Exploring the structure-activity relationship of Mincle ligands

By

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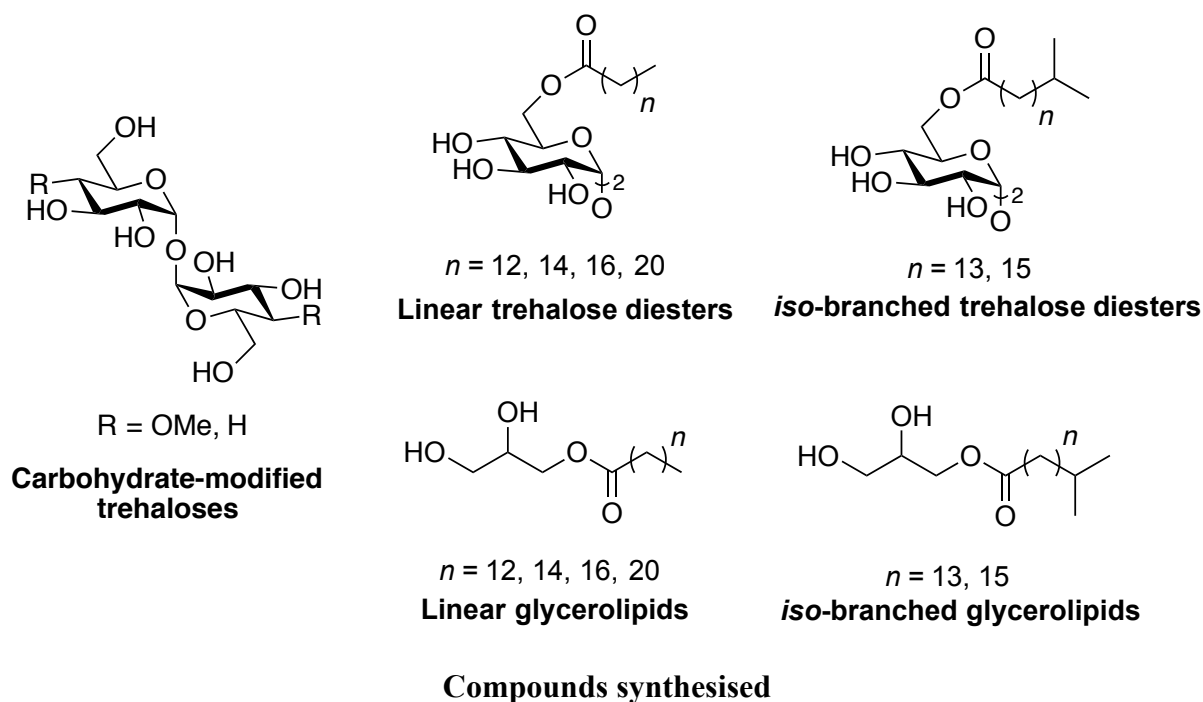
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Abstract

Mincle is a C-type lectin that plays a critical role in the body's innate immune response to bacteria and fungi. Compounds identified as ligands for Mincle include trehalose and glycerol esters of complex long chain fatty acids. Harnessing its ability to activate the immune system, trehalose dimycolate has been used as a vaccine adjuvant and for anti-cancer treatment, highlighting the potential use of Mincle immunomodulators in a medical context. To further understand the receptor-ligand interactions and optimise biological activity, the structure-activity relationship of Mincle ligands was investigated through the synthesis and biological evaluation of a series of modified ligands. The preparation of carbohydrate modified trehalose dibehenates was attempted in order to assess the importance of hydroxyl-protein interactions, and despite the syntheses being incomplete, improvements on literature methodologies for the regioselective modification of α,α′-D-trehalose were developed. Simplified analogues of the glycerol based natural products identified as Mincle ligands containing straight chain and *iso*-branched lipids were prepared to evaluate the significance of the branch and establish whether there is a relationship between lipid length and Mincle activation. The corresponding trehalose diester analogues were also synthesised to gauge the capacity of the protein to tolerate changes in the carbohydrate portion of ligands.

R = OMe, H
Carbohydrate-modified trehaloses

n = 12, 14, 16, 20
Linear trehalose diesters

n = 13, 15
***iso*-branched trehalose diesters**

n = 12, 14, 16, 20
Linear glycerolipids

n = 13, 15
***iso*-branched glycerolipids**

Compounds synthesised

Preliminary biological testing of the glycerol monoesters and trehalose diesters confirmed suggestions in the literature that glycerolipids are not capable of activating murine macrophages, however the trehalose glycolipids were found to be active. The glycolipids displayed the expected correlation between lipid length and macrophage activation, with the exception of 6,6′-di-*O*-tetradecanoate-α,α′-D-trehalose, which induced the production of a disproportionately high quantity of the cytokine IL-1β. The *iso*-branched compounds were found to be significantly more active than their linear analogues, hypothesised as being due to enhanced binding to the hydrophobic groove in Mincle. Assays should be repeated with Mincle knockout macrophages in order to confirm that the activation observed is due to Mincle-dependent signaling, and furthermore, human macrophages should be employed in order to elicit responses to the glycerolipids and hence allow for assessment of their structure-activity relationship. Overall, the biological results obtained, though preliminary, have demonstrated that the addition of a *iso*-branch at the end of the lipid of trehalose diesters increases their immunomodulatory activity, and that the trend between lipid length and biological activity may be bimodal. These novel findings indicate a new and simple method for increasing the biological activity of trehalose diesters, potentially applicable to adjuvancy and anti-cancer treatments.

Acknowledgements

The journey towards this Masters degree has been long and interesting, and there are a number of people whom I would like to acknowledge for their role in helping making it as smooth as possible. First, to my supervisors Dr Bridget Stocker and Dr Mattie Timmer, thank you for your time, your energy, and all you have taught me. You have helped me develop so much as a scientist and I will always remember your commitment and work ethic. Next, to all the past and current lab members – Alex Hunt-Painter, Amy Foster, Amy Lynch, Ben Deeble, Billy Mills, Charlotte Waghorne, Chriselle Braganza, Hilary Corkran, Jaimé Hill, Janice Cheng, Jessie Bird, Kris Santoso, Kristel Kodar, Stefan Munneke, and Thomas Teunissen – thank you for your help, companionship, music suggestions, and putting up with me when I was stressed and more than a bit manic. In particular, I want to thank those who have played a big role in guiding, educating, and supporting me in my project, specifically Jessie and Amy F., without whom I don't know I would have coped. I will never forget the friendship and support you offered me in good times and bad. My project has drawn on methodology developed by two old lab members, Stefan Munneke and Ashna Khan, so thank you, both of you, for your previous hard work. Furthermore, Amy F., Billy Mills, and particularly Kristel Kodar have carried out biological testing for me and provided advice besides, enabling this project to begin assessing the activity of my compounds. Thank you for giving up your valuable time for this bio-ignorant chemist, you've certainly increased my respect for the art of pipetting if nothing else! Alongside group members, there are a number of other people within the School of Chemical and Physical Sciences at VUW who have helped me immensely. Jackie King, Teresa Gen, Gordon Heeley, Joanne Harvey, Rob Keyzers, and most especially Ian Vorster: thank you all for your chemistry advice and help with instrumentation. Additionally, Martyn Coles kindly collected my X-Ray crystal structure for me, probably the most exciting piece of data I received! Outside university (yes, I do have a life outside uni), much love to my friends for introducing me to a whole variety of new and interesting music, books, TV, and more. You have helped keep me sane and not totally consumed by my work, which has been invaluable. Also to my extended family and family friends, thank you for your support and interest, even when you couldn't understand any of the words I use when I talk about my project. Last, but certainly not least, to my mum, thank you, thank you, thank you. Without your love and support I wouldn't have made it through this in one piece. Good food, emotional support, and the comforts of home go a long way. I'm proud of you and I look forward to us handing in our PhDs together.

Glossary

AIBN: Azobisisobutyronitrile

bMincle: Bovine Mincle

BMM: Bone marrow derived macrophages

Bn: Benzyl

BSA: Bis(trimethylsilyl)acetamide

Bu: Butyl

Bz: Benzoyl

CLR: C-type lectin receptor

COSY: Correlation spectroscopy

CRD: Carbohydrate recognition domain

CSA: Camphorsulfonic acid

CTL: C-type lectin

DC: Dendritic cell

DCM: Dichloromethane

DMAP: 4-Dimethylaminopyridine

DMF: *N,N*-dimethylformamide

DMSO: Dimethylsulfoxide

EA: Ethyl acetate

EDCI: 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide

ELISA: Enzyme-linked immunosorbent assay

ESI: Electron-spray ionisation

Et: Ethyl

FcRγ: Fragment, crystallisable region receptor gamma

GMB: Glycerol monobehenate

GM-CSF: Granulocyte-macrophage colony-stimulating factor

GMCM: Glycerol monocorynomycolate

GMM: Glycerol monomycolate

HBTU: *N,N,N′,N′*-tetramethyl-O-(benzotriazol-1-yl)uronium hexafluorophosphate

HDMS: Hexamethyldisilazide

HMBC: Heteronuclear multiple-bond correlation spectroscopy

hMincle: Human Mincle

HRMS: High resolution mass spectrometry

HSQC: Heteronuclear single quantum coherence spectroscopy

Il: Interleukin

IR: Infrared spectroscopy

ITAM: Immunoreceptor tyrosine-based activation motif

LPS: Lipopolysaccharide

Malt1: Mucosa associated lymphoid tissue lymphoma translocation gene 1

Me: Methyl

Mincle$^{-/-}$: Mincle knockout

Mins: Minutes

Mpl: Monophospholipid

Mtb: *Mycobacterium tuberculosis*

NFAT/GFP: Nuclear factor of activated T-cells/Green fluorescent protein

NF-κB: Nuclear factor kappa-light-chain-enhancer of activated B cells

NMR: Nuclear magnetic resonance

PAMP: Pathogen associated molecular pattern

PBS: Phosphate-buffered saline

PE: Petroleum ether

Pyr: Pyridine

Quant: Quantitative

Syk: Spleen tyrosine kinase

TBAF: Tetrabutylammonium fluoride

TBAI: Tetrabutylammonium iodide

TBTU: N,N,N',N'-tetramethyl-O-(benzotriazol-1-yl)uranium tetrafluoroborate

TDB: Trehalose dibehenate

TDCM: Trehalose dicorynmycolate

TDE: Trehalose diester

TDM: Trehalose dimycolate

TFA: Trifluoroacetic acid

TFAA: Trifluoroacetic anhydride

TGFβ: Transforming growth factor beta

THF: Tetrahydrofuran

TLR: Toll-like receptor

TMCM: Trehalose monocorynomycolate

TMS: Trimethylsilyl

TNF: Tumour necrosis factor

Table of Contents

1 Introduction

## 1.1 The innate immune system

The human immune system is comprised of two branches, the innate and the adaptive, which work together to protect the body from harmful foreign invasion by pathogens. The adaptive immune system is developed over the course of infection, and assembles a library of memory cells responsible for mounting a more effective response to a future challenge by the pathogen.[1] In contrast, the innate immune system provides an immediate, non-specific response that acts as the first line of defence against an invading pathogen. The innate immune system is comprised of a number of characteristic cell types, specifically: mast cells, basophils and eosinophils (granular), natural killer cells, and the phagocytic macrophages, neutrophils and dendritic cells (DCs).[1] An integral role of the innate immune system is recognising foreign material (e.g. bacteria), and mounting an appropriate response. As part of this, antigen-presenting cells (namely DCs and macrophages) express pattern-recognition receptors (PRRs), whose role it is to bind pathogen-associated molecular patterns (PAMPs), and activate the wider immune system through the release of inflammatory cytokines. This response leads to the mounting of a defense against infections.

A sub-family of the PRRs are the C-type lectin receptors (CLRs), which are tasked with binding carbohydrates though conserved amino acids motifs in a Ca^{2+}-dependent manner. CLRs play a critical role in antifungal and antibiotic immunity, and also contribute to homeostasis, autoimmunity, allergy, and the recognition of dead cells and tumours.[2-5] At least eight CLRs, including Dectin 1, Dectin 2, macrophage inducible C-type lectin (Mincle), macrophage C-type lectin (MCL), and dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN), have been identified in humans, with overlapping and complementary molecular recognition capabilities.[5,6] Given the multi-faceted role these receptors play in the immune system, research has focused on further understanding their roles and utilising their immunomodulatory activity for medicinal purposes.

1.1.1 Mincle

The macrophage inducible C-type lectin, (Mincle, also known as Clec4e) is a transmembrane receptor that is expressed by macrophages, DCs, and monocytes upon stimulation.[7,8] Mincle

is an innate PRR that recognises pathogenic fungi[9,10] and mycobacteria,[11] and its presence has been linked to protection against infection.[12] Binding of a ligand to Mincle triggers an immune response through the activation of the speleen tyroking kinase (Syk) - Card9 - Bc110 - Mucosa associated lymphoid tissue lymphoma translocation gene 1 (Malt1) pathway, first by inducing phosphorylation of the immunoreceptor tyrosine-based activating motif (ITAM) in the Fcγ receptor (Figure 1).[13] Phosphorylation of ITAM provides a binding site for Syk, the association of which activates Card9, which promotes expression of cytokines such as tumour necrosis factor (TNF) and interleukin (IL) - 6.[13] Furthermore, translocation of transcription factor nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) to the nuclear is promoted, which leads to the production of IL-23. Cytokine expression drives the adaptive immune response, and in particular, the production of T_h1 and T_h17 polarised helper T cells, (by IL-12 and IL-6 and Transforming growth factor beta (TGFβ), IL-23, and IL-1β, respectively),[14] which act to recruit phagocytes to the site of infection.[1]
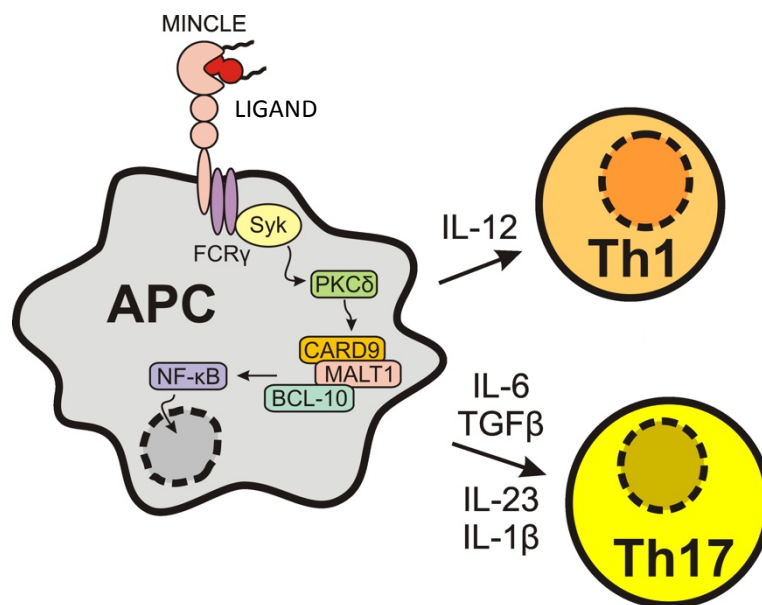

Figure 1: Immunomodulatory effects of Mincle activation: Binding of a ligand leads to activation of the Syk-Card9 pathway, cytokine production, and production of Th1 and Th17 cells.

1.1.2   MCL

Macrophage C-type lectin (also known as MCL, Clec4d, and Dectin-3) is a CLR that is constitutively expressed on myeloid derived cells such as macrophages, DCs, and neutrophils.[15,16] MCL has been shown to activate Syk and mediate phagocytosis and

inflammatory cytokine production.[17] As with Mincle, activating signals are transduced through the Fcγ receptor, and MCL deficient mice display reduced immune responses to mycobacteria due to impaired pathogen recognition capacity.[16] The presence of MCL is critical to the inducible expression of Mincle, hence there appears to be a degree of interaction between the two CLRs.[16] Consequently, it has been suggested that MCL and Mincle form a heterodimer at the macrophage cell surface, with Mincle bridging MCL and Fragment, crystallisable region receptor gamma (FcRγ) (Figure 2).[15,18] Furthermore, examining the correlation between Mincle and MCL on the surface of DCs suggests that MCL magnifies Mincle-dependent signaling through protein-protein interactions in the stalk region of the receptors.[19] Notwithstanding, contradictory data has emerged from other studies.[20]
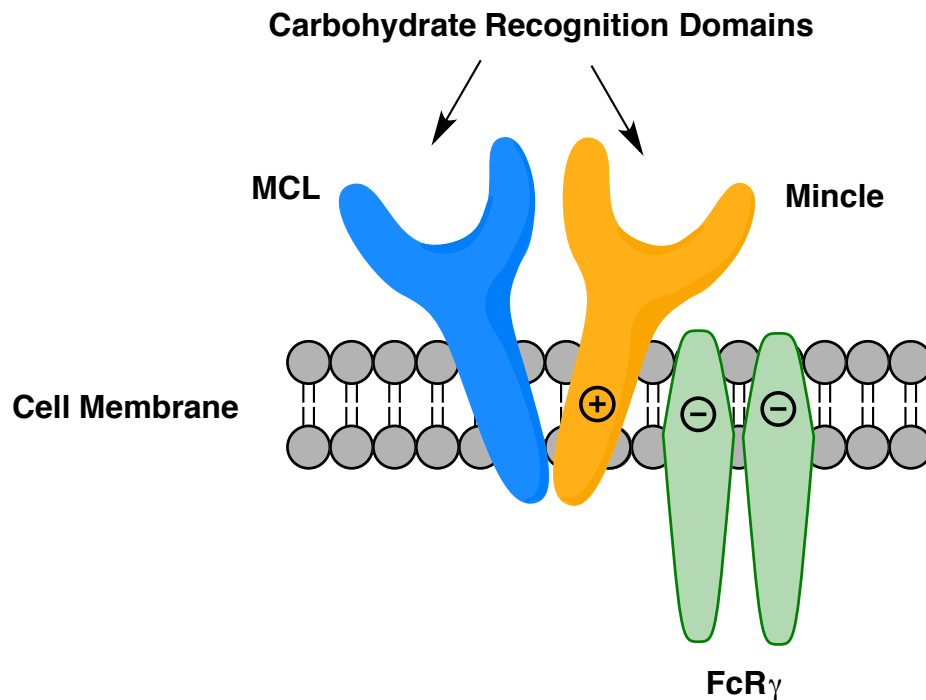
Figure 2: Proposed MCL and Mincle heterodimer at the cell surface, adjacent to FcRγ for signal transduction (adapted from Yamasaki[15]).

1.2 Mincle ligands

Glycolipids have been the primary class of compounds that have been identified as being capable of binding to and activating Mincle; namely trehalose, mannose, and gentiobiose glycolipids, in addition to a simplified glycerol conjugate and one steroid.[21]
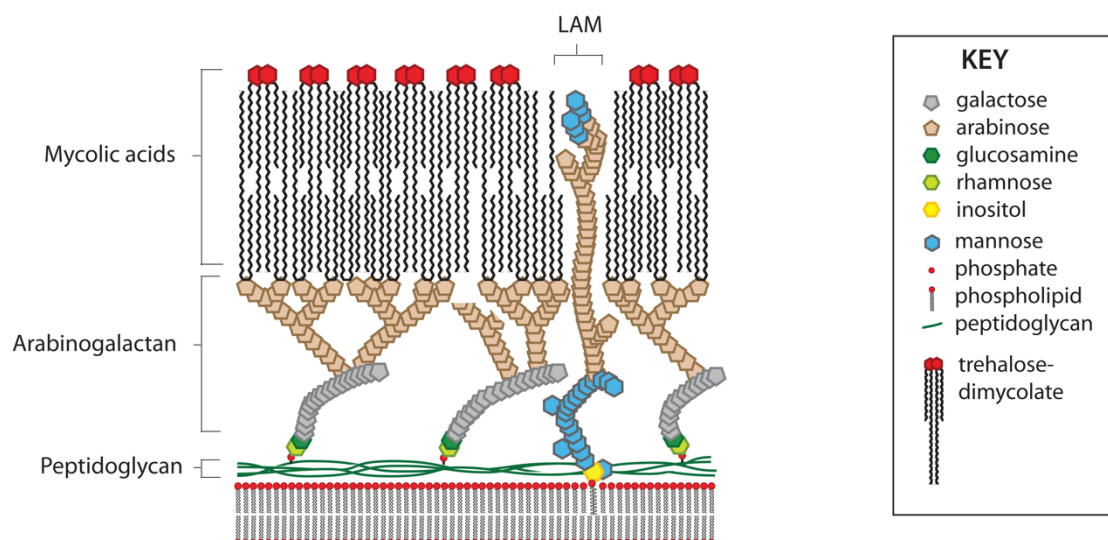
1.2.1 Trehalose glycolipids

The trehalose glycolipids are a group of structurally related compounds that are produced by a number of organisms, including species from the *Mycobacteria* and *Corynebacteria* genera.[12,22] Mincle recognises these ligands in a cation-dependent manner through a carbohydrate recognition domain (CRD) in the extracellular region of the protein.[11,23] Trehalose glycolipids comprise an α,α′-D-trehalose core, with lipid esterified to one or more of the sugar hydroxyls (Figure 3). The number, positioning, and functionality of the lipid chains varies between the different glycolipids. For example, trehalose dimycolates (TDMs, **1**) contain a branched lipid that may include a variety of different functional groups, such as methoxides, ketones, epoxides, and *cis*-cyclopropanes. The mycolic acid alkyl chain also varies between sixty and ninety carbons in length, and contains at least two chiral centers. In contrast, trehalose dicorynomycolate (TDCM, **2**) and monomycolate (TMCM, **3**) contain branched lipids similar to those present in TDM **1**, however the chains possess no additional functionality.
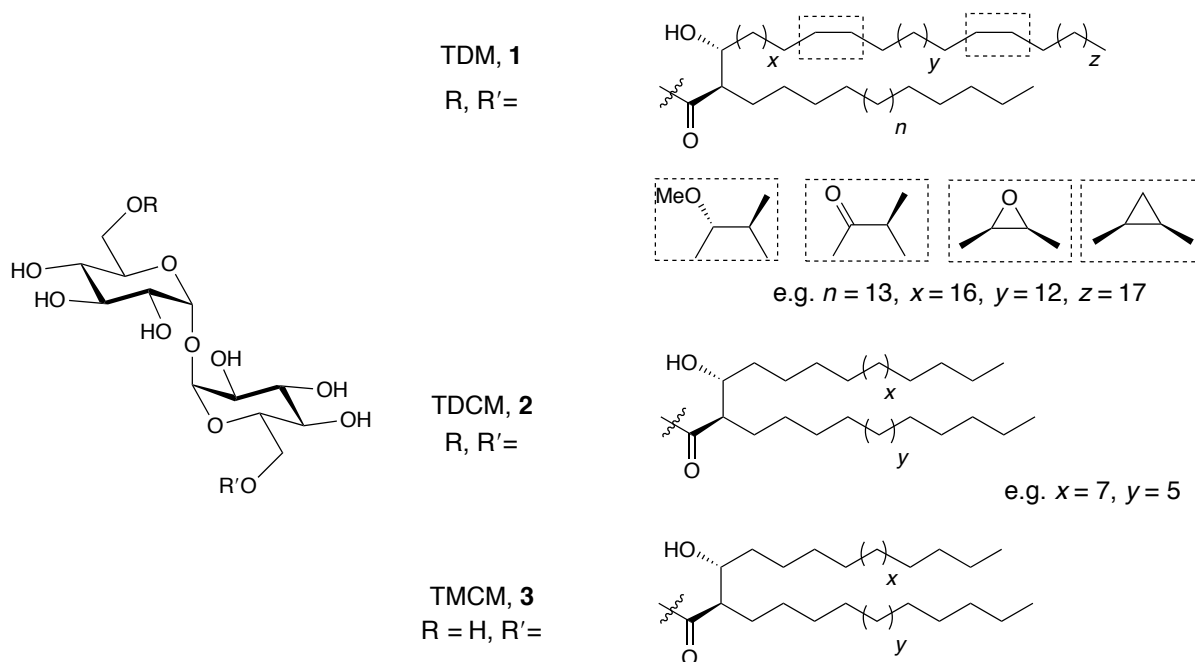
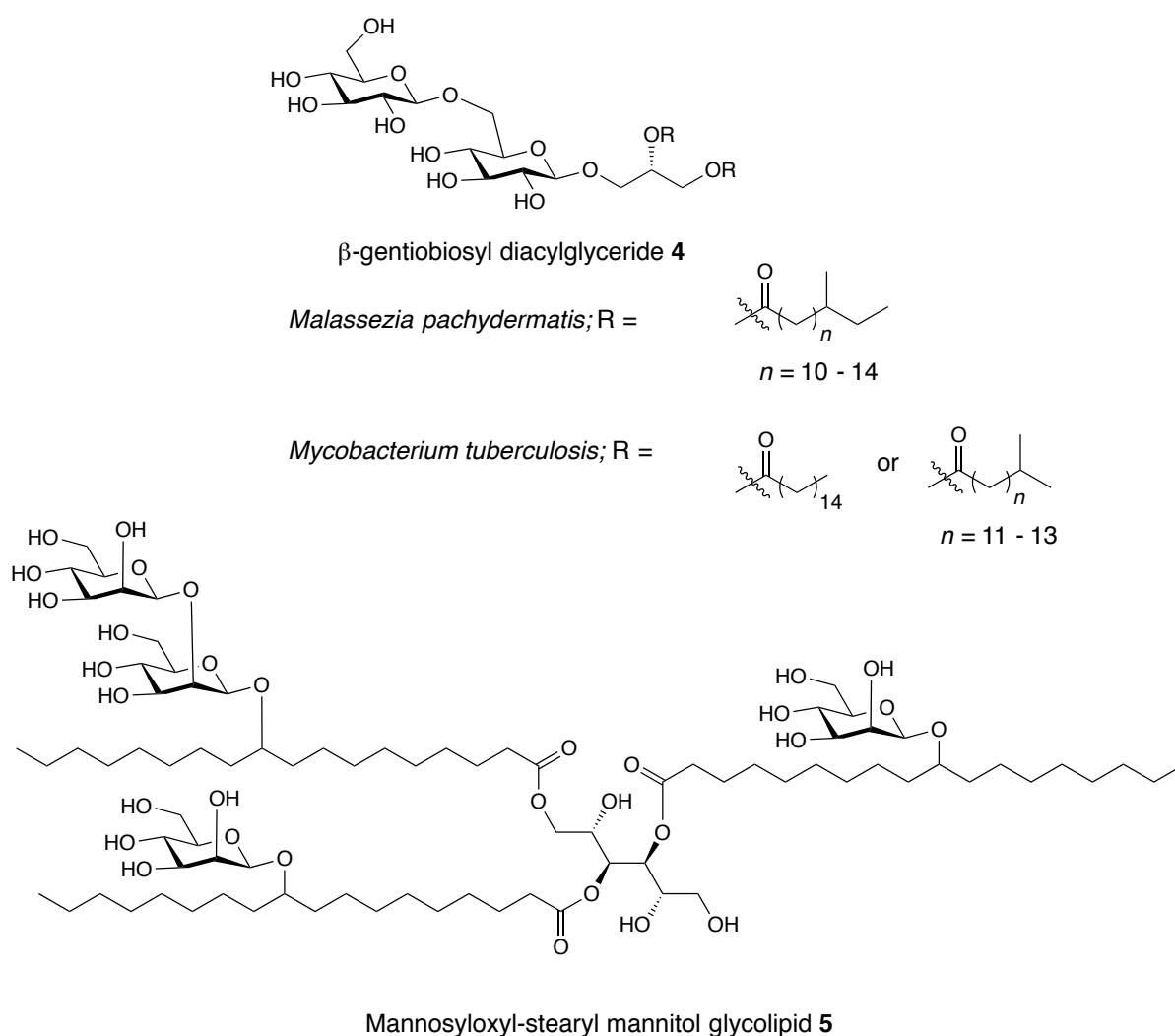**Figure 3:** Trehalose glycolipids known to activate Mincle

TDM is the most abundant glycolipid in the hydrophobic mycobacterial cell wall, found alongside arabinogalactan, peptidoglycan and lipoarabinomannan. (Figure 4) When discovered, TDM was identified as 'cord factor' due to the ability of the material to cause serpentine, cord-like mycobacterial cell growth.[24] Indeed, cord factor was the first compound isolated from *M. tuberculosis* (*Mtb*) that showed immunostimulatory behaviour.[25] Although essential for the survival of the mycobacteria within the phagosome of the host cells,[26] TDM is recognised by the immune system of the host as an indicator of infection.[27] Mincle expression is critical to the specific immune response evoked by TDM stimulation, hence TDM is a direct ligand for Mincle.[28] Similarly, TDCM and TMCM have been shown to signal through Mincle through assessing the stimulation of nuclear factor of activated T cells-green fluorescent protein (NFAT-GFP) transfected with Mincle.[29]

Figure 4: Structure of the mycobacterial cell wall (adapted from Dangerfield *et al*. with permission)[30]
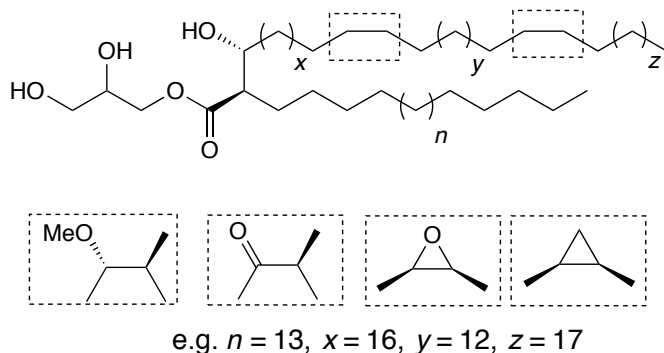
1.2.2 Other glycolipids

Apart from in trehalose glycolipids, long-chain fatty acids have also been found esterified to β-gentobiose via *S*-glycerol (**4**), with this conjugate also identified as binding to and activating Mincle (Figure 5). These glycolipids contain *ante-iso* or *iso* branched lipids, depending on the organism (and hence biosynthetic machinery) of origin.[29,31] Further, a complex mannosyloxy-stearyl mannitol glycolipid (**5**) extracted from *Mycobacteria pachdermatis* has also been found to signal through Mincle with a potency similar to that of TDM.[10]

β-gentiobiosyl diacylglyceride **4**

Malassezia pachydermatis; R =

n = 10 - 14

Mycobacterium tuberculosis; R =

or

n = 11 - 13

Mannosyloxyl-stearyl mannitol glycolipid **5**

Figure 5: Gentiobiose and mannose glycolipid Mincle ligands

1.2.3 Non-glycolipids ligands

A limited number of non-glycolipid ligands for Mincle have been identified, as shown in Figure 6. Glycerol monomycolate (GMM, **7**) is a mycobacterial lipid capable of stimulating innate immune cells to produce inflammatory cytokines,[32] induces local infiltration by immune cells upon skin injection,[33,34] and is recognised by transfectant cell lines expressing human, though not mouse Mincle.[33] The last Mincle ligand identified to date is crystalline cholesterol, which is an endogenous ligand capable of activating the innate immune system.[35]

e.g. $n = 13$, $x = 16$, $y = 12$, $z = 17$

GroMM, **7**
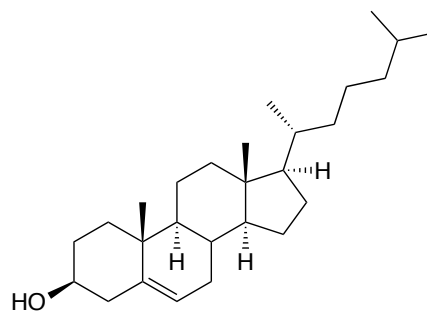
Cholesterol, **8**

Figure 6: Non-glycolipids identified as Mincle ligands

1.3   Immunotherapy

Immunotherapy involves manipulation of the immune system, with the aim of preventing or curing disease.   The most widely known and used application of immunotherapy is vaccination, which revolutionised modern medicine in the 20th century, though use for the treatment of cancer is also well established. Activating the immune system through use of Mincle ligands have proved useful in both of these applications.

1.3.1   Cancer immunotherapy

The utilisation of the immune system to combat cancer in an affected patient is known as cancer immunotherapy, and the strategy mostly focuses on invoking an immune response against tumour tissue.[1] The therapy was pioneered in the late nineteenth century by William Coley, who experimented with the use of bacteria to stimulate the production of phagocytic immune cells, which can kill tumour cells.[36] Although met with mixed success, Coley's toxins acted as a proof of concept and his work has inspired more nuanced approaches based on a greater understanding of the immune system. There are a number of cancers for which immunotherapy is currently a standard treatment, such as advanced prostate cancer[36] and non-muscle invasive bladder cancer.[37]

1.3.2 Vaccination

The modern era of vaccination is usually defined as commencing with Edward Jenner's efforts towards smallpox immunisation. In the late 18th century, Jenner discovered that the exposure of patients to the related virus cowpox (vaccinia) lead to the development of resistance to the commonly fatal disease smallpox.[1] Continued efforts towards smallpox vaccination over the following two centuries eventually led to the World Health Organisation declaring the disease eradicated in 1979.[38]

Immunisation against a specific disease exposes both the innate and adaptive arms of the immune system to pathogenic antigens, which results in the development of immunological memory.[1] Consequently, upon re-exposure of the body to the same pathogen, a stronger immune response can be mounted in a shorter amount of time, compared to the naïve response. At the cellular level, the delivery of the vaccine to the innate immune system triggers the activation of the adaptive immune response though antigen presentation to T- and B-cells.[1] Antigen presentation leads to clonal expansion and the development of memory T- and B-cells, which possess surface receptors specific to the antigen. Once established, memory cells persist long after the antigen has been cleared from the body and are responsible for the enhanced immune response observed upon secondary exposure to the pathogen.[1]

1.3.3 Adjuvancy

Along with the antigenic component, many vaccines, especially those comprising isolates separated from the whole organism, include an adjuvant in order to boost the efficacy of the vaccine. Adjuvants can act through a number of mechanisms, such as hastening the production of a robust immune response to the antigen, helping increase the duration of the immune response, inducing local mucosal responses, generating antibodies with an increased affinity for the appropriate receptor, and helping to promote the production of cytotoxic T lymphocytes (CTLs).[39] Only a very few adjuvants are currently licensed for use in prophylactic vaccines, and these generally act to enhance antibody responses. Although these adjuvants are valuable, there remains a large number of diseases and population subgroups for whom vaccination is not effective, and it is thought that the development of adjuvants which operate through alternative modes of action will help address this problem.[39] Prime

candidates for new adjuvants are molecules that activate the innate immune system through binding to PRRs on an antigen presenting cell (APC), such as a dendritic cell or macrophage. Upon activation of the APC, the antigen is recognised by $CD4^+$ and $CD8^+$ T cells, which leads to clonal expansion, and the production of CTLs and memory cells.[39] Examples of compounds which have shown promise in this capacity include ligands for the toll-like receptors (TLRs, a class of PRR), such as lipopolysaccharide (LPS), monophospholipid (MPL), synthetic lipopeptide Pam_3Cys and CpG.[40] Likewise, the mycobacterial isolate TDM (**1**), which as previously discussed activates the PRR Mincle, has been used as an adjuvant.[41-47] It is thought that fine-tuning the structure of this PAMP could allow for the discovery of an even better, clinically useable adjuvant.

1.4 Structure-activity relationship

In order to optimise the activity of Mincle ligands as immunotherapeutic agents, investigating the relationship between the structure and activity is required. Two approaches have been taken in the literature to studying this relationship: synthesising analogues of identified ligands and assessing their activity; and obtaining information on the ligand binding region of the protein through collecting a crystal structure of the protein and ideally with the ligand co-crystallised in the active site.
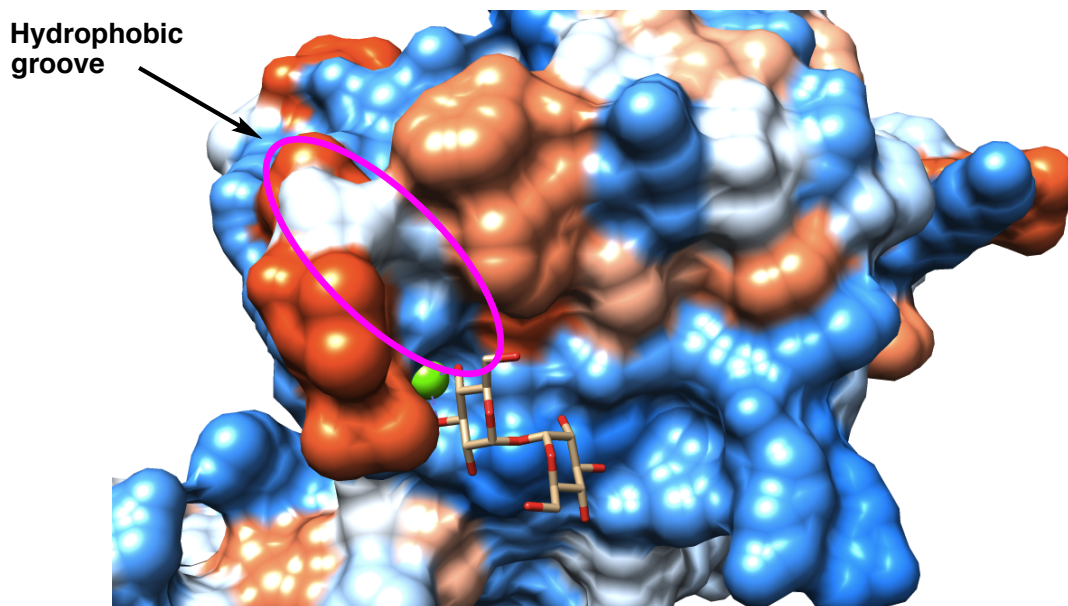
1.4.1 Crystal structure studies

The crystal structures of both human[7] and bovine[45] Mincle have recently been published and provide valuable insight into the mode of binding of ligands such as TDM (**1**) to these CLRs. The bovine receptor (bMincle) was successfully crystallised with trehalose (**9**) bound, (Figure 7)[45] whereas human Mincle (hMincle) was studied either ligand free or with citrate bound.[7] Mincle binds multiple calcium ions, one of which is involved in carbohydrate recognition through a Ca^{2+} mediated binding network (Figure 7).[7,45] The overall structure of Mincle is similar to that of other CLRs, but possesses a unique conformation in the CRD. Furthermore, the amino acid sequence determined for the binding site from these two studies generally concur, although there are minor discrepancies that may reflect the differences in crystallisation conditions or might be due to interspecies differences, despite a close homology between human and bovine Mincle.[48]
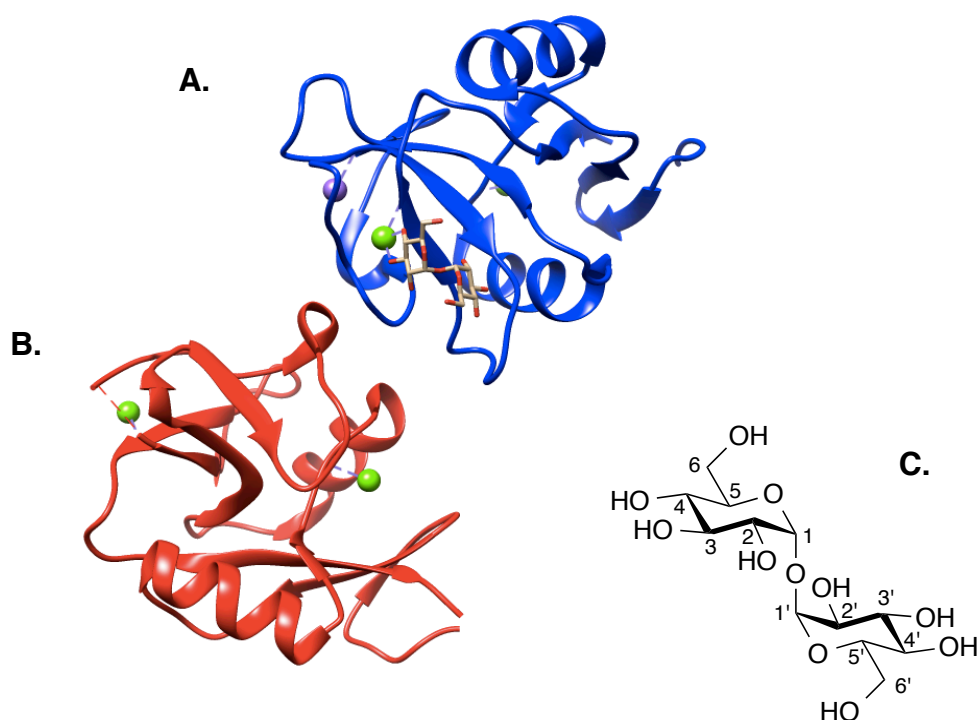
Figure 7: Carbohydrate recognition domain of bMincle with trehalose bound: **A.** Carbohydrate binding portion of bMincle CRD, **B:** Non-carbohydrate binding monomer of bMincle CRD, **C.** Trehalose, **8**

Adjacent to the CRD of Mincle is a region of amino acid residues that form a shallow hydrophobic groove, thought to be involved with binding the lipid chain of TDM and other ligands. In bMincle, this region contains Phe197 and Phe198 on one side, and Leu172 and Val173 on the other (Figure 8).[45] In hMincle, the hydrophobic region encompasses Val195, Thr196, Phe198, Leu199, Tyr201 and Phe202.[7]

Figure 8: Surface map of bMincle hydrophobic groove (red: hydrophobic, blue: hydrophilic)

Although no crystal structures have been obtained of Mincle complexed to a molecule capable of activating the protein, mutagenesis studies indicate that the aforementioned amino acids in the hydrophobic groove are directly involved with binding to trehalose glycolipids.[7,45] Changing the functionality of the amino acid at any of the key residues identified above reduces the capacity of bMincle to bind trehalose glycolipids, as assessed through binding competition assays using trehalose mono-octanoate as a model glycolipid.[45]

The Ca^{2+} ion-mediated binding in Mincle involves the hydroxyls on the 3- and 4-positions of one glucose moiety of trehalose (**8**), which form coordination bonds to the Ca^{2+} and hydrogen bonds to some of the amino acid side chains that ligate the calcium (Figure 9). In bMincle, these amino acid residues are Glu176, Asn192, Glu168 and Asn170, whereas in the hMincle, the Glu169, Pro170 and Asn171 motif (commonly conserved among CLRs) is implicated in the binding of one of the glucose residues of α,α′-D-trehalose.[7,45]
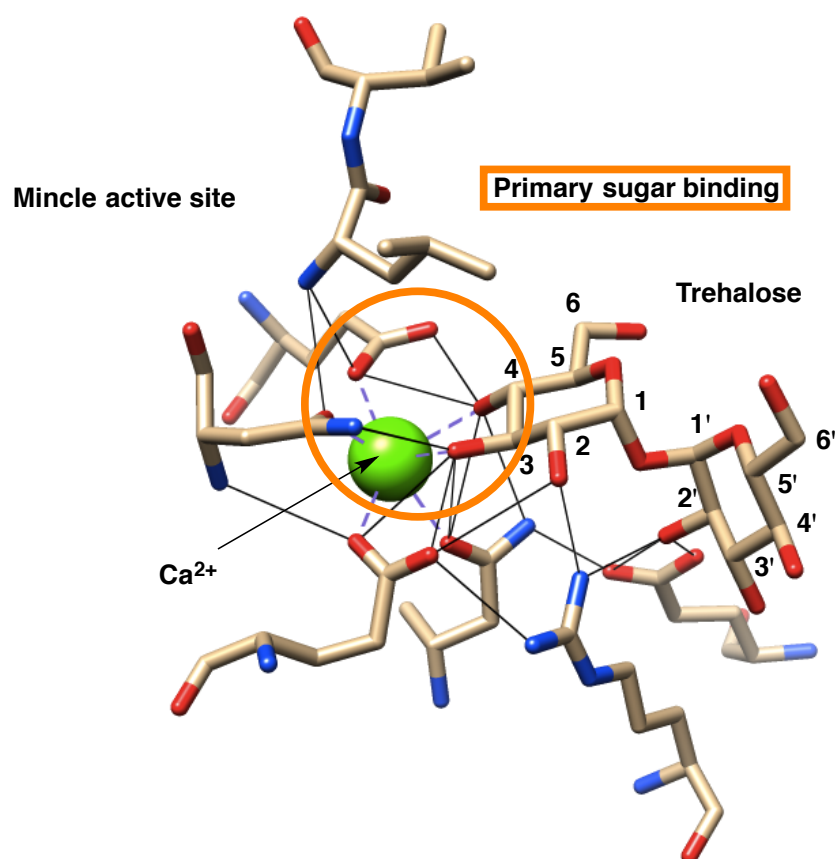
Figure 9: Ca^{2+} dependent binding network with 3-OH and 4-OH of trehalose (bMincle, dashed line: co-ordination bond, solid line: hydrogen bond)

Lastly, the active site of bMincle revealed a secondary carbohydrate–active site residue interaction that was not reported for hMincle, as the ligand used for crystallisation in hMincle would not participate in binding at this site. The 2′-OH of the glucose moiety of trehalose (**9**) not involved in the primary binding participates in a cooperative hydrogen bonding network with Arg182 and Glu135, bridging the two side chains (Figure 10).[45] The presence of this secondary sugar-binding interaction results in a 36-fold stronger binding of trehalose compared to glucose.[45]
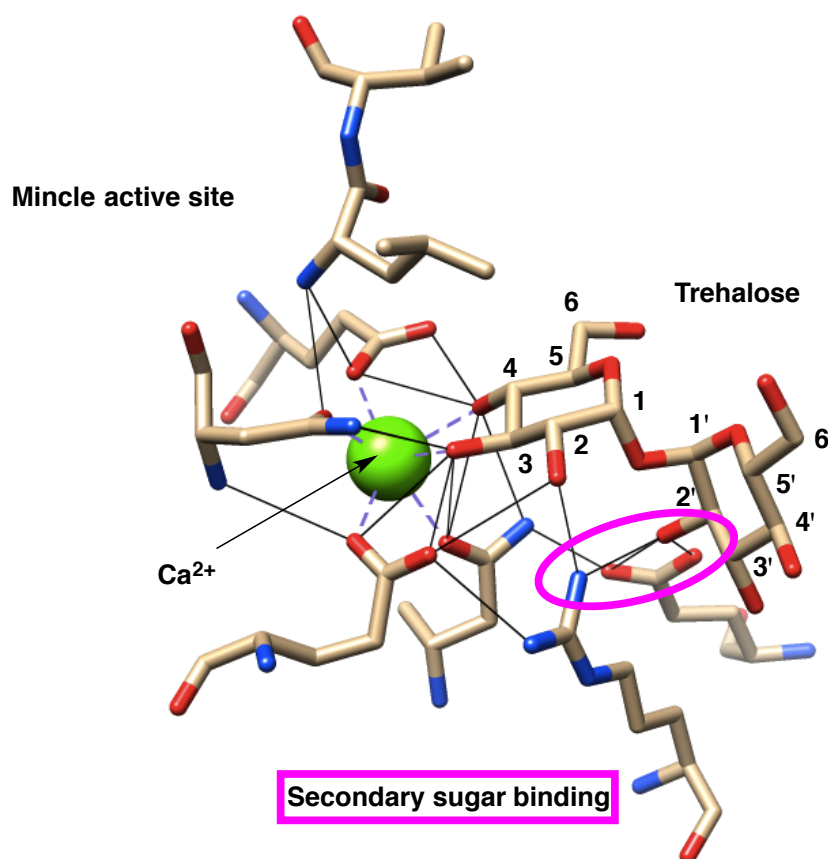
Figure 10: Hydrogen bonding network with 2′-OH of trehalose (bMincle, co-ordination bond, solid line: hydrogen bond)

1.4.2 Synthetic studies

In addition to studying the structure-activity relationship of Mincle ligands through crystal structure analysis, the synthesis of analogs of a number of natural products has been carried out. Studies have generally found a dependence on lipid length on activity, though some variability in the carbohydrate core appears to be tolerated.

1.4.2.1 Trehalose glycolipids

Binding competition studies included in the crystal structure studies using trehalose glycolipids with simple, short fatty acid chains showed that the glycolipids have a greater affinity for binding to both bovine and human Mincle than trehalose, and there is also a direct

proportionality between lipid length and binding affinity.[7,45] This finding is consistent with the work of Khan *et al.* who synthesised a variety of trehalose glycolipids with native and non-native lipid chains and assessed their biological activity.[22] A series of linear trehalose diesters (TDEs) with lipids of four (**9**), seven (**10**), ten (**11**), eighteen (**12**), twenty (**13**), twenty two (**14**), and twenty six (**15**) carbons were prepared, (Figure 11) and it was found that a chain length of at least 18-20 carbons was required to stimulate the cytokine production by macrophages.[49] Furthermore, the most active of these diesters, TDB (trehalose dibehenate, **14**), was found to activate immune cells in a manner similar to TDM.[12,13] Additionally, mono-ester analogues of two of the most active trehalose diesters (**16** and **17**, with C_{22} and C_{26} lipids respectively) were synthesised and found to activate macrophages in a Mincle dependent manner.[50]

Diesters
9: *n* = 2
10: *n* = 5
11:*n* = 8
12: *n* = 16
13: *n* = 18
14: *n* = 20
15: *n* = 24

R=R′=

Monoesters
16: *n* = 20, R′=H
17:*n* = 24, R′=H

R=
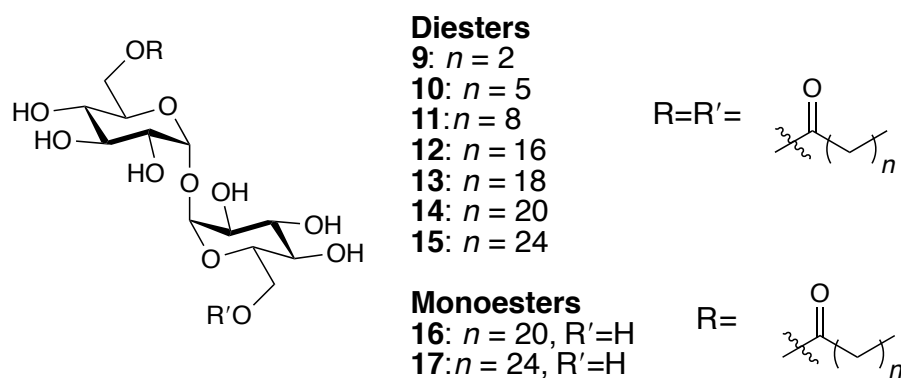
Figure 11: Linear TDEs reported in the literature

1.4.2.2 Other glycolipids

Richardson *et al.* investigated the structure-activity relationship of β-gentobiose **4** through truncation of the molecule.[31] First, the acyl groups on the glycerol were removed, followed by truncation of the entire glycerol group and replacement with a lipid. Both of the modifications abolished signaling through Mincle, assessed by NFAT-GFP reporter cells transfected with Mincle, indicating that the presence of both lipid and glycerol was required for Mincle activation. Next, the lipid structure was investigated through the esterification of a series of *iso*-branched and linear fatty acids to the glycerol moiety, and while all of the branched structures (**18a - c**) weakly activated mMincle, none activated hMincle (Figure 12). The longer chain linear compounds (**18d - e**) both activated mMincle and **18e** (C_{12} chain) also

activated hMincle, whilst the shorter linear lipids (**18f – g**) activated neither receptor. Next, the gentiobiose moiety was reduced to a glucose unit and two different lipids lengths were esterified to the glycerol (**19**). The branched compound **19a** activated both mMincle and hMincle, whereas linear C_{12} diester **19b** activated only mMincle. The trends evident from this study are that the truncation of disaccharide to monosaccharide is well tolerated, that medium length acyl chains are more active than short, and species differences in response exist.
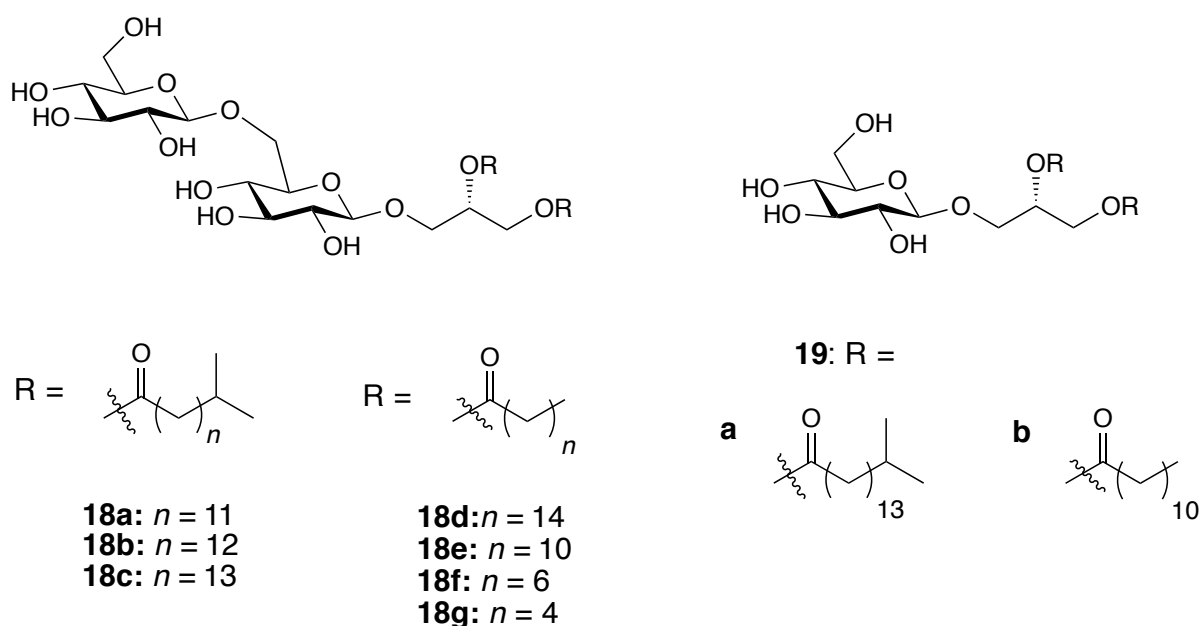
Figure 12: Truncated gentiobiose structures investigated as Mincle ligands

Continuing the truncation of gentiobiose **4**, a glucose monoester of enantiopure corynomycolic acid was synthesised, and it was found that the branched corynomycolate **20a** could effectively activate both human and mouse Mincle (Figure 13). In comparison, linear glucose behenate **20b** could not activate either receptor, which is in contrast to the trends evident for trehalose glycolipids, whereby trehalose dibehenate has similar activity levels to the mycolate and corynomycolate.[29] The glycerol portion of the gentiobiose natural product was also been studied by van der Peet *et al.* through the synthesis of monocorynomycolate **21a**. Glycerol monomycolate (GMCM) **21a** was shown to signal through human, but not mouse Mincle, and further, the *S*-isomer was found to be more active than the *R*-isomer.[29] In a separate study, the immunomodulatory activity of racemic glycerol monomycolate (GMM, **21b**) and behenate (GMB, **22**) was assessed, and both were found to signal through Mincle.[33]

GMM was shown to only activate hMincle and not mMincle, whilst GMB signaled very weakly through mMincle and strongly through hMincle.[33] Both studies indicate that drastic simplifications in both the lipid and carbohydrate portions of the naturally derived Mincle ligand can be performed whilst still retaining biological activity.
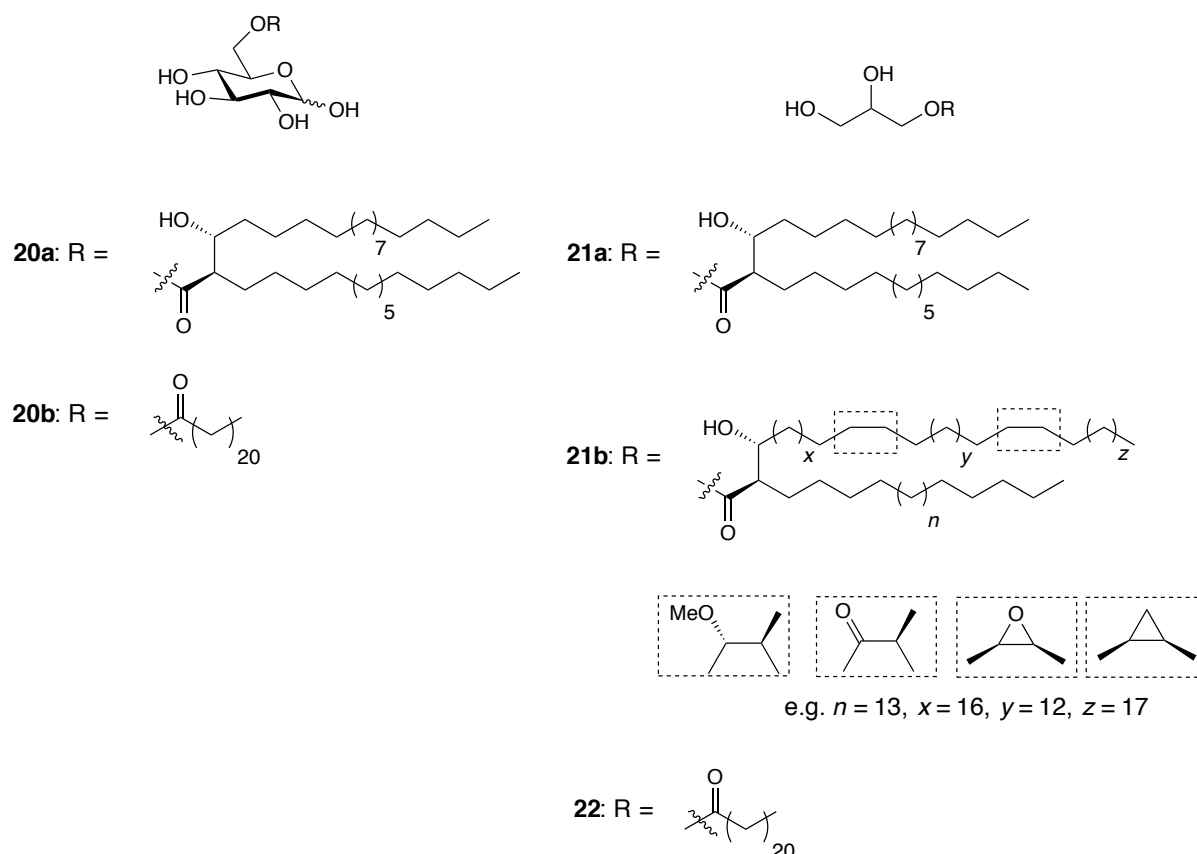
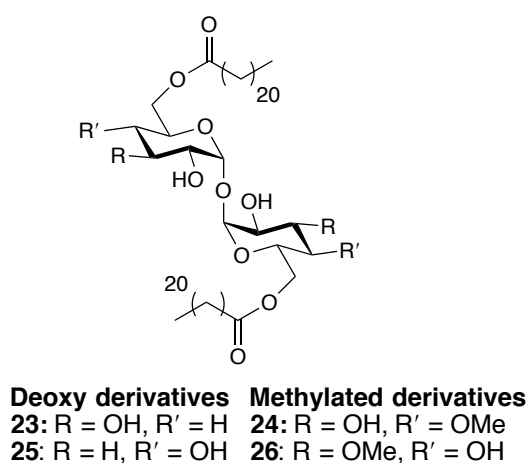**Figure 13:** Glucose and glycerol monoesters investigated as Mincle ligands

1.4.3   Proposed targets

Although there have been a number of studies published in the past few years looking at the structure-activity relationship of Mincle ligands, a number of gaps remain in understanding of how molecules bind to the active site of Mincle, and what structural features are required for biological activity. This thesis will take a two-pronged synthetic approach to furthering knowledge about the structure-activity relationship of Mincle ligands: studying the effect of modification to the carbohydrate structure of trehalose glycolipids; and the effect of *iso*-branching and lipid length on glycerolipids as compared to trehalose glycolipids.

1.4.3.1 Carbohydrate-modified trehalose glycolipids

Although several works have been published studying the effect of varying the lipid length of trehalose glycolipids, no sugar modified trehalose glycolipids have been reported. Given the potential importance of the sugar-active site interactions identified in the human and bovine Mincle crystal structure studies, it would be prudent to study how modification to the trehalose sugar influences macrophage activation.

Using trehalose dibehenate as a starting point, systematic modifications will be made to assess the relationship between the immunomodulatory activity of trehalose glycolipids and their carbohydrate structure. Since crystal structure studies of the receptor Mincle have revealed interactions between the 3- and 4-OH of the sugar and the CRD of the receptors, altering the functionality at these positions of the trehalose residue should be informative. To this end, a series of modified trehalose dibehenate targets are proposed (Figure 14), each with a symmetric modification to the 3- or 4-hydroxyls of the sugar residues. Both deoxygenation (**23** and **25**) and methylation (**24** and **26**) of the hydroxyls are anticipated to reduce the capacity of the glycolipid to participate in binding to the active site of the CRD of Mincle.

Deoxy derivatives **Methylated derivatives**
23: R = OH, R′ = H   **24:** R = OH, R′ = OMe
25: R = H, R′ = OH   **26**: R = OMe, R′ = OH

Figure 14: Target carbohydrate-modified trehalose glycolipids

1.4.3.2 Glycerolipids

The only glycerolipids reported in the context of Mincle activation have contained long chain fatty acids, with either linear lipids (behenate) or branched, functionalised lipids (corynomycolate and mycolate). Given the dependence of lipid length on the biological activity of the trehalose glycolipids, it seems prudent to investigate the biological activity of glycerol monoesters of shorter chain lipids. Additionally, the synthesis of glycerol mono-esters with *iso*-branched fatty acids would help complete the study on the structure-activity relationship of truncated gentiobiose Mincle ligands. To this end, two complementary series of glycerolipids have been proposed, each chain containing 14 - 22 carbons long (Figure 15). The linear series will comprise glycerol mono-tetradecanoate (**27**), hexadecanoate **28**, octadecanoate **29** and docosonoate **22**, with the last acting as a positive control, given its activity has already been established. The branched series will contain *iso*-branched analogues of the shorter three linear glycerolipids, explicitly glycerol mono-(13-methyltetradecanoate) (**30**), 15-methylhexadecanoate **31**, and 17-methyloctadecanoate **32**. These lipids were chosen due to their inclusion in or similarity to those included in the *M. tuberculosis* derived gentiobiose ligands found to activate Mincle.[10]

27: $n = 12$
28: $n = 14$
29: $n = 16$
22: $n = 20$

30: $n = 11$
31: $n = 13$
32: $n = 15$
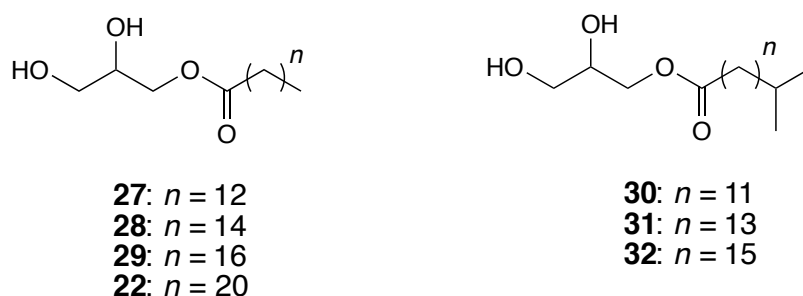
Figure 15: Straight- and branched-chain glycerol monoester targets

Although it has been shown by van der Peet *et al.* that the *S*-isomer of glycerol monocorynomycolate was more active then the *R*-isomer,[29] for the sake of synthetic ease the racemic forms of the above targets will be synthesised. If preliminary results proved interesting, each isomer could be individually prepared through the use of an enantiopure glycerol starting material.

In order to use as a point of comparison to the previously unstudied glycerol monoesters in biological assays, the corresponding linear and branched esters of trehalose will also be synthesised (Figure 16). Most of these linear TDEs have not previously been investigated, although they fall within the range of lipid lengths investigated by Khan *et al.*[49] The *iso*-branched targets containing a total of 15, 17, and 19 carbons (**35 - 57**) are in fact simplified analogues of another group of trehalose glycolipids isolated from the nematode *C. elegans,* called maradolipids.[51] These compounds are 6,6′-*O*-diesters of trehalose, with lipids generally comprising one unsaturated and one *iso*-branched fatty acid, of about 14 – 18 carbons in length, and have not been previously tested for immunomodulatory activity.

