2010-07-09

Synthesis of Glycolipids and Evaluation of Their NKT Cell Stimulatory Properties

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SYNTHESIS OF GLYCOLIPIDS AND EVALUATION OF THEIR NKT CELL STIMULATORY PROPERTIES

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

Doctor of Philosophy

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August 2010

ABSTRACT

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Natural killer T (NKT) cells are a subset of T cells that modify a variety of immune responses. NKT cells recognize glycolipid antigen presented by a molecule called CD1d, a nonclassical antigen-presenting molecule. The best known subset of CD1d-dependent NKT cells expresses an invariant T cell receptor Vα (TCR-α) chain. These are referred to as type I or invariant NKT (*i*NKT) cells. When stimulated by a glycolipid, NKT cells rapidly release large amounts of cytokines. Cytokines released by NKT cells can induce either Th1 or Th2 responses. Th1 cytokines are effective in regulating bacterial, parasitic, and viral infections. But Th1 responses are also involved in some autoimmune diseases, such as type 1 diabetes, lupus, rheumatoid arthritis, and allergen-induced asthma. Th2 cytokines can attenuate proinflammatory Th1 responses and therefore prevent some autoimmune diseases.

Lysosomal processing and CD1d loading is very important in regulating the stimulatory properties of antigens. Analogs of KRN7000, with small molecules appended on C6" position of the galactose portion, do not significantly change stimulation of NKT cells. The question is if the substitution at this position would influence the lysosomal processing. Two sets of mono- and disaccharides with and without substitution at C6" position were prepared and evaluated the NKT cell stimulatory properties. The substitution at the C6" position of the sugar moiety of glycolipids do not significantly impact the stimulatory properties of glycolipids and their processing in lysosome. Small changes at C6" are well tolerated.

A double bond in the acyl chain and modification of the C6" functional group helped the glycolipid loading into CD1d and NKT cells stimulation. PBS57 is 100 times more active than KRN 7000 in stimulation of NKT cells responses *in vitro* and *in vivo*. This improvement is probably due to increasing solubility and improving binding ability with the CD1d.

Keywords: NKT cells, CD1d, Glycolipid

ACKNOWLEDGEMENTS

My dissertation and research work was developed with the help of Dr. Savage, the active involvement of my committee members and the support of my family. I would like to acknowledge the contributions that were made by the following people:

I express my sincere gratitude to my advisor, Dr. Savage, who gave me valuable opinions, support and encouragement for the successful completion of my dissertation and research work. With his suggestions and critical comments, the research work has been compelling.

I also wish to express my thankfulness to my committee members, Dr. Steven L. Castle, Dr. Merritt B. Andrus, Dr. Matt A. Peterson, and Dr. Roger G. Harrison for their kindness in revising my dissertation.

I would also like to thank all the members working in Dr. Savage's group. I am grateful to Dr. Shenglou Deng, Mr. Yanshu Feng and Dr. Xiangtian Long for providing valuable assistance to my research work. I am grateful to Mr. Brian Anderson for help with editing grammar errors in the dissertation.

Last but not least, a big warm hug to Mr. Lei Pei, my husband, for supporting and encouraging me all these years.

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LIST OF ABBREVIATIONS

APC	Antigen presenting cell
BMDC	Bone marrow-derived dendritic cell
CD1d/TD	Tail-deleted CD1d
DCC	N,N'- Dicyclohexylcarbodimide
EAE	Experimental autoimmune encephalomyelitis
Hexb B	Hexosaminidase B
HOBt	N-Hydroxybenzotrizole
IFN-γ	Interferon-γ
IL	Interleukin
LPS	Lipopolysaccharides
LTP	Lipid transfer protein
MHC	Major histocompatibility complex
NBD	7-Nitrobenzo-2-oxa-1,3-diazole
NKTCell	Natural killer T cell
NPC1	Neimann-Pick type C1 protein
RBL	Rat basophilic leukaemia
TCR	T cell receptor
Th	T helper cell

CHAPTER 1.

DEVELOPMENT AND FUNCTION OF NATURAL KILLER T CELLS

1.1 What are NKT cells

Natural killer T (NKT) cells are a subset of T cells as they express a T cell receptor (TCR).[1] NKT cells also share some characteristics of NK cells (such as NK1.1 and CD161). Unlike conventional T cells that recognize peptides bound to polymorphic major histocompatibility complex (MHC) class I or class II molecules, NKT cells recognize glycolipid antigen presented by a molecule called CD1d, a nonclassical antigen-presenting molecule.

The best known subset of CD1d-dependent NKT cells expresses an invariant T cell receptor Vα (TCR-α) chain. These are referred to as type I or invariant NKT cells (iNKT) cells. *i*NKT cells have a rearrangement of the TCR (Vα14-Jα18 in mice and Vα24-Jα18 in humans).[2-6] *i*NKT cells are found at the highest frequency in the liver, constituting 10-40% of the total lymphocytes. They typically are approximately 1% or less of the lymphocytes in thymus, bone marrow, spleen, lung, and blood. When stimulated, *i*NKT cells rapidly produce large quantities of cytokines and chemokines, which can be recognized by other cells of the immune system and enhance the function of dendritic cells, NK cells and B cells, as well as conventional CD4+ and CD8+ T cells.[7-9]

1.2 The history of NKT cells

In 1987, three independent groups reported a distinct subset of αβTCR + T cells in mice

that expressed intermediate instead of high levels of TCR.[10-12] At the same time, other groups reported a subset of αβ- TCR+ T cells that expressed NK1.1, a C-lectin type NK receptor.[13-14] Collectively, these lymphocytes were characterized in mice as cells that express both a T cell antigen receptor (TCR) and NK 1.1.

This definition was not entirely satisfactory. Further evidence showed that TCRs on these cells recognize glycolipids associated with the monomorphic MHC-like CD1d molecule. This is different from TCRs on the conventional T cells because these cells recognize small peptides bound to polymorphic MHC class I or class II molecules.[15]

Most NKT cells have an invariant Vα14-Jα18 rearrangement. It is widely accepted that mouse NKT cells are characterized by CD1d reactivity, an invariant Vα14-Jα18 TCR α-chain, a TCR β-chain biased towards Vβ8.2, Vβ2 and Vβ 7, glycolipid-antigen reactivity expression of NK-cell and memory T-cell markers, and potent cytokine-producing capacity. In humans, NKT cells are characterized by an invariant Vα24-Jα18 TCR α-chain, a Vβ11 TCR β-chain.

1.3 CD1d-glycolipid complexes

CD1 molecules are cell surface glycoproteins which have similar structure to classic MHC molecules.[16] Based on the amino acid sequence in the α1 and α2 domains, CD1 proteins are classified into two groups, group 1 (CD1a-c) and group 2 (CD1d). Although humans express all family members, mice only have CD1d genes. CD1 molecules consist of two chains: β_2-microglobulin (β_2M) light chain, a component of MHC type-I protein and a heavy chain containing three extracellular domains (α1-α3) (**Figure 1.1**). The hydrophobic binding site in CD1 forms channels (CD1b) or pockets (mouse CD1d) which can

accommodate hydrocarbon chains of lipids.[17-18] The complementarity determining residues (CDRs), loops of the TCR that contact with the CD1d-glycolipid complex, are responsible for antigen binding. There is a narrow opening between the α-helices which allows the polar moieties of the lipid exposed at the CD1d surface for recognition by CDRS of the TCRs.

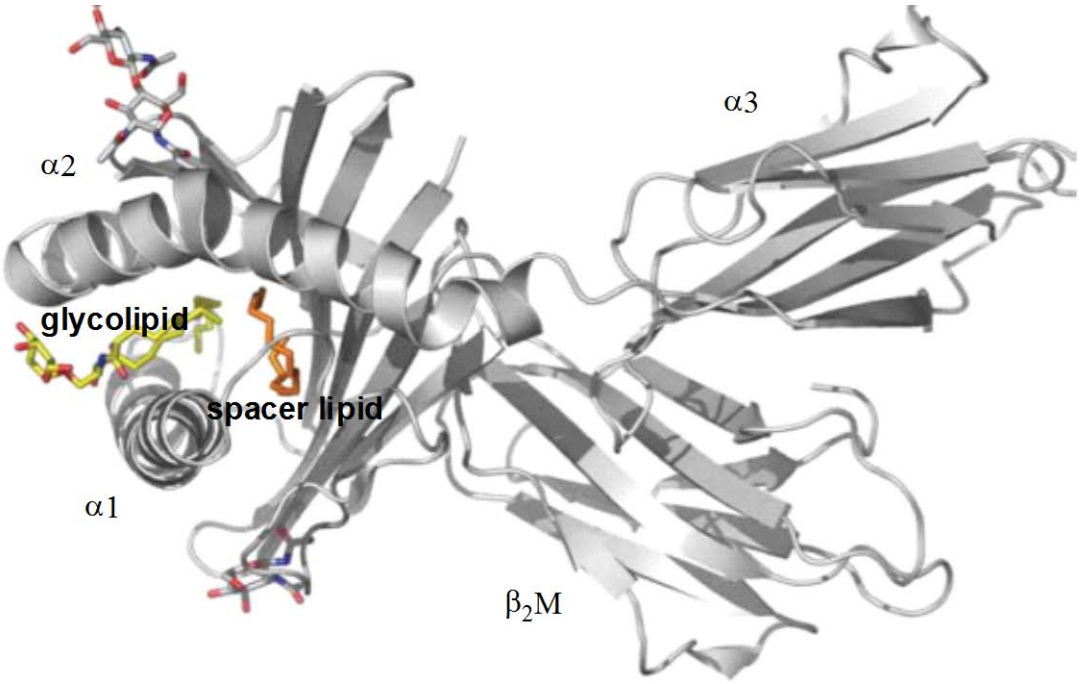

Figure 1.1. Glycolipid-mouse CD1d complex. Ribbon represent the protein backbone of a mouse CD1d.19 Reproduced from ref. 19. Copyright 2005 Nature Immunology Publishing Group.)

Although the existence of CD1 molecules was reported in 1979, it was only during the past ten years that an interest in the function of CD1 proteins has emerged.[20-21] Unlike the evolutionarily MHC class I and II molecules, which present peptide antigens, CD1 molecules whose groove is highly hydrophobic present lipid antigens to T cells. CD1 molecules can present a variety of both exogenous (e.g., mycobacterial lipid antigens) and endogenous lipid and glycolipid antigens to T cells. CD1 group 1 molecules present lipid antigens, such as mycobacterial cell wall components, to CD1-specific T cells, whereas CD1 group 2 molecules present lipids or glycolipids to NKT cells.

When activated by CD1d restricted antigens, NKT cells can influence and regulate a wide range of immune responses and pathogenic processes. NKT cells can rapidly produce large amounts of cytokines and chemokines in one to two hours, including immunomodulatory T helper cell type 2 (Th2) and inflammatory (Th1) cytokines. (**Figure 1.2**) Th2 cytokines include interleukin-4 (IL-4) and IL-13, Th1 cytokines include interferon-γ (IFN-γ) and IL-2.[22]

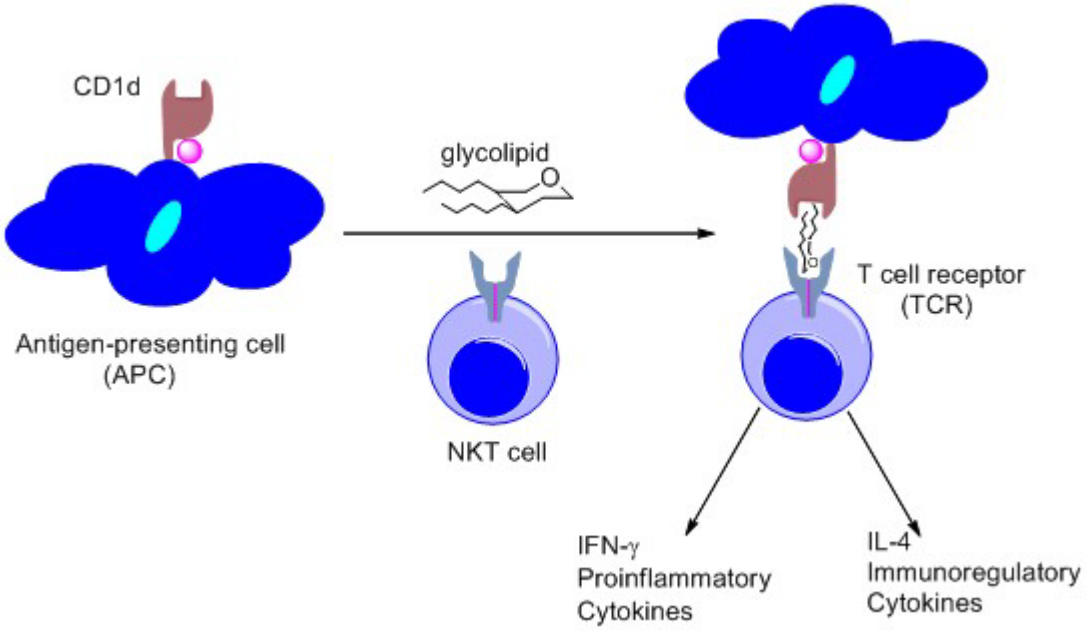

Figure 1.2. NKT cells stimulated by CD1d-glycolipid complex.

Responses induced by Th1 cytokines are effective in regulating bacterial, parasitic and viral infections. But Th1 responses are also involved in some autoimmune diseases, such as type 1 diabetes, lupus, rheumatoid arthritis, and allergen-induced asthma.[23-24] Th2 cytokines can attenuate proinflammatory Th1 responses and therefore prevent some autoimmune diseases. A confusing aspect of NKT cells responses is that both Th1 and Th2 cytokines can be produced upon stimulation.[25] This phenomenon can be seen in experimental autoimmune encephalomyelitis (EAE), a Th1 cell-mediated autoimmune disease.[26] When Th2 cytokines are inhibited in mice with EAE, the disease is enhanced. Conversely, when Th1 cytokines are

prevented from being produced, the disease is ameliorated. If the production of both cytokines is unrestricted, Th1 and Th2 responses often offset each other. It is reasonable for a given compound to induce a Th1 or Th2 bias for the treatment of a disease. For treating infection and tumors, a Th1 response is useful, and a Th2 response is desirable to prevent the autoimmune diseases.

1.4 Glycolipids as NKT cells antigens.

NKT cells can be stimulated by various kinds of glycolipids derived from not only mammals but also from bacteria or marine organisms.

1.4.1 Exogenous antigen: KRN7000

In 1995, agelasphins, were isolated from an extract of a marine sponge, *Agelas mauritianu*.[27-28] Koezuka *et al.* at the Pharmaceutical Research Laboratory of Kirin Brewery found that these glycolipids had antitumor activity through stimulation of the immune system (**Figure 1.3**).[29]

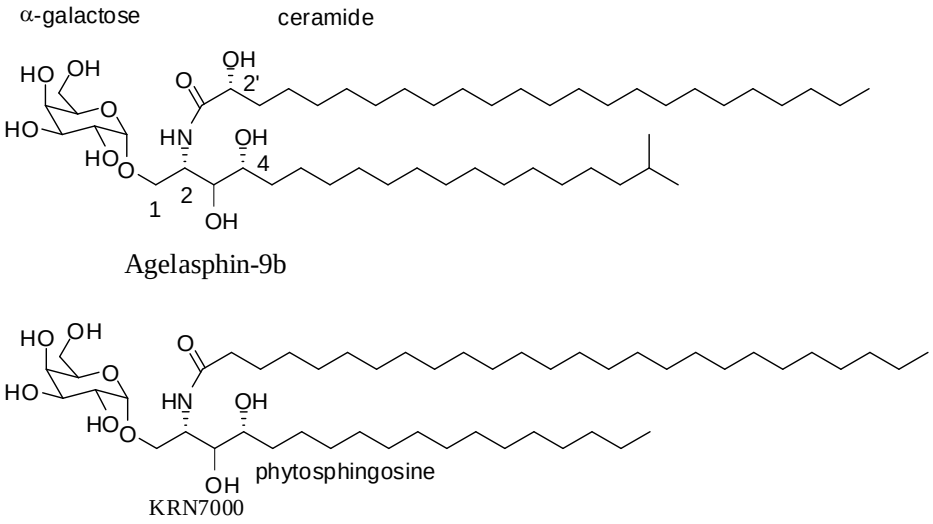

Figure 1.3. Structure of Agelasphin-9b isolated from *Agelas mauritianus* and the structure of KRN7000.

These glycolipids are typically composed of two parts: a sugar head and a ceramide part which consists of a fatty acid and sphingoid chain. A glycosidic bond is formed between the hemiacetal group of a saccharide and the primary hydroxyl group of the sphingoid. The glycosidic bond of glycolipids in higher organisms is typically β. In mammals, the sphingoid is unsaturated and is called sphingosine. This made the discovery of agelasphins unusual, because these agelasphins most had α-glycosidic linkages and saturated sphingoids known as phytosphingosine (**Figure1.3**).[27-29]

Structure-activity studies on the antitumor activity of the agelasphins led to the development of KRN7000, a synthetic variant of agelasphin. The focus was on the effect of structural differences on the antitumor activity.[30] It seems that ceramide part plays an important role and the structure of phytosphingosine is optimized. The studies showed that the C2' hydroxyl group was not required for the compound to be functional, while the C3 hydroxyl group is the necessary component (**Figure 1.3**). Changing the branched phytosphingine chain to straight chain was acceptable. All these modification led to the development of KRN7000. Treatment of NKT cells with KRN7000 can significantly inhibit tumor growth in the mouse liver, inhibit disease in diabetes-prone mice and demonstrate strong antimalaria activity.[31-34] It was the principal glycolipid used in the study of NKT cell stimulation.

1.4.2 Antitumor effects of KRN7000 in mice with hepatic metastases of Colon26 Cells

KRN7000 has significant antitumor activity in mice with hepatic metastasis of Colon26 tumors.[31] Nakagawa *et al.* examined the antitumor activity of KRN7000 in mice with

Colon26 liver metastasis. When treated with a broad range dose of KRN7000, the mice's liver weight did not increase. The cure rate among these animals was greater than 60%. In contrast, the increased rate of control mice liver reached 3.5-fold that of normal mice after 3 days and all these animals died after 40 days. These results suggest that KRN7000 has the ability to inhibit the development of hepatic metastasis of melanomas.

1.4.3 Anti-malaria activity mediated by KRN7000 in the development of murine malaria

Administration of a single relatively small dose of KRN7000 strongly inhibited development of the liver stages of the rodent malaria parasites *Plasmodium yoelii* and *Plasmodium berghei*.[34] 20-30% of mice lymphoid cells consist of NKT cells. After being introduced into mammalian hosts by mosquito bites, malaria parasites immediately invade hepatocytes. The study of Gloria *et al.* presents clear evidence of a protective role of activated NKT cells against murine malaria. Although the precise mechanism of the antimalaria effect of KRN7000 remains unclear, IFN-γ is essential for protection. KRN7000, when bound to CD1d, stimulates rapid IFN-γ cytokine production.

1.4.4 KRN7000 inhibits allergic airway inflammation

Several studies have suggested that Th1 cytokines, such as IFN-γ or IL-12, inhibit antigen-induced airway inflammation and airway hyper responsiveness (AHR), and Th2 cytokine expression in a murine model of asthma.[35-36] Matsuda et al. found that a single injection of KRN7000 almost completely abrogated an infiltrate with eosinophils in the lung and reduced AHR.[37] This inhibition of allergic inflammation was accompanied by a

significant decrease in the levels of IL-4, IL-5, and IL-13 in bronchoalveolar lavage fluid. These results suggested that KRN7000 prevented AHR and allergic airway inflammation possibly by INF-γ production. Their work suggested the therapeutic possibility of KRN7000 in the treatment of asthma.

1.4.5 Analogs of KRN7000 as antigens for CD1d-restricted NKT cells.

In order to further understand the mechanism of CD1d-restricted T cell activation, investigators have synthesized a number of glycolipids and tested them for NKT cell activation (Figure 3-6). [38-39] These included glycolipids of bacterial origin, variations of sulfatide, KRN7000 analogs modified on the acyl group, and the C-glycoside analogue of KRN7000.

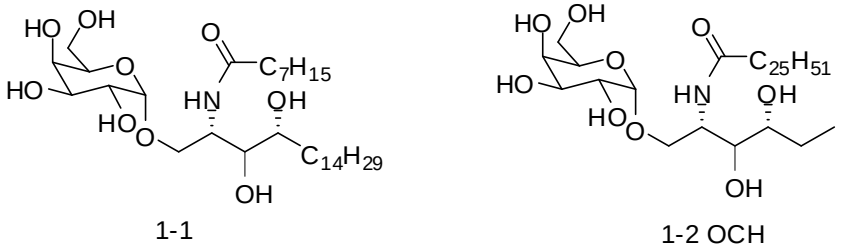

Figure 1.4. Structures of KRN7000 analogs.

These studies reveal that truncation of the fatty acyl group or the phytosphingosine group allows for more selective Th2 response.[38] (**Figure 1.4**) OCH, a phytosphingosine truncated analogue of KRN7000, selectively induces Th2 cytokines.[39-40] OCH, due to its short lipid chain, was less stable when binding to the CD1d molecule compared with KRN7000 and exerted short-lived stimulation of NKT cells. IFN-γ production by NKT cells required longer TCR stimulation than was required for IL-4 production. Taken together, these results suggest that sustained TCR stimulation leads to the production of IFN-γ, whereas short-term

activation results in preferential production of IL-4 by NKT cells.

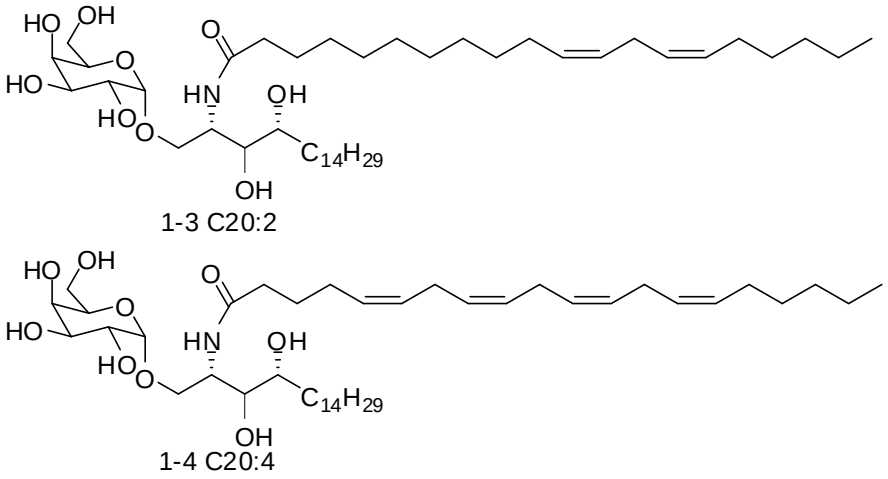

Figure 1.5. Structures of KRN7000 analogs with unsaturated fatty acid chain.

The introduction of double bonds into the fatty acyl chain modifies the outcome of Vα14*i* NKT cell activation. Analogues C20:2 and C20:4 seemed to bias Th2 responses, with diminished IFN-γ production and reduced Vα14*i* NKT cell expansion (**Figure 1.5**).[41]

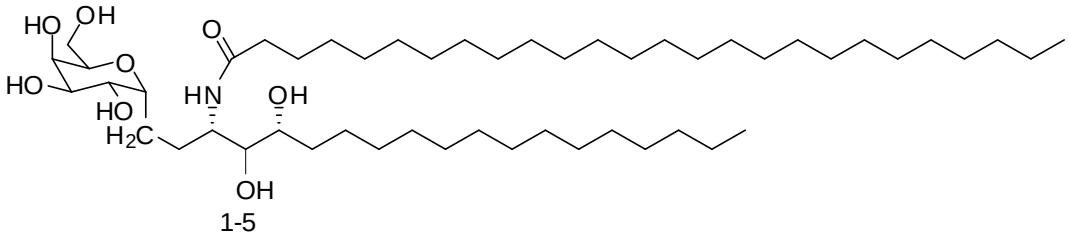

Figure 1.6. Structure of C-glycoside analogue of KRN7000

The C-glycoside analogue of KRN7000 seems to be biased toward Th1 responses (**Figure 1.6**).[42-43] Schmieg *et al.* reported that a synthetic C-glycoside analogue of KRN7000, α-C-Galcer, stimulated an enhanced Th1-type response in mice. The difference comes from the replacement of O, a polar hydrogen bond acceptor, with CH_2, a nonpolar group. The CH_2 can not participate in hydrogen bonds. This change in hydrogen bonding may cause an altered structure of the CD1d-α-C-GalCer complex and a change in its affinity with the NKT cell's TCR compared with KRN7000. Such altered binding would likely result in

differential recognition of the CD1d-α-C-GalCer complex by the TCR of Vα14 NKT cells and qualitatively different signal transduction events in both NKT cells and DCs. Such differential signal transduction would result in the altered response characterized by low IL-4 and prolonged IL-12 and IFN-γ.

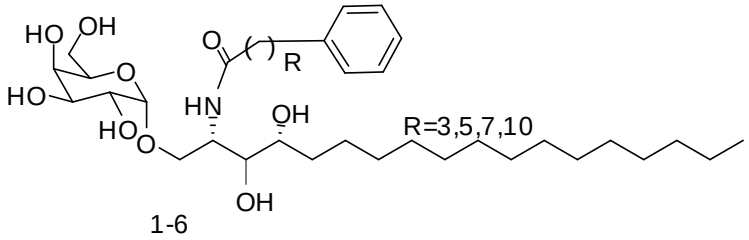

Figure 1.7. Structures of KRN7000 analogs with an aromatic group chain

The introduction of an aromatic group to the fatty acyl chain greatly enhanced IFN-γ/IL-4 secretion (**Figure1.7**).[44] Fujio et al. designed and synthesized various fatty acyl chain analogues. Of these compounds, the longer alkyl chain analogues are 4 times more potent and biased for IFN-γ secretion. These results show that it is possible to tune the Th1/Th2 cytokine ratio by the introduction of a terminal aromatic group.

1.4.6 Natural endogenous antigens

The first glycolipid recognized by NKT cells as an antigen was extracted from marine sponges. It is unlikely that these glycolipids are natural antigens for NKT cells, because it is not likely that mice and humans have T-cell populations selected for their recognition of marine sponge antigens. A key question is whether there exist equivalent mammalian glycolipid antigens, and if they exist, whether they are involved in NKT cell stimulation.

Without successful recognition of the endogenous antigen, the NKT cells would undergo apoptosis. Therefore, the way to determine if the natural antigen is present is to observe the

presence or absence of NKT cells. Joyce *et al.* found that a cell line lacking the enzyme β-D-glucosylceramide synthase, an enzyme involved in the production of β-glucosylceramide, can not stimulate NKT cells (**Figure 1.8**).[45] But mice lacking β-glucosylceramide could produce NKT cells in a manner comparable with the wild type mice. These results suggest that the endogenous antigen might be a derivative of β-glucosylceramide.

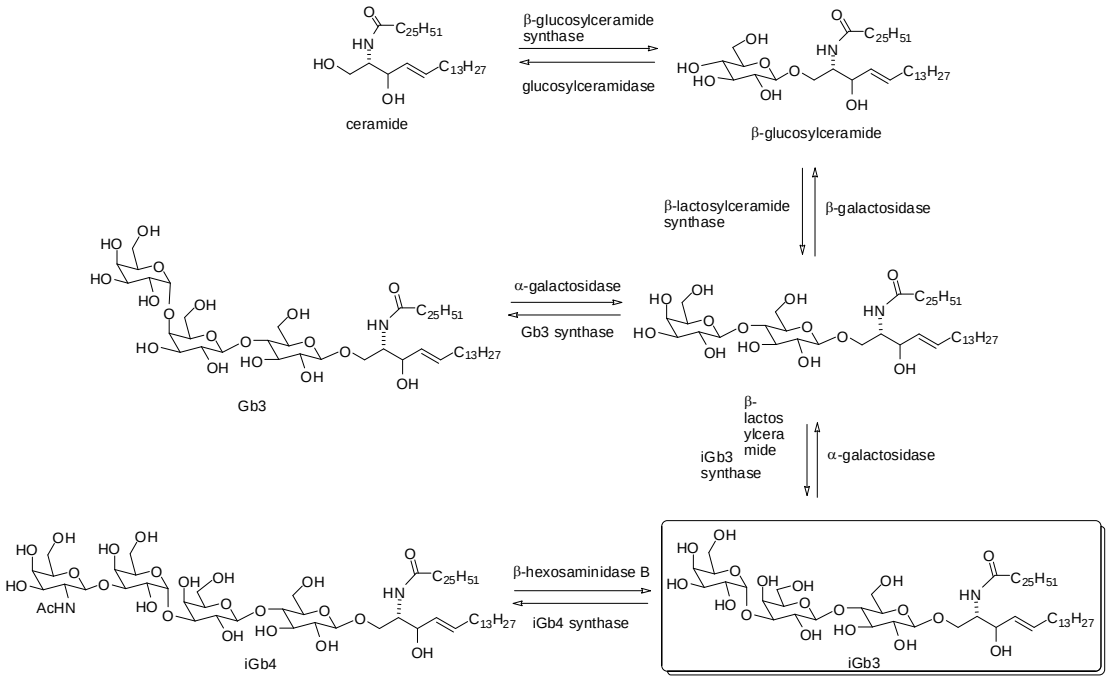

Figure 1.8. Glycolipid processing pathways associated with iGb4.[45-46]

Zhou *et al.* reported that mice deficient in β-hexosaminidase (Hexb) B, a lysosomal glycosphingolipid degrading enzyme, showed a severe reduction in the number of Vα-14 NKT cells.[46] β-Hexb cleaves N-acetylgalactosamine (GalNAc) groups from the glycolipids and GalNac is found in many endogenous glycolipids, such as globotrihexosylceramide Gb4 and isoglobotrihexosylceramide iGb4 (**Figure 1.8**). After testing the abilities to stimulate NKT cells, only iGb3 and iGb4 can stimulate NKT cells, both Gb3 and β-glucosylceramide caused no stimulation. But iGb4 can not stimulate NKT cells without the presence of β-Hexb, indicating that removing of the GalNAc group must occur to stimulate NKT cells. These

results suggested that iGb3 might be the endogenous antigen. In spite of the identification of iGb3 as an endogenous antigen for NKT cells, the information about iGb3-CD1d-TCR interactions remains unclear and whether iGb3 is unique or just one of many other ligands that activate NKT cells is the next project to be studied.

1.4.7 Natural exogenous antigens

The ability of a multi-cellular organism to defend itself against invasion by pathogen, such as bacteria, fungi, viruses, etc., depends on its mounting immune responses. All mammals have inborn defense mechanisms called innate immunity. The lipopolysaccharides (LPS) are the main outer surface membrane components present in most Gram-negative bacteria. LPS can not be produced by human cells and act as stimulators of innate immunity. However, there are still many Gram-negative bacteria that do not have LPS. The problem is how innate immune system detects this kind of bacteria.

Both Zahringer *et al.* and Kawasaki *et al.* studied the cell wall components of these Gram-negative, non-LPS-producing bacteria and explored the mechanism of innate immunity against these bacteria. Their work led to the discovery of another natural source of glycolipids, the *Sphingomonadaceae* family of non-LPS-producing Gram-negative α-aproteobacteria.[47-50] Zahringer *et al.* reported two novel glycosphingolipids isolated from *Sphingomonas paucimobilis*, termed GSL-1 and GSL-4A.[47, 50] The glycosidic bonds of these bacterial glycolipids are α-anomers and they have C_{18} sphinganine or C_{21} sphinganine with a *cis* double bond or cyclopropyl group instead of phytospingsine chains. In addition, the carbohydrate moieties contain either glucuronic or galacturonic aicds. The relative

stereochemistry of the sphinganine portion of GSLs was shown in initial reports as *erythro* but the absolute configuration was not established. Generally speaking, sphingosines and sphinganines isolated from biological sources have D-*erythro* configurations (derived from L-serine), the stereochemistry of GSLs was as shown in **Figure 1.9**.

The ability of these glycolipids to stimulate NKT cells was tested. Both CD1d-deficient mice and NKT-deficient mice have a higher bacterial load than wild-type mice after exposure to *Sphingomonas*.[51] These results show that NKT cells can provide protection from certain bacteria by recognizing glycolipid antigens.

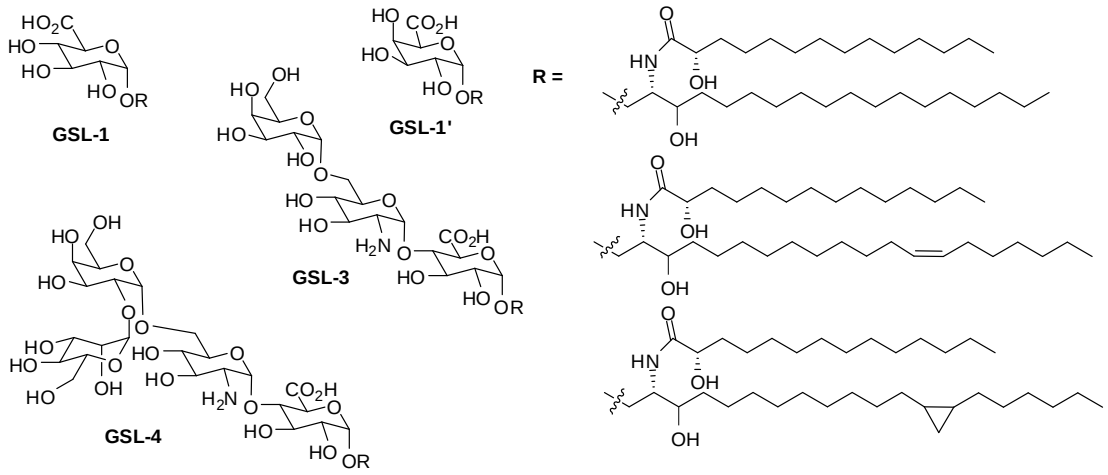

Figure 1.9. Structure of glycolipids isolated from the *Sphingomonadacaea* family.

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CHAPTER 2.

PREPARATION OF DIGLYCOSYLCERAMIDES AND EVALUATION OF THEIR NKT CELL STIMULATORY PROPERTIES

2.1 Introduction

NKT cell responses to glycolipids are mediated by CD1d, which acts as lipid-binding and presenting molecules. CD1d is expressed on the surface of antigen-presenting cells (APCs), such as dendritic cell, B cells and macrophages. CD1d has hydrophobic pockets which can accommodate lipid chains up to 30 carbons.[1] This binding of glycolipids with CD1d results in a complex in which the lipid chains are buried in the binding pockets while the carbohydrate epitopes of glycolipids protrude into the solvent and can be recognized by the TCRs of NKT cells. The glycolipids with relatively simple structures can be envisioned to fit comfortably into the space between the CD1d and TCRs of NKT cells.

But how are glycolipids with complicated oligosaccharide head-groups presented by CD1d and stimulate NKT cells? Processing is an important aspect of glycolipid presentation by CD1d, which can occur in the lysosomes of APCs.[2] Glycolipids are usually trafficked to lysosomes in APCs, and exposed to a collection of glycosidases, that can truncate oligoglycosylceramides to monoglycosylceramides. Lysosomes are also the place where CD1d is loaded with glycolipids, and the cell surface is the place where CD1d presents glycolipids to TCRs of NKT cells. The processed glycolipids are transported to the surface of the APCs with the help of CD1d. CD1d is cycled between lysosomes and the cell surface. Tail-deleted CD1d (CD1d/TD) lacking the peptide sequence required for routing to lysosome

can not effectively cycle between the cell surface and lysosomes. Most CD1d/TD molecules accumulate on the cell surface.[3] The processed glycolipids in the lysosomes can not be loaded into the CD1d/TD. Consequently, CD1d/TD is useful to observe the influence of lysosomal glycolipid processing on stimulation of NKT cells.

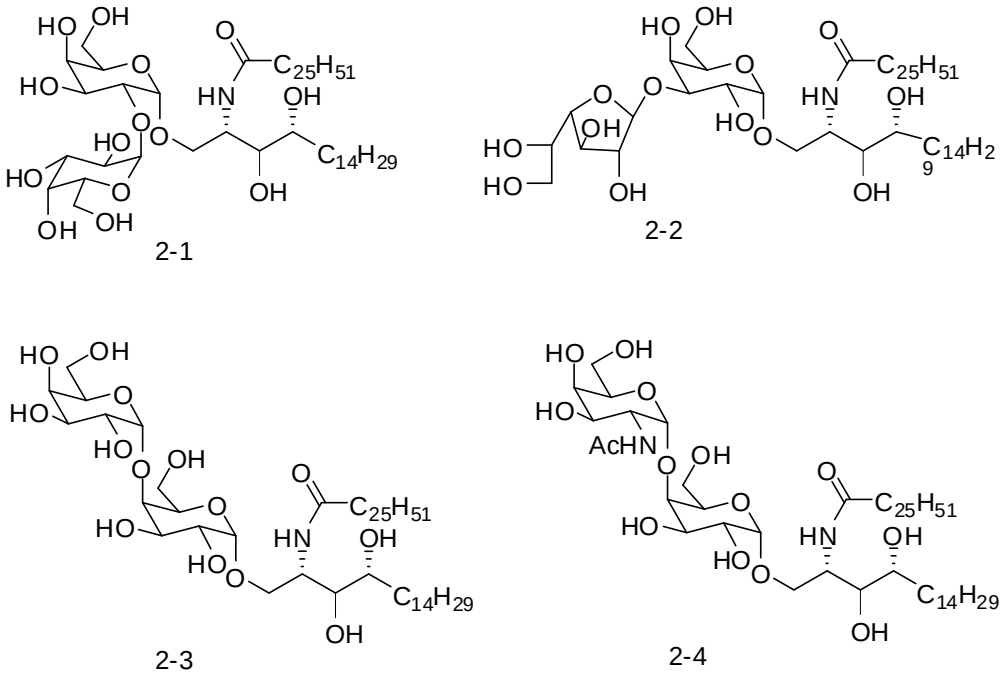


Figure 2.1. Structures of glycolipids **2-1, -2-4.**

The work of Prigozy *et al.* provides a model system in which enzymatic processing is required for some complicated glycolipids recognized by TCRs of NKT cells.[4] Prigozy *et al.* compared KRN7000, which does not need processing to be recognized with the analogs of KRN7000 containing a second sugar. Their work showed that disaccharide **2-1** (**Figure 2.1**) was not antigenic for NKT cell hybridomas when incubated with CD1d/TD. But dissacharide **2-1** can be presented by wild-type CD1d and stimulate NKT cells. A lysosomal hydrolase, α-galactosidase A, removes the terminal galactose residue of dissacharide **2-1**, and converts it to the corresponding KRN7000, which is recognizable by TCRs.[4] Prigozy *et al.* showed

another dissacharide **2-2** with a similar processing requirement. Zhou *et al.* also reported that dissacharides **2-3** and **2-4** must be processed to KRN7000 to stimulate NKT cells (**Figure 2.2**).

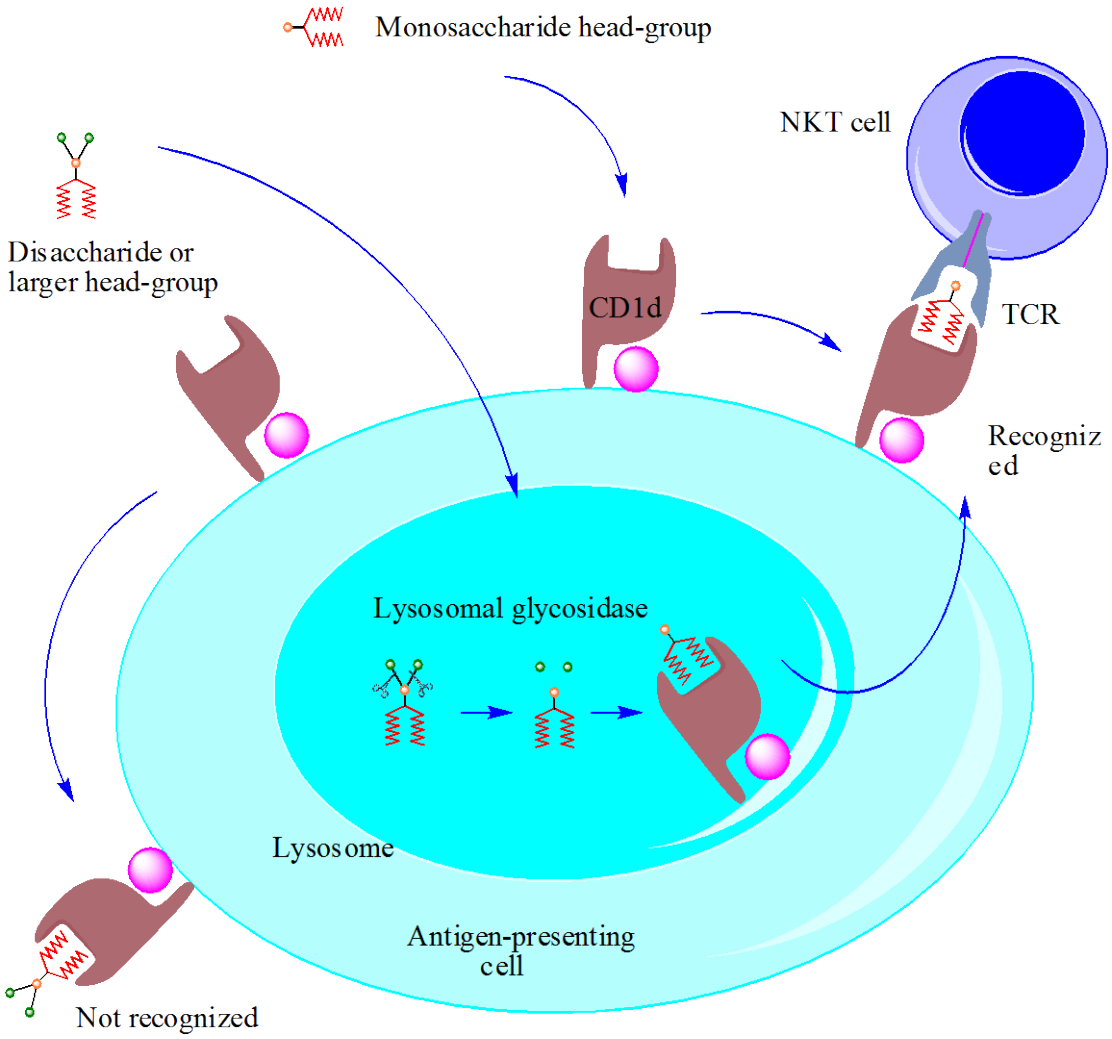


Figure 2.2. Lysosomal processing is required for glycolipids recognized by TCRs.

In the case of glycolipids with small molecules appended at the C6" position, it is clear that they do not significantly change stimulation of NKT cells.[5] However, the influence of C6" substitution on lysosome processing needs to be determined. Based on this idea, we designed and synthesized a series of diglycosylceramides with and without substitution on the C6" position of the galactose (**Figure 2.3**).

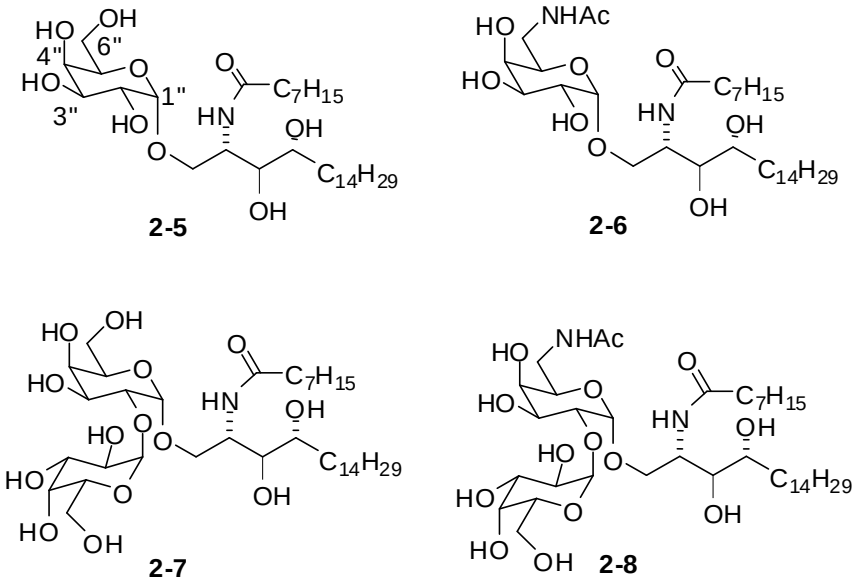


Figure 2.3. Structures of glycolipids used in the study of lysosomal processing.

The solubility of most glycolipids presented by CD1d and recognized by NKT cells is very low. Consequently, the presentation of glycolipids largely depends on a series of lipid transfer proteins (LTPs).[6] Zajonc *et al.* compared the interaction of **2-5** and KRN7000 with CD1d.[7] Compound **2-5** is loaded into CD1d much more rapidly than KRN7000. Adding lipid-transfer proteins, such as mouse saposin or mouse GM2 activator substantially improved the loading of KRN7000 to CD1d but did not influence the kinetic activity of **2-5**. The reason can be explained by the crystal structure of the CD1d-**2-5** complex.

As was mentioned in Chapter 1, the basic structure of CD1d is composed of the three heavy chain domains (α1-α3) and β_2-microglobulin. A narrow deep binding groove forms between the α1, α2 helices and the β-strands. The hydrophobic binding groove is divided into two pockets, A' and F'. The acyl chain of **2-5** is inserted into the A' pocket and the phytosphingosine into the F' pocket. The galactose domain of **2-5** lies at the boundary between the A' and F' pockets. Both chains are oriented perpendicular to the β-sheet platform. The acyl chains are stabilized by Van der Waals interactions between the aromatic

residues of the pockets (e.g., Tyr73 of A' pocket, Phe77 and Trp133 of F' pocket) and the glycolipids (**Figure 2.4**).

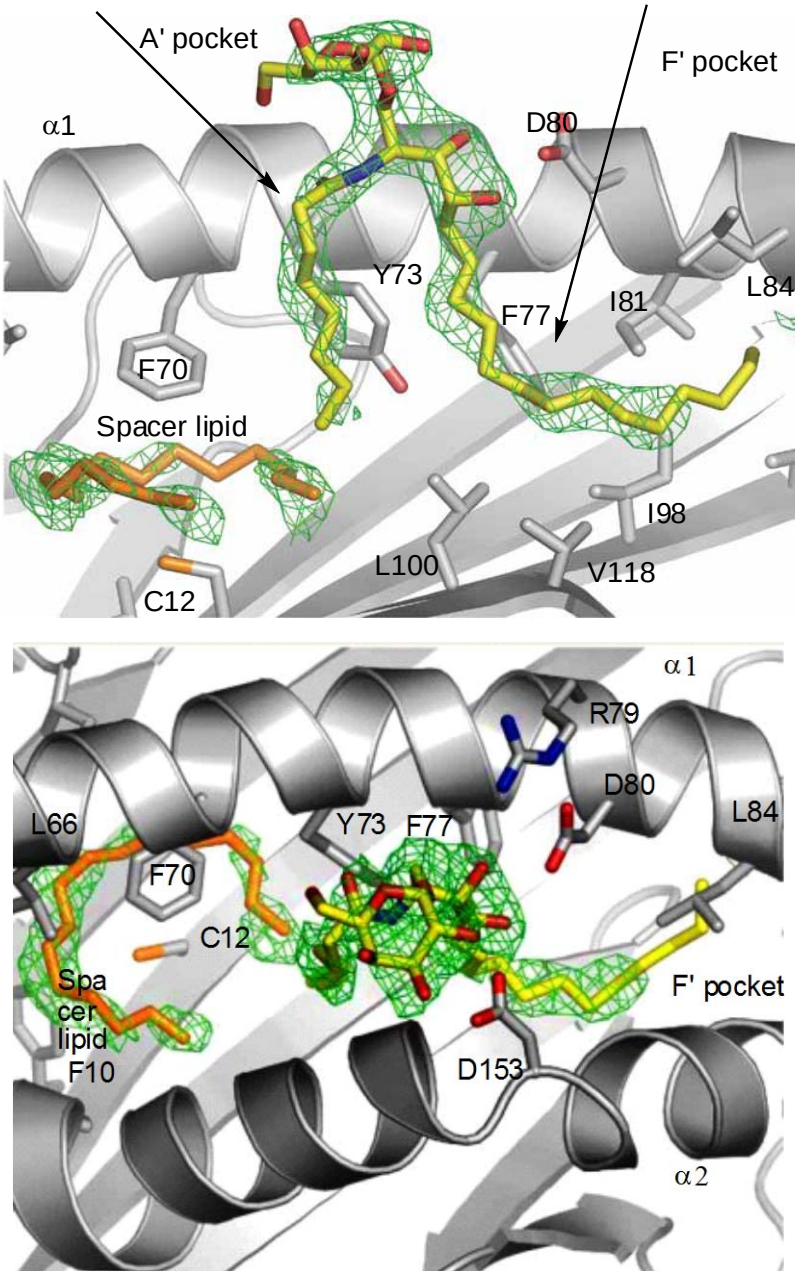


Figure 2.4. Structure of **2-5** in the mouse CD1d. (Upper one is the side view, lower one is the Top view. Reproduced from ref. 7. Copyright 2005 Nature Immunology Publishing Group.)

