

THE SYNTHESIS AND BIOLOGICAL EVALUATION
OF GLYCOSPHINGOLIPIDS FOR
IMPROVED CANCER IMMUNOTHERAPY

By

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Abstract

The immune system plays a crucial role in providing the first line of defence against invading pathogens such as bacteria, viruses and parasites. It is activated when immune cells known as dendritic cells (DCs) detect specific molecules that are foreign to the host, and present them to T cells. This in turn causes the activation of T cells, which marks the start of an immune response leading to the clearance of the invader. The pathogen-derived molecules recognised by the immune cells are typically peptides and their role as activators of the immune system is well established. While T cells were originally thought to only recognise peptide antigens, it is now evident that T cells are also able to recognise non-peptide antigens. Recognition of non-peptide antigens confers protection against pathogens that have cell surfaces that are highly functionalised with carbohydrate moieties, such as glycolipids, glycopeptides and polysaccharides.

Specifically, glycosphingolipids (GSLs) can activate invariant Natural Killer T (iNKT) cells via their T cell receptor (TCR) when presented by the CD1d molecule found on the surface of DCs. α -Galactosylceramide (α -GalCer **1**, Figure 1), a synthetic analogue of a GSL extracted from the marine sponge *Agelas mauritanus*, was discovered to be a potent stimulator of iNKT cells when presented by CD1d.

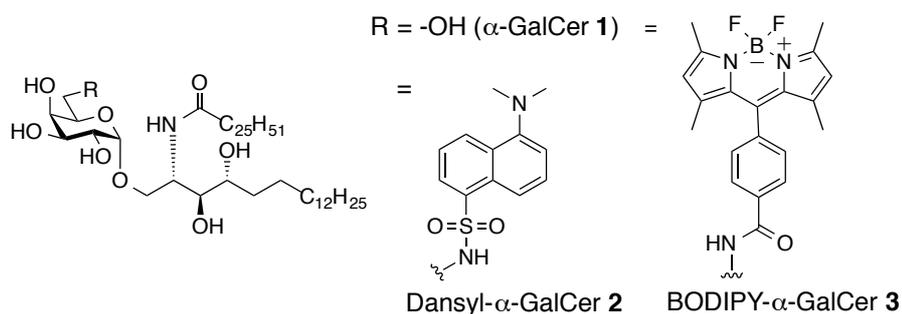


Figure 1. α -GalCer and fluorescent probes.

α -GalCer is currently being used in clinical trials as an adjuvant to boost the activation of immune cells during cancer immunotherapy. Although the molecular interaction of α -GalCer with CD1d and iNKT cells is well established, it is not

fully understood how the glycolipid interacts with different subsets of DCs. Greater understanding of the fate of the glycolipid during cancer immunotherapy will provide crucial information on how the current therapy can be improved.

In this thesis, the design and synthesis of two fluorescent α -GalCer probes, dansyl- α -GalCer (**2**, Figure 1) and BODIPY- α -GalCer (**3**, Figure 1) is reported. Dansyl- α -GalCer was able to activate DCs and iNKT cells in a similar fashion to the parent glycolipid α -GalCer. Its activity was CD1d-dependent and DCs that have taken up α -GalCer *in vitro* can be detected by flow cytometry. Unfortunately, the fluorescence of dansyl- α -GalCer was too weak to be detected by fluorescent microscopy due to photobleaching of the dye. Accordingly, another α -GalCer probe bearing a brighter fluorescent group, BODIPY, was synthesised. The α -GalCer probes were made via two synthetic strategies and the benefits and shortcomings of each synthetic route are discussed.

Isoglobotrihexosylceramide (**4**, iGb3, Figure 2) is another GSL known to activate iNKT cells. Like α -GalCer, it is presented by DCs in the context of a CD1d molecule. iGb3 contains a sphingosine lipid backbone β -linked to a trisaccharide head group, which is in contrast to the α -linked phytosphingosine lipid found on α -GalCer. Despite the structural difference, iGb3 can stimulate iNKT cells, though to a lesser extent than α -GalCer. The intriguing activity of iGb3 provides a platform to further investigate the molecular interactions between CD1d, glycolipid and TCR of iNKT cell.

The crystal structure of iGb3 in complex with mouse CD1d and TCR of mouse iNKT cell show compelling evidence that the terminal galactose moiety is crucial for the observed activity and this is attributed to the hydrogen bond between the 6'''-OH and Thr159 on the CD1d. To unambiguously determine the importance of the hydrogen bond conferred by 6'''-OH, 6'''-deoxy-iGb3-sphingosine (**5**, Figure 2) was synthesised. 6'''-deoxy-iGb3-sphinganine **6** was also synthesised to study the role of the double bond on the sphingosine backbone. A novel synthetic route for the synthesis of iGb3 analogues was established. This allowed for the expedient synthesis of 6'''-deoxy-iGb3 derivatives that will subsequently be

crystallised with CD1d and TCR of iNKT cell, to provide further insight into the structural requirements of β -linked GSLs.

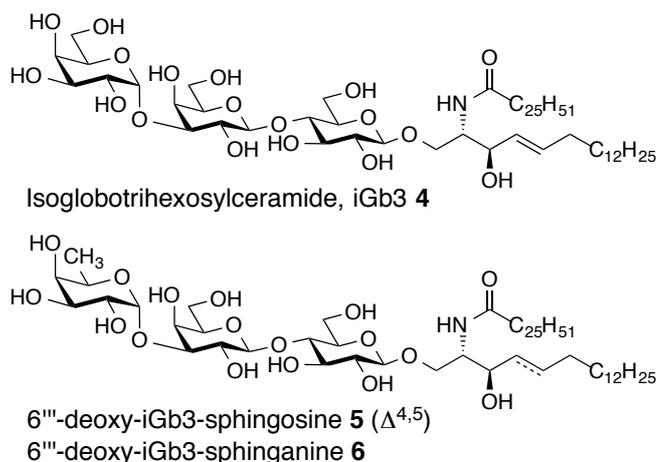


Figure 2. iGb3 and 6'''-deoxy-iGb3 derivatives.

Studies have also revealed that the length and saturation of the *N*-acyl chain of GSLs greatly influences their activity. It is speculated that varying the length of the acyl chain affects the processing and loading of the glycolipid onto CD1d and also TCR binding affinity. To this end, the syntheses of a series of acyl chain analogues of iGb3, including the shorter chain homologue C12:0 **7** (Figure 3) and the unsaturated C20:2 derivative **8** are reported. A divergent synthetic route was employed, whereby a common intermediate from the synthesis of 6'''-deoxy-iGb3 was used. This allowed for efficient syntheses of the acyl chain analogues that will facilitate a greater understanding of the structure-activity relationships.

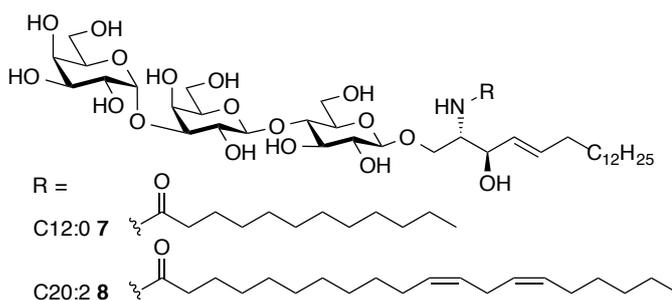


Figure 3. Acyl chain analogues of iGb3.

Taken together, the GSLs synthesised provide crucial insight into how they modulate the immune system and will guide future optimisation of cancer immunotherapy regimes.

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List of Abbreviations

α -GalCer	α -galactosylceramide	GSL	glycosphingolipid
Ac	acetyl	h	hour(s)
AcOH	acetic acid	HBV	hepatitis B virus
APC	antigen presenting cells	hDC	human DC
aq.	Aqueous	HBTU	<i>N,N,N',N'</i> -Tetramethyl-O-(1 <i>H</i> -benzotriazol-1-yl)uronium
β -GalCer	β -galactosylceramide		hexafluorophosphate
β -GlcCer	β -glucosylceramide	HMBC	heteronuclear multiple quantum coherence
β -LacCer	β -lactosylceramide	HMPA	hexamethylphosphoramide
Bn	benzyl	HPLC	High pressure liquid Chromatography
Boc	<i>tert</i> -butyloxycarbonyl	HPV	human papilloma virus
br	broad	hNKT	human iNKT
Bz	benzoyl	HRMS	high-resolution mass spectrometry
Calcd.	calculated	HSQC	heteronuclear single quantum coherence
CD	cluster of differentiation	HT-2	murine T-helper cell-derived line
COSY	correlated NMR spectrum	Hz	hertz
CTL	cytotoxic T lymphocyte	IFN	interferon
CTLA-4	cytotoxic T-lymphocyte Antigen 4	iGb3	isoglobotrihexosylceramide
d	doublet	IL	interleukin
dd	doublet of doublets	IMDM	Iscove's Modified Dulbecco's Medium
DAB	1,4-dideoxy-1,4-imino-D-arabinitol	Imid.	imidazole
DBU	1,8-diazabicyclo[5.4.0]undec-7-ene	iNKT	invariant natural killer T
DC	dendritic cell	<i>iPr</i>	<i>iso</i> -propyl
DCM	dichloromethane	IR	infrared spectroscopy
DiPEA	diisopropylethylamine	<i>i.v.</i>	intra venous
DMAP	4-(dimethylamino)pyridine	<i>J</i>	coupling constant
DMDP	2,5-dihydroxymethyl-3,4-dihydropyrrolidine	KO	knock out
DMF	dimethylformamide	LSD	lysosomal storage disorder
DMSO	dimethyl sulfoxide	m	multiplet
DNA	deoxyribonucleic acid	Me	methyl
DPA	arabinofuranosyl-1-mono phosphoryl-decaprenol	MHC	major histocompatibility complex
EDCI	1-ethyl-3-(3-dimethylaminopropyl) carbodiimide	MHz	mega hertz
eq.	equivalents	MIC	minimum inhibitory concentration
ER	endoplasmic reticulum	min	minutes
ESI	electron spray ionization	mNKT	mouse iNKT
Et	ethyl		
FITC	fluorescein isothiocyanate		
Gb3	globotrihexosylceramide		

Mp	melting point	rt	room temperature
Ms	mesyl	s	singlet
<i>m/z</i>	mass to charge ratio	sat.	saturated
NBD	7-Nitrobenz-2-oxa-1,3-diazol	SE	Standard Error
NBS	<i>N</i> -bromosuccinimide	<i>t</i>	triplet
NIS	<i>N</i> -iodosuccinimide	TAA	Tumour associated antigen
NK	natural killer	TB	tuberculosis
NMR	nuclear magnetic resonance	<i>t</i> -Bu	<i>tert</i> -butyl
Obsd.	observed	TCR	T cell receptor
PBMC	peripheral blood mononuclear cells	TES	triethylsilane
PyBOP	(Benzotriazol-1-yloxy) tripyrrolidinophosphonium hexafluorophosphate	TFA	trifluoroacetic acid
PBS	phosphate-buffered saline	THF	tetrahydrofuran
PD-1	programmed death 1	TLC	thin layer chromatography
PE	phycoerythrin	TLR	toll-like receptor
Ph	phenyl	TMSOTf	Trimethylsilyl methanesulfonate
ppm	parts per million	Tf	trifluoromethanesulfonyl
<i>p</i> TsOH	<i>para</i> -toluenesulfonic acid	TNF	tumour necrosis factor
RBC	red blood cell	Tr	triphenylmethyl
RPMI	Roswell Park Memorial Institute		

Chapter 1. Introduction

The term cancer refers to a family of diseases characterised by the uncontrolled growth and spread of abnormal cells. Cancer can be caused by inherited genetic factors or environmental (external) factors and it remains a leading cause of death worldwide, claiming 7.6 million lives in 2008.¹ In New Zealand, approximately 21,000 people were diagnosed with cancer in 2010.² Treatment of cancer by surgical removal of the tumour can be traced back to ancient Egyptian times (1600 BC), as documented in the Edwin Smith Papyrus,^{3,4} while radiation therapy came into practice after the discovery of X-rays for medical diagnostics in 1896.⁵⁻⁷ In the 20th century, the need to treat World War I soldiers suffering from hematologic malignancies caused by nitrogen mustard gases prompted the development of chemical agents to treat cancer – a treatment referred to as chemotherapy.⁸ To this day, surgery, radiotherapy and chemotherapy continue to play a major role in the treatment of cancer, however, such practices often fail to induce complete tumour remission and are associated with unpleasant side effects.

1.1 Tumour immunology

The immune system plays a key role in homeostasis in the body. It responds in a regulated fashion to pathogens, but is trained not to react against self-tissues, a phenomenon known as self-tolerance.⁹ In the 1950s, the scientific community generally accepted the theory that lymphocytes that are responsive to self-tissue were deleted in prenatal life during the development of the immune system.¹⁰ While self-tolerance is beneficial in the prevention of autoimmune diseases such as cystic fibrosis and rheumatoid arthritis, tumours often use this mechanism to evade immune recognition, which results in a weak natural protection against malignant tumours. In the 1960s, however, it was recognised that tumours, though being self-derived, contain tumour-associated antigens (TAAs), which the body's lymphocytes can detect – a concept called immunosurveillance.¹¹

The importance of immunosurveillance in maintaining the health of an individual can be illustrated in many ways. For example, renal transplant patients receiving immunosuppressive treatment to prevent transplant rejection face a greater risk of developing skin cancer when exposed to UV rays from the sun.¹² Also, the ability of the immune system to recognise specific TAAs is demonstrated by the ability of mice, which upon treatment of the first tumour challenge, were able to reject a second tumour challenge with the same tumour.¹³⁻¹⁶ Moreover, malignant tumours that are highly genetically unstable often generate a vast number of TAAs, which have not been seen by the host.^{17,18} This provides a range of candidate antigens for immune recognition. Nonetheless, due to the dynamic ability of cancerous cells to mutate and evade immunological checkpoints, the body's natural immune response is rarely robust enough to fight against aggressive tumours.¹⁹

1.2 Antigen presentation of tumour-associated peptides

Antigen presenting cells (APCs) patrol the body in the blood circulation and play a crucial role in sending signals to other immune cells upon encountering an antigen, thereby activating the immune system. There are two classes of APCs; professional and non-professional APCs, which are both capable of presenting peptide antigens. Professional APCs are very efficient at internalising antigens and displaying them on their cell surface. Dendritic cells (DCs) are an example of professional APCs and these specialised cells are distinct from non-professional APCs because they are capable of activating the immune cells that they present the antigen to.²⁰

When an immature DC encounters an antigen, it matures and migrates to a nearby lymph node (Figure 1.1). At the lymph node, the DC cross presents the peptide antigen on a cell surface receptor, known as Major Histocompatibility Complex I (MHC I), to naïve T cells.^{21,22} These naïve T cells express T cell receptors (TCRs) on their cell surface and can recognise the peptide presented by MHC I. The interaction between TCR with MHC I and peptide antigen is highly specific and marks the initiation of an immune response, whereby naïve T cells become activated to produce the cytokine IL-2, causing them to proliferate and

differentiate into mature, effector T cells. In addition to the formation of the TCR-MHC I-peptide trimer, the interaction of co-stimulatory molecules on DCs and naïve T cells are also necessary for the maturation of T cells.^{23,24} The effector T cells then migrate from the lymph node to the site of infection and respond by eliminating the infection or by releasing other soluble mediators to recruit other immune cells. Different classes of T cells play distinct roles in the immune system and are often characterised by the expression of cell surface markers. CD8+ T cells, also known as cytotoxic T lymphocytes (CTLs), express the cluster of differentiation (CD) 8 protein on their surface and represent an important subset of T cells because they are capable of directly killing pathogens or tumour cells. The induction of CTLs is thus desired in cancer immunotherapy as CTLs are highly effective at killing tumour cells by releasing cytotoxic proteins such as perforin and granzymes.²⁵

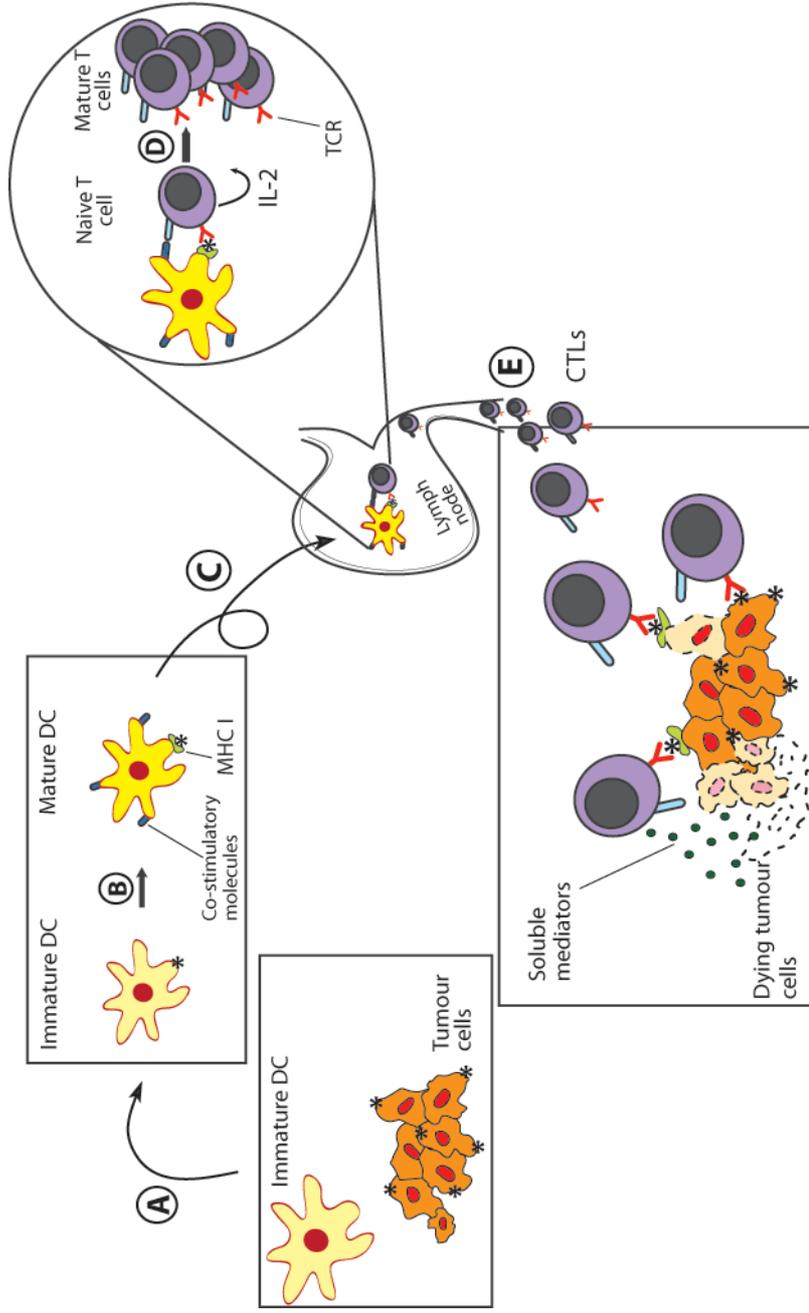


Figure 1.1 **A.** An immature DC encounters and internalises tumour cells. **B.** As the DC matures, it processes the tumour proteins into smaller fragments and cross-presents these peptide fragments on MHC I. The mature DC also expresses co-stimulatory molecules (e.g. CD86, CD40). **C.** The mature DC migrates to the lymph node and presents the antigen to a naïve T cell. **D.** The naïve T cell releases the cytokine IL-2, which drives the naïve T cells to proliferate and differentiate into mature cytotoxic T cells (CTL). **E.** CTLs migrate from the lymph node to the tumour site and release soluble mediators that directly kill the tumour cells, or sends recruitment signals to other immune cells.

1.3 Cancer immunotherapy

Cancer immunotherapy is a promising new type of anti-cancer treatment that employs a patient's own immune system to fight cancers. The strategy for immunotherapy is to improve the immune response to tumours with the ultimate goal being the induction of a tumour-specific immune response. Although William B. Coley pioneered cancer immunotherapy more than 100 years ago by giving cancer patients bacterial infections to invoke their immune system,²⁶ this method of treatment was subsequently abandoned because his results were not reproducible and other treatments for cancer became more mainstream. Nonetheless, in the mid-1980s a resurrection of cancer immunotherapy began. Since then, a greater understanding of the immune system aided by advances in technology has led to a boost in confidence, and the potential of cancer immunotherapy has become more widely accepted in the scientific and clinical community. The ability of the immune system to react in a very specific manner, hence with minimal side effects, makes it a promising treatment for malignant diseases. Moreover, the immune system is able to generate long-lasting tumour immunity when activated appropriately.

Cancer vaccines can be broadly classified into two types:

1. **Prophylactic (preventive) vaccine:** which prevents the onset of cancer in healthy people.
2. **Therapeutic vaccine:** which treats existing cancers by enhancing immune responses in patients.

In light of the successful immunisation against diseases such as polio and smallpox, many researchers are attempting to replicate similar immunisation strategies for malignant diseases. This led to the birth of prophylactic cancer vaccines effective against the hepatitis B virus (HBV) and the human papilloma virus (HPV), which are the causative agents for liver and cervical cancers, respectively.²⁷⁻³⁰ Though it would be ideal to develop prophylactic vaccines against all cancers, at present, this is unfeasible because of the vast number of rapidly mutating cancers.

In addition to preventive vaccinations, various immunotherapy regimes have been developed to prime and boost a patient's immune system so that it can attack existing malignant tumours. Some therapeutic vaccination strategies currently under investigation include DNA vaccination, monoclonal antibody treatment, adoptive cellular therapy, peptide-based immunotherapy and of particular interest to us, dendritic cell (DC)-based vaccination.³¹⁻⁴³ These are summarised in Table 1.1.

Table 1.1. Therapeutic vaccinations currently under investigation.

Vaccination	Description
DNA vaccine	Naked DNA that encodes for a tumour antigen (protein) is injected intramuscularly or intradermally into a cancer patient. Cells in the body temporarily absorb the DNA vaccine and produce the protein of interest. This leads to the priming of the immune response against the target protein. ³¹⁻³³
Monoclonal antibody treatment	A passive immunotherapy regime that involves the administration of an antibody that specifically targets molecules that are either tumour cell surface markers (<i>e.g.</i> CTLA-4 or PD-1) or are directly responsible for tumour growth (<i>e.g.</i> endothelial growth factor receptor). ³⁴⁻³⁶
Adoptive cellular therapy	T lymphocytes or tumour-infiltrating lymphocytes are harvested from a patient, expanded <i>in vitro</i> with tumour antigens and cytokines and readministered to the patient. These effector T cells attack tumour cells in the patient. ^{37,38}
Peptide-based immunotherapy	Tumour-specific peptide fragments of defined sequence are administered to the patient. The immune system mounts a response against the peptide vaccine. ³⁹⁻⁴¹
Dendritic cell-based vaccination	Dendritic cells (DCs) are harvested from the patient, pulsed (<i>ie.</i> incubated) with tumour antigens and sometimes adjuvants and reinjected into the patient. This effectively teaches the DCs to recognise the tumour (Section 1.4). ^{42,43}

In April 2010, the US Food and Drug Administration (FDA) approved the first anti-cancer therapy vaccine, which is a DC-based cancer immunotherapy (Provenge) against prostate cancer.⁴⁴ Shortly after this, a monoclonal antibody was developed against a receptor found on the surface of T cells, known as the cytotoxic T-lymphocyte antigen 4 (CTLA-4) that is responsible for immunosuppression.^{35,45} Treatment of metastatic melanoma with the anti-CTLA-4 is highly promising and was approved by the US FDA in 2011, under the name ipilimumab. In addition, there are other immunotherapies in the pipeline, such as anti-programmed death (PD)-1, which block the molecules expressed on dying T cells known as PD-1 and it is currently being tested in clinical trials.^{36,46-48} Taken as a whole, the recent breakthroughs in cancer immunotherapy will pave the way for the release of many other immunotherapies into the market in the future.

1.4 DC-based cancer immunotherapy

In a typical DC-based immunotherapy programme, tumour cells are extracted from a patient and apoptosis (programmed cell death) is induced (Figure 1.2A). As previously discussed, tumour cells aberrantly express proteins which can serve as antigens for a T cell mediated anti-tumour immune response.²⁰ At the same time, white blood cells containing precursors (peripheral blood mononuclear cells) to DCs are obtained from the same patient via a process called leukapheresis. The DC precursors are incubated *in vitro* with tumour cells that have undergone apoptosis and a cocktail of activation factors, which leads to DC maturation. The primed DCs are then reinjected into the patient to generate a systemic anti-tumour response.

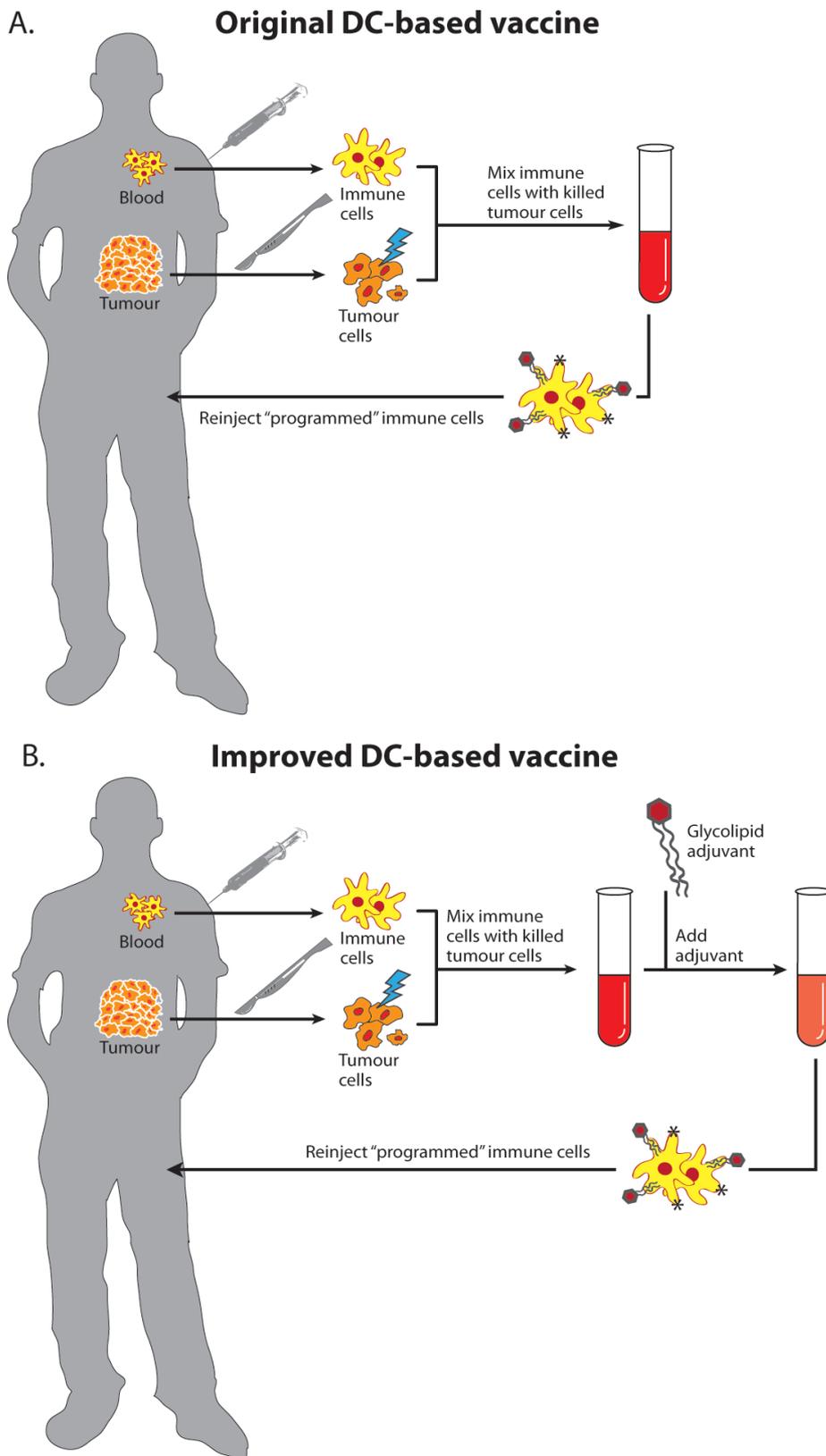


Figure 1.2. **A.** Original DC-based immunotherapy regime. **B.** Improved regime incorporating a glycolipid adjuvant.

One may ask why the favourable anti-tumour immune response is not generated spontaneously *in vivo* considering that both DCs and tumour antigens are present in the body. It has been shown that most DC precursors found in or near malignant tissues are poor antigen presenters because they fail to be activated, possibly due to the immunosuppressive environment of the tumour.^{49,50} However, if the DC precursors are activated under *in vitro* culture conditions, they express vital co-stimulatory molecules (*e.g.* CD40, CD80 and CD86) and acquire the capacity to present antigens and activate naïve T cells.

Though tumour regression has been observed in a number of patients treated by this DC-based immunotherapy regime, the immune response generated can be further improved.⁵¹ It has been found that the addition of certain glycolipids, acting as adjuvants, boosts the immune response to elicit enhanced anti-tumour activity.⁵¹ Accordingly, an improved DC-based vaccination regime has been developed which involves the isolation of tumour cells and immune cells from the patient, apoptosis of the tumour cells by radiation, combination of the cells with a glycolipid adjuvant and reinjection of this mixture into the patient (Figure 1.2B). This leads to the stimulation of an enhanced tumour-specific response.

The original DC vaccination strategy, whereby DCs pulsed with peptide *in vitro* are reinjected into the patient, has been proven to generate a positive outcome in melanoma patients.⁵² The use of glycolipids in cancer immunotherapy has reached clinical trials but the glycolipid adjuvant has only ever been administered on its own,⁵³ or administered after it was pre-loaded onto DCs.⁵⁴⁻⁵⁸ The improved protocol which incorporates the priming of DCs with both the peptide as well as the glycolipid adjuvant has yet to be tested in a clinical setting.

1.5 DC-based vaccination at the molecular level

1.5.1 Overview

An explanation of the cancer immunotherapy protocol at the molecular level is given in Figure 1.3. Here the peptide antigen from the apoptosed tumour cell is presented on the MHC I molecule of the DC (as discussed in Section 1.2) and the presented antigen is then recognised by cytotoxic T lymphocytes (CTLs), which become activated to release soluble mediators that directly target the tumour, or trigger a cascade of immune activation leading to an anti-tumour response.⁵⁹ This anti-tumour immune response can be modulated by the addition of a glycolipid adjuvant (for a comprehensive review see Wu et. al.).⁶⁰ The glycolipid adjuvant is also presented on the surface of the DC, this time via another protein called CD1d. The CD1d-glycolipid complex in turn is detected by invariant natural killer T (iNKT) cells, a subset of T cells that play a crucial role in regulating immune responses.⁶⁰⁻⁶⁵ Upon stimulation, iNKT cells release cytokines that can promote either a favourable or unfavourable anti-tumour immune response, generally termed as T helper (Th) 1 or Th2 response, respectively.⁶⁶ The ‘Th1’ response is characterised by the release of proinflammatory cytokines, such as interferon (IFN)- γ and interleukin (IL)-12, which promote CTL and natural killer (NK) cell activation and therefore enhance the anti-tumour response. The ‘Th2’ response is immunomodulatory and produces cytokines such as IL-4, which are vital to prevent autoimmune diseases such as type-I diabetes and multiple sclerosis. However, activation of the Th2 response also leads to suppression of T cell mediated anti-tumour immunity. Hence, in developing adjuvants for anti-cancer immunotherapy, a glycolipid that is capable of selectively inducing a ‘Th1’ response is desired.

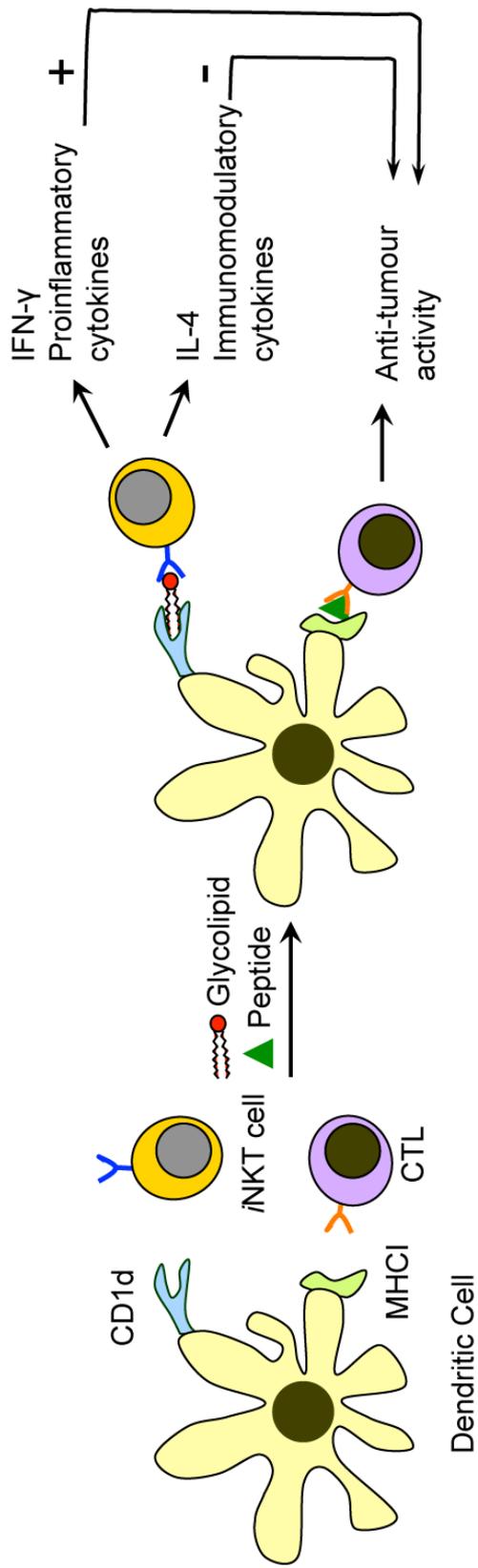


Figure 1.3. A tumour peptide is presented by the dendritic cell (DC) via the MHC class I molecule and this activates cytotoxic T lymphocytes (CTL) towards anti-tumour activity. At the same time, presentation of the glycolipid by CD1d activates invariant natural killer T (iNKT) cells, which elicit a Th1 proinflammatory or a Th2 immunomodulatory response. The former acts to enhance the anti-tumour immune response towards the peptide antigen while the latter suppresses it.

1.5.2 α -Galactosyl ceramide – A potent glycosphingolipid adjuvant

In 1993, the pharmaceutical division of Kirin Breweries isolated a series of novel α -galactosyl ceramides from the marine sponge *Agelas mauritianus*.^{67,68} Of these glycosphingolipids, agelasphin-9b (AGL-9b, **9**, Figure 1.4), exhibited potent anti-tumour activity in *in vivo* models of several murine tumour cells and later served as the parent compound for subsequent analogue syntheses and structure-activity studies.⁶⁹ The term “glycosphingolipid” refers to a class of glycolipids that contain the sphingoid base. AGL-9b is a glycosphingolipid that consists of a galactosyl moiety α -linked to a ceramide portion that is made up of an *N*-acylated phytosphingosine backbone. During the course of this structure-activity work, KRN7000 (**1**) was found to have similar anti-tumour activity to **9** and, due to its easier synthesis, was deemed the more suitable candidate for clinical use. The abolishment of both the terminal methyl group on the phytosphingosine backbone and the hydroxyl group on the α -position of the acyl chain did not affect the anti-tumour activity of the glycolipid. Since then, KRN7000, now widely known as α -galactosyl ceramide (α -GalCer), has been reported to have potential in the treatment of several diseases including cancer, malaria, type-I diabetes, and multiple sclerosis. It has been found to exert its therapeutic activity via its ability to bind to CD1d and activate iNKT cells.^{70,71} This discovery was remarkable and provided the first evidence that glycolipids, like their protein counterparts, can be presented by APCs and recognised by T cells to invoke an immune response. The potential for α -GalCer to treat various diseases lies in its potent ability to stimulate iNKT cells, which subsequently activates the Th1 and the Th2 response. Given the therapeutic potential of α -GalCer, much effort has been spent in identifying other ligands that bind to CD1d and activate iNKT cells.^{60,69,72-80}

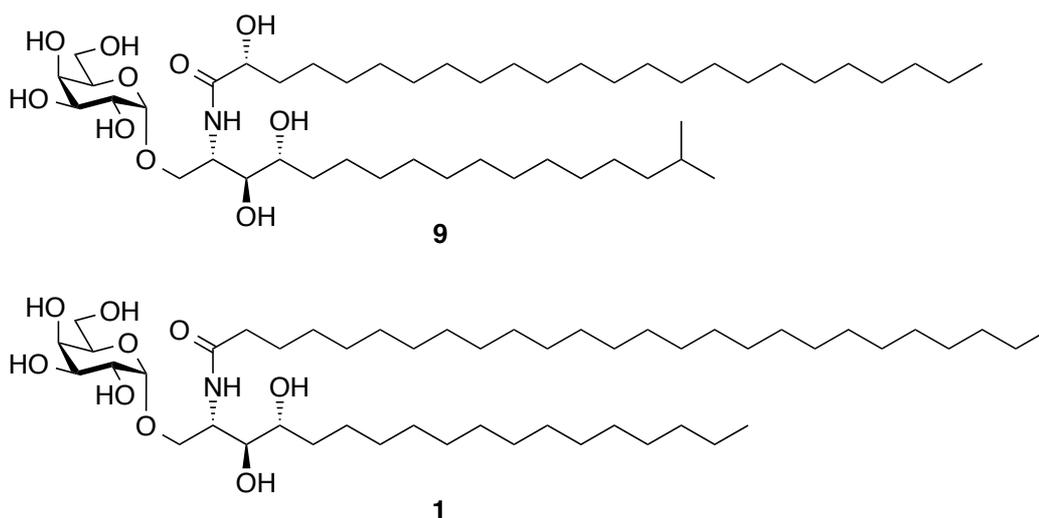


Figure 1.4. Agelasphin 9b **9** and α -GalCer **1**.

1.5.3 CD1d

The CD1d protein is a member of a family of transmembrane glycoproteins consisting of a heavy chain with three extracellular domains (α 1, α 2 and α 3) non-covalently linked to a β ₂-microglobulin (β ₂m) chain.⁸¹ The non-polar amino acids in the α 1 and α 2 chains form two hydrophobic pockets, A' and F' that can accommodate the long lipid tails of a glycolipid, leaving the sugar head group exposed as depicted in Figure 1.5A. The protruding carbohydrate portion is then recognised by iNKT cells, via their TCR. The trimolecular interaction between the CD1d, α -GalCer and TCR initiates a cascade of intracellular and extracellular signalling that results in the activation of iNKT cells. The precise molecular interaction of CD1d with glycolipid was elucidated when Rossjohn and co-workers crystallised a human iNKT cell TCR (blue and yellow) in complex with human CD1d (green) loaded with α -GalCer (pink) (Figure 1.5B).⁸² In addition, crystal structures of mouse and human CD1d reveal that this protein is conserved across these two species (Figure 1.5C), whereby the human CD1d is shown in grey and the mouse CD1d in green.⁸³

Given this similarity, it is generally accepted that the murine model is suitable when studying the potency of various analogues of α -GalCer, however, considering the subtle, but non-negligible variation in DC subsets between mice and humans,⁸⁴ care should be taken, and these glycosphingolipids should always be cross-tested against both species. The varying glycosphingolipid activities of triglycosyl ceramides such as isoglobotrihexosylceramide (iGb3) are attributed to the difference in CD1d structure between mice and humans.⁸⁵ In contrast, differences in activities observed for glycosphingolipids that possess one sugar head group (*i.e.* α -GalCer analogues) have been traced back to the variation in the TCR structure rather than the CD1d molecule.⁸⁶⁻⁸⁸

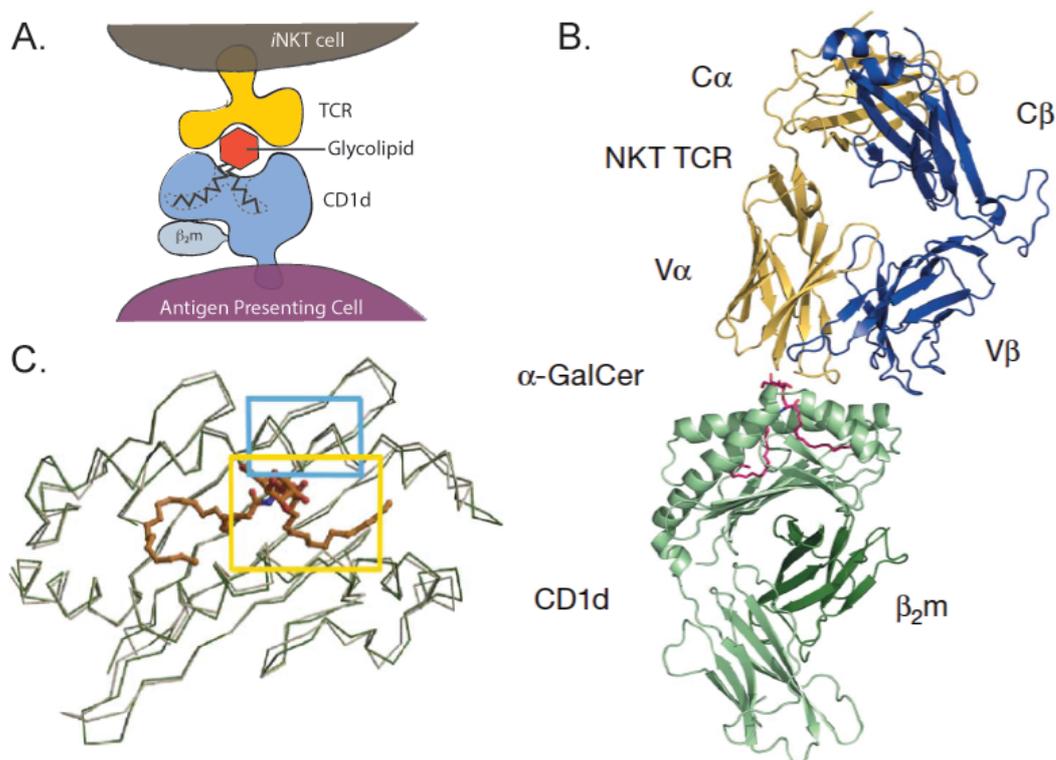


Figure 1.5. **A.** Representation of CD1d–glycolipid–TCR interaction during glycolipid presentation. **B.** Crystal structure of human NKT TCR (blue and yellow) interacting with CD1d (green)-glycolipid (pink) complex.⁸² (Adapted by permission from Macmillan Publishers Ltd: Nature Immunology, 2007, 448, 44-49, ©2007) **C.** Structural comparison of human (grey) and mouse (green) CD1d with α -GalCer bound (orange).⁸³ (Adapted by permission from Macmillan Publishers Ltd: Nature Immunology, 2005, 6, 819-826, ©2005)

1.5.4 iNKT cells

iNKT cells represent less than 1% of the total T cell population in humans and are a heterogeneous population of cells that express an invariant TCR and the natural killer (NK) 1.1 marker on the cell surface.^{61,63} They are commonly found in the thymus, spleen, liver and bone marrow.⁸⁹ Unlike CD4⁺ and CD8⁺ T cells, which recognise peptide antigens, iNKT cells are restricted by the CD1d molecule and can only recognise glycolipids presented by CD1d. iNKT cells play an important role in bridging innate and adaptive immunity,⁸⁹ for they not only have a key role in the activation of the immediate innate immune response, but also affect the subsequent, longer-lasting, adaptive immunity involving B and T cells.⁹⁰⁻⁹² It is this unique characteristic that makes iNKT cells such a potent cell type with great potential in cancer immunotherapy. Of note, structure activity relationship studies and crystal structure analyses, have revealed that subtle differences exist between the TCR structures of humans and mice and can sometimes lead to species-selective activity of glycolipids.⁸⁶⁻⁸⁸

1.6 Fluorescent α -GalCer probes to study cellular uptake and trafficking

A comprehensive understanding of the effect of the glycosphingolipids on the immune system is crucial for the development of an effective DC-based cancer immunotherapy utilising α -GalCer or analogues thereof. The study of these glycosphingolipids in terms of their ability to be presented by CD1d molecules on DCs and to iNKT cells to produce specific cytokine profiles is important to provide information on how the structures of the glycosphingolipids can be altered to improve immune responses. In addition, it is important to understand the pharmacokinetics of glycosphingolipid adjuvants once they are administered as part of a cancer immunotherapy regime.

Within the human body, there are different subsets of DCs, residing in different compartments *in vivo* that play specific and sometimes overlapping roles in the immune system.^{93,94} Studies have shown that targeting of α -GalCer adjuvants to these specific subsets of DCs (such as the CD8 α + DCs) can lead to enhanced anti-tumour responses.^{95,96} One of the methods to identify and quantify subsets of DCs that have taken up glycolipids and are responsible for the observed immune activation is by using a fluorescently labelled antibody that binds to the CD1d-glycolipid complex.⁹⁷⁻¹⁰¹ Unfortunately, these monoclonal antibodies are only useful in systems where α -GalCer is bound to CD1d and are less applicable when trying to study glycolipid trafficking. Therefore, an alternative approach is to tag the glycolipid itself with a fluorophore to directly visualise the glycolipid and its interaction with different immune cells. As such, a number of α -GalCer-derivatives containing a fluorophore¹⁰²⁻¹⁰⁴ or a biotinylated probe^{103,105,106} have been prepared. Though the ability of α -GalCer to bind to CD1d and activate iNKT cells has been robustly studied, scant information is available on inter- and intracellular lipid trafficking and how this influences CD1d presentation and iNKT cell activation.¹⁰⁷ Many groups have thus focussed on derivatising the lipid portion of α -GalCer with a reporter probe in order to address such issues, as

dansylated α -GalCer derivative **2** (Figure 1.7).¹⁰³ As such, biotin-labelled α -GalCer **13**¹⁰³ and BODIPY-labelled α -GalCer **14**¹⁰⁴ were also synthesised, with extensive confocal microscopy studies being performed on the latter to understand the intracellular trafficking on this glycolipid between endosomal compartments within a cell. These findings illustrate the versatility of using α -GalCer derivatives with fluorophores attached on the 6' position of the carbohydrate moiety. Therefore, attachment of small fluorescent groups at the 6'-position of α -GalCer will provide useful probes to track and visualise these glycolipids *in vitro* and *in vivo*.

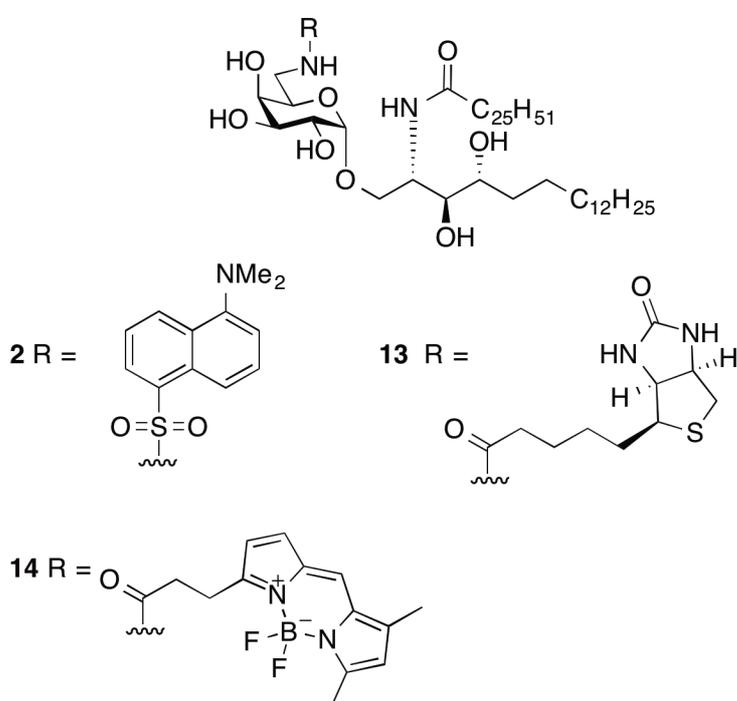


Figure 1.7. 6'-labelled α -GalCer.

1.7 β -linked glycolipids

In a general sense, the potent activity of exogenous glycolipids that stimulate the immune system requires an α -linkage between the sugar moiety and the lipid portion. For example, bacterial glycolipids such as α -glucosyldiacylglycerol from *Streptococcus pneumoniae* and α -glycuronosylceramides from *Sphingomonas spp.* have α -linkages and typically, β -analogues of immunoactive bacterial glycolipids have significantly lower stimulatory activity compared to their α -linked counterparts.^{71,116-119} Indeed, the potent immunostimulatory activity attributed to α -GalCer is significantly reduced when the glycosidic linkage is converted to give β -GalCer. In spite of this, β -linked glycolipids represent an important class of glycolipids because mammalian cells produce β -linked rather than α -linked glycolipids.

iNKT cells represent a potent subset of T cells in both the innate and adaptive immune response and accordingly, much effort has focused on understanding iNKT cell development. The development of iNKT cells occurs in the thymus, where a T cell with a TCR that can recognise CD1d bound to a self glycolipid is positively selected. Mice lacking in the CD1d molecule (CD1d knockout mice) do not have iNKT cells,^{63,120} while mice with impaired intracellular CD1d trafficking have abnormal iNKT cell development.¹²¹ Because all endogenous glycolipids in mammalian cells are β -linked, this class of glycolipids has been studied extensively to understand how they shape the iNKT cell repertoire. That said, the exact identity of the self β -glycolipid remains elusive and it is unclear as to whether one or more glycolipids are responsible for the positive iNKT cell selection in the thymus.^{61,62}

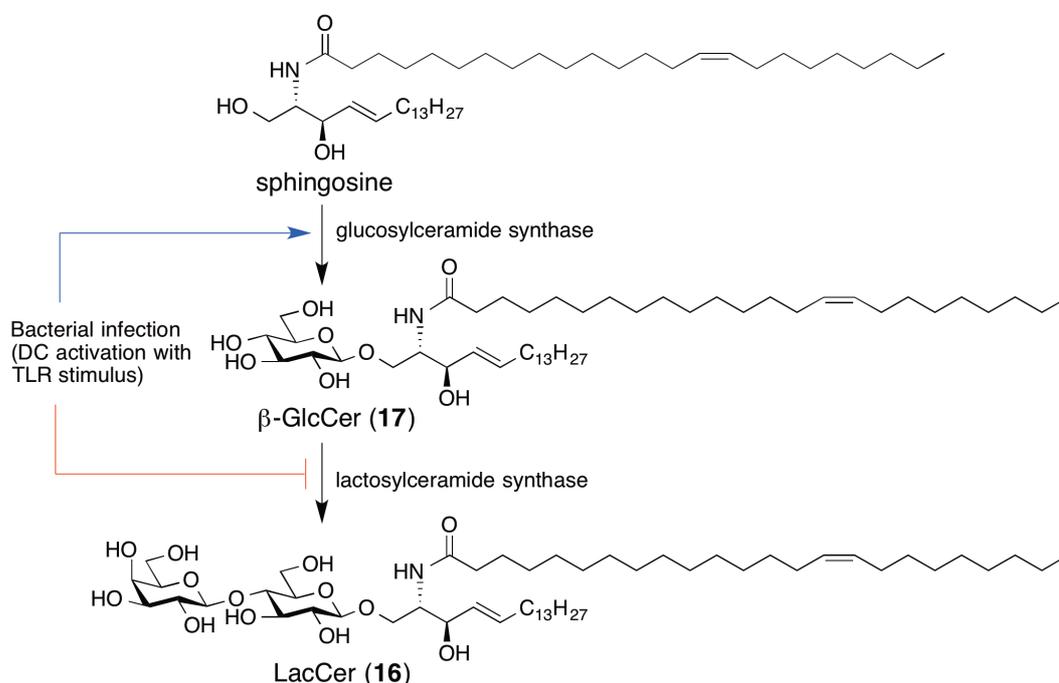
From a structural point of view, in a CD1d-glycolipid complex, the sugar headgroup of α -linked monoglycosyl ceramides wraps closely to the α 2-helix of CD1d in a parallel orientation, whereas the sugar headgroup of β -linked monoglycosyl ceramides protrude from CD1d at an angle perpendicular to the α 2-

helix. For example, β -galactosylceramide (β -GalCer), β -glucosylceramide (β -GlcCer) and isoglobotrihexosylceramide (iGb3) are implicated in the development and activity of iNKT cells.¹²² The ability of the TCR to accommodate this difference is a source of fascination for many structural immunologists and much research is still underway to accurately determine the extent of their influence on iNKT cell biology.

1.7.1 Isoglobotrihexosylceramide (iGb3)

Isoglobotrihexosylceramide (iGb3, **4** Scheme 1.1) is a glycosphingolipid containing a trisaccharide headgroup attached to a sphingosine ceramide via a β -linkage. The prospect of iGb3 acting as the self antigen responsible for iNKT cell development was first reported by Bendelac and co-workers in 2004. Upon observing that mice lacking in β -hexosaminidase B have severely reduced number of iNKT cells, they synthesised and tested iGb3 to assess if it was indeed the endogenous ligand important for iNKT cell development.¹²³ β -Hexosaminidase B is the lysosomal enzyme responsible for cleaving terminal β -linked GalNAc residues from mammalian glycosphingolipids (GSLs) and one of the substrates, iGb4 (**15**) is converted to iGb3 (**4**) by this enzyme. While Bendelac and co-workers did not directly purify and characterise iGb3 from mammalian tissues, they were able to show that synthetic iGb3 stimulates the expansion of human iNKT cells from peripheral blood mononuclear cells (PBMCs), and the cytokine levels (IFN- γ and IL-4) were comparable to that of α -GalCer.¹²³ They suggested that iGb3 was the major self glycolipid responsible for the selection of iNKT cells in the thymus of mammalian cells.

More recent findings by Brennan *et al.* revealed that β -glucosylceramide (β -GlcCer, **17**, Scheme 1.2) is an important self glycolipid whose expression is upregulated upon DC activation.¹²⁹ During an infection, toll-like receptor (TLR)-mediated activation of DCs leads to the upregulation of the enzyme glucosylceramide synthase that catalyses the synthesis of β -GlcCer (**17**) while the enzyme lactosylceramide synthase that converts β -GlcCer (**17**) to LacCer (**16**) is downregulated. The increased accumulation and presentation of β -GlcCer (**17**) by CD1d, in combination with the TLR-dependant production of cytokine IL-12, stimulates iNKT cells to produce IFN- γ . Brennan *et al.* also showed that the composition of the *N*-acyl lipid dictates the activity of β -GlcCer (**17**) and that the most abundant form found in mouse lymphoid organs is the C24:1 variant. This study, along with others thus illustrates that β -linked self glycolipids exert their effect not only during the development of iNKT cells, but that they are also responsible for iNKT cell activation during infection.^{101,130-134} The challenge remains to attribute the contribution of self β -linked glycolipids and foreign α -linked glycolipids on the immunological profile during an infection.



Scheme 1.2. Biosynthetic pathway for β -GlcCer (**17**).

1.7.2. Analogues of iGb3

Several trisaccharide analogues of CD1d-binding iGb3 have been synthesised to probe the structural requirements of CD1d-binding β -linked glycosphingolipids. Modifications have been made to both the carbohydrate portion of the glycosphingolipid and to the lipid backbone. For example, Xia *et al.* synthesised two iGb3 analogues iGb3-phytosphingosine (**18**) and iGb3-sphinganine (**19**), each of which contain a modified ceramide backbone (Figure 1.8).¹³⁵ In subsequent studies, they discovered that iGb3-phytosphingosine (**18**), which bears the same lipid backbone as α -GalCer, was better at stimulating iNKT cells compared to the parent iGb3 (**4**) which bears a sphingosine backbone. Drawing from existing crystal structure studies of α -GalCer with CD1d, the authors suggested that the 4-OH in phytosphingosine is able to provide an additional H-bond with the F' pocket of CD1d. However, it is important to note that this higher activity was only observed at low concentrations of the glycolipids (10 – 100 ng/mL). In addition, iGb3-sphinganine (**19**) also stimulated iNKT cells efficiently and it was rationalised that the absence of the *E*-alkene reduces the rigidity, of the lipid backbone, allowing for easier loading into the F' pocket of CD1d.¹³⁵

Savage and co-workers have also synthesised analogues of iGb3 and Gb3 with an α -linkage between the proximal sugar and lipid portion, α iGb3 (**20**) and α Gb3 (**21**), and showed that akin to other monoglycosyl ceramides, the α -versions were able to activate iNKT cells more effectively than their β -counterparts.¹³⁶

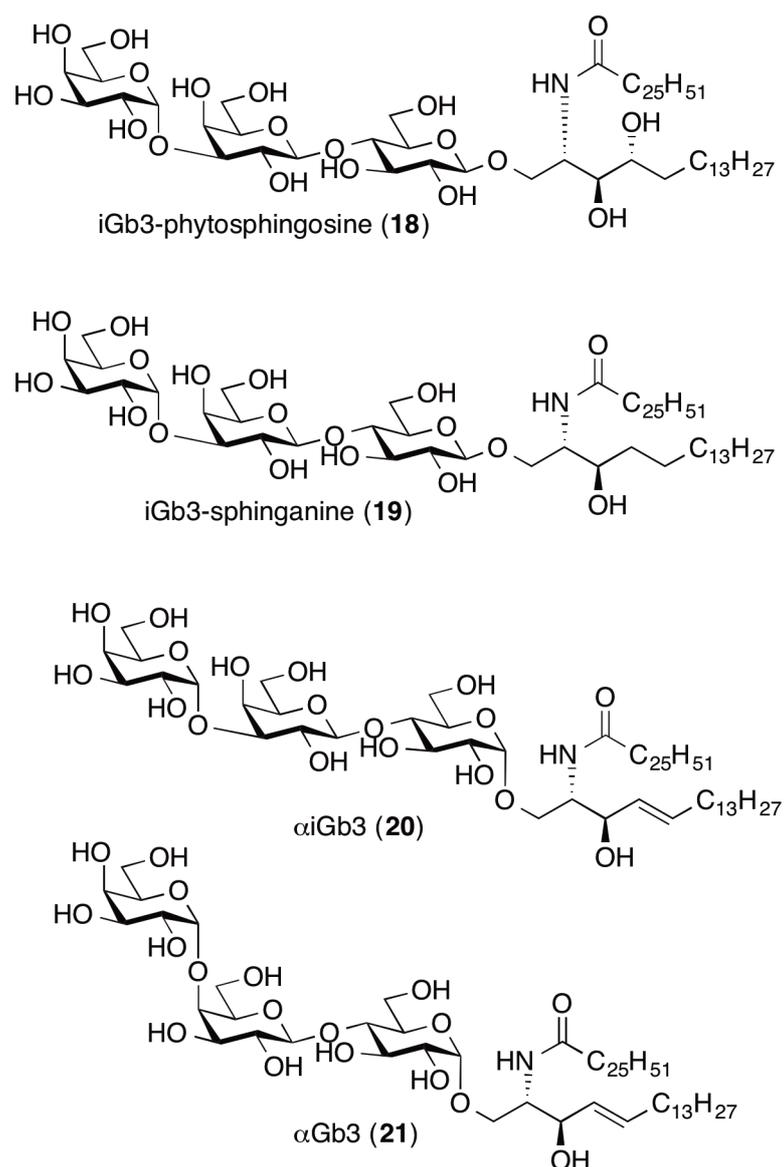


Figure 1.8. Analogues of iGb3.^{135,136}

Despite the studies by Xia *et. al.* and the earlier work by Bendelac and co-workers,^{123,135} the ability of iGb3 to stimulate iNKT cells was still surprising as most known iNKT cell ligands were monoglycosyl ceramides. While it was already known that some diglycosyl ceramides, such as Gal(α1,2)-α-GalCer (**22**) (Figure 1.9) needed to be processed into the monoglycosyl version before iNKT cells can be stimulated.¹²³ Here, the terminal galactose moiety of Gal(α1,2)-α-GalCer (**22**) is hydrolysed by α-galactosidase A in the lysosomal compartment of APCs, to give α-GalCer, which is then loaded onto the CD1d and presented to an

iNKT cell. While iGb3 can be processed into smaller fragments such as β -LacCer (**23**) and β -GlcCer (**24**) and stimulate iNKT cells, iGb3 was found to be able to activate iNKT cells in the absence of processing.^{117,123,137} Interestingly, a regioisomer of iGb3 that is found in mammalian cells, globotrihexosyl ceramide (Gb3, **25**), varying only in the α 1,4 linkage of the terminal galactose, was not able to stimulate iNKT cells.^{123,138} This indicated that the linkage of the terminal sugar is crucial for the observed activity of iGb3.

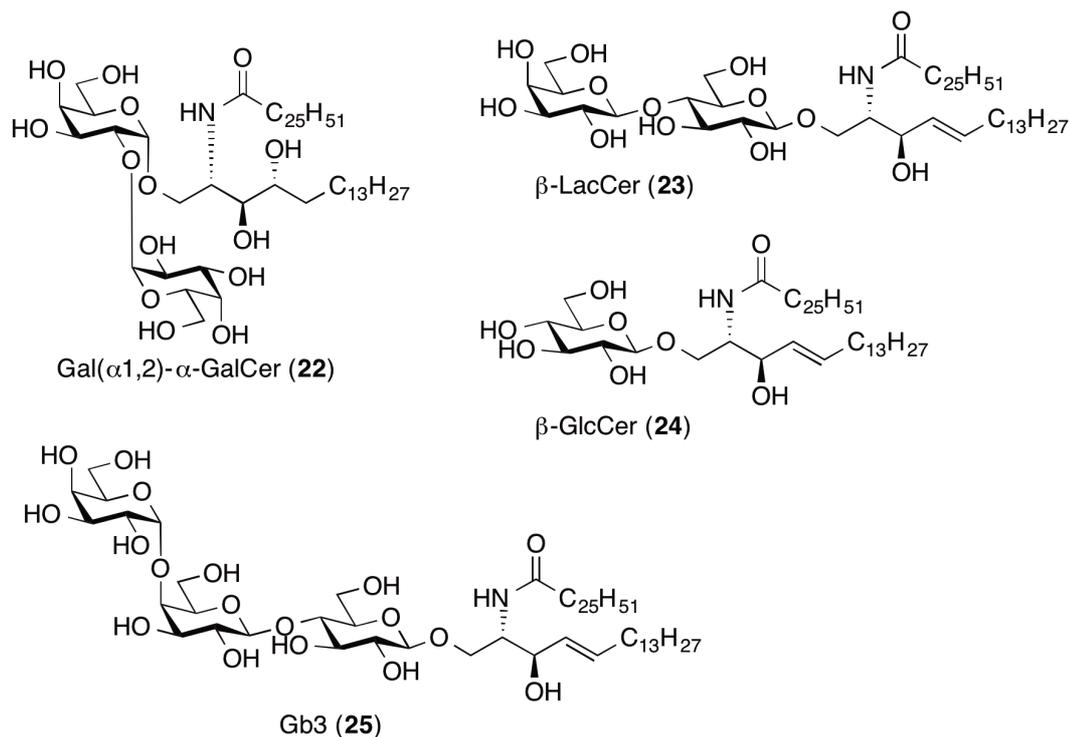
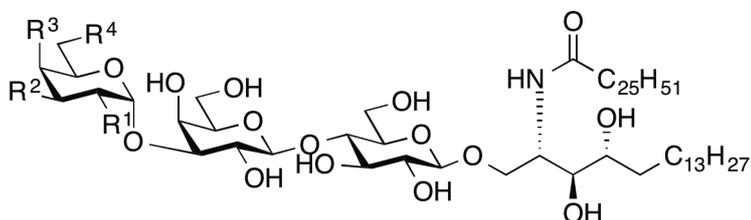


Figure 1.9. Analogues of iGb3.

To probe the effect of the terminal galactose residue on the activity of iGb3, Chen *et al.* synthesised a series of dehydroxylated analogues of iGb3, whereby the hydroxyl groups on the terminal galactose were systematically removed to give 2'''-deoxy- (**26**), 3'''-deoxy- (**27**), 4'''-deoxy- (**28**) and 6'''-deoxy-iGb3 (**29**) (Figure 1.10).¹³⁹ Of note, a phytosphingosine backbone was incorporated instead of the sphingosine lipid found on the original iGb3, with the intention of creating glycolipids with more potent iNKT cell stimulatory property. Testing of this series of iGb3 analogues revealed that the removal of hydroxyl groups at the 4'''- and 6'''-position did not significantly hamper the ability to stimulate iNKT cells,

but the absence of the 2'''-OH or 3'''-OH reduced the agonist effect considerably. Thus, these studies further confirmed that the terminal sugar is critical for the activity of iGb3 and analogues thereof.



2'''-deoxy-iGb3-phytosphingosine (**26**): R¹ = H, R² = OH, R³ = OH, R⁴ = OH

3'''-deoxy-iGb3-phytosphingosine (**27**): R¹ = OH, R² = H, R³ = OH, R⁴ = OH

4'''-deoxy-iGb3-phytosphingosine (**28**): R¹ = OH, R² = OH, R³ = H, R⁴ = OH

6'''-deoxy-iGb3-phytosphingosine (**29**): R¹ = OH, R² = OH, R³ = OH, R⁴ = H

Figure 1.10. Deoxy analogues of iGb3 with phytosphingosine backbones.¹³⁹

1.8 Crystal structures of β -linked glycolipids

Building from their seminal work in solving the crystal structure of α -GalCer bound to CD1d, Rossjohn and co-workers solved the crystal structure of a mouse iNKT cell TCR in complex with mCD1d- β -GalCer and showed that the TCR docked onto the CD1d- β -GalCer complex in a very similar manner to CD1d- α -GalCer (Figure 1.11).¹³⁸ It is well established that when the lipid portion of α -GalCer is embedded within the hydrophobic cavity of CD1d, the sugar headgroup that is exposed lies flat (parallel) against the α 2-helix of CD1d.⁸² One could anticipate that the sugar headgroup of β -GalCer protrudes out of the CD1d pocket so that it is perpendicular to the α 2-helix, however, surprisingly, Rossjohn and co-workers observed that when the iNKT TCR engages with the CD1d- β -GalCer complex, the protruding sugar headgroup is “bulldozed” so that it lies flat against the α 2-helix of CD1d, and hence occupies the space in a manner more similar to α -GalCer.¹³⁸ Of note, the hydrogen bond network between the β -linked galactose and the α 2-helix of CD1d were analogous to the interactions found between CD1d and α -GalCer, and moreover, the interactions between the iNKT TCR and CD1d were also conserved. Taken together, these crystal structures provide a molecular explanation for the ability of structurally distinct β -linked glycolipids to maintain their function as iNKT cell agonists. Nonetheless, the iNKT TCR has a lower affinity for β -GalCer compared to α -GalCer, suggesting that an “energetic penalty” is imposed for “flattening” the β -linked glycolipid.¹³⁸

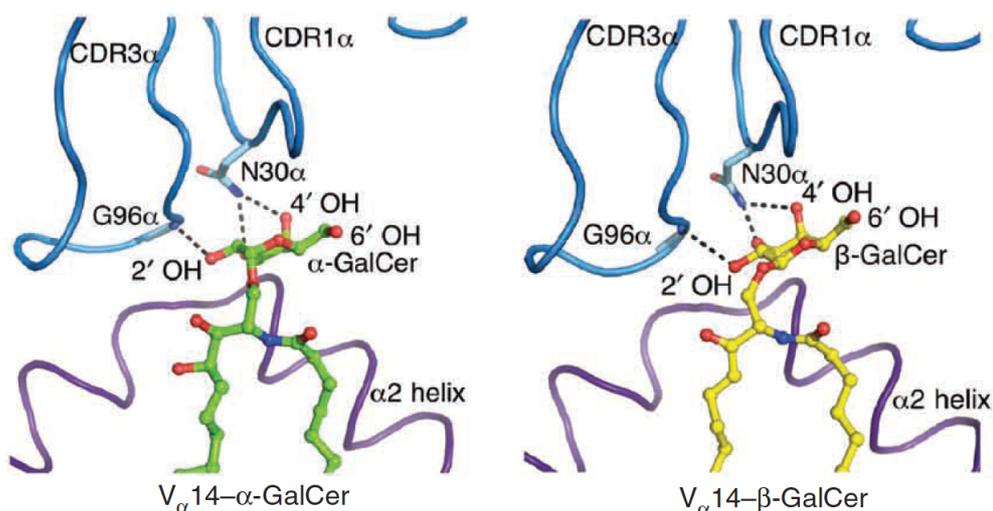


Figure 1.11. Crystal structure of mCD1d and TCR of iNKT cell with α -GalCer (in green) and β -GalCer (in yellow).¹³⁸ (Adapted by permission from Macmillan Publishers Ltd: Nature Immunology, 2011, 12, 827-833, ©2011)

The crystal structures of β -linked monoglycosyl ceramides formed the foundation for further studies of β -linked glycolipids with more than one sugar headgroup. In 2011, Yu *et al.* and Rossjohn and co-workers both reported the crystal structure of mouse iNKT cell TCR in complex with mCD1d-iGb3 and their results were in agreement.^{137,138} Both groups observed the flattening of the trisaccharide headgroup against the α 2-helix of CD1d, similar to that observed for β -GalCer (Figure 1.12). The proximal glycosidic bond is “contorted” to resemble the α -like conformation, with the β -Glc itself forming interactions with both CD1d and the iNKT cell TCR. The middle β -Gal also establishes many contacts with CD1d, and has some contact with the TCR. The terminal α -Gal moiety, on the other hand, only interacts with the α 2-helix of CD1d and not the TCR. Here, the terminal sugar is anchored by the hydrogen bond between the 6'''-OH with Thr159 and the van der Waals interaction of 4'''-OH and 6'''-OH with Met162 of CD1d. In the absence of the terminal α -Gal moiety, as in β -LacCer (**23**), the agonist activity of the glycolipid is abolished. In addition, a structural isomer Gb3 (**25**), does not stimulate iNKT cells because the terminal sugar (α 1,4-Gal) is oriented away from CD1d and does not form the interaction required to stabilise the trisaccharide headgroup. The loss of key interactions in the absence of the terminal α 1,3-linked

galactose results in the compromised avidity of β -LacCer (**23**) and Gb3 (**25**). Therefore, the engagement of the terminal sugar of iGb3 with CD1d is the prerequisite for the formation of a stable glycolipid conformation, giving rise to the iNKT cell agonist effect.¹³⁸

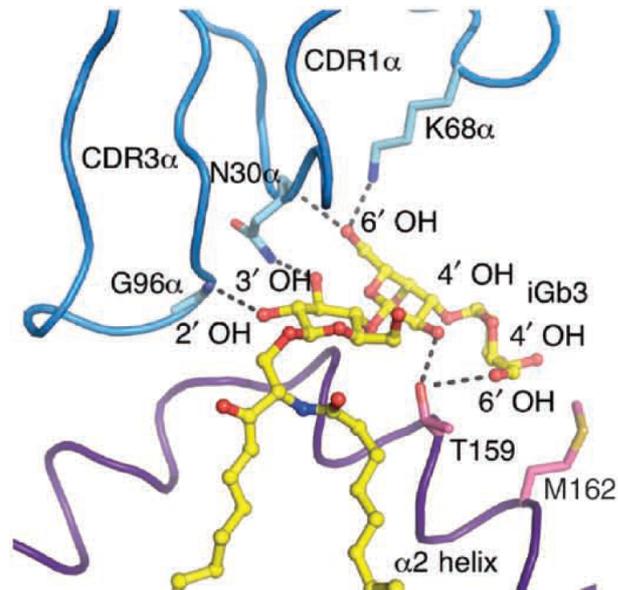


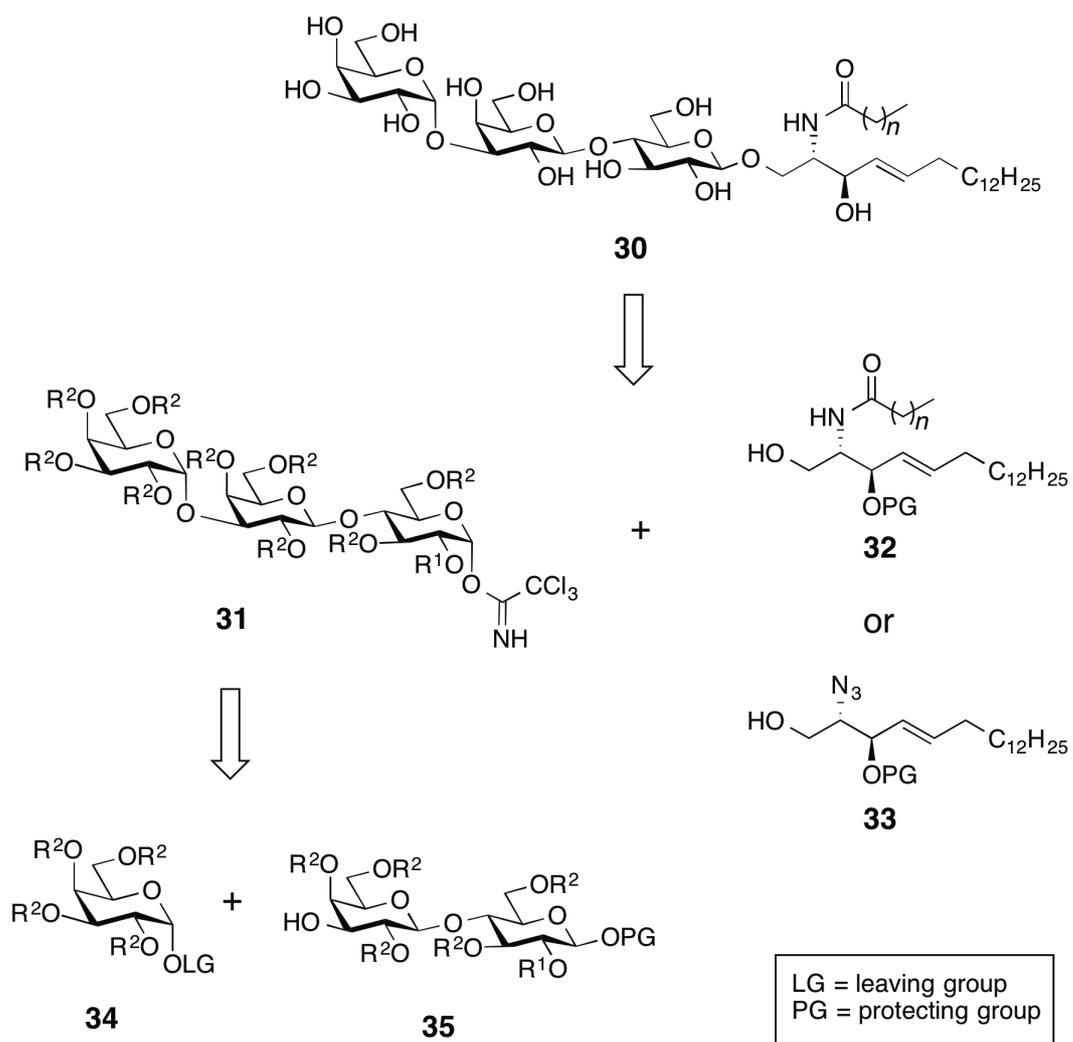
Figure 1.12. Crystal structure of mCD1d and TCR of iNKT cell with iGb3.¹³⁸
(Adapted by permission from Macmillan Publishers Ltd: Nature Immunology, 2011, 12, 827-833, ©2011)

1.9 The significance of understanding β -linked self glycolipids

The ability of iNKT cells to act as potent immune cells both for the innate and the adaptive immune response has driven much research to focus on developing glycolipid ligands that can invoke their activation. Current knowledge about CD1d presentation of glycolipids to iNKT cells and the associated downstream effects is largely attributed to the exogenous glycolipid α -GalCer, however there is a growing appreciation of the ways in which β -linked self glycolipids modulate the immune response. Not only are β -linked self glycolipids responsible for the development of iNKT cells in the thymus,^{128,129} they also act as direct agonists during infection.^{101,131,133,134,140,141} Accordingly, the synergy of structure-activity-relationship studies and crystal structure analyses will provide a fuller picture of how β -linked glycolipids exert their effect on the immune system, with the view that this will then allow for the rational design of β -glycolipids for the treatment of diseases such as infections, autoimmune diseases and cancer.

1.10 Synthetic strategies for iGb3 and related analogues

The first synthesis of trihexosyl ceramides dates back to 1987, when Koike *et al.* synthesised the iGb3 with a C24 *N*-acyl lipid along with Gb3 also bearing the C24 lipid (Scheme 1.3, $n = 22$).¹⁴² The key step in this synthetic route was the coupling of a trisaccharide donor with a trichloroacetimidate leaving group **31** and the ceramide acceptor **32**. The trisaccharide donor **31** was in turn obtained by coupling galactose donor **34** with a suitably protected lactose acceptor **35**. It is important to note that the protecting group used at the 2-OH (R¹O-) of lactose must be one that is capable of participating during the final glycosidation with the lipid acceptor **32** to facilitate the formation of the β -anomer. Schmidt and co-workers subsequently synthesised another iGb3 analogue with the *N*-palmitoyl (C16) lipid ($n = 14$) using a very similar strategy, except that the trisaccharide donor was coupled in a stepwise fashion, first to the sphingosine acceptor, followed by functionalisation with the C16 acyl lipid.¹⁴³ It was not until 2006, two years after iGb3 bearing a hexacosanoyl (C26, $n = 24$) lipid was first reported to be an important endogenous glycolipid for iNKT cell biology, that a flurry of syntheses of the prototypical iGb3-C26 analogue **4** were reported.^{144,145} Since then, the synthesis of various other derivatives such as the C18 homologue,¹⁴⁶ thio-iGb3,¹⁴⁷ 6'''-deoxy analogues **26-29**,¹³⁹ phytosphingosine **18** and sphinganine **19** derivatives¹³⁵ and α iGb3 analogue **20**¹³⁹ have been published. Generally, the routes employed involve the coupling of a trisaccharide imidate donor with either a full ceramide lipid acceptor **32** or a 2-azido-sphingolipid acceptor **33**. The alternative approach by first coupling the lactose donor to the ceramide backbone, followed by linkage of the terminal sugar has not been reported. While complete chemical synthesis of this glycolipid is the most common, some have also used enzymatic syntheses either to make the trisaccharide,¹⁴⁵ or to furnish LacCer with the desired terminal galactose, in a highly stereospecific fashion.¹⁴⁸



Scheme 1.3. Strategies for the synthesis iGb3 and analogues.

1.11 Thesis outline

The research described in this thesis focuses on the synthesis of glycosphingolipids that can modulate the immune response, particularly in a cancer setting. To this end, the development of a variety of glycolipid probes which can be used as fluorescent tools to track the uptake and trafficking of α -GalCer will be discussed, as will the synthesis of iGb3 derivatives that can be used in structure-activity studies to better understand the effects of these glycosphingolipids on iNKT cell activation. A brief outline of each thesis chapter is given below.

Chapter 2 describes the synthesis of the first α -GalCer fluorescent probe, Dansyl- α -GalCer **2** (Figure 1.13), which contains the dansyl fluorophore at the 6'-position of α -GalCer. A new and highly efficient route for the synthesis of this probe is presented, and the ability of the probe to activate the immune response, as compared to the parent compound α -GalCer, is also discussed. Finally, the ability of the probe to act as a fluorescent tool to track the *in vitro* uptake of the glycolipids is also discussed.

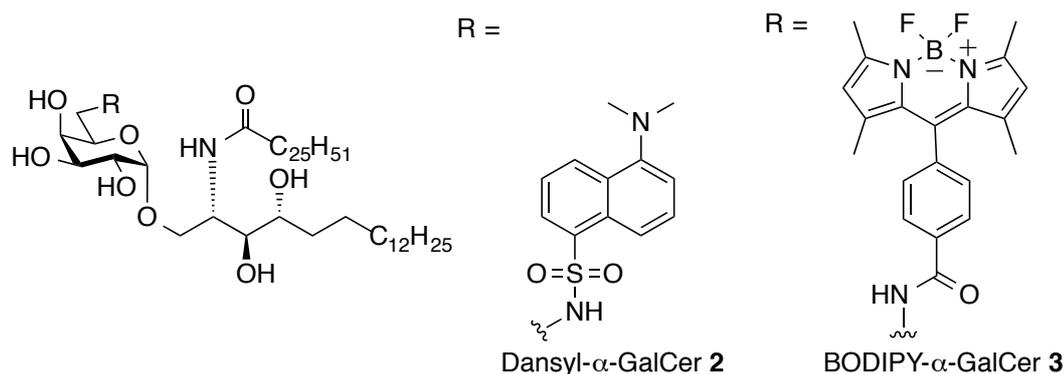


Figure 1.13. Fluorescent α -GalCer probes

Chapter 3 then continues on with the development of fluorescent α -GalCer probes and discusses the synthesis of BODIPY- α -GalCer **3**, which contains the BODIPY fluorophore at the 6'-position (Figure 1.13). A synthetic strategy, which

is different to that used for the synthesis of dansyl- α -GalCer, is presented and it is envisioned that the brighter BODIPY fluorophore will extend the range of biological experiments that can be undertaken using this probe. Finally, chapter 3 also discusses some of the limitations of the BODIPY fluorophore, the steps that need to be taken to ensure that the BODIPY- α -GalCer **3** probe does not degrade, and initial studies into exploring the photo-reactivity of this fluorescent group.

Chapter 4 describes the synthesis of the first series of iGb3 derivatives, namely the 6'''-deoxy analogues with either the sphingosine **5** or sphinganine **6** lipid backbone (Figure 1.14). This is the first time that such 6'''-deoxy homologues of iGb3 have been prepared and, in association with our collaborators, the subsequent biological evaluation and crystal structure studies using these substrates will allow for an assessment of the effect of the 6'''-OH on the activity of iGb3, and also, how the lipid backbone effects glycolipid loading and iNKT cell activation. The route chosen for the synthesis of iGb3 sphingosine **5** or sphinganine **6** is highly efficient and moreover, allows for a convergent strategy that can be used to prepare not only sugar analogues, such as those discussed in this chapter, but also *N*-acyl chain derivatives. This is the first time that such a convergent route for the synthesis of iGb3 analogues will be described.

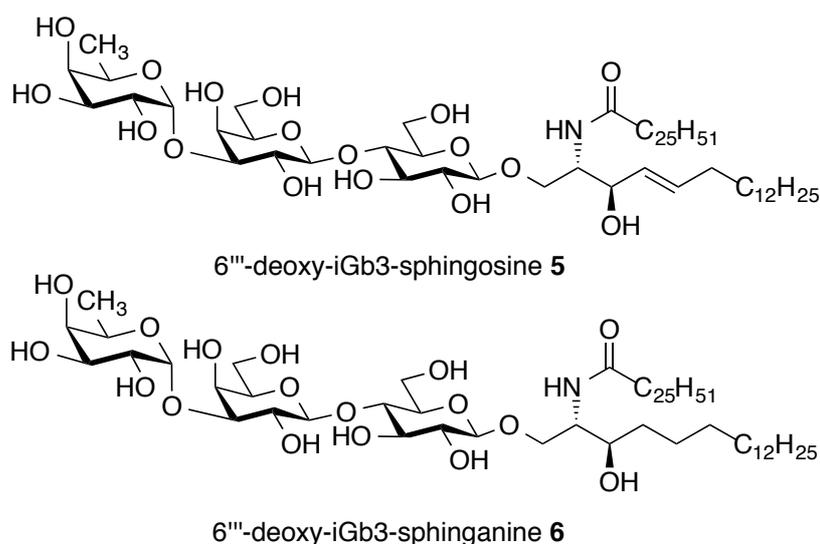


Figure 1.14. Target 6'''-deoxy-iGb3 analogues.

Chapter 5 describes the synthesis of two acyl chain homologues of iGb3. Key in the methodology is the use of a common intermediate presented in Chapter 4, which allows for the preparation of two *N*-acyl derivatives, namely the C12 **7** and C20:2 **8** homologues (Figure 1.15). The challenges and effectiveness of the synthetic route are presented, which can then be compared to the route used for the 6''-deoxy analogues. It is envisioned that the *N*-acyl analogues, when compared to iGb3 itself, will allow for the effect of lipid length and saturation on iNKT cell activation to be determined.

