2013-05-15

Synthesis and Immunological Evaluation of Type I, Type II, and gamma delta NKT Cell Antigens

Brian L. Anderson
Brigham Young University - Provo

Synthesis and Immunological Evaluation of Type I, Type II, and $\gamma\delta$ NKT Cell Antigens

Brian L. Anderson

A dissertation submitted to the faculty of
Brigham Young University
in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

Paul Savage, Chair
Merritt Andrus
Steve Castle
Steven Goates
Matt Peterson

Department of Chemistry and Biochemistry

Brigham Young University

May 2013

ABSTRACT

Synthesis and Immunological Evaluation of Type I, Type II,
and $\gamma\delta$ NKT Cell Antigens

Brian L. Anderson
Department of Chemistry and Biochemistry
Doctor of Philosophy

The purpose of the immune system is to protect our bodies from infection. One way it accomplishes this task is through the presentation of foreign pathogens to NKT cells. After an antigen is presented to the T cell receptor, activated NKT cells quickly release soluble chemical signals, termed chemokines and cytokines, that modulate the response of the immune system. Due to the immunological relevance of NKT cell activation, we developed and synthesised non-natural analogs of immunostimulatory type I, II, and $\gamma\delta$ NKT cell antigens. The immunological evaluations of these analogs resulted in identification of sulfatide as a $\gamma\delta$ NKT cell antigen, along with the characterization of these newly discovered sulfatide-reactive $\gamma\delta$ NKT cell line. During sulfatide structure activity relationship studies, a novel azido-sulfatide analog was synthesized to traffick and image sulfatide in vivo. These studies demonstrated that sulfatide accumulated in the late endosome/lysosome. In conjunction with previous studies, this observation explains the persistence of CD1d-restricted T cells with high affinity for this antigen in healthy individuals. Finally, stimulatory assays were performed on a panel of synthesized lyso-glycosylceramides. This led to the discovery of stimulatory type I NKT cell antigens, α-psychosine and α-glucopsychosine.

Keywords: sulfatide, glycolipid, carbohydrate, synthesis, organic, chemistry, immunology, trafficking, analogs

ACKNOWLEDGMENTS

First and foremost, I want to acknowledge Dr. Savage's role in my doctoral studies and more importantly my professional development. Since day one of my doctoral studies, I was given all the tools, resources, and most importantly trust to carry out my own projects. This format of learning was at first difficult and accompanied by a variety of growing pains. After one such experience, Dr. Savage taught me that these difficulties are not just secular lessons, but life lessons. That particular experience was the turning point of my PhD training. The point when I left my undergrad mentality, and started to mature into a responsible and capable researcher. Also, Dr. Savage is the best teacher I have ever observed and I am grateful to have had the opportunity to learn from his teaching style.

Along with Dr. Savage, Dr. Burt has been an exceptional mentor. He always performed above and beyond his responsibilities to mentor me. His teaching and training of NMR, gave me the self-confidence that I lacked at a crucial time during my PhD experience. I consider him a mentor and a friend.

I also want to acknowledge our lab's role at teaching me the essential tools of organic chemistry. Dr. Deng, especially, has been a fantastic mentor. He has acquired the skill to patiently answer our synthetic questions and respond in a non-condescending way. This trait has fostered a fantastic learning environment in our lab. Along with Dr. Deng, Vinod Chaudhary, Sara Mata, YuBo Li, Dr. Gu, Xiantian Long, and many others have helped me along the way. I cherish their support and friendship and I wish them the best in life.

I also want to acknowledge my loving wife for her support during these past few years. She personifies love and support. She never complains when I come home late for work, when she bears the brunt of the work of rearing our children and maintaining our home, or when I have to work on Saturday. Through unselfish acts of service and love, she has enabled me to work hard and also mature into a better human being.

Contents

Table of Contents **iv**

List of Figures **vi**

1 Invariant Natural Killer T Cells Recognize Glycolipid Antigens **1**
1.1 Blood facilitates immune responses . . . 2
1.1.1 B and T lymphocytes recognize a diverse array of antigens . . . 4
1.2 Natural killer T cells . . . 5
1.2.1 *i*NKT cells recognize lipid-bound CD1 . . . 6
1.2.2 Natural Killer T cells are integral parts of the immune system . . . 8
1.3 *i*NKT cells recognize lipid antigens . . . 10
1.3.1 The model *i*NKT antigen: α-GalCer . . . 10
1.3.2 Isolation and characterization of natural antigens . . . 12
1.3.3 Structure-activity analyses of α-GalCer . . . 15
1.3.4 Immunological and therapeutic importance of NKT cells . . . 17
1.4 Conclusion . . . 18

2 Synthesis and Evaluation of Sulfatide analogs: a $\gamma\delta$ T cell antigen **20**
2.1 Introduction . . . 20
2.2 Synthesis of Sulfatide analogs . . . 23
2.2.1 Synthesis of sulfatide **2.1** and the phytosphingosine analog of sulfatide **2.2** 23
2.2.2 Synthesis of 6″-sulfo-sulfatide variants **2.23** and **2.24** . . . 28
2.2.3 Synthesis of N-acyl sulfatide variant **2.33** . . . 30
2.2.4 Synthesis of sulfonate sulfatide analog **2.41** . . . 32
2.2.5 Synthesis of 4″-deoxy sulfatide analog **2.49** . . . 34
2.3 Results and Discussion . . . 35
2.3.1 c24:1 sulfatide is a potent NKT cell antigen . . . 35
2.3.2 N-acyl sulfatide analog is a comparable CD1d antigen and the sulfonate moiety is tolerated . . . 39
2.3.3 Identification of CD1d-sulfatide-specific Vδ1+ T cells among fresh PBMCs in healthy individuals . . . 40
2.3.4 Antigen-specific recognition of CD1d molecules by Vδ1+ $\gamma\delta$ T cells Clones 43

2.3.5 Progress towards solving the crystal structure of the $\gamma\delta$ TCR-sulfatide-CD1d complex . . . 44
2.4 Concluding remarks . . . 45
2.5 Experimental Procedures . . . 45
2.6 Immunological methods . . . 97

3 Synthesis and Trafficking Studies of a BODIPY-Appended Sulfatide Analog 101
3.1 Introduction . . . 101
3.2 Synthesis of 6-azido-sulfatide **3.1** . . . 103
3.3 Results . . . 104
3.3.1 Presentation of sulfatide by CD1d-expressing cells . . . 104
3.3.2 Discussion . . . 107
3.4 Experimental Procedures . . . 108
3.5 Immunological experimentals . . . 113

4 Synthesis and Evaluation of Lyso Glycolipid Analogs 115
4.1 Introduction . . . 115
4.2 Synthesis of lyso-ceramides . . . 119
4.2.1 Synthesis of β-psychosine **4.1a** and β-glucopsychosine **4.2a** . . . 119
4.2.2 Synthesis of α-psychosine **4.3a** and α-glucopsychosine **4.4a** . . . 123
4.2.3 Synthesis of the β-sphingo-psychosine diastereomer **4.5** and the β-sphingo-glucopsychosine analog **4.6** . . . 125
4.2.4 Synthesis of N-acyl β-sphingo-psychosine (**4.7**) and β-sphingo-glucopsychosine analogs (**4.8**) . . . 128
4.3 Results and Discussion . . . 131
4.3.1 α-psychosine and α-glucopsychosine stimulate *i*NKT cells . . . 131
4.3.2 The L363 antibody binds to α-psychosine and α-glucopsychosine . . . 131
4.3.3 Observed anomerization from β-sphingopsychosine to α-sphingopsychosine 133
4.4 Conclusion . . . 136
4.5 Experimental Procedures . . . 137
4.6 Immunological Methods . . . 176

Bibliography 178

A List of Abbreviations 186

B NMR spectra 189

List of Figures

1.1 Functions of the immunological components of the blood 3

1.2 Antigen recognition of subsets of T cells 6

1.3 α-GalCer-CD1d complex 7

1.4 NKT cells modulate the innate and adaptive immune responses 9

1.5 Representative structures of an agelasphin and KRN7000 11

1.6 Representative structures of natural and synthetic *i*NKT cell antigens 13

2.1 Representative structure of sulfatide 21

2.2 Initial sulfatide analog targets 36

2.3 Stimulatory activities of the sulfatide isoforms 38

2.4 Immunological evaluation of **2.41, 2.33,** and **2.1b** 39

2.5 Majority of CD1d-sulfatide specific T cells are $\gamma\delta$ 42

3.1 Novel azido-sulfatide variant **3.1** and BODIPY-appended sulfatide variant **3.2** . . . 103

3.2 Sulfatide is localized in the Lysosome 106

4.1 Mechanism of glycosylation anomerization of GluCer (24:1) 117

4.2 High performance TLC of fractionated commercial β-GluCer 118

4.3 Representative structure of β-GalCer and β-psychosine 118

4.4 Initial synthetic lyso targets 120

4.5 Formation of the diastereomeric sphingosine precursor **2.7b** 125

4.6 Acetyl migration mechanism under acidic conditions 127

4.7 L363 binds α-galactosyl and -glucosylceramide-CD1 complexes 133

4.8 Proposed mechanism of spontaneous anomerization of β-GluCer(24:1) 134

4.9 Anomerization of β-sphingopsychosine to α-sphingopsychosine 136

List of Schemes

2.1 Representative synthesis of sulfatide **2.11d** 24

2.2 Representative synthesis of sulfatide **2.1d** 26

2.3 Representative synthesis of sulfatide **2.2d** 27

2.4 Representative synthesis of sulfatide **2.23** 29

2.5 Representative synthesis of sulfatide **2.24** 30

2.6 Representative synthesis of sulfatide variant **2.33** 31

2.7 Representative synthesis of sulfonate sulfatide analog **2.41** 33

2.8 Representative synthesis of 4″-deoxy sulfatide analog **2.49** 35

3.1 Representative synthesis of sulfatide **3.1** 104

4.1 Synthesis of phytosphingosine acceptor **4.11** 121

4.2 Synthesis of β-psychosine **4.1a** and β-glucopsychosine **4.2a** 122

4.3 Synthesis of α-psychosine **4.3a** and α-glucopsychosine **4.4a** 124

4.4 Synthesis of β-sphingo-GluCer diastereomer **4.5** 126

4.5 Synthesis of β-sphingo-GluCer analog **4.6** 128

4.6 Synthesis of N-acyl β-sphingo-psychosine (**4.7**) and β-sphingo-glucopsychosine analogs (**4.8**) 130

Chapter 1

Invariant Natural Killer T Cells Recognize Glycolipid Antigens

A highly discriminatory immune system is essential for our survival as humans. Without an immune system, a variety of invading viral, bacterial, parasitic, and infectious pathogens would take advantage of the rich sources of nutrients provided by our bodies. Our immune system has evolved an exceptional set of mechanisms that can not only differentiate between destructive foreign pathogens and our own cells, but also between beneficial foreign entities (e.g., gut flora, fetus) and foreign pathogens. These mechanisms are categorized into innate immunity and adaptive immunity. Innate immune responses are nonspecific and target a large range of conserved microbial and pathogen components, including proteins, nucleic acids, and carbohydrates. In contrast, adaptive immune responses are specific to a foreign antigen and remain at rest until they encounter a foreign pathogen. Despite the rich complexity of these two mechanisms and the overall effi-

ciency of the immune system in fighting off viral, bacterial, and parasitic attack, the human body is nonetheless susceptible to infection. Indeed, there are even destructive foreign pathogens that routinely elude the detection of the human immune system. One clinical approach to this dilemma is to activate the native immune response toward otherwise undetected foreign pathogens. An advantage of this approach is that the immune system's existing (and efficient) evolutionary mechanisms are leveraged to eliminate pathogens and disease. Thus, the development of immunostimulatory compounds is an active and promising field of scientific research. This dissertation addresses the development of non-natural analogs of immunostimulatory compounds.[1–3]

1.1 Blood facilitates immune responses

The principal function of our immune system is to protect against infection. To fulfill this purpose, the immune system utilizes a range of cells, tissues, and organs that not only work together to target, mark, and eliminate foreign pathogens, but also develop immunity to future infection. The primary immunological organs are the bone marrow and the thymus (where the majority of immunological cells are developed). Since the immune system needs to be able to find and respond to infection quickly, these cells use immunological tissues and media (i.e., blood, lymphatic tissues, and the spleen) to quickly respond throughout the entire body.[1]

Three key immune components are found in the blood: antibodies, plasma, and hematopoietic cells. Blood is thus a vital immunological medium. Antibodies, or immunoglobulins, have the ability to bind specifically to foreign cells/antigens. The hallmark of the immune system is to be

able to distinguish between foreign antigen and self-antigen (antigen produced by and necessary to the proper functioning of the body), and mount an immune response to the former. Plasma is the main liquid component of the blood, allowing the immune system to transport a variety of immunological cells quickly throughout the body. Plasma also consists of clotting factors that can help localize hematopoietic cells at the site of an infection that circulate throughout the body. Lastly, blood also contains many hematopoietic cells, classified into myeloid, lymphoid, and erythroid lineages (Figure 1.1). These cells have varied function ranging from antimicrobial activity, inflammation, and blood clotting, to cell mediated immunity.[1,2] Lymphoid cells, in particular, are developed to recognize proteins, substructures, and moieties of invading pathogens.

Cellular components of blood		
Type	Subtype	Immune functions
Myeloid	Monocyte	Antigen pesentation, Inflammation, ADCC
	Macrophage	Antigen presentation, Inflammation, ADCC
	Neutrophil	Antimicrobial activity, Inflammation, ADCC
	Basophil	IgE responsive
	Mast Cell	IgE responsive, Allergy responses
	Eosinophil	Asthma, Allergy responses, Parasitic defenses
Lymphoid	T Lymphocyte	Cell-mediated Immunity
	B Lymphocyte	Humoral Immunity
Erythroid	Erythrocite	Removal of antibody complexes
	Platelet	Clotting, Inflammation
Noncellular components of blood		
	Clotting Factors	Inflammation
	Complement	Innate Immunity, Regulation of immune function
	Immunoglobulins	Humoral Immunity

Figure 1.1 Functions of the immunological components of the blood. Adapted from Pier et. al.[1]

1.1.1 B and T lymphocytes recognize a diverse array of antigens

Lymphoid cells are key to the immune system's ability to respond to nearly infinite types of pathogens. They can be subdivided into cells causing humoral immunity and cell-mediated immunity. Humoral immunity involves the activation of B lymphocytes. B lymphocytes have surface B-cell receptors (BCRs) that recognize a diverse array of antigens. B cells are also unique in their ability to secrete forms of their BCRs (often referred to as antibodies or immunoglobulins).[1,4] Antibodies gain antigen diversity by somatic recombination of variable and joining DNA segments encoding their BCR. Even though each B cell only presents one BCR, diversity in the B cell population allows B cells to recognize a near infinite number of distinct antigens.[5]

Cell mediated immunity is carried out by T lymphocytes and large granular lymphocytes. T lymphocytes develop in the bone marrow initially but fully develop in the thymus. They also have cell surface binding receptors called T cell receptors (TCRs), but unlike B cells they do not produce antibodies. Their TCRs are comprised of an α and β or γ and δ chains.[6] They also recognize a diverse array of antigens due to special genes that undergo genetic rearrangements during T cell development. Segments of these genes are spliced from their genomic DNA, which results in distinct rearranged genes. There are 10^{12} combinations of TCR proteins due to this process.[1,2]

Large granular lymphocytes, termed natural killer (NK) cells, are also involved in cell-mediated immunity. These cells do not have TCRs or BCRs but have other cell surface proteins that recognize cell surface changes on tumor cells and virally infected cells. These cells damage infected cells and mark them for apoptosis.[7]

1.2 Natural killer T cells

Three different populations of T cells are developed for specific immune functions, differing primarily by their cell surface receptors. T cells that express the CD4 coreceptor on their surface (CD4+ T cells) are termed Helper T cells. They increase the activity of other immunological cells through the release of cytokines (soluble mediators of the immune system). CD4+ cells recognize peptides bound to the transmembrane antigen presenting molecule (APM) major histocompatibility complex (MHC) class II presented on antigen presenting cells such as dendritic cells.[1,2,6]

The second population of T cells, the cytotoxic T cells (CTLs), express the CD8 coreceptor (CD8+ cells). These cells recognize peptides that are complexed to MHC class I proteins, which are found on all nucleated cells of the body. Many times the MHC I protein is complexed with endogenous or self-peptide. When a CTL comes in contact with self-peptide, the CTL recognizes it as self and no immune response is triggered. When foreign peptide is presented by MHC class I, CTLs become activated and induce lysis of the cell, thereby eliminating the infected cell. Not only can CTLs mark and destroy foreign intracellular pathogens, they also mark and destroy aberrant self-cells that might be harmful and possibly give rise to cancer.[1,2,6]

In 1987, two groups reported a third subset of CD4- CD8- double negative T cells.[8] Originally these cells were termed natural killer T (NKT) cells because they expressed NK1.1 and/or CD161 receptors, natural killer c-lectin receptors, along with a TCR that overexpressed a specific vβ8 gene segment. These cells are found in the thymus, liver, bone marrow, spleen, and peripheral blood. Subsequent studies found that these T cells have an $\alpha\beta$-TCR that did not recognize peptides via MHC class I or II proteins, but instead recognized lipid containing antigens that were bound to

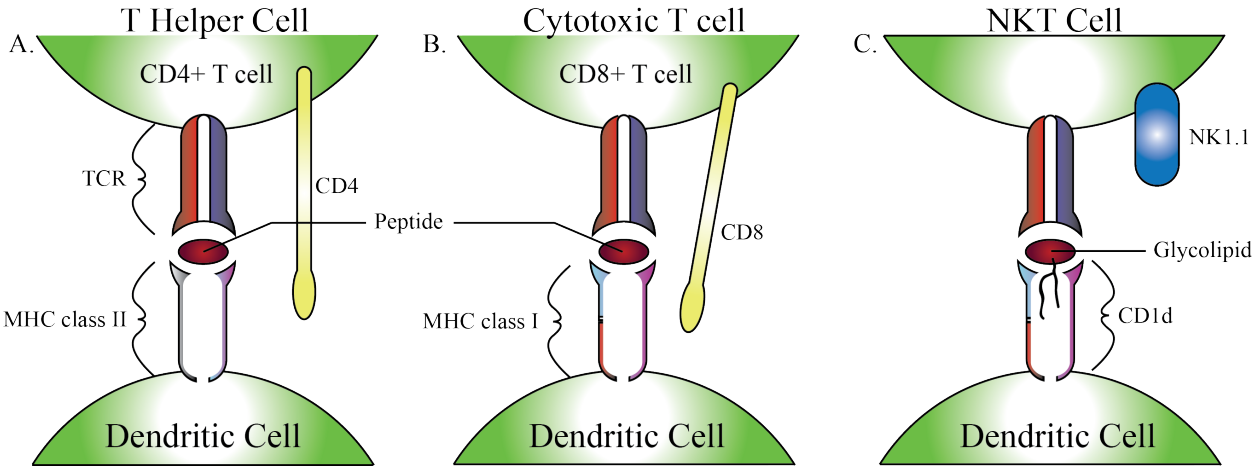


Figure 1.2 Antigen recognition of subsets of T cells. A) CD4+ T-helper cell TCR recognition of peptide presented by MHC class II. B) CD8+ CTL cell TCR recognition of peptide presented by MHC class I. C) TCR recognition of CD1d-presented glycolipid to a CD4-CD8- NKT cell. Adapted from Tupin et. al.[6]

a non-classical MHC class I like protein called CD1.[9] Since then, other NKT cells that are CD4+ or CD8+ have been isolated and characterized.[10–12]

1.2.1 *i*NKT cells recognize lipid-bound CD1

NKT cells express a much more limited range of TCRs than B cells, TH cells, and CTLs. The most studied subpopulation of NKT cells, invariant NKT cells (*i*NKT or Type 1 NKT cells), have a TCR with an invariant Vα14-Jα18 rearrangement in mice and a surprisingly homologous Vα24-Jα18 rearrangement in humans. In addition, the TCR has a restricted selection of β TCR chains (Vβ11 in humans). The second major population of NKT cells, type 2 NKT cells, do not have an invariant TCR-α chain but instead have a semi-invariant TCR-α chain that is over represented by Vα3 and Vα8 chain segments.[10,12–17]

NKT cells recognize lipid-containing antigens that are bound to CD1, the non-classical

MHC-like APM. CD1 molecules are cell surface glycoproteins that are expressed on B-cells, dendritic cells, macrophages, hepatocytes, and epithelial cells. They consist of two chains: a heavy chain comprised of three extracellular domains ($\alpha 1 - \alpha 3$) and β_2 microglobulin (β_2 microglobulin is also found in MHC class I molecules).[5,18]

The CD1 family comprises five isoforms in humans (CD1a-e). These isoforms are also subdivided into groups based on their sequence similarity: group 1 comprises CD1a-c, group 2 only consists of CD1d, and group 3 consists of CD1e.[18,19] A characteristic of type 1 and type 2 NKT cells is that they are CD1d restricted. Less abundant T cell lineages recognize lipid based antigens for the other CD1 isoforms. These isoforms recycle through intracellular compartments where they sample multiple lipid antigens that are present or are trafficked to different endocytic compartments.[20] For example, CD1a trafficks through early and recycling endosomes on its way to the cell surface, whereas CD1b and CD1d are localized in the late endosome and lysosome where microbial lipids accumulate during infections.[12,13,21]

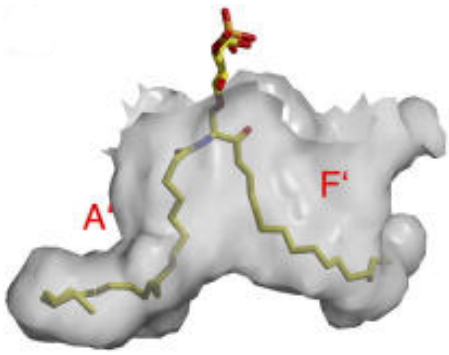


Figure 1.3 α-GalCer-CD1d complex. The fatty acyl chain binds into the A′ pocket, whereas the sphingosine chain binds into the F′ pocket. Reproduced from Zajonc et. al.[21] Copyright 2005 Nature Immunology Publishing Group

CD1 proteins have very different binding grooves than their MHC counterparts. The groove on CD1 is narrower, deeper, and more hydrophobic. This makes the binding site well-suited for docking long lipid tails. The antigen binding groove forms two hydrophobic pockets, termed the A′ and F′ pockets (Figure 1.3). Long carbon lipid tails can fit into the F′ and the A′ pockets.[3,13] For example, α-galactosylsylceramide (α-GalCer), the characteristic glycolipid antigen of *i*NKT cells, has its sphingosine tail bind into the F′ pocket, its fatty acyl chain binds into the A′ pocket, leaving the carbohydrate portion bound by an extensive hydrogen bonding network that positions and extends the sugar head for interaction with the TCR of *i*NKT cells.[5,13,19,21]

1.2.2 Natural Killer T cells are integral parts of the immune system

After stimulation from a CD1-bound antigen, NKT cells can produce, within hours, large amounts of cytokines. The released cytokines promote two different immune responses. One group of cytokines (including interleukin (IL)-2, inferferon-γ,and tumor necrosis factor-α) leads to a proinflammatory T helper 1 (TH1) response. TH1 responses are employed to combat and control invading bacterial, viral, and parasitic infections. Cytokines, such as IL-4, IL-5, IL-6, IL-10, and IL-13, promote an immunoregulatory T helper 2 (TH2) response. Many autoimmune diseases such as type 1 diabetes, multiple sclerosis, lupus, and rheumatoid arthritis are TH2-mediated diseases.[5,9,17,19,22,23]

Not only can NKT cells directly modulate the immune system but they can also indirectly modulate innate and adaptive immune responses (Figure 1.4). Released cytokines can activate adaptive cells such as T and B cells, and innate cells such as dendritic cells and NK cells.[22,24] These

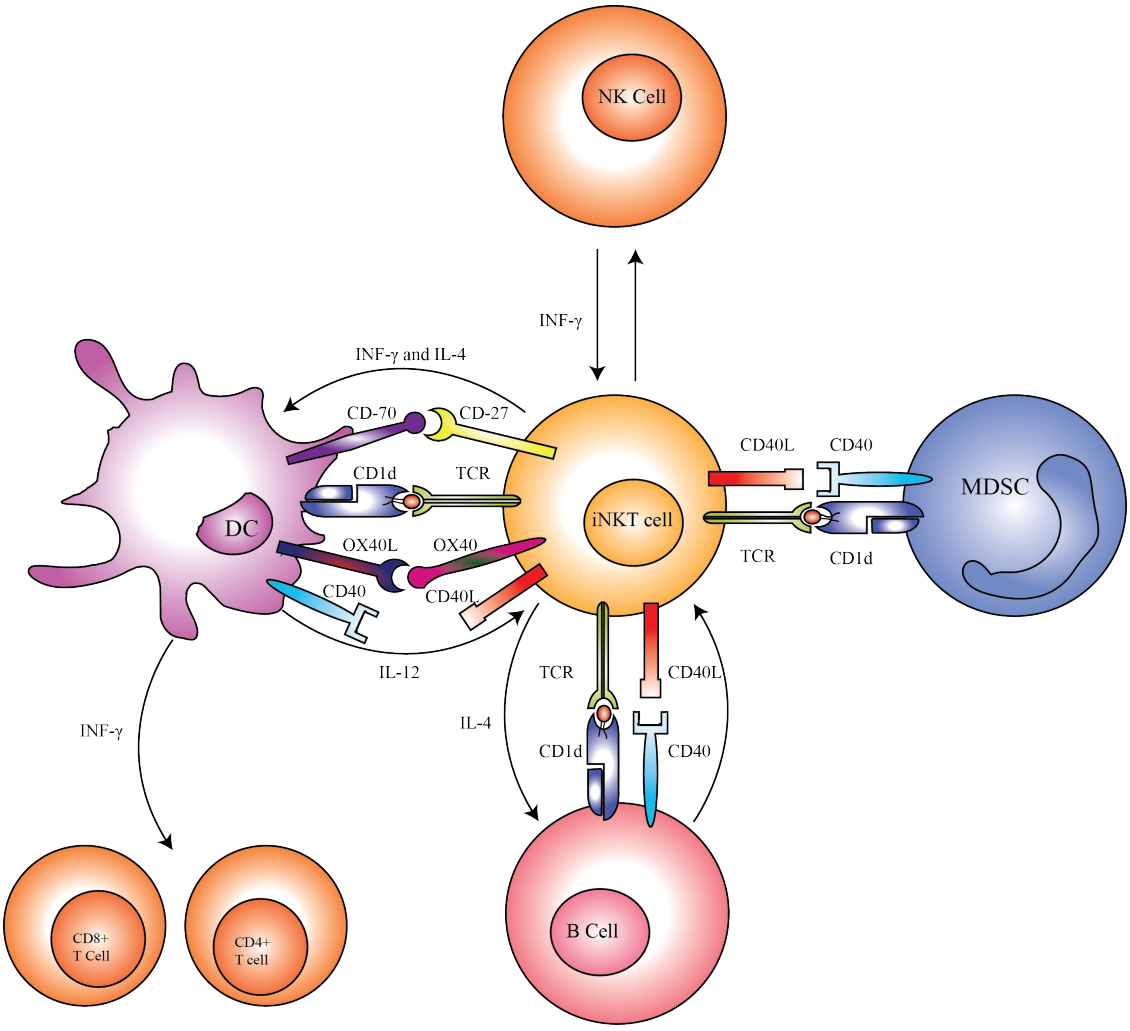


Figure 1.4 NKT cells modulate the innate and adaptive immune responses. Illustration depicts NKT cells activating T and B cells, dendritic cells, MDSCs, and NK cells. Activation signals can be received through cell surface receptors, such as the TCR recognizing lipid-bound CD1, costimulatory receptors (CD40, CD70, OX40), or cytokines. Adapted from Cerundolo et. al.[13]

bidirectional signals can be received through cell surface receptors, such as the TCR recognizing lipid-bound CD1, costimulatory receptors (CD40, CD70, OX40), or cytokines. For example, activation of NKT cells results in rapid maturation of dendritic cells and B cells. Activated NKT cells can also counterbalance the suppressive effects of myeloid-derived suppressor cells (MDSCs).[13,23]

1.3 *i*NKT cells recognize lipid antigens

As stated earlier, *i*NKTs were the first NKT cells to be discovered. Consequently *i*NKT cells have become the foundation for all NKT cell research. A brief historical background and review of the discovery and advancements in the field of *i*NKT cell research is essential to illustrate the fundamental principles and theories that helped shape our research.

1.3.1 The model *i*NKT antigen: α-GalCer

In 1993, Kirin Pharmaceuticals, a pharmaceutical subsidiary of Kirin Brewery inc., screened for marine natural products for anti-tumor activity. Their efforts led to the isolation and characterization of glycolipids termed "agelasphins," as shown in Figure 1.5. Agelasphins were an exciting discovery because these isolated glycolipids had an α-linked glycosidic bond, instead of a β-linked bond (β-linked glycolipids had already been found in mammals and other higher organisms).[25,26]

At the time Kirin pharmaceuticals focused on structure-activity relationships of the lipid moiety to elucidate and then enhance the anti-tumor properties of agelasphins. These initial analogs showed that the C2 hydroxyl (see Figure 1.5 for glycolipid numbering) did not significantly im-

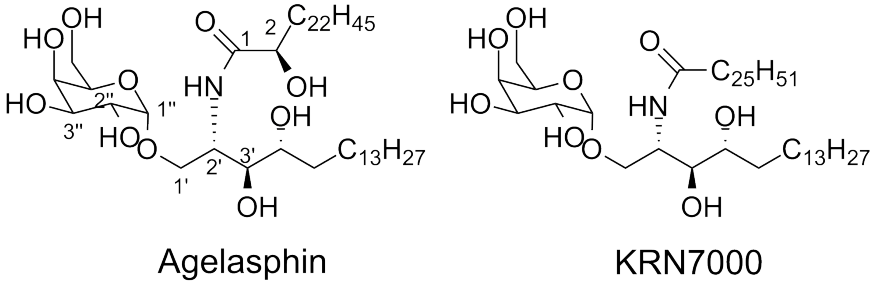


Figure 1.5 Representative structures of an agelasphin and KRN7000

pact the anti-tumor properties, the C4′ hydroxyl had very slight impact, and the C3′ hydroxyl was essential for the anti-tumor characteristics. These studies also produced a synthetically simplified agelasphin analog called KRN7000, also known as α-galactosylceramide (α-GalCer).[25,27] Over the next several years the immunoregulatory role of α-GalCer with *i*NKT cells became more apparent. Consequently, this analog became the model and primary *i*NKT cell antigen in the study of *i*NKT cell stimulation (Figure 1.5).[3]

Although α-GalCer is the model *i*NKT cell antigen, it has two limitations that inhibit its therapeutic effectiveness. First, after *i*NKT cell stimulation from CD1d-bound α-GalCer, the immune system releases a bevy of TH1 and TH2 cytokines that counteract each others' abilities to modulate the immune system in a deliberate fashion. Second, the massive amount of cytokines also leads to *i*NKT cell anergy, or inactivation of the *i*NKT cell.[28] Because of these limitations, a desire to find more effective *i*NKT cell antigens has ensued. Through understanding the antigen specificity of *i*NKT cells, research groups have manipulated immunomodulatory properties of *i*NKT cells to varying success. Research has been roughly divided into three main foci: isolation and characterization of natural antigens, structure-activity analyses, and the study of the immunological and therapeutic importance of *i*NKT cells.

1.3.2 Isolation and characterization of natural antigens

Figure 1.6 shows a variety of natural *i*NKT cell antigens that have been identified over the last decade. These natural antigens are categorized as endogenous antigens (antigens synthesized in our bodies) or exogenous antigens (those synthesized from other organisms).

Endogenous antigens

Researchers have spent the past decade attempting to find "the" endogenous antigen that our body uses for positive selection in the thymus. During development of *i*NKT cells, an immature thymocyte must interact with CD1 bound with an endogenous antigen. Without this antigen, our body does not allow maturation of the *i*NKT cell.[18] To date, no α-linked glycolipids have been found in humans, so efforts were focused on finding β-linked endogenous antigens.[26] In 2004, Zhuo *et al*. presented lysosomal isoglobotrihexosylceramide (iGb3) as an endogenous antigen and likely selecting antigen candidate for *i*NKT cells.[29] The fact that iGb3 activated most *i*NKT cells *in vitro*, combined with the data that the lack of iGb3 in mice resulted in a dramatic decrease of *i*NKT cells *in vivo*, made a good argument for iGB3.[15] Initially this claim was followed by controversy because it was believed that humans lacked the enzyme responsible for synthesis of iGb3. The support for iGb3 came via two papers that described the detection of iGb3 in human cells via ion-trap mass spectrometry.[23]

Although iGb3's purpose as the predominant selecting antigen during *i*NKT cell development has been questioned, iGb3 is still the most potent endogenous antigen discovered to date. Other endogenous antigen candidates that have been isolated are sulfatide, a 3-sulfo-galactosylceramide

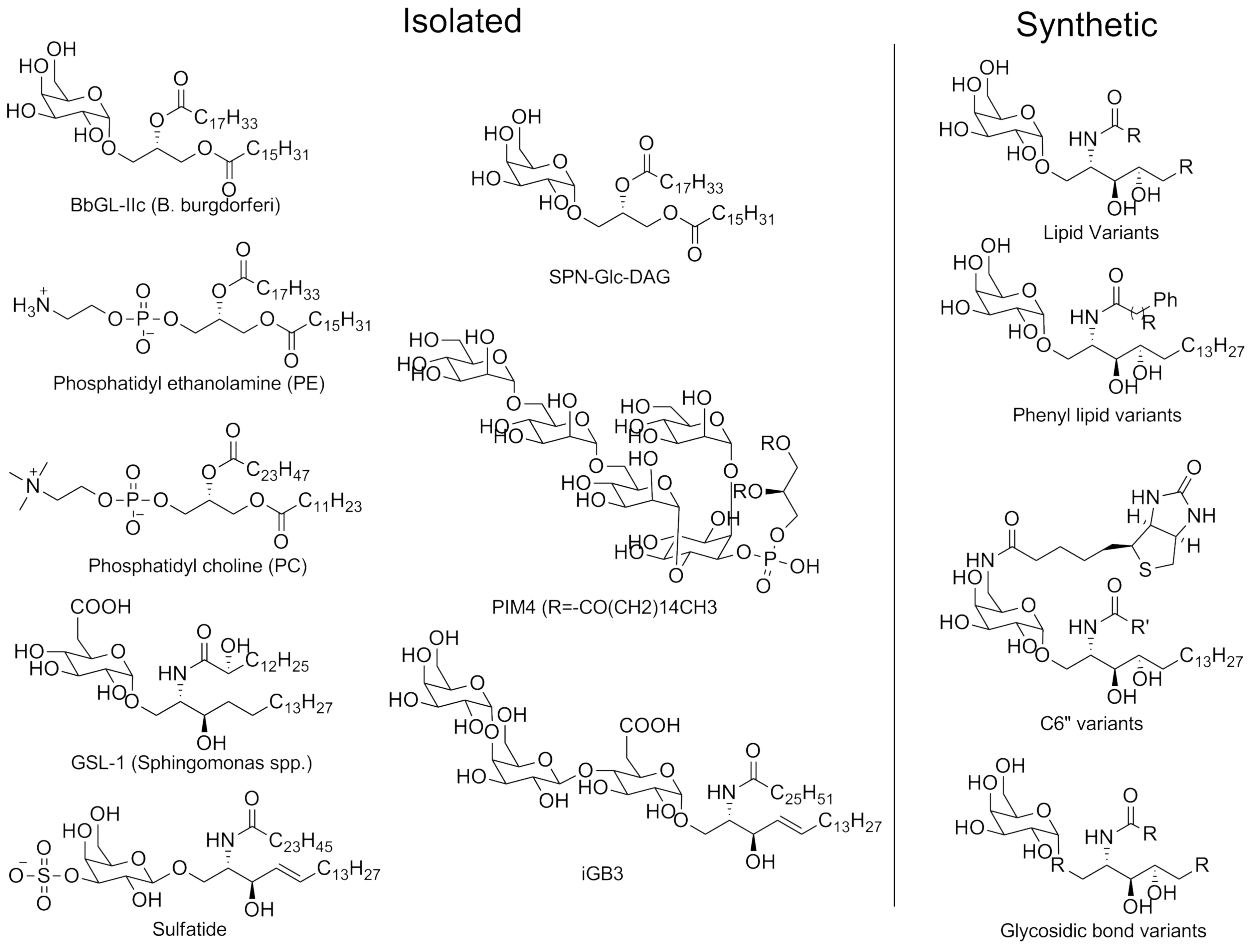


Figure 1.6 Representative structures of natural and synthetic *i*NKT cell antigens

that is found in the myelin sheaths of the brain, and phosphotidyl choline (PC) and tumor-cell extracted phosphotidyl ethanolamine (PE). These antigens do bind to CD1d but they are very weak antigens or type 2 antigens.

Exogenous antigens

Since one of the purposes of the immune system is to fight off invading bacteria, it would be logical to assume that *i*NKT cells have exogenous bacterial antigens. In recent years, immunologists

and chemists have found a variety of these natural exogenous antigens. Recently, diacylglycerol compounds were isolated from the the causative agent of lyme disease, *B. burgdorferi*. These *B. burgdorferi* compounds, shown in Figure 1.6, demonstrated a direct role in host defense in conjunction with *i*NKT cells.[20] These glycerols have an α-linked carbohydrate moiety and two fatty acids of varied length and saturation.[9] The degree of saturation plays a key role in their immunological potency reinforcing the structure-activity studies from Zhou *et al.*[30] Interestingly, unlike α-GalCer, the lipids of these glycerols can bind in either the F$'$ or A$'$ pockets.[20]

Similar to the *B. burgdorferi* glycerols, three more diacyl glycerol natural antigens have been isolated (Figure 1.6). PC and PE from extracted cypress pollen was shown to bind to CD1d and CD1a, similar to their endogenous counterparts. PIM_4, one of the first reported *i*NKT cell associated bacterial glycolipids, was isolated from *Mycobacterium bovis bacillus* and showed *i*NKT cell recognition also.[3,13,22]

The TCR of *i*NKT cells also recognizes a series of glycolipid antigens that were isolated from the *Sphingomonadaceae* family of bacteria, called the GSLs.[22,31] The structure of GSL-1 is represented in Figure 1.6. There is also a GSL-2 (synthetic), GSL-3, and GSL-4 which are di, tri, and tetrasaccharides respectively. Even though the GSLs are very similar to α-GalCer, they do not stimulate NKT cells as well. Crystal structures of the TCR-GSL1-CD1d complex showed that the lack of the C4$'$-hydroxyl results in an alternative hydrogen-bonding network (in comparison to α-GalCer), which showed a slight lateral shift of the galactosyl head group. This slight lateral shift is thought to explain the difference in antigenicity between GSL-1 and KRN7000.[9,31,32]

1.3.3 Structure-activity analyses of α-GalCer

Because of α-GalCer potency, many structure-activity relationship studies have been conducted over the years to determine the TCR-glycolipid-CD1d specificity. These analyses have focused on three moieties: the phytosphingosine scaffold, the galactosyl head group, and the lipid chains. The following subsections will discuss a non-exhaustive list of many of the key analyses performed.

Modification of phytosphingosine scaffold

As discussed in section 1.3.1, Kirin Breweries conducted the first structure-activity studies that found the importance of the $3'$-hydroxyl and the minimal impact of the $4'$-hydroxyl (see Figure 1.5 for glycolipid numbering). The majority of studies have verified these findings. For example, Kronenberg *et al.* verified Kirin's work by making a $3'$-deoxy variant; this variant could not bind to CD1d.[33] Another group replaced the amide with a triazole moiety; this triazole variant stimulated comparably to α-GalCer, proving that the amide hydrogen was not essential to binding.[34] The other main analyses replaced the phytosphingosine subunit with scaffolding found in other natural antigens, namely sphingosine (GSLs), diacyl glycerol (*B. burgdorferi*, PE, PC, and serine-based lipids (a non-natural ceramide mimic).[3,19,35] Another noted analog replaced the oxygen glycosidic bond with a carbon-carbon bond. This analog showed an increased immunostimulatory response against malaria and metastatic melanoma (in mice) in comparison to α-GalCer.[13,28] In recent years, these analyses have been verified from the solved crystal structures of CD1d bound to the appropriate antigen. These comparison studies truly solidified the importance of the 3'-hydroxyl, and demonstrated small shifts that occur without the 4'-hydroxyl that consequently gives

α-GalCer stronger binding affinity and better stimulatory activity.[3,19]

Modification of the sugar headgroup

The Koezuka group, at Kirin pharmaceuticals, synthesized a large array of analogs that explored the structure-activity relationships of the galactosyl headgroup. Their major findings were gleaned from a series of disaccharides that demonstrated that α-linked sugars were potent but β-linked antigens gave no stimulatory response. Furthermore, their mannose analog gave no response, suggesting the importance of the 2$''$-hydroxyl (see Figure 1.5 for glycolipid numbering).[27,28] The mannose results led many groups to explore the importance of the 2$''$-hydroxyl. 2$''$-fluoro, 2$''$-deoxy, and 2$''$-acetoamino analogs nullified the stimulatory activity.[19,28] Whereas analogs that explored 4$''$-hydroxyl and the 6$''$-hydroxyl variants were well tolerated. Notably, Liu et al. were able to substitute the 6$''$-hydroxyl with an acetamide group, that matched and exceeded α-GalCers potency.[36] Along with well tolerated biotinylated and fluorophoranated analogs that are used for glycolipid trafficking and CD1d loading studies.[19,37] The aforementioned crystal structure analyses further proved the importance of the 2$''$-hydroxyl and showed why modification at the 6$''$ and 4$''$-hydroxyl are well tolerated. The 2$''$-hydroxyl strongly hydrogen bonds with an aspartamine from CD1d to position the headgroup for TCR presentation. The 2$''$-hydroxyl analogs hamper the alcohol's ability to bind and position the sugar, whereas the 6$''$ and 4$''$-hydroxyl analogs are well tolerated because they protude out and away from the CD1d complex.[38]

Modification of lipids

The SAR studies that have focused on the lipid portion of α-GalCer have found a very interesting and useful observation. Lipid chain length biases the balance of TH1 and TH2 cytokines released. Zhou *et al*. showed that shortened lipid chains biased a TH2 cytokine release profile whereas longer lipid chain lengths biased a TH1 response.[30] These findings along with Miyake and coworkers study present compelling evidence that INF-γ production requires longer TCR stimulation than IL-4 production. The shorter lipid chain length analogs do not form as stable complexes with CD1d than the longer chain analogs, due to the lack of additional hydrophobic interactions in the F$'$ pocket.[30,39] These analogs stimulate the TCR for a short amount of time in comparison to the more stable longer lipid antigens. Chi-Huey Wong and collaborators were able to verify this by making lipid tails with terminal aromatic groups to enhance hydrophobic interactions. Their analogs biased a TH1 response and were even more potent than α-GalCer.[19,40,41]

1.3.4 Immunological and therapeutic importance of NKT cells

The therapeutic potential of *i*NKT cells lies in their ability to modulate or activate the immune system through the rapid release of TH1 and TH2 cytokines. Endogenous and exogenous antigens are currently being modified to provide antigens that polarize cytokine release to either a TH1 or a TH2 response. This interest stems from years of well-documented research that has focused on finding out what diseases are affected by *i*NKT cells.[3] These studies have generally done one of three things to survey *i*NKT cell involvement in murine and human diseases: (1) observed the *i*NKT cell numbers, (2) monitored the effect of CD1d or *i*NKT cell depletion on the disease,

or (3) administered α-GalCer to see its effect on the disease. For example, when studying two separate Type 1 diabetes animal models, there were lower concentrations of *i*NKT cells in the spleen, liver and thymus, one study ameliorated the disease with lack of CD1d/*i*NKT cells, whereas administration of α-GalCer improved the diseased mice.[15] In this way, *i*NKT cells have been implicated in many autoimmune diseases such as type 1 diabetes, multiple sclerosis, rheumatic arthritis, and asthma.

One of the first applications of *i*NKT cell antigens has been as vaccine adjuvants. The classic live attenuated pathogens (or whole inactivated organisms) and the current recombinant virus vaccine strategies have had limited success. Many times these vaccines fail to promote a strong enough response to cause immunity to the virus in question. Advances in vaccination strategies has allowed the design of highly specific synthetic protein vaccines. As an adjuvant, a NKT cell antigen could cause a strong enough immune response to promote immunity. This hypothesis has shown promise by a variety of groups by co-injection of α-GalCer with an antigen. This caused an increased immune response for the co-antigen. Because of the limitations of α-GalCer (section 1.3.1), more *i*NKT cell adjuvant candidates are necessary for the successful adjuvant application.[13]

1.4 Conclusion

This dissertation addresses key advances in the understanding of antigen specificity of type I, type II, and $\gamma\delta$ NKT cells. It builds upon previous SAR studies to further the goal of finding and/or understanding NKT cell antigens, as well as present data of a new sulfatide-reactive CD1d-

restricted $\gamma\delta$ T cell line derived from the blood of healthy individuals.

Chapter 2

Synthesis and Evaluation of Sulfatide analogs: a $\gamma\delta$ T cell antigen

2.1 Introduction

Sulfatide, introduced in section 1.3.2, was the first isolated sulfated glycolipid. Although isolated from human brain in 1884, it was first chemically characterized in 1962.[42,43] As shown in figure 2.1, sulfatide is a galactosylceramide with a sphingosine scaffold (outlined in blue) and a sulfate at the 3″-hydroxyl position. The biosynthesis of sulfatide starts in the endoplasmic reticulum where UDP-galactose is added onto ceramide via the enzyme UDP-galactose:ceramide galactosyltransferase (CGT; EC 2.4.1.45). Galactosyl ceramide is transported from the endoplasmic reticulum to the golgi apparatus where 3′phosphoadenosine - 5′phosphosulfate:cerobroside sulfotransferase (CST; EC 2.8.2.11) sulfates the 3″-hydroxyl position of galactosylceramide.[42]

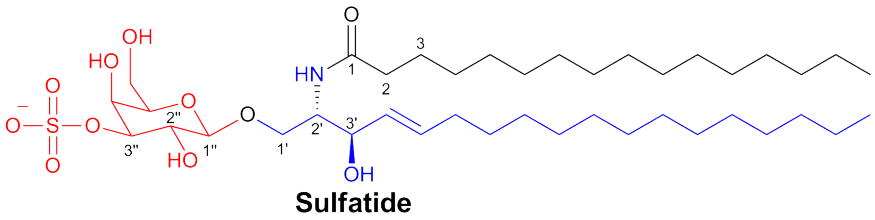


Figure 2.1 Representative structure of sulfatide

Sulfatide is widely expressed throughout the body and has been found in neural tissue, the islets of langerhans (pancreas), kidneys, and retina.[44] Sulfatide is found in a variety of isoforms that vary in the amide chain length and degree of saturation (a minor portion of sulfatide also can have hydroxylated acyl chains).[45] Most sulfatide found in humans have the following fatty acyl chains c16:0, 18:0, 18:1, 20:0, 22:0, 24:0, and 24:1 (chain length:degree of saturation). Distinct ratios of sulfatide isoforms have been reported in different areas in the body. For example, c16:0 sulfatide is predominantly found in the pancreas, whereas longer chain sulfatides (c24:1 and c24:0) are more abundant in the brain.[44]

As discussed in section 1.3.2, sulfatide is an endogenous NKT cell antigen. Unlike α-Galcer, sulfatide has a binding affinity to multiple CD1 moieties and has been shown to bind to CD1a, b, and c. Upon stimulation with sulfatides, multiple CD1a-c-specific T cell lines have been isolated from the blood of healthy individuals.[46,47] Work presented by Vipin Kumar's group also found a CD1d-dependent sulfatide-reactive T cell population in naive mice. This work also defined the subpopulation as mostly (90%) CD4+CD8- T cells with the majority lacking the NK1.1 marker. They also gave clear evidence that the subpopulation did not bind α-GalCer which further characterized these T cells as type II NKT cells. Another notable finding was the observation that

the concentration of sulfatides increased in the absence of the enzyme CGT, suggesting that the autoreactive cells were normally eliminated by exposure to self antigen.[48]

Since the discovery of sulfatide-reactive T cells, subsequent research has implicated their involvement in tumor immunity, experimental autoimmune encephalomyelitis (EAE), multiple sclerosis (MS), and type 1 diabetes.[49] We were particularly interested in the potential pathological relevance of sulfatide-specific T cells in EAE and MS because of the reported increased frequency of sulfatide in the blood of patients with multiple sclerosis and also in the lesions of EAE in mice.[46,48,50] As conditions, MS and EAE are associated with demyelination via attack of the myelin sheaths of the axons of the central nervous system's.[51] Because the most abundant component of myelin sheaths are glycolipids, with one fifth of the total myelin glycolipids being sulfatide, subsequent release of sulfatides may expand T cells reactive to self-lipids and further aggravate the immunopathological process.[46,52]

In an attempt to enumerate and characterize CD1d-sulfatide-specific T cells in fresh human blood, instead of previously reported mouse blood, in an unbiased manner (i.e., without prior expansion of cultured cell lines), we used tetramers for direct enrichment of CD1d-sulfatide specific T cells. Additionally, we synthesized a series of sulfatide analogs that mimicked previous SAR study analogs for Type I NKT cells in order to find out the structural requirements necessary for NKT cell stimulation. Finally, we solved the crystal structure of the $\gamma\delta$ TCR-sulfatide-CD1d complex.

2.2 Synthesis of Sulfatide analogs

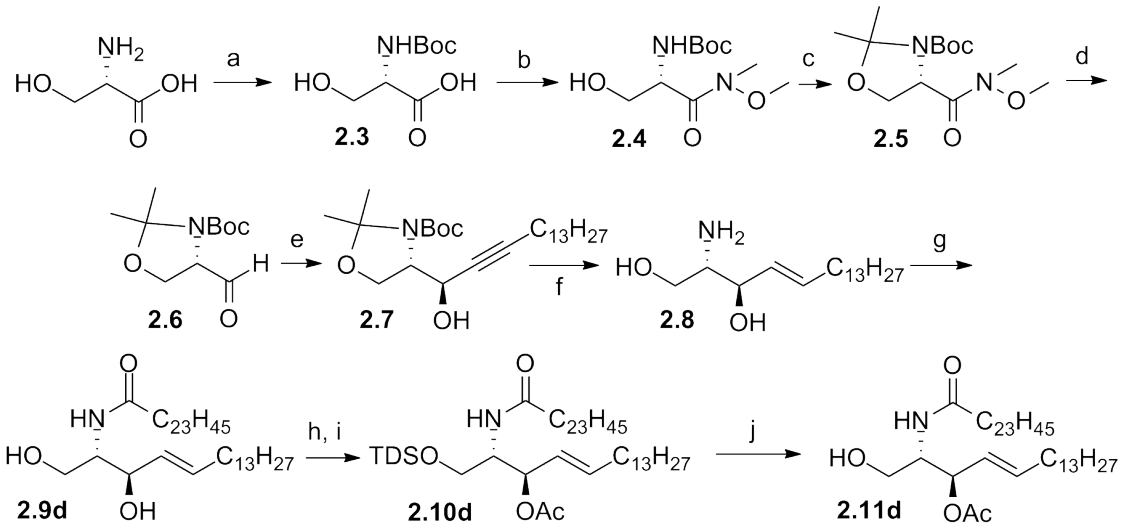
To facilitate our collaborative research with Albert Bendelac and Erin Adams, at the University of Chicago, seventeen sulfatide analogs were designed. With these variants in hand, we explored the effect of lipid chain length on the immunological impact of sulfatides, evaluated the positioning of the sulfate, mimicked the addition of acetamide in the highly potent PBS57 NKT cell antigen, developed a nonhydrolyzable sulfonated β-galactosylceramide, trafficked and imaged sulfatide (Chapter 3), and carried out binding studies on a series of deoxy sulfatide variants. Most immunological studies utilize isolated or semi-synthetic sulfatide, which has sometimes been shown to be contaminated, our work has allowed evaluation of pure and consistent sulfatide throughout this study. The following section outlines the synthetic routes towards these sulfatide analogs.

2.2.1 Synthesis of sulfatide 2.1 and the phytosphingosine analog of sulfatide 2.2

Sulfatide synthesis strategies include three different aspects: (1) the preparation of the sphingosine scaffold acceptor, (2) the selective sulfation of the 3″-hydroxyl, and (3) the glycosylation of the galactosyl donor with the sphingosine acceptor. (1) We closely followed the synthesis of Xing *et al*. using ceramide as an acceptor instead of azido-sphingosine.[53] Although traditionally plagued by poor yields during glycosylation, a ceramide acceptor produced a convergent route suitable for our varying syntheses. (2) The decision of orthogonal protection of the 3″-hydroxyl (towards subsequent deprotection then sulfation) was influenced by a concern of reproducibility between

separate sulfatide batches sythesized prior to this work in our lab. Another method that exists is the use of dibutyl tin oxide, followed by selective sulfation at the 3″-position with sulfur trioxide. This method, developed by Flitsch *et al.*, is a useful method that shortens the synthesis significantly, but has the slight disadvantage of sulfating the 6″-hydroxyl position. We ultimately decided to follow the previously adapted synthesis of sulfatide first, which later proved fruitful as nearly all the sulfatide variants share the common synthetic building block **2.14**. (3) The last decision of glycosylation conditions is many times chosen by preference or experience, the majority of the glycosylations were carried out under the well-established Schmidt coupling reaction conditions.

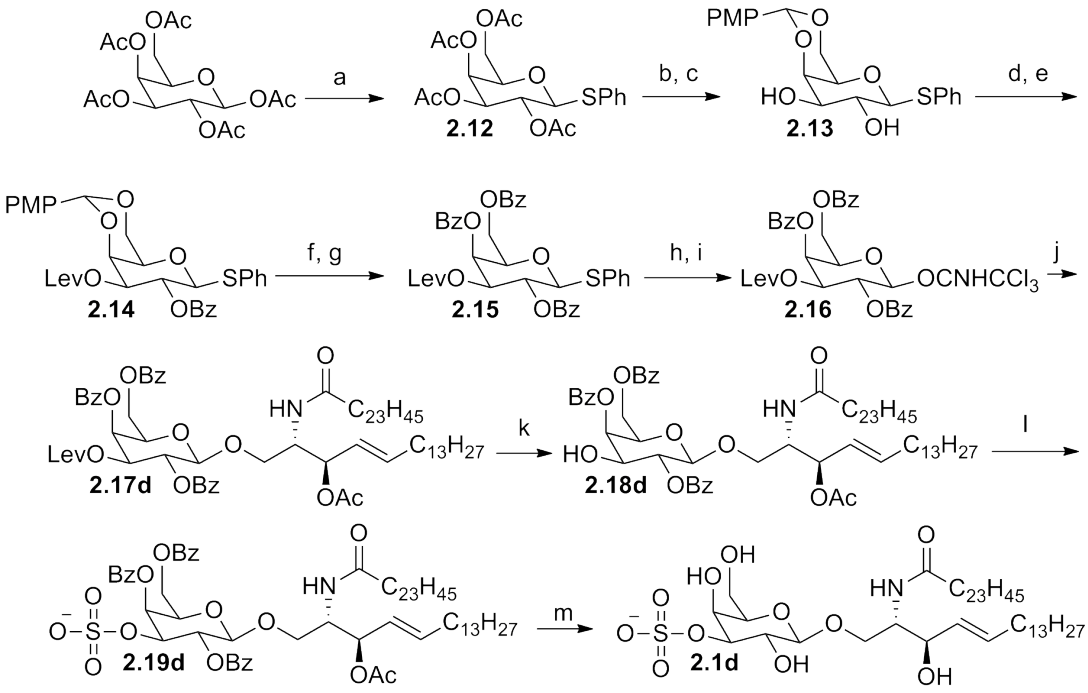
Synthesis of ceramide 2.11d



Reagents and conditions: a. Boc_2O, NaOH, dioxane, 0 °C ; b. NHA, NMM, EDCI, DCM; c. DMP, TsOH, DMF, 55% (3 steps); d. LAH,THF, 0 °C, 93%; e. n-BuLi, pentadecyne, THF, -23 °C, 41%; f. Li, ethylamine, THF, -78 °C, 88% (small scale); g. EDCI, HOBt, nervonic acid, THF; h. TDS-Cl, py; i. Ac_2O, DMAP, py, 71% (2 steps); j. HF(aq), ACN, DCM, 97%

Scheme 2.1 Representative synthesis of sulfatide **2.11d**

The amine of commercially available L-serine was protected with Boc anhydride in order for the successful conversion of serine's carboxylic acid into a Weinreb amide. Amide **2.4** was further protected with dimethoxy propane. Fully protected amide **2.5**, was reduced with lithium aluminum hydride to form "Garner's aldehyde (**2.6**)." The addition of lithium 1-pentadecyne to the aldehyde gives excellent stereoselectivity (15:1) towards the desired propargylic alcohol **2.7**. The absence of chelating agents allows the pentadecyne to attack the Re face in accordance with the Felkin-Ahn model. As reported by Garner *et al*., formation of sphingosine **2.8** was achieved by subjecting the propargylic alcohol to the strong reducing conditions of lithium in liquid ethyl amine.[54] This not only reduced the alkyne but it deprotected the Boc and the dimethoxy propane groups. To verify that we synthesized and isolated the correct propargylic alcohol. We took a small portion of **2.8** and commercially available isolated sphingosine, peracylated them, and compared them by ^{1}H NMR. Once the structure of sphingosine was verified, traditional peptide couplings were performed to form the desired amides from the appropriate acid (e.g. nervonic acid (24:1)) to form ceramide **2.9d**. Selective thexyl dimethyl silyl protection of the primary alcohol and subsequent protection of the secondary alcohol led to compound **2.10d**. Selective deprotection of the silyl group with hydrofluoric acid provided glycosylation acceptor **2.11d** (Note: this reaction if done slowly, left overnight, heated up, or purified slowly can and will result in migration of the 2-acetyl group to the primary alcohol. It is essential that the chemist quench this reaction immediately after completion).



Reagents and conditions: a. $BF_3(OEt)_2$, PhSH, DCM, 0 ^oC, 92%; b.NaOMe, MeOH; c. PMP-acetal, DMF, TsOH, 50 ^oC, 60%; d. levulinic acid, DMAP, EDCI, DCM, 0 ^oC; e. bz-cl, py, 78%; f. AcOH:H_2O; g. bz-Cl, py, 80%; h. NBS, H_20, Acetone; i. K_2CO_3, Cl_3CCN, DCM, 89%; j. 4A M.S., **2.11d**, TMSOTf, DCM,0 ^oC, 48%; k. hydrazine, AcOH, MeOH, THF, 95%; l. SO_3-Py, py, 80%; m. NaOMe, THF, MeOH, 76%.

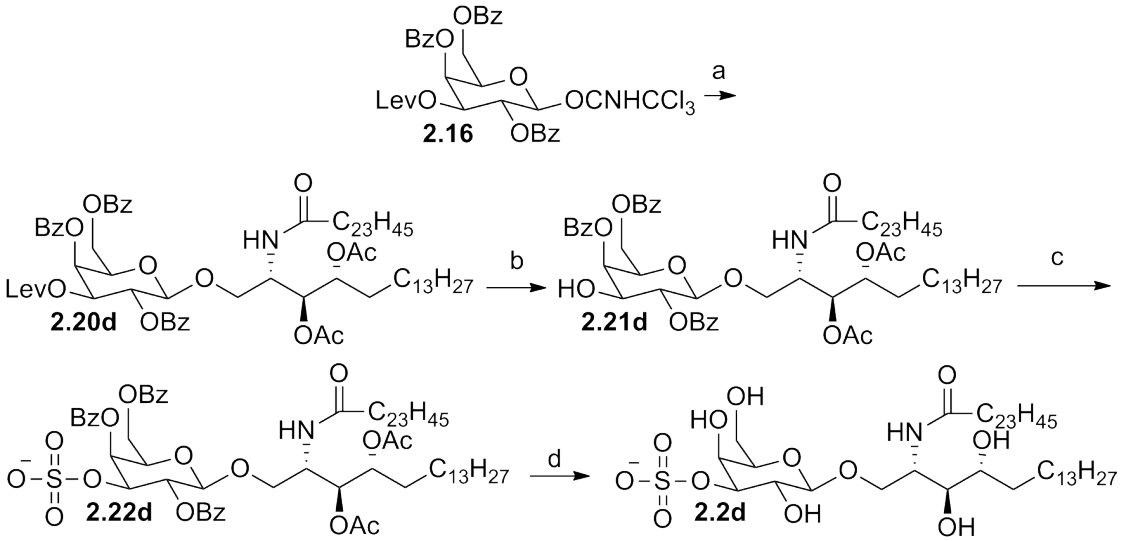
Scheme 2.2 Representative synthesis of sulfatide **2.1d**

Synthesis of sulfatide 2.1d

The synthesis of the Schmidt glycosolation donor **2.16,** outlined in scheme 2.1, was an adapted synthesis from Dr. Nin Ying's dissertation at this institution. Starting with commercially available β-D-pentacetate-glucose, the anomeric position was protected with thiophenol. Global deprotection of the acetate protecting groups, via sodium methoxide in methanol, followed by protection of the 4-hydroxyl and the 6-hydroxyl with anisaldehyde dimethyl acetal afforded compound **2.13**. Exploiting the nucleophilicity difference between the 3-hydroxyl and the 2-hydroxyl, stepwise addition of levulinic acid followed by benzoyl chloride, installed the orthogonal Lev group on the

3-hydroxyl position (**2.14**). The para-methoxy phenyl group was removed under acidic conditions, followed by the installation of benzoyl protecting groups to make compound **2.15**. Fully protected galactose **2.15**, was transformed into coupling donor **2.16** through a traditional 2-step process that replaces the thiophenol with a trichloroacetonitrile moiety. Schmidt coupling conditions were carried out with donor **2.16** and acceptor **2.11d** to yield glycolipid **2.17d**. Orthogonal hydrolysis of the levulinic ester with hydrazine and acetic acid, gave alcohol **2.18d**. The 3-hydroxyl was used to form the sulfated glycolipid **2.19d** in the presence of sulfur-trioxide pyridine complex. Finally, **2.19d** was globally deprotected with fresh sodium methoxide in methanol and tetrahydrofuran (THF) to form sulfatide **2.1d**. The following procedure was repeated with four different ceramide acceptors, differing in their acyl chain length and saturation (c8:0, 16:0, 24:0, 24:1).

Synthesis of phytosphingosine sulfatide analog 2.2d



Reagents and conditions: a. 4A M.S., appropriate ceramide, TMSOTf, DCM, 0 °C, 37%; b. hydrazine, AcOH, MeOH, THF, 91%; c. SO_3-Py, py, 70%; d. NaOMe, THF, MeOH, 93%.

Scheme 2.3 Representative synthesis of sulfatide **2.2d**

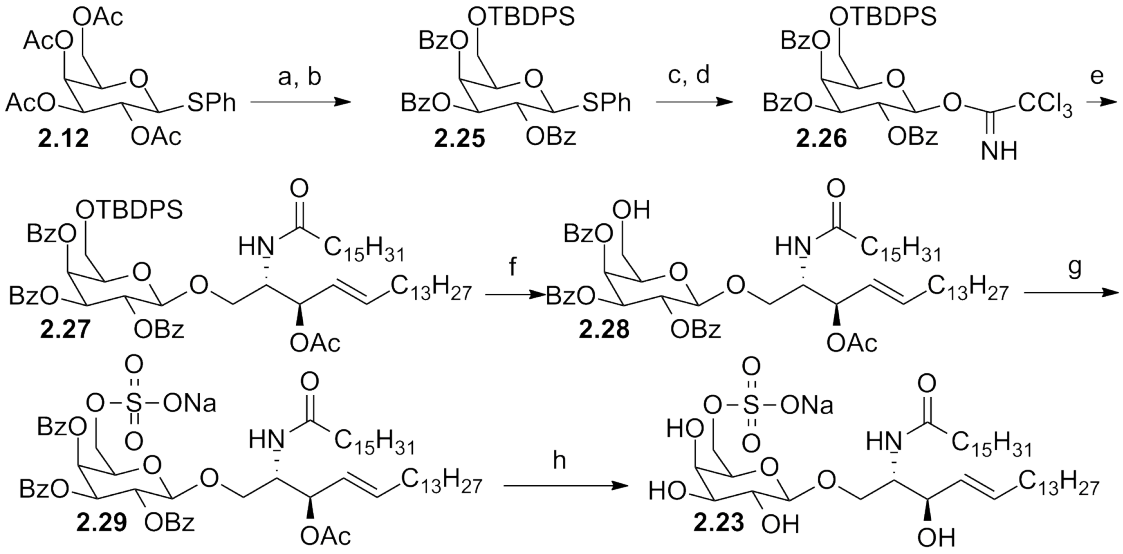
While synthesizing the sphingosine series of sulfatide, we also synthesized four phytosphingosine sulfatides (**2.2a-d**). These variants only differ by the presence of a 4′-hydroxyl instead of a double bond between the 4′ and 5′ carbon of the sphingosine scaffold. Yang Liu, while a graduate student at BYU, provided all four phytosphyingosine ceramides (prepared from commercially available phytosphingosine following the same ceramide route). These ceramides were coupled under Schmidt conditions to give the desired glycolipids. Figure 2.3 shows the abbreviated synthesis of these sulfatides.

2.2.2 Synthesis of 6′′-sulfo-sulfatide variants 2.23 and 2.24

During the initial synthesis of sulfatide, we developed a theory concerning the origins of a reproduciblity problem between previous batches of sulfatide (synthesized from two other graduates in our lab). We postulated that under basic conditions, migration of the sulfate to other alcohols may be possible and subsequently produce different stimulatory results. Since migration to the primary 6′′-hydroxyl would be the most likely by-product, we devised a synthesis for 6′′-sulfo sulfatide variants. The synthesis is represented in scheme 2.4. To install a sulfate at the 6′′-hydroxyl, donor **2.26**, had to be synthesized. This was carried out starting from **2.12**. Crude deprotected thiophenyl galactose was dissolved in DMF and heated in the presence of tert-butylchlorodiphenylsilane (TBDPS-Cl) in order to protect the primary alcohol at C6. After successful silyl ether formation the remaining hydroxyl groups were protected with benzoyl chloride to ultimately form compound **2.25**. The same series of steps outlined in Scheme 2.2, were followed to form the donor and subsequently the glycolipid. Under Schmidt reaction conditions, the glycolipid formed very sluggishly, presumably

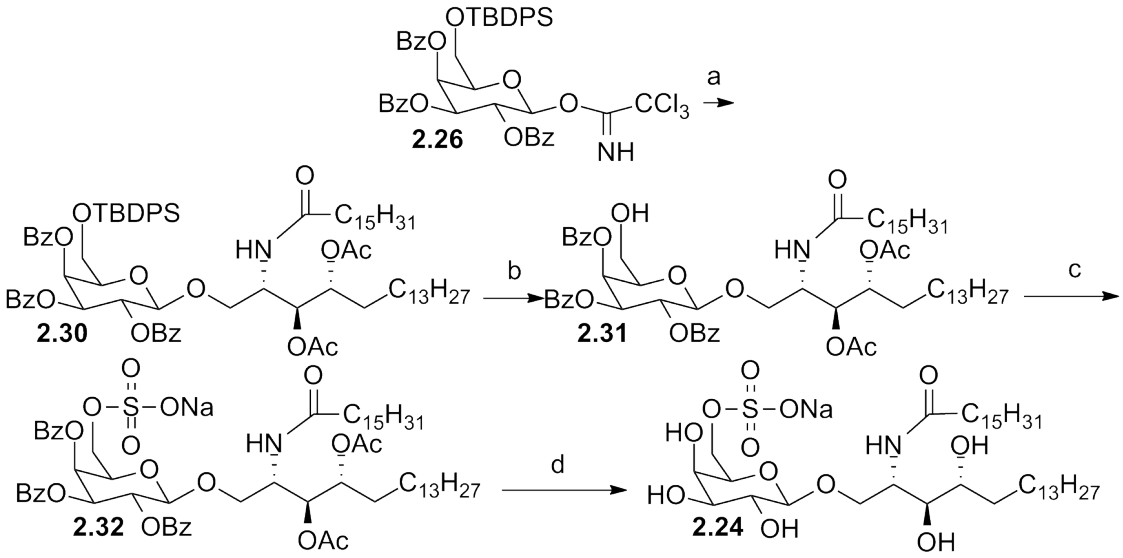
due to the bulky TBDPS group that hindered attack at the anomeric position, and favored ortho-ester formation. After glycosylation, the silyl group was deprotected with hydrofluoric acid. The final installation of the sulfate and global deprotection followed the synthetic route of **2.1d** to give 6″-sulfo-β-galactosylceramide **2.23**.

We also synthesized a phytosphingosine 6″-sulfo sulfatide variant from donor **2.26** with the appropriate palmitic phytosphingosine acceptor. The partial synthesis of **2.24d** is represented in Scheme 2.5.



Reagents and conditions: a. NaOMe, MeOH; b. 1) TBDPS-Cl, py, 2) Bz-Cl, 73%; c. NBS, H_2O, Acetone; d. K_2CO_3, Cl_3CCN, DCM, 98%; e. 4Å M.S., ceramide, TMSOTf, DCM, 0 ^oC; f. hydrazine, AcOH, MeOH, THF, 21% (two steps); g. SO_3-Py, py, 94%; h. NaOMe, THF, MeOH, 72%.

Scheme 2.4 Representative synthesis of sulfatide **2.23**



Reagents and conditions: a. 4Å M.S., ceramide, TMSOTf, DCM, 0 °C; b. hydrazine, AcOH, MeOH, THF, 21% (two steps); c. SO_3-Py, py, 81%; d. NaOMe, THF, MeOH, 72%.

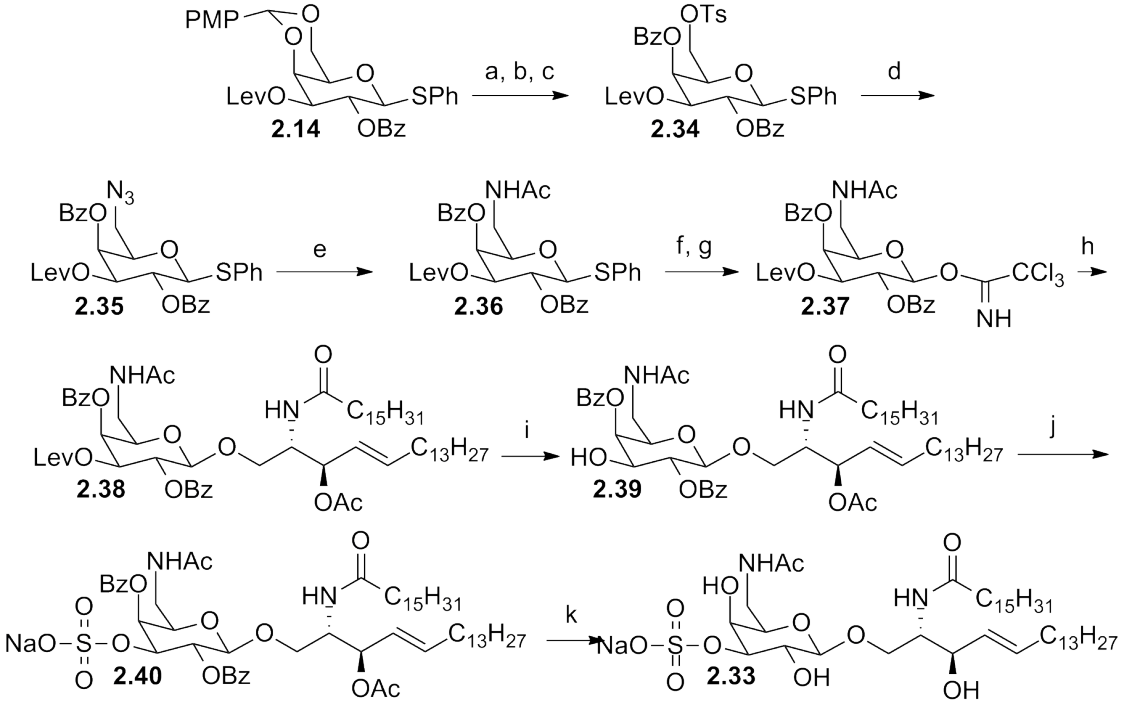
Scheme 2.5 Representative synthesis of sulfatide **2.24**

2.2.3 Synthesis of N-acyl sulfatide variant 2.33

Our immunological studies involved initially working with Type II sulfatide-reactive CD1d-restricted NKT cells. Our strategy, while waiting for biological data, involved mimicking previous SAR studies of *i*NKT cells. One of the most potent *i*NKT cell antigens ever developed, is PBS57. This analog (PBS57) differs only by replacement of a hydroxyl by an amide instead of the 6″-hydroxyl. This small change resulted in a significant increase in stimulatory activity.[36] Since type I and II NKT cells are both presented by CD1d, a similar sulfatide analog (**2.33**) became a logical target.

