to the high specificity of this reaction, functional group protection and deprotection was not required and the triazole ring, formed after ligation, was thought to facilitate some protein-binding interactions. CC probes are commonly used since the 'reporter' group in this case is either the alkyne or azide moiety, which is much smaller than other reporter groups (e.g. biotin, fluorophores) and thus does not hamper the interaction between the probe and protein. Fuwa *et al.* used CC to synthesise a diazirine based benzodiazepine probe **91** (Figure 23), in order to study the enzyme γ -secretase.¹⁶⁶ CC was also used by Yao and co-workers, to synthesise probe **92** for the detection of all four plasmesins in the malarial parasite *Plasmodium falciparum*.¹⁶⁷

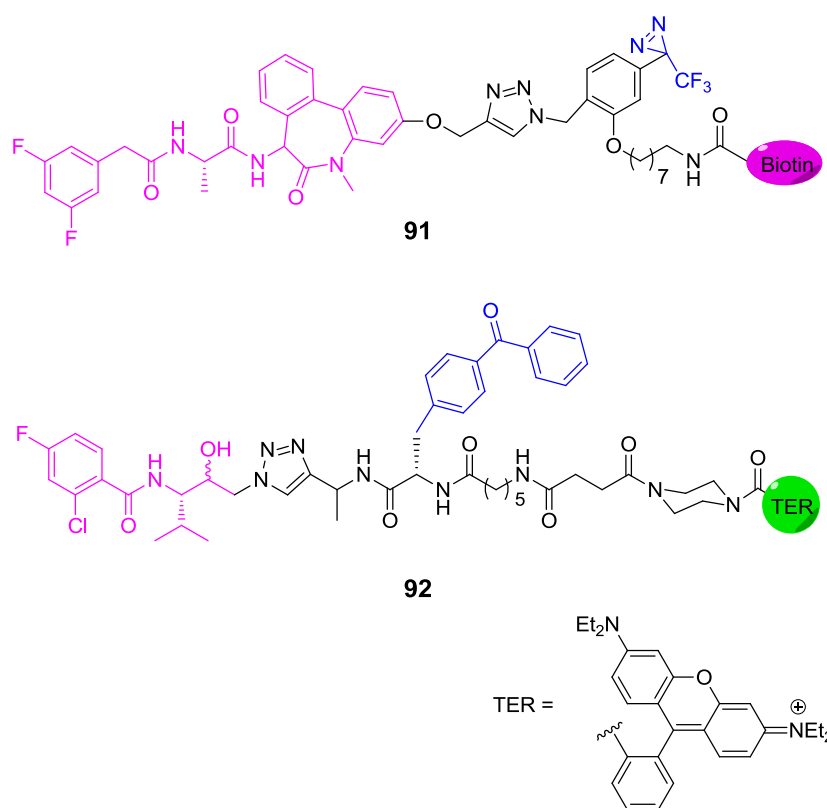


Figure 23. CC-based A/BP probes.

1.7.3 Elucidation of bound enzymes

Once the proteins have been ‘trapped’ by the probe, the complex is isolated, digested using trypsin and the peptide fragments are characterised using analytical platforms like gel electrophoresis (*e.g.* SDS-PAGE) and liquid chromatography-mass spectrometry (LC-MS).¹⁴⁶ Trypsin is commonly used for peptide digestion as it is known to cleave peptide chains at the carboxyl side of lysine and arginine residues specifically, except when they are attached to a proline residue. In gel electrophoresis, the probe-treated proteome is first resolved using one- (1D) or two-dimensional (2D) polyacrylamide gel electrophoresis (PAGE), which differentiates peptide fragments based on their molecular weights, and the labelled proteins are visualised by in-gel fluorescence scanning (for fluorescent probes) or avidin blotting (for biotinylated probes). In LC-MS analysis, after digestion with trypsin, the streptavidin enriched peptides are analysed using LC-MS/MS.

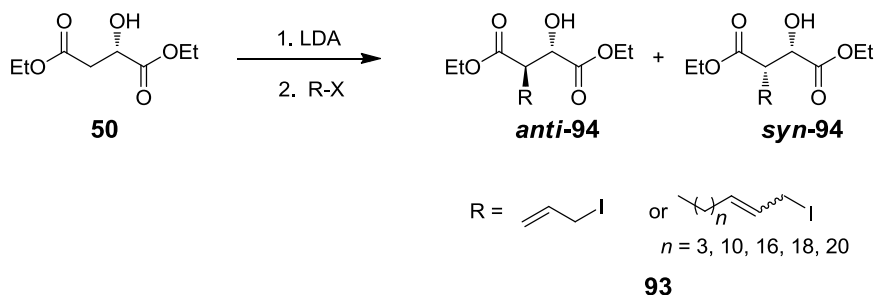
1.8 Thesis Outline

The work described in this thesis focuses on trehalose glycolipids, particularly the synthesis and biological testing of these compounds. In **Chapter 2**, investigations into the synthesis of trehalose diesters of fatty acids (TDEs, **14**, Figure 24) with varying lipid lengths and a discussion on the different synthetic strategies employed in order to obtain all required compounds will be presented. This will be followed by the biological evaluation of TDEs by way of macrophage activation studies and subsequent investigation of structural requirements of trehalose glycolipids for the activation of macrophages in **Chapter 3**. The effect of TDEs on cytotoxicity would also be analysed and correlations made to the previously observed anti-tumour activity of these compounds.



Figure 24. TDEs

In **Chapter 4**, the development of an improved method for the α -alkylation of β -hydroxy-esters will be presented (Scheme 14). Here, it will be illustrated that diethyl malate (**50**) is more effectively alkylated using allylic iodides, **93**, to yield α -alkylated- β -hydroxy esters, **94**, when compared to alkyl iodides, in the Fráter-Seebach alkylation methodology. This method is commonly used in the synthesis of TDMs and TDCMs and thus our methodology could lead to more effective synthesis of these glycolipids. This will be followed by the use of this improved methodology for the synthesis of a trehalose monocorynomycolate (TMCM) analogue (**95**, Figure 25) in **Chapter 5** in order to set the conditions required for the synthesis of other TMCMs and related TDCM glycolipids.



Scheme 14. Improved methodology for Fráter-Seebach alkylation

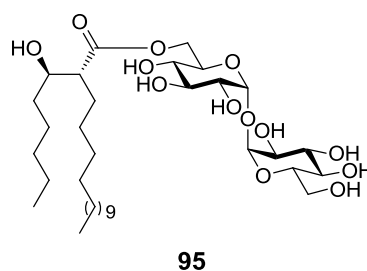


Figure 25. TCMCM analogue

Chapter 6 would focus on the synthesis of an A/BPP probe (**96**, Figure 26), which can be used to elucidate the proteins that TDMs bind to upon interaction with host cells. The probe will consist of a benzophenone trap and an alkyne tag group and together with the reactive trehalose moiety has all the components needed in an A/BP probe.

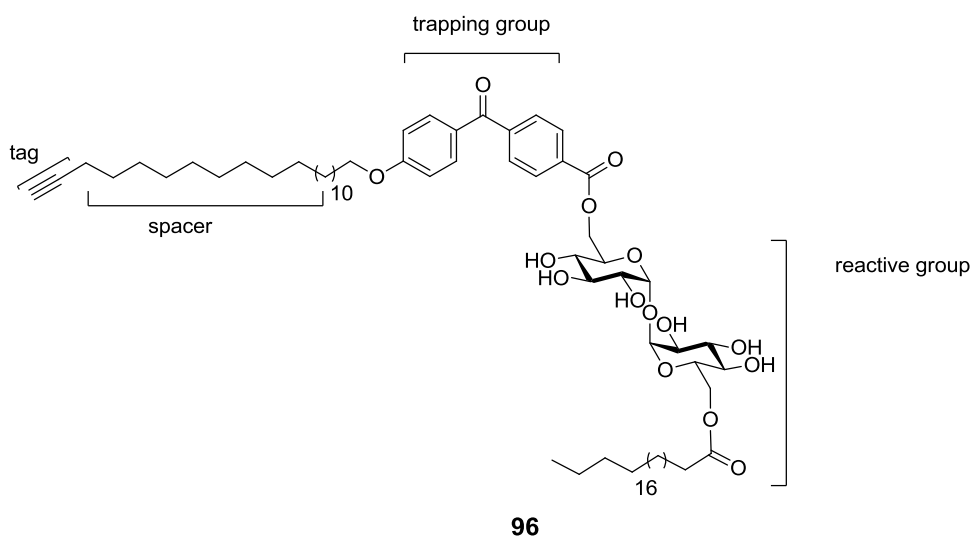


Figure 26. A/BPP probe

Chapter 7 would comprise of a summary of this thesis and some of the future work which will be carried out. Unless otherwise stated, all work was performed by the candidate.

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2

TDE Synthesis¹

2.1 Introduction

Trehalose diesters of fatty acids (TDEs, **1**, Figure 1) are the simplest form of trehalose glycolipids that have been studied for many decades for their biological activities such as anti-tumour, anti-bacterial and macrophage activation properties.^{2,3,4} Perhaps the most well known member of the TDE family is the C22 diester, trehalose dibehenate (TDB) that has shown comparable macrophage activation properties to the more complex trehalose dimycolates (TDMs).⁵ This observation has renewed interest in these glycolipids and one area of interest for us was to investigate the effect that lipid length has on the ability of TDEs to activate macrophages and lead to subsequent activation of the immune system.

To correlate the lipid length to the biological activity of these glycolipids (see Chapter 3 for biological analysis), we planned to synthesise TDEs containing short

(C4), medium (C7, C10), long (C18, C20 and C22) and very long (C26) aliphatic lipid chains (Figure 1).

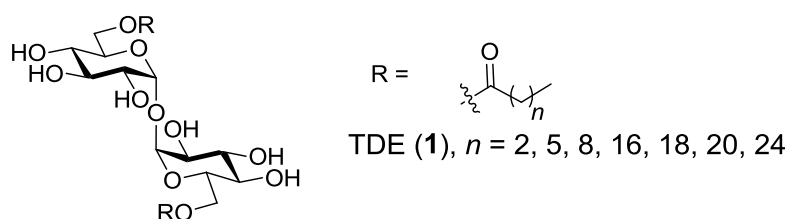


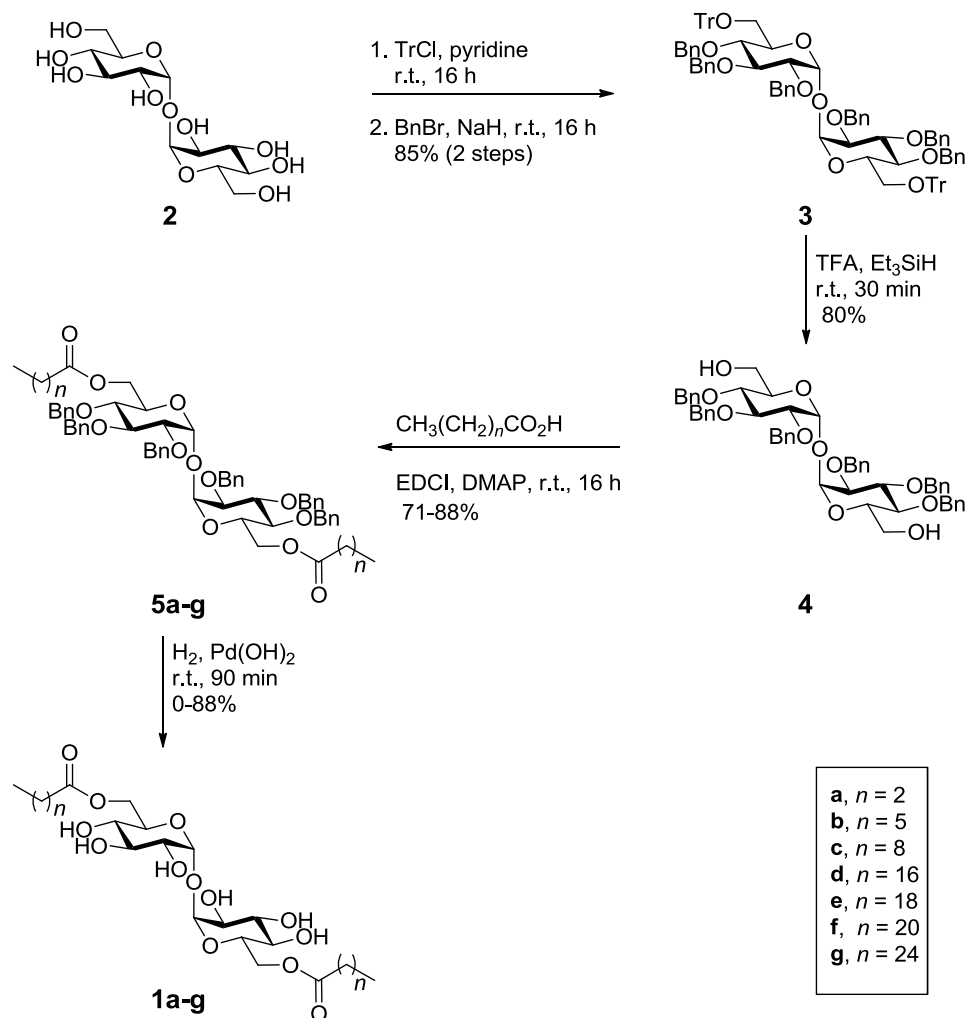
Figure 1. Target TDEs **1**

2.2 TDE Synthesis

2.2.1 Initial Strategy

In order to synthesise a range of TDEs, we envisaged a single synthetic strategy that could be used for the synthesis of all these related compounds. To this end, commercially available α,α' -D-trehalose (**2**) was selectively tritylated at the 6 and 6' positions using trityl chloride and pyridine,⁶ perbenzylated⁷ using benzyl bromide and sodium hydride to form the fully protected compound **3** in 85% over two steps (Scheme 1). The trityl groups were subsequently removed using trifluoroacetic acid (TFA) and triethylsilane resulting in diol **4** in good (80%) yield. Diol **4** was then subjected to esterification with various fatty acids using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDCI) and 4-dimethylamino-pyridine (DMAP) to form the fully protected diacyl trehaloses **5a-g**. Most of the carboxylic acids were sourced commercially, except for docosanoic acid which was obtained after Jones oxidation of docosanol.⁸ The optimum conditions for the esterification reaction were found to be 1 equiv. of diol **4**, 4.4 equiv. of the acid, 6.6 equiv. of EDCI, and 0.15 equiv of DMAP with the reaction being stirred overnight. However, it was noted that longer reaction times were required for the longer lipids (Table 1). With the protected diacyl trehaloses **5** in hand, attempts were then made to remove the benzyl protecting groups so as to generate the target TDEs **1**. Initially, Pd/C in $\text{CHCl}_3\text{:MeOH}$ (3:2) was used for the reaction; however, no product formation was

seen with only starting material retrieved from the reaction mixture. Next, catalytic $\text{Pd}(\text{OH})_2$ was used and the debenzylolation proceeded uneventfully for the C4–C18 derivatives (**5a–d**, Table 1, Entries 1–4). Unfortunately, the extension of this methodology to the C20–C26 homologues (**5e–g**, Entries 5–7) was unsuccessful and only product degradation was observed.



Scheme 1. Initial synthetic strategy

Table 1. Formation of TDEs

Entry	C _n	Coupling Conditions EDCI = 6.6 equiv., DMAP = 0.15 equiv CH ₂ Cl ₂	Yield-Coupling (%)	Debenzylation Conditions	Yield-Debenzylation (%)
1	2	C4 acid = 4.4 equiv., overnight, r.t.	88	Pd(OH) ₂ , CHCl ₃ :MeOH 3:2, 1 atm	62
2	5	C7 acid = 4.4 equiv., overnight, r.t.	85	Pd(OH) ₂ , CHCl ₃ :MeOH 3:2, 1 atm	67
3	8	C10 acid = 4.4 equiv., overnight, r.t.	83	Pd(OH) ₂ , CHCl ₃ :MeOH 3:2, 1 atm	63
4	16	C18 acid = 4.4 equiv., overnight, r.t.	85	Pd(OH) ₂ , CHCl ₃ :MeOH 3:2, 1 atm	88
5	18	C20 acid = 4.4 equiv., overnight, r.t.	80	Pd(OH) ₂ , CHCl ₃ :MeOH 3:2, 1 atm	0
6	20	C22 acid = 4.4 equiv., 2 days, r.t.	78	Various (see Table 2)	0
7	24	C26 acid = 4.4 equiv., 7 days, r.t.	71	Various (see Table 2)	0

The key reason for the low reactivity of the longer chain TDEs was attributed to the low solubility of these compounds leading to reduced reactivity. Hence different catalysts, solvent systems, reaction temperatures and pressures were used, but at best, only minor amounts of partially debenzylated products were observed (Table 2). This result was disappointing, however, the sluggish hydrogenation of benzyl protected long-chain glycolipids has been reported previously.⁹ The lack of reactivity of the long chain diacyl trehaloses **5e-g** towards hydrogenation led us to adopt an alternative approach for the preparation of the C20–C26 TDEs focusing on using another protecting group for the trehalose unit.

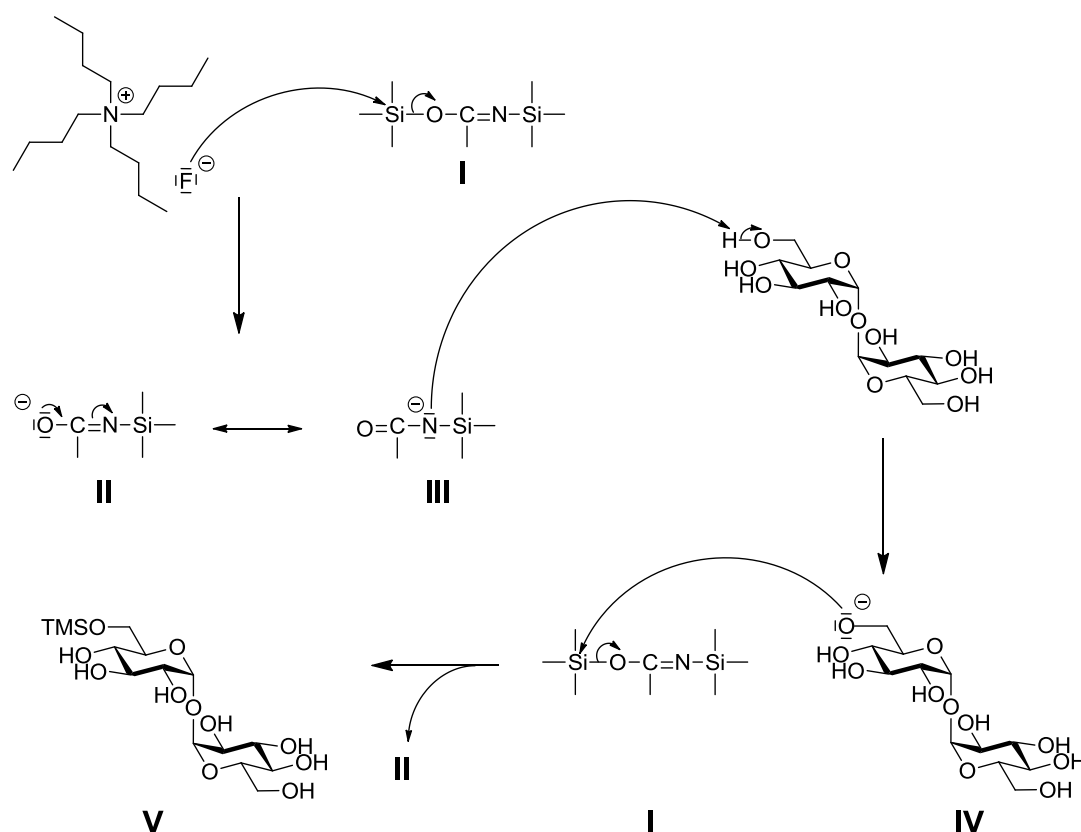
Table 2. Attempted debenzylation conditions

Entry	C _n	Debenzylation Conditions	Results
1	20	Pd(OH) ₂ , CHCl ₃ :MeOH 3:2, 1 atm at r.t. over 3 days	Degradation (loss of lipid chain) and starting material
2	20	Pd(OH) ₂ , CHCl ₃ :MeOH 3:2, 15 psi at r.t. overnight	Degradation
3	20	Pd/C, EtOH:EtOAc:CHCl ₃ 1:1:1, 15 psi at r.t. overnight	partially-debenzylated products
4	20	Pd/C, THF:EtOH:EtOAc 1:1:1, 1 atm at 50 °C, over 2 days	Degradation
5	24	Pd(OH) ₂ , CHCl ₃ :MeOH 3:2, 1 atm at r.t. over 3 days	Degradation
6	24	Pd/C, THF:EtOH:EtOAc 1:1:1, 1 atm at 50 °C over 4 days	Degradation

2.2.2 Alternate Synthetic Strategy

With a view to using protecting groups that could be more readily removed at the final stage in the synthesis, we employed the strategy of Toubiana and co-workers to prepare 2,2',3,3',4,4'-hexa-*O*-trimethylsilyl- α,α -trehalose.¹⁰ This elegant two-step one-pot synthesis of hexa-silylated trehalose was proposed by Johnson¹¹ and the reaction proceeds through per-silylation of the trehalose moiety followed by the removal of the more labile primary trimethylsilyl (TMS) ethers using potassium carbonate. The reaction is initiated with the attack on the silyl atom in *N,O*-bis-trimethylsilylacetamide (BSA) (**I**) by the fluoride anion of tetra-butylammonium fluoride (TBAF), generating intermediate **II** (Scheme 2). The preference for attack on this silyl group is due to the stability of the resulting intermediate **II**, attributed to resonance stabilization of the negative charge on the oxygen in **II** by the nitrogen as

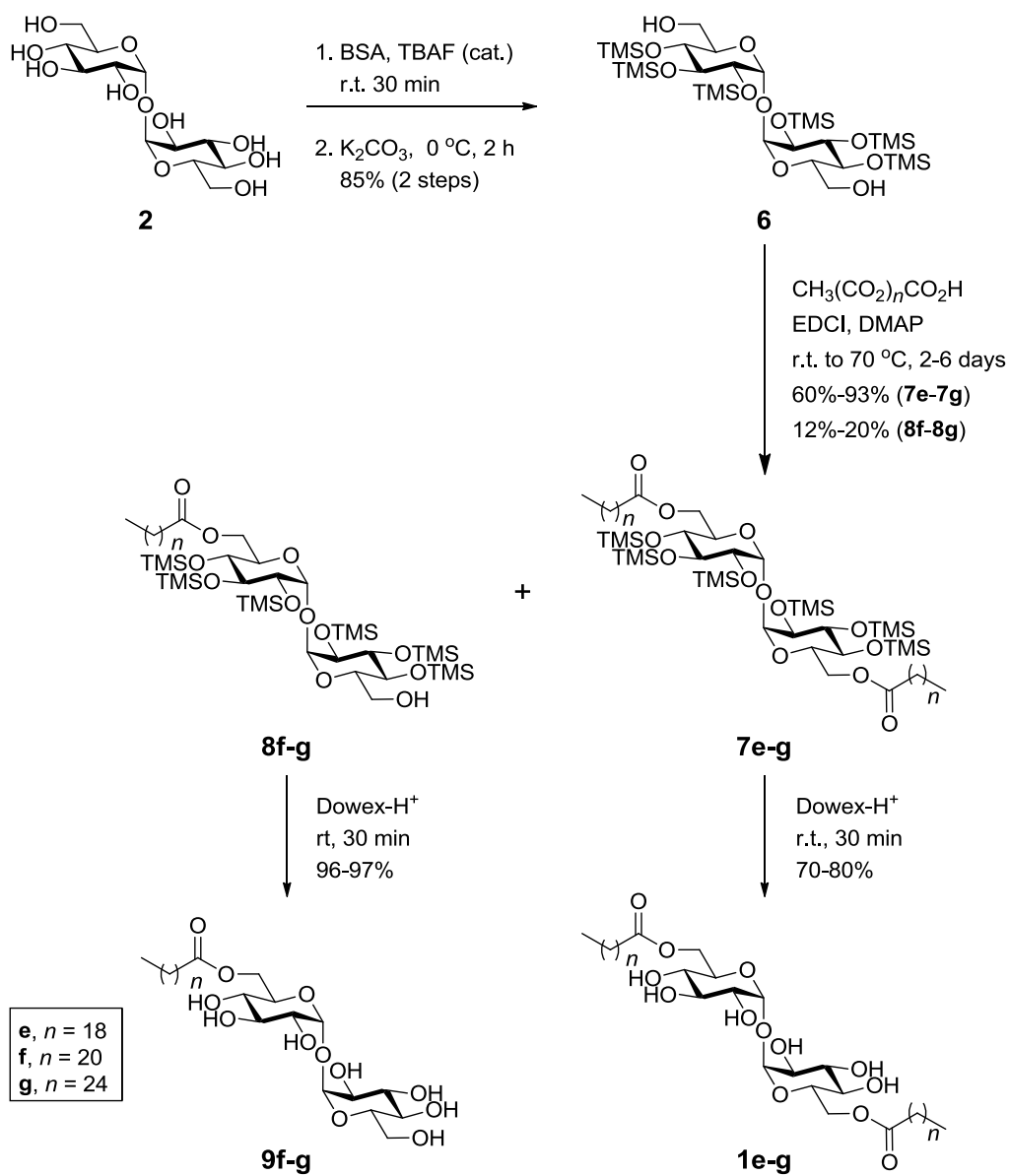
shown in intermediate **III**. The negatively charged nitrogen in intermediate **III** abstracts a proton from one of the trehalose hydroxyls. The resulting negatively charged oxygen in **IV** then attacks the silyl group in another molecule of BSA (**I**) leading to the formation of a TMS ether (**V**) and regeneration of the BSA intermediate (**II**).



Scheme 2. Reaction mechanism for BSA mediated silylation of trehalose

Accordingly, α,α -D-trehalose (**2**) was treated with BSA and catalytic TBAF, followed by K_2CO_3 to generate diol **6** in a good (85%) yield over two steps (Scheme 3). Diol **6** was then coupled to the longer chain carboxylic acids using the protocols outlined in Table 3. Formation of the C20 trehalose diester ($n = 18$) proceeded uneventfully mediated by EDCI and DMAP in CH_2Cl_2 to give trehalose diester **7e** in 93% yield (Table 3, Entry 1). Surprisingly, extension of this methodology to the C22 homologue proved unsuccessful with none of the desired trehalose diester **7f**

observed even after the use of excess acid, the addition of further equivalents of EDCI and DMAP, or refluxing of the reaction mixture (Entries 2-5). In all instances, only minor (<40%) amounts of the corresponding mono-ester were observed. Although, a change of solvent to dimethylformamide (DMF) did not lead to any improvement (Entries 4 and 5), the use of toluene and heating the reaction at 70 °C for 2 days saw complete conversion of starting material and the desired trehalose diester **7f** was isolated in 86% yield (Entry 6). These conditions were then used for the synthesis of the C26 trehalose diester **7g**, which was formed in a respectable (60%) yield although a longer reaction time (6 days) was required to see complete consumption of starting material (entry 7). The TMS-groups were then readily removed following subjection of the diesters to Dowex-H⁺ resin to yield the target TDEs (**1e-g**) in 80%, 80% and 70% yield, respectively.



Scheme 3. Alternate synthetic strategy used

Table 3. Esterification reaction using diol **6**

Entry	Cn	Conditions	Product	Yield ^[a]
EDCI (6.6 equiv.), DMAP (0.15 equiv.),				
1	18	CH ₃ (CH ₂) ₁₈ CO ₂ H (4.4 equiv.), CH ₂ Cl ₂ , rt, 18 h	7e	93%
2	20	CH ₃ (CH ₂) ₂₀ CO ₂ H (4.4 equiv.), CH ₂ Cl ₂ , rt, 18 h	7f	0% ^[b]
3	20	CH ₃ (CH ₂) ₂₀ CO ₂ H (4.4 equiv.), CH ₂ Cl ₂ , reflux, 48 h	7f	0% ^[b]
4	20	CH ₃ (CH ₂) ₂₀ CO ₂ H (4.4 equiv.), DMF, rt, 48 h	7f	0% ^[b]
5	20	CH ₃ (CH ₂) ₂₀ CO ₂ H (4.4 equiv.), DMF, 50 °C, 48 h	7f	0% ^[b]
6	20	CH ₃ (CH ₂) ₂₀ CO ₂ H (4.4 equiv.), toluene, 70 °C, 48 h	7f	86%
7	24	CH ₃ (CH ₂) ₂₄ CO ₂ H (4.4 equiv.), toluene, 70 °C, 6 days	7g	60%

[a] Isolated yield

[b] Only monoester (12–40%) was observed.

In addition to the protected TDEs, the esterification reaction also yielded about 12–20% of the protected trehalose monoesters of fatty acids (TMEs, **8f** and **8g**). Both the protected TMEs were more polar than the corresponding trehalose diesters **7f** and **7g**, making it possible to separate the monoesters from the diesters through silica gel flash chromatography. The protected TMEs were then deprotected using Dowex-H⁺ resin to yield the target TMEs **9f** and **9g** in good yields.

2.2.3 Isolation of final compounds and characterisation

For C4–C22 TDEs, the compounds were readily purified using silica gel flash chromatography, with the compounds eluting in 1–3% MeOH in EtOAc. For the purification of C26 TME (**9g**) and TDE (**1g**) silica gel chromatography was not ideal for the separation of compounds from impurities. Reversed phase chromatography gave better results with the C26 TME eluting in MeOH, however, the C26 TDE

could only be eluted using pyridine in reversed phase chromatography. The final compounds were obtained as white solids and NMR analyses of the glycolipids comprising of 18 carbon lipid chains or lower were carried out in a mixture of deuterated chloroform and methanol. For the longer TDEs and TMEs, pyridine was used as the NMR solvent.

The TDEs and TMEs were characterised based on NMR and mass spectral analysis. For the C26 TME (**9g**, Figure 2 and 3), the chemical shifts for the anomeric protons were different in the ^1H NMR spectrum, with two doublet resonances observed at 5.84 ($J = 3.9$ Hz) and 5.82 ppm ($J = 3.9$ Hz), indicating that the trehalose molecule was no longer symmetrical. In addition, the protons at 6 and 6' positions had very different chemical shifts indicative of different groups attached to the carbons at these positions. Integral comparison of the α -protons of the lipid chain and the anomeric protons indicated a 1:1 ratio. An HMBC correlation was found between the carbonyl carbon at 173.8 ppm and the protons at the 6 position but not at the 6' position and this together with the mass spectral analysis [HRMS(ESI) m/z calcd. for $[\text{C}_{38}\text{H}_{72}\text{O}_{12}+\text{Na}]^+$: 743.4916, obsd.: 743.4907] unequivocally established that only one of the primary positions in the trehalose moiety was esterified. On the other hand, for the C26 TDE (**1g**, Figure 4 and 5), the ^1H NMR spectrum showed only one doublet resonance at 5.78 ppm ($J = 3.7$ Hz) for the anomeric protons and using COSY correlations the resonances for the protons at 6a/6a' and 6b/6b' positions were found to being 4.87 ppm (m) and 4.82 ppm (dd, $J = 11.7$ Hz, $J = 5.4$ Hz,) with both showing HMBC correlations to the carbonyl carbon at 173.8 ppm. Here, integral comparison of the α -protons of the lipid chain and the anomeric protons clearly indicated a 2:1 ratio indicating that lipid chains were attached at both the 6 and 6' position of the trehalose. Mass spectral analysis of the product was in agreement with the expected mass [HRMS(ESI) m/z calcd. for $[\text{C}_{64}\text{H}_{122}\text{O}_{12} + \text{Na}]^+$: 1121.8783, obsd.: 1121.8764]. Full assignments for all the compounds were made on the basis of 2D NMR (COSY, HSQC and HMBC) (see experimental for full assignment).

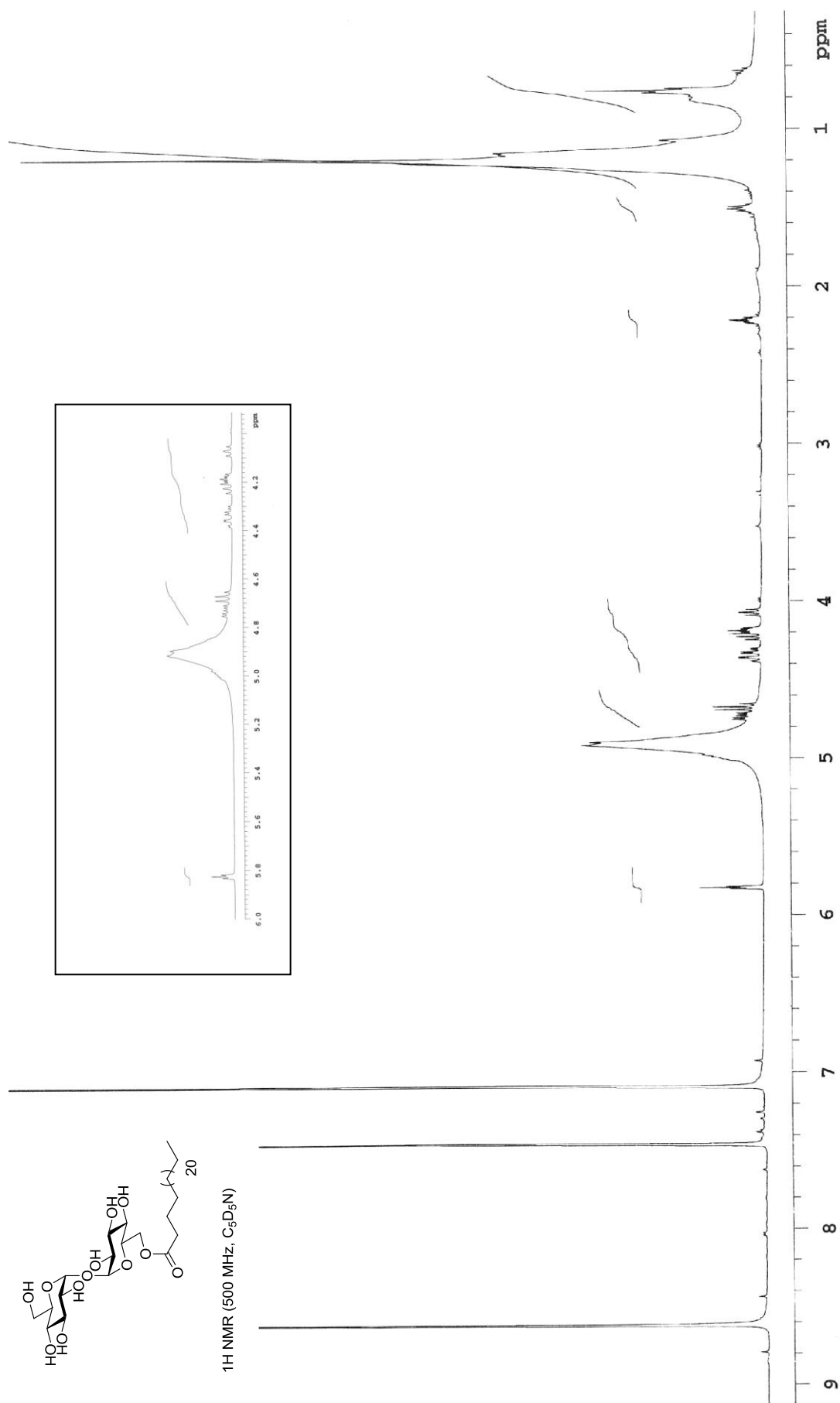


Figure 2. ^1H NMR spectrum of C26 TME (**9g**)

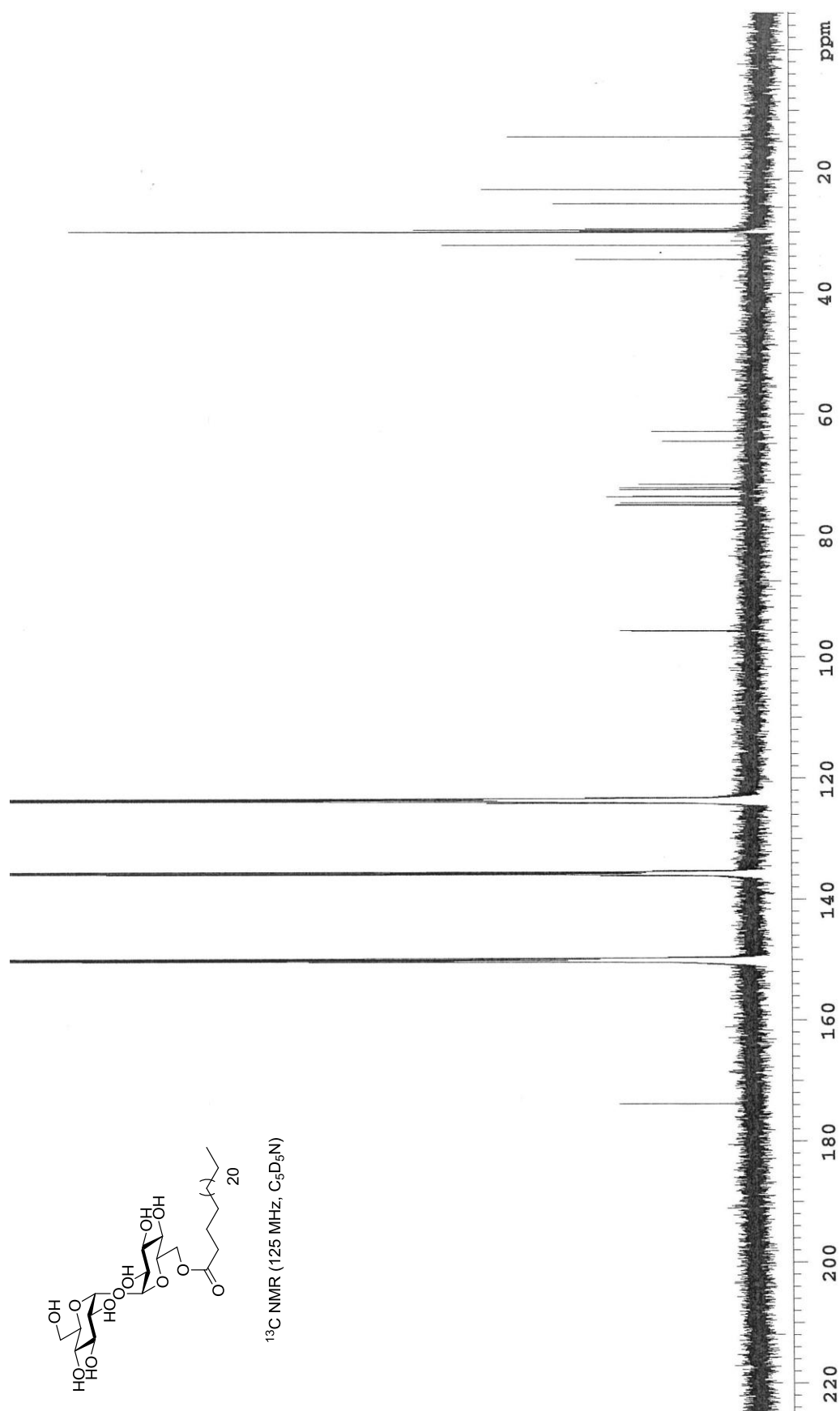


Figure 3. ^{13}C NMR spectrum of C26 TME (**9g**)

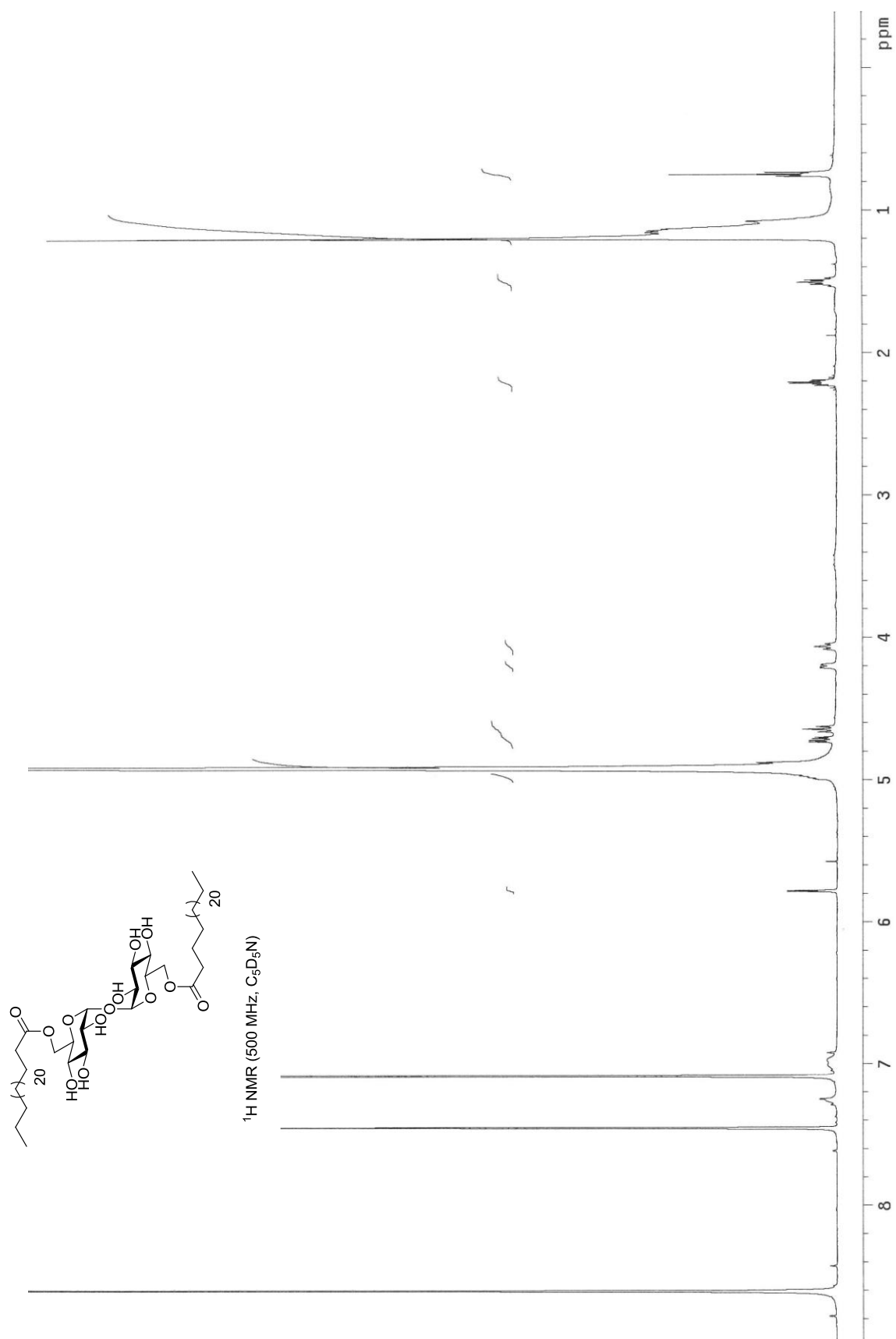


Figure 4. ^1H NMR spectrum of C26 TDE (**1g**)

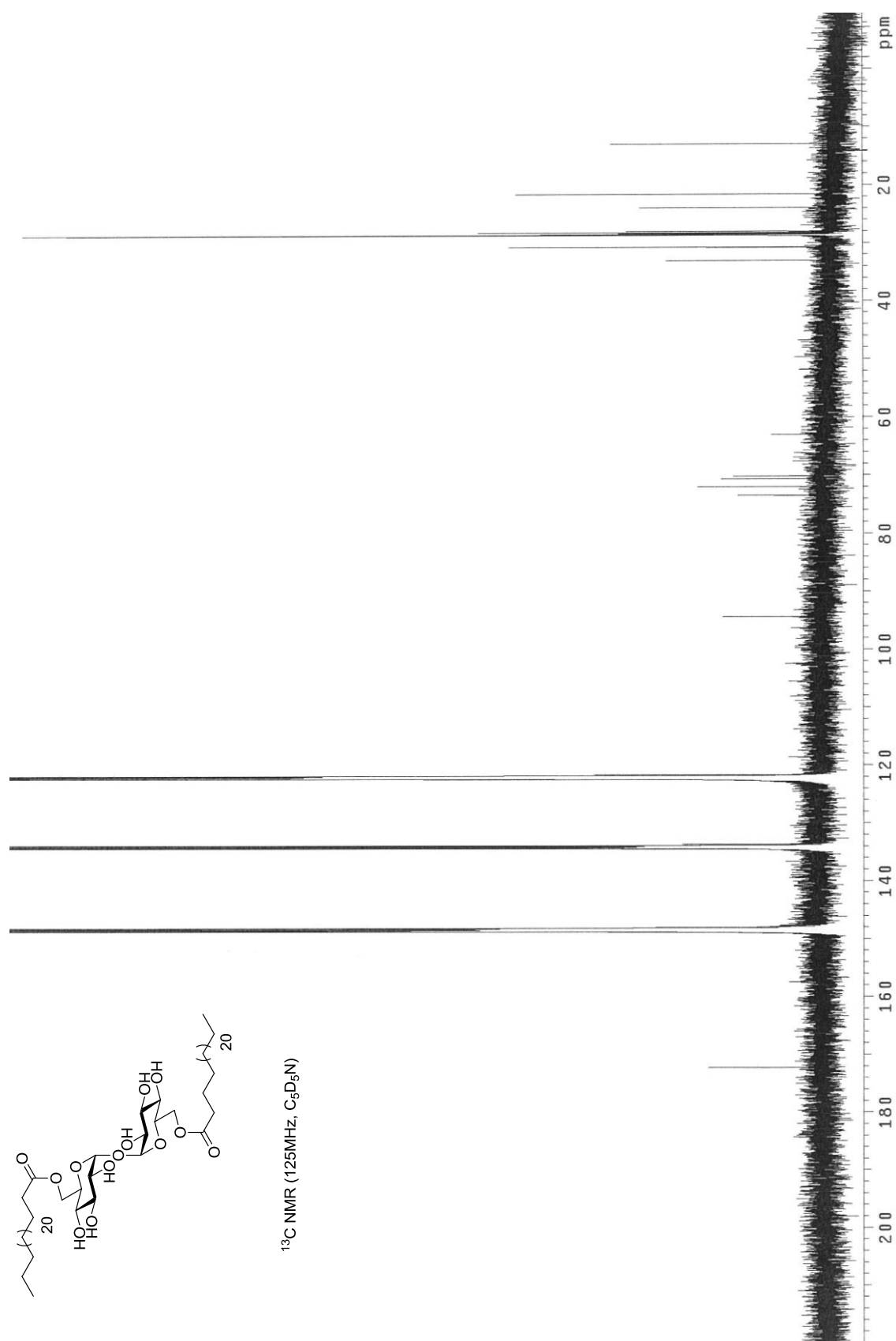


Figure 5. ^{13}C NMR spectrum of C26 TDE (**1g**)

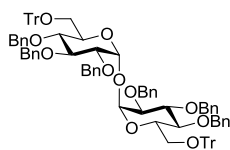
2.3 Conclusion

In this work, 7 TDEs ranging from C4-C26 were prepared, along with the C22 and C26 TMEs. Two different synthetic strategies were used to obtain these final compounds with the use of TMS protecting groups deemed more favourable in the synthesis of these trehalose glycolipids.

2.4 Experimental Section

Unless otherwise stated, all reactions were performed under N₂. Prior to use, pyridine was dried and stored over 4 Å molecular sieves (4 Å MS), CH₂Cl₂ was distilled from P₂O₅ and toluene was dried and stored under Na wire. D-(+)-Trehalose dihydrate (Sigma), trityl chloride (Acros), anhydrous DMF (Acros), TBAI (Riedel-de Haen), NaH (Avocado Research Chemicals, 60% dispersion in mineral oil), Et₃SiH (Sigma), TFA (Aldrich), EDCI (Aldrich), DMAP (Merck), n-butyric acid (BDH), heptanoic acid (BDH), decanoic acid (Merck), stearic acid (ICN Pharmaceuticals), arachidic acid (Sigma), 1-docosanol (Fluka), hexacosanoic acid (Acros), Pd (OH)₂/C (Aldrich, 20 wt%), TBAF (Aldrich), *N,O*-bis(trimethylsilyl)acetamide (Fluka), Dowex-H⁺ (Supelco), anhydrous Et₂O (Biolab), EtOAc (Panreac), petroleum ether (Pure Science), anhydrous MeOH (Panreac), isopropanol (Pure Science), CHCl₃ (Panreac), NaHCO₃ (Pure Science), K₂CO₃ (Pure Science), MgSO₄ (Pure Science) and NaCl (Panreac) were used as received. All solvents were removed through evaporation under reduced pressure. Reactions were monitored by TLC analysis on Macherey-Nagel silica gel coated plastic sheets (0.20 mm with fluorescent indicator UV₂₅₄) via detection by UV-absorption (254 nm) and by dipping in 10% H₂SO₄ in EtOH followed by charring at 150 °C. Column chromatography was performed using Pure Science silica gel (40-63 µm). AccuBOND II ODS-C18 (Agilent) was used for reversed phase chromatography. High resolution mass spectra were recorded on a Waters Q-TOF PremierTM Tandem Mass Spectrometer using positive electro-spray ionisation. Optical rotations were recorded on a Perkin-Elmer 241 polarimeter or Autopol II (Rudolph Research Analytical) at 589 nm (sodium D-line). Infrared spectra were recorded as thin films using a Bruker Tensor 27 FTIR spectrometer equipped with an Attenuated Total Reflectance (ATR) sampling accessory and are

reported in wave numbers (cm^{-1}). Nuclear magnetic resonance spectra were at 20 °C in $\text{C}_5\text{D}_5\text{N}$, CD_3OD or CDCl_3 using a Varian INOVA operating at 500 MHz. Chemical shifts are given in ppm (δ) relative to solvent residual peak. NMR peak assignments were made using COSY, HSQC and HMBC 2D experiments. Melting points were obtained using a Gallenkamp Melting Point Apparatus.

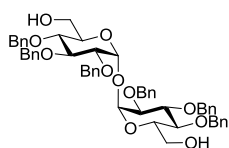


2,2',3,3',4,4'-Hexa-*O*-benzyl-6,6'-di-*O*-trityl- α,α' -D-trehalose (3). α,α' -Trehalose **2** (5.26 g, 14.6 mmol) was co-evaporated with pyridine (3 x 20 mL) and then dissolved in 30 mL of pyridine. Trityl chloride (28.5 g, 102.2

mmol) was added and the resulting mixture stirred at 40 °C under nitrogen for 16 h after which dry methanol (40 mL) was added and the reaction stirred for a further 0.5 h. The solvent was removed under reduced pressure to give a pale yellow solid. Diethyl ether (100 mL) was added to the crude product and the resulting suspension filtered through a Büchner filter. The solid product was repeatedly washed with diethyl ether until free from triphenylmethyl chloride, as gauged by TLC. The solid was then washed with 3% NaHCO_3 and dried under vacuum and stored in a dessicator.

6,6'-Di-*O*-trityl-trehalose (2.57 g, 3.12 mmol) was co-evaporated with anhydrous DMF (3 x 5 mL) and dissolved in 15 mL of DMF. Catalytic TBAI (103 mg, 0.28 mmol) was added and the resulting solution cooled down to 0 °C. Benzyl bromide (3.14 mL, 28.1 mmol) was added slowly to the reaction mixture followed by portion-wise addition of sodium hydride (60% dispersion in mineral oil) (1.86 g, 46.7 mmol), and the reaction was allowed to warm up to room temperature. After stirring for 2 h, the reaction was quenched with MeOH (30 mL), stirred vigorously for 10 min and concentrated *in vacuo*. The resulting crude mixture was dissolved in Et_2O (200 mL), washed with water (3 x 50 mL) and brine (50 mL), dried (MgSO_4), filtered and concentrated *in vacuo*. The title compound was obtained after silica gel flash chromatography eluting in 15:1 (v/v) Pet. Ether/ EtOAc , as a white crystals (3.62 g, 2.65 mmol, 85% over 2 steps). Mp 91.0–94.0 °C; R_f = 0.5 (4/1, PE/EA, v/v); $[\alpha]_D^{23} = +60.5$ (c = 1.0 CHCl_3); IR (film): 3019, 2878, 2212, 1265, 1214, 744, 668 cm^{-1} ; ^1H

NMR (500 MHz, CDCl₃) δ 6.76–7.42 (m, 60H, H_{arom}), 5.47 (d, $J_{1,2}$ = 3.7 Hz, 2H, H-1), 4.92 (d, $J_{a,b}$ = 10.7 Hz, 2H, CH-a, 3-*O*-Bn), 4.80 (d, $J_{a,b}$ = 10.7 Hz, 2H, CH-b, 3-*O*-Bn), 4.72 (d, $J_{a,b}$ = 10.3 Hz, 2H, CH-a, 4-*O*-Bn), 4.67 (d, $J_{a,b}$ = 11.9 Hz, CH-b, 2-*O*-Bn), 4.66 (d, $J_{a,b}$ = 11.9 Hz, 2H, CH-a, 2-*O*-Bn), 4.23 (d, $J_{a,b}$ = 10.3 Hz, 2H, CH-a, 4-*O*-Bn), 4.17 (bd, $J_{4,5}$ = 9.6 Hz, 2H, H-5), 3.97 (t, $J_{2,3}$ = $J_{3,4}$ = 9.6 Hz, 2H, H-3), 3.87 (t, $J_{3,4}$ = $J_{4,5}$ = 9.6 Hz, 2H, H-4), 3.72 (dd, $J_{2,3}$ = 9.6 Hz, $J_{1,2}$ = 3.7 Hz, 2H, H-2), 3.33 (dd, $J_{6a,6b}$ = 10.3 Hz, $J_{5,6a}$ = 2.7 Hz, 2H, H-6a), 3.06 (dd, $J_{6a,6b}$ = 10.3 Hz, $J_{5,6b}$ = 1.7 Hz, 2H, H-6b); ¹³C NMR (125 MHz, CDCl₃) δ 143.8, 138.7, 138.2, 138.0 (*Ci*-Tr and Bn), 128.9, 128.4, 128.2, 128.1, 128.1, 127.8, 127.6, 127.5, 127.1, 126.9, 126.8 (CH_{arom}), 94.9 (C-1), 81.8 (C-3), 80.3 (C-2), 77.8 (C-4), 75.9 (CH₂, 3-*O*-Bn), 75.1 (CH₂, 4-*O*-Bn), 72.5 (CH₂, 2-*O*-Bn), 70.7 (C-5) and 62.2 (C-6); HRMS(ESI) m/z calcd. for [C₉₂H₈₆O₁₁+Na]⁺: 1389.6068, obsd.: 1389.6072.



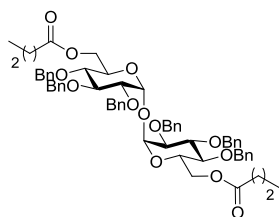
2,2',3,3',4,4'-Hexa-*O*-benzyl- α,α' -D-

trehalose (4). Et₃SiH (0.75 mL, 4.74 mmol) was added to the hexa-*O*-benzyl-di-*O*-trityl-trehalose (3.24 g, 2.37 mmol) (co-evaporated 3 x with toluene) in dry CH₂Cl₂ (30 mL). 10%

TFA solution was then added drop-wise to the reaction mixture until the yellow colour no longer appeared. The reaction was completed in 30 min, (as determined *via* TLC monitoring) after which MeOH (6 mL), saturated NaHCO₃ (20 mL) and brine (20 mL) was added. The aqueous layer was extracted with CH₂Cl₂ (2 x 50 mL) and the combined organic layer was dried (MgSO₄), filtered and concentrated *in vacuo*. The residue was subjected to silica gel flash chromatography and eluted with 1:1 (v/v) Pet. Ether/EtOAc to afford the title compound **4** as a colourless viscous oil (1.675 g, 1.90 mmol, 80%). R_f = 0.38 (PE/EA, 1/1, v/v); $[\alpha]_D^{23}$ = +68.0 (c = 1.0, CHCl₃); IR (film): 3465, 3067, 3016, 2928, 2875, 1360, 1215, 1155, 1095, 1068, 1027 cm⁻¹; ¹H NMR (500 MHz, CDCl₃) δ 7.27–7.53 (m, 30H, H_{arom}), 5.13 (d, $J_{1,2}$ = 3.6 Hz, 2H, H-1), 5.01 (d, $J_{a,b}$ = 11.0 Hz, 2H, CH-a, 3-*O*-Bn), 4.89 (d, $J_{a,b}$ = 11.0 Hz, 2H, CH-b, 3-*O*-Bn), 4.88 (d, $J_{a,b}$ = 11.0 Hz, 2H, CH-a, 4-*O*-Bn), 4.72 (d, $J_{a,b}$ = 11.7 Hz, 2H, CH-a, 2-*O*-Bn), 4.67 (d, $J_{a,b}$ = 11.7 Hz, 2H, CH-b, 2-*O*-Bn), 4.65 (d, $J_{a,b}$ = 11.0 Hz, 2H, CH-b, 4-*O*-Bn), 4.08 (m, 4H, H-3 and H-5), 3.55–3.60 (m, 6H, H-4 and H-6a,b), 3.54 (dd, $J_{2,3}$ = 10.0 Hz, $J_{1,2}$ = 3.6 Hz, 2H, H-2); ¹³C NMR (125 MHz,

CDCl₃) δ 138.8 (C-*i*, 3-*O*-Bn), 138.2 (C-*i*, 4-*O*-Bn), 138.0 (C-*i*, 2-*O*-Bn), 128.9, 128.5, 128.4, 128.1, 127.9, 127.7, 127.6, 127.5 (CH_{arom}), 94.1 (C-1), 81.6 (C-3), 79.5 (C-2), 77.3 (C-4), 75.6 (CH₂, 3-*O*-Bn), 75.1 (CH₂, 4-*O*-Bn), 73.0 (CH₂, 2-*O*-Bn), 71.3 (C-5) and 61.5 (C-6); HRMS(ESI) m/z calcd. for [C₅₄H₅₈O₁₁+Na]⁺: 905.3877, obsd.: 905.3881.

General Procedure for Esterification. To a solution of diol **4** (1 mmol, 1 equiv.) in DCM (10 mL) the carboxylic acid (4.4 mmol, 4.4 equiv.), EDCI (6.6 mmol, 6.6 equiv.) and DMAP (0.15 mmol, 0.15 equiv.) were added. The reaction was stirred at room temperature overnight, after which the resulting precipitate was removed from the mixture by filtration and washed with cold DCM. The combined organic layers were washed with water (30 mL), dried (MgSO₄), filtered and concentrated *in vacuo*. The residue was purified using silica gel flash chromatography and the eluent given.

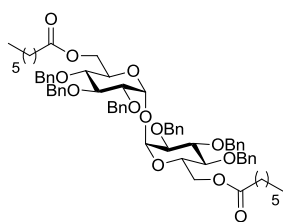


2,2',3,3',4,4'-Hexa-*O*-benzyl-

6,6'-di-*O*-butanoyl- α,α' -D-trehalose (5a**).** By subjecting diol **4** (100 mg, 0.11 mmol), butyric acid (44 mg, 0.50 mmol), EDCI (143 mg, 0.73 mmol) and DMAP (2.06 mg, 0.002 mmol) to the general procedure for esterification,

the title compound was obtained (eluting with 5:1 (v/v) Pet. Ether/EtOAc) as a colourless oil (101.8 mg, 0.097 mmol, 88%). R_f = 0.76 (PE/EA, 1/1, v/v); $[\alpha]^{18}_D$ = +90.25 (c = 1.0, CHCl₃); IR (film): 3028, 2934, 1733, 1497, 1454, 1359, 1215, 1158, 1070, 1028, 995, 747, 697, 667 cm⁻¹; ¹H NMR (500 MHz, CDCl₃) δ 7.25–7.38 (m, 30H, H_{arom}), 5.17 (d, $J_{1,2}$ = 3.5 Hz, 2H, H-1), 5.01 (d, $J_{a,b}$ = 10.5 Hz, 2H, CH-a, 3-*O*-Bn), 4.87 (d, $J_{a,b}$ = 10.5 Hz, 2H, CH-b, 3-*O*-Bn), 4.87 (d, $J_{a,b}$ = 10.6 Hz, 2H, CH-a, 4-*O*-Bn), 4.72 (d, $J_{a,b}$ = 11.9 Hz, 2H, CH-a, 2-*O*-Bn), 4.69 (d, $J_{a,b}$ = 11.9 Hz, 2H, CH-b, 2-*O*-Bn), 4.52 (d, $J_{a,b}$ = 10.6 Hz, 2H, CH-b, 4-*O*-Bn), 4.23 (bd, $J_{4,5}$ = 9.5 Hz, 2H, H-5), 4.15 (dd, $J_{6a,6b}$ = 12.2 Hz, $J_{5,6a}$ = 3.4 Hz, 2H, H-6a), 4.06 (dd, $J_{6a,6b}$ = 12.2 Hz, $J_{5,6b}$ = 2.4 Hz, 2H, H-6b), 4.05 (t, $J_{2,3}$ = $J_{3,4}$ = 9.3 Hz, H-3), 3.55 (dd, $J_{2,3}$ = 9.3 Hz, $J_{1,2}$ = 3.5 Hz, H-2), 3.53 (t, $J_{3,4}$ = $J_{4,5}$ = 9.5 Hz, 2H, H-4), 2.24 (t, $J_{8,9}$ = 6.9 Hz, 4H, H-8), 1.63–1.60 (m, 4H, H-9), 0.91 (t, $J_{9,10}$ = 7.3 Hz, 6H, H-10); ¹³C NMR (125 MHz,

CDCl₃) δ 173.3 (C-7), 138.6 (C-*i*, 3-*O*-Bn), 137.8 (C-*i*, 4-*O*-Bn), 137.8 (C-*i*, 2-*O*-Bn), 128.53, 128.5, 128.1, 128.0, 127.8, 127.7, 127.5 (CH_{arom}), 94.0 (C-1), 81.6 (C-3), 79.3 (C-2), 77.5 (C-4), 75.7 (CH₂, 3-*O*-Bn), 75.2 (CH₂, 4-*O*-Bn), 72.9 (CH₂, 2-*O*-Bn), 69.1 (C-5), 62.5 (C-6), 36.0 (C-8), 18.4 (C-9), 13.6 (C-10); HRMS(ESI) m/z calcd for [C₆₂H₇₀O₁₃+Na]⁺: 1045.4714, obsd.: 1045.4720.

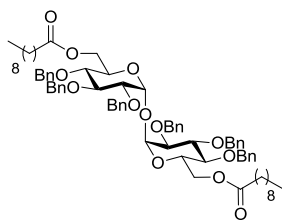


2,2',3,3',4,4'-Hexa-*O*-

benzyl-6,6'-di-*O*-heptanoyl- α,α' -D-trehalose (**5b**). By

subjecting diol **4** (0.41 g, 0.46 mmol), heptanoic acid (0.29 mL, 2.02 mmol), EDCI (0.58 g, 3.03 mmol) and DMAP (0.008 g, 0.07 mmol) to the general procedure

for esterification, the title compound was obtained (eluting in 10:1 (v/v) Pet. Ether/EtOAc) as a colourless oil (0.43 g, 0.39 mmol, 85%). R_f = 0.8 (PE/EA, 1/1, v/v); $[\alpha]_D^{18}$ = +69.8 (c = 1.0, CHCl₃); IR (film): 3018, 2981, 1732, 1454, 1215, 1159, 1069, 996, 747, 697, 667 cm⁻¹; ¹H NMR (500 MHz, CDCl₃) δ 7.25–7.38 (m, 30H, H_{arom}), 5.17 (d, $J_{1,2}$ = 3.6 Hz, 2H, H-1), 5.01 (d, $J_{a,b}$ = 10.7 Hz, 2H, CH-*a*, 3-*O*-Bn), 4.87 (d, $J_{a,b}$ = 10.7 Hz, 2H, CH-*b*, 3-*O*-Bn), 4.87 (d, $J_{a,b}$ = 10.5 Hz, 2H, CH-*a*, 4-*O*-Bn), 4.72 (d, $J_{a,b}$ = 11.9 Hz, 2H, CH-*a*, 2-*O*-Bn), 4.69 (d, $J_{a,b}$ = 11.9 Hz, 2H, CH-*b*, 2-*O*-Bn), 4.52 (d, $J_{a,b}$ = 10.5 Hz, 2H, CH-*b*, 4-*O*-Bn), 4.23 (bd, $J_{4,5}$ = 9.6 Hz, 2H, H-5), 4.15 (dd, $J_{6a,6b}$ = 12.5 Hz, $J_{5,6a}$ = 3.5 Hz, 2H, H-6a), 4.06 (dd, $J_{6a,6b}$ = 12.5 Hz, $J_{5,6b}$ = 2.4 Hz, 2H, H-6b), 4.05 (t, $J_{2,3}$ = $J_{3,4}$ = 9.6 Hz, H-3), 3.58 (dd, $J_{2,3}$ = 9.6 Hz, $J_{1,2}$ = 3.6 Hz, H-2), 3.54 (t, $J_{3,4}$ = $J_{4,5}$ = 9.6 Hz, 2H, H-4), 2.25 (t, $J_{8,9}$ = 7.1 Hz, 4H, H-8), 1.60–1.56 (m, 4H, H-9), 1.29–1.22 (m, 12H, H-10–H-12), 0.86 (t, $J_{12,13}$ = 6.6 Hz, 6H, H-13); ¹³C NMR (125 MHz, CDCl₃) δ 173.5 (C-7), 138.6 (C-*i*, 3-*O*-Bn), 137.9 (C-*i*, 4-*O*-Bn), 137.8 (C-*i*, 2-*O*-Bn), 128.53, 128.5, 128.1, 128.0, 127.8, 127.7, 127.5 (CH_{arom}), 94.0 (C-1), 81.6 (C-3), 79.3 (C-2), 77.5 (C-4), 75.7 (CH₂, 3-*O*-Bn), 75.2 (CH₂, 4-*O*-Bn), 72.9 (CH₂, 2-*O*-Bn), 69.1 (C-5), 62.5 (C-6), 34.1 (C-8), 28.8, 24.8, 22.5, 18.4 (C-9–C-12), 14.0 (C-13); HRMS(ESI) m/z calcd. for [C₆₈H₈₂O₁₃+Na]⁺: 1129.5653, obsd.: 1129.5646.

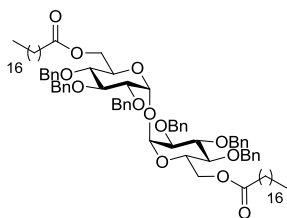


2,2',3,3',4,4'-Hexa-O-

benzyl-6,6'-di-O-decanoyl- α,α' -D-trehalose (5c).

By subjecting diol **4** (0.47 g, 0.53 mmol), decanoic acid (0.40 g, 2.35 mmol), EDCI (0.68 g, 3.52 mmol) and

DMAP (0.01 g, 0.08 mmol) to the general procedure for esterification, the title compound was obtained (eluting in 10:1 (v/v) Pet. Ether/EtOAc) as a yellow oil (0.53 g, 0.45 mmol, 83%). $R_f = 0.74$ (PE/EA, 1/1, v/v); $[\alpha]^{18}_D = +70.0$ ($c = 1.0$, CHCl_3); IR (film): 2927, 1732, 1454, 1215, 1157, 1070, 996, 747, 697, 667 cm^{-1} ; ^1H NMR (500 MHz, CDCl_3) δ 7.38–7.26 (m, 30H, H_{arom}), 5.17 (d, $J_{1,2} = 3.4$ Hz, 2H, H-1), 5.01 (d, $J_{a,b} = 10.7$ Hz, 2H, CH-a, 3-O-Bn and), 4.88 (d, $J_{a,b} = 10.7$ Hz, 2H, CH-b, 3-O-Bn), 4.86 (d, $J_{a,b} = 10.8$ Hz, 2H, CH-a, 4-O-Bn), 4.72 (d, $J_{a,b} = 11.8$ Hz, 2H, CH-a, 2-O-Bn), 4.68 (d, $J_{a,b} = 11.8$ Hz, 2H, CH-b, 2-O-Bn), 4.52 (d, $J_{a,b} = 10.8$ Hz, 2H, CH-b, 4-O-Bn), 4.24–4.21 (m, 2H, H-5), 4.15 (dd, $J_{6a,6b} = 12.2$ Hz, $J_{5,6a} = 3.7$ Hz, 2H, H-6a), 4.05 (dd, $J_{6a,6b} = 12.2$ Hz, $J_{5,6b} = 3.1$ Hz, 2H, H-6b), 4.04 (t, $J_{2,3} = J_{3,4} = 9.3$ Hz, H-3), 3.57 (dd, $J_{2,3} = 9.3$ Hz, $J_{1,2} = 3.4$ Hz, H-2), 3.54 (t, $J_{3,4} = J_{4,5} = 9.3$ Hz, 2H, H-4), 2.25 (t, $J_{8,9} = 7.3$ Hz, 4H, H-8), 1.58–1.55 (m, 4H, H-9), 1.29–1.22 (m, 24H, H-10–H-15), 0.87 (t, $J_{15,16} = 7.0$ Hz, 6H, H-16); ^{13}C NMR (125 MHz, CDCl_3) δ 173.5 (C-7), 138.6 (C-i, 3-O-Bn), 137.9 (C-i, 4-O-Bn), 137.8 (C-i, 2-O-Bn), 128.53, 128.5, 128.1, 128.0, 127.8, 127.7, 127.5 (CH_{arom}), 94.0 (C-1), 81.6 (C-3), 79.3 (C-2), 77.5 (C-4), 75.7 (CH_2 , 3-O-Bn), 75.2 (CH_2 , 4-O-Bn), 72.9 (CH_2 , 2-O-Bn), 69.1 (C-5), 62.5 (C-6), 34.1 (C-8), 31.8, 29.4, 29.2, 29.1, 24.9, 22.7 (C-9–C-15) and 14.1 (C-16); HRMS(ESI) m/z calcd. for $[\text{C}_{74}\text{H}_{94}\text{O}_{13} + \text{Na}]^+$: 1213.6592, obsd.: 1213.6591.



2,2',3,3',4,4'-Hexa-O-benzyl-

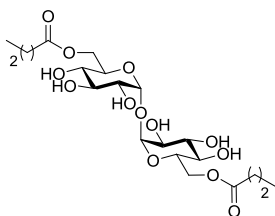
6,6'-di-O-stearoyl- α,α' -D-trehalose (5d).

By subjecting diol **4** (0.21 g, 0.24 mmol), stearic acid (0.31 g, 1.07 mmol), EDCI (0.31 g, 1.61 mmol) and DMAP (0.005 g, 0.04 mmol) to the general procedure for esterification, the title compound was obtained (eluting in 10:1 (v/v)

Pet. Ether/EtOAc) as a colourless oil (0.30 g, 0.21 mmol, 85%). $R_f = 0.79$ (PE/EA, 2/1, v/v); $[\alpha]^{18}_D = +61.8$ ($c = 1.0$, CHCl_3); IR (film): 3055, 2926, 2855, 1732, 1455,

1265, 1157, 1071, 998, 896, 733, 701, 669 cm^{-1} ; ^1H NMR (500 MHz, CDCl_3) δ 7.38–7.26 (m, 30H, H_{arom}), 5.17 (d, $J_{1,2} = 3.4$ Hz, 2H, H-1), 5.01 (d, $J_{\text{a,b}} = 10.9$ Hz, 2H, CH-a, 3-*O*-Bn), 4.87 (d, $J_{\text{a,b}} = 10.9$ Hz, 2H, CH-b, 3-*O*-Bn), 4.87 (d, $J_{\text{a,b}} = 10.5$ Hz, 2H, CH-a, 4-*O*-Bn), 4.72 (d, $J_{\text{a,b}} = 11.8$ Hz, 2H, CH-a, 2-*O*-Bn), 4.69 (d, $J_{\text{a,b}} = 11.8$ Hz, 2H, CH-b, 2-*O*-Bn), 4.52 (d, $J_{\text{a,b}} = 10.5$ Hz, 2H, CH-b, 4-*O*-Bn), 4.23 (bd, $J_{4,5} = 9.2$ Hz, 2H, H-5), 4.16 (dd, $J_{6\text{a},6\text{b}} = 12.4$ Hz, $J_{5,6\text{a}} = 3.7$ Hz, 2H, H-6a), 4.05 (dd, $J_{6\text{a},6\text{b}} = 12.4$ Hz, $J_{5,6\text{b}} = 2.1$ Hz, 2H, H-6b), 4.06 (t, $J_{2,3} = J_{3,4} = 9.2$ Hz, H-3), 3.57 (dd, $J_{2,3} = 9.2$ Hz, $J_{1,2} = 3.4$ Hz, H-2), 3.54 (t, $J_{3,4} = J_{4,5} = 9.2$ Hz, 2H, H-4), 2.24 (t, $J_{8,9} = 7.3$ Hz, 4H, H-8), 1.59–1.56 (m, 4H, H-9), 1.31–1.23 (m, 56H, H-10–H-23), 0.88 (t, $J_{23,24} = 7.0$ Hz, 6H, H-24); ^{13}C NMR (125 MHz, CDCl_3) δ 173.5 (C-7), 138.6 (C-*i*, 3-*O*-Bn), 137.9 (C-*i*, 4-*O*-Bn), 137.9 (C-*i*, 2-*O*-Bn), 128.5, 128.4, 128.1, 128.0, 127.8, 127.5 (CH_{arom}), 94.1 (C-1), 81.6 (C-3), 79.3 (C-2), 77.5 (C-4), 75.7 (CH_2 , 3-*O*-Bn), 75.2 (CH_2 , 4-*O*-Bn), 72.9 (CH_2 , 2-*O*-Bn), 69.1 (C-5), 62.5 (C-6), 34.1 (C-8), 31.9, 30.9, 29.7, 29.6, 29.5, 29.4, 29.3, 29.2, 29.1, 24.8, 22.7 (C-9–C-23) and 14.1 (C-24); HRMS(ESI) m/z calcd. for $[\text{C}_{90}\text{H}_{126}\text{O}_{12} + \text{Na}]^+$: 1437.9096, obsd.: 1437.9098.

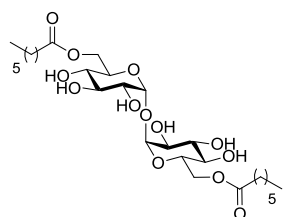
General procedure for debenzylation. $\text{Pd}(\text{OH})_2$ (2.5 mol%) was added to a solution of the benzylated trehalose diesters (1 mmol) in a mixture of $\text{CHCl}_3/\text{MeOH}$ (10 mL, 3/2, v/v) and stirred at room temperature under a steam of H_2 (gas) at 1 atm for 7 h. The reaction mixture was then filtered through Celite, the Celite washed thoroughly with $\text{CHCl}_3/\text{MeOH}$ (20 mL, 3/2, v/v) and the filtrate concentrated *in vacuo*. The product was purified using silica gel flash chromatography and the eluent given.



6,6'-Di-*O*-butanoyl- α,α' -D-

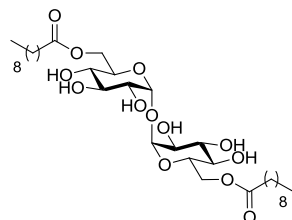
trehalose (1a). By subjecting dibutyl trehalose **5a** (180 mg, 0.17 mmol) to the procedure for debenzylation, trehalose diester **1a** was obtained (eluting in 95:5 (v/v) EtOAc/MeOH) as a white powder (52.9 mg, 0.10 mmol, 62%). $R_f = 0.2$ (EtOAc); $[\alpha]^{24}_{\text{D}} = +132.75$ ($c = 1.0$, MeOH); IR (film): 3318, 2943, 2832, 1732, 1449, 1114, 1022 cm^{-1} ; ^1H NMR (500 MHz, CD_3OD) δ 5.04 (d, $J_{1,2} = 3.6$ Hz, 2H, H-1), 4.36 (dd, $J_{6\text{a},6\text{b}} = 11.9$ Hz, $J_{5,6\text{a}} = 2.0$

Hz, 2H, H-6a), 4.20 (dd, $J_{6a,6b} = 11.9$ Hz, $J_{5,6b} = 5.1$ Hz, 2H, H-6b), 4.04–4.00 (m, 2H, H-5), 3.78 (t, $J_{2,3} = J_{3,4} = 9.2$ Hz, H-3), 3.46 (dd, $J_{2,3} = 9.2$ Hz, $J_{1,2} = 3.6$ Hz, 2H, H-2), 3.35 (t, $J_{3,4} = J_{4,5} = 9.2$ Hz, 2H, H-4), 2.33 (t, $J_{8,9} = 7.3$ Hz, 4H, H-8), 1.65 (pentet, $J_{\alpha,\beta} = J_{\beta,\gamma} = 7.3$ Hz, 4H, H-9), 0.96 (t, $J_{9,10} = 7.3$ Hz, 6H, H-10); ^{13}C NMR (125 MHz, CD_3OD) δ 173.9 (C-7), 93.9 (C-1), 73.1 (C-3), 71.7 (C-2), 70.4 (C-4), 70.1 (C-5), 62.9 (C-6), 36.8 (C-8), 18.0 (C-9), 12.5 (C-10); HRMS(ESI) m/z calcd. for $[\text{C}_{20}\text{H}_{34}\text{O}_{13} + \text{Na}]^+$: 505.1897, obsd.: 505.1891.



6,6'-Di-*O*-heptanoyl- α,α' -D-trehalose (1b). By subjecting diheptyl trehalose **5b** (105.6 mg, 0.096 mmol) to the general procedure for debenzylation, trehalose diester **1b** was obtained (eluting in 95:5 (v/v) EtOAc/MeOH) as a white solid (35.9 mg, 0.064 mmol,

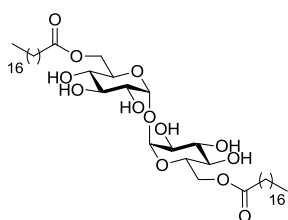
67%). $R_f = 0.75$ (EtOAc/MeOH, 9/1, v/v); $[\alpha]_D^{24} = +70.8$ ($c = 1.0$, EtOH); IR (film): 3319, 2943, 2831, 1735, 1448, 1113, 1022 cm^{-1} ; ^1H NMR (500 MHz, CD_3OD) δ 5.04 (d, $J_{1,2} = 3.1$ Hz, 2H, H-1), 4.36 (dd, $J_{6a,6b} = 11.7$ Hz, $J_{5,6a} = 2.0$ Hz, 2H, H-6a), 4.20 (dd, $J_{6a,6b} = 11.7$ Hz, $J_{5,6b} = 4.9$ Hz, 2H, H-6b), 4.03–4.00 (m, 2H, H-5), 3.88 (t, $J_{2,3} = J_{3,4} = 9.3$ Hz, H-3), 3.47 (dd, $J_{2,3} = 9.3$ Hz, $J_{1,2} = 3.1$ Hz, 2H, H-2), 3.34 (t, $J_{3,4} = J_{4,5} = 9.3$ Hz, 2H, H-4), 2.35 (t, $J_{8,9} = 7.4$ Hz, 4H, H-8), 1.62 (pentet, $J_{\alpha,\beta} = J_{\beta,\gamma} = 7.3$ Hz, 4H, H-9), 1.36–1.32 (m, 12H, H-10–H-12), 0.96 (t, $J_{12,13} = 7.3$ Hz, 6H, H-13); ^{13}C NMR (125 MHz, CD_3OD) δ 174.1 (C-7), 93.8 (C-1), 73.1 (C-3), 71.7 (C-2), 70.5 (C-4), 70.1 (C-5), 63.0 (C-6), 33.6 (C-8), 31.3, 28.5, 24.6, 22.2 (C-9–C-12), 13.0 (C-13); HRMS(ESI) m/z calcd. for $[\text{C}_{26}\text{H}_{46}\text{O}_{13} + \text{Na}]^+$: 589.2836, obsd.: 589.2837.



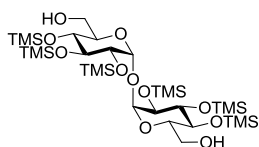
6,6'-Di-*O*-decanoyl- α,α' -D-trehalose (1c). By subjecting didecanoyl trehalose **5c** (119 mg, 0.10 mmol) to the general procedure for debenzylation, trehalose diester **1c** was obtained (eluting in 95:5 (v/v) EtOAc/MeOH) as a white solid (40.9 mg, 0.063 mmol, 63%). $R_f = 0.5$

(EA/MeOH, 9/1, v/v); $[\alpha]_D^{24} = +111.3$ ($c = 1.0$, EtOH); IR (film): 3320, 2973, 2882, 1736, 1379, 1275, 1088, 1046, 880 cm^{-1} ; ^1H NMR (500 MHz, CD_3OD) δ 5.05 (d, $J_{1,2}$

= 3.9 Hz, 2H, H-1), 4.35 (dd, $J_{6a,6b} = 11.9$ Hz, $J_{5,6a} = 2.0$ Hz, 2H, H-6a), 4.20 (dd, $J_{6a,6b} = 11.9$ Hz, $J_{5,6b} = 5.1$ Hz, 2H, H-6b), 4.05–4.01 (m, 2H, H-5), 3.78 (t, $J_{2,3} = J_{3,4} = 9.1$ Hz, H-3), 3.47 (dd, $J_{2,3} = 9.1$ Hz, $J_{1,2} = 3.9$ Hz, 2H, H-2), 3.34 (t, $J_{3,4} = J_{4,5} = 9.1$ Hz, 2H, H-4), 2.35 (t, $J_{8,9} = 7.5$ Hz, 4H, H-8), 1.62 (pentet, $J_{\alpha,\beta} = J_{\beta,\gamma} = 7.1$ Hz, 4H, H-9), 1.33–1.30 (m, 24H, H-10–H-15), 0.90 (t, $J_{15,16} = 7.3$ Hz, 6H, H-16); ^{13}C NMR (125 MHz, CD_3OD) δ 174.1 (C-7), 93.8 (C-1), 73.1 (C-3), 71.2 (C-2), 70.5 (C-4), 70.1 (C-5), 63.0 (C-6), 33.7 (C-8), 31.7, 29.2, 29.0, 28.8, 24.7, 22.3 (C-9–C-15), 13.1 (C-16); HRMS(ESI) m/z calcd. for $[\text{C}_{32}\text{H}_{58}\text{O}_{13} + \text{Na}]^+$: 673.3775, obsd.: 673.3773.

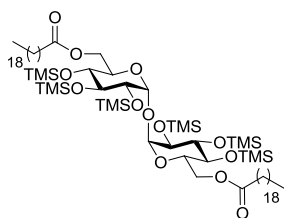


6,6'-Di-*O*-stearoyl- α,α' -D-trehalose (1d). By subjecting distearoyl trehalose **5d** (112.1 mg, 0.079 mmol) to the general procedure for debenzoylation, trehalose diester **1d** was obtained after silica gel chromatography eluting in 95:5 (v/v) EtOAc/MeOH as a white solid (60.9 mg, 0.070 mmol, 88%). $R_f = 0.64$ (EA/MeOH, 9/1, v/v); $[\alpha]_D^{22} = +16.0$ ($c = 1.0$, EtOH); IR (film): 3330, 2919, 2850, 2835, 1660, 1453, 1109, 697 cm^{-1} ; ^1H NMR (500 MHz, CD_3OD) δ 5.06 (d, $J_{1,2} = 3.7$ Hz, 2H, H-1), 4.35 (dd, $J_{6a,6b} = 11.4$ Hz, $J_{5,6a} = 2.0$ Hz, 2H, H-6a), 4.21 (dd, $J_{6a,6b} = 11.4$ Hz, $J_{5,6b} = 5.1$ Hz, 2H, H-6b), 4.02–3.98 (m, 2H, H-5), 3.77 (t, $J_{2,3} = J_{3,4} = 9.3$ Hz, H-3), 3.48 (dd, $J_{2,3} = 9.3$ Hz, $J_{1,2} = 3.7$ Hz, 2H, H-2), 3.34 (t, $J_{3,4} = J_{4,5} = 9.3$ Hz, 2H, H-4), 2.34 (t, $J_{8,9} = 7.4$ Hz, 4H, H-8), 1.62 (pentet, $J_{\alpha,\beta} = J_{\beta,\gamma} = 7.1$ Hz, 4H, H-9), 1.32–1.27 (m, 56H, H-10–H-23), 0.89 (t, $J_{23,24} = 7.3$ Hz, 6H, H-24); ^{13}C NMR (125 MHz, CD_3OD) δ 174.2 (C-7), 93.7 (C-1), 73.2 (C-3), 71.7 (C-2), 70.4 (C-4), 70.0 (C-5), 63.1 (C-6), 33.8 (C-8), 31.7, 29.5, 29.4, 29.3, 29.1, 29.1, 28.9, 24.7, 22.4 (C-9–C-23), 13.1 (C-24); HRMS(ESI) m/z calcd. for $[\text{C}_{48}\text{H}_{90}\text{O}_{13} + \text{Na}]^+$: 897.6279, obsd.: 897.6279.



2,2',3,3',4,4'-Hexa-*O*-trimethylsilyl- α,α' -D-trehalose (6). α,α' -D-Trehalose dihydrate **2** (0.25 g, 0.66 mmol) was co-evaporated with anhydrous DMF (3 x 5 mL) and then dissolved in DMF (1 mL). To this solution, *N,O*-bis(trimethylsilyl)acetamide (1.4 mL, 5.70 mmol) and TBAF (0.04 mL, 0.04 mmol)

were added and the reaction stirred at room temperature for 1.5 h. The reaction was quenched with 0.25 mL iso-propanol and the resulting solution was diluted with 15 mL MeOH and cooled down to 0 °C. K₂CO₃ solution (0.091 g in 20 mL MeOH) was added to the reaction mixture and stirring at 0 °C was continued for 2 h. The reaction mixture was concentrated *in vacuo* and the resulting residue partitioned between ether and brine. The aqueous layer was extracted twice more with diethyl ether (30 mL x 2) and the combined organic layer dried (MgSO₄) and concentrated *in vacuo*. Flash chromatography of the crude product gave diol **6**, eluting in 4:1 (v/v) Pet. Ether/EtOAc, as a white solid (0.43 g, 0.56 mmol, 85%). *R_f* = 0.29 (PE/EA, 3/1, v/v); $[\alpha]_D^{18} = +109$ (*c* = 1.0, CHCl₃), Lit¹¹ $[\alpha]_D^{25} = +103$; IR (film): 3628, 3019, 2958, 1558, 1541, 1387, 1250, 1215, 1166, 1109, 1075, 1005 cm⁻¹; ¹H NMR (500 MHz, CDCl₃) δ 4.91 (d, *J*_{1,2} = 3.1 Hz, 2H, H-1), 3.90 (t, *J*_{2,3} = *J*_{3,4} = 9.0 Hz, 2H, H-3), 3.88–3.84 (m, 2H, H-5), 3.72 (dd, *J*_{6a,6b} = 11.8 Hz, *J*_{5,6a} = 2.7 Hz, 2H, H-6a), 3.70, (dd, *J*_{6a,6b} = 11.8 Hz, *J*_{5,6b} = 3.9 Hz 2H, H-6b), 3.49 (t, *J*_{3,4} = *J*_{4,5} = 9.1 Hz, 2H, H-4), 3.42 (dd, *J*_{2,3} = 9.0 Hz, *J*_{1,2} = 3.1 Hz, 2H, H-2), 0.17, 0.15, 0.13 (3 s, 54H, CH₃, TMS); ¹³C NMR (125 MHz, CDCl₃) δ 94.6 (C-1), 73.3 (C-3), 73.0 (C-2), 72.8 (C-5), 71.4 (C-4), 61.7 (C-6), 1.0, 0.8, 0.07 (TMS); HRMS(ESI) *m/z* calcd. for [C₃₀H₇₀O₁₁Si₆+Na]⁺: 797.3431, obsd.:797.3424.

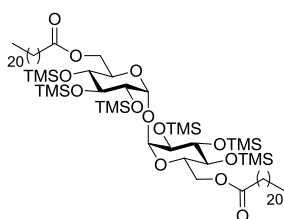


2,2',3,3',4,4'-Hexa-O-trimethylsilyl-6,6'-di-O-eicosanoyl- α,α' -D-trehalose (7e**).** To a solution of diol **6** (37.6 mg, 0.049 mmol) and arachidic acid (84.6 mg, 0.22 mmol) in anhydrous CH₂Cl₂ (3 mL) was added EDCI (61.4 mg, 0.32 mmol) and DMAP (8.8 mg, 0.001 mmol)

and the reaction stirred at room temperature for 2 nights. The resulting precipitate was removed by filtration and washed thoroughly with EtOAc (2 x 10 mL). The combined organic layers were washed with water (20 mL), brine (20 mL), dried (MgSO₄), filtered and concentrated *in vacuo*. Purification of the crude product by silica gel flash chromatography gave the title compound **7e**, eluting in 50:1 (v/v) Pet. Ether/EtOAc, as pale yellow oil (61.5 mg, 0.045 mmol, 93%). *R_f* = 0.83 (PE/EtOAc, 5/1, v/v); $[\alpha]_D^{18} = +45.0$ (*c* = 0.1 CHCl₃); IR (film): 3016, 2970, 2924, 2853, 1739, 1010, 966, 843 cm⁻¹; ¹H NMR (500 MHz, CDCl₃) δ 4.92 (d, *J*_{1,2} = 3.2 Hz, 2H, H-1),

4.28 (dd, $J_{6a,6b} = 12.0$ Hz, $J_{5,6a} = 2.2$ Hz, 2H, H-6a), 4.06 (dd, $J_{6a,6b} = 12.0$ Hz, $J_{5,6b} = 4.4$ Hz, 2H, H-6b), 4.06–3.99 (m, 2H, H-5), 3.90 (t, $J_{2,3} = J_{3,4} = 9.0$ Hz, 2H, H-3), 3.49 (t, $J_{3,4} = J_{4,5} = 9.0$ Hz, 2H, H-4), 3.45 (dd, $J_{2,3} = 9.0$ Hz, $J_{1,2} = 3.2$ Hz, 2H, H-2), 2.37 (dt, $J_{8a,8b} = 13.7$ Hz, $J_{8a,9} = 7.6$ Hz, 2H, H-8a), 2.36 (dt, $J_{8a,8b} = 13.7$ Hz, $J_{8b,9} = 7.5$ Hz, 2H, H-8b), 1.66–1.60 (m, 4H, H-9), 1.30–1.26 (m, 64H, H-10–H-25), 0.89 (t, $J_{25,26} = 7.1$ Hz, H-26), 0.16, 0.141, 0.137 (3 s, 54H, CH₃, TMS); ¹³C NMR (125 MHz, CDCl₃) δ 173.4 (C-7), 94.4 (C-1), 73.5 (C-3), 72.6 (C-2), 71.9 (C-4), 70.7 (C-5), 63.3 (C-6), 34.2 (C-8), 31.9, 29.71, 29.67, 29.5, 29.4, 29.3, 29.2, 29.1, 24.8, 22.7 (C-9–C-25), 14.1 (C-26), 1.1, 0.9, 0.2 (TMS) HRMS(ESI) m/z calcd. for [C₇₀H₁₄₆O₁₃Si₆+Na]⁺: 1385.9277, obsd.: 1385.9282.

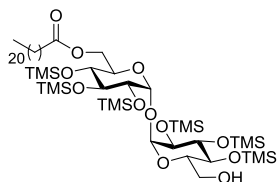
General Procedure for Esterification in Toluene. To a solution of diol **6** (1 mmol, 1 equiv.) and the carboxylic acid (4.4 mmol, 4.4 equiv.) in dry toluene (5 mL), EDCI (6.6 mmol, 6.6 equiv.) and DMAP (0.15 mmol, 0.15 equiv.) were added. The reaction was stirred at 70 °C until analysis by TLC showed no further formation of product. The resulting precipitate was removed by filtration and the precipitate washed thoroughly with EtOAc (2 x 50 mL). The combined organic layers were washed with water (50 mL) and brine (40 mL), dried (MgSO₄), filtered and concentrated *in vacuo*. The product was purified by silica gel flash chromatography using the eluent given.