33: *n* = 12
34: *n* = 14
12: *n* = 16
14: *n* = 20

35: *n* = 11
36: *n* = 13
37: *n* = 15
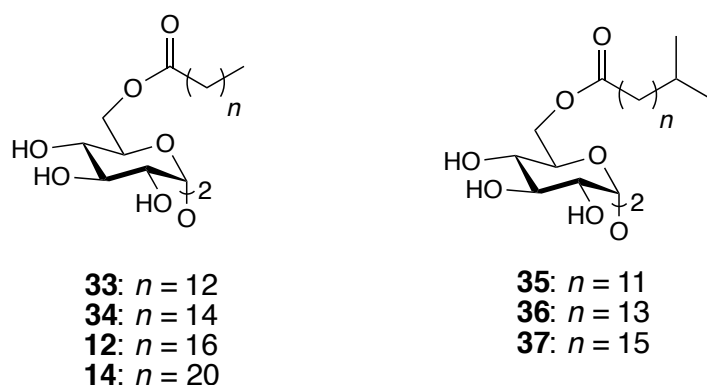
Figure 16: Linear and branched trehalose diester targets

Previous work in the field of maradolipid synthesis has generated a number of symmetric and asymmetric trehalose diesters. Oleic acid, 9,10-cyclopropyloleic acid, tetradecanoic acid, hexadecanoic acid, 13-methyltetradecanoic acid, and 15-methylhexadecanoic acid, have all been esterified at the primary positions by means of enzymatic coupling,[52] protecting-group mediated coupling,[53,54] or protecting-group-free coupling methods (Figure 17).[55]
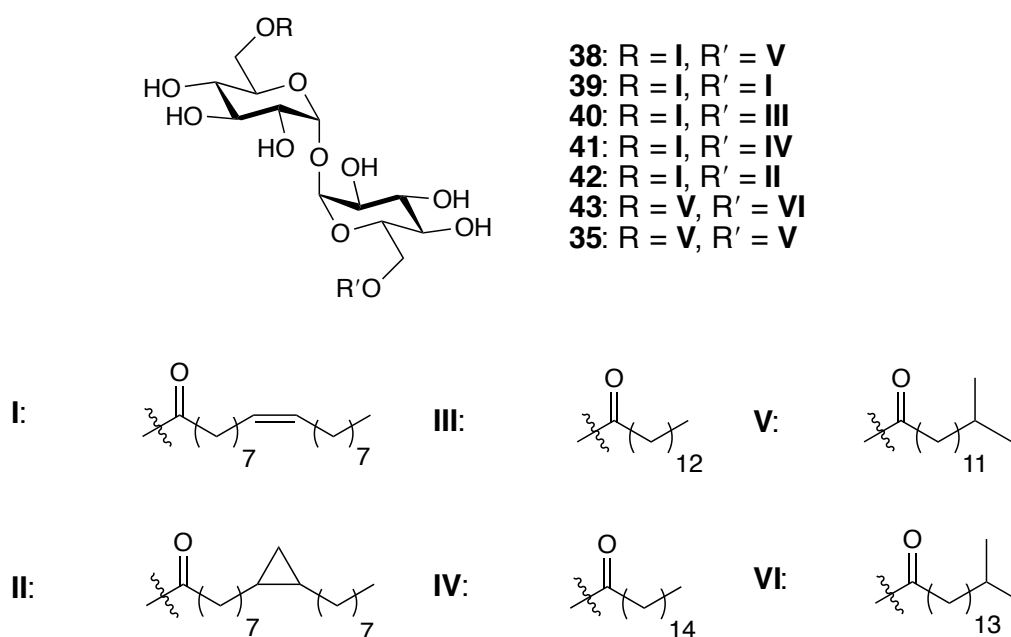
Figure 17: Previously synthesised maradolipids

1.5 Retrosynthetic analysis

1.5.1 Carbohydrate-modified trehalose glycolipids

The modifications planned for the trehalose core of TDB are deoxygenation and methylation, both of which are anticipated to alter the capacity of the sugar to participate in the binding with the CRD of Mincle, by either eliminating or masking the polar hydroxyl group on the 3- or 4-position of trehalose. For each target, the appropriate hydroxyl on the sugar must be chemically distinguished from the other secondary alcohols present in the molecule, which may prove challenging.[56] The overall retrosynthetic analysis is described in Scheme 1. Here, the target glycolipids **23 - 26** can be prepared from the esterification of suitably functionalised trehalose core (**45 - 48**) and behenic acid (**44**) through Steglich esterification.[57] The trehalose core is, in turn, prepared from α,α′-D-trehalose using a series of selective, orthogonal protecting group manipulations.
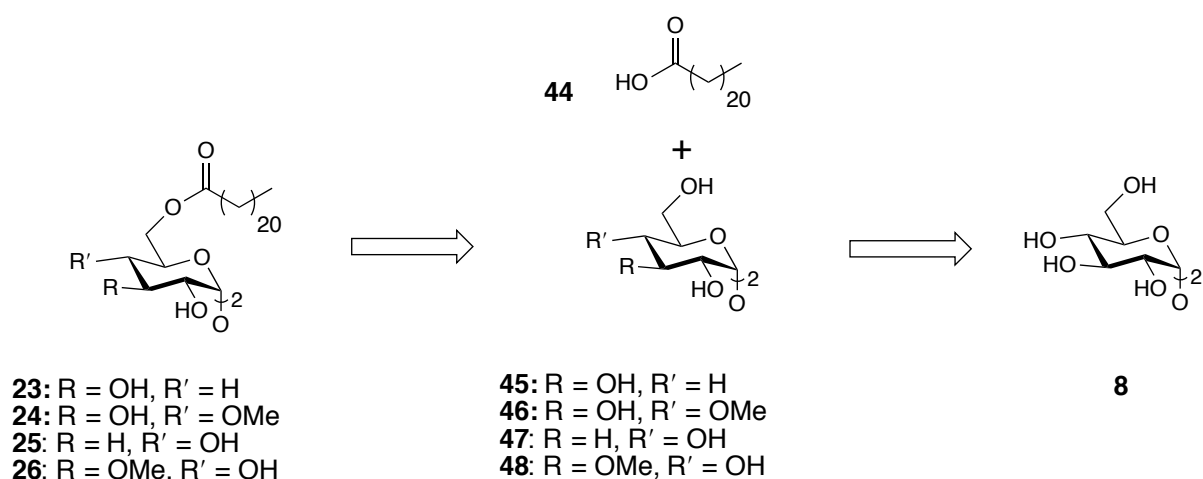
Scheme 1: Retrosynthetic analysis of trehalose esterification

Commercially available α,α′-D-trehalose will be used as the starting carbohydrate for all targets, as it contains all of the desired stereochemistry. It is also possible to construct synthetic trehalose analogues using two glucose monomers, but as the synthetic formation of the α,α′ glycosidic linkage is challenging,[56] this approach will not be used. Recently, an enzymatic process for the formation of α,α′-D-trehalose analogues from glucose-UDP and a modified glucose unit has been reported,[58] hence if the planned modifications to trehalose prove unfeasible, then this approach could also be explored.

1.5.1.1 Modifications to the 4-position

As shown in Scheme 2, trimethylsilyl (TMS) protected targets **49** and **50** can be prepared from the corresponding 4-modified sugars **45** and **46** through protection of the 2-, 3-, and 6-hydroxyls with bis(trimethylsilyl)acetamide (BSA) then selective deprotection of the more labile primary silyl ethers.[49,59,60] Carbohydrates **45** and **46** can be prepared from hexa-benzylated intermediate **51** by modification to the 4-position (deoxygenation or methylation), then hydrogenolytic benzyl deprotection.[56] 2,3,6,2′,3′,6′-Hexa-O-benzyl trehalose (**51**) can be made from the key building block benzylidene trehalose **52** via benzylation of the 2- and 3-hydroxyls and reductive regioselective opening of the benzylidene protecting group to install the benzyl at the 6-position.[56,61] Finally, 4,6,4′,6′-di-O-benzylidene trehalose (**52**) can be prepared from α,α′-D-trehalose (**8**) by protection of the 4- and 6-hydroxyls with benzylidene groups.[53,62-67]
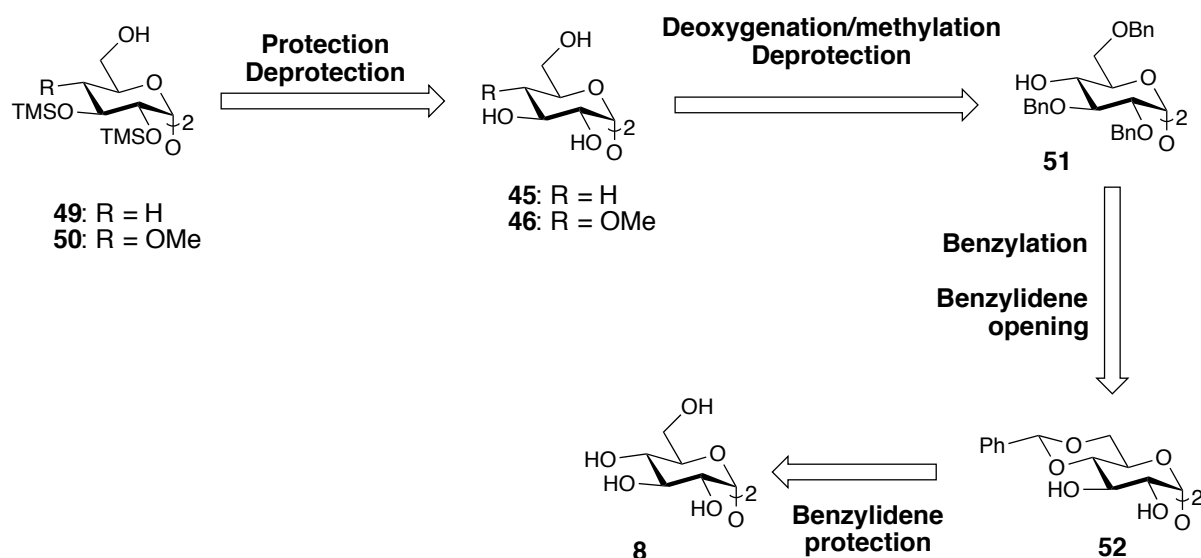
Scheme 2: Retrosynthetic analysis of modification to the 4-position of trehalose

1.5.1.2 Modifications to the 3-position

TMS protected targets modified at the 3-position (**49** and **50**) can be prepared from the corresponding unprotected modified sugars **47** and **48** through protection of all available hydroxyls with BSA then selective deprotection of the primary positions (Scheme 3). In turn, 3-modified trehaloses **47** and **48** can be made from 2,2′-di-*O*-benzoyl trehalose **55** by either deoxygenation or methylation,[56,68] followed by global deprotection. 2,4,6 protected trehalose **55** can be prepared from 4,6,4′,6′-di-*O*-benzylidene trehalose **52**,[56] which can be made from α,α′-D-trehalose (**8**) by protection of the 4- and 6-hydroxyls with benzylidene groups.
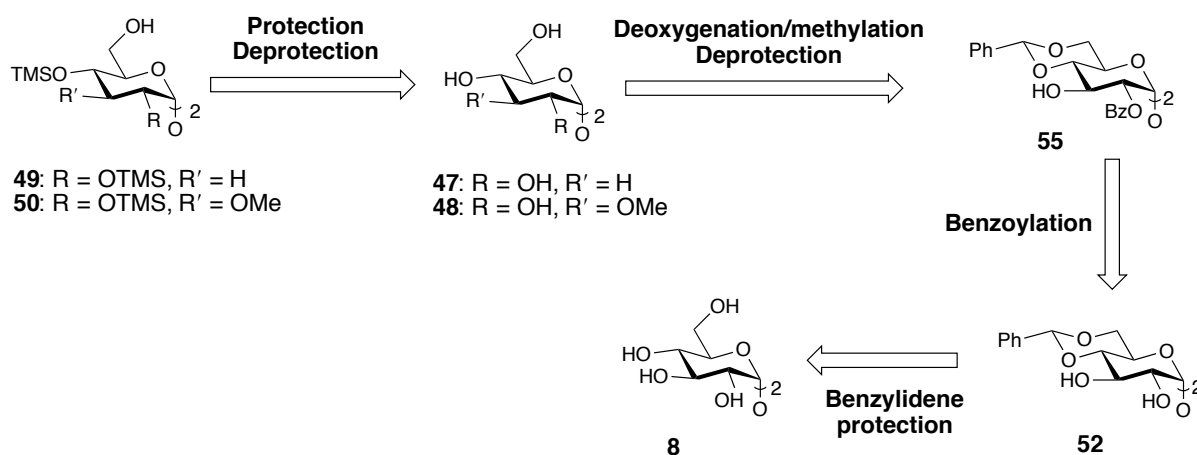
Scheme 3: Retrosynthetic analysis of modification to the 3-positions of trehalose

1.5.2 Glycerolipids

Both linear (**27 – 29**, **22**) and branched (**30 – 32**) glycerol monoesters can be prepared from commercially available 1,2-*O*-isopropylidene glycerol **56** and the appropriate linear (**57 – 59**, **44**) or branched (**61 – 62**; retrosynthesis *vide infra*) carboxylic acid through Steglich esterification using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDCI) and 4-dimethlyamino pyridine (DMAP).[57,69-75] followed by deprotection using acetic acid (Scheme 4).[70,76]

27: $n = 12$	**56**	**57**: $n = 12$
28: $n = 14$		**58**: $n = 14$
29: $n = 16$		**59**: $n = 16$
22: $n = 20$		**44**: $n = 20$

30: $n = 11$	**56**	**60**: $n = 11$
31: $n = 13$		**61**: $n = 13$
32: $n = 15$		**62**: $n = 15$
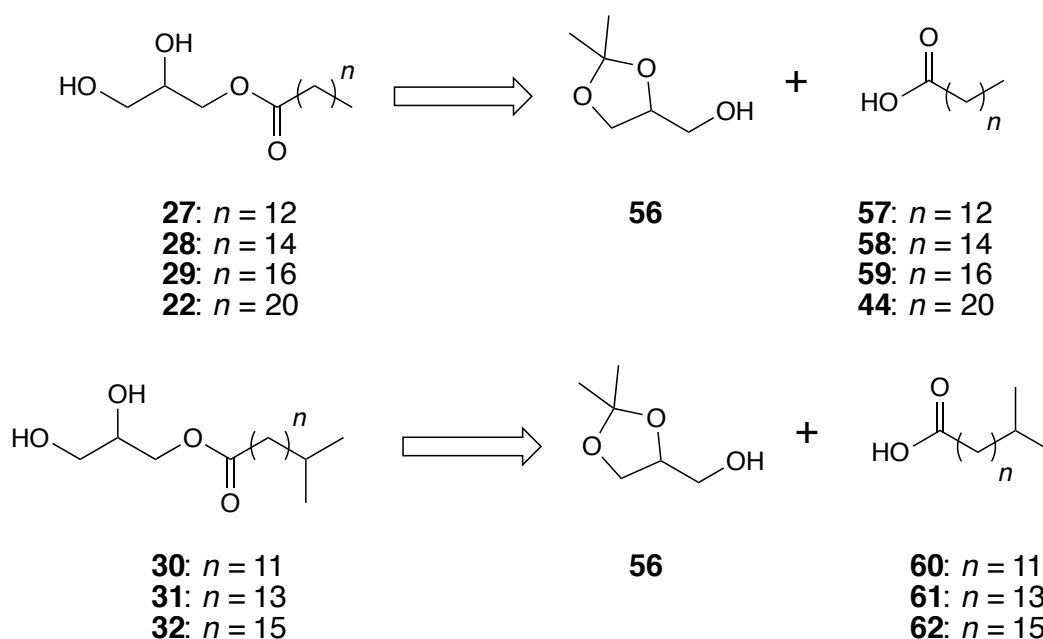
Scheme 4: Retrosynthetic analysis of glycerolipid esterification

1.5.3 Maradolipids

The maradolipid analogues of the glycerolipid targets described above can be prepared in an identical fashion to those of the other trehalose glycolipids previously discussed (Scheme 5). α,α'-D-trehalose can be TMS protected at the secondary hydroxyls, then esterified to the appropriate linear (**57 - 59**, **44**) or branched (**61 - 62**; retrosynthesis *vide infra*) carboxylic acid using EDCI and DMAP, then the protected glycolipid deprotected using Dowex/H$^+$.[49,60]

33: $n = 12$
34: $n = 14$
12: $n = 16$
14: $n = 20$

8

57: $n = 12$
58: $n = 14$
59: $n = 16$
44: $n = 20$

35: $n = 11$
36: $n = 13$
37: $n = 15$

8

60: $n = 11$
61: $n = 13$
62: $n = 15$
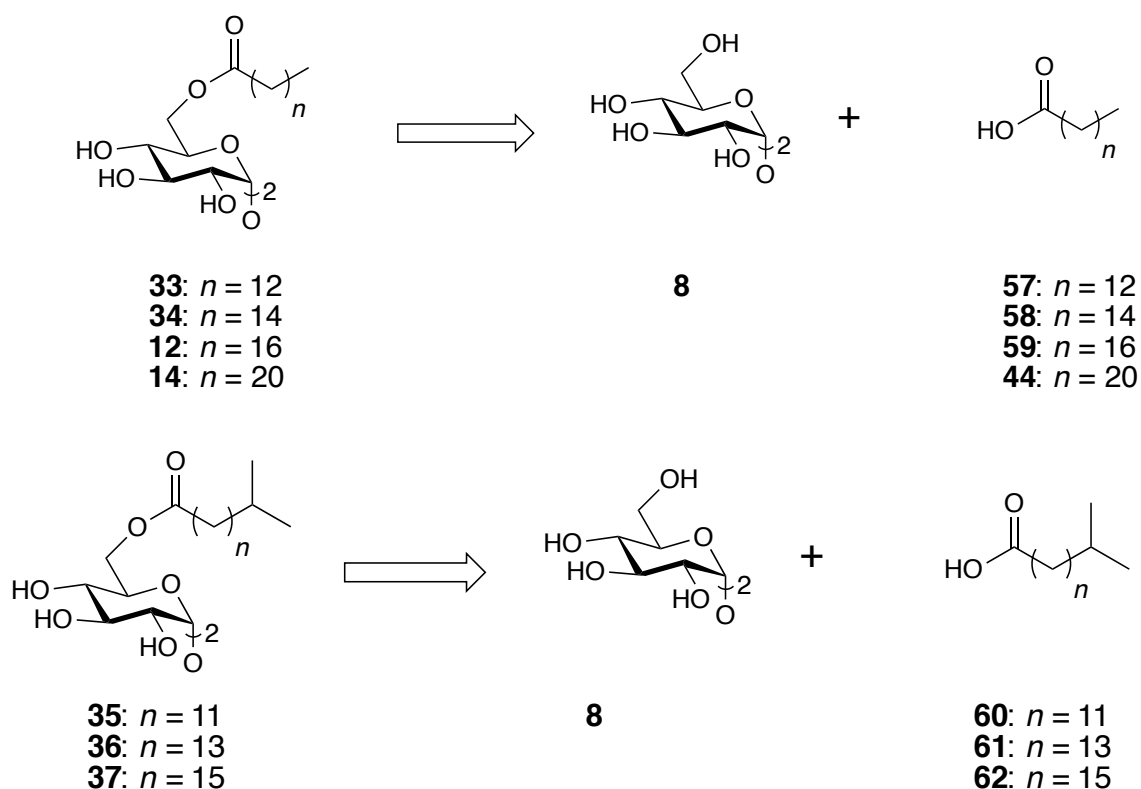
Scheme 5: Retrosynthetic analysis of maradolipid esterification

1.5.4 Iso-branched lipids

None of the *iso*-branched lipids required for this project (**60 - 62**) were practicable to buy from commercial sources, hence required synthesis from reasonably priced starting materials. The linear synthesis of C$_{16+1}$, C$_{15+1}$, and C$_{14+1}$ acids from 15-cyclopentadecanolide has recently been published, and this methodology will be used for this project.[77] To this end, 13-methyltetradecanoic acid (**60**) can be prepared from 14-methylpentadec-1-ene (**63**) through

permanganate oxidation (Scheme 6). Alkene **63** can in turn be made from 14-methylpentadecanoic acid (**64**) by reduction of the carboxylic acid to an alcohol, formation of a monothiocarbonate and elimination of the terminal leaving group. 15-Methylhexadecanoic acid (**61**) can be prepared from 15-methylhexadec-1-ene (**65**) by permanganate oxidation as for the longer-chain analogue **60**, and alkene **65** in turn can be made from 15-methylhexadecan-1-ol (**66**) by thiocarbonate formation and reduction. Alcohol **66** can be prepared from 15-cyclopentadecanolide (**67**) by Grignard addition of two methyl groups, then selective reduction of the tertiary alcohol initially formed. Finally, 15-methylhexadecanoic acid **61** can be made from alkene **66** by permanganate oxidation.
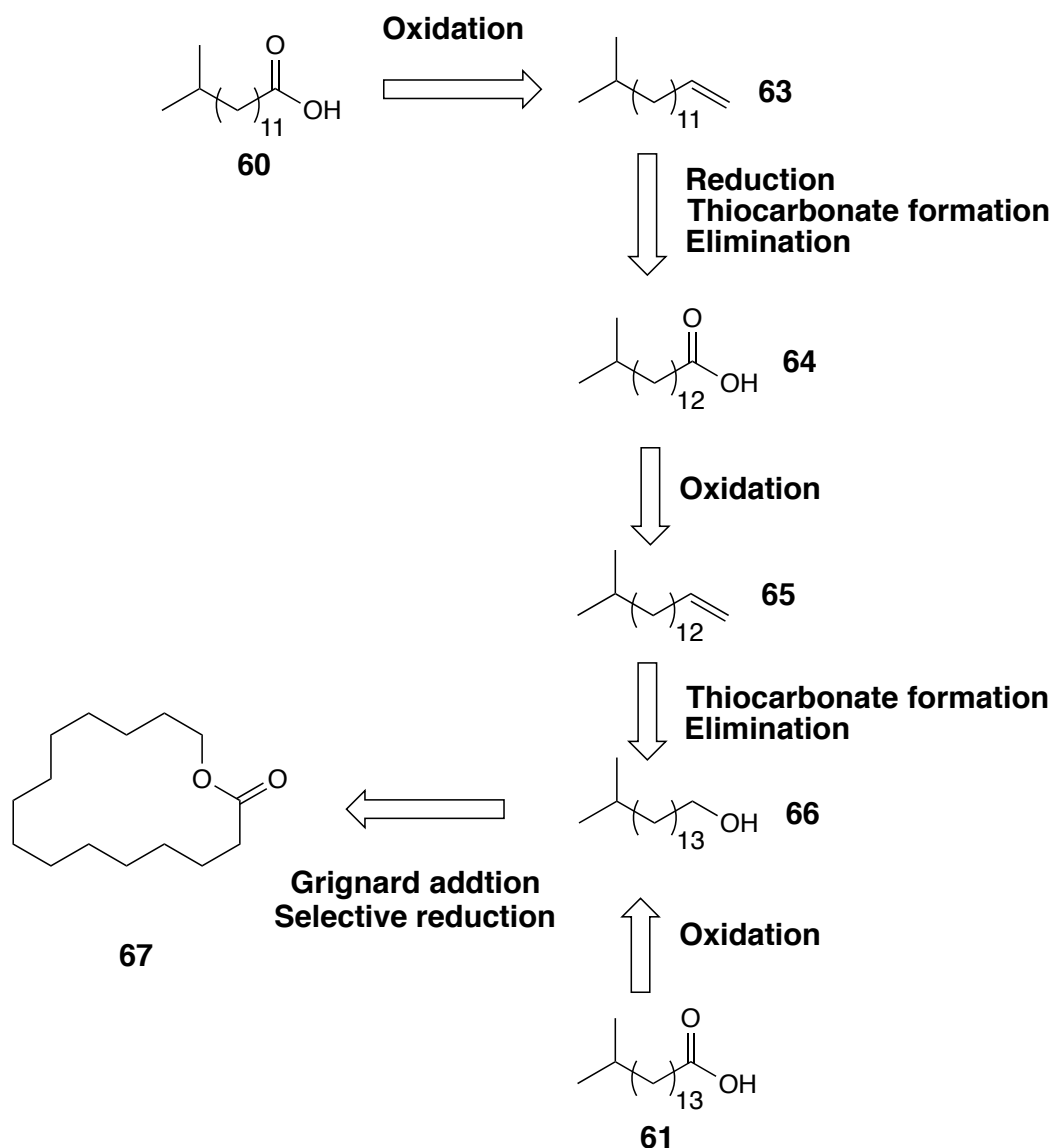
Scheme 6: Retrosynthetic analysis of Grignard-based iso-lipid synthesis

In order to synthesise 17-methyloctadecanoic acid (**62**), 15-cyclopentadecanolide (**67**) can once again be used as a starting material. The C_{18+1} acid can be prepared from isobutyraldehyde (**69**) and 15-bromopentadecanoic acid (**68**) through Wittig reaction (Scheme 7).[78] Carboxylic acid **68** can in turn be made from lactone **67** through ring-opening bromination of the lactone.[79]
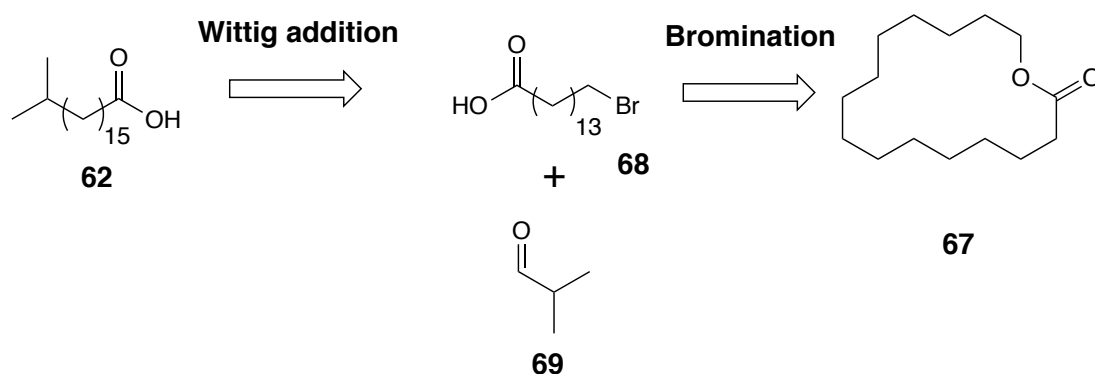
Scheme 7: Retrosynthetic analysis of Wittig-based iso-lipid synthesis

1.6   Biological testing

To assess the impact of the structural modifications made to the naturally occurring ligands on Mincle activation, biological assays will be carried out on the target compounds produced. Murine bone marrow derived macrophages (BMMs), which express mMincle on their cell surface, will be used to assess the activity of the carbohydrate-modified TDEs. The use of a mouse model for human Mincle activity using trehalose glycolipids has been shown to be valid, with a large degree of homology in gene sequence and closely-paralleled response profiles to short-chain TDEs.[80] BMMs will be produced from bone marrow cells by differentiation using granulocyte-macrophage colony-stimulating factor (GM-CSF).[81] On stimulation with a Mincle ligand such as TDB, BMMs produce a variety of cytokines and cellular mediators, measurable using an enzyme-linked immunosorbent assay (ELISA), which operates on the basis of antibody recognition of the cytokine of interest (i.e. TNF-α, IL-6, and IL-1β).[1] In contrast, BMMs that do not express Mincle (Mincle^{-/-}) will produce no measurable response when stimulated with TDB, and hence can be used to verify that the ligand was stimulating the immune system by binding to Mincle. Given the difference in activity for the glycerol monoesters between mMincle and hMincle reported in literature, a

different approach will need to be taken for the glycerolipid and maradolipid targets. Initially, the compounds produced will be tested against murine BMMs, as the glycerol monoesters in literature were only tested against NFAT-GFP reporter cell lines expressing mMincle. This assay will eliminate the possibility of differences between natural macrophages and transfectant cell lines, which do not adequately represent the *in vivo* macrophage given the lack of any other native proteins or receptors. Lastly, immunomodulatory activity in mouse and human macrophages will be compared in order to assess the response difference between the species'. Mincle$^{-/-}$ cells will be used to confirm that any biological activity detected is as a result of activation of Mincle, as opposed to a different receptor.

2 Results and Discussion

2.1 Synthesis of carbohydrate-modified trehalose glycolipids

2.1.1 Synthesis of TDB

Before commencing studies on the effect of modification to the 3- and 4-positions of trehalose glycolipids on their biological activity, the synthesis of TDB was first undertaken to establish the methodology that would be used for esterification of sugar to lipid (Scheme 8).[49] Initially, per-silylation of trehalose was performed, using BSA and catalytic tetrabutylammonium fluoride (TBAF) to introduce the TMS protecting groups. Mildly basic conditions were used to selectively remove the more labile primary TMS group, affording hexa-TMS trehalose **70** in a 58% yield over two steps.[59,60,82] Spectroscopic analysis of the deprotection reaction confirmed that it had proceeded with the desired selectivity, whereby the data corresponded to those published.[49]
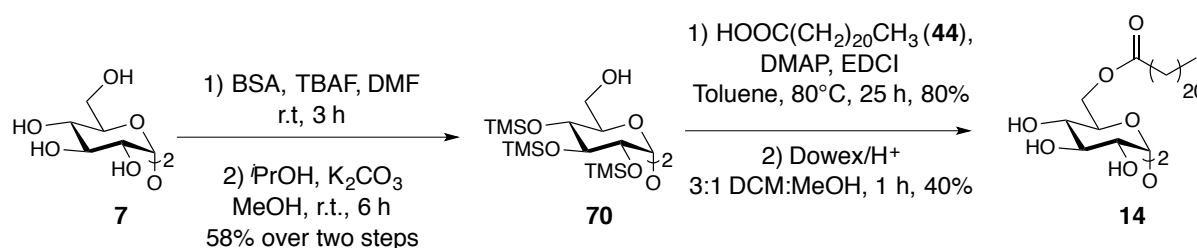
Scheme 8: Synthesis of TDB

Next, esterification of hexa-TMS trehalose **70** to behenic acid (**44**) was carried out using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDCI) and DMAP as promoters.[49] The mechanism for the coupling is shown below (Scheme 9). Here, DMAP (**I**) deprotonates the carboxylic acid **II** to give carboxylate **III**, which then attacks the electrophilic carbon center of EDCI **IV**. This condensation gives anion **V**, which is protonated by an equivalent of the conjugate acid of DMAP (**VI**). The carbodiimide functionality increases the electrophilicity, and accordingly nucleophilic attack on **VII** by the alcohol **VIII** occurs.[57] This reaction leads to the formation of a tetrahedral intermediate (**X**), which collapses to give the desired ester

XI and the leaving group **XII**. Finally, anion **XII** is protonated by a further equivalent of cationic **VI** to give diamide **XIII** and regenerate the catalytic DMAP.
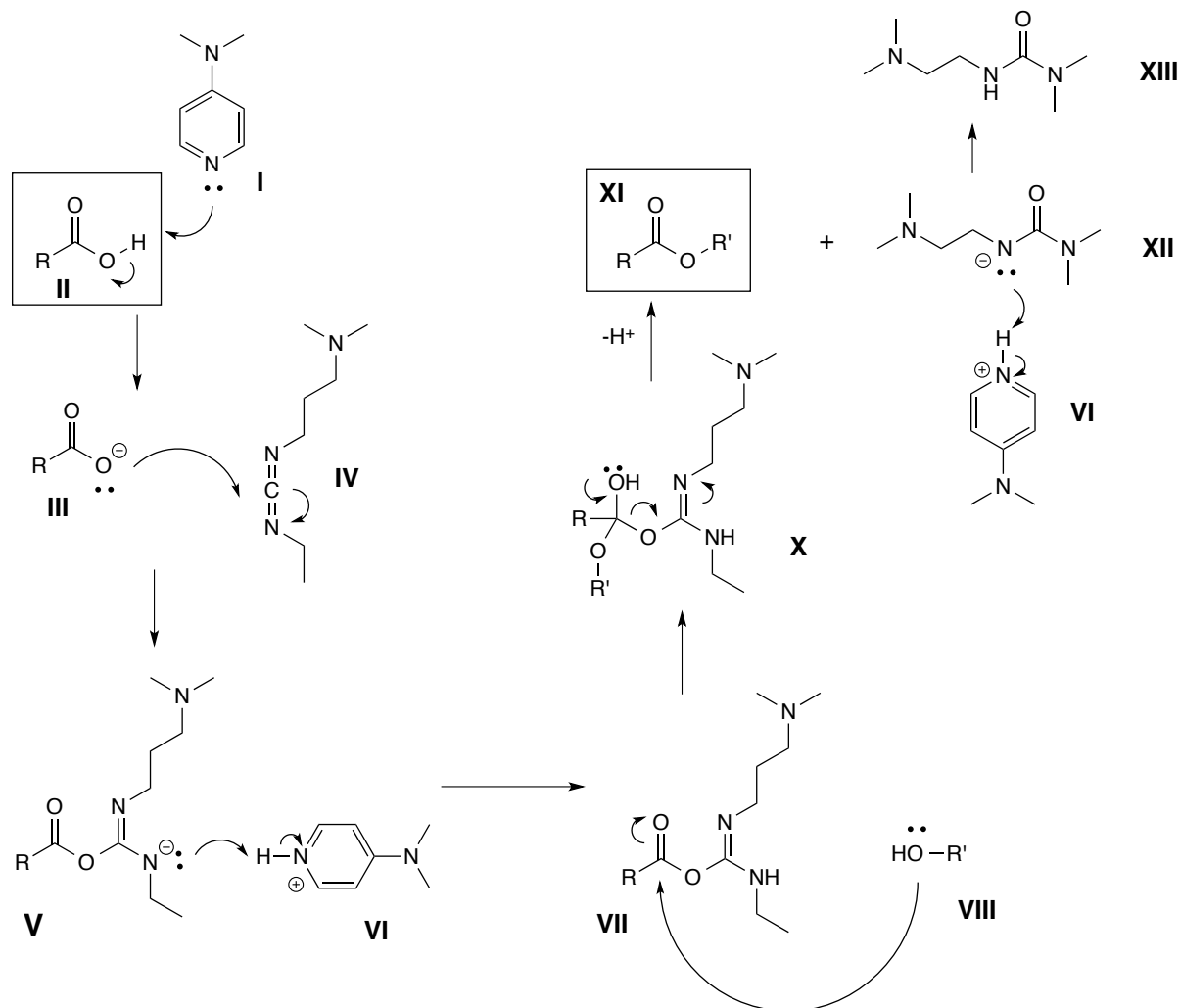
Scheme 9: Mechanism of EDCI and DMAP promoted esterification

The diester **14** was isolated in a good (80%) yield, then finally deprotection of the TMS groups was performed, yielding TDB in a moderate yield. The final product was purified by silica gel flash chromatography (1:0 - 94:6 EA/MeOH, *v/v*) though it was found that heating of the column was required to ensure dissolution of the crude product and hence elution.

The formation of TDB was confirmed through analysis of NMR and MS spectra, whereby data obtained corresponded to reported literature for this known compound.[49] Only one set of

sugar protons were observed in the ^{1}H NMR spectrum, indicating a symmetric sugar, which, in combination with the integration of the lipid peaks, suggested the presence of a diester. Heteronuclear multiple-bond correlation spectroscopy (HMBC) analysis showed correlations between the carbonyl of the ester and both the lipid chain and the 6-position of the sugar, confirming that the ester linkage has indeed been formed (Figure 18). Lastly, high-resolution mass spectrometry (HRMS) confirmed the mass of the product, demonstrating that TDB (**6**) had been successfully synthesised and isolated (*m/z* calculated for $[C_{56}H_{107}O_{13} + NH_4]^+$: 1004.7972, found 1004.7972).
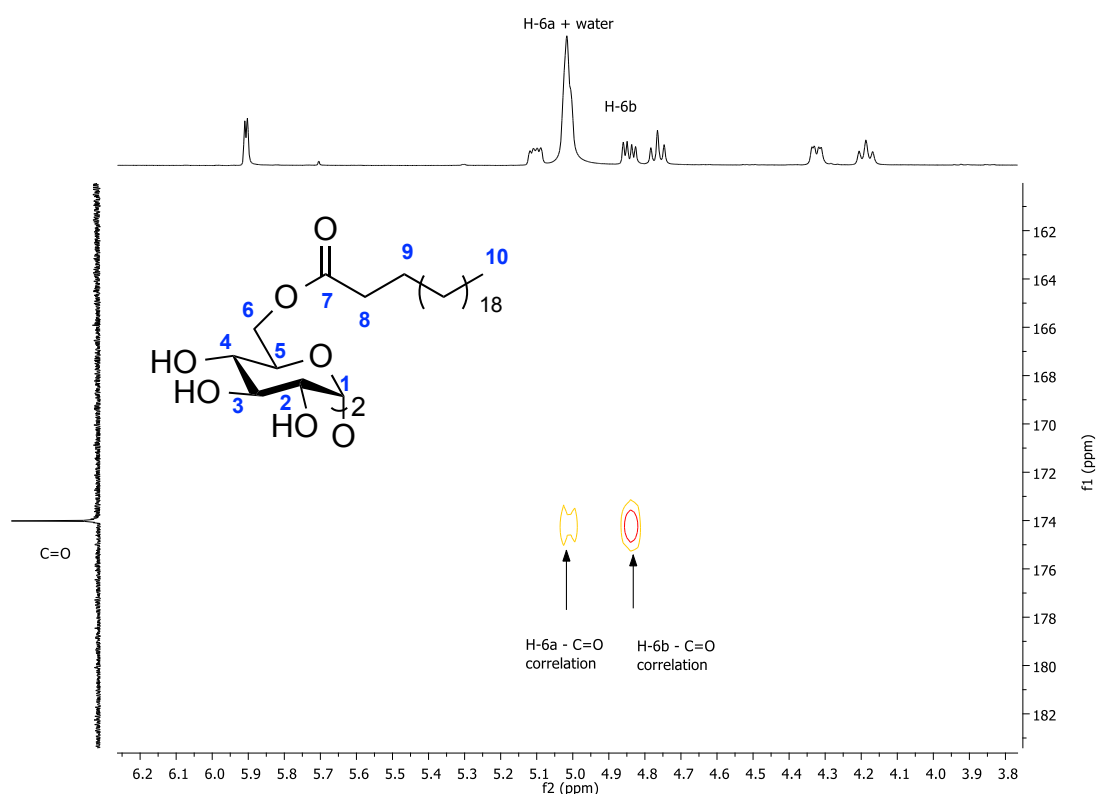
Figure 18: Carbonyl region of the HMBC spectra for diester **14**

2.1.2 Synthesis of 4-modified TDBs

2.1.2.1 Synthesis of 2,2′,3,3′,6,6′-O-hexabenzyl-α,α′-D-trehalose

Having successfully prepared TDB, efforts were then made to prepare the 4,4′-modified derivatives. In order to chemically distinguish the 4-position of trehalose from the other secondary alcohols, a three-step strategy was proposed (Scheme 10). Here, the 4,4′- and 6,6′-position of α,α′-D-trehalose (**8**) were first protected with a benzylidene group in excellent yield (97%) to give benzylidene-protected **52**.[53,62,64,66,67]
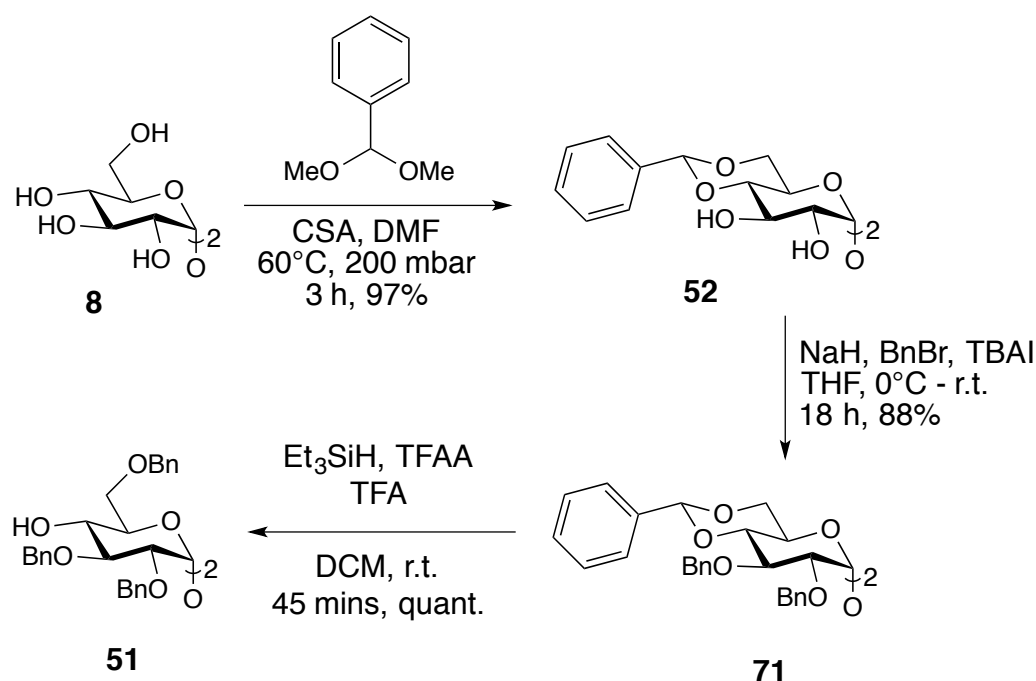
Scheme 10: Synthesis of 2,2′,3,3′,6,6′-O-hexabenzyl-α,α′-D-trehalose **51**

Next, benzylidene protected **52** was benzylated in excellent yield (88%), using sodium hydride and tetrabutylammonium iodide (TBAI) to increase reactivity.[56,63] Sodium hydride deprotonates the free hydroxyls of tetraol **52**, whilst TBAI replaces the bromine on benzyl bromide with an iodide, increasing the electrophilicity. The last step in the synthesis was the regioselective opening of the benzylidene acetal to give 2,2′,3,3′,6,6′-hexa-O-benzyl **51**, with the 4- and 4′-hydroxyls as the sole unprotected hydroxyl on the sugar. The ring-opening conditions involving the use of triethylsilane (SiEt$_3$H) and trifluoroacetic acid (TFA) have

been previously reported for a variety of simple[83,84] and complex benzylidene-protected glucosides,[85,86] but not trehalose. Only two methods for the elective opening of a 4,6-benzylidene acetal have been reported for trehalose.[56,61] Wessel *et al.* employed a two-step procedure, that first involved forming a tin acetal with the 4,4′- and 6,6′-oxygens, then selectively benzylating the 6-position using benzyl bromide (BnBr) and tetrabutylammonium bromide (Bu$_4$NBr), which gave the desired product in a 83% yield, albeit requiring 57 hours of heating at 80°C. Lin, van Halbeek and Bertozzi used a different procedure, but also struggled with the sluggishness of the reaction. Here, the benzylidene acetal was directly opened, employing sodium cyanoborohydride as the reductant in combination with triflic acid to promote ring opening. The authors could not drive the reaction to completion, and the yield was reported over two steps with the next step (thiocarbonyl formation) yielding only 9% for the desired product. In contrast to these reported methods, using SiEtH$_3$ in combination with TFA and trifluoroacetic anhydride (TFAA) lead to complete reaction in under an hour, furnishing the desired product **51** in quantitative (quant.) yield.

The regioselectivity that leads to the exclusive formation of the benzyl ether group on the 6-position can be explained by examining the mechanism of benzylidene acetal opening.[87,88] As shown in Scheme 11, the first step is protonation of the 4-O, which is more favourable than protonation at the 6-O due to greater inductive stabilisation of the secondary position. Once cation **I** has been formed, the acetal collapses to form oxocarbenium **II**. The final step is hydride reduction of **II** to give 6,6′-*O*-benzyl **51**.
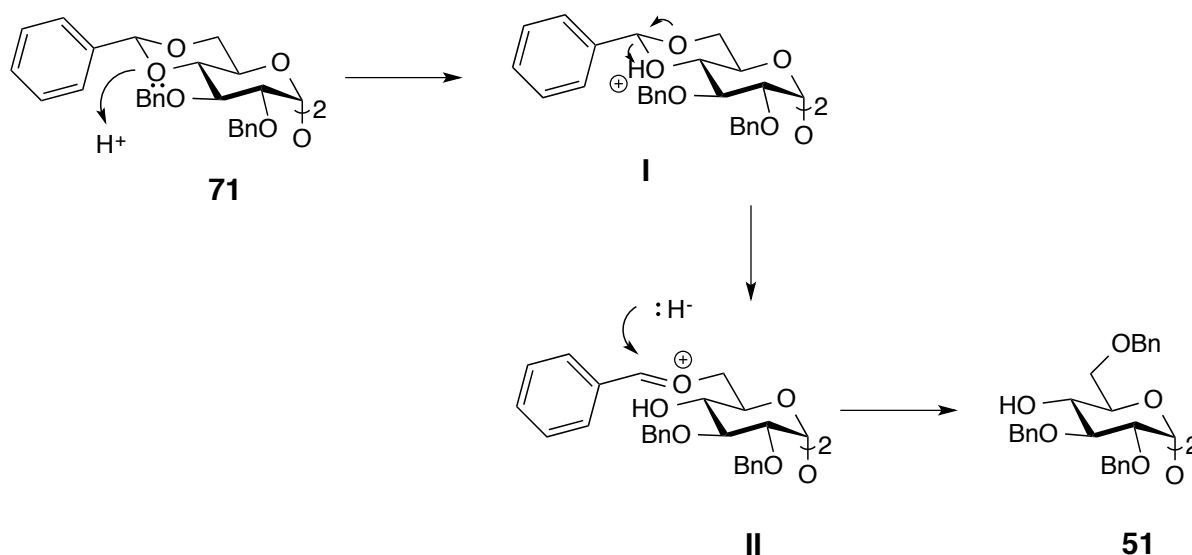
Scheme 11: Mechanism of regioselective benzylidene opening[87]

The transformation of fully protected **71** to 2,2′,3,3′,6,6′-benzyl **51** was shown to proceed with the desired selectivity through analysis of the HMBC spectrum, where correlations between the benzyl methylene protons and the 2, 3, and 6 protons were observed (Figure 19).
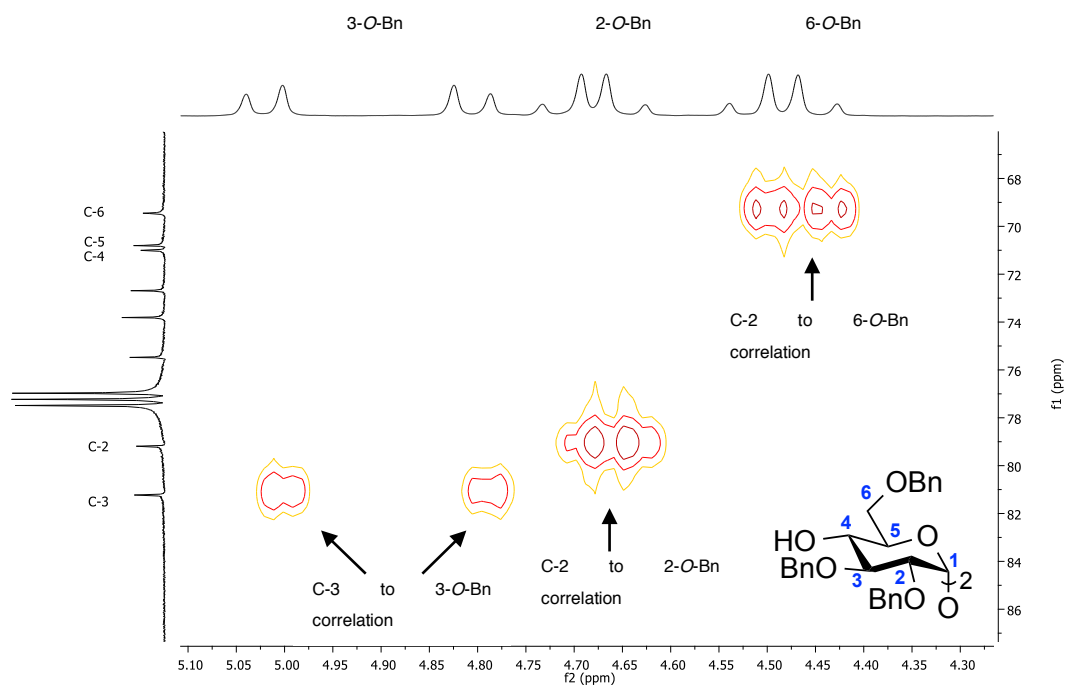
Figure 19: HMBC spectrum of 2,3,6-*O*-Bn trehalose **51**

2.1.2.2 Synthesis of 4-deoxy TDB

With hexabenzylated trehalose **51** in hand, Barton-McCombie deoxygenation was carried out at the 4-position. The Barton-McCombie deoxygenation is a two-step method that produces an alkyl group from an alcohol via a thiocarbonyl intermediate, and has previously been used for the selective modification of trehalose at the 2-, 3-, and 4-positions.[56,89] This reaction first proceeds through attack of the alcohol (**I**) onto the nucleophilic carbon of a thiocarbonyl reagent (**II**) such as carbon disulfide, generating a xanthate intermediate (**IV**) via a tetrahedral intermediate (**III**, Scheme 12).[89] In the second step, radical tin based chemistry is used to substitute the xanthate for a hydrogen atom. First, AIBN (azobisisobutyronitrile, **V**) undergoes homolytic cleavage to form radical nitrile **VI**, which then abstracts an H• from tributyltin hydride (**VII**), generating tin radical **VIII**. Xanthate **IV** can then react with **VIII** to give tin-xanthate adduct **IX** in addition to an equivalent of alkyl radical **X**. Finally, radical **X** abstracts a H• from a second equivalent of SnBu$_3$H (**VII**), forming tin radical **VIII** and the desired alkyl product **XI** (Scheme 12).
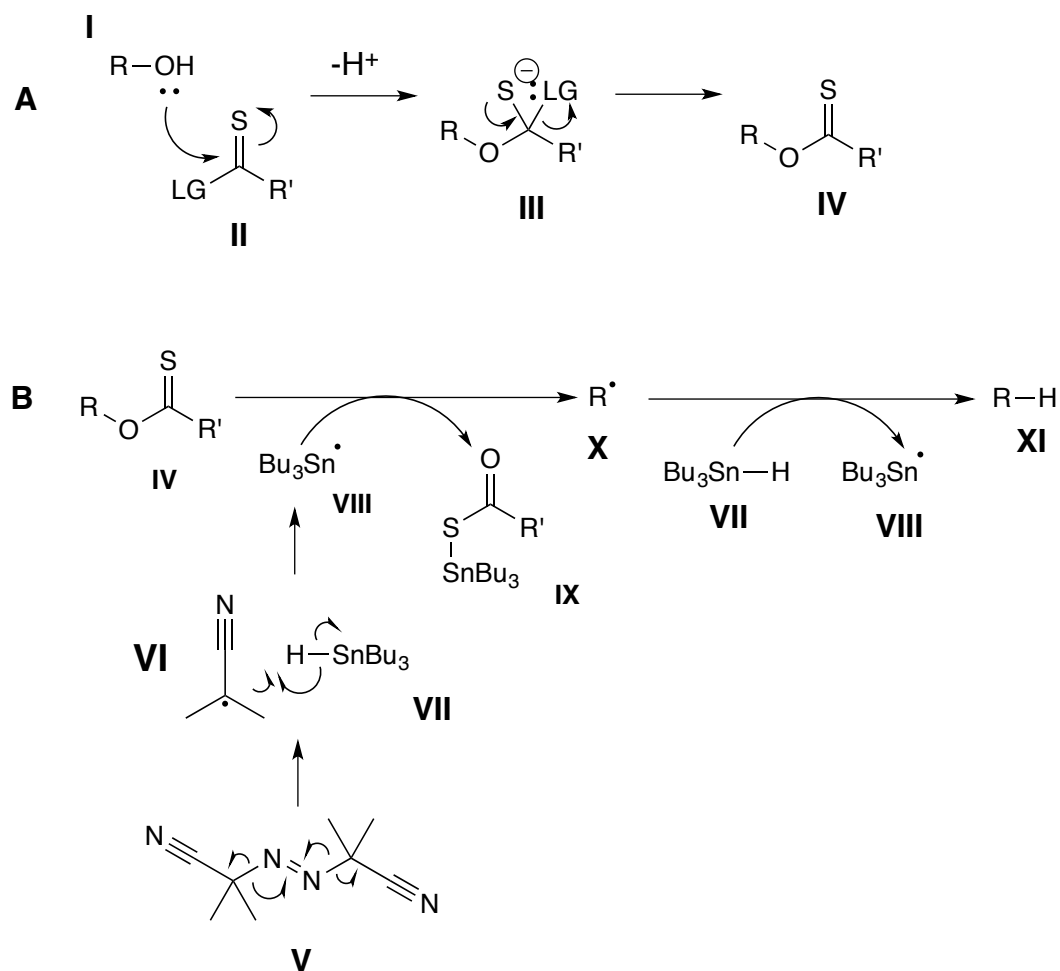
Scheme 12: Mechanism of Barton-McCombie deoxygenation. **A:** Xanthate formation, **B:** Radical reduction[89]

In order to form the *S*-methylxanthate moeity, each hydroxyl was first deprotonated by sodium hydride, then the alkoxide can nucleophically attacked carbon disulfide (Scheme 13). Lastly, methyl iodide was added, which acts as an electrophilic trap for the singly bonded sulfur of the xanthate. Upon reaction of both hydroxyls in diol **51**, xanthate **72** was formed in good (85%) yield.
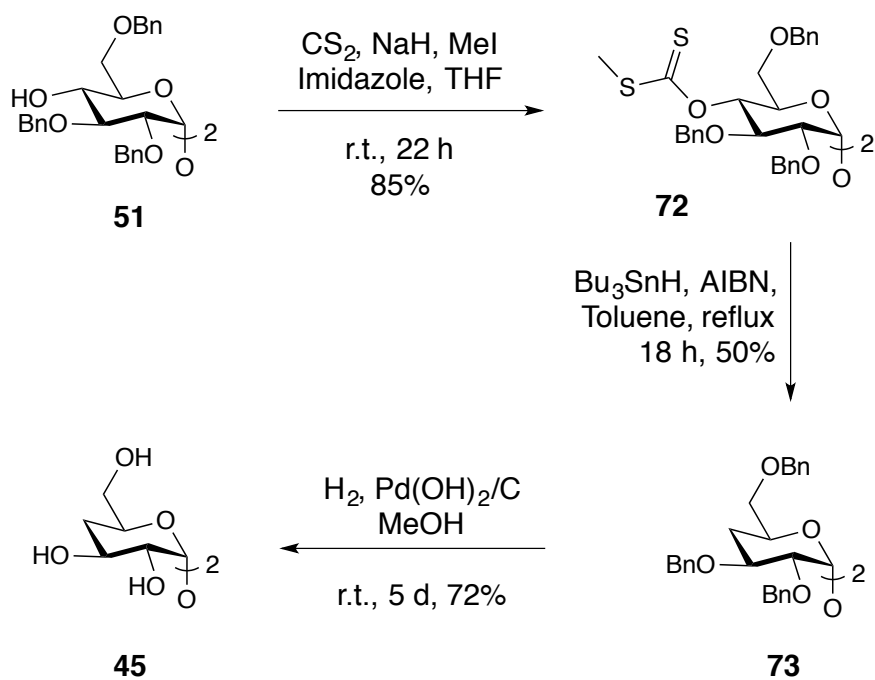
Scheme 13: Synthesis of 4-deoxy trehalose **45**

Xanthate **72** is a bright yellow, vile smelling oil whose structure was supported by ^{1}H NMR (Figure 20), whereby the proton at the 4-position is shifted significantly downfield by the deshielding effect of the thiocarbonyl (3.68 ppm in **51** to 6.10 ppm in **71**).
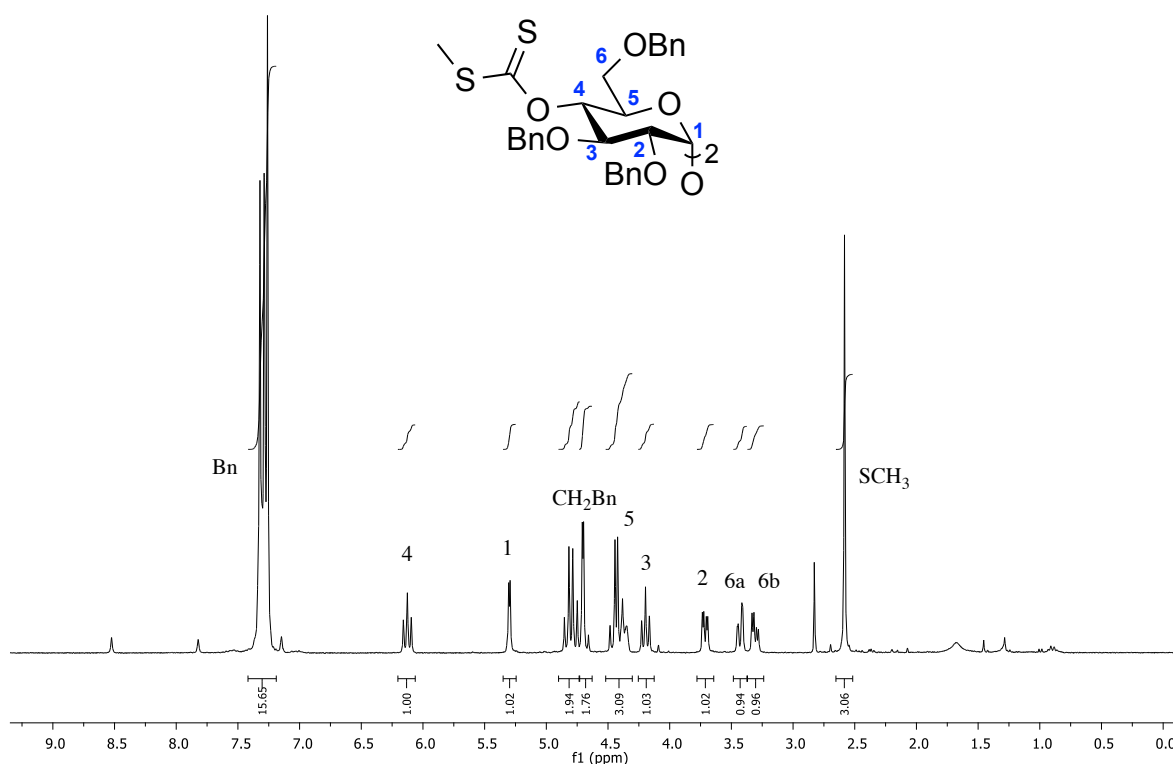
Figure 20: ^{1}H NMR section of 4-xanthate **72** (500 MHz, CDCl$_3$)

With thiocarbonyl **72** in hand, the xanthates on the 4- and 4′-positions were then displaced by a hydrogen, effecting deoxygenation. Whilst the first step of the deoxygenation had proceeded with a good yield (85%), the second step was completed in moderate yield (50%), yielding di-deoxy trehalose **73**. The radical deoxygenation could not be followed by TLC analysis, as both the starting material and product of the reaction eluted identically in all solvent systems trialled. Instead, an aliquot of the reaction was taken, concentrated under reduced pressure, and submitted for ^{1}H NMR, where the disappearance of starting material could be easily followed by the disappearance of the triplet at 6.10 ppm, assigned to the deshielded H-4 of xanthate **72**. Following purification by silica gel flash chromatography, the structure of deoxygenated sugar **73** was characterised by NMR analysis. Firstly, with reference to the COSY spectrum (Figure 21), the two H-4s can be clearly identified by correlation to H-3 and H-5, with their upfield chemical shift (2.11 and 1.59 ppm) due to the successful deoxygenation.
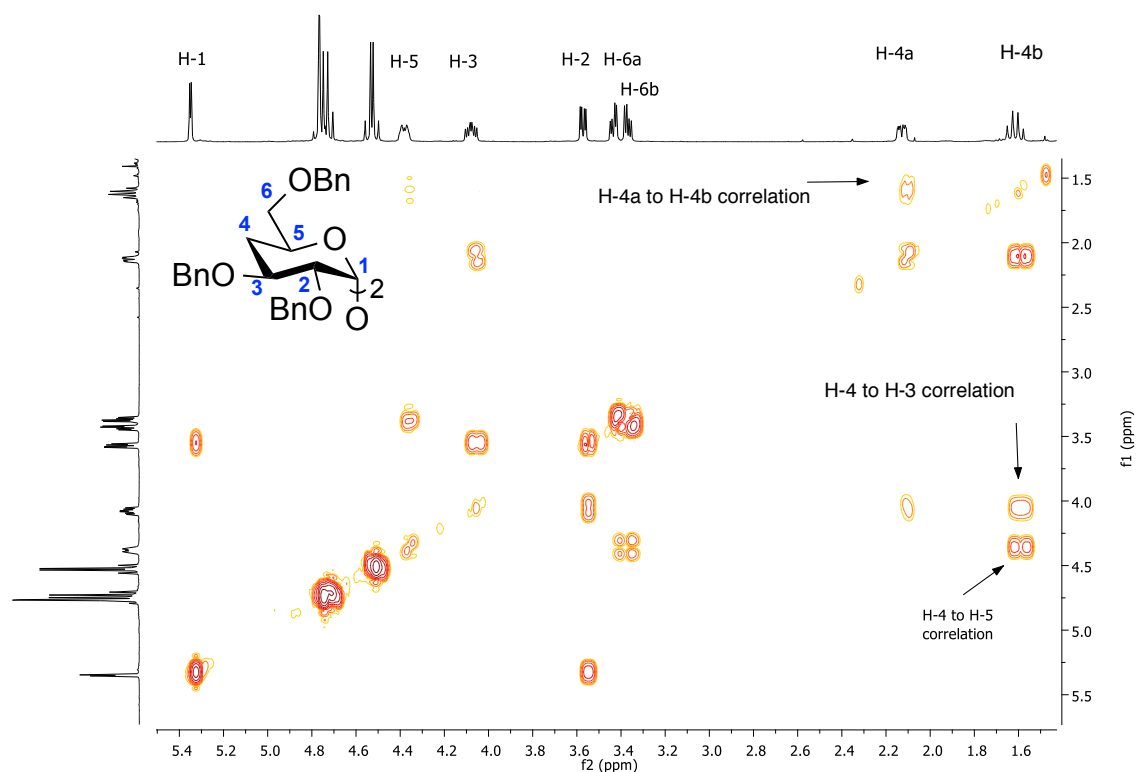
Figure 21: COSY spectrum of 2,3,6-*O*-Bn-4-deoxy trehalose **73**

With the two H-4's identified, they can be distinguished as axial and equatorial based on the vicinal coupling constants trends, by utilising the relationship relating dihedral angle between vicinal protons and $^3J_{HH}$ coupling constant as first described by Karplus.[90,91] The signal at 2.11 ppm has vicinal coupling of 5.1 and 2.2 Hz, whilst the 1.59 ppm peak has a coupling of 12.1 Hz. These *J* values are consistent with those predicted, allowing assignment of the 2.11 ppm peak as equatorial (predicted $^3J_{HH}$ = 4.5 Hz, 45° dihedral angles) and the 1.51 ppm peak as axial (predicted $^3J_{HH}$ = 15 Hz, 180° dihedral angles) (Figure 22). The final step towards the synthesis of deoxygenated trehalose **45** was the deprotection of the benzyl groups, which proceeded uneventfully to yield 4,4′-dideoxy-trehalose in good yield (72%).
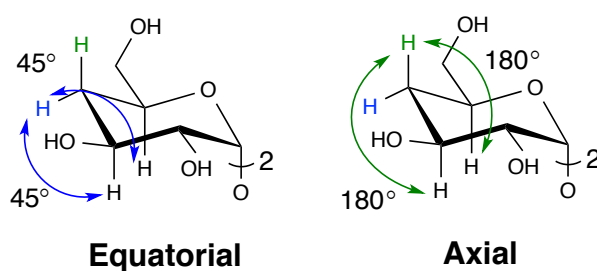
Figure 22: Dihedral angles of axial and equatorial 4-H's of 4-deoxy trehalose **45**

Esterification of carbohydrate **45** to behenic acid (**44**) was then attempted in the same manner as for TDB (Scheme 14, Route A), however it was found that the modification at the 4-position had a marked effect on the silylation. Although the silyl groups could readily be installed on all positions, exposure of the per-silylated 4-deoxy intermediate to the same conditions used *en route* to the synthesis of TDB **14** led to the non-selective removal of TMS groups. Attempts to use a lower temperature or more neutral pH in order to afford selectivity were unsuccessful. It was hypothesised that the use of the triethylsilyl protecting group, with greater steric bulk and hence base stability than trimethylsilyl, would enhance the difference in reactivity between primary and secondary oxygens, however this hypothesis was not borne out. Given the failure of the silyl-protecting group mediated coupling method, attention turned to protecting-group free coupling methods (Scheme 14, Route B).
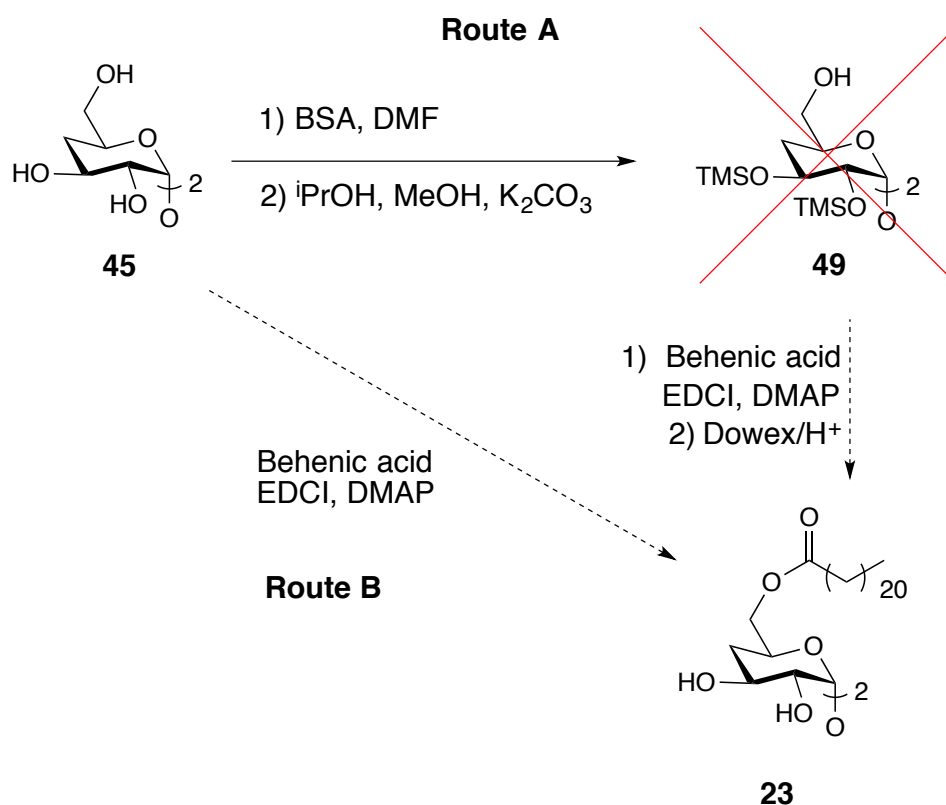
Scheme 14: Attempted synthesis of 4,4′-dideoxy trehalose dibehenate **23**

Trehalose 6,6′-diesters of short saturated and unsaturated lipids have been synthesised via protecting group free coupling of trehalose to the fatty acid.[55] This approach relies upon the greater reactivity of the primary alcohol and employs peptide-coupling reagent *N,N,N′,N′*-tetramethyl-O-(benzotriazol-1-yl)uronium tetrafluoroborate (TBTU) to affect the coupling, however this strategy has not previously been applied to lipids longer than 18 carbons.