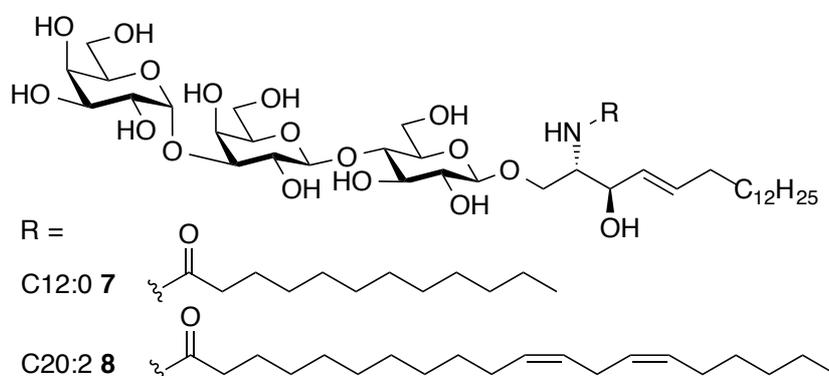


Figure 1.15. Target acyl chain homologues of iGb3.

Finally, **Chapter 6** summarises the research performed in this thesis and highlights some future directions for each project area.

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Chapter 2. The synthesis and biological evaluation of dansyl α -galactosylceramide as a fluorescent probe for the monitoring of glycolipid uptake by cells

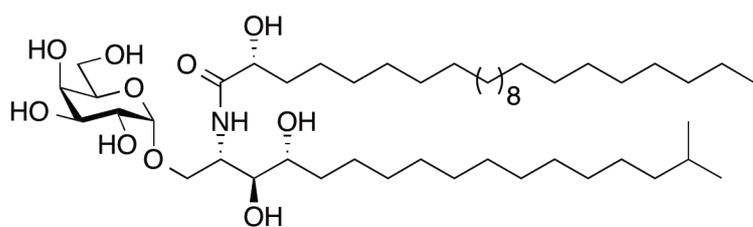
This chapter is an edited version of the following publication:

Cheng, J. M. H.; Chee, S. H.; Knight, D. A.; Acha-Orbea, H.; Hermans, I. F.; Timmer, M. S. M.; Stocker, B. L. 'An improved synthesis of dansylated α -galactosylceramide and its use as a fluorescent probe for the monitoring of glycolipid uptake by cells.' *Carbohydr. Res.* **2011**, *346*, 914-926.

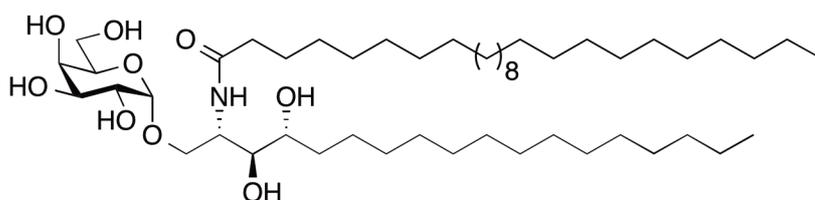
As the principal author, I carried out all the chemical transformations described in this paper and the biological testings were performed at the Malaghan Institute of Medical Research under the supervision of I. F. H. and D. A. K. and I played a major role in the writing this paper.

2.1 Introduction

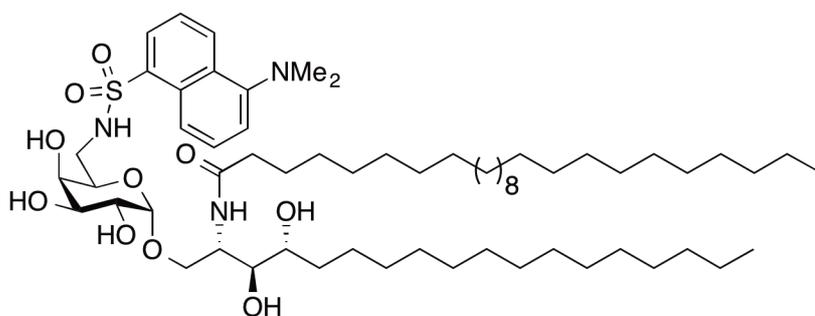
The potent glycosphingolipid adjuvant, α -galactosylceramide (α -GalCer **1**, Figure 2.1) is a structural analogue of agelasphin-9b (AGL-9b, **9**) that was isolated from the marine sponge *Agelas mauritianus*.¹⁻³ α -GalCer is able to activate a subset of T cells known as invariant natural killer T (*i*NKT) cells when it is presented in the context of CD1d [a member of the CD1 family of proteins found on the surface of antigen presenting cells (APCs)].⁴⁻⁶ *i*NKT cells represent a potent type of T cell, and the ability for glycosphingolipids to modulate their activation makes them useful targets for the treatment of several diseases including cancer, malaria, type I diabetes, and multiple sclerosis.



Agelasphin-9b (AGL-9b, **9**)



KRN7000 or α -Galactosyl ceramide (α -GalCer, **1**)



Dansyl α -GalCer **2**

Figure 2.1. α -Galactosyl ceramide and derivatives.

Given the therapeutic potential of α -GalCer, much effort has been spent in developing robust routes for its synthesis.^{3,7-10} In addition, a number of α -GalCer-derivatives containing a fluorophore¹¹⁻¹³ or a biotinylated probe^{11,14,15} have been prepared with the objective of using these substrates to better understand the mechanism of *i*NKT cell activation by α -GalCer.^{13,16,17} Though the ability of α -GalCer to bind to CD1d and activate *i*NKT cells has been robustly studied, only little information is known about lipid trafficking and how this influences presentation by CD1d and activation of *i*NKT cells.¹⁸ Accordingly, many groups have focussed on modifying the lipid portion of α -GalCer with a reporter probe in order to address such issues.¹²⁻¹⁴ However, as CD1d binds lipid chains within deep hydrophobic pockets,¹⁹ it has been proposed that the addition of a label on the

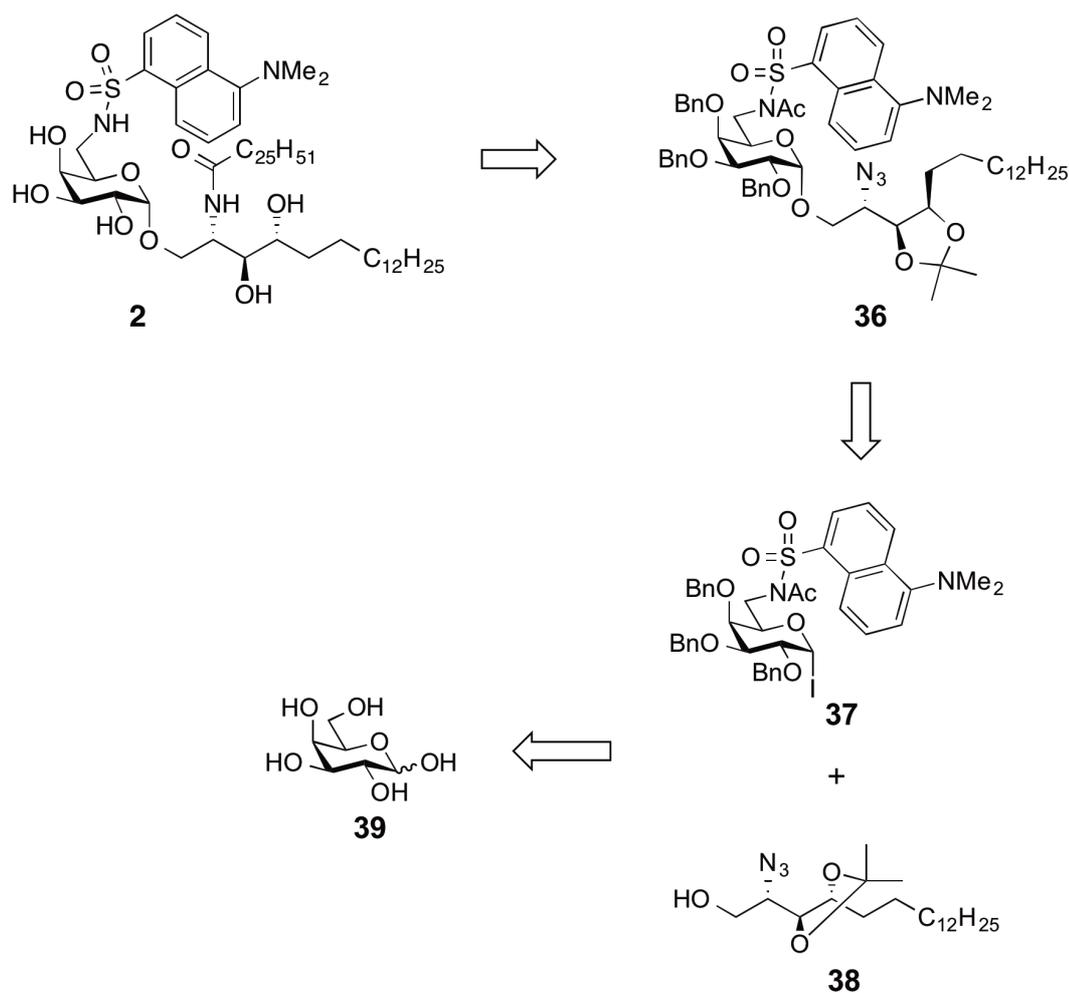
lipid may interfere with α -GalCer-CD1d association,¹⁷ and moreover, may influence intracellular trafficking.^{18,20-22} Conversely, modelling of the CD1d- α -GalCer complex suggests that the hydroxyl groups at C4'' and C6'' on galactose are not involved in complex formation.²³ This theory is also supported by the preparation of an α -GalCer analogue containing an additional α -linked galactose at the C6'' position which was shown to stimulate *i*NKT cells without the need for processing.²⁴ Taken as a whole, it is generally accepted that the CD1d-glycolipid-NKT cell receptor interaction tolerates the appendage of small molecules at C6'' and that this is an ideal position to attach a fluorescent reporter group. Indeed, this was the conclusion made by Zhou *et al.* who developed a strategy for the preparation of dansylated α -GalCer derivative **2**.¹¹

Despite there being much interest in α -GalCer, the need for a more efficient synthesis of an appropriately labelled fluorescent α -GalCer derivative remains. An improved strategy for the synthesis of the dansylated α -GalCer derivative **2** (Figure 2.1) is needed to facilitate studies of the mechanism by which α -GalCer is transferred to resident dendritic cells (DCs) during cancer immunotherapy.^{25,26} The objective was to achieve a robust synthesis of **2** with high reaction yields throughout, and to assess the ability of **2** to both activate *i*NKT cells and to serve as a fluorescent reporter group. The results of these studies are reported herein.

2.2 Synthesis of dansyl α -GalCer

Retrosynthesis

The retrosynthetic analysis for the preparation of dansylated α -GalCer **2** is presented in Scheme 2.1. **2** was prepared from the mono-lipidated derivative **36**, itself formed via the coupling of dansylated galactosyl iodide donor **37** to the phytosphingosine backbone **38**. Donor **37** and acceptor **38** are in turn both readily prepared from D-galactose (**39**). The reasoning behind these key disconnects are two-fold. First, though it is possible to couple the complete ceramide lipid backbone to a suitably protected galactose donor, the ceramide is a particularly poor acceptor and yields for these glycosylations are typically modest (*ca.* 25-55%). This is a general phenomenon largely independent of the type of galactose donor used.^{8,15,27-31} There are also advantages to be gained by incorporating the fluorescent dansyl group at an early stage in the synthesis for it has been well documented that molecules containing a chromophore are more easily monitored by TLC and purified by flash column chromatography.³² Accordingly, in contrast to other syntheses of fluorescent α -GalCer probes,¹¹⁻¹³ a glycosylation reaction involving the use of a fluorescent galactose donor, such as **37** was employed.

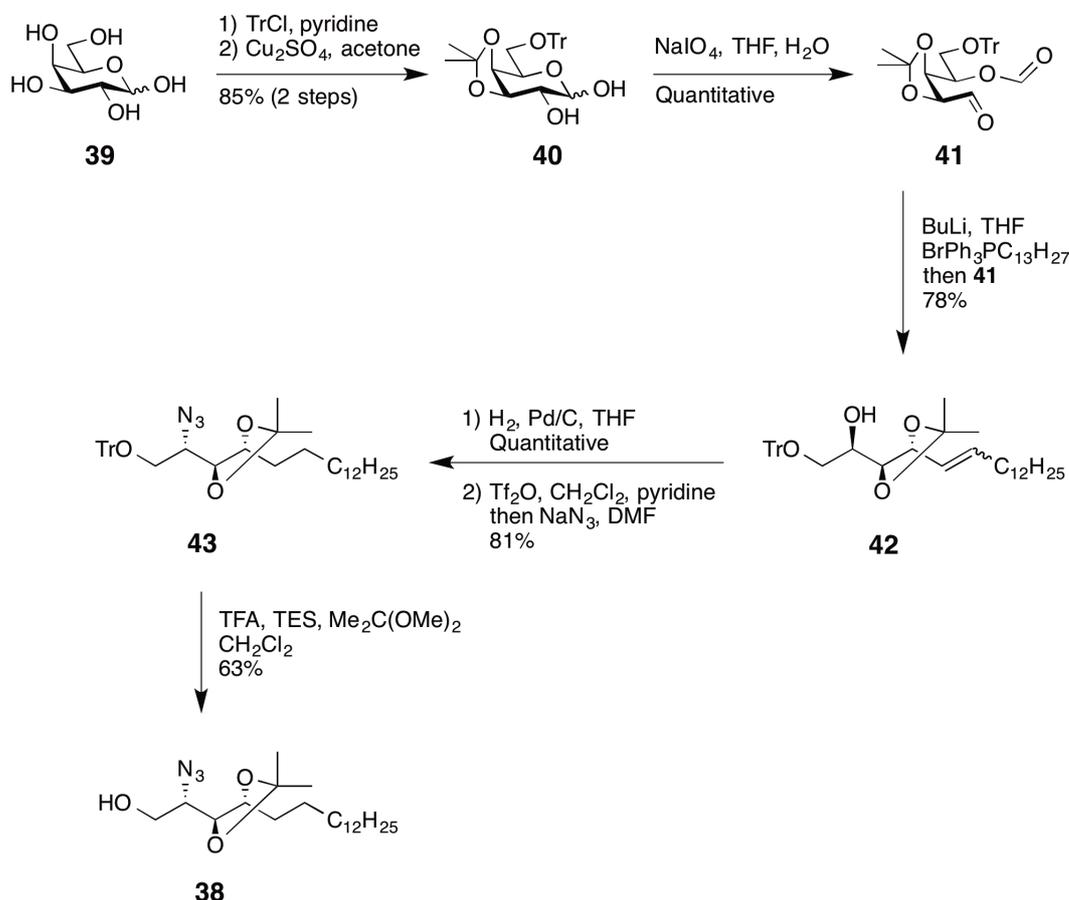


Scheme 2.1. Retrosynthetic analysis for the preparation of dansylated α -GalCer **2**.

Synthesis of phytosphingosine acceptor **6.**

With the synthetic strategy in place, the first goal was the preparation of the phytosphingosine backbone **38** (Scheme 2.2). Here, synthesis of the lipid portion commenced with the selective tritylation of D-galactose (**39**) at the primary position,³³ followed by installation of an isopropylidene at the 3- and 4-positions to give the diol **40** in 85% (over two steps).³⁴ Diol **40** was then treated with a solution of NaIO₄ in THF/H₂O, which resulted in cleavage of the diol and formation of the formate ester **41** in quantitative yield. As a temporary protecting group, the formate at the 4-position was a pivotal step in this strategy as it prevented cyclisation to the corresponding D-lyxofuranose derivative, which gave

only modest yields (50-60%) when subject to the subsequent Wittig reaction. Treatment of ester **41** with excess ylide (BuLi, 2.4 equiv., phosphonium bromide, 2.5 equiv.), however, gave the corresponding alkene **42** in good (78%) yield and in an *E:Z* ratio of approximately 1:10 based on ¹H NMR analysis.



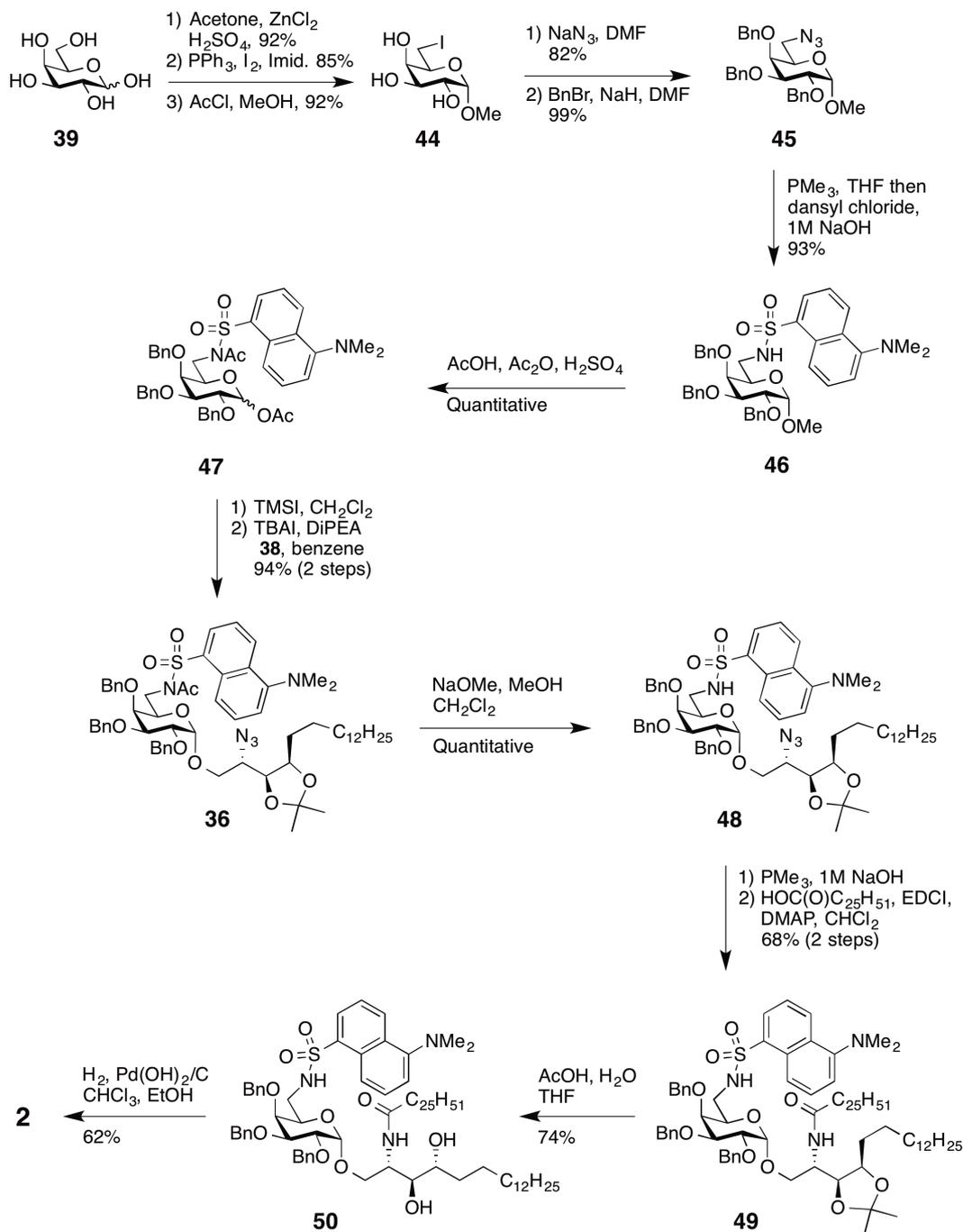
Scheme 2.2. Synthesis of phytosphingosine acceptor **38**.

Subsequent conversion of alkene **42** to the required phytosphingosine backbone then followed via quantitative hydrogenation, using 1.5 wt% Pd/C, and treatment with triflic anhydride to give the intermediate triflate, which was converted to azide **43** *in situ*. During the installation of the azide functionality, it was found that the best yields were obtained when the reaction was preformed at -15 °C as higher temperatures resulted in the elimination of the triflate and the formation of the corresponding olefinic by-product. Selective removal of the trityl ether in the presence of the isopropylidene group was then attempted. This transformation has

proven problematic in the past,⁷ and resulted in the authors adopting a 4-step deprotection/reprotection strategy to generate a modified 3-*O*, 4-*O*-benzylated phytosphingosine derivative. In view of the lability of the isopropylidene group, azide **43** was treated with TFA and TES, then added 2,2-dimethoxypropane to the reaction vessel to reinstall the isopropylidene, should it be cleaved under the acidic reaction conditions. Indeed, using this approach, phytosphingosine **38** was obtained in a respectable 63% yield. Numerous attempts were then made to further optimise the reaction including the addition of MeOH (to scavenge the liberated TES cation, which was sometimes found to be present on the 1-position of the lipid), however, this did not lead to any improvements in yield. The use of boron trichloride or formic acid to cleave the trityl group resulted in a lower yield of product. Despite this, in sum, the core phytosphingosine backbone **38** was prepared in 7-steps and in a good overall yield of 34%. This represents one of the most efficient syntheses of the phytosphingosine backbone to date.^{35,36}

Synthesis of dansylated galactose donor and dansyl- α -GalCer

Having completed the synthesis of the required phytosphingosine lipid, attention was turned to the preparation of the dansylated galactose donor. The synthesis of the carbohydrate portion commenced with the formation of the common azide (**45**)¹¹ using a modified sequence of reactions that first led to the formation of iodide **44** via the selective installation of isopropylidenes at the 1,2 and 3,4 positions of D-galactose (**39**), subsequent installation of the iodide at the primary position, and then simultaneous cleavage of the isopropylidenes and Fisher glycosylation using a 5% solution of AcCl in MeOH (Scheme 2.3). This route provided a convenient means by which to prepare gram quantities of iodide **45** in excellent overall yield (72%, 3 steps) and without the need for column chromatography. The iodide in **44** was then displaced to give the intermediate azide, which was subsequently benzylated to give the fully protected azide **45**, again in excellent overall yield.



Scheme 2.3. Synthesis of dansyl- α -GalCer **2**.

With the objective of installing the fluorescent group at an early stage in the synthesis, the dansyl group was then incorporated onto the galactose residue **45** in a two-step one-pot procedure involving the reduction of the azide to an amine

using Staudinger conditions¹⁰ and subsequent treatment with dansyl chloride in the presence of 1M NaOH. Using this strategy, dansylated galactose **46** was prepared in excellent (93%) yield. Dansylated galactose **46** is a bright-green oil, and as such, is readily identified during flash column chromatography whereby the yellow-green band can be followed by eye during the purification process. Furthermore, incorporation of the dansyl fluorophore also aids in the monitoring of the reaction by TLC analysis with **46** glowing bright white under UV light ($\lambda = 254$ nm). Treatment of **46** with a solution of AcOH, Ac₂O and H₂SO₄ at 0 °C for 3 h then resulted in the quantitative formation of acetate **47**. Here, it should be noted that in addition to the conversion of the anomeric methoxy to the acetate, the amine of the dansyl group was also acetylated. Though not optimal, the over-acetylation of dansyl groups has been previously reported³⁷ and it was envisaged that the superfluous acetyl could be readily removed during a subsequent step of the synthesis. A dramatic change in colour was also observed upon monitoring the reaction by TLC analysis ($\lambda = 254$ nm), with the methyl glycoside **46** fluorescing bright white, whilst the corresponding acetate **47** was mauve (see supporting information). As with all dansylated compounds prepared, acetate **47** was a fluorescent green colour, which allowed for its ready purification by flash column chromatography and illustrates the added value of the early incorporation of the dansyl-group into the synthetic plan.

Forging ahead, glycosyl donor **47** was converted to the corresponding glycosyl iodide via methodology based on seminal work by Gervay-Hague.³⁸⁻⁴¹ Though a number of galactosyl donors have been prepared and coupled to the phytosphingosine backbone *en route* to the preparation of α -GalCer,^{7,9,10,27-29,42-45} few of these glycosylation reactions have been reported to be both high-yielding and highly α -selective.^{38,46-48} To this end, the glycosyl iodide route of Gervay-Hague was employed and glycolipid **36** was prepared in 94% yield and as only the α -anomer, as determined by a ¹H $J_{1,2}$ coupling constant of 3.2 Hz and a ¹³C-¹H (¹ J_{CH}) coupling of 172.2 Hz.⁴⁹

With glycolipid **36** in hand, the next step was the installation of the C26 acyl chain. First, the acetyl on the sulfonimide was removed using Zemplén

conditions⁵⁰ to give the desired sulfonamide **48** in quantitative yield. Both glycolipid **36** and sulfonamide **48** had identical R_f values (0.40; PE/EA, 2/1, v/v), though were different colours when observed via TLC analysis. With glycolipid **36** being mauve in colour (UV light, $\lambda = 254$ nm) and sulfonamide **48** bright white, this greatly aided in the monitoring of the reaction. The isopropylidene group was then removed and the diol product subjected to hydrogenation conditions using $\text{Pd}(\text{OH})_2/\text{C}$ in the presence of HCl in an attempt to simultaneously remove the benzyl groups and reduce the azide to an amine. Numerous attempts were made to achieve this transformation, yet despite the addition of HCl to protonate the resultant amine and prevent poisoning of the palladium catalyst, only partial debenylation was observed. In view of this, an alternative strategy was used whereby the azide in **48** was first reduced to the amine, using Staudinger methodology, then coupled the product to hexacosanoic acid via an EDCI/DMAP condensation protocol to give fully protected dansylated α -GalCer **49** in good (68%) overall yield. Removal of the isopropylidene group occurred smoothly to give **50** in 74% yield. Finally, global deprotection, again using $\text{Pd}(\text{OH})_2/\text{C}$ as the catalyst, gave dansyl α -GalCer **2** in 62% yield, which glowed bright white under UV light ($\lambda = 366$ nm). Though glycolipids such as **2** have been reported to be notoriously difficult to solubilise and hence assign,^{51,52} using a variety of 2D NMR spectroscopy techniques (COSY, HSQC, HMBC) with spectra recorded in pyridine- d_5 , all ^{13}C and ^1H resonances were fully assigned. Furthermore, the dansyl group of **2** allowed for the ready detection of the compound by way of HPLC analysis (see supporting information). Thus, in summary, dansyl α -GalCer **2** was prepared in 14 linear steps from the commercially available and inexpensive D-galactose and in 16% overall yield. This represents a highly efficient synthesis of this important biological probe and is comparable to the work of Zhou *et al.* whereby the probe was prepared in <10% yield from 6-azido-6-deoxy-1,2:3,4-di-*O*-isopropylidene- α -D-galactopyranose.¹¹

2.3 The effects of dansyl- α -GalCer on the stimulation of iNKT cells *in vitro* and *in vivo*

Having developed an efficient synthesis of dansyl α -GalCer **2**, the next task was to investigate whether **2** could activate *i*NKT cells in a manner similar to that of its parent compound, α -GalCer (**1**).^{6,7,9,11,28,30,45} This was achieved by analysing levels of the cytokine IL-2 released by the V α 14⁺ *i*NKT cell hybridoma⁴ DN32.D3 following *in vitro* stimulation with the glycolipid compounds and by examining the expression of the co-stimulatory molecule, CD86, on splenic DC and B cells after injection of the compounds into mice.⁹ The latter assay is based on the observation that CD86 is upregulated as a consequence of *i*NKT cells interacting with, and subsequently driving, DC and B cell maturation.^{53,54}

To assess the ability of **2** to stimulate DN32.D3 *i*NKT cell hybridoma cells, titrated amounts of dansyl α -GalCer **2**, or α -GalCer **1**, were incubated for 24 h with the hybridoma cells in the presence of a constant number of murine DCs from the immortalised line, DC2114,⁵⁵ and supernatants were then collected and analysed for IL-2. Proliferation of the IL-2-dependent cell line HT-2 was used as a read-out of IL-2 levels. As illustrated (Figure 2.2A), both α -GalCer **1** and dansyl α -GalCer **2** stimulated release of IL-2 in a dose-dependent manner, with α -GalCer **1** driving a higher level of this cytokine. This observation is consistent with literature precedent for weaker cytokine profiles generated by similar C6''-substituted α -GalCer derivatives, but is nonetheless indicative that dansyl α -GalCer **2** is indeed capable of *i*NKT cell activation.^{11,15} The ability of dansyl α -GalCer **2** to stimulate *i*NKT cells *in vivo* was assessed by injecting mice with 200 ng of dansyl α -GalCer **2** or α -GalCer **1**, and then determining the expression of the maturation marker CD86 on the surface of splenic B cells and DCs by flow cytometry 20 h later. As shown, administration of fluorescent analogue **2** stimulated the maturation of B cells and DCs (Figure 2.2B) with activities resembling that of the parent compound **1**. To confirm that this *in vivo* activity was mediated by *i*NKT cells, the experiment was repeated in CD1d deficient

mice. Here, no up-regulation of CD86 was observed on DCs (Figure 2.2C), confirming that dansyl α -GalCer **2**, like α -GalCer **1**, activates APCs by stimulating *i*NKT cells in a CD1d dependent manner.

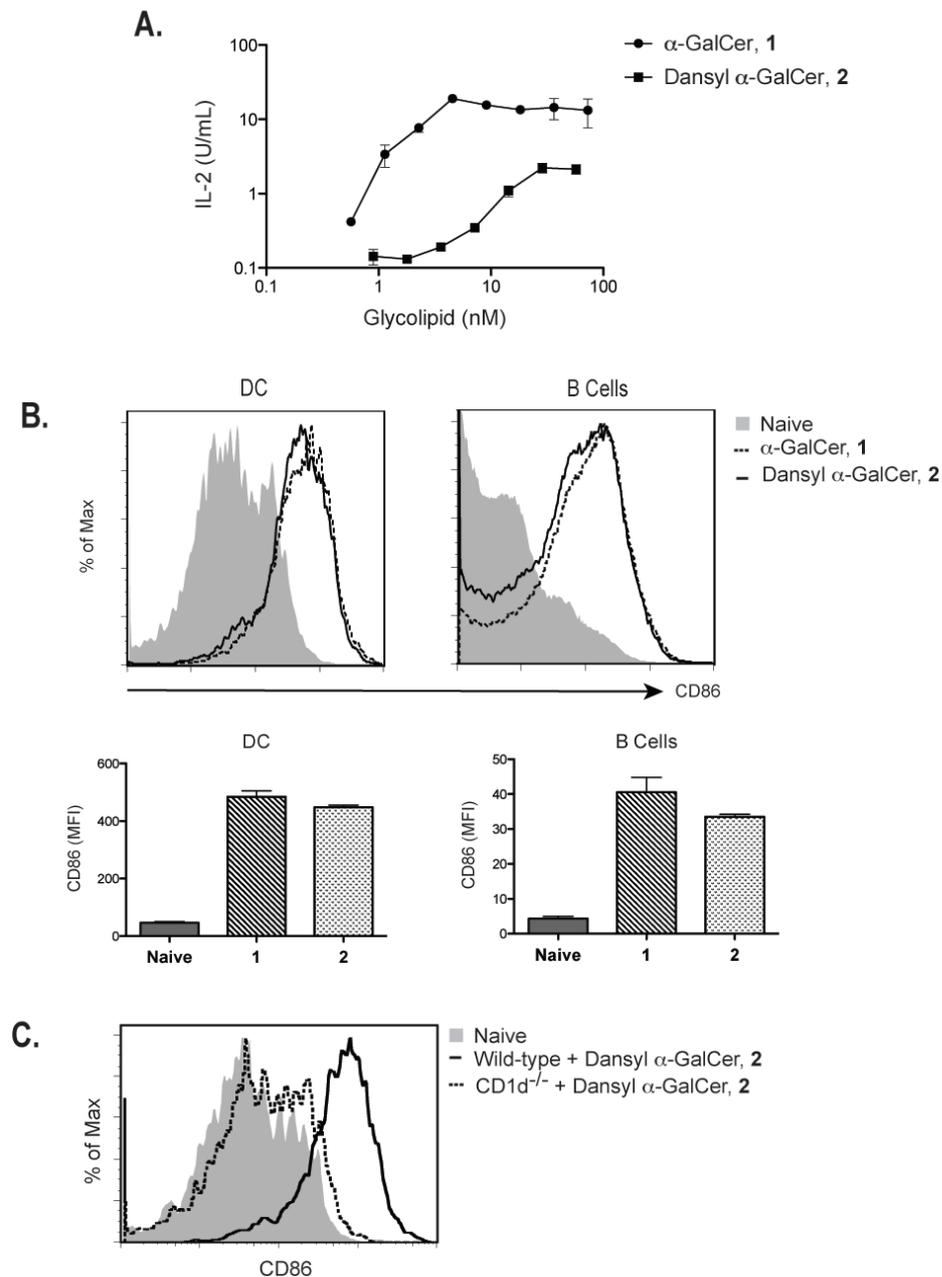


Figure 2.2. Dansyl α -GalCer 2 stimulates *i*NKT cells *in vitro* and *in vivo*. (A). Production of IL-2 by *i*NKT cells (DN32.D3) in the presence of titrated doses of α -GalCer 1 or dansyl α -GalCer 2; (B) Dansyl α -GalCer 2 drives the maturation of splenic DCs and B cells *in vivo*. C57BL/6 mice were injected intravenously with 200 ng of α -GalCer 1 ($n=3$) or dansyl α -GalCer 2 ($n=3$), and spleens were removed 20 h after injection for antibody labelling and flow cytometry. The expression of CD86 on CD11c⁺ dendritic cells and B220 B cells were assessed; (C) 200 ng of dansyl α -GalCer 2 was injected intravenously into wild type (C57BL/6) and CD1d^{-/-} mice, and spleens were removed 20 h after injection for antibody labelling and flow cytometry. The spleen of an untreated wild type (C57BL/6) mouse was used as control. The expression of CD86 on CD11c⁺ dendritic cells was assessed. Mean fluorescence intensities (MFI) \pm SD are presented.

2.4 Uptake of α -GalCer by dendritic cells

Having established that dansyl α -GalCer **2** binds to CD1d and activates *i*NKT cells in a manner similar to that of α -GalCer **1**, the ability of dansyl α -GalCer **2** to function as a fluorescent reporter group was investigated. This has previously not been reported. The fluorescence quantum yield of dansyl α -GalCer **2** (ϕ_f) was found to be 0.22 as determined via a relative method against the standard, quinine sulfate ($\phi_f = 0.54$ in 1M NaOH). The wavelengths of maximum absorbance and emission of **2** were determined by spectrofluorimetry to be 343 nm and 510 nm, respectively, and these maxima were used in further analyses. The DC cell line, DC2114 was incubated with 4 μ g of dansyl α -GalCer **2** for 24 h and uptake of the fluorescent glycolipid was analysed by flow cytometry (Figure 3). A distinct shift in mean fluorescence intensity was observed for cells treated with dansyl α -GalCer **2**, but not for those treated with α -GalCer **1** or controls (untreated and vehicle), illustrating the ability of dansyl α -GalCer **2** to be used as a fluorescent probe to detect sub-populations of DCs that present the glycolipid.

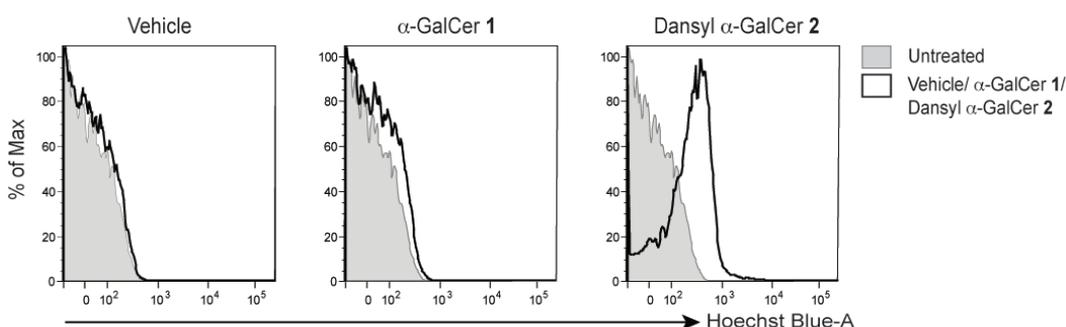


Figure 2.3. Cells that have taken up dansyl α -GalCer **2** can be detected by flow cytometry. 1×10^6 cells (DC2114 cell line) were incubated for 24 h with 4 μ g of dansyl α -GalCer **2**, α -GalCer **1** or vehicle only. Cells were excited with the UV laser, and detected by the Hoechst Blue-A detector.

2.5 Visualisation of dansyl α -GalCer by fluorescence microscopy

Following the successful detection of dendritic cells that have taken up the fluorescent glycolipid by flow cytometry, the next objective was to visualise the location of glycolipid when incubated with dendritic cells. Initially, the DC2114 cell line was incubated at a concentration similar to that used for flow cytometry (1×10^6 cells incubated with 4 μg of dansyl α -GalCer **2**). Unfortunately, the fluorescence could not be detected, despite increasing the concentration of glycolipid by two-fold. In addition, two other cell types, bone marrow derived dendritic cells (BMDCs) and A20, a murine B lymphoma cell line used in *in vivo* vaccination, were also incubated with dansyl α -GalCer **2** but again the fluorescence was no different when compared to the negative control containing cells only. When glycolipid concentrations that well exceed what would be relevant in *in vitro* and *in vivo* experiments were used, slight fluorescence was observed, but this rapidly disappeared due to quenching of the dye. Similarly, the probe was also quenched too rapidly when cells that have taken up the glycolipid was visualised under a confocal microscope. Efforts to amplify the fluorescence of dansyl α -GalCer **2** by using the secondary antibody, anti-dansyl antibody was also met with little success. Unfortunately, while dansyl α -GalCer **2** behaved in a very similar manner to the parent glycolipid α -GalCer **1**, it could not be visualised at the concentrations typically used in an *in vivo* setting.

2.6 Conclusion

In summary, an efficient synthesis of the biologically important probe, dansyl α -GalCer **2**. *En route* to the synthesis of this glycolipid, a highly efficient synthesis of the phytosphingosine lipid acceptor was developed. In addition, it was found that the early incorporation of the fluorescent group greatly aids in the monitoring and purification of reaction products. The ability of dansyl α -GalCer **2** to activate *i*NKT cells and to be used as a fluorescent probe for the analysis of glycolipid uptake by DCs has also been illustrated. Unfortunately, the fluorophore was too weak and underwent photobleaching when subjected to the UV laser of the fluorescent microscope.

Given the importance of α -GalCer **1**, it is anticipated that similar derivatives with a fluorophore attached on the 6''-position will find wide application as a molecular tool to better understand the trafficking of α -GalCer and glycolipid presentation by CD1d. Extension of this work is presented in Chapter 3.

2.7 Experimental

2.7.1 General procedure

Unless otherwise stated all reactions were performed under N₂. Prior to use, THF (Pancreac) was distilled from LiAlH₄, pyridine was dried over 4Å molecular sieves (4Å MS), acetone (Pure Science) was dried over 3Å MS, DCM (Pancreac) was distilled from P₂O₅, TMSI (Aldrich) was distilled from antimony powder (M&B) and stored over Cu(s) powder (Hopkin & Williams), and H₂O and benzene (Fisher Scientific) were distilled. Trityl chloride (Acros), CuSO₄ (Fluka), NaIO₄ (M&B), anhydrous Et₂O (Pancreac), *n*-BuLi (Aldrich), 1-bromotridecane (Aldrich), PPh₃ (Merck), Pd/C (Aldrich, 10 wt%), Tf₂O (Aldrich), NaN₃ (BDH), anhydrous DMF (Acros), TFA (Aldrich), triethylsilane (Fluka), Me₂C(OMe)₂ (Aldrich), ZnCl₂ (Aldrich), H₂SO₄ (Lab-Scan), I₂ (BDH), imidazole (Aldrich), AcCl (B&M), BnBr (Fluka), NaH (Avocado Research Chemicals, 60% dispersion in mineral oil), PMe₃ (Aldrich, 1M in THF), dansyl chloride (Acros), AcOH (Ajax Finechem), Ac₂O (Peking Reagent), TBAI (Riedel-de Haen), DiPEA (Aldrich), NaOMe (Janssen Chimica), C₂₅H₅₁COOH (Acros), EDCI (Aldrich), DMAP (Merck), Pd(OH)₂/C (Aldrich, 20 wt%), EtOAc (Pancreac), hexanes (Fisher Scientific), petroleum ether (Pure Science), MeOH (Pure Science), CHCl₃ (Pancreac), EtOH (absolute, Pure Science), NaOH (Pure Science), NaHCO₃ (Pure Science), NaCl (Pancreac) were used as received. All solvents were removed by evaporation under reduced pressure. Reactions were monitored by TLC-analysis on Macherey-Nagel silica gel coated plastic sheets (0.20 mm, with fluorescent indicator UV₂₅₄) with detection by UV-absorption (short wave UV – 254 nm; long wave UV – 366 nm), by dipping in 10% H₂SO₄ in EtOH followed by charring at ~150 °C, by dipping in I₂ in silica, or by dipping into a solution of ninhydrin in EtOH followed by charring at ~150 °C. Column chromatography was performed on Pure Science silica gel (40-63 micron). AccuBOND II ODS-C18 (Agilent) was used for reverse phase chromatography. High-resolution mass spectra were recorded on a Waters Q-TOF Premier™ Tandem Mass Spectrometer using positive electro-spray ionisation. Optical rotations were recorded using a Perkin-Elmer 241 polarimeter or Autopol II (Rudolph Research Analytical) at 589 nm

(sodium D-line). Infrared spectra were recorded as thin films using a Bruker Tensor 27 FTIR spectrometer equipped with an Attenuated Total Reflectance (ATR) sampling accessory and are reported in wave numbers (cm^{-1}). Nuclear magnetic resonance spectra were recorded at 20 °C in D_2O or CDCl_3 using either a Varian INOVA operating at 500 MHz or Varian VNMRS operating at 600 MHz. Chemical shifts are given in ppm (δ) relative to TMS. NMR peak assignments were made using COSY, HSQC and HMBC 2D experiments. Melting points were obtained using the Gallenkamp Melting Point Apparatus. Spectrofluorimetry measurements were carried out on the Horiba Jobin-Yvon Fluorolog-3 spectrofluorometer using the three dimensional (3D) scanning mode. High-pressure liquid chromatography was performed on Waters 600 liquid chromatograph with a photodiode array detector (Waters 2996) and a C-18 column (Waters Xbridge 5 μm).

3,4-*O*-Isopropylidene-6-*O*-triphenylmethyl-D-galactopyranoside (40).

Trityl chloride (16.4 g, 58.8 mmol) was added to a solution of D-galactose (**39**) (10 g, 55.5 mmol) in pyridine (150 mL). The reaction mixture was stirred at 50 °C for 48 h and concentrated *in vacuo*. The resulting residue was purified by chromatography through a short silica column and the tritylated galactose was eluted with acetone and crystallised from EtOH to afford the tritylated galactose (23.4 g, 55.4 mmol, quant.) [Rf: 0.17 (EtOAc)] as a white powder. To a solution of tritylated galactose (15 g, 35.5 mmol) in anhydrous acetone (750 mL) was added copper sulfate (60 g, 380 mmol). After stirring the reaction mixture for 3 d at room temperature, carbon was then added and the solution filtered through celite. The filtrate was concentrated *in vacuo* and purified by flash column chromatography. Elution with 1:1 (v/v) hexanes/EtOAc afforded the title compound **40** (13.9 g, 30.1 mmol, 85%, 2 steps) as a clear oil. R_f: 0.65 (EtOAc); $[\alpha]_{\text{D}}^{20} = +18.0^\circ$ ($c = 1.0$, CHCl_3); IR (film) 3406, 3059, 3034, 2986, 2936, 1597, 1491, 1449, 1380, 1219, 1162, 1132, 1079, 1032, 909, 872, 814, 776, 732, 705 cm^{-1} ; ^1H NMR (500 MHz, CDCl_3) δ 7.48–7.46 (m, 6H, CPh₃, CH-*o*), 7.30 (t, $J_{\text{o,m}} = J_{\text{m,p}} = 7.4$ Hz, 6H, CPh₃, CH-*m*), 7.26–7.23 (m, 3H, CPh₃, CH-*p*), 5.23 (t, $J_{1,2} = J_{1,\text{OH}} = 3.7$ Hz, 1H, H-1 α), 4.34–4.29 (m, 3H, H-3, H-4, H-5), 3.87–3.85 (m, 1H, H-2), 3.02 (dd, $J_{6\text{a},6\text{b}} = 9.1$ Hz, $J_{6\text{a},5} = 6.4$ Hz, 1H, H-6a), 3.33 (dd, $J_{6\text{a},6\text{b}} = 9.1$ Hz,

$J_{6b,5} = 6.4$ Hz, 1H, H-6a), 3.02 (d, $J_{OH,1} = 3.6$ Hz, 1H, 1-OH), 2.45–2.44 (m, 1H, 2-OH), 1.44 (s, 3H, CH₃ *iPr*), 1.36 (s, 3H, CH₃ *iPr*); ¹³C NMR (125 MHz, CDCl₃) δ 143.9 (C-*i*, CPh₃), 128.8 (C-*o*, CPh₃), 127.8 (C-*m*, CPh₃), 127.0 (C-*p*, CPh₃), 109.5 (C_q *iPr*), 91.2 (C-1), 86.8 (C_q CPh₃), 75.4 (C-3), 72.9 (C-4), 69.1 (C-2), 67.9 (C-5), 62.8 (C-6), 27.4, 25.7 (2 x CH₃ *iPr*); HRMS(ESI) *m/z* calcd. for [C₂₈H₃₀O₆+Na]⁺: 485.1940, obsd.: 485.1949.

4-O-Formyl-2,3-O-isopropylidene-5-O-triphenylmethyl-D-lyxose (41). A solution of NaIO₄ (0.46 g, 2.14 mmol) in H₂O (4 mL) was added to **40** (0.66 g, 1.42 mmol) dissolved in 4 mL THF at 0 °C. After stirring at room temperature overnight, the reaction mixture was diluted with EtOAc (15 mL) and washed with water (20 mL), saturated NaHCO₃ solution (20 mL) and brine (20 mL), dried (MgSO₄), filtered and concentrated *in vacuo* to afford the title compound **41** (0.65 g, 1.42 mmol, quant.) as a clear oil, which was carried through without further purification. R_f: 0.48 (PE/EA, 1/1, v/v); IR (film) 3450, 3060, 3025, 2989, 2938, 2360, 2341, 2252, 1732, 1597, 1491, 1449, 1383, 1218, 1160, 1076, 987, 907, 764, 729, 701, 648 cm⁻¹; ¹H NMR (500 MHz, CDCl₃) δ 9.60 (d, $J_{1,2} = 2.4$ Hz, 1H, H-1), 8.00 (s, 1H, HCO₂), 7.44–7.41 (m, 6H, CPh₃, CH-*o*), 7.34–7.30 (m, 6H, CPh₃, CH-*m*), 7.27–7.24 (m, 3H, CPh₃, CH-*p*), 5.18 (q, $J_{3,4} = J_{4,5a} = J_{4,5b} = 4.9$ Hz, 1H, H-4), 4.74 (dd, $J_{2,3} = 7.7$ Hz, $J_{3,4} = 4.2$ Hz, 1H, H-3), 4.31 (dd, $J_{2,3} = 7.7$ Hz, $J_{1,2} = 2.4$ Hz, 1H, H-2), 3.40 (dd, $J_{5a,5b} = 9.8$ Hz, $J_{4,5a} = 5.6$ Hz, 1H, H-5a), 3.37 (dd, $J_{5a,5b} = 9.8$ Hz, $J_{4,5b} = 5.6$ Hz, 1H, H-5b), 1.56 (s, 3H, *iPr* CH₃), 1.42 (s, 3H, *iPr* CH₃); ¹³C NMR (125 MHz, CDCl₃) δ 201.1 (C-1), 159.7 (C-6), 128.6 (CH-*o*, CPh₃), 127.9 (CH-*m*, CPh₃), 127.2 (CH-*p*, CPh₃), 111.1 (C_q *iPr*), 87.2 (C_q CPh₃), 80.2 (C-2), 77.0 (C-3), 69.5 (C-4), 61.8 (C-5), 26.8, 25.1 (2 x CH₃ *iPr*); HRMS(ESI) *m/z* calcd. for [C₂₈H₂₈O₆+Na]⁺: 483.1784, obsd.: 483.1793.

3,4-O-Isopropylidene-1-O-triphenylmethyl-D-arabino-octadec-5-en-1,2,3,4-tetraol (42). A mixture of 1-bromotridecane (7.0 mL, 27.4 mmol) and triphenylphosphine (7.18 g, 27.4 mmol) was heated at 140 °C under N₂ atmosphere for 5 h. The reaction mixture was allowed to cool to room temperature and formed a solid gel. The gel was dissolved in boiling dry acetone (36 mL) and dry diethyl ether (87 mL) was added and the compound left to crystallise, which

greatly improved in subsequent batches via the use of seed crystals. The crystals were filtered under N₂ atmosphere to afford BrPh₃PC₁₃H₂₇ (11.1 g, 21.1 mol, 77%) as white needles. *n*-BuLi (6.6 mL, 13.2 mmol) was added to BrPh₃PC₁₃H₂₇ (7.0 g, 13.7 mmol) dissolved in distilled THF (130 mL) at -78 °C and stirred for 15 min. To the bright orange mixture, a solution of formate ester **41** (co-evaporated with dry toluene x 3) in THF (55 mL) was added. The reaction mixture was stirred at -78 °C for 1 h, then warmed to room temperature and stirred for a further 3.5 h before being quenched with NH₄Cl solution (150 mL), concentrated, and redissolved in ethyl acetate (100 mL). The organic layer was washed with saturated NaHCO₃ solution (100 mL), water (100 mL) and brine (100 mL), dried (MgSO₄), filtered and concentrated *in vacuo*. The resulting residue was purified by silica gel column chromatography and the desired product eluted with 8:1 (v/v) petroleum ether/EtOAc to afford alkene **42** as a clear oil (2.57 g, 4.29 mmol, 78%) in an approximate *E:Z*, 1:10. R_f: 0.33 (PE/EA, 5/1, v/v); [α]_D²⁰ = -22.2° (*c* = 1.0, CHCl₃); IR (film) 3559, 3058, 3023, 2924, 2854, 1598, 1491, 1449, 1380, 1214, 1162, 1073, 898, 880, 763, 746, 705 cm⁻¹; ¹H NMR (500 MHz, CDCl₃) δ 7.48–7.44 (m, 6H, CPh₃, CH-*o*), 7.33–7.29 (m, 6H, CPh₃, CH-*m*), 7.26–7.23 (m, 3H, CPh₃, CH-*p*), 5.59 (dd, *J*_{5,6} = 11.3 Hz, *J*_{6,7} = 6.0 Hz, 1H, H-6), 5.55 (dd, *J*_{5,6} = 11.3 Hz, *J*_{4,5} = 7.7 Hz, 1H, H-5), 4.92 (dd, *J*_{4,5} = 7.7 Hz, *J*_{3,4} = 6.9 Hz, 1H, H-4), 4.23 (dd, *J*_{3,4} = 6.9 Hz, *J*_{2,3} = 4.3 Hz, 1H, H-3), 3.77 (pent, *J*_{1,2} = *J*_{2,3} = *J*_{2,OH} = 5.4 Hz, 1H, H-2), 3.18 (dd, *J*_{1a,1b} = 9.4 Hz, *J*_{1a,2} = 5.0 Hz, 1H, H-1a), 3.13 (dd, *J*_{1a,1b} = 9.4 Hz, *J*_{1b,2} = 6.4 Hz, 1H, H-1b), 2.03–1.98 (m, 1H, H-7a), 1.82–1.77 (m, 1H, H-7a), 1.51 (s, 3H, CH₃ *i*Pr), 1.41 (s, 3H, CH₃ *i*Pr), 1.33–1.23 (m, 20H, H-8–H-17), 0.90 (t, *J*_{17,18} = 7.0 Hz, 3H, H-18); ¹³C NMR (125 MHz, CDCl₃) δ 143.9 (C-*i*, CPh₃), 135.4 (C-6), 128.7 (CH-*o*, CPh₃), 127.8 (CH-*m*, CPh₃), 127.0 (CH-*p*, CPh₃), 125.0 (C-5), 108.4 (C_q *i*Pr), 86.7 (C_q CPh₃), 77.5 (C-3), 73.0 (C-4), 69.2 (C-2), 65.0 (C-1), 31.9, 29.70, 29.67, 29.62, 29.52, 29.50, 29.4, 29.3, 27.6, 22.7 (C-7–C-17), 27.4, 25.1 (2 x CH₃ *i*Pr), 14.2 (C-18); HRMS(ESI) *m/z* calcd. for [C₄₀H₅₄O₄+Na]⁺: 621.3920, obsd.: 621.3926.

2-Azido-3,4-*O*-isopropylidene-1-*O*-triphenylmethyl-D-*arabino*-octadecane-1,3,4-triol (43). Pd/C (1.5 wt %) was added to a solution of **42** (2.52 g, 4.21 mmol) in distilled THF (42 mL) and stirred under H₂ (g) overnight at room temperature. The reaction mixture was then filtered through celite and concentrated to afford 3,4-*O*-Isopropylidene-1-*O*-triphenylmethyl-D-*arabino*-octadecane-1,2,3,4-tetraol as a clear oil (2.53 g, 4.21 mmol, quant.), which was used without further purification. *R*_f: 0.44 (PE/EA, 5/1, v/v); [α]_D²⁰ = -4.0° (*c* = 1.0, CHCl₃); IR (film) 3560, 3060, 3023, 2923, 2853, 1598, 1491, 1449, 1379, 1216, 1073, 899, 872, 762, 746, 705 cm⁻¹; ¹H NMR (500 MHz, CDCl₃) δ 7.47 (d, *J*_{o,m} = 7.7 Hz, 6H, CPh₃, CH-*o*), 7.31 (t, *J*_{o,m} = *J*_{m,p} = 7.7 Hz, 6H, CPh₃, CH-*m*), 7.27–7.23 (m, 3H, CPh₃, CH-*p*), 4.15 (dd, *J*_{2,3} = 6.0 Hz, *J*_{3,4} = 3.8 Hz, 1H, H-3), 4.11–4.07 (m, 1H, H-4), 3.74 (dt, *J*_{1,2} = 9.5 Hz, *J*_{2,3} = 6.0 Hz, 1H, H-2), 3.24–3.19 (m, 2H, H-1a, H-1b), 2.34 (d, *J*_{OH,2} = 5.9 Hz, 1H, OH), 1.71–1.64 (m, 2H, H-5), 1.51–1.28 (m, 24H, H-6–H-17), 1.46 (s, 3H, *i*Pr CH₃), 1.37 (s, 3H, *i*Pr CH₃), 0.89 (t, *J*_{17,18} = 6.9 Hz, 3H, H-18); ¹³C NMR (125 MHz, CDCl₃) δ 143.9 (C-*i*, CPh₃), 128.7 (CH-*o*, CPh₃), 127.8 (CH-*m*, CPh₃), 127.1 (CH-*p*, CPh₃), 107.7 (C_q *i*Pr), 86.8 (C_q CPh₃), 77.5 (C-4), 77.0 (C-3), 69.0 (C-2), 65.2 (C-1), 31.9, 29.72, 29.68, 29.63, 29.62, 29.57, 29.39, 26.8, 22.7 (C-5–C-17), 27.4, 25.2 (2 x CH₃ *i*Pr), 14.2 (C-18); HRMS(ESI) *m/z* calcd. for [C₄₀H₅₆O₄+Na]⁺: 623.4076, obsd.: 623.4080. 3,4-*O*-Isopropylidene-1-*O*-triphenylmethyl-D-*arabino*-octadecane-1,2,3,4-tetraol (1.59 g, 2.65 mmol) was then co-evaporated with dry toluene (x 3), dissolved in a mixture of dry DCM (8 mL) and distilled pyridine (0.54 mL) and cooled to -15 °C. Triflic anhydride (0.67 mL, 3.97 mmol) was added dropwise over 5 mins and stirred at -15 °C for 45 mins to afford the triflate intermediate [*R*_f: 0.56 (PE/EA, 5/1, v/v)]. Dry DMF (27 mL) was cooled to -15 °C and added to the reaction mixture, followed by the addition of sodium azide (0.69 g, 10.61 mmol). The reaction mixture was then warmed to room temperature, stirred overnight, and quenched by adding ice water (120 mL). The desired product was extracted with EtOAc (2 x 150 mL) and the combined organic layers washed with saturated NaHCO₃ solution (200 mL), water (200 mL) and brine (200 mL), dried (MgSO₄), filtered and concentrated *in vacuo*. The residue was purified by silica gel flash column chromatography and eluted with 1.5% EtOAc/hexanes to afford the title compound **43** (1.35 g, 2.16 mmol, 81%) as an amorphous white solid. *R*_f: 0.61

(PE/EA, 5/1, v/v); $[\alpha]_{\text{D}}^{25} = +7.0^{\circ}$ ($c = 1.0$, CHCl_3), Lit.⁵⁸ $[\alpha]_{\text{D}}^{26} = +9.0^{\circ}$ ($c = 1.0$, CHCl_3); IR (film) 3060, 2923, 2853, 2097, 1598, 1491, 1379, 1370, 1246, 1219, 1156, 1073, 1034, 900, 869, 762, 744, 703 cm^{-1} ; ^1H NMR (500 MHz, CDCl_3) δ 7.51 (d, $J_{\text{o,m}} = 7.3$ Hz, 6H, CPh_3 , CH-*o*), 7.32 (t, $J_{\text{o,m}} = J_{\text{m,p}} = 7.6$ Hz, 6H, CPh_3 , CH-*m*), 7.26 (d, $J_{\text{o,m}} = 7.1$ Hz, 3H, CPh_3 , CH-*p*), 4.13–4.09 (m, 1H, H-4), 3.89 (dd, $J_{2,3} = 9.5$ Hz, $J_{3,4} = 5.5$ Hz, 1H, H-3), 3.55 (dd, $J_{1\text{a},1\text{b}} = 9.9$ Hz, $J_{1\text{a},2} = 2.5$ Hz, 1H, H-1a), 3.50 (dt, $J_{2,3} = 9.5$ Hz, $J_{1\text{a},2} = 2.5$ Hz, 1H, H-2), 3.37 (dd, $J_{1\text{a},1\text{b}} = 9.9$ Hz, $J_{1\text{b},2} = 7.8$ Hz, 1H, H-1b), 1.59–1.28 (m, 26H, H-5–H-17), 1.27 (s, 3H, *iPr* CH_3), 1.24 (s, 3H, *iPr* CH_3), 0.90 (t, $J_{17,18} = 6.9$ Hz, 3H, H-18); ^{13}C NMR (125 MHz, CDCl_3) δ 143.8 (C-*i*, CPh_3), 128.7 (CH-*o*, CPh_3), 127.9 (CH-*m*, CPh_3), 127.1 (CH-*p*, CPh_3), 108.1 (C_q CPh_3), 87.3 (C_q *iPr*), 77.9 (C-4), 75.9 (C-3), 64.6 (C-1), 60.8 (C-2), 32.0, 29.73, 29.69, 29.66, 29.64, 29.57, 29.4, 29.3, 26.5, 22.7 (C-5–C-17), 28.0, 25.7 (2 x CH_3 *iPr*), 14.2 (C-18); HRMS(ESI) m/z calcd. for $[\text{C}_{40}\text{H}_{55}\text{N}_3\text{O}_3+\text{Na}]^+$: 648.4141, obsd.: 648.4145.

2-Azido-3,4-*O*-isopropylidene-D-ribo-octadecane-1,3,4-triol (38). Triethylsilane (91.8 μL , 0.58 mmol) was added to a stirred solution of azide **43** (181 mg, 0.289 mmol) (co-evaporated with dry toluene x 3) in dry DCM (5 mL) at 0 $^{\circ}\text{C}$. Trifluoroacetic acid (TFA) (90 μL , 1.17 mmol) was added very slowly, allowing the reaction mixture to return to a colourless solution before adding the next drop. After stirring for 2 min, 2,2-dimethoxypropane (7 μL , 0.057 mmol) was added and the reaction mixture stirred for a further 1 min before being quenched with saturated NaHCO_3 solution (50 mL). The product was extracted with DCM (2 x 50 mL) and the combined organic layers washed with water (80 mL) and brine (80 mL), dried (MgSO_4), filtered and concentrated *in vacuo*. The resulting residue was purified by silica gel flash column chromatography and eluted with 5:1 (v/v) petroleum ether/EtOAc to afford the title compound **38** (69.3 mg, 0.18 mmol, 63%) as an amorphous white solid. R_f : 0.21 (PE/EA, 5/1, v/v); $[\alpha]_{\text{D}}^{20} = +24.0^{\circ}$ ($c = 1.0$, CHCl_3); IR (film) 3426, 2923, 2853, 2098, 1465, 1370, 1247, 1220, 1170, 1063, 909, 869, 734 cm^{-1} ; ^1H NMR (500 MHz, CDCl_3) δ 4.21–4.17 (m, $J_{3,4} = 5.7$ Hz, H-4), 4.0 (ddd, $J_{1\text{a},1\text{b}} = 11.8$ Hz, $J_{1\text{a},\text{OH}} = 5.1$ Hz, $J_{1\text{a},2} = 4.4$ Hz, 1H, H-1a), 3.97 (dd, $J_{2,3} = 9.6$ Hz, $J_{3,4} = 5.7$ Hz, 1H, H-3), 3.87 (ddd, $J_{1\text{a},1\text{b}} = 11.8$ Hz, $J_{1\text{b},\text{OH}} = 6.6$ Hz, $J_{1\text{b},2} = 5.6$ Hz, 1H, H-1b), 3.48 (ddd, $J_{2,3} = 9.6$ Hz, $J_{1\text{b},2} = 5.6$ Hz, $J_{1\text{a},2} = 4.4$

Hz, 1H, H-2), 2.20 (dd, $J_{1b,OH} = 6.6$ Hz, $J_{1a,OH} = 5.1$ Hz, 1H, OH), 1.63–1.55 (m, 3H, H-5a, H-5b, H-6a), 1.43 (s, 3H, *i*Pr CH₃), 1.34 (s, 3H, *i*Pr CH₃), 1.41–1.26 (m, 23H, H-6b, H-7–H-17), 0.88 (t, $J_{17,18} = 7.0$ Hz, 3H, H-18); ¹³C NMR (125 MHz, CDCl₃) δ 108.4 (C_q *i*Pr), 77.7 (C-4), 76.7 (C-3), 63.9 (C-1), 61.2 (C-2), 31.9, 29.70, 29.69, 29.68, 29.66, 29.65, 29.60, 29.58, 29.53, 29.39, 29.36, 26.52, 22.7 (C-5–C-17), 28.0, 25.5 (2 x CH₃ *i*Pr), 14.1 (C-18); HRMS(ESI) *m/z* calcd. for [C₂₁H₄₁N₃O₃+Na]⁺: 406.3046, obsd.: 406.3049.

Methyl 6-deoxy-6-iodo- α -D-galactopyranoside (44). To a mixture of 1,2:3,4-di-*O*-isopropylidene- α -D-galactopyranose^{59,60} (2.6 g, 10 mmol), PPh₃ (3.93 g, 15 mmol) and imidazole (1.36 g, 20 mmol) in dry THF (100 mL), was added iodine (3.81 g, 15 mmol) in small portions. After refluxing for 1 h, the reaction mixture was cooled to room temperature, and quenched by the addition of 10% aq. Na₂S₂O₄. The product was extracted with EtOAc, and the combined organic layers were washed with brine, dried (MgSO₄), filtered and concentrated. Distillation of the residue gave 6-deoxy-6-iodo-1,2:3,4-di-*O*-isopropylidene- α -D-galactopyranose as a yellow oil (3.14 g, 8.48 mmol, 85%). AcCl (1.8 mL) was added dropwise to a mixture of 6-deoxy-6-iodo-1,2:3,4-di-*O*-isopropylidene- α -D-galactopyranose (3.14 g, 8.48 mmol) in MeOH (60 mL). After stirring for 2 d, the mixture was concentrated *in vacuo* and the residue crystallised from MeOH, to give the title compound **44** (2.37 g, 7.80 mmol, 92%) as white crystals. Mp 175.0–175.6 °C, Lit.⁵⁷ 162 °C; R_f = 0.43 (MeOH/EtOAc, 1/9, v/v); [α]_D²¹ = +129.0° (*c* = 1.0, H₂O), [α]_D²¹ = +122.1° (*c* = 1.0, MeOH), Lit.⁵⁷ [α]_D²² = +142.0° (*c* = 1.0, H₂O); IR (film) 3354, 3227, 3007, 2953, 2935, 2906, 2836, 1454, 1417, 1360, 1347, 1297, 1257, 1200, 1145, 1132, 1102, 1078, 1026, 999, 935, 886, 854, 790, 721, 691, 657 cm⁻¹; ¹H NMR (500 MHz, D₂O) δ 4.69 (d, $J_{1,2} = 3.6$ Hz, 1H, H-1), 3.97 (dd, $J_{3,4} = 3.3$, $J_{4,5} = 1.2$ Hz, 1H, H-4), 3.90 (ddd, $J_{4,5} = 1.2$ Hz, $J_{5,6a} = 5.3$, $J_{5,6b} = 8.0$ Hz 1H, H-5), 3.75 (dd, $J_{1,2} = 3.6$, $J_{2,3} = 10.2$ Hz, 1H, H-2), 3.69 (dd, $J_{2,3} = 10.2$, $J_{3,4} = 3.3$ Hz, 1H, H-3), 3.46 (s, 3H, OMe), 3.37 (dd, $J_{5,6a} = 5.3$, $J_{6a,6b} = 10.2$ Hz, 1H, H-6a), 3.33 (dd, $J_{5,6b} = 8.0$, $J_{6a,6b} = 10.2$ Hz, 1H, H-6b); ¹³C NMR (125 MHz, D₂O) δ 100.2 (C-1), 71.4 (C-5), 70.4 (C-4), 69.9 (C-2), 68.4 (C-3), 54.6 (OMe), 2.3 (C-6). HRMS(ESI) *m/z* calcd. for [C₇H₁₃IO₅+Na]⁺: 326.9700, obsd.: 326.9709.

Methyl 6-azido-2,3,4-tri-*O*-benzyl-6-deoxy- α -D-galactopyranoside (45).

Sodium azide (4.28 g, 0.066 mmol) was added to iodide **44** (5.01 g, 0.016 mmol) dissolved in dry DMF (300 mL). After stirring overnight at room temperature, MeOH (150 mL) was added and the reaction mixture was concentrated *in vacuo*. The residue was purified by silica gel flash column chromatography and the product eluted with 8% MeOH/DCM. The residue was crystallised from methanol to afford methyl 6-azido-6-deoxy- α -D-galactopyranoside (2.96 g, 14 mmol, 82%) as white crystals. Mp 173.8–174.1 °C, Lit.⁶⁰ 172–173 °C; R_f = 0.43 (MeOH/EtOAc, 1/9, v/v); $[\alpha]_D^{23}$ = +144.0° (c = 1.0, H₂O), Lit.⁶¹ $[\alpha]_D^{22}$ = +154.0° (c = 0.8, H₂O); IR (film) 3371, 3238, 2934, 2091, 1642, 1459, 1349, 1297, 1245, 1137, 1107, 1080, 1029, 961, 791, 730, 698 cm⁻¹; ¹H NMR (500 MHz, CDCl₃) δ 4.73 (d, $J_{1,2}$ = 3.7 Hz, 1H, H-1), 3.90 (ddd, $J_{5,6a}$ = 8.8 Hz, $J_{5,6b}$ = 4.0 Hz, $J_{4,5}$ = 1.1 Hz, 1H, H-5), 3.79 (dd, $J_{3,4}$ = 3.3 Hz, $J_{4,5}$ = 1.1 Hz, 1H, H-4), 3.77 (dd, $J_{2,3}$ = 10.1 Hz, $J_{1,2}$ = 3.7 Hz, 1H, H-2), 3.72 (dd, $J_{2,3}$ = 10.1 Hz, $J_{3,4}$ = 3.3 Hz, 1H, H-3), 3.58 (dd, $J_{6a,6b}$ = 12.8 Hz, $J_{5,6a}$ = 8.8 Hz, 1H, H-6a), 3.44 (s, 3H, OCH₃), 3.27 (dd, $J_{6a,6b}$ = 12.8 Hz, $J_{5,6b}$ = 4.0 Hz, 1H, H-6b); ¹³C NMR (125 MHz, CDCl₃) δ 100.2 (C-1), 70.0, 69.83, 69.77 (C-3, C-4, C-5), 68.6 (C-2), 54.3 (OCH₃), 51.3 (C-6); HRMS(ESI) m/z calcd. for [C₇H₁₃N₃O₅+Na]⁺: 242.0753, obsd.: 242.0749. Methyl 6-azido-6-deoxy- α -D-galactopyranose (1.53 g, 6.98 mmol) was then co-evaporated from dry DMF (x 1), dissolved in dry DMF (150 mL) and cooled to 0 °C. Benzyl bromide (3.3 mL, 27.9 mmol) was added followed by sodium hydride (60% in oil suspension) (1.40 g, 34.9 mmol) and the reaction mixture allowed to warm to rt. After stirring for 2 h, the reaction was quenched with MeOH (20 mL) and concentrated *in vacuo*. The residue was taken up in EtOAc (100 mL) and the mixture was washed with water (100 mL), saturated NaHCO₃ solution (100 mL), water (100 mL) and brine (100 mL), dried (MgSO₄), filtered and concentrated *in vacuo*. The residue was purified by silica gel flash column chromatography and eluted with 20:1 (v/v) petroleum ether/EtOAc to afford the title compound **45** (3.37 g, 6.88 mmol, 99%) as a clear oil. R_f : 0.72 (PE/EA, 1/1, v/v); $[\alpha]_D^{20}$ = +4.6° (c = 1.0, CHCl₃); IR (film) 2937, 2098, 1453, 1351, 1124, 1094, 1045, 789, 735 cm⁻¹; ¹H NMR (500 MHz, CDCl₃) δ 7.44–7.31 (m, 15H, H_{arom}), 5.03 (d, $J_{a,b}$ = 11.4 Hz, 1H, CH-a, 4-*O*-Bn), 4.93 (d, $J_{a,b}$ = 11.7 Hz, 1H, CH-a, 3-*O*-Bn), 4.87 (d,

$J_{a,b} = 12.1$ Hz, 1H, CH-a, 2-*O*-Bn), 4.78 (d, $J_{a,b} = 11.7$ Hz, 1H, CH-b, 3-*O*-Bn), 4.72 (d, $J_{a,b} = 12.1$ Hz, 1H, CH-b, 2-*O*-Bn), 4.72 (d, $J_{1,2} = 3.7$ Hz, 1H, H-1), 5.03 (d, $J_{a,b} = 11.4$ Hz, 1H, CH-b, 4-*O*-Bn), 4.07 (dd, $J_{2,3} = 10.1$ Hz, $J_{1,2} = 3.7$ Hz, 1H, H-2), 3.96 (dd, $J_{2,3} = 10.1$ Hz, $J_{3,4} = 2.6$ Hz, 1H, H-3), 3.83 (dd, $J_{5,6a} = 8.3$ Hz, $J_{5,6b} = 4.7$ Hz, 1H, H-5), 3.80 (d, $J_{3,4} = 2.6$ Hz, 1H, H-4), 3.53 (dd, $J_{6a,6b} = 12.7$ Hz, $J_{5,6a} = 8.3$ Hz, 1H, H-6a), 3.43 (s, 3H, OCH₃), 2.94 (dd, $J_{6a,6b} = 12.7$ Hz, $J_{5,6b} = 4.7$ Hz, 1H, H-6b); ¹³C NMR (125 MHz, CDCl₃) δ 138.7 (C-*i*, 3-*O*-Bn), 138.4 (C-*i*, 2-*O*-Bn), 138.1 (C-*i*, 4-*O*-Bn), 128.52, 128.50, 128.4, 128.1, 128.0, 127.8, 127.7, 127.6 (8 x CH_{arom}), 98.8 (C-1), 79.0 (C-3), 76.3 (C-2), 75.3 (C-4), 74.6 (CH₂, 4-*O*-Bn), 73.70, 73.65 (2 x CH₂, 2-*O*-Bn, 3-*O*-Bn), 69.8 (C-5), 55.5 (OCH₃), 51.5 (C-6); HRMS(ESI) *m/z* calcd. for [C₂₈H₃₁N₃O₅+Na]⁺: 512.2161, obsd.: 512.2159.

Methyl 2,3,4-tri-*O*-benzyl-6-deoxy-6-[*N*-(5-[dimethylamino]naphth-1-ylsulfonyl)amido]- α -D-galactopyranoside (46). Trimethylphosphine (1M in THF) (10.2 mL, 10.2 mmol) was added to a solution of **45** (1.0 g, 2.04 mmol) (co-evaporated with toluene x 3) in distilled THF (10 mL) at 0 °C. After stirring for 15 min at 0 °C and 45 min at rt, the reaction mixture was cooled to 0 °C and dansyl chloride (1.38 g, 5.11 mmol) was added followed by the addition of NaOH (1M, 8.17 mL, 8.17 mmol) over 10 min. The reaction was then allowed to warm to rt and after 6 h, further dansyl chloride (0.83 g, 3.06 mmol) and NaOH (1M, 2.04 mL, 2.04 mmol) was added. After stirring for a further 16 h, the solution was diluted with EtOAc (50 mL), and the organic layer washed with water (50 mL) and brine (50 mL), dried (MgSO₄), filtered and concentrated *in vacuo*. The residue was purified by silica gel flash column chromatography and eluted with 1:1 (v/v) petroleum ether/EtOAc to afford the title compound **46** (1.32 g, 1.89 mmol, 93%) as a lime green foam. *R_f*: 0.61 (PE/EA, 1/1, v/v); Glows white on TLC ($\lambda = 254$ nm); $[\alpha]_D^{19} = +9.2^\circ$ (*c* = 1.0, CHCl₃); IR (film) 3293, 3063, 3030, 2988, 2349, 1588, 1574, 1497, 1326, 1200, 1143, 791, 735, 698 cm⁻¹; ¹H NMR (500 MHz, CDCl₃) δ 8.55 (d, $J_{CH-2,CH-3} = 8.8$ Hz, 1H, CH-2 Dan), 8.13 (d, $J_{CH-3,CH-4} = 7.4$ Hz, 1H, CH-4 Dan), 8.08 (d, $J_{CH-7,CH-8} = 8.7$ Hz, 1H, CH-8 Dan), 7.54 (dd, $J_{CH-7,CH-8} = 8.7$ Hz, $J_{CH-6,CH-7} = 7.7$ Hz, 1H, CH-7 Dan), 7.50 (d, $J_{CH-2,CH-3} = 8.8$ Hz, 1H, CH-8 Dan), 7.38–7.26 (m, 15H, H_{arom}), 7.20–7.17 (m, 3H, H_{arom}, CH-6

Dan), 4.84 (d, $J_{a,b} = 11.7$ Hz, 1H, CH-a, 4-*O*-Bn), 4.77 (d, $J_{a,b} = 12.0$ Hz, 1H, CH-a, 2-*O*-Bn), 4.76 (d, $J_{a,b} = 11.5$ Hz, 1H, CH-a, 3-*O*-Bn), 4.61 (d, $J_{a,b} = 12.0$ Hz, 2H, CH-b, 2-*O*-Bn, CH-b, 3-*O*-Bn), 4.47 (d, $J_{1,2} = 3.8$ Hz, 1H, H-1), 4.29 (dd, $J_{NH,6a} = 8.4$ Hz, $J_{NH,6b} = 4.6$ Hz, 1H, NH Dan), 3.86 (dd, $J_{1,2} = 3.8$ Hz, $J_{2,3} = 9.4$ Hz, 1H, H-2), 3.65 (dd, $J_{2,3} = 9.4$ Hz, $J_{3,4} = 2.7$ Hz, 1H, H-3), 3.64 (bs, 1H, H-4), 3.48 (t, $J_{5,6} = 6.6$ Hz, 1H, H-5), 3.23 (s, 3H, OCH₃), 2.99–2.77 (m, 2H, H-6a, H-6b), 2.88 (s, 6H, NMe₂); ¹³C NMR (125 MHz, CDCl₃) δ 138.5, 138.3 (2 x C-*i*, 2-*O*-Bn, 3-*O*-Bn), 137.9 (C-*i*, 4-*O*-Bn), 134.9 (C-4a Dan), 130.4 (C-2 Dan), 129.5, 128.9, 128.6, 128.5, 128.4, 128.3, 128.1, 127.8, 127.7, 127.6 (10 x CH_{arom}), 123.4 (C-3 Dan), 118.9 (C-8 Dan), 115.4 (C-6 Dan), 98.7 (C-1), 79.0 (C-3), 76.1 (C-2), 74.1 (CH₂, 4-*O*-Bn), 73.7 (C-4), 73.63, 73.59 (2 x CH₂, 2-*O*-Bn, 3-*O*-Bn), 68.5 (C-5), 55.4 (OCH₃), 45.5 (NMe₂), 43.3 (C-6); HRMS(ESI) *m/z* calcd. for [C₄₀H₄₄N₂O₇S+Na]⁺: 719.2756, obsd.: 719.2760.

1-*O*-Acetyl-2,3,4-tri-*O*-benzyl-6-deoxy-6-[*N*-(5-[dimethylamino]naphth-1-ylsulfonyl)acetimido]- α -D-galactopyranoside (47). Concentrated sulfuric acid (63.1 μ L, 1.18 mmol) was added dropwise to a solution of **46** (550 mg, 0.79 mmol) in acetic anhydride (2.4 mL, 25.3 mmol) and acetic acid (0.32 mL, 5.53 mmol) at 0 °C. After stirring at 0 °C for 1 h and at rt for 2 h, the solution was diluted with DCM (50 mL) and washed with water (50 mL), saturated NaHCO₃ solution (50 mL) and brine (100 mL), dried (MgSO₄), filtered and concentrated *in vacuo*. The residue was purified by silica gel flash column chromatography and eluted with 3:1 (v/v) petroleum ether/EtOAc to afford the title compound **47** (605 mg, 0.79 mmol) as a lime green foam. *R*_f: 0.66 (PE/EA, 1/1, v/v); Glows mauve on TLC ($\lambda = 254$ nm); $[\alpha]_D^{23} = +65.8^\circ$ ($c = 1.0$, CHCl₃); IR (film) 3064, 3030, 2940, 2872, 1750, 1705, 1454, 1342, 1229, 1135, 1102, 1051, 1009, 788, 739, 699 cm⁻¹; ¹H NMR (500 MHz, CDCl₃) δ 8.64 (bs, 1H, CH-2 Dan), 8.30 (d, $J_{CH-3,CH-4} = 7.4$ Hz, 1H, CH-4 Dan), 7.85 (bs, 1H, CH-8 Dan), 7.58–7.53 (m, 2H, CH-3, CH-7 Dan), 7.44–7.29 (m, 15H, H_{arom}), 7.23 (bs, 1H, CH-6 Dan), 6.35 (d, $J_{1,2} = 3.7$ Hz, 1H, H-1), 5.11 (d, $J_{a,b} = 11.6$ Hz, 1H, CH-a, 4-*O*-Bn), 4.91 (d, $J_{a,b} = 11.6$ Hz, 1H, CH-a, 3-*O*-Bn), 4.82 (d, $J_{a,b} = 11.6$ Hz, 1H, CH-b, 3-*O*-Bn), 4.72 (s, 2H, CH-a, CH-b, 2-*O*-Bn), 4.69 (d, $J_{a,b} = 11.6$ Hz, 1H, CH-b, 4-*O*-Bn), 4.29 (dd, $J_{5,6a} = 8.6$ Hz, $J_{5,6b} = 2.5$ Hz, 1H, H-5), 4.18 (dd, $J_{2,3} = 10.0$ Hz, $J_{1,2} = 3.7$ Hz, 1H, H-2), 4.10

(dd, $J_{6a,6b} = 15.2$ Hz, $J_{5,6} = 8.6$ Hz, 1H, H-6a), 4.07 (bd, $J_{3,4} = 2.6$ Hz, 1H, H-4), 3.96 (dd, $J_{2,3} = 10.0$ Hz, $J_{3,4} = 2.6$ Hz, 1H, H-3), 3.85 (dd, $J_{6a,6b} = 15.2$ Hz, $J_{5,6b} = 2.5$ Hz, 1H, H-6b), 2.93 (s, 6H, NMe₂), 2.18 (s, 3H, NAc), 2.15 (s, 3H, OAc); ¹³C NMR (125 MHz, CDCl₃) δ 170.9 (C=O NAc), 169.7 (C=O OAc), 138.4 (C-*i*, 3-*O*-Bn), 138.3 (C-*i*, 4-*O*-Bn), 137.9 (C-*i*, 2-*O*-Bn), 134.7 (C-4a Dan), 131.2 (C-2 Dan), 129.2 (C-1/C-8a Dan), 128.53, 128.50, 128.45, 128.42, 128.40, 128.38, 128.01, 127.94, 127.89, 127.86, 127.82, 127.74, 127.72, 127.61, 127.5 (15 x CH_{arom}), 123.5 (C-3 Dan), 115.5 (C-6 Dan), 90.2 (C-1), 78.9 (C-3), 75.2 (C-2), 74.9 (C-4), 74.6 (CH₂, 4-*O*-Bn), 73.5 (CH₂, 3-*O*-Bn), 73.4 (CH₂, 2-*O*-Bn), 72.1 (C-5), 47.7 (C-6), 45.5 (NMe₂), 24.7 (NAc), 21.2 (OAc), HRMS(ESI) *m/z* calcd. for [C₄₃H₄₆N₂O₉S+Na]⁺: 789.2822, obsd.: 789.2819.

2-Azido-3,4-*O*-isopropylidene-1-*O*-(2,3,4-tri-*O*-benzyl-6-deoxy-6-[*N*-(5-[dimethylamino]naphth-1-ylsulfonyl)acetimido]- α -D-galactopyranosyl)-D-ribo-octadecane-1,3,4-triol (36). Iodotrimethylsilane (65.3 μ L, 0.46 mmol) was added to a solution of **47** (237 mg, 0.31 mmol) (co-evaporated with toluene x 3) in dry DCM (2 mL) at 0 °C. After stirring for 1 h at 0 °C, the reaction mixture was concentrated *in vacuo*, azeotroped with toluene (x 3) and redissolved in dry benzene (3 mL). In a separate flask, molecular sieves (4Å, 50 mg), TBAI (342 mg, 0.93 mmol) and DiPEA (53.8 μ L, 0.31 mmol) were added to a solution of the lipid acceptor **38** (39.1 mg, 0.10 mmol) (co-evaporated from toluene x 3) in dry benzene (1 mL) at room temperature. After stirring the reaction mixture at 70 °C for 15 min, the glycosyl iodide was cannulated into the flask containing the lipid acceptor and stirred at 70 °C for 20 h. The reaction mixture was then diluted with EtOAc (10 mL), cooled to 0 °C and filtered. The filtrate was concentrated and the resulting residue purified by silica gel flash column chromatography. Elution with 7:1 (v/v) hexanes/EtOAc afforded the desired glycolipid **36** (103.9 mg, 0.095 mmol, 94%) as a lime green oil. *R*_f: 0.40 (PE/EA, 2/1, v/v); Glows mauve on TLC ($\lambda = 254$ nm); $[\alpha]_D^{24} = +40.9^\circ$ (*c* = 1.0, CHCl₃); IR (film) 3064, 3031, 2923, 2853, 2789, 2099, 1704, 1572, 1455, 1347, 1231, 1147, 1096, 1045, 787, 735, 697 cm⁻¹; ¹H NMR (500 MHz, CDCl₃) δ 8.58 (d, $J_{CH-2,CH-3} = 8.6$ Hz, 1H, CH-2 Dan), 8.29 (d, $J_{CH-3,CH-4} = 7.3$ Hz, 1H, CH-4 Dan), 7.75 (d, $J_{CH-7,CH-8} = 8.6$ Hz, 1H, CH-8 Dan), 7.53 (t, $J_{CH-2,CH-3} = J_{CH-7,CH-8} = 8.6$ Hz, 2H, CH-3, CH-7 Dan), 7.43–7.2.8

(m, 15H, H_{arom}), 7.19 (d, $J_{\text{CH-6,CH-7}} = 7.5$ Hz, 1H, CH-6 Dan), 5.11 (d, $J_{\text{a,b}} = 11.7$ Hz, 1H, CH-a, 4'-O-Bn), 5.00 (d, $J_{1,2} = 3.2$ Hz, 1H, H-1), 4.91 (d, $J_{\text{a,b}} = 11.7$ Hz, 1H, CH-a, 2'-O-Bn), 4.80 (d, $J_{\text{a,b}} = 12.0$ Hz, 1H, CH-a, 3'-O-Bn), 4.79 (d, $J_{\text{a,b}} = 11.7$ Hz, 1H, CH-b, 2'-O-Bn), 4.74 (d, $J_{\text{a,b}} = 12.0$ Hz, 1H, CH-b, 3'-O-Bn), 4.68 (d, $J_{\text{a,b}} = 11.7$ Hz, 1H, CH-b, 4'-O-Bn), 4.25–4.18 (m, 3H, H-1a, H-5', H-3), 4.15–4.05 (m, 4H, H-6'a, H-4, H-3', H-2'), 4.00 (bs, 1H, H-4'), 3.85 (dd, $J_{6\text{a}',6\text{b}' } = 14.8$ Hz, $J_{5,6\text{b}' } = 2.9$ Hz, 1H, H-6'b), 3.74 (dd, $J_{1\text{a},1\text{b}} = 10.8$ Hz, $J_{1\text{b},2} = 5.2$ Hz, 1H, H-1b), 3.41 (ddd, $J_{2,3} = 9.5$ Hz, $J_{1\text{a},2} = 5.2$ Hz, $J_{1\text{b},2} = 2.5$ Hz, 1H, H-2), 2.89 (s, 6H, NMe₂), 2.18 (s, 3H, NAc), 1.67–1.54 (m, 4H, H-5, H-6), 1.43 (s, 3H, CH₃ *i*Pr), 1.30 (s, 3H, CH₃ *i*Pr), 1.41–1.27 (m, 22H, H-7–H-17), 0.89 (t, $J_{17,18} = 7.0$ Hz, 3H, H-18); ¹³C NMR (125 MHz, CDCl₃) δ 170.8 (C=O NAc), 152.2 (C-5 Dan), 138.9 (C-*i*, 3'-O-Bn), 138.8 (C-*i*, 2'-O-Bn), 138.6 (C-*i*, 4'-O-Bn), 134.5 (C-4a Dan), 131.5 (C-2 Dan), 131.4 (C-4 Dan), 129.9, (C-1 Dan), 129.6 (C-8a Dan), 128.7 (C-7 Dan), 128.43, 128.40, 128.37, 128.2, 127.7, 127.6, 127.53, 127.52, 127.43 (9 x CH_{arom}), 123.1 (C-3 Dan), 117.8 (C-8 Dan), 115.2 (C-6 Dan), 108.1 (C_q *i*Pr), 98.5 (C-1'), 78.8 (C-2'), 77.8 (C-4), 76.5 (C-3'), 75.5 (C-4'), 75.0 (C-3), 74.5 (CH₂, 4'-O-Bn), 73.7 (CH₂, 2'-O-Bn), 72.7 (CH₂, 3'-O-Bn), 70.0 (C-5'), 69.2 (C-1), 59.3 (C-2), 47.8 (C-6'), 45.4 (NMe₂), 31.9, 29.71, 29.68, 29.67, 29.66, 29.64, 29.61, 29.4, 26.6, 22.7 (C-5–C-17), 28.3, 25.8 (2 x CH₃ *i*Pr), 24.8 (NAc), 14.1 (C-18); HRMS(ESI) *m/z* calcd. for [C₆₂H₈₃N₅O₁₀S+Na]⁺: 1112.5758, obsd.: 1112.5767.