The phytosphingosine domain fully occupies the F' pocket, which is able to accommodate a maximum chain length of 18 carbons.[8] Although the fatty acid chain terminates at the entrance of the A' pocket, a linear hydrophobic compound (**Figure 2.4**)

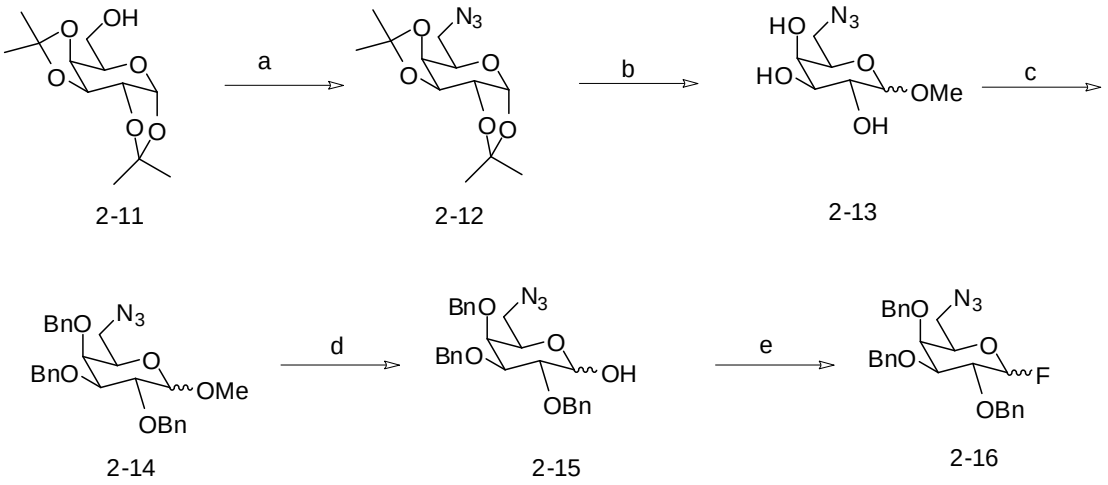
exists in the channel, which acts as a spacer lipid to stabilize the hydrophobic binding pockets in the absence of a longer fatty acid chain, such as in KRN7000.[7] The short fatty acid chain can bind CD1d immediately, while KRN7000 requires loss of spacer lipid and makes loading more dependent on LTPs. So in this paper, we used the C8 fatty acid chain instead of C26 chain of KRN7000 to remove the possible influences of LTPs and focus on activities of lysosomal glycosidases.[6, 9]

The compounds we prepared are shown in Figure **2.3**. In the earlier studies, we have already known the importance of processing the disaccharide into the corresponding monosaccharide in the stimulating NKT cells. It would be expected that the short fatty acid chain analogs of disaccharide **2-7** have the same effect. We can compare the stimulation difference between compounds **2-5** and **2-7** by wild-type CD1d and CD1d/TD. Monosaccharide **2-5** would be expected to bind the CD1d directly and stimulate NKT cells comparably with wild-type CD1d and CD1d/TD. The dissacharide **2-7**, which would need processing to the monosaccharide prior to stimulate NKT cells, would stimulate NKT cells less than **2-5** by using CD1d/TD. If the compound **2-8** does not significantly influence lysosomal processing, the same trend should be observed. Compoud **2-8** should lose stimulatory properties with CD1d/TD.

2.2 Results and discussion.

The synthesis galactosyl donor **2-16** was prepared according to a published method

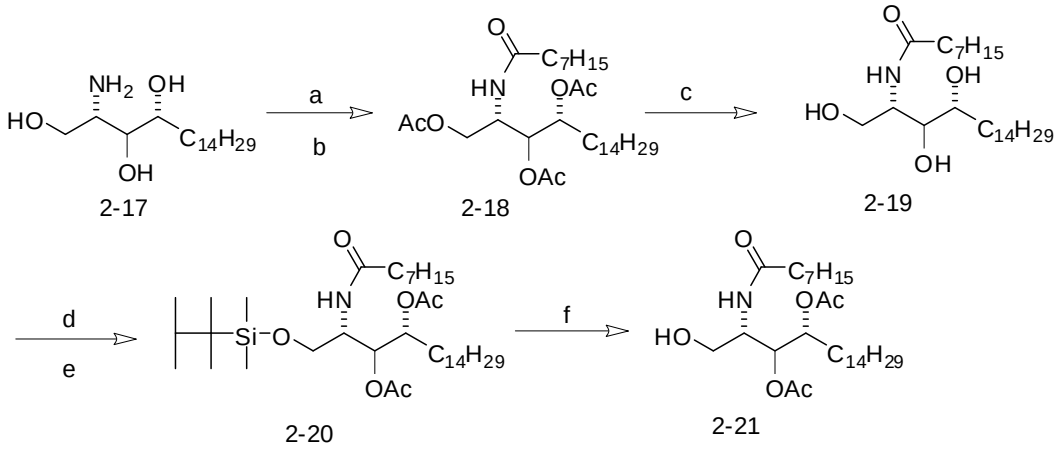
(**Scheme 2.1**).[5] The first step was to incorporate an azide at C6" position to substitute the –OH group using the Mitsunobu reaction.[10] The actetonides were hydrolyzed with concomitant methylgalactosid formation. The following steps were to form benzyl esters at C2", C3" and C4". The fluoride donor **2-16** was synthesized by treating **2-15** with DAST for half an hour.[11]



Reagents: (a) PPh_3, DPPA, DIAD(79% yield); (b) AcCl, MeOH (81% yield); (c) BnBr, NaH, DMF (47% yield); (d) AcOH, HCl (69% yield); (e) DAST, CH_2Cl_2(87% yield)

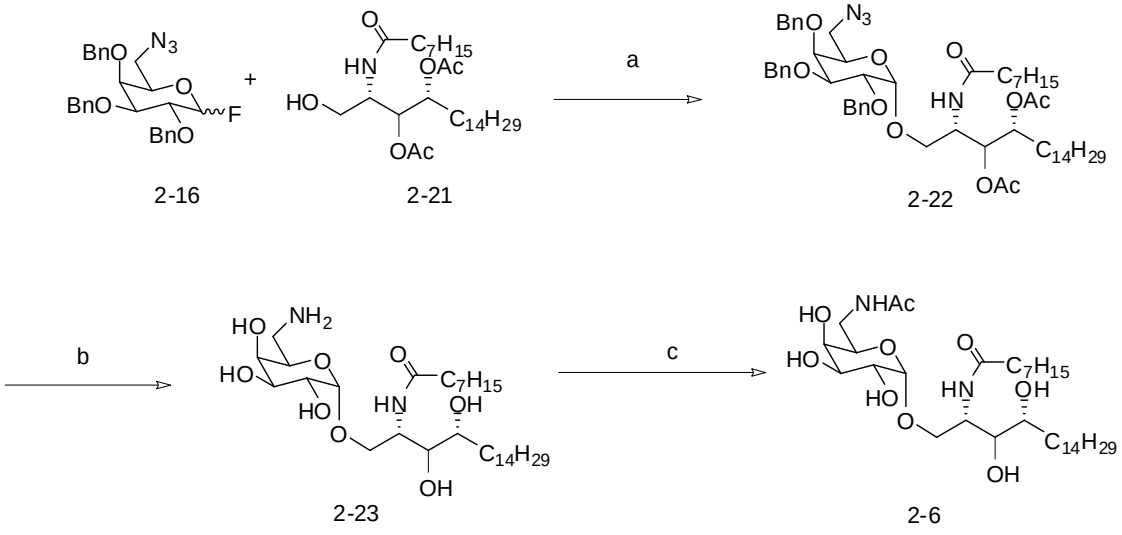
Scheme 2.1. Synthesis of galactose donor **2-16**.

Phytosphingosine **2-17** was coupled with octanoic acid using HOBt/DCC conditions (**Scheme 2.2**). These unprotected ceramides were hard to isolate due to their amphiphilic nature, which could not be purified without triacetylation (**2-18**). Peracetylation of phytosphingosine proceeded at a much higher yield. After purification, the peracetyl ceramides completed deacetylation with sodium methoxide in methanol. We selectively silylated the primary alcohol with dimethylthexylsilyl chloride (TDSCl) in pyridine, then diacetylated the secondary alcohols with Ac_2O and DMAP. Removal of the silyl ether was rapidly achieved in aqueous HF/THF solution.



Scheme 2.2. Synthesis of Ceramide **2-21.**

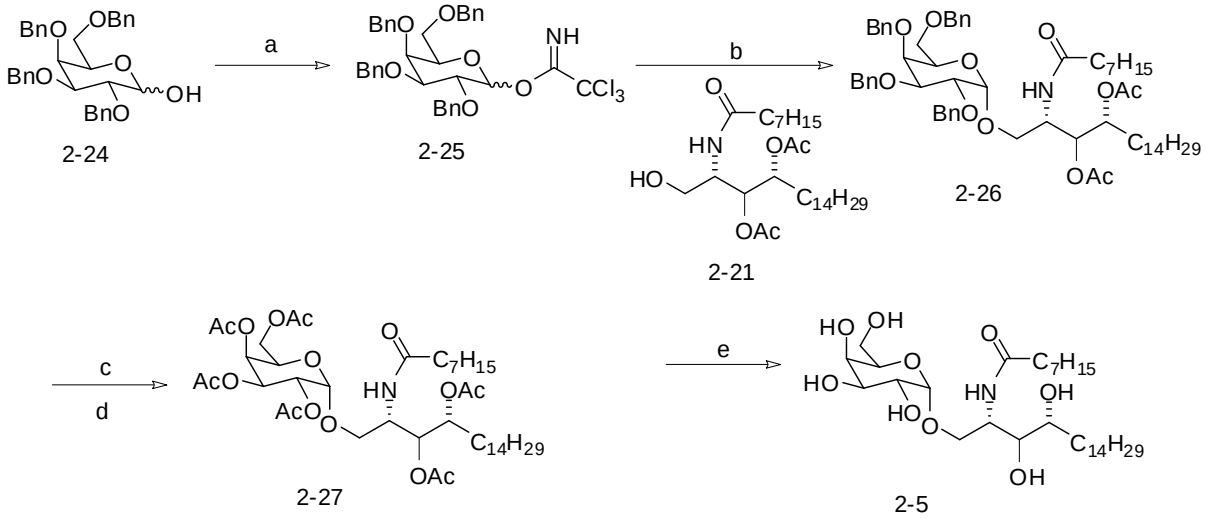
We must be careful at the workup and purification steps, because it was found that migration of the acetyl group occurred when either the acid was not fully neutralized by aqueous $NaHCO_3$ or it took too much time on the column.



Scheme 2.3. Synthesis of 2-6.

The glycolipid **2-22** was synthesized through the method provided by Mukaiyama ($AgClO_4$, $SnCl_2$, 4Å molecular sieves, anhydrous CH_2Cl_2).[12] The yield was not very satisfing (54%), while the published yields in Mukaiyama's group can reach 91% (**Scheme 2.3**). The difference may be caused by the azide functional group of galactose donors. The major

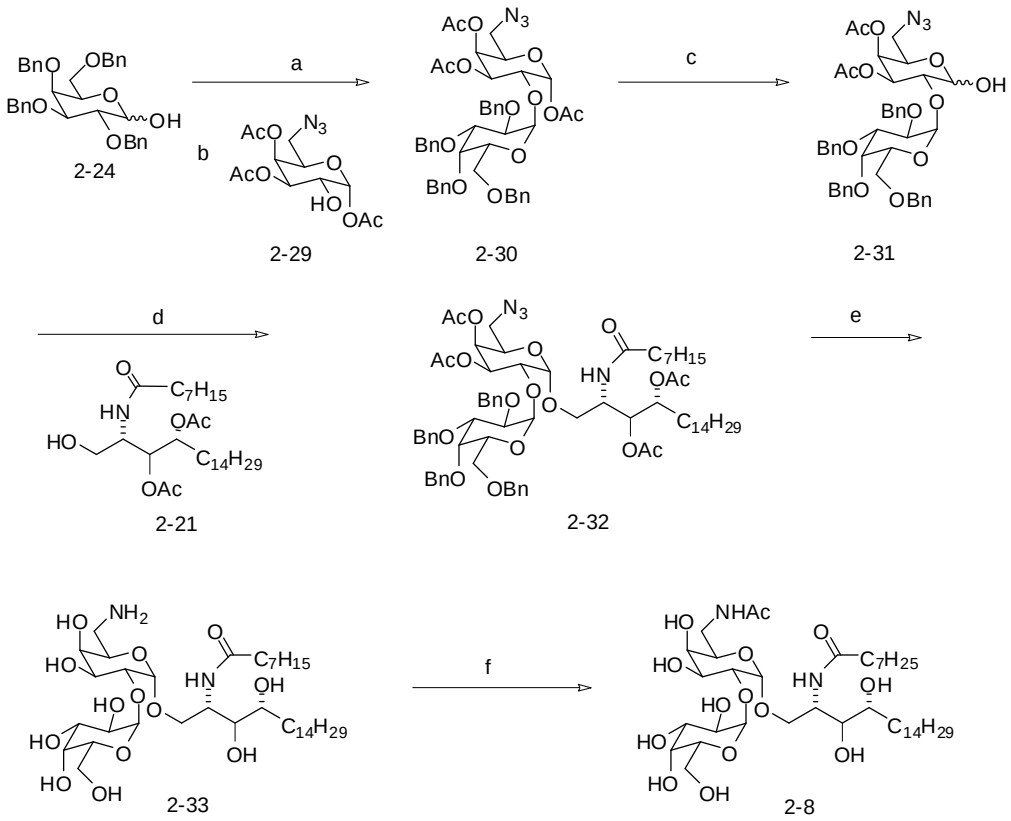
product of this coupling reaction was the α-anomer, followed by reduction of the azide and removal of the protecting group giving **2-23.**[13] Reaction of **2-23** with acetic acid which was activated by N-Hydroxybenzotrizole (HOBt) and N,N'- Dicyclohexylcarbodimide (DCC) gave reasonable yields of the corresponding amides **2-6** (**Scheme 2.3**).



Reagents (yields in parentheses): a) K_2CO_3, trichloroacetonitrile; b) TMSOTf, DCM (37-41% for 2 steps); c) Pt/C,H_2, THF/MeOH; d) Ac_2O, Pyridine (65-71% for 2 steps); e) NaOMe, MeOH (67-71%).

Scheme 2.4. Synthesis of **2-5.**

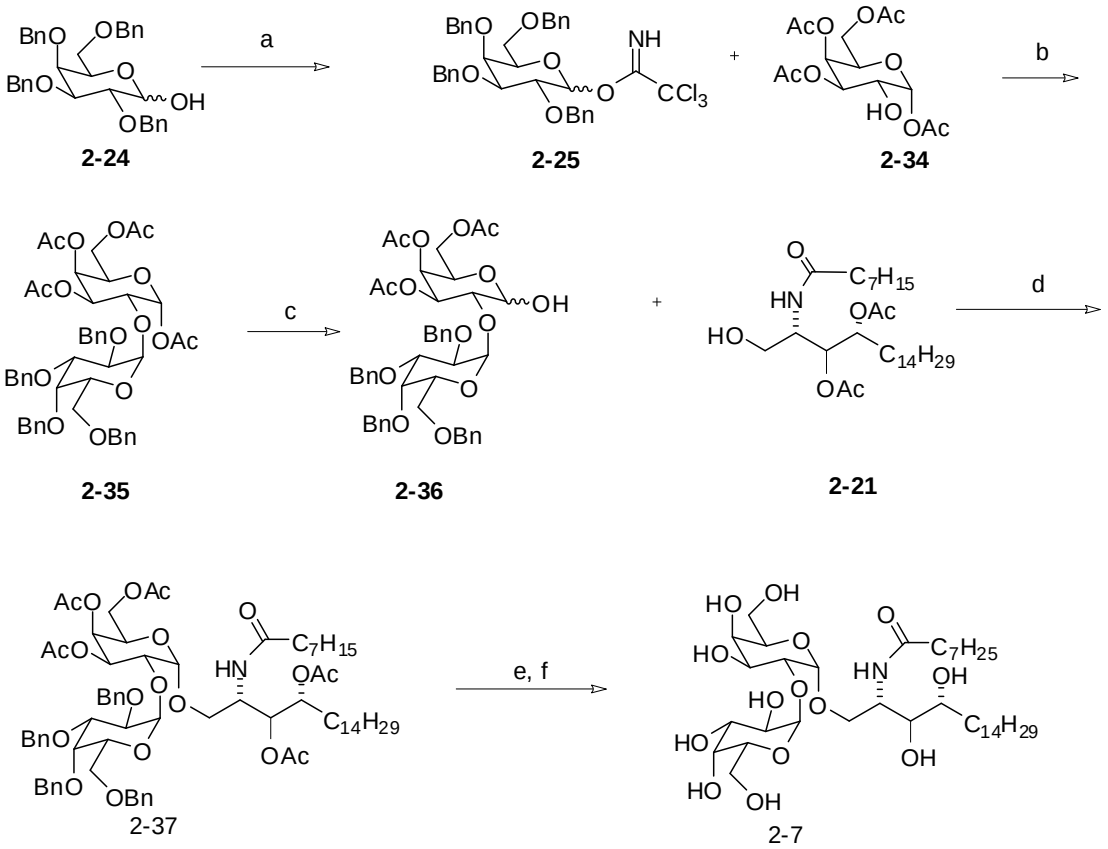
Coupling of **2-24** and **2-21** was achieved with the trichloroacetimidate method.[14] The stereochemistry of newly generated glycosidic bond in **2-26** was alpha as determined by ^{1}H and 2D COSY NMR spectra. Hydogenation of the benzyl groups followed by acylation gave **2-27**. Final compound **2-5** can be obtained in a good yield by treating **2-27** with sodium methoxide in methanol.



Reagents (yields in parentheses): a) K_2CO_3, trichloroacetonitrile; b) TMSOTf, DCM (37% for 2 steps); c) $H_2NCH_2CH_2NH_2$/AcOH (64%); d) diphenylsulfoxide, Tf_2O, tri-*t*-butylpyrimidine, (53%); e) Na/NH_3, (27%); f) AcOH, DCC, HOBt, (22%).

Scheme 2.6. Synthesis of **2-8.**

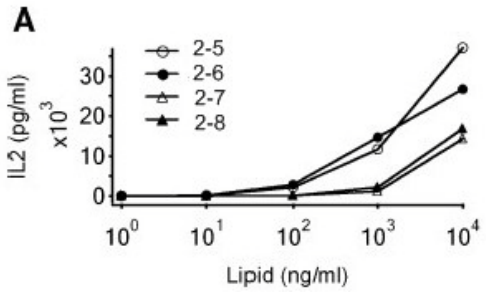
Coupling of donor **2-24** with galactose **2-29** gave predominantly the α-anomers **2-30** under -15^{o}C. Selective deprotection of anomeric ester using ethylene diamine/acetic acid afforded donor **2-31**. Glycosyl bond was formed between **2-31** and **2-21** using Ph_2SO/Tf_2O as promoter to give glycoceramide **2-32**, followed by deprotection and reduction of azide with the Sodium in liquid ammonium under -78^{o}C get the final product compound **2-8** (**Scheme 2.6**). A similar approach can be used in the synthesis of the amide compound **2-6 (Scheme 2.7)**.



Reagents (yields in parentheses): a: K_2CO_3, trichloroacetonitrile; b: TMSOTf, DCM (40% for 2 steps); c: $H_2NCH_2CH_2NH_2$/AcOH, rt, 24 h, (82%); d: diphenylsulfoxide, Tf_2O, tri-t - butylpyrimidine, (71%); e: Pd/C (10%), THF, MeOH (70%); f: MeONa, THF, MeOH (79%)

Scheme 2.7. Synthesis of **2-7.**

Synthesis of **2-37** used the same method as **2-32**. Pd/C catalyzed hydrogenation of **2-37** at high pressure (500 psi) slowly gave the corresponding tetraols with high yield, which, after routine work-up, was directly subject to deprotection by utilization of sodium methoxide, generating desired new antigen **2-7**. The structures of all synthesized compounds were confirmed by spectroscopic methods (**Scheme 2.7**).



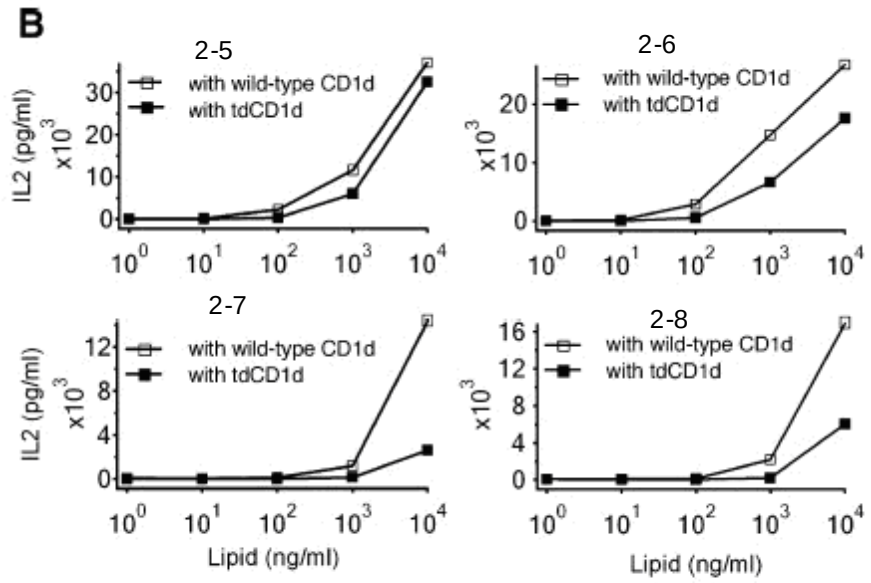


Figure 2.5. (**A**) Comparison of stimulatory activity on mouse Vα14*i* NKT hybridoma DN3.2.D3 by murine bone marrow-derived dentritic cells in the presence of different concentrations of synthesized glycoceramides. (**B**) Comparison of stimulatory activity of different antigens with wild-type CD1d and CD1d/TD.

The diagrams of both **2.5A** and **2.5B** show the mean release of IL-2 (pg/mL) in cell culture supernatants determined by ELISA. The NKT cell stimulatory properties of proposed compound were studied by NKT cell hybridoma and dendritic cells (DCs). DCs which act as APCs generate a well-established lysosome and can effectively truncate many oligosaccharides to monosaccharides. From the **Figure 2.5A**, we can see all four glycolipids stimulated NKT cells in a good manner.

To further establish the dependency of the disaccharides on lysosomal processing, dendritic cells with CD1d/TD were used as antigen-presenting cells. Because CD1d/TD can not effectively process the glycolipids in lysosomes, it was expected that stimulation with **2-7** and **2-8** would be decreased. As shown in **Figure 2.5B**, this trend was observed, while the

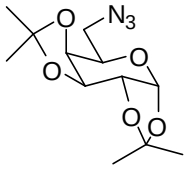
monoglycosylceramides were less dependent on CD1d.

2.3 Conclusion

Lysosomal processing and CD1d loading is very important in regulating the stimulatory properties of antigens. In the earlier studies, analogs of KRN7000 with small molecules appended on C6” position of the galactose portion can stimulate NKT cells comparably with the KRN7000. Furthermore, we want to know if the substitution at this position would influence the lysosomal processing. Two sets of mono- and disaccharides with and without substitution at C6” position were prepared and evaluated the NKT cell stimulatory properties. The substitution at the C6” position of the sugar moiety of glycolipids do not significantly impact the stimulatory properties of glycolipids and their processing in lysosome. Small changes at C6” are well tolerated. Further studies on how the C6” substitution glycolipids stimulate NKT cells are ongoing.

Experimental Procedures

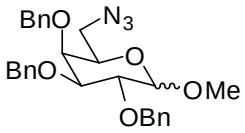
Materials and General Methods.^{1}H NMR and ^{13}C NMR, were recorded on Varian Unity 500 MHz or Varian Unity 300 MHz NMR spectrometers using 99.8% $CDCl_3$ with 0.05% v/v TMS, 99.8% CD_3OD with 0.05% v/v TMS. Mass spectrometric data were obtained on JEOL SX 102 A spectrometer or Agilent 1100 series spectrometer. Flash chromatography was performed using 230-400 mesh silica gel. Thin layer chromatography was performed on aluminumbacked, 254 nm UV-active plates with a silica gel particle size of 250 m. Chemicals were obtained from Sigma and Aldrich and were used as received unless otherwise noted.



2-12

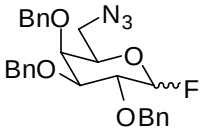
Preparation of 2-12. Compound **2-11** (3.00 g, 11.3 mmol) was dissolved in THF (20 mL), cooled to 0°C, and PPh_3 (5.95 g, 22.6 mmol) was added into the system, followed by DIAD (5 mL, 22.6mmol), then DPPA (3.7 mL, 22.6 mmol), the mixture was allowed warm to room temperature and the was stirred overnight. The mixture was concentrated under reduce pressure, dissolved in EtOAc (200 mL), washed with 5% HCl (80 mL), then washed with saturated $NaHCO_3$, the extracts were concentrated in vacuo, and the product was purified by column chromatography (SiO_2, 1:5 EtOAc: hexanes) giving a clear glass **2-12** (2.61 g, 79% yield). NMR (^{1}H, $CDCl_3$) δ5.55 (d, *J* = 5.0 Hz, 1 H), 4.63 (dd, *J* = 2.5, 8.0 Hz, 1 H), 4.34 (dd, *J* = 2.5, 5.0 Hz, 1 H), 4.20 (dd, *J* = 2.0, 8.0 Hz, 1 H), 3.93-3.90 (m, 1 H), 3.51 (dd, *J* = 9.0, 12.5 Hz, 1 H), 3.36 (dd, *J* = 5.5, 13.0 Hz, 1 H), 1.55 (s, 3 H), 1.46 (s, 3 H), 1.34 (s, 3 H); ^{13}C NMR (500 Hz, $CDCl_3$) δ109.8, 108.9, 96.5, 77.2, 77.0, 67.17, 50.8, 26.2, 26.1, 25.1, 24.6.

(can not obtain the mass data)



2-14

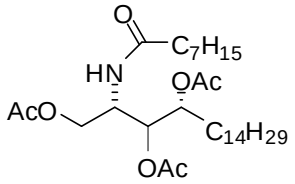
Preparation of 2-14. Compound **2-12** (3.71 g, 13.0 mmol) was dissolved in MeOH (40 mL), cooled to 0°C, and AcCl (8.6 mL) was added. The mixture was allowed warm to room temperature and was stirred overnight. The solvent was removed under reduced pressure, and the residue was purified by chromatography (SiO_2, 10% MeOH in CH_2Cl_2) yielding **2-13** as a white solid (2.31 g, 81% yield). To a solution of **2-13** (1.5 g, 6.9 mmol) in DMF (60 mL) was added sodium hydride in oil (1.26 g, 60% in mineral oil). The mixture was stirred for 5 min at 0°C, benzyl bromide (4.9 mL, 41.4 mmol) was added dropwise, the stirring was continued for 12 h at room temperature, methanol (10 mL) was added. The solvent was removed by vacuo and extracted by CH_2Cl_2. The extract was washed with 2M HCl and water, dried (Na_2SO_4) and concentrated. Column chromatography (SiO_2, EtOAc:hexanes 1:6) gave **2-14** (1.6 g, 47%). NMR (^{1}H, CDCl3) δ 7.40-7.25 (m, 15 H), 5.02 -4.62 (m, 7 H), 4.14 -3.76 (m, 4 H), 3.57-3.48 (m, 1 H), 3.39 (s, 3 H), 2.94 (dd, *J* = 2.4, 4.4 Hz, 1 H); NMR (^{13}C, CDCl3) δ 138.65, 138.58, 138.34, 128.68, 128.61, 128.32, 128.12, 128.01, 127.87, 127.78, 99.01, 79.16, 76.48, 75.45, 74.81, 73.89, 69.98, 55.71, 51.64; HRFAB-MS (thioglycerol + H^+ matrix) *m/e* ($[M + H]^+$) 490.2347(3.6%), calcd 490.2342.



2-16

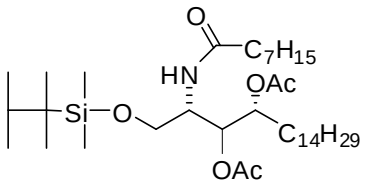
Preparation of 2-16. Compound **2-14** (1.6 g, 3.2 mmol) was dissolved in Acetic acid: 6M

HCl (50 mL: 7 mL). The mixture was stirred at 85°C for 1 h, and the solution was concentrated. The product was extracted with chloroform (100 mL), washed with cold water (2 x 50 mL), and the combined extracts were dried over Na_2SO_4 and concentrated in vacuo. After chromatography (SiO_2, EtOAc:hexanes 1:4), compound **2-15** (1g, 69% yield) was obtained as a clear oil. NMR (^1H, $CDCl_3$) δ 7.39- 7.28 (m, 15 H), 6.38 (d, *J* = 3.5 Hz, 1 H), 5.02- 4.58 (m, 6 H), 4.17 (dd, *J* = 11.0, 4.0 Hz, 1 H), 3.91-3.88 (m, 3 H), 3.47 (dd, *J* = 12.5, 7.0 Hz, 1 H), 3.15 (dd, *J* = 12.5, 7.0 Hz, 1 H), 2.12 (s, 3 H);NMR (^{13}C, $CDCl_3$) δ 169.55, 138.59, 138.13, 138.00, 128.68, 128.62, 128.57, 128.56, 128.53, 128.51, 128.18, 128.13, 128.10, 128.03, 127.98, 127.85, 127.79, 127.60, 90.65, 78.67, 75.45, 75.31, 74.95, 74.69, 74.60, 74.42, 73.57, 73.53, 71.89, 50.85, 21.28; HRFAB-MS (thioglycerol +Na^+ matrix) *m/e* ($[M + Na]^+$) 540.2112(100%), calcd 540.2111. Compound **2-15** (3.09g, 5.7 mmol) was dissolved in 200 mL of CH_2Cl_2, followed by dropwise addition of DAST (0.906 mL), the solution was stirred at room temperature for 30 min before the reaction was quenched with H_2O (30 mL). The mixture was then diluted with CH_2Cl_2 and washed with water and brine, the organic layer was dried and concentrated in vacuo. The desired product **2-16** (2.7 g, 87% yield) was obtained as a clear oil after chromatography (SiO_2, EtOAc:hexanes 1:6). NMR (^1H, $CDCl_3$) δ 7.40- 7.25 (m, 15 H), 5.63 (dd, *J* = 54.0, 2.5 Hz, 1 H), 5.00 (d, *J* = 11.5 Hz, 1 H), 4.88- 4.72 (m, 4 H), 4.61 (d, *J* = 11.0 Hz, 1 H), 4.01- 3.88 (m, 4 H), 3.51 (dd, *J* = 12.5, 7.5 Hz, 1 H), 3.13 (dd, *J* = 12. 0, 6.0 Hz, 1 H); NMR (^{13}C ,$CDCl_3$) δ 138.38, 138.09, 138.07, 128.74, 128.71,128.66, 128.56, 128.23, 128.20, 128.16, 128.04, 127.80, 107.12, 105.32, 78.45, 75.85, 75.67, 74.99, 74.48, 73.99, 73.67, 72.14, 72.11, 50.96; HRFAB-MS (thioglycerol + Na^+ matrix) *m/e* ($[M + Na]^+$) 500.1956(100%), calcd 500.1962.



2-18

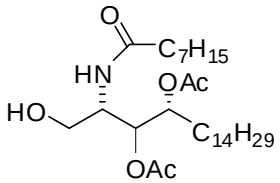
Preparation of 2-18. Octanoic acid (3 g, 20.8 mmol) was dissolved in anhydrous THF (100 mL) followed by HOBt (3.06 g, 22.9 mmol) and DCC (4.72 g, 22.9 mmol). The mixture was stirred for 1 h. Phytosphingsine (7.2g, 22.9 mmol) dissolved in THF and pyridine was added to the reaction mixture and stirred for 12 h, then added acetic anhydride (9 mL), triethylamine (9 mL), DMAP (300 mg), stirred 2 h. The solvent was removed by vacuo. Pure triacetate **4-10** (9.5 g, 80.0%) was isolated by chromatography (SiO_2, EtOAc:hexanes 1:8). NMR (^{1}H, CDCl3) δ 6.14 (d, *J* = 9.5, 1 H), 5.34 (m, 2 H), 5.12 (dd, *J* = 3, 8.5 Hz, 1 H), 4.93 (m, 1 H), 4.50(m, 1 H), 4.29 (dd, *J* = 5, 11.5 Hz, 1 H), 4.0 (dd, *J* = 3.0, 11.5 Hz, 1 H), 2.21 (t, *J* = 7.5 Hz, 2 H), 2.08 (s, 3 H), 2.05-1.99 (m, 10 H), 1.66-1.60 (m, 4 H), 1.33-1.22 (m, 32 H), 0.89 (t, *J* = 7.0 Hz, 6 H); NMR (^{13}C ,CDCl3) δ 178.25, 173.19, 171.31, 170.99, 170.20, 129.99, 73.15, 71.92, 63.05, 60.52, 47.50, 36.81, 34.11, 32.05, 29.91, 29.83, 29.80, 29.74, 29.67, 29.51, 29.45, 29.39, 29.26, 28.10, 27.33, 25.80, 25.68, 24.92, 22.82, 21.13, 20.88, 20.84, 14.30, 14.24. HRFAB-MS (thioglycerol + Na^+ matrix) *m/e* ([M + Na]$^+$) 592.4187 (100%), calcd 592.4189.



2-20

Preparation of 2-20. Adding sodium metal (460 mg, 20 mmol) to MeOH (200 mL) Triacetate **2-18** (9.5 g, 16.0 mmol) was deacetylated by dissolving in upper solvent, stirred

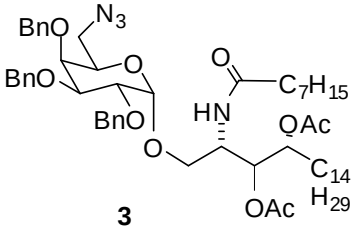
for 1 h and centrifuged (3000 rpm, 5 m) to isolate the solid triol product. The solvent was removed, the solid rinsed with fresh MeOH (160 mL) to remove any remaining base, and centrifuged again, then repeated twice. After removal of the solvent, the crude white solid **2-19** (6.3 g, 89%) was dried under vacuo. The triol **2-19** (6.3g, 14.2 mmol) was dissolved in pyridine (15 mL), then adding dimethylthexylsilyl chloride 2.8 mL , 14.2 mmol), after 5 min, the reaction was monitored by TLC, if the reaction was not complete, just add another 2.8 mL, until the starting material disappeared, acetic anhydride (5.36 mL, 56.8 mmol), DMAP (500 mg) was added into the system, stirred 2 h, Purification by chromatography (SiO2, EtOAc:Hexanes 1:20) yielded **2-20** (7.90 g, 84%) a clear oil. ^{1}H NMR ($CDCl_3$, 500 MHz) δ 5.77 (d, *J* = 9.5 Hz, 1 H), 5.08 (dd, *J* = 10.0, 3.0 Hz, 1 H), 4.76 (dt, *J* = 10.5, 3.0 Hz, 1 H), 4.18 (tt, *J* = 9.5, 3.0 Hz, 1 H), 3.49 (dd, *J* = 10.0, 2.0 Hz, 1 H), 3.40 (dd, *J* = 10.0, 3.0 Hz, 1 H), 2.05 (dt, *J* = 7.5, 2.0 Hz, 2 H), 1.94 (s, 3 H), 1.88 (s, 3 H), 1.58-1.42 (m, 4 H), 1.13 (br s, 32 H), 0.78-0.69 (m, 19 H), -0.09 (d , *J* = 9.0 Hz, 6 H); ^{13}C NMR ($CDCl_3$, 125 MHz) δ173.43, 172.81, 171.28, 169.81, 73.79, 71.55, 61.48, 49.34, 37.18, 34.45, 32,13, 31.89, 29.91, 29.87, 29.84, 29.81, 29.57, 29.44, 29.22, 27.92, 25.92, 25.38, 22.90, 22.82, 21.26, 21.07, 20.46, 20.45, 20.35, 18.79, 18.71, 14.32, 14.27, -1.25, -3.57, -3.669; HRMS (FAB) *m/z* for $C_{38}H_{75}NNaO_6Si$ ([M+Na]+) 692.5303 (100%), calc. 692.5301



2-21

Preparation of 2-21. Compound **2-20** (7.9 g, 11.9 mmol) was dislove in THF (10 mL) in a centrifuge tube (plastic), followed by adding aqueous HF (5 mL), check the TLC, if the

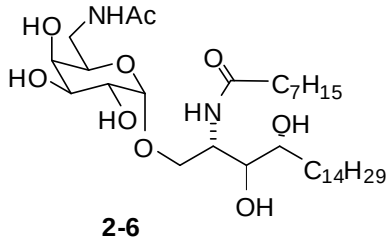
reation was not completed, just added more THF and aqueous HF. The mixture was pour into the saturated $NaHCO_3$ (200 mL), then extracted with EtOAc (100 mL x 2), the organic layer was dried and concentrated, then purified by chromatography (SiO2, EtOAc:Hexanes 1:2) to afford a white solid **2-21** (3.07 g, 49%). ^{1}H NMR ($CDCl_3$, 500 MHz) δ 6.27 (d, *J* = 9.0 Hz, 1 H), 5.04 (dd, *J* = 10.0, 3.0 Hz, 1 H), 4.96 (dt, *J* = 10.5, 3.0 Hz, 1 H), 4.16 (tt, *J* = 9.5, 3.0 Hz, 1 H), 3.62-3.53 (m, 2 H), 2.22 (t, *J* = 7.5 Hz, 2 H), 2.15 (s, 3 H), 2.04 (s, 3 H), 1.78-1.58 (m, 4 H), 1.28 (br s, 32 H), 0.88 (dt, *J* = 7.0, 2.5 Hz, 6 H); ^{13}C NMR ($CDCl_3$, 125 MHz) δ173.14, 171.61, 170.95, 73.13, 73.01, 61.56, 49.58, 36.77, 31.91, 31.67, 29.68, 29.65, 29.61, 29.57, 29.35, 29.22, 28.98, 27.92, 25.69, 25.67, 22.67, 22.59, 21.01, 20.88, 14.10, 14.05; HRMS (FAB) *m/z* for $C_{30}H_{57}NNaO_6$ ([M+Na]+) 550.4113 (100%), calc. 550.4111



3

Preparation of 2-22. Compound **2-21** (340 mg, 0.63 mmol) and coumpound **2-16** (661 mg, 1.25 mmol) were dissolved in dry CH_2Cl_2 (30 ml), and powdered 4Å molecular sieves (1.80 g) were added, cooled to 0°C, stired for 10 min, $AgClO_4$ (390 mg, 1.88 mmol), $SnCl_2$ (357 mg, 1.88 mmol) were introduced into the solution with the protection of light. The mixture was allowed to warm to room temperature with stirring over 3 h under N_2, then filtered through Celite, washed with CH_2Cl_2. The combined filtrate was concentrated under reduced pressure. The residue was purified by chromatography (SiO_2, EtOAc:Hex 1:7) to give compound **2-22** (357 mg, 54%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.41-7.25 (m, 15 H), 6.44 (d , *J* = 9.5 Hz, 1 H), 5.19 (dd, *J* = 9.5, 2.5 Hz, 1 H), 4.96 (d, *J* = 11.0 Hz, 1 H), 4.92 (dt, *J* = 10.5, 3.0, 1 H), 4.83 (d,

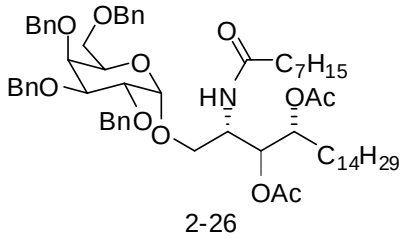
J = 12.0, 1 H), 4.78-4.73 (m, 3 H), 4.67 (d, *J* = 11.5 Hz, 1 H), 4.58 (d, *J* = 11.5 Hz, 1 H), 4.35 (tt, *J* = 6.0, 3.5 Hz, 1 H), 4.01 (dd, *J* = 9.5, 3.5 Hz, 1 H), 3.93 (dd, *J* = 8.0, 5.0 Hz, 1H), 3.87-3.82 (m, 3 H), 3.61-3.55 (m, 2 H), 3.06 (dd, *J* = 12.5, 5.0 Hz, 1 H), 2.13 (t, *J* = 7.5 Hz, 2 H), 2.04 (s, 1 H), 2.00 (s, 1 H), 1.71-1.58 (m, 4 H), 1.31-1.15 (m, 32 H), δ0.89-0.85 (m, 6 H). ^{13}C NMR ($CDCl_3$, 125 MHz) δ 172.82, 170.81, 138.50, 138.22, 137.96, 128.47, 128.37, 128.00, 127.91, 127.78, 127.58, 127.51, 99.79, 78.49, 76.36, 74.73, 74.61, 73.50, 73.40, 73.05, 71.57, 70.31, 69.99, 51.23, 48.19. 36.65, 31.87, 31.87, 31.65, 29.63, 29.59, 29.56, 29.52, 29.30, 29.26, 29.20, 28.97, 27.62, 25.60, 25.56, 22.63, 22.56, 20.94, 20.84, 14.06, 14.02. HRMS (ESI) calcd for $C_{57}H_{84}N_4O_{10}Na$ $[M+Na]^+$: 1007.60797, found: 1007.60625.



2-6

Preparation of 2-6. Na° (155 mg, 6.73 mmol) was added to liquid NH_3 (40 mL) under N_2 at -78°C, stirred for 5 min. Compound **2-22** (333 mg, 0.34 mmol) was dissolved in dry THF (2 mL). The solution was added to the blue liquid NH_3, stirred for 5h. The reaction was quenched with MeOH. After the ammonia was romoved, the solution was concetrated, run a plug to give compound **2-23** (81 mg, 40%). HRMS (ESI) calcd for $C_{32}H_{65}N_2O_8$ $[M+H]^+$: 605.47354, found: 605.47385. AcOH (3.8 µL, 0.07 mmol) was added into THF (1 mL), HOBt (9.5 mg, 0.07 mmol) and DCC (14.4 mg, 0.07 mmol) were also added, stirred for 1 h. **2-23** (20mg, 0.03 mmol) were introduced into the solution. The solution was stirred at room temperature for 8 h then concentrated *in vacuo.* Flash chromatography (SiO_2, MeOH:CH_2Cl_2 1:99) was performed to afford the product as a white solid **2-6** (6.6 mg, 31%). NMR (^{1}H,

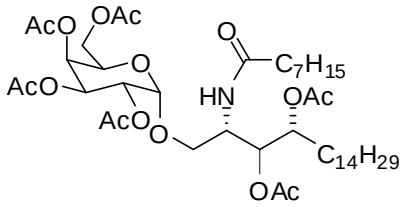
MeOD:$CDCl_3$/10:90) δ 4.88 (d, *J* = 3.5 Hz, 1 H), 4.17 (d, *J* = 3.5 Hz, 1 H), 3.85 (dd, J = 11.0, 5.0 Hz, 1 H), 3.79-3.69 (m, 4 H), 3.65 (dd, J = 10.5, 5.0 Hz, 1 H), 3.57-3.53 (m, 3 H), 3.35 (brs, 1 H), 3.23 (dd, J = 14.0, 7.5, Hz, 1 H), 2.20 (t, J = 7.5 Hz, 2 H), 1.98 (s, 3 H), 1.67-1.55 (m, 4 H), 1.37-1.21 (m, 32 H), 0.89-0.87 (m, 6 H). ^{13}C NMR (125 MHz, MeOD:$CDCl_3$/10:90) δ 174.64, 172.63, 99.62, 74.65, 72.00, 69.95, 69.32, 68.96, 68.85, 66.99, 50.51, 49.27, 49.10, 48.93. 48.76, 48.59, 48.42, 48.25, 39.82, 36.47, 32.64, 31.93, 31.70, 29.81, 29.71, 29.66, 29.37, 29.30, 29.05, 25.90, 25.86, 22.67, 22.61, 22.35, 13.96, 10.91. HRMS (ESI) calcd for $C_{34}H_{67}N_2O_9$ $[M+H]^+$: 647.48411, found: 647.48523.



2-26

Preparation of 2-26. 2-24 (300 mg, 0.56 mmol) was dissolved in trichloroacetonitrile (2 mL), K_2CO_3 (100 mg) was also been added into the solvent, stirred for 4h, the reaction was checked by TLC. The activated donor was passed through filter paper. The solvent was concentrated *in vacuo*. To a solution of activated donor in CH_2Cl_2 (5 mL), were added **2-21** (123mg, 0.28mmol) and 4Å molecular sieves (400 mg), stirred for 1 h. Trimethylsilyl trifluoromethanesulfonate (100.0 µL, 0.56 mmol) was added drop wise at 0^{o}C. The reaction was stirred at 0^{o}C for 3 h. The molecular sieves were removed by filtration through filter paper. The solvent was removed *in vacuo*. Purification by column chromatography on silical gel afforded **2-26** (117 mg, 40%) as a colorless oil. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.39-7.22 (m, 20 H), 6.72 (d , *J* = 10.0 Hz, 1 H), 5.25 (dd, *J* = 9.5, 2.0 Hz, 1 H), 4.98 (d, *J* = 10.0 Hz, 1 H), 4.88 (d, *J* = 11.5, 1 H), 4.80-4.64 (m, 5 H), 4.55-4.52 (m, 2 H), 4.45 (d, *J* = 12.0 Hz, 1 H),

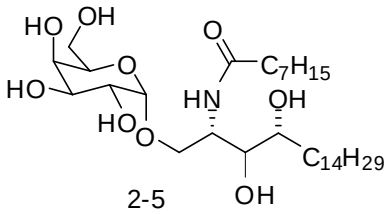
4.33-4.27 (m, 1 H), 4.07-3.84 (m, 5 H), 3.56 (t, *J* = 7.5 Hz, 1 H), 3.51 (t, *J* = 11.5 Hz, 1 H), 3.35-2.32 (m, 1 H), 2.13-2.04(m, 2 H), 2.02 (s, 3 H), 1.97 (s, 3 H), 1.69-1.55 (m, 4 H), 1.32-1.14 (m, 32 H), δ0.89-0.84 (m, 6 H). ^{13}C NMR ($CDCl_3$, 125 MHz) δ 173.11, 170.71, 170.06 138.77, 138.50, 138.40, 137.82, 128.52 128.48, 128.33, 128.12, 128.01, 127.86, 127.82, 127.62, 100.59, 78.74, 76.69, 74.83, 74.74, 73.55, 73.53, 73.23, 73.14, 71.55, 71.06, 70.43, 69.40, 48.36, 36.61, 32.03, 31.83, 29.79, 29.76, 29.72, 29.46, 29.44, 29.36, 29.19, 27.88, 25.75, 25.69, 22.80. 22.74, 21.11, 21.07, 14.25, 14.21. HRFAB-MS (thioglycerol + Na^+ matrix) *m/e* ([M + Na]$^+$) 1072.6490 (100.0%), calcd 1072.6490



2-27

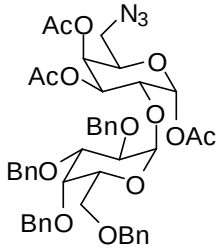
Preparation of 2-27. Compound **2-26** (117 mg, 0.111 mmol) was dissolved in a mixture of THF (5 mL) and Methanol (5 mL). To this solution was added Pd/C (10%, 50 mg), The mixture was then subjected to hydrogenation at 500 psi with stirring for 12 h, followed by filtration through silica gel pad and concentration on rotavapor. The crude mixture was treated with pyridine (5 mL), acetic anhydride (3 mL) and DMAP (50 mg). The desired product **2-27** (61.83 mg, 65%) was purified through column. ^{1}H NMR ($CDCl_3$, 125 MHz) δ 6.20 (d, *J* = 10.0 Hz, 1 H), 5.46 (d, *J* = 2.5 Hz, 1 H), 5.31-5.25 (m, 2 H), 5.16-5.13 (m, 1 H), 4.91-4.86 (m, 1 H), 4.37 (t, *J* = 9.5 Hz, 1 H), 4.13-4.01 (m, 3 H), 3.66 (d, *J* = 10.5 Hz, 1 H), 3.42 (d, *J* = 10.5 Hz, 1 H), 2.32-2.26 (m, 2 H), 2.14 (s, 3 H), 2.11 (s, 3 H), 2.07 (s, 3 H), 2.04 (s, 3 H), 2.00 (s, 3 H), 1.99 (s, 3 H), 1.67-1.60 (m, 4 H), 1.38-1.21 (m, 32 H), 0.89-0.86 (m, 6 H); ^{13}C NMR ($CDCl_3$, 125 MHz) δ173.07, 171.27, 170.87, 170.61, 170.36, 170.30, 169.89,

97.34, 73.50, 71.65, 68.04, 67.63, 67.60, 67.46, 66.84, 61.95, 47.91, 36.90, 32.09, 31.87, 29.86, 29.83, 29.79, 29.53, 29.46, 29.43, 29.21, 27.41, 25.89, 25.80, 22.86, 22.80, 21.15, 20.91, 20.84, 20.79, 20.77, 14.30, 14.25; HRFAB-MS (thioglycerol + Na^+ matrix) *m/e* ([M + $Na]^+$) 880.5035 (97.6%), calcd 880.5034.