The synthesis of **2.33** is outlined in Scheme 2.6, and benefits from using the same building block (**2.13**) as the previous syntheses. The PMP group in **2.13** was removed in acetic acid and water. The primary alcohol was selectively protected with tosyl chloride, followed by protection

of the 4-hydroxyl with benzoyl chloride to form compound **2.34**. Introduction of sodium azide replacing the tosyl group with an azido moiety at the sixth position. Stepwise azide reduction and amide formation (Ph_3P, water followed by acetyl chloride in pyridine) led to multiple migratory biproducts between the Lev and the other benzoyl groups. A one step amide formation with ruthenium trichloride and thioacetic acid proved particularly effective towards the synthesis of **2.36**.[55] The final steps of the synthesis of **2.33** followed the route outlined in scheme 2.2 without any further complications.

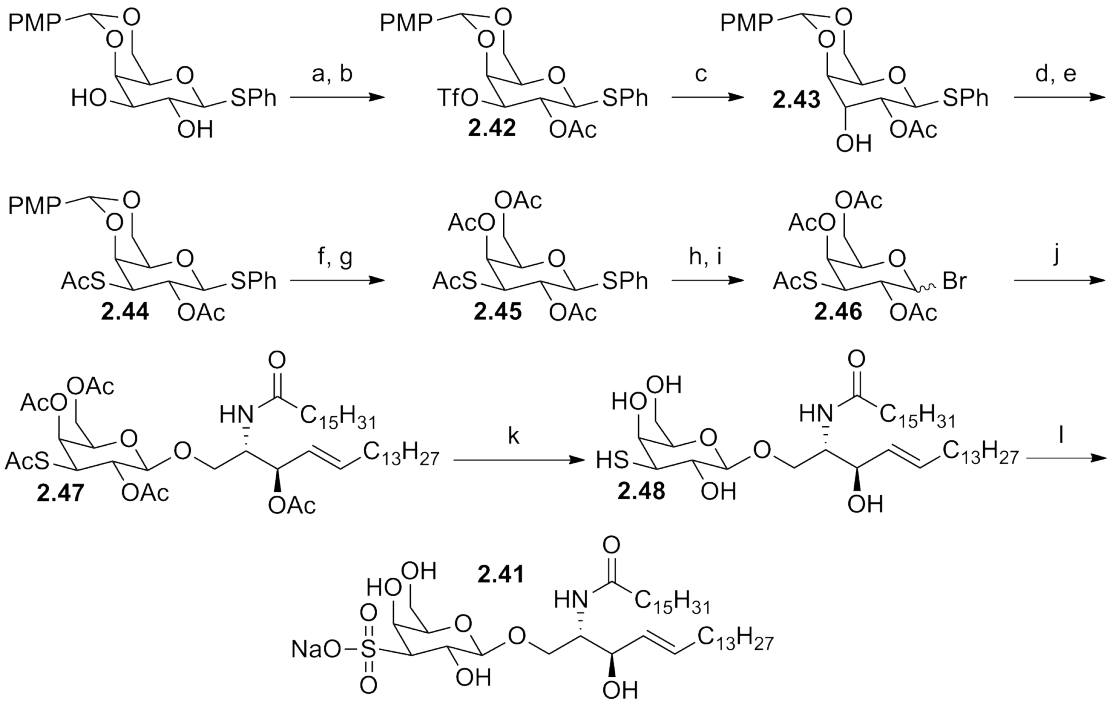


Reagents and conditions: a. AcOH:H_2O; b. TsCl, py; c. Bz-Cl, py, 52% (25% **2.15**); d. NaN_3, DMF, 100 ^oC, 67%; e. $RuCl_3$, thioacetic acid, 2,6 lutidine, MeOH, 60%; f. NBS, H_2O, Acetone; g. K_2CO_3, Cl_3CCN, DCM, 65%; h. 4Å M.S., ceramide, TMSOTf, DCM, 0 ^oC, 19%; i. hydrazine, AcOH, MeOH, THF; j. SO_3-py, 63% (over two steps); k. NaOMe, THF, MeOH, 66%.

Scheme 2.6 Representative synthesis of sulfatide variant **2.33**

2.2.4 Synthesis of sulfonate sulfatide analog 2.41

The biosynthesis of sulfatide was briefly discussed in section 2.1, and a brief discussion of the degradation of sulfatide is necessary to justify the synthesis of **2.41**. Once sulfatide is in the lysosome, arylsulfatase A (ASA: EC3.1.6.8) specifically hydrolyzes the sulfate at the $3''$-hydroxyl position. It has been shown that ASA employs the help of sphingolipid activator protein-1, also referred to as Saposin B, to extract sulfatide from membranes thereby making sulfatide accessible to ASA for degradation.[42] Our theory was to synthesize a sulfonate analog (**2.41**), this analog would benefit from a sulfur-carbon bond that would make degradation from sulfatide to ceramide via ASA not possible. We postulated that this analog would therefore have a longer period of stimulation to the NKT cells, resulting in a larger stimulatory response.



Reagents and conditions: a. Tf_2O, py, DCM, 0 ^oC; b. AcCl, py; c. tetrabutylammonium nitrate, DMF, 60 ^oC, 54 % (over three steps); d. Tf_2O, py; e. potassium thioacetate, DMF, 50 ^oC, 80%; f. AcOH:H_2O; g. Ac_2O, DMAP, py, 73 %; h. $BF_3(OEt)_2$, AcOH, ACN; i. HBr/AcOH, DCM, 0 ^oC, 70%; j. AgOTf, 4Å M.S., DCM, 46%; k. NaOMe, MeOH, 63%; l. NaOAc, H_2O_2, HOAc,13%.

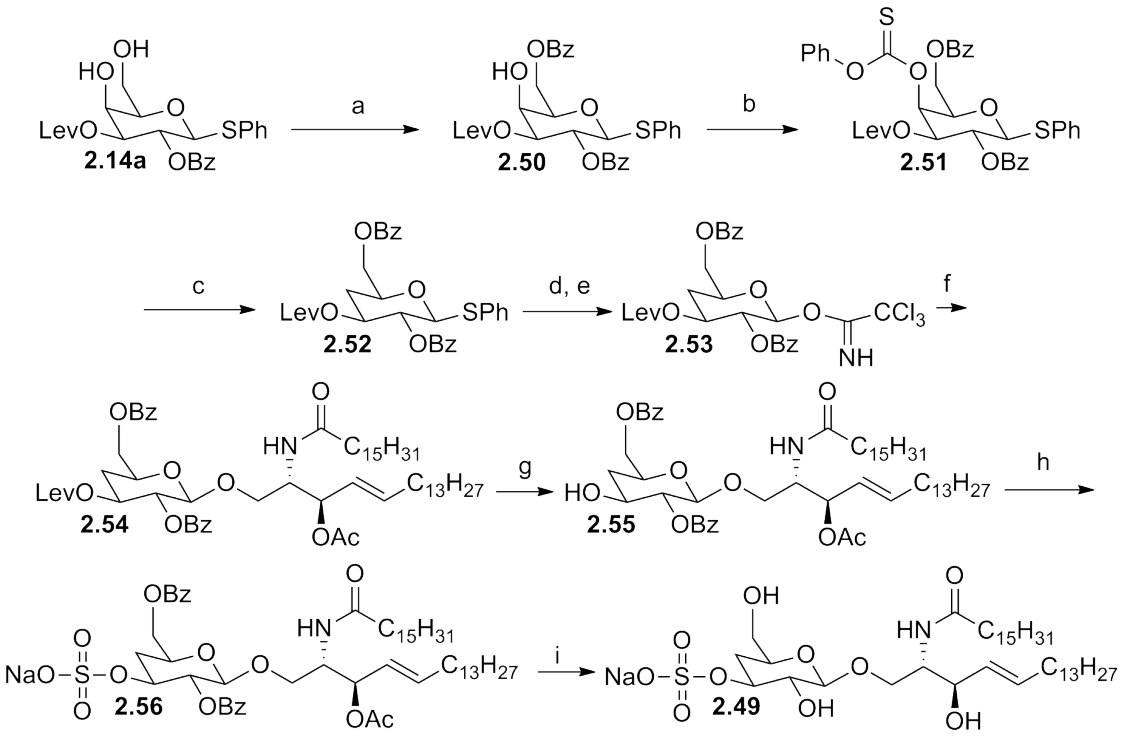
Scheme 2.7 Representative synthesis of sulfonate sulfatide analog **2.41**

Scheme 2.7, outlines the synthesis of this novel sulfonate glycolipid. Compound **2.42** was prepared by utilizing the nucleophilicity difference between the 3-hydroxyl and the 2-hydroxyl. Therefore, stepwise addition of triflic anhydride followed by acyl chloride, installed the triflate group on the 3-hydroxyl position. In the presence of tetrabutyl ammonium nitrate and heat, the triflate was replaced with an axial alcohol to give allose adduct **2.43**. This alcohol was transformed to a triflate, so that potassium thioacetate could add via an S_N2 reaction to afford compound **2.44**. The PMP group was deprotected under acidic conditions, followed by the installation of benzoyl protecting groups to make compound **2.45**. After unsuccessful glycosylation attempts with Schmidt

coupling conditions, compound **2.46** was synthesized as a Koenigs-Knorr donor. Under Koenigs-Knorr conditions, glycolipid **2.47** was coupled in excellent yield. Global deprotection of **2.47** with sodium methoxide in methanol and THF afforded thiol **2.48**. The final sulfonate formation with sodium acetate, acetic acid, and hydrogen peroxide suffered from a difficult separation and poor yield, nevertheless compound **2.41** was finally obtained.

2.2.5 Synthesis of 4″-deoxy sulfatide analog 2.49

Shortly before our initial immunological results were published, Erin Adams, a structural biologist, joined our collaboration. She was very interested in our findings with $\gamma\delta$ T cells (this is discussed in further detail in section 2.3). With her, our goal was to provide pure sulfatide so her group could solve the crystal structure of the $\gamma\delta$ TCR-sulfatide-CD1d complex. Along with this, we decided to make additional analogs of sulfatide so we could observe any relevant changes in the crystal structure in comparison to sulfatide. To date, only **2.49** has been synthesized, as well as varying degrees of progress towards a 6″-deoxy, and 2″-deoxy analosg. Schematic 2.8 outlines the synthesis of 4″-deoxy-3″-sulfo sulfatide. Starting from sulfatide's **2.14a**, the primary 6-hydroxyl was selectively protected quantitatively with benzoyl chloride to yield compound **2.52**. The next two steps followed classical Barton deoxygenation conditions to give us the 4-deoxy compound **2.54**. The rest of the synthesis follows the same sulfatide synthesis as previously described without any additional difficulties.



Reagents and conditions: a. bz-cl, py, 99%; b. phenyl chlorothionoformate, DMAP, DCM; c. $(Bu)_3Sn$-H, AIBN, benzene, 80 ^oC, 91% (2 steps); d. NBS, H_2O, Acetone; j. K_2CO_3, Cl_3CCN, DCM, 82%; k. 4Å M.S., ceramide, TMSOTf, DCM, 0 ^oC, 57%; l. hydrazine, AcOH, MeOH, THF, 85% ; m. SO_3-Py, py, 91%; o. NaOMe, THF, MeOH, 96%.

Scheme 2.8 Representative synthesis of 4″-deoxy sulfatide analog **2.49**

2.3 Results and Discussion

2.3.1 c24:1 sulfatide is a potent NKT cell antigen

All sulfatide variants were gauged on their ability to stimulate NKT cells by measuring their IL-2, IL-4, TNF-α and/or INF-γ production. Hela cells expressing WT CD1d molecules were pulsed with bovine sulfatide (obtained from Matreya, LLC; Pleasant Gap, PA) or synthetic sulfatide overnight at the indicated concentrations. Cells were washed and cocultured with sulfatide-

specific $\alpha\beta$ T cells (for sulfatide) overnight. All cytokine concentrations were measured in the supernatants. All representative data were acquired in triplicates.

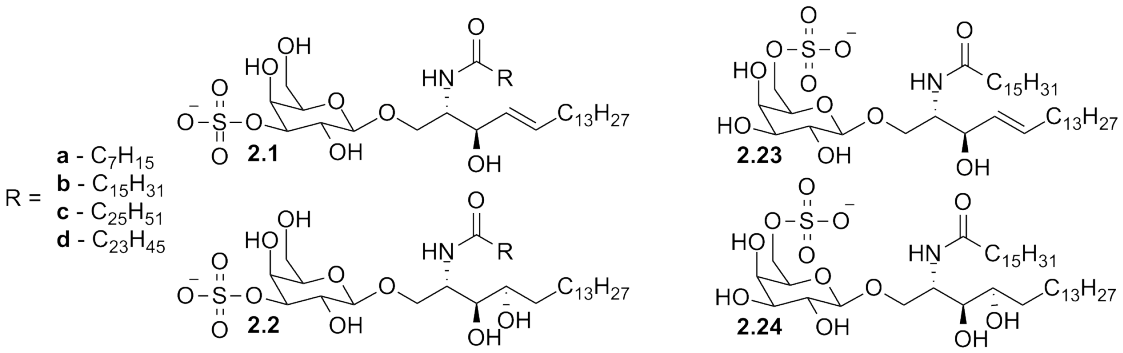


Figure 2.2 Initial sulfatide analog targets

Our first series of variants were synthesized in order to understand the structural activity relationships of the fatty acid chain length, the sphingosine scaffold, and the positioning of the sulfate. Preliminary immunological studies used an isolated sulfatide mixture from bovine brain as an indicator of a Type II NKT cell antigen. The approximate sulfatide isoform ratios (provided by the company) informed us that of the twenty sulfatide isoforms in the complex mixture, nearly 50% of the sulfatide was composed of the c24:1 and c24:0 isoforms. We decided to focus our efforts on synthesizing the ten variants represented in figure 2.2. Our first results (not shown) compared the immunostimulatory activity between the small chain sulfatides **2.1a** vs. **2.2a**. Not surprisingly, neither stimulated NKT cells, presumably due to their poor loading with CD1d. The lack of hydrophobic interactions in the F′ pocket, inhibited their loading capacity and consequently sulfatide's ability to be presented to the TCR. Figure 2.3 (A) represents the first relevant data gleaned from this series of variants. The results, as expected, indicted that cytokine release is dependent on lipid concentration. Compound **2.1b** had significantly higher IL-4 and INF-γ production than **2.2b**,

which suggests that the 4′-hydroxyl of the phytosphingosine chain makes an unfavorable interaction in comparison to the 3′-4′ double bond present in the sphingosine scaffold. The comparisons between **2.1c** vs **2.2c** and **2.1d** vs **2.2d** showed similar results (analysis carried out be the Bendelac lab).

To elucidate the stimulatory activities of the distinct sulfatide isoforms, we compared the stimulatory activity of **2.1a-d**. Figure 2.3(B), shows us a comparison of the stimulatory activity of **2.1b** to **2.1d**. This identified that the most potent NKT cell stimulator that was tested was the nervonic acid sulfatide. Also, **2.1d** is more potent than the bovine mixture, indicating that it is the immunodominant sufatide presented in the bovine mixture. This claim has been verified by the published work of Blomqvist *et al*. During our studies, they published a more in depth investigation on the stimulatory activity of the distinct sulfatide isoforms. They not only concluded that the most potent sulfatide isoform was the nervonic acid sulfatide, but they found that a lysed version of sulfatide, was a significantly more potent Type II $\alpha\beta$-NKT cell antigen.[56]

As discussed in section 2.2.2, we synthesized two 6′′-sulfo-sulfatides (16:0) to verify if the sulfate positioning had an effect on the stimulatory properties of sulfatide. Data in figure 2.3(C), demonstrated that the sulfate at the 3′′-hydroxyl postion is crucial to TCR stimulation, as **2.23** did not stimulate at all. Though not graphically presented, **2.24** also did not show any appreciable release of cytokines post-presentation.

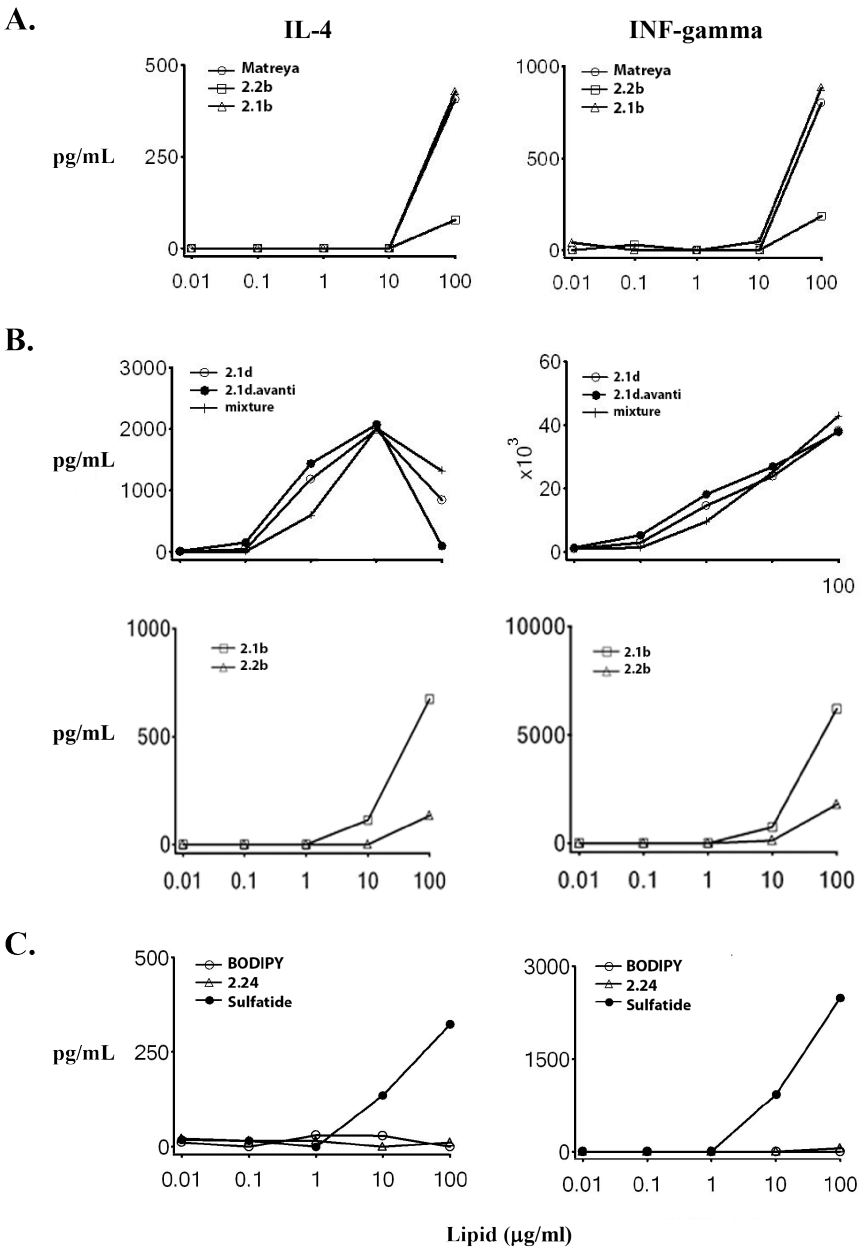


Figure 2.3 Stimulatory activities of the sulfatide isoforms. (A) Compound **2.1b** had significantly higher IL-4 and INF-γ production than **2.2b**, suggesting an unfavorable interaction occurs with the 4′-hydroxyl of the phytosphingosine chain. (B) Nervonic acid isoform of sulfatide stimulated NKT cells more than the other tested isoforms. (C) 6″-sulfo-sulfatide did not stimulate NKT cells, thus the sulfate at the 3″-hydroxyl postion is crucial to TCR stimulation.

2.3.2 N-acyl sulfatide analog is a comparable CD1d antigen and the sulfonate moiety is tolerated

In 2006, Liu. *et al*. presented a novel α-GalCer analog. They modified the 6″-hydroxyl position to an amide so that they could make a more soluble NKT cell antigen. This resulted in a variant that stimulated NKT cells at a much lower concentration, presumably due to the better solubility.[36] Though the sulfatide does not have solubility problems similar to α-GalCer, we synthesized a PBS57-like variant **2.33**. Upon completion, **2.33** was tested and compared to the stimulatory results of **2.1b**. As shown in figure 2.4, **2.33** gave comparably stimulatory activity to its sulfatide counterpart. Though we did not improve the activity, the maintained stimulatory effect supports the ability to modify the 6″-hydroxyl in sulfatide, similar to reported *i*NKT cell counterparts.[37]

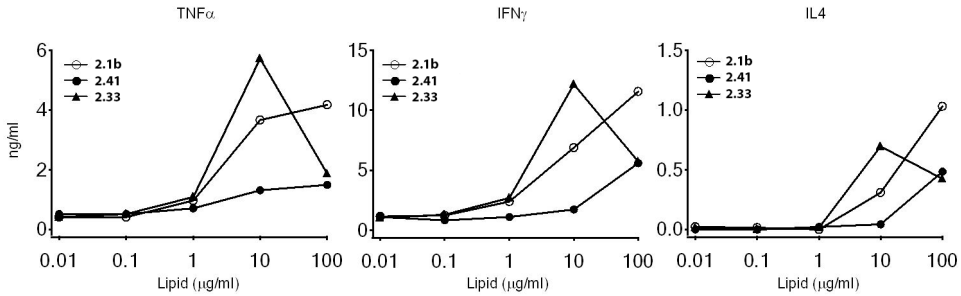


Figure 2.4 Dose response (TNF-α, INF-γ, IL-2) of NKT cells to sulfatide analogs **2.41, 2.33,** and **2.1b**(control).

Figure 2.4 also shows the stimulatory activity of sulfonate **2.41**. Though there is a substantial decrease in activity, we were excited to see that compound **2.41** retained the ability to stimulate $\alpha\beta$ T cells *in vitro*. *in vivo* testing of compound **2.41**, has not been completed. The full impact of having the sulfonate instead of the sulfate at the 3″ position. Since the sulfonate will not be removed

enzymatically, the degradation of this analog will be significantly slowed. Longer stimulation of NKT cells with this analog might result in a substantial inflammatory immune response.

2.3.3 Identification of CD1d-sulfatide-specific Vδ1+ T cells among fresh PBMCs in healthy individuals

We first assessed the lipid-loading conditions required to generate CD1d-sulfatide complexes. As shown by their altered migration on a native isoelectrofocusing (IEF) gel, recombinant human CD1d molecules were efficiently loaded after 1 h of incubation with a ten-fold molar excess of sulfatide in pH 7.2 HEPES-buffered saline, without requirement for lipid transfer protein (2.5(A)). We used these loading conditions prior to adding streptavidin in order to generate sulfatide-loaded human CD1d tetramers. These tetramers were used in flow cytometry experiments to obtain an unbiased representation of the existing population of sulfatide-specific CD1d-restricted T cells in healthy individuals. The frequency of CD1d-sulfatide+ cells among CD3+ T cells was under 10^{-4} in the PBMCs of all individuals examined (2.5(B)). Because it is typically difficult to distinguish genuine staining from background at this low frequency, 10^8 PBMCs were MACS-enriched using CD1d-sulfatide tetramers, a procedure that allowed the recovery of 10^5 cells, of which 0.6-5.7% were CD1d-sulfatide+ CD3+ T cells in different individuals examined (Figure 2.5(B-C)). Surprisingly, these cells rarely expressed the $\alpha\beta$ TCR, but were mostly $\gamma\delta$ T cells that used the Vδ1 chain. On average, in all seven individuals examined, 83% of the tetramer-positive cells (range 48-100%) were $\gamma\delta$ T cells that expressed the same Vδ1 variable gene segment (Fig. 1C). In another individual (no. 4) for which Vδ1 staining was not available, RT-PCR detected Vδ1 TCR chain expression

among 200 sorted tetramer+ cells. Most circulating $\gamma\delta$ T cells in humans express the Vγ9-Vδ2 TCR, representing 3% of adult blood T cells on average, whereas Vδ1 T cells represent only 0.8% of T cells.[57] As shown in Figure 2.5(D), the CD1d-sulfatide-specific T cells represented less than 1% of these circulating Vδ1 T cells. Thus, the frequency of CD1d-sulfatide-specific Vδ1+ T cells was generally very low, well under 10^{-4} in all the healthy subjects examined.[46]

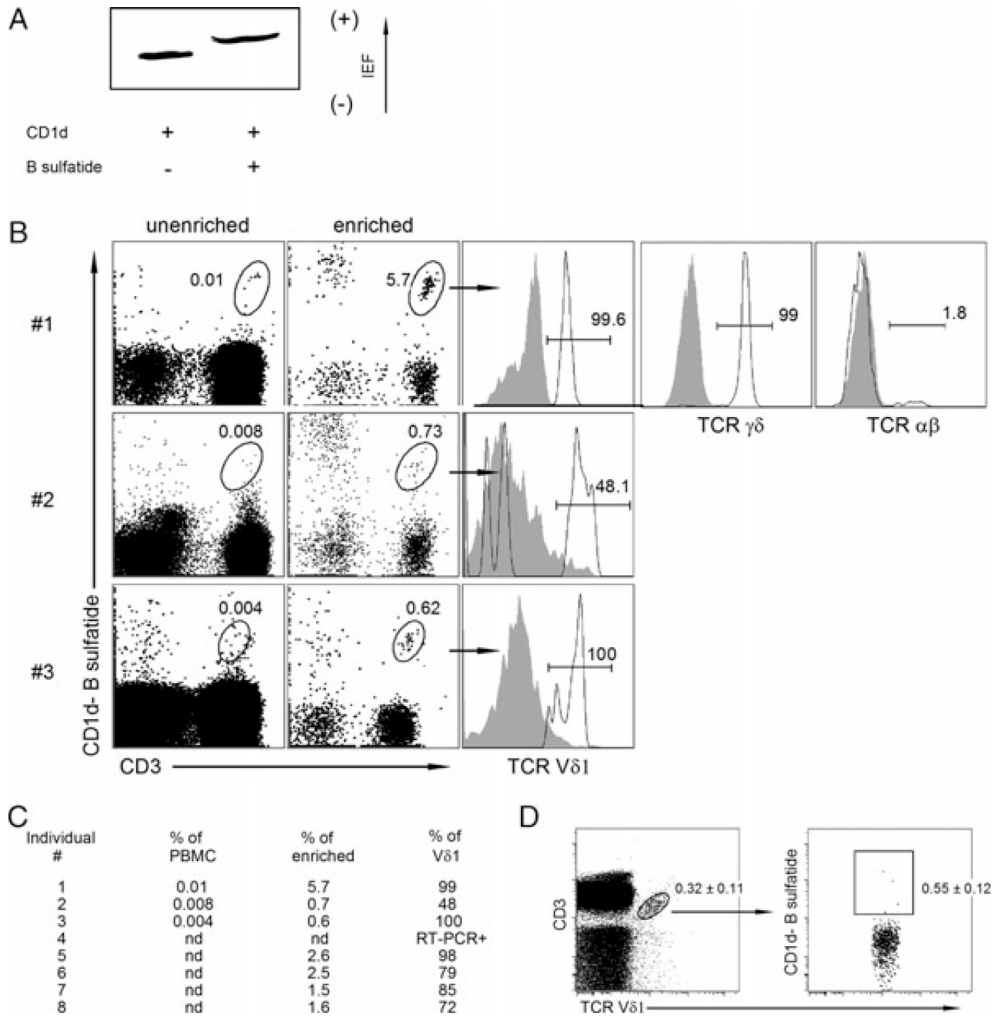


Figure 2.5 CD1d-sulfatide tetramer staining and MACS enrichment of healthy PBMCs. (A) Loading of human CD1d molecules with purified bovine sulfatide demonstrated by native isoelectrofocusing (IEF). Data are representative of two independent experiments. (B) CD3/tetramer staining before and after tetramer MACS enrichment in PBMCs from three healthy subjects. Starting population included 1 x 10^8 PBMCs, with 1 x 10^5 cells typically recovered after MACS enrichment. Gated CD3+ tetramer+ cells were further characterized for TCR$\alpha\beta$, TCR$\gamma\delta$, and Vδ1 expression. Numbers indicate percentages in the gated population. Data are representative of one to five experiments per individual. (C) Frequency of CD1d sulfatide tetramer+ CD3+ PBMCs in eight healthy individuals; nd, not determined; for individual no. 4, presence of Vδ1 T cells was inferred from the amplification of a Vδ1-Jδ1 sequence by RT-PCR after CD1d-sulfatide tetramer MACS enrichment followed by FACS sorting of 200 tetramer+ cells. (D) Frequency of CD1d-sulfatide tetramer+ cells among gated CD3+ Vδ1+ cells PBMCs. Values indicate average percentage +/- SEM of six individuals examined.[46]

2.3.4 Antigen-specific recognition of CD1d molecules by Vδ1+ $\gamma\delta$ T cells Clones

DP10.7 and AB18.1, clonally expanded Vδ1+ $\gamma\delta$ T cells derived from the blood of healthy individuals, demonstrated specificity for sulfatide apparent from positive tetramer staining by purified bovine sulfatide as well as by our synthetic sulfatide c26:0. This study showed no staining by CD1d-αGalCer or unloaded tetramers. Furthermore, tetramer staining was specifically inhibited by preincubation with anti-TCR $\gamma\delta$ but not by isotype control antibody. Tetramer staining did not decay significantly over an extended period of 6 h both at ice cold temperature and at 37°C, suggesting substantial affinity of the Vδ1 TCR for the CD1d-sulfatide complex. Interestingly, CD1d-α-GalCer tetramers exhibited even lower binding than unloaded CD1d, further supporting the antigen-specific recognition of CD1d-lipid complexes.[46]

The question of the antigenic specificity of $\gamma\delta$ T cells, in particular whether putative antigens are directly recognized or are presented by MHC-like molecules remains largely unresolved.[58] With the exception of the MHC class I-like ligand T10, whose direct recognition in the absence of associated peptide was demonstrated at the biochemical and structural level, other $\gamma\delta$ T-cell antigens have not been fully characterized.[59] Because of the cellular readout of the tetramer-staining experiments, the apparent interaction between the Vδ1 TCRs, CD1d, and sulfatide might be indirect, involving some other cellular product. We generated recombinant DP10.7 and AB18.1 TCRs to probe direct binding with CD1d sulfatide complexes in a cell-free system. After incubation for 15 min with equimolar amounts of CD1d-sulfatide complexes, both TCRs demonstrated di-

rect binding as shown by the formation of complexes in native gel electrophoresis. Importantly, both TCRs required sulfatide loading for binding CD1d, providing direct proof of antigen-specific recognition in a cell-free system.[46]

2.3.5 Progress towards solving the crystal structure of the $\gamma\delta$ TCR-sulfatide-CD1d complex

After the immunological results were obtained (outlined in sections 2.3.3 and 2.3.4); Dr. Erin Adams and her group focused their efforts on understanding the $\gamma\delta$ TCR-sulfatide-CD1d molecular interactions. Their main goal was to solve the TCR-sulfatide-CD1d crystal structure. They first spent a year trying to get good crystals to form using commercial sulfatide with little luck. A key problem with commercial sulfatide is impurity and variablity among batches. We were able to provide clean and consistent sulfatidefor the study.

We then sent out two batches of sulfatide (C24:1) to them. These batches of sulfatide loaded CD1d better than Matreya's samples and ultimately facilitated the crystallography process. $\gamma\delta$ TCR-CD1d-sulfatide crystals have been obtained and recently the crystal structure has been solved.

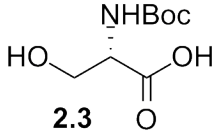
We have also utilized isoelectric focusing gels to compare the binding of different synthetic sulfatides (**2.1b, 2.1d, 2.49, 3.1**) to CD1d. As expected, the 4-deoxy sulfatide (**2.49**) binds just as well as **2.1d**. Finally, we used surface plasmon resonance (SPR) to quantify the effect of bound lipid on CD1d recognition with $\gamma\delta$ T cells. Using β-GalCer as a control, we have convincing data that sulfatide recognizes both DP10.7 and AB18.1 $\gamma\delta$ TCR clones.

2.4 Concluding remarks

Our collaboration underscores the value of utilizing synthetic chemistry to further the understanding of NKT cell biology. As further evidenced by these results, our group has provided cleaner and more consistent glycolipids than the commercial counterparts. This has been essential for the molecular biologists in progressing the structural $\gamma\delta$ T cell field. Also, we have been able to synthesize multiple analogs that broadened the necessary structural requirements for sulfatide.[46]

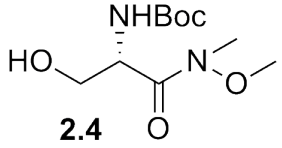
Our immunological findings highlight the value of unbiased studies of fresh antigen-specific T cells using MHC or CD1 tetramers in humans over in vitro expanded cell lines. While the origin and function of the Vδ1+ CD1d-sulfatide reactive T cells remain to be elucidated, especially in the context of demyelinating processes, the current findings, together with reported examples of blood- or gut-derived Vδ1 T-cell clones with specificity for CD1 molecules,[60,61] support the emerging notion that a large fraction of this enigmatic $\gamma\delta$ T-cell population may be specialized for recognition of lipids presented by CD1 family members. In addition, our biochemical studies constitute the first direct demonstration of antigen presentation by MHC-like molecules to human $\gamma\delta$ T cells, a longstanding hypothesis which has received surprisingly little support over the years.[46]

2.5 Experimental Procedures



2.3

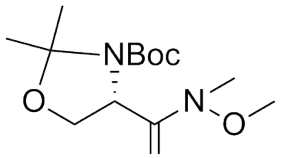
Preparation of 2.3: Commercially available D-serine (40.0g, 381 mmol) was dissolved in 1 M NaOH (700 mL) and dioxane (350 mL). This solution was cooled to 0°C (ice bath). After cooling was achieved, boc anhydride (100g, 419 mmol) was dissolved in the remaining dioxane (350 mL) and added dropwise to the solution. The reaction slowly warmed to room temperature overnight. The next day the dioxane was evaporated off via rotary evaporator. The resultant NaOH solution was washed with diethyl ether (3 x 250 mL). The aqueous layer was saved and acidified by introduction of concentrated hydrochloric acid (12.1 M HCl) to pH 2.0. After acidification, the solution was once again washed with diethyl ether (3 x 250 mL). The organic layer was dried over sodium sulfate ($NaSO_4$), concentrated via rotary evaporator, and placed on a high vacuum system in preparation for the second step of the reaction. This compound does not stain well on TLC or show up well in mass spec, so monitoring this reaction is very difficult to accomplish.



2.4

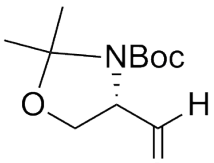
Preparation of 2.4: The dried pale-yellow syrup was dissolved in DCM (500 mL) and cooled to -10°C (acetone:ice bath). 4-methyl morpholine (83.8 mL, 762 mmol) and N,O-dimethyl hydroxyamine-HCl (37.2 g, 381 mmol) were added to the round bottom flask and stirred for ten minutes. Over the time period of fifteen minutes, 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDCI)(73.0 g, 381 mmol) was added portionwise. This reaction stirred for another 30 min after which time the reaction was removed from the ice bath and maintained at room temperature for two more hours. Ice cold 1 M HCl was added to quench the reaction and extract and

subsequently discard the excess EDCI. The organic layer was rebasified with a saturated sodium bicarbonate ($NaHCO_3$) wash, dried over $NaSO_4$, concentrated, and dried with a high vacuum pump in preparation for the next step. The following compound was synthesized via the experimental procedure outlined from Garner et al.[54] After isolation, the compound was affirmed via the comparison of the published NMR spectroscopic data.



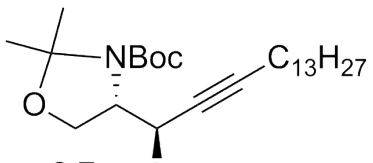
2.5

Preparation of 2.5: The solid pale yellow material (**2.4**), dimethoxy propane (39.7 g, 381 mmol), and boron trifluoride diethyl etherate (7 mL, 57.2 mmol) were dissolved in acetone and stirred at room temperature overnight. The reaction was quenched with triethylamine. After removal of the acetone (600 mL), the resultant residue was loaded onto a silica gel column and purified by a 10%, 40%, and 60% (EtOAc:Hexane) eluent method. A pale yellow syrup was isolated (60.0 g, 55% yield, over three steps). This compound does not stain well on TLC or show up well in mass spec, so monitoring this reaction is very difficult to accomplish. The following compound was synthesized via the experimental procedure outlined from Garner et al.[54] After isolation, the compound was affirmed via the comparison of the published NMR spectroscopic data.



2.6

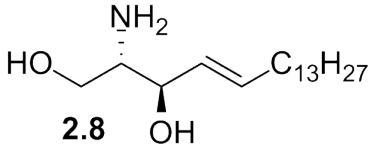
Preparation of 2.6: All glassware was flame dried under a vacuum to evaporate off any microscopic water that had collected in the flasks from moisture in the air. Purified amide **2.5** (28.0 g, 97.3 mmol) was washed with toluene three times in order to azeotrope water from the product. This was dissolved in anhydrous THF (300 mL) and cooled to 0°C. In a separate RBF, LAH (3.70 g, 97.3 mmol) was dissolved in anhydrous THF (100 mL) and also cooled to 0°C. The dissolved LAH was transferred to the RBF via cannula dropwise over a few minutes. The reaction temperature was maintained for two hours and monitored by thin layer chromatography (TLC). After starting material was consumed, indicated by the emergence of a dark yellow spot on TLC, the reaction was stopped by the slow addition of saturated ammonium chloride. The excess THF was evaporated off and the salty solution was washed with diethyl ether (3 x 250 mL). The diethyl ether washes were dried over $NaSO_4$, concentrated, and subjected to flash chromatography (10% EtOAc/Hexane column). The resulting product was a viscous clear syrup (41.0 g, 93%). The following compound was synthesized via the experimental procedure outlined from Garner et al.[54] After isolation, the compound was affirmed via the comparison of the published NMR spectroscopic data.



2.7

Preparation of 2.7: All glassware was flame dried under a vacuum to evaporate off any microscopic water that had collected in the flasks from moisture in the air. 1-pentadecyne (32.6 mL, 124 mmol) was diluted with anhydrous THF (800 mL) and cooled to -23°C by the piece-wise

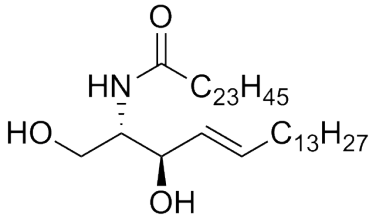
addition of dry ice into a bath of acetonitrile. N-butyl lithium (43 mL, 108 mmol) was added dropwise and the cloudy mixture was stirred at -23°C for one hour. To the solution was added dropwise via cannula a solution of compound **2.6** (19.0 g, 82.9 mmol) in anhydrous THF (50 mL). After one more hour the reaction was complete and subsequently quenched with saturated ammonium chloride. The excess THF was evaporated off and the salty solution was washed with ethyl acetate (3 x 200 mL). The ethyl acetate washes were dried over $NaSO_4$, concentrated, and subjected to flash chromatography (5% EtOAc/Hexane column). The resulting product was a clear yellow syrup (15.0 g, 41%). The following compound was synthesized via the experimental procedure outlined from Garner et al.[54] After isolation, the compound was affirmed via the comparison of the published NMR spectroscopic data.



2.8

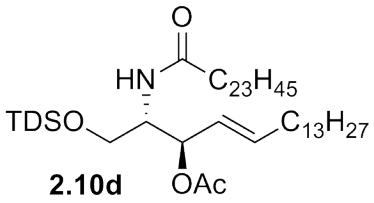
Preparation of 2.8: All glassware was flame dried under a vacuum to evaporate off any microscopic water that had collected in the flasks from moisture in the air. Ethyl amine (100 mL) was condensed in a two-neck flame-dried RBF that was cooled to -78°C (acetone:dry ice bath). Hexane-washed lithium (1.70 g, 246 mmol) was added to the ethyl amine and stirred for half an hour. After the solution turned a dark blue color, compound **2.7** (7.16 g, 16.4 mmol) was dissolved in anhydrous THF (150 mL), cooled to -78°C, and transferred via cannula into the dissolving metal solution. The reaction was maintained at -78°C for four hours. The reaction was quenched with solid ammonium chloride and raised to room temperature (at which point the solution turned

a creamy yellow color). The quenched reaction stirred overnight in a hood to allow any excess ethyl amine to evaporate. The next day the excess THF was removed via rotary evaporator and the slurry was dissolved in water and extracted with diethyl ether (4 x 300 mL). This ether layer was dried over $NaSO_4$, impregnated with silica gel, and loaded dry onto a column. A very polar eluent method (0.5:5:95, 1:10:90, 1:15:85 ammonium hydroxide:MeOH:DCM) was employed to purify the compound (4.30 g, 88%). HRMS (ESI) calcd for $C_{18}H_{37}NO_2$ [M+H]+: 299.2824, found: 300.3042. The following compound was synthesized via the experimental procedure outlined from Garner et al.[54] After isolation, the compound was affirmed via the comparison of the published NMR spectroscopic data. Due to differential shifts in the amine 1H, and surrounding 1H chemical shifts, we bought isolated sphingosine, peracylated it, and compared it to some peracylated synthetic sphingosine. This comparision affirmed that the major product was the desired diasteriomer.



Preparation of 2.9d: EDCI (1.06 g, 5.51 mmol), hydroxybenzotriazol (HOBt)(745 mg, 5.51 mmol), and nervonic acid (2.02 g, 5.51 mmol) were combined in a RBF and diluted in anhydrous THF (75 mL). The reactants did not dissolve well so the RBF was heated with a heat gun for a few seconds to allow more dissolution of the compounds. This mixture was stirred for one hour at room temperature. Compound **2.8** (1.50 g, 5.01 mmol) was dissolved in anhydrous THF (25

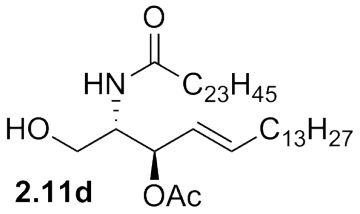
mL) and added to the reaction mixture. The reaction was left at room temperature overnight. The next morning the THF was evaporated via rotary evaporator, the resultant yellow solid was dissolved in water, washed with DCM (3 x 100 mL), and dried over $NaSO_4$. The organic layer was concentrated down and subjected to flash chromatography (50%, 70% EtOAc/Hexane eluent, then 5%, 10% MeOH/DCM). HRMS (ESI) calcd for $C_{42}H_{81}NO_3$ [M+H]+: 647.6216, found: 648.6289.



2.10d

Preparation of 2.10d: Partially purified compound **2.9d** (Theoretical 5.01 mmol) was dissolved in a minimal amount of anhydrous pyridine (10 mL). Chloro(dimethyl)thexylsilane (TDS-Cl) (0.983 mL, 5.01 mmol) was added to the reaction flask. The reaction was monitored by mass spec for formation of the monosilated product. Every hour, more TDS-Cl (0.983 mL, 5.01 mmol) was added as needed. Once the starting material was completely consumed or presence of the disilated product was found, excess acetic anhydride (1.89 mL, 20.0 mmol) was added. After positive confirmation of completion of the reaction, the pyridine was evaporated via a high vacuum rotary evaporator. The dark syrup was dissolved in water and washed with DCM. The organic layer was concentrated down and subjected to flash chromatography (5% EtOAc/Hexane). The column yielded a white powder compound (2.80 g, 71% over three steps). 1H NMR (300 MHz, Chloroform-d) δ 5.75 (m, 1H), 5.64 (d, J = 9.4 Hz, 1H), 5.45 - 5.21 (m, 3H), 4.21 (m, 1H), 3.73 (dd, J = 10.2, 2.8 Hz, 1H), 3.56 (dd, J = 10.2, 4.1 Hz, 1H), 2.14 (t, J = 7.6 Hz, 2H), 2.02 (q, J = 6.2, 5.2 Hz, 5H), 1.60 (m, 4H), 1.41 - 1.13 (m, 58H), 0.88 (m, 18H), 0.08 (s, 3H), 0.00 (s, 3H). HRMS

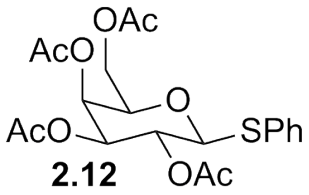
(ESI) calcd for $C_{52}H_{101}NO_4Si$ [M+H]+: 831.7500, found: 832.7614.



2.11d

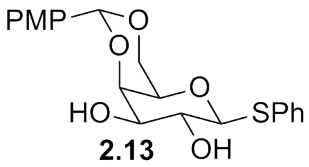
Preparation of 2.11d: Compound **2.10d** (245 mg, 0.295 mmol) was dissolved in DCM (5 mL) and acetonitrile (ACN)(30 mL) and placed in a plastic centrifuge tube (with the appropriate stir bar). To this tube was added 1 mL of hydrofluoric acid (48%). The reaction was monitored by TLC for formation of product **2.11d**. Every thirty minutes, more hydrofluoric acid was added as needed. Once the starting material was completely consumed, the reaction was quickly and carefully quenched on a bed of solid $NaHCO_3$ in a separate plastic container (equipped with the appropriate stir bar). Water, DCM, and solid $NaHCO_3$ were slowly added until a neutral or basic pH was reached. The water/DCM workup was filtered to remove excess undissolved $NaHCO_3$. The water was washed with DCM (6 x 100 mL) and the organic layer was dried over $NaSO_4$, concentrated (water bath was 30°C or below to minimize acetal migration), and subjected to flash chromatography (5% EtOAc/Hexane). The column yielded a white powder compound (210 mg, 99%). Note: this reaction if done slowly, left overnight, heated up, or purified slowly can and will result in migration of the 2-acetyl group to the primary alcohol. It is essential that the chemist quench this reaction immediately after completion. 1H NMR (500 MHz, Chloroform-d) δ 5.94 (d, J = 8.4 Hz, 1H), 5.77 (dt, J = 14.2, 6.7 Hz, 2H), 5.50 - 5.43 (m, 1H), 5.35 (t, J = 4.8 Hz, 2H), 5.28 (t, J = 7.4 Hz, 1H), 4.16 - 4.09 (m, 1H), 3.65 (brs, 2H), 2.74 (brs, 1H), 2.17 (td, J = 7.4, 4.2 Hz,

2H), 2.11 (s, 3H), 2.02 (p, J = 7.9, 6.9 Hz, 6H), 1.61 (q, J = 7.2 Hz, 2H), 1.40 - 1.16 (m, 56H), 0.88 (t, J = 6.8 Hz, 6H). HRMS (ESI) calcd for $C_{44}H_{83}NO_4$ [M+H]+: 689.6322, found: 690.6459.



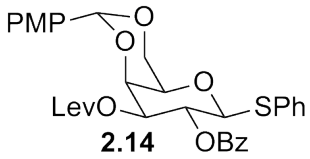
2.12

Preparation of 2.12: Pentaacetate-d-galactose (25.0 g, 64 mmol) was dissolved in DCM (200 mL) and placed in an ice bath. Phenyl thiol (13 mL, 130 mmol) was added and the reaction mixture and was stirred for fifteen minutes, followed by dropwise addition of boron trifluoride diethyl etherate (9.5 mL, 77 mmol). The temperature was maintained for three hours. The reaction vessel warmed to room temperature and stirred overnight. After quenching the reaction with triethylamine, the solvent was evaporated and bubbled through bleach to remove and oxidize any excess phenyl thiol. The resulting yellow syrup was washed with water and extracted with DCM (3 x 150 mL). The organic layer was dried over $NaSO_4$, concentrated via rotary evaporator, and the residual syrup was purified on SiO_2 (40% EtOAc:Hexane). A clear brown syrup was recovered (26.0 g, 92%) 1H NMR (500 MHz, Chloroform-d) δ 7.55 - 7.49 (m, 2H), 7.35 - 7.29 (m, 3H), 5.42 (dd, J = 3.3, 1.1 Hz, 1H), 5.25 (t, J = 10.0 Hz, 1H), 5.05 (dd, J = 10.0, 3.3 Hz, 1H), 4.72 (d, J = 10.0 Hz, 1H), 4.20 (dd, J = 11.3, 7.0 Hz, 1H), 4.12 (dd, J = 11.4, 6.2 Hz, 1H), 3.98 - 3.92 (m, 1H), 2.11 (d, J = 12.3 Hz, 6H), 2.05 (s, 3H), 1.98 (s, 3H); 13C NMR (126 MHz, CDCl3) δ 170.37, 170.18, 170.05, 169.43, 132.54, 132.45, 128.89, 128.15, 86.63, 77.26, 77.01, 76.76, 74.40, 71.99, 67.23, 67.19, 61.62, 20.86, 20.68, 20.65, 20.60.



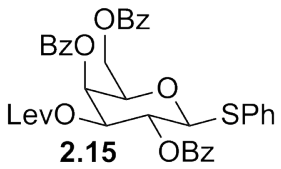
2.13

Preparation of 2.13: Compound **2.12** (26.0 g, 59.1 mmol) was dissolved in methanol (300 mL), followed by the addition of 1 M sodium methoxide (NaOMe)(10 mL). Sixteen hours later the reaction was quenched with an excessive amount of Amberlite (highly acidic beads) and stirred for thirty minutes. The reaction was filtered, washed with MeOH, and concentrated in a 500 mL RBF. The clear red syrup was further dissolved in dry DMF (200 mL) and the pH of the solution was lowered to 2.5 by the addition of tosylic acid. The reaction was stirred for two hours on a rotary evaporator (50°C) after adding anisaldehyde dimethyl acetal (13.0 mL, 76.3 mmol). The mixture was quenched with triethylamine and the DMF was pulled off with a high vacuum rotary evaporator. This resultant syrup was washed with water and extracted with EtOAc (3 x 150 mL). The concentrated and dried ($NaSO_4$) organic layer was subjected to flash chromatography (50%, 70% EtOAc:Hexane, 10% MeOH). The resulting product was a white powder (13,890 mg, 60% over two steps).1H NMR (500 MHz, Chloroform-d) δ 7.72 - 7.66 (m, 2H), 7.36 - 7.24 (m, 6H), 6.91 - 6.85 (m, 2H), 5.47 (s, 1H), 4.54 - 4.48 (m, 1H), 4.37 (dd, J = 12.5, 1.6 Hz, 1H), 4.23 - 4.18 (m, 1H), 4.02 (dd, J = 12.5, 1.8 Hz, 1H), 3.72 - 3.66 (m, 2H), 3.55 (q, J = 1.5 Hz, 1H). 13C NMR (126 MHz, Chloroform-d) δ 160.34, 133.79, 130.70, 130.14, 128.95, 128.23, 127.84, 113.58, 101.31, 87.01, 77.26, 77.21, 77.01, 76.76, 75.31, 73.83, 70.08, 69.27, 68.88, 55.35. HRMS (ESI) calcd for $C_{20}H_{22}O_6S$ [M+H]+: 390.1137, found: 1391.1197.



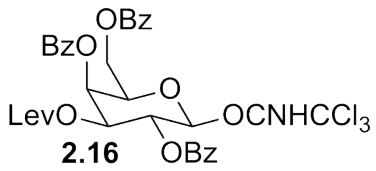
2.14

Preparation of 2.14: Compound **2.13** (6.50 g, 16.7 mmol) was dissolved in dry DCM (200 mL) and the solution was cooled to 0°C. Levulinic acid (2.04 mL, 20.0 mmol) and dimethylaminopyridine (DMAP)(937 mg, 8.35 mmol) were added and the reaction was allowed to stir for fifteen minutes, followed by the inclusion of EDCI (4.80 g, 25.1 mmol). The reaction was carefully monitored for completion or formation of dilevulinic protected byproduct by mass spectrometry (M.S.) and TLC. When the starting material was consumed, excess pyridine (30 mL) and benzoyl chloride (BzCl)(9.68 L, 83.5 mmol) were added to the reaction. The next morning the pyridine was pulled off via high vacuum rotary evaporation and the brown syrup was was washed with water and extracted with DCM (3 x 150mL). The organic layer was dried over $NaSO_4$, concentrated via rotary evaporator, and the residual syrup was purified on SiO_2 (30%, 40% EtOAc:Hexane). A white powder was recovered (5.61 g, 78%). 1H NMR (500 MHz, Chloroform-d) δ 8.05 - 7.99 (m, 2H), 7.62 - 7.55 (m, 3H), 7.50 - 7.42 (m, 2H), 7.38 - 7.22 (m, 6H), 6.92 - 6.86 (m, 2H), 5.57 (d, J = 9.9 Hz, 1H), 5.45 (s, 1H), 5.18 (dd, J = 10.0, 3.4 Hz, 1H), 4.86 (d, J = 9.8 Hz, 1H), 4.42 - 4.35 (m, 2H), 4.03 (dd, J = 12.3, 1.6 Hz, 1H), 3.63 (q, J = 1.4 Hz, 1H), 2.62 - 2.35 (m, 4H); 13C NMR (126 MHz, Chloroform-d) δ 206.10, 172.02, 164.90, 160.20, 133.85, 133.26, 131.23, 130.20, 129.86, 129.66, 128.89, 128.76, 128.45, 128.17, 127.91, 113.48, 101.03, 85.33, 78.56, 77.27, 77.02, 76.76, 73.55, 73.25, 69.84, 69.05, 67.29, 67.10, 55.35, 37.77, 29.44, 28.26. HRMS (ESI) calcd for $C_{32}H_{32}O_9S$ [M+H]+: 592.1767, found: 593.1840.



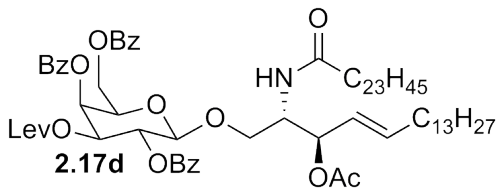
2.15

Preparation of 2.15: A solution of **2.14** (1.70 g, 2.94 mmol), water (20 mL), and glacial acetic acid (80 mL) was stirred overnight at room temperature. Upon completion the water and acetic acid were pulled off via high vacuum rotary evaporation. The light yellow slurry was quenched with saturated $NaHCO_3$ and the product was extracted with EtOAc (3 x 50 mL). The organic phase was dried over $NaSO_4$, concentrated, and the yellow syrup was loaded onto a bed of SiO_2 and was partially purified with a 2.5%, 5% MeOH: DCM eluent wash. This stable diol intermediate was dissolved in pyridine (40 mL). Benzoyl chloride (4.10 mL, 29.38 mmol) was added and the reaction mixture was stirred for sixteen hours. The pyridine was removed via high vacuum rotary evaporator. The dark brown residue was dissolved in water and washed with DCM (3 x 50 mL). The resultant syrup was loaded onto a silica gel column and the product was eluted out in 30% EtOAc:Hexanes. A clear light brown syrup was collected (1.60 g, 80%). 1H NMR (500 MHz, Chloroform-d) δ 8.06 - 7.94 (m, 5H), 7.66 - 7.53 (m, 4H), 7.51 - 7.40 (m, 5H), 7.35 - 7.29 (m, 2H), 7.28 - 7.20 (m, 4H), 5.84 (dd, J = 3.3, 1.1 Hz, 1H), 5.58 (t, J = 9.9 Hz, 1H), 5.38 (dd, J = 9.9, 3.3 Hz, 1H), 4.96 (d, J = 10.0 Hz, 1H), 4.60 (dd, J = 11.5, 7.1 Hz, 1H), 4.40 (dd, J = 11.5, 5.5 Hz, 1H), 4.33 - 4.26 (m, 1H), 2.59 - 2.27 (m, 4H). 13C NMR (126 MHz, Chloroform-d) δ 206.10, 172.02, 164.90, 160.20, 133.85, 133.26, 131.23, 130.20, 129.86, 129.66, 128.76, 128.45, 128.17, 127.91, 113.48, 101.03, 85.33, 77.27, 77.02, 76.76, 73.55, 73.25, 69.84, 69.05, 67.29, 67.10, 55.35, 37.77, 29.44, 28.26. HRMS (ESI) calcd for $C_{38}H_{34}O_{10}S$ [M+NH4]+: 682.1873, found: 700.2211.



2.16

Preparation of 2.16: Fully protected galactose **2.15** (600 mg, 0.879 mmol) was dissolved in an acetone/water solution (20mL/3mL), followed by the portion wise addition of NBS (469 mg, 2.64 mmol). Subsequent equivalents of NBS were added until the reaction was completed (monitored by TLC). Upon completion, the reaction was quenched with solid $NaHCO_3$, and the acetone was evaporated off. The remaining mixture was dissolved in water, washed with DCM (3 x 50 mL), dried with $NaSO_4$, concentrated, and subjected to flash chromatography (30% EtOAc:Hexanes). The white powder (440 mg, 0.746 mmol) was dissolved in DCM (25 mL). Solid potassium carbonate (1320 mg, 9.55 mmol) was added, followed by the addition of excess trichloroacetonitrile (1.12 mL, 11.2 mmol). Once setup, the reaction was stirred for sixteen hours at room temperature. In the morning, the potassium carbonate was filtered and washed with EtOAc (100 mL). The filtrate was dried off at or below 30°C en vacuo. This pale yellow solid was loaded onto a bed of silica gel and was eluted with 25% EtOAc/Hexane. The two anomeric isomers were collected, combined, and concentrated at or below 30°C (424 mg, 89% from two steps). HRMS (ESI) calcd for $C_{34}H_{30}Cl_3NO_{11}$ [M+H]+: 733.0884, found: 573.2294 (fragment).

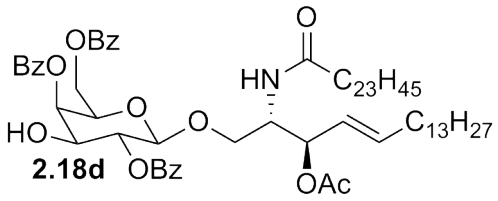


2.17d

Preparation of 2.17d: With the donor synthesized, each sulfatide product was made by

the coupling of the appropriate ceramide followed by the deprotection of the levulinic protecting group, installation of the sulfate, and finally the global removal of the esters to make the appropriate sulfatide. Due to the shared synthetic route, one synthetic series will be described followed by the analytical data of the final sulfatides. Donor **2.16** (150 mg, 0.205 mmol) was stirred for one hour at room temperature in the presence of ceramide **2.10** (156 mg, 0.226 mmol), 4 angstrom molecular sieves (400 mg), and dry DCM (5 mL). The vessel was cooled to -10°C (acetone/ice bath), followed by the dropwise addition of trimethylsilyltriflate (9 μL, 51.3 μmol). The reaction temperature was maintained for two hours then quenched by the dropwise addition of TEA. The molecular sieves were filtered and washed with EtOAc by means of a plug silica gel column. The filtrate was dried over $NaSO_4$, concentrated, and subsequently loaded onto a silica gel column. Purification was achieved with a 20%, 30% EtOAc/Hexane eluent system (124 mg, 48%). 1H NMR (500 MHz, Chloroform-d) δ 8.17 (d, J = 7.7 Hz, 2H), 8.00 (dd, J = 7.8, 4.5 Hz, 3H), 7.64 (d, J = 7.5 Hz, 1H), 7.55 (m, J = 15.6, 7.4 Hz, 4H), 7.44 (q, J = 7.5 Hz, 5H), 5.83 (m, 2H), 5.73 (s, 1H), 5.60 (d, J = 9.2 Hz, 1H), 5.57 - 5.51 (s, 1H), 5.42 - 5.27 (m, 5H), 4.69 (d, J = 7.8 Hz, 1H), 4.60 (d, J = 11.3, 1H), 4.37 (dd, J = 11.3, 6.2 Hz, 1H), 4.31 (m, J = 10.4, 7.1, 3.5 Hz, 1H), 4.24 (m, 1H), 4.10 (m, 1H), 3.57 (m, 2H), 2.68 - 2.30 (m, 6H), 2.11 - 1.84 (m, 12H), 1.76 (t, J = 7.7 Hz, 2H), 1.28 (m, J = 20.6, 7.4 Hz, 50H), 0.88 (t, J = 6.8 Hz, 6H). 13C NMR (126 MHz, Chloroform-d) δ 205.86, 205.69, 205.67, 172.61, 172.59, 171.78, 171.73, 171.67, 169.70, 169.65, 166.01, 165.99, 165.97, 165.73, 165.65, 165.56, 165.36, 137.46, 137.03, 133.74, 133.58, 133.51, 133.43, 133.35, 133.17, 130.07, 130.03, 130.01, 130.00, 129.94, 129.90, 129.88, 129.85, 129.79, 129.77, 129.74, 129.50, 129.31, 129.28, 129.20, 128.99, 128.94, 128.77, 128.70, 128.65, 128.61, 128.56, 128.53, 128.50,

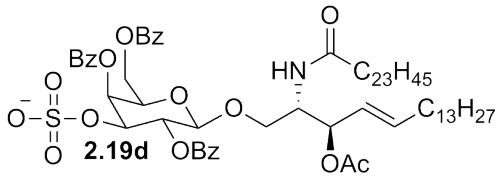
128.49, 128.46, 128.43, 128.41, 128.39, 128.37, 124.86, 124.72, 101.73, 101.13, 90.93, 77.30, 77.25, 77.05, 76.79, 73.59, 71.33, 71.27, 70.99, 69.86, 69.68, 69.40, 69.15, 67.84, 67.70, 67.37, 66.54, 62.34, 62.03, 60.38, 50.30, 50.18, 37.80, 37.71, 37.70, 36.82, 36.46, 32.32, 32.14, 31.93, 31.91, 29.83, 29.81, 29.78, 29.73, 29.71, 29.68, 29.66, 29.62, 29.59, 29.54, 29.53, 29.51, 29.49, 29.47, 29.45, 29.43, 29.41, 29.39, 29.38, 29.36, 29.32, 29.28, 29.24, 29.21, 29.02, 28.83, 27.95, 27.87, 27.25, 27.23, 27.21, 25.65, 25.49, 22.70, 22.69, 21.10, 21.04, 14.20, 14.18, 14.14, 14.13, 14.12. HRMS (ESI) calcd for $C_{76}H_{111}NO_{14}S$ [M+H]+: 1261.8005, found: 391.1197.



2.18d

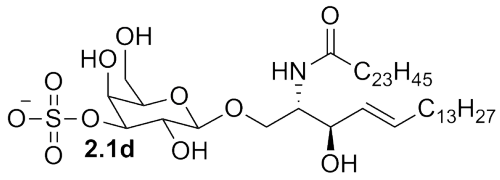
Preparation of 2.18d: A 1 M solution of hydrazine was freshly prepared in dry tetrahydrafuran (THF). A portion (77 μL, 77 μmol) of this was stirred with glacial acetic acid (102 μL, 102 μmol) for five minutes. This mixture was added to a RBF that contained compound **2.17d** and dry THF:Methanol (10:1, 11 mL). After one hour the reaction was completed by the observance of product and the complete disappearance of starting material via M.S. The solvent was dried of en vacuo at or below 30°C. The white slurry was quenched with saturated $NaHCO_3$ solution, followed by the extraction of product in DCM washes (3 x 15 mL). The DCM washes were dried over $NaSO_4$, evaporated, and purified via flash chromatography (30% EtOAc:Hexane). The white powder product collected was isolated in excellent yield (65 mg, 95%). 1H NMR (500 MHz, Chloroform-d) δ 8.16 (d, J = 7.8 Hz, 2H), 8.03 (m, 4H), 7.65 - 7.40 (m, 9H), 5.75 - 5.69 (m, 1H), 5.62 (d, J = 9.1 Hz, 1H), 5.40 - 5.24 (m, 5H), 4.65 (d, J = 7.8 Hz, 1H), 4.57 (dd, J = 11.4, 6.8

Hz, 2H), 4.40 (dd, J = 11.5, 6.0 Hz, 2H), 4.35 - 4.26 (m, 1H), 4.20 - 4.08 (m, 3H), 4.06 (dd, J = 9.9, 3.4 Hz, 1H), 3.59 (dd, J = 9.9, 3.8 Hz, 1H), 2.88 - 2.84 (m, 1H), 1.96 (m, 11H), 1.77 (t, J = 7.6, 2H), 1.40 - 1.05 (m, 54H), 0.88 (t, J = 6.8 Hz, 6H). 13C NMR (126 MHz, Chloroform-d) δ 172.67, 172.65, 171.15, 169.81, 169.72, 166.72, 166.37, 166.35, 166.06, 137.00, 133.67, 133.63, 133.57, 133.53, 133.33, 130.15, 130.12, 130.08, 130.03, 129.92, 129.90, 129.87, 129.86, 129.85, 129.81, 129.77, 129.72, 129.66, 129.45, 129.40, 129.08, 129.02, 128.67, 128.60, 128.57, 128.53, 128.49, 128.46, 128.43, 128.40, 128.36, 124.81, 124.71, 101.48, 100.83, 77.30, 77.24, 77.04, 76.79, 73.57, 73.54, 73.44, 71.55, 71.50, 71.41, 71.39, 70.41, 67.34, 62.45, 60.39, 50.37, 36.80, 36.49, 32.31, 32.13, 31.94, 31.91, 29.81, 29.78, 29.74, 29.73, 29.71, 29.68, 29.66, 29.62, 29.58, 29.55, 29.53, 29.51, 29.46, 29.41, 29.38, 29.36, 29.33, 29.31, 29.28, 29.25, 29.21, 29.18, 29.00, 28.83, 27.23, 27.22, 25.64, 25.51, 22.70, 21.14, 21.06, 21.03, 14.19, 14.17, 14.12. HRMS (ESI) calcd for $C_{71}H_{105}NO_{12}$ [M+H]+: 1163.7637, found: 1164.7714.



Preparation of 2.19d: Glycolipid **2.18d** (65 mg, 55.9 μmol) was dissolved in dry pyridine (1.30 ml) followed by the addition of sulfur trioxide pyridine complex (125 mg, 0.782 mmol). The reaction was stirred at room temperature for fourteen hours, followed by TLC (indicating the completion of the reaction). The solvent was removed en vacuo, and the remaining slurry was dissolved in saturated $NaHCO_3$, washed with DCM (3 x 10 mL), dried ($NaSO_4$), and concentrated. The residue was chromatographed (SiO_2, methanol/DCM 5%, 10%) to afford product **2.19d** (55

mg, 80%). 1H NMR (500 MHz, Methanol-d4) δ 8.16 - 8.08 (m, 3H), 8.01 (d, J = 7.6 Hz, 2H), 7.66 - 7.39 (m, 10H), 6.11 (d, J = 3.3 Hz, 1H), 5.63 (dd, J = 14.5, 7.2 Hz, 1H), 5.57 (dd, J = 10.2, 7.8 Hz, 1H), 5.37 - 5.24 (m, 5H), 4.95 (dd, J = 10.2, 3.4 Hz, 2H), 4.90 (d, J = 7.9 Hz, 2H), 4.32 - 4.25 (m, 2H), 3.92 (dd, J = 10.1, 5.7 Hz, 2H), 3.66 (dd, J = 10.0, 5.1 Hz, 2H), 2.02 (q, J = 6.4 Hz, 5H), 1.95 (t, J = 7.5 Hz, 2H), 1.90 (d, J = 8.1 Hz, 6H), 1.48 - 1.14 (m, 54H), 0.89 (t, J = 6.8 Hz, 6H). 13C NMR (126 MHz, Methanol-d4) δ 175.99, 171.51, 167.54, 167.46, 167.03, 138.22, 134.57, 134.37, 134.32, 131.37, 131.23, 131.10, 131.02, 130.98, 130.89, 130.87, 130.78, 129.69, 129.58, 129.48, 125.73, 101.95, 76.54, 74.96, 72.99, 71.85, 71.20, 64.27, 51.91, 49.54, 49.37, 49.20, 49.03, 48.86, 48.69, 48.52, 37.08, 33.37, 33.13, 33.11, 30.88, 30.87, 30.84, 30.82, 30.72, 30.68, 30.66, 30.55, 30.50, 30.39, 30.36, 30.31, 30.21, 30.08, 28.18, 28.16, 27.01, 23.79, 21.16, 14.53. HRMS (ESI) calcd for $C_{71}H_{104}NO_{15}S$- [M+H]+: 1242.7132, found: 1244.7279.



Preparation of 2.1d: Sulfated glycolipid **2.19d** (15 mg, 12.1 μmol) was dissolved in an anhydrous 1:1 solution of THF and methanol (5 mL). Freshly prepared 1 M sodium methoxide was added (0.150 mL) to the reaction flask and the reaction was stirred for one hour at room temperature. Reaction completion was verified via mass spectrometry and TLC. Upon completion, the solvent was removed en vacuo and the resulting residue was washed with water (3 x 2 mL), and diethyl ether (3 x 2 mL) (solid was washed via centrifugation, the solvents washes were decanted off and saved, just in case of partial dissolution in water). The purified compound was a white

powder (8.17 mg, 76%). 1H NMR (500 MHz, DMSO-d6) δ 7.44 (d, J = 9.0 Hz, 1H), 5.51 (dt, J = 13.9, 6.6 Hz, 1H), 5.32 (m, 3H), 5.07 (d, J = 2.6 Hz, 1H), 4.83 (d, J = 5.4 Hz, 1H), 4.57 (t, J = 5.7 Hz, 1H), 4.41 (d, J = 4.6 Hz, 1H), 4.14 (d, J = 7.6 Hz, 1H), 3.97 - 3.89 (m, 3H), 3.74 (tt, J = 8.8, 4.5 Hz, 1H), 3.56 - 3.33 (m, 6H), 1.96 (m, 8H), 1.42 (d, J = 6.6 Hz, 2H), 1.22 (s, 54H), 0.84 (t, J = 6.8 Hz, 6H). 13C NMR (126 MHz, DMSO-d6) δ 172.17, 131.73, 131.69, 130.07, 104.77, 79.47, 75.35, 71.39, 69.71, 69.26, 66.68, 60.55, 53.66, 40.48, 40.41, 40.32, 40.24, 40.15, 40.07, 39.98, 39.81, 39.65, 39.48, 36.08, 32.23, 31.76, 31.74, 29.60, 29.58, 29.56, 29.53, 29.51, 29.46, 29.30, 29.28, 29.24, 29.19, 29.16, 29.15, 29.03, 29.00, 27.00, 26.98, 25.82, 22.55, 14.38. HRMS (ESI) calcd for $C_{48}H_{90}NO_{11}S$- [M+H](Neg. mode): 888.6240, found: 888.6293.

NMR and M.S. data of Compound 2.1a

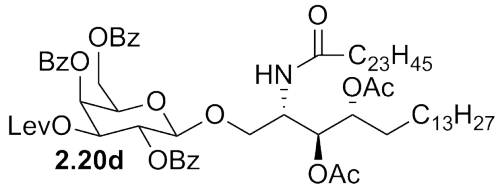
1H NMR (500 MHz, DMSO-d6) δ 7.47 (d, J = 8.7 Hz, 1H), 5.52 (dt, J = 14.8, 6.6 Hz, 1H), 5.41 - 5.32 (m, 1H), 5.06 (d, J = 2.6 Hz, 1H), 4.85 (d, J = 5.4 Hz, 1H), 4.59 (dd, J = 6.3, 5.1 Hz, 1H), 4.43 (d, J = 4.6 Hz, 1H), 4.14 (d, J = 7.7 Hz, 1H), 3.97 - 3.85 (m, 3H), 3.76 (m, 1H), 3.53 - 3.47 (m, 3H), 3.47 - 3.38 (m, 3H), 3.35 (m, 1H) 2.01 (t, J = 7.5 Hz, 2H), 1.92 (m, 2H), 1.44 (q, J = 7.2, 6.7 Hz, 2H), 1.22 (m, 30H), 0.84 (t, J = 6.9 Hz, 6H). 13C NMR (126 MHz, DMSO-d6) δ 172.25, 131.72, 104.73, 79.48, 75.36, 71.41, 69.69, 66.67, 63.52, 60.54, 36.05, 32.21, 31.75, 31.70, 29.55, 29.51, 29.46, 29.22, 29.18, 29.16, 29.11, 29.06, 25.80, 22.57, 22.55, 14.41. HRMS (ESI) calcd for $C_{32}H_{60}NO_{11}S$- [M+H]+: 666.3893, found: 668.4038.

NMR data of Compound 2.1b

1H NMR (500 MHz, DMSO-d6) δ 7.47 (d, J = 8.7 Hz, 1H), 5.50 (dd, J = 14.6, 7.3 Hz, 1H), 5.34 (dd, J = 15.5, 7.1 Hz, 1H), 4.83 (d, J = 5.4 Hz, 1H), 4.58 (t, J = 5.9 Hz, 1H), 4.41 (d, J =

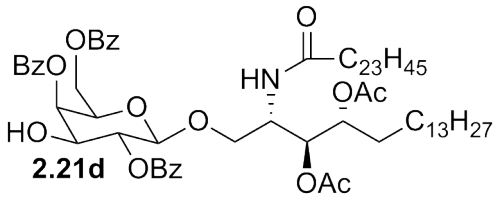
4.6 Hz, 1H), 4.13 (d, J = 7.7 Hz, 1H), 3.93 (m, 3H), 3.74 (m, 1H), 3.52 - 3.46 (m, 3H), 3.43 (m, 3H), 3.33 (m, 2H), 2.00 (t, J = 7.4 Hz, 2H), 1.91 (m, 2H), 1.47 - 1.42 (m, 2H), 1.40 (m, 2H), 1.21 (m, 46H), 0.83 (t, J = 6.8 Hz, 6H). 13C NMR (126 MHz, DMSO-d6) δ 172.22, 131.72, 131.69, 104.71, 79.46, 75.35, 71.41, 69.67, 69.29, 66.67, 60.53, 53.64, 40.47, 40.39, 40.30, 40.22, 40.13, 40.06, 39.97, 39.89, 39.80, 39.63, 39.47, 36.08, 32.24, 31.77, 31.76, 29.61, 29.60, 29.57, 29.55, 29.51, 29.49, 29.46, 29.44, 29.26, 29.24, 29.20, 29.18, 25.82, 22.55, 22.54, 14.37. HRMS (ESI) calcd for $C_{40}H_{76}NO_{11}$S- [M+H]+: 778.5145, found: 780.5313.