2-Azido-3,4-O-isopropylidene-1-O-(2,3,4-tri-O-benzyl-6-deoxy-6-[N-(5-dimethylamino)naphth-1-ylsulfonyl)amido]-α-D-galactopyranosyl)-D-ribo-octadecane-1,3,4-triol (48). Glycolipid **36** (42.5 mg, 0.039 mmol) was dissolved in a mixture of methanol/DCM (3:1, v/v) and to this was added NaOMe until the solution reached pH 9.0. After stirring at room temperature for 3 d in the dark, while maintaining pH 9.0 via the addition of NaOMe. The solution was then diluted with EtOAc (15 mL), and washed with saturated NH₄Cl solution (20 mL), water (20 mL) and brine (20 mL). The organic layer was dried (MgSO₄), filtered and concentrated *in vacuo* to afford the title compound **48** (41 mg, quant.) as a lime green oil, which was used without further purification. R_f: 0.40 (PE/EA, 2/1, v/v); Glows white on TLC (λ = 254 nm); [α]_D²¹ = +10.5° (*c* = 1.0, CHCl₃); IR (film) 3309, 3031, 2923, 2853, 2360, 2099, 1574, 1454, 1328, 1220, 1145, 1094,

1047, 791, 734, 697 cm^{-1} ; ^1H NMR (500 MHz, CDCl_3) δ 8.55 (d, $J_{\text{CH-2,CH-3 Dan}} = 8.4$ Hz, 1H, CH-2 Dan), 8.17 (d, $J_{\text{CH-3,CH-4 Dan}} = 7.3$ Hz, 1H, CH-4 Dan), 8.09 (d, $J_{\text{CH-7,CH-8 Dan}} = 8.7$ Hz, 1H, CH-8 Dan), 7.56 (dd, $J_{\text{CH-7,CH-8 Dan}} = 8.7$ Hz, $J_{\text{CH-6,CH-7 Dan}} = 7.6$ Hz, 1H, CH-7 Dan), 7.53 (dd, $J_{\text{CH-2,CH-3 Dan}} = 8.4$ Hz, $J_{\text{CH-3,CH-4 Dan}} = 7.3$ Hz, 1H, CH-3 Dan), 7.38–7.26 (m, 13H, H_{arom}), 7.20–7.18 (m, 3H, H_{arom} , CH-6 Dan), 4.86 (d, $J_{\text{a,b}} = 11.8$ Hz, 1H, CH-a, 4'-O-Bn), 4.79 (d, $J_{\text{a,b}} = 12.0$ Hz, 1H, CH-a, 3'-O-Bn), 4.77 (d, $J_{1,2} = 3.6$ Hz, 1H, H-1'), 4.75 (d, $J_{\text{a,b}} = 11.9$ Hz, 1H, CH-a, 2-O-Bn), 4.66 (d, $J_{\text{a,b}} = 11.9$ Hz, 1H, CH-b, 2-O-Bn), 4.63 (d, $J_{\text{a,b}} = 12.0$ Hz, 1H, CH-b, 3-O-Bn), 4.53 (d, $J_{\text{a,b}} = 11.8$ Hz, 1H, CH-b, 4-O-Bn), 4.34 (dd, $J_{6'b, \text{NH}} = 8.3$ Hz, $J_{6'a, \text{NH}} = 4.5$ Hz, 1H, NH), 4.13 (p, $J_{4,5} = 4.2$ Hz, 1H, H-4), 4.04 (dd, $J_{2,3} = 9.8$ Hz, $J_{3,4} = 5.4$ Hz, 1H, H-3), 3.95 (dd, $J_{1a,1b} = 10.6$ Hz, $J_{1a,2} = 2.5$ Hz, 1H, H-1a), 3.91 (dd, $J_{2',3'} = 10.1$ Hz, $J_{1,2} = 3.6$ Hz, 1H, H-2'), 3.79 (dd, $J_{2',3'} = 10.1$ Hz, $J_{3',4'} = 2.7$ Hz, 1H, H-3'), 3.70 (bs, 1H, H-4'), 3.69–3.68 (m, 1H, H-5'), 3.61 (dd, $J_{1a,1b} = 10.6$ Hz, $J_{1b,2} = 6.4$ Hz, 1H, H-1b), 3.38 (ddd, $J_{2,3} = 9.8$ Hz, $J_{1b,2} = 6.4$ Hz, $J_{1a,2} = 2.5$ Hz, 1H, H-2), 2.92–2.81 (dd, $J_{5,6'a} = 7.1$ Hz, $J_{6'a, \text{NH}} = 4.5$ Hz, 1H, H-6'a), 2.83 (ddd, $J_{6'a, 6'b} = 13.6$ Hz, $J_{6'b, \text{NH}} = 8.3$ Hz, $J_{5,6'b} = 6.1$ Hz, 1H, H-6'b), 2.89 (s, 6H, NMe_2), 1.64–1.27 (m, 26H, H-5–H-17), 1.45 (s, 3H, CH_3 *iPr*), 1.31 (s, 3H, CH_3 *iPr*), 0.89 (t, $J_{17,18} = 7.0$ Hz, 3H, H-18); ^{13}C NMR (125 MHz, CDCl_3) δ 152.1 (C-5 Dan), 138.7, 138.6 (2 x C-*i*, 2'-O-Bn, 3'-O-Bn), 138.0 (C-*i*, 4'-O-Bn), 134.7 (C-4a Dan), 130.5 (C-2 Dan), 129.9 (C-1/C-8a Dan), 129.6 (C-4 Dan), 129.5 (C-1/C-8a Dan), 128.9, 128.6, 128.4 (3 x CH_{arom}) 128.30 (C-7 Dan), 128.25, 128.22, 127.64, 127.63, 127.54, 127.48 (6 x CH_{arom}), 123.1 (C-3 Dan), 118.8 (C-8 Dan), 115.2 (C-6 Dan), 108.3 (C_q *iPr*), 98.5 (C-1'), 78.6 (C-3'), 77.8 (C-4), 76.3 (C-2'), 75.2 (C-3), 74.1 (CH_2 , 4'-O-Bn), 73.8 (C-4'), 73.7 (CH_2 , 3'-O-Bn), 72.9 (CH_2 , 2'-O-Bn), 69.5 (C-1), 69.2 (C-5'), 59.5 (C-2), 45.4 (NMe_2), 43.3 (C-6'), 31.9, 29.72, 29.69, 29.68, 29.64, 29.61, 29.4, 29.3, 26.6, 22.7 (C-5–C-17), 28.3, 25.7 (2 x CH_3 *iPr*), 14.1 (C-18); HRMS(ESI) m/z calcd. for $[\text{C}_{60}\text{H}_{81}\text{N}_5\text{O}_9\text{S}+\text{Na}]^+$: 1070.5653, obsd.: 1070.5657.

1-*O*-(2,3,4-tri-*O*-benzyl-6-deoxy-6-[*N*-(5-[dimethylamino]naph-1-ylsulfonyl)amido]- α -D-galactopyranosyl)-2-hexacosanoylamido-3,4-*O*-isopropylidene-D-ribo-octadecane-1,3,4-triol (49). Trimethylphosphine (1M in THF) (0.17 mL, 0.17 mmol) was added to a solution of **48** (34.7 mg, 0.033 mmol) (co-evaporated with toluene x 3) in distilled THF (0.2 mL) at 0 °C. After stirring the reaction mixture for 15 min at 0 °C and 45 min at rt, the solution was cooled to 0 °C and 1M NaOH (aq) solution (0.33 mL, 0.33 mmol) added drop wise. The reaction mixture was then stirred at rt for 21 h, then diluted with EtOAc (20 mL), washed with water (2 x 20 mL) and brine (20 mL), dried (MgSO₄) and filtered. Concentration *in vacuo* afforded the amine [*R*_f: 0.11 (Toluene:EtOAc, 3/2, v/v)] as a green oil, which was used without further purification. A solution of hexacosanoic acid (33.5 mg, 0.084 mmol), EDCI (16.2 mg, 0.084 mmol), and DMAP (0.4 mg, 0.0034 mmol) in dry DCM (1 mL) was added to the amine dissolved in dry DCM (1 mL). After stirring at room temperature for 43 h, the reaction mixture was diluted with EtOAc (20 mL) and washed with saturated NaHCO₃ solution (20 mL), water (20 mL) and brine (20 mL), dried (MgSO₄) and filtered. The filtrate was concentrated *in vacuo* and the residue was purified by silica gel column chromatography. Elution with 5:1 (v/v) hexanes/EtOAc afforded the desired product **49** (31.9 mg, 0.023 mmol, 68%) as a lime green oil. *R*_f: 0.79 (Toluene/EtOAc, 3/2, v/v); Glows white on TLC ($\lambda = 254$ nm); [α]_D²² = +4.3° (*c* = 1.0, CHCl₃); IR (film) 2923, 2853, 2361, 1650, 1541, 1455, 1330, 1218, 1145, 1092, 1052, 791, 697 cm⁻¹; ¹H NMR (500 MHz, CDCl₃) δ 8.55 (d, *J*_{CH-2,CH-3} = 7.4 Hz, 1H, CH-2 Dan), 8.19 (bs, 1H, CH-4 Dan), 8.16 (d, *J*_{CH-7,CH-8} = 7.3 Hz, 1H, CH-8 Dan), 7.56 (t, *J*_{CH-6,CH-7} = *J*_{CH-7,CH-8} = 8.1 Hz, 1H, CH-7 Dan), 7.52 (t, *J*_{CH-2,CH-3} = *J*_{CH-3,CH-4} = 8.0 Hz, 1H, CH-3 Dan), 7.41–7.23 (m, 15H, H_{arom}), 7.20 (d, *J*_{CH-6,CH-7} = 7.1 Hz, 1H, CH-6 Dan), 5.89 (d, *J*_{NH,2} = 9.5 Hz, 1H, NH C₂₆), 4.92 (bs, 1H, NH Dan), 4.88 (d, *J*_{a,b} = 11.5 Hz, 1H, CH-a, 4''-*O*-Bn), 4.89 (d, *J*_{1,2} = 3.6 Hz, 1H, H-1''), 4.79 (d, *J*_{a,b} = 11.4 Hz, 1H, CH-a, 2''-*O*-Bn), 4.76 (d, *J*_{a,b} = 11.7 Hz, 1H, CH-a, 3''-*O*-Bn), 4.71 (d, *J*_{a,b} = 11.7 Hz, 1H, CH-b, 3''-*O*-Bn), 4.64 (d, *J*_{a,b} = 11.4 Hz, 1H, CH-b, 2''-*O*-Bn), 4.53 (d, *J*_{a,b} = 11.5 Hz, 1H, CH-b, 4''-*O*-Bn), 4.18–4.14 (m, 1H, H-2), 4.07 (t, *J*_{2,3} = *J*_{3,4} = 6.9 Hz, 1H, H-3), 4.04–4.01 (m, 1H, H-4), 3.96 (dd, *J*_{2'',3''} = 10.0 Hz, *J*_{1'',2''} = 3.6 Hz, 1H, H-2''), 3.85 (dd, *J*_{1a,1b} = 11.6 Hz, *J*_{1a,2} = 4.6 Hz, 1H, H-1a), 3.82 (bs, 1H, H-4''), 3.77 (dd, *J*_{2'',3''} = 10.0 Hz, *J*_{3'',4''} =

2.7 Hz, 1H, H-3''), 3.71 (t, $J_{5'',6''a} = 6.6$ Hz, 1H, H-5''), 3.59 (dd, $J_{1a,1b} = 11.6$ Hz, $J_{1b,2} = 2.6$ Hz, 1H, H-1b), 2.90 (s, 6H, NMe₂), 2.87–2.85 (m, 2H, H-6''a, H-6''b), 2.14 (dt, $J_{2'a,2'b} = 14.7$ Hz, $J_{2'a,3'c} = 7.5$ Hz, 1H, H-2'a), 2.06 (dt, $J_{2'a,2'b} = 14.7$ Hz, $J_{2'a,3'c} = 7.5$ Hz, 1H, H-2'b), 1.71 (bs, 2H, CH₂, H-5), 1.59 (m, 2H, H-3'), 1.47–1.43 (m, 68H, CH₂, H-6 – H-17, H-4'–H-25'), 1.43 (s, 3H, CH₃ *i*Pr), 1.32 (s, 3H, CH₃ *i*Pr), 0.89 (t, $J_{17,18} = J_{25',26'} = 7.0$ Hz, 6H, H-18, H-26'); ¹³C NMR (125 MHz, CDCl₃) δ 172.6 (C=O), 151.8 (C-5 Dan), 138.4 (C-*i*, 3''-O-Bn), 138.2 (C-*i*, 2''-O-Bn), 138.1 (C-*i*, 4''-O-Bn), 134.7 (C-4a/C-8a Dan), 130.3 (C-2 Dan), 129.8 (C-4a/C-8a Dan), 129.6 (C-8 Dan), 128.8, 128.72, 128.66, 128.60, 128.56, 128.5, 128.4, 128.15, 128.1, 128.0, 127.94, 127.88, 127.7, 127.6, 127.5 (15 x CH_{arom}), 128.21 (C-7 Dan), 123.3 (C-3 Dan), 119.1 (C-1 Dan), 115.2 (C-6 Dan), 107.8 (C_q *i*Pr), 99.3 (C-1''), 79.1 (C-3''), 77.7 (C-4), 76.6 (C-3), 76.5 (C-2''), 74.2 (CH₂, 4''-O-Bn), 73.7 (CH₂, 2''-O-Bn), 73.4 (C-4''), 73.0 (CH₂, 3''-O-Bn), 69.4 (C-1), 69.1 (C-5''), 49.1 (C-2), 45.5 (NMe₂), 43.3 (C-6''), 36.9 (C-2'), 31.95, 31.94, 31.6, 29.8, 29.73, 29.71, 29.70, 29.67, 29.65, 29.50, 29.47, 29.42, 29.39, 29.37, 28.83, 26.8, 22.7 (C-5–C-17, C-3'–C-25'), 27.8, 25.73 (2 x CH₃ *i*Pr), 25.72 (CH₂, C-3''), 14.1 (2 x CH₃, C-18, C-26'); HRMS(ESI) *m/z* calcd. for [C₈₆H₁₃₃N₃O₁₀S+Na]⁺: 1422.9609, obsd.: 1422.9615.

1-O-(2,3,4-tri-O-benzyl-6-deoxy-6-[N-(5-[dimethylamino]naph-1-ylsulfonyl)amido]-α-D-galactopyranosyl)-2-hexacosanoylamido-D-ribo-

octadecane-1,3,4-triol (50). Glycolipid **49** (31.9 mg, 0.023 mmol) was dissolved in a mixture of 8:4:1 (v/v/v) AcOH/H₂O/toluene and stirred at 50 °C for 1 d after which analysis by TLC revealed presence of starting material. The reaction mixture was concentrated *in vacuo* (to remove the acetone by product), then redissolved in the same mixture of solvents and stirred for a further day. This procedure was repeated until all starting material was converted to product as indicated by TLC analysis. The reaction mixture was then concentrated *in vacuo* and the residue was purified by silica gel flash column chromatography. Elution with 1:2 (v/v) hexanes/EtOAc afforded the diol **50** (23.0 mg, 0.017 mmol, 74%) as a lime green oil. *R_f*: 0.22 (PE/EA, 3/2, v/v); Glows white on TLC (λ = 254 nm); [α]_D²¹ = +13.7° (*c* = 1.0, CHCl₃); IR (film) 3357, 3032, 2922, 2852, 2360, 1643, 1536, 1498, 1312, 1143, 1092, 1050, 909, 789, 733, 697 cm⁻¹; ¹H NMR (500

MHz, CDCl₃) δ 8.54 (d, $J_{\text{CH-2,CH-3}} = 8.5$ Hz, 1H, CH-2 Dan), 8.10 (d, $J_{\text{CH-3,CH-4}} = 7.4$ Hz, 1H, CH-4 Dan), 8.07 (d, $J_{\text{CH-7,CH-8}} = 8.6$ Hz, 1H, CH-8 Dan), 7.56 (dd, $J_{\text{CH-7,CH-8}} = 8.6$ Hz, $J_{\text{CH-6,CH-7}} = 7.8$ Hz, 1H, CH-7 Dan), 7.51 (dd, $J_{\text{CH-2,CH-3}} = 8.5$ Hz, $J_{\text{CH-3,CH-4}} = 7.4$ Hz, 1H, CH-3 Dan), 7.41–7.27 (m, 13H, H_{arom}), 7.22–7.18 (m, 3H, H_{arom}, CH-6 Dan), 6.31 (d, $J_{\text{NH,2}} = 8.3$ Hz, 1H, NH C₂₆), 4.88 (d, $J_{\text{a,b}} = 11.7$ Hz, 1H, CH-a, 4''-O-Bn), 4.83 (d, $J_{\text{a,b}} = 11.7$ Hz, 1H, CH-a, 2''-O-Bn), 4.81 (d, $J_{\text{a,b}} = 11.2$ Hz, 1H, CH-a, 3''-O-Bn), 4.75 (d, $J_{\text{a,b}} = 11.2$ Hz, 1H, CH-b, 3'-O-Bn), 4.74 (d, $J_{1'',2''} = 3.7$ Hz, 1H, H-1''), 4.66 (d, $J_{\text{a,b}} = 11.7$ Hz, 1H, CH-b, 2''-O-Bn), 4.53 (d, $J_{\text{a,b}} = 11.7$ Hz, 1H, CH-b, 4''-O-Bn), 4.51–4.49 (m, 1H, NH Dan), 4.30 (dd, $J_{2,3} = 8.1$ Hz, $J_{1\text{a/b},2} = 3.7$ Hz, 1H, H-2), 3.95 (dd, $J_{2'',3''} = 10.0$ Hz, $J_{1'',2''} = 3.7$ Hz, 1H, H-2''), 3.87–3.84 (m, 3H, H-1a, H-4'', H-5''), 3.80 (dd, $J_{2'',3''} = 10.0$ Hz, $J_{3'',4''} = 2.4$ Hz, 1H, H-3''), 3.76 (dd, $J_{1\text{a},1\text{b}} = 10.5$ Hz, $J_{1\text{b},2} = 4.1$ Hz, 1H, H-1b), 3.65–3.64 (m, 1H, 3-OH), 3.54–3.51 (m, 2H, H-3, H-4), 2.89 (s, 6H, NMe₂), 2.83 (ddd, $J_{6''\text{a},6''\text{b}} = 14.1$ Hz, $J_{5,6''\text{a}} = 7.1$ Hz, $J_{6''\text{a},\text{NH}} = 4.4$ Hz, 1H, H-6''a), 2.77 (ddd, $J_{6''\text{a},6''\text{b}} = 14.1$ Hz, $J_{6''\text{b},\text{NH}} = 8.1$ Hz, $J_{5,6''\text{b}} = 6.1$ Hz, 1H, H-6''b), 2.56–2.55 (m, 1H, 4-OH), 2.25 (dt, $J_{2'\text{a},2'\text{b}} = 14.9$ Hz, $J_{2'\text{a},3'} = 7.5$ Hz, 1H, H-2'a), 2.19 (dt, $J_{2'\text{a},2'\text{b}} = 14.9$ Hz, $J_{2'\text{b},3'} = 7.5$ Hz, 1H, H-2'b), 1.67–1.15 (m, 74H, CH₂, H-5–H-17, H-3'–H-25'), 0.89 (t, $J_{17,18} = J_{25',26'} = 7.0$ Hz, 6H, H-18, H-26'); ¹³C NMR (125 MHz, CDCl₃) δ 173.5 (C=O), 152.0 (C-5 Dan), 138.2 (C-i, 3''-O-Bn), 137.9 (C-i, 4''-O-Bn), 137.8 (C-i, 2''-O-Bn), 134.1 (C-4a Dan), 130.6 (C-2 Dan), 129.9 (C-1 Dan), 129.6 (C-4 Dan), 129.5 (C-8a Dan), 128.9, 128.6, 128.54, 128.52, 128.2, 128.05, 128.00, 127.8, 127.5 (9 x CH_{arom}), 128.4 (C-7 Dan), 123.1 (C-3 Dan), 118.8 (C-8 Dan), 115.2 (C-6 Dan), 98.8 (C-1''), 79.4 (C-3''), 76.3 (C-3), 75.9 (C-2''), 74.2 (CH₂, 4''-O-Bn), 74.0 (CH₂, 2''-O-Bn), 73.3 (CH₂, 3''-O-Bn), 73.2 (C-4), 73.1 (C-4''), 69.6 (C-5''), 69.2 (C-1), 49.9 (C-2), 45.4 (NMe₂), 43.2 (C-6''), 36.8 (C-2'), 33.5 (C-5), 31.9, 29.73, 29.71, 29.68, 29.67, 29.6, 29.44, 29.38, 25.9, 25.7, 22.7 (C-6–C-17, C-3'–C-25'), 14.1 (2 x CH₃, C-18, C-26'); HRMS(ESI) *m/z* calcd. for [C₈₃H₁₂₉N₃O₁₀S+Na]⁺: 1382.9296, obsd.: 1382.9296.

1-O-[6-deoxy-6-[N-(5-[dimethylamino]naphth-1-ylsulfonyl)amido]- α -D-galactopyranosyl]-2-hexacosanoylamido-D-ribo-octadecane-1,3,4-triol (2).

Pd(OH)₂/C (2.5 mol %) was added to a solution of diol **50** (9.9 mg, 0.0073 mmol) in a mixture of CHCl₃/EtOH (1 mL, 3/2, v/v) and the reaction stirred under H₂ (g) for 17 h at rt. The reaction mixture was then filtered through Celite, the Celite washed thoroughly with CHCl₃/EtOH (3/2, v/v), and the filtrate concentrated *in vacuo*. The residue was purified by silica gel flash column chromatography (10% MeOH/DCM), followed by reverse phase chromatography (ODS-C18 resin, product eluted with MeOH), and finally silica gel column chromatography by elution with 5% MeOH/DCM. This afforded the target compound **2** (4.9 mg, 4.49 μ mol, 62%) as a yellow oil. R_f: 0.22 (DCM/MeOH, 92/8, v/v); Glows white on TLC ($\lambda = 366$ nm); $[\alpha]_D^{22} = +40.0^\circ$ ($c = 0.1$, pyridine); IR (film) 3320, 2918, 2851, 2359, 1653, 1634, 1560, 1457, 1310, 1144, 1030, 791, 684 cm⁻¹; UV $\lambda_{\text{max}}^{\text{Abs}} = 343$ nm, $\lambda_{\text{max}}^{\text{Em}} = 510$ nm; ¹H NMR (600 MHz, Pyridine-*d*₅) δ 9.04 (d, $J_{\text{CH-2,CH-3}} = 8.7$ Hz, 1H, CH-2 Dan), 8.61–8.57 (m, 2H, NH C₂₆, CH-4 Dan), 8.54 (d, $J_{\text{CH-7,CH-8}} = 8.6$ Hz, 1H, CH-8 Dan), 7.54 (dd, $J_{\text{CH-7,CH-8}} = 8.6$ Hz, $J_{\text{CH-6,CH-7}} = 7.4$ Hz, 1H, CH-7 Dan), 7.51 (dd, $J_{\text{CH-2,CH-3}} = 8.7$ Hz, $J_{\text{CH-3,CH-4}} = 7.6$ Hz, 1H, CH-3 Dan), 7.12 (d, $J_{\text{CH-6,CH-7}} = 7.4$ Hz, 1H, CH-6 Dan), 5.45 (d, $J_{1'',2''} = 3.9$ Hz, 1H, H-1''), 5.23 (m, 1H, H-2), 4.54 (dd, $J_{1a,1b} = 10.9$ Hz, $J_{1a,2} = 5.6$ Hz, 1H, H-1a), 4.52 (dd, $J_{2'',3''} = 9.9$ Hz, $J_{1'',2''} = 3.9$ Hz, 1H, H-2''), 4.51 (t, $J_{5,6a} = J_{5,6b} = 6.6$ Hz, 1H, H-5''), 4.35–4.30 (m, 3H, H-3, H-4, H-4''), 4.26 (dd, $J_{2'',3''} = 9.9$ Hz, $J_{3'',4''} = 3.3$ Hz, 1H, H-3''), 4.19 (dd, $J_{1a,1b} = 10.9$ Hz, $J_{1b,2} = 4.8$ Hz, 1H, H-1b), 3.85 (bs, 2H, H-6''a, H-6''b), 2.50 (dt, $J_{2'a,2'b} = 14.8$ Hz, $J_{2'a,3'} = 7.8$ Hz, 1H, H-2'a), 2.46 (dt, $J_{2'a,2'b} = 14.8$ Hz, $J_{2'b,3'} = 7.8$ Hz, 1H, H-2'b), 2.34–1.24 (m, 72H, CH₂, H-5–H-17, H-3'–H-25'), 0.86 (t, $J_{17,18} = J_{25',26'} = 7.0$ Hz, 6H, H-18, H-26'); ¹³C NMR (150 MHz, Pyridine-*d*₅) δ 173.7 (C=O), 152.4 (C-5 Dan), 137.8 (C-4a Dan), 130.8 (C-1 Dan), 130.6 (C-8a Dan), 130.3 (C-8 Dan), 129.3 (C-4 Dan), 128.5 (C-3 Dan), 123.5 (C-7 Dan), 120.7 (C-2 Dan), 115.9 (C-6 Dan), 101.4 (C-1''), 76.8 (C-3), 72.8 (C-4), 71.42 (C-5''), 71.37 (C-3''), 71.2 (C-4''), 70.2 (C-2''), 68.5 (C-1), 51.5 (C-2), 45.52 (NMe₂), 45.16 (C-6''), 37.1 (C-2'), 34.6 (C-5), 32.42, 32.41, 30.8, 30.7, 30.5, 30.4, 30.34, 30.33, 30.32, 30.31, 30.29, 30.27, 30.26, 30.23, 30.21, 30.19, 30.14, 30.10, 29.92, 29.90, 26.8, 26.7, 23.24, 23.23 (C-6–C-17, C-3'–C-

25'), 14.6 (2 x CH₃, C-18, C-26'); HRMS(ESI) *m/z* calcd. for [C₆₂H₁₁₁N₃O₁₀S+Na]⁺: 1112.7888, obsd.: 1112.7878.

2.7.2 Materials and methods for cell proliferation (IL-2) assay, in vivo DC maturation assay and in vitro treatment of dendritic cells

Mice. Breeding pairs of the inbred strains C57BL/6 were obtained from The Jackson Laboratories and from the Animal Resource Centre. All mice were maintained in the Biomedical Research Unit of the Malaghan Institute of Medical Research. CD1d^{-/-} mice, which are devoid of CD1d-restricted *i*NKT cells, were also used.⁶² Experiments were approved by the NZ national animal ethics committee and performed according to established national guidelines.

Solubilisation of glycolipid. α -GalCer (Industrial Research Ltd, New Zealand) and synthesised glycolipids were tested to be endotoxin-free at the sensitivity of 0.125 EU/mL with an endotoxin kit (Pyrotell, Limulus Amebocyte Lysate). Each glycolipid (1 mg) was dissolved in 200 μ L of CHCl₃/MeOH/H₂O (10:10:3), heated at 37°C for 15 min followed by sonication for 10 min. The solution was then diluted to 200 μ g/mL in 0.5% Tween/phosphate-buffered saline (PBS) and left to sit at -4 °C overnight. The glycolipid was then heated at 80 °C for 5 min followed by sonication for 5 min then cooled at 0 °C for 5 mins (x 2) and left to sit at -4 °C overnight.

Cell proliferation (IL-2) assay. Dendritic cells (DC2114;⁵³ 2.5x10⁴ cells) and murine NKT hybridoma cells (DN32.D3;⁴ 1x10⁵ cells) were incubated with various concentration of glycolipid (α -GalCer 1: 583 nM–0.569 nM; Dansyl α -GalCer 2: 458 nM–0.447 nM in two fold dilution) in 200 μ L of complete medium consisting of Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 2 mM glutamine (Invitrogen), 1% penicillin-streptomycin (Invitrogen), 5x10⁻⁵ M 2-mercaptoethanol (Invitrogen) and 5% foetal bovine serum (Sigma-Aldrich) at 37 °C for 24 h. 100 μ L of supernatant was added to 5x10³ HT-2 cells in a flat-bottom 96-well plate, incubated at 37 °C for 24 h and incorporation of

[³H]thymidine over the final 6 h was measured with a beta-counter (Wallac). A standard curve was constructed by incubating HT-2 cells with known concentrations of IL-2 (200–0 U/mL in five fold dilution). Each sample was analysed in triplicate.

Antigen presenting cell maturation assay. Glycolipid diluted to 2 µg/mL and 200 ng (in 100 µL) was administered *i.v.* to groups of C57BL/6 mice ($n = 3$) and spleens were removed 20 h later. Spleens were teased through a cell strainer, red blood cells (RBC) were lysed with RBC lysis buffer followed by antibody labelling for CD11c (clone HL3; BD Pharmingen), B220 (clone RA3-6B2 conjugated to A488; BD Pharmingen), CD86 (clone GL1 conjugated to PE; eBioscience) and propidium iodide (BD Pharmingen) and analysis by flow cytometry (BD FACSort). The same protocol was used for assessing expression of CD86 on CD11c⁺ dendritic cells by CD1d^{-/-} mice.

Uptake of glycolipid by dendritic cells *in vitro*. 1×10^6 DC2114 cells were incubated with 4 µg of glycolipid in 1 mL cIMDM in a flat-bottom 6-well plate at 37°C for 24 h. Cells were resuspended in FACS buffer, stained with viability dye (PI; BD Pharmingen) and analysed by flow cytometry (BD LSRII SORP; UV laser, filter 525/50, Hoechst Blue detector)

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Chapter 3. Synthesis of BODIPY- α -galactosylceramide

3.1 Introduction

Fluorescently labelled α -galactosylceramide (α -GalCer) probes (eg. Dansyl- α -GalCer **2** and BODIPY- α -GalCer **3**, Figure 3.1) are valuable tools that can be used to further understand the mechanism of iNKT cell activation by α -GalCer (**1**). While crystal structure analysis and the results from structure activity relationships provide a clear understanding of the specific molecular interactions between α -GalCer, CD1d and TCR, which gives rise to the activation of iNKT cells, little is known about the trafficking of α -GalCer.

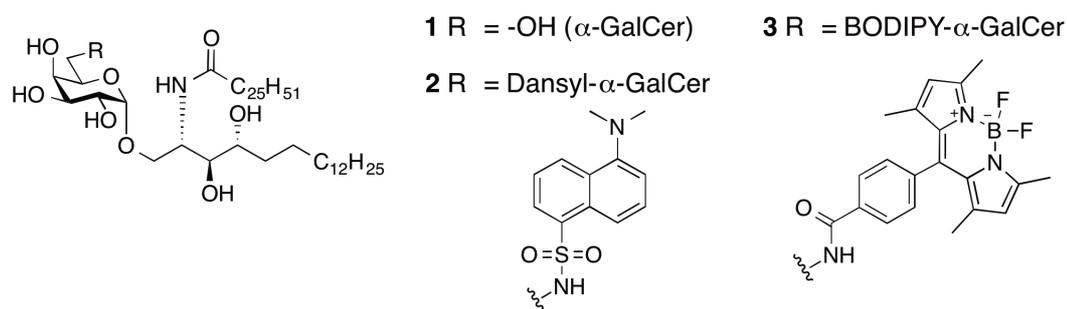


Figure 3.1. α -GalCer **1**, dansyl- α -GalCer **2** and BODIPY- α -GalCer **3**.

Tagging α -GalCer with a fluorophore will allow for further understanding about the behaviour of the glycolipid during cancer immunotherapy. The value of fluorescently labeled α -galactosylceramide has been illustrated in Chapter 2, whereby the synthesis and biological evaluation of dansyl- α -GalCer **2** were described. While the uptake of dansyl- α -GalCer **2** by dendritic cells (DCs) can be monitored by flow cytometry, the dansyl fluorophore is not bright enough to be detected by fluorescent and confocal microscopy. This is because the fluorescent glycolipid underwent rapid photobleaching when it was subjected under the UV laser used to excite the fluorophore. Moreover, it was found that when DCs were

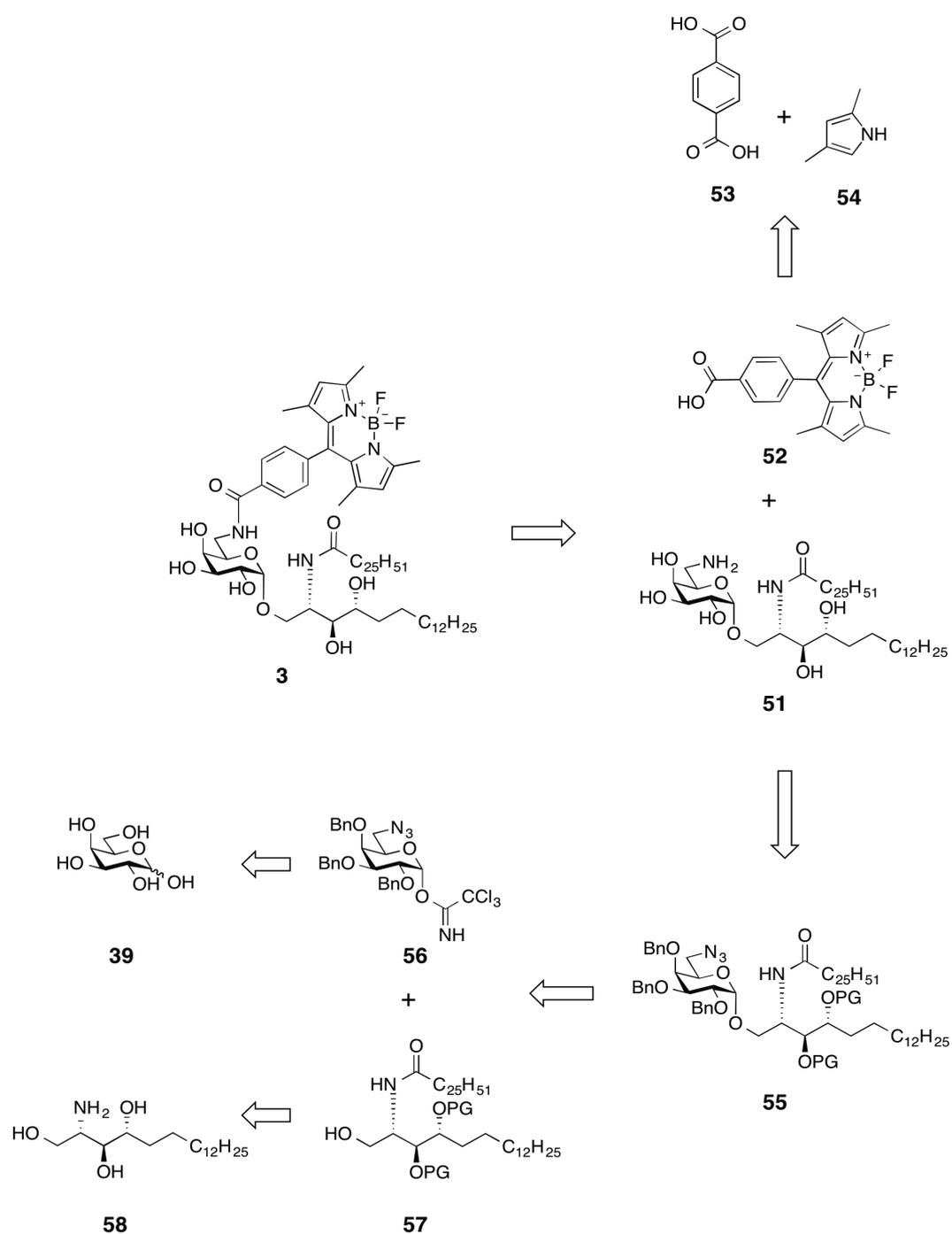
isolated from mice injected with dansyl- α -GalCer **2**, and analysed, no glycolipid could be detected by flow cytometry. Therefore, to meet the need for a brighter fluorophore, boron dipyrromethene (BODIPY)- α -GalCer **3** was developed. BODIPY is a boron dipyrromethene dye that has a wavelength of maximum absorbance at around 500 nm and emits light at 510 nm. It was chosen as a fluorophore due to its higher fluorescence quantum yield ($\Phi = 0.40 - 0.60$) compared to the dansyl group ($\Phi = 0.20$).¹

3.2 Synthesis of BODIPY- α -galactosylceramide

Retrosynthesis

To synthesise BODIPY- α -GalCer **3**, a strategy that differed to that used for the synthesis of dansyl- α -GalCer **2** was undertaken. Instead of the early incorporation of the fluorescent group onto the galactose sugar head group, the route was designed such that the BODIPY group was introduced at the end of the synthesis, by coupling BODIPY-COOH **52** with the fully deprotected glycolipid, 6'-amino- α -GalCer **51** (Scheme 3.1). It was envisaged that the development of the late stage intermediate, 6'-amino- α -GalCer **51** will be useful not only for making BODIPY- α -GalCer, but also for attaching other functionalities to the 6'-position of α -GalCer, such as other fluorescent groups, biotin tags, or peptides.²⁻⁷ The BODIPY-COOH **52** could in turn be synthesised in six steps from terephthalic acid **53** and dimethylpyrrole **54** using a strategy developed in the Stocker-Timmer group,^{1,8} while the deprotected glycolipid **51** could be obtained from 6'-azido- α -GalCer **55** via removal of the ceramide protecting groups with simultaneous hydrogenolysis of the benzyl ethers and reduction of the azide to an amine. The fully protected glycolipid **55** could be made by coupling galactosyl imidate **56**, itself obtained from D-galactose **39**,^{9,10} with a suitably protected lipid acceptor **57**, prepared in a few steps from phytosphingosine **58**. In contrast to the synthetic route employed for the synthesis of dansyl- α -GalCer **2**, the glycosylation strategy employed for the preparation of BODIPY- α -GalCer **3** would involve the coupling of galactose donor **56** with the complete ceramide acceptor **57**. While the yields of glycosylations with *N*-acylated ceramide acceptors are typically lower (ca. 25-55%) compared to coupling with the ceramide backbone, this strategy is necessary

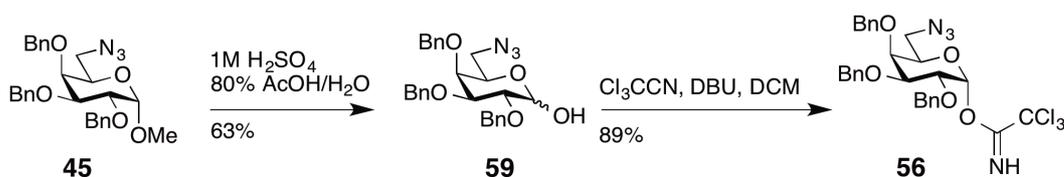
to avoid the presence of two amine groups, which would be difficult to selectively acylate after coupling.⁷



Scheme 3.1. Retrosynthetic scheme for BODIPY- α -GalCer **3**.

Synthesis of 6-azido-galactosyl imidate donor **56**.

The synthesis of BODIPY- α -GalCer **3** commenced with the preparation of galactosyl imidate donor **56** (Scheme 3.2). Here, methyl 6-azido galactoside **45**, a convenient intermediate previously described in Chapter 2 (Scheme 2.3), was converted to lactol **59** via acid-mediated hydrolysis of the anomeric methyl group. The trichloroacetimidate moiety was installed via the treatment of lactol **59** with trichloroacetonitrile in the presence of DBU to provide imidate donor **56**, as the α -anomer only, in 89% yield. Taken together, galactosyl imidate donor **56** was made from D-galactose (**39**) in 33% overall yield over seven steps, which is more efficient compared to the route reported by Li *et al.*, which produced the imidate donor **56** in 11% overall yield over seven steps, starting from a later stage intermediate, 1-S- β -D-galactosyl toluene.¹⁰

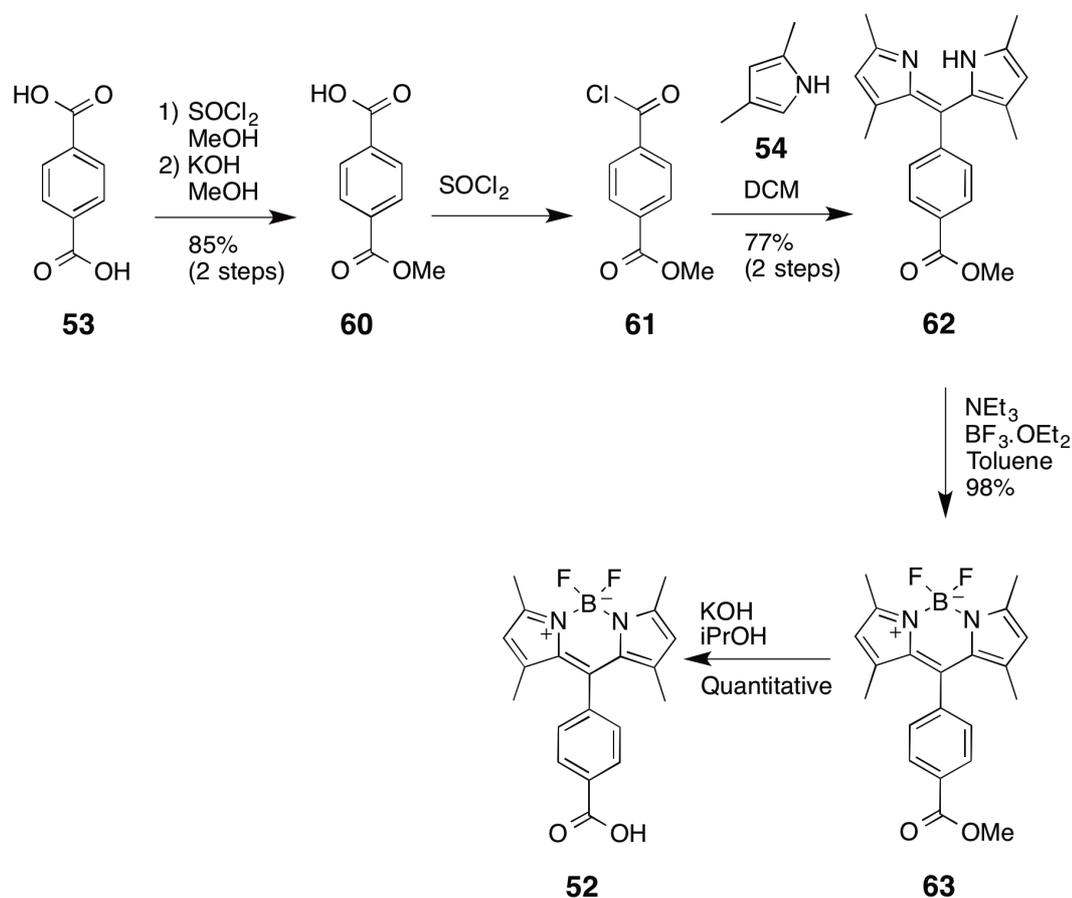


Scheme 3.2. Synthesis of 6-azido-galactosyl imidate donor **56**.

Synthesis of BODIPY-COOH **52**

A robust and efficient route for the synthesis of BODIPY-COOH **52** was developed by Ms. Stephanie Chee within the group (Scheme 3.3).¹¹ Briefly, the synthesis began with the methylation and partial hydrolysis of terephthalic acid **53** to give monoester **60** in 85% yield over two steps. Next, the acid chloride **61** was generated in the presence of thionyl chloride, followed immediately by condensation with dimethylpyrrole **54** to form dipyrrole **62** in 77% yield over two steps. The boron functionality was then introduced by reacting dipyrrole **62** with BF₃.OEt₂ to give BODIPY ester **63** in an excellent 98% yield. Finally, hydrolysis of the methyl ester under basic conditions afforded BODIPY-COOH **52** in quantitative yield. For this thesis, methyl ester **63** was kindly provided by Ms.

Stephanie Chee, which upon hydrolysis afforded the desired BODIPY-COOH **52** in identical yield to that previously reported.¹¹

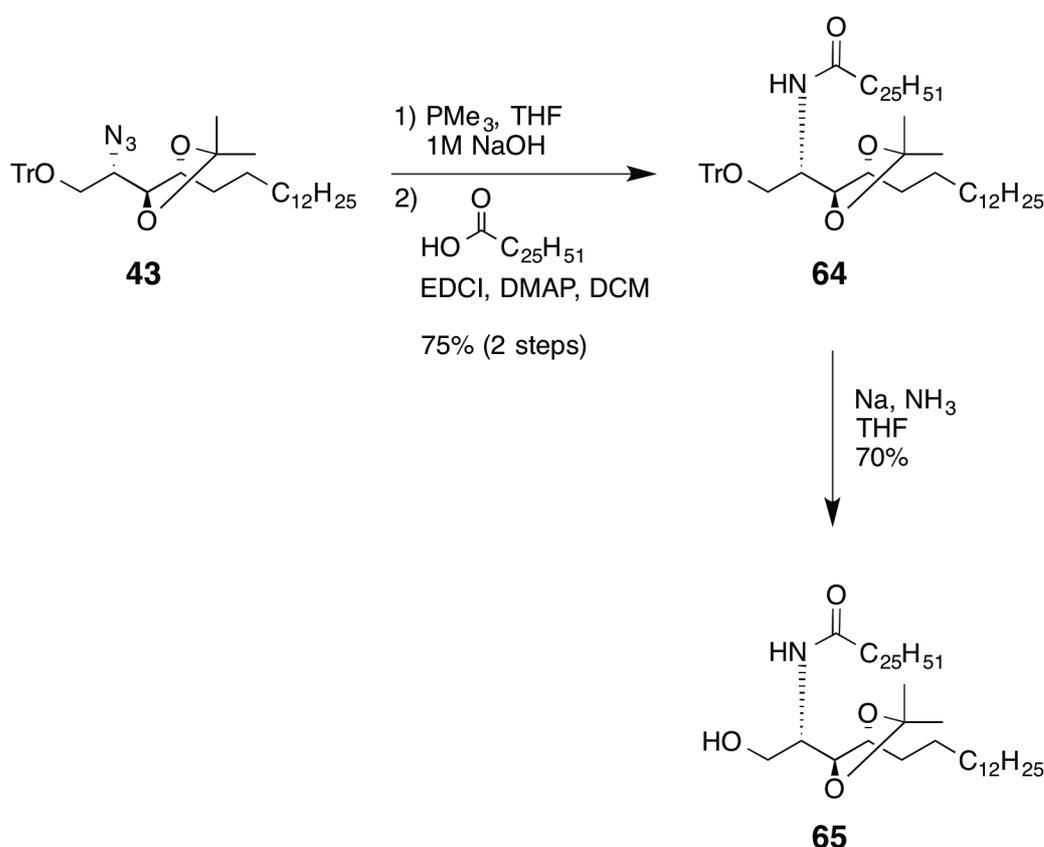


Scheme 3.3. Synthesis of BODIPY-COOH **52**.¹¹

Synthesis of 3,4-isopropylidene protected ceramide acceptor **65 and coupling to galactosyl donor **56****

With the sugar building block and BODIPY-COOH in hand, attention then turned to the synthesis of the ceramide acceptor. Again, an existing intermediate from the established route for the synthesis of dansyl- α -GalCer **2**, namely the 2-azido-3,4-isopropylidene protected phytosphingosine **43** (Chapter 2), was used (Scheme 3.4).⁹ The azide functionality in **43** was reduced using trimethylphosphine and 1M NaOH and the resultant amine coupled to hexacosanoic acid using an EDCI/DMAP-mediated protocol to give the fully protected lipid **64**. Next, the

selective removal of the trityl group was attempted under acidic conditions, but despite the controlled and careful addition of AcOH or TFA, hydrolysis of the isopropylidene protecting group could not be prevented. Therefore, a Birch reaction was used as an alternative method to selectively cleave the trityl group, which yielded the 3,4-isopropylidene protected acceptor **65** in 70% yield. Here, it should be noted that the synthesis of acceptor **65** starting from 2-azido phytosphingosine, which has previously been described by Schmidt and co-workers,^{12,13} is a shorter and more direct route than the one used herein, however intermediate **43** was chosen as it was readily available from previous work with dansyl- α -GalCer **2**.⁹

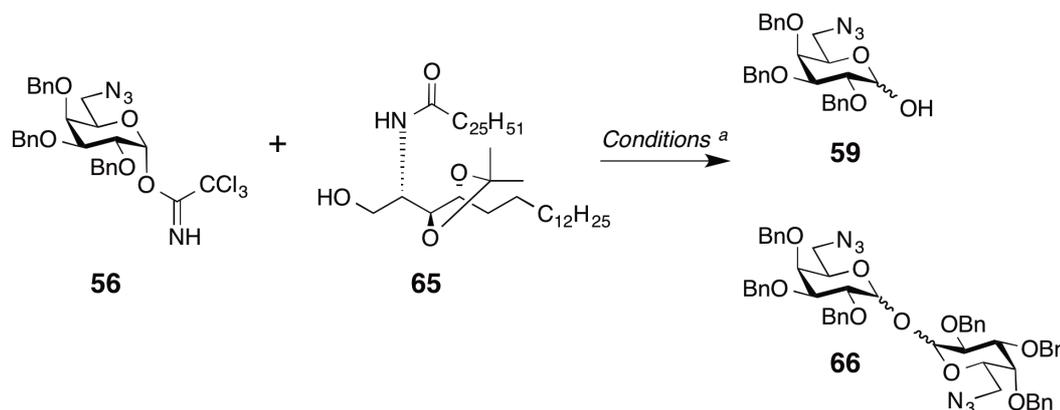


Scheme 3.4. Synthesis of 3,4-isopropylidene protected ceramide acceptor **65**.

With acceptor **65** in hand, attempts were then made to perform the glycosylation reaction using imidate donor **56** and TMSOTf as the promoter. Glycosylation reactions with imidate donors using TMSOTf as the activator are typically performed at temperatures of 0 °C or lower,¹⁴ however, when the coupling was attempted with a 2:1 mixture of DCM/THF as the solvent (Table 3.1, Entry 1), the

reaction mixture containing both donor and acceptor appeared cloudy, indicating poor solubility of the lipid acceptor. As a result, no desired glycolipid was observed and instead the hydrolysed donor **59** was obtained upon workup of the reaction, along with the recovery of uncoupled acceptor **65**.

Table 3.1. Glycosylation reaction between imidate donor **56** and 3,4-isopropylidene acceptor **65**.



Entry	Activator	Solvent ^b	Temperature	Product ^c
1	TMSOTf	DCM/THF (2/1)	0 °C to rt	59
2	TMSOTf	Toluene/DCM/DMF (2/1/0.1)	rt	59
3	TMSOTf	Toluene/DCM (2/1)	30 °C	59 + 66
4	TMSOTf	Et ₂ O/THF (5/1)	rt	59 + 66
5	BF ₃ ·OEt ₂	Et ₂ O/THF (5/1)	-20 °C	59 + 66

^a Donor and acceptor were co-evaporated with dry toluene (x3) and dissolved in the solvent and stirred with 4Å molecular sieves for 30 mins. Then the reaction was set to the desired temperature and the promoter was added slowly.

^b Solvent and ratio (v/v) in parentheses.

^c Uncoupled lipid acceptor **65** was recovered from all unsuccessful coupling reactions.

During co-evaporation of the donor and acceptor prior to the glycosylation reactions, it was observed that toluene, which was used as the solvent for azeotropic removal of water, was able to dissolve the lipid acceptor well. Therefore, the glycosylation was attempted using a mixture of toluene/DCM/DMF (2/1/0.1, v/v/v) as the solvent (Entry 2). While the reaction mixture was completely clear at room temperature, unfortunately the glycosylation was

unsuccessful, presumably because the highly reactive donor was being activated at a temperature that was too high. Again only the hydrolysed donor **59** was obtained. Similarly, glycosylation with toluene and DCM as a solvent at 30 °C was to no avail and gave 1,1'-digalactoside **66** (Entry 3). While all effort was made to ensure that dry conditions were used for the sensitive coupling reactions, unfortunately the severe unreactivity of the lipid acceptor **65** meant that trace amounts of water in the reaction mixture led to the formation of lactol **59**, which then reacted with another molecule of imidate **56** to form 1,1'-disaccharide **66**. A diethyl ether/THF solvent system, which is commonly used in coupling reactions between lipid acceptors and glycoside donors,¹⁵ but again the lipid acceptor was only soluble at room temperature and as a result, gave 1,1'-digalactoside **66** (Entry 4). In accordance with Wong and co-workers' procedure,¹⁵ a milder promoter BF₃.OEt₂ was used with the same solvent system, though disappointingly, this also only gave a mixture of lactol **59** and 1,1'-digalactoside **66** (Entry 5). From this series of glycosylation attempts, it was concluded that the solubility of the ceramide acceptor **65**, and hence its poor reactivity, was a major issue and that even trace amounts of water would thus preferentially react with the imidate donor **56** to form the lactol. Here, it should also be noted that although Schmidt and co-workers have reported the synthesis of lipid acceptor **65**, it has not been used directly as a glycosyl acceptor and instead was converted to a 1-phosphite derivative for the synthesis of glycosphosphatidylinositols (GPIs).^{12,13}

Synthesis of benzoyl protected ceramide acceptor 57 and coupling to galactosyl donor 56

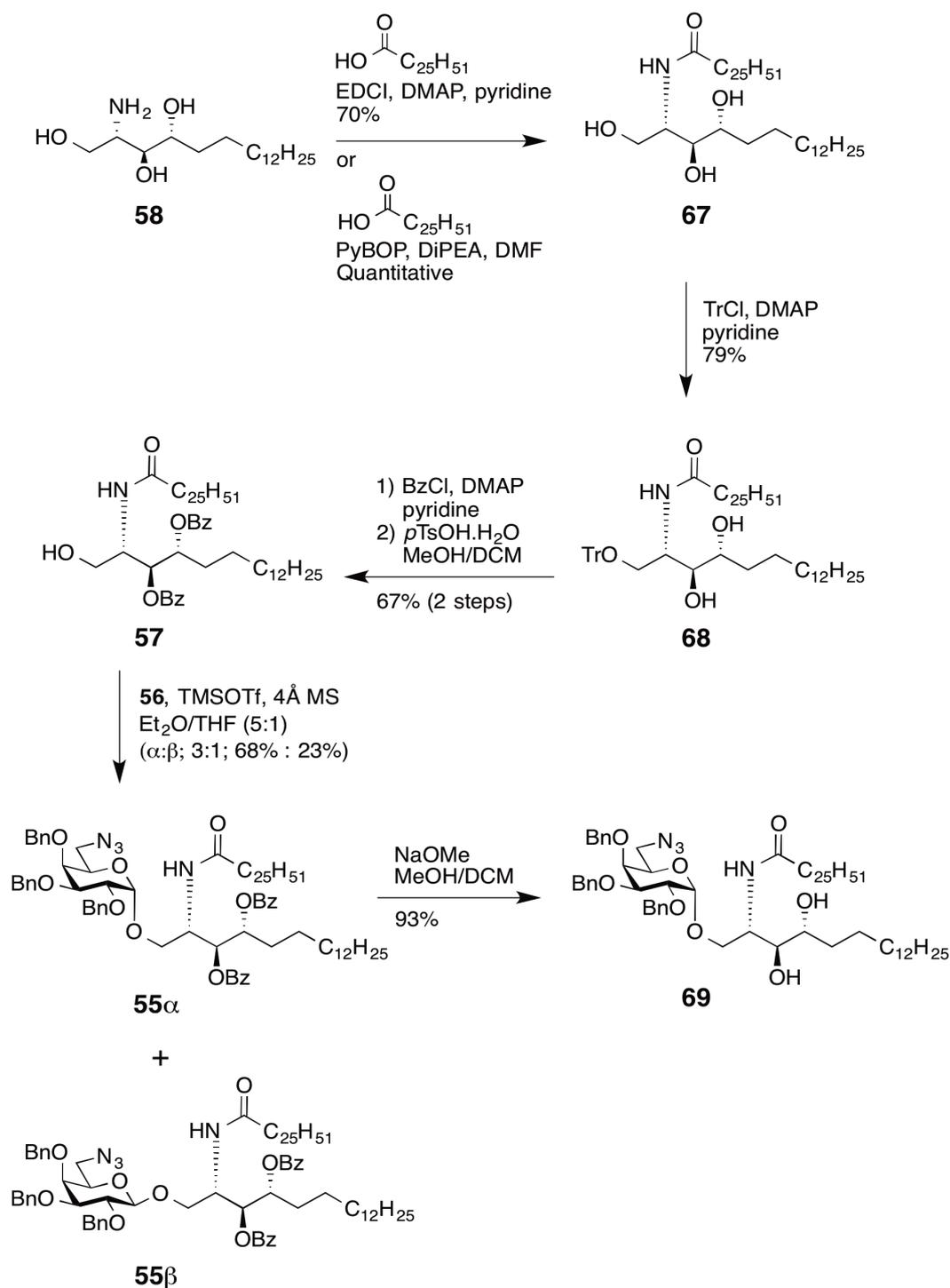
Due to the poor reactivity of isopropylidene protected lipid acceptor **65**, alternative protecting groups on the phytosphingosine backbone were evaluated. Xia *et al.* reported on the successful coupling of the lipid acceptor when the 3- and 4-hydroxyls were protected as either benzyl ethers or benzoate esters (yield 57-59%).¹⁶ It was therefore proposed that a change of protecting groups would aid with the solubility of the lipid, and thus the glycosylation reaction. Furthermore, Xia *et al.* observed that when the 3- and 4- hydroxyls of the phytosphingosine backbone were protected with benzyl groups, upon glycosylation with a

galactosyl imidate donor, the β -coupled glycolipid was obtained. This was a consequence of the high reactivity of the acceptor due to the arming effect of the electron-donating benzyl groups. Alternatively, when the secondary alcohols were protected as benzoate esters, only the α -isomer was obtained as the major product. From the above results, it was therefore proposed that the glycosylation reaction would be more successful if a benzoate-protected lipid was used.

To this end, the synthesis of the target *N*-acylated phytosphingosine lipid began with a similar approach to that previously reported, whereby commercially available phytosphingosine **58** was first *N*-acylated at the 2-position with hexacosanoic (C26) acid (Scheme 3.5).^{16,17} Various strategies such as direct coupling of the amine **58** and the C26 acid with EDCI¹⁸⁻²¹ as the coupling agent or coupling to the C26 acid chloride²⁰ or the C26-*N*-hydroxysuccinimidyl ester^{16,22} have been employed to obtain the trihydroxy ceramide **67**.

Initial attempts to carry out this reaction according to Gervay-Hague and co-workers' protocol utilizing EDCI as a coupling agent in the presence of DMAP afforded trihydroxy ceramide **67** in 70% yield (Scheme 3.5).¹⁸ During the reaction, it was observed that the C26 acid was poorly soluble in pyridine solvent used. The poor solubility of the lipid leading to its lower reactivity, as well as the ability of EDCI to facilitate the formation of both esters and amides were suspected to be the reason for this average yield. Therefore, to improve the yield of the reaction, an alternative coupling agent selective only for the acylation of amines but not alcohols, PyBOP, in the presence of DiPEA and DMF was used. Due to the difficulty in monitoring the disappearance of the starting materials, phytosphingosine **67** and C26 acid by TLC, two reactions were set up and allowed to stir for 1 day or 3 days, which upon purification by crystallisation afforded the ceramide **67** in 37% and 73%, respectively. The purity of the lipid was confirmed by ¹H NMR. It was concluded that the reaction proceeded slowly, and so when it was allowed to react for 7 days, the triol **67** was obtained in quantitative yield. This is the first report of PyBOP being used as the coupling agent for phytosphingosine-based ceramides and has by far the best yield compared to other coupling protocols available.^{16,19-24}

Next, the primary hydroxyl was regioselectively protected as a trityl ether to give diol **68** in 79% yield. Subsequent benzylation of the secondary hydroxyls, followed by the selective removal of the trityl group then afforded the ceramide acceptor **57** in 67% over two steps.^{16,23} This strategy thus allowed for the synthesis of the complete ceramide acceptor in an excellent overall yield of 53% over four steps. The results obtained are comparable to Xia and co-workers' route, which began with succinimide-activated acyl lipid (77% and 65% overall yield over five steps for the C26 and C8 lipids, respectively)^{16,17} and Morita *et al.*'s route, which began with 2-azido-tri-*O*-benzyl-phytosphingosine (42% overall yield over 5 steps).²³

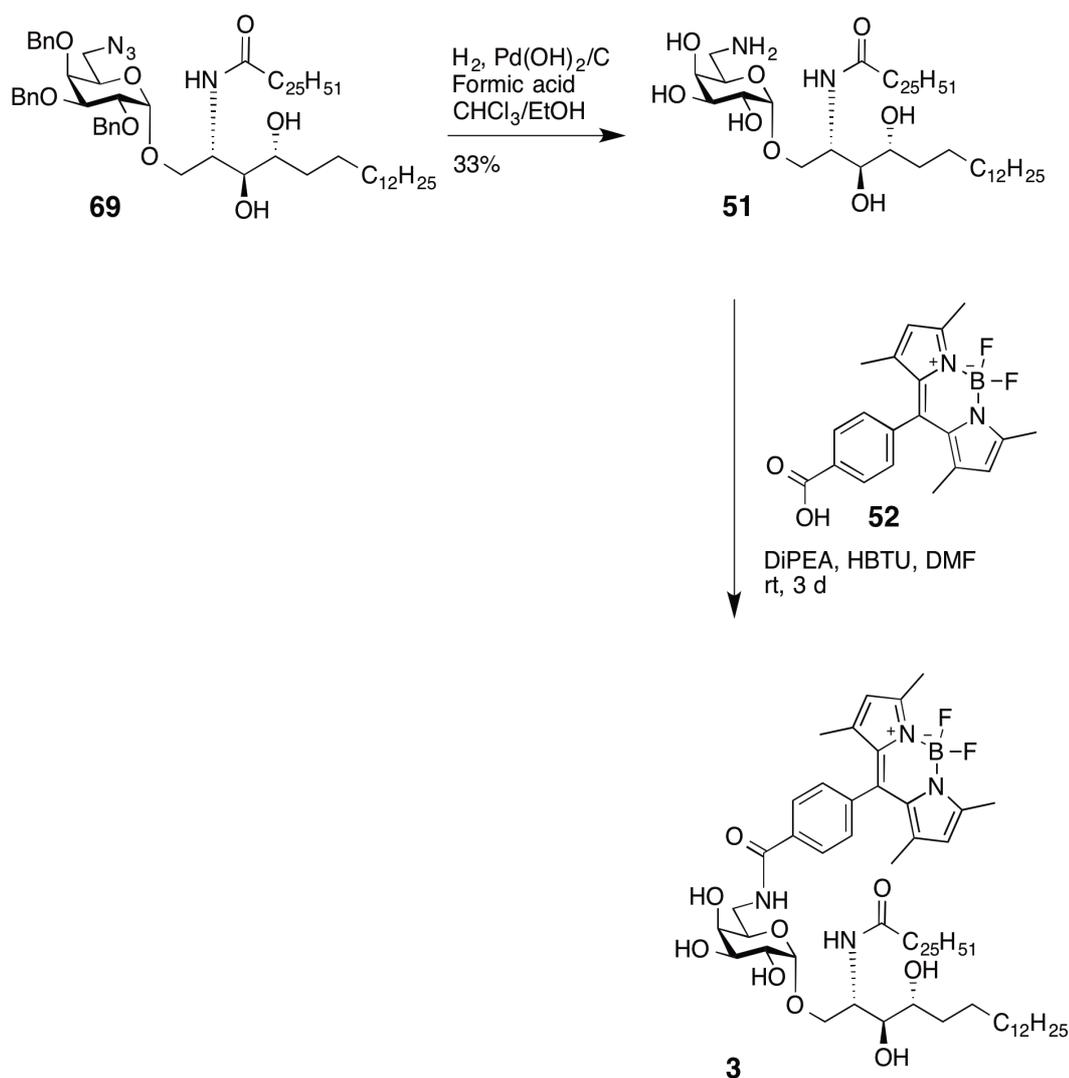


Scheme 3.5. Synthesis of α -GalCer diol **69**.

With the benzoate protected lipid acceptor **57** and the galactose donor **56** in hand, the glycosylation was then attempted using TMSOTf as the activator. It was found that 0 °C was the lowest temperature that could be used for optimal activation of

the trichloroacetimidate donor **56**, while keeping the highly lipophilic lipid acceptor **57** dissolved. Indeed, this afforded the protected 6'-azido glycolipid **55** in excellent yield (91%) and satisfactory α -selectivity ($\alpha:\beta = 3:1$), as confirmed by a coupling constant of 3.6 Hz for the anomeric signal in the ^1H NMR and a one bond coupling ($^1J_{\text{CH}}$) of 168.6 Hz for the α -anomer.²⁵ In contrast, the β -anomer had an anomeric coupling constant of 7.8 Hz and a one bond CH-coupling of 159.4 Hz. Due to the poor solubility of the lipid, glycosylation reactions using the complete ceramide acceptor typically result in a modest yield (ca. 40-50%) for the α -anomer,^{16,19} thus the results observed here were encouraging, particularly as the α and β -anomers could be isolated in 68% and 23% yields, respectively. The removal of the protecting groups on α -linked glycolipid **55** was initially attempted with a Birch reduction, which would remove the benzyl groups as well as the benzoyl groups upon quenching of the reaction with methanol to form sodium methoxide *in situ*. However, difficulty in isolating the product upon neutralisation with Dowex+H⁺ meant that stepwise deprotection was needed and the 3- and 4-*O*-benzoyl groups were first removed under basic conditions to afford the diol glycolipid **69** in an excellent yield of 93%.

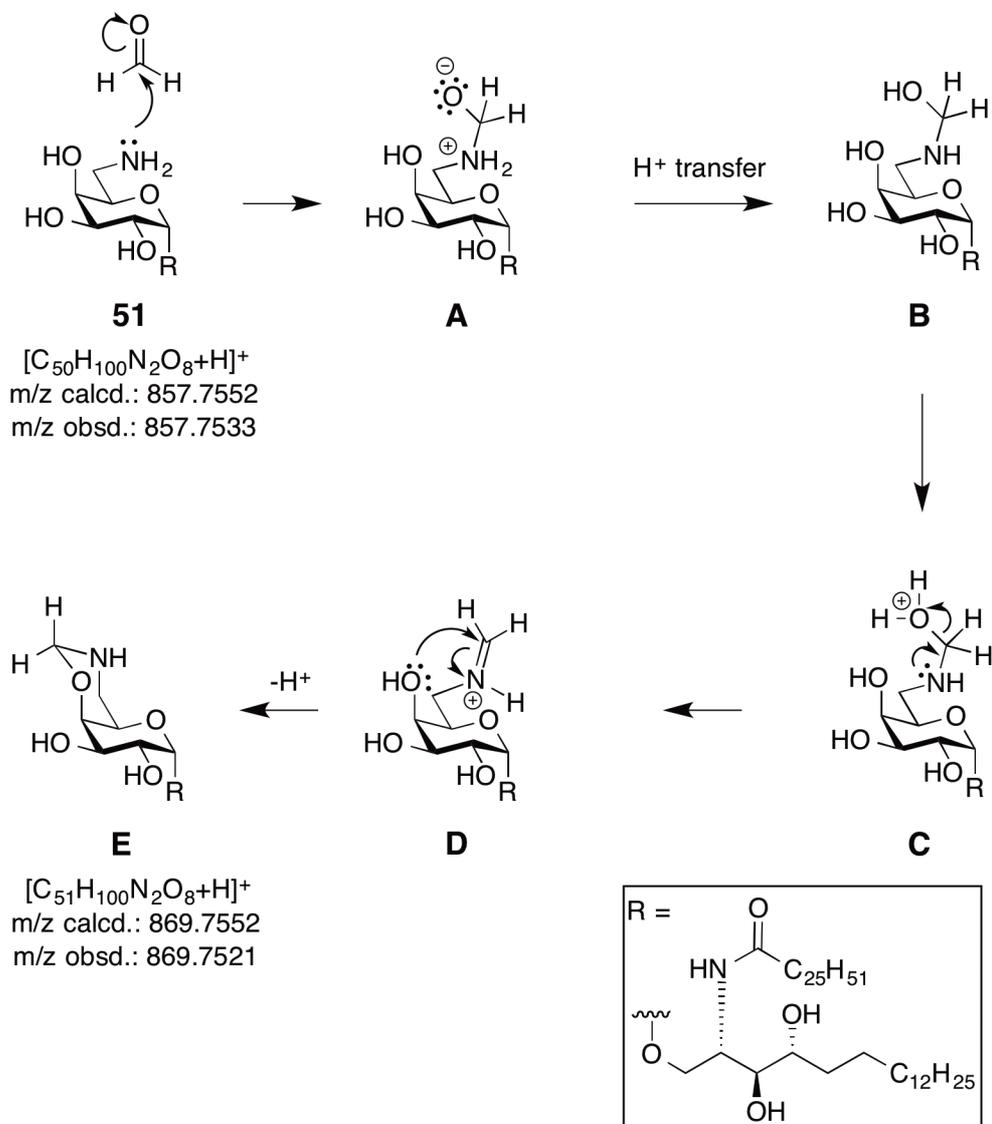
Next, the simultaneous hydrogenolysis of the three benzyl ethers and the reduction of the azide to the amine was carried out using Pearlman's catalyst with the addition of 5% formic acid to prevent catalyst poisoning by the amine that is formed during the reaction (Scheme 3.6). While the reaction appeared to proceed to completion by TLC analysis, the isolation and purification of amine **51** proved difficult. Despite using copious amounts of CHCl₃/EtOH/MeOH and pyridine to wash the celite plug that was used to filter the catalyst upon completion of the reaction, only a small amount of material was recovered. The difficulty in isolating the glycolipid from the reaction mixture was attributed to the poor solubility of the fully deprotected 6'-amino glycolipid **51** and its affinity for the palladium hydroxide on carbon catalyst. Initial attempts to directly couple the crude mixture of amine **51** to BODIPY-COOH **52** was unsuccessful, as confirmed by the absence of new product when the reaction mixture was analysed by TLC. Indeed, both starting materials were re-isolated.



Scheme 3.6. Synthesis of BODIPY- α -GalCer **3** using BODIPY-COOH **52**.

To understand why the coupling reaction was unsuccessful, some of the crude mixture of glycolipid **51** that was used in the coupling reaction was analysed by mass spectrometry. The presence of the desired product 6'-amino- α -GalCer **51** was detected (m/z calcd. for $[\text{C}_{50}\text{H}_{100}\text{N}_2\text{O}_8+\text{H}]^+$: 857.7552, obsd.: 857.7533) along with an unexpected m/z of 869.7521. Upon careful analysis, this was attributed to the formation of *cis*-decalin **E** (Scheme 3.7) due to the presence of trace formaldehyde present in the methanol that was used to wash, filter and transfer the glycolipid (m/z calcd. for $[\text{C}_{51}\text{H}_{100}\text{N}_2\text{O}_8+\text{H}]^+$: 869.7552, obsd.: 869.7521). The mechanism for the formation of *cis*-decalin **E** is depicted in Scheme 3.7, whereby the amine **51** attacks a molecule of formaldehyde to form alcohol **B**, which upon

elimination of water gives the iminium intermediate **D**. Subsequent attack of the 4-OH on the iminium carbon followed by the loss of H⁺ generates the *cis*-decalin by-product **E**.



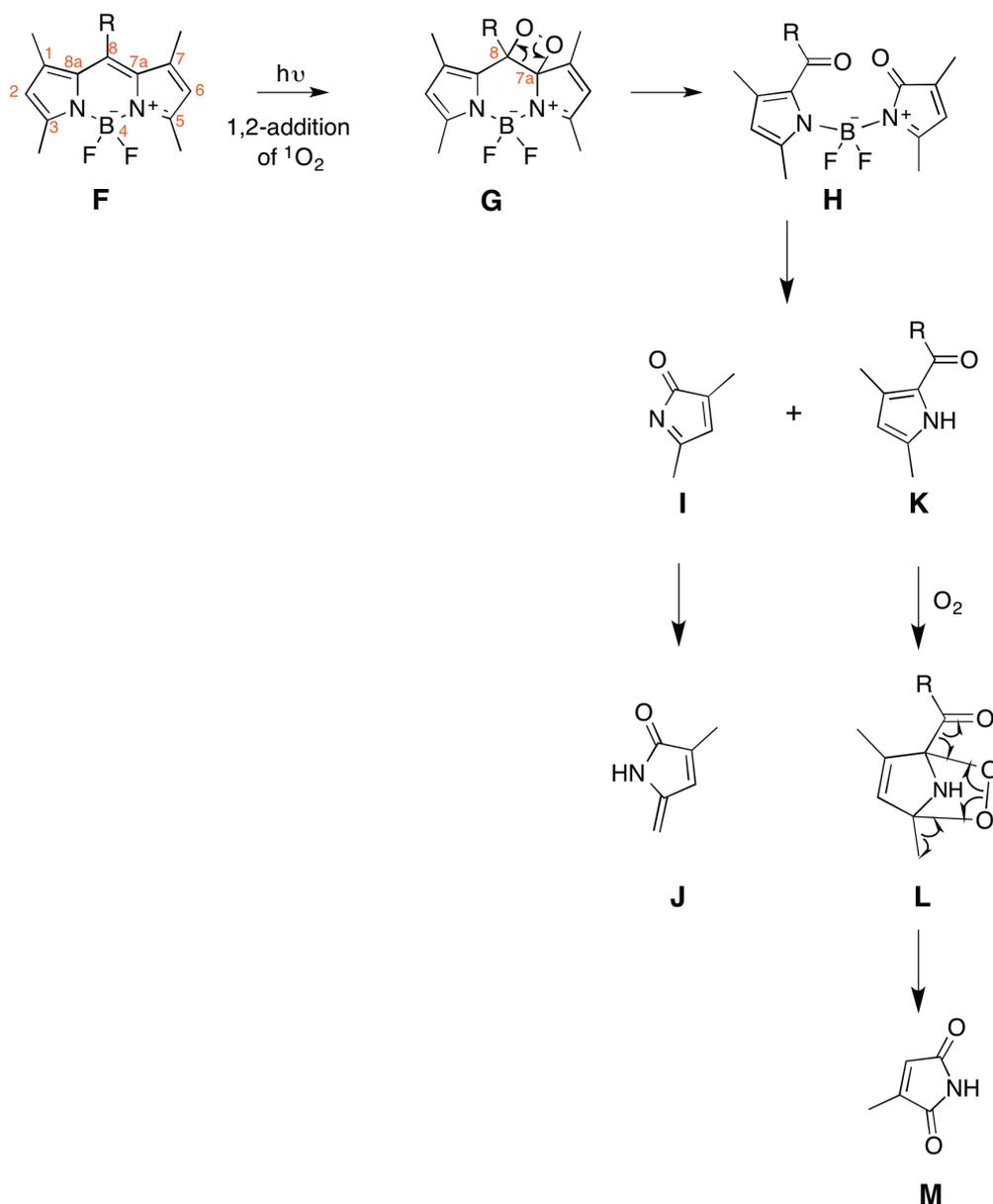
Scheme 3.7. Mechanism for the formation of *cis*-decalin **E** by-product.

In an attempt to regenerate the free amine (ie. to hydrolyse the *cis*-decalin **E**), the crude mixture was co-evaporated with 1M HCl (aq). Though somewhat successful, unfortunately, the reaction did not go to completion. Careful purification of the mixture of amine **51** and *cis*-decalin **E** by silica gel column chromatography using a solvent system of DCM, MeOH, EtOH and NH₃ was also unsuccessful and instead caused the formation of ammonium chloride salt when

the ammonia in the eluting solvent reacted with trace HCl from the hydrolysis step. Efforts to separate the salt from the glycolipid by reverse phase column chromatography were futile as the glycolipid was insoluble in water and had to be loaded onto the column with a few drops of pyridine, which caused the product to be eluted together with the salts. Removal of the salts by dialysis was also attempted, but again proved unviable because the glycolipid was only soluble in a mixture of DCM/MeOH/water (2/1/5, v/v/v), which was not compatible with the cellulose dialysis tube. Fortunately, most of the ammonium chloride was successfully removed by crystallisation from a mixture of warm 30% MeOH in DCM and filtered. Due to the challenges in obtaining pure 6'-amino- α -GalCer (ie. without salts), it was decided that the semi-pure mixture would be used for the subsequent coupling to BODIPY-COOH **52** and the yield for conversion of diol **69** to fully deprotected amine **51** was estimated at 33%. The identity of amine **51** was confirmed by HRMS and the ^1H NMR data matched that reported by Zhou *et al.*² The synthetic approach used here is similar to Zhou *et al.*'s strategy whereby a galactosyl donor is coupled to the complete ceramide acceptor leading to the generation of the fully deprotected 6'-amino- α -GalCer **51** and subsequent functionalisation with a fluorophore. However, the protecting group strategy used in the synthesis reported herein allowed for the simultaneous removal of benzyl protecting groups and azide reduction leading to the generation of amine **51** in comparable overall yield of 21% over 3 steps from the ceramide lipid acceptor. In contrast, Zhou *et al.* reported an overall yield of 19% over 4 steps from the ceramide acceptor.²

Next, amine **51** was coupled to BODIPY-COOH **52** using HBTU as the coupling agent (Scheme 3.6). The reaction appeared promising based on TLC analysis, however, to obtain BODIPY- α -GalCer **3** of sufficient purity and free from the coupling reagents and by-products, repeated purification by silica gel flash column chromatography was required. Unfortunately, all attempts to purify BODIPY- α -GalCer **3** proved futile. Indeed, the more time taken to purify the desired product, the more decomposition was observed.