2,2',3,3',4,4'-Hexa-O-trimethylsilyl-6,6'-di-O-docosanoyl- α,α' -D-trehalose (7f). By subjecting diol **6** (106.1 mg, 0.14 mmol), docosanoic acid (205 mg, 0.60 mmol), EDCI (177 mg, 0.92 mmol) and DMAP (2.5 mg, 0.02 mmol) to the general procedure for esterification (2

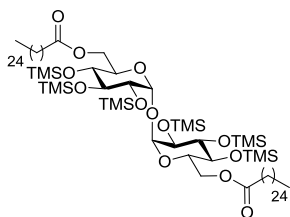
days), the title compound **7f** was obtained (eluting in 50:1 (v/v) Pet. Ether/ EtOAc) as a white solid (170 mg, 0.12 mmol, 86%). $R_f = 0.84$ (PE/EA, 5/1, v/v); $[\alpha]_D^{18} = +46.1$ ($c = 0.1$ CHCl₃); IR (film): 2970, 2926, 2854, 1737, 1046, 1032, 993, 755, 704 cm⁻¹; ¹H NMR (500 MHz, CDCl₃) δ 4.93 (d, $J_{1,2} = 2.9$ Hz, 2H, H-1), 4.29 (dd, $J_{6a,6b} = 11.9$ Hz, $J_{5,6a} = 2.2$ Hz, 2H, H-6a), 4.07 (dd, $J_{6a,6b} = 12.0$ Hz, $J_{5,6b} = 4.7$ Hz, 2H, H-

6b), 4.02–3.98 (m, 2H, H-5), 3.91 (t, $J_{2,3} = J_{3,4} = 9.0$ Hz, 2H, H-3), 3.49 (t, $J_{3,4} = J_{4,5} = 9.0$ Hz, 2H, H-4), 3.45 (dd, $J_{2,3} = 9.0$ Hz, $J_{1,2} = 2.9$ Hz, 2H, H-2), 2.37 (dt, $J_{8a,8b} = 13.1$ Hz, $J_{8a,9} = 7.3$ Hz, 2H, H-8a), 2.36 (dt, $J_{8a,8b} = 13.1$ Hz $J_{8b,9} = 7.4$ Hz, 2H, H-8b), 1.64–1.58 (m, 4H, H-9), 1.34–1.22 (m, 72H, H-10–H-27), 0.89 (t, $J_{27,28} = 6.8$ Hz, H-28), 0.16, 0.141, 0.137 (3 s, 54H, CH₃, TMS); ¹³C NMR (125 MHz, CDCl₃) δ173.8 (C-7), 94.4 (C-1), 73.5 (C-3), 72.6 (C-2), 71.9 (C-4), 70.7 (C-5), 63.8 (C-6), 34.2 (C-8), 31.9, 29.71, 29.67, 29.6, 29.5, 29.4, 29.3, 29.2, 24.8, 22.7 (C-9–C-27), 14.1 (C-28), 1.1, 0.9, 0.2 (TMS); HRMS (ESI) m/z calcd. for [C₇₄H₁₅₄O₁₃Si₆+Na]⁺: 1441.9903, obsd.: 1441.9901.



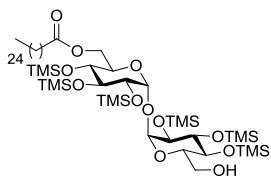
2,2',3,3',4,4'-Hexa-*O*-trimethylsilyl-6-docosanoyl-6'-hydroxy- α,α' -D-trehalose (8f). The title compound **8f** was obtained (eluting in Hex: EtOAc 20:1) as a white solid (20 mg, 0.018 mmol, 12%). $R_f = 0.55$ (PE/EA, 5/1, v/v); $[\alpha]_D^{18} = +20.2$ ($c = 1.0$, CHCl₃); IR (film): 3017,

2923, 2852, 1737, 1372, 1250, 1076, 1007, 965, cm⁻¹; ¹H NMR (500 MHz, CDCl₃) δ 4.94 (d, $J_{1/1',2/2'} = 3.5$ Hz, 1H, H-1/1'), 4.92 (d, $J_{1/1',2/2'} = 3.7$ Hz, 1H, H-1/1'), 4.30 (dd, $J_{6a,6b} = 12.0$ Hz, $J_{5,6a} = 2.2$ Hz, 1H, H-6a), 4.07 (dd, $J_{6a,6b} = 12.0$ Hz, $J_{5,6b} = 4.6$ Hz, 1H, H-6b), 4.03–4.00 (m, 1H, H-5), 3.92 (t, $J_{2/2',3/3'} = J_{3/3',4/4'} = 8.8$ Hz, 1H, H-3/3'), 3.91 (t, $J_{2/2',3/3'} = J_{3/3',4/4'} = 9.0$ Hz, 1H, H-3/3'), 3.85 (dt, $J_{4',5'} = 9.5$ Hz, $J_{5',6a'} = J_{5',6b'} = 3.5$ Hz, 1H, H-5'), 3.86–3.83 (m, 2H, H-6a' and H-6b'), 3.49 (t, $J_{3,4} = J_{4,5} = 9.0$ Hz, 1H, H-4), 3.47 (t, $J_{3',4'} = J_{4',5'} = 9.0$ Hz, 1H, H-4'), 3.44 (dd, $J_{2,3/2',3'} = 9.0$ Hz, $J_{1,2/1',2'} = 3.5$ Hz, 1H, H-2/2'), 3.43 (dd, $J_{2,3/2',3'} = 9.0$ Hz, $J_{1,2/1',2'} = 3.5$ Hz, 1H, H-2/2'), 2.35 (dt, $J_{8a,8b} = 13.2$ Hz, $J_{8a,9} = 7.3$ Hz, 1H, H-8a), 2.34 (dd, $J_{8a,8b} = 13.2$ Hz, $J_{8b,9} = 7.4$ Hz, 1H, H-8b), 1.66–1.57 (m, 2H, H-9), 1.34–1.26 (m, 36H, H-10–H-27), 0.89 (t, $J_{27,28} = 7.1$ Hz, 3H, H-28), 0.17, 0.16, 0.15, 0.14, 0.13 (6 s, 54H, CH₃, TMS); ¹³C NMR (125 MHz, CDCl₃) δ173.8 (C-7), 94.5 (C-1/1'), 94.4 (C-1/1'), 73.5 (C-3/3'), 73.3 (C-3/3'), 72.9 (C-2/2'), 72.8 (C-5'), 72.6 (C-2/2'), 71.9 (C-4), 71.4 (C-4'), 70.8 (C-5), 63.3 (C-6), 61.7 (C-6'), 34.2 (C-8), 31.9, 29.70, 29.66, 29.60, 29.5, 29.4, 29.3, 29.2, 24.8, 22.7 (C-9–C-27), 14.1 (C-28), 1.04, 1.00, 0.89, 0.85, 0.14, 0.10 (TMS) HRMS (ESI) m/z calcd. for [C₅₂H₁₁₂O₁₂Si₆+Na]⁺: 1119.6662, obsd.: 1119.6667.



2,2',3,3',4,4'-Hexa-*O*-trimethylsilyl-6,6'-di-*O*-hexacosanoyl- α,α' -D-trehalose (7g). By subjecting diol **6** (17 mg, 0.022 mmol), hexacosanoic acid (37.9 mg, 0.096 mmol), EDCI (27.7 mg, 0.14 mmol) and DMAP (0.39 mg, 0.003 mmol) to the general procedure for

esterification (6 days), the title compound **7g** was obtained (eluting in 50:1 (v/v) Pet. Ether/ EtOAc) as a white solid (20 mg, 0.013 mmol, 60%). $R_f = 0.83$ (PE/EA, 5/1, v/v); $[\alpha]_D^{18} = +43.2$ ($c = 1.0$, CHCl_3); IR (film): 3016, 2970, 2919, 2851, 1739, 966, 899, 760 cm^{-1} ; ^1H NMR (500 MHz, CDCl_3) δ 4.92 (d, $J_{1,2} = 2.9$ Hz, 2H, H-1), 4.28 (dd, $J_{6a,6b} = 11.7$ Hz, $J_{5,6a} = 2.2$ Hz, 2H, H-6a), 4.06 (dd, $J_{6a,6b} = 11.7$ Hz, $J_{5,6b} = 4.7$ Hz, 2H, H-6b), 4.02–3.98 (m, 2H, H-5), 3.91 (t, $J_{2,3} = J_{3,4} = 9.0$ Hz, 2H, H-3), 3.48 (t, $J_{3,4} = J_{4,5} = 9.0$ Hz, 2H, H-4), 3.45 (dd, $J_{2,3} = 9.0$ Hz, $J_{1,2} = 2.9$ Hz, 2H, H-2), 2.36 (dt, $J_{8a,8b} = 13.6$ Hz, $J_{8a,9} = 7.3$ Hz, 2H, H-8a), 2.34 (dt, $J_{8a,8b} = 13.6$ Hz, $J_{8b,9} = 7.0$ Hz, 2H, H-8b), 1.64–1.60 (m, 4H, H-9), 1.34–1.18 (m, 88H, H-10–H-31), 0.89 (t, $J_{31,32} = 6.7$ Hz, H-32), 0.16, 0.14, 0.137 (3 s, 54H, CH_3 , TMS); ^{13}C NMR (125 MHz, CDCl_3) δ 174.0 (C-7), 94.4 (C-1), 73.5 (C-3), 72.6 (C-2), 71.9 (C-4), 70.7 (C-5), 63.3 (C-6), 34.2 (C-8), 31.9, 29.71, 29.67, 29.6, 29.5, 29.4, 29.31, 29.28, 29.2, 24.8, 22.7 (C-9–C-31), 14.1 (C-32), 1.1, 0.9, 0.2 (TMS); HRMS (ESI) m/z calcd. for $[\text{C}_{82}\text{H}_{170}\text{O}_{13}\text{Si}_6+\text{Na}]^+$: 1554.1155, obsd.: 1554.1166.

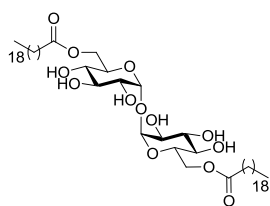


2,2',3,3',4,4'-Hexa-*O*-trimethylsilyl-6-hexacosanoyl-6'-hydroxy- α,α' -D-trehalose (8g). The title compound was obtained after (eluting in Hex: EtOAc 20:1) a colourless oil (5 mg, 0.005 mmol, 20%). $R_f = 0.61$ (PE/EA, 5/1, v/v);

$[\alpha]_D^{18} = 23.2$ ($c = 1.0$ CHCl_3); IR (film): 3400, 2920, 2851, 1739, 1457, 1166, 1077, 1008, 843, 760 cm^{-1} ; ^1H NMR (500 MHz, CDCl_3) δ 4.93 (d, $J_{1/1',2/2'} = 3.5$ Hz, 1H, H-1/1'), 4.92 (d, $J_{1/1',2/2'} = 3.5$ Hz, 1H, H-1/1'), 4.30 (dd, $J_{6a,6b} = 11.7$ Hz, $J_{5,6a} = 2.2$ Hz, 1H, H-6a), 4.07 (dd, $J_{6a,6b} = 11.7$ Hz, $J_{5,6b} = 4.7$ Hz, 1H, H-6b), 4.03–3.99 (m, 1H, H-5), 3.91 (t, $J_{2/2',3/3'} = J_{3/3',4/4'} = 9.0$ Hz, 1H, H-3/3'), 3.90 (t, $J_{2/2',3/3'} = J_{3/3',4/4'} = 9.0$ Hz,

1H, H-3/3'), 3.85 (dt, $J_{4',5'} = 9.6$ Hz, $J_{5',6a'} = J_{5',6b'} = 3.4$ Hz, 1H, H-5'), 3.71 (dd, $J_{6a',6b'} = 11.7$ Hz, $J_{5',6a'} = 3.0$ Hz, 1H, H-6a'), 3.69 (dd, $J_{6a',6b'} = 11.7$ Hz, $J_{5',6b'} = 3.9$ Hz, 1H, H-6b'), 3.49 (t, $J_{3,4} = J_{4,5} = 9.0$ Hz, 1H, H-4), 3.48 (t, $J_{3',4'} = J_{4',5'} = 9.0$ Hz, 1H, H-4'), 3.44 (dd, $J_{2,3/2',3'} = 9.0$ Hz, $J_{1,2/1',2'} = 3.5$ Hz, 1H, H-2/2'), 3.43 (dd, $J_{2,3/2',3'} = 9.0$ Hz, $J_{1,2/1',2'} = 3.5$ Hz, 1H, H-2/2'), 2.36 (dt, $J_{8a,8b} = 12.5$ Hz, $J_{8a,9} = 7.1$ Hz, 1H, H-8a), 2.34 (dt, $J_{8a,8b} = 12.5$, $J_{8b,9} = 7.0$ Hz, 1H, H-8b), 1.63 (pentet, $J_{\alpha,\beta} = J_{\beta,\gamma} = 6.8$ Hz, 2H, H-9), 1.38–1.20 (m, 44H, H-10–H-31), 0.89 (t, $J_{31,32} = 6.8$ Hz, 3H, H-32), 0.17, 0.16, 0.15, 0.14, 0.13 (5 s, 54H, CH₃, TMS); ¹³C NMR (125 MHz, CDCl₃) δ 173.8 (C-7), 94.5 (C-1/1'), 94.4 (C-1/1'), 73.5 (C-3/3'), 73.3 (C-3/3'), 72.9 (C-2/2'), 72.8 (C-5'), 72.6 (C-2/2'), 71.9 (C-4), 71.4 (C-4'), 70.8 (C-5), 63.3 (C-6), 61.7 (C-6'), 34.2 (C-8), 31.9, 31.4, 30.3, 30.2, 29.70, 29.68, 29.67, 29.60, 29.5, 29.4, 29.3, 29.2, 24.8, 22.7 (C-9–C-31), 14.1 (C-32), 1.05, 1.00, 0.89, 0.85, 0.14, 0.10 (C-TMS); HRMS(ESI) m/z calcd. for [C₅₆H₁₂₀O₁₂Si₆+Na]⁺: 1175.7288, obsd.: 1175.7285.

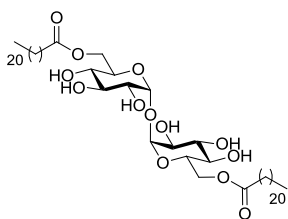
General Procedure for Desilylation. To a solution of the trehalose diester **7e–7g** or trehalose monoester **8f–8g** (1 mmol) in 5 mL CH₂Cl₂:MeOH mixture (3/1, v/v) Dowex-H⁺ (10% by weight) was added and the reaction stirred at room temperature. After 30 min, the reaction mixture was filtered and concentrated *in vacuo*, and the resulting residue subjected to column chromatography (straight phase or reverse phase) using the eluent given in order to obtain the fully deprotected compounds.



6,6'-Di-O-eicosanoyl- α,α' -D-trehalose (1e). By subjecting trehalose diester **7e** (13 mg, 0.010 mmol) to the general procedure for desilylation, the title compound **1e** was obtained after silica gel flash chromatography eluting in 98:2 (v/v) EtOAc/MeOH as a white solid (7.5

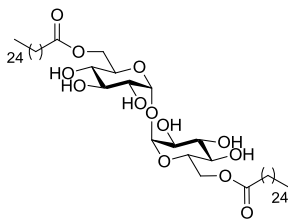
mg, 0.008 mmol, 80%). $R_f = 0.47$ (EA/MeOH, 95/5, v/v); $[\alpha]_D^{18} = +29.8$ ($c = 0.1$, C₅H₅N); IR (film); 3567, 3459, 2967, 2926, 1737, 1016, 846, 750 cm⁻¹; ¹H NMR (500 MHz, C₅H₅N) δ 5.79 (d, $J_{1,2} = 3.6$ Hz, 2H, H-1), 5.00–4.97 (m, 2H, H-5), 4.90 (m, 2H, H-6a), 4.72 (dd, $J_{6a,6b} = 11.7$ Hz, $J_{5,6b} = 5.4$ Hz, 2H, H-6b), 4.64 (t, $J_{2,3} = J_{3,4} = 9.0$ Hz, 2H, H-3), 4.20 (bd, $J_{2,3} = 9.0$ Hz, 2H, H-2), 4.07 (t, $J_{3,4} = J_{4,5} = 9.0$ Hz, 2H,

H-4), 2.22 (dt, $J_{8a,8b} = 12.2$ Hz, $J_{8a,9} = 7.9$ Hz, 2H, H-8a), 2.20 (dt, $J_{8a,8b} = 12.2$ Hz, $J_{8b,9} = 7.3$ Hz, 2H, H-8b), 1.51 (pentet, $J_{\alpha,\beta} = J_{\beta,\gamma} = 7.3$ Hz, 4H, H-9), 1.19–1.09 (m, 64H, H-10–H-25), 0.75 (t, $J_{25,26} = 6.8$ Hz, 6H, H-26); ^{13}C NMR (125 MHz, $\text{C}_5\text{H}_5\text{N}$) δ 173.7 (C-7), 95.9 (C-1), 75.0 (C-3), 73.5 (C-2), 72.2 (C-4), 71.7 (C-5), 64.5 (C-6), 34.5 (C-8), 32.2, 30.09, 30.05, 30.0, 29.9, 29.7, 29.5, 25.4, 23.0 (C-9–C-25), 14.3 (C-26); HRMS(ESI) m/z calcd. for $[\text{C}_{52}\text{H}_{98}\text{O}_{13}+\text{Na}]^+$: 953.6905, obsd.: 953.6904.



6,6'-Di-*O*-docosanoyl- α,α' -D-trehalose (1f). By subjecting trehalose diester **7f** (42 mg, 0.030 mmol) to the general procedure for desilylation, the title compound **1f** was obtained after silica gel flash chromatography eluting in 98:2 (v/v) EtOAc/MeOH as a

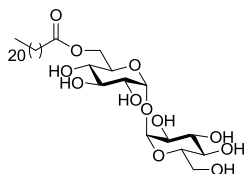
white solid (23.3 mg, 0.024 mmol, 80%) $R_f = 0.52$ (EA/MeOH, 95/5, v/v); $[\alpha]_D^{25} = +1.3$ ($c = 0.1$ $\text{C}_5\text{H}_5\text{N}$); IR (film): 3324, 2944, 2832, 1737, 1449, 1216, 1114, 1022, 737 cm^{-1} ; ^1H NMR (500 MHz, $\text{C}_5\text{H}_5\text{N}$) δ 5.78 (d, $J_{1,2} = 3.4$ Hz, 2H, H-1), 5.00–4.97 (m, 2H, H-5), 4.89 (m, 2H, H-6a), 4.73 (dd, $J_{6a,6b} = 11.7$ Hz, $J_{5,6b} = 5.3$ Hz, 2H, H-6b), 4.64 (t, $J_{2,3} = J_{3,4} = 9.6$ Hz, 2H, H-3), 4.20 (bd, $J_{2,3} = 9.6$ Hz, 2H, H-2), 4.07 (t, $J_{3,4} = J_{4,5} = 9.6$ Hz, 2H, H-4), 2.22 (dt, $J_{8a,8b} = 11.7$ Hz, $J_{8a,9} = 7.5$ Hz, 2H, H-8a), 2.18 (dt, $J_{8a,8b} = 11.7$ Hz, $J_{8b,9} = 7.2$ Hz, 2H, H-8b), 1.51 (pentet, $J_{\alpha,\beta} = J_{\beta,\gamma} = 7.1$ Hz, 4H, H-9), 1.20–1.14 (m, 72H, H-10–H-27), 0.76 (t, $J_{27,28} = 6.8$ Hz, 6H, H-28); ^{13}C NMR (125 MHz, $\text{C}_5\text{H}_5\text{N}$) δ 173.8 (C-7), 95.9 (C-1), 75.0 (C-3), 73.5 (C-2), 72.1 (C-4), 71.7 (C-5), 64.5 (C-6), 34.5 (C-8), 32.3, 30.14, 30.09, 30.0, 29.9, 29.72, 29.69, 29.5, 25.4, 23.0 (C-9–C-27), 14.4 (C-28); HRMS(ESI) m/z calcd. for $[\text{C}_{56}\text{H}_{106}\text{O}_{13}+\text{Na}]^+$: 1009.7531, obsd.: 1009.7532.



6,6'-Di-*O*-hexacosanoyl- α,α' -D-trehalose (1g). By subjecting trehalose diester **7g** (3 mg, 0.002 mmol) to the general procedure for desilylation, the title compound **1g** was obtained after reverse phase chromatography eluting in pyridine as a white solid

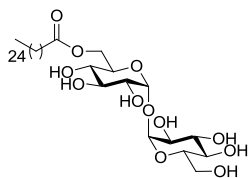
(1.53 mg, 0.0014 mmol, 70%). $R_f = 0.52$ (EA/MeOH, 95/5, v/v); $[\alpha]_D^{25} = +0.7$ ($c =$

0.1, C₅H₅N); IR (film) cm⁻¹ 3361, 2923, 2851, 2215, 1733, 1181, 1107, 1022; ¹H NMR (500 MHz, C₅H₅N) δ 5.78 (d, *J*_{1,2} = 3.7 Hz, 2H, H-1), 4.98–4.96 (m, 2H, H-5), 4.87 (m, 2H, H-6a), 4.72 (dd, *J*_{6a,6b} = 11.7 Hz, *J*_{5,6a} = 5.4 Hz, 2H, H-6b), 4.64 (t, *J*_{2,3} = *J*_{3,4} = 9.0 Hz, 2H, H-3), 4.21 (dd, *J*_{2,3} = 9.0 Hz, *J*_{1,2} = 3.7 Hz, 2H, H-2), 4.01 (t, *J*_{3,4} = *J*_{4,5} = 9.0 Hz, 2H, H-4), 2.23 (dt, *J*_{8a,8b} = 12.2 Hz, *J*_{8a,9} = 7.5 Hz, 2H, H-8a), 2.17 (dt, *J*_{8a,8b} = 12.2 Hz, *J*_{8a,9} = 7.5 Hz, 2H, H-8b), 1.50 (pentet, *J*_{α,β} = *J*_{β,γ} = 7.4 Hz, 4H, H-9), 1.26–1.04 (m, 88H, H-10–H-31), 0.73 (t, *J*_{31,32} = 6.8 Hz, 6H, H-32); ¹³C NMR (125 MHz, C₅H₅N) δ 173.8 (C-7), 95.9 (C-1), 75.0 (C-3), 73.5 (C-2), 72.1 (C-4), 71.7 (C-5), 64.5 (C-6), 34.5 (C-8), 32.2, 30.17, 30.16, 30.12, 30.07, 30.04, 29.9, 29.74, 29.72, 29.5, 25.4, 23.0 (C-9–C-31), 14.4 (C-32); HRMS(ESI) *m/z* calcd. for [C₆₄H₁₂₂O₁₂ + Na]⁺: 1121.8783, obsd.: 1121.8764.



6-O-Docosanoyl-α,α'-D-trehalose (9f). By subjecting trehalose monoester **8f** (5.6 mg, 0.005 mmol) to the procedure described above, the title compound **9f** was obtained after flash chromatography (eluting in 2% MeOH) as a white solid (3.2 mg, 0.0048 mmol, 96%). *R*_f = 0.37 (5% MeOH in EtOAc); [α]_D²³ = 0.6 (*c* = 0.1, C₅H₅N); IR (film) IR (film); 3350, 2917, 2926, 1642, 1443, 1021, 942 cm⁻¹; ¹H NMR (500 MHz, C₅H₅N) δ 5.84 (d, *J*_{1/1',2/2'} = 3.7 Hz, 1H, H-1/1'), 5.83 (d, *J*_{1/1',2/2'} = 3.7 Hz, 1H, H-1/1'), 5.04–5.00 (m, 1H, H-5), 4.91 (dd, *J*_{6a,6b} = 11.7 Hz, *J*_{5,6a} = 1.7 Hz, 1H, H-6a), 4.87–4.84 (m, 1H, H-5'), 4.73 (dd, *J*_{6a,6b} = 11.7 Hz, *J*_{5,6b} = 5.1 Hz, 1H, H-6b), 4.71 (t, *J*_{2/2',3/3'} = *J*_{3/3',4/4'} = 9.5 Hz, 1H, H-3/3'), 4.69 (t, *J*_{2/2',3/3'} = *J*_{3/3',4/4'} = 9.0 Hz, 1H, H-3/3'), 4.39 (dd, *J*_{6a',6b'} = 12.0 Hz, *J*_{5',6a'} = 2.7 Hz, 1H, H-6a'), 4.31 (dd, *J*_{6a',6b'} = 12.0 Hz, *J*_{5',6a'} = 4.9 Hz, 1H, H-6b'), 4.20 (dd, *J*_{2,3/2',3'} = 9.5 Hz, *J*_{1,2/1',2'} = 3.7 Hz, 1H, H-2/2'), 4.19 (dd, *J*_{3',4'} = *J*_{4',5'} = 9.5 Hz, 1H, H-4') 4.18 (dd, *J*_{2,3/2',3'} = 9.5 Hz, *J*_{1,2/1',2'} = 3.2 Hz, 1H, H-2/2'), 4.08 (t, *J*_{3',4'} = *J*_{4',5'} = 9.5 Hz, 1H, H-4), 2.24 (dt, *J*_{8a,8b} = 11.4 Hz, *J*_{8a,9} = 7.5 Hz, 1H, H-8a), 2.23 (dt, *J*_{8a,8b} = 11.4 Hz, *J*_{8b,9} = 7.4 Hz, 1H, H-8b), 1.52 (pentet, *J*_{α,β} = *J*_{β,γ} = 7.6 Hz, 2H, H-9), 1.20–1.08 (m, 36H, H-10–H-27), 0.73 (t, *J*_{27,28} = 7.1 Hz, 3H, H-28); ¹³C NMR (125 MHz, C₅H₅N) δ 173.9 (C-7), 95.9 (C-1/1'), 95.7 (C-1/1'), 75.1 (C-3/3'), 74.9 (C-3/3'), 74.6 (C-5'), 73.6 (C-2/2'), 73.5 (C-2/2'), 72.4 (C-4'), 72.1 (C-4), 71.5 (C-5), 64.5 (C-6),

62.8 (C-6'), 34.4 (C-8), 32.3, 30.7, 28.62, 28.57, 28.52, 28.36, 28.20, 28.19, 27.98, 23.88, (C-9–C-27), 14.4 (C-28); HRMS(ESI) m/z calcd. for $[C_{34}H_{64}O_{12}+Na]^+$: 687.4290, obsd.: 687.4287.



6-*O*-Hexacosanoyl- α,α' -D-trehalose (9g). By subjecting trehalose monoester **8g** (5 mg, 0.004 mmol) to the procedure described above, the title compound **9g** was obtained after reverse phase chromatography (eluting in MeOH) as a white solid (3 mg, 0.004 mmol, 97%) R_f = 0.38 (5% MeOH in

EtOAc); $[\alpha]_D^{25}$ = 2.0 (c = 0.1, $CHCl_3$); IR (film) 3322, 2917, 2932, 1659, 1449, 1022, 736 cm^{-1} ; 1H NMR (500 MHz, C_5H_5N) δ 5.84 (d, $J_{1/1',2/2'}$ = 3.9 Hz, 1H, H-1/1'), 5.82 (d, $J_{1/1',2/2'}$ = 3.9 Hz, 1H, H-1/1'), 5.01–5.91 (m, 1H, H-5), 4.89 (dd, $J_{6a,6b}$ = 11.5 Hz, $J_{5,6a}$ = 1.9 Hz, 1H, H-6a), 4.89–4.86 (m, 1H, H-5'), 4.73 (dd, $J_{6a,6b}$ = 11.5 Hz, $J_{5,6b}$ = 5.2 Hz, 1H, H-6b), 4.70 (t, $J_{2/2',3/3'} = J_{3/3',4/4'} = 8.9$ Hz, 1H, H-3/3'), 4.68 (t, $J_{2/2',3/3'} = J_{3/3',4/4'} = 8.9$ Hz, 1H, H-3/3'), 4.37 (dd, $J_{6a',6b'}$ = 11.7 Hz, $J_{5',6a'}$ = 2.5 Hz, 1H, H-6a'), 4.31 (dd, $J_{6a',6b'}$ = 11.7 Hz, $J_{5',6a'}$ = 4.9 Hz, 1H, H-6b'), 4.22 (dd, $J_{2,3/2',3'}$ = 9.4 Hz, $J_{1,2/1',2'}$ = 3.9 Hz, 1H, H-2/2'), 4.19 (dd, $J_{3',4'} = J_{4',5'} = 9.4$ Hz, 1H, H-4'), 4.18 (dd, $J_{2,3/2',3'}$ = 9.4 Hz, $J_{1,2/1',2'}$ = 3.9 Hz, 1H, H-2/2'), 4.08 (t, $J_{3',4'} = J_{4',5'} = 9.4$ Hz, 1H, H-4), 2.22 (dt, $J_{8a,8b}$ = 11.5 Hz, $J_{8a,9}$ = 7.4 Hz, 1H, H-8a), 2.21 (dd, $J_{8a,8b}$ = 11.5 Hz, $J_{8b,9}$ = 7.4 Hz, 1H, H-8b), 1.51 (pentet, $J_{\alpha,\beta} = J_{\beta,\gamma} = 7.3$ Hz, 2H, H-9), 1.27–1.06 (m, 36H, H-10–H-31), 0.74 (t, $J_{31,32} = 7.1$ Hz, 3H, H-32); ^{13}C NMR (125 MHz, C_5H_5N) δ 173.8 (C-7), 95.8 (C-1/1'), 95.7 (C-1/1'), 75.1 (C-3/3'), 74.9 (C-3/3'), 74.6 (C-5'), 73.7 (C-2/2'), 73.5 (C-2/2'), 72.4 (C-4'), 72.1 (C-4), 71.6 (C-5), 64.5 (C-6), 62.9 (C-6'), 34.5 (C-8), 32.2, 30.1, 30.1, 30.04, 30.01, 29.9, 29.71, 29.69, 29.48, 25.4, 23.0 30.7, 28.62, 28.57, 28.52, 28.36, 28.20, 28.19, 27.98, 23.88 (C-9–C-31), 14.4 (C-32); HRMS(ESI) m/z calcd. for $[C_{38}H_{72}O_{12}+Na]^+$: 743.4916, obsd.: 743.4907.

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3

TDE Biological Testing

3.1 Introduction

Although the biological properties of trehalose dimycolates (TDMs, **1**, Figure 1) and trehalose diesters of fatty acids (TDEs, **2**) have been studied for many years, it was only in 2009 that TDM and its simpler analogue, trehalose dibehenate (TDB, **2f**), were found to activate macrophages *via* the Macrophage Inducible C-type Lectin ‘Mincle’.^{1,2,3} It has been suggested that Mincle binds to the ester and carbohydrate groups in trehalose derivatives,^{1,4} however, to date, no comprehensive structure-activity studies have been performed. In view of this, we were interested in determining the structural requirements of trehalose glycolipids for the activation of macrophages by way of the Mincle receptor. We were also interested in investigating how TDE structure influences cytotoxicity and whether this correlates to the previously observed anti-tumour activity of these compounds.⁵⁻⁹

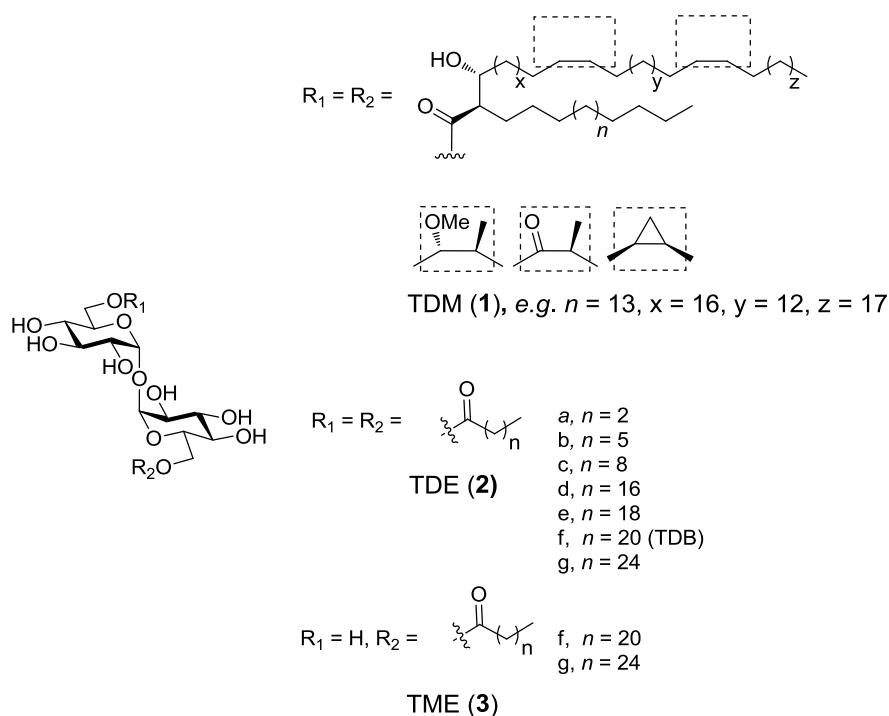


Figure 1. TDM (1), TDEs (2) and TMEs (3)

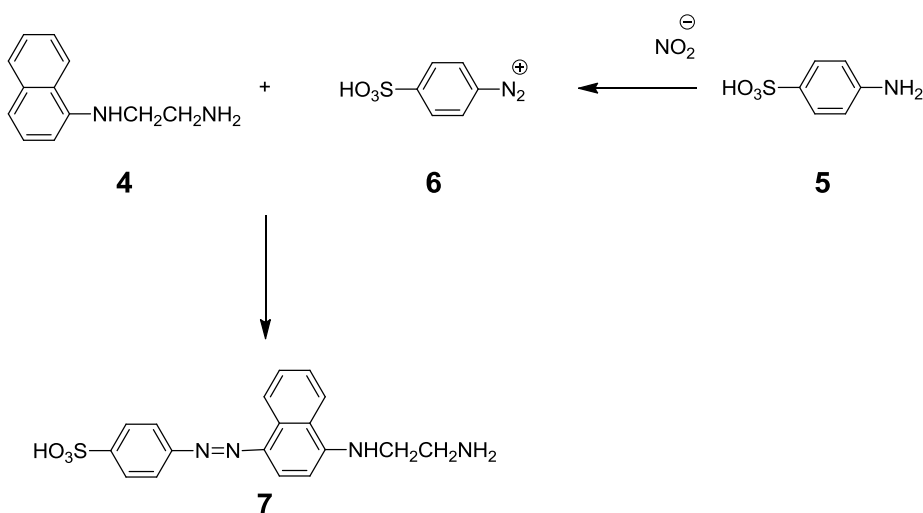
To meet the aforementioned objectives, TDEs of different lipid length (**2a-g**, Figure 1) were tested for their ability to activate macrophages, as evidenced by nitric oxide (NO) and cytokine production [interleukin (IL)-6 and IL-1 β].^{10,11} The mechanism of activation was then explored by determining whether Mincle^{-/-} or specific Toll-like-receptor (TLR)^{-/-} macrophages are activated in the presence of TDEs **2a-g**. In addition to these studies, the requirements for Mincle binding were further probed by testing trehalose monoesters of fatty acids (TMEs), such as **3f-g**, in similar macrophage activation assays. Finally, the cytotoxicity of each TDE was determined and two representative TDEs were subsequently tested in an *in vivo* tumour model. The results of these studies and their implications are discussed in this chapter.

3.2 Results and Discussion

3.2.1 Bone Marrow Macrophage (BMM) Assay Optimisation

Macrophages are the first line of defense for the host organism against bacterial infection.^{12,13} Since TDB (**2f**) has been shown to bind to Mincle on macrophages and lead to macrophage activation we wanted to investigate if other TDEs can also lead to macrophage activation as evidenced by the production of NO and cytokines such as IL-6 and IL-1 β . NO is commonly produced by activated macrophages and is the radical responsible for cytotoxic effects.^{14,15} Macrophages release IL-6 in response to specific microbial molecules known as pattern associated molecular patterns (PAMPs) for example, trehalose glycolipids, which are recognised by pattern recognition receptors. IL-6 can act both as a pro-inflammatory and anti-inflammatory cytokine. IL-1 β is produced by activated macrophages as a pro-protein which is processed to its active form by caspase 1. It is an important mediator of the inflammatory response and has been shown to be involved in cell proliferation and differentiation. Thus using NO and cytokine IL-6 and IL-1 β readouts, the activation state of macrophages can be deduced. Initial experiments were done using TDB as a model compound to set up the parameters for analysis of these glycolipids in a bone marrow macrophage (BMM) assay.

NO production is commonly analysed using Griess assay, which is a chemical test that detects the amount of nitrite present by spontaneous oxidation of NO under physiological conditions.^{16,17} Griess reagent consists of *N*-(1-naphthyl)ethylenediamine (**4**) and sulfanilic acid (**5**) (Scheme 1). The sulfanilic acid in the presence of nitrite anion forms the diazonium salt, **6** which further reacts with *N*-(1-naphthyl)ethylenediamine (**4**) to form the azo dye **7**, detectable at 548 nm.



Scheme 1. Griess reaction

The first parameter to establish was the number of bone marrow (BM) cells required to be derivatised into macrophages in order to get a response from these compounds. To this end, BM cells were isolated from C57BL/6 mice, plated out in 24-well plates at 2.5×10^5 cell/mL, 5×10^5 cells/mL, 1×10^6 cells/mL and 2×10^6 cells/mL to a total of 1 mL in each well, and differentiated into macrophages over 10 days (see experimental section for more details). On day 10, the BMM were primed with IFN- γ (10 ng/mL) for 3 hours after which time they were incubated with TDB at four concentrations (50, 10, 2 and 0.4 $\mu\text{g/mL}$), and lipopolysaccharide (LPS, 100 ng/mL) which was used as positive control. IFN- γ priming is commonly used to skew naïve macrophages more towards a Th1 phenotype (classically activated macrophages).^{18,19} As the TDEs and TMEs are similar to the bacterial cell wall component TDMs, a Th1 response is more akin to that which would be observed when considering the immune response to the whole bacteria.²⁰ The supernatants were analysed for NO production using Griess assay at 96 hours (Figure 2). In all cases, good dose response curves were observed when starting with the four different concentrations of cells. We opted for the lower number of cells (250 000) because this enabled more assays to be performed per animal.

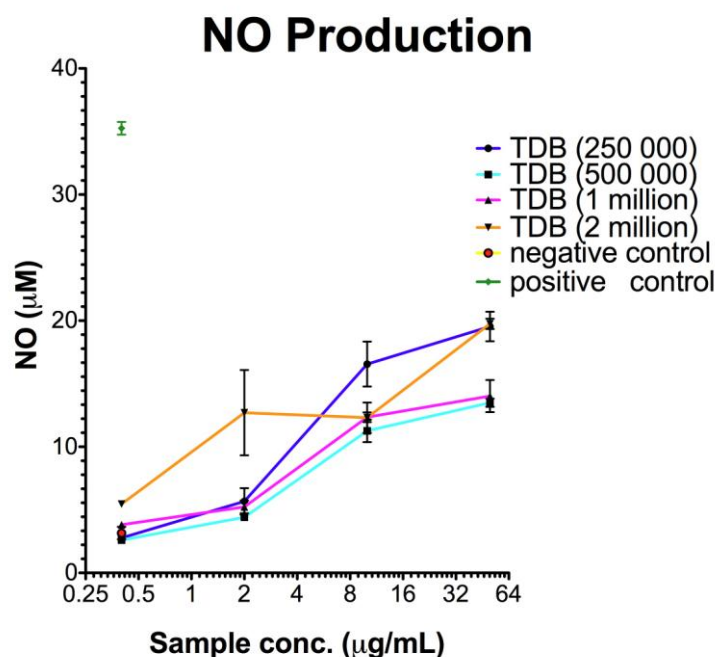


Figure 2. NO production by TDB-treated BMM. Bone marrow cells were plated out at four different concentrations, differentiated into macrophages over 10 days, primed with 10 ng/mL IFN- γ and stimulated with TDB (50, 10, 2 and 0.4 μ g/mL) or LPS (100 ng/mL) for 96 h, after which levels of NO production in the supernatants were analysed.

The next parameters analysed were the amounts of IFN- γ and TDB required to activate macrophages. Thus BMM were cultured, incubated with IFN- γ at 10 ng/mL and 20 ng/mL for 3 hours, after which time they were incubated with TDB at six concentrations (80, 40, 20, 10, 5 and 2.5 μ g/mL) and LPS (100 ng/mL). The supernatants were analysed after 96 hours and as illustrated in Figure 3, more consistent NO readings were obtained when the macrophages were primed with 10 ng/mL IFN- γ followed by stimulation with TDB at either 20 or 40 μ g/mL. Thus future experiments were performed using 10 ng/mL IFN- γ for priming and samples at 20 and 40 μ g/mL.

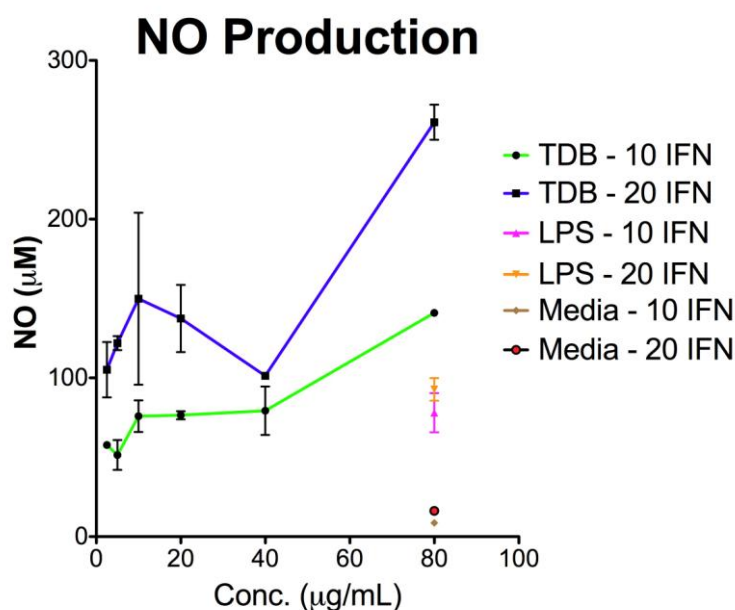


Figure 3. NO production by TDB-treated BMM following IFN- γ priming at different concentrations. BMM were primed with IFN- γ (10 or 20 ng/mL), followed by stimulation with TDB (80, 40, 20, 10, 5 and 2.5 μ g/mL) or LPS (100 ng/mL) and the supernatants analysed for NO production after 96 h.

In order to determine the optimum time-point for cytokine read-outs, a time-course experiment for cytokine analysis was undertaken. To this end, BMM were stimulated with TDB (20 μ g/mL) and the supernatants analysed for IL-1 β and IL-6 production at 24, 48, 72 and 96 hours by enzyme-linked immunosorbent assay (ELISA) [Cytokine analysis was performed by Rene McLaughlin, a PhD student in Dr Jacquie Harper's group at MIMR]. The induction of IL-6 and IL-1 β by TDB (**2f**) reached a plateau at 72 h (Figure 4A and 4B). As with NO production, TDB (**2f**) induced production of IL-6 and IL-1 β , exhibited delayed kinetics and was weaker than LPS induced production.

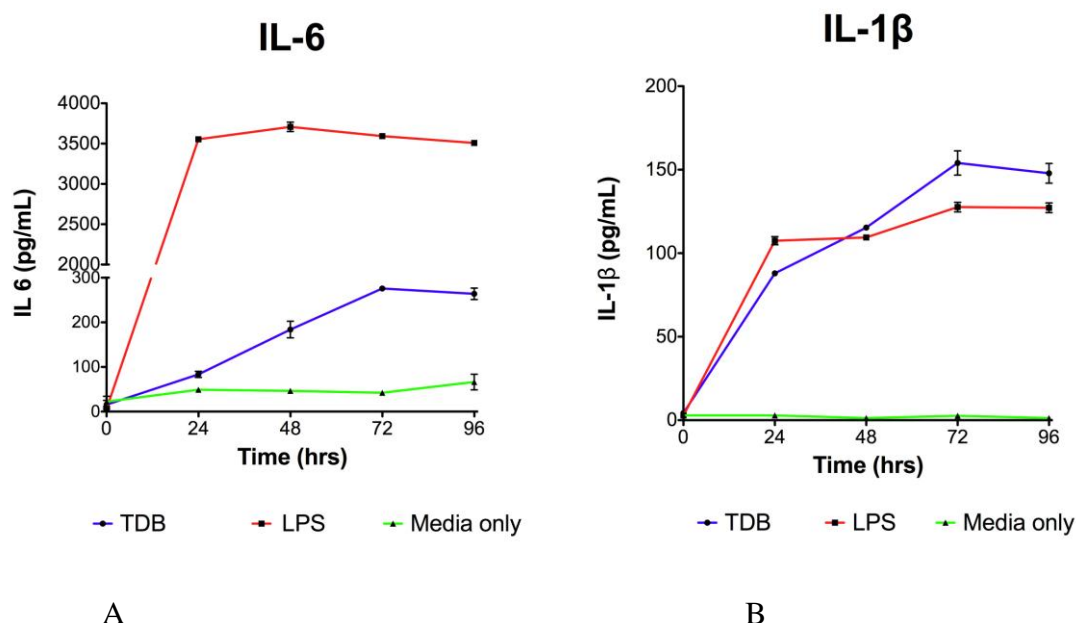


Figure 4. Cytokine production by TDB-treated BMM. BMM were treated with TDB (20 $\mu\text{g/mL}$) or LPS (100 ng/mL). At 24, 48, 72 and 96 h the supernatants were analysed for A) IL-6 and B) IL-1 β levels by ELISA.

3.2.2 Understanding how trehalose lipid length affects macrophage activation

With the BMM assay set-up, all the synthesised TDEs were tested for their ability to activate macrophages. Here, IFN- γ primed BMM were treated with 20 $\mu\text{g/mL}$ of TDEs, and at different time points supernatants were collected for NO analysis (Figure 5A). Trehalose and the uncoupled C22 (behenic) acid were used as controls in the experiment. The longer chain TDEs **2e-g** all induced NO production, with NO levels reaching a plateau at 96 h (C22 > C20 > C26). The shorter TDEs (C4-C10) were inactive. The C18 analogue led to a small but significant ($p < 0.05$) increase in NO production. Trehalose and the uncoupled C22 (behenic) acid showed no activity. To determine the influence of TDE concentration on NO production, BMM were then incubated with 20 $\mu\text{g/mL}$ and 40 $\mu\text{g/mL}$ of each compound and NO production measured at 96 h (Figure 5B). For those TDEs that stimulated BMM (**2e-g**), there was a modest increase in NO production with increased glycolipid concentration.

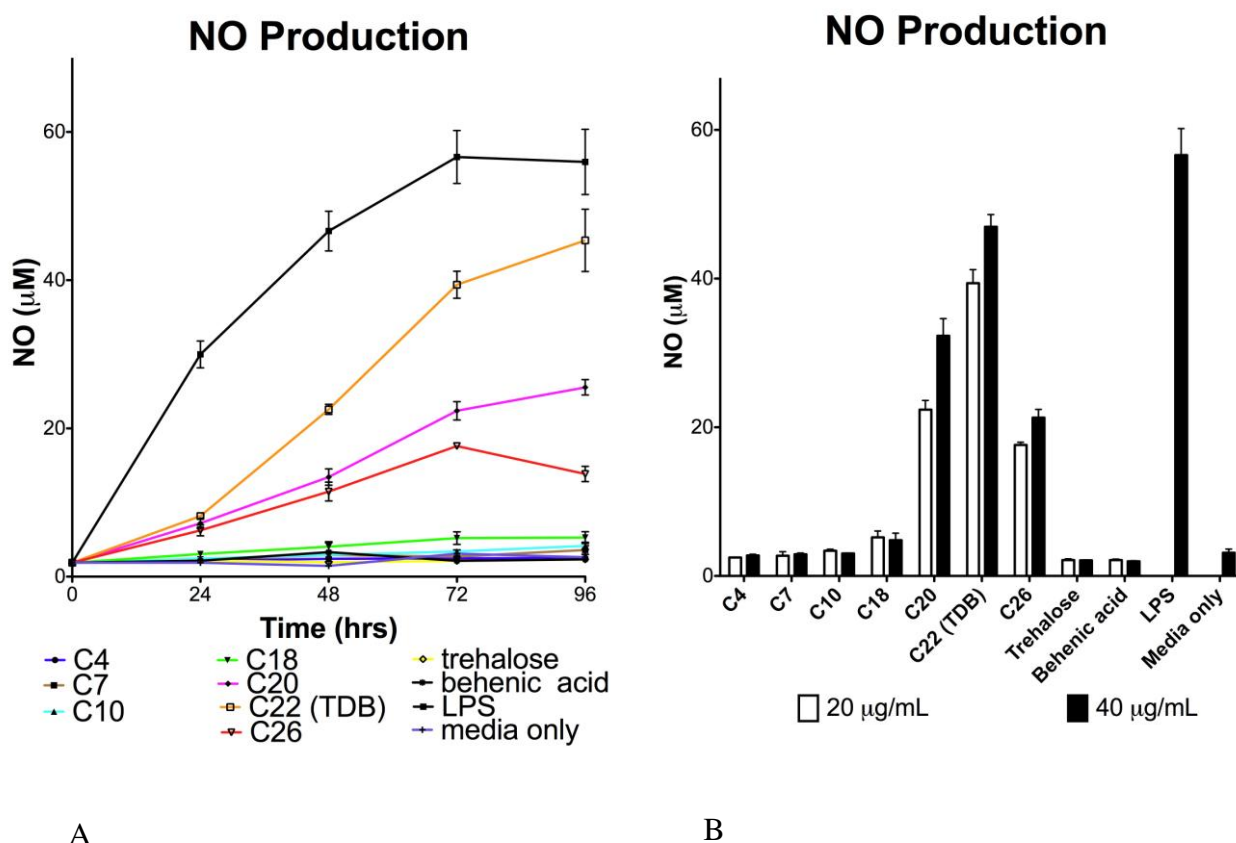


Figure 5. NO production by TDE-treated BMM. A) BMM were stimulated with TDEs (20 $\mu\text{g/mL}$) or with LPS (100 ng/mL). At 24, 48, 72 and 96 h the supernatants were analysed for NO production using Griess assay. B) BMM were treated with TDEs, trehalose or behenic acid (40 $\mu\text{g/mL}$ and 20 $\mu\text{g/mL}$) or with LPS (100 ng/mL) and NO production was determined at 96 h. The mean and SD of triplicate samples from a representative experiment of two are shown.

The levels of IL-6 and IL-1 β production were also measured at 96 hours for all the TDEs. In both cases, only the longer TDEs (C22~C26>C20) induced significant cytokine production (Figure 6A and 6B). TDEs induced lower levels of IL-6 production by the BMMs when compared to LPS. The shorter lipid chain analogues (C4-C10) showed no activity.

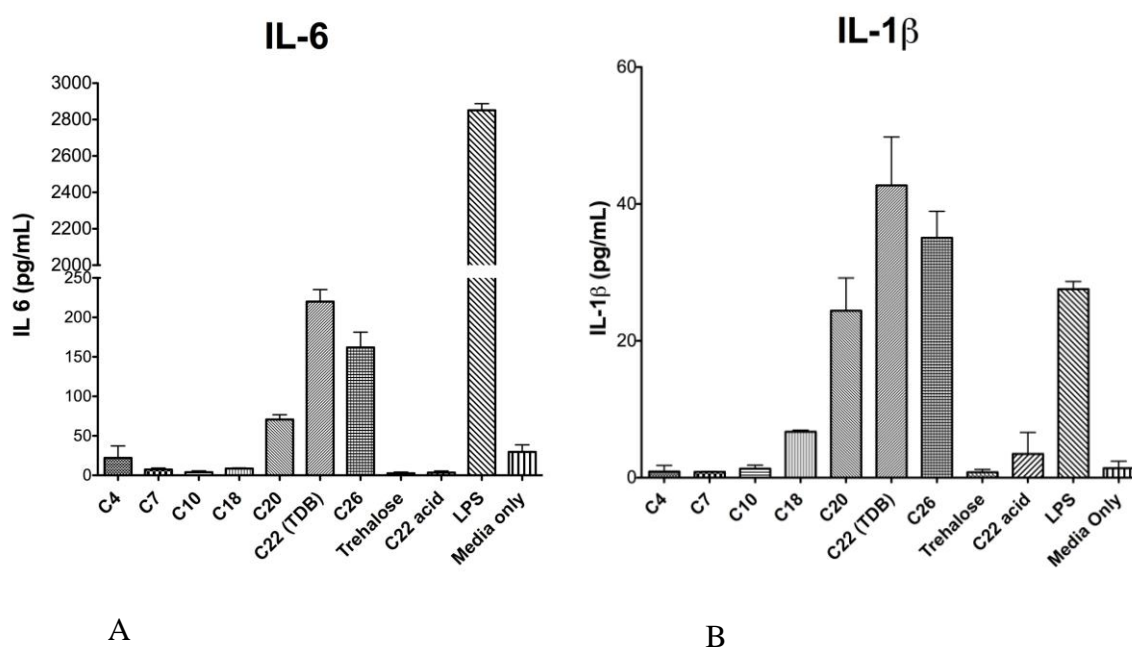


Figure 6. Cytokine production by TDE-treated BMM. BMM were stimulated with TDEs (20 $\mu\text{g/mL}$) or with LPS (100 ng/mL) for 96 h, and the supernatants were analysed for the accumulation of A) IL-6 and B) IL-1 β by ELISA. The mean and SD of triplicate samples from a representative experiment of two are shown.

In this work, the importance of the lipid chain length on trehalose in inducing macrophage activation is clearly emphasised. Taken as a whole, these results indicate that longer lipids ($\geq\text{C18}$) are required for the activation of macrophages by diacyltrehalose glycolipids whereas TDEs with short chain lipids ($<\text{C18}$) are inactive. In addition to the lipid length, the ester linkage was also found to be an important moiety required for the recognition of trehalose glycolipids by macrophages since both trehalose and behenic acid on their own were unable to induce macrophage activation. These observations are interesting as they provide insight into why *M. tb*, which expresses long lipid chain TDMs, triggers an innate immune response in the host upon invasion.

3.2.3 On One Leg: Trehalose Mono-Esters (TMEs) Activate Macrophages in a Mincle-Dependant Manner

As highlighted previously, diacyltrehalose glycolipids, in the form of trehalose 6,6'-dimycolates [TDMs (1), Figure 1], are the most abundant cell surface glycolipid from *M. tb* and related mycobacteria, and have important immunomodulatory functions in *M. tb* pathogenicity.^{21,22,23,24} In particular, the pivotal role of TDMs in granuloma formation and in the prevention of phagosome-lysosome fusion, thus facilitating bacterial survival within the host macrophages^{25,26} has led to much interest in the mechanism by which TDMs interact with macrophages. Initially it was proposed that TDMs regulate the immune response by binding to the cluster of differentiation (CD) 1 isoform CD1d on macrophages, thus allowing for subsequent glycolipid presentation to Natural Killer (NK) T cells,^{27,28} however, further studies using CD1d^{-/-} mice revealed that the activity of TDMs was independent of CD1d interaction.²⁹ It was not until 2009 that Ishikawa and co-workers¹ and Lang and co-workers² independently identified Mincle as a receptor for TDMs. Lang then followed on from these studies to illustrate that TDM-Mincle interaction induces a SyK-Card9-Bcl1-Malt1 signalling pathway,³ and furthermore, that the simple straight-chain TDM analogue TDB (2f) also activates macrophages in a manner similar to the parent esters. This work proved revolutionary, not only because the binding of TDMs/TDB to Mincle provides insight into the mechanisms by which *M. tb* interacts with its host cell, but also because the Th-1 type cytokine response generated by macrophages upon stimulation with trehalose glycolipids may be better harnessed in the development of *M. tb* vaccine adjuvants^{30,31} if more knowledge about the requirements of TDM-Mincle binding are known. To date, however, the limited availability of the appropriate Mincle^{-/-} murine models has meant that much in this field remains unanswered.

Mincle, (also known as Clec4e and Clec9)^{32,33} is a trans-membrane protein found on cells of the myeloid lineage (predominantly macrophages) and was discovered just over ten years ago by Akira and co-workers.³⁴ Mincle has an extracellular Ca²⁺-dependent carbohydrate recognition domain and is highly conserved between mice and humans with 85% protein similarity,³⁴ however, to date, no tertiary structure is

known. In addition to the trehalose diesters, Mincle is also a receptor for *Candida albicans*³⁵ and the pathogenic fungus *Malassezia*.³⁶ It has been proposed that Mincle binds to α -linked mannose containing glycolipids in these organisms, although no specific agonists were identified. Moreover, Mincle does not recognise α -1,2 mannose containing glycolipids from *mycobacterium* cell wall extracts.^{3,4}

To better understand the influence that trehalose diesters have on the immune response, and in particular, the manner in which they bind to macrophages the structural requirements for TDE/Mincle binding was explored. In the earlier studies reported here, it has been shown how lipid length affects the innate immune response of macrophages to trehalose diesters and observed that long lipids (\geq C18) are required for the activation of macrophages.³⁷ The mechanism of activation, however, was not investigated, although others have suggested that trehalose diesters bind to Mincle *via* their carbohydrate and ester groups.^{3,4} Herein, the structural requirements for the binding of TDEs with Mincle, including unprecedented insight into the number of esters required for macrophage activation and the mechanisms of activation is reported.

3.2.3a Trehalose mono-esters of carbon length \geq C20 activate macrophages

To understand more about the structural requirements for the binding of trehalose esters to Mincle, the ability of trehalose mono-esters of fatty acids (TMEs, **3**, Figure 1) to activate macrophages was investigated. Given that long lipids (\geq C18) are required for macrophage activation,³⁷ C22 TMEs (**3f**) and C26 TME (**3g**), and along with C22 TDE (TDB, **2f**, as positive control) were prepared and the ability of each of these glycolipids to activate BMMs derived from C57BL/6 mice, as determined by NO and cytokine production (Figure 8) was explored. As illustrated, NO production by the BMMs in response to C22 TME (**3f**) and C26 TME (**3g**) (20 μ g/mL) increased over the 96 h time period, although at a much slower rate when compared to the toll-like receptor (TLR) 4 ligand, LPS (Figure 7A). At 24 h, C22 TMEs (**3f**) and C26 TME (**3g**) and TDB (**2f**) all induced similar levels of NO, however by 96 h, levels of this intracellular mediator were lower for C22 TME (**3f**) and C26 TME (**3g**) (with

3g<3f). The influence of glycolipid concentration on NO production was then investigated and BMMs were incubated with 20 and 40 µg/mL of the TMEs/TDB and NO levels recorded at 96 h. As illustrated (Figure 7B), a modest increase in NO production with increased glycolipid concentration was observed for both the mono- and di-esters.

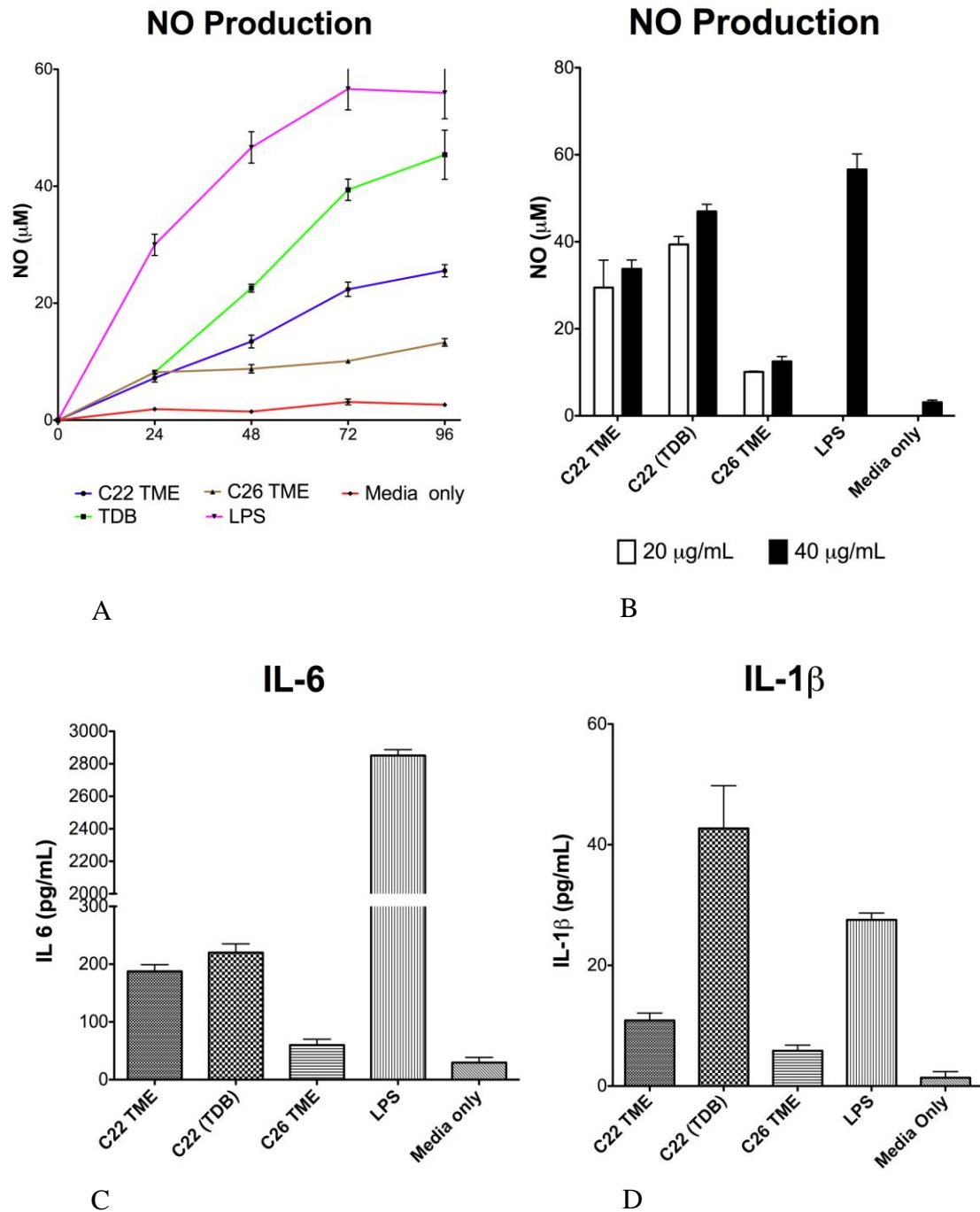


Figure 7. NO and cytokines IL-6 and IL-1 β production by TDB/TME-treated BMM. A) BMM were stimulated with TMEs, TDB (20 μ g/mL) or with LPS (100 ng/mL). At 24, 48, 72 and 96 h the supernatants were analysed for NO accumulation. B) BMM were treated with TMEs, TDB (40 μ g/mL or 20 μ g/mL) or with LPS (100 ng/mL) and NO production was determined at 96 h. BMM were stimulated with TMEs, TDB (20 μ g/mL) or LPS (100 ng/mL) for 96 h, and the supernatants were analysed for the accumulation of C) IL-6 and D) IL-1 β by ELISA. For all graphs, the mean and SD of triplicate samples from a representative experiment of two are shown.

Having established that C22 and C26 TMEs (**3f** and **3g**), stimulate the production of NO by BMMs, the effect of these mono-esters on the production of the intracellular cytokines, IL-6 and IL-1 β was then investigated. To this end, BMMs were stimulated with the trehalose esters and the levels of each cytokine measured at 96 h, which was previously determined as being the optimum time-point.³⁷ Surprisingly, levels of IL-6 were similar for C22 TME (**3f**) and the corresponding C22 TDE (**2f**), while treatment of the BMMs with C26 TME (**3g**) also led to IL-6 production, albeit at lower levels (Figure 7C). Production of IL-1 β was slightly lower for C22 and C26 TME (**3f** and **3g**), when compared to both TDB (**2f**) and LPS (Figure 7D). Taken as a whole, these observations are interesting and suggest that trehalose structure can influence the relative ratios of cytokines produced. Moreover, the first evidence to suggest that trehalose mono-esters also activate macrophages is provided, which is surprising and contrary to current ideas about the requirements for trehalose glycolipid/Mincle interactions.^{1,4}

3.2.3b Mincle is a key cellular mediator in the activation of macrophages by trehalose mono- and di-esters

In the earlier studies the effect of lipid length on the activation of BMMs by TDEs was demonstrated; however, no insight was provided for the mechanism of this phenomenon.³⁷ Although one could assume that all linear trehalose diesters that activate BMMs do so *via* Mincle [as established by Lang and co-workers for TDB (**2f**)],² this hypothesis needs to be explored. Similarly, the manner by which trehalose C22 and C26 TME, (**3f** and **3g**), activate BMMs also needs to be determined. To this end, the ability of C20 and C22 TDEs (**2e** and **2f**) and C22 and C26 TMEs (**3f** and **3g**) to activate Mincle^{-/-} BMMs was investigated. Given that other C-type lectins, such as Dectin-1, act synergistically with other receptors (*e.g.*, TLR2 and TLR4),³⁸ we also explored the ability of the trehalose esters to activate TLR2/4^{-/-} BMMs. This work was carried out by Dr. Faustin Kamena at Max Plank Institute, Berlin, Germany.

Using the NO BMM assay developed above, C22 and C26 TMEs (**3f** and **3g**), and C20 TDE (**2e**), C22 TDE (**2f**) and C4 TDE, (**2a**, as a negative control),³⁷ were tested

for their ability to activate BMMs (Figure 8). In this experiment, the C20 TDE (**2e**) did not lead to the generation of NO by wild-type BMMs, however, TDB (**2f**) did. What is more important, however, is that both C22 (**3f**) and C26 (**3g**) TMEs were able to activate wild-type BMMs but not Mincle^{-/-} BMMs, thus highlighting the importance of this receptor for the activation of macrophages by trehalose monoesters. The ability of C22 and C26 TMEs, (**3f** and **3g**), and TDB (**2f**) to activate TLR2/4^{-/-} macrophages, also suggests that neither of these TLRs binds to the trehalose glycolipids. This finding is supported by Lang's earlier studies where it was illustrated that both TDM and TDB were able to activate BMMs from MyD88^{-/-} mice, as measured by NO and cytokine production.² Here, it should be noted however, that care needs to be taken in the interpretation of our TLR2 data because the control (PamCys – a TLR2 ligand) led to the activation of TLR2/4^{-/-} BMMs. This unusual result could either be due to too high levels of PamCys being used in the assay or the PamCys being contaminated. These assays will be repeated in due course to better explain this phenomenon.

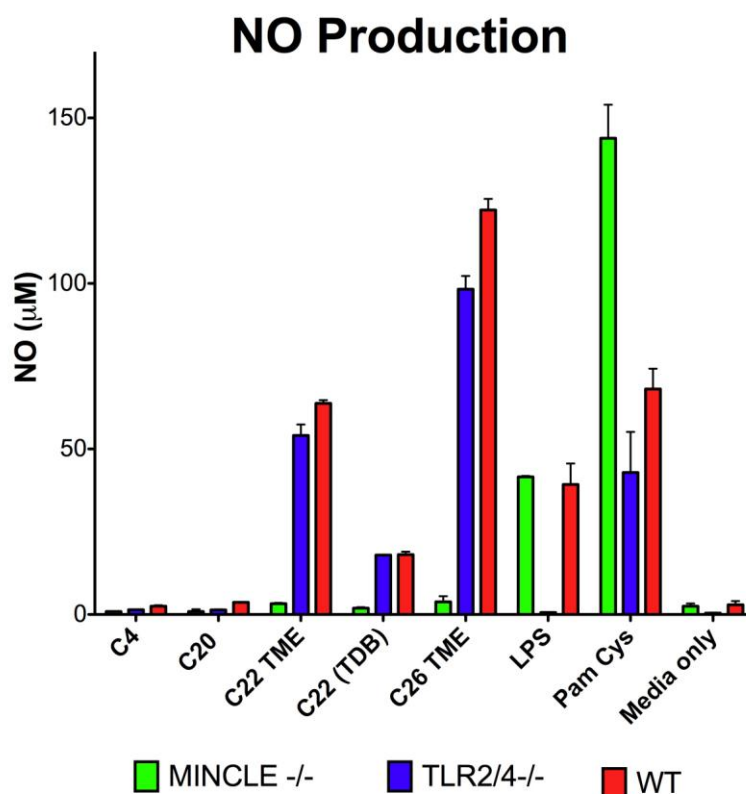


Figure 8. NO production from TDE/TME-treated BMM derived from *Mincle*^{-/-}, *TLR2/4*^{-/-} and wild type mice. BMM were primed with IFN- γ (10 ng/mL) and stimulated with C4 TDE (**2a**), C20 TDE (**2e**), C22 TDE (**2f**) C22 TME (**3f**), and C26 TME (**3g**) at 40 μ g/mL, LPS (10 ng/mL) or PamCys (10 ng/mL) and NO measured after 72 h.

Although there are still a number of experiments to be performed using the trehalose mono- and di-esters (including the testing of further analogues in the BMM assays), results obtained to date, point to a number of interesting facts. First, the ability of trehalose diesters to activate BMMs appears to be a general phenomenon of all diesters which have a lipid length \geq C20, with the mechanism of macrophage activation being Mincle, but not TLR, dependent. While earlier studies by Giesel *et al.*³⁹ and Bowdish *et al.*⁴⁰ had implicated TLRs (including TLR2) in TDM-binding and recognition, the studies by Ishikawa,¹ Lang,² and now the results obtained during this research, point to a mechanism of macrophage activation by TDEs that is independent of TLRs. To add to this argument, the ability of the TDEs and TMEs to

activate BMMs in the absence of other TLRs (*e.g.* by using TLR9^{-/-}, Myd88^{-/-} BMMs *etc.*) will be further explored.

In addition to providing insight into the mechanism of action for a range of TDE's, we have also illustrated that C22 and C26 TMEs (**3f** and **3g**) activate macrophages via Mincle. Although, it is generally thought that the induction of intracellular signalling by macrophages in response to trehalose esters requires two esters to bind to and activate Mincle,^{3,4} through this work, however, it has been demonstrated that only one ester group is necessary, and by doing so, a new sub-class of Mincle ligands have been uncovered, which will provide potential for the future development of agonists for this receptor.

3.2.3c Trehalose diesters (TDEs) can prime macrophages

To engage a full immune response, macrophages are typically primed prior to the addition of any subsequent immunomodulatory compound. Priming of macrophages is often performed using IFN- γ , which causes an increase in antigen presentation and lysosome activity of macrophages and skews naïve macrophages towards a Th1 phenotype (classically activated macrophages).^{18,19} The TLR4 ligand LPS has also shown potential to be used as a priming agent for macrophages, making macrophages sensitive to induction of pro-inflammatory cytokines in the presence of secondary stimuli.⁴¹ The identification of a new class of compounds that can prime macrophages in a TLR-independent manner, however, provides a new way to enhance the immune response and may lead to the identification of new combinations of therapies to generate the desired immunological response.

To this end, the ability of TDE's to activate macrophages with different levels of IFN- γ priming was explored. Preliminary work in this area was performed by Lang and co-workers,² whereby BMMs were incubated with TDM and TDB (20 μ g/mL) and found to produce NO, albeit at a lower level than the IFN- γ primed (20 ng/mL) BMMs. In the current study, however, the ability of unprimed BMMs to generate NO and cytokines (*e.g.* IL-6) upon exposure to a variety of TDE's was investigated. Accordingly, BMMs were stimulated with different concentrations of IFN- γ (0, 10

and 20 ng/mL) 3 h prior to the addition of TDB (**2f**) and the supernatant analysed for NO and IL-6. As illustrated (Figure 9A), in the absence of IFN- γ priming, there was no NO production by the BMMs in response to TDB (**2f**), however, IL-6 production for the unprimed BMMs was similar to that for BMMs primed with 10 ng/mL of IFN- γ (Figure 9B).

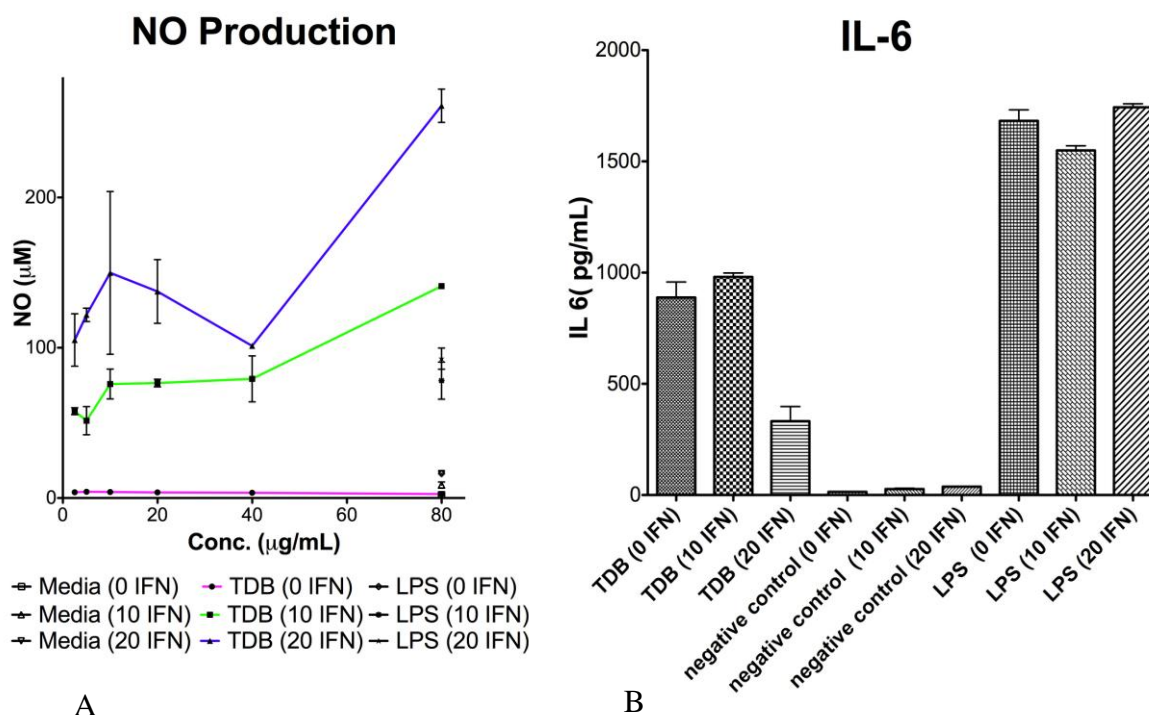


Figure 9. The effect of priming with IFN- γ on BMM stimulated with TDB. BMM were primed with IFN- γ (0, 10 or 20 ng/mL) for 3 h followed by stimulation with TDB (80, 40, 20, 10, 5, 2.5 μ g/mL) or LPS (100 ng/mL); A) The supernatants were analysed for NO production after 96 h. B) The supernatants from BMM stimulated with TDB (20 μ g/mL), LPS (100 ng/mL) were analysed for IL-6 production at 96 h.

From the above preliminary results, it is apparent that TDB (**2f**) is able to activate macrophages independent of IFN- γ priming. There are, however, some differences between the results reported here and those obtained by Weringhaus *et al.* who showed that in the absence of IFN- γ priming, macrophages were able to induce modest NO production in response to 20 μ g/mL of TDB (**2f**).² That said, no investigations into the levels of cytokines produced by unprimed macrophages in response to TDB (**2f**) were reported.²

To further explore the potential of trehalose diesters as priming agents, there are a number of experiments that should follow. First, a variety of TMEs and TDEs should be tested for their ability to activate BMMs (without priming) to determine whether this is a response unique to TDB (**2f**) or more widely applicable. Moreover, it should be determined whether BMMs treated with LPS show greater activity following priming with TDB (**2f**). By performing this assay, the potential of trehalose glycolipids to act as priming agents can be more readily assessed. Indeed, if increased BMM activation is observed following TDB priming, a variety of other immunostimulatory molecules could then be tested following TDB (or similar trehalose glycolipid) priming, thus opening-up a whole new avenue of research and means by which to generate an enhanced immune response.

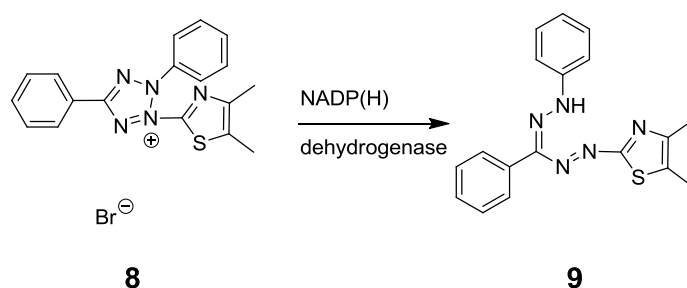
3.2.4 Application of TMEs and TDEs in cancer therapy

It has been known for some time that trehalose glycolipids have potential in the treatment of solid tumours,⁵⁻⁹ however, the mechanism of action and the means by which to develop an optimum anti-tumour response are poorly understood. This is in part due to the variety of assays that have been performed, which involve either intravenous (*i.v.*), intra-tumoural, subcutaneous or oral administration of different trehalose diesters (C2-C22) in different vehicles (*e.g.* as saline solutions or as oil emulsions with or without the addition of LPS), and in different tumour models.^{5,7,9,43-47}

From an analysis of the literature, it would appear as though use of an oil vehicle leads to a better anti-tumour effect,^{42,44} as does the addition of LPS,^{7,9,45} however, these conclusions can only be reached by considering experiments that were performed using similar assays and where only one variable was altered at a time. Given this, it is difficult to determine whether there is a particular trehalose lipid length that leads to an optimum anti-tumour response. For example, trehalose diesters with lipids of lengths C8, C10, C18, C22 have all shown anti-tumour potential, depending on the assay used. Moreover, it is not known whether the anti-tumour effect of the trehalose esters is due to their cytotoxic or immunomodulatory effects (or both). Given that previously it has been observed that only trehalose esters with lipid length \geq C18 were able to activate BMMs,³⁷ the mechanism by which

trehalose esters exert their anti-tumour activity was explored next, particularly since solid tumours contain large numbers of the immunosuppressive tumour associated macrophages (TAMs).⁴⁶

To determine whether the anti-cancer activities of trehalose esters are due to general cytotoxicity, the compounds were tested in an *in vitro* MTT viability assay using a human hepatocellular carcinoma (HepG2) cell line. HepG2 is a liver cancer cell line commonly used to study liver metabolism and toxicity. The MTT assay assesses cell viability by determining whether the yellow tetrazolium salt MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (**8**) is reduced to the purple formazan product **9** by NADPH in the presence of dehydrogenase, which occurs during normal cellular respiration (Scheme 2). In the presence of a cytotoxic agent however, cellular growth is suppressed and no or limited colour change of the dye occurs. Cell viability is then expressed in a percentage of MTT reduction with respect to an untreated control.



Scheme 2. MTT reduction

The results of the MTT assay are depicted in Figure 10 [Assays performed by Dr James Baty, MIMR]. None of the tested trehalose glycolipids showed appreciable cytotoxicity at concentration under 50 μM (data not shown). At the two highest concentrations (50 and 150 μM), however, the more lipophilic compounds, the C26 monoester and the C20, C22 and C26 diesters of trehalose, appeared to be slightly cytotoxic, in a concentration dependant manner. Interestingly, the C22 TME did not follow this trend, as it did not show any cytotoxicity at the concentrations tested.

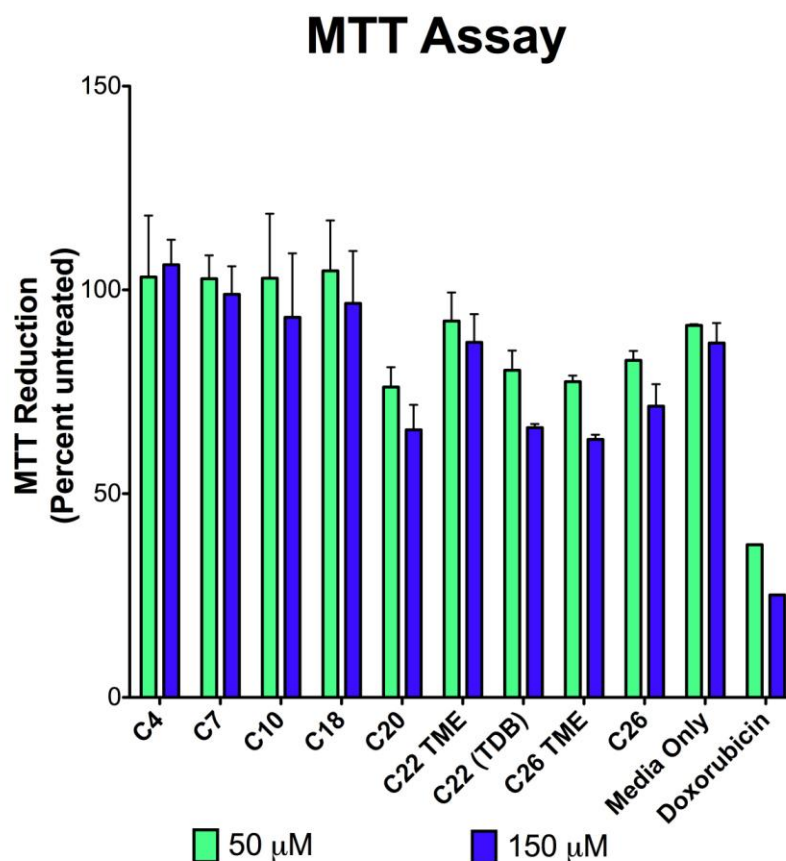


Figure 10. Cytotoxicities of TMEs and TDEs against HepG cell line. HepG cells were stimulated with TDEs, TMEs, Doxorubicin (positive control) at 50 and 150 μ M for 2 days after which time 20 μ L MTT was added, followed by addition of SDS lysing buffer after 2 hours. The cells were incubated a further overnight and absorbance were measured in a FLUOstar OPTIMA plate reader at 570 nm.

Future Directions

Though investigations into understanding the mechanism by which trehalose diesters exert their anti-cancer activity is very much in its infancy, from the analysis it is apparent that when the trehalose esters are administered in PBS they exhibit poor cytotoxicities therefore direct killing of cancer cells by the TDMs is unlikely. It has, however, been shown that the same TDEs are able to activate BMMs, leading to the generation of NO and cytokines (such as IL-1 β and IL-6), which suggests an ability to induce a Th1 immune response in macrophages. Given this, one could suggest that

the anti-tumour activity of the trehalose diesters is due to the ability of the compounds to invoke the systemic activation of macrophages, which could lead to tumour regression, or potentially switch the phenotype of the immunosuppressive TAMs to that of the tumour suppressive M1 phenotype (a phenotypic ‘switch’ known to occur via the agency of Th-1 cytokines such as tumour necrosis factor (TNF)- α and IL-12.^{46,47} Indeed, if this is true, then improved anti-tumour immunomodulators may be generated through the development of trehalose esters that lead to an optimum ‘Th-1’ response.

To further substantiate this hypothesis, however, numerous other experiments need to be performed. First, the cytotoxicity of the trehalose esters should be determined when administered in an oil vehicle. Perhaps the cytotoxicity of the compounds depends on the mode of administration, as has been suggested in earlier studies whereby the co-presence of an oil vehicle was necessary for anti-tumour activity.^{42,44} It has also been suggested that oil primarily functions as a carrier of the glycolipids to the tumour site,⁴⁴ and that the lack of activity of trehalose diesters when administered in aqueous media is due to micelle formation, thus rendering the trehalose esters biologically inert.⁴³ During the course of this thesis, however, some provisional *in vivo* results were obtained which suggest that the trehalose diesters (when administered as a solution in 2% DMSO and PBS) do exhibit an ability to reduce tumour growth [Assay performed by Sabine Kuhn, MIMR]. As this experiment was only performed once, it is too premature to present the work here, however, as some tumour regression was observed, it would therefore be instructive to determine the critical micelle concentration of our diesters. This can readily be achieved by fluorescent measurements of the trehalose glycolipids at 25°C, using pyrene or pyrene-3-carboxaldehyde as the probe.⁴⁸ Following on from this, the *in vivo* assays can then be repeated using a selection of trehalose glycolipids. Here, a number of variables can be considered including glycolipid concentration, tumour type,⁵ and the co-administration of LPS.^{7,9,45,49} If significant tumour regression is observed using any one combination of reagents, this experiment could then be repeated using Mincle^{-/-} mice to understand more about the mechanism of action.

Finally, whether trehalose esters have the ability to switch the phenotype of macrophages, for example from TAM to M1 (thus leading to better tumour regression),^{46,47,50,51} could be investigated by following the protocols of Duluc *et al.*⁵² and Eriksson *et al.*⁴⁷ Here, TAMs would be generated from naïve macrophages (obtained from BM cells) by culturing them for 48 hours in complete medium containing 30% medium from tumour cultures. Subsequent treatment with the TDEs followed by analysis of immunosuppressive and pro-tumoural mediators [*e.g.* IL-10, Vascular endothelial growth factor (VEGF) and Chemokine ligand (CCL) 18] would then be used to determine if phenotype switching occurs. Should macrophage phenotype switching be observed, these studies can then be repeated using Mincle^{-/-} mice to understand more about the mechanism of action.

3.3 Conclusion

From the first part of this work, it is clearly evident that long chain trehalose diesters (\geq C18) are required to activate macrophages. The activation of macrophages was measured using NO and cytokine IL-6 and IL-1 β read-outs. In addition the C22 and C26 TMEs were also shown to activate macrophages, thus unveiling a new sub-class of compounds which have macrophage activation abilities. The work with Mincle^{-/-} and TLR2/4^{-/-} BMM clearly showed that Mincle is the key receptor on macrophages for TDEs and TMEs binding. The recognition of TDEs and TMEs is independent of TLR2 and 4 receptors and further testing using other TLR^{-/-} and MyD88^{-/-} BMM needs to be performed to rule out involvement of other TLR receptors. TDB has also shown potential to be used as a priming agent, leading to the discovery of a new class of compounds, independent of TLR binding, that can be used to generate an enhanced immune response.

The anti-tumour activity of the TDEs and TMEs was also probed and results from the cytotoxicity assay using HepG2 cell line showed that these compounds were only slightly cytotoxic; however, preliminary *in-vivo* results showed that both the C10 and C22 TDE have anti-tumour activities. Thus the mechanism by which the trehalose glycolipids exert anti-tumour activity needs to be further investigated.

3.4 Experimental

Material and methods for Biological Assays: C57BL/6 male mice were bred and housed in a conventional animal facility at the Malaghan Institute of Medical Research, Wellington, New Zealand. All animals used for the experiments were aged between 8-10 weeks. All experimental procedures were approved by the Victoria University Animal Ethics Committee in accordance with their guidelines for the care of animals. All synthetic compounds were confirmed to be free of endotoxin, at a sensitivity of ≤ 0.125 EU/mL by testing them in Limulus ameobocyte lysate (LAL) assay using an endotoxin kit (Pyrotell, Limulus Amebocyte Lysate).

Generation of bone marrow derived macrophages (BMMs): Bone marrow cells were collected from the tibia and femur of C57BL/6, Mincle^{-/-} or TLR2/4^{-/-} mice and were cultured (250 000 cells/mL) in Iscove's Modified Dulbecco's Media (IMDM-Gibco) supplemented with 5% FBS (Gibco), 1% Penicillin streptomycin (Gibco) and 55 μ M 2-mercaptoethanol (Invitrogen) containing 10 ng/mL GM-CSF (clone X63/GM-CSF murine cells). Cells were incubated at 37 °C (5% CO₂) and the media changed on days 2, 5 and 7. On day 10, the media was removed and the BMM were primed with 10ng/mL IFN- γ (Peprotech) for 3 h prior to the addition of the compounds.²

BMM Assay: TDEs, TMEs, trehalose, and docasanoic acid stock solutions were prepared (2.5 mg/mL in PBS containing 2% DMSO), vortexed and warmed to 50 °C for 30 min (x 3) to ensure complete solubilisation of the compounds prior to administration to BMM cultures. BMM cultures were then treated with 20 or 40 μ g/mL of TDE, TMEs, trehalose, or behenic acid, with LPS (100 ng/mL) as a positive control and media only as a negative control for the times indicated. Supernatants were collected and tested immediately for NO levels or stored at -80 °C for subsequent cytokine analysis.

NO and cytokine analysis: NO levels in supernatants were determined using the Griess assay. Levels of IL-6 and IL-1 β cytokine in the supernatants sandwich ELISA (R&D Systems and BD Biosciences respectively), as per manufacturer's instructions.

HepG2 cell culture: HepG2 cells were maintained in RPMI supplemented with 10% (v/v) fetal calf serum (FCS), 2 mM glutamate, 100 U/mL penicillin, and 100 μ g/mL streptomycin and were kept at 37 °C in a humidified incubator maintained at 5% CO₂.

Cell Metabolism MTT assay: Cell metabolism activity was measured using the 2-day MTT assay as previously described and reviewed.^{53,54} Doxorubicin was used as a positive control in these experiments. HepG2 cells were plated into 96-well plates in the presence of different concentration of the TDEs and TMEs (final cell concentration = 0.1×10^6 cells/mL in complete RPMI) and incubated for 48 h (37 °C, 5% CO₂). 20 μ L of MTT (2.5 mg/mL in HBSS) was then added to each well and the cells incubated for 2 h before adding 100 μ L of SDS lysing buffer (10%)w/v SDS, 45% v/v dimethylformamide/H₂O, pH 4.7) and further overnight incubation. The absorbance at 570 nm was measured in a FLUOstar OPTIMA plate reader (BMG Labtechnologies Pty. Germany). All assays were performed in triplicate and the mean result recorded.

Murine tumour and compound preparation: EG.7-OVA (thymoma) cells were cultured and washed with PBS and resuspended in PBS to a final concentration of 1×10^6 cell/mL. C10 and C22 TDEs stock solutions were prepared (3.0 mg/mL in PBS containing 2% DMSO), vortexed and warmed to 50 °C for 30 min (x3) to ensure complete solubilisation of the compounds prior to administration.

Treatment of mice with established tumour transplants: On day 1 groups of 5 mice received 100 μL of viable thymoma cells subcutaneously, followed by 50 μL of TDEs, 2% DMSO in PBS (negative control) or 2×10^6 CFU *M. smegmatis* (positive control). At day 2, 4, 6 and 8 thereafter, the same groups were given intra-tumoural injection of TDEs, 2% DMSO in PBS or 2×10^6 CFU *M. smegmatis*.

Evaluation of tumour growth: The tumour size for individual mice were measured (using calipers) every 2 days until the tumour reached 150 mm^2

Statistical Analysis: Statistical significance was determined by student's t-test.

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4

An Improved Fráter-Seebach Alkylation¹

4.1 Introduction

Fráter-Seebach alkylation is a key step commonly used in the synthesis of trehalose dimycolates (TDMs, **1**, Figure 1) and trehalose dicorynomycolate (TDCM, **2**) to install the α -chain in the mycolic acid and corynomycolic acid fragments, respectively. However, using this methodology for installing long alkyl chains at the α -position of β -hydroxy esters has been met with mixed success. In order to improve the efficacy of this reaction for the installation of long alkyl chains, we developed a strategy whereby allyl halides are used as electrophiles.

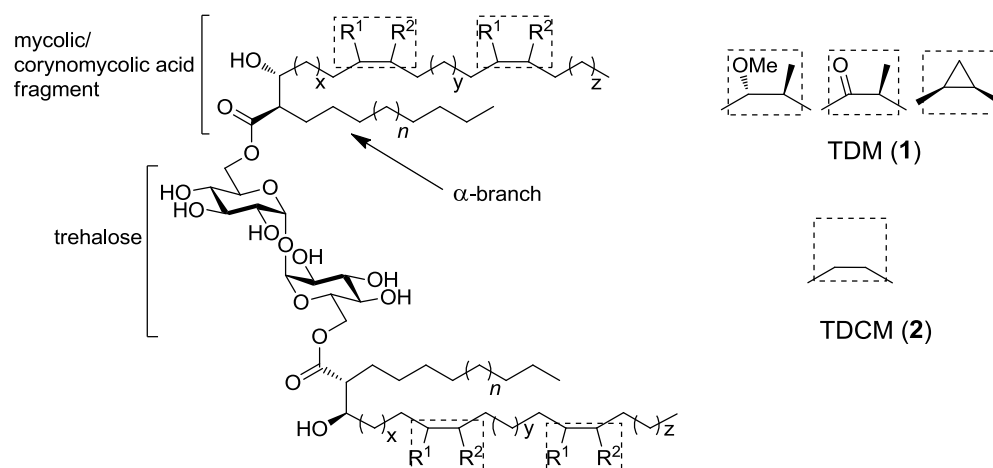
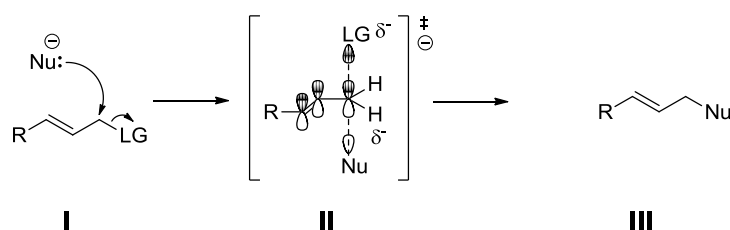


Figure 1. TDM (1) and TDCM (2)

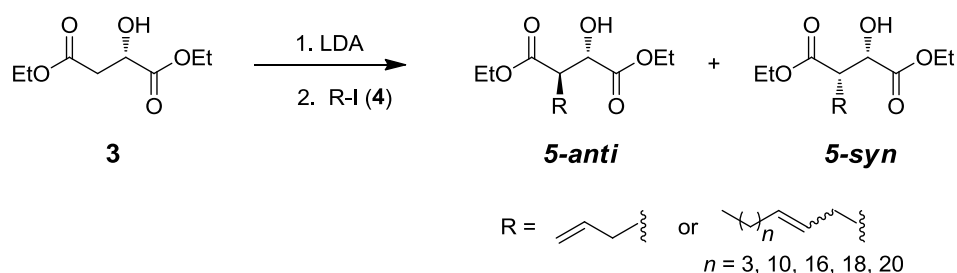
Allyl halides are known to have excellent reactivity, which arises from stereoelectronic interactions between the σ^*_{C-X} orbital and the adjacent π system.^{2,3} In the transition state for the S_N2 reaction of an allylic halide (**I**, Scheme 1), the overlap between the p orbital developed on the carbon at which displacement occurs and the p orbitals of the conjugated double bond allows for electron density of the π system to be delocalised over the three carbon atoms involved in the allylic system (**II**). This, in turn, leads to a lower transition state energy when compared to that of the corresponding saturated halide, resulting in higher yield of the product (**III**).