NMR data of Compound 2.1c Due to a server outage, various spectroscopic data was unable to be recovered (department wide), due to this missing data should be here.



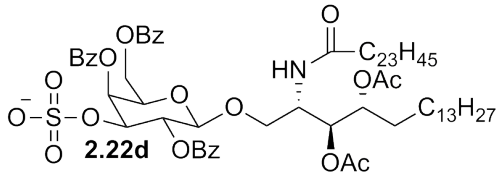
Preparation of 2.20d: With the donor **2.16** synthesized, each sulfatide product was made by the coupling of the appropriate ceramide followed by the removal of the levulinic protecting group, installation of the sulfate, and finally the global removal of the esters to make the appropriate sulfatide. Due to the shared synthetic route, one synthetic series will be described followed by the analytical data of the final sulfatides. Donor **2.16** (150 mg, 0.205 mmol) was stirred for one hour at room temperature in the presence of ceramide **2.10** (168 mg, 0.226 mmol), 4 angstrom (400 mg), and dry DCM (5 mL). The vessel was cooled to -10°C (acetone/ice bath), followed by the dropwise addition of trimethylsilyltriflate (9 μL, 51.3 μmol). The reaction temperature was maintained for two hours then quenched by the dropwise addition of TEA. The M.S. were filtered

and washed with EtOAc by means of a plug silica gel column. The filtrate was dried over $NaSO_4$, concentrated, and subsequently loaded onto a silica gel column. Purification was achieved with a 20%, 30% EtOAc/Hexane eluent system (100 mg, 37%). 1H NMR (500 MHz, Chloroform-d) δ 8.20 - 8.14 (m, 2H), 8.00 (m, 4H), 7.67 - 7.61 (m, 2H), 7.61 - 7.49 (m, 4H), 7.45 (m, 3H), 5.95 (d, J = 9.3 Hz, 1H), 5.82 (dd, J = 3.2, 1.1 Hz, 1H), 5.50 (dd, J = 10.3, 7.7 Hz, 1H), 5.40 - 5.31 (m, 3H), 5.13 (dd, J = 8.0, 3.1 Hz, 1H), 4.95 (dt, J = 10.1, 3.2 Hz, 1H), 4.68 (d, J = 7.7 Hz, 1H), 4.59 (dd, J = 11.3, 6.6 Hz, 1H), 4.40 - 4.31 (m, 2H), 4.22 (m, 1H), 4.06 (dd, J = 9.7, 2.8 Hz, 1H), 3.56 (dd, J = 9.6, 3.3 Hz, 1H), 2.63 - 2.46 (m, 3H), 2.46 - 2.32 (m, 3H), 2.06 - 1.98 (m, 8H), 1.93 (s, 2H), 1.88 - 1.71 (m, 4H), 1.65 (s, 4H), 1.46 - 1.05 (m, 52H), 0.88 (t, J = 6.9 Hz, 6H). 13C NMR (126 MHz, Chloroform-d) δ 205.71, 172.73, 171.74, 170.80, 169.61, 165.96, 165.60, 165.32, 133.74, 133.56, 133.38, 130.12, 129.89, 129.82, 129.73, 129.29, 129.19, 128.91, 128.77, 128.59, 128.52, 100.74, 77.26, 77.21, 77.01, 76.75, 73.09, 72.34, 71.22, 70.95, 69.83, 67.74, 67.00, 62.05, 47.45, 37.71, 36.35, 31.92, 29.82, 29.78, 29.75, 29.74, 29.72, 29.70, 29.67, 29.63, 29.57, 29.53, 29.38, 29.35, 29.33, 29.21, 28.53, 27.88, 27.24, 27.22, 25.61, 25.38, 22.70, 22.69, 21.07, 20.78, 14.12. HRMS (ESI) calcd for $C_{78}H_{115}NO_{16}$ [M+H]+: 1321.8216, found: 1322.8216.

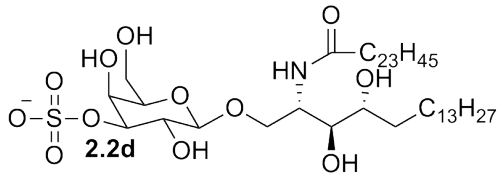


Preparation of 2.21d: A 1 M solution of hydrazine was freshly prepared in dry THF. A portion (59 μL, 59 μmol) of this was stirred with glacial acetic acid (78 μL, 78 μmol) for five minutes. This mixture was added to a RBF that contained compound **2.20d** (60 mg, 0.045 mmol)

and dry THF:Methanol (10:1, 11 mL). After one hour the reaction was completed by the observance of product and the complete disappearance of starting material via M.S. The solvent was dried of en vacuo at or below 30°C. The white slurry was quenched with saturated $NaHCO_3$ solution, followed by the extraction of product in DCM washes (3 x 15 mL). The DCM washes were dried over $NaSO_4$, evaporated, and purified via flash chromatography (30% EtOAc:Hexane). The white powder product collected was isolated in excellent yield (50 mg, 91%). 1H NMR (500 MHz, Chloroform-d) δ 8.20 - 8.14 (m, 2H), 8.08 - 7.99 (m, 4H), 7.67 - 7.41 (m, 8H), 5.93 (d, J = 9.2 Hz, 1H), 5.76 (dd, J = 3.5, 1.1 Hz, 1H), 5.38 - 5.27 (m, 3H), 5.11 (dd, J = 8.0, 3.2 Hz, 1H), 4.93 (dt, J = 10.0, 3.2 Hz, 1H), 4.66 (d, J = 7.8 Hz, 1H), 4.56 (dd, J = 11.4, 6.8 Hz, 2H), 4.37 (m, 3H), 4.19 - 4.08 (m, 3H), 4.04 (dd, J = 9.6, 3.0 Hz, 1H), 3.60 (dd, J = 9.6, 3.3 Hz, 1H), 2.65 (d, J = 5.8 Hz, 2H), 2.12 - 1.97 (m, 12H), 1.64 (d, J = 18.7 Hz, 2H), 1.58 - 1.52 (m, 2H), 1.46 - 1.13 (m, 54H), 0.88 (t, J = 6.9 Hz, 6H). 13C NMR (126 MHz, Chloroform-d) δ 172.77, 170.83, 169.62, 166.50, 166.26, 166.05, 133.71, 133.62, 133.37, 130.19, 129.90, 129.89, 129.84, 129.72, 129.41, 129.32, 128.97, 128.71, 128.58, 128.52, 100.43, 77.27, 77.22, 77.01, 76.76, 73.58, 73.08, 72.36, 71.56, 71.45, 70.26, 67.04, 62.40, 60.39, 47.55, 36.39, 31.94, 31.92, 29.82, 29.79, 29.75, 29.73, 29.72, 29.71, 29.69, 29.68, 29.67, 29.63, 29.57, 29.54, 29.38, 29.33, 29.21, 28.52, 27.24, 27.23, 25.60, 25.40, 22.70, 21.06, 20.74, 14.21, 14.13. HRMS (ESI) calcd for $C_{73}H_{109}NO_{14}$ [M+H]+: 1223.7848, found: 1224.8077.



Preparation of 2.22d: Glycolipid **2.18d** (40 mg, 33.0 μmol) was dissolved in dry pyridine (8 mL) followed by the addition of sulfur trioxide pyridine complex (72.8 mg, 0.456 mmol). The reaction was stirred at room temperature for fourteen hours, followed by TLC (indicating the completion of the reaction). The solvent was removed en vacuo, and the remaining slurry was dissolved in saturated $NaHCO_3$, washed with DCM (3 x 10 mL), dried ($NaSO_4$), and concentrated. The residue was chromatographed (SiO_2, methanol/DCM 5%, 10%) to afford product **2.19d** (30 mg, 70%). 1H NMR (500 MHz, Methanol-d4) δ 8.12 (m, 4H), 8.03 - 7.99 (m, 2H), 7.67 - 7.40 (m, 9H), 6.10 (d, J = 3.2 Hz, 1H), 5.54 (dd, J = 10.2, 7.9 Hz, 1H), 5.34 (dd, J = 5.5, 4.3 Hz, 2H), 5.17 (dd, J = 8.4, 3.3 Hz, 1H), 4.98 - 4.88 (m, 3H), 4.47 - 4.36 (m, 3H), 4.28 (m, 1H), 3.96 - 3.89 (m, 1H), 3.73 (dd, J = 10.2, 3.4 Hz, 1H), 2.03 (q, J = 6.4 Hz, 4H), 1.96 (s, 3H), 1.93 (s, 3H), 1.86 - 1.79 (m, 2H), 1.66 - 1.38 (m, 6H), 1.37 - 1.11 (m, 56H), 0.89 (t, J = 6.9 Hz, 6H). 13C NMR (126 MHz, Methanol-d4) δ 174.27, 170.97, 170.01, 166.11, 166.02, 165.52, 132.91, 129.90, 129.77, 129.67, 129.56, 129.52, 129.46, 129.43, 129.42, 129.33, 128.25, 128.12, 128.03, 100.13, 72.56, 71.51, 70.38, 62.86, 48.08, 48.03, 47.91, 47.85, 47.74, 47.57, 47.40, 47.23, 47.06, 35.54, 31.68, 31.66, 29.42, 29.38, 29.34, 29.30, 29.23, 29.20, 29.09, 29.05, 29.03, 28.93, 28.91, 28.84, 28.81, 27.87, 26.71, 26.70, 25.40, 25.12, 22.34, 19.54, 19.40, 13.06. HRMS (ESI) calcd for $C_{73}H_{108}NO_{17}S$-[M+H]+: 1302.7343, found: 1304.8101.



Preparation of 2.2d: Sulfated glycolipid **2.22d** (4 mg, 12.1 μmol) was dissolved in an

anhydrous 1:1 solution of THF and methanol (3 mL). Freshly prepared 1 M sodium methoxide was added (80 μL) to the reaction flask and the reaction was stirred for one hour at room temperature. Reaction completion was verified via mass spectrometry and TLC. Upon completion, the solvent was removed en vacuo and the resulting residue was washed with water (3 x 1 mL), and diethyl ether (3 x 2 mL) (solid was washed via centrifugation, the solvents washes were decanted off and saved, just in case of partial dissolution in water). The purified compound was a white powder (2.60 mg, 93%). 1H NMR (500 MHz, DMSO-d6) δ 7.57 (d, J = 8.7 Hz, 1H), 5.30 (td, J = 4.4, 2.1 Hz, 2H), 5.03 (d, J = 2.5 Hz, 1H), 4.54 (m, 2H), 4.41 (d, J = 4.8 Hz, 1H), 4.13 (m, 2H), 3.92 (dd, J = 8.9, 3.5 Hz, 3H), 3.86 (t, J = 5.0 Hz, 1H), 3.55 - 3.46 (m, 2H), 3.46 - 3.38 (m, 3H), 3.34 (m, 3H), 2.04 (td, J = 7.3, 3.7 Hz, 3H), 1.96 (q, J = 6.4 Hz, 4H), 1.43 (m, 5H), 1.31 - 1.16 (m, 52H), 0.83 (t, J = 6.8 Hz, 6H). Due to a server outage, various spectroscopic data was unable to be recovered (department wide), due to this missing data should be here. HRMS (ESI) calcd for $C_{48}H_{92}NO_{12}S$-[M+H]+: 906.6346, found: 908.6496.

NMR data of Compound 2.2a

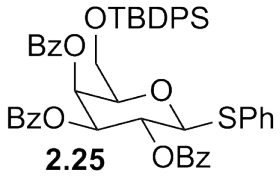
1H NMR (500 MHz, DMSO-d6) δ 7.59 (d, J = 8.9 Hz, 1H), 5.05 (d, J = 2.5 Hz, 1H), 4.60 (t, J = 5.4 Hz, 2H), 4.44 (d, J = 4.8 Hz, 1H), 4.17 (m, 2H), 3.97 - 3.89 (m, 3H), 3.85 (dd, J = 10.3, 5.7 Hz, 1H), 3.57 - 3.47 (m, 3H), 3.47 - 3.30 (m, 4H), 2.05 (t, J = 7.3 Hz, 2H), 1.45 (m, 3H), 1.22 (m, 31H), 0.84 (td, J = 7.0, 1.2 Hz, 6H). 13C NMR (126 MHz, DMSO-d6) δ 172.33, 104.58, 79.47, 75.37, 74.44, 71.04, 69.65, 69.27, 66.68, 60.53, 50.75, 40.47, 40.39, 40.30, 40.22, 40.13, 40.06, 39.97, 39.89, 39.80, 39.70, 39.63, 39.47, 36.01, 31.78, 31.75, 31.68, 29.68, 29.66, 29.57, 29.55, 29.52, 29.47, 29.16, 29.12, 29.03, 25.87, 25.82, 22.56, 22.54, 14.41. HRMS (ESI) calcd for

$C_{32}H_{62}NO_{12}S$- [M+H]+: 684.3998, found: 686.4190.

NMR data of Compound 2.2b

1H NMR (500 MHz, DMSO-d6) δ 7.18 (d, J = 8.8 Hz, 1H), 4.64 (d, J = 2.5 Hz, 1H), 4.19 - 4.12 (m, 2H), 4.02 (d, J = 4.6 Hz, 1H), 3.75 (dd, J = 10.5, 7.2 Hz, 2H), 3.53 (d, J = 8.5 Hz, 3H), 3.47 (s, 1H), 3.15 - 3.10 (m, 3H), 3.08 - 3.01 (m, 3H), 2.91 (m, 1H), 1.65 (td, J = 7.1, 3.1 Hz, 2H), 1.08 - 1.01 (m, 6H), 0.83 (m, 44H), 0.44 (t, J = 6.6 Hz, 6H). (not correctly shifted to solvent). 13C NMR (126 MHz, DMSO-d6). δ 172.29, 104.61, 79.46, 75.38, 74.28, 71.02, 69.67, 69.37, 66.70, 60.55, 50.79, 40.57, 40.48, 40.40, 40.31, 40.24, 40.14, 40.07, 39.98, 39.90, 39.81, 39.64, 39.48, 36.00, 31.75, 31.51, 29.70, 29.68, 29.61, 29.59, 29.58, 29.56, 29.54, 29.53, 29.48, 29.42, 29.40, 29.17, 25.91, 25.81, 22.55, 22.54, 14.39. HRMS (ESI) calcd for $C_{40}H_{78}NO_{12}S$- [M+H]+: 796.5250, found: 798.5396.

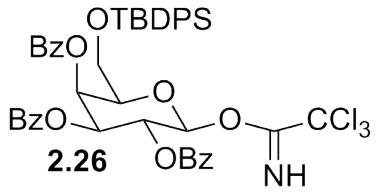
NMR data of Compound 2.2c Due to a server outage, various spectroscopic data was unable to be recovered (department wide), due to this missing data should be here.



2.25

Preparation of 2.25: Diacylated intermediate **2.13a**, isolated during the synthesis of **2.13**, (5.00 g, 18.4 mmol) was dissolved in DMF (150 mL), followed by the addition of imidazole (5.00 g, 73.5 mmol), and tert-butylchlorodiphenylsilane (9.55 mL, 36.8). The reaction was warmed to 50°C. After disappearence of the starting material (via TLC), the reaction was quenched with methanol and the reaction was concentrated by high pressure vacuum rotary evaporation. Pyridine

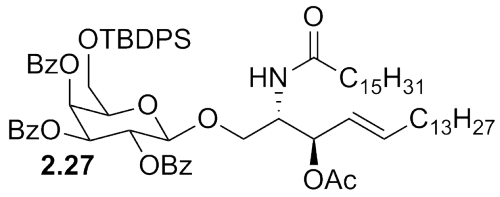
(50 mL) was then added to dissolve the reaction mixture, followed by the addition of benzoyl chloride (8.54 mL, 73.6 mmol) and DMAP (225 mg, 1.84 mmol). After completion, the reaction was concentrated by high pressure vacuum rotary evaporation. A $NaHCO_3$/DCM (3 x 50 mL) workup was carried out, dried ($NaSO_4$), concentrated, and loaded onto a silica gel column. This very non-polar product was purified via a pure hexane, 5%, 10 % EtOAc:Hexane eluent system. 1H NMR (500 MHz, Chloroform-d) δ 7.95 (dd, J = 7.8, 3.7 Hz, 2H), 7.86 - 7.19 (m, 28H), 6.04 (q, J = 3.3 Hz, 1H), 5.70 - 5.55 (m, 2H), 4.96 (dd, J = 9.7, 3.6 Hz, 1H), 4.12 (m, 1H), 3.88 (m, 1H), 3.78 (m, 1H), 1.00 (d, J = 3.6 Hz, 9H).



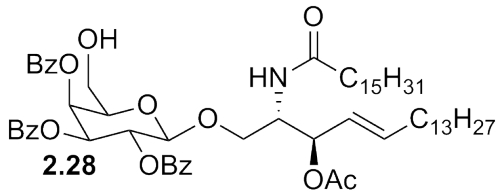
2.26

Preparation of 2.26: Fully protected compound **2.25** (500 mg, 0.608 mmol) was dissolved in an acetone/water solution (20mL/3mL), followed by the portion wise addition of NBS (325 mg, 1.82 mmol). Subsequent equivalents of NBS were added until the reaction was completed (monitored by TLC). Upon completion, the reaction was quenched with solid $NaHCO_3$, and the acetone was evaporated off. The remaining mixture was dissolved in water, washed with DCM (3 x 50 mL), dried with $NaSO_4$, concentrated, and subjected to flash chromatography (20% EtOAc:Hexanes). The white powder (437 mg, 0.599 mmol) was dissolved in DCM (25 mL). Solid potassium carbonate (1320 mg, 9.55 mmol) was added, followed by the addition of excess trichloroacetonitrile (1.20 mL, 11.98 mmol). Once setup, the reaction was stirred for sixteen hours at room temperature. In the morning, the potassium carbonate was filtered and washed with EtOAc (100 mL). The filtrate

was dried off at or below 30°C en vacuo. This pale yellow solid was loaded onto a bed of silica gel and was eluted with 15% EtOAc/Hexane. The two anomeric isomers were collected, combined, and concentrated at or below 30°C (500 mg, 98% from two steps).

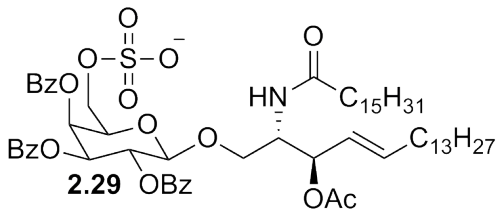


Preparation of 2.27: Donor **2.26** (240 mg, 0.275 mmol), palmitic ceramide (145 mg, 0.250 mmol), and 4 angstrom molecular sieves (600 mg) were stirred together for one hour in dry DCM (5 mL). The vessel was cooled to -10°C (acetone/ice bath), followed by the dropwise addition of trimethylsilyltriflate (25 μL, 0.141 mmol). The reaction temperature was maintained for two hours then quenched by the dropwise addition of TEA. The molecular sieves were filtered and washed with EtOAc by means of a plug silica gel column. The filtrate was concentrated and subsequently loaded onto a silica gel column. Purification was achieved with a 10%, 15% EtOAc/Hexane eluent system. Very bad separation! Concentrated partially purified product and preceded with the next step. HRMS (ESI) calcd for $C_{79}H_{109}NO_{12}Si$ [M+H]+: 1291.7719, found: 1292.7719.

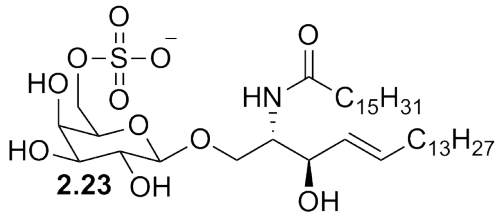


Preparation of 2.28: Compound **2.27** was dissolved in DCM (5 mL) and ACN(30 mL) and placed in a plastic centrifuge tube (with the appropriate stir bar). To this tube was added 10 mL

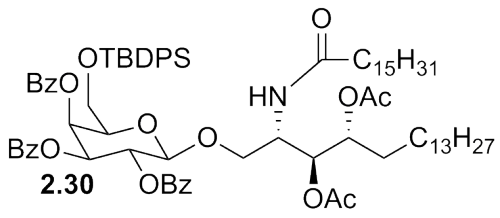
of hydrofluoric acid (48%). The reaction was monitored by TLC for formation of product **2.11**. Every thirty minutes, more hydrofluoric acid was added as needed. Once the starting material was completely consumed, the reaction was quickly and carefully quenched on a bed of solid $NaHCO_3$ in a separate plastic container (equipped with the appropriate stir bar). Water, DCM, and solid $NaHCO_3$ were slowly added until a neutral or basic pH was reached. The water/DCM workup was filtered to remove excess undissolved $NaHCO_3$. The water was washed with DCM (6 x 100 mL) and the organic layer was dried over $NaSO_4$, concentrated (water bath was 30°C or below to minimize acetal migration), and subjected to flash chromatography (40%, 50% EtOAc/Hexane). The column yielded a white powder compound (65 mg, 21% over two steps). Note: this reaction if done slowly, left overnight, heated up, or purified slowly can and will result in migration of the 2-acetyl group to the primary alcohol. It is essential that the chemist quench this reaction immediately after completion. 1H NMR (500 MHz, Chloroform-d) δ 8.12 (dd, J = 8.2, 1.4 Hz, 2H), 8.00 - 7.94 (m, 2H), 7.80 (dd, J = 8.2, 1.5 Hz, 2H), 7.67 - 7.59 (m, 1H), 7.56 - 7.47 (m, 3H), 7.47 - 7.36 (m, 3H), 7.25 (t, J = 7.8 Hz, 2H), 5.84 - 5.71 (m, 3H), 5.68 - 5.57 (m, 2H), 5.44 - 5.34 (m, 2H), 4.76 (d, J = 7.8 Hz, 1H), 4.35 (d, J = 6.3 Hz, 1H), 4.02 (q, J = 6.8 Hz, 2H), 3.87 - 3.83 (m, 1H), 3.70 - 3.61 (m, 2H), 2.54 (s, 1H), 2.07 - 1.95 (m, 5H), 1.86 (t, J = 7.6 Hz, 2H), 1.48 - 1.11 (m, 32H), 0.88 (t, J = 6.8 Hz, 6H). 13C NMR (126 MHz, Chloroform-d) δ 172.75, 170.11, 166.55, 165.47, 165.43, 137.22, 133.77, 133.50, 133.35, 130.11, 129.73, 129.71, 129.15, 128.84, 128.74, 128.69, 128.54, 128.33, 124.62, 101.53, 77.27, 77.02, 76.77, 74.36, 73.82, 71.49, 70.25, 68.87, 67.91, 60.90, 50.65, 36.56, 32.33, 31.93, 29.72, 29.71, 29.69, 29.67, 29.66, 29.56, 29.53, 29.37, 29.28, 29.23, 29.01, 25.53, 22.70, 21.21, 14.12.



Preparation of 2.29: Glycolipid **2.28** (47 mg, 0.045 mmol) was dissolved in dry pyridine (3 mL) followed by the addition of sulfur trioxide pyridine complex (108 mg, 0.679 mmol). The reaction was stirred at room temperature for fourteen hours, followed by TLC (indicating the completion of the reaction). The solvent was removed en vacuo, and the remaining slurry was dissolved in DCM, washed with saturated $NaHCO_3$, dried ($NaSO_4$), and concentrated. The residue was chromatographed (SiO_2, MeOH/DCM 5%, 10%) to afford product **2.32** (47 mg, 94%). 1H NMR (500 MHz, Methanol-d4) δ 8.09 - 8.03 (m, 2H), 7.98 - 7.92 (m, 2H), 7.74 - 7.62 (m, 3H), 7.53 (m, 3H), 7.42 (m, 3H), 7.25 (t, J = 7.9 Hz, 2H), 5.92 (d, J = 3.1 Hz, 1H), 5.75 - 5.64 (m, 3H), 5.42 - 5.28 (m, 2H), 5.01 (d, J = 7.5 Hz, 1H), 4.47 (t, J = 6.3 Hz, 1H), 4.32 (m, 1H), 4.25 - 4.14 (m, 2H), 4.05 (m, 1H), 3.77 - 3.71 (m, 1H), 2.05 - 1.91 (m, 5H), 1.47 (m, 2H), 1.34 - 1.18 (m, 32H), 0.89 (t, J = 6.9 Hz, 6H). 13C NMR (126 MHz, Methanol-d4) δ 174.74, 170.28, 165.57, 165.15, 136.72, 133.33, 133.02, 129.49, 129.34, 129.24, 129.22, 129.11, 128.90, 128.40, 128.20, 127.97, 124.39, 100.15, 73.48, 71.94, 71.61, 70.21, 68.57, 66.99, 65.41, 50.85, 50.76, 48.09, 47.92, 47.75, 47.58, 47.41, 47.24, 47.07, 35.74, 35.68, 31.93, 31.67, 29.42, 29.41, 29.40, 29.38, 29.36, 29.27, 29.22, 29.08, 29.07, 28.87, 28.80, 28.65, 25.63, 22.33, 19.87, 13.05.

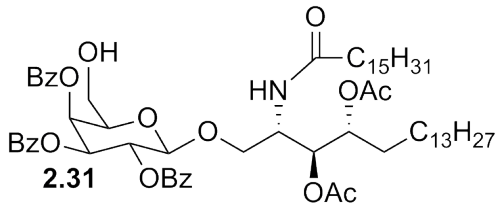


Preparation of 2.23: Sulfated glycolipid **2.29** (15 mg, 0.0126 mmol) was dissolved in an anhydrous 1:1 solution of THF and methanol (3 mL). Freshly prepared 1 M sodium methoxide was added (0.100 mL) to the reaction flask and the reaction was stirred for one hour at room temperature. Reaction completion was verified via mass spectrometry and TLC. Upon completion, the solvent was removed en vacuo and the resulting residue was washed with water (3 x 1 mL), and diethyl ether (3 x 2 mL) (solid was washed via centrifugation, the solvents washes were decanted off and saved, just in case of partial dissolution in water). The purified compound was a white powder (7 mg, 72%). 1H NMR (500 MHz, DMSO-d6) δ 7.51 (d, J = 9.1 Hz, 1H), 5.37 - 5.31 (m, 1H), 4.90 - 4.81 (m, 2H), 4.64 (d, J = 5.2 Hz, 1H), 4.47 (d, J = 4.7 Hz, 1H), 3.95 (d, J = 4.9 Hz, 1H), 3.88 - 3.72 (m, 3H), 3.57 (t, J = 3.9 Hz, 1H), 3.52 (t, J = 6.2 Hz, 1H), 3.39 - 3.32 (m, 2H), 3.30 - 3.23 (m, 3H), 2.00 (q, J = 7.2 Hz, 2H), 1.95 - 1.86 (m, 2H), 1.42 (m, 3H), 1.22 (m, 45H), 0.83 (t, J = 6.9 Hz, 6H). Due to a server outage, various spectroscopic data was unable to be recovered (department wide), due to this missing data should be here.



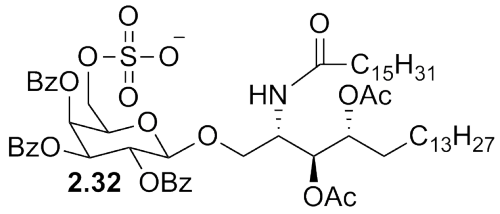
Preparation of 2.30: Donor **2.26** (307 mg, 0.351 mmol), palmitic ceramide (239 mg, 0.320

mmol), and 4 angstrom molecular sieves (700 mg) were stirred together for one hour in dry DCM (5 mL). The vessel was cooled to -10°C (acetone/ice bath), followed by the dropwise addition of trimethylsilyltriflate (0.032 mL, 0.176 mmol). The reaction temperature was maintained for two hours then quenched by the dropwise addition of triethyl amine. The molecular sieves were filtered and washed with EtOAc by means of a plug silica gel column. The filtrate was concentrated and subsequently loaded onto a silica gel column. Purification was achieved with a 10%, 15% EtOAc/Hexane eluent system. Very bad separation! Concentrated partially purified product and did the next step. HRMS (ESI) calcd for $C_{81}H_{113}NO_{14}Si$ [M+H]+: 1351.7930, found: 1352.8007.



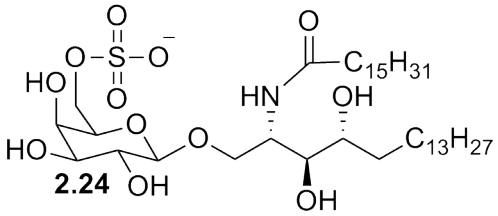
Preparation of 2.31: Compound **2.30** was dissolved in DCM (5 mL) and ACN (30 mL) and placed in a plastic centrifuge tube (with the appropriate stir bar). To this tube was added 5 mL of hydrofluoric acid (48%). The reaction was monitored by TLC for formation of product **2.11**. Every thirty minutes, more hydrofluoric acid was added as needed. Once the starting material was completely consumed, the reaction was quickly and carefully quenched on a bed of solid $NaHCO_3$ in a separate plastic container (equipped with the appropriate stir bar). Water, DCM, and solid $NaHCO_3$ were slowly added until a neutral or basic pH was reached. The water/DCM workup was filtered to remove excess undissolved $NaHCO_3$. The water was washed with DCM (6 x 100 mL) and the organic layer was dried over $NaSO_4$, concentrated (water bath was 30°C or below to minimize acetal migration), and subjected to flash chromatography (40%, 50% EtOAc/Hexane).

The column yielded a white powder compound (82 mg, 21% over two steps). Note: this reaction if done slowly, left overnight, heated up, or purified slowly can and will result in migration of the 2-acetyl group to the primary alcohol. It is essential that the chemist quench this reaction immediately after completion. 1H NMR (500 MHz, Chloroform-d) δ 8.16 - 8.10 (m, 2H), 8.00 - 7.94 (m, 2H), 7.82 - 7.76 (m, 2H), 7.67 - 7.60 (m, 1H), 7.52 (m, 3H), 7.47 - 7.35 (m, 3H), 7.28 - 7.21 (m, 2H), 6.03 (d, J = 9.3 Hz, 1H), 5.81 (dd, J = 3.5, 1.0 Hz, 1H), 5.74 (dd, J = 10.5, 7.8 Hz, 1H), 5.60 (dd, J = 10.4, 3.4 Hz, 1H), 5.20 (dd, J = 7.9, 3.4 Hz, 1H), 5.00 (dt, J = 10.0, 3.2 Hz, 1H), 4.73 (d, J = 7.8 Hz, 1H), 4.38 (m, 1H), 4.29 (dt, J = 13.7, 8.3 Hz, 2H), 4.04 - 3.91 (m, 2H), 3.83 (dt, J = 12.8, 6.6 Hz, 1H), 3.70 - 3.61 (m, 2H), 3.08 (t, J = 7.1 Hz, 1H), 2.07 (s, 3H), 2.04 (d, J = 1.4 Hz, 3H), 1.98 - 1.82 (m, 2H), 1.70 - 1.37 (m, 4H), 1.36 - 1.09 (m, 46H), 0.88 (td, J = 6.7, 3.3 Hz, 6H). 13C NMR (126 MHz, Chloroform-d) δ 222.79, 172.80, 171.13, 170.97, 170.22, 166.45, 165.47, 165.42, 133.76, 133.49, 133.35, 130.90, 130.14, 129.74, 129.71, 129.14, 128.83, 128.80, 128.74, 128.71, 128.53, 128.32, 101.05, 77.27, 77.22, 77.02, 76.77, 74.33, 73.05, 72.68, 71.40, 70.24, 68.80, 67.44, 61.01, 60.39, 47.71, 36.46, 31.94, 29.73, 29.71, 29.68, 29.63, 29.61, 29.56, 29.37, 29.34, 29.23, 28.42, 25.55, 25.44, 22.70, 21.07, 21.05, 20.86, 14.20, 14.12. HRMS (ESI) calcd for $C_{65}H_{95}NO_{14}$ [M+H]+: 1113.6753, found: 1114.6828.

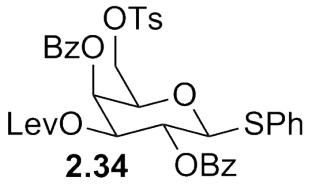


Preparation of 2.32: Glycolipid **2.31** (40 mg, 0.036 mmol) was dissolved in dry pyridine

(2.5 mL) followed by the addition of sulfur trioxide pyridine complex (86 mg, 0.539 mmol). The reaction was stirred at room temperature for fourteen hours, followed by TLC (indicating the completion of the reaction). The solvent was removed en vacuo, and the remaining slurry was dissolved in DCM, washed with saturated $NaHCO_3$, dried ($NaSO_4$), and concentrated. The residue was chromatographed (SiO_2, MeOH/DCM 5%, 10%) to afford product **2.32** (35 mg, 81%). (Aggregation in the NMR spectra was observed) 1H NMR (500 MHz, Methanol-d4) δ 8.10 (dq, J = 11.8, 7.5, 6.7 Hz, 2H), 7.97 (dq, J = 11.4, 7.4, 6.4 Hz, 2H), 7.71 (m, 2H), 7.55 (q, J = 8.2, 7.8 Hz, 3H), 7.43 (m, 3H), 7.27 (dt, J = 12.0, 7.7 Hz, 2H), 5.94 (dd, J = 17.1, 7.7 Hz, 1H), 5.68 (dq, J = 20.1, 9.4, 8.5 Hz, 2H), 5.28 - 5.19 (m, 1H), 4.48 (dt, J = 11.7, 7.0 Hz, 1H), 4.40 - 4.32 (m, 1H), 4.27 - 4.13 (m, 2H), 4.07 (q, J = 9.4, 8.1 Hz, 1H), 3.78 (dd, J = 16.2, 9.4 Hz, 1H), 3.33 (s, 2H), 2.16 - 2.00 (m, 6H), 1.89 (d, J = 7.6 Hz, 2H), 1.65 (m, 2H), 1.55 - 1.40 (m, 2H), 1.34 - 1.25 (m, 48H), 0.90 (p, J = 7.9, 7.3 Hz, 6H). 13C NMR (126 MHz, Methanol-d4) δ 173.66, 170.35, 169.56, 164.90, 164.83, 164.46, 132.62, 132.46, 132.31, 128.84, 128.63, 128.56, 128.54, 128.48, 128.41, 128.19, 127.67, 127.55, 127.49, 127.26, 99.14, 71.98, 71.33, 70.83, 70.76, 69.55, 67.82, 66.28, 64.67, 47.46, 47.39, 47.28, 47.22, 47.16, 47.10, 47.05, 46.93, 46.88, 46.76, 46.71, 46.61, 46.55, 46.53, 46.43, 46.37, 34.86, 31.04, 30.95, 28.76, 28.71, 28.65, 28.59, 28.52, 28.42, 28.37, 28.34, 28.15, 28.12, 27.21, 24.74, 24.46, 21.69, 21.63, 18.94, 18.92, 13.09, 12.37. HRMS (ESI) calcd for $C_{65}H_{94}NO_{17}S$- [M+H]+: 1192.6248, found: 1194.6446.



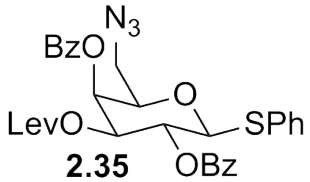
Preparation of 2.24: Sulfated glycolipid **2.32** (35 mg, 0.0294 mmol) was dissolved in an anhydrous 1:1 solution of THF and methanol (8 mL). Freshly prepared 1 M sodium methoxide was added (0.400 mL) to the reaction flask and the reaction was stirred for one hour at room temperature. Reaction completion was verified via mass spectrometry and TLC. Upon completion, the solvent was removed en vacuo and the resulting residue was washed with water (3 x 2 mL), and diethyl ether (3 x 2 mL) (solid was washed via centrifugation, the solvents washes were decanted off and saved, just in case of partial dissolution in water). The purified compound was a white powder (16.8 mg, 72%). 1H NMR (500 MHz, DMSO-d6) δ 7.69 (d, J = 9.1 Hz, 1H), 4.86 - 4.84 (m, 1H), 4.73 - 4.70 (m, 1H), 4.62 (d, J = 5.7 Hz, 1H), 4.53 (d, J = 4.6 Hz, 1H), 4.24 (d, J = 7.0 Hz, 1H), 4.12 - 4.07 (m, 1H), 3.95 - 3.80 (m, 3H), 3.66 - 3.51 (m, 3H), 3.36 - 3.29 (m, 2H), 2.11 (m, 2H), 1.54 - 1.43 (m, 4H), 1.28 (m, 44H), 0.90 (t, J = 6.9 Hz, 6H). 13C NMR (126 MHz, DMSO-d6) δ 172.47, 104.79, 74.08, 73.43, 73.31, 71.03, 71.02, 70.08, 68.83, 65.67, 50.71, 40.48, 40.40, 40.31, 40.23, 40.14, 40.07, 39.98, 39.90, 39.81, 39.64, 39.48, 36.01, 31.75, 31.37, 29.72, 29.69, 29.62, 29.61, 29.59, 29.57, 29.55, 29.53, 29.49, 29.44, 29.17, 29.15, 25.92, 25.86, 22.55, 14.38. HRMS (ESI) calcd for $C_{40}H_{78}NO_{12}S$- [M+H]+: 796.5250, found: 798.5397.



2.34

Preparation of 2.34: Compound **2.14** (2.25 g, 3.80 mmol) was diluted in a 4:1 glacial acetic acid:water solution (100 mL) and stirred overnight at room temperature. The next morning the solvent was removed en vacuo and the remaining white slurry was dissolved in DCM (50 mL)

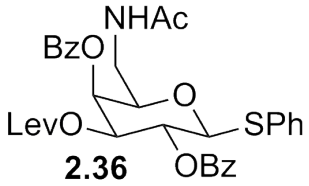
and added to a saturated $NaHSO_4$ solution (50 mL). The aqueous phase was extracted with DCM (2 x 25 mL), followed by the organic layer being dried ($NaSO_4$) and concentrated under reduced pressure. The off white solid was dissolved in dry pyridine (100 mL), followed by the addition of tosyl chloride (1.93 g, 10.1 mmol) at 0°C. The reaction warmed to room temperature after three hours and the reaction stirred overnight. In the morning, benzoyl chloride (1.76 mL, 15.2 mmol) was added. Two hours later the solvent was removed via a high vacuum rotary evaporator, followed by a DCM (3 x 75 mL):water (75 mL) workup. The dark brown slurry was subjected to flash chromatography (25% EtOAc:Hexane) to give pure product **2.34** (1.91 g, 52%) and useful dibenzoylated product (25% yield). 1H NMR (500 MHz, Chloroform-d) δ 8.04 - 7.97 (m, 2H), 7.84 - 7.78 (m, 2H), 7.71 (d, J = 8.1 Hz, 2H), 7.65 - 7.56 (m, 2H), 7.54 - 7.26 (m, 9H), 7.20 (d, J = 8.0 Hz, 2H), 5.70 - 5.65 (m, 1H), 5.45 (t, J = 9.9 Hz, 1H), 5.28 (dd, J = 10.0, 3.2 Hz, 1H), 4.87 (d, J = 9.9 Hz, 1H), 4.27 - 4.15 (m, 2H), 4.02 (dd, J = 9.9, 5.5 Hz, 1H), 2.49 (qt, J = 18.4, 6.9 Hz, 2H), 2.32 (d, J = 3.6 Hz, 2H), 2.04 (s, 3H), 1.91 (s, 3H). 13C NMR (126 MHz, Chloroform-d) δ 205.67, 171.56, 165.15, 165.06, 145.11, 134.22, 133.98, 133.64, 133.46, 132.06, 130.91, 129.97, 129.89, 129.88, 129.23, 128.86, 128.82, 128.64, 128.56, 128.49, 128.44, 127.97, 110.00, 85.77, 77.28, 77.23, 77.03, 76.77, 74.63, 72.31, 67.63, 67.48, 66.64, 60.38, 37.64, 29.36, 27.86, 21.61, 21.05, 14.20. HRMS (ESI) calcd for $C_{38}H_{36}O_{11}S$ [M+Na]+: 732.1699, found: 750.2032.



2.35

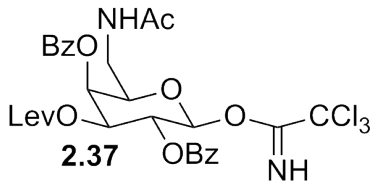
Preparation of 2.35: Compound **2.34** (1.80 g, 2.46 mmol) and sodium azide (959 mg, 14.8

mmol) were dissolved in a 9:1 solution of DMF:water (40 mL). The RBF (connected to a condenser) was heated to 100°C. This reaction was completed in one hour (evidenced by TLC). The water and DMF were pulled off on a high vacuum rotary evaporator, followed by a water:DCM (3 x 50 mL) workup. The dried ($NaSO_4$) and concentrated organic layer was loaded onto a silica gel column (25% EtOAc:Hexane) (991 mg, 67%). 1H NMR (500 MHz, Chloroform-d) δ 8.07 - 7.94 (m, 2H), 7.93 - 7.87 (m, 2H), 7.67 - 7.55 (m, 4H), 7.55 - 7.28 (m, 7H), 5.66 (d, J = 3.2 Hz, 1H), 5.54 (t, J = 9.9 Hz, 1H), 5.31 (dd, J = 10.0, 3.2 Hz, 1H), 4.91 (d, J = 9.9 Hz, 1H), 4.05 - 3.98 (m, 1H), 3.57 (dd, J = 13.0, 8.0 Hz, 1H), 3.27 (dd, J = 13.0, 4.6 Hz, 1H), 2.58 - 2.26 (m, 4H), 1.91 (s, 3H). 13C NMR (126 MHz, Chloroform-d) δ 205.65, 171.67, 165.49, 165.13, 134.62, 133.75, 133.47, 130.63, 130.00, 129.91, 129.32, 128.87, 128.71, 128.68, 128.65, 128.52, 85.72, 77.27, 77.21, 77.01, 76.76, 76.47, 72.57, 68.55, 67.64, 50.98, 37.67, 29.35, 27.91.

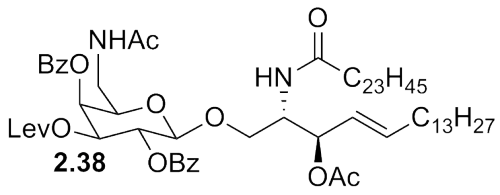


2.36

Preparation of 2.36: Azide **2.35** (730 mg, 1.21 mmol) was dissolved in 10 mL of MeOH. To this flask was added 0.213 mL of thioacetic acid (3.03 mmol), 0.350 mL of 2,6-lutidine (3.03 mmol), and 126 mg of ruthenium trichloride (0.605 mmol). The reaction was stirred for 18 hours. The ruthenium trichloride was filtered off and the dark red solution was concentrated en vacuo and loaded onto a silica gel column (50%, 70% EtOAc:Hexane, then 5%, 10% MeOH:DCM elution profile). The isolated compound was formed in 60% yield (445 mg).

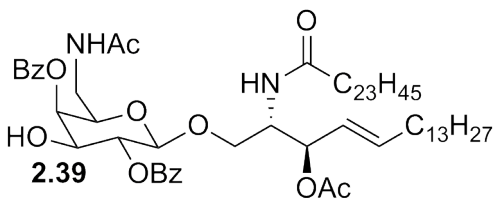


Preparation of 2.37: Amide **2.36** (427 mg, 0.688 mmol) was dissolved in an acetone/water solution (22mL/3mL), followed by the portion wise addition of NBS (367 mg, 2.06 mmol). Subsequent equivalents of NBS were added until the reaction was completed (monitored by TLC). Upon completion, the reaction was quenched with solid $NaHCO_3$, and the acetone was evaporated off. The remaining mixture was dissolved in water (50 mL), washed with DCM (3 x 50 mL), dried with $NaSO_4$, concentrated, and subjected to flash chromatography (5% MeOH:DCM). The white powder (437 mg, 0.599 mmol) was dissolved in DCM (25 mL). Solid potassium carbonate (1.50 g, 10.9 mmol) was added, followed by the addition of excess trichloroacetonitrile (1.03 mL, 10.3 mmol). Once setup, the reaction was stirred for sixteen hours at room temperature. In the morning, the potassium carbonate was filtered and washed with EtOAc (100 mL). The filtrate was dried off at or below 30°C en vacuo. This pale yellow solid was loaded onto a bed of silica gel and was eluted with 2.5%, 5 % MeOH:DCM. The two anomeric isomers were collected, combined, and concentrated at or below 30°C (300 mg, 65% from two steps).



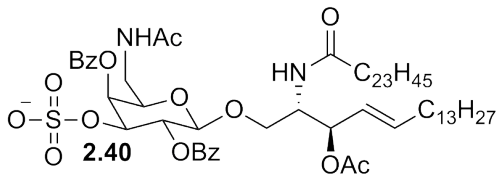
Preparation of 2.38: Donor **2.37** (200 mg, 0.298 mmol), nervonic ceramide (230 mg, 0.397

mmol), and 4 angstrom molecular sieves (700 mg) were stirred together for one hour in dry DCM (6 mL). The vessel was cooled to -10°C (acetone/ice bath), followed by the dropwise addition of trimethylsilyltriflate (0.032 μL, 0.176 mmol). The reaction temperature was maintained for two hours then quenched by the dropwise addition of triethyl amine. The molecular sieves were filtered and washed with EtOAc by means of a plug silica gel column. The filtrate was concentrated and subsequently loaded onto a silica gel column. Purification was achieved with a 70%, 80% EtOAc/Hexane eluent system. Isolated 60 mgs of coupled product (19% yield). 1H NMR (500 MHz, Chloroform-d) δ 8.16 (d, J = 7.8 Hz, 2H), 8.01 (d, J = 7.8 Hz, 2H), 7.66 (t, J = 7.5 Hz, 1H), 7.55 (m, 3H), 7.45 (t, J = 7.7 Hz, 2H), 5.76 (dt, J = 14.2, 6.6 Hz, 1H), 5.65 (d, J = 3.5 Hz, 1H), 5.59 - 5.48 (m, 2H), 5.40 - 5.22 (m, 3H), 4.68 (d, J = 7.8 Hz, 1H), 4.31 (m, 1H), 4.13 (dd, J = 9.8, 3.0 Hz, 1H), 4.02 (dd, J = 8.6, 3.9 Hz, 1H), 3.64 - 3.55 (m, 2H), 3.22 (dd, J = 13.1, 3.9 Hz, 1H), 2.60 - 2.46 (m, 2H), 2.46 - 2.29 (m, 2H), 2.08 - 1.91 (m, 9H), 1.70 (q, J = 7.5 Hz, 4H), 1.42 - 1.22 (m, 48), 0.88 (t, J = 6.8 Hz, 6H). 13C NMR (126 MHz, Chloroform-d) δ 205.66, 172.56, 171.74, 169.87, 165.73, 165.35, 137.18, 133.89, 133.65, 130.08, 129.94, 129.81, 129.12, 128.82, 128.76, 128.73, 128.64, 128.55, 128.52, 124.85, 101.18, 77.28, 77.23, 77.03, 76.77, 73.79, 73.52, 70.91, 69.73, 68.51, 67.55, 50.75, 50.12, 37.78, 37.70, 37.68, 36.41, 32.32, 31.94, 31.93, 29.73, 29.70, 29.68, 29.66, 29.57, 29.54, 29.51, 29.50, 29.38, 29.29, 29.20, 29.00, 27.92, 27.85, 25.47, 22.70, 21.18, 14.13. HRMS (ESI) calcd for $C_{63}H_{96}N_2O_{13}$ [M+H]+: 1088.6912, found: 1089.6925.

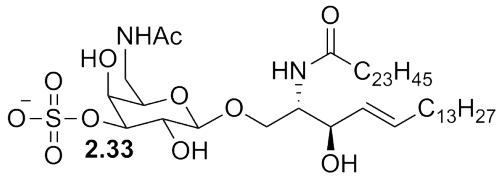


2.39

Preparation of 2.39: A 1 M solution of hydrazine was freshly prepared in dry THF. A portion (48 μL, 48 μmol) of this was stirred with glacial acetic acid (64 μL, 64 μmol) for five minutes. This mixture was added to a RBF that contained compound **2.20d** (40 mg, 0.037 mmol) and dry THF:Methanol (10:1, 11 mL). After one hour the reaction was completed by the observance of product and the complete disappearance of starting material via M.S. The solvent was dried of en vacuo at or below 30°C. The white slurry was quenched with saturated $NaHCO_3$ solution, followed by the extraction of product in DCM washes (3 x 15 mL). The DCM washes were dried over $NaSO_4$, evaporated, and purified via flash chromatography (70%, 80% EtOAc:Hexane). This compound did not separate well from the starting material. The mixture was dried off and used in the next step. 1H NMR (500 MHz, Chloroform-d) δ 8.15 (d, J = 7.7 Hz, 2H), 8.05 (d, J = 7.7 Hz, 2H), 7.68 - 7.48 (m, 5H), 7.46 (t, J = 7.7 Hz, 2H), 5.75 (dt, J = 14.3, 6.8 Hz, 1H), 5.58 - 5.53 (m, 2H), 5.39 - 5.30 (m, 2H), 5.26 (t, J = 7.3 Hz, 1H), 4.66 (d, J = 7.8 Hz, 1H), 4.32 (dq, J = 10.2, 3.6 Hz, 1H), 4.12 (ddd, J = 13.1, 9.9, 3.5 Hz, 2H), 3.94 (dd, J = 8.6, 3.7 Hz, 1H), 3.67 - 3.48 (m, 3H), 3.25 (dd, J = 13.1, 3.6 Hz, 2H), 1.98 (d, J = 18.6 Hz, 5H), 1.73 (t, J = 7.6 Hz, 3H), 1.41 - 1.04 (m, 45H), 0.88 (t, J = 6.9 Hz, 6H). 13C NMR (126 MHz, Chloroform-d) δ 172.61, 172.53, 169.88, 166.50, 166.40, 137.19, 133.87, 133.69, 130.13, 129.82, 129.25, 128.76, 128.74, 128.70, 128.63, 128.58, 124.77, 100.82, 77.27, 77.22, 77.02, 76.76, 73.90, 73.78, 73.40, 71.35, 71.04, 67.54, 51.02, 50.19, 36.46, 36.42, 32.30, 31.94, 29.73, 29.69, 29.68, 29.65, 29.56, 29.53, 29.50, 29.45, 29.37, 29.33, 29.28, 29.19, 28.98, 25.49, 22.70, 21.15, 14.12. HRMS (ESI) calcd for $C_{58}H_{90}N_2O_{11}$ [M+H]+: 990.6545, found: 991.6784.

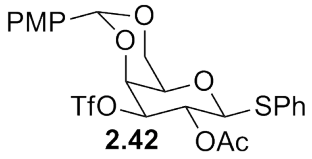


Preparation of 2.40: Partially purified glycolipid **2.37** (theoretical-0.037 mmol) was dissolved in dry pyridine (2 mL) followed by the addition of sulfur trioxide pyridine complex (868 mg, 0.555 mmol). The reaction was stirred at room temperature for fourteen hours, followed by TLC (indicating the completion of the reaction). The solvent was removed en vacuo, and the remaining slurry was dissolved in DCM, washed with saturated $NaHCO_3$, dried ($NaSO_4$), and concentrated. The residue was chromatographed (SiO_2, MeOH/DCM 5%, 10%, 15%) to afford product **2.32** (25 mg, 63% over two steps). 1H NMR (500 MHz, Methanol-d4) δ 8.17 - 8.08 (m, 4H), 7.65 (t, J = 7.5 Hz, 2H), 7.56 (m, 4H), 7.46 (t, J = 7.7 Hz, 3H), 5.93 (d, J = 3.3 Hz, 1H), 5.65 (dt, J = 14.2, 6.8 Hz, 2H), 5.53 (dd, J = 10.2, 7.9 Hz, 2H), 5.39 - 5.24 (m, 3H), 4.89 (dd, J = 13.9, 7.4 Hz, 2H), 4.35 - 4.27 (m, 2H), 4.19 (dd, J = 8.4, 3.6 Hz, 2H), 3.97 (dd, J = 10.1, 5.8 Hz, 2H), 3.67 (dd, J = 10.1, 5.4 Hz, 2H), 3.44 (dd, J = 13.1, 8.4 Hz, 2H), 3.35 (dd, J = 8.8, 4.3 Hz, 2H), 2.00 - 1.89 (m, 9H), 1.42 (m, 4H), 1.28 (d, J = 6.2 Hz, 48H), 0.90 (t, J = 6.9 Hz, 6H). 13C NMR (126 MHz, Methanol-d4) δ 175.99, 171.63, 167.42, 167.14, 138.26, 134.66, 134.36, 131.35, 131.26, 131.09, 130.88, 129.73, 129.51, 125.63, 101.75, 76.44, 75.15, 74.95, 71.81, 67.93, 52.39, 51.68, 49.54, 49.37, 49.20, 49.03, 48.86, 48.85, 48.69, 48.52, 37.04, 33.38, 33.13, 30.87, 30.86, 30.84, 30.82, 30.80, 30.71, 30.66, 30.54, 30.53, 30.51, 30.33, 30.20, 30.07, 26.99, 23.79, 21.23, 14.50.



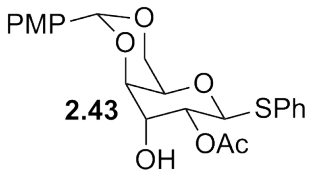
Preparation of 2.33: Sulfated glycolipid **2.32** (25 mg, 0.0234 mmol) was dissolved in an anhydrous 1:1 solution of THF and methanol (5 mL). Freshly prepared 1 M sodium methoxide was added (0.200 mL) to the reaction flask and the reaction was stirred for one hour at room temperature. Reaction completion was verified via mass spectrometry and TLC. Upon completion, the solvent was removed en vacuo and the resulting residue was washed with water (3 x 2 mL), and diethyl ether (3 x 2 mL) (solid was washed via centrifugation, the solvents washes were decanted off and saved, just in case of partial dissolution in water). The purified compound was a white powder (12.5 mg, 66%). 1H NMR (500 MHz, DMSO-d6) δ 7.40 (d, J = 9.0 Hz, 1H), 5.51 (dt, J = 15.5, 6.6 Hz, 2H), 5.38 - 5.30 (m, 2H), 5.07 (d, J = 2.6 Hz, 1H), 4.82 (d, J = 5.4 Hz, 1H), 4.70 (d, J = 4.9 Hz, 1H), 4.21 (d, J = 7.7 Hz, 1H), 4.02 - 3.92 (m, 2H), 3.87 (t, J = 4.5 Hz, 2H), 3.79 - 3.73 (m, 1H), 3.64 (dd, J = 8.7, 4.1 Hz, 1H), 3.52 - 3.38 (m, 4H), 3.23 - 3.18 (m, 1H), 2.00 (s, 3H), 1.95 - 1.86 (m, 2H), 1.50 - 1.36 (m, 4H), 1.22 (m, 46H), 0.83 (t, J = 6.8 Hz, 6H). 13C NMR (126 MHz, Chloroform-d) δ 172.79, 170.90, 170.01, 165.78, 165.75, 165.65, 165.51, 165.09, 164.92, 133.57, 133.48, 133.43, 133.30, 133.21, 129.90, 129.87, 129.83, 129.79, 129.76, 129.73, 129.69, 129.27, 129.06, 128.88, 128.84, 128.80, 128.66, 128.49, 128.44, 128.30, 128.28, 128.26, 101.08, 100.92, 77.28, 77.02, 76.77, 76.43, 74.73, 73.47, 73.03, 72.72, 72.52, 72.39, 72.17, 72.05, 71.78, 69.88, 69.43, 68.32, 67.85, 63.20, 61.35, 47.47, 36.28, 34.13, 31.93, 29.76, 29.75, 29.73, 29.71, 29.68, 29.66, 29.64, 29.61, 29.58, 29.53, 29.49, 29.44, 29.37, 29.29, 29.21, 29.15, 28.27, 25.80, 25.61,

25.42, 24.84, 22.70, 21.08, 20.98, 20.89, 14.13. HRMS (ESI) calcd for $C_{42}H_{79}N_2O_{11}$S- [M+H]+: 819.5410, found: 820.5321.



2.42

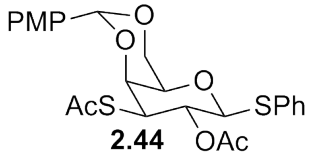
Preparation of 2.42: Diacylated intermediate **2.13a**, isolated during the synthesis of **2.13**, (4.23 g, 10.8 mmol) was dissolved in dry DCM (50 mL), followed by the addition of pyridine (3.50 mL, 43.4 mmol). The reaction was cooled to -10°C. Triflic anhydride (2.01 mL, 11.9 mmol) was added dropwise to the cooled solution. After disappearence of the starting material (via TLC), acyl chloride (2.31 mL, 32.5 mmol) was added to the reaction. After forty minutes the reaction was allowed to warm to room temperature. One hour later the reaction was diluted with saturated $NaHCO_3$ (50 mL), and washed with DCM (3 x 50 mL). The organic layer was dried over $NaSO_4$, concentrated (water bath was 30°C or below).



2.43

Preparation of 2.43: The crude solid **2.42** was dissolved in dry DMF (50 mL), followed by the addition of tetrabutylammonium nitrate (9.34 g, 33.4 mmol). This mixture was stirred overnight at 60°C. The next morning, the mixture was dissolved in cold saturated sodium chloride (50 mL) and washed with DCM (3 x 50 mL). The organic layer was dried over $NaSO_4$, concentrated, and subjected to flash chromatography (35% EtOAc:Hexane). The three step process yielded 2.5 g

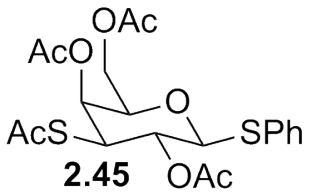
of product (54%). Due to a server outage, various spectroscopic data was unable to be recovered (department wide), due to this missing data should be here.



2.44

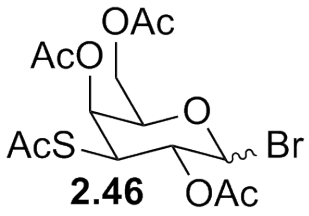
Preparation of 2.44: Alcohol **2.43** (550 mg, 1.27 mmol) and dry pyridine (5.13 mL, 63.6 mmol) were dissolved in dry DCM (20 mL). The vessel was cooled to -10°C, at which point triflic anhydride (0.642 mL, 3.82 mmol) was added dropwise. After two hours the reaction was warmed to room temperature, two hours later the reaction mixture was diluted with DCM (25 mL) and 1 M HCl (50 mL). The mixture was washed with DCM (2 x 50 mL) and the organic phase was collected. The organic layer was then subjected to a saturated $NaHCO_4$ (100 mL) : DCM workup (2 x 50 mL). The organic layer was dried over $NaSO_4$, concentrated, and dried over high vacuum in preparation for the next step. Crude **2.44** was dissolved in dry DMF (20 mL). Potassium thioacetate (581 mg, 5.09 mmol) was added and the reaction was stirred overnight at 50°C. The next day the solvent was pulled off via high vacuum rotary evaporator, the dark reddish brown sludge was dissolved in water (100 mL) and washed with DCM (3 x 50 mL). The organic layer was dried over $NaSO_4$, concentrated, and subjected to flash chromatography (25% EtOAc:Hexane). A light brown powder was isolated. (500 mg, 80% over two steps). 1H NMR (500 MHz, Chloroform-d) δ 7.62 - 7.55 (m, 2H), 7.34 - 7.22 (m, 5H), 6.91 - 6.84 (m, 2H), 5.43 (s, 1H), 5.14 (t, J = 9.9 Hz, 1H), 4.78 (d, J = 9.5 Hz, 1H), 4.34 (dd, J = 12.3, 1.6 Hz, 1H), 4.05 (d, J = 10.1 Hz, 2H), 3.99 (dd, J = 12.4, 1.7 Hz, 1H), 3.66 (q, J = 1.4 Hz, 1H), 2.31 (s, 3H), 2.05 (s, 3H). 13C NMR (126 MHz, Chloroform-d)

δ 194.83, 169.23, 160.16, 133.35, 131.71, 130.01, 128.76, 127.95, 127.71, 113.51, 101.27, 86.93, 77.27, 77.21, 77.01, 76.76, 75.62, 71.19, 69.04, 66.73, 55.34, 48.41, 30.54, 21.05, 20.82, 14.20.



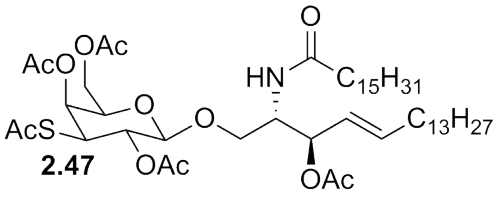
2.45

Preparation of 2.45: A solution of **2.44** (1,620 mg, 3.31 mmol), water (10 mL), and glacial acetic acid (40 mL) was stirred overnight at room temperature. Upon completion the water and acetic acid were pulled off via high vacuum rotary evaporation. The light yellow slurry was quenched with excess saturated $NaHCO_3$ and the product was extracted with DCM (3 x 50 mL). The organic phase was dried over $NaSO_4$, concentrated, and then dissolved in pyridine (50 mL). Acetic anhydride (1.25 mL, 13.2 mmol) and DMAP (40 mg, 0.331 mmol) were added and the reaction mixture was stirred for sixteen hours. The pyridine was removed via high vacuum rotary evaporator. The dark brown residue was dissolved in water and washed with DCM (3 x 50 mL). The resultant syrup was loaded onto a silica gel column and the product was eluted out in 30% EtOAc:Hexanes. A clear light brown syrup was collected (1100 mg, 73%). 1H NMR (500 MHz, Chloroform-d) Due to a server outage, various spectroscopic data was unable to be recovered (department wide), due to this missing data should be here. [M+NH4]+: 456.0913, found: 474.1305.



2.46

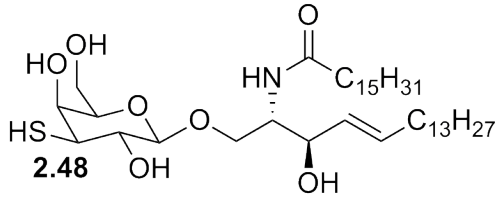
Preparation of 2.46: Thioacetate **2.45** (568 mg, 1.25 mmol) was dissolved in acetic acid (20 mL). One equivalent of N-iodosuccinimide (280 mg, 1.25 mmol) was added. The reaction was monitored via TLC. More N-iodosuccinimide was added every thirty minutes if necessary. Once complete, The acetic acid was pulled off via a high vacuum rotary evaporator. The yellow solid was subjected to a $NaHCO_3$ (50 mL) : DCM (3 x 50 mL) workup. The organic layer was dried over sodium sulfate ($NaSO_4$), concentrated via rotary evaporator, and placed on a high vacuum system in preparation for the second step of the reaction. This intermediate was dissolved in dry DCM (15 mL), followed by the slow addition of hydrobromic acid solution (33 wt. % in acetic acid)(6 mL). Upon completion, the acetic acid/HBr/DCM was pulled off via a high vacuum rotary evaporator. The solid was subjected to a $NaHCO_3$ (50 mL) : DCM (3 x 50 mL) workup. The organic layer was dried over sodium sulfate ($NaSO_4$), concentrated via rotary evaporator, and subjected to flash chromatography (20%, 25% EtOAc:Hexane). The product was stored in the fridge for the next reaction (346 mg, 70%).



2.47

Preparation of 2.47: Bromide donor **2.46** (143 mg, 0.336 mmol), palmitic ceramide (130

mg, 0.224 mmol), and 4 angstrom molecular sieves (500 mg) were stirred together in DCM (8 mL) at 0°C. After one hour, silver triflate (87 mg, 0.336 mmol) was added to the reaction. After four hours, the reaction was filtered through a bed of silica gel (washed with EtOAc). The filtrate was concentrated, and subjected to flash chromatography (30% EtOAc:Hexane). The isolated product was a white powder (95 mg, 46%). 1H NMR (500 MHz, Chloroform-d) δ 5.77 (dt, J = 15.4, 6.7 Hz, 1H), 5.67 (d, J = 9.1 Hz, 1H), 5.37 (m, 1H), 5.29 (dd, J = 3.2, 1.2 Hz, 1H), 5.25 (t, J = 7.0 Hz, 1H), 4.99 (dd, J = 11.8, 7.6 Hz, 1H), 4.49 (d, J = 7.7 Hz, 1H), 4.32 (m, 1H), 4.16 - 4.06 (m, 2H), 4.06 - 3.95 (m, 2H), 3.95 - 3.89 (m, 1H), 3.59 (dd, J = 10.1, 4.5 Hz, 1H), 2.32 (s, 3H), 2.15 (s, 3H), 2.04 (t, J = 4.6 Hz, 15H), 1.62 - 1.56 (m, 2H), 1.37 - 1.20 (m, 47H), 0.88 (t, J = 6.9 Hz, 6H). 13C NMR (126 MHz, Chloroform-d) δ 193.68, 172.65, 171.13, 170.37, 169.90, 169.62, 137.10, 124.61, 102.02, 77.26, 77.21, 77.01, 76.76, 73.77, 73.28, 68.64, 67.85, 66.95, 61.80, 60.39, 50.53, 46.79, 36.85, 32.33, 31.93, 30.51, 29.72, 29.69, 29.67, 29.66, 29.56, 29.52, 29.44, 29.37, 29.34, 29.28, 29.00, 25.70, 22.70, 21.13, 21.05, 20.74, 20.70, 20.59, 14.21, 14.12.

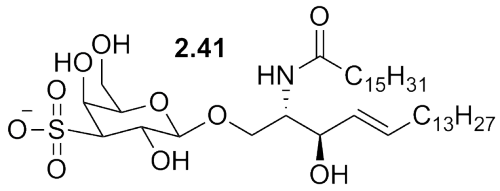


2.48

Preparation of 2.48: Glycolipid **2.47** (83 mg, 0.0897 mmol) was dissolved in an anhydrous 1:1 solution of THF and methanol (10 mL). Freshly prepared 1 M sodium methoxide was added (0.300 mL) to the reaction flask and the reaction was stirred for one hour at room temperature. Reaction completion was verified via mass spectrometry and TLC. Upon completion, the solvent was removed en vacuo and the resulting residue was subjected to flash chromatography (5%, 10%,

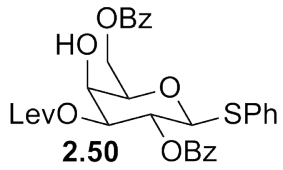
20% MeOH:DCM). The purified compound was a white powder (40 mg, 63%). 1H NMR (500 MHz, DMSO-d6) δ 7.41 (d, J = 9.1 Hz, 1H), 5.51 (dd, J = 14.7, 7.3 Hz, 1H), 5.34 (dd, J = 15.4, 7.2 Hz, 1H), 5.13 (d, J = 4.4 Hz, 1H), 4.79 (d, J = 5.5 Hz, 1H), 4.69 (d, J = 6.5 Hz, 1H), 4.54 (t, J = 5.6 Hz, 1H), 4.07 (d, J = 7.4 Hz, 1H), 4.00 - 3.91 (m, 2H), 3.91 - 3.84 (m, 1H), 3.78 (m, 1H), 3.49 (m, 3H), 3.43 - 3.30 (m, 3H), 2.95 (dd, J = 11.2, 2.9 Hz, 1H), 1.97 (dt, J = 42.8, 6.8 Hz, 6H), 1.22 (m, 46H), 0.84 (t, J = 6.7 Hz, 6H). 13C NMR (126 MHz, DMSO-d6) δ 172.32, 131.89, 131.81, 106.02, 77.94, 71.16, 68.76, 66.59, 61.24, 60.36, 53.50, 40.66, 40.57, 40.49, 40.40, 40.33, 40.24, 40.16, 40.07, 39.99, 39.90, 39.73, 39.64, 39.57, 36.07, 32.19, 31.73, 29.57, 29.56, 29.52, 29.51, 29.47, 29.45, 29.42, 29.25, 29.23, 29.14, 25.82, 22.51, 14.33.

2.41



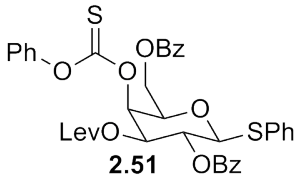
Preparation of 2.41: To a solution of **2.48** (30 mg, 42.0 μmol), sodium acetate (3.44 mg, 42.0 μmol), and acetic acid (1.5 mL) was added 33 % wt./v. hydrogen peroxide (35 μL, 0.377 mmol). The reaction was maintained at 80°C for five hours at which point the solution was concentrated and subjected to flash chromatography. A very polar eluent method (0.5:5:95, 1:10:90, 1:20:80 ammonium hydroxide:MeOH:DCM) was employed to purify the compound (4 mg, 13 %) (had to do multiple columns to purify the compound, each column resulted in a loss of material, 13 % yield is indictive of the pure isolated compound). 1H NMR (500 MHz, DMSO-d6) δ 7.45 (d, J = 8.9 Hz, 1H), 5.57 (dd, J = 14.5, 7.4 Hz, 1H), 5.42 (dd, J = 15.5, 6.9 Hz, 1H), 5.18 (s, 1H), 4.92 - 4.83 (m, 2H), 4.66 (d, J = 6.0 Hz, 1H), 4.28 (d, J = 7.4 Hz, 1H), 4.06 (d, J = 2.4 Hz, 1H), 3.97 (m,

2H), 3.86 - 3.72 (m, 3H), 3.61 - 3.43 (m, 4H), 2.03 (m, 4H), 1.49 (m, 2H), 1.29 (m, 46H), 0.91 (t, J = 6.8 Hz, 6H). 13C NMR (126 MHz, DMSO-d6) δ 172.15, 131.65, 131.62, 109.99, 105.01, 77.93, 71.60, 68.89, 66.90, 64.82, 62.63, 60.41, 53.79, 40.57, 40.48, 40.41, 40.31, 40.24, 40.15, 40.07, 39.98, 39.90, 39.81, 39.65, 39.48, 36.07, 32.21, 31.75, 29.58, 29.54, 29.52, 29.49, 29.47, 29.40, 29.22, 29.19, 29.17, 29.16, 25.76, 22.54, 14.39. HRMS (ESI) calcd for $C_{40}H_{76}NO_{10}S$- [M+H]+: 762.5195, found: 764.5095.



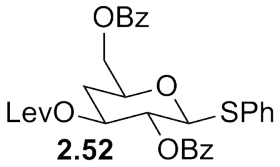
2.50

Preparation of 2.50: Compound **2.14a** (1.00 g, 2.11 mmol) was dissolved in dry pyridine (20 mL) at 0°C. One equivalent of benzoyl chloride was added to the flask. Upon completion, the solvent was removed in vacuo. The brown syrup was dissolved in water, washed with DCM (3 x 50 mL), dried with $NaSO_4$, concentrated, and subjected to flash chromatography. A pale clear syrup was collected (1.21 g, 99%). 1H NMR (500 MHz, Chloroform-d) δ 8.04 (m, 4H), 7.63 - 7.55 (m, 2H), 7.46 (t, J = 7.3 Hz, 6H), 7.22 - 7.16 (m, 1H), 7.11 (t, J = 7.7 Hz, 2H), 5.64 (t, J = 9.9 Hz, 1H), 5.10 (dd, J = 9.8, 3.1 Hz, 1H), 4.89 (d, J = 10.1 Hz, 1H), 4.66 (t, J = 6.3 Hz, 2H), 4.31 (t, J = 3.5 Hz, 1H), 4.07 - 4.01 (m, 1H), 2.98 (d, J = 4.6 Hz, 1H), 2.56 - 2.47 (m, 2H), 2.47 - 2.38 (m, 2H), 2.12 (s, 3H). 13C NMR (126 MHz, Chloroform-d) δ 207.82, 171.75, 166.31, 165.26, 133.30, 133.20, 133.18, 132.10, 129.88, 129.77, 129.75, 129.52, 128.78, 128.44, 128.41, 127.74, 86.87, 77.24, 76.98, 76.73, 76.16, 75.07, 67.92, 66.96, 63.64, 38.24, 29.62, 28.27.