As a fluorescent dye, BODIPY has many advantages including its high quantum yield and relatively small size.¹ It is also fairly inert, although some decomposition of the dye has been observed under rather harsh photo-oxidation conditions. For example, some early analyses of the dye in the 1970's revealed that complete fragmentation can occur when a slow stream of oxygen is allowed to bubble through a solution of 3,5,3',5'-tetramethyl-4,4'-diethyldipyrrylmethene exposed to photolysis under a halogen lamp (500W).^{26,27} Similar photo-oxidation was proposed to occur on boron-containing dipyrromethenes **F** (Scheme 3.8).²⁸ Here, addition of a singlet oxygen species across the double bond at C-7a and C-8 of the BODIPY core gave dioxetanes **G**, which subsequently broke down to the ring opened intermediates **H**, which upon hydrolysis of the boron-nitrogen bonds produce dimethylketopyrroles **I** and ketones **K**. Tautomerisation of dimethylketopyrroles **I** then leads to the formation of lactam **J**, while further oxidation of ketone **K** produces 3-methylmaleimide **M** via dealkylation of the endoperoxide intermediate **L**.^{28,29}

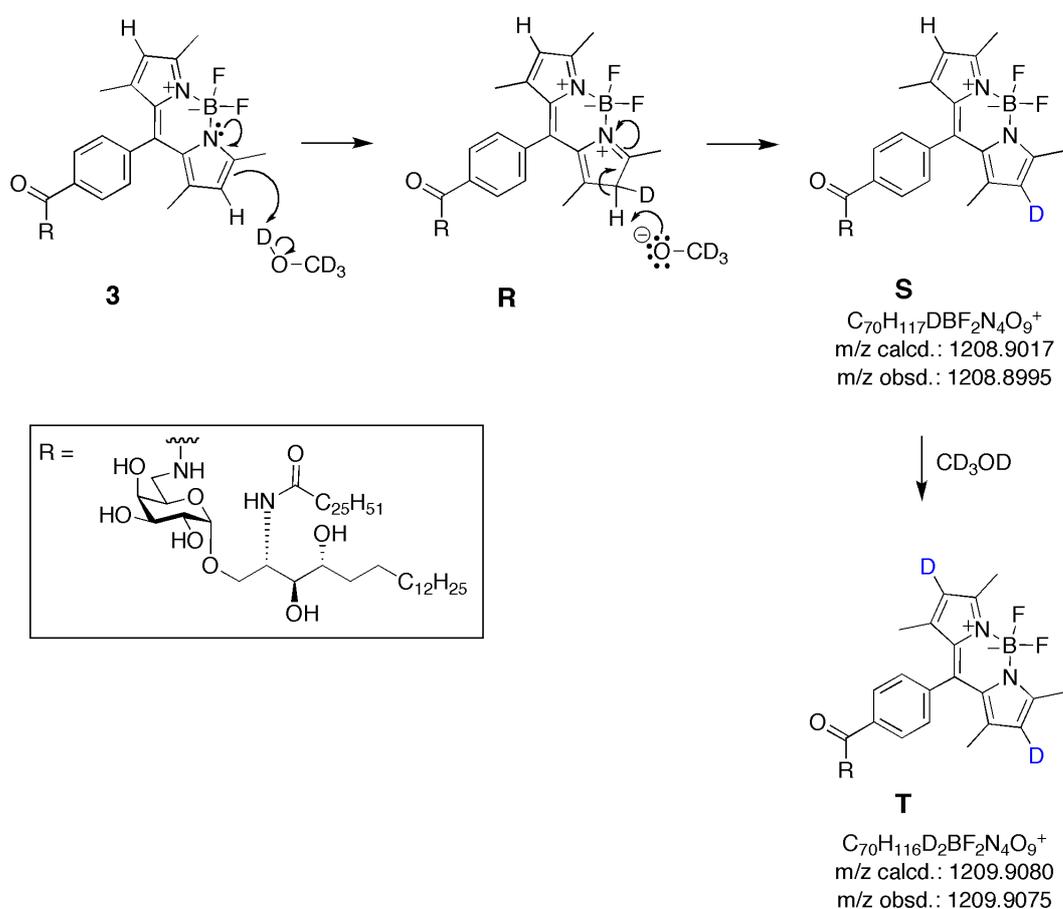


Scheme 3.8. Proposed mechanism of photo-oxidation of BODIPY core following photolysis with halogen lamp and O_2 stream.²⁸

The degradation of the BODIPY dye under more ambient conditions, however, has not been previously reported, yet TLC, HRMS and 1H NMR analysis of the product mixture following the repeated purification of BODIPY- α -GalCer **3** revealed the presence of several species. Given the small quantities of material available, it was difficult to conclusively prove the structures of the degradation products, however, by TLC, all products had very similar R_f values. Notably, analysis by HRMS revealed two by-products containing either one or two chlorine atoms, as evidenced by characteristic isotopic distributions arising from ^{35}Cl and ^{37}Cl atoms. Further analysis revealed the $[M+H]^+$ peak of 1241.8541, which

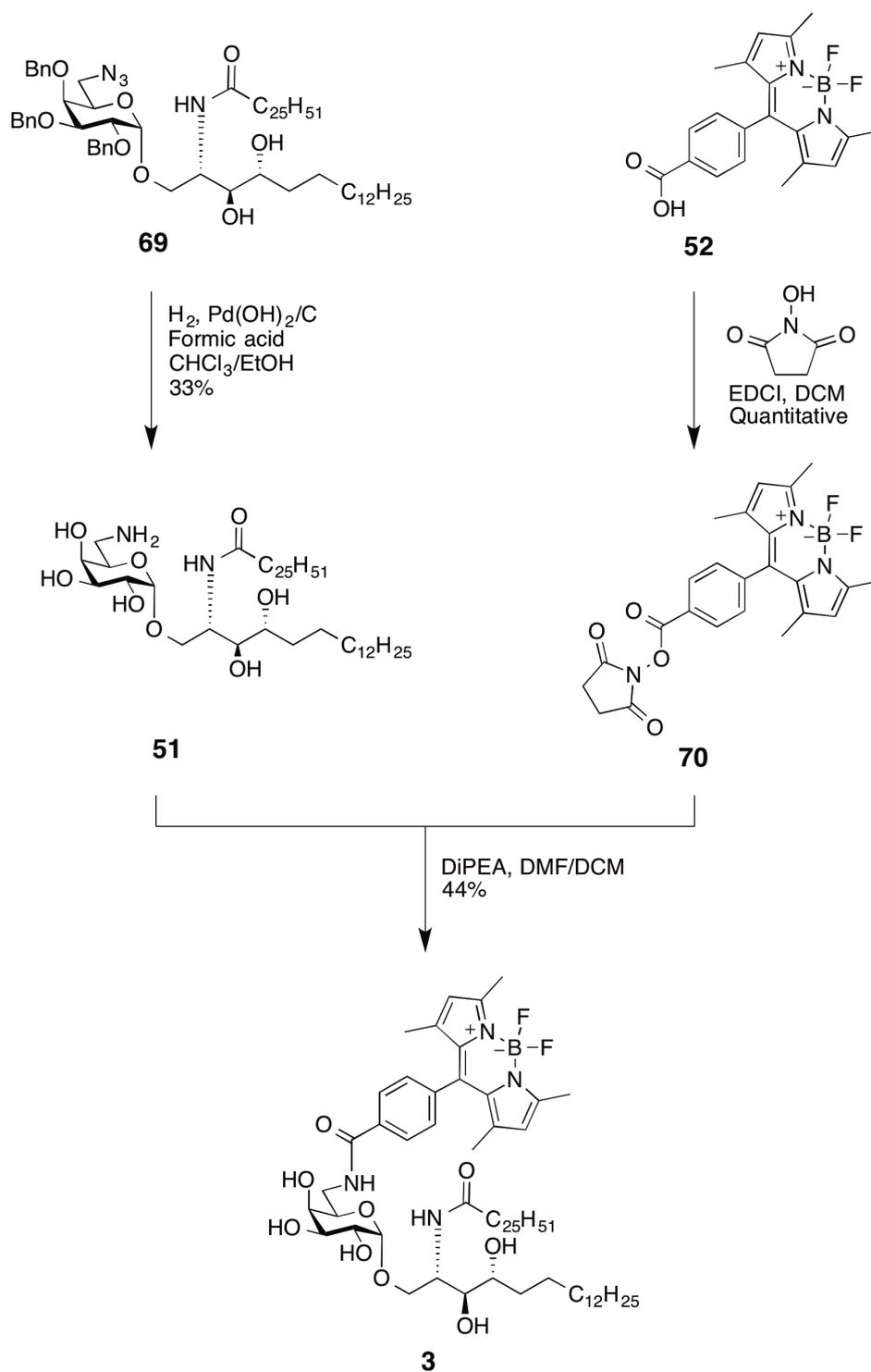
correlated to the calculated value of 1241.8565 for $C_{70}H_{117}BClF_2N_4O_9^+$. Thus, it has been tentatively proposed that BODIPY- α -GalCer **3** undergoes photo-oxidation with subsequent addition of Cl^- , which is formed from the decomposition of the NMR solvent $CDCl_3$, to yield the chlorinated adduct **Q** (Scheme 3.9). In this proposed mechanism, a singlet oxygen species is added across C-3 and C-8a of the BODIPY core to give endoperoxide **N**, which subsequently breaks down to give adduct **O** that is set up for attack by Cl^- at the 2-position. Loss of H_2O_2 then reinstates the aromaticity of the BODIPY core to produce the chlorinated BODIPY- α -GalCer **Q**. While there is a possibility of chlorination at the 3- and 5- methyl carbons of the BODIPY core, this is unlikely to be the by-product in this case because halogenation at the 3- and 5-positions typically occurs in the presence of a cationic halogen, such as *N*-bromosuccinimide, to form a 3-brominated BODIPY core.³⁰

In addition to the oxidised chlorinated adduct, which was determined by HRMS, ^1H NMR of the reaction mixture of BODIPY- α -GalCer **3** after repeated purification also revealed that the proton signal belonging to H-2 and H-6 of the pyrrole ring was significantly reduced compared to the other signals. Upon analysis by mass spectrometry, two masses corresponding to the substitution of one and two hydrogen(s) with deuterium(s) were detected (Scheme 3.10). This increase in mass came about due to the insolubility of the BODIPY- α -GalCer **3** in CDCl_3 alone, which necessitated the need for a mixture of 10% deuterated methanol (CD_3OD) in CDCl_3 for NMR analyses. Electrophilic aromatic substitution is most likely to occur at the 2- and 6- positions of the BODIPY core, giving rise to deuteration at one ($\rightarrow\text{S}$) or both ($\rightarrow\text{T}$) of these sites. The protic nature of CD_3OD means that a D^+ is a readily available electrophile. Accordingly, it was thus determined that non-protic deuterated solvents, such as pyridine- d_5 , should be used as the NMR solvent for future analyses.



Scheme 3.10. Deuteration of BODIPY- α -GalCer **3**.

To circumvent the aforementioned issues and to develop a BODIPY dye that would allow for easier purification (and thus limit exposure to light and oxygen), BODIPY-COOH **52** was derivatised as the *N*-hydroxysuccinimidyl ester **70** (Scheme 3.11). In this way, only a base, such as DiPEA, would need to be added to the reaction vessel, while the cleaved *N*-hydroxysuccinimide could be more readily separated from the desired BODIPY- α -GalCer **3**. Indeed, the coupling of the amine **51** with BODIPY-NHS **70** proceeded smoothly and the product could be readily purified by silica gel flash column chromatography (gradient from 1% to 3% MeOH in DCM) to afford BODIPY- α -GalCer **3** as a red solid in 44% yield. This compromised yield is largely attributed to the loss of material when purifying the fully deprotected amine **51**, as the 44% yield obtained is comparable to amide couplings performed on the 6' position of the α -GalCer which typically range from 33-53% yield.^{2,3} Unfortunately, limited quantities of fully protected 6'-azido- α -GalCer **55** prevented further optimisation of the last two steps of this route. Nonetheless, enough quantity of the final target BODIPY- α -GalCer **3** was obtained for the purpose of characterisation. The identity of the BODIPY- α -GalCer **3** was confirmed by ¹H and ¹³C NMR and mass spectrometry analysis (m/z calcd. for [C₇₀H₁₁₇BF₂N₄O₉+H]⁺: 1207.8954, obsd.: 1207.8958), including the characteristic three-bond correlation (HMBC) between H-6' and carbonyl carbon of BODIPY amide, and there was no evidence of oxidized, chlorinated or deuterated by-products by NMR or HRMS. BODIPY- α -GalCer **3** has subsequently been sent to collaborators Dr. Hans van der Vliet and co-workers for use as a fluorescent probe to study DC processing of α -GalCer, and also to determine the loading of glycolipids on tumour cells.



Scheme 3.11. Synthesis of BODIPY- α -GalCer **3** using BODIPY-NHS **70**.

3.3 Conclusion

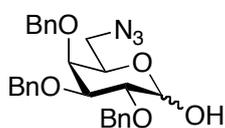
In conclusion BODIPY- α -GalCer **3** was successfully synthesised from the fully deprotected amine **51** and BODIPY-NHS **70**. It was discovered that the solubility of the ceramide lipid acceptor **57** was crucial for its reactivity in the glycosylation with imidate donor **56**. Though the glycosylation yield obtained when using the *N*-acylated ceramide acceptor is generally lower compared to the yields when the phytosphingosine acceptor, remarkably a good 68% yield of the desired α -anomer was observed. Moreover, the synthetic strategy employed here is more convergent and provides the fully deprotected glycolipid **51** bearing a reactive amine on the 6'-position that can be readily derivatised. The main challenge, however, is the purification of the amine glycolipid **51**, of which can be optimised with the availability of more of the fully protected glycolipid **55**. In the course of purification of the final product, BODIPY- α -GalCer **3**, limitations due to the sensitivity of the dye to photo-oxidation were also discovered. Indeed, this is an area that is currently undergoing further investigation in the group to provide more conclusive evidence for the oxidation products observed. BODIPY- α -GalCer **3** has been sent to collaborators who will use the probe to study the role of α -GalCer in cancer immunotherapy in more depth.

3.4 Experimental

General procedure

Unless otherwise stated all reactions were performed under N₂. Prior to use, THF (Pancreac) was distilled from sodium and benzophenone, pyridine was distilled and dried over 4Å molecular sieves (4Å MS), DCM (Pancreac) was distilled from P₂O₅, and H₂O and benzene (Fisher Scientific) were distilled. Trityl chloride (Acros), anhydrous Et₂O (Pancreac), PPh₃ (Merck), Pd(OH)₂/C (Aldrich, 20 wt%), anhydrous DMF (Acros), TFA (Aldrich), trichloroacetonitrile (Aldrich), triethylsilane (Fluka), TMSOTf (Aldrich), H₂SO₄ (Lab-Scan), Formic acid (Aldrich), AcCl (B&M), BnBr (Fluka), PMe₃ (Aldrich, 1M in THF), AcOH (Ajax Finechem), Ac₂O (Peking Reagent), TMSOTf (Aldrich), DiPEA (Aldrich),

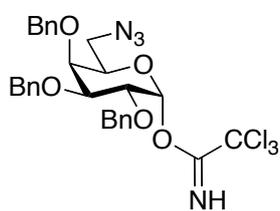
NaOMe (Janssen Chimica), phytosphingosine (TCI), C₂₅H₅₁COOH (Acros), BzCl (Aldrich, distilled and stored under argon), HBTU (Acros), PyBOP (Aldrich), EDCI (Aldrich), DMAP (Merck), sodium (Aldrich), Pd(OH)₂/C (Aldrich, 20 wt%), EtOAc (Pancreac), hexanes (Fisher Scientific), petroleum ether (Pure Science), MeOH (Pure Science), CHCl₃ (Pancreac), EtOH (absolute, Pure Science), NaOH (Pure Science), NaHCO₃ (Pure Science), NaCl (Pancreac) were used as received. All solvents were removed by evaporation under reduced pressure. Reactions were monitored by TLC-analysis on Macherey-Nagel silica gel coated plastic sheets (0.20 mm, with fluorescent indicator UV₂₅₄) with detection by UV-absorption (short wave UV – 254 nm; long wave UV – 366 nm), by dipping in 10% H₂SO₄ in EtOH followed by charring at ~150 °C, by dipping in I₂ in silica, or by dipping into a solution of ninhydrin in EtOH followed by charring at ~150 °C. Column chromatography was performed on Pure Science silica gel (40-63 micron). AccuBOND II ODS-C18 (Agilent) was used for reverse phase chromatography. Sephadex[®] LH-20 (Aldrich) was used for size exclusion chromatography. Infrared spectra were recorded as thin films using a Bruker Tensor 27 FTIR spectrometer equipped with an Attenuated Total Reflectance (ATR) sampling accessory and are reported in wave numbers (cm⁻¹). Nuclear magnetic resonance spectra were recorded at 20 °C in CD₃OD, CDCl₃, or pyridine-d₅ using either a Varian INOVA operating at 500 MHz or Varian VNMRs operating at 600 MHz. Chemical shifts are given in ppm (δ) relative to TMS. NMR peak assignments were made using COSY, HSQC and HMBC 2D experiments.



6-Azido-2,3,4-tri-*O*-benzyl-6-deoxy- α -D-galactose (59)

Methyl 6-azido-2,3,4-tri-*O*-benzyl-6-deoxy- α -D-galactoside **45**^{2,9} (360 mg, 0.74 mmol) was dissolved in aqueous acetic acid (11 mL, 80% v/v) in water followed by the addition of 1M H₂SO₄ (2.72 mL, 2.72 mmol). The reaction was stirred at 100 °C for 4.5 h, then 120 °C for a further 1.5 h. The reaction mixture was poured into ice water and extracted with EtOAc (2 x 50 mL) and the combined organic layers were washed with water (100 mL), saturated NaHCO₃ solution (2 x 100 mL) and brine (100 mL), dried (MgSO₄), filtered and concentrated *in vacuo*. The residue was purified by silica gel flash

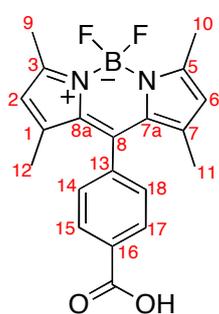
column chromatography and eluted with 5:1 (v/v) petroleum ether/EtOAc to afford lactol **59** (219 mg, 0.46 mmol, 63%) as a clear oil. R_f : 0.43 (PE/EA, 2:1, v/v); $[\alpha]_D^{25} = +9.0^\circ$ (c = 1.0, CHCl₃); IR (film) 3410, 3064, 3031, 2871, 2101, 1497, 1454, 1362, 1281, 1214, 1062, 1027, 912, 736, 697 cm⁻¹; ¹H NMR (500 MHz, CDCl₃) δ 7.45–7.31 (m, 15H, H_{arom}), 5.31 (t, $J_{1,2} = J_{1,OH} = 2.6$ Hz, 1H, H-1), 5.03 (d, $J_{a,b} = 11.2$ Hz, 1H, CH-a, 4-*O*-Bn), 4.88 (d, $J_{a,b} = 12.0$ Hz, 1H, CH-a, 2-*O*-Bn), 4.86 (d, $J_{a,b} = 11.8$ Hz, 1H, CH-a, 3-*O*-Bn), 4.81 (d, $J_{a,b} = 12.0$ Hz, 1H, CH-b, 2-*O*-Bn), 4.74 (d, $J_{a,b} = 11.8$ Hz, 1H, CH-b, 3-*O*-Bn), 4.64 (d, $J_{a,b} = 11.2$ Hz, 1H, CH-b, 4-*O*-Bn), 4.08–4.06 (m, 2H, H-2, H-5), 3.97 (dd, $J_{2,3} = 9.9$ Hz, $J_{3,4} = 2.6$ Hz, 1H, H-3), 3.86 (bs, 1H, H-4), 3.53 (dd, $J_{6a,6b} = 12.5$ Hz, $J_{5,6a} = 7.6$ Hz, 1H, H-6a), 3.33 (d, $J_{1,OH} = 2.6$ Hz, 1H, OH), 3.13 (dd, $J_{6a,6b} = 12.5$ Hz, $J_{5,6b} = 5.9$ Hz, 1H, H-6b); ¹³C NMR (125 MHz, CDCl₃) δ 138.5 (C-*i*, 2-*O*-Bn), 138.2 (C-*i*, 4-*O*-Bn), 138.1 (C-*i*, 3-*O*-Bn), 128.6, 128.53, 128.47, 128.2, 128.1, 128.0, 127.8, 127.6 (9 x C_{arom}), 91.9 (C-1), 78.6 (C-3), 76.5 (C-2), 74.74 (C-4), 74.67 (CH₂, 4-*O*-Bn), 73.6 (CH₂, 2-*O*-Bn), 73.3 (CH₂, 3-*O*-Bn), 69.7 (C-5), 51.2 (C-6); HRMS(ESI) m/z calcd. for [C₂₇H₂₉N₃O₅+Na]⁺: 498.1999, obsd.: 498.2005.



O-(6-Azido-2,3,4-tri-*O*-benzyl-6-deoxy- α -D-galactosyl) trichloroacetimidate (56**)**

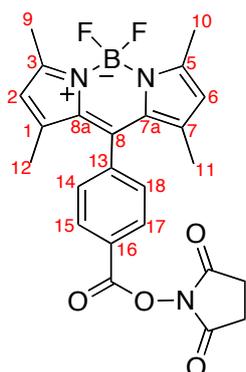
Trichloroacetonitrile (448 μ L, 4.47 mmol) and DBU (100 μ L, 0.67 mmol) were added to lactol **59** (213 mg, 0.45 mmol) (co-evaporated with toluene x 3) in dry CH₂Cl₂ (5 mL). After stirring for 1.5 h at rt, the reaction mixture was concentrated *in vacuo* and purified by silica gel flash column chromatography (silica gel was pre-washed with 1% triethylamine in 20:1 v/v petroleum ether/EtOAc). Elution with 20:1 (v/v) petroleum ether/EtOAc afforded imidate **56** (246 mg, 0.40 mmol, 89%) as a clear oil. R_f : 0.62 (PE/EA, 2:1, v/v); $[\alpha]_D^{24} = +34.0^\circ$ (c = 1.0, CHCl₃); IR (film) 3338, 3064, 3031, 2916, 2101, 1732, 1672, 1497, 1454, 1351, 1287, 1107, 1071, 1027, 838, 794, 736, 697 cm⁻¹; ¹H NMR (500 MHz, CDCl₃) δ 8.61 (s, 1H, NH), 7.41–7.29 (m, 15H, H_{arom}), 6.56 (d, $J_{1,2} = 3.3$ Hz, 1H, H-1), 5.06 (d, $J_{a,b} = 11.3$ Hz, 1H, CH-a, 4-*O*-Bn), 4.91 (d, $J_{a,b} = 11.8$ Hz, 1H, CH-a, 3-*O*-Bn), 4.81 (d, $J_{a,b} = 11.8$ Hz, 1H, CH-b, 3-*O*-Bn), 4.78 (bs, 2H, CH-a, CH-b, 2-*O*-Bn), 4.65 (d, $J_{a,b} = 11.3$ Hz, 1H, CH-b, 4-*O*-Bn), 4.27 (dd, $J_{2,3} = 10.0$ Hz, $J_{1,2} = 3.3$ Hz, 1H, H-2), 4.06–4.05

(m, 2H, H-3, H-5), 3.92 (bs, 1H, H-4), 3.53 (dd, $J_{6a,6b} = 12.6$ Hz, $J_{5,6a} = 7.4$ Hz, 1H, H-6a), 3.14 (dd, $J_{6a,6b} = 12.6$ Hz, $J_{5,6b} = 5.8$ Hz, 1H, H-6b); ^{13}C NMR (125 MHz, CDCl_3) δ 161.1 ($\text{C}=\text{NH}$), 138.4 (C-*i*, 3-*O*-Bn), 138.3 (C-*i*, 2-*O*-Bn), 138.1 (C-*i*, 4-*O*-Bn), 128.54, 128.52, 128.51 (3 x C-*m*, 2-*O*-Bn, 3-*O*-Bn, 4-*O*-Bn), 128.4 (C-*o*, 4-*O*-Bn), 127.8 (C-*m*, 3-*O*-Bn), 127.6 (C-*m*, 2-*O*-Bn), 128.1, 127.9, 127.7 (3 x C-*p*, 2-*O*-Bn, 3-*O*-Bn, 4-*O*-Bn), 94.9 (C-1), 78.0 (C-3), 75.8 (C-2), 74.9 (CH_2 , 4-*O*-Bn), 74.7 (C-4), 73.6 (CH_2 , 3-*O*-Bn), 73.1 (CH_2 , 2-*O*-Bn), 72.4 (C-5), 50.9 (C-6); HRMS(ESI) m/z calcd. for $[\text{C}_{29}\text{H}_{29}\text{N}_4\text{O}_5\text{Cl}_3+\text{Na}]^+$: 641.1096, obsd.: 641.1113.



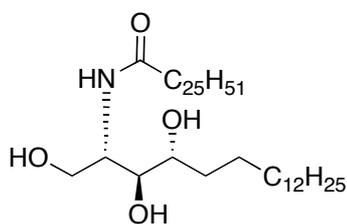
4-(4,4-Difluoro-1,3,5,7-tetramethyl-3a,4a-diaza-4-bora-s-indacen-8-yl)benzoic acid (52)

To a solution of the methyl ester **63**¹¹ (0.148 g, 0.387 mmol) in isopropanol (15 mL) under an argon atmosphere, an aqueous solution of 0.1M KOH (15 mL) was added. The reaction mixture was stirred at room temperature until complete consumption of **63** by TLC analysis was observed. The reaction mixture was then acidified with H^+ dowex until pH=2-3, filtered and evaporated to dryness *in vacuo*. The residue was purified by silica gel gradient column chromatography ($\text{CH}_2\text{Cl}_2/\text{MeOH}$, 99:1 to 96:4, v/v) to afford the BODIPY-COOH **52** (0.142 g, 0.387 mmol, quantitative). The obtained red-brown solid is stored in the dark. Mp 182.5–182.8 °C; $R_f = 0.74$ (MeOH/EtOAc, 1/4, v/v); IR (film) 2924, 1723, 1544, 1510, 1309, 1194, 1157, 983, 739 cm^{-1} ; ^1H NMR (500 MHz, CDCl_3) δ 8.25 (d, $J_{14,15} = J_{17,18} = 8.5$ Hz, 8.2 Hz, 2H, H-15, H-17), 7.44 (d, $J_{14,15} = J_{17,18} = 8.2$, 2H, H-14, H-18), 6.01 (s, 2H, H-2, H-6), 2.56 (s, 6H, H-9, H-10), 1.37 (s, 6H, H-11, H-12); ^{13}C NMR (125 MHz, CDCl_3) δ 130.9 (C-15, C-17), 128.5 (C-14, C-18), 121.5 (C-2), 14.6 (C-9, C-10, C-11, C-12); HRMS(ESI) m/z calcd. for $[\text{C}_{20}\text{H}_{19}\text{BF}_2\text{N}_2\text{O}_2\text{Na}]^+$: 391.1405, obsd.: 391.1413.



2,5-Dioxopyrrolidin-1-yl-4-(4,4-difluoro-1,3,5,7-tetramethyl-3a,4a-diaza-4-bora-s-indacen-8-yl)benzoate (70)

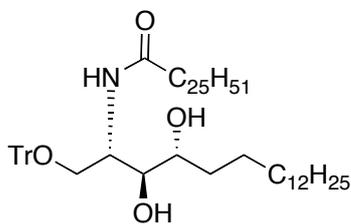
To a solution of BODIPY-COOH **52** (60.4 mg, 0.164 mmol) in CH₂Cl₂ (6.5 ml) was added NHS (37.8 mg, 0.328 mmol) and EDCI (62.9 mg, 0.328 mmol) and the solution stirred in the dark overnight. After completion of the reaction by TLC analysis, the mixture is diluted with distilled water, extracted with CH₂Cl₂ (2 x 25 mL) and washed with brine (1 x 25 mL). The combined organic phase was dried over anhydrous MgSO₄, filtered and concentrated *in vacuo*. Purification of the crude residue by silica gel gradient column chromatography (petroleum ether to CH₂Cl₂) afforded BODIPY-NHS **70** (76.0 mg, 0.163 mmol, quantitative) as a red solid. *R_f* = 0.83 (MeOH/EtOAc, 1/4, v/v); ¹H NMR (500 MHz, CDCl₃) δ 8.29 (d, *J*_{14,15} = *J*_{17,18} = 8.5 Hz, 2H, H-15, H-17), 7.51 (d, *J*_{14,15} = *J*_{17,18} = 8.5, 2H, H-14, H-18), 6.02 (s, 2H, H-2, H-6), 3.50 (s, 4H, CH₂ NHS x 2), 2.57 (s, 6H, H-9, H-10), 1.39 (s, 6H, H-11, H-12); ¹³C NMR (125 MHz, CDCl₃) δ 169.2 (C=O NHS), 160.3 (C=O BOD), 156.2 (C-3, C-5), 142.1 (C-1, C-7), 142.0 (C-13), 139.3 (C-16), 131.3 (C-15, C-17), 131.1 (C-7a, C-8a), 129.0 (C-14, C-18), 125.8 (C-8), 121.7 (C-2, C-6), 53.4 (CH₂ NHS x 2), 14.8 (C-9, C-10, C-11, C-12); HRMS(ESI) *m/z* calcd. for [C₂₄H₂₃BF₂N₃O₄]⁺: 466.1744, obsd.: 466.1740.



(2*S*,3*S*,4*R*)-2-Hexacosanoylamido-D-ribo-octadecane-1,3,4-triol (67)

Phytosphingosine **58** (200 mg, 0.63 mmol) and hexacosanoic acid (275 mg, 0.69 mmol) were co-evaporated together with toluene (x 3) and dry DMF (6.5 mL) was added followed by PyBOP (656 mg, 1.26 mmol) and DiPEA (0.28 mL, 1.58 mmol). The white suspension was stirred at rt for 7 d, at which time the solvent was removed under reduced pressure and the desired product crystallised from EtOAc/MeOH (30 mL, 1:1, v/v). The suspension was spun down in a centrifuge, the filtrate removed and the white solid washed twice with

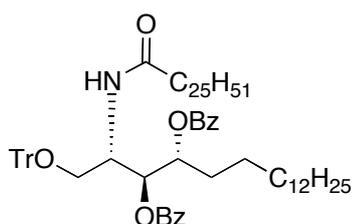
EtOAc/MeOH, before it was dried *in vacuo* to afford triol **67** as a white solid (440 mg, 0.63 mmol, quantitative). R_f : 0.60 (15% MeOH/CHCl₃, v/v, charred with cerium ammonium molybdate dip); $[\alpha]_D^{25} = -2.9^\circ$ (c = 1.0, pyridine); IR (film) 3313, 2956, 2917, 2850, 1642, 1543, 1471, 1086, 1036, 981, 717, 573 cm⁻¹; ¹H NMR (600 MHz, 10% CD₃OD in CDCl₃) 6.90 (d, $J_{NH,2} = 8.2$ Hz, 1H, NH), 3.99 (dd, $J_{NH,2} = 8.2$ Hz, $J_{1a,2} = J_{1b,2} = 4.5$ Hz, 1H, H-2), 3.71 (dd, $J_{1a,1b} = 11.4$ Hz, $J_{1a,2} = 4.5$ Hz, 1H, H-1a), 3.61 (dd, $J_{1a,1b} = 11.4$ Hz, $J_{1b,2} = 4.5$ Hz, 1H, H-1b), 3.49–3.42 (m, 2H, H-3, H-4), 2.13 (t, $J_{\alpha,\beta} = 7.6$ Hz, 2H, CH₂-α), 1.56–1.51 (m, 2H, CH₂-β), 1.36–1.30 (m, 2H, H-5), 1.61–1.56 (m, 1H, H-6a), 1.48–1.44 (m, 1H, H-6b), 1.23–1.81 (m, 66H, H-7–H-17, H-γ–H-(ω-1)), 0.80 (t, $J_{17,18} = J_{\omega-1,\omega} = 7.0$ Hz, 6H, H-18, H-ω); ¹³C NMR (150 MHz, 10% CD₃OD in CDCl₃) δ 174.6 (HNC=O), 75.6 (C-3), 72.4 (C-4), 61.1 (C-1), 51.9 (C-2), 36.5 (C-α), 33.0 (C-6), 31.84, 29.64, 29.62, 29.60, 29.57, 29.56, 29.45, 29.30, 29.26, 29.23, 22.58 (C-7–C-17, C-γ–C-(ω-1)), 25.77, 25.72 (C-5, C-β), 13.9 (C-18, C-ω); HRMS(ESI) m/z calcd. for [C₄₄H₈₉NO₄+H]⁺: 696.6864, obsd.: 696.6837.



(2S,3S,4R)-2-Hexacosanoylamido-1-triphenylmethoxy-octadecane-3,4-diol (68)

Trityl chloride (213 mg, 0.76 mmol) and DMAP (1.9 mg, 0.015 mmol) were added to triol **67** (106 mg, 0.15 mmol) (co-evaporated with toluene x 3) dissolved in dry pyridine (1.2 mL). The reaction was stirred for 20 h at rt, at which point further trityl chloride (128 mg, 0.46 mmol) and pyridine (0.7 mL) were added. Stirring was continued for 4 h, then at 40 °C for 5 h to ensure complete conversion as monitored by TLC. The reaction mixture was then diluted with warm CHCl₃ (50 mL), washed with brine (30 mL), dried (MgSO₄), filtered and concentrated *in vacuo*. The residue was purified by silica gel flash column chromatography and eluted with 0.3% MeOH/CHCl₃ to afford diol **68** (113 mg, 0.12 mmol, 79%) as a clear oil. R_f : 0.53 (PE/EA, 2:1, v/v); $[\alpha]_D^{26} = +17.0^\circ$ (c = 1.0, CHCl₃); IR (film) 3305, 2918, 2850, 1643, 1536, 1491, 1468, 1449, 1217, 1071, 900, 760, 704, 633 cm⁻¹; ¹H NMR (500 MHz, CDCl₃) δ 7.42 (d, $J_{CH-o,CH-m} = 7.3$ Hz, 6H, CPh₃, CH-*o*), 7.32 (t, $J_{CH-o,CH-m} = J_{CH-m,CH-p} = 7.6$ Hz, 6H, CPh₃, CH-*m*), 7.25 (t, $J_{CH-m,CH-p} = 7.2$ Hz, 3H, CPh₃, CH-*p*), 6.11 (d, $J_{NH,2} = 8.3$ Hz, 1H,

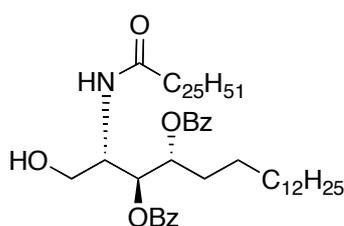
NH), 4.36 (dd, $J_{\text{NH},2} = 8.3$ Hz, $J_{1a,2} = J_{1b,2} = 4.1$ Hz, 1H, H-2), 3.60–3.59 (m, 1H, H-3), 3.51 (dd, $J_{1a,1b} = 10.0$ Hz, $J_{1a,2} = 4.1$ Hz, 1H, H-1a), 3.40–3.36 (m, 1H, H-4), 3.35 (dd, $J_{1a,1b} = 10.0$ Hz, $J_{1b,2} = 4.1$ Hz, 1H, H-1b), 3.23 (d, $J_{3,3\text{-OH}} = 8.6$ Hz, 1H, 3-OH), 2.46 (d, $J_{4,4\text{-OH}} = 4.1$ Hz, 1H, 4-OH), 2.15 (t, $J_{\alpha,\beta} = 7.7$ Hz, 2H, CH₂- α), 1.90–1.64 (m, 1H, H-5a), 1.64–1.58 (m, 2H, CH₂- β), 1.48–1.38 (m, 3H, H-6, H-5b), 1.32–1.15 (m, 66H, H-7–H-17, H- γ –H-(ω -1)), 0.89 (t, $J_{17,18} = J_{\omega-1,\omega} = 6.8$ Hz, 6H, H-18, H- ω); ¹³C NMR (125 MHz, CDCl₃) δ 173.3 (HNC=O), 143.3 (C-*i*, CPh₃), 128.6 (C-*o*, CPh₃), 128.2 (C-*m*, CPh₃), 127.5 (C-*p*, CPh₃), 87.8 (C_q CPh₃), 75.8 (C-3), 73.3 (C-4), 63.0 (C-1), 50.5 (C-2), 37.0 (C- α), 33.4 (C-5), 32.1, 29.84, 20.83, 29.82, 29.80, 29.79, 29.76, 29.6, 29.54, 29.50, 29.49, 29.48, 22.8 (C-7–C-17, C- γ –C-(ω -1)), 26.0 (C-6), 25.9 (C- β), 14.3 (C-18, C- ω); HRMS(ESI) *m/z* calcd. for [C₆₃H₁₀₃NO₄+Na]⁺: 960.7779, obsd.: 960.7781.



(2*S*,3*S*,4*R*)-3,4-Dibenzoyloxy-2-hexacosanoylamido-1-triphenylmethoxy-octadecane

Benzoyl chloride (94 μ L, 0.81 mmol) and DMAP (1.6 mg, 0.013 mmol) were added to diol **68** (126 mg, 0.13 mmol) (co-evaporated with toluene x 3) dissolved in dry pyridine (1.3 mL) and stirred at rt for 15 h. The pyridine was removed *in vacuo*, the residue redissolved in EtOAc (70 mL) and the organic layer then washed with water (70 mL), saturated NaHCO₃ solution (70 mL), brine (70 mL), dried (MgSO₄), filtered and concentrated *in vacuo*. This afforded the title compound, which was used without further purification. *R*_f: 0.49 (PE/EA, 5:1, v/v); $[\alpha]_{\text{D}}^{26} = +12.0^{\circ}$ (*c* = 1.0, CHCl₃); IR (film) 2923, 2853, 1719, 1671, 1540, 1450, 1315, 1280, 1109, 1069, 1027, 757, 707, 632 cm⁻¹; ¹H NMR (500 MHz, CDCl₃) δ 7.96 (d, $J_{\text{CH-}o,\text{CH-}m} = 7.5$ Hz, 2H, CH-*o*, 4-*O*-Bz), 7.89 (d, $J_{\text{CH-}o,\text{CH-}m} = 7.5$ Hz, 2H, CH-*o*, 3-*O*-Bz), 7.59 (t, $J_{\text{CH-}m,\text{CH-}p} = 7.3$ Hz, 1H, CH-*p*, 3-*O*-Bz), 7.55 (t, $J_{\text{CH-}m,\text{CH-}p} = 7.6$ Hz, 1H, CH-*p*, 4-*O*-Bz), 7.42 (t, $J_{\text{CH-}o,\text{CH-}m} = J_{\text{CH-}m,\text{CH-}p} = 8.3$ Hz, 2H, CH-*m*, 3-*O*-Bz), 7.41 (t, $J_{\text{CH-}o,\text{CH-}m} = J_{\text{CH-}m,\text{CH-}p} = 8.3$ Hz, 2H, CH-*m*, 4-*O*-Bz), 7.31–7.30 (m, 6H, CPh₃, CH-*o*), 7.15–7.20 (m, 9H, CPh₃, CH-*m*, CH-*p*), 6.03 (d, $J_{\text{NH},2} = 9.3$ Hz, 1H, NH), 5.81 (dd, $J_{2,3} = 9.1$ Hz, $J_{3,4} = 2.6$ Hz, 1H, H-3), 5.35 (dt, $J_{4,5} = 10.1$ Hz, $J_{3,4} = 2.6$ Hz, 1H, H-4), 4.59 (tt, $J_{2,\text{NH}} = J_{2,3} = 9.3$ Hz, $J_{1a,2} = J_{1b,2} = 3.2$ Hz, 1H, H-2), 3.34 (dd,

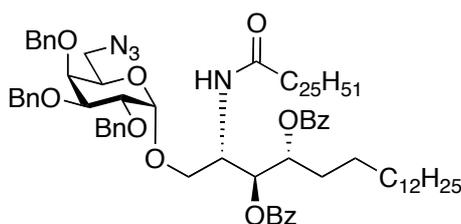
$J_{1a,1b} = 9.9$ Hz, $J_{1a,2} = 3.2$ Hz, 1H, H-1a), 3.29 (dd, $J_{1a,1b} = 9.9$ Hz, $J_{1b,2} = 3.2$ Hz, 1H, H-1b), 2.18 (dt, $J_{a,b} = 14.5$ Hz, $J_{\alpha,\beta} = 7.7$ Hz, 2H, CH₂- α), 1.93–1.81 (m, 2H, H-5), 1.68–1.58 (m, 2H, CH₂- β), 1.40–1.14 (m, 68H, H-6–H-17, H- γ –H-(ω -1)), 0.89 (t, $J_{17,18} = J_{\omega-1,\omega} = 7.0$ Hz, 6H, H-18, H- ω); ¹³C NMR (125 MHz, CDCl₃) δ 172.8 (HNC=O), 166.4 (C=O, 4-*O*-Bz), 165.0 (C=O, 3-*O*-Bz), 143.3 (C-*i*, CPh₃), 133.1 (C-*p*, 3-*O*-Bz), 132.9 (C-*p*, 4-*O*-Bz), 130.2 (C-*i*, 4-*O*-Bz), 129.82 (C-*i*, 3-*O*-Bz), 129.78 (C-*o*, 3-*O*-Bz), 129.75 (C-*o*, 4-*O*-Bz), 128.5 (C-*o*, CPh₃), 128.40 (C-*m*, 3-*O*-Bz), 128.35 (C-*m*, 4-*O*-Bz), 127.8 (C-*m*, CPh₃), 127.0 (C-*p*, CPh₃), 86.8 (C_q CPh₃), 74.2 (C-4), 72.7 (C-3), 61.6 (C-1), 48.5 (C-2), 36.9 (C- α), 31.9, 29.74, 29.73, 29.72, 29.69, 29.68, 29.67, 29.66, 29.63, 29.58, 29.55, 29.53, 29.45, 29.39, 29.38, 25.72 (C-6–C-17, C- γ –C-(ω -1)), 28.4 (C-5), 25.70 (C- β), 14.1 (C-18, C- ω); HRMS(ESI) *m/z* calcd. for [C₇₇H₁₁₁NO₆+Na]⁺: 1168.8304, obsd.: 1168.8302.



(2S,3S,4R)-3,4-Dibenzoyloxy-2-hexacosanoylamido-octadecan-1-ol (57)

(2S,3S,4R)-3,4-Dibenzoyloxy-2-hexacosanoylamido-1-triphenylmethoxy-octadecane was dissolved in 1:1 (v/v) MeOH/CH₂Cl₂ and *p*TsOH.H₂O (7.7 mg, 0.040 mmol) was added. The reaction was stirred for 20 h at rt, followed by the addition of further *p*TsOH.H₂O (7.7 mg, 0.040 mmol), and stirring was continued for 6 h. The reaction mixture was diluted with EtOAc (70 mL), washed with saturated NaHCO₃ solution (2 x 70 mL), brine (70 mL), dried (MgSO₄), filtered and concentrated *in vacuo*. The crude mixture was purified by silica gel flash column chromatography and eluted with MeOH/CH₂Cl₂ (3%, v/v) to afford the lipid acceptor **57** as an amorphous white solid (81 mg, 0.090 mmol, 67% over two steps). *R_f*: 0.25 (PE/EA, 2:1, v/v); [α]_D²⁷ = +54.0° (c = 1.0, CHCl₃); IR (film) 2918, 2850, 1721, 1649, 1541, 1468, 1315, 1280, 1177, 1111, 1070, 1027, 757, 710 cm⁻¹; ¹H NMR (500 MHz, CDCl₃) δ 8.05 (d, $J_{CH-o,CH-m} = 7.7$ Hz, 2H, CH-*o*, 3-*O*-Bz), 7.94 (d, $J_{CH-o,CH-m} = 7.5$ Hz, 2H, CH-*o*, 4-*O*-Bz), 7.63 (t, $J_{CH-m,CH-p} = 7.7$ Hz, 1H, CH-*p*, 3-*O*-Bz), 7.52 (t, $J_{CH-m,CH-p} = 7.5$ Hz, 1H, CH-*p*, 4-*O*-Bz), 7.49 (t, $J_{CH-m,CH-p} = J_{CH-o,CH-m} = 7.7$ Hz, 2H, CH-*m*, 3-*O*-Bz), 7.37 (t, $J_{CH-m,CH-p} = J_{CH-o,CH-m} = 7.5$ Hz, 2H, CH-*m*, 4-*O*-Bz), 6.48 (d, $J_{NH,2} = 9.3$ Hz, 1H, NH),

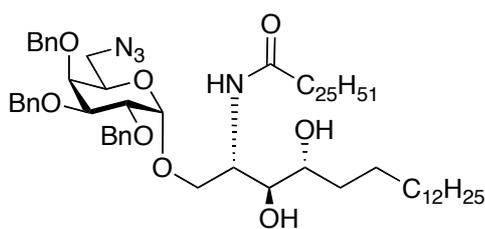
5.44 (dd, $J_{2,3} = 9.6$ Hz, $J_{3,4} = 2.3$ Hz, 1H, H-3), 5.37 (dt, $J_{4,5} = 9.1$ Hz, $J_{3,4} = 2.9$ Hz, 1H, H-4), 4.40 (t, $J_{2,3} = 9.6$ Hz, 1H, H-2), 3.68–3.59 (m, 2H, H-1a, H-1b), 2.91 (dd, $J_{\text{OH},1a} = 8.5$ Hz, $J_{\text{OH},1b} = 5.1$ Hz, 1H, OH), 2.28 (t, $J_{\alpha,\beta} = 7.6$ Hz, 2H, CH₂- α), 2.03–2.00 (m, 2H, H-5), 1.72–1.66 (m, 2H, CH₂- β), 1.45–1.13 (m, 68H, H-6–H-17, H- γ –H-(ω -1)), 0.88 (t, $J_{17,18} = J_{\omega-1,\omega} = 7.0$ Hz, 6H, H-18, H- ω); ¹³C NMR (125 MHz, CDCl₃) δ 173.3 (HNC=O), 167.1 (C=O, 3-*O*-Bz), 166.3 (C=O, 4-*O*-Bz), 133.8 (C-*p*, 3-*O*-Bz), 133.1 (C-*p*, 4-*O*-Bz), 130.0 (C-*o*, 3-*O*-Bz), 129.9 (C-*i*, 4-*O*-Bz), 129.6 (C-*o*, 4-*O*-Bz), 129.1 (C-*i*, 3-*O*-Bz), 128.7 (C-*m*, 3-*O*-Bz), 128.4 (C-*m*, 4-*O*-Bz), 74.0 (C-4), 73.6 (C-3), 61.6 (C-1), 49.9 (C-2), 36.9 (C- α), 31.9, 29.73, 29.71, 29.68, 29.66, 29.65, 29.60, 29.58, 29.55, 29.40, 29.37, 29.34, 25.85, 22.7 (C-6–C-17, C- γ –C-(ω -1)), 28.4 (C-5), 25.75 (C- β), 14.1 (C-18, C- ω); HRMS(ESI) *m/z* calcd. for [C₅₈H₉₇NO₆+Na]⁺: 926.7208, obsd.: 926.7220.



(2*S*,3*S*,4*R*)-3,4-Dibenzoyloxy-1-(2,3,4-tri-*O*-benzyl-6-deoxy-6-azido- α -D-galactopyranosyloxy)-2-hexacosanoylamido-octadecane (55**)**

Imidate donor **56** (88 mg, 0.14 mmol) and lipid acceptor **57** (64 mg, 0.071 mmol) were co-evaporated with toluene (x 3), dissolved in dry Et₂O/THF (2.7 mL, 5:1, v/v) and stirred with activated 4Å molecular sieves for 1 h. The reaction mixture was cooled to 0 °C, freshly distilled TMSOTf (stock of 0.055M in Et₂O, 76.8 μ L, 0.0042 mmol) was added dropwise and the resulting solution stirred at 0 °C for 1.5 h. The reaction was quenched with triethylamine (50 μ L, 0.36 mmol), diluted with EtOAc (20 mL), filtered through a celite pad and concentrated *in vacuo*. The residue was first purified by size exclusion column chromatography (LH20, CH₂Cl₂/MeOH, 1:1, v/v) to isolate a mixture of α - and β -coupled glycolipid, which was further purified by silica gel gradient column chromatography (petroleum ether/EtOAc, 15:1 to 5:1, v/v) to give the α -coupled glycolipid **55 α** as a colourless oil (65.7 mg, 0.048 mmol, 68%) and the β -coupled glycolipid **55 β** (22.6 mg, 0.017 mmol, 23%) also as a colourless oil. α : *R_f*: 0.58 (8% EA/Toluene); $[\alpha]_D^{27} = -36.0^\circ$ (*c* = 1.0, CHCl₃); IR (film) 3063, 3030, 2922, 2852, 2362, 2338, 2102, 1722, 1673,

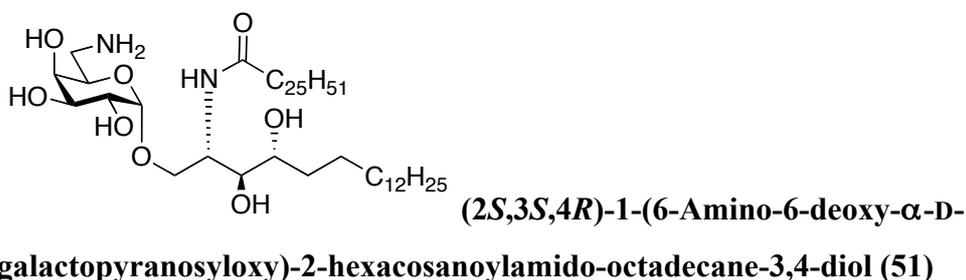
1530, 1453, 1278, 1095, 1068, 1027, 756, 711, 699 cm^{-1} ; ^1H NMR (500 MHz, CDCl_3) δ 8.03 (d, $J_{\text{CH-}o,\text{CH-}m} = 7.1$ Hz, 2H, CH-*o*, 3-*O*-Bz), 7.96 (d, $J_{\text{CH-}o,\text{CH-}m} = 7.0$ Hz, 2H, CH-*o*, 4-*O*-Bz), 7.60 (t, $J_{\text{CH-}m,\text{CH-}p} = 7.5$ Hz, 1H, CH-*p*, 3-*O*-Bz), 7.54 (t, $J_{\text{CH-}m,\text{CH-}p} = 7.5$ Hz, 1H, CH-*p*, 4-*O*-Bz), 7.48–7.19 (m, 19H, H_{arom}), 6.66 (d, $J_{\text{NH},2} = 9.5$ Hz, 1H, NH), 5.64 (dd, $J_{2,3} = 9.3$ Hz, $J_{2,3} = 2.7$ Hz, 1H, H-3), 5.39–5.36 (m, 1H, H-4), 4.96 (d, $J_{a,b} = 11.5$ Hz, 1H, CH-*a*, 4'-*O*-Bn), 4.78 (d, $J_{a,b} = 11.7$ Hz, 1H, CH-*a*, 3'-*O*-Bn), 4.75 (d, $J_{1',2'} = 3.6$ Hz, 1H, H-1'), 4.78 (d, $J_{a,b} = 11.7$ Hz, 1H, CH-*a*, 3'-*O*-Bn), 4.68 (d, $J_{a,b} = 11.7$ Hz, 1H, CH-*b*, 3'-*O*-Bn), 4.64 (s, 2H, CH-*a*, CH-*b*, 2'-*O*-Bn), 4.61 (t, $J_{1,2} = 3.2$ Hz, 1H, H-2), 4.57 (d, $J_{a,b} = 11.5$ Hz, 1H, CH-*b*, 4'-*O*-Bn), 3.98 (dd, $J_{2',3'} = 10.1$ Hz, $J_{1',2'} = 3.6$ Hz, 1H, H-2'), 3.93 (dd, $J_{5',6'a} = 8.3$ Hz, $J_{5',6'b} = 5.2$ Hz, 1H, H-5'), 3.90 (dd, $J_{1a,1b} = 11.6$ Hz, $J_{1a,2} = 3.2$ Hz, 1H, H-1a), 3.83 (dd, $J_{2',3'} = 10.1$ Hz, $J_{3',4'} = 2.7$ Hz, 1H, H-3'), 3.78 (bs, 1H, H-4'), 3.67 (dd, $J_{1a,1b} = 11.6$ Hz, $J_{1b,2} = 3.2$ Hz, 1H, H-1b), 3.55 (dd, $J_{6'a,6'b} = 12.4$ Hz, $J_{5',6'a} = 8.3$ Hz, 1H, H-6'a), 3.04 (dd, $J_{6'a,6'b} = 12.4$ Hz, $J_{5',6'b} = 5.2$ Hz, 1H, H-6'a), 2.20 (t, $J_{\alpha,\beta} = 7.6$ Hz, 2H, $\text{CH}_2\text{-}\alpha$), 1.95–1.90 (m, 2H, H-5), 1.67–1.64 (m, 2H, H β), 1.39–1.21 (m, 68H, H-6–H-17, H- γ -H-(ω -1)), 0.89 (t, $J_{17,18} = J_{\omega-1,\omega} = 6.9$ Hz, 6H, H-18, H- ω); ^{13}C NMR (125 MHz, CDCl_3) δ 173.1 (HNC=O), 166.2 (C=O, 4-*O*-Bz), 165.3 (C=O, 3-*O*-Bz), 138.6 (C-*i*, 3'-*O*-Bn), 138.2 (C-*i*, 2'-*O*-Bn), 138.0 (C-*i*, 4'-*O*-Bn), 133.3 (C-*p*, 3-*O*-Bz), 132.9 (C-*p*, 4-*O*-Bz), 130.1, 129.8 (C-*i*, 3-*O*-Bz, 4-*O*-Bz), 129.8 (C-*o*, 3-*O*-Bz), 129.8 (C-*o*, 4-*O*-Bz), 128.6, 128.5, 128.42, 128.41, 128.34, 128.32, 128.1, 128.0, 127.7, 127.62, 127.56 (21 x CH_{arom}), 99.7 (C-1', $^1J_{\text{CH}} = 168.5$ Hz), 78.5 (C-3'), 76.4 (C-2'), 74.8 (C-4'), 74.6 (CH_2 , 4'-*O*-Bn), 73.8 (C-4), 73.5 (CH_2 , 3'-*O*-Bn), 73.3 (CH_2 , 2'-*O*-Bn), 72.4 (C-3), 70.2 (C-5'), 69.7 (C-1), 51.3 (C-6'), 48.6 (C-2), 36.8 (C- α), 28.3 (C-5), 25.8 (C- β), 31.9, 29.8, 29.72, 29.70, 29.67, 29.64, 29.61, 29.60, 29.56, 29.50, 29.4, 25.7, 22.7 (C-6–C-17, C- γ -C-(ω -1)), 14.1 (C-18, C- ω); HRMS(ESI) m/z calcd. for $[\text{C}_{85}\text{H}_{124}\text{N}_4\text{O}_{10}+\text{H}]^+$: 1361.9390, obsd.: 1361.9399.



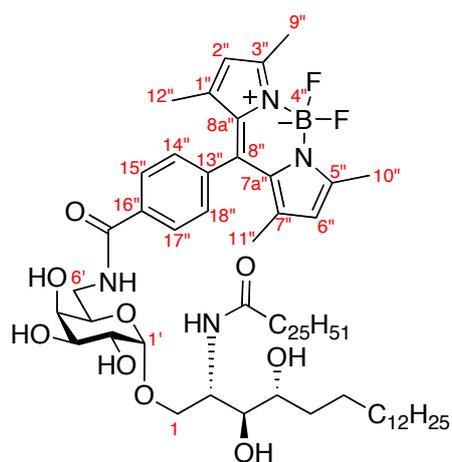
**(2*S*,3*S*,4*R*)-2-Hexacosanoylamido-
(2,3,4-tri-*O*-benzyl-6-deoxy-6-azido- α -
D-galactopyranosyloxy)-octadecane-
3,4-diol (**69**)**

Glycolipid **55 α** (66 mg, 0.048 mmol) was dissolved in MeOH/CH₂Cl₂ (3 mL, 2:1 (v/v)) and sodium methoxide was added until the reaction mixture reached pH 9.0. After stirring for 12 h at rt, the reaction was quenched with Dowex-H⁺, filtered and concentrated under reduced pressure. The residue was purified by silica gel gradient column chromatography (petroleum ether/EtOAc, 10:1 to 3:1, v/v) to afford glycolipid diol **69** as an amorphous white solid (52 mg, 0.045 mmol, 93%). *R*_f: 0.28 (PE/EtOAc, 2:1, v/v); [α]_D²⁴ = +6.0° (c = 1.0, CHCl₃); IR (film) 3332, 3065, 3032, 2918, 2850, 2100, 1717, 1636, 1623, 1542, 1469, 1455, 1350, 1284, 1216, 1078, 1041, 909, 757, 696 cm⁻¹; ¹H NMR (500 MHz, CDCl₃) δ 7.40–7.29 (m, 15H, H_{arom}), 6.29 (d, *J*_{NH,2} = 8.3 Hz, 1H, NH), 4.98 (d, *J*_{a,b} = 11.3 Hz, 1H, CH-a, 4'-*O*-Bn), 4.884 (d, *J*_{a,b} = 11.6 Hz, 1H, CH-a, 2'-*O*-Bn), 4.885 (d, *J*_{1,2} = 3.7 Hz, 1H, H-1'), 4.82 (d, *J*_{a,b} = 11.7 Hz, 1H, CH-a, 3'-*O*-Bn), 4.78 (d, *J*_{a,b} = 11.7 Hz, 1H, CH-b, 3'-*O*-Bn), 4.70 (d, *J*_{a,b} = 11.7 Hz, 1H, CH-b, 2'-*O*-Bn), 4.59 (d, *J*_{a,b} = 11.3 Hz, 1H, CH-b, 4'-*O*-Bn), 4.27 (dd, *J*_{NH,2} = 8.3 Hz, *J*_{1a,2} = 3.4 Hz, 1H, H-2), 4.05 (dd, *J*_{2',3'} = 10.0 Hz, *J*_{1',2'} = 3.7 Hz, 1H, H-2'), 3.93 (dd, *J*_{1a,1b} = 10.4 Hz, *J*_{1a,2} = 3.4 Hz, 1H, H-1a), 3.86 (dd, *J*_{2',3'} = 10.0 Hz, *J*_{3',4'} = 2.4 Hz, 1H, H-3'), 3.84–3.82 (m, 2H, H-1b, H-4'), 3.73 (t, *J*_{5',6'} = 6.6 Hz, 1H, H-5'), 3.52–3.46 (m, 3H, H-4, H-6'a, H-3), 3.03 (dd, *J*_{6'a,6'b} = 12.6 Hz, *J*_{5',6'b} = 5.5 Hz, 1H, H-6'b), 2.16 (t, *J* _{α,β} = 7.6 Hz, 2H, CH₂- α), 1.60–1.50 (m, 3H, CH₂- β a, H-5), 1.48–1.21 (m, 69H, H-6–H-17, H- β b–H-(ω -1)), 0.88 (t, *J*_{17,18} = *J* _{$\omega-1,\omega$} = 7.0 Hz, 6H, H-18, H- ω); ¹³C NMR (125 MHz, CDCl₃) δ 173.9 (HNC=O), 138.2 (C-*i*, 3'-*O*-Bn), 138.0 (C-*i*, 4'-*O*-Bn), 137.8 (C-*i*, 2'-*O*-Bn), 128.69, 128.67, 128.6, 128.5, 128.3, 128.2, 128.1, 128.0, 127.7 (15 x CH_{arom}), 99.0 (C-1'), 79.3 (C-3'), 76.3 (C-3), 75.9 (C-2'), 74.8 (CH₂, 4'-*O*-Bn), 74.5 (C-4'), 74.4 (CH₂, 2'-*O*-Bn), 73.5 (C-4), 73.2 (CH₂, 3'-*O*-Bn), 70.4 (C-5'), 69.9 (C-1), 51.2 (C-6'), 49.2 (C-2), 37.0 (C- α), 33.5, 32.1, 29.9, 29.85, 29.81, 29.7, 29.6, 29.51, 29.46, 22.8 (C-6–C-17, C- γ –C-(ω -1)), 26.0, 25.9 (C-5, C- β), 14.3 (C-18,

C- ω); HRMS(ESI) m/z calcd. for $[C_{71}H_{117}N_4O_8+H]^+$: 1153.8866, obsd.: 1153.8871.



To a solution of glycolipid diol **69** (25.1 mg, 0.022 mmol) dissolved in $CHCl_3/EtOH$ (2.5 ml, 3:2, v/v) was added formic acid (0.13 mL, 0.0034 mmol) and $Pd(OH)_2/C$ (20 wt% loading, 50 wt% H_2O , 36 mg, 0.033 mmol) and the reaction stirred under H_2 (g) for 15 h at rt. The reaction mixture was filtered through a celite pad and washed successively with $CHCl_3/EtOH/MeOH$ (20 mL, 3:1:1, v/v/v) and pyridine (10 mL) and concentrated *in vacuo*. The residue was semi-purified by silica gel column chromatography ($CH_2Cl_2 \rightarrow CH_2Cl_2/MeOH/EtOH/NH_3$ (35% aq) \rightarrow 55:2:2:1 \rightarrow 35:2:2:1 \rightarrow 15:2:2:1, v/v/v/v) to afford amine **51** as an amorphous white solid (6.2 mg, 0.0072 mmol, 33%). R_f : 0.50 ($DCM/MeOH/EtOH/NH_3 \cdot H_2O$, 5:2:2:1, v/v/v/v); 1H NMR (500 MHz, 10% CD_3OD in $CDCl_3$) δ 4.84 (d, $J_{1',2'} = 3.9$ Hz, 1H, H-1'), 4.12 (dd, $J_{NH,2} = 9.1$ Hz, $J_{1a,2} = J_{1b,2} = 4.9$ Hz, 1H, H-2), 3.82–3.79 (m, 2H, H-1a, H-4'), 4.74–4.73 (m, 1H, H-5'), 3.71 (dd, $J_{2',3'} = 10.1$ Hz, $J_{1',2'} = 3.9$ Hz, 1H, H-2'), 3.63 (dd, $J_{2',3'} = 10.1$ Hz, $J_{3',4'} = 3.4$ Hz, 1H, H-3'), 3.53 (dd, $J_{1a,1b} = 10.6$ Hz, $J_{1b,2} = 4.9$ Hz, 1H, H-1b), 3.47–3.42 (m, 2H, H-3, H-4), 3.01 (dd, $J_{6'a,6'b} = 13.0$ Hz, $J_{5,6'a} = 7.6$ Hz, 1H, H-6'a), 2.85 (dd, $J_{6'a,6'b} = 13.0$ Hz, $J_{5,6'b} = 3.6$ Hz, 1H, H-6'b), 2.12 (t, $J_{\alpha,\beta} = 7.6$ Hz, 2H, $CH_2-\alpha$), 1.63–1.47 (m, 4H, $CH_2-\beta$, H-5), 1.30–1.06 (m, 68H, H-6–H-17, H- γ –H-($\omega-1$)), 0.80 (t, $J_{17,18} = J_{\omega-1,\omega} = 6.9$ Hz, 6H, H-18, H- ω); HRMS(ESI) m/z calcd. for $[C_{50}H_{100}N_2O_8+H]^+$: 857.7552, obsd.: 857.7533.



(2*S*,3*S*,4*R*)-1-(6-Deoxy-6-[4-(4,4-difluoro-1,3,5,7-tetramethyl-3a,4a-diaza-4-bora-s-indacen-8-yl)benzylamido])- α -D-galactopyranosyloxy)-2-hexacosanoylamido-octadecane-3,4-diol (3**)**

Amine **51** (4.1 mg, 0.0048 mmol) was co-evaporated with DMF (x 3), dissolved in dry DMF (0.6 mL) and the solution was warmed to 45 °C followed by the addition of DiPEA (3.3 μ L, 0.019 mmol). BODIPY-NHS **70** (9.2 mg, 0.020 mmol) was co-evaporated with DMF (x 3) and added as a solution in DMF (0.6 mL) and stirred for 1 h at 45 °C, at which point additional BODIPY-NHS **70** (4.6 mg, 0.010 mmol) in dry CH₂Cl₂ (0.6 mL) and DiPEA (3.3 μ L, 0.019 mmol) was added and the reaction mixture stirred for further 14 h at 45 °C. The reaction mixture was then concentrated *in vacuo* and purified immediately by silica gel gradient column chromatography (CH₂Cl₂ to 3% MeOH in CH₂Cl₂) to afford BODIPY- α -GalCer **3** as a red solid (2.5 mg, 0.0021 mmol, 44%). *R*_f: 0.38 (10% MeOH/DCM, v/v); IR (film) 3346, 2851, 1642, 1631, 1608, 1547, 1513, 1468, 1450, 1409, 1308, 1192, 1157, 1071, 1044, 983, 759, 700, 635 cm⁻¹; ¹H NMR (600 MHz, pyridine-d₅) δ 9.74 (t, $J_{6'a,NH} = J_{6'b,NH} = 5.6$ Hz, 1H, NH-BOD), 8.67 (d, $J_{2,NH} = 8.5$ Hz, 1H, NH-lipid), 8.46 (d, $J_{14'',15''} = J_{17'',18''} = 8.2$ Hz, 2H, H-15'', H-17''), 7.34 (d, $J_{14'',15''} = J_{17'',18''} = 8.2$ Hz, 2H, H-14'', H-18''), 5.99 (s, 2H, H-2'', H-6''), 5.57 (d, $J_{1',2'} = 3.9$ Hz, 1H, H-1'), 5.29–5.27 (m, 1H, H-2), 4.70 (t, $J_{5',6'a} = J_{5',6'b} = 6.3$ Hz, 1H, H-5'), 4.68–4.65 (2H, H-1a, H-2'), 4.49 (dd, $J_{5',6'a} = 6.3$ Hz, $J_{6'a,6'b} = 13.4$ Hz, 1H, H-6'a), 4.46 (d, $J_{3',4'} = 1.3$ Hz, 1H, H-4'), 4.40–4.37 (m, 2H, H-1b, H-3'), 4.35–4.23 (m, 2H, H-3, H-4), 4.22–4.19 (m, 1H, H-6'b), 2.71 (s, 6H, H-9'', H-10''), 2.48 (t, $J_{\alpha,\beta} = 5.6$ Hz, 2H, CH₂- α), 2.30–2.27 (m, 1H, CH₂), 1.97–1.91 (m, 3H, H-5, CH₂), 1.85–1.80 (m, 3H, CH₂- β , CH₂), 1.71–1.69 (m, 2H, CH₂), 1.36 (s, 6H, H-11'', H-12''), 1.45–1.17 (m, 63H, H-6–H-17, H- γ -H-(ω -1)), 0.88 (t, 6H, H-18, H- ω); ¹³C NMR (150 MHz, pyridine-d₅) δ 173.7 (HNC=O lipid), 168.1 (HNC=O BOD), 156.2 (C-3'', C-5''), 143.7 (C-7a'', C-8a''), 141.9 (C-8''), 138.1 (C-13''), 136.9 (C-16''), 131.8 (C-1'', C-7''), 129.3 (C-15'', C-17''), 128.8 (C-14'', C-18''),

122.2 (C-2'', C-6''), 77.1 (C-3/C-4), 72.9 (C-3/C-4), 71.64 (C-3'), 71.57 (C-4'), 70.8 (C-5'), 70.4 (C-2'), 69.1 (C-1), 51.8 (C-2), 72.3 (C-6'), 37.2 (C- α), 34.7, 32.5, 30.8, 30.5, 30.41, 30.40, 30.39, 30.37, 30.36, 30.34, 30.33, 30.29, 30.27, 30.26, 30.18, 30.14, 29.98, 29.97, 23.29 (C-6-C-17, C- γ -C-(ω -1)), 26.89 (C-5), 26.79 (C- β), 15.1, 14.9 (C-9'', C-10'', C-11'', C-12''), 14.6 (C-18, C- ω); HRMS(ESI) m/z calcd. for $[\text{C}_{70}\text{H}_{117}\text{BF}_2\text{N}_4\text{O}_9+\text{H}]^+$: 1207.8954, obsd.: 1207.8958.

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iNKT cell is able to “bulldoze” the protruding headgroup so that it sits flat against the α 2-helix of CD1d.^{2,3} The internal glycosidic bond is thus forced to resemble the α -conformation. Indeed, the crystal structure studies reveal that the terminal galactose sugar forms crucial interactions with CD1d. Specifically, the 6'''-hydroxyl of iGb3 forms a hydrogen bond with Thr159 of CD1d, and both the 4'''- and 6'''-hydroxyls partake in van der Waals interactions with Thr159 and Met162 (Figure 4.2).

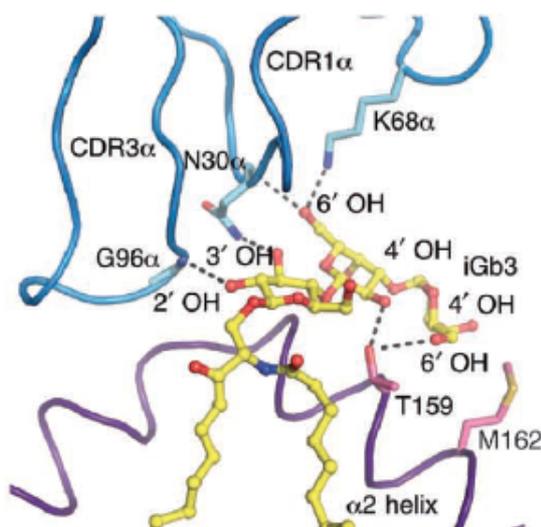


Figure 4.2. Crystal structure of iNKT cell TCR (blue) in engagement with iGb3 (yellow) and murine CD1d (purple).² (Adapted by permission from Macmillan Publishers Ltd: Nature Immunology, 2011, 12, 827-833, ©2011)

To unambiguously ascertain the importance of the 6'''-hydroxyl in iGb3 binding and the activation of iNKT cells, the synthesis of 6'''-deoxy-iGb3-sphingosine **5** (Figure 4.3) was envisioned so that its activity as an iNKT cell ligand can be established. 6'''-deoxy-iGb3-sphingosine **5** will also allow for crystal structure studies to be performed by the groups of Jamie Rossjohn and Dale Godfrey with whom we are collaborating. A similar derivative, the 6'''-deoxy-iGb3-phytosphingosine,⁴ which contains the lipid found in α -GalCer, has been synthesised by Chen *et al.* but no crystal structure data was presented. Herein, however, it is envisioned that our target 6'''-deoxy homologue **5**, bearing the same sphingosine backbone as the parent iGb3, will be a more useful tool to

systematically probe the role of the terminal sugar without changing the lipid portion.

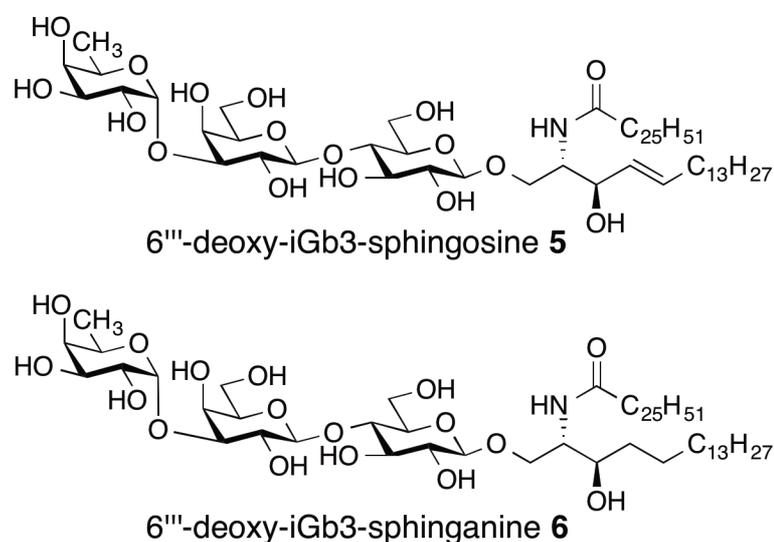


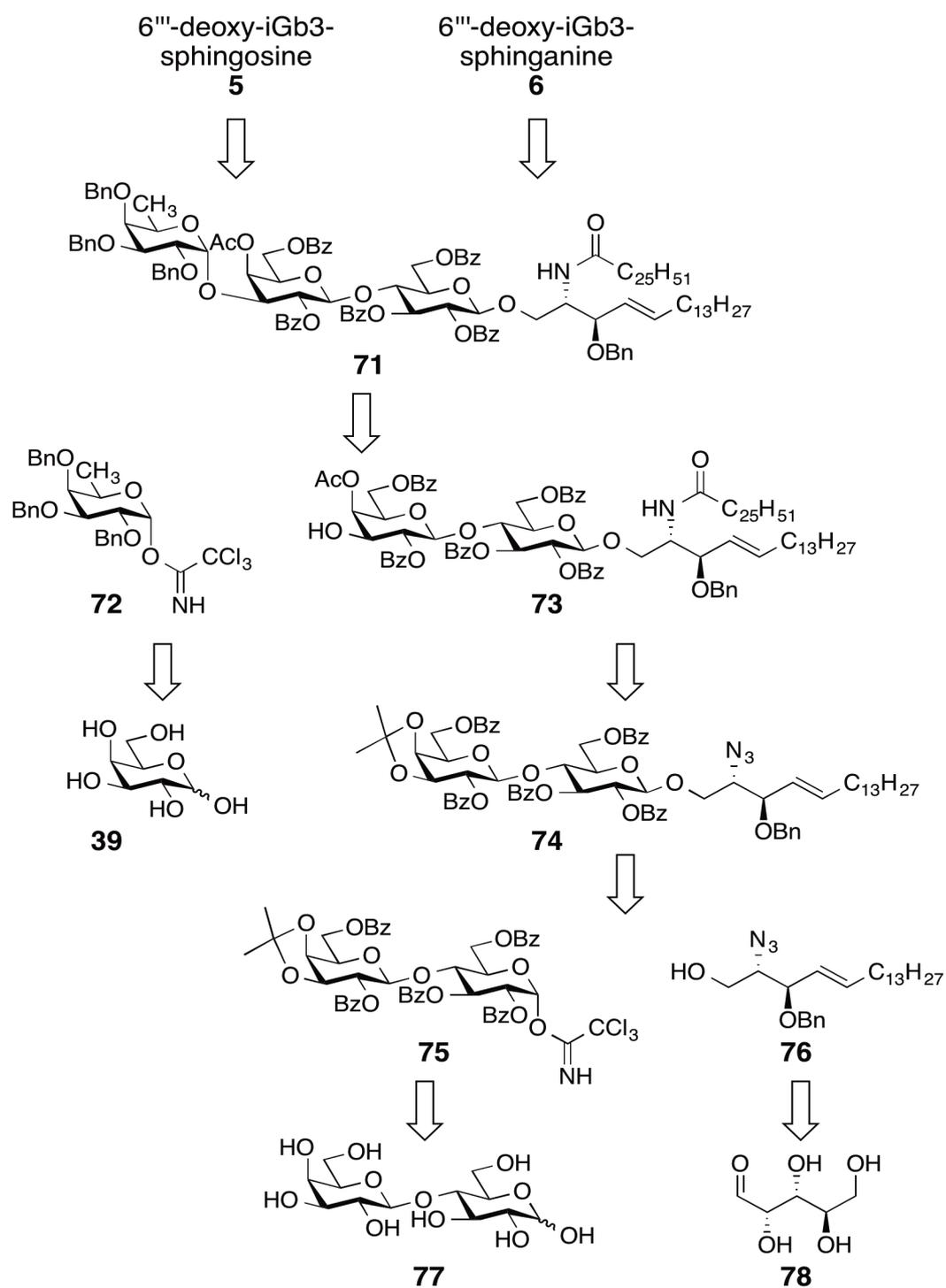
Figure 4.3. Target iGb3 analogues, **5** and **6**.

In addition to the 6'''-deoxy homologue **5**, the 6'''-deoxy-iGb3-sphinganine **6** (Figure 4.3) is also a useful tool to determine if removal of the rigid *E*-alkene on the lipid backbone alters activity of the glycolipid. Hereby, it should also be noted that the iGb3-sphinganine analogue (with galactose as the terminal sugar) has been synthesised and found to activate iNKT cells more efficiently than the original iGb3-sphingosine.⁵ Various studies have also shown that changes to the lipid backbone of a glycolipid can alter the presentation of the sugar head group.⁶⁻⁸ Thus, the biological assessment of 6'''-deoxy-iGb3-sphinganine **6** will help determine if the loss of rigidity in the lipid backbone will compensate for the loss of H-bond through the 6'''-hydroxyl of the terminal sugar.

4.2 Synthesis of 6'''-deoxy homologues of iGb3

Retrosynthesis

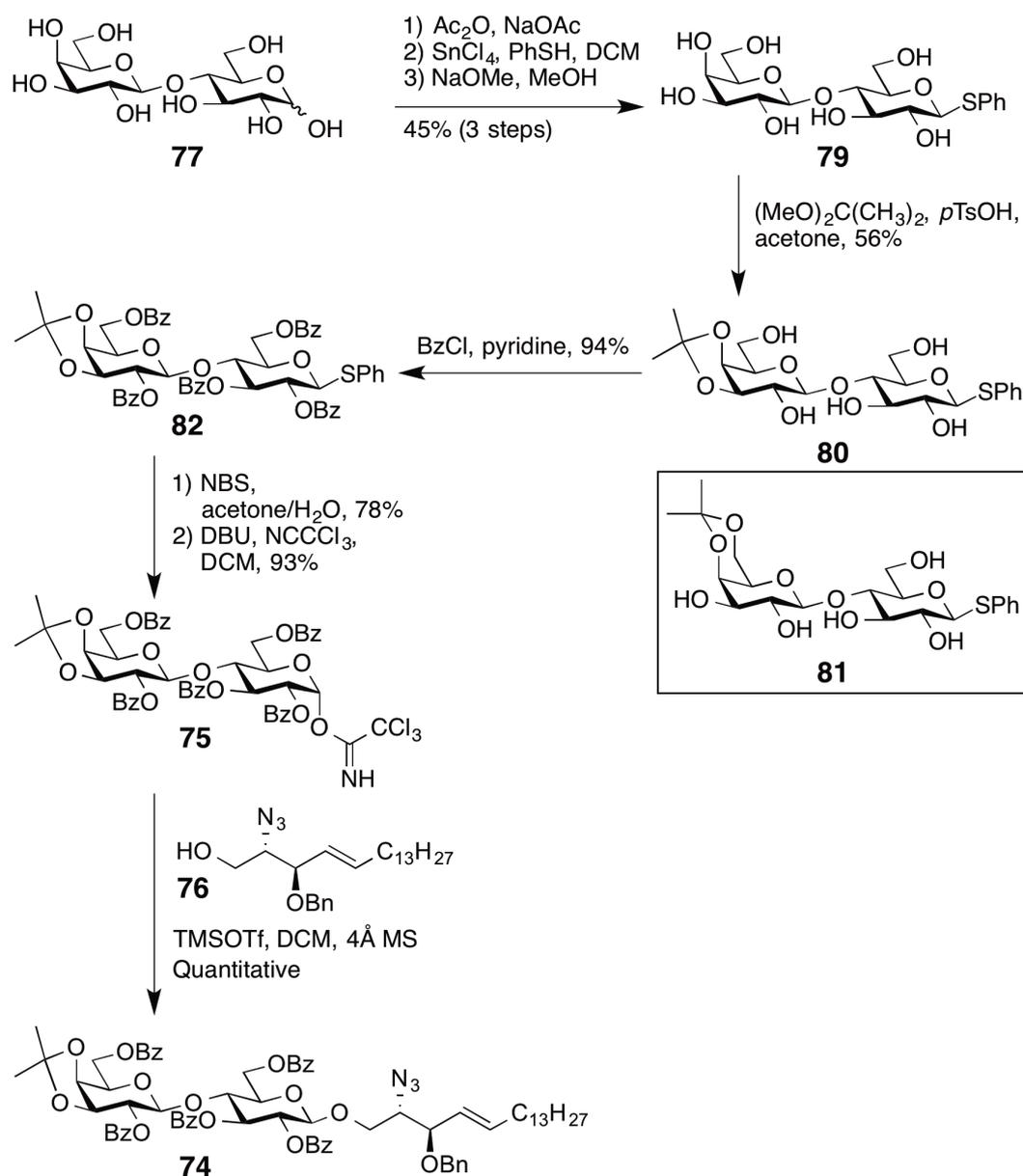
To synthesise 6'''-deoxy-iGb3-sphingosine **5** and 6'''-deoxy-iGb3-sphinganine **6** a convergent strategy was employed whereby the fully protected 6'''-deoxy-iGb3-sphingosine derivative **71** could be selectively deprotected to give either target (Scheme 4.1). Here, global deprotection via Birch reduction will afford sphingosine **5** with the double bond still intact, while catalytic hydrogenation followed by hydrolysis will afford sphinganine **6**. The fully protected glycolipid **71** could in turn be obtained via the glycosylation of lactosylceramide (LacCer) acceptor **73** with the D-fucosyl donor **72**, itself obtained in several steps from D-galactose **39**. LacCer acceptor **73** could be obtained from lactosyl 2-azido-sphingosine **74** via coupling with hexacosanoic acid followed by hydrolysis of the isopropylidene protecting group and selective acetylation of the 4''-OH. Lactosyl 2-azido-sphingosine **74** could be formed by the coupling of lactosyl donor **75** and sphingosine acceptor **76**. Finally, the lactosyl donor **75** could be prepared from D-lactose (**77**) and the sphingosine acceptor **76** from D-arabinose (**78**) according to published procedures.^{9,10}



Scheme 4.1. Retrosynthesis of target 6'''-deoxy-iGb3 sphingosine **5** and 6'''-deoxy-iGb3-sphinganine **6**.

Synthesis of lactosylceramide (LacCer) acceptor

The synthesis of 6'''-deoxy-iGb3-sphingosine **5** and 6'''-deoxy-iGb3-sphinganine **6** began with the preparation of the appropriately protected lactosyl 2-azido sphingosine intermediate **74** (Scheme 4.2). To this end, D-lactose (**77**) was peracetylated, followed by the conversion of the anomeric acetate to a thiophenol group and removal of the remaining acetate groups under Zemplén conditions to give thiolactoside **79** as a crystalline solid according to literature procedure.^{9,11-14} Thiolactoside **79** was then subjected to 2,2-dimethoxypropane and a catalytic amount of *p*-toluenesulfonic acid (*p*TsOH) to afford the selectively protected 3',4'-ketal **80**. While these conditions generally yield the thermodynamically favoured 5-membered 3',4'-ketal **80**, some of the kinetic 4',6'-derivative **81** was also obtained.¹⁴⁻¹⁶ Efforts to drive the exclusive formation of the 3',4'-ketal **80** by allowing for a longer reaction time (3 days) was to no avail. Moreover, Xing *et al.* and Chan *et al.* also showed that performing the reaction at warmer temperatures did not increase the yield of the desired product significantly.^{9,17} Fortunately, the 3',4'-*O*-isopropylidene derivative **80** could be isolated in 56% yield from the mixture by crystallisation. Subsequent protection of the free hydroxyl groups as benzoate esters afforded the fully protected thioglycoside **82** in 94% yield, again as crystalline material. Next, the anomeric thiophenol was hydrolysed with NBS in aqueous acetone^{9,18} followed by the installation of the trichloroacetimidate leaving group^{9,16,19} to give lactosyl imidate donor **75** in 73% yield over the two steps. Spectral data of lactosyl imidate donor **75** matched that previously reported,^{9,16} which was obtained from D-lactose (**77**) via the use of either an anomeric *p*-toluenethiol group⁹ or an anomeric hexyldimethylsilyl group.¹⁶

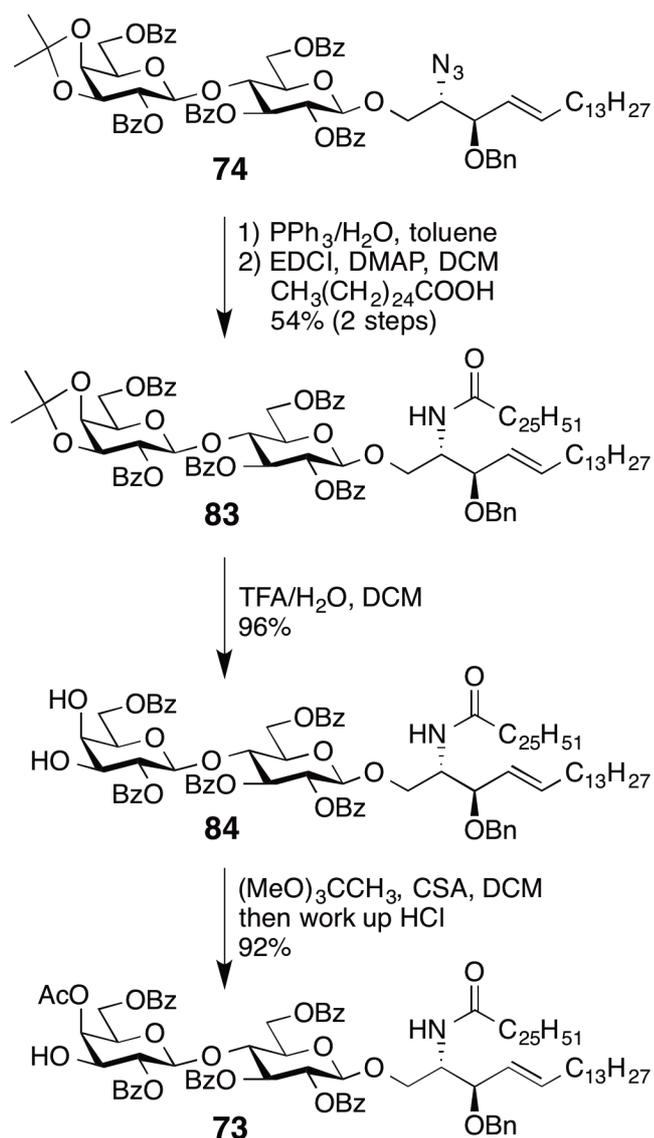


Scheme 4.2. Synthesis of lactosyl 2-azido-sphingosine **74**.

With the lactosyl imidate donor **75** in hand, a coupling reaction to sphingosine acceptor **76** was then performed (Scheme 4.2), whereby sphingosine acceptor **76** was prepared from D-arabinose (**78**), in nine steps using a strategy previously developed in our group.¹⁰ The glycosylation reaction utilised TMSOTf as a promoter and proceeded smoothly to give lactosyl 2-azido sphingosine **74** in quantitative yield.⁹ ^1H NMR analysis of the resulting glycolipid revealed a coupling constant of 7.8 Hz between H-1' and H-2', which confirms the β -selectivity of the reaction. This stereoselectivity was enabled by the participation of the benzoyl protecting group at C2. Interestingly, when Xing *et al.* coupled the

identical lactosyl donor to the full ceramide sphingosine acceptor bearing a C18 acyl lipid under similar activating conditions, the yield was only 60%, presumably due to poorer solubility of the dilipid acceptor.⁹

Following the coupling of the lipid, the azide on the sphingosine backbone was reduced with PPh₃ to produce the corresponding amine, which was used without further purification in the condensation with hexacosanoic acid using an EDCI/DMAP-mediated protocol (Scheme 4.3). Under these conditions the fully protected LacCer **83** was produced in 54% yield over two steps. The acetonide on the 3''- and 4''-positions was then removed via hydrolysis under acidic conditions to afford diol **84** in an excellent 96% yield. The regioselective protection of the 4''-OH was then performed by using trimethyl orthoacetate and camphorsulfonic acid to first install a methylorthoacetate protecting group across the 3''-OH and 4''-OH, which subsequently ring opened during an acidic workup to afford the LacCer acceptor **73**.



Scheme 4.3. Synthesis of LacCer acceptor **73**.

The position of the acetate ester was confirmed, firstly by the chemical shift of the hydrogens at the 3'' and 4''-positions. As expected, because of the electron withdrawing nature of ester protecting groups, the narrow doublet with a small coupling constant ($J_{3'',4''} = 3.5$ Hz,) characteristic of H-4'' is located downfield (5.22 ppm) compared to the doublet of doublets ($J_{2'',3''} = 9.8$ Hz, $J_{3'',4''} = 3.5$ Hz) for H-3'', which is located at 3.82 ppm. Additionally, HMBC correlation between the carbonyl carbon of the acetate and the proton at the 4''-position unambiguously confirms the acetylation of the 4''-OH (Figure 4.4).