Scheme 1. Transition state for allyl halide substitution via an S_N2 reaction

Accordingly, we proposed that a Fráter-Seebach alkylation using highly reactive long chain allylic iodides would lead to more efficient α -alkylations when compared to the alkyl halide counterpart. The hydroxy-ester of choice was diethyl malate (**3**) which could be derivatised at a later stage to form the corynomycolic or mycolic

acids for the synthesis for TDCMs and TDMs respectively. A range of allylic iodides (**4**) were used to form alkylated esters **5** of varying lipid lengths (Scheme 2).

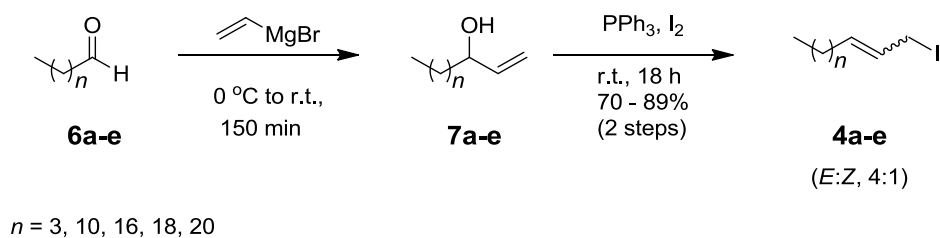


Scheme 2. Proposed Fráter-Seebach alkylation

4.2 Results and Discussion

4.2.1 Synthesis of allylic iodides

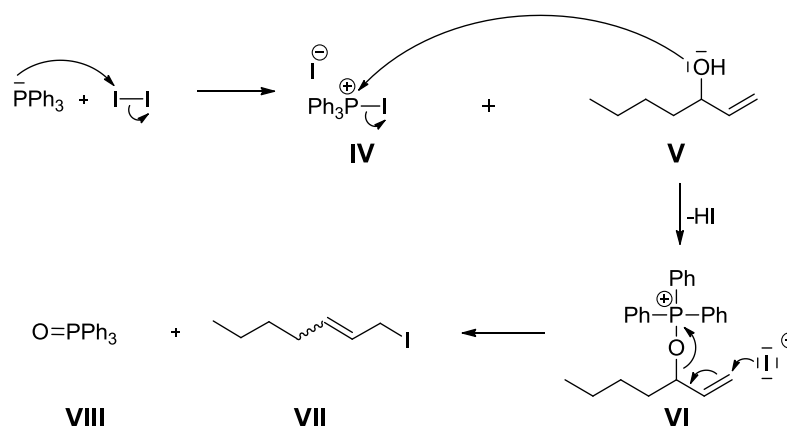
To prepare the required allylic iodides, an efficient two-step strategy was proposed, that first involved the Grignard addition of vinyl magnesium bromide to an aldehyde³ and a subsequent $\text{S}_{\text{N}}2'$ substitution of the *in situ* generated alkoxytriphenylphosphonium iodide intermediate (Scheme 3).⁴ The starting aldehydes, pentanal **6a** and dodecanal **6b**, were sourced commercially, while octadecanal **6c**, eicosanal **6d**, and docosanal **6e** were prepared *via* a pyridinium chlorochromate (PCC) oxidation of the corresponding alcohols.⁵ In case of pentanal and dodecanal, the Grignard reaction was carried out smoothly in THF with 1M vinyl magnesium bromide solution, to give the corresponding allylic alcohols **7a-b** in good yields. However, when longer aldehydes **6c-6e** were used, the yield for this reaction was quite low, with reduction of the aldehyde to the corresponding alcohols also observed. In order to reduce this by-product formation, the solvent was replaced with toluene, which had been previously reported to minimise aldehyde reduction.⁶ Indeed, a small improvement in product yield was observed, however, the by-product was still present. Other reasons for this by-product formation were attributed to the lower reactivity of long chain aldehydes, and the quality of the reagents used. Thus, in an attempt to improve the latter, vinyl magnesium bromide was prepared *in situ*⁷ and the reaction was performed at 0 °C in toluene, which did indeed lead to improved yields (71–89%) of the allylic alcohols **7c-7e**.



Scheme 3. Synthesis of representative allylic iodides

The intermediate allylic alcohols **7a-e** were then subjected to a short silica gel plug and used immediately in the iodination reaction, as they are prone to decomposition.⁴ Treatment of each alcohol with a solution of triphenylphosphine and iodine in THF gave the corresponding allylic iodides, **4a** ($n = 3$), **4b** ($n = 10$), **4c** ($n = 16$), **4d** ($n = 18$) and **4e** ($n = 20$) in 89%, 81%, 70%, 71% and 73% yield, respectively, over two steps. As anticipated, each of the allylic iodides **4a-e** were found to be an inseparable isomeric mixture consisting mainly of the *E*-isomer (*E*:*Z* *ca.* 4:1), as determined by the 18.6 Hz *vs* 8.5 Hz coupling constant for the alkene protons in the ¹H NMR of the crude reaction mixtures. Spectroscopically, a very minor (<5%) amount of the corresponding 1,3-diene elimination product was also observed during the reaction.

The iodination of allylic alcohol **4a-e** could be asserted to occur *via* a S_N2' mechanism (Scheme 4).³ Here, the *in situ* generated iodophosphonium iodide (**IV**), undergoes a nucleophilic attack by alcohol **V**, which results in the generation of an alkoxytriphenylphosphonium iodide (**VI**). S_N2' attack of the iodide then leads to the generation of the desired internal alkene (**VII**), with triphenylphosphine oxide (**VIII**) being generated as a by-product.



Scheme 4. Mechanism for the $\text{S}_{\text{N}}2'$ reaction

4.2.2 Fráter-Seebach Alkylation

With the necessary allylic halides in hand, we set out to investigate their potential in a Fráter-Seebach alkylation. Thus, diethyl malate **3**, was prepared via esterification of the commercially available L-malic acid in a good (95%) yield,⁸ and then alkylated with the allylic halides or their saturated counterparts (Table 1). The saturated alkyl iodides were prepared via the iodination of the corresponding alcohols or, in the case of iodo-tetradecane, via cross halogenation of bromo-tetradecane. Overall yields were, in general, similar to those for the preparation of the allylic iodides. The alkylations were performed in THF using 2.5 equivalents of lithium diisopropylamide (LDA, generated *in situ* at $-78\text{ }^{\circ}\text{C}$), to which was added 1 equivalent of diethyl malate **3**, followed by 1.5 equivalents of the alkyl or alkenyl halide.

Our first study involved the alkylation of diethyl malate **3**, using either allyl bromide (entry 1), or allyl iodide (entry 2), in the absence of hexamethylphosphoramide (HMPA). As illustrated, both reactions proceeded smoothly to give *anti*-**5a** as the predominant diastereomer in respectable yield. Use of heptyl iodide, however resulted in a decreased yield with **5b** being obtained in 37% following purification by flash column chromatography (entry 3). There was no other identifiable product obtained from the reaction, and although excess alkyl halide could be recovered from the reaction mixture, diethyl malate **3** had decomposed. On the other hand,

alkylation with the C7 allylic iodide, 1-iodo-hept-2-ene (**4a**), resulted in an improved (55%) yield of the desired α -alkylated β -hydroxy ester with a diastereoselectivity of 9:1; *anti:syn* (entry 4). This result was encouraging and demonstrated the improved reactivity of the allylic system.

Entry	R-X	<i>n</i>	Conditions ^[a]	Major Product	Yield ^[b]	Ratio <i>anti:syn</i> ^[c,d]
1		-	A	5a	57%	14:1
2		-	A	5a	52%	9:1
3		3	A	5b	37%	7:1
4		3	A	5c	55%	9:1
5		10	A	5d	15%	9:1
6		10	A	5d	7%	7:1
7		10	B	5d	35%	7:1
8		10	A	5e	51%	10:1
9		10	B	5e	45%	9:1
10		16	A	5f	5%	6:1
11		16	A	5g	48%	9:1
12		18	A	5h	8%	6:1
13		18	A	5i	42%	9:1
14		20	A	5j	41%	9:1

[b] Isolated yield following purification by flash chromatography

[d] Used as a mixture of isomers (*E:Z* *ca.* 4:1)

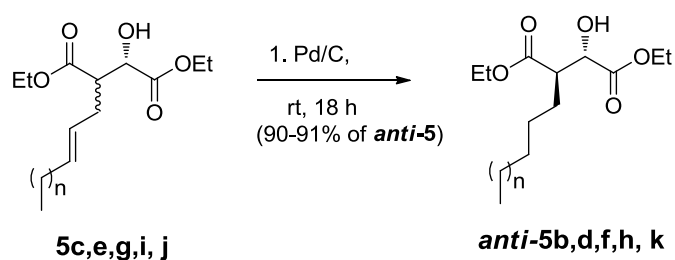
When either 1-iodotetradecane or 1-bromotetradecane was used as the electrophile, very poor yield of the corresponding α -alkylated ester **5d** was observed (entries 5 and 6). In an attempt to improve these yields, HMPA was added to the reaction vessel as a co-solvent. HMPA has been shown to enhance S_N2 reactions via its ability to strongly solvate cations, and has been used by Utaka *et al.*⁹ and Fujisawa *et al.*¹⁰ to perform α -alkylations using 1-iodotetradecane. Indeed, we also observed an improvement in reaction yield (35%) when HMPA was added (entry 7). These results were then compared to similar reactions using the corresponding C14 allylic iodide. In the absence of HMPA, the use of 1-iodotetradec-2-ene (entry 8) gave an improved 51% yield of ester **5e** (*cf.* entry 5). Surprisingly, the addition of HMPA to the reaction mixture (entry 9) did not further improve the yield (*cf.* entry 8). In view of the toxicity of this reagent and the lack of improvement in reaction yield, HMPA was not used in subsequent alkylations.

The difficulties in obtaining good yields when using long chain alkyl iodides in the Fráter-Seebach alkylation was further demonstrated when attempts were made to alkylate diethyl malate **3** using 1-iodoeicosane (entry 10). In our hands, this reaction resulted in a dismal 5% yield. The general problem we encountered when performing this α -alkylation was that the reaction was very slow and required the gradual warming of the reaction mixture from -78 °C to 0 °C for any product formation to be observed via TLC. Unfortunately, the temperature at which the reaction commenced (*ca.* -20 °C) was also the temperature at which diethyl malate **3** began to decompose. Many changes to solvent, reaction time, and equivalents of reagents were then made in an attempt to improve this yield, but without success. A change to iodo-eicos-2-ene, however, gave the desired α -alkylated ester **5g** in a much improved 49% yield (entry 11). Although some decomposition of diethyl malate **3** was observed, we were satisfied with the final yield of the ester *anti*-**5g**, given the difficulty of this α -alkylation. Attempts at performing the Fráter-Seebach alkylation using 1-iododocosane gave a mere 8% yield of **5h** (entry 12), while the use of iodo-docos-2-ene as an electrophile improved the yield of the alkylation to 42% (entry 13). The methodology was then used to synthesise ester *anti*-**5j**, which contains the most common mycolic acid α -chain length. Gratifyingly, the yield for this reaction was a respectable 41% (entry 14). In all examples, the diastereoselectivity of the allylic

alkylations was excellent (*ca.* 9:1, *anti:syn*), and was higher than those observed for the corresponding saturated alkyl halides.

4.2.3 Hydrogenation

Having demonstrated the benefit of allylic halides in the Fráter-Seebach alkylation, we then set about to hydrogenate the α,β -unsaturated esters (Scheme 4). The mixtures of diastereomers *anti:syn* ($n = 3, 10, 16, 18, 20$) were thus hydrogenated in the presence of catalytic (5 wt%) Pd/C (Scheme 5). Following hydrogenation, the diastereomers were readily separable by flash column chromatography and the desired esters *anti-5b* ($n = 3$), *anti-5d* ($n = 10$), *anti-5f* ($n = 16$), *anti-5h* ($n = 18$) and *anti-5k* ($n = 20$) were isolated as pure compounds in excellent yields (90-91%).

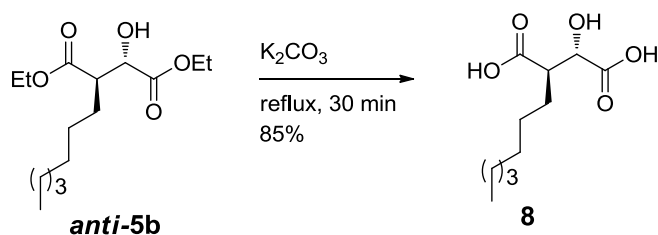


Scheme 5. Hydrogenation of α,β -unsaturated esters

4.2.4 Diastereoselectivity

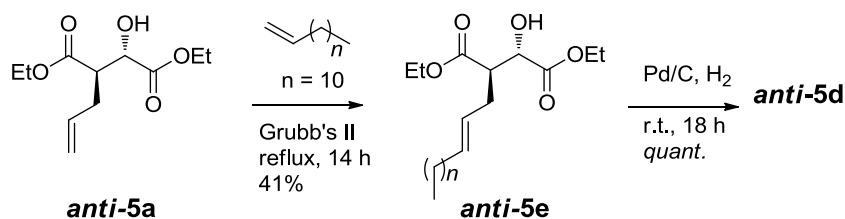
In an attempt to prove that the major diastereomer obtained from the Fráter-Seebach reaction was indeed the *anti*-isomer, we set about hydrolysing ester *anti-5b* to form the di-acid **8**. This could potentially be crystallised, enabling the use of X-ray crystallography to determine the absolute stereochemistry of the compound. We opted to use this method as crystallisation of the hydrolysed product of malic ester **5a** had been reported in Seebach's earlier work.¹¹ To this end, ester *anti-5b* (obtained after hydrogenation of **5c**) was subjected to hydrolysis with potassium carbonate in MeOH:H₂O (1:1) in order to form di-acid **8** (Scheme 6). The crude product was obtained as a white solid, however, despite several attempts, compound **8** could not be crystallised, with a waxy solid being obtained in most instances. This may be due

to the lipid chain at the α -position of the molecule, and thus another method of distereoselectivity determination was investigated.



Scheme 6. Hydrolysis of product **5b**

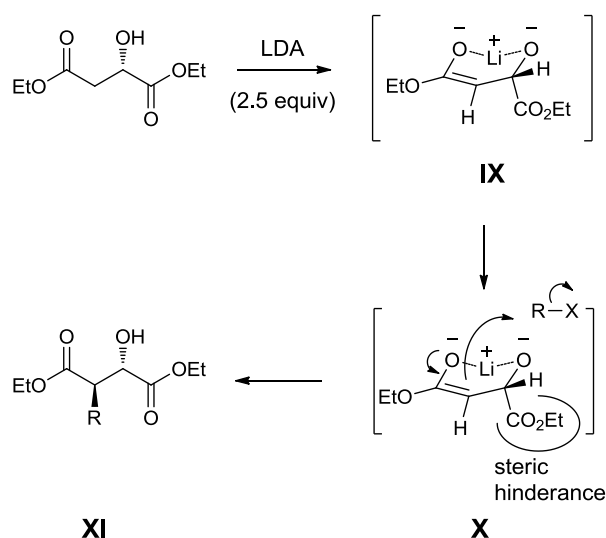
We envisaged using cross metathesis as a means of deducing the stereochemistry of the alkylated compounds. Thus, Grubbs cross metathesis reaction¹² was performed between allylic ester *anti*-**5a** (of known $2S$, $3R$ stereochemistry),¹¹ and 1-tridecene to give the unsaturated ester *anti*-**5e** in 41% yield and in an *E*:*Z* ratio of 4:1 (Scheme 7). In addition, the homo-dimerisation product of 1-tridecene was observed along with unreacted allylic ester *anti*-**5a**, which accounted for the remainder of the material. Hydrogenation of ester *anti*-**5e** then gave a product that exhibited spectral data consistent with those obtained from the direct alkylation of ethyl (*S*)-malate **5** with 1-iodotetradecane (Table 1). This unequivocally established that the major isomer from the allylic alkylation was the anticipated $2S$, $3R$ -ester.



Scheme 7. Confirming the stereochemistry of the alkylated products

To explain the preferential formation of the $2S$, $3R$ diastereomer, the rigid cyclic intermediate that is formed during the course of reaction needs to be considered (Scheme 8). More than two equivalents of LDA are added to the β -hydroxy ester,

whereby, one equivalent is used to deprotonate the hydroxyl, which results in the formation of a monoanion where the negatively charged oxygen is countered by the lithium cation, while the second equivalent of LDA leads to the formation of an ester enolate where the lithium cation bridges the two negative charges via a six-membered cyclic intermediate **IX**. Regeneration of the carbonyl then allows the enolate **X**, to attack the alkyl halide, R-X, at the face opposite to the ester which is adjacent to the β -hydroxyl substituent due to steric hindrance thus leading to the anti-diastereomer **XI**.¹³ It should also be noted that greater reactivity of the allylic system leads to higher diastereoselectivities, due to the ability of the reactions to be performed at lower temperatures.



Scheme 8. Explanation of stereoselectivity in the Fráter-Seebach alkylation

4.2.5 Product Characterisation

The Fráter-Seebach reaction products were characterised by NMR and mass spectral analyses. As shown for compound **5j** (Figure 2 and 3), the distinct resonance for the alkylated products included peaks at 5.56 ppm (dt, $J = 15.1$ Hz, $J = 6.8$ Hz) and 5.39 ppm (dt, $J = 15.1$ Hz, $J = 6.5$ Hz) for the alkene protons. In addition, the resonance at 2.91 ppm (ddd, $J = 9.1$ Hz, $J = 9.1$ Hz, $J = 3.2$ Hz) integrated for one proton and had COSY correlations to α -protons of the lipid chain at 2.58–2.53 ppm (m) and at 2.38 ppm (ddd, $J = 14.3$ Hz, $J = 8.3$ Hz, $J = 8.3$ Hz), indicating that alkylation has occurred at the desired position. Furthermore, the different signals for the two α -protons of the

lipid chain were indicative of an adjacent chiral centre. A clear HMBC between the resonance at 5.39 ppm and C-3 at 48.6 ppm, established the position of the respective allylic protons. Mass spectral analysis of the product was in agreement the expected mass; [HRMS(ESI) m/z calcd. for $[C_{32}H_{60}O_5+Na]^+$: 547.4338, obsd.: 547.4344.] and full assignment was made on the basis of 2D NMR (COSY, HSQC and HMBC) (see experimental for full assignment). With the saturated alkylated products as shown for **5k** (Figure 4 and 5), key peaks in the 1H NMR included resonance at 2.84 (td, $J = 7.3$ Hz, $J = 3.7$ Hz) integrating for one proton and the protons from the α -chains at 1.83 (ddt, $J = 13.9$ Hz, $J = 7.6$ Hz, $J = 7.6$ Hz), and 1.66 ppm (ddt, $J = 13.9$ Hz, $J = 7.1$ Hz, $J = 7.1$ Hz). HMBC correlation between the α -protons and carbonyl peak at 172.9 ppm established the resonances for one of the carbonyl carbons. Mass spectral analysis of the product was in agreement with the expected mass; [HRMS(ESI) m/z calcd. for $[C_{32}H_{62}O_5+Na]^+$: 549.4495, obsd.: 549.4490] $^+$ and full assignment was made on the basis of 2D NMR (COSY, HSQC and HMBC) (see experimental for full assignment).

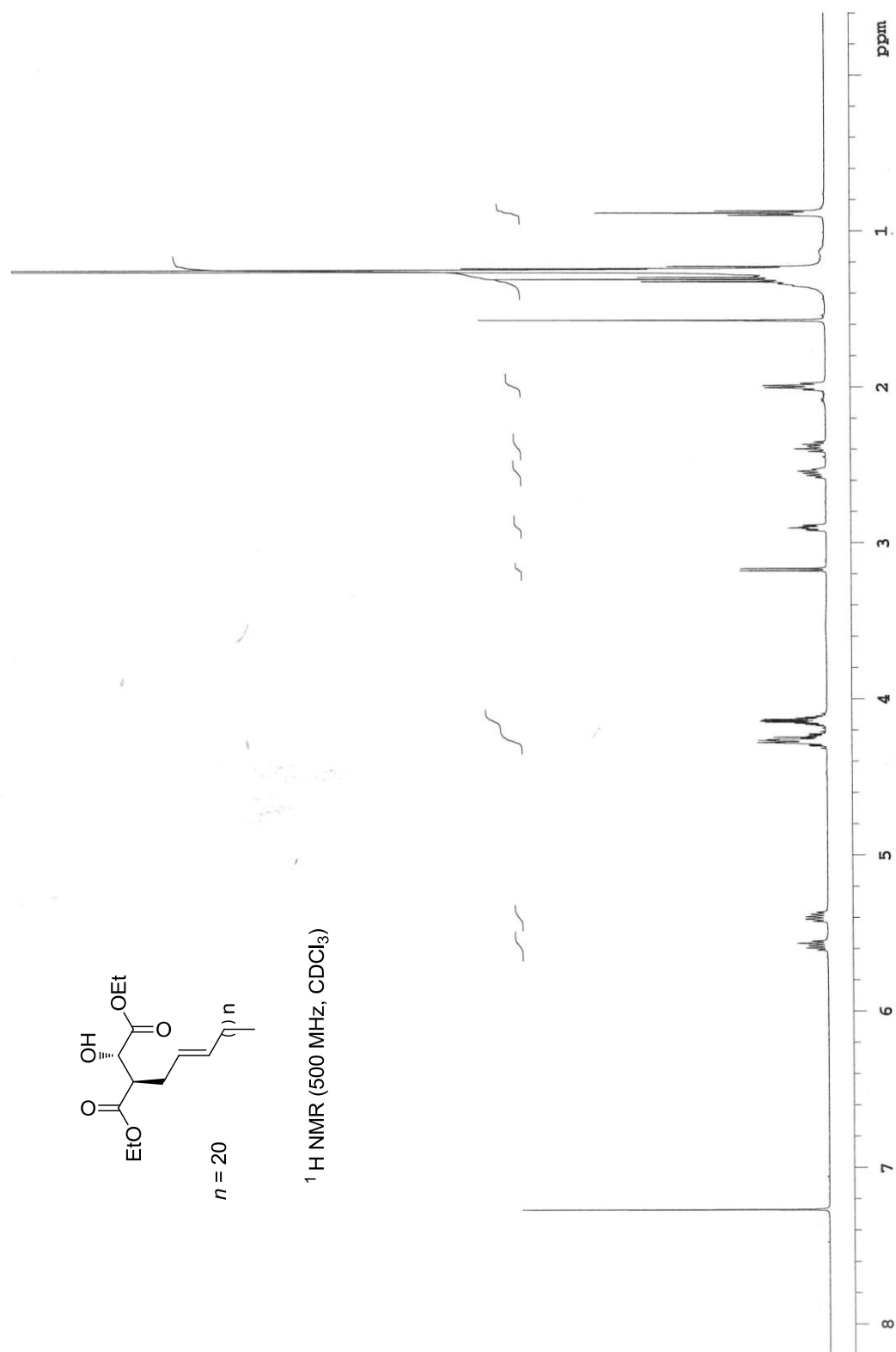


Figure 2. ^1H NMR spectrum of product **5j**

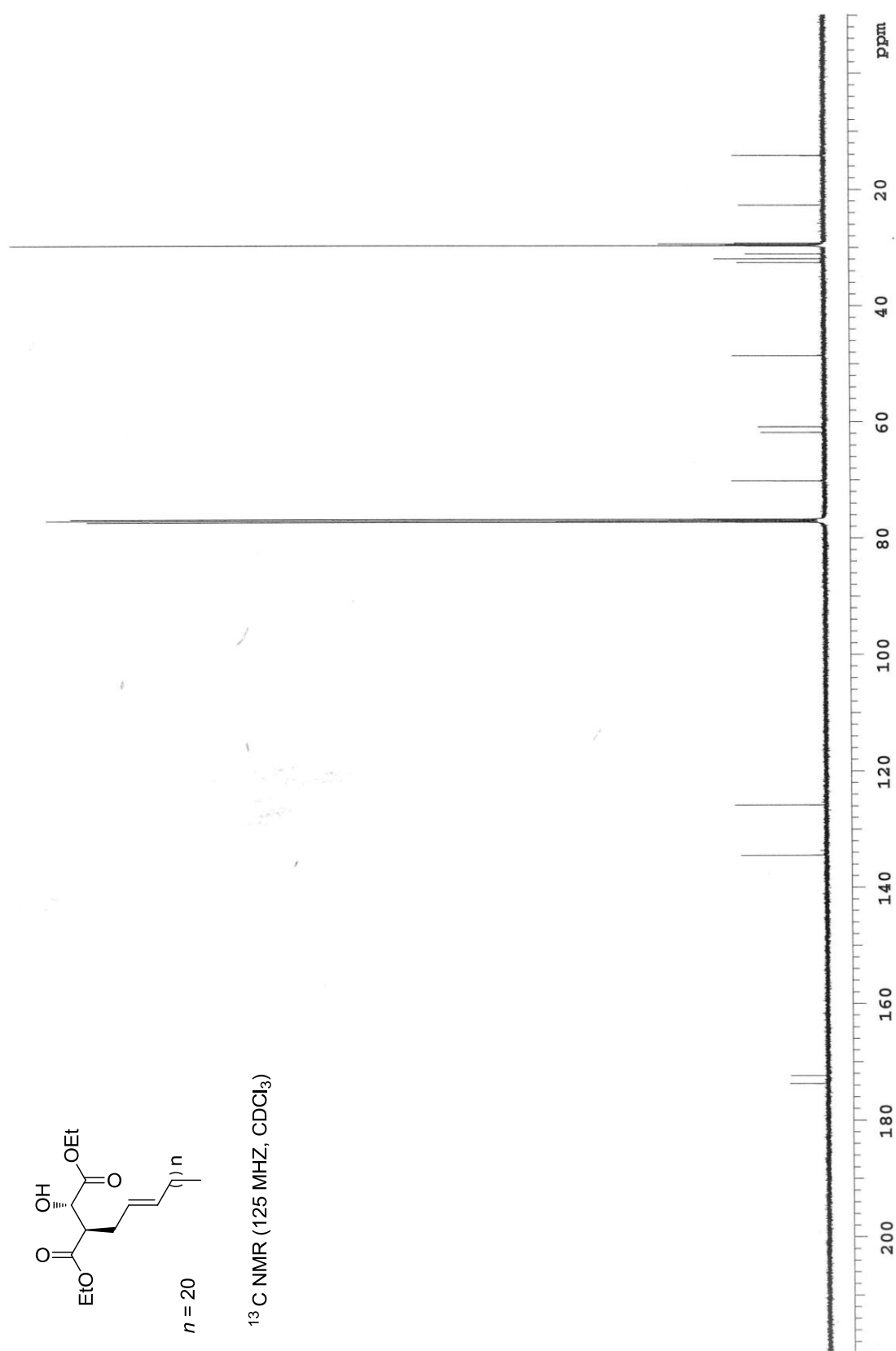


Figure 3. ^{13}C NMR spectrum for product **5j**

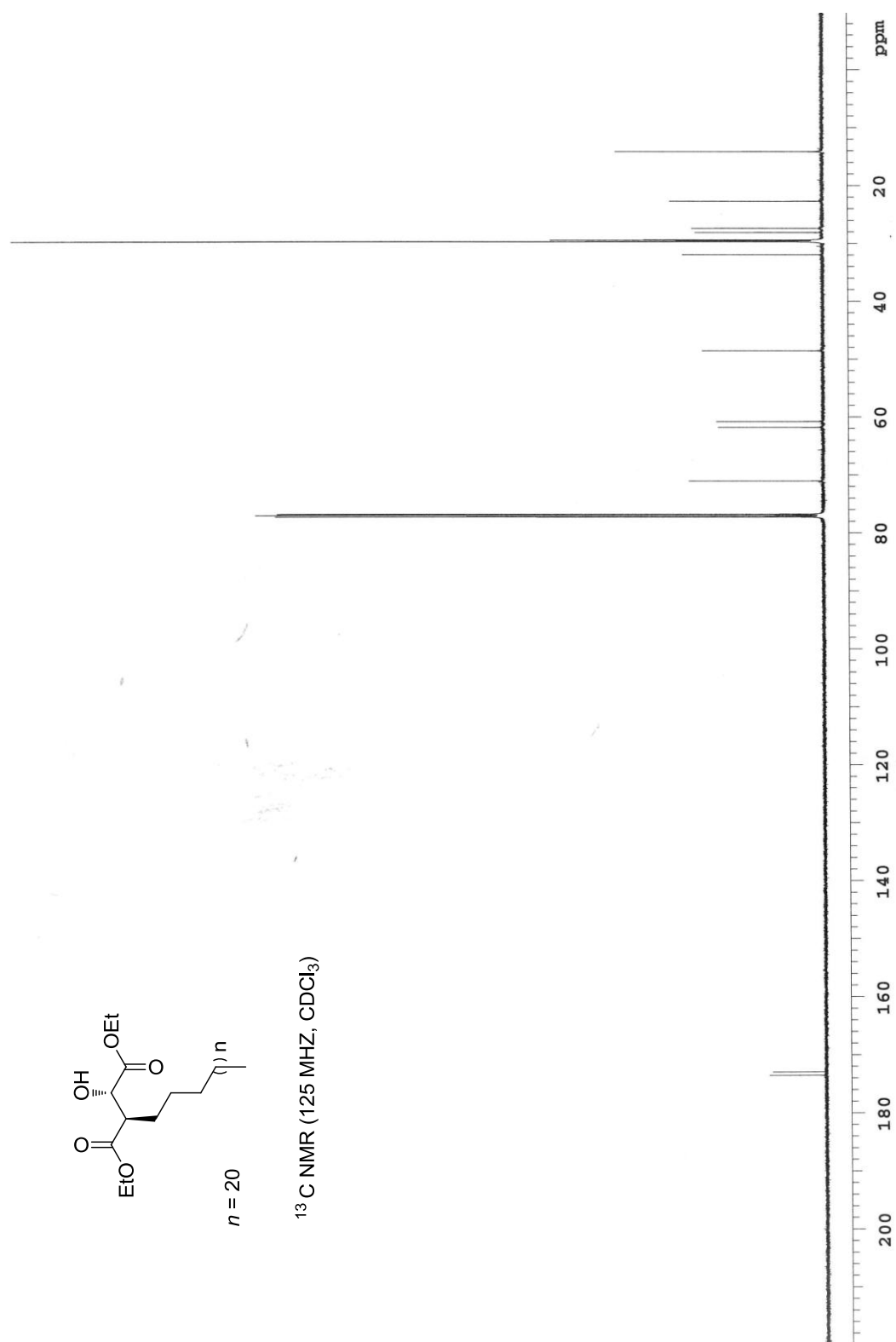


Figure 5. ^{13}C NMR spectrum of product **5k**

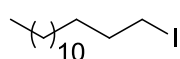
4.3 Conclusion

We have developed an improved methodology for the synthesis of long chain α -alkylated β -hydroxy esters via the use of allylic halides in the Fráter-Seebach alkylation. This allylic alkylation is highly diastereoselective, and when compared to similar reactions using the corresponding saturated alkyl halides, gives superior yields. The prerequisite allylic iodides are themselves readily prepared in two steps and in good yield from the corresponding aldehydes, while the unsaturated α -alkylated β -hydroxy esters can be readily hydrogenated in excellent yield. The final α -alkylated β -hydroxy esters are, in turn, valuable synthetic intermediates for the preparation of a number of biologically important lipids.

4.4 Experimental

Unless stated otherwise all reactions were performed under N₂. Prior to use, THF was distilled from Na wire and benzophenone, toluene was dried and stored under Na wire, CH₂Cl₂ was distilled from P₂O₅ and acetone was dried with K₂CO₃, distilled and stored over 4Å MS. HMPA was dried with CaH₂, distilled and stored under nitrogen, di-isopropylamine was dried with NaOH, distilled and stored under nitrogen, allyl bromide, allyl iodide and heptyl iodide were dried with MgSO₄, distilled and stored under nitrogen. L-(–)-Malic acid (Sigma), bromotetradecane (Fluka), 1-docosanol (Aldrich), 1-eicosanol (Alfa Aesar), 1M vinylmagnesium bromide (Aldrich) pentanal (Koch-Light Laboratories), *n*-BuLi (Aldrich, 2M solution in hexanes), dodecanal (Aldrich), octadecanol (BDH), PPh₃ (Merck), I₂ (Unilab), Pd/C (Aldrich), H₂SO₄ (Lab-Scan), ethanol (Pure Science), anhydrous Et₂O (Biolab), EtOAc (Panreac), petroleum ether (Pure Science), CHCl₃ (Panreac), NaHCO₃ (Pure Science), K₂CO₃ (Pure Science), Na₂S₂O₃ (Panreac), MgSO₄ (Pure Science), NaCl (Panreac) were used as received. 1-Iodotetradecane¹⁴ and L-diethyl malate⁸ were prepared using literature procedures. All solvents were removed via evaporation at reduced pressure. Reactions were monitored by TLC analysis on Macherey-Nagel silica gel coated plastic sheets (0.20 mm, Polygram SIL G/UV254) by coating with a solution of 5% KMnO₄ and 1% NaIO₄ in H₂O followed by heating. Column chromatography was performed using Pure Science silica gel (40-63 μ m). High

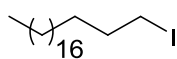
resolution mass spectra were recorded on a Waters Q-TOF PremierTM Tandem Mass Spectrometer using positive electro-spray ionisation and atmospheric-pressure chemical ionisation. Optical rotations were recorded on a Perkin-Elmer 241 polarimeter or Autopol II (Rudolph Research Analytical) at 589 nm (sodium D-line). Infrared spectra were recorded as thin films using a Bruker Tensor 27 FTIR spectrometer equipped with an Attenuated Total Reflectance (ATR) sampling accessory and are reported in wave numbers (cm⁻¹). Nuclear magnetic resonance spectra were recorded at 20 °C in CDCl₃ using a Varian INOVA operating at 500 MHz. Chemical shifts are given in ppm (δ) relative to solvent residual peak. NMR peak assignments were made using COSY, HSQC and HMBC 2D experiments.



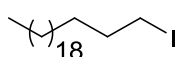
1-Iodotetradecane.¹⁵ Sodium iodide (10.81 g, 90.1 mmol) was added to a stirring solution of 1-bromotetradecane (10.0 g, 36.1 mmol) in acetone (20 mL). The reaction mixture was refluxed under nitrogen for 48 h, after which the mixture was filtered and concentrated *in vacuo*. The resulting residue was dissolved in diethyl ether and washed with sat. Na₂S₂O₃, brine dried (MgSO₄) and concentrated *in vacuo*. The product was obtained after silica gel flash chromatography (eluting in petroleum ether) as colourless oil (13.7g, 84.7 mmol, 94%). *R_f* = 0.82 (PE/EA, 2/1, v/v); IR (film) 2921, 2852, 1176, 720 cm⁻¹; ¹H NMR (500 MHz, CDCl₃) δ 3.19 (t, *J* = 7.1 Hz, 2H, H-1), 1.83 (pentet, *J*_{α,β} = *J*_{β,γ} = 7.1 Hz, 2H, H-2), 1.40–1.36 (m, 2H, H-3), 1.28–1.20 (m, 20H, H-4–H-13), 0.89 (t, *J* = 7.1 Hz, 3H, H-14); ¹³C NMR (125 MHz, CDCl₃) δ 33.5 (C-2), 31.9, 30.5, 29.70, 29.66, 29.63, 29.57, 29.44, 29.37, 28.56, 22.7 (C-3–C-13), 14.1 (C-14), 7.3 (C-1).

General Procedure for the synthesis of alkyl iodides from the corresponding alcohol. PPh₃ (12 mmol, 1.2 equiv.) was added to a solution of the appropriate alcohol (10 mmol, 1 equiv.) in dry THF (100 mL), followed by imidazole (18 mmol, 1.8 equiv.). The flask was then lowered into a water bath and I₂ (13 mmol, 1.3 equiv.) was added slowly to maintain the temperature at *ca.* 25 °C. The resulting mixture was refluxed for 5 h, after which water was added to quench the reaction and the mixture was extracted with DCM. The organic layer was washed with Na₂S₂O₃, water and

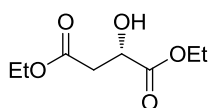
brine, dried (MgSO₄) and concentrated *in vacuo*. The product was purified using silica gel column chromatography and the eluent given.



1-Iodoeicosane. By subjecting 1-eicosanol (1.0g, 3.35 mmol) to the general procedure for the synthesis of alkyl iodides, 1-iodoeicosane was obtained (eluting in petroleum ether) as a white solid (0.95 g, 2.35 mmol, 70%). $R_f = 0.80$ (PE/EA, 2/1, v/v); IR (film) 2920, 2851, 917, 783 cm⁻¹; ¹H NMR (500 MHz, CDCl₃) δ 3.20 (t, $J = 7.1$ Hz, 2H, H-1), 1.83 (pentet, $J_{\alpha,\beta} = J_{\beta,\gamma} = 7.1$ Hz, 2H, H-2), 1.44–1.37 (m, 2H, H-3), 1.31–1.70 (m, 32H, H-4–H-19), 0.90 (t, $J = 6.9$ Hz, 3H, H-20); ¹³C NMR (125 MHz, CDCl₃) δ 33.6 (C-2), 32.0, 30.5, 29.73, 29.71, 29.69, 29.65, 29.58, 29.45, 29.40, 28.58, 22.7 (C-3–C-19), 14.2 (C-20), 7.3 (C-1).

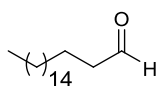


1-Iododocosane. By subjecting 1-docosanol (1.0 g, 3.06 mmol) to the general procedure for synthesis of alkyl iodides, 1-iododocosane was obtained (eluting in petroleum ether) as a white solid (0.8 g, 1.84 mmol, 60%). $R_f = 0.79$ (PE/EA, 2/1, v/v); IR (film): 2915, 2848, 1165, 740 cm⁻¹; ¹H NMR (500 MHz, CDCl₃) δ 3.19 (t, $J = 7.1$ Hz, 2H, H-1), 1.83 (pentet, $J_{\alpha\beta} = J_{\beta\gamma} = 7.4$ Hz, 2H, H-2), 1.40–1.36 (m, 2H, H-3), 1.33–1.26 (m, 36H, H-4–H-21), 0.89 (t, $J = 6.8$ Hz, 3H, H-22); ¹³C NMR (125 MHz, CDCl₃) δ 33.6 (C-2), 31.9, 30.5, 29.70, 29.67, 29.63, 29.56, 29.43, 29.37, 28.56, 26.06, 22.7 (C-3–C-21), 14.1 (C-22), 7.4 (C-1).



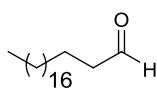
Diethyl-L-malate (3). ⁸ L-Malic acid (15 g, 112 mmol) was dissolved in ethanol and conc. H₂SO₄ (0.36 ml, 89.9 mmol) was added. The reaction mixture was refluxed for 72 h, after which the reaction was stopped and the solvent evaporated *in vacuo*. The light yellow residue was purified via silica gel column chromatography (5:1, petroleum ether/EtOAc, v/v) to afford diethyl malate **3** as a colourless oil (20.2 g, 106 mmol, 94.9%). $R_f = 0.40$ PE/EA, 5/1, v/v); $[\alpha]_D^{23} = -6.3$ ($c = 1.0$, CHCl₃); IR (film): 3481,

2984, 2940, 1731, 1447, 1373, 1267, 1177, 1099, 1023, 859, 735 cm^{-1} ; ^1H NMR (500 MHz, CDCl_3) δ 4.49 (ddd, $J_{2,3a} = J_{2,3b} = J_{2,\text{OH}} = 5.3$ Hz, 1H, H-2), 4.32–4.25 (m, 2H, CH_2O -1), 4.18 (q, $J = 7.2$ Hz, 2H, CH_2O -4), 3.21 (d, $J_{2,\text{OH}} = 5.3$ Hz, 1H, OH), 2.86 (dd, $J_{3a,3b} = 16.3$ Hz, $J_{2,3a} = 4.5$ Hz, 1H, H-3a), 2.79 (dd, $J_{3a,3b} = 16.3$ Hz, $J_{2,3b} = 6.0$ Hz, 1H, H-3b), 1.31 (t, $J = 7.2$ Hz, 3H, CH_3 , 1-OEt), 1.28 (t, $J = 7.2$ Hz, 3H, CH_3 , 4-OEt); ^{13}C NMR (125 MHz, CDCl_3) δ 173.4 (C-1), 170.5 (C-4), 67.3 (C-2), 62.1 (CH_2O , 1-OEt), 61.0 (CH_2O , 4-OEt), 38.7 (C-3), 14.1 (CH_3 , 1-OEt), 14.1 (CH_3 , 4-OEt); HRMS(ESI) m/z calcd. for $[\text{C}_8\text{H}_{14}\text{O}_5 + \text{Na}]^+$: 213.0739, obsd.: 213.0737.



1-Octadecanal (6c). 1-Octadecanol (5.00 g, 18.5 mmol) was added to a solution of PCC (7.97 g, 37 mmol) and molecular sieves \AA (5.6 g) in DCM (90 mL) under nitrogen and the reaction mixture

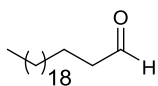
stirred at r.t. for 6 h. The mixture was then reduced *in vacuo* and the resulting oil subjected to silica gel flash chromatography with 1-octadecanal eluting in petroleum ether/EtOAc 20:1 (v/v) as white solid (4.86 g, 18.1 mmol, 98%). $R_f = 0.78$ (PE/EA, 2/1, v/v); IR (film) 2953, 2913, 2848, 1711, 1275 cm^{-1} ; ^1H NMR (500 MHz, CDCl_3) δ 9.75 (t, $J = 1.9$ Hz, 1H, H-1), 2.40 (dt, $J_{2a,2b} = 14.7$ Hz, $J_{2,3} = 7.4$ Hz, 2H, H-2), 1.61 (pentet, $J_{\alpha,\beta} = J_{\beta,\gamma} = 7.3$ Hz, 2H, H-3), 1.32–1.24 (m, 28H, H-4–H-17), 0.86 (t, $J = 7.2$ Hz, 3H, H-18); ^{13}C NMR (125 MHz, CDCl_3) δ 202.9 (C-1), 43.9 (C-2), 31.9 (C-3), 29.70, 29.69, 29.68, 29.66, 29.63, 29.60, 29.57, 29.42, 29.36, 29.35, 29.2, 22.7, 22.1 (C-4–C-17), 14.1 (C-18).



1-Eicosanal (6d). 1-Eicosanol (5.00 g, 16.7 mmol) was added to a solution of PCC (7.22 g, 33.5 mmol) and molecular sieves 4\AA (5.0 g) in DCM (80 mL) under nitrogen and the reaction mixture stirred

at r.t. for 6 h. The reaction mixture was then reduced *in vacuo* and the resulting oil subjected to silica gel flash chromatography with 1-eicosanal eluting in petroleum ether/EtOAc, 20:1 (v/v) as white solid (4.45 g, 15.0 mmol, 90%). $R_f = 0.82$ (PE/EA, 2/1, v/v); IR (film): 2913, 2849, 1711, 1264 cm^{-1} ; ^1H NMR (500 MHz, CDCl_3) δ 9.78 (t, $J = 1.9$ Hz, 1H, H-1), 2.43 (dt, $J_{2a,2b} = 14.8$ Hz, $J_{2,3} = 7.4$ Hz, 2H, H-2), 1.64 (pentet, $J_{\alpha,\beta} = J_{\beta,\gamma} = 7.5$ Hz, 2H, H-3), 1.30–1.25 (m, 32H, H-4–H-19), 0.89 (t, $J = 7.1$

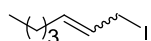
Hz, 3H, H-20); ^{13}C NMR (125 MHz, CDCl_3) δ 203.0 (C-1), 43.9 (C-2), 31.9 (C-3), 29.70, 29.68, 29.68, 29.66, 29.63, 29.58, 29.42, 29.36, 29.35, 29.2, 22.7, 22.1 (C-4–C-19), 14.1 (C-20).



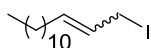
1-Docosanal (6e). 1-Docosanol (2.00 g, 6.12 mmol) was added to a solution of PCC (2.64 g, 12.2 mmol) and molecular sieves 4Å (1.84 g) in DCM (40 mL) under nitrogen and the reaction mixture stirred at r.t. for 6 h. The reaction mixture was then reduced *in vacuo* and the resulting oil subjected to silica gel flash chromatography with 1-docosanal eluting in petroleum ether/EtOAc, 20:1 (v/v) as white solid (1.75 g, 5.38 mmol, 88%). R_f = 0.66 (PE/EA, 2/1, v/v); IR (film): 2913, 2849, 1711, 1264 cm^{-1} ; ^1H NMR (500 MHz, CDCl_3) δ 9.77 (t, J = 1.7 Hz, 1H, H-1), 2.43 (dt, $J_{2a,2b}$ = 13.2 Hz, $J_{2,3}$ = 7.6 Hz, 2H, H-2), 1.63 (pentet, $J_{\alpha,\beta}$ = $J_{\beta,\gamma}$ = 7.1 Hz, 2H, H-3), 1.30–1.26 (m, 36H, H-4–H-21), 0.89 (t, J = 6.8 Hz, 3H, H-22); ^{13}C NMR (125 MHz, CDCl_3) δ 203.0 (C-1), 44.0 (C-2), 31.8 (C-3), 29.70, 29.69, 29.68, 29.66, 29.63, 29.60, 29.42, 29.38, 29.35, 29.2, 22.7, 22.1 (C-4–C-21), 14.1 (C-22).

General procedure for the synthesis of allylic iodides. To a solution of aldehyde (10 mmol, 1.0 equiv.) in dry THF (50 mL), 1M vinyl magnesium bromide in THF (15 mmol, 1.5 equiv.) was added dropwise at 0 °C. For the longer lipids (**4c-4e**), freshly prepared vinyl magnesium bromide was used and the aldehyde was dissolved in toluene instead of THF. The mixture was stirred for 90 min at 0 °C, followed by 1 h at room temperature. The reaction was then quenched by the addition of NH_4Cl , extracted with Et_2O (3 x 50 mL) and the combined organic layers were dried over MgSO_4 , filtered and concentrated *in vacuo* to give the allylic alcohol as a viscous oil which was used without further purification. To a solution of PPh_3 (15 mmol, 1.5 equiv.) in CH_2Cl_2 (15 mL) was added iodine (15 mmol, 1.5 equiv.) and the mixture stirred at room temperature for 5 min. A solution of the crude allylic alcohol (10 mmol, 1 equiv.) in CH_2Cl_2 (30 mL) was then added and the mixture stirred at r.t. overnight. The reaction mixture was diluted with Et_2O and successively washed with sat. $\text{Na}_2\text{S}_2\text{O}_3$, (3 x until the organic layer was free of iodine), water, saturated sodium

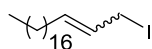
bicarbonate solution and brine. The organic layer was dried over MgSO_4 , filtered and concentrated *in vacuo*. Purification of the crude residue by silica gel flash column chromatography (petroleum ether) afforded the allylic iodide.



1-Iodo-hept-2-ene (4a). By subjecting pentanal (10.0 g, 116 mmol) to the general procedure for the synthesis of allylic iodides, iodoheptene **4a** was obtained (eluting in petroleum ether) as a pale yellow oil (23.0 g, 103 mmol, 89% over 2 steps). $R_f = 0.71$ (PE/EA, 2/1, v/v); IR (film): 2956, 2928, 2859, 1458, 970, 741 cm^{-1} ; ^1H NMR (500 MHz, CDCl_3) δ 5.74–5.71 (m, 2H, H-2 and H-3), 3.91–3.88 (m, 2H, H-1a and H-1b), 2.04 (dd, $J_{3,4} = J_{4,5} = 7.0$ Hz, 2H, H-4a and H-4b), 1.39–1.24 (m, 4H, H-5 and H-6), 0.82 (t, $J_{6,7} = 7.2$ Hz, 3H, CH_3 -7); ^{13}C NMR (125 MHz, CDCl_3) δ 135.3 (C-2), 127.8 (C-3), 31.7, 30.9, 22.1 (C-4–C-6), 13.9 (C-7), 7.1 (C-1); HRMS(APCI) m/z calcd. for $[\text{C}_7\text{H}_{13}\text{I}+\text{H}]^+$: 225.0140, obsd.: 225.0149.

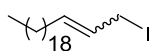


1-Iodo-tetradec-2-ene (4b). By subjecting dodecanal (1.00 g, 5.42 mmol) to the general procedure for the synthesis of allylic iodides, iodo-tetradecene **4b** was obtained (eluting in petroleum ether) as a pale yellow oil (1.40 g, 4.39 mmol, 81% over 2 steps). $R_f = 0.76$ (PE/EA, 2/1, v/v); IR (film): 2955, 2922, 2852, 1464, 961, 721 cm^{-1} ; ^1H NMR (500 MHz, CDCl_3) δ 5.73–5.71 (m, 2H, H-2 and H-3), 3.90–3.88 (m, 2H, H-1a and H-1b), 2.03 (dd, $J_{3,4} = J_{4,5} = 7.2$ Hz, 2H, H-4a and H-4b), 1.39–1.27 (m, 18H, H-5–H-13), 0.82 (t, $J_{6,7} = 7.2$ Hz, 3H, CH_3 -14); ^{13}C NMR (125 MHz, CDCl_3) δ 135.4 (C-2), 127.7 (C-3), 32.0, 31.9, 29.65, 29.62, 29.60, 29.4, 29.3, 29.1, 28.8, 22.7, 31.7 (C-4–C-13), 14.1 (C-14), 7.1 (C-1); HRMS(APCI) m/z calcd. for $[\text{C}_{14}\text{H}_{27}\text{I}+\text{H}]^+$: 323.1236, obsd.: 323.1242.

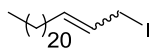


1-Iodo-eicos-2-ene (4c). By subjecting octadecanal (1.69 g, 6.32 mmol) to the general procedure for the synthesis of allylic iodides, iodo-eicosene **4c** was obtained (eluting in petroleum ether) as a white solid (1.80 g, 4.42 mmol, 70% over 2 steps). $R_f = 0.86$ (PE/EA, 2/1, v/v); IR

(film): 2924, 2853, 1464, 961, 738 cm^{-1} ; ^1H NMR (500 MHz, CDCl_3) δ 5.73–5.71 (m, 2H, H-2 and H-3), 3.89–3.87 (m, 2H, H-1a and H-1b), 2.03 (dd, $J_{3,4} = J_{4,5} = 7.2$ Hz, 2H, H-4a and H-4b), 1.44–1.22 (m, 30H, H-5–H-19), 0.89 (t, $J_{6,7} = 6.7$ Hz, 3H, CH_3 -20); ^{13}C NMR (125 MHz, CDCl_3) δ 135.4 (C-2), 127.8 (C-3), 32.1, 31.9, 29.71, 29.68, 29.67, 29.65, 29.6, 29.50, 29.45, 29.3, 29.1, 28.8, 28.7, 26.8, 22.7 (C-4–C-19), 14.1 (C-20), 7.1 (C-1); HRMS(APCI) m/z calcd. for $[\text{C}_{20}\text{H}_{39}\text{I}+\text{H}]^+$: 407.2175, obsd.: 407.2168.

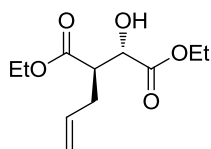


1-Iodo-docos-2-ene (4d). By subjecting eicosanal (1.20 g, 4.05 mmol) to the general procedure for the synthesis of allylic iodides, iodo-docosene **4d** was obtained (eluting in petroleum ether) as a white solid (1.25 g, 2.87 mmol, 71 % over 2 steps). $R_f = 0.95$ (PE/EA, 2/1, v/v); IR (film): 2922, 2852, 1464, 962, 740 cm^{-1} ; ^1H NMR (500 MHz, CDCl_3) δ 5.72–5.71 (m, 2H, H-2 and H-3), 3.88–3.87 (m, 2H, H-1a and H-1b), 2.01 (dd, $J_{3,4} = J_{4,5} = 7.2$ Hz, 2H, H-4a and H-4b), 1.43–1.28 (m, 34H, H-5–H-21), 0.89 (t, $J_{6,7} = 6.7$ Hz, 3H, CH_3 -22); ^{13}C NMR (125 MHz, CDCl_3) δ 135.4 (C-2), 127.7 (C-3), 32.0, 31.9, 29.70, 29.67, 29.66, 29.65, 29.58, 29.5, 29.44, 29.36, 29.3, 29.1, 28.8, 28.7, 26.9, 26.8, 22.7 (C-4–C-21), 14.1 (C-22), 7.1 (C-1); HRMS(APCI) m/z calcd. for $[\text{C}_{22}\text{H}_{43}\text{I}+\text{H}]^+$: 435.2488, obsd.: 435.2494.



1-Iodo-tetracos-2-ene (4e). By subjecting docosanal (3.50 g, 10.8 mmol) to the general procedure for the synthesis of allylic iodides, iodo-tetracosene **4e** was obtained (eluting in petroleum ether) as a white solid (3.63 g, 7.87 mmol, 73.2 % over 2 steps). $R_f = 0.95$ (PE/EA, 2/1, v/v); IR (film): 2915, 2848, 1713, 1471, 739 cm^{-1} ; ^1H NMR (500 MHz, CDCl_3) δ 5.73–5.71 (m, 2H, H-2 and H-3), 3.89–3.88 (m, 2H, H-1a and H-1b), 2.02 (dd, $J_{3,4} = J_{4,5} = 6.8$ Hz, 2H, H-4a and H-4b), 1.37–1.26 (m, 38H, H-5–H-23), 0.89 (t, $J_{6,7} = 7.1$ Hz, 3H, CH_3 -24); ^{13}C NMR (125 MHz, CDCl_3) δ 135.4 (C-2), 127.8 (C-3), 32.0, 31.9, 29.72, 29.69, 29.68, 29.67, 29.60, 29.47, 29.39, 29.1, 28.8, 22.7 (C-4–C-23), 14.1 (C-24), 7.1 (C-1); HRMS(APCI) m/z calcd. for $[\text{C}_{24}\text{H}_{47}\text{I}+\text{H}]^+$: 463.2801, obsd.: 463.2805.

General Procedure for the Synthesis of α -Alkenyl- β -Hydroxy-Diesters. *n*-BuLi (11.5 mL, 2.0M solution in hexanes, 23.0 mmol) was added to a solution of diisopropylamine (3.6 mL, 25.0 mmol) in THF (20 mL) at 0 °C. After 30 min the solution was cooled down to -78 °C and diethyl malate **5** (1.9 g, 10 mmol) in THF (20 mL) was added. The resulting mixture was stirred at -78 °C for 1 h, warmed up to -20 °C over 1 h and stirred at -20 °C for 20 min. After cooling down to -78 °C, the allylic iodide (15.0 mmol, 1.5 equiv.) in THF (20 mL) was added dropwise and the reaction was stirred for 1 h at -50 °C and then a further 1h during which time the temperature increased to -20 °C. The reaction was then quenched using NH₄Cl (sat.) and extracted with EtOAc (3 x 100 mL). The combined organic layers were washed with water (30 mL) and brine (30 mL), dried (MgSO₄), filtered and the solvent removed *in vacuo* to yield a crude yellow oil. Purification of the residue, using silica gel flash column chromatography and the eluent given, gave the alkylated product.



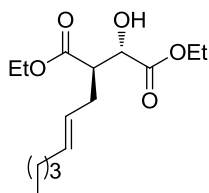
Ethyl 2*S*-hydroxy-3*R*-ethoxycarbonyl-hex-4-enoate (*anti*-5a**).**

By reacting diethyl malate **3** (3.96 g, 20.9 mmol) with allyl bromide (2.64 mL, 31.25 mmol) according to the general procedure for the synthesis of α -alkenyl- β -hydroxy-diesters,

hexenoate **5a** was obtained (eluting in 10:1, petroleum

ether/EtOAc, v/v) as a colourless oil (2.72 g, 11.9 mmol, 57%, *anti:syn* = 14:1). R_f = 0.39 (PE/EA, 2/1, v/v); $[\alpha]_D^{23}$ = +13.0 (c = 2.23, CHCl₃), Lit^[34] $[\alpha]_D^{20}$ = +11.3 (c = 2.23, CHCl₃); IR (film): 3079, 2982, 2961, 2924, 1733, 1643, 1132, 1099, 1031, 736, 703 cm⁻¹; ¹H NMR (500 MHz, CDCl₃) δ 5.75 (ddt, $J_{5,6\text{-trans}}$ = 17.1 Hz, $J_{5,6\text{-cis}}$ = 10.2 Hz, $J_{4a,5}$ = $J_{4b,5}$ = 7.5 Hz, 1H, H-5), 5.11 (d, $J_{5,6\text{-trans}}$ = 17.1 Hz, 1H, H-6-*cis*), 5.05 (d, $J_{5,6\text{-trans}}$ = 10.2 Hz, 1H, H-6-*trans*), 4.25–4.14 (m, 3H, CH₂O-1 and H-2), 4.12–4.01 (m, 2H, 3-CO₂CH₂), 2.90 (m, 1H, H-3), 2.56 (ddd, $J_{4a,4b}$ = 14.4 Hz, $J_{3,4a}$ = $J_{4a,5}$ = 7.5 Hz, 1H, H-4a), 2.38 (ddd, $J_{4a,4b}$ = 14.4 Hz, $J_{3,4b}$ = $J_{4b,5}$ = 7.5 Hz, 1H, H-4b), 1.24 (t, J = 7.1 Hz, 3H, CH₃, 1-OEt), 1.17 (t, J = 7.0 Hz, 3H, CH₃, 3-CO₂Et); ¹³C NMR (125 MHz, CDCl₃) δ 173.6 (C-1), 172.0 (3-CO₂), 134.9 (C-5), 118.0 (C-6), 70.2 (C-2), 61.9 (CH₂O, 1-OEt), 61.0 (CH₂O, 3-CO₂CH₂), 48.1 (C-3), 32.2 (C-4), 14.1 (CH₃, 1-

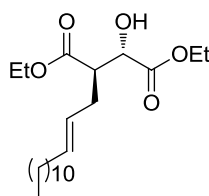
OEt), 14.1 (CH₃, 3-CO₂Et); HRMS(ESI) m/z calcd. for [C₁₁H₁₈O₅+Na]⁺: 253.1052, obsd.: 253.1055.



Ethyl 2*S*-hydroxy-3*R*-ethoxycarbonyl-decen-5-enoate (*anti*-

5c). By reacting diethyl malate **3** (3.98 g, 20.9 mmol) with 1-iodo-hept-2-ene (7.03 g, 31.42 mmol) according to the general procedure for the synthesis of α -alkenyl- β -hydroxy-diester, decenoate **5c** was obtained (eluting in 15:1, petroleum ether/EtOAc, v/v) as a colourless oil (3.28 g, 11.5 mmol, 55%,

anti:syn = 9:1). R_f = 0.42 (PE/EA 2/1, v/v); $[\alpha]_D^{23}$ = +19.0 (c = 1.0, CHCl₃); IR (film): 3234, 2983, 2960, 2929, 1734, 1466, 1374, 1198, 861, 736 cm⁻¹; ¹H NMR (500 MHz, CDCl₃) δ 5.57 (dt, $J_{5,6}$ = 15.2 Hz, $J_{6,7a} = J_{6,7b}$ = 6.6 Hz, 1H, H-6), 5.39 (dt, $J_{5,6}$ = 15.2 Hz, $J_{4a,5} = J_{4b,5}$ = 6.2 Hz, 1H, H-5), 4.21–4.31 (m, 3H, CH₂O-1 and H-2), 4.16–4.10 (m, 2H, 3-CO₂CH₂), 2.90 (ddd, $J_{3,4a} = J_{3,4b}$ = 8.3 Hz, $J_{2,3}$ = 3.2 Hz, 1H, H-3), 2.58–2.51 (m, 1H, H-4a), 2.38 (ddd, $J_{4a,4b}$ = 14.4 Hz, $J_{3,4b}$ = 8.3 Hz, $J_{4b,5}$ = 6.2 Hz, 1H, H-4b), 2.01 (q, $J_{6,7} = J_{7,8}$ = 6.6 Hz, 2H, H-7), 1.33–1.27 (m, 4H, H-8 and H-9), 1.24 (t, J = 7.3 Hz, 6H, 2 x CH₃), 0.88 (t, J = 7.2 Hz, 3H, CH₃-10); ¹³C NMR (125 MHz, CDCl₃) δ 173.7 (C-1), 172.3 (3-CO₂), 134.4 (C-6), 125.9 (C-5), 70.2 (C-2), 61.8 (CH₂O, 1-OEt), 60.8 (CH₂O, 3-CO₂CH₂), 48.6 (C-3), 32.2 (C-4), 31.5 (C-7), 31.1 (C-8), 22.2 (C-9), 14.2 (C-10), 14.1 (CH₃, 1-OEt), 14.1 (CH₃, 3-CO₂Et); HRMS(ESI) m/z calcd. for [C₁₅H₂₆O₅+Na]⁺: 309.1678, obsd.: 309.1682.



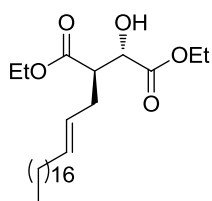
Ethyl 2*S*-hydroxy-3*R*-ethoxycarbonyl-heptadecen-5-enoate (*anti*-

5e). By reacting diethyl malate **3** (3.99 g, 21.0 mmol) with 1-iodo-tetradec-2-ene (10.14 g, 31.5 mmol) according to the general procedure for the synthesis of α -alkenyl- β -hydroxy-diester, heptadecenoate **5e** was obtained (eluting in 15:1,

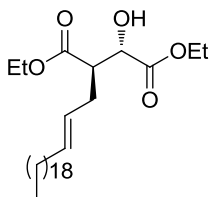
petroleum ether/EtOAc, v/v) as a colourless oil (4.11 g, 10.7 mmol, 51%, *anti:syn* = 10:1). R_f = 0.5 (PE/EA, 2/1, v/v); $[\alpha]_D^{23}$ = +2.0 (c = 0.1, CHCl₃); IR (film): 3522, 2924, 2853, 1737, 1219, 1032, 772 cm⁻¹; ¹H NMR (500 MHz, CDCl₃) δ 5.57 (dt, $J_{5,6}$ = 15.1 Hz, $J_{6,7a} = J_{6,7b}$ = 7.1 Hz, 1H, H-6), 5.39 (dt, $J_{5,6}$ = 15.1 Hz, $J_{4a,5} = J_{4b,5}$ = 7.2

Hz, 1H, H-5), 4.31–4.20 (m, 3H, CH₂O-1 and H-2), 4.16–4.11 (m, 2H, 3-CO₂CH₂), 3.18 (d, $J_{2,\text{OH}} = 7.4\text{ Hz}$, 1H, OH), 2.90 (ddd, $J_{3,4a} = J_{3,4b} = 8.3\text{ Hz}$, $J_{2,3} = 3.2\text{ Hz}$, 1H, H-3), 2.58–2.51 (m, 1H, H-4a), 2.38 (ddd, $J_{4a,4b} = 14.2\text{ Hz}$, $J_{3,4b} = 8.3\text{ Hz}$, $J_{4b,5} = 7.2\text{ Hz}$, 1H, H-4b), 1.99 (q, $J_{6,7} = J_{7,8} = 7.1\text{ Hz}$, 2H, H-7), 1.32–1.23 (m, 24H, H-8–H-16 and 2 x CH₃), 0.88 (t, $J = 7.2\text{ Hz}$, 3H, CH₃-17); ¹³C NMR (125 MHz, CDCl₃) δ 173.7 (C-1), 172.3 (3-CO₂), 134.4 (C-6), 125.8 (C-5), 70.2 (C-2), 61.8 (CH₂O, 1-OEt), 60.8 (CH₂O, 3-CO₂CH₂), 48.6 (C-3), 32.2 (C-4), 31.9 (C-7), 31.1 (C-8), 29.7, 29.6, 29.6, 29.5, 29.4, 29.3, 29.2, 22.68 (C-9–C-16), 14.1 (C-17), 14.1 (CH₃, 1-OEt), 14.1 (CH₃, 3-CO₂Et); HRMS(ESI) m/z calcd. for [C₂₂H₄₀O₅+Na]⁺: 407.2773, obsd.: 407.2781.

Ethyl 2S-hydroxy-3R-ethoxycarbonyl-tricos-5-enoate (*anti*-5g). By reacting



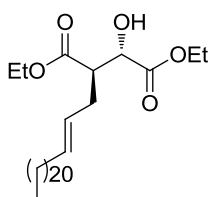
diethyl malate **3** (0.40 g, 2.11 mmol) with 1-iodo-eicos-2-ene (1.28 g, 3.16 mmol) according to the general procedure for the synthesis of α-alkenyl-β-hydroxy-diester, tricosenoate **5g** was obtained (eluting in 15:1, petroleum ether/EtOAc, v/v) as white solid (0.48 g, 1.03 mmol, 49%, *anti:syn* = 9:1). R_f = 0.49 (PE/EA, 2/1, v/v); $[\alpha]_D^{22} = +6.0$ ($c = 1.0$, CHCl₃); IR (film): 3517, 2924, 2853, 1737, 1219, 1032, 970, 772, cm⁻¹; ¹H NMR (500 MHz, CDCl₃) δ 5.56 (dt, $J_{5,6} = 15.4\text{ Hz}$, $J_{6,7a} = J_{6,7b} = 6.6\text{ Hz}$, 1H, H-6), 5.40 (dt, $J_{5,6} = 15.4\text{ Hz}$, $J_{4a,5} = J_{4b,5} = 6.6\text{ Hz}$, 1H, H-5), 4.31–4.22 (m, 3H, CH₂O-1 and H-2), 4.19–4.10 (m, 2H, 3-CO₂CH₂), 2.90 (ddd, $J_{3,4a} = J_{3,4b} = 8.8\text{ Hz}$, $J_{2,3} = 3.2\text{ Hz}$, 1H, H-3), 2.58–2.52 (m, 1H, H-4a), 2.38 (ddd, $J_{4a,4b} = 15.2\text{ Hz}$, $J_{3,4b} = J_{4b,5} = 8.8\text{ Hz}$, 1H, H-4b), 1.99 (q, $J_{6,7} = J_{7,8} = 7.1\text{ Hz}$, 2H, H-7a,b), 1.32–1.23 (m, 36H, H-8–H-22 and 2 x CH₃), 0.89 (t, $J = 7.1\text{ Hz}$, 3H, CH₃-23); ¹³C NMR (125 MHz, CDCl₃) δ 173.7 (C-1), 172.3 (3-CO₂), 134.4 (C-6), 125.8 (C-5), 70.2 (C-2), 61.8 (CH₂O, 1-OEt), 60.8 (CH₂O, 3-CO₂CH₂), 48.6 (C-3), 32.2 (C-4), 31.9 (C-7), 31.1 (C-8), 29.7, 29.66, 29.65, 29.5, 29.4, 29.2, 22.7 (C-9–C-22), 14.1 (C-23), 14.1 (CH₃, 1-OEt), 14.1 (CH₃, 3-CO₂Et); HRMS(ESI) m/z calcd. for [C₂₈H₅₂O₅+Na]⁺: 491.3712, obsd.: 491.3706.



Ethyl 2*S*-hydroxy-3*R*-ethoxycarbonyl-pentacos-5-enoate

(*anti*-**5i**). By reacting diethyl malate **3** (0.53 g, 2.77 mmol) with 1-iodo-docos-2-ene (1.80 g, 4.15 mmol) according to the general procedure for the synthesis of α -alkenyl- β -hydroxydiesters, pentacosenoate **5i** was obtained (eluting in 15:1, petroleum ether/EtOAc, v/v) as a white solid (0.57 g, 1.16

mmol, 42%, *anti:syn* = 9:1). R_f = 0.6 (PE/EA, 2/1, v/v); $[\alpha]_D^{23}$ = +5.0 (c = 1.0, CHCl₃); IR (film): 3523, 2916, 2849, 1735, 1373, 1213, 1032, 969, 756 cm⁻¹; ¹H NMR (500 MHz, CDCl₃) δ 5.56 (dt, $J_{5,6}$ = 15.1 Hz, $J_{6,7a} = J_{6,7b}$ = 6.9 Hz, 1H, H-6), 5.39 (dt, $J_{5,6}$ = 15.1 Hz, $J_{4a,5} = J_{4b,5}$ = 7.3 Hz, 1H, H-5), 4.30–4.22 (m, 3H, CH₂O-1 and H-2), 4.14–4.10 (m, 2H, 3-CO₂CH₂), 3.18 (d, $J_{2,OH}$ = 6.9 Hz, 1H, OH), 2.90 (ddd, $J_{3,4a} = J_{3,4b}$ = 8.3 Hz, $J_{2,3}$ = 3.2 Hz, 1H, H-3), 2.58–2.52 (m, 1H, H-4a), 2.38 (ddd, $J_{4a,4b}$ = 14.1 Hz, $J_{3,4a} = J_{4a,5}$ = 7.8 Hz, 1H, H-4b), 1.99 (q, $J_{6,7} = J_{7,8}$ = 7.1 Hz, 2H, H-7a,b), 1.33–1.21 (m, 40H, H-8–H-24 and 2 x CH₃), 0.88 (t, J = 7.2 Hz, 3H, CH₃-25); ¹³C NMR (125 MHz, CDCl₃) δ 173.7 (C-1), 172.3 (3-CO₂), 134.5 (C-6), 125.8 (C-5), 70.2 (C-2), 61.8 (CH₂O, 1-OEt), 60.8 (CH₂O, 3-CO₂CH₂), 48.6 (C-3), 32.5 (C-4), 31.9 (C-7), 31.1 (C-8), 29.7, 29.66, 29.64, 29.5, 29.4, 29.2, 22.7 (C-9–C-24), 14.1 (C-25), 14.1 (CH₃, 1-OEt), 14.1 (CH₃, 3-CO₂Et); HRMS(ESI) m/z calcd. for [C₃₀H₅₆O₅+Na]⁺: 519.4025, obsd.: 519.4029.



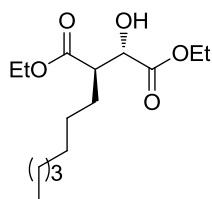
Ethyl 2*S*-hydroxy-3*R*-ethoxycarbonyl-heptacos-5-enoate

(*anti*-**5j**). By reacting diethyl malate **3** (0.55 g, 2.89 mmol) with 1-iodo-tetracos-2-ene (2.0 g, 4.32 mmol) according to the general procedure for the synthesis of α -alkenyl- β -hydroxydiesters, heptacosenoate **5j** was obtained (eluting in 15:1, petroleum ether/EtOAc, v/v) as a white solid (0.62 g, 1.19 mmol, 41%, *anti:syn* =

9:1). R_f = 0.58 (PE/EA, 2/1, v/v); $[\alpha]_D^{22}$ = +0.1 (c = 1.0, CHCl₃); IR (film): 3351, 2922, 2852, 1736, 1376, 1215, 1042, 756 cm⁻¹; ¹H NMR (500 MHz, CDCl₃) δ 5.56 (dt, $J_{5,6}$ = 15.1 Hz, $J_{6,7a} = J_{6,7b}$ = 6.8 Hz, 1H, H-6), 5.39 (dt, $J_{5,6}$ = 15.1 Hz, $J_{4a,5} = J_{4b,5}$ = 6.5 Hz, 1H, H-5), 4.30–4.22 (m, 3H, CH₂O-1 and H-2), 4.16–4.12 (m, 2H, 3-CO₂CH₂), 3.17 (d, $J_{2,OH}$ = 7.3 Hz, 1H, OH), 2.91 (ddd, $J_{3,4a} = J_{3,4b}$ = 9.1 Hz, $J_{2,3}$ = 3.2 Hz, 1H, H-3), 2.58–2.53 (m, 1H, H-4a), 2.38 (ddd, $J_{4a,4b}$ = 14.3 Hz, $J_{3,4a} = J_{4a,5}$ = 8.3

Hz, 1H, H-4b), 2.00 (q, $J_{6,7} = J_{7,8} = 7.1$ Hz, 2H, H-7a,b), 1.37–1.18 (m, 44H, H-8–H-26 and 2 x CH₃), 0.88 (t, $J = 6.8$ Hz, 3H, CH₃-27); ¹³C NMR (125 MHz, CDCl₃) δ 173.7 (C-1), 172.3 (3-CO₂), 134.5 (C-6), 125.8 (C-5), 70.2 (C-2), 61.8 (CH₂O, 1-OEt), 60.8 (CH₂O, 3-CO₂CH₂), 48.6 (C-3), 32.6 (C-4), 31.9 (C-7), 31.1 (C-8), 29.71, 29.67, 29.65, 29.58, 29.52, 29.37, 29.2, 22.7 (C-9–C-26), 14.2 (C-27), 14.1 (CH₃, 1-OEt), 14.1 (CH₃, 3-CO₂Et); HRMS(ESI) m/z calcd. for [C₃₂H₆₀O₅+Na]⁺: 547.4338, obsd.: 547.4344.

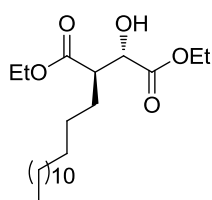
General Procedure for the Synthesis of α -Alkyl- β -Hydroxy-Diesters. *n*-BuLi (11.5 mL, 2.0M solution in hexanes, 23.0 mmol) was added to a solution of diisopropylamine (3.6 mL, 25.0 mmol) in THF (20 mL) at 0 °C. After 30 min, the solution was cooled down to -78 °C and diethyl malate **5** (1.9 g, 10 mmol) in THF (20 mL) was added. The resulting mixture was stirred at -78 °C for 1 h, warmed up to -20 °C over 1 h and stirred at -20 °C for 20 min. After cooling down to -78 °C, the alkyl iodide (15.0 mmol, 1 equiv.) in THF (20 mL) was added dropwise and the reaction stirred for 2 h, during which time the temperature increased to 0 °C and stirred for a further 2 h at room temperature. The reaction was quenched using NH₄Cl (sat.) and extracted with EtOAc (3 x 100 mL). The combined organic layers were washed with water (30 mL) and brine (30 mL), dried (MgSO₄), filtered and the solvent removed *in vacuo* to yield a crude yellow oil. Purification of the residue using silica gel flash column chromatography and the eluent given, gave the alkylated product.



Ethyl 2*S*-hydroxy-3*R*-ethoxycarbonyl-decanoate (*anti*-5b**).**

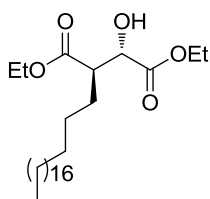
By reacting diethyl malate **3** (3.97 g, 20.9 mmol) with 1-iodoheptane (5.13 mL, 31.3 mmol) according to the general procedure for the synthesis of α -alkylated- β -hydroxy-diesters, decanoate **5b** was obtained (eluting in 15:1, petroleum ether/EtOAc, v/v) as a colourless oil (2.24 g, 7.4 mmol, 37%, *anti:syn* = 7:1). R_f = 0.73 (PE/EA, 2/1, v/v); $[\alpha]_D^{23} = +0.7$ ($c = 0.1$, CHCl₃); IR (film): 3522, 3020, 2928, 2857, 1734, 1376, 1216, 1027, 754, 668 cm⁻¹; ¹H NMR (500 MHz, CDCl₃) δ 4.31–4.21 (m, 3H, CH₂O-1 and H-2), 4.14 (q, $J_{a,b} = 7.1$ Hz, 2H, CH₂O, 3-CO₂CH₂), 3.19

(d, $J_{2,\text{OH}} = 7.6$ Hz, 1H, OH), 2.84 (td, $J_{3,4} = 7.1$ Hz, $J_{2,3} = 3.7$ Hz, 1H, H-3), 1.85 (ddt, $J_{4a,4b} = 14.9$ Hz, $J_{3,4a} = J_{4a,5a} = J_{4a,5b} = 7.1$ Hz, 1H, H-4a), 1.66 (ddt, $J_{4a,4b} = 14.9$ Hz, $J_{3,4b} = J_{4b,5a} = J_{4b,5b} = 6.8$ Hz, 1H, H-4b), 1.40–1.24 (m, 16H, H-5–H-9, 2 x CH₃), 0.89 (t, $J = 6.9$ Hz, 3H, CH₃-10); ¹³C NMR (125 MHz, CDCl₃) δ 173.5 (C-1), 172.9 (3-CO₂), 71.1 (C-2), 61.8 (CH₂O, 1-OEt), 60.8 (CH₂O, 3-CO₂CH₂), 48.6 (C-3), 31.8 (C-4), 29.4, 29.1, 28.1, 27.4, 22.6 (C-5–C-9), 14.1 (C-10), 14.1 (CH₃, 1-OEt), 14.1 (CH₃, 3-CO₂Et); HRMS(ESI) m/z calcd. For [C₁₅H₂₈O₅+Na]⁺: 311.1833, obsd.: 311.1834.



Ethyl 2S-hydroxy-3R-ethoxycarbonyl-heptadecanoate (*anti*-5d).

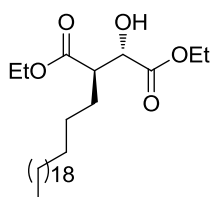
By reacting diethyl malate **3** (0.44 g, 2.35 mmol) with 1-iodotetradecane (1.14 g, 3.53 mmol) according to the general procedure for the synthesis of α -alkyl- β -hydroxy-diester, heptadecanoate **5d** was obtained (eluting in 15:1, petroleum ether/EtOAc, v/v) as a colourless oil (0.14 g, 0.34 mmol, 15%, *anti:syn* = 9:1). R_f = 0.66 (PE/EA, 2/1, v/v); $[\alpha]_D^{23} = +4.0$ ($c = 1.0$, CHCl₃); IR (film): 3514, 3020, 2925, 2584, 1735, 1466, 1369, 1216, 755 cm⁻¹; ¹H NMR (500 MHz, CDCl₃) δ 4.29–4.22 (m, 3H, CH₂O-1 and H-2), 4.14 (q, $J = 7.1$ Hz, 2H, 3-CO₂CH₃), 3.19 (d, $J_{2,\text{OH}} = 7.6$ Hz, 1H, OH), 2.84 (td, $J_{3,4} = 7.3$ Hz, $J_{2,3} = 3.5$ Hz, 1H, H-3), 1.83 (ddt, $J_{4a,4b} = 14.0$ Hz, $J_{3,4a} = J_{4a,5a} = J_{4a,5b} = 7.0$ Hz, 1H, H-4a), 1.66 (ddt, $J_{4a,4b} = 14.0$ Hz, $J_{3,4b} = J_{4b,5a} = J_{4b,5b} = 7.2$ Hz, 1H, H-4b), 1.45–1.21 (m, 30H, H-5–H-16 and 2 x CH₃), 0.87 (t, $J = 6.4$ Hz, 3H, CH₃-17); ¹³C NMR (125 MHz, CDCl₃) δ 173.5 (C-1), 172.9 (3-CO₂), 71.1 (C-2), 61.8 (CH₂O, 1-OEt), 60.8 (CH₂O, 3-CO₂CH₂), 48.6 (C-3), 31.9 (C-4), 29.7, 29.7, 29.7, 29.7, 29.6, 29.6, 29.4, 28.1, 27.4, 22.7 (C-5–C-16), 14.2 (C-17), 14.1 (CH₃, 1-OEt), 14.1 (CH₃, 3-CO₂Et); HRMS(ESI) m/z calcd. For [C₂₂H₄₂O₅+Na]⁺: 409.2931, obsd.: 409.2928.



Ethyl 2S-hydroxy-3R-ethoxycarbonyl-tricosanoate (*anti*-5f).

By reacting diethyl malate **3** (0.22 g, 1.15 mmol) with 1-iodoeicosane (0.70 g, 1.72 mmol) according to the general procedure for the synthesis of α -alkyl- β -hydroxy-diester,

tricosanoate **5f** was obtained (eluting in 15:1, petroleum ether/EtOAc, v/v) as a white solid (0.02 g, 0.05 mmol, 4%, *anti:syn* = 6:1). R_f = 0.58 (PE/EA, 2/1, v/v); $[\alpha]_D^{22}$ = +2.0 (c = 1.0, CHCl₃); IR (film): 2918, 2850, 1737, 1252, 1182, 1099, 1030, 837, 757, cm⁻¹; ¹H NMR (500 MHz, CDCl₃) δ 4.31–4.21 (m, 3H, CH₂O-1 and H-2), 4.18–4.12 (m, 2H, 3-CO₂CH₃), 3.20 (d, $J_{2,OH}$ = 7.8 Hz, 1H, OH), 2.84 (td, $J_{3,4}$ = 7.2 Hz, $J_{2,3}$ = 3.7 Hz, 1H, H-3), 1.83 (ddt, $J_{4a,4b}$ = 14.4 Hz, $J_{3,4a}$ = $J_{4a,5a}$ = $J_{4a,5b}$ = 7.8 Hz, 1H, H-4a), 1.66 (ddt, $J_{4a,4b}$ = 14.4 Hz, $J_{3,4b}$ = $J_{4b,5a}$ = $J_{4b,5b}$ = 7.4 Hz, 1H, H-4b), 1.39–1.23 (m, 42H, H-5–H-22 and 2 x CH₃), 0.89 (t, J = 6.9 Hz, 3H, CH₃-23); ¹³C NMR (125 MHz, CDCl₃) δ 173.5 (C-1), 172.9 (3-CO₂), 71.0 (C-2), 61.8 (CH₂O, 1-OEt), 60.8 (CH₂O, 3-CO₂CH₂), 48.6 (C-3), 31.9 (C-4), 29.68, 29.66, 29.64, 29.62, 29.5, 29.4, 29.3, 28.1, 27.4, 22.7 (C-5–C-22), 14.2 (C-23), 14.1 (CH₃, 1-OEt), 14.1 (CH₃, 3-CO₂Et); HRMS(ESI) m/z calcd. For [C₂₈H₅₄O₅+Na]⁺: 493.3869, obsd.: 493.3871.



Ethyl 2S-hydroxy-3R-ethoxycarbonyl-pentacosanoate

(*anti*-**5h**). By reacting diethyl malate **3** (0.22 g, 1.16 mmol) with 1-iododocosane (0.78 g, 1.77 mmol) according to the general procedure for the synthesis of α -alkyl- β -hydroxydiesters, pentacosanoate **5h** was obtained (eluting in 15:1,

petroleum ether/EtOAc, v/v) as a white solid (0.05 g, 0.096 mmol, 8%, *anti:syn* = 6:1). R_f = 0.58 (PE/EA, 2/1, v/v); $[\alpha]_D^{22}$ = +3.0 (c = 0.1, CHCl₃); IR (film) 3473, 2917, 2850, 1737, 1599, 2032, 837, 758, 721, 668 cm⁻¹; ¹H NMR (500 MHz, CDCl₃) δ 4.30–4.21 (m, 3H, CH₂O-1 and H-2), 4.14 (m, 2H, 3-CO₂CH₂), 3.20 (d, $J_{2,OH}$ = 7.6 Hz, 1H, 2-OH), 2.84 (td, $J_{3,4}$ = 7.6 Hz, $J_{2,3}$ = 3.7 Hz, 1H, H-3), 1.83 (ddt, $J_{4a,4b}$ = 14.2 Hz, $J_{3,4a}$ = $J_{4a,5a}$ = $J_{4a,5b}$ = 7.2 Hz, 1H, H-4a), 1.66 (ddt, $J_{4a,4b}$ = 14.2 Hz, $J_{3,4b}$ = $J_{4b,5a}$ = $J_{4b,5b}$ = 7.2 Hz, 1H, H-4b), 1.40–1.24 (m, 46H, H-5–H-24, 2 x CH₃), 0.89 (t, J = 6.6 Hz, 3H, CH₃-25); ¹³C NMR (125 MHz, CDCl₃) δ 173.5 (C-1), 172.9 (3-CO₂), 71.1 (C-2), 61.8 (CH₂O, 1-OEt), 60.8 (CH₂O, 3-CO₂CH₂), 48.6 (C-3), 31.9 (C-4), 29.7, 29.7, 29.6, 29.4, 29.4, 28.1, 27.4, 22.7 (C-5–C-24), 14.2 (C-25), 14.1 (CH₃, 1-OEt), 14.1 (CH₃, 3-CO₂Et); HRMS(ESI) m/z calcd. For [C₃₀H₅₈O₅+Na]⁺: 521.4182, obsd.: 521.4180.

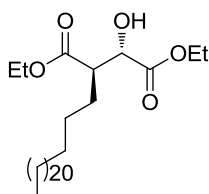
General Procedure for Hydrogenation. Pd/C (5 wt%) was added to a solution of the allylic alkylated diester (1 mmol) in a mixture of CH₂Cl₂/MeOH (10 mL, 1/1, v/v) and stirred at room temperature under H₂ (gas) overnight. The reaction mixture was filtered through Celite, the Celite washed thoroughly with CH₂Cl₂/MeOH (20 mL, 1/1, v/v) and the filtrate concentrated *in vacuo*. The product was purified using silica gel flash chromatography and the eluent given.

Ethyl 2S-hydroxy-3R-ethoxycarbonyl-decanoate (*anti*-5b). By subjecting decenoate (*anti:syn* 9:1) **5c** (2.01 g, 6.99 mmol) to the general procedure for hydrogenation pure *anti*-**5b** was obtained (eluting in 15:1 petroleum ether/EtOAc, v/v) as a colourless oil (1.81 g, 6.29 mmol, 90%). The spectral data matched that reported earlier.

Ethyl 2S-hydroxy-3R-ethoxycarbonyl-heptadecanoate (*anti*-5d). By subjecting heptadecenoate (*anti:syn* 10:1) **5e** (3.60 g, 9.38 mmol) to the general procedure for hydrogenation pure *anti*-**5d** was obtained (eluting in 15:1, petroleum ether/EtOAc, v/v) as a colourless oil (3.29 g, 8.52 mmol, 91%). The spectral data matched that reported earlier.

Ethyl 2S-hydroxy-3R-ethoxycarbonyl-tricosanoate (*anti*-5f). By subjecting tricosenoate (*anti:syn* 9:1) **5g** (0.40g, 0.85 mmol) to the general procedure for hydrogenation pure *anti*-**5f** was obtained (eluting in 15:1, petroleum ether/EtOAc, v/v) as a white solid (0.36 g, 0.77 mmol, 90%). The spectral data matched that reported earlier.

Ethyl 2S-hydroxy-3R-ethoxycarbonyl-pentacosanoate (*anti*-5h). By subjecting pentacosenoate (*anti:syn* 9:1) **5i** (0.80 g, 1.61 mmol) to the general procedure for hydrogenation pure *anti*-**5h** was obtained (eluting in 15:1, petroleum ether/EtOAc, v/v) as a white solid (0.72 g, 1.45 mmol, 90%). The spectral data matched that reported earlier.



Ethyl 2S-hydroxy-3R-ethoxycarbonyl-heptacosanoate (*anti*-5k). By subjecting heptacosenoate (*anti:syn* 9:1) **5j** (0.11 g, 0.21 mmol) to the general procedure for hydrogenation pure

anti-**5k** was obtained (eluting in 15:1, petroleum ether/EtOAc, v/v) as a white solid (0.10 g, 0.19 mmol, 90%). $R_f = 0.54$ (PE/EA, 2/1, v/v); $[\alpha]_D^{22} = +2.0$ ($c = 0.1$, CHCl₃); IR (film) 3473, 2917, 2850, 1737, 1208, 1029, 771, 668 cm⁻¹; ¹H NMR (500 MHz, CDCl₃) δ 4.30–4.20 (m, 3H, CH₂O-1 and H-2), 4.18–4.12 (m, 2H, 3-CO₂CH₂), 3.20 (d, $J_{2,OH} = 7.9$ Hz, 1H, 2-OH), 2.84 (td, $J_{3,4} = 7.3$ Hz, $J_{2,3} = 3.7$ Hz, 1H, H-3), 1.83 (ddt, $J_{4a,4b} = 13.9$ Hz, $J_{3,4a} = J_{4a,5a} = J_{4a,5b} = 7.6$ Hz, 1H, H-4a), 1.66 (ddt, $J_{4a,4b} = 13.9$ Hz, $J_{3,4b} = J_{4b,5a} = J_{4b,5b} = 7.1$ Hz, 1H, H-4b), 1.43–1.24 (m, 50H, H-5–H-26, 2 x CH₃), 0.89 (t, $J = 6.8$ Hz, 3H, CH₃-27); ¹³C NMR (125 MHz, CDCl₃) δ 173.5 (C-1), 172.9 (3-CO₂), 71.0 (C-2), 61.8 (CH₂O, 1-OEt), 60.8 (CH₂O, 3-CO₂CH₂), 48.6 (C-3), 31.9 (C-4), 29.7, 29.68, 29.67, 29.65, 29.58, 29.45, 29.37, 28.1, 27.4, 22.7 (C-5–C-26), 14.2 (C-27), 14.1 (CH₃, 1-OEt and CH₃, 3-CO₂Et); HRMS(ESI) m/z calcd. For [C₃₂H₆₂O₅+Na]⁺: 549.4495, obsd.: 549.4490.

Cross metathesis for the preparation of ethyl-2*S*-hydroxy-3*R*-ethoxycarbonyl-heptadecanoate (*anti*-**5d**). To a solution of compound *anti*-**5a** (0.106 g, 0.46 mmol) in CH₂Cl₂ (2.5 mL), was added Grubbs second generation catalyst (0.02 g, 0.023 mmol) followed by 1-tridecene (0.11 mL, 0.46 mmol) and the reaction mixture refluxed under nitrogen for 14 hours before being cooled down and reduced *in vacuo*. The resulting crude product was columned yielding the heptadecenoate *anti*-**5e** (eluting in 15:1, petroleum ether/EtOAc, v/v) as a colourless oil (0.07 g, 0.19 mmol, 41%). Subsequent hydrogenation of *anti*-**5e** according to the above general procedure, then yielded pure *anti*-**5d** (0.07 g, 0.18 mmol, 99 %) as a colourless oil. The spectral data matched that previously determined as reported herein.

General procedure for alkylation using HMPA as a solvent. *n*-BuLi (11.5 mL, 2.0M solution in hexanes, 23.0 mmol) was added to a solution of di-isopropylamine (3.6 mL, 25.0 mmol) in THF (20 mL) at 0 °C. After 30 min the solution was cooled down to -78 °C and diethyl malate **3** (1.9 g, 10 mmol) in THF (20 mL) was added. The resulting mixture was stirred at -78 °C for 1 h, warmed up to -20 °C over 1 h and stirred at -20 °C for 20 min. After cooling down to -78 °C, the appropriate iodide (15.0 mmol, 1.5 equiv.) in HMPA (20 mL) was added dropwise and the reaction was stirred for 1 h at -50 °C and then a further 1h during which time the temperature

increased to 0 °C . The reaction was then quenched using NH₄Cl (sat.) and extracted with EtOAc (3 x 100 mL). The combined organic layers were washed with water (30 mL) and brine (30 mL), dried (MgSO₄), filtered and the solvent removed *in vacuo* to yield a crude yellow oil. Purification of the residue, using silica gel flash column chromatography and the eluent given, gave the alkylated product.