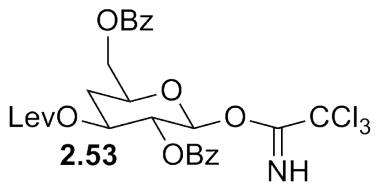
2.51

Preparation of 2.51: Compound **2.50** (510 mg, 0.882 mmol) was dissolved in DCM (10 mL), followed by the addition of phenyl chlorothionoformate (244 μL, 1.76 mmol) and DMAP (301 mg, 2.47 mmol). After completion, a $NaHCO_3$/DCM(3 x 50 mL) workup was carried out, dried ($NaSO_4$), concentrated, and loaded onto a silica gel column (eluent 20% EtOAc:Hexanes).



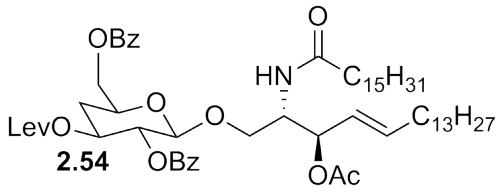
2.52

Preparation of 2.52: The recovered powder was dissolved in dry benzene (5 mL) in a flame-dried RBF that was connected to a condenser. Tributyl tin hydride (376 μL, 1.42 mmol) was added to the reaction vessel, and the mixture was degassed and stirred for 15 minutes. After the 15 minutes, AIBN (58 mg, 0.353 mmol) was added and the reaction was heated to 80°C. After two hours, the reaction was cooled to room temperature, the solvent was evaporated off, and the syrup was subjected to a a $NaHCO_3$/DCM (3 x 50 mL) workup. The resultant syrup was loaded onto a bed of silica gel and separated by a 20% EtOAc:Hexane wash. The solid formed was collected and analyzed (450 mg, 91% over two steps).



2.53

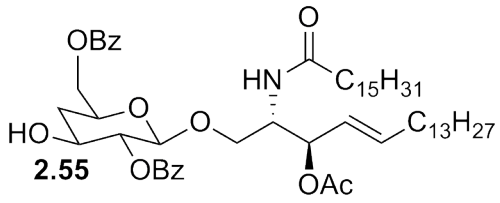
Preparation of 2.53: Fully protected galactose **2.52** (180 mg, 0.320 mmol) was dissolved in an acetone/water solution (20mL/3mL), followed by the portion wise addition of NBS (170 mg, 0.961 mmol). Subsequent equivalents of NBS were added until the reaction was completed (monitored by TLC). Upon completion, the reaction was quenched with solid $NaHCO_3$, and the acetone was evaporated off. The remaining mixture was dissolved in water, washed with DCM (3 x 50 mL), dried with $NaSO_4$, concentrated, and subjected to flash chromatography (30% EtOAc:Hexanes). The white powder was dissolved in DCM (25 mL). Solid potassium carbonate (700 mg) was added, followed by the addition of excess trichloroacetonitrile (0.640 mL, 6.4 mmol). Once setup, the reaction was stirred for sixteen hours at room temperature. In the morning, the potassium carbonate was filtered and washed with EtOAc (100 mL). The filtrate was dried off at or below 30°C en vacuo. This pale yellow solid was loaded onto a bed of silica gel and was eluted with 25% EtOAc/Hexane. The two anomeric isomers were collected, combined, and concentrated at or below 30°C (160 mg, 82% from two steps).



2.54

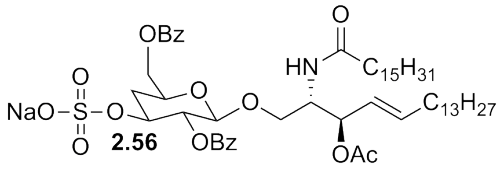
Preparation of 2.54: Donor **2.53** (160 mg, 0.261 mmol) was stirred for one hour at room temperature in the presence of ceramide **2.11d** (216 mg, 0.313 mmol), 4 angstrom molecular sieves (750 mg), and dry DCM (5 mL). The vessel was cooled to -10°C (acetone/ice bath), followed by the dropwise addition of trimethylsilyltriflate (23 μL, 0.130 mmol). The reaction temperature was maintained for two hours then quenched by the dropwise addition of TEA. The molecular sieves

were filtered and washed with EtOAc by means of a plug silica gel column. The filtrate was dried over $NaSO_4$, concentrated, and subsequently loaded onto a silica gel column. Purification was achieved with a 20%, 30% EtOAc/Hexane eluent system (170 mg, 57%). 1H NMR (500 MHz, Chloroform-d) δ 8.04 (dd, J = 19.3, 7.8 Hz, 4H), 7.58 (td, J = 7.2, 4.6 Hz, 2H), 7.46 (q, J = 8.3 Hz, 4H), 5.70 (d, J = 15.3 Hz, 1H), 5.55 (d, J = 9.2 Hz, 1H), 5.37 - 5.27 (m, 3H), 5.25 - 5.13 (m, 3H), 4.52 (d, J = 7.6 Hz, 1H), 4.42 (dd, J = 10.5, 5.0 Hz, 2H), 4.24 (m, 1H), 4.04 - 3.94 (m, 2H), 3.49 (dd, J = 9.9, 3.8 Hz, 1H), 2.66 - 2.57 (m, 2H), 2.54 - 2.47 (m, 2H), 2.06 - 1.91 (m, 10H), 1.78 - 1.68 (m, 4H), 1.39 - 1.18 (m, 56H), 0.87 (t, J = 6.8 Hz, 6H). 13C NMR (126 MHz, Chloroform-d) δ 205.86, 172.52, 171.92, 169.69, 166.13, 165.45, 136.91, 133.42, 133.26, 129.87, 129.85, 129.74, 129.68, 129.62, 129.43, 128.54, 128.49, 128.47, 128.43, 124.82, 101.04, 77.24, 77.18, 76.98, 76.73, 73.58, 72.90, 70.43, 69.75, 67.22, 65.74, 60.34, 50.28, 37.77, 36.39, 32.64, 32.24, 31.90, 31.88, 29.78, 29.75, 29.71, 29.69, 29.65, 29.62, 29.59, 29.55, 29.50, 29.36, 29.34, 29.29, 29.24, 29.17, 28.96, 28.04, 27.21, 27.19, 25.45, 22.66, 21.00, 14.17, 14.08. HRMS (ESI) calcd for $C_{69}H_{107}NO_{12}$ [M+H]+: 1141.7793, found: 1142.7913.



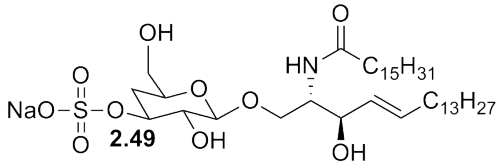
Preparation of 2.55: A 1 M solution of hydrazine was freshly prepared in dry THF. A portion of this was stirred with glacial acetic acid for five minutes. This mixture was added to a RBF that contained compound **2.54** (170 mg, 0.148 mmol) and dry THF:Methanol (10:1, 11 mL). After one hour the reaction was completed by the observance of product and the complete

disappearance of starting material via M.S. The solvent was dried off en vacuo at or below 30°C. The white slurry was quenched with saturated $NaHCO_3$ solution, followed by the extraction of product in DCM washes (3 x 15 mL). The DCM washes were dried over $NaSO_4$, evaporated, and purified via flash chromatography (30% EtOAc:Hexane). The white powder product collected was isolated in excellent yield (130 mg, 85%). 1H NMR (500 MHz, Chloroform-d) δ 8.05 (dd, J = 7.9, 3.4 Hz, 4H), 7.58 (t, J = 7.4 Hz, 2H), 7.45 (q, J = 7.2 Hz, 4H), 5.73 - 5.64 (m, 1H), 5.59 (d, J = 9.1 Hz, 1H), 5.34 (t, J = 4.8 Hz, 2H), 5.22 (t, J = 7.3 Hz, 1H), 4.92 (t, J = 8.4 Hz, 1H), 4.50 (d, J = 7.7 Hz, 1H), 4.42 (t, J = 5.2 Hz, 2H), 4.25 (m, 1H), 4.00 (m, 2H), 3.94 - 3.88 (m, 1H), 3.52 (dd, J = 10.0, 3.8 Hz, 1H), 2.05 - 1.97 (m, 5H), 1.97 - 1.91 (m, 4H), 1.73 (m, 2H), 1.39 - 1.20 (m, 56H), 0.87 (t, J = 6.7 Hz, 6H). δ 172.67, 169.72, 166.56, 166.21, 136.90, 133.52, 133.24, 129.87, 129.84, 129.76, 129.70, 129.67, 129.65, 129.49, 128.55, 128.46, 124.75, 100.75, 77.24, 77.08, 76.99, 76.74, 73.58, 69.96, 69.76, 67.14, 65.99, 50.37, 36.43, 35.41, 32.24, 31.90, 31.87, 29.77, 29.74, 29.70, 29.69, 29.67, 29.64, 29.61, 29.58, 29.54, 29.49, 29.35, 29.33, 29.29, 29.24, 29.22, 29.16, 28.95, 27.20, 27.19, 25.48, 22.65, 20.97, 14.07. HRMS (ESI) calcd for $C_{64}H_{101}NO_{10}$ [M+H]+: 1043.7425, found: 1044.7501.



Preparation of 2.56: Glycolipid **2.55** (130 mg, 0.125 mmol) was dissolved in dry pyridine (5 mL) followed by the addition of sulfur trioxide pyridine complex (397 mg, 2.49 mmol). The reaction was stirred at room temperature for fourteen hours, followed by TLC (indicating the

completion of the reaction). The solvent was removed en vacuo, and the remaining slurry was dissolved in saturated $NaHCO_3$, washed with DCM (3 x 10 mL), dried ($NaSO_4$), and concentrated. The residue was chromatographed (SiO_2, methanol/DCM 5%, 10%) to afford product **2.19d** (127 mg, 91%). 1H NMR (500 MHz, Methanol-d4) δ 8.07 (t, J = 8.5 Hz, 4H), 7.60 (dt, J = 19.7, 7.4 Hz, 2H), 7.47 (dt, J = 21.1, 7.7 Hz, 4H), 5.60 (dd, J = 14.7, 7.2 Hz, 1H), 5.33 (q, J = 7.1, 6.0 Hz, 1H), 5.29 - 5.20 (m, 2H), 5.03 (t, J = 8.7 Hz, 2H), 4.87 (m, 6H), 4.75 (td, J = 10.7, 5.4 Hz, 2H), 4.68 (d, J = 7.9 Hz, 1H), 4.45 (q, J = 4.9, 4.0 Hz, 2H), 4.29 - 4.19 (m, 2H), 4.06 (dd, J = 11.5, 5.6 Hz, 2H), 3.85 (dd, J = 10.1, 5.6 Hz, 2H), 3.56 (dd, J = 10.0, 5.2 Hz, 1H), 3.32 (d, J = 19.5 Hz, 6H), 2.67 - 2.58 (m, 2H), 2.03 (q, J = 6.4 Hz, 5H), 1.95 - 1.84 (m, 8H), 1.42 (dd, J = 16.2, 7.8 Hz, 2H), 1.39 - 1.14 (m, 60H), 0.89 (t, J = 6.7 Hz, 6H). 13C NMR (126 MHz, Methanol-d4) δ 174.45, 170.10, 166.22, 166.08, 136.73, 132.96, 132.77, 130.09, 129.81, 129.69, 129.44, 129.42, 129.26, 128.24, 128.01, 124.27, 100.54, 74.76, 73.55, 73.36, 69.76, 66.49, 65.87, 50.44, 48.44, 48.10, 48.04, 47.93, 47.87, 47.76, 47.70, 47.59, 47.53, 47.42, 47.25, 47.07, 35.66, 35.61, 33.57, 31.89, 31.68, 31.65, 29.43, 29.42, 29.40, 29.38, 29.37, 29.35, 29.27, 29.22, 29.21, 29.20, 29.08, 29.06, 29.04, 28.94, 28.91, 28.85, 28.77, 28.75, 28.63, 26.73, 26.72, 25.53, 22.33, 19.69, 13.07. HRMS (ESI) calcd for $C_{64}H_{100}NO_{13}S$- [M]+: 1122.6921, found: 1122.6849 (neg ion mode).



Preparation of 2.49: Sulfated glycolipid **2.19d** (97 mg, 0.0864 mmol) was dissolved in an anhydrous 1:1 solution of THF and methanol (5 mL). Freshly prepared 1 M sodium methoxide was

added (0.3 mL) to the reaction flask and the reaction was stirred for one hour at room temperature. Reaction completion was verified via mass spectrometry and TLC. Upon completion, the solvent was removed en vacuo and the resulting residue was washed with water (3 x 2 mL), and diethyl ether (3 x 2 mL) (solid was washed via centrifugation, the solvents washes were decanted off and saved, just in case of partial dissolution in water). The purified compound was a white powder (72 mg, 96%). 1H NMR (500 MHz, DMSO-d6) δ 7.44 (d, J = 8.9 Hz, 1H), 5.50 (dd, J = 14.6, 7.4 Hz, 1H), 5.31 (dt, J = 10.0, 5.9 Hz, 3H), 4.84 (s, 1H), 4.70 (s, 1H), 4.18 - 4.07 (m, 2H), 3.86 (t, J = 7.7 Hz, 1H), 3.73 (dt, J = 8.4, 4.2 Hz, 2H), 3.41 - 3.36 (m, 2H), 3.31 (m, 1H), 3.04 (t, J = 8.3 Hz, 1H), 1.96 (m, 4H), 1.41 (m, 2H), 1.21 (m, 56H), 0.83 (t, J = 6.8 Hz, 6H). 13C NMR (126 MHz, DMSO-d6) δ 172.19, 131.70, 131.67, 130.06, 130.05, 104.46, 76.57, 74.27, 72.48, 71.44, 69.26, 64.08, 53.71, 40.61, 40.52, 40.45, 40.35, 40.28, 40.19, 40.11, 40.02, 39.94, 39.85, 39.69, 39.59, 39.52, 36.09, 34.18, 32.21, 31.75, 31.72, 29.57, 29.56, 29.53, 29.50, 29.48, 29.42, 29.39, 29.29, 29.27, 29.23, 29.19, 29.16, 29.13, 29.03, 28.99, 27.00, 26.98, 25.80, 22.53, 14.36. HRMS (ESI) calcd for $C_{48}H_{90}NO_{10}S$- [M]+: 872.6291, found: 872.6181 (neg ion mode).

2.6 Immunological methods

Note: all immunological testing was carried out under the direction of Albert Bendelac and Erin Adams at the University of Chicago.

Human subjects

Blood samples were obtained following a protocol approved by the Institutional Review

Board. Written informed consent was received from all participants prior to inclusion in the study.[46]

CD1d production and sulfatide loading

Human CD1d was expressed by the baculovirus method in Hi5 insect cells. Purified CD1d at 1 mg/mL was incubated for 1 hour with a 10-fold molar excess of bovine brain sulfatides (Matreya) in HEPES-buffered saline, pH 7.2, at 37 C. 4 μg of sulfatide-loaded CD1d, and 4 μg of unloaded control CD1d, were run on an pH 3-9 isoelectroic-focusing gel (GE Healthcare) using the PhastGel system (GE Healthcare).[46]

Flow cytometry

CD1d-lipid tetramers were produced by incubating 0.011 mg of CD1d with 0.0017 mg of lipid antigens in 0.020 mL PBS at 37°C overnight. CD1d-lipid complexes were then incubated with streptavidin-APC (Invitrogen, CA) for 2 hours at room temperature in a 0.110 mL reaction at 4:1 molar ratio.[46]

Fluorochrome labeled monoclonal antibodies against human CD3 (HIT3a), TCR Vδ1 (TS8.2), TCR $\alpha\beta$ (IP26), and TCR $\gamma\delta$(B1) were purchased from eBioscience, BD Biosciences, Immunotech, and Thermo Scientific. Samples were analyzed on an LSRII (BD Bioscience), or sorted on a FACSAria (BD Bioscience) with doublet exclusion and DAPI staining of dead cells in most experiments. Data was analyzed using FlowJo (Tree Star).[46]

For CD1d-Sulfatide+ cells enrichment, human PBMCs were isolated from blood by centrifugation over Ficoll-Paque (GE healthcare, NJ, USA). Cells stained with allophycocyanin conjugated CD1d-Sulfatide tetramer on ice for 1 hour, before enrichment with anti-allophycocyanin

conjugated beads and the autoMACS cell separator (Miltenyi Biotech).[46]

Generation of CD1d-sulfatide tetramer+ clones

CD1d-sulfatide tetramer+ cells were enriched by MACS and sorted as single cells in 96 well plates with feeder cells consisting of irradiated (4500 rad) allogeneic PBMC ($5x10^5$/well) and irradiated (10,000 rad) EBV transformed cells (5x103/well) in RPMI1640 medium containing 8% FCS (Biowest), 2% AB human serum (Atlanta Biologicals), 0.055 mM 2-ME, 2mM L-glutamine (Cellgro), 1mM sodium pyruvate (Invitrogen), 1mM nonessential amino acids (Invitrogen), 0.001 mg/ml PHA and 100 U/ml human IL-2. Cells were restimulated every 2-3 weeks according to the same protocol.[46]

TCR sequencing

Total RNA was isolated from sorted CD1d-sulfatide+CD3+Vδ1+ cells using a combination of Trizol (Invitrogen) and the RNeasy Mini Kit (Qiagen) and was reverse-transcribed with Superscript III (Invitrogen) using oligo-d(T). TCR sequences were amplified by PCR with the following primers (18): 5′-ctgtcaacttcaagaaagcagcgaaa -3′ (Vδ1), 5′- tgggagagatgacaatagcaggatc -3′ (Cδ), 5′- cgcaaggacaaggaacaacttgagatt -3′ (Vγ2), 5′- ctatgacgtctccaccgcaagg -3′ (Vγ3), 5′- cggaagcacaaggaacttgagaat -3′ (Vγ4), 5′- ggtggagctggatattgatactacga -3′ (Vγ5), 5′- gcaagcacagggaagagccttaaattta -3′ (Vγ8), 5′- tggtgaagtcatacagttcctggtg -3′ (Vγ9), and 5′- gaatcgtgttgctcttcttttcttgcc -3′ (Cγ). For sequencing, PCR products were subcloned using Topo TA Cloning Kit (Invitrogen, CA) and randomly selected clones were processed for sequencing (Applied Biosystems 3730XL). Sequences were analysed using the IMGT tools (http://imgt.cines.fr/).[46]

Recombinant TCRs and CD1d-binding assay

The DP10.7 TCR γ and δ variable domains were fused to the TCR constant domains from β and α chains, respectively, and the hybrid ectodomains were cloned into the pET28a vector and expressed in BL21 Escherichia coli cells. The resultant inclusion bodies were solubilized in 7 M guanidine HCl and refolded as a heterodimer by dilution with 50 mM Tris pH 8.0 in the presence of 10:1 reduced:oxidized glutathione (Sigma). The protein was purified by Ni-NTA chromatography (Qiagen) followed by Superdex 200 size exclusion chromatography (Amersham). For production by the baculovirus method in Hi5 insect cells, both the DP10.7 TCR and AB18.1 γ and δ variable domains were fused to $\alpha\beta$ TCR constant domains, as above, then cloned into the pACGP67a vector (Pharmingen) and produced as a heterodimer by the baculovirus expression system in High FiveTM insect cells as described [13]. Human CD1d and β2M were cloned into the pACGP67a vector for baculovirus expression in High FiveTM cells, with CD1d containing a C-terminal 6x His tag, allowing for heterodimer purification over Ni-NTA resin. CD1d was purified by Superdex 200 size exclusion chromatography, loaded with a tenfold molar excess of bovine brain sulfatides (Matreya) and then purified by MonoQ anion exchange chromatography (Amersham). For the native gels, equimolar amounts of the TCRs and either unloaded or sulfatide-loaded CD1d were combined at a total protein concentration of 1 mg/mL and incubated for 15 min at RT. A total of 0.004 mL of the complexes were loaded on a 12.5% homogenous gel (GE Healthcare), along with the same amount of noncomplexed TCRs and CD1d proteins as controls. The gels were run using the PhastGel system (Pharmacia Biotech) and stained with PhastGel Blue R (Amersham) at 0.02% in 3:1:6 methanol:acetic acid:water. Gels were destained with 3:1:6 methanol:acetic acid:water for imaging.[46]

Chapter 3

Synthesis and Trafficking Studies of a BODIPY-Appended Sulfatide Analog

3.1 Introduction

The presence of sulfatide-specific T cells in the blood of healthy individuals, presented in chapter 2, raised several interesting questions. How could these autoreactive T cells escape thymic deletion, especially since high concentrations of sulfatide were reproducibly measured in healthy human serum?[59,62] Are these T cells responsible for the increased frequency of sulfatide-reactive T cells in the blood of MS patients? Do they play a significant role in MS pathogenesis? These results and subsequent questions motivated research in this area.

We were particularly interested in solving the paradoxical presence of high-affinity TCR sulfatide-reactive $\gamma\delta$ T cells. During our investigation, Cernadas *et al.* published research suggest-

ing that the trafficking pattern of CD1b, unlike that of CD1a, impaired sulfatide presentation.[63] As described in section 1.2.1, CD1a trafficks through early and recycling endosomes on its way to the cell surface, whereas CD1b and CD1d are localized in the late endosome and lysosome where microbial lipids accumulate during infections.[12,13,21] It has been shown that CD1a does not contain a tyrosine based cytoplasmic tail motif unlike the other isoforms of CD1. It has been suggested that this difference allows CD1a to traffick distinctly from CD1b and d.[63]

Motivated by their preliminary findings that dendritic cells pulsed with sulfatide maintained the ability to stimulate CD1a four times longer than CD1b, Cernadas *et al.* used CD1b/a chimeric molecules, CD1a molecules that expressed the cytoplasmic tail of CD1b, to traffick sulfatide. Direct staining of exogenously added sulfatide with a sulfatide-specific monoclonal antibody demonstrated sulfatides accumulate exclusively in the early endocytic compartment rather than in the late endosome/lysosome, an intriguing observation that led to their hypothesis that defective presentation by CD1b, which recycled to the late endosome/lysosome, results from a mismatch of trafficking patterns.[63] An alternative explanation might be that the trafficking of CD1-sulfatide complexes to the late endosome/lysosome impaired their expression on the cell surface, for example due to fast dissociation in the acidic environment. It is also possible that the sulfatide-specific antibody may not be able to detect sulfatide in the lysosome secondary to antigenic epitope changes. This paper's surprising conclusion motivated us to test the intracellular trafficking of human CD1d to hopefully understand the posed paradoxical sulfatide-reactive T cell question.

With the synthesized sulfatides from chapter 2, we were able to do similar immunological testing of sulfatide with chimeric CD1d/a molecules instead of CD1b/a chimeric molecules. We

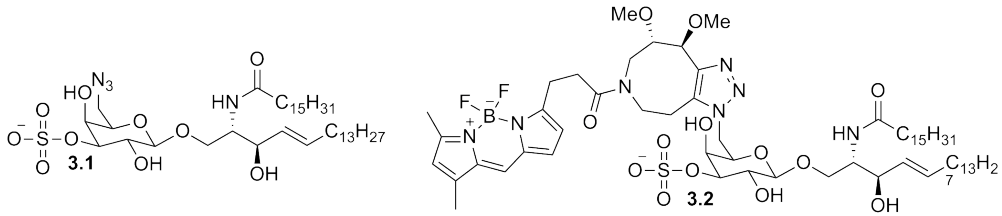


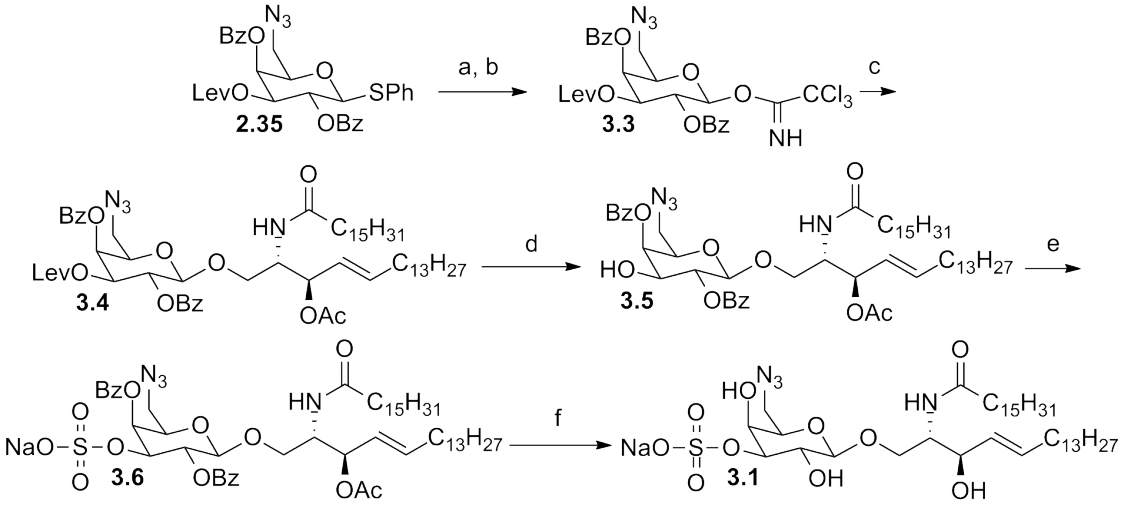
Figure 3.1 Novel azido-sulfatide variant **3.1** and BODIPY-appended sulfatide variant **3.2**

also synthesized a novel synthetic variant of sulfatide, azido-sulfatide (with an azide on C″6, Figure 3.1). Unlike the sulfatide-specific antibody method, azido-sulfatide can be imaged post trafficking in the presence of a BODIPY appended-alkyne (synthesized in house, also commerically available). The strained eight-membered ring allows for quantitative copper-free click chemistry to the azido-sulfatide to give BODIPY-appended sulfatide variant **3.2**. This florescence detected method showed that azido-sulfatide accumulated in the late endosome/lysosome compartment like CD1d. Despite correction of the trafficking mismatch, azido-sulfatide was still poorly presented by CD1d compared with CD1d/a. We conclude therefore that defective presentation of sulfatide by CD1d could be a consequence of the recycling of CD1d-sulfatide complexes in late endosome/lysosome compartments.

3.2 Synthesis of 6-azido-sulfatide 3.1

The synthetic design for **3.1** was quite simple since we had previously installed an azide at the 6-hydroxyl position during the synthesis of **2.34**. Scheme 3.1 demonstrates that we simply converted **2.35** into a Schmidt glycosylation donor. **3.2** was coupled with a c(16:0) ceramide to yield **3.3**. Due to the disarming effect of the azide, the coupling reaction suffered from extremely poor yields. Suf-

ficient **3.3** was isolated, though, to continue the synthesis. Next, the levulinic group was selectively removed with hydrazine and acid to give 3″-hydroxyl compound **3.4**. In dry pyridine, compound **3.4** formed fully protected 3″-sulfo-sulfatide **3.5** in the presence of sulfur trioxide-pyridine complex. Global removal with sodium methoxide in THF and methanol afforded 6″-azido-sulfatide **3.1**.



Reagents and conditions: a. NBS, H_2O, Acetone; b. K_2CO_3, Cl_3CCN, DCM, 43% (two steps); c. 4Å M.S., ceramide, TMSOTf, DCM, 0 ^oC, 14%; d. hydrazine, AcOH, MeOH, THF, 94%; e. SO_3-Py, py, 77%; f. NaOMe, THF, MeOH, 44%.

Scheme 3.1 Representative synthesis of sulfatide **3.1**

3.3 Results

3.3.1 Presentation of sulfatide by CD1d-expressing cells

Our first set of experiments compared the stimulatory properties of a variety of our sulfatides bound to either wild-type CD1d or chimeric CD1d/a. Indeed, Figure 3.2(A) shows that every CD1d/a

bound sulfatide tested had enhanced presentation and stimulation when compared to wild-type CD1d. In contrast, and as expected, presentation of α-GalCer by CD1d/a molecules to an NKT cell clone was greatly impaired. The CD1d/a study did not verify if a mismatch of trafficking patterns trafficked CD1-sulfatide complexes to the late endosome/lysosome instead of the lysosome. We tested the hypothesis that sulfatide presentation on the cell surface was impaired by imaging our novel synthetic variant of sulfatide, azido-sulfatide (**3.1**), which, unlike sulfatide, accumulated in the late endosome/lysosome compartment like CD1d (Figure 3.2(B)). Localization of azido-sulfatide in the lysosome and late endosome is most likely due to fast dissociation of sulfatide from CD1d in the acidic environment of the lysosome. Our data affirms that the sulfatide-specific antibody did not detect sulfatide in the lysosome most likely to antigenic epitope changes. Despite correction of the trafficking mismatch of CD1, azido-sulfatide was still poorly presented by CD1d compared with CD1d/a (Figure 3.2(A)). These data therefore conclude that defective presentation of sulfatide by CD1d could be a consequence of the recycling of CD1d-sulfatide complexes in late endosome/lysosome compartments.

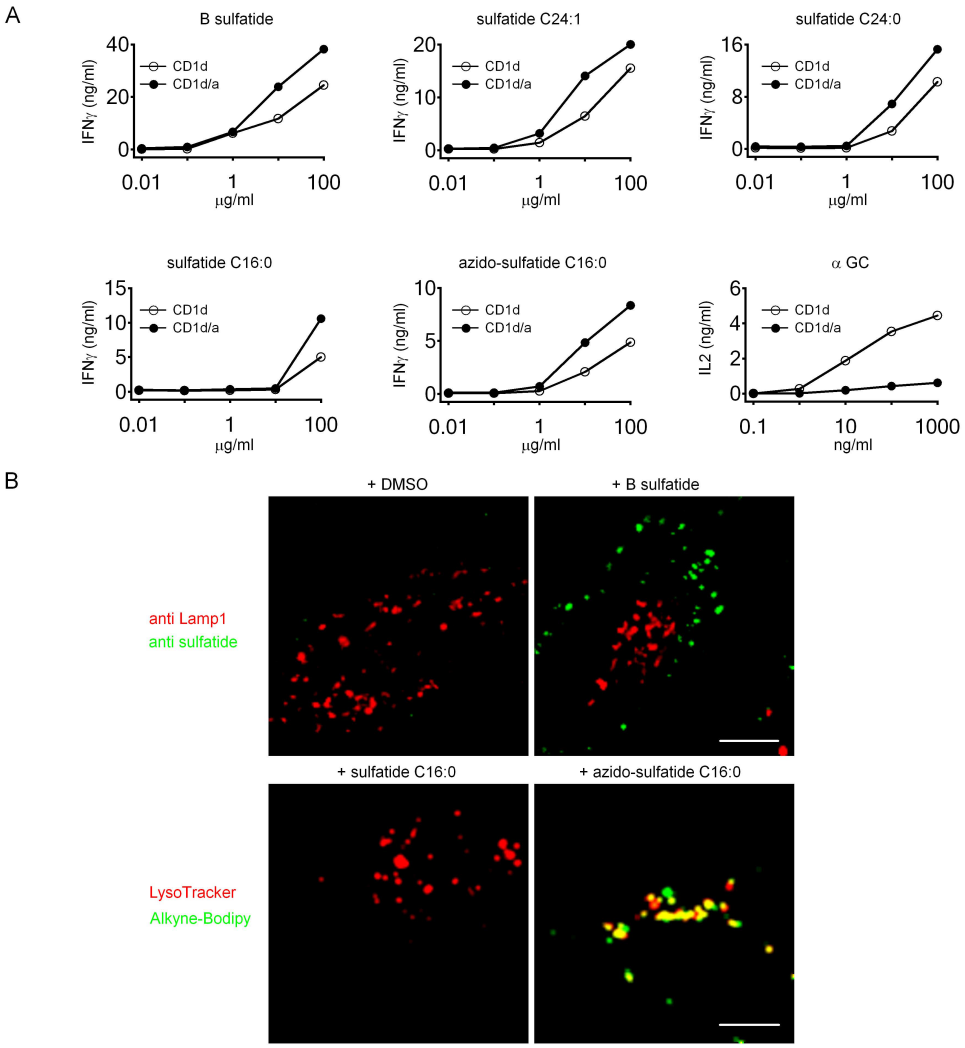


Figure 3.2 (A) Hela cells expressing WT CD1d molecules or chimeric CD1d/a molecules were pulsed with BOVINE sulfatide or αGC overnight at indicated concentrations. Cells were washed and cocultured with sulfatide specific $\gamma\delta$ T cells (for sulfatide) or mouse hybridoma DN32.D3 (crossreactive to human CD1d-αGalCer) overnight. Human IFNγ (human T cell clone) or mouse IL2 (hybridoma) were measured in supernatants. Results are representative of three experiments where the concentration of sulfatide giving 50% of maximum stimulation was 3.6 fold less for CD1d/a than for CD1d on average. (B) Top row, intracellular location of exogenously administered bovine sulfatide (5 μg/ml) incubated for 24 hours with Hela.CD1d cells before staining with anti-sulfatide Ab O4 (green) and anti-Lamp1 (red). Results represent two separate experiments. Bottom row, Hela.CD1d cells incubated with sulfatide C16:0 or azido-sulfatide C16:0 (each at 5 μg/ml) for 24 hours before staining with alkyne-Bodipy (green) and LysoTracker (red). (Results represent two separate experiments. Bars, 5 μM.

3.3.2 Discussion

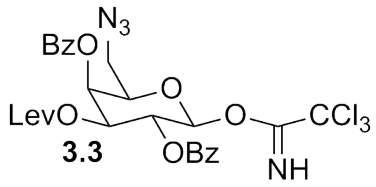
From an immunopathological perspective, it was remarkable that autoreactive T cells could be brightly stained by tetramers and self-antigens. The slow decay of tetramer staining further predicted that the affinity of interactions between the Vδ1 TCRs and their target antigens would be highly significant. Indeed, SPR studies (section 2.3.5) from the two cloned $\gamma\delta$ TCRs measured affinities for our CD1d-sulfatide complexes in the micromolar range.

The presence of TCRs with such high affinity for self antigens was especially intriguing since sulfatides are abundantly represented not only in the brain and other organs, but also in the normal serum of human and other mammalian species where studies have reported concentrations reaching the micromolar range.[59,62] These considerations coupled with Cernadas's report that CD1b poorly presented sulfatide when compared to CD1a, led us to investigate the cell biology of sulfatide loading onto CD1d molecules.[63] Our chimeric CD1d/a results mirrored previously reported results for chimeric CD1b/a. With the help of its fused intracytoplasmic CD1a tail, CD1d/a showed superior presentation of sulfatide compared with wild type CD1d. Thus, the trafficking patterns of CD1d and CD1b impaired their ability to present sulfatide at the cell surface. This property was independent of the accumulation of sulfatide in early endocytic compartment because it was also observed with azidosulfatide, a novel sulfatide analog which accumulated in the lysosome.

Therefore, we propose that the main impediment to sulfatide presentation by CD1d is the trafficking of this glycoprotein to the late endosome/lysosome. This conclusion is consistent with previous demonstrations that sulfatides are efficiently loaded at the cell surface,[47] and suggest that the CD1d-sulfatide complexes may be promptly dissociated in the lysosomal compartment. Irre-

spective of the fine mechanism involved, the decreased presentation of sulfatide by CD1 molecules recycling to late endosome and lysosome mitigates the autoimmune potential of sulfatide reactive T cells and may explain the persistence of CD1d-restricted T cells with high affinity for this antigen in healthy individuals. In contrast, we speculate that high affinity CD1d-sulfatide-specific T cells might be deleted because they would cross the threshold of tolerance in the presence of high endogenous levels of sulfatide. Thus, tetramer-based approaches to identify sulfatide-specific T cells might be more appropriate for CD1b- and CD1d- restricted T cells than for CD1a-restricted T cells.

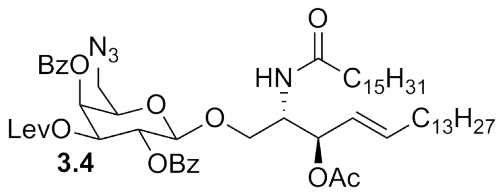
3.4 Experimental Procedures



3.3

Preparation of 3.3: Fully protected galactose **2.35** (300 mg, 0.587 mmol) was dissolved in an acetone/water solution (17mL/2mL), followed by the portion wise addition of NBS (313 mg, 1.76 mmol). Subsequent equivalents of NBS were added until the reaction was completed (monitored by TLC). Upon completion, the reaction was quenched with solid $NaHCO_3$, and the acetone was evaporated off. The remaining mixture was dissolved in water, washed with DCM (3 x 50 mL), the organic layer was dried with $NaSO_4$, concentrated, and subjected to flash chromatography (30% EtOAc:Hexanes). The partially purified white powder was dissolved in trichloroacetonitrile (18

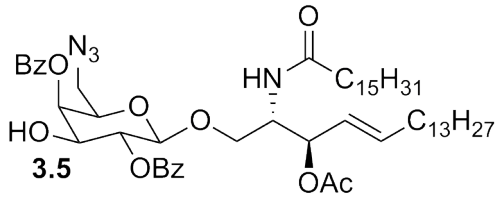
mL). Solid potassium carbonate (1.50 g, 10.9 mmol) was added. Once setup, the reaction was stirred for sixteen hours at room temperature. In the morning, the potassium carbonate was filtered and washed with EtOAc (100 mL). The filtrate was dried off at or below 30°C en vacuo. This pale yellow solid was loaded onto a bed of silica gel and was eluted with 25% EtOAc/Hexane. The two anomeric isomers were collected, combined, and concentrated at or below 30°C (161 mg, 43% from two steps).



3.4

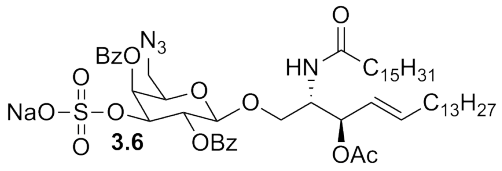
Preparation of 3.4: Donor **3.2** (161 mg, 0.252 mmol), palmitic ceramide (133 mg, 0.023 mmol), and 4 angstrom M.S. (600 mg) were stirred together for one hour in dry DCM (5 mL). The vessel was cooled to -10°C (acetone/ice bath), followed by the dropwise addition of trimethylsilyltriflate (0.025 mL, 0.141 mmol). The reaction temperature was maintained for two hours then quenched by the dropwise addition of triethyl amine. The molecular sieves were filtered and washed with EtOAc by means of a plug silica gel column. The filtrate was concentrated and subsequently loaded onto a silica gel column. Purification was achieved with a 20%, 25%, 30% EtOAc/Hexane eluent system. A white powder was collected (35 mg, 14%). 1H NMR (500 MHz, Chloroform-d) δ 8.15 (t, J = 7.0 Hz, 2H), 8.01 (d, J = 7.8 Hz, 2H), 7.65 (d, J = 7.5 Hz, 1H), 7.62 - 7.42 (m, 5H), 5.80 - 5.71 (m, 1H), 5.65 (d, J = 3.5 Hz, 1H), 5.60 - 5.49 (m, 2H), 5.34 (m, 2H), 5.27 (t, J = 7.4 Hz, 1H), 4.68 (d, J = 7.8 Hz, 1H), 4.32 (m, 1H), 4.13 (dd, J = 9.7, 3.0 Hz, 1H), 4.02 (dd, J = 8.6, 3.9 Hz, 1H), 3.63 - 3.55 (m, 2H), 3.22 (dd, J = 13.2, 3.9 Hz, 1H), 2.54 (dt, J

= 19.7, 6.8 Hz, 2H), 2.38 (dt, J = 14.0, 6.7 Hz, 2H), 2.02 (s, 3H), 1.99 (d, J = 6.4 Hz, 2H), 1.93 (s, 3H), 1.70 (t, J = 7.7 Hz, 2H), 1.41 - 1.12 (m, 46H), 0.88 (t, J = 6.8 Hz, 6H). 13C NMR (126 MHz, Chloroform-d) δ 205.66, 172.56, 171.74, 169.87, 165.73, 165.35, 137.18, 133.89, 133.65, 130.08, 129.94, 129.81, 129.12, 128.82, 128.76, 128.73, 128.64, 128.55, 128.52, 124.85, 101.18, 77.28, 77.23, 77.03, 76.77, 73.79, 73.52, 70.91, 69.73, 68.51, 67.55, 50.75, 50.12, 37.78, 37.70, 37.68, 36.41, 32.32, 31.94, 31.93, 29.73, 29.70, 29.68, 29.66, 29.57, 29.54, 29.51, 29.50, 29.38, 29.29, 29.20, 29.00, 27.92, 27.85, 25.47, 22.70, 21.18, 14.13. HRMS (ESI) calcd for $C_{61}H_{92}N_4O_{12}$ [M+NH4]+: 1072.6712, found: 1089.6925.



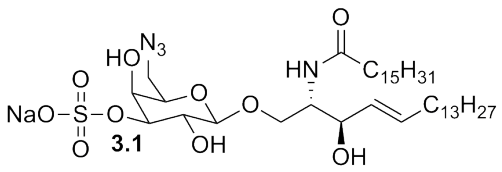
Preparation of 3.5: A 1 M solution of hydrazine was freshly prepared in dry THF. A portion (44 μL, 44 μmol) of this was stirred with glacial acetic acid (59 μL, 59 μmol) for five minutes. This mixture was added to a RBF that contained compound **3.4** (35 mg, 32.6 μmol) and dry THF:Methanol (10:1, 5.5 mL). After one hour the reaction was completed by the observance of product and the complete disappearance of starting material via M.S. The solvent was dried of en vacuo at or below 25°C. The white slurry was quenched with saturated $NaHCO_3$ solution, followed by the extraction of product in DCM washes (3 x 15 mL). The DCM washes were dried over $NaSO_4$, evaporated, and purified via flash chromatography (30% EtOAc:Hexane). The white powder product collected was isolated in excellent yield (30 mg, 94%). 1H NMR (500 MHz, Chloroform-d) δ 8.15 (d, J = 7.7 Hz, 2H), 8.05 (d, J = 7.7 Hz, 2H), 7.62 (dt, J = 23.1, 7.5 Hz, 2H),

7.53 (t, J = 7.6 Hz, 2H), 7.46 (t, J = 7.7 Hz, 2H), 5.78 - 5.71 (m, 1H), 5.60 - 5.53 (m, 2H), 5.38 - 5.30 (m, 2H), 5.26 (t, J = 7.3 Hz, 1H), 4.66 (d, J = 7.8 Hz, 1H), 4.32 (m, 1H), 4.12 (ddd, J = 13.1, 9.9, 3.5 Hz, 2H), 3.94 (dd, J = 8.7, 3.7 Hz, 1H), 3.61 (dd, J = 9.9, 4.0 Hz, 1H), 3.55 (dd, J = 13.2, 8.6 Hz, 1H), 3.25 (dd, J = 13.1, 3.6 Hz, 1H), 2.00 (s, 3H), 1.97 (d, J = 7.5 Hz, 2H), 1.73 (m, J = 7.7 Hz, 2H), 1.25 (dd, J = 9.2, 4.6 Hz, 48H), 0.88 (t, J = 6.8 Hz, 6H). 13C NMR (126 MHz, Chloroform-d) δ 172.61, 172.53, 169.88, 166.50, 166.40, 137.19, 133.87, 133.69, 130.13, 129.82, 129.25, 128.76, 128.74, 128.70, 128.63, 128.58, 124.77, 100.82, 77.27, 77.22, 77.02, 76.76, 73.90, 73.78, 73.40, 71.35, 71.04, 67.54, 51.02, 50.19, 36.46, 36.42, 32.30, 31.94, 29.73, 29.69, 29.68, 29.65, 29.56, 29.53, 29.50, 29.45, 29.37, 29.33, 29.28, 29.19, 28.98, 25.49, 22.70, 21.15, 14.12. HRMS (ESI) calcd for $C_{56}H_{86}N_4O_{10}$ [M+NH4]+: 974.6344, found: 975.6420.



Preparation of 3.6: Glycolipid **3.5** (30 mg, 30.8 μmol) was dissolved in dry pyridine (2 mL) followed by the addition of sulfur trioxide pyridine complex (73 mg, 0.462 mmol). The reaction was stirred at room temperature for fourteen hours, followed by TLC (indicating the completion of the reaction). The solvent was removed en vacuo, and the remaining slurry was dissolved in DCM, washed with saturated $NaHCO_3$, dried ($NaSO_4$), and concentrated. The residue was chromatographed (SiO_2, MeOH/DCM 5%, 10%) to afford product **2.32** (25 mg, 77%). 1H NMR (500 MHz, Methanol-d4) δ 8.17 - 8.08 (m, 3H), 7.69 - 7.62 (m, 1H), 7.56 (m, J = 25.0, 7.5 Hz, 4H), 7.46 (m, J = 7.7 Hz, 2H), 5.93 (d, J = 3.3 Hz, 1H), 5.64 (m, 1H), 5.53 (dd, J = 10.2, 7.8 Hz,

1H), 5.28 (m, 1H), 4.94 - 4.86 (m, 2H), 4.35 - 4.28 (m, 1H), 4.19 (dd, J = 8.5, 3.6 Hz, 1H), 3.97 (dd, J = 10.1, 5.8 Hz, 1H), 3.67 (dd, J = 10.1, 5.4 Hz, 1H), 3.44 (dd, J = 13.1, 8.4 Hz, 1H), 3.34 (dd, J = 13.2, 3.7 Hz, 2H), 1.98 - 1.89 (m, 5H), 1.41 (m, J = 14.4, 7.1 Hz, 4H), 1.28 (m, J = 5.5 Hz, 46H), 0.90 (t, J = 6.9 Hz, 6H). 13C NMR (126 MHz, Methanol-d4) δ 175.99, 171.63, 167.42, 167.14, 138.26, 134.66, 134.36, 131.35, 131.26, 131.09, 130.88, 129.73, 129.71, 129.51, 125.63, 101.75, 76.44, 75.15, 74.95, 71.81, 71.66, 67.93, 52.39, 51.68, 49.54, 49.52, 49.49, 49.37, 49.35, 49.34, 49.32, 49.30, 49.25, 49.20, 49.17, 49.16, 49.15, 49.13, 49.12, 49.10, 49.09, 49.08, 49.07, 49.03, 49.00, 48.98, 48.97, 48.96, 48.86, 48.85, 48.83, 48.81, 48.81, 48.80, 48.72, 48.69, 48.66, 48.65, 48.64, 48.52, 37.04, 33.38, 33.13, 30.87, 30.86, 30.84, 30.82, 30.80, 30.78, 30.76, 30.74, 30.71, 30.69, 30.66, 30.54, 30.53, 30.51, 30.33, 30.20, 30.07, 26.99, 23.79, 21.23, 14.50.



Preparation of 3.1: Sulfated glycolipid **3.5** (12 mg, 11.4 μmol) was dissolved in an anhydrous 1:1 solution of THF and methanol (3 mL). Freshly prepared 1 M sodium methoxide was added (0.100 mL) to the reaction flask and the reaction was stirred for one hour at room temperature. Reaction completion was verified via mass spectrometry and TLC. Upon completion, the solvent was removed en vacuo and the resulting residue was washed with water (3 x 1 mL), and diethyl ether (3 x 2 mL) (solid was washed via centrifugation, the solvents washes were decanted off and saved, just in case of partial dissolution in water). The purified compound was a white powder (4 mg, 44%). 1H NMR (500 MHz, DMSO-d6) δ 7.40 (d, J = 8.5, 4.1 Hz, 1H), 5.50 (m,

1H), 5.36 (m, 1H), 5.07 (d, J = 2.6 Hz, 1H), 4.82 (d, J = 5.4 Hz, 1H), 4.70 (d, J = 4.9 Hz, 1H), 4.21 (d, J = 7.7 Hz, 1H), 4.01 - 3.91 (m, 2H), 3.87 (m, 2H), 3.77 (m, 1H), 3.66 - 3.62 (m, 1H), 3.51 - 3.36 (m, 3H), 3.21 (dd, J = 12.8, 4.0 Hz, 1H), 2.00 (t, J = 7.4 Hz, 2H), 1.93 - 1.89 (m, 2H), 1.46 - 1.37 (m, 2H), 1.22 (m, 46H), 0.83 (t, J = 6.8 Hz, 6H). 13C NMR (126 MHz, DMSO-d6) δ 172.22, 131.79, 131.68, 129.50, 104.41, 78.88, 73.93, 71.41, 69.45, 69.10, 67.53, 63.53, 53.50, 51.31, 40.57, 40.48, 40.41, 40.32, 40.24, 40.15, 40.07, 39.98, 39.90, 39.81, 39.65, 39.48, 36.07, 32.22, 31.75, 29.58, 29.56, 29.54, 29.53, 29.49, 29.47, 29.42, 29.36, 29.23, 29.19, 29.17, 29.14, 25.80, 22.54, 14.39, 14.38. HRMS (ESI) calcd for $C_{40}H_{75}N_4O_{10}$S- [M+H](neg ion): 803.5209, found: 803.5671.

3.5 Immunological experimentals

Note: all immunological testing was carried out under the direction of Albert Bendelac and Erin Adams at the University of Chicago.

Confocal microscopy

Hela.CD1d cells (a gift of Dr. Steven Porcelli, were pulsed with 20 μg/ml sulfatide overnight, washed and fixed with 4% paraformaldehyde for 20 minutes. Cells were permeabilized with 0.05% saponin and blocked with 10% donkey serum for 30 minutes. Sulfatide was stained antibody O4 (mouse IgM supernatant, generously provided by Dr. Joan Boggs, Hospital for Sick Children, Toronto) and anti-Lamp1 (10 μg/ml; Abcam, MA) for 1 hour. Secondary antibodies were goat anti mouse IgM DyLight 549 and donkey anti-rabbit IgG (H+L) DyLight 649 diluted 1:200 and

added for 30 minutes at room temperature. For confocal imaging of azido-sulfatide, azido-sulfatide C16:0 (5 μg/ml) was added to Hela.CD1d cells overnight. After washing, cells were incubated with Alkyne-Bodipy (5 μg/ml) in culture medium for 1h at 37°C and LysoTracker Red (0.001 mM; Invitrogen, CA) was added for the last 10 minutes. Samples were examined by confocal microscopy (FV1000, Olympus) with a 60x oil objective. Data were analyzed using Adobe Photoshop and ImageJ.

Sulfatide stimulation

Hela.CD1d and Hela.CD1d/a cells (provided by Dr. Steven Porcelli, Albert Einstein College of Medicine, NY) and sorted to express similar levels of surface CD1d were plated at $5x10^4$ cells per well and pulsed with lipids at different concentrations overnight, washed and cocultured with sulfatide-specific $\alpha\beta$ T cells ($1x10^5$ per well) or DN32.D3 ($5x10^4$ cells per well) overnight. Cytokines released in medium were measured by BD Cytometric Beads Array human Th1/Th2/Th17 kit and mouse IL2 Flex Set.

Chapter 4

Synthesis and Evaluation of Lyso Glycolipid Analogs

4.1 Introduction

As introduced in Chapter 1, over the past decade, multiple NKT cell antigens have been discovered. However, after attempting to verify and reproduce the proposed stimulatory activity, many antigenic NKT cell candidates have given inconsistent results. For example, Fischer *et al.* presented PIM_4 (Figure 1.6), isolated from *Mycobacterium bovis bacillus*, as an exogenous NKT cell antigen.[19] However, two years later, Kinjo *et al.* synthesized PIM_4 and found that it did not stimulate NKT cells.[64] In 2004, Ortaldo *et al.* presented results suggesting that β-GalCer was a type I NKT cell antigen.[65] Also a recent publication in *Nature Immunology*, reported the stimulatory properties of β-glucosylceramide (β-GluCer).[66] In contrast, our labs have used these proposed

antigens as negative controls (i.e., they do not stimulate NKT cells).

Surprised by the recently published results that β-glucosylceramides were endogenous NKT cell antigens, we and our collaborators began experimentation to attempt to explain the disparity between the Brenner group's observation that β-GluCer stimulated NKT cells and our multiple observations that β-GluCer showed no stimulatory activity.[66] The β-GluCer used by the Brenner group (C12 and C24:1) was synthetic and was obtained from Avanti Polar Lipids Inc. We purchased a small amount of β-GluCer (24:1) from Avanti to see if we could reproduce the reported results. In support of the published results, the β-GluCer from Avanti weakly stimulated NKT cells (unpublished data, Luc Teyton's lab).

One possible explanation for this result is that there are stimulatory contaminations present in the commercial sample. Considering both β-GluCer and β-GalCer, likely contaminants are the alpha anomers, that is, the potent NKT cell antigens, α-GluCer and α-GalCer respectively.[23] Generally, before glycosylation, the 2-hydroxyl on the sugar is protected with an ester that can participate in anchimeric assistance (Figure 4.1). During glycosylation, this ester intramolecularly attacks the 1-carbon to give species **3**. In theory, the reversible conversion of **2** to **3** effectively shields the α-anomer from forming. However, in practice, small amounts of α-anomer are always formed during glycosylation. In the synthesis of β-GalCer and β-GluCer, global removal of the ester protecting groups occurs directly after glycosylation. Although the anomers are diastereomers of each other, their similar polarities make complete separation of the two difficult. Therefore, α-anomer contamination is not only possible, but is routinely observed.

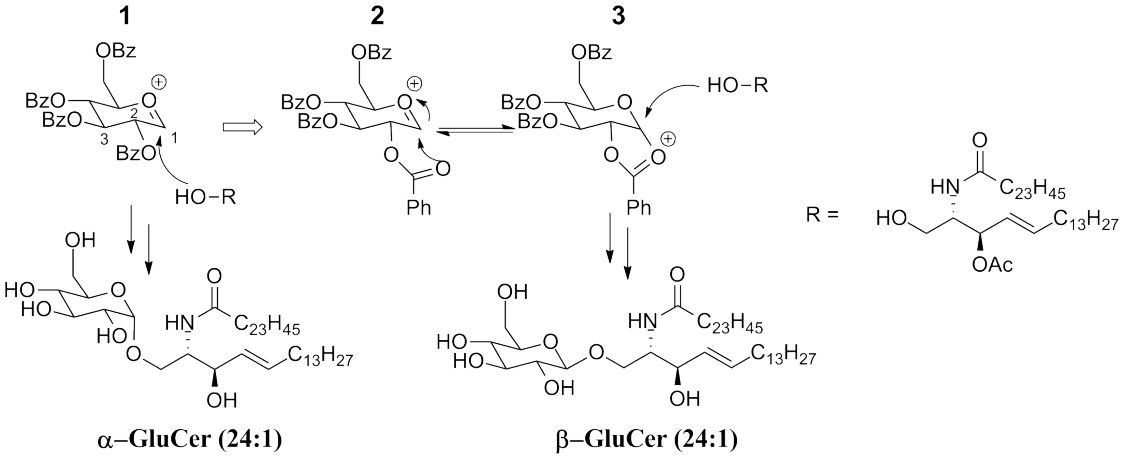


Figure 4.1 Mechanism of glycosylation anomerization of GluCer (24:1). Intramolecular attack at the anomeric position (**2**) by the 2-benzoyl ester forms species **3**. Interconversion between compound **1** and compound **3**, promotes nucleophilic attack to form the β-glycolipid.

Due to the likelihood of α-anomer contamination, we further analyzed the Avanti sample by NMR to assess purity. NMR analysis did not detect any α contaminants or any relevant contaminations. Due to the high potency of the α-anomer, the detection limits of NMR are insufficient to prove the lack of α-GluCer. We then subjected the sample to flash chromatography and we fractionated the sample by differing eluents with increasing polarity (5% MeOH/DCM, 10% MeOH/DCM, 20% MeOH/DCM, 40% MeOH/DCM, 25:65:2 MeOH:DCM:H_2O, 25:65:4 MeOH:DCM:H_2O, and 65:25:10 MeOH:DCM:H_2O)). These fractions were sent to the Teyton lab (The Scripps Research Institute), where high performance TLC was carried out (Figure 4.2). Each fraction was then tested for stimulatory activity, with multiple fractions producing substantial stimulatory responses, even those fractions lacking β-GluCer (unpublished data).

High resolution mass spectrometry analysis of the fractions that showed stimulatory activity (fractions 6 and 7) suggested the presence of lysed version of α- or β-GluCer. Lyso-ceramides,

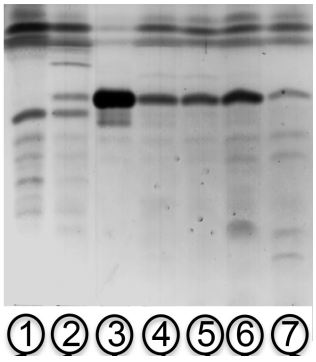


Figure 4.2 High performance TLC of fractionated commercial β-GluCer. Each numbered lane(10 μg of material per lane) roughly represents the following eluents (in ascending order) 5% MeOH/DCM, 10% MeOH/DCM, 20% MeOH/DCM, 40% MeOH/DCM, 25:65:2 MeOH:DCM:H_2O, 25:65:4 MeOH:DCM:H_2O, and 65:25:10 MeOH:DCM:H_2O

as shown in Figure 4.3, are ceramides that are lacking the fatty acyl chain, the common name for the lyso version of β-GluCer is β-glucopsychosine (lyso version of β-GalCer = β-psychosine). In vivo, lyso-ceramides are formed via ceramidase enzymes that produce lyso-gluco and lyso-galactosylceramides. In vivo, five ceramidases have been found, two expressed in the lysosome (acid ceramidase (ASAH1) and N-acylamidehydrolase (NAAA)) and three others expressed in epidermal cells (alkaline ceramidase 1-3).[67,68] Poor handling of glycolipids could be a source of the small β-glucopsychosine contaminations.

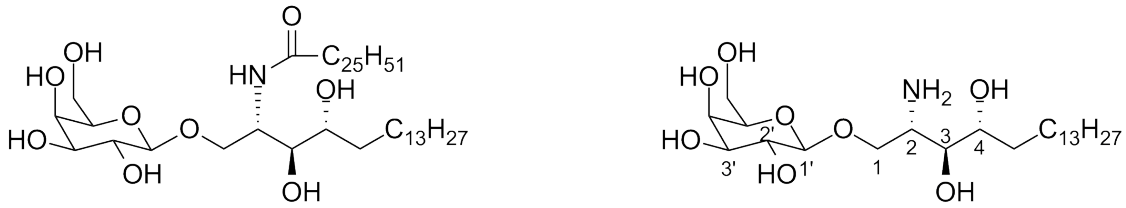


Figure 4.3 Representative structure of β-GalCer and β-psychosine

Following this preliminary analysis of the commercial β-GluCer, we wanted to test if lyso-

glycosylceramides were NKT cell antigens. We hypothesized that if lyso-glycosylceramides are stimulatory, then the observed stimulatory activity of β-GalCer and β-GluCer was due to enzymatic degradants (i.e. enzymatically degraded lyso-glycosylceramides) in the samples, but that if lyso-glycosylceramides are not stimulatory, then the observed stimulatory activity was due to chemical contaminants (i.e. contaminants introduced during glycolipid synthesis) present in the tested samples. To test this hypothesis we synthesized a broad panel of lyso-gluco and lyso-galactosylceramides (as well as new batches of β-Gal and -GluCer and α-Gal and GluCer) and tested them for stimulatory activity. Figure 4.4 represents the initial synthetic targets. α- and β-psychosine and glucopsychosine were selected as plausible lyso degradants of α and β-Gal and -GluCer (as well as their sphingosine counterparts). We chose compound **4.5** and compound **4.6** as lysed versions of possible chemical contaminants. Compound **4.5** would be made if the diastereomer of sphingosine were used during the synthesis of α-GalCer(24:1), and compound **4.6** would be formed if the 2-hydroxyl group was the unprotected alcohol in the glycosylation acceptor. Lastly, compounds **4.7** and **4.8** were synthesized lysed β-anomers of the potent NKT cell antigen, PBS57.

4.2 Synthesis of lyso-ceramides

4.2.1 Synthesis of β-psychosine 4.1a and β-glucopsychosine 4.2a

The syntheses of lyso glycolipids are relatively straightforward. Starting with commercially available phytosphingosine (Scheme 4.1), we performed a copper catalyzed diazo transfer to oxidize

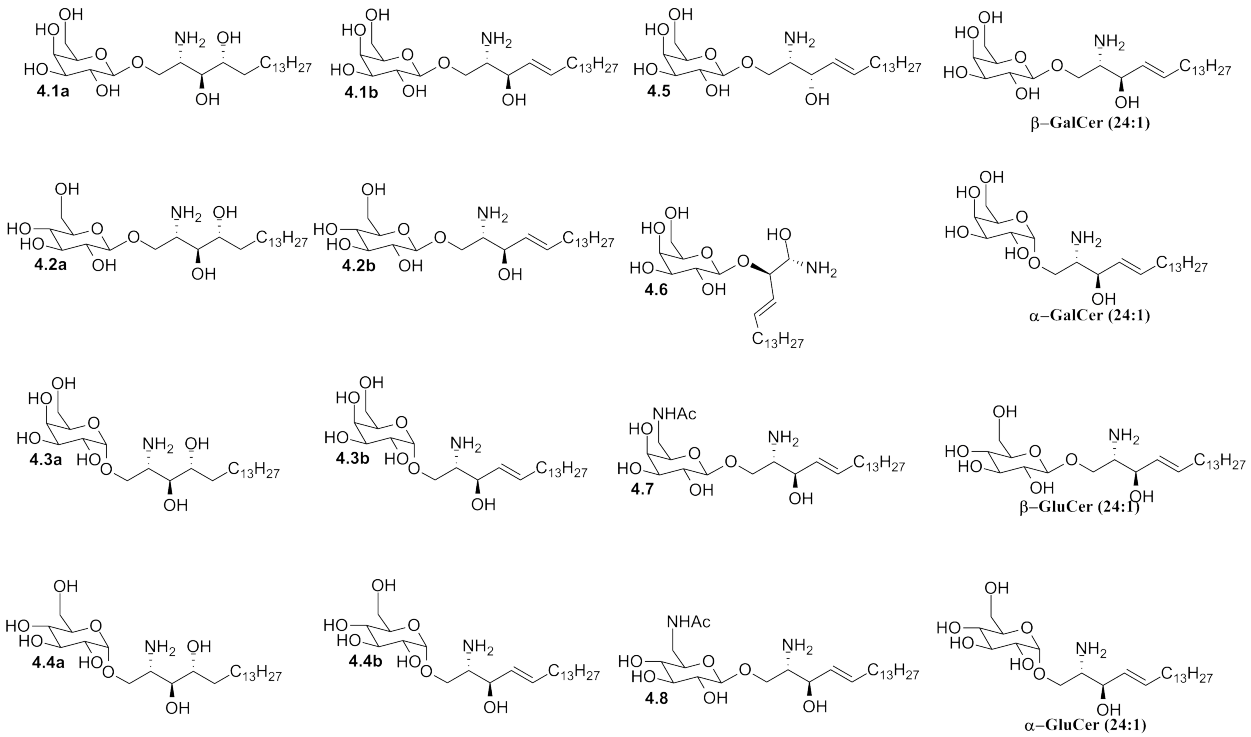
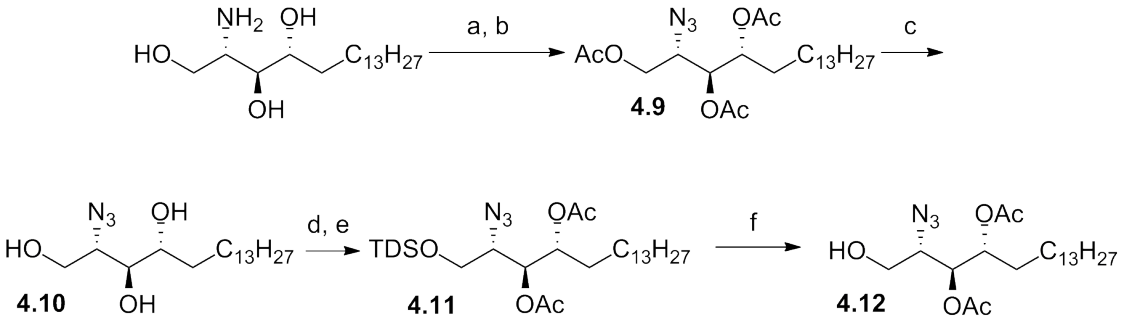


Figure 4.4 Initial synthetic lyso targets

the amine to an azide.[69] To simplify the purification process, a quick workup was performed followed by the addition of pyridine, DMAP, and acetic anhydride. These two steps yielded acylated compound **4.9**. Removal of the esters with sodium methoxide resulted in azide **4.10**. Dissolving **4.10** in a minimal amount of pyridine at room temperature and carefullying adding TDS-Cl, selectively protected the primary alcohol of the azido-phytosphingosine. After complete conversion to the primary alcohol protected product, addition of excess acetic anhydride afforded fully protected phytosphingosine **4.11**. Acidic cleavage of the silyl ether, produced our azido-phytosphingosine coupling acceptor **4.12** (used in the synthesis of **4.1a** - **4.4a** (Note: this reaction if done slowly, left overnight, heated up, or purified slowly can and will result in migration of the 2-acetyl group to the primary alcohol. It is essential that the chemist quench this reaction immediately after comple-

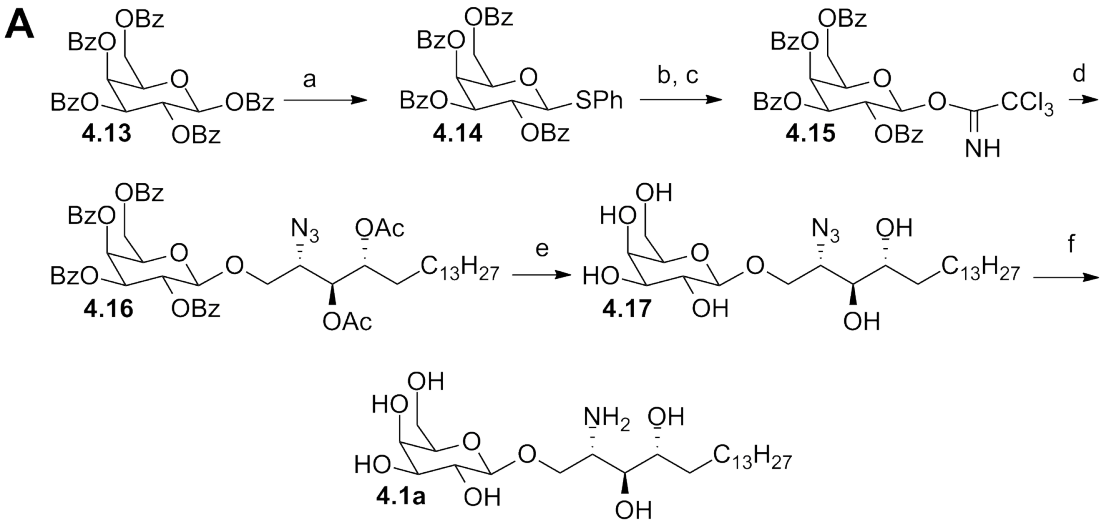
tion.)).



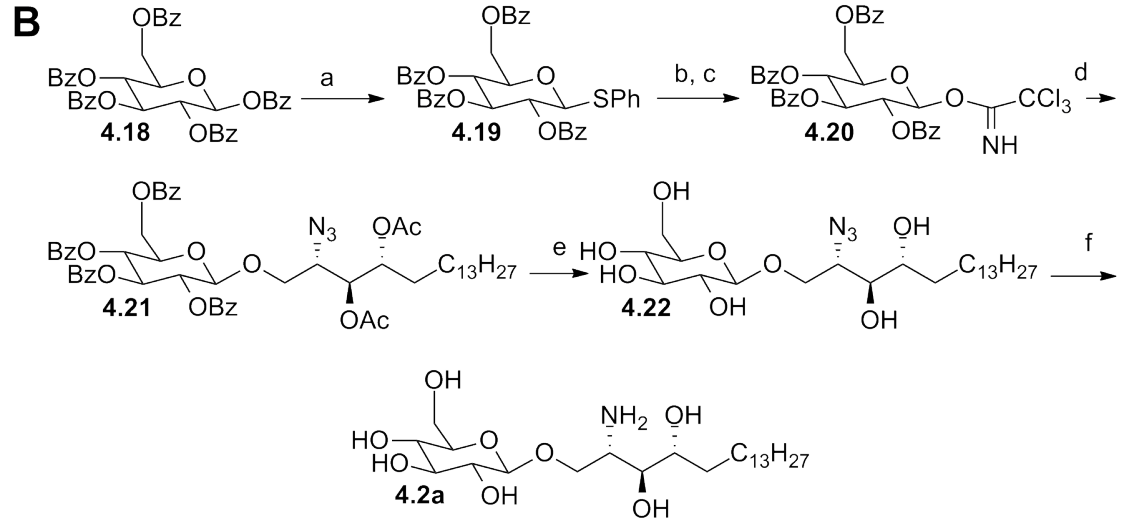
Reagents and conditions: a. TfN_3, K_2CO_3, $CuSO_4$-hydrate, H_2O, MeOH; b. Ac_2O, py, 80% c. NaOMe, MeOH:THF; d. TDS-Cl, py; e. Ac_2O, DMAP, py, 78%; f. HF(aq), ACN, DCM, 76%

Scheme 4.1 Synthesis of phytosphingosine acceptor **4.11**

Unlike the synthesis of sulfatide, the sugar donor is straightforward to synthesize (Scheme 4.2(A)). After obtaining undesirable glycosylation yields from a peracylated galactosyl Schmidt donor, we opted for a more activated donor by synthesizing the perbenzoylated Schmidt donor **4.15**. This was synthesized by installation of a thio-phenol at the anomeric position, followed by a traditional 2-step process that replaced the thiophenol with a trichloroacetimidate moiety. Schmidt coupling conditions[70] were carried out towards the successful conversion of donor **4.15** and acceptor **4.12** to glycolipid **4.16**. The $2'$-benzoate participated in anchimeric assistance during glycosylation, which effectively shielded the bottom face of the sugar to give primarily the β anomer. Global removal of the ester protecting groups cleanly afforded compound **4.17**. Reduction of the azide with palladium hydroxide and hydrogen gave the final β-psychosine **4.1a**. The synthesis of **4.2a** was uncomplicated due to its mirrored synthetic route to **4.1a**. Scheme 4.2(B) outlines the synthesis of β-glucopsychosine.



Reagents and conditions: a. $BF_3(OEt)_2$, PhSH, DCM, 0 °C; b. NBS, H_2O, Acetone; c. K_2CO_3, Cl_3CCN, DCM, 79%; d. 4Å M.S., **4.12**, TMSOTf, DCM, 0 °C, 53%; e. NaOMe, THF,MeOH, 83%; f. $Pd(OH)_2$, H_2, MeOH, THF,72%.

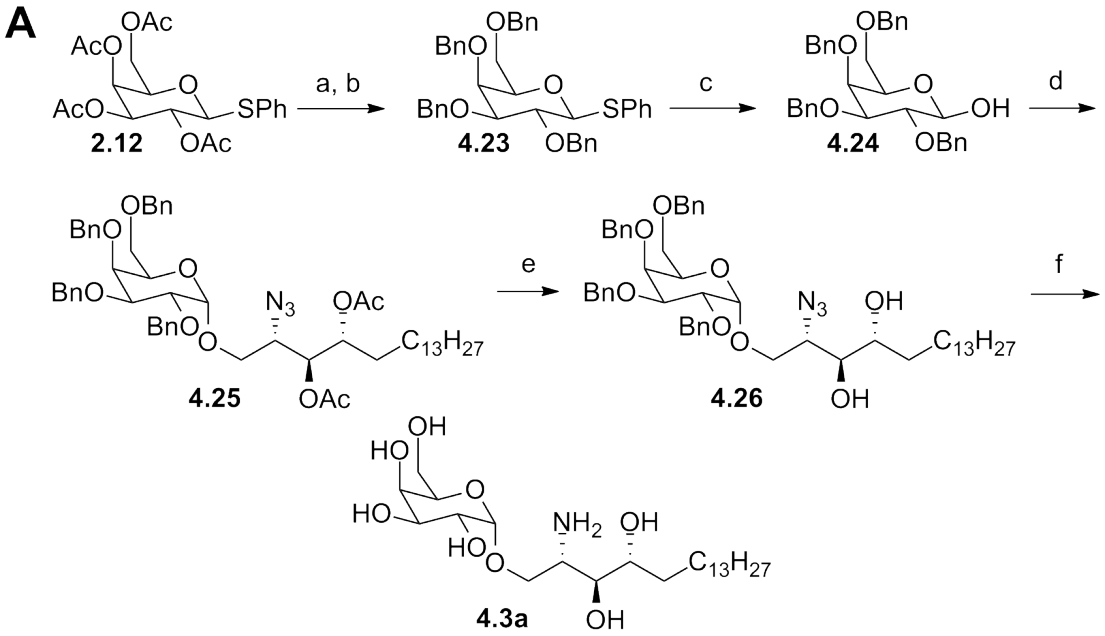


Reagents and conditions: a. $BF_3(OEt)_2$, PhSH, DCM, 0 °C; b. NBS, H_2O, Acetone; c. K_2CO_3, Cl_3CCN, DCM, 83%; d. 4Å M.S., **4.12**, TMSOTf, DCM, 0 °C, 75%; e. NaOMe, THF,MeOH, 98%; f. $Pd(OH)_2$, H_2, MeOH, THF, 77%.