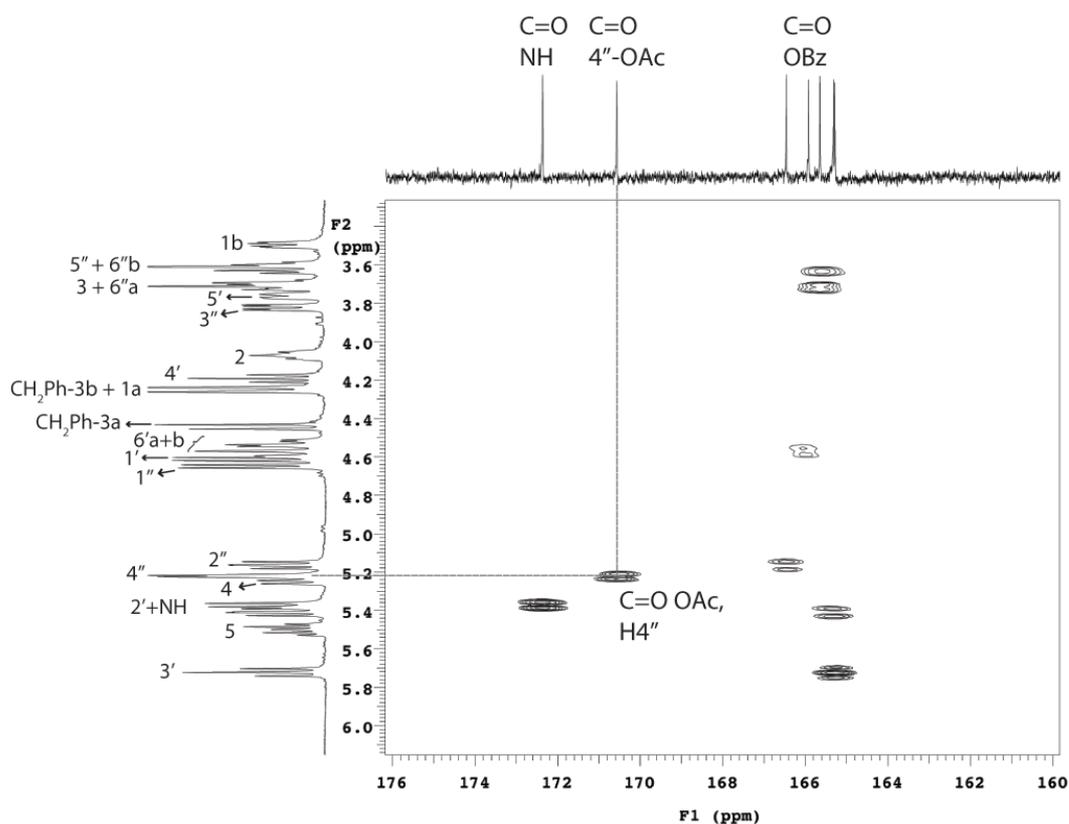
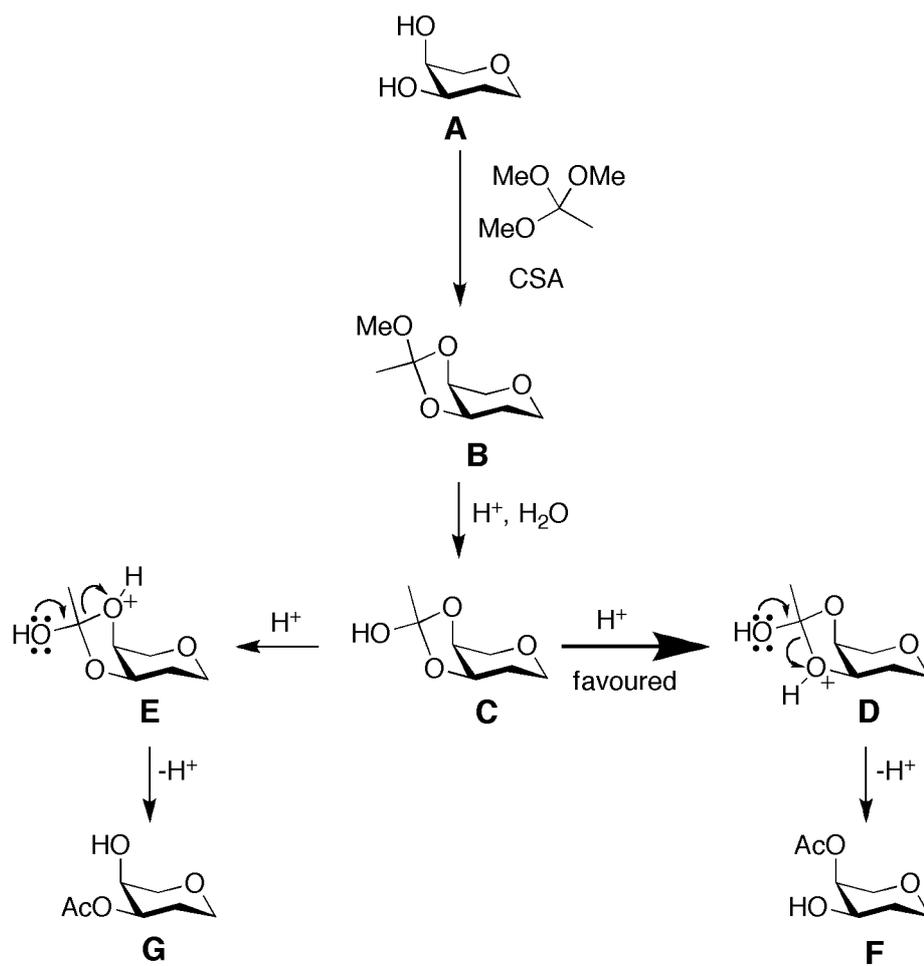


Figure 4.4. HMBC of LacCer acceptor **73** confirming acetylation at position 4''.

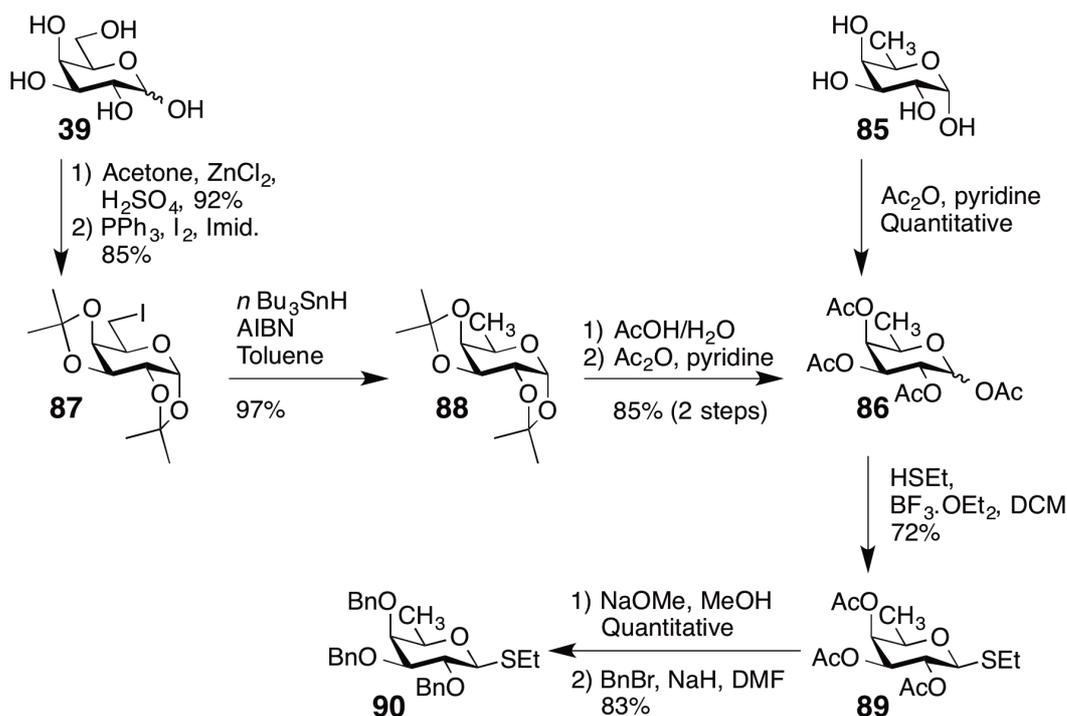
This versatile approach to acetylate the axial 4-OH over equatorial 3-OH in a *cis*-diol system was first reported by Lemieux *et al.*²⁰ and the selectivity can be rationalised by stereoelectronic effects.^{21,22} During acidic workup, the methoxy group of intermediate **B** is substituted by water (to give tetrahedral intermediate **C**), and subsequent protonation of either the equatorial oxygen at C-3 (**D**) or the axial oxygen at C-4 (**E**) determines the formation of the 4-OAc (**F**) or 3-OAc (**G**) product, respectively (Scheme 4.4). The less sterically hindered oxygen at the equatorial position is preferentially protonated over the axial oxygen,²³ leading to the selective acetylation at the 4-position (**F**), giving rise to LacCer acceptor **73**. In summary, the LacCer acceptor **73** was prepared in twelve steps from D-lactose (**77**) in 8% overall yield.



Scheme 4.4. Regioselective acetylation of the 3,4-*cis*-diol system of galactose.

Synthesis of thioethyl D-fucoside donor

Having successfully prepared the LacCer acceptor **73**, the next synthetic target was the β -thioethyl fucose donor **90** (Scheme 4.5). Glycosylation reactions incorporating both D- or L-fucose donors are notorious for poor reaction yields due to the increased acid lability of the α -fucosyl linkage.²⁴⁻²⁶ Therefore, mild activating conditions are required for their glycosidation. It was envisioned that β -thioethyl fucose donor **90**,^{27,28} which can be activated under milder conditions using CuBr_2 and Pr_4NBr , will circumvent the problem of low yields. This glycosidation protocol was previously optimised within the group from Ogawa's method.²⁹



Scheme 4.5. Synthesis of β -thioethyl fucose donor **90**.

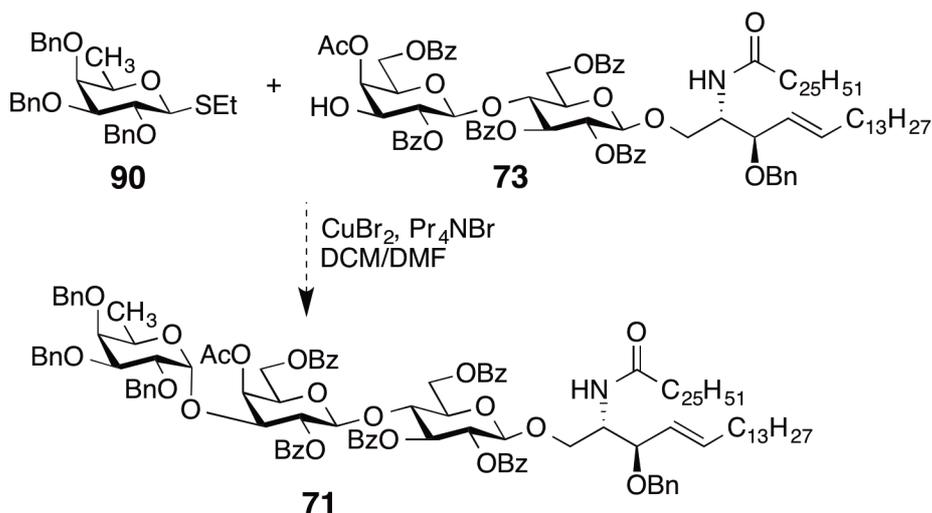
The synthesis of the D-fucose donor building block began with peracetylated D-fucose **86**, which can be obtained in quantitative yield by subjecting D-fucose (**85**) to acetic anhydride and pyridine. However, due to the high cost of the starting

material D-fucose (**85**), an alternative route to make the peracetylated D-fucose intermediate **86**, starting from D-galactose (**39**) was developed. Iodogalactoside **86** can be made according to methods described earlier (Chapter 2, Scheme 2.3) whereby the 1,2 and 3,4 positions of D-galactose (**39**) were protected with isopropylidenes followed by the installation of an iodide at the primary position via a modified Appel reaction.³⁰ Next, reduction of the iodide under conditions akin to the Barton-McCombie deoxygenation, whereby the radical initiator AIBN and reducing agent tributyltin hydride were used, provided 6-deoxy galactose **88** in an excellent 97% yield.³¹ Subsequent hydrolysis of the isopropylidene groups under acidic conditions, followed by peracetylation gave D-fucose tetraacetate **86** in 85% yield over two steps. According to this route, D-fucose tetraacetate **86** was made from D-galactose **39** in 65% yield over five steps. The robust methodology for the synthesis of D-fucose intermediates, along with the significantly reduced cost of D-galactose (60 times cheaper than D-fucose) provides great incentive to start with the more readily available D-galactose.

Following this, β -thiofucoside **89** was prepared by converting the anomeric acetate to the thioethyl functionality by using boron trifluoride etherate as the Lewis acid (72% yield). The remaining acetate protecting groups were then hydrolysed under Zemplén conditions and the resultant free hydroxyls benzylated to give benzylated β -thiofucoside donor **90** in 83% yield over two steps.

Attempted coupling of thioethyl fucoside 90 donor and LacCer acceptor 73

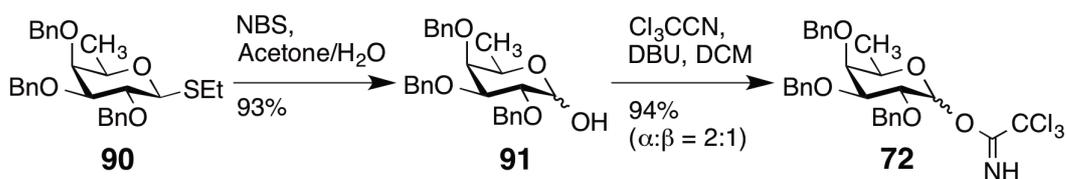
With the thiofucose donor **90** and LacCer acceptor **73** in hand, the coupling reaction was attempted by using CuBr_2 and Pr_4NBr , but to no avail (Scheme 4.6). No desired product was observed, instead, the self-coupled donor 1,1'-difucose and unreacted acceptor **73** were obtained. It was speculated that perhaps the lipophilic acceptor was not as nucleophilic while the activated fucose donor was highly reactive, thus leading to the 1,1'-difucose by-product in the presence of trace amount of water.



Scheme 4.6. Attempted coupling of thioethyl donor **90** and LacCer acceptor **73**.

Modification to the trichloroacetimidate donor **72**

Therefore, an alternative donor, the trichloroacetimidate was synthesised in two steps from the thiofucoside donor **90** (Scheme 4.7). Hydrolysis of the anomeric thioethyl group in the presence of NBS and acetone¹⁸ afforded the corresponding lactol **91** in 93% yield. The anomeric hydroxyl was then converted to a trichloroacetimidate group in an excellent 94% yield to afford the desired fucose donor **72**.³² Here, both α and β anomers were generated in a 2:1 ratio, and they were separable by silica gel column chromatography. Of note, due to the prevalence of L-fucose in biological systems, only L-fucosyl imidate donors have been reported. This work represents the first synthesis of the D-fucosyl imidate. As expected, the ¹H and ¹³C NMR data of α -D-fucosyl imidate **72** were identical to that of the enantiomer, α -L-fucosyl imidate,³³ and the optical rotation was equal in value but opposite in magnitude ($[\alpha]_{\text{D}}^{23}$ α -D-fucosyl imidate **72** = +58°; $c = 1.0$, CHCl₃; $[\alpha]_{\text{D}}^{20}$ α -L-fucosyl imidate = -60°; $c = 1.0$, CHCl₃³³)

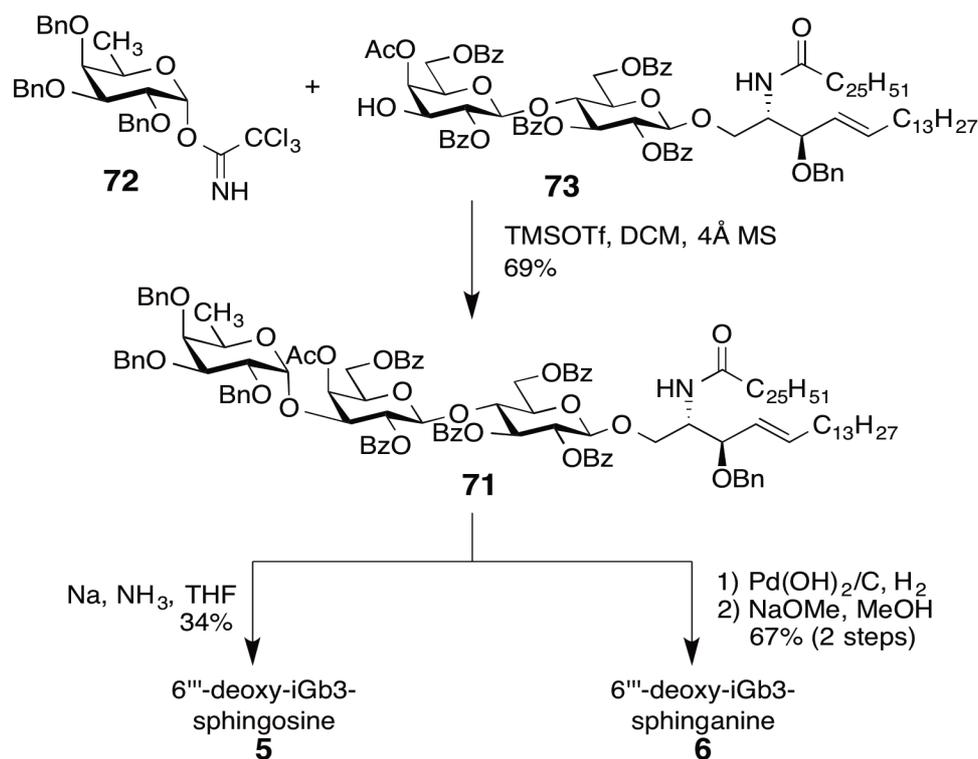


Scheme 4.7. Synthesis of trichloroacetimidate fucose donor **72**.

Coupling of trichloroacetimidate donor **72 and LacCer acceptor **73** and global deprotection**

Though coupling reactions using either β -L-fucosyl imidate^{34,35} or α/β mixtures of L-fucosyl imidate³⁶ have been reported, only the α -anomer of D-fucosyl imidate **72** was used in the glycosylation reaction with LacCer acceptor **73** so as to follow Schmidt's original coupling conditions,³³ and to eliminate any ambiguity in the effects of starting with a mixture of anomers on α/β -selectivity of the coupling reaction.

The coupling of α -fucosyl imidate donor **72** and the LacCer acceptor **73** was performed with TMSOTf as the catalyst (Scheme 4.8).³³ Gratifyingly, this reaction proceeded smoothly to afford the fully protected 6'''-deoxy-iGb3 **71** in good 69% yield.



Scheme 4.8. Total syntheses of 6'''-deoxy-iGb3-sphingosine **5** and 6'''-deoxy-iGb3-sphinganine **6**.

Analysis of the product by ¹H NMR revealed that the coupling constant of H-1''' at the newly formed anomeric centre was 3.5 Hz, confirming the formation of the

α -anomer. No β -coupled product was detected and unreacted acceptor **73** was recovered. Three bond correlations of key glycosidic bonds from HMBC analysis are shown in Figure 4.5. Specifically, the correlation between C-3'' and H-1''' confirms the attachment of the terminal fucose moiety to the 3'' position of LacCer. In addition, an HMBC correlation between H-4'' and carbonyl carbon of the acetate further confirms that the LacCer was glycosylated at the 3'' (Figure 4.6).

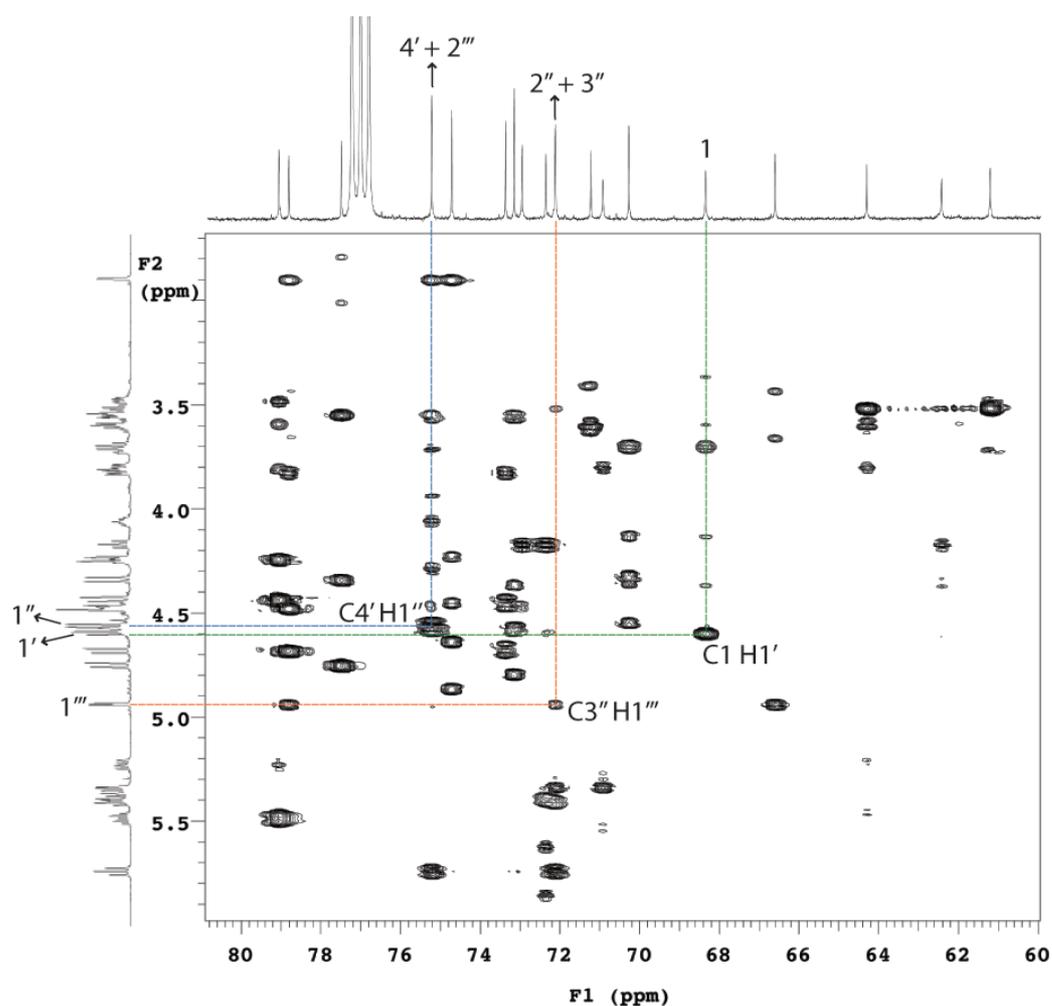


Figure 4.5. HMBC spectrum of 6'''-deoxy-iGb3 **71** confirming the fucosylation of LacCer **73** at the 3''-position.

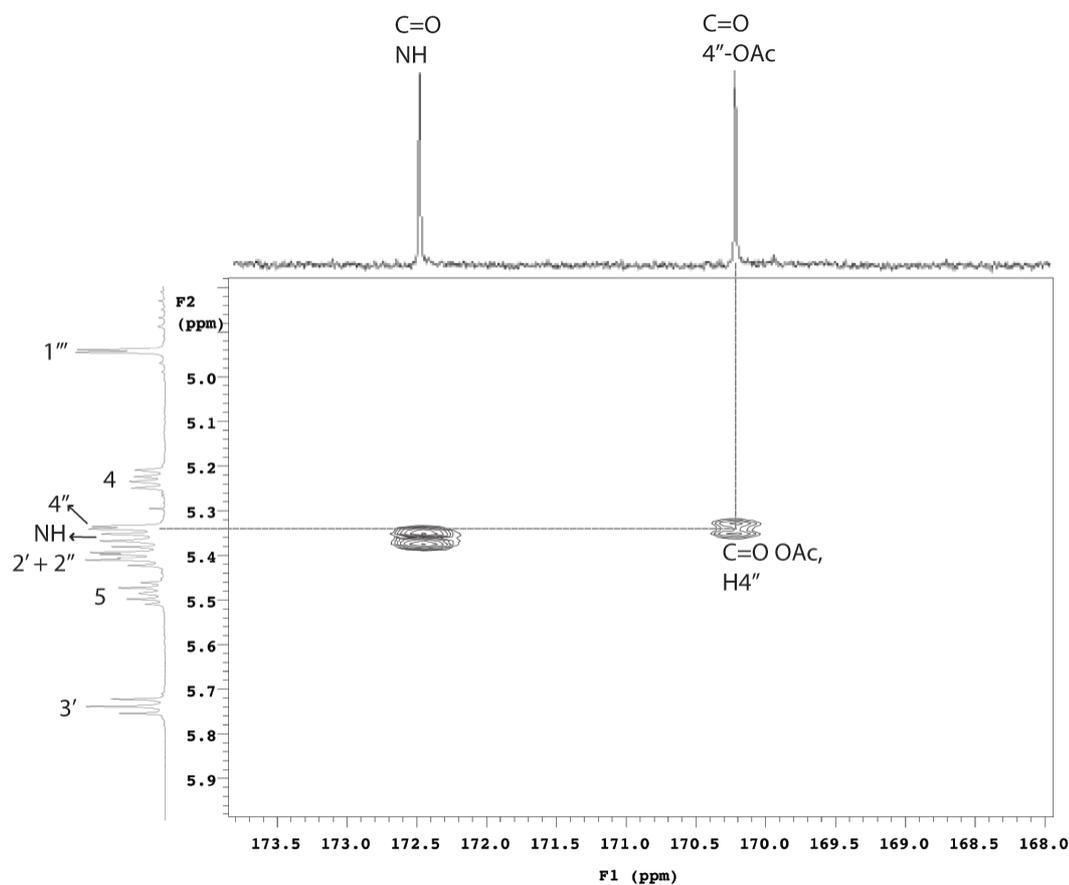


Figure 4.6. HMBC of 6'''-deoxy-iGb3 **71** confirming acetylation at position 4''.

With the fully protected 6'''-deoxy-iGb3 **71** at hand, global deprotection under Birch conditions was performed and this allowed for the removal of all ether and ester protecting groups without reducing the alkene. As large amounts of ammonium salts are produced in the Birch reduction, purification by a reverse phase chromatography on a C18 column was attempted, but to no avail as the poor solubility of the amphiphilic glycolipid made the loading of the column rather difficult. Fortunately, upon careful purification by silica gel column chromatography, the target 6'''-deoxy-iGb3-sphingosine **5** could be isolated in 34% yield. While the yield of this final step was modest, limited quantities of the fully protected glycolipid **71** prevented further optimisation of this procedure.

Alternatively, hydrogenation with Pearlman's catalyst followed by the hydrolysis of the esters (acetate and benzoates) under Zemplén condition afforded 6'''-deoxy-iGb3-sphinganine **6** in 67% yield over two steps. In contrast to the sphingosine derivative **5**, purification of the fully deprotected sphinganine

analogue **6** was more straightforward as the glycolipid could be obtained by precipitating the glycolipid with methanol, followed by centrifugation of the mixture to form a glycolipid pellet and removal of the sodium methoxide-containing methanol.

Full characterisation by mass spectrometry, infrared spectroscopy, ^1H and ^{13}C NMR confirmed the structure and identity of both target glycolipids. Specifically, analysis by mass spectrometry confirmed the identity of 6'''-deoxy-iGb3-sphingosine **5** (HRMS ESI m/z calcd. for $[\text{C}_{62}\text{H}_{117}\text{NO}_{17}+\text{H}]^+$: 1148.8394, obsd.: 1148.8374) and 6'''-deoxy-iGb3-sphinganine **6** (m/z calcd. for $[\text{C}_{62}\text{H}_{120}\text{NO}_{17}+\text{H}]^+$: 1150.8551, obsd.: 1150.8553). The key difference between the two target glycolipids is the presence of the *E*-alkene in sphingosine **5**, which was confirmed via NMR spectroscopy by the signature doublet of doublets (H-4) and doublet of triplets (H-5) at 6.04 ppm and 5.93 ppm, respectively and their corresponding carbon signal at 132.6 ppm and 133.0 ppm. ^1H and ^{13}C NMR data is in agreement with other iGb3 analogues such as the 6'''-deoxy-iGb3-phytosphingosine⁴ and the iGb3-sphinganine analogue.⁵

4.3 Conclusion

In this work, two analogues of iGb3 were synthesised. An iGb3 derivative lacking the 6'''-OH on the terminal galactose sugar, 6'''-deoxy-iGb3-sphingosine **5** was synthesised in 2% overall yield over 14 steps (longest linear sequence). A further derivative, 6'''-deoxy-iGb3-sphinganine **6**, which lacks the alkene on the ceramide backbone was made in 4% overall yield over 15 steps (longest linear sequence). The highlight of this synthesis was the high yielding coupling between lactosyl imidate donor and sphingosine acceptor, which proceeded with excellent β -selectivity. The selective acetylation of the 4''-position of LacCer also allowed for the regioselective fucosylation of the 3'' position, which proceeded smoothly when the trichloroacetimidate donor was employed. These 6'''-deoxy-iGb3 derivatives will allow for future assessment of their structure activity relationship in terms of iNKT cell activation, as well as analysis of structural interaction with the TCR of iNKT cells and CD1d by X-ray crystallography.

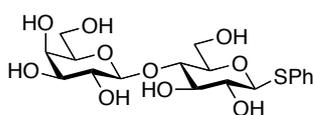
4.4 Experimental

General procedure

Unless otherwise stated all reactions were performed under argon. Prior to use, THF (Pancreac) was distilled from sodium and benzophenone, pyridine was distilled and dried over 4Å molecular sieves (4Å MS), DCM (Pancreac) was distilled from P₂O₅, and H₂O and benzene (Fisher Scientific) were distilled. SnCl₄ (Aldrich), PhSH (Koch-Light Laboratories), benzaldehyde dimethyl acetal (Aldrich), Me₂C(OMe)₂ (Aldrich), NBS (Aldrich), DBU (Merck), CSA (Acros), *n*Bu₃SnCl (Aldrich), AIBN (Aldrich), D-fucose (Aldrich), D-lactose (Aldrich), trityl chloride (Acros), anhydrous Et₂O (Pancreac), PPh₃ (Aldrich), Pd(OH)₂/C (Aldrich, 20 wt%), anhydrous DMF (Acros), TFA (Aldrich), *p*TsOH (Aldrich), TMSOTf (Aldrich), H₂SO₄ (Lab-Scan), formic acid (Aldrich), AcCl (Aldrich), BnBr (Fluka), PMe₃ (Aldrich, 1M in THF), AcOH (Ajax Finechem), Ac₂O (Peking Reagent), TMSOTf (Aldrich), DiPEA (Aldrich), NaOMe (Janssen Chimica), trichloroacetonitrile (Aldrich), C₂₅H₅₁COOH (Acros), BzCl (Aldrich, distilled and stored under argon), HBTU (Acros), PyBOP (Aldrich), EDCI (Aldrich), DMAP (Merck), sodium (Aldrich), trimethyl orthoacetate (Aldrich), LiAlH₄ (Aldrich), EtOAc (Pancreac), hexanes (Fisher Scientific), petroleum ether (Pure Science), MeOH (Pure Science), CHCl₃ (Pancreac), EtOH (absolute, Pure Science), NaOH (Pure Science), NaHCO₃ (Pure Science), NaCl (Pancreac), NH₃ (BOC gasses) were used as received. All solvents were removed by evaporation under reduced pressure. Reactions were monitored by TLC-analysis on Macherey-Nagel silica gel coated plastic sheets (0.20 mm, with fluorescent indicator UV₂₅₄) with detection by UV-absorption (short wave UV – 254 nm; long wave UV – 366 nm), by dipping in 10% H₂SO₄ in EtOH followed by charring at ~150 °C, by dipping in I₂ in silica, or by dipping into a solution of ninhydrin in EtOH followed by charring at ~150 °C. Column chromatography was performed on Pure Science silica gel (40-63 micron). AccuBOND II ODS-C18 (Agilent) was used for reverse phase chromatography. Infrared spectra were recorded as thin films using a Bruker Tensor 27 FTIR spectrometer equipped with an Attenuated Total

Reflectance (ATR) sampling accessory and are reported in wave numbers (cm^{-1}). Nuclear magnetic resonance spectra were recorded at 20 °C in CD_3OD , CDCl_3 , or pyridine- d_5 using either a Varian INOVA operating at 500 MHz or Varian VNMRs operating at 600 MHz. Chemical shifts are given in ppm (δ) relative to TMS. NMR peak assignments were made using COSY, HSQC and HMBC 2D experiments.

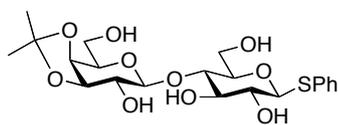
Phenyl 4-*O*-(β -D-galactopyranosyl)-1-thio- β -D-glucopyranoside (**79**)



A solution of sodium acetate (20 g, 244 mmol) in acetic anhydride (100 mL, 1058 mmol) was heated to reflux and D-lactose (**77**) (40 g, 117 mmol) was added in small portions over 30 mins. The resulting mixture was refluxed for 2 h, after which it was cooled to rt and poured over ice and stirred for 1 h. The white solid was filtered and washed with H_2O (100 mL). The filtrate was dissolved in Et_2O (800 mL) and the organic layer was washed with H_2O (2 x 800 mL), dried (MgSO_4), filtered and concentrated *in vacuo* to afford peracetylated D-lactose as a white solid (55 g, 81 mmol, 70%). Peracetylated D-lactose (17 g, 25 mmol) was dissolved in CH_2Cl_2 (125 mL) and thiophenol (2.09 mL, 30 mmol) was added. The reaction mixture was then cooled to 0 °C, SnCl_4 (289 μL , 2.5 mmol) added and the resultant mixture stirred at rt for 4 d. The reaction mixture was diluted with CH_2Cl_2 (200 mL), quenched with sat. aq. NaHCO_3 (150 mL) and sat. aq. KF (150 mL) and the organic layer was isolated from the mixture, and further washed with sat. aq. KF (150 mL), sat. aq. NaHCO_3 (150 mL), H_2O (150 mL), brine (150 mL) and dried (MgSO_4), filtered and concentrated *in vacuo*. Crystallisation of the crude mixture from EtOAc /petroleum ether (9/1, v/v) afforded phenyl 4-*O*-(2,3,4,6-tetra-*O*-acetyl- β -D-galactopyranosyl)-2,3,6-tri-*O*-acetyl-1-thio- β -D-glucopyranoside as a white solid (11.6 g, 16 mmol, 64%). Phenyl 4-*O*-(2,3,4,6-tetra-*O*-acetyl- β -D-galactopyranosyl)-2,3,6-tri-*O*-acetyl-1-thio- β -D-glucopyranoside (5 g, 6.86 mmol) was dissolved in MeOH (55 mL), and NaOMe was added until the pH of the reaction mixture reached 9.0. The resulting mixture was stirred at rt for 20 h, after which the reaction mixture was neutralised with Dowex- H^+ , filtered and concentrated *in vacuo*. The residue was crystallised from EtOH/MeOH (9.5/0.5, v/v) to afford thiolactoside **79**^{11,12,14,37} (2.8 g, 6.55 mmol,

quantitative) as a white solid. Thiolactoside **79** was obtained in 45% overall yield in 3 steps from D-lactose (**77**). Mp 218.2–219.9 °C;¹¹ R_f : 0.02 (DCM/MeOH, 5.7/1, v/v); $[\alpha]_D^{25} = -40.0^\circ$ (c = 1.0, H₂O);¹¹ IR (film) 3360, 2884, 1644, 1583, 1479, 1439, 1373, 1278, 1117, 1069, 1020, 889, 822, 784, 743, 691 cm⁻¹; ¹H NMR (600 MHz, D₂O) δ 7.55–7.53 (m, 2H, CH-*o*), 7.39–7.33 (m, 3H, CH-*m*, CH-*p*), 4.78 (d, $J_{1,2} = 9.1$ Hz, 1H, H-1), 4.40 (d, $J_{1',2'} = 7.8$ Hz, 1H, H-1'), 3.91 (dd, $J_{6a,6b} = 12.6$ Hz, $J_{5,6a} = 1.7$ Hz, 1H, H-6a), 3.87 (d, $J_{3',4'} = 3.4$ Hz, 1H, H-4'), 3.76 (dd, $J_{6a,6b} = 12.6$ Hz, $J_{5,6b} = 5.0$ Hz, 1H, H-6b), 3.76–3.70 (m, 2H, H-6'a, H-6'b), 3.69–3.66 (m, 1H, H-5'), 3.64–3.63 (m, 2H, H-3, H-4), 3.61 (dd, $J_{2',3'} = 9.9$ Hz, $J_{3',4'} = 3.4$ Hz, 1H, H-3'), 3.59–3.57 (m, 1H, H-5), 3.49 (dd, $J_{2',3'} = 9.9$ Hz, $J_{1',2'} = 8.0$ Hz, 1H, H-2'), 3.37 (t, $J_{1,2} = 9.1$ Hz, 1H, H-2); ¹³C NMR (150 MHz, D₂O) δ 131.9 (C-*i*), 131.6, 129.3 (C-*o*, C-*m*), 128.1 (C-*p*), 102.8 (C-1'), 87.1 (C-1), 78.7 (C-5), 77.8 (C-4), 75.8 (C-3), 75.3 (C-5'), 72.5 (C-3'), 71.4 (C-2), 70.9 (C-2'), 68.5 (C-4'), 61.0 (C-6'), 60.0 (C-6); HRMS(ESI) m/z calcd. for [C₁₈H₂₆O₁₀S+Na]⁺: 457.1139, obsd.: 457.1148.

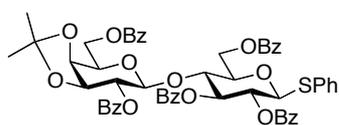
Phenyl 4-O-(3,4-O-isopropylidene- β -D-galactopyranosyl)-1-thio- β -D-glucopyranoside (80**)**



To a solution of thiolactoside **79** (1.98 g, 4.56 mmol) in dry DMF (17 mL) and dry acetone (34 mL), 2,2-dimethoxypropane (923 μ L) and *p*TsOH (81 mg, 0.427 mmol) were added and the resulting solution stirred for 3 d at rt. The solution was quenched with NEt₃ and concentrated under reduced pressure and the residue was then crystallised from hot ethanol to give title compound **80** as a white crystalline product (1.14 g, 2.40 mmol, 56%). Mp 203.2–203.9 °C R_f : 0.30 (DCM/MeOH, 9/1, v/v); $[\alpha]_D^{25} = -27.0^\circ$ (c = 1.0, MeOH); IR (film) 3364, 2946, 2836, 2073, 1653, 1450, 1222, 1119, 1078, 1023, 977, 873, 737 cm⁻¹; ¹H NMR (500 MHz, CDCl₃/CD₃OD, 1/1, v/v) δ 7.52–7.51 (m, 2H, CH-*o*), 7.30–7.23 (m, 3H, CH-*m*, CH-*p*), 4.58 (d, $J_{1,2} = 9.8$ Hz, 1H, H-1), 4.31 (d, $J_{1',2'} = 8.3$ Hz, 1H, H-1'), 4.12 (dd, $J_{3',4'} = 5.6$ Hz, $J_{4',5'} = 2.2$ Hz, 1H, H-4'), 4.03 (dd, $J_{2',3'} = 7.5$ Hz, $J_{3',4'} = 5.6$ Hz, 1H, H-3'), 3.91–3.88 (m, 1H, H-5'), 3.86–3.73 (m, 4H, H-6a, H-6b, H-6'a, H-6'b), 3.57 (t, $J_{2,3} = J_{3,4} = 8.7$ Hz, 1H, H-3), 3.53 (t, $J_{3,4} = J_{4,5} = 8.7$ Hz, 1H, H-4), 3.46 (t, $J_{2',3'} = 7.5$ Hz, 1H, H-2'), 3.43–3.39 (m, 1H, H-5), 3.33–3.29

(m, 1H, H-2), 1.47 (s, 3H, CH₃ *i*Pr), 1.31 (s, 3H, CH₃ *i*Pr); ¹³C NMR (125 MHz, CDCl₃/CD₃OD, 1/1, v/v) δ 132.6 (C-*i*), 131.8, 128.5 (C-*o*, C-*m*), 127.3 (C-*p*), 109.9 (C_q *i*Pr), 102.7 (C-1'), 87.6 (C-1), 79.7 (C-4), 79.1 (C-3'), 78.5 (C-5), 76.2 (C-3), 73.9 (C-5'), 73.4 (C-4'), 72.7 (C-2'), 71.7 (C-2), 61.1, 60.9 (C-6/C-6'), 27.4, 25.6 (2 x CH₃ *i*Pr); HRMS(ESI) *m/z* calcd. for [C₂₁H₃₀O₁₀S+Na]⁺: 497.1452, obsd.: 497.1461. Spectral data of 3',4'-isopropylidene protected thiolactoside **Error! Reference source not found.** matched that previously reported.¹⁷

Phenyl 4-*O*-(2,6-di-*O*-benzoyl-3,4-*O*-isopropylidene-β-D-galactopyranosyl)-2,3,6-tri-*O*-benzoyl-1-thio-β-D-glucopyranoside (82**)**

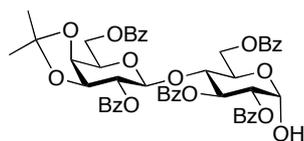


3',4'-isopropylidene protected thiolactoside **80**^{14,38}
(1.10 g, 2.31 mmol) was co-evaporated with toluene

(x3) and dissolved in pyridine (23 mL). Benzoyl chloride (5.9 mL, 50.86 mmol) and DMAP (0.14 g, 1.16 mmol) were added and the reaction was stirred at rt for 15 h. The reaction mixture was diluted with EtOAc (100 mL), washed with sat. aq. NaHCO₃ (3 x 100 mL), H₂O (100 mL) and brine (100 mL), dried (MgSO₄), filtered and concentrated under reduced pressure. The product was crystallised from petroleum ether/EtOAc (2/1, v/v) to give the fully protected lactoside **82** as white fluffy crystals (1.98 g, 1.99 mmol, 86%), and the remainder mother liquor was purified by silica flash chromatography (petroleum ether/EtOAc, 3/1, v/v) to afford more product (0.18 g, 0.18 mmol, 8%). *R_f*: 0.56 (PE/EA, 1/1, v/v); [α]_D²⁵ = +40.0° (c = 1.0, CHCl₃); IR (film) 3064, 2988, 2941, 1723, 1602, 1451, 1315, 1265, 1177, 1110, 1083, 1069, 1027, 1000, 753, 708 cm⁻¹; ¹H NMR (500 MHz, CDCl₃) δ 8.07 (d, *J*_{CH-*o*,CH-*m*} = 7.8 Hz, 2H, CH-*o*, OBz), 8.00 (d, *J*_{CH-*o*,CH-*m*} = 7.8 Hz, 2H, CH-*o*, OBz), 7.96 (d, *J*_{CH-*o*,CH-*m*} = 8.0 Hz, 4H, 2 x CH-*o*, OBz), 7.92 (d, *J*_{CH-*o*,CH-*m*} = 7.8 Hz, 2H, CH-*o*, OBz), 7.62–7.09 (m, 20H, H_{arom}), 5.73 (t, *J*_{3,4} = 9.5 Hz, 1H, H-3), 5.40 (t, *J*_{1,2} = 9.9 Hz, 1H, H-2), 5.13 (t, *J*_{1',2'} = 7.6 Hz, 1H, H-2'), 4.88 (d, *J*_{1,2} = 9.9 Hz, 1H, H-1), 4.65 (d, *J*_{6a,6b} = 12.0 Hz, 1H, H-6a), 4.59 (d, *J*_{1',2'} = 7.6 Hz, 1H, H-1'), 4.47 (dd, *J*_{6a,6b} = 12.0 Hz, *J*_{5,6b} = 5.1 Hz, 1H, H-6b), 4.26–4.21 (m, 2H, H-3', H-6'a), 4.11 (t, *J*_{3,4} = 9.5 Hz, 1H, H-4), 4.09 (d, *J*_{4',5'} = 5.8 Hz, 1H, H-4'), 3.89 (dd, *J*_{5,6a} = 9.6 Hz, *J*_{4,5} = 5.0 Hz, 1H, H-5), 3.83–3.81 (m, 1H, H-

5'), 3.66 (dd, $J_{6'a,6'b} = 11.2$ Hz, $J_{6'a,6'b} = 7.6$ Hz, 1H, H-6'b), 1.52 (s, 3H, CH₃ *i*Pr), 1.25 (s, 3H, CH₃ *i*Pr); ¹³C NMR (125 MHz, CDCl₃) δ 166.1 (C=O, 6'-*O*-Bn), 166.0 (C=O, 6-*O*-Bn), 165.7 (C=O, 3-*O*-Bn), 165.3 (C=O, 2-*O*-Bn), 165.0 (C=O, 2'-*O*-Bn), 133.52, 133.41, 133.30, 133.11, 133.07 (C-*p*, 5 x OBz), 132.0 (C-*i*, SPh), 130.31, 130.03, 130.00, 129.93, 129.86, 129.72, 129.67, 129.47, 129.40, 128.96, 128.83, 128.63, 128.59, 128.54, 128.53, 128.27, 128.24 (30 x CH_{arom}), 111.0 (C_q *i*Pr), 100.4 (C-1'), 86.0 (C-1), 77.4 (C-3'), 77.3 (C-5), 75.5 (C-4), 74.0 (C-3), 73.8 (C-2'), 73.3 (C-4'), 71.5 (C-5'), 70.6 (C-2), 63.0 (C-6), 62.9 (C-6'), 27.6, 26.3 (2 x CH₃ *i*Pr); HRMS(ESI) *m/z* calcd. for [C₅₆H₅₀O₁₅S+Na]⁺: 1017.2763, obsd.: 1017.2774.

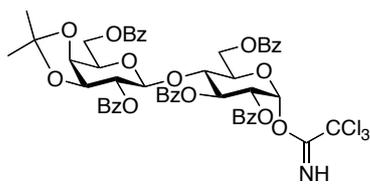
4-*O*-(2,6-Di-*O*-benzoyl-3,4-*O*-isopropylidene-β-D-galactopyranosyl-2,3,6-tri-*O*-benzoyl-α-D-glucopyranose (82a)



NBS (1.86 g, 10.45 mmol) was added to fully protected lactoside **82** (2.60 g, 2.61 mmol) dissolved in acetone/H₂O (9/1, v/v, 52 mL), and the mixture was stirred at rt for 50 mins. The reaction was quenched with sat. aq. NaHCO₃ (10 mL), diluted with EtOAc (80 mL), washed with sat. aq. Na₂S₂O₃ (100 mL) and brine (100 mL), dried (MgSO₄), filtered and concentrated under reduced pressure. The crude mixture was purified by gradient flash chromatography (petroleum ether/EtOAc, 5/1 to 2/1, v/v) to afford title compound **82a** as a clear oil (1.84 g, 2.04 mmol, 78%). For long term storage, the lactol can be crystallised from petroleum ether/EtOAc (2/1, v/v) to give white fluffy crystals. *R_f*: 0.50 (PE/EtOAc, 1/1, v/v); [α]_D²⁴ = +71.0° (c = 1.0, CHCl₃); IR (film) 3064, 1720, 1602, 1452, 1374, 1316, 1265, 1222, 1177, 1109, 1069, 999, 755, 687 cm⁻¹; ¹H NMR (500 MHz, CDCl₃) δ 8.08 (d, $J_{CH-o,CH-m} = 7.3$ Hz, 2H, CH-*o*, OBz), 8.07–7.95 (m, 8H, 4 x CH-*o*, OBz), 7.62–7.26 (m, 15H, H_{arom}), 6.07 (t, $J_{2,3} = J_{3,4} = 10.0$ Hz, 1H, H-3), 5.58 (t, $J_{1,2} = 3.6$ Hz, 1H, H-1), 5.19 (dd, $J_{2,3} = 10.0$ Hz, $J_{1,2} = 3.6$ Hz, 1H, H-2), 5.16 (t, $J_{1',2'} = J_{2',3'} = 7.2$ Hz, 1H, H-2'), 4.70 (d, $J_{1',2'} = 7.4$ Hz, 1H, H-1'), 4.60 (dd, $J_{6a,6b} = 12.2$ Hz, $J_{5,6a} = 1.6$ Hz, 1H, H-6a), 4.52 (dd, $J_{6a,6b} = 12.2$ Hz, $J_{5,6b} = 3.4$ Hz, 1H, H-6b), 4.40–4.38 (m, 1H, H-5), 4.32 (dd, $J_{6'a,6'b} = 15.3$ Hz, $J_{5,6'a} = 8.7$ Hz, 1H, H-6'a), 4.27 (t, $J_{3',4'} = 6.1$ Hz, 1H, H-3'), 4.20 (t, $J_{3,4} = 10.0$ Hz, 1H, H-4), 4.13–4.11 (m, 1H, H-4'), 3.90–3.85

(m, 2H, H-5', H-6'b), 2.90 (d, $J_{1,\text{OH}} = 3.6$ Hz, 1H, OH), 1.51 (s, 3H, CH₃ *iPr*), 1.26 (s, 3H, CH₃ *iPr*); ¹³C NMR (125 MHz, CDCl₃) δ 166.0, 166.0 (C=O, 6-*O*-Bn, 6'-*O*-Bn), 165.9 (C=O, 2-*O*-Bn), 165.6 (C=O, 3-*O*-Bn), 165.0 (C=O, 2'-*O*-Bn), 133.4, 133.4, 133.2, 133.1, 132.9 (C-*p*, 5 x OBz), 130.04, 129.91, 129.77, 129.70, 129.65, 129.59, 129.52, 129.33, 128.98, 128.60, 128.46, 128.43, 128.36, 128.15 (25 x CH_{arom}), 110.9 (C_q *iPr*), 100.0 (C-1'), 90.3 (C-1), 77.1 (C-3'), 75.4 (C-4), 73.6 (C-2'), 73.6 (C-4'), 72.2 (C-2), 71.3 (C-5'), 69.5 (C-3), 68.6 (C-5), 63.0 (C-6'), 62.5 (C-6), 27.4, 26.1 (2 x CH₃ *iPr*); HRMS(ESI) *m/z* calcd. for [C₅₀H₄₆O₁₆+Na]⁺: 925.2678, obsd.: 925.2681. Spectral data of lactol **82a** matched that previously reported.⁹

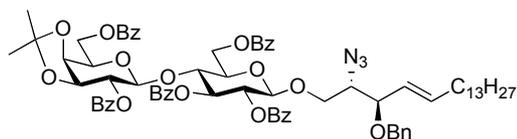
***O*-(4-*O*-(2,6-Di-*O*-benzoyl-3,4-*O*-isopropylidene-β-D-galactopyranosyl)-2,3,6-tri-*O*-benzoyl-α-D-glucopyranosyl) trichloroacetimidate (**75**)**



Lactol **82a** was co-evaporated with toluene (x3) and dissolved in dry CH₂Cl₂ (6 mL). Trichloroacetonitrile (1.13 mL, 11.30 mmol) and DBU (84 μL, 0.56 mmol) were added and the reaction mixture was stirred at rt for 1 h. Upon completion, the reaction mixture was concentrated and purified immediately by flash column chromatography (petroleum ether/EtOAc/CH₂Cl₂/NEt₃, 6/1/1/0.08, v/v) to afford imidate **75** as a colourless foam (1.08g, 1.03 mmol, 92%). *R_f*: 0.54 (PE/EA, 1/1, v/v); [α]_D²⁴ = +57.0° (c = 1.0, CHCl₃); IR (film) 3336, 3065, 2986, 2938, 1725, 1677, 1452, 1315, 1265, 1177, 1109, 1070, 1026, 797, 755, 708 cm⁻¹; ¹H NMR (500 MHz, CDCl₃) δ 8.55 (s, 1H, NH), 8.07 (d, $J_{\text{CH-}o,\text{CH-}m} = 7.1$ Hz, 2H, CH-*o*, 6'-*O*-Bz), 8.02 (t, $J_{\text{CH-}o,\text{CH-}m} = 8.6$ Hz, 4H, 2 x CH-*o*, OBz), 7.95 (t, $J_{\text{CH-}o,\text{CH-}m} = 8.7$ Hz, 4H, 2 x CH-*o*, OBz), 7.64–7.30 (m, 15H, H_{arom}), 6.69 (d, $J_{1,2} = 3.4$ Hz, 1H, H-1), 6.10 (t, $J_{3,4} = 9.2$ Hz, 1H, H-3), 5.50 (dd, $J_{2,3} = 10.2$ Hz, $J_{1,2} = 3.4$ Hz, 1H, H-2), 5.16 (t, $J_{2',3'} = 6.9$ Hz, 1H, H-2'), 4.70 (d, $J_{1',2'} = 7.6$ Hz, 1H, H-1'), 4.59 (d, $J_{6a,6b} = 11.8$ Hz, 1H, H-6a), 4.52 (d, $J_{6a,6b} = 11.8$ Hz, 1H, H-6b), 4.31–4.24 (m, 4H, H-4, H-5, H-6'a, H-3'), 4.11 (d, $J_{3',4'} = J_{4',5'} = 4.4$ Hz, 1H, H-4'), 3.81–3.78 (m, 2H, H-5', H-6'b), 1.50 (s, 3H, CH₃ *iPr*), 1.25 (s, 3H, CH₃ *iPr*); ¹³C NMR (125 MHz, CDCl₃) δ 165.9 (C=O, 6'-*O*-Bn), 165.7 (C=O, 6-*O*-Bn), 165.5 (C=O, 2-*O*-Bn), 165.4 (C=O, 3-*O*-Bn),

165.0 (C=O, 2'-O-Bn), 160.7 (C=NH), 133.5, 133.4, 133.3, 133.2, 133.1 (C-*p*, 5 x OBz), 130.1, 129.94, 129.86, 129.78, 129.74, 129.54, 129.50, 129.29, 128.68, 128.63, 128.47, 128.43, 128.41, 128.21 (25 x CH_{arom}), 110.8 (C_q *i*Pr), 100.7 (C-1'), 93.1 (C-1), 90.7 (CCl₃), 77.0 (C-3'), 75.1 (C-4), 73.7 (C-2'), 73.0 (C-4'), 71.4 (C-5), 71.3 (C-5'), 70.6 (C-2), 70.1 (C-3), 62.8 (C-6'), 62.1 (C-6), 27.3, 26.1 (2 x CH₃ *i*Pr); HRMS(ESI) *m/z* calcd. for [C₅₂H₄₆NO₁₆Cl₃+H]⁺: 1046.1955, obsd.: 1046.1917. Spectral data of lactosyl imidate donor **75** matched that previously reported.^{9,16}

(2*S*,3*R*,4*E*)-2-Azido-1-(4-*O*-(2,6-di-*O*-benzoyl-3,4-*O*-isopropylidene-β-*D*-galactopyranosyl)-2,3,6-tri-*O*-benzoyl-β-*D*-glucopyranosyloxy)-3-benzyloxy-octadec-4-ene (74**)**

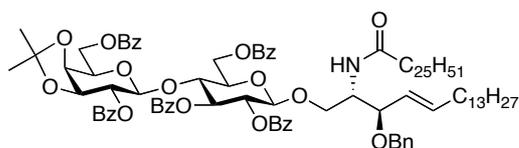


A solution of imidate donor **75** (973 mg, 0.93 mmol) and lipid acceptor **76** (300 mg, 0.72 mmol), co-evaporated

with dry toluene (x3), was dissolved in dry CH₂Cl₂ (6 mL) and 4 Å molecular sieves were added. This mixture was cooled to -20 °C and a solution of TMSOTf in CH₂Cl₂ (1.38 mmol/mL, 157 μL, 0.22 mmol) was added slowly dropwise, the resulting solution was stirred 1.5 h at -20 °C until TLC analysis showed complete consumption of lipid acceptor **76**. The solution was quenched with NEt₃ (400 μL, 2.87 mmol) and concentrated under reduced pressure. The resulting oil was purified by gradient flash chromatography (petroleum ether/EtOAc, 10/1 to 5/1, v/v) to give glycolipid **74** as a colourless oil (936 mg, 0.72 mmol, Quantitative). *R_f*: 0.49 (PE/EA, 2/1, v/v); [α]_D²⁵ = +10.0° (c = 1.0, CH₂Cl₂); IR (film) 3440, 3297, 3067, 2925, 2854, 2361, 2342, 2101, 1720, 1602, 1452, 1373, 1315, 1264, 1177, 1109, 1068, 1027, 825, 707 cm⁻¹; ¹H NMR (500 MHz, CDCl₃) δ 8.10 (d, *J*_{CH-*o*,CH-*m*} = 7.3 Hz, 2H, CH-*o*, OBz), 8.03 (d, *J*_{CH-*o*,CH-*m*} = 7.3 Hz, 2H, CH-*o*, OBz), 8.00 (d, *J*_{CH-*o*,CH-*m*} = 7.3 Hz, 2H, CH-*o*, OBz), 7.96–7.94 (m, 4H, 2 x CH-*o*, OBz), 7.64–7.19 (m, 20H, H_{arom}), 5.73 (t, *J*_{2',3'} = 9.4 Hz, 1H, H-3'), 5.46–5.39 (m, 2H, H-2', H-5), 5.40 (t, *J*_{5,6} = 6.7 Hz, 1H, H-5), 5.26 (dd, *J*_{4,5} = 15.6 Hz, *J*_{3,4} = 8.6 Hz, 1H, H-4), 5.14 (t, *J*_{2'',3''} = 7.3 Hz, 1H, H-2''), 4.68 (d, *J*_{1',2'} = 7.8 Hz, 1H, H-1'), 4.60–4.58 (m, 2H, H-1'', H-6'a), 4.47 (dd, *J*_{6'a,6'b} = 12.3 Hz, *J*_{5',6'b} = 4.2 Hz, 1H, H-6'b), 4.41 (d, *J*_{a,b} = 12.0 Hz, 1H, CH-a, 3-*O*-Bn), 4.25–4.20 (m, 3H, H-4',

H-3'', H-6''a), 4.15 (d, $J_{a,b} = 12.0$ Hz, 1H, CH-b, 3-*O*-Bn), 4.07 (dd, $J_{4'',5''} = 5.6$ Hz, $J_{3'',4''} = 1.9$ Hz, 1H, H-4''), 3.90 (dd, $J_{1a,1b} = 10.3$ Hz, $J_{1a,2} = 5.7$ Hz, 1H, H-1a), 3.83–3.77 (m, 2H, H-5', H-5''), 3.73 (dd, $J_{3,4} = 8.6$ Hz, $J_{2,3} = 5.7$ Hz, 1H, H-3), 3.65 (dd, $J_{6'',a,6''b} = 11.5$ Hz, $J_{5,6''b} = 7.4$ Hz, 1H, H-6''b), 3.60 (dd, $J_{1,2} = 11.2$ Hz, $J_{2,3} = 5.7$ Hz, 1H, H-2), 3.50 (dd, $J_{1a,1b} = 10.3$ Hz, $J_{1b,2} = 5.7$ Hz, 1H, H-1b), 1.93–1.91 (m, 2H, H-6), 1.52 (s, 3H, CH₃ *i*Pr), 1.32–1.26 (m, 25H, CH₃ *i*Pr, H-7–H-17), 0.89 (t, $J_{17,18} = 7.0$ Hz, 3H, H-18); ¹³C NMR (125 MHz, CDCl₃) δ 166.0 (C=O, 6''-*O*-Bn), 165.9 (C=O, 6'-*O*-Bn), 165.7 (C=O, 3'-*O*-Bn), 165.1 (C=O, 2'-*O*-Bn), 165.0 (C=O, 2''-*O*-Bn), 138.3 (C-5), 138.1 (C-*i*, 3-*O*-Bn), 133.4, 133.3, 133.24, 133.21, 133.0 (C-*p*, 5 x OBz), 129.88, 129.85, 129.82, 129.8, 129.6, 129.5, 129.35, 129.28, 128.7, 128.449, 128.44, 128.37, 128.27, 128.17, 127.53, 127.45, 127.44 (30 x CH_{arom}), 125.4 (C-4), 110.9 (C_q *i*Pr), 101.0 (C-1'), 100.2 (C-1''), 79.6 (C-3), 77.0 (C-3''), 75.3 (C-4'), 73.6 (C-2''), 73.2 (C-4''), 73.0 (C-5'), 72.7 (C-3'), 71.8 (C-2'), 71.3 (C-5''), 70.0 (CH₂, 3-*O*-Bn), 68.5 (C-1), 63.7 (C-2), 62.8 (C-6''), 62.5 (C-6'), 32.3 (C-6), 27.4, 26.2 (2 x CH₃ *i*Pr), 31.9, 29.70, 29.69, 29.68, 29.66, 29.63, 29.42, 29.37, 29.16, 28.9, 22.7 (C-7–C-17), 14.1 (C-18); HRMS(ESI) *m/z* calcd. for [C₇₅H₈₅N₃O₁₇+Na]⁺: 1322.5771, obsd.: 1322.5764.

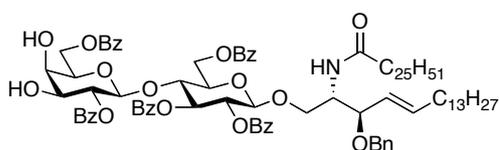
(2*S*,3*R*,4*E*)-1-(4-*O*-(2,6-Di-*O*-benzoyl-3,4-*O*-isopropylidene-β-D-galactopyranosyl)-2,3,6-tri-*O*-benzoyl-β-D-glucopyranosyloxy)-3-benzyloxy-2-hexacosanoylamido-octadecen-4-ene (83)



To a solution of glycolipid azide **74** (100 mg, 0.077 mmol) in toluene (1 mL) triphenylphosphine (40 mg, 0.15 mmol), and distilled water (10 drops) were added, the solution was warmed to 80 °C and stirred overnight. The reaction mixture was then cooled to room temperature, diluted with EtOAc, washed with sat. aq. NH₄Cl, dried (MgSO₄), filtered and concentrated under reduced pressure to give a colourless oil which was used without further purification. The oil was co-evaporated twice with dry toluene then suspended in DCM (2 mL), EDCI (73 mg, 0.383 mmol), DMAP (30 mg, 0.246 mmol), and hexacosanoic acid (151 mg, 0.383 mmol) were added and the resulting solution stirred over three nights at room temperature. The reaction mixture was then purified directly by gradient flash chromatography (petroleum

ether/EtOAc, 10/1 to 2/1, v/v) to give **83** as a colourless oil (92 mg, 0.056 mmol, 72% over two steps). R_f : 0.55 (PE/EA, 2/1, v/v); $[\alpha]_D^{22} = +16.0^\circ$ ($c = 1.0$, CH_2Cl_2); IR (film) 3323, 3063, 3034, 2921, 2852, 2361, 2341, 1971, 1724, 1646, 1602, 1531, 1452, 1267, 1112, 1068, 1027, 707 cm^{-1} ; $^1\text{H NMR}$ (500 MHz, CDCl_3) δ 8.09 (d, $J_{\text{CH-}o,\text{CH-}m} = 7.3$ Hz, 2H, CH-*o*, OBz), 8.02 (d, $J_{\text{CH-}o,\text{CH-}m} = 7.3$ Hz, 2H, CH-*o*, OBz), 8.00 (d, $J_{\text{CH-}o,\text{CH-}m} = 7.6$ Hz, 2H, CH-*o*, OBz), 7.96 (d, $J_{\text{CH-}o,\text{CH-}m} = 7.5$ Hz, 2H, CH-*o*, OBz), 7.92 (d, $J_{\text{CH-}o,\text{CH-}m} = 7.3$ Hz, 2H, CH-*o*, OBz), 7.62–7.11 (m, 20H, H_{arom}), 5.73 (t, $J_{2',3'} = 9.4$ Hz, 1H, H-3'), 5.49 (dt, $J_{4,5} = 15.3$ Hz, $J_{5,6} = 6.9$ Hz, 1H, H-5), 5.393 (dd, $J_{2',3'} = 9.6$ Hz, $J_{1',2'} = 8.0$ Hz, 1H, H-2'), 5.392 (d, $J_{\text{NH},2} = 9.1$ Hz, 1H, NH C₂₆), 5.24 (dd, $J_{4,5} = 15.3$ Hz, $J_{3,4} = 8.5$ Hz, 1H, H-4), 5.14 (t, $J_{1'',2''} = 7.2$ Hz, 1H, H-2''), 4.60–4.55 (m, 3H, H-1', H-1'', H-6'a), 4.48 (dd, $J_{6'a,6'b} = 12.3$ Hz, $J_{5',6'} = 4.0$ Hz, 1H, H-6'b), 4.44 (d, $J_{a,b} = 11.5$ Hz, 1H, CH-a, 3-*O*-Bn), 4.26–4.19 (m, 5H, H-1a, H-4', H-3'', H-6'a, CH-b, 3-*O*-Bn), 4.09–4.05 (m, 2H, H-2, H-4''), 3.81–3.79 (m, 1H, H-5''), 3.75–3.69 (m, 3H, H-5', H-6'b, H-3), 3.47 (dd, $J_{1a,1b} = 9.5$ Hz, $J_{1b,2} = 3.4$ Hz, 1H, H-1b), 1.95–1.92 (m, 2H, H-6), 1.71–1.61 (m, 2H, $\text{CH}_2\text{-}\alpha$), 1.52 (s, 3H, CH_3 *iPr*), 1.55–1.03 (m, 71H, H-7–H-17, H- β –H-(ω -1), CH_3 *iPr*), 0.88 (t, $J_{17,18} = J_{\omega-1,\omega} = 6.9$ Hz, 6H, H-18, H- ω); $^{13}\text{C NMR}$ (125 MHz, CDCl_3) δ 172.4 (HNC=O), 166.0 (C=O, 6''-*O*-Bn), 165.8 (C=O, 6'-*O*-Bn), 165.6 (C=O, 3'-*O*-Bn), 165.3 (C=O, 2'-*O*-Bn), 165.0 (C=O, 2''-*O*-Bn), 138.3 (C-*i*, 3-*O*-Bn), 137.0 (C-5), 133.5, 133.4, 133.3, 133.2, 133.1 (C-*p*, 5 x OBz), 129.9, 129.81, 129.78, 129.7, 129.6, 129.5, 129.3, 129.1, 128.7, 128.6, 128.5, 128.4, 128.2, 127.6, 127.4 (30 x CH_{arom}), 127.3 (C-4), 110.9 (C_q *iPr*), 101.3 (C-1'), 100.1 (C-1''), 79.1 (C-3), 77.0 (C-3''), 75.2 (C-4'), 73.6 (C-2''), 73.1 (C-4''), 73.0 (C-5'), 72.4 (C-3'), 72.3 (C-2'), 71.3 (C-5''), 70.3 (CH_2 , 3-*O*-Bn), 68.3 (C-1), 62.8 (C-6''), 62.6 (C-6'), 51.2 (C-2), 36.4 (C- α), 32.2 (C-6), 31.9, 29.75, 29.73, 29.71, 29.70, 29.66, 29.56, 29.54, 29.40, 29.38, 29.37, 29.27, 29.23, 22.70 (C-7–C-17, C- γ –C-(ω -1)), 27.4, 26.1 (2 x CH_3 *iPr*), 25.5 (C- β), 14.1 (C-18, C- ω); HRMS(ESI) m/z calcd. for $[\text{C}_{101}\text{H}_{137}\text{NO}_{18}+\text{Na}]^+$: 1674.9728, obsd.: 1674.9717.

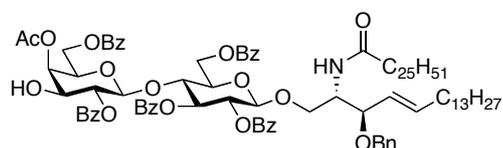
(2*S*,3*R*,4*E*)-1-(4-*O*-(2,6-Di-*O*-benzoyl- β -D-galactopyranosyl)-2,3,6-tri-*O*-benzoyl- β -D-glucopyranosyloxy)-3-benzyloxy-2-hexacosanoylamido-octadec-4-ene (84**)**



To a solution of fully protected lactosyl ceramide **83** (226 mg, 0.14 mmol) in CH₂Cl₂ (5 mL) was added TFA/H₂O solution (1/1, v/v, 0.5 mL) and the resulting solution was stirred at room temperature for 12 h. The solution was diluted with ethyl acetate and the organic layer was then washed with sat. aq. NaHCO₃ and brine, dried (MgSO₄), filtered and concentrated under reduced pressure. The colourless oil was then purified by gradient flash chromatography (petroleum ether/EtOAc, 5/1 to 1/1, v/v) to give diol **84** as a colourless oil (213 mg, 0.13 mmol, 96%). R_f: 0.08 (PE/EA, 2/1, v/v); [α]_D²⁵ = +15.0° (c = 1.0, CH₂Cl₂); IR (film) 3064, 2922, 2852, 2357, 1724, 1512, 1452, 1267, 1176, 1110, 1068, 1028, 708 cm⁻¹; ¹H NMR (500 MHz, CDCl₃) δ 8.07 (d, $J_{\text{CH-}o,\text{CH-}m}$ = 7.3 Hz, 2H, CH-*o*, 2''-*O*-Bz), 8.00 (t, $J_{\text{CH-}o,\text{CH-}m}$ = 8.7 Hz, 4H, CH-*o*, OBz), 7.94 (t, $J_{\text{CH-}o,\text{CH-}m}$ = 8.7 Hz, 4H, 2 x CH-*o*, OBz), 7.59–7.14 (m, 20H, H_{arom}), 5.66 (t, $J_{2',3'} = J_{3',4'} = 9.4$ Hz, 1H, H-3'), 5.50 (dt, $J_{4,5} = 15.4$ Hz, $J_{5,6} = 6.6$ Hz, 1H, H-5), 5.40–5.36 (m, 2H, H-2', NH), 5.29 (t, $J_{1'',2''} = 7.8$ Hz, 1H, H-2''), 5.23 (dd, $J_{4,5} = 15.4$ Hz, $J_{3,4} = 8.7$ Hz, 1H, H-4), 4.59 (d, $J_{1'',2''} = 7.8$ Hz, 1H, H-1''), 4.56 (d, $J_{1',2'} = 7.8$ Hz, 1H, H-1'), 4.53 (bs, 2H, H-6'a, H-6'b), 4.44 (d, $J_{a,b} = 11.5$ Hz, 1H, CH-a, 3-*O*-Bn), 4.24 (d, $J_{a,b} = 11.5$ Hz, 1H, CH-b, 3-*O*-Bn), 4.23–4.22 (m, 1H, H-1a), (t, $J_{3',4'} = 9.4$ Hz, 1H, H-4'), 4.08–4.05 (m, 1H, H-2), 4.00 (dd, $J_{6''a,6''b} = 11.3$ Hz, $J_{5,6''a} = 6.8$ Hz, 1H, H-6''a), 3.81 (bs, 1H, H-4''), 3.73–3.67 (m, 3H, H-3, H-5', H-3''), 3.63 (dd, $J_{6''a,6''b} = 11.3$ Hz, $J_{5,6''b} = 6.2$ Hz, 1H, H-6''b), 3.48–3.47 (m, 2H, H-1b, H-5'), 1.95–1.92 (m, 2H, H-6), 1.68–1.60 (m, 2H, CH₂- α), 1.37–0.90 (m, 68H, H-7–H-17, H- β –H-(ω -1)), 0.88 (t, $J_{17,18} = J_{\omega,\omega+1} = 6.9$ Hz, 6H, H-18, H- ω); ¹³C NMR (125 MHz, CDCl₃) δ 172.4 (HNC=O), 166.4 (C=O, 2''-*O*-Bn), 166.1 (C=O, 6''-*O*-Bn), 165.9 (C=O, 6'-*O*-Bn), 165.8 (C=O, 3'-*O*-Bn), 165.3 (C=O, 2'-*O*-Bn), 138.3 (C-*i*, 3-*O*-Bn), 137.0 (C-5), 133.5, 133.4, 133.3 (C-*p*, 5 x OBz), 129.9, 129.8, 129.7, 129.64, 129.56, 129.54, 129.47, 129.1,

129.0, 128.59, 128.56, 128.51, 128.4, 128.2, 127.6, 127.4 (30 x CH_{arom}), 127.3 (C-4), 101.1 (C-1'), 100.8 (C-1''), 79.1 (C-3), 75.9 (C-4'), 73.7 (C-2''), 73.0 (C-5'), 72.7 (C-3'), 72.6 (C-3''), 72.5 (C-5''), 72.1 (C-2'), 70.3 (CH₂, 3-O-Bn), 68.5 (C-4''), 68.3 (C-1), 62.6 (C-6'), 61.8 (C-6''), 51.2 (C-2), 36.4 (C-α), 32.2 (C-6), 31.9, 29.72, 29.71, 29.68, 29.66, 29.56, 29.54, 29.40, 29.37, 29.27, 29.23, 22.7 (C-7-C-17, C-γ-C-(ω-1)), 25.5 (C-β), 14.1 (C-18, C-ω); HRMS(ESI) m/z calcd. for [C₉₈H₁₃₄NO₁₈+H]⁺: 1612.9595, obsd.: 1612.9612.

(2S,3R,4E)-1-(4-O-(4-O-Acetyl-2,6-di-O-benzoyl-β-D-galactopyranosyl)-2,3,6-tri-O-benzoyl-β-D-glucopyranosyloxy)-3-benzyloxy-2-hexacosanoylamido-octadec-4-ene (73)

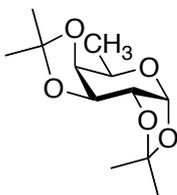


Diol **84** (181 mg, 0.11 mmol) was co-evaporated with toluene (x3) and dissolved in dry CH₂Cl₂ (1.1 mL).

Trimethyl orthoacetate (84 μL, 0.67 mmol) and CSA (52 mg, 0.22 mmol) were added and the reaction mixture was stirred at rt for 16 h. The reaction mixture was diluted with EtOAc (30 mL), washed with 1M HCl solution (3 x 30 mL), sat. aq. NaHCO₃ (30 mL) and brine (30 mL), dried (MgSO₄), filtered and concentrated *in vacuo*. The residue was purified by gradient flash chromatography (petroleum ether/EtOAc, 10/1 to 1/1, v/v) to afford acetate **73** as a clear oil (170 mg, 0.26 mmol, 92%). R_f: 0.24 (PE/EA, 2/1, v/v); [α]_D²⁴ = +4.0° (c = 1.0, CH₂Cl₂); IR (film) 3325, 3064, 2923, 2853, 2359, 1966, 1726, 1452, 1372, 1265, 1177, 1108, 1094, 1069, 1027, 975, 736, 708 cm⁻¹; ¹H NMR (500 MHz, CDCl₃) δ 8.06 (d, J_{CH-o,CH-m} = 7.8 Hz, 2H, CH-o, OBz), 8.03 (d, J_{CH-o,CH-m} = 7.6 Hz, 4H, 2 x CH-o, OBz), 7.96 (d, J_{CH-o,CH-m} = 7.3 Hz, 2H, CH-o, OBz), 7.96 (d, J_{CH-o,CH-m} = 7.8 Hz, 2H, CH-o, OBz), 7.60–7.13 (m, 20H, H_{arom}), 5.72 (t, J_{2',3'} = J_{3',4'} = 9.7 Hz, 1H, H-3'), 5.50 (dt, J_{4,5} = 15.6 Hz, J_{5,6} = 6.6 Hz, 1H, H-5), 5.41 (dd, J_{1',2'} = 8.0 Hz, J_{2',3'} = 9.7 Hz, 1H, H-2'), 5.37 (d, J_{NH,2'} = 9.3 Hz, 1H, NH), 5.26–5.22 (m, 1H, H-4), 5.22 (d, J_{3'',4''} = 3.5 Hz, 1H, H-4''), 5.16 (dd, J_{2'',3''} = 9.8 Hz, J_{1'',2''} = 8.0 Hz, 1H, H-2''), 4.65 (d, J_{1'',2''} = 8.0 Hz, 1H, H-1''), 4.61 (d, J_{1',2'} = 8.0 Hz, 1H, H-1'), 4.60–4.51 (m, 2H, H-6'a, H-6'b), 4.44 (d, J_{a,b} = 11.5 Hz, 1H, CH-a, 3-O-Bn), 4.25 (d, J_{a,b} = 11.5 Hz, 1H, CH-b, 3-O-Bn), 4.19 (t, J_{3',4'} = 9.7 Hz, 1H, H-4'), 4.07 (m, 1H, H-2), 3.82 (dd, J_{2'',3''} = 9.8 Hz, J_{3'',4''} = 3.5 Hz, 1H, H-3''), 3.76 (d, J_{4',5'} =

7.8 Hz, 1H, H-5'), 3.73–3.68 (m, 2H, H-3, H-6''a), 3.64–3.59 (m, 2H, H-5'', H-6''b), 3.50 (dd, $J_{1a,1b} = 9.8$ Hz, $J_{1b,2} = 3.5$ Hz, 1H, H-1b), 1.99 (s, 3H, OAc), 1.98–1.92 (m, 2H, H-6), 1.69–1.60 (m, 2H, CH₂-α), 1.37–1.03 (m, 68H, H-7–H-17, H-β–H-(ω-1)), 0.88 (t, $J_{17,18} = J_{\omega-1,\omega} = 6.9$ Hz, 6H, H-18, H-ω); ¹³C NMR (125 MHz, CDCl₃) δ 172.4 (HNC=O), 170.6 (C=O, OAc), 166.5 (C=O, 2''-O-Bn), 165.9 (C=O, 6'-O-Bn), 165.6 (C=O, 6''-O-Bn), 165.3 (C=O, 2'-O-Bn), 165.3 (C=O, 3'-O-Bn), 138.3 (C-*i*, 3-O-Bn), 137.0 (C-5), 133.6, 133.5, 133.4, 133.2 (C-*p*, 5 x OBz), 129.89, 120.82, 129.76, 129.62, 129.51, 129.41, 129.06, 128.9, 128.6, 128.58, 128.26, 128.19, 127.63, 127.40, 127.30 (30 x CH_{arom}), 101.3 (C-1'), 100.3 (C-1''), 79.1 (C-3), 75.3 (C-4'), 73.5 (C-2''), 73.0 (C-5'), 72.4 (C-3'), 72.1 (C-2'), 71.5 (C-3''), 71.1 (C-5''), 70.3 (CH₂, 3-O-Bn), 69.3 (C-4''), 68.3 (C-1), 62.5 (C-6'), 61.2 (C-6''), 21.1 (C-2), 36.4 (C-α), 32.2 (C-6), 31.92, 29.72, 29.70, 29.68, 29.66, 29.55, 29.54, 29.39, 29.37, 29.36, 29.26, 29.22, 25.45, 22.69 (C-7–C-17, C-β–C-(ω-1)), 20.6 (OAc), 14.1 (C-18, C-ω); HRMS(ESI) *m/z* calcd. for [C₁₀₀H₁₃₅NO₁₉+Na]⁺: 1676.9521, obsd.: 1676.9526.

6-Deoxy-1,2:3,4-di-*O*-isopropylidene-α-D-galactopyranose (**88**)³¹



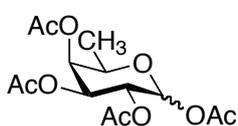
LiAlH₄ (350 mg, 9.22 mmol) was carefully added to a solution of Bu₃SnCl (3 g, 9.22 mmol) in dry Et₂O (20 mL) at 0 °C. The reaction mixture was stirred at rt for 3 h, and ice water (10 mL) was added and stirred for a further 5 mins. The mixture was

filtered through a celite pad and the organic layer washed with H₂O (2 x 20 mL), dried (MgSO₄) and concentrated *in vacuo* to give Bu₃SnH as an oil, which was set aside for the next step.

6-Deoxy-6-iodo-1,2:3,4-di-*O*-isopropylidene-α-D-galactopyranose, **87**^{30,39,40} (1.0 g, 2.84 mmol) was co-evaporated with toluene (x3) and dissolved in toluene (1.8 mL). Bu₃SnH (1.53 mL, 5.67 mmol) and AIBN (70 mg, 0.43 mmol) were added and stirred at 100 °C for 1.5 h, after which the reaction mixture was diluted with EtOAc (80 mL), the organic layer washed with water (80 mL) and brine (80 mL), dried (MgSO₄), filtered and concentrated *in vacuo*. The resultant oil was purified by gradient flash chromatography (petroleum ether to petroleum ether/EtOAc, 30/1 to 3/1, v/v) to afford 6-deoxy-galactose **88** as a clear oil (672 mg, 2.75 mmol, 97%). *R*_f: 0.27 (PE/EA, 10/1, v/v); [α]_D²⁵ = -37.0° (c = 1.0, CHCl₃); IR (film)

3027, 2930, 1369, 1321, 1230, 1170, 1045, 1020, 904, 669 cm^{-1} ; ^1H NMR (500 MHz, CDCl_3) δ 5.51 (d, $J_{1,2} = 5.1$ Hz, 1H, H-1), 4.58 (dd, $J_{3,4} = 8.0$ Hz, $J_{2,3} = 2.2$ Hz, 1H, H-3), 4.27 (dd, $J_{1,2} = 5.1$ Hz, $J_{2,3} = 2.2$ Hz, 1H, H-2), 4.07 (dd, $J_{3,4} = 8.0$ Hz, $J_{4,5} = 1.8$ Hz, 1H, H-4), 3.90 (dq, $J_{5,6} = 6.6$ Hz, $J_{4,5} = 1.8$ Hz, 1H, H-5), 1.51 (s, 3H, CH_3 *iPr*), 1.45 (s, 3H, CH_3 *iPr*), 1.34 (s, 3H, CH_3 *iPr*), 1.32 (s, 3H, CH_3 *iPr*), 1.24 (d, $J_{5,6} = 6.5$ Hz, 1H, H-6); ^{13}C NMR (125 MHz, CDCl_3) δ 108.9 (C_q *iPr*-3,4), 108.2 (C_q *iPr*-1,2), 96.5 (C-1), 73.5 (C-4), 70.9 (C-3), 70.3 (C-2), 63.4 (C-5), 26.0 (2 x CH_3 *iPr*), 26.0 (CH_3 *iPr*), 24.9 (CH_3 *iPr*), 17.5 (C-6); HRMS(ESI) m/z calcd. for $[\text{C}_{12}\text{H}_{20}\text{O}_5 + \text{NH}_4]^+$: 262.1649, obsd.: 262.1655.

1,2,3,4-Tetra-*O*-acetyl-6-deoxy- α/β -D-galactopyranose (**86**)



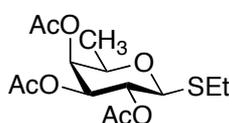
α -D-Fucose **85** (250 mg, 1.52 mmol) was dissolved in pyridine (7.6 mL) and Ac_2O (4.6 mL, 48.6 mmol) was added. The reaction was stirred for 14 h at rt and diluted with CH_2Cl_2 , washed with H_2O (2 x 50 mL), sat. aq. NaHCO_3 (50 mL), brine (50 mL) and dried over MgSO_4 . The MgSO_4 was filtered off, the filtrate concentrated *in vacuo* and purified by gradient flash chromatography (petroleum ether/ EtOAc , 10:1 to 3:1, v/v) to afford an α/β mixture (1:1.4) of peracetylated D-fucose **86** as a clear oil (506 mg, 1.52 mmol, Quantitative).

*Alternative method starting from 6-deoxy-1,2:3,4-di-O-isopropylidene- α -D-galactopyranose, **88**³¹:*

A solution of $\text{AcOH}/\text{H}_2\text{O}$ (4/1, v/v, 5 mL) was added to 6-deoxy galactoside **88** and stirred for 19 h, after which the reaction mixture was warmed to 105 $^\circ\text{C}$ and stirred for further 2 h. The reaction mixture was concentrated, and the residue co-evaporated with pyridine (3 x 8 mL), dissolved in pyridine (5 mL) and Ac_2O (0.46 mL, 4.86 mmol). After stirring at rt for 16 h, the reaction mixture was taken up in EtOAc (50 mL), washed with 0.5 M HCl solution (50 mL), sat. aq. NaHCO_3 (50 mL), water (50 mL) and brine (50 mL), dried (MgSO_4), filtered and concentrated *in vacuo*. Purification by gradient flash chromatography (petroleum ether/ EtOAc , 10:1 to 3:1, v/v) afforded an α/β mixture (1:5) of peracetylated D-fucose **86** as a clear oil (273 mg, 0.82 mmol, 85%). R_f : 0.51 (PE/EA, 1/2, v/v); $[\alpha]_D^{25} = 59.0^\circ$ ($c = 1.0$, CHCl_3); IR (film) 3027, 2942, 2880, 1746, 1433, 1369, 1321, 1212, 1167,

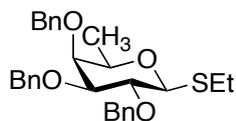
1052, 1023, 904, 668 cm^{-1} ; ^1H NMR (500 MHz, CDCl_3) δ 5.66 (d, $J_{1,2} = 5.7$ Hz, 1H, H-1), 5.29 (t, $J_{2,3} = 10.3$ Hz, 1H, H-2), 5.25 (d, $J_{3,4} = 3.2$ Hz, 1H, H-4), 5.05 (dd, $J_{2,3} = 10.3$ Hz, $J_{3,4} = 3.2$ Hz, 1H, H-3), 3.94 (q, $J_{5,6} = 6.4$ Hz, 1H, H-5), 2.17 (s, 3H, 4-OAc), 2.09 (s, 3H, 1-OAc), 2.02 (s, 3H, 2-OAc), 1.97 (s, 3H, 3-OAc), 1.20 (d, $J_{5,6} = 6.4$ Hz, 1H, H-6); ^{13}C NMR (125 MHz, CDCl_3) δ 170.5 (C=O, 4-OAc), 170.0 (C=O, 3-OAc), 169.5 (C=O, 2-OAc), 169.2 (C=O, 1-OAc), 92.1 (C-1), 71.2 (C-3), 70.2 (C-5), 69.9 (C-4), 67.9 (C-2), 20.8 (1-OAc), 20.7 (2-OAc), 20.63 (4-OAc), 20.56 (3-OAc), 15.9 (C-6); HRMS(ESI) m/z calcd. for $[\text{C}_{14}\text{H}_{20}\text{O}_9+\text{NH}_4]^+$: 350.1446, obsd.: 350.1450.