2-5

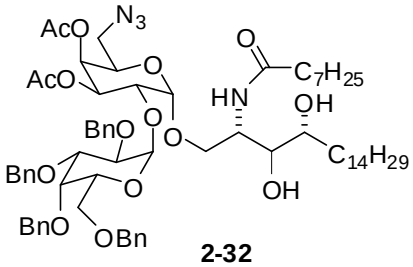
Preparation of 2-5. Adding sodium metal (5 mg) to MeOH (5 mL). **2-27** (61.83 mg, 0.072 mmol) was deacetylated by dissolving in solvent, stirred for 1 h and centrifuged (3000 rpm, 5 min) to isolate the solid product **2-5** (29mg, 67%). NMR (^1H, 10% MeOD in $CDCl_3$, 500 MHz), 4.91 (d, *J* = 3.5 Hz, 1 H), 4.14-4.08 (m, 1 H), 3.95 (d, *J* = 2.5 Hz, 1 H), 3.83-3.56 (m, 10 H), 2.20 (t, *J* = 7.5 Hz, 2 H), 1.64-1.53 (m, 4 H), 1.34-1.228 (m, 32 H), 0.87 (t, *J* = 7.0 Hz, 6 H); NMR (^{13}C , 10% MeOD in $CDCl_3$) δ174.52, 99.36, 73.76, 71.89, 70.58, 69.87, 69.60, 68.60, 67.36. 61.46, 50.32, 49.41, 49.25, 49.16, 48.99, 48.82, 48.71, 48.21, 36.33, 31.84, 31.64, 31.46, 29.76, 29.74, 29.68, 29.66, 29.59, 29.27, 29.21, 29.02, 25.89, 25.79, 22.60, 22.55, 13.99, 13.92. HRFAB-MS (thioglycerol + Na^+ matrix) *m/e* ([M + $Na]^+$) 628.4400 (95.4%), calcd 628.4401.



2-30

Preparation of 2-30. 2-24 (87mg, 0.16 mmol) was dissolved in trichloroacetonitrile (2 mL),

K_2CO_3 (100 mg) was also been added into the solvent, stirred for 4h, the reaction was checked by TLC. The activated donor was passed through filter paper. The solvent was concentrated in vacuo. To a solution of activated donor in CH_2Cl_2 (3 mL), were added **2-29** and 4Å molecular sieves (200 mg), stirred for 1h. Trimethylsilyl trifluoromethanesulfonate (28.5 µL, 0.16 mmol) was added drop wise at -15°C. The reaction was stirred at 15°C for 14 h. The molecular sieves were removed by filtration through filter paper. The solvent was removed *in vacuo*. Purification by column chromatography on silical gel (EtOAc/Hex, 1:1-2:1) afforded **2-30** (50.9 mg. 37%) as a colorless oil. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.40-7.23 (m , 20 H), 6.44 (d, *J* = 4.0 Hz, 1 H), 5.45 (dd, *J* = 3.5, 1.2 Hz, 1 H), 5.27 (dd, *J* = 10.5, 3.5 Hz, 1 H), 4.95-4.71 (m, 4 H), 4.63 (d, *J* = 24.0 Hz, 1 H), 4.54 (d, *J* = 24.0 Hz, 1 H), 4.43-4.40 (m, 2 H), 4.21 (dd, *J* = 10.5, 4.0 Hz, 1 H), 4.15-3.90 (m, 5 H), 3.85 (dd, *J* = 9.5, 4.5 Hz, 1 H), 3.54-3.89 (m, 3 H), 3.18 (dd, *J* = 12.5, 5.5 Hz, 1 H), 2.05 (s, 3 H), 2.02 (s, 3 H), 1.92 (s, 3 H). ^{13}C NMR ($CDCl_3$, 125 MHz) δ 170.31, 170.25, 169.42, 138.73, 138.58, 137.95, 128.60, 128.47, 128.43, 128.39, 128.20, 128.05, 127.81, 127.74, 127.65, 99.75, 89.06, 78.86, 75.58, 74.96, 73.73, 70.15, 69.81, 69.18, 68.97, 68.86, 68.49, 60.60, 50.50, 29.90, 21.26, 20.88, 20.78, 20.64, 14.39. HRMS (ESI) calcd for $C_{46}H_{51}N_3Na\ O_{13}$ $[M+Na]^+$: 876.33141, found: 876.33220.

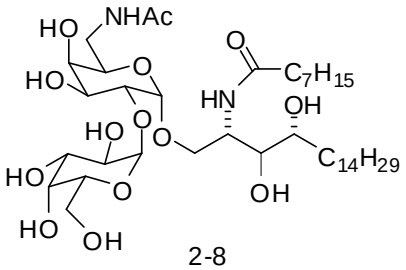


2-32

Preparation of 2-32. Ethylene diamine (1.6 mL) was added to acetic acid (2 mL) at 0°C and the solution was stirred at 0°C until the mixture turned into white solid. The solid was added

into a solution of **2-30** (50.9 mg, 0.06 mmol) in THF (2 mL), stirred for 8 h. The product was extracted with CH_2Cl_2 (3 X 10 mL), and the combined organic layers were washed with brine (10 mL), and concentrated. Purification of the residue by column chromatography (EtOAc:Hex, 1:4) gave **2-31** (31 mg, 64%) as a colorless oil. HRMS (ESI) calcd for $C_{44}H_{53}N_4O_{12}$ $[M+NH_4]^+$: 829.36545, found: 829.36438. To a solution of **2-31** were added BSP (66 mg, 0.32 mmol), TTBP (156 mg, 0.63 mmol), 3Å molecular sieves (800 mg). The reaction mixture was stirred at room temperature for 30 min, cooled to -60^{o}C, Tf_2O (45.8μL) were added. The resulting mixture was allowed to warm slowly to -40^{o}C. **2-21** was introduced into the system, warmed to room temperature. The reaction was quenched with Et_3N (0.8 mL). The molecular sieves were filtered. The mixture was diluted with EtOAc, then washed with brine (2 X 10 mL), dried (Na_2SO_4) and concentrated. The residue was purified by silica gel flash chromatography (EtOAc:Hex, 1:6) to give the **2-32** (146 mg, 53%). ^{1}H NMR (500 MHz, $CDCl_3$) δ7.83-7.23 (m , 20 H), 6.35 (d, *J* = 8.5, 1 H), 5.36 (d, *J* = 3.5 Hz, 1 H), 5.18 (dd, *J* = 9.5, 3.5 Hz, 1 H), 5.13 (dd, *J* = 8.0, 2.5 Hz, 1 H), 4.92-4.68 (m, 8 H), 4.52 (d, *J* = 5.12 Hz, 1 H), 4.40 (dd, *J* = 20.0, 11.5 Hz, 2 H), 4.29-4.27 (m , 1 H), 4.20 (dd, *J* = 8.0, 4.0 Hz, 1 H), 4.04-4.87 (m, 5 H), 3.66-3.44 (m, 5 H) 3.23 (dd, *J* = 13.0, 4.5 Hz, 1 H), 2.20-2.11 (m, 2 H), 2.05 (s, 3H), 2.00 (s, 3 H), 1.88 (s, 3 H), 1.67 (s, 3 H), 1.62-1.56 (m, 4 H), 1.30-1.11 (m, 32 H), 0.90-085 (m, 6 H). ^{13}C NMR ($CDCl_3$, 125 MHz) δ 173.53, 171.05, 170.33, 170.225, 170.04, 138.75, 138.59, 138.43, 137.90, 128.83, 128.69,128.65, 128.50, 128.39, 128.38, 128.33, 128.16, 128.10, 127.88, 127.45, 99.07, 98.04, 79.18, 75.64, 75.07, 74.42, 74.17, 73.77, 73.22, 72.55, 72.43, 72.34, 70.37, 70.07, 69.34, 68.64, 68.50, 59.67, 51.05, 48.66, 39.81, 36.66, 32.18, 31.95, 29.95, 29.91, 29.75, 29.62, 29.54, 29.35, 28.43,

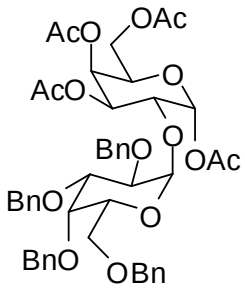
27.93, 25.91, 25.86, 22.95, 22.89, 21.22, 21.06, 20.82, 14.38. HRMS (ESI) calcd for $C_{74}H_{105}N_4O_{17}$ $[M+H]^+$: 1321.74692, found: 1321.74339.



2-8

Preparation of 2-8. To a liquid NH_3 (20 mL) under N_2 at -78°C, were added Na°(51 mg, 2.21 mmol), stirred for 5 min. The color of solvent changed to dark blue. Compound **2-32** (146 mg, 0.34 mmol) was dissolved in dry THF (1.0 mL). The solution was added into the solvent, stirred for 5 h. The reaction was quenched with MeOH. The solution was diluted with MeOH. A few drops of water were added into it. The product will precipitate. Using the centrifuge machine, **2-33** (23mg, 27%) was obtained. HRMS (ESI) calcd for $C_{38}H_{75}N_2O_{13}$ $[M+H]^+$: 767.52637, found: 767.52468. To a solution of Acetic acid (3.4 µL, 0.06 mmol) in THF (1 mL), HOBt (8.11 mg, 0.06 mmol) and DCC (12.4 mg, 0.06 mmol) were added, stirred for 1h. **2-33** (23mg, 0.03 mmol) were introduced into the solution. The solution was stirred at room temperature for 8 h then concentrated *in vacuo* and purified by silica gel flash chromatography (SiO_2, MeOH:CH_2Cl_2 1:99) to give compound **2-8** (5.4 mg, 22%) as a white solid. ^{1}H NMR (500 MHz, MeOD:$CDCl_3$/10:90) δ 5.02 (t, *J* = 3.5 Hz, 2 H), 4.15-4.13 (m, 2 H), 3.93 (brs,2 H), 3.86-3.54 (m, 12 H), 3.26-3.18 (m, 1 H), 2.24 (t, *J* = 7.0 Hz, 2 H), 1.99 (s, 3 H), 1.62-1.55 (m, 4 H), 1.42-1.23 (m, 32 H), 0.90-0.87 (m, 6 H). ^{13}C NMR (500 MHz, MeOD:$CDCl_3$/10:90) δ 174.56, 172.71, 97.55, 97.06, 74.35, 74.08, 72.23, 70.99, 69.87, 69.69, 68.91, 68.78, 68.08, 67.32, 61.95, 49.16, 48.99, 48.82, 48.65, 48.48, 48.31, 48.14, 40.03, 39.97, 36.31, 31.88, 31.69, 29.67, 29.27, 29.05, 25.91, 22.62, 13.87. HRMS (ESI) calcd for

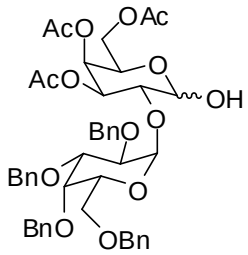
$C_{40}H_{77}N_2O_{14}$ $[M+H]^+$: 809.53693, found: 809.53550.



2-35

Preparation of 2-35. 2,3,4,6-tetra-*O*-benzyl-D-galactose **2-24** (61.5 mg, 0.114 mmol) and freshly powdered potassium carbonate (300 mg) were stirred in trichloroacetonitrile (5 mL) for 2 h. the solvent was removed in vacuo, and the activated donor was passed through a silica gel plug using a mixture of ethyl acetate and hexane as eluent. The solvent was removed on rotavapor under reduced pressure. The obtained residue was further dissolved in anhydrous DCM (8 mL) and combined with glycosyl acceptor **2-34**, 1,3,4,6-tetra-*O*-acetyl-α-D-galactose (39.6 mg, 0.113 mmol). To this mixture was added 4 Å molecular sieves (50 mg). The reaction mixture was kept stirring at room temperature for 30 min, and then cooled to 0 °C. TMSOTf (20.1 μL, 0.113 mmol) was added drop wise. The mixture was allowed to stir for another 8 h at room temperature. The solvent was removed and the remaining slurry was chromatographed on silica gel column (EtOAc/hexane, 1/3~1/2). The product **2-35** was obtained as a colorless oil (39.6 mg, 0.0455 mmol) in 40% yield of 2 steps. ^{1}H NMR ($CDCl_3$, 500 MHz): δ 7.38-7.22 (m, 20 H), 6.43 (d, J = 3.4 Hz, 1 H), 5.46 (d, J = 2.00 Hz, 1 H), 5.28 (dd, J = 3.40, 10.70 Hz, 1 H), 4.96-4.31 (m, 9 H), 4.29 (ddd, J = 1.00, 6.90 Hz, 1 H), 4.24 (dd, J = 3.50, 10.30 Hz, 1 H), 4.10-4.08 (m, 2 H), 4.04-4.01 (m, 2 H), 3.95 (d, J = 2.00 Hz, 1 H), 3.87 (dd, J = 3.00, 10.3 Hz, 1 H), 3.55 (dd, J = 7.30, 8.80 Hz, 1 H), 3.47 (dd, J = 5.80, 8.70 Hz, 1 H), 2.20 (s, 3 H), 2.03 (s, 3 H), 1.92 (s, 3 H), 1.91 (s, 3 H). ^{13}C

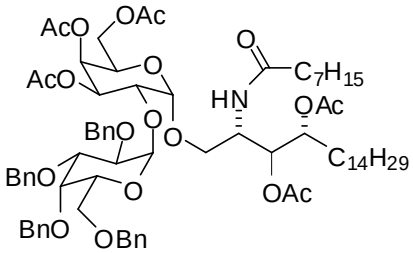
NMR ($CDCl_3$, 125 MHz): δ 170.60, 170.40, 170.30, 169.50, 138.80, 138.71, 138.62, 137.93, 128.64, 128.61, 128.5, 128.46, 128.40, 18.20, 128.09, 128.01, 127.81, 127.73, 127.70, 127.64, 96.70, 89.23, 78.89, 75.48, 74.88, 74.85, 73.68, 73.70, 73.00, 70.09, 69.06, 68.87, 68.78, 68.50, 67.66, 61.48, 20.89, 20.85, 20.80, 20.57. HRFAB-MS (thioglycerol + Na^+ matrix) m/z: calcd: 893.3358 (100%); found: 893.3361.



2-36

Preparation of 2-36. The starting material **2-35** (450 mg, 0.517 mmol) was dissolved in anhydrous THF (30 mL). To this solution was added prepared salt (1 g) which was formed by mixing ethylene diamine (1.6 mL) with acetic acid (2 mL) at 0 ºC for 15 minutes. The reaction mixture was stirred at room temperature for 24 hours. TLC showed that no starting material was observed. The mixture was then concentrated, diluted with DCM, washed with water. The organic phase was dried over anhydrous sodium sulfate. After filtration and concentration, the crude mixture was further purified by silical gel column affording 350 mg of sticky oil as a mixture of anomers (ratio: 1/1 by ^1H NMR). ^1H NMR ($CDCl_3$, 500 MHz): δ 7.31-7.18 (m, 20 H), 5.32 (dd, *J* = 1.00, 3.50 Hz), 5.27 (dd, *J* = 1.00, 3.50 Hz), 5.194 (d, *J* = 3.50 Hz), 5.15 (dd, *J* = 3.50, 9.50 Hz), 4.96 (d, *J* = 3.50 Hz), 4.92 (dd, *J* = 3.50, 10.50 Hz), 4.85 (d, *J* = 4.50 Hz), 4.825 (d, *J* = 4.50 Hz), 4.81 (d, *J* = 7.50 Hz), 4.29 (d, *J* = 7.50 Hz), 4.71-4.66 (m), 4.61 (d, *J* = 12.00 Hz), 4.48 (d, *J* = 6.50 Hz), 4.46 (d, *J* = 6.50 Hz), 4.36-4.28 (m), 4.10-3.82 (m), 3.62 (dd, *J* = 7.00, 10.00 Hz), 3.45-3.38 (m), 3.334 (dd, *J* = 15.00, 8.50

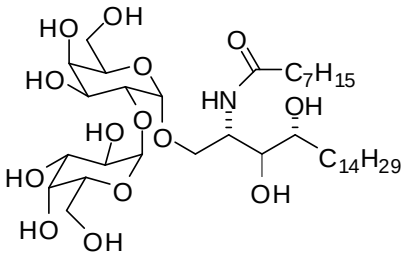
Hz), 2.03 (s), 2.00 (s), 1.98 (s), 1.97 (s), 1.83 (s), 1.79 (s). ^{13}C NMR ($CDCl_3$, 125 MHz): δ 170.81, 170.73, 170.58, 170.52, 170.43, 138.72, 138.59, 138.48, 137.89, 137.77, 137.52, 128.93, 128.87, 128.82, 128.72, 128.64, 128.55, 128.52, 128.38, 128.25, 128.18, 128.14, 127.99, 127.96, 127.69, 100.66, 97.51, 97.29, 91.01, 79.69, 79.61, 79.42, 76.28, 75.31, 75.11, 75.04, 74.89, 74.75, 74.56, 74.44, 73.76, 73.49, 72.94, 72.77, 71.32, 71.13, 70.67, 70.38, 68.96, 68.79, 68.55, 68.18, 67.90, 67.18, 62.04, 61.79, 21.11, 20.99, 20.96, 20.86, 20.75. HRMS (ESI) calcd for $C_{46}H_{56}NO_{14}$ $[M+NH_4]^+$: 846.36953, found: 846.38508.



2-37

Preparation of 2-37. A solution of donor **2-36** (72 mg, 0.087 mmol), diphenylsulfoxide (44 mg, 0.22 mmol) and 2,4,6- tri-*tert*-butylpyrimidine (75.63 mg, 0.3045 mmol) in dichloromethane (3 mL) was stirred over 150 mg of activated 3 Å MS for 30 min. The mixture was cooled to -60˚C before triflic acid anhydride (20.5 µL, 0.122 mmol) was added. The mixture was warmed to -40˚C over 45 min, followed by addition of acceptor (44.15 mg, 0.087 mmol) in dichloromethane (1 mL). The reaction mixture was warmed to 0˚C and quenched with Et_3N (0.4 mL). The solids were filtered off and the filtrate was concentrated. Purification by SiO_2 column chromatography (1:3, EtOAc/hexane) afforded desired product **2-37** as colorless oil (82 mg, 70.5%). ^{1}H NMR ($CDCl_3$, 500 MHz): δ 7.31-7.15 (m, 20 H), 6.25 (d, J = 8.50 Hz, 1 H), 5.343 (dd, J = 1.50, 3.50 Hz, 1 H), 5.13 (dd, J = 3.00, 10.50 Hz, 1 H), 5.07 (dd, J = 3.50, 9.00 Hz, 1 H), 4.85 (tt, J = 3.50, 10.00 Hz, 1 H), 4.82 (br s, 1 H), 4.80

(d, *J* = 8.50 Hz, 1 H), 4.79 (dd, *J* = 4.00 Hz, 1 H), 4.76 (d, *J* = 8.00 Hz, 1 H), 4.75 (d, *J* = 4.00 Hz, 1 H), 4.67 (q, *J* = 12.00, 2 H), 4.64 (d, *J* = 12.00 Hz, 1 H), 4.45 (d, *J* = 12.00 Hz, 1 H), 4.32 (q, *J* = 11.50 Hz, 2 H), 4.23-4.17 (m, 1 H), 4.178 (tt, *J* = 0.50, 7.00 Hz, 1 H), 4.05 (dd, *J* = 6.50, 11.00 Hz, 1 H), 4.00-3.91 (m, 4 H), 3.82 (dd, *J* = 3.00, 10.50 Hz, 1 H), 3.61 (dd, *J* = 3.00, 10.50 Hz, 1 H), 3.49 (dd, *J* = 6.50, 11.00 Hz, 1 H), 3.46 (t, *J* = 7.50 Hz, 1 H), 3.35 (dd, *J* = 5.50, 9.00 Hz, 1 H), 2.10-1.85 (m, 2 H), 1.99 (s, 3 H), 1.95 (s, 3 H), 1.91 (s, 6 H), 1.81(s, 3 H), 1.63-1.44 (m, 4 H), 1.22-1.14 (m, 32 H), 0.80 (t, *J* = 7.00 Hz, 3 H), 0.78 (t, *J* = 7.00 Hz, 3 H). ^{13}C NMR ($CDCl_3$, 125 MHz): δ 173.54, 170.91, 170.74, 170.34, 170.14, 170.10, 138.76, 138.62, 138.46, 137.90, 128.82, 128.67, 128.65, 128.48, 128.38, 128.29, 128.15, 128.10, 127.87, 127.39, 98.99, 97.92, 79.26, 75.57, 75.05, 74.39, 74.12, 73.77, 73.13, 72.51, 72.45, 72.31, 70.03, 69.68, 68.65, 68.57, 67.35, 62.02, 48.71, 36.60, 32.17, 31.94, 29.95, 29.91, 29.61, 29.52, 29.36, 28.62, 25.83, 22.94, 22.89, 21.21, 21.05, 20.97, 20.84, 14.38, 14.34. HRMS (ESI) calcd for $C_{76}H_{111}N_2O_9$ $[M+NH_4]^+$: 1355.77756, found: 1355.79127.



2-7

Preparation of 2-7. The starting material **2-37** (42 mg, 0.0314 mmol) was dissolved in a mixture of anhydrous THF (6 mL) and Methanol (1 mL). To this solution was added Pd/C (10%, 28 mg), The mixture was then subjected to hydrogenation at 500 psi with stirring for 3 days, followed by filtration through silica gel pad and concentration on rotavapor. The crude mixture was purified by silica gel column using a mixture of methanol and DCM, affording

21.6 mg of desired product as a sticky oil in 70.4% yield. The product's structure was confirmed by HRESI-MS (calcd for $C_{48}H_{83}NNaO_{19}$ $[M+Na]^+$:1000.54515, found: 1000.57734). The obtained product (21.6 mg, 0.022 mmol) was further dissolved in a mixture of anhydrous THF (2.5 mL) and methanol (2.5 mL). A solution of NaOMe in methanol (1M, 0.1 mL) was added to the prepared solution. After 6 hrs' stirring, TLC showed that the starting material was consumed completely. Acetic acid (2 drops) was added and stirred for additional 10 min. the mixture was then concentrated and purified by silica gel column using a mixture of methanol and DCM, affording 13.5 mg of product **2-7** as a white solid in 79% yield. ^{1}H NMR ($CDCl_3/CD_3OD$ (3/1), 500 MHz): δ 5.03 (d, J = 3.50 Hz, 1 H), 5.00 (d, J = 2.00 Hz, 1 H), 4.17-4.13 (m, 2 H), 4.00 (d, J = 3.00 Hz, 1 H), 3.94 (dd, J = 3.50, 10.50 Hz, 1 H), 3.93 (br s, 1 H), 3.89 (dd, J = 3.50, 10.50 Hz, 1 H), 3.86-3.82 (m, 2 H), 3.81-3.65 (m, 7 H), 3.63-3.60 (m, 1 H), 2.22 (t, J = 7.00 Hz, 2 H), 1.62-1.55 (m, 4 H), 1.42-1.17 (m, 32 H), 0.89 (t, J = 7.00 Hz, 3 H), 0.88 (t, J = 7.00 Hz, 3 H). ^{13}C NMR (CDCl*3, 125 MHz): δ* 163.71, 102.13, 101.42, 79.17, 78.42, 76.44, 75.17, 74.61, 74.25, 74.00, 73.77, 72.88, 72.45, 71.78, 66.19, 65.77, 40.45, 35.99, 33.78, 33.44, 33.15, 30.17, 29.99, 26.74, 18.01, 17.93. HRMS (ESI) calcd for $C_{38}H7_3NaO_{14}$ $[M+Na]^+$: 790.49233, found: 790.49802.

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CHAPTER 3.

SYNTHESIS OF *N*-APPENDED-6”-AMINO- GALACTOSYLCERAMIDES AND NKT CELL STIMULATING PROPERTIES

3.1 Introduction

Until now, we are still not very clear how the different structure of glycolipid influences the CD1d and NKT cell receptor binding. Although it is obvious that glycolipids must be presented by CD1d to be recognized by NKT cells, the precise pathway needs to be determined.

This glycolipid presentation pathway is very complex. It has been shown that glycolipids are endocytosed by APCs with the help of extracellular lipid carrier proteins such as apolipoprotiein E.[1] Prigozy *et al*. verified the importance of lysosomes in that some disaccharides need to be processed by glycosidases to the corresponding monosaccharides to stimulate NKT cells.[2] Lipid transfer proteins such as the GM2 activator protein and saposins are required to help glycolipids to be loaded into CD1d in endosome and finally glycolipids are transported to the cell surface to be presented to NKT cells.[1-6] These CD1d-glycolipids-TCRs complexes cause the stimulation of NKT cells.

The challenges to understand the roles of glycolipid structure are lacking direct observation of celluar trafficking of glycolipids and difficulties to quantify their association with CD1d and TCRs. One of the most likely solutions to this problem is to label glycolipids with flurophores or small molecules. It is possible to observe the antigens at very low concentration and measure the association of CD1d and TCRs through this method.[7] It will

also help to identify the location that foreign antigens localize at before being endocytosed by APCs.

3.1.1 Modification of the KRN7000

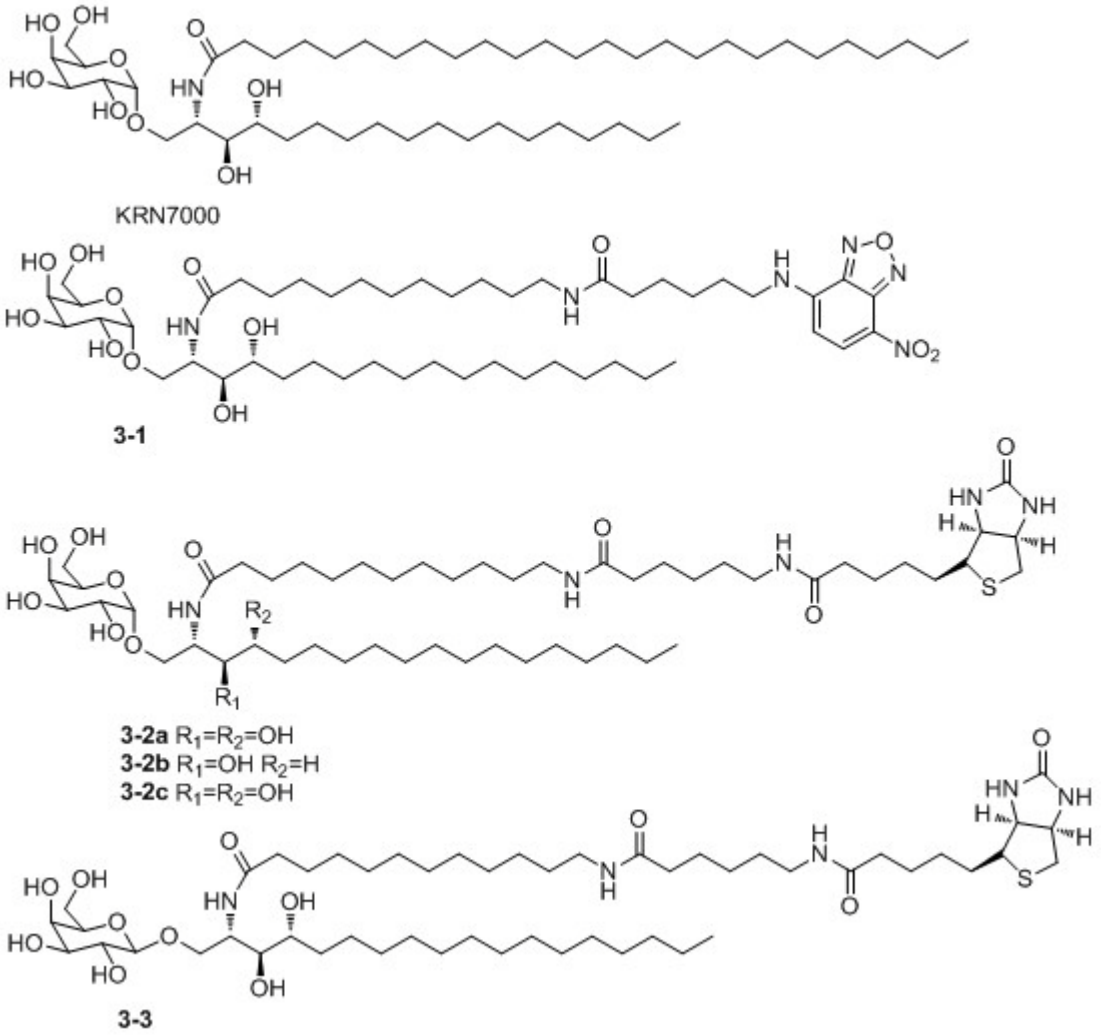


Figure 3.1. Structure of KRN7000 analogs with labels at the end of the lipid chains.

The first question is to choose an appropriate location to substitute with the labels. The ideal location is the place that has the minimal effect on the glycolipid trafficking and processing. Analogs of KRN7000 with biotin (**3-2**) and the NBD (**3-1**) were synthesized and tested for NKT cell proliferation. Sakai *et. al.* prepared analogs of KRN7000 with labels (fluorophore[8] or biotin[9]) at the end of fatty acid chains of the ceramides (**Figure 3.1**).

Sakai *et al.* evaluated their binding affinities to mouse CD1d and human CD1d molecules. Compared with KRN7000, the uptake of ^{3}H-thymidine in these biotinylated compounds significantly decreased. They found both **3-2a** and **3-3** can bind to CD1d, and their binding activities were comparable. This provided the first evidence for the earlier prediction that the sugar linkage does not significantly influence CD1d binding but is more likely to affect the TCR contact.[10] It also showed that **3-2b** significantly stimulated the proliferation of spleen cells at concentrations of 10 and 100 ng/mL, and its potency was a little weaker than **3-2a**, whereas **3-2a**-**3-2c** stimulated only slightly better than that of vehicle alone at lower concentration.[9] They also reported that analogue **3-1**, bearing NBD probe, induced a greater uptake of ^{3}H-thymidine than KRN7000.[8]

But the result may not accurately represent the degree of Vα14i interaction. Measurement of nucleoside assimilation would include proliferation of cells not only those restricted by CD1d molecules but also NKT cell subpopulations that have different function from the classical invariant NKT cells.[11] A new approach, measurement of cytokine release from the T cell hybridoma DN32D3, which express the canonical Vα-Jα281/Vβ8 TCR, showed that modification of the fatty acid chain with the biotin group made that a weaker antigen.[12] In dose-dependent experiments with mCD1d APCs, biotinylated compound **3-2a** gave a 1.5-2-fold lower stimulation than KRN7000.

The crystal structure of CD1d has two deep hydrophobic pockets.[13] The lipid chains will fit into the pockets. The presence of bulky groups at the end of the fatty acid chains would likely hinder them from entering the proper positions and perhaps this binding would not be recognizable by a NKT cell. Incorporation of labels at the end of the lipid chain may

interfere with the CD1d binding, thus influencing the biological activities of the KRN7000 analogs.

Therefore, the following focus is on changing the sugar moiety. Kawano *et al.* reported that □KRN7000 and αglucosylceramide (α-GlcCer) stimulated Vα14 NKT cells readily, while β-galactosylceramide are inactive, suggested that the α-anomeric conformation is important.[10] α-mannosylceramide, whose hydroxyl group configuration at C2" position is axial, showed no stimulatory activity. So the configuration of the 2-hydroxyl group is also important (**Figure 3.2**).[14]

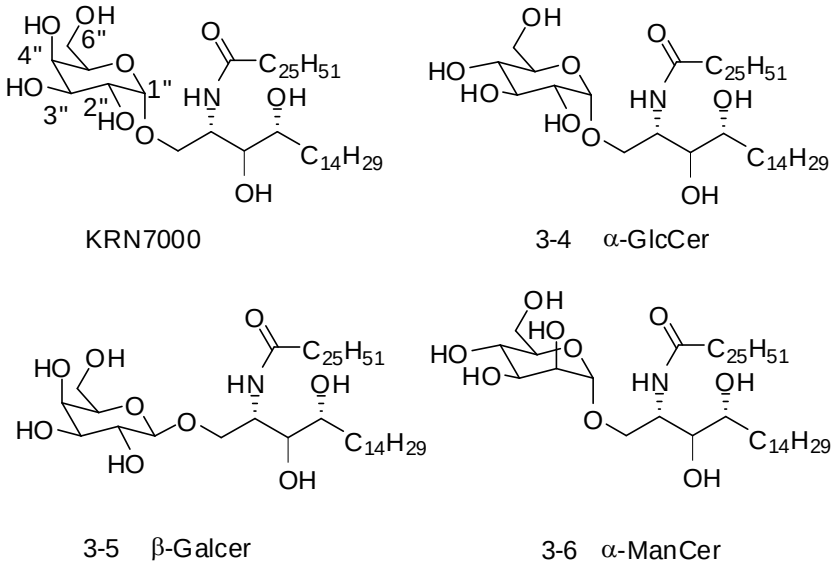


Figure 3.2. Structures of the compound 3-4 to 3-6.

The crystal structure of CD1d complexes of KRN7000 and related glycolipids illustrated that seven H-bonds are formed between CD1d with the hydroxyl groups and amide group of the glycolipid (**Figure 3.3**).[13] The lipid chains of the glycolipid point down into the binding pockets of the CD1d and the galactose head group is exposed to the CD1d surface between the α1 and α2 helices. The ceramide domain is stabilized by the hydrogen bonds between the amide nitrogen with Thr 156, 3 - and 4 - hydroxyl groups with Arg79 and Asp80. The α2

helix is involved mainly in hydrogen bonding to the galactose head group. The 2”- and 3”-hydroxyl groups of galactose are stabilized through the hydrogen bonds with Asp153 of the CD1d molecule (**Figure 3.3**).

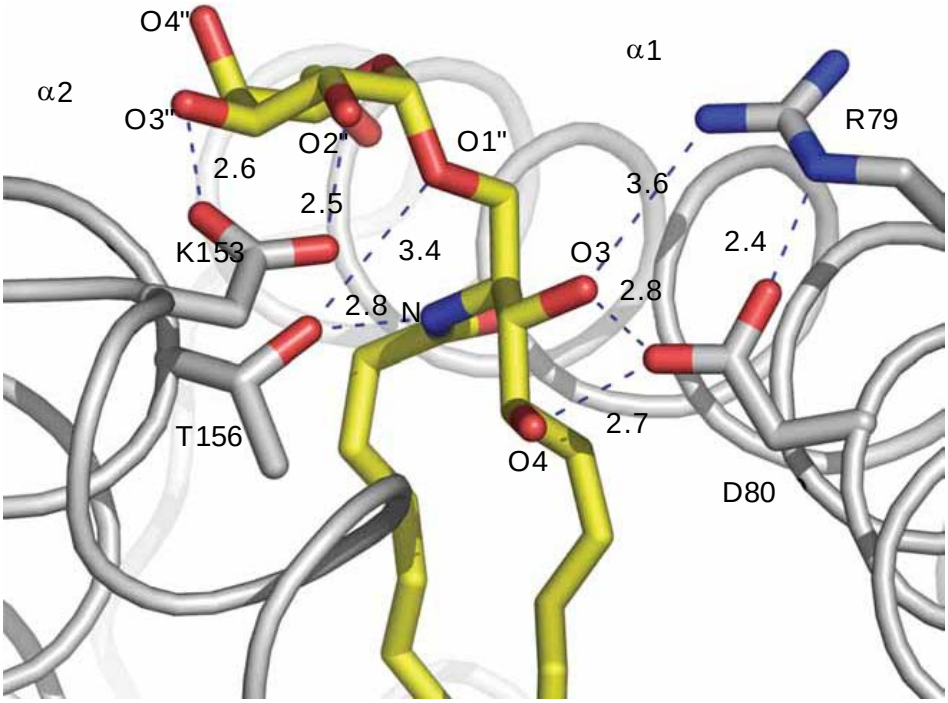


Figure 3.3. Hydrogen bond network between mouse CD1d and a glycolipid antigen. Dash lines represent hydrogen bonds. (Reproduced from ref. 13. Copyright 2005 Nature Immunology Publishing Group.)

So, the additional sugars at C2” and C3” would interfere with the hydrogen bonds. This makes the C4” and C6” potential positions to be substituted with labeled compounds. But from a recent study, the hydroxyl groups at C2”, C3”, and C4” positions of the galactose head group form hydrogen bonds with the α-chain of TCR.[15] It is clear that modification at C2”, C3” and C4” impair CD1d binding and simulation of NKT cells.

Kawano *et. al.* showed that the analogs of KRN7000 substituted with sugars at the C2”, C3”, or C6” positions stimulated NKT cells comparably with KRN7000.[10] While Prigozy *et.*

al. reported that the disaccharide substituted at the C2" and C3" positions can not stimulate NKT cells without removal of the terminal sugar to afford the corresponding galactose.[2] However, only 1-6 linked disaccharide can stimulate NKT cells without requiring cellular processing for NKT cell stimulation,[2] which suggested that the glycolipid-CD1d-TCR interaction tolerates a small molecule at C6" on the galactose. This was confirmed by the observation that small fluorophores and biotin appended at C6" position were tolerated in glycolipid-CD1d-TCR interaction **(Figure 3.4**).[7]

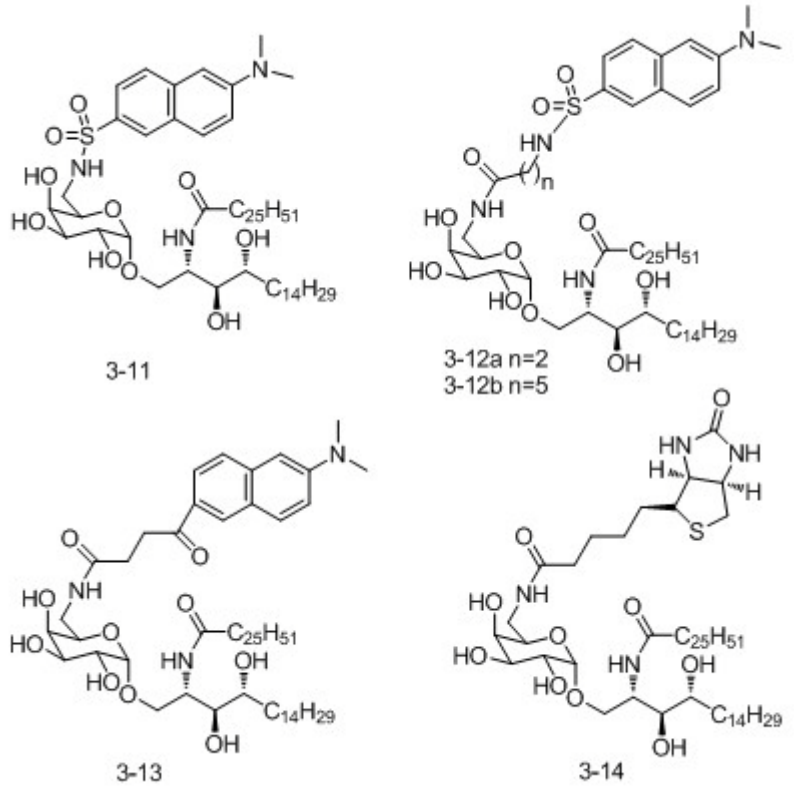


Figure 3.4. The structures of labeled compounds

The small molecules appended to KRN7000 reported by Zhou *et al.* included dansyl, prodan derivative, and biotin (**Figure 3.4**). Because it is not known how flexible a label will influence the CD1d and T cell receptor binding, three dansylated molecules (**3-11** to **3-12**) were made with varied chain lengths attached to galactose. Both dansyl (**3-11**) and prodan

(**3-13**) are fluorophores with high quantum yields, which will change the emission wavelength with increasing polarity of the solvent environment.[16] A remarkable shift is shown upon changing the solvent polarity, the spectral maxima extends from 401 nm (cyclohexane) to 531 nm (water). The environment of the fluorophore labeled compounds probably changes after contact with the CD1d. Thus, this binding should be visualized by changing the emission wavelength.

The abilities to stimulate NKT cells were studied by measuring IL-2 production through an immobilized CD1d assay (**Figure 3.5**).[11] Fluorophore-appended compound **3-11**, **3-12b**, and **2-13** showed high stimulatory effects at 1000 ng/mL compared with KRN7000, though **3-11** and **3-13** appeared to be less efficient at 100 ng/mL (**Figure 3.5**). (The tests for each compound were repeated at least three times) Biotinylated **3-14** slightly but more efficiently produced a larger quantity of IL-2 than KRN7000 (**Figure 3.5**).

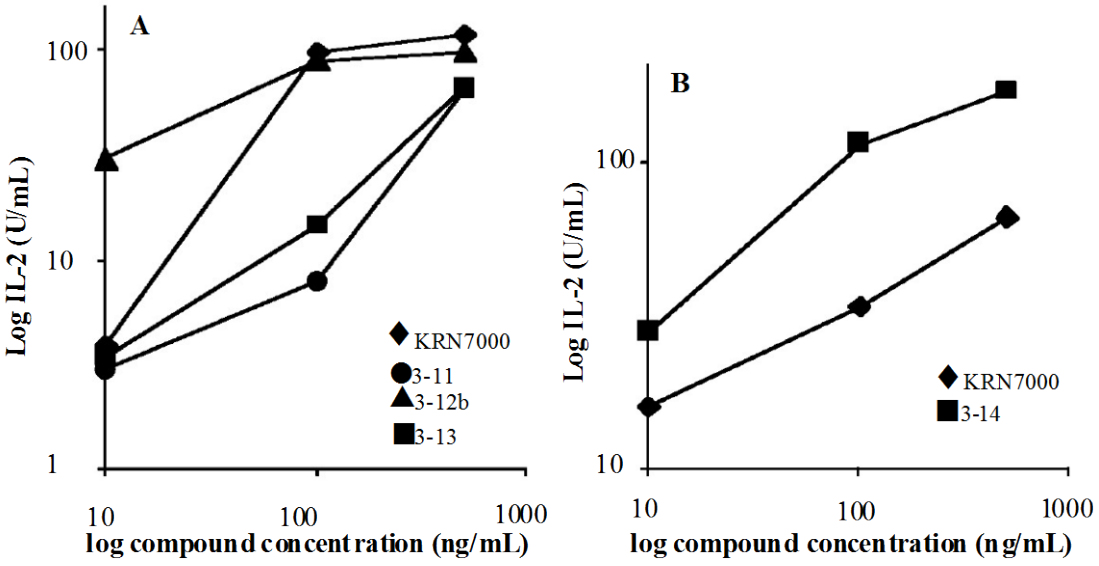


Figure 3.5. NKT cell stimulatory activity of compounds with fluorophore-appended (**A**) and biotin-appended (**B**).

An interesting finding is that attachment of a dansyl group directly at C6 or through a

tether did not cause a significant changing of stimulatory properties, although slightly better results can be seen in **2-6** whose tether between the bulky group and glycolipid was five carbons. The studies of Zhou *et. al.* suggest that compounds with modification at the C6" position may be helpful to clarify the effect of structural features on NKT cell stimulation.[7]

In order to further study the interactions between carbohydrate and T cell receptor, we designed and synthesized compounds, **3-15** to **3-20**, that have different molecular labels at the C6" position and varied lipid chain lengths. The labels included BODIPY, biotin-X , NBD and NBD-X (**Figure 3.6**).

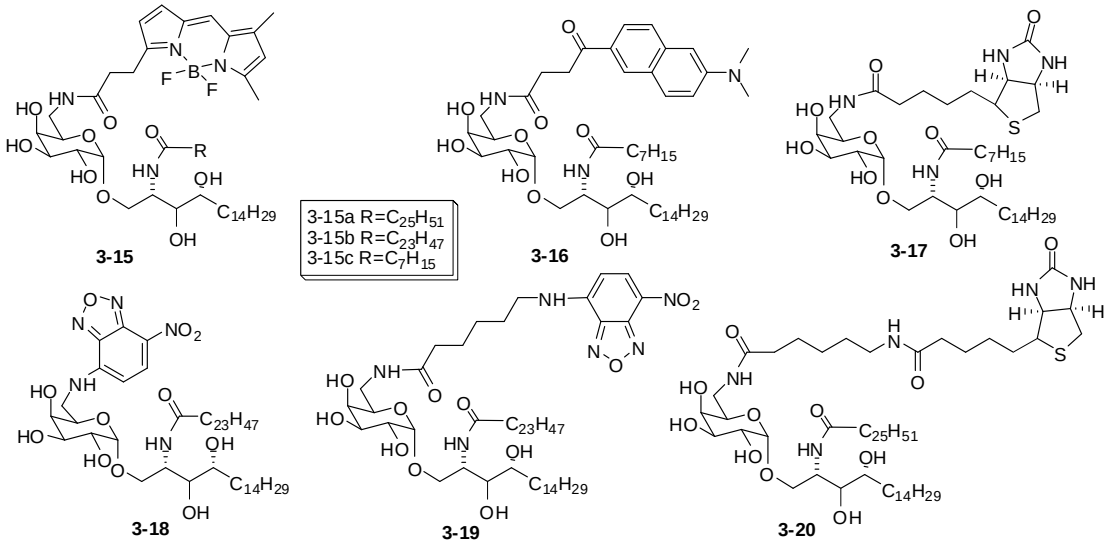


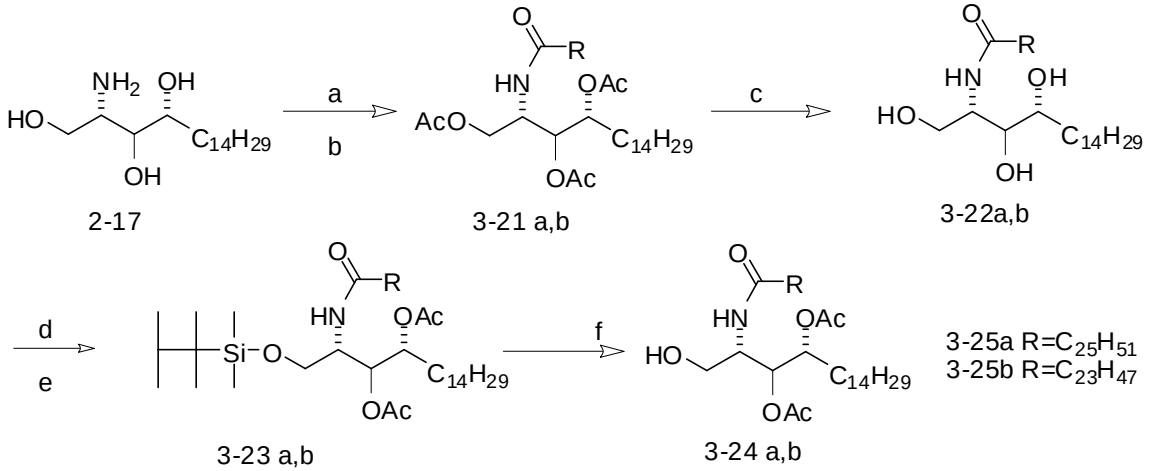
Figure 3.6. Structures of 3-15 to 3-20.

Using compounds labeled with NBD (**3-18**) and NBD-X **(3-19**), which are smaller than BODIPY (**3-15**) and prodon (**3-16**), may decrease the impact of fluorophore on trafficking. Compound **2-5**, the short lipid chain length analog of KRN7000, has an increased Th2 cytokine release profile compared with KRN7000.[13] However, the structures of the CD1d-glycolipid complexes were almost identical at the TCR interface.[13] There must be other explanations that account for the bias. The difference in glycolipid trafficking may

cause the variation in NKT cell responses. So we synthesized not only longer fatty acid chain labeled compounds but also shorter fatty acid chain labeled compounds (**Figure 3.6**). We also synthesized **3-20 (3-19)**, which have a longer tether than **3-14 (3-18)**, to determine how bulky the labels will tolerate the NKT cell stimulation.

