Novel Glycolipids in CD1d-mediated Immunity:
Synthesis of New Agonists of CD1d

by

Justyna Wojno

A thesis submitted to the
University of Birmingham
for the degree of
DOCTOR OF PHILOSOPHY

School of Chemistry
College of Engineering and Physical Sciences
University of Birmingham
February 2012
For my Family
Abstract

A detailed understanding of the human immune system is crucial for developing novel pharmaceuticals to fight a range of diseases. The glycolipid \( \alpha \)-galactosyl ceramide, \( \alpha \)-GalCer, has been shown to stimulate the proliferation of murine spleen cells and activate the immune system. Stimulation occurs through binding of the glycolipid to the protein CD1d. Subsequent presentation of the CD1d–glycolipid complex to invariant Natural Killer T cells (iNKT cells) initiates the proliferation of a host of cytokines leading to an immune response. The therapeutic potential of \( \alpha \)-GalCer is currently being explored; however the induction of both Th1 and Th2 cytokines by this agent is likely to limit its therapeutic application. The crystal structure of human CD1d in complex with \( \alpha \)-GalCer reveals a key hydrogen bond between the N-H of the amide group of the glycolipid and the side-chain O-H of Thr 154 located on the \( \alpha \)2 helix of the CD1d molecule; however, the carbonyl group of the amide is not directly involved in binding. Carbonyl group replacements should therefore behave similarly but may involve more selective iNKT cell induction as well as improved hydrolytic stability. Significantly, analogues of \( \alpha \)-GalCer have been shown to induce iNKT cell-derived cytokines more selectively through a skewed Th1-Th2 response. To date, very few alterations around the amide bond have been explored. To investigate its importance in iNKT cell stimulation, a range of \( \alpha \)-GalCer and threitol ceramide (ThrCer) analogues has been synthesised in which the amide functionality in these two leads has been replaced with
different carbonyl functional groups. These compounds have been tested for iNKT cell induction and in particular their Th1/Th2 response, which determined their therapeutic potential. Labelled derivatives of α-GalCer and ThrCer have also been designed and synthesised to find application in lipid trafficking studies.
Declaration

The work recorded in this thesis was carried out in the School of Biosciences at the University of Birmingham, U.K. during the period of November 2007 to March 2011. The work in this thesis is original except where acknowledged by reference.

No portion of this work is being, or has been submitted for a degree, diploma or any other qualification at any other university.
Acknowledgements

I wish to thank my supervisors Dr Liam R. Cox and Prof. Gurdyal S. Besra for offering me a PhD position at University of Birmingham, helping me with my work with patience throughout the time it took me to complete my research and write the thesis. The time I spent working with you has been an extraordinary experience that far exceeded my expectations, strongly shaped my research interests and influenced my future career objectives.

This thesis is dedicated to my family in Poland but also to all members of Besra and Cox groups, who became my colleagues, close friends and everyday companions. It is impossible to fit in one page all your names and precious moments of laughter to/and tears we had together that I shall remember and cherish for a long time to come. Nevertheless, my biggest thanks go to Natacha, Vee, Helen, Luke, Renate, Apoo, Usha, Peter the Russian, Hemza, Yoel, John-Paul, Oona, Cristian, Athina, Becky, Arun, Anaxi, Ting, English Pete, George, Albi, Sara, Mimi, Amrita, Monika, Kiran, Shipra, Liz, Reenette. I could not wish for a better company!

I am very greatful to my Glasgow favourites Dominika, Nick, Sylvain, Julian and Mike for their huge support and making me smile a lot.
## Contents

Abstract \hspace{1cm} ii  
Declaration \hspace{1cm} iv  
Acknowledgements \hspace{1cm} v  
Table of contents \hspace{1cm} vi  
List of abbreviations \hspace{1cm} ix  
Published work associated with this thesis \hspace{1cm} xii

## Table of contents

**Chapter 1**  
1. Introduction \hspace{1cm} 2  
  1.1 The immune system, an overview \hspace{1cm} 2  
  1.2 Invariant natural killer T (iNKT) cells \hspace{1cm} 4  
  1.3 CD1: lipid antigen-presenting molecules \hspace{1cm} 8  
  1.4 α-Galactosylceramide: the most potent activator of iNKT cells \hspace{1cm} 13  
  1.5 Crystal structure of the α-GalCer-CD1d complex \hspace{1cm} 18  
  1.6 Crystal structure of the α-GalCer–CD1d–TCR ternary complex \hspace{1cm} 22  
  1.7 Other agonists of CD1d \hspace{1cm} 27  
    1.7.1 Natural self-lipids presented by CD1d \hspace{1cm} 27  
    1.7.2 Synthetic analogues of α-galactosylceramide \hspace{1cm} 30  
      1.7.2.1 Sphingosine base derivatives \hspace{1cm} 31  
      1.7.2.2 N-acyl chain derivatives \hspace{1cm} 33  
      1.7.2.3 Carbohydrate modifications \hspace{1cm} 36  
      1.7.2.4 Glycosidic bond derivatives \hspace{1cm} 39  
  1.8 Aims and objectives \hspace{1cm} 41
Chapter 2

2. Amide analogues of α-galactosylceramide and threitol ceramide
   2.1 Interactions between the amide moiety of α-GalCer and amino acid residues of the iNKT TCR and CD1d protein in the human TCR–hCD1d–α-GalCer ternary complex
   2.2 Reported analogues of α-GalCer with variations around the amide group
   2.3 Target compounds and the rationale for replacing the amide bond
   2.4 Retrosynthetic analysis
   2.5 First-generation Synthesis of α-GalCer
   2.6 Thioamide analogue
   2.7 Synthesis of urea analogue of α-GalCer
   2.8 Second-generation Synthesis of α-GalCer
   2.9 Synthesis of carbamate analogue of α-GalCer
   2.10 Synthesis of Threitol Ceramide
   2.11 Synthesis of thioamide analogue of ThrCer
   2.12 Synthesis of urea analogue of ThrCer
   2.13 Synthesis of carbamate analogue of ThrCer
   2.14 Biological evaluation of glycolipid antigens: thioamide, urea and carbamate analogues of α-GalCer and ThrCer
   2.15 Conclusion and Future Work

Chapter 3

3. Synthesis and functional activity of labelled α-GalCer, ThrCer and their unsaturated analogues
   3.1 Target compounds
   3.2 Retrosynthetic analysis
   3.3 Synthesis of biotinylated ThrCer
   3.4 Biological evaluation of biotinylated ThrCer
   3.5 Synthesis of ThrCer(C26:0) labelled with Alexa 488
   3.6 Synthesis of α-GalCer(C26:0) labelled with Alexa 488
3.7 Synthesis of α-GalCer(C20:2) labelled with Alexa 594 135
3.8 Synthesis of ThrCer(C20:2) labelled with Alexa 594 145
3.9 Biological evaluation of Alexa-labelled glycolipids 148
3.10 Conclusions and future work 151

Chapter 4

4. Synthesis and biological activity of Galp-α-(1→2)-ThrCer 155
   4.1 Introduction to Galp-α-(1→2)-ThrCer 155
   4.2 Retrosynthetic analysis 158
   4.3 Synthesis of Galp-α-(1→2)-ThrCer 160
   4.4 Biological evaluation of Galp-α-(1→2)-ThrCer 169
   4.5 Conclusions and future work 172

Chapter 5

5. Experimental section 174

Chapter 6

6. References 298
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>%</td>
<td>percent</td>
</tr>
<tr>
<td>°C</td>
<td>degrees centigrade</td>
</tr>
<tr>
<td>Å</td>
<td>angstrom</td>
</tr>
<tr>
<td>Ac</td>
<td>acyl, acetyl</td>
</tr>
<tr>
<td>APC</td>
<td>antigen presenting cell</td>
</tr>
<tr>
<td>Ar</td>
<td>aromatic, aryl</td>
</tr>
<tr>
<td>Asp</td>
<td>aspartic acid</td>
</tr>
<tr>
<td>β₂m</td>
<td>beta 2 microglobulin</td>
</tr>
<tr>
<td>Bn</td>
<td>benzyl (CH₂Ph)</td>
</tr>
<tr>
<td>BODIPY</td>
<td>boron dipyrromethane difluoride</td>
</tr>
<tr>
<td>b.p.</td>
<td>boiling point</td>
</tr>
<tr>
<td>Bu</td>
<td>butyl</td>
</tr>
<tr>
<td>CD1</td>
<td>cluster of differentiation 1</td>
</tr>
<tr>
<td>Cys</td>
<td>cysteine</td>
</tr>
<tr>
<td>DC</td>
<td>dendritic cell</td>
</tr>
<tr>
<td>DDM</td>
<td>didehydroxymycobactin</td>
</tr>
<tr>
<td>DGJ</td>
<td>1-deoxygalactonojirimycin</td>
</tr>
<tr>
<td>DMF</td>
<td>N,N-dimethylformamide</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethylsulfoxide</td>
</tr>
<tr>
<td>DN</td>
<td>double negative: CD4-CD8-</td>
</tr>
<tr>
<td>DIPEA</td>
<td>diisopropylethylamine</td>
</tr>
<tr>
<td>DP</td>
<td>double positive</td>
</tr>
<tr>
<td>DTBMP</td>
<td>2,6-di-tert-butyl-4-methylpyridine</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>Et</td>
<td>ethyl</td>
</tr>
<tr>
<td>FACS</td>
<td>fluorescence-activated cell sorting</td>
</tr>
<tr>
<td>FCC</td>
<td>flash column chromatography</td>
</tr>
<tr>
<td>g</td>
<td>grammes</td>
</tr>
<tr>
<td>GSL</td>
<td>glycosphingolipid</td>
</tr>
</tbody>
</table>
h  hours
hCD1d  human CD1d
HMBC  heteronuclear multiple bond correlation
HSQC  heteronuclear single quantum correlation
IFN  interferon
IL  interleukin
iNKT Cell  invariant natural killer T cell
(also referred to as CD1d-restricted T cell)
IR  infrared
$K_d$  dissociation constant
LTP  lipid transfer protein
M  molar
m  milli
mCD1  mouse CD1
Me  methyl
MFI  mean fluorescent intensity
MHC  major histocompatibility complex
MHz  mega-hertz
min  minute
ml  millilitres
n  nano
NBS  $N$-bromosuccinimide
NIS  $N$-iodosuccinimide
NK  natural killer
NKT  natural killer T
NMR  nuclear magnetic resonance
PE  1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine
PG  phosphatidylglycerol
Phe  phenylalanine
PI  phosphatidylinositol
$p$-Ts  para-toluenesulfonyl
py  pyridine
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>RT</td>
<td>room temperature</td>
</tr>
<tr>
<td>s</td>
<td>second</td>
</tr>
<tr>
<td>SPR</td>
<td>surface plasmon resonance</td>
</tr>
<tr>
<td>t</td>
<td>time</td>
</tr>
<tr>
<td>TBAF</td>
<td>tetrabutylammonium fluoride</td>
</tr>
<tr>
<td>TBAI</td>
<td>tetrabutylammonium iodide</td>
</tr>
<tr>
<td>TBDPS</td>
<td>tert butyl diphenylsilyl</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>Tf</td>
<td>trifluoromethanesulfonyle</td>
</tr>
<tr>
<td>Th</td>
<td>T helper</td>
</tr>
<tr>
<td>THF</td>
<td>tetrahydrofuran</td>
</tr>
<tr>
<td>Thr</td>
<td>threonine</td>
</tr>
<tr>
<td>ThrCer</td>
<td>threitol ceramide</td>
</tr>
<tr>
<td>TLC</td>
<td>thin layer chromatography</td>
</tr>
<tr>
<td>TMU</td>
<td>tetramethylurea</td>
</tr>
<tr>
<td>Tr</td>
<td>trityl</td>
</tr>
<tr>
<td>wt</td>
<td>wild type</td>
</tr>
<tr>
<td>α-GalCer</td>
<td>alpha-galactosylceramide</td>
</tr>
<tr>
<td>α-GlcCer</td>
<td>alpha-glucosylceramide</td>
</tr>
<tr>
<td>α-ManCer</td>
<td>alpha-mannosylceramide</td>
</tr>
<tr>
<td>μ</td>
<td>micro</td>
</tr>
</tbody>
</table>
Published work associated with this thesis


Chapter 1

Introduction
1. Introduction

1.1. The immune system, an overview

The immune system is a very complex “machinery” that involves many different proteins interacting with each other. In humans first line of defence against invaders such as viruses, bacteria and parasites consists of physical barriers, namely skin and mucous membranes that line our digestive, respiratory and reproductive tracts. The innate immune system serves as a second line of defence, providing a quick response to common invaders.\(^1\) The innate immune system is a universal and ancient form of host defense against infection. Innate immune recognition relies on a limited number of germline-encoded receptors. These receptors have evolved to recognise the conserved products of microbial metabolism, which are produced by microbial pathogens, but not by the host. Recognition of these molecular structures allows the immune system to distinguish infectious non-self from non-infectious self. Toll-like receptors (TLR) play a major role in pathogen recognition and initiation of inflammatory and immune responses.\(^2\) The weapons of the innate immune system include the complement proteins, professional phagocytes, and natural killer (NK) cells.

The adaptive immune system involves great variability and rearrangement of receptor gene segments, and therefore provides specific recognition of foreign antigens, immunological memory of infection, and pathogen-specific adaptor proteins. However, the adaptive immune response is also responsible for allergy, autoimmunity, and the rejection of tissue grafts.
Innate immunity is responsible for most inflammatory responses which are triggered by macrophages, polymorphonuclear leukocytes, and mast cells through their innate immune receptors. Adaptive immunity supports the innate immune system in specific recognition of proteins, carbohydrates, lipids, nucleic acids, and pathogens, using the same activated, but not antigen-specific, effector cells generated by innate immune recognition. Thus the two systems are intimately linked in their use of the same effector cells. The key differences between the innate and adaptive immune systems are summarised in Table 1.1.

<table>
<thead>
<tr>
<th>Property</th>
<th>Innate immune system</th>
<th>Adaptive immune system</th>
</tr>
</thead>
<tbody>
<tr>
<td>Receptors</td>
<td>Fixed in genome</td>
<td>Encoded in gene segments</td>
</tr>
<tr>
<td></td>
<td>Rearrangement is not necessary</td>
<td>Rearrangement is necessary</td>
</tr>
<tr>
<td>Distribution</td>
<td>Non-clonal,</td>
<td>Clonal</td>
</tr>
<tr>
<td></td>
<td>All cells of a class identical</td>
<td>All cells of a class distinct</td>
</tr>
<tr>
<td>Recognition</td>
<td>Conserved molecular patterns (lipopolisaccharides, mannans, glycans)</td>
<td>Details of molecular structure (proteins, peptides, carbohydrates)</td>
</tr>
<tr>
<td>Self Non-self discrimination</td>
<td>Perfect: selected over evolutionary time</td>
<td>Imperfect: selected in individual somatic cells</td>
</tr>
<tr>
<td>Action time</td>
<td>Immediate activation of effectors</td>
<td>Delayed activation of effectors</td>
</tr>
<tr>
<td>Response</td>
<td>Co-stimulatory molecules</td>
<td>Clonal expansion or anergy</td>
</tr>
<tr>
<td></td>
<td>Cytokines (IL-1β, IL-6)</td>
<td>IL-2</td>
</tr>
<tr>
<td></td>
<td>Chemokines (IL-8)</td>
<td>Effector cytokines: (IL-4, IFNγ)</td>
</tr>
</tbody>
</table>

Table 1.1. Differences between innate and adaptive immunity. Table adapted from ref 2.
1.2. Invariant natural killer T (iNKT) cells

Natural killer T cells (NKT cells) are a major population of innate-like T-lymphocytes. These multifunctional lymphocytes can mediate an immune response to tumours and play an important role in the host's defense mechanism against microbial infections as well as a function in maintaining immune tolerance.\(^3\)

NKT cells possess properties of the natural killer (NK) cells of the innate immune system, and of traditional T cells of the adaptive immune system. They mature in the thymus and co-express the αβ T cell antigen receptor (TCR) and NK receptors NK1.1 or NKR-P1A. In contrast to the αβ receptors of traditional T cells, which are incredibly diverse, the repertoire of receptors expressed by NKT cells is quite limited. Traditional T cells recognise peptides presented by either class I or class II major histocompatibility complex (MHC) molecules, whereas NKT cells recognise lipids and glycolipids presented by CD1 proteins (Figure 1.1).\(^1\)

Four categories of T cell have been referred to as natural killer T cells. Of these, the most abundant and well-studied are so-called type 1 iNKT cells, also referred to as invariant natural killer T cells (iNKT cells) because of their expression of an antigen-specific T-cell receptor (TCR). iNKT cells are unique lymphocytes that bridge the innate and adaptive immune system. The invariant α chain of the iNKT cell TCR consists of a canonical Vα14–Jα18 (V – variable, J – joining region) chain in mice, and a homologous Vα24–Jα18 chain in humans, which is paired with a more variable β chain (Vβ2, Vβ7, Vβ8 in mice, and Vβ11 in humans).\(^4\)
Figure 1.1. **Schematic view of the ligand-specificity of iNKT cells compared with conventional T cells.** Peptide antigens presented by MHC class I molecules are recognised by diverse T-cell receptors (TCRs) expressed by CD8+ T cells, while peptide antigens presented by MHC class II molecules are recognised by diverse TCRs expressed by CD4+ T cells. Semi-invariant Vα14i TCRs expressed by iNKT cells recognise glycolipids presented by CD1 proteins.

Figure adapted from ref. 5. Permission to reproduce figure was obtained from Elsevier through RightsLink® - licence number 2812640815855.

iNKT cells are mainly found in the thymus, liver and bone marrow. They are also present in smaller amounts in the spleen and the peripheral blood, but are rare in the lymph nodes.6-8 The maturation of iNKT cells begins in the thymus, where an uncommitted precursor thymocyte rearranges and expresses a CD1d-specific Vα14i TCR at the double-positive (DP) stage, and is then positively selected by
CD1d-expressing DP thymocytes (Figure 1.2).\textsuperscript{6} Positively selected Vα14i T cells mature into double-negative (DN) and CD4\textsuperscript{+} T cells, whilst the expression of CD8 cells induces negative selection by increasing the affinity of the TCR for CD1d. After selection, NK1.1\textsuperscript{+}Vα14i T cells are exported to the periphery of the spleen or liver,\textsuperscript{7,8} where a hypothetical second signal is required for the acquisition of the mature NK1.1\textsuperscript{+} phenotype (Figure 1.2).

**Figure 1.2.** Schematic view of the development of Vα14i T cells in the thymus. T cell receptor (TCR) with random αβ rearrangement is first expressed by an uncommitted precursor thymocyte at the double-positive (DP) stage. DP cells with CD1d-specific Vα14i α-chain (Vα14–Jα18) are
positively selected by CD1d-expressing DP thymocytes. They mature into double-negative (DN) and CD4+ T cells and are exported to peripheral organs where a hypothetical second signal is required for the expression of mature iNKT cells. Positive selection of T cells with TCRs that are specific for MHC–peptide complexes leads to maturation of mainstream CD4+ and CD8+ T cells.

Figure adapted from ref 9. Permission to reproduce figure was obtained from Elsevier through RightsLink® - licence number 2812641351351.

Mouse Vα14i and human Vα24i T cells have cytolytic activity,10,11 and they rapidly induce the expression of various cytokines — including IL-4 and interferon-γ (IFNγ) — after TCR stimulation. Vα14i T-cell-derived cytokines can, in turn, activate several other cell types, including NK cells,12,13 conventional T cells14, macrophages15 and B cells,16 and recruit myeloid dendritic cells (DCs).17 iNKT cells are able to require T helper 1 (Th1) or Th2 type of cytokine response. The Th1 pattern includes pro-inflammatory cytokines (typified by IFNγ), whereas the Th2 pattern includes regulatory cytokines such as IL-4, IL-5 and IL-13.9
1.3. **CD1: lipid antigen-presenting molecules**

CD1 molecules are a family of highly conserved antigen-presenting proteins that are similar in function to classic MHC molecules. However, whilst classic MHC molecules present peptide antigens to receptors located on T cells, CD1 molecules are able to bind and display a variety of lipids and glycolipids, which they then present to receptors located on CD1-restricted T cells.\(^{18,19}\) Based on similarities between their nucleotide and amino acid sequences, the five known isoforms of the CD1 family have been divided into two groups, Group I (CD1a, CD1b, CD1c, and CD1e present in humans) and Group II (CD1d present in humans and mice).\(^{20}\) In humans, one copy of each of the five CD1 molecules is encoded on chromosome 1\(^{21}\) in the order CD1d, CD1a, CD1c, CD1b, CD1e (Figure 1.3.A). Mice and rats appear to have duplicated CD1d (Figure 1.3.B) and it is postulated that the genes for CD1a, CD1b, CD1c and CD1e have been lost in a chromosomal translocation event.\(^{22}\)

![Diagram](image)

**Figure 1.3.** (A) The order of CD1 proteins encoded on human chromosome 1. (B) Duplicated CD1d proteins located on mouse chromosome 3.
CD1 proteins are heterodimers and most closely resemble class I MHC molecules in their overall structure. A transmembrane heavy chain, comprising α1, α2 and α3 domains, is non-covalently associated with β2-microglobulin (β2m). The antigen-binding sites of CD1 molecules are deep hydrophobic pockets that accommodate the alkyl chains of lipid antigens, leaving the more polar parts of the antigen accessible at the molecular surface for recognition by T-cell receptors.

Antigens presented by CD1 molecules range from endogenous cellular lipids and foreign lipids derived from intracellular parasites, to extracellular lipids of self or foreign origin. CD1a has been found to bind and present glycolipids and mycobacterial lipopeptides such as the sphingolipid sulfatide (1, a sulfate ester of β-galactosylceramide) and didehydroxymycobactin (2) (DDM). The CD1b isoform is able to bind lipids with very long alkyl chains (e.g. C80), such as the mycolic acids, which are important components of mycobacterial cell walls. CD1c has been shown to present mycobacterial isoprenoid lipids (3), which contain unusual alkyl chains consisting of repeating branched, saturated units. Some lipids, including phospholipids and sphingolipids, have been shown to bind multiple CD1 isoforms including CD1a, CD1b, CD1c and CD1d. The CD1e molecule differs significantly from other CD1 family members. It is the only protein to be converted into a soluble lysosomal form, and in contrast to other CD1 isoforms, is not expressed at the cell surface. However, CD1e remains immunologically relevant and is required for the presentation of the mycobacterial PIM6 antigen by CD1b to specific T cells and for the in vitro conversion of PIM6 to PIM2 by α-mannosidase.
iNKT cells characteristically express a semi-invariant TCR that specifically recognises the CD1 family member, CD1d. The finding that both mouse and human iNKT cells were autoreactive to cells expressing CD1d, resulted in considerable research in this CD1 subset.\textsuperscript{31}
Intracellular trafficking of mouse and human CD1d is depicted in Figure 1.5. CD1d molecules are synthesised in the endoplasmatic reticulum (ER) and are transported through the Golgi and then directly to the plasma membrane via the secretory pathway. At the cell surface, nascent CD1d molecules are internalised via clathrin-cloated pits – and directed either through the recycling endosomal system or deeper in the endocytic system to lysosomal antigen loading compartments (MIIC), before being re-exported to the cell surface. The route of intracellular trafficking appears to depend largely on the nature of the cytoplasmic tails that bind the adaptor protein (AP)-3, directing their trafficking to sorting endosomes. Murine CD1d contains tyrosine-based motifs and is efficiently directed toward late endosomal MIIC compartments, whereas human CD1d, which cannot bind (AP)-3 is mainly distributed in the early endosomal system. Additionally, a small fraction of human CD1d molecules physically associate with MHC class II molecules during trafficking and are transported through the endosomal system to MIICs prior to their expression on the cell surface (Figure 1.5).
Figure 1.5. **Overview of CD1d intracellular trafficking.** CD1d molecule is synthesised in the endoplasmic reticulum (ER). Both human and mouse CD1d are transported through the Golgi to the cell surface and internalised via clathrin-coated pits before being directed into early endosomes. Human CD1d is next re-exported directly to the cell surface, while mouse CD1d and rare human CD1d associated with MHC class II molecule are trafficked deeper into late endosomes or lysosomes (MIIC) before reaching plasma membrane.  

32
1.4. \(\alpha\)-Galactosylceramide: the most potent activator of iNKT cells

\(\alpha\)-Galactosylceramide (\(\alpha\)-GalCer) is one of the most potent agonists of CD1d and the strongest activator of human and mouse iNKT cells. \(\alpha\)-GalCer was first discovered by the Kirin Pharmaceutical Research Corporation during a screen for agelasphins (AGLs) that could prevent metastasis of transplanted tumours to the livers of mice. A series of agelasphins was originally isolated from the marine sponge *Agelas mauritianus*, collected in the Okinawa Sea.\(^{34}\) Although a number of \(\beta\)-galactosylceramides were already known, the AGLs were the first cerebrosides with an \(\alpha\)-galactosyl linkage (Figure 1.6). Very interestingly all AGLs showed antitumour activity.

\[
\begin{align*}
4, \text{agelasphin-7a} & : R = -(\text{CH}_2)_1\text{CH}_3 \\
5, \text{agelasphin-9a} & : -(\text{CH}_2)_2\text{CH}_3 \\
6, \text{agelasphin-9b} & : -(\text{CH}_2)_1\text{CH}(\text{CH}_3)_2 \\
7, \text{agelasphin-11} & : -(\text{CH}_2)\text{CH}(\text{CH}_3)_2\text{C}_2\text{H}_5
\end{align*}
\]

**Figure 1.6.** Agelasphins isolated from the marine sponge *Agelas mauritianus*.

\(\alpha\)-GalCer 8 is a synthetic glycolipid (also referred to as KRN7000), and is a derivative of the isolated agelasphin AGL-9b 6, which was developed for
experimental studies and clinical trials (Figure 1.7). The α-GalCer structure consists of a galactopyranose combined with a ceramide through an α-glycosidic linkage. The ceramide part comprises a phytosphingosine base, which is N-acylated with hexacosanoic acid (Figure 1.7).

![Diagram of α-GalCer](image)

**Figure 1.7.** Structure of α-galactosylceramide (α-GalCer) 8.

The affinity of CD1d to α-GalCer was first measured by Naidenko et al. in SPR assay,\(^{35}\) in which biotinylated α-GalCer was immobilized on a BIACore chip and CD1d was passed on the lipid, followed by affinity measurements. The dissociation data from this experiment were fitted onto a 2-component dissociation model with the following results (Table 1.2):
<table>
<thead>
<tr>
<th>CD1 type</th>
<th>Ligand</th>
<th>$K_{on}$ (M$^{-1}$ s$^{-1}$)</th>
<th>$K_{off}$ fast (s$^{-1}$)</th>
<th>$K_{off}$ slow (s$^{-1}$)</th>
<th>$K_d$ (nM) fast</th>
<th>$K_d$ (nM) slow</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse</td>
<td>Bio-α-GalCer</td>
<td>6.3 x 10$^4$</td>
<td>0.1</td>
<td>4 x 10$^{-3}$</td>
<td>1587.3</td>
<td>63.5</td>
</tr>
<tr>
<td>Human</td>
<td>Bio-α-GalCer</td>
<td>1.0 x 10$^5$</td>
<td>0.08</td>
<td>4 x 10$^{-3}$</td>
<td>800.0</td>
<td>40</td>
</tr>
</tbody>
</table>

Table 1.2. Dissociation data from SPR assay$^{35}$ with human and mouse CD1d protein and biotinylated α-GalCer.

McCarthy et al.$^{36}$ reported half-life of α-GalCer in CD1d – 593 min measured in SPR half-life assay in which biotinylated human CD1d was refolded with α-GalCer and other antigens (shorter acyl and/or sphingosine chains) and loaded onto a BIAcore chip. The loading was monitored by 9B antibody that recognises the galactose headgroup and CD1d.

Although the expression of α-GalCer in marine sponges could not be linked with any physiologically relevant function, the striking properties of this ligand have provided early support for the hypothesis that the conserved TCRs of NKT cells evolved to recognise conserved lipids. It is widely assumed that α-GalCer is only a mimic of the natural ligand that is recognised by Vα14i and Vα24i T cells, as this lipid is not present in mammals and nearly all natural glycolipids are β-linked glucosylceramides.$^{37}$ Nevertheless, α-GalCer very effectively stimulates iNKT cells in a CD1d-dependent manner, resulting in the rapid production of large amounts of
Th1 (IFNγ) and Th2 (IL-4) types of cytokines (Figure 1.8), and the expansion of antigen-specific T- and B cell responses.\textsuperscript{12,16,38}

\textbf{Figure 1.8.} Activation of iNKT cells results in the release of large amounts of different cytokines, strongly activating the immune system.

Efficient stimulation of the immune system by α-GalCer made this antigen a promising adjuvant for the generation of optimal therapeutic and vaccination strategies against a range of infectious diseases and cancer. Clinical trials with soluble α-GalCer or α-GalCer-pulsed antigen-presenting cells (APCs) aimed at in
vivo reconstitution and activation of human NKT cells have been carried out\textsuperscript{39-41} and provided both promising and challenging results. Recent phase I studies on patients with lung cancer,\textsuperscript{42} as well as those with recurrent head and neck cancer,\textsuperscript{43} demonstrated the safety and effectiveness of $\alpha$-GalCer-pulsed APCs in anti-tumour therapy.
1.5. **Crystal structure of the α-GalCer-CD1d complex**

Crystal structures of mouse and human CD1d, with and without α-GalCer, have been determined and show how the glycolipids bind inside the protein (Figure 1.9).

![Crystal structure diagram](image)

**Figure 1.9.** Cartoon (A) and surface (B) representation of the crystal structure of the hCD1d-α-GalCer complex. The galactose head group is exposed for recognition by the iNKT T cell receptor. Figure generated from 1ZT4 with Pymol.
The CD1d protein is formed of three domains, namely α1, α2 and α3, comprising the heavy chain, which is non-covalently associated with β2-microglobulin (β2m). Both murine and human CD1d molecules contain a large, hydrophobic pocket in the centre of the α1-α2 domain. This antigen-binding groove has two channels that meet at the surface of the protein. Lipid chains of a dialkyl glycolipid are then held inside the CD1d molecule by hydrophobic interactions. The acyl chain (26 carbon atoms) of α-GalCer occupies the longer pocket A’, leaving the shorter C18 sphingosine chain to occupy the F’ channel. Both lipid tails in α-GalCer terminate at the end of the pockets A’ and F’ thereby determining the maximum number of atoms in each chain (Figure 1.10). This finding explains why α-GalCer is one of the most potent agonists of CD1d and binds with one of the highest affinities and also why other antigens with longer lipid tails are not suitable since the longer lipid chains cannot fit in the binding pockets. Dialkyl glycolipids with fewer atoms in either of the chains are unable to fully occupy the hydrophobic binding pockets, resulting in an increased rate of lipid dissociation from the CD1d molecule.

The observed mode of glycolipid binding leaves the polar galactopyranose head group exposed over the lipid-binding groove for recognition by the iNKT TCR receptor. Analysis of the crystal structure of the α-GalCer-CD1d complex has identified key hydrogen bonds between the glycolipid and protein (Figure 1.11). The role of these hydrogen bonds is not only to stabilise the glycolipid, but also critically to orient the sugar moiety in such a fashion that it can be recognised by the TCR.
The oxygen atom 1’-O of the α-GalCer glycosidic forms a hydrogen bond with the side-chain OH of Thr154 (Thr 156 in mouse CD1d), which is located on the α2 helix of the protein. The 2’-OH of the galactose forms a second hydrogen bond to the side-chain of Asp151 (Asp153 in mouse CD1d), which is also located on the α2 helix. The 3-OH hydroxyl group of the phytosphingosine chain is hydrogen-bonded to the α1 helix Asp80 whereas Asp 80 of mouse CD1d binds the 3-OH and 4-OH hydroxyl groups of the phytosphingosine. In combination, these hydrogen bonds anchor the exposed sugar polar head group in a position parallel to the plane of the α helices, which is crucial for the antigenicity of α-GalCer (Figure 1.11).
Figure 1.11. Hydrogen bond formation between the amino acid residues of hCD1d and α-GalCer found in the hCD1d-α-GalCer complex. Figure generated from 1ZT4 with Pymol.
1.6 Crystal structure of the α-GalCer–CD1d–TCR ternary complex

A co-crystal structure of human CD1d–α-GalCer with the human iNKT T cell receptor (Figure 1.12) has also been described (3.2 Å resolution) and reveals valuable insight into how the TCR recognises the glycolipid-protein complex. The structure shows the T cell receptor locating almost parallel to the long axis of the CD1d–α-GalCer binding cleft and positioned over the extreme end of the CD1d molecule, directly above the F' pocket, which is occupied by the sphingosine chain.

A semi-invariant human NKT cell TCR comprises an invariant α-chain and CDR3α loop (Vα24–Ja18, where V is ‘variable’ and J is ‘joining’) and a restricted Vβ11-containing β-chain repertoire (Figure 1.13). The ternary structure shows that the α-chain contributes more interactions with the CD1d-α-GalCer complex than does the β-chain (approximately 82 versus 32 contacts, respectively), and also provides more buried surface areas (BSA) (65.5% versus 34.5%, respectively).
**Figure 1.12.** Crystal structure of the iNKT TCR–α-GalCer–hCD1d ternary complex. Cartoon representation was obtained with PyMol from 2PO6.46
Lipid recognition by the T cell receptor occurs through the exposed polar head group of α-GalCer. Contacts with the glycolipid are mediated by the CDR1α and CDR3α loops of the TCR. The invariant TCR α-chain forms three hydrogen bonds with the 2', 3' and 4' hydroxyl groups of the galactose ring: the 2' and 4' hydroxyl groups of the galactose are H-bonded to Gly96 and Phe29, respectively, whilst on the main chain, the 3' hydroxyl forms a hydrogen bond with the side-chain OH in Ser30 (Figure 1.14).
Figure 1.14. α-GalCer-mediated interactions with iNKT T cell receptor.

Figure adapted from ref 46. Permission to reproduce figure was obtained from Nature through RightsLink® - licence number 2812180982041.

The formation of these three hydrogen bonds appears to be important for the recognition of the glycolipid-antigen-presenting complex and activation of iNKT cells. Moreover these observations explain why α-mannosylceramide (α-ManCer) does not activate iNKT cells. The 2’ and 4’ hydroxyl groups in mannose have a different (2’-axial and 4’-equatorial) orientations when compared with galactose, which results in the loss of two key hydrogen bonds with the TCR. Thus whilst it is
likely that α-ManCer binds to CD1d, the resulting complex is not recognised by the TCR and so does not lead to iNKT cell activation.

The α-glycosidic linkage is also important for TCR recognition. The corresponding β-anomer, β-galactosylceramide, was recently shown to activate iNKT cells \textit{in vivo} and in a CD1d-dependent manner;\textsuperscript{48} however it is a significantly less potent agonist than α-GalCer. It has been postulated that the β-GalCer binds in a more perpendicular orientation, which disrupts contacts with the α-chain.
1.7 Other agonists of CD1d

Since the discovery of α-GalCer and subsequent detailed evaluation of its biological activity, a large number of natural and other synthetic agonists of CD1d have been examined. It has become clear that the immune responses that these analogues can trigger differ from those elicited by α-GalCer, not only quantitatively but also qualitatively. These observations have had a profound impact on our understanding of the biology of iNKT cells, which is crucial for developing effective iNKT-cell-based therapies.

1.7.1 Natural self-lipids presented by CD1d

A number of CD1d antigens have been found among endogenous self phospholipids and self glycosphingolipids. These are able to activate both mouse and human iNKT cells. Their autoreactivity occurs through the recognition of cellular lipids associated with CD1d proteins within cells.

A representative example of a natural agonist is the glycosphingolipid isoglobotrihexosylceramide 9 (iGb3) (Figure 1.15).\textsuperscript{49} iGb3, which is generated in lysosomal compartments, was found to activate human and murine iNKT cells \textit{in vitro}; however when compared with the most potent synthetic CD1d agonist (α-GalCer), it is at least 5 orders of magnitude less active.\textsuperscript{49} Recognition of the much
larger trisaccharide head group by the TCR is possible thanks to its peripherally located CDR3β loop, which provides plasticity to accommodate different antigens.\textsuperscript{46} Interestingly, it was shown that iNKT cells are not stimulated by Gb3 (the iGb3 analogue with an α1-4-linked terminal galactose), which suggests that the specific orientation of the terminal sugar is crucial for the formation of the CD1d-glycolipid-TCR ternary complex.\textsuperscript{46} It remains unclear whether or not iGb3 can serve as a physiologically relevant ligand \textit{in vivo}. To be stimulated, human iNKT cells require lysosomal processing of self antigens.\textsuperscript{50,51} Moreover, in contrast to mouse, iGb3 is not found in humans, owing to the lack of functional genes for iGb3 synthase, which is required for its biosynthesis.\textsuperscript{52}

![iGb3](image1.png)

**Figure 1.15.** Structure of the natural self-lipid, isoglobotrihexosylceramide (iGB3).

Self phospholipid antigens form another group of natural lipid ligands for CD1d. Among these are phosphatidylinositol (PI), phosphatidylglycerol (PG), phosphatidylethanolamine (PE) and phosphatidylcholine (PC) (Figure 1.16). Whilst these polar lipids were also found to bind murine CD1d, they displayed limited capability to activate murine iNKT hybridoma cells.\textsuperscript{53-57} In another study of the cellular ligands of hCD1d such as PE \textbf{10}, PC \textbf{11} also showed little or no
antigenicity, although it was suggested that lipids found in cell membranes may play an important role in the folding and stabilisation of CD1 molecules and hence modulate the ability of other more antigenic lipids to load on to CD1d molecules.\textsuperscript{58}

\begin{center}
\begin{figure}
\centering
\includegraphics[width=\textwidth]{Figure1.16.png}
\caption{Structure of natural phospholipids, phosphatidylethanolamine (PE) and phosphatidylcholine (PC).}
\end{figure}
\end{center}

A recent study of lysophosphatidylcholine (LPC) shows that this monoacyl form of PC also serves as a natural phospholipid ligand for NKT cells. LPC was found among lipids extracted from the plasma of multiple myeloma patients and proved to bind CD1d dimers and activate type II NKT cells (Vα24 Vβ11) present in human blood.
This self phospholipid is postulated to be an endogenous signal for cellular stress or damage, as higher levels of LPC are detected in asthma, allergic and autoimmune inflammation and human cancers such as multiple myeloma.\textsuperscript{59} LPC has consequently gained an increased interest and a recent study of Fox \textit{et al.}\textsuperscript{58} also suggests that NKT cells might be stimulated by physiological lipid signalling pathways to carry out their homeostatic immunoregulatory functions.

1.7.2 Synthetic analogues of $\alpha$-galactosylceramide

Even though $\alpha$-GalCer is the most potent agonist of iNKT cells, it may not be an optimal ligand for generating an antitumour or immunomodulatory effect. The iNKT cell activation by $\alpha$-GalCer leads to the release of both Th1- and Th2-type cytokines. A number of different modifications have been applied to the KRN7000 structure resulting in significant changes in the profiles of cytokine production following iNKT cell activation. Finding the right balance in the Th1 \textit{versus} Th2 cytokine profile is very important as these two types of cytokine result in two different immune responses.
1.7.2.1 Sphingosine base derivatives

Analogues of α-GalCer with modifications in the phytosphingosine base have been extensively studied and provided researchers with a set of useful SAR (structure-activity relationship) information long before the crystal structure of CD1d with bound α-GalCer was disclosed.

Phytosphingosine is structurally similar to naturally occurring sphingosine: both molecules have C18 hydrocarbon chains; however, sphingosine lacks the hydroxyl group at C4 and bears a trans double bond between C4 and C5 instead of the fully saturated backbone found in phytosphingosine. The importance of the two hydroxyl groups, at C3 and C4 in the phytosphingosine base in α-GalCer structure has been confirmed. Kronenberg and co-workers reported that 4-deoxy α-GalCer binds to murine CD1d but is not recognised by human CD1d. 3,4-Dideoxy-α-GalCer was not antigenic in both mouse and human models. These findings were later rationalised, when it was shown that the 3-OH group forms a hydrogen bond to Asp80 in both mouse and human CD1d, whilst the OH group at the 4 position participates in hydrogen bond formation only in mCD1d.

OCH 13 is an analogue of α-GalCer, which induces a Th2 bias of NKT cells through the predominant production of IL-4 cytokines. In this CD1d agonist, the sphingosine base is truncated from 18 to 9 carbons and the acyl chain is reduced to 24 instead of 26 carbons (Figure 1.18). OCH is five- to tenfold less active in inducing cell proliferation and produces less IFNγ but more IL-4 in vitro, when compared with α-GalCer. These observations have been confirmed in experiments.
in vivo where IL-4 levels in serum remained unaffected for both agonists, but the amount of IFNγ detected for the OCH analogue was much lower. Even when much lower doses of α-GalCer are administered, the ratios of IL-4 / IFNγ remains almost unchanged, proving that the distinct cytokine polarising effect of OCH cannot be obtained by simply lowering the amount of α-GalCer. Despite being a less active agonist in inducing NKT cell proliferation, OCH displays a different type of activity to α-GalCer. OCH is now commonly used as a control in biological assays for IL-4 production, when compared with new agonists. Other analogues with a truncated phytosphingosine chain ranging from 9 to 15 carbons have been reported and were also found to increase the release of IL-4 in both murine and human iNKT cells.63 These Th2-biasing analogues may find a future application in the treatment of different autoimmune diseases.

The origin of the Th2 bias for OCH and related analogues remains unclear. However, it has been postulated that the shorter sphingosine length destabilises the CD1d-glycolipid complex. As the release of IFNγ by iNKT cells needs a longer exposure to the CD1d-glycolipid complex (usually measured after 18 hours), lower levels of IFNγ are observed when compared with α-GalCer.

Attaching an aromatic ring to the end of a truncated sphingosine chain has been shown to result in a potent iNKT cell activator that induces a more Th1-biased cytokine response (Figure 1.18).64 It is suggested that C13 14 binds to CD1d with higher affinity thanks to the added aromatic substituent in the phytosphingosine
Chapter 1

Introduction

chain, which increases the time that C13 is in contact with CD1d resulting in higher levels of IFNγ upon iNKT cell activation.

13, OCH

14, C13

Figure 1.18. Structure of OCH 13 and C13, 14 analogues of α-GalCer.

1.7.2.2 N-acyl chain derivatives

The length and degree of unsaturation in the N-acyl chain of α-GalCer was another point of focus in the search of new agonists of CD1d protein. C10:0 15 (Figure 1.19) is an analogue with significantly shorter N-acyl chain, bearing 10 instead of 26 carbons in the chain. This modification in the agonist’s structure was found to bias Th2 type response and weaken the activation of iNKT cells.65
α-GalCer-C20:2 \(19\) has two double bonds in the acyl chain at carbon 11 and 14 (Figure 1.19) and like compound \(15\) preferentially induces IL-4 production being another example of Th2 type agonist of CD1d.\(^6\) However, in contrary to α-GalCer, it does not require endosomal localisation of CD1d for efficient loading and presentation.\(^6\)

Other modifications in the \(N\)-acyl chain involved incorporation of the C20 prostaglandin B1 chain (α-GalCer-PGB1 \(16\)), aromatic rings (α-GalCer-C11Ph \(17\)), 4-fluorophenylacetate group (α-GalCer-4FPA \(18\)) or arachidonic acid (α-GalCer-C20:4 \(20\)) (Figure 1.19). These analogues also showed Th2-skewing of the cytokine profile generated by iNKT cell activation and are less dependent on endosomal loading for presentation by CD1d when compared with α-GalCer which produces mixed Th1 and Th2 cytokine profile.

It is suggested that the polarised iNKT cell cytokine production biased towards Th2-type response is an effect of direct loading of these agoniststs into CD1d molecules at the cell surface of APC.
Figure 1.19. Structures of representative N-acyl chain analogues of α-GalCer.
1.7.2.3 Carbohydrate modifications

α-GalCer analogues with modified sugar unit are an important group of new agonists of CD1d. The activity of α-glucosylceramide (α-GlcCer) 21 in mouse spleen iNKT cell activation assay was found to be similar to that of α-GalCer 8 (Figure 1.20). Only configuration of 4-hydroxyl group (equatorial) in α-GlcCer 21 varies from structure of α-GalCer 8 (axial), this indicated that the 4-hydroxyl configuration of the sugar was not essential for bioactivity. However, α-mannosylceramide (α-ManCer) 22 (Figure 1.20) with the 2-hydroxyl group in an axial configuration as compared to an equatorial orientation in α-GalCer 8 or α-GlcCer 21, was found to be inactive. The configuration of the 2-hydroxyl group on the sugar moiety proved to be crucial to activate iNKT cells.68 These observations were later explained by determining the crystal structures of mouse and human CD1d-α-GalCer-TCR ternary complexes, which revealed that the equatorial position of the galactose 2′-OH in α-GalCer forms a hydrogen bond with the side-chain of Asp151 in hCD1d (Asp153 in mCD1d) and the resulting adopted orientation of the sugar is crucial for the recognition by the iNKT TCR.
Trappeniers et al. reported few analogues of α-GalCer with modifications at the galactose 6'-position.\textsuperscript{69} The crystal structure analysis of human and mouse CD1d-α-GalCer complexes showed that the 6'-hydroxyl group of the galactose in α-GalCer does not take part in a hydrogen bond formation between α-GalCer and CD1d. Analogue 23 (Figure 1.20) in which 1-naphtyl group attached to 6'ureido-6'deoxy-α-GalCer, was found to stimulate a Th1-cytokine biased response with production of IFNγ cytokine similar to α-GalCer.

The threitolceramide ThrCer 24 (Figure 1.20) is a truncated non-glycosidic analogue of α-GalCer. In this compound the labile α-glycosidic linkage is replaced with an ether bond to gain metabolic stability \textit{in vivo}. A galactose ring has been replaced with an acyclic sugar but with retained relative and absolute stereochemistry. ThrCer 24 proved to be a less potent agonist of CD1d when compared with α-GalCer, however it does not cause the lysis of dendritic cells.\textsuperscript{70}
Figure 1.20. Representative carbohydrate derivatives of α-GalCer.
1.7.2.4 Glycosidic bond derivatives

The C-glycosyl derivative, α-C-GalCer 25 (Figure 1.21), was found to be a more potent agonist than α-GalCer in mouse model of malaria and melastatic melanoma, but also showed induced Th1-biased cytokine production.\(^7\) In this analogue the oxygen atom of the α-GalCer glycosidic bond (a polar hydrogen acceptor) has been replaced with a non-polar CH\(_2\), which cannot participate in hydrogen bonding. This modification has been proposed to cause the glycolipid to sit differently in the CD1d groove thereby changing the structure of the complex and its affinity for the iNKT TCR, and may also allow for free rotation of the “glycoside” bond connecting the galactose and ceramide portions of the molecule.\(^71,72\) Greater levels of IFN-γ production might be also a result of higher stability of this analogue \textit{in vivo}, as C-glycosidic linkage has increased resistance to enzymatic degradation.

Recently, the same research group (R. W. Franck and co-workers) reported a series of α-C-GalCer analogues and found \(E\)-alkene-linked C-glycoside analogues \textit{e.g.} GK 109 26 (Figure 1.21) were potent human iNKT-cell stimulants that also induce Th1-biased cytokine production relative to α-GalCer.\(^73\) It was proposed that replacement of the CH\(_2\) glycosidic linkage with the \(E\)-alkene linker may result in fixed orientation of the polar head group that is beneficial for iNKT cell TCR recognition.\(^73\)
These findings highlight the flexibility of the iNKT cell TCR, which in spite of its relatively limited diversity appears to be capable of different modes of recognition that enable responses to a wide range of CD1d-presented ligands.

Figure 1.21. Representative glycosidic bond derivatives of α-GalCer.
1.8 Aims and objectives

Recent years have shown a growing interest in understanding the biology of iNKT cells that in turn could translate into successful immune therapies. Numerous α-GalCer analogues have been synthesised and tested in order to find a potent iNKT cell agonist that could selectively induce beneficial cytokine profile.

To date, very few alterations around the amide bond of α-GalCer have been explored. To investigate its importance in iNKT cell stimulation, we decided to synthesise a range of α-GalCer and ThrCer analogues, in which the amide group in our lead has been replaced with different carbonyl functional groups. These compounds would then be tested for iNKT cell induction and in particular their Th1/Th2 response, which in turn would determine their therapeutic potential.

The second part of the project will focus on additional modifications and the synthesis of labelled derivatives, which will find application in intracellular trafficking studies.
Chapter 2

Amide Analogues of $\alpha$-Galactosylceramide

and Threitol Ceramide
Chapter 2  Amide Analogues of α-Galactosylceramide and Threitol Ceramide

2. Amide Analogues of α-Galactosylceramide and Threitol Ceramide

2.1. Interactions between the amide moiety of α-GalCer and amino acid residues of the iNKT TCR and CD1d protein in the human TCR–hCD1d–α-GalCer ternary complex

The first crystal structures of the hCD1d–α-GalCer complex, and the TCR–hCD1d–α-GalCer ternary complex, discussed in the Introduction (Sections 1.5 and 1.6), did not reveal any direct hydrogen bonds between the amide group of α-GalCer and amino acid residues of CD1d or the iNKT cell TCR. Recently however, Pellici et al. reported a crystal structure of the human NKT cell TCR–hCD1d–α-GalCer complex at improved (2.5 Å) resolution (Borg et al. reported the crystal structure of the NKT cell TCR–hCD1d–α-GalCer complex at 3.2 Å resolution). From this better-resolved structure, the carbonyl group of the amide functionality in α-GalCer was found to form a hydrogen bond with a water molecule, which in turn, formed a hydrogen bond with the backbone carbonyl of isoleucine-69 (Ile69) in the CD1d molecule.
Figure 2.1. Cartoon representation of polar contacts in the iNKT cell TCR–hCD1d–α-GalCer complex (α-GalCer – green sticks, CD1d – blue cartoon). Figure generated from 3HUJ.\textsuperscript{74}

The structural analyses made by Pellici et al. also identified a direct hydrogen bond between the N-H of the amide group of α-GalCer and the hydroxyl group in the side-chain of the threonine-154 (Thr154) residue located on the α-2 helix of the human CD1d molecule (Thr156 in mouse CD1d). We postulated that this interaction might play an important role in positioning α-GalCer in the right conformation for subsequent recognition by the iNKT cell TCR receptor.
Figure 2.2. Cartoon representation of the hydrogen bond interaction between the –NH of α-GalCer (pink sticks), and the Thr154 residue of hCD1d (green cartoon). Figure generated from 3HUJ.74

Figure 2.3. Interactions between the amide moiety of α-GalCer and amino acid residues of hCD1d in the iNKT cell TCR–hCD1d–α-GalCer ternary complex revealed by the crystal structure determined at 2.5 Å resolution.74
2.2. Reported analogues of α-GalCer with variations around the amide group

To date, only a few analogues of α-GalCer have been synthesised, in which the amide group has been replaced by other functional groups. Lee et al.\textsuperscript{75} replaced the amide moiety in α-GalCer with a 1,2,3-triazole. They reported a series of analogues containing this heteroaromatic unit and possessing different lengths of the lipid chain ranging from 6 to 26 carbon atoms \textbf{27-32} (Figure 2.4).

![Figure 2.4](image)

\textbf{Figure 2.4.} A series of 1,2,3-triazole-containing analogues of α-GalCer \textbf{27-32}.

The 1,2,3-triazole functionality exhibits structural similarities to the ubiquitous amide moiety found in natural systems,\textsuperscript{76} however in contrast to amides, triazoles are much more stable to hydrolysis, as well as to oxidising and reducing conditions \textit{in vivo}.\textsuperscript{77} In a similar fashion to the amide group, a 1,4-disubstituted 1,2,3-triazole can also participate in hydrogen bond formation (Figure 2.5). However, whilst primary and secondary amides can function as hydrogen bond donors and acceptors, two of three nitrogen atoms of 1,2,3-triazoles can only ever serve as hydrogen bond acceptors.
Figure 2.5. Hydrogen bond formation and dipole-dipole interactions between amides (A) and 1,2,3-triazoles (B) and a hydroxyl group.

The bioisosteric replacement of the amide moiety of α-GalCer with a 1,2,3-triazole unit is potentially interesting as the resulting modified glycolipids should survive a wider range of conditions in vivo and therefore last longer. Activation of iNKT cells with the C-glycosyl analogue of α-GalCer (discussed in the Introduction in Chapter 1.7.2.4), results in greater production of IFNγ (Th1-type) and lower amounts of IL-4 (Th2-type). Since IFNγ requires a longer release time than IL-4, it has been postulated that the longer exposure time of the CD1d-glycolipid complex to iNKT cell TCR might account for this Th1-biased cytokine response.

Biological evaluation of glycolipids 27-32 in vitro (with the use of mouse splenocytes), however, showed a Th2 bias for all analogues. Analogue 31 in particular, which bears a saturated 24-carbon alkyl chain, produced higher levels of IL-4 and less IFNγ when compared with α-GalCer. Subsequent in vivo studies confirmed the in vitro observations for triazoles 30-32, which all possess longer alkyl chains (n = 21, 22, 23); however those analogues with short- and medium-chain lengths were unable to stimulate iNKT cells in vivo.
Recently, Mori and co-workers investigated the replacement of the amide bond in α-GalCer with ether 33 and ester groups 34. In both cases, the formation of the hydrogen bond with the Thr154 residue of human CD1d (and Thr156 for mouse CD1d) may still be possible in these analogues with relatively little structural reorganisation; the oxygen atom of the ether or ester group, which replaced the N-H of the amide group of α-GalCer, might now act as a hydrogen bond acceptor to the hydrogen of the hydroxyl group in the side-chain of Thr154.

![Figure 2.6](image)

**Figure 2.6.** α-GalCer analogues where ether and ester groups have been introduced in place of the amide linkage found in α-GalCer.