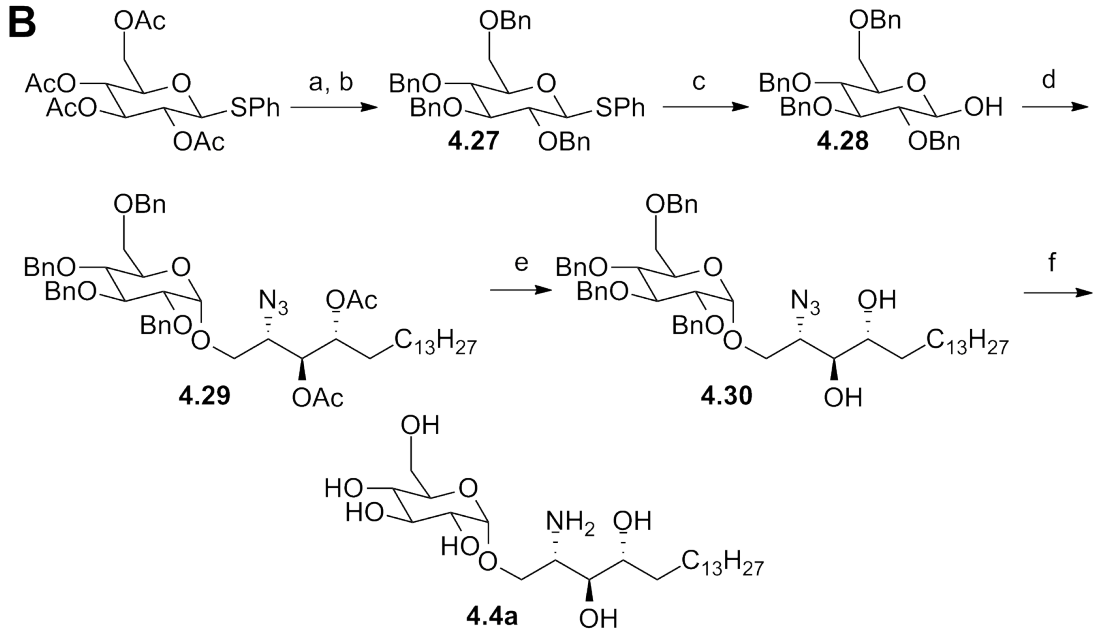
Scheme 4.2 Synthesis of β-psychosine **4.1a** and β-glucopsychosine **4.2a**

4.2.2 Synthesis of α-psychosine 4.3a and α-glucopsychosine 4.4a

The synthesis of **4.3a** (Scheme 4.3(A)) started from compound **2.12**. Removal of the acetates with sodium methoxide left naked hydroxyls that were protected with benzyl bromide to give perbenzylated compound **2.23**. Transformation of the thio phenyl to a hydroxyl group at the anomeric position was quickly accomplished with n-bromosuccinimide with water and acetone as the solvent. Using donor **4.24** and acceptor **4.12** under David Gin's coupling conditions[71] resulted in compound **4.25**. In this case the anomeric effect biased towards the α-anomer product **4.25**. The acetyl groups were removed with sodium methoxide in methanol. One-pot removal of the benzyl groups and reduction of the azide were initially attempted with traditional hydogenation conditions (palladium on carbon, H_2, 350 p.s.i.). These classic conditions reduced the azide, but the newly formed amine poisoned the catalyst. Switching to palladium hydroxide alleviated this problem, and succesfully produced α-psychosine **4.3a**. Scheme 4.3(B) outlines the synthesis of β-glucopsychosine **4.4a**. The synthetic route is identical to that of compound **4.3a**.



Reagents and conditions: a. NaOMe, MeOH; b. Bn-Br, NaH, TBAI, 0 ^{o}C; c. NBS, H_2O, Acetone, 75%; d. 3Å M.S., Tf_2O, **4.12**, DCM, 0 ^{o}C, 76%; e. NaOMe, THF, MeOH, 96%; f. $Pd(OH)_2$, H_2, MeOH, THF, 57%.



Reagents and conditions: a. NaOMe, MeOH; b. Bn-Br, NaH, TBAI, 0 ^{o}C; c. NBS, H_2O, Acetone, 84%; d. 3Å M.S., Tf_2O, **4.12**, DCM, 0 ^{o}C, 68%; e. NaOMe, THF, MeOH, 91%; f. $Pd(OH)_2$, H_2, MeOH, THF, 57%.

Scheme 4.3 Synthesis of α-psychosine **4.3a** and α-glucopsychosine **4.4a**

4.2.3 Synthesis of the β-sphingo-psychosine diastereomer 4.5 and the β-sphingo-glucopsychosine analog 4.6

As discussed in section 2.2.1, during the synthesis of ceramide a small amount of the diastereomer (**2.7b**) is formed. The absence of chelating agents causes the pentadecyne to attack the Re face in accordance with the Felkin-Ahn model. Small amounts of pentadecyne attack the Si face and form the anti-Felkin-Ahn product **2.7b**.

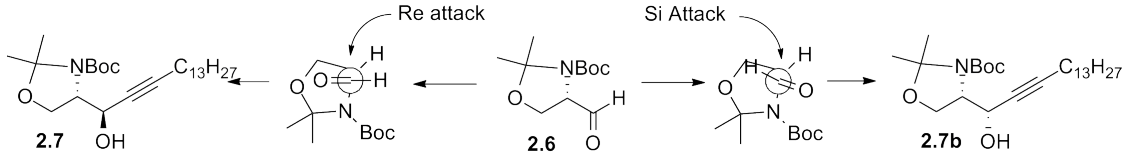
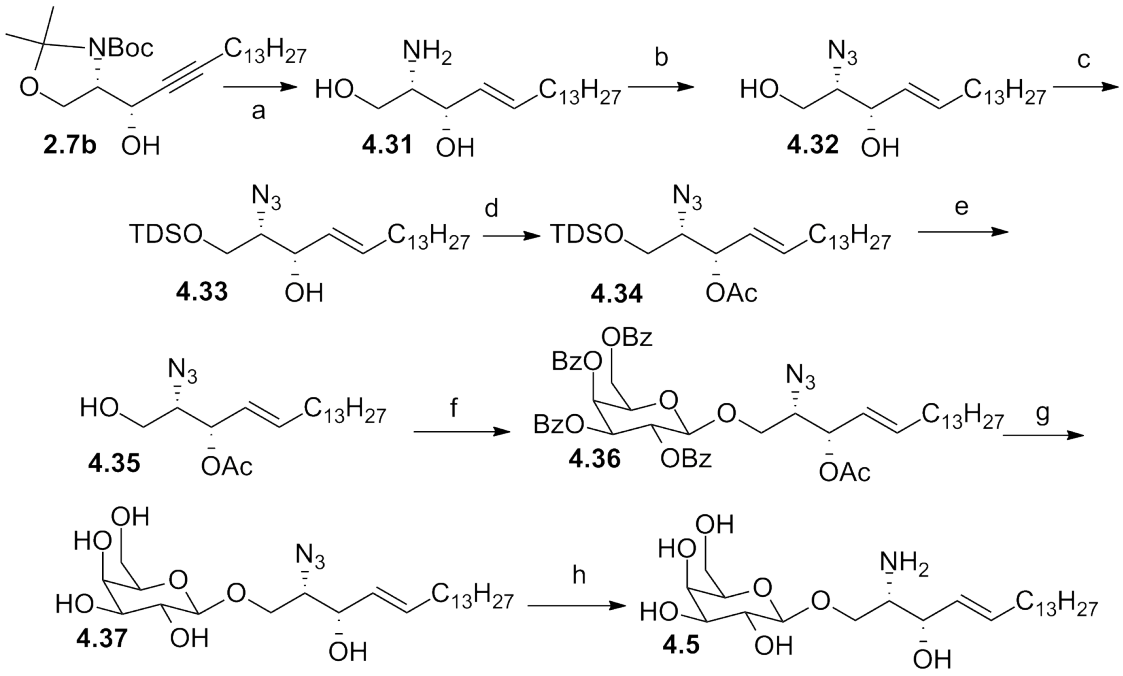


Figure 4.5 Formation of the diastereomeric sphingosine precursor **2.7b**. The absence of chelating agents causes the pentadecyne to attack the Re face in accordance with the Felkin-Ahn model to give compound **2.7**. Small amounts of pentadecyne attack the Si face and form the anti-Felkin-Ahn product **2.7b**.

With isolated compound **2.7b** in hand, we subjected it to dissolving metal conditions to give sphingosine **4.31**. The amine of **4.31** was oxidized to an azide via a copper-catalyzed diazo transfer reaction.[69] TDS-Cl selectively protected the primary alcohol of the azido-phytosphingosine, followed by the addition of excess acetic anhydride which gave fully protected sphingosine **4.34**. Hydrofluoric acid efficiently cleaved the TDS group to produce the desired azido-sphingosine coupling acceptor **4.35** (see procedure for discussion of the migration problem). We followed Schmidt glycoslation conditions[70] and subsequent deprotections of the ester protecting groups, which gave compound **4.37**. Due to the double bond present in the sphingosine, a zinc reduction step was employed instead of a hydrogenation to afford compound **4.5**.



Reagents and conditions: a. Li, ethylamine, THF, -78 ^{o}C, 94%; b. TfN_3, K_2CO_3, $CuSO_4$-hydrate, H_2O, MeOH, 70%; c. TDS-Cl, py; d. Ac_2O, DMAP, py, (82% two steps); e. HF(aq), ACN, DCM, 67%; f. 4Å M.S., **4.15**, TMSOTf, DCM, 0 ^{o}C, 77%; g. NaOMe, THF, MeOH, 88%; h. Zn, AcOH, NaOH, MeOH, THF, 87%.

Scheme 4.4 Synthesis of β-sphingo-GluCer diastereomer **4.5**

Although a very unlikely synthetic contaminant, we decided to synthesize the β-sphingo-psychosine analog **4.6** nonetheless. This compound could be formed in two different ways. If during the selective protection of the primary hydroxyl, the secondary alcohol was instead protected, then after acetylation and acidic cleavage of the silyl ether, donor **2.11m** would be formed. The other, and more likely way to form acceptor **2.11m** would be acetyl migration (via a stable 6-membered ring intermediate) during the workup, purification, or storage (Figure 4.6. The latter migration is routinely observed.

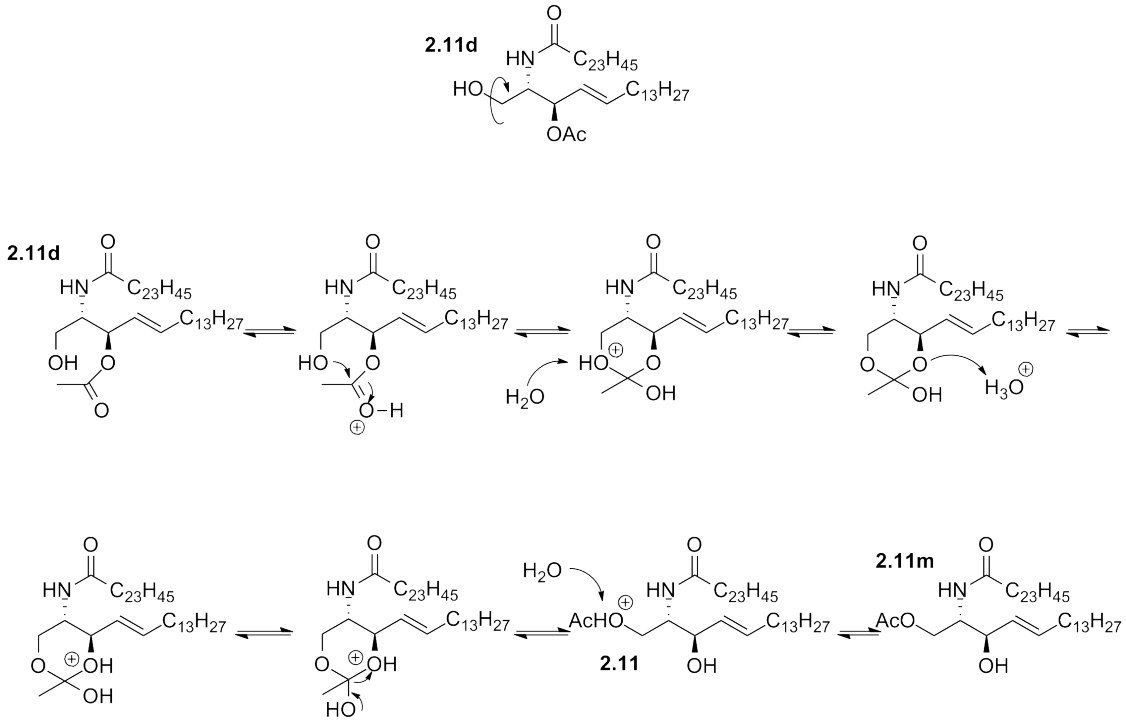
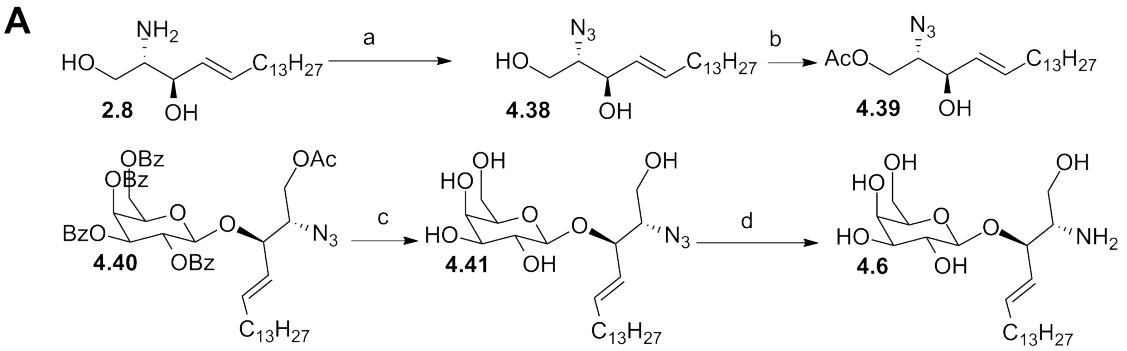
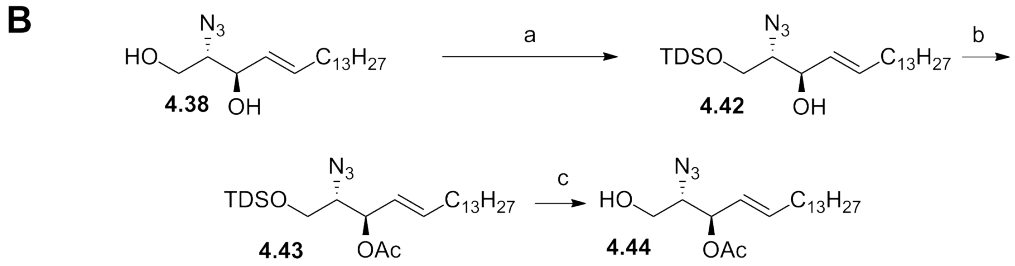


Figure 4.6 Acetyl migration mechanism under acidic conditions.

This synthesis of the appropriate acceptor required azide formation,[69] acetylation, and removal of the acetates from compound **2.8** to give compound **4.38**. Next, we protected the primary hydroxyl with one equivalent of acetic anhydride, which gave us acceptor **4.39**. Following Schmidt coupling conditions,[70] with donor **4.15**, the desired glycolipid (**4.40**) was formed. Global deprotection of the esters, followed by azide reduction afforded compound **4.6**.



Reagents and conditions: a. TfN_3, K_2CO_3, $CuSO_4$-hydrate, H_2O, MeOH, 45%(over two steps); b. Ac_2O, py; c. 4Å M.S., **4.15**, TMSOTf, DCM, 0 °C, 99%; d. NaOMe, THF, MeOH, 79%; k. Zn, AcOH, MeOH, THF, NaOH, 73%.



Reagents and conditions: a. TDS-Cl, py; b. Ac_2O, DMAP, py, 80%(over two steps); c. HF(aq), ACN, DCM, 92%..

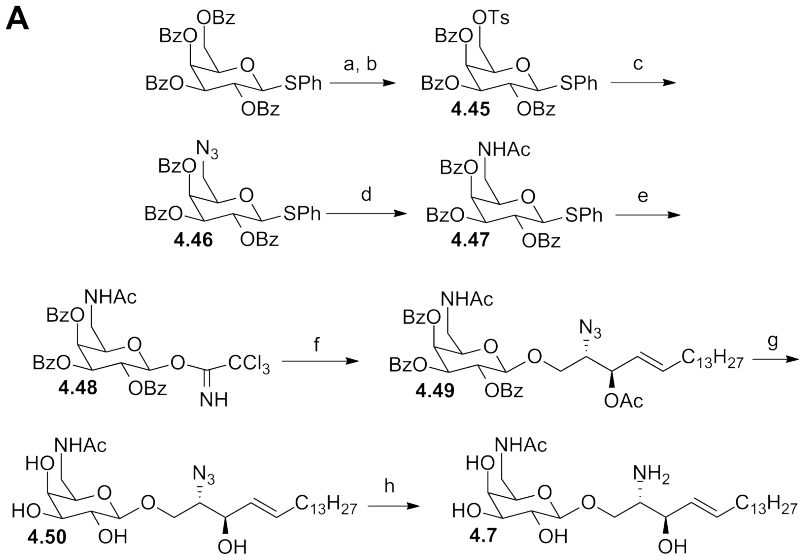
Scheme 4.5 Synthesis of β-sphingo-GluCer analog **4.6**

During the synthesis of acceptor **4.39**, we selectively protected the primary hydroxyl of **4.38** with TDS, acetylated the 3-hydroxyl, and removed the silyl ether group to give us azido-sphingosine acceptor **4.44**. This acceptor was used in the synthesis of **4.7** and **4.8**.

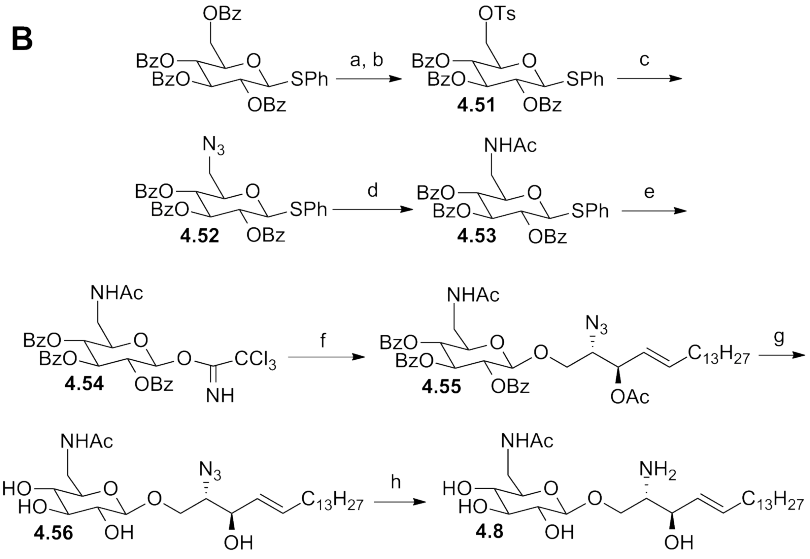
4.2.4 Synthesis of N-acyl β-sphingo-psychosine (4.7) and β-sphingo-glucopsychosine analogs (4.8)

The synthesis of N-acyl β-sphingo-psychosine (**4.7**) and β-sphingo-glucopsychosine analogs (**4.8**) is outlined in Scheme 4.6. Due to the similarities of the synthetic routes of **4.7** and **4.8**, only

Scheme 4.6(A) will be described. Starting from compound **2.13**, we removed the acetyl groups with sodium methoxide in methanol. After quenching and drying off the solvent, we installed a tosyl group at the 6-hydroxyl position, followed by global protection with benzoyl chloride to afford compound **4.45**. An azido moiety replaced the tosyl group via an S_N2 reaction. We utilized ruthenium trichloride and thioacetic acid to convert the azide to an N-acyl moiety to give compound **4.47**. With **4.47** in hand, we transformed it to a Schmidt coupling donor (**4.48**), and coupled it to acceptor **4.44**. The glycolipid was globally deprotected, followed by reduction of the azide to give lyso-galactosylceramide **4.7**. Scheme 4.6(B) followed the same synthetic route towards the synthesis of lyso-glucosylceramide **4.8**.



Reagents and conditions: a. NaOMe, MeOH; b. 1)TsCl, py; 2)BzCl; c. NaN_3, DMF, 80%(over 3 steps); d. $RuCl_3$, MeOH, thiacetic acid, 41%; e. 1) NBS, H_2O, Acetone; 2) K_2CO_3, Cl_3CCN, DCM, 92%; f. 4Å M.S., **4.44**, TMSOTf, DCM, 0 °C; g. NaOMe, THF, MeOH, 46%(over 2 steps); f. Zn, AcOH, NaOH, 83%.



Reagents and conditions: a. NaOMe, MeOH; b. 1)TsCl, py; 2)BzCl; c. NaN_3, DMF, 71%(over 3 steps); d. $RuCl_3$, MeOH, thiacetic acid, 59%; e. 1) NBS, H_2O, Acetone; 2) K_2CO_3, Cl_3CCN, DCM, 75%; f. 4Å M.S., **4.44**, TMSOTf, DCM, 0 °C, 35%; g. NaOMe, THF, MeOH, 93%; f. Zn, AcOH, NaOH, 76%.

Scheme 4.6 Synthesis of N-acyl β-sphingo-psychosine (**4.7**) and β-sphingo-glucopsychosine analogs (**4.8**)

4.3 Results and Discussion

4.3.1 α-psychosine and α-glucopsychosine stimulate *i*NKT cells

All proposed lyso-glycosylceramides were successfully synthesized. These compounds were subjected to a *i*NKT cell activation assay to test for *i*NKT cell stimulatory properties. A mouse *i*NKT cell hybridoma DN32.D3 was incubated with each synthetic lyso-glycosylceramide and DC3.2 cells, a dendritic cell line expressing CD1d. Twenty-four hours later, cell culture supernatents were collected and analyzed for IL-2 concentrations. Each assay was performed at the Scripps Research Institute, under the direction of Luc Teyton. Of the eight lyso-glycosylceramides tested, only α-psychosine (**4.3a**) and α-glucopsychosine (**4.4a**) provided potent stimulatory activity.

4.3.2 The L363 antibody binds to α-psychosine and α-glucopsychosine

After the initial analysis of the commercial β-GluCer, Teyton and coworkers incubated the sample with an antibody called L363. Discovered by Porcelli and coworkers, L363, along with L317, are monoclonal antibodies specific to the CD1d-α-GalCer complex. They showed no reactivity with non-loaded CD1d, nor did they bind to CD1d-bound iGB3.[72] Teyton and coworkers observed that treatment of the commercial β-GluCer with L363 effectively blocked the stimulatory activity (unpublished data). These results would suggest that an α-glycolipid was the stimulatory contaminant in the commercial samples.

To verify these results, we examined the binding of L363 antibody against our panel of α and β gluco- and galactosylceramides via surface plasmon resonance. Our results, presented in

Figure 4.7, show that L363 binds not only to α-GalCer (CD1d-bound), but also to α-psychosine, α-glucopsychosine, and α-GluCer. No binding affinity to L363 was observed with their β counterparts. We can also glean from the data that L363 has a strong preference for galactose when compared to glucose. Recently, the crystal structure of CD1d-α-GalCer in complex with L363 was solved. A study of the crystal structure provides rationals for our experimental data. For example, L363 forms a hydrogen bonding interaction with the 4$''$-hydroxyl group of α-GalCer, this hydrogen bond most likely explains why galactose binds better than glucose.[73] These binding data, combined with previous studies,[72,73] further characterize the antigen repertoire of L363 and further validate that the commercial β-GluCer has an α contaminant.

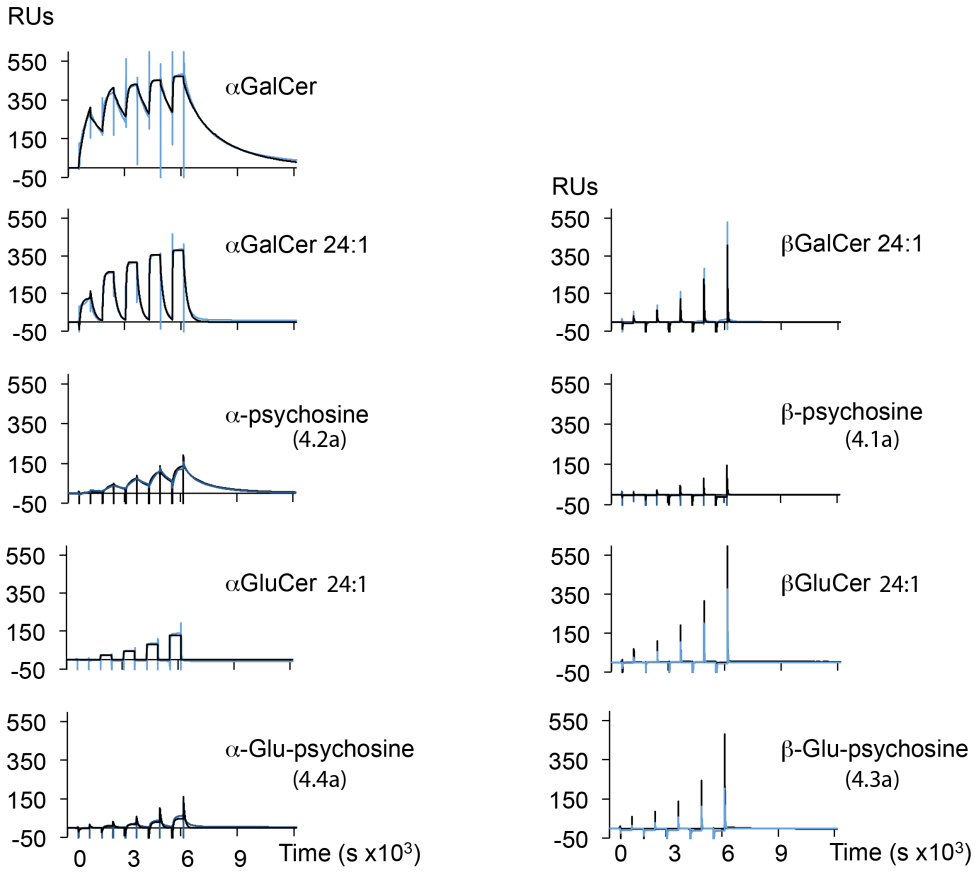


Figure 4.7 L363 binds α-galactosyl and -glucosylceramide-CD1d complexes. SPR binding data of various synthetic glycolipids. All tested α-linked glycolipids showed measurable binding to the L363 antibody, while no measurable binding affinity was observed for the various β-glycosylceramides. These analyses were performed using the Biacore T200 Biaevaluation global analysis software using subtracted sensorgrams (L363 antibody - Control antibody).

4.3.3 Observed anomerization from β-sphingopsychosine to α-sphingopsychosine

We have observed an interesting phenomenon with synthetic β-GluCer prepared in our laboratory. Initially, NKT cell assays performed on the batch resulted in no observed stimulatory activity. After being stored for a long period of time (6 months), NKT cell assays were again performed

resulting in stimulation of NKT cells. Taken in context with our reported analysis of the commercial β-GluCer, this phenomenon posed an alternative explanation for the presence of an α-contaminant. Can β-glycosylceramides undergo spontaneous anomerization? Figure 4.8, shows a proposed mechanism of this process. Typically at neutral pH, anomerization has not been reported to occur to glycolipids, but under acidic conditions anomerization could occur. In our proposed mechanism, the amide proton interacts with a hydrogen-bonded water molecule which allows the carbohydrate ring to open and the formed hydroxide to be stabilized by the amide proton. Reformation of the water molecule, and attack at the anomeric position, would produce either the α or β-GalCer (24:1), depending on what face of the aldehyde was attacked.

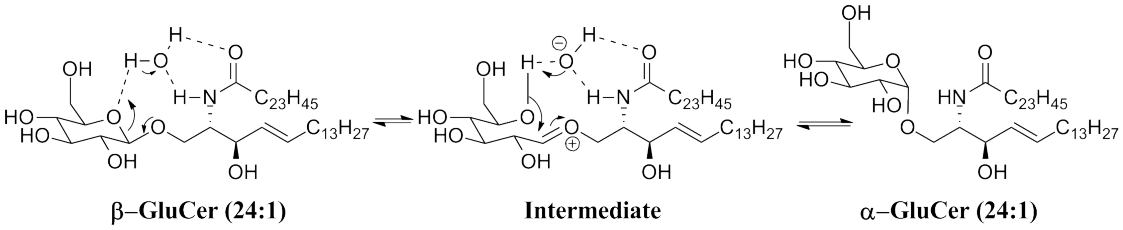


Figure 4.8 Proposed mechanism of spontaneous anomerization of β-GluCer(24:1)

If we could positively observe spontaneous anomerization, not only would this be a viable means of alpha-contamination, but a possible occurrence in the body. Introduced in section 4.1, β-psychosine and β-glucopsychosine are synthesized in vivo via ceramidases expressed in the lysosome.[67,68] It is possible that under physiologically-unique controlled conditions (acidic environment of the lysosome) anomerization might occur. With the knowledge that α-psychosine and α-glucopsychosine are NKT cell antigens, and that CD1d is localized in the lysosome, these antigens may be endogenous and could play a pivitol role in NKT cell biology.

To address the viability of anomerization, we performed annomerization studies on β-GalCer (24:1, sphingosine), and β-sphingopsychosine. We dissolved β-GalCer (24:1, sphingosine), and β-sphingopsychosine in three different solvent systems and at pH 4.5 and pH 7 (25:75 DMSO:pH 7 buffered water, 25:75 DMSO:pH 4.5 buffered water, 12.5:12.5:75 MeOH:THF:pH 7 buffered water, 12.5:12.5:75 MeOH:THF:pH 4.5 bluffered water, 0.5:99.5 Tween 20:pH 7 buffered water, and 0.5:99.5 Tween 20:pH 4.5 buffered water). We chose pH 7 and pH 4.5 to mimic physiological neutral pH and the pH of the lysosome, respectively.[67] These samples were stirred and heated at 37°C for six days. After a six day period, the samples were tested via the NKT cell assay described in section 4.3.1. Figure 4.9 shows the only stimulatory results observed. These data show that anomerization of the β-sphingopsychosine to the α-sphingopsychosine occured in DMSO at pH 4.5. These preliminary results warrant further experimentation into spontaneous anomerization. If anomerization is consistently observed in this forthcoming study, then further sub-cellular localization, degradation, and trafficking studies will need to be pursued to verify if anomerization is a mode of action in the production of α-lyso-ceramides in vivo.

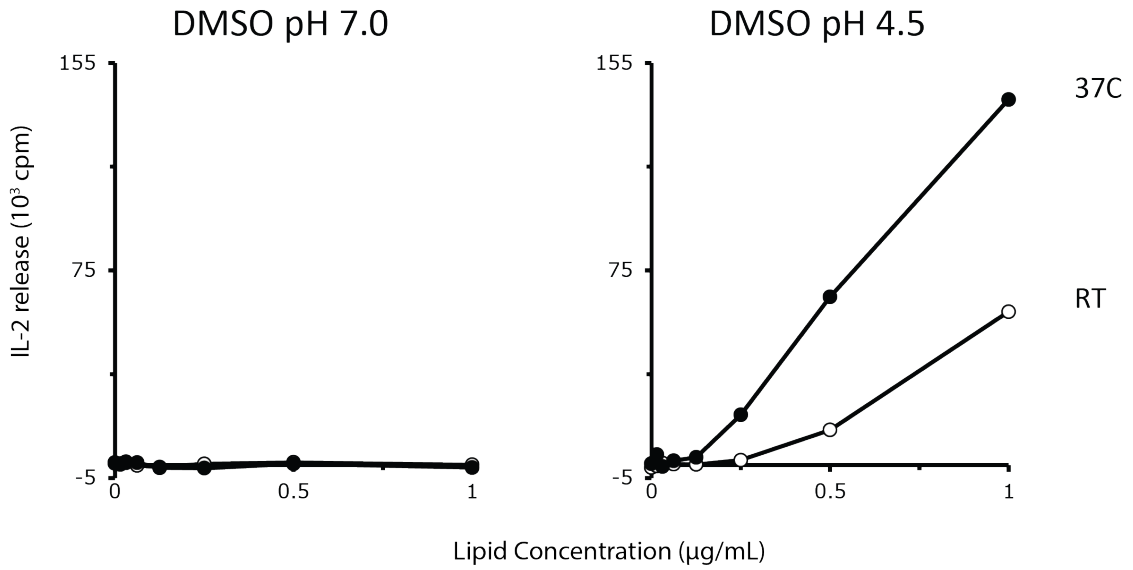


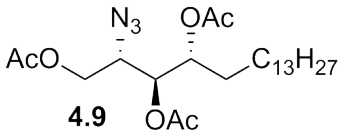
Figure 4.9 Data shows that β-sphingopsychosine loaded onto DC3.2 cells stimulated DN32.D3 T cells. The analyzed concentrationof IL-2 in the supernatents is represented.

4.4 Conclusion

Purification and analysis of commercially available β-GluCer directed us towards the discovery of stimulatory NKT cell antigens, α-psychosine, and α-glucopsychosine. L363, a CD1d-α-GalCer specific antibody, blocked the stimulatory activity of Avanti's β-GluCer (unpublished data acquired at the Teyton lab). These data led us to speculate that a stimulatory α-linked contaminant was present in the commercial β-GluCer sample. To verify this idea, SPR binding studies were performed on the binding affinity of CD1d-bound synthetic glycosylceramides with the L363 antibody. These studies revealed that α-psychosine, α-glucopsychosine, and α-GluCer all measurably bind to the L363 antibody, whereas no binding affinity was observed with their β counterparts. Following this study, we wanted to see if anomerization between α- and β-GalCer (24:1, sphingo-

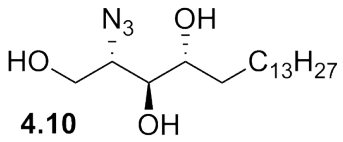
sine) as well as α- and β-sphingopsychosine were possible. Preliminary data suggest that under acidic conditions anomerization of β-sphingopsychosine to α-sphingopsychosine occurs. These studies warrant further investigations to determine the presence and/or role of α-psychosine and α-glucopsychosine in vivo.

4.5 Experimental Procedures



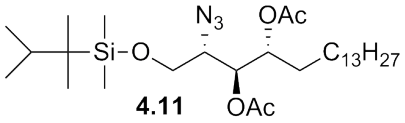
4.9

Preparation of 4.9: First, fresh triflic azide was made by the following procedure. Triflic anhydride (3.18 mL, 18.9 mmol) and sodium azide (6.14 g, 94.5 mmol) were dissolved in water (20 mL) and DCM (20 mL) at 0°C for two hours. Dissolved copper sulfate hydrate (151 mg, 0.947 mmol) and potassium carbonate (1.95 g, 14.2 mmol) in water (65 mL). This light blue solution was added to a large 2 liter flask that contained phytosphingosine (3.00 g, 9.46 mmol), DCM (100 mL) and MeOH (900 mL). To this reaction was added dropwise the triflic azide (just the DCM layer). The reaction was left overnight. In the morning the solvent was pulled off. To the blue slush was added acetic anhydride (8.04 mL, 85.2 mol), DMAP (288 mg, 2.37 mmol), and pyridine (200 mL). After a few hours the reaction was completed. The pyridine was pulled off via high vacuum rotary evaporator. A EtOAc:water (3 x 100 mL:100 mL) workup was performed. The organic layer was dried over $NaSO_4$, concentrated via rotary evaporator, and the residual solid was purified on SiO_2 (20% EtOAc:Hexane). A white powder was recovered (3.16 g, 71% over two steps).



4.10

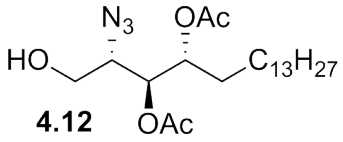
Preparation of 4.10: Azide **4.9** (1.88 g, 4.00 mmol) was dissolved in an anhydrous 1:1 solution of THF and methanol (25 mL).Freshly prepared 1 M sodium methoxide was added (0.5 mL) to the reaction flask and the reaction was stirred for one hour at room temperature. Reaction completion was verified via mass spectrometry and TLC. Upon completion, the solvent was removed en vacuo and the resulting residue was loaded onto a bed of silica gel and subjected to chromatography (5%, 10% MeOH:DCM). The purified compound was a white powder (1.25 g, 90%).



4.11

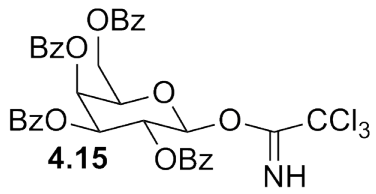
Preparation of 4.11: Compound **4.10** (2.16 g, 4.61 mmol) was dissolved in a minimal amount of anhydrous pyridine (40 mL). Chloro(dimethyl)thexylsilane (1.35 mL, 6.91 mmol) was added to the reaction flask. The reaction was monitored by mass spec for formation of the monosilated product. Every hour, more chloro(dimethyl)thexylsilane was added as needed. Once the starting material was completely consumed or presence of the di or tri silated was found, excess acetic anhydride (2.61 mL, 21.8 mmol) was added. After positive confirmation of completion of the reaction, the pyridine was evaporated via a high vacuum rotary evaporator. The dark syrup was dissolved in water and washed with DCM. The organic layer was concentrated down and subjected to flash chromatography (5%, 10% EtOAc/Hexane). The column yielded a white powder

compound (2.00 g, 78%).

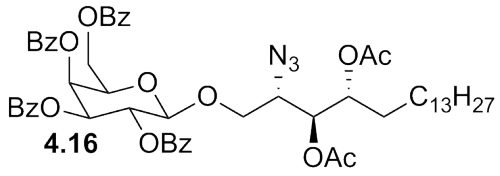


4.12

Preparation of 4.12: Compound **4.11** (250 mg, 0.452 mmol) was dissolved in DCM (3 mL) and ACN (27 mL) and placed in a plastic centrifuge tube (with the appropriate stir bar). To this tube was added 3 mL of hydrofluoric acid (48%). The reaction was monitored by TLC for formation of product **2.11**. Every thirty minutes, more hydrofluoric acid was added as needed. Once the starting material was completely consumed, the reaction was quickly and carefully quenched on a bed of solid $NaHCO_3$ in a separate plastic container (equipped with the appropriate stir bar). Water, DCM, and solid $NaHCO_3$ were slowly added until a neutral or basic pH was reached. The water/DCM workup was filtered to remove excess undissolved $NaHCO_3$. The water was washed with DCM (6 x 100 mL) and the organic layer was dried over $NaSO_4$, concentrated (water bath was 30°C or below to minimize acetate migration), and subjected to flash chromatography (20%, 25% EtOAc/Hexane). The column yielded a white powder compound (146 mg, 76%). Note: this reaction if done slowly, left overnight, heated up, or purified slowly can and will result in migration of the 2-acetyl group to the primary alcohol. It is essential that the chemist quench this reaction immediately after completion. 1H NMR (300 MHz, Chloroform-d) δ 5.23 - 5.10 (m, 2H), 3.90 (dd, J = 7.6, 3.8 Hz, 1H), 3.75 - 3.67 (m, 1H), 3.66 - 3.57 (m, 1H), 2.15 (s, 3H), 2.10 (s, 3H), 1.67 (s, 2H), 1.29 (m, 25H), 0.96 - 0.86 (m, 3H).

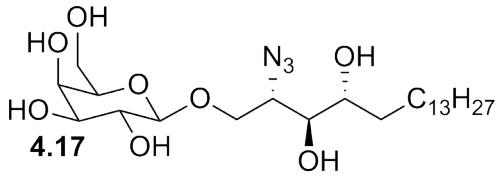


Preparation of 4.15: Fully protected galactose **4.14** (200 mg, 0.291 mmol) was dissolved in an acetone/water solution (20mL/3mL), followed by the portion wise addition of NBS (0.291 mmol). Subsequent equivalents of NBS were added until the reaction was completed (monitored by TLC). Upon completion, the reaction was quenched with solid $NaHCO_3$, and the acetone was evaporated off. The compound was dissolved in DCM (25 mL). Solid potassium carbonate (750 mg) was added, followed by the addition of excess trichloroacetonitrile (291 μL, 2.91 mmol). Once setup, the reaction was stirred for sixteen hours at room temperature. In the morning, the potassium carbonate was filtered and washed with EtOAc (100 mL). The filtrate was dried off at or below 30°C en vacuo. This pale yellow solid was loaded onto a bed of silica gel and was eluted with 15% EtOAc/Hexane. The two anomeric isomers were collected, combined, and concentrated at or below 30°C (170 mg, 79% from two steps)..



Preparation of 4.16: Donor **4.15** (100 mg, 0.135 mmol), azide **4.12** (50 mg, 0.113 mmol), and 4 angstrom molecular sieves (450 mg) were stirred together for one hour in dry DCM (5 mL). The vessel was cooled to -10°C (acetone/ice bath), followed by the dropwise addition of trimethylsilyltriflate (13 μL, 0.176 mmol). The reaction temperature was maintained for two hours

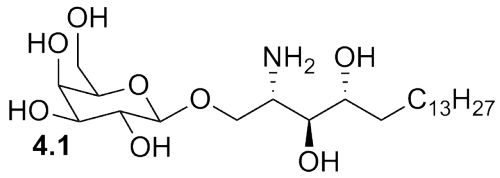
then quenched by the dropwise addition of triethyl amine. The M.S. were filtered and washed with EtOAc by means of a plug silica gel column. The filtrate was concentrated and subsequently loaded onto a silica gel column. Purification was achieved with a 10%, 20% EtOAc/Hexane eluent system. Isolated 60 mgs of coupled product (53% yield). 1H NMR (500 MHz, Chloroform-d) δ 8.13 - 8.06 (m, 2H), 8.05 - 7.98 (m, 2H), 7.98 - 7.93 (m, 1H), 7.81 - 7.74 (m, 2H), 7.66 - 7.59 (m, 1H), 7.56 (td, J = 7.3, 1.3 Hz, 1H), 7.54 - 7.34 (m, 6H), 7.29 - 7.20 (m, 2H), 5.99 (d, J = 1.2 Hz, 1H), 5.81 (dd, J = 10.3, 7.9 Hz, 1H), 5.61 (dd, J = 10.4, 3.4 Hz, 1H), 5.11 - 5.02 (m, 2H), 4.91 (d, J = 7.9 Hz, 1H), 4.69 (dd, J = 11.2, 6.3 Hz, 1H), 4.47 - 4.31 (m, 2H), 4.16 - 4.04 (m, 1H), 3.93 (dd, J = 9.9, 2.9 Hz, 1H), 3.70 (m, 1H), 2.03 (m, J = 9.2 Hz, 6H), 1.98 (s, 3H), 1.55 (d, J = 6.7 Hz, 2H), 1.26 (m, 24H), 0.88 (t, J = 6.9 Hz, 3H). 13C NMR (126 MHz, Chloroform-d) δ 171.14, 170.23, 169.45, 166.02, 165.56, 165.49, 165.01, 133.61, 133.32, 133.29, 133.20, 130.05, 129.79, 129.76, 129.34, 128.93, 128.70, 128.67, 128.65, 128.52, 128.49, 128.48, 128.32, 128.30, 128.27, 100.77, 77.25, 77.00, 76.80, 76.74, 72.34, 71.93, 71.67, 71.41, 69.58, 68.48, 67.93, 61.90, 60.39, 60.23, 31.91, 29.68, 29.66, 29.64, 29.62, 29.55, 29.51, 29.46, 29.35, 29.32, 25.35, 22.68, 21.04, 20.85, 20.72, 14.18, 14.11. HRMS (ESI) calcd for $C_{56}H_{67}N_3O_{14}$ [M+H]+: 1005.4623, found: 1023.4751.



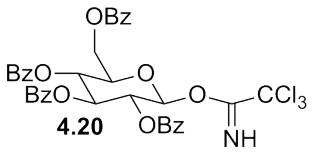
4.17

Preparation of 4.17: Azide **4.16** (60 mg, 0.0597 mmol) was dissolved in 20 mL of anhydrous methyl alcohol. Freshly prepared 1 M sodium methoxide was added to the reaction flask and

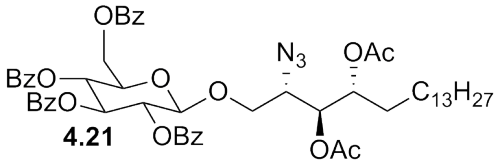
the reaction was stirred for one hour at room temperature. Reaction completion was verified via mass spectromety and TLC. Upon completion, the solvent was removed en vacuo and the resulting residue was subjected to flash chromatography (20% MeOH:DCM). The purified compound was a white powder (25 mg, 83%). 1H NMR (500 MHz, Methanol-d4) δ 4.28 (d, J = 7.6 Hz, 1H), 4.11 - 4.06 (m, 1H), 3.97 - 3.90 (m, 1H), 3.86 - 3.70 (m, 4H), 3.59 - 3.44 (m, 5H), 1.57 (q, J = 8.2, 7.1 Hz, 2H), 1.41 - 1.26 (m, 24H), 0.89 (t, J = 6.8 Hz, 3H). HRMS (ESI) calcd for $C_{24}H_{47}N_3O_8$ [M+H]+: 505.3633, found: 523.3736.



Preparation of 4.1: $Pd(OH)_2$ (a spatula tip) was added to a solution of compound **4.17** (25 mg, 0.0495 mmol) in MeOH:$CHCl_3$ (4 mL, 1:1) at rt. The reaction vessel was charged with H_2 gas (3x vacuum-H_2 flushes) and the resulting mixture stirred overnight. Filtration through Celite and removal of the solvent under reduced pressure left a residue which was purified by flash column chromatography (40% MeOH in $CHCl_3$) to afford psychosine **4.2**(17 mg, 72%) as a white powder. 1H NMR (500 MHz, Methanol-d4) δ 4.32 (d, J = 7.3 Hz, 1H), 4.09 - 4.04 (m, 2H), 3.84 (d, J = 2.9 Hz, 1H), 3.75 (m, 2H), 3.67 (dt, J = 8.6, 4.7 Hz, 1H), 3.62 - 3.48 (m, 4H), 3.48 - 3.44 (m, 1H), 1.56 (m, 2H), 1.41 - 1.26 (m, 24H), 0.89 (t, J = 6.8 Hz, 3H). 13C NMR (126 MHz, Methanol-d4) δ 103.11, 75.50, 73.25, 71.89, 71.81, 71.03, 68.92, 65.27, 61.22, 53.64, 48.11, 48.05, 47.94, 47.88, 47.77, 47.71, 47.68, 47.60, 47.43, 47.26, 47.09, 33.99, 31.65, 29.40, 29.37, 29.34, 29.05, 24.85, 22.31, 13.03. HRMS (ESI) calcd for $C_{24}H_{49}NO_8$ [M+H]+: 479.3458, found: 480.3529.

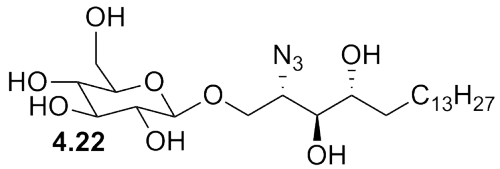


Preparation of 4.20: Fully protected glucose **4.19** (288 mg, 0.419 mmol) was dissolved in an acetone/water solution (20mL/3mL), followed by the portion wise addition of NBS (0.419 mmol). Subsequent equivalents of NBS were added until the reaction was completed (monitored by TLC). Upon completion, the reaction was quenched with solid $NaHCO_3$, and the acetone was evaporated off. The resultant slurry was dissolved in DCM (25 mL). Solid potassium carbonate (750 mg) was added, followed by the addition of excess trichloroacetonitrile (503 μL, 5.03 mmol). Once setup, the reaction was stirred for sixteen hours at room temperature. In the morning, the potassium carbonate was filtered and washed with EtOAc (100 mL). The filtrate was dried off at or below 30°C en vacuo. This pale yellow solid was loaded onto a bed of silica gel and was eluted with 15% EtOAc/Hexane. The two anomeric isomers were collected, combined, and concentrated at or below 30°C (257 mg, 83% from two steps).



Preparation of 4.21: Donor **4.20** (125 mg, 0.169 mmol), azide **4.12** (108 mg, 0.254 mmol), and 4 angstrom molecular sieves (500 mg) were stirred together for one hour in dry DCM (5 mL). The vessel was cooled to -10°C (acetone/ice bath), followed by the dropwise addition of trimethylsilyltriflate (16 μL, 0.0.845 mmol). The reaction temperature was maintained for two

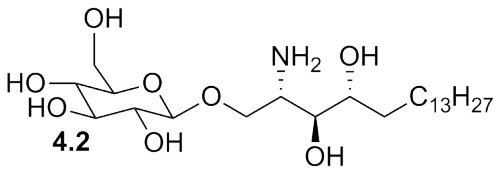
hours then quenched by the dropwise addition of TEA. The molecular sieves were filtered and washed with EtOAc by means of a plug silica gel column. The filtrate was concentrated and subsequently loaded onto a silica gel column. Purification was achieved with a 15%, 20% EtOAc/Hexane eluent system. 127 mgs of coupled product was isolated (75% yield). 1H NMR (500 MHz, Chloroform-d) δ 8.06 - 8.00 (m, 2H), 7.93 (m, 4H), 7.85 - 7.79 (m, 2H), 7.59 - 7.46 (m, 3H), 7.46 - 7.24 (m, 7H), 5.91 (t, J = 9.6 Hz, 1H), 5.70 (t, J = 9.7 Hz, 1H), 5.54 (dd, J = 9.7, 7.8 Hz, 1H), 5.08 - 4.98 (m, 2H), 4.93 (d, J = 7.8 Hz, 1H), 4.65 (dd, J = 12.2, 3.2 Hz, 1H), 4.51 (dd, J = 12.2, 5.1 Hz, 1H), 4.18 (m, 1H), 4.03 (dd, J = 10.5, 7.7 Hz, 1H), 3.88 (dd, J = 10.5, 3.1 Hz, 1H), 3.66 (m, 1H), 1.98 (m, J = 14.2 Hz, 6H), 1.52 (q, J = 6.6, 6.0 Hz, 2H), 1.24 (d, J = 8.9 Hz, 24H), 0.88 (t, J = 6.8 Hz, 3H). 13C NMR (126 MHz, Chloroform-d) δ 170.21, 169.45, 166.10, 165.78, 165.11, 164.87, 133.44, 133.24, 133.19, 133.15, 129.82, 129.75, 129.73, 129.51, 129.27, 128.72, 128.39, 128.30, 128.27, 100.59, 77.25, 77.20, 76.99, 76.74, 72.82, 72.40, 72.29, 71.96, 71.67, 69.52, 68.61, 62.99, 60.22, 31.92, 29.69, 29.66, 29.65, 29.62, 29.55, 29.46, 29.35, 29.31, 25.33, 22.69, 20.84, 20.66, 14.12. HRMS (ESI) calcd for $C_{56}H_{67}N_3O_{14}$ [M+H]+: 1005.4623, found: 1023.4756.



4.22

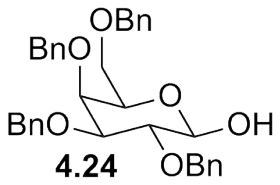
Preparation of 4.22: Azide **4.20** (75 mg, 0.0746 mmol) was dissolved in 20 mL of anhydrous MeOH. Freshly prepared 1 M sodium methoxide was added to the reaction flask and the reaction was stirred for one hour at room temperature. Reaction completion was verified via mass

spectrometry and TLC. Upon completion, the solvent was removed in vacuo and the resulting residue was subjected to flash chromatography (20% MeOH:DCM). The purified compound was a white powder (37.0 mg, 98%). 1H NMR (300 MHz, Methanol-d4) δ 4.35 (d, J = 7.7 Hz, 1H), 4.13 (dd, J = 10.7, 7.9 Hz, 1H), 4.03 - 3.78 (m, 3H), 3.77 - 3.66 (m, 1H), 3.64 - 3.54 (m, 2H), 3.46 - 3.18 (m, 4H), 1.76 - 1.66 (m, 1H), 1.66 - 1.53 (m, 2H), 1.34 (d, J = 11.2 Hz, 23H), 0.98 - 0.87 (m, 3H). 13C NMR (75 MHz, Methanol-d4) δ 102.86, 76.66, 76.63, 74.53, 73.67, 71.24, 70.16, 68.26, 62.88, 61.32, 48.46, 48.18, 47.89, 47.61, 47.33, 47.04, 46.76, 32.57, 31.70, 29.42, 29.39, 29.10, 25.33, 22.36, 13.07. HRMS (ESI) calcd for $C_{24}H_{47}N_3O_8$ [M+H]+: 505.3633, found: 523.3610.



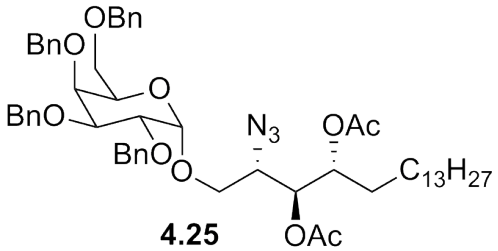
Preparation of 4.2: $Pd(OH)_2$ (a spatula tip) was added to a solution of compound **4.22** (37 mg, 0.130 mmol) in MeOH:$CHCl_3$ (4 mL, 1:1) at rt. The reaction vessel was charged with H_2 gas (3x vacuum-H_2 flushes) and the resulting mixture stirred overnight. Filtration through Celite and removal of the solvent under reduced pressure left a residue which was purified by flash column chromatography (40% MeOH in $CHCl_3$) to afford psychosine **4.2** (27 mg, 77%) as a white powder. 1H NMR (500 MHz, Methanol-d4) δ 4.35 (d, J = 7.8 Hz, 1H), 4.06 (d, J = 6.6 Hz, 2H), 3.90 (dd, J = 11.8, 2.2 Hz, 1H), 3.67 (dd, J = 11.7, 5.6 Hz, 2H), 3.58 (dd, J = 8.9, 4.2 Hz, 1H), 3.46 (dt, J = 8.7, 4.2 Hz, 1H), 3.42 - 3.21 (m, 4H), 1.55 (m, 2H), 1.41 - 1.26 (m, 24H), 0.89 (t, J = 6.9 Hz, 3H). 13C NMR (126 MHz, Methanol-d4) δ 102.63, 76.66, 76.38, 73.42, 71.91, 71.81, 70.05, 65.38, 61.05, 53.48, 48.21, 48.09, 48.04, 47.92, 47.88, 47.87, 47.85, 47.82, 47.79, 47.75, 47.73, 47.71,

47.69, 47.69, 47.68, 47.67, 47.66, 47.64, 47.61, 47.58, 47.53, 47.52, 47.50, 47.45, 47.41, 47.24, 47.07, 33.98, 31.65, 29.40, 29.37, 29.35, 29.33, 29.05, 24.83, 22.31, 13.02. HRMS (ESI) calcd for $C_{24}H_{49}NO_8$ [M+H]+: 479.3458, found: 480.3518.



4.24

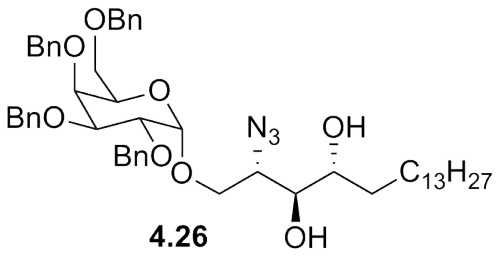
Preparation of 4.24: Fully protected galactose **4.23** (936 mg, 1.58 mmol) was dissolved in an acetone/water solution (30mL/5mL) and cooled to -10°C (Due to the armed sugar, this reaction has to be done at cold temperatures, or one will end up with a variety of byproducts), followed by the portion wise addition of NBS (141 mg, 0.79 mmol). Subsequent equivalents of NBS were added until the reaction was completed (monitored by TLC). Upon completion, the reaction was quenched with solid $NaHCO_3$, and the acetone was evaporated off. The remaining mixture was dissolved in water, washed with DCM (3 x 50 mL), dried with $NaSO_4$, concentrated, and subjected to flash chromatography (30% EtOAc:Hexanes). The white powder was dried collected for the next reaction (600 mg, 75%).



4.25

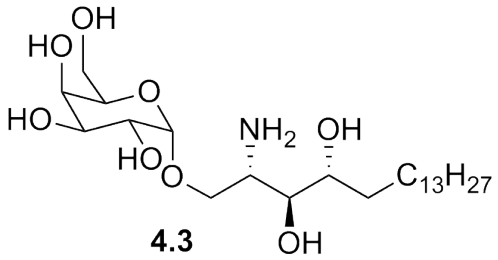
Preparation of 4.25: Under nitrogen, 2,4,6-Tri-tert-butylpyrimidine (204 mg, 0.820 mmol), phenyl sulfoxide (118 mg, 0.585 mmol), and 3 angstrom molocular sieves (250 mg) were added

to a stirred solution of donor **4.24** (151 mg, 0.281 mmol) dissolved in 4.5 mL of anhydrous DCM. The combined mixture was stirred at room temperature for one hour. The flask was then cooled to -60°C, at which point triflic anhydride (0.055 mL, 0.328 mmol) was added dropwise to the solution. The vessel was allowed to warm to -40°C at which point acceptor **4.12** (100 mg, 0.234 mmol) dissolved in 0.5 mL of DCM was added dropwise. The reaction then warmed up to room temperature and was quenched by the addition of TEA. Filtration through a bed of silica gel (ethyl acetate as the eluent) followed by removal of the solvent left a residue which was purified by flash column chromatography (15% ethyl acetate in hexane) to afford glycolipid **4.25** as a white powder (170 mg, 76%). 1H NMR (500 MHz, Chloroform-d) δ 7.41 - 7.23 (m, 20H), 5.12 - 5.05 (m, 2H), 4.94 (d, J = 11.5 Hz, 1H), 4.89 - 4.67 (m, 6H), 4.56 (d, J = 11.5 Hz, 1H), 4.46 (d, J = 11.9 Hz, 1H), 4.40 (d, J = 11.9 Hz, 1H), 4.04 (dd, J = 9.7, 3.6 Hz, 1H), 4.00 - 3.91 (m, 3H), 3.88 (dd, J = 10.9, 2.9 Hz, 1H), 3.80 (dd, J = 5.8, 2.9 Hz, 1H), 3.60 (dd, J = 10.9, 8.4 Hz, 1H), 3.55 - 3.42 (m, 3H), 2.01 (s, J = 3.9 Hz, 6H), 1.57 (d, J = 4.7 Hz, 2H), 1.25 (d, J = 5.5 Hz, 23H), 0.88 (t, J = 6.8 Hz, 3H). 13C NMR (126 MHz, Chloroform-d) δ 170.25, 169.64, 138.83, 138.67, 138.54, 137.92, 128.37, 128.32, 128.28, 128.26, 128.21, 127.82, 127.72, 127.70, 127.59, 127.57, 127.51, 127.43, 99.07, 78.64, 77.26, 77.20, 77.00, 76.75, 76.45, 75.15, 74.71, 73.41, 73.27, 73.17, 72.26, 72.18, 69.99, 69.22, 68.41, 60.84, 31.92, 29.69, 29.67, 29.65, 29.63, 29.56, 29.51, 29.48, 29.36, 25.31, 22.69, 20.92, 20.71, 14.13. HRMS (ESI) calcd for $C_{56}H_{75}N_3O_{10}$ [M+H]+: 949.5452, found: 967.5865.



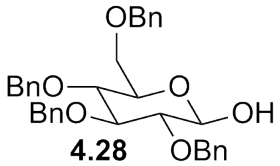
4.26

Preparation of 4.26: Azide **4.25** (81 mg, 85.3 μmol) was dissolved in 20 mL of anhydrous MeOH. Freshly prepared 1 M sodium methoxide was added to the reaction flask and the reaction was stirred for one hour at room temperature. Reaction completion was verified via mass specromety and TLC. Upon completion, the solvent was removed in vacuo and the resulting residue was subjected to flash chromatography (20% ethyl acetate:hexane). The purified compound was a white powder (71 mg, 96%). 1H NMR (500 MHz, Chloroform-d) δ 7.69 - 7.62 (m, 5H), 7.50 - 7.40 (m, 8H), 7.40 - 7.23 (m, 7H), 4.91 (dd, J = 11.6, 8.4 Hz, 2H), 4.81 - 4.63 (m, 4H), 4.59 - 4.46 (m, 2H), 4.42 (d, J = 11.9 Hz, 1H), 4.16 (dd, J = 10.6, 3.4 Hz, 1H), 4.08 - 3.89 (m, 4H), 3.81 (dd, J = 10.6, 3.8 Hz, 1H), 3.77 - 3.72 (m, 1H), 3.60 (m, 1H), 3.56 - 3.44 (m, 2H), 3.39 (d, J = 7.1 Hz, 1H), 2.31 (d, J = 5.3 Hz, 1H), 1.52 (m, 2H), 1.25 (s, 24H), 0.88 (t, J = 6.8 Hz, 3H). 13C NMR (126 MHz, Chloroform-d) δ 145.60, 138.45, 138.32, 137.91, 137.84, 131.03, 129.31, 129.29, 129.29, 129.28, 129.21, 128.45, 128.41, 128.39, 128.23, 128.19, 128.13, 127.95, 127.80, 127.73, 127.63, 127.61, 127.58, 124.82, 124.77, 124.69, 99.22, 79.21, 77.26, 77.20, 77.00, 76.75, 75.83, 75.22, 74.77, 74.68, 74.21, 73.41, 72.91, 72.80, 70.05, 69.13, 68.85, 59.97, 32.68, 31.92, 29.70, 29.68, 29.66, 29.65, 29.64, 29.36, 25.82, 22.69, 14.13. HRMS (ESI) calcd for $C_{52}H_{71}N_3O_8$ [M+H]+: 865.5241, found: 883.5685.



4.3

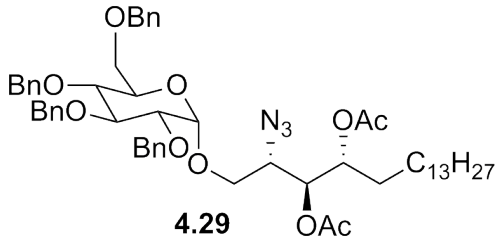
Preparation of 4.3: $Pd(OH)_2$ (a spatula tip) was added to a solution of compound **4.26** (120 mg, 0.130 mmol) in MeOH:$CHCl_3$ (5 mL, 1:1) at rt. The reaction vessel was charged with H_2 gas (3x vacuum-H_2 flushes) and the resulting mixture stirred overnight. Filtration through Celite and removal of the solvent under reduced pressure left a residue which was purified by flash column chromatography (40% MeOH in $CHCl_3$) to afford compound **4.3** (35 mg, 57%) as a white powder. (aggreagation was observed in the NMR spectra) 1H NMR (500 MHz, Pyridine-d5) δ 5.43 (s, 1H), 4.99 (dd, J = 10.8, 3.5 Hz, 1H), 4.76 (m, 1H), 4.66 (m, 3H), 4.54 (s, 1H), 4.51 - 4.32 (m, 5H), 4.18 - 4.11 (m, 1H), 3.62 (d, J = 3.3 Hz, 1H), 2.26 (m, J = 7.1, 3.9 Hz, 1H), 1.89 - 1.82 (m, 1H), 1.79 (dd, J = 9.2, 4.3 Hz, 1H), 1.64 - 1.58 (m, 1H), 1.24 (d, J = 11.4 Hz, 21H), 0.87 (t, J = 6.9 Hz, 3H). 13C NMR (126 MHz, pyridine) δ 100.18, 72.63, 71.66, 71.01, 69.93, 69.34, 69.27, 64.50, 61.15, 53.41, 34.02, 30.70, 28.79, 28.64, 28.57, 28.51, 28.50, 28.20, 28.19, 24.67, 21.52, 12.87. HRMS (ESI) calcd for $C_{24}H_{49}NO_8$ [M+H]+: 479.3458, found: 480.3446.



4.28

Preparation of 4.28: Fully protected glucose **4.27** (2.38 g, 3.76 mmol) was dissolved in an acetone/water solution (40mL/5mL) and cooled to -10°C, followed by the portion wise addition

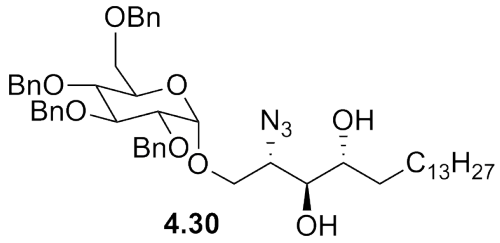
of NBS (335 mg, 1.88 mmol). Subsequent equivalents of NBS were added until the reaction was completed (monitored by TLC). Upon completion, the reaction was quenched with solid $NaHCO_3$, and the acetone was evaporated off. The remaining mixture was dissolved in water, washed with DCM (3 x 50 mL), dried with $NaSO_4$, concentrated, and subjected to flash chromatography (10% EtOAc:Hexanes). The white powder was dried collected for the next reaction (1.70 g, 84%).



4.29

Preparation of 4.29: 2,4,6-Tri-tert-butylpyrimidine (204 mg, 0.820 mmol), phenyl sulfoxide (118 mg, 0.585 mmol), and 3 angstrom molocular sieves (250 mg) were added to a stirred solution of donor **4.28** (151 mg, 0.281 mmol) dissolved in 4.5 mL of anhydrous DCM. The combined mixture was stirred at room temperature for one hour. The flask was then cooled to -60°C, at which point triflic anhydride (55 microliter, 0.328 mmol) was added dropwise to the solution. The vessel was allowed to warm to -40°C at which point ceramide **4.12** (100 mg, 0.234 mmol), dissolved in 0.5 mL of DCM, was added dropwise. The reaction then warmed up to room temperature and was quenched by the addition of TEA. Filtration through a bed of silica gel (ethyl acetate as the eluent) followed by removal of the solvent left a residue which was purified by flash column chromatography (15% EtOAc:Hexanes) to afford glycolipid **4.29** as a white powder (150 mg, 68%). 1H NMR (500 MHz, Chloroform-d) δ 7.69 - 7.62 (m, 6H), 7.50 - 7.40 (m, 9H), 7.39 - 7.23 (m, 5H), 5.12 (t, J = 5.6 Hz, 2H), 4.92 (d, J = 11.1 Hz, 2H), 4.86 - 4.70 (m, 4H), 4.63 - 4.51

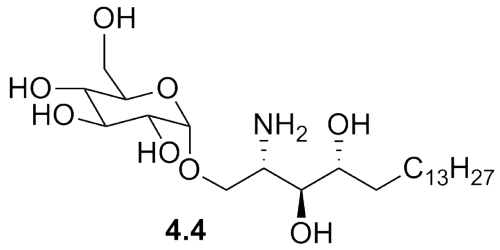
(m, 3H), 4.41 (d, J = 7.7 Hz, 1H), 4.16 - 4.03 (m, 2H), 3.80 - 3.68 (m, 3H), 3.68 - 3.57 (m, 3H), 3.51 - 3.43 (m, 2H), 2.09 - 2.00 (m, 6H), 1.64 - 1.57 (m, 2H), 1.36 - 1.22 (m, 24H), 0.88 (t, J = 6.8 Hz, 3H). 13C NMR (126 MHz, Chloroform-d) δ 170.25, 169.59, 145.62, 138.55, 138.50, 138.06, 138.02, 131.04, 131.03, 129.48, 129.37, 129.34, 129.32, 129.30, 129.27, 128.38, 128.37, 128.35, 128.34, 127.99, 127.98, 127.94, 127.92, 127.86, 127.77, 127.74, 127.72, 127.70, 127.63, 127.60, 127.55, 124.85, 124.80, 124.78, 124.77, 124.76, 124.75, 124.73, 124.67, 109.99, 103.15, 84.58, 82.28, 77.71, 77.28, 77.22, 77.02, 76.77, 75.61, 75.03, 74.98, 73.47, 73.44, 72.40, 71.84, 68.83, 68.48, 60.75, 60.37, 31.92, 29.69, 29.67, 29.65, 29.63, 29.59, 29.56, 29.48, 29.41, 29.35, 25.38, 25.35, 22.69, 21.04, 20.93, 20.78, 14.20, 14.12. HRMS (ESI) calcd for $C_{56}H_{75}N_3O_{10}$ [M+H]+: 949.5452, found: 967.5740.



4.30

Preparation of 4.30: Azide **4.29** was dissolved in 20 mL of anhydrous methyl alcohol. Freshly prepared 1 M sodium methoxide was added to the reaction flask and the reaction was stirred for one hour at room temperature. Reaction completion was verified via mass spectrometry and TLC. Upon completion, the solvent was removed in vacuo and the resulting residue was subjected to flash chromatography (20% ethyl acetate:hexane). The purified compound was a white powder (132 mg, 91%). 1H NMR (500 MHz, Chloroform-d) δ 7.66 (dd, J = 7.8, 1.8 Hz, 1H), 7.49 - 7.43 (m, 1H), 7.39 - 7.24 (m, 16H), 7.18 - 7.11 (m, 2H), 4.95 - 4.88 (m, 1H), 4.83 (dd, J = 11.2, 8.7 Hz,

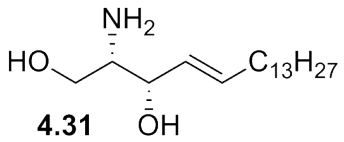
3H), 4.72 (d, J = 3.7 Hz, 1H), 4.62 (dd, J = 21.2, 12.1 Hz, 2H), 4.54 - 4.44 (m, 3H), 4.17 - 4.09 (m, 2H), 3.95 (t, J = 9.3 Hz, 1H), 3.83 (m, 1H), 3.79 - 3.54 (m, 6H), 3.06 (d, J = 5.8 Hz, 1H), 2.39 (d, J = 5.2 Hz, 1H), 1.63 - 1.56 (m, 2H), 1.55 - 1.48 (m, 1H), 1.41 (m, 2H), 1.35 - 1.23 (m, 22H), 0.89 (t, J = 6.9 Hz, 3H). 13C NMR (126 MHz, Chloroform-d) δ 138.50, 138.14, 137.76, 137.72, 131.04, 129.31, 128.55, 128.54, 128.44, 128.39, 128.38, 128.36, 128.34, 128.11, 128.08, 128.07, 127.94, 127.90, 127.78, 127.76, 127.73, 127.71, 127.65, 127.63, 98.44, 82.05, 79.33, 77.49, 77.26, 77.21, 77.00, 76.75, 75.73, 74.97, 74.81, 73.83, 73.48, 72.48, 70.87, 68.28, 68.14, 60.49, 60.38, 32.61, 31.92, 29.70, 29.68, 29.66, 29.64, 29.36, 25.81, 22.69, 21.04, 14.19, 14.12. HRMS (ESI) calcd for $C_{52}H_{71}N_3O_8$ [M+H]+: 865.5241, found: 883.5621.



4.4

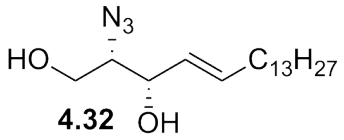
Preparation of 4.4: $Pd(OH)_2$ (a spatula tip) was added to a solution of azide **4.29** (35 mg, 0.0405 mmol) in MeOH:$CHCl_3$ (5 mL, 1:1) at rt. The reaction vessel was charged with H_2 gas (3x vacuum-H_2 flushes) and the resulting mixture stirred overnight. Filtration through Celite and removal of the solvent under reduced pressure left a residue which was purified by flash column chromatography (40% MeOH in $CHCl_3$) to afford amine **4.4** (35 mg, 57%) as a white powder. (aggreagation was observed in the NMR) 1H NMR (500 MHz, Pyridine-d5) δ 5.42 (d, J = 3.7 Hz, 1H), 4.94 (dd, J = 10.7, 3.5 Hz, 1H), 4.78 (t, J = 9.2 Hz, 1H), 4.73 - 4.64 (m, 2H), 4.50 - 4.34 (m, 3H), 4.29 (dd, J = 11.8, 5.3 Hz, 1H), 4.19 (t, J = 9.3 Hz, 1H), 4.12 (m, 2H), 3.62 (s, 1H), 2.27 -

2.22 (m, 1H), 1.88 - 1.80 (m, 1H), 1.80 - 1.72 (m, 1H), 1.58 (m, 1H), 1.42 - 1.21 (m, 22H), 0.88 (t, J = 6.8 Hz, 3H). 13C NMR (126 MHz, pyridine) δ 100.05, 73.72, 73.19, 72.56, 72.52, 70.96, 70.39, 64.65, 61.12, 53.46, 33.98, 30.70, 28.78, 28.63, 28.57, 28.50, 28.19, 24.66, 21.52, 12.87. HRMS (ESI) calcd for $C_{24}H_{49}NO_8$ [M+H]+: 479.3458, found: 480.3437



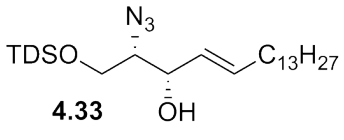
4.31

Preparation of 4.31: All glassware was flame dried under a vacuum to evaporate off any microscopic water that had collected in the flasks from moisture in the air. Ethyl amine (10 mL) was condensed in a two-neck flame-dried RBF that was cooled to -78°C (acetone:dry ice bath). Hexane-washed lithium (117 mg, 16.9 mmol) was added to the ethyl amine and stirred for half an hour. After the solution turned a dark blue color, compound **2.7** (492 mg, 1.13 mmol) was dissolved in anhydrous THF (15 mL), cooled to -78°C, and transferred via cannula into the dissolving metal solution. The reaction was maintained at -78°C for four hours. The reaction was quenched with solid ammonium chloride and raised to room temperature (at which point the solution turned a creamy yellow color). The quenched reaction stirred overnight in a hood to allow any excess ethyl amine to evaporate. The next day the excess THF was removed via rotary evaporator and the slurry was dissolved in water and extracted with diethyl ether (4 x 25 mL). This ether layer was dried over $NaSO_4$, impregnated with silica gel, and loaded dry onto a column. A very polar eluent method (0.5:5:95, 1:10:90, 1:15:85 ammonium hydroxide:MeOH:DCM) was employed to purify the compound (317 mg, 94%).