3.2 Results and discussion

The preparations of ceramides **3-25a,b** used the same method as **2-21** (**Scheme 3.1).**



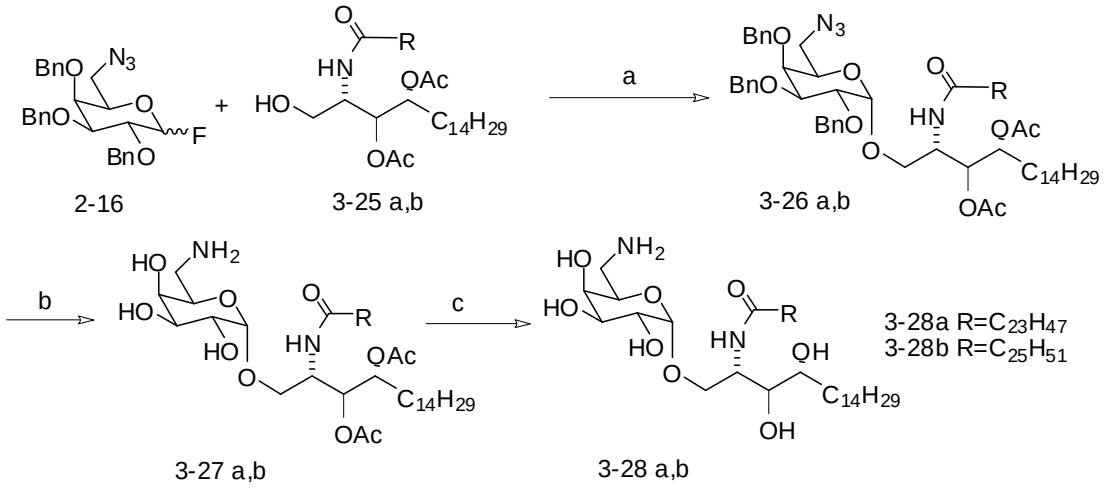
Reagents and conditions: a) RCO_2H, DCC, HOBt, THF; b) Ac_2O, DMAP, Et_3N, THF, 43-80%; c) NaOMe, MeOH; d) TDSCl, pyridine; e) Ac_2O, Pyridine, DMAP, 70-72%; f) HF (aq.), THF, 46-53%.

Scheme 3.1. Synthesis of ceramides **3-25a,b**.

Phytosphingosine **2-17** was coupled with fatty acid using HOBt/DCC conditions, followed by peracetylation of ceramide. Deacetylation of compound with sodium methoxide gave very pure **3-22** in white solid powder. Removal of the silyl ether was done with aqueous HF/THF solution yielding the ceramide acceptor **3.24**.

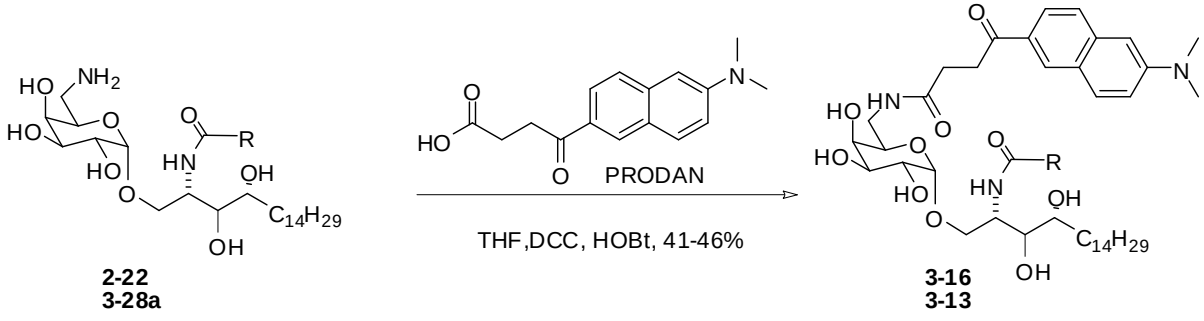
The fellowing steps were to make **3-28 a-c**, the 6- amino- 6- deoxygalactosylceramide (**Scheme 3.2**). The glycolipids (**3-26**) were synthesized ins the same manner as **2-22** method.[17] Reduction of the azide functional group and removal of the benzyl protecting group gave **3-27**. Deprotection of **3-27** was done by putting them directly into the methanol

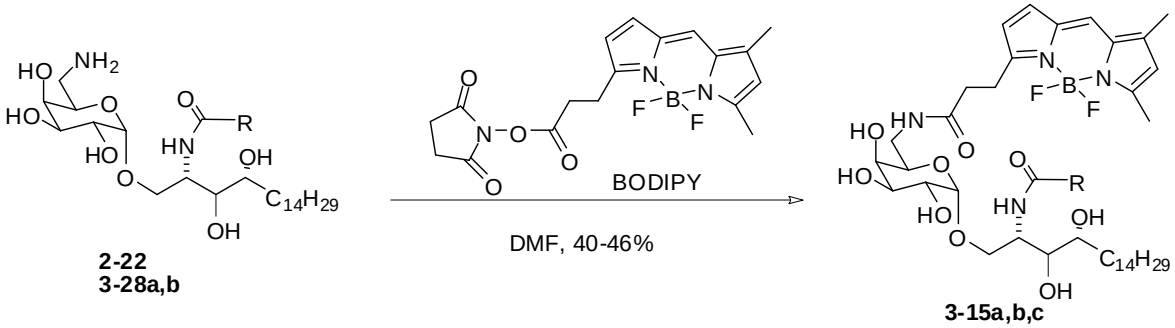
and water solutions to remove the acetyl group, yielding **3-28** as a white precipitate.

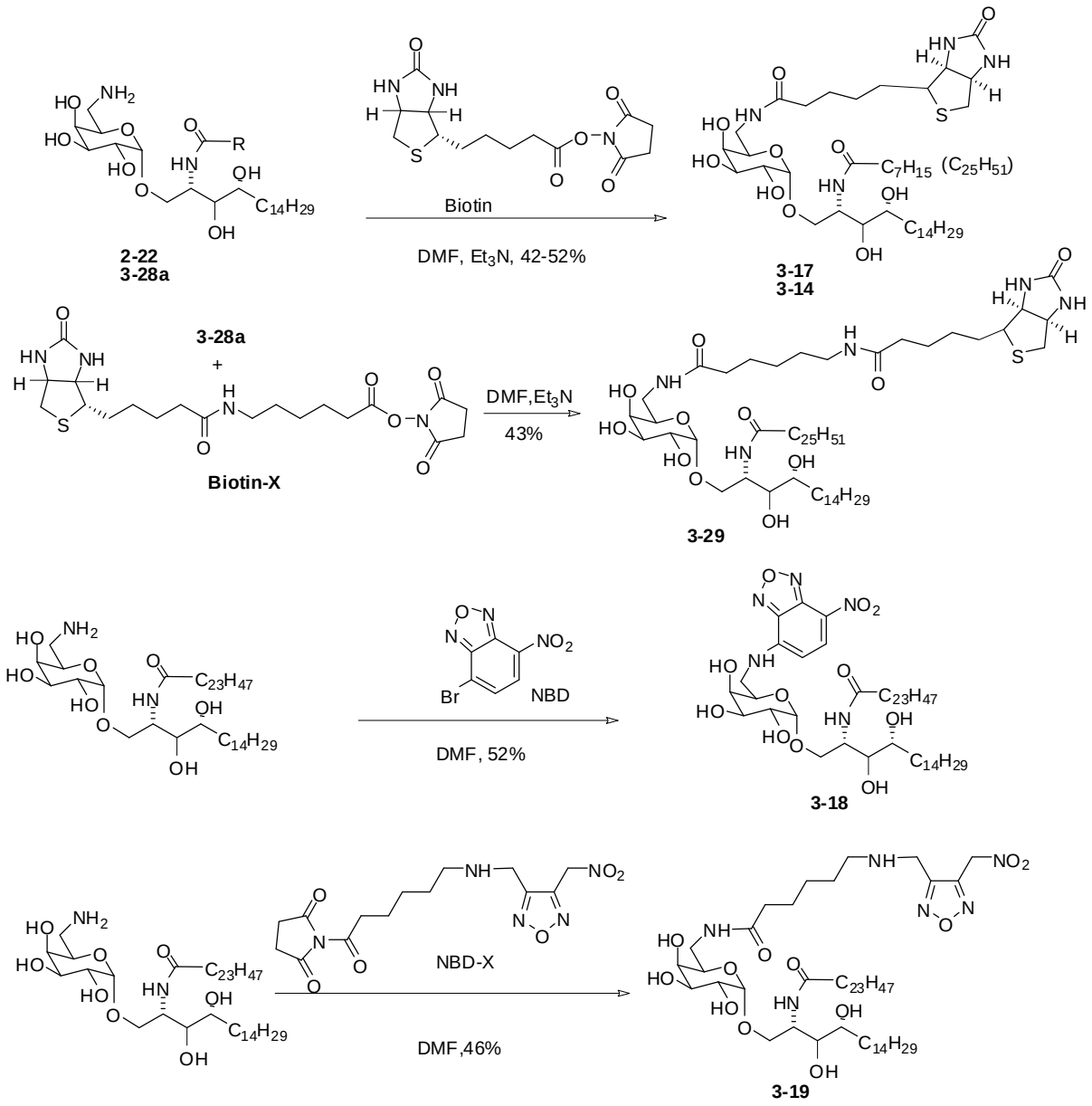


Scheme 3.2. Syntheses of compound **3-28.**

The activated *N*-hydroxysuccinimidyl esters of the appendages can directly couple with amine **2-22** and **3-28** in DMF or THF. The crude compounds can be purified directly on silica gel with modest yields (40-52%) without requiring aqueous workup (**Scheme 3.3**)







Scheme 3.3. Syntheses of *N*-appended-6”-amino-galactosylceramides.

.Fluorescent analogs of KRN7000 were studied to better understand glycolipids trafficking and processing. Niemann-Pick Type C1 (NPC1) is a late endosomal/lysosomal transmembrane protein that plays a role in transport of glycolipids and cholesterol. The large majority of mutations in the NPC1 gene described have been associated with severe cellular lipid trafficking impairment. Sagiv *et al.* reported that mice with NPC1 deficiencies showed a

massive loss of Va14i NKT cells and there was a block in glycolipid transport from late endosome (LE) to lysosome, which is important for NKT cells loading onto CD1d.[18] To study trafficking, cells were incubated with prodanylated **3-13**, at 37°C, for overnight. The cells included $NPC1^{+/+}$ or $NPC1^{-/-}$ bone marrow-derived dendritic cells (BMDC).

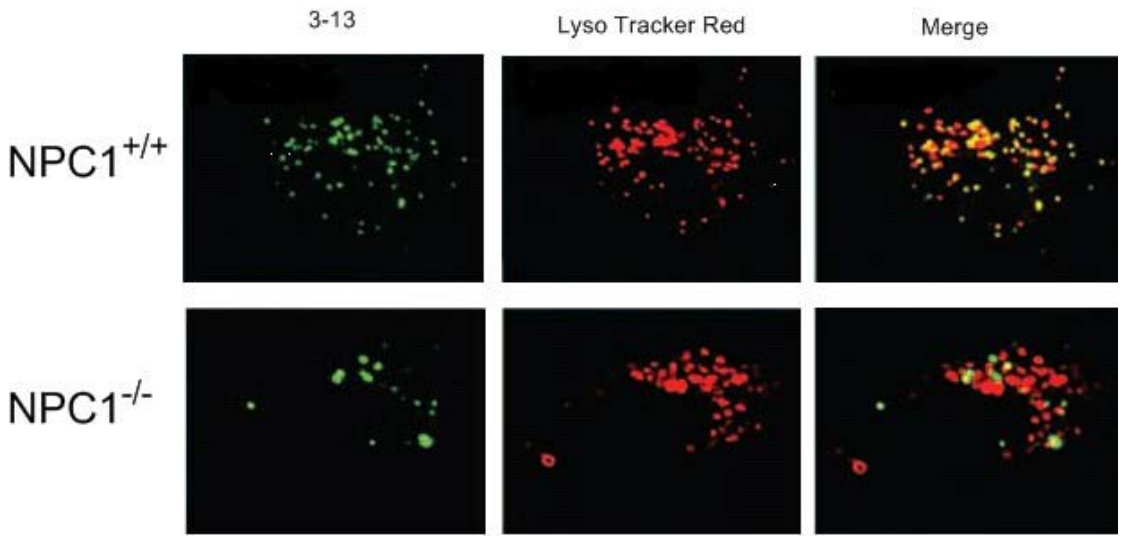


Figure 3.7. Trafficking of **3-13** in NPC1+/+ and NPC-/- BMDCs.[18]

Confocal microscopy was used to visualize lipid transport. Lyso Tracker Red was used to stain the lysosome and it can not stain endosome (**Figure 3.7**). The addition of red to green can get yellow. If there showed yellow, that meant **3-13** (green) may enter the lysosome (red) for loading onto CD1d. Therefore, **3-13** is a useful tool for studying the lipid transport from LE to lysosome. **3-13** accumulated in the lysosomal compartment of $NPC1^{+/+}$ BMDCs, while it failed to reach the lysosome of $PNC1^{-/-}$ BMCDs even over 24 h.

The information of the intracellular trafficking of CD1d and the mechanisms of lipid loading is well understood, but the intracellular trafficking of lipid antigens and location remains unclear. Furthermore, dynamics of CD1-lipid complex formation and dissociation need to be resolved. To visualize the site and kinetics of CD1d loading by KRN7000 analogs,

rat basophilic leukaemia (RBL) cells were incubated with BODIPY-labeled compounds, **3-15a** and **3-15c** (**Figure 3.8**).[19] Confocal microscropy studies were used to visualize the staining of the cell.

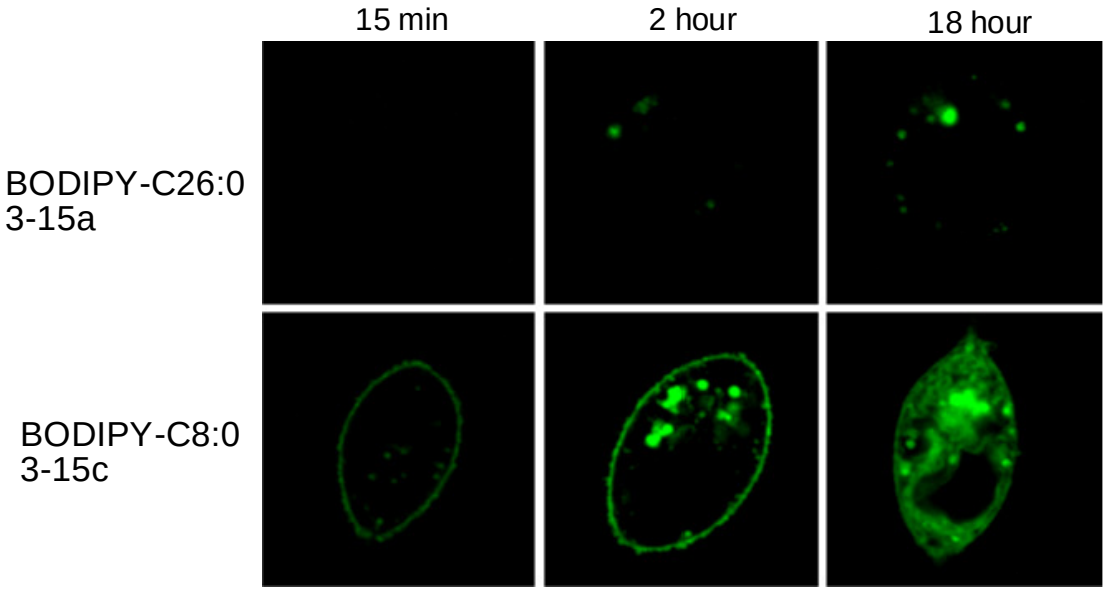


Figure 3.8. RBL. CD1d cells incubated with BODIPY-C26:0 or BODIPY-C8:0[19]

3-15c can be found to appear at the plasma membrane and intracellular vesicles as soon as 15 min after incubation, while **3-15a** only appeared after 2 h and in much lower amounts than **3-15c**.

To model the conditions leading to dissociation of CD1d-**3-15a,c** complexes in lysosome, Bai *et. al.* used a competitor lipid naturally present in the lysosome, glycosphingolipid GT1b, at different pH conditions, in a cell-free assay (**Figure 3.9**). The complexes of both were stable at either neutral or acidic conditions without introduction of GT1b. (**Figure 3.9** Left) If we use the GT1b, the complex of CD1d-**3-15a** will dissociate within less than 1 min. But the complex of CD1d-**3-15c** remained stable (**Figure 3.9** Right).

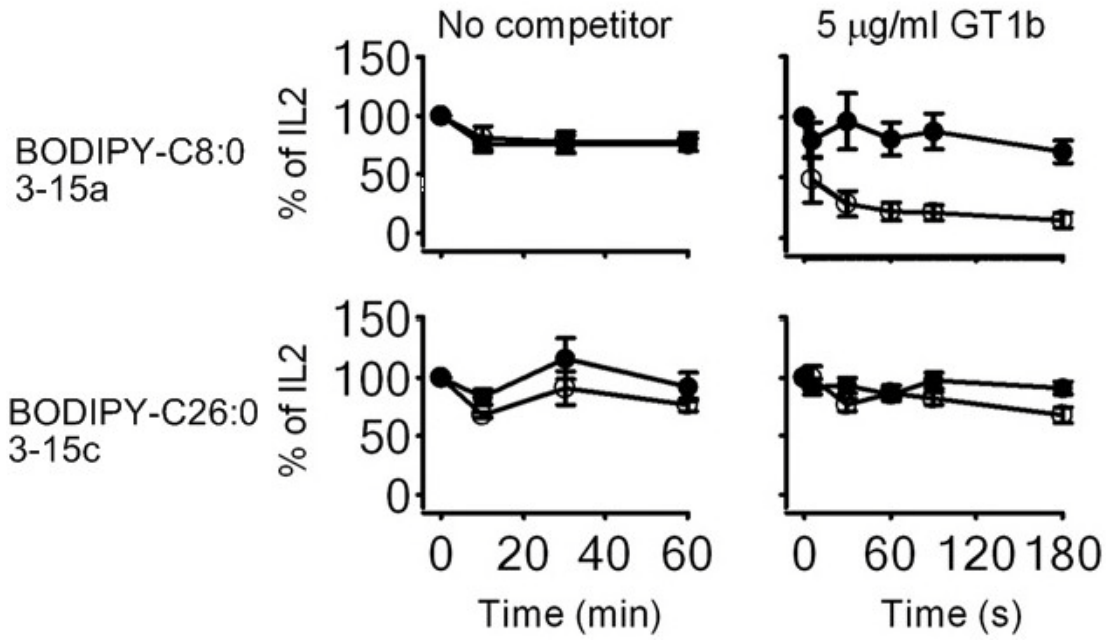


Figure 3.9. Influence of pH and Free lipids on Dissociation of CD1d-lipid complexes.[19]

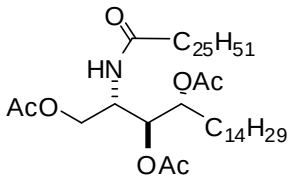
3.3 Discussions:

A confocal microscopy study of prodan-appended **3-13** found that GSLs must be transported from the LE to the lysosome to be loaded into CD1d and thus to stimulate NKT cells. In cell free assays, the fast dissociation of CD1d with short lipid variant of KRN7000 occurred under acidic conditions with addition of an endogenous competitor lipid. These findings may help to explain the decreased stimulatory properties of short KRN7000 variant.

Our future work may include incorporating radio labels at C6 position, which will help us in monitoring substrate trafficking.

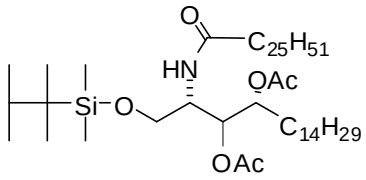
Experimental Procedures

Materials and General Methods.^{1}H NMR and ^{13}C NMR, were recorded on Varian Unity 500 MHz or Varian Unity 300 MHz NMR spectrometers using 99.8% CDCl3 with 0.05% v/v TMS, 99.8% CD3OD with 0.05% v/v TMS. Mass spectrometric data were obtained on JEOL SX 102 A spectrometer or Agilent 1100 series spectrometer. Flash chromatography was performed using 230-400 mesh silica gel. Thin layer chromatography was performed on aluminumbacked, 254 nm UV-active plates with a silica gel particle size of 250 □m. Chemicals were obtained from Sigma and Aldrich and were used as received unless otherwise noted.



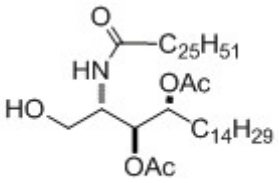
3-21a

Preparation of 3-21a. Same procedure as synthesis of **2-18** (80 %). ^{1}H NMR ($CDCl_3$, 500 MHz) δ 5.91 (d, *J* = 9.5, 1 H), 5.09 (dd, *J* = 8.5, 3.0 Hz, 2 H), 4.92 (dt, *J* = 3.0, 10.5 Hz, 1 H), 4.48 (m, 1 H), 4.28 (dd, *J* = 5.5, 4.5 Hz, 1 H), 3.98 (dd, *J* = 3.0, 11.5 Hz, 1 H), 2.19 (t, *J* = 7.5 Hz, 2 H), 2.06 (s, 3 H), 2.03 (s, 3 H), 2.02 (s, 3 H), 1.64-1.60 (m, 4 H), 1.32-1.18 (m, 68 H), 0.86 (t, *J* = 6.5 Hz, 6 H); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 172.98, 171.24, 171.01, 170.20, 73.15, 72.17, 63.09, 47.59, 36.93, 32.11, 29.89, 29.79, 29.72, 29.55, 29.49, 29.4, 29.43, 28.30, 25.82, 25.74, 22.82, 21.20, 20.95, 20.92, 14.30. HRFAB-MS (thioglycerol + Na^+ matrix) *m/e* ($[M + Na]^+$) 814.6526(100%), calcd 814.6537.



3-23a

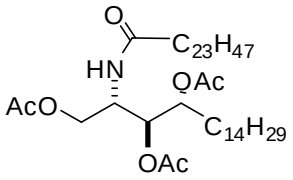
Preparation of 3-23a. Same procedure as synthesis of **2-20** (70 % for 2 steps). ^{1}H NMR ($CDCl_3$, 500 MHz) δ 5.90 (d, *J* = 10.0, 1 H), 5.22 (d, *J* = 9.5, 1 H), 4.90 (d, *J* = 10.0 Hz, 1 H), 4.21 (t, *J* = 9.5 Hz, 1 H), 3.64-3.53 (m, 2 H), 2.19 (t, *J* = 7.5 Hz, 2 H), 2.07 (s, 3 H), 2.02 (s, 3 H), 1.79-1.56 (m, 4 H), 1.40-1.14 (m, 68 H), 0.91-0.79 (m, 18 H), 0.06-0.01 (m, 6 H). ^{13}C NMR ($CDCl_3$, 125 MHz) δ 172.81, 171.28, 169.82, 73.65, 71.80, 71.55, 61.50, 49.34, 37.20, 34.45, 32.16, 29.93, 29.88, 29.72, 29.86, 29.83, 29.74, 29.59, 29.52, 27.94, 25.94, 25.40, 22.92, 21.27, 21.06, 20.47, 18.72, 14.34, 6.13, 0.21, -3.55, -3.64; HRMS (ESI) calcd for $C_{54}H_{106}NO_6Si$ $[M+H]^+$ 892.77839, found 892.78177.



3-25a

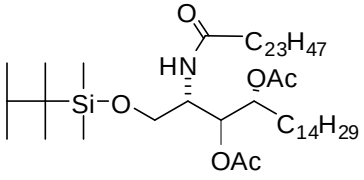
Preparation of 3-25a. Same procedure as synthesis of **2-21** (53%). ^{1}H NMR ($CDCl_3$, 500 MHz) δ 6.21 (d, *J* = 9.2 Hz, 1 H), 5.05 (dd, *J* = 9.3, 2.4 Hz, 1 H), 4.96 (dt, *J* = 10.3, 2.4 Hz, 1 H), 4.18 (tt, *J* = 9.3, 2.9 Hz, 1 H), 4.30 (dd, *J* = 11.2, 4.9 Hz, 1 H), 3.58 (m, 2 H), 2.72 (br s, 1 Hz), 2.22 (t, *J* = 7.6 Hz, 2 H), 2.15 (s, 3 H), 2.04 (s, 3 H), 1.70-1.58 (m, 4 H), 1.25 (brs, 68 H), 0.88 (t, *J* = 6.8 Hz, 6 H); ^{13}C NMR ($CDCl_3$, 125 MHz) 173.43, 171.81, 171.26, 73.37, 72.01, 61.72, 49.73, 36.97, 32.13, 29.91, 29.80, 29.73, 29.58, 29.51, 28.03, 25.91, 22.90, 21.25,

21.10, 14.33; HRMS (FAB) *m/z* for $C_{50}H_{93}NNaO_6$ ([M+Na]+) 802.68930 (100%), calc. 802.68951.



3-21b

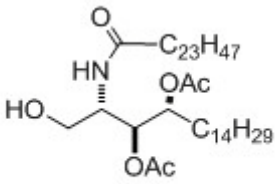
Preparation of 3-21b. Same procedure as synthesis of **3-21a**, substituting with lignoceric acid (43%). ^{1}H NMR ($CDCl_3$, 500 MHz) δ 6.05 (d, *J* = 9.5 Hz, 1 H), 5.11 (dd, *J* = 8.4, 3.1 Hz, 1 H), 4.93 (dt, *J* = 9.7, 3.2 Hz, 1 H), 4.49 (m, 1 H), 4.29 (dd, *J* = 11.6, 5.0 Hz, 1 H), 3.99 (dd, *J* = 11.7, 2.9 97 Hz, 1 H), 2.20 (t, *J* = 7.6 Hz, 2 H), 2.07 (s, 3 H), 2.04 (s, 6 H), 1.75-1.54 (m, 4 H), 1.25 (br s, 64 H), 0.88 (t, *J* = 6.6 Hz, 6 H); ^{13}C NMR ($CDCl_3$, 125 MHz) 173.03, 171.33, 171.02, 170.28, 73.18, 72.19, 63.10, 47.61, 37.00, 32.15, 29.75, 29.58, 29.51, 28.34, 25.85, 25.77, 22.92, 21.27, 21.00, 14.34; HRMS (FAB) *m/z* for $C_{43}H_{82}NO_7$ ([M+H]+) 724.6211 (100%), calc. 724.6086.



3-23b

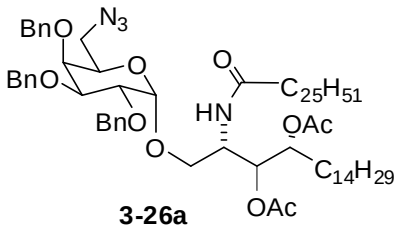
Preparation of 3-23b. Same procedure as synthesis of **3-23a** (72%). ^{1}H NMR ($CDCl_3$, 300 MHz) δ 6.44 (d, *J* = 9.3 Hz, 1 H), 5.06 (dd, *J* = 9.3, 2.4 Hz, 1 H), 4.95 (dt, *J* = 9.9, 2.8 Hz, 1 H), 4.17 (tt, *J* = 9.4, 2.6 Hz, 1 H), 3.57 (m, 2 H), 2.80 (t, *J* = 6.5 Hz, 1 H), 2.22 (t, *J* = 7.6 Hz, 2 H), 2.14 (s, 3 H), 2.04 (s, 3 H), 1.78-1.56 (m, 4 H), 1.25 (br s, 64 H), 0.88 (t, *J* = 6.6 Hz, 6

H); ^{13}C NMR ($CDCl_3$, 75 MHz) 73.39, 171.67, 171.31, 73.42, 72.90, 61.69, 101 49.76, 36.96, 32.12, 32.10, 29.91, 29.86, 29.77, 29.73, 29.58, 29.56, 29.51, 28.00, 25.91, 22.88, 21.24, 21.08, 14.31; HRMS (FAB) *m/z* for $C_{41}H_{79}NNaO_6$ ($[M+Na]^+$) 704.5798 (100%), calc. 704.5805.



3-25b

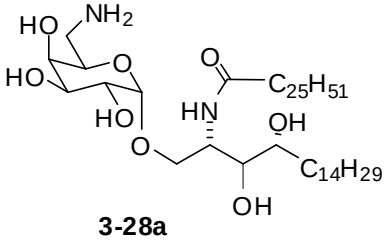
Preparation of 3-25b. Same procedure as synthesis of **3-25a** (46%). ^{1}H NMR ($CDCl_3$, 500 MHz) δ 6.22 (d, *J* = 9.0 Hz, 1 H), 5.03 (dd, *J* = 9.0, 2.5 Hz, 1 H), 4.97 (dt, *J* = 10.5, 3.0 Hz, 1 H), 4.16 (tt, *J* = 9.5, 3.0 Hz, 1 H), 3.58 (m, 2 H), 2.22 (t, *J* = 7.5 Hz, 2 H), 2.15 (s, 3 H), 2.04 (s, 3 H), 1.70-1.52 (m, 4 H), 1.25 (brs, 64 H), 0.88 (t, *J* = 7.0 Hz, 6 H); ^{13}C NMR ($CDCl_3$, 125 MHz) 173.37, 171.92, 171.13, 73.34, 61.81, 49.81, 37.02, 32.15, 29.93, 29.88, 29.73, 29.59, 29.51, 28.17, 25.94, 25.91, 22.91, 21.25, 21.11, 14.34; HRMS (FAB) *m/z* for $C_{50}H_{93}NNaO_6$ ($[M+Na]^+$) 774.6584 (100%), calc. 774.6588.



3-26a

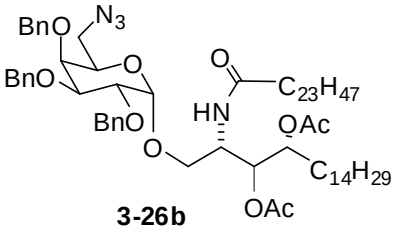
Preparation of 3-26a. Same procedure as synthesis of **2-22** (52%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.41-7.25 (m, 15 H), 6.59 (d , *J* = 10.0 Hz, 1 H), 5.21 (dd, *J* = 9.5, 2.5 Hz, 1 H), 4.96 (d, *J* = 11.5 Hz, 1 H), 4.92 (dt, *J* = 10.5, 3.0, 1 H), 4.83 (d, *J* = 11.5, 1 H), 4.78-4.73 (m, 3 H), 4.68 (d, *J* = 11.5 Hz, 1 H), 4.58 (d, *J* = 11.5 Hz, 1 H), 4.36 (tt, *J* = 6.0, 3.5 Hz, 1 H),

4.03 (dd, *J* = 9.5, 3.5 Hz, 1 H), 3.93 (dd, *J* = 8.0, 5.0 Hz, 1H), 3.89-3.83 (m, 3 H), 3.62-3.56 (m, 2 H), 3.06 (dd, *J* = 12.5, 5.0 Hz, 1 H), 2.15 (t, *J* = 7.5 Hz, 2 H), 2.06 (s, 3 H), 2.02 (s, 3 H), 1.71-1.58 (m, 4 H), 1.31-1.15 (m, 68 H), δ0.89-0.85 (m, 6 H). ^{13}C NMR ($CDCl_3$, 125 MHz) δ 173.12, 171.09, 170.27, 138.68, 138.34, 138.12, 128.73, 128.59. 128.27, 128.15, 128.03, 127.82, 127.75, 99.89, 78.66, 76.54, 74.86, 74.81, 73.70, 73.63, 73.82, 71.57, 70.50, 70.02, 51.40, 48.33, 36.87, 32.10. 29.88, 29.77, 29.60. 29.55, 29.50, 27.68, 25.88, 25.79, 22.87, 21.17, 21.09, 14.31. HRMS (FAB) *m/z* $C_{75}H_{120}N_4NaO_{10}$ ([M+Na]+) 1259.8907 (100%), calc. 1259.8902.



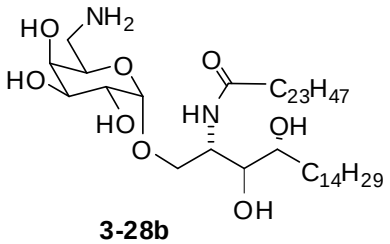
3-28a

Preparation of 3-28a. Same procedure as synthesis of **2-23** (39%). ^{1}H NMR ($CDCl_3$:CD_3OD 95:5) δ 4.91 (d, *J* = 4.0 Hz, 1 H), 4.21 (m, 1 H), 3.88 (m, 2 H), 3.80 (dd, *J* = 10.0, 3.5 Hz, 1 H), 3.75 (m, 1 H), 3.70 (dd, *J* = 10.0, 3.5 Hz, 1 H), 3.62-3.51 (m, 10 H), 3.06 (dd, *J* = 13.0, 7.5 Hz, 1 H), 2.90 (dd, *J* = 13.0, 4.0 Hz, 1 H), 2.19 (t, *J* = 8.0 Hz, 2 H), 1.68-1.25 (m, 73 H), 0.88 (t, *J* = 7.0 Hz, 6 H); ^{13}C NMR (CDCl3:CD3OD 95:5) 174.36, 99.75, 75.17, 72.06, 70.84, 70.37, 70.22, 68.92, 67.31, 50.36, 42.40, 36.62, 33.02, 31.97, 29.83, 29.77, 29.74, 29.71, 29.70, 29.61, 29.46, 29.42, 25.90, 25.87, 22.73, 14.11; HRMS (FAB) *m/z* $C_{50}H_{100}N_2NaO_8$ ($[M+Na]^+$) 879.7384 (100%), calc. 879.7372.



3-26b

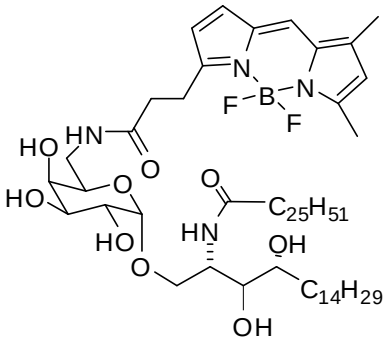
Preparation of 3-26b. Same procedure as synthesis of **3-26a** (47%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.41-7.25 (m, 15 H), 6.49 (d , *J* = 10.0 Hz, 1 H), 5.21 (dd, *J* = 9.5, 2.5 Hz, 1 H), 4.98-4.90 (m, 2 H), 4.83 (d, *J* = 11.5, 1 H), 4.75 (t, *J* = 11.5, 3 H), 4.63 (d, *J* = 11.5, 1 H), 4.53 (d, *J* = 11.5 Hz, 1 H), 4.35 (tt, *J* = 10.0, 2.5 Hz, 1 H), 4.01 (dd, *J* = 9.5, 3.5 Hz, 1 H), 3.95 (dd, *J* = 8.0, 5.0 Hz, 1H), 3.89-3.83 (m, 3 H), 3.61-3.51 (m, 2 H), 3.04 (dd, *J* = 12.5, 5.0 Hz, 1 H), 2.15 (t, *J* = 7.5 Hz, 2 H), 2.06 (s, 3 H), 2.00 (s, 3 H), 1.71-1.58 (m, 4 H), 1.31-1.15 (m, 64 H), δ0.89-0.85 (m, 6 H). ^{13}C NMR ($CDCl_3$, 125 MHz) δ 173.11, 171.04, 170.25, 138.64, 138.31, 138.11, 128.67, 128.49. 128.17, 128.05, 128.01, 127.92, 127.54, 99.82, 78.61, 76.51, 74.86, 74.71, 73.80, 73.33, 71.53, 70.51, 70.01, 51.50, 48.43, 36.97, 32.13. 29.78, 29.67, 29.50. 29.41, 29.41, 27.64, 25.82, 25.73, 22.81, 21.14, 21.03, 14.34. HRMS (FAB) *m/z* $C_{73}H_{117}N_4O_{10}$ ($[M+H]^+$) 1209.8776 (99.3%), calc. 1209.8770.



3-28b

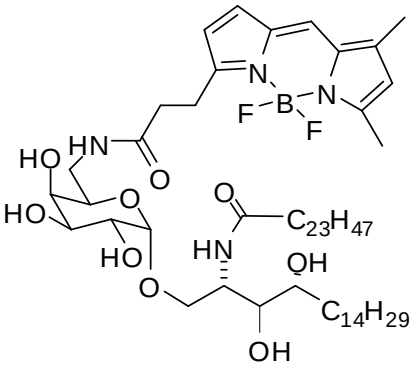
Preparation of 3-28b. Same procedure as synthesis of **2-23** (36%). ^{1}H NMR (500 MHz, $CDCl_3$:CD_3OD 95:5) δ 4.91 (d, *J* = 3.5 Hz, 1 H), 4.21 (m, 1 H), 3.86 (m, 2 H), 3.80 (dd, *J* = 10.0, 3.5 Hz, 1 H), 3.75-3.69 (m, 2 H), 3.63 (dd, *J* = 10.0, 4.5 Hz, 1 H), 3.59-3.54 (m , 2 H), 2.95 (dd, *J* = 13.0, 4.0 Hz, 1 H), 2.79 (dd, *J* = 12.5, 3.5 Hz, 1 H), 2.20 (t, *J* = 8.0 Hz, 2 H), 1.68-1.56 (m, 4 H), 1.36-1.21 (m, 64 H), 0.88 (t, *J* = 7.0 Hz, 6 H); ^{13}C NMR (125 MHz, CDCl3:CD3OD 95:5) 174.50, 99.45, 74.26, 71.80, 70.89, 70.36, 70.06, 68.66, 66.86, 50.35,

48.91, 48.74, 48.57, 48.40, 48.23, 41.97, 36.34, 32.07, 31.82, 29.63, 29.58, 29.38, 29.30, 29.26, 25.81, 25.78, 22.56, 13.88; HRMS (FAB) *m/z* $C_{48}H_{96}N_2NaO_8$ ([M+Na]+) 851.7054 (100%), calc. 851.7064.



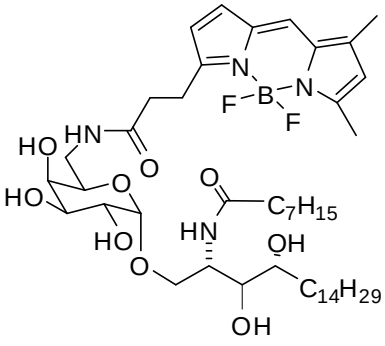
3-15a

Preparation of 3-15a. Compound **3-28a** (15.7 mg, 0.026 mmol) and BODIPY (5.0 mg, 0.013 mmol) were dissolved in dry DMF (1.5 mL), stirred for overnight. The solvent was concentrated in vacuo. Flash chromatography (SiO_2, MeOH:$CHCl_2$ 2:98) was performed to afford the product as a green solid **3-15a** (4.1mg, 36.3%). ^{1}H NMR (MeOD/$CDCl_3$, 500 MHz) δ 7.38 (s, 2 H), 7.16 (s, 1 H), 6.92 (d, *J* = 4.0 Hz, 1 H), 6.30 (d, *J* = 4.0 Hz, 1 H), 6.15 (s, 1 H), 4.86 (d, *J* = 4.0 Hz, 1 H), 4.15-4.13 (m, 1 H), 3.92-3.75 (m, 4 H), 3.72-3.68 (m, 1 H), 3.63 (dd, *J* = 10.0, 4.5 Hz, 1 H), 3.60-3.53 (m, 2 H), 3.51 (t, *J* = 7.0 Hz, 1 H), 3.27-3.22 (m, 1 H), 2.64 (t, *J* = 7.0 Hz, 2 H), 2.55 (s, 3 H), 2.27 (s, 3 H), 2.17 (t, *J* = 7.5 Hz, 2 H), 1.61-1.51 (m, 4 H), 1.43-1.13 (m, 34 H), 0.88 (t, *J* = 7.0 Hz, 6 H); ^{13}C NMR (MeOD/$CDCl_3$, 125 MHz) δ 174.71, 173.99, 160.53, 157.08, 144.43, 135.36, 133.46, 128.47, 124.17, 120.67, 116.98, 99.59, 74.45, 72.09, 69.91, 69.27, 69.25, 68.90, 68.82, 67.14, 50.51, 39.71, 36.51, 35.26, 32.30, 32.01, 29.82, 29.78, 29.56, 29.45, 25.96, 24.70, 22.76, 14.85, 14.11, 11.28; HRMS (ESI) calcd for $C_{64}H_{113}BF_2N_4NaO_9$[M+Na]$^+$: 1153.84609, found: 1153.84720.



3-15b

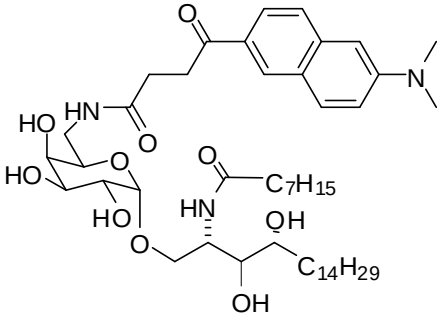
Preparation of 3-15b. Same procedure as synthesis of **3-15a** (46%). ^{1}H NMR (MeOD/$CDCl_3$, 500 MHz) δ 7.17 (s, 1 H), 6.93 (d, *J* = 4.0 Hz, 1 H), 6.29 (d, *J* = 4.0 Hz, 1 H), 6.11 (s, 1 H), 4.84 (d, *J* = 4.0 Hz, 1 H), 4.14 (d, *J* = 4.0, 1 H), 3.82-3.75 (m, 4 H), 3.71 (dd, *J* = 10.0, 3.0 Hz, 1 H), 3.63 (dd, *J* = 11.0, 4.5 Hz, 1 H), 3.68-3.53 (m, 2 H), 3.50 (t, *J* = 7.0 Hz, 1 H), 3.22-3.21 (m, 1 H), 2.63(t, *J* = 8.5 Hz, 2 H), 2.54 (s, 3 H), 2.27 (s, 3 H), 2.17 (t, *J* = 7.5 Hz, 2 H), 1.66-1.48 (m, 4 H), 1.41-1.10 (m, 64 H), 0.88 (t, *J* = 6.0 Hz, 6 H); NMR (^{13}C, 5% CD_3OD in $CDCl_3$) δ 174.46, 173.99, 160.52, 157.09, 144.43, 128.06, 124.35, 121.72, 116.49, 99.58, 76.88, 74.45, 72.09, 69.90, 69.25, 68.89, 68.81 67.14, 62.15, 50.50, 49.41, 49.24, 49.07, 48.90, 48.72, 48.55, 48.38, 39.70, 36.51, 35.26, 32.29, 32.01, 29.82, 29.78, 29.56, 29.44, 25.95, 24.70, 22.75, 14.85, 14.10, 11.27.; HRFAB-MS (thioglycerol + Na+ matrix) *m/e* ($[M + Na]^+$) 1125.8158(100%), calcd 1125.8153.



3-15c

Preparation of 3-15c. Same procedure as synthesis of **3-15a** (41%).; ^{1}H NMR

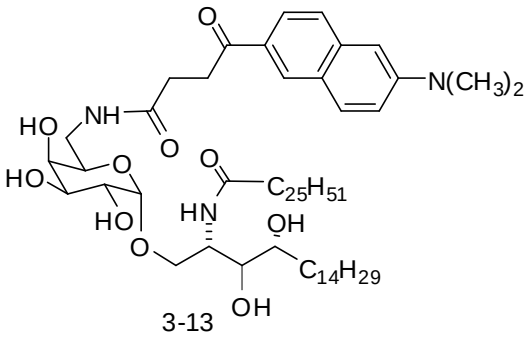
(MeOD/$CDCl_3$, 500 MHz) δ 7.41 (s, 2 H), 7.18 (s, 1 H), 6.93 (d, *J* = 4.0 Hz, 1 H), 6.31 (d, *J* = 4.0 Hz, 1 H), 6.16 (s, 1 H), 4.87 (d, *J* = 3.5 Hz, 1 H), 4.15-4.13 (m, 1 H), 3.82-3.75 (m, 4 H), 3.71 (dd, *J* = 10.0, 3.0 Hz, 1 H), 3.63 (dd, *J* = 11.0, 4.5 Hz, 1 H), 3.58-3.53 (m, 2 H), 3.51 (t, *J* = 7.0 Hz, 1 H), 3.28-3.22 (m, 1 H), 2.64 (t, *J* = 7.5 Hz, 2 H), 2.55 (s, 3 H), 2.28 (s, 3 H), 2.18 (t, *J* = 7.5 Hz, 2 H), 1.62-1.52 (m, 4 H), 1.41-1.10 (m, 34 H), 0.88 (t, *J* = 6.0 Hz, 3 H), 0.87 (t, *J* = 6.0 Hz, 3 H); ^{13}C NMR (MeOD/$CDCl_3$, 125 MHz) δ 174.73, 173.99, 160.49, 157.03, 144.45, 135.32, 133.43, 128.46, 124.18, 120.64, 116.90, 99.54, 74.27, 71.98, 70.40, 69.84, 69.30, 68.90, 68.73, 67.00, 50.47, 39.71, 36.42, 35.15, 32.12, 31.98, 31.77, 29.78, 29.72, 29.42, 29.34, 29.11, 25.91, 24.62, 22.73, 22.66, 14.79, 14.07, 14.00, 11.23; HRMS (ESI) calcd for $C_{46}H_{77}BF_2N_4NaO_9[M+H]^+$: 879.58244, found: 879.58390.



3-16

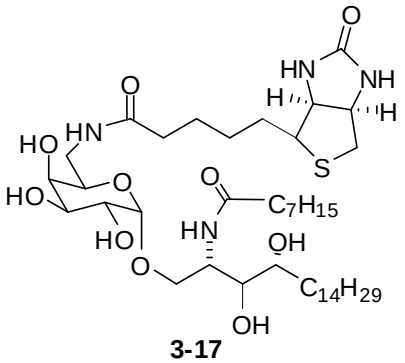
Preparation of 3-16. PRODAN (6.6 mg, 0.018 mmol) was added to a solution of **2-22** (5.0 mg, 0.0058 mmol) in pyridine (1 mL), and the mixture was stirred for 12 h. The pyridine was removed *in vacuo*, and the product was purified by column chromatography (SiO_2, 10% MeOH in CH_2Cl_2) giving **3-16** (3.0 mg, 46% yield). NMR (1H, 5% CD_3OD in CDCl3) δ 7.89 (dd, *J* = 9.0, 2.0 Hz, 1 H), 7.81 (d, *J* = 8.5 Hz, 1 H), 7.63 (d, *J* = 9.5 Hz, 1 H), 7.19 (dd, *J* = 9.5, 2.0 Hz, 1 H), 6.87 (d, *J* = 2.5 Hz, 1 H), 4.88 (d, *J* = 4.0 Hz, 1 H), 4.19 (dd, *J* = 9.0, 4.5 Hz, 1 H), 3.89 (dd, *J* = 12.0, 5.0 Hz, 1 H), 3.82 – 3.38 (m, 9 H), 3.24 (dd, *J* = 13.5, 6.5 Hz, 1 H), 3.19 (s, 6 H), 2.65 (t, *J* = 6.5 Hz, 2 H), 2.18 (m, 2H), 1.65 – 1.23 (m, 38 H), 0.87 (t, *J* = 7.0

Hz, 6 H); NMR (^{13}C, 5% CD_3OD in $CDCl_3$) 198.86, 174.38, 174.26, 150.37, 137.83, 130.75, 130.19, 129.67, 126.20, 124.91, 124.11, 116.29, 105.16, 99.45, 94.76, 74.75, 72.16, 69.80, 68.79, 67.27, 52.27, 50.31, 49.66, 49.49, 49.31, 49.14, 48.97, 48.80, 48.63, 40.30, 39.46, 36.44, 33.39, 32.66, 32.12, 31.85, 31.62, 29.94, 29.72, 29.65, 29.59, 29.29, 29.21, 28.96, 25.79, 24.85, 22.60, 22.53, 14.00, 13.93; HRFAB-MS (thioglycerol + Na+ matrix) *m/e* ([M + Na]+) 880.5673 (100%), calcd 880.5663.



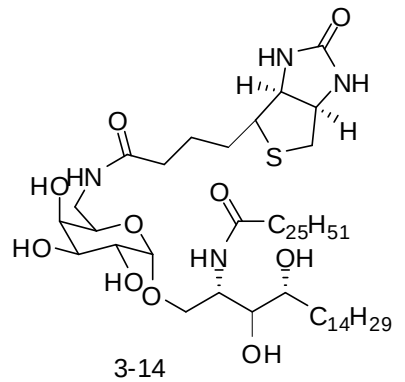
3-13

Preparation of 3-13. Same procedure as synthesis of **3-16** (41%). ^{1}H NMR ($CDCl_3$:CD_3OD 95:5, 500 MHz) δ 8.36 (m, 1 H), 7.89 (dd, *J* = 8.0, 1.5 Hz, 1 H), 7.81 (d, *J* = 8.5 Hz, 1 H), 7.64 (d, *J* = 8.5 Hz, 1 H), 7.18 (dd, *J* = 8.5, 2.5 Hz, 1 H), 7.01 (d, *J* = 8.0 Hz, 1 H), 6.86 (d, *J* = 2.5 Hz, 1 H), 4.89 (d, *J* = 4.0 Hz, 1 H), 4.19 (m, 1 H), 3.92 (dd, *J* =10.0, 4.5 Hz, 1 H), 3.82-3.38 (m, 9 H), 3.22 (dd, *J* = 13.5, 6.0 Hz, 1 H), 3.19 (s, 6 H), 2.65 (t, *J* = 7.0 Hz, 2 H), 2.34 (br s, 5 H), 2.18 (m, 2H), 2.05-2.08 (m, 2 H), 1.65-1.23 (m, 72 H), 0.87 (t, *J* = 7.0 Hz, 6 H); ^{13}C NMR ($CDCl_3$:CD_3OD 9:1) 199.01, 174.46, 150.56, 138.06, 130.98, 130.42, 129.86, 126.43, 125.10, 124.35, 121.72, 116.49, 110.59, 105.36, 99.72, 84.07, 75.29, 72.47, 70.11, 69.23, 68.99, 68.69, 67.80, 60.06, 50.56, 40.54, 36.76, 33.62, 33.11, 32.07, 30.23, 29.86, 29.85, 29.81, 29.80, 29.71, 29.57, 29.51, 25.99, 22.83, 14.25; HRMS (FAB) *m/z* $C_{66}H_{115}N_3NaO_{10}S$ ([M+Na]+) 1132.8484 (100%), calc. 1132.8475.