The ether analogues, which lacked both the N-H and the carbonyl oxygen of the amide in α-GalCer were unable to activate iNKT cells. Glycolipids with an ester group, however, were able to activate iNKT cells although with significantly reduced activity compared to α-GalCer: IFNγ production was 10% of that shown by α-GalCer; IL-4 production was one-third to two-thirds of that shown by α-GalCer. These results further support the importance of the amide N-H in α-GalCer, and its ability to act as a hydrogen bond donor to the Thr154 residue of hCD1d, for iNKT cell activation.
2.3. Target compounds and the rationale for replacing the amide bond

We were also interested in investigating further modifications around the amide bond. In light of the apparent importance of the amide N-H in CD1d binding, and in contrast to other studies, we decided to retain the NH in the structure, and replace the amide moiety with related functional groups, namely a thioamide, urea and carbamate. Our target molecules are shown below in Figure 2.7.

![Chemical structures](image_url)

**Figure 2.7.** Target molecules, α-GalCer and three analogues in which the amide has been replaced with other functional groups.

We also decided to make similar changes to ThrCer, the non-glycosidic analogue of α-GalCer (see Introduction Chapter 1.7.2.3) and the four target molecules based on this scaffold are presented in Figure 2.8.
We postulated that these changes would be interesting for a number of reasons. First, the applied modifications could result in increased metabolic stability, as these functional groups should survive a wider range of conditions \textit{in vivo}, being less prone to enzyme-mediated hydrolysis.\textsuperscript{79-81} Second, the introduction of different heteroatoms could alter the hydrogen bonding network. The $pK_a$ values of the N-H in a urea, amide, carbamate and thioamide are summarised in Figure 2.9. The urea has the highest $pK_a$ value and is less acidic than an amide, whereas the N-H in a carbamate and thioamide is more acidic and should be a better hydrogen donor.\textsuperscript{82,83}
Chapter 2  Amide Analogues of α-Galactosylceramide and Threitol Ceramide

![Chemical Structures](image)

$pK_a = 26 - 27$  \hspace{1cm} $pK_a = 24.2$  \hspace{1cm} $pK_a = 20.8$  \hspace{1cm} $pK_a = 13.3$

**Figure 2.9.** Comparison of $pK_a$ values (in DMSO) for amide, carbamate, urea and thioamide.\cite{82,83}

The formation of additional hydrogen bonds is possible for the carbamate analogue, where the additional oxygen atom is a potential hydrogen bond acceptor. Likewise, the urea analogue possesses an additional N-H which could serve as another hydrogen bond donor. In the case of the thioamide analogue, we postulated that the larger\footnote{The atomic volume of a sulfur atom is 1.66 times larger (24.4/14.7=1.66) than oxygen (van der Waals radius of a sulfur atom $r_W = 1.80 \text{Å}$, for oxygen $r_W = 1.52 \text{Å}$, hydrogen $r_W = 1.20 \text{Å}$)}, lipophilic sulfur atom might also change the rate of glycolipid loading / deloading from CD1d and increase the stability of the CD1d-glycolipid-TCR complex, since this more hydrophobic analogue of α-GalCer, might be buried “deeper” in the hydrophobic binding groove of CD1d, leading to a more stable CD1d-glycolipid-TCR complex.
Chapter 2  Amide Analogues of α-Galactosylceramide and Threitol Ceramide

A

\[
\begin{align*}
\text{N} & \quad \text{O} \\
\text{H} &
\end{align*}
\]

B

\[
\begin{align*}
\text{N} & \quad \text{S} \\
\text{H} &
\end{align*}
\]

\(r_w = 1.52 \, \text{Å}\)

\(r_w = 1.80 \, \text{Å}\)

\(r_w\) - van der Waals radius

Figure 2.10. Sphere representation of the primary amide \(N\)-methyl-acetamide (A) and \(N\)-methyl-thioacetamide (B), indicating a difference in atomic volume between the carbonyl oxygen and sulfur atoms. (oxygen – red, sulfur – yellow, nitrogen – blue, carbon – grey, hydrogen – white).

These modifications have not been applied to glycosyl ceramide structures, although similar modifications to the amide bond in a ceramide lipid were recently reported\(^{84-87}\) after this synthetic work had been completed. In this study, De Libero and co-workers prepared a range of sphingolipids and ceramides with different carbonyl functions (Figure 2.11). Compounds 41-45 were tested for iNKT cell activation, but none showed significant activity. A competition assay was designed
to test the ability of these ceramides to compete with α-GalCer for binding to CD1d and prevent T cell activation. Only compound 43 bearing a 1,2,3-triazole and thioamide function was found to compete with α-GalCer in *in vitro* studies, albeit only at 15-fold molar excess over α-GalCer; the same result was not replicated in *in vivo* assays, even when a large excess of this ceramide was used. In light of the structural differences, principally the absence of a sugar residue, further comparisons of these ceramide analogues with our α-GalCer and ThrCer derivatives are difficult.

**Figure 2.11.** Modification of the amide group of 7-oxaceramides.\(^8\)
2.4. Retrosynthetic analysis

The retrosynthesis of \( \alpha \)-GalCer 8 and its three analogues 35, 36 and 37 is outlined in Scheme 2.1. In our approach we wanted to prepare a common precursor, namely amine 46, which could be functionalised at a late stage of the synthesis with acyl chloride 47, isocyanate 48 and mixed carbonate 49, to provide three of our target molecules after deprotection. We identified \( \beta \)-galactose pentaacetate 53 and phytosphingosine 54 as the starting materials for our synthesis; these could be readily converted into the two reacting partners required for the glycosylation reaction, which would unite the two fragments. Galactose 51 would then serve as the glycosyl donor and phytosphingosine 52 as the acceptor. Reduction of the azide in the resulting glycoside product 50 would afford our common precursor, amine 46.
Scheme 2.1. Retrosynthetic analysis of α-GalCer 8 and thioamide 35, urea 36 and carbamate 37.
2.5. First-Generation Synthesis of α-GalCer

We first decided to prepare α-GalCer, as this compound would be needed as the control against which our new analogues 35, 36 and 37 would be compared in the biological assays. Moreover, this first synthesis would help us to identify any limitations (low-yielding reactions, poor choice of reagents and protecting groups) in our planned approach towards advanced intermediate amine 46, as well as the final deprotection step, before embarking on the synthesis of the new analogues.

The synthesis of 2,3,4,6-tetra-O-benzyl-D-galactose 51 began with commercially available β-galactose pentaacetate, which, on activation with BF$_3$·OEt$_2$ at 0 °C in the presence of PhSH, afforded thioglycoside 55 in excellent yield as an inconsequential 1:1 mixture of anomers (Scheme 2.2). The acetate groups in 55 were then removed under Zemplén conditions to afford the fully deprotected thioglycoside 56. This deprotection method allowed a simple work-up, which consisted of neutralising the reaction mixture with an acidic Dowex ion exchange resin followed by filtration of the resin and removal of the solvent under reduced pressure. The very polar tetraol 56 was collected as a white foam without any need for further purification. Subsequent re-protection of the alcohols in 56 as benzyl ethers provided thioglycoside 57, which was then hydrolysed with N-iodosuccinimide in an acetone/water mixture (10:1) to yield the hemiacetal 51 as our sugar donor precursor as a 1:1 mixture of diastereoisomers. The product was characterised by HRMS, and the 1:1 ratio of diastereoisomers was evidenced.
in the $^1$H NMR spectrum by integrating the OH proton resonances for the two anomers, which appeared at 2.93 ppm and 3.15 ppm. The IR spectrum also showed an OH stretch as further evidence for the thioglycoside hydrolysis.

![Scheme 2.2. Synthesis of 2,3,4,6-tetra-$O$-benzyl-D-galactose 51.](image)

Phytosphingosine 54 served as the starting material for the glycosyl acceptor 52 whose synthesis is summarised in Scheme 2.3. This commercially available amine 54 was first converted into the corresponding azide 58 in a diazo transfer reaction. Imidazole-1-sulfonyl azide hydrochloride was used as the “diazo donor” in this copper(II)-catalysed reaction, which proceeded in excellent yield. This reaction had been carried out previously in our group using trifluoromethanesulfonyl azide (TfN$_3$) as the “diazo donor”. However, disadvantages of using TfN$_3$ as a reagent include the explosive nature of neat
TfN₃, and its poor shelf-life, which necessitates its preparation immediately prior to use. Moreover, the preparation of TfN₃ involves the use of expensive trifluoromethanesulfonic anhydride and the removal of trifluoromethanesulfonamide from the reaction mixture, which complicates the work-up procedure. In contrast, imidazole-1-sulfonyl azide hydrochloride is cheap to prepare, more robust, easy to store (in its crystalline form) and provides a much safer alternative to TfN₃.

Scheme 2.3. Synthesis of azido alcohol 52.

Selective tritylation of the less hindered primary alcohol in 58 afforded trityl ether 59 in 60% yield. The low yield of this reaction was a result of by-product formation. MS and NMR spectroscopic analysis of the side-products, isolated from the crude reaction mixture by flash column chromatography, showed that a major side-
Chapter 2  
Amide Analogues of α-Galactosylceramide and Threitol Ceramide

product was a di-trityl ether, in which the primary and one of the secondary alcohols had reacted with the trityl chloride. Diol 59 was then treated with sodium hydride to provide the corresponding di-alkoxide, which was reacted with 2.2 equiv of benzyl bromide to give the dibenzylated derivative 60 in 80% yield. The desired glycosyl acceptor 5296-98 was finally obtained after selective deprotection of the trityl ether with p-TsOH.
A key challenge in our synthesis was the selective formation of the α-glycosidic bond in our target galactosides. To this end, we chose to employ the halide ion-catalysed α-glycosylation methodology developed originally by Lemieux and co-workers,\textsuperscript{97-99} and shown to be a useful entry into α-galactosides by Kobayashi and co-workers.\textsuperscript{100,101} Scheme 2.4 illustrates the mechanism of this interesting α-glycosylation reaction.

In the first step, a 2-O-benzyl-1-hydroxy sugar\textsuperscript{†} reacts with CBr$_4$ and Ph$_3$P, the so-called Appel reagents,\textsuperscript{102,103} to generate a glycosyl bromide. Under the reaction conditions, and in the presence of an excess of nucleophilic bromide anions (Bu$_4$NBr) and N,N-tetramethyl urea (TMU), the more stable α-glycosyl bromide equilibrates with the highly reactive β-anomer. The acceptor alcohol (R-OH) then reacts preferentially with the more reactive β-glycosyl bromide in an S$_{N}2$ fashion to provide the α-glycoside product. TMU and bromide anions play a critical role in the glycosylation, in ensuring the isomerisation of the α- and β-glycosyl bromides is much faster than their reaction with the glycosyl acceptor.

The labile β-glycosyl bromide will also react with adventitious water to generate the starting 2-O-benzyl-1-hydroxy sugar. However, CBr$_4$ and Ph$_3$P are employed in excess to ensure any hydrolysed donor is recycled. The Appel reagents also capture any adventitious water. Kobayashi and co-workers\textsuperscript{100} found that 3 molar equivalents of CBr$_4$ and Ph$_3$P were optimal. Under these conditions, in situ formation of the glycosyl bromide and glycosylation proceeded without having to

\textsuperscript{†} The key requirement for the glycosyl donor in this α-selective glycosylation is a non-participating protecting group on the C-2 position.
pay special attention to carrying out the reaction under strictly anhydrous conditions.

\[ \text{Scheme 2.4. Overview of one-pot dehydrative } \alpha\text{-glycosylation with Appel agents.} \]
In our hands, when glycosyl donor 51 (2,3,4,6-tetra-O-benzyl-D-galactose) was treated with CBr₄ and PPh₃ at r.t., the formation of the glycosyl bromide, as monitored by TLC analysis, was complete within three hours. In the next step, the acceptor 52, TMU and Bu₄NBr were added to the reaction mixture. Work-up after 72 hours provided the desired α-galactoside 50 as a single anomer.

Scheme 2.5. Synthesis of α-galactoside 50.

In our first attempt at glycosylation, the reported procedure was followed closely, with three equivalents of acceptor 52 being employed as directed. However, instead of leaving the reaction for 8 days, the reaction mixture was worked-up after only 3 days, as TLC analysis showed no further consumption of the starting materials after that time. Unfortunately, it proved difficult to separate the α-glycosyl product from the starting materials (bromide of the donor 51 and the acceptor 52), which compromised the isolated yield (42%) of α-galactoside 50. Table 2.1 summarises the modifications we applied in order to improve matters.
Chapter 2  
Amide Analogues of α-Galactosylceramide and Threitol Ceramide

<table>
<thead>
<tr>
<th>entry</th>
<th>acceptor 52 [equiv]</th>
<th>scale [mmol of 51]</th>
<th>3 Å MS used</th>
<th>recovery of 52 [%]</th>
<th>isolated yield of 50 α-glycoside [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3.0</td>
<td>0.37</td>
<td>no</td>
<td>43</td>
<td>42</td>
</tr>
<tr>
<td>2</td>
<td>1.5</td>
<td>0.37</td>
<td>yes</td>
<td>44</td>
<td>64</td>
</tr>
<tr>
<td>3</td>
<td>1.5</td>
<td>1.85</td>
<td>yes</td>
<td>45</td>
<td>62</td>
</tr>
<tr>
<td>4</td>
<td>1.5</td>
<td>3.50</td>
<td>yes</td>
<td>41</td>
<td>64</td>
</tr>
</tbody>
</table>

Table 2.1. Summary of modifications applied to the literature Kobayashi α-glycosylation.

First, to facilitate the chromatographic separation of the α-glycosyl product 50 from the starting acceptor, we decreased the amount of alcohol 52 from 3.0 to 1.5 equivalents and were pleased to observe that this modification did not result in a lower isolated yield, nor in a longer reaction time. Under these conditions, chromatographic purification of 50 was now possible. The inclusion of molecular sieves also helped matters. In combination, these changes led to a significantly improved isolated yield (64%) without affecting the reaction time (72 h). Moreover, it was now possible to perform the reaction on larger scale (3.50 mmol) without observing any deleterious effect on either the isolated yield or the stereoselectivity. With these modifications, we were able to access significant quantities of glycoside 50 for further study.

‡ 45% of the unreacted acceptor 52 was also recovered.
Chapter 2  Amide Analogues of α-Galactosylceramide and Threitol Ceramide

The stereochemistry of the glycosidic linkage was unambiguously assigned by NMR spectroscopic analysis. The easiest signal to find in the $^{13}$C NMR spectrum of 50 is the anomic carbon, which appears at $\delta$ 98.6 ppm. From this, the attached H-1’ proton was then identified by 2-D chemical shift-correlated spectroscopy (HSQC). Measuring the coupling constant of this anomic proton from the $^1$H NMR spectrum [δ 4.91 ppm (1H, d, $J_{1,2}$ 3.5 Hz, H-1’)], confirmed the assignment as the α-glycoside. The β-anomer would be expected to show a larger coupling constant in the range 8-12 Hz, associated with trans diaxial axial-axial coupling (Figure 2.12). An axial-equatorial coupling constant is much smaller, usually in the range 2-5 Hz.

![Figure 2.12](image)

Figure 2.12. $J_{1,2}$ coupling constants for α- and β-glycosyl products.
The azido group in glycoside 50 was next reduced with trimethylphosphine in a Staudinger reaction\textsuperscript{105,106} to give amine 46 in 72\% yield (Scheme 2.6). In the first step of the proposed mechanism of this Staudinger reaction (Scheme 2.7),\textsuperscript{107} a phosphine, usually Ph\textsubscript{3}P, reacts with the azide function to form a phosphazide intermediate, which then liberates nitrogen gas in the formation of an iminophosphorane.

\begin{center}
\begin{tikzpicture}
\node[draw] (a) {50};
\node[draw, right of=a, xshift=3cm] (b) {46, 72\%};
\draw[->] (a) -- (b) node[midway, above] {PMe\textsubscript{3} \text{ wet THF}};
\end{tikzpicture}
\end{center}

\textbf{Scheme 2.6.} Synthesis of common precursor amine 46.

In the presence of water (wet solvent or aqueous work-up) the iminophosphorane is hydrolysed to give the desired amine and a phosphine oxide by-product. Although triphenylphosphine is a stable and easy-to-handle reagent, the resulting by-product, triphenylphosphine oxide, can be difficult to remove in the work-up. We therefore chose to use trimethylphosphine instead as this reacts with azides in the same fashion;\textsuperscript{94} however, the trimethylphosphine oxide by-product is a volatile solid, which can be removed from the crude product under reduced pressure.\textsuperscript{108}
Chapter 2  
Amide Analogues of α-Galactosylceramide and Threitol Ceramide

Scheme 2.7.  
The general mechanism of the Staudinger reaction.

Amine 46 was next reacted with hexacosanoyl chloride 47 in the presence of NEt₃ to give protected α-GalCer 62 in 85% yield (Scheme 2.8). The acid chloride was freshly prepared from hexacosanoic acid with oxalyl chloride in the absence of solvent, and could be used without purification.

Scheme 2.8.  
Synthesis of amide 62.
Finally, hydrogenolysis of the benzyl groups in the presence of palladium over charcoal afforded the desired product 8 in 68% yield (Scheme 2.9). We modified the literature conditions of this debenzylation reaction,\textsuperscript{104} with THF being used as the solvent instead of the reported CH\textsubscript{2}Cl\textsubscript{2}/EtOH. Reaction in THF led to a much faster reaction, which was usually complete within 24 hours. In contrast, reaction in CH\textsubscript{2}Cl\textsubscript{2}/EtOH was very slow, with benzylated material still present even after 72 hours. Moreover, with this solvent system, the reaction mixture required repeated filtration followed by addition of “fresh” catalyst to achieve complete removal of the benzyl protecting groups.

Scheme 2.9. Hydrogenolysis of 62 led to target compound 8.

Having prepared the advanced intermediate, amine 46, and successfully used it to synthesise our first target compound – α-GalCer 8, we were ready to move on to our target α-GalCer analogues. Although we had not encountered any major problems in the synthesis of α-GalCer, we felt further improvements might still be made, particularly to the final deprotection step in the synthesis.
2.6. Thioamide analogue

With α-GalCer 8 in hand, three steps were required to access the desired thioamide 35 (Scheme 2.12). The key step in this synthesis would involve thionation of the amide and we chose to investigate Lawesson’s reagent 63 as a thionating agent in the first instance. Lawesson’s reagent\textsuperscript{109} is widely used for converting carbonyl compounds such as amides, ketones, lactams and lactones into their thiocarbonyl analogues.\textsuperscript{110,111} The reaction proceeds under mild conditions and is now generally preferred over the use of phosphorus pentasulfide (P\textsubscript{2}S\textsubscript{5}), which requires boiling toluene, pyridine or xylene as solvent, a large excess of the thionating agent and long reaction times.\textsuperscript{109,112}

The mechanism of the thionation reaction using Lawesson’s reagent 63 is depicted in Scheme 2.10. Lawesson’s reagent is in equilibrium with a highly reactive dithiophosphine ylide, which reacts with a carbonyl-containing compound to form a thiaoxaphosphetane.\textsuperscript{110} The driving force for the reaction involves the formation of a stable P=O bond in the final cycloreversion step, which is similar to the Wittig reaction.
There are very few examples of direct thionation of amides in the presence of free hydroxyl groups. Nishio et al. investigated the thionation of $\beta$-, $\delta$-, and $\kappa$-hydroxy amides 64, 67 and 70, respectively, with Lawesson’s reagent. Various products were formed in all three cases, including $\alpha$-$\beta$-unsaturated thioamide 65, mercaptoamide 66, thiopheneimine 68 and thiones 69 and 71 (Scheme 2.11).
In light of these results, we were not surprised to observe that performing the thionation of α-GalCer 8 without protecting the hydroxyl groups, resulted in the formation of a range of products. A large amount of unreacted starting material was also present in the reaction mixture, probably owing to the low solubility of the starting material in toluene. Moreover, separating the small amounts of the desired thioamide 35 from the excess of unreacted 8 proved to be difficult owing to their similar behaviour on silica.

A more effective synthesis of thioamide 35 is outlined in Scheme 2.12. We first decided to protect the hydroxyl groups in 8 as acetates, expecting only the amide
The amide group would react with the Lawesson’s reagent at 80 °C in toluene. Thionation of esters is possible using Lawesson’s reagent, but the reaction is usually performed in xylene at 140 °C. Peracetylation of α-GalCer afforded 72 in 94% yield. We were pleased to observe that reaction of 72 with one equivalent of Lawesson’s reagent in toluene at 80 °C for four hours afforded thioamide 73 in 85% yield. Deprotection of the acetyl groups under Zemplén conditions afforded thioamide 35 in 90% yield.

Scheme 2.12. Synthesis of thioamide analogue 35.
2.7. Synthesis of urea analogue of α-GalCer

Formation of the urea analogue 36 from amine 46 required the synthesis of an appropriate isocyanate. We envisaged this would be accessed by a Curtius rearrangement on the corresponding acid azide.\textsuperscript{117,118} Use of hexacosanoic acid 61 as the starting point would lead to a urea product containing 27 atoms (26 carbons and one nitrogen) in the acyl chain. Since CD1d does not readily accommodate acyl chain lengths longer than the C26 chain found in α-GalCer,\textsuperscript{119} we chose to start with tetracosanoic acid 74 as this would be processed through to a urea product containing 25 atoms in the acyl chain (24 carbons and one nitrogen). Since the α-GalCer analogue containing a C\textsubscript{24} acyl chain displays similar biological activity to α-GalCer 8,\textsuperscript{68} we expected any differences in biological activity between a urea analogue containing 25 atoms in the acyl chain (\textit{i.e.} 36), and α-GalCer 8, would be attributable to an amide-urea switch and not a result of the slightly truncated acyl chain.

The preparation of isocyanate 48 and urea analogue 36 is summarised in Scheme 2.13. Conversion of the starting tetracosanoic acid 74 to the corresponding acid chloride 75 using the (COCl)\textsubscript{2} procedure,\textsuperscript{120} followed by treatment with NaN\textsubscript{3} led to the acid azide 76,\textsuperscript{121} which underwent Curtius rearrangement\textsuperscript{121} on heating in toluene at reflux for four hours to provide the desired isocyanate 48. These three steps were performed as a sequence on the same day, without purification of any intermediates to minimise their possible decomposition. Crude isocyanate 48 was
reacted with amine 46 to provide urea 77 in 68% yield (Scheme 2.13). The product was characterised by HRMS and the quaternary resonance corresponding to the carbonyl C=O of the urea was observed at δ 158.8 ppm in the $^{13}$C NMR spectrum. The IR spectrum also showed a C=O stretch at 1670 cm$^{-1}$, providing further evidence of successful functionalisation of amine 77. Hydrogenolysis of the benzyl groups in urea 77 under our standard conditions, effected global deprotection and afforded our next target, urea 36.

**Scheme 2.13.** Synthesis of urea analogue 36.
2.8. Second-Generation Synthesis of α-GalCer

Whilst benzyl ethers are very commonly employed as protecting groups, particularly in carbohydrate chemistry, conformational effects can mean that some groups are particularly stubborn to remove under hydrogenolysis conditions. Indeed, this proved to be the case with one of the benzyl ethers in the phytosphingosine unit of amide 62 and urea 77. Debenzylation was often slow, and frequently required filtration of the reaction mixture and addition of fresh catalyst to effect complete removal of all benzyl protecting groups.

We envisaged that we could facilitate the hydrogenolysis step in our synthesis by finding alternative protecting groups for the two secondary alcohols in the phytosphingosine acceptor. To this end, we chose to investigate a modified phytosphingosine acceptor 78 in our glycosylation. Whilst the use of an acetal to protect the internal 1,2-diol in our phytosphingosine acceptor would likely necessitate a two-step deprotection post glycosylation, we expected the debenzylation step would be easier, and no problems with a late-stage acetal hydrolysis. Moreover, this additional deprotection step would be mitigated by a more straightforward two-step synthesis of acceptor 78 from phytosphingosine 58 (Scheme 2.14),\textsuperscript{55,90} compared with the three-step synthesis required to access dibenzyl ether 52.\textsuperscript{96}
Although the choice of donor/acceptor pairs can impact on the stereoselectivity of glycosylation reactions, we were pleased to observe that galactoside 79 could be accessed in similarly good yield and once again with complete α-stereoselectivity under our optimised conditions (Scheme 2.15). As an unexpected bonus, purification of 79 proved to be less laborious than for 50, and probably goes some way to accounting for the slightly higher (71%) isolated yield of the product. The product was confirmed by HRMS and the characteristic α-anomeric proton in the $^1$H NMR spectrum at 4.93 ppm, which appeared as a doublet with a small coupling constant (1H, d, $J_{1,2}$ 3.6 Hz, H-1'). The characteristic α-anomeric carbon resonance at 98.8 ppm in the $^{13}$C NMR spectrum provided further diagnostic data.
Staudinger reduction provided amine 80, which was acylated, as before, to provide amide 81 (Scheme 2.16). In the next step, the acetal protecting group was hydrolysed with TFA and after evaporation of the volatiles from the reaction mixture, the crude diol product was debenzylated to provide α-GalCer 8. As expected, hydrogenolysis of four instead of six benzyl ether groups proved to be much faster, taking eight hours instead of the 22 hours required to debenzylate 62.

**Scheme 2.16.** Second-generation synthesis of α-GalCer 8.
2.9. Synthesis of carbamate analogue of α-GalCer

Carbamates are traditionally prepared either by functionalisation of an alcohol with an isocyanate, or by reacting an amine with a chloroformate or synthetic equivalent. Chloroformates are moisture-sensitive, and their preparation requires reaction of an alcohol with difficult-to-handle and highly toxic phosgene or slightly less dangerous triphosgene. We therefore chose to investigate an alternative approach reported by Ghosh et al.\textsuperscript{122} In this report, Ghosh showed that alcohols react with commercially available \textit{N},\textit{N}'-disuccinimidyl carbonate in the presence of triethylamine to afford stable and easy-to-handle mixed carbonates, which can then be reacted with an amine to provide the corresponding carbamate.

![Scheme 2.17](image)

\textbf{Scheme 2.17.} Retrosynthetic analysis for carbamate.
Our next target was the carbamate analogue of α-GalCer 37 (Scheme 2.18). To maintain the optimum length of 26 atoms in the lipid tail, our carbamate analogue required a 24-carbon alkyl chain. We therefore started from tetracosanoic acid 74, which was reduced to alcohol 82 with LiAlH₄ in THF at reflux (Scheme 2.19). Reaction of 1-tetracosanol with 1.5 equivalents of \( N,N' \)-disuccinimidyl carbonate 83 in the presence of 2.5 equivalents of triethylamine afforded mixed carbonate 49 in almost quantitative yield. The product was characterised by HRMS. Further evidence came from the \( ^{13} \)C NMR spectrum, which showed a quaternary carbon resonance characteristic for the carbonate carbonyl group at δ 151.6 ppm in addition to a resonance at δ 168.7 ppm corresponding to the imide carbonyls. Mixed carbonate 49 was then reacted with amine 80, to provide carbamate 84 in very good yield (Scheme 2.19). Formation of the product was confirmed by HRMS and by the chemical shift of the quaternary carbon in the \( ^{13} \)C NMR spectrum, which appeared at δ 159.9 ppm. The IR spectrum also showed a C=O stretch at
1689 cm\(^{-1}\) as further evidence of carbamate formation. Finally, the protecting groups in carbamate 84 were removed uneventfully in a two-step acetal hydrolysis / debenzylation sequence to provide our final target 37 (Scheme 2.19).

Scheme 2.19. Synthesis of carbamate 37.
2.10. Synthesis of Threitol Ceramide

Threitol ceramide (ThrCer) 24 is a non-glycosidic analogue of α-GalCer (described previously in Chapter 1.7.2.3). Like α-GalCer, it does not display a biased Th1/Th2 cytokine response and compared with α-GalCer, it is a less potent agonist of CD1d; however it successfully activates iNKT cells without causing their overstimulation, thereby overcoming the problematic iNKT cell activation-induced anergy associated with α-GalCer.123

The synthesis of ThrCer 24 has been recently reported by Reddy et al.70 and by our research group.124 The protecting group strategy employed in both these approaches is slightly different to the synthetic route we chose to follow. We envisaged ThrCer and its three analogues would again be accessed from a common precursor amine 85, which could then be functionalised in the same fashion as for the α-GalCer series (Scheme 2.20). Amine 85 would be accessed from the corresponding azide 86, which can be further disconnected to the previously prepared primary alcohol 78 (the acceptor in our improved second-generation glycosylation reaction) and commercially available (+)-2,3-O-isopropylidene-L-threitol 88.
Chapter 2  Amide Analogues of α-Galactosylceramide and Threitol Ceramide

Scheme 2.20. Retrosynthetic analysis of ThrCer 24 and its analogues, thioamide 38, urea 39 and carbamate 40.
The protecting groups in the azide 86 were carefully selected to provide an intermediate suitable for the synthesis of ThrCer and its three analogues. Reddy et al.70 used a similar azide 89 in their synthesis of ThrCer, only the primary alcohol on the threitol moiety was protected as a benzyl ether, whilst in our previous report, the same primary alcohol was protected with a bulky TBDPS silyl ether, and the internal 1,2-diol of the threitol unit as a benzylidene acetal 90.

![Diagram](image)

**Scheme 2.21.** Comparison of protecting group strategy employed in the synthesis of ThrCer.

In our approach, deprotection of all five hydroxyl groups was envisaged as the last step of the synthesis. We wanted to avoid benzyl ethers as these usually require a hydrogenolysis deprotection step, which would prevent us from incorporating
unsaturated acyl chains. We also wanted to protect the two 1,2-diols as isopropylidene acetals and avoid a benzylidene acetal, as this introduces an additional stereocentre, which would render characterisation of the resulting products more difficult. We first considered protecting the primary alcohol of threitol as its tetrahydropyranyl ether 91, as this would allow us to perform a deprotection of all five hydroxyl groups in a single step; however, like for the benzylidene acetal, the THP-protected derivatives would likely result in diastereoisomeric mixtures. We therefore opted for a bulky TBDPS silyl ether in azide 86. Whilst this protecting group would entail a two-step deprotection sequence, it might be advantageous to employ an orthogonal protecting group, should we wish to selectively functionalise the primary alcohol of the threitol moiety at a later date.

The synthesis of our precursor amine 85 is summarised in Scheme 2.22. Starting from commercially available (+)-2,3-O-isopropylidene-L-threitol 88, reaction with equimolar quantities of tBuPh₂SiCl afforded the corresponding mono-silyl ether 92, which was further reacted with triflic anhydride in pyridine to provide triflate 87. This intermediate served as our electrophilic coupling partner for an etherification with nucleophilic coupling partner 78, which was the acceptor we had employed previously in our second-generation glycosylation reaction (see Section 2.9 of this Chapter).
Scheme 2.22. Synthesis of the advanced intermediate amine 85.

Treatment of alcohol 78 with NaH afforded the corresponding sodium alkoxide 93, which underwent smooth reaction with triflate 87 to provide the desired ether 86 in 80% yield. Staudinger reduction of the resulting azide using PMe$_3$ provided amine 85 (Scheme 2.22), and thence straightforward access to ThrCer and its three analogues, using the same approaches that were developed for the thioamide 35, urea 36 and carbamate 37 analogues of α-GalCer.
The synthesis of ThrCer 24 is summarised in Scheme 2.23. Amine 85 was first N-acylated with hexacosanoyl chloride 47 to provide amide 94. The product 94 was confirmed by HRMS. Key spectroscopic signals included the amide carbonyl stretch in the IR spectrum at 1646 cm$^{-1}$. The amide functionality was further evidenced in the $^{13}$C NMR spectrum, which showed the characteristic quaternary carbon environment of a carbonyl at $\delta$ 172.4 ppm. In the next steps, silyl ether deprotection with TBAF, followed by acid-catalysed hydrolysis of the acetals provided our target molecule ThrCer 24 as confirmed by HRMS. Analysis of the $^1$H- and $^{13}$C NMR spectra also confirmed the successful removal of the TBDPS silyl ether and two acetals. The IR spectrum showed the OH stretch as further evidence of hydroxyl groups.

Scheme 2.23. Synthesis of ThrCer 24.
2.11. Synthesis of thioamide analogue of ThrCer

Scheme 2.24 presents the synthesis of the thioamide 38 analogue of ThrCer, which was prepared in the same fashion as the thioamide analogue of α-GalCer 35 (Chapter 2.6). ThrCer 24 was first peracetylated to give 95, which underwent thionation, using Lawesson’s reagent, to provide thioamide 96 in good yield. Acetate deprotection with sodium methoxide in methanol provided our target compound 38.

Scheme 2.24. Synthesis of the thioamide analogue of ThrCer 38.
We also wanted to investigate an alternative approach to thioamide 38, and perform thionation on the early-stage intermediate amide 94 to minimise handling of the fully deprotected ThrCer 24. Our alternative approach is summarised in Scheme 2.25. We were pleased to observe that thionation of amide 94 proceeded uneventfully. The product 97 was confirmed by HRMS and spectroscopic data, which provided evidence for the thiocarbonyl C=S group in the $^{13}$C NMR spectrum at δ 205.5 ppm. Silyl ether deprotection, followed by hydrolysis of the two isopropylidene acetals, provided the final product 38.

Scheme 2.25. Alternative synthesis of thioamide analogue of ThrCer 38 starting from amide 94.
2.12. Synthesis of urea analogue of ThrCer

Reaction of amine 85 with tricosanyl isocyanate 48 (prepared as previously discussed in Chapter 2.7) in toluene at 80 °C furnished urea 98, which underwent our standard two-step deprotection sequence to afford urea analogue 39 in a satisfactory yield. This final compound was insoluble at r.t. in the chloroform / methanol mixture normally used to solubilise these glycolipids for NMR spectroscopic analysis. Fortunately, running the spectra at 40 °C prevented product precipitation and allowed us to obtain good quality data.

\[ \text{Scheme 2.26. Synthesis of 39, the urea analogue of ThrCer.} \]
2.13. Synthesis of carbamate analogue of ThrCer

Treatment of amine 85 with the mixed carbonate 49 derived from the reaction of tetracosanoyl alcohol with $N,N'$-disuccinimidyl carbonate (discussed in Chapter 2.9), provided carbamate 99. After aqueous work-up and silica column chromatography, the product was confirmed by HRMS. Formation of the carbamate function was further evidenced in the $^{13}$C NMR spectrum, which revealed a quaternary carbon resonance at 156.0 ppm and a C=O stretch at 1687 cm$^{-1}$ in the IR spectrum. Our standard two-step deprotection sequence provided
carbamate analogue 40. Carbamate 40 was also found to precipitate from a 2:1 chloroform / methanol mixture at r.t. and $^1$H- and $^{13}$C NMR experiments were performed at 40 °C in order to obtain good quality data.

Having prepared our target compounds 8, 24, 35-40, these were next submitted for biological testing which was carried out in the group of Prof. Vincenzo Cerundolo by Dr John-Paul Jukes and Dr Hemza Ghadbane at the Weatherall Institute of Molecular Medicine in Oxford, UK.
2.14. Biological evaluation of glycolipid antigens: thioamide, urea and carbamate analogues of α-GalCer and ThrCer

In a preliminary screen, our newly synthesised α-GalCer and ThrCer thioamide, urea and carbamate analogues were tested for their ability to stimulate the iNKT cell hybridoma DN32§, following pulsing of C1R-mCD1d** cells with various concentrations of ligands. The concentration of interleukin-2 (IL-2) in the supernatant, released after iNKT cell activation, was measured using an enzyme-linked immunosorbent assay (ELISA) (Figure 2.13 A, B).

Encouragingly, the results of these experiments demonstrated that ThrCer thioamide 38 and ThrCer carbamate 40 induced increased activation compared with ThrCer 24; however the ThrCer urea analogue 39 led to weak stimulation (Figure 2.13 A). A similar hierarchy was observed for the α-GalCer analogues (Figures 2.13 B), although the differences, particularly at high concentration, were less pronounced (Figure 2.13 B).

§ DN32 - the Vα14+ NKT cell hybridoma cell lines.
** C1R-mCD1d - mouse CD1d-expressing C1R cells.
Figure 2.13. **Activation of murine iNKT cells using thioamide, urea and carbamate analogues of ThrCer and α-GalCer.** C1R-mCD1d cells were pulsed with various concentrations of ThrCer analogues (A) and α-GalCer analogues (B) as indicated and used to stimulate the DN32 hybridoma in vitro. The supernatants were tested for the presence of IL-2.

To confirm these findings, human iNKT cells were co-cultured for 40 hours with C1R-hCD1d cells that had been pulsed with 100 ng/ml, 10 ng/ml or 1 ng/ml vehicle, ThrCer 24, ThrCer thioamide 38, ThrCer urea 39, ThrCer carbamate 40 (Figure 2.14 A) and α-GalCer 8, α-GalCer thioamide 35, α-GalCer urea 36, α-GalCer carbamate 37 (Figure 2. 14 B). Cytokine production was again determined by ELISA. The ThrCer analogues stimulated human iNKT cells and in agreement with murine iNKT cell data, the weakest ligand at 100 ng/ml was ThrCer urea 39, although in this experiment, both the ThrCer thioamide 38 and ThrCer carbamate...
Amide Analogues of α-Galactosylceramide and Threitol Ceramide

40 analogues were comparable to ThrCer 24 (Figure 2.14 A). All the α-GalCer analogues stimulated human iNKT cells showing almost the same activity at 100 ng/ml when compared with α-GalCer, while at 10 ng/ml they stimulated iNKT cells stronger than α-GalCer. Interestingly at that concentration α-GalCer thioamide analogue 35 was found to be 2-fold more potent than α-GalCer 8. Unlike the ThrCer analogues, all three α-GalCer analogues 35, 36 and 37 were active even at low concentrations (Figures 2.14 A, B).

---

**Figure 2.14.** Activation of human iNKT cells using thioamide, urea and carbamate analogues of ThrCer and α-GalCer. C1R-hCD1d cells were pulsed with various concentrations of ThrCer analogues (A) and α-GalCer analogues (B) as indicated and used to stimulate human iNKT cells *in vitro*. The supernatants were tested for IFNγ.
Since the two urea derivatives 36 and 39 displayed the weakest activity in our in vitro experiments, further studies focused solely on the thioamide and carbamate derivatives of ThrCer and α-GalCer. These analogues were further investigated in vivo, alongside the parent compounds and Th2-biasing molecule OCH 13, specifically to assess their ability to effect DC maturation as well as their Th1/Th2 cytokine response profile. To this end, 1 µg lipid was injected intravenously into mice. After 2 h, the mice were tail-bled and IL-4 levels in the serum measured by ELISA (Figure 2.15). At 18 h, the mice were sacrificed and blood serum levels of IFNγ were measured by ELISA (Figure 2.15).

The in vivo experiments for the α-GalCer analogues show that the thioamide and carbamate derivatives, 35 and 37, respectively, both display a slightly more Th1-biasing cytokine response than α-GalCer 8, resulting from a reduction in IL-4 production relative to the parent α-GalCer 8, rather than an increase in IFNγ production, which was similar to that generated by α-GalCer 8. Results for the ThrCer derivatives were more interesting in that these displayed a similar but more pronounced trend. Compared with α-GalCer 8, ThrCer 24 is a weaker activator of iNKT cells, although it displays a similar Th1-Th2 cytokine profile. Very interestingly ThrCer thioamide 38 and ThrCer carbamate 40 displayed no IL-4 production, when assayed at 2 h; however they showed levels of IFNγ production at 18 h that were higher than those shown for ThrCer 24 and only four times lower than that displayed by α-GalCer 8. Thus ThrCer analogues 38 and 40 appear to be promising Th1-biasing glycolipids.
Figure 2.15. IL-4 and IFN-γ levels in blood serum taken at the indicated times after injection of mice with glycolipid.
Finally, bacterially-expressed hCD1d and beta-2-microglobulin (β2m) molecules were refolded with the thioamide, and carbamate analogues of both α-GalCer and ThrCer by oxidative refolding chromatography, and then biotinylated as described previously for use in surface plasmon resonance (SPR) experiments (BIAcore 3000 instrument). The urea analogues of α-GalCer and ThrCer could not be refolded, so no SPR data are available for these analogues.

Soluble human iNKT TCR was prepared as described by McCarthy et al. SPR experiments were used to measure the affinity and kinetics of human iNKT cell TCRs for hCD1d loaded with α-GalCer, ThrCer, and their thioamide and carbamate analogues (Figure 2.16). To this end, increasing concentrations of TCR were injected for 5 seconds over the indicated complex immobilised on the BIAcore chip until the specific binding reached its plateau. $K_d$ and $B_{max}$ were calculated by fitting the data using a non-linear regression binding kinetics model (GraphPad Prism) (Figure 2.16). Kinetic measurements for the $k_{off}$ were calculated using BIAevaluation software kit; $k_{on}$ values were calculated from the experimental $k_{off}$ and $K_d$ (Figure 2.16 C).

In terms of binding affinity of the TCR for glycolipid-loaded hCD1d, $K_d$ values for the TCR–carbamate–hCD1d complexes in both series were comparable to those measured for the parent compounds. The iNKT TCR exhibited slightly lower binding affinity for hCD1d loaded with α-GalCer-thioamide compared with hCD1d/α-GalCer, whilst binding affinity for hCD1d loaded with ThrCer-thioamide was interestingly an order of magnitude lower than that for hCD1d/ThrCer. The binding kinetics experiment showed that these lower binding affinities are a
consequence of a faster off rate and a slower on rate of the TCR–thioamide–hCD1d complex compared to CD1d complexes with the parent molecules (Figure 2.16 C). This observation is comparable to the kinetics displayed by the Th2-biasing α-GalCer analogue, OCH 13.127 These iNKT cell TCR binding affinity data for the CD1d/glycolipid complexes do not show a clear correlation with the observed cytokine response.

<table>
<thead>
<tr>
<th>Lipid on CD1d</th>
<th>Experimental $K_d$ (μM)</th>
<th>Experimental $k_{off}$ (s$^{-1}$)</th>
<th>Calculated $k_{on}$ (M$^{-1}$ s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ThrCer</td>
<td>4.57±0.12±0.12</td>
<td>1.18±0.034</td>
<td></td>
</tr>
<tr>
<td>α-GalCer</td>
<td>2.19±0.07±0.07</td>
<td>0.56±0.008</td>
<td></td>
</tr>
<tr>
<td>ThrCer Thioamide</td>
<td>36.05±0.96±0.96</td>
<td>1.88±0.045</td>
<td></td>
</tr>
<tr>
<td>α-GalCer Thioamide</td>
<td>4.20±0.15±0.15</td>
<td>0.53±0.011</td>
<td></td>
</tr>
<tr>
<td>ThrCer Carbamate</td>
<td>4.62±0.13±0.13</td>
<td>1.91±0.072</td>
<td></td>
</tr>
<tr>
<td>α-GalCer Carbamate</td>
<td>1.72±0.10±0.10</td>
<td>0.91±0.035</td>
<td></td>
</tr>
</tbody>
</table>
Chapter 2  Amide Analogues of α-Galactosylceramide and Threitol Ceramide

<table>
<thead>
<tr>
<th></th>
<th>Binding Affinity (μM)</th>
<th>Kinetics (h⁻¹)</th>
<th>Metabolism (h⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ThrCer, 24</td>
<td>4.57 ± 0.12</td>
<td>1.18 ± 0.034</td>
<td>2.58 x 10⁵ ± 0.010 x 10⁵</td>
</tr>
<tr>
<td>ThrCer thioamide, 38</td>
<td>36.06 ± 0.96</td>
<td>1.88 ± 0.045</td>
<td>5.2 x 10⁵ ± 0.26 x 10⁴</td>
</tr>
<tr>
<td>ThrCer carbamate, 40</td>
<td>4.60 ± 0.13</td>
<td>1.19 ± 0.072</td>
<td>2.59 x 10⁵ ± 0.017 x 10⁵</td>
</tr>
<tr>
<td>α-GalCer, 8</td>
<td>2.19 ± 0.07</td>
<td>0.565 ± 0.008</td>
<td>2.58 x 10⁵ ± 0.009 x 10⁵</td>
</tr>
<tr>
<td>α-GalCer thioamide, 35</td>
<td>4.20 ± 0.15</td>
<td>0.537 ± 0.011</td>
<td>1.28 x 10⁵ ± 0.05 x 10⁵</td>
</tr>
<tr>
<td>α-GalCer carbamate, 37</td>
<td>1.72 ± 0.10</td>
<td>0.919 ± 0.035</td>
<td>5.34 x 10⁵ ± 0.37 x 10⁵</td>
</tr>
</tbody>
</table>

Figure 2.16. Binding affinities (left) and kinetics (right) of the iNKT TCR for hCD1d molecules loaded with α-GalCer and ThrCer analogues measured using surface plasmon resonance (SPR). ThrCer analogues (A) and α-GalCer analogues (B).

A recent study by Sullivan et al. made a direct comparison between the Th2-biasing OCH glycolipid 13 and the Th1-biasing C-glycosyl analogue of α-GalCer 25,127 The group showed that both OCH and the C-glycosyl analogue of α-GalCer displayed weaker interactions than α-GalCer with iNKT cell TCRs. The authors attributed differences in cytokine response profiles to other factors including their differing pharmacokinetics properties, and their ability to transactivate other cytokine-releasing T-lymphocytes such as NK cells. When such transactivation requires prolonged lifetime after initial injection, metabolically more stable
glycolipid analogues should function better. A similar observation was made by Taniguchi and co-workers with the neoglycolipid α-carba-GalCer, which also induces a Th1-biased cytokine response profile upon iNKT cell activation.\textsuperscript{128} Having replaced the amide residue with metabolically more stable functionality, we expected all three structural analogues, and in particular the non-glycosidic threitol derivatives 38, 39 and 40, which are not susceptible to glycosidase-mediated hydrolysis, would exhibit prolonged lifetimes \textit{in vivo}, and for this reason, tentatively postulated that this might lead to Th1-biasing antigens. This hypothesis appears to have been borne out, at least in part, in the ThrCer series with the thioamide and carbamate analogues displaying a cytokine profile that is biased towards Th1. In both α-Gal and Thr series, the urea analogues displayed poor activity and we were unable to obtain binding and kinetics data for these two substrates, which may suggest that the additional NH incorporated into the acyl chain disrupts glycolipid binding and subsequent presentation.
2.15. Conclusion and Future Work

We have designed and synthesised two advanced intermediate amines 46 and 78, which provided us with easy access to α-GalCer 8 and ThrCer 24 and their thioamide, urea and carbamate analogues.

All of the analogues were found to stimulate iNKT cells by CD1d-mediated presentation to varying degrees; however, the thioamide and carbamate analogues of ThrCer were of particular interest in that they elicited a strongly polarised in vivo Th1 response in mice.

ThrCer thioamide 38 may be useful for future investigation since it has the ability to activate both murine and human iNKT cells and elicited a strongly polarised in vivo Th1 response in mice. Its low binding affinity for CD1d is analogous to the truncated form of α-GalCer, OCH which has been shown to be rapidly presented on CD1d molecules, compared to α-GalCer. The analysis of the crystal structure of the iNKT TCR-hCD1d-α-GalCer ternary complex suggests that whilst NH in ThrCer thioamide 38 should be able to partake in a strong hydrogen bond with the side-chain hydroxyl of Thr156 in mCD1d (and Thr154 in hCD1d), we expected any hydrogen bonding with a bridging water molecule would be weaker. It is also possible that one of the water molecules might be displaced due to the increased size of the sulfur atom. Attempts are currently on-going to crystallise CD1d with ThrCer 26 and ThrCer thioamide 38 with to understand the mechanism by which the addition of thioamide influences CD1d-TCR interactions.
It would be interesting to investigate further why the urea analogues of \(\alpha\)-GalCer and ThrCer showed very weak iNKT cell stimulation. One possibility is that the additional N-H present in the urea can form a hydrogen bond with one of the amino acid residues of CD1d and change the conformation of the glycolipid-CD1d complex which is no longer recognised by iNKT TCR. To test this hypothesis the \(N\)-methyl urea analogue of \(\alpha\)-GalCer and ThrCer should be prepared (Figure 2.17).

Our findings might suggest that increased metabolic stability of the glycolipid is important for favouring a Th1-biased cytokine response. For that reason we also considered moving away from the carboxylic acid derivatives and introducing aromatic and heterocyclic rings into the glycolipid structure that could mimic amide functionality. These amide derivatives would be less conformationally flexible and also more stable \textit{in vivo}. We also envisage the preparation of the thioamide analogue of the \(C\)-glycosyl \(\alpha\)-GalCer derivative, which stimulates a Th1-biased cytokine response (see Chapter 1.7.2.4), would be worthwhile. Direct comparison of the altered biological activity of this analogue with our ThrCer thioamide 38 could provide us with more information on the relation between glycolipid structure and Th1/Th2 bias.
Figure 2.17. Possible future targets.
Chapter 3

Synthesis and Functional Activity of Labelled α-GalCer, ThrCer and Their Unsaturated Analogues
3. Synthesis and functional activity of labelled α-GalCer, ThrCer and their unsaturated analogues

3.1. Target compounds

The mechanisms leading to stimulation of biased Th1/Th2 responses by α-GalCer analogues remain poorly understood. As discussed earlier (see Chapter 1.7.2.1), one hypothesis states that for Th2 type agonists, like OCH 13, low levels of IFNγ are observed due to the low affinity of the CD1d-OCH complex for the iNKT cell TCR. However, another Th2-biasing agonist, α-GalCer(C20:2) 19, when complexed with mCD1d or hCD1d, has been shown to have high binding affinity for the iNKT cell TCR, similar to that displayed by the most potent CD1d agonist α-GalCer 8, a molecule which does not show significant skewing in its cytokine profile.

Another hypothesis points at differences in uptake and presentation of various glycolipids. Studies carried out by van den Elzen and co-workers revealed that α-GalCer loading into CD1d occurs mainly in endosomes and requires uptake by endocytosis through a process that is facilitated by extracellular lipid carrying proteins such as apolipoprotein E (ApoE). Further studies showed that α-GalCer loading and presentation is also facilitated by lipid transfer proteins (LTPs) such as saposins and GM2 activator protein and requires acidic pH. Yu and co-workers demonstrated that Th2-biasing α-GalCer(C20:2) 19 loads directly into cell-surface CD1d and does not require intracellular loading, which leads to much faster presentation. Figure 3.1 outlines the proposed mechanism.
leading to stimulation of biased cytokine responses by α-GalCer and its analogues.

**Figure 3.1.** Proposed mechanism leading to stimulation of Th2-biased cytokine responses by α-GalCer and its analogues. α-GalCer requires intracellular processing and loading into CD1d protein in late endosomes. α-GalCer(C20:2), on the contrary, binds to CD1d at the cell surface without endosomal processing and activates iNKT cells resulting in Th2 type response.

Figure adapted from ref 3. Permission to reproduce figure was obtained from *Elsevier* through RightsLink® - licence number 2812141231176.
To further study intracellular trafficking of exogenous glycolipids and to better understand the relationship between the cytokine profile of iNKT agonists and their differences in loading and presentation, we desired a series of labelled derivatives of α-GalCer, ThrCer and their unsaturated C20:2 analogues (Figure 3.2). Our first target was biotinylated ThrCer 106. The synthesis and biological evaluation of this molecule would first confirm that the location of the label and linker did not impact on functional activity, as it is important that labelled analogues mimic the unlabelled glycolipids closely to provide useful information.

Having confirmed the biotin label did not significantly modify functional activity of ThrCer, we would then prepare α-GalCer derivative 108 and α-GalCer(C20:2) derivative 109, each bearing differently coloured fluorescent labels. The same modifications would be applied in the ThrCer series. A green label would be attached to α-GalCer and ThrCer, and a red label to their unsaturated analogues α-GalCer(C20:2) and ThrCer(C20:2). Incubating cell lines with a pair of differently fluorescent α-GalCer derivatives, and separately with similar ThrCer analogues, would allow us to use multicolour fluorescence detection. The resulting cellular images should reveal the location and distribution of the different CD1d bound glycolipids and allow their direct comparison. For example, we would expect to see red colouration at the cell surface where the presentation of α-GalCer(C20:2) occurs and green areas in the intracellular compartments and at the cell surface, since α-GalCer(C26) needs to be first internalised before presentation to iNKT TCR can take place.
Chapter 3  Synthesis and Functional Activity of Labelled α-GalCer, ThrCer and Their Unsaturated Analogues

Figure 3.2.  Target molecules: biotinylated ThrCer 106, ThrCer Alexa 488 107, α-GalCer Alexa 488 108, α-GalCer Alexa 594 109, ThrCer Alexa 594 110.
Alexa Fluor® dyes, although very expensive, are now frequently used for conjugates with histological applications, providing the high fluorescence quantum yield and high photostability required to detect biological structures with excellent selectivity and sensitivity.\textsuperscript{133} Figure 3.3 outlines alternative dyes commonly used in fluorescence microscopy, flow cytometry, and nucleic acid-based detection: cyanine dye Cy3 \textsuperscript{111}, Oregon Green® \textsuperscript{112} from the rhodamine class and a boron-dipyrromethane example, namely BODIPY TR \textsuperscript{113}. However Alexa Fluor® dyes and their bioconjugates have more desirable properties including resistance to photobleaching, aqueous solubility, relative insensitivity to pH changes, high molar extinction coefficients and favourable quantum yields.\textsuperscript{134}

![Figure 3.3](image)

**Figure 3.3.** Examples of commonly used fluorescent dyes: cyanine Cy3 \textsuperscript{111}, rhodamine Oregon Green \textsuperscript{112} and BODIPY TR \textsuperscript{113}.