Before attempting direct coupling with 4,4′-dideoxy trehalose **45**, the conditions reported by Paul *et al.* were first applied to the synthesis of TDB in order to assess viability with behenic acid. The optimised conditions used for the longest lipid reported (oleic acid) were used as a starting point for the direct coupling of trehalose to behenic acid. Reaction with *N,N,N′,N′*-tetramethyl-O-(benzotriazol-1-yl)uronium hexafluorophosphate (HBTU) at room temperature in pyridine for 10 days did not produce any glycolipid product however (as evidenced by TLC analysis in 2:9:9 methanol/ethyl acetate (EA)/dichloromethane (DCM) (*v/v/v*)), and even after heating to 70°C for 11 days only an 8.5% yield of the desired product **14** was obtained. Heating a new reaction with HBTU from the beginning did not appear to improve

reaction progress, so the addition of esterification promoter EDCI was trialed. This combination of coupling reagents led to a slight improvement in yield, up to 10.5%. Using just EDCI, a 27% yield of glycolipid **14** was obtained after 8 days reaction at 70°C, which was sufficient to consider the route viable.

The direct synthesis of 4,4′-dideoxy TDB **23** was attempted using EDCI, however, the use of a non-protic and less nucleophilic solvent was also examined, utilising the less polar nature of **45** as opposed to trehalose **7**, with the aim of improving reactivity by limiting solvent interference. Thus, tetrahydrofuran (THF) was trialled, with the addition of DMAP to aid reaction, however it was found that asymmetric, over-esterified products were being formed. It was thought that this was due to unforeseen limited solubility of the carbohydrate **45** in hot THF, whilst the lipid was fully soluble. This would have lead to any sugar that dissolved meeting a very high lipid concentration in solution and hence reacting with more than two equivalents of activated lipid faster than more sugar could dissolve. In an effort to combat this, pyridine was returned to as the solvent of choice, in which **45** was fully soluble. Preliminary experiments using these conditions showed similar results to those obtained in TDB synthesis, however insufficient material was procured to perform full characterisation of the products obtained. Unfortunately, time limitations did not allow for repeating the reaction on a larger scale, hence **23** could not be obtained.

2.1.2.3   Synthesis of 4-O-Me TDB

In addition to using 2,2′,3,3′,6,6′-*O*-hexabenzyl-α,α′-D-trehalose (**51**) for the preparation of the 4,4′-dideoxy trehalose derivatives, this intermediate can also be methylated in order to explore how the incorporation of a methoxy group at the 4- and 4′- positions of TDB influences Mincle binding and activation. Accordingly, the methylation of diol **51** was carried out using methyl iodide, sodium hydride and *N,N*-dimethylformamide (DMF) to give 4,4′-di-*O*-Me trehalose **74** in good (75%) yield, consistent with literature precedence for similar methylations on trehalose (Scheme 15).[68]
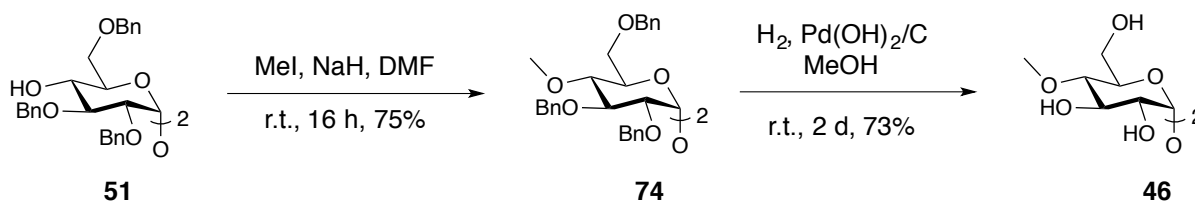
Scheme 15: Synthesis of 4-*O*-Me trehalose **46**

Evidence of successful methylation was provided by ^{1}H and HMBC NMR spectra, where a singlet integrating for three protons was observed in the ^{1}H NMR spectrum this resonance in turn demonstrated an HMBC correlation to the proton attached to C-4 of the sugar (Figure 23).
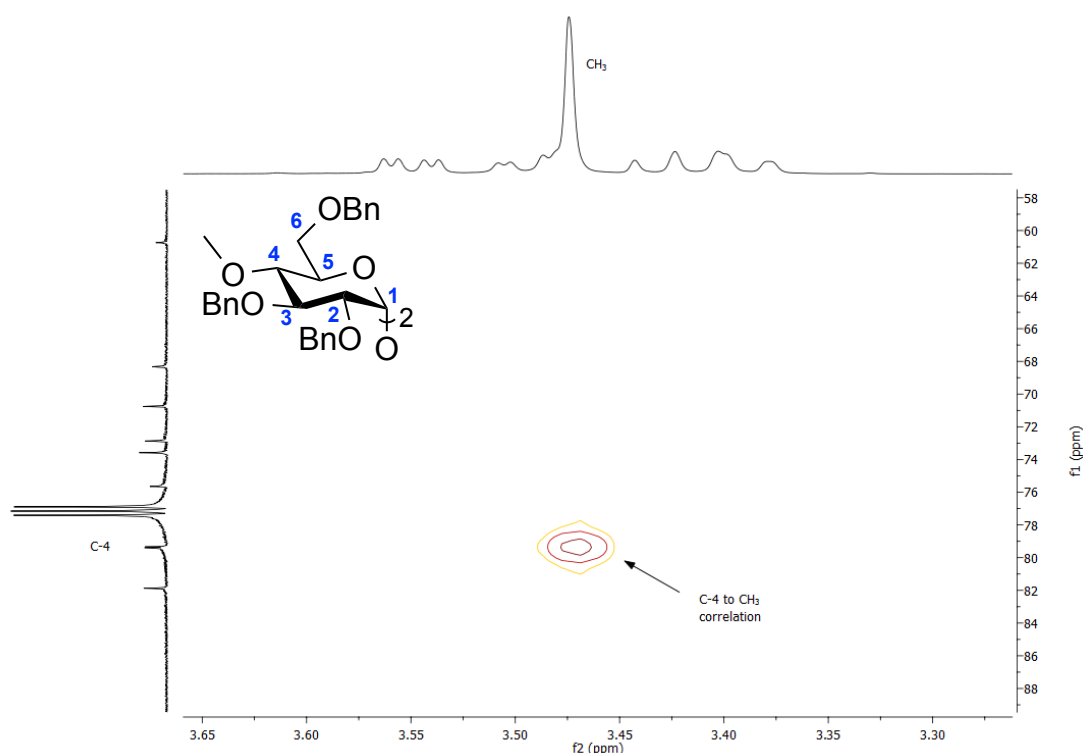
Figure 23: HMBC spectrum of 2,2′,3,3′,6,6′-hexa-*O*-Bn-4,4′-di-*O*-Me trehalose **74**

The deprotection of hexa-benzylated **74** was then carried out using heterogenous palladium catalyzed hydrogenolysis, with the reaction being followed by TLC analysis in 4:1EA/MeOH, *v/v*. The starting material and intermediates with some benzyl groups still

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attached run at the solvent front, whereas product runs with an R_F of 0.29. The reaction proceeded smoothly to give 4,4′-O-Me trehalose **46** in good yield (73%).

Similar to the previously discussed 4-deoxy esterification, silylation proved to not be a feasible route (Scheme 16, Route A). Initial attempts at direct coupling in THF led to the formation of products such as 4,4′-di-O-methyl-penta-O-behenate-α,α′-D-trehalose (structure evidenced by HMB correlations between the multiple carbonyl carbons, ∂ 174.3 − 173.9 (C_5D_5N), and five of the protons on the asymmetric sugar), (Scheme 16, Route B), and whilst small-scale trials in pyridine appeared promising, insufficient material was obtained to allow for purification and hence biological testing. Time constraints unfortunately meant that more starting material could not be prepared, so the reaction could not be repeated on a larger scale.
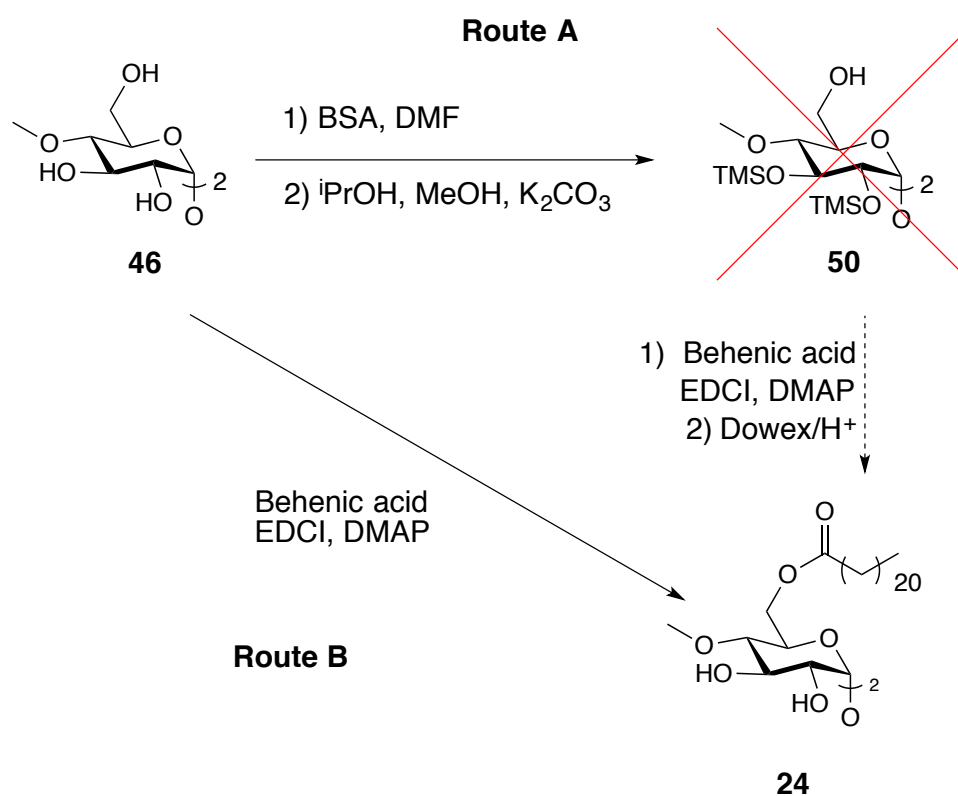
Scheme 16: Attempted synthesis of 4,4′-O-Me trehalose dibehenate **24**

2.1.3 Synthesis of 3-modified TDBs

2.1.3.1 Synthesis of 4,6,4′,6′-di-O-benzylidene-2,2′-di-O-benzoyl-trehalose

Once 4,6-benzylidene trehalose **52** was prepared, efforts also turned to preparing the derivatives with the functional group at the 3-position altered. The first step towards this goal was the regioselective protection of the 2-hydroxyl of 4,6-benzylidene trehalose (**52**) with a benzoyl group (Bz) to yield protected carbohydrate **55** (Scheme 17).
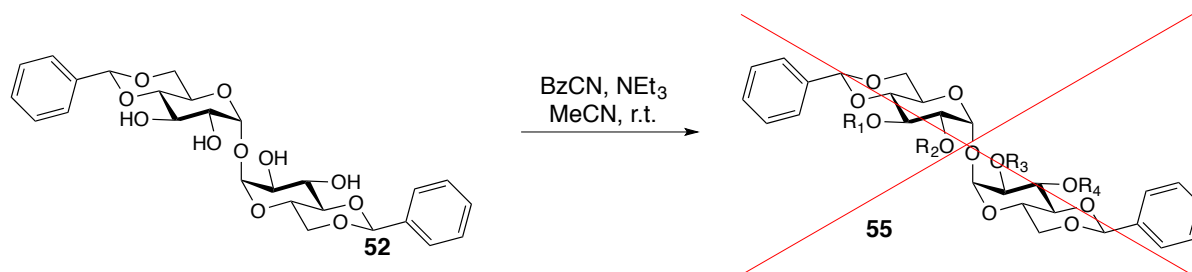
Scheme 17: Benzoylation of 4,6-benzylidene trehalose **52**

Initially the conditions employed by Lin *et al.*[56] were used for the attempted synthesis of 2,2′-di-*O*-benzoyl **55**, however using the conditions reported, a complex mixture of tetra-, tri-, di-, and mono-benzoylated products were formed, along with recovered starting material. The reaction was repeated several times to no avail (Table 1, Entry 1). Numerous other conditions were then trialed, including the addition of 2.4 equivalents of benzoyl chloride under basic conditions, however this led to the formation of a single product, 2,3,2′,3′-tetra-*O*-benzoyl-4,6,4′,6′-di-*O*-benzylidene-α,α′-D-trehalose (Table 1, Entry 2). It was postulated that this could be due to the protected starting material **52** having lower solubility in the solvents trialed than the products, hence the addition of one benzoyl group may increase solubility and therefore reactivity. Silver oxide was then added in an attempt to give selectivity, nonetheless only 2,3,2′,3′-tetra-*O*-benzoyl-4,6,4′,6′-di-O-benzylidene-α,α′-D-trehalose was recovered (Entry 3).[92] With nickel (II) chloride as a catalyst,[93] a mixture of benzoylated products was formed (Entries 4 and 5), which was also the case when THF was used as the solvent with no

catalyst added (Entry 6). With the failure of these conditions to improve on the selectivity of the reaction, attempts returned to the original Bertozzi conditions.

Entry	Bz reagent	# Eq	Temp (°C)	Catalyst	Solvent	Result
1	BzCN	2.0	r.t.	Et$_3$N	MeCN	Mix[a]
2	BzCl	2.4	-35 – 0	N/A	Pyr[b]	Tetra[c]
3	BzCl	2.4	-40 – 40	Ag$_2$O	THF	Tetra[b]
4	BzCl	2.2	r.t.	NiCl$_2$[d]	THF	Mix[a]
5	BzCl	1.8	r.t.	NiCl$_2$[e]	THF	Mix[a]
6	BzCl	2.2	Reflux	N/A	THF	Mix[a]

[a]Complex mixture of products formed; [b]pyridine; [c]2,3,2′,3′-tetra-*O*-benzoyl-4,6,4′,6′-di-*O*-benzylidene-α,α′-D-trehalose formed; [d]used as received; [e]freshly dehydrated

Table 1: Attempted synthesis of dibenzoylated **55**

As shown below in Table 2, initially it was hoped that reducing the reaction time from the reported two hours might allow for more of the desired product to be isolated, however a near-identical mixture of products was formed (Entry 1). Silica gel flash chromatography (1:0 – 4:1 toluene/EA, *v/v*) allowed for separation of some of the isomers, however, in the case of Entry 1, no clean 2,2′-benzoyl product could be isolated, only a mixture of diesters, in a 28% yield. An attempt to decrease the temperature of the reaction from room temperature to 0°C was made in order to increase selectivity, however this proved unfeasible due to the limited solubility of the carbohydrate starting material, and hence the concentration of the reaction solution was instead halved from the original conditions (Entry 2), and it was found that an increased proportion of the diesters could be isolated, although there was not any improvement in regioselectivity. Next, attention was turned to the number of equivalents of triethylamine added to the reaction, with 0.36 equivalents used by Lin *et al.*[56] It was found that, while some NEt$_3$ is necessary for benzoylation, drastically decreasing the amount added

has a positive impact on the reactivity. Reducing the number of equivalents of NEt$_3$ while conserving other reaction conditions did not initially improve the yield or selectivity (Entry 3), however, returning to the reaction concentration to that reported in Lin *et al.* was rewarded with an increase in diester yield to 41 mol% (Entry 4). Further decreasing the concentration of triethylamine to 1.3 mol% further increased the yield (Entry 5), and although a mixture of diesters was formed, analysis of the ^{1}H NMR of the crude product mixture clearly demonstrated that the desired 2,2′-*O*-benzoyl-4,6,4′,6′-di-*O*-benzylidene-α,α′-D-trehalose (**55**) was formed as the major isomer. Slightly increasing the amount of base (Entry 6) increased the yield to 53%, slightly higher than that originally reported.[56] Moreover, it was discovered that the desired product could be purified by fractional crystallisation from acetonitrile at -20°C, allowing for purification of 2,2′-di-*O*-benzoyl trehalose **55** from the reaction mixture in a completely pure form. Lastly, using a higher NEt$_3$ concentration with this new purification method in hand (Entry 7) led to a decrease in yield of product **55**, confirming the optimum base loading is indeed around 0.5 mol%.

Entry	Time (min)	NEt$_3$ (equiv.)	Conc[a]	Product (isolated yield)
1	25	0.36	50 mg/mL	Mix diesters[b] (28%)
2	120	0.31	25 mg/mL	Mix diesters[b] (36%)
3	60	0.1	25 mg/mL	Mix diesters[b] (28%)
4	20	0.05	50 mg/mL	Mix diesters[b] (41%)
5	15	0.0013	40 mg/mL	Mix diesters[b] (56%)
6	210	0.005	50 mg/mL	**55** (53%)
7	60	0.0225	50 mg/mL	**55** (40%)

[a]concentration of **52** in the reaction; [b]inseparable mixture of di-benzoylated isomers isolated

Table 2: Optimisation of benzoylation conditions

To confirm that the benzoyl groups in protected carbohydrate **55** had been selectively installed at the 2- and 2′-positions, a number of analytical techniques were employed. Firstly, both the ^{1}H and ^{13}C NMR spectra indicated that only one isomer was present in the crystallised product, due to the presence of the correct number of chemical environments (Figure 24).
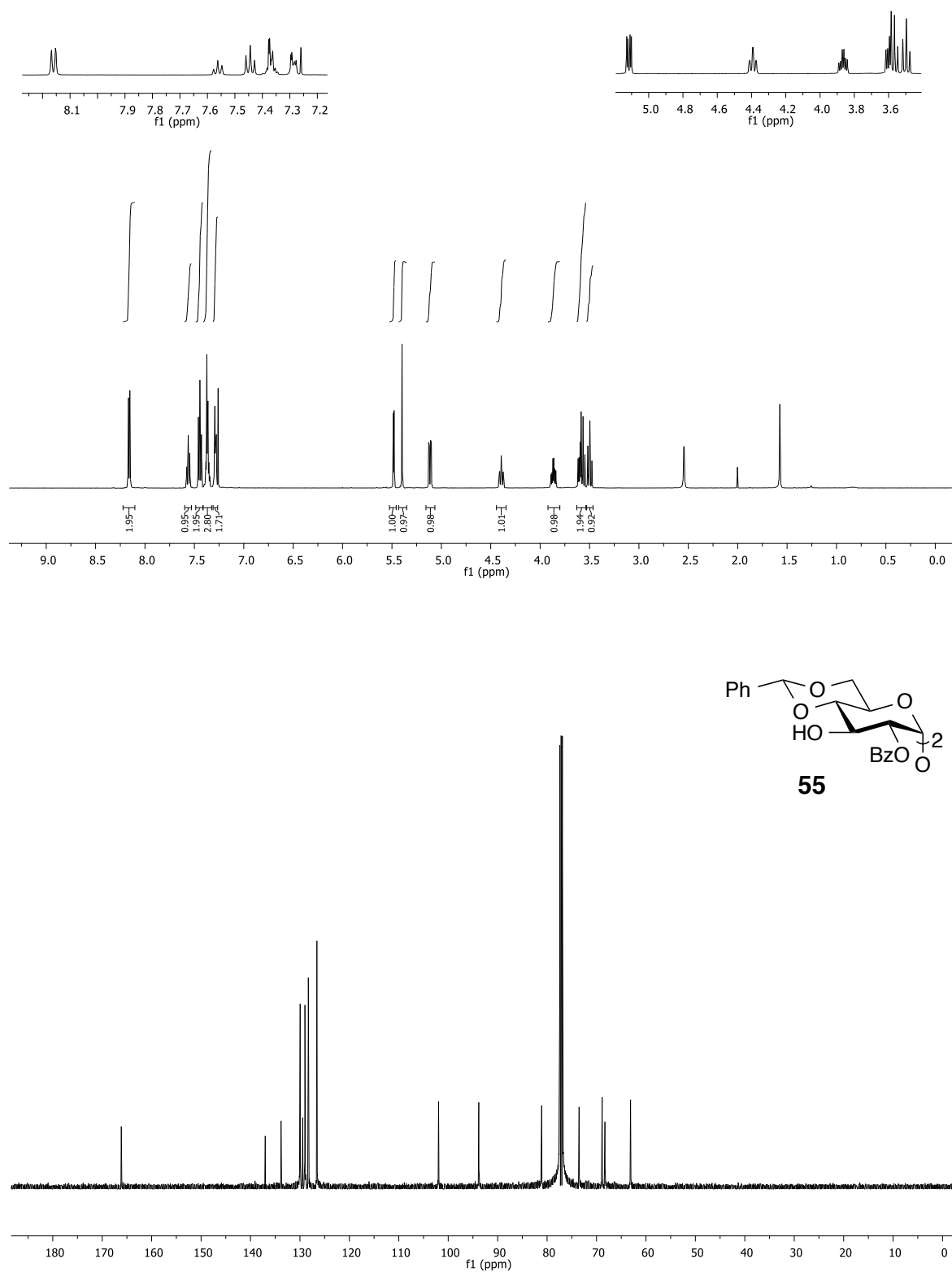
Figure 24: ^{1}H and ^{13}C NMR spectra of 2,2′-Bz **55**

Correlation spectroscopy (COSY) analysis allowed for the identification of the 2/2′ and 3/3′-protons of the (symmetric) sugar (through correlations from the anomeric proton, distinguishable by chemical shift, Figure 25). Moreover, the proton at the 2/2′-position was shifted downfield compared to H-3 of **52** (∂ 5.12 ppm versus 4.39), which is consistent with it being attached to the same carbon as the electron withdrawing benzoyl ester. In addition, an HMBC between the carbonyl carbon of the benzoyl and exclusively the 2/2′-proton provided further evidence for the positioning of the benzoyl at the 2/2′ carbons of the sugar (Figure 26)
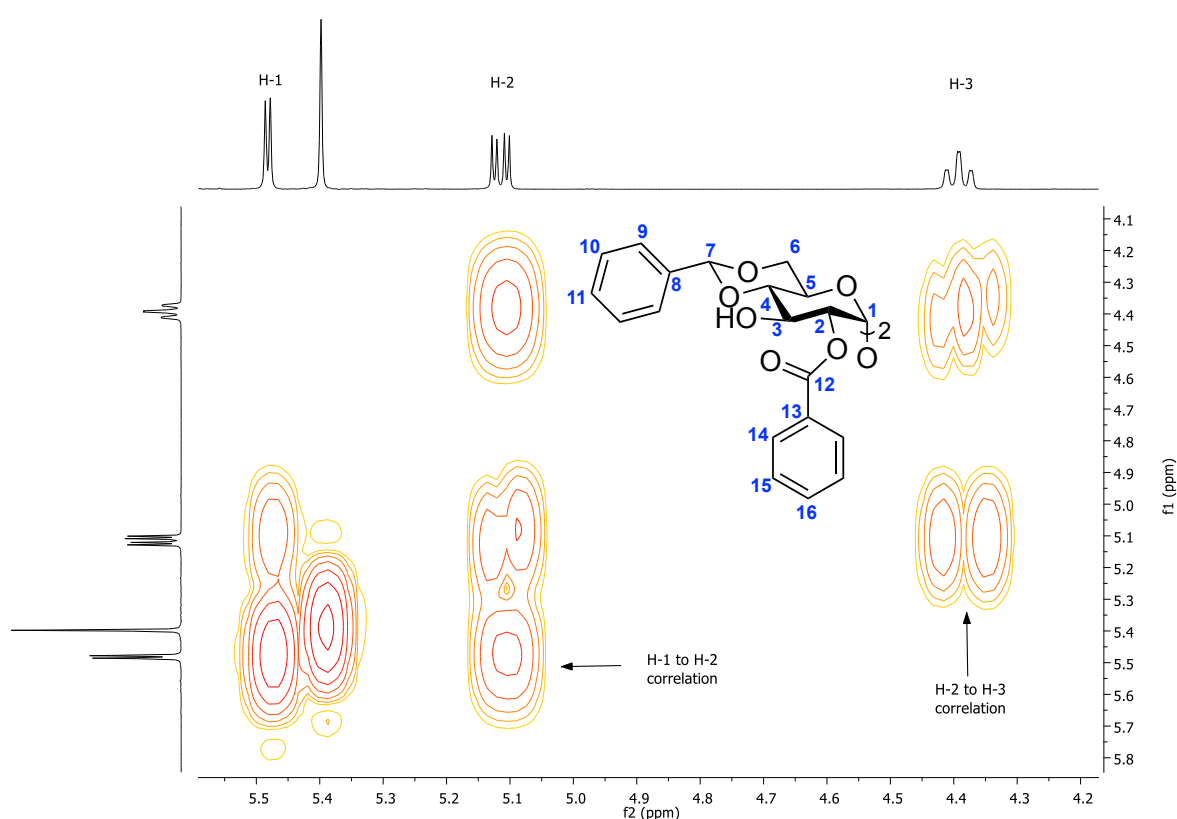
Figure 25: COSY section of dibenzoylated **55**
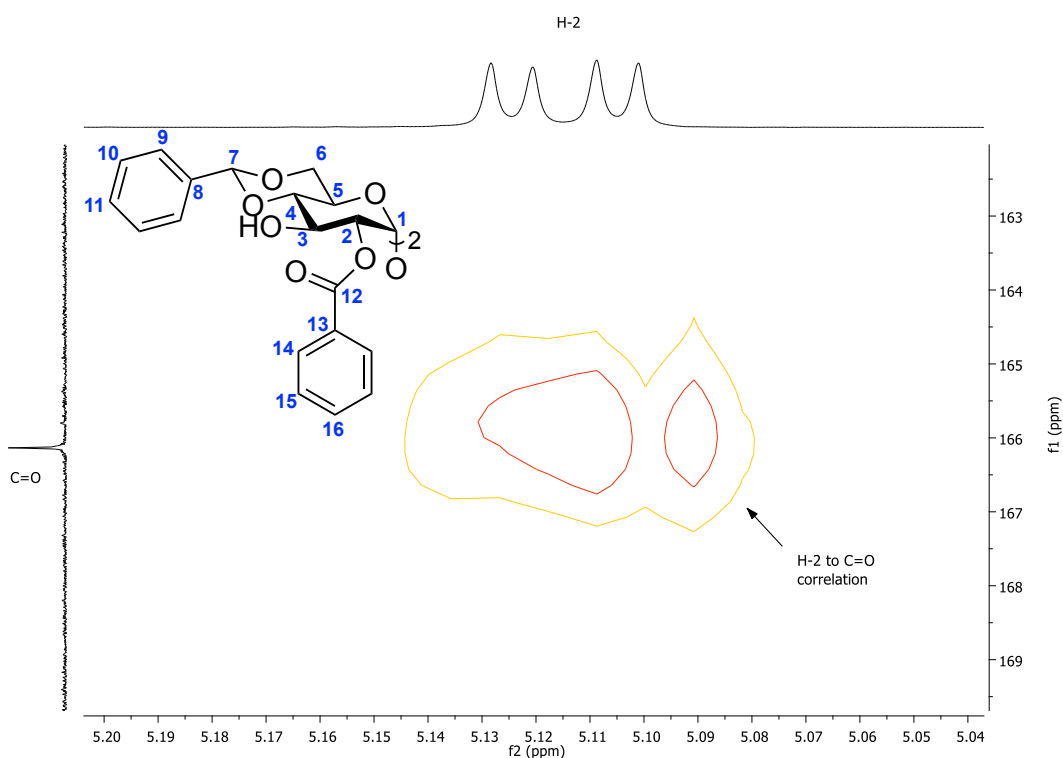
Figure 26: HMBC section of dibenzoylated **55**

Final proof of the structure of dibenzoylated **55** was provided in the form of a single crystal X-ray diffraction experiment, obtained at Victoria University of Wellington by Associate Professor Martyn Coles, and the data recorded analysed and solved by myself using shelxl and optimised using Olex2. As shown in Figure 27, a benzoyl group is located on the 2-position on both halves of the chiral, symmetric sugar.
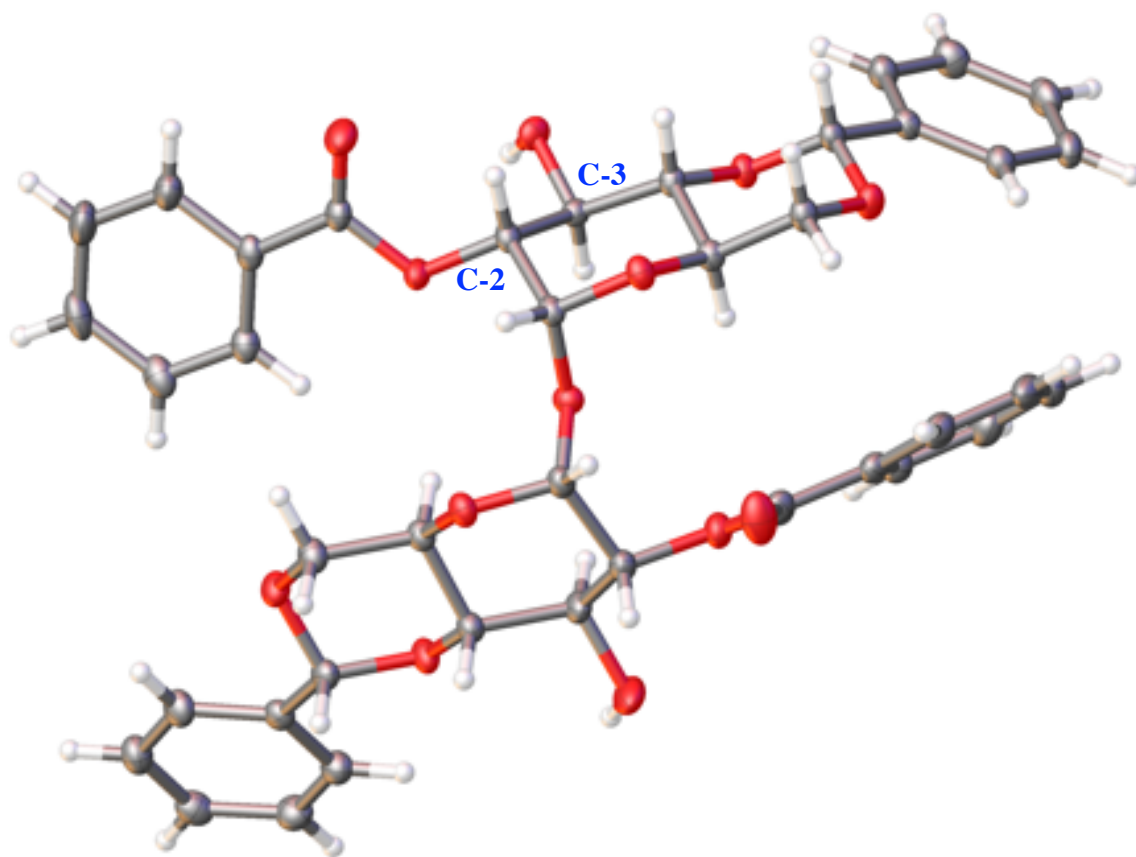
Figure 27: Single crystal X-ray diffraction pattern of dibenzoylated **55**. Thermal ellipsoids shown at 50% probability level, R1 = 3.06%

2.1.3.2 Synthesis of 3-deoxy TDB

With the selectively protected trehalose **55** in hand, deoxygenation was attempted in the same manner as at the 4-position, however it was found that the strongly basic conditions used lead to migration of the benzoyl group and hence an inseparable mixture of products were formed in addition to 3-xanthate **75** (Scheme 18). In order to minimise the possibility of migration, milder conditions were used, utilising similar methodology to that reported by Lin *et al.*[56] Diol **55** was reacted with DMAP and phenyl chlorothionoformate to successfully form a monothiocarbonate **76** as at least the primary isomer, although complete purification and hence characterisation was not carried out. Deoxygenation was then attempted in the same manner as for the 4-position (identical to that used by Lin *et al.*), however no reaction was observed, and unfortunately time restrictions did not allow for optimisation of this procedure.
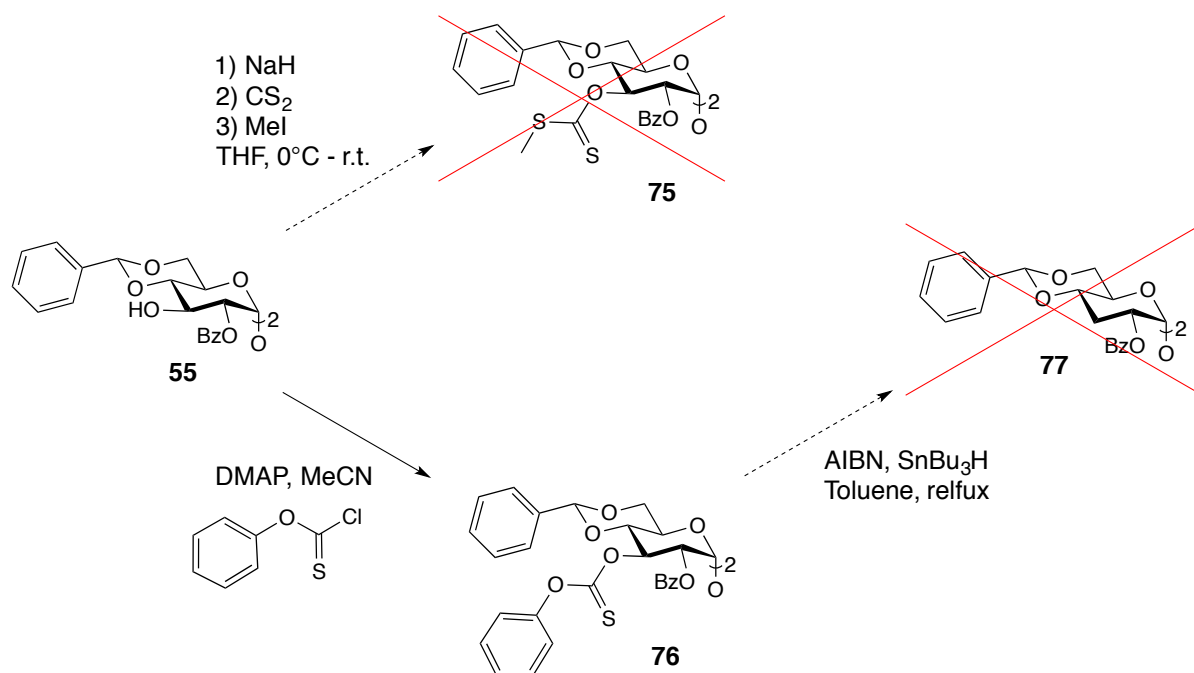
Scheme 18: Attempted synthesis of 3-deoxy trehalose

2.1.3.3 Synthesis of 3-O-Me TDB

Similar to deoxygenation of diol **55**, methylation proved challenging due to unwanted benzoyl migration being observed upon addition of a base, as evidenced by the isolation of regioisomers of 3-*O*-Me **78** (Scheme 19).
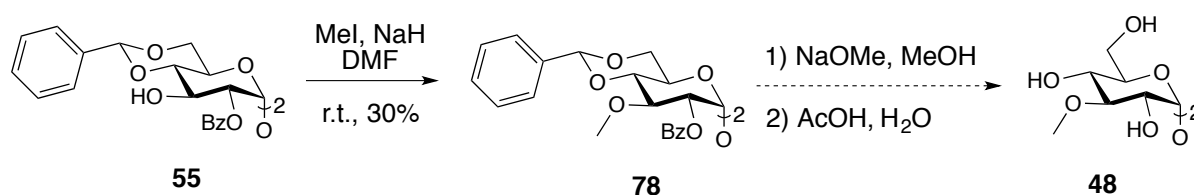
Scheme 19: Methylation of 2-Bz **55**

Whilst the desired product dimethylated **78** could be formed as the major product, a significant proportion of the starting material underwent migration, and reduction of the temperature to 0 or -20 °C lead to an even greater degree of migration, whilst increasing the temperature to reflux lead to the formation of 2,2′,3,3′-tetra-*O*-methyl-4,4′,6,6′-di-*O*-benzylidene-α,α′-D-trehalose. Use of halophilic Ag$_2$O as a non-basic promotor of

methylation did not lead to any reaction whatsoever, hence the best yield that could be obtained was the moderate (30%) yield obtained at room temperature with NaH. Unfortunately, insufficient material was obtained to carry through with deprotection and coupling of the 3,3′-modified trehalose, and time constraints meant that more starting material could not be prepared in order to repeat and optimise the reaction.

2.2   Synthesis of maradolipids and glycerolipids

2.2.1   Synthesis of *iso*-branched fatty acids

Before the maradolipid and glycerol targets could be prepared, the iso-branched fatty acids first required synthesis. Two strategies were taken to make the *iso*-branched fatty acids - one for lipids possessing 15 - 17 carbons in total, and another for the C_{18+1} lipid.

The synthesis of 17-methyloctadecanoic acid (C_{18+1} acid, **62**) was first undertaken using methodology developed by Stefan Munneke (Unpublished work). 15-Cyclopentadecanolide (**67**) was used to generate 15-methylbromopentadecanoic acid **79**[94-97] in good yield (84%), which was then be submitted to Wittig conditions with isobutyraldehyde to produce 17-methyloctadecan-15-enoic acid (**80**) in moderate yield (44%) (Scheme 20). The formation of the alkene could clearly be seen in the ^{1}H NMR spectrum, with the protons attached to the alkene giving signals considerably downfield (∂ 5.20 ppm) than the rest of the lipid (∂ 2.34 – 0.94 ppm). Lastly, hydrogenation of alkene **80** leads to the formation of 17-methyloctadecanoic acid **62** in good yield (68%).
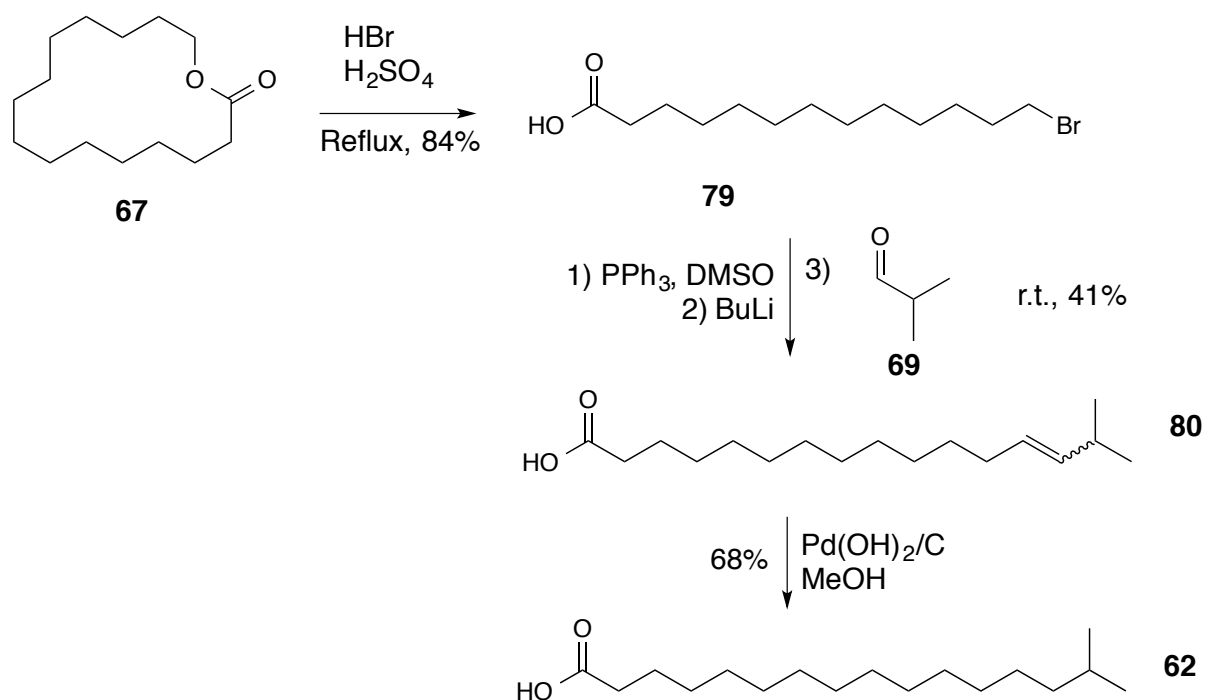
Scheme 20: Synthesis of 17-methyloctadecanoic acid **62**

To synthesise the shorter lipids, a strategy reported by Richardson and Williams was employed.[77] As shown in Scheme 21, a Grignard reaction was first carried out on cyclopentadecanolide (**67**) using methyl magnesiumiodide, resulting in the addition of two carbon units and opening of the lactone in good yield (85%). Evidence for the formation of diol **81** was provided by ^{1}H NMR, and in particular, the successful installation of the *iso*-branched terminus can be confirmed by the upfield doublet integrating for 6 protons (∂ 1.20 ppm).
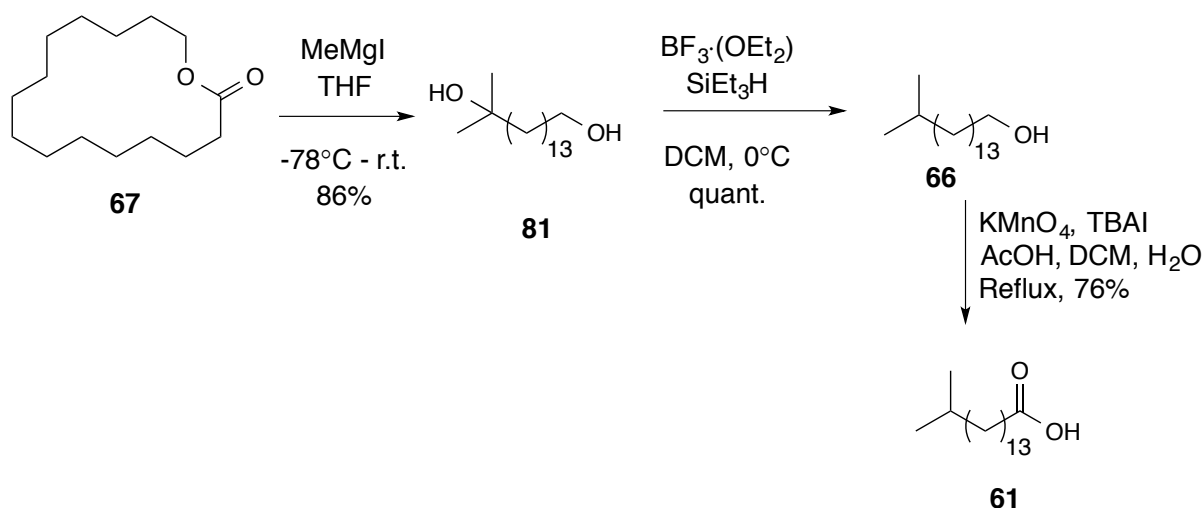
Scheme 21: Synthesis of 15-methylhexadecanoic acid **61**[77]

The next step was the selective deoxygenation of diol (**81**) at the tertiary position in quantitative yield, using boron trifluoroetherate as a Lewis acid. The mechanism for this reaction involves a simple reduction, whereby departure of the tertiary hydroxyl is promoted by complexation to the Lewis acid (**I**) (Scheme 21). Following leaving group removal, a tertiary carbocation (**II**) is formed, which is considerably more stable than the primary carbocation that would have formed if the primary hydroxyl had departed. It is this increased stability which results in the selectivity observed, yielding mono-ol **66** after hydride addition.
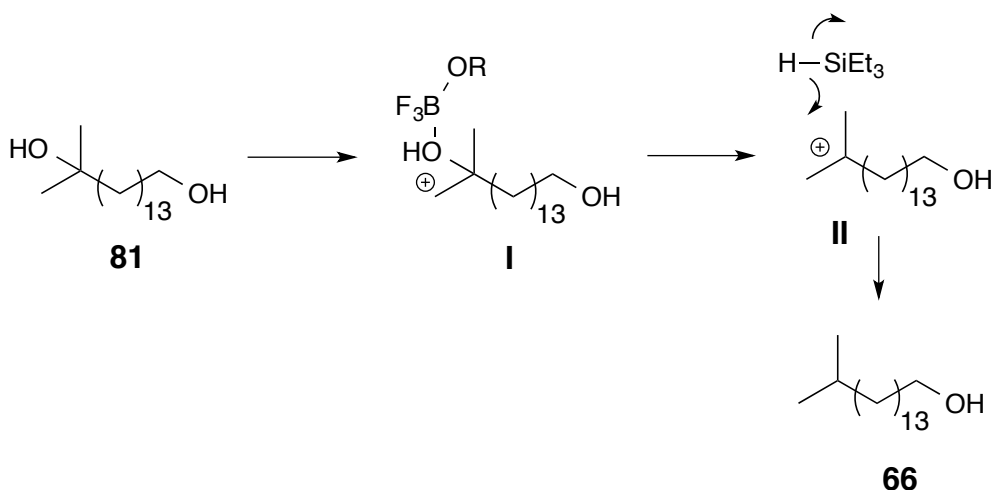
Scheme 22: Mechanism of selective reduction of **78**

Finally, the terminal alcohol (**66**) was oxidised via a biphasic reaction to give C_{16+1} acid (**61**) in good yield. The successful transformation to a carboxylic acid was verified using ^{13}C nuclear magnetic resonance (NMR), where a new, significantly down-shifted carbon signal appears upon oxidation, consistent with the formation of a carbonyl (∂ 178.5 ppm). In order to synthesise the shorter lipids in this series, the mono-ol (**66**) was transformed into a *S*-methylthiocarbonyl (**82**) via deprotonation by NaH, nucleophilic attack on carbon disulfide, and finally methylation of the anionic sulfur (Scheme 23).[77] Although the monothiocarbonate was not purified, the ^{1}H NMR spectrum showed a signal at ∂ 4.58 ppm, which is characteristic of the α-proton of the deshielding thiocarbonyl, as well as a singlet at ∂ 2.51 ppm integrating to three protons (SCH₃). Thiocarbonate **82** was then heated, eliminating the monothiocarbonate on the terminal carbon, yielding alkene (**65**) in a moderate yield (51%). Permanganate cleavage of the alkene then gave 14-methylpentadecanoic acid (**63**) in a moderate yield (41%).
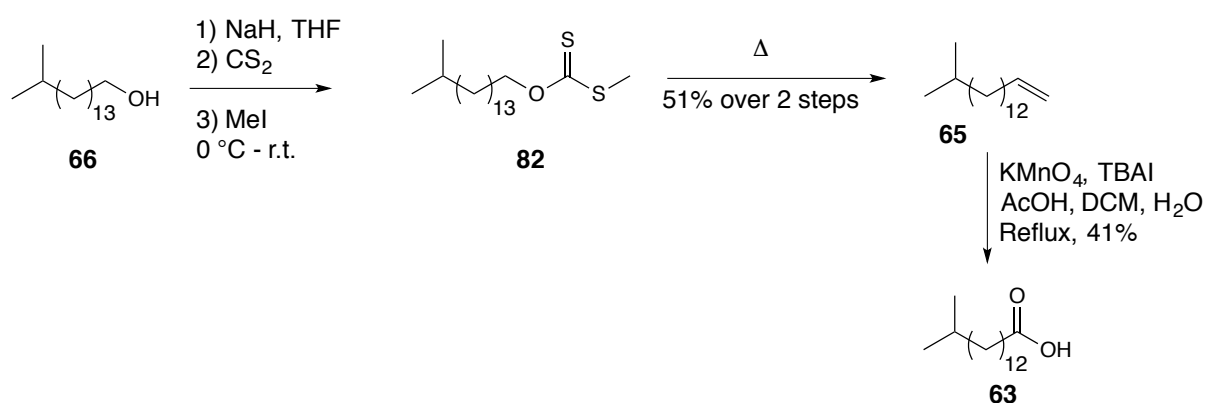
Scheme 23: Synthesis of 14-methylpentadecanoic acid **63**[77]

With 14-methylpentadecanoic acid **63** in hand, the carboxylic acid was reduced using borane dimethylsulfide to give alcohol (**83**) in good yield (68%, Scheme 24). The disappearance of the ^{13}C signal at 178.5 ppm indicated removal of the carbonyl functional group. Next, xanthate (**84**) formation and elimination was carried out as discussed above, yielding alkene **65** in only a poor yield (18%). Time constraints did not allow for optimisation of the yield. Alkene **65** was clearly identified by ^{1}H NMR, where the deshielding alkene functionality shifts the α-and β-terminal protons downfield (∂ 5.82, and 5.00 & 4.93 ppm, respectively), with the characteristically large vicinal alkene coupling constant of 17 Hz. Lastly, oxidative

cleavage was carried out on alkene **65** to give 13-methyltetradecanoic acid **63** (in moderate (41%) yield, where disappearance of the alkene ^{1}H signals was consistent with successful reaction, however this proceeded in poor yield (13%).
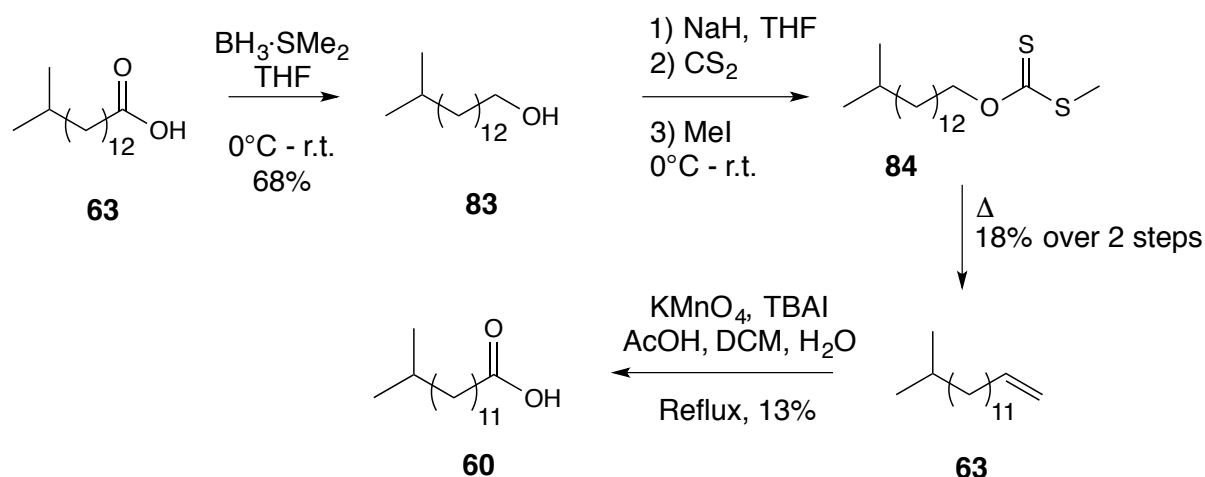
Scheme 24: Synthesis of 13-methyltetradecanoic acid **60**[77]

Due to time constraints, optimisation of the aforementioned route to 13-methyltetradecanoic acid (**60**) could not be performed, hence utilising the Wittig methodology for shorter chain lipids was explored. 11-Bromoundecanoic acid was reacted with triphenylphosphine to form ylide **85**,[79] then butyl lithium was added to deprotonate the lipid (Scheme 25). Next, *iso*-butyraldehyde was added, and a Wittig reaction was carried out between the two species, resulting in the formation of alkene **86**.[78] Finally, hydrogenation of the semi-purified alkene was carried out, yielding 13-methyltetradecanoic acid (**60**) in trace amounts. The poor yield obtained for this reaction was due to the formation of byproducts during the Wittig step, and unfortunately time constraints did not allow for optimisation of this reaction, and insufficient quantities of C_{14+1} acid **60** could be obtained to proceed with coupling to trehalose or glycerol.
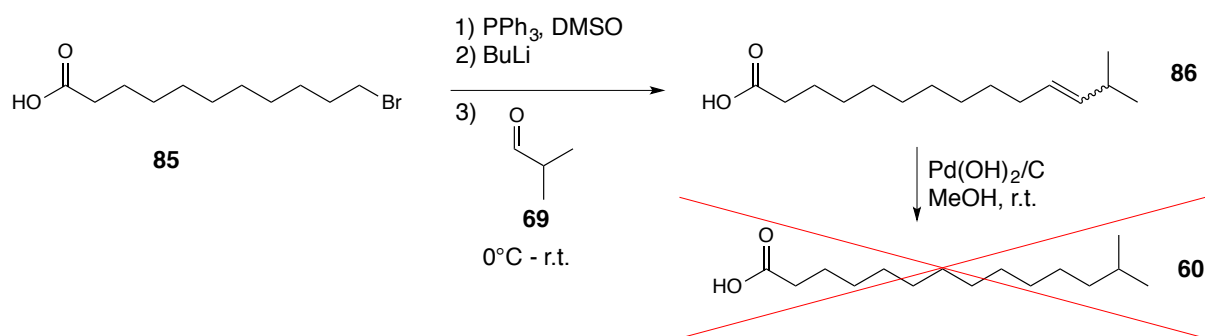
Scheme 25: Alternative synthesis of 13-methyltetradecanoic acid **60**

2.2.2 Synthesis of maradolipid analogues

2.2.2.1 Synthesis of straight-chain TDEs

In order to thoroughly investigate the effect of *iso*-branching of the lipid chain on the biological properties of the trehalose glycolipid, the straight chain equivalents of the maradolipids targets were first prepared (Scheme 26). Previously synthesised hexa-TMS trehalose **70** was reacted with the appropriate carboxylic acid in toluene, with the addition of EDCI and DMAP to promote esterification in accordance with methodology developed by Khan.[49] Tetradecanoic (myristic), hexadecanoic (palmitic), and octadecanoic (stearic) acids coupled smoothly to give diesters **33**, **34**, and **12** after deprotection of the TMS groups (using acidic Dowex/H^{+} resin) in moderate yields (43, 46, and 29%, respectively). The success of these reactions was determined using NMR and MS, as discussed previously for TDB **14**.

33: $n = 12$, **43%**
34: $n = 14$, **46%**
12: $n = 16$, **29%**
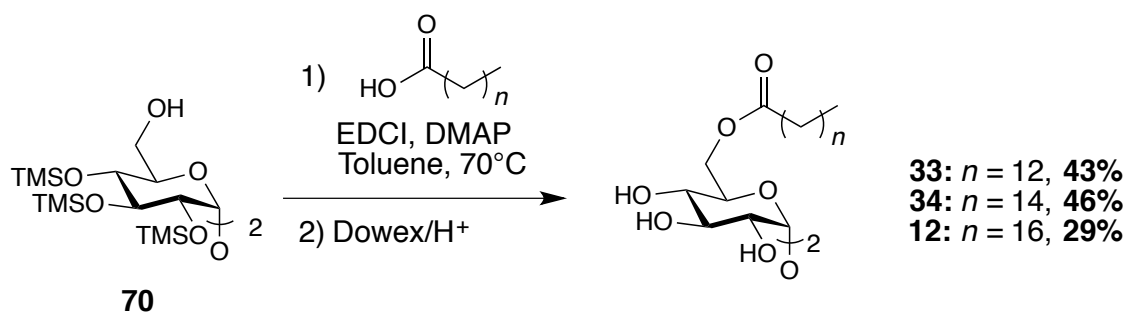
Scheme 26: Synthesis of C_{14}, C_{16} and C_{18} diesters of trehalose

2.2.2.2 Synthesis of branched-chain TDEs

The synthesis of the corresponding *iso*-branched glycolipids was attempted in an identical manner to the straight chain targets (Scheme 27), with the C_{16+1} and C_{18+1} lipids coupling smoothly to produce targets **36** and **37** in moderate to good yields after deprotection (53 and 29%, respectively). In contrast, the coupling of C_{14+1} acid to hexa-TMS trehalose **70** proved challenging, primarily yielding the undesired mono-ester product. Insufficient 13-methyltetradecanoic acid (**60**) was available to optimise the coupling, hence deprotected target **35** could not be produced. The success of these reactions was determined using NMR and MS, as discussed previously for TDB **14**.
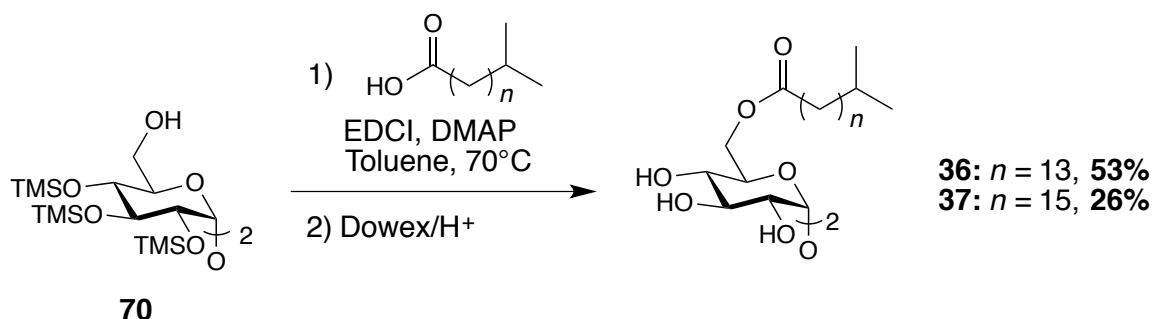
Scheme 27: Synthesis of iso-branched trehalose glycolipids

It should here be noted that the synthesis of C_{15+1} acid (**64**) was previously completed, however this lipid was not coupled to trehalose due to the fact that only fatty acids with an even number of carbons in the longest branch are commonly found in nature, due to the biosynthetic pathways involved.[98]

2.2.3 Synthesis of Glycerolipids

2.2.3.1 Synthesis of straight-chain esters

The straight-chain glycerol monoester targets **27** - **29** and **22** were prepared in two steps from the corresponding fatty acid and racemic isopropylidene-protected glycerol **56** (Scheme 28). First, a Steiglich esterification was performed, followed by removal of the isopropylidene

protecting group by acidic hydrolysis using 4:1 *v/v* acetic acid/water, with evidence of successful deprotection provided by disappearance of the two singlets at 1.44 and 1.37 ppm.
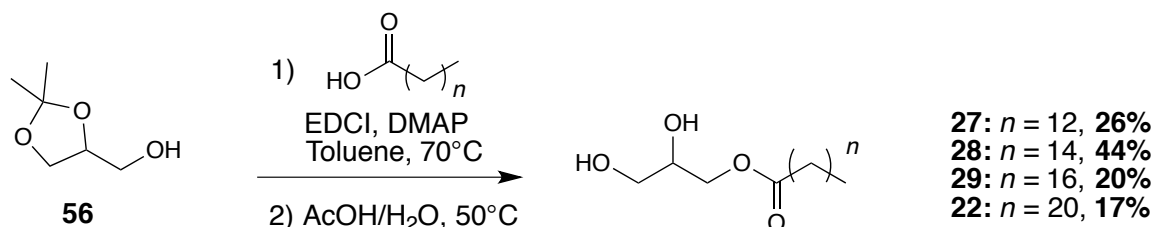
Scheme 28: Coupling of straight-chain glycerol monoesters **71 - 74**

Successful formation of the new C-O bond was evidenced by HMB correlation between the carbonyl carbon at 171.4 ppm and the adjacent glycerol protons at 4.71 and 4.65 ppm (Figure 28, for glycerol monotetradecanoate **27**). Furthermore, HRMS was used to confirm the mass of the product, indicating that tetradecanoate **27** had indeed been formed (HRMS ESI *m/z* calculated for $[C_{17}H_{34}O_4 + NH_4]^+$: 320.2795, found 320.2802).
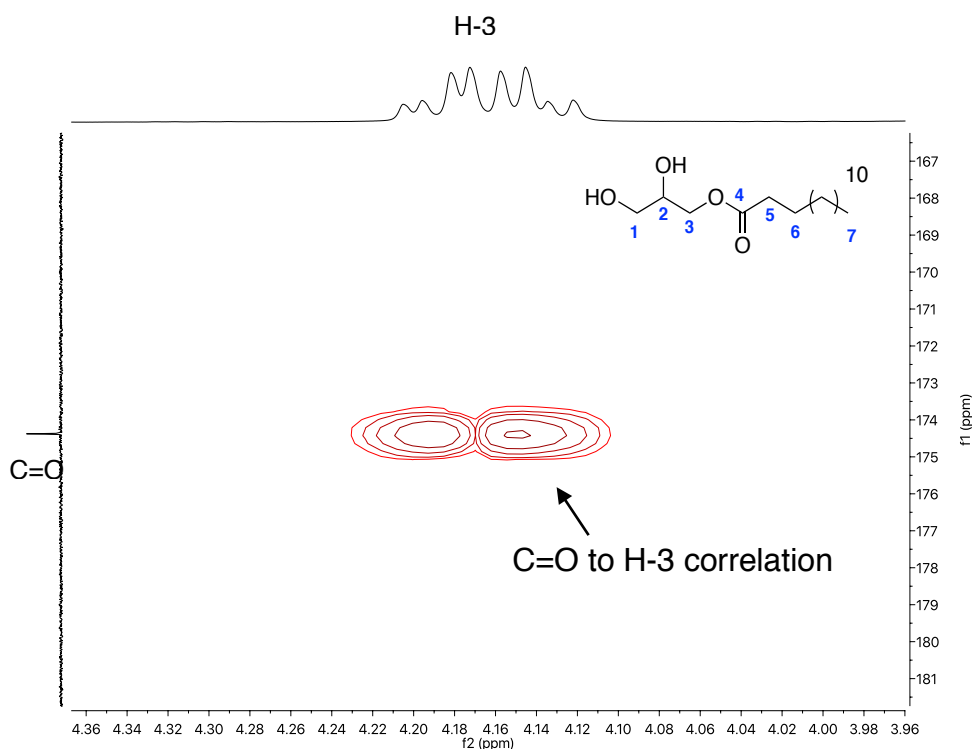
Figure 28: Carbonyl region of the HMBC spectrum of glycerol tetradecanoate **27**

The deprotection reaction had to be carried out at 50°C due to a lack of solubility at lower temperatures, however, over two steps the yields obtained were only moderate to poor (17 - 44%), thought to be due to collapse of the ester linkage. Time constraints did not allow for optimisation of these reactions.