Ethyl 2,3,4-tri-*O*-acetyl-6-deoxy-1-thio- β -D-galactopyranoside (**89**)



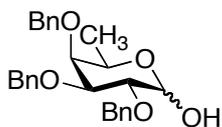
Peracetylated D-fucose **86** (620 mg, 1.87 mmol) was co-evaporated with toluene (x3) and dissolved in dry CH_2Cl_2 (10 mL). Ethanethiol (0.41 mL, 5.60 mmol) was added at rt, followed by freshly distilled 48% $\text{BF}_3\cdot\text{OEt}_2$ (0.71 mL, 2.80 mmol) and stirred for 2 h. The reaction mixture was diluted with CH_2Cl_2 (80 mL) and washed with H_2O (2 x 50 mL), sat. aq. NaHCO_3 (50 mL), brine (50 mL) and dried over MgSO_4 and filtered. The concentrated filtrate was purified by flash column chromatography (petroleum ether/EtOAc, 5:1, v/v) to give β -thiofucoside **89** as a clear oil (452 mg, 1.35 mmol, 72%). R_f : 0.55 (PE/EA, 1/1, v/v); $[\alpha]_D^{25} = +49.0^\circ$ (c = 1.0, CHCl_3); IR (film) 3023, 2904, 1746, 1331, 1245, 1218, 1084, 1055, 1020, 863, 749, 667 cm^{-1} ; ^1H NMR (500 MHz, CDCl_3) δ 5.23 (d, $J_{3,4} = 3.5$ Hz, 1H, H-4), 5.17 (t, $J_{1,2} = J_{2,3} = 10.0$ Hz, 1H, H-2), 5.00 (dd, $J_{2,3} = 10.0$ Hz, $J_{3,4} = 3.5$ Hz, 1H, H-3), 4.42 (d, $J_{1,2} = 10.0$ Hz, 1H, H-1), 3.79 (q, $J_{5,6} = 6.4$ Hz, 1H, H-5), 2.75–2.62 (m, 2H, CH_2CH_3), 2.13 (s, 3H, 4-OAc), 2.02 (s, 3H, 2-OAc), 1.94 (s, 3H, 3-OAc), 1.23 (t, $J_{\text{CH}_2,\text{CH}_3} = 7.5$ Hz, 3H, CH_2CH_3), 1.17 (d, $J_{5,6} = 6.6$ Hz, 3H, H-6); ^{13}C NMR (125 MHz, CDCl_3) δ 170.6 (C=O, 4-OAc), 170.1 (C=O, 3-OAc), 169.6 (C=O, 2-OAc), 83.5 (C-1), 73.1 (C-5), 72.3 (C-3), 70.4 (C-4), 67.3 (C-2), 24.1 (CH_2CH_3), 20.8 (2-OAc), 20.7 (4-OAc), 20.6 (3-OAc), 16.4 (C-6), 14.7 (CH_2CH_3); HRMS(ESI) m/z calcd. for $[\text{C}_{14}\text{H}_{22}\text{O}_7\text{S}+\text{NH}_4]^+$: 352.1424, obsd.: 352.1430.

Ethyl 2,3,4-tri-*O*-benzyl-6-deoxy-1-thio- β -D-galactopyranoside (**90**)²⁸



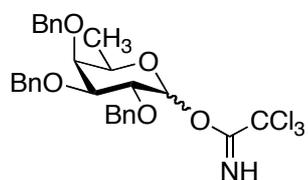
β -thiofucoside **89** (389 mg, 1.16 mmol) was dissolved in methanol and NaOMe was added until the solution reached pH 9.0. The reaction mixture was stirred at rt for 1h, neutralized with Dowex H⁺, filtered and concentrated to give the pure triol as a colourless oil (242 mg, 1.16 mmol), which was used without further purification. The triol (243 mg, 1.16 mmol) was co-evaporated with toluene (x3) and dissolved in dry DMF (12 mL) and benzyl bromide (0.55 mL, 4.66 mmol) was added. The mixture was cooled to 0 °C and NaH (60% in oil suspension, 233 mg, 5.82 mmol) was added. The reaction was stirred for 17 h at rt, quenched with MeOH (5 mL) and concentrated to remove the DMF. The crude oil was redissolved in EtOAc (50 mL), and washed with sat. aq. NaHCO₃ (50 mL) and brine (50 mL), dried (MgSO₄), filtered and concentrated *in vacuo*. The residue was purified by gradient flash chromatography (petroleum ether/EtOAc, 20:1 to 10:1, v/v) to afford benzylated thiofucoside **90** as a clear oil (464 mg, 0.97 mmol, 83%). R_f: 0.73 (PE/EA, 2/1, v/v); [α]_D²⁷ = -6.0° (c = 1.0, CHCl₃); IR (film) 3064, 2978, 2868, 1606, 1497, 1454, 1357, 1208, 1124, 1087, 1066, 1047, 875, 745, 732, 697 cm⁻¹; ¹H NMR (500 MHz, CDCl₃) δ 7.44–7.29 (m, 15H, H_{arom}), 5.03 (d, $J_{a,b}$ = 11.8 Hz, 1H, CH-a, 4-*O*-Bn), 4.93 (d, $J_{a,b}$ = 10.3 Hz, 1H, CH-a, 2-*O*-Bn), 4.84 (d, $J_{a,b}$ = 10.3 Hz, 1H, CH-b, 2-*O*-Bn), 4.80 (d, $J_{a,b}$ = 12.0 Hz, 1H, CH-a, 3-*O*-Bn), 4.77 (d, $J_{a,b}$ = 12.0 Hz, 1H, CH-b, 3-*O*-Bn), 4.73 (d, $J_{a,b}$ = 11.8 Hz, 1H, CH-b, 4-*O*-Bn), 4.43 (d, $J_{1,2}$ = 9.5 Hz, 1H, H-1), 3.86 (t, $J_{1,2} = J_{2,3} = 9.5$ Hz, 1H, H-2), 3.64 (d, $J_{3,4} = 2.6$ Hz, 1H, H-4), 3.60 (dd, $J_{2,3} = 9.5$ Hz, $J_{3,4} = 2.6$ Hz, 1H, H-3), 3.51 (q, $J_{5,6} = 6.4$ Hz, 1H, H-5), 2.84–2.71 (m, 2H, CH₂CH₃), 1.33 (t, $J_{CH_2,CH_3} = 7.5$ Hz, 2H, CH₂CH₃), 1.24 (d, $J_{5,6} = 6.4$ Hz, 1H, H-6); ¹³C NMR (125 MHz, CDCl₃) δ 138.7 (C-*i*, 4-*O*-Bn), 138.5 (C-*i*, 3-*O*-Bn), 138.4 (C-*i*, 2-*O*-Bn), 128.6, 128.5, 128.34, 128.29, 128.22, 128.19, 128.0, 127.73, 127.69, 127.59, 127.54 (15 x CH_{arom}), 85.0 (C-1), 84.5 (C-3), 78.4 (C-2), 76.5 (C-4), 75.7 (CH₂, 2-*O*-Bn), 75.6 (C-5, CH₂, 4-*O*-Bn), 72.9 (CH₂, 3-*O*-Bn), 24.7 (CH₂CH₃), 17.3 (C-6), 15.0 (CH₂CH₃); HRMS(ESI) m/z calcd. for [C₂₉H₃₄NO₄S+NH₄]⁺: 496.2516, obsd.: 496.2517.

2,3,4-Tri-*O*-benzyl-6-deoxy- α/β -D-galactopyranose (**91**)⁴¹



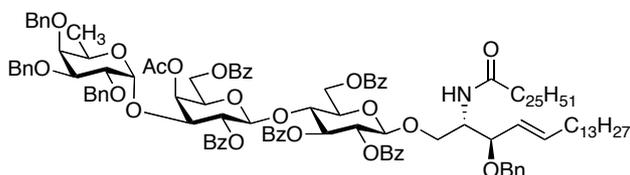
Thiofucoside **90** (183 mg, 0.38 mmol) was dissolved in acetone/H₂O (7.6 mL, 9:1, v/v) and NBS (238 mg, 1.34 mmol) was added. The reaction mixture was stirred at rt for 10 mins and diluted with ethyl acetate (30 mL) and the organic layer was then washed with sat. aq. NaHCO₃ (30 mL), and brine (30 mL), dried (MgSO₄), filtered and concentrated under reduced pressure. The residue was purified by gradient flash chromatography (petroleum ether/EtOAc, 2:1, v/v) to afford an α/β mixture (2.5:1) of lactol **91** as a colourless oil (154 mg, 0.35 mmol, 93%). *R*_f: 0.16 (PE/EA, 2/1, v/v); $[\alpha]_D^{27} = +18.0^\circ$ (c = 1.0, CHCl₃, value obtained for 2.5:1 α/β mixture); IR (film) 3398, 3063, 3030, 2877, 1497, 1454, 1359, 1309, 1211, 1170, 1091, 1061, 1027, 950, 912, 815, 734, 696, 666 cm⁻¹; α : ¹H NMR (500 MHz, CDCl₃) δ 7.41–7.28 (m, 15H, H_{arom}), 5.27 (d, *J*_{1,2} = 3.7 Hz, 1H, H-1), 4.98 (d, *J*_{a,b} = 11.6 Hz, 1H, CH-a, 4-*O*-Bn), 4.84 (d, *J*_{a,b} = 11.5 Hz, 1H, CH-a, 3-*O*-Bn), 4.82 (d, *J*_{a,b} = 10.5 Hz, 1H, CH-a, 2-*O*-Bn), 4.76 (d, *J*_{a,b} = 11.5 Hz, 1H, CH-b, 3-*O*-Bn), 4.72 (d, *J*_{a,b} = 10.5 Hz, 1H, CH-b, 2-*O*-Bn), 4.67 (d, *J*_{a,b} = 11.7 Hz, 1H, CH-b, 4-*O*-Bn), 4.10 (q, *J*_{5,6} = 6.5 Hz, 1H, H-5), 4.04 (dd, *J*_{2,3} = 9.9 Hz, *J*_{1,2} = 3.7 Hz, 1H, H-2), 3.90 (dd, *J*_{2,3} = 9.9 Hz, *J*_{3,4} = 2.6 Hz, 1H, H-3), 3.67 (bs, 1H, H-4), 1.14 (d, *J*_{5,6} = 6.5 Hz, 1H, H-6); ¹³C NMR (125 MHz, CDCl₃) δ 138.7 (C-*i*, 3-*O*-Bn), 138.5 (C-*i*, 4-*O*-Bn), 138.2 (C-*i*, 2-*O*-Bn), 128.47, 128.45, 128.44, 128.35, 128.26, 128.21, 128.1, 127.8, 127.69, 127.68, 127.62, 127.60, 127.5 (15 x CH_{arom}), 91.9 (C-1), 79.1 (C-3), 77.4 (C-4), 76.5 (C-2), 74.8 (CH₂, 4-*O*-Bn), 73.5 (CH₂, 2-*O*-Bn), 73.0 (CH₂, 3-*O*-Bn), 66.7 (C-5), 16.8 (C-6); HRMS(ESI) *m/z* calcd. for [C₂₇H₃₀O₅+NH₄]⁺: 452.2431, obsd.: 452.2436.

***O*-(2,3,4-Tri-*O*-benzyl-6-deoxy- α/β -D-galactopyranosyl) trichloroacetimidate (72)^{32,33}**



Lactol **91** (153 mg, 0.35 mmol) was co-evaporated with toluene (x3) and dissolved in dry CH₂Cl₂ (3.5 mL). DBU (79 μ L, 0.53 mmol) and trichloroacetonitrile (353 μ L, 3.52 mmol) were added at rt and the mixture was stirred for 1 h. The reaction mixture was concentrated *in vacuo* and the residue purified by gradient flash chromatography (1% NEt₃ + petroleum ether to petroleum ether/EtOAc, 20:1, v/v). Both the α -isomer **72** (126 mg, 0.22 mmol, 62%) and the β -isomer (66 mg, 0.11 mmol, 32%) were obtained as clear oils. R_f: 0.61 (PE/EA, 2/1, v/v); α : $[\alpha]_D^{23} = +58.0^\circ$ (c = 1.0, CHCl₃); IR (film) 3343, 30064, 3030, 2935, 2904, 2874, 1731, 1669, 1603, 1497, 1454, 1356, 1293, 1216, 1103, 1066, 1027, 968, 943, 838, 794, 735, 697, 644 cm⁻¹; ¹H NMR (500 MHz, CDCl₃) δ 8.53 (s, 1H, NH), 7.41–7.29 (m, 15H, H_{arom}), 6.55 (d, $J_{1,2} = 3.4$ Hz, 1H, H-1), 5.04 (d, $J_{a,b} = 11.5$ Hz, 1H, CH-a, 4-*O*-Bn), 4.89 (d, $J_{a,b} = 11.7$ Hz, 1H, CH-a, 3-*O*-Bn), 4.80 – 4.78 (m, 3H, CH-b, 3-*O*-Bn, CH-a, CH-b, 2-*O*-Bn), 4.71 (d, $J_{a,b} = 11.5$ Hz, 1H, CH-b, 4-*O*-Bn), 4.27 (dd, $J_{2,3} = 10.0$ Hz, $J_{1,2} = 3.4$ Hz, 1H, H-2), 4.12 (q, $J_{5,6} = 6.4$ Hz, 1H, H-5), 4.06 (dd, $J_{2,3} = 10.0$ Hz, $J_{3,4} = 2.6$ Hz, 1H, H-3), 3.74 (bs, 1H, H-4), 1.19 (d, $J_{5,6} = 6.4$ Hz, 1H, H-6); ¹³C NMR (125 MHz, CDCl₃) δ 161.3 (C=NH), 138.6 (C-*i*, 3-*O*-Bn), 138.5 (C-*i*, 2-*O*-Bn), 138.4 (C-*i*, 4-*O*-Bn), 128.5, 128.42, 128.37, 128.29, 128.26, 127.74, 127.70, 127.6, 127.5 (15 x CH_{arom}), 95.3 (C-1), 91.6 (CCl₃), 78.3 (C-3), 77.3 (C-4), 75.8 (C-2), 75.0 (CH₂, 4-*O*-Bn), 73.2 (CH₂, 3-*O*-Bn), 72.9 (CH₂, 2-*O*-Bn), 69.6 (C-5), 16.7 (C-6); HRMS(ESI) m/z calcd. for [C₂₉H₃₀NO₅Cl₃+Na]⁺: 600.1082, obsd.: 600.1092.

(2*S*,3*R*,4*E*)-1-(4-*O*-(4-*O*-Acetyl-2,6-di-*O*-benzoyl-3-*O*-(2,3,4-tri-*O*-benzyl-6-deoxy- α -D-galactopyranosyl)- β -D-galactopyranosyl)-2,3,6-tri-*O*-benzoyl- β -D-glucopyranosyloxy)-3-benzyloxy-2-hexacosanoylamido-octadec-4-ene (71)

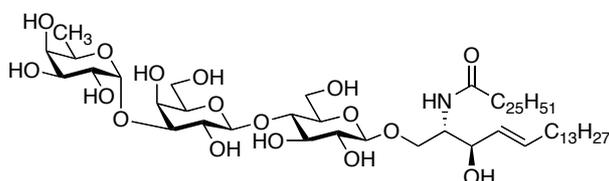


Fucosyl imidate donor **72** (32 mg, 0.055 mmol) and lactosyl ceramide acceptor **73** (44.7 mg, 0.027 mmol) were co-

evaporated with toluene (x3), dissolved in dry CH₂Cl₂ (1 mL) and stirred with activated 4Å molecular sieves for 3 h. The reaction mixture was cooled to -40 °C, and a solution of TMSOTf in CH₂Cl₂ (0.28 mmol/mL, 9.8 μL, 0.0027 mmol) was added and stirred for 1 h. The reaction was warmed to -20 °C and further TMSOTf solution (0.28 mmol/mL, 5.9 μL, 0.0016 mmol) was added. The reaction mixture was allowed to warm slowly to rt while stirring overnight (20 h), upon which it was quenched with NEt₃ (40 μL, 0.29 mmol), filtered and concentrated *in vacuo*. The resultant oil was purified by gradient flash chromatography (3% to 8 % EtOAc in toluene) to afford the fully protected triglycosyl ceramide **71** as a clear oil (38.6 mg, 0.019 mmol, 69%). *R*_f: 0.49 (PE/EA, 2/1, v/v); [α]_D²⁴ = +12.0° (c = 1.0, CHCl₃); IR (film) 3091, 2923, 2853, 1730, 1672, 1602, 1497, 1453, 1364, 1315, 1267, 1176, 1097, 1069, 1028, 978, 755, 710, 641 cm⁻¹; ¹H NMR (600 MHz, CDCl₃) δ 8.02 (d, *J*_{CH-*o*,CH-*m*} = 7.3 Hz, 2H, CH-*o*, 6''-*O*-Bz), 8.01 (d, *J*_{CH-*o*,CH-*m*} = 7.0 Hz, 2H, CH-*o*, 3'-*O*-Bz), 7.98 (d, *J*_{CH-*o*,CH-*m*} = 7.1 Hz, 2H, CH-*o*, 2'-*O*-Bz), 7.95 (d, *J*_{CH-*o*,CH-*m*} = 7.1 Hz, 2H, CH-*o*, 2''-*O*-Bz), 7.93 (d, *J*_{CH-*o*,CH-*m*} = 7.1 Hz, 2H, CH-*o*, 6'-*O*-Bz), 7.61–7.13 (m, 35H, H_{arom}), 5.74 (t, *J*_{3',4'} = 9.6 Hz, 1H, H-3'), 5.49 (dt, *J*_{4,5} = 15.3 Hz, *J*_{5,6} = 6.7 Hz, 1H, H-5), 5.41 (t, *J*_{1',2'} = *J*_{2',3'} = 7.5 Hz, 1H, H-2'), 5.40 (t, *J*_{1'',2''} = *J*_{2'',3''} = 7.5 Hz, 1H, H-2''), 5.36 (d, *J*_{NH,2'} = 9.1 Hz, 1H, NH), 5.34 (d, *J*_{3'',4''} = 3.4 Hz, 1H, H-4''), 5.23 (dt, *J*_{4,5} = 15.3 Hz, *J*_{3,4} = 8.8 Hz, 1H, H-4), 4.94 (d, *J*_{1''',2'''} = 3.5 Hz, 1H, H-1'''), 4.75 (d, *J*_{a,b} = 11.3 Hz, 1H, CH-a, 4'''-*O*-Bn), 4.72 (d, *J*_{a,b} = 11.9 Hz, 1H, CH-a, 3'''-*O*-Bn), 4.60 (d, *J*_{1',2'} = 7.9 Hz, 1H, H-1'), 4.56 (d, *J*_{1'',2''} = 8.2 Hz, 1H, H-1''), 4.58–4.54 (m, 2H, CH-a, CH-b, 2'''-*O*-Bn), 4.48 (d, *J*_{a,b} = 11.9 Hz, 1H, CH-b, 3'''-*O*-Bn), 4.48–4.47 (m, 2H, H-6'a, H-6'b), 4.44 (d, *J*_{a,b} = 11.5 Hz, 1H, CH-a, 3-*O*-Bn), 4.34 (d, *J*_{a,b} = 11.3 Hz, 1H, CH-b, 4'''-*O*-Bn), 4.25 (dd, *J*_{1a,1b} = 9.8 Hz,

$J_{1a,2} = 2.7$ Hz, 1H, H-1a), 4.24 (d, $J_{a,b} = 11.5$ Hz, 1H, CH-b, 3-*O*-Bn), 4.17 (t, $J_{3',4'}$ = 9.6 Hz, 1H, H-4'), 4.09–4.05 (m, 1H, H-2), 3.83 (dd, $J_{2'',3''} = 10.2$ Hz, $J_{1'',2''} = 3.5$ Hz, 1H, H-2''), 3.80 (dd, $J_{2'',3''} = 10.3$ Hz, $J_{3'',4''} = 3.4$ Hz, 1H, H-3''), 3.74–3.69 (m, 2H, H-5', H-3), 3.62–3.59 (m, 2H, H-6''a, H-6''b), 3.57–3.55 (m, 2H, H-5'', H-3''), 3.52 (t, $J_{5'',6''} = 6.9$ Hz, 1H, H-5''), 3.48 (dd, $J_{1a,1b} = 9.8$ Hz, $J_{1a,2} = 3.7$ Hz, 1H, H-1b), 2.90 (d, $J_{3'',4''} = 0.9$ Hz, 1H, H-4''), 1.96–1.92 (m, 2H, H-6), 1.70–1.62 (m, 2H, CH₂-α), 1.63 (s, 3H, OAc), 1.31–1.04 (m, 68H, H-7–H-17, H-β–H-(ω-1)), 0.88 (t, $J_{17,18} = J_{\omega-1,\omega} = 7.0$ Hz, 6H, H-18, H-ω), 0.79 (d, $J_{5'',6''} = 6.5$ Hz, 3H, H-6''); ¹³C NMR (150 MHz, CDCl₃) δ 172.5 (HNC=O), 170.2 (C=O, OAc), 166.0 (C=O, 6'-*O*-Bn), 165.8 (C=O, 6''-*O*-Bn), 165.5 (C=O, 2''-*O*-Bn), 165.3 (C=O, 3'-*O*-Bn), 164.5 (C=O, 2'-*O*-Bn), 139.0 (C-*i*, 3'''-*O*-Bn), 138.7 (C-*i*, 2'''-*O*-Bn), 138.5 (C-*i*, 4'''-*O*-Bn), 138.4 (C-*i*, 3-*O*-Bn), 137.2 (C-5), 133.7, 133.6, 133.55, 133.52, 133.3 (C-*p*, 5 x OBz), 129.97, 129.89, 129.87, 129.78, 129.67, 129.60, 129.48, 129.18, 128.75, 128.71, 128.57, 128.44, 128.34, 128.32, 128.22, 128.17, 128.09, 128.05, 127.85, 127.76, 127.61, 127.59, 127.57, 127.52, 127.41 (45 x CH_{arom}), 127.44 (C-4), 101.5 (C-1'), 100.9 (C-1''), 94.2 (C-1'''), 79.2 (C-3), 79.0 (C-3'''), 77.6 (C-4'''), 75.4 (C-4', C-2''), 74.9 (CH₂, 4'''-*O*-Bn), 73.5 (CH₂, 2'''-*O*-Bn), 73.3 (CH₂, 3'''-*O*-Bn), 73.1 (C-5'), 72.5 (C-3'), 72.3 (C-2'', C-3''), 71.4 (C-5''), 71.1 (C-2'), 70.4 (CH₂, 3-*O*-Bn), 68.5 (C-1), 66.7 (C-5'''), 64.4 (C-4'), 62.5 (C-6'), 61.3 (C-6''), 51.3 (C-2), 36.6 (C-α), 32.4 (C-6), 32.07, 30.17, 29.89, 29.87, 29.85, 29.82, 29.80, 29.70, 29.68, 29.53, 29.52, 29.51, 29.41, 29.39, 29.36, 27.22, 25.58, 22.84 (C-7–C-17, C-β–C-(ω-1)), 20.4 (OAc), 16.2 (C-6'''), 14.3 (C-18, C-ω); HRMS(ESI) *m/z* calcd. for [C₁₂₇H₁₆₃NO₂₃+Na]⁺: 2093.1508, obsd.: 2093.1463.

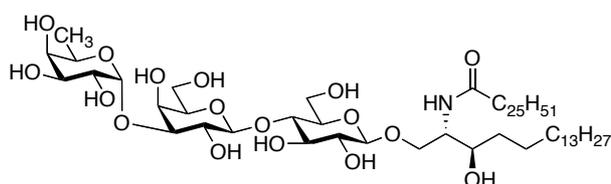
(2*S*,3*R*,4*E*)-1-(4-*O*-(3-*O*-(6-Deoxy- α -D-galactopyranosyl)- β -D-galactopyranosyl)- β -D-glucopyranosyloxy)-2-hexacosanoylamido-3-hydroxy-octadec-4-ene (5)



Fully protected triglycosyl ceramide **71** (33 mg, 0.016 mmol) was dissolved in dry THF (2 mL) and NH₃ (10 mL) was condensed into the reaction vessel at -78 °C. Small pieces of Na (s) were added carefully until the solution remained deep blue and the reaction mixture was stirred for 30 mins. The reaction was then quenched with a few drops of MeOH and Na (s) was added again until the deep blue colour persisted, and the reaction mixture stirred for a further 30 mins. The reaction was quenched with 10 mL MeOH, and the reaction warmed slowly to rt to allow the ammonia to evaporate. Trace ammonia was removed with an Ar stream. The reaction mixture was quenched to pH 7 with Dowex-H⁺, filtered and washed with pyridine and concentrated *in vacuo*. The resultant oil was purified by gradient flash chromatography (DCM/MeOH, 20/1 to 10/1, v/v) to afford fully deprotected 6'''-deoxy-iGb3-sphingosine **5** as an amorphous white solid (6.2 mg, 0.0054 mmol, 34%). *R*_f: 0.29 (DCM/MeOH, 5.7/1, v/v); [α]_D²⁷ = +37.0° (c = 0.1, pyridine); IR (film) 3402, 2922, 1653, 1558, 1541, 1458, 1134, 1099, 1072, 1038, 890, 814, 791, 694, 648, 633 cm⁻¹; ¹H NMR (500 MHz, pyridine-d₅) δ 8.45 (d, *J*_{2,NH} = 8.2 Hz, 1H, NH), 6.04 (dd, *J*_{4,5} = 15.2 Hz, *J*_{3,4} = 16.4 Hz, 1H, H-4), 5.93 (dt, *J*_{4,5} = 15.2 Hz, *J*_{5,6} = 6.9 Hz, 1H, H-5), 5.59 (d, *J*_{1'',2''} = 3.7 Hz, 1H, H-1''), 5.11 (d, *J*_{1',2'} = 7.9 Hz, 1H, H-1'), 4.95 (q, *J*_{5'',6''} = 6.7 Hz, 1H, H-5''), 4.89 (d, *J*_{1',2'} = 7.9 Hz, 1H, H-1'), 4.81–4.79 (m, 3H, H-1a, H-2, H-3), 4.66 (dd, *J*_{2'',3''} = 9.9 Hz, *J*_{1'',2''} = 3.7 Hz, 1H, H-2''), 4.59 (d, *J*_{3',4'} = 2.1 Hz, 1H, H-4'), 4.56–4.53 (m, 2H, H-2'', H-3''), 4.50–4.44 (m, 3H, H-6'a, H-6'b, H-6'a), 4.34 (dd, *J*_{6'a,6'b} = 10.9 Hz, *J*_{5',6'b} = 3.4 Hz, 1H, H-6'b), 4.30–4.23 (m, 3H, H-4', H-3'', H-3'), 4.18–4.17 (m, 1H, H-1b), 4.14 (bs, 1H, H-4''), 4.10–4.05 (m, 2H, H-5'', H-2'), 3.87–3.85 (m, 1H, H-5'), 2.46 (t, *J* _{α,β} = 7.4 Hz, 2H, CH₂- α), 2.08 (dd, *J*_{6,7} = 13.9 Hz, *J*_{5,6} = 6.9 Hz, 2H, H-6), 1.87–1.81 (m, 2H, CH₂- β), 1.59 (d, *J*_{5'',6''} = 6.6 Hz, 3H, H-6''), 1.41–1.27 (m, 66H, H-7–H-17, H- γ –H-(ω -1)), 0.90–0.86 (m, 6H, H-

18, H- ω); ^{13}C NMR (125 MHz, pyridine- d_5) δ 173.7 (HNC=O), 133.0 (C-5), 132.6 (C-4), 105.84, 105.79 (C-1', C-1''), 97.9 (C-1'''), 82.4 (C-4'), 80.4 (C-3''), 77.00 (C-3'), 76.95 (C-5''), 76.8 (C-5'), 75.1 (C-2'), 73.8 (C-4'''), 73.0 (C-3), 72.0 (C-3'''), 70.8 (C-1, C-2''), 70.4 (C-2'''), 68.1 (C-5'''), 66.3 (C-4'), 62.3 (C-6'), 62.2 (C-6''), 55.2 (C-2), 37.3 (C- α), 33.1 (C-6), 26.8 (C- β), 32.48, 32.46, 30.38, 30.36, 30.33, 30.25, 30.14, 30.07, 29.98, 29.94, 23.29, 23.27 (C-7–C-17, C- γ –C-(ω -1)), 17.6 (C-6'''), 14.6 (C-18, C- ω); HRMS(ESI) m/z calcd. for $[\text{C}_{62}\text{H}_{117}\text{NO}_{17}+\text{H}]^+$: 1148.8394, obsd.: 1148.8374.

(2*S*,3*R*,4*E*)-1-(4-*O*-(3-*O*-(6-Deoxy- α -D-galactopyranosyl)- β -D-galactopyranosyl)- β -D-glucopyranosyloxy)-2-hexacosanoylamido-3-hydroxy-octadecane (6)



$\text{Pd}(\text{OH})_2/\text{C}$ (2 mol%) was added to a solution of fully protected triglycosyl ceramide **71** (22 mg, 0.010 mmol) dissolved in

$\text{CHCl}_3/\text{EtOH}$ (3/2, v/v). The reaction mixture was stirred under H_2 atmosphere for 4 h, filtered through a celite pad and concentrated under reduced pressure. The resultant oil was purified by gradient flash chromatography (2.5% to 5% MeOH in DCM) to afford debenzylated 6'''-deoxy-iGb3-sphinganine (13.4 mg, 0.0078 mmol, 75%), which was subsequently dissolved in MeOH/DCM (2/1, v/v, 4.5 mL). NaOMe was added until the reaction mixture reached pH 9 and stirred at rt for 17 h. The reaction mixture was warmed to 40 °C and stirred for further 3 h, then neutralised to pH 7 with Dowex- H^+ . The resin was removed by filtration and washed successively with pyridine and concentrated under reduced pressure. MeOH (5 mL) was added to the crude mixture and cooled to -4 °C to give a suspension of glycolipid in solution. The precipitate was collected by centrifuge and washed with MeOH (2 x 5 mL), to afford the fully deprotected 6'''-deoxy-iGb3-sphinganine **6** as an amorphous white solid (6.1 mg, 0.0053 mmol, 70%). More desired product was precipitated from the combined MeOH supernatant (1.7 mg, 0.0015 mmol, 19%). The total yield of 6'''-deoxy-iGb3-sphinganine **6** was 89% (7.8 mg, 0.0068 mmol). R_f : 0.23 (DCM/MeOH, 5/1, v/v); $[\alpha]_D^{27} = +42.0^\circ$ ($c = 0.1$, pyridine); IR (film) 3342, 2919, 2850, 1626, 1551, 1468, 1379, 1165, 1081,

1038, 899, 813, 778, 722, 677, 651, 632 cm^{-1} ; ^1H NMR (500 MHz, pyridine- d_5) δ 8.45 (d, $J_{2,\text{NH}} = 9.0$ Hz, 1H, NH), 7.64 (d, $J_{2',\text{OH}} = 3.0$ Hz, 1H, H-2'-OH), 7.36 (d, $J_{2''/3''',\text{OH}} = 5.5$ Hz, 1H, H-2''/3'''-OH), 7.08 (d, $J_{2''',\text{OH}} = 5.5$ Hz, 1H, H-2'''-OH), 6.68 (d, $J_{6',\text{OH}} = 5.0$ Hz, 1H, H-6'-OH), 6.56 (d, $J_{2''/3''',\text{OH}} = 5.4$ Hz, 1H, H-2''/3'''-OH), 6.48 (d, $J_{6',\text{OH}} = 6.2$ Hz, 1H, H-6'-OH), 6.30 (d, $J_{3,\text{OH}} = 6.3$ Hz, 1H, H-3-OH), 6.26 (d, $J_{4''',\text{OH}} = 3.8$ Hz, 1H, H-4'''-OH), 6.18 (s, 1H, H-3'-OH), 5.83 (s, 1H, H-4''-OH), 5.60 (d, $J_{1''',2'''} = 3.3$ Hz, 1H, H-1'''), 5.10 (d, $J_{1'',2''} = 8.0$ Hz, 1H, H-1''), 4.95 (q, $J_{5''',6'''} = 6.5$ Hz, 1H, H-5'''), 4.89 (d, $J_{1',2'} = 7.6$ Hz, 1H, H-1'), 4.80 (dd, $J_{1a,1b} = 10.4$ Hz, $J_{1a,2} = 4.4$ Hz, 1H, H-1a), 4.73–4.69 (m, 1H, H-2), 4.68–4.64 (m, 1H, H-2''), 4.59 (bs, 1H, H-4''), 4.57–4.53 (m, 2H, H-2'', H-3'''), 4.51–4.49 (m, 2H, H-6'a, H-6'b), 4.46 (dd, $J_{6'a,6'b} = 12.2$ Hz, $J_{5,6'a} = 5.6$ Hz, 1H, H-6'a), 4.37–4.33 (m, 1H, H-6''b), 4.29–4.21 (m, 4H, H-3', H-4', H-3'', H-3), 4.18 (dd, $J_{1a,1b} = 10.4$ Hz, $J_{1b,2} = 3.2$ Hz, 1H, H-1b), 4.14 (bs, 1H, H-4'''), 4.10–4.06 (m, 2H, H-5'', H-2'), 4.89–4.87 (m, 1H, H-5'), 2.48 (t, $J_{\alpha,\beta} = 7.4$ Hz, 2H, $\text{CH}_2\text{-}\alpha$), 1.96–1.80 (m, 4H, H-4, $\text{CH}_2\text{-}\beta$), 1.60 (d, $J_{5''',6'''} = 6.5$ Hz, 3H, H-6'''), 1.41–1.27 (m, 70H, H-5–H-17, H- γ –H-(ω -1)), 0.89–0.87 (m, 6H, H-18, H- ω); ^{13}C NMR (125 MHz, pyridine- d_5) δ 173.6 (HNC=O), 105.9 (C-1', C-1''), 98.0 (C-1'''), 82.5 (C-4'), 80.5 (C-3''), 77.1 (C-3'), 77.0 (C-5''), 76.9 (C-5'), 75.1 (C-2'), 73.8 (C-4'''), 72.0 (C-3'''), 71.7 (C-3), 71.1 (C-1), 70.9 (C-2''), 70.4 (C-2'''), 68.1 (C-5'''), 66.3 (C-4'), 62.4 (C-6'), 62.2 (C-6''), 55.3 (C-2), 37.3 (C- α), 35.3 (C-4), 26.9 (C- β), 32.49, 32.48, 30.59, 30.48, 30.40, 30.38, 30.30, 30.27, 30.22, 30.12, 29.99, 29.96, 26.79, 23.30, 23.29 (C-7–C-17, C- γ –C-(ω -1)), 17.6 (C-6'''), 14.6 (C-18, C- ω); HRMS(ESI) m/z calcd. for $[\text{C}_{62}\text{H}_{119}\text{NO}_{17}+\text{H}]^+$: 1150.8551, obsd.: 1150.8553.

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Chapter 5. Synthesis of acyl chain homologues of iGb3

5.1 Introduction

The *N*-acyl lipids of CD1d-binding glycolipids have been reported to impact glycolipid activity by altering the ability of the glycolipids to activate iNKT cells and also by affecting the cytokine profile induced.¹⁻⁶ The acyl lipid lodges itself into the hydrophobic A'-pocket of CD1d^{7,8} and along with the ceramide backbone, is responsible for the positioning of the sugar headgroup for recognition by the iNKT cell TCR.⁵ In addition, changes to the lipid portion of α -GalCer have been suggested to affect its solubility, leading to variations in *in vivo* distribution and pharmacology of the glycolipids.³ This could alter glycolipid processing and presentation by various subsets of dendritic cells, ultimately resulting in the specific iNKT cell activation.

While the influence of α -GalCer analogues with acyl chains of varying length, saturation and substitution patterns have been extensively studied,^{1,2,4-6,9} no systematic analysis has been carried out for *N*-acyl chain homologues of isoglobotrihexosylceramide, iGb3, **4** (Figure 5.1). To date, the short acyl chain derivatives of iGb3 **4** (i.e. C8 and C18 derivatives), have been used for crystallographic analysis, but they have not been compared side-by-side for their structure activity relationships. The C26 acyl lipid is notorious for being poorly soluble, therefore, in the synthesis of glycosphingolipids, the original C26 acyl chain, as found in α -GalCer and iGb3, is often substituted by shorter variants (C16, C18, C24). This presumably has the advantage of increasing the yield of the amide coupling with the more readily soluble fatty acids, and consequently, of improving the solubility of the final glycosphingolipids, which assists with purification and characterisation.

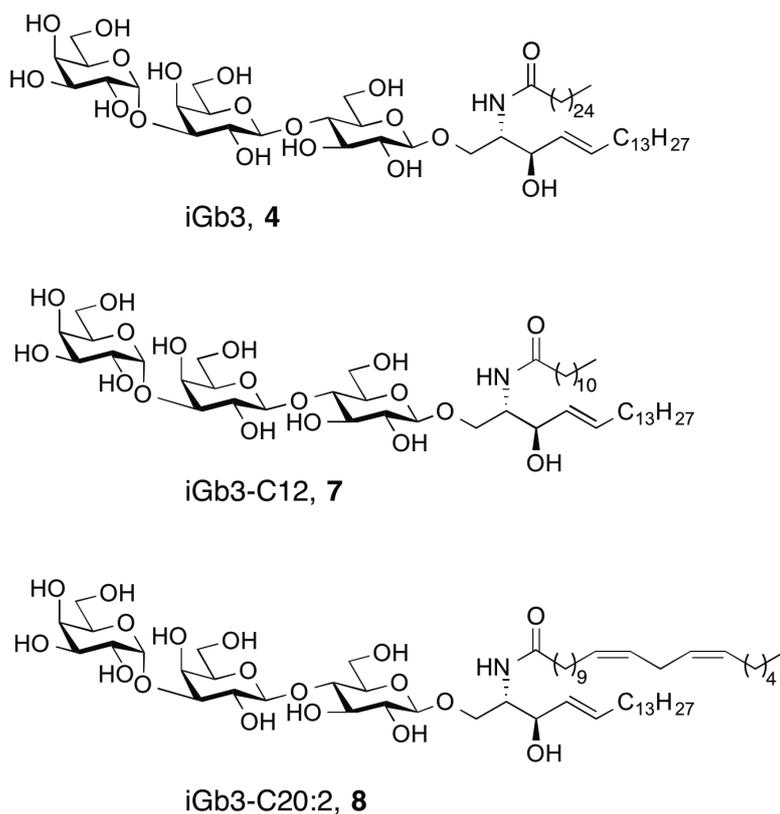


Figure 5.1. iGb3 (**4**) and acyl chain homologues, iGb3-C12 (**7**) and iGb3-C20:2 (**8**).

Access to a panel of acyl chain homologues of iGb3 (**4**) will provide a useful means by which to probe the structure activity relationship between iNKT cell activation and acyl lipid length and saturation. To this end, the novel iGb3-C12 analogue **7** will provide a platform to further test how the truncated lipid can affect the orientation of the sugar headgroup and hence activity (Figure 5.1). While shorter acyl chain homologues of iGb3 such as the C8,^{10,11} C16,¹² C18,¹³ and C24¹⁴ homologues have previously been made, no systematic structure activity relationship studies were performed on this series. In addition, α -GalCer with a C20:2 acyl lipid has been found to have superior activity compared to the saturated analogue C20, and can skew the immune system towards an anti-inflammatory (Th2) cytokine response.^{2,15,16} Accordingly, the unprecedented C20:2 derivative of iGb3 **8**, will be prepared. Through synthesis, subsequent structure activity relationship studies and crystal structure analysis, it will thus be possible to understand the influence that the unsaturated acyl chain has on the presentation of triglycosylceramides.

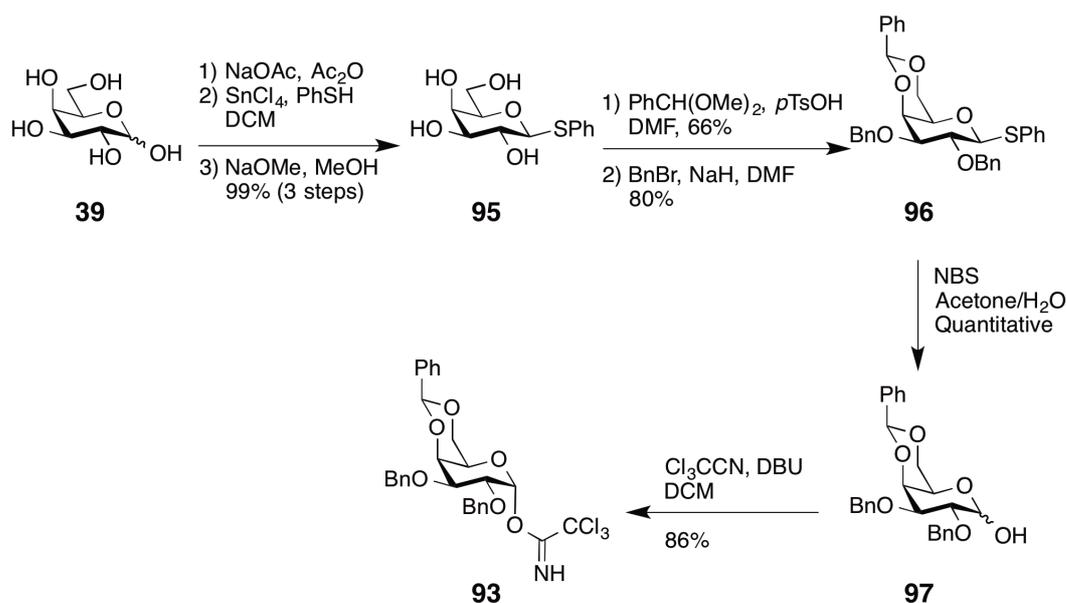
One other point to keep in mind is that while α -GalCer remains the glycolipid adjuvant of choice in DC based cancer immunotherapy, the activity of this glycolipid is so potent that anergy can be induced.¹⁷⁻²⁰ Thus, while it is a long way off, there is also the potential to use iGb3 analogues in cancer immunotherapy or, through structural modification and the judicious induction of a different cytokine profile, as a therapy for diseases other than cancer, such as infectious diseases and autoimmune diseases.²¹⁻²³ Furthermore, there has been increasing appreciation for the role that β -linked glycolipids play in activating iNKT cells, beyond just serving as the endogenous ligands responsible for positive selection of iNKT cells.²⁴ Brigl *et al.* convincingly illustrated that in a microbial infection setting, iNKT cells were able to respond to inflammatory stimulus in the absence of known microbial-derived ligands for iNKT TCR.²⁵ Innate stimulus such as the TLR signal stimulates the production of IL-12, causing cytokine-mediated activation of iNKT cells, presumably through the activity of endogenous glycolipids. While the exact identity of the self-glycolipids remains to be determined, β -glucosylceramide (β -GlcCer) was isolated in abundance and was shown to accumulate in the lymphoid tissues of mice and humans during infection.²⁶ Much remains to be answered about the exact role of β -linked self glycolipids, but these findings point towards the possibility of using β -linked glycolipids, such as iGb3 analogues, to activate iNKT cells not just for cancer, but also for other disease settings.

5.2 Synthesis of acyl chain homologues of iGb3

Retrosynthesis

To synthesise the acyl chain homologues of iGb3, iGb3-C12 **7** and iGb3-C20:2 **8**, a highly convergent strategy was chosen, whereby both glycolipids **7** and **8** could be obtained from the triglycosyl 2-azido-sphingosine intermediate **92** via azide reduction, amide coupling with the desired fatty acid, then global deprotection via Birch reduction (Scheme 5.1). In turn, azide **92** could be made by the glycosylation reaction between galactosyl imidate donor **93** and lactosylsphingosine (LacSphing) acceptor **94**, while the galactosyl imidate donor could be obtained in several steps from D-galactose (**39**). Finally, the LacSphing acceptor **94** could be obtained from lactosyl 2-azido-sphingosine **74**, where the synthesis of lactosyl 2-azido-sphingosine **74** is described in Chapter 4 (Scheme 4.2). This strategy differs from the approach used to synthesise 6'''-deoxy-iGb3, in that the complete trisaccharide core is prepared prior to the addition of *N*-acyl chain. The advantage of this approach is that the late stage azide intermediate **92** could be functionalised with desired fatty acids, followed immediately by global deprotection to afford the target iGb3 analogues.

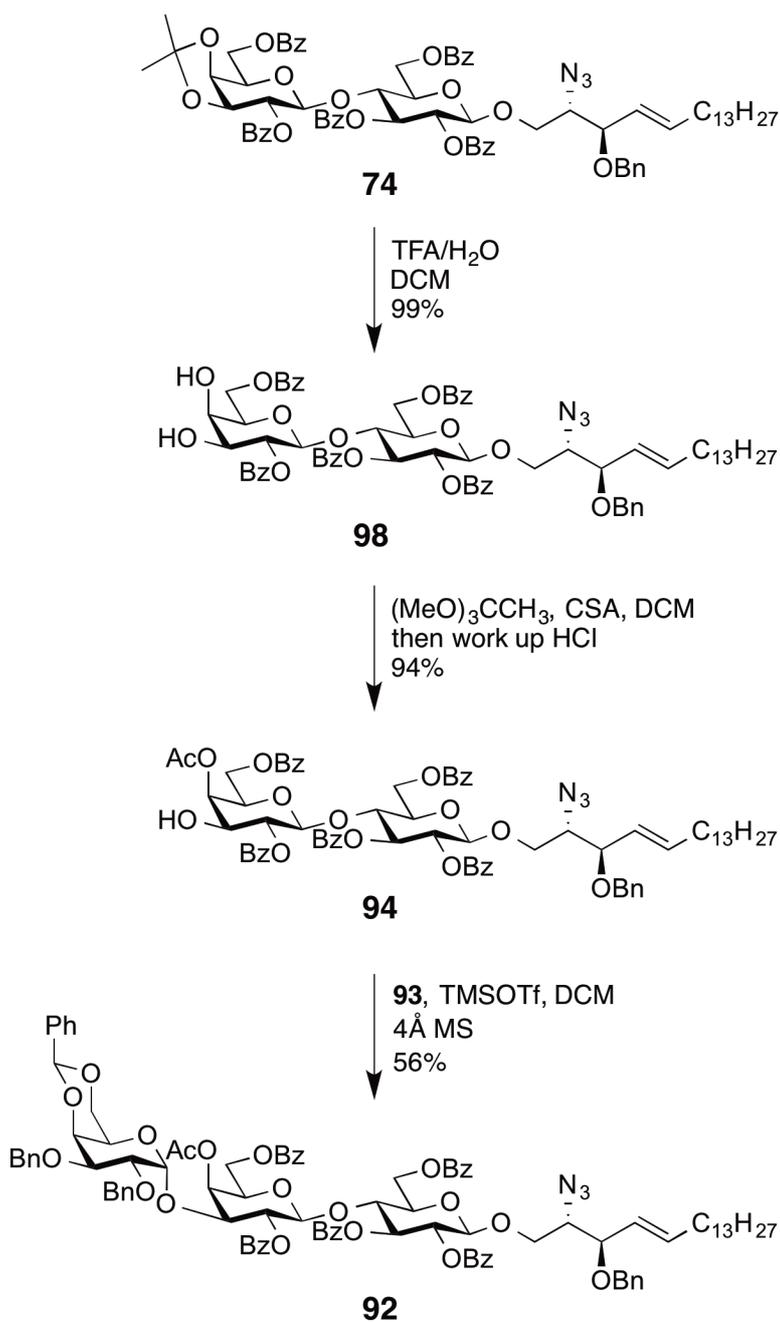
methoxide in methanol to afford thiogalactoside **95**. Next, the 4- and 6-hydroxyls were selectively protected with a benzylidene group, which was previously anticipated to favour α -glycosylation,^{28,32,33} and the remaining hydroxyls were then benzylated to afford the fully protected thiogalactoside **96** in 53% yield over two steps. Initial attempts at synthesising iGb3 by other members of our group using a thiol donor to attach the terminal sugar led to poor yields and a mixture of products,³⁴ so accordingly, an imidate donor was chosen. To this end, the anomeric thiophenol group in **96** was hydrolysed using NBS in the presence of acetone and water to provide lactol **97** in quantitative yield.³⁵ The anomeric hydroxyl was then converted into a trichloroacetimidate group to afford galactosyl imidate donor **93** as crystalline material in 86% yield. In summary, the galactose donor building block **93** was made from D-galactose (**39**) in seven steps and in an overall yield of 45%, which is comparable to Wong and co-workers' synthesis via the 1-*S-p*-methylphenyl intermediate.²⁸



Scheme 5.2. Synthesis of galactosyl imidate donor **93**.

Triglycosyl 2-azido-sphingosine 92

With the galactose building block in hand, attention was then turned towards the synthesis of triglycosyl 2-azido-sphingosine **92**, which commenced with lactosyl 2-azido-sphingosine **74**, an intermediate from the synthesis of 6'''-deoxy-iGb3 analogues (cf. Chapter 4, Scheme 4.2). Accordingly, the isopropylidene group in **74** was first hydrolysed in the presence of acid to afford diol **98** in an excellent 99% yield (Scheme 5.3). Next, the 4''-OH was regioselectively protected as an acetate by employing the same strategy used in Chapter 4, whereby trimethyl orthoacetate and camphorsulfonic acid were used to first install a methyl orthoacetate protecting group at 3''-OH and 4''-OH, which subsequently ring-opened upon acidic workup. In this way, LacSphing acceptor **94** was prepared in an excellent 94% yield. The successful acetylation at the 4''-position was confirmed by a HMBC between the carbonyl carbon of the acetate and H-4''.



Scheme 5.3. Synthesis of triglycosyl 2-azido-sphingosine **92**.

Using galactose donor **93** and the LacSphing acceptor **94** a glycosylation reaction was then performed with TMSOTf as the promoter. Initially, the reaction was carried out at at $-40\text{ }^{\circ}\text{C}$, however this proved unsuccessful and no desired product was formed. When the reaction was repeated at warmer temperatures (activation at $-20\text{ }^{\circ}\text{C}$ followed by stirring from $0\text{ }^{\circ}\text{C}$ to rt over 2.5 h) the desired α -isomer of **92** was formed (56% yield), however, a significant amount (ca. 40%) of unreacted acceptor **94** was also isolated. Despite efforts to push the reaction to completion

by sequentially adding more equivalents of the donor to the reaction mixture, the glycosylation yield could not be improved. Indeed, the best yields were obtained when the donor was added in one portion. The glycosylation at the 3'' position was confirmed by HMBC between C-3'' and H-1''' (Figure 5.2).

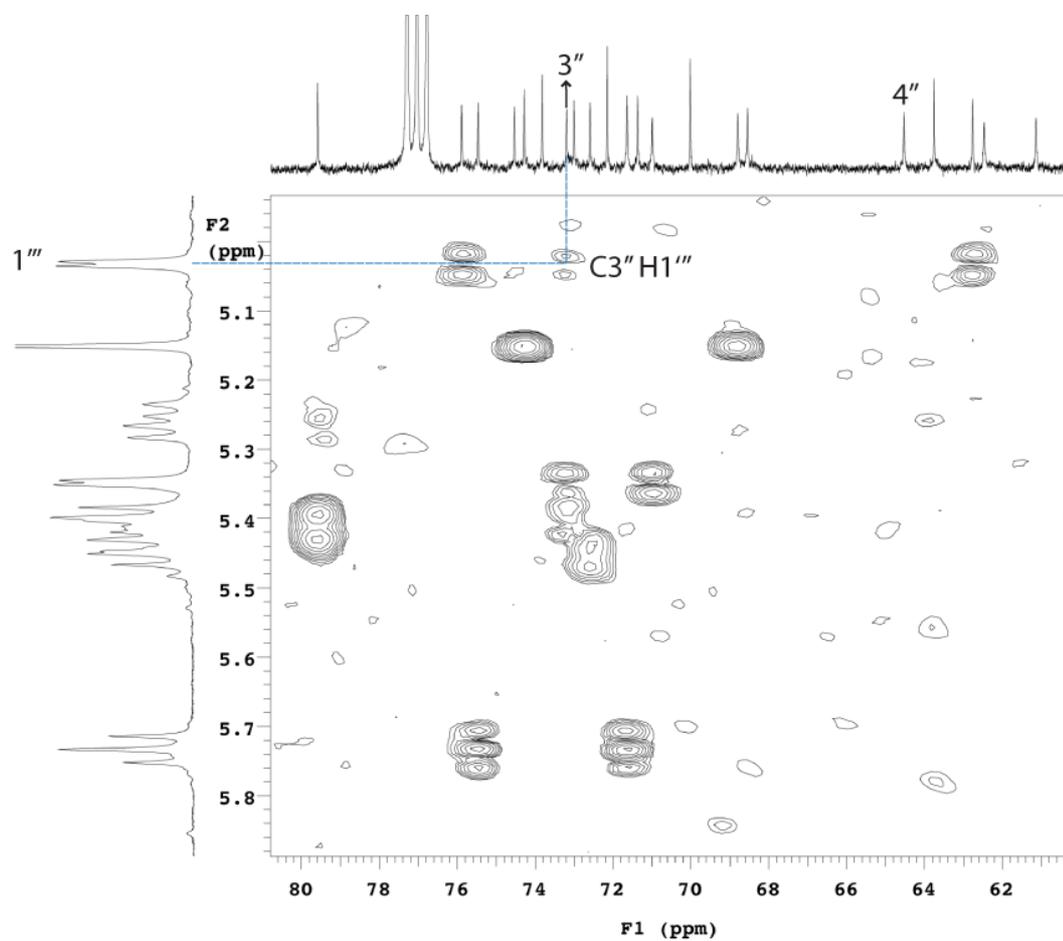
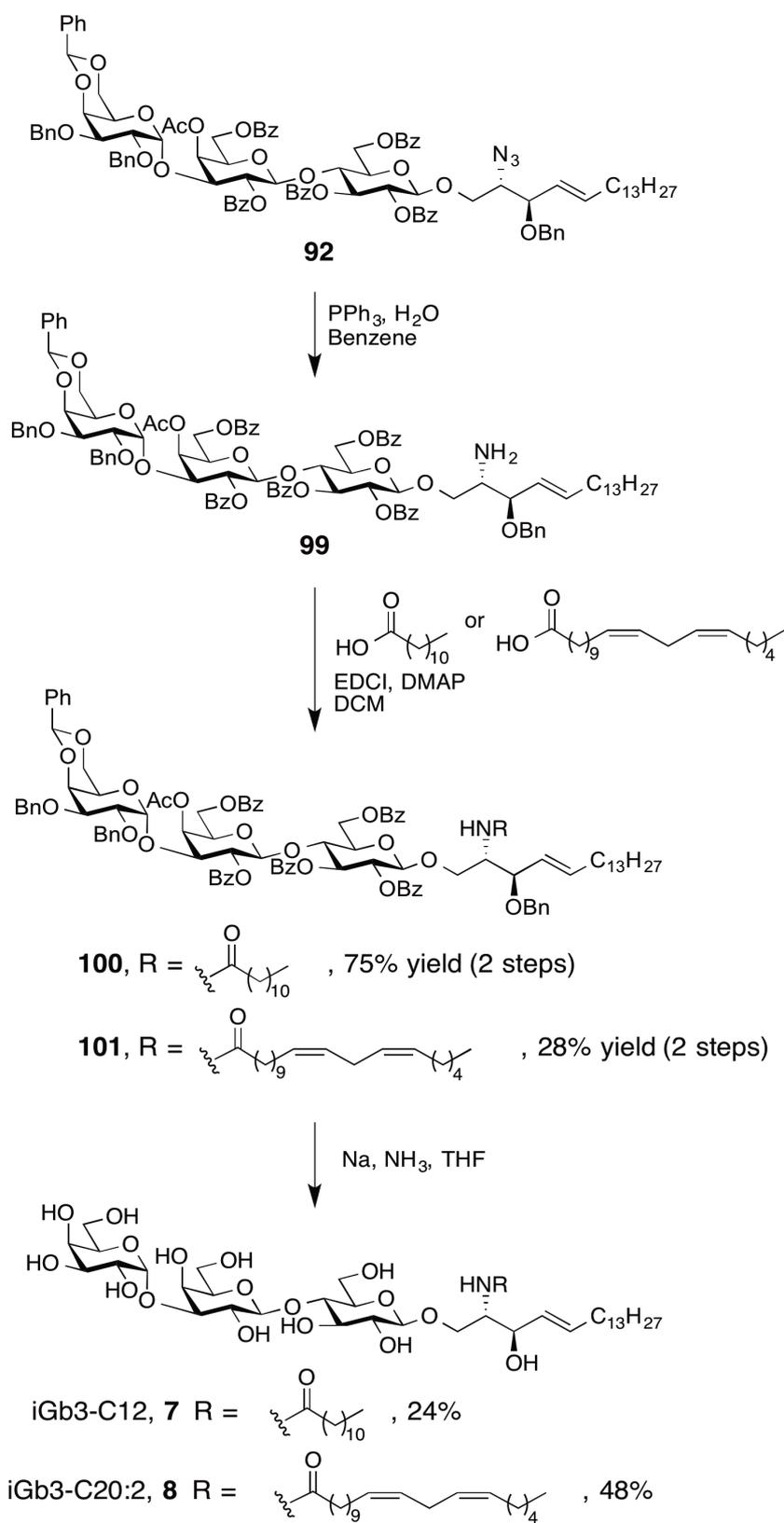


Figure 5.2. HMBC spectrum of triglycosyl 2-azido-sphingosine **92**.

Coupling of acyl lipids and global deprotection

Having prepared triglycosyl 2-azido-sphingosine **92**, the next step was to functionalise the sphingosine backbone with fatty acids bearing the C12 or the C20:2 lipid. The reduction of the azide to the amine using triphenylphosphine and water was then undertaken according to previously published procedures,^{36,37} and this provided amine **99**, which was used without further purification (Scheme 5.4). Dodecanoic (C12) acid was coupled to the amine by using an EDCI/DMAP coupling protocol to provide the fully protected iGb3-C12 **100** in an excellent 75% yield over two steps. An identical coupling strategy was carried out for the C20:2 analogue, whereby azide reduction to the amine **99**, followed by immediate coupling with 11Z,14Z-eicosadienoic (C20:2) acid afforded the fully protected iGb3-C20:2 **101** in 28% yield over two steps. The lower yield of this second acylation is in part attributed to the poorer solubility of the C20:2 lipid compared to the shorter chain C12 variant, which causes it to be less reactive. In addition, it has also been reported that skipped dienes are particularly prone to autoxidation to form conjugated diene hydroperoxides.^{38,39} Extensive studies on linoleic (C18:2) acid, which has the same unsaturation pattern as the C20:2 acid, have shown that 1,3-conjugated diene hydroperoxides are formed from the incorporation of atmospheric oxygen.^{39,40} Exposure to oxygen from the atmosphere for too long leads to autoxidation products as observed for the starting material C20:2 acid whereby *m/z* corresponding to the 1,3-conjugated diene alcohol (*m/z* calcd. for C₂₀H₃₅O₃⁻: 323.2592, obsd.: 323.2587) and the 1,3-conjugated diene ketone (*m/z* calcd. for C₂₀H₃₃O₃⁻: 321.2435, obsd.: 321.2435) were detected by mass spectrometry. Effort to minimise the degradation of the C20:2 glycolipid by autoxidation was taken by ensuring that the compounds are always kept under an inert, oxygen-free atmosphere. It should also be noted that none of the by-products were in the purified fractions of the fully protected iGb3-C20:2 **101**.



Scheme 5.4. Coupling of acyl lipid and global deprotection to form iGb3-C12 (**7**) and iGb3-C20:2 (**8**).

Next, the global deprotection of the fully protected iGb3-C12 **100** was carried out with a Birch reaction to remove all ether and ester protecting groups, but leaving the alkene intact. The reaction was performed under standard conditions for glycolipids,^{41,42} yet surprisingly, mass spectrometry analysis of the crude reaction mixture before and after quenching with Dowex-H⁺ revealed the presence the desired product iGb3-C12 (**7**) (m/z calcd. for [C₄₈H₈₉NO₁₈+H]⁺: 968.6152, obsd.: 968.6159) along with the hydrolysed by-products LacCer-C12 (m/z calcd. for [C₄₂H₇₈NO₁₃+H]⁺: 806.5624, obsd.: 806.5632), GlcCer-C12 (m/z calcd. for [C₃₆H₆₈NO₈+H]⁺: 644.5096, obsd.: 644.5100) and Cer-C12 (m/z calcd. for [C₃₀H₅₈NO₃+H]⁺: 482.4568, obsd.: 482.4573). As glycosidic linkages are generally considered to be stable under basic conditions, this result was surprising. The yields of Birch reactions performed on glycolipids varies,^{41,42} and indeed, others have reported modest yields,⁴¹⁻⁴³ fragmentation under Birch conditions has not been widely reported, though the cleavage of β-mannosyl glycosidic bonds have been observed by several others.^{44,45} Further systematic studies need to be undertaken to understand why this hydrolysis occurred and it would be valuable if others were to comment on observed by-products to assist with understanding the mechanisms that are taking place.

Despite the formation of the by-products, purification of the crude residue by silica gel column chromatography, followed by reverse phase column chromatography afforded the desired target iGb3-C12 **7** in 24% yield. GlcCer-C12 was also isolated (34% yield) along with a mixture of semi-deprotected glycolipid (with intact benzylidene and the benzyl protecting groups) as identified by mass spectrometry (m/z calcd. for [C₇₆H₁₁₁NO₃+H]⁺: 1326.7874, obsd.: 1326.7851). The incomplete deprotection of the ether protecting groups was puzzling as the Birch protocol employed involved two rounds of addition of sodium, with quenching by methanol in between each addition of sodium, however due to the limited availability of the intermediate triglycosyl 2-azidosphingosine **92**, this reaction was not further optimised. Nonetheless, the desired target iGb3-C12 **7** was obtained in sufficient quantities for characterisation and biological evaluation.

The same global deprotection procedure was applied to the fully protected iGb3-C20:2 **101**. Again, like the C12 analogue **100**, careful mass spectrometry analysis of the crude reaction mixture before and after neutralisation with Dowex-H⁺ revealed the presence of the desired product iGb3-C20:2 (**8**) (m/z calcd. for [C₅₆H₁₀₁NO₁₈+H]⁺: 1076.7091, obsd.: 1076.7090) along with by-products from hydrolysis of the glycosidic linkages, namely LacCer-C20:2 (m/z calcd. for [C₅₀H₉₁NO₁₃+H]⁺: 914.6563, obsd.: 914.6575), GlcCer-C20:2 (m/z calcd. for [C₄₄H₈₁NO₈+H]⁺: 752.6035, obsd.: 752.6042) and Cer-C20:2 (m/z calcd. for [C₃₈H₇₁NO₃+H]⁺: 590.5507, obsd.:m/z 590.5524). The incompletely deprotected intermediate with ether groups intact were not detected. Purification of the crude reaction mixture by silica gel column chromatography afforded the desired target iGb3-C20:2 (**8**) in 48% yield.

Having successfully prepared the C12 and C20:2 iGb3 homologues, and in Chapter 4 the 6'''-deoxy derivatives, it would be interesting to compare the syntheses. Three key transformations for both routes are the acyl chain coupling, the terminal sugar coupling and the formation of the 3''-OH glycolipid acceptor, and as the yields for these key transformations in both strategies are very comparable, it is difficult to say whether one of these approaches is better. The advantage to the synthetic strategy employed across these two different series of iGb3 homologues, however, is the fact that the late stage key intermediate, lactosyl 2-azido-sphingosine glycolipid **74**, is easily accessible from D-lactose and can be functionalised accordingly to obtain both terminal sugar homologues and acyl chain homologues from a common intermediate. Taken as a whole, the strategy here differs from all other iGb3 syntheses previously reported, whereby a trisaccharide donor is coupled directly to either the complete ceramide^{41,46} or in two steps, to the sphingosine backbone followed by *N*-acylation of the lipid backbone.^{37,47,48} While these reported strategies may be more convergent for the synthesis of one target, the route designed in this work is robust and versatile for the synthesis of multiple targets of either the terminal sugar or the acyl chain series.

5.3 Conclusion

In summary, two novel acyl chain homologues of iGb3, iGb3-C12 (**7**) and iGb3-C20:2 (**8**) were synthesised in 2% and 1% overall yield, respectively, over 12 steps from D-lactose (longest linear sequence). Key to the synthesis is the coupling of the terminal galactose sugar donor **93** to the lactosylsphingosine (LacSphing) acceptor **94**, which proceeded in good selectivity giving only the α -isomer in 56% yield. Acceptor **94** itself was synthesised from the common intermediate lactosyl 2-azido sphingosine **74**, which was used for the synthesis of 6''-deoxy-iGb3 analogues described in Chapter 4. In addition, the triglycosyl sphingosine intermediate **92** represents a useful intermediate for functionalisation with desired acyl chains, as was illustrated in this work, whereby the C12 and C20:2 lipids were coupled. It was observed that amide coupling with shorter chain homologues (i.e. C12), proceeds more smoothly compared to their longer chain counterparts, such as the C20:2, and also the C26 lipid used the Chapter 4. In addition, the sensitivity of C20:2 lipid to autoxidation means that extra care had to be taken when working with this lipid. From this work, the successful synthesis of these two acyl chain homologues will allow for further structure activity relationship studies to be performed by our collaborators so that more insight can be gained on the influence of lipid length and saturation patterns for iNKT cell activity. Moreover, crystal structure studies of these glycolipids in complex with CD1d and TCR of iNKT cells will shed light on the favourable or unfavourable interactions giving rise to the activity observed.

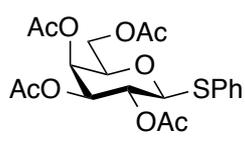
5.4 Experimental

General procedure

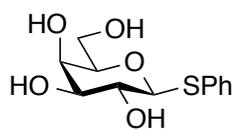
Unless otherwise stated all reactions were performed under argon. Prior to use, THF (Pancreac) was distilled from sodium and benzophenone, pyridine was distilled and dried over 4Å molecular sieves (4Å MS), DCM (Pancreac) was distilled from P₂O₅, and H₂O and benzene (Fisher Scientific) were distilled. SnCl₄ (Aldrich), PhSH (Koch-Light Laboratories), benzaldehyde dimethyl acetal (Aldrich), Me₂C(OMe)₂ (Aldrich), NBS (Aldrich), DBU (Merck), CSA (Acros), *n*Bu₃SnCl (Aldrich), AIBN (Aldrich), D-fucose (Aldrich), D-lactose (Aldrich), trityl chloride (Acros), anhydrous Et₂O (Pancreac), PPh₃ (Aldrich), Pd(OH)₂/C (Aldrich, 20 wt%), anhydrous DMF (Acros), TFA (Aldrich), *p*TsOH (Aldrich), TMSOTf (Aldrich), H₂SO₄ (Lab-Scan), formic acid (Aldrich), AcCl (Aldrich), BnBr (Fluka), PMe₃ (Aldrich, 1M in THF), AcOH (Ajax Finechem), Ac₂O (Peking Reagent), TMSOTf (Aldrich), DiPEA (Aldrich), NaOMe (Janssen Chimica), trichloroacetonitrile (Aldrich), lauric acid (Hopkin & Williams), 11Z,14Z-eicosadienoic acid (Allichem LLC), BzCl (Aldrich, distilled and stored under argon), HBTU (Acros), PyBOP (Aldrich), EDCI (Aldrich), DMAP (Merck), sodium (Aldrich), trimethyl orthoacetate (Aldrich), LiAlH₄ (Aldrich), EtOAc (Pancreac), hexanes (Fisher Scientific), petroleum ether (Pure Science), MeOH (Pure Science), CHCl₃ (Pancreac), EtOH (absolute, Pure Science), NaOH (Pure Science), NaHCO₃ (Pure Science), NaCl (Pancreac), NH₃ (BOC gases) were used as received. All solvents were removed by evaporation under reduced pressure. Reactions were monitored by TLC-analysis on Macherey-Nagel silica gel coated plastic sheets (0.20 mm, with fluorescent indicator UV₂₅₄) with detection by UV-absorption (short wave UV – 254 nm; long wave UV – 366 nm), by dipping in 10% H₂SO₄ in EtOH followed by charring at ~150 °C, by dipping in I₂ in silica, or by dipping into a solution of ninhydrin in EtOH followed by charring at ~150 °C. Column chromatography was performed on Pure Science silica gel (40-63 micron). AccuBOND II ODS-C18 (Agilent) was used for reverse phase chromatography. Infrared spectra were recorded as thin films using a Bruker Tensor 27 FTIR spectrometer equipped with an Attenuated Total

Reflectance (ATR) sampling accessory and are reported in wave numbers (cm^{-1}). Nuclear magnetic resonance spectra were recorded at 20 °C in CD_3OD , CDCl_3 , or pyridine- d_5 using either a Varian INOVA operating at 500 MHz or Varian VNMRs operating at 600 MHz. Chemical shifts are given in ppm (δ) relative to TMS. NMR peak assignments were made using COSY, HSQC and HMBC 2D experiments.

Phenyl 2,3,4,6-tetra-*O*-acetyl-1-thio- β -D-galactopyranoside

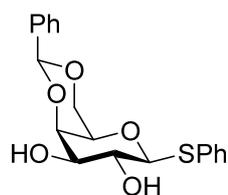

 1,2,3,4,6-penta-*O*-acetyl- β -D-galactopyranose (20 g, 51.24 mmol) was co-evaporated with toluene (x2), dissolved in CH_2Cl_2 (150 mL) and cooled to 0 °C. Thiophenol (6.3 mL, 61.42 mmol) and tin(IV) chloride (0.6 mL, 5.12 mmol) was added to the reaction mixture under argon and stirred for 3 h. The reaction was quenched with a 3M solution of potassium fluoride (100 mL, 6 equiv.) and the organic layer was washed with water, saturated sodium bicarbonate solution, brine and dried over MgSO_4 , filtered and concentrated *in vacuo*. The residue was taken up in ethyl acetate and passed through a plug of silica gel to remove residual tin by-products. This afforded title compound as a yellow oil (23.9 g). R_f : 0.73 (PE/EA, 1/1, v/v); $[\alpha]_D^{23} = +4.0^\circ$ ($c = 1.0$, CHCl_3); IR (thin film) 3060, 2917, 2849, 2400, 2000, 1747, 1583, 1480, 1439, 1369, 1200, 1054, 917, 736, 702 cm^{-1} ; ^1H NMR (500 MHz, CDCl_3) δ 7.51 (m, 2H, CH-*o* Ph), 7.31 (m, 3H, CH-*m* Ph), 5.41 (d, 1H, $J_{3,4} = 3.4$ Hz, H-4), 5.24 (t, 1H, $J_{1,2} = J_{2,3} = 10.0$ Hz, H-2), 5.05 (dd, 1H, $J_{2,3} = 10.0$ Hz, $J_{3,4} = 3.4$ Hz, H-3), 4.72 (d, 1H, $J_{1,2} = 10.0$ Hz, H-1), 4.18 (dd, 1H, $J_{6a,6b} = 11.4$ Hz, $J_{5,6a} = 7.1$ Hz, H-6a), 4.11 (dd, 1H, $J_{6a,6b} = 11.4$ Hz, $J_{5,6b} = 6.2$ Hz, H-6b), 3.94 (t, 1H, $J_{5,6a} = 7.1$ Hz, $J_{5,6b} = 6.2$ Hz, H-5), 2.11 (s, 3H, COCH_3), 2.09 (s, 3H, COCH_3), 2.04 (s, 3H, COCH_3), 1.97 (s, 3H, COCH_3); ^{13}C NMR (500 MHz, CDCl_3) δ 170.4, 170.2, 170.1, 169.4 (COCH_3), 132.5 (CH-*o* Ph) 132.5 (C-*i* Ph), 128.9 (CH-*m* Ph), 128.2 (CH-*p* Ph), 86.6 (C-1), 74.4 (C-5), 72.0 (C-3), 67.22, 67.20 (C-2 and C-4), 61.6 (C-6), 20.8 (COCH_3), 20.7 (COCH_3), 20.64 (COCH_3), 20.59 (COCH_3); HRMS(ESI) m/z calcd. for $[\text{C}_{20}\text{H}_{24}\text{O}_9\text{S}+\text{Na}]^+$: 463.1034, obsd.: 463.1034.

Phenyl 1-thio- β -D-galactopyranoside (**95**)



Sodium methoxide was added slowly to a suspension of phenyl 2,3,4,6-tetra-*O*-acetyl-1-thio- β -D-galactopyranoside (23.9 g) in methanol (200 mL) until the solution reached pH 13. The reaction mixture was stirred for 4 h and neutralised with Dowex-H⁺. The resin was removed by filtration and the methanolic solution was concentrated to afford **95** as a white solid (13.9 g, 99% over two steps), which was used without further purification. *R*_f: 0.37 (EA/MeOH, 9/1, v/v); $[\alpha]_{\text{D}}^{21} = -37.0^\circ$ (c = 0.1, MeOH); IR (thin film) 3383, 2895, 1583, 1480, 1439, 1057, 865, 743, 692 cm⁻¹; ¹H NMR (500 MHz, CD₃OD) δ 7.56–7.53 (m, 2H, CH-*o* Ph), 7.31–7.27 (m, 2H, CH-*m* Ph), 7.24–7.22 (m, 1H, CH-*p* Ph), 4.60 (d, 1H, *J*_{1,2} = 9.7 Hz, H-1), 3.91 (bs, 1H, H-4), 3.77 (dd, 1H, *J*_{6a,6b} = 11.5 Hz, *J*_{5,6a} = 7.0 Hz, H-6a), 3.72 (dd, 1H, *J*_{6a,6b} = 11.5 Hz, *J*_{5,6b} = 5.5 Hz, H-6b), 3.60 (t, 1H, *J*_{1,2} = *J*_{2,3} = 9.7 Hz, H-2), 3.56 (t, 1H, *J*_{4,5} = *J*_{5,6b} = 5.5 Hz, H-5), 3.49 (dd, 1H, *J*_{2,3} = 9.5 Hz, *J*_{3,4} = 3.0 Hz, H-3); ¹³C NMR (500 MHz, CD₃OD) δ 135.9 (C*i* SPh) 131.9 (CH-*o* Ph), 129.8 (CH-*m* Ph), 127.9 (CH-*p* Ph), 90.2 (C-1), 80.5 (C-5), 76.2 (C-3), 70.9 (C-2), 70.3 (C-4), 62.6 (C-6); HRMS(ESI) *m/z* calcd. for [C₁₂H₁₆O₅S+Na]⁺: 295.0611, obsd.: 295.0613.

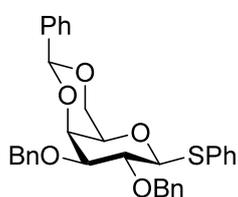
Phenyl 4,6-*O*-benzylidene-1-thio- β -D-galactopyranoside



Thioglycoside **95** (13.9 g, 51.0 mmol) was co-evaporated with dry DMF (x2) and dissolved in dry DMF (180 mL). Under an argon atmosphere, benzaldehyde dimethyl acetal (11.5 mL, 76.6 mmol) was added followed by *p*TsOH (1.0 g, 5.1 mmol) and the reaction mixture was stirred at rt overnight. The reaction mixture was neutralised with triethylamine, concentrated *in vacuo* and redissolved in ethyl acetate. The organic layer was washed with water, sat. aq. NaHCO₃, brine, dried over MgSO₄, filtered and concentrated *in vacuo*. Crystallisation from ethyl acetate/petroleum ether (1:2, v/v) afforded title compound as fluffy white crystals (12.2 g, 33.8 mmol, 66%). Mp 154–156 °C; *R*_f: 0.69 (EA/MeOH, 9/1, v/v); $[\alpha]_{\text{D}}^{22} = -34.5^\circ$ (c = 1.0, CHCl₃); IR (thin film) 3401, 3061, 2976, 2869, 1583, 1480, 1451, 1439, 1362, 1098, 1070, 1041, 1027, 925, 898, 866, 734, 696 cm⁻¹; ¹H NMR (500 MHz, CDCl₃) δ 7.70 (m, 2H, aromatic H), 7.40–7.29 (m, 8 H, aromatic H), 5.52 (s, 1H, CH-Ph), 4.52 (d, 1H, *J*_{1,2} = 9.0 Hz,

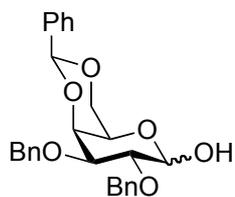
H-1), 4.40 (d, 1H, $J_{6a,6b} = 12.5$ Hz, H-6a) 4.22 (d, 1H, $J_{3,4} = 1.5$ Hz, H-4), 4.05 (dd, 1H, $J_{6a,6b} = 12.5$ Hz, $J_{5,6b} = 1.0$ Hz, H-6b), 3.71-3.70 (m, 2H, H-2, and H-3), 3.57 (bs, 1H, H-5), 2.60 (s, 1H, HO-3), 2.58 (d, 1H, $J = 8.5$ Hz, HO-2); ^{13}C NMR (500 MHz, CDCl_3) δ 137.5, 130.6 (C-*i* Ph), 133.8, 129.4, 129.0, 128.3, 126.5 (8 x CH_{arom}), 101.4 (PhCHO_2), 87.0 (C-1), 75.3 (C-4), 73.8 (C-2), 70.1 (C-5), 69.3 (C-6), 68.8 (C-3); HRMS(ESI) m/z calcd. for $[\text{C}_{19}\text{H}_{20}\text{O}_5\text{S}+\text{Na}]^+$: 383.0924, obsd.: 383.0927.

Phenyl 2,3-*O*-benzyl-4,6-benzylidene-1-thio- β -D-galactopyranoside (**96**)



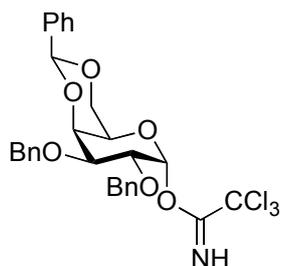
Phenyl 4,6-*O*-benzylidene-1-thio- β -D-galactopyranoside (2 g, 5.55 mmol) was dissolved in dry DMF (25 mL) and benzyl bromide (1.6 mL, 13.3 mmol) was added under nitrogen. The reaction mixture was cooled to 0 °C and sodium hydride (60% in oil suspension) (0.72 g, 18.0 mmol) was added slowly. The reaction mixture was stirred overnight at rt after which time methanol (15 mL) was added slowly to quench the reaction. The reaction mixture was diluted with diethyl ether and ethyl acetate and washed with water, sat. aq. NaHCO_3 , brine and dried over MgSO_4 . The organic layer was concentrate *in vacuo* and the product was crystallised from ethyl acetate/petroleum ether to afford **96** as white fluffy crystals (2.39 g, 4.42 mmol, 80%). Mp 171.2–171.9 °C; R_f : 0.49 (PE/EA, 3/1, v/v); $[\alpha]_{\text{D}}^{22} = -18.9^\circ$ ($c = 1.0$, CHCl_3); IR (thin film) 2817, 2849, 1977, 1584, 1453, 1399, 1092, 1057, 1026, 815, 759, 732, 696 cm^{-1} ; ^1H NMR (500 MHz, CDCl_3) δ 7.73–7.71 (m, 2H, CH-*o* PhCHO_2), 7.55–7.53 (m, 2H, CH-*o* SPh), 7.43–7.19 (m, 16H, aromatic H), 5.50 (s, 1H, PhCHO_2), 4.72 (m, 4H, 2 x CH_2Ph), 4.63 (d, 1H, $J_{1,2} = 9.3$ Hz, H-1), 4.39 (d, 1H, $J_{6a,6b} = 12.5$ Hz, H-6a), 4.17 (d, 1H, $J_{3,4} = 3.4$ Hz, H-4), 4.01 (dd, 1H, $J = 1.3, 12.0$ Hz, H-6a), 3.91 (t, 1H, $J_{1,2} = J_{2,3} = 9.3$ Hz, H-2), 3.64 (dd, $J_{2,3} = 9.3$ Hz, 1H, $J_{3,4} = 3.4$ Hz, H-3), 3.44 (bs, 1H, H-5); ^{13}C NMR (500 MHz, CDCl_3) δ 138.5, 138.1, 137.9 (C-*i* Ph), 132.8 (CH-*o* SPh), 132.7, 129.1, 128.9, 128.4, 128.4, 128.24, 128.19, 127.8, 127.7, 127.5, 126.6 (19 x CH_{arom}), 101.4 (PhCHO_2), 86.5 (C-1), 81.4 (C-3), 75.5 (2- OCH_2Ph), 75.4 (C-2), 73.7 (C-4), 71.9 (3- OCH_2Ph), 69.9 (C-5), 69.4 (C-6); HRMS(ESI) m/z calcd. for $[\text{C}_{19}\text{H}_{20}\text{O}_5\text{S}+\text{Na}]^+$: 563.1868, obsd.: 563.1871.

2,3-Di-*O*-benzyl-4,6-*O*-benzylidene-*D*-galactose (**97**)



NBS (1.73 g, 9.71 mmol) was added to a solution of thioglycoside **96** (1.5 g, 2.77 mmol) in acetone (111 mL) and water (5.5 mL), and the reaction was stirred at room temperature for 10 min. The reaction mixture was diluted with EtOAc (80 ml), and washed sat. aq. Na₂S₂O₃ (80 mL), water (80 mL) and brine (80 mL), dried over MgSO₄, filtered and concentrated under reduced pressure. The resulting oil was purified by gradient flash chromatography (Petroleum ether/EtOAc, 5/1 to 0/100, v/v) then crystallised (Petroleum ether/EtOAc, 2/1, v/v) to give **97** as fluffy white crystals (1.24 g, 2.76 mmol, Quantitative). $R_f = 0.08$ (PE/EA, 5/1, v/v). Mp = 165.5–166.4 °C; $[\alpha]_D^{24} = +41.0$ ($c = 1.0$, CHCl₃). IR (film) 3408, 3064, 3032, 2865, 1454, 1400, 1364, 1249, 1097, 1052, 1027, 996, 909, 732, 697 cm⁻¹; ¹H NMR (500 MHz, CDCl₃) δ 7.57–7.53 (m, 3H, aromatics), 7.43–7.29 (m, 12H, aromatics), 5.50 (s, 1H, CH-benzylidene), 5.38 (s, 1H, H-1), 4.93–4.86 (m, 1H, CH-a, 2-*O*-Bn), 4.82–4.76 (m, 2H, CH-a, CH-b, 3-*O*-Bn), 4.73–4.69 (m, 1H, CH-a, 2-*O*-Bn), 4.24 (d, $J_{6a,6b} = 12.5$ Hz, 1H, H-6a), 4.23 (s, 1H, H-4), 4.07 (dd, $J_{1,2} = 3.4$, $J_{2,3} = 9.7$ Hz, 1H, H-2), 4.03 (d, $J_{6a,6b} = 12.5$ Hz, 1H, H-6b), 3.98 (dd, $J_{3,4} = 3.4$, $J_{2,3} = 9.7$ Hz, 1H, H-3), 3.88 (s, 1H, H-5), 2.90 (s, 1H, 1-OH); ¹³C NMR (125 MHz, CDCl₃) δ 138.5, 138.2, 137.8, 128.9, 128.7, 128.6, 128.5, 128.45, 128.39, 128.21, 128.18, 128.14, 128.1, 128.9, 128.8, 128.7, 126.3 (18 x CH_{arom}), 101.1 (PhCHO₂), 92.2 (C1), 75.73 (C2), 75.71 (C3), 74.3 (C4), 73.9 (2-OCH₂Ph), 71.8 (3-OCH₂Ph), 69.5 (C6), 62.8 (C5); HRMS(ESI) m/z calcd. for [C₂₇H₂₈O₆+Na]⁺: 471.1778, obsd.: 471.1774.