We first had to identify a label attachment point which would be common for all our target molecules. Chemical labelling studies of α-GalCer \textsuperscript{8} have previously introduced the label at either the terminus of the N-acyl chain\textsuperscript{135-137} or at the C(6)OH of the galactose residue,\textsuperscript{138,139} both of which were deemed unsuitable for our purposes. We argued that a ThrCer analogue possessing a biotin label (and
any other for that matter) at the terminus of the N-acyl chain would result in the label being buried deep inside the A’ pocket of the protein molecule (assuming such an analogue still binds in a similar conformation to ThrCer) and therefore unavailable for streptavidin or anti-biotin Ab recognition. Appending a label to the sugar residue in ThrCer was also rejected since all three alcohol groups in this truncated sugar unit are important for TCR recognition. We therefore considered alternative sites in the glycolipid, which would preserve binding and at the same time allow the biotin label to be recognised by some type of reporter group. Although an X-ray structure of the ThrCer-CD1d complex has so far eluded us, our working hypothesis assumes that ThrCer 24 adopts a similar bound conformation as does α-GalCer 8 in the CD1d molecule;\textsuperscript{45,46,74} thus the N-acyl chain of ThrCer occupies the hydrophobic A’ pocket of the protein and the ceramide base the less voluminous F’ pocket, leaving the polar sugar residue surface-exposed for TCR recognition. An examination of the available X-ray structures of α-GalCer\textsuperscript{45} and various other glycolipids bound to CD1d\textsuperscript{44,127,140,141} and the related iNKT cell TCR ternary complexes,\textsuperscript{46,74,142} encouraged us to consider the α-methylene unit in the N-acyl chain as a potential derivatisation site. Moreover, of the first set of glycolipid CD1d agonists, which were isolated from the marine sponge Agelas mauritianus,\textsuperscript{34} AGL-548 is a potent synthetic analogue.\textsuperscript{143} This glycolipid is structurally identical to α-GalCer apart from it possessing a hydroxyl group in the α-position (S configuration) of the N-acyl chain. Early studies had shown that deleting this hydroxyl functionality (which generates α-GalCer 8) has no significant impact on activity.\textsuperscript{143} Analysis of the X-ray structures of the α-GalCer-CD1d complex\textsuperscript{45} and the α-GalCer-CD1d-TCR complex\textsuperscript{46,74} revealed this site in the
glycolipid lies close to the surface of the CD1d molecule in the glycolipid-protein-TCR complex. Based on this analysis, we decided to introduce our labels into the α-position of the N-acyl chain of all our glycolipids.

Figure 3.4. Chosen label attachment point (pointed with white arrow) at the α-position of the acyl chain of α-GalCer, showing that this part of the molecule is not buried inside the CD1d protein in the α-GalCer-CD1d complex (A) and is also exposed in the iNKT TCR–α-GalCer–CD1d ternary complex (B, C). Surfaces: grey – CD1d, purple – iNKT TCR, cyan – β2m. (A – generated from 1ZT4, B, C – generated from 2PO6 with Pymol).
We chose not to couple our labels directly to the glycolipid (junction of three components of the α-GalCer-CD1d-TCR complex), and instead attach these through a linker to minimise their impact on binding to CD1d protein, and recognition by the iNKT TCR. Owing to its widespread use elsewhere for similar purposes an oligo(ethylene glycol) spacer group was chosen to separate the biotin label from the ‘active’ ThrCer portion of the molecule.\textsuperscript{144-146} Such a linker would also impart favourable solubility properties on the late-stage products in a range of organic solvents.\textsuperscript{147,148} A triethylene glycol spacer unit was deemed to be of sufficient length to ensure the label in our targets would extend away from the TCR recognition site so as not to interrupt antigen presentation, and allow its own uninhibited recognition by streptavidin (in the case of biotin).
3.2. Retrosynthetic analysis

The general retrosynthetic analysis of labelled glycolipids 106, 107, 108, 109 and 110 is outlined in Scheme 3.1. Considering the very high unit cost of the fluorescent labels and to minimise difficult-to-handle, water-soluble labelled products, we decided to install the label of choice in the final step of the synthesis.

We identified α-azido fatty acid chlorides and the previously prepared precursor amine 85 and modified (PMB protecting groups instead of Bn in galactose ring) amine 80 (see Chapter 2) as the starting materials for all of our syntheses. The resulting α-azido amide would then be reacted with the alkyne-terminated oligoethylene glycol linker 114 in a “click” reaction to provide the desired 1,2,3-triazole, which after global deprotection, would provide the primary amine ready to be coupled with a commercially available, amine-reactive biotin or fluorophore derivative leading to our target compounds.
Scheme 3.1. Retrosynthetic analysis of biotinylated ThrCer 106, ThrCer and α-GalCer derivatives 107 and 108 labelled with green Alexa 488 fluorescent label; and α-GalCer(C20:2) and ThrCer(C20:2) derivatives 109, 110 labelled with red Alexa 594.
3.3. Synthesis of biotinylated ThrCer

Biotinylation of ThrCer had been performed before in our research group;\textsuperscript{149} however, we wanted to refine the previous synthesis of 106 to provide a late-stage intermediate amine 122, which could be coupled with any amine-reactive label such as the $N$-succinimidyl ester of biotin 123 or an activated ester of a fluorescent label such as Alexa Fluor\textsuperscript{®}.

We first had to prepare $\alpha$-azido hexacosanoic acid 116, which would also be used in the synthesis of our target labelled molecules 107 and 108.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{scheme32.png}
\caption{Synthesis of $\alpha$-azido hexacosanoyl chloride 117.}
\end{figure}
α-Azido hexacosanoic acid 116 was prepared in two steps from hexacosanoic acid 61 (Scheme 3.2). Thus treatment of acid 61 with bromine in the presence of red phosphorus provided the corresponding α-bromo acid bromide, and then α-bromo acid 115 after aqueous work up, which was converted into α-azido acid 116 by reaction with NaN₃ in DMF. Treatment of acid 116 with oxalyl chloride generated the corresponding acid chloride 117, which was reacted directly with amine 118 to provide the desired amide 119 as an inseparable 1:1 mixture of epimers (Scheme 3.3).

![Scheme 3.3. Synthesis of amide 119.](image)

Linker 114 was prepared in four steps starting from commercially available 2-[2-(2-chloroethoxy)ethoxy]ethanol 120, which was first reacted with NaN₃ to provide the corresponding azide, in which the hydroxyl group was then treated with NaH and
reacted directly with propargyl bromide to give alkyne 121 in 70% overall yield (Scheme 3.3). The product was characterised by HRMS and the quaternary resonance corresponding to the alkynyl C≡CH was observed at δ 158.8 ppm in the $^{13}$C NMR spectrum. The IR spectrum showed a strong N$_3$ stretch at 2099 cm$^{-1}$ providing further evidence of successful formation of the product 121. Staudinger reduction of the azido group under our standard conditions provided the corresponding amine, which was next N-Boc-protected to give linker 114 in 82% yield after two steps. The product 114 was confirmed by HRMS. Key spectroscopic signals included the carbamate carbonyl stretch in the IR spectrum at 1706 cm$^{-1}$. The carbamate functionality was further evidenced in the $^{13}$C NMR spectrum, which showed the characteristic quaternary carbon environment of a carbonyl at δ 154.2 ppm.

Click reaction\textsuperscript{152,153} of azide 119 with alkyne 114 proceeded uneventfully and with complete regioselectivity to afford the desired 1,4-disubstituted 1,2,3-triazole 121, which was isolated as a 1:1 mixture of epimers. At this stage we were able to observe two epimers by TLC analysis; however, their chromatographic separation was not possible. Formation of the product was confirmed by HRMS. Further evidence came from the $^{13}$C NMR spectrum, which showed a quaternary carbon resonance at $\delta$ 145.4 ppm and a CH resonance at $\delta$ 122.3 ppm characteristic for the 1,4-disubstituted 1,2,3-triazole. From this, the proton of the triazole ring H-1‴ was then identified by 2-D chemical shift-correlated spectroscopy (HSQC) and appeared as two broad singlets in the $^1$H NMR spectrum in the region of 7.71-7.75 ppm for one epimer and in the region of 7.76-7.82 ppm for second epimer. Final global deprotection with trifluoroacetic acid provided the corresponding amine 122, which underwent smooth coupling with the N-hydroxysuccinimide activated ester of biotin 123 to provide our target\textsuperscript{154} biotinylated ThrCer 106 as a 1:1 epimeric mixture (Scheme 3.7). Both epimers were now separable by careful column chromatography on silica, which provided us with 5 mg of the less polar epimer (epimer A) and 4 mg of the more polar epimer (epimer B).
Scheme 3.7. Synthesis of biotinylated ThrCer 106.
The epimeric mixture 106 and the single epimers A and B were submitted for biological testing, which was carried out in the group of Prof. Vincenzo Cerundolo by Dr John-Paul Jukes and Dr Hemza Ghadbane at the Weatherall Institute of Molecular Medicine in Oxford, UK.
3.4. Biological evaluation of biotinylated ThrCer

The epimeric mixture and single epimers of biotinylated ThrCer 106 were next tested separately for their ability to be loaded on to hCD1d or mCD1d molecules as detected by the binding of tetrameric iNKT cell TCR (Figure 3.3 A) or the stimulation of murine iNKT cells (Figure 3.3 B). Whilst the mixture of epimers behaved similarly to ThrCer 24, when tested separately, it was apparent that the two epimers displayed different activity: iNKT cell TCR recognition of the CD1d–single epimer complex was noticeably weaker when the less polar (on silica) epimer (epimer A) was employed (Figure 3.3 A). These results were confirmed using mCD1d C1R cells and murine iNKT cell hybridomas (DN32) where the differences between epimer A and B became even more apparent (Figure 3.3 B). As a consequence of the difference in functional activity between the two epimers, the biological results using the 1:1 mixture were similar to those using purified epimer B, and for this reason, all subsequent experiments described below were performed with the more readily accessed epimeric mixture of 106.
Figure 3.3. iNKT cell recognition of ThrCer 24 and biotinylated ThrCer 106, epimer A, epimer B and 1:1 mixture of epimers A+B. (A) The recognition of 24 and 106 by human iNKT TCR tetramer was assessed by FACS following co-incubation of fluorescent human iNKT cell TCR and hCD1d C1R cells loaded with indicated lipids. (B) The activation of iNKT cells (DN32) was examined following overnight culture with mCD1d C1R cells pulsed with 24 and 106. IL-2 production in supernatant was measured by ELISA after 24 h.
Functional experiments were next performed with both ThrCer 24 and biotinylated ThrCer 106 to assess whether or not the presence of the biotin moiety linked to ThrCer alters optimal loading on to CD1d molecules and presentation to human iNKT cells. To this end, we first analysed the ability of CD1d+ target cells pulsed with both compounds to be recognised by human iNKT cells. The results of these experiments are summarised in Figure 3.4 and demonstrate that both ThrCer 24 and the active epimer of biotinylated ThrCer 106 are capable of sensitising iNKT cells to a similar level, as defined by IFNγ secretion (analysed after 36 h by ELISA70 (Figure 3.3)). We extended these results by employing a second protocol based on the use of a soluble human iNKT cell TCR tetramer,36 which was used as a staining reagent for CD1d+ target cells pulsed with a range of concentrations of biotinylated ThrCer 106 (Figure 3.3). These results again indicate that the biotin label and linker in the active epimer of 106 do not alter optimal loading on to CD1d molecules, since the biotin moiety remains available for binding to streptavidin and anti-biotin (Figure 3.3 A/B), and still allows iNKT TCR tetramer binding (Figure 3.4). Significantly, it is possible to co-localise the iNKT TCR tetramer and biotin moiety using streptavidin (Figure 3.5).
Chapter 3  Synthesis and Functional Activity of Labelled α-GalCer, ThrCer and Their Unsaturated Analogues

Figure 3.4. Biotinylated ThrCer 106 activates human iNKT cells in vitro. A human iNKT cell line was incubated with C1R hCD1d cells pulsed with ThrCer 24 (black squares) or biotinylated ThrCer 106 (1:1 epimeric mixture) (open squares) at various concentrations. IFNγ secretion was analysed after 36 h by ELISA.
Figure 3.5. Detection of biotinylated ThrCer 106 (1:1 epimeric mixture) on the surface of APCs. C1R hCD1d cells were pulsed with the indicated concentrations of biotinylated ThrCer for 16 h and then stained with a) fluorescent streptavidin (APC), b) anti-biotin Ab (FITC), c) soluble iNKT TCR tetramer (APC). Panels a, b, and c depict histogram overlays of the indicated fluorescence intensities. The dot plot shown in panel d depicts a double staining with the soluble iNKT TCR (APC) and anti-biotin Ab (FITC).

At this stage of the study, we did not know the absolute stereochemistry at the triazole tethering site of the more active epimer although our previous analysis of the crystal structure of the α-GalCer-CD1d-TCR ternary complex\textsuperscript{46,74} suggested that substituting the pro-S hydrogen in the α-methylene unit would exert fewer
conformational changes on the iNKT cell TCR-CD1d-glycolipid complex and also allow the biotin label to protrude away from this complex to allow its recognition.

![Diagram of pro-S and pro-R hydrogens in α-GalCer](image)

**Figure 3.6.** (A) Representation of the pro-S hydrogen and pro-R hydrogens in the α-GalCer structure. (B) Pro-S hydrogen in the α-position of the N-acyl chain of α-GalCer pointing away from the interior of the CD1d protein in the crystal structure of the iNKT TCR – α-GalCer – hCD1d ternary complex. Cartoon representation was obtained with PyMol from 2PO6.46
In summary, we have shown that attaching a biotin label through a tri(ethylene glycol) spacer to the α-position of the N-acyl chain in ThrCer 24 generates a functionally active labelled analogue 106. Whilst introducing a label at this site necessarily introduces a new stereogenic centre into the molecule, and therefore a mixture of diastereoisomers when racemic azido acid 116 is employed, these two epimers can be separated by chromatography. Although the two epimers display different functional behaviour, the relatively large difference between the two means that the more active epimer 106 B dominates the behaviour of the epimeric mixture, and obviates the need to use the diastereoisomerically pure active epimer for biological studies.

More generally, this study has shown that the α-site of the N-acyl chain should be a viable location for introducing labels into other ceramide-based CD1d agonists (and potentially other bioactive glycolipids). Furthermore, whilst this study involved the introduction of a biotin label, our synthetic strategy has been designed to allow the late-stage incorporation of other labels (e.g. fluorophores, lumophores) and we were now ready to prepare Alexa-labelled derivatives of ThrCer and α-GalCer.
3.5. Synthesis of ThrCer(C26:0) labelled with Alexa 488

With advanced intermediate amine 122 in hand we were also ready to perform a coupling reaction with an amine-reactive fluorescent label. To prepare Alexa 488 labelled ThrCer 107 we chose to use the commercially available Alexa Fluor® 488 carboxylic acid, 2,3,5,6-tetrafluorophenyl ester 124, which unfortunately comes with a high cost of £230 per 1 mg. Our biggest challenge would be to perform the coupling reaction efficiently on small scale; however we knew that providing 0.5-1 mg of labelled compound would be sufficient for our collaborators to carry out many biological assays.

Amine 122 was reacted with Alexa 488 active ester 124 in the presence of NEt₃ in DMF to provide the final product 107 in 73% yield (1.2 mg of isolated product). Owing to the small amount of isolated material, its high molecular mass (Figure 3.2), the charged nature of the material and the fact that it was an epimeric mixture, analysis of the ¹H NMR spectra proved to be very difficult. TLC analysis however showed two bright fluorescent products that were not contaminated with any of the starting materials and gave a positive test for glycolipids with Molisch’s reagent (α-naphtol solution 10 wt. % in ethanol) and lipid stain (phosphomolybdic acid solution 5 wt. % in ethanol).
Chapter 3  Synthesis and Functional Activity of Labelled α-GalCer, ThrCer and Their Unsaturated Analogues

3.6. Synthesis of α-GalCer(C26:0) labelled with Alexa 488

To access the corresponding α-GalCer derivative labelled with Alexa 488, we first considered using our previously prepared amine 80 (See Chapter 2.8), which could be coupled with α-azido hexacosanoyl chloride and then processed as before after removal of the protecting groups. However, the intermediate amine 80 bears four benzyl ether groups whose removal would likely cause a problem in our next target 109, which incorporates a skipped diene in the acyl chain. We therefore decided to further optimise our glycosylation reaction, and investigate the use of a new galactosyl donor containing PMB protecting groups, which we envisaged could be removed selectively under acidic conditions.

p-Methoxybenzyl (PMB) ethers have been used widely in carbohydrate chemistry. These electron-rich ethers can be selectively removed by oxidation with, for example, 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ), without affecting alkenes, acetals, epoxides and silyl ethers. They can also be cleaved with trifluoroacetic acid (TFA) without affecting azido groups, acetates and O-glycosidic linkages in oligosaccharides. For our purposes, the employment of PMB groups in the glycosyl donor would result in a potentially more efficient approach to amine 131. In analogy with amine 122, a final deprotection step with TFA would remove four PMB groups, the isopropylidene acetal and the Boc group.

Our new donor precursor, 2,3,4,6-tetra-O-p-methoxybenzyl-galactose 126, was prepared in two steps (Scheme 3.9). Previously prepared tetraol 56 (see Chapter 2.2) was first treated with NaH and the resulting alkoxide was reacted with p-
methoxybenzyl chloride to afford thioglycoside 125 in 90% yield. This step required the reaction temperature to be raised to 60 °C for the reaction to go to completion, whereas with benzyl bromide, r.t. had been sufficient to generate the tetra benzyl ether in almost quantitative yield. The thioglycoside 125 was then hydrolysed with N-bromosuccinimide in an acetone/water mixture (10:1) to yield the hemiacetal 126 as a 1:1 mixture of diastereoisomers.\(^{158}\)

We were pleased to find that the glycosylation reaction of donor precursor 126 with acceptor 78 under our standard conditions resulted in glycoside 127 with complete \(\alpha\)-selectivity and in 70% yield (Scheme 3.9). The product was confirmed by HRMS and the characteristic \(\alpha\)-anomeric proton in the \(^1\)H NMR spectrum at \(\delta\) 4.93 ppm, which appeared as a doublet with a small coupling constant (1H, d, \(J_{1,2}\) 3.6 Hz, H-1′). The \(^{13}\)C NMR spectrum also showed the diagnostic \(\alpha\)-anomeric carbon resonance at \(\delta\) 98.8 ppm.

![Scheme 3.9](image)

**Scheme 3.9.** Synthesis of galactoside 127 using new donor 126.
The next steps involved a Staudinger reaction to afford amine 128, which was acylated with acid chloride 117 to provide amide 129 as a 1:1 mixture of epimers (Scheme 3.10). Formation of the product 129 was evidenced by HRMS and in the $^{13}$C NMR spectrum, two characteristic amide C=O quaternary resonances were observed for both epimers at $\delta$ 168.6 and $\delta$ 168.9 ppm. From this, the H-2'' protons were identified by 2-D chemical shift-correlated spectroscopy (HMBC) and the resonances for the corresponding C(2'') carbons for both epimers at $\delta$ 64.2 and $\delta$ 64.5 ppm (by HSQC). The IR spectrum showed a characteristic azido stretch at 2100 cm$^{-1}$, and a carbonyl amide C=O stretch at 1720 cm$^{-1}$ provided further evidence of successful functionalisation of amine 128 with $\alpha$-azido-hexacosanoyl chloride 117. Click reaction performed on azide 129 with linker 114 resulted in the desired 1,2,3-triazole 130 as a 1:1 mixture of epimers.
Scheme 3.10. Synthesis of 1,4-disubstituted 1,2,3-triazole 130.

Scheme 3.11 outlines the synthesis of target molecule 108. Treatment of 130 with TFA afforded amine 131, which was purified by chromatography and confirmed by HRMS. Amine 131 was next reacted with the 2,3,5,6-tetrafluorophenyl ester of Alexa Fluor® 488 124 in the presence of NEt$_3$ in DMF. This reaction was performed, as previously, on 1 mg (Alexa Fluor® 488) scale. The product 108 was purified from the starting materials by flash column chromatography using a cartridge pre-packed with 100 mg of silica. Working on such small scale and with a mixture of epimers prevented us from obtaining full characterisation data of the product; however, we were able to confirm the formation of the product by HRMS.
Scheme 3.11. Synthesis of target molecule α-GalCer derivative labelled with Alexa 488 108.
To summarise, we further optimised our glycosylation reaction by introducing 2,3,4,6-tetra-O-p-methoxybenzyl-galactose \textbf{126} as a new donor precursor. Since all the protecting groups in the glycoside product can be removed (in a single step) under acidic conditions, this modification opened up the possibility of coupling unsaturated carboxylic acids, which forms the subject of the next Section.
3.7. Synthesis of α-GalCer(C20:2) labelled with Alexa 594

With amine 128 in hand we could now focus on the preparation of α-azido-eicosadienoic carboxylic acid. The high cost of cis-11,14-eicosadienoic acid (circa £100 / 100 mg, SIGMA-ALDRICH) encouraged us to develop a synthesis of α-azido-eicosadienoic acid from the much cheaper linoleic acid (£43 / 5 g, SIGMA-ALDRICH). For convenience, the α-azido acid would be synthesised in racemic form in the first instance, which would necessarily generate a final product as a mixture of a diastereoisomers. If necessary, an enantioselective route would be investigated at a later stage.

The retrosynthetic analysis of (Z,Z)-2-azido-eicosa-11,14-dienoic acid 132 is outlined in Scheme 3.12. Starting from inexpensive cis-9,12-linoleic acid 138 requires the addition of two carbons to the 18-carbon alkyl chain. These would be introduced sequentially, proceeding via aldehyde 135 (19-carbons in length), which would undergo a Strecker reaction to provide the corresponding α-amino-nitrile 134 (20 carbons in total) and after subsequent hydrolysis, α-amino-eicosadienoic acid 133. The final step in our approach would require an amine-azide conversion in a diazo transfer reaction. Goddard-Borger et al. optimised this reaction for the preparation of α-azido acids from natural α-amino acids and therefore, we presumed it would also work as a final step in our synthesis of 132.
The Strecker reaction\textsuperscript{159,160} is a well-known method for preparing α-amino nitriles, from which the corresponding α-amino acids can be obtained by hydrolysis of the nitrile. Furthermore enantioselective variants of the reaction, employing chiral auxiliaries\textsuperscript{161,162} or asymmetric catalysts,\textsuperscript{163,164} opens up the possibility of rendering our route to 132 enantioselective at a later stage.

Our synthesis of α-azido acid 132, began with the conversion of linoleic acid 138 into its methyl ester, which was then reduced with LiAlH\textsubscript{4} to the corresponding alcohol 139.\textsuperscript{165} In the next step, 139 was reacted with the Appel reagents (CBr\textsubscript{4}, PPh\textsubscript{3}) to provide bromide 137,\textsuperscript{165} which, upon treatment with NaCN in DMSO,\textsuperscript{166} afforded nitrile 136. DIBAL-H reduction, and subsequent hydrolysis of the intermediate imine, afforded aldehyde 135.\textsuperscript{167} The product was confirmed by HRMS and further evidenced by the \textsuperscript{13}C NMR spectrum, which showed the characteristic C=O resonance of an aldehyde at δ 202.8 ppm, and by the \textsuperscript{1}H NMR spectrum, in which the aldehyde proton appeared as a triplet [δ 9.77 ppm (1H, t, J 1.9, CHO)]. IR analysis revealed an aldehyde C=O stretch at 1742 cm\textsuperscript{-1}. 


In the next step, aldehyde 135 was converted to α-aminonitrile 134 using the Strecker reaction. The general mechanism of this transformation is summarised in Scheme 3.14. The first steps involve in situ generation of HCN from the cyanide salt and ammonium chloride and acid-catalysed imine formation. Cyanide anions react more rapidly with the iminium species than with the starting aldehyde, to generate an α-aminonitrile, which can then be hydrolysed to the corresponding α-amino acid.
Scheme 3.14. The general mechanism of the Strecker reaction.

Aldehyde 135 underwent a one-pot Strecker reaction\textsuperscript{168} to afford racemic α-aminonitrile 134 in a satisfactory 75% yield. The product was confirmed by HRMS. The \textsuperscript{13}C NMR spectrum of 134 showed a quaternary carbon resonance for the nitrile group at δ 122.2 ppm and only one CH resonance in the alkyl region at δ 43.4 ppm. The product was further evidenced in the IR spectrum with a characteristic C≡N stretch at 2246 cm\textsuperscript{-1} and a broad amine stretch at 3343 cm\textsuperscript{-1}. Nitrile 134 was then hydrolysed under basic conditions\textsuperscript{169} by heating with 40% aqueous potassium hydroxide solution for 8 hours, followed by work-up with 10% aqueous HCl solution, to provide crude α-amino acid 133, which was used in the next step without further purification. The final step involved conversion of 133 into the corresponding α-azido acid 132,\textsuperscript{90} which was evidenced by HRMS and confirmed by \textsuperscript{13}C NMR spectroscopic analysis, where the quaternary carbon resonance for the carboxylic acid group appeared at δ 176.1 ppm and the CH resonance for the α-carbon at δ 61.7 ppm. The IR spectrum showed a C=O stretch...
Chapter 3  Synthesis and Functional Activity of Labelled α-GalCer, ThrCer and Their Unsaturated Analogues

at 1719 cm\(^{-1}\), a characteristic azide stretch at 2108 cm\(^{-1}\) and a very broad stretch for the O-H group in the range of 3400-2500 cm\(^{-1}\), providing further evidence of successful formation of target molecule 132. The two \(cis\) double bonds remained unchanged throughout the synthesis, and always appeared in the \(^{13}\)C NMR spectra as four CH resonances in the region of \(\delta\) 128-130 ppm.

We next converted carboxylic acid 132 into the corresponding acid chloride 140 under mild reaction conditions (Scheme 3.15). Here we wanted to avoid heating the acid in neat oxalyl chloride\(^{\dagger\dagger}\), conditions which might affect the skipped diene present in the acyl chain. Acid 132 was therefore reacted with two equivalents of oxalyl chloride in the presence of catalytic amount of DMF in dichloromethane at 0 °C.\(^{170}\)

\[ \text{HO} \quad \text{N}_3 \quad \text{7} \quad \text{4} \quad \text{Cl} \quad \text{N}_3 \quad \text{7} \quad \text{4} \]

132 \[ \text{CH}_2\text{Cl}_2, 0 ^\circ\text{C} \]

140, quant.

Scheme 3.15. Synthesis of acid chloride 140.

\(^{\dagger\dagger}\) We had employed these conditions for the conversion of hexacosanoic acid 61 and \(\alpha\)-azido-hexacosanoic acid 132 into the corresponding acid chlorides.
Previously prepared intermediate amine 128 was successfully acylated with the resulting acyl chloride 140, affording amide 141 in 85% yield as a 1:1 mixture of epimers. Formation of the product 141 was evidenced by HRMS and in the $^{13}$C NMR spectrum by two characteristic amide C=O quaternary resonances for both epimers at $\delta$ 168.8 and $\delta$ 168.9 ppm. From this, the H-2'' protons were identified by 2-D chemical shift-correlated spectroscopy (HMBC) and the resonances for the corresponding C(2'') carbons for both epimers at $\delta$ 64.3 and $\delta$ 64.6 ppm (by HSQC). Four CH resonances for the two double bonds were identified in the $^{13}$C NMR spectrum at $\delta$ 127.9, 128.8, 130.0, 130.2 ppm. The IR spectrum showed a characteristic azido stretch at 2103 cm$^{-1}$ and a carbonyl amide C=O stretch at 1683 cm$^{-1}$, providing further evidence of successful functionalisation of amine 128 with $\alpha$-azido acid chloride 140. Azide 141 underwent click reaction with linker 114, to afford the desired 1,2,3-triazole 142 as a 1:1 mixture of epimers.
Scheme 3.15. Synthesis of advanced intermediate amine 143.
Treatment of 142 with TFA and purification by chromatography resulted in amine 143, as confirmed by HRMS, which was reacted with the succinimidyl ester of Alexa Fluor® 594 carboxylic acid 144 in the presence of NEt₃ in DMF. This reaction was performed as previously on 1 mg (1.2 μmol of Alexa Fluor® 594) scale. The amide product 109 was purified from the starting materials by flash column chromatography using a cartridge prepacked with 100 mg of silica. As was the case for 107 and 108, the small scale of this reaction, working with a mixture of epimers and now regioisomers‡‡ prevented us from obtaining full characterisation data of the product 109; however in the preliminary biological experiment this labelled glycolipid was found to activate iNKT cells in a similar way as its unlabelled derivative.

‡‡ Alexa Fluor® 594 succinimidyl ester is only available as a mixture of 5- and 6-regioisomers, which after reaction with our amine 143 results in formation of four possible products adding even more complexity to the spectroscopic data.
Scheme 3.16. Synthesis of α-GalCer(C20:2) derivative 109 labelled with Alexa 594.
3.8. Synthesis of ThrCer(C20:2) labelled with Alexa 594

Our last target molecule ThrCer(C20:2) labelled with Alexa 594 110 was prepared in four steps as for α-GalCer derivative 109. The synthesis of 110 is outlined in Schemes 3.17 and 3.18. Amine 118 was first N-acylated with acid chloride 140 to provide amide 145 as a 1:1 mixture of epimers. The product 145 was confirmed by HRMS. Key spectroscopic signals included the amide carbonyl stretch in the IR spectrum at 1660 cm\(^{-1}\) and a characteristic azido stretch at 2100 cm\(^{-1}\). The amide functionality was further evidenced in the \(^{13}\)C NMR spectrum, which showed two characteristic quaternary carbon environments of a carbonyl for both epimers at \(\delta\) 168.9 ppm and at 168.9 ppm. Click reaction of azide 145 and previously prepared alkyne 114 proceeded uneventfully and with complete regioselectivity to afford the desired 1,4-disubstituted triazole 146 as a 1:1 mixture of epimers. Treatment of 146 with TFA afforded amine 147, which was purified by chromatography and confirmed by HRMS.
Scheme 3.17. Synthesis of ThrCer(C20:2) derivative 110 labelled with Alexa 594.
Chapter 3  Synthesis and Functional Activity of Labelled α-GalCer, ThrCer and Their Unsaturated Analogues

Amine 147 was then reacted with the succinimidyl ester of Alexa Fluor® 594 carboxylic acid 144 in the presence of NEt₃ in DMF. This reaction was again performed on 1 mg (Alexa Fluor® 594) scale. The amide product 110 was purified from the starting materials by flash column chromatography using a cartridge pre-packed with 100 mg of silica. As was the case for 107, the small scale of this reaction, working with a mixture of epimers and regioisomers prevented us from obtaining full characterisation data of the product 110; however in the preliminary biological experiment this labelled glycolipid was also found to activate iNKT cells in a similar way as its unlabelled derivative ThrCer(C20:2).

Alexa labelled glycolipids 107, 108, 109 and 110 were submitted for biological testing, which was carried out in the group of Prof. Vincenzo Cerundolo by Dr John-Paul Jukes and Dr Hemza Ghadbane at the Weatherall Institute of Molecular Medicine in Oxford, UK.
3.9. Biological evaluation of Alexa-labelled glycolipids

The Alexa-conjugated α-GalCer and ThrCer (C26 and C20:2) compounds 107-110 were tested for their ability to stimulate iNKT cells. Splenocytes from C57 BL/6 mice were pulsed with various concentrations of ligands. The concentration of IFNγ in the supernatant released after iNKT cell activation was measured using ELISA. The results of these experiments demonstrated that Alexa-conjugated α-GalCer activated iNKT cells, although this was considerably weaker than the parent compound (Figure 3.7 A). Moreover, the Alexa-conjugated ThrCer were found to be inactive when cultured with C57 BL/6 splenocytes (Figure 3.7 A).

In parallel, the Alexa-conjugated α-GalCer and ThrCer (C26 and C20:2) compounds 107-110 were further tested for their ability to bind to biotinylated tetramerised human iNKT TCR, as previously described by McCarthy et al.36 Thus, C1R-hCD1d cells were pulsed with lipids to determine the binding affinity of iNKT TCR tetramer (as determined by MFI) by FACS.

The Alexa 594-labelled α-GalCer(C20:2) 109 was shown to bind as well as the parent compound 19 although the Alexa 488-conjugated α-GalCer(C26) 108 bound considerably weaker than its parent compound α-GalCer 8 (Figure 3.7 B). Although the Alexa-conjugated ThrCer derivatives were found to be inactive when cultured with C57 BL/6 splenocytes (Figure 3.7 A), the binding assay showed only moderate binding with the Alexa 594-labelled ThrCer(C20:2) 110, and this was
considerably weaker for the Alexa 488-conjugated ThrCer(C26) 107, when compared to the parent compound (Figure 3.6 B).

![Graphs showing IFN-γ production and MFI]  

Figure 3.7. Testing the activation of murine iNKT cells using Alexa-labelled α-GalCer. Splenocytes from C57BL/6 mice were cultured for 48 hours in the presence of vehicle, α-GalCer(C26) 8, α-GalCer(C26) Alexa 488 108, α-GalCer(C20:2) 19 and α-GalCer(C20:2) Alexa 594 109 in vitro (A). The supernatants were analysed for the presence of IFNγ by ELISA. C1R-hCD1d cells were pulsed with vehicle, α-GalCer(C26) 8, α-GalCer(C26) Alexa 488 108, α-GalCer(C20:2) 19 and α-GalCer(C20:2) Alexa 594 109 to determine the binding affinity using biotinylated tetramerised iNKT TCR. Mean fluorescent intensity (MFI) as determined by fluorescence-activated cell sorting (FACS).
Chapter 3  Synthesis and Functional Activity of Labelled \( \alpha \)-GalCer, ThrCer and Their Unsaturated Analogues

Figure 3.8. Testing the activation of murine iNKT cells using Alexa-labelled ThrCer. Splenocytes from C57BL/6 mice were cultured for 48 hours in the presence of vehicle, ThrCer (C26), ThrCer-Alexa 488 (C26), ThrCer (C20:2) and ThrCer-Alexa 594 (C20:2) in vitro (A). The supernatants were analysed for the presence of IFN-\( \gamma \) by ELISA. C1R-hCD1d cells were pulsed with vehicle, ThrCer (C26), ThrCer-Alexa 488 (C26), ThrCer (C20:2) and ThrCer-Alexa 594 (C20:2) to determine the binding affinity using biotinylated tetramerised iNKT TCR. Mean fluorescent intensity (MFI) as determined by FACS (B).

Alexa-594-conjugated \( \alpha \)-GalCer(C20:2) \textbf{109} showed comparable binding to hCD1d as the parent compound (C20:2) and was able to stimulate murine iNKT cells. However, the Alexa-594-conjugated ThrCer, despite binding in a cell-free system to hCD1d was inactive when tested with murine iNKT cells. Both Alexa-488-conjugated \( \alpha \)-GalCer and ThrCer were either significantly weaker (\( \alpha \)-GalCer) or
inactive (ThrCer) in comparison to their corresponding parent compound in both assays.

At this stage it is not clear why saturated derivatives 107 and 108 failed to activate iNKT cells whilst their unsaturated analogues showed activity and binding similar (or better) than the parent unlabelled compounds. It is unclear whether the Alexa-488-conjugated analogues compromised binding and hence activation of iNKT cells or whether these compounds require C20:2 ceramides for binding and activity. Future work will assess the choice of the label and its effect on the solubility of the resulting conjugates. It was previously reported by Moqbel and co-workers\textsuperscript{171} that in some cases the negative charge of fluorophores may cause a non-specific electrostatic interaction with positively charged cell structures. In these few cases, conventional neutral dyes, such as BODIPY FL, rhodamine, or Texas Red, may be better alternatives.\textsuperscript{133} Alexa 488 is doubly negative charged with diethylammonium counter ions, while Alexa 596 comes as a zwitterion. Based on the information provided in the introduction of this Chapter, it may be that the Alexa 594-labelled glycolipids are more easily presented to iNKT TCR at the surface of the cell, while Alexa 488-labelled glycolipids, like α-GalCer 8, require additional intracellular processing, which proves to be difficult for labelled compounds.
3.10. Conclusions and future work

We successfully synthesised biotinylated ThrCer 106 and showed that attaching a biotin label through a tri(ethylene glycol) spacer to the α-position of the N-acyl chain in ThrCer 24 generates a functionally active labelled analogue 106. Whilst introducing a label at this site necessarily introduces a new stereogenic centre into the molecule, and therefore a mixture of diastereoisomers when racemic azido acid 116 is employed, these two epimers can be separated by careful chromatography.

Continuation of this work in our group has since led to an enantioselective synthesis of (S)-2-azido-hexacosanoic acid, which provided biotinylated ThrCer 106 as a single epimer of known configuration, allowing us to confirm the identity of the more active epimer B. Gratifyingly, this more active diastereoisomer corresponded to the more polar epimer prepared using racemic α-azido acid 116, which is in complete agreement with our predictions that this epimer should better orient the label away from the TCR and therefore have minimal impact on iNKT cell activity.

The synthesis of ThrCer and α-GalCer analogues containing Alexa fluorescent labels proved to be challenging in respect to the small scale of the final step. However, we were able to provide two labelled derivatives that showed activity towards iNKT cells and which might be useful for in vivo imaging. The labelled derivatives of α-GalCer and ThrCer need to be further optimised and alternative labels will probably need to be examined. Ongoing work in the group involves
labelling α-GalCer and ThrCer with red Alexa 594, which should allow us to test whether or not the green Alexa 488 was the problem with α-GalCer 108 and ThrCer 107.
Chapter 4

Synthesis and biological activity of Gal\(\beta\)-\(\alpha\)-(1\(\rightarrow\)2)-ThrCer
4. Synthesis and biological activity of Galp-α-(1→2)-ThrCer

4.1. Introduction to Galp-α-(1→2)-ThrCer

Among the numerous analogues of α-GalCer, Galp-α-(1→2)-Galp-α-1-Cer 148 (Figure 4.1) has proven to be a valuable tool for studying iNKT cell-mediated immunity. Galp-α-(1→2)-Galp-α-1-Cer 148 is a derivative of α-GalCer 8, in which the 2′O-position of the galactose moiety is α-glycosylated with a second D-galactose unit. Determination of the crystal structure of the iNKT TCR–hCD1d–α-GalCer complex 46 revealed an important role for the free hydroxyl group in the C-2′-position of α-GalCer (see Chapter 1.6), as this forms a hydrogen bond to the side-chain carboxylate of Asp151, which is crucial for the antigenicity of α-GalCer.

![Figure 4.1. Structure of Galp-α-(1→2)-Galp-α-1-Cer 148.](image)

Although some disaccharide glycosphingolipid antigens can be recognised by iNKT TCR without prior processing, the responses to Galp-α-(1→2)-Galp-α-1-Cer 148 require removal of the terminal galactose to permit interaction with the TCR. 172 In this case, a lysosomal enzyme, α-galactosidase A, is responsible for hydrolysing the terminal glycoside and generating the antigenic α-GalCer 8.
As it requires intracellular processing, Galp-α-(1→2)-Galp-α-1-Cer 148 has proven to be a very useful tool to assess the lipid antigen presenting capacity of professional antigen presenting cells, like dendritic cells. Indeed, it has been shown that cells bearing mutations in enzymes involved in glycosphingolipid processing have altered processing capacity of Galp-α-(1→2)-Galp-α-1-Cer 148 because of engulfment and lysosomal storage. We have observed that presentation of Galp-α-(1→2)-Galp-α-1-Cer requires lipid transfer proteins belonging to the family of saposins. THP1 cells in which prosaposin has been inactivated by lentiviral transduction of shRNA sequences, are impaired in presentation of Galp-α-(1→2)-Galp-α-1-Cer 148 and partly of α-GalCer 8 but not of α-GalCer(C20:2) 19, which is efficiently loaded from the extracellular milieu. To further investigate the role of saposins in CD1d-mediated lipid antigen presentation, THP1 prosaposin deficient cells were pulsed with the non-glycosidic α-GalCer analogue ThrCer 24. In addition to being sensitive to cellular fixation, these experiments showed that this lipid is also dependent on saposin activity. We wished to extend these results using a galactosylated derivative of ThrCer.

The synthesis of Galp-α-(1→2)-Galp-α-1-Cer 148 has been recently reported. We decided to prepare the analogous Galp-α-(1→2)-ThrCer 149 (Figure 4.2), which we hoped would provide a similarly useful molecular tool for studying the biological pathways of this important non-glycosidic analogue of α-GalCer. In particular, this glycosylated ThrCer analogue would be useful for studying the kinetics and cellular site of glycolipid loading, which is known to play a critical role in dictating the outcome of iNKT cell activation.
Figure 4.2. Structure of target molecule Galp-α-(1→2)-ThrCer 149.
4.2. Retrosynthetic analysis

The retrosynthesis of Galp-α-(1→2)-ThrCer 149 is summarised in Scheme 4.1. We envisaged that the first step in the synthesis of our target molecule would involve formation of the disaccharide 153, which would be obtained from commercially available methyl 3,4-O-isopropylidene-L-threonate 154 and 2,3,4,6-tetra-O-p-methoxybenzyl-D-galactose 126. The key challenge in this step would involve the stereoselective synthesis of the α-glycosidic linkage using a relatively hindered secondary alcohol as the glycosyl acceptor. Reduction of the ester to the primary alcohol in the resulting disaccharide, and formation of the corresponding triflate 152 would then allow a Williamson etherification with a suitably protected phytosphingosine base 78. Unmasking the amine 150 and acylation with hexacosanoyl chloride 47 would complete the assembly of the four fragments, leaving a one-step global deprotection of the four PMB ether protecting groups and two acetals to deliver our target molecule 149.
Scheme 4.1. Retrosynthetic analysis of Galp-α-(1→2)-ThrCer 149.
4.3. Synthesis of Galp-α-(1→2)-ThrCer

In Chapter 3.2 we demonstrated that PMB-protected galactose 126 worked well as the donor in a highly α-stereoselective glycosylation. Reaction of PMB-protected galactose 126 with methyl 3,4-O-isopropylidene-L-threonate 154 under our standard glycosylation conditions also provided the desired α-glycoside product 155. Unfortunately a second α-linked§§ disaccharide, with the same molecular weight, was also observed in the crude reaction mixture. Although we were unable to separate the two compounds by flash column chromatography, very careful analysis of the NMR spectra of the mixture revealed this second disaccharide product to be α-galactoside 156. Whilst this compound was the minor product when the reaction was performed on small scale (e.g. 0.5 mmol, ratio 155:156, 3:2), it predominated when the glycosylation was scaled up (e.g. 1.85 mmol scale, ratio 155:156, 1:3).

§§ The small vicinal coupling constant between H-1’ and H-2’ in this disaccharide by-product allowed assignment of the α-configuration [δH 5.09 ppm (1H, d, J1,2 3.7 Hz, H-1’)].
Scheme 4.2. Synthesis of disaccharide 155.

The easiest resonance to assign in the $^{13}$C NMR spectrum of glycosides is the anomeric carbon (C-1’), which usually appears in the region 90-110 ppm. We found two resonances in that region when we analysed a 1:3 mixture of disaccharides 155 and 156: one resonance for the minor product appeared at $\delta$ 96.0 ppm, and a second at $\delta$ 98.2 ppm for the major component of the mixture. From this, the corresponding anomeric protons [δ 5.09 ppm (1H, d, $J$ 3.7, H-1’)] and δ 4.91 ppm (1H, d, $J_{1,2}$ 3.5 Hz, H-1’), respectively] were then identified by 2-D chemical shift-correlated spectroscopy (HSQC). Both doublet resonances have small $J_{1,2}$ coupling constants confirming an α-glycosidic linkage in both products. An HMBC experiment revealed strong cross-peaks of $^3J_{C-H}$ coupling for both anomeric carbons, which correlated to two different proton environments in the threitol portion of the molecule (Figure 4.3). The anomeric carbon at $\delta$ 98.2 correlated to a proton resonance in the $^1$H NMR spectrum [δ 3.65 ppm (1H, dd, $J$...
which was assigned by HSQC and COSY experiments as C(4)HₐHₖ of the undesired 156 disaccharide. In the same way, the resonance at δ 96.1 ppm correlated to the H2 resonance [δ 4.27 ppm (1H, d, J 7.0)]. Thus the major product in this mixture was the undesired disaccharide 156.

![Figure 4.3](image)

**Figure 4.3.** HMBC was crucial for assigning the minor 155 and major 156 disaccharides in the mixture.

Aside from generating the reactive glycosyl bromide in situ, the Appel agents (PPh₃ and CBr₄) in the glycosylation reaction serve two other useful roles: they function as a scavenger for adventitious water, and when used in excess, are capable of re-generating the active donor from any product that is trapped initially by water rather than the acceptor. However, one of the by-products from this dehydrative glycosylation pathway is HBr, which in our case, presumably catalyses the translational isomerisation of the isopropylidene acetal in 154 from the terminal to the internal 1,2-diol, allowing the remaining primary alcohol to function as a (more reactive) second glycosyl acceptor (Scheme 4.3). Fortunately, we were able to suppress completely this undesirable side-reaction by including Hünig’s base as an acid scavenger in the glycosylation reaction mixture. Under
these modified conditions, the desired disaccharide 155 was isolated in a satisfactory 45% yield as a single α-stereoisomer.

\[
\text{Scheme 4.3. Proposed mechanism of the acid-catalysed isopropylidene acetal migration in 154.}
\]

With disaccharide 155 in hand, reduction of the ester with LiAlH\(_4\) afforded primary alcohol 153 in 92% yield. We next decided to combine disaccharide 153 with a phytosphingosine coupling partner using a Williamson etherification. Applying the same method as had been used successfully in the synthesis of ThrCer 24 (see Chapter 2.7), we envisaged converting the primary alcohol of the disaccharide 153 into the corresponding triflate and reacting this with the alkoxide of phytosphingosine 78 would deliver our desired product.
Scheme 4.4. Unsuccessful attempt at triflate formation using alcohol 153.

Unfortunately the corresponding triflate 152 proved to be unstable (Scheme 4.4) and we were unable to effect Williamson ether formation. The colour of the reaction mixture turned from colourless to black immediately after adding an equimolar amount of triflic anhydride to the solution of 153 and 2 equivalents of acid scavenger, 2,6-di-tert-butyl-4-methylpyridine (DTBMP), at 0 °C in dichloromethane. TLC analysis of the resulting reaction mixture revealed the formation of more than four by-products, which were difficult to separate. The $^1$H NMR spectrum of the crude reaction mixture was also too complicated to assign any of the by-products; however, a significant reduction of resonances in the aromatic proton region, when compared with the $^1$H NMR spectrum of the starting material 153, suggested partial deprotection of the PMB groups.
We next wanted to check whether or not the etherification step would work if the triflate were formed on the phytosphingosine instead. Applying the same reaction conditions as before, but this time using alcohol 78, TLC analysis of the reaction mixture showed the formation of a less polar product, suggesting that triflate formation had been successful. However, its reaction with the sodium alkoxide of the alcohol in disaccharide 153 provided none of the desired etherification product.

**Scheme 4.5.** Reversing the electrophile and nucleophile coupling partners failed to provide the desired ether product.

At this juncture, whilst a range of other coupling partners could have been investigated, we had concurrently been investigating the glycosylation reaction using 2,3,4,6-tetra-O-benzyl-D-galactose 51 as the latent glycosyl donor. Under our optimised reaction conditions, where Hünig’s base was employed as an acid scavenger to prevent acetal migration in the threonate acceptor, the desired disaccharide 157 was obtained in a moderate 45% yield, again as a single α-anomer (Scheme 4.6). We were not surprised that the isolated yield was lower than for the α-selective glycosylation reaction employed in the synthesis of α-GalCer (64%, see Chapter 2.3) as the sterically hindered secondary alcohol of acceptor 154 is less reactive than the primary alcohol of phytosphingosine 78.
Moreover, separating the product 157 from the unreacted starting materials proved to be difficult, and prevented us from using an excess of the acceptor, which could also account for the observed lower yield.

LiAlH$_4$ effected reduction of the ester in 157 to provide alcohol 158 in 95% yield, which was converted into triflate 159 by treatment with Tf$_2$O in the presence of the acid scavenger, 2,6-di-tert-butyl-4-methylpyridine (DTBMP). As expected, triflate 159 proved to be more stable than its analogue containing PMB protecting groups; however it was still used directly and without purification in the etherification step with the sodium alkoxide of alcohol 78 (Scheme 4.7).$^{124}$
Using this pairing, the desired ether 160 was isolated in 72% over the two steps from alcohol 78. Further processing to the target was straightforward: Staudinger reduction\(^{105,124}\) of the azide in 160 provided the corresponding amine 161 in 86% yield, which was acylated with hexacosanoyl chloride 47, prepared as before, from the reaction of hexacosanoic acid with oxalyl chloride,\(^{120}\) to provide amide 162 in 85% yield. A two-step deprotection involving acetal hydrolysis with aqueous trifluoroacetic acid, followed by hydrogenolysis of the remaining benzyl ethers, delivered our target Galp-\(\alpha\)-(1→2)-ThrCer 149 in 75% yield over the two steps (Scheme 4.7).
Our target compound Galp-α-(1→2)-ThrCer 149, was submitted for biological testing. This work was carried out in the group of Prof. Vincenzo Cerundolo by Dr Mariolina Salio at the Weatherall Institute of Molecular Medicine in Oxford, UK.
4.4. **Biological evaluation of Galp-α-(1→2)-ThrCer**

Human monocyte-derived dendritic cells (DC) (50000/well) were pulsed with the indicated concentrations of lipids and then a human iNKT cell line was added (30000 cell/well). Supernatants were harvested after 36 hours and assayed for human IFNγ by ELISA.

![Figure 4.4](image_url)

**Figure 4.4.** Activation of human iNKT cells using Galp-α-(1→2)-Galp-α-1-Cer, Galp-α-(1→2)-ThrCer, ThrCer, α-GalCer. C1R-hCD1d cells were pulsed with various concentrations of Galp-α-(1→2)-Galp-α-1-Cer 148, Galp-α-(1→2)-ThrCer 149, ThrCer 24, α-GalCer 8 as indicated and used to stimulate human iNKT cells in vitro. The supernatants were tested for IFNγ.
These results demonstrate that Galp-α-(1→2)-ThrCer is presented by DC, albeit weakly, and recognised by iNKT cells.

In another experiment, DC were differentiated from bone marrow precursors of wild type and CD1d/NKT deficient mice for 4 days in 1 ng/ml GM-CSF. DC (33000/well) were pulsed for 16 hours with 20 ng/ml α-GalCer, 200 ng/ml Galp-α-(1→2)-Galp-α-1-Cer, 200 ng/ml ThrCer, 1 µg/ml Galp-α-(1→2)-ThrCer in the presence or absence of 1mM 1-deoxygalactonojirimycin (DGJ) to inhibit α-galactosidase A activity. The cells were extensively washed and incubated with the iNKT hybridoma DN32 (30000 cell/well). Supernatants were harvested after 36 hours and IL-2 secretion measured by ELISA.

![Figure 4.5](image)

**Figure 4.5.** Activation of murine iNKT cells using Galp-α-(1→2)-Galp-α-1-Cer, Galp-α-(1→2)-ThrCer, ThrCer, α-GalCer. C1R-mCD1d cells were pulsed with various concentrations of Galp-α-(1→2)-Galp-α-1-Cer 148, Galp-α-(1→2)-ThrCer 149, ThrCer 24, α-GalCer 8 as indicated and used to stimulate the DN32 hybridoma *in vitro*. The supernatants were tested for the presence of IL-2.
These results demonstrate that Galp-α-(1→2)-ThrCer is processed in an α-galactosidase A-dependent manner, as presentation is abrogated by treatment with DGJ mice (which are deficient in α-galactosidase A).
4.5. Conclusions and future work

We have successfully completed a stereoselective synthesis of the target molecule Galp-α-(1→2)-ThrCer 149. In the key step in our synthesis, namely the α-glycosylation, we found that incorporation of an acid scavenger was important to prevent acetal migration in the acceptor 154.

Preliminary immunological assays have shown that this compound is active and requires intracellular processing by an α-galactosidase enzyme, which requires as cofactor lipid transfer proteins belonging to the saposin family.
Chapter 5

Experimental Section
5. Experimental Section

5.1 General Experimental

Optical rotations were measured using an Optical Activity PolAAr2001 automatic polarimeter. Melting points were determined using open capillaries on a Gallenkamp MPD350 melting point apparatus and are uncorrected. Infrared spectra were recorded either neat as thin films between NaCl discs, on a Perkin Elmer 1600 FTIR spectrometer, or neat on a Perkin Elmer Spectrum 100 fitted with a universal ATR accessory. The intensity of each band is described as s (strong), m (medium) or w (weak), and with the prefix v (very) and suffix br (broad) where appropriate. $^1$H-NMR spectra were recorded at 500 MHz, 400 MHz, or 300 MHz, using Bruker DRX 500, Bruker AMX 400, Bruker AV 400, Bruker AV 300 and Bruker AC 300 spectrometers. $^{13}$C-NMR spectra were recorded at 125 MHz, 100 MHz, or 75 MHz, respectively, using Bruker DRX 500, Bruker AMX 400, Bruker AV 400, Bruker AV 300 and Bruker AC 300 spectrometers. Chemical shifts are reported as values (ppm) referenced to the following solvent signals: CHCl$_3$, $\delta_1$ 7.26; CDCl$_3$, $\delta_2$ 77.0; CH$_3$OH, $\delta_3$ 3.34; CD$_3$OD, $\delta_4$ 49.9. The term "stack" is used to describe a region where resonances arising from non-equivalent nuclei are coincident, and multiplet, m, to describe a region where resonances arising from a single nucleus (or equivalent nuclei) are coincident but coupling constants cannot be readily assigned. Mass spectra were recorded on a Micromass LCT spectrometer utilising electrospray ionisation (and a MeOH mobile phase), and are reported as ($m/z$ (%)). HRMS were recorded on a Micromass LCT spectrometer using a lock mass incorporated into the mobile phase.
All reagents were obtained from commercial sources and used without further purification unless stated otherwise. Anhydrous solvents were purchased from Sigma-Aldrich UK, stored over 4 Å molecular sieves and under an Ar atmosphere. All solutions are aqueous and saturated unless stated otherwise. Reactions were monitored by TLC using pre-coated aluminium-backed ICN silica plates (60A F$_{254}$) and visualised by UV detection (at 254 nm) and staining with 5% phosphomolybdic acid in EtOH (MPA spray). Column chromatography was performed on Merck silica gel (particle size 40-63 m mesh) or Fluka 60 (40-60 m mesh) silica gel.
(2S,3S,4R)-2-Azido-octadecane-1,3,4-triol (58)

Amine 54 (5.00 g, 15.6 mmol), CuSO₄·5H₂O (40 mg, 0.16 mmol), and K₂CO₃ (3.23 g, 23.4 mmol) were dissolved in H₂O (50 mL) and the mixture stirred for 10 min at r.t. A solution of imidazole-1-sulfonyl azide hydrochloride⁹⁰ (6.00 g, 28.1 mmol, CAUTION: potential explosion risk during purification of this reagent) in CH₂Cl₂ (50 mL) was then added with vigorous stirring. CH₃OH (150 mL) was then added over 30 min through a dropping funnel. After 20 min, the resulting slurry became a transparent pale blue solution, which was stirred vigorously at r.t. for a further 18 h. The mixture was then diluted with CHCl₃ (400 mL). The phases were separated and the organic phase was extracted with H₂O (2 × 350 mL). The combined organic phases were dried with Na₂SO₄ and the volatiles were removed under reduced pressure. The crude azide product was re-dissolved in a CHCl₃ / CH₂Cl₂ mixture (5:1, 16 mL) and filtered through a silica plug, washing with EtOAc / acetone (9:1) until complete elution of the product. Removal of the solvent under reduced pressure afforded azide 58 as a white solid (4.95 g, 91%): \( R_f = 0.7 \) (eluent: EtOAc); mp 92 – 94 °C, (lit. mp 90 °C);¹⁷⁶ \([\alpha]_D^{22} + 9.6 \) (c = 1.0, CHCl₃) (lit. \([\alpha]_D^{25} + 10 \) (c = 1, CHCl₃);¹⁷⁷ \( v_{max}(\text{film}) / \text{cm}^{-1} \) 3261 br (OH), 2916s, 2847s, 2096s (N₃), 1590w, 1461m, 1248m, 1099w, 1058s, 1008m, 923w, 857m, 723s; \( \delta_H(300 \text{ MHz, CDCl}_3/\text{CD}_3\text{OD}, 2:1) \) 0.66 (t, J 6.5, 3H, CH₃CH₂), 0.95-1.22 (stack, 24H, CH₂), 1.37-1.42 (stack, 2H, CH(OH)CH₂CH₂), 3.34-3.39 (stack, 3H), 3.58 (dd, J 6.1, 5.7,
Chapter 5  Experimental Section

1H), 3.72 (dd, J 3.9, 3.7, 1H); δ(C(75 MHz, CDCl₃/CD₃OD, 2:1) 14.4 (CH₃, CH₂CH₃), 23.7 (CH₂), 26.7 (CH₂), 30.5 (CH₂), 30.7 (CH₂), 30.8 (CH₂), 33.1 (CH₂), 33.9 (CH₂), 62.5 (CH₂, CH₂OH), 66.6 (CH, CHN₃), 72.9 (CH, CHOH), 76.0 (CH, CHOH), some overlap in the methylene resonances; MS (TOF ES+) m/z 366.2 ([M + Na]⁺, 100%); HRMS (TOF ES+) calcd for C₁₈H₃₇N₃O₃Na [M + Na]⁺ 366.2733, found 366.2728.