2.2.3.2 Synthesis of branched-chain esters

With the straight-chain esters in hand, the corresponding *iso*-branched glycerolipids were prepared using the same esterification and deprotection methodology (Scheme 29). As with the TMS-trehalose coupling, C_{14+1} acid did not couple smoothly, and could not be optimised due to lack of material. The reactions with C_{16+1} and C_{18+1} acids, however, did proceed as desired, and following acidic isopropylidene deprotection, glycerol monoesters **31** and **32** were produced.
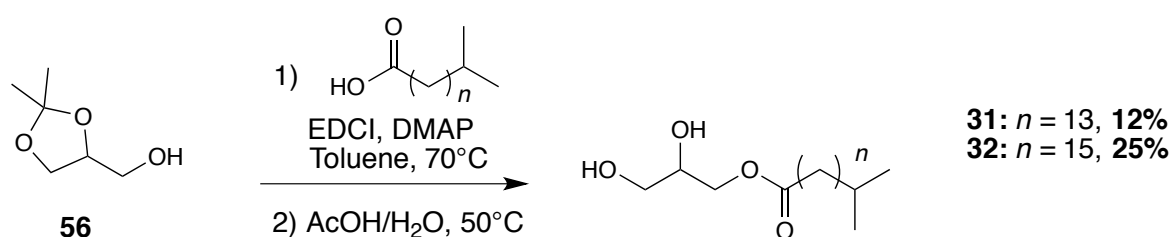
Scheme 29: Synthesis of branched glycerolipids **30 - 32**

Confirmation that the ester functionality has successfully been installed was provided by NMR and MS, as discussed above for the straight-chain analogues. Unfortunately, the yields over two steps were poor (12 and 25%, respectively), and time constraints did not allow for optimisation.

2.3   Biological evaluation

2.3.1   Carbohydrate-modified trehalose glycolipids

As discussed above, none of the target carbohydrate-modified trehalose glycolipids could be made in any appreciable quantity, hence were not able to undergo biological testing.

2.3.2   Maradolipids and glycerolipids

Since all of the straight chain glycerol monoesters (**27 – 29**, **22**) and trehalose diesters (**33 - 34, 12, 14**) and all but the C_{14+1} branched esters were successfully prepared and purified (GMEs **31-32** and TDEs **36 - 37**), evaluation of the biological activity of this series of compounds was performed (Figure 30). GM-CSF differentiated bone marrow derived macrophages were prepared by William Mills (Immunoglycomics Research Group, VUW), then stimulated with 5 mM solutions of the compounds (98:2 phosphate buffered saline (PBS)/dimethylsulfoxide (DMSO), *v/v*) by Dr. Kristel Kodar (Immunoglycomics Research Group, VUW). Untreated cells were used as a negative control, and those stimulated with LPS (100 ng/mL) were used as a positive control.

IL-1β ELISA on straight-chain and iso-branched TDEs and GMEs
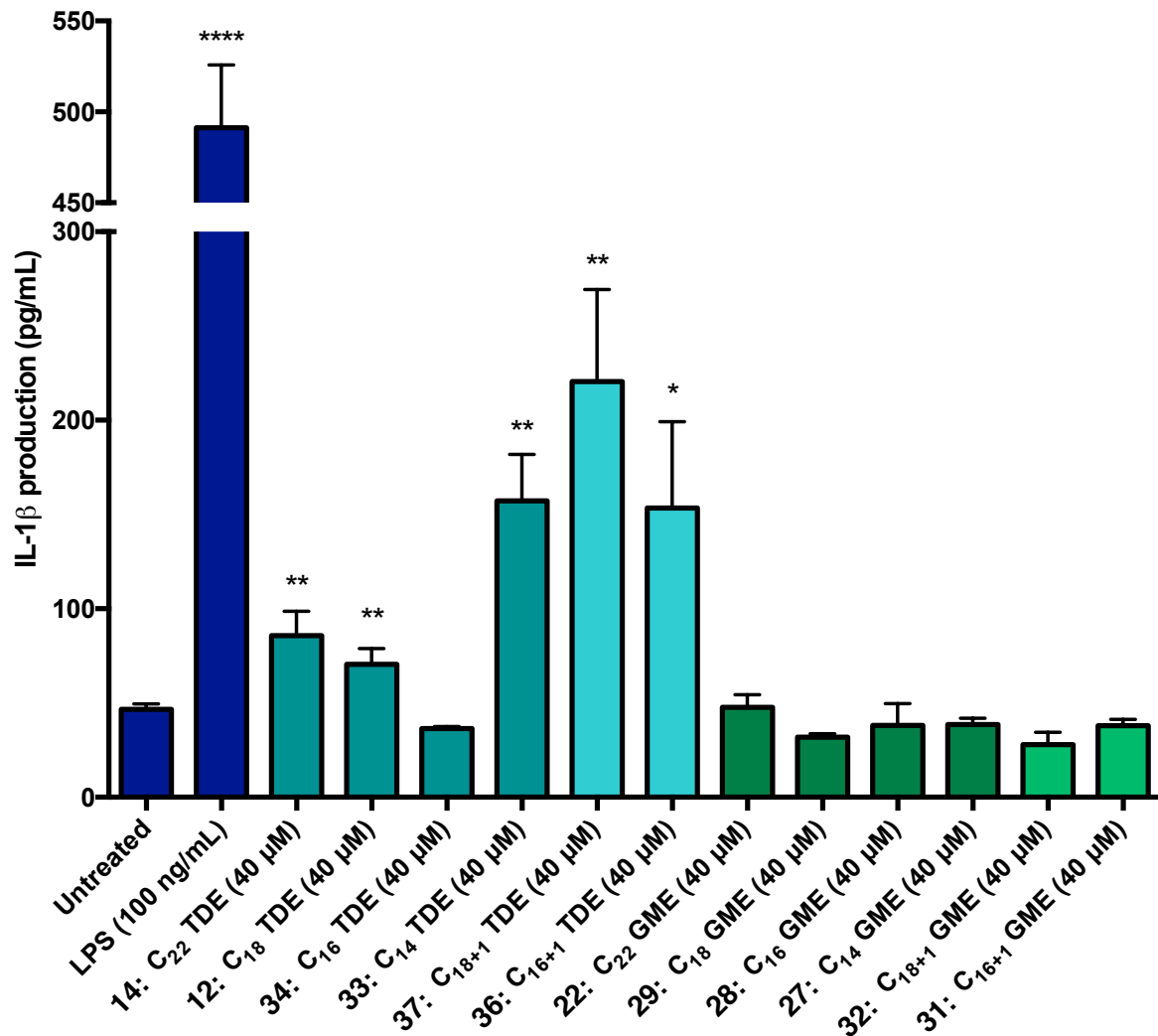
Figure 29: IL-1β production by bone marrow derived macrophages stimulated with glycerol monoesters (GMEs) and trehalose diesters (TDEs); *,**,**** = statistically different from untreated cells at the $p < 0.05$, $p < 0.01$, $p < 0.001$ level, respectively.

Macrophage activation was assessed through ELISA measurement of IL-Iβ production (performed by Dr. Kristel Kodar), and it was found that none of the glycerol monoesters activated murine macrophages. This result confirms the conclusions drawn by Hattori *et al.* whereby glycerol monomycolate and monobehenate were not capable of signaling through mMincle transfected NFAT-GFP reporter cells.[33] No trends could be discerned with lipid length or *iso*-branching due to the general lack of macrophage activation. In contrast,

stimulation of the BMMs with the trehalose glycolipids evaluated did induce production of IL-1β. The straight chain diesters followed the expected trends of increasing activity with longer lipid length, with the marked exception of tetradecanoate **33**. Trehalose dibehenate **14** showed greater activation at the 40 μM concentration than the dioctadecanoate **12**, which displayed greater activation than the hexadecanoate **34**, whose activity was not statistically different from untreated cells. This trend is consistent with that obtained by Khan *et al.* using the same cell line, though measuring different cytokines and using different concentrations of glycolipids.[49] Unexpectedly, the C_{14} diester induced the production of significantly higher levels of IL-1β than any of the other linear glycolipids. This compound has not previously been used to stimulate BMMs, so there are no literature results to compare this response to. Given that diesters of both longer and shorter lipids have been studied, and the trend in activity well established,[49] it seems possible that the activity of **33** is due to experimental error. Of particular concern is the possibility of this shorter chain glycolipid being more soluble in PBS than its longer chain counterparts, thereby increasing the concentration of diester that reached the cells and hence increasing response. In the future, this could be countered by decreasing the concentration of the stock solutions and increasing the proportion of DMSO present, both of which should aid dissolution of all of the trehalose glycolipids and eliminate the possibility of differential solubility affecting results.

The *iso*-branched trehalose diesters **36** and **37** both induced strong production of IL-1β, more so than their straight chain analogues **34** and **12** ($p < 0.05$ and $p < 0.01$, respectively). These results are completely unprecedented, and suggest that the addition of an *iso*-branch at the end of the lipid chain increases macrophage activation. The next step in exploring this structure-activity relationship would be to repeat the above assay using murine BMMs from Mincle deficient mice, which would establish whether or not the macrophage activation observed was due to Mincle activation. If this was the case, the increased activity could be due to enhanced interactions with the hydrophobic groove adjacent to the Mincle CRD. Unfortunately, time constraints meant that this assay could not be performed. Furthermore, the use of human macrophages, which would be expected to respond to stimulation by the glycerol monoesters and thereby allow for analysis of their structure-activity relationship, was also not possible due to time delays in obtaining ethics approval for these assays.

3   Conclusions and Future Directions

3.1   Carbohydrate-modified trehalose glycolipids

To commence synthesis, 2,3,6-benzyl trehalose (**51**) was prepared in excellent yield (85%) over three steps from α,α′-D-trehalose, significantly improving on literature procedures for the regioselective opening of the 4,6-benzylidene group to a 4-*O*-benzyl ether. This intermediate was used to synthesise 4-deoxy trehalose (**23**) in moderate yield (31%) over three steps by using the Barton-McCombie deoxygenation. Similarly, 4-*O*-Me (**24**) trehalose was synthesised from regioselectively protected **51** in good yield (54%) over two steps. In both cases, the methodology used for modification at the 4-hydroxyl had not previously been applied to trehalose, and was shown to be an improvement on reported methods. Synthesis of the behenate diesters of these compounds via a TMS protected carbohydrate intermediate was attempted, however it was discovered that modification at the 4-hydroxyl leads to the primary silyl group not being able to be selectively removed. Therefore, protecting-group free esterification was attempted, and although initial results appeared promising, insufficient quantities of 4-deoxy (**23**) or 4-*O*-Me (**24**) trehalose dibehenate could be obtained for biological testing. In the future, larger-scale attempts at the direct coupling should be made, which should yield enough material to purify, characterise, and test for biological activity

4,6-Benzylidene trehalose **52**, which was generated in the synthesis of the 4-modified trehaloses, was used as the starting material for the preparation of targets **46** and **48** with a deoxygenated or methylated 3-hydroxyl, respectively. Extensive optimisation of reaction conditions was necessary in order to selectively generate 2-Bz **55**, but eventually led to an improvement on literature yields. Methylation and deoxygenation at the 3-position of **55** was then attempted, however it was found that the basic conditions required for both reactions led to migration of the benzoyl group and hence the generation of a complex mixture of products. Protected 3-*O*-Me **72** could be generated in poor yield (30%), however insufficient material could be obtained to proceed with global deprotection. In the future, the use of a weaker base, such as sodium hexamethyldisilazide (NaHDMS), should be trialled, or alternatively, the use of a different protecting group on the 2-OH could be investigated.

3.2 Maradolipids and glycerolipids

17-Methyloctadecanoic acid (**62**) was synthesised in three steps from 15-pentadecanolide (**67**) in moderate yield (25%), utilising a Wittig reaction with isobutyraldehyde (**69**) to install the *iso*-branching at the end of the lipid. Lactone **67** was also used to prepare 15-methylhexadecanoic acid **61** by Grignard reaction with methylmagnesium iodide in good yield over three steps (65%). The synthesis of 13-methyltetradecanoic acid (**60**) was then attempted from the intermediate 15-methylhexadecan-1-ol (**66**) through two rounds of sequential xanthate formation, elimination, and oxidation, however only a very poor yield was obtained (0.3% over 7 steps). To improve this, applying the Wittig methodology to 11-bromoundecanoic acid (**84**) was attempted, however the formation of side products limited the yield severely and insufficient product could be obtained to couple to glycerol or trehalose. In the future, optimising the order of reagent addition, along with finer control of temperature and basicity could be used in order to avoid the formation of byproducts in the Wittig reaction.

Hexa-TMS trehalose (**70**) was successfully coupled to C_{14}, C_{16}, C_{18}, and C_{22} acids in moderate to good yields (19 - 46% over two steps), and likewise C_{16+1} and C_{18+1} acids coupled smoothly (53% and 26% over two steps, respectively). Although acceptable, these yields could be improved on in the future. In particular, there appears to be a relationship between increasing lipid length and decreasing yields, presumably due to issues introduced by the decreasing polarity of the glycolipid. It may be that the highly amphiphilic nature of the compounds makes purification challenging, with material left undissolved on a silica gel column. This problem could be avoided by either trialing the use of a different mobile phase, or utilising a different method for purification, such as reversed phase or size exclusion chromatography. Despite issues with yield, the branched glycolipids **36** and **37** are novel compounds, with above syntheses the first reported for these compounds.

1,2-*O*-isopropylidene glycerol (**56**) was coupled to the same lipids as above in very similar yields. The linear acids gave yields of 17 - 44%, and the *iso*-branched only gave yields of 12 - 25%. Although not novel, the branched esters (**31** and **32**) have only previously been isolated from bacteria, hence these syntheses are the first reported. All of these yields could be improved upon, in particular by optimising the conditions used for deprotection of the

isopropylidene group. The use of TFA, as opposed to acetic acid, and the incorporation of a less polar solvent such as THF could improve yields by minimising loss of the ester group and hence product recovery.

As a preliminary assessment of their structure-activity relationship, the series of glycerol monoesters and trehalose diesters synthesised were tested for their ability to activate murine BMMs. None of the glycerolipids tested were able to active these cells, whereas almost all of the trehalose glycolipids did induce production of the cytokine IL-1β. The C_{22}, C_{18}, and C_{16} diesters displayed the expected trend of decreasing activity with decreasing lipid length, however this was not continued with the C_{14} analogue. This could be due to experimental error, such as problems with glycolipid solubility, or this ester could in fact have an unexpectedly large capacity to activate macrophages. At the concentration tested, the *iso*-branched diesters induced the production of much more IL-1β than their linear counterparts, though this needs to be confirmed by repeating the assays. Future work should also include testing these compounds with Mincle deficient BMMs, in order to confirm that the activation observed is due to binding to Mincle. Additionally, repeating the assays using human macrophages should be performed, in order to elicit a response to the glycerol monoesters, and hence allow for assessment of their structure activity relationship.

4 Experimental

4.1 Chemical Synthesis

Prior to use, DCM was distilled from P_2O_5, THF was distilled from Na, MeOH was distilled, MeI was distilled, MeCN was distilled from CaH, Dowex/H^+ was washed with water, methanol, ethanol, and 6 M HCl, NEt_3 was distilled over KOH, toluene was dried and stored over Na wire, and the following solvents were distilled from drum: acetone, ethyl acetate and petroleum ether (PE). α,α'-D-Trehalose dihydrate (Sigma), behenic acid (BDH Biochem), $CDCl_3$ (Aldrich), C_5D_5N (Cambridge Isotope Labs), D_2O (Apollo Scientific), CD_3OD (Roth), EtOH (Fisher), dried $MgSO_4$ (Pure Science), NaCl (Chem Solute), seasand purified by acid and calcined (Chem Solute), H_2SO_4 (Pancreac), Et_2O (LabServ), DMF extra dry over molecular sieves (Acros Organics), TBAF (Aldrich, 1.0 M solution in THF), BSA (Aldrich), NaH (Aldrich, 60% dispersion in mineral oil), $Pd(OH)_2$/C (Acros Organics), $SnBu_3H$ (Merck), K_2CO_3 (Pancreac), KF (Riedel-de Haën), NH_4Cl (Loba Chemie), TBAI (Riedel-de Haën), $SiEt_3H$ (Aldrich), DMAP (Merck), EDCI (Sigma-Aldrich), AcOH (Pancreac), TFAA (Riedel-de Haën), BnBr (Aldrich), $NaHCO_3$ (Pure Science), HCl (Chem Solute), CS_2 (M&B), isopropanol (Fischer Scientific), sephadex LH-20 (Sigma), Dowex/H^+ (Serva), benzylaldehyde dimethyl acetal (Aldrich), CSA (Aldrich), TFA (ChemImpex), imidazole (Apollo Scientific), AIBN (Aldrich), activated charcoal (BDH), celite (Pure Science), BzCN (Acros), 15-cyclopentadecanolide (Acros Organics), MeMgI (Aldrich), $BF_3 \cdot (OEt)_2$ (Janssen Chimica), $KMnO_4$ (AnalR), Na_2SO_3 (Merck), $BSMe_2$ (Aldrich), 11-bromoundecanoic acid (Aldrich), PPh_3 (Acros Organics), BuLi (2M in cyclohexane, Alridch), isobutyraldehyde (Aldrich), C_5H_5N (Pancreac), tetradecanoic acid (Sigma), octadecanoic acid (ICN Pharmaceuticals), hexadecanoic acid (BDH) and isopropylidene glycerol (BDH Chemicals) were used as received. Reactions were monitored by TLC analysis on Macherey-Nagel silica gel coated plastic sheets (0.20 mm with fluorescent indicator UV_{254}) via detection by UV-absorbtion (254 nm) where relevant and dipping in 10% H_2SO_4 in EtOH or a solution of $KMnO_4$ (0.05 M), K_2CO_3 (0.4 M), and NaOH (0.06%) in water, followed by charring at 150 °C. Column chromatography was performed using Pure Science silica gel (40 – 63 microns), and AccuBOND II ODS-C8 (Aglient) was used for reversed phase chromatography. High resolution mass spectra were recorded on a Waters Q-TOF Premier^{TM} Tandem Mass Spectrometer using positive or negative electro-spray ionisation (ESI). Optical

rotations were recorded on a Autopol II (Rudolph Rearsch Analytical) at 589 nm. Infrared (IR) spectra were recorded as thin films using either a Bruker Platinum-ATR spectrometer or a PerkinElmer Spectrum One FT-IR Spectrometer and are reported in wave numbers (cm^{-1}). Nuclear magnetic resonance spectra were at 20 °C in CDCl$_3$, C$_5$D$_5$N, D$_2$O, or CD$_3$OD using a Varian INOVA operating at 500 MHz. Chemical shifts are given in ppm (δ) relative to the solvent residue peak. NMR peak assignments were made using COSY, heteronuclear single quantum coherence spectroscopy (HSQC), and HMBC 2D experiments.

2,2′,3,3′,4,4′- hexa-*O*-trimethylsilyl-α,α′-D-trehalose (70)
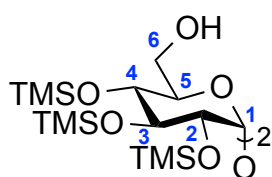
Protected trehalose **70** was prepared according to an adapted procedure of Khan *et al*.[49] α,α′-D-trehalose dihydrate (2.96 g, 7.83 mmol) was co-evaporated[49] with 20 mL dry DMF, then dissolved in 20 mL dry DMF. BSA (20 mL, 0.079 mmol) and TBAF (0.6 mL, 1.0 M in THF, 0.6 mmol) were added, and the resulting yellow solution was stirred under argon for 3 hours. The reaction was then opened to air and quenched with 1 mL of isopropanol, then diluted with 100 mL methanol. K$_2$CO$_3$ (204 mg, 1.47 mmol) was then added and the solution stirred for 3 hours before adding sufficient acetic acid to neutralise the reaction. The methanol and isopropanol was then removed *in vacuo* to leave crude product dissolved in DMF. This yellow oil was then diluted with Et$_2$O and extracted with NaHCO$_3$ (aq), brine, and water to give the partially purified product as a white solid. Purification was then carried out by silica gel flash column chromatography (20:1 - 0:1 PE/EA, *v/v*) to yield 2,2′,3,3′,4,4′-hexa-*O*-trimethylsilyl-α,α′-D-trehalose as a white solid (3.56 g, 4.59 mmol, 58%). R$_F$ = 0.25 (9:1 PE/EA, *v/v*); [α]$^{22.7}_{589}$ = +103 (CH$_2$Cl$_2$, c = 10 mg/mL); IR (film): 2959, 1250, 1075, 870, 836, 747 cm^{-1}; ^{1}H NMR (500 MHz, CDCl$_3$) δ 4.90 (d, $^3J_{1,2}$ = 2.4 Hz, 2H, H-1), 3.89 (t, $^3J_{3,2}$ = $^3J_{3,4}$ = 9.2 Hz, 2H, H-3), 3.85 (d, $^3J_{5,4}$ = 9.5 Hz, 2H, H-5), 3.72 (d, $^2J_{6a,6b}$ = 10.8 Hz, 2H, H-6a), 3.68 (dd, $^2J_{6b,6a}$ = 12.3 Hz, $^3J_{6b,5}$ = 3.5 Hz, 2H, H-6b), 3.47 (t, $^3J_{4,3}$ = $^3J_{4,5}$ = 9.3 Hz, 2H, H-4), 3.42 (dd, $^3J_{2,3}$ = 9.3 Hz, $^3J_{2,1}$ = 2.8 Hz, 2H, H-2), 0.16 (s, 18H, Si(CH$_3$)$_3$), 0.14 (s, 18H, Si(CH$_3$)$_3$), 0.12 (s, 18H, Si(CH$_3$)$_3$); ^{13}C NMR (125 MHz, CDCl$_3$) δ 94.8 (C-1), 73.5 (C-3), 73.1 (C-5), 72.9 (C-2), 71.5 (C-4), 61.8 (C-6), 1.15 (Si(CH$_3$)$_3$), 1.00 (Si(CH$_3$)$_3$), 0.22 (Si(CH$_3$)$_3$); HRMS (ESI) *m/z* calculated for [C$_{30}$H$_{70}$O$_{11}$Si$_6$ +NH$_4$]$^+$ 792.3872, found 792.3094. The data recorded was consistent with the published literature.[60]

6,6′-di-O-docosanoate-α,α′-D-trehalose (14)
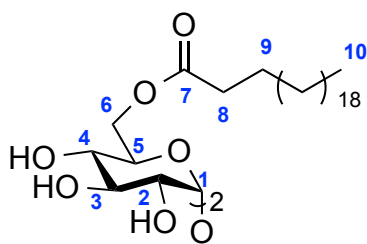
Trehalose glycolipid **14** was prepared according to an adapted procedure from Khan.[49] 2,2′,3,3′,4,4′- hexa-O-trimethylsilyl-α,α′-D-trehalose (51 mg, 0.0657 mmol) and docosanoic acid (101 mg, 0.296 mmol) were co-evaporated together with dry toluene, then suspended in 1 mL dry toluene under an atmosphere of argon. To the reaction mixture, EDCI (57.2 mg, 0.403 mmol) and DMAP (10.3 mg, 0.0844 mmol) were added, and the resultant suspension heated with stirring to 80°C for 23 hours, at which time TLC analysis in 9:1 PE/EA (*v/v*) showed reaction completion. The reaction was cooled, diluted with Et_2O (5 mL), then washed with $NaHCO_3$ (aq) (5 mL), NaCl (aq) (5 mL), and H_2O (5 mL), and the combined aqueous phases re-extracted with 5 mL Et_2O. The combined organic phases were dried with anhydrous magnesium sulfate, filtered, and concentrated to give a colourless oil. The crude product was purified by size exclusion chromatography (Sephadex LH-20, 1:1 DCM/MeOH, *v/v*), yielding 2, 2′,3,3′,4,4′- hexa-O-trimethylsilyl-6,6′-di-O-docosanoate-α,α′-D-trehalose as a white solid (74.5 mg, 0.0657 mmol, 80%).

2,2′,3,3′,4,4′- hexa-O-trimethylsilyl-6,6′-di-O-docosacanoate-α,α′-D-trehalose (93.3 mg, 0.0657 mmol) was dissolved in 2 mL of 3:1 DCM:MeOH (*v/v*) and Dowex/H^+ was added. The reaction was allowed to proceed without stirring for 60 mins, at which time TLC analysis in 9:1 PE/EA (*v/v*) showed reaction completion. The resin was filtered off and the colourless solution was concentrated to give a white solid. Flash chromatography was carried out to purify the product (1:0 – 9:1, EA/MeOH, *v/v*), yielding 6,6′-di-O-docosocanoate-α,α′-D-trehalose (26.1 mg, 0.0262 mmol, 40%) as a white solid. R_F = 0.54 (EA:MeOH, 4:1, *v/v*); $[\alpha]^{19.3}_{589}$ = 43.6 (c = 10 mg/mL, C_5H_5N); (IR (film): 3361, 2922, 2849, 1735, 1154, 1016 cm^{-1}; ^1H NMR (500 MHz, C_5D_5N) δ 5.91 (d, $^3J_{1,2}$ = 3.6 Hz, 2H, H-1), 5.10 (dd, $^3J_{5,4}$ = 10.2 Hz, $^3J_{5,6b}$ = 4.6 Hz, 2H, H-5), 5.02 (m, H_2O + H-6a), 4.84 (dd, $^2J_{6b,6a}$ = 12.1 Hz, $^3J_{6b,5}$ = 5.6 Hz, 2H, H-6b), 4.77 (t, $^3J_{3,2}$ = $^3J_{3,4}$ = 9.3 Hz, 2H, H-3), 4.33 (dd, $^3J_{2,3}$ = 9.5 Hz, $^3J_{2,1}$ = 3.2, 2H, H-2), 4.19 (t, $^3J_{3,2}$ = $^3J_{3,4}$ = 9.3 Hz, 2H, H-4), 2.34 (m, 6H, H-8), 1.64 (pent, J = 7.6 Hz, 6H, H-9), 1.33 (m, 72H, CH_2), 0.89 (t, J = 6.4 Hz, 6H, H-10); ^{13}C NMR (125 MHz, C_5D_5N) δ 174.0 (C-7), 96.1 (C-1) 75.2 (C-3), 73.7 (C-2), 72.4 (C-4), 71.9 (C-5), 64.7 (C-6), 34.8 (C-8), 32.5 (CH_2), 30.40 (CH_2), 30.38 (CH_2), 30.35 (CH_2), 30.30 (CH_2), 30.29 (CH_2), 30.1 (CH_2), 29.98 (CH_2), 29.96 (CH_2), 29.7 (CH_2), 25.6 (C-9), 23.3 (CH_2), 14.7 (C-10). HRMS (ESI) *m/z*

calculated for $[C_{56}H_{107}O_{13} + NH_4]^+$: 1004.7972, found 1004.7972. The data recorded was consistent with the published literature.[49]

4,4′,6,6′-di-*O*-benzylidene-α,α′-D-trehalose (52)
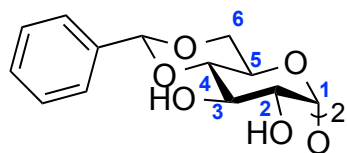
Benylidene trehalose **52** was prepared in an adapted procedure of Sarpe & Kulkarni.[53] α,α′-D-trehalose dihydrate (4.55 g, 12.0 mmol) was co-evaporated with ethanol, twice with distilled methanol, and twice with dry DMF (100 mL each), then dissolved in 100 mL of dry DMF and benzaldehyde dimethylacetal (4.6 mL, 30.1 mmol) and camphirfulfonic acid (CSA, 59.3 mg, 0.602 mmol) were added. The resulting colourless solution was reacted *in vacuo* (200 mbar) at 60°C for 3 hours, then concentrated nearly to dryness *in vacuo*, and the resulting pale yellow syrup was dissolved in 100 mL distilled methanol and dry loaded onto 20 mL silica gel. Isocratic column flash chromatography with EA was then used for purification, yielding pure 4,4′,6,6′-di-*O*-benzylidene-α,α′-D-trehalose, which after triple trituration with water to remove residual DMF, appeared as a white crystalline material (6.04 g, 11.7 mmol, 97%). R_F = 0.84 (4:1 EA:MeOH, *v/v*); $[\alpha]^{24.5}_{589}$ = +84.08 (c = 50 mg/mL, EtOH); IR (film): 3618, 3595, 2979, 2901, 1057 cm^{-1}; ^{1}H NMR (500 MHz, CD$_3$OD) δ 7.50 (m, 4H, *o*-H$_{Ar}$), 7.33 (m, 6H, *m*+*p*-H$_{Ar}$), 5.57 (s, 2H, H-CO$_2$Ar), 5.13 (d, $^3J_{1,2}$ = 4.2 Hz, 2H, H-1), 4.23 (dd, $^2J_{6a,6b}$ = 10.0 Hz, $^3J_{6b,5}$ = 4.9 Hz, 2H, H-6a), 4.13 (td, $^3J_{5,6b}$ = $^3J_{5,4}$ = 9.9 Hz, $^3J_{5,6a}$ = 4.9 Hz, 2H, H-5), 4.03 (t, $^3J_{3,2}$ = $^3J_{3,4}$ = 9.3 Hz, 2H, H-3), 3.73 (t, $^2J_{6b,6a}$ = $^3J_{6b,5}$ = 10.2 Hz, 2H, H-6b), 3.63 (dd, $^3J_{2,3}$ = 9.6 Hz, $^3J_{2,1}$ = 3.8 Hz, 2H, H-2), 3.49 (t, $^3J_{4,3}$ = $^3J_{4,5}$ = 9.5 Hz, 2H, H-4); ^{13}C NMR (125 MHz, CD$_3$OD) δ 139.2 (*i*-C$_{Ar}$), 129.9 (*p*-C$_{Ar}$), 129.0 (*m*-C$_{Ar}$), 127.5 (*m*-C$_{Ar}$), 103.1 (CO$_2$Ar), 96.4 (C-1), 83.0 (C-4), 73.8 (C-2), 71.5 (C-3), 69.9 (C-6), 64.2 (C-5); HRMS ESI *m/z* calculated for $[C_{26}H_{30}O_{11} + H]^+$: 519.1861, found 519.1884. Data recorded was consistent with the published literature.[64]

2,2′,3,3′-tetra-*O*-benzyl-4,4′,6,6′-di-*O*-benzylidene-α,α′-D-trehalose (71)
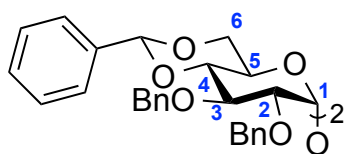
Fully protected trehalose **71** was prepared in an adapted procedure of Hui & Chang.[63] 4,4′,6,6′-di-*O*-benzylidene-α,α′-D-trehalose (**23**, 1.78 g, 3.44 mmol) was co-evaporated with dry toluene, ethanol and dry THF (25 mL each), then dissolved in 25 mL of dry THF. Sodium hydride (1.3888 g, 34.72 mmol) was added to the colourless solution, and the resulting grey

suspension was stirred under argon for 60 min at 0°C, followed by addition of TBAI (265 mg, 0.716 mmol) and BnBr (2.0 mL, 17 mmol). The ensuing white suspension was stirred for 19 hours, then quenched with MeOH (1 mL), diluted with EA (25 mL) and neutralised with aqueous HCl (5 mL) and washed with water (50 mL). The organic phase was extracted with brine and water (50 mL each), and the combined aqueous phases re-extracted with EA (2 x 100 mL). The combined organic phases were dried using MgSO$_4$, filtered, and concentrated *in vacuo* to give a yellow oil. The crude product was purified by silica gel column chromatography (95:5 - 72:28 PE/EA, *v/v*) yielding 2,2′,3,3′-tetra-*O*-benzyl 4,4′,6,6′-di-*O*-benzylidene-α,α′-D-trehalose as a white crystalline solid (2.64 g, 3.02 mmol, 88%). R$_F$ = 0.39 (PE:EA, 3:1, *v/v*); $[\alpha]^{24.5}_{589}$ = +46.9 (c = 50 mg/mL, CHCl$_3$,); IR (film): 2923, 2870, 2050, 1086, 984 cm^{-1}; ^{1}H NMR (500 MHz, CDCl$_3$): δ 7.50 - 7.28 (m, 30 H, H$_{Ar}$), 5.55 (s, 2H, H-CO$_2$Ar), 5.11(dm, $^3J_{1,2}$ = 3.2 Hz, 2H, H-1), 4.95 (d, $^2J_{a,b}$ = 10.9 Hz, 2H, CH-a, 3-*O*-Bn), 4.84 (m, 4H, CH-b, 3-*O*-Bn + CH-a, 2-*O*-Bn), 4.73 (d, $^2J_{a,b}$ = 12.0 Hz, 2H, CH-b, 2-*O*-Bn), 4.27 (dt, $^3J_{5,4}$ = $^3J_{5,6a}$ = 9.9 Hz, $^3J_{5,6b}$ = 4.8 Hz, 2H, H-5), 4.16 - 4.10 (m, 4H, H-3 + H-6a), 3.68 - 3.60 (m, 6H, H-6b + H-4 + H-2); ^{13}C NMR (125 MHz, CDCl$_3$) δ 138.9 - 137.7 (C$_{Ar}$), 129.0 - 126.3 (CH$_{Ar}$), 101.4 (CO$_2$Ar), 95.1 (C-1), 82.5 (C-4), 78.9 (C-2), 78.8 (C-3), 75.5 (CH$_2$, 3-*O*-Bn), 74.0 (CH$_2$, 2-*O*-Bn), 69.1 (C-6), 63.1 (C-5); HRMS ESI *m/z* calculated for $[C_{54}H_{58}O_9 + NH_4]^+$: 896.4004, found 896.4039. Data recorded was consistent with literature values.[63]

2,2′,3,3′,6,6′-hexa-*O*-benzyl-α,α′-D-trehalose (51)
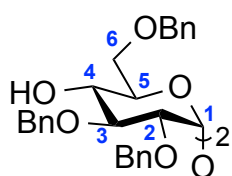
2,2′,3,3′-*O*-tetrabenzyl-4,4′,6,6′-*O*-dibenzylidene-α,α′-D-trehalose (306 mg, 0.348 mmol) was co-evaporated three times with dry toluene (3 mL each), then dissolved in 2 mL dry DCM. Et$_3$SiH (0.66 mL, 4.1 mmol), TFAA (0.10 mL, 0.68 mmol), and TFA (0.27 mL, 3.4 mmol) were added, and the reaction stirred under argon for 30 min at 0°C, then 15 mins at room temperature. The reaction was then quenched with aqueous sodium bicarbonate, the layers were separated, and the aqueous phase was re-extracted with DCM. The combined organic phases were dried with MgSO$_4$, filtered, and concentrated *in vacuo* to give 2,2′,3,3′,6,6′-hexa-*O*-benzyl-α,α′-D-trehalose as a colourless oil (312 mg, quantitative). R$_F$: 0.51 (2:1 PE/EA, *v/v*); $[\alpha]^{24.5}_{589}$ = +71.26 (c = 100 mg/mL, CHCl$_3$,); IR (film): 3435, 3066, 3014, 2954, 2913, 2875, 1455, 1216, 1153, 1059, 751 cm^{-1}; ^{1}H NMR (500 MHz, CDCl$_3$) δ 7.39 -

7.24 (m, 30H, H$_{Ar}$), 5.24 (d, $^3J_{1,2}$ = 3.2 Hz, 2H, H-1), 5.01 (d, $^2J_{a,b}$ = 11.2 Hz, 2H, CH-a, 3-O-Bn), 4.80 (d, $^2J_{b,a}$ = 11.2 Hz, 2H, CH-b, 3-O-Bn), 4.70 (d, $^2J_{a,b}$ = 12.3 Hz, 2H, CH-a, 2-O-Bn), 4.64 (d, $^2J_{b,a}$ = 12.2 Hz, 2H, CH-b, 2-O-Bn), 4.51 (d, $^2J_{a,b}$ = 12.0 Hz, 2H, CH-a, 6-O-Bn), 4.45 (d, $^2J_{b,a}$ = 12.2 Hz, 2H, CH-b, 6-O-Bn), 4.12 (td, $^3J_{5,4}$ = $^3J_{5,6a}$ = 8.8 Hz, $^3J_{5,6b}$ = 3.3 Hz, 2H, H-5), 3.88 (t, $^3J_{3,2}$ = $^3J_{3,4}$ = 9.3 Hz, 2H, H-3), 3.68 (t, $^3J_{4,3}$ = $^3J_{4,5}$ = 9.4 Hz, 2H, H-4), 3.57 (dd, $^3J_{2,3}$ = 9.2 Hz, $^3J_{2,1}$ = 3.1 Hz, 2H, H-2), 3.52 (dd, $^2J_{6a,6b}$ = 10.8 Hz, $^3J_{6a,5}$ = 4.2 Hz, 2H, H-6a), 3.47 (dd, $^2J_{6b,6a}$ = 10.2 Hz, $^3J_{6b,5}$ = 2.9 Hz, 2H, H-6b); ^{13}C NMR (125 MHz, CDCl$_3$) δ 138.9 - 138.1 (C$_{Ar}$), 128.7 - 127.6 (C$_{Ar}$), 94.3 (C-1), 81.2 (C-3), 79.1 (C-2), 75.4 (CH$_2$, 3-O-Bn), 73.7 (CH$_2$, 6-O-Bn), 72.6 (CH$_2$, 2-O-Bn), 70.9 (C-4), 70.7 (C-5), 69.4 (C-6); HRMS ESI m/z calculated for [C$_{54}$H$_{58}$O$_{11}$ + NH$_4$]$^+$: 900.4317, found 900.4355. Data recorded was consistent with literature values.[61]

2,2′,3,3′,6,6′-hexa-O-benzyl-4,4′-di-S-methylthiocarbonyl-α,α′-D-trehalose (72)
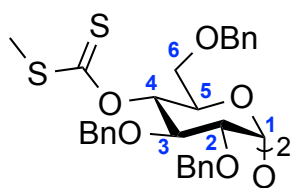
2,2′,3,3′,6,6′-hexa-O-benzyl-α,α′-D-trehalose (541 mg, 0.613 mmol), was co-evaporated in toluene (3 × 3 mL) then dissolved in dry THF (5 mL) then cooled on ice. To this yellow solution, sodium hydride (60% in oil, 181 mg, 4.52 mmol) was added portion-wise, interspersed with imidazole (30.0 mg, 0.441 mmol) were added and the resulting yellow suspension was stirred for 30 mins, then allowed to warm to room temperature. Carbon disulfide (0.22 mL, 3.7 mmol) and methyl iodide (0.25 mL, 3.7 mmol) were then added and the reaction left to stir for 30 minutes. TLC analysis (2:1 PE/EA, v/v) at this point indicated incomplete reaction, so a further 331.7 mg NaH (8.29 mmol) was added. After 3 hours, 0.5 mL CS$_2$ was added (8.4 mmol), then after an additional 30 min an extra 5 mL dry THF was added to enhance solubility, followed by 0.55 mL MeI (8.8 mmol). The reaction was left to stir for 17 hours, then the reaction mixture was diluted with DCM (5 mL) and extracted with saturated aqueous ammonium chloride (10 mL) and water (10 mL). The combined aqueous phases were re-extracted with DCM (2 × 10 mL). The combined organic phases were then dried over anhydrous magnesium sulfate, filtered, and concentrated under reduced pressure to afford a yellow oil. This was purified by normal phase flash chromatography (3:1 PE/EA, v/v), yielding 2,2′,3,3′,6,6′-hexa-O-benzyl-4,4′-di-S-methylthiocarbonyl-α,α′-D-trehalose (555 mg, 0.523 mmol, 85%) as a yellow oil. R$_F$: 0.66 (2:1 PE/EA, v/v); [α]$^{24.5}_{589}$ = +90.32 (c = 50 mg/mL, CHCl$_3$); IR (film): 3014, 2933, 2866, 1216, 749 cm^{-1}; ^{1}H NMR (500 MHz, CDCl$_3$) δ 7.30 - 7.23 (m, 30H, H$_{Ar}$), 6.10 (t, J$_{4,3/5}$ = 9.0 Hz, 2H, H-4),

5.27 (d, $^3J_{1,2}$ = 3.5 Hz, 2H, H-1), 4.80 (d, $^2J_{a,b}$ = 10.8 Hz, 2H, CH-a, 3-O-Bn), 4.74 (d, $^2J_{b,a}$ = 10.8 Hz, 2H, CH-b, 3-O-Bn), 4.70 (d, $^2J_{a,b}$ = 11.9 Hz, 2H, CH-a, 2-O-Bn), 4.66 (d, $^2J_{b,a}$ = 11.2 Hz, 2H, CH-b, 2-O-Bn), 4.43 (d, $^2J_{a,b}$ = 11.5 Hz, 2H, CH-a, 6-O-Bn), 4.38 (d, $^2J_{b,a}$ = 11.9 Hz, 2H, CH-b, 6-O-Bn), 4.34 (m, 2H, H-5), 4.17 (t, $^3J_{3,2}$ = $^3J_{3,4}$ = 9.2 Hz, 2H, H-3), 3.69 (dd, $^3J_{2,3}$ = 9.3 Hz, $^3J_{2,1}$ = 3.5 Hz, 2H, H-2), 3.40 (dd, $^2J_{6a,6b}$ =11.1 Hz, $^3J_{6a,5}$ = 2.6 Hz, 2H, H-6a), 3.28 (dd, $^2J_{6b,6a}$ = 11.0 Hz, $^3J_{6b,5}$ = 4.2 Hz, 2H, H-6b), 2.55 (s, 6H, S-CH$_3$); ^{13}C NMR (125 MHz, CDCl$_3$) δ 215.4 (CO(S)S), 138.2 (*i*-C, 3-O-Bn), 138.0 (*i*-C, 2-O-Bn), 137.8 (*i*-C, 6-O-Bn), 128.4 - 127.4 (C$_{Ar}$), 93.2 (C-1), 79.2 (C-3), 79.0 (C-4) 78.9 (C-2), 75.2 (CH$_2$, 3-O-Bn), 73.7 (CH$_2$, 6-O-Bn), 73.3 (CH$_2$, 2-O-Bn), 69.3 (C-5), 68.3 (C-6), 19.5 (S-CH$_3$); HRMS ESI *m/z* calculated for [C$_{58}$H$_{62}$O$_{11}$S$_4$ + NH$_4$]$^+$: 1080.3513, found 1080.3545.

2,2′,3,3′,6,6′-hexa-O-benzyl-4,4′-dideoxy-α,α′-D-trehalose (73)
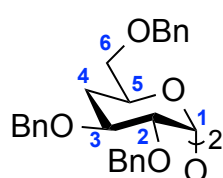
2,2′,3,3′,6,6′-O-hexabenzyl-4,4′-dimethylthiocarbonyl-α,α′-D-trehalose (555 mg, 0.523 mmol) was co-evaporated in toluene (3 × 15 mL) then dissolved in 10 mL toluene. To this yellow solution, azobisisobutyronitrile (33.7 mg, 0.205 mmol) and tributyl tin hydride (1.4 mL, 5.2 mmol) were added and the resulting colourless solution was heated to reflux. After 25 min, the reaction had turned black, and an NMR aliquot indicated full starting material consumption, hence the reaction was cooled. Activated charcoal and additional DCM were added, and the reaction left to stir for 18 hours. The black suspension was then filtered over celite, concentrated under reduced pressure, then the colourless oil purified by repeated silica gel flash column chromatography (10:1 - 3:1 PE/EA, *v/v*) to yield 2,2′,3,3′,6,6′-hexa-O-benzyl-4,4′-dideoxy-α,α′-D-trehalose (235 mg, 0.261 mmol, 50%) as a colourless oil. R$_F$: 0.66 (2:1 PE/EA, *v/v*); [α]$^{24.5}_{589}$ = +115.44 (c = 100 mg/mL, CHCl$_3$); IR (film): 3064, 3030, 2925, 2863, 1455, 1367, 1084, 1069, 985, 750 cm^{-1}; ^{1}H NMR (500 MHz, CDCl$_3$) δ 7.39 - 7.25 (m, 30H, H$_{Ar}$), 5.33 (d, $^3J_{1,2}$ = 3.4 Hz, 2H, H-1), 4.77 - 4.63 (m, 8H, CH$_2$ 3-O-Bn + CH$_2$ 2-O-Bn), 4.53 (d, $^2J_{a,b}$ = 12.2 Hz, 2H, CH-a, 6-O-Bn), 4.49 (d, $^2J_{b,a}$ = 12.0 Hz, 2H, CH-b, 6-O-Bn), 4.36 (m, 2H, H-5), 4.06 (td, $^3J_{3,2}$ = $^3J_{3,4ax}$ = 10.3 Hz, $^3J_{3,4eq}$ = 4.9 Hz, 2H, H-3), 3.55 (dd, $^3J_{2,3}$ = 9.5 Hz, $^3J_{2,1}$ = 3.4 Hz, 2H, H-2), 3.42 (dd, $^2J_{6a,6b}$ = 10.2 Hz, $^3J_{6a,5}$ = 3.9 Hz, 2H, H-6a), 3.35 (dd, $^2J_{6b,6a}$ = 10.2 Hz, $^3J_{6b,5}$ = 5.1 Hz, 2H, H-6b), 2.11 (ddd, $^2J_{4eq/4ax}$ = 12.9 Hz, $^3J_{4eq,5}$ = 5.1 Hz, $^3J_{4eq,3}$ = 2.2 Hz, 2H, H-4$_{eq}$), 1.59 (q, $^2J_{4ax/4eq}$ = $^3J_{4ax,5}$ = $^3J_{4ax,3}$ = 12.1 Hz, 2H, H-4$_{ax}$); ^{13}C NMR (500 MHz, CDCl$_3$) δ 139.1 - 127.5 (C$_{Ar}$), 94.1 (C-1),

80.3 (C-2), 76.9 (C-3), 75.3 (CH$_2$, 6-O-Bn), 73.6 (CH$_2$, 2-O-Bn), 72.4 (C-6), 72.1 (CH$_2$, 3-O-Bn), 67.4 (C-5), 33.6 (C-4); HRMS ESI m/z calculated for [C$_{54}$H$_{58}$O$_9$ + NH$_4$]$^+$: 868.4419, found 868.4440.

4,4′-dideoxy-α,α′-D-trehalose (45)
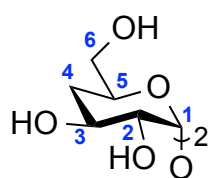
4-deoxy trehalose **45** was prepared in an adapted procedure of Lin $et\ al.$[56] 2,2′,3,3′,6,6′-hexa-O-benzyl-4,4′-dideoxy-α,α′-D-trehalose (319 mg, 0.4482 mmol) was co-evaporated thrice in toluene, then dissolved in dry methanol 5 mL). To this colourless solution, catalytic Pd(OH)$_2$/C was added and hydrogen bubbled through the grey suspension. The reaction was left to stir for a total of 5 days, over which period additional quantities of palladium were added upon suspension clumping and new hydrogen balloons were added as necessary. Once TLC analysis in 2:1 PE/EA (v/v) indicated complete reaction, the suspension was filtered through Celite and concentrated under reduced pressure to crude product as a white solid. Purification was carried out using reverse phase chromatography (0:1 - 1:0, MeOH/H$_2$O, v/v), yielding 4,4′-dideoxy-α,α′-D-trehalose (101 mg, 324 mmol, 72%) as a white solid. R$_F$: 0.67 (EA:MeOH, 4:1, v/v); IR (film): 3403, 2950, 2901, 2850, 1407, 1113, 802 cm^{-1}; ^{1}H NMR (500 MHz, D$_2$O) δ 5.19 (d, $^3J_{1,2}$ = 3.9 Hz, 2H, H-1), 4.07 (m, 4H, H-5 +H-3), 3.65 (dd, $^2J_{6a,6b}$ = 12.3 Hz, $^3J_{6a,5}$ = 3.0 Hz, 2H, H-6a), 3.56 (dd, $^2J_{6b,6a}$ = 12.2 Hz, $^3J_{6b,5}$ = 6.4 Hz, 2H, H-6b), 3.53 (dd, $^3J_{2,3}$ = 9.3 Hz, $^3J_{2,1}$ = 2.9 Hz, 2H, H-2), 1.99 (dd, $^2J_{4eq,4ax}$ = 13.1 Hz, $^3J_{4eq,5}$ = 3.5 Hz, 2H, H-4$_{eq}$), 1.45 (q, $^2J_{4ax,4eq}$ = $^3J_{4ax,5}$ = 11.8 Hz, 2H, H-4$_{ax}$); ^{13}C NMR (125 MHz, D$_2$O) δ 93.6 (C-1), 72.8 (C-2), 69.0 (C-5), 66.6 (C-3), 65.5 (C-6), 34.1 (C-4); HRMS ESI m/z calculated for [C$_{12}$H$_{22}$O$_9$ + NH$_4$ + H]$^{2+}$: 329.1675, found 329.1682. Data recorded was consistent with the published literature.[56]

2,2′,3,3′,6,6′-hexa-O-benzyl-4,4′-di-O-Me-α,α′-D-trehalose (74)
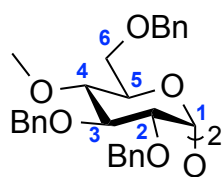
2,2′,3,3′,6,6′-hexa-O-benzyl-α,α′-D-trehalose (303 mg, 0.343 mmol) was co-evaporated twice in dry toluene, then dissolved in 5 mL dry DMF and 5 mL MeI. Sodium hydride (137 mg, 3.43 mmol) was added to the orange solution, forming a light yellow suspension. After 35 mins, an additional portion of NaH (127 mg, 3.18 mmol) was added, forming an offwhite suspension, and the

reaction left to stir for 4.5 hours, when TLC analysis in 2:1 PE/EA (*v/v*) indicated complete reaction. The reaction was quenched with MeOH (0.5 mL), diluted with 75 mL DCM and extracted with $NH_4Cl_{(aq)}$, $NaCl_{(aq)}$, and H_2O (75 mL each). The combined aqueous phases were re-extracted with 2 x 75 mL DCM, and the organic phases combined and dried with anhydrous magnesium sulfate, filtered, and concentrated *in vacuo*. The resulting colourless oil was purified by silica gel flash chromatography column (1:0 - 3:1 PE/EA, *v/v*) to give 2,2′,3,3′,6,6′-hexa-*O*-benzyl-4,4′-di-*O*-Me-α,α′-D-trehalose as a colourless oil (237.5 mg, 0.257 mmol, 75%). R_F: 0.59 (1:1 PE/EA, *v/v*); $[\alpha]^{24.2}_{589}$ = +114 (c = 1.0 mg/mL, CH_2Cl_2,); IR (film): 3088, 3063, 3030, 2930, 1496, 1453, 1364, 1208, 1156, 1098, 997, 734, 697 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.40 - 7.25 (m, 30H, H_{Ar}), 5.19 (d, $^3J_{1,2}$ = 3.4 Hz, 2H, H-1), 4.97 (d, $^2J_{a,b}$ = 10.7 Hz, 2H, CH-a, 3-*O*-Bn), 4.86 (d, $^2J_{b,a}$ = 10.8 Hz, 2H, CH-b, 3-*O*-Bn), 4.67 (m, 4H, CH-a + CH-b, 2-*O*-Bn), 4.58 (d, $^2J_{a,b}$ = 12,2 Hz, 2H, CH-a, 6-*O*-Bn), 4.43 (d, $^2J_{b,a}$ = 11.9 Hz, 2H, CH-b, 6-*O*-Bn), 4.09 (m, 2H, H-5), 3.93 (t, $^3J_{3,2}$ = $^3J_{3,4}$ = 9.4 Hz, 2H, H-3), 3.54 (dd, $^3J_{2,3}$ = 9.7 Hz, $^3J_{2,1}$ = 3.6 Hz, 2H, H-2), 3.50 (m, 8H, H-4 + CH_3), 3.43 (d, $^2J_{6a,6b}$ = 9.8 Hz, 2H, H-6a), 3.39 (dd, $^2J_{6b,6a}$ =10.8 Hz, $^3J_{6b,5}$ = 1.4 Hz, 2H, H-6b); ^{13}C NMR (500 MHz, $CDCl_3$) δ 139.1 - 127.6 (C_{Ar}), 94.8 (C-1), 81.9 (C-3), 79.40 (C-2/4), 79.35 (C-24), 75.7 (CH_2, 3-*O*-Bn), 73.6 (CH_2, 6-*O*-Bn), 72.9 (C-2), 70.8 (C-5), 68.3 (C-6), 60.7 (CH_3); HRMS ESI *m/z* calculated for $[C_{56}H_{62}O_{11} + Na]^+$: 933.4184, found 933.4214.

4,4′-di-*O*-Me-α,α′-D-trehalose (46)
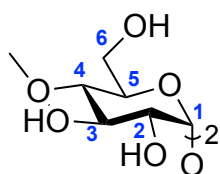
2,2′,3,3′,6,6′-hexa-*O*-benzyl-4,4′-di-*O*-Me-α,α′-D-trehalose (103 mg, 0.113 mmol) was dissolved in 10 mL distilled methanol, $Pd(OH)_2$/C (54.0 mg, 52 wt%) was added, and the reaction stirred under an atmosphere of H_2 for 20 hours. The resulting grey suspension was filtered through a column of Celite to give a white solid. The crude product was purified by normal phase chromatography on silica gel (1:0 - 0:1 EA/MeOH, *v/v*) to yield 4,4′-di-*O*-Me-α,α′-D-trehalose as a white solid (29.4 mg, 0.0794 mmol, 70%). R_F: 0.29 (4:1 EA/MeOH, *v/v*); $[\alpha]^{22.7}_{589}$ = +169 (c = 1.0 mg/mL, MeOH); IR (film): 3362, 2934, 2841, 1647, 1451, 1376, 1125, 1070, 993 cm^{-1}. ^{1}H NMR (500 MHz, CD_3OD) δ 5.07 (d, $^3J_{1,2}$ = 3.9 Hz, 2H, H-1), 3.86 (t, $^3J_{3,2}$ = $^3J_{3,4}$ = 9.3 Hz, 2H, H-3), 3.80 (ddd, $^3J_{5,4}$ = 10.0 Hz, $^3J_{5,6b}$ = 4.1 Hz, $^3J_{5,6a}$ = 1.9 Hz, 2H, H-5), 3.75 (dd, $^2J_{6a,6b}$ =11.9 Hz, $^3J_{6a,5}$ = 2.2 Hz, 2H, H-6a), 3.66 (dd, $^2J_{6b,6a}$ =12.0 Hz, $^3J_{6b,5}$ = 4.7 Hz, 2H, H-6b), 3.56 (s, 6H, CH_3), 3.46 (dd, $^3J_{2,3}$ = 9.7 Hz, $^3J_{2,1}$ = 3.6 Hz, 2H,

H-2), 3.12 (t, $^3J_{4,3} = {}^3J_{4,2}$ = 9.3 Hz, 2H, H-4); ^{13}C NMR (125 MHz, CD$_3$OD) δ 95.0 (C-1), 81.1 (C-4), 74.9 (C-2/3), 73.3 (C-5), 72.8 (C-2/3), 62.1 (CH$_3$), 60.8 (C-6); HRMS ESI *m/z* calculated for $[C_{14}H_{26}O_{11} + Na]^+$: 393.1367, found 393.1382.

2,2′-di-*O*-benzoyl-4,4′,6,6′-di-*O*-benzylidene-α,α′-D-trehalose (55)
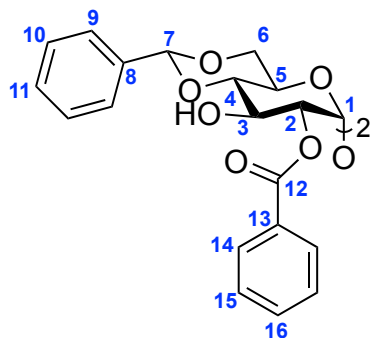
Regioselectively protected trehalose **55** was prepared using an adapted procedure from Lin *et al.*[56] 4,4′,6,6′-di-*O*-benzylidene-α,α′-D-trehalose (526 mg, 1.01 mmol) was co-evaporated twice with distilled MeOH, once with dry toluene and once with distilled MeCN (25 mL each), then dissolved in 20 mL distilled MeCN. Benzoyl cyanide (267 mg, 2.04 mmol) was added, followed by NEt$_3$ (0.5% in MeCN, 0.14 mL, 0.00507 mmol) and the colourless solution left to stir for an hour, in which time white solid precipitated. TLC analysis in 4:1 toluene/EA (*v/v*) indicated incomplete reaction, so a further 0.14 mL NEt$_3$ (0.5% in MeCN, 0.00507 mmol) was added and the reaction stirred for 90 mins, followed by heating to 49°C for 10 mins. The suspension was then filtered, with the solid comprising pure 2,2′-di-*O*-benzoyl-4,4′,6,6′-di-*O*-benzylidene-α,α′-D-trehalose. The mother liquor was concentrated in *vacuo* and recrystallised from MeCN at -20°C to yield more pure product as a crystalline white solid (total 392 mg, 0.529 mmol, 53%). R$_F$: 0.15 (4:1 toluene/EA, *v/v*); $[α]^{25.8}_{589}$ = +108 (c = 1.0 mg/mL, CH$_2$Cl$_2$,); IR (film): 3039, 3071, 2933, 2862, 1722, 1270, 1090, 992, 699 cm^{-1}; ^{1}H NMR (500 MHz, CDCl$_3$) δ 8.16 (dd, $^3J_{14,15}$ = 8.2 Hz, $^4J_{14,16}$ = 1.2 Hz, 4H, H-14), 7.56 (tt, $^3J_{16,15}$ = 7.4 Hz, $^4J_{16,14}$ = 1.3 Hz, 2H, H-16), 7.45 (t, $^3J_{9,10}$ = 7.9 Hz, 4H, H-9), 7.37 (m, 6H, H-15 + H-11), 7.29 (m, 4H, H-10), 5.48 (d, $^3J_{1,2}$ = 4.0 Hz, 2H, H-1), 5.40 (s, 2H, H-7), 5.12 (dd, $^3J_{2,3}$ = 10.0 Hz, $^3J_{2,1}$ = 4.0 Hz, 2H, H-2), 4.39 (td, $^3J_{3,2} = {}^3J_{3,4}$ = 9.7 Hz, $^4J_{3,5}$ = 2.1 Hz, 2H, H-3), 3.87 (td, $^3J_{5,4} = {}^3J_{5,6b}$ = 10.1 Hz, $^3J_{5,6a}$ = 4.8 Hz, 2H, H-5), 3.60 (dd, $^2J_{6a,6b}$ = 10.2 Hz, $^3J_{6a,5}$ = 5.2 Hz, 2H, H-6a), 3.57 (t, $^3J_{4,3} = {}^3J_{4,5}$ = 9.6 Hz, 2H, H-4), 3.50 (t, $^2J_{6b,6a} = {}^3J_{6b,5}$ = 10.2 Hz, 2H, H-6b); ^{13}C NMR (125 MHz, CDCl$_3$) δ 166.1 (C-12), 137.0 (C-13), 133.8 (C-14), 130.0 (C-16), 129.4 (C-11), 129.0 (C-9/10), 128.9 (C-9/10), 128.3 (C-15), 126.6 (C-8), 102.0 (C-1), 93.8 (C-7), 81.1 (C-4), 73.5 (C-2), 68.9 (C-6), 68.3 (C-3), 63.1 (C-5); HRMS ESI *m/z* calculated for $[C_{40}H_{38}O_{13} + NH_4]^+$: 744.2651, found 744.2661. Data recorded was consistent with literature values.[56]

15-bromopentadecanoic acid (79)
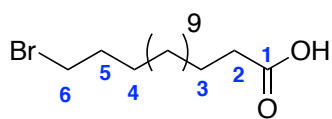
Carboxylic acid **79** was prepared using an adapted procedure of Davey & Hayman.[95] 15-Cyclopentadecanolide (2.03 g, 8.45 mol), HBr (33% in AcOH, 7 mL), and H_2SO_4 (1 mL) were combined and heated to reflux for 5 hours then cooled to room temperature. At this point, the orange solution was diluted with 30 mL H_2O and 50 mL Et_2O and the layers separated. The organic layer was washed with 50 mL brine and the combined aqueous layers were re-extracted with 2 x 50 mL Et_2O. The combined organic phases were dried with anhydrous magnesium sulfate, filtered, and concentrated *in vacuo* to give a yellow solid. The crude product was recrystallised from DCM/PE to yield 15-bromopentadecanoic acid as a crystalline white solid (2.26 g, 7.06 mmol, 84%). R_F: 0.32 (9:1 PE/EA, *v/v*); M.p.: 70.7 - 70.9 °C (literature m.p. 66 - 67 °C)[101]; IR (film) ν 3043, 2915, 2848, 1712, 1695, 1472, 1199, 894, 650 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$) δ 3.41 (t, $^3J_{6,5}$ = 7.1 Hz, 2H, H-6), 2.35 (t, $^3J_{2,3}$ = 7.3 Hz, 2H, H-2), 1.85 (pent, $^3J_{5,4}$ = $^3J_{5,6}$ = 7.2 Hz, 2H, H-5), 1.63 (pent, $^3J_{3,2}$ = $^3J_{3,4}$ = 7.2 Hz, 2H, H-3), 1.42 (pent, $^3J_{4,5}$ = $^3J_{4,CH2}$ = 7.2 Hz, 2H, H-4), 1.26 (br s, 18 H, CH_2); ^{13}C NMR (125 MHz, $CDCl_3$) δ 179.7 (C-1), 34.2 (C-6), 34.1 (C-2), 33.0 (C-5), 29.73 (CH_2), 29.67 (CH_2), 29.6 (CH_2), 29.4 (CH_2), 29.2 (CH_2), 28.9 (CH_2), 28.3 (C-4), 24.8 (C-3); HRMS (ESI) *m/z* calculated for $[C_{15}H_{29}BrO_2 - H]^-$: 319.1278, found 319.1278. Data recorded matched that available.[95]

17-methyloctadec-14-enoic acid (80)
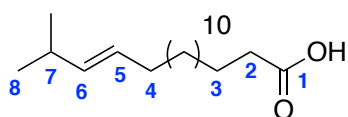
15-Bromopentadecanoic acid (741 mg, 2.32 mmol) and triphenylphosphine (669 mg, 2.55 mmol) were combined and heated under Ar to 180°C for 2 hours, then cooled to room temperature and suspended in 2 mL dry toluene and 15 mL dry THF. Sodium hydride (151 mg, 3.78 mmol) was added and the reaction stirred for 10 mins, then 0.26 mL isobutyraldehyde (2.90 mmol) was added and the suspension stirred for another 10 mins. Butyl lithium (2.0 M in cyclohexane, 1.8 mL, 3.6 mmol) was slowly added over the course of an hour, with each addition leading to an orange colouration appearing in the reaction. At this time, the reaction was nearly colourless, but TLC analysis (3:1, toluene/EA, *v/v*) indicated incomplete reaction, so a further 0.9 mL BuLi (1.8 mmol) was added, and the reaction stirred for 2 hours. The orange reaction was still incomplete, so a final portion of isobutyraldehyde

(0.1 mL, 1.11 mmol) was added, rendering the reaction colourless. The suspension was diluted with 50 mL DCM, and washed with 50 mL 1M HCl and 50 mL 1M HCl:brine (1:9, *v/v*). The combined aqueous layers were re-extracted with 2 x 50 mL DCM, then the combined organic phases dried with anhydrous magnesium sulfate, filtered, then concentrated to give a yellow oil. The crude product was purified by silica gel flash chromatography (1:0 - 3:1 PE/EA, *v/v*) followed be recrystallisation from acetone to yield 17-methyloctadec-14-enoic acid as a white solid (284 mg, 0.959 mmol, 41%). R_F = 0.13 (toluene); IR (film) ν 2998, 2923, 2853, 1708, 1462, 1411, 932, 739 cm^{-1}; ^{1}H NMR (500 MHz, CDCl$_3$) δ 5.20 (m, 2H, H-5 + H-6), 2.59 (sex, $^3J_{7,8}$ = 7.3 Hz, 1H, H-7), 2.35 (t, $^3J_{2,3}$ = 7.7 Hz, 2H, H-2), 2.02 (q, $^3J_{4,5}$ = $^3J_{4,CH2}$ = 6.7 Hz, 2H, H-4), 1.63 (pent, $^3J_{3,2}$ = $^3J_{3,CH2}$ = 6.7 Hz, 2H, H-3), 1.26 (br s, 20 H, CH$_2$), 0.94 (dd, $^3J_{8,7}$ = 6.8 Hz, $^4J_{8,6}$ = 1.2 Hz, 6H, H-8); ^{13}C NMR (125 MHz, CDCl$_3$) δ 179.7 (C-1), 137.6 (C-6), 127.7 (C-5), 34.1 (C-2), 30.1 (CH$_2$), 29.83 (CH$_2$), 29.80 (CH$_2$), 29.78 (CH$_2$), 29.70 (CH$_2$), 29.6 (CH$_2$), 29.5 (CH$_2$), 29.4 (C-4), 29.2 (CH$_2$), 27.4 (CH$_2$), 26.6 (C-7), 24.8 (C-3), 23.4 (C-8).