Ethyl 2*S*-hydroxy-3*R*-ethoxycarbonyl-heptadecanoate (*anti*-5d**).** By reacting diethyl malate **3** (0.47 g, 2.45 mmol) with 1-iodotetradecane (1.19 g, 3.69 mmol) according to the general procedure for alkylation described above, heptadecanoate **5d** was obtained (eluting in 15:1, petroleum ether/EtOAc, v/v) as a colourless oil (0.32 g, 0.83 mmol, 35%, *anti:syn* = 7:1). The spectral data matched that reported earlier.

Ethyl 2*S*-hydroxy-3*R*-ethoxycarbonyl-heptadecen-5-enoate (*anti*-5e**).** By reacting diethyl malate **3** (0.45 g, 2.35 mmol) with 1-iodo-tetradec-2-ene (1.14 g, 3.53 mmol) according to the general procedure described above, heptadecenoate **5e** was obtained (eluting in 15:1, petroleum ether/EtOAc, v/v) as a colourless oil (0.40 g, 1.05 mmol, 45%, *anti:syn* = 9:1). The spectral data matched that reported earlier.

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5

TMCM Synthesis

5.1 Introduction

Trehalose dicorynomycolates (TDCMs, **1**, Figure 1) are naturally occurring glycolipids found in *Corynebacteria* which exhibit important immunological properties such as adjuvant^{1,2} and tumor regression activities.³ With recent advances in the studies of the biological potential of trehalose glycolipids, interest in TDCMs has been renewed. Accordingly, there have been a number of synthetic routes reported in literature for accessing TDCMs and their analogues^{4,5,6} (see Chapter 1, Section 1.5.3b). We envisaged, however, that these routes could be improved and thus make this important class of compound more readily accessible. With the improved Fráter-Seebach methodology already developed (Chapter 4), it was proposed that the product from this alkylation could form a key intermediate *en route* to the synthesis of TDCM and analogues. For the purpose of the work presented herein, the synthesis of trehalose monocorynomycolate (TMCM, **2**) was undertaken

in order to determine other key methodologies that then may be used in the synthesis of further TDCMs. Attempts to prepare diester **3** will also be discussed.

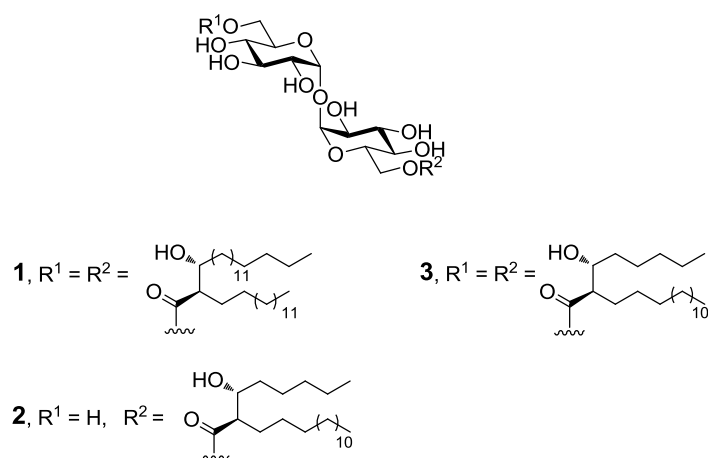
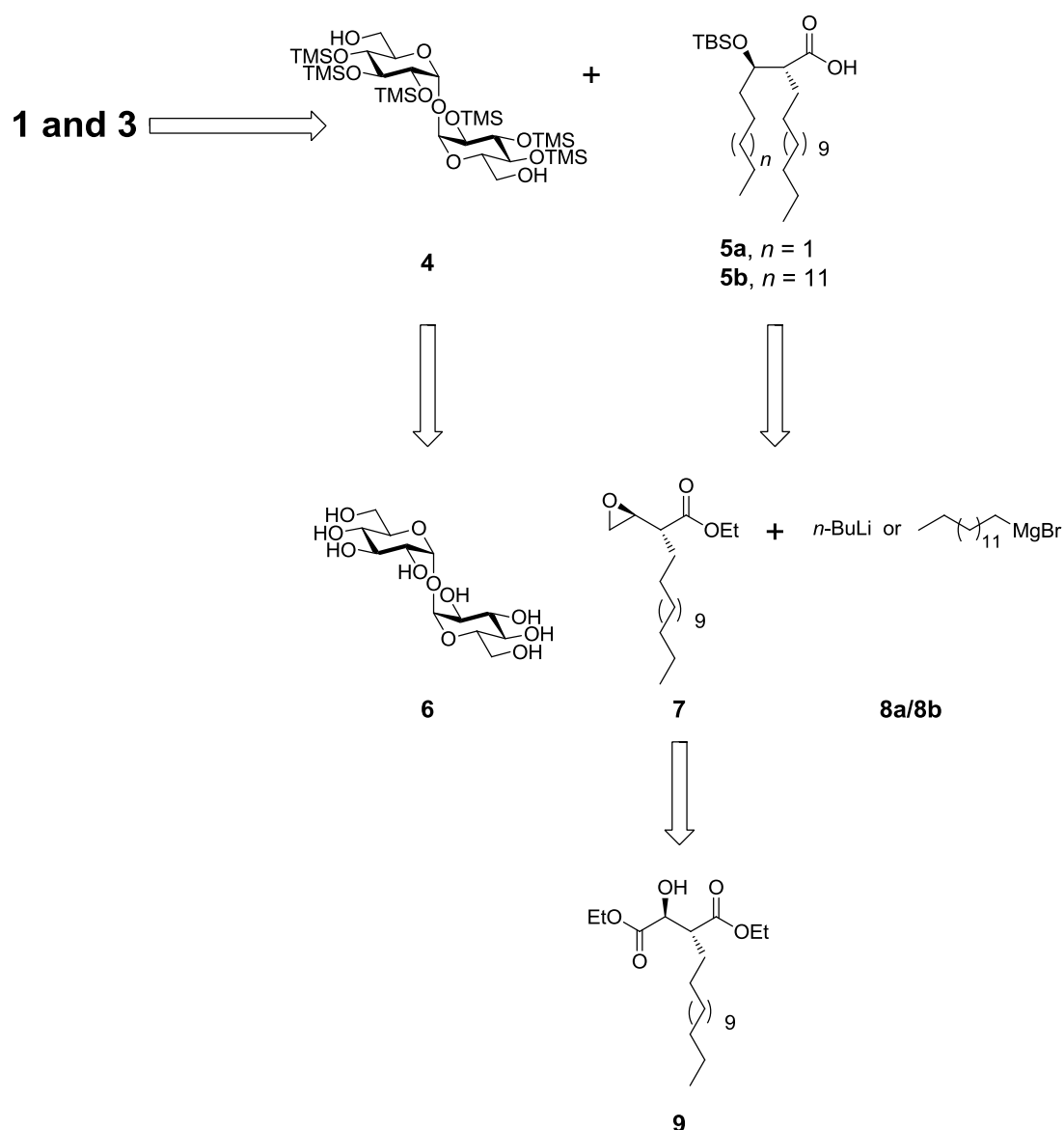


Figure 1. TDCM (**1**), TMCM analogue (**2**) and TDCM analogue (**3**)

5.2 TMCM and TDCM Synthesis

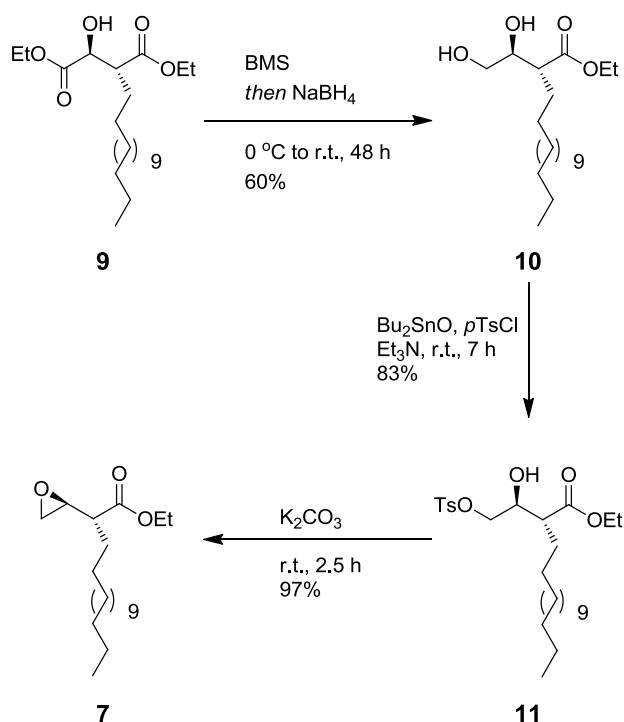
The retrosynthetic strategy for the synthesis of TDCMs **1** and **3** is presented (Scheme 1). Here, a convergent strategy was proposed whereby the two key intermediates, TMS trehalose **4** and *tert*-butyldimethylsilyl (TBS) protected corynomycolic acid **5a** or **5b** could be coupled to obtain the mono or diester as required. As previously illustrated (Chapter 2), it was envisioned that the stoichiometry of acid would determine whether the mono- or diester would be formed. TMS trehalose **4**, in turn, could be synthesised through the previously reported one-pot two-step silylation process starting with commercially available α,α -trehalose (**6**).⁷ To prepare TBS corynomycolic acids **5a** and **5b**, a copper-mediated Grignard reaction could be performed between epoxide **7** and either commercially available *n*-BuLi (**8a**) or readily synthesised tetradecylmagnesium bromide (**8b**). Epoxide **7** could be accessed in three steps from malic ester **9** according to our previously reported Fráter-Seebach alkylation methodology (see Chapter 4).



Scheme 1. Retrosynthesis for TDCMs **1** and **3**.

To prepare the key TBS corynomycolic acid **5**, our synthetic efforts commenced with the borane dimethylsulfide (BMS) reduction of malic ester **9** in order to selectively reduce the ethyl ester adjacent to the hydroxyl group.⁸ This reaction led to diol **10** in a respectable 60% yield and as the only observed product (Scheme 2). The primary hydroxyl in **10** was then transformed into a tosylate *via* reaction of **10** with TsCl, Et₃N in the presence of catalytic dibutyltin oxide. Subjecting tosylate **11** to basic

conditions then resulted in the formation of epoxide **7** in good (81%) yield over two steps.

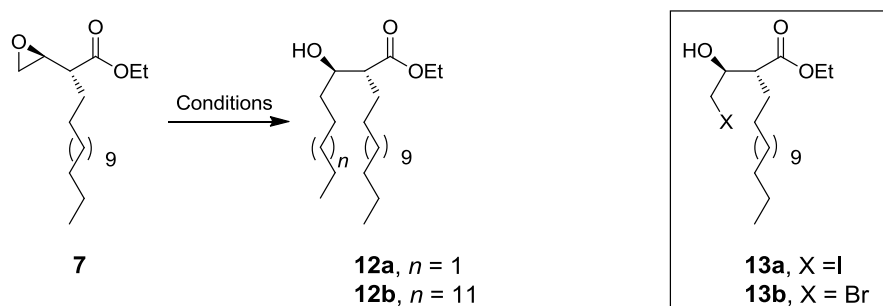


Scheme 2. Synthesis of epoxide **7**

With epoxide **7** in hand, we next attempted to prepare corynomycolic ester **12** using a copper-mediated Grignard reaction to open the epoxide.^{9,10} Copper-mediated Grignard reactions are versatile tools for carbon-carbon bond formation^{11,12,13} and are carried out in the presence of copper salts such as CuCN, CuI, CuCl and CuBr, of which CuI is most commonly used.¹⁴ Accordingly, epoxide **7** was first subjected to reaction with CuI (2.5 equiv.) and *n*-BuLi (5 equiv.) in THF (Table 1, Entry 1). Unfortunately, the major product from this reaction was halohydrin **13a** with none of the desired corynomycolic ester **12a** being obtained. This was not entirely unexpected as the formation of similar halo-products have been reported in literature.¹⁵ In an attempt to restrict formation of **13a**, the amount of CuI was reduced (0.15 equiv.), however, the halohydrin was still the only product obtained from the reaction mixture (Entry 2). Alexakis *et al.* had previously reported that use of a Lewis acid could reduce the formation of halohydrin products in the nucleophilic

ring opening of epoxides.¹⁶ Thus, BF₃.Et₂O was used in the reaction (Entry 3). Although, a faster reaction was observed, again only halohydrin **13a** was isolated. In view of this, we then opted to use CuCN as the copper reagent (Entry 4). Fortunately, this reaction was successful with complete consumption of starting material seen after 4 hours and only the desired alkylated product **12a** was observed (71% yield).

Table 1. Copper mediated Grignard reaction



Entry	Conditions	Results	Yield(%) ^[a]
1	CuI (2.5 equiv.), <i>n</i> -BuLi (5 equiv.), THF, -35 °C, 4 h	13a only	60%
2	CuI (0.1 equiv.), <i>n</i> -BuLi (2 equiv.), THF, -35 °C, 4 h	13a only	10%
3	CuI (2.5 equiv.), <i>n</i> -BuLi (5 equiv.), BF ₃ .OEt (5 equiv.), THF, -35 °C, 2 h	13a only	60%
4	CuCN (2 equiv.), <i>n</i> -BuLi (4 equiv.), THF, -50 to -20 °C, 4 h	12a only	71%
5	CuCN (2 equiv.), C14MgBr (4 equiv. in THF), THF, -50 to 0 °C, 5 h	13b only	60%
6	CuCN (2 equiv.), C14Li (4 equiv. in THF), THF, -50 to 0 °C, 8 h	Starting material	80% ^[b]
7	CuCN (2 equiv.), C14Li (4 equiv. in Et ₂ O), -50 to 0 °C, 8 h	Starting material unidentified by-product (trace)	80% ^[b]
8	CuCN (2 equiv.), C14Li (4 equiv. in toluene), THF, -50 to 0 °C, 8 h	Starting material	80% ^[b]
9	CuCN (2 equiv.), C14Li (4 equiv. in cyclohexane), THF, -50 to 0 °C, 8 h	Starting material	80% ^[b]

[a] Isolated yields

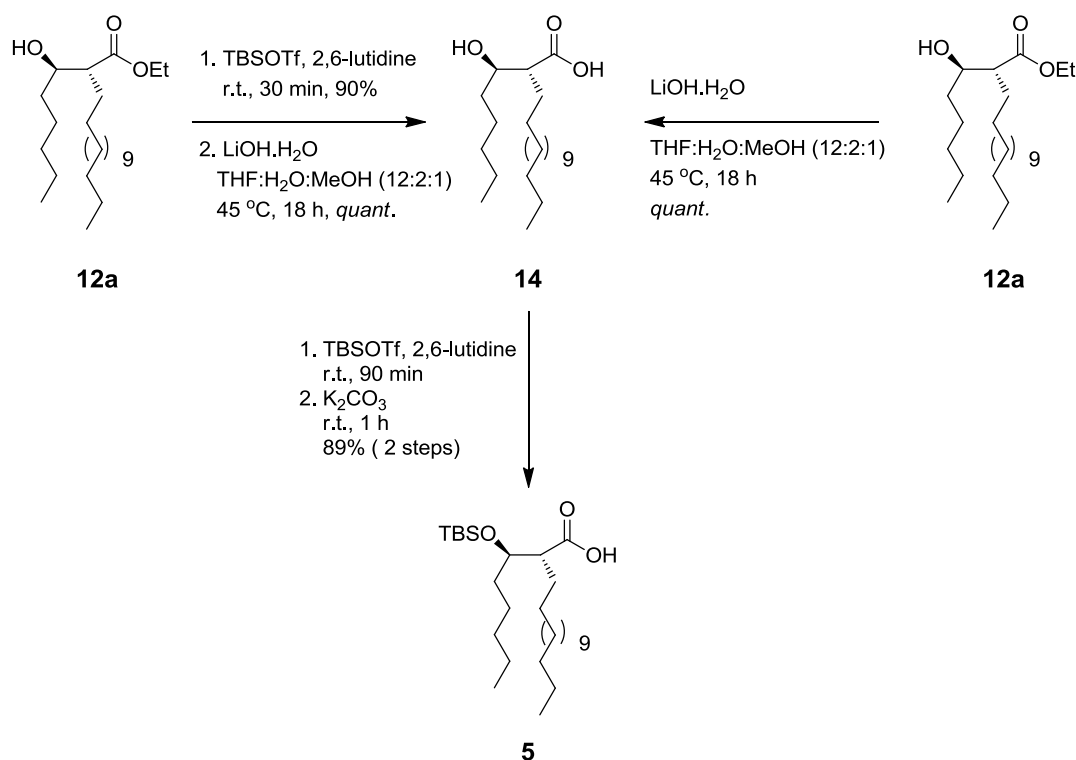
[b] Yield of recovered starting material

Having successfully synthesised the butylated product **12a**, our attention then turned to the use of copper-mediated Grignard reaction to install the C14 lipid. Thus, tetradecylmagnesium bromide (prepared *via* reaction of bromo-tetradecane and magnesium)¹⁷ was subjected to Grignard reaction with epoxide **7** and CuCN (Entry 5). Unfortunately, the only product obtained from the reaction was halohydrin **13b**. We then opted to use tetradecyllithium (C14Li) in the alkylation so as to avoid formation of any halogenated by-products. To this end, C14Li was prepared as a solution in THF (*via* reaction of tetradecylbromide and Li),¹⁸ and then added to a solution of epoxide **7** in THF (Entry 6). Unfortunately, no formation of **12b** occurred and only starting material was isolated. A change of reaction solvent (entries 7–9) fared no better, with only starting material recovered.

In the interest of time, we proceeded using the butylated adduct **12a** in the synthesis of a short-chain TDCM. It is proposed, however, that the longer alkyl chains could be installed *via* the use of the more reactive alkyne lithium in this reaction.¹⁹ Though adding an additional step (reduction of the triple bond) following alkylation, this strategy could still allow for a highly competitive synthesis of the TDCMs.

Continuing on with the butylated adduct **12a**, a TBS protection of the hydroxyl group in **12a** (using TBSCl and imidazole in DMF) was attempted. This reaction, however, was not successful with starting material and only minor amounts (<10%) of the desired product formed. A change of solvent (CH₂Cl₂), prolonged reaction times, heating the reaction mixture, or the addition of DMAP as a catalyst did not improve the product yield. As the hydroxyl is in a secondary position with adjacent lipid groups, steric hindrance could be a potential cause for the poor yield in the protection step. In order to improve the yield, **12a** was subjected to the more reactive TBSOTf (prepared *via* reaction of TfOH and TBSCl)²⁰ and 2,6-lutidine in CH₂Cl₂. Gratifyingly, the reaction was complete in 30 min and the silylated product obtained in high (90%) yield (Scheme 3). Hydrolysis of the ethyl ester using LiOH was then attempted so as to generate protected corynomycolic acid **5**. Unfortunately, this reaction was not chemo-selective and loss of the TBS group also occurred to give corynomycolic acid **14**.

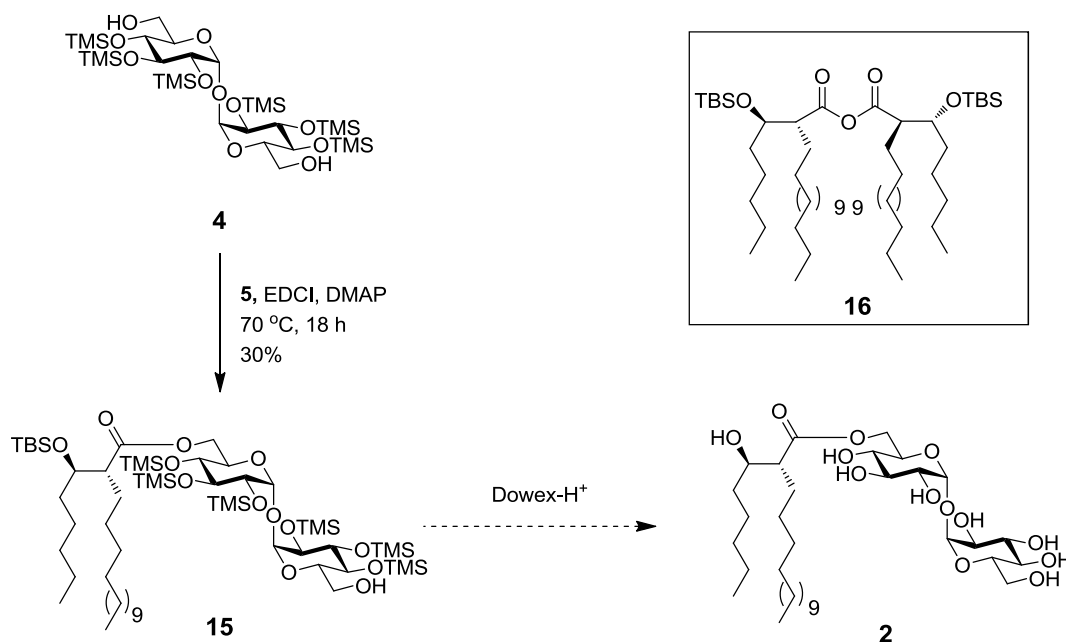
Given this, we resorted to a different strategy whereby corynomycolic ester **12a** would be first hydrolysed, then bis-silylated.²¹ The bis-silylated product could then be subjected to milder basic conditions in order to selectively remove the more labile TBS-ester leading to protected corynomycolic acid **5**. To this end, corynomycolic ester **12a** was treated with LiOH and acid **14** was obtained in quantitative yields (Scheme 3). Acid **14** was then treated with TBSOTf and 2,6-lutidine to give the bis-silylated product which, upon treatment with K₂CO₃, resulted in the desired TBS corynomycolic acid **5** in good (89%) yield over two steps.



Scheme 3. Synthesis of TBS protected corynomycolic acid **5**.

With the TBS corynomycolic acid **5** in hand, our attention turned to the coupling of this acid to the trehalose core. TMS trehalose **4** was synthesised using Johnson's protocol⁷ (see Chapter 2), and subjected to esterification with acid **5** (4.4 equiv.) in the presence of EDCI (6.6 equiv.) and DMAP (1.6 equiv.) (Scheme 4). It was proposed that these conditions would lead to the diester (as observed with the linear trehalose diesters (TDEs) series, Chapter 2). Indeed after stirring at 70 °C overnight,

complete consumption of acid **5** was noted and two higher running spots (compared to TMS trehalose **4**) were observed during TLC analysis of the reaction mixture, which were thought to be the mono and diesterified products. One of the products obtained was the protected TMCM **15** (30% yield), unfortunately, the other product was not the expected diester but tentatively assigned corynomycolic anhydride **16**. The structure of anhydride **16** was assigned based on ^1H NMR spectral data. In addition, treatment of product **16** with K_2CO_3 , yielded acid **5**, which is in agreement with the product expected from the reaction of an anhydride with a base. Time did not permit further optimisation of the reaction to increase yield of **15**, or indeed, to investigate ways in which to synthesise the desired diester. It is envisioned that a change of solvent and prolonged reaction times could help to improve this reaction. Anhydrides are known to react with alcohols in the presence of EDCI and DMAP²² and thus addition of further equivalents of these reagents could potentially drive the esterification further. In order to form the final TMCM compound **2**, TMCM **15** would be deprotected using Dowex- H^+ .



Scheme 4. Synthesis of protected TMCM analogue **15**.

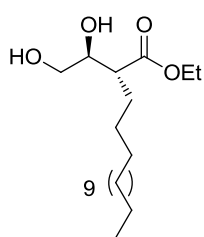
5.3 Conclusion and Future work

The synthesis of a fully protected truncated TMCM has been achieved. One of the key steps in the synthesis was a copper mediated Grignard reaction for the formation of the corynomycolic acid moiety. Difficulties were observed when attempts were made to install a longer alkyl lipid chain (C14) using this alkylation and one way to solve this would be *via* the use of the more reactive lithiated C14 alkyne in this reaction. Functional group manipulations were then performed and the desired silyl ether was obtained in good overall yields, however, esterification proved more difficult and only the protected TMCM along with the corynomycolic anhydride were isolated. To overcome this, it is proposed that prolonged reaction times and addition of further equivalents of EDCI and DMAP to the reaction mixture is required to drive the reaction further. Once these issues are resolved, it is then proposed that this general synthetic strategy can be used for the synthesis of a variety of TDCMs.

5.4 Experimental

Unless stated otherwise all reactions were performed under N₂. Prior to use, THF was distilled from Na wire and benzophenone, toluene was dried and stored under Na wire and TfOH (flurochem) was distilled. BH₃.Me₂S (1M in THF, Acros), NaBH₄ (Aldrich), dibutyltin oxide (Aldrich), tosyl chloride (Aldrich), *n*-BuLi (Aldrich, 2M solution in hexanes), imidiazole (Apollo), TBSCl (Apollo), 2,6-lutidine (Fluka), CuCN (Ajax chemicals), LiOH.H₂O (Riedel-de-Haën), EDCI (Aldrich), DMAP (Merck), anhydrous DMF (Acros), 33% HCl (Panreac), ethanol (Pure Science), methanol (Panreac), EtOAc (Panreac), petroleum ether (Pure Science), NaHCO₃ (Pure Science), K₂CO₃ (Pure Science), MgSO₄ (Pure Science), NaCl (Panreac) were used as received. All solvents were removed via evaporation at reduced pressure. Reactions were monitored by TLC analysis on Macherey-Nagel silica gel coated plastic sheets (0.20 mm, Polygram SIL G/UV254) by coating with a solution of 5%

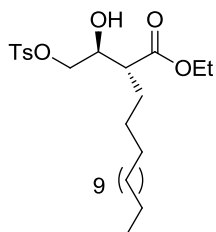
KMnO₄ and 1% NaIO₄ in H₂O followed by heating or by dipping in 10% H₂SO₄ in EtOH followed by charring at ~150 °C. Column chromatography was performed using Pure Science silica gel (40-63 μm). High resolution mass spectra were recorded on a Waters Q-TOF PremierTM Tandem Mass Spectrometer using positive electro-spray ionisation. Optical rotations were recorded on a Perkin-Elmer 241 polarimeter or Autopol II (Rudolph Research Analytical) at 589 nm (sodium D-line). Infrared spectra were recorded as thin films using a Bruker Tensor 27 FTIR spectrometer equipped with an Attenuated Total Reflectance (ATR) sampling accessory and are reported in wave numbers (cm⁻¹). Nuclear magnetic resonance spectra were recorded at 20 °C in CDCl₃ using a Varian INOVA operating at 500 MHz. Chemical shifts are given in ppm (δ) relative to solvent residual peak. NMR peak assignments were made using COSY, HSQC and HMBC 2D experiments.



3R-Ethoxycarbonyl-2S-hydroxy-heptadecanol (10). Borane dimethylsulfide complex (0.68 mL, 1M in THF, 0.68 mmol) was slowly added to a solution of malic ester **9** (0.24 g, 0.62 mmol) in THF (2 mL). The reaction was stirred at r.t. overnight after which time it was cooled to 0 °C and NaBH₄ (0.002 g,

0.04 mmol) was added. The reaction was allowed to warm to room temperature overnight, before being quenched with Dowex-H⁺ (10% by weight) and then ethanol (3 mL) was added. The reaction mixture was filtered, washed (EtOH) and concentrated *in vacuo*. The residue was dissolved in toluene:ethanol (1:1, 5 mL) and concentrated (repeated 3 x) in order to remove ethanol and boron as B(OEt)₃. The resulting crude oil was purified using silica gel flash column chromatography (eluting in 1:1, petroleum ether/EtOAc, v/v) to give the title compound **10** as a colourless oil (0.13 g, 0.37 mmol, 60%). *R*_f = 0.30 (PE/EA, 1/1, v/v); [α]_D²⁰ = 4.0 (*c* = 0.1, CHCl₃); IR (film) 2922, 2852, 1734, 1176, 756 cm⁻¹; ¹H NMR (500 MHz, CDCl₃) δ 4.23–4.10 (m, 2H, 3-CO₂CH₂), 3.83–3.80 (m, 1H, H-2), 3.69 (dd, *J*_{1a,1b} = 11.0 Hz, *J*_{1a,2} = 6.8 Hz, 1H, H-1a), 3.55 (dd, *J*_{1a,1b} = 11.0 Hz, *J*_{1b,2} = 6.8 Hz, 1H, H-1b), 3.11 (d, *J*_{2,OH} = 3.4 Hz, C2-OH), 2.53 (dt, *J*_{2,3} = *J*_{3,4a} = *J*_{3,4b} = 8.0 Hz, 1H, H-3), 2.13 (bs, 1H, C1-OH), 1.69–1.60 (m, 1H, H-4a), 1.59–1.55 (m, 1H, H-4b), 1.31–1.28

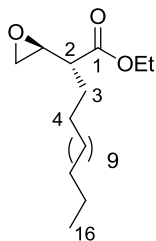
(m, 27H, H-5–H-16 and OCH_2CH_3), 0.88 (t, $J_{16,17} = 7.1$ Hz, 3H, CH_3 -17); ^{13}C NMR (125 MHz, CDCl_3) δ 175.5 (3- CO_2), 72.7 (C-2), 65.0 (C-1), 60.8 (CH_2O , 3- CO_2CH_2), 47.5 (C-3), 31.9, 29.68, 29.67, 29.65, 29.61, 29.5, 29.44, 29.39, 29.36, 29.34, 27.14, 22.69 (C-4–C-16), 14.2 (C-17), 14.1 (CH_3 , 3- CO_2Et); HRMS(ESI) m/z calcd. for $[\text{C}_{20}\text{H}_{40}\text{O}_4+\text{Na}]^+$: 367.2824, obsd.: 367.2820.



3R-Ethoxycarbonyl-2S-hydroxy-

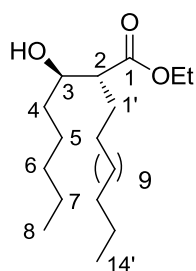
heptadecanoyl *p*-toluenesulfonate (11). To a solution of diol **10** (0.29 g, 0.84 mmol) in CH_2Cl_2 (5 mL), Bu_2SnO (0.004, 0.016 mmol), TsCl (0.18 g, 1.01 mmol), and Et_3N (0.14 mL, 1.01 mmol) were added. The resulting reaction mixture was

stirred at r.t. for 2.5 h, after which time brine was added and the resulting solution extracted with CH_2Cl_2 (3 x 30 mL). The combined organic layers were dried with MgSO_4 and concentrated *in vacuo*. The resulting crude solid was purified using silica gel flash column chromatography (eluting in 5:1, petroleum ether/ EtOAc , v/v) to give the title compound as a white solid (0.35 g, 0.70 mmol, 84%). $R_f = 0.56$ (PE/ EA , 1/1, v/v); $[\alpha]_D^{20} = 2.0$ ($c = 1.0$, CHCl_3); IR (film) 2925, 1539, 1265, 1176, 753 cm^{-1} ; ^1H NMR (500 MHz, CDCl_3) δ 7.80 (d, $J = 8.3$ Hz, 2H, H_{arom}), 7.35 (d, $J = 8.1$ Hz, 2H, H_{arom}), 4.20–4.12 (m, 2H, 3- CO_2CH_2), 4.05 (d, $J_{1,2} = 4.9$ Hz, 2H, H-1), 3.9 (dt, $J_{1,2} = J_{2,3} = 4.9$ Hz, 1H, H-2), 2.55 (m, 1H, H-3), 2.46 (s, 3H, $\text{C}_{\text{ar}}\text{-CH}_3$), 1.70–1.64 (m, 1H, H-4a), 1.53–1.43 (m, 1H, H-4b), 1.30–1.25 (m, 27H, H-5–H-16, OCH_2CH_3), 0.88 (t, $J_{16,17} = 6.4$ Hz, 3H, CH_3 -17); ^{13}C NMR (125 MHz, CDCl_3) δ 174.8 (3- CO_2), 145.1 ($\text{C}_{\text{ar}}\text{-CH}_3$), 132.5 ($\text{C}_{\text{ar}}\text{-SO}_2$), 129.9 (C_{ar}), 128.0 (C_{ar}), 71.6 (C-1), 70.0 (C-2), 60.8 (CH_2O , 3- CO_2CH_2), 47.0 (C-3), 31.9, 29.69, 29.68, 29.66, 29.61, 29.53, 29.36, 29.27, 27.11, 22.69, 21.67 (C-4–C-16, $\text{C}_{\text{ar}}\text{-CH}_3$), 14.14 (C-17), 14.10 (CH_3 , 3- CO_2Et); HRMS(ESI) m/z calcd. for $[\text{C}_{27}\text{H}_{46}\text{O}_6\text{S}+\text{Na}]^+$: 521.2913, obsd.: 521.2908.



Ethyl (2R)-2-[(R)-oxiranyl]-hexadecanoate (7). To a solution of tosylate **11** (0.32 g, 0.64 mmol) in MeOH (7 mL) was added K_2CO_3 (0.11 g, 0.76 mmol) and the reaction stirred at r.t. for 2 h after which time NH_4Cl (20 mL) was added. The resulting solution was extracted with EtOAc (3 x 30 mL), the combined organic layers were dried with $MgSO_4$, concentrated *in vacuo* and crude solid was

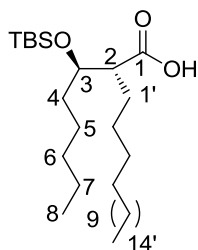
purified using silica gel flash column chromatography (eluting in 20:1, petroleum ether/EtOAc, v/v) to give the title compound as a white solid (0.20 g, 0.61 mmol, 97%). $R_f = 0.73$ (PE/EA, 1/1, v/v); $[\alpha]_D^{20} = 1.0$ ($c = 0.1$, $CHCl_3$); IR (film) 2825, 1733, 1464, 1203, 668 cm^{-1} ; 1H NMR (500 MHz, $CDCl_3$) δ 4.20 (q, $J = 7.1$ Hz, 2H, 1- OCH_2), 3.17–3.10 (m, 1H, 2-CH), 2.83 (dd, $J_{CHa,CHb} = 8.8$ Hz, $J_{2-CH,2-CHCHa} = 4.1$ Hz, 1H, 2-CHCHaHb), 2.56 (dd, $J_{CHa,CHb} = 8.8$ Hz, $J_{2-CH,2-CHCHb} = 4.1$ Hz, 1H, 2-CHCHbH), 2.13 (dt, $J_{2,3} = J_{2-CH} = 8.0$ Hz, 1H, H-2), 1.75–1.69 (m, 1H, H-3a), 1.62–1.55 (m, 1H, H-3b), 1.30–1.25 (m, 27H, H-4–H-15, 1- OCH_2CH_3), 0.88 (t, $J_{15,16} = 6.6$ Hz, 3H, CH_3 -16); ^{13}C NMR (125 MHz, $CDCl_3$) δ 173.6 (C-1), 60.8 (CH_2O , 1- CO_2CH_2), 52.7 (2- $CHCH_2$), 49.0 (C-2), 46.4 (2- $CHCH_2$), 31.9, 29.69, 29.67, 29.65, 29.61, 29.53, 29.47, 29.37, 29.36, 29.08, 27.23, 22.69 (C-3–C-15), 14.3 (C-16), 14.1 (CH_3 , 1-OEt); HRMS(ESI) m/z calcd. for $[C_{20}H_{38}O_3+Na]^+$: 349.2719, obsd.: 349.2713.



Ethyl-(2R,3R)-3-hydroxy-2-tetradecyl-octanoate (12a). A solution of $CuCN$ (13 mg, 0.14 mmol) in THF (0.3 mL) was cooled to -50 $^{\circ}C$ and $BuLi$ (2M in cyclohexane, 0.14 mL, 0.28 mmol) was added dropwise to the solution. The resulting reaction mixture was stirred for 30 min after which time the temperature increased to -20 $^{\circ}C$. The reaction was then cooled to

-50 $^{\circ}C$ and epoxide **7** (23 mg, 0.070 mmol) in THF (0.5 mL) was added. The reaction mixture was stirred for a further 2 h during which time the temperature increased to -20 $^{\circ}C$, then quenched with NH_4Cl , extracted with EtOAc (3 x 20 mL), and the combined organic layers were washed with water, brine, dried with $MgSO_4$ and concentrated *in vacuo*. The resulting crude solid was purified using silica gel flash

column chromatography (eluting in 20:1, petroleum ether/EtOAc, v/v) to give the title compound as a white solid (19 mg, 0.050 mmol, 71%). $R_f = 0.41$ (PE/EA, 5/1, v/v); $[\alpha]_D^{20} = 8.0$ ($c = 1.0$, CHCl_3); IR (film) 2924, 2854, 1714, 754, 689 cm^{-1} ; ^1H NMR (500 MHz, CDCl_3) δ 4.18 (q, $J = 7.3$ Hz, 2H, 3- CO_2CH_2), 3.72–3.61 (m, 1H, H-3), 2.51 (d, $J_{3,\text{OH}} = 8.5$ Hz, 1H, 3-OH), 2.41 (ddd, $J_{2,3} = 9.1$ Hz, $J_{1a',2} = J_{1b',2} = 5.1$ Hz, 1H, H-2), 1.72 (ddt, $J_{1a',1b'} = 13.2$ Hz, $J_{1a',2} = J_{1a',2a'} = J_{1a',2a'} = 8.6$ Hz, 1H, H-1a'), 1.58 (ddt, $J_{1a',1b'} = 13.2$ Hz, $J_{1b',2} = J_{1b',2a'} = J_{1b',2a'} = 8.2$ Hz, 1H, H-1b'), 1.46 (m, 2H, H-4), 1.39–1.16 (m, 33H, H-5–H-7, H-2'–H-13', OCH_2CH_3), 0.88 (t, $J_{7,8} = J_{13',14'} = 7.1$ Hz, 6H, CH_3 -8 and 14'); ^{13}C NMR (125 MHz, CDCl_3) δ 175.9 (C-1), 72.3 (C-3), 60.4 (CH_2O , 1-OEt), 50.8 (C-2), 35.7 (C-4), 31.9, 31.7, 29.70, 29.69, 29.67, 29.63, 29.57, 29.50, 29.44, 29.37, 27.36, 25.43, 22.7, 22.60 (C-5–C-7 and C-1'–C-13'), 14.3, 14.1, 14.0 (C-8, C-14' and 1-OEt); HRMS(ESI) m/z calcd. for $[\text{C}_{24}\text{H}_{48}\text{O}_3 + \text{Na}]^+$: 407.3501, obsd.: 407.3508.



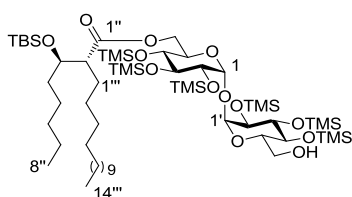
(2R,3R)-3-Tert-butyldimethylsilyloxy-2-tetradecyl-octanoic

acid (5). A solution of corynomycolic ester **12a** (15 mg, 0.039 mmol) in THF:MeOH:H₂O (12:2:1, 2 mL) was treated with LiOH.H₂O (54 mg, 1.28 mmol) and the resulting reaction mixture was heated at 45 °C overnight (18 h) after which time it was acidified with 1M HCl (pH = 3) and diluted with EtOAc (10

mL). The organic layer was washed with water, brine, dried with MgSO_4 and concentrated *in vacuo*. This gave corynomycolic acid **14** in quantitative yield, which was used without further purification.

TBSOTf (0.09 mL, 0.39 mmol) and 2,6-lutidine (0.07 mL, 0.59 mmol) was added to a solution of corynomycolic acid **14** (14 mg, 0.039 mmol) in CH_2Cl_2 (1 mL) and the reaction was stirred at r.t. for 90 min after which time complete conversion of the starting material to the bis-silyl product was observed. The solvent was then removed *in vacuo* and the residue diluted with EtOAc (10 mL), washed with 0.1 M HCl (3 x 10 mL), water (10 mL), brine (10 mL), dried with MgSO_4 and filtered. Following

removal of the solvent *in vacuo*, the crude product was then dissolved in CH₂Cl₂ and treated with 10% K₂CO₃ in water (0.05 mL). The reaction mixture was stirred at r.t. for 1 h after which time it was neutralised with 1 M HCl (pH = 3) and extracted with EtOAc (3 x 20 mL). The combined organic layer was washed with water, brine, dried with MgSO₄ and concentrated *in vacuo*. The title compound was obtained after silica gel flash column chromatography (eluting in 50:1, petroleum ether/EtOAc, v/v) to give the title compound as a pale yellow oil (16.4 mg, 0.035 mmol, 89% over 2 steps). *R*_f = 0.38 (PE/EA, 10/1, v/v); [α]_D¹⁷ = 20.0 (*c* = 0.1, CHCl₃); IR (film) 2924, 2854, 1708, 1254, 774 cm⁻¹; ¹H NMR (500 MHz, CDCl₃) δ 3.82 (dt, *J*_{2,3} = *J*_{3,4a} = *J*_{3,4b} = 7.3 Hz, 1H, H-3), 2.56–2.46 (m, 1H, H-2), 1.72 (ddt, *J*_{1a',1b'} = 14.0 Hz, *J*_{1a',2} = *J*_{1a',2a'} = *J*_{1a',2b'} = 6.7 Hz, 1H, H-1a'), 1.59 (ddt, *J*_{4a,4b} = 13.5 Hz, *J*_{3,4a} = *J*_{4a,5} = 6.6 Hz, 1H, H-4a), 1.57 (ddt, *J*_{4a,4b} = 13.5 Hz, *J*_{3,4b} = *J*_{4b,5} = 6.6 Hz, 1H, H-4b), 1.51 (ddt, *J*_{1a',1b'} = 14.0 Hz, *J*_{1b',2} = *J*_{1b',2a'} = *J*_{1b',2b'} = 6.7 Hz, 1H, H-1b'), 1.34–1.26 (m, 33H, H-5–H-7, H-2'–H-13', OCH₂CH₃), 0.92 (s, 9H, Si(CH₃)₃), 0.87 (t, *J*_{7,8} = *J*_{13',14'} = 7.1 Hz, 6H, CH₃-8 and 14'), 0.14 (s, 3H, Si(CH₃)), 0.13 (s, 3H, Si(CH₃)); ¹³C NMR (125 MHz, CDCl₃) δ 175.8 (C-1), 73.6 (C-3), 50.2 (C-2), 35.5 (C-4), 31.9, 31.8, 29.8, 29.70, 29.69, 29.66, 29.63, 29.55, 29.45, 29.40, 29.41, 29.37, 27.44, 25.85, 24.64, 22.70, 22.51 (C-5–C-7 and C-1'–C-13'), 25.72 (SiC(CH₃)₃), 17.9 (SiC(CH₃)₃), 14.1, 14.0 (C-8 and C-14'), -4.3, -4.9 (SiCH₃); HRMS(ESI) *m/z* calcd. for [C₂₈H₅₈O₃Si+Na]⁺: 493.4047, obsd.: 493.4059.



**2,2',3,3',4,4'-Hexa-
O-trimethylsilyl-6-O-[(2*R*,3*R*)-3-*tert*-
butyldimethylsilyloxy-2-tetradecyl-octanoyl]-
 α,α' -D-trehalose (15).** To a solution of TMS
trehalose **4** (4.0 mg, 0.005 mmol) and protected

corynomycolic acid **5** (10.3 mg, 0.022 mmol) in toluene (1.5 mL), EDCI (6.0 mg, 0.033 mmol) and DMAP (1.0 mg, 0.008 mmol) were added and the reaction was stirred at 70 °C overnight after which time the resulting precipitate was filtered off and washed with EtOAc (3 x 10 mL). The combined organic layers were washed with water (10 mL), brine (10 mL) and concentrated *in vacuo*. Silica gel flash

chromatography of the crude product resulted in protected TCMC **15**, (eluting in 30:1 (v/v) Pet. Ether/EtOAc), as a colourless oil (1.84 mg, 0.015 mmol, 30%). R_f = 0.53 (PE/EA, 5/1, v/v); $[\alpha]_D^{18}$ = +60 (c = 0.05, CHCl_3); IR (film): 3627, 3019, 2959, 1721, 1463, 1278, 1070 cm^{-1} ; ^1H NMR (500 MHz, CDCl_3) δ 4.90 (d, $J_{1,2/1',2'}$ = 3.0 Hz, 1H, H-1/1'), 4.83 (d, $J_{1,2/1',2'}$ = 3.0 Hz, 1H, H-1/1'), 4.59 (dd, $J_{6a,6b}$ = 11.6 Hz, $J_{5,6a}$ = 2.3 Hz, 2H, H-6a), 4.33 (dd, $J_{6a,6b}$ = 11.6 Hz, $J_{5,6b}$ = 4.1 Hz, 2H, H-6b), 3.97 (ddd, $J_{4,5}$ = 9.0 Hz, $J_{5,6a}$ = 2.3 Hz, $J_{5,6b}$ = 4.1 Hz, 1H, H-5), 3.93 (dt, $J_{2'',3''}$ = $J_{3'',4''}$ = 6.0 Hz, 1H, H-3''), 3.88 (dd, $J_{2,3/2',3'}$ = $J_{3,4/3',4'}$ = 9.0 Hz, 1H, H-3), 3.84–3.82 (m, 1H, H-5'), 3.72–3.65 (m, 2H, H-6a' and 6b'), 3.47 (dd, $J_{3,4/3',4'}$ = $J_{4,5/4',5'}$ = 9.0 Hz, 2H, H-4 and 4'), 3.42 (dd, $J_{1,2/1',2'}$ = 3.0 Hz, $J_{2,3/2',3'}$ = 9.0 Hz, 1H, H-2/2'), 3.38 (dd, $J_{1,2/1',2'}$ = 3.0 Hz, $J_{2,3/2',3'}$ = 9.0 Hz, 1H, H-2/2'), 2.55 (ddd, $J_{2'',3''}$ = 9.4 Hz, $J_{1a'',2''}$ = $J_{1b'',2''}$ = 4.8 Hz, 1H, H-2''), 1.72 (dt, $J_{3'',4''}$ = $J_{4'',5''}$ = 5.3 Hz, 2H, H-4''), 1.60–1.52 (m, 2H, H-1'''), 1.33–1.26 (m, 30H, H-5''–H-7'', H-2'''–H-13'''), 0.93 (s, 9H, $\text{Si}(\text{CH}_3)_3$), 0.167, 0.154, 0.149, 0.120, 0.059 (6 s, 60H, CH_3 , TMS and TBS); ^{13}C NMR (125 MHz, CDCl_3) 174.1 (C-1''), 94.5 (C-1/1'), 94.4 (C-1/1'), 73.40 (C-3/3'), 73.39 (C-3/3'), 72.72 (C-3''), 72.83 (C-5'), 72.76 (C-2/2'), 72.72 (C-2/2'), 71.9 (C-4/4'), 71.4 (C-4/4'), 70.7 (C-5), 62.42 (C-6), 61.65 (C-6'), 51.8 (C-2''), 33.30, 31.92, 31.43, 31.00, 30.17, 29.79, 29.70, 29.67, 29.53, 29.38, 28.12, 26.94, 26.30, 25.81, 24.50, 22.70, 22.63, 22.06 (C-4''–C-7'' and C-1'''–C-13''') 25.81 ($\text{SiC}(\text{CH}_3)_3$), 18.0 ($\text{SiC}(\text{CH}_3)_3$), 14.14, 14.04 (C-8'' and C-14'''), 1.05, 1.01, 0.92, 0.86, 0.17, 0.04 (SiCH_3 , TMS), -4.3, -4.9 (SiCH_3 , TBS); HRMS (ESI) m/z calcd. for $[\text{C}_{58}\text{H}_{126}\text{O}_{13}\text{Si}_7+\text{Na}]^+$: 1249.7476, obsd.: 1249.7482.

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AfBP Probe Synthesis and Biological Testing

6.1 Introduction

To understand more about the manner in which trehalose dimycolates (TDMs) interact with cells of the immune system, we developed the Affinity Based Proteome (AfBP) probe **1**, which is structurally similar to the parent TDMs whilst also containing a benzophenone trap and an alkyne tag group. This particular trap and tag system has been chosen due to its small size and hydrophobic character and thus its ability to best mimic the TDM lipid. Our probe has been developed so as to leave the bioactive carbohydrate portion of the TDM unfunctionalised.

Previous studies have shown that TDM, as well as its simpler synthetic analogue trehalose dibehenate (TDB), binds to macrophage inducible C-type lectin (Mincle) in order to induce an immune response,^{1,2} however, to date no other receptors for TDMs have been determined. Accordingly, by synthesising probe **1** and using it for Affinity Based Proteome Profiling (AfBPP), other proteins that TDMs bind to may be identified. The focus of this chapter is the synthesis of AfBP probe **1**. The probe

binding and protein identification studies will be performed by our collaborator Dr. Faustin Kamena at a later date.

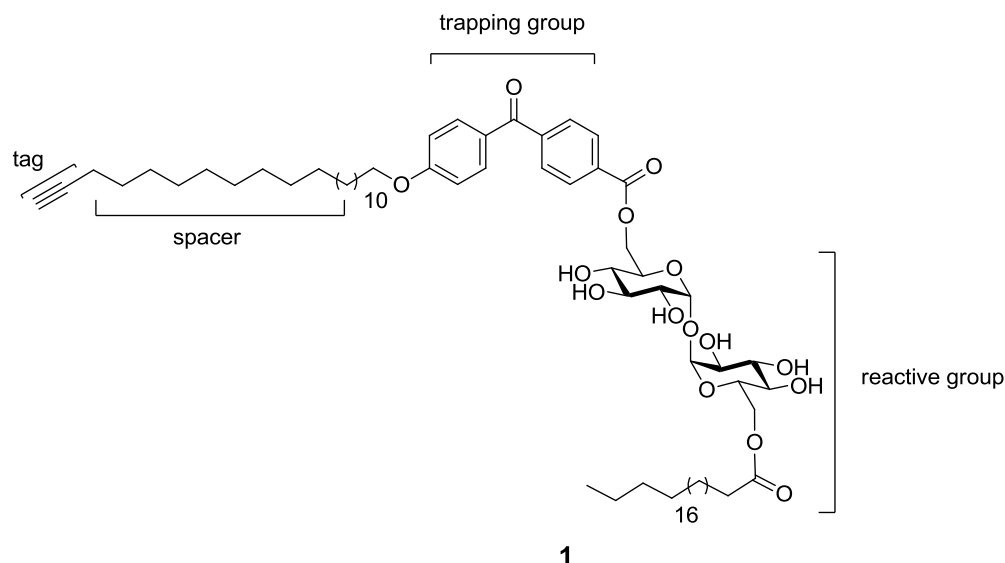
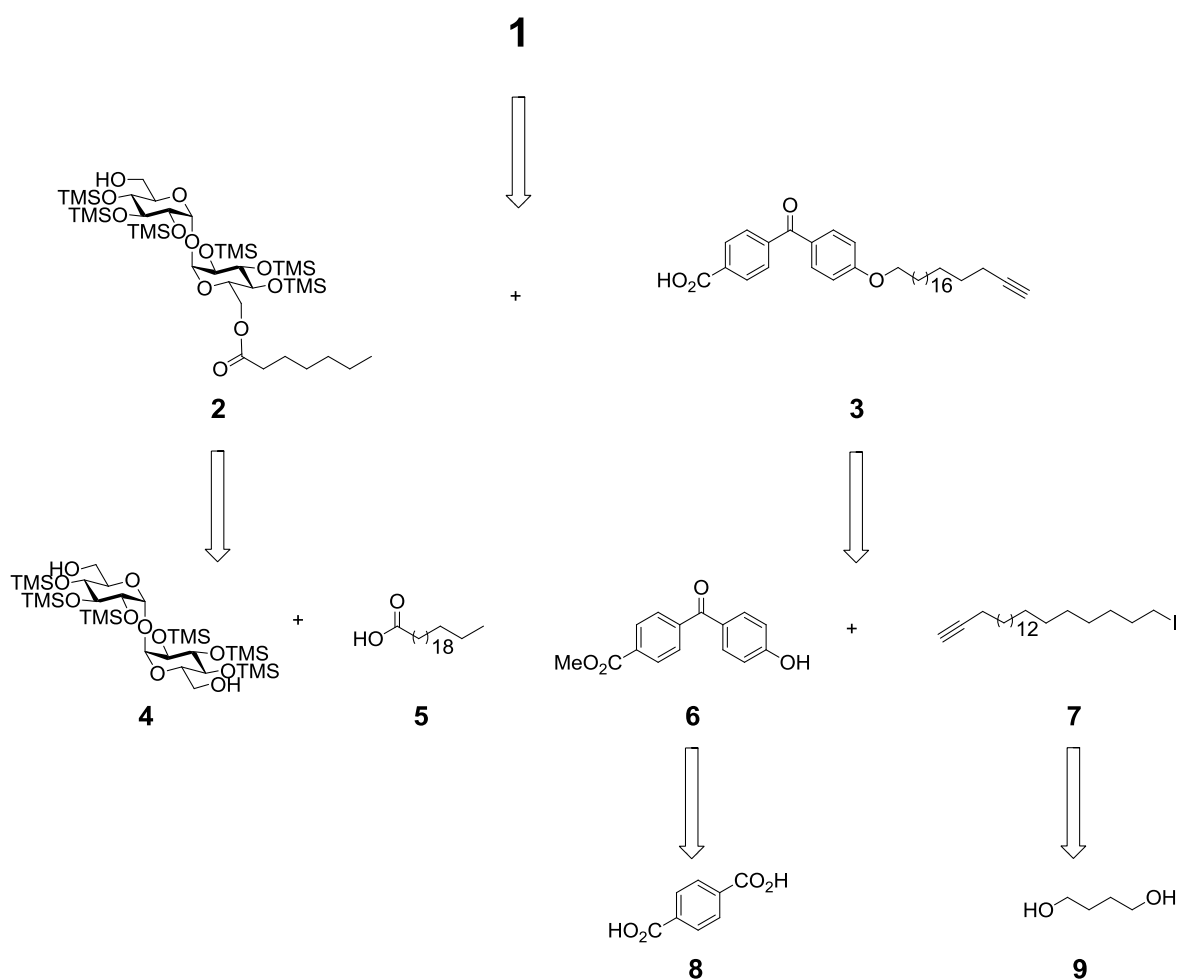


Figure 1. Proposed A/BP Probe **1**

6.2 TDM Probe Synthesis

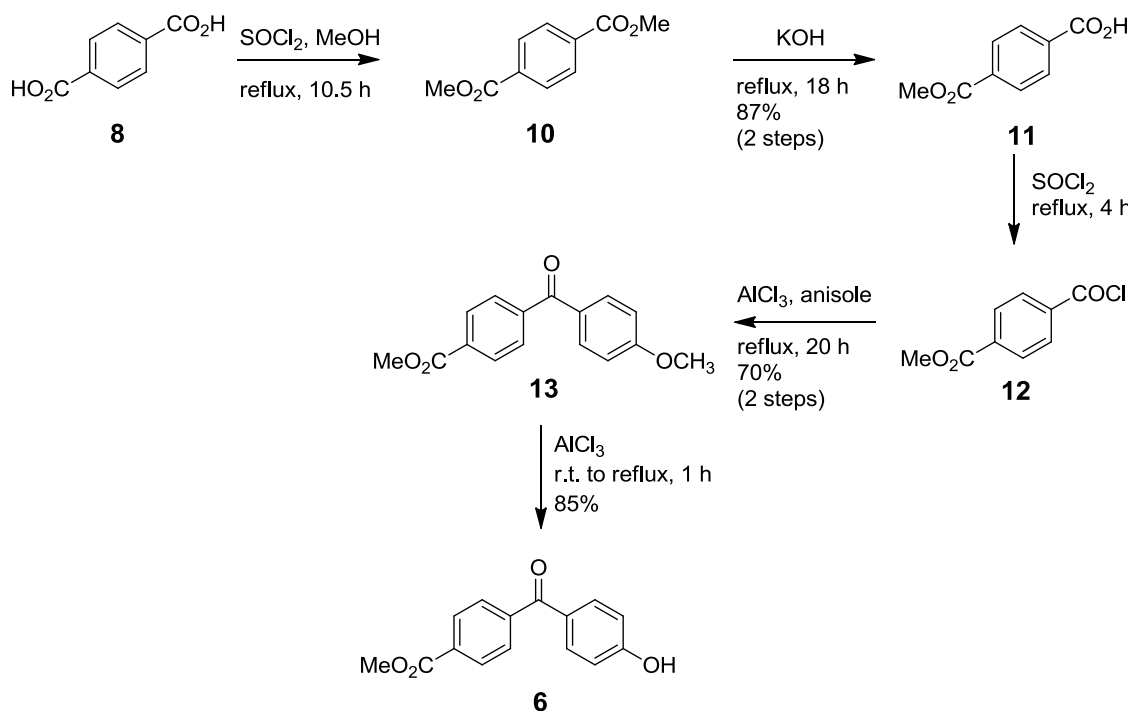
For the synthesis of probe **1**, a convergent strategy was planned whereby the two intermediates, TMS mono-trehalose ester **2** and carboxylic acid **3** would be coupled followed by deprotection to obtain the final compound (Scheme 1). The TMS mono-trehalose ester **2** could be obtained through esterification of diol **4** with behenic acid (**5**). Compound **4**, in turn, could be synthesised *via* a one-pot two-step silylation process starting with commercially available α,α -D-trehalose.³ To prepare the trap and tag, carboxylic acid **3** could be accessed *via* the alkylation of benzophenone **6** with iodide **7**, with compound **6** prepared in five steps from commercially available terephthalic acid (**8**) and iodide **7** synthesised in five steps from 1,4-butanediol (**9**).



Scheme 1. Retrosynthesis of target AfBP probe **1**

To prepare benzophenone **6**, terephthalic acid **8** was first converted to dimethyl ester **10** via reaction with thionyl chloride and MeOH (Scheme 2). Hydrolysis of one of the methyl ester groups using potassium hydroxide (1.1 equiv.) gave monoacid **11** in good (87%) yield over two steps.⁴ Acid **11** was then converted to acid chloride **12** by reacting it with SOCl_2 . Due to the instability of acid chloride **12**, it was immediately subjected to Friedel-Crafts acylation with anisole in the presence of aluminium chloride in an attempt to form the *para*-substituted benzophenone compound **13**. The literature procedure involved addition of AlCl_3 in one portion and in our hand, this led to a yield of only 40% which was comparable to the reported yield of 42%.⁵ The yield of the product could be significantly increased to 70% by portion-wise addition

of AlCl_3 during the course of the reaction. About 5% of the expected *ortho*-substituted product was also formed which was inseparable from the *para*-substituted product. However, the *para*- and *ortho*-substituted products could be separated after demethylation of benzophenone **13** (*vide infra*). The major product was identified as *para*-substituted benzophenone **13** based on the proton, COSY, and HMBC spectral analysis. The four protons of the anisole moiety in the *para*-product are in two different chemical environments, and as expected only two distinguished resonances were observed for them, as opposed to four resonances which would be expected for the *ortho*-substituted product.



Scheme 2. Synthesis of benzophenone **6**

Having successfully prepared benzophenone ester **13**, the next step in our strategy was demethylation to generate the target benzophenone **6**, ready for coupling to the alkynyl iodide **7**. To this end, benzophenone **13** was treated with trimethylsilyl iodide (1.1 equiv.),⁶ however, despite several attempts this strategy did not eventuate in

product formation and only starting material was recovered from the reaction. Use of boron trichloride (4 equiv.) was also not successful with starting material retrieved from the reaction. Next, AlCl_3 (4 equiv.) was used, according to the more recent procedure of Hartmann and co-workers.⁷ This resulted in *c.a.* 30% of the desired product along with unreacted starting material. Fortunately, by increasing the amount of AlCl_3 (6 equiv.), and adding it in two portions during the reaction, greatly enhanced the yield, with benzophenone ester **6** obtained in good (85%) yield. The ^1H NMR spectra of compound **6** (Figure 2), showed one singlet at δ 3.98 ppm as compared to the two singlets at δ 3.98 and δ 3.91 ppm for the methyl ester precursor **13**. ^{13}C NMR spectral analysis showed a downfield shift of 3.6 ppm for the aromatic carbon attached to the hydroxyl group in compound **6** when compared to the same carbon in **13**. A shift of 1.5 ppm for the adjacent aromatic carbons also provided evidence that demethylation occurred. The chemical shifts of the other carbons in compounds **6** and **13** were within 0.5 ppm of each other. Mass spectral analysis of the product was in agreement of the expected mass, [HRMS calcd. for $[\text{C}_{15}\text{H}_{12}\text{O}_4+\text{Na}]^+$: 279.0626, obsd.: 279.0633] and full assignment was made on the basis of 2D NMR (COSY, HSQC and HMBC) (see experimental for full assignment).

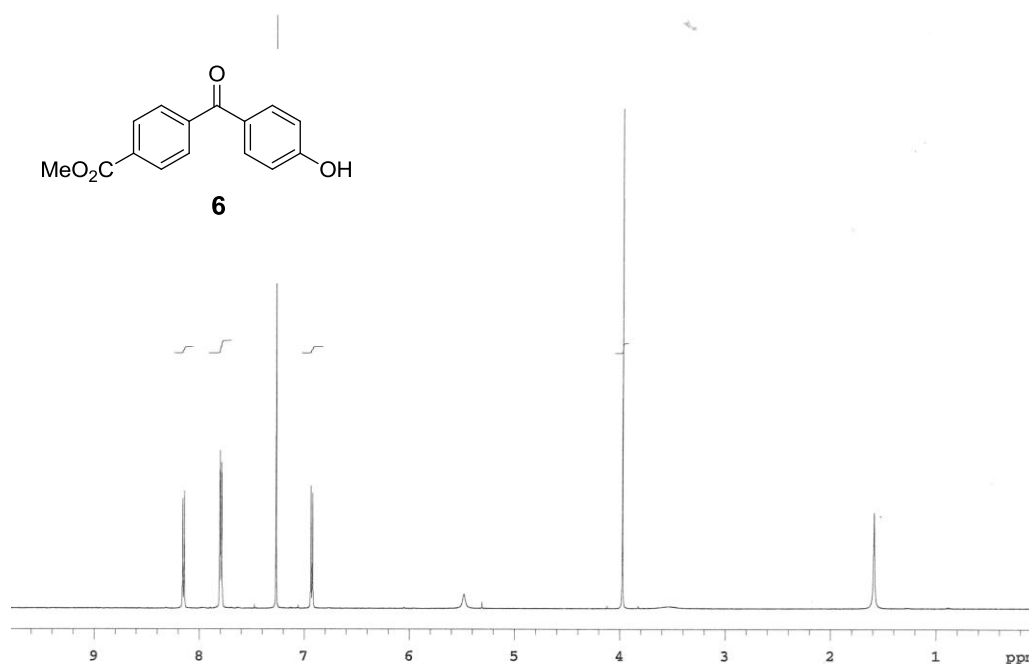
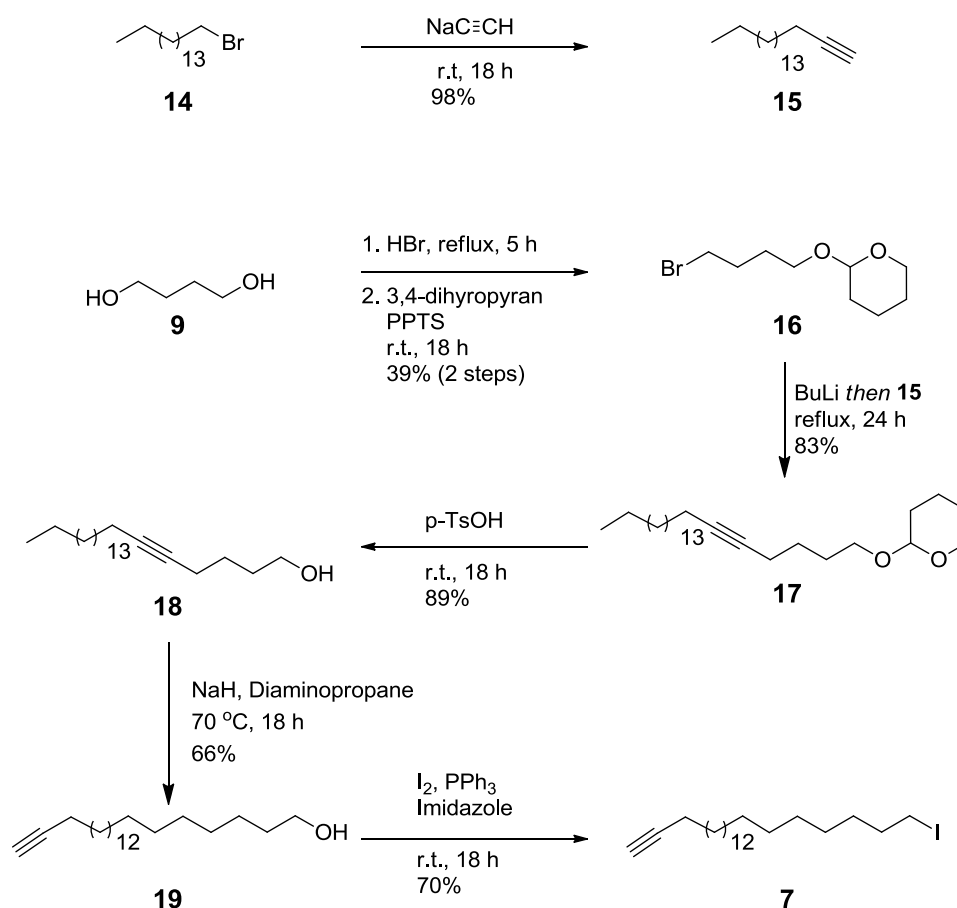


Figure 2. ^1H NMR spectrum of benzophenone **6**

To prepare the iodide **7**, a convergent synthetic strategy was proposed (Scheme 3). First, bromo-hexadecane (**14**) was reacted with sodium acetylide in order to form 1-octadecyne (**15**). The concentration of the reaction mixture proved critical in obtaining optimal yields for this reaction, with the use of either THF or DMF at 5 mL/mmol resulting in no product formation and starting material being recovered from the reaction mixture. However, when the reaction was carried out in DMF at 1.1 mL/mmol,⁸ 98% yield of alkyne **15** was achieved. The other key intermediate required for the synthesis of iodide **7** was the THP-protected bromide **16**. To this end, 1,4-butanediol (**9**) was reacted with hydrogen bromide under Dean-Stark conditions to form corresponding mono-substituted bromide.⁹ Though this reaction was low yielding (*c.a.* 40%) the amount of product obtained was comparable to literature when either toluene or benzene was used as a solvent. As TLC of the reaction mixture showed complete consumption of the starting material, one possible reason for this low yield could be due to further reaction of the mono-substituted bromide with HBr resulting in formation of THF, which could have been lost during work-up and subsequent removal of the solvent *in vacuo*. Treatment of the desired mono-brominated intermediate with 3,4-dihydropyran and pyridinium *para*-toluenesulfonate (PPTS), resulted in formation of tetrahydropyran (THP) ether **16** in excellent (97%) yield.¹⁰

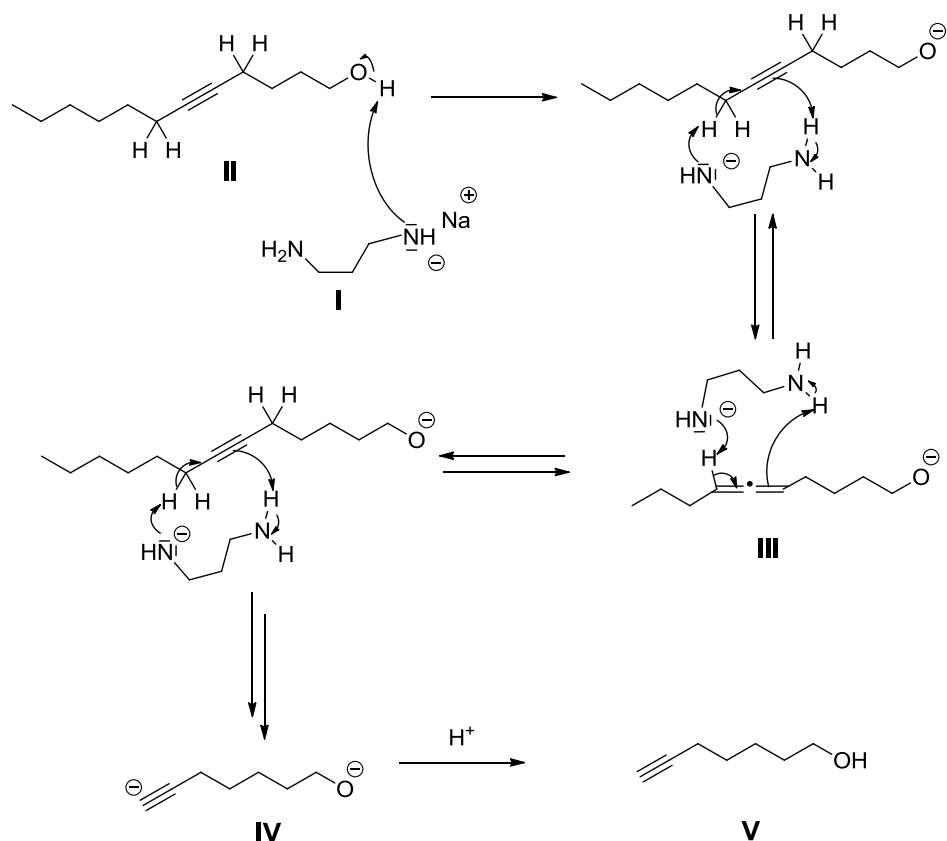


Scheme 3. Synthesis of iodide **7**

With the required THP ether **16** in hand, a BuLi-mediated alkylation with octadecyne **15** was then attempted. This proceeded smoothly with alkyne **17** being formed in a high (83%) yield. Subsequent removal of the THP protecting group in **17** using *para*-toluenesulfonic acid (PTSA) resulted in alcohol **18**, which was then isomerised by way of a zipper reaction to form alcohol **19**.

The mechanism of the zipper reaction can be explained by a series of allene/alkyne interconversions (Scheme 4).¹¹ The first step in the process involves reacting a strong base (*e.g.* NaH) with diaminopropane to form sodium 3-propylamide (**I**), which subsequently deprotonates the hydroxyl moiety in alcohol **II**. This deprotonation is followed by a concerted mechanism whereby the distal carbon adjacent to the alkyne is deprotonated, and allene **III** is formed. The reaction then goes through a series of allene/alkyne interconversions all of which are in equilibrium. The final product is

trapped as a very stable alkyne anion (**IV**) which is protonated in the work-up to form the terminal alkyne product (**V**). The stability of alkyne anion **IV** is attributed to the presence of the two negative charges on opposite end of the molecule, minimising charge repulsion and thus enhancing the stability of the anion.



Scheme 4. Zipper reaction for isomerisation of triple bonds

Comparison of the ^1H NMR spectral data for alcohol **18** and **19** clearly illustrates the migration of the triple bond (Figure 3). In the ^1H NMR spectrum for alcohol **18**, characteristic peaks include triplets at 3.68 ppm ($J = 6.4$ Hz) for H-1 and at 0.89 ppm ($J = 7.0$ Hz) for H-22 and the two resonances at 2.20 ppm (tt, $J = 7.1$ Hz, $^5J = 2.2$ Hz) and 2.14 ppm (tt, $J = 6.9$ Hz, $^5J = 2.2$ Hz) for H-4 and H-7 corresponding to the two methyl protons flanking the triple bond. In the spectrum for alcohol **19**, however, isomerisation of the triple bond is clearly evident by the presence of a triplet at 1.94 ppm ($^4J = 2.6$ Hz) accounting for the terminal alkyne proton and the absence of the peak at approximately 0.89 ppm, (correlating to H-22 in **18**). To complete the

synthesis of target iodide **7**, alcohol **19** was then treated with iodine, triphenylphosphine, and imidazole to give **7** in 70% yield.

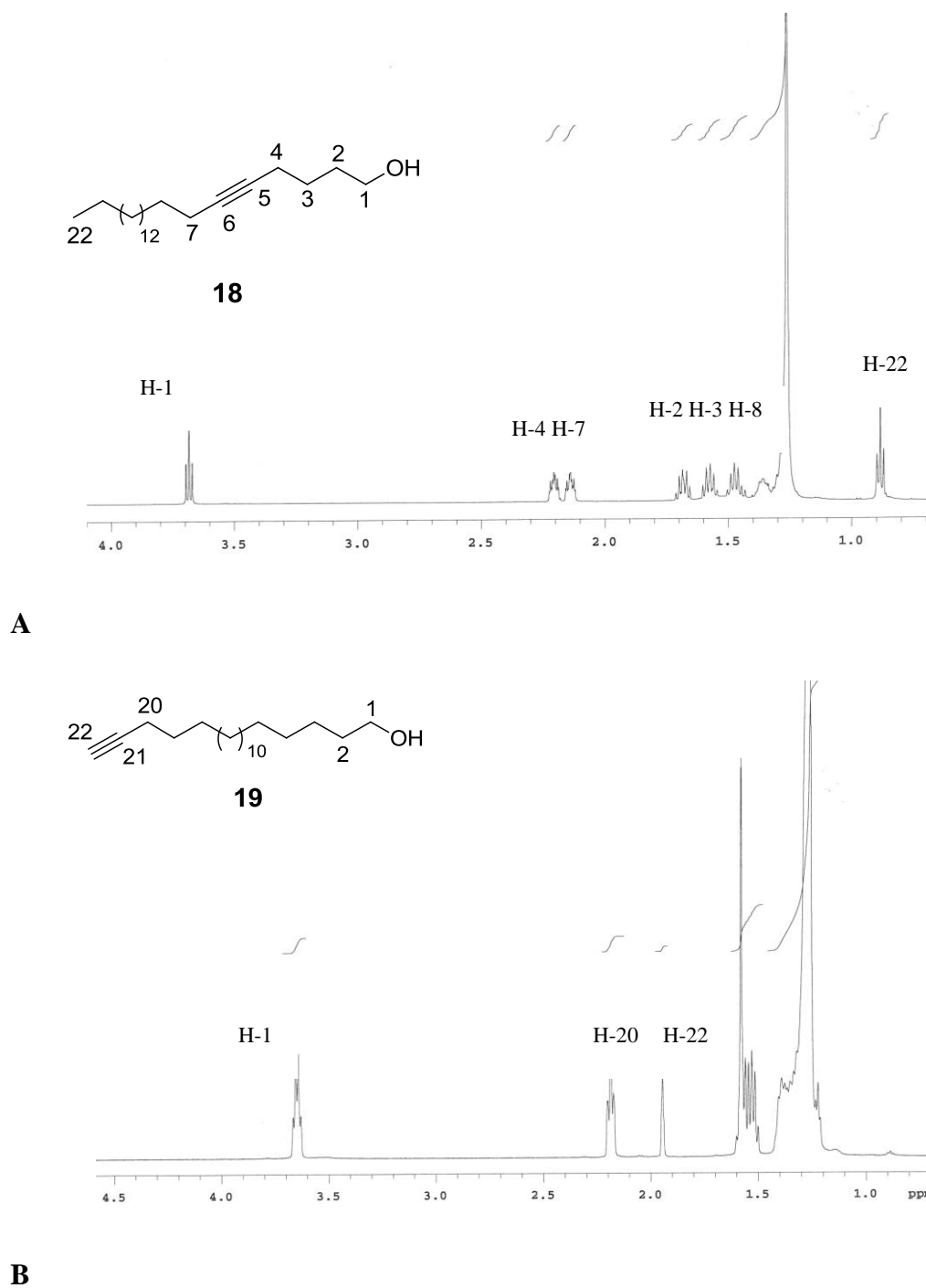
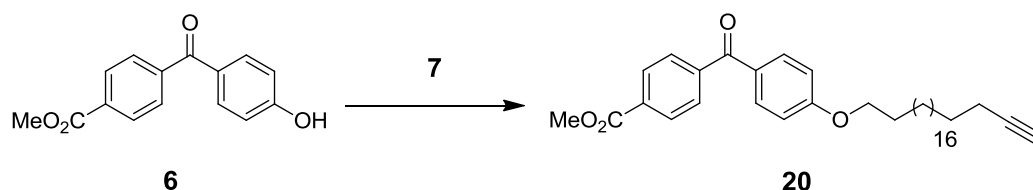


Figure 3. ^1H NMR spectra of compounds **18** and **19**

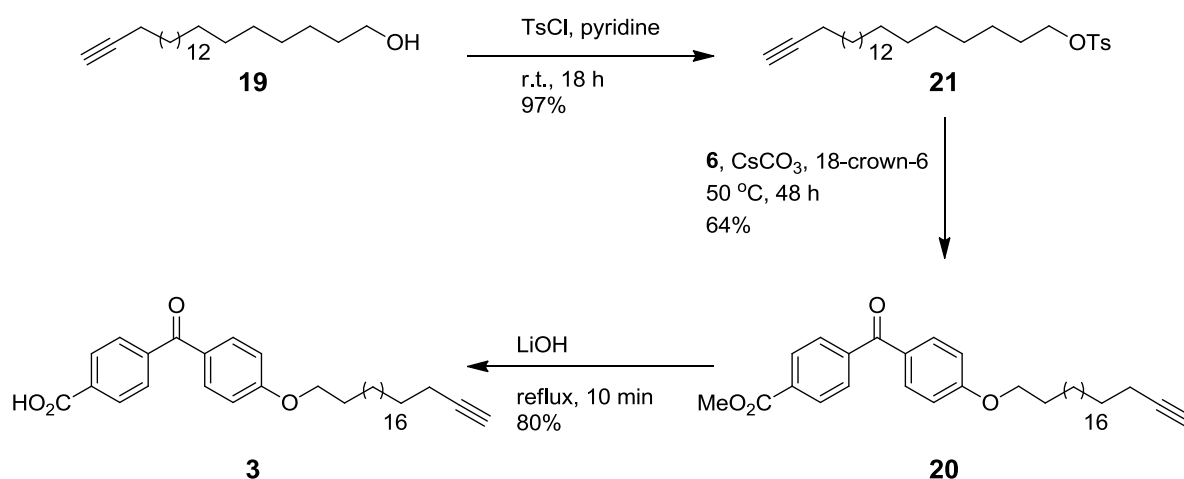
Having prepared iodide **7**, coupling with benzophenone **6** was then attempted (Table 1). First, sodium hydride (1.1 equiv.) was used as a base and the reaction was carried out in toluene (entry 1). Unfortunately, this only gave 10% of the alkylated product **20**, with mainly starting material being obtained from the reaction mixture. Increasing the amount of NaH (2.2 equiv.) did not lead to any significant improvement in the yield (entry 2) and in addition, degradation of the alkyl iodide **7** was observed. Use of K₂CO₃ along with 18-crown-6 ether also did not lead to any significant increase in yield (entry 3), however with caesium carbonate a small increase in product yield was achieved (entry 4). It was thought that one of the reasons for this low yield could be the low solubility of compound **6** in toluene, therefore the solvent system was changed to toluene:DMF (1:1) (entry 5). Indeed this led to an increased yield (24%), though as the result was still modest we turned our attention to the use of a lipid containing a different leaving group.

Table 1. Alkylation conditions for coupling benzophenone **6** and iodide **7**



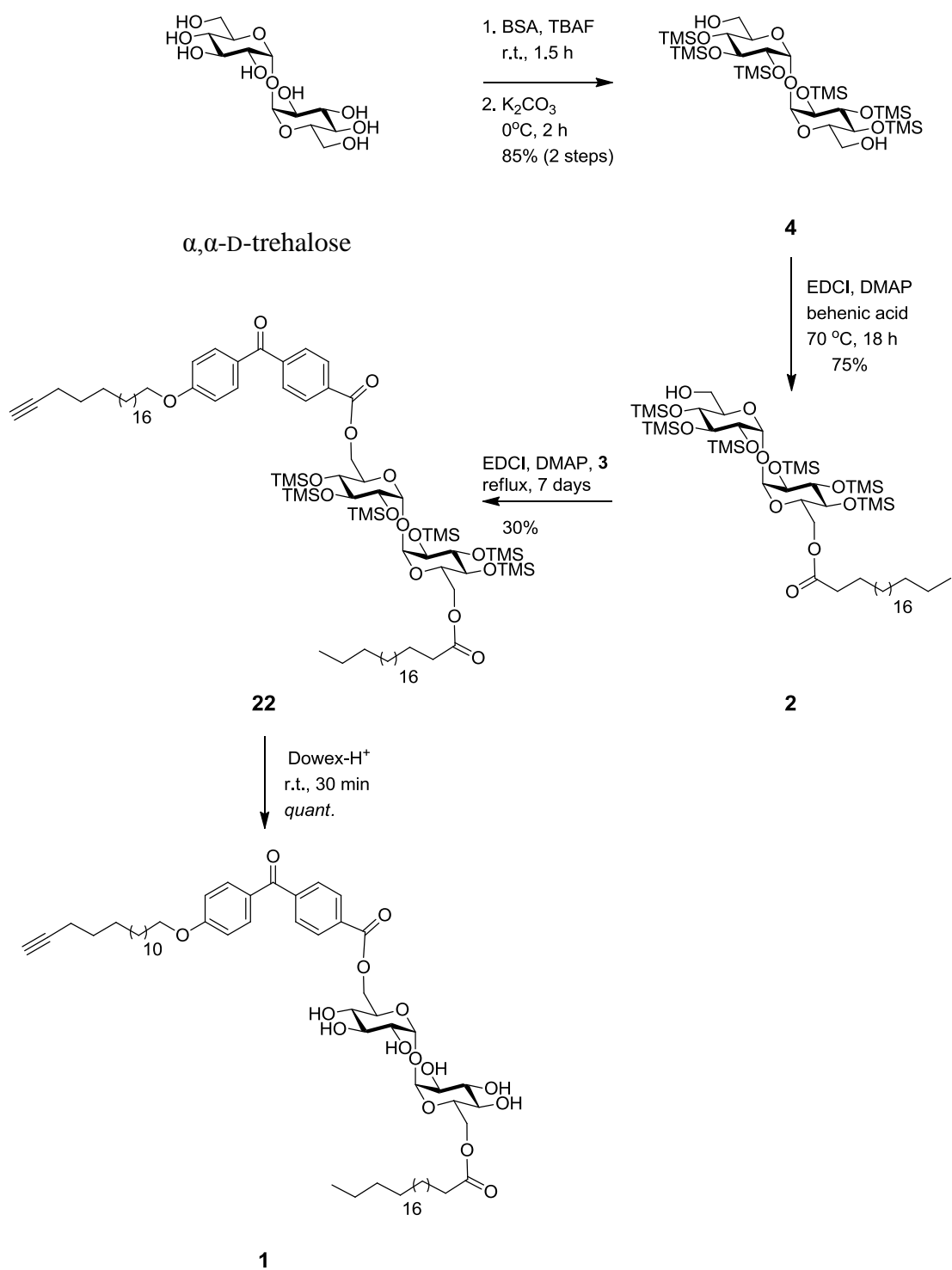
Entry	Conditions	Product yield (%)
1	NaH (1.1 equiv.), toluene, r.t.	10
2	NaH (2.2 equiv.), toluene, r.t to 70 °C	12
3	K ₂ CO ₃ (1.1 equiv.), 18-crown-6, toluene, 70 °C	12
4	CsCO ₃ (1.1 equiv.), 18-crown-6, toluene, 70 °C	16
5	CsCO ₃ (1.1 equiv.), 18-crown-6, DMF:toluene 1:1, 50 °C	24

It was proposed that a triflate group, a good leaving group, would make the adjacent carbon electrophile ‘hard’ which is desired in this instance since in *O*-alkylation the nucleophile is an oxygen atom which is a ‘hard’ nucleophile. Accordingly, alcohol **19** was reacted with triflic anhydride and pyridine, with TLC analysis of the reaction mixture after 45 minutes indicating complete consumption of starting material. Unfortunately, the triflate product could not be purified with hydrolysis of the product to alcohol **19** observed after silica gel column chromatography. At this point the tosylate leaving group was chosen, as in addition to being a good leaving group, it is more stable than the triflate group. Thus alcohol **19** was reacted with tosyl chloride and pyridine¹² providing tosylate **21** in 97% yield (Scheme 5). Tosylate **21** was then subjected to alkylation with benzophenone **6** in the presence of NaH (1.1 equiv.). This resulted in slightly improved but still modest 30% yield of the alkylated product **20**. However, a change of base to CsCO₃ (1.1 equiv.), along with the addition of 18-crown-6 ether, resulted in good (64%) product yield, which was deemed sufficient to continue on with the synthesis. Subsequent hydrolysis of the methyl ester in product **20** with LiOH, led to carboxylic acid **3** in good (80%) yield. Here, it should be noted that the hydrolysis was performed under reflux to ensure complete solubilisation of the starting material. In addition, the reaction needed to be stopped following complete consumption of the starting material to prevent product degradation.



Scheme 5. Synthesis of benzophenone building block **3**

With the synthesis of the benzophenone building block **3** established, our focus then turned to the synthesis of the trehalose building block **2**. Using a protocol previously discussed (see Chapter 2), commercially available α,α -D-trehalose was per-silylated using *N,O*-bis-trimethylsilylacetamide (BSA) and catalytic tetra-butyl ammonium fluoride (TBAF), and the more labile primary trimethylsilyl (TMS) ethers were then selectively removed *via* the addition of K_2CO_3 to give the trehalose diol **4** (Scheme 6). Esterification of diol **4** with behenic acid **5** (obtained from Jones oxidation of 1-docosanol), *via* the mediation of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDCI) and 4-dimethylamino-pyridine (DMAP) led to the mono-trehalose ester **2** in 75% yield. Compound **2** was then subjected to a second esterification, this time using benzophenone **3**. Here, it was interesting to note that this coupling was quite slow with the reaction only occurring when the mixture was refluxed in toluene over seven days. Even then, only a modest (30%) yield of the protected probe **22** was obtained, which was then subjected to Dowex- H^+ in order to liberate target AfBP **1** in quantitative yield.



Scheme 6. Total synthesis of A β BP probe **1**

The ^1H NMR spectrum of A/BP probe **1** clearly displayed all the required resonances (Figure 4). The key peaks in this instance are the two anomeric signals which were present as doublets at 5.84 (d, $J = 3.6$ Hz) and 5.83 ppm (d, $J = 3.6$ Hz) indicative of the asymmetrical nature of the trehalose moiety. The presence of the aromatic resonances for the benzophenone protons are clearly evident as doublets at 8.20 ($J = 8.2$ Hz), 7.86 ($J = 8.8$ Hz), 7.68 ($J = 8.2$ Hz) and 7.06 ppm ($J = 8.8$ Hz). The terminal alkyne proton had a triplet resonance at 2.68 ppm ($^4J = 2.7$ Hz) with the ether protons present as a triplet at 3.96 ppm ($J = 6.3$ Hz). Mass spectral analysis of the product was in agreement with the expected mass, [HRMS(ESI) m/z calcd. for $[\text{C}_{70}\text{H}_{112}\text{O}_{15}+\text{Na}]^+$:1215.7893, obsd.:1215.7897] and full assignment was made on the basis of 2D NMR (COSY, HSQC and HMBC) (see experimental for full assignment). The NMR analysis was carried out using deuterated pyridine as the solvent due to the amphiphilic nature of the molecule which made solubilisation of the compound difficult in any other solvent system.

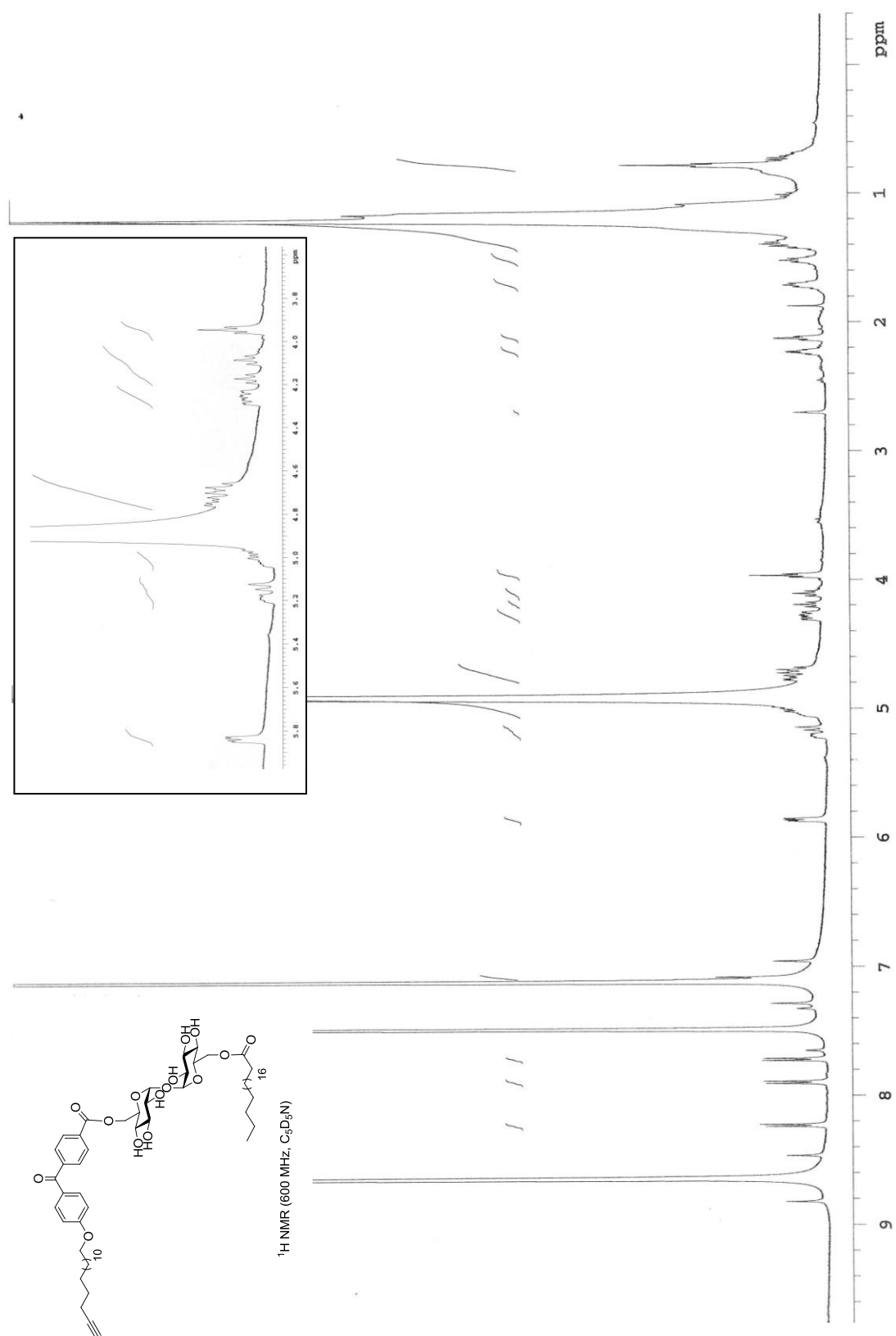


Figure 4. ^1H NMR spectrum of A/BP probe 1

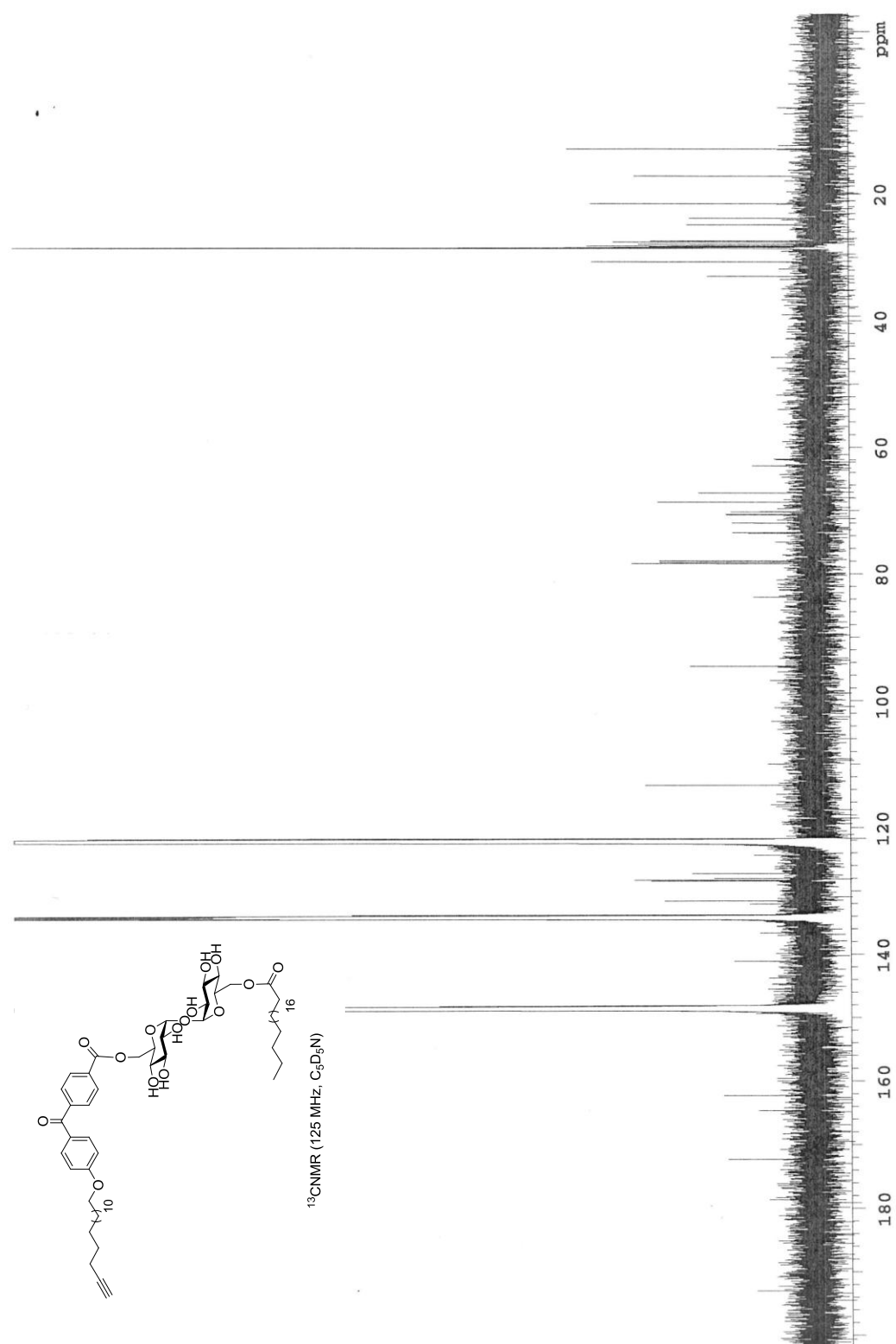


Figure 5. ^{13}C NMR spectrum of A/BP probe 1

6.3 Biological Testing

With AfBP probe **1** in hand, the ability of the probe to activate bone marrow macrophages (BMM) was investigated. To this end, bone marrow cells were isolated from mice and plated out in 24 well plate at 2.5×10^5 cell/mL to a total of 1 mL in each well and differentiated into macrophages over 10 days (see experimental section for more details). The macrophages were primed with 10 ng/mL IFN- γ for 3 hours after which time they were stimulated with probe **1** or C22 trehalose diester (C22 TDE) at 20 μ g/mL and 40 μ g/mL. LPS was used as a positive control in the experiments. The supernatants were analysed after 96 hours for nitric oxide (NO) production (Figure 6). As seen, the probe did activate macrophages in a concentration dependent manner. Although the activation by probe **1** was lower than that by the C22 TDE, it was still significant ($p = 0.005$ at 40 μ g/mL).