Data were in agreement with those reported in the literature.¹²⁴,¹⁷⁶

(2S,3S,4R)-2-Azido-1-O-trityl-octadecane-1,3,4-triol (59)

TrCl (4.43 g, 15.9 mmol) was added to a solution of alcohol 58 (5.20 g, 15.5 mmol) in pyridine (30 mL) at r.t. The reaction mixture was stirred for 21 h at r.t. and then the solvent was removed under reduced pressure to provide the crude product. Purification by flash column chromatography (15% EtOAc in hexane) afforded trityl ether 59 as a pale yellow oil (5.45 g, 60%): Rᵣ = 0.3 (15% EtOAc in hexane); [α]_D¹⁸

177
Chapter 5  Experimental Section

\[ \alpha D_20^20 = +8.9 \ (c = 1, \ CHCl_3), ^{96} \ \nu_{max}(film)/cm^{-1} 3291 \ (br \ OH), 2915s, 2848s, 2096m (N_3), 1463m, 1447m, 1249m, 1216w, 1152w, 1099w, 1071m, 1059w, 1010w, 929w, 909w, 757s, 723m, 700s, 667w; \ \alpha H(300 MHz, CDCI_3) 0.88 \ (t, J 7.0, 3H, CH_3CH_2), 1.18-1.34 \ (stack, 23H), 1.36-1.57 \ (stack, 3H), 1.81 \ (d, J 5.7, 1H, OH), 2.35 \ (d, J 5.0, 1H, OH), 3.38-3.45 \ (m, 1H), 3.49-3.59 \ (stack, 2H), 3.60-3.69 \ (stack, 2H), 7.20-7.37 \ (stack, 9H, Ph), 7.40-7.51 \ (stack, 6H, Ph); \ \alpha C(75 MHz, CDCl_3) 14.1 \ (CH_3, CH_3CH_2), 22.7 \ (CH_2), 25.6 \ (CH_2), [29.4, 29.6, 29.7 \ (CH_2, resonance overlap)], 31.7 \ (CH_2), 31.9 \ (CH_2), 62.3 \ (CH, CHN_3), 63.5 \ (CH_2, CH_2OTr), 72.2 \ (CH, CHOH), 74.2 \ (CH, CHOH), 87.8 \ (C, CPh_3), [127.3, 128.0, 128.5, (CH, Ph)], 137.4 \ (C, ipso Ph); MS (TOF ES+) m/z 608.5 ([M + Na]^+, 100%); HRMS (TOF ES+) calcd for C_{37}H_{51}N_3O_3Na [M + Na]^+ 608.3828, found 608.3836.

Data were in agreement with those reported in the literature.\(^{96}\)
NaH (1.095 g of a 60 wt % suspension in mineral oil, 18.24 mmol) was added to a solution of alcohol 59 (4.45 g, 7.60 mmol) in DMF (38 mL) at r.t. The reaction mixture was stirred for 30 min and then cooled to 0 °C. BnBr (1.99 mL, 16.7 mmol) was added dropwise over 3 min and the reaction mixture was stirred for 15 h at r.t. H₂O (40 mL) was then added and the mixture was extracted with Et₂O (2 × 20 mL) and then dried over Na₂SO₄. The drying agent was removed by filtration and the filtrate concentrated under reduced pressure. Purification of the residue by flash column chromatography (5% EtOAc in hexane) afforded dibenzyl ether 60 as a yellow oil (4.66 g, 80%): \( R_f = 0.3 \) (5% EtOAc in hexane); \( \alpha_\text{D}^{18} = +4.9 \) (c = 1, CHCl₃) (lit. \( \alpha_\text{D}^{20} = +6.4 \) (c = 1, CHCl₃)); \( \nu_{\text{max}}(\text{film})/\text{cm}^{-1} \) 2923s, 2853s, 1491m, 1449m, 1315w, 1265w, 1209w, 1065s, 899w, 744m, 696s, 632m; \( \delta_\text{H} \) (300 MHz, CDCl₃) 0.88 (t, \( J = 6.9 \) Hz, 3H, CH₃CH₂), 1.14-1.34 (stack, 23H), 1.35-1.62 (stack, 3H), 3.32-3.41 (stack, 2H), 3.43-3.57 (stack, 3H), 4.37-4.60 (stack, 4H, CH₂Ph), 7.04-7.11 (stack, 3H, Ph), 7.14-7.32 (stack, 15H, Ph), 7.35-7.47 (stack, 7H, Ph); \( \delta_\text{C} \) (75 MHz, CDCl₃) 14.1 (CH₃, CH₃CH₂), 22.7 (CH₂), 25.3 (CH₂), [29.4, 29.7 (CH₂, resonance overlap)], 31.9 (CH₂), 63.2 (CH, CHN₃), 64.3 (CH₂,}
CH$_2$OTr), 72.0 (CH$_2$, CH$_2$Ph), 73.5 (CH$_2$, CH$_2$Ph), 79.2 (CH, CHO$_2$Bn), 79.4 (CH, CHO$_2$Bn), 87.2 (C, CPh$_3$), [127.0, 127.5, 127.6, 127.8, 128.2, 128.3, 128.7 (CH, Ph, resonance overlap)], 137.9 (C, ipso Ph), 138.3 (C, ipso Ph), 143.7 (C, ipso Ph); MS (TOF ES+) m/z 788.5 ([M + Na]$^+$, 100%); HRMS (TOF ES+) calcd for C$_{51}$H$_{63}$N$_3$O$_3$Na [M + Na]$^+$ 788.4767, found 788.4774.

Data were in agreement with those reported in the literature.$^{96}$

(2S,3S,4R)-2-Azido-3,4-di-O-benzyl-octadecane-1,3,4-triol (52)

A solution of trityl ether 60 (4.61 g, 6.02 mmol) and p-TsOH (170 mg, 0.90 mmol) in CH$_2$Cl$_2$ / MeOH (60 mL, 3:1) was stirred for 16 h at r.t. The reaction mixture was then washed with NaHCO$_3$ solution (20 mL). The phases were separated and the aqueous phase was extracted with EtOAc (2 × 20 mL) and dried over Na$_2$SO$_4$. The drying agent was removed by filtration and the filtrate concentrated under reduced pressure. Purification of the residue by flash column chromatography
Chapter 5  Experimental Section

(10% EtOAc in hexane) afforded alcohol 52 as a colourless oil (84%, 2.65 g): \( R_f = 0.2 \) (10% EtOAc in hexane); \([\alpha]_D^{17} = + 5.5 \) (c 1, CHCl\(_3\)) (lit. \([\alpha]_D^{20} = + 5.7 \) (c = 1, CHCl\(_3\))\));\(^{96}\) \( \nu_{max}(\text{film})/\text{cm}^{-1} \) 3428m (br, OH), 3088w, 3063m, 3030m, 2603, 2532w, 2496w, 2366w, 2335w, 2099s (N\(_3\)), 1944w, 1887w, 1810w, 1741w, 1682w, 1644w, 1606w, 1496m, 1454s, 1326m, 1264m, 1208m, 1071s, 1028s, 912w, 844w, 735s, 697s, 608m; \( \delta_H(300 \text{ MHz, CDCl}_3) \) 0.89 (t, \( J = 6.4 \), 3H, CH\(_3\)), 1.18-1.48 (stack, 24H, alkyl chain), 1.50-1.62 (m, 1H), 1.63-1.75 (m, 1H), 2.55 (app t, \( J = 6.6 \), 1H, OH), 3.61-3.74 (stack, 3H), 3.75-3.84 (m, 1H), 3.86-3.95 (m, 1H), 4.55-4.65 (stack, 2H, OCH\(_2\)Ph), 4.66-4.74 (stack, 2H, OCH\(_2\)Ph), 7.31-7.37 (stack, 10H, Ph); \( \delta_C(75 \text{ MHz, CDCl}_3) \) 14.1 (CH\(_3\), CH\(_2\)CH\(_3\)), [22.6, 25.4, 29.3, 29.6, 30.1, 31.9 (CH\(_2\), alkyl chain, resonance overlap)], 62.1 (CH\(_2\), CH\(_2\)OH), 63.1 (CH, CHN\(_3\)), 72.3 (CH\(_2\), CH\(_2\)Ph), 73.5 (CH\(_2\), CH\(_2\)Ph), 78.9 (CH, CHO\(_\text{Bn}\)), 80.1 (CH, CHO\(_\text{Bn}\)), [127.7, 127.9, 128.0, 128.3, 128.4 (CH, Ph, resonance overlap)], 137.4 (C, ipso Ph), 137.9 (C, ipso Ph); MS (TOF ES+) \( m/z \) 546.3 ([M + Na]*, 100%); HRMS (TOF ES+) calcd for C\(_{32}\)H\(_{49}\)N\(_3\)O\(_3\)Na [M + Na]* 546.3672, found 546.3657.

Data were in agreement with those reported in the literature.\(^{96}\)
(2S,3S,4R)-2-Azido-3,4-O-isopropylidene-octadecane-1,3,4-triol (78)

98% H$_2$SO$_4$ (200 µL) was added dropwise over 2 min to a solution of triol 58 (509 mg, 1.49 mmol) in dry acetone (10 mL) at 0 °C. After 30 min, the reaction mixture was carefully poured into saturated NaOH solution (1 mL), and then evaporated to dryness under reduced pressure. The residue was extracted with EtOAc (15 mL). The organic phase was washed with brine (3 mL), and then dried over Na$_2$SO$_4$. Concentration of the filtrate under reduced pressure and purification of the residue by flash column chromatography afforded acetal 78 as a white, low-melting point, amorphous solid (412 mg, 72%): $R_f = 0.3$ (25% EtOAc in hexane); $[\alpha]_D^{21} = +27.5$ (c = 1.0, CHCl$_3$) (lit. $[\alpha]_D^{22} = +23$ (c = 1.0, CHCl$_3$); $^1$H NMR (300 MHz, CDCl$_3$) 0.88 (t, $J = 6.0$, 3H, CH$_2$CH$_3$), 1.26-1.30 (stack, 23H), 1.34 (s, 3H, 1 × C(CH$_3$)$_2$), 1.43 (s, 3H, 1 × C(CH$_3$)$_2$), 1.50-1.63 (stack, 3H), 2.08 (app. t, $J = 6.0$, 1H, CH$_2$OH), 3.44-3.50 (m, 1H), 3.83-3.91 (m, 1H), 3.94-4.03 (stack, 2H), 4.15-4.21 (m, 1H); $^1$C NMR (75 MHz, CDCl$_3$) 14.1 (CH$_3$, CH$_2$CH$_3$), 25.5 (CH$_3$, 1 × C(CH$_3$)$_2$), 26.5 (CH$_2$), 28.0 (CH$_3$, 1 × C(CH$_3$)$_2$), 29.35 (CH$_2$), 29.41 (CH$_2$), 29.53 (CH$_2$), 29.58 (CH$_2$), 29.59 (CH$_2$), 29.65 (CH$_2$), 29.68 (CH$_2$), 29.69 (CH$_2$), 31.9 (CH$_2$), 61.2 (CH, CHN$_3$), 63.9 (CH$_2$, CH$_2$OH), 76.6 (CH, CHO), 77.7 (CH, CHO), 108 (C, C(CH$_3$)$_2$), some overlap in the methylene resonances; MS (TOF...
ES+) $m/z$ 406.2 ([M + Na]$^+$, 100%); HRMS (TOF ES+) calcd for C$_{21}$H$_{41}$N$_3$O$_3$Na [M + Na]$^+$ 406.3046, found 406.3033.

Data were in agreement with those reported in the literature.$^{124}$

(2S,3R,4R)-2-Azido-3,4-di-O-benzyl-1-O-(2',3',4',6'-tetra-O-benzyl-α-D-galactopyranosyl)octadecane-1,3,4-triol (50)

Ph$_3$P (1.46 g, 5.55 mmol) and CBr$_4$ (1.84 g, 5.55 mmol) were added sequentially to a solution of 2,3,4,6-tetra-O-benzyl-D-galactose 51 (1.00 g, 1.85 mmol) in CH$_2$Cl$_2$ (10 mL) at r.t. The reaction mixture was stirred for 3 h. In separate flasks, a solution of tetramethyl urea (TMU) (1.2 mL) and Bu$_4$NBr (1.79 g, 5.55 mmol) in CH$_2$Cl$_2$ (5 mL), and a solution of azide 52 (1.46 g, 2.78 mmol) in CH$_2$Cl$_2$ (5 mL), were stirred over activated 3 Å MS for 30 min, after which time, these solutions were added dropwise (15 min) via syringe sequentially (TMU/Bu$_4$NBr solution first) to the solution containing the glycosyl donor. The reaction mixture was stirred at r.t. for 3 d until the donor was no longer being consumed (as judged by TLC).
reaction mixture was then filtered through a silica plug, washed with CH$_2$Cl$_2$ (1.2 L) and concentrated under reduced pressure to provide the crude product. Purification of the residue by flash column chromatography (8% EtOAc in hexane) afforded glycoside 50 as a colourless oil (1.21 g, 62%, $\alpha$-anomer only): $R_f = 0.3$ (8% EtOAc in hexane); [$\alpha$]$_D^{18} + 22 \ (c\ 1.4, \ CH_2Cl_2)\ (lit\ [\alpha]_D^{20} = + 26\ (c\ 1.4, \ CH_2Cl_2))$; $\gamma_{\text{max}}$(film)/cm$^{-1}$ 2988s, 2972s, 2923s, 2854m, 2097m (N$_3$), 1721m, 1704m, 1602w, 1585w, 1545m, 1405m, 1394m, 1380m, 1314m, 1262s, 1155m, 1066s, 1056s, 1027s, 904m, 805m, 734s, 711s; $\delta_H$ (500 MHz, CDCl$_3$) 0.90 (3H, t, $J_6.9$, CH$_2$C$_6$H$_5$), 1.20-1.35 (23H, stack, alkyl chain methylenes), 1.35-1.45 (m, 1H, alkyl chain CH$_3$H$_b$), 1.50-1.58 (m, 1H, C(5)H$_a$H$_b$), 1.63-1.71 (m, 1H, C(5)H$_a$H$_b$), 3.47-3.54 (stack, 2H, C(6')H$_a$H$_b$), 3.60-3.63 (m, 1H, H-4), 3.71-3.76 (stack, 2H, C(1)H$_a$H$_b$, H-2, H-3), 3.94-3.98 (stack, 2H, H-4', H-5'), 3.98-4.04 (stack, 2H, H-3', C(1)H$_a$H$_b$), 4.08 (dd, $J_{10.0, 3.5}$, 1H, H-2'), 4.37 (A of AB, $J_{11.8, 1H}$, C(6')OCH$_3$H$_a$H$_b$Ph), 4.45 (B of AB, $J_{11.8, 1H}$, C(6')OCH$_3$H$_a$Ph), 4.48 (A of AB, $J_{11.6, 1H}$, C(4)OCH$_3$H$_a$H$_b$Ph), 4.57-4.59 (stack, 2H, C(4)OCH$_3$H$_a$Ph, C(4')OCH$_3$H$_a$H$_b$Ph), 4.63 (A of AB, $J_{11.3, 1H}$, C(3)OCH$_3$H$_a$H$_b$Ph), 4.67 (B of AB, $J_{11.3, 1H}$, C(3)OCH$_3$H$_a$Ph), 4.69 (A of AB, $J_{12.0, 1H}$, C(2')OCH$_3$H$_a$Ph), 4.74 (A of AB, $J_{11.5, 1H}$, C(3')OCH$_3$H$_a$H$_b$Ph), 4.81 (B of AB, $J_{12.0, 1H}$, C(2')OCH$_3$H$_a$Ph), 4.84 (B of AB, $J_{11.5, 1H}$, C(3')OCH$_3$H$_a$Ph), 4.91 (d, $J_{3.5, 1H}$, H-1'), 4.95 (d, $J_{11.5, 1H}$, C(4')OCH$_3$H$_a$Ph), 7.22-7.40 (stack, 30H, 6 × Ph); $\delta_C$(125 MHz, CDCl$_3$) 14.1 (CH$_3$, CH$_2$CH$_3$), [22.6, 25.3, 29.3, 29.6, 29.9, 31.9 (CH$_2$, alkyl chain, resonance overlap)], 61.9 (CH, C-2), 68.4 (CH$_2$, C-1), 68.9 (CH$_2$, C-6'), 69.6 (CH, C-5'), 71.9 (CH$_2$, C(4)OCH$_2$Ph), 73.0 (CH$_2$, C(3')OCH$_2$Ph), 73.1 (CH$_2$, C(2')OCH$_2$Ph), 73.3 (CH$_2$, C(6')OCH$_2$Ph), 73.6 (CH$_2$, C(3)OCH$_2$Ph), 74.7 (CH$_2$, C(4')OCH$_2$Ph), 75.0
(CH, C(4')), 76.3 (CH, C(2')), 78.7 (CH, C(3')), 78.8 (CH, C(3)), 79.2 (CH, C(4)),
98.6 (CH, C(1')), [127.3, 127.4, 127.56, 127.60, 127.7, 127.8, 128.1, 128.2 (CH, Ph, resonance overlap)], 137.9 (C, ipso Ph), 138.0 (C, ipso Ph), 138.3 (C, ipso Ph), 138.58 (C, ipso Ph), 138.63 (C, ipso Ph), 138.7 (C, ipso Ph); MS (TOF ES+) m/z 1068.8 ([M + Na]^+), 100%; HRMS (TOF ES+) 1068.6063 [M + Na]^+, C_{66}H_{83}N_{3}O_{8}Na requires 1068.6078; and then unreacted azide 52 was also recovered (445 mg, 45%).

Data were in agreement with those reported in the literature for 50 prepared by a different route.\textsuperscript{178}

\begin{center}
(2S,3S,4R)-2-Amino-3,4-di-O-benzyl-1-O-(2',3',4',6'-tetra-O-benzyl-\textalpha-D-galactopyranosyl)octadecane-1,3,4-triol (46)
\end{center}

\begin{center}
\includegraphics[width=0.2\textwidth]{46.png}
\end{center}

\(\text{PMe}_3 (455 \mu\text{L of a 1.0 M soln in THF, 0.46 mmol})\) was added dropwise over 5 min to a solution of azide 50 (433 mg, 0.414 mmol) in THF (3.5 mL). The reaction mixture was stirred at r.t. for 4 h after which time, \(\text{H}_2\text{O} (3 \text{ mL})\) was added. The
reaction mixture was stirred for 1 h and then concentrated under reduced pressure. The residual H$_2$O was removed by co-evaporation with toluene (3 × 3 mL) to provide the crude product. Purification of the residue by flash column chromatography (35% EtOAc in hexane) afforded amine 46 as a white solid (300 mg, 72%), which was used directly without further purification. Selected data: $R_f = 0.3$ (35% EtOAc in hexane); MS (TOF ES+) $m/z$ 1020.5 ([M + H]$^+$, 100%); HRMS (TOF ES+) calcd for C$_{66}$H$_{86}$NO$_8$ [M + H]$^+$ 1020.6353, found 1020.6357.

\[(2S,3S,4R)-3,4-Di-O-benzyl-1-O-(2',3',4',6'-tetra-O-benzyl-\alpha-D-galacto-pyranosyl)-2-(hexacosanoylamino)octadecane-1,3,4-triol\] (62)

A screw-capped glass tube containing a solution of hexacosanoic acid 61 (240 mg, 0.580 mmol) in (COCl)$_2$ (2.0 mL, 23 mmol) was closed tightly and heated at 70 °C for 2 h. After 2 h, the volatiles were removed under a stream of N$_2$ and the residual solvent removed on the vacuum line (1 h) to provide hexacosanoyl chloride 47 as a pale yellow oil, which was used directly in the next step without further purification (265 mg, quant.): a solution of freshly prepared hexacosanoyl
chloride (265 mg, 0.58 mmol) in CH₂Cl₂ (1.5 mL) was added dropwise over 2 min to an ice-cooled solution of amine 46 (500 mg, 0.49 mmol) and NEt₃ (136 µL, 0.98 mmol) in CH₂Cl₂ (3.5 mL) at 0 ºC. The reaction mixture was stirred at r.t. for 8 h and then diluted with CH₂Cl₂ (20 mL), washed sequentially with NaHCO₃ solution (20 mL), brine (4 mL) and then dried over Na₂SO₄. The drying agent was removed by filtration and the filtrate concentrated under reduced pressure. Purification of the residue by flash column chromatography (12% EtOAc in hexane) afforded amide 62 as a white solid (582 mg, 85%): $R_f = 0.3$ (15% EtOAc in hexane); $[\alpha]_D^{20} = + 31.2$ (c 1, CHCl₃) (lit. $[\alpha]_D^{24} = + 18.8$ (c 0.9, CHCl₃);¹⁷⁹ mp 75 – 76 ºC (lit. mp 74 – 75 ºC);¹⁷⁹ $\nu_{\text{max}}$(film)/cm⁻¹ 2918s, 2850s, 1647m (C=O), 1531w, 1496w 1469m, 1454m, 1349w, 1206w, 1115s, 1058s, 952m, 776w, 735s, 695s; $\delta_H$(300 MHz, CDCl₃) 0.88 (6H, t, $J = 6.9$, 2 × CH₂CH₃), 1.16-1.34 (69H, stack, alkyl chain methylenes), 1.37-1.57 (2H, stack), 1.58-1.72 (1H, m), 1.85-2.00 (2H, stack), 3.36-3.43 (1H, m), 3.44-3.53 (2H, stack), 3.69-3.76 (1H, m), 3.83-3.96 (4H, stack), 3.99-4.08 (2H, stack), 4.09-4.20 (1H, m), 4.32-4.47 (2H, stack), 4.48-4.67 (4H, stack), 4.70-4.86 (6H, stack), 4.92 (1H, d, $J = 11.7$), 6.12 (1H, d, $J = 8.7$, NH), 7.20-7.37 (30H, stack); $\delta_C$(75 MHz, CDCl₃) 14.1 (CH₃), 14.7 (CH₃), [22.7, 25.7, 26.1, 29.3, 29.4, 29.7, 31.9, 36.7 (CH₂, alkyl chain, resonance overlap)], 50.3 (CH), 69.6 (CH₂), 69.96 (CH₂), 70.05 (CH), 71.7 (CH₂), 71.8 (CH₂), 72.9 (CH₂), 73.4 (CH₂), 73.6 (CH₂), 73.7 (CH), 74.8 (CH₂), 74.9 (CH), 78.6 (CH), 78.9 (CH), 80.1 (CH), 99.6 (CH, C-1'), [127.4, 127.6, 127.8, 128.2, 128.3 (CH, Ph, resonance overlap)], [137.5, 138.4, 138.5, 138.6 (C, ipso Ph, resonance overlap)], 172.8 (C, C=O); MS (TOF ES⁺) m/z 1421.6 ([M + Na]⁺, 100%).
Data were in agreement with those reported in the literature for 62 prepared by a different route.\textsuperscript{179}

**General procedure for catalytic hydrogenolysis:** Pd(OH)$_2$/C or Pd/C (0.05 eq per benzyl group) was added to a solution of the benzylated compound in THF (0.01 M). H$_2$ gas was bubbled through the stirred suspension. The progress of the reaction was monitored by TLC. On completion, the reaction mixture was filtered through a plug of Celite, washed with THF and then CHCl$_3$/MeOH (90/10, v/v), and concentrated under reduced pressure to provide the crude product, which was purified by flash column chromatography.
(2S,3S,4R)-1-O-(α-D-Galactopyranosyl)-2-(hexacosanoylamino)octadecane-1,3,4-triol (α-GalCer, 8)

Experimental details for the conversion of 62 to 8

Amide 8 (α-GalCer) was prepared from perbenzylated amide 62 (180 mg, 0.129 mmol) and Pd/C (85 mg, 10% wet) in THF (10 mL) according to the general procedure. After 22 h, work-up and purification by flash column chromatography (8% MeOH in CHCl₃) afforded amide 8 as an amorphous white solid (75 mg, 68%).

Experimental details for the conversion of 81 to 8

TFA (150 µL) was added dropwise over 1 min to a solution of acetal 81 (120 mg, 0.095 mmol) in CH₂Cl₂ / H₂O (10:1, 0.9 mL). After stirring for 2 h at r.t., the reaction mixture was concentrated under reduced pressure and the residual TFA was removed by co-evaporation with Et₂O (3 × 3 mL) to provide the crude acetal hydrolysis product as a white solid (116 mg, quant.), which was then treated with Pd(OH)₂/C (30 mg, 10% wet) in THF (10 mL) according to the general hydrogenolysis procedure. After 8 h, work-up and purification by flash column
chromatography (8% MeOH in CHCl₃) afforded amide 8 as a white solid (61 mg, 75%): $R_f = 0.3$ (10% MeOH in CHCl₃); $[\alpha]_D^{20} = +15.2$ (c 1, 2:1 CHCl₃/CH₃OH) (lit. $[\alpha]_D^{23} = +43.6$ (c 1, pyridine));¹⁴³ mp 188 – 189 °C (lit. mp 189 – 190 °C);¹⁸⁰

$\nu_{max}(\text{film})$ / cm⁻¹ 3313br (OH), 2918s, 2850s, 1642m (C=O), 1548w, 1468m, 1261m, 1027s, 800s, 721m, 600w; $\delta_t$(400 MHz, 2:1 CDCl₃/CD₃OD) 0.83 (6H, t, $J$ 6.7, 2 × CH₂CH₃), 1.16-1.40 (68H, stack, alkyl chain methylenes), 1.45-1.63 (4H, stack), 2.16 (2H, app. t, $J$ 7.8), 3.46-3.57 (2H, stack), 3.60-3.79 (6H, stack), 3.80-3.87 (1H, m), 3.90 (1H, d, $J$ 2.5), 4.11-4.18 (1H, m), 4.86 (1H, d, $J$ 3.7), OH and NH resonances not observed; $\delta_c$(100 MHz, 2:1 CDCl₃/CD₃OD) 14.3 (CH₃, 2 × CH₂CH₃), 23.1 (CH₂), 26.29 (CH₂), 26.32 (CH₂), [29.8, 29.9, 30.0, 30.09, 30.13, 30.2 (CH₂, alkyl chain, resonance overlap)], 32.4 (CH₂), 32.9 (CH₂), 36.9 (CH₂), 50.9 (CH), 62.3 (CH₂), 67.8 (CH₂), 69.4 (CH), 70.3 (CH), 70.8 (CH), 71.2 (CH), 72.5 (CH), 75.1 (CH), 100.2 (CH), 175.1 (C, C=O); MS (TOF ES+) m/z 880.7 ([M + Na]⁺, 100%); HRMS (TOF ES+) calcd for C₅₀H₉₉NO₉Na [M + Na]⁺ 880.7218, found 880.7198.

Data were in agreement with those reported in the literature.¹⁸⁰
(2S,3S,4R)-3,4-Di-O-acetyl-1-O-(2',3',4',6'-tetra-O-acetyl-a-D-galactopyranosyl)-2- (hexacosanoylamino)octadecane-1,3,4-triol (72)

Ac₂O (300 µL, 3.2 mmol) was added dropwise over 1 min to a solution of α-GalCer 8 (90 mg, 0.11 mmol) in pyridine (2 mL) and the reaction mixture was stirred at r.t. for 10 h, after which time, the volatiles were removed under reduced pressure. The residue was diluted with CH₂Cl₂ (10 mL), washed sequentially with H₂O (5 mL), NaHCO₃ solution (10 mL), brine (3 mL) and then dried over Na₂SO₄. The drying agent was removed by filtration and the filtrate was concentrated under reduced pressure. The crude product was purified by flash column chromatography (25% EtOAc in hexane) to afford hexa-acetate 72 as a white solid (110 mg, 94%): 

\[ R_f = 0.3 \text{ (20\% EtOAc in hexane) } \]
\[ [\alpha]_{20}^D = +8.4 \text{ (c 0.5, CHCl}_3) \]
\[ \text{mp 43 – 44 °C; } \]
\[ \nu_{\text{max}}(\text{film}) / \text{cm}^{-1} \]
\[ 1745s \text{ (C=O), 1683w (C=O); } \]
\[ \delta_{\text{H}}(75 \text{ MHz, CDCl}_3) 0.87 \text{ (t, J 6.7, 6H, 2 } \times \text{ CH}_2\text{CH}_3), \]
\[ 1.12-1.40 \text{ (stack, 68H, alkyl chain methylenes), } \]
\[ 1.56-1.72 \text{ (stack, 4H), } \]
\[ 1.98 \text{ (s, 3H, C(O)CH}_3), \]
\[ 1.99 \text{ (s, 3H, C(O)CH}_3), \]
\[ 2.04 \text{ (s, 3H, C(O)CH}_3), \]
\[ 2.06 \text{ (s, 3H, C(O)CH}_3), \]
\[ 2.09 \text{ (s, 3H, C(O)CH}_3), \]
\[ 2.13 \text{ (s, 3H, C(O)CH}_3), \]
\[ 2.27 \text{ (t, J 7.4, 2H), } \]
\[ 3.39 \text{ (dd, J 10.6, 2.2, 1H), } \]
\[ 3.64 \text{ (dd, J 10.7, 2.6, 1H), } \]
\[ 3.97-4.14 \text{ (stack, 4H), } \]
\[ 4.31-4.41 \text{ (m, 1H), } \]
\[ 4.90 \text{ (d, J 3.7, 1H), } \]
\[ 5.13 \text{ (dd, J 10.8, 3.7, 1H), } \]
\[ 5.25-5.36 \text{ (stack, 2H), } \]
\[ 5.44 \text{ (d, J 3.1, 1H), } \]
\[ 6.39 \text{ (d, J 9.7, 1H); } \]
\[ \delta_{\text{C}}(100 \text{ MHz, CDCl}_3) 14.1 \text{ (CH}_3, 2 \times \text{ CH}_2\text{CH}_3), \]
[20.60, 20.66, 20.72, (CH$_3$, C(O)CH$_3$), resonance overlap], 20.1 (CH$_3$, C(O)CH$_3$),
22.7 (CH$_2$), 25.6 (CH$_2$), 25.7 (CH$_2$), [29.29, 29.35, 29.40, 29.7 (CH$_2$, alkyl chain
methylenes, resonance overlap)], 31.9 (CH$_2$), 36.7 (CH$_2$), 47.8 (CH), 61.8 (CH$_2$),
66.7 (CH), 67.2 (CH$_2$), 67.5 (CH), 67.9 (CH), 70.5 (CH), 73.4 (CH), 97.1 (CH),
169.7 (C), 170.1 (C), 170.4 (C), 170.7 (C), 171.1 (C), 172.9 (C), some resonance
overlap in C=O region; MS (TOF ES+) m/z 1132.8 ([M + Na]$^+$, 100%); HRMS (TOF
ES+) calcd for C$_{62}$H$_{111}$NO$_{15}$Na [M + Na]$^+$ 1132.7851, found 1132.7860.

(2S,3S,4R)-3,4-Di-O-acetyl-1-O-(2',3',4',6'-tetra-O-acetyl-α-D-galactopyranosyl)-2-(hexacosanethioylamino)octadecane-1,3,4-triol (73)

Lawesson’s reagent (60 mg, 0.15 mmol) was added to a solution of amide 72 (110
mg, 0.10 mmol) in toluene (2 mL) at r.t. The reaction mixture was stirred at 80 ºC
for 4 h and then the solvent was removed under reduced pressure. The residue
was diluted with CH$_2$Cl$_2$ (10 mL), washed sequentially with H$_2$O (5 mL), NaHCO$_3$
solution (10 mL), brine (2 mL) and then dried over Na$_2$SO$_4$. The drying agent was
removed by filtration and the filtrate was concentrated under reduced pressure. Purification of the residue by flash column chromatography (20% EtOAc in hexane) provided thioamide 73 as a pale yellow solid (96 mg, 85%): $R_f = 0.3$ (15% EtOAc in hexane); $[\alpha]^{20}_D = +46.0$ (c 1, CHCl$_3$); mp 47 – 48 °C; $\nu_{\text{max}}$(film) / cm$^{-1}$ 1747s (C=O); $\delta_h$ (500 MHz, CDCl$_3$) 0.87 (t, $J = 6.8$, 6H, 2 × CH$_2$CH$_3$), 1.21-1.37 (stack, 68H, alkyl chain methylenes), 1.58-1.69 (stack, 2H, H-5), 1.71-1.82 (stack, 2H, H-3”), 1.99 (s, 3H, C(O)CH$_3$), 2.00 (s, 3H, C(O)CH$_3$), 2.03 (s, 3H, C(O)CH$_3$), 2.07 (s, 3H, C(O)CH$_3$), 2.08 (s, 3H, C(O)CH$_3$), 2.12 (s, 3H, C(O)CH$_3$), 2.66-2.78 (stack, 2H, H-2”), 3.40 (dd, $J = 10.7$, 1.9, 1H, C(1)H$_a$H$_b$), 3.65 (dd, $J = 10.7$, 2.7, 1H, C(1)H$_a$H$_b$), 3.97-4.07 (stack, 2H, C(6’)H$_a$H$_b$, H-5’), 4.08-4.14 (m, 1H, C(6’)H$_a$H$_b$), 4.75-4.81 (m, 1H, H-4), 4.93 (d, $J = 3.6$, 1H, H-1’), 5.08-5.16 (stack, 2H, (including 5.10 (dd, $J = 10.8$, 3.7, 1H, H-2’)), H-2’, H-2), 5.37 (dd, $J = 10.8$, 3.4, 1H, H-3’), 5.40-5.42 (m, 1H, H-4’), 5.49 (dd, $J = 10.0$, 2.4, 1H, H-3), 8.66 (d, $J = 9.2$, 1H, N-H); $\delta_c$(125 MHz, CDCl$_3$) 11.11 (CH$_3$, 2 × CH$_2$CH$_3$), [20.50, 20.54, 20.62, 20.66, 20.95 (CH$_3$, C(O)CH$_3$), resonance overlap], [22.6, 25.5, 27.5, 28.9, 29.2, 29.3, 29.4, 29.5, 29.7, 31.9 (CH$_2$, alkyl chain methylenes, resonance overlap]), 47.0 (CH$_2$, C-2”), 53.7 (CH, C-2), 61.7 (CH$_2$, C-6’), 65.3 (CH$_2$, C-1), 67.0 (CH, C-5’), 67.3 (CH, C-3’), 67.8 (CH, C-2’), 68.0 (CH, C-4’), 69.8 (CH, C-3), 73.6 (CH, C-4), 96.8 (CH, C-1’), 169.7 (C, C(3)C=O), 170.0 (C, C(4’)C=O), 170.3 (C, C(3’)C=O), 170.4 (C, C(6’)C=O), 170.5 (C, C(2’)C=O), 171.5 (C, C(4)C=O), 207.0 (C, C=S); MS (TOF ES+) $m/z$ 1148.9 ([M + Na]$^+$, 100%); HRMS (TOF ES+) calcd for C$_{62}$H$_{111}$NO$_{14}$SNa [M + Na]$^+$ 1148.7623, found 1148.7631.
(2S,3S,4R)-1-O-(α-D-Galactopyranosyl)-2-(hexacosanethioylamino)-octadecane-1,3,4-triol (35)

NaOMe (10 μL of a 0.5 M soln in MeOH, 0.005 mmol) was added to a solution of hexa-acetate 73 (25 mg, 0.022 mmol) in MeOH (2.5 mL). After stirring at r.t. for 2 h, the reaction mixture was neutralised by the addition of acidic ion-exchange resin (Dowex H CR-S, pre-washed with MeOH (100 mL) and CHCl₃ (50 mL)). The solution was filtered and the resin washed with MeOH (25 mL) and CHCl₃/MeOH (25 mL, 9:1). The filtrate was concentrated under reduced pressure. Purification of the residue by flash column chromatography (8% MeOH in CHCl₃) provided thioamide 35 as a pale yellow solid (17 mg, 90%): \( R_f = 0.2 \) (8% MeOH in CHCl₃); \([\alpha]^{20}_D = +43.2 \) (c 1, CHCl₃:CH₃OH, 2:1); mp 136 – 137 °C; \( \nu_{\text{max}} \) (film) / cm\(^{-1}\) 3368m br (O–H); \( \delta_{\text{H}} \) (400 MHz, CDCl₃:CD₃OD, 2:1) 0.84 (t, J 6.9, 6H, 2 × CH₂CH₃), 1.14-1.44 (stack, 69H, alkyl chain methylenes), 1.45-1.78 (stack, 3H), 2.56-2.66 (stack, 2H), 3.50-3.58 (m, 1H), 3.65-3.83 (stack, 8H), 3.90 (d, J 2.9, 1H), 3.96 (dd, J 10.9, 4.3, 1H), 4.85 (app. q, J 4.3, 1H) 4.94 (d, J 3.7, 1H), OH resonances not observed; \( \delta_{\text{C}} \) (100 MHz, CDCl₃:CD₃OD, 2:1) 14.3 (CH₃, 2 × CH₂CH₃), 23.1 (CH₂), 26.4 (CH₂), 29.6 (CH₂), [29.9, 30.0, 30.1, 30.17, 30.21 (CH₂, alkyl chain, resonance overlap)], 32.4 (CH₂), 32.7 (CH₂), 47.1 (CH₂), 56.8 (CH), 62.3 (CH₂), 66.8 (CH₂), 69.5 (CH),
70.4 (CH), 70.8 (CH), 71.4 (CH), 72.6 (CH), 73.9 (CH), 100.2 (CH), 206.1 (C, C=S); MS (TOF ES+) m/z 896.8 ([M + Na]+, 100%); HRMS (TOF ES+) calcd for C_{50}H_{99}NO_{8}SNa [M + Na]^+ 896.6989, found 896.6998.

(2S,3S,4R)-2-Azido-3,4-O-isopropylidene-1-O-(2’,3’,4’,6’-tetra-O-benzyl-α-D-galactopyranosyl)octadecane-1,3,4-triol (79)

Ph\textsubscript{3}P (1.46 g, 5.55 mmol) and CBr\textsubscript{4} (1.84 g, 5.55 mmol) were added sequentially to a solution of 2,3,4,6-tetra-O-benzyl-D-galactose \textbf{51} (1.00 g, 1.85 mmol) in CH\textsubscript{2}Cl\textsubscript{2} (10 mL) at r.t. The reaction mixture was stirred for 3 h. In separate flasks, a solution of tetramethylurea (TMU) (1.2 mL) and Bu\textsubscript{4}NBr (1.79 g, 5.55 mmol) in CH\textsubscript{2}Cl\textsubscript{2} (5 mL), and a solution of azide \textbf{78} (1.07 g, 2.78 mmol) in CH\textsubscript{2}Cl\textsubscript{2} (5 mL) were stirred over activated 3 Å MS for 30 min, after which time, these solutions were added dropwise (15 min) and sequentially (TMU/Bu\textsubscript{4}NBr solution first) to the solution containing the glycosyl donor. The reaction mixture was stirred at r.t. for 3 d until there was no evidence by TLC that the donor was still being consumed. The reaction mixture was then filtered through a silica plug, washing with CH\textsubscript{2}Cl\textsubscript{2} (1.2
L) and then concentrated under reduced pressure. Purification of the residue by flash column chromatography (10% EtOAc in hexane) afforded glycoside 79 as a colourless oil (1.56 g, 71%, α-anomer only): $R_f = 0.2$ (10% EtOAc in hexane); $[\alpha]_D^{22} = +24.8$ (c 1, CHCl$_3$); lit. $[\alpha]_D^{rt} = +32.1$ (c 2.0, CHCl$_3$); $1^8$ $v_{\text{max}}$(film) / cm$^{-1}$ 2924s, 2854s, 2099s (N$_3$), 1497w, 1455m, 1370m, 1249m, 1220m, 1110s, 1059s, 735s, 697s; $\delta_H$(500 MHz, CDCl$_3$) 0.87 (t, $J = 6.8$, 3H, CH$_2$CH$_3$), 1.20-1.42 (stack, 29H, alkyl chain methylenes, C(CH$_3$)$_2$), 1.47-1.64 (stack, 3H), 3.44-3.54 (stack, 3H, C(6')H$_2$, H-2), 3.71 (dd, $J = 10.8$, 6.7, 1H, C(1)H$_a$H$_b$, H-3), 3.91-3.94 (m, 1H, H-4'), 3.95-4.12 (stack, 6H, H-2', H-3', H-5', C(1)H$_a$H$_b$, H-3, H-4), 4.39 (A of AB, $J = 11.9$, 1H, OCH$_a$H$_b$Ph), 4.47 (B of AB, $J = 11.9$, 1H, OCH$_a$H$_b$Ph), 4.70 (A of AB, $J = 12.0$, 1H, OCH$_a$H$_b$Ph), 4.71 (A of AB, $J = 11.8$, 1H, OCH$_a$H$_b$Ph), 4.79 (B of AB, $J = 12.0$, 1H, OCH$_a$H$_b$Ph), 4.84 (B of AB, $J = 11.8$, 1H, OCH$_a$H$_b$Ph), 4.93 (d, $J = 3.6$, 1H, H-1'), 4.94 (B of AB, $J = 11.5$, 1H, OCH$_a$H$_b$Ph), 7.22-7.33 (stack, 16H, Ph), 7.36-7.38 (stack, 4H, Ph); $\delta_C$(125 MHz, CDCl$_3$) 14.1 (CH$_3$, CH$_2$CH$_3$), 22.7 (CH$_2$), 25.7 (CH$_3$, 1 × C(CH$_3$)$_2$), 26.6 (CH$_2$), 28.1 (CH$_3$, 1 × C(CH$_3$)$_2$), [29.3, 29.60, 29.65, 29.69 (CH$_2$, alkyl chain, resonance overlap)], 31.9 (CH$_2$), 59.8 (CH, C-2), 69.1 (CH$_2$, C-6'), 69.6 (CH$_2$, C-1), 69.9 (CH), 72.9 (CH$_2$, CH$_2$Ph), 73.3 (CH$_2$, CH$_2$Ph), 73.4 (CH$_2$, CH$_2$Ph), 74.7 (CH$_2$, CH$_2$Ph), 75.3 (CH, C-4'), 75.4 (CH), 76.6 (CH), 77.8 (CH, C-4), 78.7 (CH), 98.8 (CH, C-1'), 108.2 (C, C(CH$_3$)$_2$), [127.4, 127.5, 127.60, 127.64, 127.7 (CH, Ph, resonance overlap)], [128.20, 128.25, 128.29, 128.35 (CH, Ph, resonance overlap)], 138 (C, ipso Ph), 138.7 (C, ipso Ph), 138.9 (C, 2 × ipso Ph, resonance overlap); MS (TOF ES+) $m/z$ 928.7 ([M+Na]$^+$, 100%); HRMS (TOF ES+) 928.5470 [M+Na]$^+$. $C_{55}H_{75}N_3O_8$Na requires 928.5452. The unreacted azide was also recovered (394 mg, 37%).
Data were in agreement with those reported in the literature for 79 prepared by a different route.\(^{180}\)

\[(2S,3S,4R)-2\text{-Amino-3,4-\text{O}-isopropylidene-1-O-}(2',3',4',6'\text{-tetra-\text{O}-benzyl-\text{α-D-galactopyranosyl}})\text{octadecane-1,3,4-triol (80)}\]

\[\text{PMe}_3 (930 \mu\text{L of a 1.0 M soln in THF, 0.93 mmol}) \text{ was added dropwise over 5 min to a solution of azide 79 (700 mg, 0.77 mmol) in THF (7 mL). The reaction mixture was stirred at r.t. for 3 h, after which time, H}_2\text{O (0.5 mL) was added. The reaction mixture was stirred for 1 h and then concentrated under reduced pressure. The residual H}_2\text{O was removed by co-evaporation with toluene (3 \times 3 mL) to provide the crude product. Purification by flash column chromatography (25% EtOAc in hexane) afforded amine 80 as a colourless oil (632 mg, 93%): } R_f = 0.2 \text{ (25% EtOAc in hexane); } [\alpha]^{20}_D = +35.6 \text{ (c 1, CHCl}_3); \nu_{\max}(\text{film}) / \text{cm}^{-1} 3372 \text{ br (N–H); } \delta_H(300 \text{ MHz, CDCl}_3) 0.87 \text{ (t, } J 7.0, \text{ 3H, CH}_2\text{CH}_3), 1.20–1.43 \text{ (stack, 24H, alkyl chain methylenes, } 1 \times \text{ C(CH}_3)_2\text{), 1.37 (s, 3H, } 1 \times \text{ C(CH}_3)\text{), 1.42–1.57 (stack, 5H), 2.99–3.08 \text{ (m, 1H), 3.37 (dd, } J 10.1, 7.6, 1 \text{H), 3.48–3.57 (stack, 2H), 3.81–3.98}\]
Chapter 5  Experimental Section

(stack, 5H), 4.02–4.12 (stack, 2H), 4.38 (A of AB, \(J_{A-B} 11.8, 1H\)), 4.46 (B of AB, \(J_{B-A} 11.8, 1H\)), 4.57 (d, \(J 11.5, 1H\)), 4.68 (d, \(J 11.8, 1H\)), 4.71–4.83 (stack, 3H), 4.91–4.93 (stack, 2H), 7.22–7.39 (stack, 20H, Ph), NH\(_2\) not observed; \(\delta_c\) (100 MHz, CDCl\(_3\)) 14.1 (CH\(_3\), CH\(_2\)CH\(_3\)), 22.7 (CH\(_2\)), 25.9 (CH\(_3\), 1 \(\times\) C(CH\(_3\))\(_2\)), 26.2 (CH\(_2\)), 28.3 (CH\(_3\), 1 \(\times\) C(CH\(_3\))\(_2\)), [29.3, 29.7, 29.8 (CH\(_2\), alkyl chain, resonance overlap)], 31.9 (CH\(_2\)), 50.7 (CH, CHNH\(_2\)), 69.0 (CH\(_2\)), 69.5 (CH), 72.3 (CH\(_2\)), 73.0 (CH\(_2\)), 73.3 (CH\(_2\)), 73.5 (CH\(_2\)), 74.8 (CH\(_2\)), 75.0 (CH), 76.8 (CH), 77.9 (CH), 79.0 (CH), 79.1 (CH), 99.0 (CH, C-1'), 107.8 (C, C(CH\(_3\))\(_2\)), [127.4, 127.5, 127.6, 127.7, 127.8 128.2, 128.3 (CH, Ph, resonance overlap)], 138.0 (C, ipso Ph), [138.7, 138.8 (C, 3 \(\times\) ipso Ph, resonance overlap)]; MS (TOF ES+) \(m/z\) 880.8 ([M + H]\(^+\), 100%); HRMS (TOF ES+) calcd for C\(_{55}\)H\(_{78}\)NO\(_8\) [M + H]\(^+\) 880.5727, found 880.5721.
(2S,3S,4R)-2-Hexacosanoylamino-3,4-O-isopropylidene-1-O-(2',3',4',6'-tetra-O-benzyl-α-D-galactopyranosyl)octadecane-1,3,4-triol (81)

A screw-capped glass tube containing a solution of hexacosanoic acid (100 mg, 0.25 mmol) in (COCl)₂ (2.0 mL, 23 mmol) was closed tightly and heated at 70 ºC for 2 h, after which time, the volatiles were evaporated under a stream of argon and the tube then placed on a high vacuum line for at least 2 h to remove the residual volatiles. The resulting hexacosanoyl chloride was used directly without further purification: a solution of freshly prepared hexacosanoyl chloride 47 (105 mg, 0.25 mmol) in CH₂Cl₂ (0.5 mL) was added dropwise over 2 min to a solution of amine 80 (132 mg, 0.15 mmol) and NEt₃ (42 μL, 0.30 mmol) in CH₂Cl₂ (1.0 mL) at 0 ºC. The reaction mixture was stirred at r.t. for 12 h and then diluted with CH₂Cl₂ (10 mL), washed sequentially with NaHCO₃ solution (10 mL), brine (2 mL) and then dried over Na₂SO₄. The drying agent was removed by filtration and the filtrate concentrated under reduced pressure. Purification of the residue by flash column chromatography provided amide 81 as a white solid (160 mg, 85%): \( R_f = 0.3 \) (10% EtOAc in hexane); \([\alpha]_D^{20} = + 41.6 \) (c 1, CHCl₃); lit. \([\alpha]_D^{rt} = + 44.2 \) (c 0.85, CHCl₃); mp 87 – 88 ºC; \( \nu_{\text{max}} \) (film) / cm⁻¹ 2916s, 2849s, 1648m (C=O), 1541m,
Chapter 5   Experimental Section

1497w, 1469m, 1454m, 1380w, 1370w, 1250w, 1217m, 1158m, 1106s, 1044s, 859m, 881w, 859w, 791w, 733s, 721s; $\delta_{hs}(500$ MHz, CDCl$_3$) 0.87 (t, J 6.9, 6H, CH$_2$CH$_3$), 1.15-1.34 (stack, 71H, alkyl chain methylenes, $1 \times$ C(CH$_3$)$_2$), 1.39 (s, 3H, $1 \times$ C(CH$_3$)$_2$), 1.40-1.47 (stack, 2H, including C(3")H$_a$H$_b$), 1.48-1.56 (stack, 2H), 1.93-2.01 (m, 1H, C(2")H$_a$H$_b$), 2.01-2.09 (m, 1H, C(2")H$_a$H$_b$), 3.37 (dd, J 9.4, 5.7, 1H, C(6")H$_a$H$_b$), 3.54 (dd, J 9.4, 7.0, 1H, C(6")H$_a$H$_b$), 3.60 (br d, J 9.7, 1H, C(1)H$_a$H$_b$), 3.88-3.93 (stack, 3H, H-3', H-4', H-3 or H-4), 3.97 (app t, J 6.3, 1H, H-5'), 4.01-4.12 (stack, 4H, H-2', C(1)H$_a$H$_b$, H-2, H-3 or H-4), 4.36 (A of AB, $J_{A-B}$ 11.8, 1H, C(6")OCH$_a$H$_b$Ph), 4.47 (B of AB, $J_B$-A 11.8, 1H, C(6")OCH$_a$H$_b$Ph), 4.57 (A of AB, $J_{A-B}$ 11.6, 1H, C(4")OCH$_a$H$_b$Ph), 4.65 (A of AB, $J_{A-B}$ 11.5, 1H, C(2")OCH$_a$H$_b$Ph), 4.73 (A of AB, $J_{A-B}$ 11.8, 1H, C(3")OCH$_a$H$_b$Ph), 4.79 (B of AB, $J_{B-A}$ 11.5, 1H, C(2")OCH$_a$H$_b$Ph), 4.80 (B of AB, $J_B$-A 11.8, 1H, C(3")OCH$_a$H$_b$Ph), 4.89 (d, J 3.7, 1H, H-1'), 4.91 (B of AB, $J_B$-A 11.6, 1H, C(4")OCH$_a$H$_b$Ph), 6.24 (d, J 8.9, 1H, NH), 7.21-7.38 (stack, 20H, Ph); $\delta_C$(125 MHz, CDCl$_3$) 14.1 (CH$_3$, CH$_2$CH$_3$), 22.6 (CH$_2$), 25.6 (CH$_2$), 25.9 (CH$_3$, $1 \times$ C(CH$_3$)$_2$), 26.5 (CH$_2$), 28.2 (CH$_3$, $1 \times$ C(CH$_3$)$_2$), 28.9 (CH$_2$), [29.31, 29.37, 29.45, 29.55, 29.57, 29.62, 29.66, 29.68 (CH$_2$, alkyl chain, resonance overlap)], 31.9 (CH$_2$), 36.7 (CH$_2$, C-2”), 48.7 (CH, C-2), 69.5 (CH$_2$, C-6”), 69.9 (CH, C-5”), 70.6 (CH$_2$, C-1), 73.0 (CH$_2$, C(3")OCH$_2$Ph), 73.46 (CH$_2$, C(2")OCH$_2$Ph), 73.54 (CH$_2$, C(6")OCH$_2$Ph), 74.6 (CH$_2$, C(4")OCH$_2$Ph), 74.7 (CH, C-4”), 75.4 (CH, C-3 or C-4), 76.8 (CH, C-2”), 77.8 (CH, C-3 or C-4), 78.9 (CH, C-3”), 99.7 (CH, C-1”), 107.8 (C, (CH$_3$)$_2$C), [127.4, 127.5, 127.7, 127.8, 127.85, 127.91 (CH, Ph, some resonance overlap)], [128.2, 128.31, 128.34, 128.36, 128.40 (CH, Ph, some resonance overlap), 137.5 (C, ipso Ph on C-6”), 138.3 (C, ipso Ph), 138.4 (C, ipso Ph), 138.6 (C, ipso Ph on C-3”), 172.4 (C, C=O);
MS (TOF ES+) m/z 1281.0 ([M + Na]^+, 100%); HRMS (TOF ES+) calcd for C_{61}H_{127}NO_{9}Na [M + Na]^+ 1280.9409, found 1280.9417.