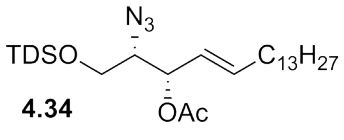
4.32

Preparation of 4.32: First, fresh triflic azide was made by the following procedure. Triflic anhydride (358 μL, 2.12 mmol) and sodium azide (689 mg, 10.6 mmol) were dissolved in water (20 mL) and DCM (20 mL) at 0°C for two hours. Dissolved copper sulfate hydrate (17 mg, 0.106 mmol) and potassium carbonate (219 mg, 1.59 mmol) in water (5 mL). This light blue solution was added to a large 2 liter flask that contained sphingosine, and MeOH (95 mL). To this reaction was added dropwise the triflic azide (just the DCM layer). The reaction was left overnight. In the morning the solvent was pulled off. Upon completion, the solvent was removed en vacuo and the resulting residue was loaded onto a bed of silica gel and subjected to chromatography (25%, 30% EtOAc:Hexanes). A white powder was recovered (243 g, 70% over two steps).



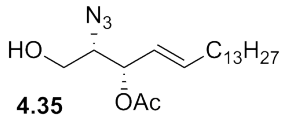
4.33

Preparation of 4.33: Compound **4.10** (240 mg, 0.738 mmol) was dissolved in a minimal amount of anhydrous pyridine (5 mL). TDS-Cl (145 μL, 0.738 mmol) was added to the reaction flask. The reaction was monitored by mass spec for formation of the mono-silated product. Every hour, more chloro(dimethyl)thexylsilane was added as needed.



4.34

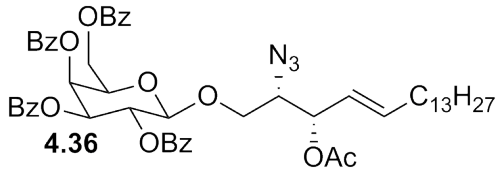
Preparation of 4.34: Once the compound **4.33** was completely consumed or presence of the di or tri silated was found, excess acetic anhydride (279 μL, 2.95 mmol) was added. After positive confirmation of completion of the reaction, the pyridine was evaporated via a high vacuum rotary evaporator. The dark syrup was dissolved in water and washed with DCM. The organic layer was concentrated down and subjected to flash chromatography (5%, 10% EtOAc/Hexane). The column yielded a white powder compound (418 mg, 82%).



4.35

Preparation of 4.35: Compound **4.34** (162 mg, 0.318 mmol) was dissolved in DCM (2 mL) and ACN (20 mL) and placed in a plastic centrifuge tube (with the appropriate stir bar). To this tube was added 0.5 mL of hydrofluoric acid (48%). The reaction was monitored by TLC for formation of product **4.35**. Every thirty minutes, more hydrofluoric acid was added as needed. Once the starting material was completely consumed, the reaction was quickly and carefully quenched on a bed of solid $NaHCO_3$ in a separate plastic container (equipped with the appropriate stir bar). Water, DCM, and solid $NaHCO_3$ were slowly added until a neutral or basic pH was reached. The water/DCM workup was filtered to remove excess undissolved $NaHCO_3$. The water was washed with DCM (6 x 25 mL) and the organic layer was dried over $NaSO_4$, concentrated (water bath was 30°C or below to minimize acetal migration), and subjected to flash chromatography (20%, 25% EtOAc/Hexane). The column yielded a white powder compound (78 mg, 67%). Note: this reaction if done slowly, left overnight, heated up, or purified slowly can and will result in migration of the 2-acetyl group to the primary alcohol. It is essential that the chemist quench this reaction

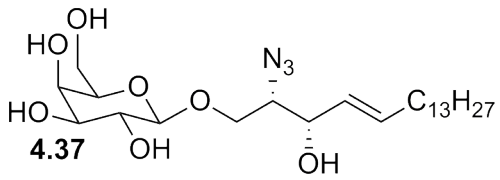
immediately after completion. 1H NMR (500 MHz, Chloroform-d) δ 5.86 (dd, J = 14.8, 6.8 Hz, 1H), 5.47 - 5.34 (m, 2H), 3.68 - 3.52 (m, 3H), 2.11 (s, 3H), 2.05 (m, 2H), 1.25 (s, 23H), 0.88 (t, J = 6.9 Hz, 3H). 13C NMR (126 MHz, Chloroform-d) δ 170.21, 138.09, 123.93, 77.24, 77.19, 76.99, 76.74, 74.32, 65.92, 61.64, 32.26, 31.91, 29.67, 29.65, 29.64, 29.56, 29.40, 29.34, 29.12, 28.65, 22.68, 21.08, 14.11.



4.36

Preparation of 4.36: Donor **4.15** (75 mg, 0.101 mmol), azide **4.35** (56 mg, 0.152 mmol), and 4 angstrom molecular sieves (300 mg) were stirred together for one hour in dry DCM (3 mL). The vessel was cooled to -10°C (acetone/ice bath), followed by the dropwise addition of trimethylsilyl-triflate (9 μL, 0.051 mmol). The reaction temperature was maintained for two hours then quenched by the dropwise addition of TEA. The molecular sieves were filtered and washed with EtOAc by means of a plug silica gel column. The filtrate was concentrated and subsequently loaded onto a silica gel column. Purification was achieved with a 10%, 15%, 20% EtOAc/Hexane eluent system. 74 mgs of coupled product was isolated (77% yield). 1H NMR (500 MHz, Chloroform-d) δ 8.10 (dt, J = 7.2, 1.4 Hz, 2H), 8.03 (dd, J = 8.1, 1.5 Hz, 2H), 7.98 - 7.91 (m, 2H), 7.78 (dd, J = 8.2, 1.4 Hz, 2H), 7.66 - 7.61 (m, 2H), 7.57 (td, J = 7.2, 1.4 Hz, 2H), 7.53 - 7.34 (m, 6H), 7.28 - 7.21 (m, 2H), 5.97 (d, J = 3.4, 1H), 5.80 (m, 1H), 5.71 (dd, J = 15.5, 6.8 Hz, 1H), 5.57 (dd, J = 10.4, 3.5 Hz, 1H), 5.29 - 5.21 (m, 1H), 4.87 (d, J = 8.0 Hz, 1H), 4.65 (dd, J = 11.4, 6.9 Hz, 1H), 4.42 (dd, J = 11.4, 6.2 Hz, 1H), 4.34 (dd, J = 8.7, 5.2 Hz, 1H), 4.30 - 4.23 (m, 1H), 4.19 - 4.03 (m, 2H), 3.76

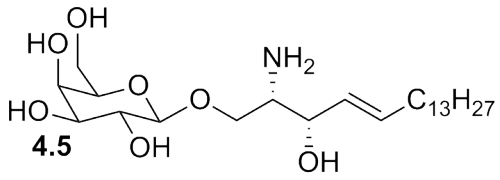
(m, 1H), 3.70 - 3.64 (m, 1H), 2.05 (m, 5H), 1.41 - 1.34 (m, 2H), 1.26 (q, J = 7.3 Hz, 22H), 0.87 (td, J = 6.9, 2.2 Hz, 3H). 13C NMR (126 MHz, Chloroform-d) δ 171.11, 170.75, 170.44, 165.96, 165.57, 165.53, 165.01, 139.96, 136.39, 133.59, 133.35, 133.25, 133.15, 130.05, 129.77, 129.74, 129.71, 129.69, 129.67, 129.46, 129.38, 128.95, 128.73, 128.67, 128.64, 128.49, 128.47, 128.41, 128.33, 128.31, 128.26, 127.22, 123.55, 97.55, 78.36, 77.25, 77.20, 77.00, 76.74, 72.63, 71.74, 71.40, 69.59, 68.06, 64.53, 63.69, 63.28, 62.98, 62.08, 60.37, 32.32, 32.28, 31.91, 29.70, 29.67, 29.65, 29.64, 29.62, 29.57, 29.44, 29.41, 29.34, 29.25, 29.15, 28.86, 28.77, 22.67, 21.03, 20.73, 20.70, 14.18, 14.10. HRMS (ESI) calcd for $C_{54}H_{63}N_3O_{12}$ [M+H]: 945.4412, found: 963.4771.



4.37

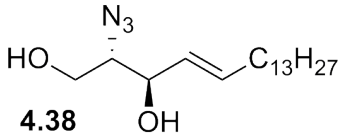
Preparation of 4.37: Azide **4.36** (64 mg, 67.6 μmol) was dissolved in 10 mL of anhydrous methyl alcohol and 5 mL of THF. Freshly prepared 1 M sodium methoxide (0.1 mL) was added to the reaction flask and the reaction was stirred for one hour at room temperature. Reaction completion was verified via mass spectrometry and TLC. Upon completion, the solvent was removed en vacuo and the resulting residue was subjected to flash chromatography (20% ethyl acetate:hexane). The purified compound was a white powder (29 mg, 88%). 1H NMR (500 MHz, Methanol-d4) δ 5.84 (dt, J = 15.4, 6.8 Hz, 1H), 4.34 (dd, J = 8.7, 6.4 Hz, 1H), 4.28 (d, J = 7.7 Hz, 1H), 3.82 - 3.66 (m, 3H), 3.62 (dd, J = 11.7, 6.8 Hz, 1H), 3.56 - 3.46 (m, 3H), 3.46 - 3.39 (m, 2H), 2.15 - 2.07 (m, 2H), 1.38 - 1.26 (m, 24H), 0.89 (t, J = 6.8 Hz, 3H). 13C NMR (126 MHz, Methanol-d4) δ 138.07, 132.10, 125.53, 99.51, 76.72, 75.35, 73.60, 70.97, 68.95, 66.69, 61.45, 61.20, 53.37, 48.20, 48.09,

48.03, 47.92, 47.89, 47.86, 47.85, 47.83, 47.81, 47.75, 47.69, 47.66, 47.58, 47.52, 47.41, 47.24, 47.07, 32.00, 31.65, 29.37, 29.35, 29.34, 29.33, 29.17, 29.05, 28.80, 28.67, 22.31, 13.01. HRMS (ESI) calcd for $C_{24}H_{45}N_3O_7$ [M+H]: 487.3258, found: 505.3642.



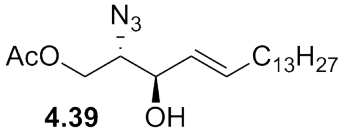
Preparation of 4.5: Compound **4.37** (29 mg, 0.0595 mmol) was dissolved in 4 mL of 1:1 THF:MeOH. 150 mg of granular zinc and 0.5 mL of glacial acetic acid were added to the flask. The mixture was sonicated for 30 minutes. Upon completion the solvent was transferred to a separate RBF and the solvent was dried off via a high vacuum rotary evaporator. Next, the compound was dissolved in 4 mL of 1:1 THF:MeOH. This solution was basified with concentrated NaOH (until pH 12) and stirred for one hour. Upon completion the solvent was dried off via a high vacuum rotary evaporator and the material was loaded onto a small silica gel column and subjected to a polar eluent system (25:65:2 MeOH:DCM:H_2O, 25:65:4 MeOH:DCM:H_2O, and 65:25:10 MeOH:DCM:H_2O). The resultant compound was a white powder (24 mg, 87%). 1H NMR (500 MHz, Methanol-d4) δ 5.89 (dt, J = 14.5, 6.8 Hz, 1H), 5.41 (dd, J = 15.5, 7.7 Hz, 1H), 4.40 (dd, J = 7.9, 4.3 Hz, 1H), 4.39 (m, 1H), 4.24 (d, J = 7.7 Hz, 1H), 3.82 - 3.72 (m, 2H), 3.72 - 3.64 (m, 2H), 3.62 - 3.57 (m, 1H), 3.57 - 3.50 (m, 1H), 3.48 - 3.41 (m, 2H), 3.06 (dt, J = 9.2, 4.6 Hz, 1H), 2.11 (q, J = 7.2 Hz, 2H), 1.43 (m, 2H), 1.30 (d, J = 9.7 Hz, 22H), 0.90 (t, J = 6.8 Hz, 3H). 13C NMR (126 MHz, Methanol-d4) δ 137.51, 124.66, 99.92, 77.22, 75.44, 73.42, 71.03, 68.96, 61.30, 60.79, 55.97, 48.19, 48.08, 48.05, 48.02, 47.91, 47.89, 47.87, 47.85, 47.77, 47.73, 47.70, 47.69, 47.68,

47.66, 47.65, 47.65, 47.63, 47.62, 47.60, 47.56, 47.54, 47.53, 47.52, 47.52, 47.51, 47.50, 47.50, 47.49, 47.48, 47.48, 47.47, 47.45, 47.44, 47.43, 47.42, 47.39, 47.36, 47.35, 47.34, 47.34, 47.33, 47.33, 47.32, 47.31, 47.29, 47.26, 47.22, 47.17, 47.07, 47.05, 32.03, 31.65, 29.38, 29.37, 29.34, 29.20, 29.05, 28.94, 28.78, 22.31, 13.00. HRMS (ESI) calcd for $C_{24}H_{47}NO_7$ [M+H]: 461.3353, found: 462.3424.



4.38

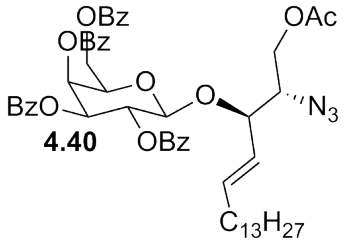
Preparation of 4.38: First, fresh triflic azide was made by the following procedure. Triflic anhydride (4.85 mL, 28.8 mmol) and sodium azide (9.36 g, 144 mmol) were dissolved in water (40 mL) and DCM (40 mL) at 0°C for two hours. Dissolved copper sulfate hydrate (230 mg, 144 mmol) and potassium carbonate (2.98 g, 21.6 mmol) in water (40 mL). This light blue solution was added to a large 2 liter flask that contained sphingosine (4.30 g, 14.4 mmol), and MeOH (950 mL). To this reaction was added dropwise the triflic azide (just the DCM layer). The reaction was left overnight. In the morning the solvent was pulled off. Upon completion, the solvent was removed en vacuo and the resulting residue was loaded onto a bed of silica gel and subjected to chromatography (30% MeOH:DCM). The purified compound was a white powder (2.40 g, 45%, over two steps(sphingosine formation)).



4.39

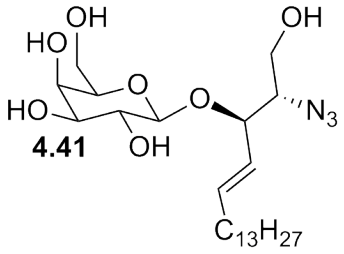
Preparation of 4.39: One equivalent of acetic anhydride (2,780 μL, 29.52 mmol) was added

to a solution of compound **4.38** in pyridine. A catalytic amount of DMAP was added to the mixture. After positive confirmation of reaction completion, the pyridine was evaporated via a high vacuum rotary evaporator. The dark syrup was dissolved in water and washed with DCM. The organic layer was concentrated down and subjected to flash chromatography (10%, 15% EtOAc/Hexane). The column yielded a white powder compound. 1H NMR (500 MHz, Chloroform-d) δ 5.85 - 5.76 (m, 1H), 5.51 (m, 1H), 4.27 (dd, J = 11.7, 3.6 Hz, 1H), 4.19 - 4.10 (m, 2H), 3.67 (m, 1H), 2.11 (s, 3H), 2.08 - 2.02 (m, 2H), 1.43 - 1.19 (m, 23H), 0.88 (t, J = 6.9 Hz, 3H). 13C NMR (126 MHz, Chloroform-d) δ 170.77, 136.44, 127.19, 77.24, 77.19, 76.99, 76.73, 72.63, 64.52, 63.69, 32.28, 31.91, 29.67, 29.66, 29.64, 29.57, 29.44, 29.34, 29.15, 28.86, 22.67, 20.73, 14.10.



Preparation of 4.40: Donor **4.15** (95 mg, 0.129 mmol), azide **4.35** (71 mg, 0.193 mmol), and 4 angstrom molecular sieves (300 mg) were stirred together for one hour in dry DCM (3 mL). The vessel was cooled to -10°C (acetone/ice bath), followed by the dropwise addition of trimethylsilyltriflate (12 μL, 0.0645 mmol). The reaction temperature was maintained for two hours then quenched by the dropwise addition of TEA. The molecular sieves were filtered and washed with EtOAc by means of a plug silica gel column. The filtrate was concentrated and subsequently loaded onto a silica gel column. Purification was achieved with a 10%, 15%, 20% EtOAc/Hexane eluent system. Isolated 120 mgs of coupled product (99% yield). 1H NMR (500

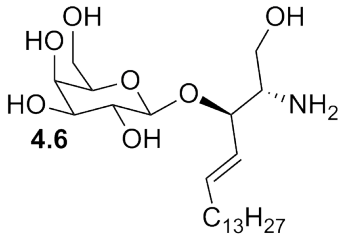
MHz, Chloroform-d) δ 8.09 (d, J = 7.6 Hz, 2H), 8.00 (dd, J = 16.8, 7.7 Hz, 4H), 7.78 (d, J = 7.7 Hz, 2H), 7.66 - 7.34 (m, 9H), 7.28 - 7.21 (m, 3H), 6.00 (d, J = 3.5 Hz, 1H), 5.81 (dd, J = 10.4, 7.8 Hz, 1H), 5.67 (dd, J = 14.8, 7.2 Hz, 1H), 5.60 (dd, J = 10.4, 3.5 Hz, 1H), 5.40 - 5.26 (m, 2H), 4.89 (d, J = 7.9 Hz, 1H), 4.69 (dd, J = 11.2, 6.4 Hz, 1H), 4.41 (dd, J = 11.3, 6.8 Hz, 1H), 4.33 (t, J = 6.7 Hz, 1H), 4.12 (q, J = 7.1 Hz, 1H), 4.00 (dd, J = 10.6, 6.4 Hz, 1H), 3.76 (dd, J = 10.6, 4.6 Hz, 1H), 3.58 (q, J = 5.5 Hz, 1H), 2.08 - 1.91 (m, 5H), 1.34 - 1.21 (m, 24H), 0.87 (t, J = 6.8 Hz, 3H). 13C NMR (126 MHz, Chloroform-d) δ 169.48, 166.00, 165.52, 165.49, 165.11, 138.08, 137.68, 133.59, 133.31, 133.27, 133.19, 130.01, 129.83, 129.77, 129.75, 129.34, 129.26, 128.97, 128.70, 128.64, 128.50, 128.47, 128.42, 128.32, 128.27, 123.94, 123.80, 101.32, 77.25, 77.20, 77.00, 76.75, 74.32, 73.67, 71.63, 71.39, 69.55, 68.25, 67.95, 65.92, 63.41, 61.85, 61.65, 60.38, 32.26, 32.21, 31.91, 29.69, 29.67, 29.66, 29.64, 29.59, 29.56, 29.40, 29.35, 29.14, 29.12, 28.65, 28.63, 22.68, 21.04, 20.98, 14.19, 14.11.



4.41

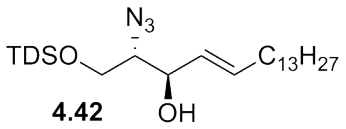
Preparation of 4.41: Azide **4.36** (120 mg, 0.127 mmol) was dissolved in 15 mL of anhydrous methyl alcohol. Freshly prepared 1 M sodium methoxide (0.5 mL) was added to the reaction flask and the reaction was stirred for one hour at room temperature. Reaction completion was verified via mass spectrometry and TLC. Upon completion, the solvent was removed en vacuo and the resulting residue was subjected to flash chromatography (5%, 10%, 15% MeOH/DCM). The puri-

fied compound was a white powder (49 mg, 79%). 1H NMR (500 MHz, Methanol-d4) δ 5.77 (dt, J = 15.9, 6.7 Hz, 1H), 5.54 (m, 1H), 4.27 - 4.17 (m, 2H), 3.94 (dd, J = 10.4, 7.3 Hz, 1H), 3.83 (d, J = 3.3 Hz, 1H), 3.74 (p, J = 4.6 Hz, 3H), 3.57 - 3.43 (m, 4H), 2.06 (q, J = 7.1 Hz, 2H), 1.45 - 1.36 (m, 2H), 1.29 (d, J = 7.5 Hz, 22H), 0.89 (t, J = 6.8 Hz, 3H). 13C NMR (126 MHz, Methanol-d4) δ 103.69, 75.29, 73.55, 71.75, 71.06, 68.83, 68.48, 65.91, 60.98, 48.10, 47.93, 47.92, 47.76, 47.58, 47.41, 47.24, 47.07, 31.96, 31.66, 29.38, 29.37, 29.34, 29.21, 29.06, 28.87, 28.80, 22.32, 13.03. HRMS (ESI) calcd for $C_{24}H_{45}N_3O_7$ [M+H]: 487.3258, found: 505.3607.



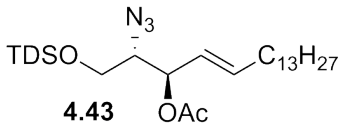
4.6

Preparation of 4.6: Compound **4.37** (49 mg, 0.101 mmol) was dissolved in 4 mL of 1:1 THF:MeOH. 300 mg of granular zinc and 0.5 mL of glacial acetic acid were added to the flask. The mixture was sonicated for 30 minutes. Upon completion the solvent was transferred to a separate RBF and the solvent was dried off via a high vacuum rotary evaporator. Next, the compound was dissolved in 4 mL of 1:1 THF:MeOH. This solution was basified with concentrated NaOH (until pH 12) and stirred for one hour. Upon completion the solvent was dried off via a high vacuum rotary evaporator and the material was loaded onto a small silica gel column and subjected to a polar eluent system (25:65:2 MeOH:DCM:H_2O, 25:65:4 MeOH:DCM:H_2O, and 65:25:10 MeOH:DCM:H_2O). The resultant compound was a white powder (34 mg, 73%). HRMS (ESI) calcd for $C_{24}H_{47}NO_7$ [M+H]: 461.3353, found: 462.3420.



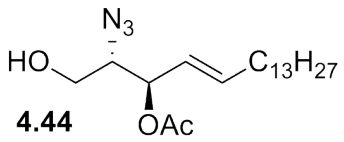
4.42

Preparation of 4.42: Compound **4.38** (2.40 g, 7.38 mmol) was dissolved in a minimal amount of anhydrous pyridine (15 mL). TDS-Cl (2.17 mL, 11.1 mmol) was added to the reaction flask. The reaction was monitored by mass spec for formation of the mono-silated product. Every hour, more chloro(dimethyl)thexylsilane was added as needed.



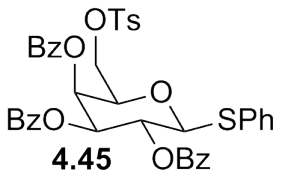
4.43

Preparation of 4.43: Once compound **4.39** was completely consumed or the presence of the di or tri silated was found, excess acetic anhydride (2.78 mL, 29.5 mmol) was added. After positive confirmation of completion of the reaction, the pyridine was evaporated via a high vacuum rotary evaporator. The dark syrup was dissolved in water and washed with DCM. The organic layer was concentrated down and subjected to flash chromatography (5%, 10% EtOAc/Hexane). The column yielded a white powder compound (3.02 g, 80%). 1H NMR (500 MHz, Chloroform-d) δ 5.81 (d, J = 15.3 Hz, 1H), 5.43 (d, J = 15.3 Hz, 1H), 5.34 (dd, J = 8.3, 3.3 Hz, 1H), 3.67 - 3.54 (m, 3H), 2.08 (s, 3H), 2.07 - 2.00 (m, 2H), 1.25 (m, 23H), 0.92 - 0.84 (m, 18H), 0.10 (d, J = 4.5 Hz, 6H). 13C NMR (126 MHz, Chloroform-d) δ 169.67, 138.25, 123.24, 77.25, 77.20, 76.99, 76.74, 73.91, 65.50, 62.43, 34.11, 32.31, 31.92, 29.68, 29.66, 29.60, 29.45, 29.36, 29.11, 28.76, 25.10, 22.69, 21.18, 20.19, 20.16, 18.47, 14.12, -3.63.



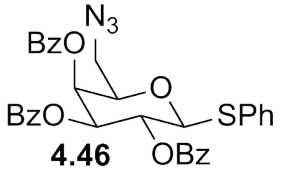
4.44

Preparation of 4.44: Compound **4.43** (3.00 g, 5.89 mmol) was dissolved in DCM (20 mL) and ACN (100 mL) and placed in a plastic centrifuge tube (with the appropriate stir bar). To this tube was added 10 mL of hydrofluoric acid (48%). The reaction was monitored by TLC for formation of product **4.35**. Every thirty minutes, more hydrofluoric acid was added as needed. Once the starting material was completely consumed, the reaction was quickly and carefully quenched on a bed of solid $NaHCO_3$ in a separate plastic container (equipped with the appropriate stir bar). Water, DCM, and solid $NaHCO_3$ were slowly added until a neutral or basic pH was reached. The water/DCM workup was filtered to remove excess undissolved $NaHCO_3$. The water was washed with DCM (6 x 25 mL) and the organic layer was dried over $NaSO_4$, concentrated (water bath was 30°C or below to minimize acetal migration), and subjected to flash chromatography (20%, 25% EtOAc/Hexane). The column yielded a white powder compound (2.00 g, 92%). Note: this reaction if done slowly, left overnight, heated up, or purified slowly can and will result in migration of the 2-acetyl group to the primary alcohol. It is essential that the chemist quench this reaction immediately after completion. 1H NMR (500 MHz, Chloroform-d) δ 5.85 (dt, J = 15.4, 6.8 Hz, 1H), 5.48 (m, 1H), 5.35 (dd, J = 8.1, 5.0 Hz, 1H), 3.72 - 3.61 (m, 2H), 3.59 - 3.52 (m, 1H), 2.09 (s, 3H), 2.05 (m, 2H), 1.42 - 1.18 (m, 23H), 0.88 (t, J = 6.9 Hz, 3H). 13C NMR (126 MHz, Chloroform-d) δ 169.91, 138.54, 123.30, 77.24, 76.99, 76.73, 74.09, 65.80, 61.91, 32.31, 29.68, 29.67, 29.65, 29.63, 29.56, 29.40, 29.33, 29.12, 29.11, 28.67, 22.67, 21.08, 14.10.



4.45

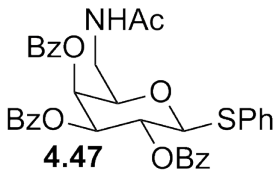
Preparation of 4.45: Compound **4.14** was diluted in methanol. To this flask was added sodium methoxide. The next morning the reaction was quenched by the addition of excess amberlight strongly acidic beads. After quenching, the beads were filtered of and the solvent was removed en vacuo. The light orange solid was dissolved in dry pyridine, followed by the addition of tosyl chloride at 0°C. The reaction warmed to room temperature after three hours and the reaction stirred overnight. In the morning, benzoyl chloride was added. Two hours later the solvent was removed via a high vacuum rotary evaporator, followed by a DCM (3 x 75 mL):water (75 mL) workup. The dark brown slurry was subjected to flash chromatography to give product **4.45**. HRMS (ESI) calcd for $C_{40}H_{34}O_{10}S_2$ [M+H]+: 738.1593, found: 725.2009.



4.46

Preparation of 4.46: Compound **4.45** (8.00 g, 10.9 mmol) and sodium azide (3.50 g, 54.3 mmol) were dissolved in a 9:1 solution of DMF:water (200 mL). The RBF (connected to a condenser) was heated to 100°C. This reaction was stirred overnight (evidenced by TLC). The water and DMF were pulled off on a high vacuum rotary evaporator, followed by a water:DCM (3 x 150 mL) workup. The dried ($NaSO_4$) and concentrated organic layer was loaded onto a silica gel column (15% EtOAc:Hexane) (7.45 g, 80%). 1H NMR (500 MHz, Chloroform-d) δ 8.02 - 7.96

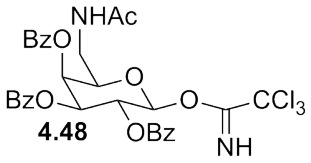
(m, 2H), 7.87 - 7.81 (m, 2H), 7.78 - 7.72 (m, 2H), 7.63 (m, 3H), 7.58 - 7.50 (m, 1H), 7.48 - 7.36 (m, 8H), 7.28 - 7.20 (m, 2H), 5.83 (dd, J = 3.2, 1.1 Hz, 1H), 5.74 (t, J = 9.9 Hz, 1H), 5.54 (dd, J = 9.9, 3.2 Hz, 1H), 5.01 (d, J = 9.9 Hz, 1H), 4.16 - 4.09 (m, 1H), 3.61 (dd, J = 13.0, 8.1 Hz, 1H), 3.32 (dd, J = 13.0, 4.3 Hz, 1H). 13C NMR (126 MHz, Chloroform-d) δ 165.48, 165.34, 165.12, 134.80, 133.65, 133.37, 133.37, 133.29, 130.39, 129.98, 129.94, 129.80, 129.76, 129.74, 129.27, 128.89, 128.84, 128.73, 128.68, 128.65, 128.60, 128.57, 128.44, 128.42, 128.28, 85.56, 77.27, 77.21, 77.01, 76.76, 76.65, 73.05, 68.72, 67.63, 51.07. HRMS (ESI) calcd for $C_{33}H_{27}N_3O_7S$ [M+H]+: 609.1570, found: 627.1952.



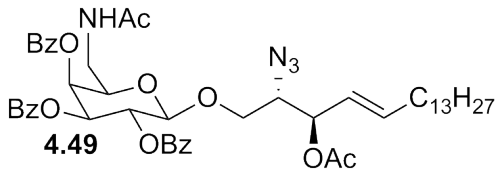
4.47

Preparation of 4.47: Azide **4.46** (1.63 g, 2.68 mmol) was dissolved in 15 mL of MeOH. To this flask was added 473 μL of thioacetic acid (6.71 mmol), 777 μL of 2,6-lutidine (671 mmol), and 278 mg of ruthenium trichloride (1.34 mmol). The reaction was stirred for 18 hours. The ruthenium trichloride was filtered off and the dark red solution was concentrated en vacuo and loaded onto a silica gel column (50%, 70% EtOAc:Hexane, then 5%, 10% MeOH:DCM elution profile). The isolated compound was formed in 41% yield (695 mg). 1H NMR (500 MHz, Chloroform-d) δ 8.01 - 7.95 (m, 2H), 7.93 - 7.87 (m, 2H), 7.79 - 7.73 (m, 2H), 7.67 - 7.50 (m, 4H), 7.50 - 7.32 (m, 7H), 7.28 - 7.19 (m, 3H), 6.01 (t, J = 6.3 Hz, 1H), 5.81 - 5.74 (m, 2H), 5.53 (dd, J = 9.9, 3.2 Hz, 1H), 5.00 (d, J = 10.0 Hz, 1H), 4.12 (m, 2H), 3.67 (dt, J = 13.6, 6.6 Hz, 1H), 3.27 (dt, J = 13.5, 6.6 Hz, 1H), 2.03 (d, J = 18.6 Hz, 3H). 13C NMR (126 MHz, Chloroform-d) δ 170.42, 166.18,

165.43, 165.19, 133.90, 133.77, 133.37, 133.30, 131.00, 130.00, 129.80, 129.78, 129.72, 129.22, 128.91, 128.63, 128.49, 128.43, 128.29, 109.99, 85.55, 77.25, 77.19, 76.99, 76.74, 75.27, 73.06, 69.17, 67.90, 60.37, 39.26, 23.29, 21.04, 14.19. HRMS (ESI) calcd for $C_{35}H_{31}NO_8S$ [M+H]+: 625.1770, found: 626.1958.

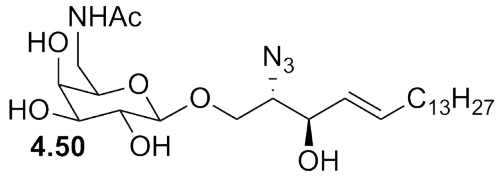


Preparation of 4.48: Amide **2.36** (685 mg, 1.10 mmol) was dissolved in an acetone/water solution (20mL/5mL), followed by the portion wise addition of NBS (600 mg, 3.30 mmol). Subsequent equivalents of NBS were added until the reaction was completed (monitored by TLC). Upon completion, the reaction was quenched with solid $NaHCO_3$, and the acetone was evaporated off. The remaining mixture was dissolved in water (50 mL), washed with DCM (3 x 50 mL), dried with $NaSO_4$, concentrated, and subjected to flash chromatography (5% MeOH:DCM). The white powder was dissolved in DCM (25 mL). Solid potassium carbonate (1.50 g, 10.9 mmol) was added, followed by the addition of excess trichloroacetonitrile (2.20 mL, 20.2 mmol). Once setup, the reaction was stirred for sixteen hours at room temperature. In the morning, the potassium carbonate was filtered and washed with EtOAc (100 mL). The filtrate was dried off at or below 30°C en vacuo. This pale yellow solid was loaded onto a bed of silica gel and was eluted with 2.5%, 5% MeOH:DCM. The two anomeric isomers were collected, combined, and concentrated at or below 30°C (684 mg, 92% from two steps). HRMS (ESI) calcd for $C_{29}H_{26}NO_8$ (fragment) [M+H]: 516.1653, found: 516.1658.



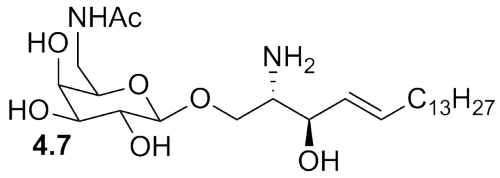
Preparation of 4.49: Donor **2.48** (53 mg, 0.0784 mmol), aceptor **4.44** (43 mg, 0.118 mmol), and 4 angstrom molecular sieves (250 mg) were stirred together for one hour in dry DCM (3 mL). The vessel was cooled to -10°C (acetone/ice bath), followed by the dropwise addition of trimethylsilyltriflate (7 μL, 0.0392 mmol). The reaction temperature was maintained for two hours then quenched by the dropwise addition of TEA. The molecular sieves were filtered and washed with EtOAc by means of a plug silica gel column. The filtrate was concentrated and subsequently loaded onto a silica gel column. Purification was achieved with a 70%, 80% EtOAc/Hexane eluent system. 1H NMR (500 MHz, Chloroform-d) δ 8.14 - 8.07 (m, 3H), 7.97 (d, J = 7.7 Hz, 2H), 7.79 (d, J = 7.7 Hz, 2H), 7.64 (t, J = 7.5 Hz, 1H), 7.51 (td, J = 7.9, 7.3, 2.7 Hz, 3H), 7.40 (dt, J = 17.6, 7.6 Hz, 3H), 7.25 (d, J = 9.7 Hz, 3H), 6.28 (t, J = 6.3 Hz, 1H), 5.82 (dd, J = 10.4, 7.9 Hz, 1H), 5.76 (d, J = 3.3 Hz, 1H), 5.64 (dd, J = 15.0, 6.8 Hz, 1H), 5.52 (dd, J = 10.4, 3.3 Hz, 1H), 5.41 - 5.27 (m, 2H), 4.78 (d, J = 8.0 Hz, 1H), 4.08 (t, J = 7.0 Hz, 1H), 3.90 (dd, J = 10.3, 7.0 Hz, 1H), 3.83 - 3.73 (m, 2H), 3.66 - 3.61 (m, 1H), 3.19 - 3.12 (m, 1H), 2.04 (s, 3H), 1.93 (q, J = 6.9 Hz, 2H), 1.63 (m, 2H), 1.33 - 1.20 (m, 20H), 0.87 (t, J = 6.8 Hz, 3H). 13C NMR (126 MHz, Chloroform-d) δ 177.05, 170.72, 169.54, 166.65, 165.53, 165.12, 138.72, 133.89, 133.37, 133.27, 130.11, 129.76, 129.73, 128.73, 128.61, 128.38, 128.32, 122.50, 101.28, 77.25, 77.20, 76.99, 76.74, 74.26, 71.74, 71.39, 69.65, 69.09, 68.51, 63.09, 38.86, 32.27, 31.91, 29.69, 29.66, 29.64, 29.59, 29.55, 29.39, 29.35, 29.16, 28.63, 23.27, 22.68, 21.08, 14.11. HRMS (ESI) calcd for $C_{49}H_{62}N_3O_{11}$ [M+H]: 882.4415,

found: 883.4361.



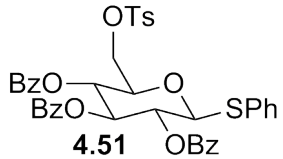
4.50

Preparation of 4.50: Azide **4.49** was dissolved in 5 mL of anhydrous methyl alcohol and 5 mL of THF. Freshly prepared 1 M sodium methoxide (0.3 mL) was added to the reaction flask and the reaction was stirred for one hour at room temperature. Reaction completion was verified via mass spectrometry and TLC. Upon completion, the solvent was removed en vacuo and the resulting residue was subjected to flash chromatography (5%, 10% MeOH:DCM). The purified compound was a white powder (19 mg, 46% over two steps). 1H NMR (500 MHz, Methanol-d4) δ 5.76 (dt, J = 15.4, 6.8 Hz, 1H), 5.52 - 5.47 (m, 1H), 4.21 (dd, J = 7.5, 3.5 Hz, 2H), 3.87 (dd, J = 10.0, 6.6 Hz, 1H), 3.75 - 3.61 (m, 3H), 3.58 - 3.42 (m, 4H), 2.07 (q, J = 7.2 Hz, 2H), 1.96 (s, 3H), 1.40 (q, J = 7.1 Hz, 2H), 1.29 (d, J = 9.6 Hz, 20H), 0.90 (t, J = 6.8 Hz, 3H). 13C NMR (126 MHz, Methanol-d4) δ 172.39, 134.46, 128.17, 103.69, 73.32, 72.99, 72.14, 70.81, 69.12, 68.61, 65.89, 48.19, 48.08, 48.02, 47.91, 47.87, 47.85, 47.81, 47.77, 47.74, 47.70, 47.70, 47.69, 47.68, 47.66, 47.64, 47.63, 47.62, 47.60, 47.59, 47.57, 47.51, 47.47, 47.43, 47.40, 47.23, 47.06, 39.84, 31.99, 31.65, 29.37, 29.35, 29.33, 29.32, 29.19, 29.05, 28.83, 28.79, 22.31, 21.11, 13.01. HRMS (ESI) calcd for $C_{26}H_{48}N_4O_7$ [M+H]: 528.3523, found: 529.3641.



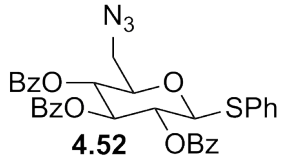
4.7

Preparation of 4.7: Compound **4.50** (19 mg, 0.0360 mmol) was dissolved in 3 mL of 1:1 THF:MeOH. 100 mg of granular zinc and 0.4 mL of glacial acetic acid were added to the flask. The mixture was sonicated for 30 minutes. Upon completion the solvent was transferred to a separate RBF and the solvent was dried off via a high vacuum rotary evaporator. Next, the compound was dissolved in 3 mL of 1:1 THF:MeOH. This solution was basified with concentrated NaOH (until pH 12) and stirred for one hour. Upon completion the solvent was dried off via a high vacuum rotary evaporator and the material was loaded onto a small silica gel column and subjected to a polar eluent system (25:65:2 MeOH:DCM:H_2O, 25:65:4 MeOH:DCM:H_2O, and 65:25:10 MeOH:DCM:H_2O). The resultant compound was a white powder (15 mg, 83%). 1H NMR (500 MHz, Methanol-d4) δ 5.78 (m, 1H), 5.50 (dd, J = 15.3, 7.1 Hz, 1H), 4.21 (d, J = 7.5 Hz, 1H), 4.09 (t, J = 6.7 Hz, 1H), 3.85 (t, J = 9.0 Hz, 1H), 3.82 - 3.71 (m, 2H), 3.57 - 3.43 (m, 4H), 3.41 - 3.34 (m, 1H), 2.10 (q, J = 7.5 Hz, 2H), 1.96 (s, 3H), 1.42 (q, J = 6.9 Hz, 2H), 1.30 (d, J = 10.1 Hz, 20H), 0.90 (t, J = 6.9 Hz, 3H). 13C NMR (126 MHz, Methanol-d4) δ 172.42, 134.43, 128.71, 103.24, 73.21, 73.19, 70.91, 69.18, 54.95, 48.20, 48.09, 48.08, 48.03, 48.03, 47.91, 47.90, 47.86, 47.84, 47.82, 47.81, 47.80, 47.79, 47.78, 47.76, 47.74, 47.71, 47.70, 47.69, 47.69, 47.68, 47.67, 47.66, 47.66, 47.65, 47.64, 47.63, 47.62, 47.61, 47.59, 47.57, 47.40, 47.23, 47.18, 47.12, 47.06, 39.89, 32.01, 31.66, 29.38, 29.35, 29.25, 29.23, 29.06, 28.96, 28.92, 22.32, 21.14, 13.02. HRMS (ESI) calcd for $C_{26}H_{50}N_2O_7$ [M+H]: 502.3618, found: 503.3739.



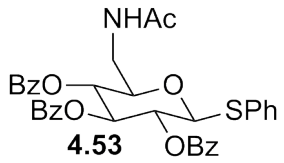
4.51

Preparation of 4.51: Compound **4.14** (7.11 g, 16.2 mmol) was diluted in methanol (100 mL). To this flask was added sodium methoxide (5 mL). The next morning the reaction was quenched by the addition of excess amberlight strongly acidic beads. After quenching, the beads were filtered of and the solvent was removed en vacuo. The light orange solid was dissolved in dry pyridine (100 mL), followed by the addition of tosyl chloride (3.08 g, 16.2) at 0°C. The reaction warmed to room temperature after three hours and the reaction stirred overnight. In the morning, benzoyl chloride (11.3 mL, 97.2 mmol) was added. Two hours later the solvent was removed via a high vacuum rotary evaporator, followed by a DCM (3 x 75 mL):water (75 mL) workup. The dark brown slurry was subjected to flash chromatography to give product **4.51**. HRMS (ESI) calcd for $C_{40}H_{34}O_{10}S_2$ [M+H]+: 738.1593, found: 725.1709.



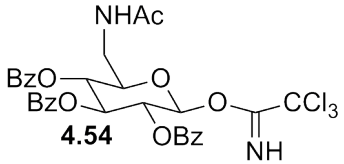
4.52

Preparation of 4.52: : Compound **4.45** and sodium azide (3.16 g, 48.6 mmol) were dissolved in a 9:1 solution of DMF:water (200 mL). The RBF (connected to a condenser) was heated to 100°C. This reaction was stirred overnight (evidenced by TLC). The water and DMF were pulled off on a high vacuum rotary evaporator, followed by a water:DCM (3 x 150 mL) workup. The dried ($NaSO_4$) and concentrated organic layer was loaded onto a silica gel column (15% EtOAc:Hexane) (7.00 g, 71% over three steps). HRMS (ESI) calcd for $C_{33}H_{27}N_3O_7S$ [M+H]+: 609.1570, found: 627.1957



4.53

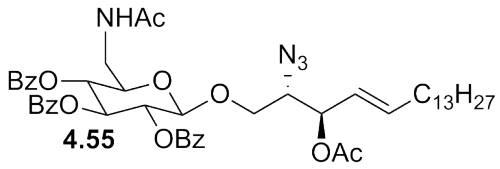
Preparation of 4.53: Azide **4.52** (183 mg, 0.300 mmol) was dissolved in 5 mL of MeOH. To this flask was added 53 μL of thioacetic acid (0.75 mmol), 87 μL of 2,6-lutidine (0.75 mmol), and 31 mg of ruthenium trichloride (0.15 mmol). The reaction was stirred for 18 hours. The ruthenium trichloride was filtered off and the dark red solution was concentrated en vacuo and loaded onto a silica gel column (50%, 70% EtOAc:Hexane, then 5%, 10% MeOH:DCM elution profile). The isolated compound was formed in 59% yield (118 mg). 1H NMR (500 MHz, Chloroform-d) δ 7.93 (m, 4H), 7.78 (dd, J = 8.1, 1.5 Hz, 2H), 7.54 - 7.44 (m, 4H), 7.42 - 7.28 (m, 7H), 7.28 - 7.20 (m, 2H), 6.05 (m, 1H), 5.87 (t, J = 9.5 Hz, 1H), 5.46 (t, J = 9.7 Hz, 1H), 5.36 (t, J = 9.8 Hz, 1H), 5.02 (d, J = 10.0 Hz, 1H), 3.96 - 3.84 (m, 2H), 3.24 - 3.16 (m, 1H), 2.02 (s, 3H). 13C NMR (126 MHz, Chloroform-d) δ 171.12, 170.03, 165.66, 165.59, 165.09, 133.82, 133.54, 133.39, 133.25, 133.22, 133.20, 133.11, 131.61, 129.86, 129.73, 129.70, 129.66, 129.06, 129.03, 129.01, 128.72, 128.61, 128.45, 128.43, 128.41, 128.39, 128.33, 128.33, 128.26, 128.22, 85.69, 77.34, 77.09, 76.83, 73.94, 70.63, 70.13, 60.37, 40.04, 23.20, 21.04, 14.18.



4.54

Preparation of 4.54: Amide **4.53** (118 mg, 0.189 mmol) was dissolved in an acetone/water solution (22mL/3mL), followed by the portion wise addition of NBS. Subsequent equivalents of

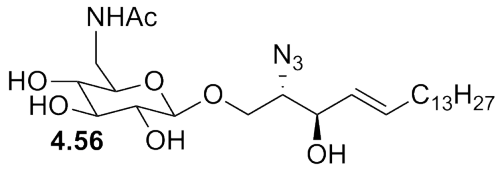
NBS were added until the reaction was completed (monitored by TLC). Upon completion, the reaction was quenched with solid $NaHCO_3$, and the acetone was evaporated off. The remaining mixture was dissolved in water (50 mL), washed with DCM (3 x 50 mL), dried with $NaSO_4$, concentrated, and subjected to flash chromatography (5% MeOH:DCM). The white powder (437 mg, 0.599 mmol) was dissolved in DCM (25 mL). Solid potassium carbonate (1.50 g, 10.9 mmol) was added, followed by the addition of excess trichloroacetonitrile (1.03 mL, 10.3 mmol). Once setup, the reaction was stirred for sixteen hours at room temperature. In the morning, the potassium carbonate was filtered and washed with EtOAc (100 mL). The filtrate was dried off at or below 30°C en vacuo. This pale yellow solid was loaded onto a bed of silica gel and was eluted with 2.5%, 5% MeOH:DCM. The two anomeric isomers were collected, combined, and concentrated at or below 30°C (95 mg, 75% from two steps). HRMS (ESI) calcd for $C_{35}H_{31}NO_8S$ [M+H]+: 625.1770, found: 626.1893.



4.55

Preparation of 4.55: Donor **2.37** (95 mg, 0.141 mmol), acceptor **4.44** (77 mg, 0.211 mmol), and 4 angstrom molecular sieves (400 mg) were stirred together for one hour in dry DCM (5 mL). The vessel was cooled to -10°C (acetone/ice bath), followed by the dropwise addition of trimethylsilyltriflate (13 μL, 70.5 μmol). The reaction temperature was maintained for two hours then quenched by the dropwise addition of TEA. The molecular sieves were filtered and washed with EtOAc by means of a plug silica gel column. The filtrate was concentrated and subsequently

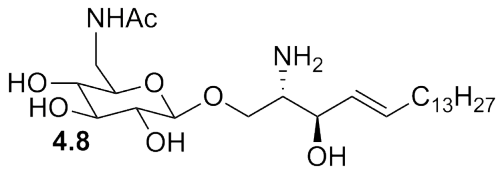
loaded onto a silica gel column. Purification was achieved with a 70%, 80% EtOAc/Hexane eluent system. Isolated 45 mgs of coupled product (36%). 1H NMR (500 MHz, Chloroform-d) δ 7.98 - 7.90 (m, 4H), 7.81 (d, J = 7.6 Hz, 2H), 7.52 (td, J = 7.2, 5.1 Hz, 2H), 7.40 (dq, J = 23.0, 7.6 Hz, 7H), 7.32 - 7.24 (m, 4H), 5.84 (t, J = 9.7 Hz, 1H), 5.54 - 5.36 (m, 4H), 4.82 (d, J = 7.9 Hz, 1H), 3.94 (m, 1H), 3.90 - 3.77 (m, 2H), 3.65 (dd, J = 10.6, 6.1 Hz, 1H), 3.55 - 3.47 (m, 1H), 2.10 - 1.94 (m, 8H), 1.34 - 1.21 (m, 24H), 0.88 (t, J = 6.8 Hz, 3H). HRMS (ESI) calcd for $C_{49}H_{62}N_3O_{11}$ [M+H]: 882.4415, found: 883.4377.



4.56

Preparation of 4.56: Azide **4.55** (45 mg, 51.0 μmol) was dissolved in 7.5 mL of anhydrous methyl alcohol and 7.5 mL of THF. Freshly prepared 1 M sodium methoxide (0.15 mL) was added to the reaction flask and the reaction was stirred for one hour at room temperature. Reaction completion was verified via mass spectrometry and TLC. Upon completion, the solvent was removed en vacuo and the resulting residue was subjected to flash chromatography (5%, 10% MeOH:DCM). The purified compound was a white powder (25 mg, 93%). 1H NMR (500 MHz, Methanol-d4) δ 5.67 (dt, J = 14.3, 6.7 Hz, 1H), 5.46 - 5.38 (m, 1H), 4.17 (d, J = 7.7 Hz, 1H), 4.09 (t, J = 6.4 Hz, 1H), 3.77 (dd, J = 10.6, 6.9 Hz, 1H), 3.64 - 3.47 (m, 3H), 3.34 - 3.16 (m, 2H), 3.07 (dt, J = 29.9, 8.9 Hz, 2H), 1.98 (q, J = 7.2 Hz, 2H), 1.88 (s, 3H), 1.31 (q, J = 7.0 Hz, 2H), 1.20 (d, J = 8.2 Hz, 22H), 0.84 - 0.77 (m, 3H). 13C NMR (126 MHz, Methanol-d4) δ 170.98, 132.94, 126.64, 101.64, 74.48, 72.97, 72.12, 70.60, 69.79, 67.24, 64.29, 46.57, 46.40, 46.34, 46.23, 46.17, 46.06, 46.00,

45.89, 45.83, 45.72, 45.55, 38.70, 30.47, 30.45, 30.14, 27.86, 27.84, 27.82, 27.81, 27.68, 27.54, 27.32, 27.27, 20.80, 19.53, 11.52. HRMS (ESI) calcd for $C_{26}H_{48}N_4O_7$ [M+H]: 528.3523, found: 529.3648.



Preparation of 4.8: Compound **4.56** (24 mg, 45.5 μmol) was dissolved in 4 mL of 1:1 THF:MeOH. 150 mg of granular zinc and 0.5 mL of glacial acetic acid were added to the flask. The mixture was sonicated for 30 minutes. Upon completion the solvent was transferred to a separate RBF and the solvent was dried off via a high vacuum rotary evaporator. Next, the compound was dissolved in 4 mL of 1:1 THF:MeOH. This solution was basified with concentrated NaOH (until pH 12) and stirred for one hour. Upon completion the solvent was dried off via a high vacuum rotary evaporator and the material was loaded onto a small silica gel column and subjected to a polar eluent system (25:65:2 MeOH:DCM:H_2O, 25:65:4 MeOH:DCM:H_2O, and 65:25:10 MeOH:DCM:H_2O). The resultant compound was a white powder (17 mg, 76%). 1H NMR (500 MHz, Methanol-d4) δ 5.79 (dt, J = 14.3, 6.8 Hz, 1H), 5.49 (dd, J = 15.3, 7.3 Hz, 1H), 4.26 (dd, J = 7.9, 3.4 Hz, 1H), 4.08 (t, J = 6.8 Hz, 1H), 3.87 - 3.76 (m, 2H), 3.59 (d, J = 2.6 Hz, 1H), 3.41 - 3.26 (m, 4H), 3.20 (dd, J = 9.3, 7.8 Hz, 1H), 3.13 (t, J = 9.3 Hz, 1H), 3.04 (q, J = 5.5, 4.4 Hz, 1H), 2.09 (q, J = 7.1 Hz, 2H), 1.97 (s, 3H), 1.42 (m, 2H), 1.30 (d, J = 10.2 Hz, 22H), 0.90 (t, J = 6.8 Hz, 3H). 13C NMR (126 MHz, Methanol-d4) δ 172.49, 134.58, 128.60, 102.83, 75.98, 74.74, 73.59, 71.38, 54.84, 48.19, 48.07, 48.07, 48.05, 48.04, 48.02, 48.01, 47.98, 47.96, 47.95, 47.94, 47.93,

47.90, 47.87, 47.85, 47.83, 47.82, 47.81, 47.80, 47.79, 47.78, 47.77, 47.76, 47.75, 47.73, 47.69, 47.69, 47.67, 47.66, 47.65, 47.63, 47.62, 47.61, 47.59, 47.56, 47.50, 47.39, 47.22, 47.05, 40.17, 31.99, 31.65, 29.36, 29.33, 29.21, 29.04, 28.95, 28.89, 22.30, 21.07, 13.00. HRMS (ESI) calcd for $C_{26}H_{50}N_2O_7$ [M+H]: 502.3618, found: 503.3712.

4.6 Immunological Methods

Note: all immunological testing was carried out under the direction of Albert Bendelac and Erin Adams at the University of Chicago.

T cell activation assay

T cell hybridoma cells were cultured in RPMI supplemented with 10% FCS, 2 mM L-glutamine, 20mM HEPES, and non-essential amino acids. Antigen presentation assays were carried out using 5-20 x 10^3 DC 3.2 cells or 1 x 10^5 splenocytes and 4 x 10^4 T cells per well in 96 well tissue culture plates in triplicates. Cell culture supernatants were collected 24 hours later for determination of IL-2 concentrations using an IL-2-dependent NK cell line reporter system.

Cells and cell lines, DC maturation

DN32.D3 and TBA.7 cells have been described extensively in other publications and are commonly used as representative of type 1 semi-invariant Vα14 NKT cells for the former, and type 2 non-Vα14 NKT cell for the latter. DC3.2 cells are a dendritic cell line expressing CD1d and susceptible to differentiation induced by TLR ligands and cytokine such as LPS and TNF. Maturation of DC3.2 was carried over periods of 16-24h. It is to be noted that beyond 24h, the

capacity of matured DC cells to stimulate NKT cells in a way that is sensitive to L363 or L317 antibody blocking diminishes. It is also important to notice that the optimal display of the ligand at the cell surface of DC3.2 cells is sensitive to cell density.

Antibodies and antibody production/purification

L363 (IgG2a) and L317 (IgG1) antibodies were a generous gift from Steve Porcelli. In most experiments, the anti-MHC class II antibodies MKD6 (anti-I-Ad, IgG2a) and 14.4.4s (anti-I-Ek, IgG2a) were used as a control. All antibodies were produced in serum-free Ultradoma media (Lonza*) in individual bioreactors. Purification was carried out on HiTrap protein A or G columns (GE Healthcare*).

Surface plasmon resonance (SPR)

A Biacore T200 instrument (GE Healthcare, USA) was used for SPR measurements. Measurements were performed using single cycle protocols to avoid repeated use of regeneration buffer on the immobilized ligands. Immobilization of target antibodies was carried out using classical amine coupling chemistry. 250 to 1,000 RU of antibody was immobilized in each flow cells. All mCD1-lipid complexes were purified after loading to ensure maximal homogeneity and avoid the presence of small amounts of aggregated material. Concentrations ranging from 1 to 10mM were used for each CD1-lipid complex. Flow cell one was used as our negative control and used for subtraction from experimental flow cells. Global analysis of subtracted sensorgrams was carried out using the T200 analysis software. Illustration was done after transfer of the data to an Excel spreadsheet.

Bibliography

[1] Pier, G. B.; Lyczak, J. B.; Wetzler, L. M. *Immunology, Infection, and Immunity*; American Society for Microbiology, 2004.

[2] Male, D.; Brostoff, J.; Roth, D. B.; Roitt, I. *Immunology*; Mosby Elsevier, 2006.

[3] Savage, P. B.; Teyton, L.; Bendelac, A. *The Royal Society of Chemistry* **2006**, *35*, 771–779.

[4] Sakaguchi, S.; Tamaguchi, T.; Nomura, T.; Ono, M. *Cell* **2008**, *133*, 775–787.

[5] Zajonc, D. M.; Kronenberg, M. *Current Opinion in Structural Biology* **2007**, *17*, 521–529.

[6] Tupin, E.; Kinjo, Y.; Kronenberg, M. *Nature Reviews Microbiology* **2007**, *5*, 405–417.

[7] Herberman, R. B.; Ortaldo, J. R. *Science* **1981**, *214*, 24–30.

[8] Godfrey, D. I.; Hammond, K. J.; Poulton, L. D.; Smyth, M. J.; Baxter, A. G. *Immunology Today* **2000**, *21*, 573–583.

[9] Kinjo, Y.; Ueno, K. *Microbiology and Immunology* **2011**, *55*, 472–482.

[10] Godfrey, D. I.; Kronenberg, M. *The Journal of Clinical Investigation* **2004**, *224*, 1379–1388.

[11] Hammond, K. J. L.; Kronenberg, M. *Current Opinion in Immunology* **2003**, *15*, 683–689.

[12] Godfrey, D. I.; Pellicci, D. G.; Patel, O.; Kjer-Nielsen, L.; McCluskey, J.; Rossjohn, J. *Seminars in Immunology* **2010**, *22*, 61–67.

[13] Cerundolo, V.; Silk, J. D.; Masri, S. H.; Salio, M. *Nature Reviews: Immunology* **2009**, *9*, 28–38.

[14] Kronenberg, M. *Annual Reviews in Immunology* **2005**, *26*, 877–900.

[15] Yu, K. O. A.; Porcelli, S. A. *2005* **Immunology Letters**, *100*, 42–55.

[16] Pellicci, D. G. et al. *Immunity* **2009**, *31*, 47–59.

[17] Van Kaer, L. *Nature Reviews* **2005**, *5*, 31–42.

[18] Hong, S.; Scherer, D. C.; Sing, N.; Mendiratta, S. K.; Serizawa, I.; Koezuka, Y.; Kaer, L. V. *Immunological Reviews* **1999**, *169*, 31–44.

[19] Wu, D.; Fujio, M.; Wong, C.-H. *Bioorganic and Medicinal Chemistry* **2008**, *16*, 1073–1083.

[20] Wang, J.; Li, Y.; Mac, T.-T.; Gibson, D.; Painter, G. F.; Kronenberg, M.; Zajonc, D. M. *Proceedings from the National Academy of Sciences* **2010**, *107*, 1535–1540.

[21] Zajonc, D. M.; Maricic, I.; Wu, D.; Halder, R.; Roy, K.; Wong, C.-H.; Kumar, V.; Wilson, I. A. *The Journal of Experimental Medicine* **2005**, *202*, 1517–1526.

[22] Tsuji, M. *Cellular and Molecular Life Sciences* **2006**, *63*, 1889–1898.

[23] Matsuda, J. L.; Mallevaey, T.; Scott-Browne, J.; Gapin, L. *Current Opinion in Immunology* **2008**, *20*, 358–368.

[24] Schneiders, F. L.; Scheper, R. J.; von Blomber, B. M. E.; Woltman, A. M.; Janssen, H. L. A.; van den Eertwegh, A. J. M.; Verheul, H. M. W.; de Gruijl, T. D.; van der Vliet, H. J. *Clinical Immunology* **2010**, *140*, 130–141.

[25] Morita, M.; Natori, T.; Akimoto, O.; Fukushima, H.; Koezuka, Y. *Bioorganic and Medicinal Chemistry Letters* **1995**, *5*, 699–704.

[26] Berkers, C. R.; Ovaa, H. *TRENDS in Pharmacological Sciences* **2005**, *26*, 252–257.

[27] Morita, M.; Motoki, K.; Akimoto, K.; Natori, T.; Sakai, T.; Sawa, E.; Yamaji, K.; Koezuka, Y.; Kobayashi, E.; Fukushima, H. *Journal of Medicinal Chemistry* **1995**, *38*, 2176–2187.

[28] Schiefner, A.; Wilson, I. A. *Current Pharmaceutical Design* **2009**, *15*, 3311–3317.

[29] Zhou, D. et al. *Science* **2004**, *306*, 1786–1789.

[30] Goff, R. D.; Gao, Y.; Mattner, J.; Zhou, D.; Yin, N.; Cantu, C.; Teyton, L.; Bendelac, A.; Savage, P. B. *Journal of the American Chemical Society* **2004**, *126*, 13602–13603.

[31] Long, X.; Deng, S.; Mattner, J.; Zang, Z.; Zhou, D.; McNary, N.; Goff, R. D.; Teyton, L.; Bendelac, A.; Savage, P. B. *Nature: Chemical Biology* **2007**, *3*, 559–564.

[32] Wu, D.; Zajonc, D. M.; Fujio, M.; Sullivan, B. A.; Kinjo, Y.; Kronenberg, M.; Wilson, I. A.; Wong, C.-H. *Proceedings from the National Academy of Sciences* **2006**, *103*, 3972–3977.

[33] Sidobre, S.; Hammond, K. J. L.; Benazet-Sidobre, L.; Maltsev, S. D.; Richardson, S. D.; Ndonye, R. M.; Howell, A. R.; Sakai, T.; Besra, G. S.; Porcelli, S. A.; Kronenberg, M. *Proceedings from the National Academy of Sciences* **2004**, *101*, 12254–12259.

[34] Kim, S.; Cho, M.; Lee, T.; Lee, S.; Min, Y., Hye; Lee, S. K. *Bioorganic and Medicinal Chemistry Letters* **2007**, *17*, 4584–4587.

[35] Fan, G.-T.; Pan, Y.-S.; Lu, K.-C.; Cheng, Y.-P.; Lin, W.-C.; Lin, S.; Lin, C.-H.; Wong, C.-H.; Fang, J.-M.; Lin, C.-C. *Tetrahedron* **2005**, *61*, 1855–1862.

[36] Liu, Y.; Goff, R. D.; Zhou, D.; Mattner, J.; Sullivan, B. A.; Khurana, A.; Cantu, C.; Ravkov, E. V.; Ibegbu, C. C.; Altman, J. D.; Teyton, L.; Bendelac, A.; Savage, P. B. *Journal of Immunological Methods* **2006**, *312*, 34–39.

[37] Zhou, X.-T.; Forestier, C.; Goff, R. D.; Li, C.; Teyton, L.; Bendelac, A.; Savage, P. B. *Organic Letters* **2002**, *4*, 1267–1270.

[38] Koch, M.; Stronge, V. S.; Shepherd, D.; Gadola, S. D.; Mathew, B.; Ritter, G.; Fersht, A. R.; Besra, G. S.; Schmidt, R. R.; Jones, E. Y.; Cerundolo, V. *Nature Immunology* **2005**, *6*, 819–826.

[39] Miyamoto, K.; Miyake, S.; Yamamura, T. *Nature* **2001**, *413*, 531–534.

[40] Schiefner, A.; Fujio, M.; Wu, D.; Wong, C.-H.; Wilson, I. A. *Journal of Molecular Biology* **2009**, *394*, 71–82.

[41] Yu, K. O. A.; Im, J. S.; Molano, A.; Dutronc, Y.; Illarionov, P. A.; Forestier, C.; Fujiwara, N.; Arias, I.; Miyake, S.; Yamamura, T.; Chang, Y.-T.; Besra, G.; Porcelli, S. A. *Proceedings from the National Academy of Sciences* **2005**, *102*, 3383–3388.

[42] Takahashi, T.; Suzuki, T. *Journal of Lipid Research* **2012**, *53*, 1437–1450.

[43] Compostella, F.; Panza, L.; Ronchetti, F. *Comptes Rendus Chimie* **2012**, *15*, 37–45.

[44] Buschard, K.; Blomqvist, M.; Osterbye, T.; Fredman, P. *Diabetologia* **2005**, *48*, 1957–1962.

[45] Kyogashima, M. K.; Tamiya-Koizumi, T.; Ehara, G.; Li, R.; Hu, A.; Hara, T.; Aoyama, R.; Kannagi, R. *Glycobiology* **2006**, *16*, 719–728.

[46] Bai, L.; Picard, D.; Anderson, B. L.; Chaudhary, V.; Luoma, A.; Jabri, B.; Adams, E. J.; Savage, P. B.; Bendelas, A. *European Journal of Immunology* **2012**, *42*, 1–6.

[47] Shamshiev, A.; Gober, H. J.; Donda, A.; Mazorra, Z.; Mori, L.; Libero, G. D. *Experimental Immunology* **2002**, *195*, 1013–1021.

[48] Jahng, A.; Maricic, I.; Aguilera, C.; Cardell, S.; Halder, R. C.; Kumar, V. *Journal of Experimental Medicine* **2004**, *199*, 947–957.

[49] Girardi, E.; Maricic, I.; Wang, J.; Mac, T.-T.; Iyer, P.; Kumar, V.; Zajonc, D. M. *Nature Immunology* **2012**, *13*, 851–857.

[50] Shamshiev, A.; Donda, A.; Carena, I.; Mori, L.; Kappos, L.; Libero, G. D. *Experimental Immunology* **1999**, *29*, 1667–1675.

[51] Boggs, J. M.; Gao, W.; Zhao, J.; Park, H.-J.; Liu, Y.; Basu, A. *Federation of European Biochemical Societies* **2010**, *584*, 1771–1778.

[52] Halder, R. C.; Jahng, A.; Maricic, I.; Kumar, V. *Neurochemical Research* **2007**, *32*, 257–262.

[53] Xing, G.-W.; Wu, D.; Poles, M. A.; Horowitz, A.; Tsuji, M.; Ho, D. D.; Wong, C.-H. *Bioorganic and Medicinal Chemistry* **2005**, *13*, 2907–2916.

[54] Garner, P.; Park, J. M.; Malecki, E. *Journal of Organic Chemistry* **1988**, *53*, 4395–4398.

[55] Fazio, F.; Wong, C.-H. *Tetrahedron Letters* **2003**, *44*, 9083–9085.

[56] Blomqvist, M.; Rhost, S.; Teneberg, S.; Lofbom, L.; Osterbye, T.; Brigl, M.; Mansson, J.-E.; Cardell, S. L. *European Journal of Immunology* **2009**, *39*, 1726–1735.

[57] Lee, P.; Putnam, A.; Benlagha, K.; Teyton, L.; Gottlieb, P.; Bendelac, A. *Journal of Clinical Investigation* **2002**, *110*, 793–800.

[58] Constantinides, M.; Picard, D.; Savage, A.; Bendelac, A. *Journal of Immunology* **2011**, *187*, 309–315.

[59] Sugiyama, E.; Hara, A.; Uemura, K. *Analytical Biochemistry* **1999**, *247*, 90–97.

[60] Wucherpfennig, K.; Newcombe, J.; Li, H.; Keddy, C.; Cuzner, M.; Hafler, D. *Proceedings from the National Academy of Sciences* **1992**, *89*, 4588–4592.

[61] Shimonkevitz, C. C. B. J. M. R., R.; Kotzin, B. *Proceedings from the National Academy of Sciences* **1993**, *90*, 923–927.

[62] Ishizuka, I. *Prog. Lipid Res.* **1997**, *36*, 245–319.

[63] Cernadas, M.; Cavallari, M.; Watts, G.; Mori, L.; De Libero, G.; Brenner, M. B. *The Journal of Immunology* **2010**, *184*, 1235–1241.

[64] Bendelac, A.; Savage, P. B.; Teyton, L. *The Annual Review of Immunology* **2007**, *25*, 297–336.

[65] Ortaldo, J. R.; Young, H. A.; Winkler-Pickett, R. T.; Bere Jr., E. W.; Murphy, W. J.; Wiltrout, R. H. *The Jounal of Immunology* **2004**, *172*, 943–953.

[66] Brennan, P. J.; Tatituri, R. V. V.; Brigl, M.; Kim, E. Y.; Tuli, A.; Sanderson, J. P.; Gadola, S. D.; Hsu, F.-F.; Besra, G. S.; Brenner, M. B. *Nature Immunology* **2011**, *12*, 1202–1211.

[67] Xu, R.; Sun, W.; Jin, J.; Obeid, L. M.; Mao, C. *The FASEB Journal* **2010**, *24*, 2507–2515.

[68] Houben, E.; Holleran, W. M.; Yaginuma, T.; Cungui Mao, C.; Obeid, L. M.; Rogiers, V.; Takagi, Y.; Elias, P. M.; Uchida, Y. *Journal of Lipid Research* **2006**, *47*, 1061–1070.

[69] Alper, P. B.; Hung, S.-C.; Wong, C.-H. *Tetrahedron Letters* **1996**, *37*, 6029–6032.

[70] Schmidt, R. R.; Michel, J. *Angew. Chem. Int. Ed. Engl.* **1980**, *19*, 731–732.

[71] Garcia, B. A.; Poole, J. L.; Gin, D. Y. *Journal of the American Chemical Society* **1997**, *119*, 7597–7598.

[72] Yu, K. O. A.; Im, J. S.; Illarionov, P. A.; Ndonye, R. M.; Howell, A. R.; Besra, G. S.; Porcelli, S. A. *Journal of Immunological Methods* **2007**, *323*, 11–23.

[73] Yu, E. D.; Girardi, E.; Wang, J.; Mac, T.-T.; Yu, K. O. A.; Van Calenbergh, S.; Porcelli, S. A.; Zajonc, D. M. *The Journal of Biological Chemistry* **2012**, *287*, 1269–1278.