The reduced activity of probe **1** when compared to C22 TDE could be due to the presence of the benzophenone group next to the trehalose sugar unit in the probe. Since it is already known that Mincle is a carbohydrate recognising receptor, the presence of the benzophenone group next to trehalose could hinder the binding of the probe to this receptor on macrophages. However, probe **1** needs to be tested in proteome profiling experiments to deduce its potential as an AfBP probe.

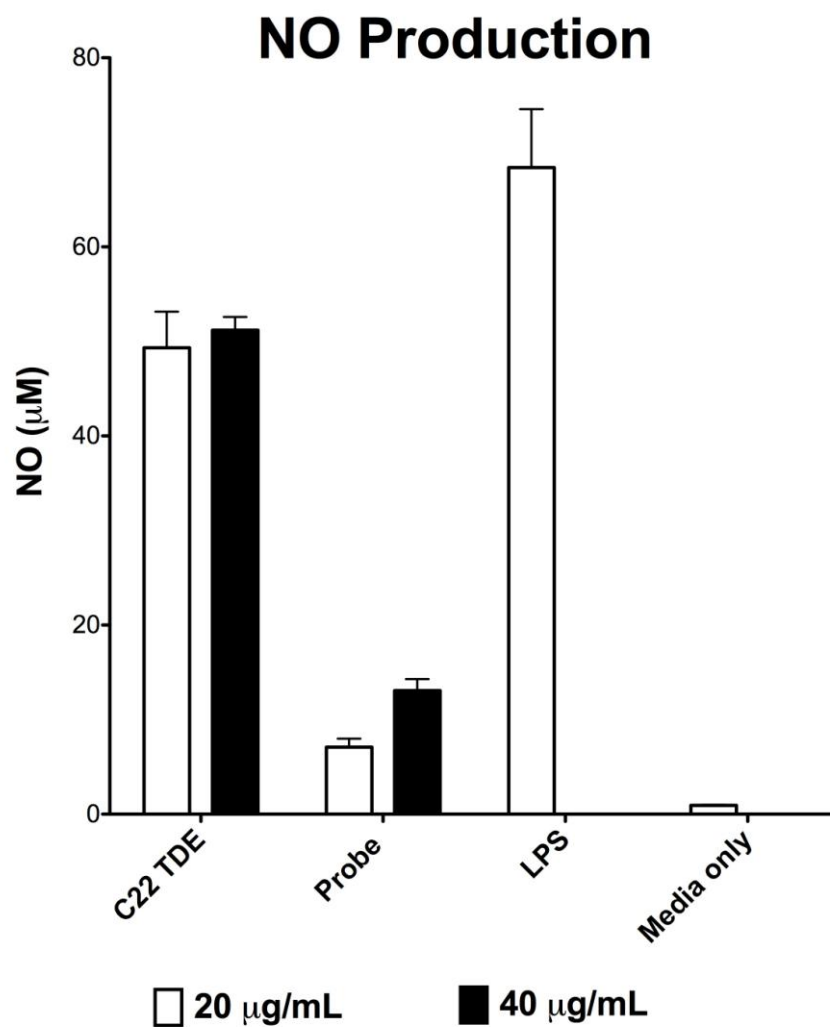


Figure 6. NO production by BMM treated with A/BP probe **1**. BMM were stimulated with 20 or 40 µg/mL of probe **1** or C22 TDE or with LPS (100 ng/mL). At 96 h the supernatants were analysed for NO using Griess assay.

6.4 Future Work

With the initial macrophage assay completed, the next step is to test the probe in proteome profiling experiments in order to deduce its usefulness for identifying proteins that trehalose glycolipids bind to. These experiments will be done *in vitro*, whereby BMM will be stimulated using probe **1**, followed by light mediated attachment of probe **1** to the protein/s it interacts with on macrophages (Figure 7). The alkyne functionality would be subjected to a click reaction with an azide linked biotin molecule. Following this, the probe-protein-azide-biotin complex would undergo streptavidin enrichment, trypsin digestion, and analysis with liquid chromatography/mass spectrometry (LC/MS) and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis in order to elucidate the identity of the linked proteins.

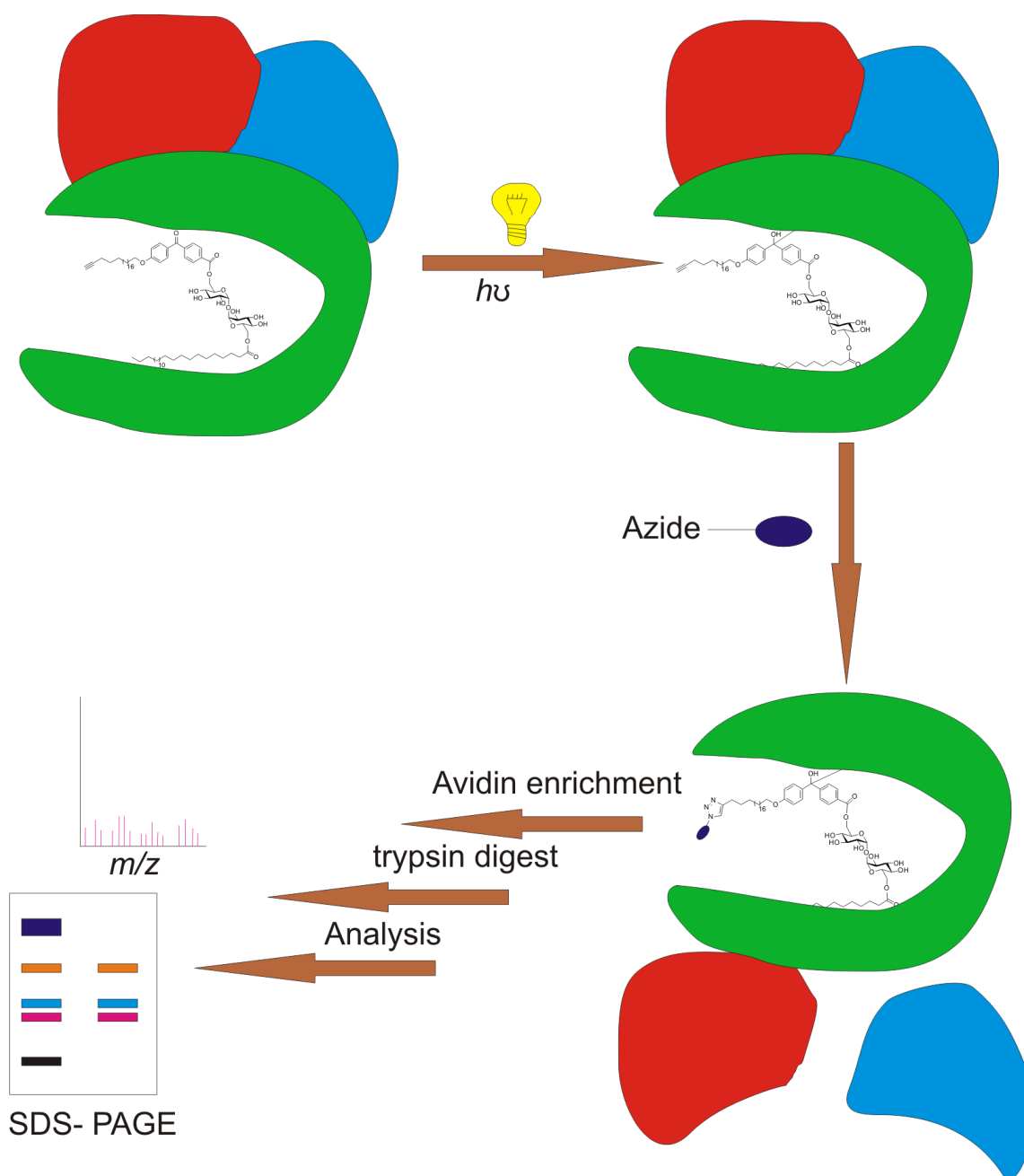


Figure 7. A/BP probe **1** in Affinity Based Profiling

6.5 Conclusion

The synthesis of an affinity based probe has been achieved using a convergent synthetic strategy in an overall 1.4% yield. The key steps in the synthesis included a Friedel-Crafts acylation for the formation of the benzophenone building group, and a zipper reaction for formation of the terminal alkyne functionality. Final coupling was achieved by way of an EDCI, DMAP-mediated esterification of the C22 trehalose mono-ester with the benzophenone based carboxylic acid. The probe was able to activate BMM albeit at lower levels than the C22 trehalose diester.

6.6 Experimental

6.6.1 Material and methods for Biological Assays

C57BL/6 male mice were bred and housed in a conventional animal facility at the Malaghan Institute of Medical Research, Wellington, New Zealand. All animals used for the experiments were aged between 8-10 weeks. All experimental procedures were approved by the Victoria University Animal Ethics Committee in accordance with their guidelines for the care of animals. Synthesised TDEs were tested to be endotoxin-free (≤ 0.125 EU/mL) with an endotoxin kit (Pyrotell, Limulus Amebocyte Lysate).

Generation of bone marrow derived macrophages (BMMs): Bone marrow cells were collected from the tibia and femur of C57BL/6 mice and were cultured (250 000 cells/mL) in Iscove's Modified Dulbecco's Media (IMDM-Gibco) supplemented with 5% FBS (Gibco), 1% Penicillin streptomycin (Gibco) and 55 μ M 2-mercaptoethanol (Invitrogen) containing 10 ng/mL GM-CSF (clone X63/GM-CSF murine cells). Cells were incubated at 37 °C (5% CO₂) and the media changed on

days 2, 5 and 7. On day 10, the media was removed and the BMM were primed with 10ng/mL IFN- γ (Peprotech) for 3 h prior to the addition of the compounds.

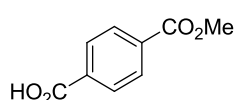
BMM Assay: C22 TDE and A/BP probe **1** stock solutions were prepared (2.5 mg/mL in PBS containing 2% DMSO), vortexed and warmed to 50 °C for 30 min (x 3) to ensure complete solubilisation of the compounds prior to administration to BMM cultures. BMM cultures were then treated with 20 or 40 μ g/mL of C22 TDE, or TDM probe **1** with LPS (100 ng/mL) as a positive control and media only as a negative control for the times indicated. Supernatants were collected and tested immediately for NO levels.

NO analysis: NO levels in supernatants were determined using the Griess assay.

6.6.2 Chemistry Experimental

Unless stated otherwise, all reactions were performed under N₂. Prior to use, THF was distilled from Na wire and benzophenone, pyridine was dried and stored over 4 Å molecular sieves (4 Å MS), toluene was dried and stored over Na wire and CH₂Cl₂ was distilled from P₂O₅. Terephthalic acid (BDH), thionyl chloride (Acros), KOH (Science), 33% HCl (Panreac), H₂SO₄ (Panreac) AlCl₃ (Sigma-Aldrich), sodium acetylide (Aldrich, 18% in xylene), bromohexadecane (BDH), 1,4-butanediol (BDH), HBr (Acros), PPTS (Aldrich), dihydropyran (Pfaltz and Bauer), *n*-BuLi (Aldrich, 2M solution in hexanes), PTSA (Sigma-Aldrich), diaminopropane (Aldrich), tosyl chloride (Aldrich), 18-crown-6 ether (Sigma-Aldrich), CrO₃ (M&B), NaH (Avocado Research Chemicals, 60% dispersion in mineral oil), D-(+)-trehalose dihydrate (Sigma), TBAF (Aldrich), BSA (Fluka), Dowex-H⁺ (Supelco), 1-docosanol (Aldrich), EDCI (Aldrich), DMAP (Merck), anhydrous DMF (Acros), anhydrous Et₂O (Biolab), EtOAc (Panreac), petroleum ether (Pure Science), CHCl₃ (Panreac), anhydrous MeOH (Panreac), isopropanol (Pure Science), NaHCO₃ (Pure Science), K₂CO₃ (Pure Science), MgSO₄ (Pure Science) and NaCl (Panreac) were used as received.

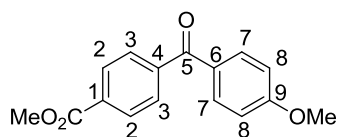
All solvents were removed *via* evaporation at reduced pressure. Reactions were monitored by TLC analysis on Macherey-Nagel silica gel coated plastic sheets (0.20 mm, Polygram SIL G/UV254) with detection by UV-absorption (short wave UV–254 nm; long wave UV–366 nm); coating with a solution of 5% KMnO₄ and 1% NaIO₄ in H₂O followed by heating or by dipping in 10% H₂SO₄ in EtOH followed by charring at ~150 °C. Column chromatography was performed using Pure Science silica gel (40–63 µm). High resolution mass spectra were recorded on a Waters Q-TOF Premier™ Tandem Mass Spectrometer using positive electro-spray ionisation. Optical rotations were recorded on a Perkin-Elmer 241 polarimeter or Autopol II (Rudolph Research Analytical) at 589 nm (sodium D-line). Infrared spectra were recorded as thin films using a Brüker Tensor 27 FTIR spectrometer equipped with an Attenuated Total Reflectance (ATR) sampling accessory and are reported in wave numbers (cm⁻¹). Nuclear magnetic resonance spectra were recorded at 20 °C in CDCl₃ or C₅D₅N using a Varian INOVA operating at 500 MHz. Chemical shifts are given in ppm (δ) relative to residual solvent peak. NMR peak assignments were made using COSY, HSQC and HMBC 2D experiments.



Monomethyl terephthalate (11). A solution of terephthalic acid (**8**) (10 g, 60.2 mmol) in MeOH (250 mL) was refluxed for 30 min after which time SOCl₂ (87.2 mL, 1.20 mol) was added and the reaction mixture was refluxed for a further 10 h. The reaction mixture was then cooled and the solvent removed *in vacuo*. The product was extracted with Et₂O (3 x 200 mL) and washed with saturated NaHCO₃ (3 x 60 mL) solution. The combined organic layers were dried with MgSO₄ and concentrated *in vacuo* to yield dimethyl terephthalate (**10**) as white solid (*quant.*) and it was used in subsequent reaction without further purification.

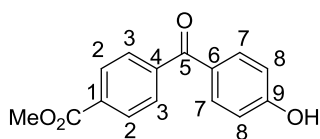
Dimethyl terephthalate (**10**) (9.77 g, 50.2 mmol) was dissolved in MeOH (165 mL) and the reaction mixture was refluxed for 30 min after which time it was cooled to r.t. followed by KOH addition (3.11 g, 55.4 mmol). The reaction was then heated at reflux overnight after which time the mixture was cooled and the solvent removed *in vacuo*. The resulting crude product was dissolved in water and washed with CH₂Cl₂

(3 x 100 mL). The aqueous layer was then neutralised with concentrated 1M HCl and the precipitate was extracted with Et₂O, dried with MgSO₄, and concentrated to yield the title compound as a white solid (7.86 g, 43.7 mmol, 87% over two steps). The spectral data obtained matched that reported in literature.⁴



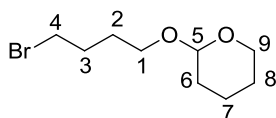
Methyl 4-(4-methoxybenzoyl)-benzoate (13). SOCl₂ (3.92 mL, 53.9 mmol) was added to acid **11** (2.44 g, 13.9 mmol) and the reaction mixture was refluxed until a

clear solution had formed (*c.a.* 4 h). The solvent was reduced *in vacuo* (under nitrogen), and the resulting acid chloride **12** (2.76 g, 13.9 mmol) was dissolved in CHCl₃ (71 mL) and anisole (1.51 mL, 13.9 mmol) and AlCl₃ (1.85 g, 13.9 mmol) were added. The reaction mixture was then refluxed for 10 h, after which time another portion of AlCl₃ (1.85 g, 13.9 mmol) was added and the mixture refluxed for a further 10 h. Following this, the solution was cooled, quenched with H₂O (100 mL), and extracted with EtOAc (3 x 80 mL). The combined organic layers were washed with brine (50 mL), dried over MgSO₄, and the solvent removed *in vacuo* to yield the crude product. The title compound was obtained after silica gel flash chromatography (eluting in 20:1, Pet. Ether/EtOAc, v/v) as a white solid (2.60 g, 9.62 mmol, 70%); *R_f* = 0.76 (EtOAc); IR (film): 2944, 2832, 1448, 1022, 737 cm⁻¹; ¹H NMR (500 MHz, CDCl₃) δ 8.15 (d, *J*_{2,3} = 8.5 Hz, 2H, H-2), 7.83 (d, *J*_{7,8} = 8.7 Hz, 2H, H-7), 7.79 (d, *J*_{2,3} = 8.5 Hz, 2H, H-3), 6.98 (d, *J*_{7,8} = 8.7 Hz, 2H, H-8), 3.98 (s, 3H, CO₂Me), 3.91 (s, 3H, OMe); ¹³C NMR (125 MHz, CDCl₃) δ 194.8 (C-5), 166.4 (CO₂Me), 163.6 (C-9), 142.2 (C-4), 132.8 (C-1), 132.6 (C-7), 129.6 (C-6), 129.4 (C-2 and C-3), 113.8 (C-8), 55.6 (-OCH₃), 52.4 (CO₂Me); HRMS(ESI) *m/z* calcd. for [C₁₆H₁₄O₄+Na]⁺: 293.0790, obsd.: 293.0790.



Methyl 4-(4-hydroxybenzoyl)-benzoate (6). AlCl₃ (1.46 g, 11.0 mmol) was added to a solution of benzophenone **13** (0.99 g, 3.66 mmol) in toluene (15 mL)

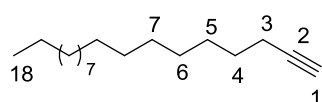
and the reaction mixture was stirred at r.t. for 30 min followed by 30 min at reflux. The reaction was then cooled to r.t., followed by further addition of AlCl_3 ((1.46 g, 11.0 mmol) and 30 min at reflux. The reaction mixture was then poured into 1:1 ice/ H_2O mixture, stirred for a further 30 min and extracted with EtOAc (3 x 50 mL). The combined organic layers were washed with brine, dried (MgSO_4), and concentrated *in vacuo*. The title compound was obtained after silica gel flash chromatography (eluting in 10:1, Pet. Ether/EtOAc, v/v) as a white solid (0.80 g, 3.13 mmol, 85%). R_f = 0.24 (2/1, PE/EA, v/v); IR (film): 3317, 2944, 2832, 1448, 1022, 741 cm^{-1} ; ^1H NMR (500 MHz, CDCl_3) δ 8.15 (d, $J_{2,3}$ = 8.3 Hz, 2H, H-2), 7.79 (d, $J_{2,3}$ = 8.3 Hz, 2H, H-3), 7.79 (d, $J_{7,8}$ = 8.5 Hz, 2H, H-7), 6.93 (d, $J_{7,8}$ = 8.5 Hz, 2H, H-8), 3.98 (s, 3H, CO_2Me); ^{13}C NMR (125 MHz, CDCl_3) δ 194.8 (C-5), 166.4 (CO_2Me), 160.0 (C-9), 142.0 (C-4), 133.0 (C-7), 132.8 (C-1), 129.8 (C-6), 129.47 (C-2), 129.46 (C-3), 115.3 (C-8), 52.4 (CO_2Me); HRMS(ESI) m/z calcd. for $[\text{C}_{15}\text{H}_{12}\text{O}_4+\text{Na}]^+$: 279.0626, obsd.: 279.0633.



2-(4-Bromobutyloxy)-tetrahydropyran (16). To a solution of 1,4-butanediol (4.46 mL, 50.3 mmol) in toluene (70 mL) was added a 48% HBr solution (6.25 mL, 55.2 mmol) and

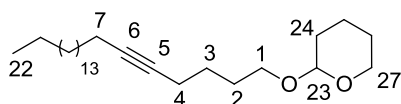
the reaction mixture was refluxed using a Dean-Stark apparatus, until all the starting material had been consumed (as determined from TLC analysis). The solution was then cooled, extracted with CH_2Cl_2 (3 x 60 mL), washed with water, dried with MgSO_4 , and the solvent removed *in vacuo* to yield 4-bromo-1-butanol (3.06 g, 20.1 mmol, 40%), which was used in the consecutive reaction without further purification. To a solution of 4-bromo-1-butanol (2.0 g, 13.0 mmol) in CH_2Cl_2 (30 mL) was added pyridinium *para*-toluenesulfonate (PPTS) (0.65 g, 2.61 mmol) and 3,4-dihydropyran (2.77 mL, 30.4 mmol) and the reaction was stirred at r.t. overnight. The reaction mixture was then quenched with H_2O (20 mL) and extracted with EtOAc (3 x 40 mL). The combined organic layers were washed with brine (30 mL), dried with MgSO_4 , and the solvent reduced *in vacuo*. The title compound was obtained after silica gel flash chromatography (eluting in 50:1, Pet. Ether/EtOAc, v/v) as a white solid (3.03 g, 12.7 mmol, 97%). R_f = 0.83 (1/1, PE/EA, v/v); IR (film): 2940, 2860,

1133, 1075, 904, 668 cm^{-1} ; ^1H NMR (500 MHz, CDCl_3) δ 4.57 (t, $J_{5,6a} = J_{5,6b} = 2.5$ Hz, 1H, H-5), 3.84 (ddd, $J_{9a,9b} = 11.0$ Hz, $J_{8a,9a} = J_{8b,9a} = 2.4$ Hz, 1H, H-9a), 3.76 (dt, $J_{1a,1b} = 9.8$ Hz, $J_{1a,2a} = J_{1a,2b} = 6.6$ Hz, H-1a), 3.52–3.48 (m, 1H, H-9b), 3.45 (t, $J_{3,4} = 7.1$ Hz, 2H, H-4), 3.41 (dt, $J_{1a,1b} = 9.8$ Hz, $J_{1b,2a} = J_{1b,2b} = 6.1$ Hz, 1H, H-1b), 1.97 (pentet, $J_{\alpha,\beta} = J_{\beta,\gamma} = 7.1$ Hz, 2H, H-3), 1.83–1.78 (m, 1H, H-7a), 1.76–1.71 (m, 1H, H-2), 1.71–1.68 (m, 1H, H-6a), 1.60–1.50 (m, 4H, H-6b, H-7b, H-8); ^{13}C NMR (125 MHz, CDCl_3) δ 98.8 (C-5), 66.5 (C-1), 62.3 (C-9), 33.8 (C-4), 30.7 (C-6), 29.8 (C-3), 28.4 (C-2), 25.4 (C-8), 19.6 (C-7); HRMS(ESI) m/z calcd. for $[\text{C}_9\text{H}_{17}\text{O}_2\text{Br}+\text{Na}]^+$: 259.0304, obsd.: 259.0318.



Octadecyne (15). A solution of bromo-hexadecane (**14**) (4.54 g, 14.9 mmol) in DMF (20 mL) was cooled to 0 °C and sodium acetylide (18% in xylene, 5.95 mL, 22.3

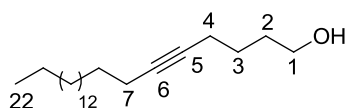
mmol) was added to it. The reaction mixture was stirred at r.t. overnight after which time it was diluted with Et_2O (100 mL), washed with water (50 mL), 1M HCl (50 mL), brine (25 mL) dried with MgSO_4 , and the solvent reduced *in vacuo*. Octadecyne (**15**) was obtained after silica gel flash chromatography (eluting in Pet. Ether), as a white solid (3.62 g, 14.5 mmol, 98%). $R_f = 0.50$ (Pet. Ether); IR (film): 3314, 2922, 2853, 2341, 1236, 668 cm^{-1} ; ^1H NMR (500 MHz, CDCl_3) δ 2.19 (dt, $J_{3a,3b} = 9.8$ Hz, $J_{3a,4} = 7.1$ Hz, 1H, H-3a), 2.18 (dt, $J_{3a,3b} = 9.8$ Hz, $J_{3b,4} = 7.1$ Hz, 1H, H-3b), 1.94 (t, $^4J_{1,3} = 2.6$ Hz, 1H, H-1), 1.55 (pentet, $J_{\alpha,\beta} = J_{\beta,\gamma} = 7.1$ Hz, 2H, H-4), 1.44–1.36 (m, 2H, H-5) 1.32–1.26 (m, 24H, H-6–H-17), 0.89 (t, $J_{17,18} = 6.8$ Hz, H-18); ^{13}C NMR (125 MHz, CDCl_3) δ 84.8 (C-2), 68.0 (C-1), 31.9, 29.70, 29.68, 29.66, 29.62, 29.52, 29.37, 29.12, 28.77, 28.50, 22.70 (C-4–C-17), 18.4 (C-3), 14.1 (C-18); HRMS(ESI) m/z calcd. for $[\text{C}_{18}\text{H}_{34}+\text{Na}]^+$: 273.2558, obsd.: 273.2550.



2-(5-Docosynyloxy)-tetrahydropyran (17). *n*-BuLi (8.28 mL, 2.0 M solution in hexanes, 16.6 mmol) was added to a solution of octadecyne (**15**)

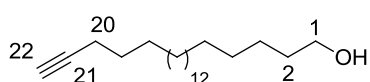
(2.07 g, 8.28 mmol) in THF (30 mL) at 0 °C and the reaction mixture was refluxed

for 10 h, after which time it was cooled to r.t. and THP ether **16** (4.97 g, 20.7 mmol) in THF (30 mL) was added. The reaction was refluxed for a further 24 h then quenched with H₂O (50 mL), extracted with Pet. Ether (3 x 60 mL), washed with brine (40 mL), dried with MgSO₄ and reduced *in vacuo*. The title compound was obtained after silica gel flash chromatography (eluting in 75:1, Pet. Ether/EtOAc, v/v) as colourless oil (2.8 g, 6.84 mmol, 83%). R_f = 0.69 (2/1, PE/EA, v/v); IR (film): 2922, 2852, 1363, 1186, 738, 704 cm⁻¹; ¹H NMR (500 MHz, CDCl₃) δ 4.58 (t, $J_{23,24a}$ = $J_{23,24b}$ = 2.9 Hz, 1H, H-23), 3.86 (ddd, $J_{27a,27b}$ = 11.1 Hz, $J_{26a,27a}$ = $J_{26b,27a}$ = 3.0 Hz, 1H, H-27a), 3.75 (dt, $J_{1a,1b}$ = 9.7 Hz, $J_{1a,2a}$ = $J_{1a,2b}$ = 3.9 Hz, 1H, H-1a), 3.51–3.48 (m, 1H, H-27b), 3.40 (dt, $J_{1a,1b}$ = 9.7 Hz, $J_{1b,2a}$ = $J_{1b,2b}$ = 3.1 Hz, 1H, H-1b), 2.19 (tt, $J_{3,4}$ = 7.1 Hz, $^5J_{4,7}$ = 2.2 Hz, 2H, H-4), 2.13 (tt, $J_{7,8}$ = 7.0 Hz, $^5J_{4,7}$ = 2.2 Hz, 2H, H-7), 1.86–1.77 (m, 1H, H-25a), 1.75–1.62 (m, 3H, H-24a and H-26), 1.61–1.50 (m, 6H, H-2, H-3, H-24b and H-25b), 1.47 (pentet, $J_{7,8}$ = $J_{8,9}$ = 7.3 Hz, 2H, H-8), 1.37–1.31 (m, 2H, H-9), 1.30–1.25 (m, 24H, H-10–H-21), 0.88 (t, $J_{1,2}$ = 6.8 Hz, 2H, H-22); ¹³C NMR (125 MHz, CDCl₃) δ 98.8 (C-23), 80.5 (C-6), 79.8 (C-5), 67.1 (C-1), 62.3 (C-27), 31.9, 30.7, 29.70, 29.68, 29.66, 29.65, 29.57, 29.4, 29.19, 29.16, 28.9, 28.9, 25.9, 25.5, 22.7 (C-2, C-3, C-8–C-21, C-24, C-26), 19.6 (C-25), 18.8 (C-7), 18.6 (C-4), 14.1 (C-22); HRMS(ESI) m/z calcd. for [C₂₇H₅₀O₂+Na]⁺: 429.3709, obsd.: 429.3713.



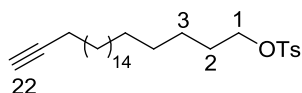
5-Docosyn-1-ol (18). To a solution of alkyne **17** (2.01 g, 4.91 mmol) in MeOH (20 mL) was added PTSA (0.28 g, 1.47 mmol) and the reaction mixture was stirred at r.t. overnight before being quenched with H₂O and extracted with EtOAc (3 x 50 mL). The combined organic layers were washed with H₂O (30 mL), brine (30 mL), dried with MgSO₄ and reduced *in vacuo*. The title compound was obtained after silica gel flash chromatography (eluting in 20:1, Pet. Ether/EtOAc, v/v) as a white solid (1.40 g, 4.34 mmol, 89%) R_f = 0.33 (5/1, PE/EA, v/v); IR (film): 2984, 1738, 1447, 1234, 1044 cm⁻¹; ¹H NMR (500 MHz, CDCl₃) δ 3.68 (t, $J_{1,2}$ = 6.4 Hz, 2H, H-1), 2.20 (tt, $J_{3,4}$ = 7.1 Hz, $^5J_{4,7}$ = 2.2 Hz, 2H, H-4), 2.14 (tt, $J_{7,8}$ = 6.9 Hz, $^5J_{4,7}$ = 2.2 Hz, 2H, H-7), 1.69 (pentet, $J_{\alpha,\beta}$ = $J_{\beta,\gamma}$ = 6.8 Hz, 2H, H-2), 1.57 (pentet, $J_{\alpha,\beta}$ = $J_{\beta,\gamma}$ = 6.9

Hz, 2H, H-3), 1.47 (pentet, $J_{\alpha,\beta} = J_{\beta,\gamma} = 7.2$ Hz, 2H, H-8), 1.40–1.32 (m, 2H, H-9), 1.29–1.22 (m, 24H, H-10–H-21), 0.89 (t, $J_{21,22} = 7.0$ Hz, 2H, H-22); ^{13}C NMR (125 MHz, CDCl_3) δ 80.8 (C-6), 79.7 (C-5), 62.6 (C-1), 31.93 (C-2), 31.88, 29.71, 29.68, 29.66, 29.65, 29.57, 29.37, 29.18, 29.15, 28.90, 25.35, 22.70 (C-3, C-8–C-21), 18.7 (C-7), 18.5 (C-4), 14.1 (C-22); HRMS(ESI) m/z calcd. for $[\text{C}_{22}\text{H}_{42}\text{O}+\text{Na}]^+$: 345.3133, obsd.: 345.3134.



21-Docosyn-1-ol (19). Diaminopropane (10 mL) was added to NaH (0.88 g, 22.0 mmol, 60% dispersion in

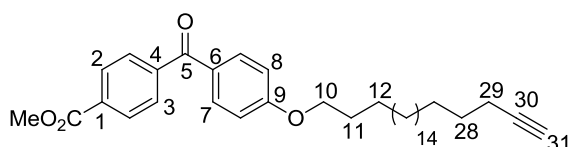
mineral oil) and the resulting mixture was stirred at 70 °C until gas evolution ceased (*c.a.* 1 h), after which time the mixture was cooled, and alcohol **18** (0.36 g, 1.10 mmol) dissolved in diaminopropane (5 mL), was added dropwise. The reaction was stirred at 70 °C overnight after which time it was quenched with H_2O and extracted with EtOAc (3 x 35 mL). The combined organic layer was then washed with water (25 mL), brine (25 mL), dried (MgSO_4) and the solvent reduced *in vacuo*. The title compound was obtained after silica gel flash chromatography (eluting in 20:1, Pet. Ether/EtOAc, v/v) as a white solid (3.1 g, 0.74 mmol, 66%) $R_f = 0.32$ (5/1, PE/EA, v/v); IR (film): 3287, 2917, 2849, 1122 cm^{-1} ; ^1H NMR (500 MHz, CDCl_3) δ 3.65 (t, $J_{1,2} = 6.6$ Hz, 2H, H-1), 2.19 (dt, $J_{20a,20b} = 11.5$ Hz, $J_{19,20a} = 7.1$ Hz, 1H, H-20a), 2.18 (dt, $J_{20a,20b} = 11.5$ Hz, $J_{19,20b} = 7.1$ Hz, 1H, H-20b), 1.94 (t, $^4J_{20,22} = 2.6$ Hz, 1H, H-22), 1.54 (pentet, $J_{\alpha,\beta} = J_{\beta,\gamma} = 6.8$ Hz, 2H, H-2), 1.51 (pentet, $J_{\alpha,\beta} = J_{\beta,\gamma} = 7.3$ Hz, 2H, H-19), 1.41–1.14 (m, 32H, H-3–H-18); ^{13}C NMR (125 MHz, CDCl_3) δ 84.8 (C-21), 68.0 (C-22), 63.1 (C-1), 32.8 (C-2) 29.70, 29.69, 29.66, 29.62, 29.51, 29.44, 29.12, 28.77, 28.50 (C-3–C-19), 22.74 (C-20); HRMS(ESI) m/z calcd. for $[\text{C}_{22}\text{H}_{42}\text{O}+\text{H}]^+$: 323.3314, obsd.: 323.3309.



21-Docosynyl *p*-toluenesulfonate (21). To a solution of alcohol **19** (0.20 g, 0.63 mmol) in CH_2Cl_2 (3 mL) at 0 °C was added pyridine (0.10 mL, 1.25 mmol) then TsCl (0.18

g, 0.94 mmol) and the reaction stirred at r.t. overnight. Et_2O (30 mL) and water (20

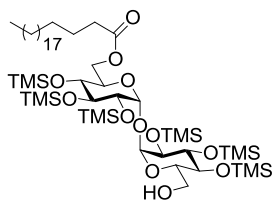
mL) were added to the reaction mixture and the organic layer was washed with 2M HCl (30 mL), followed by 5% NaHCO₃ (20 mL), water (30 mL), brine (30 mL) and then dried with MgSO₄. The solvent was removed *in vacuo* and the title compound obtained after silica gel flash chromatography (eluting in 50:1, Pet. Ether/EtOAc, v/v) as a white solid (0.28 g, 0.61 mmol, 97%). R_f = 0.46 (5/1, PE/EA, v/v); IR (film): 2917, 2850, 1472, 1173, 668 cm⁻¹; ¹H NMR (500 MHz, CDCl₃) δ 7.80 (d, J = 8.3 Hz, 2H, H_{arom}), 7.35 (d, J = 8.1 Hz, 2H, H_{arom}), 4.03 (t, $J_{1,2}$ = 6.6 Hz, 2H, H-1), 2.46 (s, 3H, CH₃), 2.19 (dt, $J_{20a,20b}$ = 13.1 Hz, $J_{19,20a}$ = 7.1 Hz, 1H, H-20a), 2.18 (dt, $J_{20a,20b}$ = 13.1 Hz, $J_{19,20b}$ = 7.1 Hz, 1H, H-20b), 1.95 (t, $^4J_{20,22}$ = 2.6 Hz, 1H, H-22), 1.64 (pentet, $J_{\alpha,\beta}$ = $J_{\beta,\gamma}$ = 6.8 Hz, 2H, H-2), 1.52 (pentet, $J_{\alpha,\beta}$ = $J_{\beta,\gamma}$ = 6.6 Hz, 2H, H-19), 1.40–1.38 (m, 2H, H-3), 1.34–1.22 (m, 30H, H-4–H-18); ¹³C NMR (125 MHz, CDCl₃) δ 144.6 (C_{ar}-CH₃), 129.8 (C_{ar}), 127.9 (C_{ar}), 84.8 (C-21), 70.7 (C-1), 68.0 (C-22), 29.70, 29.68, 29.65, 29.61, 29.50, 29.39, 29.11, 28.92, 28.81, 28.77, 28.50, 25.32 (C-2–C-19), 21.63 (CH₃), 18.4 (C-20); HRMS(ESI) m/z calcd. for [C₂₉H₄₈O₃S+Na]⁺: 499.3222, obsd.: 499.3222.



Methyl 4-[4-(21-docosynyloxy)benzoyl]-benzoate

(20). To a solution of benzophenone **6** (30.5 mg, 0.12 mmol) in 2 mL DMF was added Cs₂CO₃ (42.9 mg, 0.13 mmol), 18-crown-6-ether (37.8 mg, 0.14 mmol) and tosylate **21** (83.0 mg, 0.18 mmol) in toluene (2 mL) and the reaction was stirred at r.t. for 30 min and then heated to 50 °C over 2 nights. The reaction mixture was then cooled down, diluted with CH₂Cl₂ and water (20 mL). The aqueous layer was extracted with CH₂Cl₂ (2 x 30 mL) and the combined organic layers were dried with MgSO₄, and the solvent removed *in vacuo*. The title compound was obtained after silica gel flash chromatography (eluting in 30:1, Pet. Ether/EtOAc, v/v) as a white solid (43.0 mg, 0.08 mmol, 64 %); R_f = 0.63 (2/1, PE/EA, v/v); IR (film): 2916, 2848, 1638, 1604, 1256, 1107 cm⁻¹; ¹H NMR (500 MHz, CDCl₃) δ 8.14 (d, $J_{2,3}$ = 8.0 Hz, 2H, H-2), 7.81 (d, $J_{7,8}$ = 8.8 Hz, 2H, H-7), 7.79 (d, $J_{2,3}$ = 8.0 Hz, 2H, H-3), 6.96 (d, $J_{7,8}$ = 8.8 Hz, 2H, H-8), 4.04 (t, $J_{10,11}$ = 6.6 Hz, 2H, H-10), 3.97 (s, 3H, CO₂Me), 2.19 (tt, $J_{28,29a}$ = 7.3 Hz, $^4J_{29,31}$ = 2.4 Hz, 1H,

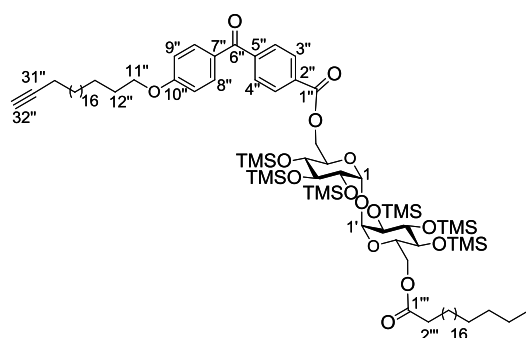
H-29a), 2.18 (tt, $J_{28,29b} = 7.1$ Hz, $^4J_{29,31} = 2.4$ Hz, 1H, H-29b), 1.94 (t, $^4J_{29,31} = 2.4$ Hz, 1H, H-31), 1.82 (pentet, $J_{9,10} = J_{10,11} = 7.1$ Hz, 2H, H-11), 1.58–1.50 (m, 2H, H-28), 1.50–1.42 (m, 2H, H-12), 1.40–1.22 (m, 30H, H-13–H-27); ^{13}C NMR (125 MHz, CDCl_3) δ 194.8 (C-5), 166.4 (CO_2Me), 163.6 (C-9), 142.2 (C-4), 132.7 (C-1), 132.6 (C-7), 129.4 (C-3), 129.4 (C-2), 129.3 (C-6), 114.2 (C-8), 84.8 (C-30), 68.4 (C-31), 68.0 (C-10), 52.4 (CO_2Me), 29.70, 29.68, 29.66, 29.62, 29.60, 29.57, 29.51, 29.36, 29.12, 29.09, 28.77, 28.50, 25.98 (C-11–C-28), 18.40 (C-29); HRMS(ESI) m/z calcd. for $[\text{C}_{37}\text{H}_{52}\text{O}_4 + \text{Na}]^+$: 583.3763, obsd.: 583.3763.



2,2',3,3',4,4'-Hexa-O-trimethylsilyl-6-docosanoyl-6'-hydroxyl, α,α' -D-trehalose (2).

To a solution of diol **4** (48.9 mg, 0.063 mmol) and behenic acid (**5**, 28.0 mg, 0.082 mmol) in dry toluene (2 mL) was added EDCI (30.2 mg, 0.16 mmol) and DMAP (1.1 mg, 0.01 mmol). The reaction was stirred at 70 °C until analysis by TLC showed no further formation of product. The resulting precipitate was removed by filtration and the precipitate washed thoroughly with EtOAc (2 x 20 mL). The combined organic layers were washed with water (20 mL) and brine (20 mL), dried (MgSO_4), filtered and concentrated *in vacuo*. The title compound was obtained after silica gel flash chromatography eluting in 20:1 (v/v) Pet. Ether/EtOAc, as colourless oil (52.0 mg, 0.047 mmol, 75%). $R_f = 0.55$ (PE/EA, 5/1, v/v); $[\alpha]_D^{18} = +20.2$ ($c = 1.0$, CHCl_3); IR (film): 3017, 2923, 2852, 2360, 2341, 1737, 1372, 1250, 1229, 1166, 1110, 1076, 1007, 965, 896, 872 cm^{-1} ; ^1H NMR (500 MHz, CDCl_3) δ 4.94 (d, $J_{1,2} = 3.5$ Hz, 1H, H-1), 4.92 (d, $J_{1',2'} = 3.6$ Hz, 1H, H-1'), 4.30 (dd, $J_{6a,6b} = 12.0$ Hz, $J_{5,6a} = 2.2$ Hz, 1H, H-6a), 4.07 (dd, $J_{6a,6b} = 12.0$ Hz, $J_{5,6b} = 4.6$ Hz, 1H, H-6b), 4.03–4.00 (m, 1H, H-5), 3.92 (t, $J_{2/2',3/3'} = J_{3/3',4/4'} = 8.8$ Hz, 1H, H-3/3'), 3.91 (t, $J_{2/2',3/3'} = J_{3/3',4/4'} = 9.0$ Hz, 1H, H-3/3'), 3.85 (dt, $J_{4',5'} = 9.5$ Hz, $J_{5',6a'} = J_{5',6b'} = 3.5$ Hz, 1H, H-5'), 3.86–3.83 (m, 2H, H-6a' and H-6b'), 3.49 (t, $J_{3,4} = J_{4,5} = 9.0$ Hz, 1H, H-4), 3.47 (t, $J_{3',4'} = J_{4',5'} = 9.0$ Hz, 1H, H-4'), 3.44 (dd, $J_{1,2/1',2'} = 3.6$ Hz, $J_{2,3/2',3'} = 9.0$ Hz, 1H, H-2/2'), 3.43 (dd, $J_{1,2/1',2'} = 3.5$ Hz, $J_{2,3/2',3'} = 9.0$ Hz, 1H, H-2/2'), 2.35 (dt, $J_{8a,8b} = 13.2$, $J_{8a,9} = 7.3$ Hz, 1H, H-8a), 2.34 (dd, $J_{8a,8b} = 13.2$, $J_{8b,9} = 7.4$ Hz,

1H, H-8b), 1.66–1.57 (m, 2H, H-9), 1.34–1.26 (m, 36H, H-10–H-27), 0.89 (t, $J = 7.1$ Hz, 3H, H-28), 0.17, 0.16, 0.15, 0.14, 0.13 (6 s, 54H, CH₃, TMS); ¹³C NMR (125 MHz, CDCl₃) δ 173.8 (C-7), 94.5 (C-1/1'), 94.4 (C-1/1'), 73.5 (C-3/3'), 73.3 (C-3/3'), 72.9 (C-2/2'), 72.8 (C-5'), 72.6 (C-2/2'), 71.9 (C-4), 71.4 (C-4'), 70.8 (C-5), 63.3 (C-6), 61.7 (C-6'), 34.2 (C-8), 31.9, 29.70, 29.66, 29.60, 29.5, 29.4, 29.3, 29.2, 24.8, 22.7 (C-9–C-27), 14.1 (C-28), 1.04, 1.00, 0.89, 0.85, 0.14, 0.10 (TMS) HRMS (ESI) m/z calcd. for [C₅₂H₁₁₂O₁₂Si₆+Na]⁺: 1119.6769, obsd.: 1119.6667.

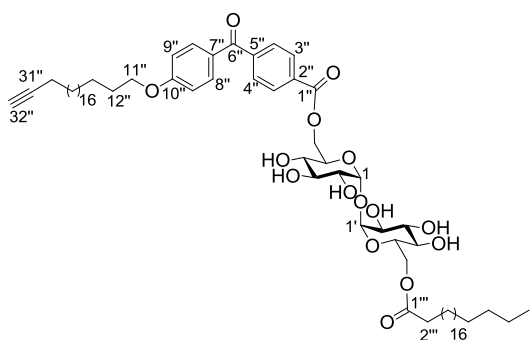


6'-O-docosanoyl-6-O-4-[4-(21-docosynyloxy)benzoyl]benzoyl-2,2',3,3',4,4'-hexa-O-trimethylsilyl- α,α' -D-trehalose (22). LiOH.H₂O (15 mg, 0.36 mmol) was added to a solution of **20** (20 mg, 0.04 mmol) in 0.5 mL

THF:H₂O:MeOH (3:1:1) and the reaction mixture was refluxed for 10 min after which it was neutralised with 0.1 M HCl (pH = 3) and extracted with EtOAc (3x 10 mL). The combined organic layers were washed with water, brine, dried (MgSO₄), filtered and concentrated *in vacuo* to yield carboxylic acid **3** as a white solid (15 mg, 0.03 mmol, 80%) which was used without further purification.

To a solution of mono-trehalose ester **2** (8.5 mg, 0.008 mmol) and carboxylic acid **3** (14 mg, 0.03 mmol) in dry toluene (1 mL) was added EDCI (6.5 mg, 0.034 mmol) and DMAP (0.2 mg, 0.001 mmol) and the reaction was refluxed for 7 days after which time the resulting precipitate was removed by filtration and the precipitate washed thoroughly with EtOAc (3 x 10 mL). The combined organic layers were washed with water (10 mL), brine (10 mL) dried with MgSO₄, filtered and concentrated *in vacuo*. The title compound was obtained after silica gel flash chromatography (eluting in 25:1, Pet. Ether/EtOAc, v/v) as colourless oil (3.5 mg, 0.002 mmol, 30%). $R_f = 0.54$ (PE/EA, 5/1, v/v); $[\alpha]_D^{22} = +0.06$ ($c = 0.1$ CHCl₃); IR (film): 3628, 2920, 1457, 720, 677 cm⁻¹; ¹H NMR (500 MHz, CDCl₃) δ 8.14 (d, $J_{3'',4''} = 8.5$ Hz, 2H, H-3''), 7.81 (d, $J_{8'',9''} = 8.8$ Hz, 2H, H-8''), 7.79 (d, $J_{3'',4''} = 8.5$ Hz, 2H, H-4''), 6.96 (d, $J_{8'',9''} = 8.8$ Hz, 2H, H-9''), 4.95 (d, $J_{1,2/1',2'} = 3.2$ Hz, 1H, H-1/1'), 4.94 (d,

$J_{1,2/1',2'} = 3.2$ Hz, 1H, H-1/1'), 4.59 (dd, $J_{6a,6b} = 12.0$ Hz, $J_{5,6a} = 2.3$ Hz, 1H, H-6a), 4.33 (dd, $J_{6a,6b} = 12.0$ Hz, $J_{5,6b} = 3.5$ Hz, 1H, H-6b), 4.27 (dd, $J_{6a',6b'} = 12.0$ Hz, $J_{5',6a'} = 2.3$ Hz, 1H, H-6a'), 4.13 (ddd, $J_{4,5} = 9.0$ Hz, $J_{5,6a} = 2.3$ Hz, $J_{5,6b} = 3.5$ Hz, 1H, H-5), 4.09 (dd, $J_{6a',6b'} = 12.0$ Hz, $J_{5',6b'} = 3.5$ Hz, 1H, H-6b'), 4.04 (t, $J_{11'',12''} = 6.4$ Hz, 2H, H-11''), 4.02–3.97 (m, 1H, H-5'), 3.96 (dd, $J_{2,3} = J_{3,4} = 9.0$ Hz, 1H, H-3), 3.92 (dd, $J_{2',3'} = J_{3',4'} = 9.0$ Hz, 1H, H-3'), 3.64 (dd, $J_{3,4} = J_{4,5} = 9.0$ Hz, 1H, H-4), 3.49 (dd, $J_{2,3/2',3'} = 9.0$ Hz, $J_{1,2/1',2'} = 3.2$ Hz, 1H, H-2/2') 3.48 (dd, $J_{3',4'} = J_{4',5'} = 9.0$ Hz, 1H, H-4'), 3.47 (dd, $J_{2,3/2',3'} = 9.0$ Hz, $J_{1,2/1',2'} = 3.2$ Hz, 1H, H-2/2'), 2.34 (dt, $J_{2a'',2b''} = 11.4$ Hz, $J_{2a'',3''} = 7.3$ Hz, 1H, H-2a''), 2.33 (dt, $J_{2a'',2b''} = 11.4$ Hz, $J_{2b'',3''} = 7.3$ Hz, 1H, H-2b''), 2.19 (dtt, $J_{30a'',30b''} = 9.9$ Hz, $J_{29'',30a''} = 7.3$ Hz, $^4J_{30a'',32''} = 2.6$ Hz, 1H, H-30a''), 2.17 (ddt, $J_{30a'',30b''} = 9.9$ Hz, $J_{29'',30b''} = 7.3$ Hz, $^4J_{30b'',32''} = 2.6$ Hz, 1H, H-30b''), 1.94 (t, $^4J_{30'',32''} = 2.6$ Hz, 1H, H-32), 1.82 (pentet, $J_{11'',12''} = J_{12'',13''} = 6.7$ Hz, 2H, H-12''), 1.64–1.52 (m, 4H, H-3''' and H-13''), 1.50–1.22 (m, 68H, H-14''–H-29'' and H-4'''–H-21'''), 0.88 (t, $J_{21''',22'''} = 7.0$ Hz, H-22'''), 0.17, 0.16, 0.15, 0.14, 0.13 (6 s, 54H, CH₃, TMS); ¹³C NMR (125 MHz, CDCl₃) δ194.5 (C-6''), 173.8 (C-1'''), 165.5 (C-1''), 163.1 (C-10''), 142.2 (C-5''), 132.4 (C-4''), 132.3 (C-2''), 129.3 (C-3''), 129.2 (C-8''), 128.7 (C-7''), 114.0 (C-9''), 94.29 (C-1/1'), 94.25 (C-1/1'), 84.6 (C-31''), 73.4 (C-3), 73.2 (C-3'), 72.44 (C-2/2'), 72.40 (C-2/2'), 71.66 (C-4), 71.60 (C-4'), 70.53 (C-5'), 70.50 (C-5), 68.2 (C-11''), 67.8 (C-32''), 63.8 (C-6), 63.0 (C-6'), 33.9 (C-2'''), 31.7, 31.2, 29.9, 29.8, 29.5, 29.43, 29.38, 29.34, 29.35, 29.27, 29.24, 29.20, 29.14, 29.1, 28.9, 28.88, 28.54, 28.26, 25.76, 24.78, 24.54, 22.47, 21.85, (C-3'''–C21''' and C-12''–C-29''), 18.17 (C-30''), 13.9 (C-22'''), 0.86, 0.83, 0.70, 0.65, 0.01, 0.00 (TMS); HRMS (ESI) *m/z* calcd. for [C₈₈H₁₆₀O₁₅Si₆+Na]⁺: 1648.0265, obsd.: 1648.0280.



6'-O-Docosanoyl-6-O-4-[4-(21-docosynyloxy)benzoyl]benzoyl- α,α' -D-trehalose (1). To a solution of the protected probe **22** (1.7 mg, 0.001 mmol) in 0.4 mL CH₂Cl₂:MeOH mixture (3/1, v/v) was added Dowex-H⁺ (10% by

weight) and the reaction stirred at room temperature. After 30 min, the reaction mixture was filtered and concentrated *in vacuo*. A/BP probe **1** was obtained after silica gel flash column chromatography (eluting in 97:3, EtOAc/MeOH, v/v) as a white solid (1.25 mg, 0.001 mmol, *quant.*). $R_f = 0.31$ (EtOAc); $[\alpha]_D^{18} = +0.6$ ($c = 0.1$, C_5H_5N); IR (film); 3567, 2970, 1739, 1365, 1217, 651 cm^{-1} ; 1H NMR (500 MHz, C_5D_5N) δ 8.20 (d, $J_{3'',4''} = 8.2$ Hz, 2H, H-3''), 7.86 (d, $J_{8'',9''} = 8.8$ Hz, 2H, H-8''), 7.68 (d, $J_{3'',4''} = 8.2$ Hz, 2H, H-4''), 7.06 (d, $J_{8'',9''} = 8.8$ Hz, 2H, H-9''), 5.84 (d, $J_{1,2/1',2'} = 3.6$ Hz, 1H, H-1/1'), 5.83 (d, $J_{1,2/1',2'} = 3.6$ Hz, 1H, H-1/1'), 5.20–5.17 (m, 1H, H-5), 5.15–4.94 (m, 5H, H-5, H-6a,b and H-6'a,b), 4.70 (dd, $J_{2,3} = J_{3,4} = 9.6$ Hz, 1H, H-3), 4.68 (dd, $J_{2',3'} = J_{3',4'} = 9.6$ Hz, 1H, H-3'), 4.28 (dd, $J_{2,3/2',3'} = 9.6$ Hz, $J_{1,2/1',2'} = 3.6$ Hz, 1H, H-2/2'), 4.24 (dd, $J_{2,3/2',3'} = 9.6$ Hz, $J_{1,2/1',2'} = 3.6$ Hz, 1H, H-2/2'), 4.09 (dd, $J_{3,4} = J_{4,5} = 9.6$ Hz, 1H, H-4), 4.09 (dd, $J_{3',4'} = J_{4',5'} = 9.6$ Hz, 1H, H-4'), 3.96 (t, $J_{11'',12''} = 6.3$ Hz, 2H, H-11''), 2.68 (t, $^4J_{30'',32''} = 2.7$ Hz, 1H, H-32''), 2.20 (dt, $J_{2a''',2b'''} = 12.2$ Hz, $J_{2a''',3'''} = 7.9$ Hz, 2H, H-2a'''), 2.19 (dt, $J_{2a''',2b'''} = 12.2$ Hz, $J_{2b''',3'''} = 7.9$ Hz, 1H, H-2b'''), 2.12 (dt, $J_{30a'',30b''} = 9.9$ Hz, $J_{29'',30a''} = 7.0$ Hz, $^4J_{30a'',32''} = 2.7$ Hz, 1H, H-30a''), 2.10 (dt, $J_{30a'',30b''} = 9.9$ Hz, $J_{29'',30b''} = 7.0$ Hz, $^4J_{30b'',32''} = 2.7$ Hz, 1H, H-30b''), 1.69 (pentet, $J_{11'',12''} = J_{12'',13''} = 6.3$ Hz, 2H, H-12''), 1.50 (pentet, $J_{2''',3'''} = J_{3''',4'''} = 6.1$ Hz, 2H, H-3'''), 1.39–1.35 (m, 2H, H-13''), 1.20–1.06 (m, 68H, H-14''–H-29'' and H-4'''–H-21'''), 0.77 (t, $J_{21''',22'''} = 6.6$ Hz, H-22'''); ^{13}C NMR (125 MHz, C_5D_5N) δ 193.0 (C-6''), 172.3 (C-1'''), 164.6 (C-1''), 162.2 (C-10''), 133.8 (C-5''), 131.7 (C-4''), 128.4 (C-2''), 128.3 (C-3''), 128.0 (C-8''), 127.3 (C-7''), 113.3 (C-9''), 94.6 (C-1 and C-1'), 83.7 (C-31''), 73.6 (C-3), 73.5 (C-3'), 72.0 (C-2/2'), 72.40 (C-2/2'), 70.70 (C-4), 70.60 (C-4'), 70.23 (C-5'), 70.16 (C-5), 68.7 (C-32''), 67.2 (C-11''), 64.3 (C-6), 62.9 (C-6'), 33.0 (C-2'''), 30.7, 28.62, 28.60, 28.54, 28.52, 28.51, 28.48, 28.38, 28.34, 28.25, 28.21, 28.16, 28.0, 27.96, 27.94, 27.58, 27.41, 24.87, 23.85, 21.54 (C-3'''–C-21''' and C-12'''–C-29''), 17.18 (C-30''), 12.9 (C-22'''); HRMS(ESI) m/z calcd. for $[C_{70}H_{112}O_{15}+Na]^+$: 1215.7893, obsd.: 1215.7897.

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7

Summary and Future Prospects

7.1 Summary

The use of bacteria in the treatment of disease was first illustrated by Coley who treated a number of cancer patients in the late 1800s using a concoction known as ‘Coley’s Toxin’ (*Streptococcal pyogenes* and *Serratia marcescens*). Following this seminal work, there has been much interest in the potential of bacterial cell wall components in the treatment of disease, as highlighted in the introduction to this thesis (**Chapter 1**). In particular, Chapter 1 focuses on the trehalose glycolipids and outlines their synthesis, biological activities, and how such glycolipids can be used to understand cellular processes [e.g. *via* Affinity Based Proteome Profiling (A/BPP)].

In **Chapter 2**, the synthesis of linear fatty acid trehalose diesters (TDEs) is reported. The different synthetic strategies used to obtain a range of TDEs and the isolation of linear fatty acid trehalose monoesters (TMEs) is described. Initial methodology involved using benzyl protecting groups in the synthesis of these glycolipids, however, with long lipid chains, the removal of the benzyl protecting groups proved

difficult. A switch to using trimethylsilyl (TMS) protecting groups yielded better results. In summary, nine TDEs and two TMEs were synthesised in good overall yields.

Chapter 3 focuses on the biological activities of the synthesised TDEs and TMEs. The importance of lipid length in inducing macrophage activation was studied along with investigations into the importance of having two lipid chains on the trehalose moiety for recognition by macrophages. It was observed that a lipid length of C18 or more is required to induce macrophage activation and one long lipid chain (\geq C22) on trehalose is sufficient for activation. This is the first time that it has been shown that TMEs are also able to activate macrophages. The synthesised TMEs and TDEs were also tested for their cytotoxicity and, interestingly, no appreciable cytotoxicity was noted. Using knock-out models, it was shown that both TDEs and TMEs are ligands for Macrophage Inducible C-type Lectin (Mincle).

In **Chapter 4**, Fráter-Seebach alkylation methodology for the synthesis of α -alkyl- β -hydroxy esters is presented. Here it was shown that allylic iodides were more effective alkylating reagents and gave better product yields as compared to their saturated alkyl halide counterparts. This was attributed to the delocalisation of the electron density over the allylic carbons in the transition state which leads to a lower transition state energy relative to the transition state of the corresponding saturated halides.

Use of the improved Fráter-Seebach alkylation for the synthesis of a fully protected trehalose monocorynomycolate (TMCM) is described in **Chapter 5**. Key steps in the synthetic route were a copper-mediated Grignard reaction for the synthesis of the corynomycolic acid moiety, selective formation of the silyl-ether and esterification to give the trehalose monoester. Problems were noted when a longer lipid chain (C14) was used for the Grignard reaction and also during the esterification reaction, whereby formation of corynomycolic anhydride was noted with no diester observed. It is envisioned that these problems, however, can be solved and that the general synthetic strategy can be used for the subsequent syntheses of a variety of TDCMs.

In **Chapter 6**, the design of an affinity based proteome (A/BP) probe for understanding the protein interactions of trehalose glycolipids is described. Incorporation of a benzophenone trap group and an alkyne tag to prepare a C22 trehalose probe was undertaken. Key synthetic steps included Friedel-Craft acylation for the formation of the benzophenone moiety and a Zipper reaction to prepare the terminal alkyne functionality. Initial biological testing of the A/BP probe was performed and it was observed that the probe led to macrophage activation (as determined by NO production) albeit at lower levels than the corresponding dibehenate derivative.

7.2 Future Prospects

7.2.1 TDEs and TMEs and associated biological activities

With our finding that long chain TMEs and TDEs are required to activate macrophages, our next step would be to understand more about the mechanism of this activation. For example, all macrophage experiments should be repeated using knock-out mice (*e.g.* Mincle^{-/-}, TLR^{-/-} and Myd88^{-/-} BMMs) so that potential receptors can be identified. The ability of the trehalose esters to form aggregates (*e.g.* micelles) and the effect that this has on macrophage activation also needs to be determined, as does the ability of the compounds to switch the macrophage phenotype from M2 (*e.g.* TAMs) to M1. The ability of these compounds to be used as priming agents should also be further explored. Such experiments, along with the appropriate references, were described in more detail in **Chapter 3**.

7.2.2 TMCM and TDCM Synthesis

Having developed an improved Fráter-Seebach alkylation methodology, the next step is to apply this to the synthesis of more complex TDCMs. Problems encountered during the synthesis of the TDCMs included the copper-mediated Grignard alkylation when longer alkyl lipids were used and difficulties in the esterification

reaction to form diesters. The use of lithiated alkyne lipids in the Grignard reaction could solve this problem and allow for the synthesis of longer chain TDCM analogues. For the esterification reaction a change of solvent, prolonged reaction times and further addition of EDCI and DMAP in order to form the diesters would be investigated as highlighted in **Chapter 5**. The TDCMs should also be tested for their biological activities – first in a macrophage activation assay, and then, for their potential as adjuvants and anti-tumour agents. By doing so, greater insight into the effects of trehalose glycolipid structure on biological function can be determined.

7.2.3 AfBP

The C22-trehalose AfBP probe (**Chapter 6**) is to be used in subsequent proteomic experiments to elucidate other receptors that the TDMs may bind to upon interaction with macrophages in the host cell. This work is to be undertaken by Dr. Faustin Kamena (Max-Planck Institute, Berlin). Depending on the results from these studies, other probes with varying trap and tag groups could then be designed and tested.

List of Publications

1. Ashna A. Khan, Janice M.H. Cheng, Bridget L. Stocker, Mattie S.M. Timmer. "Glycolipids and CD1: The Crossroad between Chemistry and Immunology" *Chemistry in NZ*, **2010**, 74, 57–62.
2. Janice M.H. Cheng, Ashna A. Khan, Bridget L. Stocker and Mattie S.M. Timmer. "Endogenous and Exogenous CD1– Binding Glycolipids" *International Journal of Carbohydrate Chemistry*, **2011**, Volume 2011 (2011), Article ID 749591.
3. Ashna A. Khan, Stephanie H. Chee, Rene J. McLaughlin, Jacquie L. Harper, Faustin Kamena, Mattie S.M. Timmer, Bridget L. Stocker. "Long-Chain Lipids are Required for the Innate Immune Recognition of Trehalose Diesters by Macrophages" *ChemBioChem*, **2011**, 12, 2572–2576.
4. Ashna A. Khan, Stephanie H. Chee, Bridget L. Stocker, Mattie S.M. Timmer. "The Synthesis of Long-Chain α -Alkyl- β -Hydroxy-Esters using Allylic Halide in a Fráter-Seebach Alkylation" *Eur. J. Org. Chem*, **2012**, 995–1002.
5. Ashna A. Khan, Bridget L. Stocker, Mattie S.M. Timmer. "Trehalose glycolipids - synthesis and biological activities" *Carbohydr. Res.* **2012**, 356, 25–36.
6. Janelle Sauvageau, Amy J. Foster, Ashna A. Khan, Stephanie H. Chee, Ian M. Sims, Mattie S.M. Timmer, Bridget L. Stocker. "Synthesis and Biological Activity of the Lipoteichoic Acid Anchor from *Streptococcus* sp. DSM 8747" *ChemBioChem*, **2012**, 13, 2416–2424.

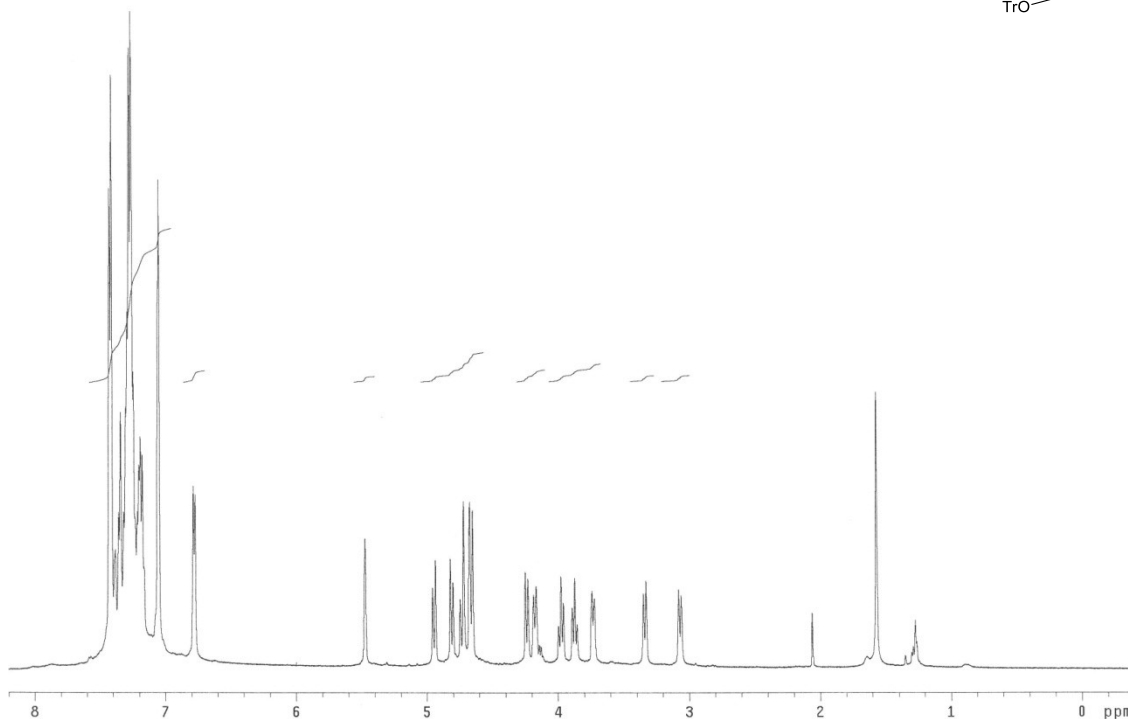
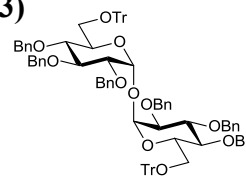
Appendix

^1H and ^{13}C NMR spectra of
synthesised compounds

Chapter 2

2,2',3,3',4,4'-Hexa-*O*-benzyl-6,6'-di-*O*-trityl- α,α' -D-trehalose (3)

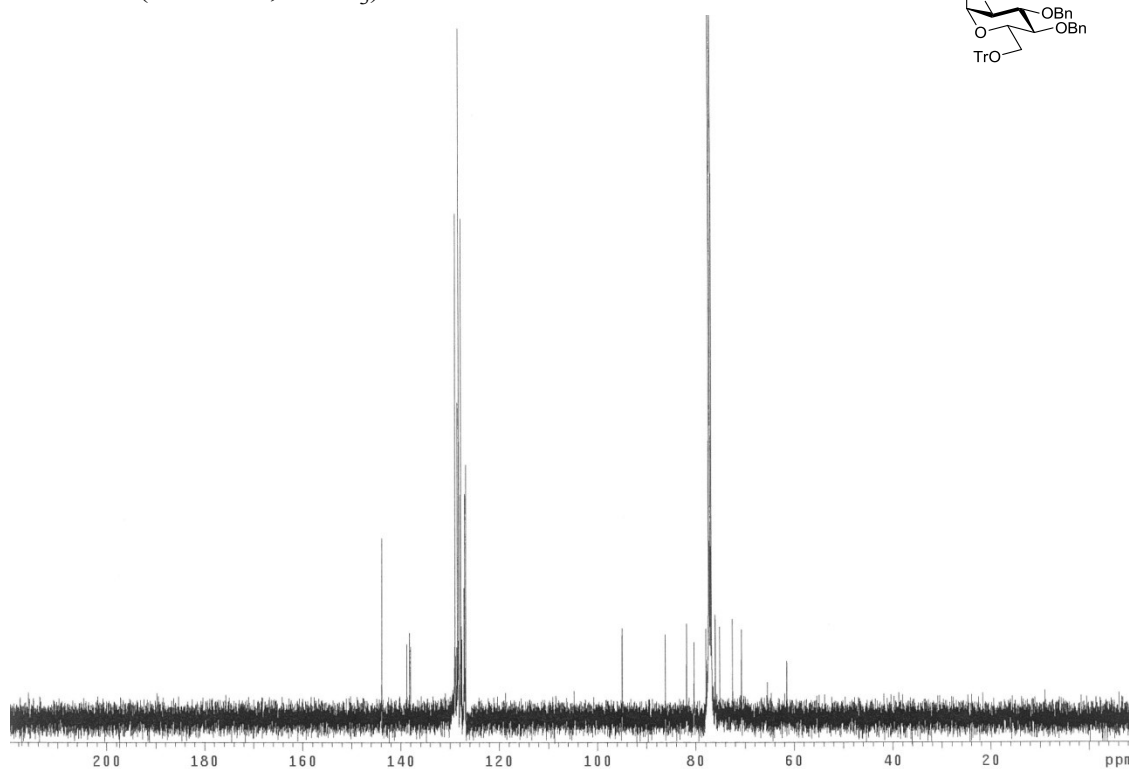
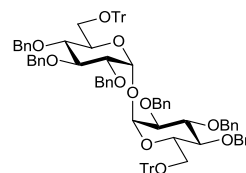
^1H NMR (500 MHz, CDCl_3)



2,2',3,3',4,4'-Hexa-*O*-benzyl-6,6'-di-*O*-trityl- α,α' -D-trehalose

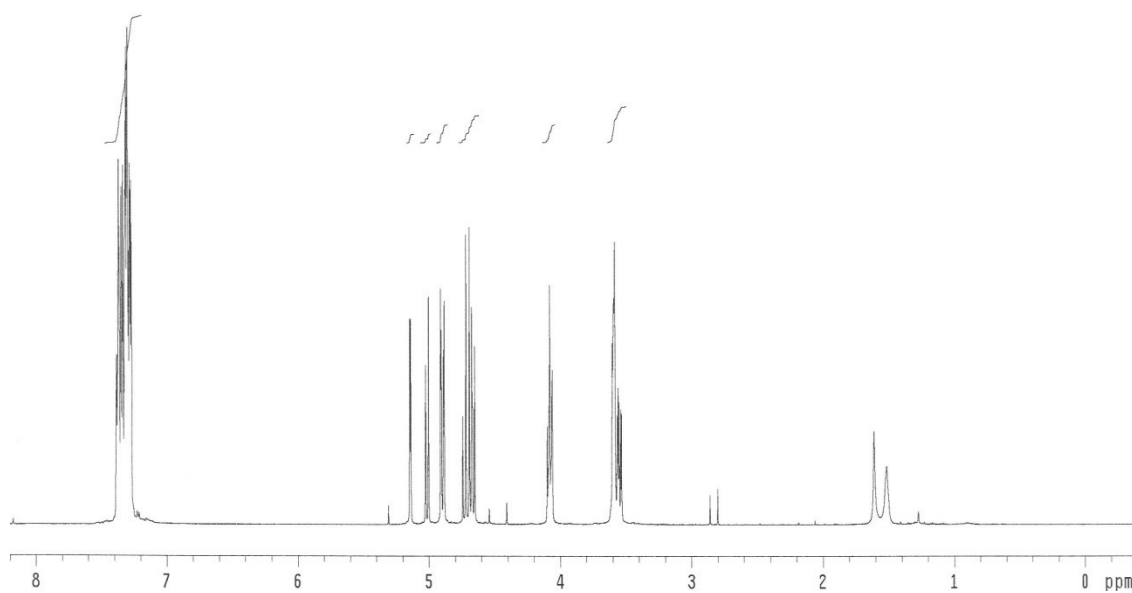
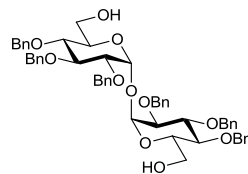
(3)

^{13}C NMR (125 MHz, CDCl_3)



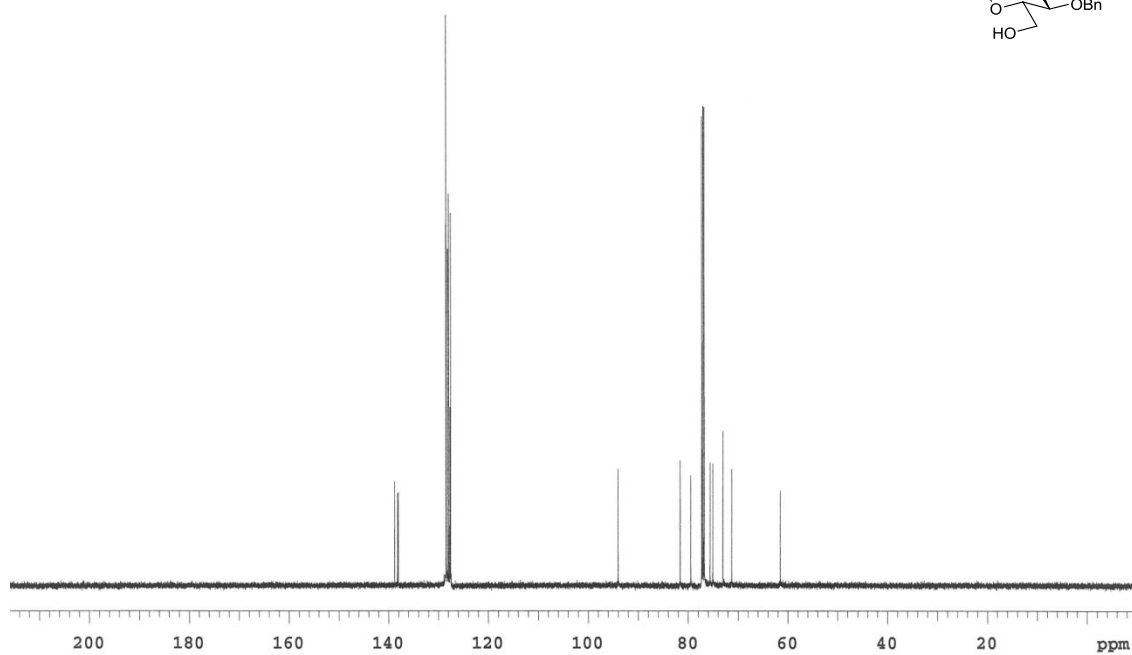
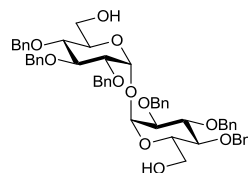
2,2',3,3',4,4'-Hexa-*O*-benzyl- α,α' -D-trehalose (4)

^1H NMR (500 MHz, CDCl_3)



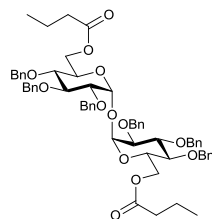
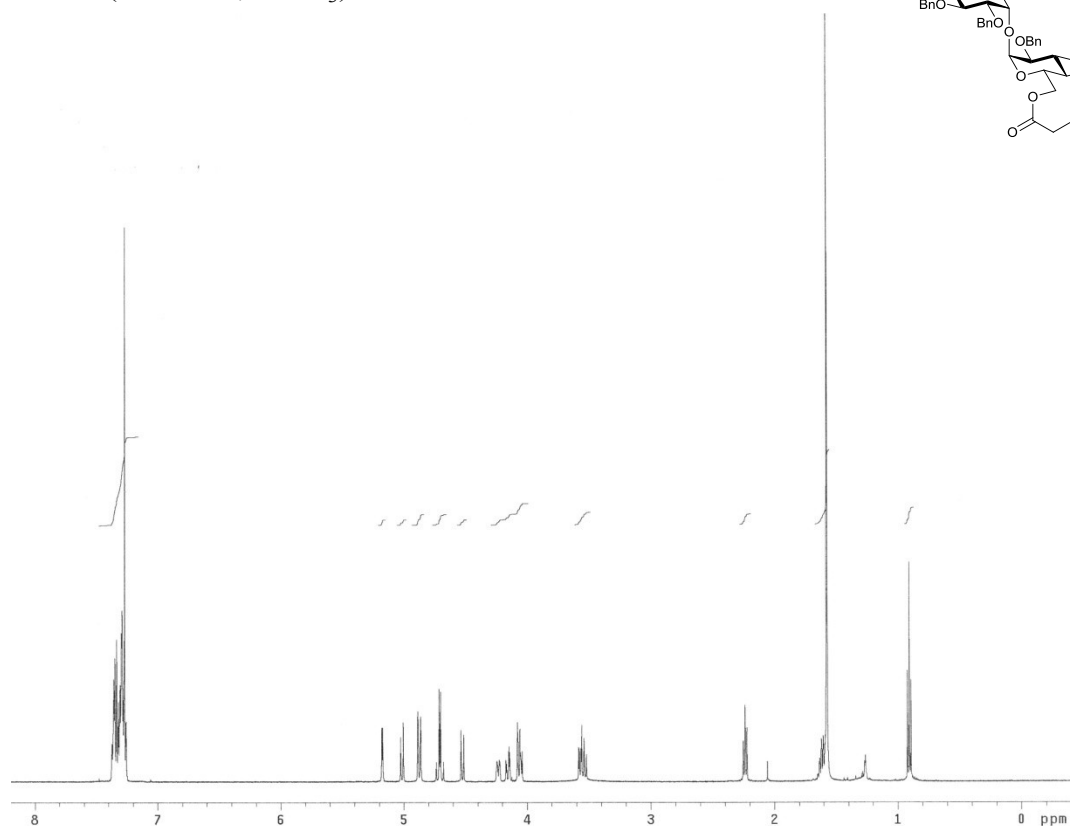
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^{13}C NMR (125 MHz, CDCl_3)



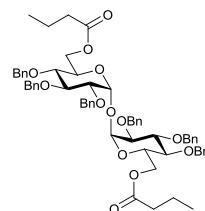
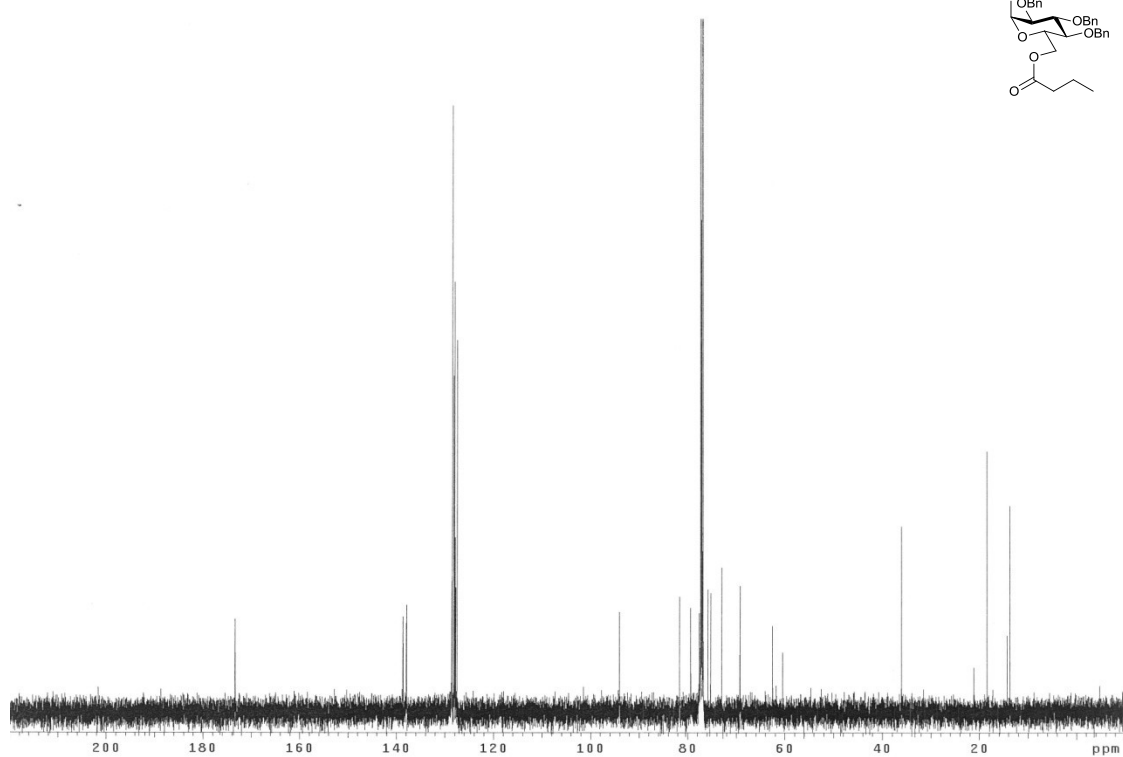
2,2',3,3',4,4'-Hexa-*O*-benzyl-6,6'-di-*O*-butanoyl- α,α' -D-trehalose (5a)

^1H NMR (500 MHz, CDCl_3)



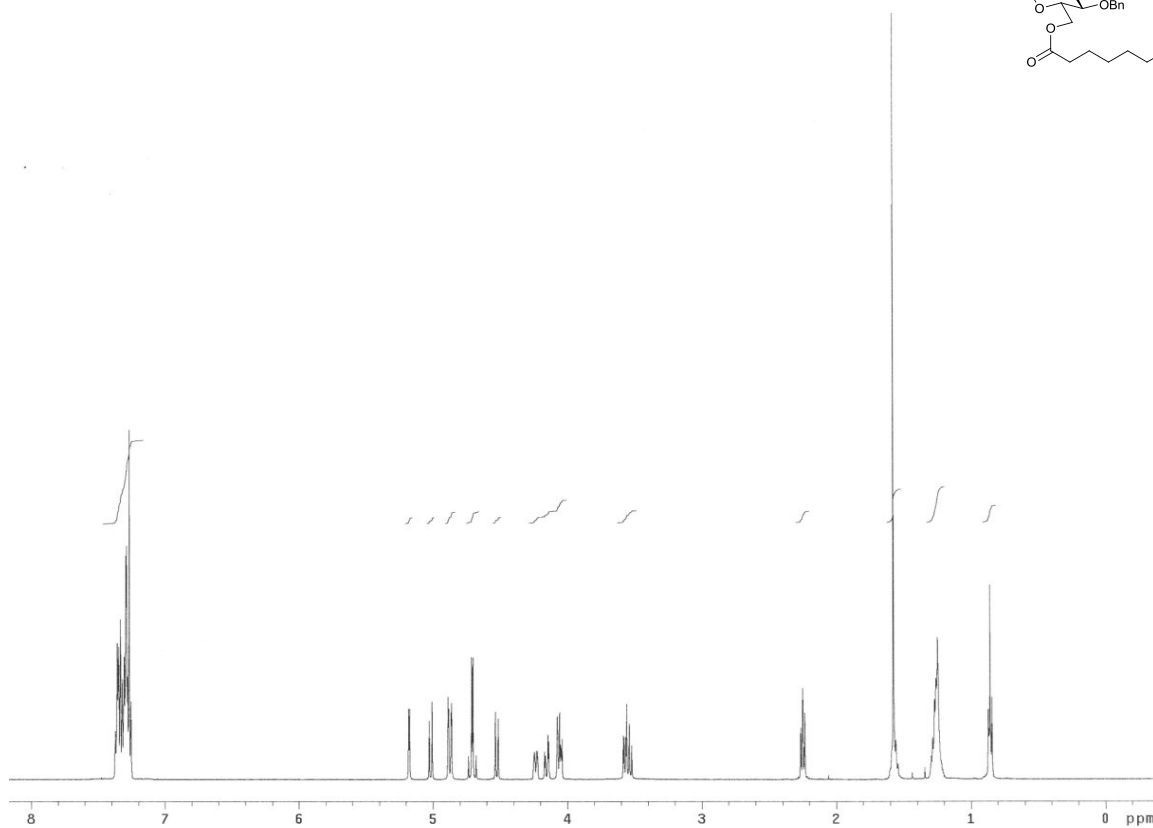
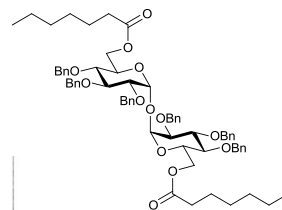
2,2',3,3',4,4'-Hexa-*O*-benzyl-6,6'-di-*O*-butanoyl- α,α' -D-trehalose (5a)

^{13}C NMR (125 MHz, CDCl_3)



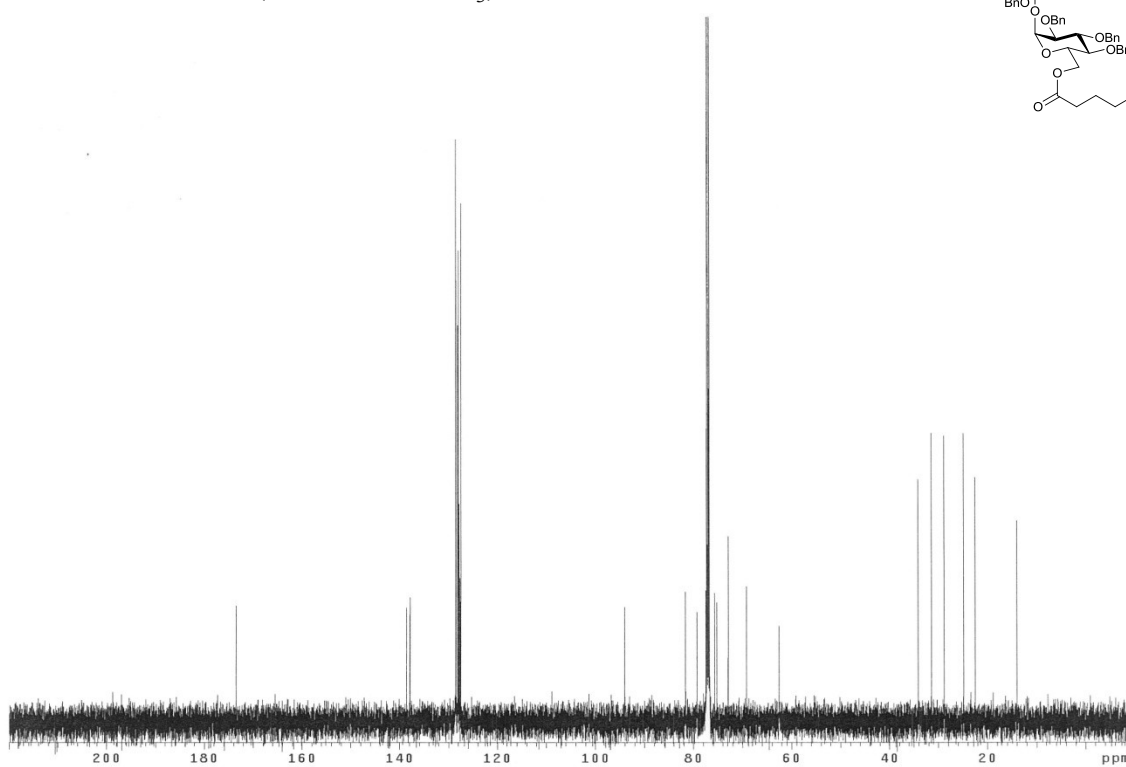
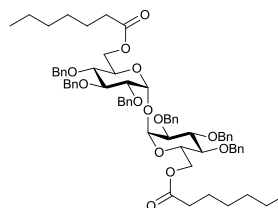
2,2',3,3',4,4'-Hexa-*O*-benzyl-6,6'-di-*O*-heptanoyl- α,α' -D-trehalose (5b)

^1H NMR (500 MHz, CDCl_3)



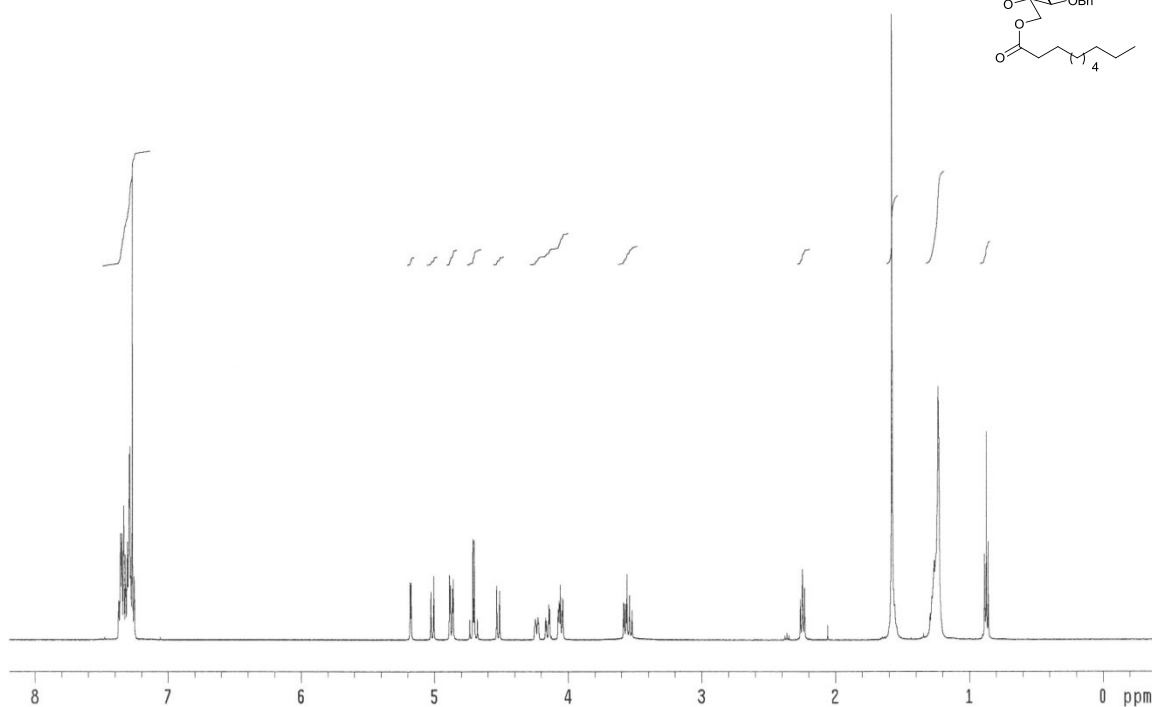
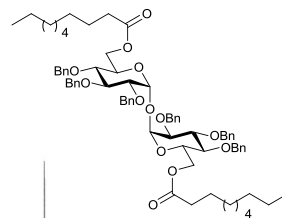
2,2',3,3',4,4'-Hexa-*O*-benzyl-6,6'-di-*O*-heptanoyl- α,α' -D-trehalose (5b)