Data were in agreement with those reported in the literature for 81 prepared using different reagents.\textsuperscript{180}

\textit{(2S,3S,4R)-3,4-Di-O-benzyl-1-O-(2',3',4',6'-tetra-O-benzyl-D-galactopyranosyl)-2-(tricosylaminocabonylamino)octadecane-1,3,4-triol (77)}

A screw-capped glass tube containing a solution of tetracosanoic acid 74 (450 mg, 1.22 mmol) in (COCl)\textsubscript{2} (2.0 mL, 23 mmol) was closed tightly and heated at 70 °C for 2 h. The volatiles were then evaporated under a stream of argon and the tube then placed on a high vacuum line for at least 2 h to remove the residual volatiles. The resulting tetracosanoyl chloride 75 was used directly in the next step without further purification: a solution of freshly prepared tetracosanoyl chloride (450 mg, 1.22 mmol) in THF (5 mL) was added dropwise over 10 min to a solution of NaN\textsubscript{3}
(300 mg, 4.62 mmol) in H₂O (0.5 mL) at 0 °C. The reaction mixture was stirred at r.t. for 5 h. The organic phase was then extracted with cold (~10 °C) THF (5 mL) and dried over Na₂SO₄. The drying agent was removed by filtration. The solvent was removed under reduced pressure to provide tetracosanoyl azide as a white solid, which was used immediately in the next step: a solution of tetracosanoyl azide (481 mg, 1.22 mmol (assuming quantitative conversion in the previous step)) in toluene (5 mL) was heated under reflux for 4 h, after which time, the reaction mixture was cooled to r.t. The resulting solution of tricosanyl isocyanate product 48 was used directly without further purification in the next step: a solution of amine 46 (150 mg, 0.147 mmol) in toluene (5 mL) was added to a solution of tricosanyl isocyanate (1.22 mmol (assuming quantitative conversion)) in toluene (5 mL) at r.t. The reaction mixture was heated under reflux for 8 h and then concentrated under reduced pressure. Purification of the residue by flash column chromatography (20% EtOAc in hexane) afforded urea 77 as a pale yellow oil (140 mg, 68% based on amine): 

\[ R_f = 0.3 \] (20% EtOAc in hexane); 

\[ [\alpha]^{20}_D = +25.2 \] (c 1, CHCl₃); \[ \nu_{\text{max}} \] (film) / cm⁻¹ 3363m (N-H), 1670m (C=O); \[ \delta_\text{t} \] (500 MHz, CDCl₃) 0.89 (t, J 7.0, 6H, 2 × CH₂CH₃), 1.10-1.39 (stack, 65H, alkyl chain methylenes), 1.39-1.51 (m, 1H, alkyl chain CH₃), 1.56-1.64 (m, 1H, C(5)H₆H₇), 1.64-1.73 (m, 1H, C(5)H₆H₇), 2.90-3.06 (stack, 2H, CH₃H₆NH), 3.33 (dd, J 9.5, 5.0, 1H, C(6′)H₆H₇), 3.55 (dd, J 9.5, 7.0, 1H, C(6′)H₆H₇), 3.59-3.64 (m, 1H, H-4), 3.73 (dd, J 10.8, 3.1, 1H, C(1)H₆H₇), 3.80-3.88 (stack, 3H, H-2, H-3, H-4′), 3.89 (dd, J 10.3, 2.7, 1H, H-3′), 3.98 (app. t, J 6.0, 1H, H-5′), 4.05 (dd, J 10.1, 3.6, 1H, H-2′), 4.11 (br dd, J 10.8, 4.5, 1H, C(1)H₆H₇), 4.38 (A of AB, Jₐ₋ₐ 12.0, 1H, C(6′)OCH₃H₆H₇Ph), 4.45-4.48 (stack, 2H, C(6′)OCH₃H₆H₇Ph, C(4)OCH₃H₆H₇Ph), 4.54 (A of AB, Jₐ₋ₐ 11.3, 1H, C(3)OCH₃H₆H₇Ph),
4.57 (A of AB, J_{A:B} 11.8, 1H, C(4')OCH\textsubscript{a}H\textsubscript{b}Ph), 4.61 (B of AB, J_{B:A} 12.6, 1H, C(4)OCH\textsubscript{a}H\textsubscript{b}Ph), 4.66 (A of AB, J_{A:B} 11.7, 1H, C(2')OCH\textsubscript{a}H\textsubscript{b}Ph), 4.75 (A of AB, J_{A:B} 11.8, 1H, C(3')OCH\textsubscript{a}H\textsubscript{b}Ph), 4.78 (B of AB, J_{B:A} 11.3, 1H, C(3)OCH\textsubscript{a}H\textsubscript{b}Ph), 4.79 (B of AB, J_{B:A} 11.8, 1H, C(3')OCH\textsubscript{a}H\textsubscript{b}Ph), 4.81 (B of AB, J_{B:A} 11.8, 1H, C(4')OCH\textsubscript{a}H\textsubscript{b}Ph), 4.87 (d, J 3.6, 1H, H-1'), 4.93 (B of AB, J_{B:A} 11.8, 1H, C(4')OCH\textsubscript{a}H\textsubscript{b}Ph), 5.00 (d, J 7.8, 1H, C(2)NH\textsubscript{f}), 7.23-7.38 (stack, 30H, 6 \times Ph), CH\textsubscript{2}NH not observed; \textit{\delta} (125 MHz, CDCl\textsubscript{3}) 14.1 (CH\textsubscript{3}, 2 \times CH\textsubscript{2}CH\textsubscript{3}), [22.7, 26.0, 26.9, 29.4, 29.7, 29.9, 30.3, 31.9 (CH\textsubscript{2}, alkyl chain methylenes, resonance overlap)], 40.3 (CH\textsubscript{2}, CH\textsubscript{2}NH), 51.7 (CH, C-2), 69.9 (CH\textsubscript{2}, C-6'), 70.1 (CH, C-5'), 71.0 (CH\textsubscript{2}, C-1), 71.9 (CH\textsubscript{2}, C(4)OCH\textsubscript{2}Ph), 73.1 (CH\textsubscript{2}, C(3')OCH\textsubscript{2}Ph), 73.4 (CH\textsubscript{2}, C(2')OCH\textsubscript{2}Ph), 73.6 (CH\textsubscript{2}, C(6')OCH\textsubscript{2}Ph), 73.8 (CH\textsubscript{2}, C(3)OCH\textsubscript{2}Ph), 74.6 (CH\textsubscript{2}, C(4')OCH\textsubscript{2}Ph), 75.0 (CH, C-3), 76.8 (CH, C-2'), 78.8 (CH, C-3'), 79.9 (CH, C-4'), 80.3 (CH, C-4), 99.8 (CH, C-1'), [127.5, 127.6, 127.67, 127.70, 127.84, 127.88, 127.9, 128.25, 128.28, 128.34, 128.37, 128.5 (CH, Ph, resonance overlap)], 137.39 (C, ipso Ph), 138.42 (C, ipso Ph), 138.48 (C, ipso Ph), 138.67 (C, ipso Ph), 138.72 (C, ipso Ph), 138.8 (C, ipso Ph), 158.8 (C, C=O); MS (TOF ES+) m/z 1408.0 ([M + Na]\textsuperscript{+}, 100%); HRMS (TOF ES+) calcd for C\textsubscript{90}H\textsubscript{132}N\textsubscript{2}O\textsubscript{9}Na [M + Na]\textsuperscript{+} 1407.9831, found 1407.9844. The unreacted amine was also recovered (40 mg, 83%).
Chapter 5  Experimental Section

(2S,3S,4R)-1-O-(α-D-Galactopyranosyl)-2-(tricosylaminocarbonylamino)-octadecane-1,3,4-triol (36)

Urea 36 was prepared from perbenzylated urea 77 (115 mg, 0.083 mmol) and Pd/C (26.5 mg, 10% wet) in THF (10 mL) according to the general procedure for catalytic hydrogenolysis. After 22 h, work-up and purification by flash column chromatography (6% MeOH in CHCl₃) afforded urea 36 as an amorphous, white solid (51 mg, 73%): $R_f = 0.23$ (6% MeOH in CHCl₃); [$\alpha$]D$^2 = +28.0$ (c 0.5, CHCl₃); mp 154 – 155 °C; $\nu$max(film) / cm$^{-1}$ 3363m (N-H), 1670m (C=O); $\delta_H$(500 MHz, CDCl₃) 0.68 (t, $J$ 6.5, 6H, $2 \times$ CH₃C₉H₃), 1.06-1.10 (stack, 62H, alkyl chain methylenes), 1.22-1.27 (stack, 2H), 1.33-1.36 (m, 1H), 1.48-1.52 (m, 1H), 1.67-1.69 (stack, 2H), 2.83-2.96 (stack, 2H, CH₂NH), 3.28-3.31 (m, 1H, H-3), 3.33-3.36 (m, 1H, H-4), 3.49-3.63 (stack, 6H, H-3’, 2 × H-6’, H-2’, H-5’, C(1)H₅H₅), 3.66 (dd, $J$ 10.5, 4.5, 1H, C(1)H₅H₅), 3.72 (br d, $J$ 2.5, 1H, H-4’), 3.97 (dd, $J$ 9.0, 4.5, 1H, H-2), 4.67 (d, $J$ 3.5, 1H, H-1’); $\delta_C$(125 MHz, CDCl₃) 13.5 (CH₃, $2 \times$ CH₂CH₃), [22.3, 25.1, 25.5, 26.6, 29.0, 29.1, 29.3, 29.4, 29.8, 31.5, 32.7 (CH₂, alkyl chain methylenes, resonance overlap)], 39.8 (CH₂, CH₂NH), 50.6 (CH, C-2), 61.5 (CH₂, C-6’), 67.6 (CH₂, C-1), 68.6 (CH, C-2’), 69.4 (CH, C-4’), 70.0 (CH, C-3’), 70.5 (CH, C-5’), 72.1 (CH, C-4), 75.2 (CH, C-3), 99.4 (CH, C-1’), 158.9 (C, C=O); MS (TOF ES+) m/z
867.9 ([M + Na]^+, 100%); HRMS (TOF ES+) calcd for C_{48}H_{96}N_{2}O_{9}Na [M + Na]^+ 867.7014, found 867.7026.

**Tetracosan-1-ol (82)**

\[
\text{HO} \quad \text{CH}_2 \quad \text{CH}_3
\]

A solution of tetracosanoic acid (2.00 g, 5.43 mmol) in THF (10 mL) was added dropwise to a suspension of LiAlH\(_4\) (310 mg, 8.14 mmol) in THF (45 mL) over 5 min at r.t. The mixture was heated under reflux for 4 h, cooled to r.t. and then quenched carefully with freshly prepared saturated Na\(_2\)SO\(_4\) solution (10 mL), followed by the addition of anhydrous MgSO\(_4\) (600 mg). The mixture was stirred vigorously for 10 min, filtered through a pad of Celite and washed with THF (2 \times 40 mL). Removal of the solvent under reduced pressure and purification of the residue by flash column chromatography (10% EtOAc in toluene) provided tetracosan-1-ol as a white solid (1.69 g, 88%): \(R_f = 0.3\) (10% EtOAc in hexane); mp 76 – 77 °C (lit. 76.0 – 76.5 °C);\(^{181}\) \(\nu_{\text{max}}\) (film) / cm\(^{-1}\) 3488 br (OH), 2974m, 2917m, 2849m, 1462w, 1356w, 1181w, 1065s, 907m, 720w, 658w, 581w; \(\delta\)\(_\text{H}(300 MHz, CDCl}_3\) 0.87 (t, \(J = 6.7\), 3H, CH\(_2\)CH\(_3\)), 1.16-1.39 (stack, 40H, alkyl chain methylenes), 1.40-1.45 (stack, 2H), 1.51-1.62 (stack, 2H), 3.64 (t, \(J = 6.6\), 2H), OH resonance not observed; \(\delta\)\(_\text{C}(75 MHz, CDCl}_3\) 14.1, (CH\(_3\), CH\(_2\)CH\(_3\)), 22.7 (CH\(_2\)).
25.7 (CH\(_2\)), 26.5 (CH\(_2\)), 28.2 (CH\(_3\)), 28.8 (CH\(_2\)), 29.1 (CH\(_2\)), [29.3, 29.4, 29.7, 29.8 (CH\(_2\), alkyl chain, resonance overlap)], 31.9 (CH\(_2\)), 32.8 (CH\(_2\)), 63.1 (CH\(_2\)); MS (TOF ES+) m/z 377.5 ([M + Na]\(^+\), 100%); HRMS (TOF ES+) calcd for C\(_{24}\)H\(_{50}\)ONa [M + Na]\(^+\) 377.3759, found 377.3755.

Data were in agreement with those reported in the literature.\(^{182}\)

**Carbonic acid, 2,5-dioxo-1-pyrrolidinyl tetracosanyl ester (49)**

\[
\text{\begin{center}
\includegraphics[width=1cm]{image.png}
\end{center}}
\]

\(N,N'\)-Disuccinimidyl carbonate 83 (190 mg, 0.75 mmol) was added to a solution of tetracosan-1-ol (177 mg, 0.50 mmol) and NEt\(_3\) (210 \(\mu\)L, 1.5 mmol) in dry CH\(_3\)CN (2.5 mL) at r.t. The resulting mixture was stirred at r.t. for 4 h and then concentrated under reduced pressure. The residue was diluted with NaHCO\(_3\) solution (10 mL) and extracted with EtOAc (2 \times 10 mL). The combined extracts were washed with brine (5 mL) and dried over Na\(_2\)SO\(_4\). Evaporation of the solvent under reduced pressure provided the corresponding mixed carbonate 49 as a white solid, which was used directly in the next step (248 mg, quant.): \(R_f = 0.3\) (25% EtOAc in hexane); \(\nu_{\text{max}}\) (film) / cm\(^{-1}\) 1711m (C=O), 1693m (C=O); \(\delta_{\text{H}}\) (300 MHz) ppm.
MHz, CDCl$_3$) 0.88 (t, J 7.0, 3H, CH$_2$CH$_3$), 1.16-1.46 (stack, 42H, alkyl chain methylenes), 1.51-1.80 (stack, 2H), 2.84 (s, 4H), 4.31 (t, J 6.6, 2H); $\delta_C$(100 MHz, CDCl$_3$) 14.1 (CH$_3$, CH$_2$CH$_3$), 22.7 (CH$_2$), 28.4 (CH$_2$), [29.1, 29.36, 29.42, 29.5, 29.7 (CH$_2$, alkyl chain, resonance overlap)], 31.9 (CH$_2$), 71.7 (CH$_2$), 151.6 (C, OC=O), 168.7 (C, NC=O); MS (TOF ES+) $m/z$ 518.5 ([M + Na]$^+$, 100%); HRMS (TOF ES+) calcd for C$_{29}$H$_{53}$NO$_5$Na [M + Na]$^+$ 518.3821, found 518.3817.

(2S,3S,4R)-3,4-O-isopropylidene-1-O-(2',3',4',6'-tetra-O-benzyl-α-D-galactopyranosyl)-2-(tetracosanyloxycarbonylamino)octadecane-1,3,4-triol (84)

A solution of carbonic acid, 2,5-dioxo-1-pyrrolidinyl tetracosanyl ester (50 mg, 0.10 mmol) in CH$_2$Cl$_2$ (0.5 mL) was added to a stirred solution of amine 80 (60 mg, 0.068 mmol) and NEt$_3$ (24 $\mu$L, 0.17 mmol) in CH$_2$Cl$_2$ (1 mL). The resulting mixture was stirred at r.t. until no mixed carbonate remained as determined by TLC (4 h). The mixture was then diluted with CH$_2$Cl$_2$ (8 mL) and washed sequentially with NaHCO$_3$ solution (10 mL) and brine (10 mL). The organic phase was dried over Na$_2$SO$_4$ and the solvent was evaporated under reduced pressure. Purification of
the residue by flash column chromatography (5% EtOAc in toluene) provided carbamate 84 as a colourless oil (70 mg, 82%): \( R_f = 0.3 \) (10% EtOAc in hexane); 
[\alpha]_D^{20} = +36.8 (c 1, CHCl_3); \( \nu_{\text{max}} \) (film) / cm\(^{-1}\) 1689m (C=O); \( \delta_t \) (500 MHz, CDCl\(_3\)) 0.88 (t, J 6.8, 6H, 2 \times CH\(_2\)CH\(_3\)), 1.24-1.34 (stack, 64H, alkyl chain methylenes, C(CH\(_3\))\(_2\)), 1.39-1.51 (stack, 8H), 1.57-1.59 (stack, 3H), 1.63-1.67 (m, 1H), 3.45 (dd, J 9.3, 6.3, 1H, C(6')\(H_aH_b\)), 3.52 (dd, J 9.3, 6.5, 1H, C(6')\(H_aH_b\)), 3.66-3.70 (m, 1H, H-3), C(1'')\(H_aH_b\)), 4.03-4.11 (stack, 3H, H-2'), C(1)\(H_aH_b\), C(1'')\(H_aH_b\)), 4.38 (A of AB, \( J_{A-B} \) 11.8, 1H, C(6')OCH\(_3\)H\(_2\)Ph), 4.49 (B of AB, \( J_{B-A} \) 11.8, 1H, C(6')OCH\(_3\)H\(_2\)Ph), 4.56 (A of AB, \( J_{A-B} \) 11.5, 1H, OCH\(_3\)H\(_2\)Ph), 4.67 (A of AB, \( J_{A-B} \) 11.7, 1H, OCH\(_3\)H\(_2\)Ph), 4.74 (A of AB, \( J_{A-B} \) 11.7, 1H, C(2')OCH\(_3\)H\(_2\)Ph) 4.78 (B of AB, \( J_{B-A} \) 11.7, 1H, OCH\(_3\)H\(_2\)Ph), 4.83 (B of AB, \( J_{B-A} \) 11.7, 1H, C(2')OCH\(_3\)H\(_2\)Ph), 4.92 (B of AB, \( J_{B-A} \) 11.5, 1H, OCH\(_3\)H\(_2\)Ph), 4.95 (d, J 3.6, 1H, H-1'), 5.32 (br d, J 9.5, 1H, NH), 7.23-7.35 (stack, 18H, Ph), 7.38-7.39 (stack, 2H, Ph); \( \delta_{C} \) (125 MHz, CDCl\(_3\)) 14.1 (CH\(_3\)), 22.7 (CH\(_2\)), 25.9 (CH\(_2\), CH\(_3\), resonance overlap), 26.5 (CH\(_2\)), 28.2 (CH\(_3\)), 28.8 (CH\(_2\)), 29.1 (CH\(_2\)), [29.3, 29.4, 29.7, 29.8 (CH\(_2\), alkyl chain, resonance overlap)], 32.0 (CH\(_2\)), 50.7 (CH), 65.1 (CH\(_2\)), 69.3 (CH\(_2\)), 69.7 (CH), 69.9 (CH\(_2\)), 73.1 (CH\(_2\)), 73.2 (CH\(_2\)), 73.6 (CH\(_2\)), 74.7 (CH\(_2\)), 74.9 (CH), 75.5 (CH), 76.8 (CH), 77.8 (CH), 79.0 (CH), 99.2 (CH, C-1'), 107.7 (C, C(CH\(_3\))\(_2\)), [127.5, 127.6, 127.8, 127.9 (CH, Ph, resonance overlap)], [128.23, 128.26, 128.34, 128.36, 128.4 (CH, Ph, resonance overlap)], 137.8 (C, ipso Ph), 138.6 (C, 2 \times ipso Ph), 138.8 (C, ipso Ph), 155.9 (C, C=O); MS (TOF ES+) \( m/z \) 1283.0 ([M + Na]\(^+\), 100%); HRMS (TOF ES+) calcd for C\(_{80}\)H\(_{125}\)NO\(_{10}\)Na [M + Na]\(^+\) 1282.9201, found 1282.9244.
(2S,3S,4R)-1-O-(α-D-Galactopyranosyl)-2-(tetracosanyloxy carbonylamino)-octadecane-1,3,4-triol (37)

TFA (120 μL) was added dropwise over 1 min to a solution of acetal 84 (60 mg, 0.048 mmol) in CH₂Cl₂ / CH₃OH (2:1, 0.6 mL). After stirring for 2 h at r.t., the reaction mixture was concentrated under reduced pressure and the residual TFA was removed by co-evaporation with Et₂O (3 × 3 mL) to provide the acetal hydrolysis product as a white solid (58 mg, quant.), which was treated with Pd(OH)₂/C (15 mg, 10% wet) and H₂ in THF (6 mL) according to the general hydrogenolysis procedure. After 6 h, work-up and purification by flash column chromatography (8% MeOH in CHCl₃) afforded carbamate 37 as an amorphous white solid (31 mg, 75%): Rᵣ = 0.3 (8% MeOH in CHCl₃); [α]₁⁸_D = +46.0 (c 1, CHCl₃); mp 166 – 167 °C; νmax (neat) / cm⁻¹ 3388 s br (OH), 1683 m (C=O); δH(500 MHz, CDCl₃:CD₃OD, 2:1) 0.63 (t, J 6.8, 6H, 2 × CH₂C₃H₃), 0.91-1.12 (stack, 66H, alkyl chain methylenes), 1.22-1.48 (stack, 4H), 3.27-3.38 (stack, 2H), 3.39-3.58 (stack, 6H), 3.64-3.75 (stack, 4H), 3.77-3.84 (m, 1H), 4.65 (d, J 3.4, 1H, H-1'), OH and NH resonances not observed; δC(125 MHz, CDCl₃:CD₃OD, 2:1) 13.4, 22.2, 25.3, 25.4, 28.6, 28.7, 28.9, 29.2, 31.4, 32.1, 51.3, 61.4, 64.8, 67.1, 68.5, 69.4, 69.9, 70.4, 71.5, 74.4, 99.3, 156.9 (significant resonance overlap in the alkyl chain
methylene resonances); MS (TOF ES+) m/z 882.4 ([M + Na]⁺, 100%); HRMS (TOF ES+) calcd for C₄₉H₉₇NO₁₀Na [M + Na]⁺ 882.7010, found 882.7000.

1-O-(tert-Butylidiphenylsilyl)-2,3-O-isopropylidene-L-threitol (92)

![Chemical Structure](image)

A solution of (+)-2,3-O-isopropylidene-L-threitol 88 (1.000 g, 6.162 mmol) in THF (15 mL) was added dropwise over 10 min to a suspension of NaH (60% dispersion in mineral oil, 271 mg, 6.782 mmol) in THF (30 mL) at 0 °C. After 30 min, a solution of tBuPh₂SiCl (1.864 g, 6.782 mmol) in THF (15 mL) was added dropwise over 20 min. After stirring at r.t. for 12 h, the reaction was quenched by the sequential addition of MeOH (5 mL) and NaHCO₃ solution (60 mL). The phases were separated and the aqueous phase was washed with CH₂Cl₂ (3 × 60 mL). The combined organic phases were washed with brine (60 mL), dried (Na₂SO₄), filtered and the solvent was evaporated under reduced pressure. The residue was purified by silica gel flash column chromatography (eluent: 15% EtOAc in hexanes) to provide silyl ether 92 as a colourless oil (2.265 g, 92%); Rᵣ = 0.25 (15% EtOAc in hexanes); [α]₀⁺²¹ = 3.5 (c 1, CHCl₃) (lit. [α]₀⁺²² = +8.5 (c 0.2, CHCl₃);¹⁸³ vₘₐₓ(film) /
cm\(^{-1}\) 3465br (OH), 3071w, 3049w, 2986m, 2931s, 2858s, 1589w, 1473m, 1463m, 1428s, 1380m, 1371m, 1246m, 1216m, 1113s, 1080s, 823m, 704s; \(\delta\)(300 MHz, CDCl\(_3\)) 1.06 (s, 9H, (CH\(_3\))\(_3\)C), 1.39 (s, 3H, (CH\(_3\))\(_2\)C), 1.41 (s, 3H, (CH\(_3\))\(_2\)C), 2.03-2.11 (m, 1H, CH\(_2\)OH), 3.61-3.86 (stack, 4H), 3.91-4.01 (m, 1H), 4.03-4.11 (m, 1H), 7.34-7.48 (stack, 6H, Ph), 7.61-7.70 (stack, 4H, Ph); \(\delta\)(100 MHz, CDCl\(_3\)) 19.1 (C, (CH\(_3\))\(_3\)C), [26.8, 26.9, 27.0 (CH\(_3\), (CH\(_3\))\(_3\)C, (CH\(_3\))\(_2\)C]), 62.5 (CH\(_2\), CH\(_2\)O), 64.1 (CH\(_2\), CH\(_2\)O), 77.4 (CH, CHO), 79.5 (CH, CHO), 109.1 (C, (CH\(_3\))\(_2\)C), 127.7 (CH, Ph), 129.8 (CH, Ph), 132.8 (C, ipso Ph), 135.5 (CH, Ph); MS (TOF ES\(^+\)) \(m/z\) 423.3 ([M + Na]\(^+\), 100%); HRMS (TOF ES\(^+\)) calcd for C\(_{23}\)H\(_{32}\)O\(_4\)SiNa [M + Na]\(^+\) 423.1968, found 423.1956.

Data were in agreement with those reported in the literature.\(^{183,184}\)
(2S,3S,4R)-2-Azido-1-O-[4\'-O-tert-butyldiphenylsilyl-2',3'-O-isopropylidene-L-threitol]-3,4-O-isopropylidene-octadecane-1,3,4-triol (86)

\[
\text{TF}_2\text{O} \quad 235 \ \mu\text{L}, \ 1.40 \ \text{mmol}
\]
was added dropwise over 10 min to a solution of alcohol 92 (561 mg, 1.40 mmol) and 2,6-di-tert-butylpyridine (346 \ \mu\text{L}, 1.54 mmol) in CH\textsubscript{2}Cl\textsubscript{2} (14 mL) at 0 °C. After 30 min, the reaction mixture was diluted with CH\textsubscript{2}Cl\textsubscript{2} (15 mL) and the resulting solution washed sequentially with cold H\textsubscript{2}O (2 \times 30 mL) and brine (10 mL), dried (Na\textsubscript{2}SO\textsubscript{4}) and filtered. Removal of the solvent under reduced pressure provided crude triflate 87 as a colourless oil [\textit{R}_f = 0.7 (15\% EtOAc in hexanes)], which was used immediately in the next etherification step. A solution of alcohol 78 (505 mg, 1.32 mmol) in THF (10 mL) was treated with NaH (60\% in mineral oil, 56.0 mg, 1.40 mmol) at 0 °C. After 1 h, a solution of triflate 87 (assuming 100\% conversion, 1.40 mmol) in THF (5 mL) was added dropwise over 5 min. The resulting solution was stirred at this temperature for 1 h and then at r.t. for 12 h. The reaction was then quenched by the addition of MeOH (2 mL) followed by NaHCO\textsubscript{3} solution (10 mL). The phases were separated and the aqueous phase was extracted with CH\textsubscript{2}Cl\textsubscript{2} (3 \times 20 mL). The combined organic fractions were washed with brine (15 mL) and dried (Na\textsubscript{2}SO\textsubscript{4}), filtered and the solvent was removed under reduced pressure. The residue was purified by flash
Chapter 5  Experimental Section

column chromatography (eluent: 5% EtOAc in hexanes) $R_f = 0.2$ (5% EtOAc in hexanes) to provide ether 86 as a colourless oil (815 mg, 80%); $R_f = 0.6$ (10% EtOAc in hexane); $[\alpha]^2_{D} = +10.0$ (c 1, CHCl$_3$); $\nu_{\text{max}}$(film) / cm$^{-1}$ 2925s, 2855s, 2098s (N$_3$), 1589w, 1463m, 1428m, 1379m, 1369s, 1218s, 1112s, 1082s, 822s, 701s; $\delta_{H}$(300 MHz, CDCl$_3$): 0.88 (t, $J$ 7.0, 3H, CH$_2$CH$_3$), 1.08 (s, 9H, C(CH$_3$)$_3$), 1.20-1.43 (stack, 35H, alkyl chain methylenes and 4 $\times$ acetonide CH$_3$), 1.49-1.63 (stack, 3H), 3.58-3.75 (stack, 4H), 3.76-3.88 (stack, 3H), 3.91-3.99 (stack, 2H), 4.10-4.24 (stack, 2H), 7.34-7.47 (stack, 6H, Ph), 7.64-7.72 (stack, 4H, Ph); $\delta_{c}$(100 MHz, CDCl$_3$) 14.1 (CH$_3$, CH$_2$CH$_3$), 19.2 (C, C(CH$_3$)$_3$), 22.7 (CH$_2$), 25.7 (CH$_3$, 1 $\times$ CH$_3$ from 1,2-anti-diol acetonide), 26.4 (CH$_2$), 26.8 (CH$_3$, 1 $\times$ CH$_3$ from 1,2-syn-diol acetonide), 27.0 (CH$_3$, C(CH$_3$)$_3$), 27.1 (CH$_3$, 1 $\times$ CH$_3$ from 1,2-syn-diol acetonide), 28.2 (CH$_3$, 1 $\times$ CH$_3$ from 1,2-anti-diol acetonide), [29.3, 29.7 (CH$_2$, alkyl chain, some resonance overlap)], 31.9 (CH$_2$), 60.0 (CH), 64.2 (CH$_2$), 72.4 (CH$_2$), 80.0 (CH$_2$), 75.8 (CH), 77.8 (CH), 77.9 (2 $\times$ CH), 108.2 (C, (CH$_3$)$_2$C), 109.4 (C, (CH$_3$)$_2$C), 127.7 (CH, Ph), 129.7 (CH, Ph), 133.2 (C, ipso Ph), 135.6 (CH, Ph); MS (TOF ES+) $m/z$ 788.7 ([M + Na]$^+$, 100%); HRMS (TOF ES+) calcd for C$_{44}$H$_{71}$N$_3$O$_6$SiNa [M + Na]$^+$ 788.5010, found 788.5015.
(2S,3S,4R)-2-Amino-1-O-[4'-O-tert-butylidiphenylsilyl-2',3'-O-isopropylidene-L-threitol]-3,4-O-isopropylidene-octadecane-1,3,4-triol (85)

PMe$_3$ (0.7 mL of a 1.0 M solution in THF, 0.7 mmol) was added dropwise over 2 min to a solution of azide 86 (500 mg, 0.65 mmol) in THF / H$_2$O (7 mL, 15:1). The reaction mixture was stirred at r.t. for 4 h and then concentrated under reduced pressure. The residual H$_2$O was removed by co-evaporation with toluene (3 x 3 mL) to provide the crude amine product. Purification of the residue by flash column chromatography (30% EtOAc in hexane) afforded amine 85 as a colourless oil (433 mg, 90%): $R_f$ = 0.3 (30% EtOAc in hexane); [$\alpha$]$^20_D$ = +45.6 (c 1, CHCl$_3$); $\nu_{\text{max}}$(film) / cm$^{-1}$ 3076w, 1113s, 1083s, 702s; $\delta_H$(300 MHz, CDCl$_3$) 0.88 (t, J 7.0, 3H, H-18), 1.08 (s, 9H, C(CH$_3$)$_3$), 1.20-1.43 (stack, 35H, alkyl chain methylenes and acetonide CH$_3$), 1.49-1.63 (stack, 3H), 3.58-3.75 (stack, 4H), 3.76-3.88 (stack, 3H), 3.91-3.99 (stack, 2H), 4.10-4.24 (stack, 2H), 7.34-7.47 (stack, 6H, Ph), 7.64-7.72 (stack, 4H, Ph); $\delta_C$(100 MHz, CDCl$_3$): 14.1 (CH$_3$, C-18), 19.2 (CH$_2$), 22.7 (CH$_2$), 25.7 (CH$_3$), 26.4 (CH$_2$), [26.8, 27.0, 27.1, 28.2 (CH$_3$, C(CH$_3$)$_2$, C(CH$_3$)$_3$, some resonance overlap], [29.3, 29.7 (CH$_2$, alkyl chain, some resonance overlap)], 31.9 (CH$_2$), 60.0 (CH), 64.2 (CH$_2$), 72.4 (CH$_2$), 80.0 (CH$_2$), 75.8 (CH), 77.8 (CH), 77.9 (2 x CH), 108.2 (C, (CH$_3$)$_2$C), 109.4 (C, (CH$_3$)$_2$C),
[127.7, 129.7 (CH, Ph, some resonance overlap)], 133.2 (C, ipso Ph), 135.6 (CH, Ph, some resonance overlap); MS (TOF ES+) m/z 740.6 ([M + H]⁺, 100%); HRMS (TOF ES+) calcd for C₄₄H₇₄NO₆Si [M + H]⁺ 740.5285, found 740.5293.

(2S,3S,4R)-1-O-[4'-O-tert-Butyldiphenylsilyl-2',3'-O-isopropylidene-L-threitol]-2-Hexacosanoylamino-3,4-O-isopropylidene-octadecane-1,3,4-triol (94)

A screw-capped glass tube containing a solution of hexacosanoic acid (163 mg, 0.41 mmol) in (COCl)₂ (2.0 mL, 23 mmol) was closed tightly and heated at 70 ºC for 2 h. The volatiles were then evaporated under a stream of argon and the tube then placed on a high vacuum line for at least 2 h to remove the residual volatiles. The resulting hexacosanoyl chloride 47 was used directly without further purification: a solution of freshly prepared hexacosanoyl chloride 47 (187 mg, 0.41 mmol) in CH₂Cl₂ (1 mL) was added dropwise over 2 min to a solution of amine 85 (250 mg, 0.34 mmol) and NEt₃ (95 µL, 0.68 mmol) in CH₂Cl₂ (3 mL) at 0 ºC. The reaction mixture was stirred at r.t. for 12 h and then diluted with CH₂Cl₂ (10 mL), washed sequentially with NaHCO₃ solution (10 mL), brine (2 mL) and then dried.
over Na₂SO₄. The drying agent was removed by filtration and the filtrate concentrated under reduced pressure. Purification of the residue by flash column chromatography provided amide 94 as a colourless oil (323 mg, 85%): \( R_f = 0.3 \) (10% EtOAc in hexane); \([\alpha]^{20}_D = +11.8 \) (c 1, CHCl₃); \( \nu_{\text{max}} \) (film) / cm\(^{-1}\) 1646m (C=O); \( \delta_\text{H} \) (300 MHz, CDCl₃) 0.88 (t, \( J = 7.0 \), 6H, 2 \( \times \) CH₂CH₃), 1.06 (s, 9H, C(CH₃)₃), 1.16-1.36 (stack, 69H, including (1.32 (s, 3H, C(CH₃)₂)), alkyl chain methylenes, 1 \( \times \) C(CH₃)₂), 1.40 (s, 3H, C(CH₃)₂), 1.41 (s, 3H, C(CH₃)₂), 1.42 (s, 3H, C(CH₃)₂), 1.45-1.66 (stack, 6H), 2.05-2.18 (stack, 2H), 3.49-3.67 (stack, 3H), 3.74-3.89 (stack, 4H), 4.00-4.23 (stack, 4H), 5.71 (br d, \( J = 9.2 \), 1H), 7.34-7.48 (stack, 6H, Ph), 7.63-7.72 (stack, 4H, Ph); \( \delta_\text{C} \) (100 MHz, CDCl₃) 14.1 (CH₃, C-18, C-26”), 22.7 (CH₂), 25.7 (CH₂), 25.8 (CH₃, C(CH₃)₂), 26.5 (CH₂), 26.9 (CH₃, C(CH₃)₃), 27.1 (CH₃, C(CH₃)₂), 27.2 (CH₃, C(CH₃)₂), 28.1 (CH₃, C(CH₃)₂), 29.1 (CH₂), [29.33, 29.37, 29.38, 29.43, 29.67, 29.68, 29.69, 29.71, 29.73 (CH₂, alkyl chain, some resonance overlap)], 31.9 (CH₂), 37.0 (CH₂), 48.2 (CH), 64.2 (CH₂), 71.3 (CH₂), 72.6 (CH₂), 76.0 (CH), 77.8 (CH), 77.9 (CH), 78.1 (CH), 107.9 (C, (CH₃)₂C), 109.5 (C, (CH₃)₂C), [127.75, 127.76, 129.79, 129.82, (CH, Ph, some resonance overlap)], 133.1 (C, ipso Ph), [135.61, 135.62 (CH, Ph, some resonance overlap)], 172.4 (C, C=O); MS (TOF ES+) \( m/z \) 1140.5 ([M + Na]⁺, 100%); HRMS (TOF ES+) calcd for C₇₀H₁₂₃NO₇Na [M + Na]⁺ 1140.8967, found 1140.8977.
(2S,3S,4R)-1-O-[4′-O-tert-Butyldiphenylsilyl-2′,3′-O-isopropylidene-L-threitol]-2-hexacosanethioylamino-3,4-O-isopropylidene-octadecane-1,3,4-triol (97)

Lawesson's reagent (81 mg, 0.2 mmol) was added to a solution of amide 94 (145 mg, 0.13 mmol) in toluene (2 mL) at r.t. The reaction mixture was stirred at 80 °C for 5 h and then the solvent was removed under reduced pressure. The residue was diluted with CH₂Cl₂ (10 mL), washed sequentially with H₂O (5 mL), NaHCO₃ solution (10 mL), brine (2 mL) and then dried over Na₂SO₄. The drying agent was removed by filtration and the filtrate was concentrated under reduced pressure. Purification of the residue by flash column chromatography (15% EtOAc in hexane) provided thioamide 97 as a pale yellow oil (130 mg, 88%): \( R_f = 0.3 \) (15% EtOAc in hexane); \( [\alpha]^{20}_D = +36.8 \) (c 0.5, CHCl₃); \( \nu_{\text{max}} \) (film) / cm⁻¹ 1258 s, 1083 s, 736 s, 702 s; \( \delta_\text{H}(300 \text{ MHz, CDCl}_3) \) 0.88 (t, J 7.0, 6H, 2 × CH₂CH₃), 1.06 (s, 9H, C(CH₃)₃), 1.17-1.35 (72H, stack, including (1.31 (s, 3H, C(CH₃)₂), alkyl chain methylenes, 1 × C(CH₃)₂), 1.39 (s, 3H, C(CH₃)₂), 1.41 (s, 3H, C(CH₃)₂), 1.42 (s, 3H, C(CH₃)₂), 1.44-1.58 (stack, 3H), 2.49-2.67 (stack, 2H), 3.49-3.58 (m, 1H), 3.61-3.72 (stack, 2H), 3.74-3.91 (stack, 5H), 4.05-4.21 (stack, 2H), 4.28-4.35 (dd, J 7.7, 5.9, 1H), 4.77-4.88 (m, 1H), 7.30-7.49 (stack, 6H, Ph), 7.60-7.75 (stack, 4H, Ph); \( \delta_\text{C}(100 \text{ MHz, CDCl}_3) \) 14.1 (CH₃, C-18, C-26”), 22.7 (CH₂), 25.6 (CH₃,
C(CH$_3$)$_2$, 26.7 (CH$_2$), 26.9 (CH$_3$, C(CH$_3$)$_3$), 27.0 (CH$_3$, C(CH$_3$)$_2$), 27.2 (CH$_3$, C(CH$_3$)$_2$), 27.7 (CH$_3$, C(CH$_3$)$_2$), 29.0 (CH$_2$), [29.37, 29.42, 29.56, 29.59, 29.61, 29.7 (CH$_2$, alkyl chain, some resonance overlap)], 31.9 (CH$_2$), 47.6 (CH$_2$), 54.6 (CH), 64.2 (CH$_2$), 69.7 (CH$_2$), 72.6 (CH$_2$), 75.5 (CH), 77.7 (CH), 77.8 (CH), 77.9 (CH), 108.1 (C, (CH$_3$)$_2$C), 109.5 (C, (CH$_3$)$_2$C), [127.8, 129.8, (CH, Ph, some resonance overlap)], 133.1 (C, ipso Ph), 135.6 (CH, Ph, some resonance overlap), 205.5 (C, C=S); MS (TOF ES$^+$) m/z 1156.8 ([M + Na]$^+$, 100%); HRMS (TOF ES$^+$) calcd for C$_{70}$H$_{123}$NO$_6$SiSNa [M + Na]$^+$ 1156.8738, found 1156.8749.

(2S,3S,4R)-1-O-[L-Threitol]-2-(hexacosanylcarbonylamino)octadecane-1,3,4-triol (24)

TBAF (1.0 M solution in THF, 120 mL, 0.12 mmol) was added to a solution of silyl ether 94 (123 mg, 0.11 mmol) in THF (1 mL) at r.t. After 4 h, NH$_4$Cl solution (10 mL) was added. The phases were separated and the aqueous phase was extracted with CH$_2$Cl$_2$ (3 × 10 mL). The solvent was removed under reduced pressure to provide the corresponding primary alcohol as a white solid (97 mg,
quart.), which was dissolved in CH$_2$Cl$_2$ / CH$_3$OH (10:1, 1.1 mL) and treated with TFA (0.5 mL; dropwise addition over 1 min). After stirring for 2 h at r.t., the reaction mixture was concentrated under reduced pressure and the residual TFA was removed by co-evaporation with Et$_2$O (3 × 4 mL). Purification of the residue by flash column chromatography (5% CH$_3$OH in CHCl$_3$) afforded ThrCer 24 as a white-yellow solid (65 mg, 74%): $R_f = 0.3$ (8% MeOH in CHCl$_3$); [α]$_D$ the insolubility of this amphiphilic compound at r.t. prevented us from obtaining reliable optical rotation data; mp 107–109 °C; $\nu$$_{max}$(neat) / cm$^{-1}$ 3308br m (O–H, N–H), 2915s, 2849s, 2098w, 1634m (C=O), 1540m, 1471m, 1108m, 1070m, 1026m, 718m; $\delta$$_{H}$(500 MHz, d$_8$-THF, 45 °C) 0.89 (app t, $J$ 6.7, 6H, 2 × CH$_2$CH$_3$), 1.25-1.63 (stack, 72H, alkyl chain), 2.12 (t, $J$ 7.7, 2H, C(O)CH$_2$), 3.40-3.47 (stack, 2H, H-3', H-4'), 3.47-3.57 (stack, 5H, C(1)H$_2$, C(4)H$_2$, H-2 or H-3), 3.59-3.63 (m, 1H, C(1')H$_a$H$_b$), 3.67-3.72 (stack, 2H, C(1')H$_a$H$_b$, H-3 or H-2), 4.14-4.18 (m, 1H, H-2'), 6.93-6.96 (m, 1H, NH); $\delta$$_{C}$(125 MHz, d$_8$-THF, 45 °C) 14.3 (CH$_3$, 2 × CH$_2$CH$_3$), 26.6 (CH$_2$), 26.8 (CH$_2$), 29.3 (CH$_2$), 30.3 (CH$_2$), 30.5 (CH$_2$), 30.59 (CH$_2$), 30.64 (CH$_2$), 30.7 (CH$_2$), 30.8 (CH$_2$), 32.8 (CH$_2$), 34.1 (CH$_2$) alkyl chain methylenes, resonance overlap], 36.9 (CH$_2$, C(O)CH$_2$), 51.8 (CH, C-2'), 64.6 (CH$_2$, C-4), 71.3 (CH, C-2 or C-3), 71.5 (CH$_2$, C-1'), 73.0 (CH, C-4'), 73.1 (CH, C-3 or C-2), 74.1 (CH$_2$, C-1), 76.7 (CH, C-3'), 173.0 (C, C=O); MS (TOF ES+) m/z 822.7 ([M+Na]$^+$, 100%); HRMS (TOF ES+) calcd for C$_{48}$H$_{97}$NO$_7$Na [M+Na]$^+$ 822.7163, found 822.7175.

Data were in agreement with those reported in the literature.$^{124}$
(2'S,3'S,4'R)-2'-Hexacosanethioylamino-1'-O-[L-threitol]-octadecane-1',3',4'-triol (38)

TBAF (1.0 M solution in THF, 120 µL, 0.12 mmol) was added to a solution of silyl ether 97 (125 mg, 0.11 mmol) in THF (1 mL) at r.t. After 4 h, NH₄Cl solution (10 mL) was added. The phases were separated and the aqueous phase was extracted with CH₂Cl₂ (3 × 10 mL). The solvent was removed under reduced pressure to provide the resulting primary alcohol as a white solid (98 mg, quant.), which was dissolved in CH₂Cl₂ / CH₃OH (10:1, 1.1 mL) and treated with TFA (0.5 mL; dropwise addition over 1 min). After stirring for 2 h at r.t., the reaction mixture was concentrated under reduced pressure and the residual TFA was removed by co-evaporation with Et₂O (3 × 4 mL). Purification of the residue by flash column chromatography (5% CH₃OH in CHCl₃) afforded pentaol 38 as a pale yellow solid (65 mg, 73%): $R_f = 0.3$ (8% CH₃OH in CHCl₃); $[\alpha]_D$ the insolubility of this amphiphilic compound at r.t. prevented us from obtaining reliable optical rotation data; mp 96 – 97 °C; $\nu_{\text{max}}$(film) / cm⁻¹ 3324s br (O–H); $\delta_H$(500 MHz, CDCl₃:CD₃OD, 2:1) 0.83 (t, $J$ 7.0, 6H, 2 × CH₂CH₃), 1.11-1.41 (stack, 69H, alkyl chain methylenes), 1.43-1.74 (stack, 3H), 2.60 (t, $J$ 8.1, 2H, C(3")H₂), 3.48-3.54 (stack, 3H, C(1')H₂, H-4), 3.55-3.63 (stack, 3H, C(4')H₂, H-3'), 3.64-3.72 (stack, 2H,
C(1)HₐHₜ, H-3), 3.74-78 (m, 1H, H-2'), 3.80-3.86 (m, 1H, C(1)HₐHₜ), 4.83-4.88 (m, 1H, H-2); 26.2 (CH₂), 29.3 (CH₂), [29.6, 29.7, 29.8, 30.0 (CH₂, alkyl chain, resonance overlap)], 32.2 (CH₂), 32.7 (CH₂, C-5), 46.9 (CH₂, C-2'), 56.1 (CH, C-2), 63.7 (CH₂, C-4'), 69.6 (CH₂, C-1), 70.6 (CH, C-2'), 72.2 (CH, C-3'), 73.0 (CH₂, C-4), 73.2 (CH₂, C-1'), 74.1 (CH, C-3), 205.9 (C, C=S); MS (TOF ES+) m/z 838.7 ([M + Na]⁺, 100%); HRMS (TOF ES+) calcd for C₅₀H₉₉NO₈SNa [M + Na]+ 838.6934, found 838.6946.