Appendix A

List of Abbreviations

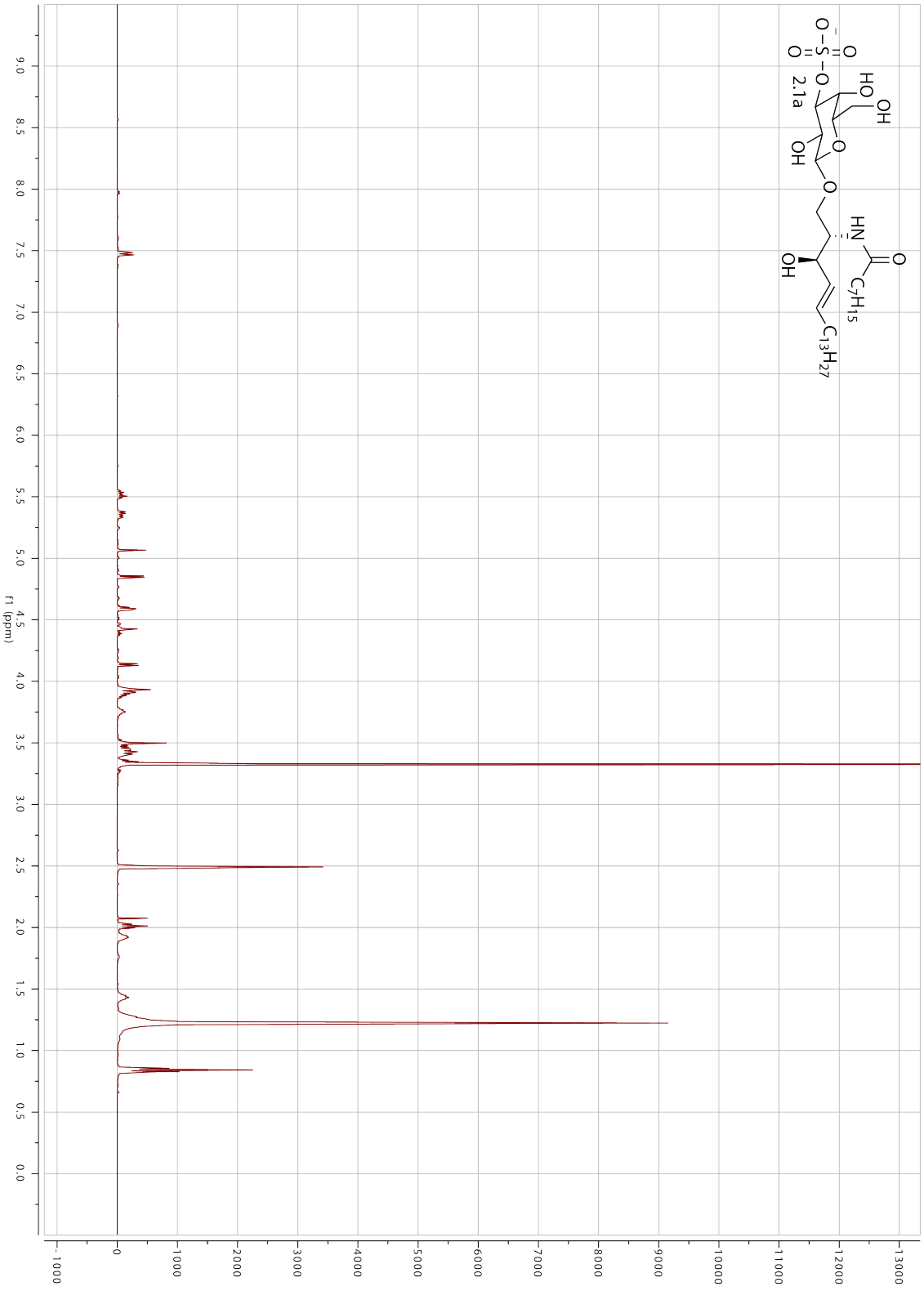
Chapter 1	xxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxx
BCR	B-Cell receptor
TCR	T-cell receptor
NK	Natural killer
CD	class of differentiation
APM	Antigen presenting molecule
MHC	Major Histocapatibilty complex
CTL	Cytotoxic T cells
NKT	Natural Killer T
iNKT	Invariant Natural Killer T
α-GalCer	α-galactosylceramide
iGB3	Isoglobotrihexosylceramide

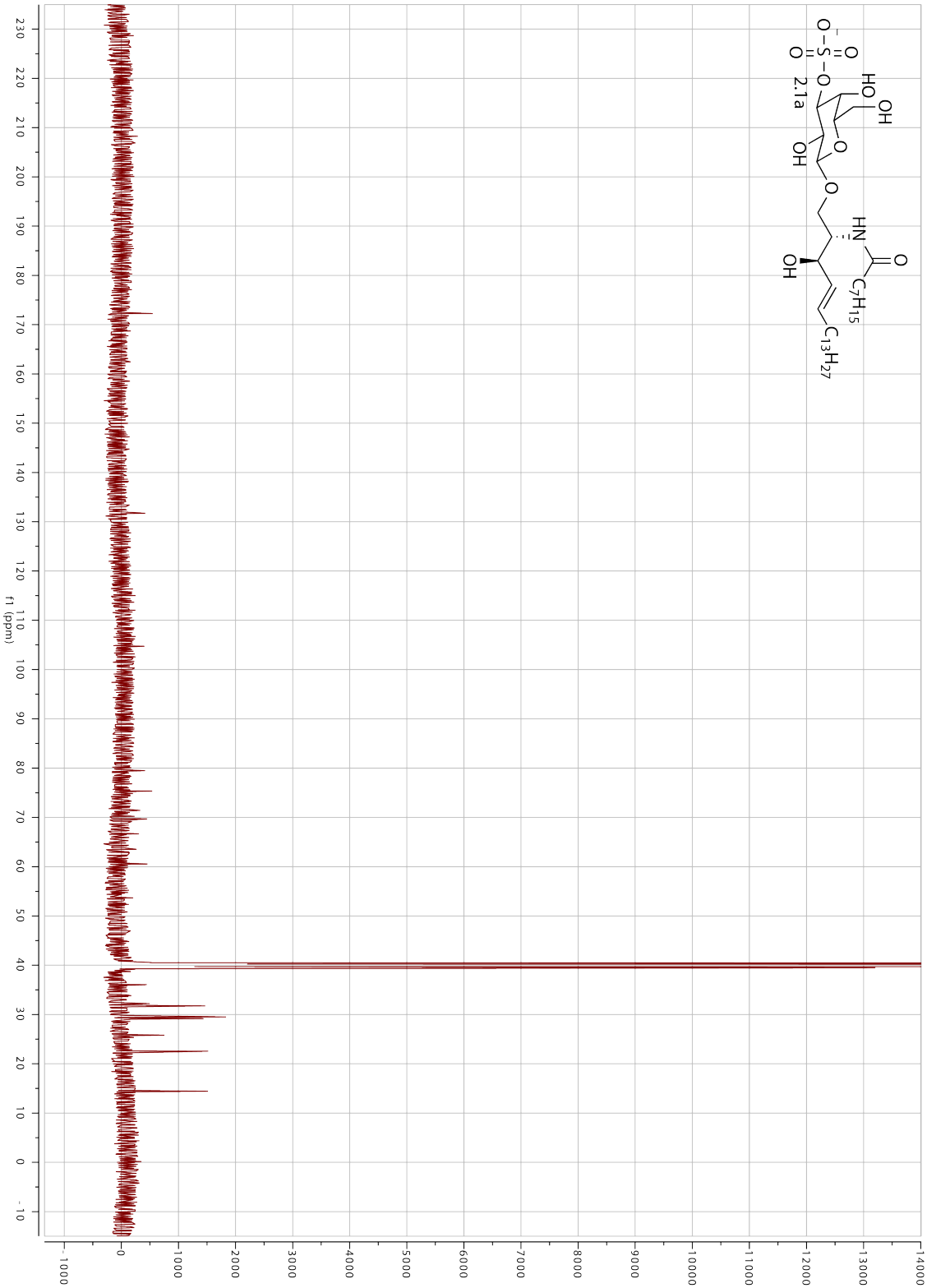
Chapter 1	xxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxx
PE	Phosphotidyl ethanolamine
PC	Phosphotidyl choline
TH	T-helper
INF	Interferon
IL	Interleukin
Chapter 2	xxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxx
CGT	Ceramide galactosyltransferase
CST	Cerobroside sulfotransferase
MS	Multiple sclerosis
EAE	Experimental autoimmune encephalomyetis
PMP	Para methoxy phenyl
ASA	Arylsulfatase A
WT	Wild type
MACS	Magnetic-activated cell sorting
FACS	Fluorescent-activated cell sorting
SPR	Surface plasmon resonance
IEF	Isoelectrofocusing
PMBC	Peripheral blood mononuclear cell
PCR	Polymerase chain reaction
SEM	Scanning Electron Microscopy

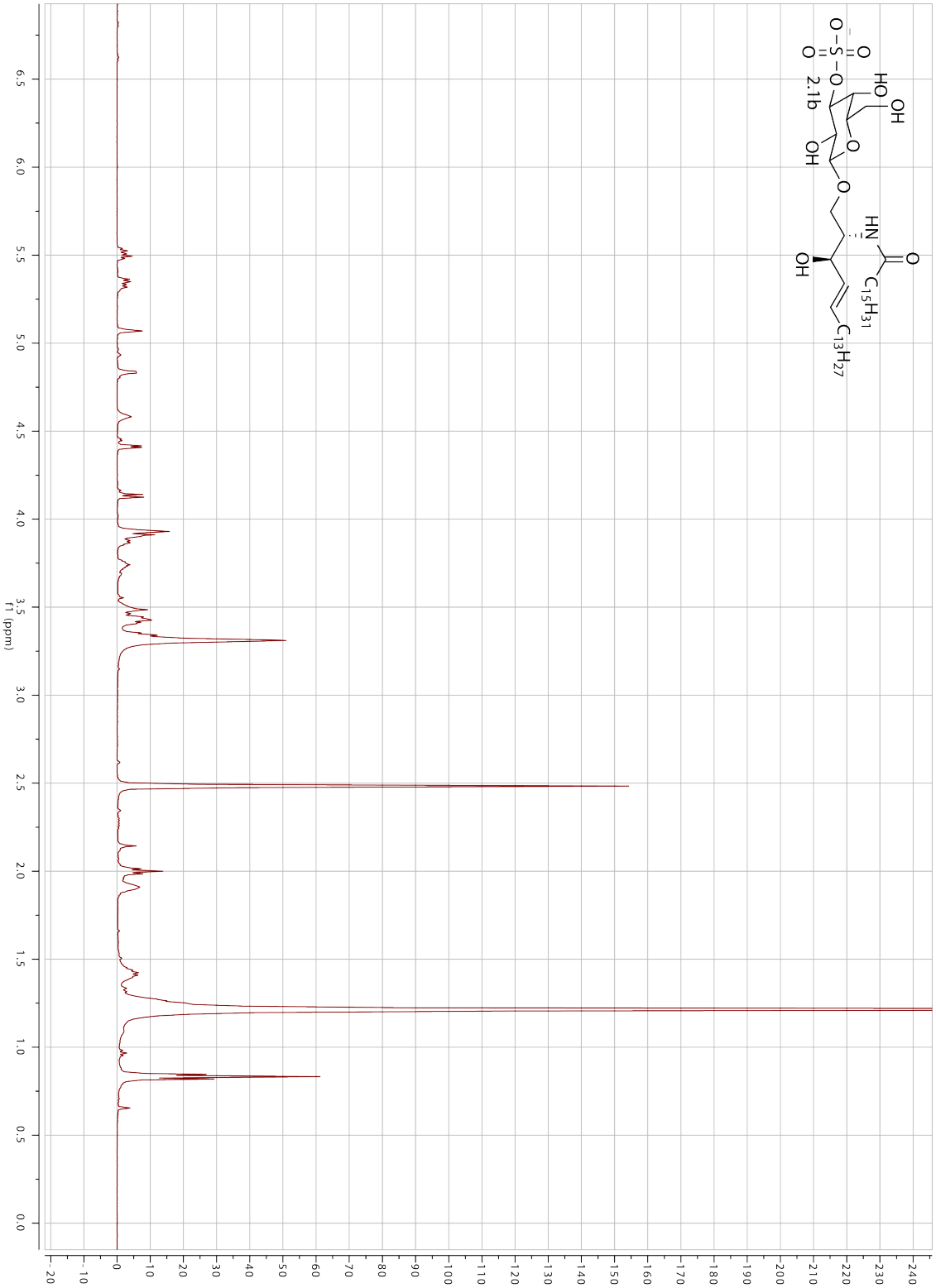
Chapter 2	xxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxx
NHA	N,O-dimethyl hydroxyamine-HCl
NMM	4-methyl morpholine
EDCI	1-ethyl-3-(3-dimethylaminopropyl) carbodiimide
DMP	Dimethoxy propane
DMF	Dimethyl formamide
LAH	Lithium aluminum hydride
THF	Tetrahydrofuran
HOBt	Hydroxybenzotriazol
TDS-Cl	Chloro(dimethyl)thexylsilane
M.S.	Mass spectrometry
RBF	Round bottom flask
DMAP	Dimethylaminopyridine
ACN	Acetonitrile
NBS	n-Bromosuccinimide
TBDPS-Cl	tert-butylchlorodiphenylsilane
Chapter 3	xxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxx
BODIPY	boron-dipyrromethene
Chapter 4	xxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxx
β-GluCer	β-glucosylceramide
ASAH1	Acid ceramidase
NAAA	N-acylamidehydrolase

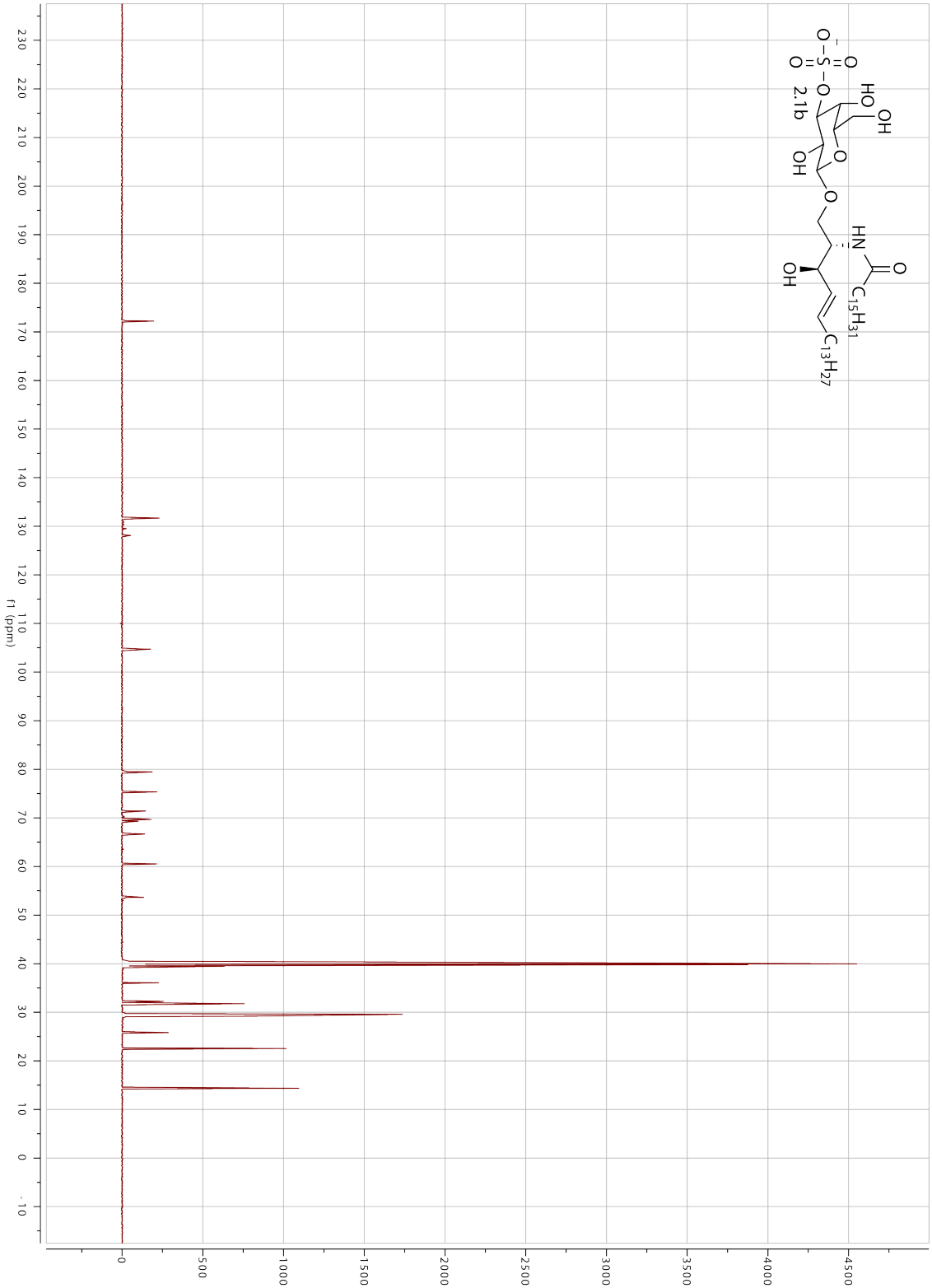
Appendix B

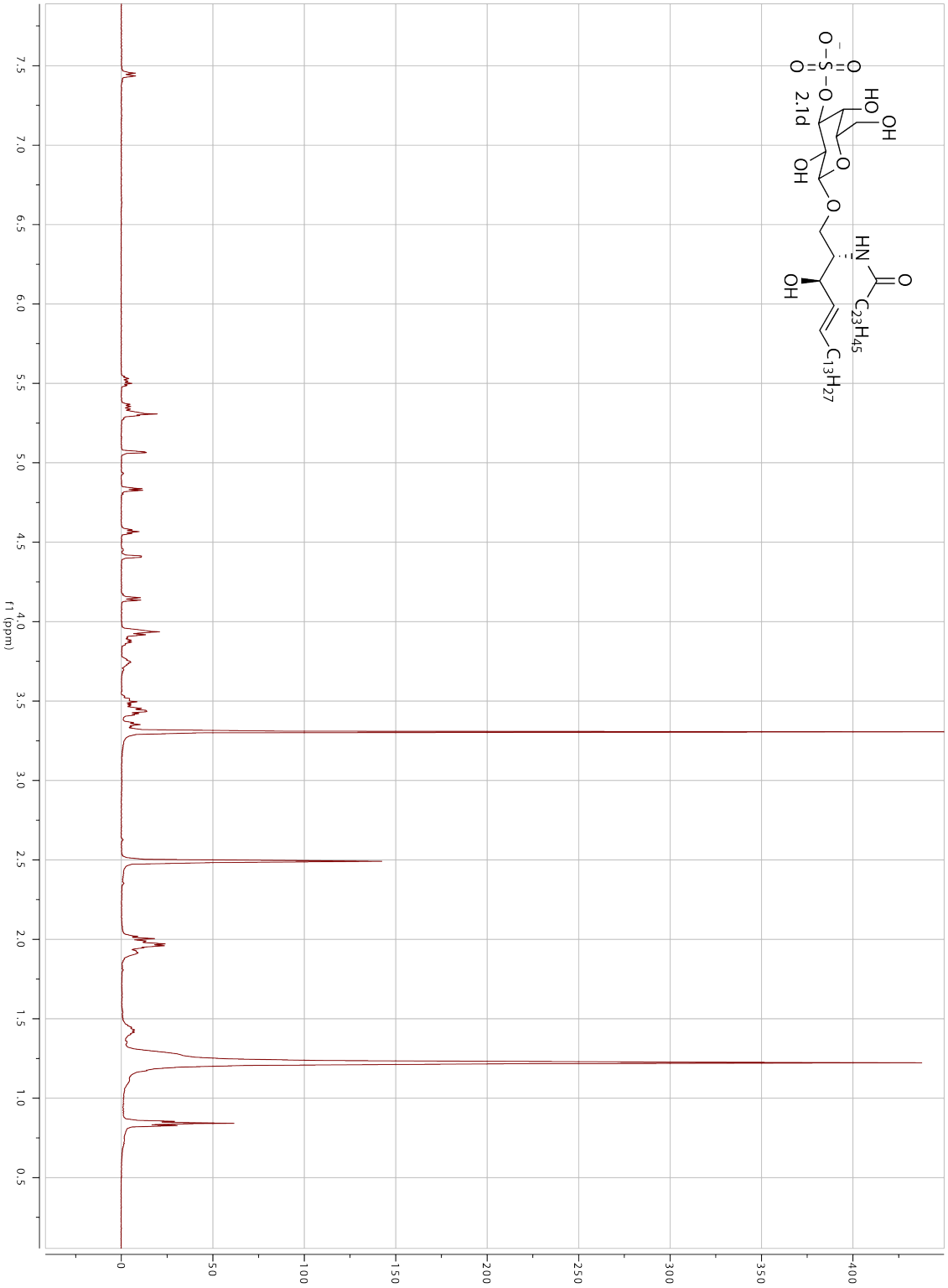
NMR spectra

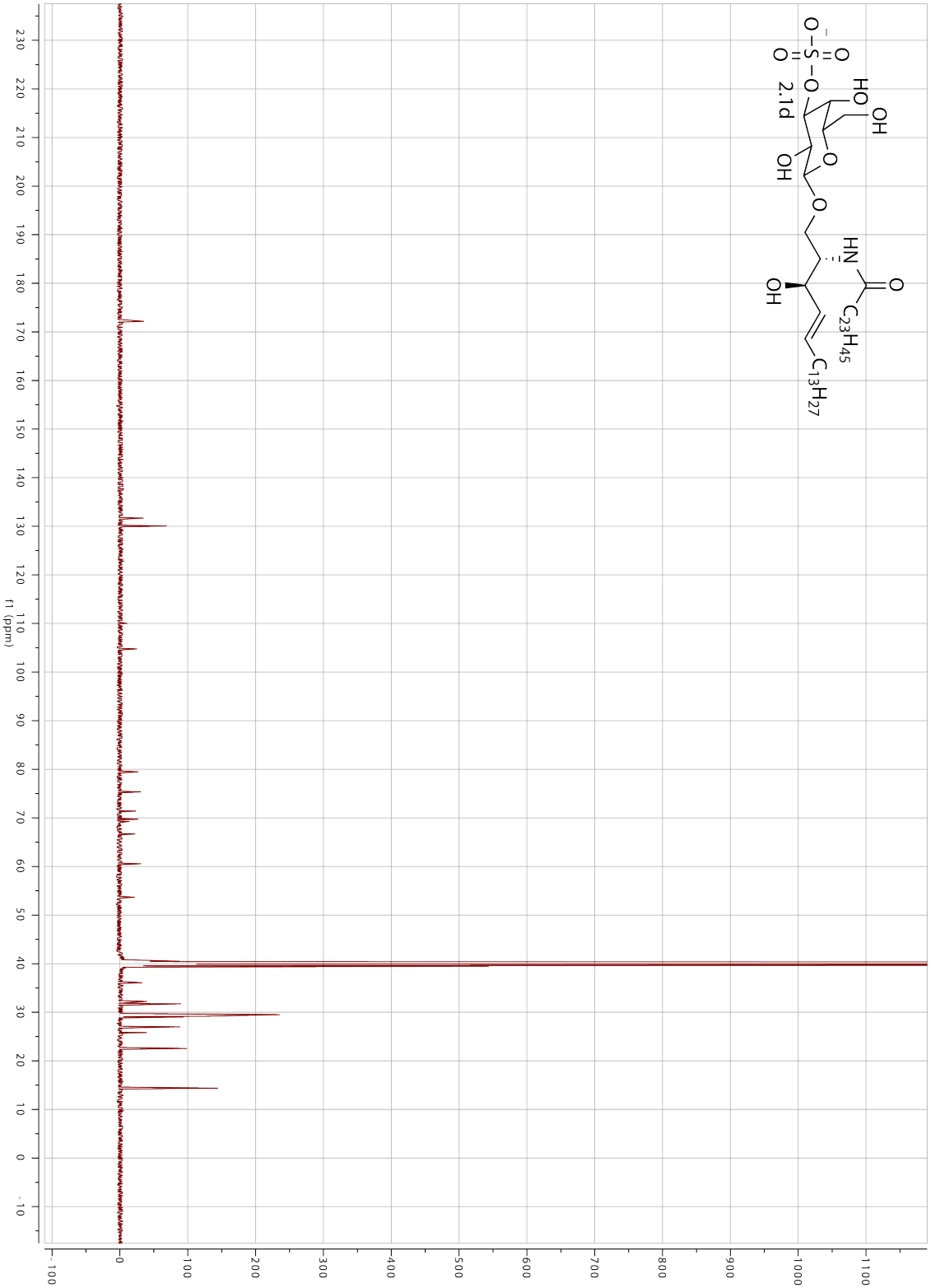

2.1a
f1 (ppm)

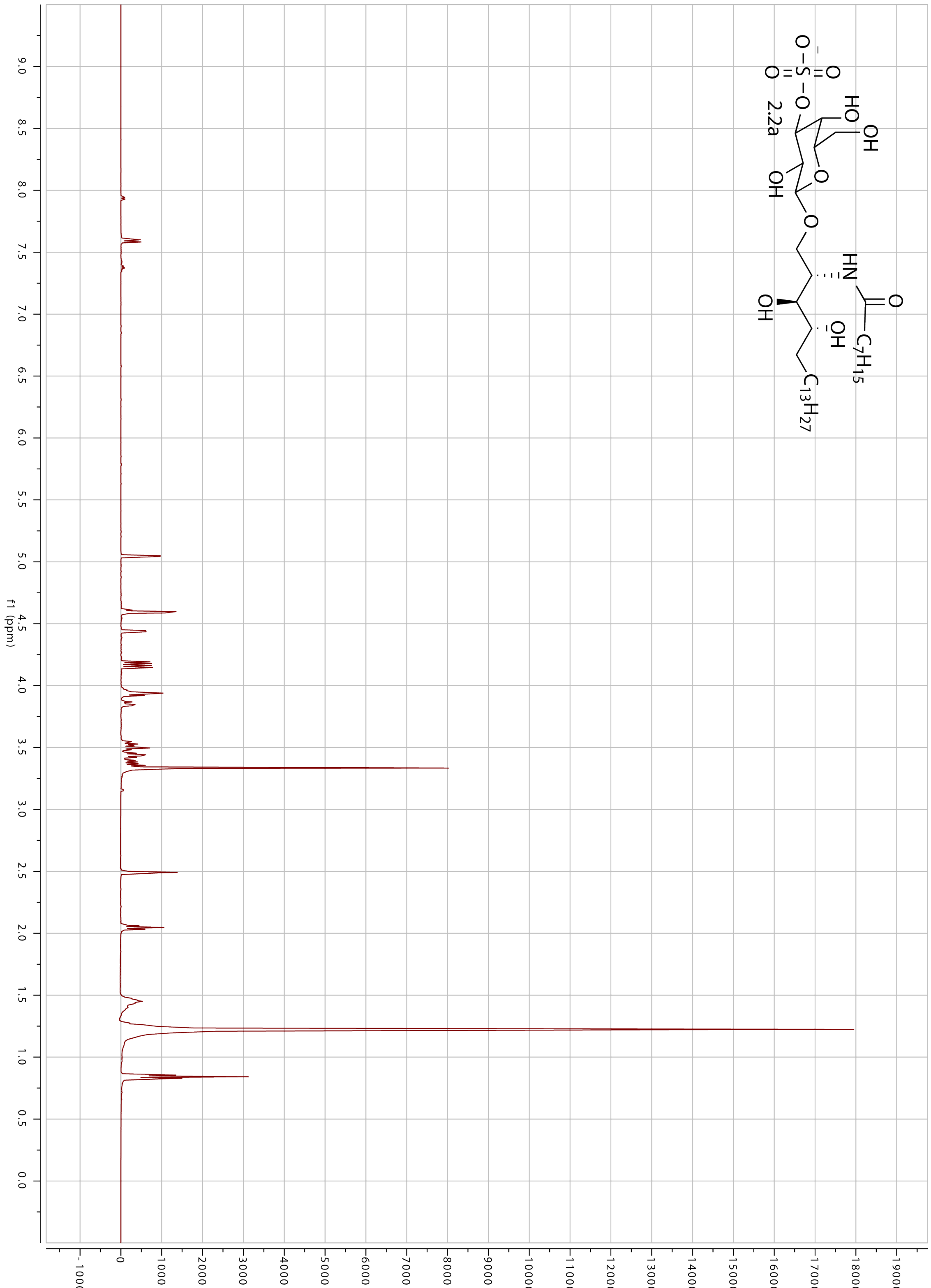

2.1a
f1 (ppm)

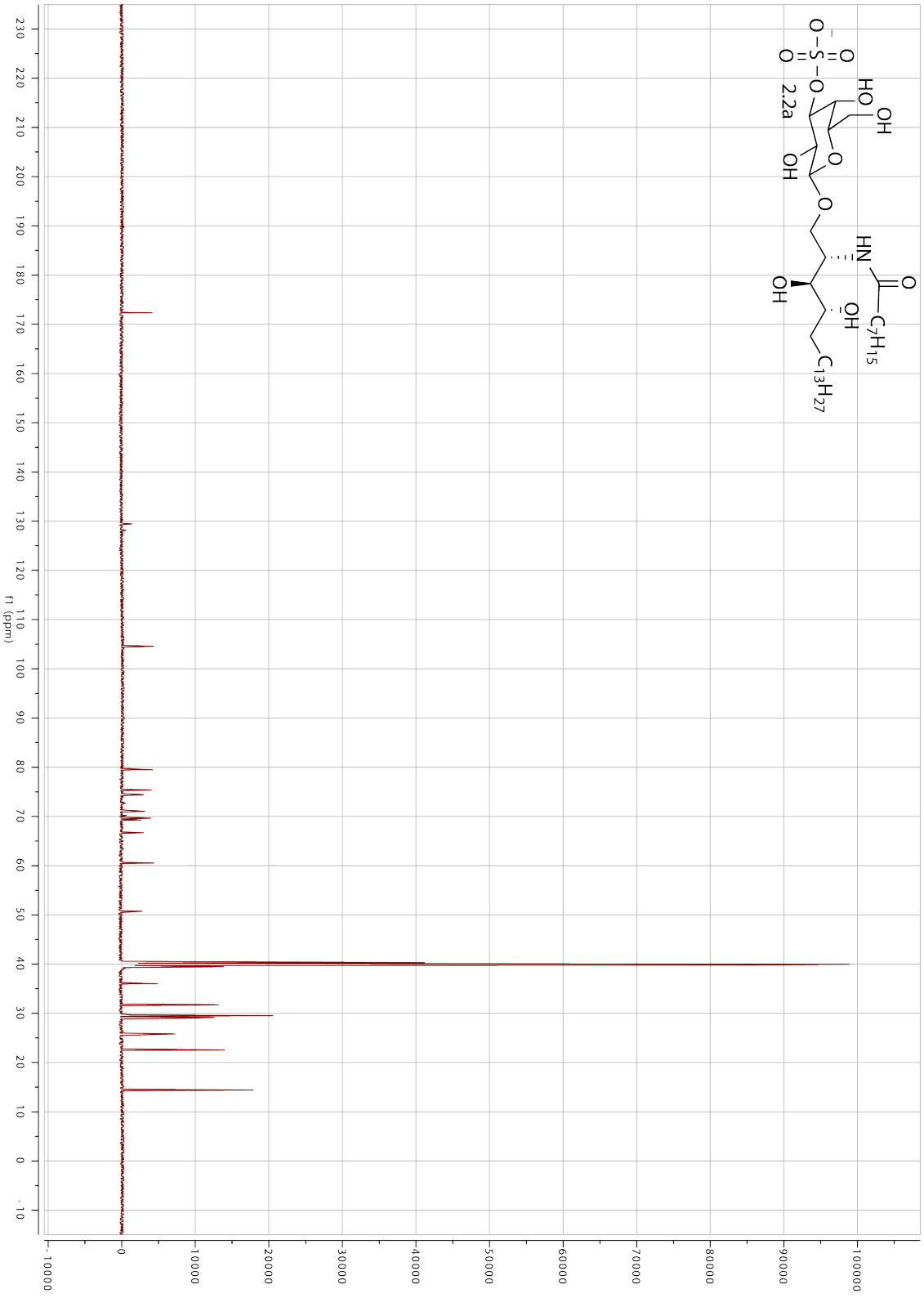

2.1b
f1 (ppm)

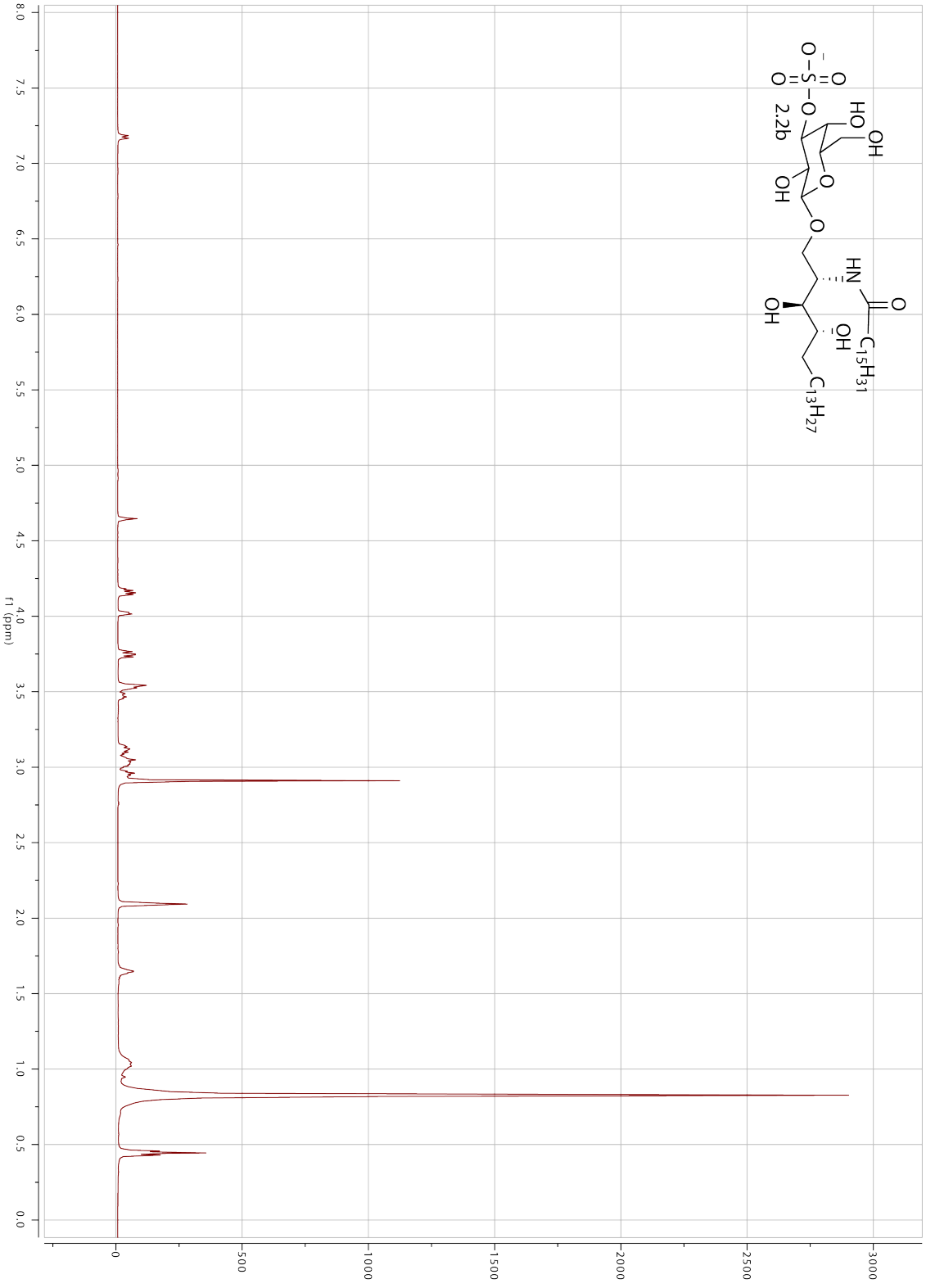

f1 (ppm)
2.1b

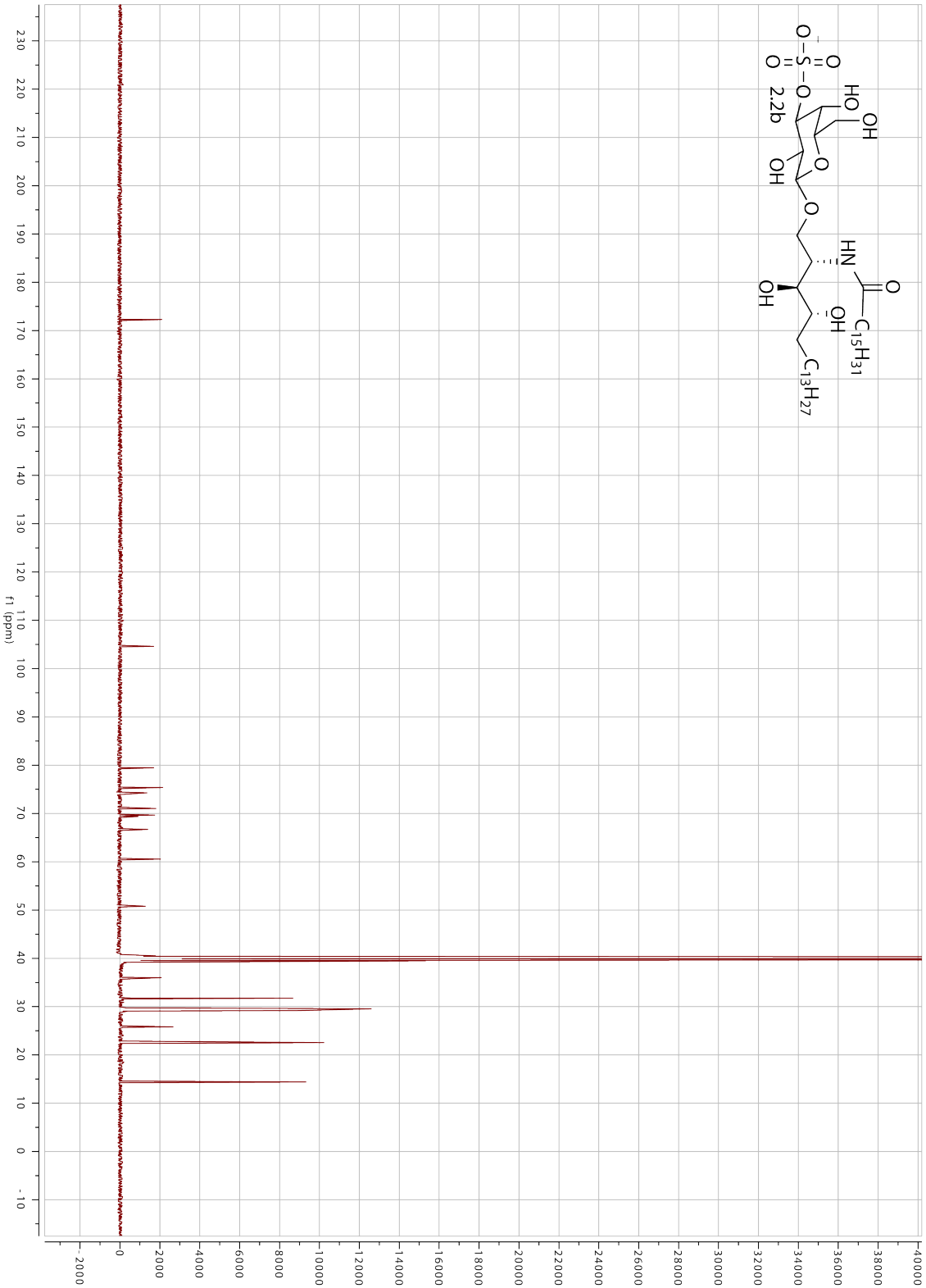

2.1d
f1 (ppm)

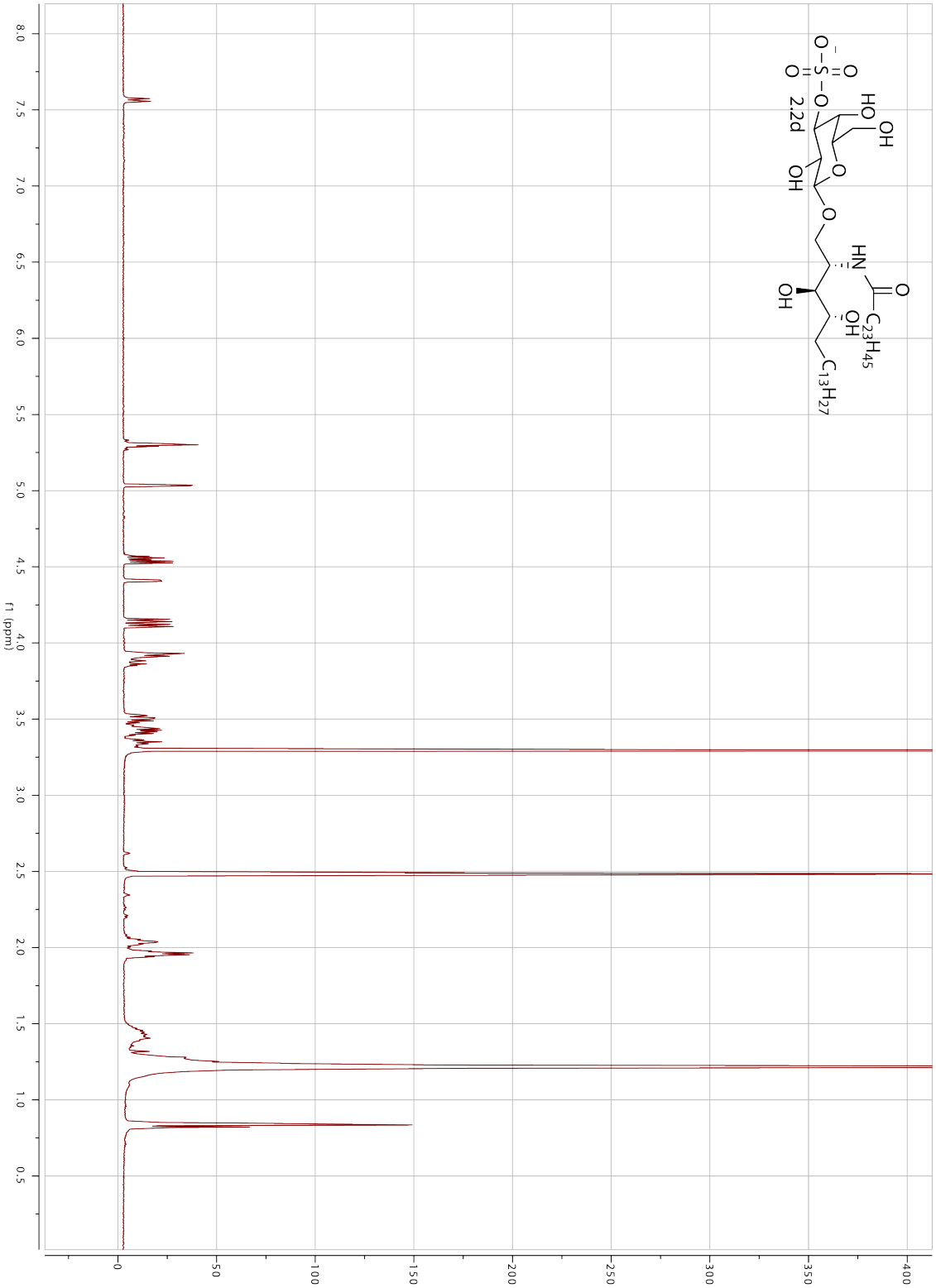

HO
OH
O
O
O
O
2.1d
OH
HN
O
OH
C23H45
C13H27
f1 (ppm)

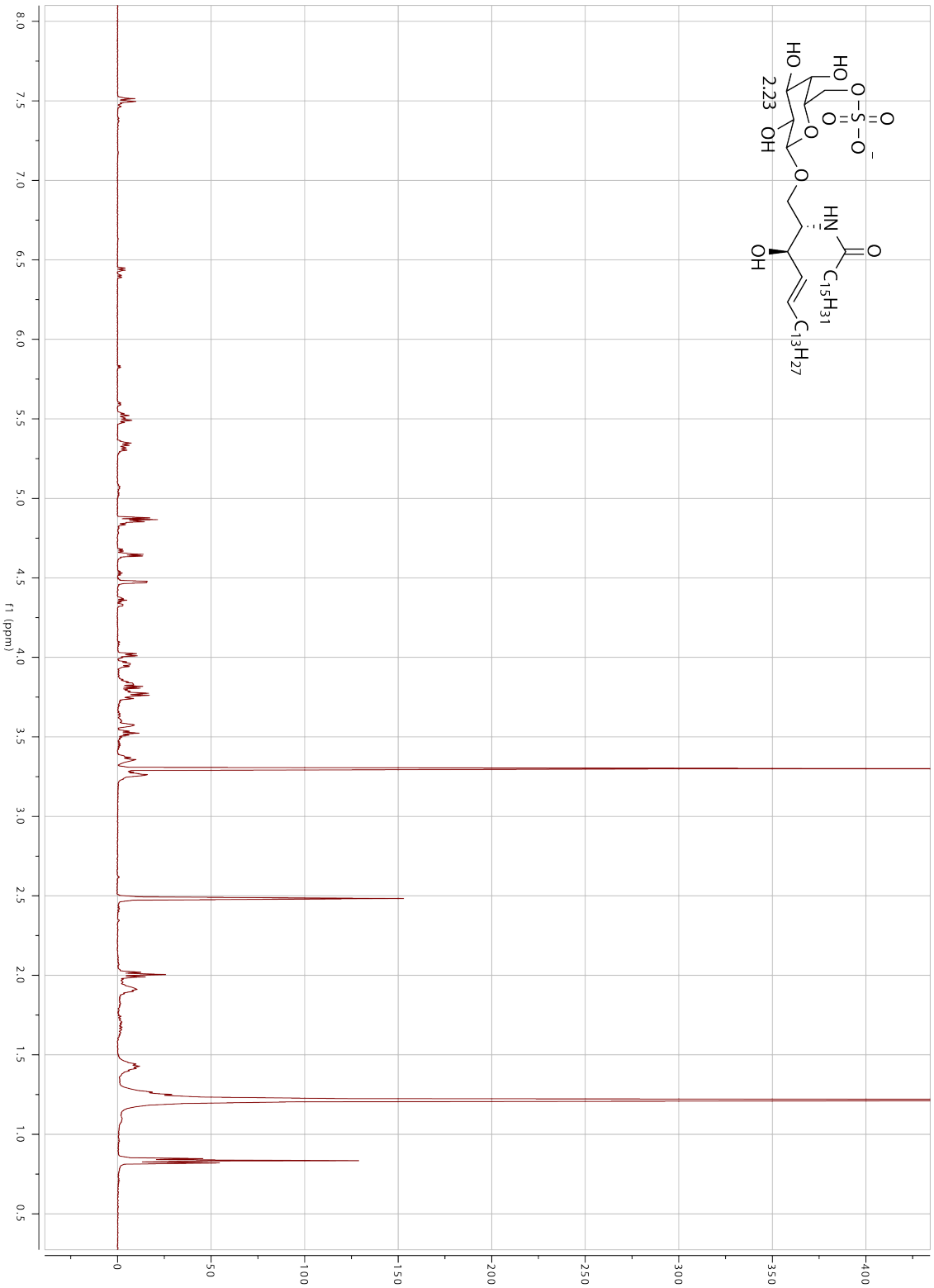

2.2a
f1 (ppm)

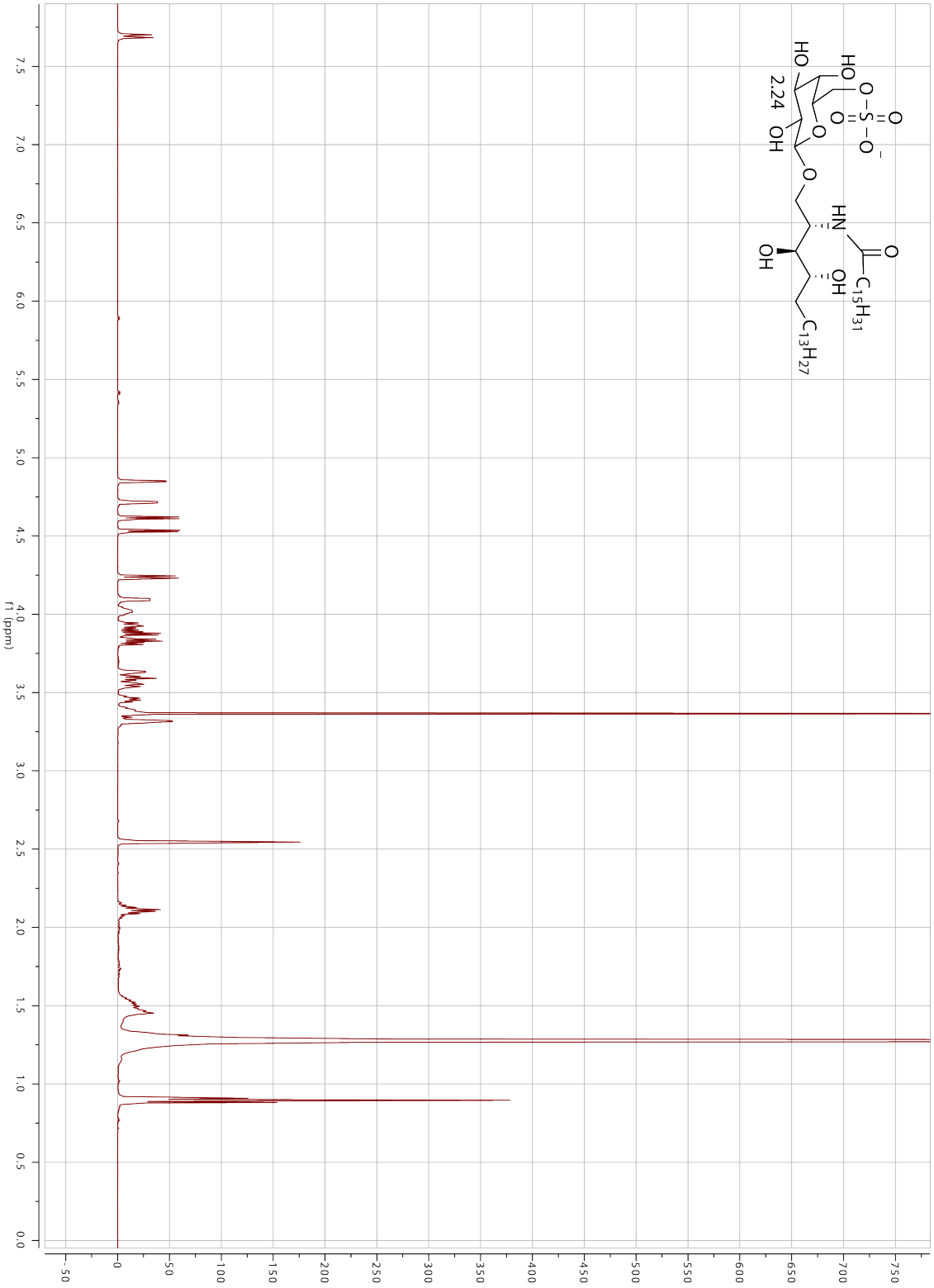

HO
OH
O
O
O
O
S
O
O
2.2a
OH
HN
O
C7H15
OH
OH
C13H27
f1 (ppm)

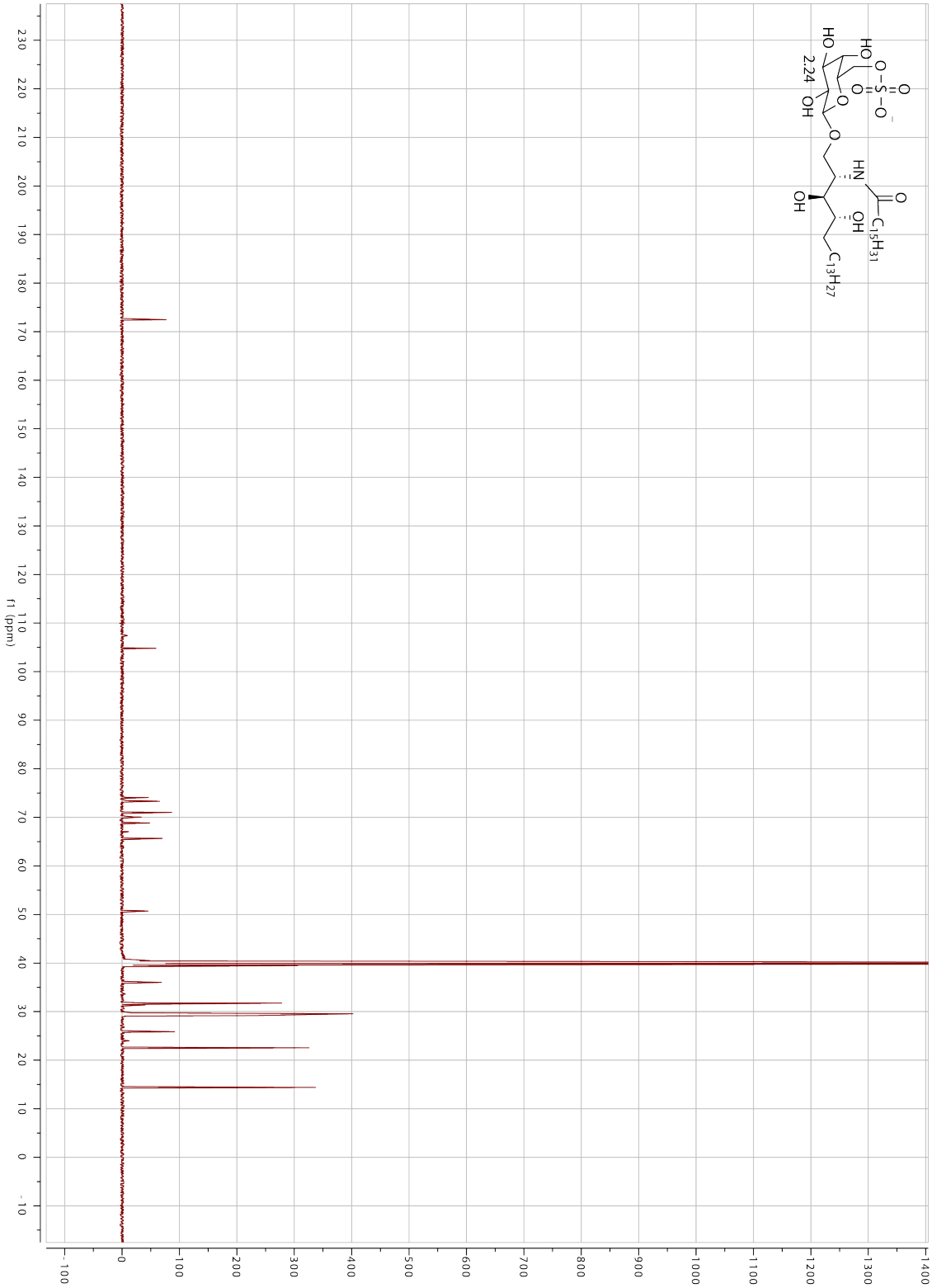

2.2b
f1 (ppm)

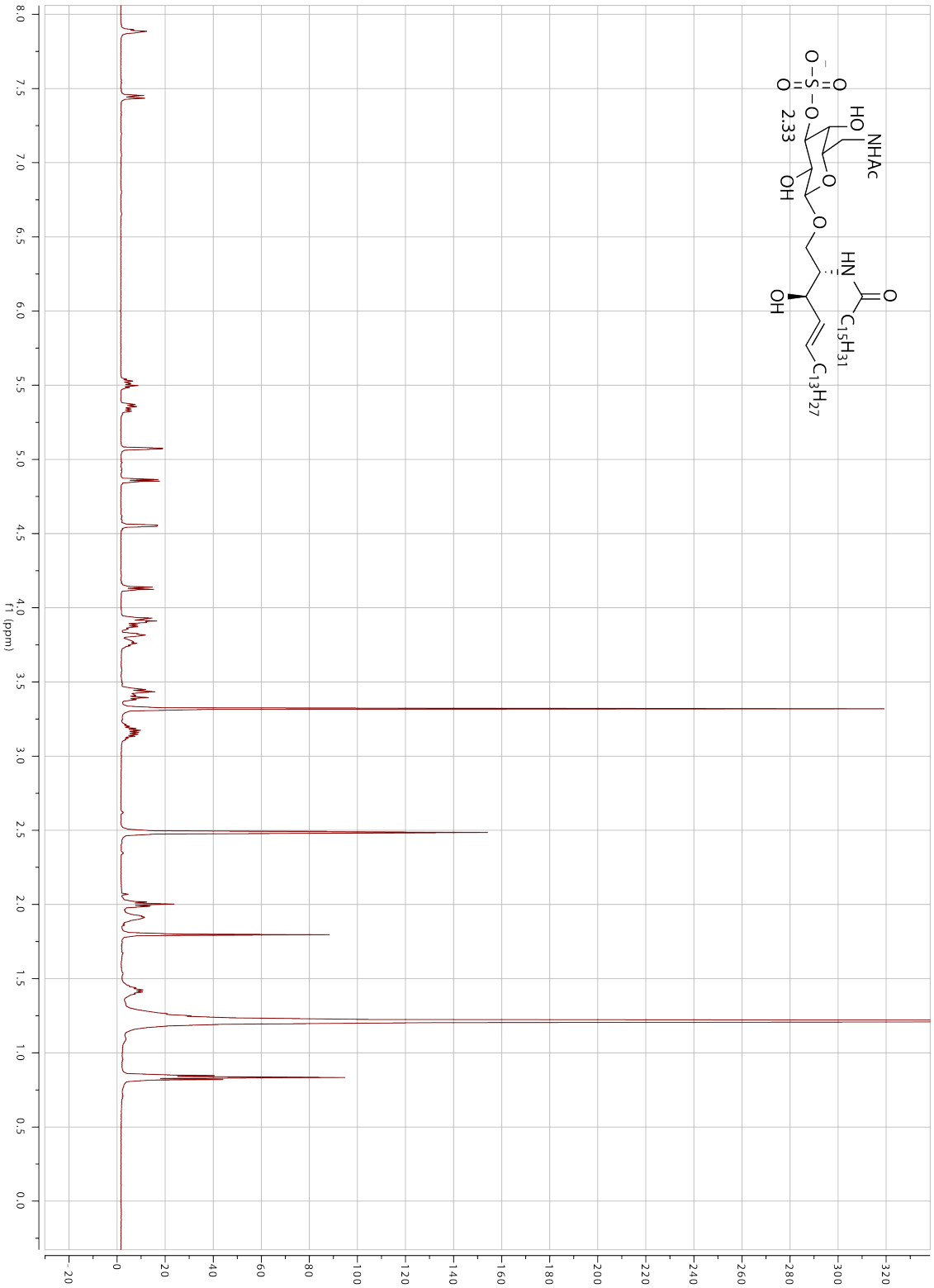

2.2b
f1 (ppm)

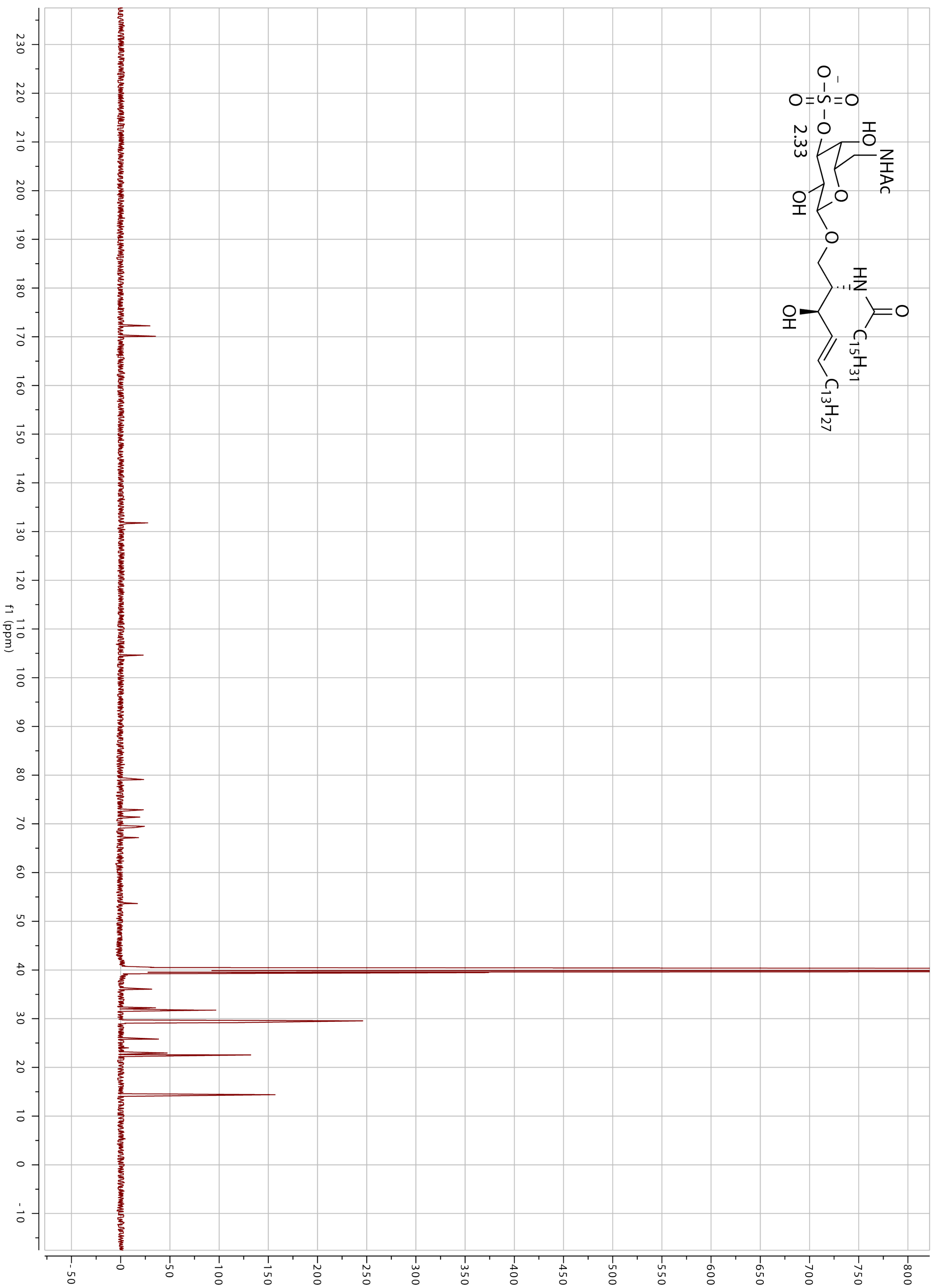

f1 (ppm)
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
50
100
150
200
250
300
350
400
2.2d
OH
HO
OH
O
O
O
O
S
O
O
HN
O
OH
OH
C23H45
C13H27

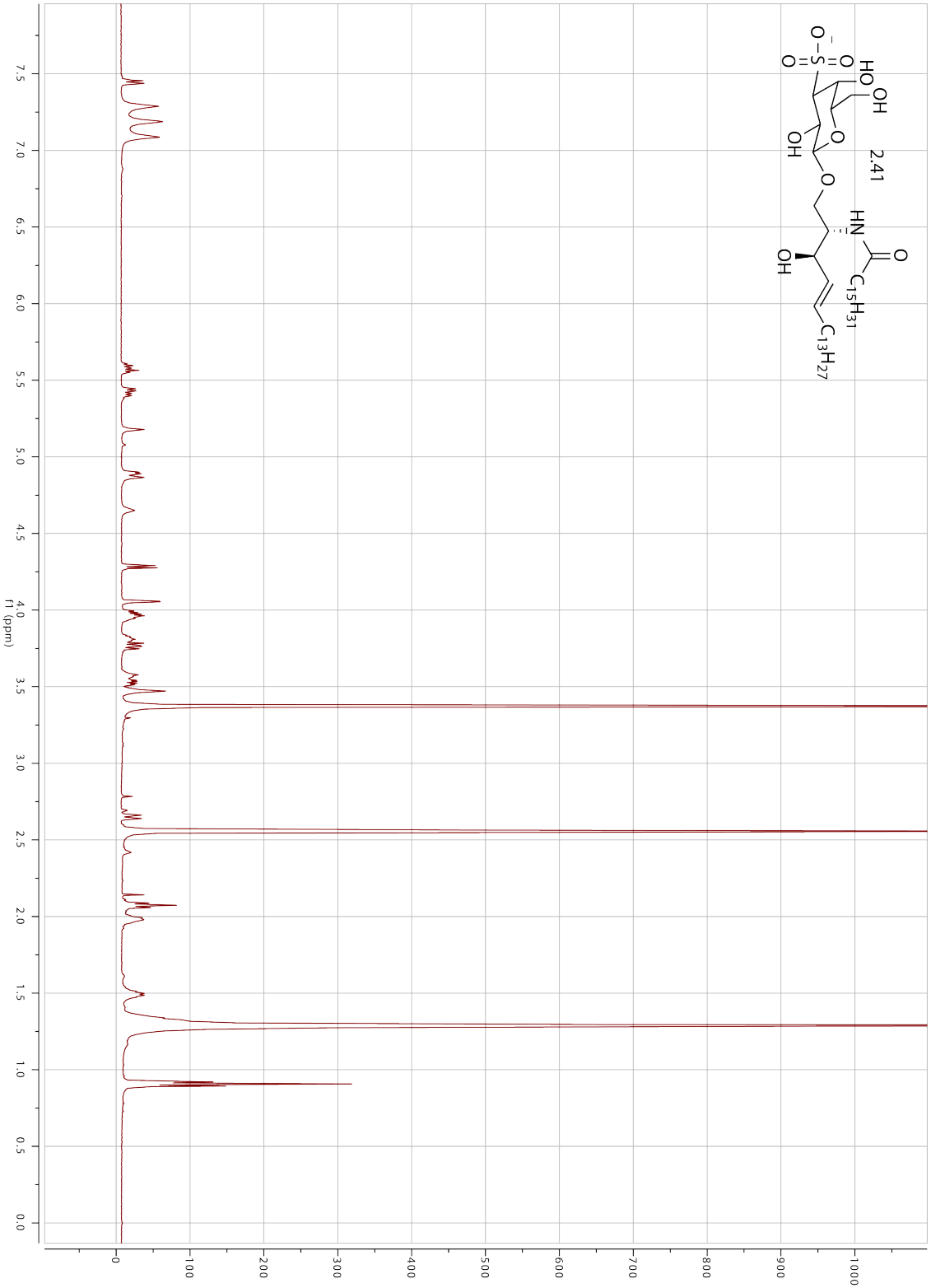

f1 (ppm)
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
50
100
150
200
250
300
350
400
HO
HO
O
O
O
O
O
O
S
O
2.23
OH
HN
O
OH
C15H31
C13H27

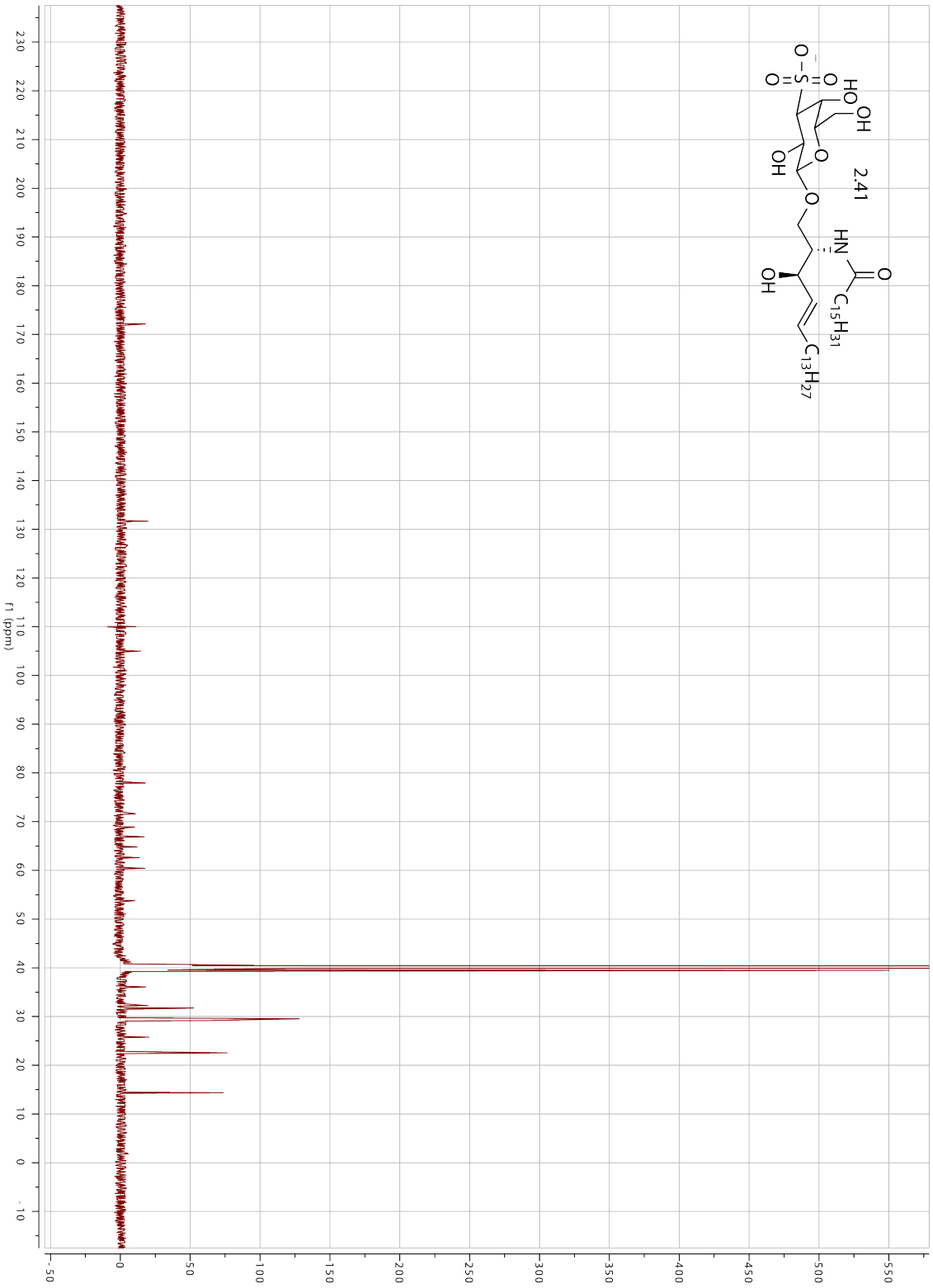

f1 (ppm)
2.24

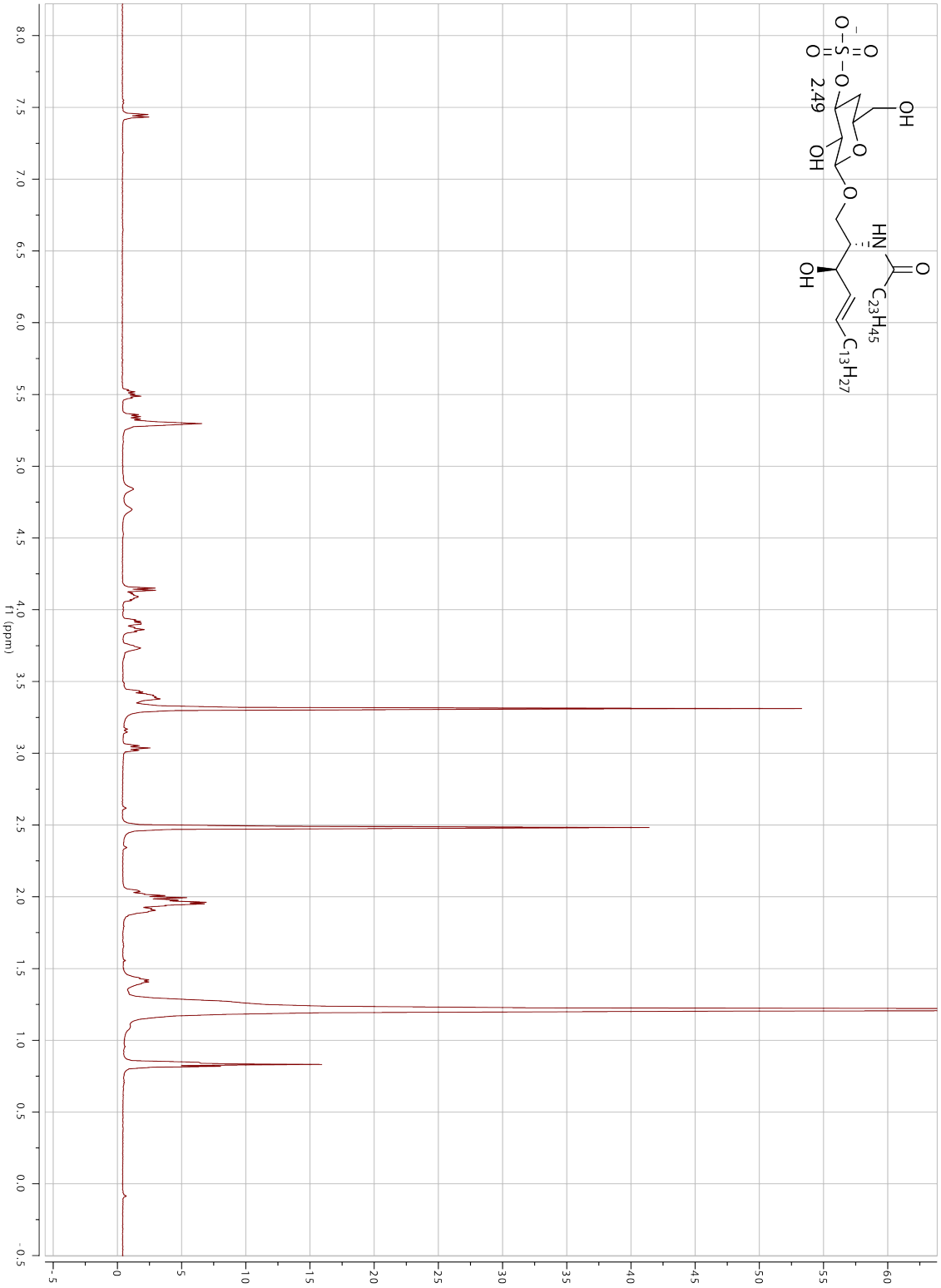

f1 (ppm)