^{13}C NMR (125 MHz, CDCl_3)



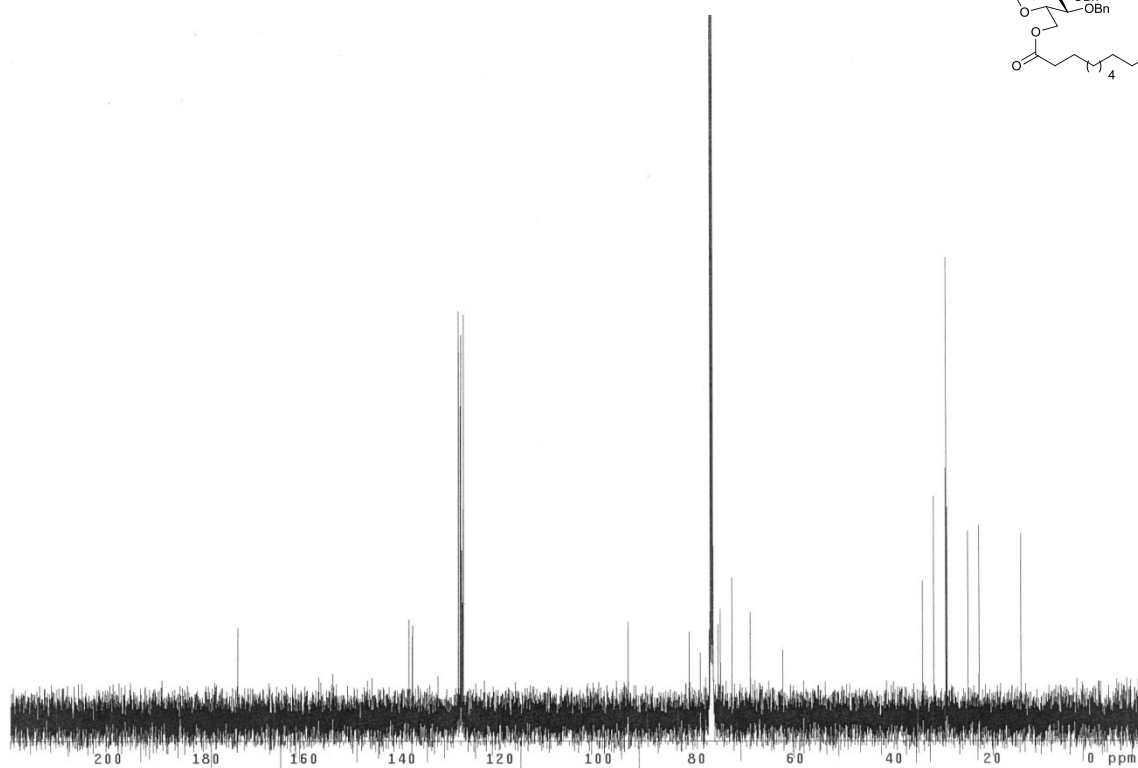
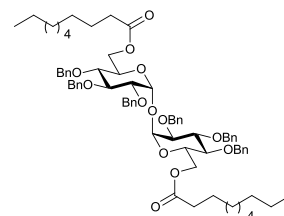
2,2',3,3',4,4'-hexa-*O*-benzyl-6,6'-di-*O*-decanoyl- α,α' -D-trehalose (5c)

^1H NMR (500 MHz, CDCl_3)



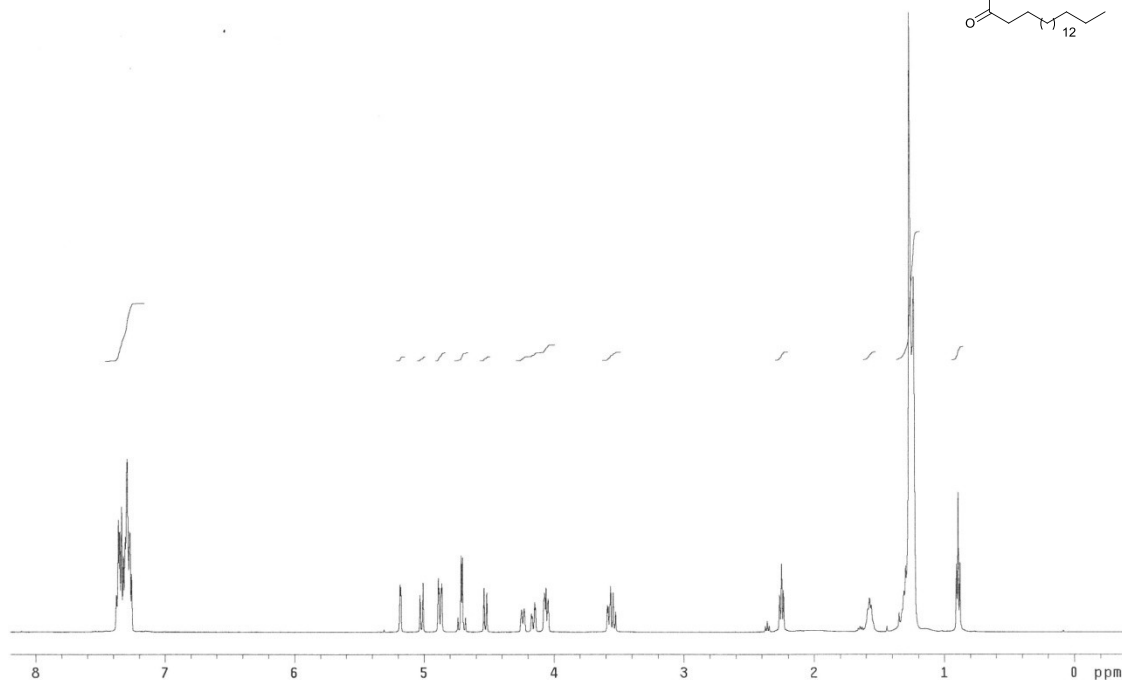
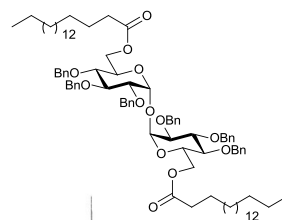
2,2',3,3',4,4'-hexa-*O*-benzyl-6,6'-di-*O*-decanoyl- α,α' -D-trehalose (5c)

^{13}C NMR (125 MHz, CDCl_3)



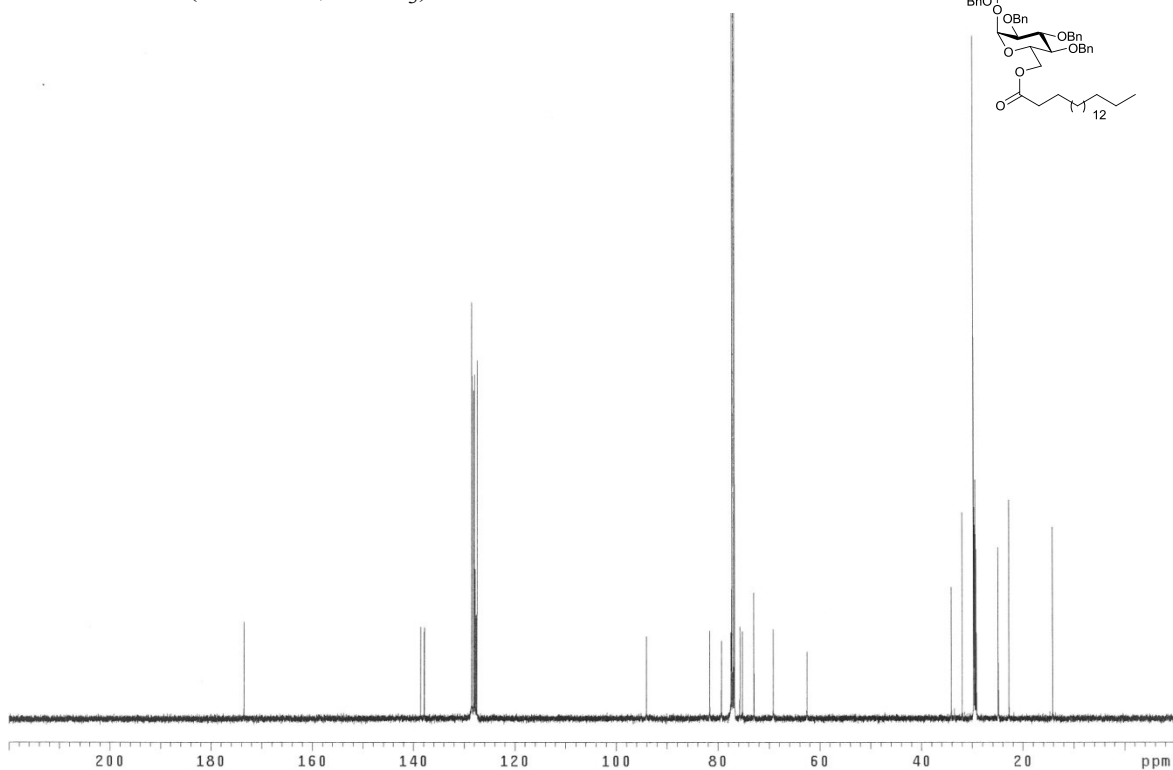
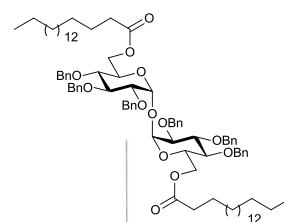
2,2',3,3',4,4'-Hexa-*O*-benzyl-6,6'-di-*O*-stearyl- α,α' -D-trehalose (5d)

^1H NMR (500 MHz, CDCl_3)



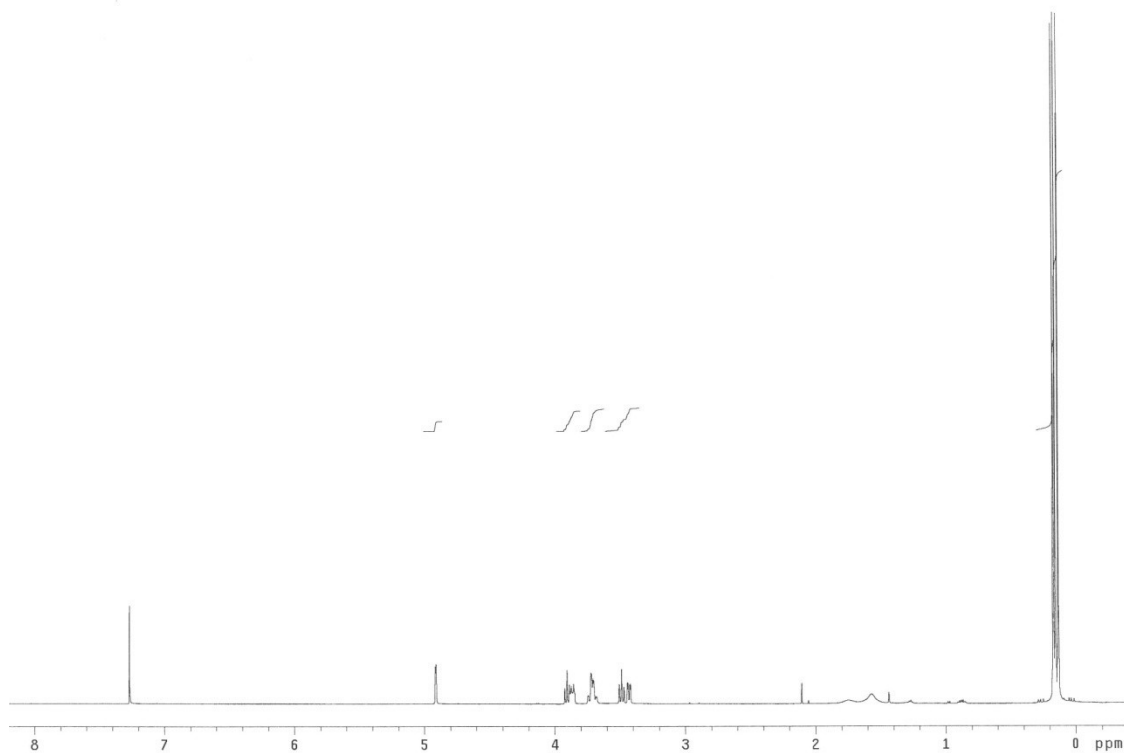
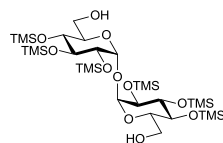
2,2',3,3',4,4'-Hexa-*O*-benzyl-6,6'-di-*O*-stearyl- α,α' -D-trehalose (5d)

^{13}C NMR (125 MHz, CDCl_3)



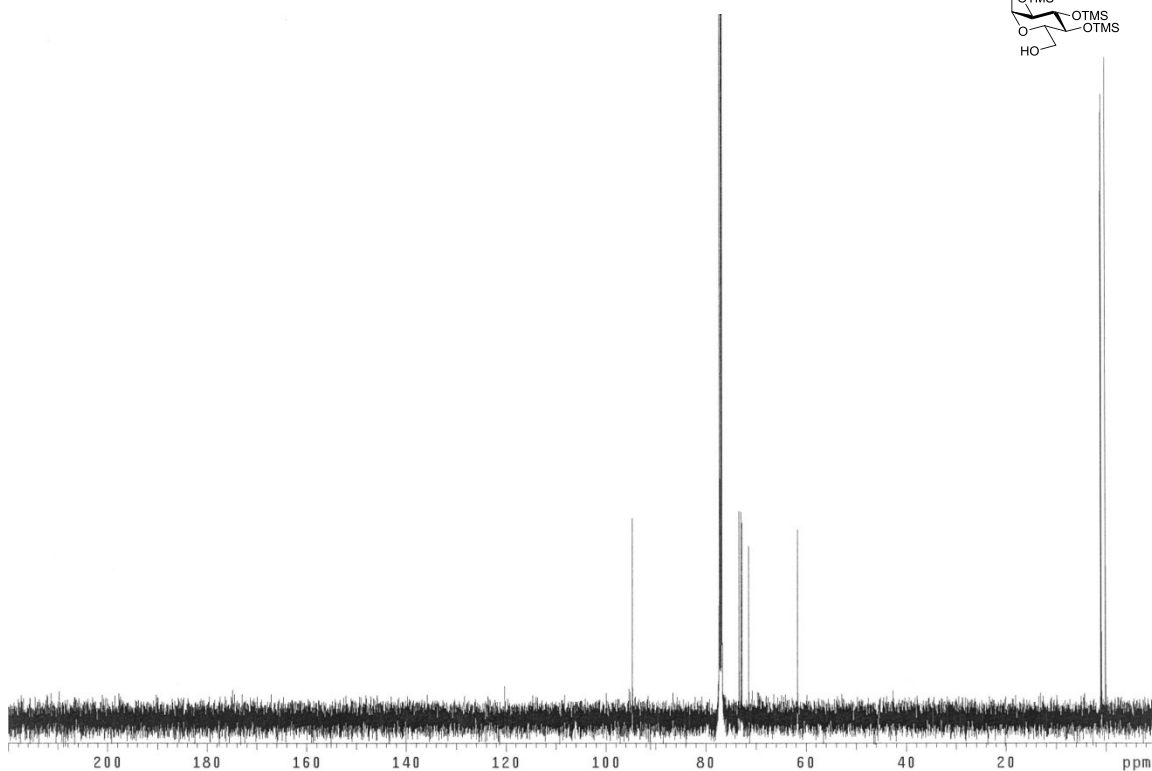
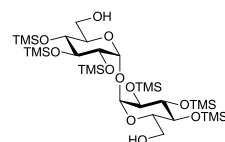
2,2',3,3',4,4'-hexa-*O*-trimethylsilyl- α,α' -D-trehalose (6)

^1H NMR (500 MHz, CDCl_3)



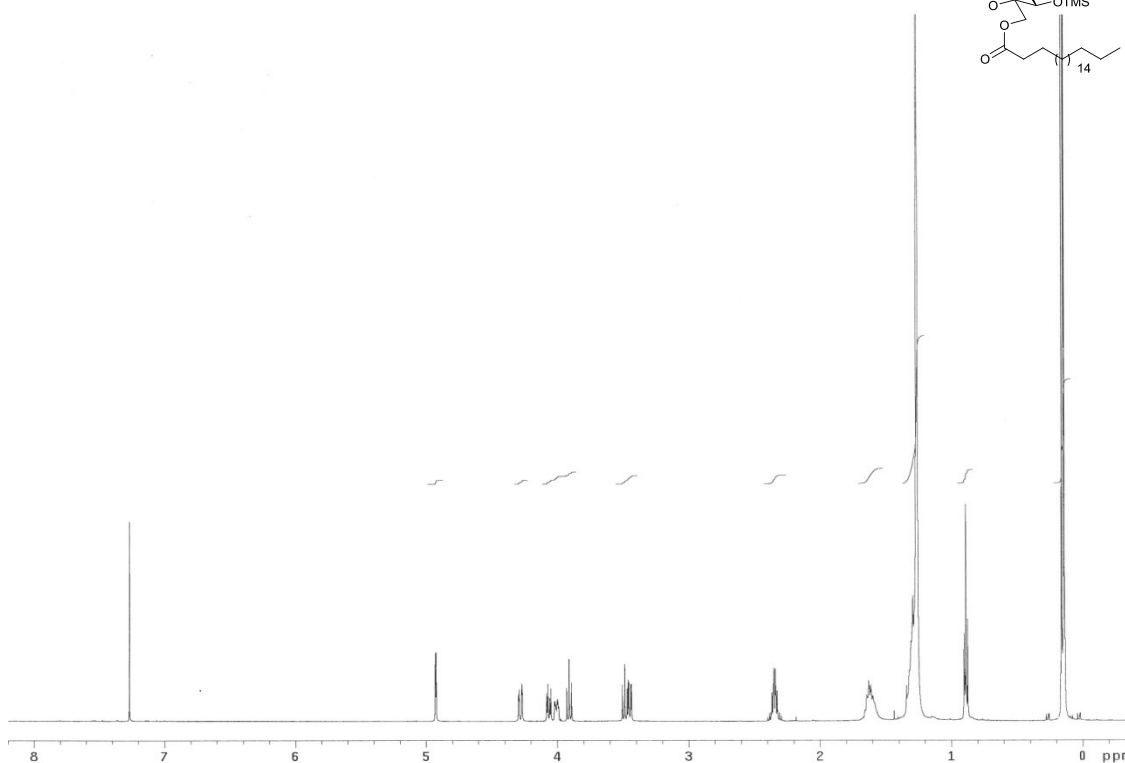
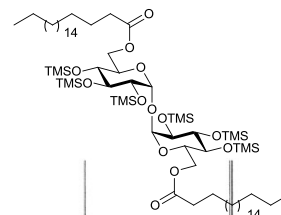
2,2',3,3',4,4'-hexa-*O*-trimethylsilyl- α,α' -D-trehalose (6)

^{13}C NMR (125 MHz, CDCl_3)



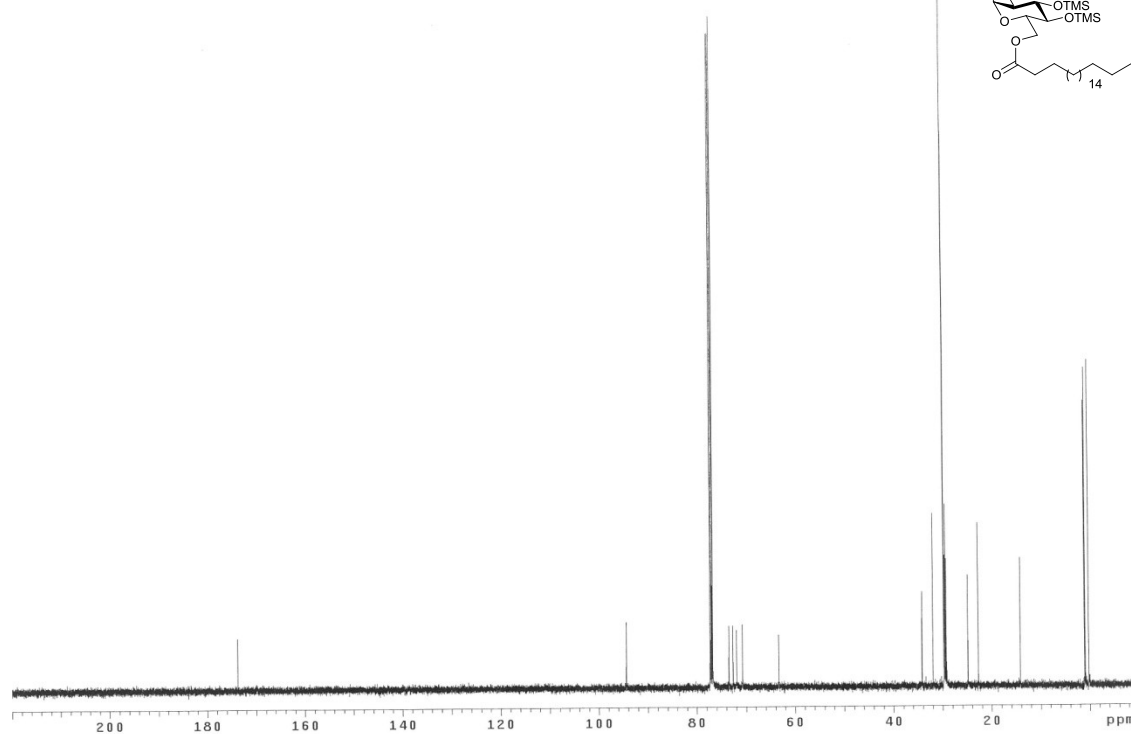
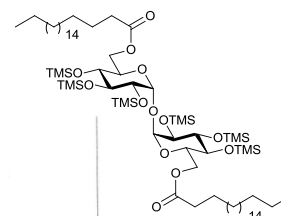
2,2',3,3',4,4'-Hexa-*O*-trimethylsilyl-6,6'-di-eicosanoyl- α,α' -D-trehalose (7e)

^1H NMR (500 MHz, CDCl_3)



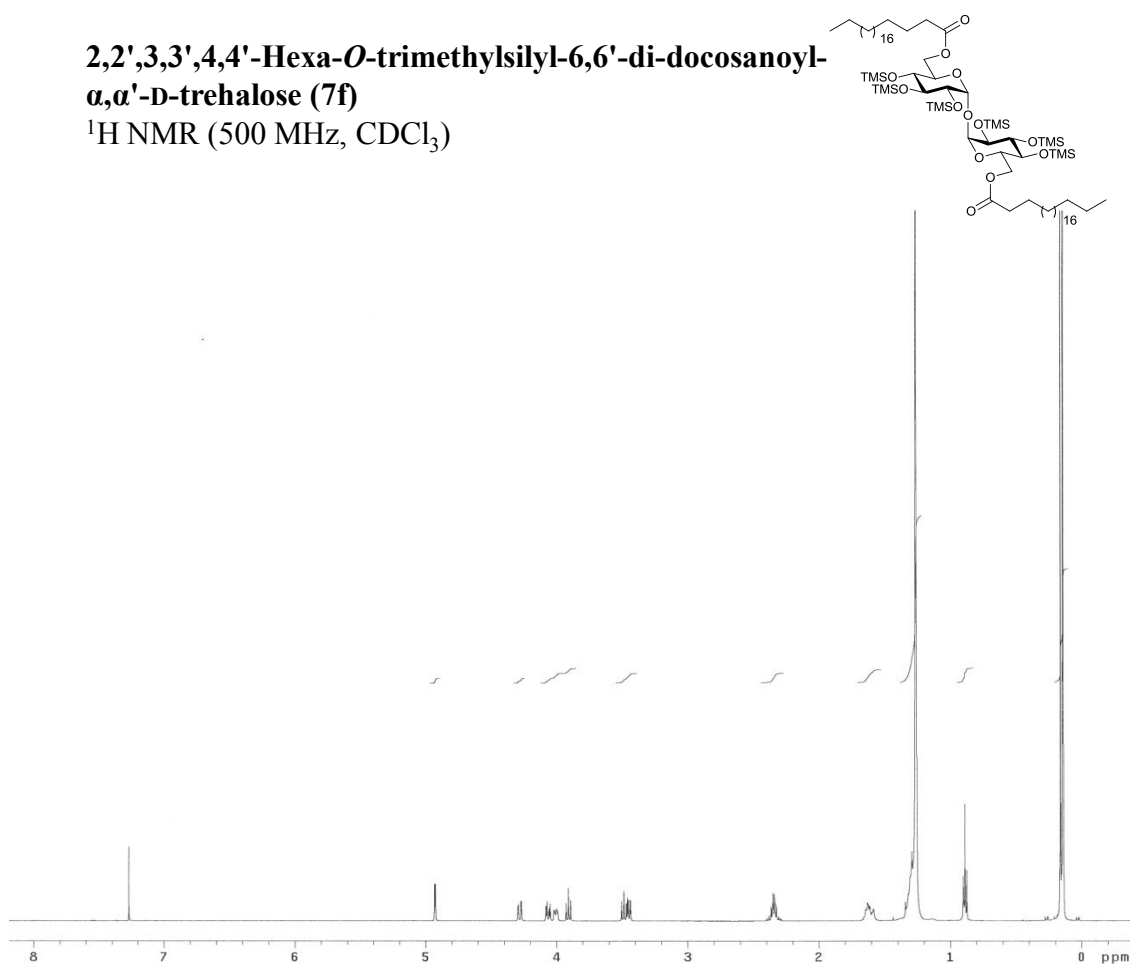
2,2',3,3',4,4'-Hexa-*O*-trimethylsilyl-6,6'-di-eicosanoyl- α,α' -D-trehalose (7e)

^{13}C NMR (125 MHz, CDCl_3)



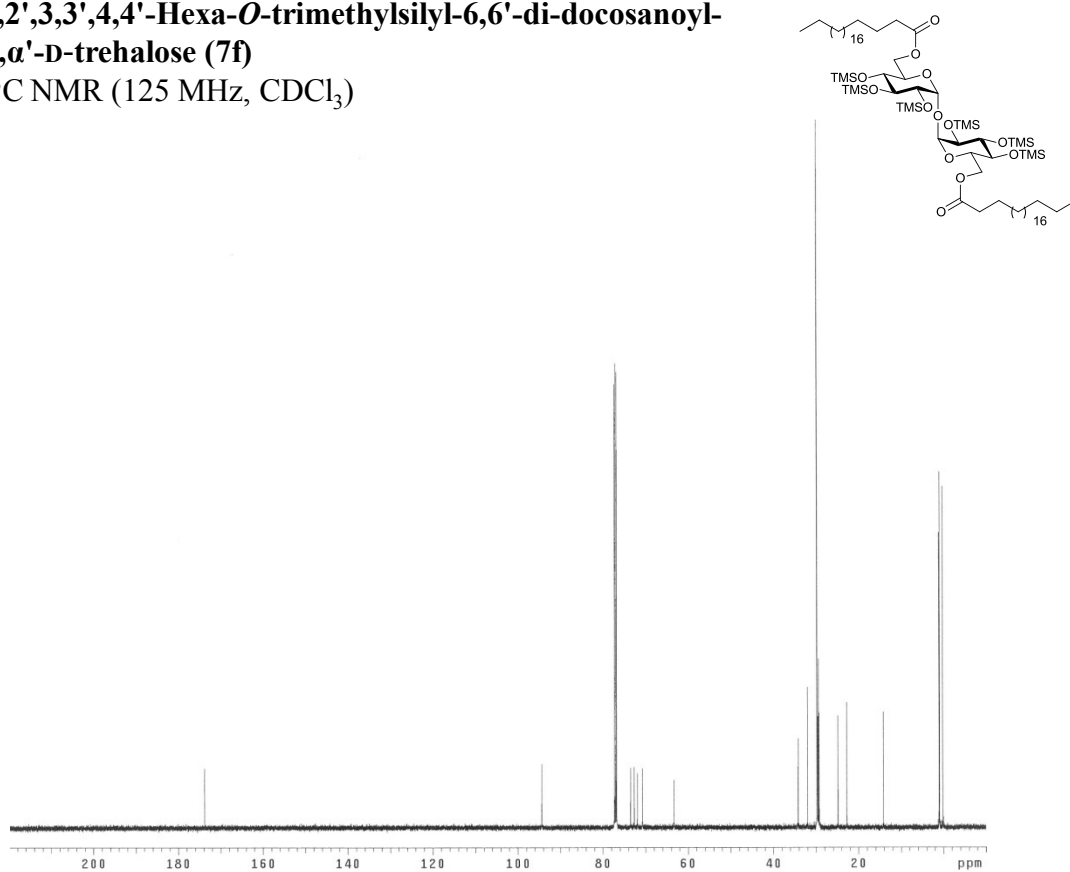
2,2',3,3',4,4'-Hexa-*O*-trimethylsilyl-6,6'-di-docosanoyl- α,α' -D-trehalose (7f)

^1H NMR (500 MHz, CDCl_3)



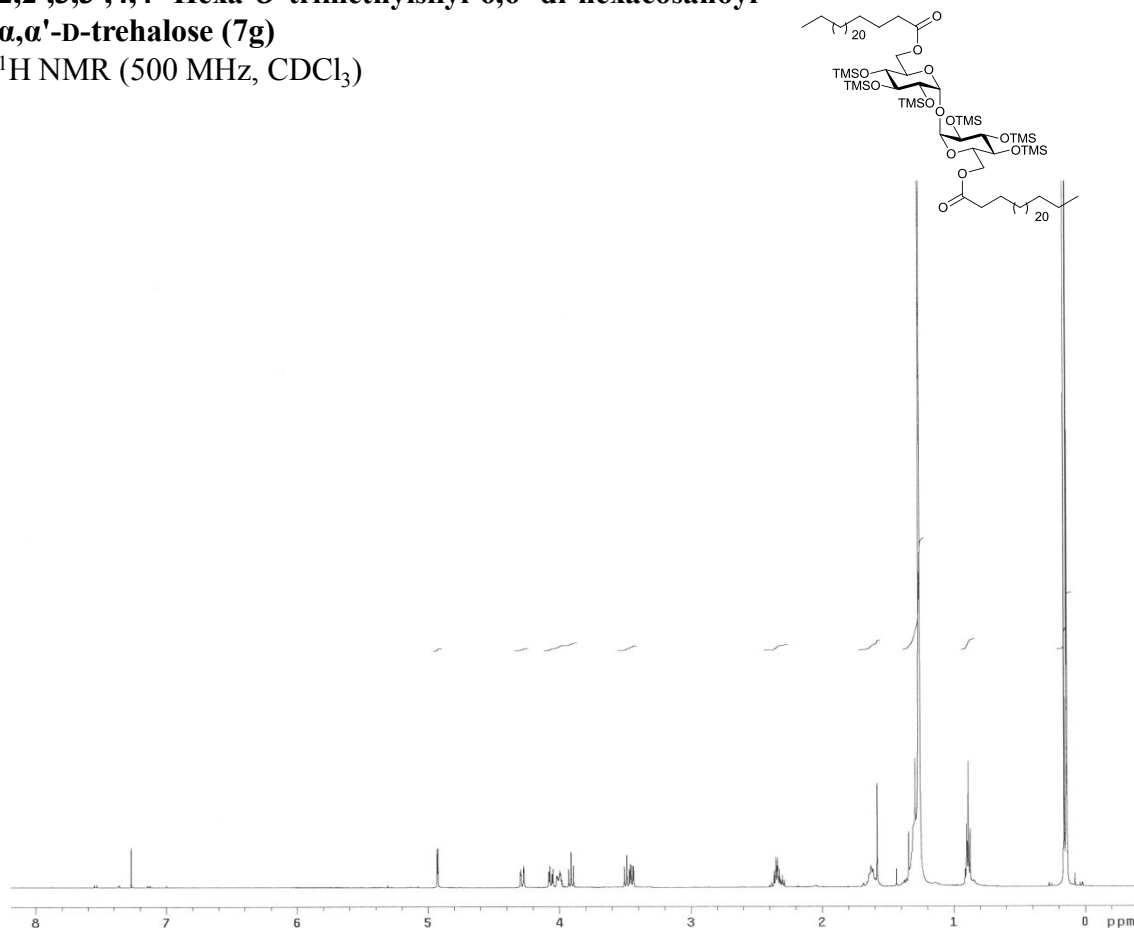
2,2',3,3',4,4'-Hexa-*O*-trimethylsilyl-6,6'-di-docosanoyl- α,α' -D-trehalose (7f)

^{13}C NMR (125 MHz, CDCl_3)



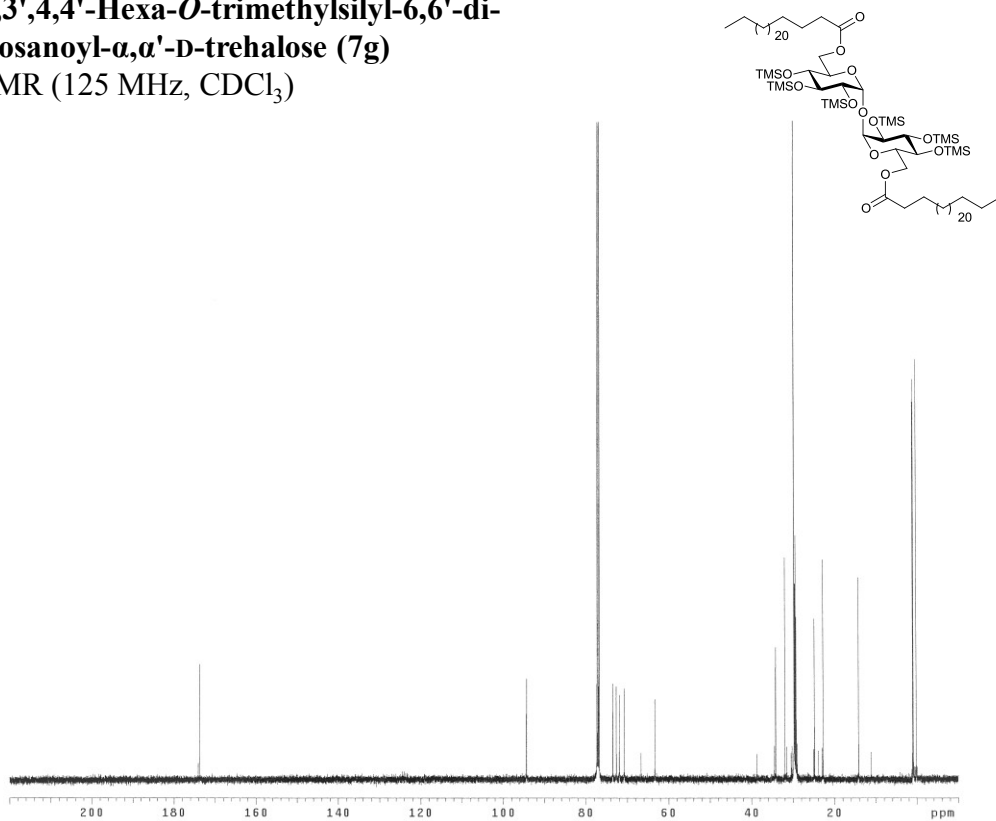
2,2',3,3',4,4'-Hexa-*O*-trimethylsilyl-6,6'-di-hexacosanoyl- α,α' -D-trehalose (7g)

^1H NMR (500 MHz, CDCl_3)



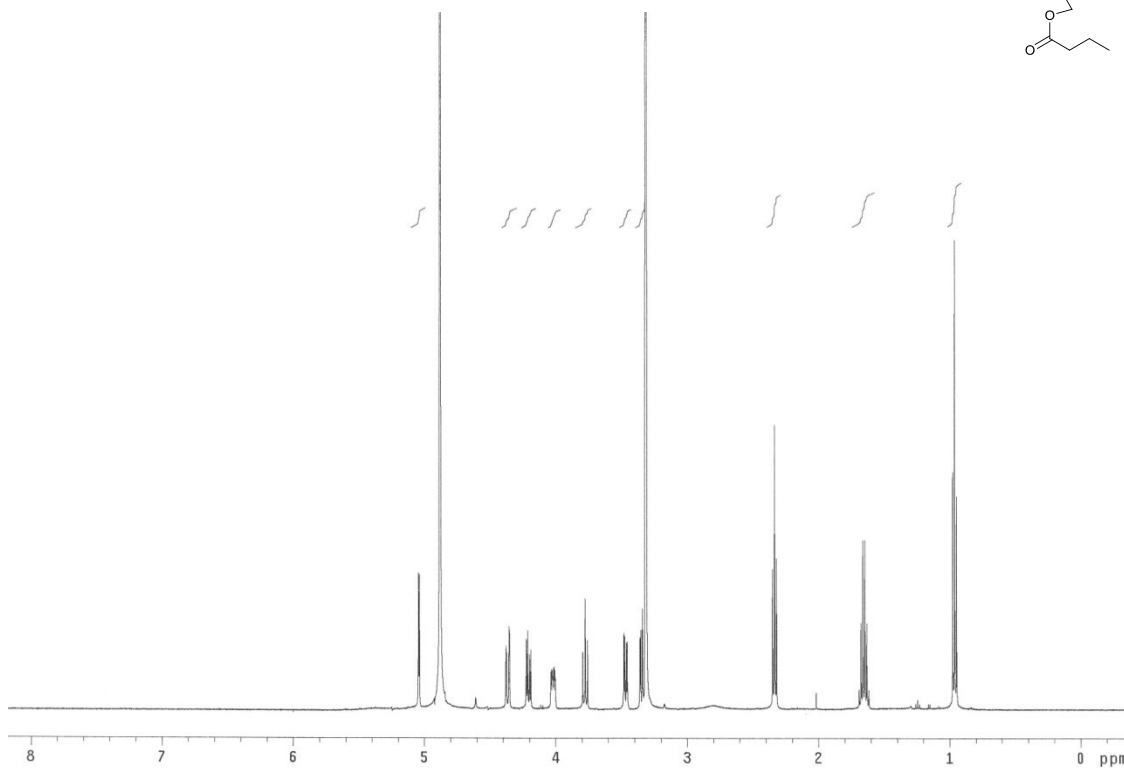
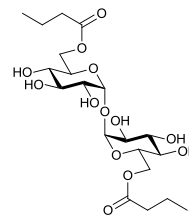
2,2',3,3',4,4'-Hexa-*O*-trimethylsilyl-6,6'-di-hexacosanoyl- α,α' -D-trehalose (7g)

^{13}C NMR (125 MHz, CDCl_3)



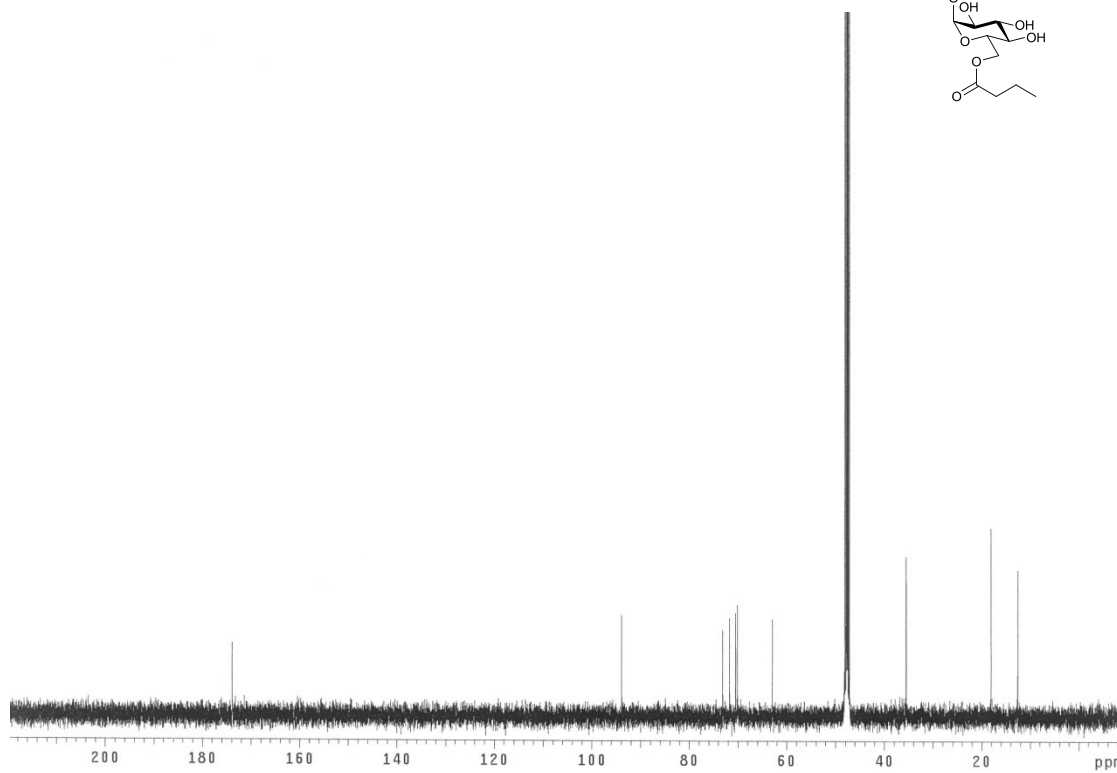
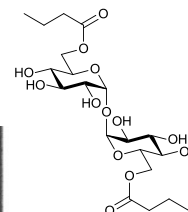
6,6'-Di-*O*-butanoyl- α,α' -D-trehalose (1a)

^1H NMR (500 MHz, CD_3OD)



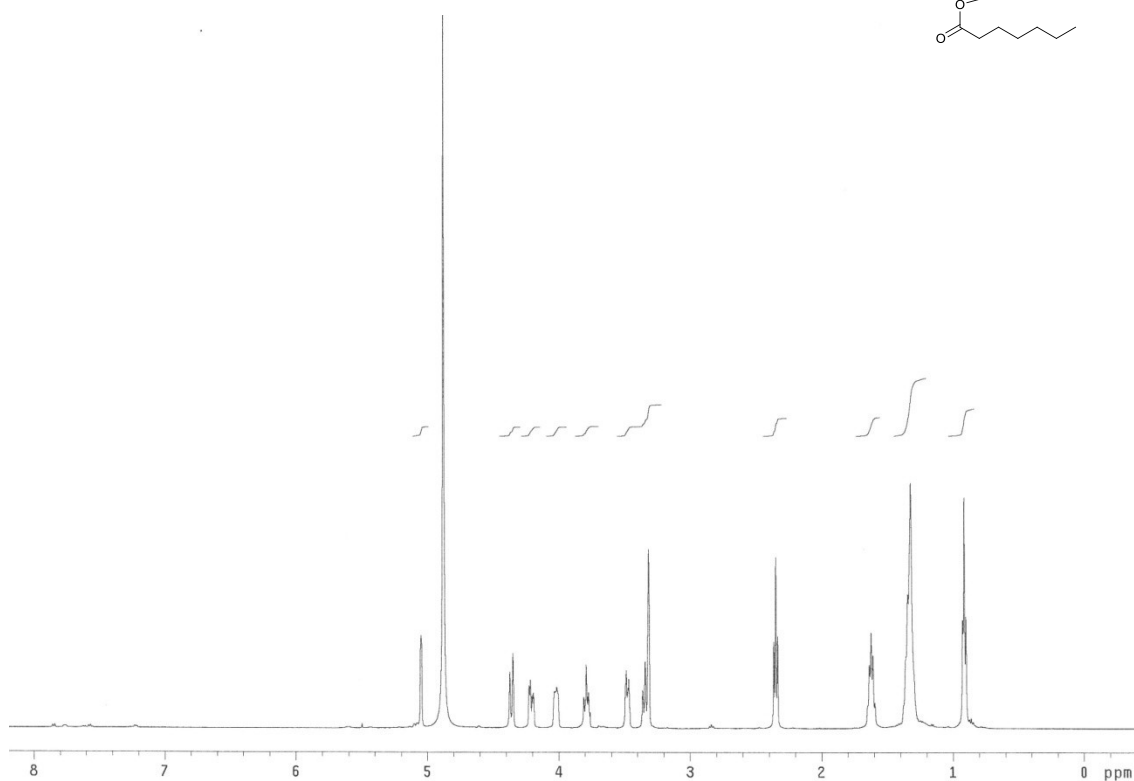
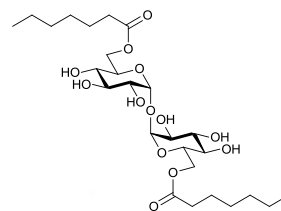
6,6'-Di-*O*-butanoyl- α,α' -D-trehalose (1a)

^{13}C NMR (125 MHz, CD_3OD)



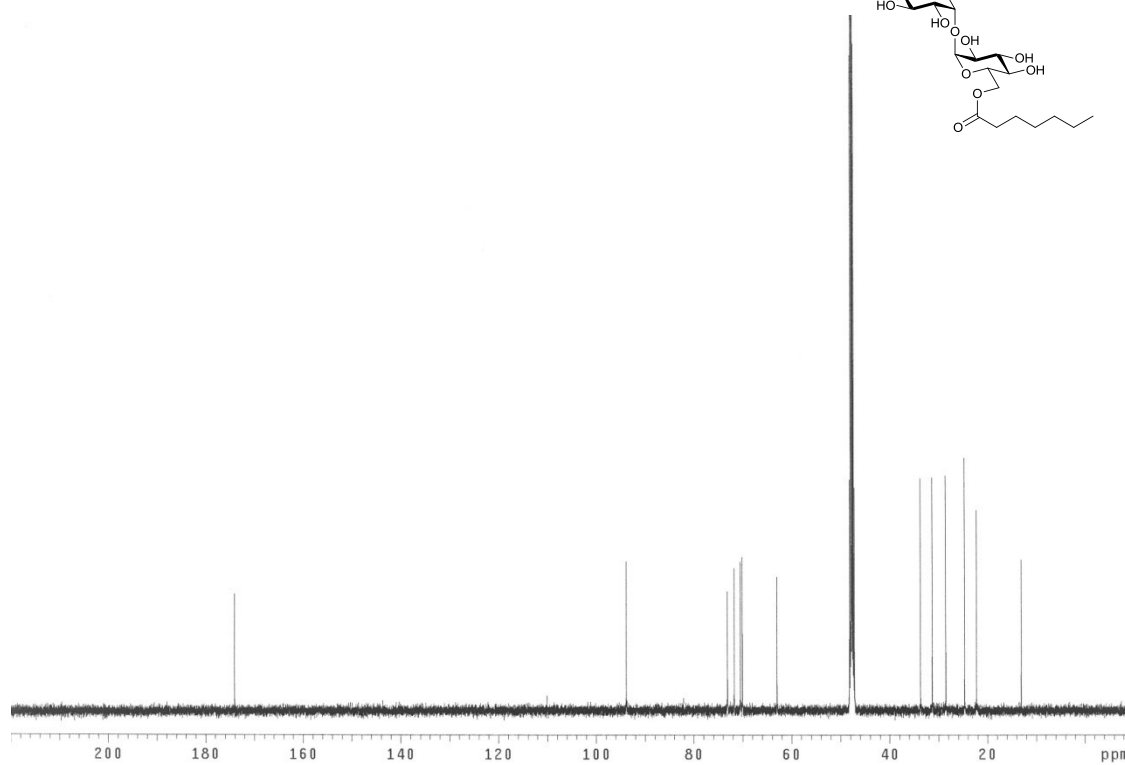
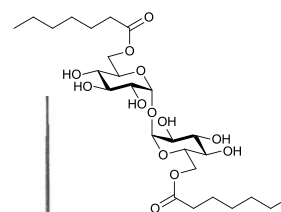
6,6'-Di-*O*-heptanoyl- α,α' -D-trehalose (1b)

^1H NMR (500 MHz, CD_3OD)



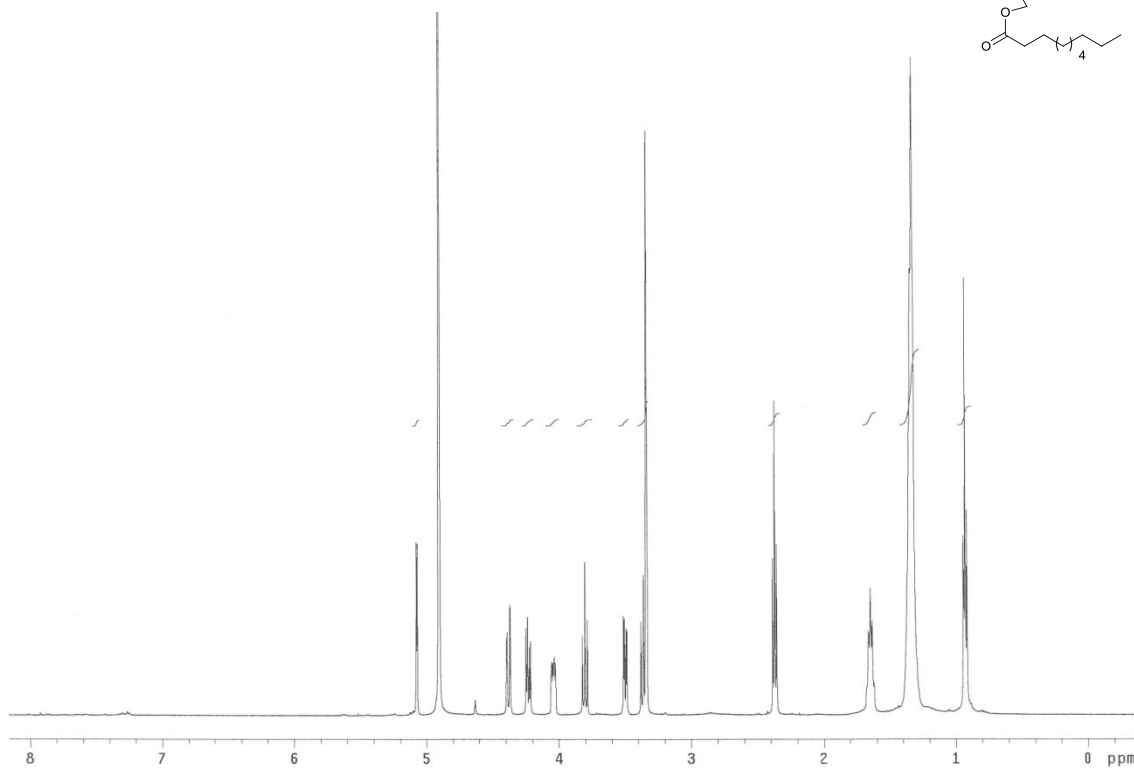
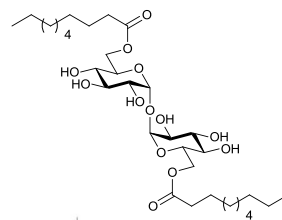
6,6'-Di-*O*-heptanoyl- α,α' -D-trehalose (1b)

^{13}C NMR (125 MHz, CD_3OD)



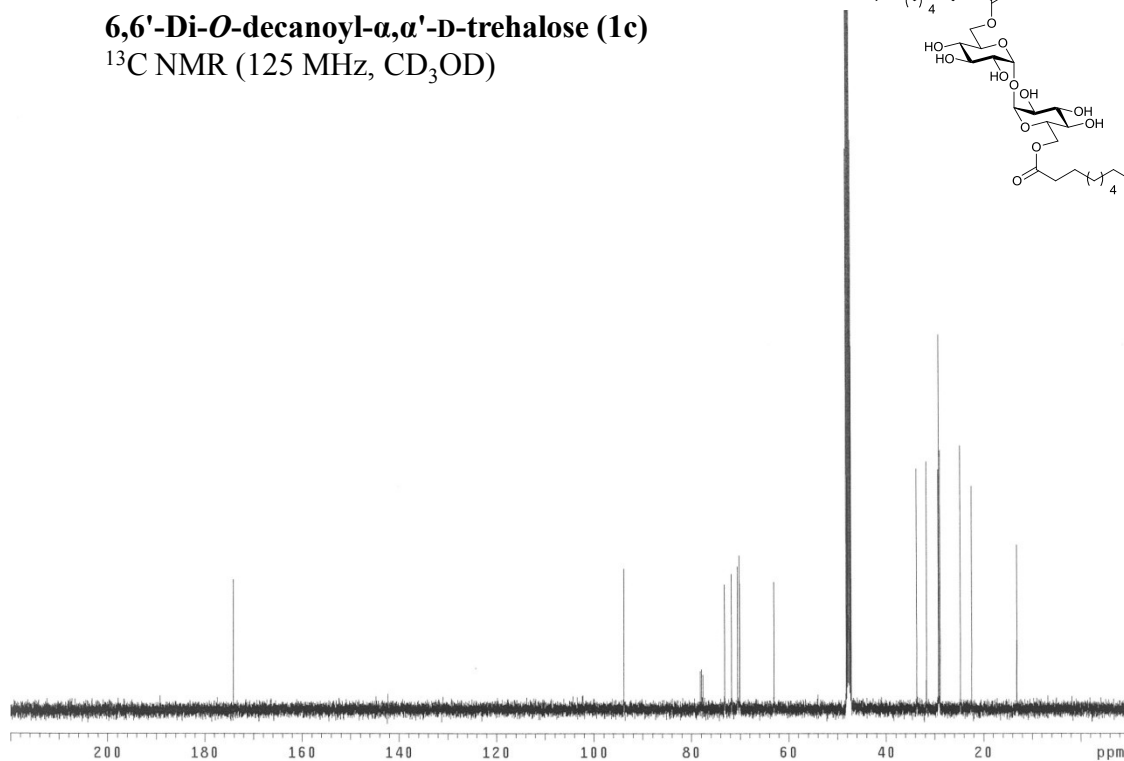
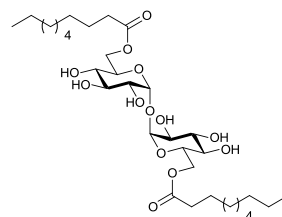
6,6'-Di-*O*-decanoyl- α,α' -D-trehalose (1c)

^1H NMR (500 MHz, CD_3OD)



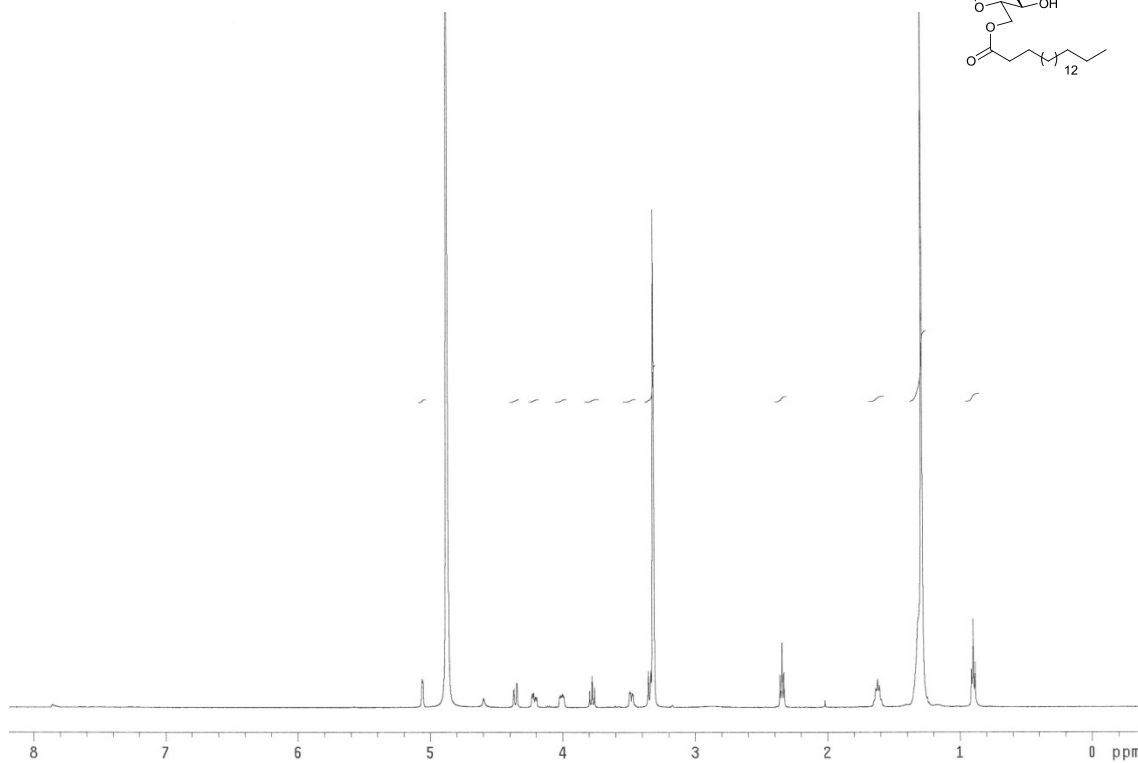
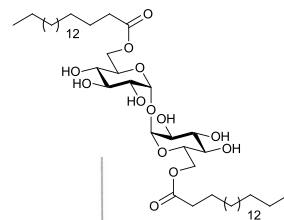
6,6'-Di-*O*-decanoyl- α,α' -D-trehalose (1c)

^{13}C NMR (125 MHz, CD_3OD)



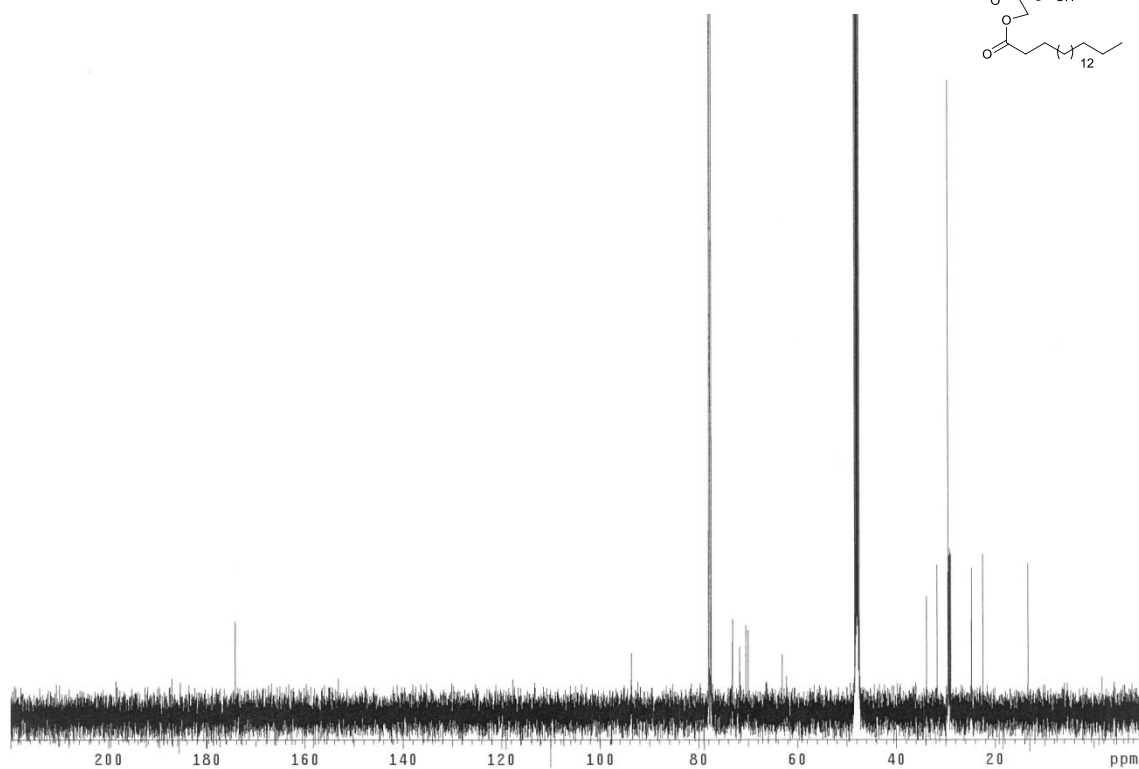
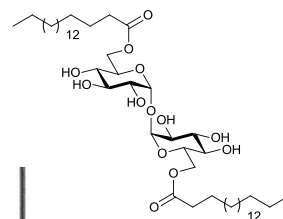
6,6'-Di-*O*-stearyl- α,α' -D-trehalose (1d)

^1H NMR (500 MHz, CD_3OD)



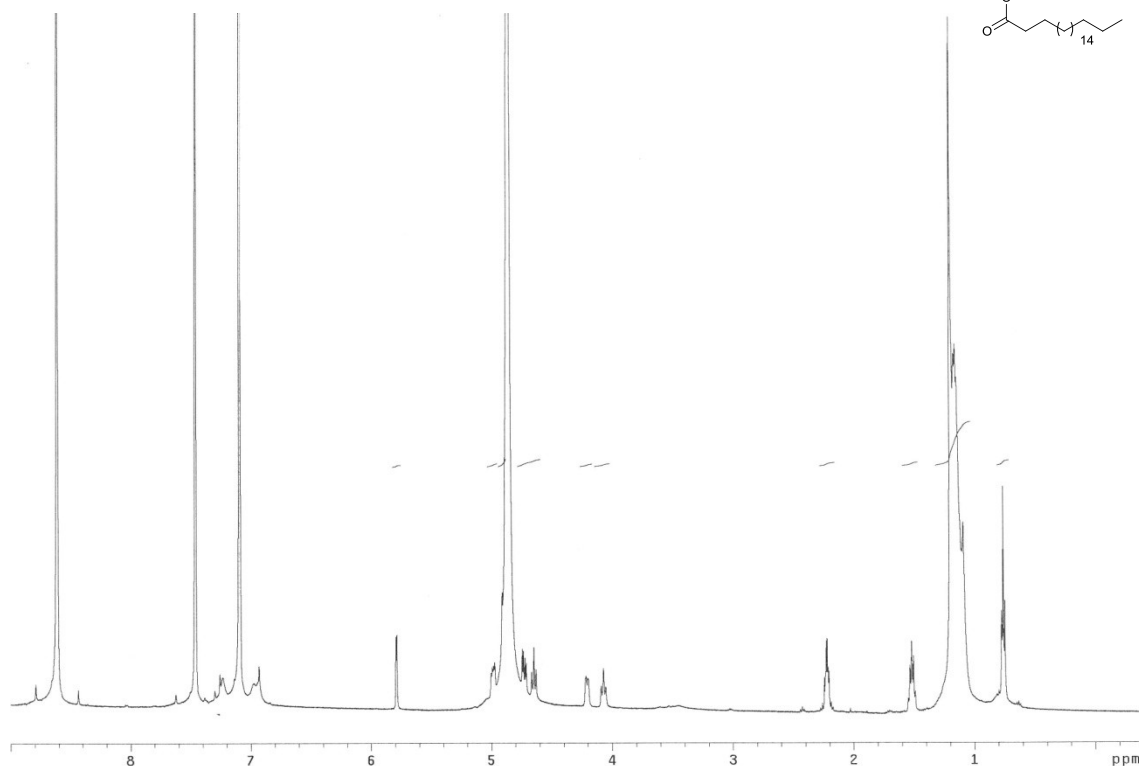
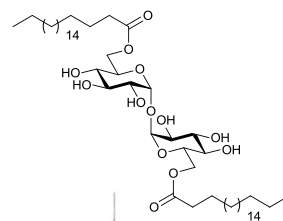
6,6'-Di-*O*-stearyl- α,α' -D-trehalose (1d)

^{13}C NMR (125 MHz, CD_3OD)



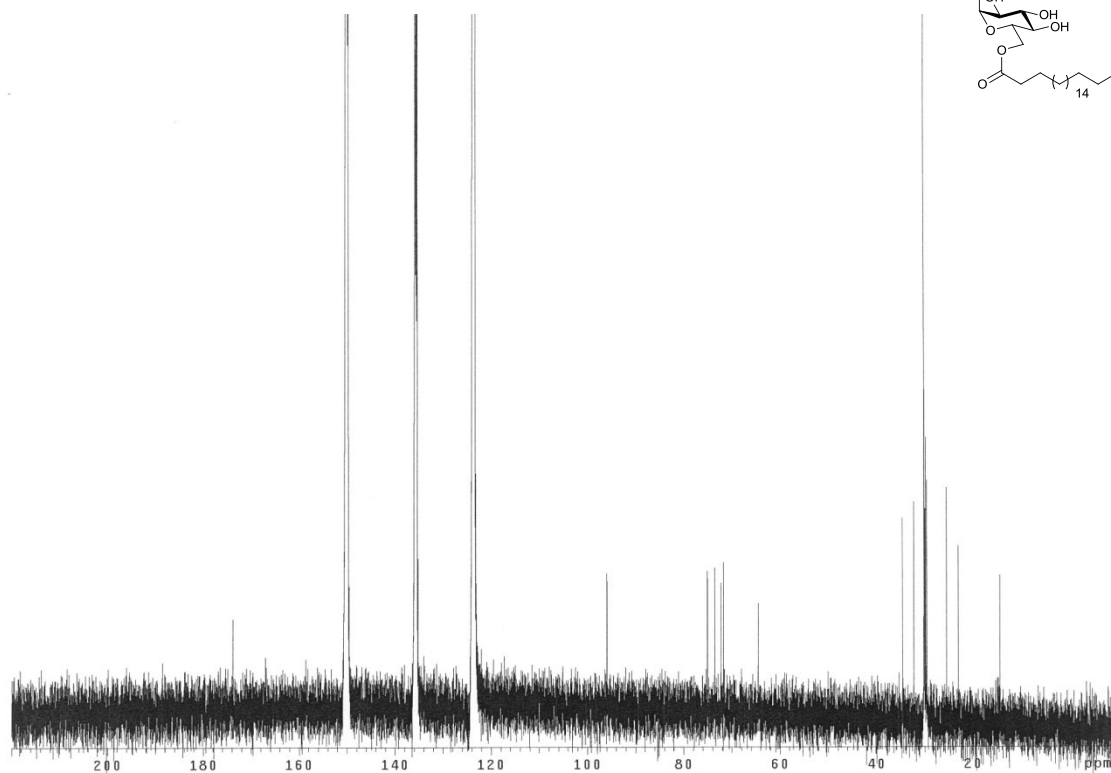
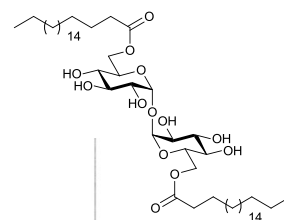
6,6'-Di-*O*-eicosanoyl- α,α' -D-trehalose (1e)

^1H NMR (500 MHz, $\text{C}_5\text{D}_5\text{N}$)



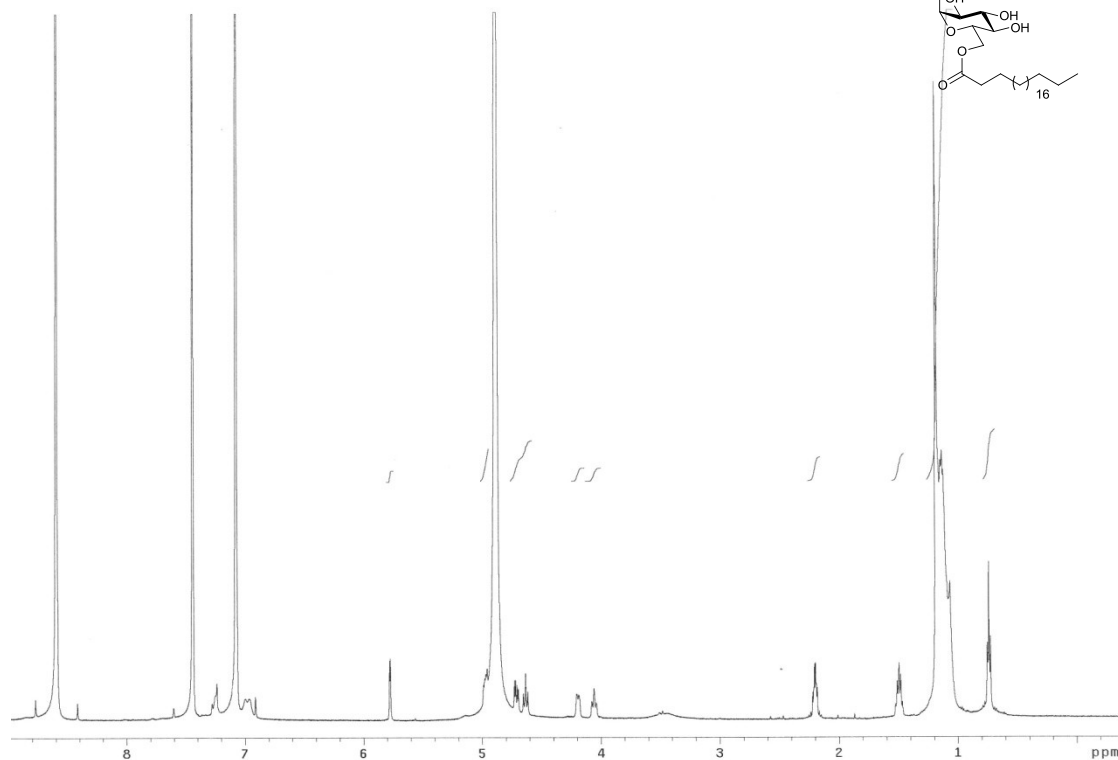
6,6'-Di-*O*-eicosanoyl- α,α' -D-trehalose (1e)

^{13}C NMR (125 MHz, $\text{C}_5\text{D}_5\text{N}$)



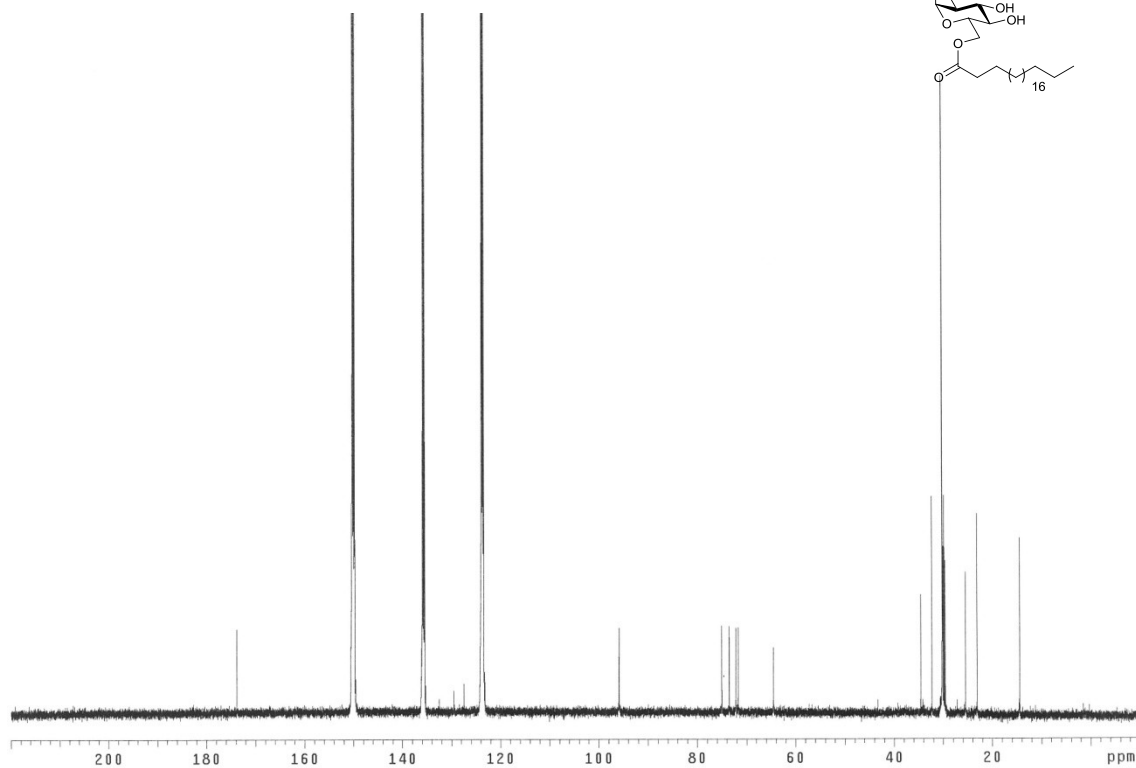
6,6'-Di-*O*-docosanoyl- α,α' -D-trehalose (1f)

^1H NMR (500 MHz, $\text{C}_5\text{D}_5\text{N}$)



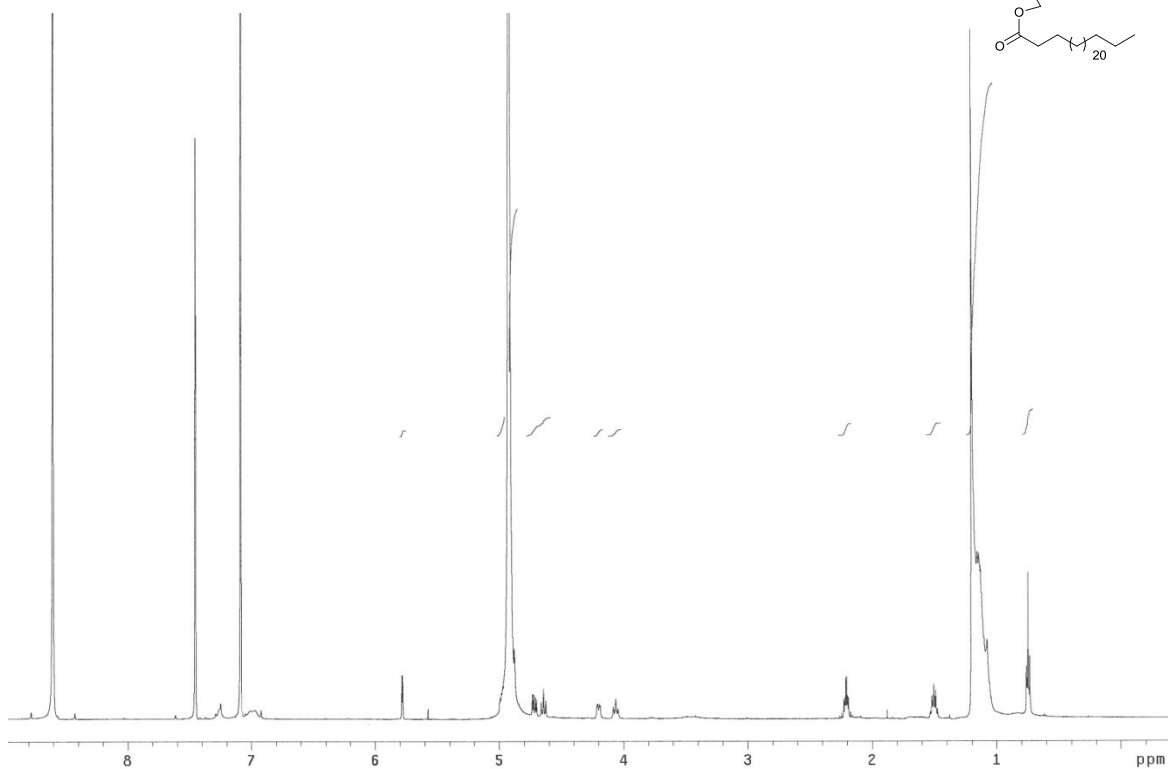
6,6'-Di-*O*-docosanoyl- α,α' -D-trehalose (1f)

^{13}C NMR (125 MHz, $\text{C}_5\text{D}_5\text{N}$)



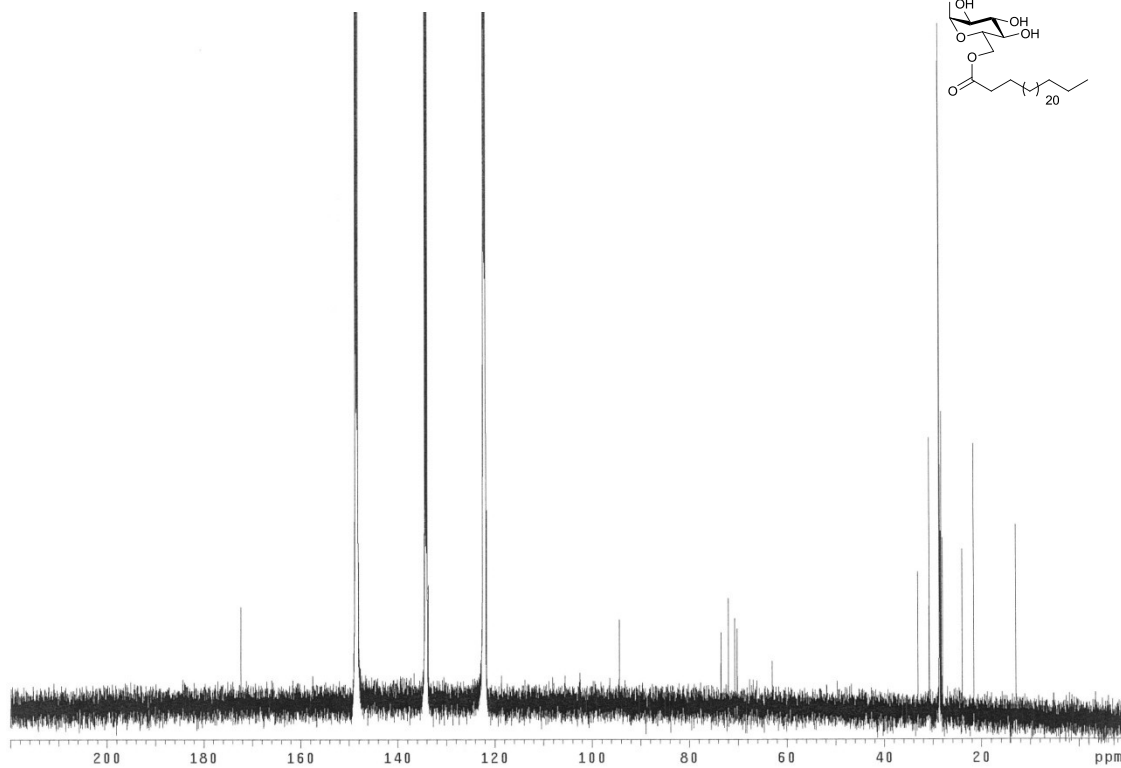
6,6'-Di-*O*-hexacosanoyl- α,α' -D-trehalose (1g)

^1H NMR (500 MHz, $\text{C}_5\text{D}_5\text{N}$)



6,6'-Di-*O*-hexacosanoyl- α,α' -D-trehalose (1g)

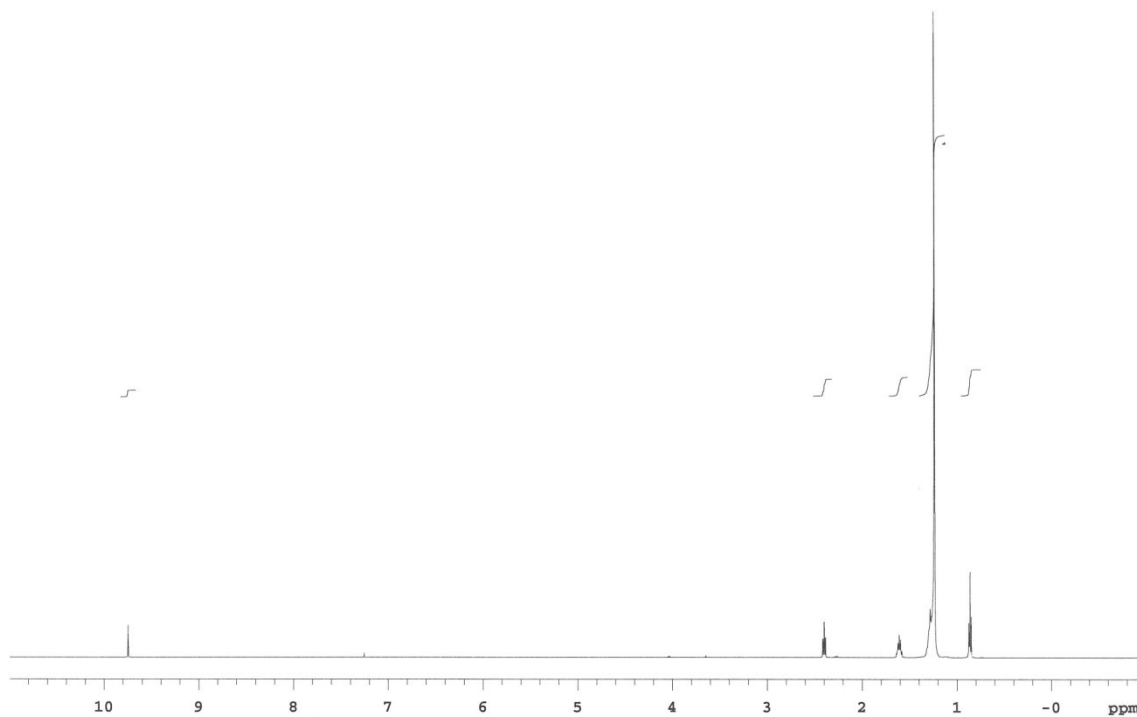
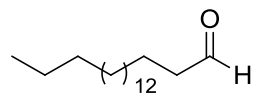
^{13}C NMR (125 MHz, $\text{C}_5\text{D}_5\text{N}$)



Chapter 4

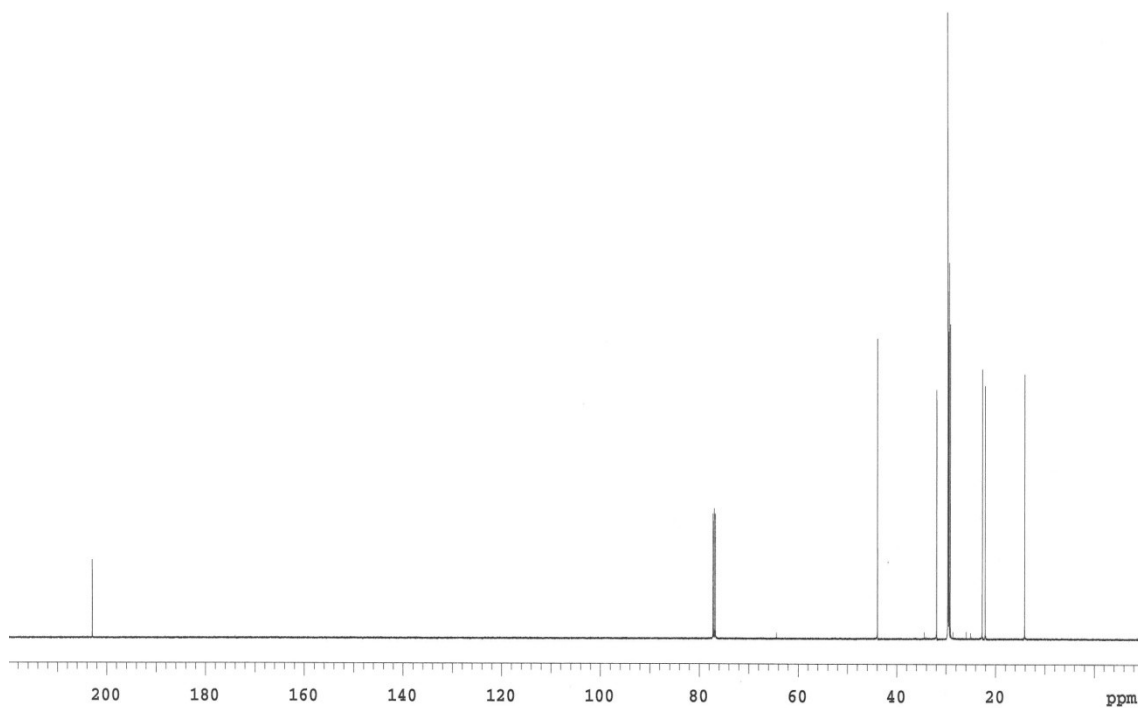
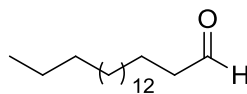
1-Octadecanal (6c)

^1H NMR (500 MHz, CDCl_3)



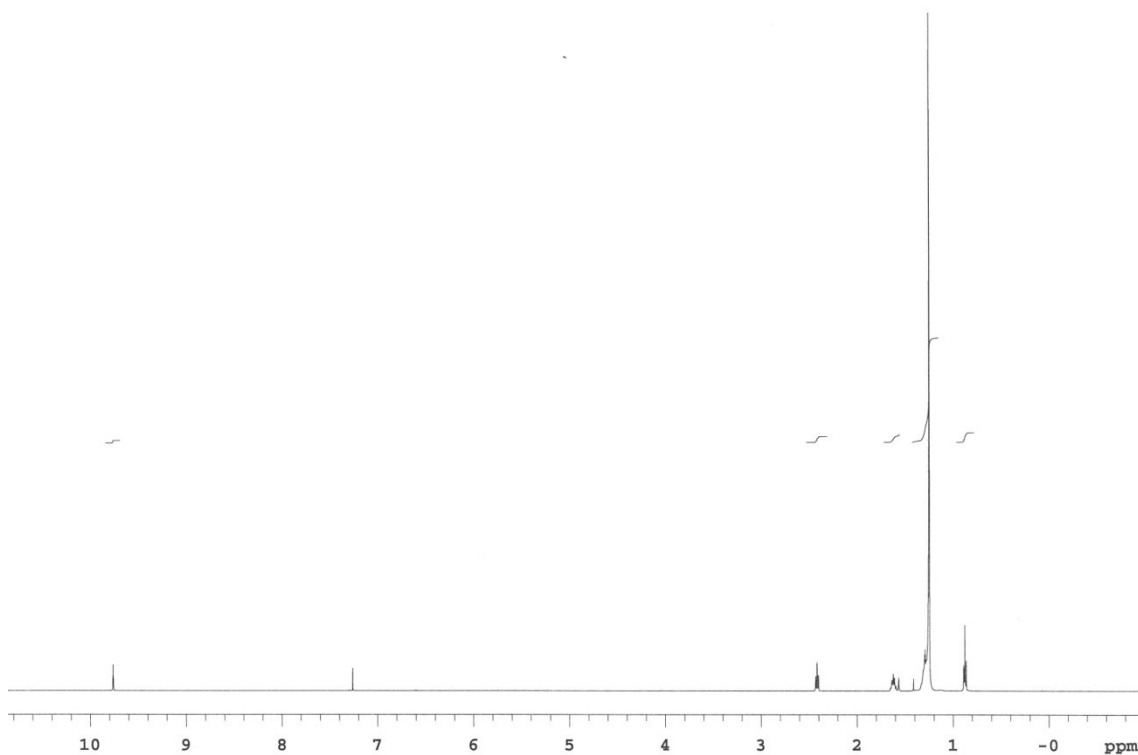
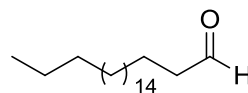
1-Octadecanal (6c)

^{13}C NMR (125 MHz, CDCl_3)



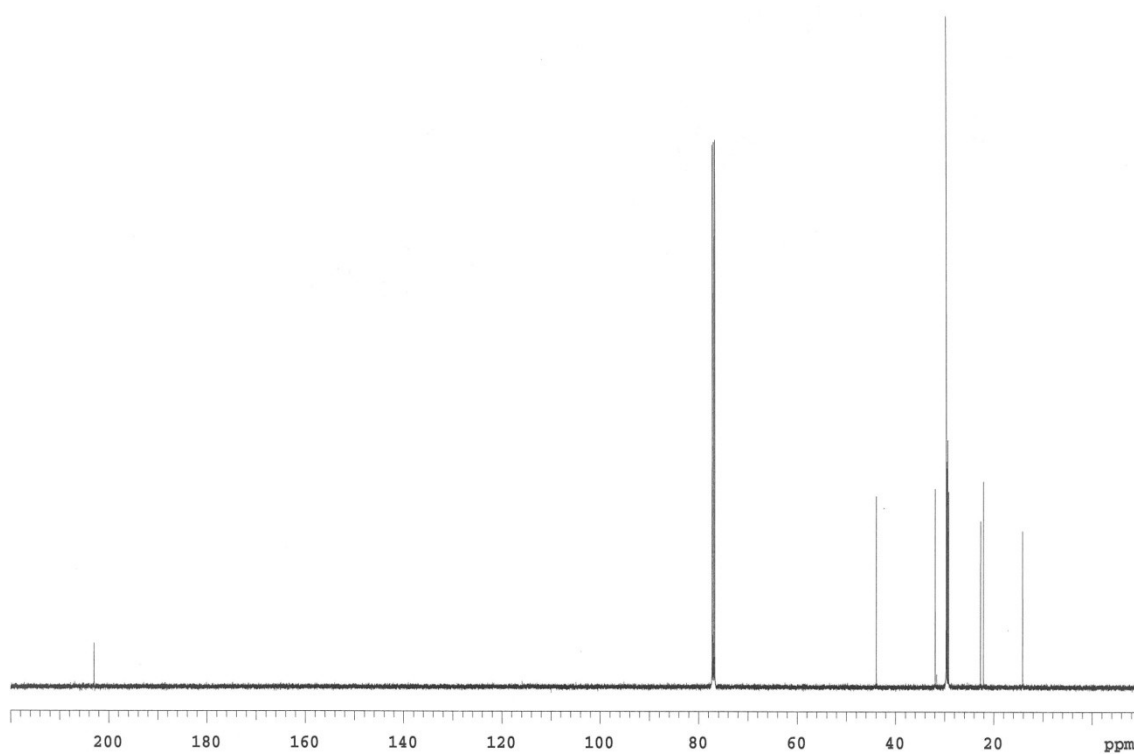
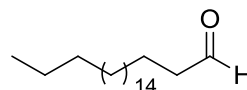
1-Eicosanal (6d)

^1H NMR (500 MHz, CDCl_3)



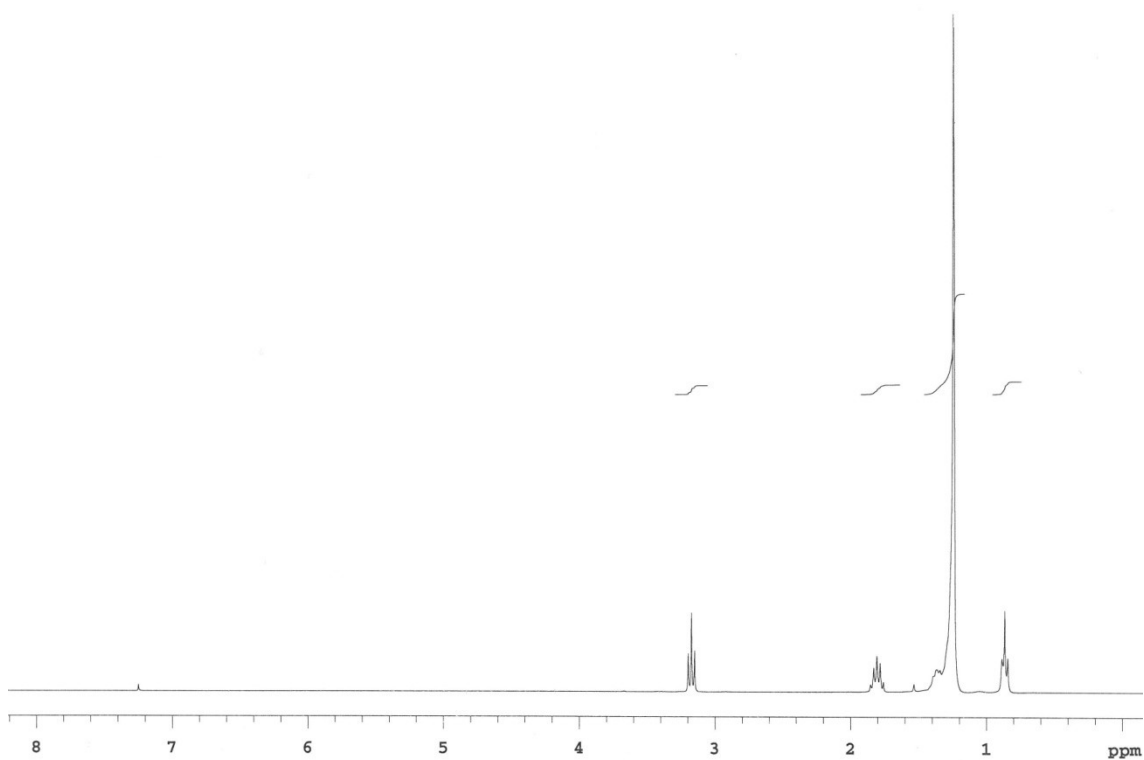
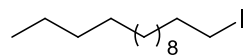
1-Eicosanal (6d)

^{13}C NMR (125 MHz, CDCl_3)