17-methyloctadecanoic acid (62)
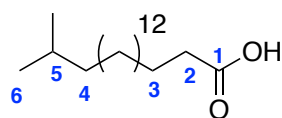
17-methyloctadec-14-enoic acid (266 mg, 0.899 mmol) was dissolved in 7 mL DCM, Pd(OH)$_2$/C (57.6 mg, 22 wt%) was added, and the black suspension was put under an atmosphere of H$_2$ and stirred for 21 hours. The suspension was filtered through celite and concentrated to give a white solid. The crude product was recrystallised from acetone to give 17-methyloctadecanoic acid as a crystalline white solid (181 mg, 0.607 mmol, 68%). R_F = 0.56 (9:1 PE/EA, *v/v*); M.p. 68.1 - 68.5°C (lit. m.p. 67.3 - 67.8°C)[102]; IR (film) ν 2917, 2849, 1703, 1470, 1264, 908, 731 cm^{-1}; ^{1}H NMR (500 MHz, CDCl$_3$) δ 2.35 (t, $^3J_{2,3}$ = 7.7 Hz, 2H, H-2), 1.64 (pent, $^3J_{3,2}$ = $^3J_{3,CH2}$ = 7.3 Hz, 2H, H-3), 1.51 (sept, $^3J_{5,6}$ = $^3J_{5,4}$ = 6.8 Hz, 1H, H-5), 1.25 (br s, 12H, CH$_2$), 0.86 (d, $^3J_{6,5}$ = 6.5 Hz, 6H, H-6); ^{13}C NMR (125 MHz, CDCl$_3$) δ 178.5 (C-1), 39.2 (C-4), 33.9 (C-2), 30.1 (CH$_2$), 29.9 (CH$_2$), 29.85 (CH$_2$), 29.82 (CH$_2$), 29.79 (CH$_2$), 29.7 (CH$_2$), 29.6 (CH$_2$), 29.4 (CH$_2$), 29.2 (CH$_2$), 28.1 (C-5), 27.6 (CH$_2$), 24.9 (C-3), 22.8 (C-6). HRMS (ESI) *m/z* calculated for [C$_{19}$H$_{38}$O$_2$ - H]$^-$: 297.2799, found 297.2807. Data recorded was consistent with literature values.[77]

15-methylhexadecane-1,15-diol (81)
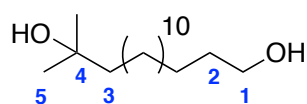
Iso-branched lipid **81** was prepared using a procedure by Richardson & Williams.[77] A solution of 15-cyclopentadecanolide (2.72 g, 11.3 mmol) in dry THF (22 mL) was cooled under Ar to -78°C, then a solution of methyl magnesium iodide in diethyl ether (3.0 M, 11.5 mL, 34.5 mmol) was added dropwise. The resulting white suspension was let warm to r.t. and stirred for 14 hours. Water, then DCM was added to the white solid formed, then the suspension was acidified through addition of AcOH until the precipitate dissolved. The layers were separated, and the aqueous phase re-extracted with DCM (2 x 50 mL), then the combined organic phase washed with brine (50 mL), then dried with magnesium sulfate, filtered, and concentrated *in vacuo* to give a yellow oil. The residue was crystallised from PE/EA to afford 15-methylhexadecane-1,15-diol as a white crystalline solid (2.67 g, 9.72 mmol, 86%). M.p. = 66.8 - 67.4 °C (literature m.p. 62°C)[99]; IR (film) ν 3342, 2914, 2847, 1470, 945 cm^{-1}; ^{1}H NMR (500 MHz, CDCl$_3$) δ 3.64 (br s, 2H, H-1), 1.56 (d, $^3J_{2,1}$ = 3.3 Hz, H, H-2), 1.44 (m, 2H, H-3), 1.26 (br s, 20H, CH$_2$), 1.20 (d, J = 3.2 Hz, 8H, H-5); ^{13}C NMR (125 MHz, CDCl$_3$) δ 71.9 (C-4), 63.1 (C-1), 44.0 (C-3), 32.8 (C-2), 30.2 (CH$_2$), 29.6 (CH$_2$), 29.2 (C-5), 25.7 (CH$_2$), 24.3 (CH$_2$); HRMS (ESI) *m/z* calculated for [C$_{17}$H$_{36}$O$_2$ + Na]$^+$: 295.2608, found 295.2618. Data collected was consistent with that reported in literature.[99]

15-methylhexadecan-1-ol (66)
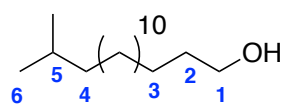
Alcohol **66** was prepared using a procedure by Richardson & Williams.[77] 15-methylhexyadecane-1,15-diol (2.67 g, 9.80 mmol) was suspended in 50 mL dry DCM, 3.13 mL (19.6 mmol) of SiHEt$_3$ was added, then the reaction was cooled to 0°C. 12 mL of freshly distilled BF$_3$(OEt)$_2$ (48%, 98.0 mmol) was added and the solution left to stir for 45 min. At this time, 40 mL ice water was added and the combined phases moved to a separating funnel. The reaction flask was rinsed three times with DCM (50 mL), and the extra solvent added to the funnel. The layers were separated and the organic phase washed with NaHCO$_3$ $_{(sat)}$ (2 x 100 mL). The organic layer was dried with anhydrous magnesium sulfate, filtered, concentrated, then crystallised from PE/EA at -10°C. Once dried, this yielded 15-methylhexadecan-1-ol as a white crystalline material (2.11 g, 8.24 mmol, 84%). IR (film) ν 3381, 2918, 2850, 1469,

1058 cm^{-1}; ^{1}H NMR (500 MHz, CDCl$_3$) δ 3.64 (br s, 2H, H-1), 1.56 (m, 5H, H-2 + H-5 + H-4), 1.25 (s, 22H, CH$_2$), 1.15 (s, 2H, CH$_2$), 0.86 (dd, $^3J_{6,5}$ = 6.6 Hz, $^4J_{6,4}$ = 2.2 Hz, 6H, H-6); ^{13}C NMR (125 MHz, CDCl$_3$) δ 110.2 (C-1), 39.2 (CH$_2$), 33.0 (C-5), 30.1 (CH$_2$), 29.88 (CH$_2$), 29.85 (CH$_2$), 29.83 (CH$_2$), 29.82 (CH$_2$), 29.78 (CH$_2$), 29.75 (CH$_2$), 29.6 (CH$_2$), 28.1 (C-5), 27.6 (CH$_2$), 25.9 (C-6). Data recorded was consistent with the published literature.[77]
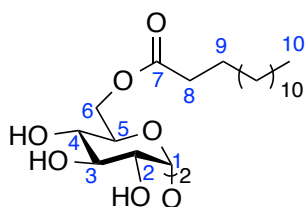
15-methylhexadecanoic acid (61)
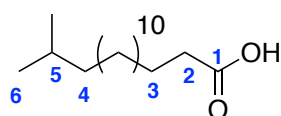
Carboxylic acid **61** was prepared using a procedure by Richardson & Williams.[77] 15-Methylhexadecan-1-ol (356 mg, 1.39 mmol), TBAI (267 mg, 0.723 mmol), and KMnO$_4$ (658 mg, 4.16 mmol) were suspended in AcOH (4 mL), DCM (20 mL), and H$_2$O (30 mL) and the resulting brown suspension heated to reflux. TLC analysis was inconclusive, hence after 18 hours, 5 M HCl (52 mL) was added, then sufficient Na$_2$SO$_3$ to dissolve the brown solid added. The layers of the reaction were separated, then the organic phase was washed with water (2 x 150 mL) and the combined organic phases extracted with DCM (2 x 150 mL). The combined organic phases were dried with anhydrous magnesium sulfate, filtered and concentrated under reduced pressure, giving an orange solid. ^{1}H NMR analysis of the solid indicated incomplete reaction, so the material was resubmitted to the same reaction conditions using TBAI (276 mg, 0.748 mmol), KMnO$_4$ (720 mg, 4.57 mmol), AcOH (4 mL), H$_2$O (10 mL), and DCM (30 mL). The biphasic brown suspension was stirred at reflux for 16 hours, then cooled and aqueous workup as before carried out. ^{1}H NMR anaylisis now indicated complete reaction, and the crude product was purified by silica gel flash chromatography (1:0 - 4:1 PE/EA *v/v*) then recrytallisation from acetone, yielding 15-methylhexadecanoic acid as a crystalline white solid (285 mg, 1.05 mmol, 76%). R$_F$: 0.56 (9:1 PE/EA, *v/v*); M.p.: 64.0 - 65.1 °C (lit m.p. 60.2 °C)[100]; IR (film) ν 2924, 2854, 1708, 905, 728 cm^{-1}; ^{1}H NMR (500 MHz, CDCl$_3$) δ 2.34 (td, $^3J_{2,3}$ = 8.0 Hz $^4J_{2,4}$ = 1.7 Hz, 2H, H-2), 1.63 (pent, $^3J_{3,2}$ = $^3J_{3,CH2}$ = 7.1 Hz, 2H, H-3), 1.51 (pent, $^3J_{5,4}$ = $^3J_{5,6}$ = 7.1 Hz, 1H, H-5), 1.25 (br s, 18H, CH$_2$), 1.15 (m, 2H, H-4), 0.86 (dd, $^3J_{6,5}$ = 6.7 Hz, $^4J_{6,CH2}$ = 2.0 Hz, 6H, H-6); ^{13}C NMR (125 MHz, CDCl$_3$) δ 178.5 (C-1), 39.0 (C-5), 33.7 (C-2), 29.9, 29.71, 29.66. 29.65, 29.62, 29.57, 29.4, 29.2, 29.1, 28.0 (C-5), 27.4, 24.7 (C-3), 22.6 (C-6); Data recorded was consistent with literature values.[77]

S-methyl-O-15-methylhexadecyl dithiocarbonate (82)
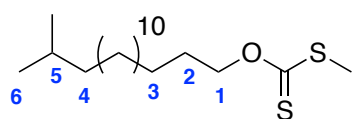
Xanthate **82** was prepared using a procedure by Richardson & Williams.[77] 15-methylhexyadecan-1-ol (1.75 g, 6.82 mmol) was co-evaporated twice with dry toluene (50 mL), then dissolved in 30 mL dry THF and cooled on ice. NaH (0.895 g, 60% dispersion in oil, 22.4 mmol) was added and the grey suspension allowed to warm to room temperature and stir for an hour. The reaction was then re-cooled to 0°C and CS_2 (0.62 mL, 10.2 mmol) was added. The resulting yellow suspension was stirred for an hour, then MeI (0.65 mL, 10.4 mmol) was added, resulting in gas formation. After 80 min, the reaction was quenched with water (50 mL), 100 mL EA was added to rinse out the flask, and the layers separated. The organic phase was washed with $NH_4Cl_{(sat)}$ (2 x 200 mL) and the combined aqueous layer was extracted with EA (1 x 200 mL). The combined organic phases were dried with anhydrous magnesium sulfate, filtered and concentrated under reduced pressure. The resulting yellow residue was crudely purified with silica gel flash column chromatography (95:5 PE/EA, *v/v*), to give semi-pure *S*-methyl-*O*-15-methylhexadecyl dithiocarbonate (2.72 g). ^{1}H NMR (500 MHz, $CDCl_3$) δ 4.58 (m, 2H, H-1), 2.50 (d, $^6J_{SCH3,1}$ = 4.4 Hz, 3H, SCH_3), 1.79 (m, 2H, H-2), 1.50 (m, 1H, H-5), 1.39 (m, 2H, H-3), 1.25 (br s, 24H, CH_2), 1.14 (m, 2H, H-4), 0.87 (m, 6H, H-6) Data recorded was consistent with literature values.[77]

15-methylhexadec-1-ene (65)
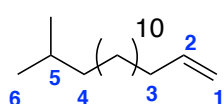
Alkene **65** was prepared using a procedure by Richardson & Williams.[77] A two-necked round bottom flask was equipped with a condenser with argon flow and a line to a bleach trap, and then charged with *S*-methyl-*O*-15-methylhexadecyl dithiocarbonate (**51**). The flask was heated for 10 min, resulting in gas evolution, then cooled and the condenser rinsed with petroleum ether. The resulting yellow solution was concentrated *in vacuo,* and the heating/rinsing procedure repeated, yielding a dark yellow oil. The oil was purified by silica gel flash column chromatography with petroleum ether, yielding 15-methylhexadec-1-ene as a light yellow oil (783 mg, 3.49 mmol, 51% over 2 steps). R_F = 0.67 (PE); ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.82 (ddd, $^3J_{2,1a}$ = 17.0 Hz, $^3J_{2,1b}$ = 10.5 Hz, $^3J_{2,3}$ = 6.8 Hz, 1H, H-2), 5.00 (d, $^3J_{1a,2}$ = 17.1 Hz, 1H, H-1a), 4.93 (d, $^3J_{1b,2}$ = 10.3 Hz, 1H, H-1b), 2.04 (q, $^3J_{3,2}$ = $^3J_{3,4}$ = 6.8 Hz, 2H, H-3), 1.51 (pent, $^3J_{5,6}$ = $^3J_{5,4}$ = 6.6 Hz, 1H, H-5), 1.38 (t, $^3J_{4,5}$ = $^3J_{4,CH2}$ = 6.9 Hz, 2H, H-4), 1.26 (s, 20H, CH_2), 1.15 (t, *J* =

6.5 Hz, 2H, CH$_2$), 0.87 (d, $^3J_{6,5}$ = 6.6 Hz, 6H, H-6); ^{13}C NMR (125 MHz, CDCl$_3$) δ 139.3 (C-2), 114.1 (C-1), 39.1 (C-5), 33.8 (C-3), 29.9 (CH$_2$), 29.73 (CH$_2$), 29.68 (CH$_2$), 29.66 (CH$_2$), 29.6 (CH$_2$), 29.5 (CH$_2$), 29.2 (CH$_2$), 29.0 (CH$_2$), 28.0 (C-5), 27.5, 22.7 (C-6). Data recorded was consistent with the published literature.[77]

14-methylpentadecanoic acid (64)
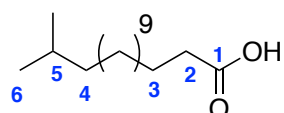
Carboxylic acid **64** was prepared using a procedure by Richardson & Williams.[77] 15-methylhexadec-1-ene (0.782 g, 3.49 mmol), TBAI (0.662 g, 1.79 mmol), and KMnO$_4$ (5.53 g, 35.0 mmol) were suspended in AcOH (11 mL), DCM (44 mL), and H$_2$O (44 mL) and the resulting brown suspension heated to reflux. After 18 hours, 5 M HCl (52 mL) was added, then sufficient Na$_2$SO$_3$ to dissolve the brown solid added. The layers of the reaction were separated, then the organic phase was washed with water (2 x 150 mL) and the combined organic phases extracted with DCM (2 x 150 mL). The combined organic phases were dried with anhydrous magnesium sulfate, filtered and concentrated under reduced pressure, giving an orange solid. The crude product was purified by two silica gel flash chromatography columns (1:0 - 1:1 PE/EA, *v/v*), giving a light yellow solid that was finally recrystallised from acetone to give 14-methylpentadecanoic acid (343 mg, 1.43 mmol, 41%). IR (film) ν 2917, 2850, 1698, 1472 cm^{-1}; ^{1}H NMR (500 MHz, CDCl$_3$) δ 2.35 (t, $^3J_{2,3}$ = 7.4 Hz, 2H, H-2), 1.63 (t, $^3J_{3,2}$ = $^3J_{3,CH2}$ = 7.0 Hz, 2H, H-3), 1.51 (pent, $^3J_{5,4}$ = $^3J_{5,6}$ = 6.4 Hz, 1H, H-5), 1.25 (br s, 18H, CH$_2$), 1.16 (m, 2H, H-4), 0.86 (d, $^3J_{6,5}$ = 6.5 Hz, 6H, H-6); ^{13}C NMR (125 MHz, CDCl$_3$) δ 178.5 (C-1), 39.5 (C-5), 33.8 (C-2), 29.9 (CH$_2$), 29.71 (CH$_2$), 29.66 (CH$_2$), 29.63(CH$_2$), 29.58 (CH$_2$), 29.4(CH$_2$), 29.2 (CH$_2$), 29.1 (CH$_2$), 28.0 (C-5), 27.4 (CH$_2$), 24.70 (C-3), 22.67 (C-6); HRMS (ESI) calculated for [C$_{16}$H$_{32}$O$_2$ - H]$^-$ 255.2330, found 255.2332. Data recorded was consistent with literature values.[77]

14-methylpentadecan-1-ol (83)
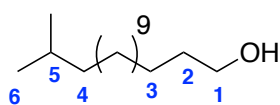
Alcohol **83** was prepared using a procedure by Richardson & Williams.[77] 14-methylpentadecanoic acid (340 mg, 1.328 mmol) was co-evaporated with dry toluene to remove water, then dissolved in 2.5 mL dry THF. The colourless solution was cooled on ice and stirred under Ar, then borane dimethylsulfide (0.16 mL) was added in a dropwise manner, resulting in gas evolution. The

reaction was left to warm to r.t. and stirred for 15 hours. After this time, the reaction was quenched at 0 °C with MeOH (0.3 mL) and water (1.1 mL), and stirred for 30 mins. Toluene (25 mL) was added, then the white suspension was evaporated to dryness *in vacuo*, then the resulting white solid was dissolved in 20 mL Et_2O and washed with brine (2 x 20 mL). The combined aqueous phases were re-extracted with Et_2O (2 x 40 mL) and the combined organic phases were dried with anhydrous magnesium sulfate. Concentration of the dried organic phase yielded a colourless oil, which was crystallised from PE/EA to afford 14-methylpentadecan-1-ol as a white crystalline solid (218 mg, 68%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 3.64 (br s, 2H, H-2), 1.56 (m, 3H, H-2 & H-14), 1.52 (m, 1H, H-5), 1.26 (s, 18H, H-4 – H-13), 1.15 (br s, 2H, H-3), 0.86 (dd, $^3J_{6,5}$ = 6.7 Hz, $^4J_{6,4}$ = 3.0 Hz, 6H, H-6); ^{13}C NMR (125 MHz, $CDCl_3$) δ 63.1 (C-1), 39.1 (C-3), 32.8 (C-2), 29.9 (CH_2), 29.7 (CH_2), 29.6 (CH_2), 29.4 (CH_2), 28.0 (CH_2), 27.4 (CH_2), 25.7 (CH_2), 22.7 (C-6); Data recorded was consistent with the published literature.[77]

S-methyl-*O*-14-methylpentadecyl dithiocarbonate (84)

Xanthate **84** was prepared using a procedure by Richardson & Williams.[77] 14-methylpentadecan-1-ol (218 mg, 0.902 mmol) was coevaporated three times with dry toluene (5 mL), then dissolved in dry THF (4 mL) and cooled on ice. NaH (130 mg, 60% dispersion in oil, 0.326 mmol) was added and the grey suspension allowed to warm to room temperature and stir for an hour. The reaction was then re-cooled to 0°C and CS_2 (0.09 mL, 1.49 mmol) was added. The resulting yellow suspension was stirred for an hour, then MeI (0.09 mL, 1.49 mmol) was added, resulting in gas formation. After 80 min, the reaction was quenched with water (50 mL), 100 mL EA was added to rinse out the flask, and the layers separated. The organic phase was washed with $NH_4Cl_{(sat)}$ (2 x 200 mL) and the combined aqueous layer was extracted with EA (1 x 200 mL). The combined organic phases were dried with anhydrous magnesium sulfate, filtered and concentrated under reduced pressure. The resulting yellow residue was crudely purified with silica gel flash column chromatography (95:5 PE/EA, *v/v*), to give semi-pure *S*-methyl-*O*-14-methylpentadecyl dithiocarbonate (271.2 mg). ^{1}H NMR (500 MHz, $CDCl_3$) δ 4.59 (t, $^3J_{1,2}$ = 6.4 Hz, 2H, H-1), 2.56 (s, 3H, CH_3), 1.80 (t, $^3J_{2,1}$ = $^3J_{2,3}$ = 7.1 Hz, 2H, H-2), 1.50 (m, 1H, H-5), 1.26 (br s, 18H, CH_2), 1.16 (m, 2H, CH_2) 0.86 (d, $^3J_{6,5}$ = 6.5 Hz, 6H, H-6).

14-methylpentadec-1-ene (63)

Alkene **63** was prepared using a procedure by Richardson & Williams.[77] A two-necked round bottom flask was equipped with a condenser with argon flow and a line to a bleach trap, and then charged with *S*-methyl-*O*-15-methylpentadecyl dithiocarbonate (**55**). The flask was heated for 10 min, resulting in gas evolution, then cooled and the condenser rinsed with petroleum ether. The resulting yellow solution (with black solid precipitate) was concentrated *in vacuo,* The oil was purified by two silica gel flash chromatography columns with petroleum ether, yielding 14-methylpentadec-1-ene as a light yellow oil (36.3 mg, 0.162 mmol, 18% over 2 steps). ^{1}H NMR (500 MHz, CDCl$_3$) δ 5.82 (ddd, $^3J_{2,1a}$ = 16.9 Hz, $^3J_{2,1b}$ = 10.5 Hz, $^3J_{2,3}$ = 6.7 Hz, 1H, H-2), 5.00 (d, $^3J_{1a,2}$ = 17.2 Hz, 1H, H-1a), 4.93 (d, $^3J_{1b,2}$ = 10.5 Hz, 1H, H-1b), 2.05 (q, $^3J_{3,2}$ = $^3J_{3,CH2}$ = 7.0 Hz, 2H, H-3), 1.52 (sept, $^3J_{5,6}$ = $^3J_{5,4}$ = 6.5 Hz, 1H, H-5), 1.39 (t, *J* = 7.0 Hz, 2H, CH$_2$), 1.27 (s, 18H, CH$_2$), 1.16 (m, 2H, H-4), 0.87 (d, $^3J_{6,5}$ = 6.6 Hz, 6H, H-6); ^{13}C NMR (125 MHz, CDCl$_3$) δ 139.5 (C-2), 114.4 (C-1), 39.1 (C-4), 33.9 (C-3), 30.0 (CH$_2$), 29.8 (CH$_2$), 29.74 (CH$_2$), 29.72 (CH$_2$), 29.71 (CH$_2$), 29.65 (CH$_2$), 29.5 (CH$_2$), 29.2 (CH$_2$), 29.0 (CH$_2$), 28.0 (C-5), 27.5 (CH$_2$), 22.7 (C-6); Data recorded was consistent with literature values.[77]

13-methyltetradecanoic acid (60)
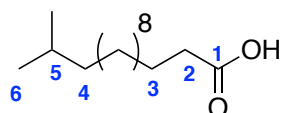
Carboxylic acid **60** was prepared using a procedure by Richardson & Williams.[77] 14-methylpentadec-1-ene (36.3 mg, 0.173 mml), TBAI (40.0 mg, 0.108 mmol), and KMnO$_4$ (302 g, 1.91 mmol) were suspended in AcOH (0.5 mL), DCM (2 mL), and H$_2$O (2 mL) and the resulting brown suspension heated to reflux. After 22 hours, 5 M HCl (2.5 mL) was added, then sufficient Na$_2$SO$_3$ to dissolve the brown solid was added. The layers of the reaction were separated, then the organic phase was washed with water (2 x 15 mL) and the combined organic phases extracted with DCM (2 x 15 mL). The combined organic phases were dried with anhydrous magnesium sulfate, filtered and concentrated under reduced pressure, giving an orange solid. The crude product was purified by two silica gel flash chromatography columns (4:1 - 1:1 PE/EA, *v/v*), giving a light yellow solid that was finally recrystallised from acetone to give 13-methyltetradecanoic acid (5.3 mg, 0.0044 mmol, 13%). IR (film) ν 2923, 2853, 1726, 1456, 1264, 1096, 736 cm^{-1}; ^{1}H NMR (500 MHz, CDCl$_3$) δ 2.35 (t, $^3J_{2,3}$ = 7.5 Hz, 2H, H-2), 1.64 (m, 2H, H-), 1.51 (m, 1H, H-5), 1.26 (br s, 16H, CH$_2$), 0.86 (d, $^3J_{6,5}$ = 6.6 Hz, 6H, H-6);

HRMS (ESI) m/z calculated for $[C_{15}H_{30}O_2 - H]^-$: 241.2173, found 241.2166. Data obtained was consistent with the published literature.[77]

6,6′-di-*O*-tetradecanoate-α,α′-D-trehalose (33)

2,2′,3,3′,4,4′-hexa-*O*-trimethylsilyl-α,α′-D-trehalose (51.2 mg, 0.066 mmol) and tetradecanoic acid (65.2 mg, 0.285 mmol), were dried by co-evaporation with dry toluene, dissolved in toluene (1.5 mL) then stirred under argon. EDCI (56.0 mg, 0.394 mmol) and DMAP (8.4 mg, 0.069 mmol) were added and the resulting colourless solution was stirred overnight. TLC analysis in 9:1 PE/EA (v/v) after 18 hours showed reaction completion. The reaction was diluted with Et$_2$O (2 mL) and washed with H$_2$O (5 mL), NaHCO$_3$ $_{(aq)}$ (5 mL), and NaCl $_{(aq)}$ (5 mL), then the aqueous phases were combined and re-extracted with Et$_2$O (5 mL). The combined organic phases were dried with anhydrous MgSO$_4$, filtered, and concentrated *in vacuo* to give a colourless oil. The crude product was purified by size exclusion chromatography using Sephadex-LH20 with a 1:1 DCM: distilled MeOH (v/v) mobile phase. Purification yielded 2,2′,3,3′,4,4′- hexa-*O*-trimethylsilyl-6,6′-di-*O*-tetradecanoate-α,α′-D-trehalose as a white solid (62.0 mg, 0.225 mmol, 79%).

2,2′,3,3′,4,4′-hexa-*O*-trimethylsilyl-6,6′-di-*O*-tetradecanoate-α,α′-D-trehalose was dissolved in 2 mL of 3:1 DCM:MeOH (v/v) and Dowex/H$^+$ was added. The reaction was allowed to proceed without stirring for 30 mins, at which time TLC analysis in 9:1 PE:EA (v/v) showed reaction completion. The resin was filtered off and the colourless solution was concentrated to give a white solid. Flash chromatography was carried out to purify the product (100:0 - 95:5 EA/MeOH, v/v), yielding 6,6′-di-*O*-tetradecanoate-α,α′-D-trehalose (21.4 mg, 54%) as a white solid. R$_F$: 0.59 (4:1, EA/MeOH, v/v); $[\alpha]^{20.1}_{589}$ = +36.0 (c = 5 mg/mL, C$_5$H$_5$N); IR (film): 3363, 2923, 2853, 1734, 1439, 1151, 1079, 987, 703 cm^{-1}; ^{1}H NMR (500 MHz, C$_5$D$_5$N) δ 5.90 (d, $^3J_{1,2}$ = 2.7 Hz, 2H, H-1), 5.10 (m, 2H, H-5), 5.01 (d, $^2J_{6a,6b}$ = 11.5 Hz, 2H, H-6a), 4.84 (dd, $^2J_{6b,6a}$ = 11.5 Hz, $^3J_{6b,5}$ = 4.9 Hz, 2H, H-6b), 4.76 (t, $^3J_{3,2}$ = $^3J_{3,4}$ = 9.1 Hz, 2H, H-3), 4.32 (dd, $^3J_{2,3}$ = 9.5 Hz, $^3J_{2,1}$ = 2.7 Hz, 2H, H-2), 4.19 (t, $^3J_{3,2}$ = $^3J_{3,4}$ = 9.0 Hz, 2H, H-4), 2.35 (m, 4H, H-8), 1.63 (pent, ^3J = 7.2 Hz, 4H, H-9), 1.25 (m, 36H, CH$_2$), 0.88 (t, $^3J_{10,CH2}$ = 5.9 Hz, 6H, H-10); ^{13}C NMR (125 MHz, C$_5$D$_5$N) δ 174.0 (C-7), 96.2 (C-1), 75.2 (C-3), 73.7

(C-2), 72.4 (C-4), 71.9 (C-5), 64.7 (C-6), 34.8 (C-8), 32.5 (CH_2), 30.33 (CH_2), 30.30 (CH_2), 30.28 (CH_2), 30.26 (CH_2), 30.1 (CH_2), 30.0 (CH_2), 29.9 (CH_2), 29.7 (CH_2), 25.6 (C-9), 23.3 (CH_2), 14.7 (C-10); HRMS (ESI) *m/z* calculated for $[C_{40}H_{74}O_{13} + NH_4]^+$: 780.5468, observed: 780.5464.

6,6′-di-*O*-hexadecanoate-α,α′-D-trehalose (34)
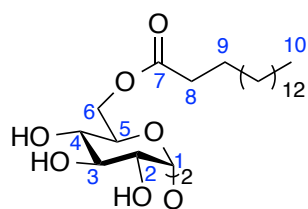
2,2′,3,3′,4,4′-hexa-*O*-trimethylsilyl-α,α′-D-trehalose (52.2 mg, 0.067 mmol) and hexadecanoic acid (77.8 mg, 0.303 mmol), were dried by co-evaporation with dry toluene, suspended in toluene (1.5 mL) then stirred under argon. EDCI (57.2 mg, 0.403 mmol) and DMAP (11.4 mg, 0.093 mmol) were added and the resulting colourless solution was stirred overnight at 80°C. TLC analysis in 9:1 PE/EA (*v/v*) after 24 hours showed reaction completion. The reaction was diluted with Et_2O (2 mL) and washed with H_2O (5 mL), $NaHCO_3$ (aq) (5 mL), and NaCl (aq) (5 mL), then the aqueous phases were combined and re-extracted with Et_2O (5 mL). The combined organic phases were dried with anhydrous $MgSO_4$, filtered, and concentrated *in vacuo* to give a colourless oil. The crude product was purified by size exclusion chromatography using Sephadex-LH20 with a 1:1 DCM/distilled MeOH (*v/v*) mobile phase, then flash chromatography (9:1, PE/EA, *v/v*). Purification yielded 2,2′,3,3′,4,4′- hexa-*O*-trimethylsilyl-6,6′-di-*O*-hexadecanoate-α,α′-D-trehalose as a white solid (65.9 mg, 0.0523 mmol, 78%).

2,2′,3,3′,4,4′-hexa-*O*-trimethylsilyl-6,6′-di-*O*-hexadecanoate-α,α′-D-trehalose was dissolved in 2 mL of 3:1 DCM/MeOH (*v/v*) and Dowex/H⁺ was added. The reaction was allowed to proceed without stirring for 90 mins, at which time TLC analysis in 9:1 PE/EA (*v/v*) showed reaction completion. The resin was filtered off and the colourless solution was concentrated to give a white solid. Flash chromatography was carried out to purify the product (EA:MeOH, 100:0 - 94:6, *v/v*), yielding 6,6′-di-*O*-hexadecanoate-α,α′-D-trehalose (25.6 mg, 59%) as a white solid. R_F = 0.59 (4:1 EA/MeOH, *v/v*); $[\alpha]^{26.7}_{589}$ = +5.2 (c = 10 mg/mL, CH_2Cl_2); IR (film): 3353, 2918, 2850, 1738, 1017 cm^{-1}; ^1H NMR (500 MHz, C_5D_5N) δ 5.91 (d, $^3J_{1,2}$ = 3.6 Hz, 2H, H-1), 5.10 (ddd, $^3J_{5,4}$ = 10.1 Hz, $^3J_{5,6b}$ = 3.7 Hz, $^3J_{5,6a}$ = 1.5 Hz, 2H, H-5), 5.01 (d, $^2J_{6a,6b}$ = 11.5 Hz, $^3J_{6a,5}$ = 1.5 Hz, 2H, H-6a), 4.84 (dd, $^2J_{6b,6a}$ = 11.7 Hz, $^3J_{6b,5}$ = 5.3 Hz, 2H, H-6b), 4.77 (t, $^3J_{3,2}$ = $^3J_{3,4}$ = 9.0 Hz, 2H, H-3), 4.32 (dd, $^3J_{2,3}$ = 9.5 Hz, $^3J_{2,1}$ =

3.6 Hz, 2H, H-2), 4.19 (t, $^3J_{4,3} = {}^3J_{4,5} = 9.3$ Hz, 2H, H-4), 2.33 (m, 4H, H-8), 1.63 (pent, $^3J =$ 7.3 Hz, 4H, H-9), 1.28 (m, 48H, CH$_2$), 0.88 (t, $^3J_{11,10} = 6.6$ Hz, 6H, H-10); ^{13}C NMR (125 MHz, C$_5$D$_5$N) δ 174.0 (C-7), 96.2 (C-1), 75.2 (C-3), 73.7 (C-2), 72.4 (C-4), 71.9 (C-5), 64.7 (C-6), 34.8 (C-8), 32.5 (CH$_2$), 30.33 (CH$_2$), 30.30 (CH$_2$), 30.28 (CH$_2$), 30.26 (CH$_2$), 30.1 (CH$_2$), 30.0 (CH$_2$), 29.9 (CH$_2$), 29.7 (CH$_2$), 25.6 (C-9), 23.3 (CH$_2$), 14.6 (C-10); HRMS (ESI) m/z calculated for [C$_{44}$H$_{82}$O$_{13}$ + NH$_4$]$^+$: 836.6094, observed: 836.6082.

6,6′-di-*O*-octadecanoic-α,α′-D-trehalose (12)
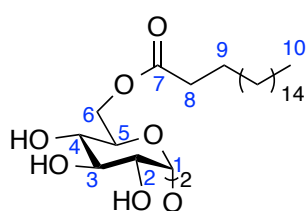
Trehalose glycolipid **12** was prepared according to an adapted procedure from Khan.[49] 2,2′,3,3′,4,4′-hexa-*O*-trimethylsilyl-α,α′-D-trehalose (58.2 mg, 0.0751 mmol) and octadecanoic acid (78.5 mg, 0.276 mmol), were dried by co-evaporation with dry toluene, suspended in toluene (1.5 mL) then stirred under argon. EDCI (52.9 mg, 0.373 mmol) and DMAP (11.6 mg, 0.095 mmol) were added and the resulting colourless solution was stirred overnight at 70°C. TLC analysis in 9:1 PE/EA (*v/v*) after 24 hours showed incomplete reaction, so a further 70.2 mg lipid (0.247 mmol), 11.5 mg DMAP (0.095 mmol), and 46.0 mg EDCI (0.343 mmol) were added and the colourless solution was stirred for a further 23 hours. At this time, the reaction was diluted with Et$_2$O (2 mL) and washed with H$_2$O (5 mL), NaHCO$_3$ (aq) (5 mL), and NaCl (aq) (5 mL), then the aqueous phases were combined and re-extracted with Et$_2$O (5 mL). The combined organic phases were dried with anhydrous MgSO$_4$, filtered, and concentrated *in vacuo* to give a colourless oil, which was progressed on to the next reaction without further purification.

2,2′,3,3′,4,4′-hexa-*O*-trimethylsilyl-6,6′-di-*O*-octadecanoate-α,α′-D-trehalose was dissolved in 4 mL of 1/1 DCM:MeOH (distilled) *v/v*, and Dowex/H$^+$ was added. The reaction was allowed to proceed without stirring for 2 hours, at which time TLC analysis in 9:1 PE/EA (*v/v*) showed reaction completion. The resin was filtered off and the colourless solution was concentrated to give a white solid. Flash chromatography was carried out to purify the product (1:0 - 4:1 EA/MeOH, *v/v*), yielding 6,6′-di-*O*-octadecanoate-α,α′-D-trehalose (19.3 mg, 0.0221 mmol, 29%) as a white solid. R$_F$ = 0.55 (4:1 EA/MeOH, *v/v*); [α]$^{29.3}_{589}$ = +48.3 (c = 10 mg/mL, acetone); IR (film): 3350, 2920, 2847, 1737, 1467, 1153, 1018 cm^{-1}; ^{1}H NMR (500 MHz, C$_5$D$_5$N) δ 6.01 (br s, 2H, OH), 5.91 (br s, 2H, H-1), 5.10 (m, 2H, H-5),

5.02 (d, $^2J_{6a,6b}$ = 11.8 Hz, 2H, H-6a), 4.85 (m, 2H, H-6b), 4.76 (td, $^3J_{3,2}$ = $^3J_{3,4}$ = 10.5 Hz, $^3J_{3,1/5}$ = 3.9 Hz, 2H, H-3), 4.33 (m, 2H, H-2), 4.19 (td, $^3J_{4,3}$ = $^3J_{4,5}$ = 9.3 Hz, $^3J_{4,2/6}$ = 3.9 Hz, 2H, H-4), 2.34 (m, 4H, H-8), 1.64 (m, 4H, H-9), 1.31 (m, 56H, CH_2), 0.89 (m, 6H, H-10); ^{13}C NMR (125 MHz, C_5D_5N) δ 174.0 (C-7), 96.2 (C-1), 75.2 (C-3), 73.7 (C-2), 72.4 (C-4), 71.9 (C-5), 64.7 (C-6), 34.8 (C-8), 32.5 (CH_2), 30.4 (CH_2), 30.3 (CH_2), 30.1 (CH_2), 30.0 (CH_2), 29.9 (CH_2), 29.7 (CH_2), 25.6 (C-9), 23.3 (CH_2), 14.6 (C-10); HRMS (ESI) *m/z* calculated for $[C_{48}9O_2O_{13} + NH_4]^+$: 892.6720, observed: 892.6720. Data recorded was consistent with the published literature.[49]

6,6′-di-*O*-(15-methylhexadecanoate)-α,α′-D-trehalose (36)
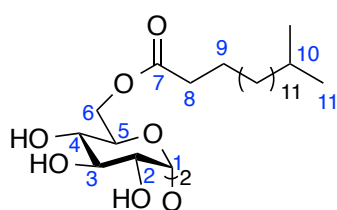
2,2′,3,3′,4,4′-hexa-*O*-trimethylsilyl-α,α-D′-trehalose (60.8 mg, 0.0785 mmol) and 15-methylhexadecanoic acid (95.3 mg, 0.352 mmol), were dried by co-evaporation with dry toluene, suspended in toluene (1.5 mL) then stirred under argon. EDCI (71.2 mg, 0.501 mmol) and DMAP (12.0 mg, 0.099 mmol) were added and the resulting colourless solution was stirred overnight at 80°C. TLC analysis in 9:1 PE/EA (*v/v*) after 24 hours showed reaction completion. The reaction was diluted with Et_2O (2 mL) and washed with H_2O (5 mL), NaHCO$_3$ (aq) (5 mL), and NaCl (aq) (5 mL), then the aqueous phases were combined and re-extracted with Et_2O (5 mL). The combined organic phases were dried with anhydrous $MgSO_4$, filtered, and concentrated *in vacuo* to give a colourless oil. The crude product was purified using flash chromatography on silica gel (8:1 PE/EA, *v/v*). Purification yielded 2,2′,3,3′,4,4′-hexa-*O*-trimethylsilyl-6,6′-di-*O*-(15-methylhexadecanoate)-α,α′-D-trehalose as a white solid (101 mg, 0.055 mmol, 70%).

2,2′,3,3′,4,4′-hexa-*O*-trimethylsilyl-6,6′-di-*O*-(15-methylhexadecanoate)-α,α′-D-trehalose was dissolved in 2 mL of 3:1 DCM/MeOH (distilled) (*v/v*), and Dowex/H$^+$ was added. The reaction was allowed to proceed without stirring for 4.5 hours, at which time TLC analysis in 9:1 PE/EA (*v/v*) showed reaction completion. The resin was filtered off and the colourless solution was concentrated to give a white solid. Flash chromatography was carried out to purify the product (1:0 - 9:1 EA/MeOH, *v/v*), yielding 6,6′-di-*O*-(15-methylhexadecanoate)-α,α′-D-trehalose (34.9 mg, 0.0412 mmol, 75%) as a white solid. R_F = 0.68 (4:1 EA/MeOH, *v/v*); $[\alpha]^{26.6}_{589}$ = +6.0 (c = 10 mg/mL, C_5H_5N); IR (film) 3355, 2954, 2923, 2853, 1738, 1464,

984 cm^{-1}; ^{1}H NMR (500 MHz, C$_5$D$_5$N) δ 5.89 (d, $^3J_{1,2}$ = 3.7 Hz, 2H, H-1), 5.09 (dd, $^3J_{5,4}$ = 10.0 Hz, $^3J_{5,6b}$ = 3.6 Hz, 2H, H-5), 5.00 (d, $^2J_{6a,6b}$ = 10.5 Hz, 2H, H-6a), 4.83 (dd, $^2J_{6b,6a}$ = 11.8 Hz, $^3J_{6b,5}$ = 5.4 Hz, 2H, H-6b), 4.75 ($^3J_{3,2}$ = $^3J_{3,4}$ = 9.0 Hz, 2H, H-3), 4.31 (dd, $^3J_{2,3}$= 9.5 Hz, $^3J_{2,1}$ = 3.7 Hz, 2H, H-2), 4.17 (t, $^3J_{3,2}$ = $^3J_{3,4}$ = 9.3 Hz, 2H, H-4), 2.33 (m, 4H, H-8), 1.63 (pent, ^3J = 7.3 Hz, 4H, H-9), 1.50 (pent, ^3J = 6.6 Hz, 2H, H-10), 1.29 (m, 44H, CH$_2$), 0.88 (m, 12H, H-11); ^{13}C NMR (125 MHz, C$_5$D$_5$N) δ 174.0 (C-7), 96.1 (C-1), 75.2 (C-3), 73.7 (C-2), 72.4 (C-4), 71.9 (C-5), 64.7 (C-6), 39.6 (CH$_2$), 34.8 (C-8), 30.6 (CH$_2$), 30.38 (CH$_2$), 30.37 (CH$_2$), 30.32 (CH$_2$), 30.28 (CH$_2$), 30.1 (CH$_2$), 29.9 (CH$_2$), 29.7 (CH$_2$), 28.6 (C-10), 28.1 (CH$_2$), 25.6 (C-9), 23.1 (C-11); HRMS (ESI) *m/z* calculated for [C$_{46}$H$_{86}$O$_{13}$ + NH$_4$]$^+$: 864.6407, found 864.6407.

6,6′-di-*O*-(17-methyloctadecanoate)-α,α′-D-trehalose (32)
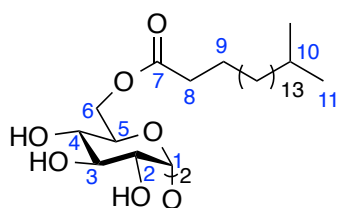
2,2′,3,3′,4,4′-hexa-*O*-trimethylsilyl-α,α′-D-trehalose (51.5 mg, 0.0665 mmol) and 17-methyloctadecanoic acid (86.4 mg, 0.290 mmol), were dried by co-evaporation with dry toluene, suspended in toluene (1.5 mL) then stirred under argon. EDCI (52.8 mg, 0.372 mmol) and DMAP (11.8 mg, 0.097 mmol) were added and the resulting colourless solution was stirred overnight at 70°C. TLC analysis in 9:1 PE/EA (*v/v*) after 24 hours showed incomplete reaction, so a further 76.6 mg lipid (0.257 mmol), 14.8 mg DMAP (0.121 mmol), and 54.4 mg EDCI (0.383 mmol) were added and the colourless solution was stirred for a further 60 mins At this time, the reaction was diluted with Et$_2$O (8 mL) and washed with H$_2$O (5 mL), NaHCO$_3$ (aq) (5 mL), and NaCl (aq) (5 mL), then the aqueous phases were combined and re-extracted with Et$_2$O (5 mL). The combined organic phases were dried with anhydrous MgSO$_4$, filtered, and concentrated *in vacuo* to give a white solid. The per-silylated trehalose glycolipid was purified by silica gel flash chromatography (1:0 - 3:1 PE/EA, *v/v*). 2,2′,3,3′,4,4′-hexa-*O*-trimethylsilyl-6,6′-di-*O*-octadecanoate-α,α′-D-trehalose was dissolved in 2 mL of 3:1 DCM/MeOH (distilled) *v/v*, and Dowex/H$^+$ was added. The reaction was allowed to proceed without stirring for 90 mins, at which time TLC analysis in 4:1 PE/EA (*v/v*) showed reaction completion. The resin was filtered off and the colourless solution was concentrated to give a white solid. Flash chromatography was carried out to purify the product (1:0 - 4:1 EA/MeOH, *v/v*), yielding 6,6′-di-*O*-(17-methyloctadecanoate)-α,α′-D-trehalose (16.9 mg, 0.0187 mmol, 29%) as a

white solid. R_F = 0.42 (9:1 EA/MeOH, *v/v*); $[\alpha]^{26.6}_{589}$ = +26 (c = 10 mg/mL, acetone); IR (film) 3371, 2923, 2853, 1744, 1466, 1152, 1079, 989 cm^{-1}; ^{1}H NMR (500 MHz, C_5D_5N) δ 5.89 (apparent s, 2H, H-1), 5.09 (m, 2H, H-5), 4.99 (m, 2H, H-6a), 4.84 (dd, $^2J_{6b,6a}$ = 12.1 Hz, $^3J_{6b,5}$ = 4.6 Hz, 2H, H-6b), 4.75 ($^3J_{3,2}$ = $^3J_{3,4}$ = 9.2 Hz, 2H, H-3), 4.32 (d, $^3J_{2,3}$ = 8.9 Hz, 2H, H-2), 4.17 (t, $^3J_{3,2}$ = $^3J_{3,4}$ = 8.0 Hz, 2H, H-4), 2.34 (m, 4H, H-8), 1.63 (t, ^3J = 8.8 Hz, 4H, H-9), 1.51 (pent, ^3J = 6.2 Hz, 2H, H-10), 1.32 (m, 52H, CH$_2$), 0.89 (dd, $^3J_{11,10}$ = 6.0 Hz, ^4J = 1.7 Hz, 12H, H-11); ^{13}C NMR (125 MHz, C_5D_5N) δ 174.0 (C-7), 96.2 (C-1), 75.2 (C-3), 73.7 (C-2), 72.4 (C-4), 71.9 (C-5), 64.7 (C-6), 39.6 (CH$_2$), 34.8 (C-8), 30.6 (CH$_2$), 30.40 (CH$_2$), 30.36 (CH$_2$), 30.31 (CH$_2$), 30.2 (CH$_2$), 30.0 (CH$_2$), 29.8 (CH$_2$), 28.6 (C-10), 28.1 (CH$_2$), 25.7 (C-9), 23.2 (C-11); HRMS (ESI) *m/z* calculated for $[C_{50}H_{94}O_{13} + NH_4]^+$: 920.7033, found 920.7033.

1-*O*-tetradecanoate-glycerol (27)
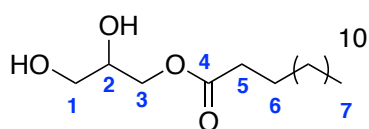
1,2-*O*-isopropylidene-glycerol (62.3 mg, 0.472 mmol) and tetradecanoic acid (241 mg, 1.04 mmol) were co-evaporated with 2 x 4 mL dry toluene then dissolved in 2 mL toluene. EDCI (173 mg, 1.22 mmol) and DMAP (37.0 mg, 0.303 mmol) were added, and the colourless solution was heated under Ar to 70°C for 18 hours. The reaction was then diluted with 5 mL diethyl ether then washed with 5 mL NaHCO$_3$ $_{(aq)}$, NaCl$_{(aq)}$, and distilled water, then the combined aqueous phases were re-extracted with 2 x 5 mL Et$_2$O. The combined organic phases were dried with anhydrous magnesium sulfate, filtered, and concentrated *in vacuo* to give the crude protected glycerolipid as a white solid which was used without further purification. The isopropylidene protected glycerolipid was suspended in 40 mL AcOH and 10 mL H$_2$O then heated to 50°C under 600 mbar of pressure for 20 mins. The colourless solution was diluted with 50 mL DCM and extracted with 50 mL each brine and distilled water. The aqueous layers were combined and re-extracted with 2 x 100 mL DCM and 1 x 100 mL EA. The combined organic phases were washed with 100 mL brine, dried with anhydrous magnesium sulfate, filtered, and concentrated to give crude product as a white solid. Purification was carried out using silica gel flash chromatography (3:1 - 1:1 PE/EA, *v/v*) to give pure 1-*O*-tetradecanoate-glycerol as a white solid (39.0 mg, 0.123 mmol, 26%). R_f = 0.28 (1:1 PE/EA, *v/v*); IR (film) 3387, 2955, 2914, 2850, 1736, 1715, 1567, 1053 cm^{-1}; ^{1}H NMR (500 MHz, CDCl$_3$) δ 4.20 (ddd, $^2J_{3a,3b}$ = 11.6 Hz, $^3J_{3a,2}$ = 4.6 Hz, $^4J_{3a,1}$ = 1.9 Hz, 1H, H-3a), 4.14 (ddd, $^2J_{3b,3a}$ = 11.6 Hz, $^3J_{3b,2}$ = 6.0 Hz, $^4J_{3b,1}$ = 1.6 Hz, 1H, H-3b),

3.93 (pent, $^3J_{2,1a} = {}^3J_{2,1b} = {}^3J_{2,3a} = {}^3J_{2,3b} = 5.10$ Hz, 1H, H-2), 3.69 (ddd, $^2J_{1a,1b} = 11.7$ Hz, $^3J_{1a,2} = 3.8$ Hz, $^4J_{1a,3} = 1.9$ Hz, 1H, H-1a), 3.60 (ddd, $^2J_{1b,1a} = 11.3$ Hz, $^3J_{1b,2} = 5.5$ Hz, $^4J_{1b,3} = 1.3$ Hz, 1H, H-1b), 2.34 (td, $^3J_{5,6} = 8.0$ Hz, $^4J_{5,CH2} = 1.6$ Hz, 2H, H-5), 1.62 (pent, $^3J_{6,5} = {}^3J_{6,CH2} = 6.8$ Hz, 2H, H-6), 1.25 (br s, 20H, CH$_2$), 0.88 (td, $^3J_{7,CH2} = 6.7$ Hz, $^4J_{7,CH2} = 1.5$ Hz, 3H, H-7); ^{13}C NMR (125 MHz, CDCl$_3$) δ 174.5 (C-4), 70.4 (C-2), 65.3 (C-3), 63.5 (C-1), 34.3 (C-5), 32.0 (CH$_2$), 29.81 (CH$_2$), 29.78 (CH$_2$), 29.7 (CH$_2$), 29.6 (CH$_2$), 29.5 (CH$_2$), 29.4 (CH$_2$), 29.3 (CH$_2$), 25.0 (C-6), 22.8 (CH$_2$), 14.3 (C-7); HRMS ESI m/z calculated for [C$_{17}$H$_{34}$O$_4$ + NH$_4$]$^+$: 320.2795, found 320.2802. Data recorded is consistent with literature values.[103]

1-*O*-hexadecanoate-glycerol (28)
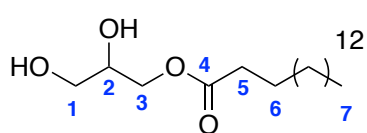
1,2-*O*-isopropylidene-glycerol (508 mg, 3.85 mmol) and hexadecanoic acid (1,458 mg, 5.70 mmol) were co-evaporated with 2 x 50 mL dry toluene then dissolved in 30 mL toluene. EDCI (1,131 mg, 7.96 mmol) and DMAP (78.7 mg, 0.645 mmol) were added, and the colourless solution was stirred at 40°C under Ar for 2.5 hours. The reaction was then diluted with 50 mL diethyl ether then washed with 50 mL NaHCO$_{3\,(aq)}$, NaCl$_{(aq)}$, and distilled water, then the combined aqueous phases were re-extracted with 2 x 50 mL Et$_2$O. The combined organic phases were dried with anhydrous magnesium sulfate, filtered, and concentrated *in vacuo* to give isopropylidene protected glycerol monohexadecanoate as a crystalline white solid. The isopropylidene protected glycerolipid was suspended in 24 mL AcOH and 6 mL H$_2$O then heated to 48°C under 500 mbar of pressure for 10 mins. The colourless solution was concentrated to give a white solid, which was purified by silica gel flash chromatography (4:1 - 1:1 PE/EA, *v/v*) to yield 1-*O*-hexadecanoate-glycerol as a white solid (582 mg, 1.68 mmol, 44%). R$_F$: 0.14 (1:1 PE/EA, *v/v*); IR (film): 3004, 2850, 1709, 1359, 1220, 529 cm^{-1}; 1H NMR (500 MHz, CDCl$_3$) δ 4.00 (m, 2H, H-3), 3.76 (m, 1H, H-2), 3.51 (dd, J = 11.3, 4.6 Hz, 1H, H-1a), 3.44 (dd, J = 11.6, 6.1 Hz, 1H, H-1b), 2.24 (t, J = 7.7 Hz, 2H, H-5), 1.51 (d, J = 6.8 Hz, 2H, H-6), 1.15 (m, 28H, CH$_2$), 0.77 (t, J = 6.7 Hz, 3H, H-7); ^{13}C NMR (125 MHz, CDCl$_3$) δ 174.5 (C-4), 69.9 (C-2), 65.1 (C-3), 63.2 (C-1), 34.1 (C-5), 31.9 (CH$_2$), 29.61 (CH$_2$), 29.58 (CH$_2$), 29.5 (CH$_2$), 29.4 (CH$_2$), 29.3 (CH$_2$), 29.2 (CH$_2$), 29.1 (CH$_2$), 24.8 (C-6), 22.6 (CH$_2$), 13.9 (C-7); HRMS (ESI) m/z calculated for [C$_{19}$H$_{38}$O$_4$ + H]$^+$: 331.2843, found 331.2845. Data recorded was consistent with literature values.[103]

1-*O*-octadecanoate-glycerol (29)
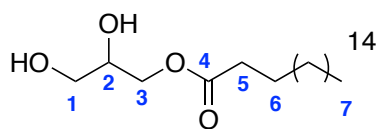
1,2-*O*-isopropylidene-glycerol (52.4 mg, 0.397 mmol) and octadecanoic acid (224 mg, 0.787 mmol) were co-evaporated with 2 x 4 mL dry toluene then dissolved in 2 mL toluene. EDCI (155 mg, 1.09 mmol) and DMAP (26.8 mg, 0.220 mmol) were added, and the colourless solution was heated under Ar to 70°C for 26 hours. The reaction was then diluted with 5 mL diethyl ether then washed with 5 mL $NaHCO_3$ (aq), $NaCl_{(aq)}$, and distilled water, then the combined aqueous phases were re-extracted with 2 x 5 mL Et_2O. The combined organic phases were dried with anhydrous magnesium sulfate, filtered, and concentrated *in vacuo* to give the crude protected glycerolipid as a white solid which was used partly purified by silica gel flash chromatography (1:0 – 30:1 PE/EA, *v/v*). The isopropylidene protected glycerolipid was suspended in 20 mL AcOH and 5 mL H_2O then heated to 50°C under 600 mbar of pressure for 20 mins. The colourless solution was quenched with $NaHCO_{3(aq)}$, diluted with 50 mL DCM and extracted with 50 mL each brine and distilled water. The aqueous layers were combined and re-extracted with 2 x 100 mL DCM and 1 x 100 mL EA. The combined organic phases were washed with 100 mL brine, dried with anhydrous magnesium sulfate, filtered, and concentrated to give crude product as a white solid. Purification was carried out using silica gel flash chromatography (3:1 - 1:1 PE/EA, *v/v*) to give pure 1-*O*-tetradecanoate-glycerol as a white solid (31.8 mg, 0.077 mmol, 20%). R_F = 0.06 (PE:EA, 4:1, *v/v*); IR (film) 3435, 3005, 2924, 2851, 1710, 1421, 1362, 1223, 1092, 530 cm^{-1}; ^{1}H NMR (500 MHz, CDCl$_3$) δ 4.19 (dd, $^2J_{3a,3b}$ = 11.5 Hz, $^3J_{3a,2}$ = 4.5 Hz, 1H, H-3a), 4.14 (dd, $^2J_{3b,3a}$ = 11.6 Hz, $^3J_{3b,2}$ = 6.3 Hz, 1H, H-3b), 3.93 (pent, $^3J_{2,3a}$ = $^3J_{2,3b}$ = $^3J_{2,1a}$ = $^3J_{2,1b}$ = 5.0 Hz, 1H, H-2), 3.69 (dd, $^2J_{1a,1b}$ = 11.7 Hz, $^3J_{1a,2}$ = 3.5 Hz, 1H, H-1a), 3.59 (dd, $^2J_{1b,1a}$ = 11.5 Hz, $^3J_{1b,2}$ = 6.0 Hz, 1H, H-1b), 2.51 (OH), 2.34 (t, $^3J_{5,6}$ = 7.7 Hz, 2H, H-5), 1.62 (pent, $^3J_{6,5}$ = $^3J_{6,CH2}$ = 7.0 Hz, 2H, H-6), 1.25 (br s, 28H, CH$_2$), 0.88 (t, $^3J_{7,6}$ = 6.5 Hz, H-7); ^{13}C NMR (125 MHz, CDCl$_3$) δ 174.5 (C-4), 70.4 (C-2), 65.3 (C-3), 63.5 (C-1), 34.3 (C-5), 32.1 (CH$_2$), 29.84 (CH$_2$), 29.82 (CH$_2$), 29.74 (CH$_2$), 29.7 (CH$_2$), 29.6 (CH$_2$), 29.5 (CH$_2$), 29.4 (CH$_2$), 29.3 (CH$_2$), 25.0 (C-6), 22.8 (CH$_2$), 14.3 (C-7); HRMS (ESI) *m/z* calculated for $[C_{21}H_{42}O_4 + NH_4]^+$: 376.3421, found 376.3411. Data recorded was consistent with the published literature.[103]

1-*O*-docosonoate-glycerol (22)

1,2-*O*-isopropylidene-glycerol (321 mg, 2.43 mmol) and docosanoic acid (1,029 mg, 3.62 mmol) were dissolved in 25 mL dry toluene. EDCI (649 mg, 74.57 mmol) and DMAP (41.5 mg, 0.340 mmol) were added, and the colourless solution was stirred at 40°C under Ar for 2.5 hours. The reaction was then diluted with 50 mL diethyl ether then washed with 50 mL NaHCO$_3$ $_{(aq)}$, NaCl$_{(aq)}$, and distilled water, then the combined aqueous phases were re-extracted with 2 x 50 mL Et$_2$O. The combined organic phases were dried with anhydrous magnesium sulfate, filtered, and concentrated *in vacuo* to a white solid. Crude product was purified using silica gel flash chromatography (95:5 PE/EA, *v/v*), then the isopropylidene protected glycerolipid was suspended in 40 mL AcOH and 10 mL H$_2$O then heated to 48°C under 600 mbar of pressure for 4.5 hours. The colourless solution was concentrated to yield 1-*O*-docosanoate-glycerol as a white solid (174 mg, 0.465 mmol, 17%). R$_F$ = 0.51 (PE/EA, 1:1, *v/v*); IR (film) ν 2916, 2849, 1730, 1264, 735 cm^{-1}; ^{1}H NMR (500 MHz, C$_5$D$_5$N) δ 4.71 (dd, $^2J_{3a,3b}$ = 11.0 Hz, $^3J_{3a,2}$ = 4.5 Hz, 1H, H-3a), 4.65 (dd, $^2J_{3b,3a}$ = 11.1 Hz, $^3J_{3b,2}$ = 6.3 Hz, 1H, H-3b), 4.40 (m, 1H, H-2), 4.12 (m, 2H, H-1), 2.37 (t, $^3J_{5,6}$ = 7.4 Hz, 2H, H-5), 2.02 (s, 1H, OH), 1.66 (t, $^3J_{6,5}$ = 6.3 Hz, 2H, H-6), 1.32 (m, 36H, CH$_2$), 0.89 (t, $^3J_{7,CH2}$ = 6.2 Hz, 3H, H-7); ^{13}C NMR (125 MHz, C$_5$D$_5$N) δ 174.1 (C-4), 71.2 (C-2), 67.1 (C-3), 64.5 (C-1), 34.8 (C-5), 32.5, 30.4, 29.7, 29.5, 29.43, 29.42, 29.38, 29.3, 29.1, 28.9, 27.8, 27.2, 25.7 (C-6), 23.3, 14.6 (C-7); HRMS (ESI) *m/z* calculated for [C$_{25}$H$_{50}$O$_4$ + NH$_4$]$^+$: 432.4047, found 432.4044.

1-*O*-(15-methylhexadecanoate)-glycerol (36)
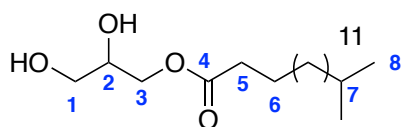
1,2-*O*-isopropylidene-glycerol (272 mg, 2.06 mmol) and 15-methylhexadecanoic acid (159 mg, 0.587 mmol) were co-evaporated with 2 x 4 mL dry toluene then dissolved in 2 mL toluene. EDCI (162.5 mg, 1.14 mmol) and DMAP (7 mg, 0.0574 mmol) were added, and the colourless solution was stirred under Ar for 18 hours. The reaction was then diluted with 5 mL diethyl ether then washed with 5 mL NaHCO$_3$ $_{(aq)}$, NaCl$_{(aq)}$, and distilled water, then the combined aqueous phases were re-extracted with 2 x 5 mL Et$_2$O. The combined organic phases were dried with anhydrous magnesium sulfate, filtered, and concentrated *in vacuo* to give a colourless oil. The crude product was purified by silica gel flash

chromatography (1:0 - 95:5 PE/EA, *v/v*) to give isopropylidene protected glycerol mono(15-methylhexadecanoate) (103 mg, 0.268 mmol, 46%). The isopropylidene protected glycerolipid was suspended in 4 mL AcOH and 1 mL H_2O then heated to 48°C under 500 mbar of pressure for 2.5 hours. The colourless solution was concentrated to give 1-*O*-(15-methylhexadecanoate)-glycerol as a white solid (50.5 mg, 0.147 mmol, 12%). $R_F = 0.37$ (1:1 PE/EA, *v/v*); IR (film) ν 3384, 2919, 2850, 1736, 1467, 1176, 1118, 1051, 721 cm^{-1}; ^{1}H NMR (500 MHz, CD$_3$OD) δ 4.14 (dd, $^2J_{3a,3b}$ = 11.6 Hz, $^3J_{3a,2}$ = 3.7 Hz, 1H, H-3a), 4.06 (dd, $^2J_{3b,3a}$ = 11.3 Hz, $^3J_{3b,2}$ = 6.2 Hz, 1H, H-3b), 3.82 (pent, $^3J_{2,3a}$ = $^3J_{2,3b}$ = $^3J_{2,1a}$ = $^3J_{2,1b}$ = 5.2 Hz, 1H, H-2), 3.55 (dd, $^3J_{1,2}$ = 5.2 Hz, $^2J_{1a,1b}$ = 2.8 Hz, 2H, H-1), 2.35 (t, $^3J_{5,6}$ = 7.5 Hz, 2H, H-5), 1.99 (s, 2H, OH), 1.62 (t, $^3J_{6,5}$ = 6.9 Hz, 2H, H-6), 1.52 (sept, $^3J_{7,8}$ = 6.7 Hz, 1H, H-7), 1.29 (br s, 22H, CH$_2$), 1.17 (m, 2H, CH$_2$), 0.88 (d, $^3J_{8,7}$ = 6.6 Hz, 6H, H-8); ^{13}C NMR (125 MHz, CD$_3$OD) δ 174.1 (C-4), 69.7 (C-2), 65.1 (C-3), 62.8 (C-1), 38.9 (CH$_2$), 33.7 (C-5), 29.7 (CH$_2$), 29.5 (CH$_2$), 29.44 (CH$_2$), 29.41 (CH$_2$), 29.37 (CH$_2$), 29.3 (CH$_2$), 29.1 (CH$_2$), 28.9 (CH$_2$), 27.9 (C-7), 27.1 (CH$_2$), 24.8 (C-6), 21.9 (C-8); HRMS (ESI) *m/z* calculated for [C$_{20}$H$_{40}$O$_4$ + NH$_4$]$^+$: 362.3265, found 362.3264. Data recorded was consistent with literature values.[104]

1-*O*-(17-methyloctadecanoate)-glycerol (37)
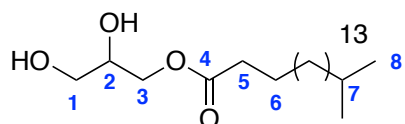
1,2-*O*-isopropylidene-glycerol (24.8 mg, 0.188 mmol) and 17-methyloctadecanoic acid (61.4 mg, 2.06 mmol) were co-evaporated with 2 x 4 mL dry toluene then dissolved in 2 mL toluene. EDCI (56.1 mg, 0.395 mmol) and DMAP (12.9 mg, 0.106 mmol) were added, and the colourless solution was heated under Ar to 70°C for 18 hours. The reaction was then diluted with 5 mL diethyl ether then washed with 5 mL NaHCO$_3$ (aq), NaCl$_{(aq)}$, and distilled water, then the combined aqueous phases were re-extracted with 2 x 5 mL Et$_2$O. The combined organic phases were dried with anhydrous magnesium sulfate, filtered, and concentrated *in vacuo* to give the crude protected glycerolipid as a white solid which was used without further purification. The isopropylidene protected glycerolipid was suspended in 24 mL AcOH and 6 mL H_2O then heated to 50°C under 600 mbar of pressure for 2.5 hours. The colourless solution was diluted with 50 mL DCM and extracted with 50 mL each brine and distilled water. The aqueous layers were combined and re-extracted with 2 x 100 mL DCM and 1 x 100 mL EA. The combined organic phases were washed with 100 mL brine, dried with anhydrous magnesium sulfate, filtered, and concentrated to give crude product as a

white solid. Purification was carried out using silica gel flash chromatography (3:1 - 1:1 PE/EA, *v/v*) to give pure 1-*O*-tetradecanoate-glycerol as a white solid (17.3 mg, 0.046 mmol, 25%). R_F = 0.17 (1:1 PE/EA, *v/v*); IR (film) 3370, 2918, 2849, 1736, 1467, 1052 cm^{-}1; ^{1}H NMR (500 MHz, CDCl$_3$) δ 4.20 (dd, $^2J_{3a,3b}$ = 12.6 Hz, $^3J_{3a,2}$ = 5.7 Hz, 1H, H-3a), 4.14 (dd, $^2J_{3b,3a}$ = 11.7 Hz, $^3J_{3b,2}$ = 6.1 Hz, 1H, H-3b), 3.93 (pent, $^3J_{2,3a}$ = $^3J_{2,3b}$ = $^3J_{2,1a}$ = $^3J_{2,1b}$ = 5.2 Hz, 1H, H-2), 3.69 (dd, $^2J_{1a,1b}$ = 11.4 Hz, $^3J_{1a,2}$ = 3.7 Hz, 1H, H-1a), 3.59 (dd, , $^2J_{1b,1a}$ = 11.4 Hz, $^3J_{1b,2}$ = 5.9 Hz, 1H, H-1b), 2.34 (t, , $^3J_{5,6}$ = 7.7 Hz, 2H, H-5), 1.62 (pent, $^3J_{6,5}$ = $^3J_{6,CH2}$ = 6.9 Hz, 2H, H-6), 1.51 (sept, $^3J_{7,8}$ = 6.8 Hz, 1H, H-7), 1.25 (br s, 24H, CH$_2$), 1.14 (m, 2H, CH$_2$), 0.86 (d, $^3J_{8,7}$ = 6.6 Hz, 6H, H-8); ^{13}C NMR (125 MHz, CDCl$_3$) δ 174.5 (C-4), 70.4 (C-2), 65.3 (C-3), 63.5 (C-1), 39.2 (CH$_2$), 34.3 (C-5), 30.1 (CH$_2$), 29.8 (CH$_2$), 29.7 (CH$_2$), 29.6 (CH$_2$), 29.4 (CH$_2$), 29.3 (CH$_2$), 28.1 (C-7), 27.6 (CH$_2$), 25.0 (C-6), 22.9 (C-8), 22.7 (C-8); HRMS (ESI) *m/z* calculated for [C$_{22}$H$_{44}$O$_4$ + NH$_4$]$^+$: 390.3578, found 390.3581. Data recorded was consistent with literature values.[104]

4.2 Biological Testing

Murine bone marrow cells were harvested and GM-CSF BMMs differentiated by William Mills and cells stimulated and supernatant analysed using ELISA by Dr. Kristel Kodar, both of the Immunoglycomics Research Group at Victoria University of Wellington. Male C57BL/6 wild-type mice (all aged between 8 and 12 weeks) were bred and housed in a conventional animal facility at the Malaghan Institute for Medical Research, Wellington, New Zealand. Experiments were approved by Victoria University Animal Ethics Committee, in accordance with their guidelines for the care of animals.