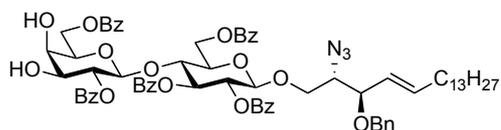
2,3-di-*O*-benzyl-4,6-*O*-benzylidene- α -*D*-galactosyl trichloroacetimidate (**93**)



To a solution of lactol **97** (240 mg, 0.535 mmol, co-evaporated 3 times dry toluene) in dry CH₂Cl₂ (5.4 mL) was added trichloroacetonitrile (537 μ L, 0.77 g, 5.35 mmol) followed by 1,8-diazabicycloundec-7-ene (120 μ L, 0.12 g, 0.80 mmol) and the resulting brown solution stirred 45 min at rt. The solution was concentrated under reduced pressure and

purified by gradient flash chromatography (Petroleum ether/EtOAc/NEt₃, 95/5/1 to 1/1/0, v/v/v) to give trichloroacetimidate **93** as a colourless oil (273 mg, 0.46 mmol, 86%). *R_f* = 0.69 (PE/EA, 1/2, v/v); *M_p* = 140.8–142.1 °C; $[\alpha]_D^{24} = +93.0$ (*c* = 1.0, CHCl₃). IR (film) 3339, 3065, 2907, 2864, 1671, 1497, 1456, 1368, 1290, 1249, 1175, 1120, 1098, 1027, 968, 846, 794, 738, 697 cm⁻¹. ¹H NMR (500 MHz, CDCl₃) δ 8.58 (s, 1H, H-N), 7.55–7.53 (m, 3H, aromatics), 7.40–7.28 (m, 15H, aromatics), 6.65 (d, *J*_{1,2} = 3.4 Hz, 1H, H-1), 5.53 (s, 1H, CH-benzylidene), 4.84 (d, *J*_{a,b} = 11.9 Hz, 1H, CH-a, 3-*O*-Bn), 4.81 (d, *J*_{a,b} = 8.8 Hz, 1H, CH-a, 2-*O*-Bn), 4.79 (d, *J*_{a,b} = 8.8 Hz, 1H, CH-b, 2-*O*-Bn), 4.77 (d, *J*_{a,b} = 11.9 Hz, 1H, CH-b, 3-*O*-Bn), 4.31–4.27 (m, 3H, H-2, H-4 and H-6a), 4.09 (dd, *J*_{3,4} = 3.5, *J*_{2,3} = 10.0 Hz, 1H, H-3), 4.02 (dd, *J*_{5,6b} = 1.5, *J*_{6a,6b} = 12.7 Hz, 1H, H-6b), 3.85 (s, 1H, H-5); ¹³C NMR (125 MHz, CDCl₃) δ 161.0 (C=NH), 138.4, 138.3, 137.6, 129.0, 128.3, 128.26, 128.2, 128.0, 127.7, 127.5, 127.4, 126.4 (18 x CH_{arom}), 101.1 (CH-benzylidene), 95.6 (C1), 91.4 (C=O), 75.1 (C2), 74.7 (C3), 74.5 (C4), 73.1 (2-OCH₂Ph), 72.2 (3-OCH₂Ph), 69.1 (C6), 65.3 (C5); HRMS(ESI) *m/z* calcd. for [C₂₉H₂₈NO₆Cl₃+Na]⁺: 614.0874, obsd.: 614.0872.

(2*S*,3*R*,4*E*)-2-Azido-1-(4-*O*-(2,6-di-*O*-benzoyl-β-*D*-galactopyranosyl)-2,3,6-tri-*O*-benzoyl-β-*D*-glucopyranosyloxy)-3-benzyloxy-octadec-4-ene (98**)**

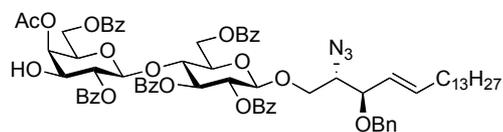


To a solution of lactosyl 2-azido-sphingosine **74** (144 mg, 0.11 mmol) in CH₂Cl₂ (4 mL) was added TFA/H₂O (1/1, v/v, 0.4 mL) and the resulting solution was stirred at room temperature for 20 h. The solution was diluted with EtOAc and the organic layer was then washed with sat. aq. NaHCO₃ (x3) and brine, dried (MgSO₄), filtered and concentrated under reduced pressure. The colourless oil was then purified by gradient flash chromatography (petroleum ether/EtOAc, 5/1 to 1/1, v/v) to give diol **98** as a colourless oil (139 mg, 0.11 mmol, 99%). *R_f*: 0.46 (PE/EA, 1/1, v/v); $[\alpha]_D^{21} = +17.0^\circ$ (*c* = 1.0, CHCl₃); IR (film) 3448, 3066, 2925, 2854, 2102, 1720, 1602, 1585, 1452, 1315, 1270, 1177, 1113, 1095, 1069, 1028, 976, 756, 708 cm⁻¹; ¹H NMR (500 MHz, CDCl₃) δ 8.07 (d, *J*_{CH-*o*,CH-*m*} = 7.8 Hz, 2H, CH-*o*, 2''-*O*-Bz), 8.02–8.00 (m, 4H, 2 x CH-*o*, OBz), 7.97 (d, *J*_{CH-*o*,CH-*m*} = 7.8 Hz, 2H, CH-*o*, OBz), 7.93 (d, *J*_{CH-*o*,CH-*m*} = 7.8 Hz, 2H, CH-*o*, 2'-*O*-Bz), 7.61–7.19 (m, 20H, H_{arom}), 5.67

(t, $J_{2',3'} = J_{3',4'} = 9.4$ Hz, 1H, H-3'), 5.46–5.39 (m, 2H, H-2', H-5), 5.30–5.23 (m, 2H, H-2'', H-4), 4.66 (d, $J_{1',2'} = 7.8$ Hz, 1H, H-1'), 4.60 (d, $J_{1'',2''} = 7.8$ Hz, 1H, H-1''), 4.59–4.58 (m, 1H, H-6'a), 4.52 (dd, $J_{6'a,6'b} = 12.0$ Hz, $J_{5',6'b} = 4.4$ Hz, 1H, H-6'b), 4.40 (d, $J_{a,b} = 11.8$ Hz, CH-a, 3-*O*-Bn), 4.19–4.14 (m, 2H, H-4', CH-b, 3-*O*-Bn), 4.04 (dd, $J_{6''a,6''b} = 11.4$ Hz, $J_{5'',6''} = 6.7$ Hz, 1H, H-6''a), 3.90 (dd, $J_{1a,1b} = 10.2$ Hz, $J_{1a,2} = 5.8$ Hz, 1H, H-1a), 3.82–3.79 (m, 1H, H-5'), 3.77 (d, $J_{3'',4''} = 3.3$ Hz, 1H, H-4''), 3.72 (dd, $J_{3,4} = 8.5$ Hz, $J_{2,3} = 5.6$ Hz, 1H, H-3), 3.66 (dd, $J_{2'',3''} = 9.8$ Hz, $J_{3'',4''} = 3.3$ Hz, 1H, H-3''), 3.60–3.56 (m, 2H, H-2, H-6''b), 3.51 (dd, $J_{1a,1b} = 10.2$ Hz, $J_{1b,2} = 5.4$ Hz, 1H, H-1b), 3.46 (t, $J_{5,6} = 6.7$ Hz, 1H, H-5'), 1.93–1.90 (m, 2H, H-6), 1.31–1.25 (m, 22H, H-7–H-17), 0.88 (t, $J_{17,18} = 7.0$ Hz, 3H, H-18); ^{13}C NMR (125 MHz, CDCl_3) δ 166.5 (C=O, 2''-*O*-Bn), 166.2 (C=O, 6''-*O*-Bn), 166.1 (C=O, 6'-*O*-Bn), 166.0 (C=O, 3'-*O*-Bn), 165.2 (C=O, 2'-*O*-Bn), 138.4 (C-5), 138.2 (C-*i*, 3-*O*-Bn), 133.6, 133.5, 133.4, 133.3 (C-*p*, 5 x OBz), 130.06, 129.95, 129.85, 129.79, 129.76, 129.70, 129.68, 129.66, 129.42, 129.22, 128.73, 128.62, 128.49, 128.39, 127.58 (30 x CH_{arom}), 125.6 (C-4), 101.0 (C-1', C-1''), 79.7 (C-3), 76.2 (C-4'), 73.8 (C-2''), 73.1 (C-3', C-5'), 72.7 (C-3'', C-5''), 71.7 (C-2'), 70.1 (CH_2 , 3-*O*-Bn), 68.6 (C-1, C-4''), 63.9 (C-2), 62.7 (C-6'), 61.9 (C-6''), 32.4 (C-6), 32.0, 29.83, 29.81, 29.80, 29.79, 29.76, 29.55, 29.49, 29.29, 29.03, 22.8 (C-7–C-17), 14.3 (C-18); HRMS(ESI) m/z calcd. for $[\text{C}_{72}\text{H}_{81}\text{N}_3\text{O}_{17}+\text{Na}]^+$: 1282.5458, obsd.: 1282.5453.

(2*S*,3*R*,4*E*)-1-(4-*O*-(4-*O*-Acetyl-2,6-di-*O*-benzoyl- β -D-galactopyranosyl)-2,3,6-tri-*O*-benzoyl- β -D-glucopyranosyloxy)-2-azido-3-benzyloxy-octadec-4-ene

(94)

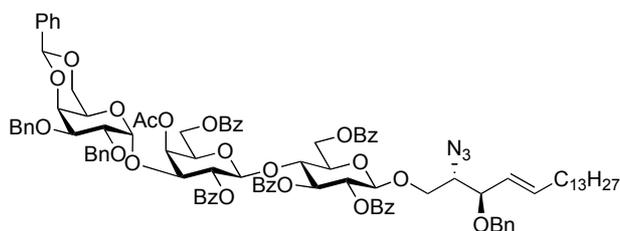


Diol **98** (62 mg, 0.049 mmol) was co-evaporated with toluene (x3) and dissolved in dry CH_2Cl_2 (0.8 mL).

Trimethyl orthoacetate (19 μL , 0.15 mmol) and CSA (5.7 mg, 0.025 mmol) were added and the reaction mixture was stirred at rt for 5 h. The reaction mixture was diluted with EtOAc (30 mL), washed with 1M HCl (30 mL x 3), sat. aq. NaHCO_3 (30 mL) and brine (30 mL), dried (MgSO_4), filtered and concentrated *in vacuo*. The residue was purified by gradient flash chromatography (petroleum ether/EtOAc, 10:1 to 3:1, v/v) to afford acetate **94** as a clear oil (59 mg, 0.046

mmol, 94%). R_f : 0.63 (PE/EA, 1/1, v/v); $[\alpha]_D^{25} = +2.0^\circ$ ($c = 1.0$, CHCl_3); IR (film) 3475, 2102, 1727, 1602, 1452, 1371, 1315, 1269, 1177, 1110, 1095, 1070, 1028, 976, 709 cm^{-1} ; ^1H NMR (500 MHz, CDCl_3) δ 8.06 (d, $J_{\text{CH-}o,\text{CH-}m} = 8.3$ Hz, 2H, CH-*o*, 2'-*O*-Bz), 8.04–8.02 (m, 4H, 2 x CH-*o*, 3'-*O*-Bz, 6''-*O*-Bz), 7.98 (d, $J_{\text{CH-}o,\text{CH-}m} = 7.8$ Hz, 2H, CH-*o*, 6'-*O*-Bz), 7.95 (d, $J_{\text{CH-}o,\text{CH-}m} = 7.8$ Hz, 2H, CH-*o*, 2'-*O*-Bz), 7.62–7.19 (m, 20H, H_{arom}), 5.72 (t, $J_{2',3'} = J_{3',4'} = 9.6$ Hz, 1H, H-3'), 5.46 (dd, $J_{2',3'} = 9.6$ Hz, $J_{1',2'} = 7.9$ Hz, 1H, H-2'), 5.41 (dt, $J_{4,5} = 15.7$ Hz, $J_{5,6} = 6.6$ Hz, 1H, H-5), 5.26 (dd, $J_{4,5} = 15.7$ Hz, $J_{3,4} = 8.6$ Hz, 1H, H-4), 5.21 (d, $J_{3'',4''} = 3.4$ Hz, 1H, H-4''), 5.17 (dd, $J_{2'',3''} = 9.8$ Hz, $J_{1'',2''} = 8.0$ Hz, 1H, H-2''), 4.69 (d, $J_{1',2'} = 7.9$ Hz, 1H, H-1'), 4.65 (d, $J_{1'',2''} = 8.0$ Hz, 1H, H-1''), 4.62 (d, $J_{6'a,6'b} = 12.0$ Hz, 1H, H-6'a), 4.53 (dd, $J_{6'a,6'b} = 12.0$ Hz, $J_{5,6'b} = 4.3$ Hz, 1H, H-6'b), 4.41 (d, $J_{a,b} = 11.7$ Hz, 1H, CH-a, 3-*O*-Bn), 4.21 (t, $J_{3',4'} = 9.6$ Hz, 1H, H-4'), 4.15 (d, $J_{a,b} = 11.7$ Hz, 1H, CH-b, 3-*O*-Bn), 3.92 (dd, $J_{1a,1b} = 10.0$ Hz, $J_{1a,2} = 5.9$ Hz, 1H, H-1a), 3.83–3.81 (m, 2H, H-5', H-3''), 3.76–3.72 (m, 2H, H-3, H-6''a), 3.61–3.56 (m, 2H, H-2, H-5''), 3.55–3.50 (m, 2H, H-6''b, H-1b), 2.01 (s, 3H, OAc), 1.98–1.91 (m, 2H, H-6), 1.32–1.21 (m, 22H, H-7–H-17), 0.88 (t, $J_{17,18} = 6.9$ Hz, 3H, H-18); ^{13}C NMR (125 MHz, CDCl_3) δ 170.7 (C=O, OAc), 166.6 (C=O, 2''-*O*-Bn), 166.1 (C=O, 6'-*O*-Bn), 165.8 (C=O, 6''-*O*-Bn), 165.5 (C=O, 3'-*O*-Bn), 165.1 (C=O, 2'-*O*-Bn), 138.4 (C-5), 138.2 (C-*i*, 3-*O*-Bn), 133.7, 133.6, 133.5, 133.4, 133.3 (C-*p*, 5 x OBz), 130.02, 130.30, 129.88, 129.74, 129.67, 129.65, 129.60, 129.43, 129.02, 128.80, 128.71, 128.70, 128.50, 128.40, 128.37, 127.58 (30 x CH_{arom}), 125.6 (C-4), 101.2 (C-1'), 100.5 (C-1''), 79.7 (C-3), 75.6 (C-4'), 73.6 (C-2''), 73.1 (C-5'), 72.8 (C-3'), 71.8 (C-2'), 71.7 (C-3''), 71.3 (C-5''), 70.1 (CH_2 , 3-*O*-Bn), 69.4 (C-4''), 68.7 (C-1), 63.9 (C-2), 62.7 (C-6'), 61.3 (C-6''), 32.4 (C-6), 32.0, 29.83, 29.81, 29.80, 29.79, 29.76, 29.55, 29.49, 29.29, 29.03, 22.8 (C-7–C-17), 20.7 (OAc), 14.3 (C-18); HRMS(ESI) m/z calcd. for $[\text{C}_{72}\text{H}_{83}\text{N}_3\text{O}_{18}+\text{NH}_4]^+$: 1319.6010, obsd.: 1319.5966.

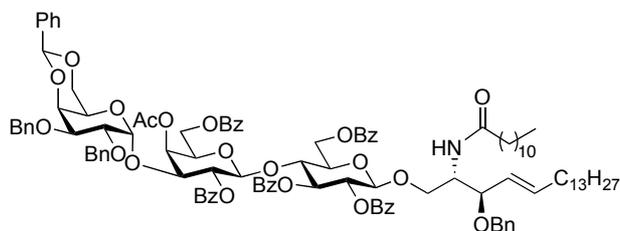
(2*S*,3*R*,4*E*)-1-(4-*O*-(4-*O*-Acetyl-2,6-di-*O*-benzoyl-3-*O*-(2,3-di-*O*-benzyl-4,6-*O*-benzylidene- α -D-galactopyranosyl)- β -D-galactopyranosyl)-2,3,6-tri-*O*-benzoyl- β -D-glucopyranosyloxy)-2-azido-3-benzyloxy-octadec-4-ene (92)



Galactosyl imidate donor **93** (165 mg, 0.278 mmol) and lactosyl 2-azido-sphingosine acceptor **94** (145 mg, 0.111 mmol) were co-evaporated with toluene (x3), dissolved in dry CH₂Cl₂ (1 mL) and stirred with activated 4Å molecular sieves for 30 mins. The reaction mixture was cooled to -20 °C, and a solution of TMSOTf in CH₂Cl₂ (0.55 mmol/mL, 50 μ L, 0.027 mmol) was added and the mixture stirred at 0 °C for 2 h. The reaction was warmed to rt and stirred for another 30 mins, upon which it was diluted with EtOAc (30 mL), washed with sat. aq. NaHCO₃ (30 mL) and brine (30 mL), dried (MgSO₄), filtered and concentrated *in vacuo*. The resultant oil was purified by gradient flash chromatography (petroleum ether/EtOAc, 10:1 to 4:1, v/v) to afford the fully protected triglycosyl ceramide **92** as a clear oil (109 mg, 0.0629 mmol, 56%). *R*_f: 0.82 (PE/EA, 1/1, v/v); [α]_D²⁶ = +58.0° (c = 1.0, CHCl₃); IR (film) 3089, 3065, 2925, 2854, 2102, 1731, 1602, 1585, 1452, 1364, 1315, 1268, 1177, 1097, 1069, 1028, 979, 755, 710 cm⁻¹; ¹H NMR (500 MHz, CDCl₃) δ 8.05–7.94 (m, 10H, 5 x CH-*o*, 2'-*O*-Bz, 3'-*O*-Bz, 6'-*O*-Bz, 2''-*O*-Bz, 6''-*O*-Bz), 7.63–7.19 (m, 35H, H_{arom}), 5.73 (t, *J*_{2',3'}} = *J*_{3',4'}} = 9.4 Hz, 1H, H-3'), 5.45 (dd, *J*_{2',3'}} = 9.4 Hz, *J*_{1',2'}} = 8.0 Hz, 1H, H-2'), 5.43–5.38 (m, 2H, H-5, H-2''), 5.35 (d, *J*_{3'',4''}} = 3.1 Hz, 1H, H-4''), 5.26 (dd, *J*_{4,5}} = 15.4 Hz, *J*_{3,4}} = 8.6 Hz, 1H, H-4), 5.15 (s, 1H, PhCHO₂), 5.03 (d, *J*_{4,5}} = 3.2 Hz, 1H, H-1'''), 4.68 (d, *J*_{1',2'}} = 8.0 Hz, 1H, H-1'), 4.65 (d, *J*_{a,b}} = 11.5 Hz, 1H, CH-a, 2'''-*O*-Bn), 4.64 (d, *J*_{a,b}} = 12.2 Hz, 1H, CH-a, 3'''-*O*-Bn), 4.59 (d, *J*_{1'',2''}} = 8.0 Hz, 1H, H-1''), 4.55 (d, *J*_{a,b}} = 11.5 Hz, 1H, CH-b, 2'''-*O*-Bn), 4.52–4.50 (m, 3H, H-6'a, H-6'b, CH-b, 3'''-*O*-Bn), 4.40 (d, *J*_{a,b}} = 11.7 Hz, 1H, CH-a, 3-*O*-Bn), 4.20–4.14 (m, 2H, H-4', CH-b, 3-*O*-Bn), 3.92 (dd, *J*_{1a,1b}} = 10.2

Hz, $J_{1a,2} = 5.8$ Hz, 1H, H-1a), 3.90–3.88 (m, 2H, H-2''', H-6'''a), 3.81–3.78 (m, 2H, H-5', H-3''), 3.73 (dd, $J_{3,4} = 8.6$ Hz, $J_{2,3} = 5.6$ Hz, 1H, H-3), 3.67–3.64 (m, 2H, H-6'''a, H-3''), 3.59 (q, $J_{1,2} = J_{2,3} = 5.5$ Hz, 1H, H-2), 3.55–3.49 (m, 3H, H-1b, H-5'', H-6''b), 3.46 (d, $J_{3''',4'''} = 2.9$ Hz, 1H, H-4'''), 3.41 (d, $J_{a,b} = 6.4$ Hz, 1H, H-6''b), 3.29 (s, 1H, H-5'''), 1.93–1.90 (2H, H-6'), 1.63 (s, 3H, OAc), 1.26–0.92 (m, 22H, H-7–H-17), 0.89 (t, $J_{17,18} = 6.9$ Hz, 3H, H-18); ^{13}C NMR (125 MHz, CDCl_3) δ 170.1 (C=O, OAc), 166.1 (C=O, 6'-O-Bn), 165.8 (C=O, 6''-O-Bn), 165.5 (C=O, 3'-O-Bn), 165.2 (C=O, 2'-O-Bn), 164.4 (C=O, 2''-O-Bn), 138.9, 138.7 (C-*i*, 2'''-O-Bn, 3'''-O-Bn), 138.4 (C-5), 138.2 (C-*i*, 3-O-Bn), 137.8 (C-*i*, benzylidene), 133.7, 133.58, 133.55, 133.4, 133.3 (C-*p*, 5 x OBz), 129.97, 129.86, 129.77, 129.70, 129.68, 129.64, 129.55, 129.44, 129.18, 129.00, 128.90, 128.80, 218.74, 128.51, 128.39, 128.33, 128.26, 128.21, 128.19, 127.72, 127.59, 127.57, 127.57, 127.50 (43 x CH_{arom}), 126.4 (C-*o*, benzylidene), 125.5 (C-4), 101.2 (C-1'), 101.0 (C-1'', CH-benzylidene), 95.4 (C-1'''), 79.7 (C-3), 76.0 (C-3'''), 75.6 (C-4'), 74.7 (C-2'''), 74.4 (C-4'''), 73.9 (CH_2 , 2'''-O-Bn), 73.3 (C-3''), 73.1 (C-5'), 72.7 (C-3'), 72.3 (CH_2 , 3'''-O-Bn), 71.8 (C-2'), 71.5 (C-5''), 71.1 (C-2''), 70.1 (CH_2 , 3-O-Bn), 68.9 (C-6'''), 68.7 (C-1), 64.6 (C-4'), 63.9 (C-2), 62.9 (C-5'''), 62.6 (C-6'), 61.3 (C-6''), 32.4 (C-6), 32.06, 29.83, 29.82, 29.80, 29.79, 29.76, 29.55, 29.49, 29.29, 29.04, 22.83 (C-7–C-17), 20.3 (OAc), 14.3 (C-18); HRMS(ESI) m/z calcd. for $[\text{C}_{101}\text{H}_{109}\text{N}_3\text{O}_{23}+\text{NH}_4]^+$: 1749.7790, obsd. 1749.7802.

(2*S*,3*R*,4*E*)-1-(4-*O*-(4-*O*-Acetyl-2,6-di-*O*-benzoyl-3-*O*-(2,3-di-*O*-benzyl-4,6-*O*-benzylidene- α -D-galactopyranosyl)- β -D-galactopyranosyl)-2,3,6-tri-*O*-benzoyl- β -D-glucopyranosyloxy)-3-benzyloxy-2-dodecanoylamido-octadec-4-ene (100)

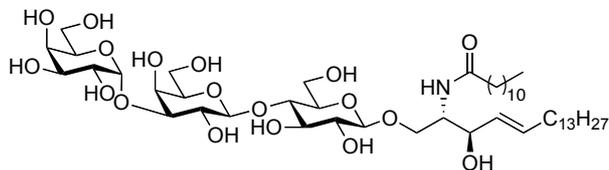


To a solution of glycolipid azide **92** (68 mg, 0.039 mmol) in dry benzene (0.8 mL) triphenylphosphine (21 mg, 0.078 mmol), and distilled water (30 μ L) were added, the solution was warmed to 45 °C and stirred overnight. The reaction mixture was then cooled to room temperature, diluted with EtOAc (20 mL), washed with sat. aq. NH_4Cl , (5 mL), dried (MgSO_4), filtered and concentrated under reduced pressure to give a colourless oil which was used without further purification. Half of the amine intermediate obtained was coupled to the C12 fatty acid as described below.

The oil was co-evaporated twice with dry toluene then suspended in DCM (1 mL), EDCI (11.3 mg, 0.0589 mmol), DMAP (9.6mg, 0.0785 mmol), and lauric acid (11.8 mg, 0.0589 mmol) were added and the resulting solution stirred over 4 days at room temperature, after which it was diluted with EtOAc (30 mL), washed with sat. aq. NaHCO_3 (30 mL) and brine (30 mL), dried (MgSO_4), filtered and concentrated *in vacuo*. The reaction mixture was then purified directly by gradient flash chromatography (petroleum ether/EtOAc, 10/1 to 2/1, v/v) to give **100** as a colourless oil (28 mg, 0.015 mmol, 75% over two steps). R_f : 0.68 (PE/EA, 1/1, v/v); $[\alpha]_D^{24} = +62.0^\circ$ (c = 0.1, CHCl_3); IR (film) 2924, 2854, 1733, 1718, 1576, 1558, 1541, 1473, 1177, 1098, 1070, 1028, 756, 633, 620 cm^{-1} ; ^1H NMR (500 MHz, CDCl_3) δ 8.04–7.93 (m, 10H, 5 x CH-*o*, 2'-*O*-Bz, 3'-*O*-Bz, 6'-*O*-Bz, 2''-*O*-Bz, 6''-*O*-Bz), 7.56–7.18 (m, 35H, H_{arom}), 5.74 (t, $J_{2',3'} = J_{3',4'} = 9.6$ Hz, 1H, H-3'), 5.49 (dt, $J_{4,5} = 15.2$ Hz, $J_{5,6} = 7.0$ Hz, 1H, H-5), 5.42–5.38 (m, 3H, H-2', H-

2'', NH), 5.36 (m, 1H, H-4''), 5.24 (dd, $J_{4,5} = 15.2$ Hz, $J_{3,4} = 8.8$ Hz, 1H, H-4),
 5.15 (s, 1H, PhCHO₂), 5.03 (d, $J_{1'',2'''} = 3.0$ Hz, 1H, H-1'''), 4.64 (d, $J_{a,b} = 11.5$
 Hz, 1H, CH-a, 3'''-O-Bn), 4.63 (d, $J_{a,b} = 12.5$ Hz, 1H, CH-a, 2'''-O-Bn), 4.61 (d,
 $J_{1',2'} = 8.0$ Hz, 1H, H-1'), 4.59 (d, $J_{1',2''} = 8.1$ Hz, 1H, H-1''), 4.51 (d, $J_{a,b} = 11.6$
 Hz, 1H, CH-b, 3'''-O-Bn), 4.49–4.43 (m, 3H, H-6'a, H-6'b, CH-b, 2'''-O-Bn),
 4.44 (d, $J_{a,b} = 11.6$ Hz, 1H, CH-a, 3-O-Bn), 4.26–4.24 (m, 2H, CH-b, 3-O-Bn, H-
 1a), 4.17 (t, $J_{2',3'} = J_{3',4'} = 9.6$ Hz, 1H, H-4'), 4.09–4.06 (m, 1H, H-2), 3.90–3.88
 (m, 2H, H-2''', H-6''a), 3.80 (dd, $J_{2'',3''} = 10.0$ Hz, $J_{3'',4''} = 3.3$ Hz, 1H, H-3''),
 3.75–3.70 (m, 2H, H-3, H-5'), 3.65 (dd, $J_{2''',3'''} = 10.0$ Hz, $J_{3''',4'''} = 3.1$ Hz, 1H, H-
 3'''), 3.62 (d, $J_{5'',6''} = 6.6$ Hz, 2H, H-6''a, H-6''b), 3.53–3.48 (m, 2H, H-1b, H-
 5''), 3.46 (d, $J_{3''',4'''} = 3.0$ Hz, 1H, H-4'''), 3.41 (d, $J_{3''',4'''} = 12.0$ Hz, 1H, H-
 6''b), 3.29 (s, 1H, H-5'''), 1.96–1.92 (m, 2H, H-6), 1.68–1.63 (m, 2H, CH₂-α),
 1.61 (s, 3H, OAc), 1.29–1.03 (m, 40H, H-7–H-17, H-β–H-(ω-1)), 0.89–0.87 (m,
 6H, H-18, H-ω); ¹³C NMR (125 MHz, CDCl₃) δ 172.5 (HNC=O), 170.1 (C=O,
 OAc), 166.1 (C=O, 6'-O-Bn), 165.8 (C=O, 6''-O-Bn), 165.5 (C=O, 3'-O-Bn),
 165.4 (C=O, 2'-O-Bn), 164.4 (C=O, 2''-O-Bn), 138.9, 138.7 (C-*i*, 2'''-O-Bn,
 3'''-O-Bn), 138.5 (C-*i*, 3-O-Bn), 137.8 (C-*i*, benzylidene), 137.2 (C-5), 133.8,
 133.6, 133.5, 133.3 (C-*p*, 5 x OBz), 132.99, 129.97, 129.89, 129.86, 129.78,
 129.69, 129.59, 129.49, 129.20, 129.17, 128.99, 128.91, 128.76, 128.74, 128.34,
 128.31, 128.24, 128.20, 128.18, 127.73, 127.71, 127.58, 127.51, 127.49, 127.44,
 126.37 (45 x CH_{arom}, C-4), 101.5 (C-1'), 101.0 (C-1''), 100.9 (CH-benzylidene),
 95.4 (C-1'''), 79.3 (C-3), 76.0 (C-3'''), 75.5 (C-4'), 74.6 (C-2'''), 74.4 (C-4'''),
 73.9 (CH₂, 3'''-O-Bn), 73.3 (C-3''), 73.1 (C-5'), 72.5 (C-3'), 72.3 (CH₂, 2'''-O-
 Bn, C-2'/C-2''), 71.5 (C-2'/C-2''), 71.1 (C-5''), 70.4 (CH₂, 3-O-Bn), 68.9 (C-
 6'''), 68.5 (C-1), 64.6 (C-4'), 62.9 (C-5'''), 62.6 (C-6'), 61.2 (C-6''), 51.4 (C-2),
 36.6 (C-α), 32.3 (C-6), 32.05, 29.84, 29.83, 29.83, 29.80, 29.78, 29.66, 29.49,
 29.39, 29.34, 25.69, 25.57, 23.99, 23.96, 22.82 (C-7–C-17, C-β–C-(ω-1)), 20.3
 (OAc), 14.3 (C-18, C-ω); HRMS(ESI) *m/z* calcd. for [C₁₁₃H₁₃₃NO₂₄+H]⁺:
 1888.9290, obsd. 1888.9282

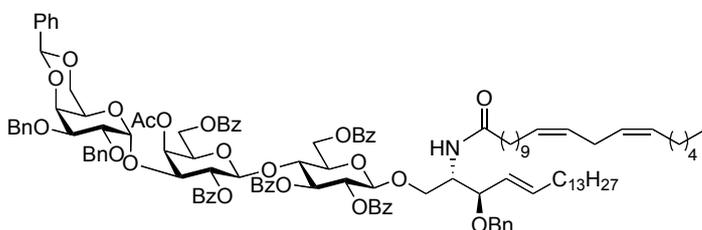
(2*S*,3*R*,4*E*)-2-Dodecanoylamido-1-(4-*O*-(3-*O*- α -D-galactopyranosyl)- β -D-galactopyranosyl)- β -D-glucopyranosyloxy)-3-hydroxy-octadec-4-ene (7)



Fully protected triglycosyl ceramide **100** (25 mg, 0.0132 mmol) was dissolved in dry THF (2 mL) and NH₃ (10 mL) was condensed into the reaction vessel at -78 °C. Small pieces of Na (s) were added carefully until the solution remained deep blue and the reaction mixture was stirred for 30 mins. The reaction was then quenched with a few drops of MeOH and Na (s) was added again until the deep blue colour persisted, and the reaction mixture stirred for a further 30 mins. The reaction was quenched with 10 mL MeOH, and the reaction warmed slowly to rt to allow the ammonia to evaporate. Trace ammonia was removed with an Ar stream. The reaction mixture was quenched to pH 7 with Dowex-H⁺, filtered and washed with pyridine and concentrated *in vacuo*. The resultant oil was purified by gradient flash chromatography (DCM/MeOH, 20/1 to 5/1, v/v) to afford fully deprotected iGb3-C12 **7** as an amorphous white solid (3.1 mg, 0.0032 mmol, 24%). R_f: 0.07 (DCM/MeOH, 5/1, v/v); [α]_D²⁴ = +29.0° (c = 0.1, pyridine); IR (film) 3347, 2956, 2922, 2852, 1641, 1552, 1466, 1377, 1266, 1150, 1076, 1029, 973, 803, 774, 720, 693, 662 cm⁻¹; ¹H NMR (600 MHz, pyridine-d₅) δ 8.45 (d, J_{2,NH} = 8.4 Hz, 1H, NH), 7.46 (d, J = 5.3 Hz, 1H, OH), 6.78 (d, J_{4,5} = 4.7 Hz, 1H, OH), 6.71, 6.64, 6.56 (3 x bs, 3H, 3 x OH), 6.51–6.49 (m, 1H, OH), 6.19 (bs, 1H, OH), 6.03 (dd, J_{4,5} = 15.4 Hz, J_{3,4} = 6.6 Hz, 1H, H-4), 5.95–5.90 (m, 1H, H-5), 5.70 (s, 1H, OH), 5.68 (d, J_{1'',2''} = 3.8 Hz, 1H, H-1''), 5.11 (d, J_{1',2'} = 7.7 Hz, 1H, H-1'), 5.08 (t, J_{5'',6''} = 6.2 Hz, 1H, H-5''), 4.90 (d, J_{1',2'} = 7.9 Hz, 1H, H-1'), 4.83–4.79 (m, 3H, H-1a, H-2, H-3), 4.75 (dd, J_{2'',3''} = 9.6 Hz, J_{1'',2''} = 3.8 Hz, 1H, H-2''), 4.69 (bs, 1H, H-4''), 4.59 (bs, 1H, H-4'), 4.57–4.43 (m, 2H, H-2'', H-3''), 4.47–4.45 (m, 3H, H-6''a, H-6''b, H-6'a), 4.34–4.31 (m, 2H, H-6'a, H-6'b), 4.28–4.23 (m, 4H, H-6'b, H-3', H-4', H-3''), 4.17 (dd, J_{1a,1b} = 9.7 Hz, J_{1b,2} = 2.9 Hz, 1H, H-1b), 4.08–4.03 (m, 2H, H-2', H-5''), 3.88–3.85 (m, 1H,

H-5'), 2.45 (t, $J_{\alpha,\beta} = 7.1$ Hz, 2H, CH₂- α), 2.07 (dd, $J_{6,7} = 14.3$ Hz, $J_{5,6} = 6.6$ Hz, 1H, H-6), 1.88–1.88 (m, 2H, CH₂- β), 1.37–1.22 (m, 38H, H-7–H-11, H- γ –H-(ω -1)), 0.88 (t, $J_{11,12} = J_{\omega-1,\omega} = 6.6$ Hz, 6H, H-12, H- ω); ¹³C NMR (125 MHz, pyridine-d₅) δ 173.7 (HNC=O), 133.0 (C-4), 132.6 (C-5), 105.8 (C-1', C-1''), 98.0 (C-1'''), 82.4 (C-4'), 80.4 (C-3'), 77.0 (C-5'), 76.92 (C-3'), 76.85 (C-5'), 75.1 (C-2'), 73.2 (C-5'''), 73.0 (C-3), 72.0 (C-3'''), 71.2 (C-4'''), 70.8 (C-1, C-2''), 70.7 (C-2'''), 66.3 (C-4''), 62.6 (C-6'''), 62.29 (C-6''), 62.17 (C-6'), 55.2 (C-2), 37.3 (C- α), 33.1 (C-6), 32.5, 30.37, 30.34, 30.31, 30.28, 30.25, 30.23, 30.21, 30.12, 30.07, 29.96, 23.28 (C7–C-11, C- γ –C-(ω -1)), 26.8 (C- β), 14.6 (C-12, C- ω); HRMS(ESI) m/z calcd. for [C₄₈H₈₉NO₁₈+H]⁺: 968.6152, obsd.: 968.6159.

(2*S*,3*R*,4*E*)-1-(4-*O*-(4-*O*-Acetyl-2,6-di-*O*-benzoyl-3-*O*-(2,3-di-*O*-benzyl-4,6-*O*-benzylidene- α -D-galactopyranosyl)- β -D-galactopyranosyl)-2,3,6-tri-*O*-benzoyl- β -D-glucopyranosyloxy)-3-benzyloxy-2-(11*Z*,14*Z*-eicosadienoylamido)-octadec-4-ene (101)

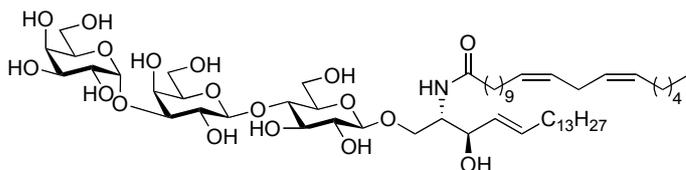


To a solution of glycolipid azide **92** (23 mg, 0.013 mmol) in dry benzene (0.8 mL) triphenylphosphine (7 mg, 0.026 mmol), and distilled water (5 μ L) were added, the solution was warmed to 45 °C and stirred overnight. The reaction mixture was then cooled to room temperature, diluted with EtOAc (15 mL), washed with sat. aq. NH₄Cl (5 mL), dried (MgSO₄), filtered and concentrated under reduced pressure to give a colourless oil which was used without further purification. The oil was co-evaporated twice with dry toluene then suspended in DCM (1 mL), EDCI (11.3 mg, 0.0589 mmol), DMAP (9.6mg, 0.0785 mmol), and 11*Z*,14*Z*-eicosadienoic acid (11.8 mg, 0.0589 mmol) were added and the resulting solution stirred over 2 days at room temperature, after which it was diluted with EtOAc (20 mL), washed with sat. aq. NaHCO₃ (20 mL) and brine (20 mL), dried

(MgSO₄), filtered and concentrated *in vacuo*. The reaction mixture was then purified directly by gradient flash chromatography (petroleum ether/EtOAc, 10/1 to 2/1, v/v) to give **101** as a colourless oil (7.5 mg, 0.0038 mmol, 28% over two steps). The product was kept under inert argon atmosphere at all time to prevent degradation by autoxidation. *R_f*: 0.39 (PE/EA, 2/1, v/v); [α]_D²⁰ = +40.0° (c = 1.0, CHCl₃); IR(film) 3062, 30111, 2956, 2926, 1730, 1453, 1438, 1270, 1177, 1119, 1070, 1028, 998, 754, 711, 634 cm⁻¹; ¹H NMR (500 MHz, CDCl₃) δ 8.04–7.93 (m, 10H, 5 x CH-*o*, 2'-*O*-Bz, 3'-*O*-Bz, 6'-*O*-Bz, 2''-*O*-Bz, 6''-*O*-Bz), 7.61–7.12 (m, 35H, H_{arom}), 5.73 (t, $J_{2',3'} = J_{3',4'} = 9.6$ Hz, 1H, H-3'), 5.49 (dt, $J_{4,5} = 15.6$ Hz, $J_{5,6} = 6.7$ Hz, 1H, H-5), 5.41–5.30 (m, 4H, H-2', H-2'', NH, H-4'), 5.23 (dd, $J_{4,5} = 15.3$ Hz, $J_{3,4} = 8.6$ Hz, 1H, H-4), 5.15 (s, 1H, PhCHO₂), 5.03 (d, $J_{1''',2'''} = 3.0$ Hz, 1H, H-1'''), 4.62 (d, $J_{a,b} = 11.3$ Hz, 1H, CH-a, 3'''-*O*-Bn), 4.63 (d, $J_{a,b} = 12.4$ Hz, 1H, CH-a, 2'''-*O*-Bn), 4.61 (d, $J_{1',2'} = 8.0$ Hz, 1H, H-1'), 4.58 (d, $J_{1',2'} = 8.0$ Hz, 1H, H-1''), 4.53 (d, $J_{a,b} = 11.8$ Hz, 1H, CH-b, 3'''-*O*-Bn), 4.49–4.47 (m, 3H, H-6'a, H-6'b, CH-b, 2'''-*O*-Bn), 4.43 (d, $J_{a,b} = 11.7$ Hz, 1H, CH-a, 3-*O*-Bn), 4.25–4.24 (m, 2H, CH-b, 3-*O*-Bn, H-1a), 4.16 (t, $J_{2',3'} = J_{3',4'} = 9.5$ Hz, 1H, H-4'), 4.08–4.05 (m, 1H, H-2), 3.89–3.87 (m, 2H, H-2''', H-6'''a), 3.79 (dd, $J_{2'',3''} = 9.9$ Hz, $J_{3'',4''} = 3.1$ Hz, 1H, H-3''), 3.74–3.69 (m, 2H, H-3, H-5'), 3.65 (dd, $J_{2''',3'''} = 10.0$ Hz, $J_{3''',4'''} = 3.1$ Hz, 1H, H-3'''), 3.61 (d, $J_{5'',6''} = 6.6$ Hz, 2H, H-6''a, H-6''b), 3.52–3.48 (m, 2H, H-1b, H-5'), 3.45 (d, $J_{3''',4'''} = 3.0$ Hz, 1H, H-4'''), 3.40 (d, $J_{3''',4'''} = 12.0$ Hz, 1H, H-6''b), 3.29 (s, 1H, H-5'''), 2.77 (t, $J_{13,14}$ (fatty acid) = $J_{12,13}$ (fatty acid) = 6.9 Hz, 2H, H-13 fatty acid), 2.05 (m, 4H, H-10, H-16 fatty acid), 1.95–1.92 (m, 2H, H-6), 1.68–1.63 (m, 2H, CH₂- α), 1.61 (s, 3H, OAc), 1.35–1.23 (m, 42H, H-7–H-17, H- β –H-(ω -1)), 0.89–0.86 (m, 6H, H-18, H- ω); ¹³C NMR (125 MHz, CDCl₃) δ 172.5 (HNC=O), 170.1 (C=O, OAc), 166.1 (C=O, 6'-*O*-Bn), 165.8 (C=O, 6''-*O*-Bn), 165.5 (C=O, 3'-*O*-Bn), 165.4 (C=O, 2'-*O*-Bn), 164.4 (C=O, 2''-*O*-Bn), 138.9, 138.7 (C-*i*, 2'''-*O*-Bn, 3'''-*O*-Bn), 138.5 (C-*i*, 3-*O*-Bn), 137.8 (C-*i*, benzylidene), 137.2 (C-5), 133.8, 133.7, 133.6, 133.5, 133.3 (C-*p*, 5 x OBz), 130.35, 13.27, 129.97, 129.91, 129.88, 129.79, 129.70, 129.67, 129.61, 129.49, 129.20, 129.16, 129.02, 128.92, 128.78, 128.76, 128.70, 128.62, 128.40, 128.36, 128.32, 128.26, 128.22, 128.19, 128.12, 128.06, 127.74, 127.73, 127.61, 127.53, 127.50, 126.39 (CH_{arom}, C-4, C-11, C-12, C-14, C-15 fatty acid), 101.5 (C-1', C-1''), 100.9 (CH-benzylidene), 95.4 (C-1'''), 79.3 (C-3), 76.0 (C-3'''),

75.5 (C-4'), 74.6 (C-2'''), 74.4 (C-4'''), 73.9 (CH₂, 3'''-O-Bn), 73.3 (C-3'''), 73.1 (C-5'), 72.5 (C-3'), 72.3 (CH₂, 2'''-O-Bn, C-2'/C-2''), 71.5 (C-2'/C-2''), 71.1 (C-5''), 70.4 (CH₂, 3-O-Bn), 69.0 (C-6'''), 68.5 (C-1), 64.6 (C-4'), 62.9 (C-5'''), 62.6 (C-6'), 61.2 (C-6''), 51.4 (C-2), 36.6 (C-2 fatty acid), 32.4 (C-6), 32.07, 31.66, 29.86, 29.85, 29.82, 29.74, 29.68, 29.66, 29.51, 29.49, 29.39, 29.37, 27.40, 27.34, 27.23, 25.77, 25.64, 25.58, 25.19, 23.92, 22.84, 22.72, 20.21 (C-7–C-17, C-3–C-10 fatty acid, C16–C19 fatty acid), 20.3 (OAc), 14.3 (C-18, C-20 fatty acid); HRMS(ESI) m/z calcd. for [C₁₂₁H₁₄₅NO₂₄+H+NH₄]²⁺: 1007.5284, obsd. 1007.5262.

(2*S*,3*R*,4*E*)-2-(11*Z*,14*Z*-Eicosadienoylamido)-1-(4-*O*-(3-*O*- α -*D*-galactopyranosyl)- β -*D*-galactopyranosyl)- β -*D*-glucopyranosyloxy)-3-hydroxyoctadec-4-ene (8)



Fully protected triglycosyl ceramide **101** (20 mg, 0.010 mmol) was dissolved in dry THF (1 mL) and NH₃ (10 mL) was condensed into the reaction vessel at -78 °C. Small pieces of Na (s) were added carefully until the solution remained deep blue and the reaction mixture was stirred for 30 mins. The reaction was then quenched with a few drops of MeOH and Na (s) was added again until the deep blue colour persisted, and the reaction mixture stirred for a further 30 mins. The reaction was quenched with 10 mL MeOH, and the reaction warmed slowly to rt to allow the ammonia to evaporate. Trace ammonia was removed with an Ar stream. The reaction mixture was quenched to pH 7 with Dowex-H⁺, filtered and washed with pyridine and concentrated *in vacuo*. The resultant oil was purified by gradient flash chromatography (DCM/MeOH, 20/1 to 5/1, v/v) to afford fully deprotected iGb3-C12 **8** as an amorphous white solid (5.2 mg, 0.0048 mmol, 48%). *R_f*: 0.13 (DCM/MeOH, 4/1, v/v); [α]_D²⁴ = +26.0° (c = 0.1, pyridine); IR (film) 3360, 3010, 2923, 2853, 1640, 1548, 1464, 1377, 1255, 1150, 1075, 1030, 971, 895, 804, 632 cm⁻¹; ¹H NMR (600 MHz, pyridine-d₅) δ 8.49 (d, *J*_{2,NH} = 8.3 Hz, 1H, NH), 6.04 (dd, *J*_{4,5} = 15.2 Hz, *J*_{3,4} = 6.3 Hz, 1H, H-4), 5.93 (dd, *J*_{4,5} = 15.2

Hz, $J_{5,6} = 6.7$ Hz, H-5), 5.68 (d, $J_{1''',2'''} = 3.6$ Hz, 1H, H-1'''), 5.54–5.48 (m, 4H, H-11, H-12, H-14, H-15 fatty acid), 5.09 (d, $J_{1'',2''} = 8.0$ Hz, 1H, H-1''), 5.06 (t, $J_{5'',6''} = 5.9$ Hz, 1H, H-5''), 4.90 (d, $J_{1',2'} = 7.9$ Hz, 1H, H-1'), 4.84–4.79 (m, 3H, H-1a, H-2, H-3), 4.76 (dd, $J_{2''',3'''} = 10.0$ Hz, $J_{1''',2'''} = 3.6$ Hz, 1H, H-2'''), 4.68 (bs, 1H, H-4'''), 4.59 (bs, 1H, H-4''), 4.58–4.52 (m, 2H, H-2'', H-3'''), 4.51–4.43 (m, 3H, H-6'''a, H-6'''b, H-6''a), 4.33 (dd, $J_{a,b} = 10.9$ Hz, $J_{a,b} = 4.9$ Hz, 2H, H-6'a, H-6''b), 4.29–4.24 (m, 4H, H-6'b, H-3', H-4', H-3''), 4.18–4.16 (m, 1H, H-1b), 4.08–4.03 (m, 2H, H-2', H-5'), 3.87–3.85 (m, 1H, H-5'), 2.93 (t, $J_{13,14}$ (fatty acid) = $J_{12,13}$ (fatty acid) = 5.3 Hz, 2H, H-13 fatty acid), 2.46 (t, $J_{\alpha,\beta} = 7.6$ Hz, 2H, H-2 fatty acid), 2.15–2.06 (m, 6H, H-10, H-16 fatty acid, H-6), 1.86–1.80 (m, 2H, CH₂- β), 1.36–1.23 (m, 42H, H-3–H-9 fatty acid, H-17–H-19 fatty acid, H-7–17), 0.89–0.86 (m, 6H, H-18, H-20 fatty acid); ¹³C NMR (125 MHz, pyridine-d₅) δ 173.7 (HNC=O), 133.0 (C-4), 132.6 (C-5), 130.8, 128.7 (C-11, C-12, C-14, C-15 fatty acid), 105.8 (C-1', C-1''), 98.0 (C-1'''), 82.3 (C-4'), 80.4 (C-3'), 76.97 (C-5'), 76.92 (C-3'), 76.82 (C-5''), 75.1 (C-2'), 73.2 (C-5'''), 73.0 (C-3), 72.0 (C-3'''), 71.2 (C-4'''), 70.8 (C-1, C-2'), 70.7 (C-2''), 66.2 (C-4''), 62.6, 62.2 (C-6''', C-6'', C-6'), 55.2 (C-2), 37.3 (C- α), 33.1 (C-6), 30.30, 30.27, 30.24, 30.21, 30.12, 30.05, 29.96, 29.94, 29.92, 29.83, 27.90, 27.79, 27.38, 26.74, 26.37, 23.27, 23.14 (C-7–C-17, C-2–C-10 fatty acid, C-16–C-19 fatty acid), 14.6, 14.56 (C-18, C-20 fatty acid); HRMS(ESI) *m/z* calcd. for [C₅₆H₁₀₁NO₁₈+H]⁺: 1076.7091, obsd.: 1076.7090).

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Chapter 6. Summary and future prospects

Glycosphingolipids play a crucial role in modulating the immune response, and in particular, the glycolipid adjuvant α -galactosyl ceramide (α -GalCer) is capable of enhancing the body's ability to detect and destroy cancerous cells during dendritic cell (DC)-based cancer immunotherapy. In stimulating an anti-cancer immune response, α -GalCer is presented by the CD1d molecule found on DCs to the TCR of iNKT cells, which was discussed in the introduction (**Chapter 1**) to this thesis. In addition to foreign α -linked glycolipids, β -linked glycosphingolipids, such as isoglobotrihexosylceramide (iGb3), can also be recognised by the TCR of NKT cells when it is bound to CD1d. Accordingly, iGb3 analogues have much potential as immunomodulatory compounds, as was described in Chapter 1.

Various analogues of α -GalCer have been synthesised with the aim of fine-tuning the immune response generated during DC-based cancer treatment. Hence, a comprehensive understanding of how α -GalCer interacts with the immune system is crucial in order to guide efforts towards improved vaccination strategies. One of the ways to study the uptake and trafficking of α -GalCer *in vitro* and *in vivo* is by using fluorescent α -GalCer probes. In **Chapter 2**, the synthesis of the fluorescent probe, dansyl- α -GalCer was described. Biological evaluation of dansyl- α -GalCer revealed that it was able to activate DCs and iNKT cells in a similar fashion to the parent glycolipid α -GalCer and its activity was CD1d-dependent. It was also shown that DCs that have taken up dansyl- α -GalCer *in vitro* were detectable by flow cytometry. Unfortunately, the fluorescence of dansyl- α -GalCer was too weak to be visualised by fluorescent microscopy due to photobleaching of the dye. Accordingly, another α -GalCer probe bearing a brighter fluorescent group, BODIPY, was synthesised, as was discussed in **Chapter 3**. The dansyl- α -GalCer and BODIPY- α -GalCer probes were made via two different synthetic strategies, which reflects the different chemistries that the respective dyes are compatible with. It is envisioned that the brighter BODIPY fluorophore will allow for its use in *in vivo* studies, however, care must be taken to avoid the photo-oxidation of the

dye upon prolonged exposure to light and oxygen. Indeed, this photo-oxidation under relatively mild conditions has not been previously reported for the BODIPY fluorophore and future work will include exploring this phenomenon further. Both dansyl- α -GalCer and BODIPY- α -GalCer were sent to our collaborator, Hans van der Vliet (VU University Medical Centre, Amsterdam) for further studies to understand glycolipid uptake by human DCs. It will be exciting to see the results from this work and how the probes have been used to address biological questions.

In **Chapter 4** the synthesis of two iGb3 analogues, 6'''-deoxy-iGb3-sphingosine and 6'''-deoxy-iGb3-sphinganine was described. The highlight of this route was the highly efficient synthesis of a lactosyl 2-azido-sphingosine acceptor which, in this chapter, was *N*-acylated then functionalised with a D-fucose moiety. The use of two different deprotection strategies performed on the late stage intermediate (i.e. the fully protected 6'''-deoxy-iGb3), allowed for the generation of 6'''-deoxy-iGb3 analogues with the sphingosine or the sphinganine lipid backbone. 6'''-deoxy-iGb3-sphingosine will be valuable to unambiguously ascertain the importance of the 6-hydroxyl group of the terminal sugar in the interaction with CD1d. The other analogue, 6'''-deoxy-iGb3-sphinganine, which has a fully saturated lipid backbone, is a valuable glycolipid to determine if the removal of the rigid *E*-alkene on the lipid backbone alters the glycolipid activity. Both glycolipids have been sent to our collaborators, Jamie Rossjohn (Monash University) and Dale Godfrey (University of Melbourne) for testing in iNKT cell assays, as well as crystal structure studies in the context of CD1d and iNKT cells.

In **Chapter 5** the synthesis of two novel iGb3 analogues bearing either the dodecanoic acid (iGb3-C12) or the di-unsaturated 11*Z*,14*Z*-eicosadienoic acid (iGb3-C20:2) was presented. The synthetic route made use of the common intermediate lactosyl 2-azido-sphingosine, previously described in Chapter 4. The key synthetic steps included the coupling of the terminal galactose sugar, which occurred in high yield, followed by functionalisation of the lipid backbone with the two desired fatty acids. The iGb3 analogues will allow for the study of structure activity relationship and crystal structure analyses, which will provide

insight into how lipid chain length and unsaturation can influence glycolipid activity.

Taken together, **Chapter 4** and **Chapter 5** describe an elegant, robust and convergent synthetic route that allows for the generation of two series of iGb3 analogues; namely the terminal sugar series and the *N*-acyl chain series. In the future, it would be valuable to extend the repertoire of these two series to further probe the structure-activity relationships. For the terminal sugar series, it would be interesting to attach D-galactose in a β 1-3 linkage, instead of the original α 1-4 linkage found in iGb3, to assess if the orientation of the terminal sugar affects how it interacts with the CD1d molecule and the TCR of iNKT cells, and subsequently the effect that this has on the quality of iNKT cell activation. In addition, it would also be valuable to substitute the terminal galactose with an *N*-acetyl galactosamine moiety to generate iGb3 analogues that are akin to gangliosides, which is another class of biologically important glycosphingolipids that contain *N*-acetylgalactosamine attached to a sialylated lactosylceramide. The effect of an *N*-acetyl group on CD1d binding and subsequent iNKT cell activation could potentially lead to an additional series of biologically active glycosphingolipids.

In the future, it would also be interesting to extend the series of acyl chain homologues by attaching fatty acids of different lengths. Of particular interest would be the saturated C20 acid which, together with the iGb3-C20:2 synthesised in Chapter 5, will provide insight into the influence that rigid double bonds have on the iNKT cell activity. These are just some of the many glycosphingolipids that can be prepared and studied in order to find an optimum glycolipid adjuvant for use in the treatment of diseases, such as cancer.

APPENDIX

List of Publications

A divergent approach to the synthesis of iGb3 sugar and lipid analogues via a lactosyl 2-azido-sphingosine intermediate

Cheng, J. M. H.; Dangerfield, E. M.; Timmer, M. S. M.; Stocker, B. L., *Org. Biomol. Chem.*, **2014**, *12*, 2729-2736.

Species-specific activity of glycolipid ligands for invariant NKT cells.

Dangerfield, E. M.; Cheng, J. M. H.; Knight, D. A.; Weinkove, R.; Dunbar, P. R.; Hermans, I. F.; Timmer, M. S. M.; Stocker, B. L., *ChemBioChem*, **2012**, *13*, 1349-1356.

An improved synthesis of dansylated α -galactosylceramide and its use as a fluorescent probe for the monitoring of glycolipid uptake by cells

Cheng, J. M. H.; Chee, S. H.; Knight, D. A.; Acha-Orbea, H.; Hermans, I. F.; Timmer, M. S. M.; Stocker, B. L., *Carbohydrate Research*, **2011**, *346*, 914-926.

Endogenous and exogenous CD1-binding glycolipids

Cheng, J. M. H.; Khan, A. K.; Stocker, B. L.; Timmer, M. S. M., *International Journal of Carbohydrate Chemistry*, **2011**, vol. 2011, Article ID 749591, 13 pages.

Rapid synthesis of 1-deoxygalactonojirimycin using a carbamate annulation

Timmer, M. S. M.; Dangerfield, E. M.; Cheng, J. M. H.; Gulab, S. A.; Stocker, B. L., *Tetrahedron Letters*, **2011**, *52*, 4803-4805.

Methyl 6-azido-6-deoxy- α -D-galactoside

Cheng, J. M. H.; Gulab, S. A.; Timmer, M. S. M.; Stocker, B. L.; Gainsford, G. J., *Acta Crystallographica Section E*, **2011**, *E67*, o1941-1942.

Methyl 6-deoxy-6-iodo- α -D-galactoside

Gulab, S. A.; Cheng, J. M. H.; Timmer, M. S. M.; Stocker, B. L.; Gainsford, G. J., *Acta Crystallographica Section E*, **2010**, *E66*, o1724.

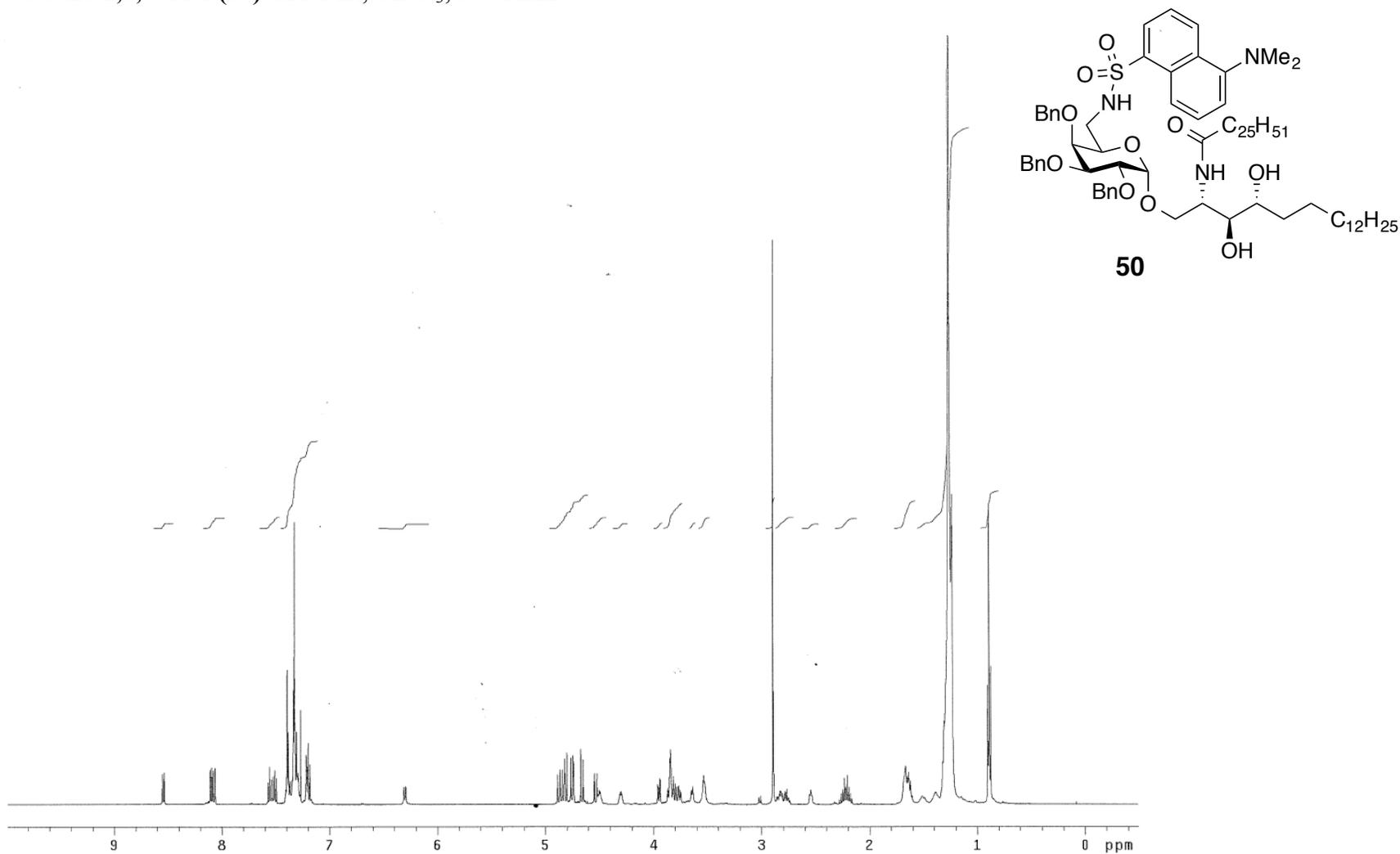
Glycolipids and CD1: The crossroad between chemistry and immunology

Khan, A. K.; Cheng, J. M. H.; Timmer, M. S. M.; Stocker, B. L., *NZIC Chemistry in New Zealand*, **2010**, *74*, 57-62.

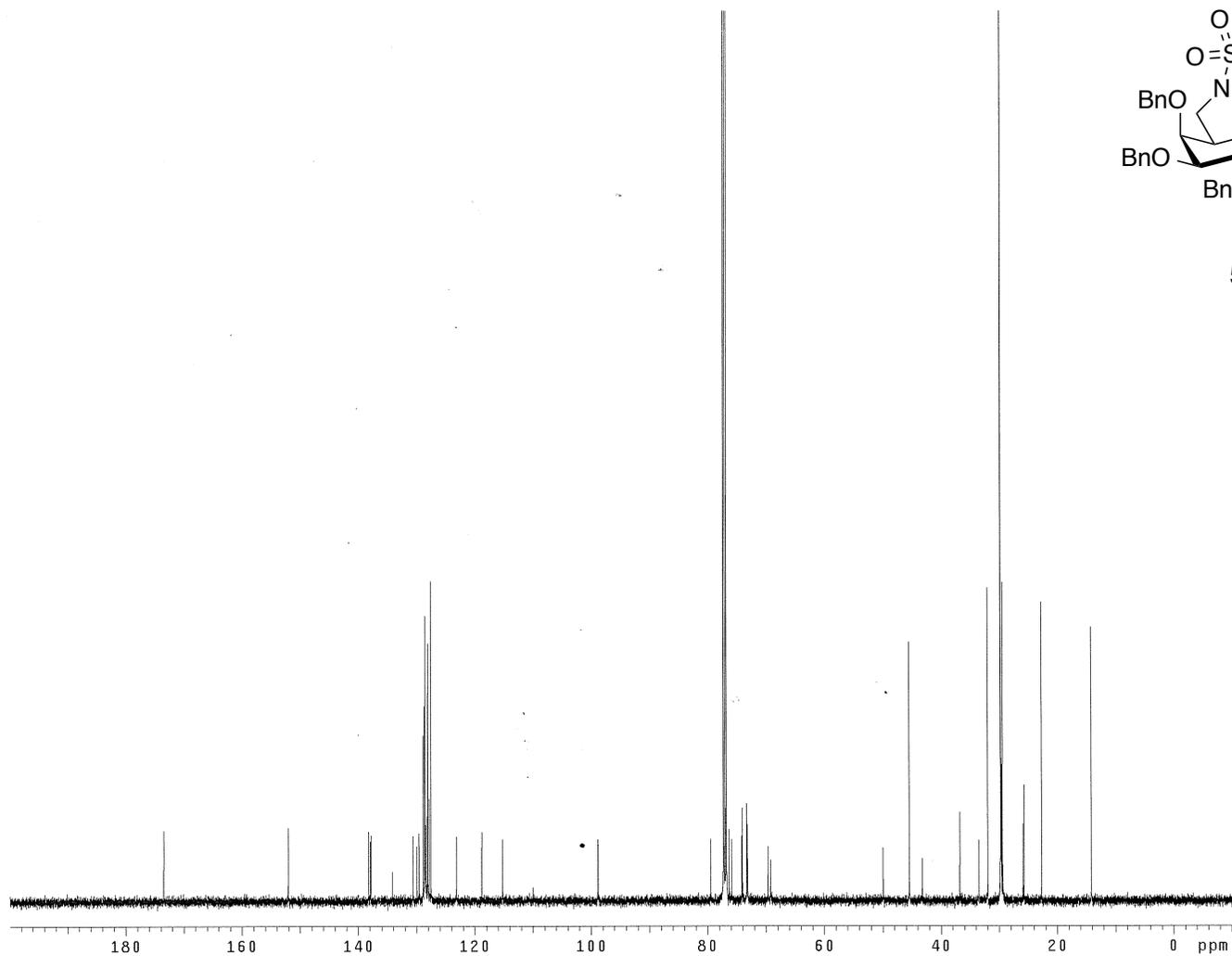
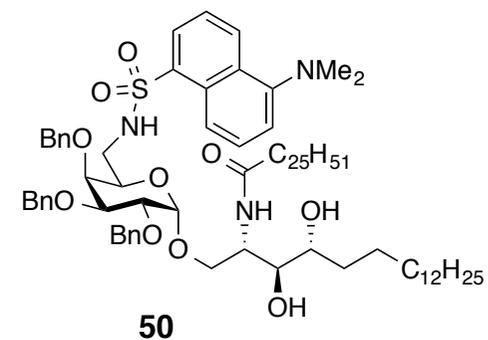
^1H & ^{13}C NMR Spectra

Chapter 2

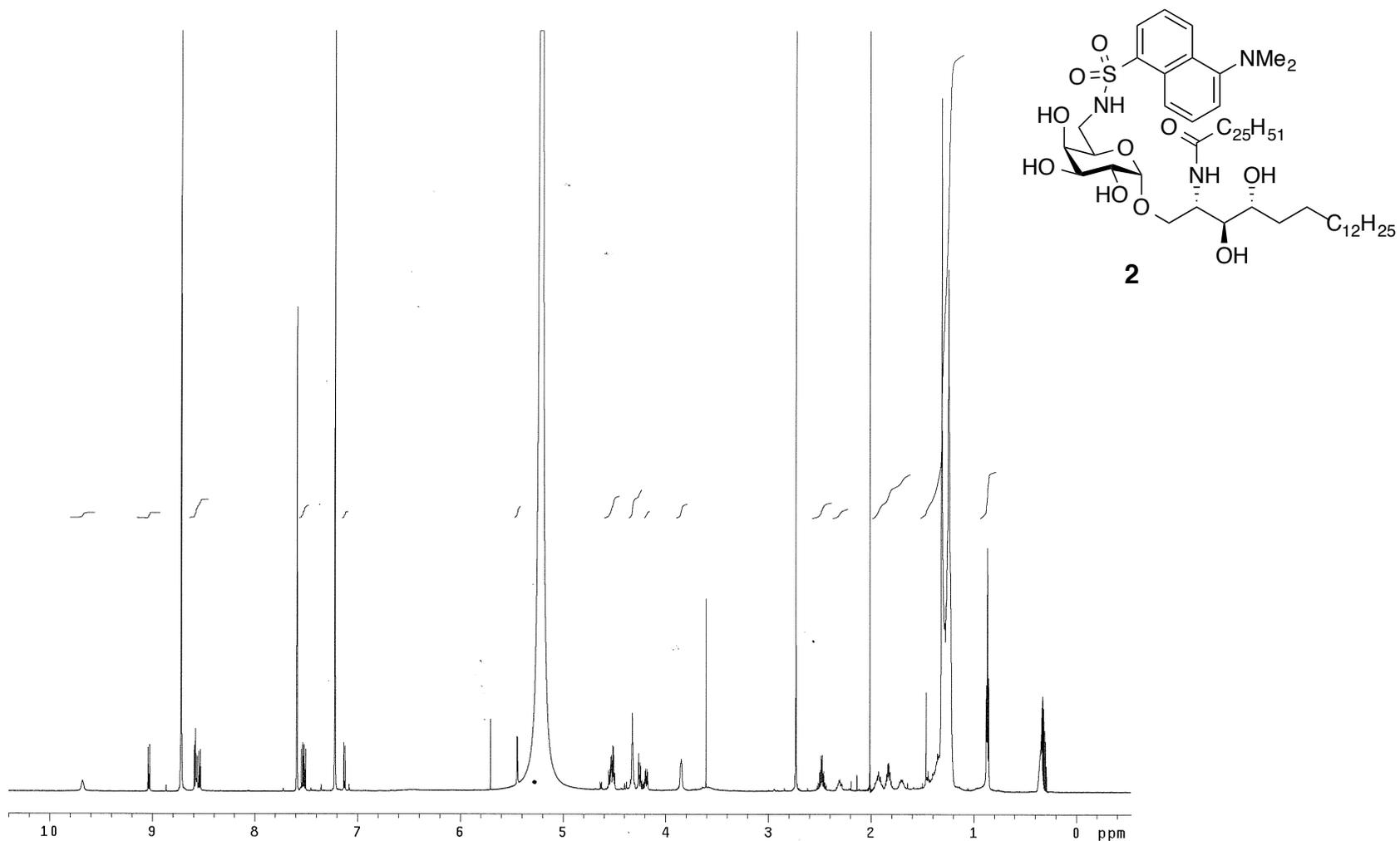
1-*O*-(2,3,4-tri-*O*-benzyl-6-deoxy-6-[*N*-(5-[dimethylamino]naph-1-ylsulfonyl)amido]- α -D-galactopyranosyl)-2-hexacosanoylamido-D-*ribo*-octadecane-1,3,4-triol (**50**) ^1H NMR, CDCl_3 , 500 MHz



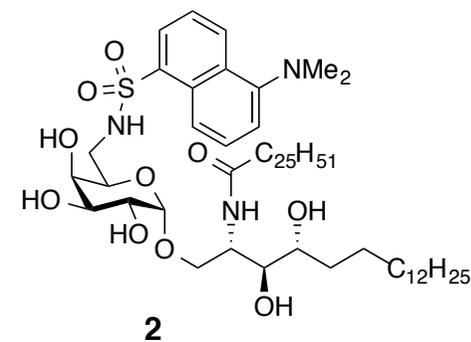
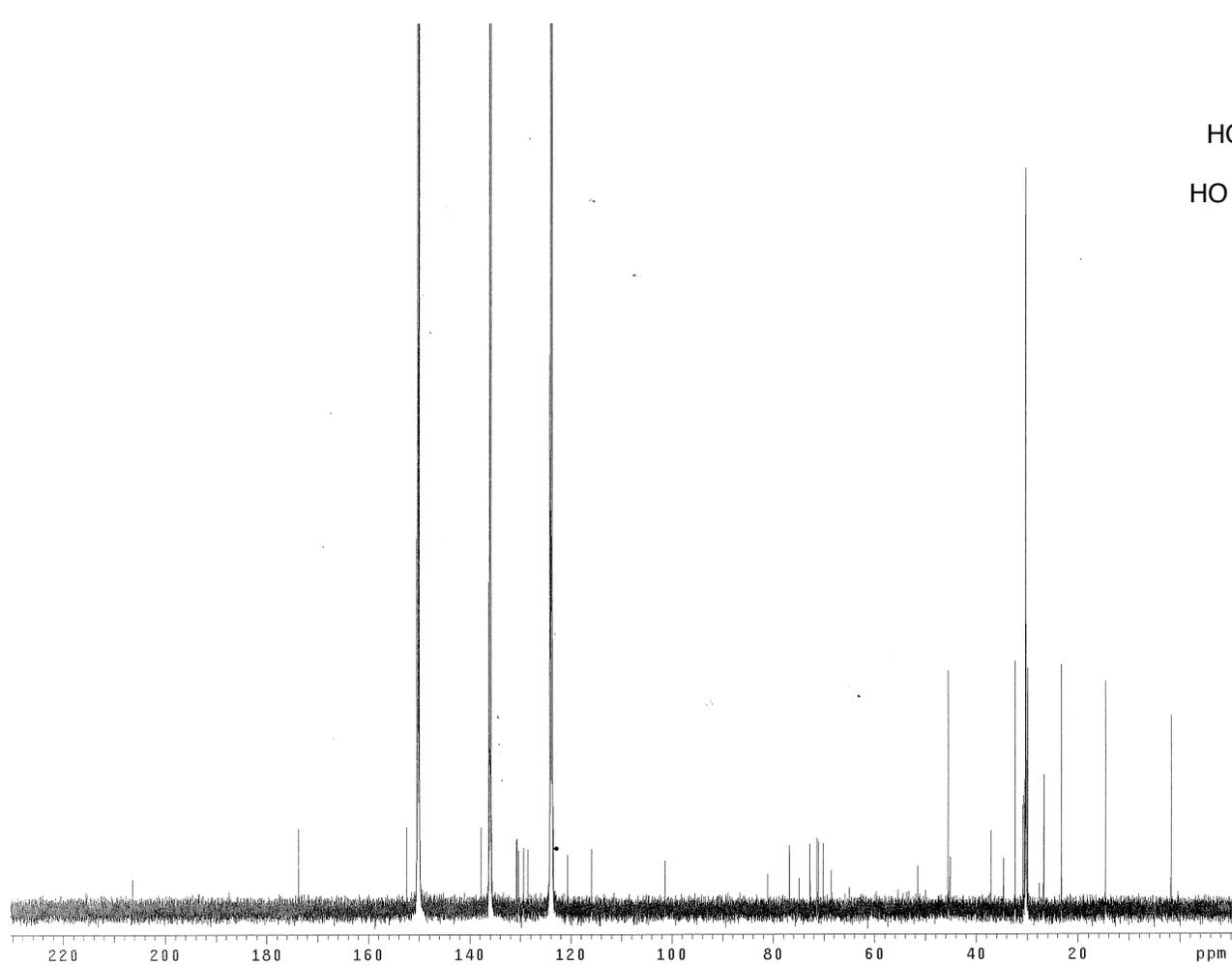
1-*O*-(2,3,4-tri-*O*-benzyl-6-deoxy-6-[*N*-(5-[dimethylamino]naph-1-ylsulfonyl)amido]- α -D-galactopyranosyl)-2-hexacosanoylamido-D-*ribo*-octadecane-1,3,4-triol (**50**) ^{13}C NMR, CDCl_3 , 125 MHz



1-*O*-[6-deoxy-6-[*N*-(5-[dimethylamino]naph-1-ylsulfonyl)amido]- α -D-galactopyranosyl]-2-hexacosanoylamido-D-ribo-octadecane-1,3,4-triol (2) ^1H NMR, pyridine- d_5 , 600 MHz



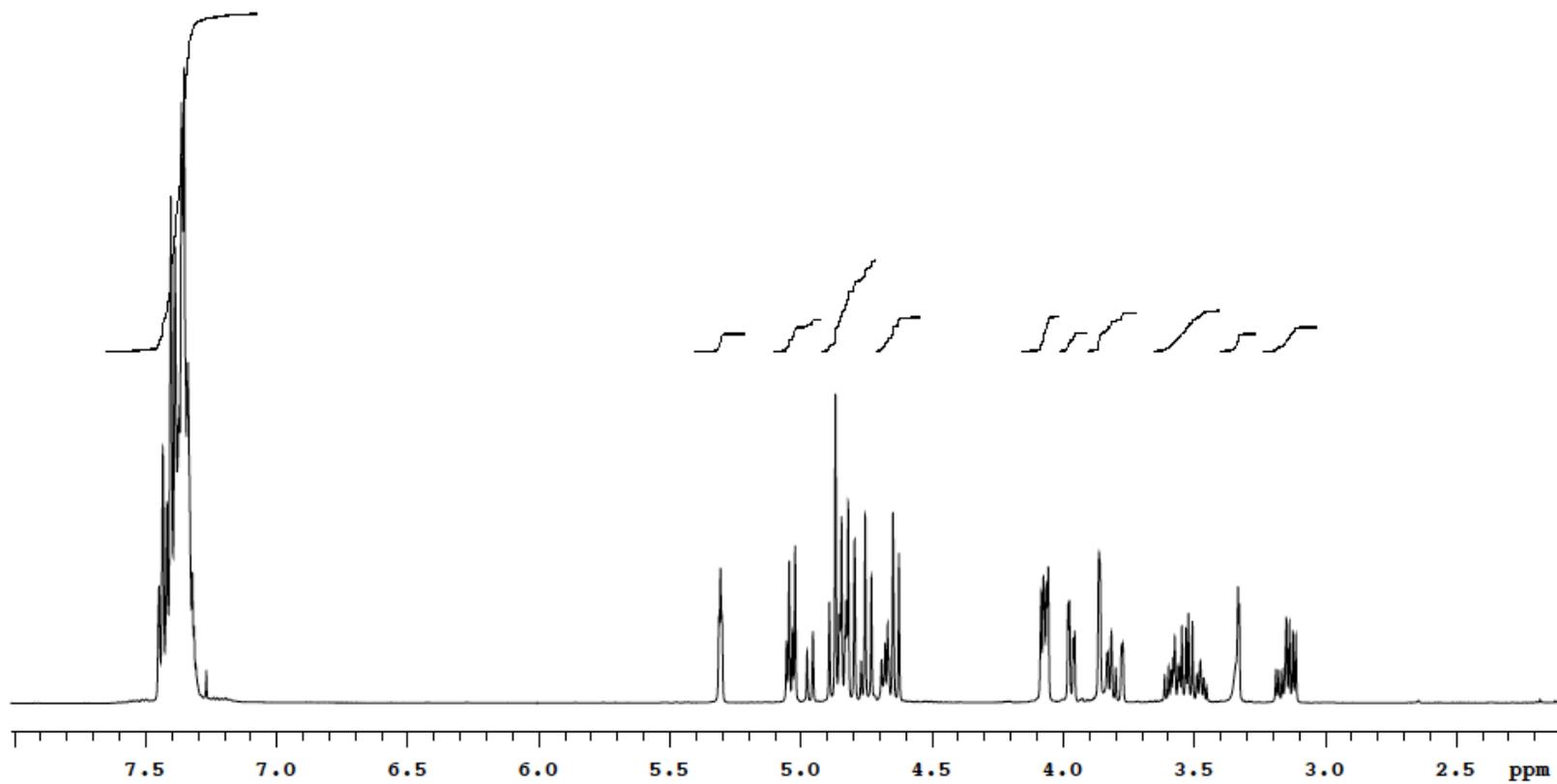
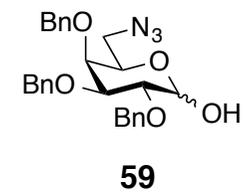
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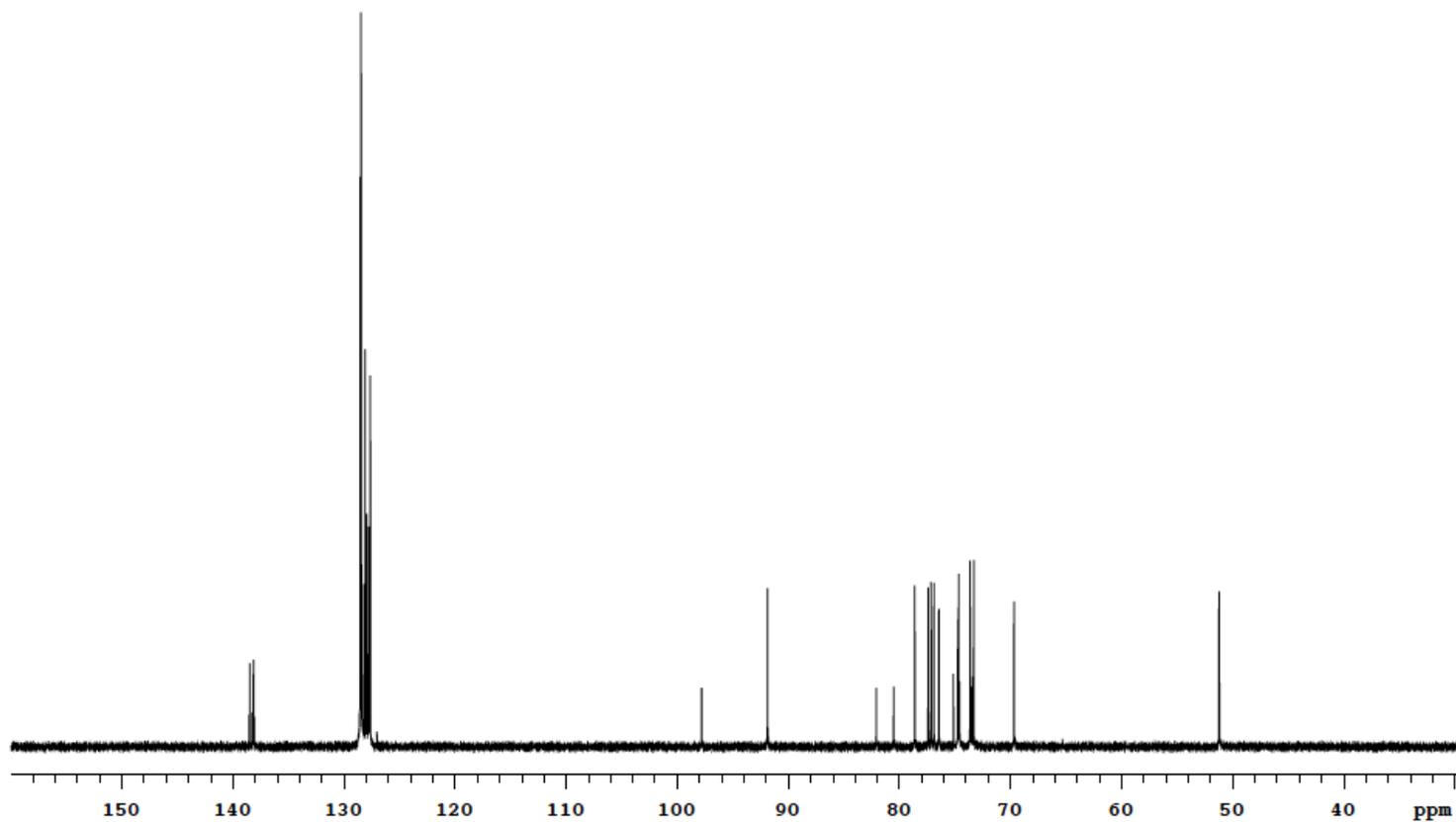
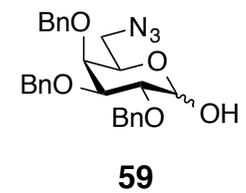
^1H & ^{13}C NMR Spectra