3-17

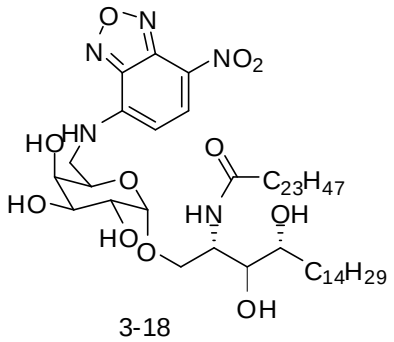
Preparation of 3-17. N-hydroxysuccinimidobiotin (5.9 mg, 0.017 mmol) and Et_3N (30 ul) were added to a solution of **2-22** (5.0 mg, 0.0058 mmol) in DMF (1.5 mL). The mixture was stirred for 12 h, and applied directly to a SiO2 column. Elution with 10% MeOH in CH2Cl2 gave the product as a clear glass (3.2 mg, 52% yield). NMR (1H, $CDCl_3$, CD_3OD) δ 8.76 (m, 1 H), 8.52 (d, *J* = 9.0 Hz, 1 H), 7.44-7.30 (m, 4 H), 5.35 (d, *J* = 4.0 Hz, 1 H), 4.56-4.31 (m, 2H), 4.23-4.10 (m, 9 H), 4.20 (m, 1 H), 3.81 (m, 1 H), 3.15 (m, 1 H), 3.06 (m, 1 H), 2.45-2.34 (m, 4 H), 2.12-26 (m, 40 H), 0.87 (t, *J* = 7.0 Hz, 6 H); NMR (^{13}C, $CDCl_3$, CD_3OD) 171.26, 169.32, 163.18, 99.41, 76.78, 72.45, 71.27, 71.19, 70.87, 70.20, 68.54, 62.37, 62.34, 55.13, 51.14, 41.12, 41.10, 37.76, 35.27, 34.44, 32.14, 30.79, 30.56, 30.23, 30.11, 28.57, 28.33, 26.65, 26.44, 26.17, 26.10, 26.00, 24.33, 22.01, 14.12; HRFAB-MS (thioglycerol + Na+ matrix) *m/e* ([M + Na]+) 854.1455(100%),calcd 854.1443



3-14

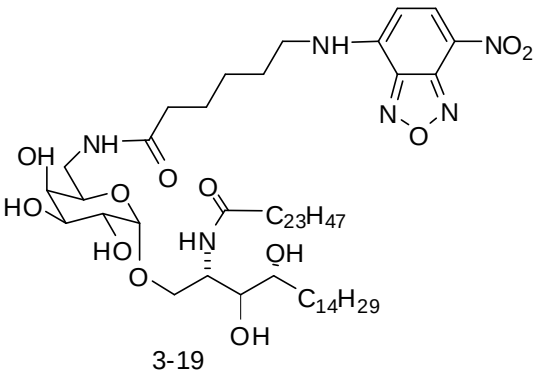
Proparation of 3-14. Same procedure as synthesis of **3-17** (42%). NMR (1H, $CDCl_3$, CD_3OD) δ 8.86 (m, 1 H), 8.61 (d, *J* = 9.0 Hz, 1 H), 7.54-7.40 (m, 4 H), 5.54 (d, *J* = 4.0 Hz, 1 H), 5.28

(br, OH), 4.66-4.61 (m, 2H), 4.56-4.50 (m, 3 H), 4.41-4.31 (m, 6 H), 4.23 (m, 1 H), 3.92 (m, 1 H), 3.27 (m, 1 H), 3.16 (m, 1 H), 3.0-2.85 (m, 10 H), 2.55-2.44 (m, 6 H), 2.32 (m, 1 H), 1.96-1.50 (m, 20 H), 1.32-1.26 (m, 44 H), 0.87 (t, *J* = 7.0 Hz, 6 H); NMR (^{13}C, $CDCl_3$, CD_3OD) 170.36, 169.46, 164.28, 101.41, 76.88, 72.57, 71.30, 71.21, 70.74, 70.10, 68.62, 62.41, 62.36, 60.57, 56.30, 56.13, 51.24, 41.22, 41.15, 36.86, 36.27, 34.52, 32.17, 30.89, 30.46, 30.23, 30.11, 29.98, 29.89, 29.85, 29.67, 29.12, 28.87, 28.53, 26.56, 26.50, 26.27, 26.16, 26.10, 24.85, 22.98, 14.33; HRFAB-MS (thioglycerol + Na+ matrix) *m/e* ([M + Na]+) 1105.8143(100%),calcd 1105.8153.



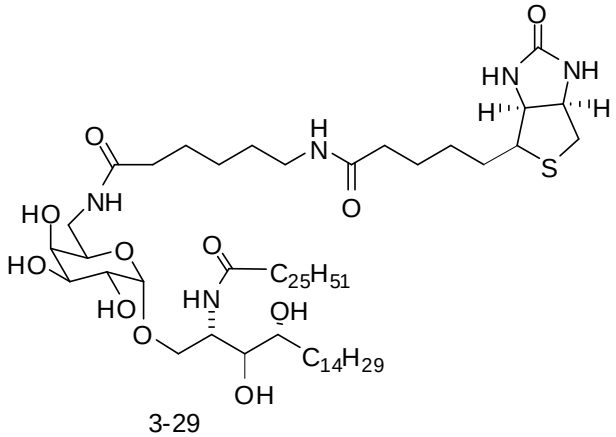
3-18

Preparation of 3-18. Same procedure as synthesis of **3-14** (52%). NMR (^{1}H, $CDCl_3$, CD_3OD) δ 8.51 (d, *J* = 9.0 Hz, 1 H), 6.36 (d, *J* = 9.0 Hz, 1 H), 4.92 (d, *J* = 4.0 Hz, 1 H), 4.17 (dd, *J* = 10.0, 3.0 Hz, 1 H), 4.08 (t, *J* = 6.5 Hz, 1 H), 3.94 (m, 1H), 3.84-3.44 (m, 6 H), 2.15 (t, *J* = 7.5 Hz, 2 H), 1.66-1.48 (m, 4 H), 1.38-1.17 (m, 64), 0.87 (t, *J* = 7.0 Hz, 6 H); NMR (^{13}C, $CDCl_3$, CD_3OD) 174.42, 127.60, 114.41, 99.50, 74.46, 72.30, 71.82, 69.94, 69.79, 68.65, 68.38, 68.11, 66.89, 63.17, 60.86, 50.01, 49.24, 49.07, 48.90, 48.73, 48.56, 48.39, 48.22, 36.33, 32.48, 31.78, 29.57, 29.44, 29.29, 29.22, 25.70, 22.53, 13.89, -0.27; HRFAB-MS (thioglycerol+Na+matrix) *m/e* ([M+Na]+) 1014.7081(100%),calcd 1014.7082.



3-19

Preparation of 3-19. *N*-Hydroxysuccinimidyl NBD-X (6.6 mg, 0.018 mmol) was added to a solution of **3-28b** (5.0 mg, 0.0058 mmol) in pyridine (1 mL) and the mixture was stirred for 12 h. The pyridine was removed in vacuo, and the product was purified by column chromatography (SiO_2, MeOH:CH_2Cl_2 9:1) giving **3-19** (3.0 mg, 46%). ^{1}H NMR (CDCl3:CD3OD 95:5, 500 MHz) δ 8.52 (d, *J* = 8.5 Hz, 1 H), 6.21 (d, *J* = 8.5 Hz, 1 H), 4.86 (d, *J* = 4.0 Hz, 1 H), 4.11 (m, 1 H), 3.78 (dd, *J* =10.0, 4.5 Hz, 1 H), 3.82-3.36 (m, 9 H), 2.24 (t, *J* = 7.5 Hz, 2 H), 1.66-1.24 (m, 74 H), 0.87 (t, *J* = 7.0 Hz, 6 H); ^{13}C NMR($CDCl_3$:CD_3OD 9:1) 174.98, 174.54, 137.18, 127.60, 114.43, 99.28, 73.88, 72.38, 71.84, 70.03, 69.65, 69.30, 68.83, 68.54, 66.82, 60.93, 50.67, 50.35, 50.13, 49.58, 48.94, 39.55, 36.31, 35.57, 31.79, 29.67, 29.60, 29.56, 29.36, 28.28, 29.23, 27.50, 26.21, 25.79, 24.91, 22.54, 13.90; HRMS (FAB) *m/z* $C_{60}H_{108}N_6NaO_{12}$ ([M+Na]+) 1127.7923 (100%), calc. 1127.7923.



3-29

Proparation of 3-29. Same procedure as synthesis of **3-14** (52% yield). NMR (1H, $CDCl_3$,

CD_3OD) δ 4.87 (d, *J* = 3.5 Hz, 1 H), 4.51 (dd, *J* = 7.5, 4.5 Hz, 1 H), 4.32 (dd, *J* = 7.5, 4.5 Hz, 1 H), 4.12 (dd, *J* = 10.0, 4.5 Hz, 1 H), 3.96-3.44 (m, 4 H), 3.28 (dd, *J* = 14.0, 7.5 Hz, 1 H), 2.23-2.18 (m, 4 H), 1.68-1.08 (m, 74), 0.87 (t, *J* = 7.0 Hz, 6 H); NMR (^{13}C, $CDCl_3$, CD_3OD) δ 175.60, 175.44, 174.80, 174.71, 164.54, 99.58, 74.27, 72.05, 69.95, 72.05, 69.95, 69.51, 69.04, 68.79, 67.10, 62.10, 60.31, 55.82, 50.60, 49.54, 49.28, 49.11, 48.94, 48.77, 48.59, 48.42, 40.45, 39.75, 39.15, 36.57, 35.97, 35.80, 32.15, 32.07, 29.97, 29.90, 29.83, 29.66, 29.57, 29.51, 28.82, 28.45, 26.19, 26.07, 25.72, 25.24, 22.82, 14.17; HRFAB-MS (thioglycerol + Na+ matrix) *m/e* ([M + Na]+) 1218.8987 (100%),calcd 1218.8994.

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CHAPTER 4.

SYNTHESIS OF THE MODIFIED ALPHA-GALACTOSYL CERAMIDE FOR STIMULATIING NATURAL KILLER T CELLS

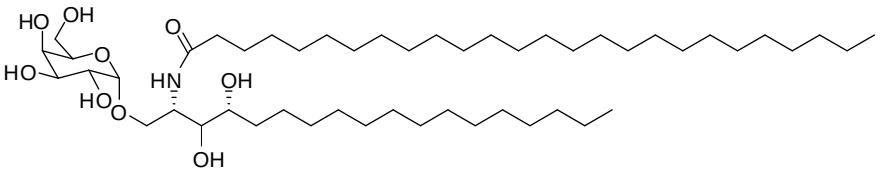
4.1 Introduction

Stimulation of NKT cells by glycolipids influences a large number of diseases.[1] When stimulated by KRN7000, cytokines released by NKT cells include those associated with both Th1 and Th2 responses.[2] Because these two responses can counteract each other, there has been considerable interest in methods that would allow a more selective activation of NKT cells. Selectively controlling either a proinflammatory or an immunomodulatory response would be medicinally useful.[3-8] The focus of these studies is to clarify the factors affecting NKT cell stimulation, including the structural features of glycolipids that influence the interactions between NKT cells and the glycolipid-CD1d complexes. The modifications are based on two principles, one is to change the combination of the glycolipid with CD1d, the other is to alter the solubility of glycolipid for biological assays.

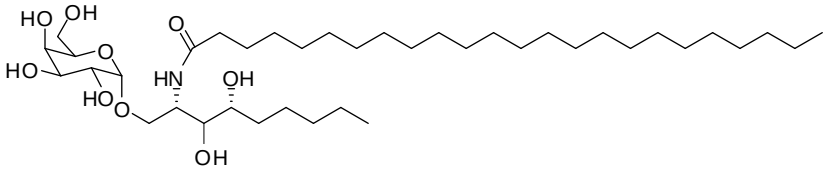
4.1.1 Effects of ceramide structure on cytokine release

Several studies have reported that modification of the structure of KRN7000 resulted in significant change in cytokine release profiles, such as shortening the ceramide fatty acid chain or deleting phytosphingosine hydroxides. Changing can cause the TCRs to recognize glycolipid in a different way or cause a less tight binding to CD1d because of an accelerated 'off rates'.[9-10] Miyamoto *et al.* reported a novel synthetic ligand (OCH), an analog of KNR7000 with a truncated sphingosine chain **(Figure 4.1**).[11] Compared with KRN7000,

injection of OCH to mice was about five to ten times less active in inducing cell proliferation and induced less IFN-γ secretion but more IL-4 secretion both *in vitro* and *in vivo*. OCH may induce a Th2 bias through a predominant production of IL-4 and lead to the suppression of experimental autoimmune encephalomyelitis, a prototype autoimmune disease mediated by Th1 cytokines. More recently, OCH has been reported to protect against diabetes in NOD mice and against collagen induced arthritis.[12-13]



KRN7000



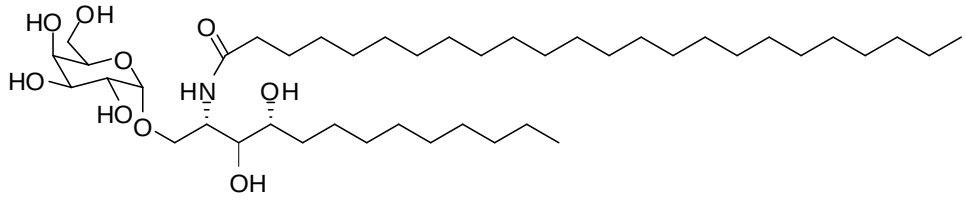
OCH

Figure 4.1. Structures of KRN7000 and OCH.

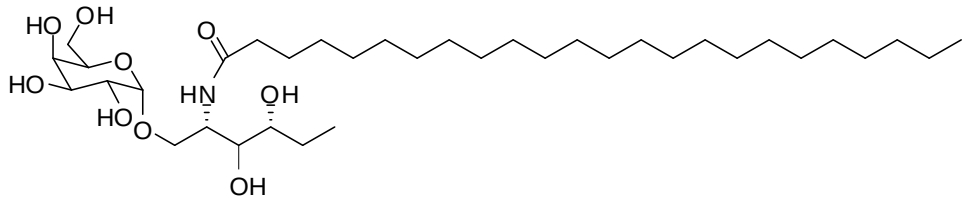
There were several proposed mechanisms to account for this inclination.[14-15] One explanation is based on the crystal structure of CD1d, which shows that CD1d has two hydrophobic pockets.[16] The lipid chains of the glycolipids fit in the pockets. OCH has a shorter phytosphingosine chain and the binding of CD1d may be less tight than KRN7000, and this combination may make the orientation of the galactose hydrophilic cap unstable. In a word, the different interaction of OCH with TCR may explain the altered Th1/Th2 balance.[10] So, changing the lipid portion will help to understand the knowledge of the NKT cell stimulatory properties.

But the extent to which lipid chain lengths affect cytokine release profiles was still unclear. In order to investigate how the different chain lengths of the phytosphingosine

moiety and the fatty acid domain influence cytokine release, Goff *et al.* investigated the cytokine release profiles of synthesized glycolipids in which the lipid chain lengths were systematically truncated.[17]



4-1



4-2

Figure 4.2. Structure of α-Galcer with varied photosphingosine chain length.

4-1 and **4-2** were prepared to study the effect of varied phytosphingosine chain length on cytokine release profiles (**Figure 4.2**). OCH was also synthesized to complete the series of α-Galcer with varied phytosphingosine lipid chains. Goff *e. al*. found that truncation of phytosphingosine lipid chain significantly increased the ratio of IL-4 to IFN-γ.

They also prepared compounds **4-3** to **4-5** with systematically truncated fatty acid chains (**Figure 4.3**). The cytokine release profiles were compared with KRN7000. Truncation of fatty acid chains caused the release of greater amounts of IL-4. The stimulation of **4-5** is weak in mouse NKT cells, possibly because it can not form a stable complex with T cell receptors. While in human NKT cells, **4-5** led to a cytokine release bias toward IL-4. The mechanism involved is similar with the theory proposed for OCH.[10]

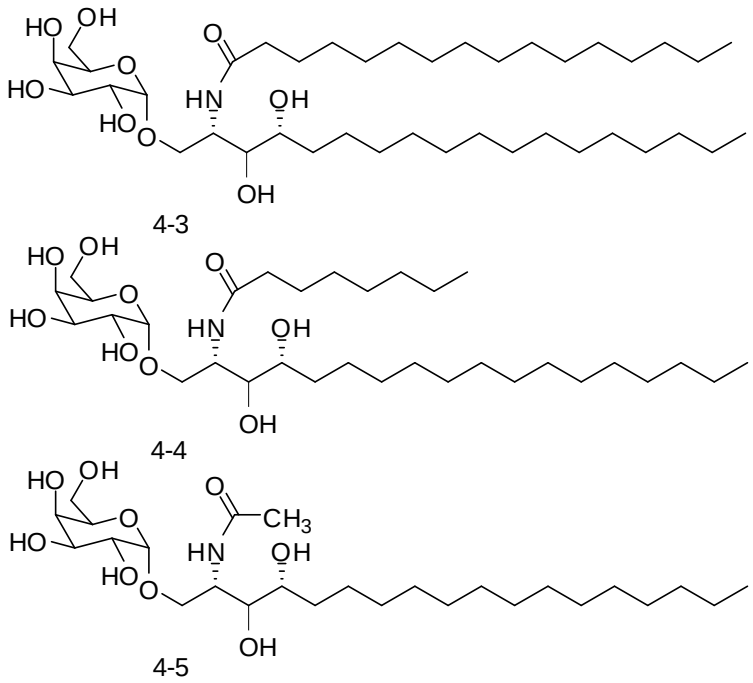


Figure 4.3. Structures of α-Galcer with varied fatty acid chain length.

In addition to truncating the fatty acid chain length, the impacts of variations in the ceramide portions were considered. Chang *et al.* synthesized a glycolipid with an aromatic ring in the phytosphingosine chain (e.g., **C13**) more effectively induced Th1 cytokines (**Figure 4.4**).[18] They proposed that **C13** could directly stimulate human dendritic cell maturation, whereas KRN7000 could not. The sphinganine analogs of KRN7000 and OCH were evaluated for the ability to induce the cytokine release profiles, and were found to be comparable with their parent compounds.[19] In another study, a 1,2,3-triazole group substituting the amide of the ceramide formed a rigid linking unit that mimics the topological and electronic features of an amide bond (**Figure 4.4**). **Triazole 8** has a 24 carbon alkyl chain linked to sphingoid base by triazole ring, which is very stable in hydrolysis and oxidative/reductive conditions.[20] The stimulatory effect of **triazole 8** is comparable to KRN7000.

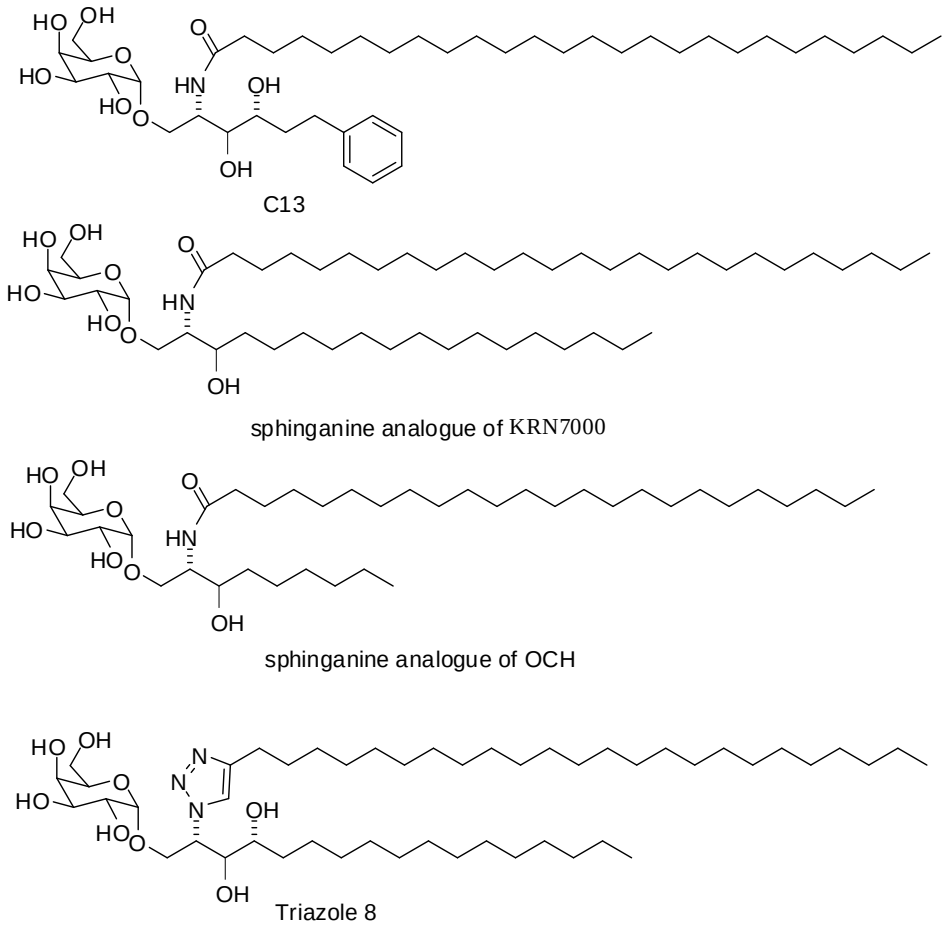


Figure 4.4. Structure of analogs of KRN7000.

As was mentioned in chapter 1, the crystal structure of CD1d shows that the binding pockets are not straight but twisted.[16] Adding bends in the fatty acid chain may facilitate loading into the CD1d and help to increase the binding with CD1d to stabilize the complex. The unsaturated alkenes with Z configuration or cyclopropyl rings could meet this requirement. Another issue is the solubility of glycolipids. Cytokine release is partly dependent of glycolipid concentration. Glycolipids are notoriously insoluble in water and most other solvents, because glycolipids are amphipathic molecules which have both hydrophilic and hydrophobic groups. For example, compound **4-4**, a short fatty acid chain analog of KRN7000, decreased the amphiphilicity and increased the solubility in water and

loaded into CD1d more efficiently. Insertion of alkenes might have the same effect as compound **4-4**.

Using unsaturated fatty acids in the ceramide moiety can achieved the goals of both increasing the binding with CD1d and raising the compound solubility. Goff *et. al.* synthesized five analogs of KRN7000 with varied points of unsaturation on the fatty acid group to study the binding ability of those fatty acid with CD1d and the solubility in DMSO solutions (**Figure 4.5**). Porcelli and coworkers found that changing the C26:0 N-acyl chain of KRN7000 with C20:2 (**4-7**) biased NKT cells toward Th2 response.[21]

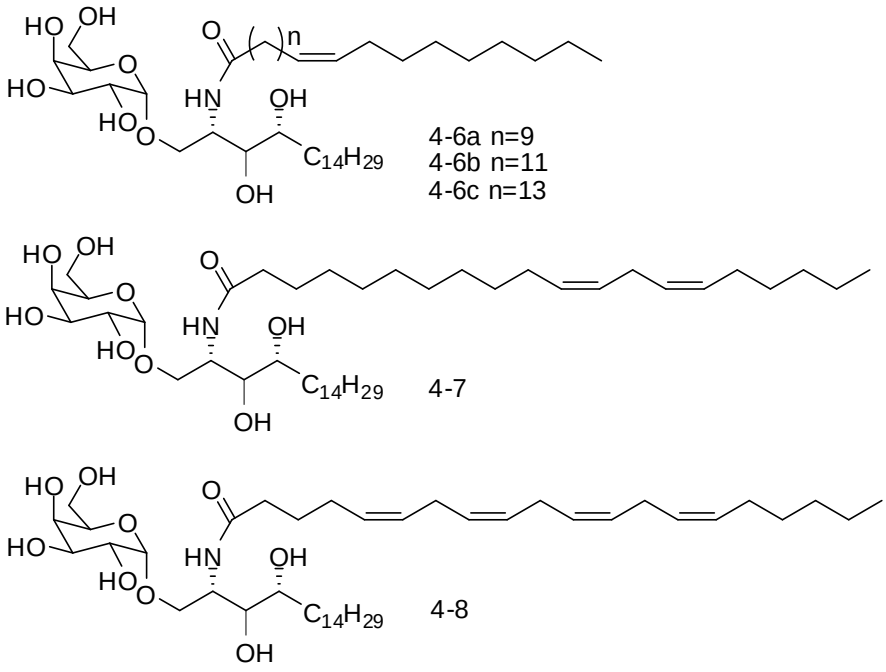
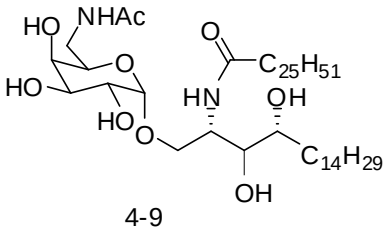


Figure 4.5. Structures of KNR7000 analogs with unsaturated fatty acid chain.

4.1.2 Modification of the sugar moiety.

Structural studies on the sugar portion of the KRN7000 showed that modification of the C6" position of the galactose was well tolerated (see chapter 3). Two disaccharide derivatives of KRN7000 were analyzed in an APC-free T cell stimulation system. The

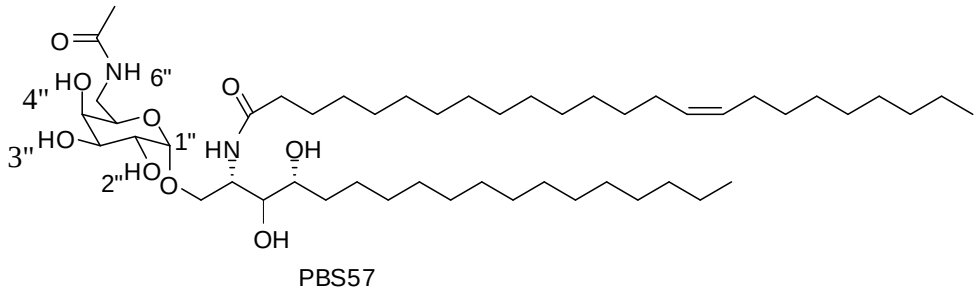
Gal(α1→6)αGalCer induced NKT cells stimulation similar to KRN7000 whereas Gal(α1→2)αGalCer was unable to stimulate NKT hybridoma cells.[22] The presentation of Gal(α1→2)αGalCer to NKT cells was dependent on the trafficking of CD1d to lysosome and to be processed by lysosomal α-galactosidase A which truncated the disaccharide to a monosaccharide. This monosaccharide can be recognized by the TCRs of the NKT cells. Preparing gylcolipids substituted at C6 position may help to understand the trafficking, processing, and presentation of glycolipids.[23] Compound **4-9**, an analog of KRN7000 with an acetamide group at C6" position, was synthesized and tested for the ability to induce NKT cell proliferation (**Figure 4.6**).



4-9

Figure 4.6. The structure of **4-9**.

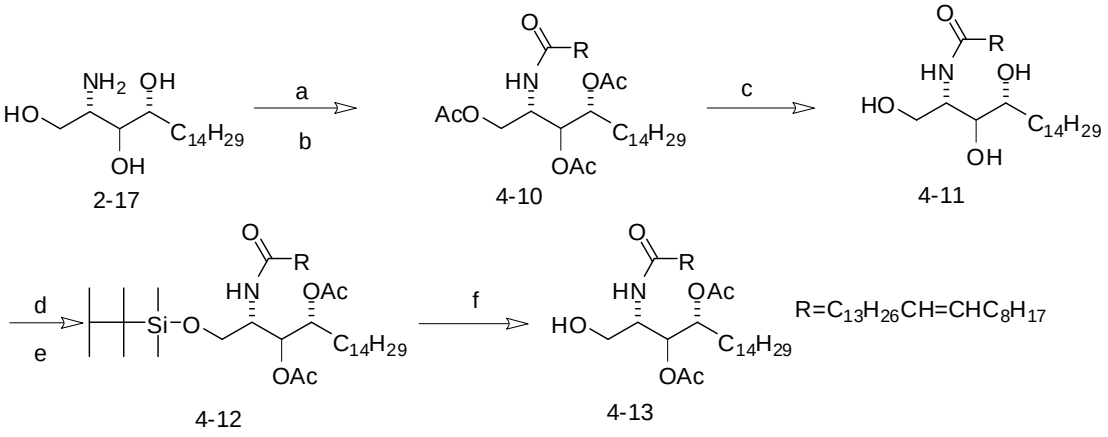
Structural studies on the analogs of KRN7000 with modification at C6 and unsaturated fatty acid chains led to the synthesis of an effective surrogate structure of KRN7000, PBS57 in Figure **4.7**.[24]



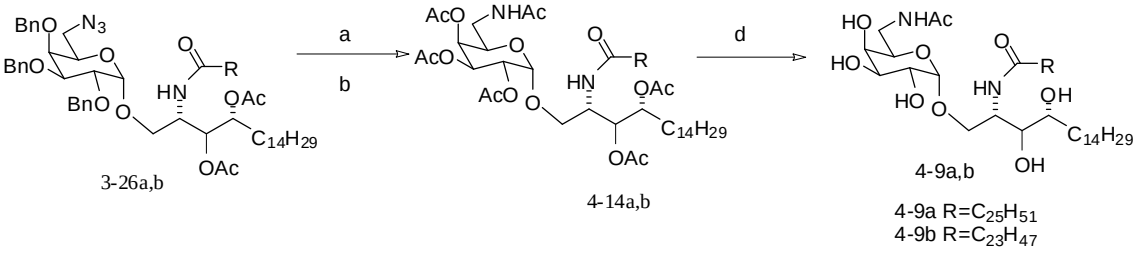
PBS57

Figure 4.7. Structure of PBS57

4.2 Results and Discussion



Scheme 4.1. Synthesis of ceramide **4-13.**

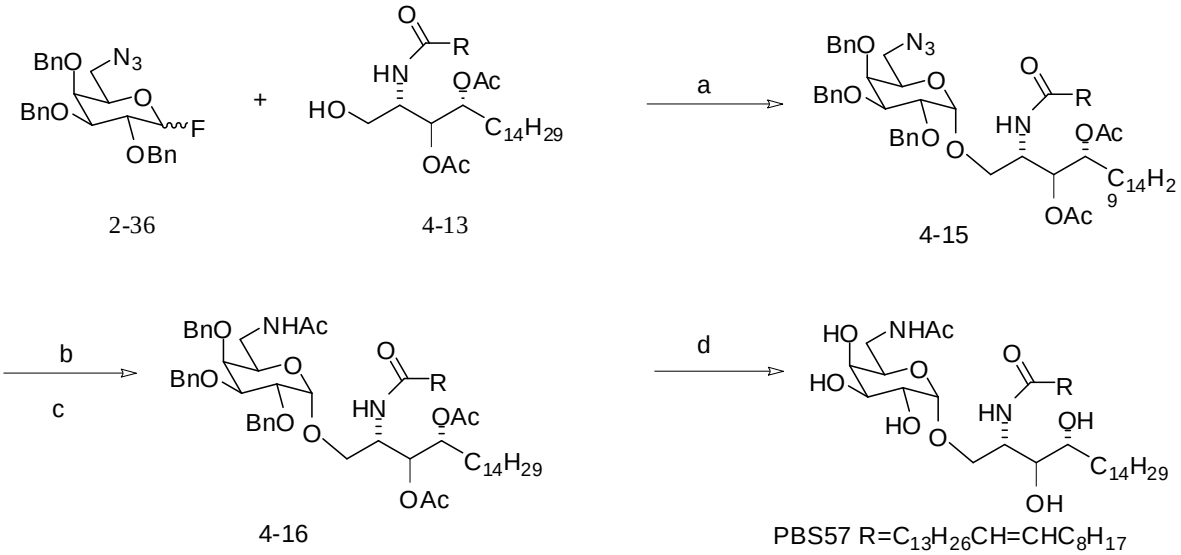


Scheme 4.2. Synthesis of glycolipids **4-9.** (The same scheme used in **2-6**)

The synthesis of **4-13** and **4-9** used the same method as was used in **2-11** (Chapter 2). Goff *et al.* tested **4-6** for their ability to stimulate NKT cells. **4-6c** induced more IFN-γ cytokines than both **4-6a** and **4-6b.** The length between the carbonyl group and double bond in the ceramide of **4-6c** seems to be most appropriate to fit in the A' pocket of the CD1d, thus the binding of **4-6c** with CD1d is better than the other two compounds. Goff *et al.* also synthesized glycolipids with polyunsaturated fatty acid chains **4-7** and **4-8,** but these two compounds were susceptible to oxidation upon exposure to air. They do not show apparently greater activity than the other unsaturated compounds. The work on the compounds with poly

unsaturated fatty acid chains thus was not investigated further.

The studies of immunological activity by compounds **4-9a**, **4-6c**, and KRN7000 showed that the former two compounds could stimulate NKT cells to a greater extent than KRN7000. This led to the design of compound PBS57 which has an acetamide group at the C6" position and a nervonic acid chain.



Reagents: a) $AgClO_4$, $SnCl_2$, CH_2Cl_2, 0°C-r.t, 56%; b) PPh_3/H_2O, THF, r.t.; c) Ac_2O, Pyridine, DMAP, 80%; d) Na, liquid NH_3, -78°C, 47%.

Scheme 4.3. Synthesis of **PBS57**.

PBS57 was obtained through the reducing metal condition because we have a double bond in an acyl chain of the ceramide. The structure of PBS57 and analogs were confirmed by ^{1}H and ^{13}C NMR and mass spectrometry.

The NKT cell stimulating properties of PBS57 were tested both in vivo and in vitro. Methods of preparing PBS57 solutions were the same as those of KRN7000, except that the solubility of PBS57 in DMSO is much higher than that of KRN7000 (20 mg/mL, <5 mg/mL, respectively). Using PBS57 thus gave a low residual concentration of DMSO. Mouse thymus and spleen were used to study the ability of PBS57 and KRN7000 to bind CD1d tetramers.

The abilities of PBS57 to load CD1d tetramers and bind to the Vα14*i* NKT cells were comparable to those of KRN7000 (**Figure 4.8**).[24]

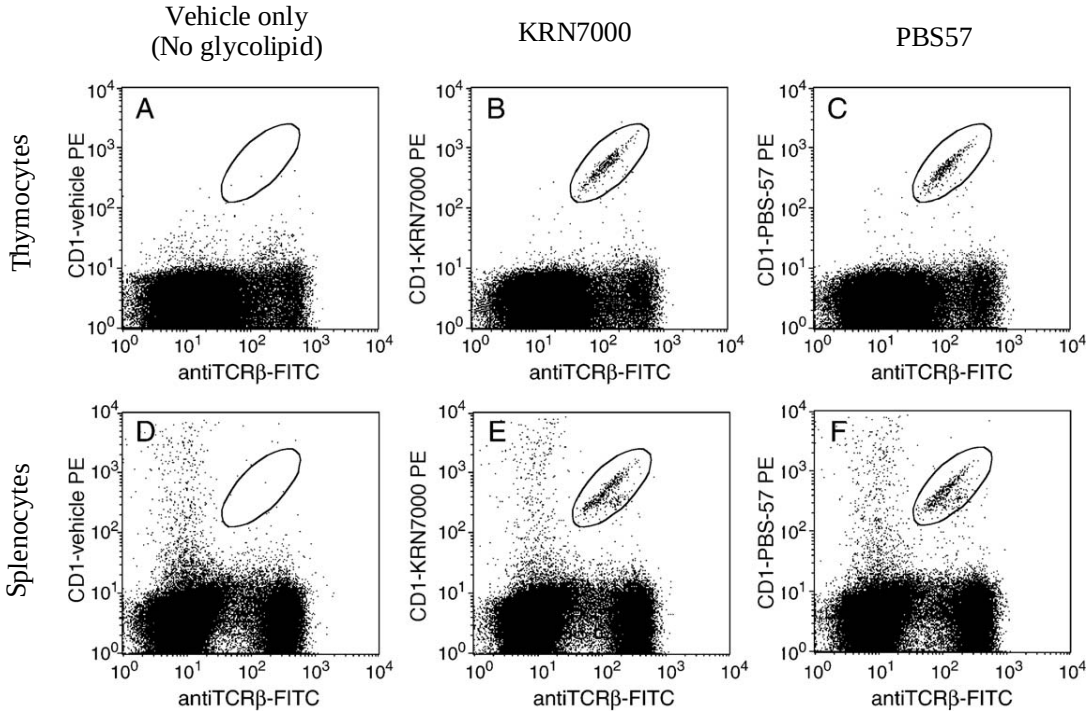


Figure 4.8. Comparative staining of Vα14*i* NKT cells from mouse thymus and spleen.[24]

In order to find out if PBS57 loaded tetramers were Vβ subunit specific in their ability to bind, NKT cell hybridomas with different TCR β chains were tested for their ability to bind PBS57 loaded CD1d tetramers. Flow cytometry results showed that PBS57 stained all hybridomas regardless of Vβ usage (**Figure 4.9**).[24]

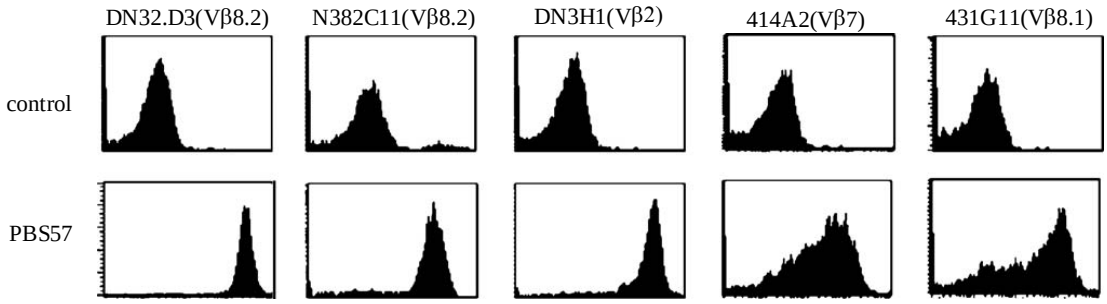


Figure 4.9. NKT cell hybridomas with different Vβ chains stained by PBS57 and non-stimulating

glycolipid (α-galactosylcholesterol).[24]

To test if PBS57 could facilitate NKT cell binding in blood samples of both human and non-human primates, PBS57 loaded mouse and human CD1d tetramers were assayed for NKT cell staining. Most of human blood samples contain sufficient NKT cells to observe staining (fourteen of seventeen samples). Among non-human primates, a majority of chimpanzee blood samples (six of ten) and rhesus macaques (four of twelve) can be observed significant NKT cell staining (**Figure 4.10**).[24] No staining was seen in pigtain macaques (six samples) or sooty mangabeys (six samples). From the hypothesis of Lee *et. al.*, this may be caused by limited population of NKT cells circulating in the blood and the small sample size.[26]

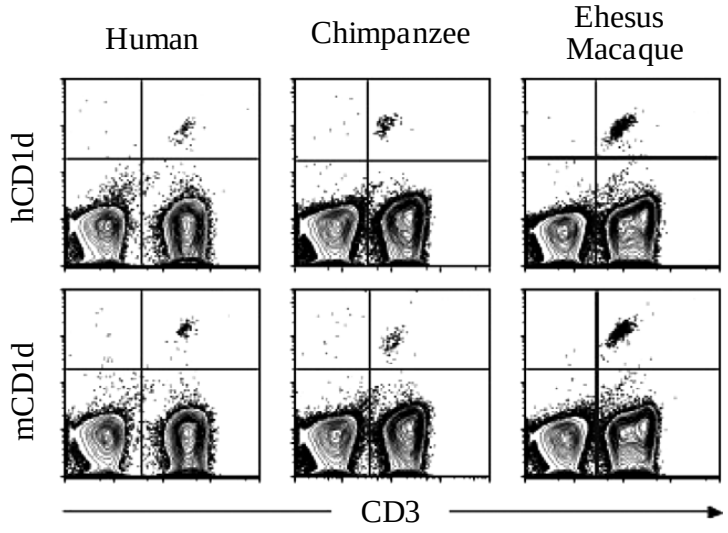


Figure 4.10. Staining of NKT cells by CD1d-tetramer loaded with PBS57.

Mouse spleen cells were used to determine the ability of PBS57 to stimulate cytokine release in vitro. We wanted to know if the cytokine release profiles were effected by the structural differences of PBS57 and KRN7000. Cytokine release of both IFN-γ and IL-4 were examined. Cytokine release results can be seen in Figure 13. The plateau of PBS57 occurred at approximately 100 pg/mL while the plateau of KRN7000 occurred at 1000 pg/mL. It

shows that PBS57 is as much as 100 times more effective than KRN7000. Both PBS 57 and KRN7000 are effective in staining NKT cells and inducing Th1 and Th2 cytokine secretion (**Figure 4.11**).

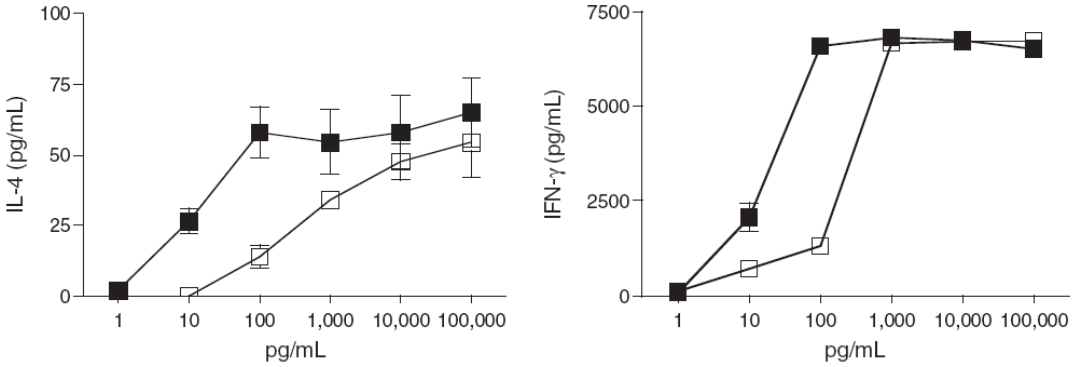


Figure 4.11. Cytokine release from B6 mouse splenocytes stimulated with ■ PBS57 and □ KRN7000. 10^5 spleen cells were incubated with the indicated concentrations of glycolipids and cytokine concentrations were measured after 48 h.[24]

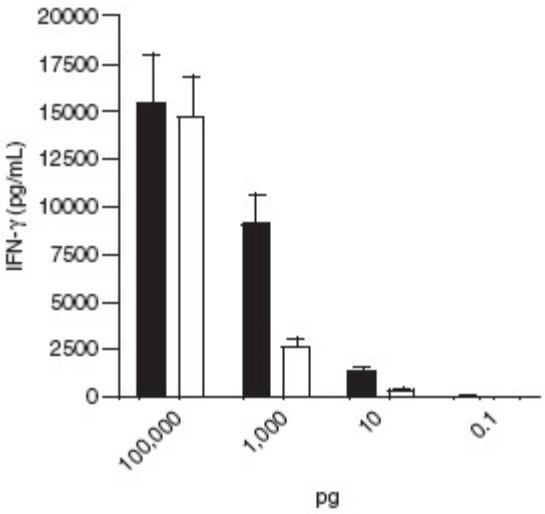


Figure 4.12. Serum IFN-γ concentrations from mice injected (i.v.) with the indicated quantities of ■ PBS57 and □ KRN7000. Cytokine concentrations were determined 24 h after injection.

Mice were used in order to prove the ability of PBS57 to simulate NKT cells in vivo. Mice were injected with different amounts of PBS57 and KRN7000 (100 μL injections with different concentrations of glycolipids). The data is shown in **Figure 4.12**. The cytokine

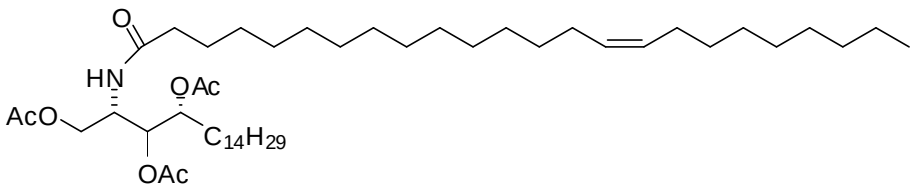
release difference in vivo is not as great as in vitro. It is probably because the solubility of PBS57 is higher than KRN7000 and easier to load into CD1d to stimulate NKT cells at lower concentrations.

4.3 Conclusion

A double bond in the acyl chain and modification of the C6” functional group helped the glycolipid loading into CD1d and NKT cells stimulation. PBS57 is as much as 100 times more active than KRN 7000 in stimulation of NKT cells responses *in vitro* and *in vivo*. This improvement is probably due to increasing solubility and improving binding ability with the CD1d.

Experimental Procedure

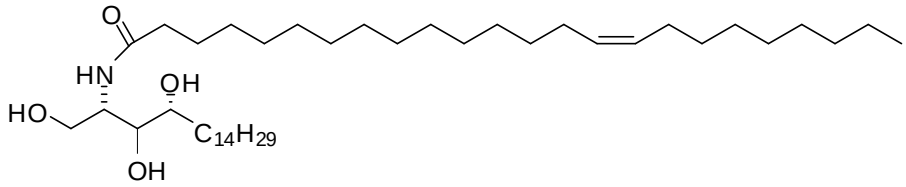
Materials and General Methods.^{1}H NMR and ^{13}C NMR, were recorded on Varian Unity 500 MHz or Varian Unity 300 MHz NMR spectrometers using 99.8% CDCl3 with 0.05% v/v TMS, 99.8% CD3OD with 0.05% v/v TMS. Mass spectrometric data were obtained on JEOL SX 102 A spectrometer or Agilent 1100 series spectrometer. Flash chromatography was performed using 230-400 mesh silica gel. Thin layer chromatography was performed on aluminumbacked, 254 nm UV-active plates with a silica gel particle size of 250 μm. Chemicals were obtained from Sigma and Aldrich and were used as received unless otherwise noted.



4-10

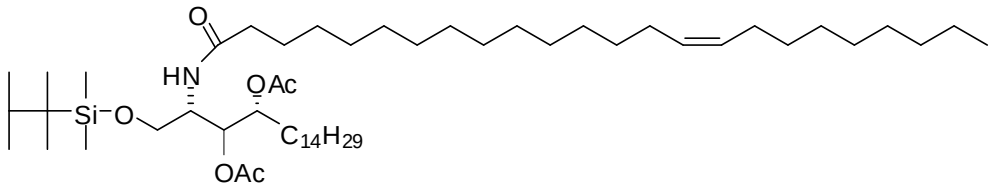
Preparation of 4-10. Nervonic acid (3 g, 8.2 mmol) was dissolved in anhydrous THF (100 mL) followed by HOBt (1.21 g, 9.0 mmol) and DCC (1.86 g, 9.0 mmol). The mixture was stirred for 1 h. Phytosphingsine dissolved in THF and pyridine was added to the reaction mixture and stirred for 12 h, then added acetic anhydride (9 mL), triethylamine (9 mL), DMAP (300 mg), stirred 2 h. The solvent was removed by vacuo. Pure triacetate **4-10** (4.8 g, 75.0%) was isolated by chromatography (SiO_2, EtOAc:hexanes 1:8). NMR (^{1}H, CDCl3) δ 6.14 (d, *J* = 9.5, 1 H), 5.34 (m, 2 H), 5.12 (dd, *J* = 3, 8.5 Hz, 1 H), 4.93 (m, 1 H), 4.50(m, 1 H), 4.29 (dd, *J* = 5, 11.5 Hz, 1 H), 4.0 (dd, *J* = 3.0, 11.5 Hz, 1 H), 2.21 (t, *J* = 7.5 Hz, 2 H), 2.08 (s, 3 H), 2.05-1.99 (m, 10 H), 1.66-1.60 (m, 4 H), 1.33-1.22 (m, 56 H), 0.89 (t, *J* = 7.0

Hz, 6 H); NMR (^{13}C ,CDCl3) δ 178.25, 173.19, 171.31, 170.99, 170.20, 129.99, 73.15, 71.92, 63.05, 60.52, 47.50, 36.81, 34.11, 32.05, 29.91, 29.83, 29.80, 29.74, 29.67, 29.51, 29.45, 29.39, 29.26, 28.10, 27.33, 25.80, 25.68, 24.92, 22.82, 21.13, 20.88, 20.84, 14.30, 14.24. HRFAB-MS (thioglycerol + Na^+ matrix) *m/e* ([M + Na]$^+$) 814.6526(100%), calcd 814.6537



4-11

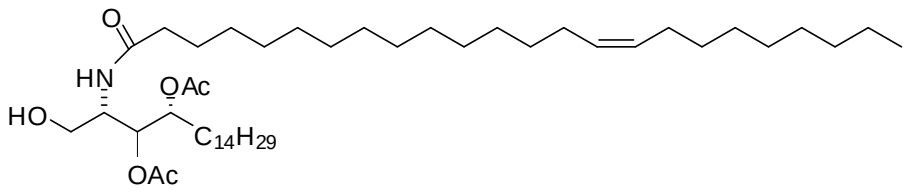
Preparation of 4-11. Adding sodium metal (230 mg, 10 mmol) to MeOH (100 mL) Triacetate **4-10** (3.4 g, 4.4 mmol) was deacetylated by dissolving in upper solvent, stirred for 1 h and centrifuged (3000 rpm, 5 m) to isolate the solid triol product. The solvent was removed, the solid rinsed with fresh MeOH (80 mL) to remove any remaining base, and centrifuged again, then repeated twice. After removal of the solvent, the crude white solid **4-11** (2.1 g, 71%) was dried under vacuo.