4.2.1 Generation of bone-marrow-derived macrophages (BMMs)

Male C57BL/6 wild type mice were sacrificed, and bone marrow cells collected from the tibias and femurs. The cells were then cultured (25,000 cells/mL) in complete in complete Roswell Park Memorial Institute medium (RPMI-1640, supplemented with 10% heat-inactivated foetal bovine serum (Gibco), 100 units/mL penicillin–streptomycin (Gibco) and 2 mm Glutamax (Gibco) containing 10 ng/mL GM-CSF (clone X63/GM-CSF murine cells)). Cells were incubated at 37°C under 5% CO$_2$ for 8 days, with the medium changed on days 3 and 6.

4.2.2 BMM Assay

Glycerol monoesters and trehalose diesters were prepared (5 mM in sterile DPBS containing 2% DMSO), vortexed, warmed to 50°C for 3 hours, and sonicated for 15 mins prior use to ensure sample homogeneity. BMMs were stimulated with the esters, LPS (100 ng/mL) as a positive control (REF) and medium-only (REF) as a negative control. After 48 hours, supernatants were collected and tested for IL-1β using Mouse IL-1β ELISA set (BD) according to the manufacturers protocol.

4.2.3 Statistical Analysis

Data were analysed for statistical significance by unpaired T-tests with equal standard deviations assumed, using Prism v6 software (GraphPad).

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6 Appendix

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

2,2′,3,3′,4,4′- hexa-*O*-trimethylsilyl-α,α′-D-trehalose (70)

^{1}H NMR, CDCl$_3$, 500 MHz
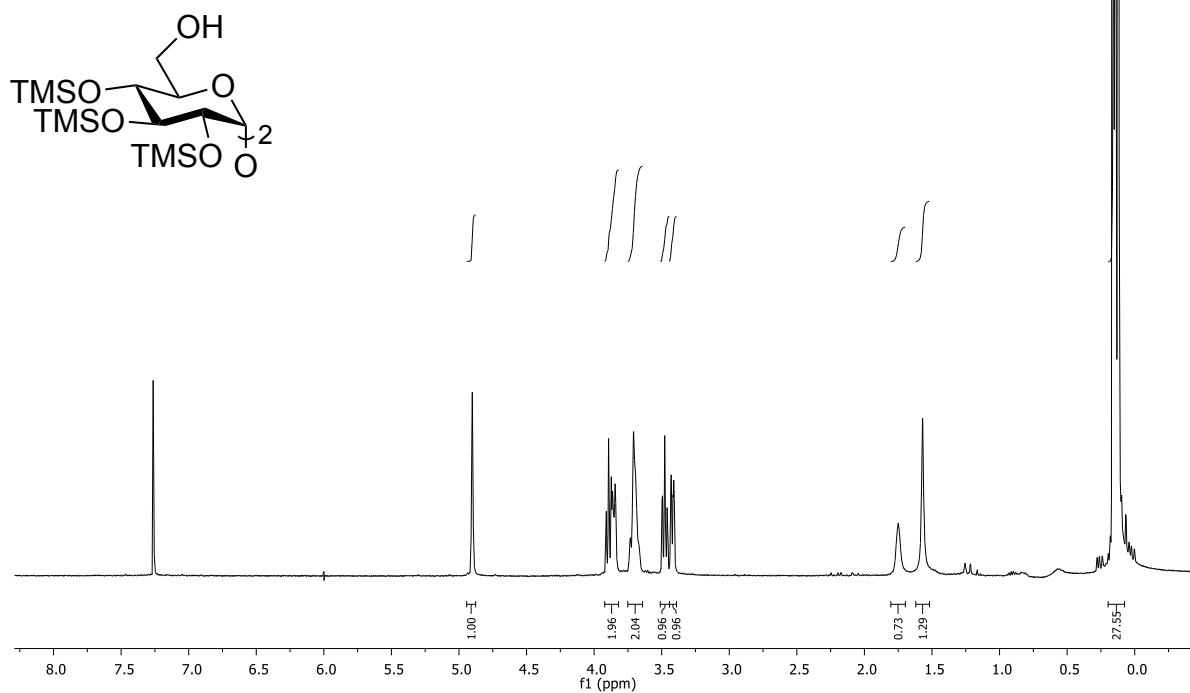
2,2′,3,3′,4,4′- hexa-*O*-trimethylsilyl-α,α′-D-trehalose (70)

^{13}C NMR, CDCl$_3$, 125 MHz
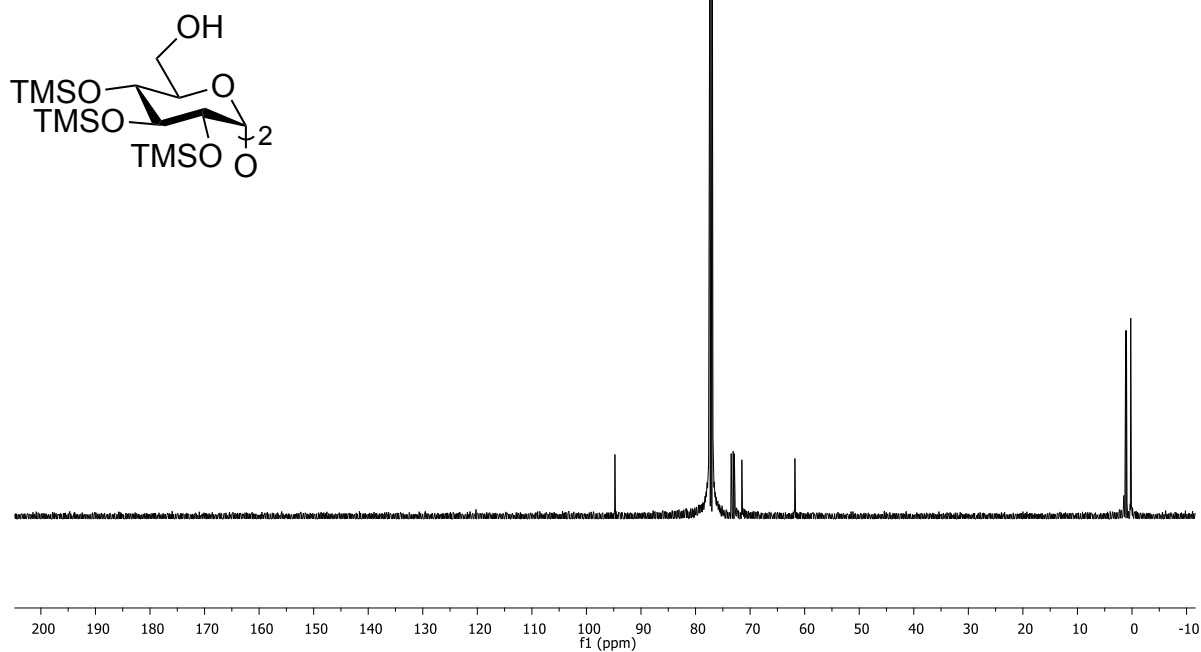
6,6′-di-*O*-docosanoate-α,α′-D-trehalose (14)

¹H NMR, C₅D₅N, 500 MHz
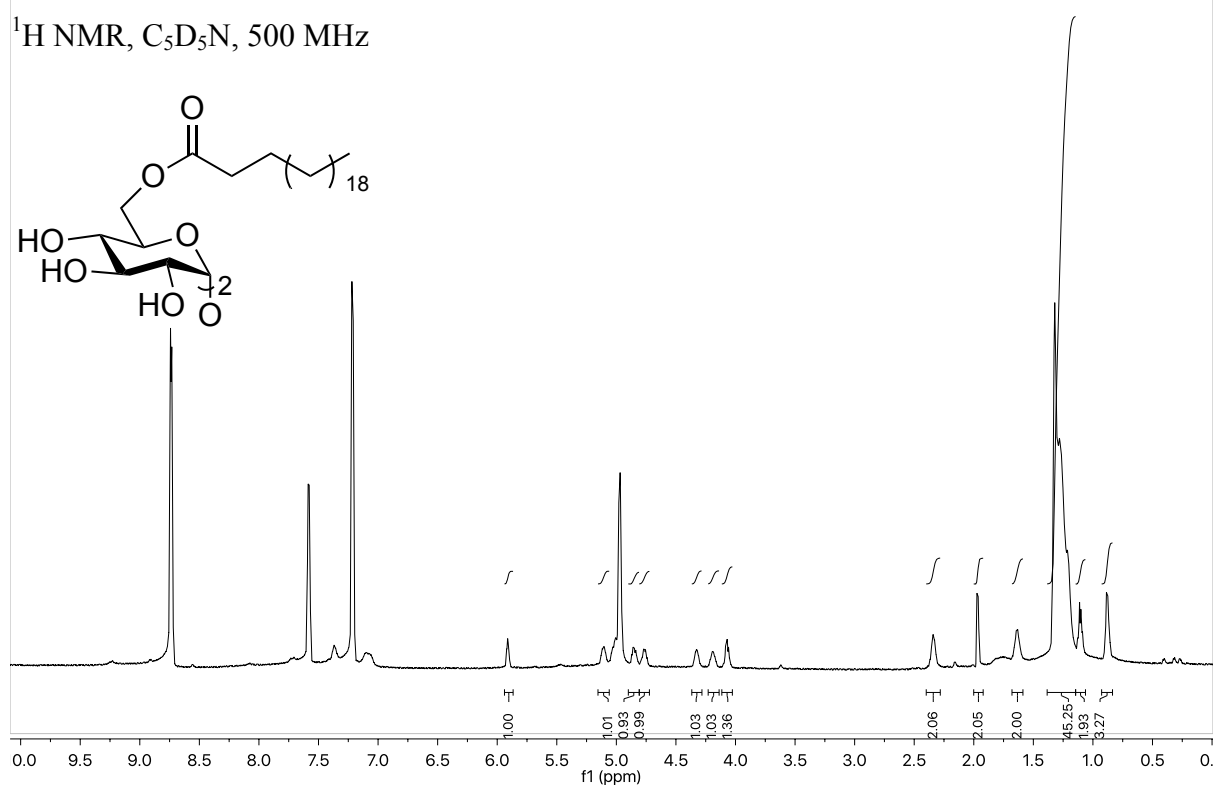
6,6′-di-*O*-docosanoate-α,α′-D-trehalose (14)

¹³C NMR, C₅D₅N, 125 MHz
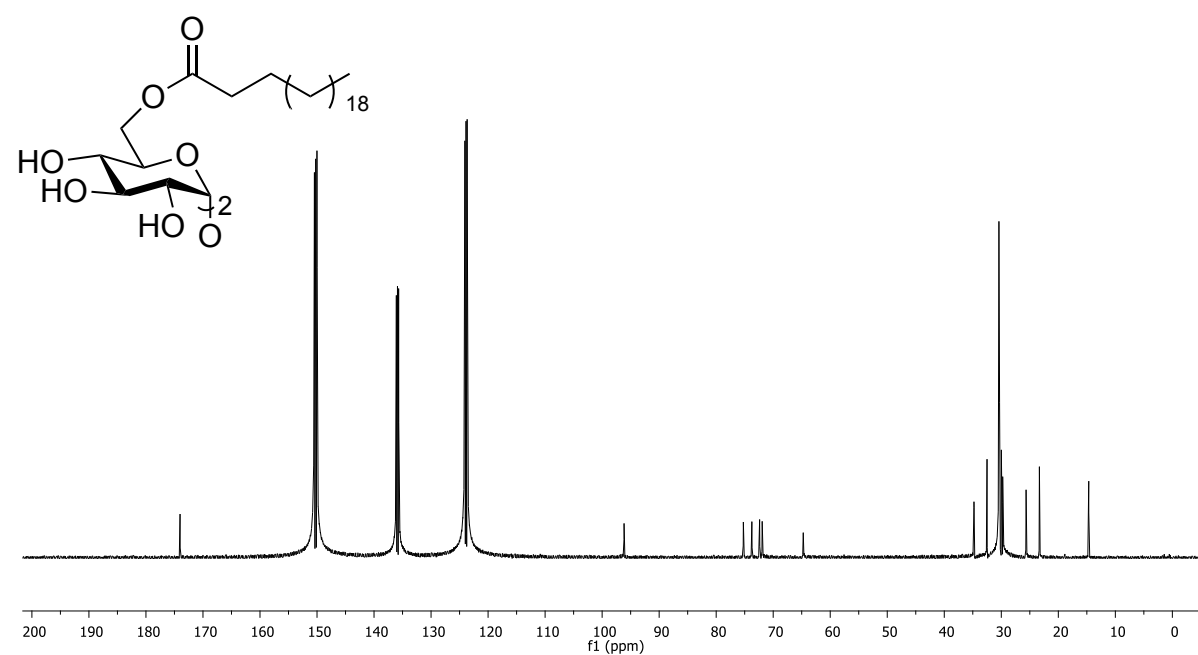
4,4′,6,6′-di-*O*-benzylidene-α,α′-D-trehalose (52)

¹H NMR, CD₃OD, 500 MHz
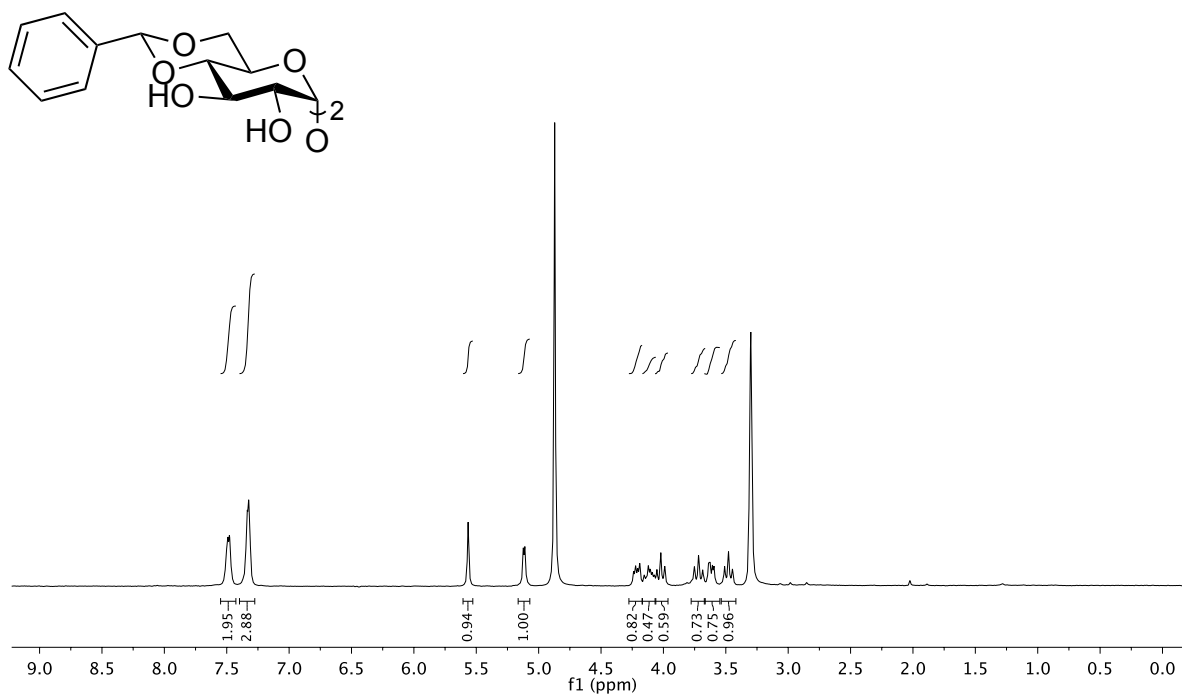
4,4′,6,6′-di-*O*-benzylidene-α,α′-D-trehalose (52)

¹³C NMR, CD₃OD, 125 MHz
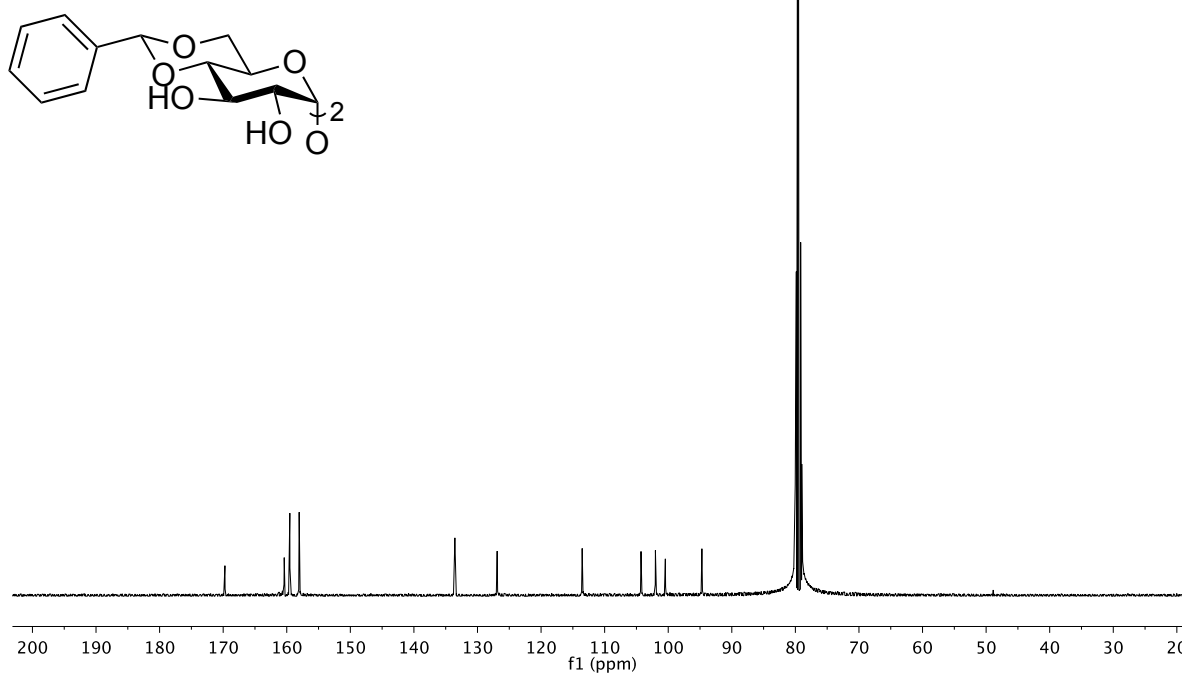
2,2′,3,3′-tetra-*O*-benzyl-4,4′,6,6′-di-*O*-benzylidene-α,α′-D-trehalose (71)

¹H NMR, CDCl₃, 500 MHz
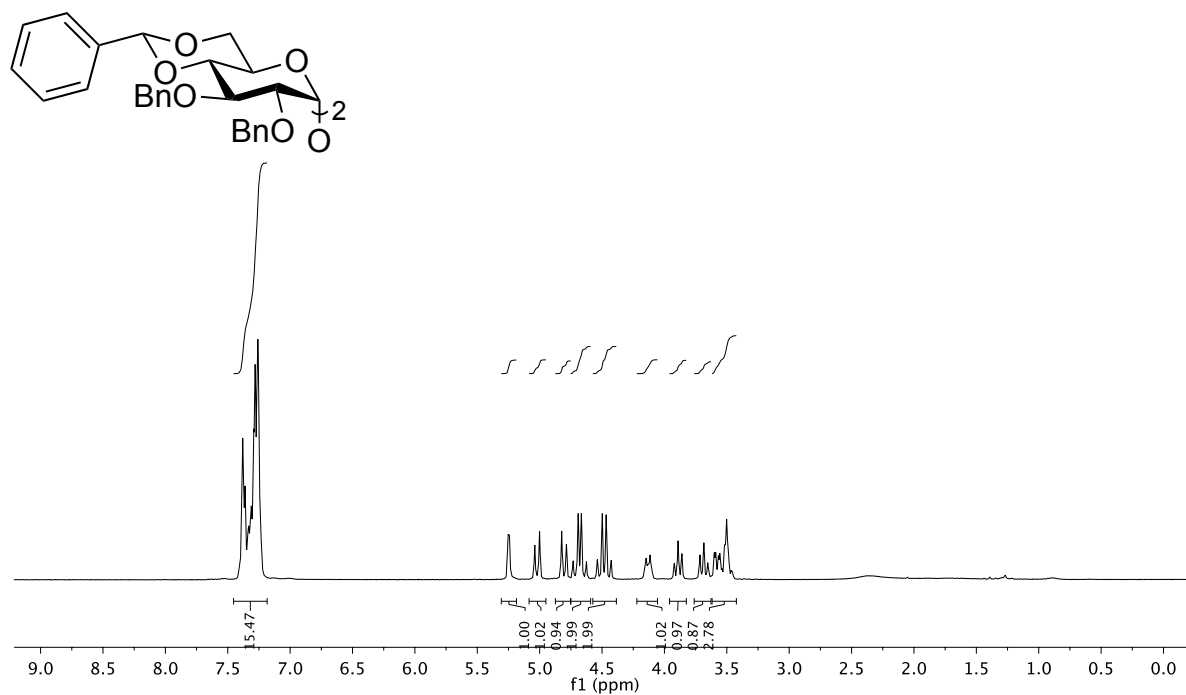
2,2′,3,3′-tetra-*O*-benzyl-4,4′,6,6′-di-*O*-benzylidene-α,α′-D-trehalose (71)

¹³C NMR, CDCl₃, 125 MHz
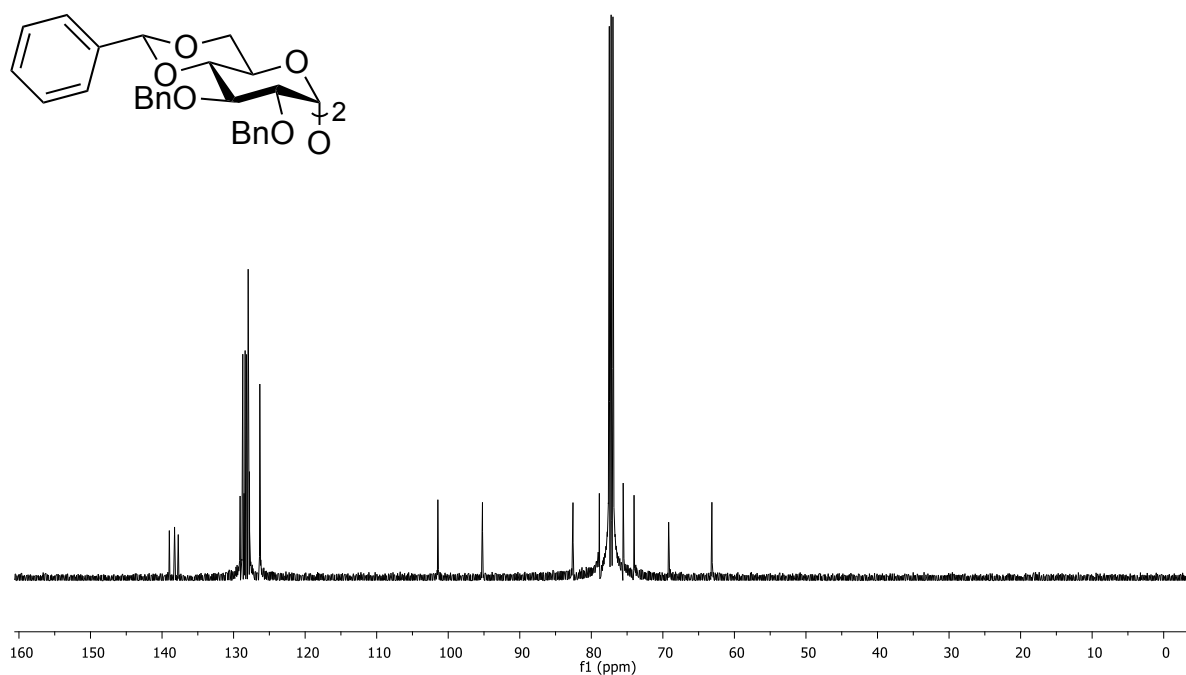
2,2′,3,3′,6,6′-hexa-*O*-benzyl-α,α′-D-trehalose (51)

¹H NMR, CDCl₃, 500 MHz
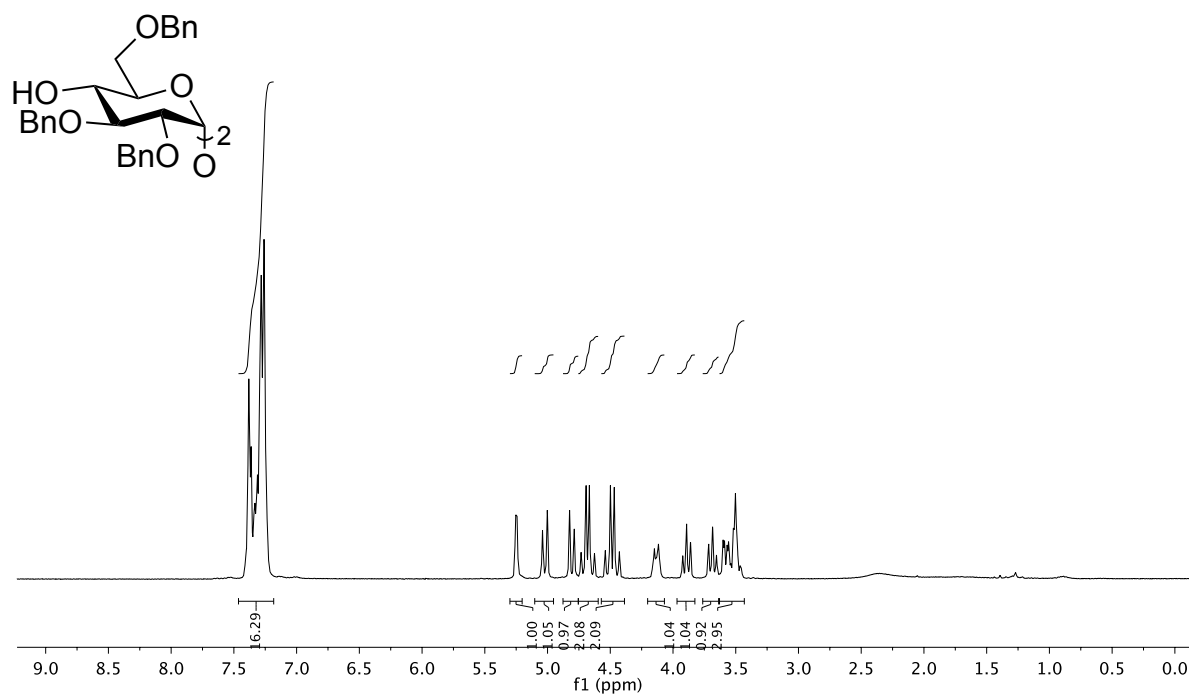
2,2′,3,3′,6,6′-hexa-*O*-benzyl-α,α′-D-trehalose (51)

¹³C NMR, CDCl₃, 125 MHz
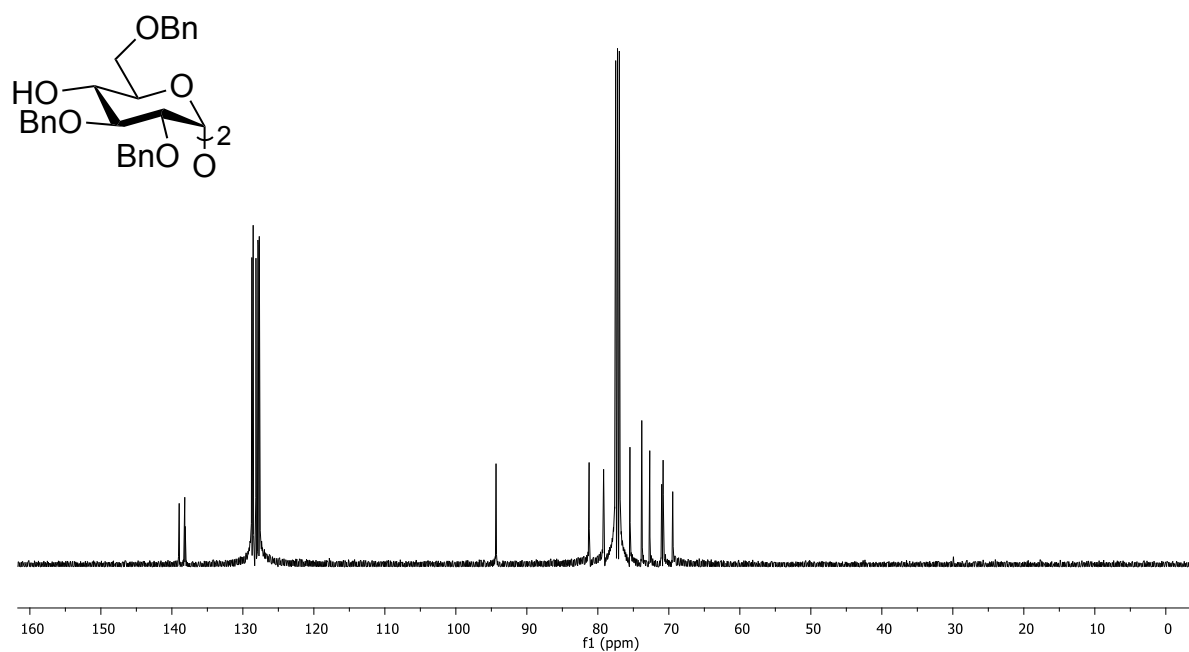
2,2′,3,3′,6,6′-hexa-*O*-benzyl-4,4′-di-*S*-methylthiocarbonyl-α,α′-D-trehalose (72)

^{1}H NMR, CDCl$_3$, 500 MHz
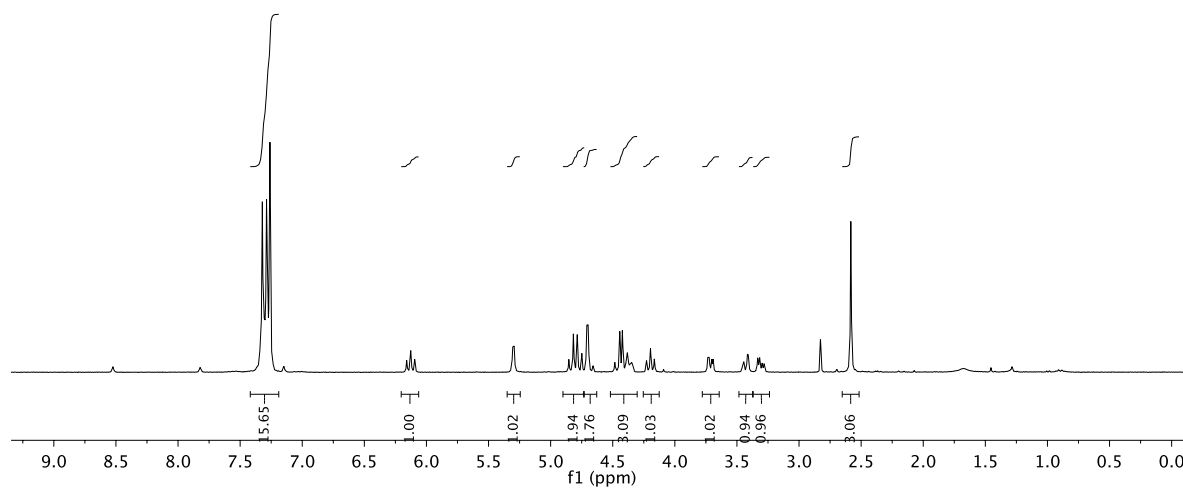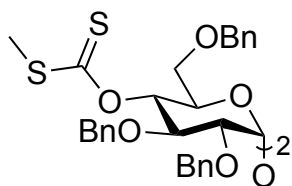
2,2′,3,3′,6,6′-hexa-*O*-benzyl-4,4′-dideoxy-α,α′-D-trehalose (73)

^{1}H NMR, CDCl$_3$, 500 MHz
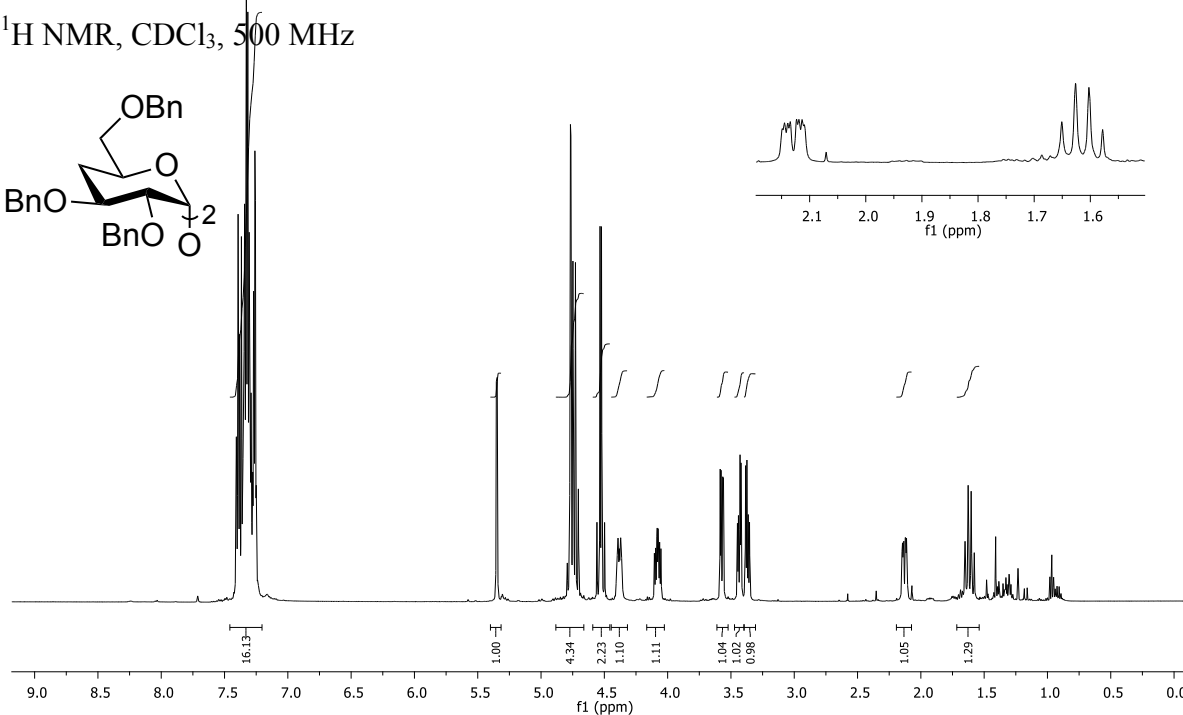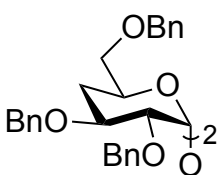
2,2′,3,3′,6,6′-hexa-*O*-benzyl-4,4′-dideoxy-α,α′-D-trehalose (73)

^{13}C NMR, CDCl$_3$, 125 MHz
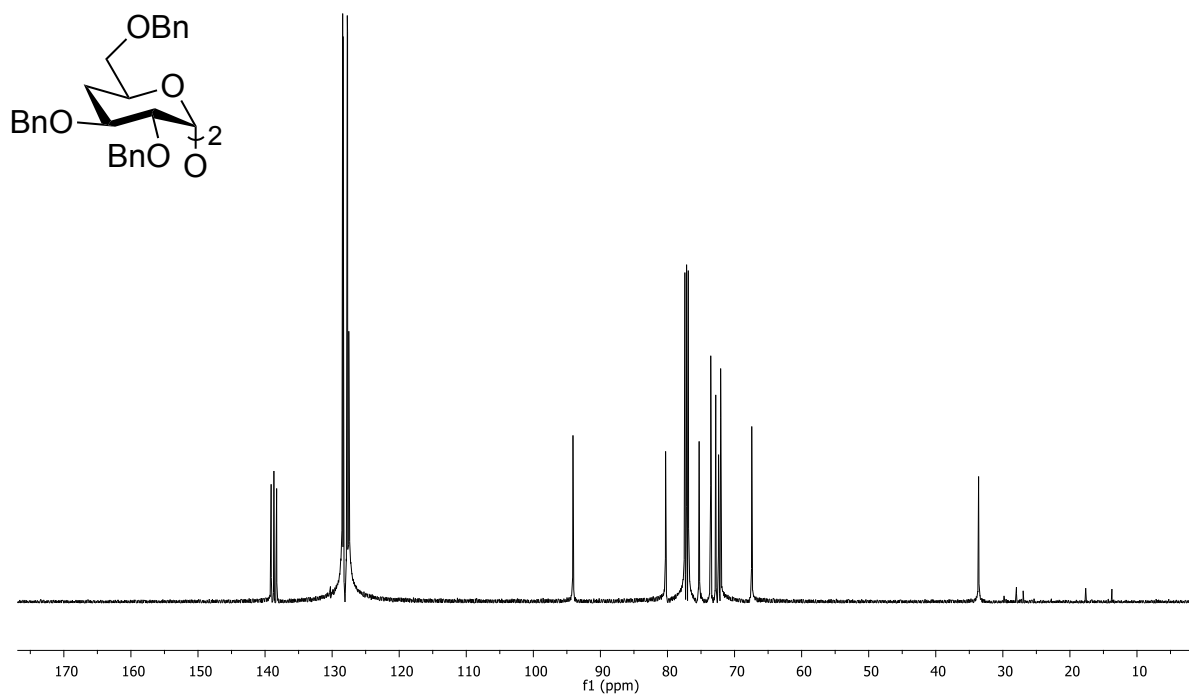
4,4′-dideoxy-α,α′-D-trehalose (45)

^{1}H NMR, D$_2$O, 500 MHz
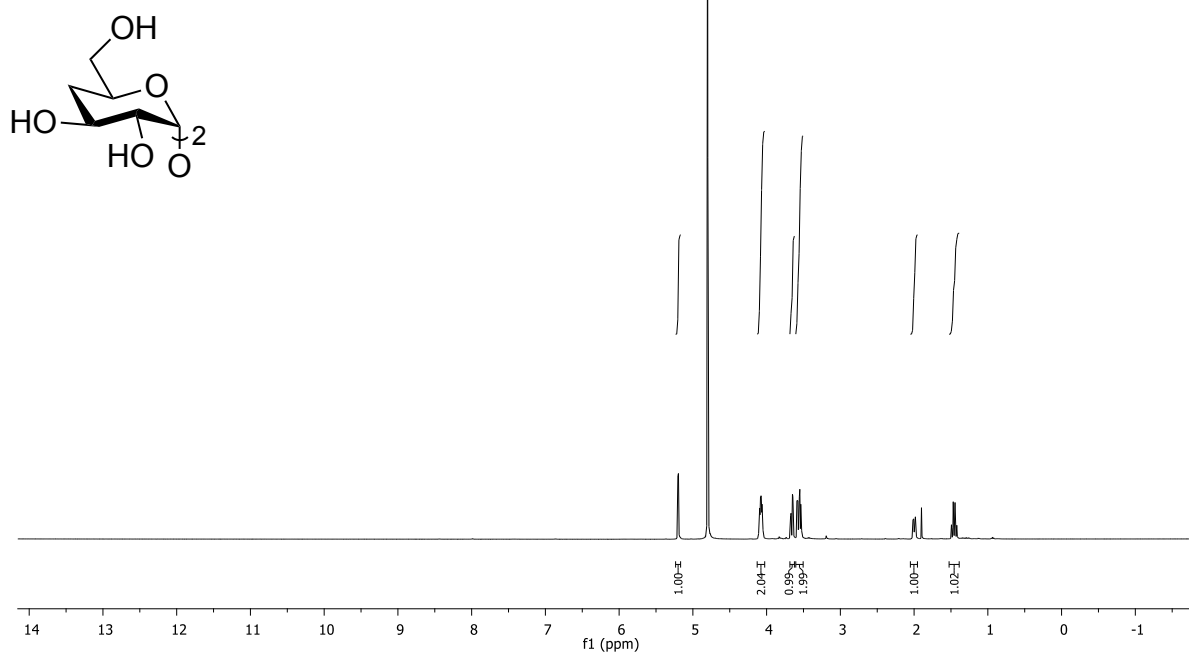
4,4′-dideoxy-α,α′-D-trehalose (45)

^{13}C NMR, D$_2$O, 125 MHz
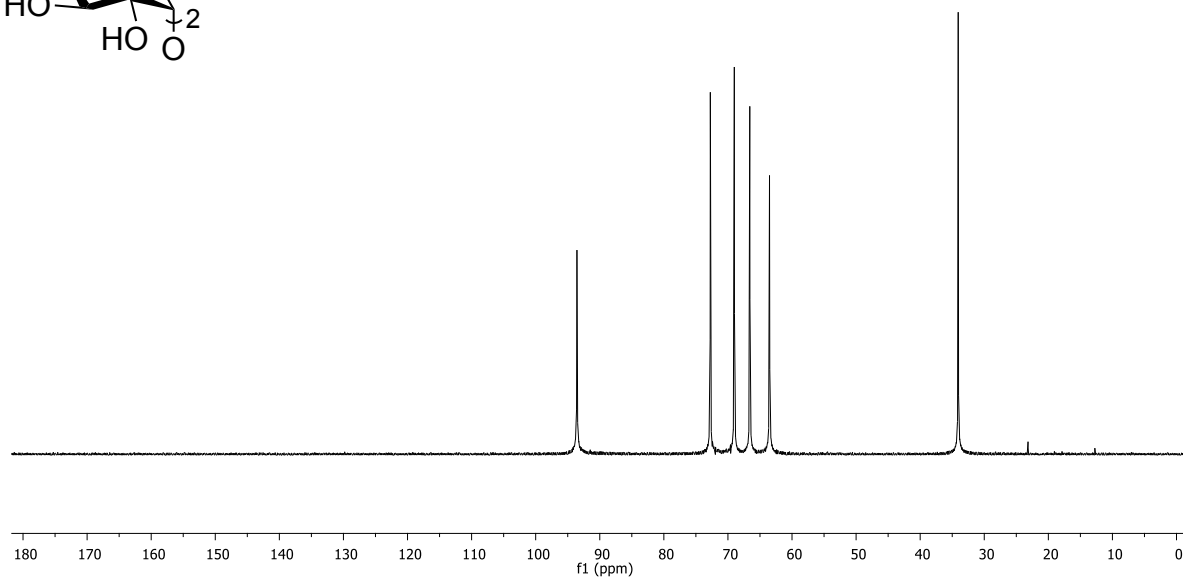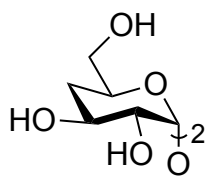
2,2′,3,3′,6,6′-hexa-*O*-benzyl-4,4′-di-*O*-Me-α,α′-D-trehalose (74)

^{1}H NMR, CDCl$_3$, 500 MHz
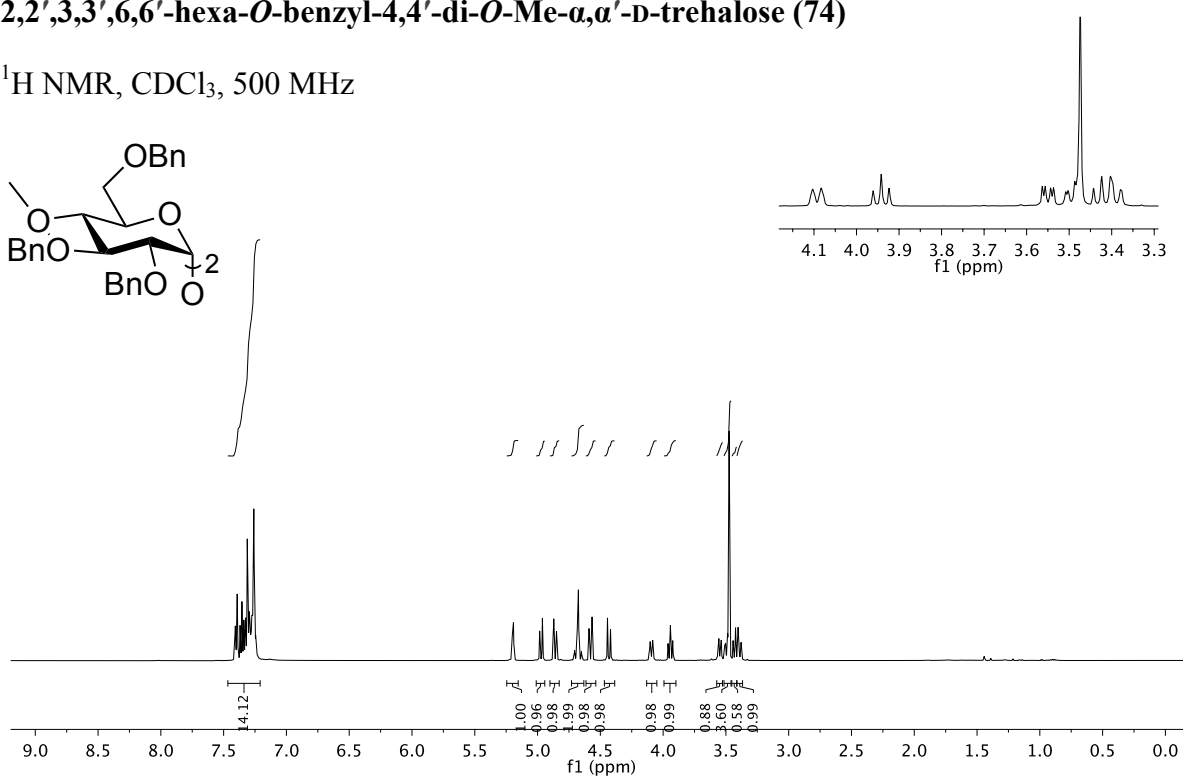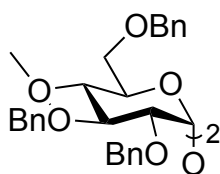
2,2′,3,3′,6,6′-hexa-*O*-benzyl-4,4′-di-*O*-Me-α,α′-D-trehalose (74)

^{13}C NMR, CDCl$_3$, 125 MHz
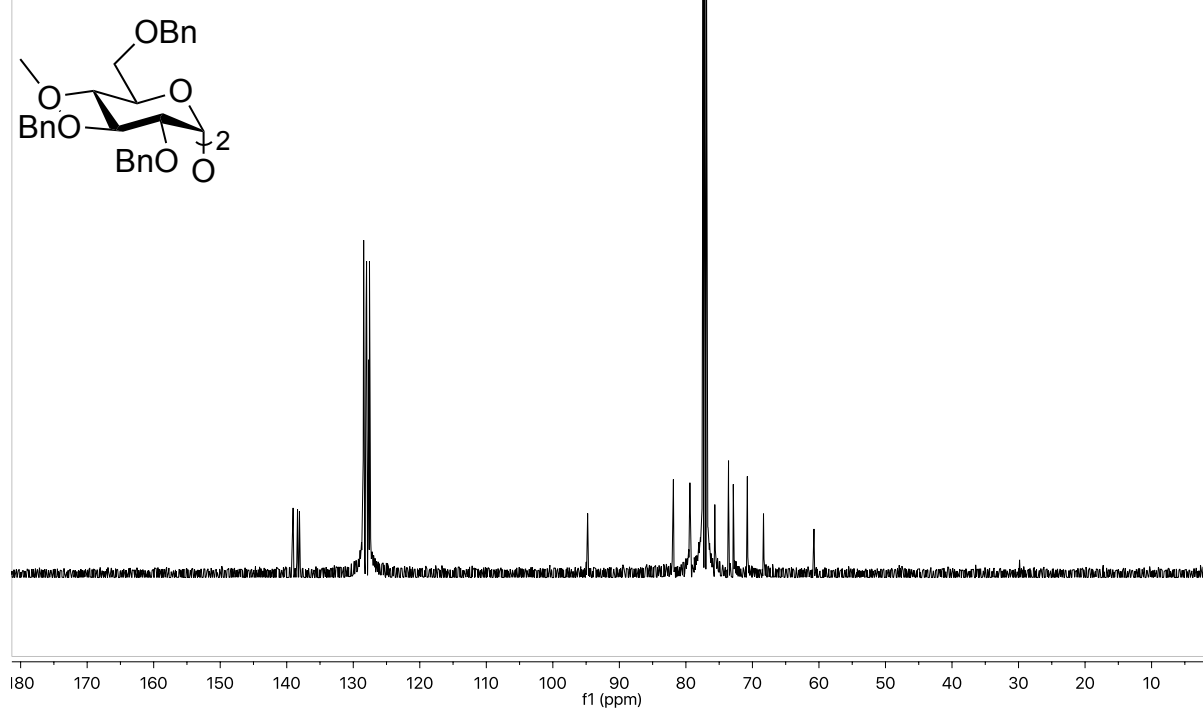
4,4′-di-*O*-Me-α,α′-D-trehalose (46)

^{1}H NMR, CD$_3$OD, 500 MHz
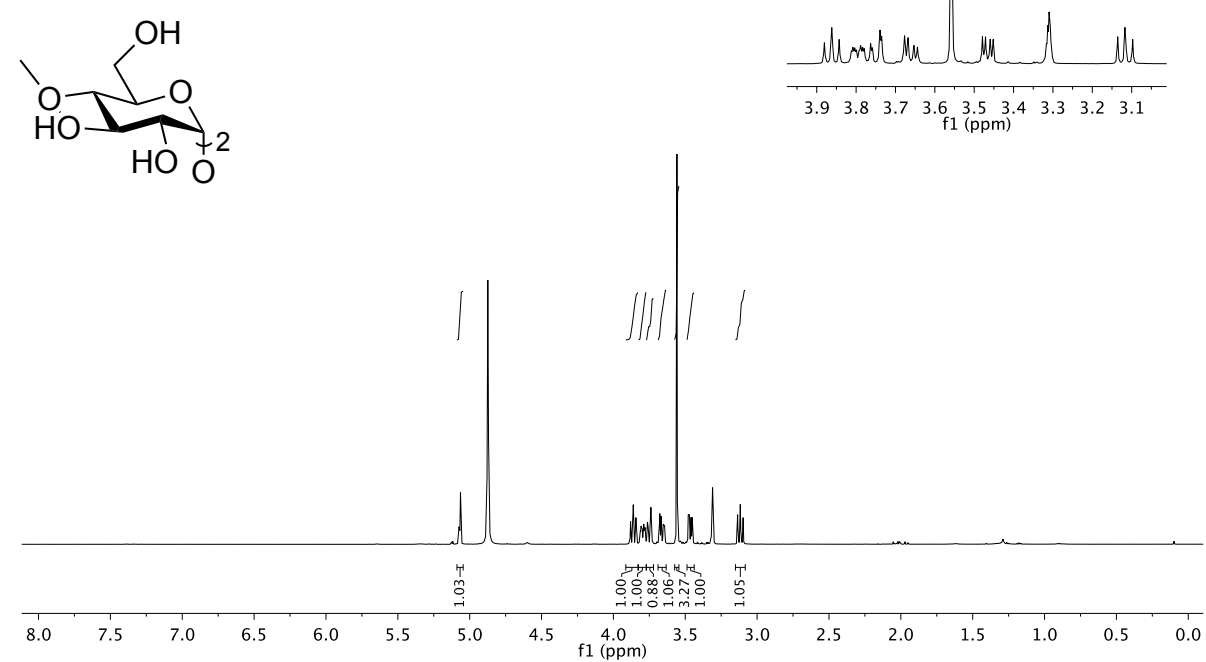
4,4′-di-*O*-Me-α,α′-D-trehalose (46)

^{13}C NMR, CD$_3$OD, 125 MHz
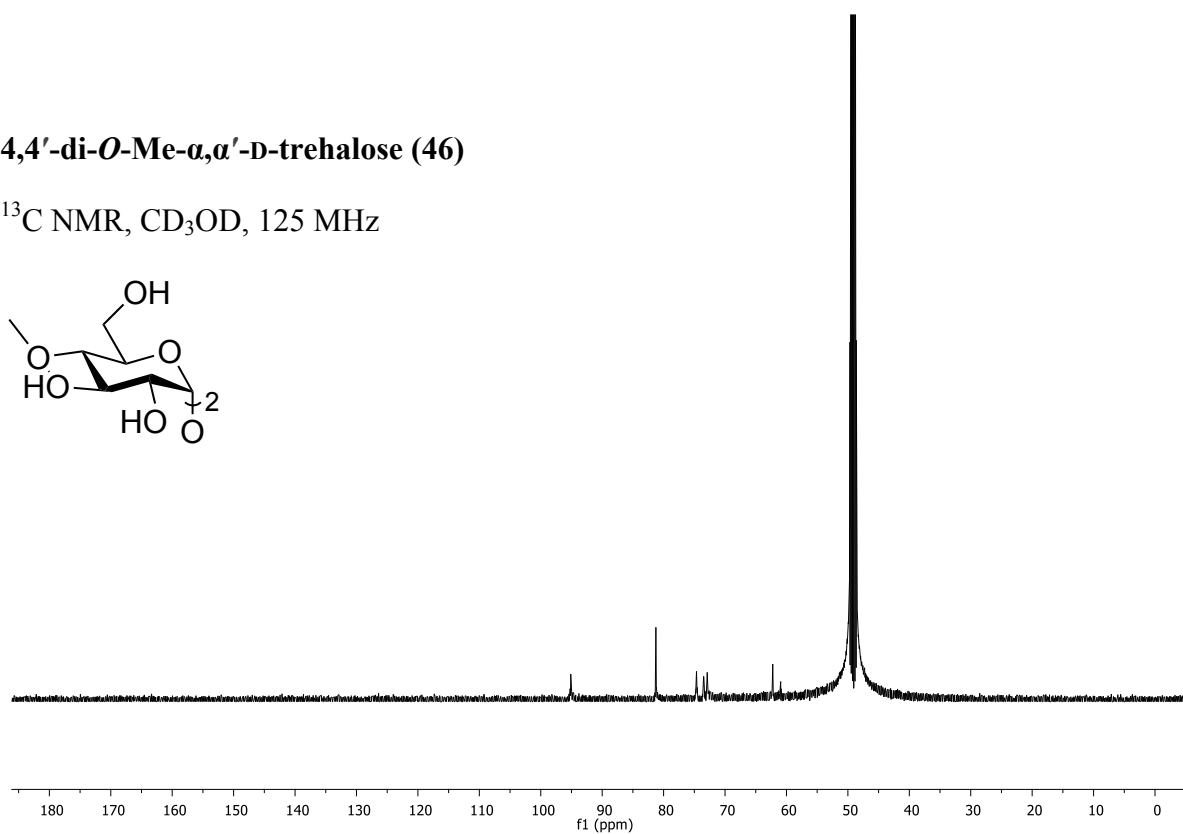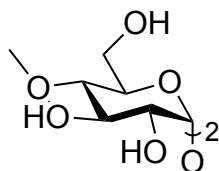
2,2′-di-*O*-benzoyl-4,4′,6,6′-di-*O*-benzylidene-α,α′-D-trehalose (55)

^{1}H NMR, CDCl$_3$, 500 MHz
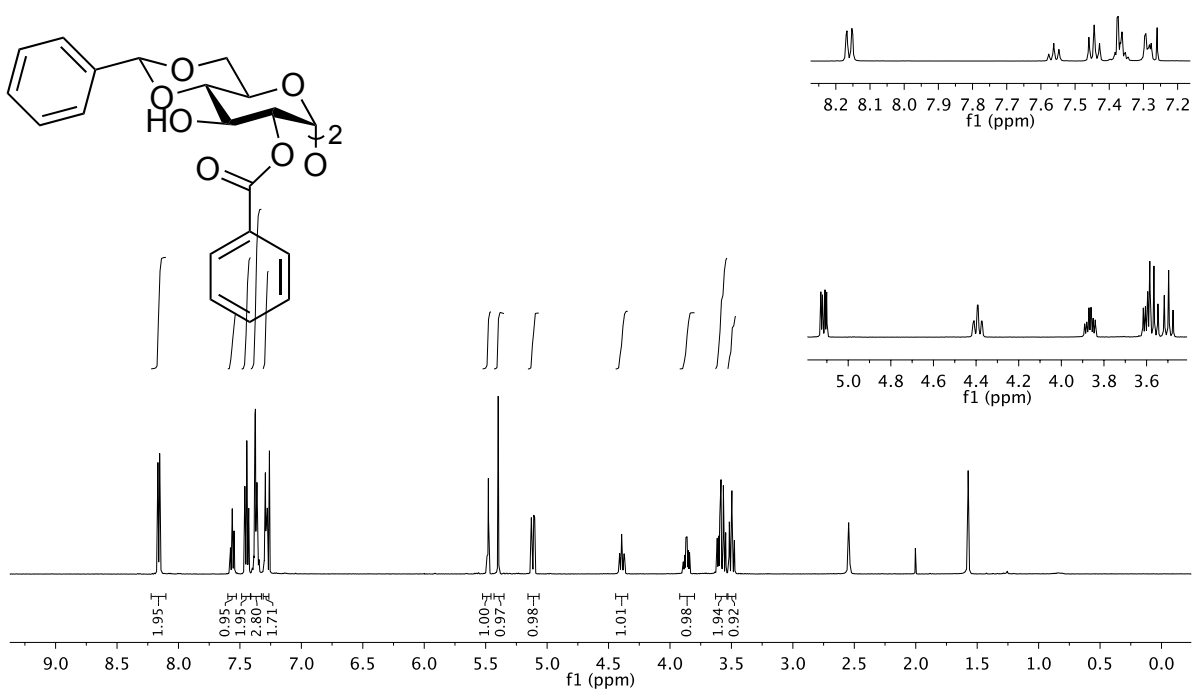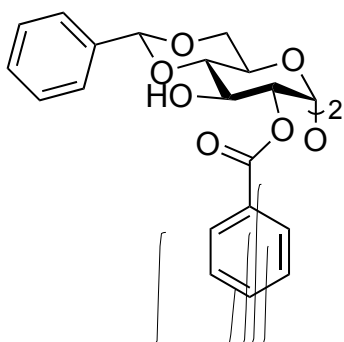
2,2′-di-*O*-benzoyl-4,4′,6,6′-di-*O*-benzylidene-α,α′-D-trehalose (55)

¹³C NMR, CDCl₃, 125 MHz
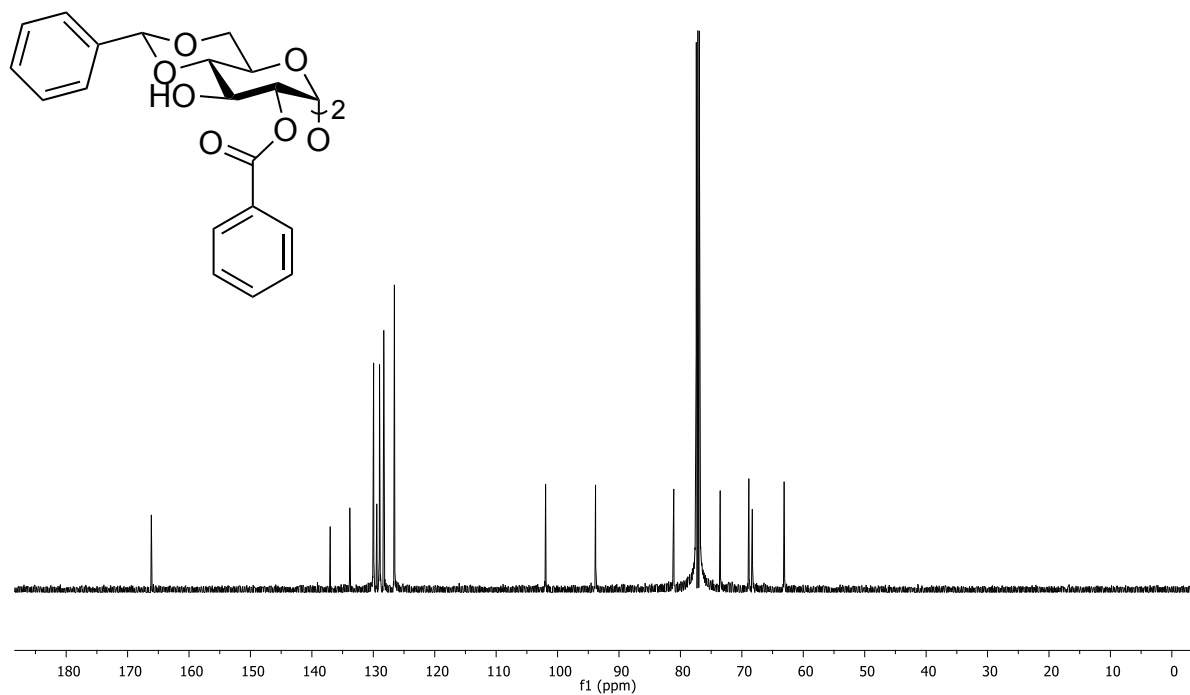
15-bromopentadecanoic acid (79)