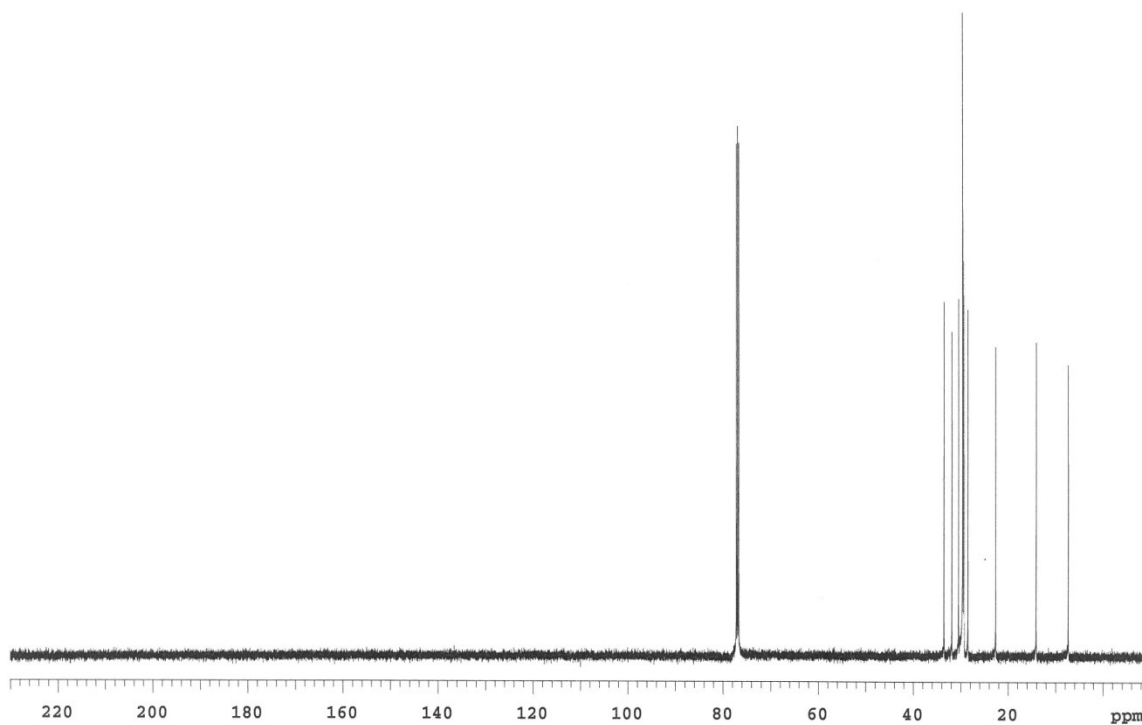
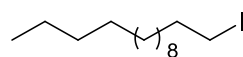
1-Iodotetradecane

^1H NMR (500 MHz, CDCl_3)



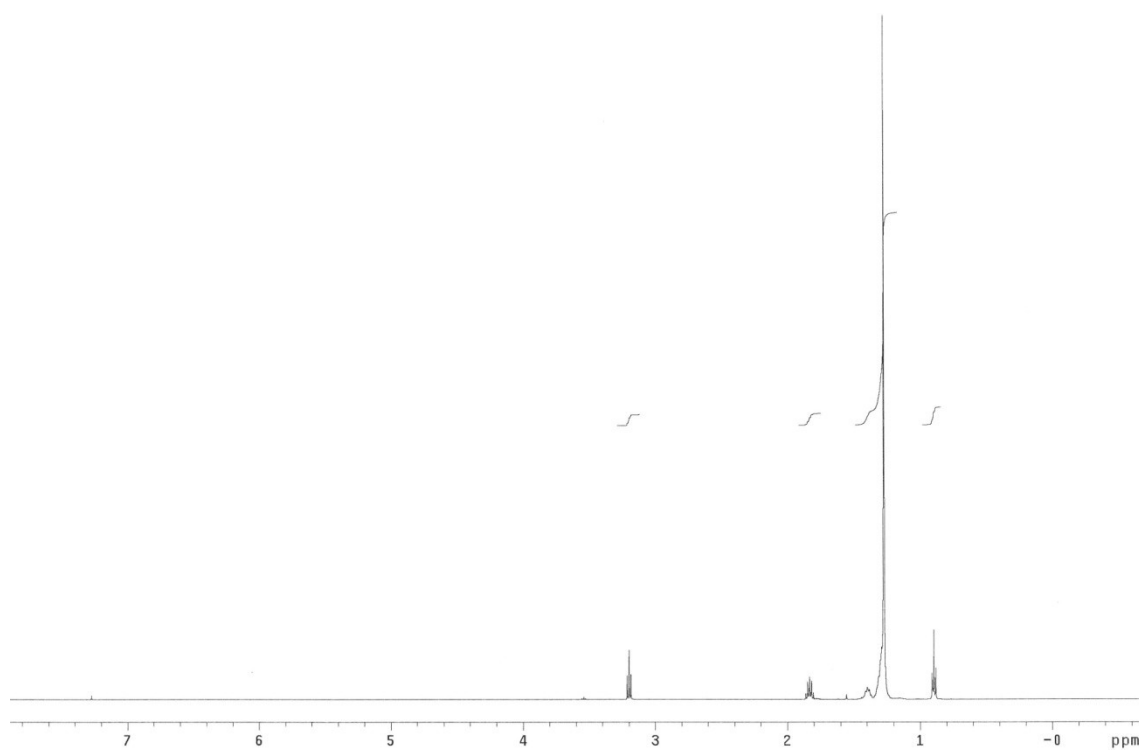
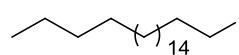
1-Iodotetradecane

^{13}C NMR (125 MHz, CDCl_3)



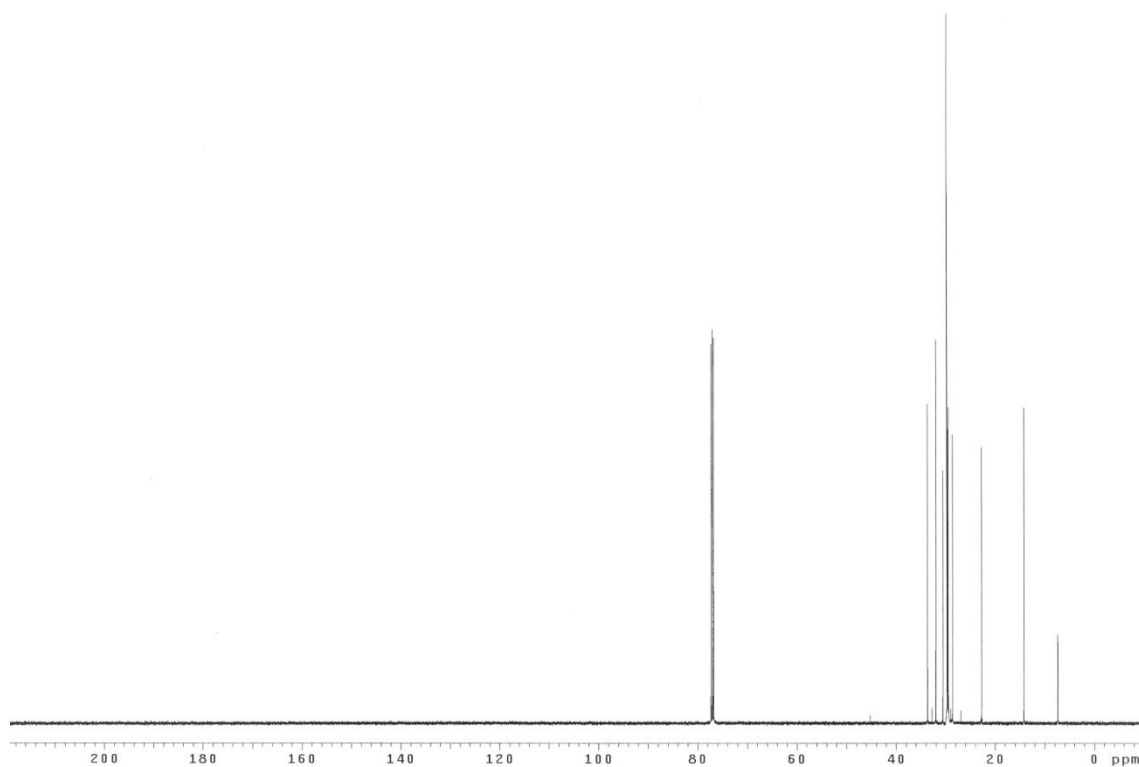
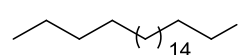
1-Iodoeicosane

^1H NMR (500 MHz, CDCl_3)



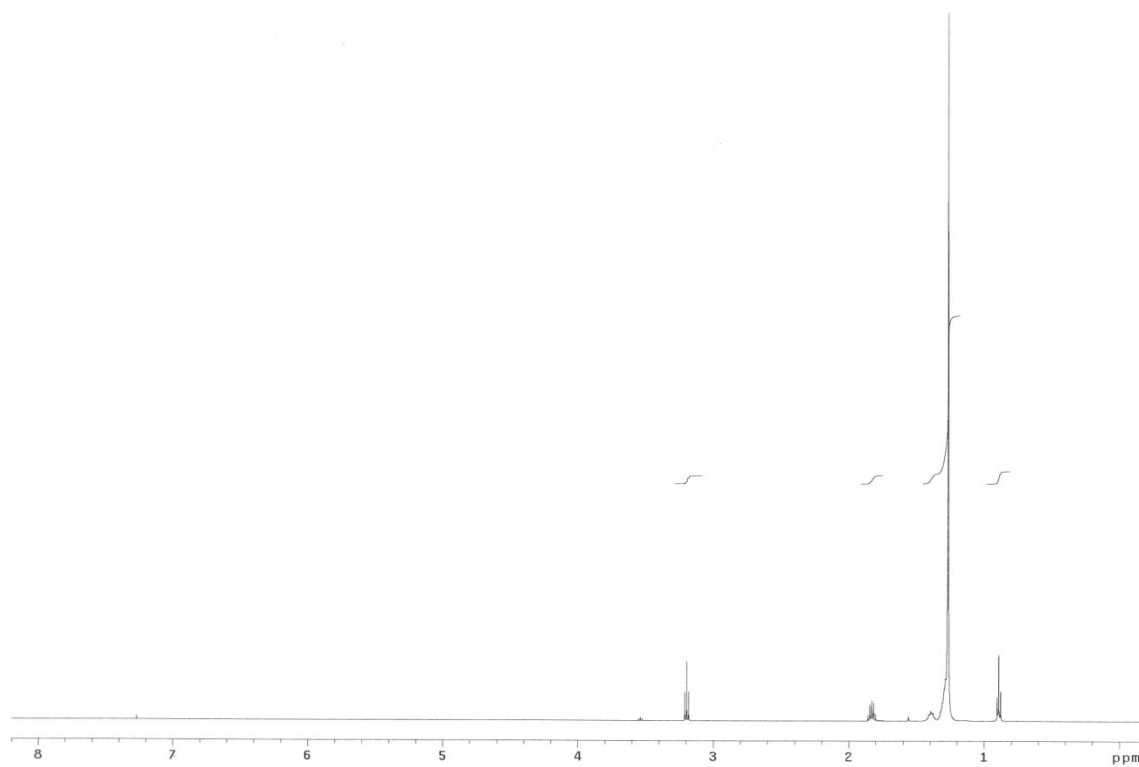
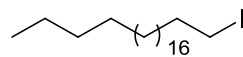
1-Iodoeicosane

^{13}C NMR (125 MHz, CDCl_3)



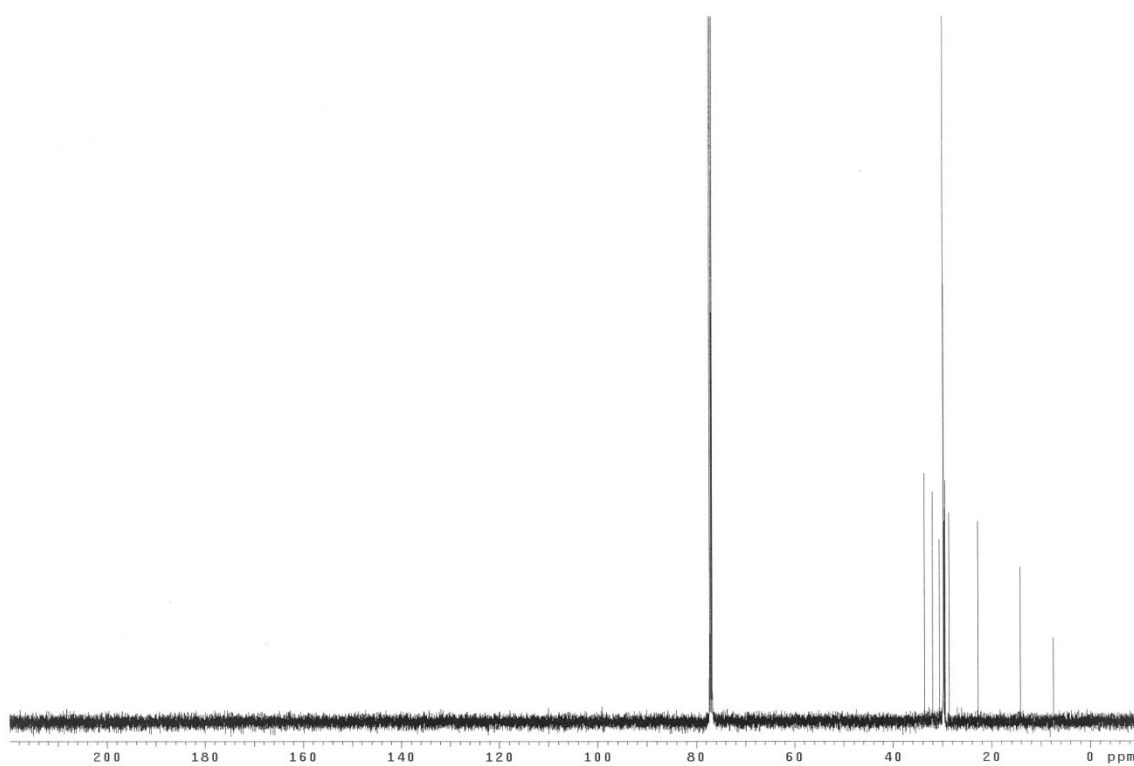
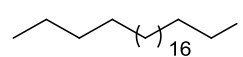
1-Iododocosane

^1H NMR (500 MHz, CDCl_3)

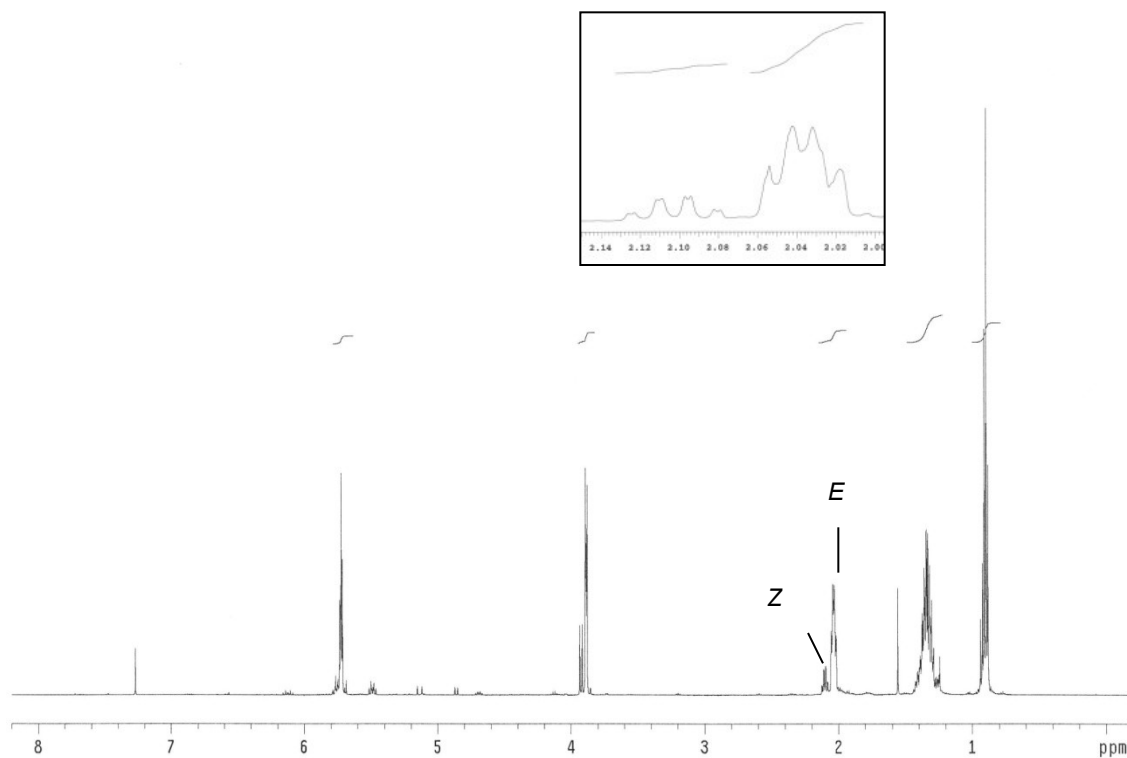
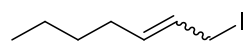


1-Iododocosane

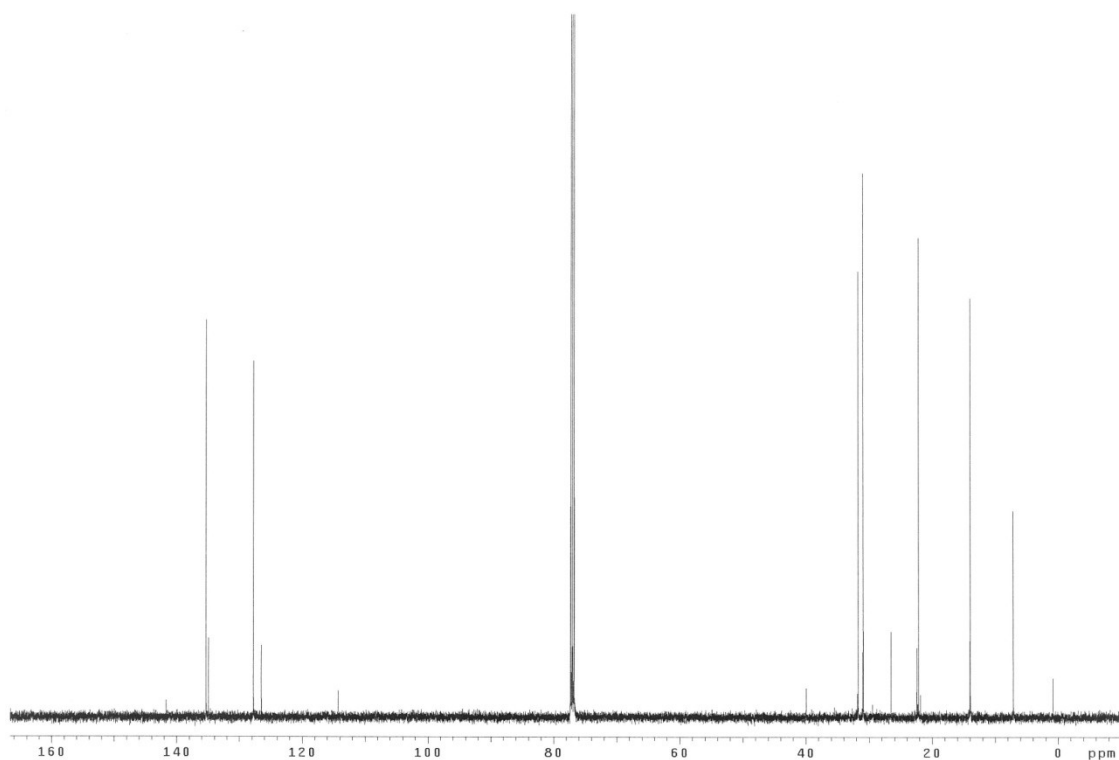
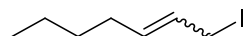
^{13}C NMR (125 MHz, CDCl_3)



1-Iodo-hept-2-ene (4a)
¹H NMR (500 MHz, CDCl₃)

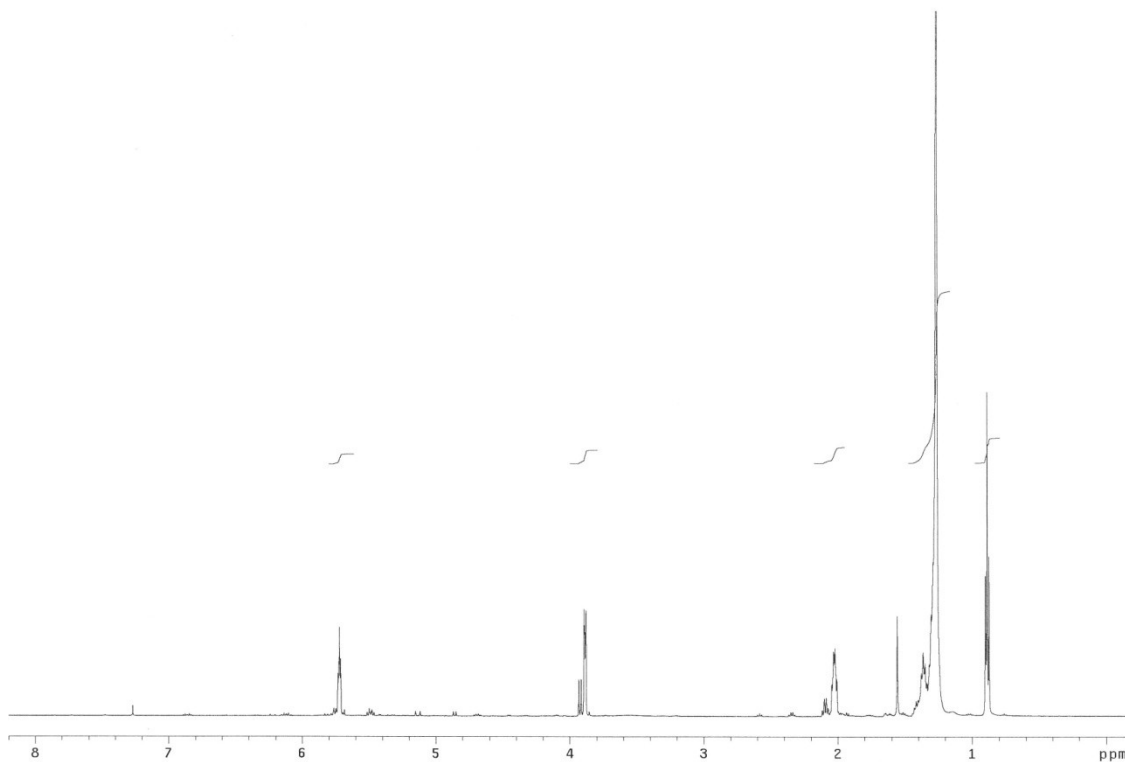
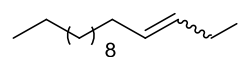


1-Iodo-hept-2-ene (4a)
¹³C NMR (125 MHz, CDCl₃)



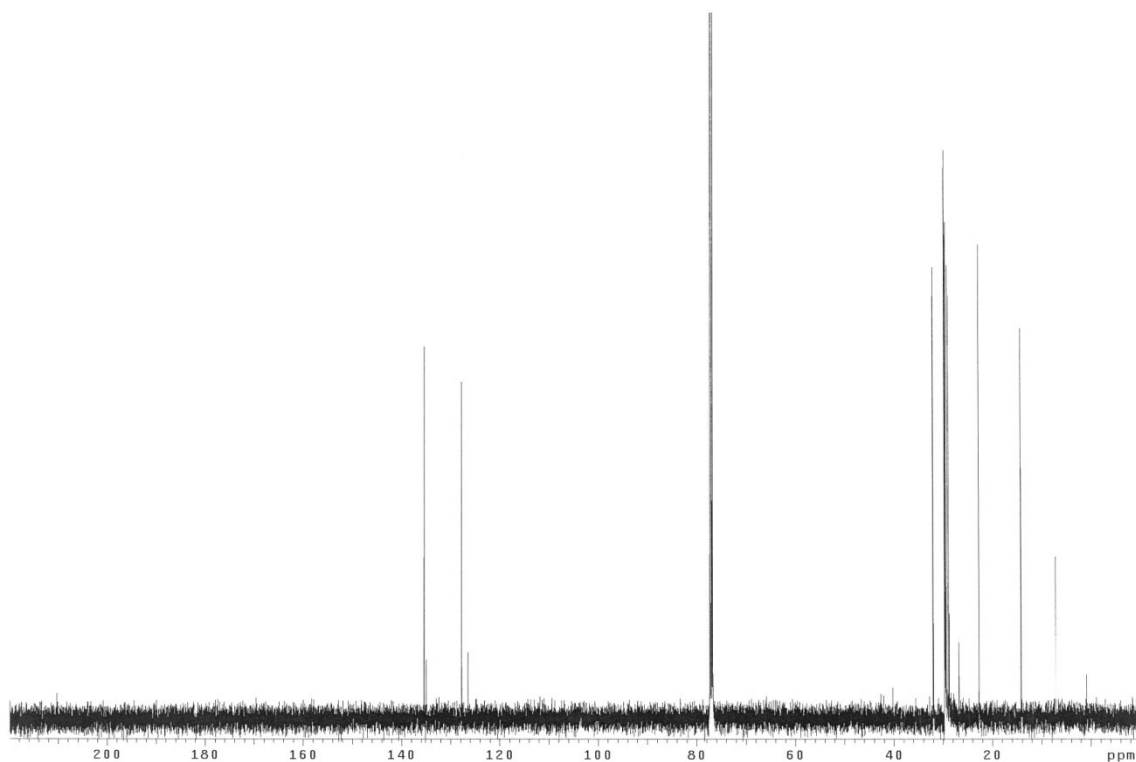
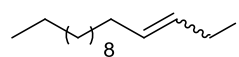
1-Iodo-tetradec-2-ene (4b)

^1H NMR (500 MHz, CDCl_3)

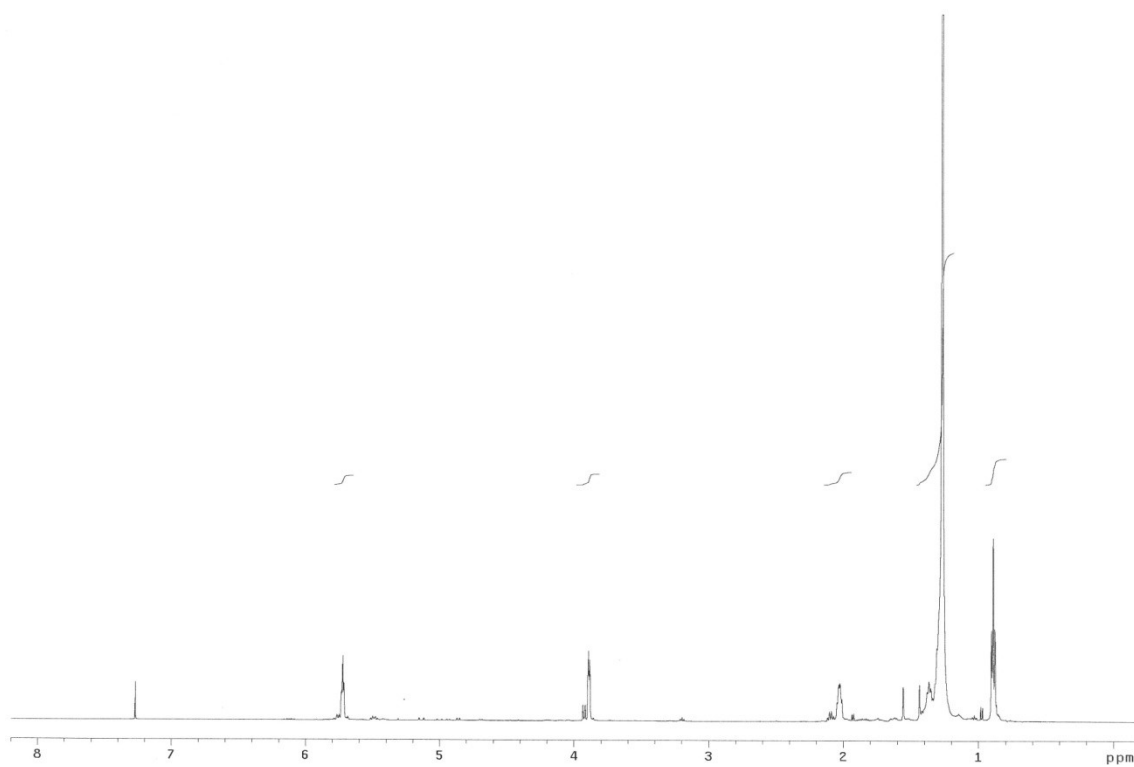
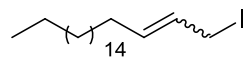


1-Iodo-tetradec-2-ene (4b)

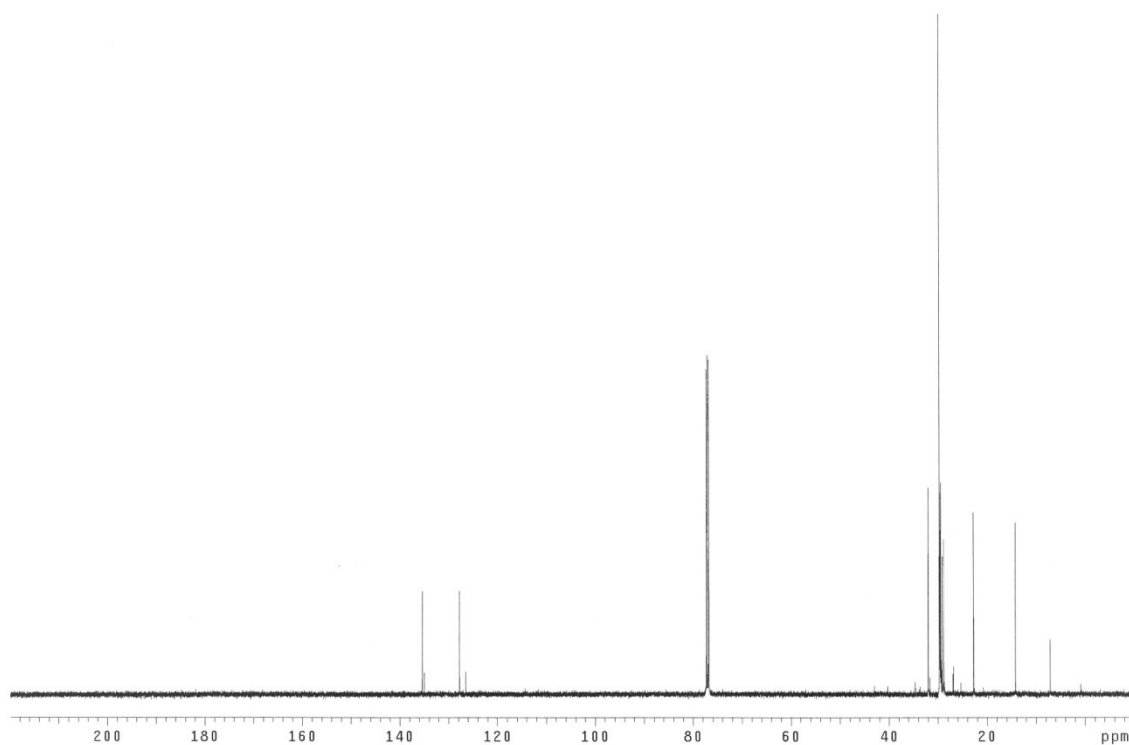
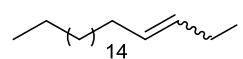
^{13}C NMR (125 MHz, CDCl_3)



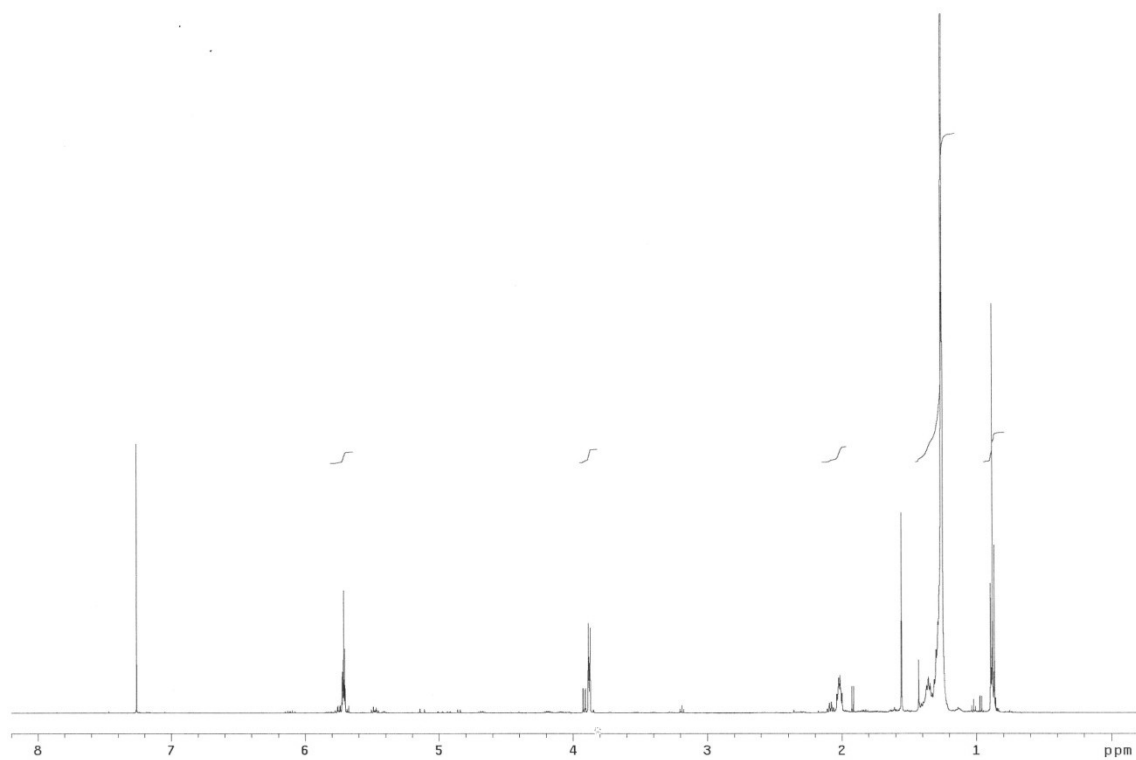
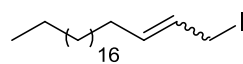
1-Iodo-eicos-2-ene (4c)
¹H NMR (500 MHz, CDCl₃)



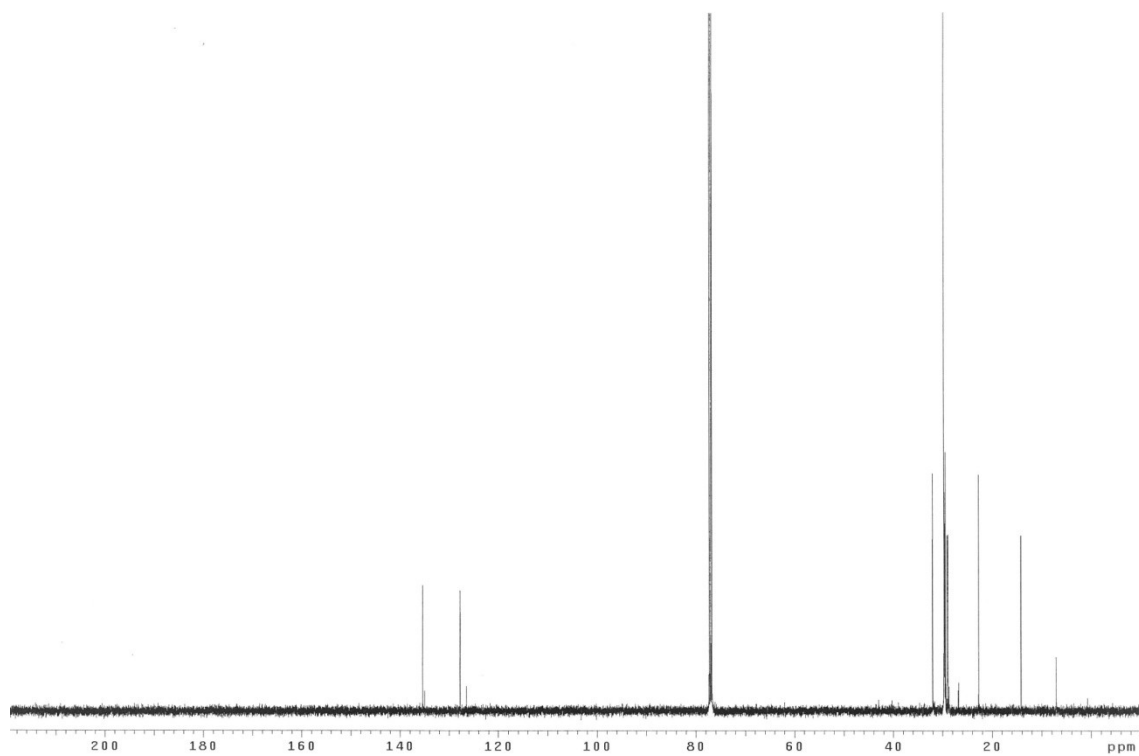
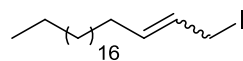
1-Iodo-eicos-2-ene (4c)
¹³C NMR (125 MHz, CDCl₃)



1-Iodo-docos-2-ene (4d)
¹H NMR (500 MHz, CDCl₃)

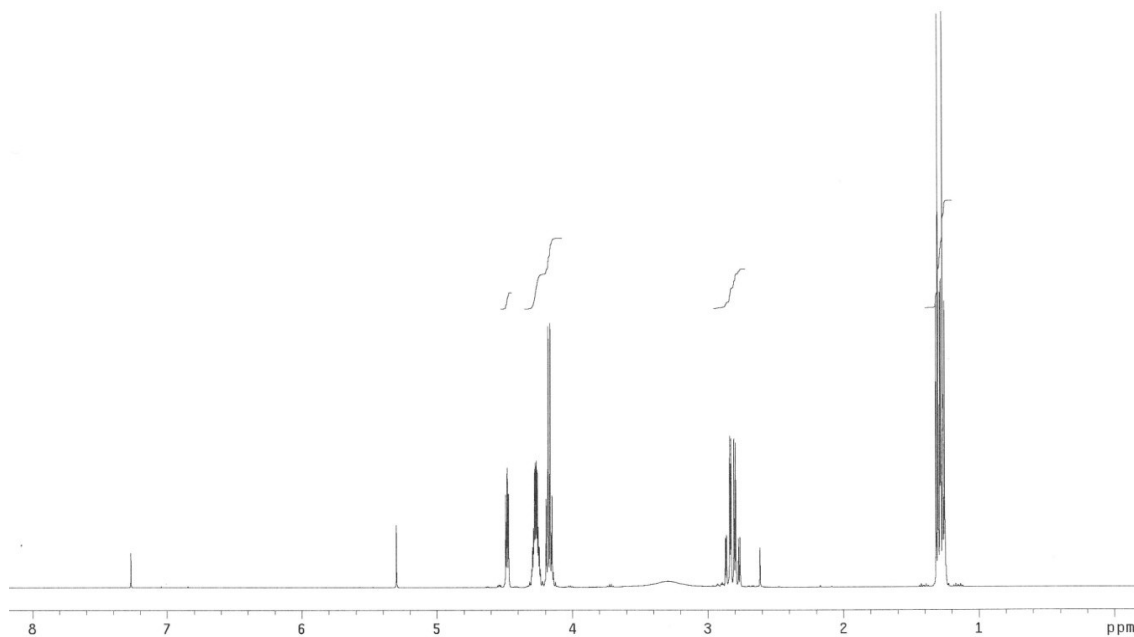
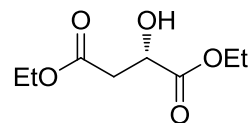


1-Iodo-docos-2-ene (4d)
¹³C NMR (125 MHz, CDCl₃)



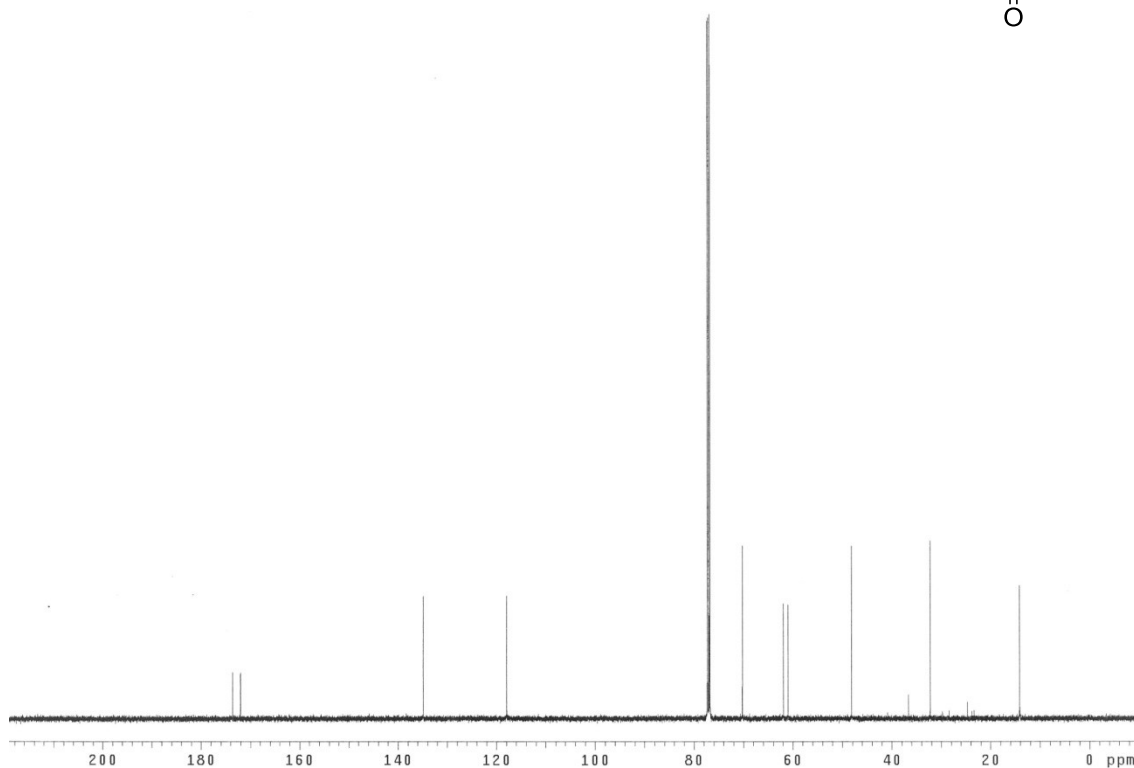
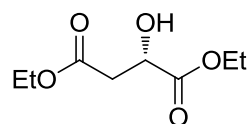
Diethyl L-malate (3)

^1H NMR (500 MHz, CDCl_3)



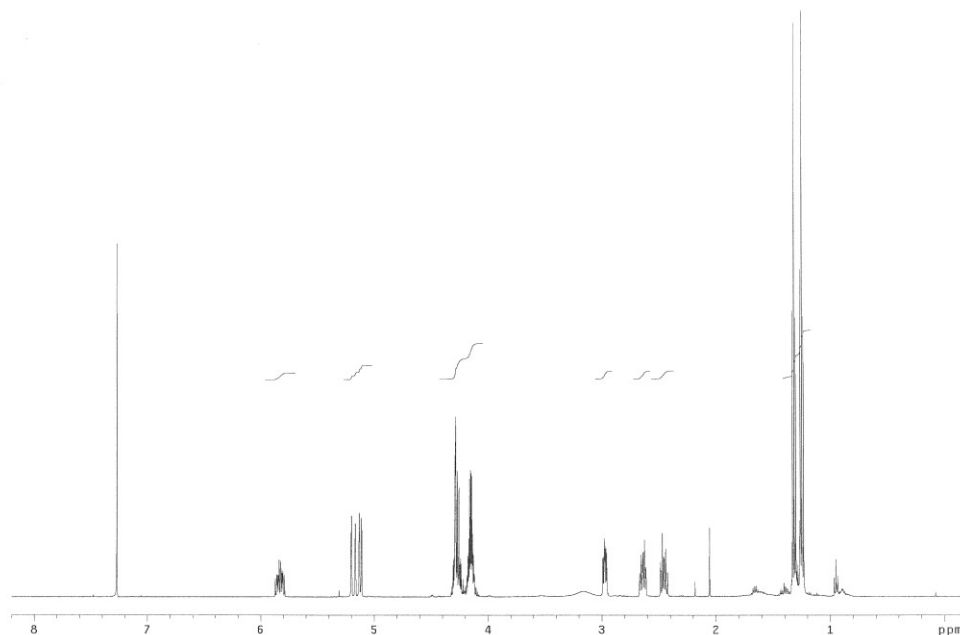
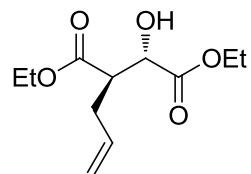
Diethyl L-malate (3)

^{13}C NMR (125 MHz, CDCl_3)



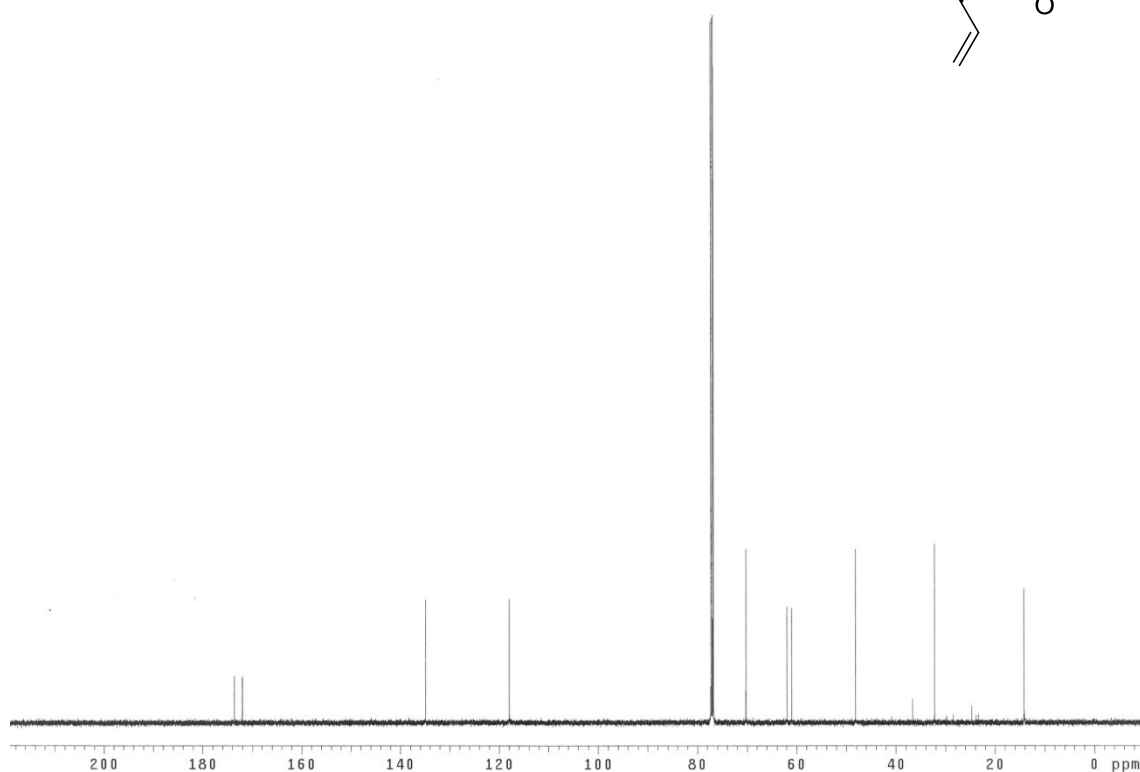
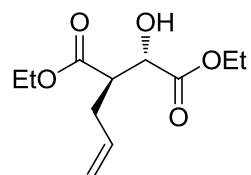
Ethyl 2*S*-hydroxy-3*R*-ethoxycarbonyl-hex-4-enoate (5a)

¹H NMR (500 MHz, CDCl₃)



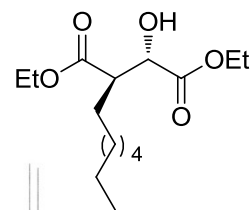
Ethyl 2*S*-hydroxy-3*R*-ethoxycarbonyl-hex-4-enoate (5a)

¹³C NMR (125 MHz, CDCl₃)

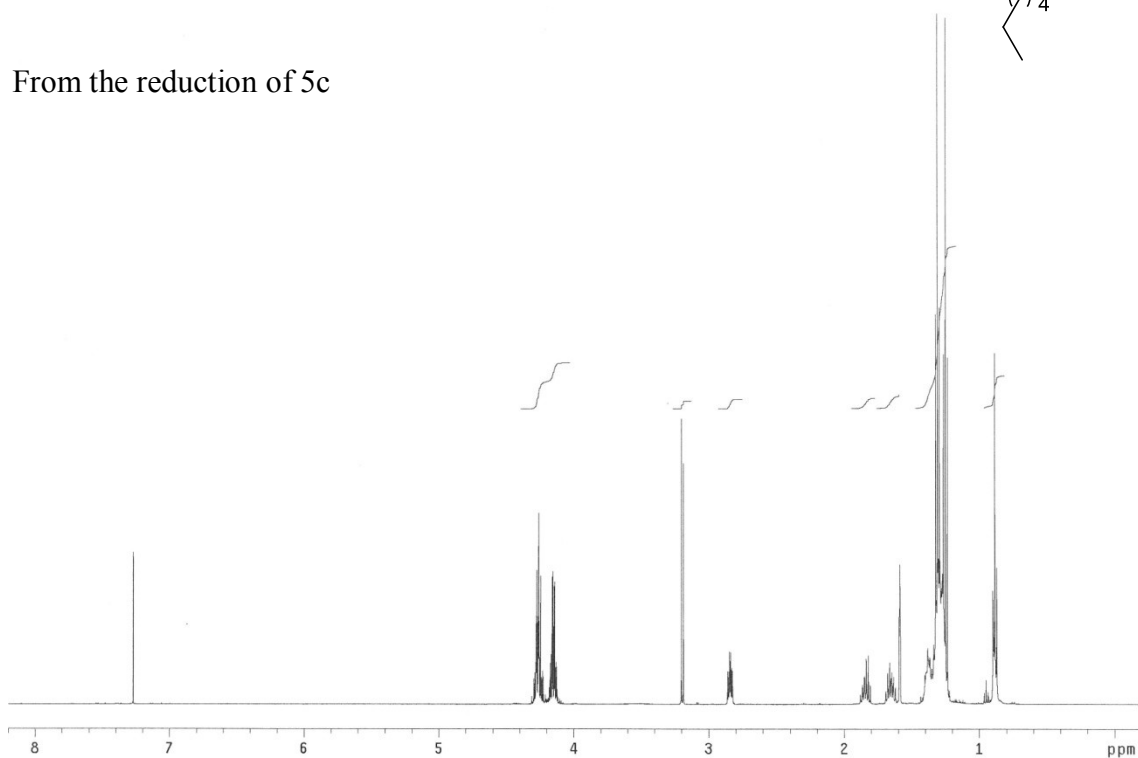


Ethyl 2*S*-hydroxy-3*R*-ethoxycarbonyl-decanoate (5b)

¹H NMR (500 MHz, CDCl₃)

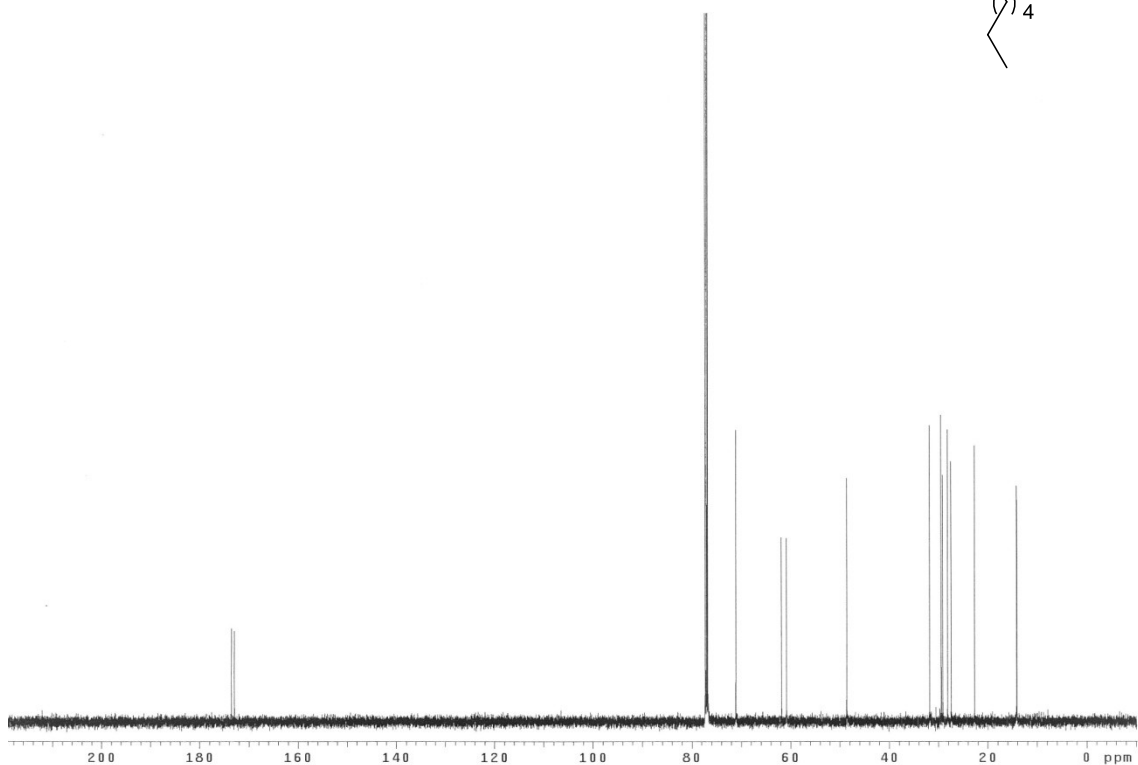
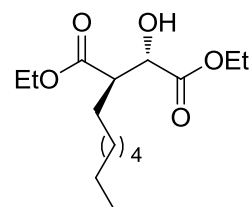


From the reduction of 5c



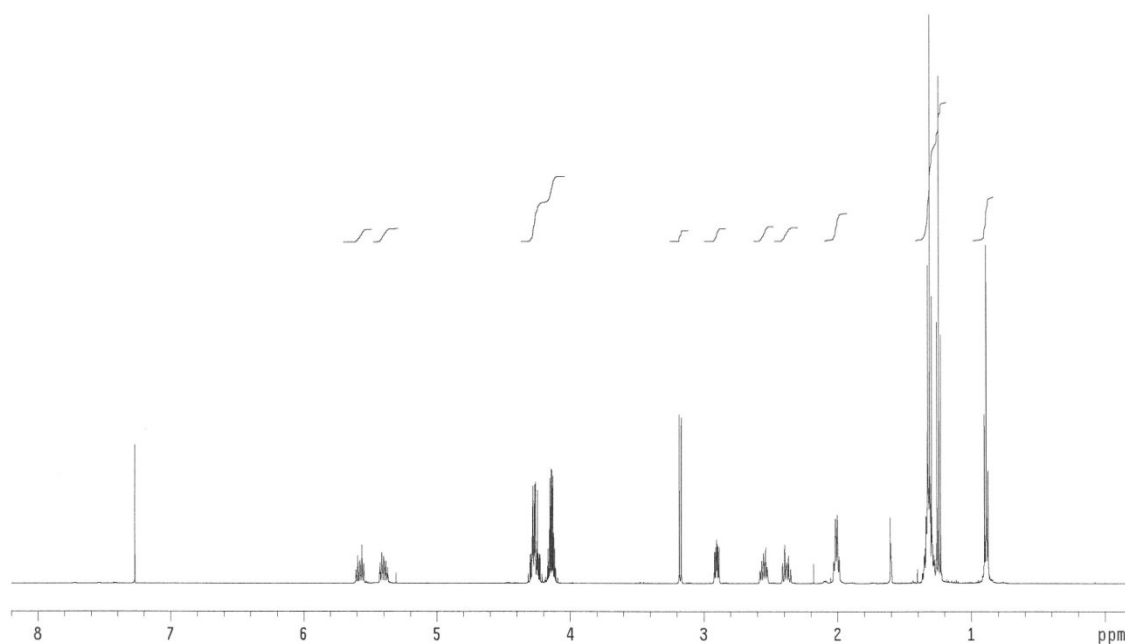
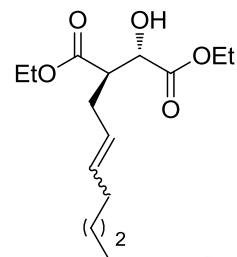
Ethyl 2*S*-hydroxy-3*R*-ethoxycarbonyl-decanoate (5b)

¹³C NMR (125 MHz, CDCl₃)



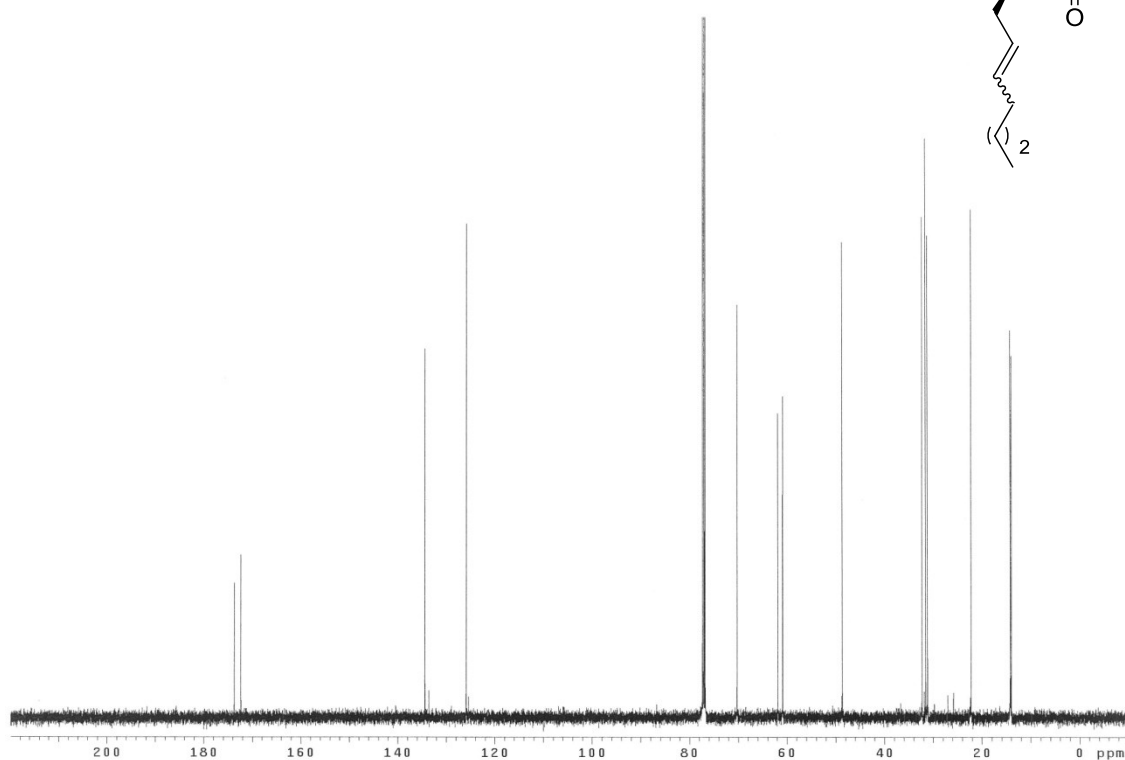
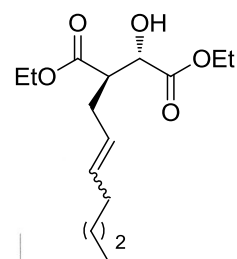
Ethyl 2*S*-hydroxy-3*R*-ethoxycarbonyl-decen-5-enoate (5c)

¹H NMR (500 MHz, CDCl₃)



Ethyl 2*S*-hydroxy-3*R*-ethoxycarbonyl-decen-5-enoate (5c)

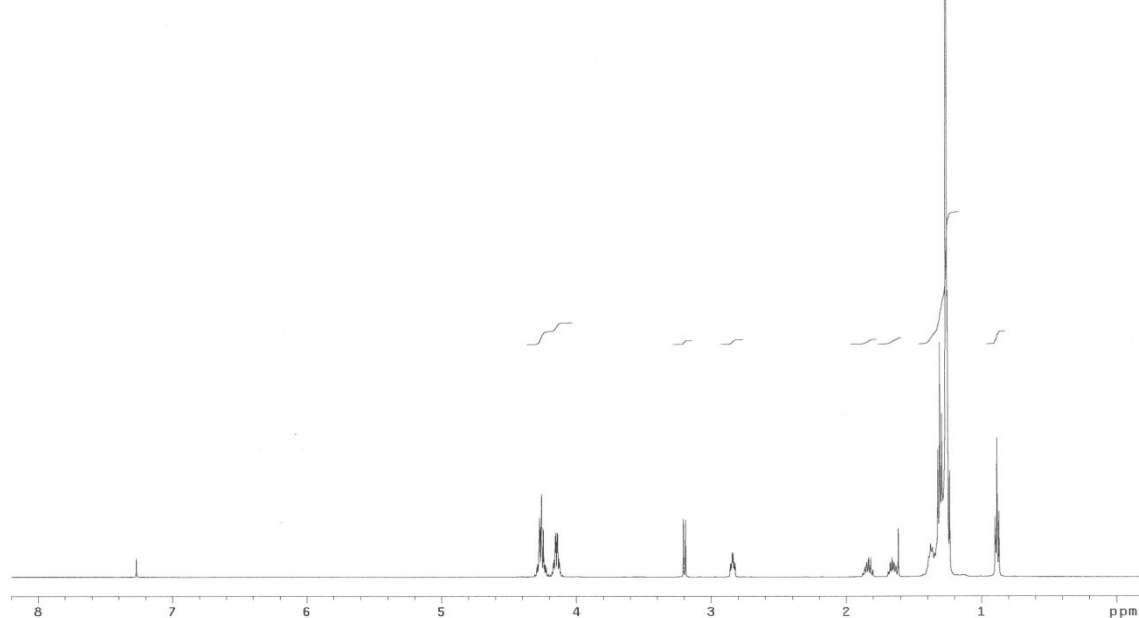
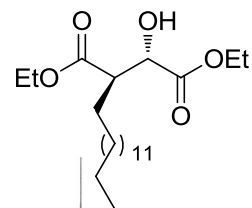
¹³C NMR (125 MHz, CDCl₃)



Ethyl 2*S*-hydroxy-3*R*-ethoxycarbonyl-heptadecanoate (5d)

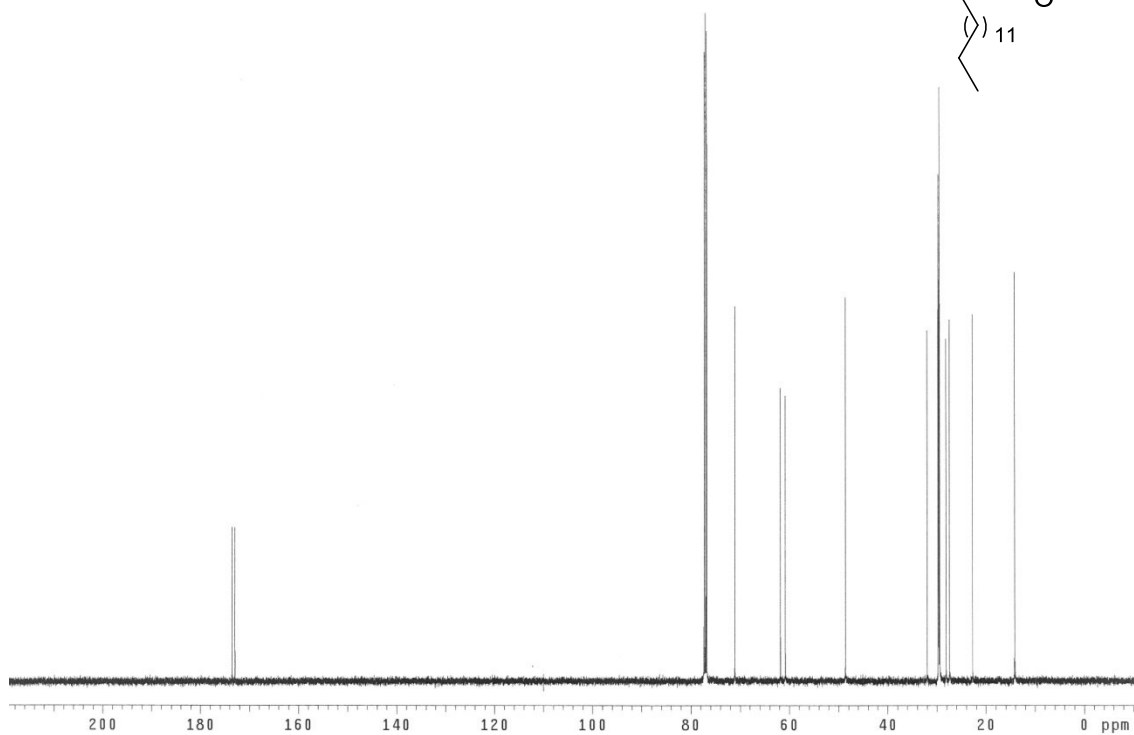
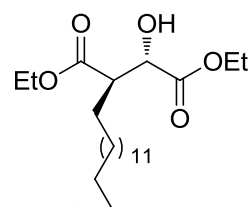
¹H NMR (500 MHz, CDCl₃)

From the reduction of 5e



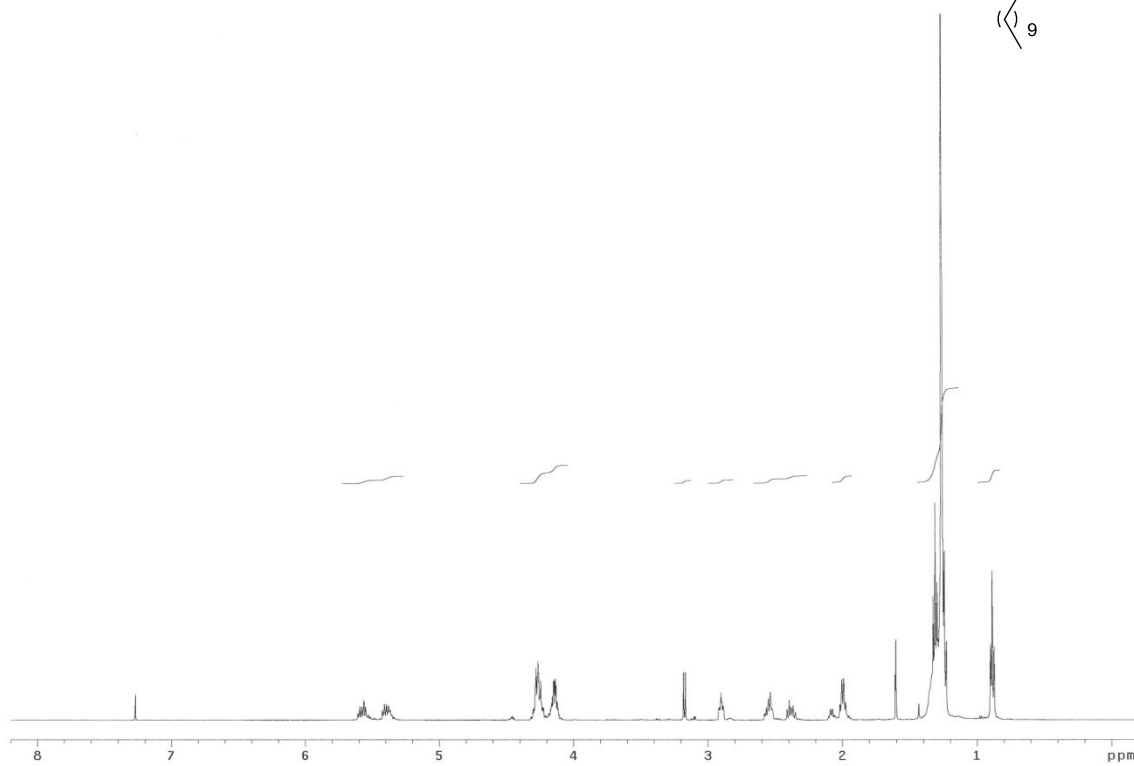
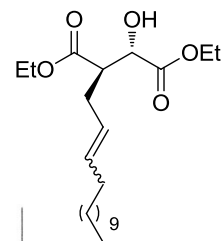
Ethyl 2*S*-hydroxy-3*R*-ethoxycarbonyl-heptadecanoate (5d)

¹³C NMR (125 MHz, CDCl₃)



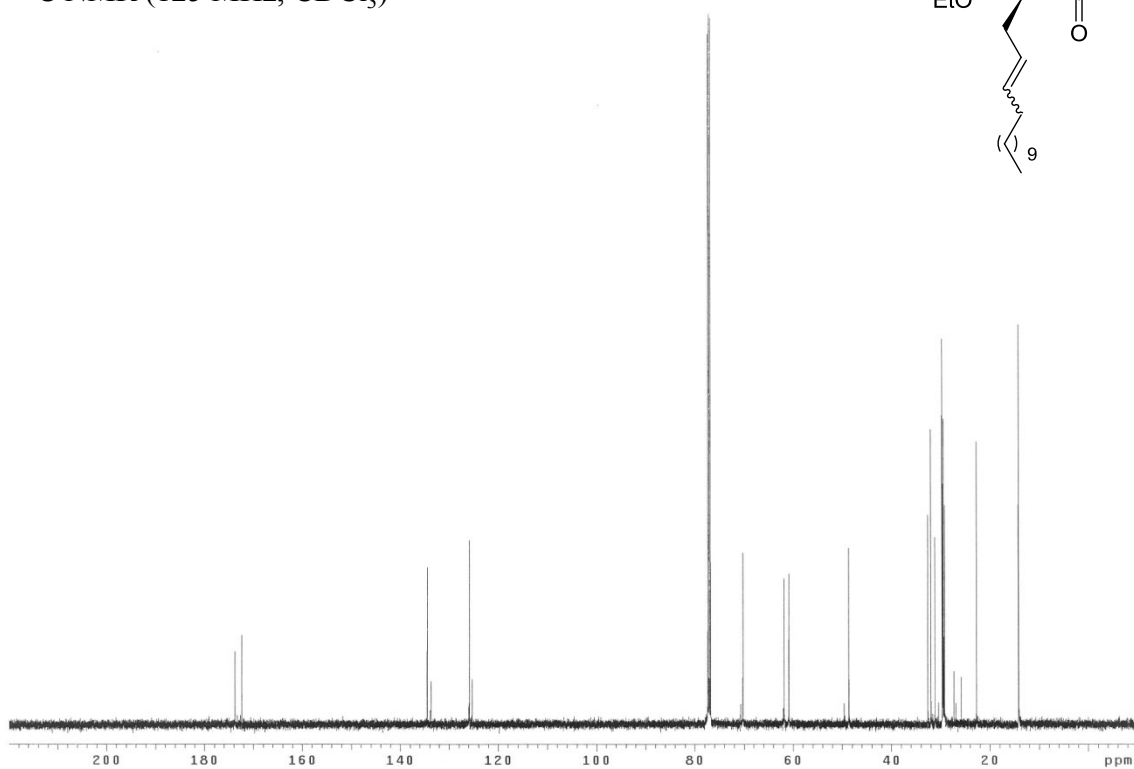
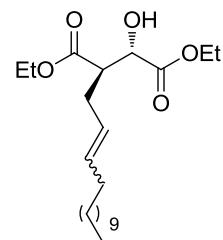
Ethyl 2*S*-hydroxy-3*R*-ethoxycarbonyl-heptadecen-5-enoate (5e)

¹H NMR (500 MHz, CDCl₃)



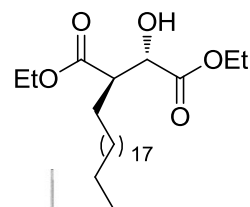
Ethyl 2*S*-hydroxy-3*R*-ethoxycarbonyl-heptadecen-5-enoate (5e)

¹³C NMR (125 MHz, CDCl₃)

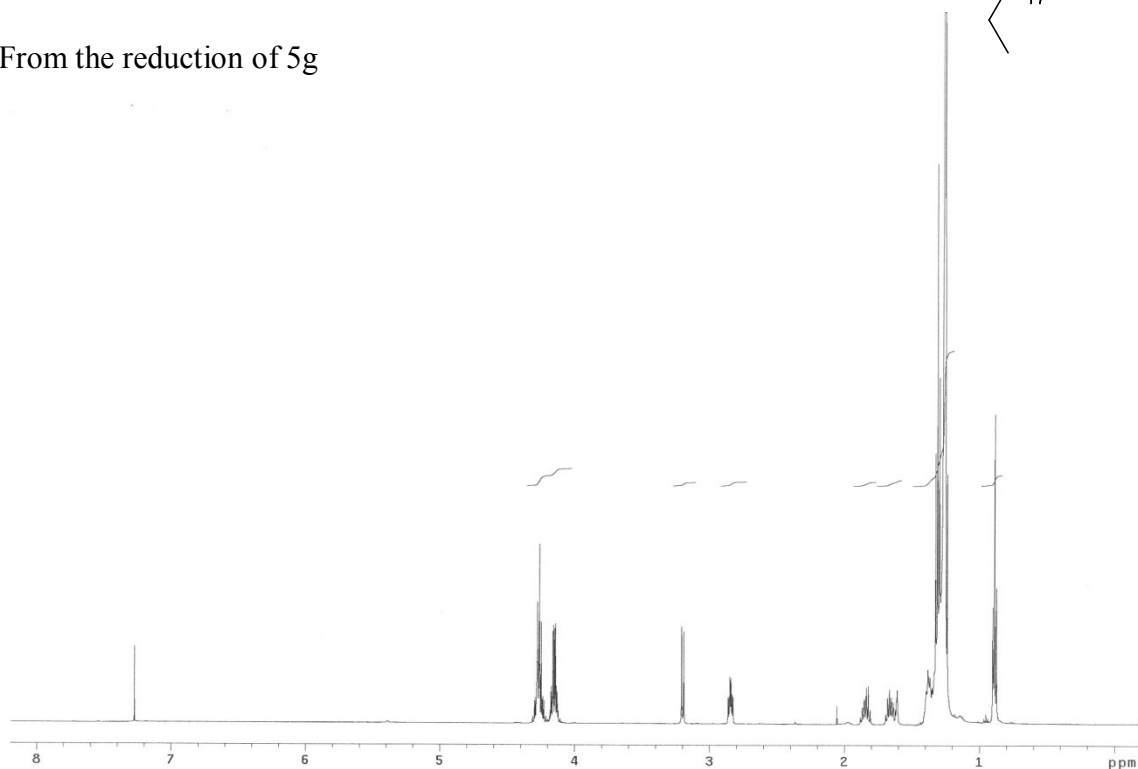


Ethyl 2*S*-hydroxy-3*R*-ethoxycarbonyl-tricosanoate (5f)

¹H NMR (500 MHz, CDCl₃)

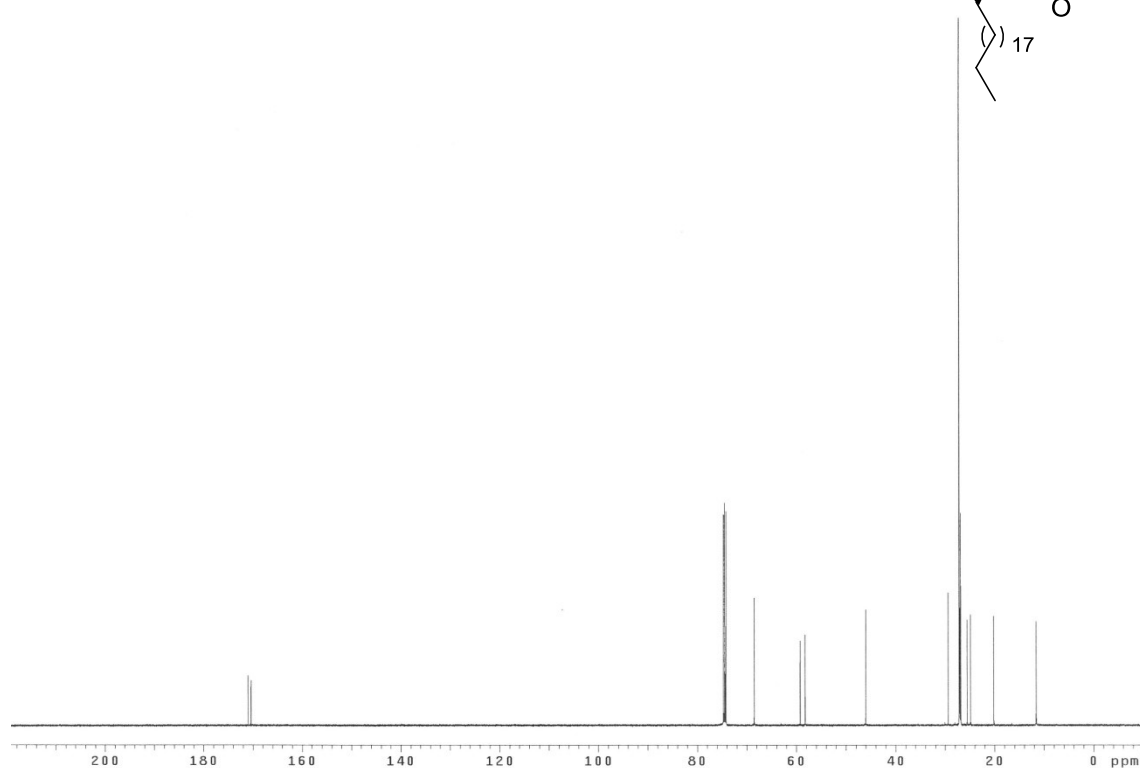
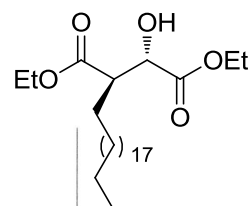


From the reduction of 5g



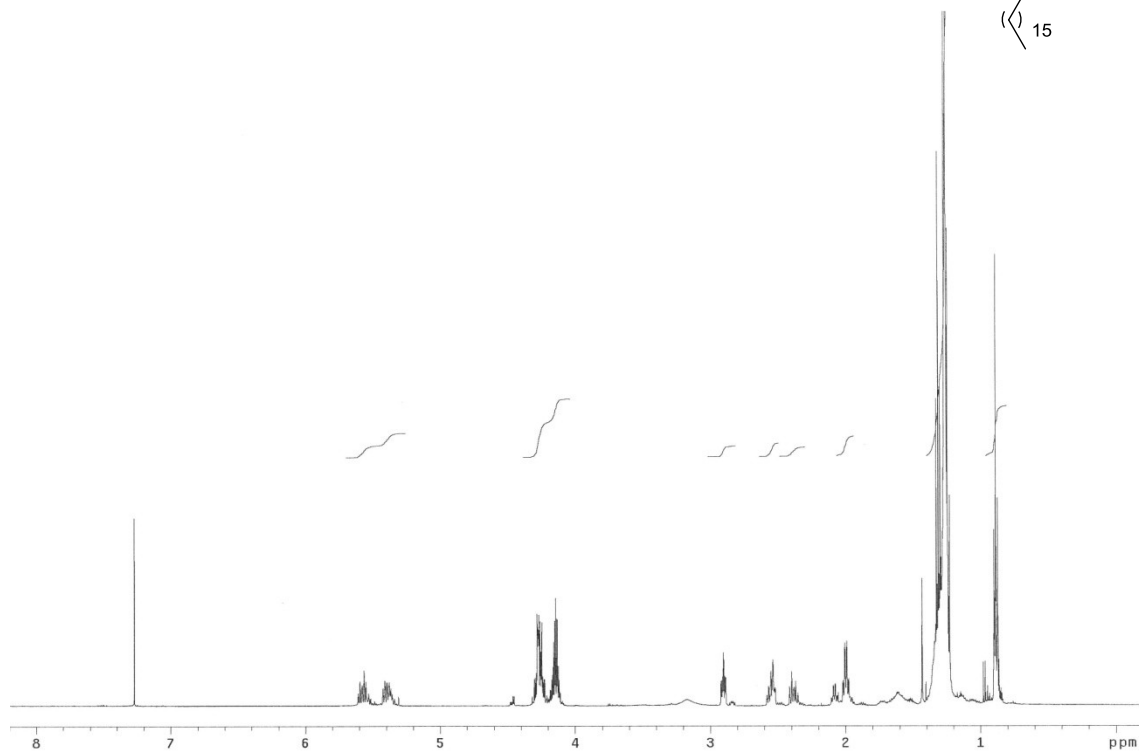
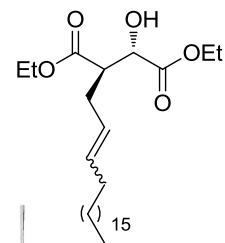
Ethyl 2*S*-hydroxy-3*R*-ethoxycarbonyl-tricosanoate (5f)

¹³C NMR (125 MHz, CDCl₃)



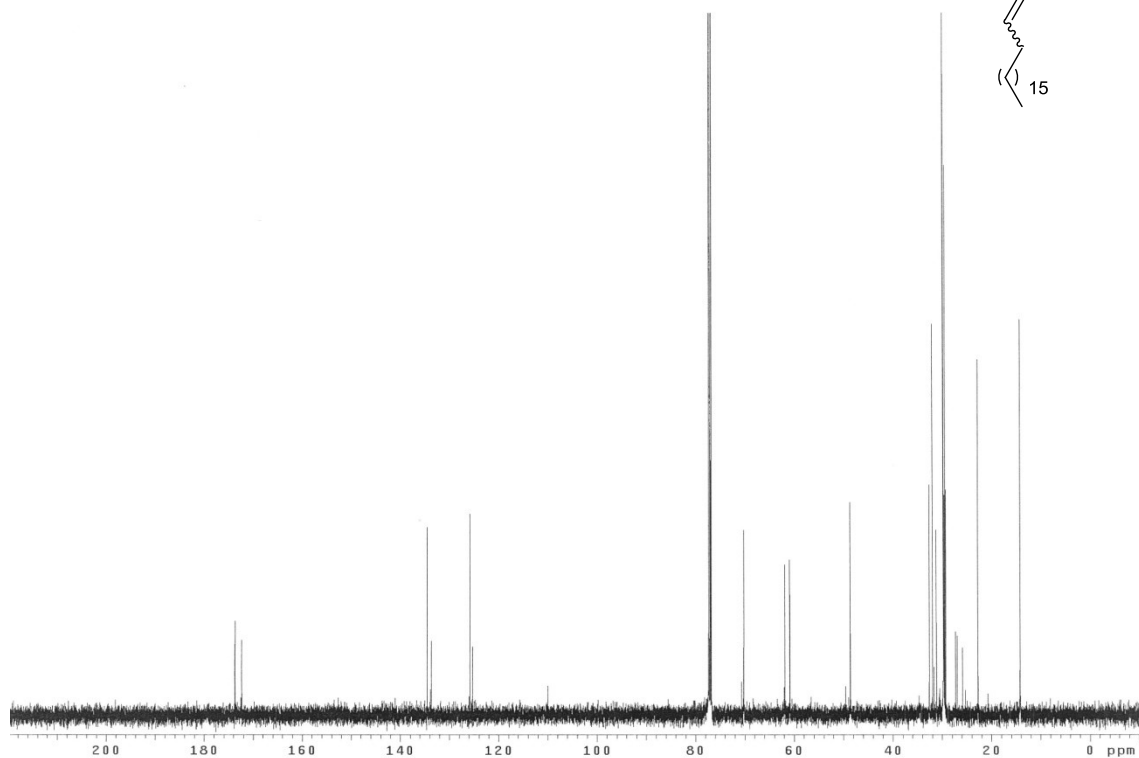
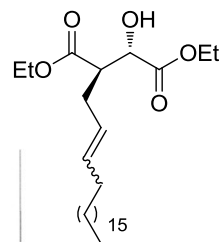
Ethyl 2*S*-hydroxy-3*R*-ethoxycarbonyl-tricos-5-enoate (5g)

¹H NMR (500 MHz, CDCl₃)



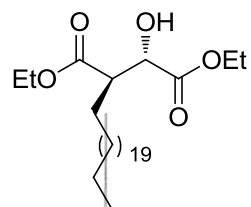
Ethyl 2*S*-hydroxy-3*R*-ethoxycarbonyl-tricos-5-enoate (5g)

¹³C NMR (125 MHz, CDCl₃)

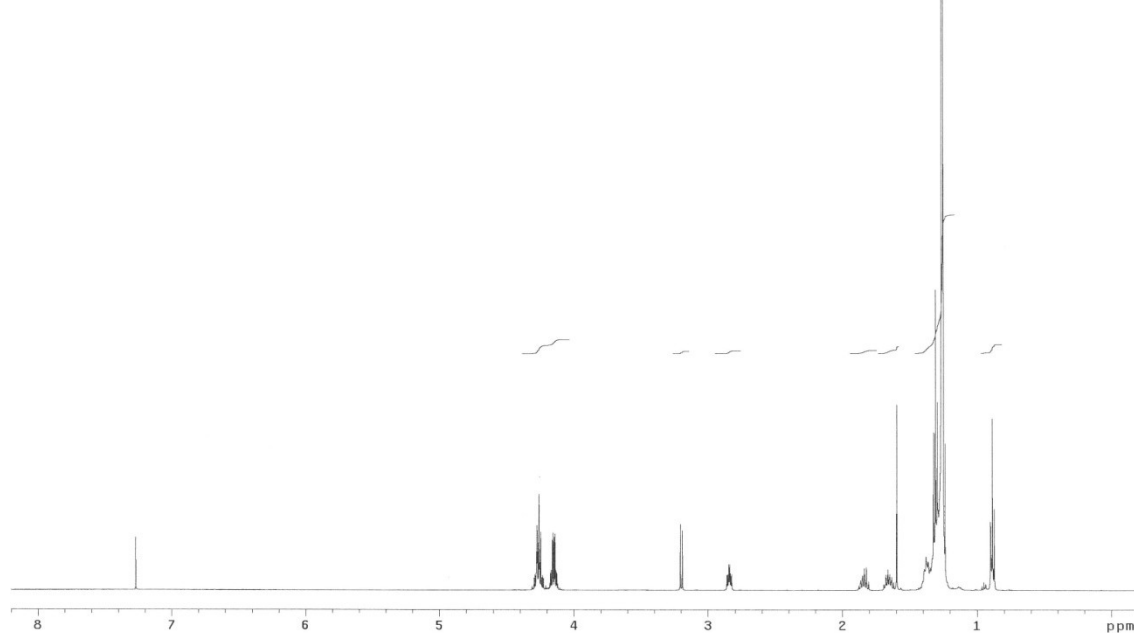


Ethyl 2*S*-hydroxy-3*R*-ethoxycarbonyl-pentacosanoate (5h)

¹H NMR (500 MHz, CDCl₃)

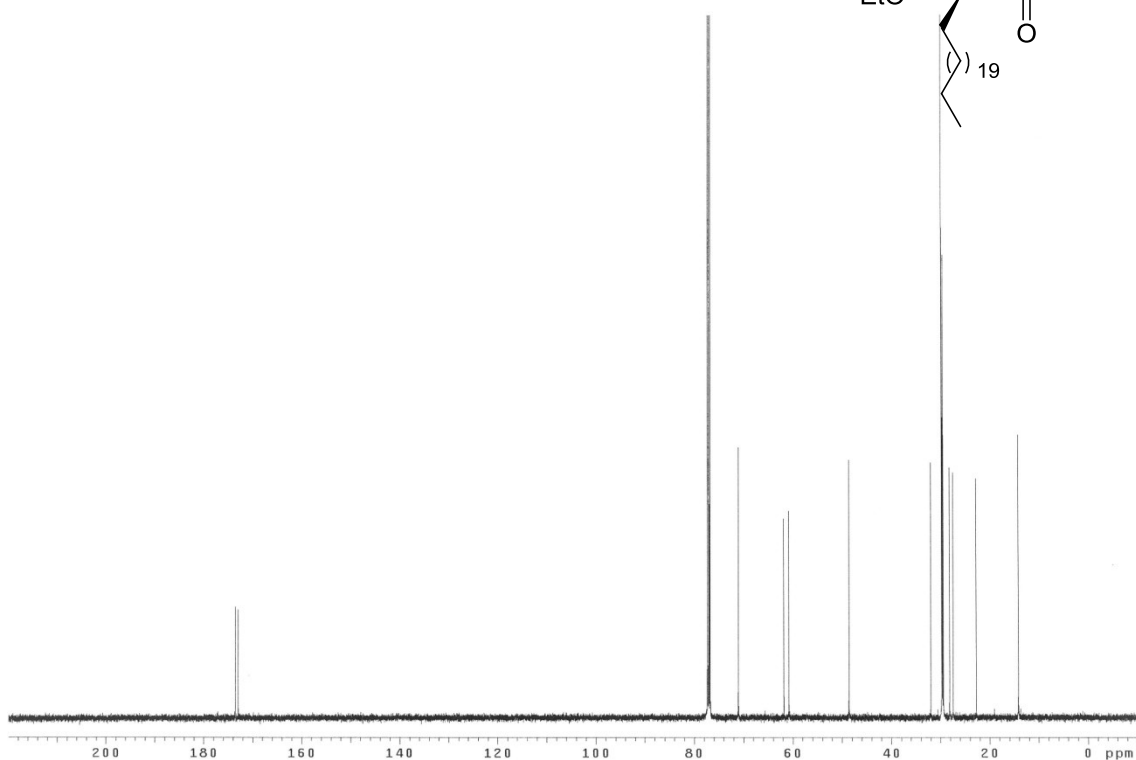
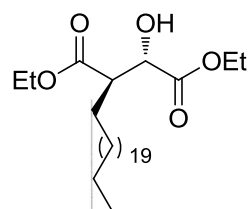


From the reduction of 5i



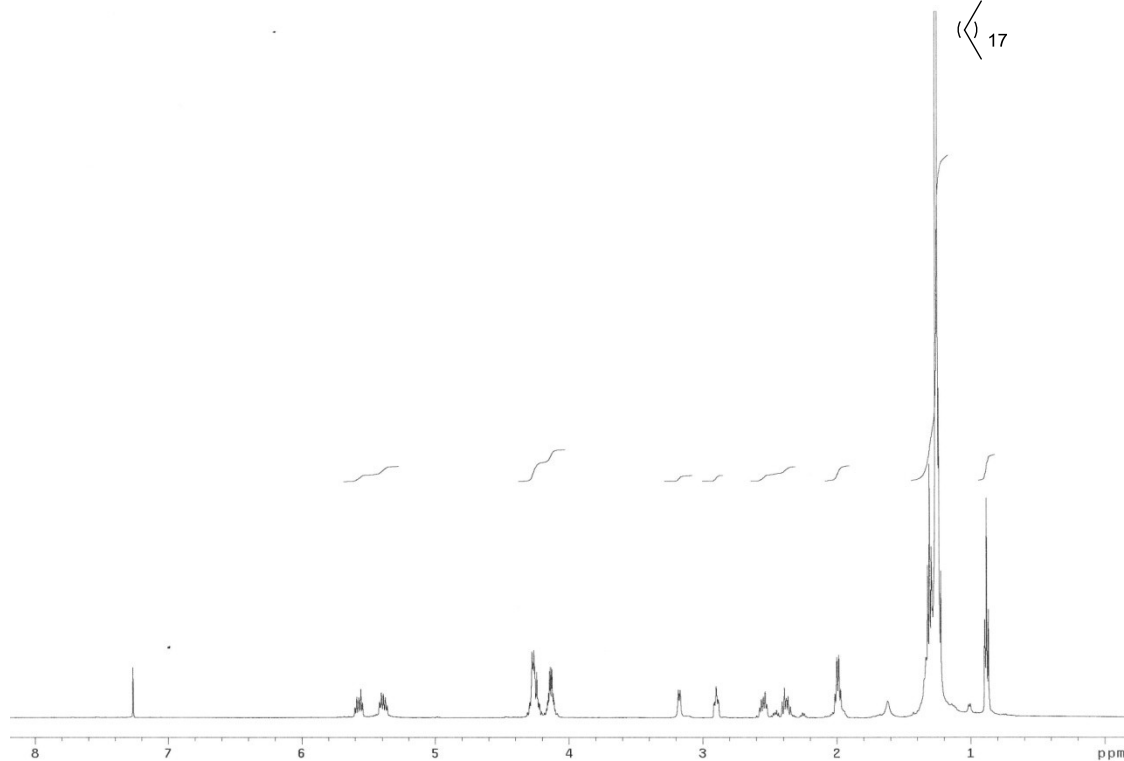
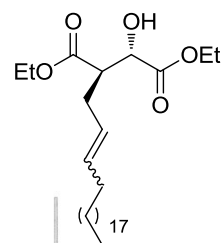
Ethyl 2*S*-hydroxy-3*R*-ethoxycarbonyl-pentacosanoate (5h)