(2'S,3'S,4'R)-3',4'-Di-O-acetyl-1'-O-(2,3,4-tri-O-acetyl-L-threitol)-2'-(hexacosa-noylamino)octadecane-1',3',4'-triol (95)

Ac₂O (420 µL, 4.5 mmol) was added dropwise over 1 min to a solution of ThrCer 24 (120 mg, 0.15 mmol) in pyridine (2 mL). The reaction mixture was stirred at r.t. for 10 h, after which time, the volatiles were removed under reduced pressure. The residue was diluted with CH₂Cl₂ (10 mL), washed sequentially with H₂O (5 mL), NaHCO₃ solution (10 mL), brine (3 mL) and then dried over Na₂SO₄. The drying
agent was removed by filtration and the filtrate was concentrated under reduced pressure. The crude product was purified by flash column chromatography (25% EtOAc in hexane) to afford hexa-acetate 95 as a white solid (144 mg, 95%): \( R_f = 0.26 \) (25\% EtOAc in hexane); \([\alpha]^{20}_D = +18.4 (c 1, CHCl_3); \nu_{\max}(film) / \text{cm}^{-1} 2917 \text{ s}, 2850 \text{ s}, 1739 \text{ s}, 1540 \text{ w}, 1371 \text{ m}, 1046 \text{ m}, 978 \text{ w}, 720 \text{ m}, 682 \text{ m}; \delta_{\nu} (500 MHz, CDCl_3) 0.88 (t, J 7.1, 6H, CH_2CH_3), 1.21-1.33 (stack, 6H, alkyl chain methylenes), 1.53-1.72 (stack, 4H, C(5')H_aH_b, C(2')H_aH_b), 2.01 (s, 3H, C(4')COCH_3), 2.05 (s, 3H, C(4)COCH_3), 2.08 (stack, 6H, C(2)COCH_3, C(3)COCH_3), 2.10 (s, 3H, C(4')COCH_3), 2.22 (t, J 7.6, 2H, C(1')H_aH_b), 3.41 (A of ABX, dd, J 9.7, 3.2, 1H, C(1')H_aH_b), 3.43-3.48 (stack, 2H, C(1)H_aH_b, C(1')H_aH_b), 3.53 (B of ABX, dd, J 10.9, 5.6, 1H, C(1)H_aH_b), 4.04 (dd, J 11.8, 6.8, 1H, C(4)H_aH_b), 4.26-4.32 (stack, 2H, C(4)H_aH_b, H2'), 4.89-4.93 (m, 1H, H4'), 5.12-5.18 (stack, 2H, H2, H3'), 5.28-5.33 (m, 1H, H3), 6.21 (1H, br d, J 9.2, NH); \( \delta_c(125 \text{ MHz}): 14.1 \) (CH, C18', C25''), 20.7 (CH, (C4)COCH_3), 20.75 (2 x CH_3, (C2)COCH_3, (C3)COCH_3), 20.8 (CH_3, (C3')COCH_3), 21.0 (CH_3, (C4')COCH_3), 22.7 (CH_2), (alkyl chain, some resonance overlap)], 25.6 (CH_2), 25.7 (CH_2, C2''), 28.0 (CH_2, C5'), [29.3-29.4 (CH_2, alkyl chain, some resonance overlap)], [29.4, 29.56, 29.58, 29.7 (CH_2, alkyl chain, some resonance overlap)], 31.9 (CH_2), 36.6 (CH_2, C1'''), 47.9 (CH, C2''), 62.2 (CH_2, C4), 69.17 (CH_2, C1), 69.24 (CH, C3), 69.66 (CH, C2), 69.72 (CH_2, C1''), 71.8 (CH, C3'), 73.2 (CH, C4'), 169.8 (C, (C3')COCH_3), 170.2 (2 x C, (C2)COCH_3, (C3)COCH_3), 170.5 (C, (C4)COCH_3), 170.8 (C, (C4')COCH_3), 173.0 (C, C=O); MS (TOF ES+) \( m/z 1032.6 ([M+Na]^+) \), 100%; HRMS (TOF ES+) calcd for C_{68}H_{120}N_2O_7SiNa ([M+Na]^+) 1032.7691, found 1032.7698.
(2'S,3'S,4'R)-3,4-Di-O-acetyl-1-O-(2,3,4-tri-O-acetyl-L-threitol)-2'-(hexacosa-thioylamino)octadecane-1',3',4'-triol (96)

[Chemical structure diagram]

Lawesson’s reagent (60 mg, 0.12 mmol) was added to a solution of amide 95 (102 mg, 0.10 mmol) in toluene (2 mL) at r.t. The reaction mixture was stirred at 80 °C for 4 h and then the solvent was removed under reduced pressure. The residue was diluted with CH₂Cl₂ (10 mL), washed sequentially with H₂O (5 mL), NaHCO₃ solution (10 mL), brine (2 mL) and then dried over Na₂SO₄. The drying agent was removed by filtration and the filtrate was concentrated under reduced pressure. Purification of the residue by flash column chromatography (20% EtOAc in hexane) provided thioamide 96 as a pale yellow solid (92 mg, 87%): $R_f = 0.26$ (20% EtOAc in hexane); $[\alpha]_{D}^{20} = +54.0$ (c 1, CHCl₃); $\delta_h$(500 MHz, CDCl₃): 0.88 (t, $J = 7.1$, 6H, CH₂CH₃), [1.19-1.36 (stack, 68H, alkyl chain methylenes)], 1.54-1.64 (m, 1H, C(5')H₆b), 1.66-1.83 (stack, 3H, C(5')H₆a, C(2'')H₆b), [2.04, 2.05, 2.08, 2.09, 2.11, (stack, 15H, 5 x COC₆H₅)], 2.63-2.74 (stack, 2H, C(1'')H₆aH₆b), 3.46 (A of ABX, dd, $J = 11.8$, 6.9, 1H, C(4)H₆aH₆b), 4.04 (A of ABX, dd, $J = 11.8$, 6.9, 1H, C(4)H₆aH₆b), 4.30 (B of ABX, dd, $J = 11.8$, 6.9, 1H, C(4)H₆aH₆b), 4.86-4.91 (m, 1H, H₄'), 5.05-5.12 (m, 1H, H2'), 5.16 (app q, $J = 10.1$, 5.2, 1H, H2), 5.28 (dd, $J = 8.0$, 3.2,
Chapter 5  
Experimental Section

1H, H3'), 5.31-5.33 (m, 1H, H3), 8.07 (br d, J 9.0, 1H, NH); δc (125 MHz): 14.1 (CH₃, C18', C25''), [20.7, 20.8, 21.0 (CH₃, 5 x COCH₃, some resonance overlap), 22.7 (CH₂), 25.5 (CH₂), 28.8 (CH₂, C5'), 29.0 (CH₂), 29.4 (CH₂, C2''), [29.5, 29.6, 29.7, (CH₂, alkyl chain, some resonance overlap)], 31.9 (CH₂), 47.2 (CH₂, C1''), 53.8 (CH, C2'), 62.2 (CH₂, C4), 68.3 (CH₂, C1'), 69.2 (CH, C3), 69.3 (CH₂, C1), 69.6 (CH, C2), 71.5 (CH, C3'), 73.1 (CH, C4'), 169.9 (C, (C3')COCH₃), 170.1 (C, (C2)COCH₃), 170.2 (C, (C3)COCH₃), 170.5 (C, (C4)COCH₃), 170.9 (C, (C4')COCH₃), 206.8 (C, C=S); MS (TOF ES+) m/z 1048.9 ([M + Na]+, 100%); HRMS (TOF ES+) calcd for C₆₈H₁₂₀N₂O₇SiNa ([M+Na]+) 1048.7463, found 1048.7466.
(2S,3S,4R)-1-O-[4'-O-tert-Butyldiphenylsilyl-2',3'-O-isopropyldiene-L-threitol]-3,4-O-isopropyldiene-2-(tetracosanyloxy carbonylamino)octadecane-1,3,4-triol (98)

A solution of carbonic acid, 2,5-dioxo-1-pyrrolidinyl tetracosanyl ester (110 mg, 0.21 mmol) in CH$_2$Cl$_2$ (0.5 mL) was added to a stirred solution of amine 85 (104 mg, 0.14 mmol) and NEt$_3$ (42 µL, 0.3 mmol) in CH$_2$Cl$_2$ (1 mL). The resulting mixture was stirred at r.t. until no mixed carbonate remained as determined by TLC (5 h). The mixture was then diluted with CH$_2$Cl$_2$ (10 mL) and washed sequentially with NaHCO$_3$ solution (10 mL) and brine (10 mL). The organic phase was dried over Na$_2$SO$_4$ and the solvent was evaporated under reduced pressure. Purification of the residue by flash column chromatography (10% EtOAc in toluene) provided carbamate 99 as a colourless oil (135 mg, 86%): $R_f = 0.3$ (10% EtOAc in hexane); $[\alpha]^{22}_D = +28.8$ (c 0.5, CHCl$_3$); $\nu_{\text{max}}$(film) / cm$^{-1}$ 1687 m (C=O); $\delta_i$(500 MHz, CDCl$_3$) 0.88 (t, $J = 7.1$, 6H, 2 x CH$_2$CH$_3$), 1.06 (s, 9H, C(CH$_3$)$_3$), 1.26-1.34 (stack, 70H, alkyl chain methylenes), 1.40 (s, 6H, C(CH$_3$)$_2$), 1.42 (s, 3H, C(CH$_3$)$_2$), 1.59 (s, 3H, C(CH$_3$)$_2$), 3.52-3.65 (stack, 3H, C(1)H$_a$H$_b$, C(1')H$_a$H$_b$), 3.74-3.81 (stack, 3H, C(4')H$_a$H$_b$, C(1)H$_a$H$_b$), 3.82-3.90 (stack, 2H, H-3', H-2), 3.98 (m, 1H, C(1")H$_a$H$_b$), 4.00-4.11 (stack, 3H, H-3, H-4, C(1")H$_a$H$_b$), 4.14-4.20 (m, 1H, H-2'), 4.97 (br d, $J = 9.5$, 1H, NH), 7.36-7.46 (stack, 6H, Ph), 7.66-7.70 (stack, 4H, Ph);
$\delta_C$ (125 MHz, CDCl$_3$) 14.1 (CH$_3$, C-18, C-24$^\prime$), 19.2 (C, (CH$_3$)$_3$CSi), 22.7 (CH$_2$), 25.8 (CH$_3$, C(CH$_3$)$_2$), 25.9 (CH$_2$), 26.4 (CH$_2$), 26.8 (CH$_3$, C(CH$_3$)$_3$), 27.0 (CH$_3$, C(CH$_3$)$_2$), 27.2 (CH$_3$, C(CH$_3$)$_2$), 28.1 (CH$_3$, C(CH$_3$)$_2$), 28.9 (CH$_2$), 29.1 (CH$_2$), 29.4 (CH$_2$), [29.6, 29.7 (CH$_2$, alkyl chains, some resonance overlap)], 31.9 (CH$_2$), 50.3 (CH, C-2), 64.1 (CH$_2$, C-4$'$), 65.2 (CH$_2$, C-1$''$), 71.6 (CH$_2$, C-1), 72.6 (CH$_2$, C-1$'$), 75.9, (CH, C-3), 77.7 (CH, C-2$'$), 77.8 (CH, C-4), 78.2 (CH, C-3$'$), 107.8 (C, (CH$_3$)$_2$C), 109.4 (C, (CH$_3$)$_2$C), [127.7, 129.75, 129.79, (CH, Ph, some resonance overlap)], 133.2 (C, ipso Ph), 135.6 (CH, Ph, some resonance overlap), 156.0 (C, C=O); MS (TOF ES$^+$) \textit{m/z} 1142.7 ([M + Na]$^+$, 100%); HRMS (TOF ES$^+$) calcd for C$_{69}$H$_{121}$NO$_8$SiNa [M + Na]$^+$ 1142.8759, found 1142.8767.
(2S,3S,4R)-2-Tetracosanyloxycarbonylamino-1-O-[L-threitol]-octadecane-1,3,4-triol (40)

TBAF (1.0 M solution in THF, 180 μL, 0.18 mmol) was added to a solution of silyl ether 99 (179 mg, 0.16 mmol) in THF (1.5 mL) at r.t. After 4 h, NH₄Cl solution (10 mL) was added. The phases were separated and the aqueous phase was extracted with CH₂Cl₂ (3 × 10 mL). The solvent was removed under reduced pressure to provide the crude primary alcohol as a white solid (141 mg, quant.), which was dissolved in CH₂Cl₂ / CH₃OH (10:1, 1.2 mL) and treated with TFA (0.6 mL; dropwise addition over 1 min). After stirring for 2 h at r.t., the reaction mixture was concentrated under reduced pressure and the residual TFA was removed by co-evaporation with Et₂O (3 × 4 mL) to provide the crude product, which was purified by flash column chromatography (10% CH₃OH in CHCl₃) to afford pentaol 40 as a pale yellow solid (90 mg, 70%): \( R_f = 0.3 \) (10% CH₃OH in CHCl₃); \([\alpha]_D\) the insolubility of this amphiphilic compound at r.t. prevented us from obtaining reliable optical rotation data; mp 55 – 56 °C; \( \nu_{\text{max}}(\text{film}) / \text{cm}^{-1} \) 3340 m br (O–H), 1683 s (C=O); \( \delta_{\text{H}}(500 \text{ MHz, CDCl₃:CD₃OD, 2:1}) \) 0.83 (t, \( J = 7.2 \), 6H, \( 2\times \text{CH₂CH₃} \)), 1.16-1.45 (stack, 65H, alkyl chain methylenes), 1.46-1.68 (stack, 5H), 3.12-3.20 (m, 1H), 3.49-3.64 (stack, 8H), 3.67-3.79 (stack, 2H), 3.85-4.07 (stack, 3H), OH
resonances not observed; $\delta_{\text{C}}$(125 MHz, CDCl$_3$:CD$_3$OD, 2:1) 14.2 (CH$_3$, 2 × CH$_2$CH$_3$), 20.1 (CH$_2$), 23.0 (CH$_2$), 24.1 (CH$_2$), 26.2 (CH$_2$), [29.5, 29.7, 30.0, 30.4, (CH$_2$, alkyl chains, some resonance overlap)], 32.3 (CH$_2$), 32.9 (CH$_2$), 52.1 (CH), 63.9 (CH$_2$), 65.8 (CH$_2$), 70.8 (CH), 71.2 (CH$_2$), 72.5 (CH), 72.8 (CH), 73.4 (CH$_2$), 75.5 (CH), 157.7 (C, C=O); MS (TOF ES+) $m/z$ 824.8 ([M + Na]$^+$, 100%); HRMS (TOF ES+) calcd for C$_{50}$H$_{99}$NO$_8$SNa [M + Na]$^+$ 809.6955, found 824.6940.

(2S,3S,4R)-1-O-[4'-O-tert-Butyldiphenylsilyl-2',3'-O-isopropylidene-L-threitol]-3,4-O-isopropylidene-2-(tricosanylaminocarbonylamino)-octadecane-1,3,4-triol (98)

A solution of amine 85 (155 mg, 0.21 mmol) in toluene (1.5 mL) was added to a solution of tricosanyl isocyanate 48 (121 mg, 0.33 mmol; prepared as reported previously (see page 198) in the synthesis of urea 77) in toluene (1 mL) at r.t. The reaction mixture was heated under reflux for 8 h and then concentrated under reduced pressure to provide the crude product. Purification of the residue by flash column chromatography (15% EtOAc in hexane) afforded urea 98 as a pale yellow
oil (186 mg, 80% based on amine): \( R_f = 0.3 \) (10% EtOAc in hexane); [\( \alpha \)]\( ^{22} \)D = +48.8 (c 1, CHCl\(_3\)); \( \nu_{\text{max}}(\text{film}) / \text{cm}^{-1} \) 1632m (C=O); \( \delta_{\text{H}} \)(500 MHz, CDCl\(_3\)) 0.88 (t, \( J = 7.1 \), 6H, 
\( 2 \times \) CH\(_2\)CH\(_3\)), 1.06 (s, 9H, C(CH\(_3\))\(_3\)), 1.21-1.32 (stack, 68H, alkyl chain methylenes), 1.39 (s, 3H, \( 1 \times \) C(CH\(_3\))\(_2\)), 1.40 (s, 3H, \( 1 \times \) C(CH\(_3\))\(_2\)), 1.41 (s, 3H, \( 1 \times \) C(CH\(_3\))\(_2\)), 1.61 (s, 3H, \( 1 \times \) C(CH\(_3\))\(_2\)), 3.03-3.17 (stack, 2H, C(1")H\(_a\)H\(_b\)), 3.53-3.60 (stack, 2H, C(1")H\(_a\)H\(_b\), C(1)H\(_a\)H\(_b\)), 3.65 (dd, \( J = 10.5 \), 3.2, 1H, C(1")H\(_a\)H\(_b\)), 3.72-3.80 (stack, 3H, C(4")H\(_a\)H\(_b\), C(1)H\(_a\)H\(_b\)), 4.04-4.11 (stack, 2H, H-3, H-4), 4.13-4.18 (m, 1H, H-2'), 4.31 (br s, 1H, CH\(_2\)NH), 4.49 (br d, \( J = 9.2 \), 1H, CHN\(_H\)), 7.37-7.46 (stack, 6H, Ph), 7.64-7.70 (stack, 4H, Ph); 
\( \delta_{\text{C}}(125 \text{ MHz, CDCl}_3) \) 14.1 (CH\(_3\), \( 2 \times \) CH\(_2\)CH\(_3\)), 19.2 (C, C(CH\(_3\))\(_3\)), 22.7 (CH\(_2\)), 25.8 (CH\(_3\), \( 1 \times \) C(CH\(_3\))\(_2\)), 26.4 (CH\(_2\)), 26.8 (CH\(_3\), C(CH\(_3\))\(_3\)), 26.9 (CH\(_2\)), 27.1 (CH\(_3\), \( 1 \times \) C(CH\(_3\))\(_2\)), 27.2 (CH\(_3\), \( 1 \times \) C(CH\(_3\))\(_2\)), 28.1 (CH\(_3\), \( 1 \times \) C(CH\(_3\))\(_2\)), 29.1 (CH\(_2\)), [29.4, 29.7 (CH\(_2\), alkyl chain, resonance overlap)], 30.2 (CH\(_2\)), 31.9 (CH\(_2\)), 40.6 (CH\(_2\), C-1''), 49.7 (CH, C-2), 64.3 (CH\(_2\), C-4'), 72.0 (CH\(_2\), C-1), 72.7 (CH\(_2\), C-1'), 76.3 (CH, C-3), 77.3 (CH, C-2'), 77.9 (CH, C-4), 78.2 (CH, C-3'), 107.8 (C, C(CH\(_3\))\(_2\)), 109.5 (C, C(CH\(_3\))\(_2\)), 127.8 (CH, Ph), 129.8 (CH, Ph), 133.1 (C, ipso Ph), 135.6 (CH, Ph), 157.4 (C, C=O); MS (TOF ES+) \( m/z \) 1127.7 ([M + Na]\(^{+}\), 100%); HRMS (TOF ES+) calcd for C\(_{68}H_{129}N_2O_7SiNa\) [M + Na]\(^{+}\) 1127.8763, found 1127.8737.
(2S,3S,4R)-1-O-[L-Threitol]-2-(tricosylaminocarbonylamino)octadecane-1,3,4-triol (39)

TBAF (1.0 M solution in THF, 170 µL, 0.17 mmol) was added to a solution of silyl ether 98 (167 mg, 0.15 mmol) in THF (1.5 mL) at r.t. After 4 h, NH₄Cl solution (10 mL) was added. The phases were separated and the aqueous phase was extracted with CH₂Cl₂ (3 × 10 mL). The solvent was removed under reduced pressure to provide a white solid (primary alcohol product, 130 mg, quant.), which was dissolved in CH₂Cl₂ / CH₃OH (10:1, 1.2 mL). TFA (0.6 mL) was added dropwise over 1 min. After stirring for 2 h at r.t., the reaction mixture was concentrated under reduced pressure and the residual TFA was removed by co-evaporation with Et₂O (3 × 4 mL) to provide the crude product, which was purified by flash column chromatography (10% CH₃OH in CHCl₃) to afford the pentaol 39 as a white solid (85 mg, 72%): \( R_f = 0.3 \) (10% CH₃OH in CHCl₃); \([\alpha]_D\) the insolvibility of this amphiphilic compound at r.t. prevented us from obtaining reliable optical rotation data; mp 131 – 132 °C; \( \nu_{\text{max}}(\text{film}) / \text{cm}^{-1} \) 3344s br (O–H), 1607s (C=O); \( \delta_h(500 \text{ MHz, CDCl}_3:\text{CD}_3\text{OD, 2:1}) \) 0.84 (t, J 6.9, 6H, 2 × CH₂CH₃), 1.15–1.55 (stack, 67H, alkyl chain methylenes), 1.58–1.69 (m, 1H), 2.99–3.15
(stack, 2H), 3.46–3.66 (stack, 8H), 3.71–3.80 (stack, 2H), 3.93–3.99 (m, 1H), OH and NH resonances not observed; $\delta_C$ (125 MHz, CDCl$_3$:CD$_3$OD, 2:1) 14.3 (CH$_3$, 2 × CH$_2$CH$_3$), 23.2 (CH$_2$), 26.4 (CH$_2$), 27.4 (CH$_2$), [29.8, 29.9, 30.1, 30.2, 30.3 (CH$_2$, alkyl chain, resonance overlap)], 30.7 (CH$_2$), 32.4 (CH$_2$), 33.5 (CH$_2$), 40.7 (CH$_2$), 51.2 (CH), 64.1 (CH$_2$), 70.7 (CH), 72.1 (CH$_2$), 72.5 (CH), 73.5 (CH), 73.9 (CH$_2$), 75.9 (CH), 159.9 (C, C=O); MS (TOF ES+) m/z 809.8 ([M + Na]$^+$, 100%); HRMS (TOF ES+) calcd for C$_{50}$H$_{99}$NO$_8$SNa [M + Na]$^+$. 809.6959, found 809.6950.

$\alpha$-Bromo hexacosanoic acid (115)

A mixture of hexacosanoic acid (600 mg, 1.51 mmol) and red phosphorus (47 mg, 1.5 mmol) was heated to 95 °C and Br$_2$ (270 µL, 5.29 mmol) was added dropwise over 2 min at this temperature with stirring. The mixture was stirred for 6 h and then cooled to r.t., diluted with Et$_2$O (30 mL) and washed with H$_2$O (30 mL). The aqueous phase was extracted with Et$_2$O (2 × 30 mL), and the combined organic fractions were washed with brine (10 mL), dried with MgSO$_4$, filtered and the solvent was removed under reduced pressure to yield the $\alpha$-bromo acid as a white solid, which was recrystallised from hexanes and used without further purification in the next reaction (481 mg, 67%). $R_f$ = 0.35 (30% EtOAc in hexane); mp 73 - 74
°C (from hexane); \( \nu_{\text{max}} \text{(film)} / \text{cm}^{-1} \): 3300-2600 v br (OH), 2915s, 2849s, 1715m (C=O), 1697s (C=O), 1477w, 1462m, 1429w, 1241w (br), 1164w, 1120w, 906w, 725m, 662m; \( \delta_d \) (300 MHz, CDCl\(_3\)) 0.88 (t, \( J = 6.8, 3\)H, CH\(_2\)CH\(_3\)), 1.21-1.33 (stack, 44H, alkyl chain), 1.95-2.21 (m, 2H, CH\(_2\)CHBr), 4.24 (app t, \( J = 7.6, 1\)H, CH\(_2\)CHBr), OH not observed; \( \delta_s \) (100 MHz, CDCl\(_3\)) 14.1 (CH\(_3\), CH\(_2\)CH\(_3\)), 22.7 (CH\(_2\)), 27.2 (CH\(_2\)), 28.8 (CH\(_2\)), [29.3, 29.4, 29.5, 29.7, 29.8 (CH\(_2\), alkyl chain, resonance overlap)], 32.0 (CH\(_2\)), 34.7 (CH\(_2\), CH\(_2\)CHBr), 45.4 (CH, CH\(_2\)CHBr), 176.0 (C, C=O); \( m/z \) (TOF ES\(^-\)) 475.3 ([M\(^{^{81}}\)Br isotopomer) – H\(^+\), 100%), 473.3 (100, [M\(^{^{79}}\)Br isotopomer) – H\(^+\)). HRMS \( m/z \) (TOF ES\(^-\)) calcd for C\(_{26}\)H\(_{50}\)BrO\(_2\) [M – H]\(^+\) 473.2994, found 473.2989.

\( \alpha \)-Azido hexacosanoic acid (116)

\[ \text{NaN}_3 (690 \text{ mg, 10.6 mmol)} \text{ was added to a solution of } \alpha \text{-bromo acid 115 (481 mg, 1.01 mmol)} \text{ in DMF (5 mL). The reaction mixture was stirred vigorously at 60 °C. After 48 h, the reaction mixture was allowed to cool to r.t., diluted with Et}_2\text{O (20 mL)} \text{ and washed with 1 M hydrochloric acid (50 mL). The phases were separated} \]
and the aqueous phase was extracted with Et₂O (2 × 25 mL). The combined organic fractions were washed with brine (20 mL) and dried with MgSO₄. Removal of the volatiles under reduced pressure and purification of the residue by flash column chromatography (eluent 15% EtOAc in toluene) afforded α-azido acid 116 as a white solid (394 mg, 60% over 2 steps from hexacosanoic acid); Rₚ = 0.2 (30% EtOAc in hexane); mp 77 - 78 °C; νₘₐₓ(neat) / cm⁻¹ 3300-2600w v br (OH), 2915s, 2849s, 2124m (N₃), 1717m (C=O), 1471m, 1256m, 717m; δₜ (300 MHz, CDCl₃) 0.88 (t, J 6.9, 3H, CH₂C₃H), 1.19-1.40 (stack, 41H, CH₂ resonances in alkyl chain), 1.41-1.54 (stack, 3H₃), 1.72-1.96 (stack, 2H, CH₂CHN₃), 4.24 (dd, J 8.3, 5.2, 1H, CH₂CHN₃), OH resonance not observed; δₜ (100 MHz, CDCl₃): 14.1 (CH₃, CH₂CH₃), 22.7 (CH₂), 25.7 (CH₂), 29.1 (CH₂), [29.4, 29.5, 29.7 (CH₂, alkyl chain, significant resonance overlap)], 31.3 (CH₂), 32.0 (CH₂, CH₂CHN₃), 61.9 (CH, CH₂CHN₃), 176.6 (C, C=O); MS (TOF ES−) m/z 436.4 ([M−1]−, 100%); HRMS (TOF ES−) calcd for C₂₆H₅₀N₃O₂ [M−1]− 436.3903, found 436.3920.
(2S,3S,4R)-2-Amino-1-O-[2',3'-O-isopropylidene-L-threitol]-3,4-O-isopropylidene-octadecane-1,3,4-triol (118)

TBAF (1.0 M solution in THF, 1.1 mL, 1.1 mmol) was added to a solution of silyl ether 85 (750 mg, 0.98 mmol) in THF (10 mL) at r.t. After 4 h, NH₄Cl solution (10 mL) was added. The phases were separated and the aqueous phase was extracted with CH₂Cl₂ (3 × 10 mL). The solvent was removed under reduced pressure and the residue purified by flash column chromatography (eluent: 25% EtOAc in hexanes) to provide alcohol 118 as a colourless oil (490 mg, 95%). Rᵣ = 0.35 (30% EtOAc in hexane); [α]D²¹ = 22.0 (c 1, CHCl₃); νmax(film) / cm⁻¹ 3487<br (OH), 2923s, 2098s (N₃), 1458w, 1370m, 1250s, 1219s, 1168m, 1058s, 989m, 846s, 707w; δᵣ(300 MHz, CDCl₃) 0.87 (t, J 6.2, 3H, CH₂CH₃), 1.22-1.34 (stack, 25H₃), 1.40-1.45, (stack, 9H₃), 1.46-1.65 (stack, 4H), 1.99 (dd, J 7.8, 4.8, 1H, OH), 3.51-4.18 (broad stack, 11H₃); δᵣ(100 MHz, CDCl₃) 14.1 (CH₃), 22.7 (CH₂), 25.6 (CH₃, (CH₃)₂C), 26.4 (CH₂), 27.0 (CH₃, (CH₃)₂C, some resonance overlap), 28.1 (CH₃, (CH₃)₂C), [29.4, 29.6 (CH₂, alkyl chain, some resonance overlap)], 31.9 (CH₂), 59.9 (CH), 62.3 (CH₂), 71.9 (CH₂), 72.9 (CH₂), 75.6 (CH), 76.0 (CH), 77.8 (CH), 79.4 (CH), 108.3 (C, (CH₃)₂C)), 109.4 (C, (CH₃)₂C)); MS (TOF ES⁺) m/z
550.5 ([M + Na], 100%); HRMS (TOF ES+) calcd for C_{28}H_{53}N_{3}O_{6}Na [M + Na]^{+} 550.3832, found 550.3853.

(2S,3S,4R)-2-[2''-Azido-hexacosanoylamino]-1-O-[2',3'-O-isopropylidene-L-threitol]octadecane-O-isopropylidene-octadecane-1,3,4-triol (119)

A solution of α-azido acid 116 (44 mg, 0.11 mmol) in (COCl)_{2} (2 mL) was heated at 70 °C. After 2 h, the volatiles were removed under a stream of N\textsubscript{2} and the residual solvent removed on the vacuum line (1 h) to provide the corresponding acid chloride 117 as a pale yellow oil, which was used directly in the next step without further purification (50 mg, quant.): A solution of freshly prepared acid chloride 117 (50 mg, 0.11 mmol) in CH\textsubscript{2}Cl\textsubscript{2} (0.6 mL) was added dropwise over 2 min to a solution of amine 118 (69 mg, 0.14 mmol) and NEt\textsubscript{3} (32 µL, 0.23 mmol) in CH\textsubscript{2}Cl\textsubscript{2} (0.8 mL) at 0 °C. The reaction mixture was stirred at rt for 12 h and then diluted with CH\textsubscript{2}Cl\textsubscript{2} (10 mL), washed sequentially with NaHCO\textsubscript{3} solution (10 mL), brine (2 mL) and then dried over Na\textsubscript{2}SO\textsubscript{4}. The drying agent was removed by filtration and
the filtrate concentrated under reduced pressure. Purification of the residue by flash column chromatography provided amide 119 as a colourless oil (92 mg, 91%, 1:1 mixture of epimers). Data on epimeric mixture: $R_f = 0.3$ (20% EtOAc in hexane); $\nu_{\text{max}}$(film) / cm$^{-1}$ 3309br (OH), 2918s, 2859s, 2109m (N$_3$), 1651m, 1534w, 1467m, 1370m, 1245m, 1218m, 1072m, 872w, 756s, 722m; $\delta_t$(500 MHz, CDCl$_3$) 0.87 (t, J 6.8, 6H, 2 x CH$_2$CH$_2$), 1.20-1.34 (stack, 6H), 1.39-1.43 (stack, 12H), 1.44-1.56 (stack, 2H), 1.74-1.94 (stack, 2H, incl. C(3")H$_2$), 2.25-2.31 (br OH), 3.52-3.61 (stack, 2H, C(1')H$_a$H$_b$ for both epimers, C(1)H$_a$H$_b$ for both epimers), 3.62-3.71 (stack, 2H, C(1')H$_a$H$_b$ for both epimers, C(4)H$_a$H$_b$ for both epimers), 3.74-3.83 (stack, 2H, C(4)H$_a$H$_b$ for both epimers, C(1')H$_a$H$_b$ for both epimers), 3.88-3.95 (stack, 1.5H, C(2")H for epimer 1, C(3)H for both epimers), 3.91-4.04 (stack, 1.5H, C(2")H for epimer 2, C(2)H for both epimers), 4.05-4.20 (stack, 3H, C(2')H for both epimers, C(3')H for both epimers, C(4')H for both epimers), 6.48-6.56 (stack, 1H, NH for both epimers); $\delta_c$(125 MHz, CDCl$_3$): 14.0 (CH$_3$, C18', C26"), 22.6 (CH$_2$), 25.2 (CH$_2$), 25.4 (CH$_2$), [25.62, 25.67 (CH$_3$, C(CH$_3$)$_2$ of 1,2-anti diol for both epimers], 26.4 (CH$_2$), 26.5 (CH$_2$), [26.90, 26.94, 26.97 (CH$_3$, C(CH$_3$)$_2$ of 1,2-syn diol for both epimers (resonance overlap))], [27.8, 27.9 (CH$_3$, C(CH$_3$)$_2$ of 1,2-anti diol for both epimers)], [29.0, 29.1, 29.16, 29.21, 29.27, 29.34, 29.42, 29.44, 29.56, 29.59, 29.6, 29.7 (CH$_2$, alkyl chain, some resonance overlap)], 31.9 (CH$_2$), 32.1 (CH$_2$), 32.2 (CH$_2$), [48.4, 48.6 (CH, C2")], [62.3, 62.4, (CH$_2$, C4)], [64.2, 64.5 (CH, C2")], 71.3 (CH$_2$, C1'), [71.71, 71.73 (CH$_2$, C1)], [75.9, 76.0 (CH, C4')], [76.4, 76.5 (CH, C2)], [77.6, 77.7 (CH, C3')], [79.2, 79.3 (CH, C3)], [108.0, 108.1 (C, (CH$_3$)$_2$C of 1,2-anti diol)], [109.28, 109.30 (C, (CH$_3$)$_2$C of 1,2-syn diol)], [168.8,
168.9 (C, C=O)]; MS (TOF ES+) m/z 943.7 [M + Na]^+, 100%; HRMS (TOF ES+)
calcd for C_{54}H_{104}N_{4}O_{7}Na [M + Na]^+ 943.7803, found 943.7800.

3-(2-(2-(2-t-butoxycarbonyl)aminoethoxy)ethoxy)ethoxy)prop-1-yn=3 (114)

Di-tert-butyl dicarbonate (120 mg, 0.56 mmol) was added to a solution of 3-(2-(2-
(2-aminoethoxy)ethoxy)ethoxy)prop-1-yn=3 (55 mg, 0.28 mmol) and NEt$_3$ (58 µL,
0.42 mmol) in CH$_3$CN (3 mL). After stirring for 6 h at r.t., the volatiles were
removed under reduced pressure and the residue was purified by flash column
chromatography (30% EtOAc in hexane) to afford Boc amide 114 as a dark yellow
oil (74 mg, 92%). $R_f = 0.2$ (30% EtOAc in hexane); $\nu_{\text{max}}$(film) / cm$^{-1}$ 3351br
, 2869m, 1706s (C=O), 1509m, 1455w, 1368m, 1249m, 1218m, 1117s, 1070s,
865w, 755s, 1391w, 1365m, 1271m, 1248m, 1170s, 1095s, 1033m, 864w, 780w;
$\alpha_{H}$(300 MHz, CDCl$_3$) 1.43 (s, 9H, C(CH$_3$_3)), 2.43 (t, J 2.4, 1H, =CH$_2$), 3.26-3.35
(stack, 2H), 3.50-3.57 (stack, 2H), 3.58-3.76 (stack, 8H), 4.18-4.23 (stack, 2H),
5.04 (br s, 1H, NH$_2$); $\alpha_{C}$(100 MHz, MeOD) 28.4 (CH$_3$, C(CH$_3$_3)), 40.4 (CH$_2$), 58.4
(CH$_2$), 69.1 (CH$_2$), 70.2 (CH$_2$), 70.2 (CH$_2$), 70.4 (CH$_2$), 70.5 (CH$_2$), 74.6 (CH), 79.2
(C), 79.6 (C), 154.2 (C, C=O); MS (TOF ES+) m/z 310.1 [M + Na]^+, 100%; HRMS (TOF ES+) calcd for C_{14}H_{25}NO_{5}Na [M + Na]^+ 310.1630, found 310.1617.

**Triazole 121**

CuSO_{4} solution (7 µL of a 0.5 M solution, 7 µmol) and sodium ascorbate solution (30 µL of a 1 M solution, 30 µmol) were added to a solution of azide 119 (65 mg, 0.074 mmol) and alkyne 114 (34 mg, 0.11 mmol) in t-BuOH / H_{2}O (1 mL, 1:1) at r.t. The reaction mixture was heated for 10 h at 50 °C and then diluted with CHCl_{3} (10 mL), and washed with brine (3 mL). The phases were separated and the aqueous layer was extracted with CHCl_{3} (2 × 5 mL). The combined organic layers were dried over Na_{2}SO_{4} and the volatiles were removed under reduced pressure. Purification of the residue by flash column chromatography (gradient: CHCl_{3} to 5% CH_{3}OH in CHCl_{3}) afforded 1,2,3-triazole 121 as a colourless oil (78 mg, 87%,
isolated as a 2:1 mixture of epimers. Data on epimeric mixture: \( R_f = 0.2^{***} \) (5% CH\(_3\)OH in CHCl\(_3\)); \( \nu_{\text{max}}(\text{film}) / \text{cm}^{-1} \) 3319br (OH), 2917s, 2850s, 1700m (C=O), 1659m (C=O), 1545w, 1471m, 1368m, 1249m, 1218m, 1117s, 1070s, 865w, 755s; \( \delta_t \) (500 MHz, CDCl\(_3\)) 0.88 (t, \( J = 6.8 \), 6H, 2 \( \times \) CH\(_2\)CH\(_3\)), 1.19-1.32 (stack, 6H, 1 \( \times \) C(CH\(_3\))\(_2\) of 1,2-anti diol), 1.38-1.40 (stack, 6H, 1 \( \times \) C(CH\(_3\))\(_2\) of 1,2-syn diol), 1.41 (s, 3H, 1 \( \times \) C(CH\(_3\))\(_2\) of 1,2-syn diol), 1.44 (s, 9H, C(CH\(_3\))\(_3\)), 2.03-2.12 (m, 1H, C(3")H\(_a\)H\(_b\)), 2.17-2.27 (m, 1H, C(3")H\(_a\)H\(_b\)), 2.40-2.72 (br s, 1H, O\(_H\)), 3.25-3.36 (stack, 2H, CH\(_2\)NH\(_{\text{Boc}}\)), 3.40-3.46 (m, 1H, C(1")H\(_a\)H\(_b\)), 3.50-3.58 (stack, 4H, CH\(_2\)CH\(_2\)NH\(_{\text{Boc}}\), C(1)H\(_b\)), 3.59-3.65 (stack, 4H), 3.65-3.69 (stack, 2H), 3.69-3.74 (stack, 3H, incl. C(4)H\(_a\)H\(_b\)), 3.74-3.79 (stack, 2H, C(4)H\(_a\)H\(_b\), C(1")H\(_a\)H\(_b\)), 3.87-3.99 (stack, 2H, incl. C(2)H), 4.00-4.07 (stack, 2H), 4.07-4.15 (m, 1H, C(2")H), 4.66-4.73 (stack, 2H, C(3")H\(_b\)), [4.95-4.98 (m, 0.34H, C(2")H for minor epimer), 5.00-5.15 (stack, 1.66H, C(2")H for major epimer, NH\(_{\text{Boc}}\)], [6.59-6.69 (m, 0.66H, CON\(_H\) for major epimer), 6.78-6.84 (m, 0.34H, CON\(_H\) for minor epimer)], [7.71-7.75 (s, 0.34H, C(1")H for minor epimer), 7.76-7.82 (s, 0.66H, C(1")H for major epimer)]; \( \delta_c \) (125 MHz, CDCl\(_3\)) 14.1 (CH\(_3\), C18", C26"), 22.7 (CH\(_2\)), 25.6 (CH\(_3\), C(CH\(_3\))\(_2\) of 1,2-anti diol), 25.8 (CH\(_2\)), 26.48 (CH\(_2\)), 26.50 (CH\(_2\)), 26.9 (CH\(_3\), C(CH\(_3\))\(_2\) of 1,2-syn diol), 27.1 (CH\(_3\), C(CH\(_3\))\(_2\) of 1,2-syn diol), 27.9 (CH\(_3\), C(CH\(_3\))\(_2\) of 1,2-anti diol), 28.4 (CH\(_3\), C(CH\(_3\))\(_3\)), [28.9, 29.0, 29.1, 29.3, 29.4, 29.5, 29.7 (CH\(_2\), alkyl chain, some resonance overlap]), 31.5 (CH\(_2\)), 31.9 (CH\(_2\)), 32.4 (CH\(_2\), C3"), 32.7 (CH\(_2\)), 40.4 (CH\(_2\), CH\(_2\)NH\(_{\text{Boc}}\)), [48.8 (CH, C2’ for minor epimer), 49.0 (CH, C2’ for major epimer)], 61.8 (CH\(_2\)), 62.7 (CH\(_2\), C4), [64.6 (CH, C2” for major epimer and CH\(_2\), C3”’, resonance overlap), 65.6 (CH, C2” for minor epimer)].

*** The two epimers can be observed by TLC; however they have very similar \( R_f \) values.
[69.9, 70.0, 70.2, 70.50, 70.52 (CH$_2$, 5 × CH$_2$O in PEG linker)], 70.9 (CH$_2$, C1'), 71.7 (CH$_2$, C1), 75.8 (CH), 76.5 (CH), 79.1 (CH), [79.4 (CH and C, incl. OC(CH$_3$)$_3$, resonance overlap)], 108.1 (C, (CH$_3$)$_2$C of 1,2-anti diol), 109.2 (C, (CH$_3$)$_2$C of 1,2-syn diol), 122.3 (CH, C1’’’), 145.4 (C, C2’’’), 156.0 (C, C=ONHBoc), 167.3 (C, C=ONH); MS (TOF ES+) m/z 1230.9 [M + Na]$^+$, 100%; HRMS (TOF ES+) m/z calcd for C$_{68}$H$_{129}$N$_5$O$_{12}$Na [M + Na]$^+$ 1230.9535, found 1230.9541.

Biotinylated ThrCer (106)

TFA (60 µL) was added dropwise over 1 min to a solution of Boc amide 121 (60 mg, 0.050 mmol) in CH$_2$Cl$_2$ / CH$_3$OH (2:1, 0.6 mL). After stirring overnight at r.t., the reaction mixture was concentrated under reduced pressure and the residual TFA was removed by co-evaporation with Et$_2$O (3 × 3 mL) to provide crude amine.
as a white solid (51 mg, quant.), which was used in the step without further purification: selected data: MS (TOF ES+) \( m/z \) 1029.1 ([M + H]\(^+\), 100%); HRMS (TOF ES+) \( m/z \) 1028.8572 [M + H]. \( \text{C}_{57}\text{H}_{114}\text{N}_5\text{O}_{10} \) requires 1028.8566.

\( \text{NEt}_3 \) (33 µL, 0.24 mmol) was added to a solution of crude amine (51 mg, 0.050 mmol) in DMF (0.5 mL). After stirring for 1 h at r.t., (+)-biotin \( \text{N}\)-hydroxysuccinimide ester 123 (10 mg, 0.029 mmol) was added. The reaction mixture was stirred for 12 h at r.t. and then the volatiles were removed under reduced pressure. Purification of the residue by flash column chromatography (eluent: first EtOAc to remove \( \text{N}\)-hydroxysuccinimide by-product, then gradient 1-10% CH\(_3\)OH in CHCl\(_3\) afforded biotinylated ThrCer 106 as a white solid (52 mg, 83%, 1:1 mixture of epimers); data on mixture unless stated otherwise: \( R_f \) = 0.3 for epimer A, \( R_f \) = 0.2 for epimer B (10% CH\(_3\)OH in CHCl\(_3\)); \( \nu_{\text{max}} \) (film) / cm\(^{-1}\) 3333br (OH), 2921m, 2852m, 1672s (C=O), 1467m, 1352w, 1203s, 1184s, 1133s, 1108w, 840m, 800m, 722s; \( \delta \) (500 MHz, CDCl\(_3\)/CD\(_3\)OD, 2:1) 0.66 (t, \( J \) 6.7, 6H, 2 \( \times \) CH\(_2\)CH\(_3\)), 0.91-1.15 (stack, 66H), 1.17-1.28 (stack, 4H), 1.32-1.55 (stack, 6H, incl. C(12")H\(_2\) (middle of stack), C(14")H\(_a\)H\(_b\) (LHS of stack), C(14")H\(_a\)H\(_b\) (RHS of stack)), 1.81-1.90 (stack, 1H, C(3")H\(_a\)H\(_b\)), 1.91-2.03 (stack, 3H, C(3")H\(_a\)H\(_b\), C(11")H\(_2\)), 2.51 (d, \( J \) 12.8, 1H, C(18")H\(_a\)H\(_b\)), 2.68-2.74 (stack, 1H, C(18") H\(_a\)H\(_b\)), 2.92-3.00 (stack, 1H, C(15")H), 3.15-3.21 (stack, 2H, C(9")H\(_2\)), 3.23-3.54 (stack, 18.5H, incl. C(1")H\(_a\)H\(_b\) for both epimers, C(4")H\(_2\)), 3.55-3.66 (stack, 1.5H, incl. C(1")H\(_a\)H\(_b\) for epimer A (LHS of stack), C(1")H\(_a\)H\(_b\) for epimer B (RHS of stack)), 3.95-4.02 (stack, 1H, C(2")H (epimer A, LHS of stack; epimer B, RHS of stack)), 4.08-4.14 (stack, 1H, C(16")H), 4.27-4.32 (stack, 1H, C(17")H), 4.45-4.49 (stack, 2H, C(3")H\(_2\)), 5.08-
5.14 (app. q, J 6.7, 1H, C(2")H), 7.79 (s, 1H, C(1'')H); δC(125 MHz, CDCl₃/CD₃OD, 2:1) 13.4 (CH₃, C18’, C26” (resonance overlap)), 22.2 (CH₂), 25.1 (CH₂, C(12’’)), 25.2 (CH₂), 25.3 (CH₂), 25.4 (CH₂), 25.5 (CH₂), 27.7 (CH₂), 28.0 (CH₂), 28.58 (CH₂), 28.63 (CH₂), [29.1, 29.2, 29.3, 29.4 (CH₂, alkyl chain, some resonance overlap]), 31.5 (CH₂), 31.9 (CH₂), 32.3 (CH₂, C(3'')), 32.5 (CH₂), 35.2 (CH₂, C(11'’)), [38.72, 38.78 (CH₂, C(9’’) for both epimers)], [39.81, 39.84 (CH₂, C(18’’)) for both epimers]), [49.8, 49.9 (CH, C(2’) for both epimers)], 55.3 (CH, C(15’’)), 59.9 (CH, C(17’’)), 61.6 (CH, C(16’’)), [63.00, 63.05 (CH₂, C(4) for both epimers]), [63.6, 63.7 (CH, C(2’) for both epimers)], [63.79, 63.83 (CH₂, C(3’’) for both epimers)], 69.2 (CH₂, C(4’’)), 69.4 (CH₂, PEG chain), [69.46, 69.51 (CH, for both epimers)], [69.54, 69.57, 69.84, 69.87, 69.90, 69.92 (3 × CH₂, PEG chain for both epimers)], [70.0, 70.1 (CH₂, C(1’) for both epimers)], [71.4, 71.5 (CH, for both epimers)], [72.0, 72.1 (CH, for both epimers)], [72.9, 73.1 (CH₂, C(1) for both epimers)], 73.8 (CH, C(3’)), [122.2, 122.3 (CH, C(1’’)) for both epimers]), [144.1, 144.2 (C, C(2’’)) for both epimers]), [163.95, 164.04 (C, NHC=ONH for both epimers]), [167.98, 168.04 (C, C(1’)) for both epimers]), [174.21, 174.23 (C, C(10’’)) for both epimers]); MS (TOF ES+) m/z 1277.1 ([M + Na]⁺, 100%); HRMS (TOF ES+) calcd for C₆₇H₁₂₇N₇O₁₂Na [M + Na]⁺ 1276.9161, found 1276.9182.

Careful column chromatography (eluent: gradient 3-8% CH₃OH in CHCl₃) on a small sample of the purified mixture of epimers allowed their separation. Selected data for epimer A – (less polar), Rᵣ = 0.3 (10% CH₃OH in CHCl₃); δH(500 MHz, CDCl₃/CD₃OD, 2:1) 0.66 (t, J 6.7, 6H, 2 × CH₂CH₂), 0.91-1.15 (stack, 66H), 1.17-1.28 (stack, 4H), 1.32-1.55 (stack, 6H, incl. C(12’’)H₂ (middle of stack),
Chapter 5  Experimental Section

C(14")H_aH_b (LHS of stack), C(14")H_aH_b (RHS of stack)), 1.81-1.90 (stack, 1H, C(3")H_aH_b), 1.91-2.03 (stack, 3H, C(3")H_aH_b, C(11")H_2), 2.51 (d, J 12.8, 1H, C(18")H_aH_b), 2.68-2.74 (m, 1H, C(18")H_aH_b), 2.92-3.00 (m, 1H, C(15")H), 3.15-3.21 (m, 2H, C(9")H_2), 3.24-3.52 (stack, 18H, incl. C(1")H_aH_b, C(4")H_2), 3.57-3.62 (stack, 2H, incl. C(1")H_aH_b), 3.97-4.00 (m, 1H, C(2")H), 4.08-4.14 (m, 1H, C(16")H), 4.27-4.32 (m, 1H, C(17")H), 4.44-4.47 (stack, 2H, C(3")H_2), 5.08-5.12 (t, J 6.9, 1H, C(2")H), 7.77 (s, 1H, C(1")H). Selected data for epimer B – (more polar), R_f = 0.2 (10% CH_3OH in CHCl_3); \delta_H (500 MHz, CDCl_3/CD_3OD, 2:1) 0.66 (t, J 6.7, 6H, 2 \times CH_2CH_3), 0.91-1.15 (stack, 66H), 1.17-1.28 (stack, 4H), 1.32-1.55 (stack, 6H, incl. C(12")H_2 (middle of stack), C(14")H_aH_b (LHS of stack), C(14")H_aH_b (RHS of stack)), 1.81-1.90 (stack, 1H, C(3")H_aH_b), 1.91-2.03 (stack, 3H, C(3")H_aH_b, C(11")H_2), 2.51 (d, 1H, J 12.8, C(18")H_aH_b), 2.68-2.74 (m, 1H, C(18")H_aH_b), 2.92-3.00 (m, 1H, C(15")H), 3.15-3.21 (stack, 2H, C(9")H_2), 3.22-3.53 (stack, 19H, incl. C(1")H_aH_b, C(4")H_2), 3.54-3.60 (m, 1H, C(1")H_aH_b), 3.93-3.98 (m, 1H, C(2")H), 4.08-4.14 (m, 1H, C(16")H), 4.27-4.32 (m, 1H, C(17")H), 4.45-4.49 (stack, 2H, C(3")H_2), 5.07-5.12 (t, J 6.8, 1H, C(2")H), 7.78 (s, 1H, C(1")H).
Alexa 488-labelled ThrCer (107)

TFA (60 µL) was added dropwise over 1 min to a solution of Boc amide 121 (60 mg, 0.050 mmol) in CH₂Cl₂ / CH₃OH (2:1, 0.6 mL). After stirring overnight at r.t., the reaction mixture was concentrated under reduced pressure and the residual TFA was removed by co-evaporation with Et₂O (3 × 3 mL) to provide crude amine 122 as a white solid (51 mg, quant.), which was used in the step without further purification: selected data: MS (TOF ES+) m/z 1029.1 ([M + H]⁺, 100%); HRMS (TOF ES+) calcd for C₅₇H₁₁₄N₅O₁₀ [M + H]⁺ 1028.8566, found 1028.8572.

NEt₃ (1 µL, 7.2 µmol) was added to a solution of crude amine 122 (2.3 mg, 2.3 µmol) in DMF (10 µL). After stirring for 1 h at r.t., Alexa Fluor® 488 carboxylic acid, 2,3,5,6-tetrafluorophenyl ester 124 (1.0 mg, 1.1 µmol) was added. The reaction mixture was stirred for 12 h at r.t. and then the volatiles were removed under reduced pressure. Purification of the residue by flash column chromatography (eluent: gradient 0-50% CH₃OH in CHCl₃) afforded compound 107 as a red solid
(1.2 mg, 73%); selected data: \( R_f = 0.3 \) for epimer A, \( R_f = 0.2 \) for epimer B in 65:35:4 CHCl₃ / MeOH / aq. NH₃.

\[(2S,3S,4R)-2\text{-Azido-3,4-}O\text{-isopropylidene-1-}O\text{-}(2',3',4',6'\text{-tetra-}O-p\text{-methoxybenzyl-D-galactopyranosyl})\text{octadecane-1,3,4-triol (127)}\]

Ph₃P (1.46 g, 5.55 mmol) and CBr₄ (1.84 g, 5.55 mmol) were added sequentially to a solution of 2,3,4,6-tetra-\( O-p \)-methoxybenzyl-D-galactose 126 (1.22 g, 1.85 mmol) in CH₂Cl₂ (10 mL) at r.t. The reaction mixture was stirred for 3 h. In separate flasks, a solution of tetramethylurea (TMU) (1.2 mL) and Bu₄NBr (1.79 g, 5.55 mmol) in CH₂Cl₂ (5 mL), and a solution of azide 78 (1.07 g, 2.78 mmol) in CH₂Cl₂ (5 mL) were stirred over activated 3 Å MS for 30 min, after which time, these solutions were added dropwise (15 min) and sequentially (TMU/Bu₄NBr solution first) to the solution containing the glycosyl donor. The reaction mixture was stirred at r.t. for 3 d until there was no evidence by TLC that the donor was still being consumed. The reaction mixture was then filtered through a silica plug, washing
with CH₂Cl₂ (1.2 L) and then concentrated under reduced pressure. Purification of
the residue by flash column chromatography (15% EtOAc in hexane) afforded
glycoside 127 as a colourless oil (1.33 g, 70%, α-anomer only): \( R_f = 0.3 \) (20%
EtOAc in hexane); \( \delta_H(300 \text{ MHz, CDCl}_3 \) 0.88 (t, \( J 6.8, 3 \text{H, CH}_2\text{CH}_3 \)), 1.20-1.42
(stack, 28H, alkyl chain methylenes, C(CH₃)₂), 1.47-1.70 (stack, 4H), 3.36-3.54
(stack, 3H), 3.70 (dd, \( J 10.8, 6.7, 1 \text{H} \)), 3.75-4.17 (stack, 19H), 4.27-4.54 (stack,
3H), 4.56-4.67 (stack, 2H), 4.68-4.79 (stack, 2H), 4.80-4.91 (stack, 2H), 6.76-6.96
(stack, 8H), 7.13-7.23 (stack, 4H), 7.24-7.37 (stack, 4H); \( \delta_C(100 \text{ MHz, CDCl}_3 \) 14.1
(CH₃, CH₂CH₃), 22.7 (CH₂), 25.7 (CH₃, 1 × C(CH₃)₂), 26.6 (CH₂), 28.1 (CH₃, 1 ×
C(CH₃)₂), 29.31 (CH₂), 29.34 (CH₂), [29.57, 29.59, 29.63, 29.64, 29.65, 29.68
(CH₂, alkyl chain, resonance overlap)], 31.9 (CH₂), [55.22, 55.24 (CH₃, 4 ×
ArOCH₃ resonance overlap)], 52.8 (CH), 68.8 (CH₂), 69.6 (CH₂), 69.8 (CH), 72.5
(CH₂), 72.9 (CH₂), 73.0 (CH₂), 74.2 (CH₂), 74.7 (CH), 75.4 (CH), 76.2 (CH), 77.8
(CH), 78.4 (CH), 98.9 (CH), 108.2 (C, C(CH₃)₂), [113.56, 113.60, 113.68, 113.73
(CH, Ar ortho to OMe)], [129.16, 129.23, 129.4, 129.9 (CH, Ar meta to OMe)],
[130.1, 130.8, 131.05, 131.09 (C, Ar para to OMe)], [159.01, 159.02, 159.1, 159.2
(C, Ar ipso to OMe)].
PMe$_3$ (1.53 mL of a 1.0 M soln in THF, 1.53 mmol) was added dropwise over 5 min to a solution of azide 127 (1.30 g, 1.27 mmol) in THF (10 mL). The reaction mixture was stirred at r.t. for 3 h, after which time, H$_2$O (0.5 mL) was added. The reaction mixture was stirred for 1 h and then concentrated under reduced pressure. The residual H$_2$O was removed by co-evaporation with toluene (3 × 3 mL) to provide the crude product. Purification by flash column chromatography (40% EtOAc in hexane) afforded amine 128 as a colourless oil (1.14 g, 90%): $R_f = 0.3$ (40% EtOAc in hexane); $\nu_{\text{max}}$(film) / cm$^{-1}$ 3380 br (N–H), 2923s, 2854s, 1497m, 1454s, 1368m, 1244m, 1216m, 1096s, 1057s, 909s, 872w, 732s, 697s; $\delta$(300 MHz, CDCl$_3$) 0.88 (t, $J$ 7.0, 3H, CH$_2$CH$_3$), 1.14-1.61 (stack, 32H, alkyl chain methylenes, C(CH$_3$)$_2$), 3.11-3.22 (m, 1H), 3.35-3.52 (stack, 3H), 3.70-4.18 (stack, 20H), 4.28-4.53 (stack, 3H), 4.54-4.78 (stack, 5H), 4.79-4.88 (stack, 2H), 6.75-6.93 (stack, 8H), 7.09-7.36 (stack, 8H); $\delta_c$(100 MHz, CDCl$_3$) 14.1 (CH$_3$, CH$_2$CH$_3$), 22.7 (CH$_2$), 25.9 (CH$_3$, 1 × C(CH$_3$)$_2$), 26.3 (CH$_2$), 28.1 (CH$_3$, 1 × C(CH$_3$)$_2$), [29.3, 29.7 (CH$_2$, alkyl chain, resonance overlap)], 31.9 (CH$_2$), 50.9 (CH), [55.2 (CH$_3$, 4 ×
ArOCH₃ resonance overlap), 68.8 (CH₂), 69.6 (CH₂), 69.8 (CH), 72.5 (CH₂), 72.9 (CH₂), 73.0 (CH₂), 74.2 (CH₂), 74.7 (CH), 75.4 (CH), 76.2 (CH), 77.8 (CH), 78.4 (CH), 98.9 (CH), 108.2 (C, C(CH₃)₂), [113.56, 113.60, 113.68, 113.73 (CH, Ar ortho to OMe)], [129.16, 129.23, 129.4, 129.9 (CH, Ar meta to OMe)], [130.1, 130.8, 131.05, 131.09 (C, Ar para to OMe)], [159.01, 159.02, 159.1, 159.2 (C, Ar ipso to OMe)].
A solution of α-azido acid 116 (88 mg, 0.22 mmol) in (COCl)$_2$ (2 mL) was heated at 70 °C. After 2 h, the volatiles were removed under a stream of N$_2$ and the residual solvent removed on the vacuum line (1 h) to provide the corresponding acid chloride 117 as a pale yellow oil, which was used directly in the next step without further purification (100 mg, quant.): A solution of freshly prepared acid chloride 117 (100 mg, 0.22 mmol) in CH$_2$Cl$_2$ (1.0 mL) was added dropwise over 2 min to a solution of amine 128 (280 mg, 0.28 mmol) and NEt$_3$ (32 µL, 0.23 mmol) in CH$_2$Cl$_2$ (2.0 mL) at 0 °C. The reaction mixture was stirred at rt for 12 h and then diluted with CH$_2$Cl$_2$ (20 mL), washed sequentially with NaHCO$_3$ solution (20 mL), brine (5 mL) and then dried over Na$_2$SO$_4$. The drying agent was removed by filtration and the filtrate concentrated under reduced pressure. Purification of the residue by flash column chromatography provided amide 129 as a colourless oil (281 mg, 90%, 1:1 mixture of epimers). Data on epimeric mixture: $R_f = 0.3$ (20% EtOAc in hexane); $\nu_{max}$(film) / cm$^{-1}$ 2922s, 2852s, 2105m (N$_3$), 1685m, 1613m, 1513s, 1465m, 1368m, 1247s, 1037s, 821s, 729s; $\delta_\alpha$(500 MHz, CDCl$_3$) 0.84-0.89 (stack,
6H, 2 × CH₂CH₃), 1.16-1.50 (stack, 56H), 1.61-1.86 (stack, 2H, C(3°)H₂), 3.34-3.40 (stack, 1H, C(6°)HₐHₜ for both epimers), 3.41-3.46 (stack, 1H, C(6°)HₐHₜ for both epimers), 3.58-3.62 (stack, 1H, C(1°)HₐHₜ for both epimers), 3.67-3.70 (m, 0.5H, C(2°)H for epimer 1), 3.74-3.81 (stack, 12H, 4 × ArOCH₃ for both epimers, and 1H, C(2°)H for epimer 2), 3.82-3.89 (stack, 3H, C(3°)H for both epimers, C(4°)H for both epimers, C(5°)H for both epimers), 3.89-4.00 (stack, 3H, C(1°)HₐHₜ for both epimers, C(4°)H for both epimers, C(2°)H for both epimers), 4.03-4.09 (stack, 1H, C(2°)H for both epimers), 4.09-4.18 (stack, 1H, C(3°)H for both epimers, C(4°)H for both epimers, C(2°)H for both epimers), 4.27 (A of AB, Jₐ₋ₐ 11.6, 1H, C(6°)OCHₐHₜAr for epimer 1), 4.28 (A of AB, Jₐ₋ₐ 11.6, 1H, C(6°)OCHₐHₜAr for epimer 2), 4.40 (B of AB, Jₐ₋ₐ 11.6, 1H, C(6°)OCHₐHₜAr for epimer 1), 4.42 (B of AB, Jₐ₋ₐ 11.6, 1H, C(6°)OCHₐHₜAr for epimer 1), 4.46 (A of AB, Jₐ₋ₐ 11.2, 1H, C(4°)OCHₐHₜAr for epimer 1), 4.47 (A of AB, Jₐ₋ₐ 11.2, 1H, C(4°)OCHₐHₜAr for epimer 2), 4.56 (A of AB, Jₐ₋ₐ 11.2, 1H, C(2°)OCHₐHₜAr for epimer 1), 4.57 (A of AB, Jₐ₋ₐ 11.2, 1H, C(2°)OCHₐHₜAr for epimer 2), 4.63 (A of AB, Jₐ₋ₐ 11.2, 1H, C(3°)OCHₐHₜAr for epimer 1), 4.64 (A of AB, Jₐ₋ₐ 11.2, 1H, C(3°)OCHₐHₜAr for epimer 2), 4.70 (B of AB, Jₐ₋ₐ 11.2, 1H, C(3°)OCHₐHₜAr for both epimers), 4.72 (B of AB, Jₐ₋ₐ 11.2, 1H, C(2°)OCHₐHₜAr for epimer 1), 4.73 (B of AB, Jₐ₋ₐ 11.2, 1H, C(2°)OCHₐHₜAr for epimer 2), 4.80 (B of AB, Jₐ₋ₐ 1H, C(4°)OCHₐHₜAr for both epimers), 4.84-4.87 (stack, 1H, C(1°)H, for both epimers), 6.74-6.89 (8H, stack, 8H, Ar), 7.13-7.18 (stack, 4H, Ar), 7.23-7.31 (stack, 4H, Ar); δC (125 MHz, CDCl₃) [14.1 (CH₃, C18°, C26°), [22.7 (CH₂)], [25.6, 25.7 (CH₂, alkyl chain (resonance overlap)), [25.9, 26.0 (CH₂, C(CH₃)₂ for both epimers)], 26.6 (CH₂), 26.7 (CH₂), [28.1, 28.2 (CH₃, C(CH₃)₂ for both epimers)], [28.96, 29.03, 29.3, 29.4, 29.5, 29.7, (CH₂, alkyl chain, some resonance overlap)], 31.9 (CH₂).
32.0 (CH$_2$), 32.1 (CH$_2$), [48.8, 49.1 (CH, C2)], [55.22, 55.24 (CH$_3$, 4 × ArOCH$_3$, resonance overlap)], [64.2, 64.5 (CH, C2")], 68.6 (CH$_2$, C1 for epimer 1), [68.88, 68.91 (CH$_2$, C6")], 69.0 (CH$_2$, C1 for epimer 2), 69.8 (CH, C5' for both epimers (resonance overlap)), [72.6, 72.7 (CH$_2$, C(3')OCH$_2$Ar), [73.08, 73.12, 73.15 (CH$_2$, C(2')OCH$_2$Ar and C(6')OCH$_2$Ar (resonance overlap))], 74.2 (CH$_2$, OCH$_2$Ar for both epimers (resonance overlap)), [74.4, 74.5 (CH, C4')], [75.1, 75.2 (CH, C3)], 76.5 (CH, C2' for both epimers (resonance overlap)), [77.69, 77.73 (CH, C4)], [78.8, 78.9 (CH, C3')], [99.0, 99.3 (CH, C1')], [107.88, 107.94 (C, (CH$_3$)$_2$C)], [113.6, 113.73, 113.75 113.77 (CH, Ar ortho to OMe)], 127.9 (CH, CH=CH), 128.0 (CH, CH=CH), [129.0, 129.4, 129.5, 129.8, 129.9, (CH, Ar meta to OMe)], 130.0 (CH, CH=CH), 130.2 (CH, CH=CH), [130.66, 130.75, 130.78, 130.9 (C, Ar para to OMe, some resonance overlap)], [159.1, 159.2, 159.3 (C, Ar ipso to OMe)], [168.6, 168.9 (C, C=O)]; MS (TOF ES+) $m/z$ 1442.4 ([M + Na]$^+$, 100%).
CuSO₄ solution (14 μL of a 0.5 M solution, 7 μmol) and sodium ascorbate solution (60 μL of a 1 M solution, 60 μmol) were added to a solution of azide 129 (65 mg, 0.15 mmol) and alkyne 114 (66 mg, 0.23 mmol) in ¹BuOH / H₂O (1 mL, 1:1) at r.t. The reaction mixture was heated for 10 h at 50 °C and then diluted with CHCl₃ (20 mL), and washed with brine (10 mL). The phases were separated and the aqueous layer was extracted with CHCl₃ (2 x 10 mL). The combined organic layers were dried over Na₂SO₄ and the volatiles were removed under reduced pressure. Purification of the residue by flash column chromatography (gradient: CHCl₃ to 5% CH₃OH in CHCl₃) afforded 1,2,3-triazole 130 as a colourless oil (231 mg, 91%, isolated as a 1:1 mixture of epimers). Data on epimeric mixture: \( R_f = 0.2^{+++} \) (40% EtOAc in hexane); \( \nu_{max}(\text{film}) / \text{cm}^{-1} \) 3343br (NH), 2923s, 2853s, 1687m (C=O), 1613m (C=O), 1586w, 1513s, 1465m, 1471m, 1367m, 1302m, 1248s, 1173s, 1093s, 1038s, 822m; \( \delta_H(500 \text{ MHz, CDCl}_3) \) 0.83-0.88 (stack, 6H, 2 × CH₂CH₃), 1.11-1.36 (stack, 69H, alkyl chain methylenes, C(CH₃)ₐ(CH₃)ₐ), 1.37-1.45 [stack,