¹H NMR, CDCl₃, 500 MHz

116
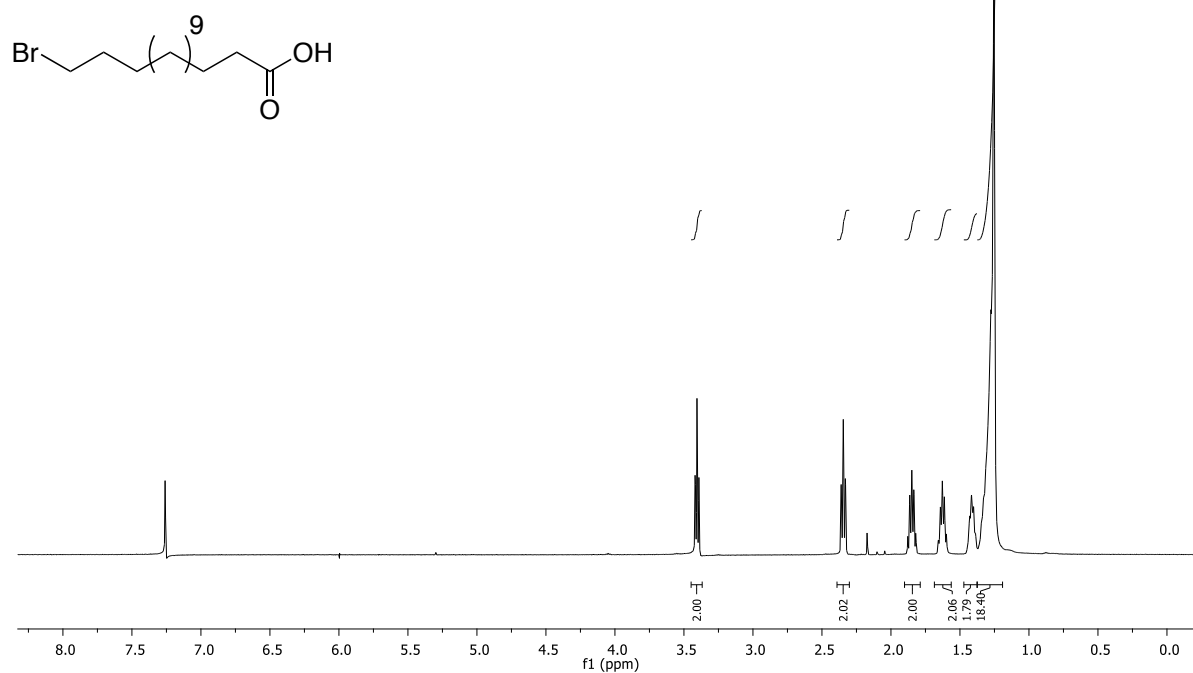
15-bromopentadecanoic acid (79)

^{13}C NMR, CDCl$_3$, 125 MHz
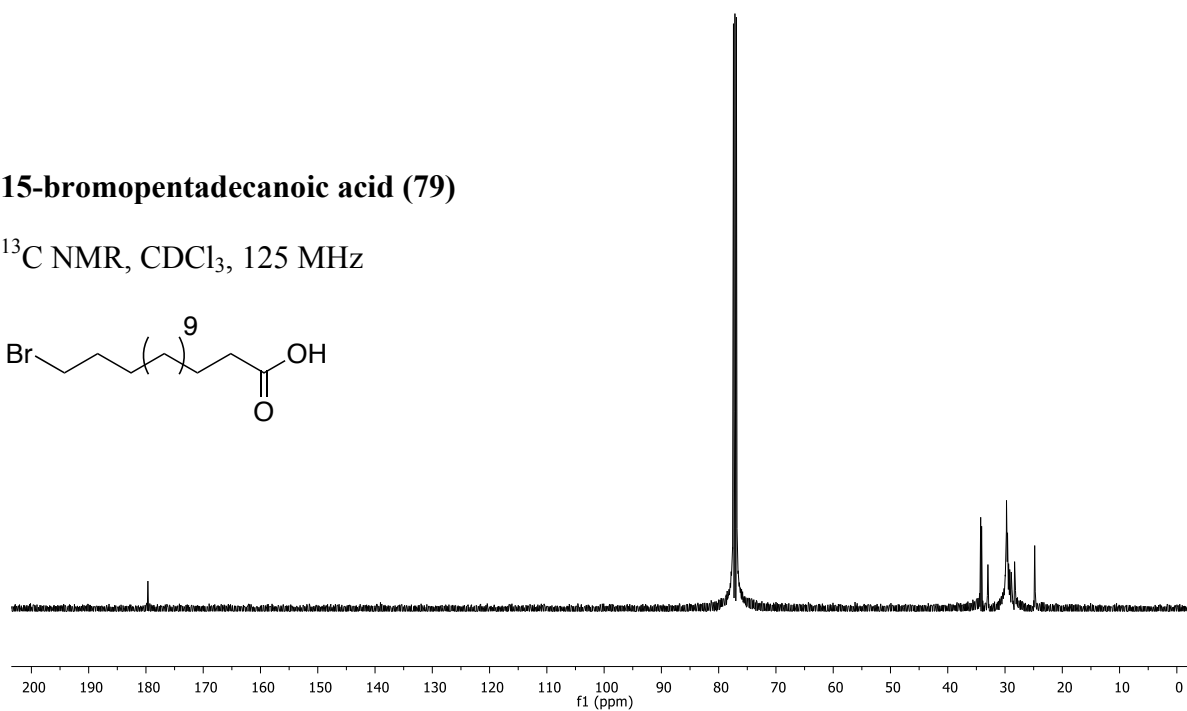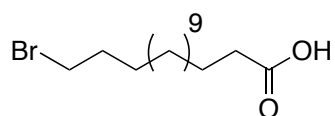
17-methyloctadec-15-enoic acid (80)

^{1}H NMR, CDCl$_3$, 500 MHz

117
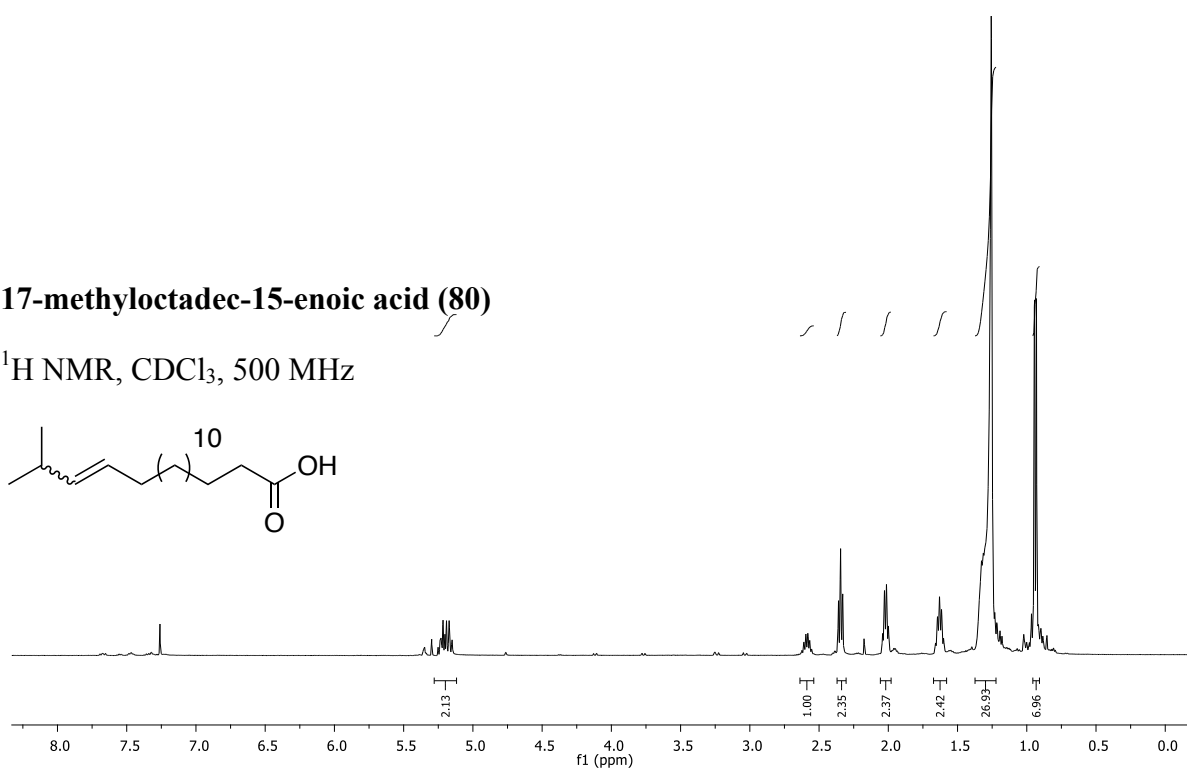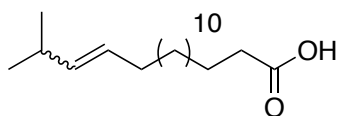
17-methyloctadec-15-enoic acid (80)

^{13}C NMR, CDCl$_3$, 125 MHz
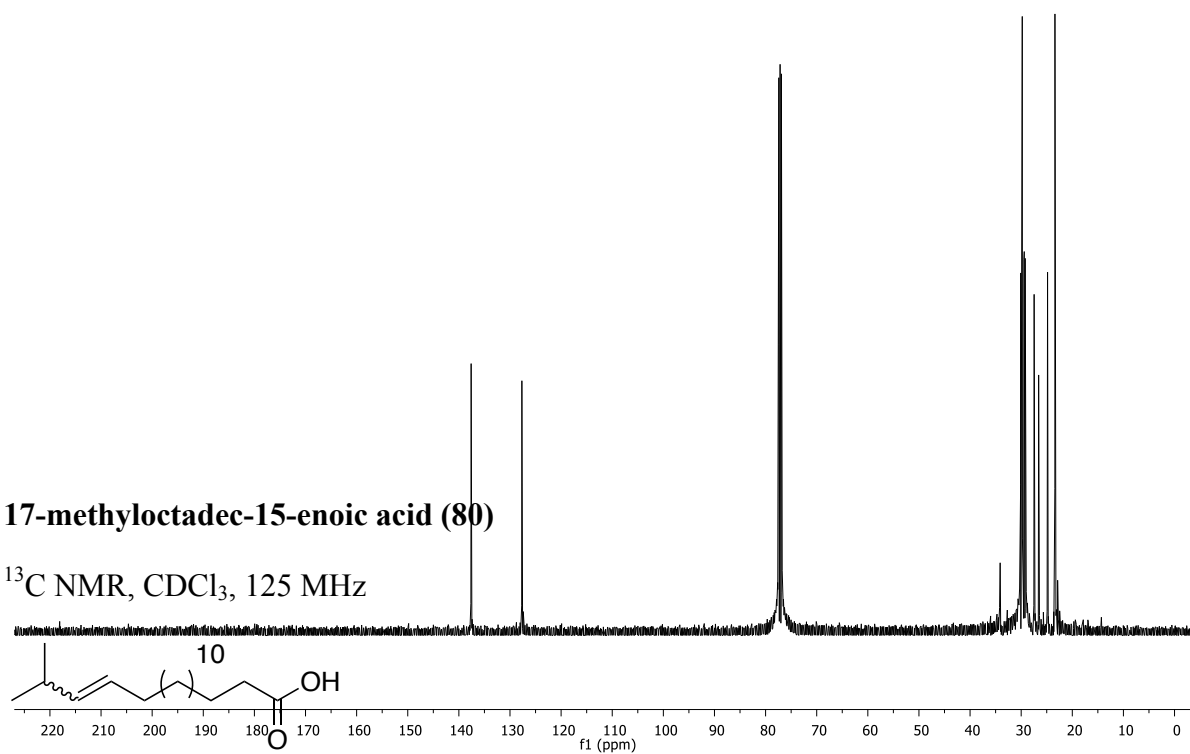
17-methyloctadecanoic acid (62)

^{1}H NMR, CDCl$_3$, 500 MHz
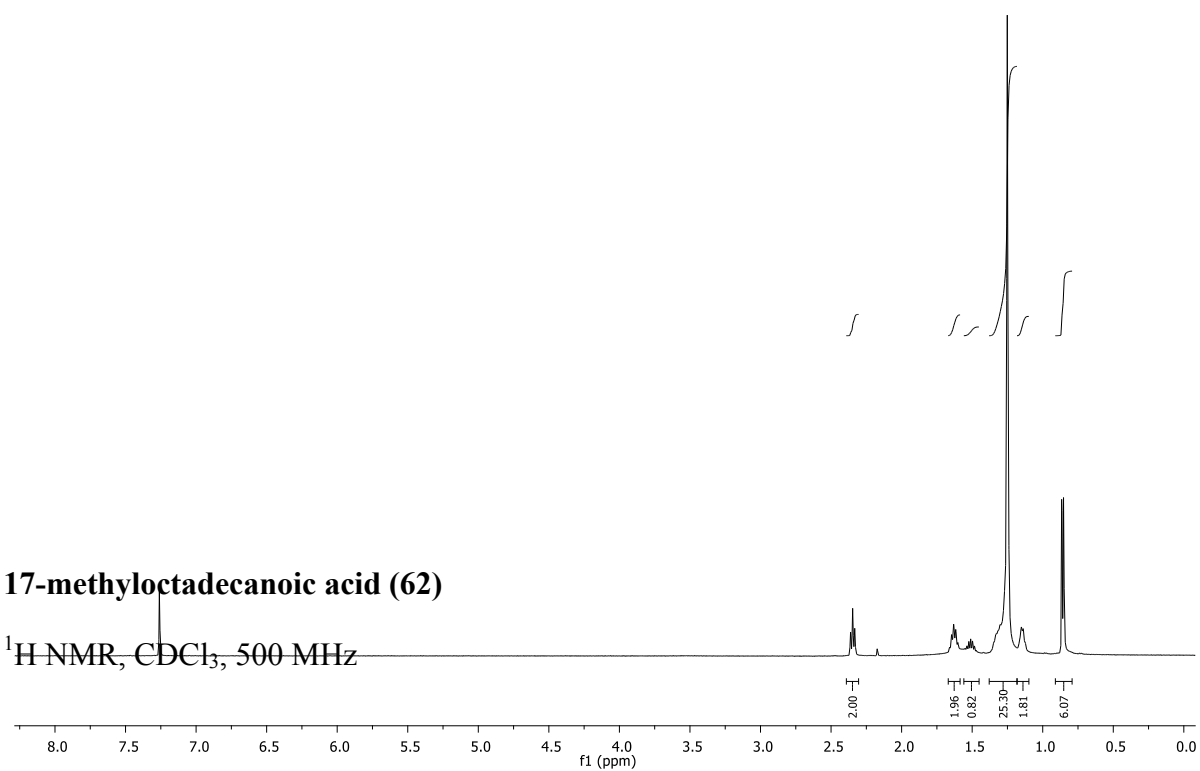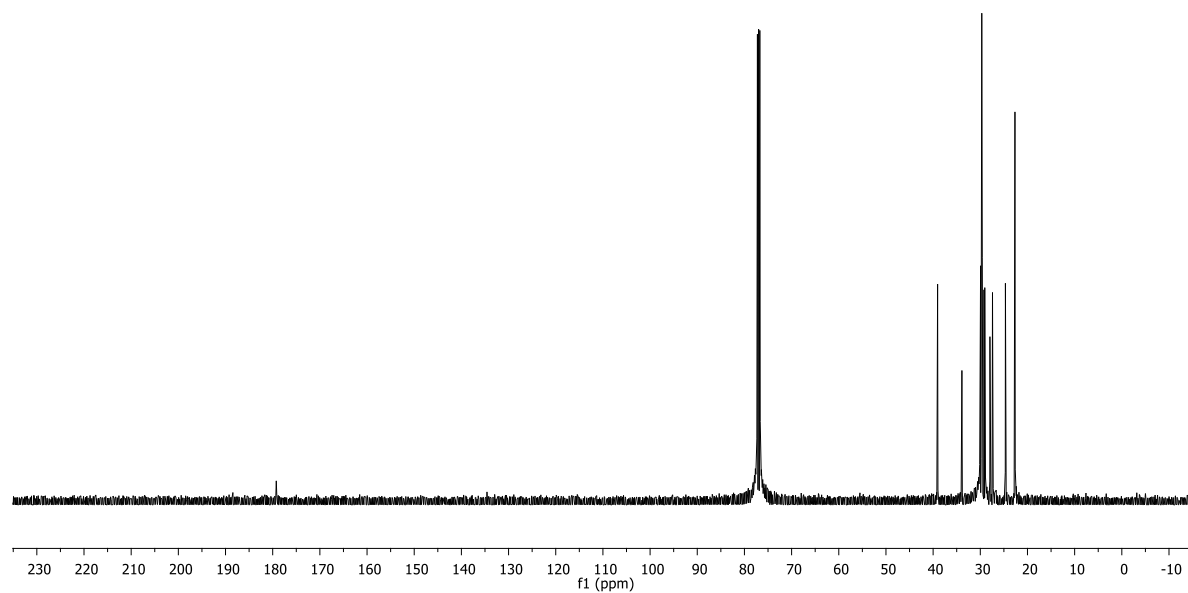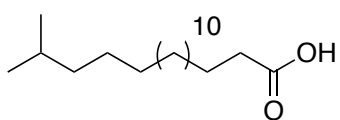
17-methyloctadecanoic acid (62)

^{13}C NMR, CDCl$_3$, 125 MHz
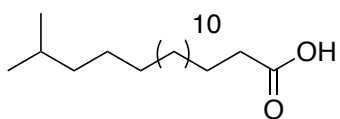
15-methylhexadecane-1,15-diol (81)

^{1}H NMR, CDCl$_3$, 500 MHz

HO—⟨⟩$_{10}$—OH
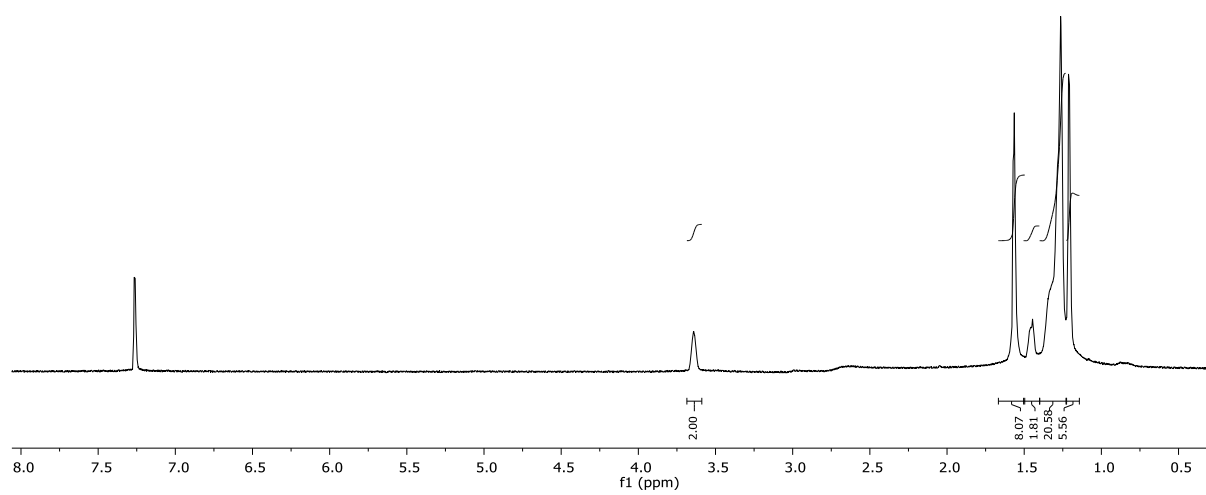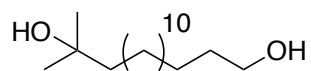
15-methylhexadecan-1-ol (66)

¹H NMR, CDCl₃, 500 MHz
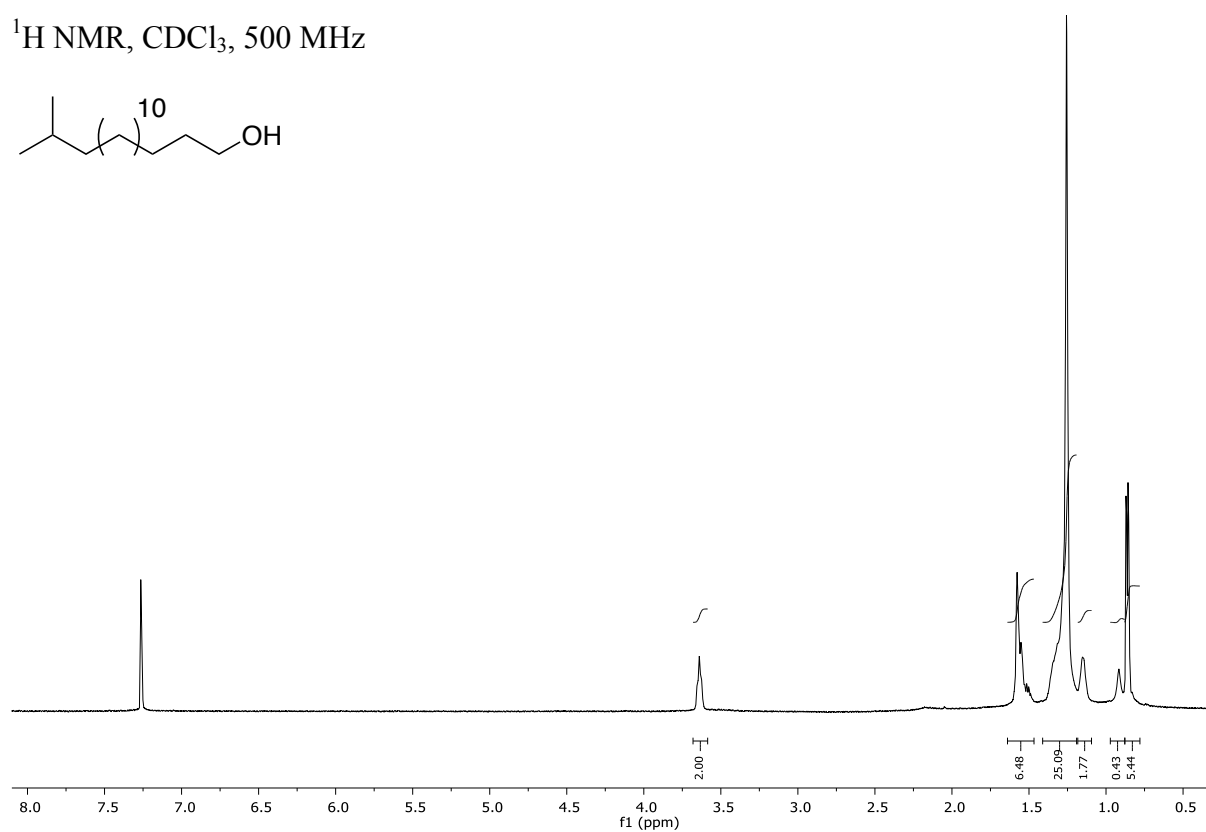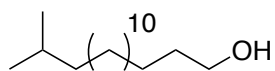
15-methylhexadecan-1-ol (66)

¹³C NMR, CDCl₃, 125 MHz
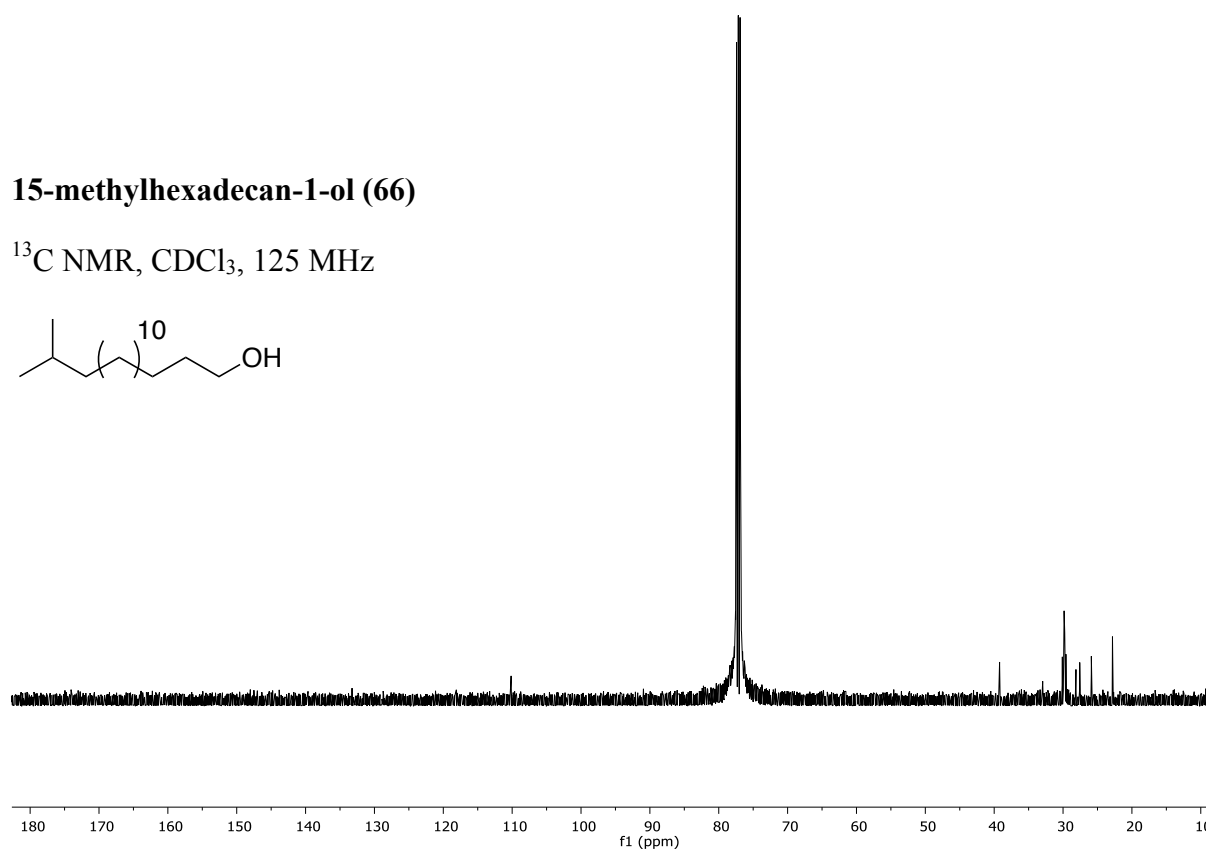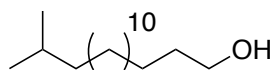
15-methylhexadecanoic acid (61)

^{1}H NMR, CDCl$_3$, 500 MHz
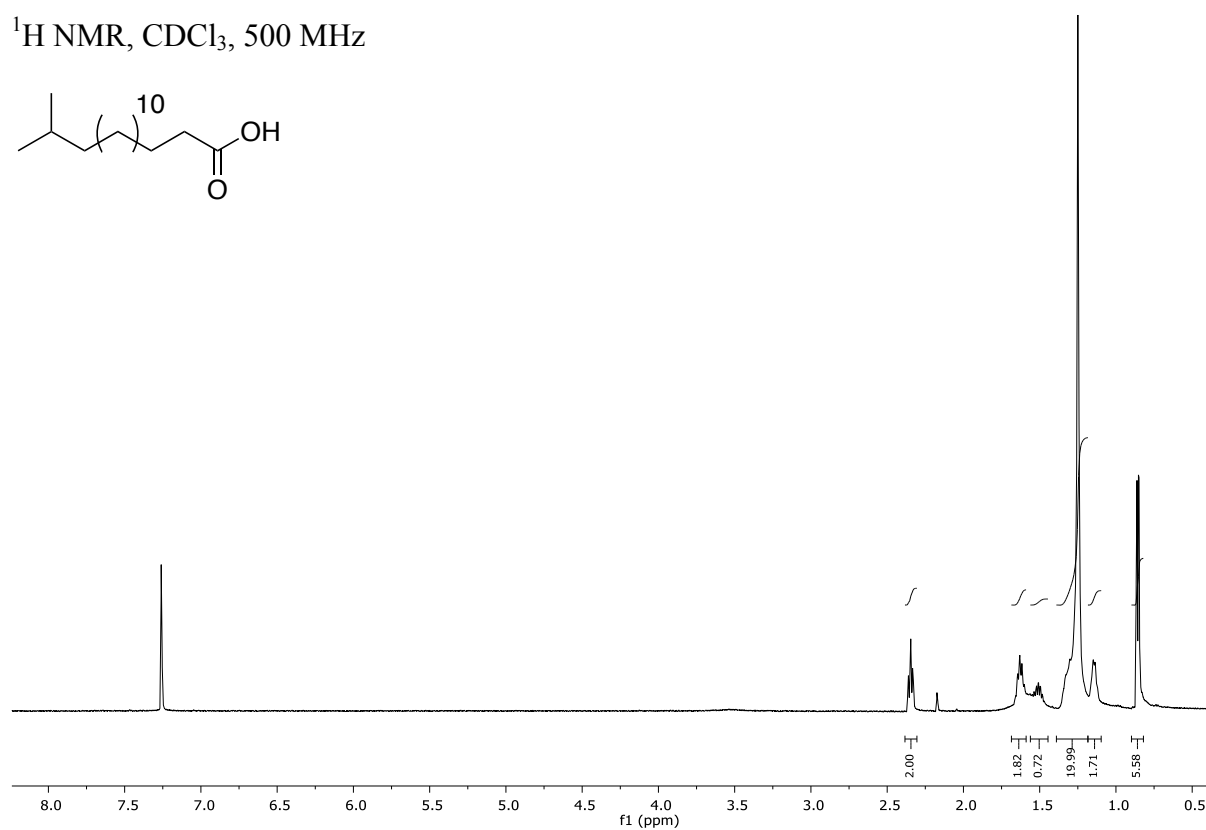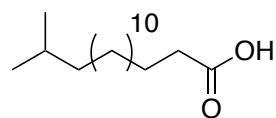
S-methyl-O-15-methylhexadecyl dithiocarbonate (82)

^{1}H NMR, CDCl$_3$, 500 MHz
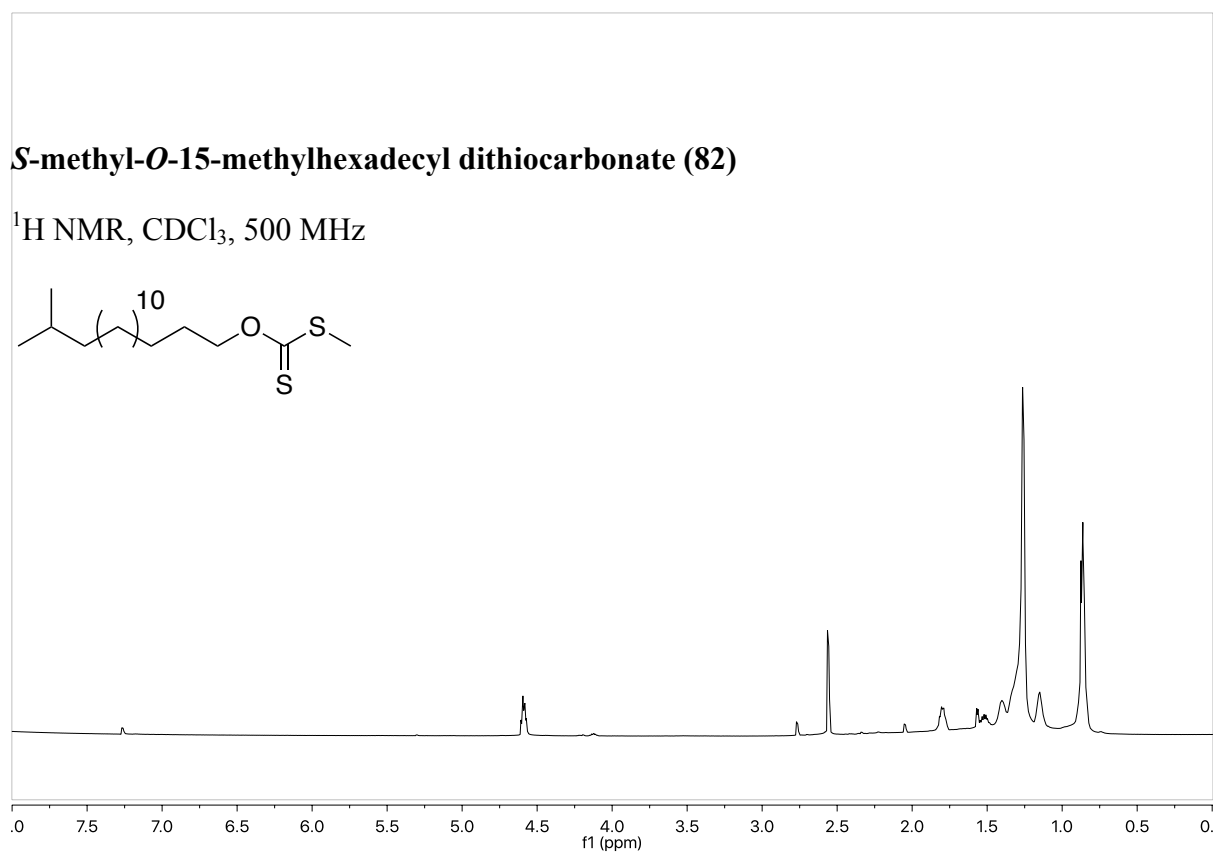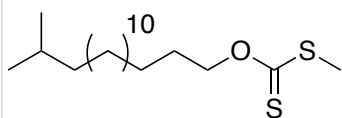
S-methyl-*O*-15-methylhexadecyl dithiocarbonate (82)

^{13}C NMR, CDCl$_3$, 125 MHz
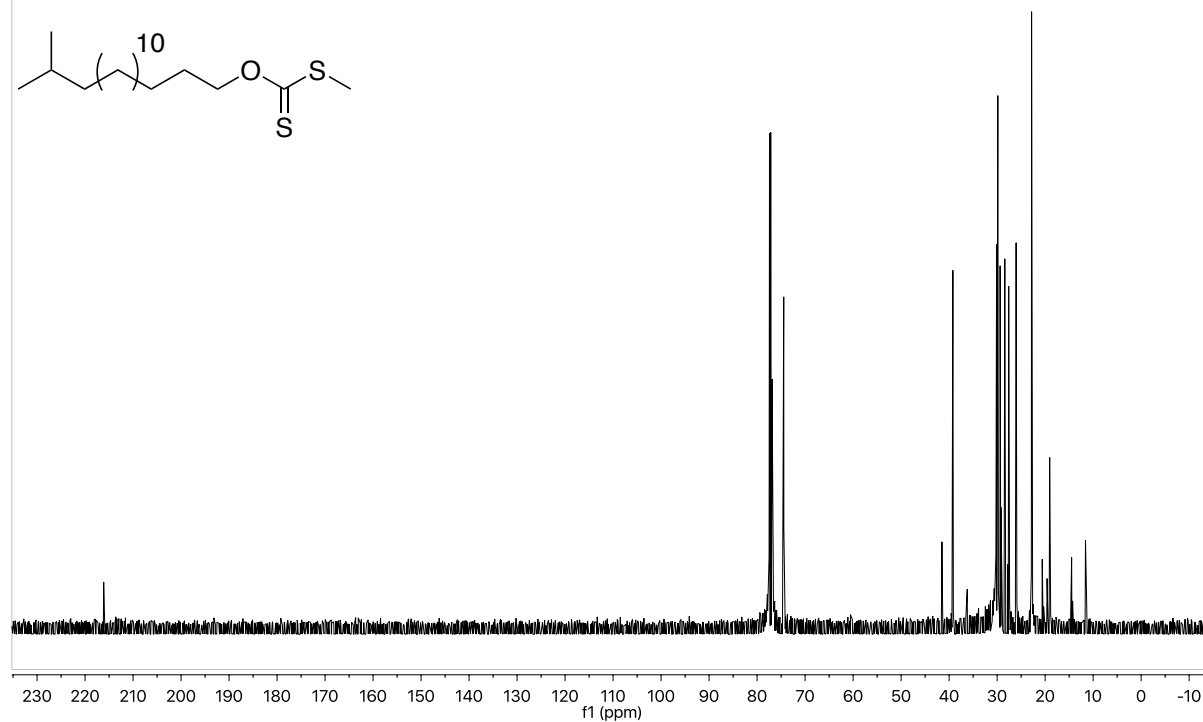
15-methylhexadec-1-ene (65)

^{1}H NMR, CDCl$_3$, 500 MHz
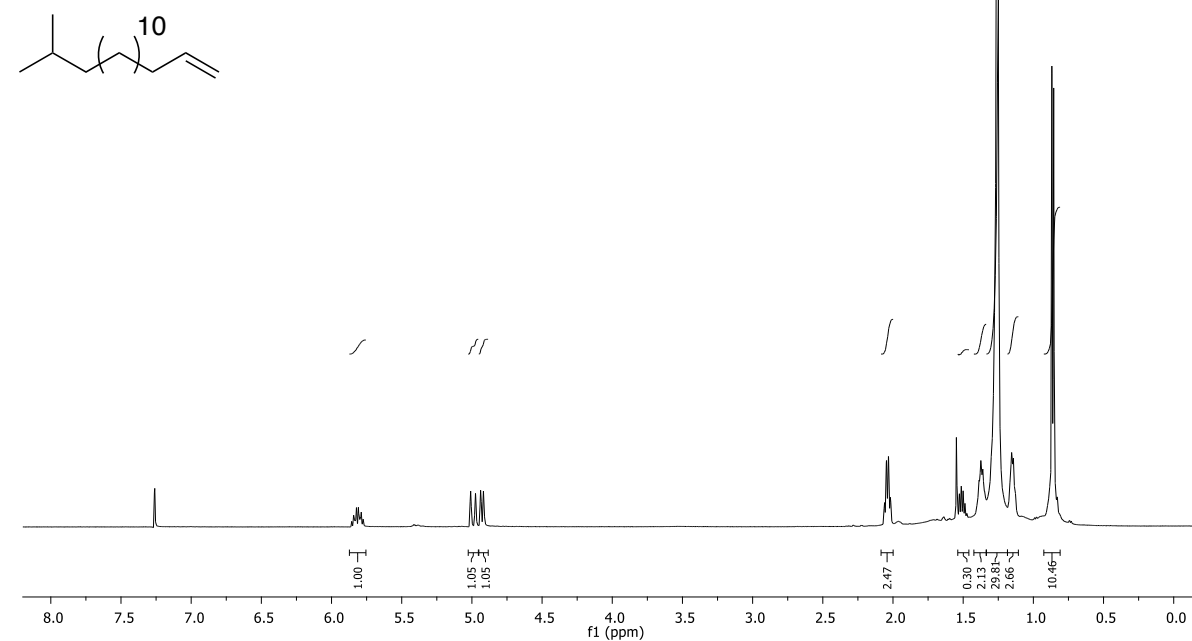
15-methylhexadec-1-ene (65)

^{13}C NMR, CDCl$_3$, 125 MHz
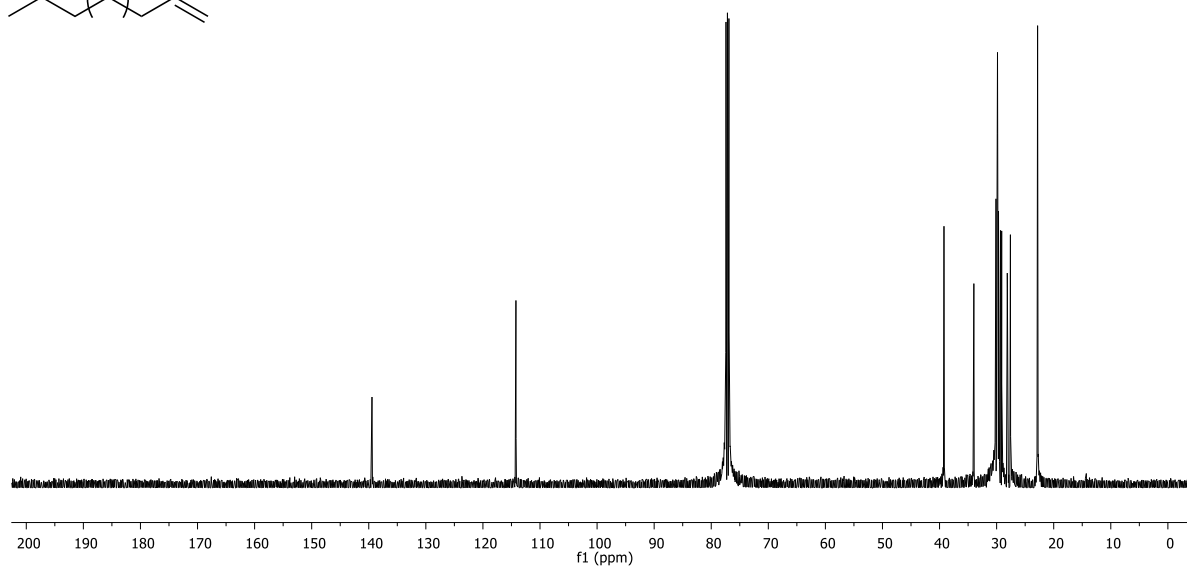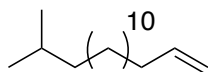
14-methylpentadecanoic acid (64)

^{1}H NMR, CDCl$_3$, 500 MHz
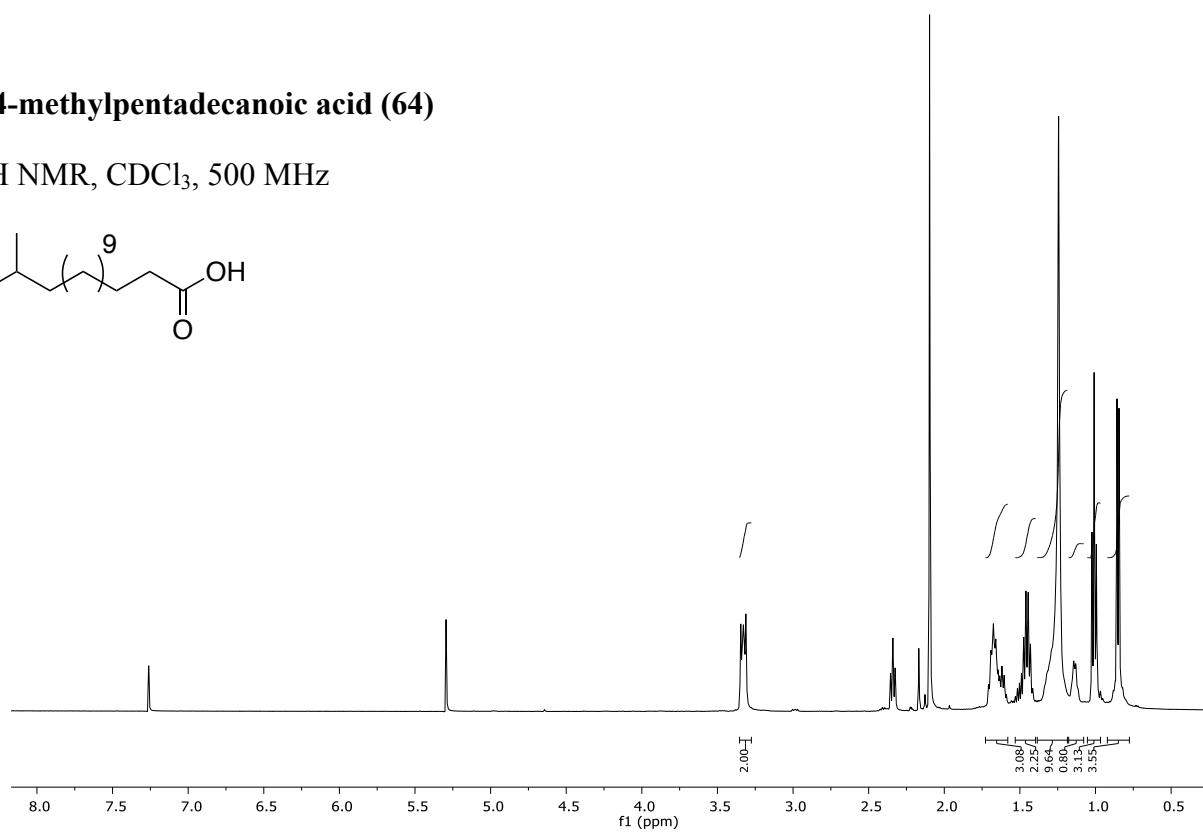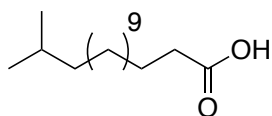
14-methylpentadecanoic acid (64)

^{12}C NMR, CDCl$_3$, 125 MHz
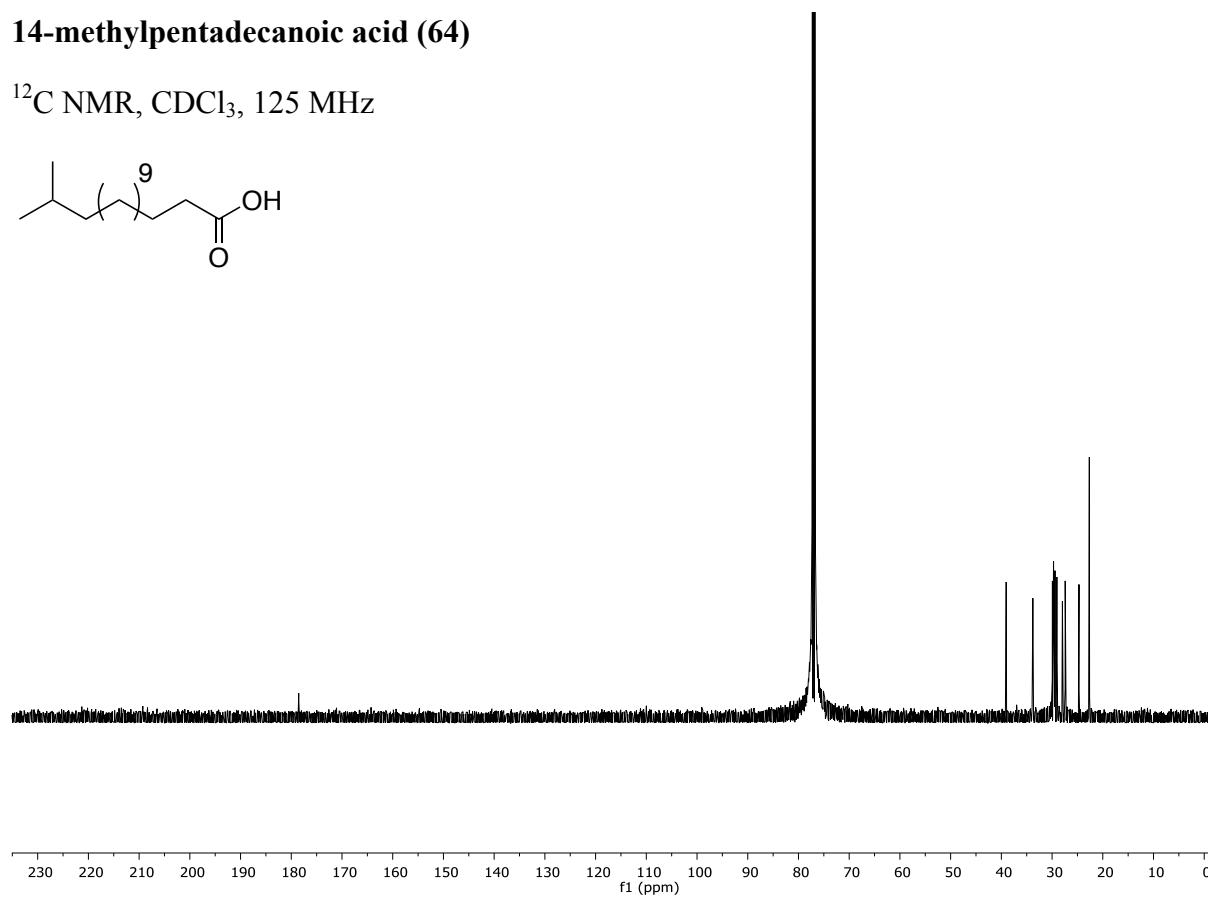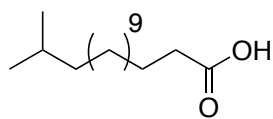
14-methylpentadecan-1-ol (83)

^{1}H NMR, CDCl$_3$, 500 MHz
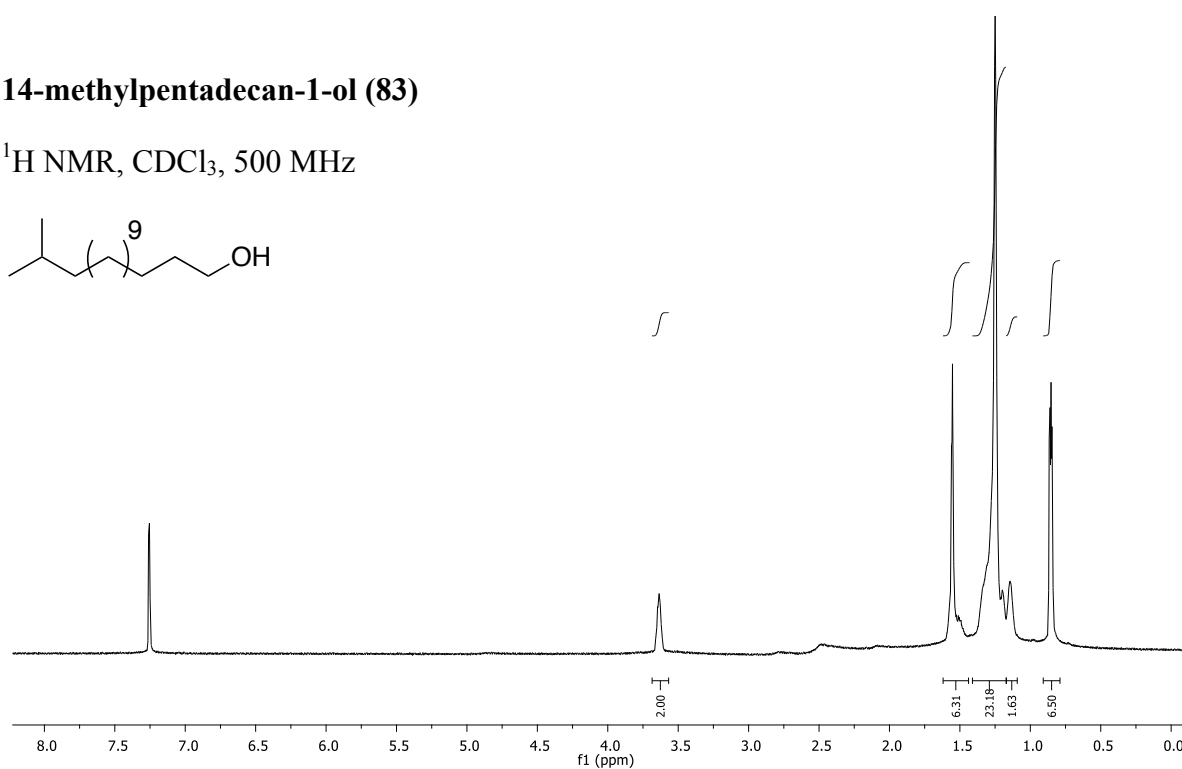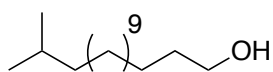
14-methylpentadecan-1-ol (83)

^{13}C NMR, CDCl$_3$, 125 MHz
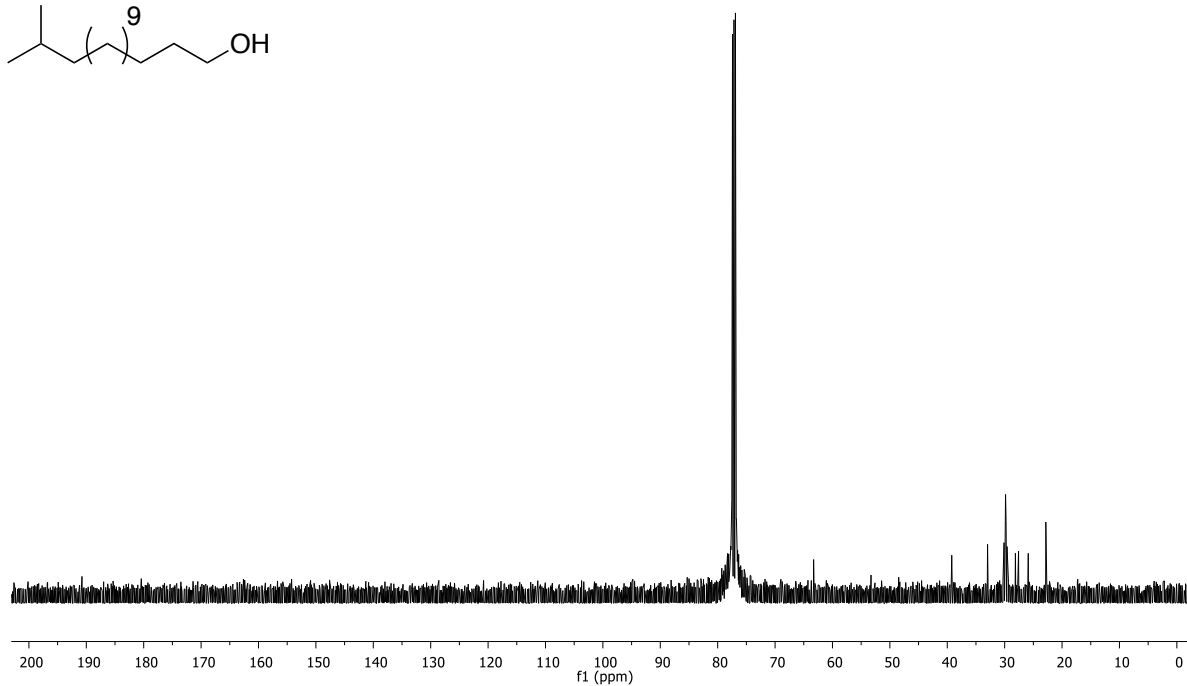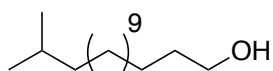
S-methyl-O-14-methylpentadecyl dithiocarbonate (84)

^{1}H NMR, CDCl$_3$, 500 MHz
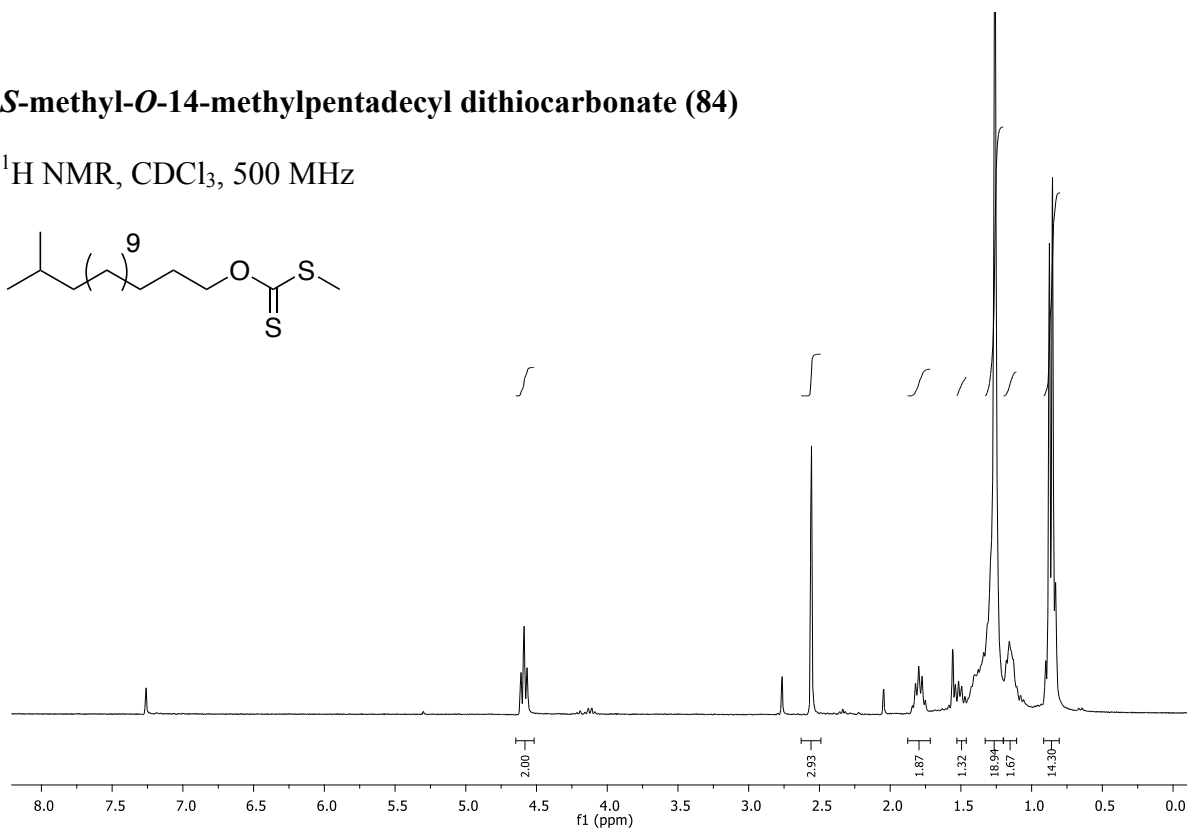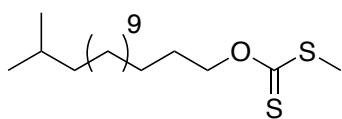
14-methylpentadec-1-ene (63)

^{1}H NMR, CDCl$_3$, 500 MHz
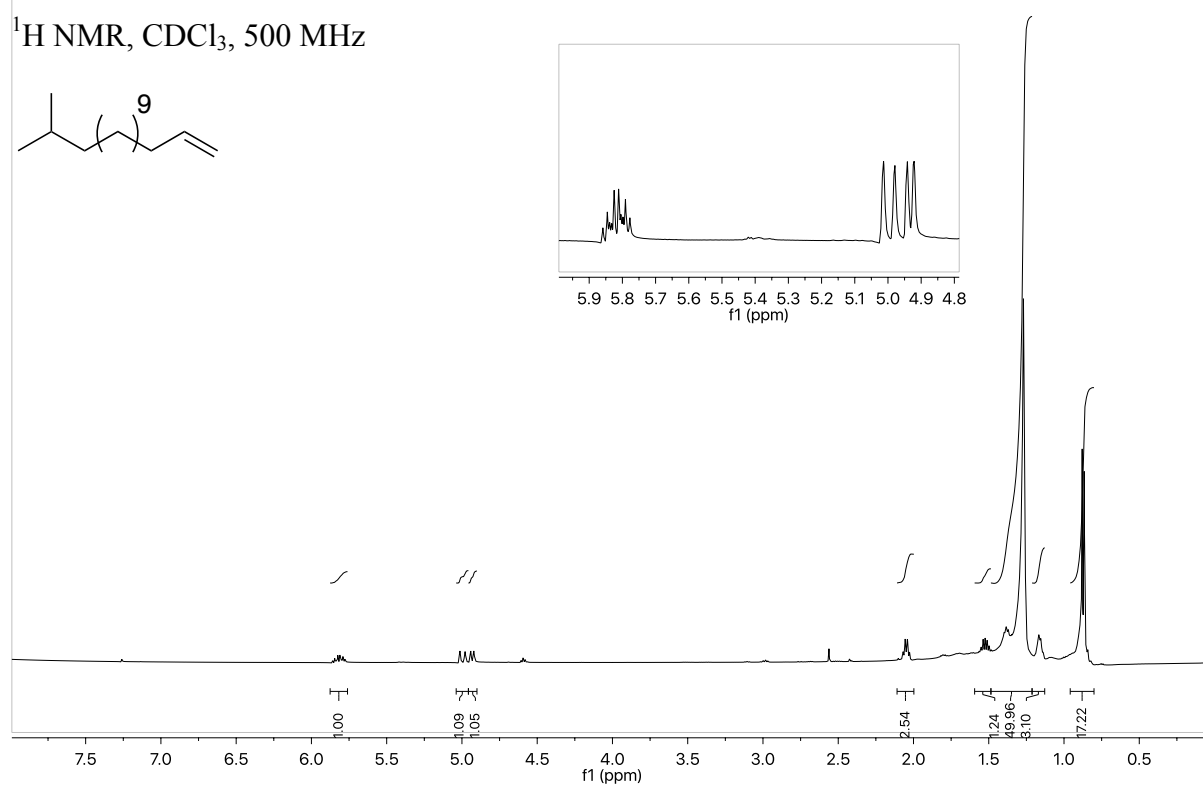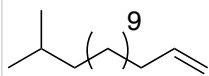
14-methylpentadec-1-ene (63)

^{13}C NMR, CDCl$_3$, 125 MHz
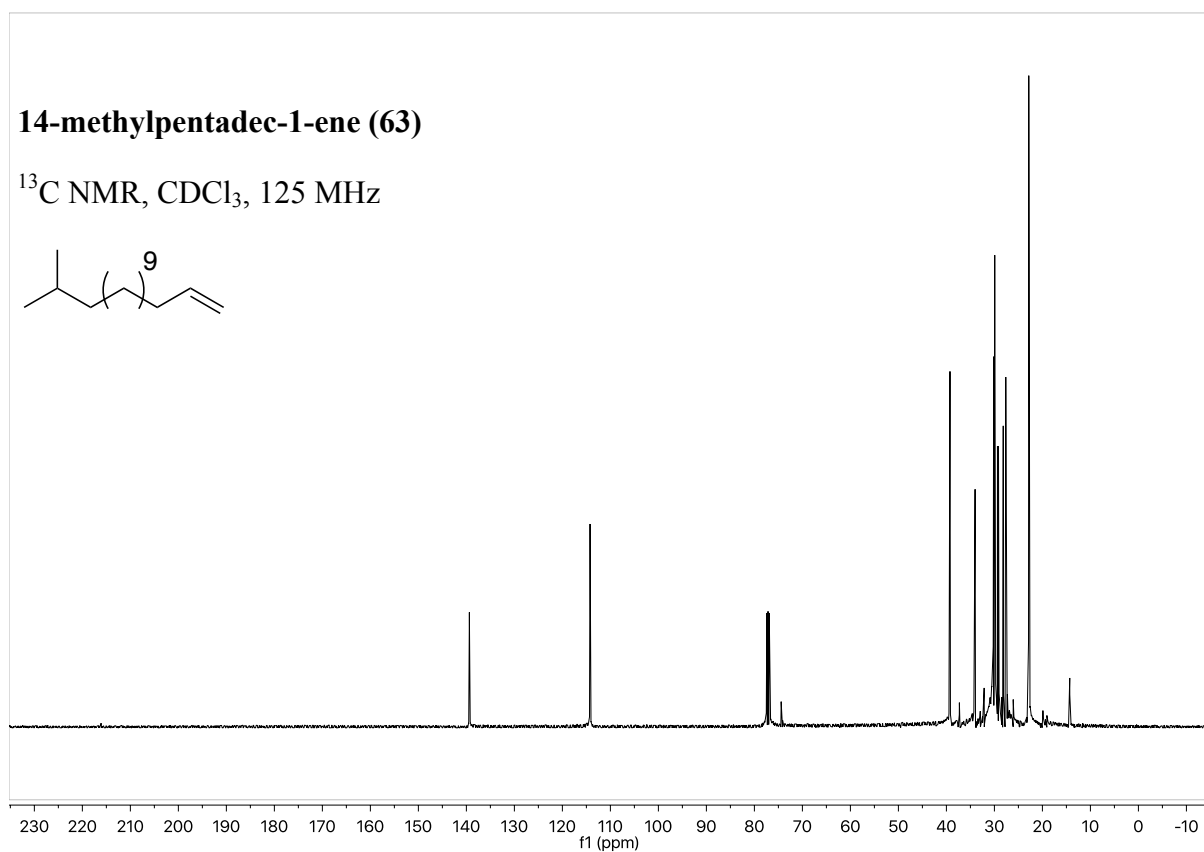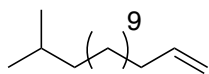
6,6′-di-*O*-tetradecanoate-α,α′-D-trehalose (33)

¹H NMR, C₅D₅N, 500 MHz
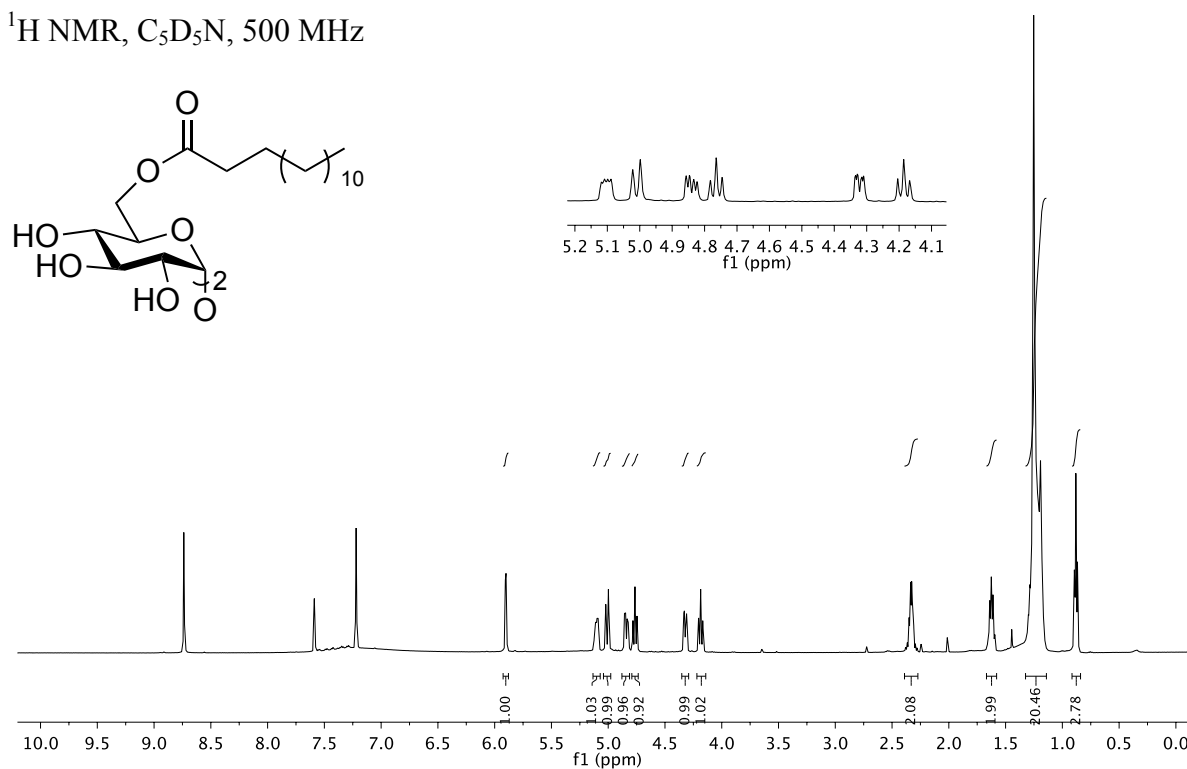
6,6′-di-*O*-tetradecanoate-α,α′-D-trehalose (33)