¹³C NMR (125 MHz, CDCl₃)



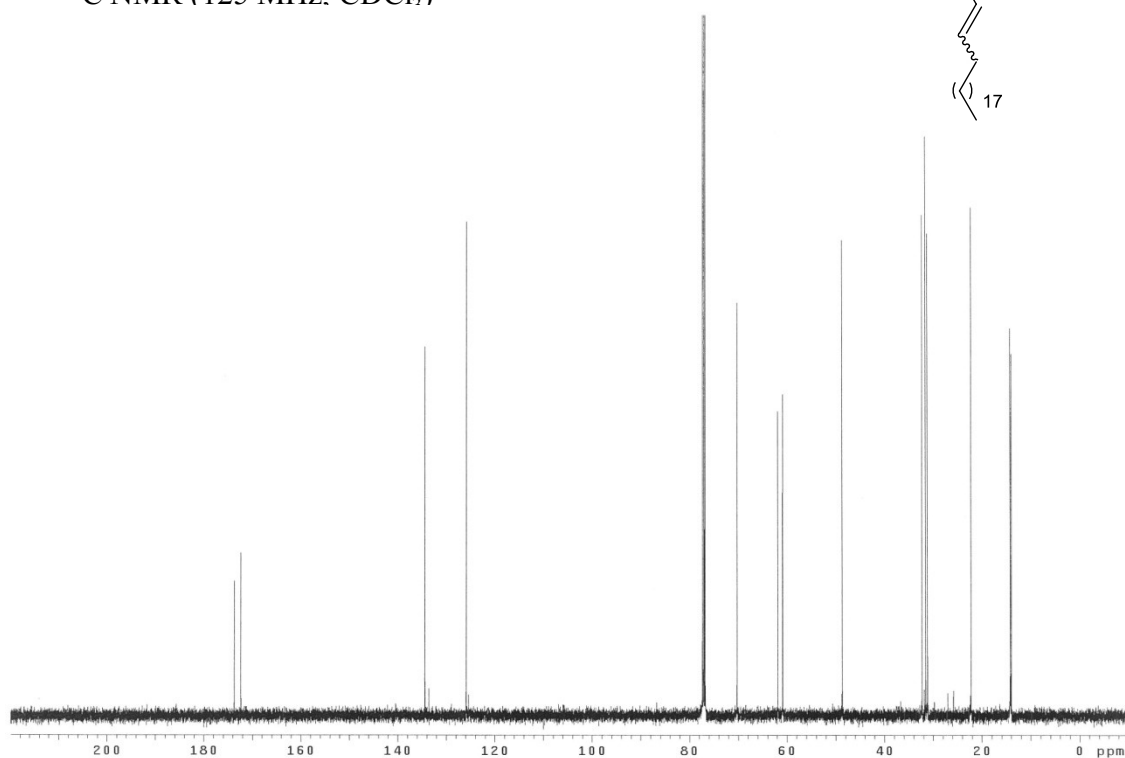
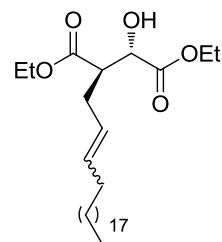
**Ethyl 2*S*-hydroxy-3*R*-ethoxycarbonyl-pentacos-5-enoate
(5i)**

^1H NMR (500 MHz, CDCl_3)



**Ethyl 2*S*-hydroxy-3*R*-ethoxycarbonyl-pentacos-5-enoate
(5i)**

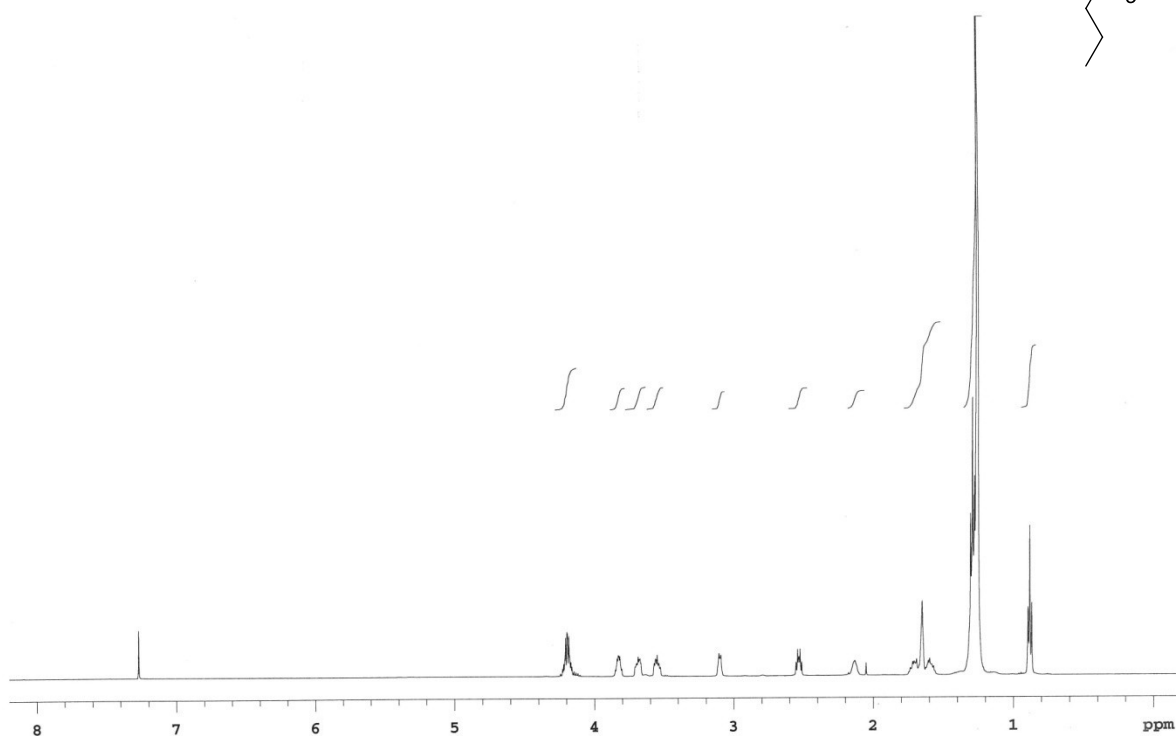
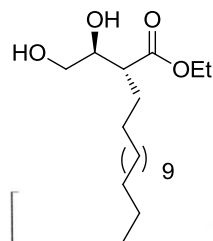
^{13}C NMR (125 MHz, CDCl_3)



Chapter 5

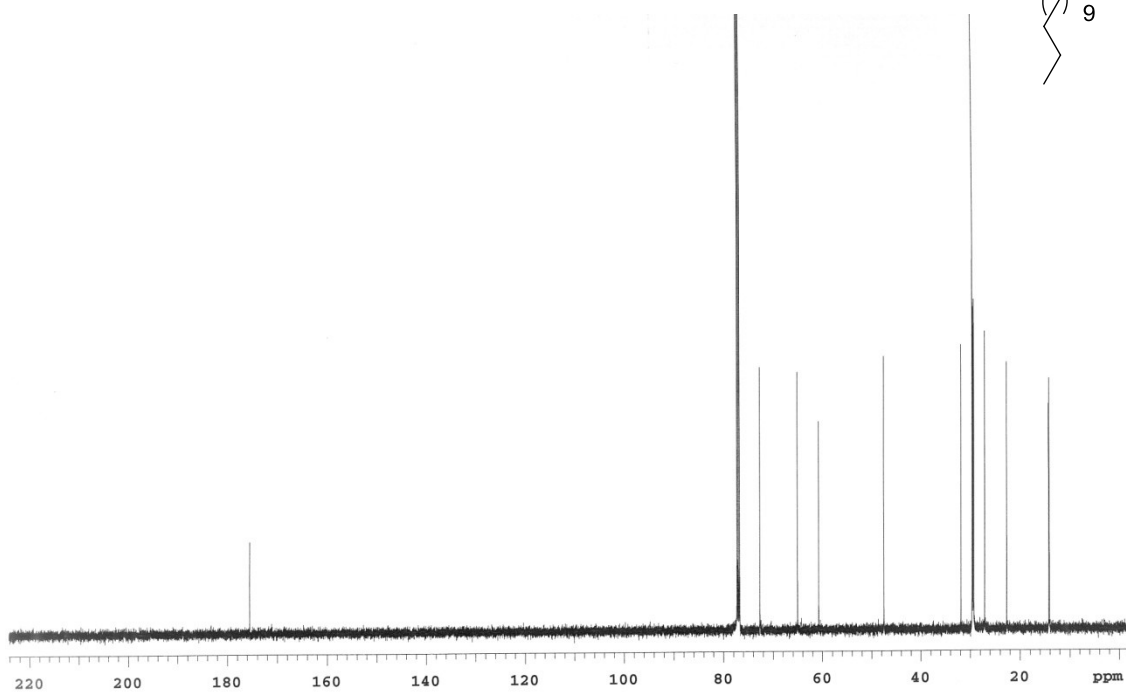
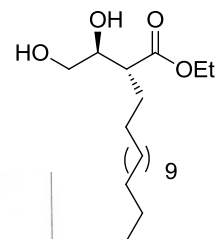
3*R*-Ethoxycarbonyl-2*S*-hydroxy-heptadecanol (10)

¹H NMR (500 MHz, CDCl₃)



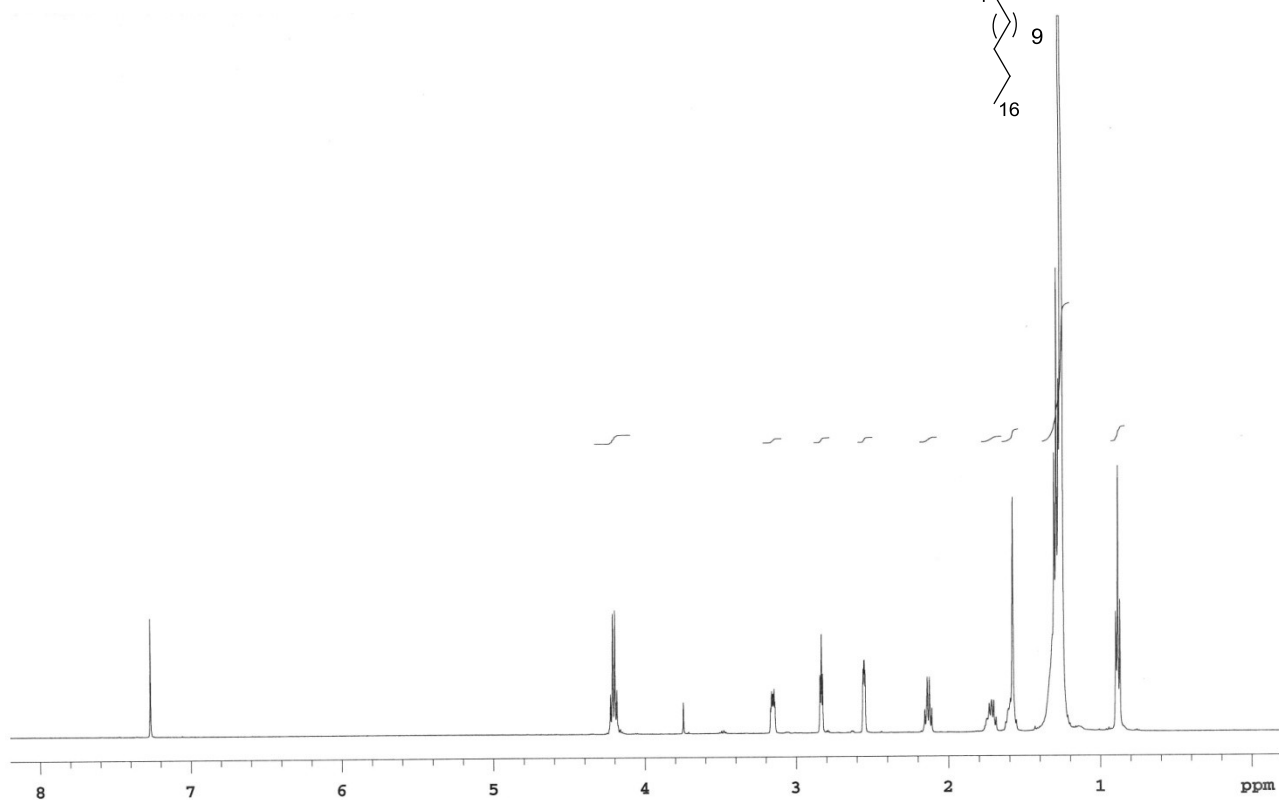
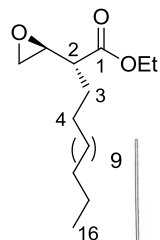
3*R*-Ethoxycarbonyl-2*S*-hydroxy-heptadecanol (10)

¹³C NMR (125 MHz, CDCl₃)



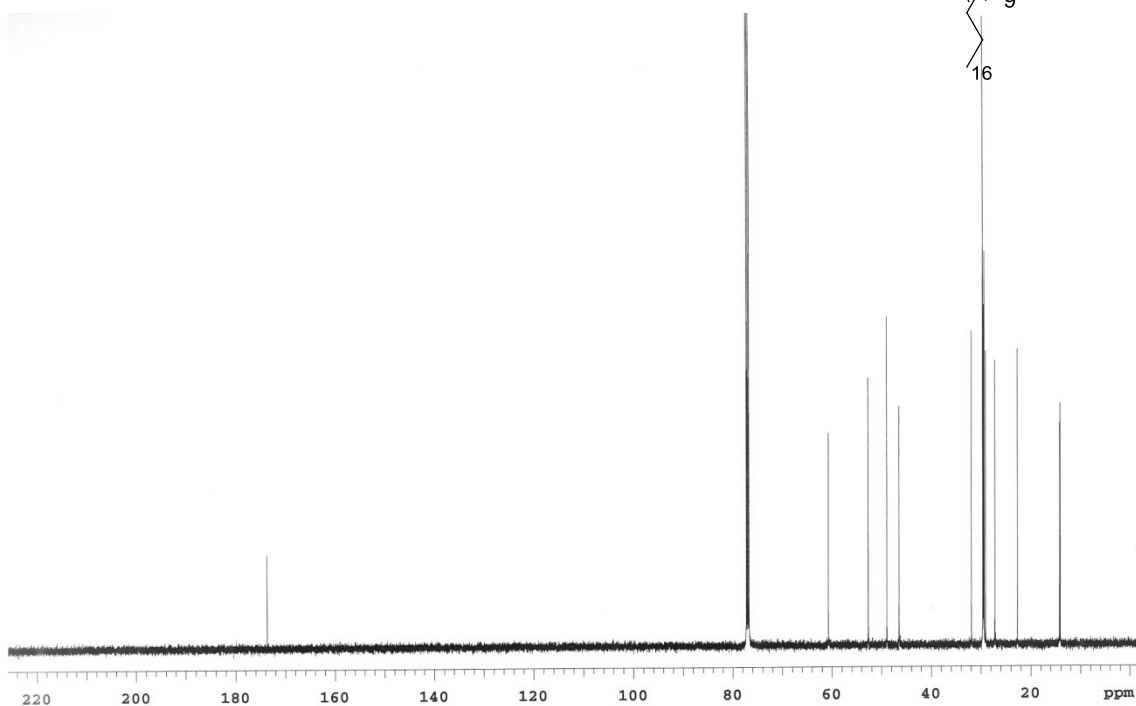
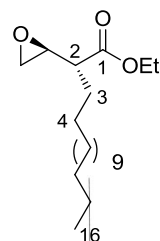
Ethyl (2*R*)-2-[(*R*)-oxiranyl]-hexadecanoate (7)

¹H NMR (500 MHz, CDCl₃)



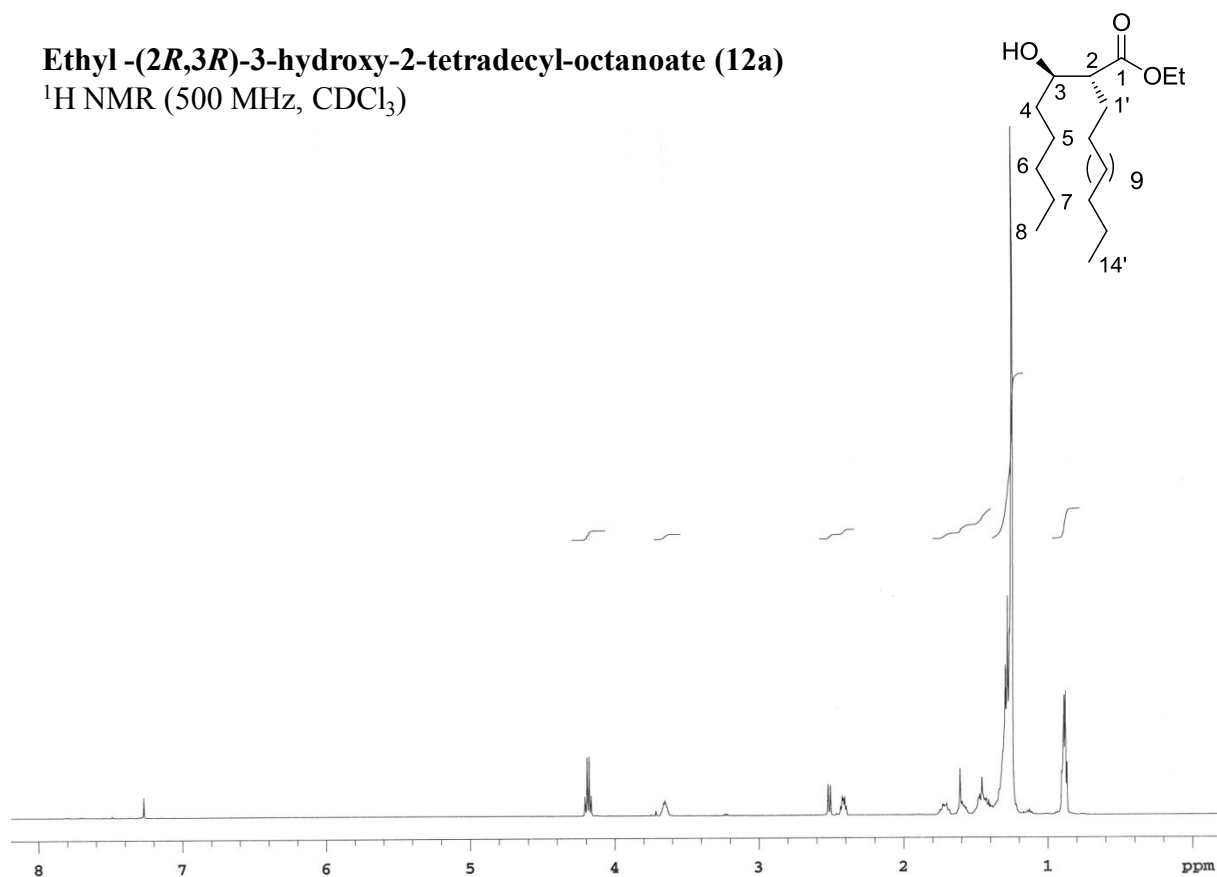
Ethyl (2*R*)-2-[(*R*)-oxiranyl]-hexadecanoate (7)

¹³C NMR (125 MHz, CDCl₃)



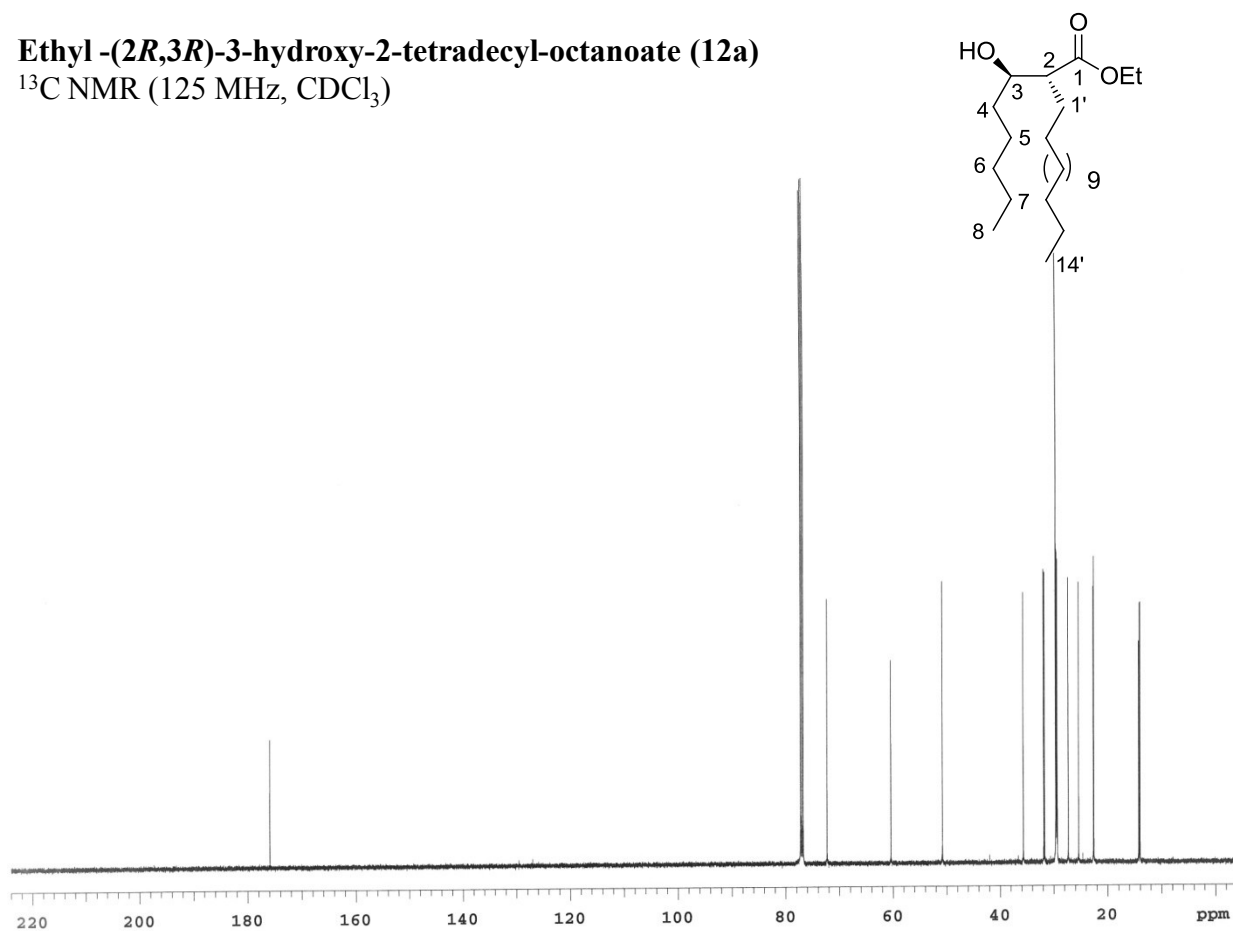
Ethyl -(2*R*,3*R*)-3-hydroxy-2-tetradecyl-octanoate (12a)

¹H NMR (500 MHz, CDCl₃)



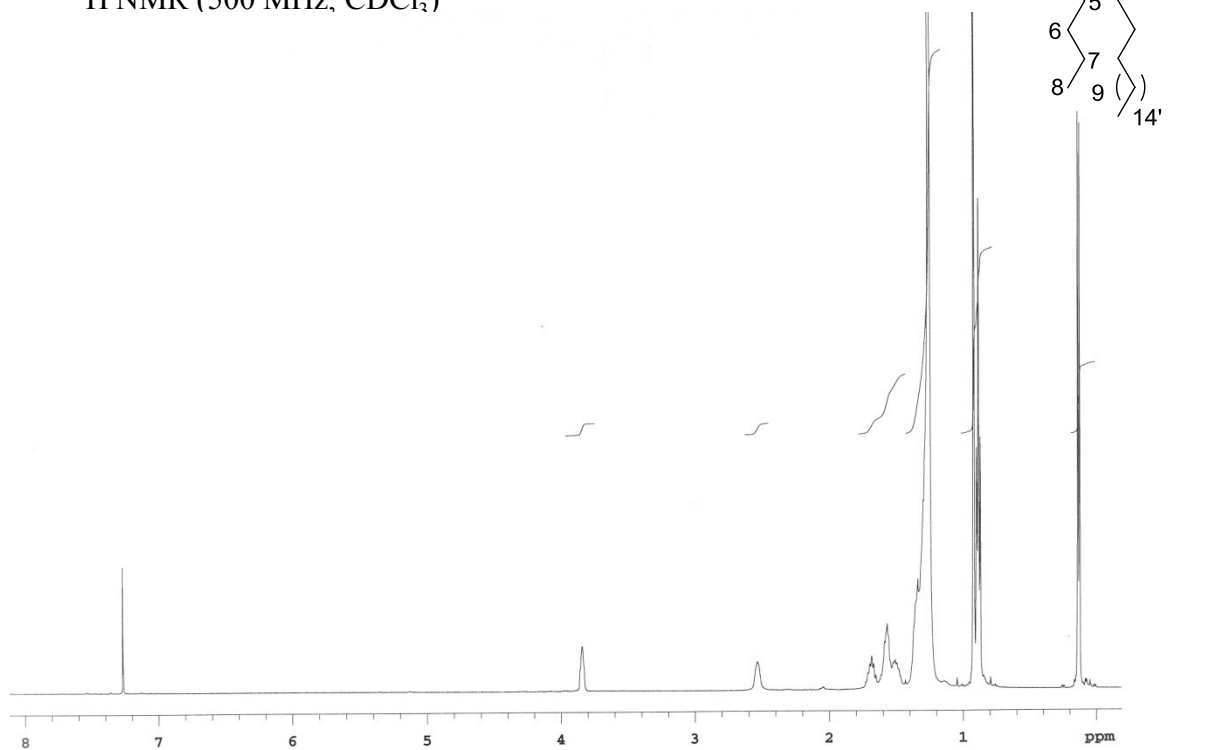
Ethyl -(2*R*,3*R*)-3-hydroxy-2-tetradecyl-octanoate (12a)

¹³C NMR (125 MHz, CDCl₃)



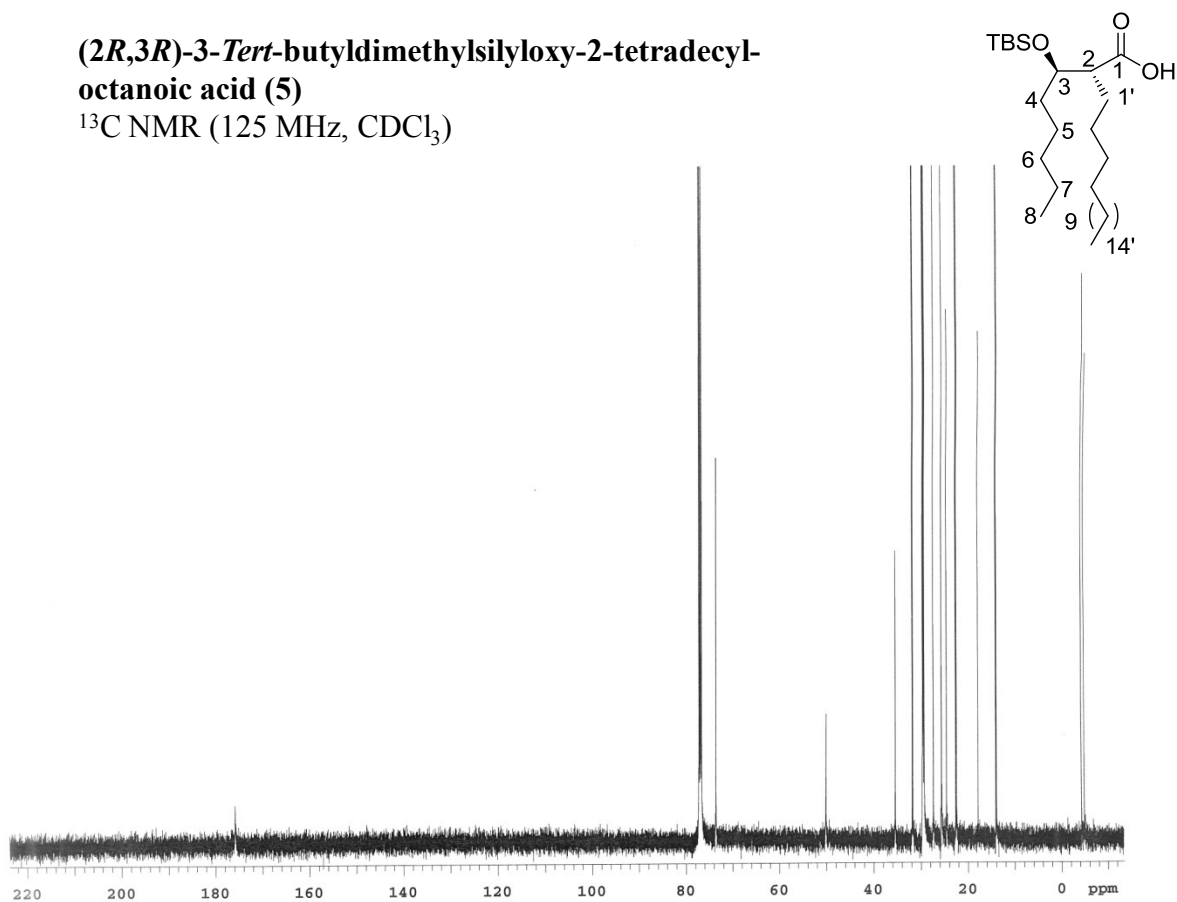
(2*R*,3*R*)-3-*Tert*-butyldimethylsilyloxy-2-tetradecyloctanoic acid (5)

¹H NMR (500 MHz, CDCl₃)

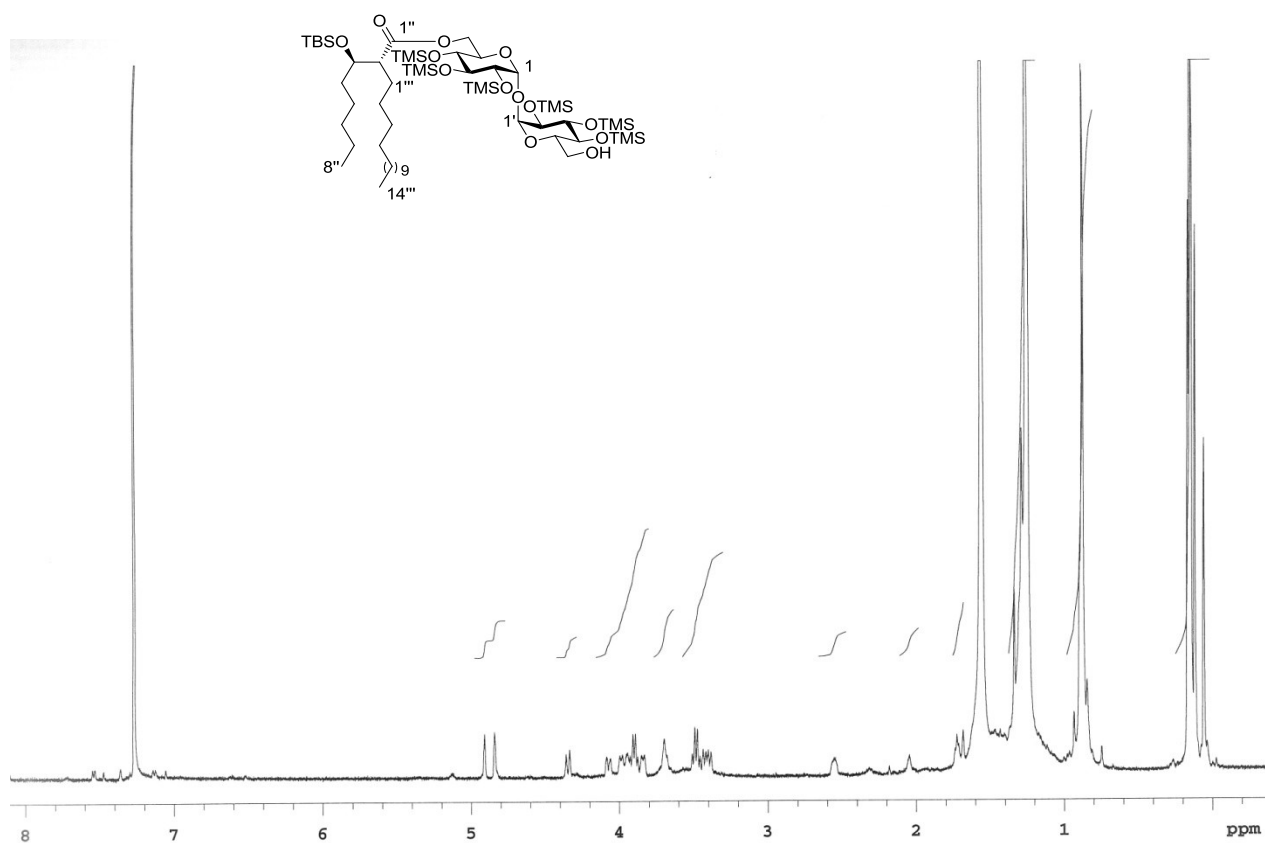


(2*R*,3*R*)-3-*Tert*-butyldimethylsilyloxy-2-tetradecyloctanoic acid (5)

¹³C NMR (125 MHz, CDCl₃)



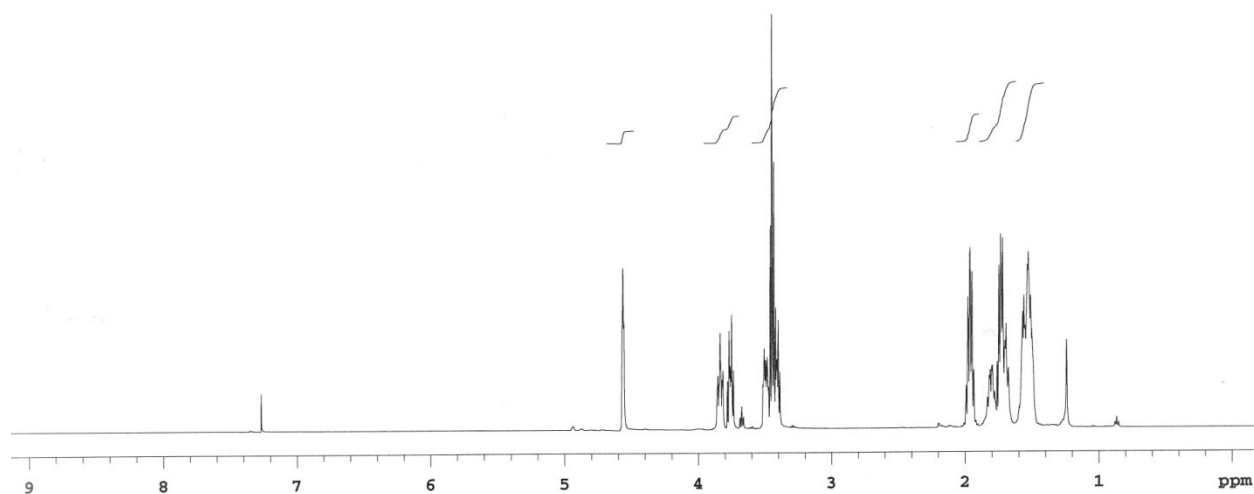
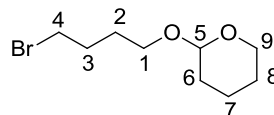
2,2',3,3',4,4'-Hexa-*O*-trimethylsilyl-6-*O*-[(2*R*,3*R*)-3-*tert*-butyldimethylsilyloxy-2-tetradecyl-octanoyl]- α,α' -D-trehalose (15)
 ^1H NMR (500 MHz, CDCl_3)



Chapter 6

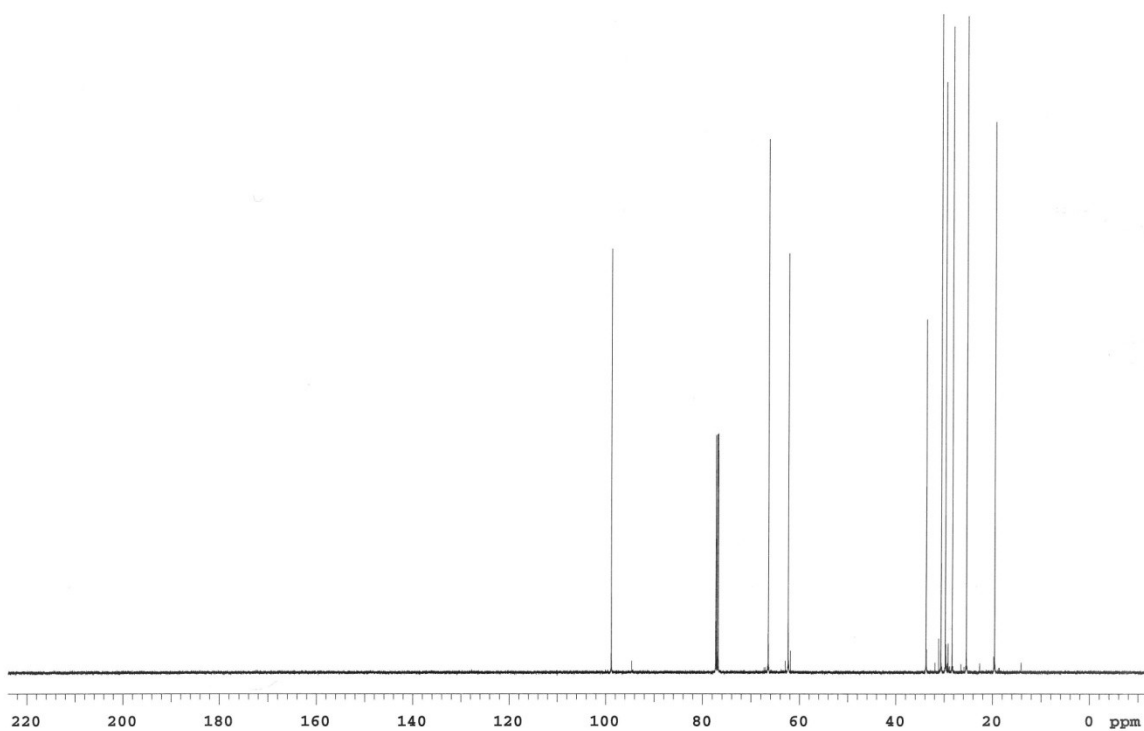
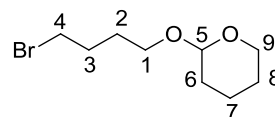
2-(4-Bromobutyloxy)-tetrahydropyran (16)

^1H NMR (500 MHz, CDCl_3)

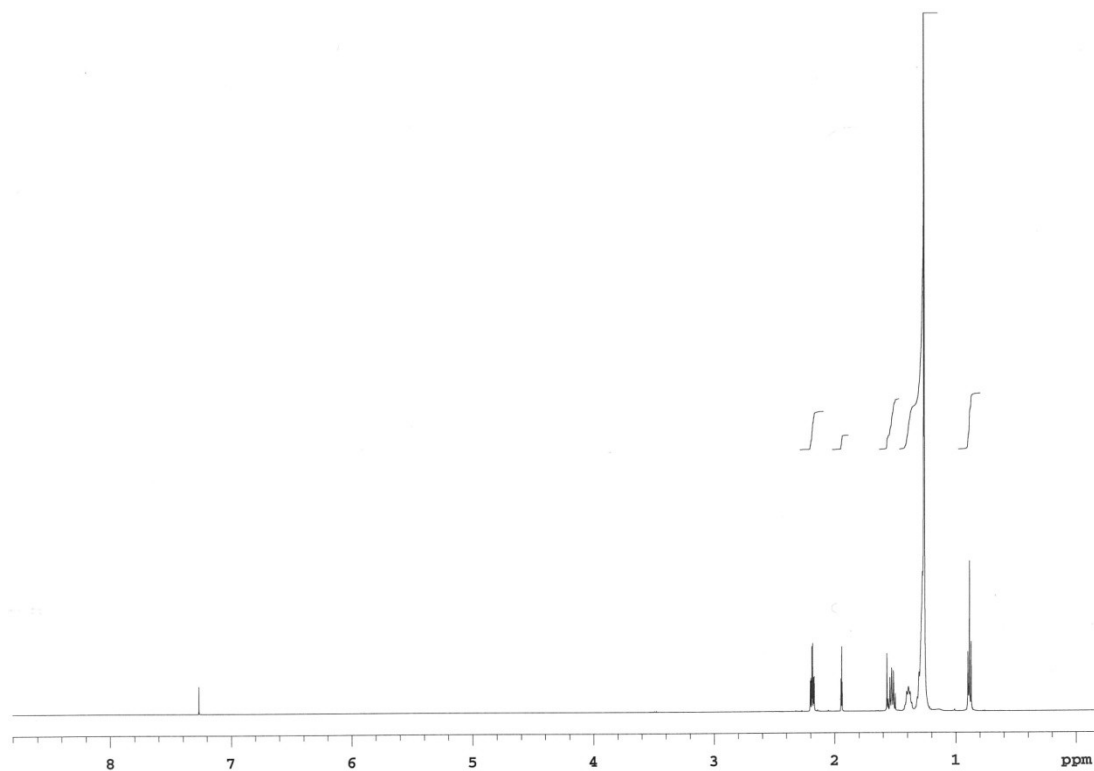
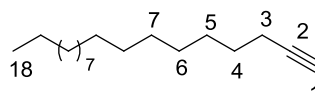


2-(4-Bromobutyloxy)-tetrahydropyran (16)

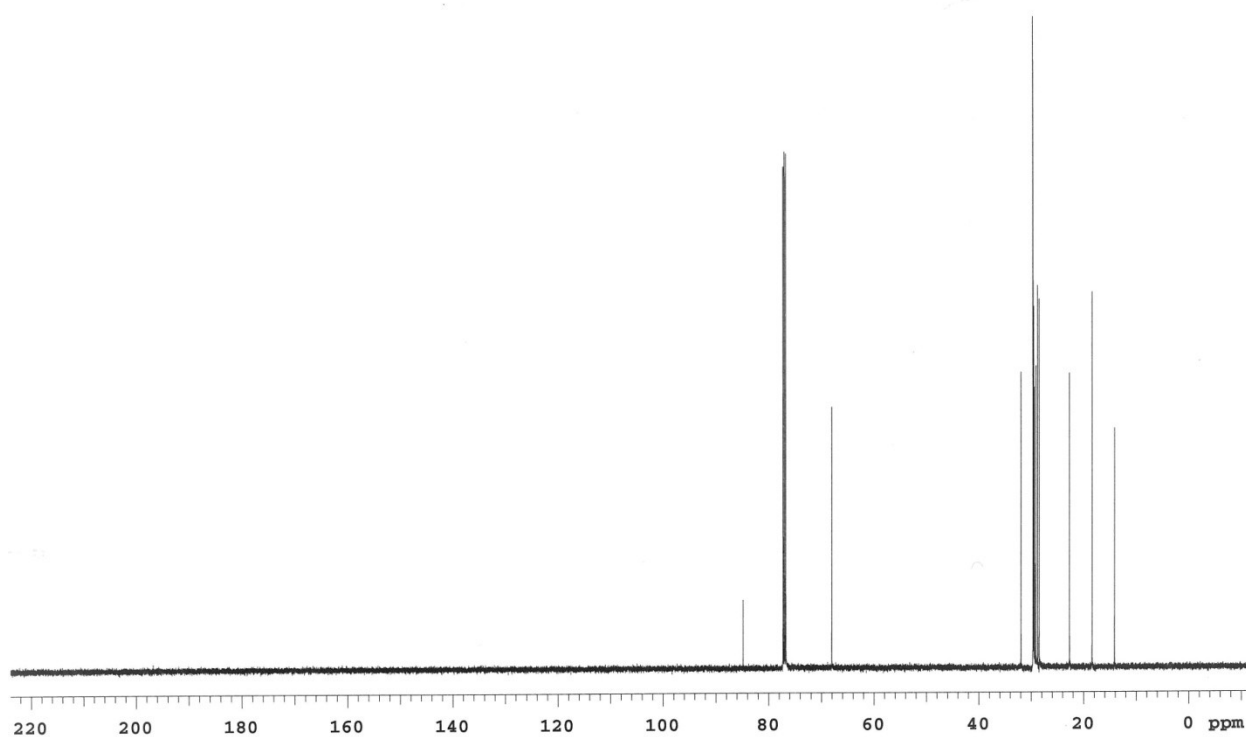
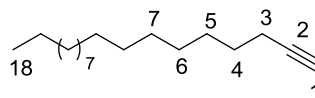
^{13}C NMR (125 MHz, CDCl_3)



Octadecyne (15)
 ^1H NMR (500 MHz, CDCl_3)

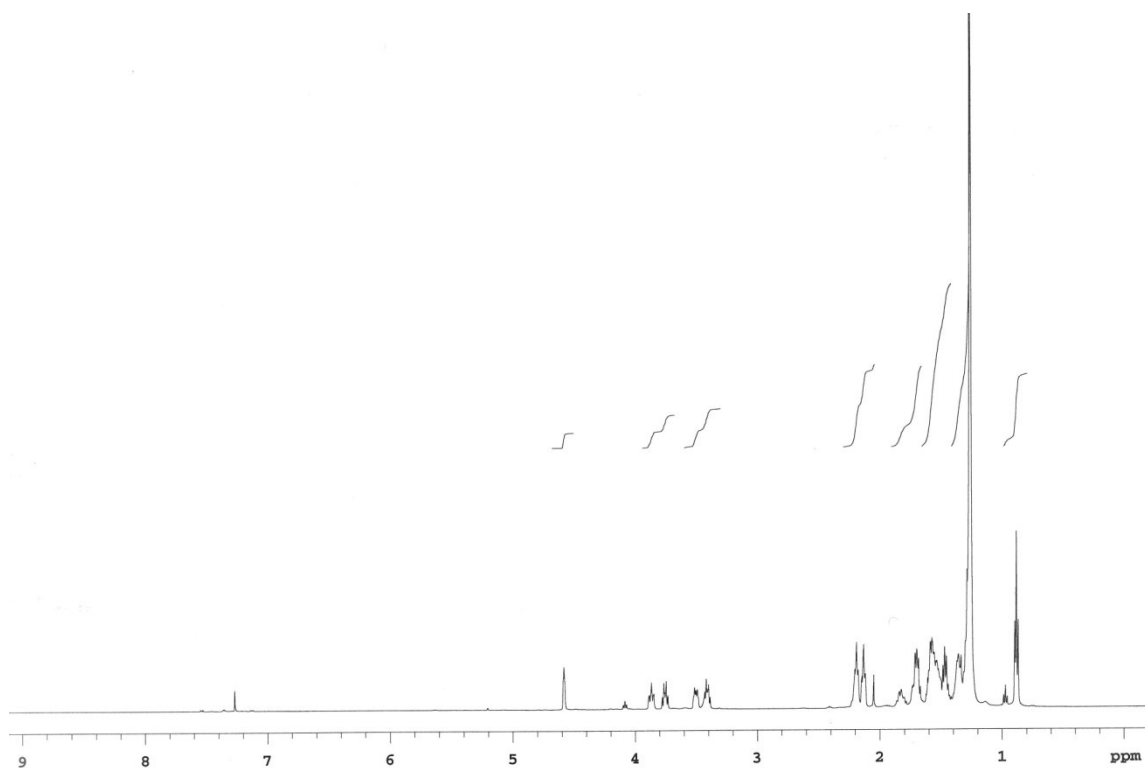
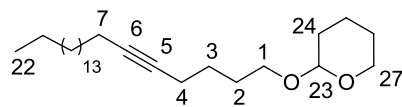


Octadecyne (15)
 ^{13}C NMR (125 MHz, CDCl_3)



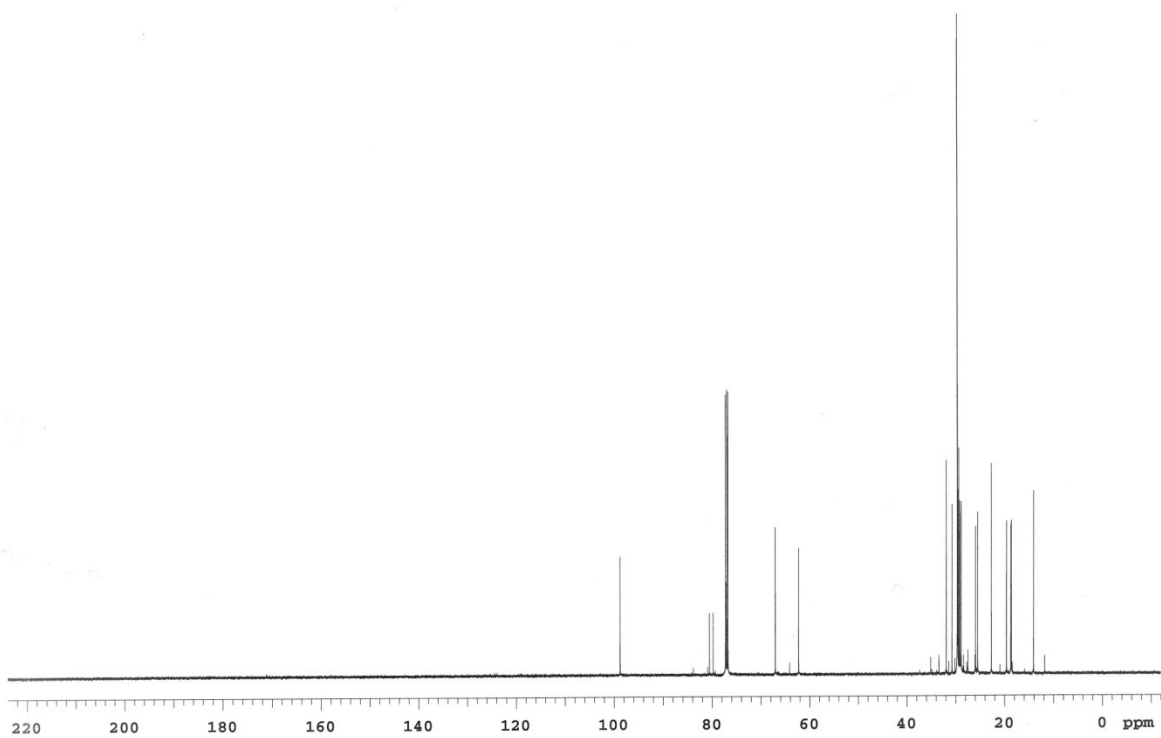
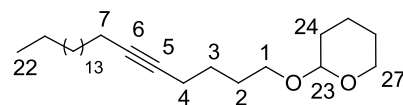
2-(5-Docosynyloxy)-tetrahydropyran (17)

^1H NMR (500 MHz, CDCl_3)



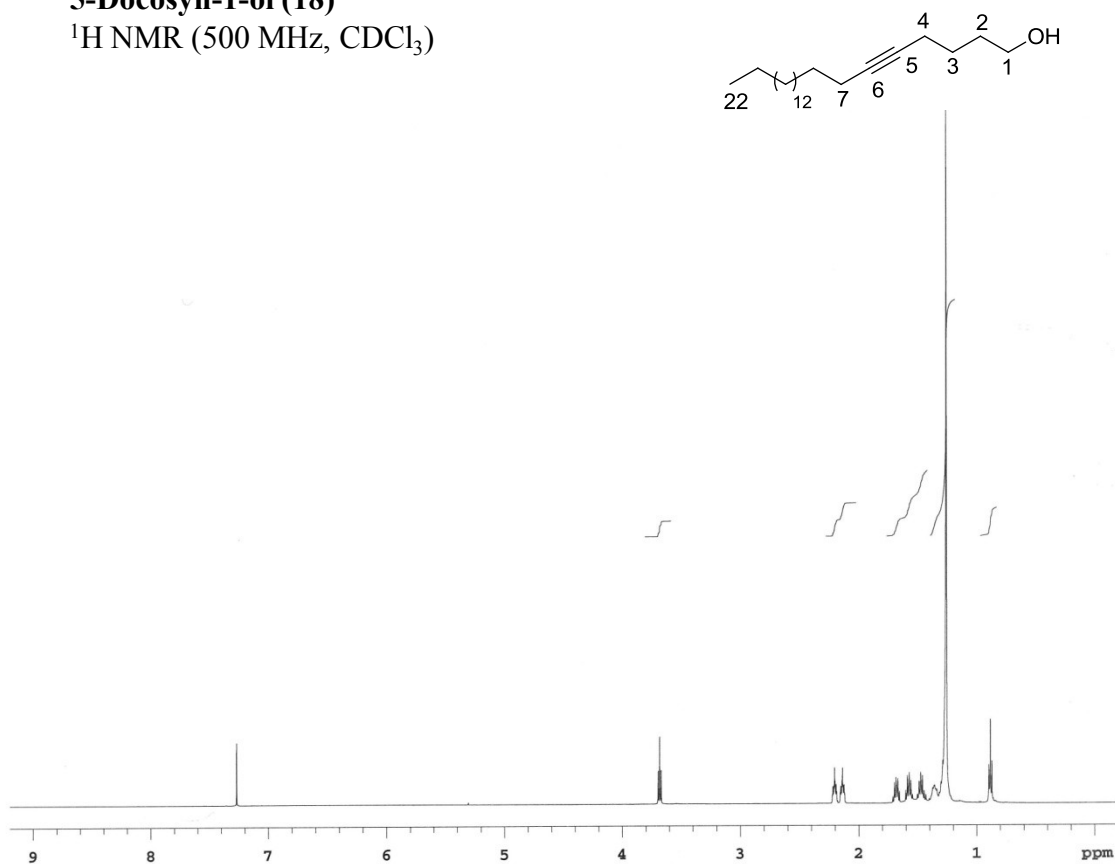
2-(5-Docosynyloxy)-tetrahydropyran (17)

^{13}C NMR (125 MHz, CDCl_3)



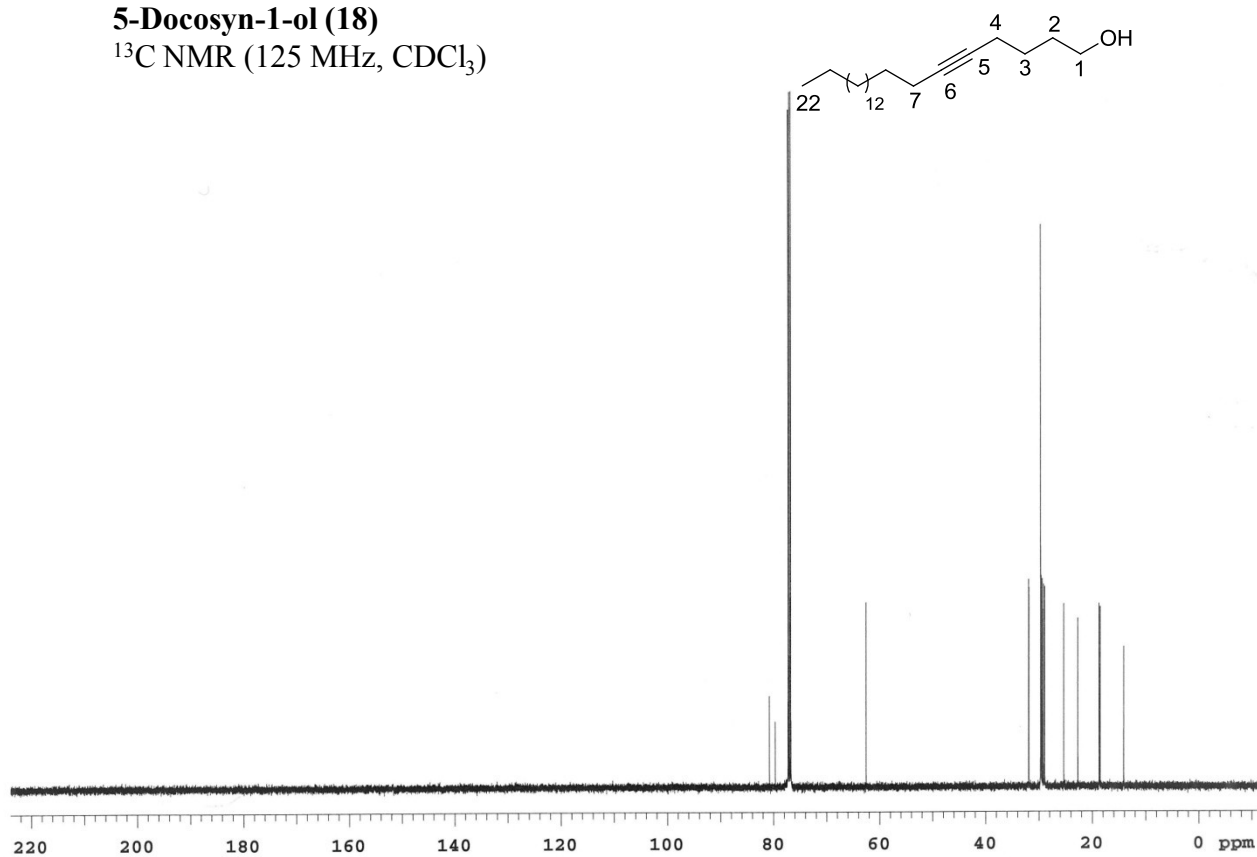
5-Docosyn-1-ol (18)

^1H NMR (500 MHz, CDCl_3)



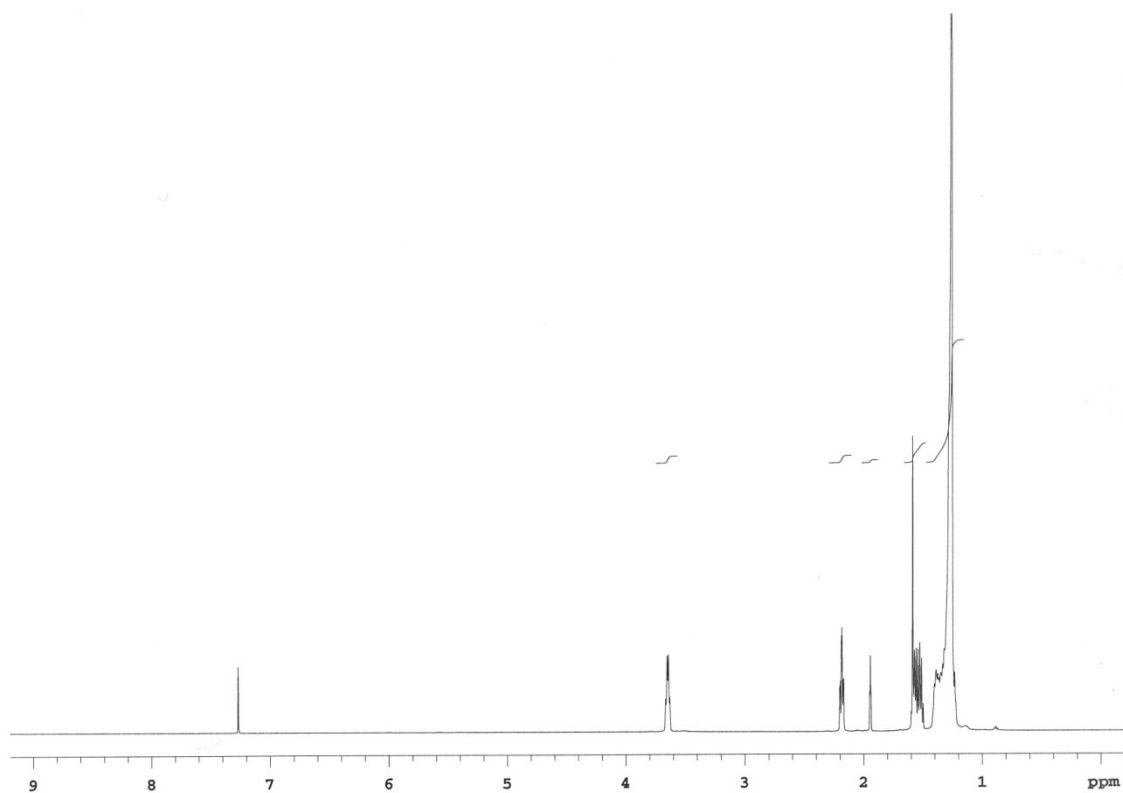
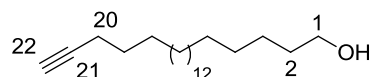
5-Docosyn-1-ol (18)

^{13}C NMR (125 MHz, CDCl_3)



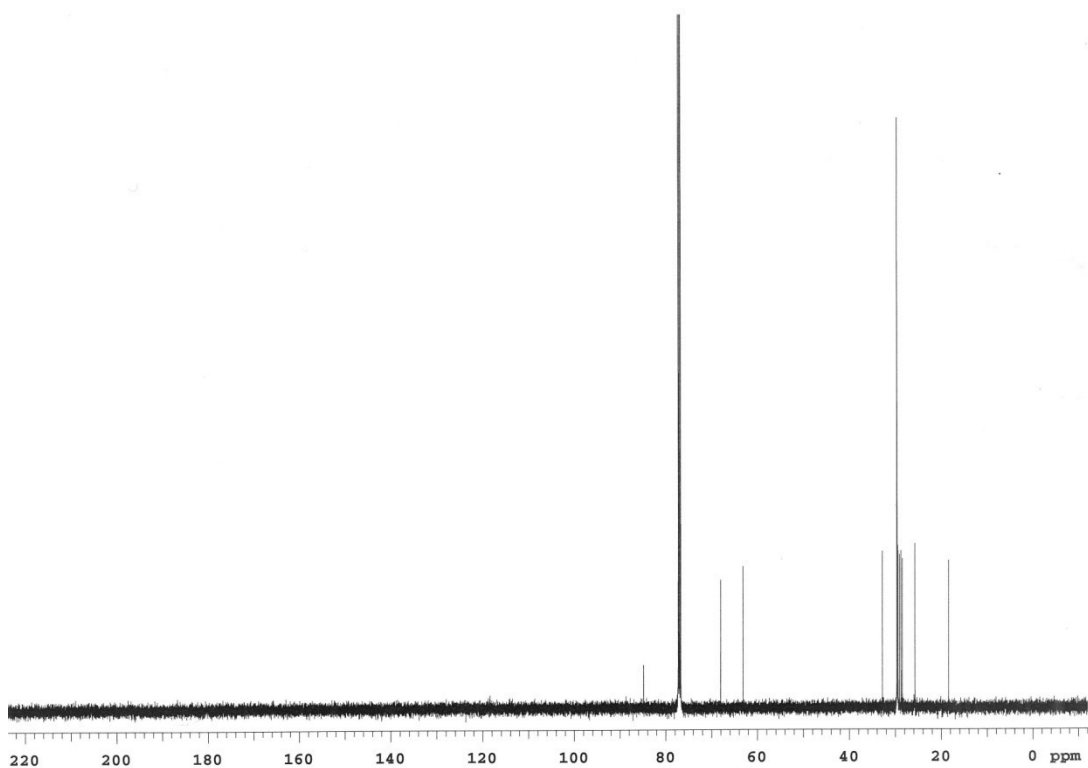
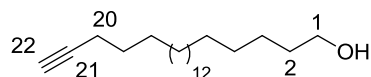
21-Docosyn-1-ol (19)

^1H NMR (500 MHz, CDCl_3)



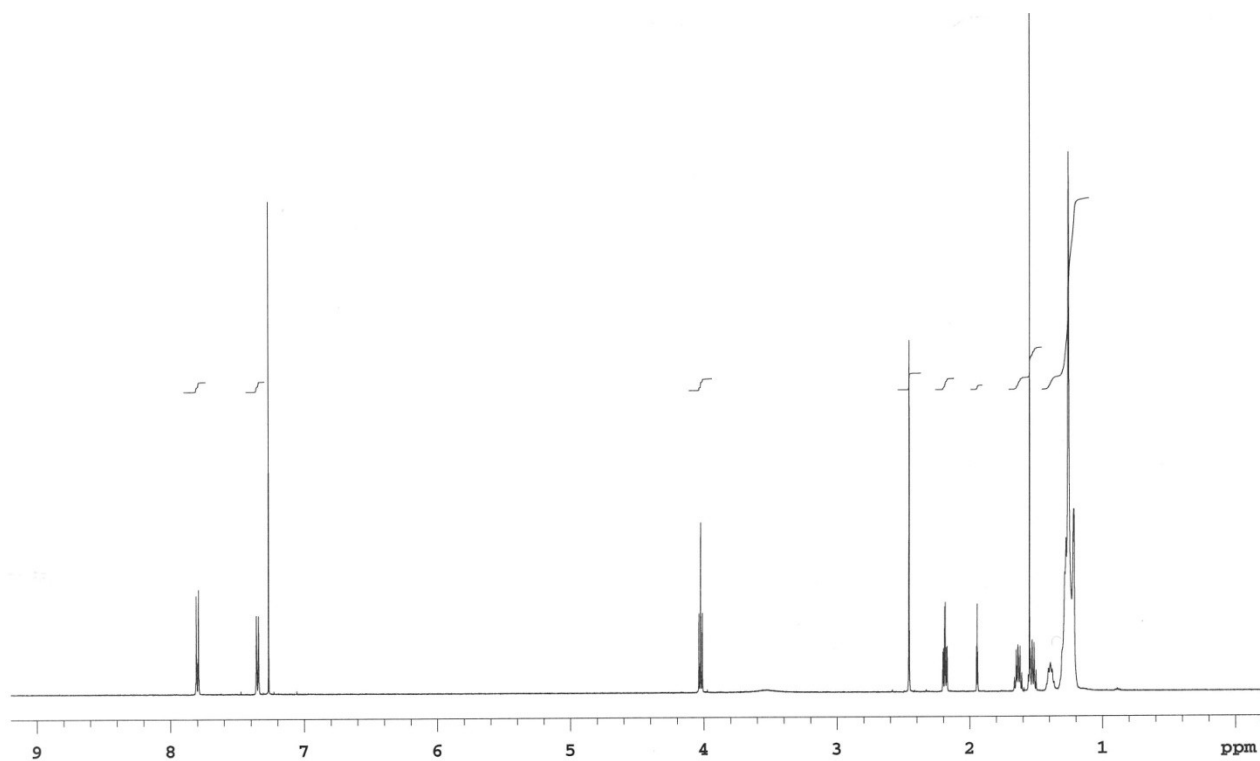
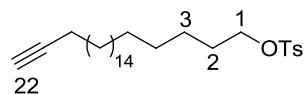
21-Docosyn-1-ol (19)

^{13}C NMR (125 MHz, CDCl_3)



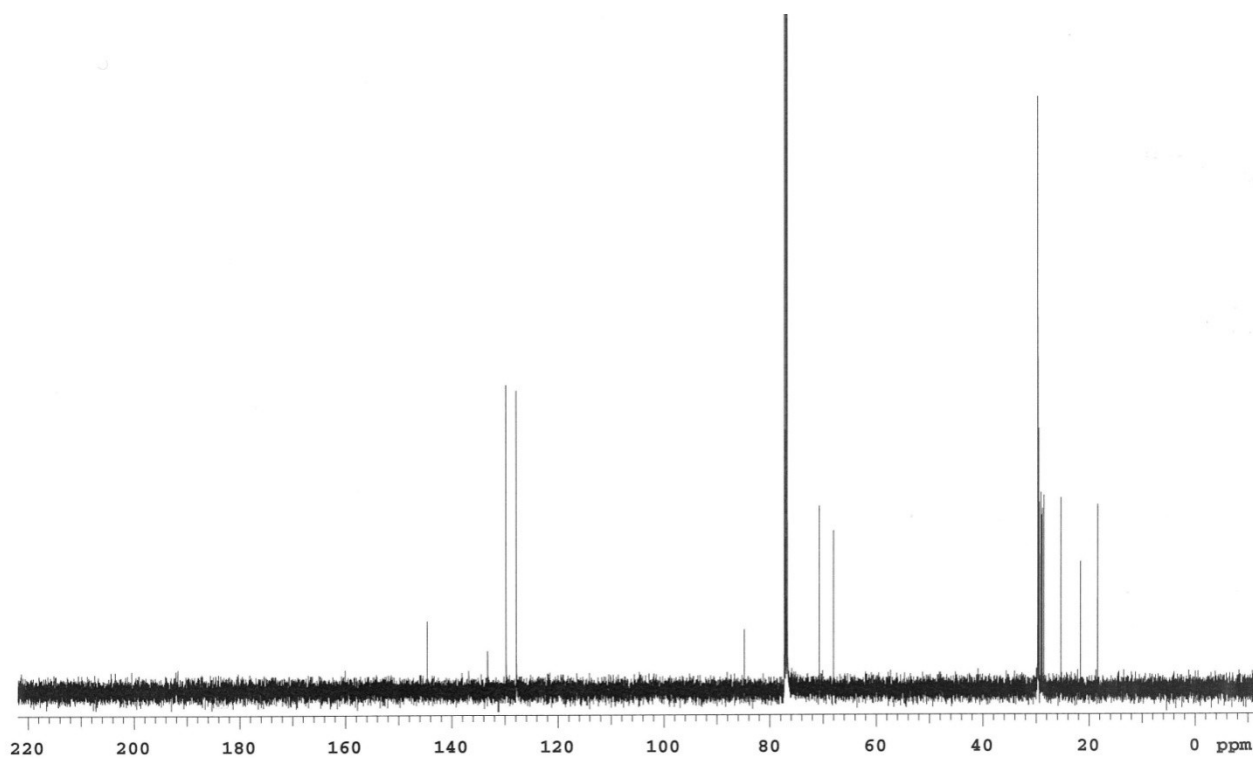
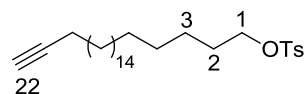
21-Docosynyl *p*-toluenesulfonate (21)

^1H NMR (500 MHz, CDCl_3)

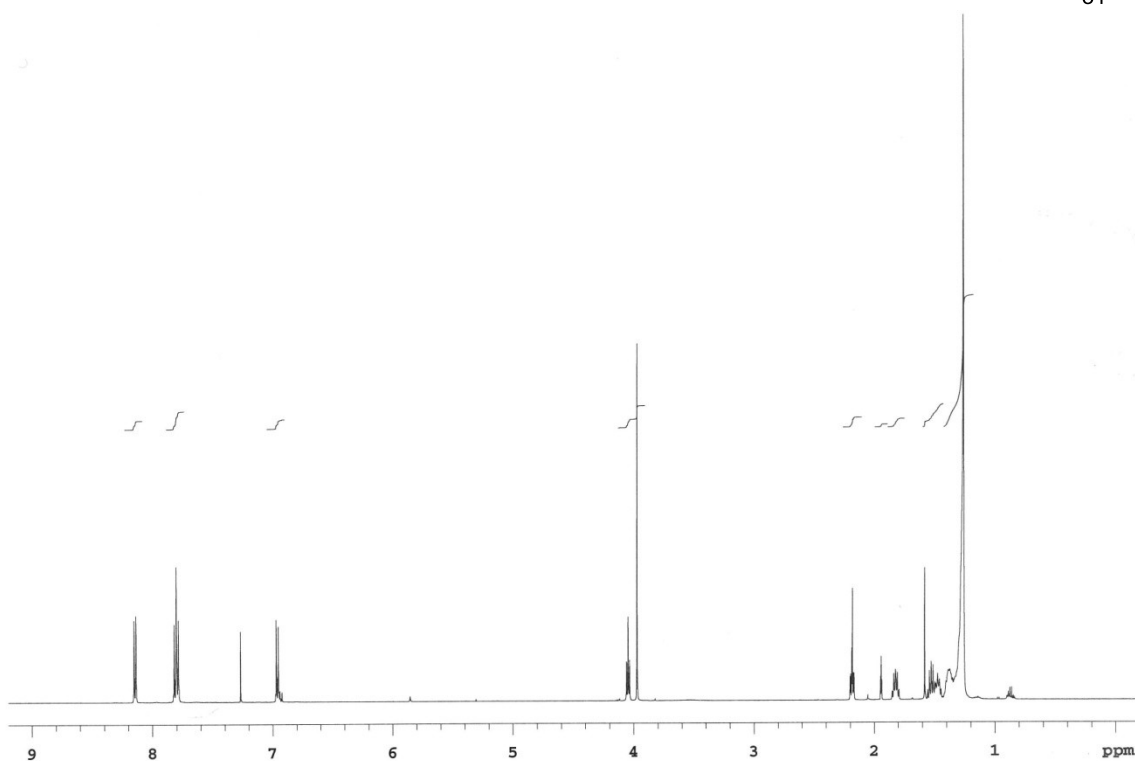
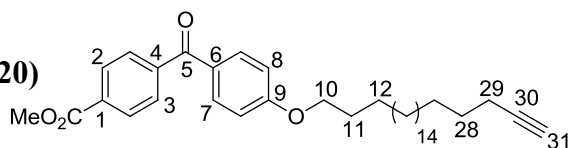


21-Docosynyl *p*-toluenesulfonate (21)

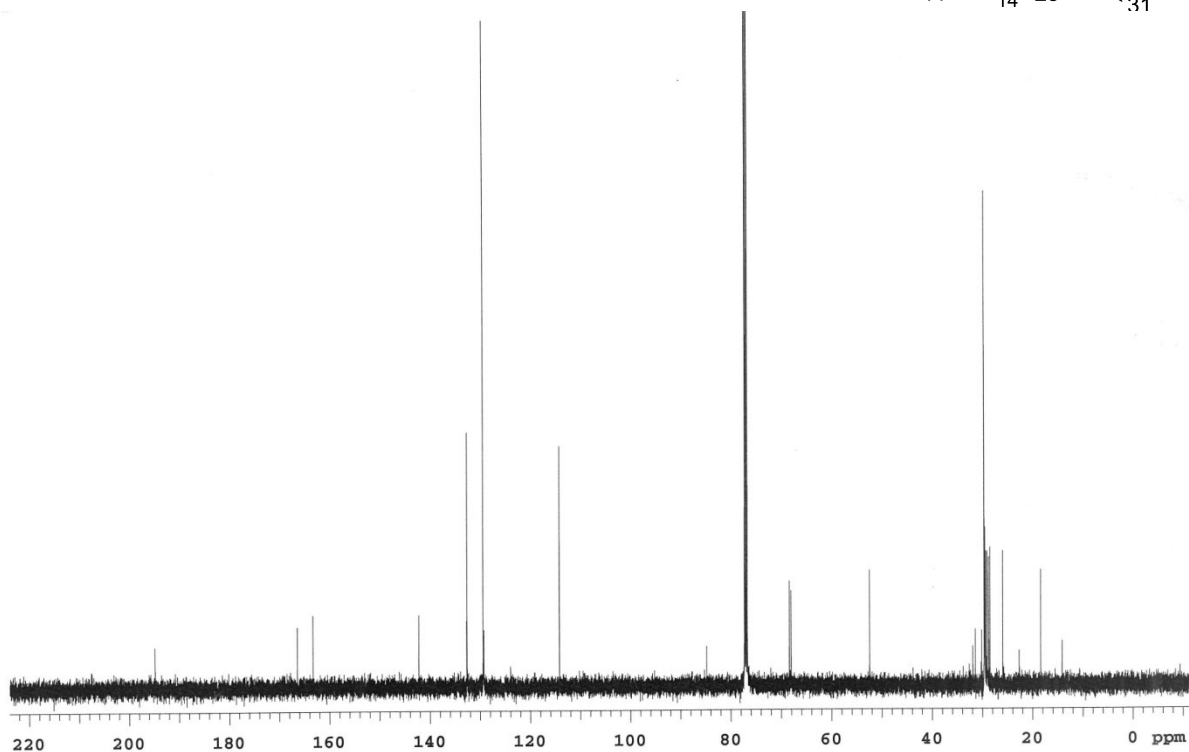
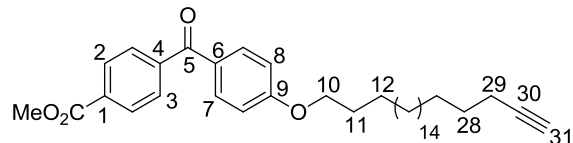
^{13}C NMR (125 MHz, CDCl_3)



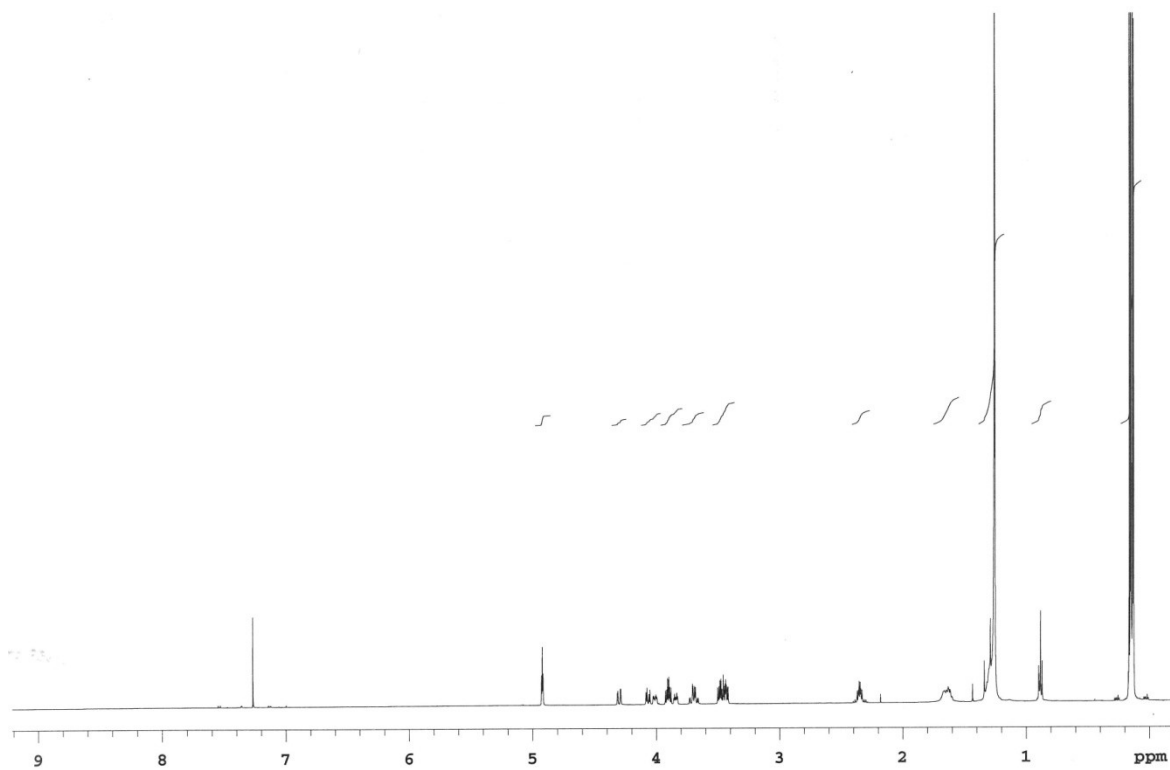
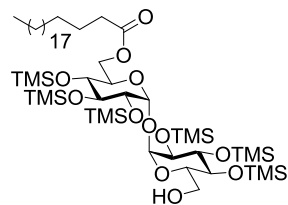
Methyl 4-[4-(21-docosynyloxy)benzoyl]-benzoate (20)
¹H NMR (500 MHz, CDCl₃)



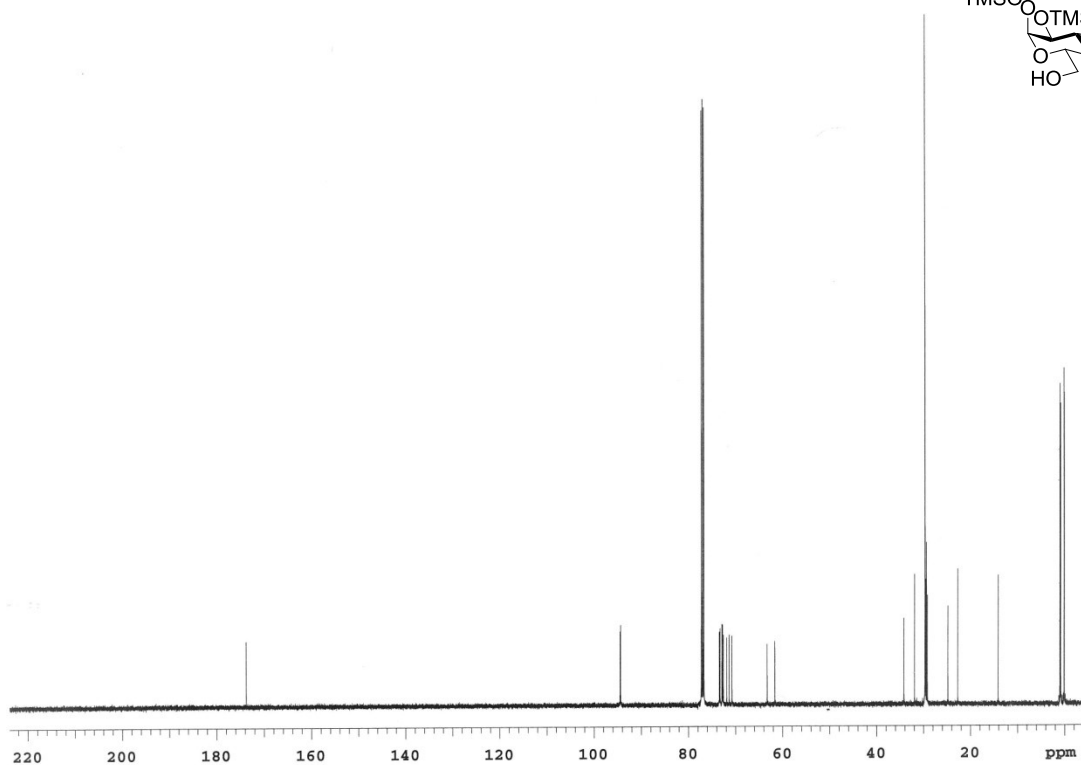
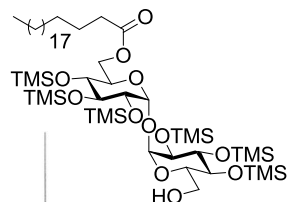
Methyl 4-[4-(21-docosynyloxy)benzoyl]-benzoate (20)
¹³C NMR (125 MHz, CDCl₃)



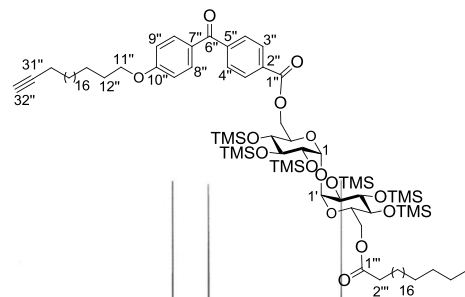
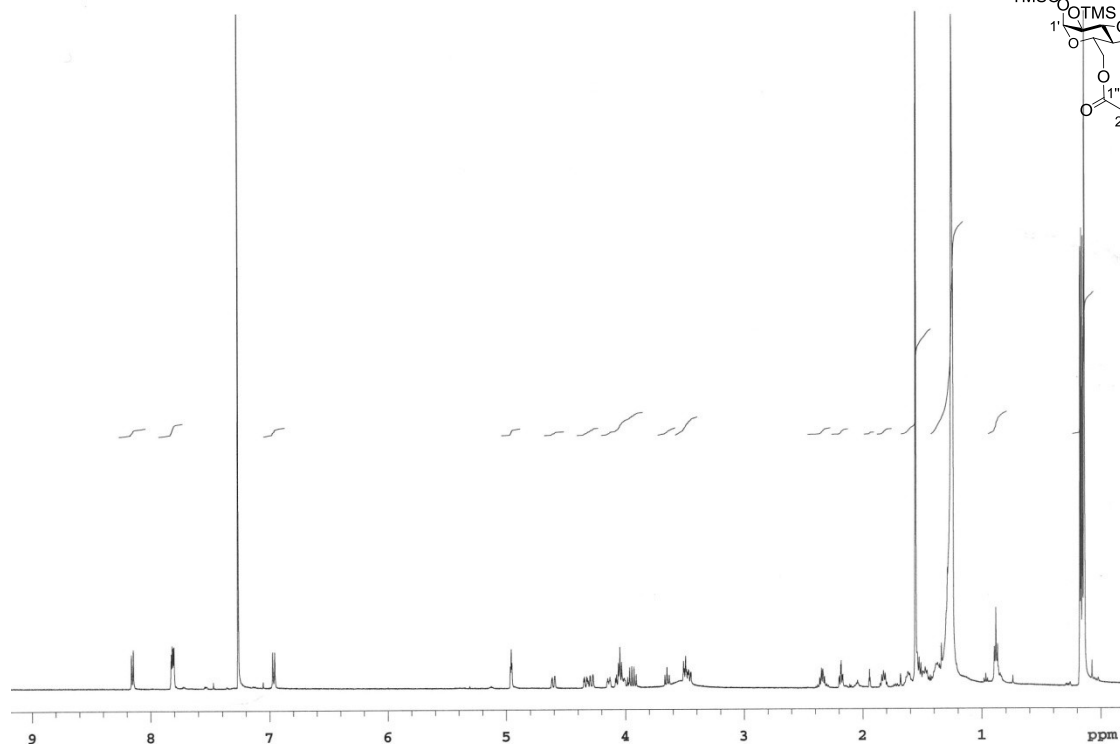
2,2',3,3',4,4'-Hexa-*O*-trimethylsilyl-6-docosanoyl-6'-hydroxyl, α,α' -D-trehalose (2)
 ^1H NMR (500 MHz, CDCl_3)



2,2',3,3',4,4'-Hexa-*O*-trimethylsilyl-6-docosanoyl-6'-hydroxyl, α,α' -D-trehalose (2).
 ^{13}C NMR (125 MHz, CD_3OD)

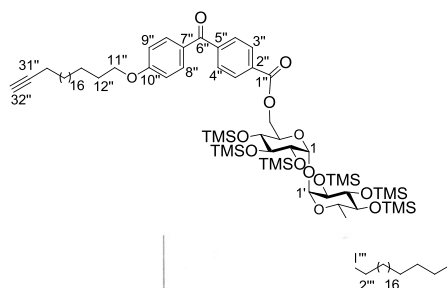
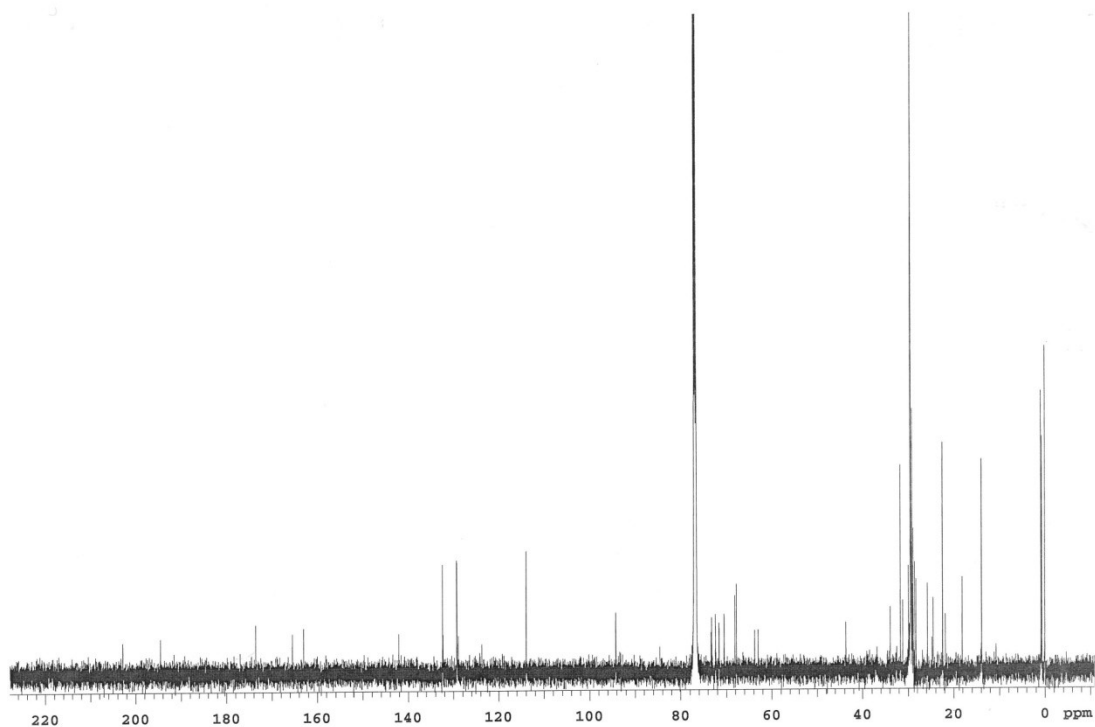


2,2',3,3',4,4'-Hexa-*O*-trimethylsilyl-6'-*O*-docosanoyl-6-*O*-4-[4-(21-docosynyloxy)benzoyl]benzoyl- α,α' -D-trehalose (22) ^1H NMR (500 MHz,



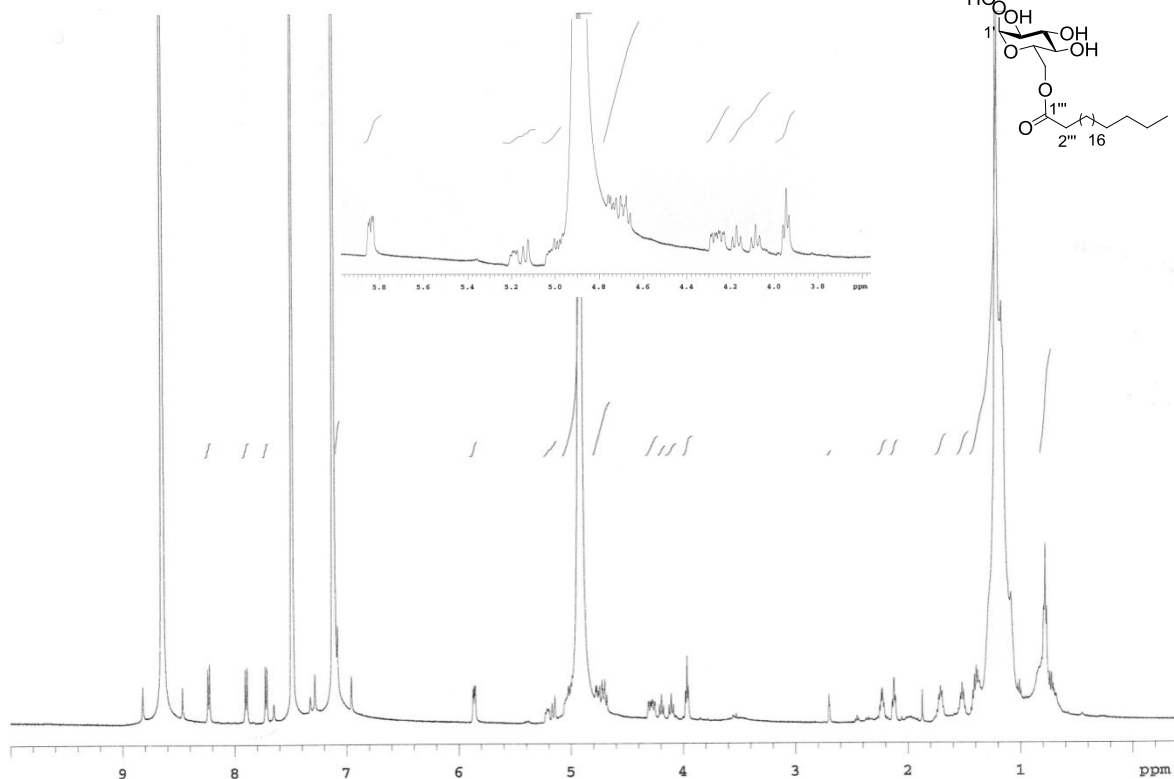
2,2',3,3',4,4'-Hexa-*O*-trimethylsilyl-6'-*O*-docosanoyl-6-*O*-4-[4-(21-docosynyloxy)benzoyl]benzoyl- α,α' -D-trehalose (22).

^{13}C NMR (125 MHz, CDCl_3)



6'-*O*-Docosanoyl-6-*O*-4-[4-(21-docosynyloxy)benzoyl]benzoyl- α,α' -D-trehalose (1)

^1H NMR (500 MHz, $\text{C}_5\text{D}_5\text{N}$)



6'-*O*-Docosanoyl-6-*O*-4-[4-(21-docosynyloxy)benzoyl]benzoyl- α,α' -D-trehalose (1)

^{13}C NMR (125 MHz, $\text{C}_5\text{D}_5\text{N}$)