4-12

Preparation of 4-12. The triol **4-11** (2.1g, 3.09 mmol) was dissolved in pyridine (15 mL), then adding dimethexylsilyl chloride (0.606 mL , 3.09 mmol), after 5 min, the reaction was monitored by TLC, if the reaction was not complete, just add another 0.606 mL, until the starting material disappeared, acetic anhydride (1.16 mL, 12.3 mmol), DMAP (200 mg) was

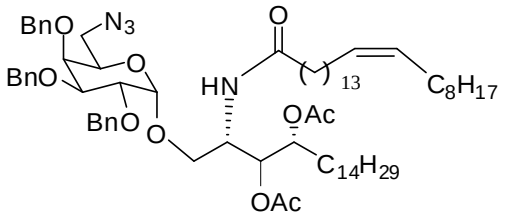
added into the system, stirred 2 h, Purification by chromatography (SiO2, EtOAc:Hexanes 1:20) yielded **4-12** (2.0 g, 80%) a clear oil. NMR (^{1}H, $CDCl_3$) δ 5.90 (d, *J* = 9.5, 1 H), 5.32-5.26 (m, 2 H), 5.19 (dd, *J* = 2.0, 10.0 Hz, 1 H), 4.86-4.83 (m, 1 H), 4.17 (t, *J* = 9.5 Hz, 1 H), 3.60-3.48 (m, 2 H), 2.15 (t, *J* = 7.0 Hz, 2 H), 2.02 (s, 3 H), 1.99-1.94 (m, 7 H), δ1.59-1.54 (m, 4 H), 1.30-1.14 (m, 56 H), 0.86-0.79 (m, 18 H), 0.01 (d , *J* = 3.0 Hz, 6 H). NMR (^{13}C ,$CDCl_3$) δ 172.64, 171.10, 169.62, 129.94, 73.65, 71.31, 61.39, 49.21, 37.04, 34.34, 32.06, 32.05, 32.06, 32.04, 29.90, 29.82, 29.72, 29.66, 29.64, 29.51, 29.26, 29.40, 27.70, 27.32, 25.83, 25.25, 22.82, 21.10, 20.91, 20.34, 20.32, 18.60, 14.22, -3.73, -3.82.; HRMS (ESI) calcd for $C_{54}H_{106}NO_6Si$ $[M+H]^+$ 892.77839, found 892.78177.



4-13

Preparation of 4-13. Compound **4-12** (2.0 g, 2.4 mmol) was dislove in THF (5 mL) in a centrifuge tube (plastic), followed by adding aqueous HF (5 mL), check the TLC, if the reation was not completed, just added more THF and aqueous HF. The mixture was pour into the saturated $NaHCO_3$ (80 mL), then extracted with EtOAc (100 mL x 2), the organic layer was drid and concentrated, then purified by chromatography (SiO2, EtOAc:Hexanes 1:2) to afford a white solid **4-13** (1.5 g, 83%). NMR (^{1}H, CDCl3) δ 6.66 (d, *J* = 9.0 Hz, 1 H), 5.34 (t, *J* = 4.0 Hz, 1 H), 5.10 (dd, *J* = 2.0, 9.0 Hz, 1 H), 4.95-4.92 (m, 1 H), 4.20-4.14 (m, 1 H), 3.61-3.55 (m, 2 H), 2.22 (t, *J* = 8.0 Hz, 2 H), 2.13 (s, 3 H), 2.04-1.99 (m, 7 H), 1.66-1.60 (m, 4 H), 1.34-1.22 (m, 56 H), 0.89-0.86 (m, 6 H). NMR (^{13}C ,$CDCl_3$) δ 173.44, 171.52, 171.45,

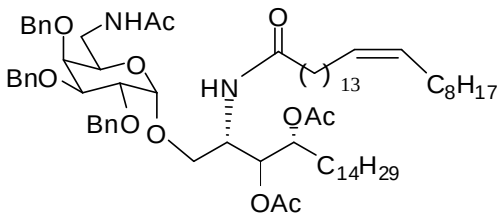
130.08, 73.54, 72.49, 61.61, 49.74, 36.93, 32.15, 32.13, 29.94, 29.90, 29.84, 29.78, 29.76, 29.64, 29.56, 27.87, 27.42, 25.97, 25.93, 22.91, 21.27, 21.09, 14,36. HRFAB-MS (thioglycerol + Na^+ matrix) *m/e* ([M + Na]$^+$) 772.6429(100%), calcd 772.6431



4-15

Preparation of 4-15. Compound **2-36** (112 mg, 0.21 mmol) and coumpound **4-12** (75 mg, 0.10 mmol) were dissolved in anhydrous CH_2Cl_2 (15 ml), and powdered 4Å molecular sieves (900 mg) were added, cooled to 0°C, stired for 10 min, $AgClO_4$ (62 mg, 0.30 mmol), $SnCl_2$ (57 mg, 0.30 mmol) were introduced into the solution with the protection of light. The mixture was allowed to warm to room temperature with stirring over 3 h under N_2, then filtered through celite, washed with CH_2Cl_2. The combined filtrate was concentrated under reduced pressure. The residue was purified by chromatography (SiO_2, EtOAc:hexanes 1:7) to give compound **4-15** (68 mg, 56%). NMR (^{1}H, $CDCl_3$) δ 7.42-7.19 (m, 15 H), 6.67 (d , *J* = 9.5 Hz, 1 H), 5.37-5.33 (m, 2 H), 5.24-5.22 (m, 1 H), 4.98 (dd, *J* = 11.0 Hz, 3.0, 1 H), 4.93 (dt, *J* = 10.5 Hz, 3.0 Hz, 1 H), 4.86-4.55 (m, 6 H), 4.37-4.31 (m, 1 H), 4.03 (dd, *J* = 10.5 Hz, 3.0 Hz, 1 H), 3.92-3.77(m, 4 H), 3.60-3.56 (m, 2 H), 3.05 (dd, *J* = 4.5 Hz, 12.5 Hz, 1 H), 2.14 (t, *J* = 8.0 Hz, 2 H), 2.06-2.00 (m, 10 H), 1.67-1.59 (m, 4 H), 1.38-1.25 (m, 56 H), 0.91-0.87 (m, 6 H); NMR (^{13}C ,$CDCl_3$) δ173.20, 171.22, 170.38, 138.76, 138.40, 138.23, 130.14, 128.78, 128.67, 128.36, 128.23, 128.11, 127.85, 127.67, 100.81, 78.73, 75.01, 74.90, 73.71, 73,42, 71.64, 70.59, 60.80, 51.50, 48.40, 36.96, 32.16, 29.96, 29.68, 29.57, 27.75, 27.46, 25,96,

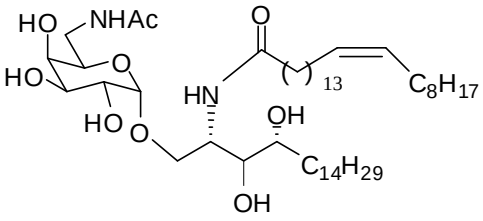
25,88, 22.94, 21.26, 21.17, 14.38; HRFAB-MS (thioglycerol + Na^+ matrix) *m/e* ($[M + Na]^+$) 1229.8448 (96.5%), calcd 1229.8433.



4-16

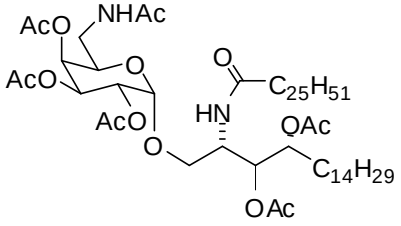
Prepartion of 4-16. T o a solution of **4-15** (68 mg, 0.056 mmol) in THF (3 ml), was added dropwised with H_2O (0.6 mL) at room temperature. Triphenylphosphine (23 mg, 0.085 mmol) was added. The reaction mixture was stirred at room temperature overnight. The solution was concentrated under reduced pressure, the residue was dissolved in 5 ml THF, Ac_2O (0.1 mL) and Et_3N (0.1 mL) was added, DMAP 5 mg was also added, stirred for 2 h, the solution was concentrated, run the column(SiO_2, EtOAc:hexanes 1:4) to give **4-16** (55 mg, 80%). NMR (^1H, $CDCl_3$) δ 7.41-7.27 (m, 15 H), 6.73 (d, *J* = 10.0 Hz, 1 H), 6.12 (t, *J* = 6.5 Hz, 1 H), 5.36-5.34 (m, 2 H), 5.24 (dd, *J* = 3.0 Hz, 13.5 Hz, 1 H), 4.96 (d, *J* = 11.5 Hz, 1 H), 4.86 (tt, *J* = 10.5, 3.0 Hz, 1 H), 4.82-4.65 (m, 6 H), 4.38 (tt, *J* = 3.0, 9.5 Hz, 1 H), 4.04 (dd, *J* = 3.0, 10.5 Hz, 1 H), 3.93 (dd, *J* = 3.0, 11.5 Hz, 1 H), 3.87-3.78 (m, 3 H), 3.53-3.46 (m, 2 H), 3.32-3.27 (m, 1 H), 2.13 (t, *J* = 8.0 Hz, 2 H), 2.06-2.00 (m, 10 H), 1.88 (s, 3 H), 1.72-1.1.57 (m, 4 H), 1.38-1.22 (m, 56 H), 0.90-0.87 (m, 6 H); NMR (^{13}C ,$CDCl_3$) δ 173.49, 171.74, 170.51, 170.40, 138.89, 138.64, 138.56, 130.13, 129.21, 128.65, 128.31, 128.11, 127.77, 100.91, 79.14, 76.64, 74.91, 73.84, 73.64, 71.32, 71.10, 70.27, 48.68, 40.24, 36.84, 32.14, 29.94, 29.90, 29.86, 29.82, 29.76, 29.68, 29.60, 29.55, 27.89, 27.45, 25.94, 25.79, 23.37, 22.92, 21.41, 21.19, 14.35; HRFAB-MS (thioglycerol + Na^+ matrix) *m/e* ($[M + Na]^+$)

1245.8634(92.8%), calcd 1245.8633.



PBS57

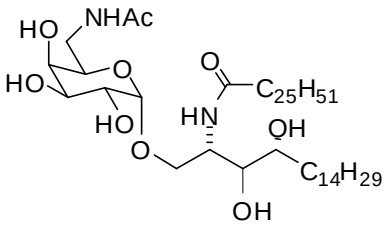
Preparation of PBS57. Na° (21 mg, 0.91mmol) was added to liquid NH_3 (20 mL) under N_2 at -78°C, stirred 5 min. The **4-16** (55 mg, 0.045 mmol) was dissolved in dry THF (2 ml). The solution was added to the blue liquid NH_3, stirred for 2 h. The reaction was quenched with MeOH. After the ammonia was removed, the solution was concentrated, The residue was purified chromatographically (SiO_2, 3% MeOH in CH_2Cl_2) to give **PBS57** (18 mg, 47%) as a white solide. NMR (^{1}H, 10% MeOD in $CDCl_3$), δ5.35 (t, *J* = 5.0 Hz, 2 H), 4.87 (d, *J* = 3.0 Hz, 1 H), 4.16-4.13 (m, 1 H), 3.85-3.51 (m, 9 H), 3.24-3.21 (m, 1 H), 2.1 (t, *J* = 7.5 Hz, 2 H), 2.03-1.98 (m, 7 H), 1.64-1.52 (m, 4 H), 1.38-1.22 (m, 56 H), 0.89-0.86(m, 6 H); NMR (^{13}C , 10% MeOD in $CDCl_3$) δ174.61, 172.60, 129.96, 99.57, 74.71, 72.16, 69.92, 69.12, 68.88, 67.14, 50.53, 49.48, 49.30, 49.13, 48.96, 48.79, 48.62, 39.70, 36.58, 32.70, 31.97, 29.83, 29.77, 29.65, 29.56, 29.50, 29.45, 29.42, 29.39, 29.36, 27.25, 25.95, 25.91, 22.72, 22.58, 14.10; HRFAB-MS (thioglycerol + Na^+ matrix) *m/e* ($[M + Na]^+$) 891.7014(100.0%), calcd 891.7014.



4-14a

Preparation of 4-14a. Compound **3-26a** (58 mg, 0.04 mmol) was dissolved in THF (5 mL)

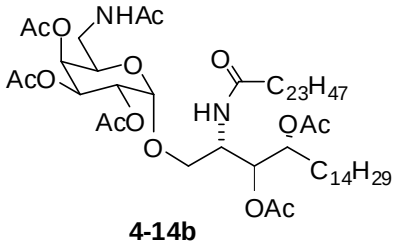
and MeOH 5 mL) and transferred to a hydrogenation vessel. Palladium on carbon (10%, 50 mg) was added to the vessel, and the vessel was subjected to H_2 (350 psi) at room temperature for 12 h. The catalyst was then removed via filtration through a celite pad, and the solvent was removed *in vacuo*. The residue was dissolved in pyridine (5 mL) and acetic anhydride (3 mL), DMAP (50 mg). The solvent was removed *in vacuo* and the residue was purified via SiO_2 chromatography to give compound **4-14a** (35.8 mg, 80%). ^{1}H NMR ($CDCl_3$, 500 MHz) δ 6.41 (d, *J* = 9.5 Hz, 1 H), 6.35 (t, *J* = 5.5 Hz, 1 H), 5.38 (d, *J* = 3.5 Hz, 1 H), 5.27 (dd, *J* = 10.5, 3.5 Hz, 1 H), 5.23 (dd, *J* = 9.0, 3.0 Hz, 1 H), 5.12 (dd, *J* = 10.5, 3.5 Hz, 1 H), 4.95 (d, *J* = 4.0 Hz, 1 H), 4.87 (dt, *J* = 10.5, 3.0 Hz, 1 H), 4.35 (tt, *J* = 9.5, 3.0 Hz, 1 H), 4.01 (t, *J* = 7.0 Hz, 1 H), 3.67-3.60 (m, 2 H), 3.39-3.33 (m, 2 H), 2.32-2.23 (m, 2 H), 2.16 (s, 3 H), 2.09 (s, 3 H), 2.02 (s, 3 H), 1.99 (s, 3 H), 1.98 (s, 3 H), 1.72-1.54 (m, 4 H), 1.39-1.13 (m, 68 H), 0.87 (t, *J* = 7.0 Hz, 6 H); ^{13}C NMR ($CDCl_3$, 125 MHz) δ173.43, 171.52, 170.66, 170.56, 170.09, 169.94, 98.36, 73.42, 71.05, 69.47, 68.58, 67.97, 67.80, 67.57, 48.42, 39.24, 36.81, 32.05, 29.83, 29.74, 29.79, 29.53, 29.49, 29.45, 29.40, 27.76, 25.82, 25.68, 23.23, 22.82, 21.20, 20.89, 20.83, 20.78, 14.25; HRMS (FAB) *m/z* for $C_{62}H_{112}N_2NaO_{14}$ ([M+Na]+) 1131.8013 (100%), calc. 1131.8011.



4-9a

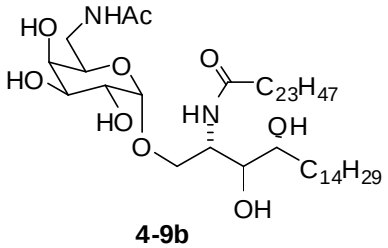
Preparation of 4-9a. Compound **4-14a** (35.8 mg, 0.032 mmol) was dissolved in MeOH (4 mL) and followed by addition of Na° (5 mg), stirred for 5 h. **4-19a** would precipitate directly in the methanol to give pure white powder (16 mg, 55%). NMR (^{1}H, 10% MeOD in $CDCl_3$,

500 MHz), 4.87 (d, *J* = 3.5 Hz, 1 H), 3.82-3.71 (m, 5 H), 3.64 (dd, *J* = 11.0, 4.5 Hz, 1 H), 3.59 (t, *J* = 5.5 Hz, 1 H), 3.56-3.48 (m, 2 H), 3.36-3.34 (m, 1 H), 3.25-3.21 (m, 1 H), 2.19 (t, *J* = 7.5 Hz, 2 H), 1.98 (s, 3 H), 1.64-1.52 (m, 4 H), 1.35-1.22 (m, 68 H), 0.88 (t, *J* = 7.0 Hz, 6 H); NMR (^{13}C , 10% MeOD in $CDCl_3$) δ174.78, 172.77, 99.54, 74.24, 72.02, 69.90, 69.44, 68.99, 68.77, 67.02, 50.55, 49.34, 49.17, 49.00, 48.83, 48.66, 48.49, 48.32, 39.84, 36.49, 32.13, 31.98, 29.79, 29.75, 29.54, 29.57, 29.46, 29.42, 25.98, 22.73, 22.39, 14.07; HRMS (ESI) calcd for $C_{52}H_{103}N_2O_9$ $[M+H]^+$ 899.76636, found 899.76624.



4-14b

Preparation of 4-14b. Same procedure as synthesis of **4-14a** (85 %). ^{1}H NMR ($CDCl_3$, 500 MHz) δ 6.36 (d, *J* = 10.0 Hz, 1 H), 6.24 (t, *J* = 6.0 Hz, 1 H), 5.37 (d, *J* = 2.5 Hz, 1 H), 5.27 (dd, *J* = 10.5, 3.0 Hz, 1 H), 5.23 (dd, *J* = 9.0, 2.5 Hz, 1 H), 5.12 (dd, *J* = 10.5, 3.5 Hz, 1 H), 4.94 (d, *J* = 3.5 Hz, 1 H), 4.86 (dt, *J* = 10.0, 3.0 Hz, 1 H), 4.34 (tt, *J* = 9.0, 3.0 Hz, 1 H), 4.00 (t, *J* = 7.0 Hz, 1 H), 3.66-3.60 (m, 2 H), 3.38-3.33 (m, 2 H), 2.30-2.21 (m, 2 H), 2.16 (s, 3 H), 2.09 (s, 3 H), 2.08 (s, 3 H), 2.02 (s, 3 H), 1.99 (s, 3 H), 1.97 (s, 3 H), 1.69-1.56 (m, 4 H), 1.39-1.21 (m, 64 H), 0.87 (t, *J* = 7.0 Hz, 6 H); ^{13}C NMR ($CDCl_3$, 125 MHz) δ173.48, 171.67, 170.66, 170.79, 170.60, 170.22, 170.06, 98.48, 73.58, 71.11, 69.58, 68.72, 68.08, 67.96, 67.65, 48.51, 39.33, 36.94, 32.16, 29.94, 29.85, 29.82, 29.64, 29.60, 29.56, 29.51, 27.85, 25.92, 25.79, 23.38, 22.93, 21.33, 21.01, 20.95, 20.91, 14.36; HRMS (FAB) *m/z* for $C_{60}H_{108}N_2NaO14$ ([M+Na]+) 1103.7696(100%), calc. 1103.7698.



4-9b

Preparation of 4-9b. Same procedure as synthesis of **4-9a** (67 %). NMR (^{1}H, 10% MeOD in $CDCl_{3,}$ 500 MHz), 4.86 (d, *J* = 3.5 Hz, 1 H), 3.82-3.72 (m, 5 H), 3.64 (dd, *J* = 10.0, 4.5 Hz, 1 H), 3.59 (t, *J* = 6.0 Hz, 1 H), 3.56-3.48 (m, 2 H), 3.25-3.21 (m, 1 H), 2.20 (t, *J* = 7.5 Hz, 2 H), 1.98 (s, 3 H), 1.64-1.50 (m, 4 H), 1.40-1.22 (m, 64 H), 0.88 (t, *J* = 7.0 Hz, 6 H); NMR (^{13}C , 10% MeOD in $CDCl_3$) δ174.78, 172.77, 99.54, 74.24, 72.02, 69.90, 69.44, 68.99, 68.77, 67.02, 50.55, 49.34, 49.17, 49.00, 48.83, 48.66, 48.49, 48.32, 39.84, 36.49, 32.13, 31.98, 29.79, 29.75, 29.54, 29.57, 29.46, 29.42, 25.98, 22.73, 22.39, 14.07; HRMS (FAB) *m/z* for $C_{34}H_{66}N_2NaO_9$ ([M+Na]+) 893.7174 (100%), calc. 893.7170.

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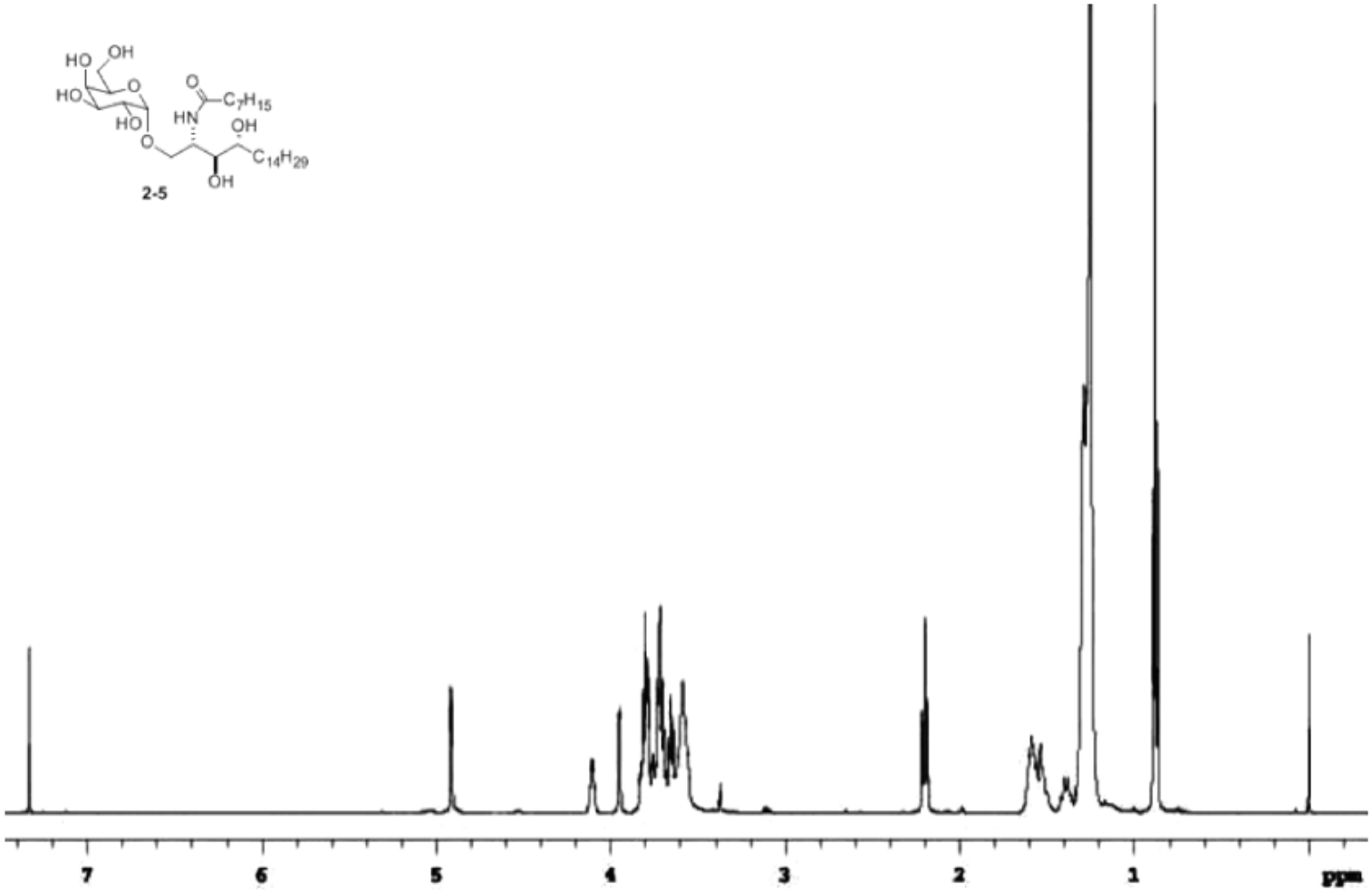
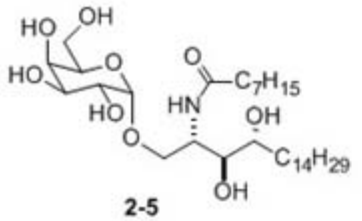
APPENDIX. ^{1}H NMR AND ^{13}C NMR SPRCTRA OF SYNTHETIC TARGETS:

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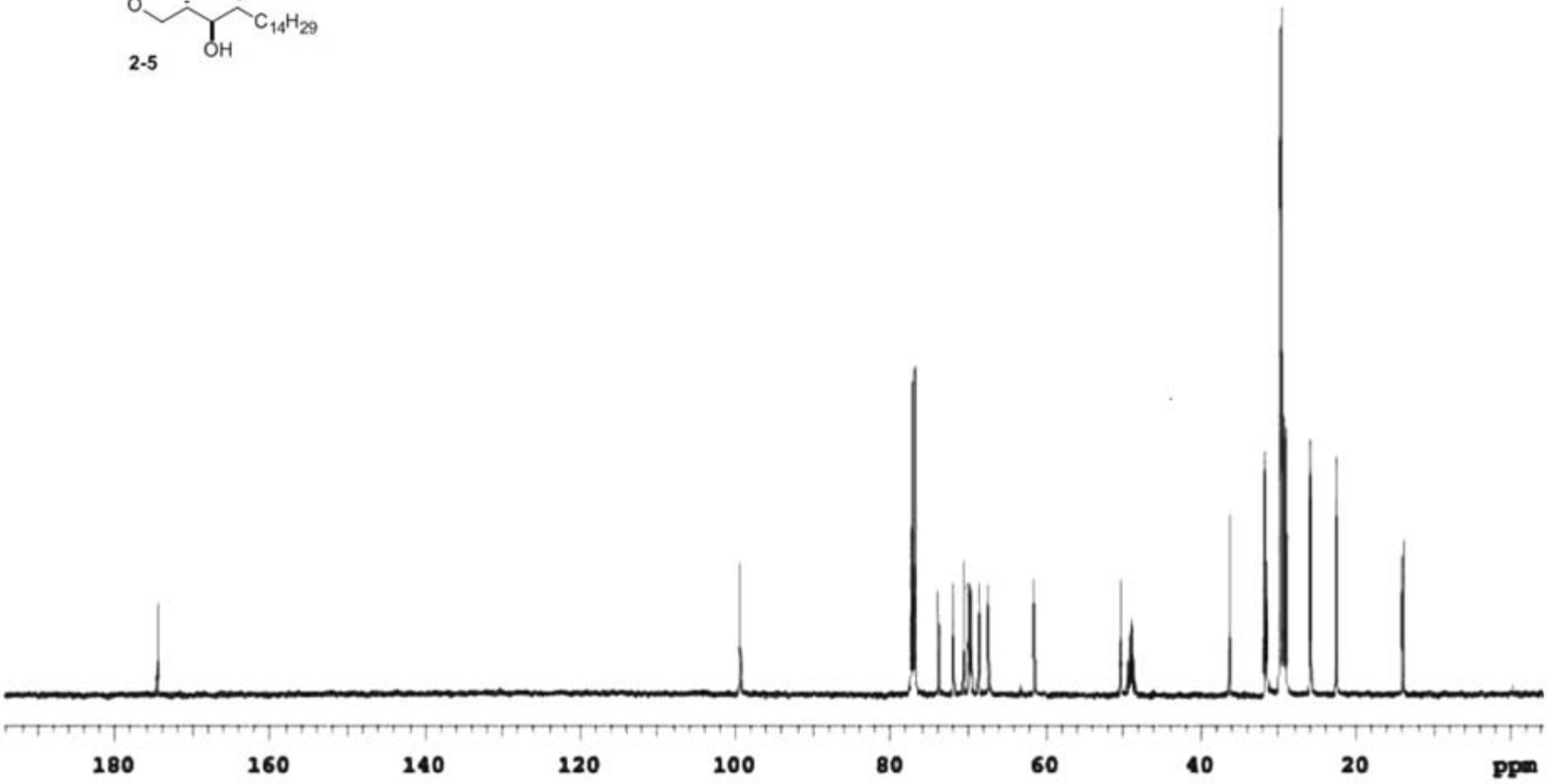
OH