+++The two epimers can be observed by TLC; however they have very similar \( R_f \) values.
16H, including C(CH₃)ₙ(CH₃)ₘ, C(CH₃)ₙ), 1.92-2.09 (stack, 2H, C(3'')H₂), 3.20-3.35 (stack, 3H, C(6'')HₙHₘ for both epimers, CH₂NHBOc), 3.39-3.46 (stack, 1H, C(6'')HₙHₘ for both epimers), 3.47-3.54 (stack, 3H, CH₂CH₂NHBOc, C(1')HₙHₘ for both epimers), 3.55-3.73 (stack, 12H, including 4 × PEG CH₂O, C(4'')H₂), 3.75-3.83 (stack, 13H, 4 × ArOC₃Hₙ for both epimers, C(3'')H for epimer 1 and C(4'')H for epimer 1), 3.84-4.16 (stack, 8H, including C(2')H for both epimers, C(3'')H for epimer 2, C(4'')H for epimer 2, C(1')HₙHₘ for both epimers, C(2')H for both epimers, C(4')H for both epimers, C(3')H for both epimers, 1 × PEG CH₂O), 4.24-4.31 (stack, 2H, A of AB, C(6'')OC₃HₙHₘ for both epimers), 4.40-4.59 (stack, 3H), 4.64-4.76 (stack, 2H), 4.76-4.82 (stack, 2H, including C(1')H, for both epimers), 4.90-4.95 (stack, 1H, C(2'')H for both epimers), 4.99-5.06 (stack, 1H, NHBOc for both epimers), 6.72-6.88 (stack, 8H, Ar), 7.10-7.18 (stack, 4H, Ar), 7.22-7.30 (stack, 4H, Ar), 7.65 (br s, 0.5H, C(1'')H for epimer 1), 7.71 (br s, 0.5H, C(1'')H for epimer 2), CONH resonance not observed; δ_C (125 MHz, CDCl₃) [14.0, 14.1 14.2 (CH₃, resonance overlap, C18, C26'')], 22.5 (CH₂), 22.7 (CH₂), [25.86, 25.89 CH₃, C(CH₃)ₙ(CH₃)ₘ], 26.5 (CH₂), 28.2 (CH₃, C(CH₃)ₙ(CH₃)ₘ), 28.4 (CH₃, C(CH₃)ₙ), 28.8 (CH₂), 29.0 (CH₂), [29.2, 29.31, 29.33, 29.4 (CH₂, resonance overlap)], [29.58, 29.63, 29.70 (CH₂, resonance overlap)], 31.5 (CH₂), 31.9 (CH₂), 33.1 (CH₂, C3''), 40.3 (CH₂, CH₂NHBOc), [49.4, 49.5 (CH, C2)], [55.21, 55.24 (CH₃, 4 × ArOCH₃, resonance overlap)], [64.3, 64.6 (CH, C2'')], 64.7 (CH₂, C3''), 68.2 (CH₂, C1), [69.1, 69.2 (CH₂, C6', resonance overlap), [70.0, 70.2, 70.4, 70.50, 70.52 (CH₂, 4 × CH₂O in PEG linker)], [72.6, 72.9 (CH₂, OCH₂Ar), [73.04, 73.12, 73.15 (CH₂, OCH₂Ar (resonance overlap))], [74.1, 74.2 (CH₂, OCH₂Ar (resonance overlap)), [74.4, 74.5 (CH, C4''), [75.1, 75.2 (CH, C3]), 76.2 (CH, C2' for both epimers}
(resonance overlap)), 77.6 (CH, C4, for both epimers (resonance overlap)), [78.8, 78.9 (CH, C3' for both epimers (resonance overlap)), 79.4 (CH, C3 and C, C(CH3)3) [98.9, 99.6 (CH, C1')], [107.87, 107.91 (C, (CH3)2C)], [113.6, 113.73, 113.75 113.77 (CH, Ar ortho to OMe)], [121.8, 122.1 (CH, C1'')], [129.0, 129.4, 129.5, 129.8, 129.9, (CH, Ar meta to OMe)], [130.6, 130.7, 130.9 (C, Ar para to OMe, some resonance overlap)], [145.4, 145.2 (C, C2'')], [159.1, 159.2, 159.3 (C, Ar ipso to OMe)], [167.31, 167.33 (C, CH2C(O)NH)], [171.1 C of Boc]; MS (TOF ES+) m/z 1729.1 [M + Na]⁺, 100%;
Chapter 5  Experimental Section

Alexa 488-labelled α-GalCer (108)

TFA (60 µL) was added dropwise over 1 min to a solution of Boc amide 121 (60 mg, 0.050 mmol) in CH₂Cl₂ / CH₃OH (2:1, 0.6 mL). After stirring overnight at r.t., the reaction mixture was concentrated under reduced pressure and the residual TFA was removed by co-evaporation with Et₂O (3 × 3 mL) to provide crude amine 122 as a white solid (51 mg, quant.), which was used in the step without further purification: selected data: MS (TOF ES+) m/z 1108.9 ([M + Na]⁺, 100%); HRMS (TOF ES+) 1108.8462 [M + Na]⁺. C₅₉H₁₁₅N₅O₁₂Na requires 1108.8440.

NEt₃ (1 µL, 7.2 µmol) was added to a solution of crude amine 122 (2.5 mg, 2.3 µmol) in DMF (10 µL). After stirring for 1 h at r.t., Alexa Fluor® 488 carboxylic acid, 2,3,5,6-tetrafluorophenyl ester 124 (1.0 mg, 1.1 µmol) was added. The reaction mixture was stirred for 12 h at r.t. and then the volatiles were removed under reduced pressure. Purification of the residue by flash column chromatography (eluent: gradient 0-50% CH₃OH in CHCl₃) afforded compound 108 as a red solid.
(1.2 mg, 83%); selected data: $R_f = 0.3$ for epimer A, $R_f = 0.2$ for epimer B in 65:35:4 CHCl$_3$ / MeOH / aq. NH$_3$; MS (TOF ES-) $m/z$ 1622.9 ([M + Na - H], 100%);

(Z,Z)-Octadeca-9,12-dien-1-ol (139)

Cs$_2$CO$_3$ (5.21 g, 16.0 mmol) was added to a solution of linoleic acid 138 (3.00 g, 10.7 mmol) in DMF (10 mL). After stirring for 20 min at r.t., MeI (735 µL, 11.8 mmol) was added to the suspension. After 2 h, the mixture was poured into H$_2$O (10 mL) and extracted with Et$_2$O (2 x 20 mL). The organic phases were washed with H$_2$O (3 x 20 mL), dried over Na$_2$SO$_4$, filtered and concentrated under reduced pressure to give the methyl ester as a colourless oil (3.15 g, 100%), which was used in the next step without further purification. A solution of methyl linoleate (3.15 g, 10.7 mmol) in Et$_2$O (20 mL) was added over 5 min via syringe to a stirred suspension of LiAlH$_4$ (0.800 g, 21.0 mmol) in Et$_2$O (20 mL). After the initial vigorous reaction had subsided, the mixture was heated at reflux for 3 h and then cooled to r.t., poured into NH$_4$Cl solution (100 mL) and H$_2$O (20 mL). The layers were separated and the aqueous layer was extracted with Et$_2$O (3 x 20 mL). The organic layer and the ethereal extracts were combined and the mixture was dried (Na$_2$SO$_4$) and concentrated under reduced pressure. The residue was
Chapter 5  Experimental Section

purified by flash column chromatography (10% EtOAc in hexane) to afford alcohol 139 as a colourless oil (2.57 g, 90%). $R_f = 0.3$ (15% EtOAc in hexane); $\nu_{\text{max}}$(film) / cm$^{-1}$ 3327 br O–H, 3010w, 2925s, 2855m, 1467m, 1378w, 1054m, 986m, 723m; $\delta_\nu$(300 MHz, CDCl$_3$) 0.86 (t, $J$ 6.8, 3H, CH$_2$CH$_3$), 1.21-1.42 (stack, 15H, alkyl chain), 1.50-1.63 (stack, 3H), 1.97-2.11 (stack, 4H, 2 × CH$_2$CH=CH), 2.72-2.82 (m, 2H, CH=CHCH$_2$CH=CH), 3.64 (t, $J$ 6.6, 2H, CH$_2$OH), 5.27-5.44 (stack, 4H, 2 × CH=CH), OH resonance not observed; $\delta_\delta$(100 MHz, CDCl$_3$) 14.0 (CH$_3$, CH$_2$CH$_3$), 22.5 (CH$_2$), 25.6 (CH$_2$), 25.7 (CH$_2$), 27.2 (CH$_2$), [29.2, 29.3, 29.4, 29.5, 29.6 (CH$_2$, alkyl chain, resonance overlap)], 31.5 (CH$_2$), 31.7 (CH$_2$), 62.9 (CH$_2$, CH$_2$OH), 127.86 (CH, CH=CH), 127.93 (CH, CH=CH), 130.0 (CH, CH=CH), 130.1 (CH, CH=CH); MS (TOF ES+) m/z 266.3 ([M+Na]$^+$, 100%); HRMS (TOF ES+) calcd for C$_{18}$H$_{34}$ONa ([M+Na]$^+$) 266.2610, found 266.2617

Data were in agreement with those reported in the literature.$^{165}$
(Z,Z)-1-Bromo-octadeca-9,12-diene (137)

CBr₄ (2.43 g, 7.34 mmol) was added in small portions to a cooled (0 °C) solution of alcohol 139 (1.85 g, 6.99 mmol) and PPh₃ (1.93 g, 7.34 mmol) in CH₂Cl₂ (35 mL). The mixture was stirred at r.t. for 16 h and then concentrated under reduced pressure. The residual solid was washed with H₂O (50 mL) and extracted with Et₂O (2 × 50 mL). The ethereal extracts were dried (Na₂SO₄) and concentrated under reduced pressure. The residue was purified by flash column chromatography (hexane) to afford bromide 137 as a colourless oil (2.51 g, 92%).

\[ R_f = 0.7 \text{(Hexane)}; \nu_{\text{max}}(\text{film}) / \text{cm}^{-1} 3008\text{m}, 2924\text{s}, 2854\text{s}, 1459\text{m}, 723\text{m}; \delta^r(300 \text{MHz, CDCl}_3) 0.89 (t, J 6.8, 3H, CH₂CH₃), 1.19-1.49 (stack, 16H, alkyl chain), 1.79-1.91 (stack, 2H), 1.98-2.12 (stack, 4H, 2 × CH₂CH=CH), 2.71-2.84 (m, 2H, CH=CHCH₂CH=CH), 3.41 (t, J 6.8, 2H, CH₂Br), 5.26-5.45 (stack, 4H, 2 × CH=CH); \delta^c(100 \text{MHz, CDCl}_3) 14.1 (CH₃, CH₂CH₃), 22.6 (CH₂), 25.6 (CH₂), 27.2 (CH₂), 28.2 (CH₂), 28.7 (CH₂), [29.2, 29.3, 29.6 (CH₂, alkyl chain, resonance overlap)], 31.5 (CH₂), 32.8 (CH₂), 33.9 (CH₂), 127.9 (CH, CH=CH), 128.0 (CH, CH=CH), 130.0 (CH, CH=CH), 130.2 (CH, CH=CH); MS (TOF ES+) \text{m/z} 328.2 ([M(\text{Br isotopomer})]^+, 100%); HRMS (TOF ES+) calcd for C₁₈H₃₃⁷⁹Br ([M(⁷⁹Br isotopomer)] ) 328.1766, found 328.1762.

Data were in agreement with those reported in the literature.\textsuperscript{165}
(Z,Z)-1-Cyano-octadeca-9,12-diene (136)

A solution of bromide 137 (1.10 g, 3.34 mmol) in DMSO (10 mL) was added over 5 min to a stirred solution of NaCN (180 mg, 3.67 mmol) in DMSO (25 mL) at 40 °C. Soon after the addition, an exothermic reaction was observed and a salt (NaBr) precipitated. The mixture was stirred and heated at 60 °C for 40 min, and then poured into ice-water (30 mL) and extracted with Et₂O (2 × 50 mL). The extract was washed sequentially with H₂O (50 mL) and brine (20 mL), dried (Na₂SO₄) and concentrated under reduced pressure. The residue was purified by flash column chromatography (3% EtOAc in hexane) to afford nitrile 136 as a colourless oil (830 mg, 90%). $R_f = 0.3$ (2% EtOAc in hexane); $v_{max}$ (film) / cm⁻¹ 3009 w, 2925 s, 2855 s, 2246 w (C≡N), 1459 m, 1398 w, 913 w, 723 s; $\delta_1$ (300 MHz, CDCl₃) 0.89 (t, J 6.9, 3H, CH₂CH₃), 1.23-1.50 (stack, 16H, alkyl chain), 1.59-1.72 (stack, 2H), 1.98-2.12 (stack, 4H, CH₂CH=CHCH₂CH=CHCH₂), 2.34 (t, J 7.1, 2H, CH₂CN), 2.73-2.81 (m, 2H, CH=CHCH₂CH=CH), 5.27-5.43 (stack, 4H, 2 × CH=CH)；$\delta_2$(100 MHz, CDCl₃) 14.0 (CH₃, CH₂CH₃), 17.0 (CH₂), 22.4 (CH₂), 25.2 (CH₂), 25.5 (CH₂), 27.0 (CH₂), 27.1 (CH₂), [28.5, 28.6, 29.0, 29.1, 29.2, 29.4 (CH₂, alkyl chain, resonance overlap)], 31.4 (CH₂), 119.6 (C, C≡N), 127.7 (CH, CH≡CH), 127.9 (CH, CH≡CH), 129.8 (CH, CH=CH), 130.0 (CH, CH=CH); MS (TOF ES⁺) m/z 298.1 ([M + Na]⁺, 100%); HRMS (TOF ES⁺) calcd for C₁₉H₃₃NNa ([M + Na]⁺) 298.2511, found 298.2500.
DIBALH (10.8 mL of a 1 M soln in CH$_2$Cl$_2$, 10.8 mmol,) was added dropwise over 10 min to a stirred solution of nitrile 136 (2.30 g, 8.35 mmol) in CH$_2$Cl$_2$ (40 mL) at $-78$ °C. The reaction mixture was stirred for 1.5 h and then quenched with iPrOH (10 mL) and H$_2$O (5 mL) at $-78$ °C before warming to r.t. Silica gel (1.00 g) was added and the resulting mixture was stirred for 1 h, before diluting with EtOAc (50 mL) and filtering through a pad of celite. The filtrate was concentrated under reduced pressure and the residue purified by flash column chromatography (2% EtOAc in hexane) to afford aldehyde 135 as a colourless oil (2.77 g, 92%). $R_f = 0.4$ (2% EtOAc in hexane); $\nu_{\text{max}}$(film) / cm$^{-1}$ 3317 br, 3009m, 2854s, 1742m (C=O), 1465m, 1374w, 1240m, 1047w, 723m; $\delta_{\text{H}}$(300 MHz, CDCl$_3$) 0.89 (t, J 6.9, 3H, CH$_2$CH$_3$), 1.23-1.42 (stack, 16H, alkyl chain), 1.57-1.70 (stack, 2H), 1.98-2.12 (stack, 4H, CH$_2$CH=CHCH$_2$CH=CHCH$_2$), 2.37-2.46 (m, 2H, CH$_2$CHO), 2.73-2.82 (m, 2H, CH=CHCH$_2$CH=CH), 5.27-5.44 (stack, 4H, 2 × CH=CH), 9.77 (t, J 1.9, 1H, CHO); $\delta_{\text{c}}$(100 MHz, CDCl$_3$): 14.1 (CH$_3$, CH$_2$CH$_3$), 22.1 (CH$_2$), 22.6 (CH$_2$), 25.7 (CH$_2$), 27.2 (CH$_2$), [29.1, 29.2, 29.3, 29.6 (CH$_2$, alkyl chain, resonance overlap)], 31.6 (CH$_2$), 43.9 (CH$_2$), 127.9 (CH, CH=CH), 128.0 (CH, CH=CH), 130.1 (CH, CH=CH), 130.2 (CH, CH=CH), 202.8 (CH, C=O); MS (TOF ES+) $m/z$ 277.2 ([M -
H]⁺, 100%); HRMS (TOF ES⁺) calcd for C₁₉H₃₃O ([M – H]⁺) 277.2531, found 277.2542.

(Z,Z)-2-Amino-1-cyano-nonadeca-10,13-diene (134)

A solution of NH₄Cl (350 mg, 6.48 mmol) in aqueous NH₃ (30% solution, 7 mL) was added to a solution of aldehyde 135 (450 mg, 1.62 mmol) in MeOH (8 mL). The reaction mixture was stirred at r.t. for 1 h before NaCN (160 mg, 3.24 mmol) was added. After 24 h, the solvent was removed under reduced pressure. The resulting solid was partitioned between EtOAc (50 mL) and H₂O (50 mL). The separated organic layer was dried over Na₂SO₄ and evaporated under reduced pressure to provide a yellowish residue, which was purified by flash column chromatography (50% EtOAc in hexane) to afford α-amino nitrile 134 as a colourless oil (370 mg, 75%). Rₑ = 0.3 (40% EtOAc in hexane); vₑₓₙₑₜ (film) / cm⁻¹: 3343 br m (NH₂), 2973m, 2927w, 2883m, 2246w (C=N), 1453w, 1380w, 1328w, 1274w, 1088m, 1046s, 880s; δₜ (300 MHz, CDCl₃): 0.89 (t, J 6.9, 3H, CH₂CH₃), 1.19-1.55 (stack, 16H, alkyl chain), 1.60-1.79 (stack, 4H), 1.97-2.11 (stack, 4H, CH₂CH=CHCH₂CH=CHCH₂), 2.77 (app. t J 5.9, 2H, CH=CHCH₂CH=CH), 3.67 (t, J 7.1, 1H, CHNH₂), 5.26-5.44 (stack, 4H, 2 × CH=CH), resonance for NH₂ not
observed; $\delta_{c}(100 \text{ MHz, CDCl}_3): 14.0 (\text{CH}_3, \text{CH}_2\text{CH}_3), 22.5 (\text{CH}_2), 25.4 (\text{CH}_2), 25.6 (\text{CH}_2), 27.1 (\text{CH}_2), [28.9, 29.2, 29.3, 29.6 (\text{CH}, \text{alkyl chain, resonance overlap})], 31.5 (\text{CH}_2), 35.3 (\text{CH}_2), 43.4 (\text{CH}, \text{CHNH}_2), 122.2 (\text{C}, \text{C}≡\text{N}), 127.9 (\text{CH}, \text{CH}=\text{CH}), 128.0 (\text{CH}, \text{CH}=\text{CH}), 130.0 (\text{CH}, \text{CH}=\text{CH}), 130.1 (\text{CH}, \text{CH}=\text{CH}); \text{MS (TOF ES+) } m/z 327.3 ([\text{M + Na}]^+, 100\%); \text{HRMS (TOF ES+) calcd for } \text{C}_{20}\text{H}_{36}\text{N}_2\text{Na} ([\text{M + Na}]^+) 327.2776, \text{found } 327.2767.

(Z,Z)-2-Amino-eicosa-11,14-dienoic acid (133)

40\% \text{Aqueous KOH solution (KOH (250 mg, 4.54 mmol) in H}_2\text{O (0.5 mL)) was added to a stirred solution of nitrile 134 (138 mg, 0.454 mmol) in EtOH (3 mL). The resulting mixture was heated at 80 °C for 8 h. After cooling the mixture to r.t., H}_2\text{O (10 mL) and MeOH (10 mL) were added sequentially. The reaction mixture was cooled to 0 °C, acidified (to pH 3.0) with hydrochloric acid (10 mL of a 10\% solution). The mixture was extracted with Et}_2\text{O (3 × 20 mL), and then the combined organic extracts were dried with Na}_2\text{SO}_4 and the volatiles evaporated under reduced pressure to afford the crude }\alpha\text{-amino acid 133 as a white solid (140 mg, 95\%), which was used in the next step without further purification. } R_f = 0.2
(20% MeOH in CHCl₃); δ (300 MHz, CD₃OD): 0.88 (t, J 7.0, 3H, CH₂CH₃), 1.18-1.59 (stack, 16H, alkyl chain), 1.72-1.92 (stack, 4H, alkyl chain), 1.93-2.10 (stack, 4H, CH₂CH=CHCH₂CH=CHCH₂), 2.70 (app. t, J 5.7, 2H, CH=CHCH₂CH=CH), 3.89 (t, J 6.1, 1H, CHNH₂), 5.19-5.36 (stack, 4H, 2 × CH=CH), 8.83 (d, J 5.4, 2H, NH₂), OH resonance not observed; δ (100 MHz, CDCl₃): 14.5 (CH₃, CH₂CH₃), 23.6 (CH₂), 25.9 (CH₂), 26.6 (CH₂), 28.2 (CH₂), [30.3, 30.4, 30.5, 30.8 (CH₂, alkyl chain, resonance overlap)], 31.5 (CH₂), 32.7 (CH₂), 54.0 (CH, CHNH₂), 128.9 (CH, CH=CH), 129.1 (CH, CH=CH), 130.9 (CH, CH=CH), 131.0 (CH, CH=CH), 171.9 (C, C=O); MS (TOF ES−) m/z 322.2 ([M – H]−, 100%); HRMS (TOF ES−) calcd for C₂₀H₃₆NO₂ ([M – H]−, 100%) 322.2746, found 322.2743.
Imidazole-1-sulfonyl azide hydrochloride\(^{90}\) (156 mg, 0.74 mmol, CAUTION: potential explosion risk during purification of this reagent) was added to a suspension of amine 133 (120 mg, 0.37 mmol), CuSO\(_4\)·5H\(_2\)O (2 mg, 2 µmol) and K\(_2\)CO\(_3\) (374 mg, 2.71 mmol) in MeOH (5 mL). The mixture was stirred at r.t. for 18 h and then concentrated under reduced pressure. H\(_2\)O (15 mL) was added to the residue which was then acidified (to pH 3.0) with hydrochloric acid (5 mL of a 10% solution). The mixture was extracted with EtOAc (3 × 10 mL). The combined organic layers were dried with Na\(_2\)SO\(_4\) and the volatiles were removed under reduced pressure. The crude product was purified by flash column chromatography (20% EtOAc in toluene) to afford α-azido acid 132 as a colourless oil (110 mg, 85%). \(R_f = 0.3\) (20% EtOAc in toluene); \(\nu_{\text{max}}\) (film) / cm\(^{-1}\) 3400-2500 w v br (OH), 2927s, 2856m, 2108s (N\(_3\)), 1719s (C=O), 1457m, 1377w, 1186m, 1080m, 907s, 732s; \(\delta_h\) (300 MHz, CDCl\(_3\)) 0.89 (t, \(J 6.9, 3H, CH_2CH_3\)), 1.23-1.52 (stack, 17H, alkyl chain), 1.53-1.70 (m, 1H, alkyl chain), 1.72-1.94 (stack, 2H, alkyl chain), 1.98-2.14 (stack, 4H, \(CH_2CH=CHCH_2CH=CHCH_2\)), 2.77 (app. t \(J 5.9, 2H, CH=CHCH_2CH=CH\)), 3.88 (dd, \(J 8.4, 5.2, 1H, CHN_3\)), 5.27-5.44 (stack, 4H, 2 × \(CH=CH\)), 10.03 (v br s, 1H, OH); \(\delta_c\) (100 MHz, CDCl\(_3\)) 14.0 (CH\(_3\), CH\(_2\)CH\(_3\)), 22.6 (CH\(_2\)), 25.6 (CH\(_2\)), 25.7 (CH\(_2\)), 27.2 (CH\(_2\)), \[28.9, 29.0, 29.2, 29.3, 29.4, 29.6, 29.7\]
(CH$_2$, alkyl chain, resonance overlap), 31.3 (CH$_2$), 31.5 (CH$_2$), 61.7 (CH, CHN$_3$),
127.9 (CH, CH=CH), 128.0 (CH, CH=CH), 130.0 (CH, CH=CH), 130.2 (CH, CH=CH), 176.1 (C, C=O); MS (TOF ES$^-$) $m/z$ 348.3 ([M – H]$^-$, 100%); HRMS (TOF ES$^-$) calcd for C$_{20}$H$_{34}$N$_3$O$_2$Na ([M – H]$^-$, 100%) 348.2651, found 348.2665.
DMF (1 µL, 13 µmol) was added to a solution of (Z,Z)-2-azido-eicosa-11,14-
dienoic acid 132 (45 mg, 0.129 mmol) in CH$_2$Cl$_2$ (1 mL) and the mixture was
cooled to 0 °C. A solution of oxalyl chloride (22 µL, 0.258 mmol) in CH$_2$Cl$_2$ (0.5'
ml) was added dropwise over 2 min. After 2 h, the volatiles were removed under
reduced pressure to provide the corresponding acid chloride 140 as a pale yellow
oil, which was used directly in the next step without further purification (48 mg, 
quant.): A solution of freshly prepared acid chloride 140 (48 mg, 0.129 mmol) in
CH$_2$Cl$_2$ (0.5 mL) was added dropwise over 2 min to a solution of amine 118 (97
mg, 019 mmol) and NEt$_3$ (36 µL, 0.26 mmol) in CH$_2$Cl$_2$ at 0 °C. The reaction
mixture was stirred for 12 h and then diluted with CH$_2$Cl$_2$ (10 mL), washed
sequentially with NaHCO$_3$ solution (10 mL), brine (2 mL) and then dried over
Na$_2$SO$_4$. The drying agent was removed by filtration and the filtrate concentrated
under reduced pressure. Purification of the residue by flash column
chromatography (20% EtOAc in hexane) provided amide 145 as a colourless oil
(97 mg, 90%, 1:1 mixture of epimers); data on epimeric mixture: $R_f = 0.3$ (25% EtOAc in hexane); $\nu_{\text{max}}$(film) / cm$^{-1}$ 3395br (OH), 2925s, 2855s, 2100m (N$_3$), 1660m (C=O), 1543w, 1465m, 1370m, 1251m, 1219m, 1080m, 1057m, 850m, 721w; $\delta_{\text{H}}$(500 MHz, CDCl$_3$) 0.84-0.88 (stack, 6H, 2 × CH$_2$CH$_3$), 1.19-1.41 (app. q, $J$ 7.2, 4H, CH$_2$CH=CHCH$_2$CH=CHCH$_2$), 1.71-1.93 (stack, 2H, C(3")H$_2$), 2.03 (app. q, $J$ 7.2, 4H, C(CH$_3$)$_2$CH=CHCH$_2$CH=CHC(CH$_3$)$_2$), 2.15-2.20 (br s, 1H, OH), 2.75 (app. t, $J$ 6.6, 2H, CH=CHCH$_2$CH=CH), 3.51-3.60 (stack, 2H, C(1")H$_a$H$_b$ for both epimers, C(1)H$_a$H$_b$ for both epimers), 3.63-3.70 (stack, 2H, C(1)H$_a$H$_b$ for both epimers, C(4)H$_a$H$_b$ for both epimers), 3.72-3.80 (stack, 2H, C(4)H$_a$H$_b$ for both epimers, C(1")H$_a$H$_b$ for both epimers), 3.87-3.93 (stack, 1.5H, C(2")H for epimer 1, C(3)H for both epimers), 3.95 (dd, $J$ 7.3, 4.6, 0.5H, C(2")H for epimer 2), 3.97-4.02 (stack, 1H, C(2)H for both epimers), 4.03-4.11 (stack, 2H, C(3')H for both epimers, C(4')H for both epimers), 4.12-4.18 (stack, 1H, C(2')H for both epimers), 5.27-5.39 (stack, 4H, 2 × CH=CH$_2$), 6.46 (d, $J$ 9.0, 0.5H, NH for epimer 1), 6.49 (d, 0.5H, $J$ 9.7, NH for epimer 2); $\delta_{\text{C}}$(125 MHz, CDCl$_3$) [14.0, 14.1 (CH$_3$, C18’, C20’)], [22.5, 22.7 (CH$_2$, C3”’)], 25.3 (CH$_2$), 25.4 (CH$_2$), 25.6 (CH$_2$, C13”), 25.7 (CH$_3$, C(CH$_3$)$_a$(CH$_3$)$_b$ of 1,2-anti diol for both epimers (resonance overlap)], 26.5 (CH$_2$), 26.6 (CH$_2$), [26.91, 26.94, 26.98 (CH$_3$, C(CH$_3$)$_2$ of 1,2-syn diol for both epimers (resonance overlap))], [27.18, 27.20 (CH$_2$, C10”’), C16”’)], [27.86, 27.93 (CH$_3$, C(CH$_3$)$_a$(CH$_3$)$_b$ of 1,2-anti diol)], 29.0 (CH$_2$), [29.16, 29.20, 29.25, 29.33, 29.4, 29.5, 29.6, 29.7 (CH$_2$, alkyl chain, some resonance overlap)], 31.5 (CH$_2$), 31.9 (CH$_2$), [32.1, 32.3 (CH$_2$, C3”)], [48.4, 48.6 (CH, C2’)], [62.4, 62.5, (CH$_2$, C4)], [64.3, 64.6 (CH, C2”)], 71.4 (CH$_2$, C1’ for both epimers (resonance overlap)), [71.71, 71.73 (CH$_2$, C1)], [76.0, 76.1 (CH, C3’ or C4’)], [76.49, 76.50 (CH, C2)], [77.6, 77.7 (CH, C4’ or C3’)], [79.2, 79.4]
(CH, C3)], [108.1, 108.2 (C, (CH)\textsubscript{2}C of 1,2-anti diol)], [109.29, 109.31 (C, (CH)\textsubscript{2}C of 1,2-syn diol)], 127.9 (CH, CH=CH), 128.0 (CH, CH=CH), 130.0 (CH, CH=CH), 130.2 (CH, CH=CH), [168.8, 168.9 (C, C=O)]; MS (TOF ES+) m/z 855.2 [M + Na]\textsuperscript{+}, 100%; HRMS (TOF ES+) 855.6579 [M + Na]\textsuperscript{+}. C\textsubscript{48}H\textsubscript{88}N\textsubscript{4}O\textsubscript{7}Na requires 855.6551.

Triazole 146

\[ \text{CuSO}_4 \text{ solution (10 } \mu\text{L of a 0.5 M aqueous solution, 10 } \mu\text{mol) and sodium ascorbate solution (40 } \mu\text{L of a 1 M aqueous solution, 40 } \mu\text{mol) were added to a solution of azide 145 (80 mg, 0.096 mmol) and alkyne 114 (33 mg, 0.115 mmol) in } ^1\text{BuOH} / \text{H}_2\text{O (1 mL, 1:1) at r.t. The reaction mixture was heated for 10 h at 50 } ^\circ\text{C and then diluted with CHCl}_3 (10 mL), and washed with brine (3 mL). The phases were separated and the aqueous layer was extracted with CHCl}_3 (2 \times 5 mL). The combined organic layers were dried over Na}_2\text{SO}_4 and the volatiles were removed under reduced pressure. Purification of the residue by flash column} \]
chromatography (gradient: 50% → 90% EtOAc in hexane) afforded 1,2,3-triazole 146 as a colourless oil (108 mg, 95%, isolated as 45 mg of a more polar epimer, 40 mg of less polar epimer and 23 mg of 1:1 mixture of two epimers). Data for less polar epimer: $R_f = 0.3$ (40% EtOAc in hexane); $[\alpha]_D^{20} = + 5.6$ (c 1, CHCl$_3$);

$\nu_{\text{max}}$(film) / cm$^{-1}$ 3316br (OH), 2924s, 2855s, 1717m (C=O), 1688s (C=O), 1515m, 1458m, 1367s, 1248s, 1220w, 1171w, 1100s, 848w, 723w; $\delta_\nu$(500 MHz, CDCl$_3$) 0.84-0.89 (stack, 6H, 2 × CH$_2$CH$_3$), 1.18-1.34 (stack, 43H, alkyl chain methylenes, C(CH$_3$)$_a$(CH$_3$)$_b$ for 1,2-anti-diol), 1.35-1.50 [stack, 22H, including (1.37 (stack, 6H, C(CH$_3$)$_a$(CH$_3$)$_b$ of 1,2-anti diol, C(CH$_3$)$_a$(CH$_3$)$_b$ of 1,2-syn diol), 1.39 (s, 3H, C(CH$_3$)$_a$(CH$_3$)$_b$ of 1,2-syn diol), 1.42 (s, 9H, C(CH$_3$)$_a$), 1.98-2.05 (stack, 4H, CH$_2$CH=CHCH$_2$CH=CHCH$_2$), 2.08-2.24 (stack, 2H, C(3")H$_2$), 2.74 (app. t, J 6.8, 2H, CH=CHCH$_2$CH=CH), 3.25-3.32 (stack, 2H, CH$_2$CH$_2$NHBOc), 3.40-3.44 (m, 1H, C(1")H$_a$H$_b$), 3.48-3.54 (stack, 4H, CH$_2$CH$_2$NHBOc, C(1)H$_2$), 3.58-3.63 (stack, 4H, PEG CH$_2$O), 3.64-3.72 (stack, 7H, C(4)H$_2$, C(1")H$_a$H$_b$, C(4"")H$_2$, 2 × PEG CH$_2$O), 3.85-3.89 (m, 1H, C(2)H), 3.90-3.95 (m, 1H, C(3)H), 3.93-4.03 (stack, 2H, C(3")H, C(4")H), 4.06-4.12 (m, 1H, C(2")H), 4.66-4.69 (stack, 2H, C(3"")H$_2$), 4.93-4.99 (m, 1H, C(2"")H), 5.02-5.09 (m, 1H, NHBOc), 5.26-5.39 (stack, 4H, 2 × CH=CHH), 6.76 (d, J 8.3, 1H, NH), 7.70 (br s, 1H, C(1"")H), OH resonance not observed; $\delta_\nu$(125 MHz, CDCl$_3$) [14.0, 14.1 (CH$_3$, C18', C26")], 22.5 (CH$_2$), 22.7 (CH$_2$), [25.6 (CH$_2$, CH=CHCH$_2$CH=CH, and CH$_3$, C(CH$_3$)$_a$(CH$_3$)$_b$ of 1,2-anti diol)], 25.9 (CH$_2$), 26.5 (CH$_2$), 26.9 (CH$_3$, C(CH$_3$)$_a$(CH$_3$)$_b$ of 1,2-syn diol), 27.1 (CH$_3$, C(CH$_3$)$_a$(CH$_3$)$_b$ of 1,2-syn diol), [27.17, 27.19 (CH$_2$, CH$_2$CH=CHCH$_2$CH=CHCH$_2$)], 27.9 (CH$_3$, C(CH$_3$)$_a$(CH$_3$)$_b$ of 1,2-anti diol), 28.4 (CH$_3$, C(CH$_3$)$_a$), 28.9 (CH$_2$), 29.0 (CH$_2$), [29.2, 29.32, 29.34, 29.4 (CH$_2$, resonance overlap)], [29.58, 29.63, 29.70 (CH$_2$,
resonance overlap]), 31.5 (CH$_2$), 31.9 (CH$_2$, C3”), 40.4 (CH$_2$, CH$_2$NHBOc), 48.8 (CH, C2’), 62.7 (CH$_2$, C4), 64.6 (CH$_2$, C3’”), 65.6 (CH, C2”), 70.0 (CH$_2$, C4’”), [70.2, 70.3, 70.50, 70.52 (CH$_2$, 4 × CH$_2$O in PEG linker)], 71.0 (CH$_2$, C1”), 71.6 (CH$_2$, C1), 75.9 (CH, C3” or C4”), 77.5 (CH, C4’ or C3’), 79.4 (CH, C3 and C, C(CH$_3$)$_3$), 108.1 (C, (CH$_3$)$_2$C of 1,2-anti diol), 109.2 (C, (CH$_3$)$_2$C of 1,2-syn diol), 123.2 (CH, C1’”), 127.9 (CH, CH=CH), 128.0 (CH, CH=CH), 145.4 (C, C2’”), 167.3 (C, CH$_2$C(O)NH), C=O of Boc not observed; MS (TOF ES+) m/z 1142.9 [M + Na]$^+$, 100%; HRMS (TOF ES+) calcd for C$_{62}$H$_{113}$N$_5$O$_{12}$Na [M + Na]$^+$ 1142.8283, found 1142.8251.

Data for more polar epimer: $R_f$ = 0.2 (40% EtOAc in hexane); $[\alpha]_D^{20}$ = + 11.2 (c 1, CHCl$_3$); $\nu_{\text{max}}$(film) / cm$^{-1}$ 3293br (OH), 2925s, 2854s, 1715m (C=O), 1685m (C=O), 1532m, 1463m, 1368m, 1249m, 1220w, 1108m, 724w; $\delta_\ell$(500 MHz, CDCl$_3$) 0.84-0.89 (stack, 6H, 2 × CH$_2$CH$_3$), 1.19-1.34 (stack, 43H, alkyl chain methylenes, C(CH$_3$)$_{a}(CH$_3$)$_{b}$ for 1,2-anti diol), 1.35-1.50 [stack, 22H, (including 1.37 (6H, stack, C(CH$_3$)$_{a}(CH$_3$)$_{b}$ of 1,2-anti diol, C(CH$_3$)$_{a}(CH$_3$)$_{b}$ of 1,2-syn diol), 1.39 (s, 3H, C(CH$_3$)$_{a}(CH$_3$)$_{b}$ of 1,2-syn diol), 1.42 (s, 9H, C(CH$_3$)$_3$)], 1.98-2.05 (stack, 5H, CH$_2$CH=CHCH$_2$CH=CHCH$_2$, C(3”)H$_a$H$_b$), 2.16-2.25 (m, 1H, C(3”)H$_a$H$_b$), 2.74 (app. t, J 6.6, 2H, CH=CHCH$_2$CH=CH), 3.24-3.33 (stack, 2H, CH$_2$NHBOc), 3.38-3.43 (m, 1H, C(1’)H$_a$H$_b$), 3.49-3.56 (stack, 4H, CH$_2$CH$_2$NHBOc, C(1)H$_2$), 3.58-3.67 (stack, 6H, PEG CH$_2$O), 3.64-3.72 (stack, 5H, C(4)H$_2$, C(1’)H$_a$H$_b$, 2 × PEG CH$_2$O), 3.89-3.97 (stack, 2H, C(2)H, C(3)H), 3.98-4.05 (stack, 2H, C(3’)H, C(4’)H), 4.05-4.11 (m, 1H, C(2’)H), 4.66-4.69 (stack, 2H, C(3”)H$_2$), 4.99-5.10 (stack, 2H, C(2”)H).
NH\textsubscript{Boc}, 5.26-5.39 (stack, 4H, 2 × CH=CH\textsubscript{2}), 6.57 (d, J 8.4, 1H, NH\textsubscript{2}), 7.75 (br s, 1H, C(1′′′)H), OH resonance not observed; \(\delta\)\textsubscript{C}(125 MHz, CDCl\textsubscript{3}) [14.0, 14.1 (CH\textsubscript{3}, C18′, C20′)]; 22.5 (CH\textsubscript{2}), 22.7 (CH\textsubscript{2}), [25.6 (CH\textsubscript{2}, CH=CHCH\textsubscript{2}CH=CH, \textit{and} CH\textsubscript{3}, C(CH\textsubscript{3})\textsubscript{a}(CH\textsubscript{3})\textsubscript{b} of 1,2-\textit{anti diol}), 25.8 (CH\textsubscript{2}), 26.5 (CH\textsubscript{2}), 26.9 (CH\textsubscript{3}, C(CH\textsubscript{3})\textsubscript{a}(CH\textsubscript{3})\textsubscript{b} of 1,2-\textit{syn diol}, 27.1 (CH\textsubscript{3}, C(CH\textsubscript{3})\textsubscript{a}(CH\textsubscript{3})\textsubscript{b} of 1,2-\textit{syn diol), [27.17, 27.19 (CH\textsubscript{2}, CH\textsubscript{2}CH=CHCH\textsubscript{2}CH=CHCH\textsubscript{2})), 27.9 (CH\textsubscript{3}, C(CH\textsubscript{3})\textsubscript{a}(CH\textsubscript{3})\textsubscript{b} of 1,2-\textit{anti diol}, 28.4 (CH\textsubscript{3}, C(CH\textsubscript{3})\textsubscript{3}), 29.0 (CH\textsubscript{2}), 29.1 (CH\textsubscript{2}), [29.22, 29.27, 29.31, 29.33, 29.4 (CH\textsubscript{2}, resonance overlap)], [29.6, 29.7 (CH\textsubscript{2}, resonance overlap)], 31.5 (CH\textsubscript{2}), 31.9 (CH\textsubscript{2}), 32.4 (CH\textsubscript{2}, C3′′′), 40.3 (CH\textsubscript{2}, CH\textsubscript{2}NH\textsubscript{Boc}), 49.0 (CH, C2′), 62.7 (CH\textsubscript{2}, C4), 64.6 (CH\textsubscript{2}, C3′′′ \textit{and CH}, C2′′), [70.0, 70.2, 70.49, 70.51 (CH\textsubscript{2}, 5 × CH\textsubscript{2}O in PEG linker, resonance overlap]), 70.9 (CH\textsubscript{2}, C1′), 71.7 (CH\textsubscript{2}, C1), 75.8 (CH, C3′ or C4′), 76.7 (CH, C2 or C3), 77.5 (CH, C4′ or C3′), 79.1 (CH, C3 or C2 and C, C(CH\textsubscript{3})\textsubscript{3}), 108.1 (C, (CH\textsubscript{3})\textsubscript{2}C of 1,2-\textit{anti diol), 109.2 (C, (CH\textsubscript{3})\textsubscript{2}C of 1,2-\textit{syn diol), 122.2 (CH, C1′′′), 127.9 (CH, CH=CH), 128.0 (CH, CH=CH\textsubscript{2}), 130.0 (CH, CH=CH), 130.2 (CH, CH=CH), 145.5 (C, C2′′′′), 156.0 (C, OC(O)NH), 167.3 (C, amide C=O);
TFA (60 µL) was added dropwise over 1 min to a solution of Boc amide 146 (60 mg, 0.050 mmol) in CH₂Cl₂ / CH₃OH (2:1, 0.6 mL). After stirring overnight at r.t., the reaction mixture was concentrated under reduced pressure and the residual TFA was removed by co-evaporation with Et₂O (3 × 3 mL) to provide crude amine 147 as a white solid (51 mg, quant.), which was used in the step without further purification.