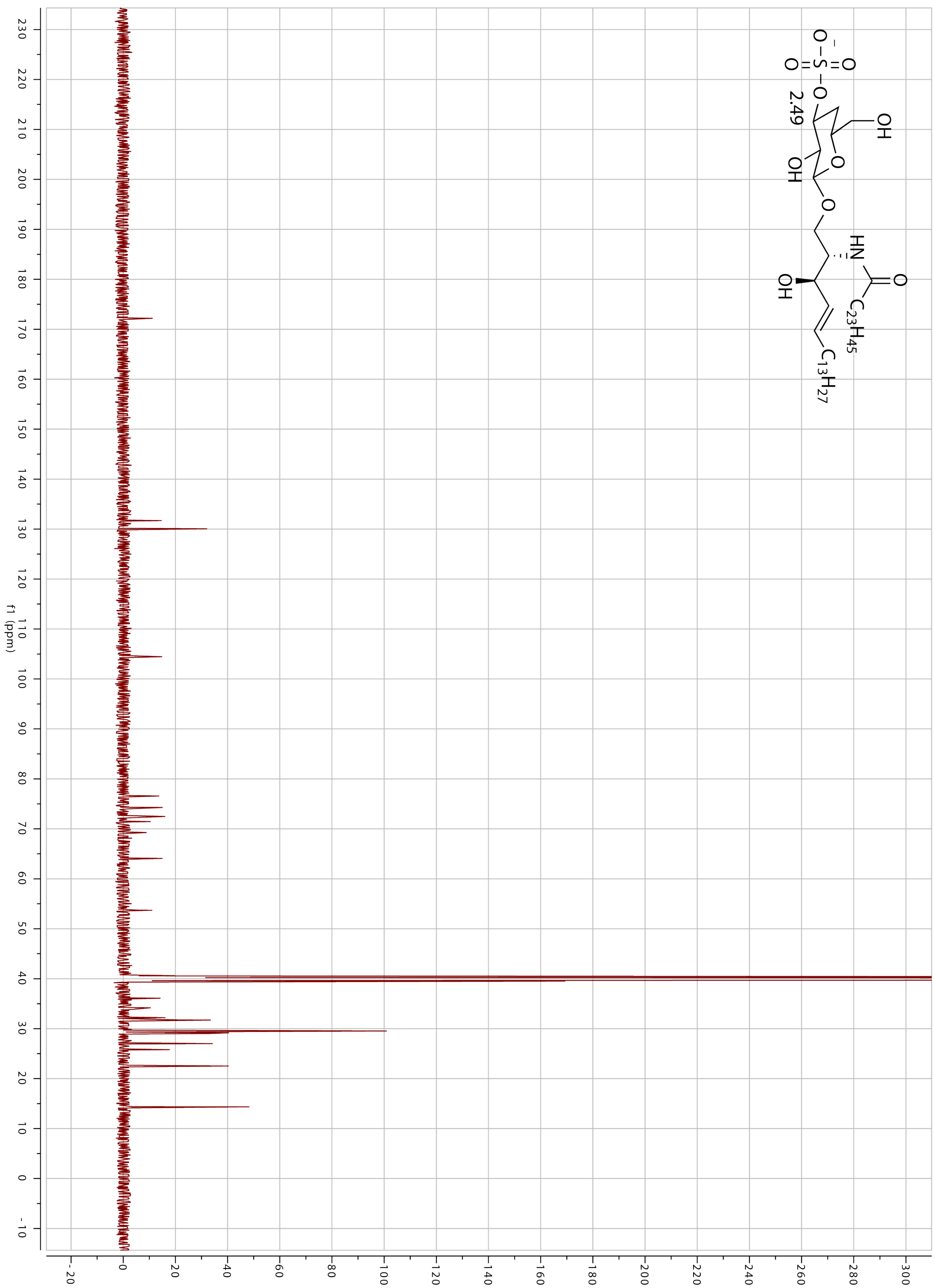

f1 (ppm)
2.33
NHAc
HO
OH
HN
OH
C15H31
C13H27

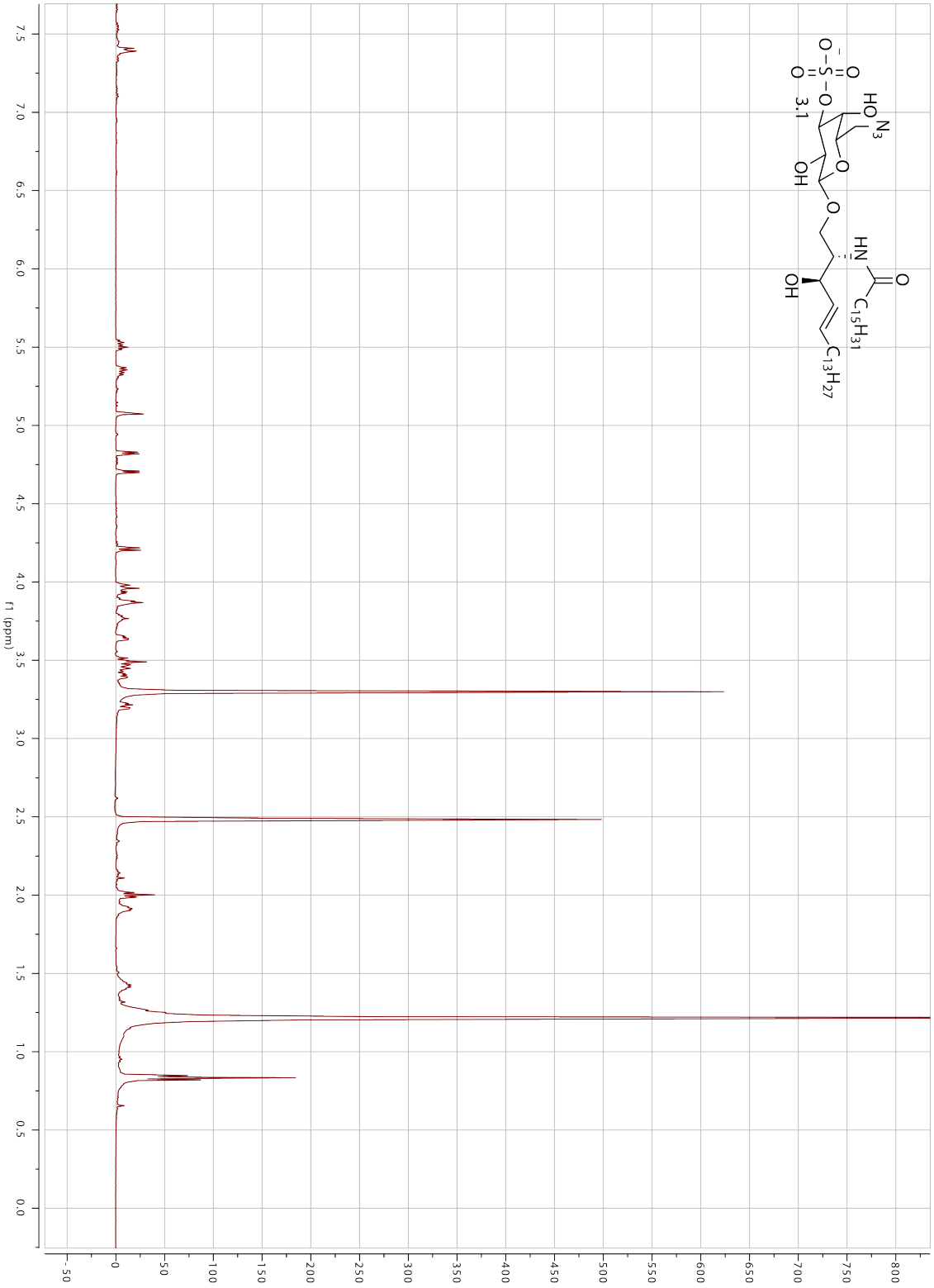

2.33
NHAc
f1 (ppm)

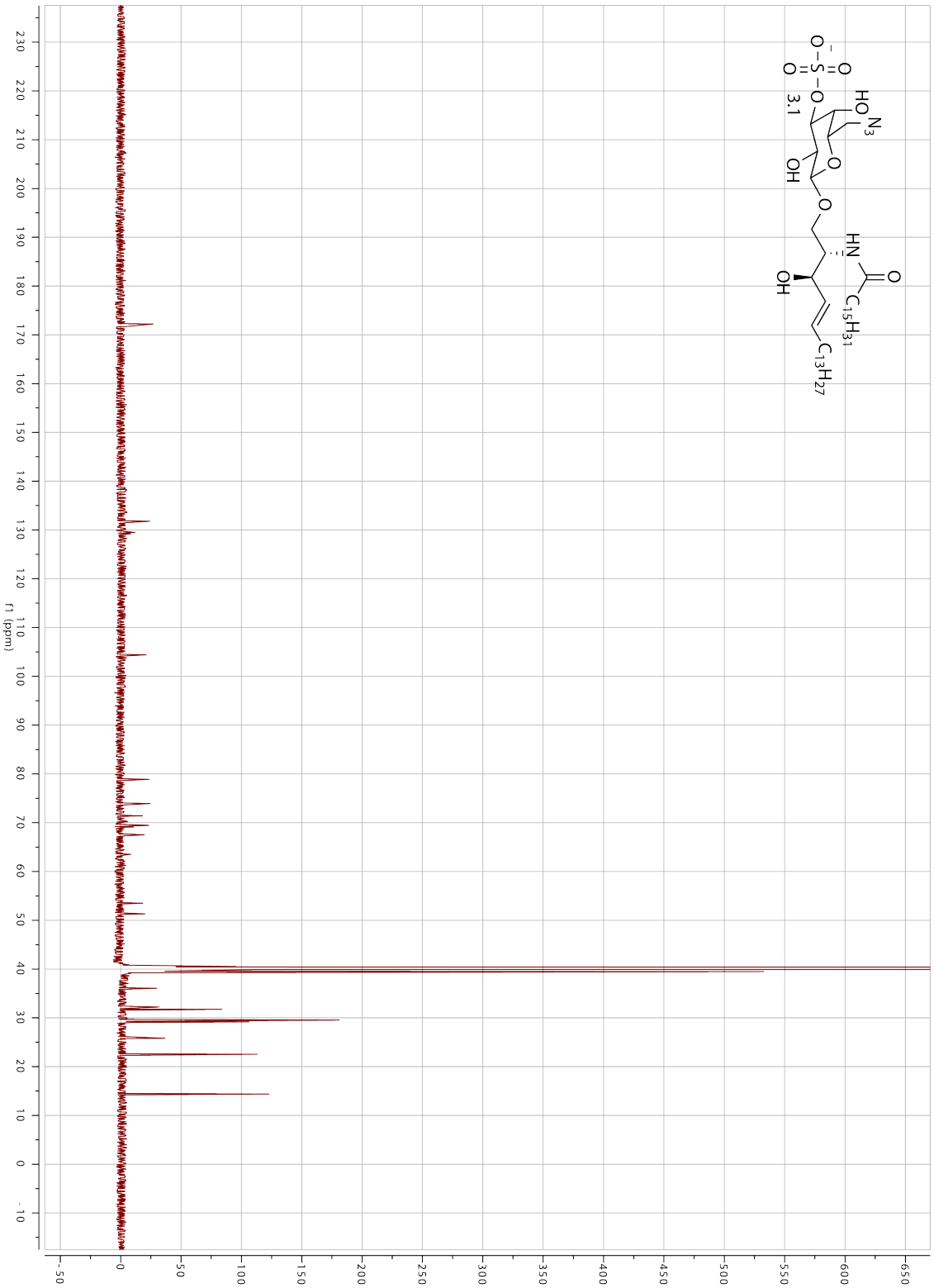

2.41
f1 (ppm)

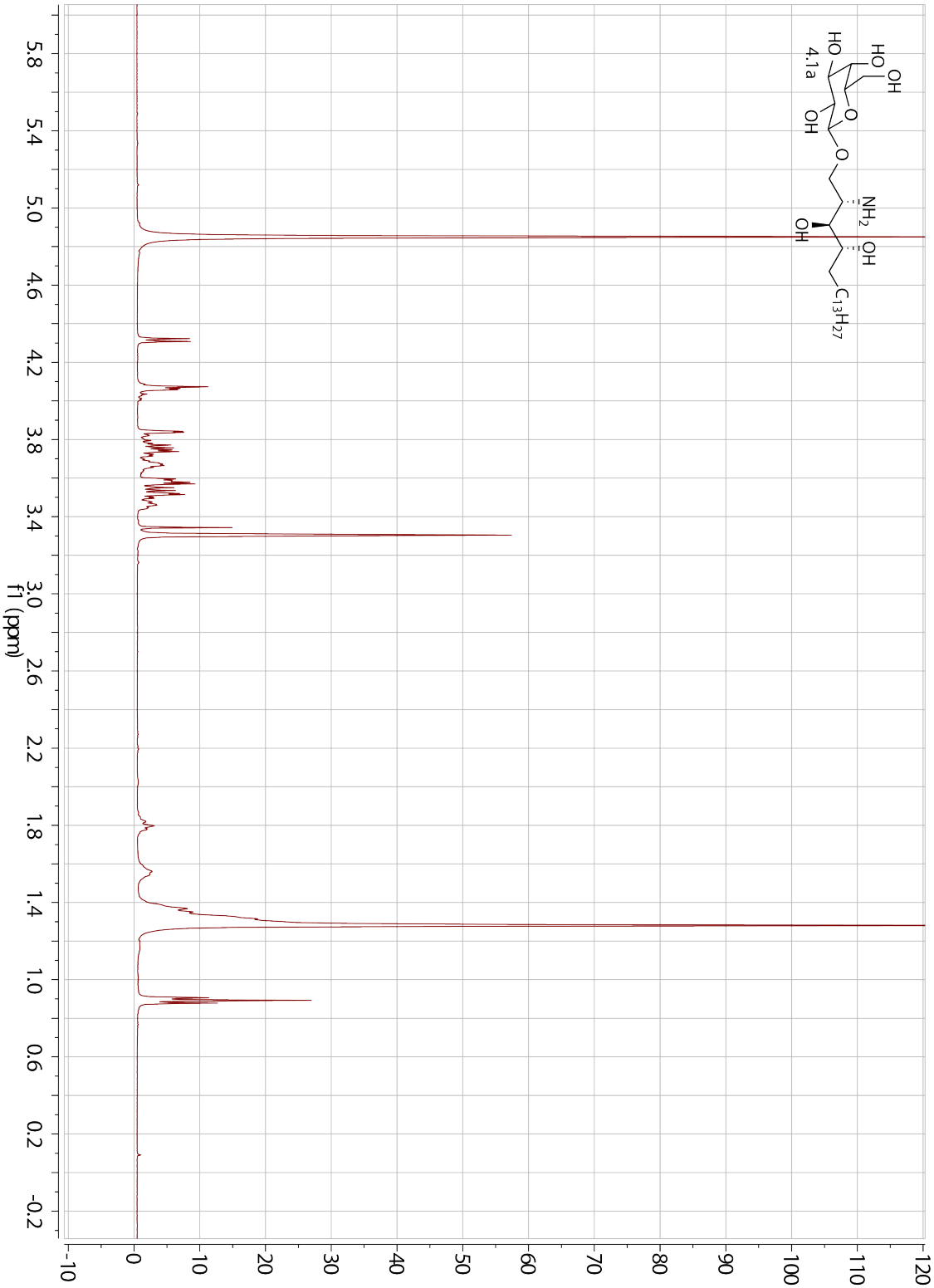

2.41
f1 (ppm)

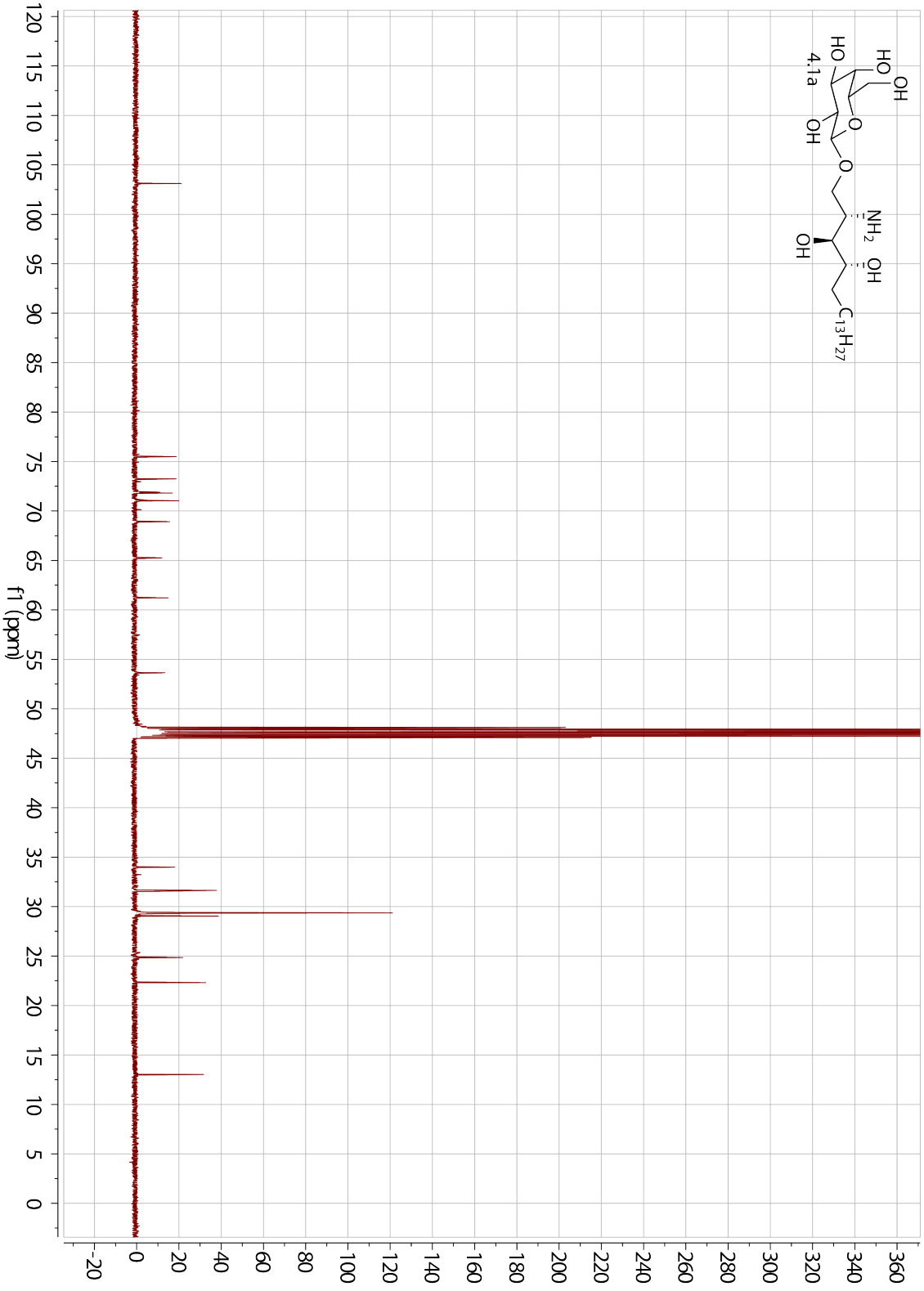

OH
O
HN
O
C23H45
C13H27
OH
OH
O
O
O
O
O
O
2.49
f1 (ppm)

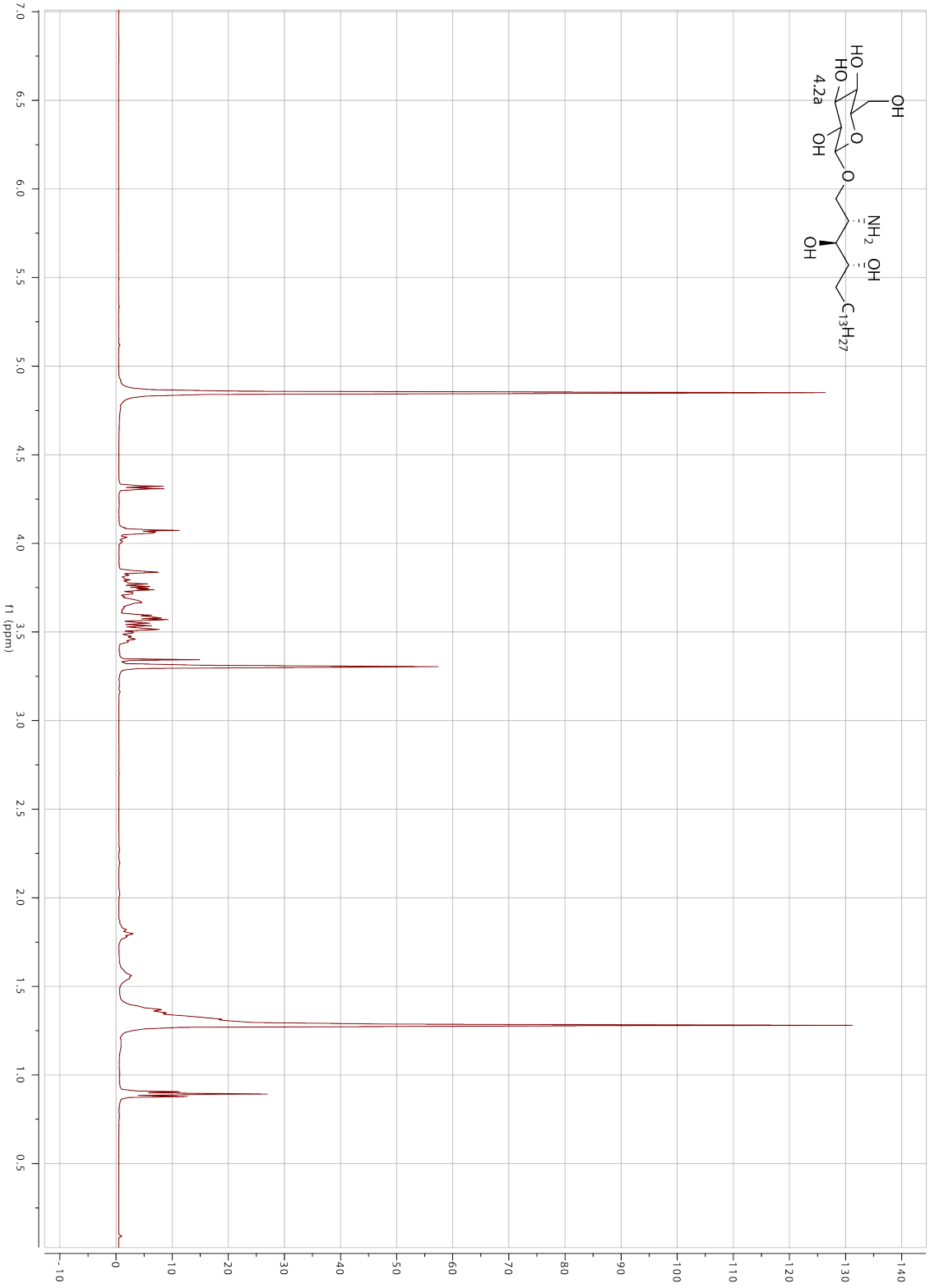

f1 (ppm)
2.49

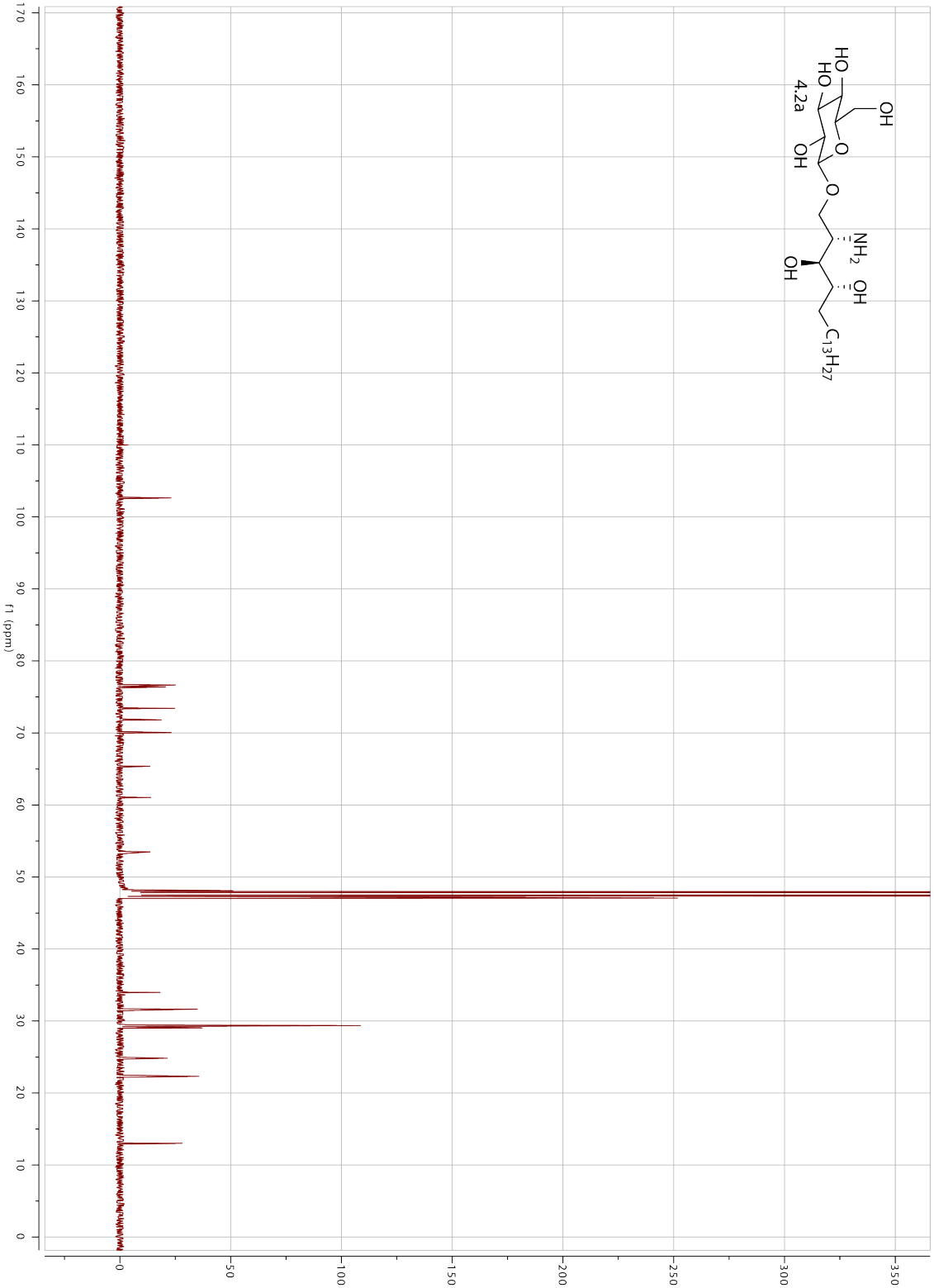

f1 (ppm)
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
-50
0
50
100
150
200
250
300
350
400
450
500
550
600
650
700
750
800
HO
N3
O
O
O
3.1
OH
HN
O
C15H31
OH
C13H27

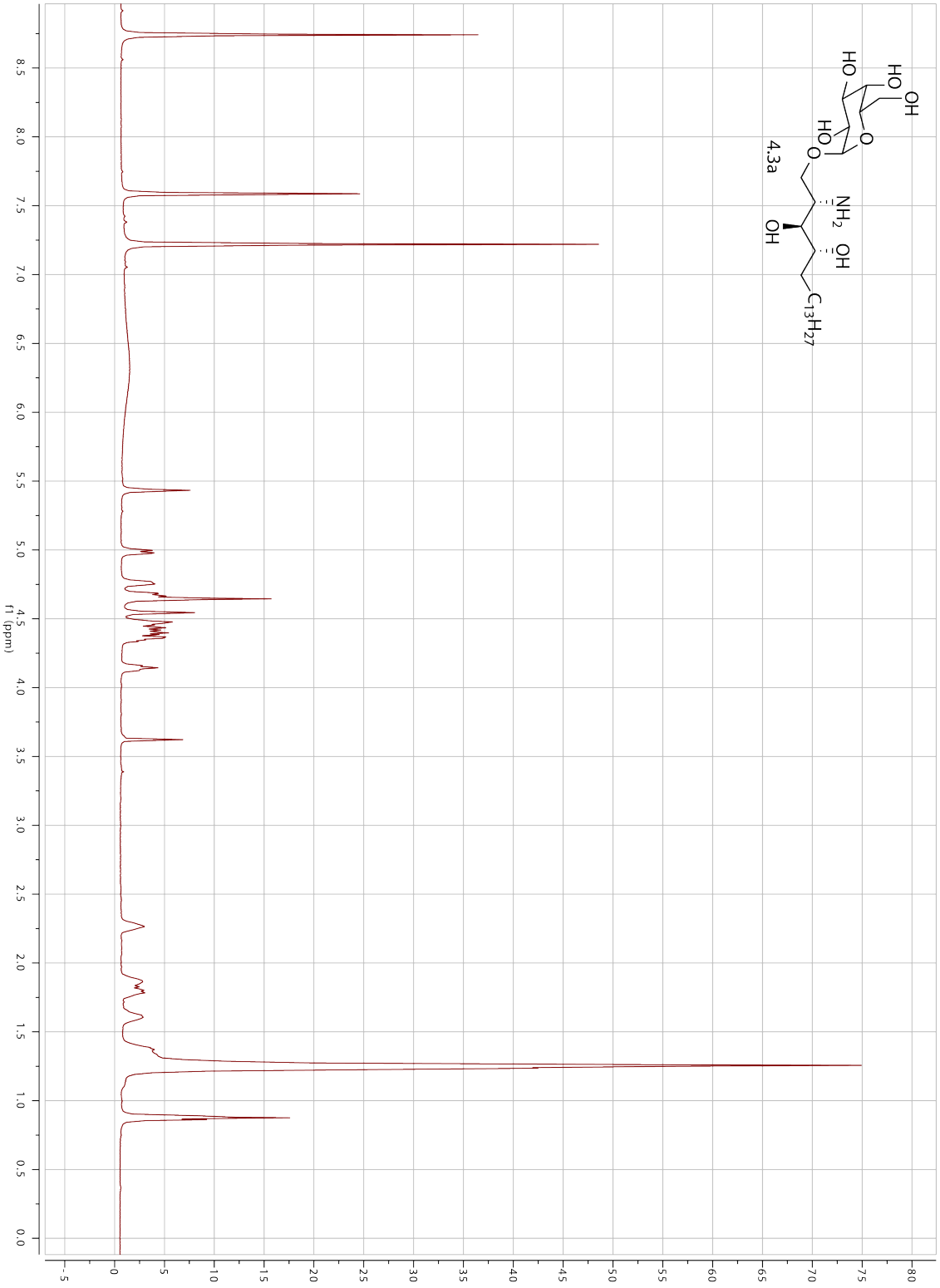

HO
N3
O
O
O
O
3.1
OH
HN
O
C15H31
OH
C13H27
f1 (ppm)

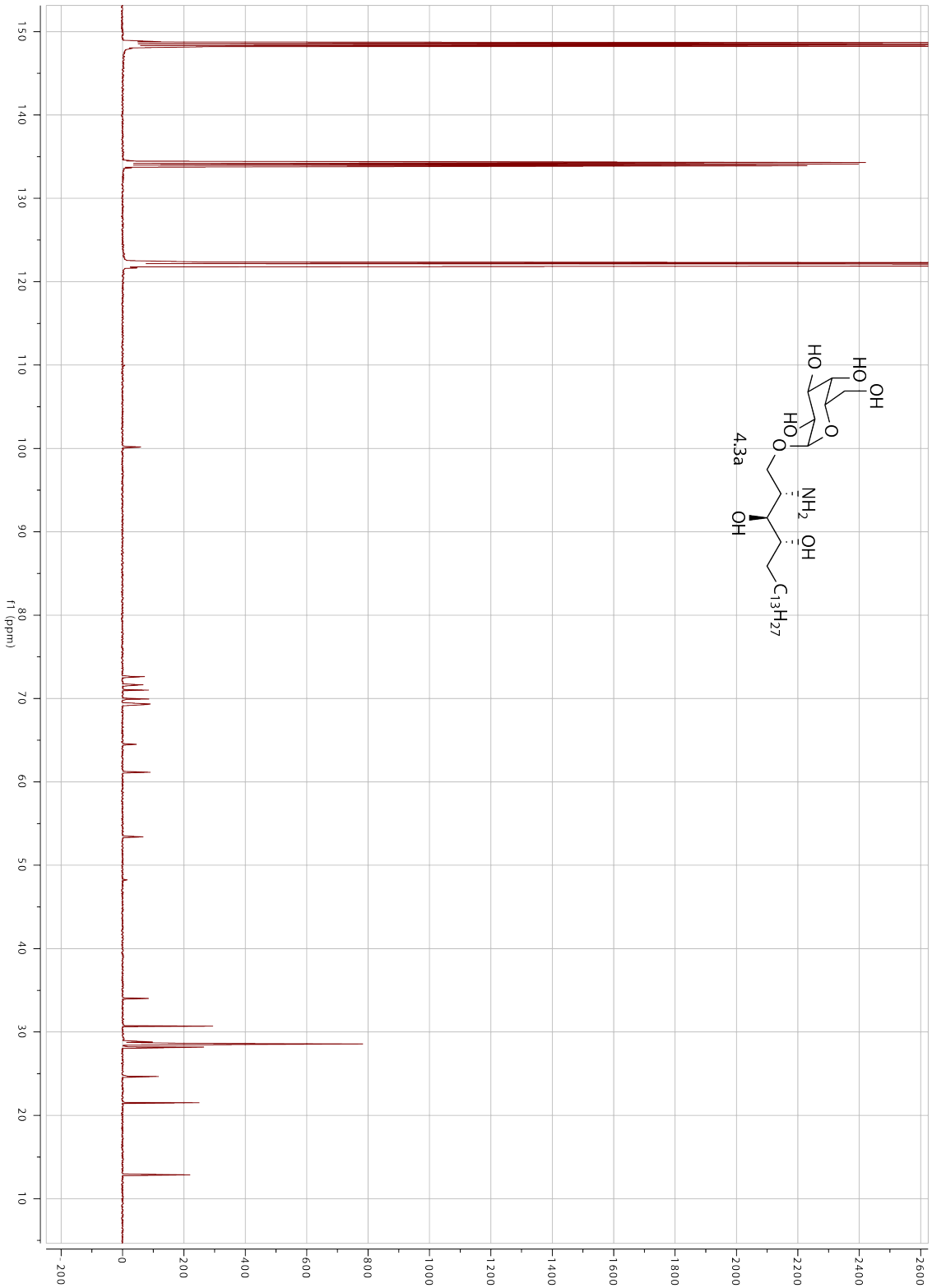

4.1a
HO
OH
HO
O
OH
O
NH2
OH
OH
C13H27
f1 (ppm)
5.8
5.4
5.0
4.6
4.2
3.8
3.4
3.0
2.6
2.2
1.8
1.4
1.0
0.6
0.2
-0.2
-10
0
10
20
30
40
50
60
70
80
90
100
110
120

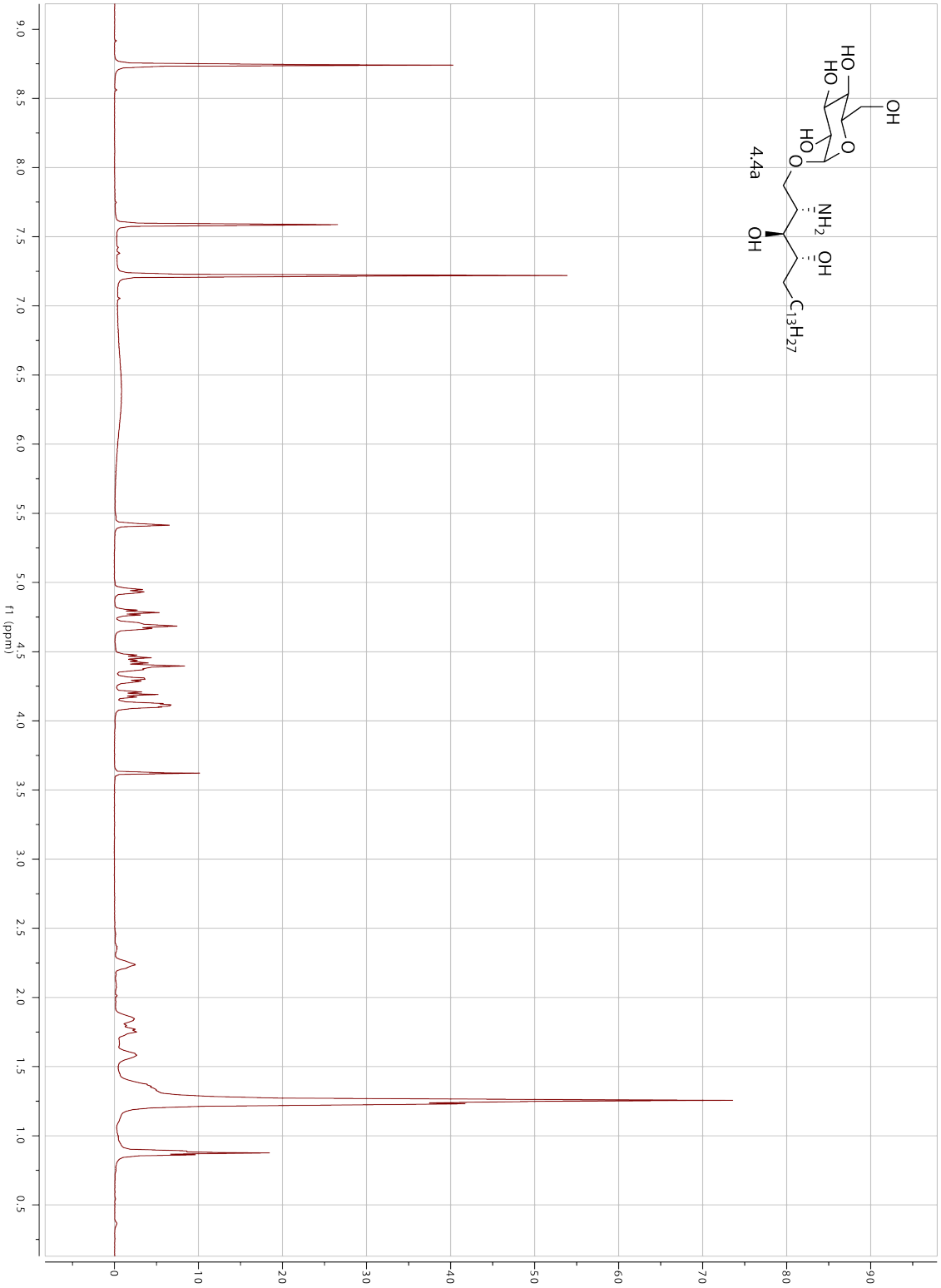

HO
OH
HO
4.1a
OH
O
O
NH2
OH
OH
C13H27
f1 (ppm)

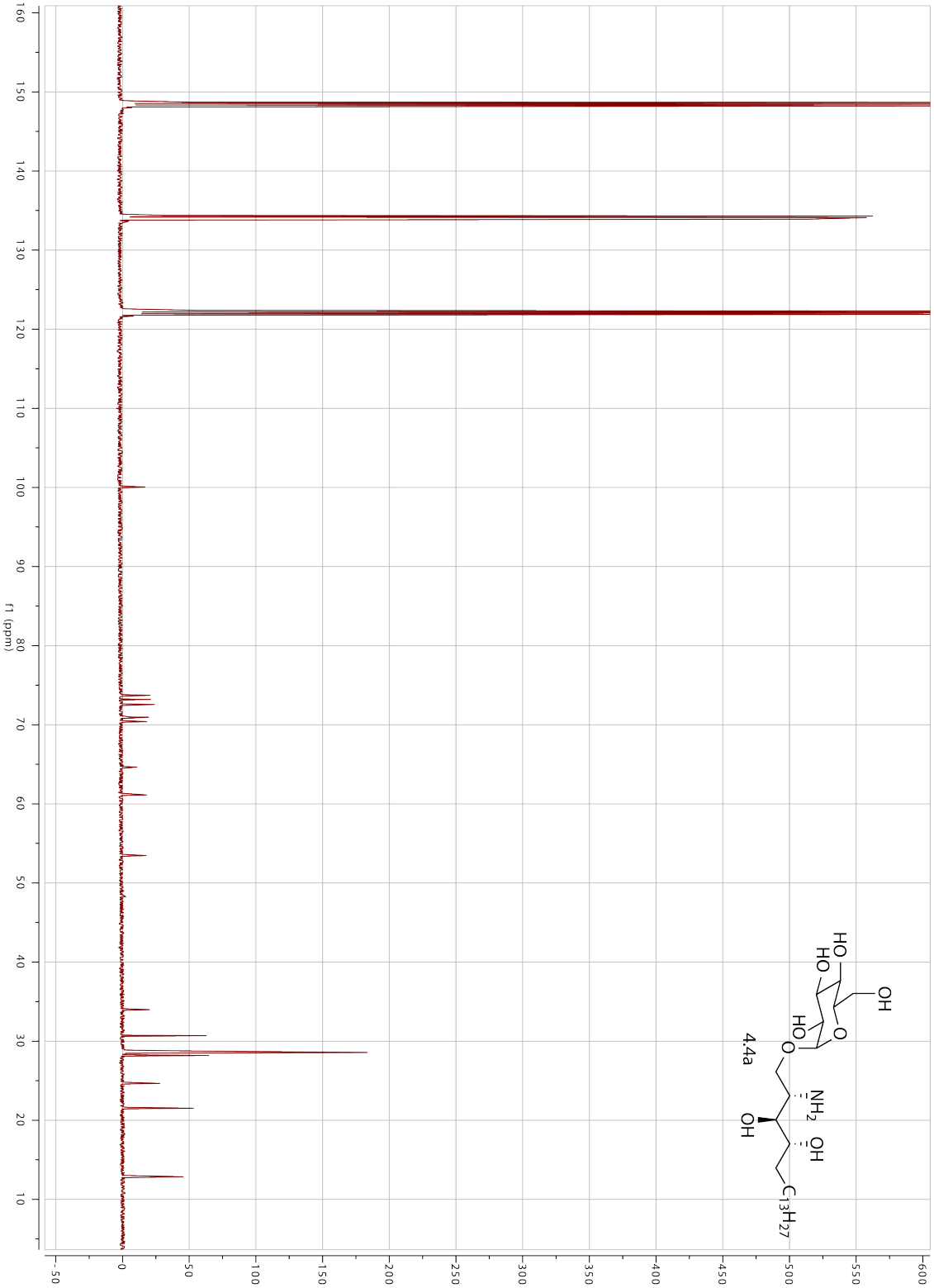

4.2a
f1 (ppm)

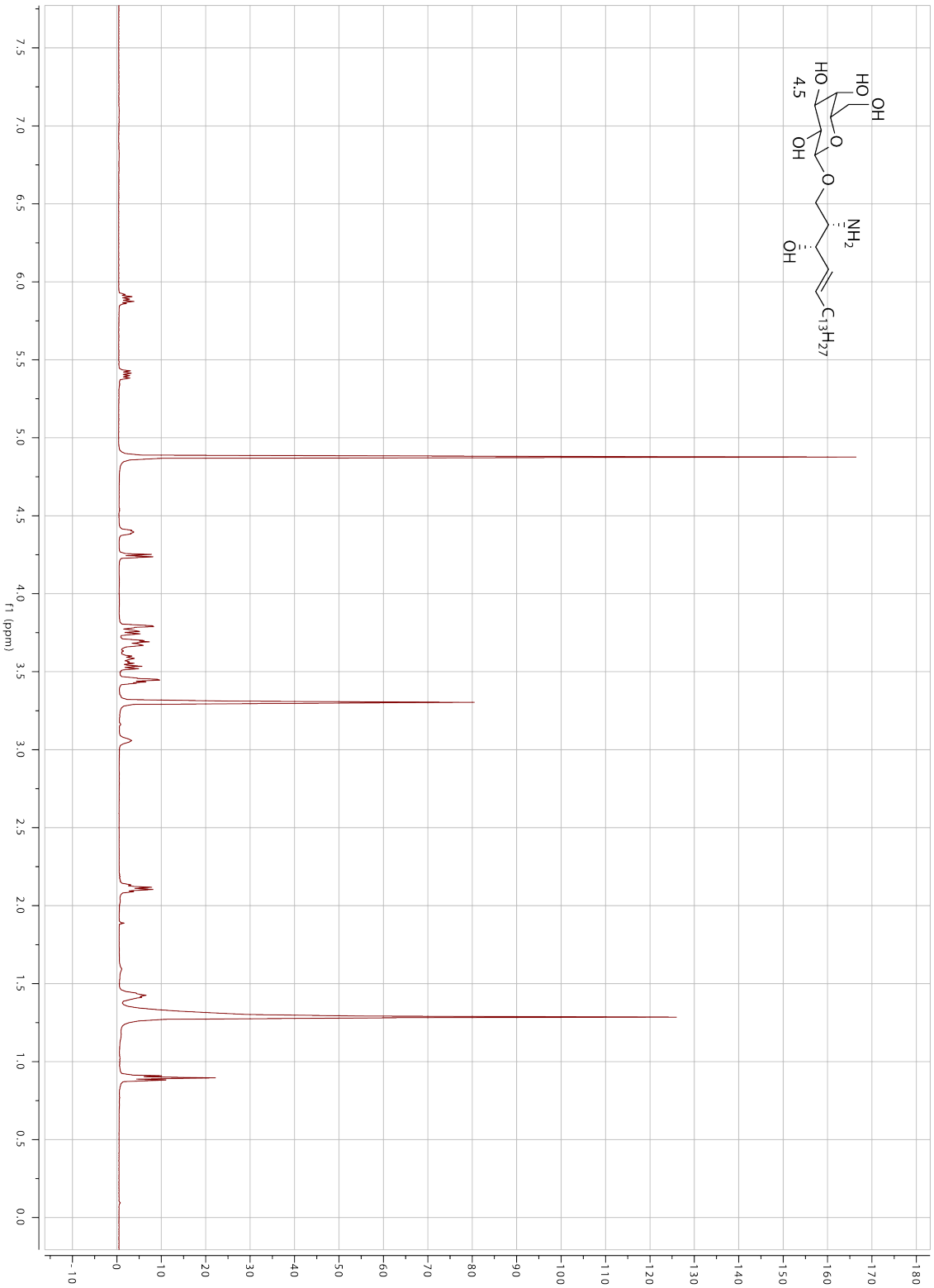

4.2a
HO
HO
OH
O
O
OH
NH2
OH
OH
C13H27
f1 (ppm)

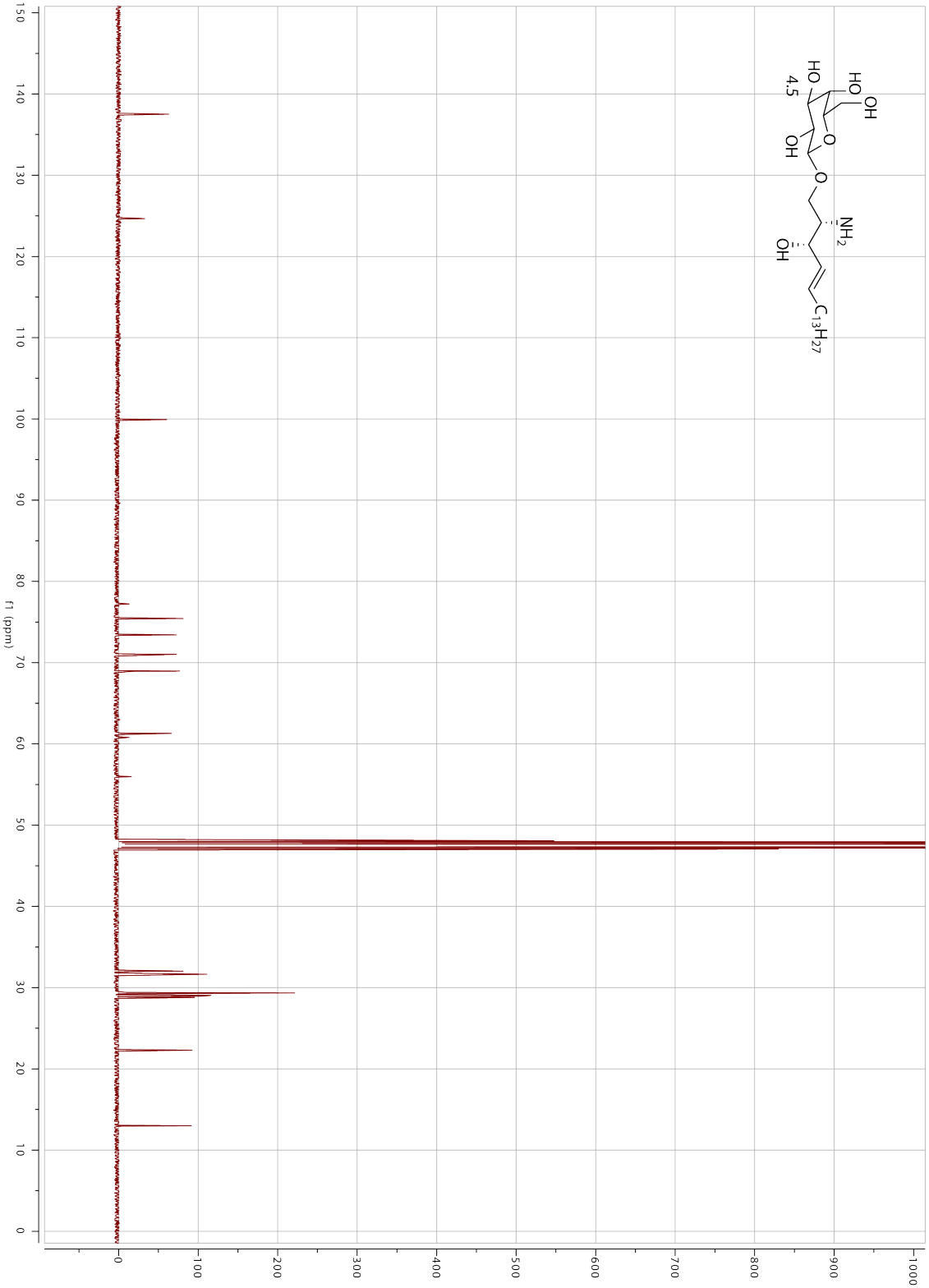

HO
HO
OH
O
HO
O
4.3a
NH2
OH
OH
C13H27
f1 (ppm)

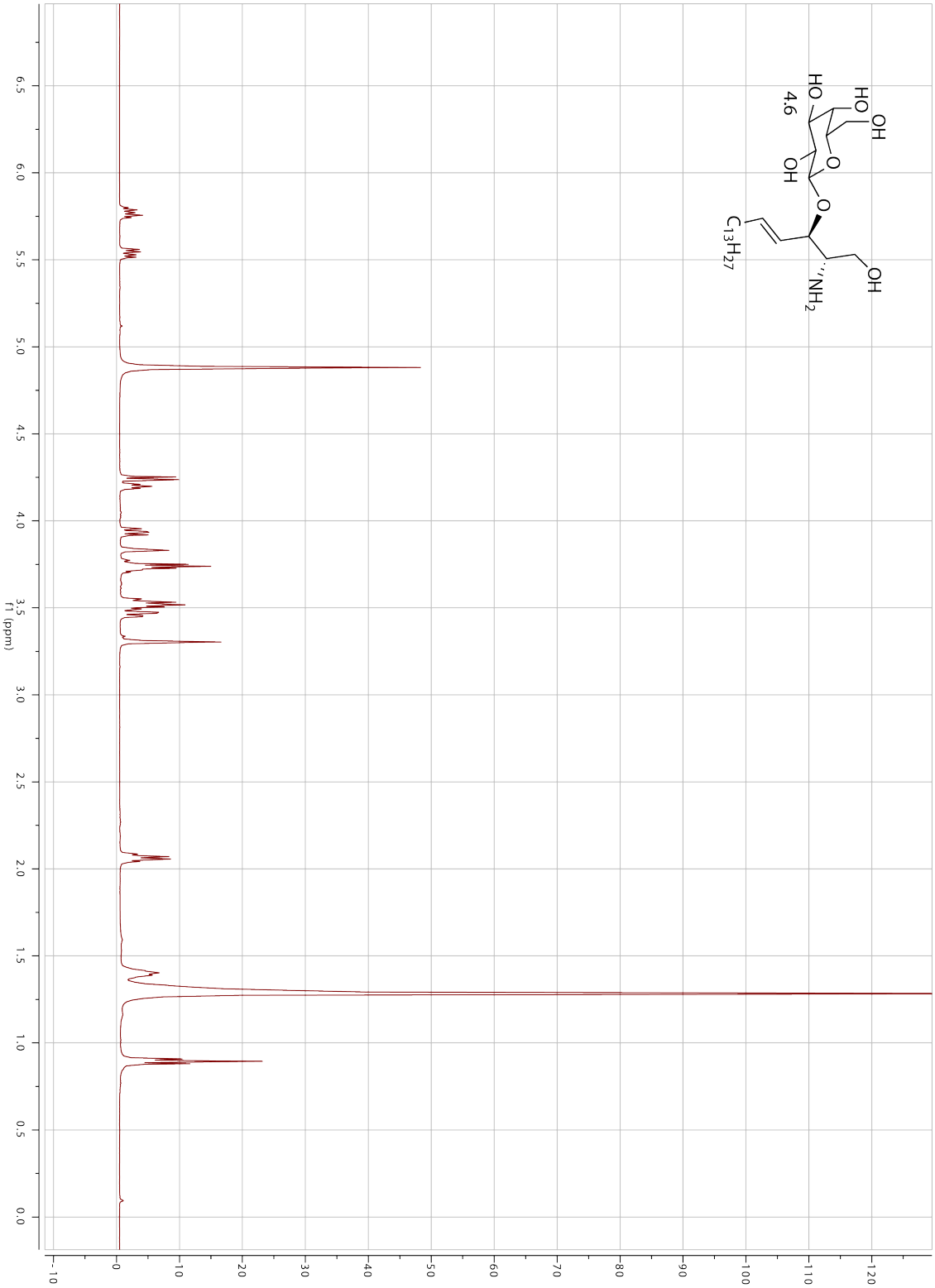

HO
HO
OH
O
HO
HO
O
4.3a
NH2
OH
OH
C13H27
f1 (ppm)

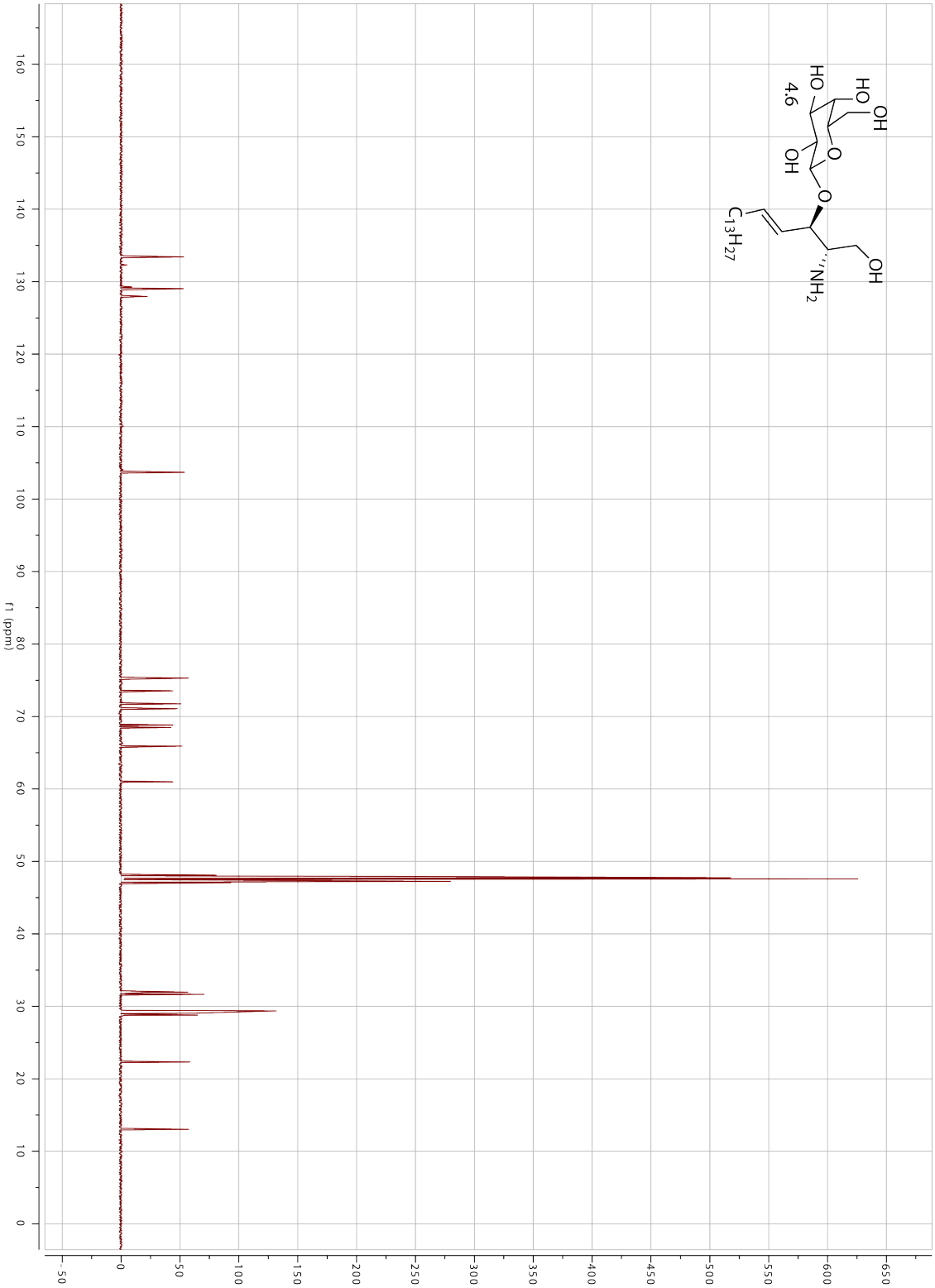

4.4a
f1 (ppm)

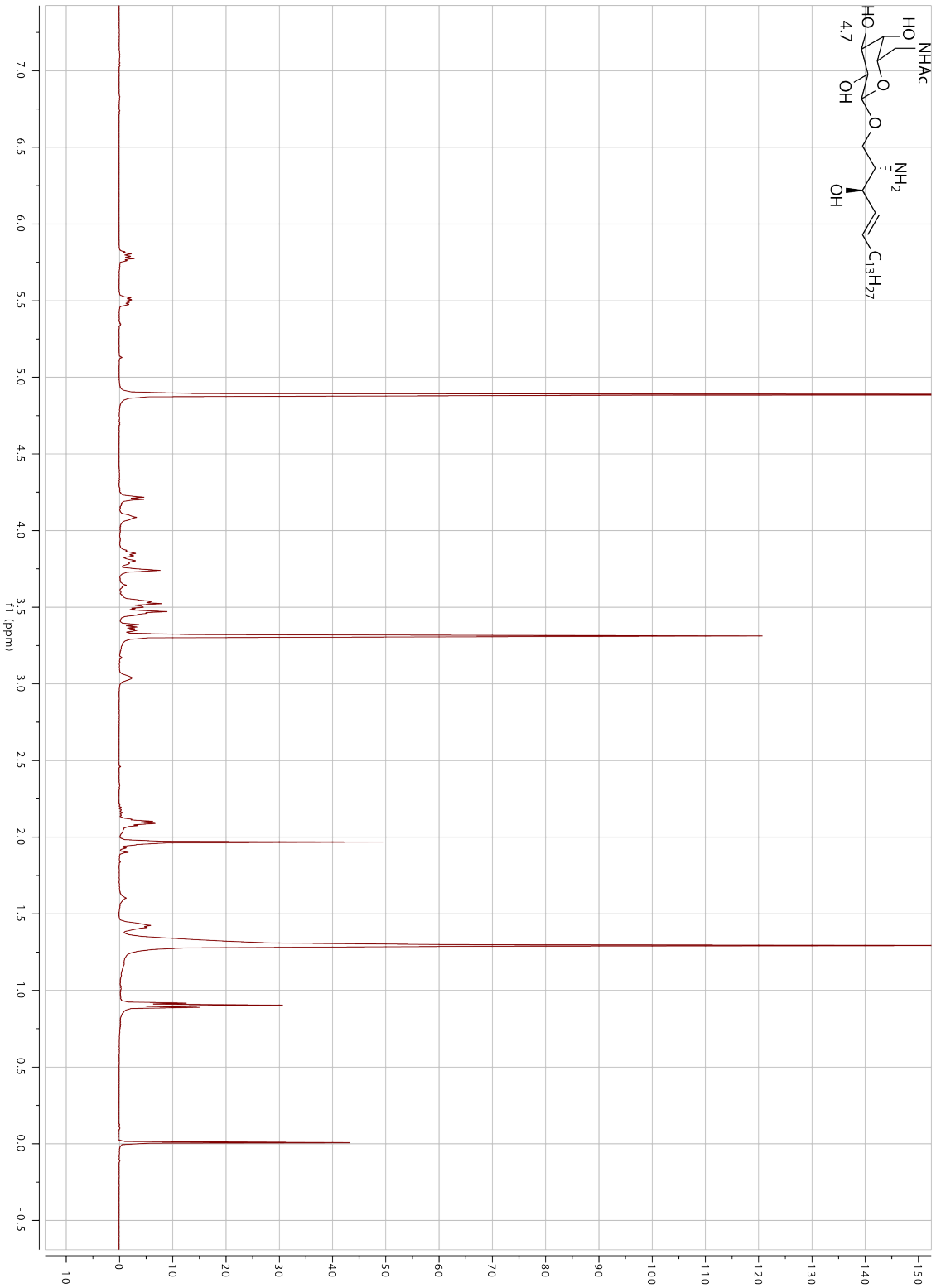

f1 (ppm)
4.4a
HO
HO
HO
O
OH
O
NH2
OH
OH
C13H27

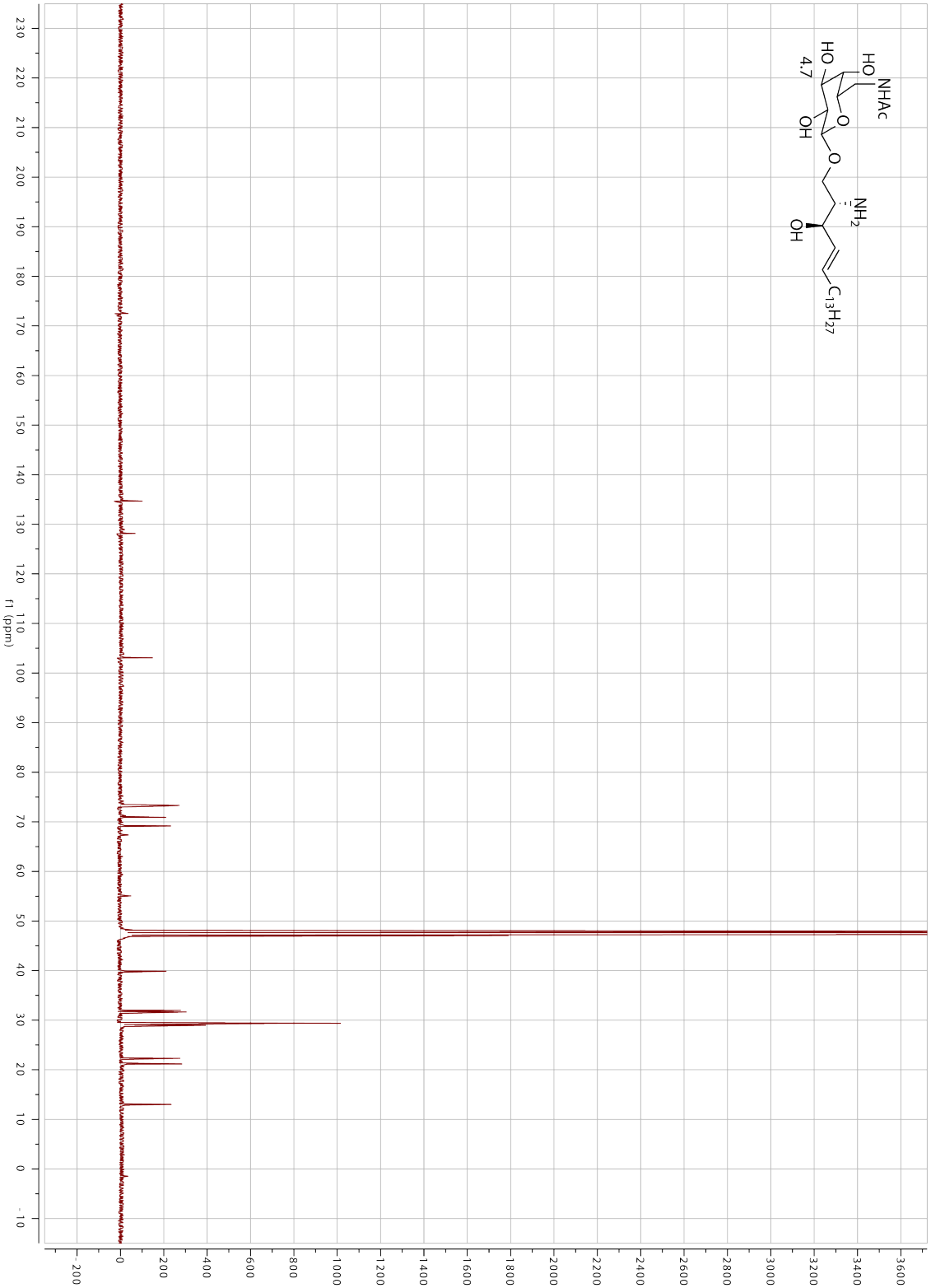

OH
HO
HO
4.5
O
OH
O
NH2
OH
C13H27
f1 (ppm)

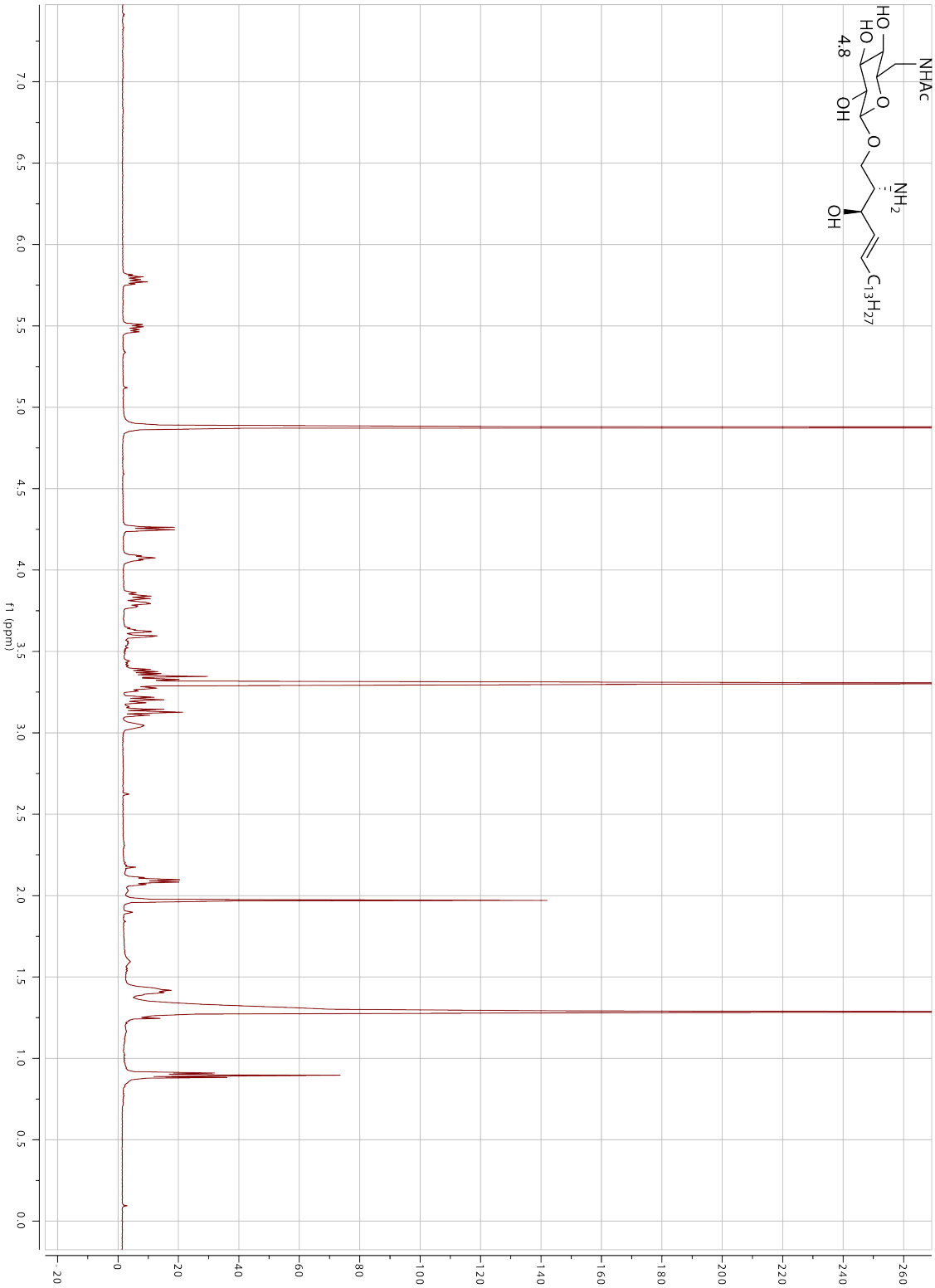

HO
OH
HO
4.5
OH
O
O
NH2
OH
C13H27
f1 (ppm)
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
0
100
200
300
400
500
600
700
800
900
1000

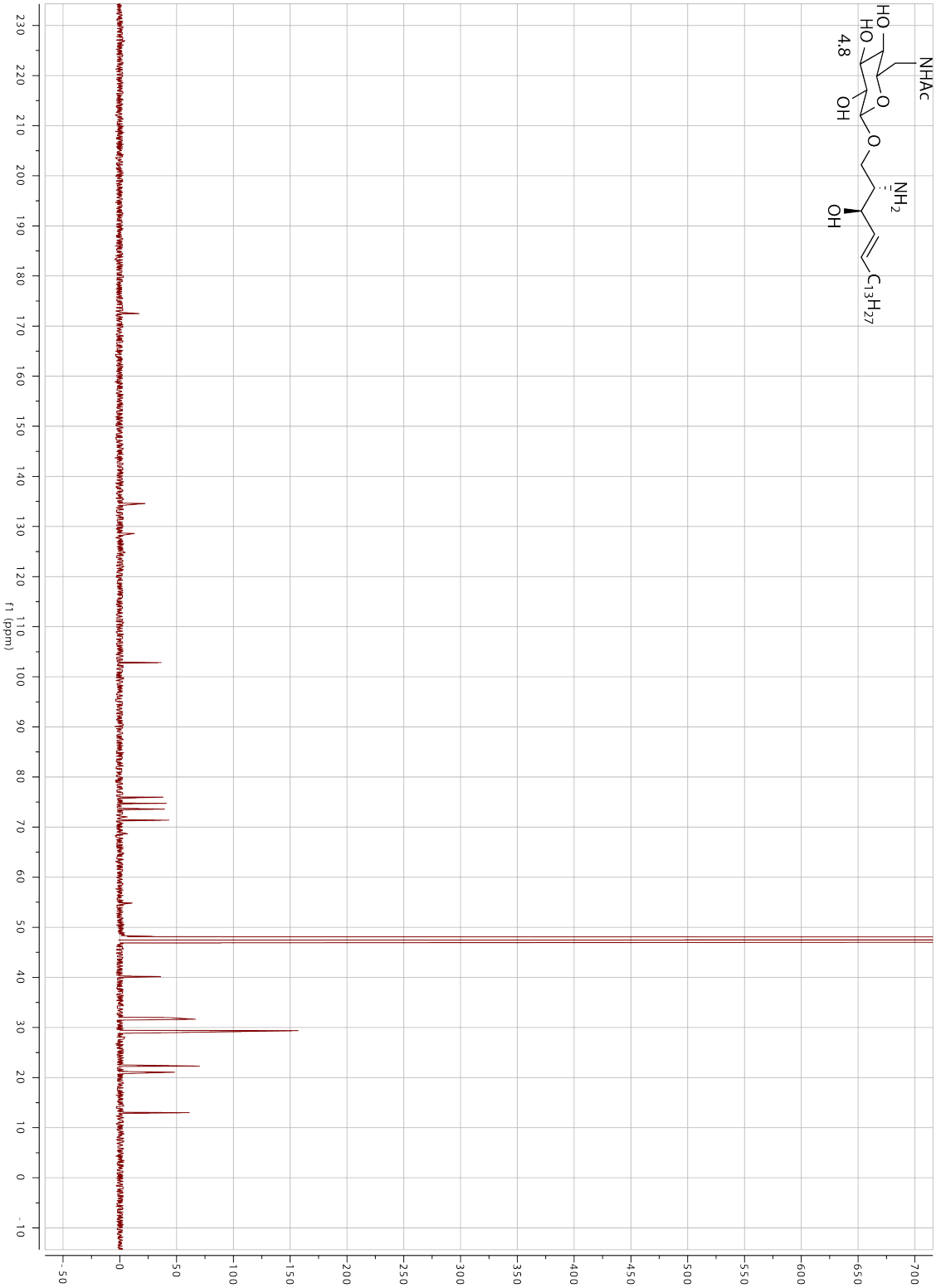

HO
OH
HO
4.6
OH
O
O
C13H27
NH2
OH
f1 (ppm)


4.6
HO
HO
OH
O
OH
O
$C_{13}H_{27}$
NH_2
OH
f1 (ppm)


NHAc
HO
HO
4.7
OH
O
O
NH2
OH
C13H27
f1 (ppm)


HO
NHAc
HO
4.7
O
OH
O
NH2
OH
C13H27
f1 (ppm)


NHAc
HO
HO
4.8
OH
O
O
NH2
OH
C13H27
f1 (ppm)


NHAc
HO
HO
4.8
OH
O
O
NH2
OH
C13H27
f1 (ppm)