2-5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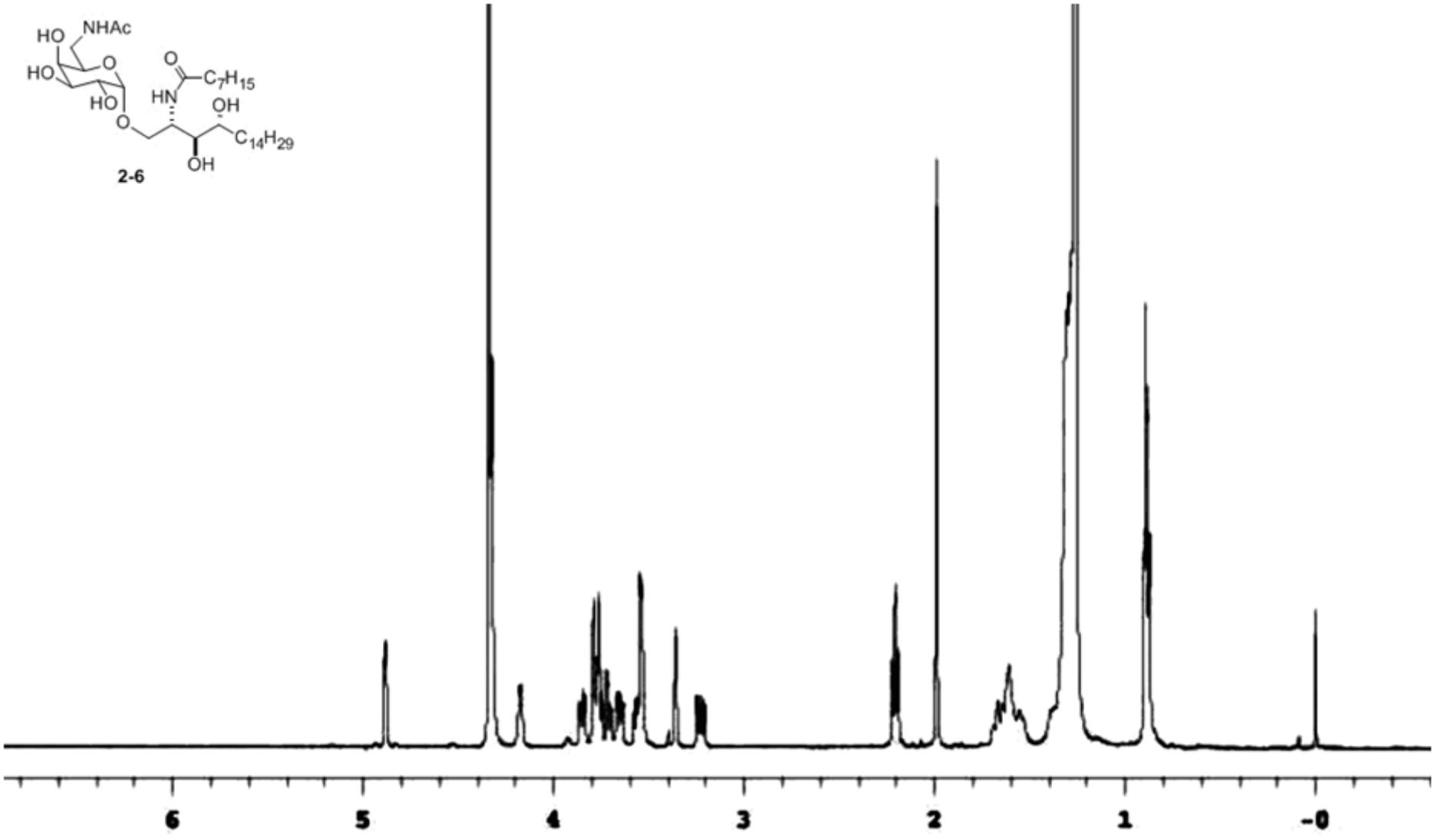
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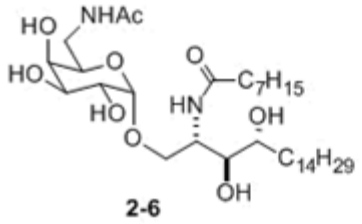
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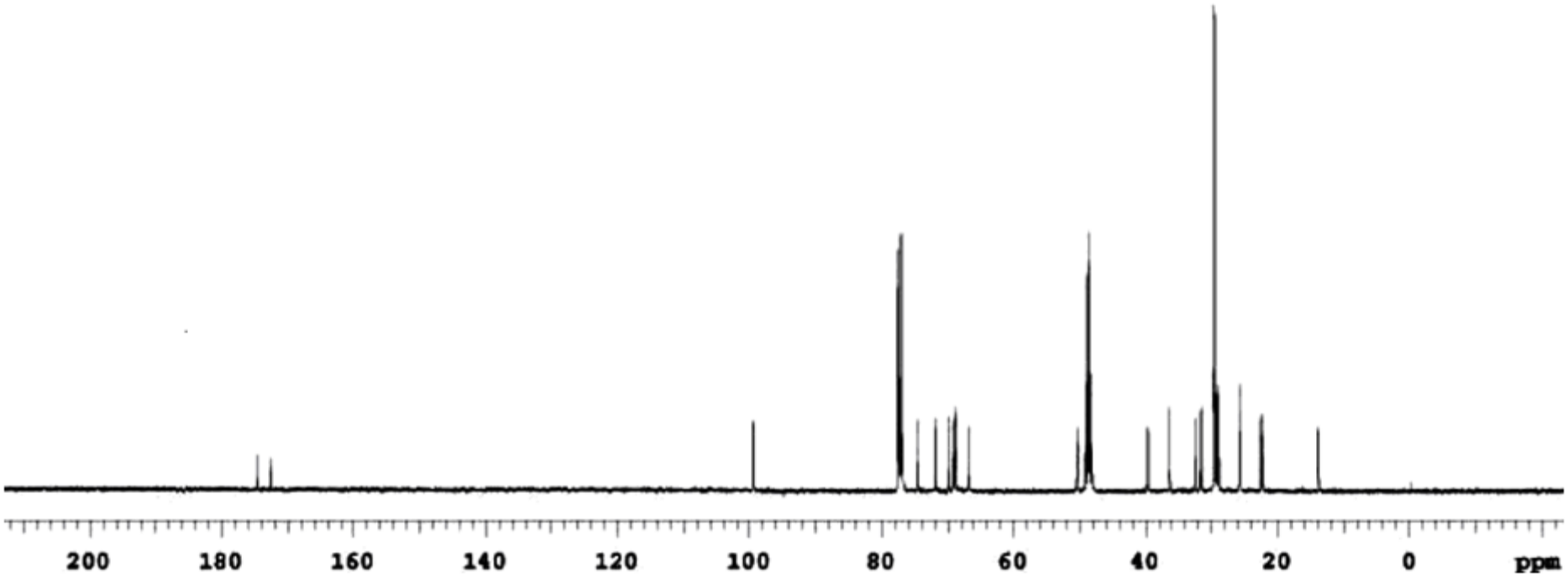
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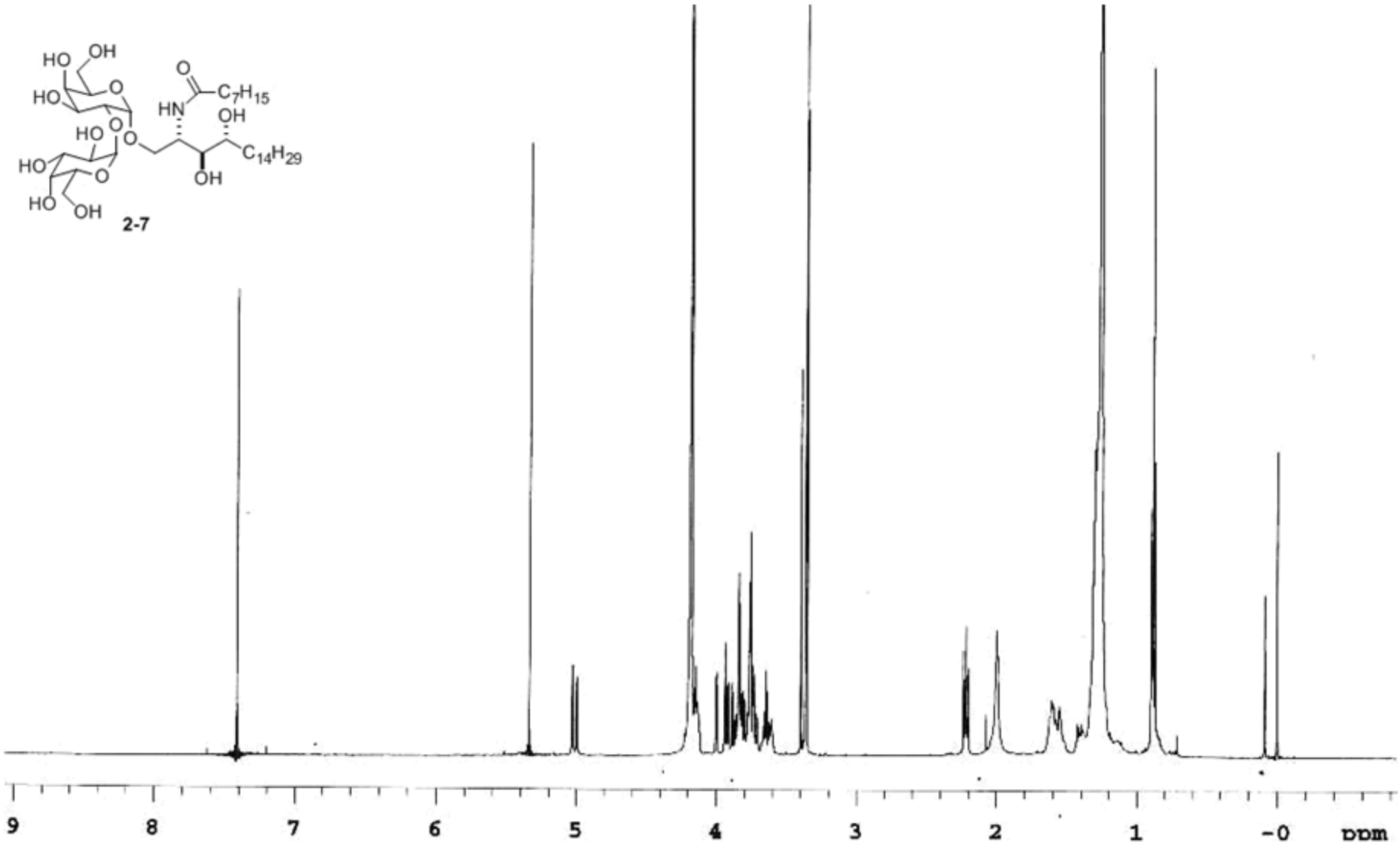
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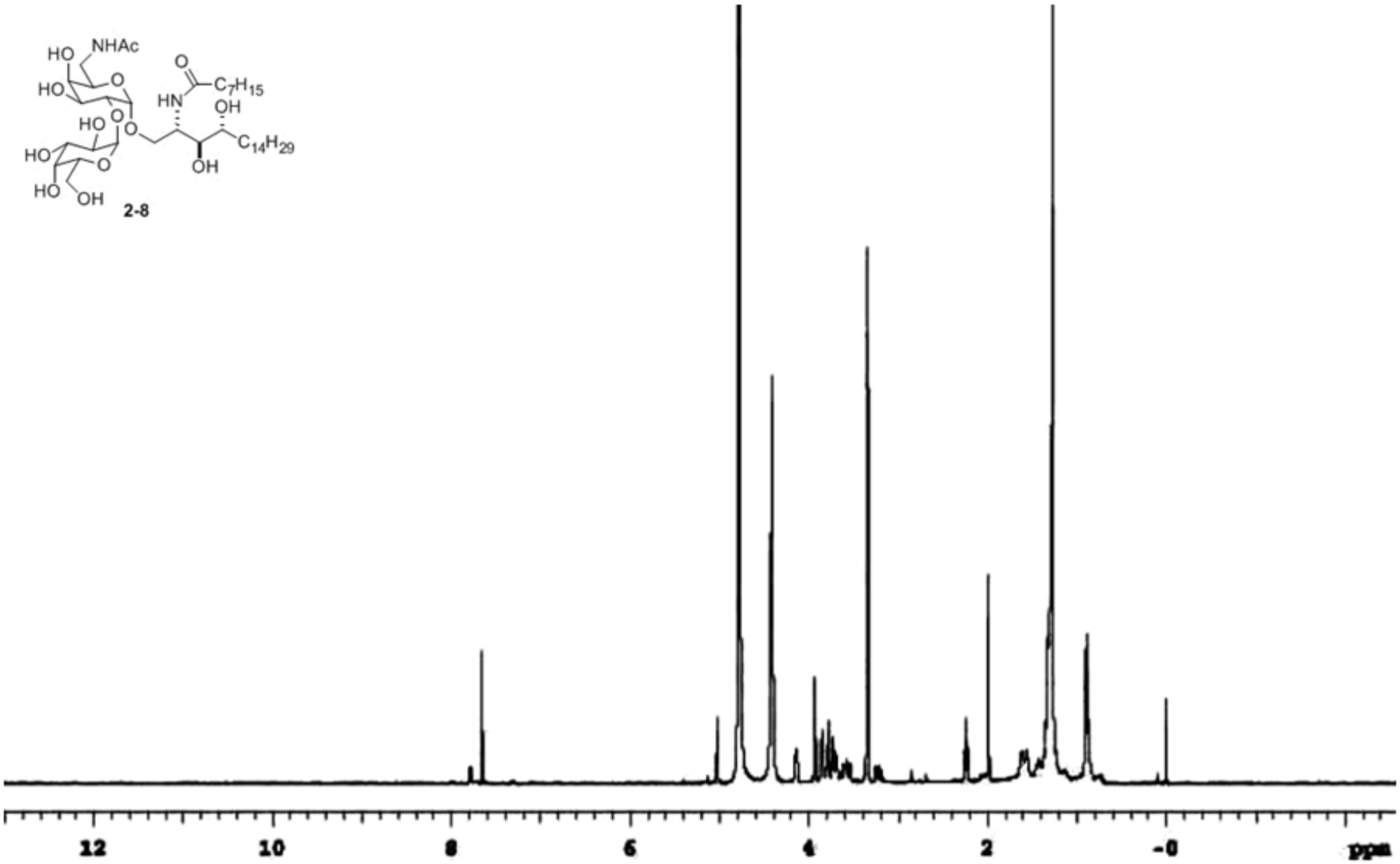
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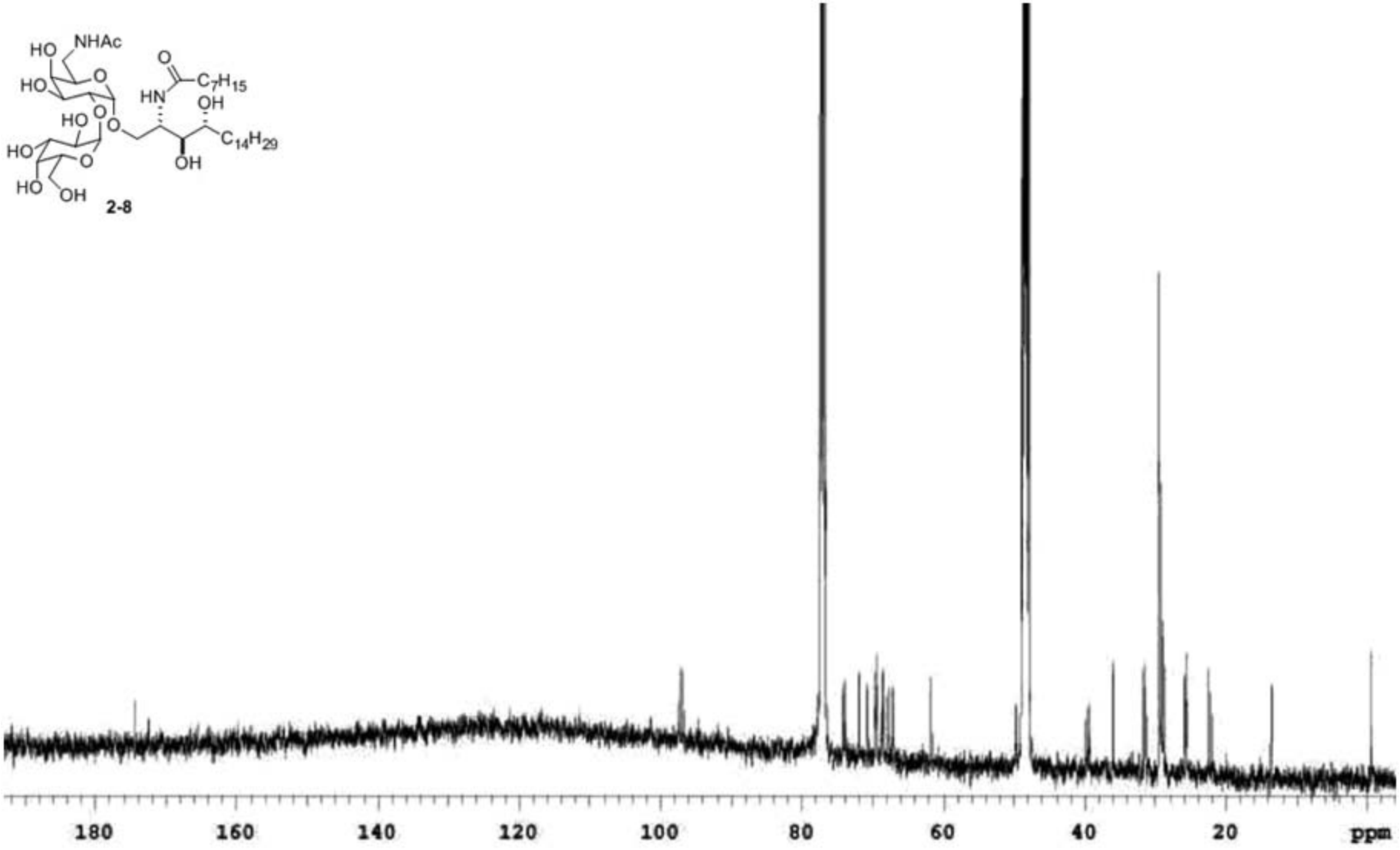
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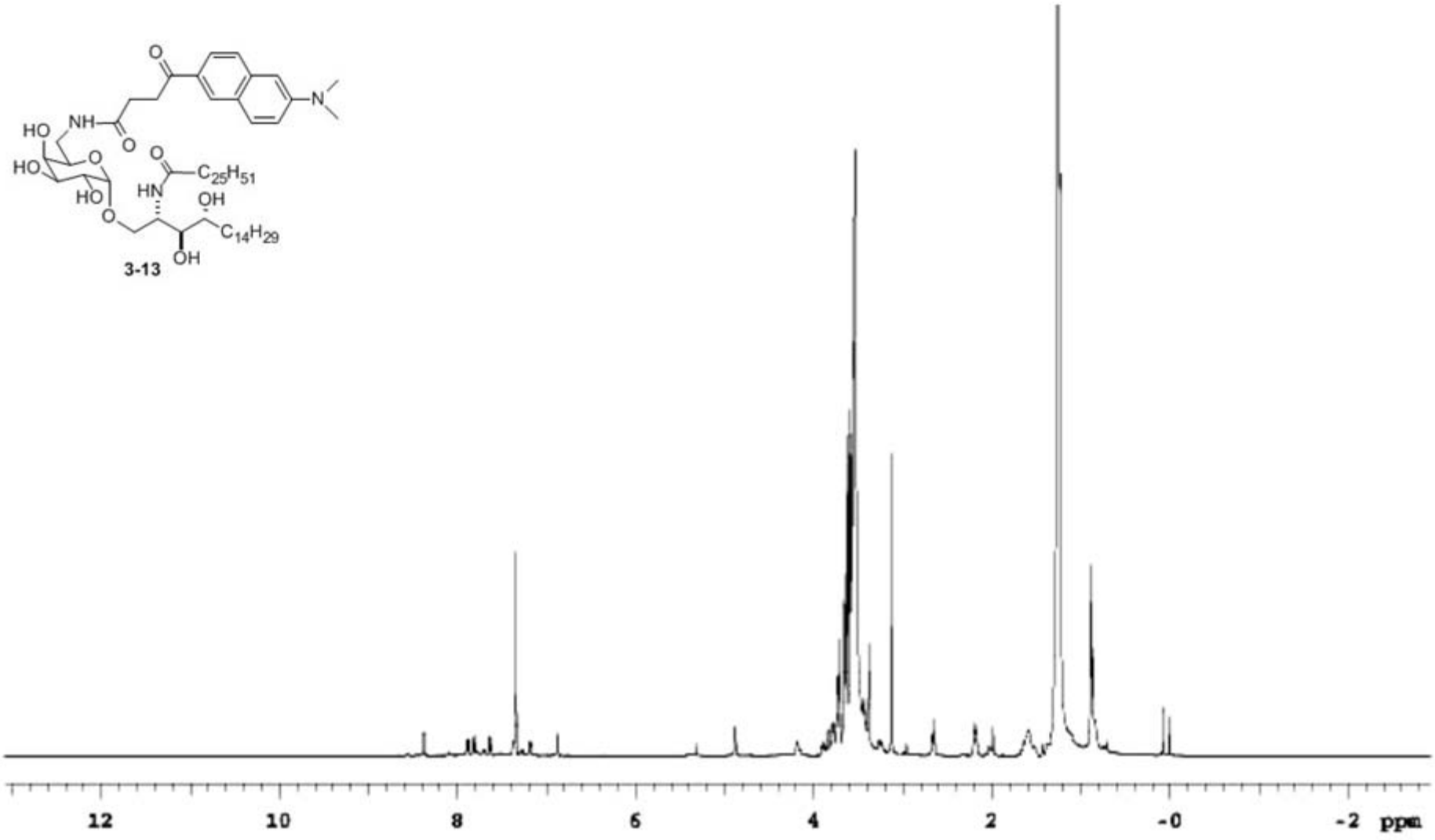
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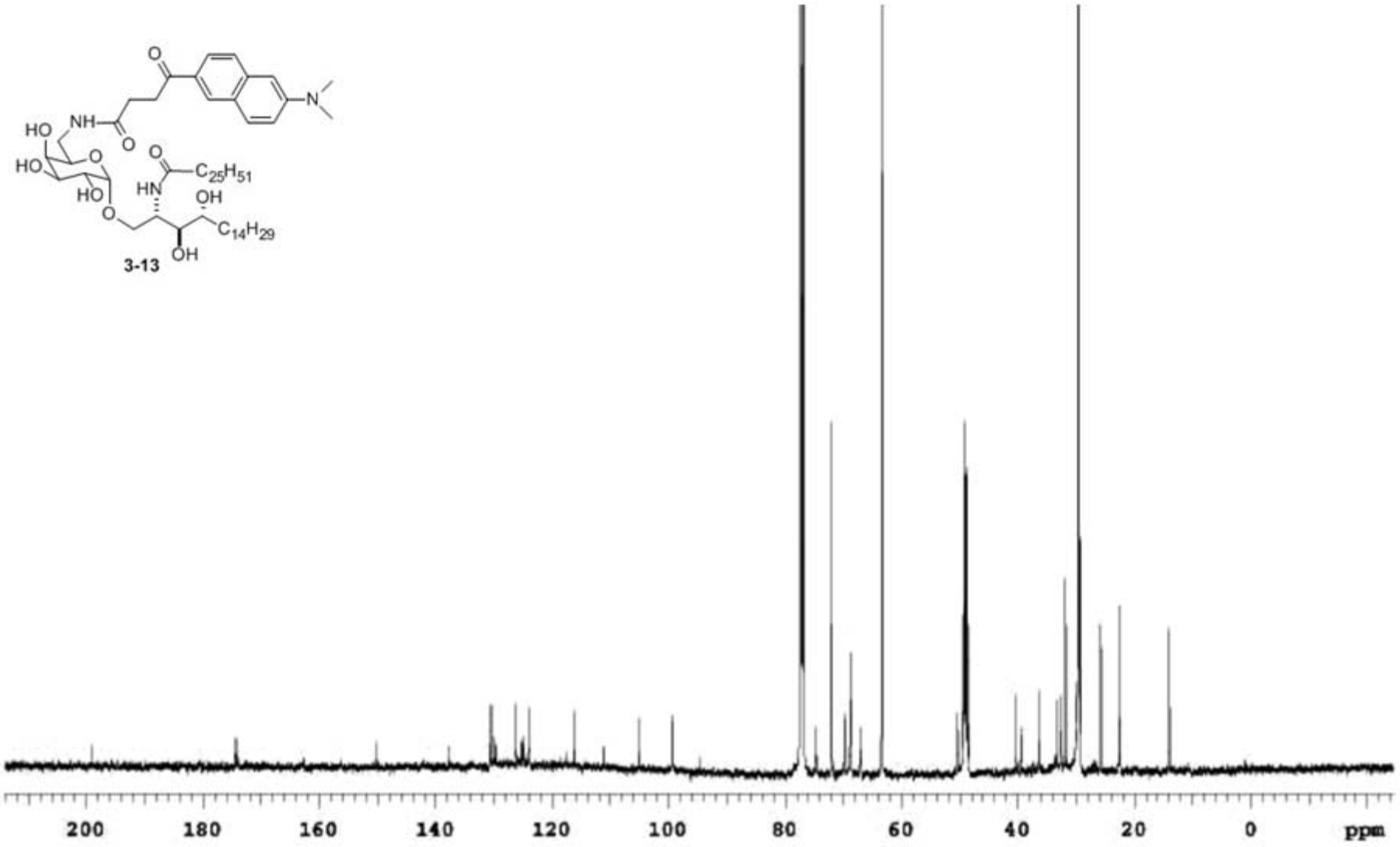
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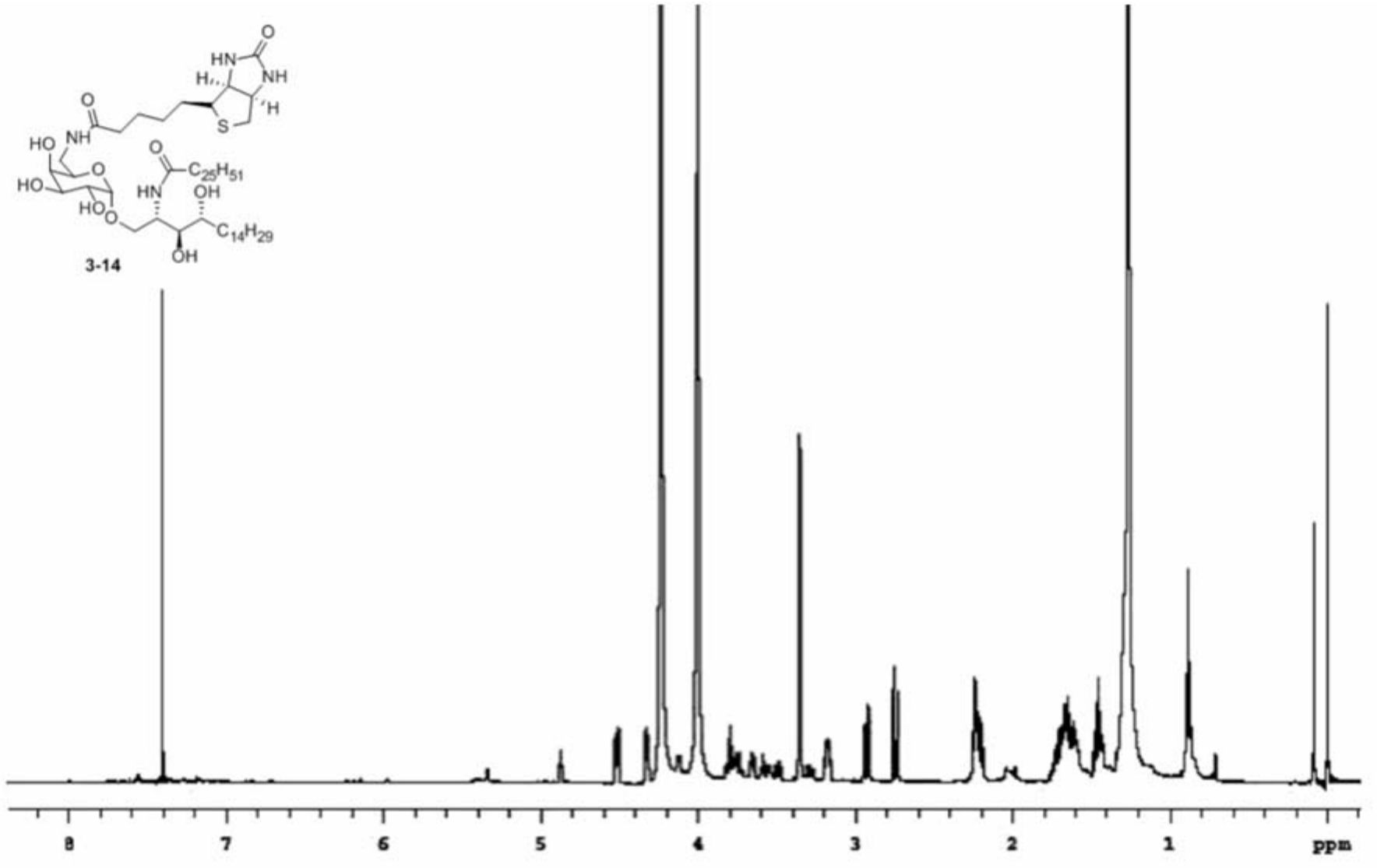
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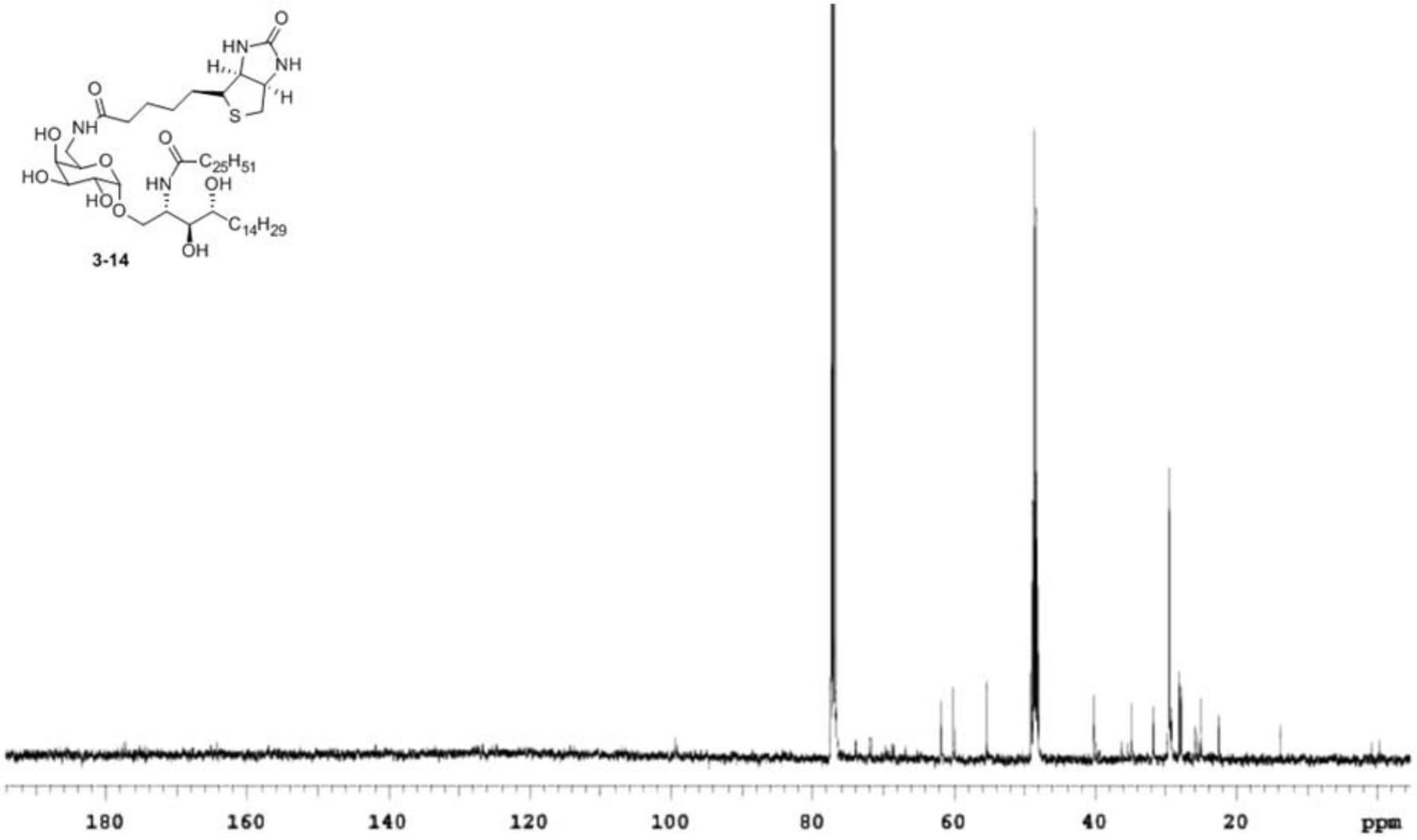
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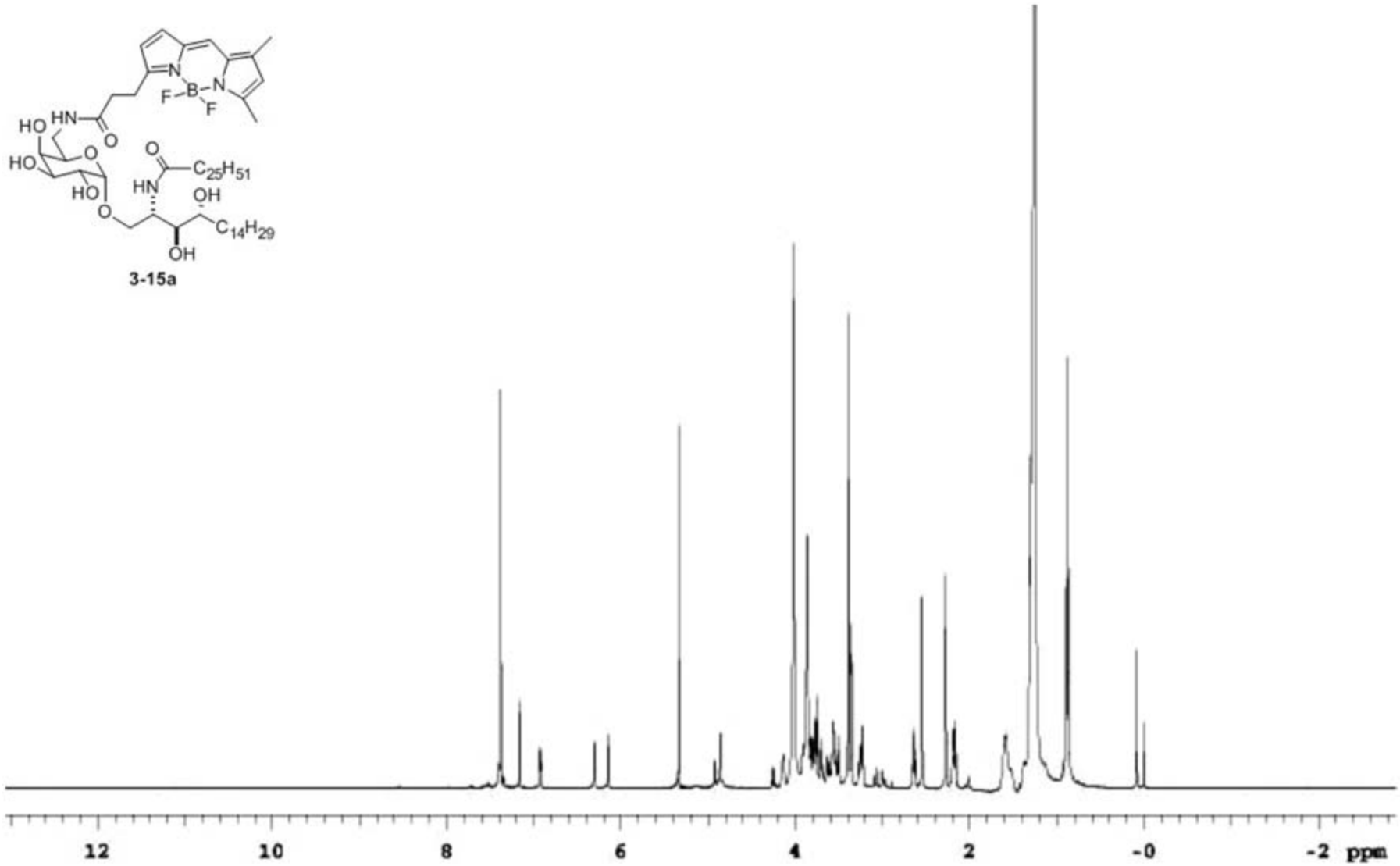
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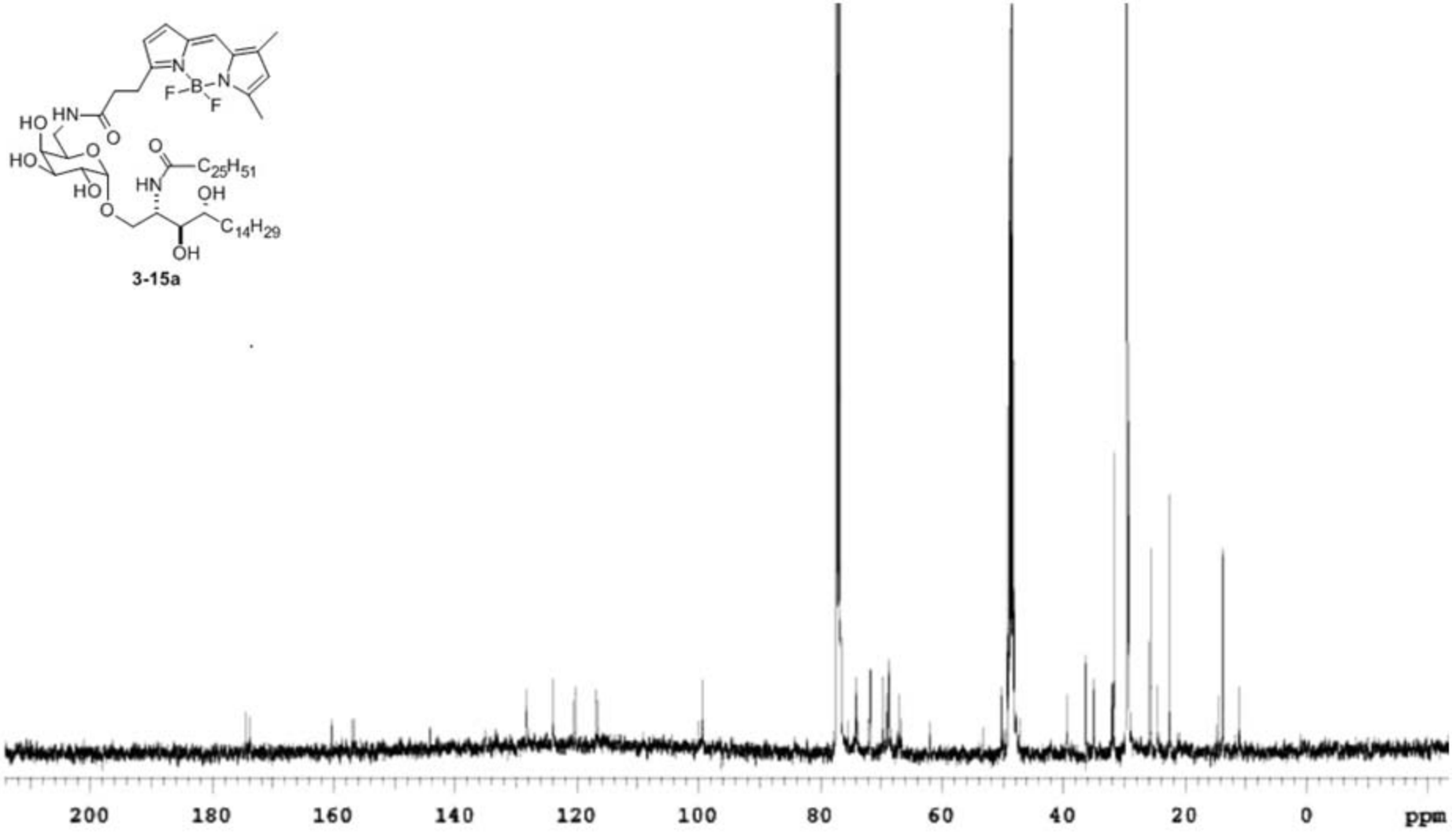
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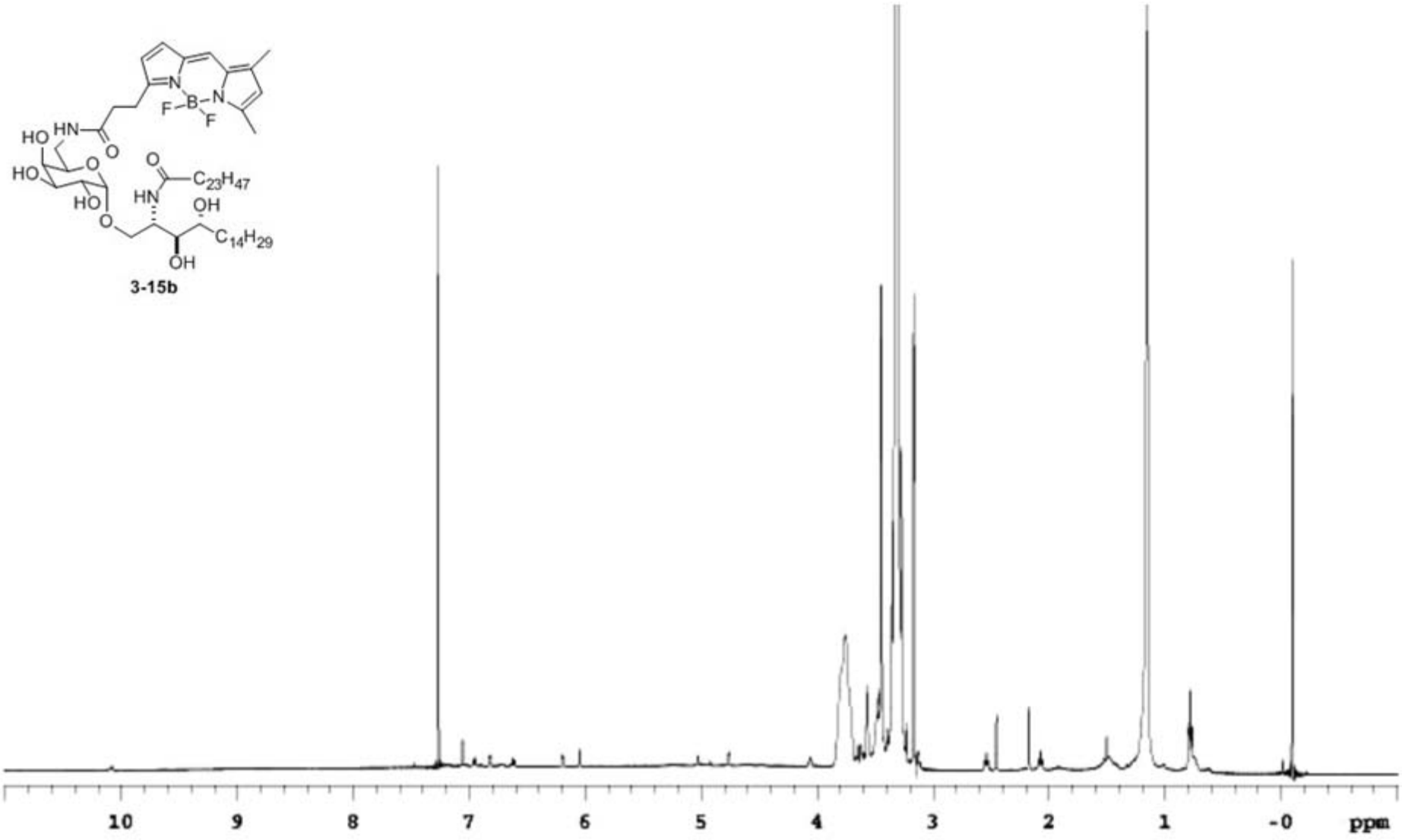
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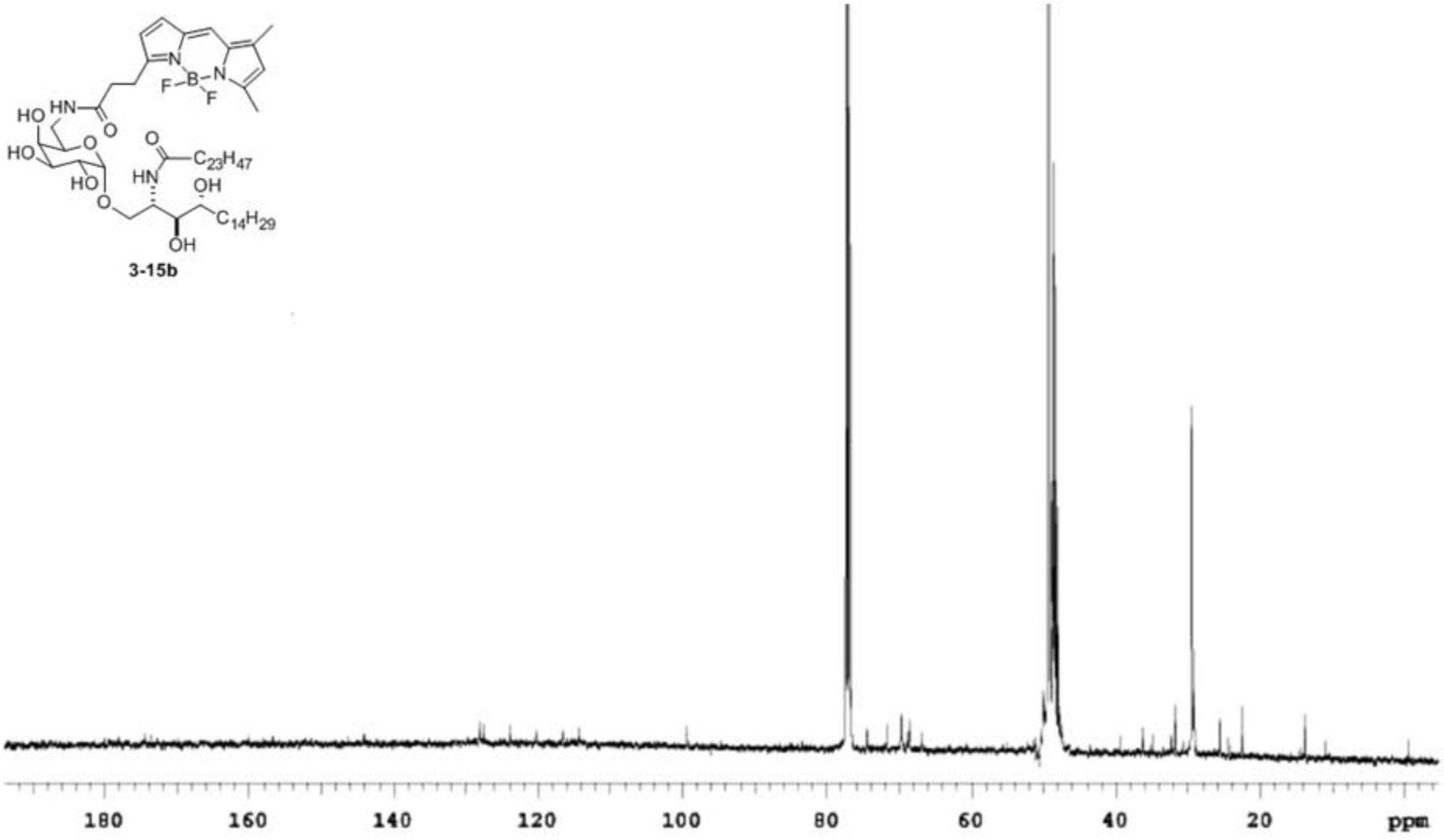
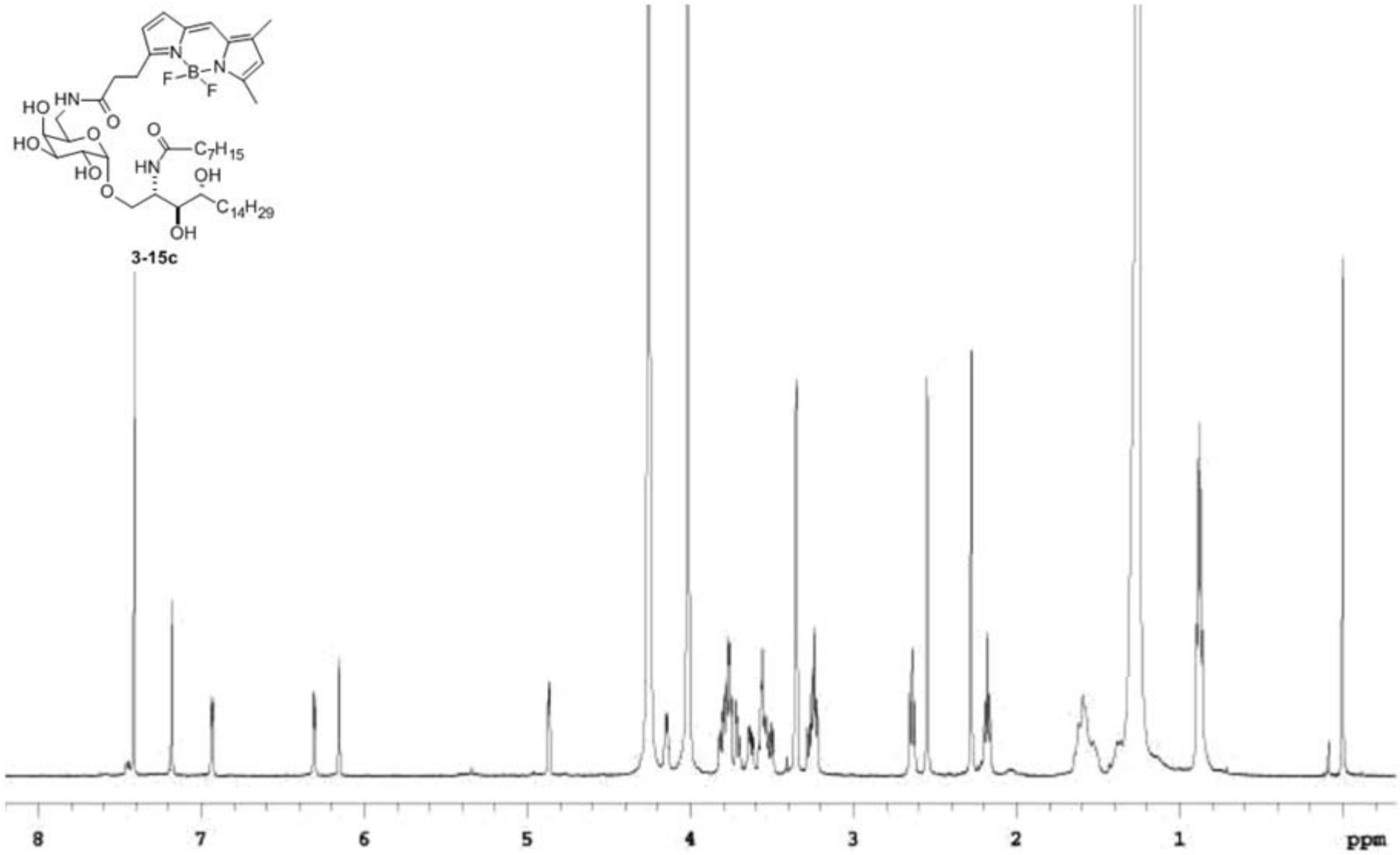
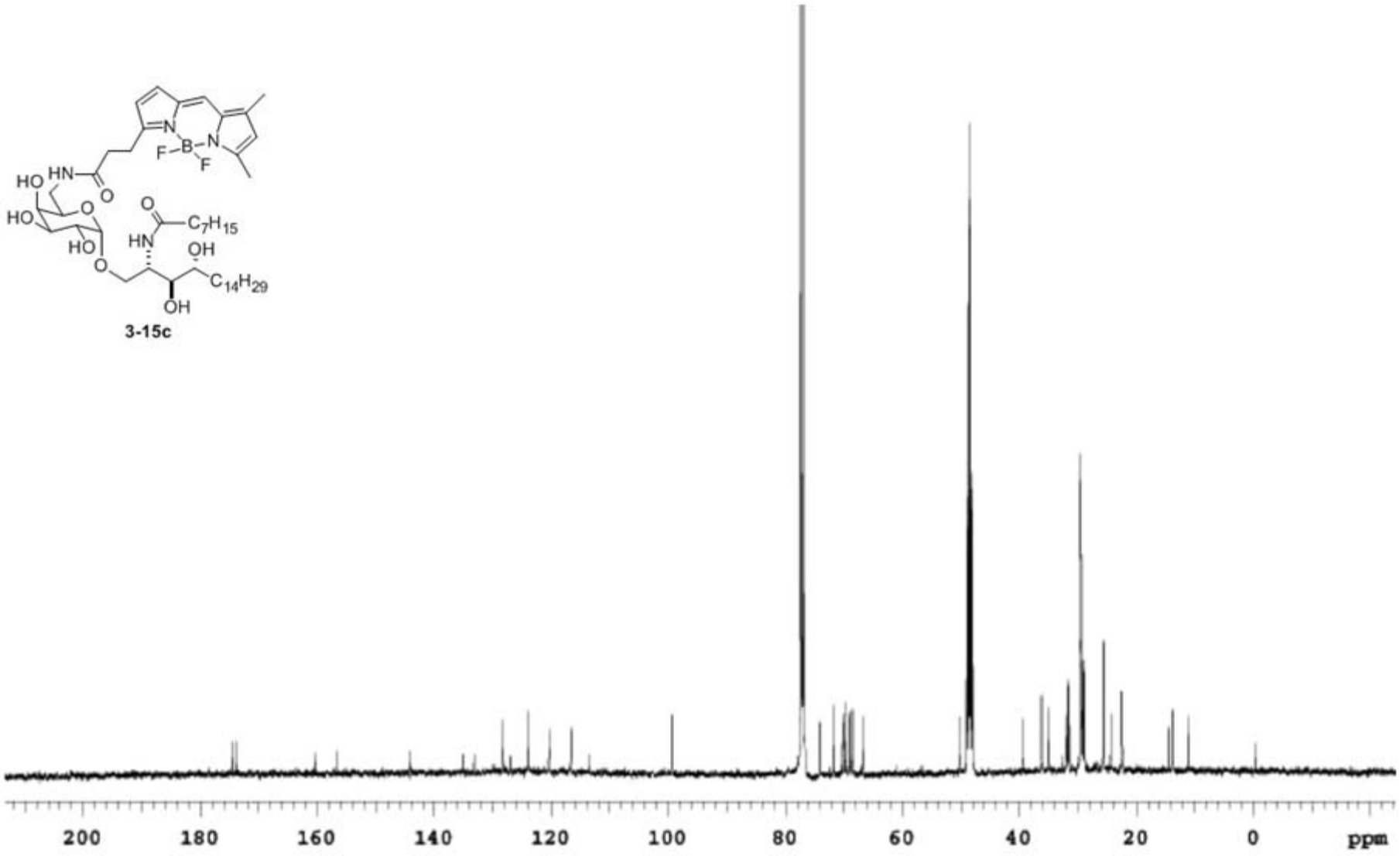
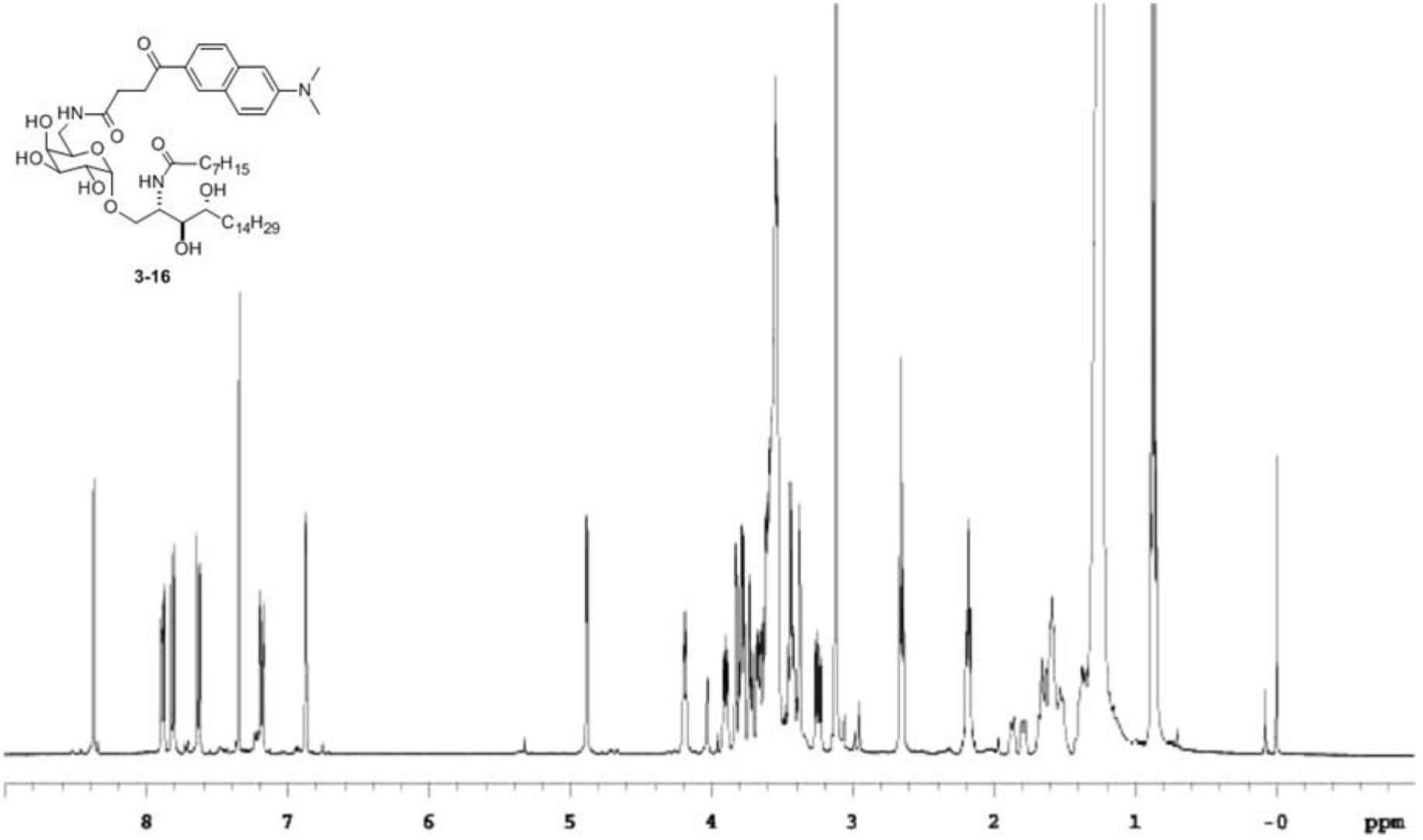
ppm