¹³C NMR, C₅D₅N, 125 MHz
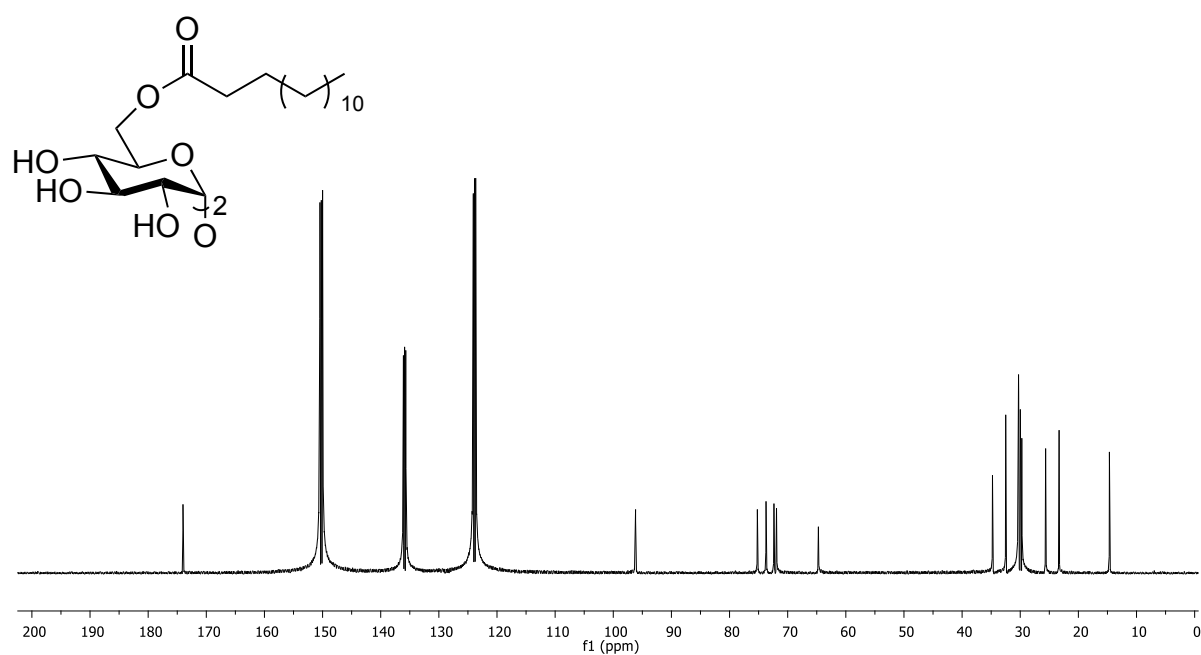
6,6′-di-*O*-hexadecanoate-α,α′-D-trehalose (34)

¹H NMR, C₅D₅N, 500 MHz
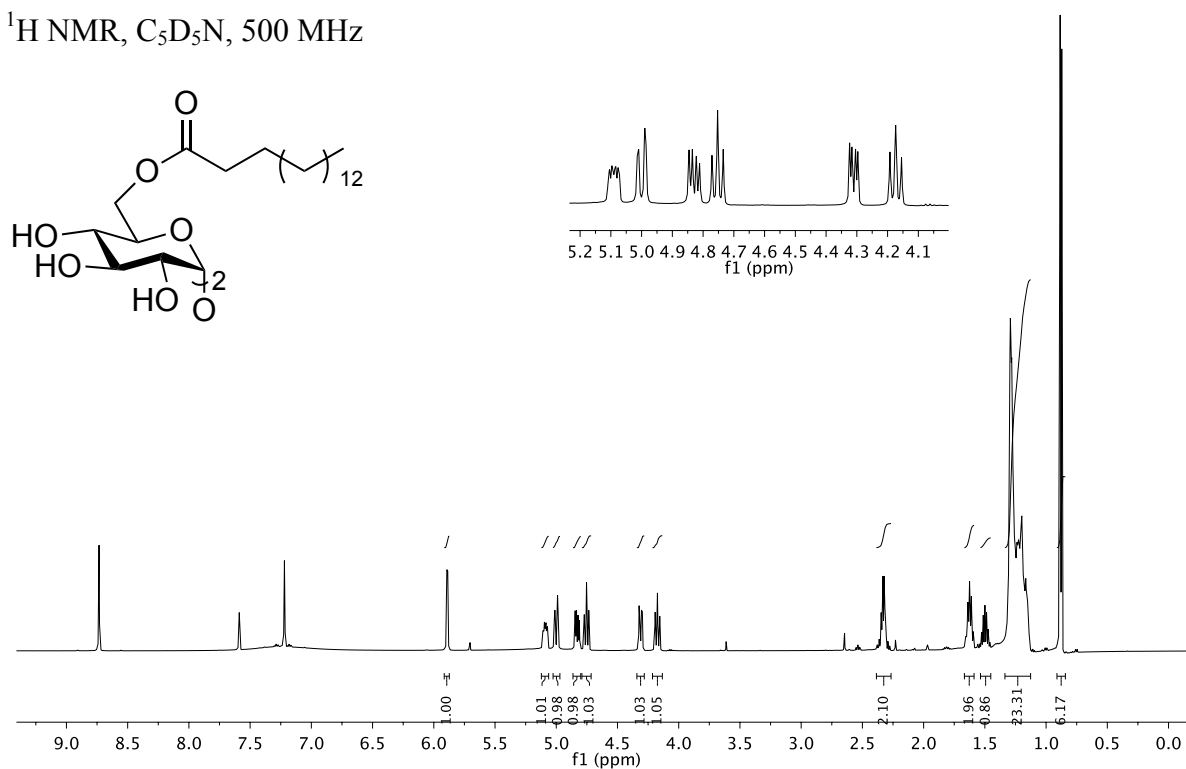
6,6′-di-*O*-hexadecanoate-α,α′-D-trehalose (34)

¹³C NMR, C₅D₅N, 125 MHz

129
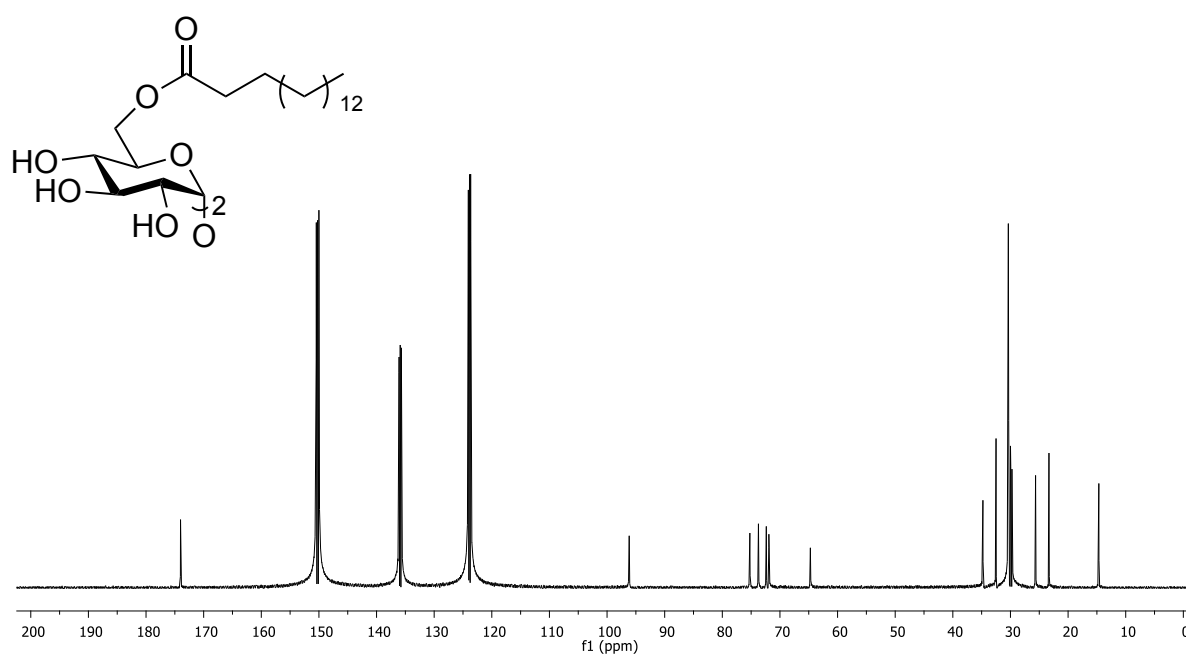
6,6′-di-*O*-octadecanoic-α,α′-D-trehalose (12)

¹H NMR, C₅D₅N, 500 MHz
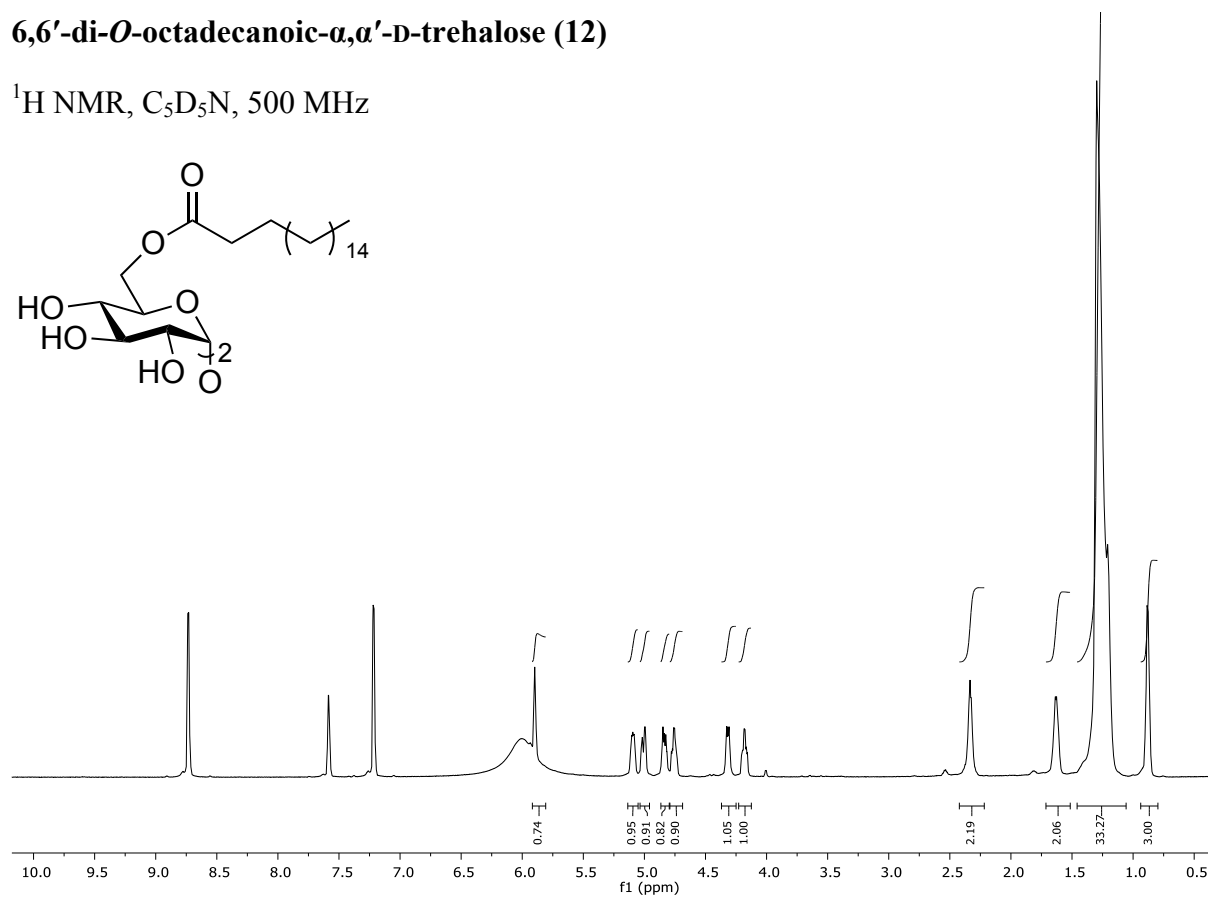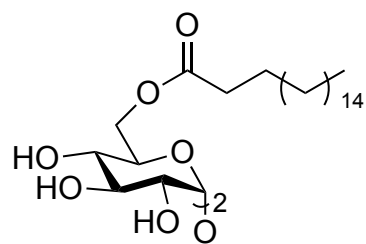
6,6′-di-*O*-octadecanoate-α,α′-D-trehalose (12)

¹³C NMR, C₅D₅N, 125 MHz

130
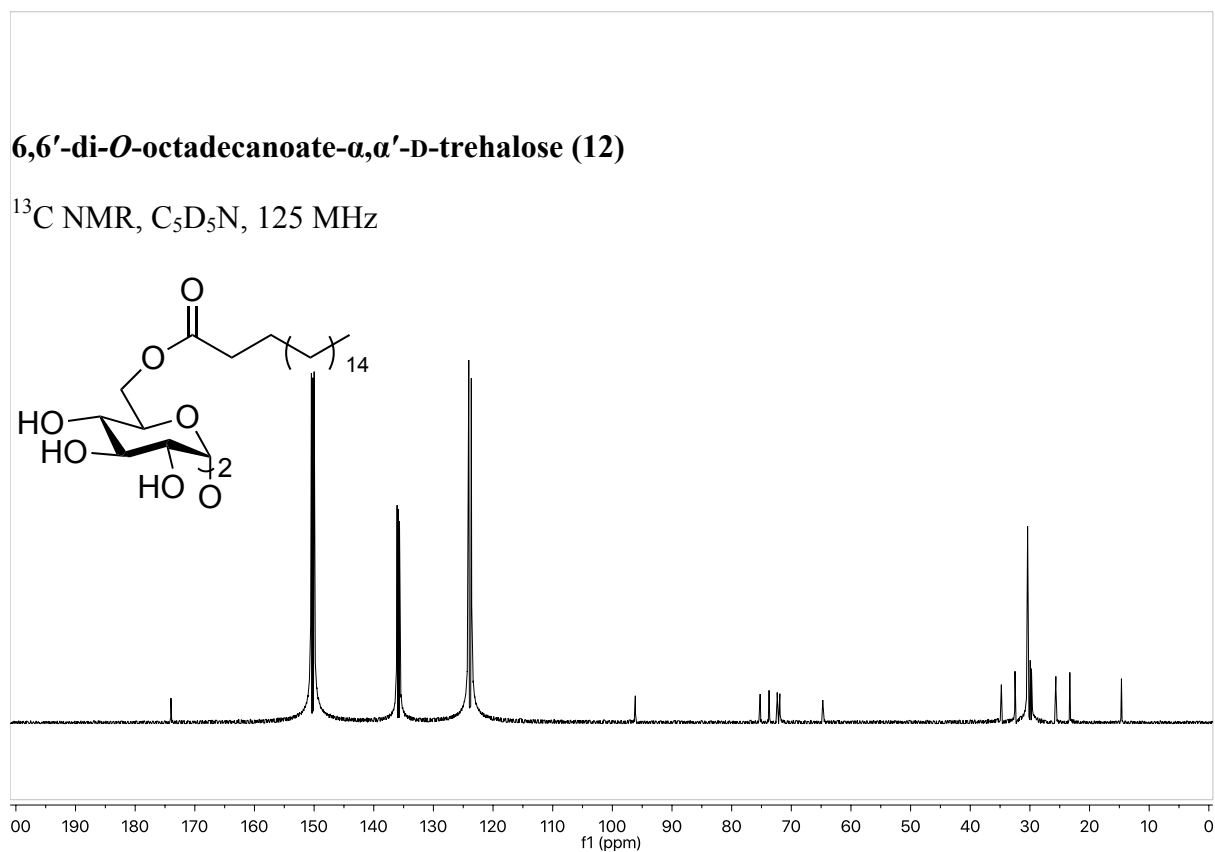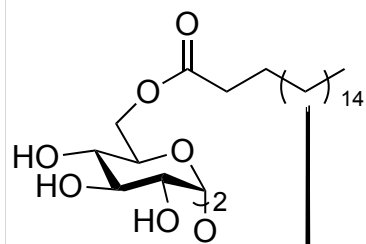
6,6′-di-*O*-(15-methylhexadecanoate)-α,α′-D-trehalose (36)

¹H NMR, C₅D₅N, 500 MHz
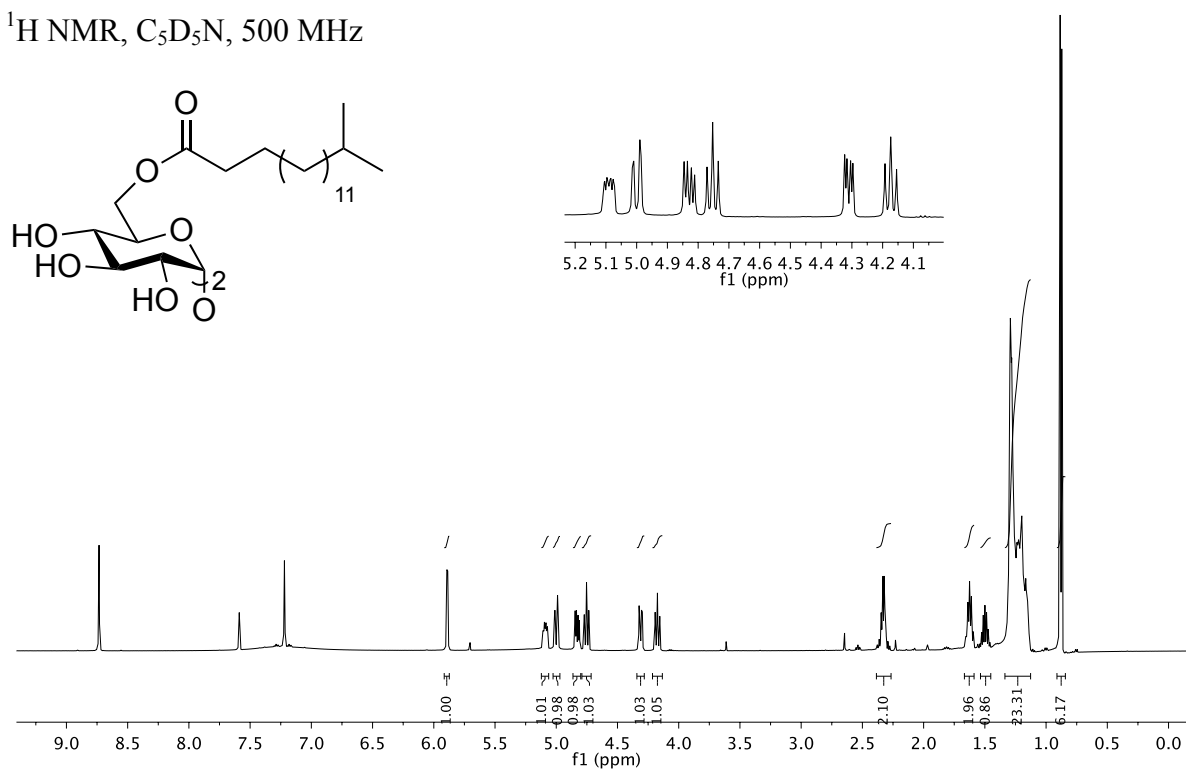
6,6′-di-*O*-(15-methylhexadecanoate)-α,α′-D-trehalose (36)

¹³C NMR, C₅D₅N, 125 MHz
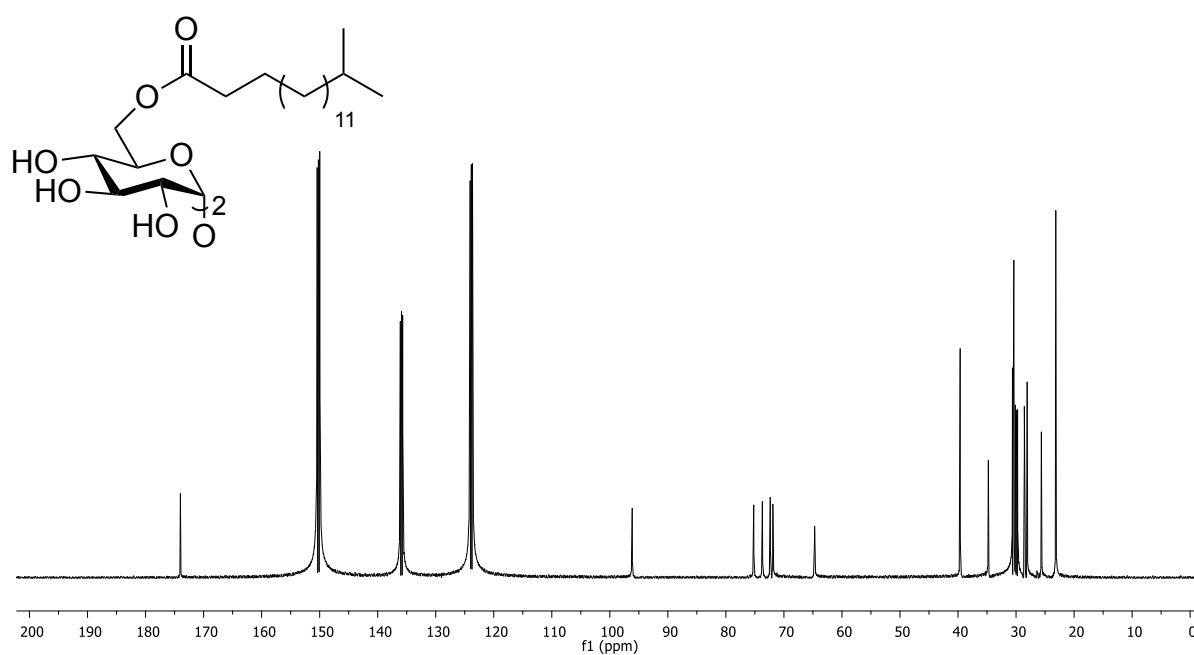
6,6′-di-*O*-(17-methyloctadecanoate)-α,α′-D-trehalose (32)

^{1}H NMR, C$_5$D$_5$N, 500 MHz
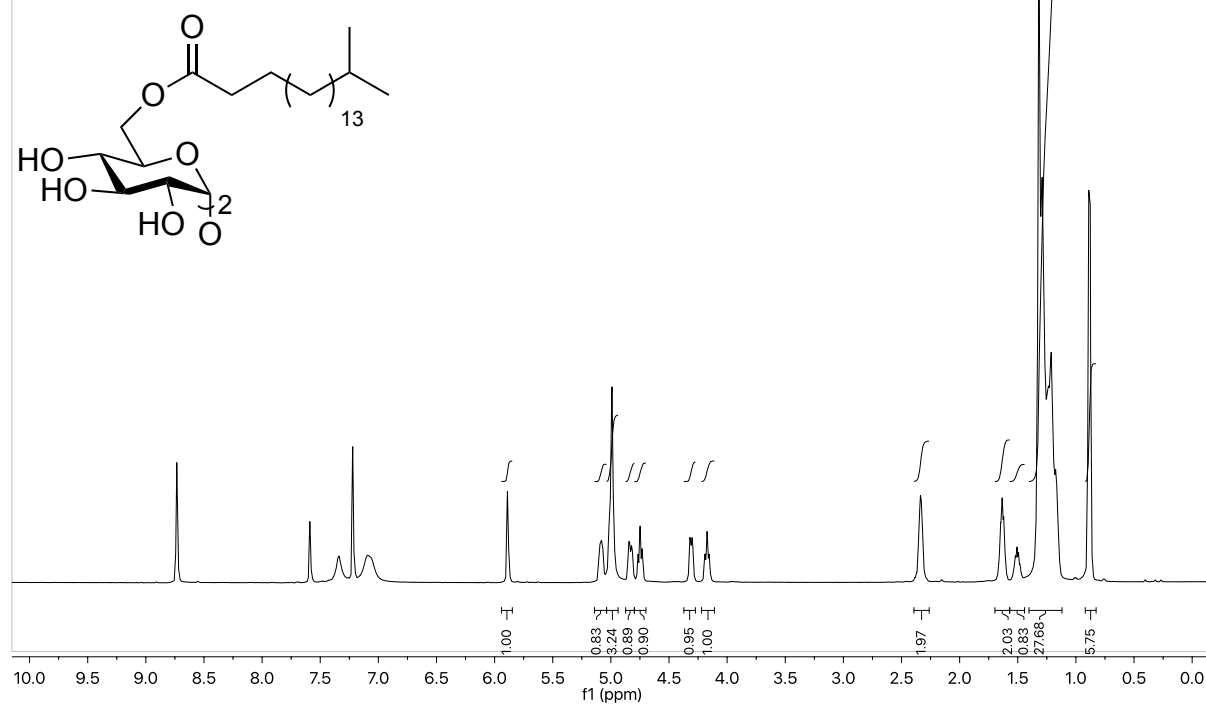
6,6′-di-*O*-(17-methyloctadecanoate)-α,α′-D-trehalose (32)

^{13}C NMR, C$_5$D$_5$N, 125 MHz

132
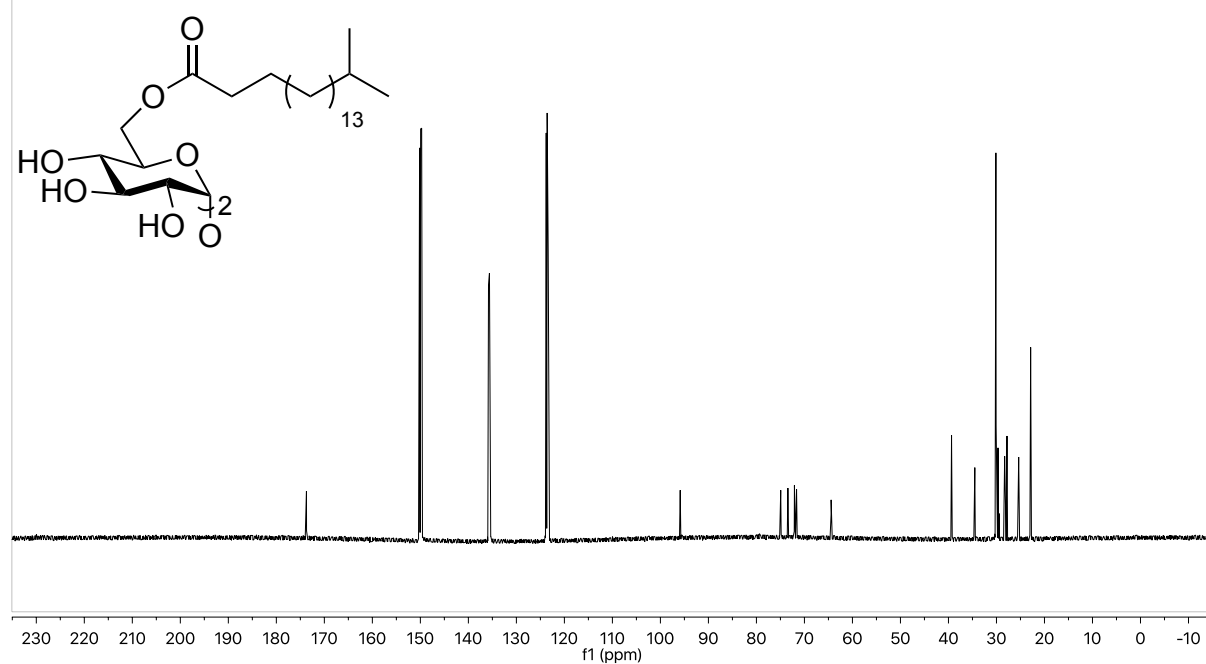
1-*O*-tetradecanoate-glycerol (27)

¹H NMR, CDCl₃, 500 MHz
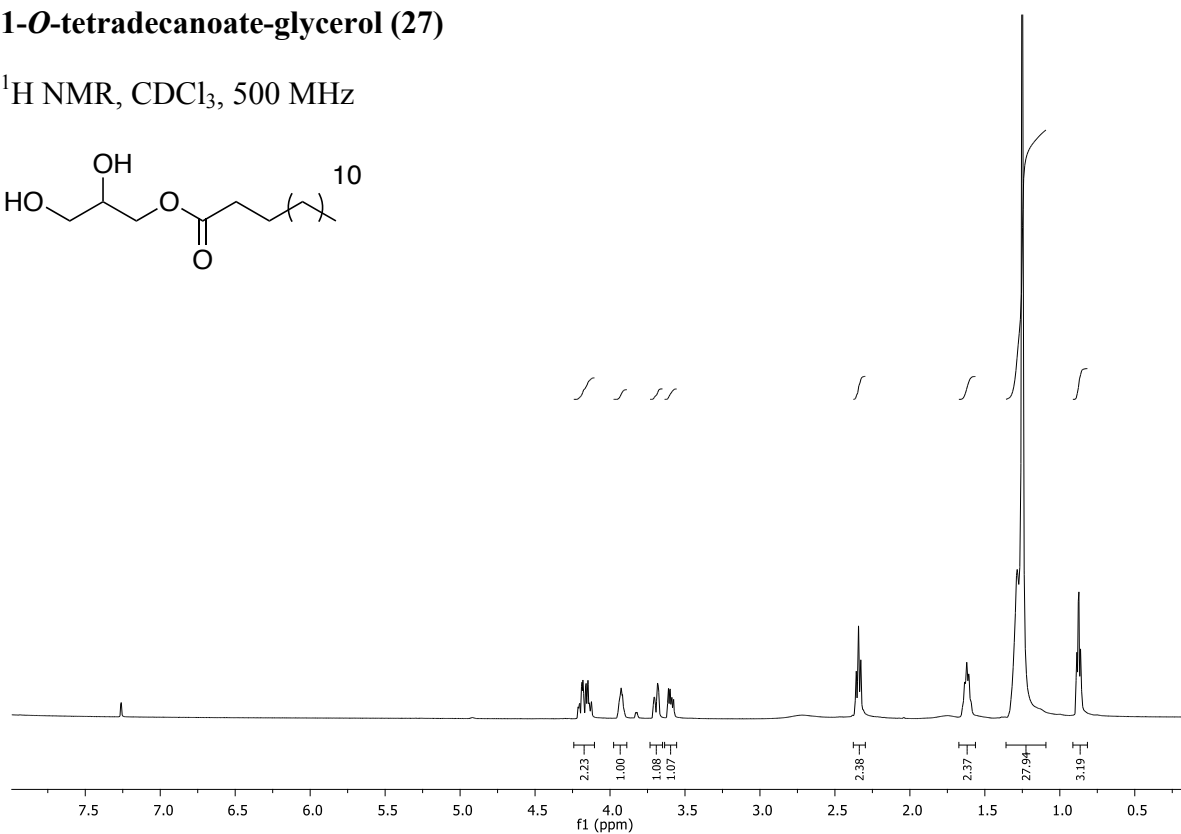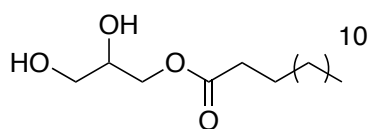
1-*O*-tetradecanoate-glycerol (27)

¹³C NMR, CDCl₃, 125 MHz

133
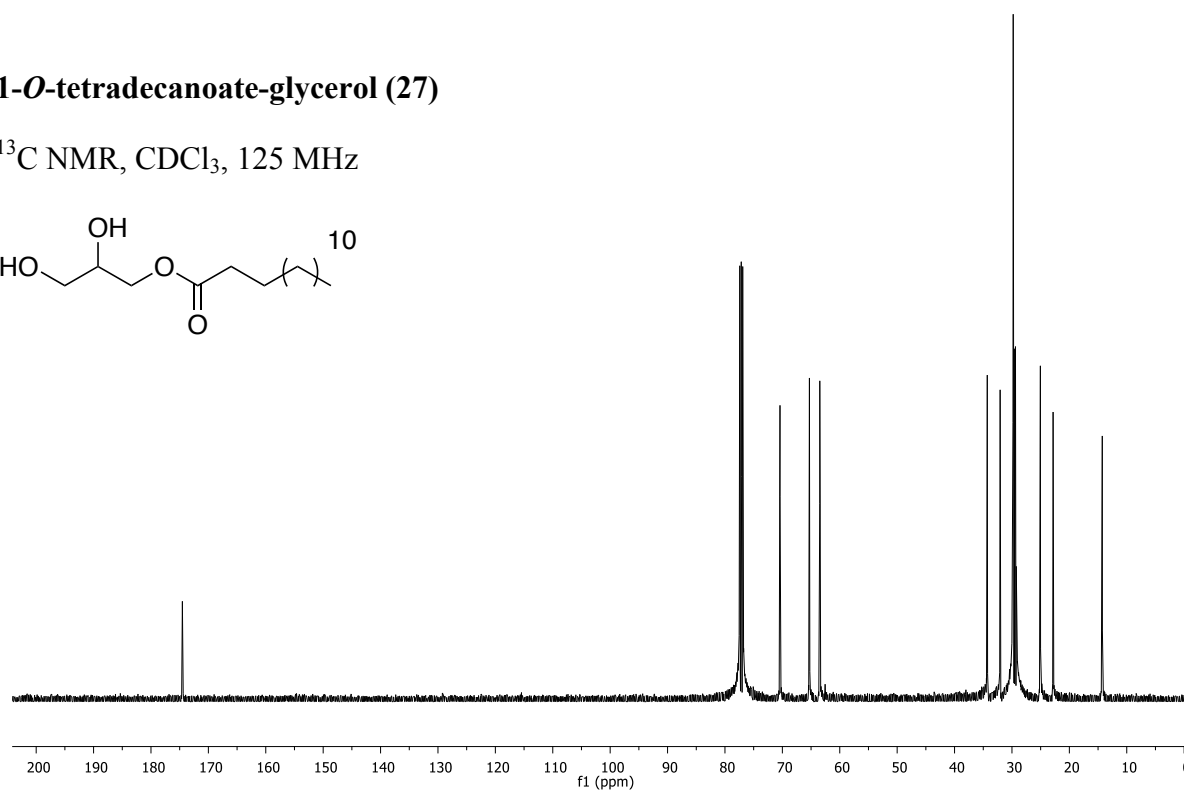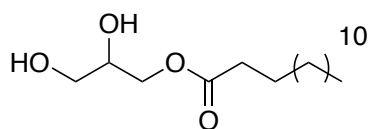
1-*O*-hexadecanoate-glycerol (28)

¹H NMR, CDCl₃, 500 MHz

0.55 0.52 1.06 0.83 0.85 2.02 6.40 26.81 3.00
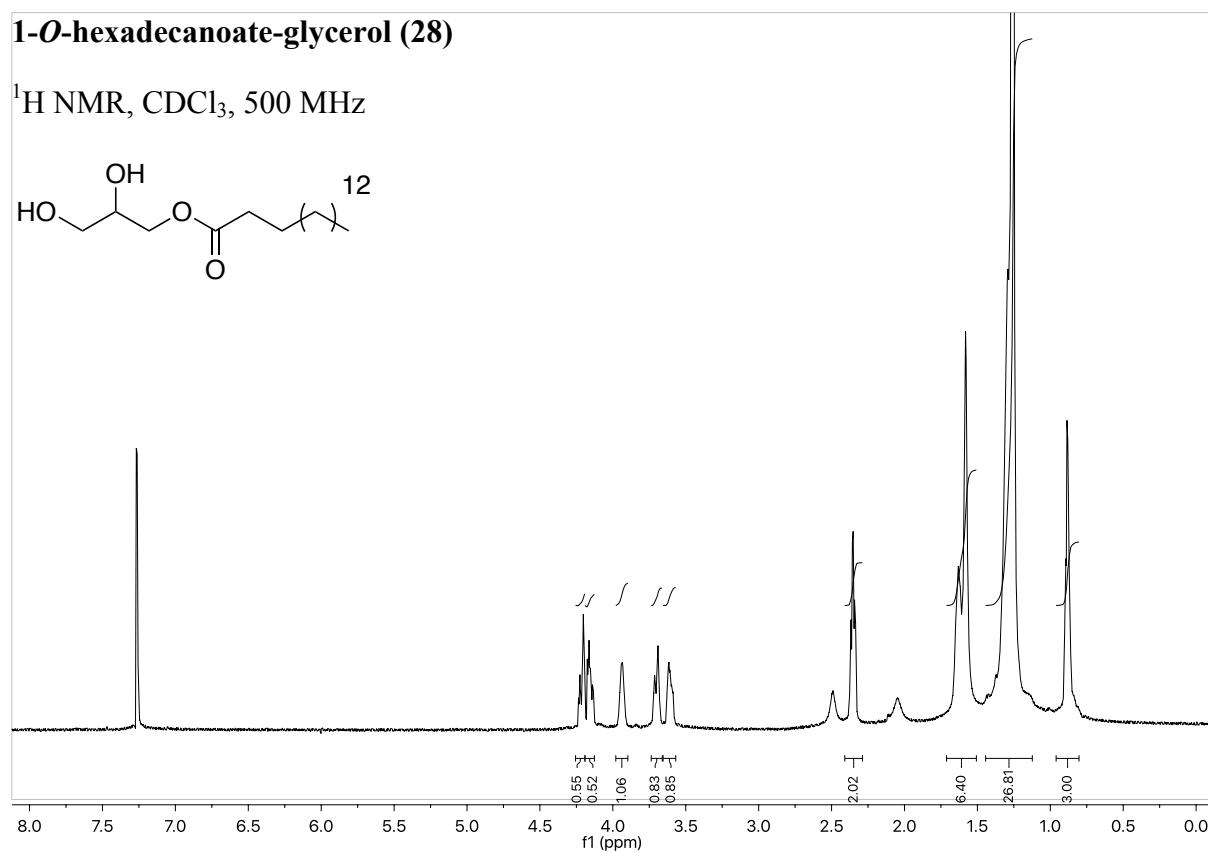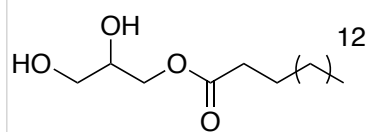
1-*O*-hexadecanoate-glycerol (28)

¹³C NMR, CDCl₃, 125 MHz
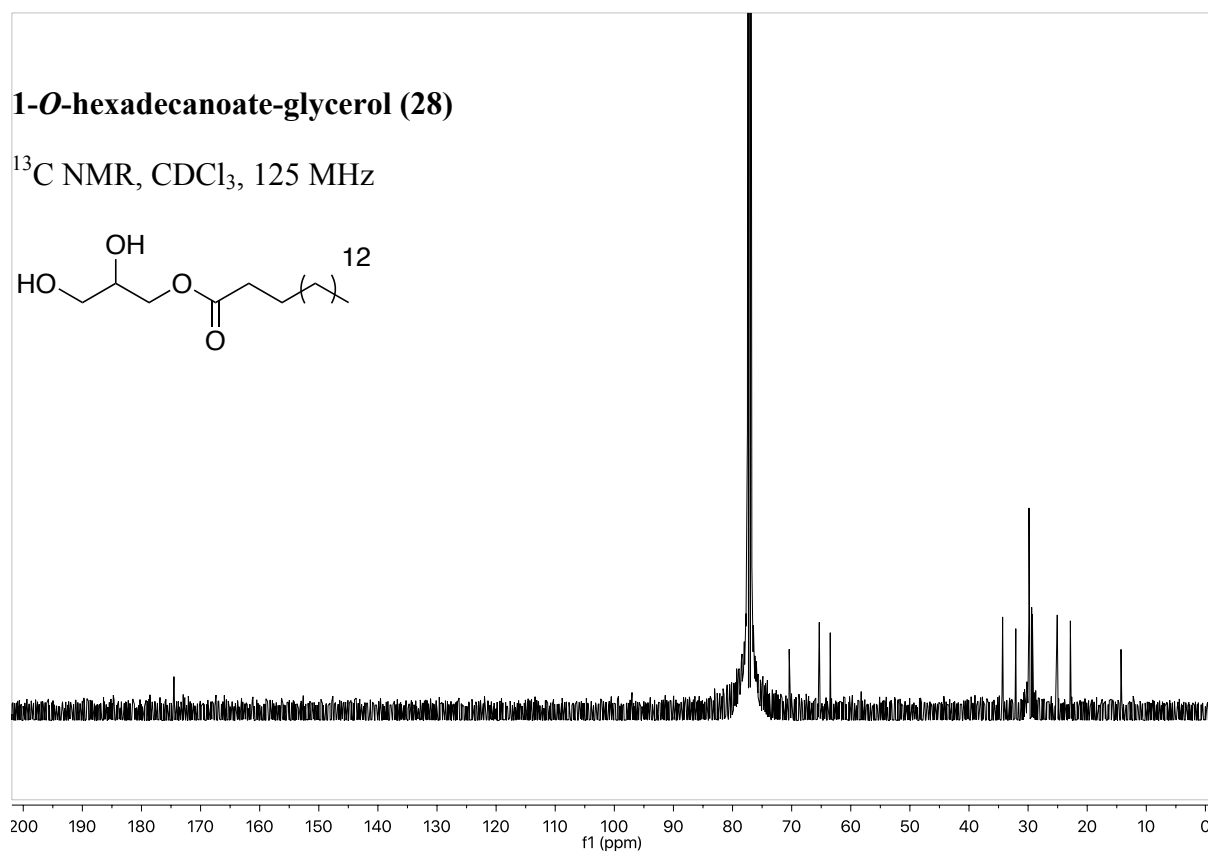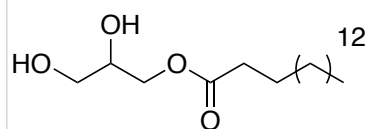
1-*O*-octadecanoate-glycerol (29)

¹H NMR, CDCl₃, 500 MHz
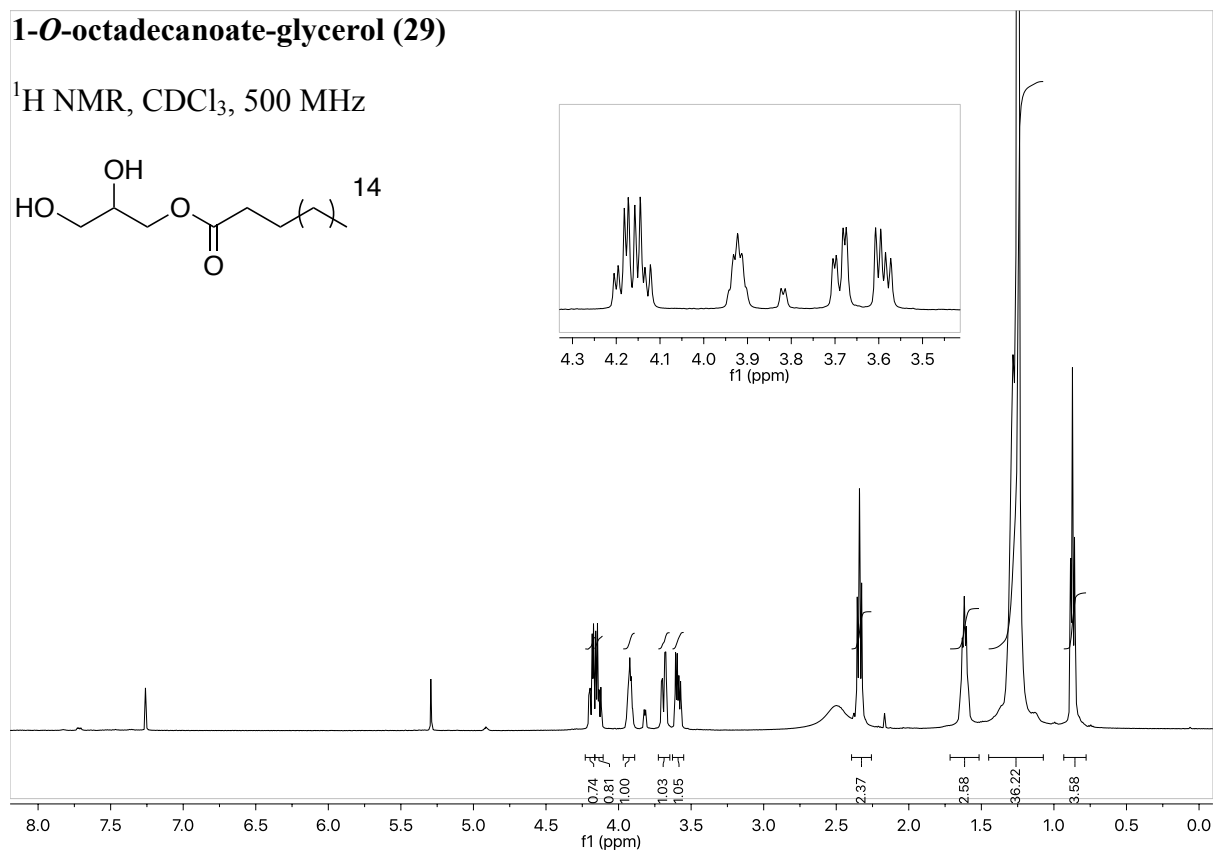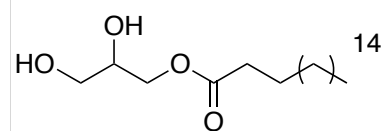
1-*O*-octadecanoate-glycerol (29)

¹³C NMR, CDCl₃, 125 MHz
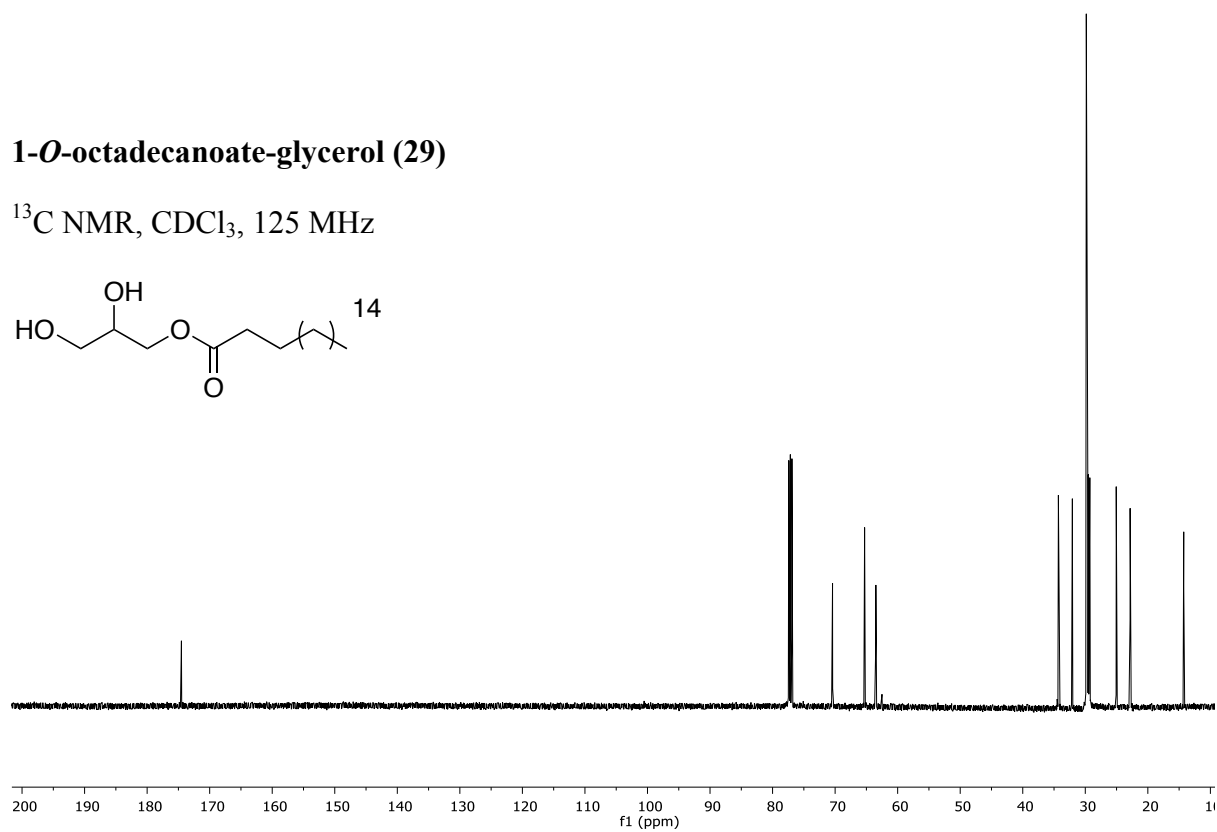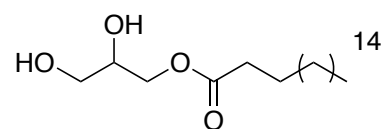
1-*O*-docosonoate-glycerol (22)

¹H NMR, CD₃OD, 500 MHz
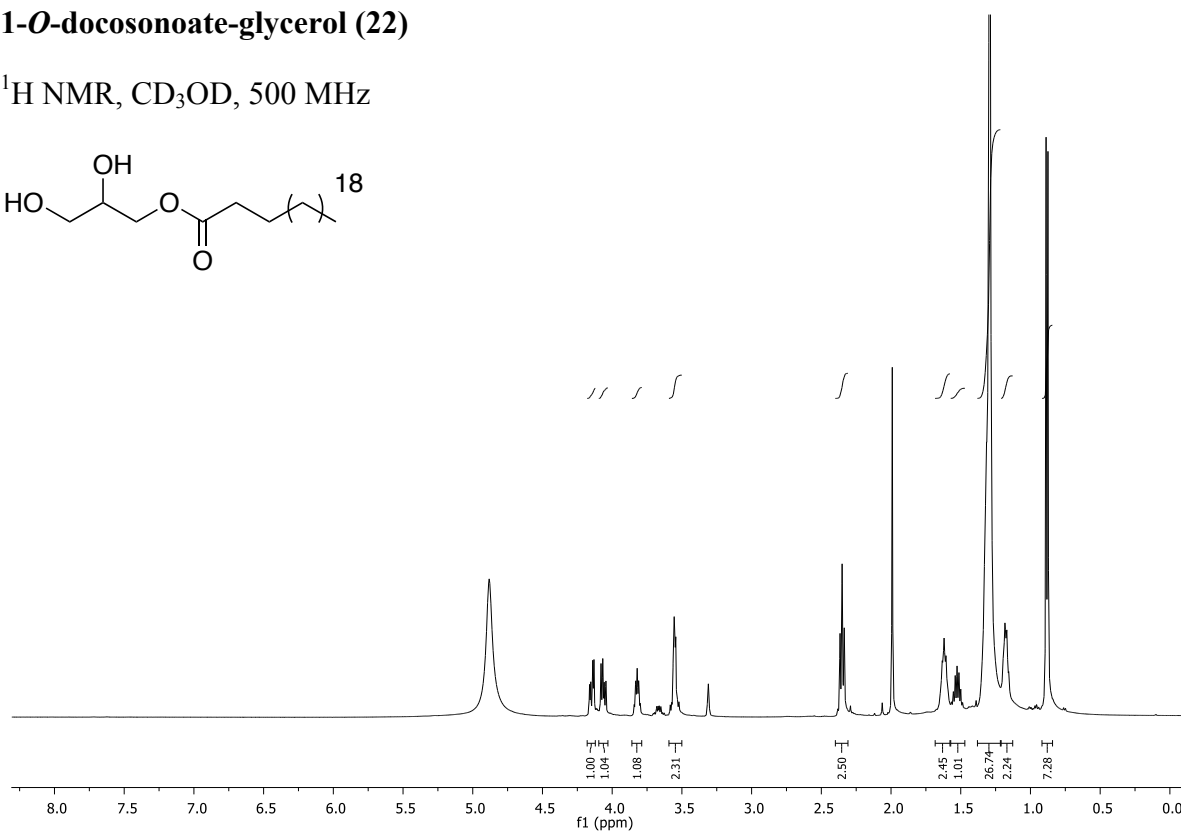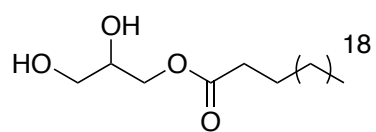
^{1}H NMR, CD$_3$OD, 500 MHz
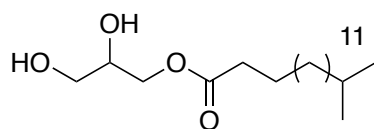

1-*O*-docosonoate-glycerol (22)

^{13}C NMR, C$_5$D$_5$N, 125 MHz
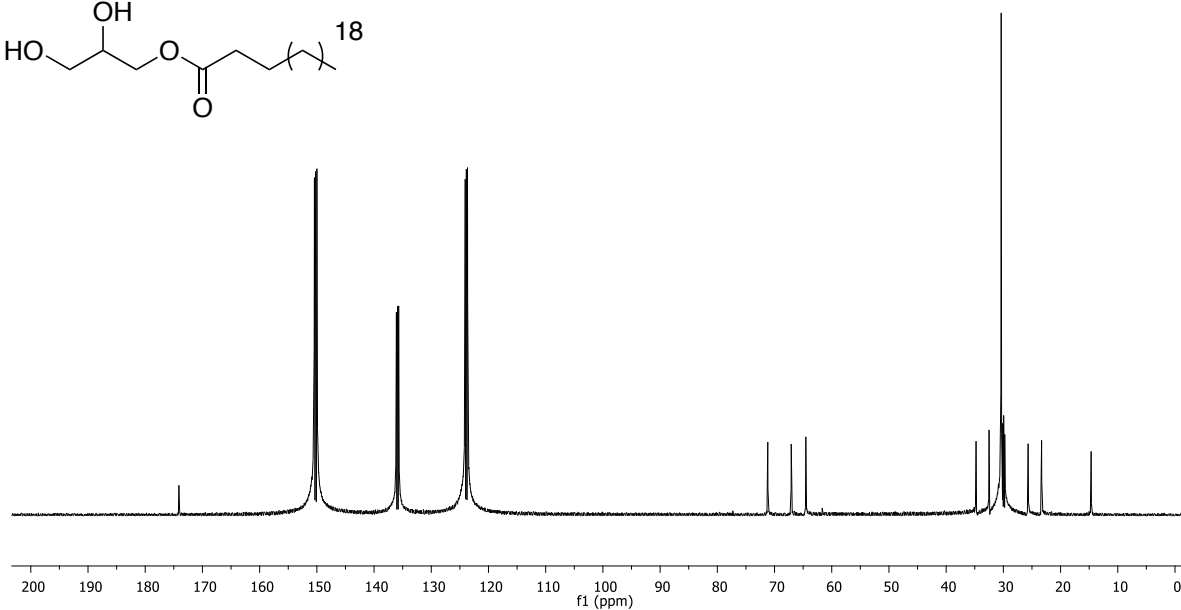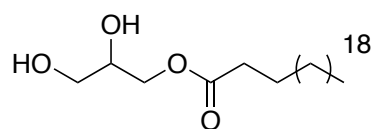
1-*O*-(15-methylhexadecanoate)-glycerol (36)

^{1}H NMR, CD$_3$OD, 500 MHz
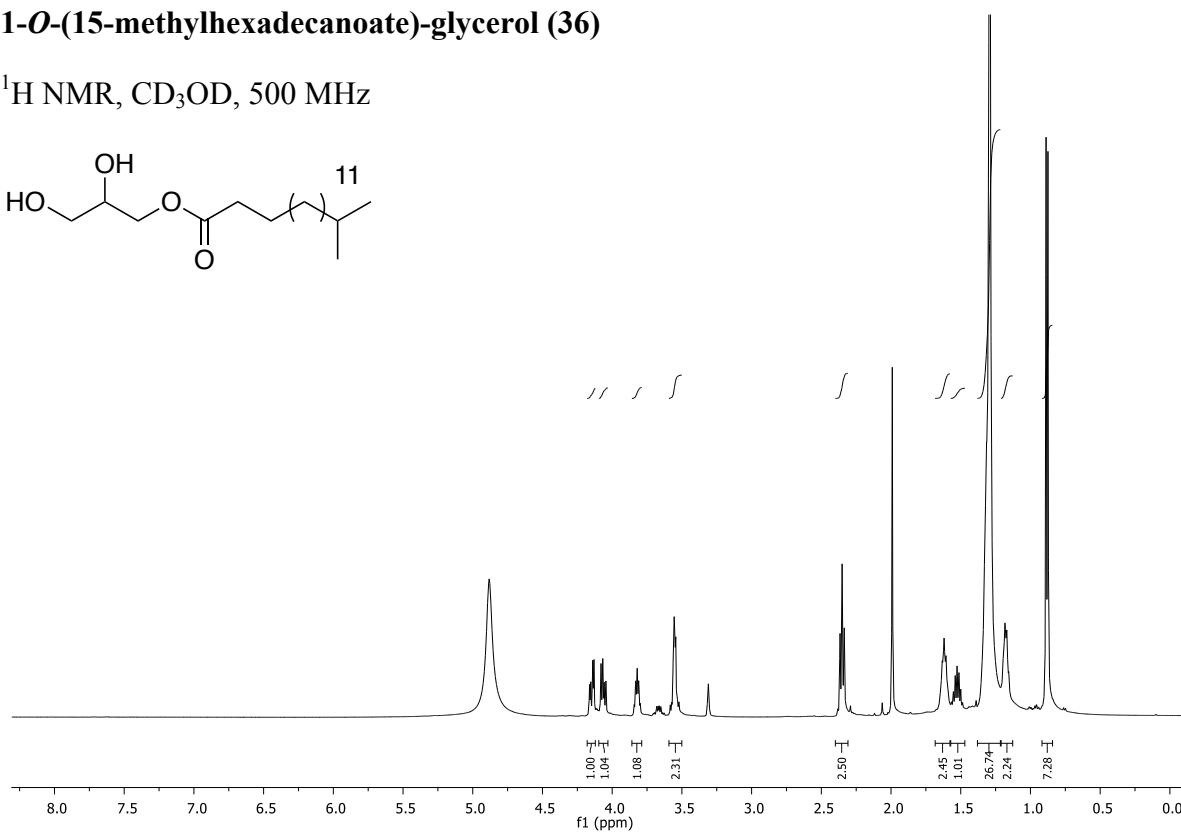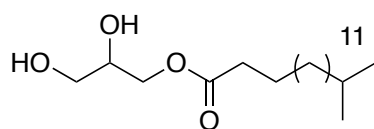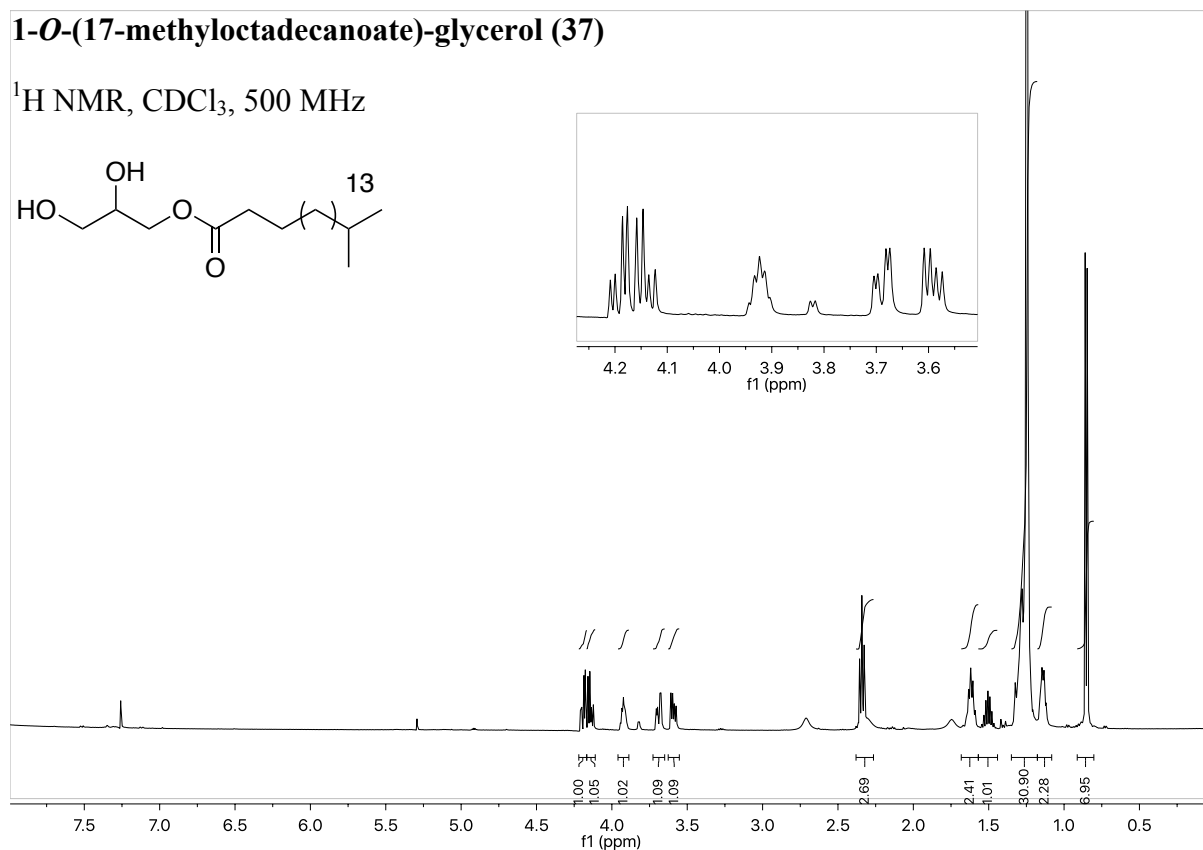

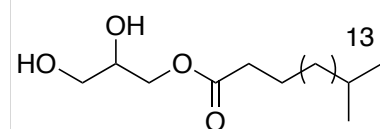
1-*O*-(15-methylhexadecanoate)-glycerol (36)

^{13}C NMR, CD$_3$OD, 125 MHz

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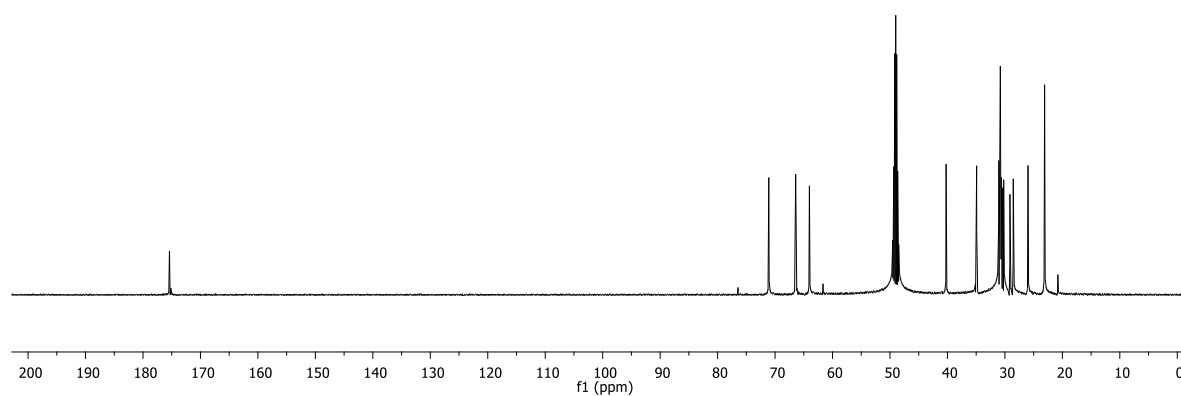
1-*O*-(17-methyloctadecanoate)-glycerol (37)

¹H NMR, CDCl₃, 500 MHz

$$^1\text{H NMR, CDCl}_3, 500 \text{ MHz}$$

1-*O*-(17-methyloctadecanoate)-glycerol (37)

¹³C NMR, CDCl₃, 125 MHz

$$^{13}\text{C NMR, CDCl}_3, 125 \text{ MHz}$$