NEt₃ (1 µL, 7.2 µmol) was added to a solution of crude amine 147 (2.1 mg, 2.3 µmol) in DMF (10 µL). After stirring for 1 h at r.t., Alexa Fluor® 488 carboxylic acid, succinimidyl ester 144 (1.2 mg, 1.1 µmol) was added. The reaction mixture was stirred for 12 h at r.t. and then the volatiles were removed under reduced pressure. Purification of the residue by flash column chromatography (eluent: gradient 0-50% CH₃OH in CHCl₃) afforded compound 110 as a dark blue solid (0.7 mg, 62%); selected data: $R_f = 0.3$ for epimer A, $R_f = 0.2$ for epimer B in 65:35:4 CHCl₃ / MeOH / aq. NH₃;
(2S,3S,4R)-2-(Z,Z)-2''-Azido-eicosa-11'',14''-dienoylamino-3,4-O-isopropylidene-1-O-(2',3',4',6'-tetra-O-benzyl-α-D-galactopyranosyl)octadecane-1,3,4-triol (141)

DMF (1 µL, 13µmol) was added to a solution of (Z,Z)-2-azido-eicosa-11,14-dienoic acid (32 mg, 0.093 mmol) in CH₂Cl₂ (1 mL) and the mixture was cooled to 0 ºC. A solution of oxalyl chloride (16 µL, 0.186 mmol) in CH₂Cl₂ (0.5 mL) was added dropwise over 2 min. After 2 h, the volatiles were removed under reduced pressure to provide the corresponding acid chloride 140 as a pale yellow oil, which was used directly in the next step without further purification (48 mg, quant.): A solution of freshly prepared acid chloride 140 (34 mg, 0.093 mmol) in CH₂Cl₂ (0.5 mL) was added dropwise over 2 min to a solution of amine 128 (57 mg, 0.062 mmol) and NEt₃ (18 µL, 0.013 mmol) in CH₂Cl₂ at 0 ºC. The reaction mixture was stirred for 12 h and then diluted with CH₂Cl₂ (10 mL), washed sequentially with NaHCO₃ solution (10 mL), brine (2 mL) and then dried over Na₂SO₄. The drying agent was removed by filtration and the filtrate concentrated under reduced pressure. Purification of the residue by flash column chromatography (20% EtOAc in hexane) provided amide 141 as a colourless oil (70 mg, 85%, 1:1 mixture of epimers); data on epimeric mixture: \( R_f = 0.3 \) (20% EtOAc in hexane); \( \nu_{\text{max}} \) (film) /
cm\(^{-1}\) 2923s, 2853m, 2103m (N\(_3\)), 1683m, (C=O), 1586w, 1513s, 1464m, 1368w, 1302m, 1247s, 1172m, 1092m, 1038s, 822m, 720w; \(\delta (500\, \text{MHz, CDCl}_3)\) 0.84-0.90 (stack, 6H, 2 × CH\(_2\)CH\(_3\)), 1.16-1.40 (stack, 54H), 1.41-1.52 (stack, 2H), 1.61-1.86 (stack, 2H, C(3")H\(_2\)), 1.99-2.07 (stack, 4H, CH\(_2\)CH=CHCH\(_2\)CH=CHCH\(_2\)), 2.73-2.78 (stack, 2H, CH=CHCH\(_2\)CH=CH), 3.33-3.41 (stack, 1H, C(6")H\(_a\)H\(_b\) for both epimers), 3.42-3.47 (stack, 1H, C(6")H\(_a\)H\(_b\) for both epimers), 3.58-3.65 (stack, 1H, C(1)H\(_a\)H\(_b\) for both epimers), 3.67-3.71 (m, 0.5H, C(2")H for epimer 1), 3.74-3.81 (stack, 1H, C(3")H\(_a\)H\(_b\) for both epimers, 3.81-3.89 (stack, 3H, C(3")H for both epimers, C(4")H for both epimers, C(5")H for both epimers), 3.90-4.01 (stack, 3H, C(1)H\(_a\)H\(_b\) for both epimers, C(4)H for both epimers, C(2")H for both epimers), 4.02-4.10 (stack, 1H, C(2)H for both epimers), 4.11-4.19 (stack, 1H, C(3)H for both epimers), 4.27 (A of AB, \(J_{A-B}\) 11.6, 1H, C(6")OCH\(_a\)H\(_b\)Ar for epimer 1), 4.28 (A of AB, \(J_{A-B}\) 11.6, 1H, C(6")OCH\(_a\)H\(_b\)Ar for epimer 2), 4.40 (B of AB, \(J_{B-A}\) 11.6, 1H, C(6")OCH\(_a\)H\(_b\)Ar for epimer 1), 4.42 (B of AB, \(J_{B-A}\) 11.6, 1H, C(6")OCH\(_a\)H\(_b\)Ar for epimer 2), 4.46 (A of AB, \(J_{A-B}\) 11.2, 1H, C(4")OCH\(_a\)H\(_b\)Ar for epimer 1), 4.47 (A of AB, \(J_{A-B}\) 11.2, 1H, C(4")OCH\(_a\)H\(_b\)Ar for epimer 2), 4.56 (A of AB, \(J_{A-B}\) 11.2, 1H, C(2")OCH\(_a\)H\(_b\)Ar for epimer 1), 4.57 (A of AB, \(J_{A-B}\) 11.2, 1H, C(2")OCH\(_a\)H\(_b\)Ar for epimer 2), 4.63 (A of AB, \(J_{A-B}\) 11.2, 1H, C(3")OCH\(_a\)H\(_b\)Ar for epimer 1), 4.64 (A of AB, \(J_{A-B}\) 11.2, 1H, C(3")OCH\(_a\)H\(_b\)Ar for epimer 2), 4.70 (B of AB, \(J_{B-A}\) 11.2, 1H, C(3")OCH\(_a\)H\(_b\)Ar for both epimers), 4.72 (B of AB, \(J_{B-A}\) 11.2, 1H, C(2")OCH\(_a\)H\(_b\)Ar for epimer 1), 4.73 (B of AB, \(J_{B-A}\) 11.2, 1H, C(2")OCH\(_a\)H\(_b\)Ar for epimer 2), 4.81 (B of AB, \(J_{B-A}\), 1H, C(4")OCH\(_a\)H\(_b\)Ar for both epimers), 4.89 (stack, 1H, C(1")H, for both epimers), 5.27-5.41 (stack, 4H, 2 × CH=CH\(_2\)), 6.75-6.89 (stack, 8H, Ar), 7.12-7.19 (stack, 4H, Ar), 7.23-7.32 (stack, 4H,
Ar); \[ \delta_{c}(125 \text{ MHz, CDCl}_3) \] [14.0, 14.1 (CH$_3$, C18', C20")], [22.6, 22.7 (CH$_2$)], [25.6, 25.7 (CH$_2$, alkyl chain and C13" (resonance overlap)], [25.9, 26.0 (CH$_3$, C(CH$_3$)$_2$ for both epimers)], 26.6 (CH$_2$), 26.7 (CH$_2$), [27.18, 27.23 (CH$_2$, C10", C16")], [28.1, 28.2 (CH$_3$, C(CH$_3$)$_2$ for both epimers)], 28.1 (CH$_2$), [28.95, 29.02, 29.25, 29.30, 29.32, 29.35, 29.38, 29.45, 29.48, 29.54, 29.57, 29.62, 29.65, 29.69, (CH$_2$, alkyl chain, some resonance overlap)], 31.5 (CH$_2$), 31.9 (CH$_2$, C3"), 32.0 (CH$_2$), 32.1 (CH$_2$), [48.8, 49.1 (CH, C2)], [55.21, 55.24 (CH$_3$, 4 \times \text{ArOCH}_3$, resonance overlap)], [64.3, 64.6 (CH, C2")], 68.6 (CH$_2$, C1 for epimer 1), [68.88, 68.91 (CH$_2$, C6")], 69.0 (CH$_2$, C1 for epimer 2), 69.8 (CH, C5' for both epimers (resonance overlap)], [72.6, 72.7 (CH$_2$, C(3')OCH$_2$Ar), [73.07, 73.12, 73.15 (CH$_2$, C(3')OCH$_2$Ar and C(3')OCH$_2$Ar (resonance overlap))], 74.2 (CH$_2$, C(4')OCH$_2$Ar for both epimers (resonance overlap)), [74.4, 74.5 (CH, C4')], [75.1, 75.2 (CH, C3)], 76.2 (CH, C2' for both epimers (resonance overlap)), [77.68, 77.72 (CH, C4)], [78.8, 78.9 (CH, C3')], [99.0, 99.2 (CH, C1')], [107.87, 107.93 (C, (CH$_3$)$_2$C)], [113.6, 113.73, 113.75 113.77 (CH, Ar ortho to OMe)], 127.9 (CH, CH=CH), 128.0 (CH, CH=CH), [129.0, 129.4, 129.5, 129.8, 129.9, (CH, Ar meta to OMe)], 130.0 (CH, CH=CH), 130.2 (CH, CH=CH), [130.6, 130.7, 130.9 (C, Ar para to OMe, some resonance overlap)], [159.1, 159.2, 159.3 (C, Ar ipso to OMe)], [168.8, 168.9 (C, C=O)]; MS (TOF ES+) m/z 1353.8 [M + Na]$^+$, 100%; HRMS (TOF ES+) calcd for C$_{79}$H$_{118}$N$_4$O$_{13}$Na [M + Na]$^+$ 1353.8593, found 1353.8610.
CuSO₄ solution (10 µL of a 0.5 M aqueous solution, 10 µmol) and sodium ascorbate solution (40 µL of a 1 M aqueous solution, 40 µmol) were added to a solution of azide 145 (100 mg, 0.075 mmol) and alkyne 114 (32 mg, 0.115 mmol) in t-BuOH / H₂O (1 mL, 1:1) at r.t. The reaction mixture was heated for 10 h at 50 °C and then diluted with CHCl₃ (10 mL), and washed with brine (3 mL). The phases were separated and the aqueous layer was extracted with CHCl₃ (2 × 5 mL). The combined organic layers were dried over Na₂SO₄ and the volatiles were removed under reduced pressure. Purification of the residue by flash column chromatography (gradient: 50% → 90% EtOAc in hexane) afforded 1,2,3-triazole 142 as a colourless oil (95 mg, 78% %, 1:1 mixture of epimers); data on epimeric mixture: \( R_f = 0.3 \) (40% EtOAc in hexane); \( \nu_{\text{max}}(\text{film}) / \text{cm}^{-1} \) 2916s, 2849m, 1789m, 1735, 1473m, 1368w, 1302m, 1206s, 1075m, 1172m, 909s, 730s; \( \delta_{\text{H}}(500 \text{ MHz, CDCl}_3) \) 0.84-0.89 (stack, 6H, 2 × CH₂CH₂), 1.12-1.36 (stack, 44H, alkyl chain methylenes, C(CH₃)₃(C(CH₃)₃)), 1.37-1.44 [stack, 16H, including C(CH₃)₃(C(CH₃)₃), C(CH₃)₃]], 1.92-2.08 (stack, 6H, CH₂CH=CHCH₂CH=CHCH₂, C(3”)H₂), 2.73 (app.
Chapter 5  Experimental Section

t, J 6.7, 2H, CH=CHCH₂CH=CH), 3.21-3.33 (stack, 3H, C(6”)HₐHₜ for both epimers, CH₂NHBOc), 3.39-3.46 (stack, 1H, C(6”)H₆Hₕ for both epimers), 3.47-3.54 (stack, 3H, CH₂CH₂NHBOc, C(1)HₗHₜ for both epimers), 3.55-3.74 (stack, 12H, including 4 × PEG CH₂O, C(4””)H₆), 3.75-3.82 (stack, 13H, 4 × ArOCH₃ for both epimers, C(3”)H for epimer 1 and C(4”)H for epimer 1), 3.84-4.16 (stack, 8H, including C(2”)H for both epimers, C(3”)H for epimer 2, C(4”)H for epimer 2, C(1)H₆Hₕ for both epimers, C(2)H for both epimers, C(4)H for both epimers, C(3)H for both epimers, 1 × PEG CH₂O), 4.17-4.21 (1H, stack), 4.24-4.31 (stack, 2H, A of AB, C(6”)OCH₃H₆Ar for both epimers), 4.40-4.59 (stack, 2H), 4.64-4.76 (stack, 2H), 4.76-4.82 (stack, 2H, including C(1”)H, for both epimers), 4.90-4.95 (stack, 1H, C(2””)H for both epimers), 4.99-5.06 (stack, 1H, NHBOc for both epimers), 5.25-5.39 (stack, 4H, 2 × CH=CH), 6.72-6.88 (stack, 8H, Ar), 7.11-7.19 (stack, 4H, Ar), 7.22-7.30 (stack, 4H, Ar), 7.66 (br s, 0.5H, C(1””)H for epimer 1), 7.72 (br s, 0.5H, C(1””)H for epimer 2), CONH resonance not observed; δ_C (125 MHz, CDCl₃) [14.0, 14.1 14.2 (CH₃, resonance overlap, C18, C20”), 22.5 (CH₂), 22.7 (CH₂), [25.6, 25.7 (CH₂, CH=CHCH₂CH=CH)], [25.86, 25.89 CH₃, C(CH₃)ₐ(CH₃)ₖ], 26.5 (CH₂), [27.18, 27.20 (CH₂, CH₂CH=CHCH₂CH=CHCH₂)], 28.2 (CH₃, C(CH₃)ₐ(CH₃)ₖ, 28.4 (CH₃, C(CH₃)ₖ), 28.8 (CH₂), 29.0 (CH₂), [29.2, 29.31, 29.33, 29.4 (CH₂, resonance overlap)], [29.58, 29.63, 29.70 (CH₂, resonance overlap)], 31.5 (CH₂), 31.9 (CH₂), 33.1 (CH₂, C3”), 40.3 (CH₂, CH₂NHBOc), [49.4, 49.5 (CH, C2)], [55.21, 55.24 (CH₃, 4 × ArOCH₃, resonance overlap)], [64.3, 64.6 (CH, C2”), 64.7 (CH₂, C3”), 68.2 (CH₂, C1), [69.1, 69.2 (CH₂, C6”, resonance overlap), [70.0, 70.2, 70.4, 70.50, 70.52 (CH₂, 4 × CH₂O in PEG linker)], [72.6, 72.9 (CH₂, OCH₂Ar), [73.04, 73.12, 73.15 (CH₂, OCH₂Ar (resonance overlap))], [74.1, 74.2
(CH₂, OCH₂Ar (resonance overlap)), [74.4, 74.5 (CH, C4')], [75.1, 75.2 (CH, C3)], 76.2 (CH, C2' for both epimers (resonance overlap)), 77.6 (CH, C4, for both epimers (resonance overlap)), [78.8, 78.9 (CH, C3' for both epimers (resonance overlap))], 79.4 (CH, C3 and C, C(CH₃)₃) [98.9, 99.6 (CH, C1')], [107.87, 107.91 (C, (CH₃)₂C)], [113.6, 113.73, 113.75 113.77 (CH, Ar ortho to OMe)], [121.8, 122.1 (CH, C1'″)], 127.9 (CH, CH=CH), 128.0 (CH, CH=CH), [129.0, 129.4, 129.5, 129.8, 129.9, (CH, Ar meta to OMe)], 130.0 (CH, CH=CH), 130.2 (CH, CH=CH), [130.6, 130.7, 130.9 (C, Ar para to OMe, some resonance overlap)], [145.4, 145.2 (C, C2'″)], [159.1, 159.2, 159.3 (C, Ar ipso to OMe)], [167.31, 167.33 (C, CH₂C(O)NH)], [171.1 C of Boc]; MS (TOF ES+) m/z 1641.0 [M + Na]^+, 100%;
TFA (60 µL) was added dropwise over 1 min to a solution of Boc amide 142 (84 mg, 0.050 mmol) in CH$_2$Cl$_2$ / CH$_3$OH (2:1, 0.6 mL). After stirring overnight at r.t., the reaction mixture was concentrated under reduced pressure and the residual TFA was removed by co-evaporation with Et$_2$O (3 x 3 mL) to provide crude amine 143 as a white solid (49 mg, quant.), which was used in the step without further purification: selected data: MS (TOF ES+) m/z 1020.8 ([M + Na]$^+$, 100%);
NEt$_3$ (1 µL, 7.2 µmol) was added to a solution of crude amine 143 (2.3 mg, 2.3 µmol) in DMF (10 µL). After stirring for 1 h at r.t., Alexa Fluor® 594 carboxylic acid, succinimidyl ester 144 (1.0 mg, 1.2 µmol) was added. The reaction mixture was stirred for 12 h at r.t. and then the volatiles were removed under reduced pressure. Purification of the residue by flash column chromatography (eluent: gradient 0-50% CH$_3$OH in CHCl$_3$) afforded compound 109 as a dark blue solid (0.9 mg, 70%);
selected data: $R_f = 0.3$ for epimer A, $R_f = 0.2$ for epimer B in 65:35:4 CHCl₃ / MeOH / aq. NH₃.

**Methyl 3',4'-O-isopropylidene-2'-O-(2,3,4,6-tetra-O-p-methoxybenzyl-D-galactopyranosyl)-(1→2)-L-threonate (155)**

![Chemical structure](image)

PPh₃ (1.46 g, 5.55 mmol) and CBr₄ (1.84 g, 5.55 mmol) were added sequentially to a solution of 2,3,4,6-tetra-O-p-methoxybenzyl-D-galactose 126 (1.22 g, 1.85 mmol) in CH₂Cl₂ (10 mL) at r.t. The reaction mixture was stirred for 3 h. In separate flasks, a solution of TMU (1.2 mL), DIPEA (1.6 mL) and Bu₄NBr (1.79 g, 5.55 mmol) in CH₂Cl₂ (5 mL), and a solution of methyl 3,4-O-isopropylidene-L-threonate 154 (493 mg, 2.59 mmol) in CH₂Cl₂ (5 mL) were stirred over activated 3 Å MS for 30 min, after which time, these solutions were added dropwise over 5 min, and sequentially (TMU/DIPEA/Bu₄NBr solution first), to the solution containing the glycosyl donor. The reaction mixture was stirred at r.t. for 2 d until there was no evidence by TLC that the donor was still being consumed. The reaction mixture
was then concentrated under reduced pressure, re-suspended in toluene (10 mL), filtered through a silica plug, washed with toluene (150 mL) and concentrated under reduced pressure to provide the crude product. Purification of the residue by flash column chromatography (20% EtOAc in hexane) afforded glycoside 153 as a colourless oil (693 mg, 45%, single α-anomer); $[\alpha]_D^{18} = + 11.2$ (c 1, CHCl$_3$); $\nu_{max}$ (film) / cm$^{-1}$: 2935 w, 2872 w, 1739 m (C=O), 1623 m, 1581 w, 1451 m, 1323 s, 1128 s, 1103 s, 1064 s, 893 m, 843 s, 736 s; $\delta_{H}$(500 MHz, CDCl$_3$) 1.30 (s, 3H, $1 \times C(CH_3)_2$), 1.35 (s, 3H, $1 \times C(CH_3)_2$), 3.40 (A of ABX, $J_{A-B}$ 8.9, $J_{A-X}$ 5.7, 1H, C(6)H$_a$H$_b$), 3.44-3.49 (m, 1H, C(6)H$_a$H$_b$), 3.72 (s, 3H, C(O)OC$_2$H$_3$), 3.76 (s, 3H, ArOC$_2$H$_3$), 3.775 (s, 3H, ArOC$_2$H$_3$), 3.778 (s, 3H, ArOC$_2$H$_3$), 3.80 (s, 3H, ArOC$_2$H$_3$), 3.90 (dd, $J_{A-X}$ 5.7, 1H, C(6)H$_a$H$_b$), 3.92-3.96 (stack, 2H, H3, H4), 4.00-4.06 (stack, 2H, H2, C(4')H$_a$H$_b$), 4.15 (app t, $J_{6.5}$, 1H, H5), 4.27 (d, $J_{7.0}$, 1H, H$^2''$), 4.30 (A of AB, $J_{A-B}$ 11.4, 1H, C(6)OCH$_a$H$_b$Ar), 4.35 (B of AB, $J_{B-A}$ 11.4, 1H, C(6)OCH$_a$H$_b$Ar), 4.41 (app. q, $J_{6.6}$, 1H, H$^3''$), 4.48 (A of AB, $J_{A-B}$ 11.2, 1H, C(4)OCH$_a$H$_b$Ar), 4.615 (A of AB, $J_{A-B}$ 11.2, 1H, C(3)OCH$_a$H$_b$Ar), 4.620 (A of AB, $J_{A-B}$ 11.4, 1H, C(2)OCH$_a$H$_b$Ar), 4.73 (B of AB, $J_{B-A}$ 11.4, 1H, C(2)OCH$_a$H$_b$Ar), 4.74 (A of AB, $J_{A-B}$ 11.2, 1H, C(3)OCH$_a$H$_b$Ar), 4.82 (B of AB, $J_{B-A}$ 11.2, 1H, C(4)OCH$_a$H$_b$Ar), 5.09 (d, $J_{3.7}$, 1H, H1), 6.73-6.87 (stack, 8H, Ar), 7.15-7.19 (stack, 4H, Ar), 7.23-7.33 (stack, 4H, Ar); $\delta_{C}$(125 MHz, CDCl$_3$) 25.3 (CH$_3$, $1 \times C(CH_3)_2$), 26.1 (CH$_3$, $1 \times C(CH_3)_2$), 52.0 (CH$_3$, C(O)OCH$_3$), 55.2 (CH$_3$, 4 $\times$ ArOCH$_3$ resonance overlap), 66.1 (CH$_2$, C4'), 68.3 (CH$_2$, C6), 69.7 (CH, C5), 72.0 (CH$_2$, C2)OCH$_2$Ar), 72.9 (CH$_2$, C(3)OCH$_2$Ar), 73.1 (CH$_2$, C(6)OCH$_2$Ar), 74.2 (CH$_2$, C(4)OCH$_2$Ar), 74.4 (CH, C4), 75.3 (CH, C3'), 75.4 (CH, C2'), 75.6 (CH, C2), 78.3 (CH, C3), 96.0 (CH, C1), 109.7 (C, C(3)OCH$_2$), [113.51, 113.54, 113.6, 113.7].
(CH, Ar ortho to OMe)], [129.0, 129.5, 129.8 (CH, Ar meta to OMe, some resonance overlap)], 130.0 (C, Ar para to OMe), 130.8 (C, Ar para to OMe), 130.9 (C, Ar para to OMe), 131.2 (C, Ar para to OMe), 158.9 (C, Ar ipso to OMe), 159.0 (C, Ar ipso to OMe), 159.1 (C, Ar ipso to OMe), 159.2 (C, Ar ipso to OMe), 169.9 (C, C=O); MS (TOF ES+) m/z 855.2 ([M + Na]+, 100%); HRMS (TOF ES+) calcd for C₄₆H₅₆O₁₄Na ([M + Na]+) 855.3568, found 855.3531.

This reaction was first performed without the addition of Hünig’s base, which led to the formation of a mixture of two glycosyl products 155 and 156. Selected data for 156:

δₜ(500 MHz, CDCl₃) selected data: 1.43 (s, 3H, 1 × C(CH₃)₂), 1.44 (s, 3H, 1 × C(CH₃)₂), 3.65 (dd, J 11.6, 5.0, 1H, C(4’)H₂H₆₂), 3.73 (s, 3H, C(O)OC₃H₃), 3.78 (s, 3H, ArOCH₃), 3.79 (s, 6H, 2 × ArOCH₃), 3.80 (s, 3H, ArOCH₃), 4.42-4.44 (m, 1H, H2’), 4.90 (d, J 3.5, 1H, H1); δₜ(125 MHz, CDCl₃) selected data: 25.9 (CH₃, 1 × C(CH₃)₂), 26.8 (CH₃, 1 × C(CH₃)₂), 52.3 (CH₃, C(O)OCH₃), 55.2 (CH₃, 4 × ArOCH₃)
resonance overlap), 67.5, 68.6, 69.5, 72.6, 72.7, 73.0, 74.2, 74.6, 75.54, 76.2, 78.4, 78.5, 98.2 (CH, C1), 111.5 (C, (CH$_3$)$_2$C), 170.9 (C, C=O).

3'4'-O-isopropylidene-2'-O-(2,3,4,6-tetra-O-p-methoxybenzyl-D-galactopyranosyl)-(1→2)-L-threitol (153)

A solution of methyl ester 155 (200 mg, 0.24 mmol) in Et$_2$O (1 mL) was added over 5 min to a stirred suspension of LiAlH$_4$ (14 mg, 0.36 mmol) in Et$_2$O (2 mL). After an initial vigorous reaction had subsided, the mixture was heated at reflux for 3 h and then cooled to r.t., diluted with Et$_2$O (10 mL) and poured into NH$_4$Cl solution (5 mL). H$_2$O (5 mL) was added and the layers were separated. The aqueous phase was extracted with Et$_2$O (3 × 10 mL). The combined organic extracts were dried (Na$_2$SO$_4$) and concentrated under reduced pressure. Purification of the residue by flash column chromatography (30% EtOAc in hexane) afforded alcohol 153 as a colourless oil (183 mg, 95%). $R_f = 0.3$ (40% EtOAc in hexane); $[\alpha]_D^{18} = +39.6$ (c 1,
CHCl$_3$; $v_{\text{max}}$(film) / cm$^{-1}$: 3465 br (O–H), 2920w, 2878w, 1497w, 1454m, 1370m, 1209m, 1078s, 1049s, 911m, 850m, 736s, 698s; $\delta$(500 MHz, CDCl$_3$) 1.25 (s, 3H, 1 $\times$ C(CH$_3$)$_2$), 1.34 (s, 3H, 1 $\times$ C(CH$_3$)$_2$), 3.40-3.49 (stack, 2H, C(6)H$_a$H$_b$), 3.57-3.61 (stack, 2H, C(1')H$_a$H$_b$), 3.61-3.68 (m, 1H, H2'), 3.766 (s, 3H, ArOC$_3$H$_3$), 3.769 (s, 3H, ArOC$_3$H$_3$), 3.774 (s, 3H, ArOC$_3$H$_3$), 3.799 (s, 3H, ArOC$_3$H$_3$), 3.84 (dd, $J$ 8.4, 5.8, 1H, C(4'')H$_a$H$_b$), 3.89-3.95 (stack, 3H, C(4'')H$_a$H$_b$, H3, H4), 4.01 (dd, $J$ 9.9, 3.8, 1H, H2), 4.08-4.12 (m, 1H, H5), 4.16 (app. q, $J$ 5.9, 1H, H3'), 4.33 (A of AB, $J_{A,B}$ 11.7, 1H, C(6)OCH$_a$H$_b$Ar), 4.39 (B of AB, $J_{B,A}$ 11.7, 1H, C(6)OCH$_a$H$_b$Ar), 4.48 (A of AB, $J_{A,B}$ 11.3, 1H, C(4)OCH$_a$H$_b$Ar), 4.62 (A of AB, $J_{A,B}$ 11.2, 1H, C(2)OCH$_a$H$_b$Ar), 4.65 (s, 2H, C(3)OCH$_2$Ar), 4.80 (B of AB, $J_{B,A}$ 11.2, 1H, C(2)OCH$_a$H$_b$Ar), 4.82 (B of AB, 1H, $J_{B,A}$ 11.3, C(4)OCH$_a$H$_b$Ar), 4.89 (d, $J$ 3.9, 1H, H1), 6.78-6.91 (stack, 8H, Ar), 7.13-7.26 (stack, 6H, Ar), 7.27-7.32 (stack, 2H, Ar); $\delta$(125 MHz, CDCl$_3$) 24.9 (CH$_3$, 1 $\times$ C(CH$_3$)$_2$), 26.3 (CH$_3$, 1 $\times$ C(CH$_3$)$_2$), 55.15 (CH$_3$, ArOCH$_3$), 55.19 (CH$_3$, 2 $\times$ ArOCH$_2$, resonance overlap), 55.22 (CH$_3$, ArOCH$_3$), 62.0 (CH$_2$, C1'), 65.4 (CH$_2$, C4'), 68.4 (CH$_2$, C6), 69.7 (CH, C5), 72.1 (CH$_2$, C(3)OCH$_2$Ar), 73.0 (CH$_2$, C(6)OCH$_2$Ar), 74.05 (CH, C4), 74.13 (CH$_2$, C(2)OCH$_2$Ar), 74.2 (CH$_2$, C(4)OCH$_2$Ph), 75.2 (CH, C3'), 75.8 (CH, C2), 79.3 (CH, C3), 81.9 (CH, C2'), 100.2 (CH, C1), 109.3 (C, (CH$_3$)$_2$C), [113.5, 113.69, 113.74, 113.8 (CH, Ar ortho to OMe)], [129.0, 129.4, 129.7, 130.0 (CH, Ar meta to OMe)], [130.6, 130.8 (C, Ar para to OMe, some resonance overlap)], [159.08, 159.11, 159.2, 159.3 (C, Ar ipso to OMe)]; MS (TOF ES+) m/z 827.1 ([M + Na]$^+$, 100%); HRMS (TOF ES+) calcd for C$_{45}$H$_{56}$O$_{13}$Na ([M + Na]$^+$) 827.3619, found 827.3621.
Methyl 3′,4′-O-isopropylidene-2-O-(2,3,4,6-tetra-O-benzyl-D-galactopyranosyl)-(1→2)-L-threonate (157)

**Experimental Section**

PPh₃ (1.46 g, 5.55 mmol) and CBr₄ (1.84 g, 5.55 mmol) were added sequentially to a solution of 2,3,4,6-tetra-O-benzyl-D-galactose 51 (1.00 g, 1.85 mmol) in CH₂Cl₂ (10 mL) at r.t. The reaction mixture was stirred for 3 h. In separate flasks, a solution of TMU (1.2 mL), DIPEA (1.6 mL) and Bu₄NBr (1.79 g, 5.55 mmol) in CH₂Cl₂ (5 mL), and a solution of methyl 3,4-O-isopropylidene-L-threonate 154 (493 mg, 2.59 mmol) in CH₂Cl₂ (5 mL) were stirred over activated 3 Å MS for 30 min, after which time, these solutions were added dropwise (over 5 min) and sequentially (TMU/DIPEA/Bu₄NBr solution first) to the solution containing the glycosyl donor. The reaction mixture was stirred at r.t. for 2 d until there was no evidence by TLC that the donor was still being consumed. The reaction mixture was then concentrated under reduced pressure, re-suspended in toluene (10 mL), filtered through a silica plug, washed with toluene (150 mL) and concentrated under reduced pressure to provide the crude product. Purification of the residue by flash column chromatography (10% EtOAc in hexane) afforded glycoside 157 as a colourless oil (593 mg, 45%, single α-anomer). \( R_f = 0.3 \) (15% EtOAc in hexane); \([\alpha]_D^{20} = +50.0 \) (c 1, CHCl₃); \( \nu_{\max}(\text{film}) / \text{cm}^{-1} \): 2991m, 2902m, 1742s
(C=O), 1613m, 1500w, 1455m, 1373m, 1266w, 1208m, 1101s, 1075s, 739m, 698m; δH(500 MHz, CDCl₃) 1.32 (s, 3H, 1 × CH₃(CH₃)₂), 1.36 (s, 3H, 1 × CH₃(CH₃)₂), 3.47 (A of ABX, Jₐ₋ₓ 9.0, Jₐ₋ₓ 5.8, 1H, C(6)HₐHₐ), 3.53-3.56 (m, 1H, C(6)HₐHₐ), 3.72 (s, 3H, OCH₃), 3.91 (dd, 1H, J 8.6, 6.3, C(4")HₐHₐ), 3.99-4.03 (stack, 2H, H₃, H₄), 4.06 (dd, J 8.6, 6.6, 1H, C(4")HₐHₐ), 4.09 (dd, J 10.1, 3.7, 1H, H₂), 4.21 (app t, J 6.6, 1H, H₅), 4.30 (d, J 7.0, 1H, H₂'), 4.38 (A of AB, Jₐ₋ₓ 11.7, 1H, C(6)OCHₐHₐPh), 4.40-4.46 [stack, 2H, (including 1H, B of AB, Jₐ₋ₓ 11.7, C(6)OCHₐHₐPh), H₃'], 4.57 (A of AB, Jₐ₋ₓ 11.5, 1H, C(4)OCHₐHₐPh), 4.71 (A of AB, Jₐ₋ₓ 11.7, 1H, C(2)OCHₐHₐPh), 4.72 (A of AB, Jₐ₋ₓ 11.7, 1H, C(3)OCHₐHₐPh), 4.82 (B of AB, Jₐ₋ₓ 11.7, 1H, C(2)OCHₐHₐPh), 4.85 (B of AB, Jₐ₋ₓ 11.7, 1H, C(3)OCHₐHₐPh), 4.92 (B of AB, Jₐ₋ₓ 11.5, 1H, C(4)OCHₐHₐPh), 5.16 (d, J 3.6, 1H, H₁), 7.23-7.41 (stack, 20H, OCH₂Ph); δC(125 MHz, CDCl₃) 25.4 (CH₃, 1 × C(CH₃)₂), 26.1 (CH₃, 1 × C(CH₃)₂), 52.1 (CH₃, OCH₃), 66.2 (CH₂, C₄), 68.6 (CH₂, C₆), 69.8 (CH, C₅), 72.5 (CH₂, C(2)OCH₂Ph), 73.3 (CH₂, C(3)OCH₂Ph), 73.5 (CH₂, C(6)OCH₂Ph), 74.8 (CH₂, C(4)OCH₂Ph), 75.0 (CH, C₄), 75.4 (CH, C₃'), 75.6 (CH, C₂'), 76.0 (CH, C₂), 78.6 (CH, C₃), 96.0 (CH, C₁), 109.8 (C, (CH₃)₂C), [127.4, 127.5, 127.7, 127.8, 127.9, 128.2, 128.3, 128.4 (CH, Ph, some resonance overlap)], 137.9 (C, ipso Ph), 138.6 (C, ipso Ph), 138.7 (C, ipso Ph), 138.9 (C, ipso Ph), 169.9 (C, C=O); MS (TOF ES+) m/z 735.1 ([M + Na]⁺, 100%); HRMS (TOF ES+) calcd for C₄₂H₄₈O₁₀Na ([M + Na]⁺) 735.3145, found 735.3148.
3',4'-O-isopropylidene-2'-O-(2,3,4,6-tetra-O-benzyl-D-galactopyranosyl)-(1→2)-L-threitol (158)

![Structure diagram](image)

A solution of methyl ester 157 (350 mg, 0.49 mmol) in Et₂O (2 mL) was added over 5 min to a stirred suspension of LiAlH₄ (28 mg, 0.74 mmol) in Et₂O (3 mL). After an initial vigorous reaction had subsided, the mixture was heated at reflux for 3 h and then cooled to r.t., poured into NH₄Cl solution (5 mL) and H₂O (5 mL). The layers were separated and the aqueous phase was extracted with Et₂O (3 × 10 mL). The organic layer and the ethereal extracts were combined and the mixture was dried (Na₂SO₄) and concentrated under reduced pressure. The residue was purified by flash column chromatography (10% EtOAc in hexane) to afford alcohol 158 as a colourless oil (320 mg, 95%). $R_f = 0.3$ (25% EtOAc in hexane); $[\alpha]_D^{21} = +33.6$ (c 1, CHCl₃); $\nu_{\text{max}}$(film) / cm⁻¹: 3471 br (O–H), 2933w, 2870w, 1497w, 1454m, 1370m, 1253w, 1210m, 1155m, 1132m, 1077s, 1046s, 910m, 889w, 850m, 733s, 696s; $\delta_H$(500 MHz, CDCl₃) 1.26 (s, 3H, 1 × C(CH₃)₂), 1.36 (s, 3H, 1 × C(CH₃)₂), 3.45-3.57 (stack, 2H, C(6)H₂), 3.58-3.64 (stack, 2H, C(1)H₂), 3.65-3.70 (m, 1H, H2'), 3.85 (dd, $J = 8.4, 5.8$, 1H, C(4')H₃H₃b), 3.92-3.96 (m, 1H, C(4')H₃H₃b), 4.00 (dd, $J = 10.0, 2.6$, 1H, H3), 4.02-4.04 (m, 1H, H4), 4.09 (dd, $J = 9.9, 3.7$, 1H, H2), 4.16-4.21
Chapter 5 Experimental Section

(stack, 2H, H3', H5), 4.42 (A of AB, $J_{A:B}$ 11.8, 1H, C(6)OCH$_a$H$_b$Ph), 4.46 (B of AB, $J_{B:A}$ 11.8, 1H, C(6)OCH$_a$H$_b$Ph), 4.58 (A of AB, $J_{A:B}$ 11.4, 1H, C(4)OCH$_a$H$_b$Ph), 4.71 (A of AB, $J_{A:B}$ 11.6, 1H, C(2)OCH$_a$H$_b$Ph), 4.76 (s, 2H, C(3)OCH$_2$Ph), 4.89 (B of AB, $J_{B:A}$ 11.6, 1H, C(2)OCH$_a$H$_b$Ph), 4.93 (B of AB, $J_{B:A}$ 11.4, 1H, C(4)OCH$_a$H$_b$Ph), 4.96 (d, $J$ 3.7, 1H, H1), 7.23-7.40 (stack, 20H, OCH$_2$Ph), OH resonance not observed; $\delta_c$(125 MHz, CDCl$_3$) 24.9 (CH$_3$, 1 × C(CH$_3$)$_2$), 26.3 (CH$_3$, 1 × C(CH$_3$)$_2$), 62.0 (CH$_2$, C1'), 65.5 (CH$_2$, C4'), 68.7 (CH$_2$, C6), 69.8 (CH, C5), 72.5 (CH$_2$, C(3)OCH$_2$Ph), 73.4 (CH$_2$, C(6)OCH$_2$Ph), 74.6 [(CH, C4) and (CH$_2$, C(2)OCH$_2$Ph) resonance overlap), 74.7 (CH$_2$, C(4)OCH$_2$Ph), 75.3 (CH, C3'), 76.2 (CH, C2), 79.5 (CH, C3), 81.9 (CH, C2'), 100.0 (CH, C1), 109.3 (C, (CH$_3$)$_2$C), [127.4, 127.6, 127.7, 128.0, 128.1, 128.2, 128.3, 128.4, 128.5 (CH, Ph, some resonance overlap)], 137.6 (C, ipso Ph on C(2)OBn), 138.0 (C, ipso Ph on C(6)OBn), 138.4 (C, ipso Ph on C(3)OBn), 138.6 (C, ipso Ph on C(4)OBn); MS (TOF ES+) m/z 707.1 ([M + Na]$^+$, 100%); HRMS (TOF ES+) calcd for C$_{41}$H$_{48}$O$_9$Na ([M+Na]$^+$) 707.3196, found 707.3231.
Tf₂O (80 µL, 0.49 mmol) was added dropwise over 2 min to a solution of alcohol 158 (320 mg, 0.47 mmol) and 2,6-di-tert-butylpyridine (120 µL, 0.52 mmol) in CH₂Cl₂ (5 mL) at 0 °C. After 30 min, the reaction mixture was diluted with CH₂Cl₂ (15 mL) and the resulting solution washed sequentially with H₂O (2 × 30 mL) and brine (10 mL), dried (Na₂SO₄) and filtered. Removal of the solvent under reduced pressure provided crude triflate 159 as a colourless oil [Rᵣ = 0.7 (25% EtOAc in hexane)], which was used immediately in the next etherification step. A solution of alcohol 78 (180 mg, 0.47 mmol) in DMF (3 mL) was treated with NaH (60% dispersion in mineral oil, 40.0 mg, 1.00 mmol) at 0 °C. After 20 min, a solution of triflate 159 (assuming 100% conversion, 0.47 mmol) in THF (2 mL) was added dropwise over 2 min. The resulting solution was stirred at this temperature for 1 h and then at r.t. for 12 h. The reaction was then quenched by the addition of MeOH (1 mL) followed by NaHCO₃ solution (5 mL) and extraction with Et₂O (3 × 10 mL). The combined organic fractions were washed with brine (10 mL) and dried.
(Na₂SO₄), filtered and the solvent was removed under reduced pressure. The residue was purified by flash column chromatography (eluent: 10% EtOAc in hexane) to provide ether 160 as a colourless oil (355 mg, 72%); \( R_f = 0.3 \) (10% EtOAc in hexane); \( \left[ \alpha \right]_D^{20} = +14.0 \) (c 1, CHCl₃); \( \nu_{\text{max}} \) (film) / cm⁻¹: 2923s, 2854m, 2098m (N₃), 1497w, 1454m, 1369m, 1249m, 1216m, 1097s, 1058s, 859w, 736m, 698s; \( \delta_{t} \) (500 MHz, CDCl₃) 0.86 (t, \( J = 6.8 \), 3H, CH₂CH₃), 1.21-1.29 (stack, 30H, alkyl chain methylenes, 2 × C(CH₃)₂), 1.33 (s, 3H, 1 × C(CH₃)₂), 1.35 (s, 3H, 1 × C(CH₃)₂), 1.39-1.55 (stack, 2H), 3.40 (ddd, \( J = 9.7, 7.3, 2.5 \), 1H, H2") 3.45-3.50 (m, 1H, H4), 4.02-4.07 (stack, 2H, H2, H3" or H4"), 4.22-4.28 (stack, 2H, H5, H3") 4.38 (A of AB, \( J_{A,B} = 11.8 \), 1H, C(6)OCH₃Ph), 4.42 (B of AB, \( J_{B,A} = 11.8 \), 1H, C(6)OCH₃Ph), 4.56 (A of AB, \( J_{A,B} = 11.5 \), 1H, C(4)OCH₃Ph), 4.71 (A of AB, \( J_{A,B} = 11.7 \), 1H, C(3)OCH₃Ph), 4.72 (A of AB, \( J_{A,B} = 12.1 \), 1H, C(2)OCH₃Ph], 4.76 (B of AB, \( J_{B,A} = 12.1 \), 1H, C(2)OCH₃Ph), 4.78 (B of AB, \( J_{B,A} = 11.7 \), 1H, C(3)OCH₃Ph), 4.91 (B of AB, \( J_{B,A} = 11.5 \), 1H, C(4)OCH₃Ph), 5.11 (d, \( J = 3.7 \), 1H, H1), 7.19-7.31 (stack, 18H, OCH₂Ph), 7.32-7.36 (stack, 2H, OCH₂Ph); \( \delta_{c} \) (125 MHz, CDCl₃) 14.1 (CH₃, CH₂CH₃), 22.7 (CH₂), 25.4 (CH₃, 1 × C(CH₃)₂), 25.7 (CH₃, 1 × C(CH₃)₂), 26.3 (CH₃, 1 × C(CH₃)₂), 26.4 (CH₂), 28.2 (CH₃, 1 × C(CH₃)₂), 29.4 (CH₂), 29.5 (CH₂), [29.6, 29.6, 29.7, 29.7 (CH₂, alkyl chain, resonance overlap)], 31.9 (CH₂), 59.9 (CH, C₂"), 65.9 (CH₂, C₄"), 68.8 (CH₂, C₆), 69.3 (CH, C₅), 71.1 (CH₂, C₁"), 72.5 (CH₂, C₁"), 72.9 (CH₂, C(3)OCH₂Ph), 73.0 (CH₂, C(2)OCH₂Ph), 73.5 (CH₂, C(6)OCH₂Ph), 74.7 (CH₂, C(4)OCH₂Ph), 75.0 (CH, C₄), 75.5 (CH, C₃" or C₄"), 76.0 (CH, C₃"), 76.4 (CH,
C2), 76.7 (CH, C2'), 77.7 (CH, C4" or C3"), 78.9 (CH, C3), 97.0 (CH, C1), 108.3 (C, (CH₃)₂C of 1,2-anti diol), 109.1 (C, (CH₃)₂C of 1,2-syn diol), [127.39, 127.41, 127.49, 127.51, 127.7, 127.8, 127.9, 128.19, 128.22, 128.3, 128.4 (CH, Ph, some resonance overlap)], 138.1 (C, ipso Ph), 138.78 (C, ipso Ph), 138.82 (C, ipso Ph), 138.9 (C, ipso Ph); m/z (TOF ES+) 1072.8 ([M + Na]⁺, 100%); HRMS m/z (TOF ES+) calcd for C₆₂H₈₇N₃O₁₁Na ([M + Na]⁺) 1072.6238, found 1072.6246.

(2"''S,3''S,4''R)-2''-Amino-1-O-[3',4'-O-isopropylidene-2'-O-(2,3,4,6-tetra-O-benzyl-D-galactopyranosyl)-(1→2)-L-threitol]-3'',4''-O-isopropylidene-octadecane-1'',3'',4''-triol (161)

![Chemical structure of 161](image)

PMe₃ (240 µL of a 1.0 M soln in THF, 0.240 mmol) was added dropwise over 5 min to a solution of azide 160 (200 mg, 0.194 mmol) in THF / H₂O (2 mL, 15:1). The reaction mixture was stirred at r.t. for 2 h and then concentrated under reduced pressure. The residual H₂O was removed by co-evaporation with toluene (3 × 3 mL) to provide the crude amine product. Purification of the residue by flash
column chromatography (40% EtOAc in hexane) afforded amine 161 as a colourless oil (175 mg, 88%); \( R_f = 0.3 \) (40% EtOAc in hexane); \( [\alpha]_D^{22} = + 8.0 \) (c 1, CHCl\(_3\)); \( \nu_{\text{max}}(\text{film}) / \text{cm}^{-1}: 3032 \text{ br (N–H)}, 2922 \text{s}, 2853 \text{m}, 1497 \text{w}, 1454 \text{m}, 1369 \text{m}, 1246 \text{m}, 1214 \text{m}, 1095 \text{s}, 1054 \text{s}, 856 \text{m}, 734 \text{s}, 697 \text{s}; \) \( \delta_H(500 \text{ MHz, CDCl}_3) \) 0.88 (t, \( J 7.1, 3\text{H}, \text{CH}_2\text{CH}_3 \)), 1.22-1.31 (stack, 30H, alkyl chain methylenes, \( 1 \times \text{C(CH}_3)_2 \) from each acetal), 1.33 (s, 3H, \( 1 \times \text{C(CH}_3)_2 \) from acetal protecting C(3”’)OH and C(4’’)OH), 1.39 (s, 3H, \( 1 \times \text{C(CH}_3)_2 \) from acetal protecting C(3’)OH and C(4’)OH), 1.46-1.60 (stack, 2H), 3.40 (dd, \( J 9.5, 5.9, 1\text{H}, \text{C}(6)\text{H}_a\text{H}_b \)), 3.47-3.57 (stack, 3H, [including 3.50 (dd, \( J 9.5, 6.7, 1\text{H}, \text{C}(6)\text{H}_a\text{H}_b \))], H2”, C(1”)H\(_a\)H\(_b \)), C(1’)H\(_a\)H\(_b \)), 3.75-3.79 (m, 1H, H2”), 3.82-3.88 (stack, 2H, C(4’)H\(_a\)H\(_b \), C(1’)H\(_a\)H\(_b \)), 3.94-4.01 (stack, 3H, H3, H4, C(4’)H\(_a\)H\(_b \)), 4.05-4.11 (stack, 2H, H2, H5), 4.16-4.21 (m, 1H, H4”), 4.34-4.41 (stack, 3H, [including 4.40 (A of AB, \( J_{A,B} 11.8, 1\text{H}, \text{C}(6)\text{OCH}_2\text{H}_b\text{Ph}) \)], H3”, H3’, C(6)OCH\(_2\)H\(_b\)Ph)), 4.46 (B of AB, \( J_{B,A} 11.8, 1\text{H}, \text{C}(6)\text{OCH}_2\text{H}_b\text{Ph}) \), 4.53 (A of AB, \( J_{A,B} 11.4, 1\text{H}, \text{C}(4)\text{OCH}_2\text{H}_b\text{Ph}) \), 4.76 (A of AB, \( J_{A,B} 11.7, 1\text{H}, \text{C}(2)\text{OCH}_2\text{H}_b\text{Ph}) \), 4.77 (A of AB, \( J_{A,B} 11.6, 1\text{H}, \text{C}(3)\text{OCH}_2\text{H}_b\text{Ph}) \), 4.85 (B of AB, \( J_{B,A} 11.6, 1\text{H}, \text{C}(3)\text{OCH}_2\text{H}_b\text{Ph}) \), 4.90 (B of AB, \( J_{B,A} 11.4, 1\text{H}, \text{C}(4)\text{OCH}_2\text{H}_b\text{Ph}) \), 4.92 (B of AB, \( J_{B,A} 11.7, 1\text{H}, \text{C}(2)\text{OCH}_2\text{H}_b\text{Ph}) \), 4.97 (d, \( J 3.7, 1\text{H}, \text{H}1 \)), 7.22-7.38 (stack, 18H, OCH\(_2\)Ph), 7.43 (br d, 2H, OCH\(_2\)Ph), NH\(_2\) resonance not observed; \( \delta_\text{C}(125 \text{ MHz, CDCl}_3) \) 14.1 (CH\(_3\), CH\(_2\)CH\(_3\)), 22.7 (CH\(_2\)), 24.5 (CH\(_3\), \( 1 \times \text{C(CH}_3)_2 \)), 24.7 (CH\(_3\), \( 1 \times \text{C(CH}_3)_2 \)), 26.1 (CH\(_3\), \( 1 \times \text{C(CH}_3)_2 \)), 26.5 (CH\(_3\), \( 1 \times \text{C(CH}_3)_2 \)), 26.9 (CH\(_2\)), [29.3, 29.4, 29.53, 29.6, 29.67, 29.70 (CH\(_2\), alkyl chain, resonance overlap)], 31.9 (CH\(_2\)), 52.1 (CH, C2”), 65.7 (CH\(_2\), C4’), 69.2 (CH\(_2\), C6), 69.6 (CH\(_2\), C1”), 70.1 (CH, C5), 70.5 (CH\(_2\), C1’), 72.7 (CH\(_2\), C(3)OCH\(_2\)Ph), 73.5
(CH₂, C(6)OCH₂Ph), 73.9 (CH₂, C(2)OCH₂Ph), 74.7 (CH, C4), 74.8 (CH₂, C(4)OCH₂Ph), 75.00 (CH, C3'), 75.04 (CH, C3''), 75.4 (CH, C2), 76.7 (CH, C4''), 77.7 (CH, C2'), 79.1 (CH, C3), 97.6 (CH, C1), 108.7 (C, (CH₃)₂C for acetal protecting C(3'')OH and C(4'')OH), 109.7 (C, (CH₃)₂C for acetal protecting C(3')OH and C(4')OH), [127.6, 127.7, 127.8, (CH, Ph, some resonance overlap)], [128.2, 128.3, 128.4 (CH, Ph, some resonance overlap)], 128.7 (CH, Ph), 137.7 (C, ipso Ph), 137.8 (C, ipso Ph), 138.4 (C, ipso Ph), 138.5 (C, ipso Ph); MS (TOF ES+) m/z 1046.5 ([M + Na]⁺, 100%), 1024.5 ([M + H]⁺, 80); HRMS (TOF ES+) m/z 1024.6521 ([M + H]⁺). C₆₂H₉₀NO₁₁ requires 1024.6514; 1046.6349 ([M + Na]⁺). C₆₂H₈₉NO₁₁Na requires 1046.6333.
A screw-capped glass tube containing a solution of hexacosanoic acid (91 mg, 0.23 mmol) in oxalyl chloride (2.0 mL, 23 mmol) was closed tightly and heated at 70 °C for 2 h. The volatiles were then evaporated under a stream of argon and the tube then placed on a high vacuum line for at least 2 h to remove the residual volatiles. The resulting acid chloride was used directly without further purification.

A solution of freshly prepared acid chloride 47 (105 mg, 0.23 mmol) in CH₂Cl₂ (0.5 mL) was added dropwise over 2 min to a solution of amine 161 (154 mg, 0.15 mmol) and NEt₃ (42 µL, 0.30 mmol) in CH₂Cl₂ (1.0 mL) at 0 °C. The reaction mixture was stirred at r.t. for 12 h and then diluted with CH₂Cl₂ (10 mL), washed sequentially with NaHCO₃ solution (10 mL), brine (2 mL) and then dried over Na₂SO₄. The drying agent was removed by filtration and the filtrate concentrated under reduced pressure. Purification of the residue by flash column chromatography provided amide 162 as a white solid (179 mg, 85%). Rᵣ = 0.3 (15% EtOAc in hexane); mp 69-70 °C; [α]D²₀ = + 41.6 (c 1, CHCl₃); νₛₐₚ₉ₐ₃(film) / cm⁻¹:
3283 br (N-H), 2919s, 2851s, 1641m (C=O), 1543w, 1497w, 1468m, 1455m, 1369m, 1246m, 1216m, 1157m, 1098s, 1064s, 860w, 735m, 698m; δ(500 MHz, CDCl₃) 0.86 (t, J 6.8, 6H, × CH₂CH₃), 1.18-1.30 (stack, 73H, alkyl chain methylenes, 1 × C(CH₃)₂ from acetal protecting C(3')OH and C(4')OH, 1 × C(CH₃)₂ from acetal protecting C(3'')OH and C(4'')OH), 1.34 (s, 3H, 1 × C(CH₃)₂ from acetal protecting C(3')OH and C(4')OH), 1.37 (s, 3H, 1 × C(CH₃)₂ from acetal protecting C(3'')OH and C(4'')OH), 1.40-1.66 (stack, 5H, alkyl chain methylenes), 2.00-2.14 (stack, 2H, C(2'')HₐHₕ), 3.41 (dd, J 9.5, 2.6, 1H, C(1'')HₐHₕ), 3.45-3.55 (stack, 4H, C(6)HₐHₕ, C(1')HₐHₕ), 3.67 (dd, J 9.5, 3.7, 1H, C(1')HₐHₕ), 3.75-3.81 (stack, 2H, H₂', C(4')HₐHₕ), 3.92-3.98 (stack, 4H, H₃, C(4')HₐHₕ, H₃”, H₄”), 4.00-4.02 (m, 1H, H₄), 4.05 (dd, J 10.1, 3.7, 1H, H₂”), 4.08-4.16 (m, 1H, H₂”), 4.20-4.26 (stack, 2H, H₅, H₃”), 4.39 (A of AB, Jₐ₋ₕ 11.8, 1H, C(6)OCHₐHₕPh), 4.42 (B of AB, Jₐ₋ₕ 11.8, 1H, C(6)OCHₐHₕPh), 4.55 (d, J 11.5, 1H, C(4)OCHₐHₕPh), 4.70 (A of AB, Jₐ₋ₕ 12.0, 1H, C(2)OCHₐHₕPh), 4.72 (A of AB, Jₐ₋ₕ 11.7, 1H, C(3)OCHₐHₕPh), 4.77 (B of AB, Jₐ₋ₕ 11.7, 1H, C(3)OCHₐHₕPh), 4.78 (B of AB, Jₐ₋ₕ 12.0, 1H, C(2)OCHₐHₕPh), 4.91 (d, J 11.5, 1H, C(4)OCHₐHₕPh), 5.05 (d, J 3.7, 1H, H₁), 5.74 (br d, J 9.3, 1H, N-H), 7.20-7.36 (stack, 20H, OCH₂Ph); δ(125 MHz, CDCl₃) 14.1 (CH₃, 2 × CH₂CH₃), 22.7 (CH₂), 25.3 (CH₃, 1 × C(CH₃)₂), 25.7(CH₂), 25.8 (CH₃, 1 × C(CH₃)₂), 26.3 (CH₃, 1 × C(CH₃)₂), 26.5 (CH₂), 28.0 (CH₃, 1 × C(CH₃)₂), 29.0 (CH₂), [29.3, 29.4, 29.5, 29.6, 29.7 (CH₂, alkyl chain, resonance overlap]), 31.9 (CH₂), 36.8 (CH₂, C₂’’), 48.1 (CH, C₂’’), 65.8 (CH₂, C₄’), 68.8 (CH₂, C₆), 69.3 (CH, C₅), 70.5 (CH₂, C₁’), 71.3 (CH₂, C₁’’), 72.8 (CH₂, C(3)OCH₂Ph), 73.2 (CH₂, C(2)OCH₂Ph), 73.5 (CH₂, C(6)OCH₂Ph), 74.7 (CH₂, C(4)OCH₂Ph), 74.8 (CH, C₄), 76.0 (CH, C₃’), 76.15 (CH, C₃” or C₄”), 76.20 (CH, C₂), 76.3 (CH, C₂’), 77.8 (CH,
C4" or C3"), 79.0 (CH, C3), 96.5 (CH, C1), 107.8 (C, (CH$_3$)$_2$C of acetal protecting C(3")OH and C(4")OH), 109.1 (C, (CH$_3$)$_2$C of acetal protecting C(3')OH and C(4')OH), [127.3, 127.4, 127.5, 127.6, 127.70, 127.75, 127.8 (CH, Ph, some resonance overlap)], [128.1, 128.2, 128.3, 128.4 (CH, Ph, some resonance overlap)], 137.9 (C, ipso Ph), 138.6 (C, ipso Ph), 138.7 (C, 2 × ipso Ph), 172.4 (C, C=O); MS (TOF ES+) m/z 1425.0 ([M + Na]$^+$, 100%); HRMS (TOF ES+) calcd for C$_{88}$H$_{139}$NO$_{12}$Na ([M+Na]$^+$) 1425.0195, found 1425.0200.

Galp-α-(1→2)-ThrCer (149)

TFA (150 µL) was added dropwise over 1 min to a solution of diacetal 162 (50 mg, 0.036 mmol) in CH$_2$Cl$_2$ / H$_2$O (10:1, 0.6 mL). After stirring for 2 h at r.t., the reaction mixture was concentrated under reduced pressure and the residual TFA was removed by co-evaporation with Et$_2$O (3 × 3 mL) to provide crude tetraol as a white solid (48 mg, quant.), which was used in the next step without further purification. Pd(OH)$_2$/C (20 mg, 0.014 mmol, 10% wet) was added to a solution of the crude benzylated compound (48 mg, 0.036 mmol) in THF/CH$_3$OH (3:1, 5 mL).
H₂ gas was bubbled through the stirred suspension for 8 h. The reaction mixture was then filtered, washed with CHCl₃/CH₃OH (8:2, 100 mL) and concentrated under reduced pressure to provide the crude product, which was purified by flash column chromatography (15% CH₃OH in CHCl₃) to afford the fully deprotected compound 149 as a white solid (26 mg, 75%). Rᵣ = 0.4 (15% CH₃OH in chloroform); mp 84-85 ºC; [α]₀²⁰ = + 56 (c 0.1, 2:1 CHCl₃/CH₃OH); νₘₚₜ(film) / cm⁻¹ 3303 v br (OH), 2944m, 2833m, 1677w (C=O), 1450w, 1217m, 1031s, 762s, 667m; δₜ (400 MHz, 2:1 CDCl₃/CD₃OD) 0.84 (t, J 6.7, 6H, 2 × CH₂CH₃), 1.15-1.33 (stack, 67H, alkyl chain methylenes), 1.34-1.65 (stack, 5H, alkyl chain methylenes), 2.20 (t, J 7.6, 2H), 3.52-3.76 (stack, 9H), 3.77-3.90 (stack, 6H), 3.92 (br d, J 3.0, 1H), 4.03-4.07 (m, 1H), 4.12-4.19 (m, 1H), 5.15 (d, J 4.1, 1H), OH resonances not observed; δₐ (100 MHz, 2:1 CDCl₃/CD₃OD) 14.1 (CH₃, 2 × CH₂CH₃), 23.1 (CH₂), 26.25 (CH₂), 26.28 (CH₂), [29.8, 29.9, 30.03, 30.06, 30.12 (CH₂, alkyl chain, resonance overlap)], 32.3 (CH₂), 32.8 (CH₂), 36.7 (CH₂), 49.8 (CH), 62.3 (CH₂), 62.9 (CH₂), 69.5 (CH), 70.1 (CH), 70.5 (CH), 71.1 (CH₂), 72.2 (CH₂), 72.3 (CH), 72.7 (CH), 73.3 (CH), 74.6 (CH), 78.1 (CH), 96.2 (CH), 175.2 (C, C=O); MS (TOF ES⁺) m/z 984.9 ([M + Na]⁺, 100%); HRMS (TOF ES⁺) calcd for C₅₄H₁₀₇NO₁₂Na ([M+Na]⁺) 984.7691, found 984.7701.
Chapter 6

References
6. References


309


(149) Garcia Diaz, Y. R. *PhD Thesis*, University of Birmingham (Birmingham, UK).


(152) Rostovtsev, V. V.; Green, L. G.; Fokin, V. V.; Sharpless, K. B. *Angew. Chem., Int. Ed.* **2002**, *41*, 2596-2599.


Chapter 6

References