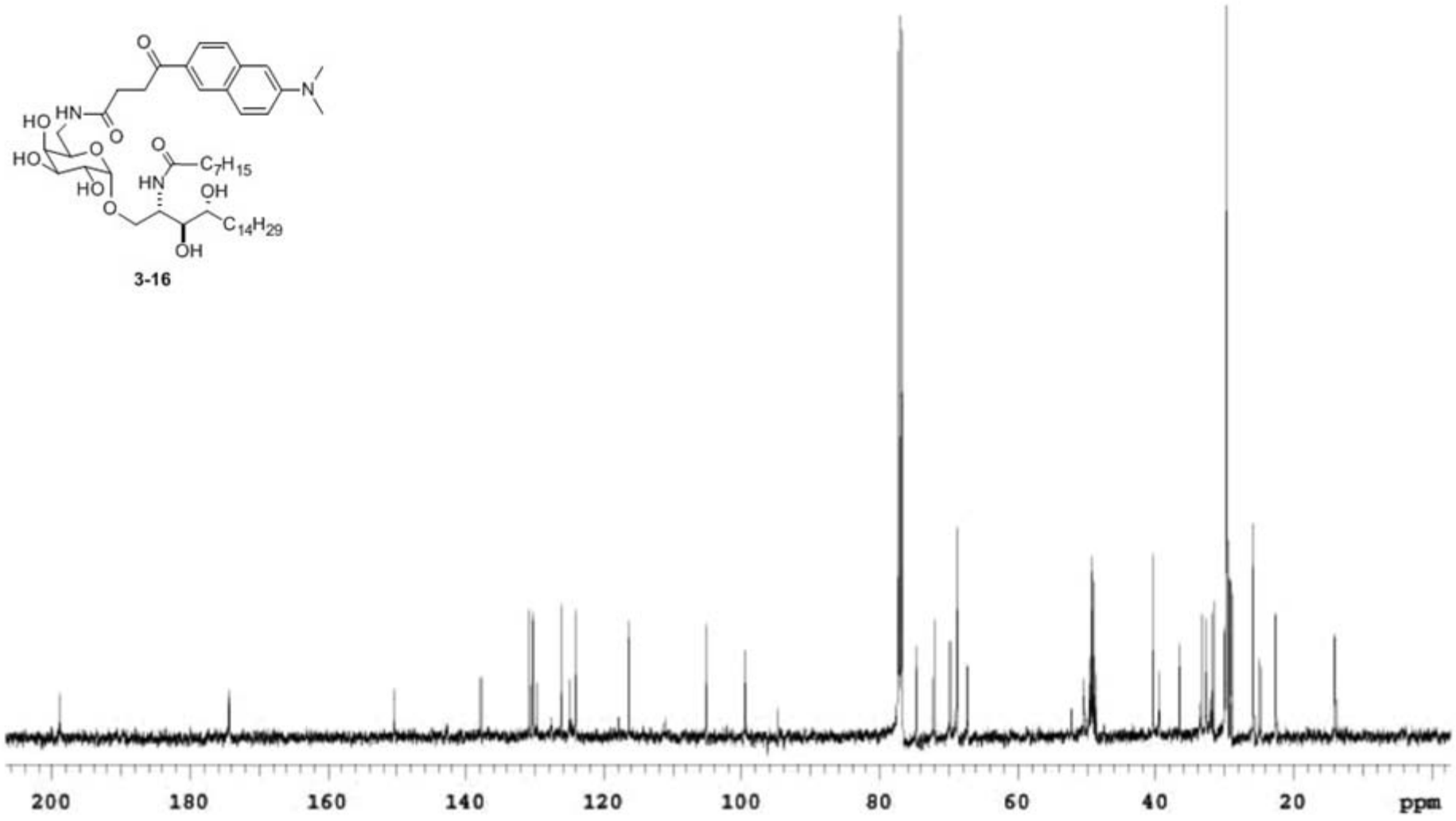

HO
HN
O
HO
O
HO
O
HN
O
$C_{23}H_{47}$
OH
$C_{14}H_{29}$
OH
F
B
F
N
N
180
160
140
120
100
80
60
40
20
ppm

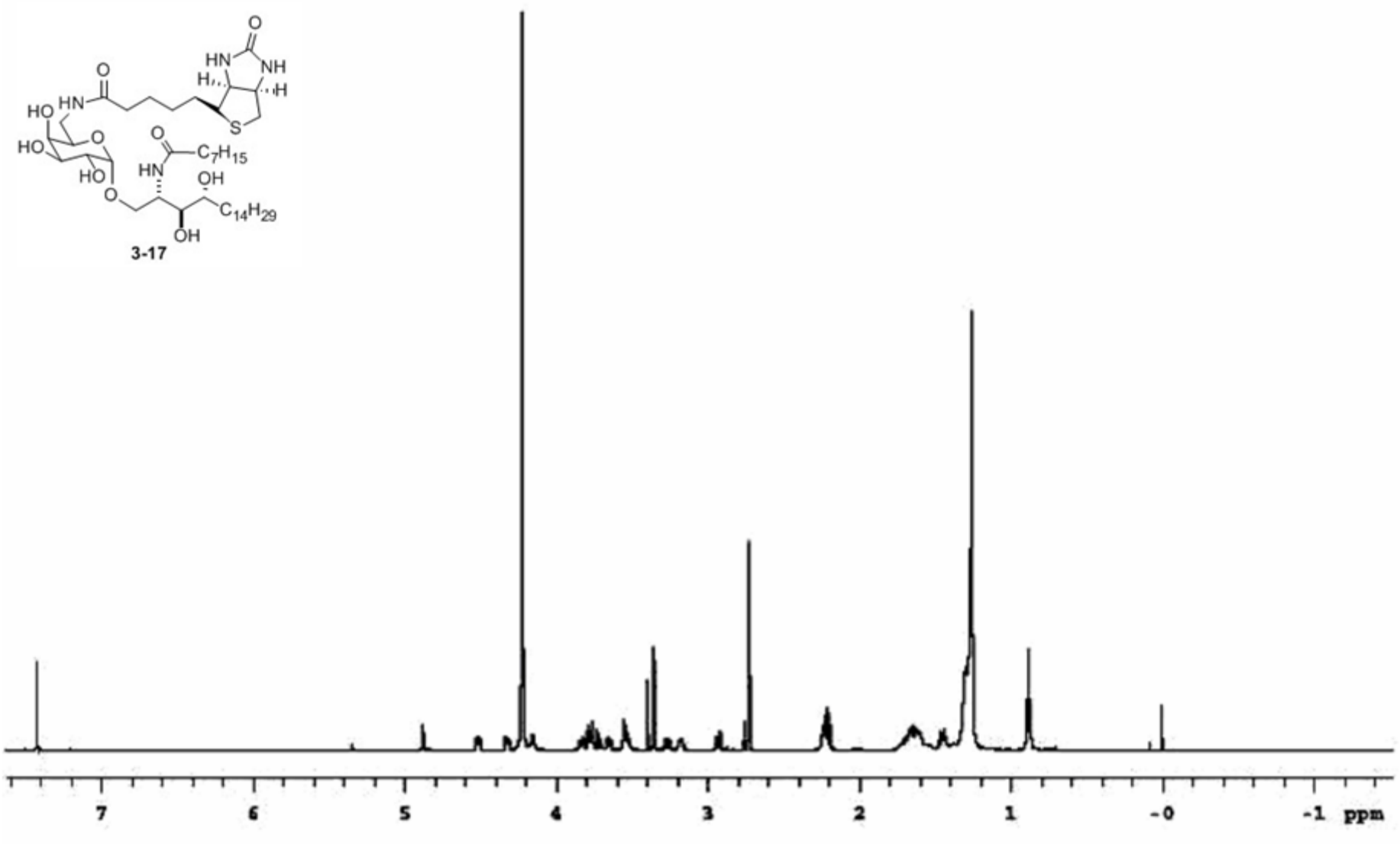
3-15b

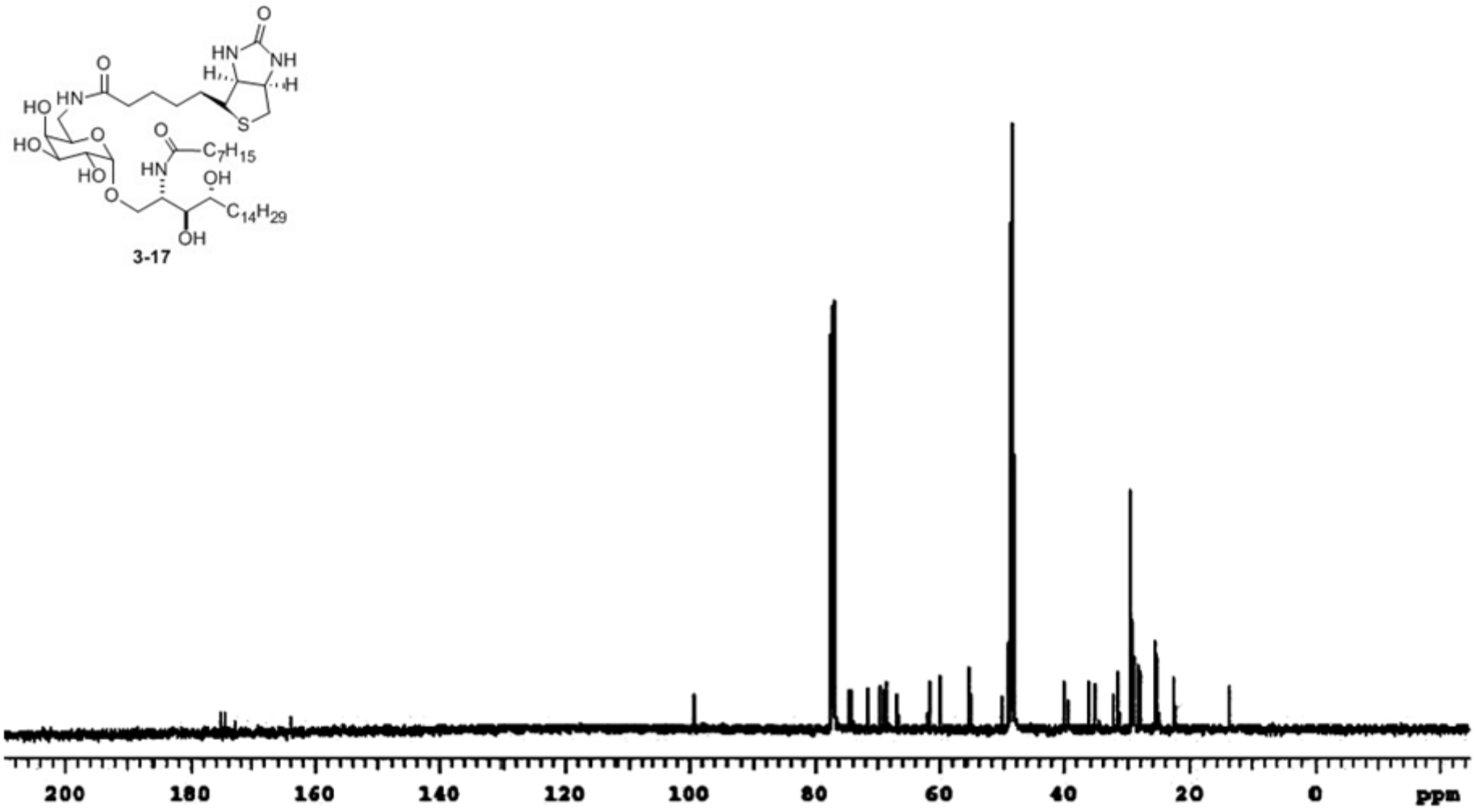

HO
HN
O
HO
O
HO
O
HN
O
C_7H_{15}
OH
$C_{14}H_{29}$
OH
N
F
B
F
N
3-15c
8
7
6
5
4
3
2
1
ppm

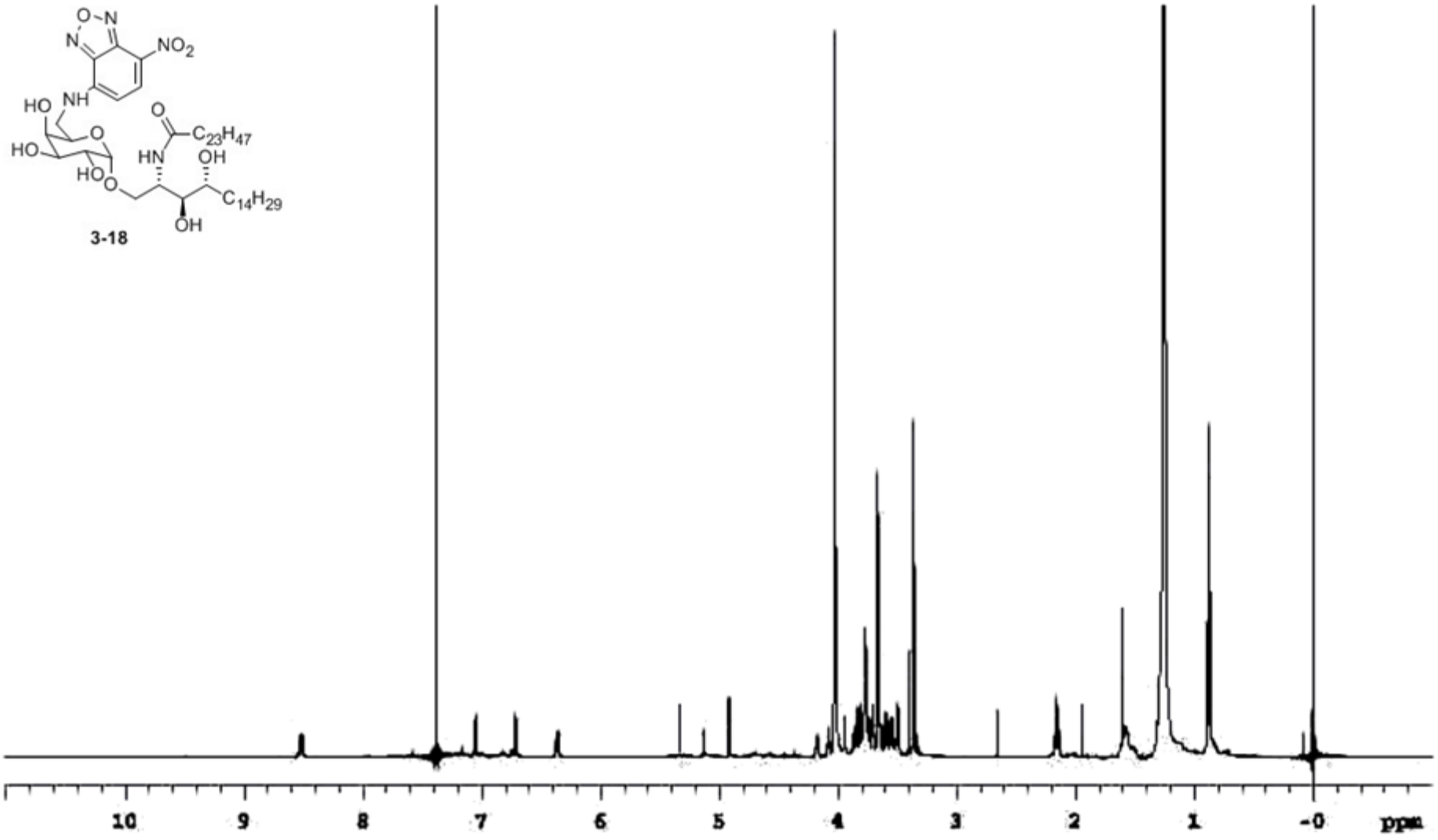

HO
HN
O
HO
O
HO
O
HN
O
C_7H_{15}
OH
$C_{14}H_{29}$
OH
F
B
F
N
N
3-15c
200
180
160
140
120
100
80
60
40
20
0
ppm

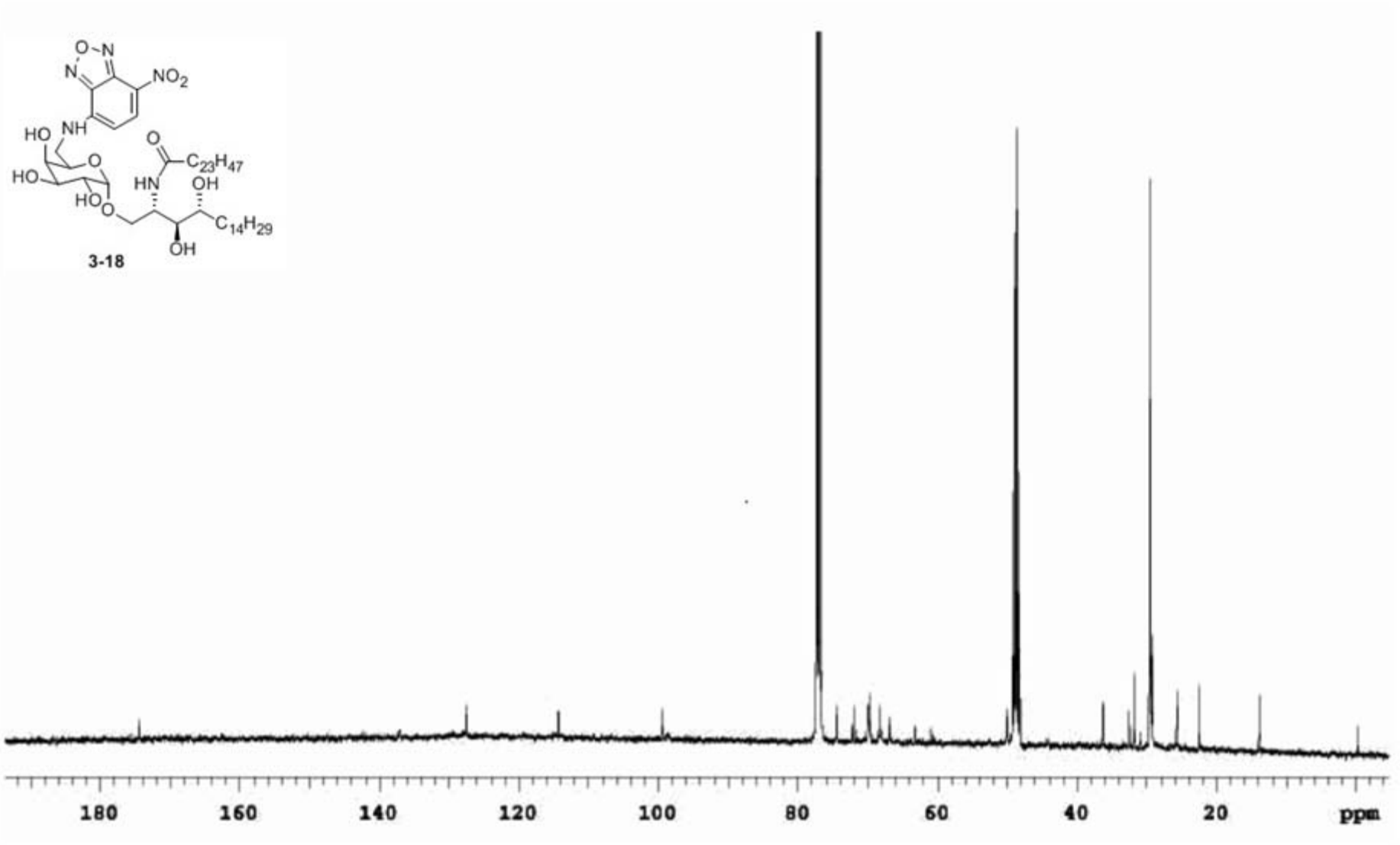

HO
HN
O
O
HO
O
HO
O
HN
O
C_7H_{15}
OH
$C_{14}H_{29}$
OH
N
3-16
8
7
6
5
4
3
2
1
-0
ppm

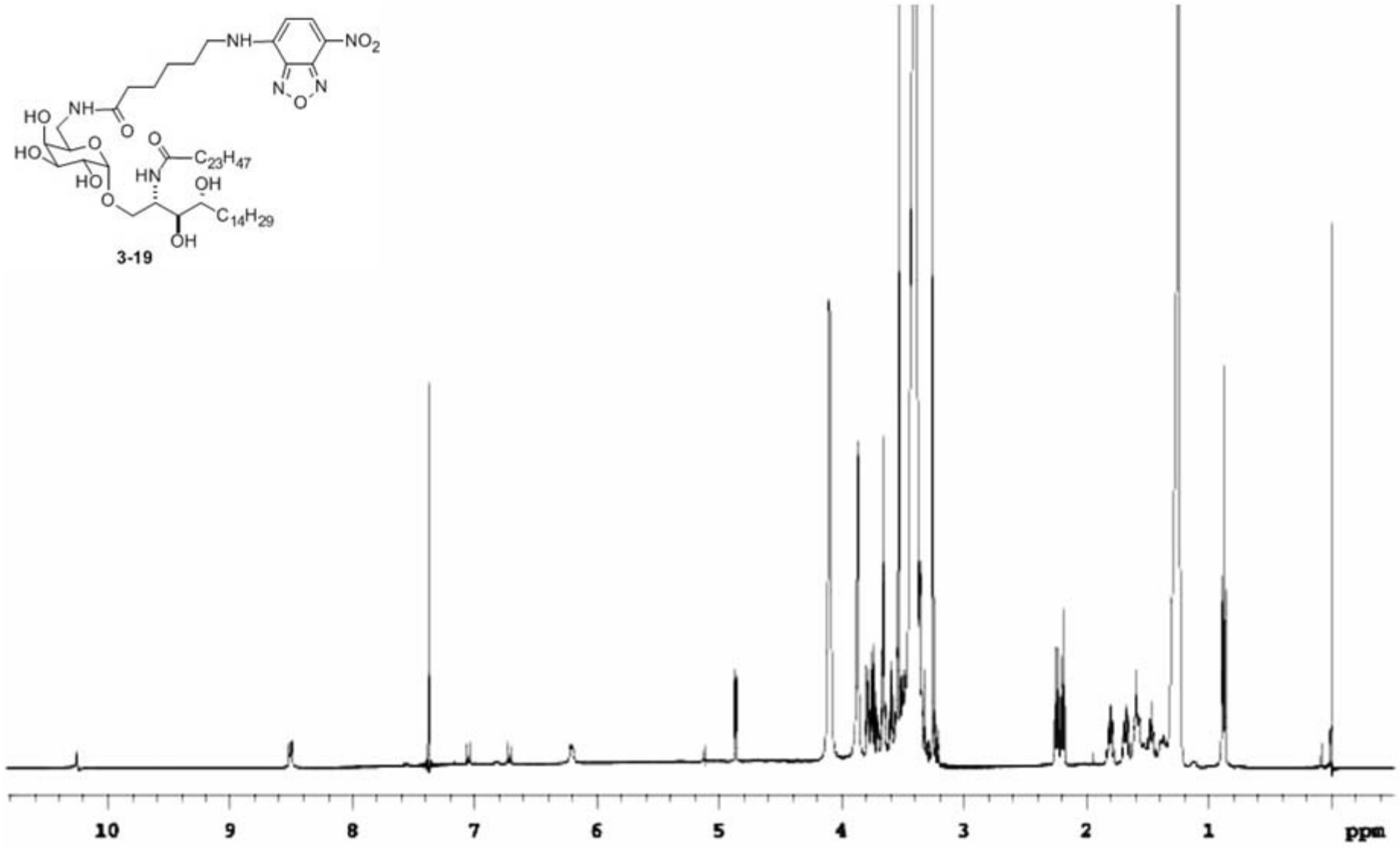

HO
HN
O
O
N
HO
O
HO
HO
O
HN
O
C7H15
OH
C14H29
OH
3-16
200
180
160
140
120
100
80
60
40
20
ppm

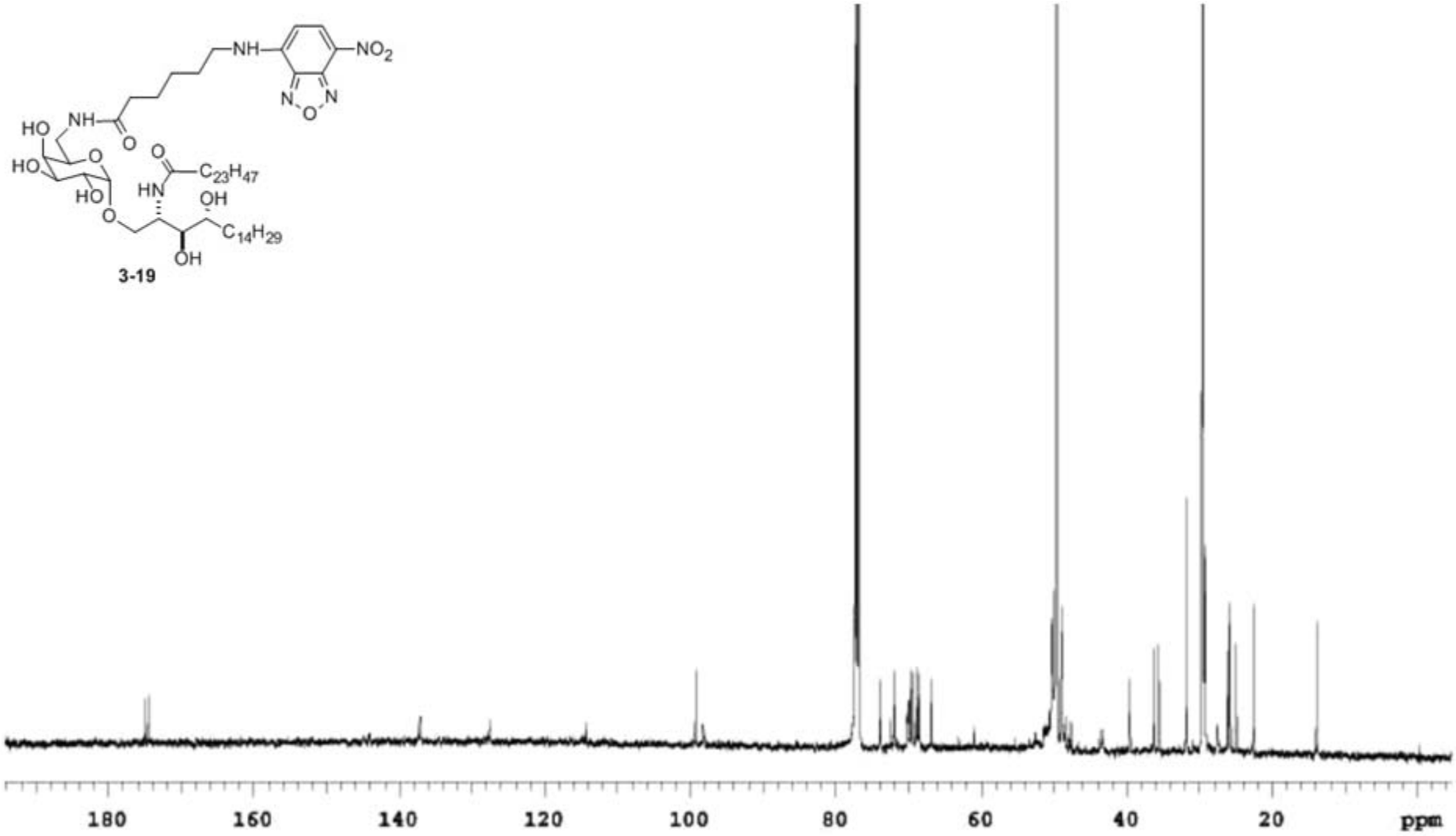

3-17
7
6
5
4
3
2
1
-0
-1 ppm

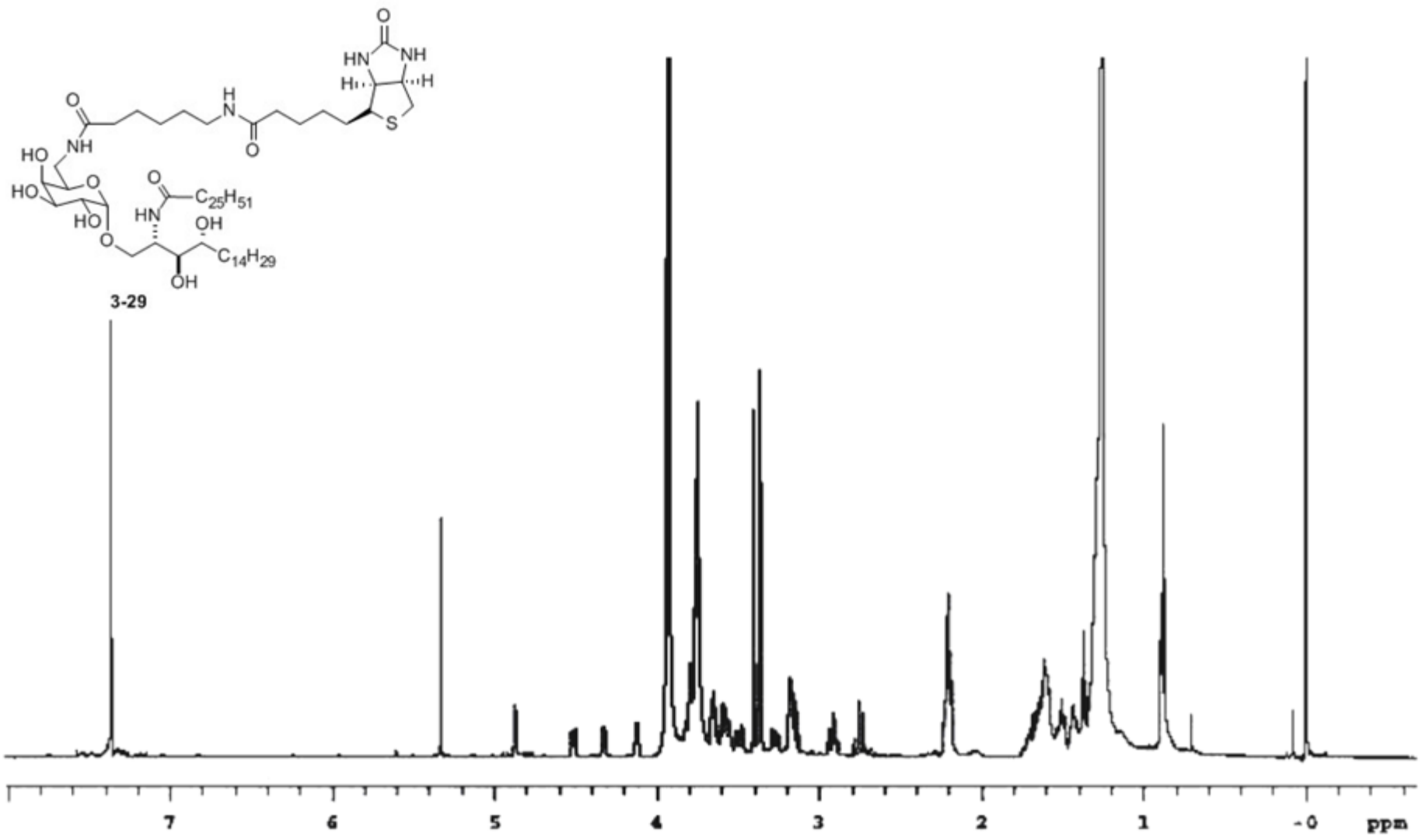

3-17
200
180
160
140
120
100
80
60
40
20
0
ppm

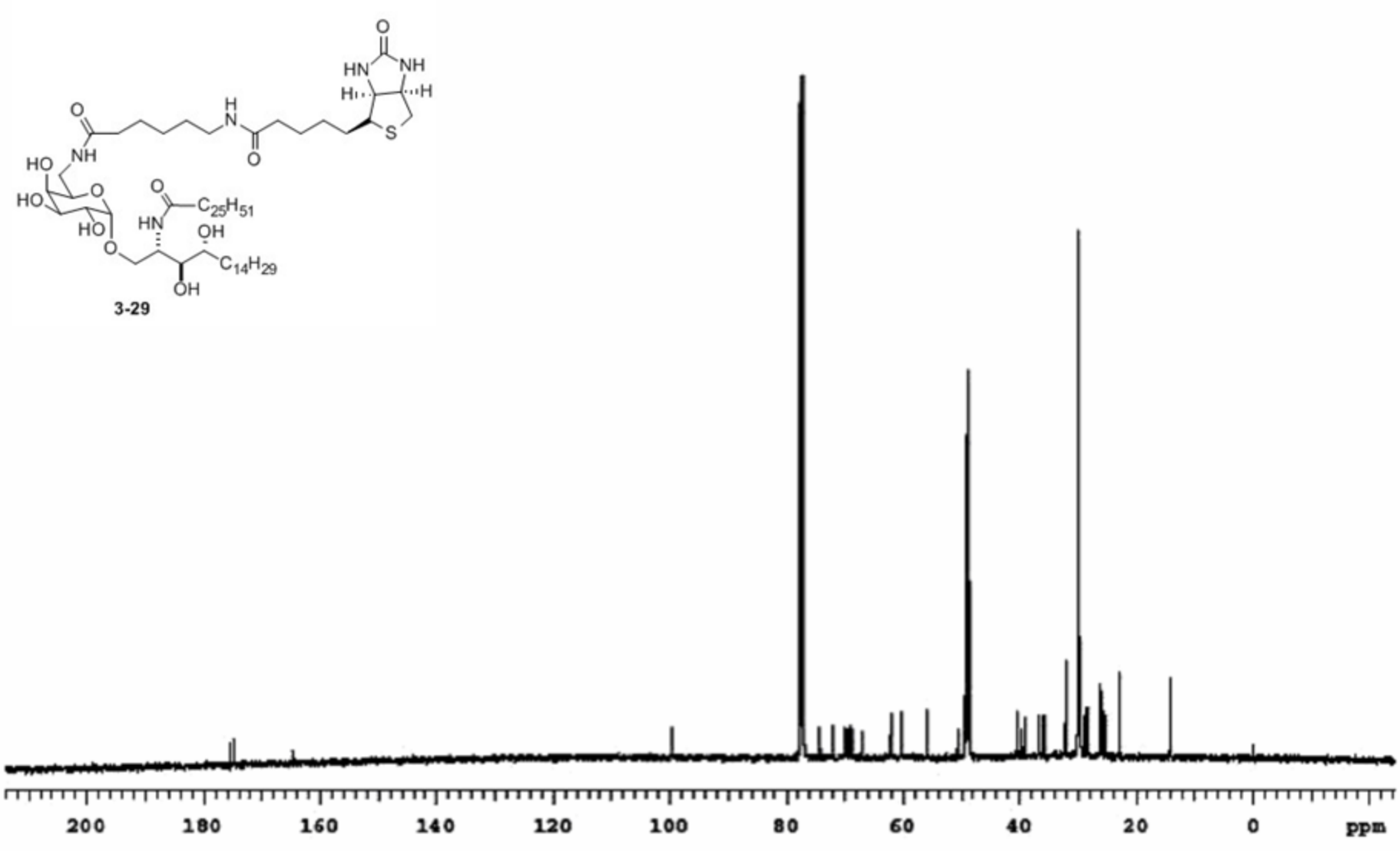

3-18
10
9
8
7
6
5
4
3
2
1
-0
ppm

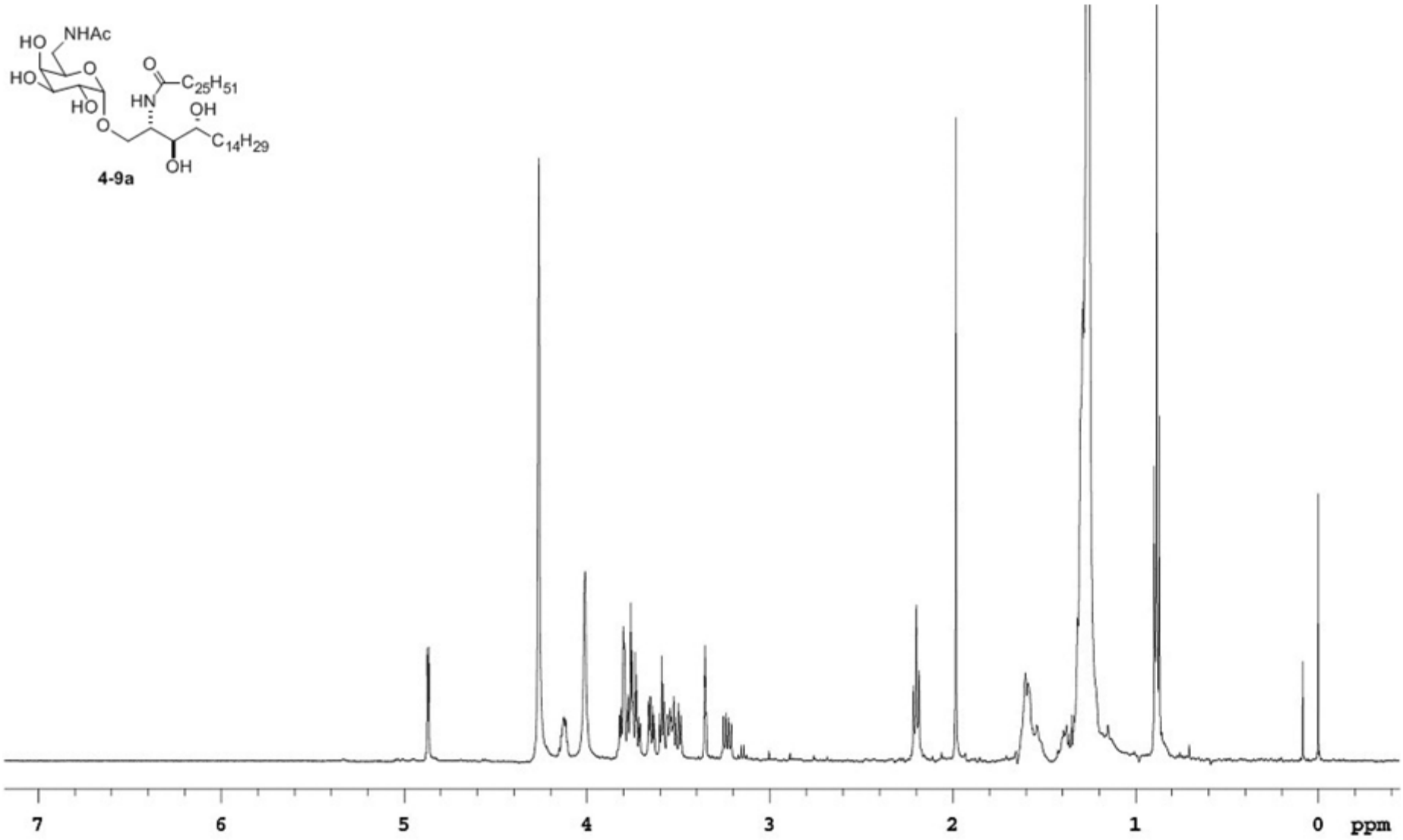

O
N
N
NO2
HO
NH
O
C23H47
HO
O
HN
OH
HO
O
C14H29
OH
3-18
180
160
140
120
100
80
60
40
20
ppm

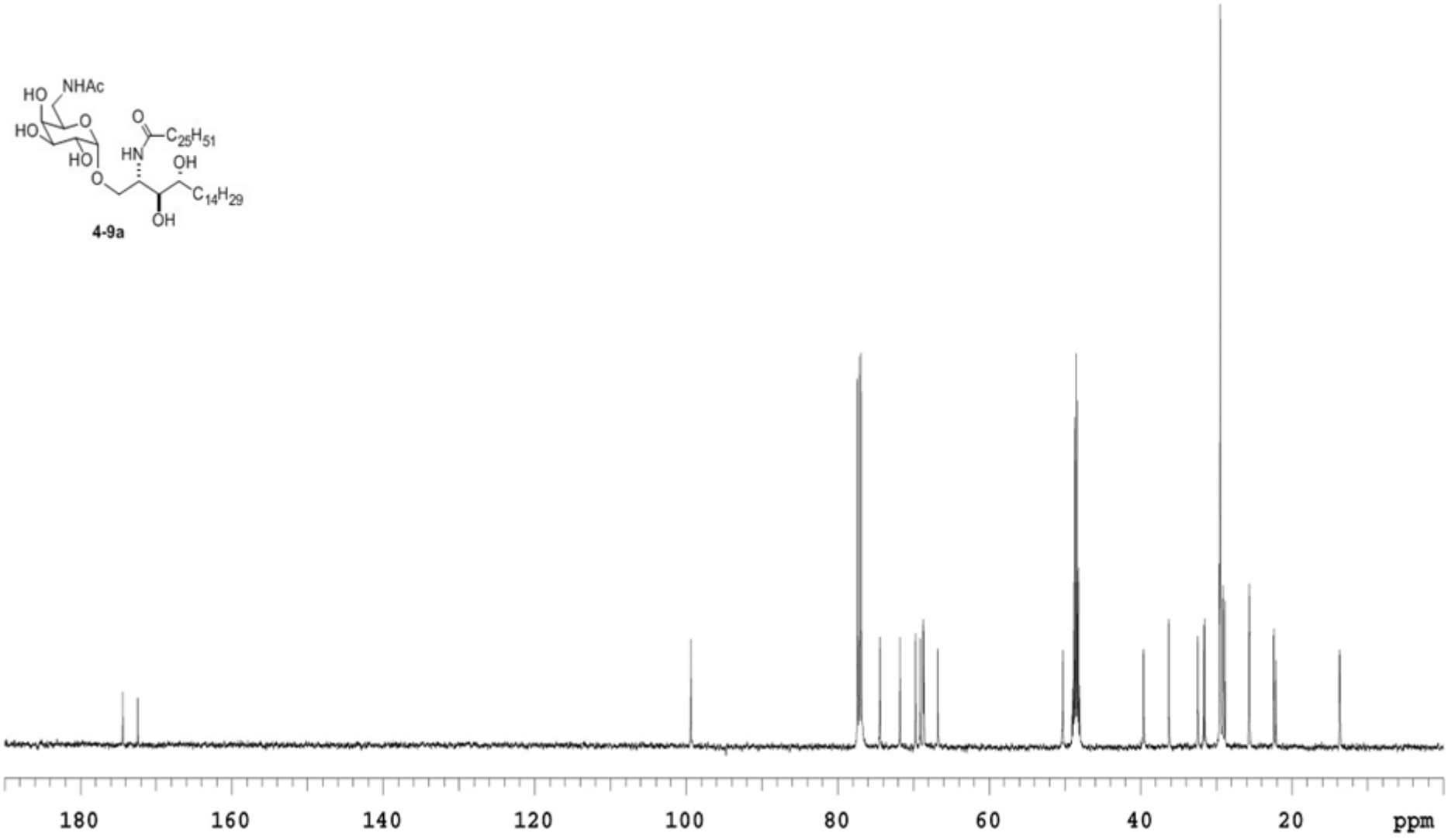

NH
NO2
N
O
N
HO
NH
O
HO
O
HO
O
O
C23H47
HN
OH
C14H29
OH
3-19
10
9
8
7
6
5
4
3
2
1
ppm

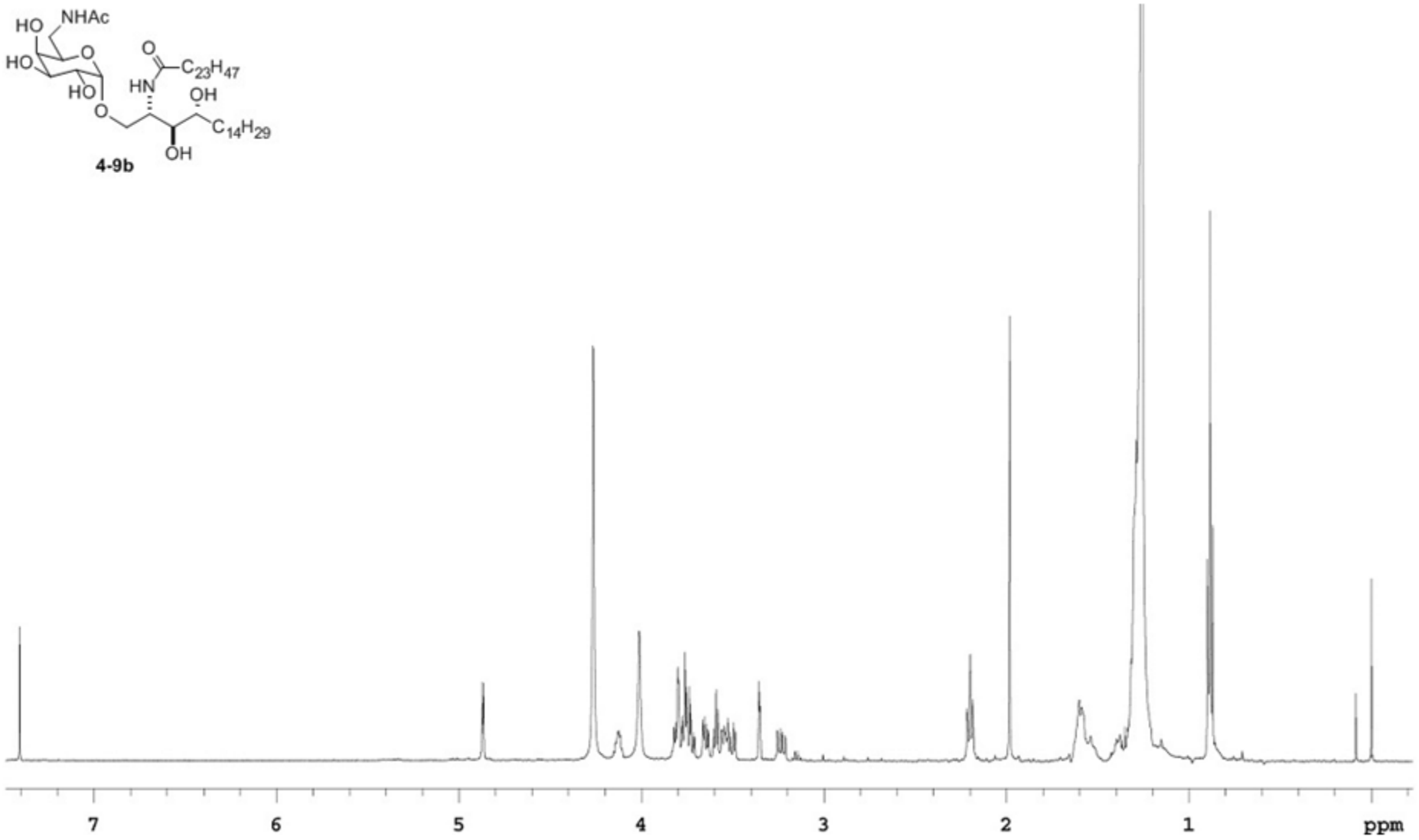

NH
NO2
N
O
N
HO
NH
O
HO
O
HO
HO
O
O
C23H47
HN
OH
C14H29
OH
3-19
180
160
140
120
100
80
60
40
20
ppm

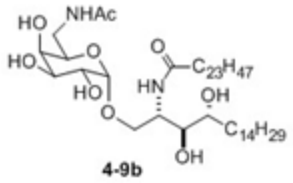

3-29
7
6
5
4
3
2
1
-0
ppm


3-29
200
180
160
140
120
100
80
60
40
20
0
ppm

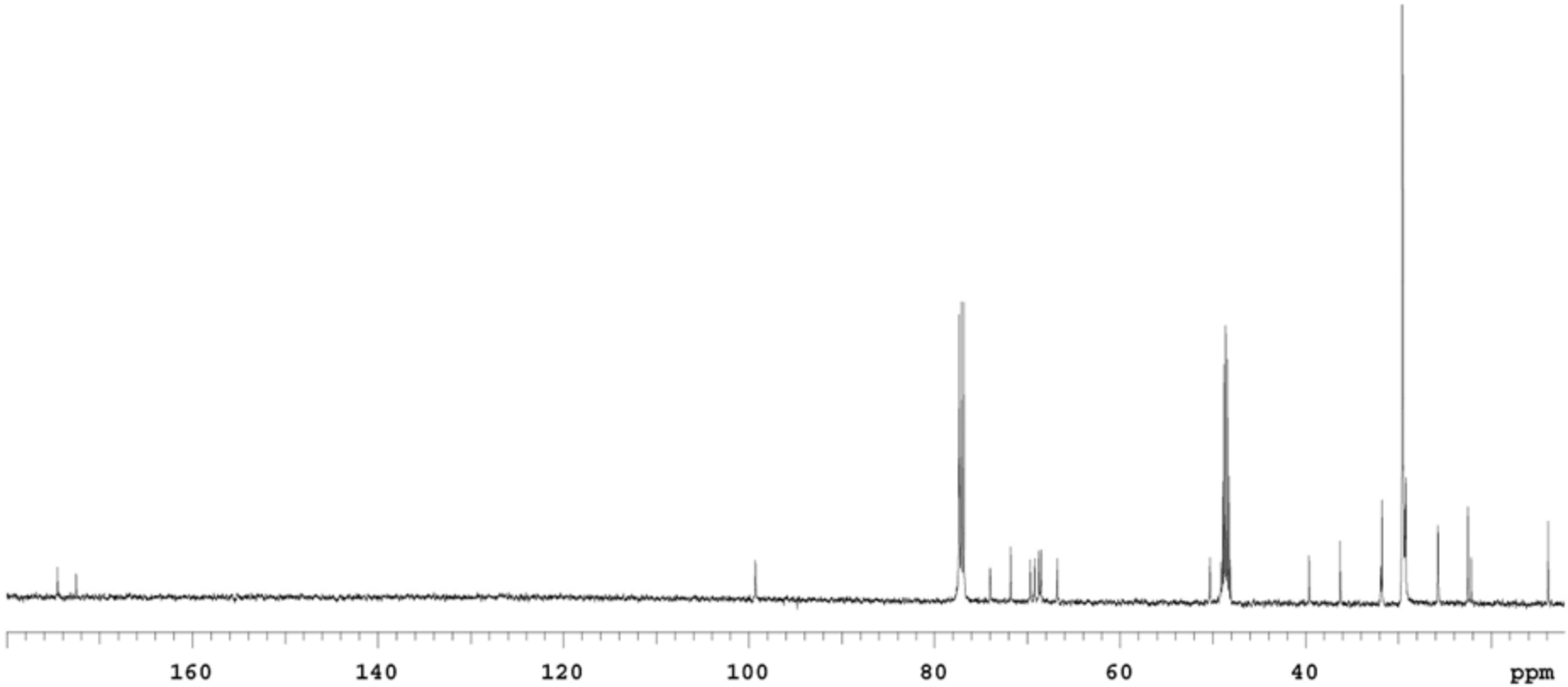

HO
NHAc
HO
O
HO
HO
O
HN
O
$C_{25}H_{51}$
OH
$C_{14}H_{29}$
OH
4-9a
7
6
5
4
3
2
1
0
ppm

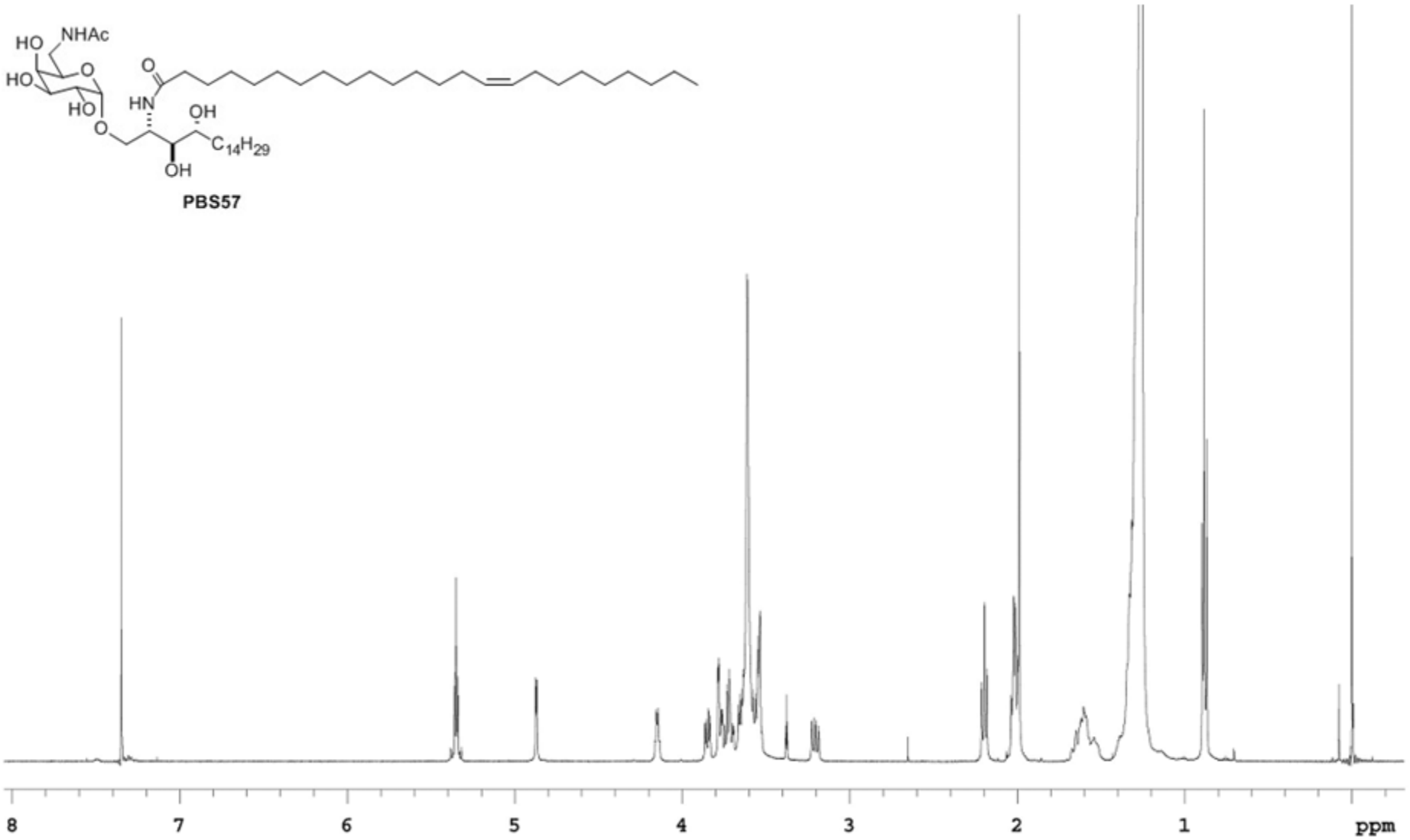

HO
NHAc
HO
O
HO
O
HN
O
$C_{25}H_{51}$
OH
$C_{14}H_{29}$
OH
4-9a
180
160
140
120
100
80
60
40
20
ppm

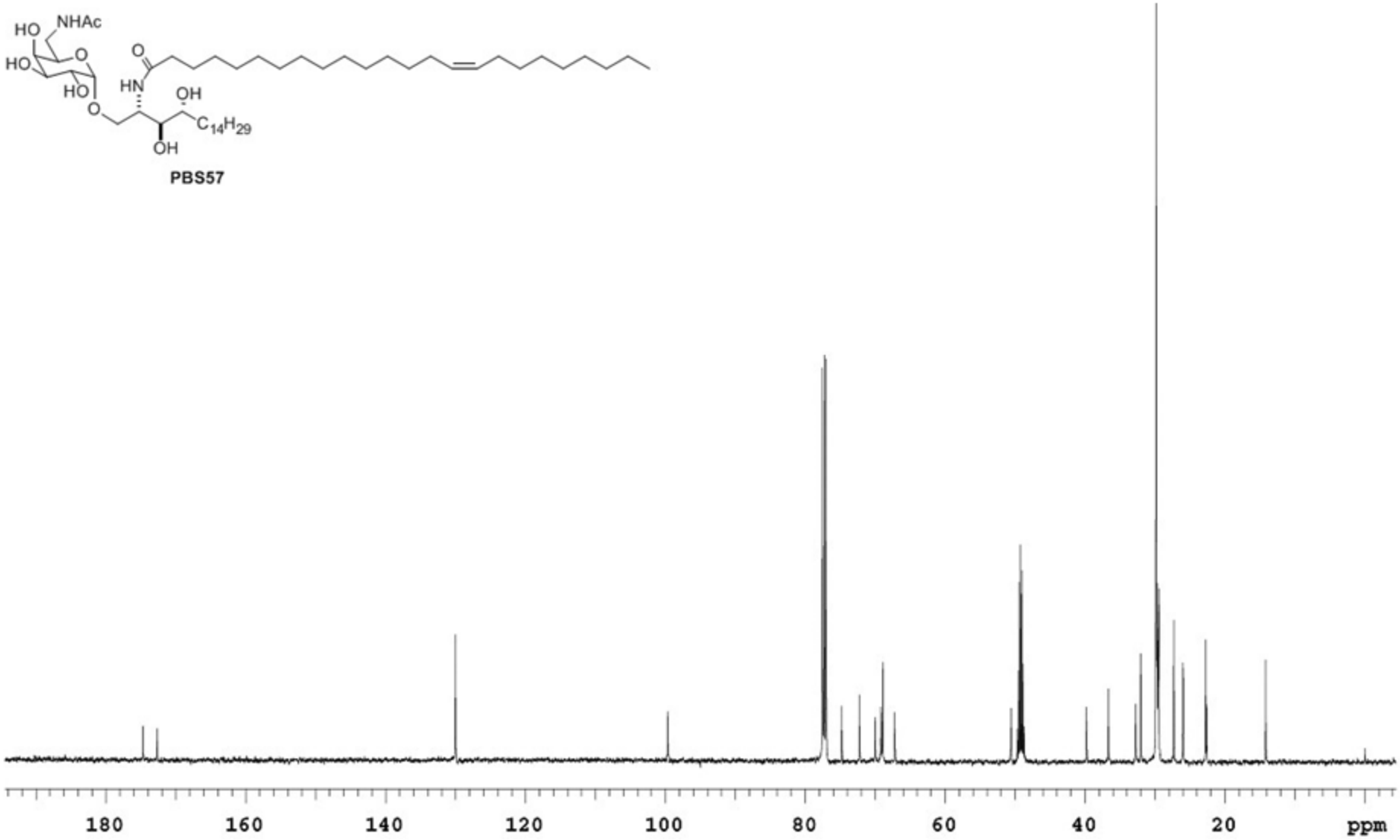

HO
NHAc
HO
HO
O
HN
O
$C_{23}H_{47}$
OH
$C_{14}H_{29}$
OH
O
4-9b
7
6
5
4
3
2
1
ppm


HO
NHAc
HO
HO
O
HN
O
$C_{23}H_{47}$
OH
$C_{14}H_{29}$
OH
O

4-9b


160
140
120
100
80
60
40
ppm


HO
NHAc
O
HO
HO
O
HN
OH
O
$C_{14}H_{29}$
OH
PBS57
8
7
6
5
4
3
2
1
ppm


HO
NHAc
O
HO
HO
O
HN
O
OH
$C_{14}H_{29}$
OH
PBS57
180
160
140
120
100
80
60
40
20
ppm