Chapter 3

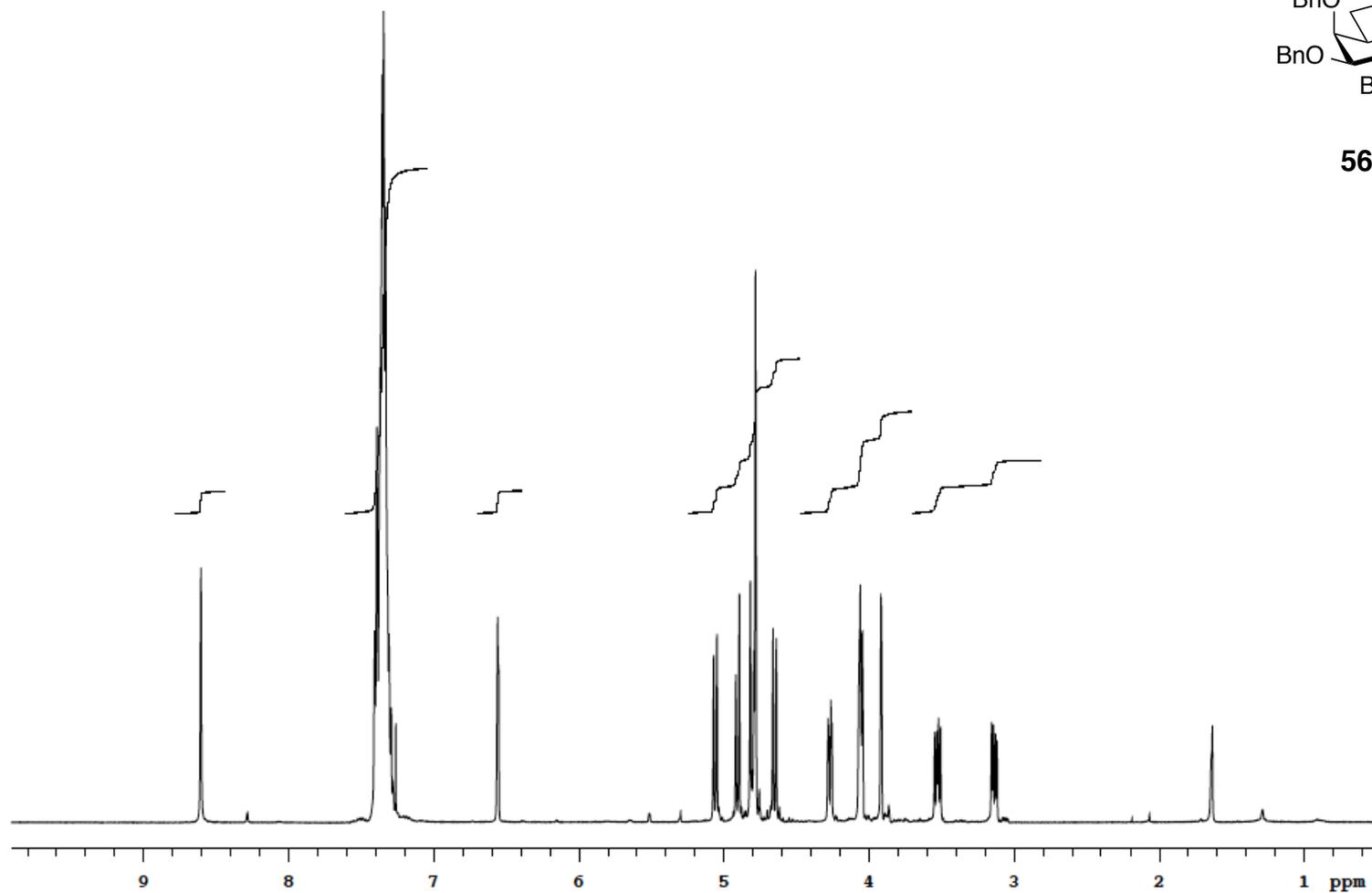
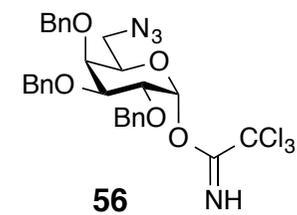
6-Azido-2,3,4-tri-*O*-benzyl-6-deoxy- α -D-galactose (**59**) ^1H NMR, CDCl_3 , 500 MHz



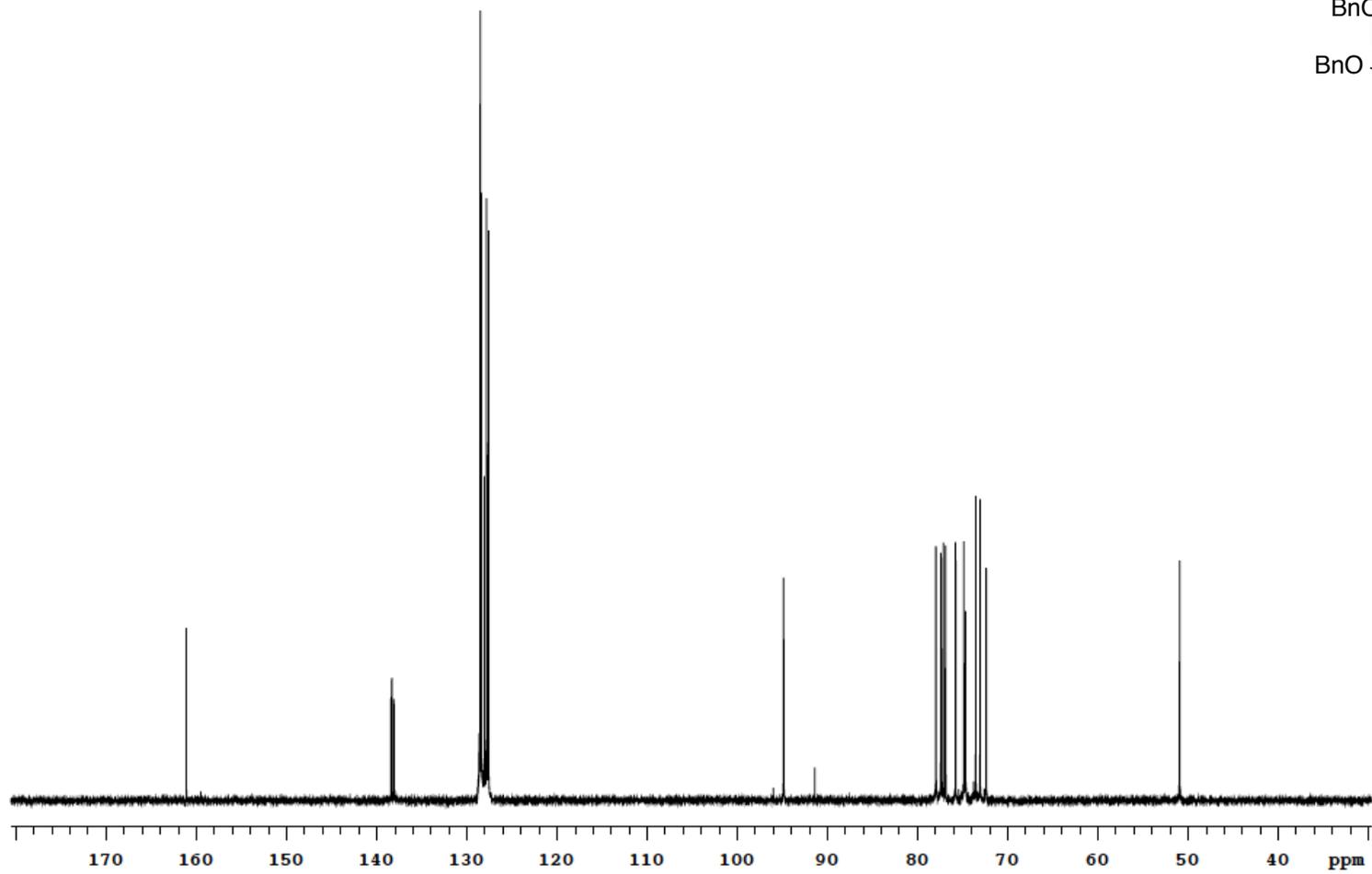
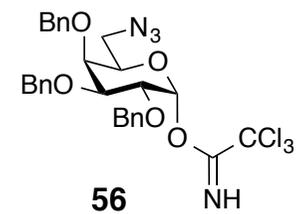
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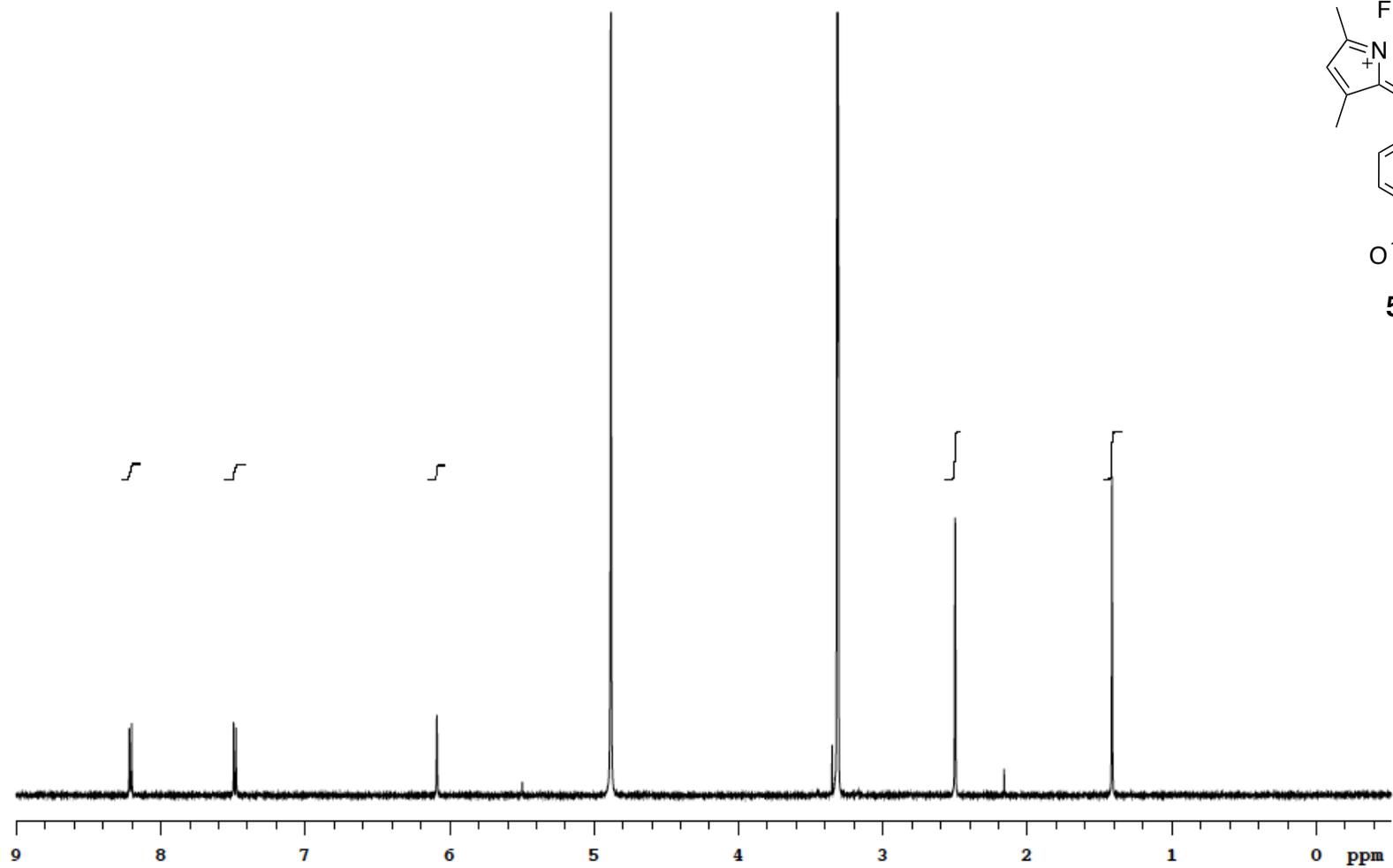
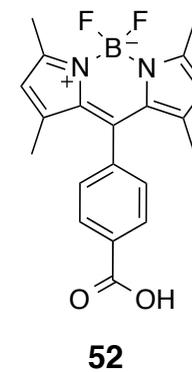
O-(6-Azido-2,3,4-tri-*O*-benzyl-6-deoxy- α -D-galactosyl) trichloroacetimidate (**56**) ^1H NMR, CDCl_3 , 500 MHz



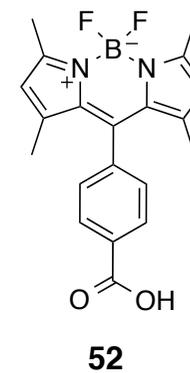
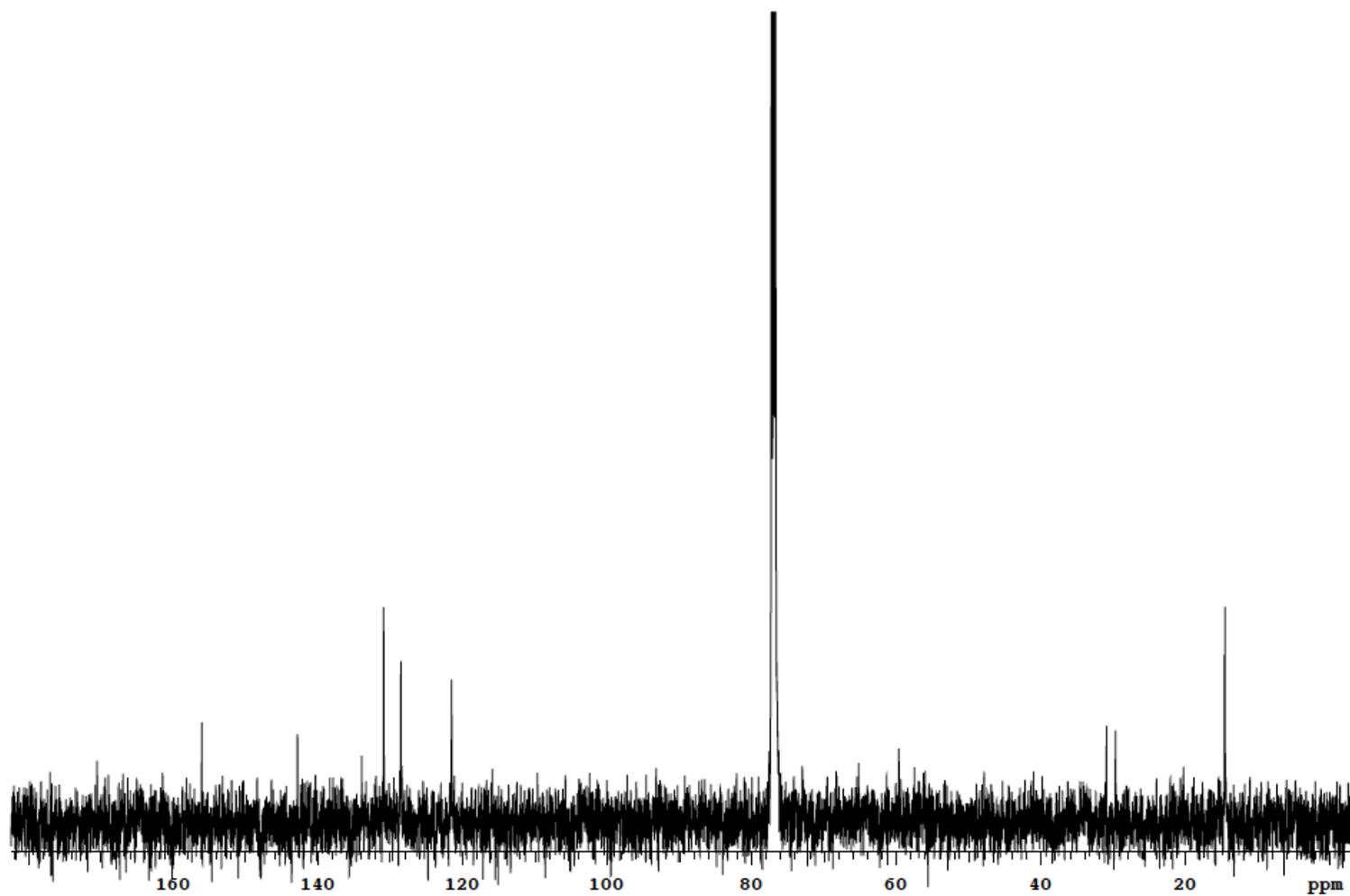
O-(6-Azido-2,3,4-tri-*O*-benzyl-6-deoxy- α -D-galactosyl) trichloroacetimidate (**56**) ^{13}C NMR, CDCl_3 , 125 MHz



4-(4,4-Difluoro-1,3,5,7-tetramethyl-3a,4a-diaza-4-bora-s-indacen-8-yl)benzoic acid (**52**) ^1H NMR, CD_3OD , 500 MHz

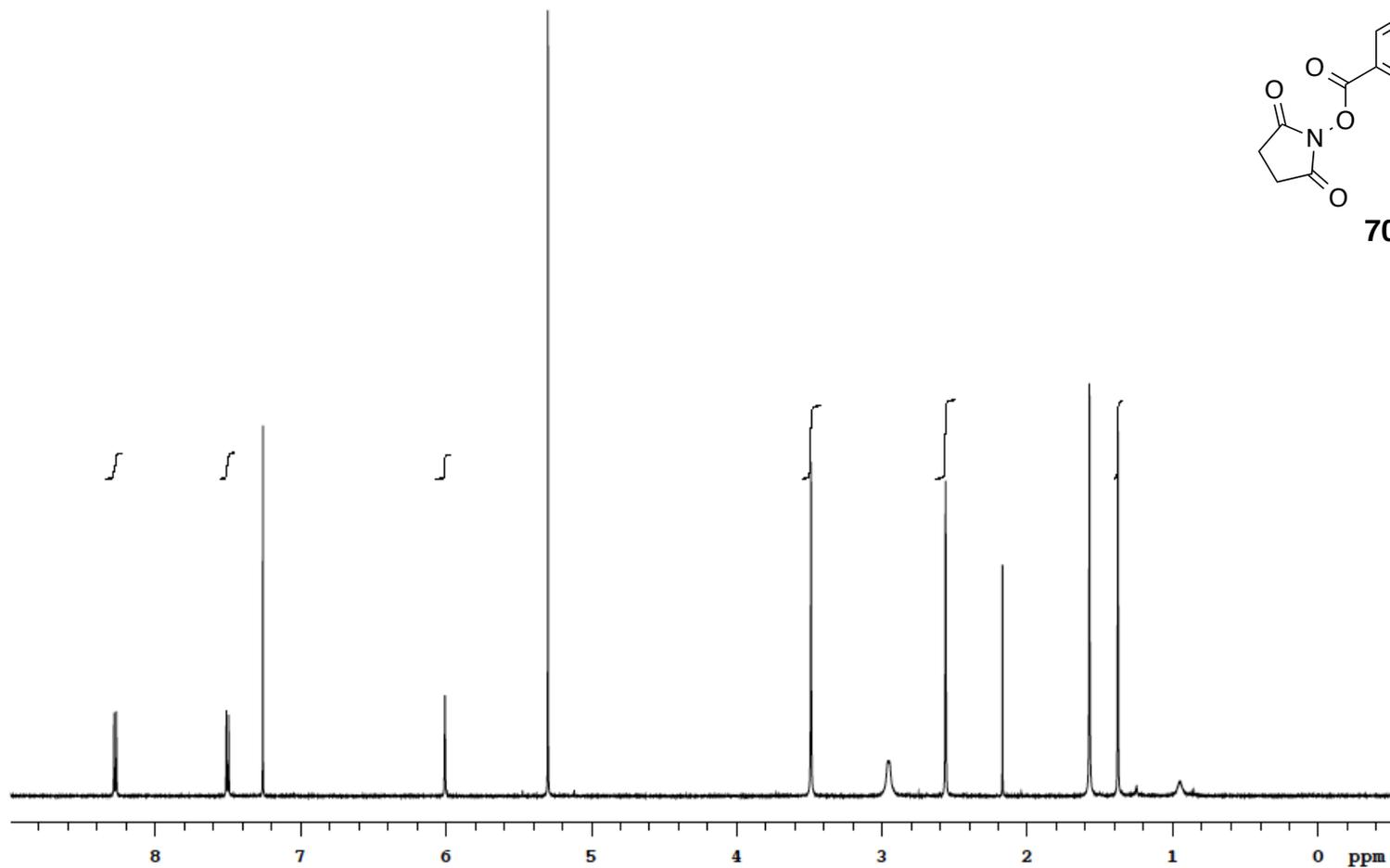
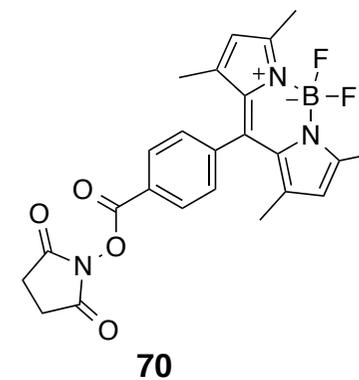


4-(4,4-Difluoro-1,3,5,7-tetramethyl-3a,4a-diaza-4-bora-s-indacen-8-yl)benzoic acid (**52**) ^{13}C NMR, CD_3OD , 125 MHz



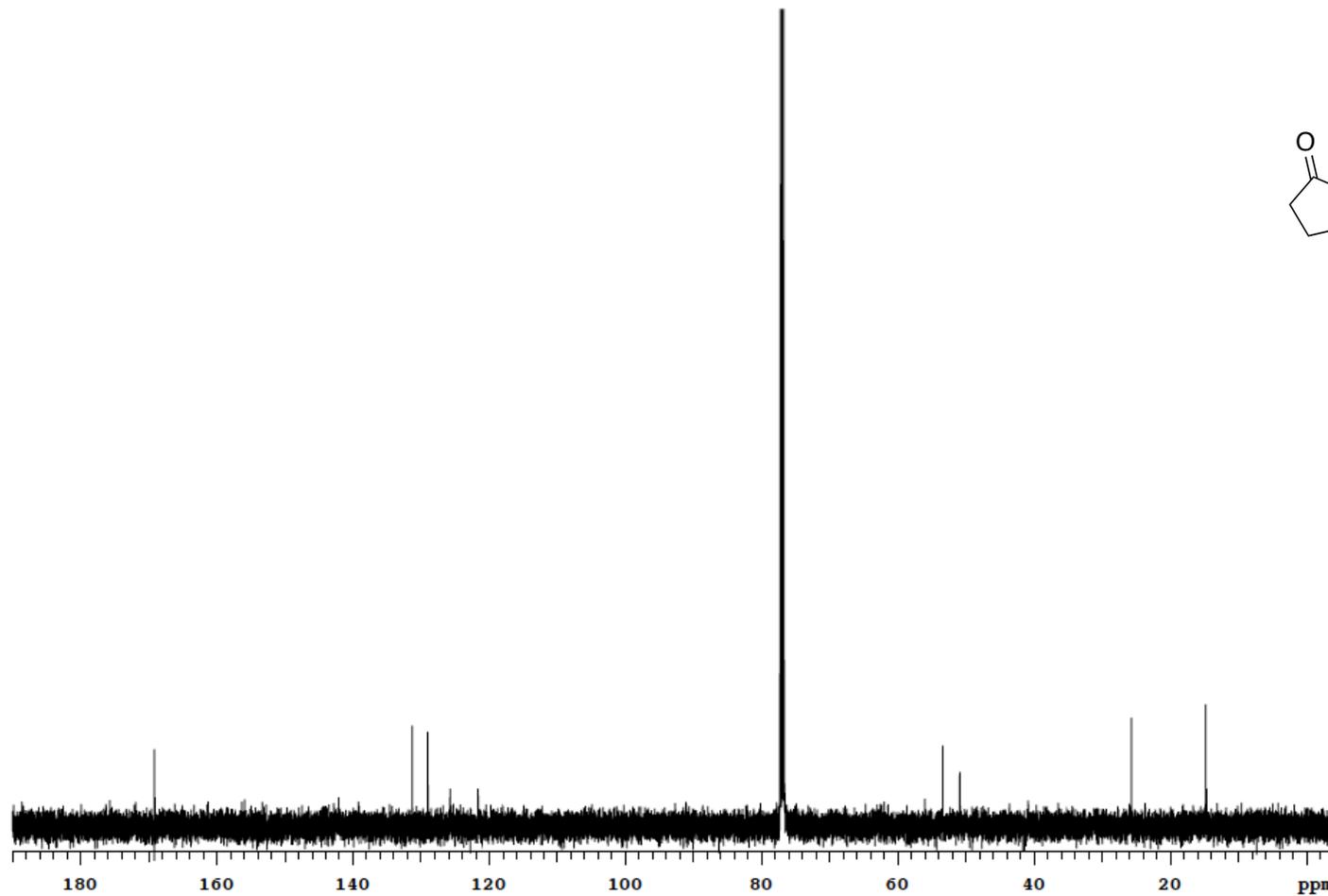
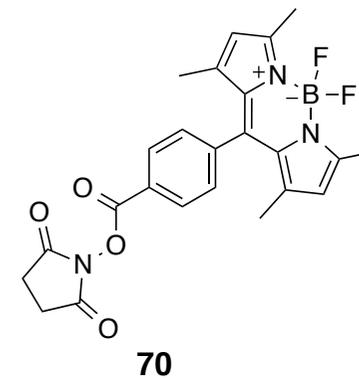
2,5-Dioxopyrrolidin-1-yl-4-(4,4-difluoro-1,3,5,7-tetramethyl-3a,4a-diaza-4-bora-s-indacen-8-yl)benzoate (70)

¹H NMR, CDCl₃, 500 MHz

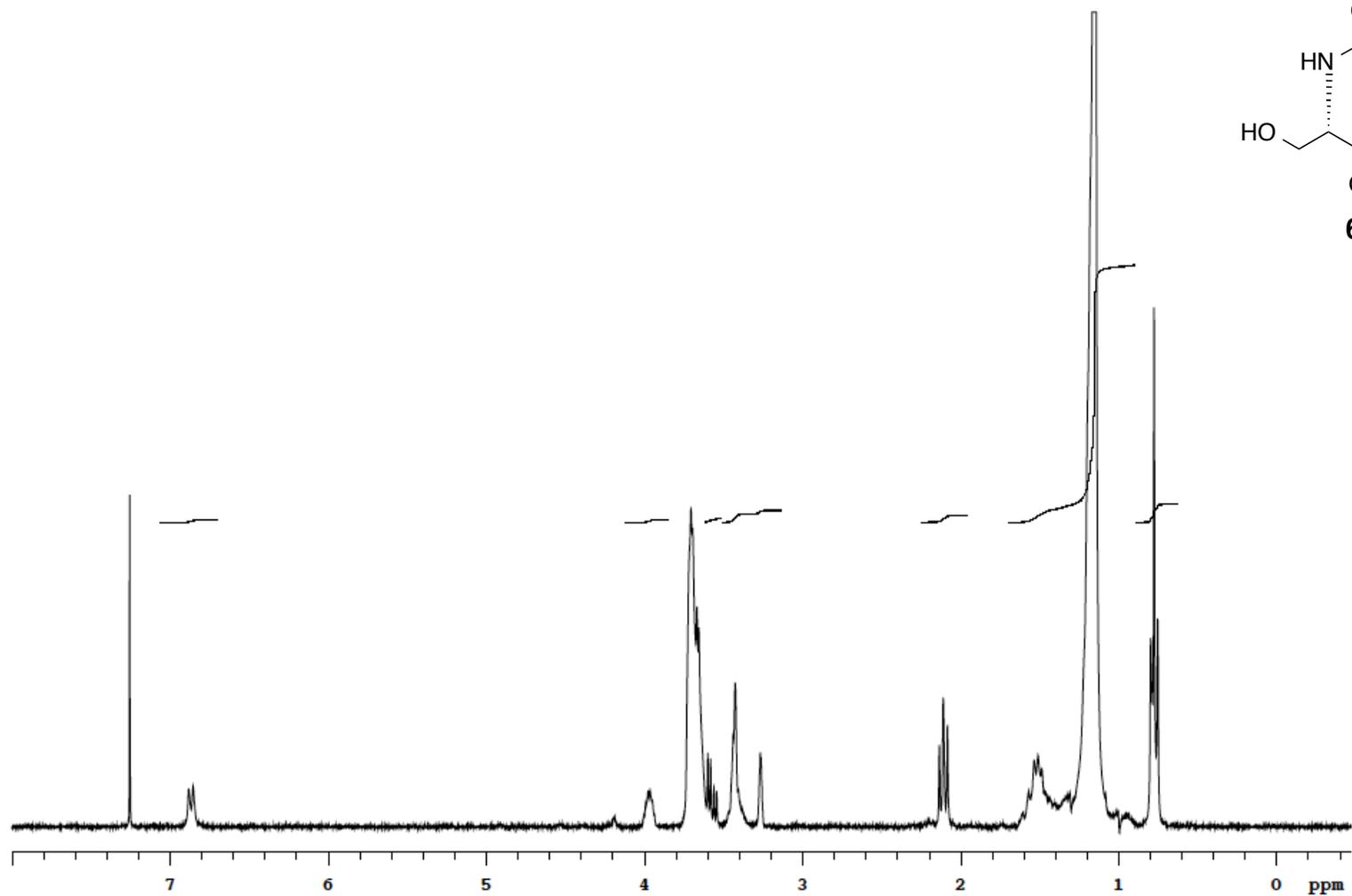
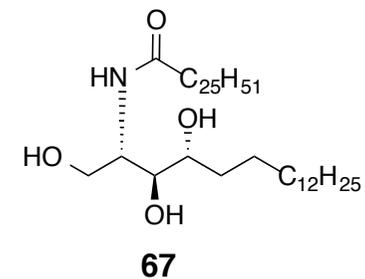


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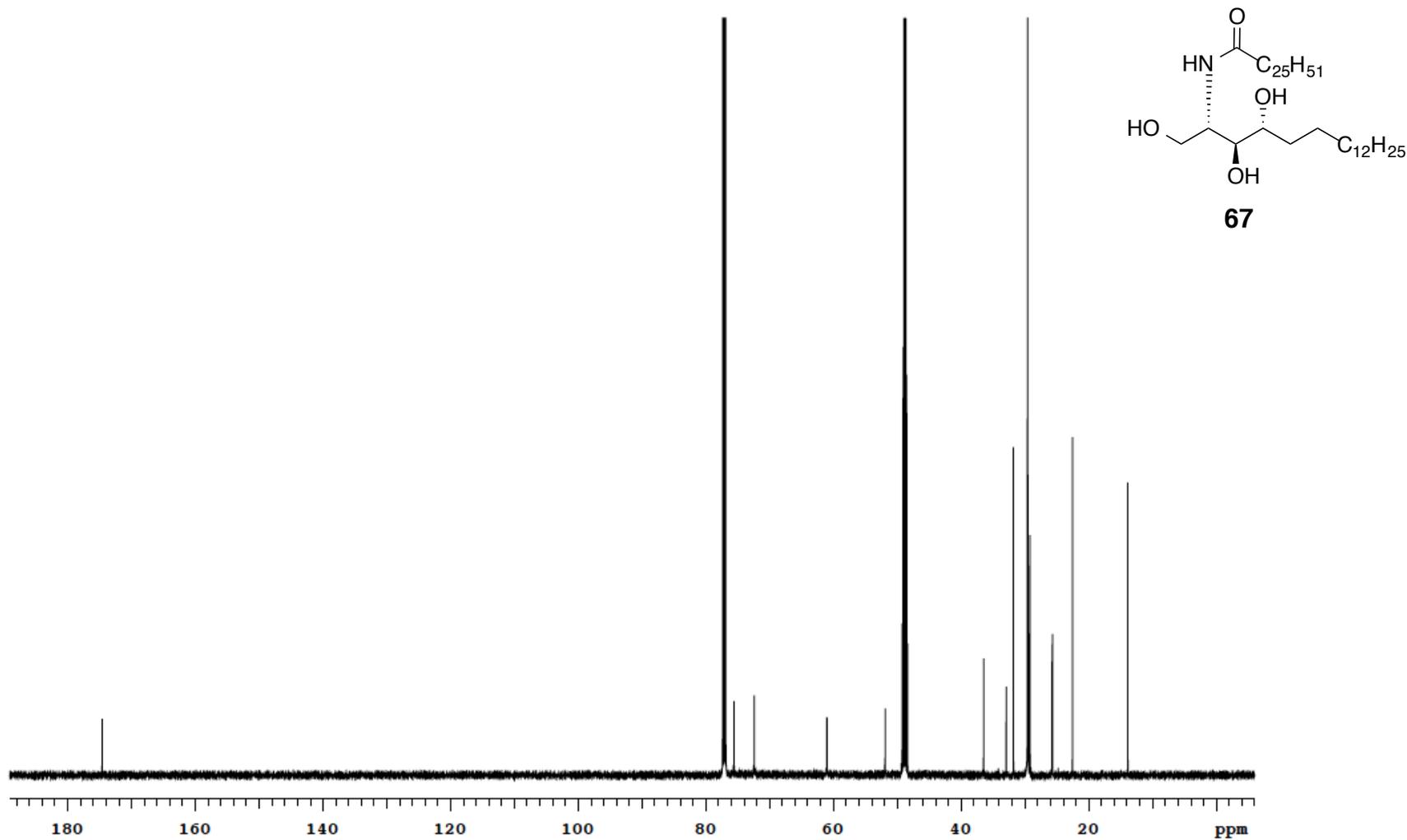
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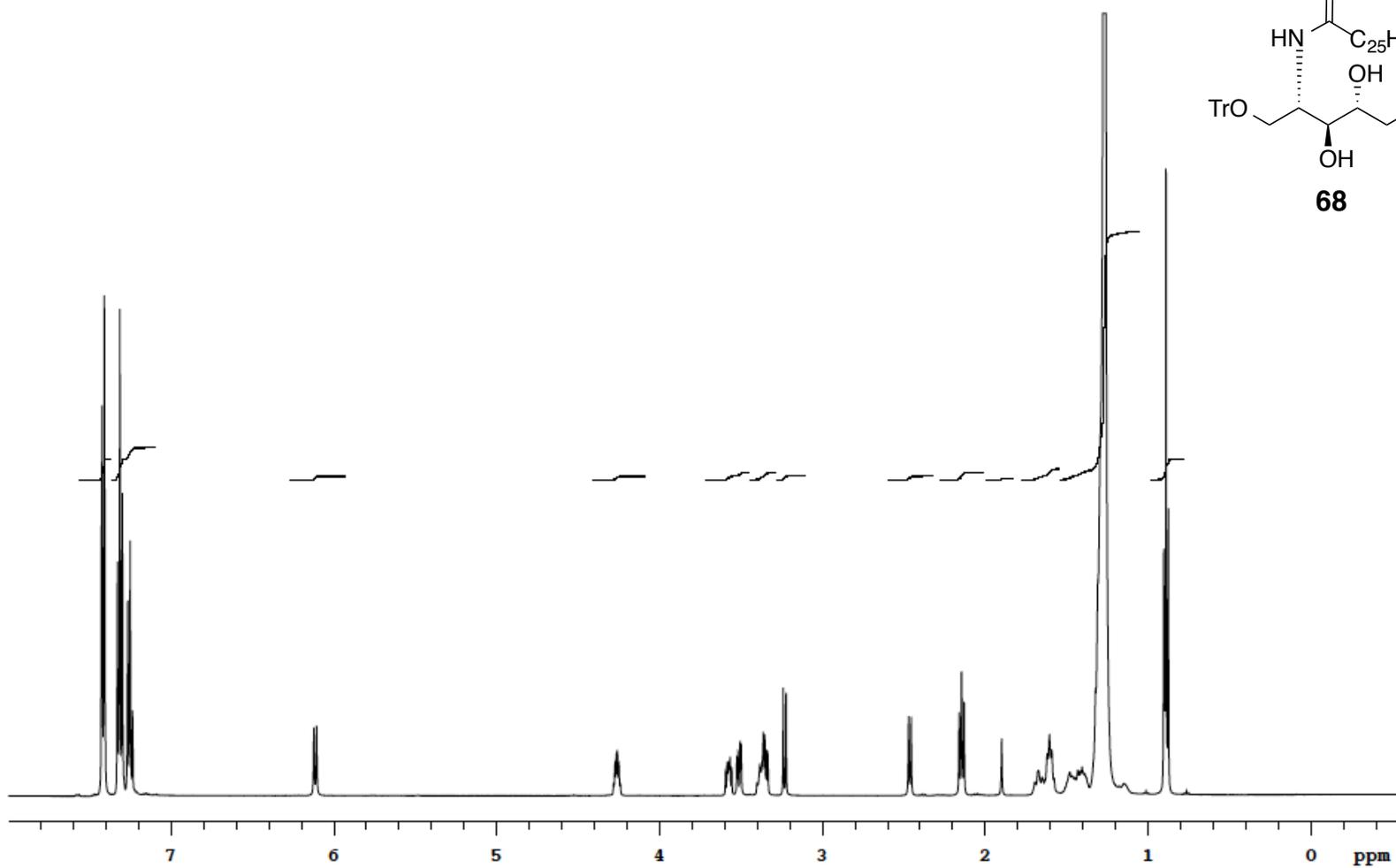
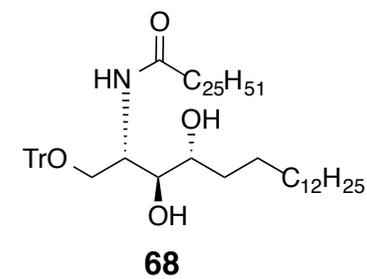
(2*S*,3*S*,4*R*)-2-Hexacosanoylamido-D-ribo-octadecane-1,3,4-triol (**67**) ^1H NMR, 10% CD_3OD in CDCl_3 , 300 MHz



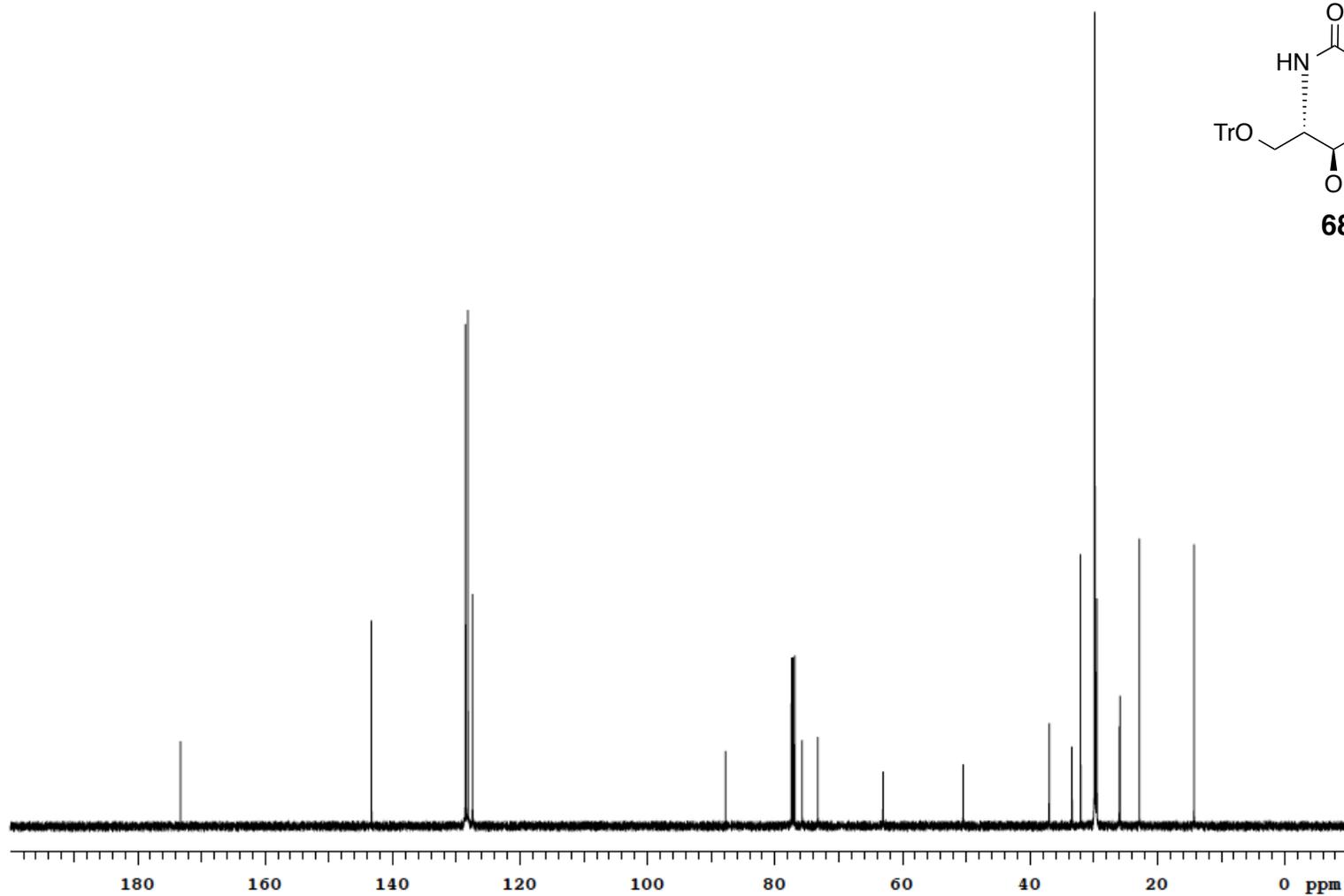
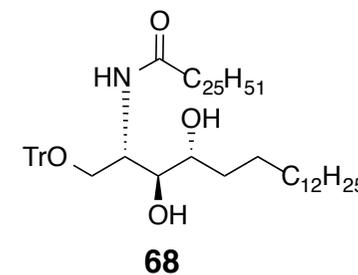
(2*S*,3*S*,4*R*)-2-Hexacosanoylamido-D-ribo-octadecane-1,3,4-triol (**67**) ^{13}C NMR, 10% CD_3OD in CDCl_3 , 125 MHz



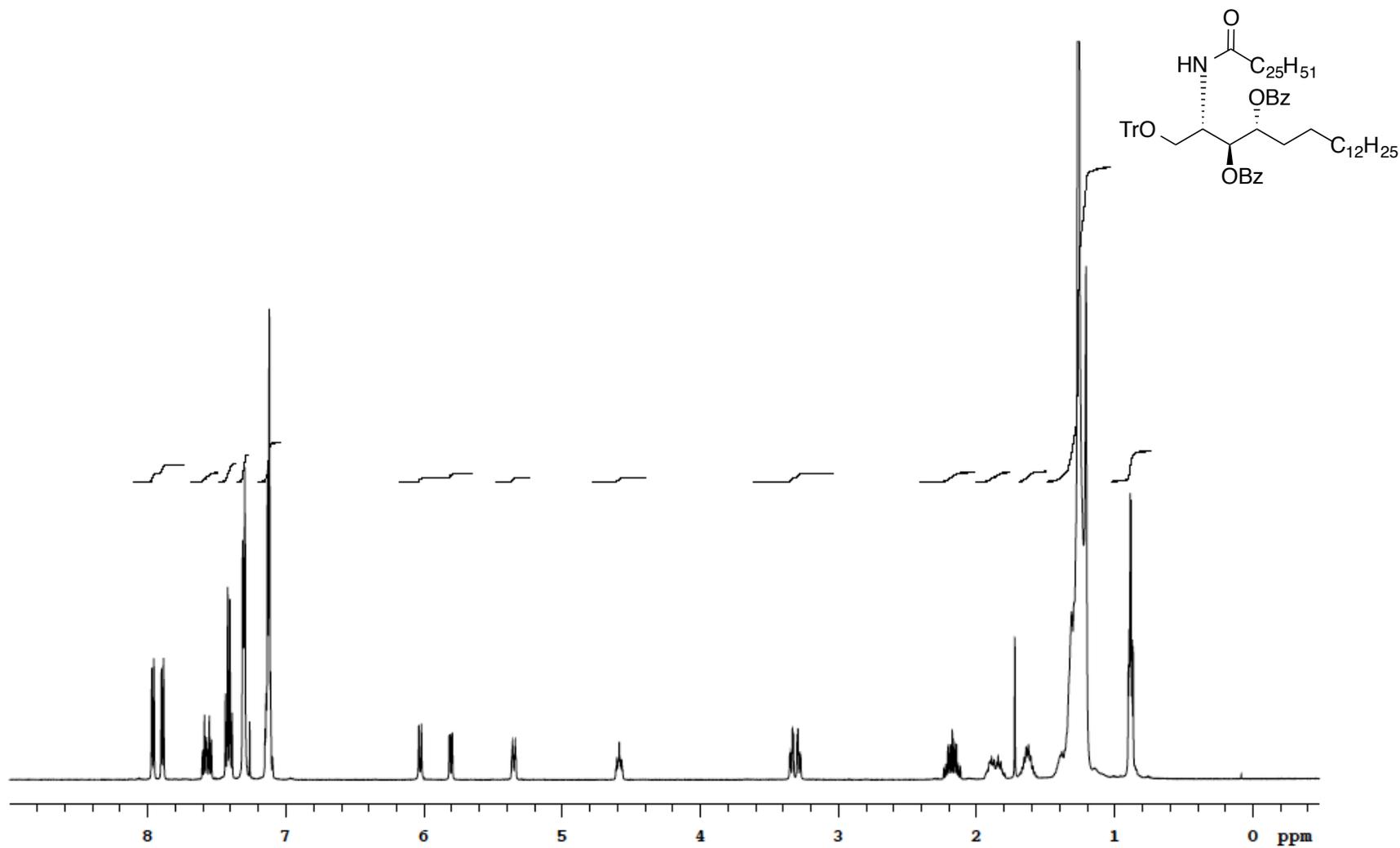
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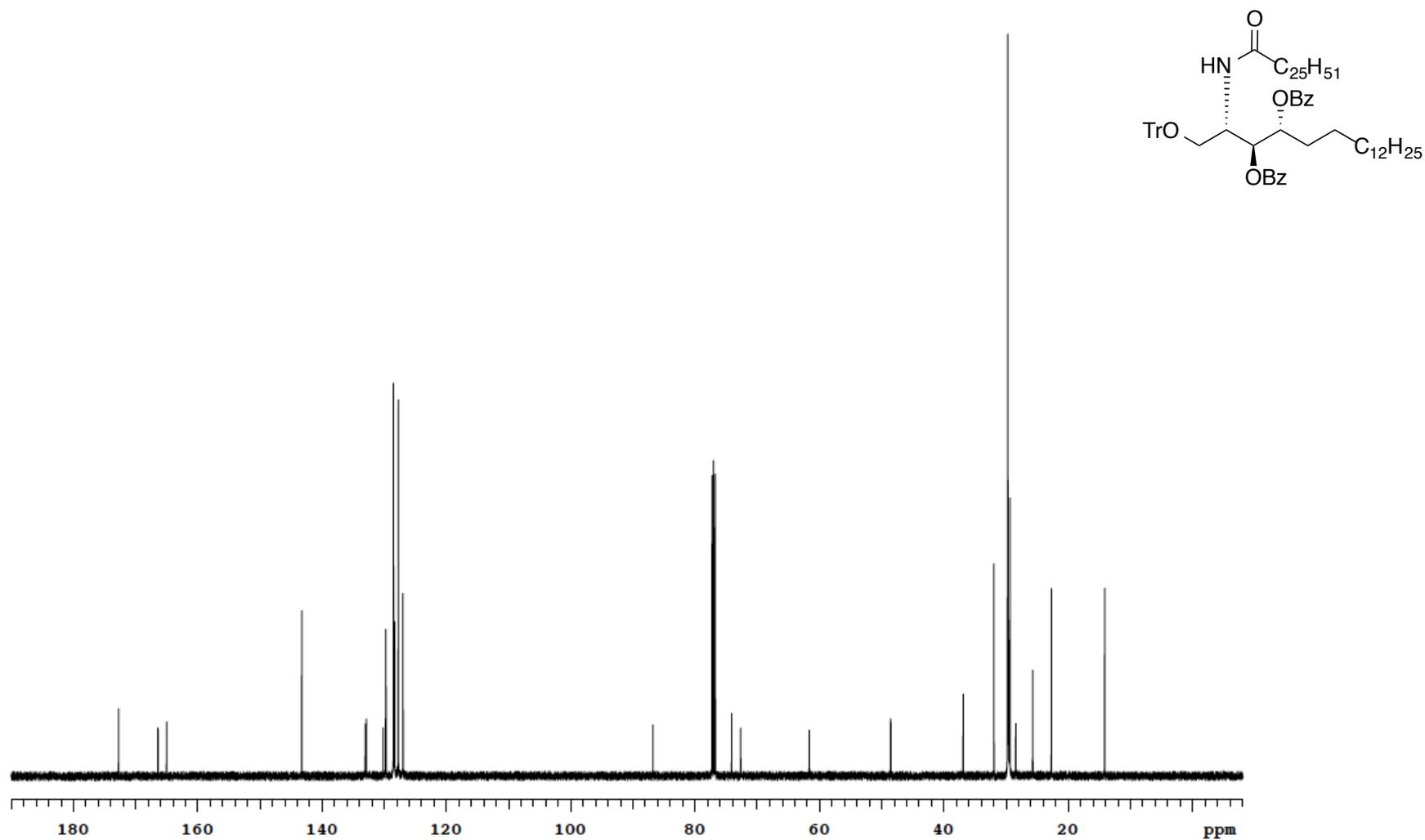
(2*S*,3*S*,4*R*)-2-Hexacosanoylamido-1-triphenylmethoxy-octadecane-3,4-diol (**68**) ^{13}C NMR, CDCl_3 , 125 MHz



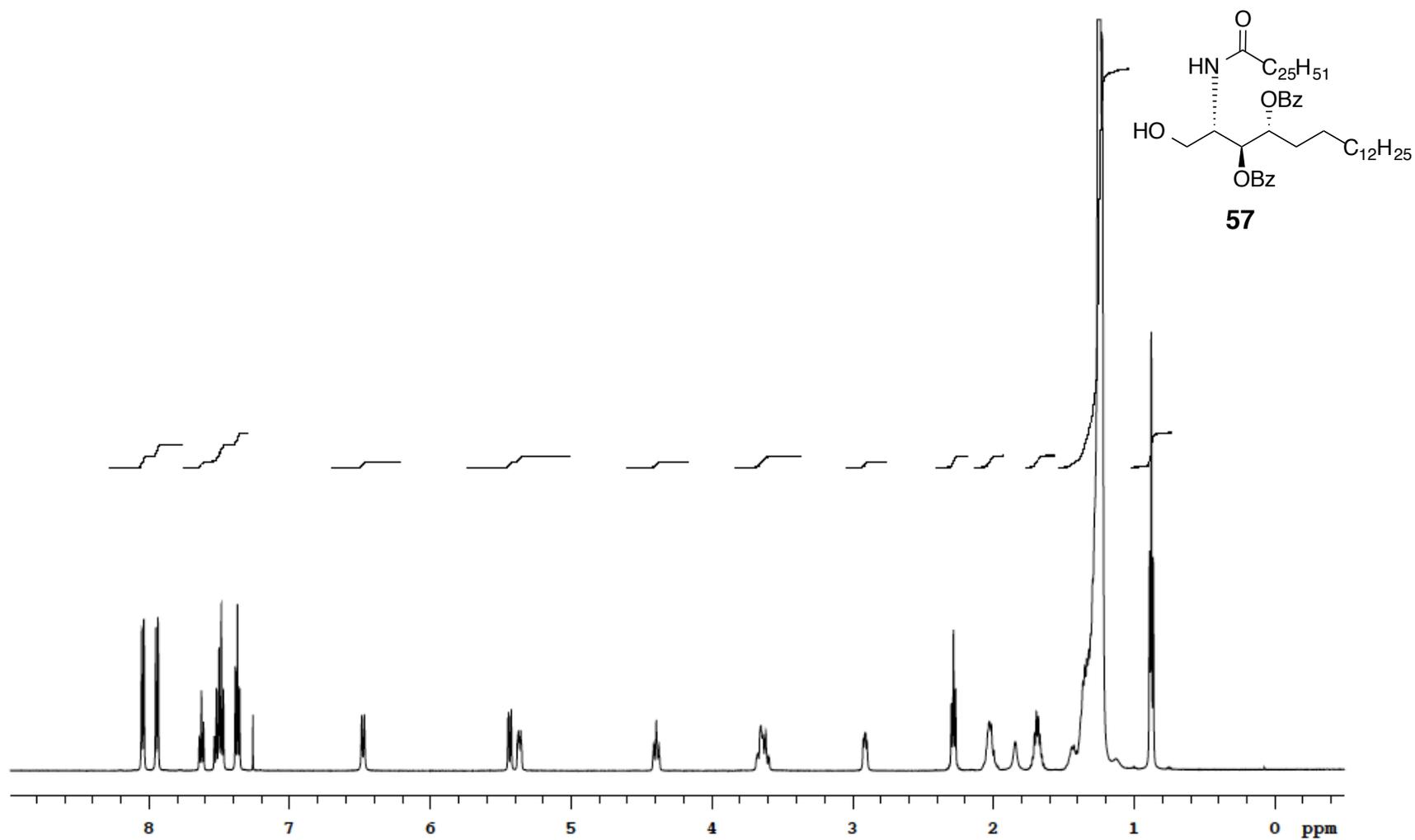
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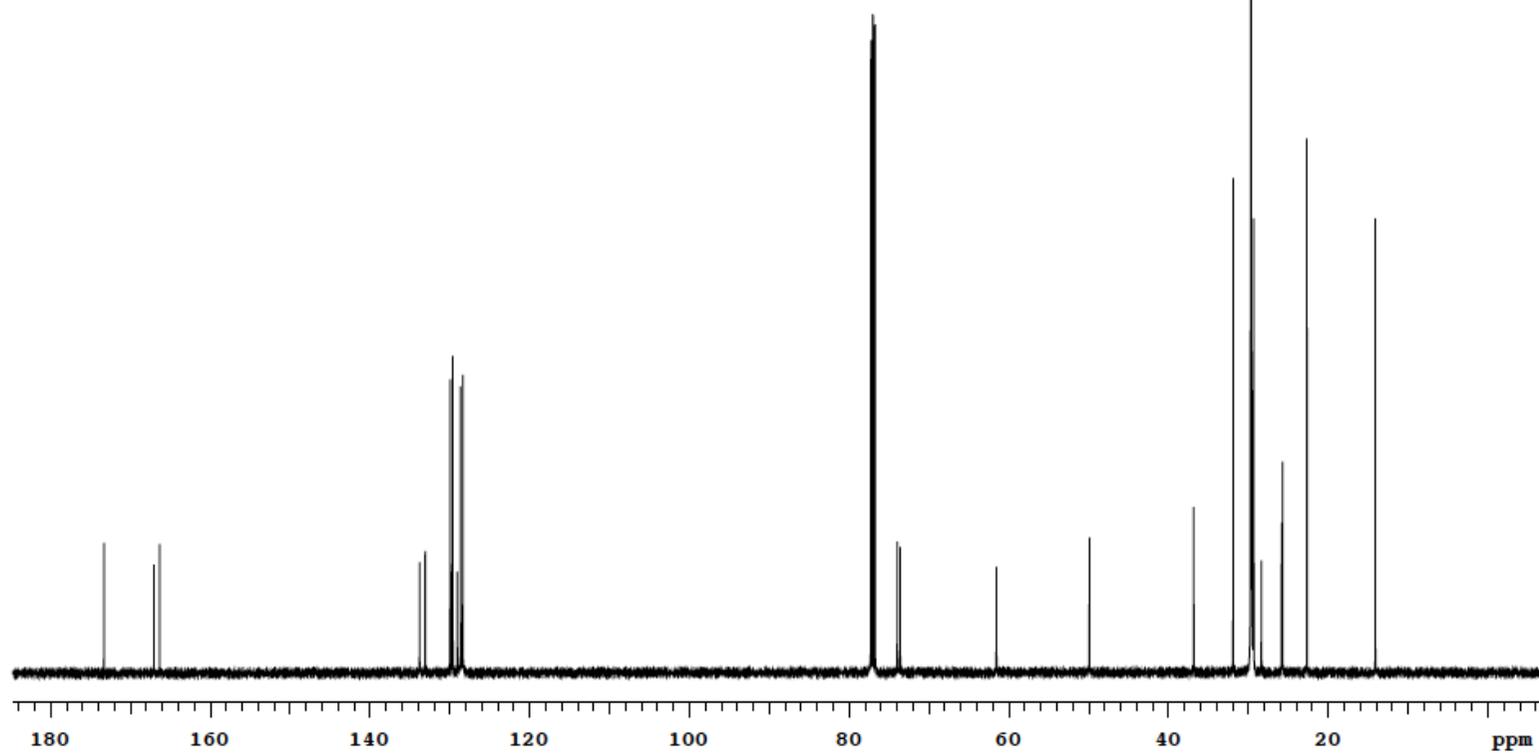
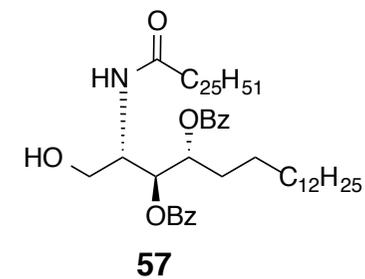
(2*S*,3*S*,4*R*)-3,4-Dibenzoyloxy-2-hexacosanoylamido-1-triphenylmethoxy-octadecane ^{13}C NMR, CDCl_3 , 125 MHz



(2*S*,3*S*,4*R*)-3,4-Dibenzoyloxy-2-hexacosanoylamido-octadecan-1-ol (**57**) ^1H NMR, CDCl_3 , 500 MHz

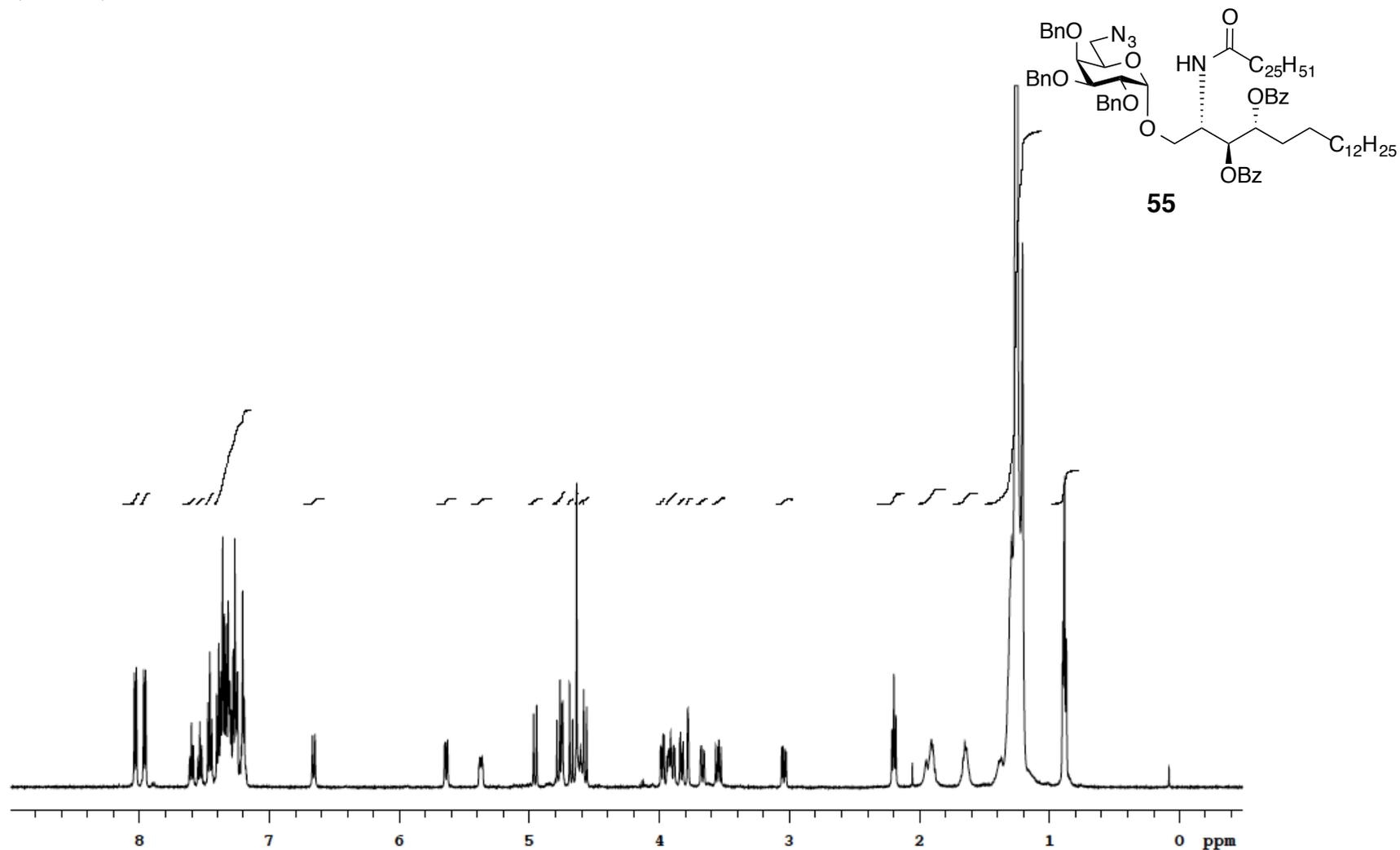


(2*S*,3*S*,4*R*)-3,4-Dibenzoyloxy-2-hexacosanoylamido-octadecan-1-ol (**57**) ^{13}C NMR, CDCl_3 , 125 MHz



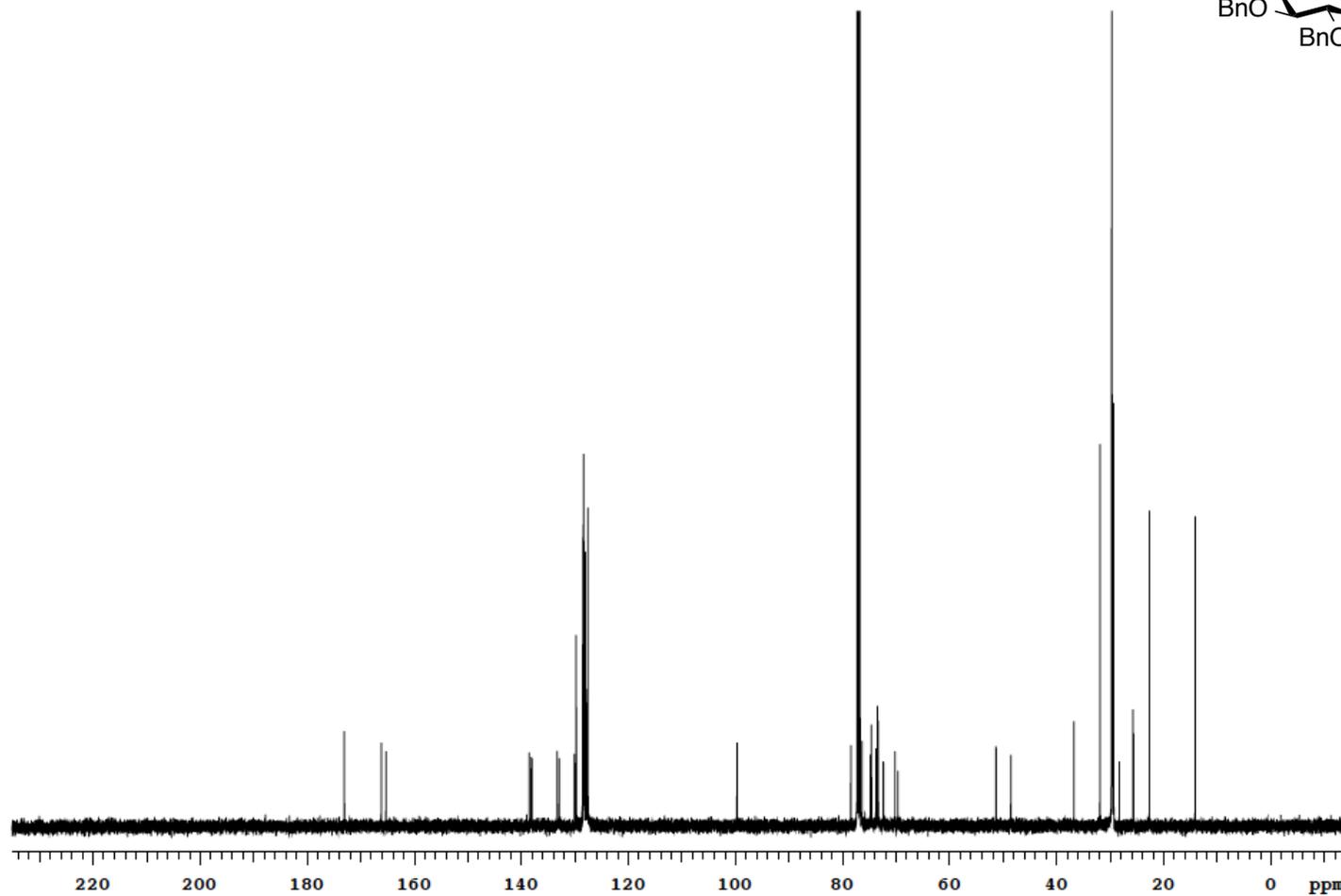
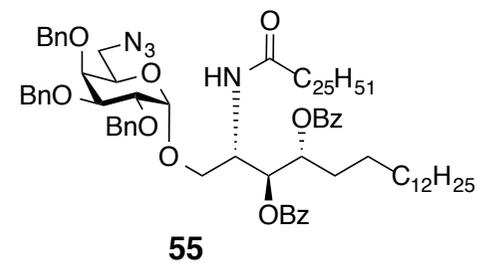
(2*S*,3*S*,4*R*)-3,4-Dibenzoyloxy-1-(2,3,4-tri-*O*-benzyl-6-deoxy-6-azido- α -D-galactopyranosyloxy)-2-hexacosanoylamido-octadecane (55)

^1H NMR, CDCl_3 , 500 MHz



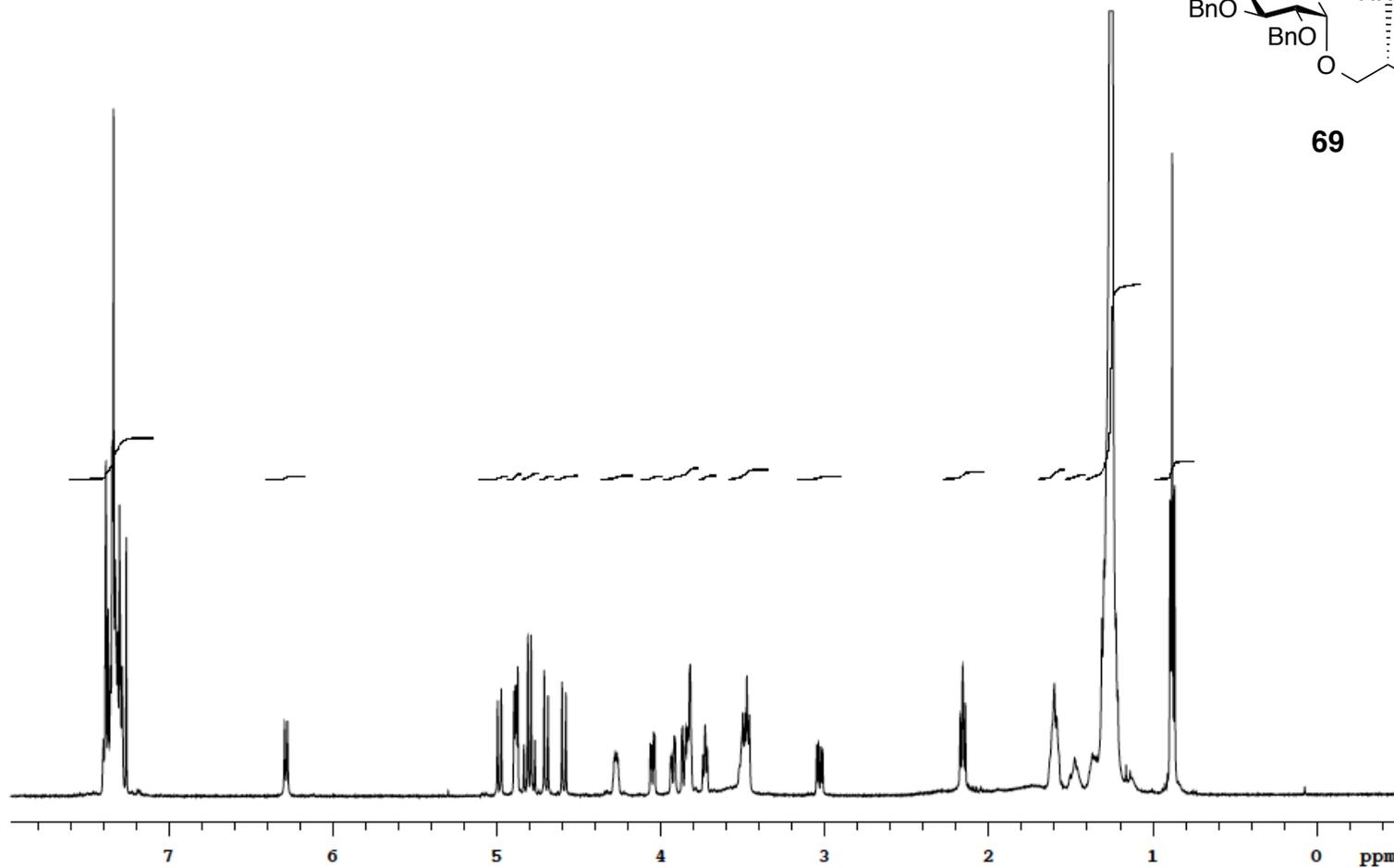
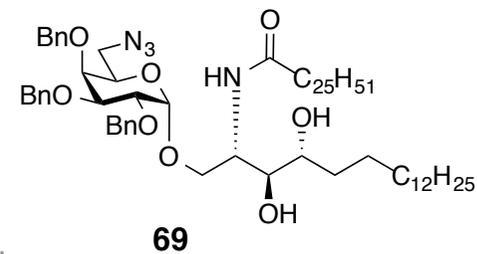
(2*S*,3*S*,4*R*)-3,4-Dibenzoyloxy-1-(2,3,4-tri-*O*-benzyl-6-deoxy-6-azido- α -D-galactopyranosyloxy)-2-hexacosanoylamido-octadecane (55)

^{13}C NMR, CDCl_3 , 125 MHz



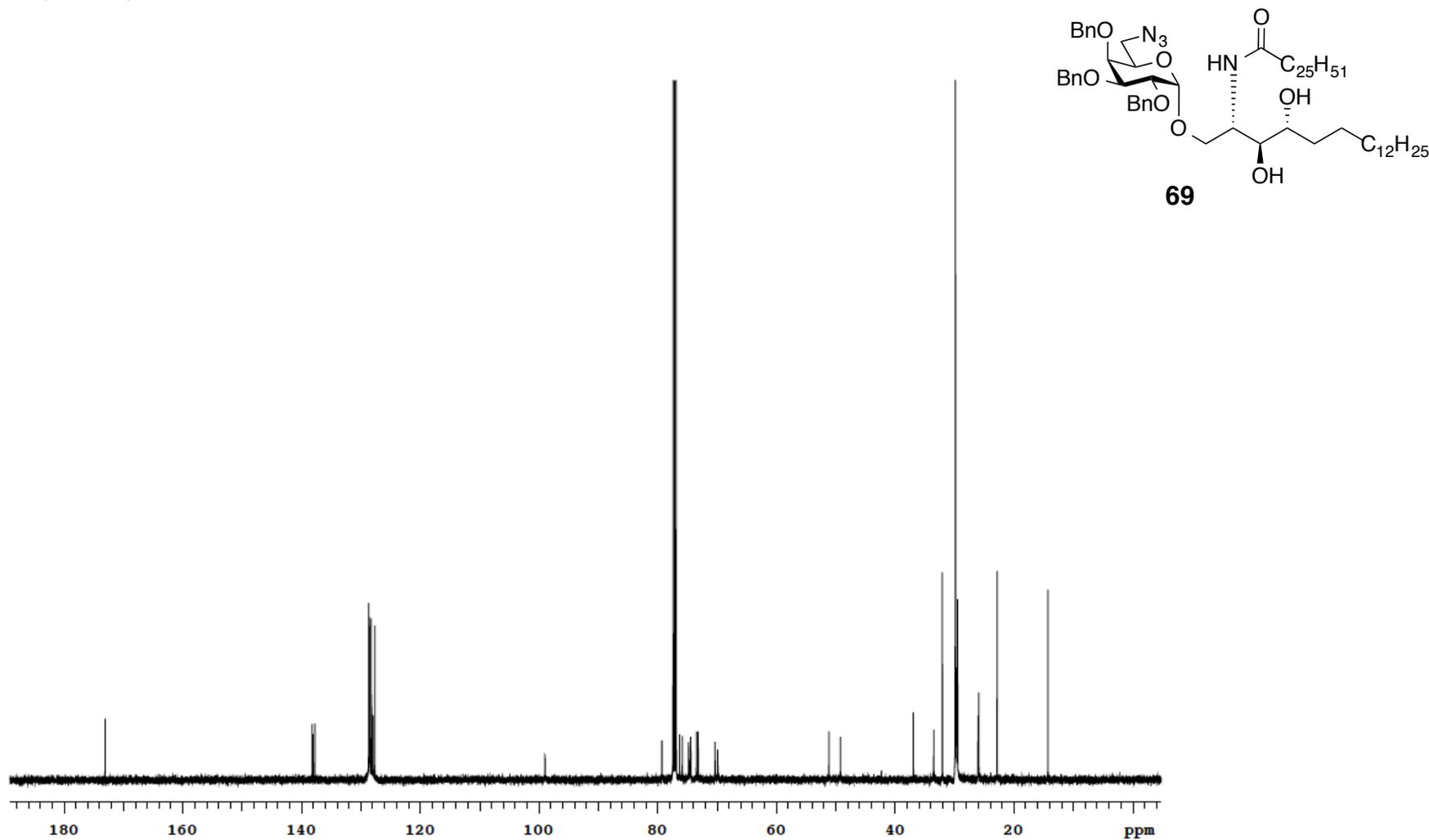
(2*S*,3*S*,4*R*)-2-Hexacosanoylamido-(2,3,4-tri-*O*-benzyl-6-deoxy-6-azido- α -D-galactopyranosyloxy)-octadecane-3,4-diol (69)

^1H NMR, CDCl_3 , 500 MHz



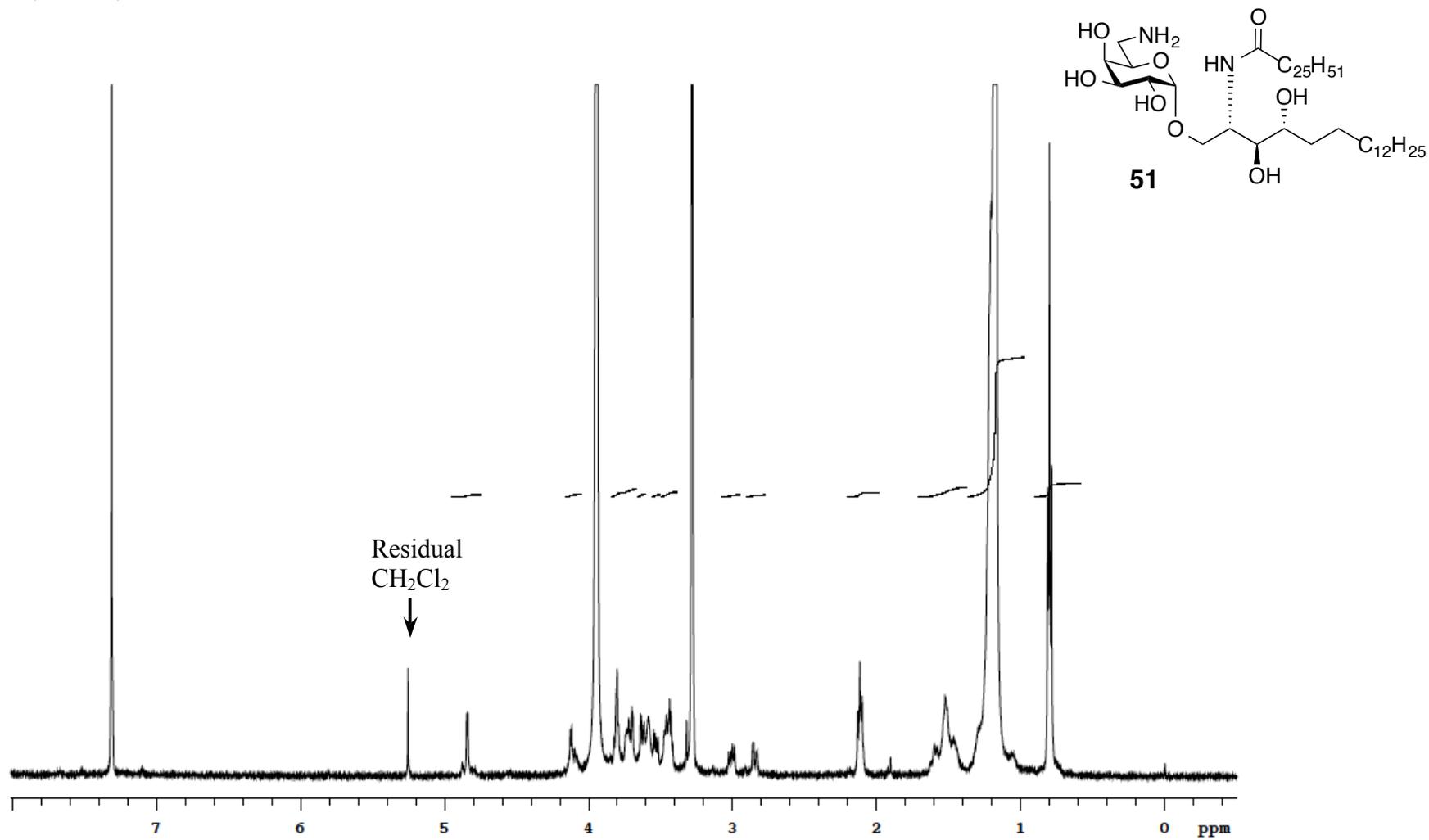
(2*S*,3*S*,4*R*)-2-Hexacosanoylamido-(2,3,4-tri-*O*-benzyl-6-deoxy-6-azido- α -D-galactopyranosyloxy)-octadecane-3,4-diol (69)

^{13}C NMR, CDCl_3 , 125 MHz

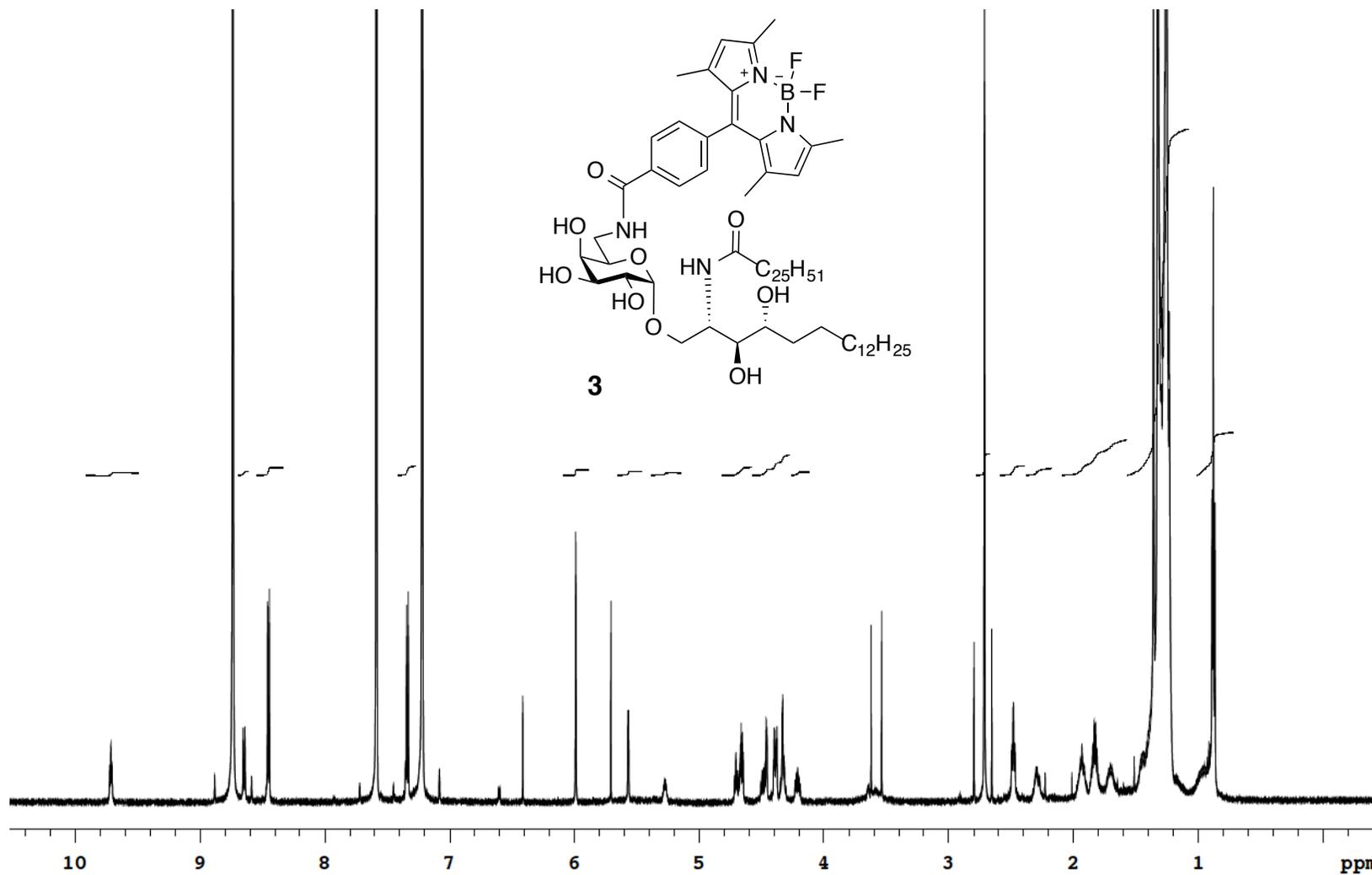


(2*S*,3*S*,4*R*)-1-(6-Amino-6-deoxy- α -D-galactopyranosyloxy)-2-hexacosanoylamido-octadecane-3,4-diol (51)

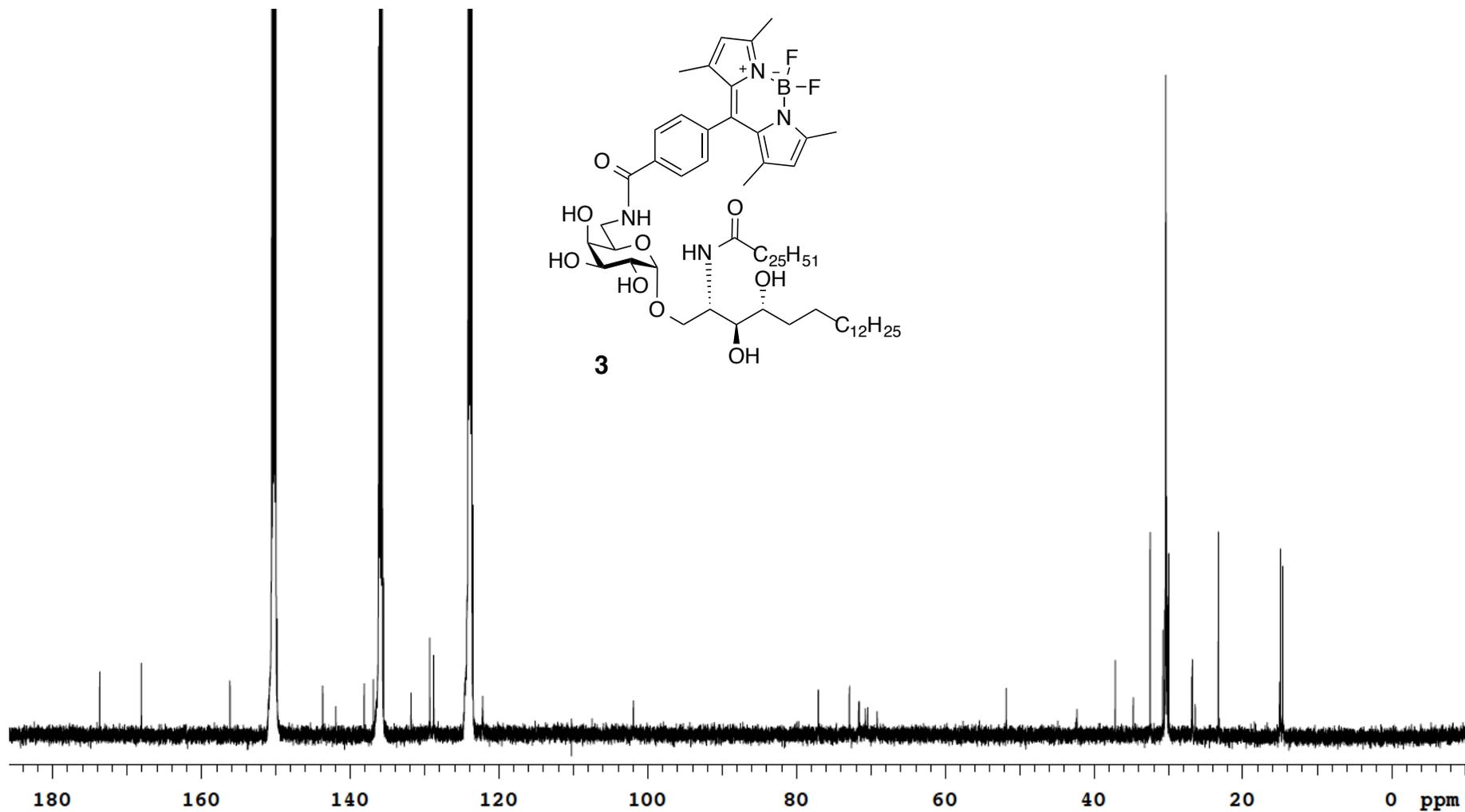
^1H NMR, CDCl_3 , 500 MHz



(2*S*,3*S*,4*R*)-1-(6-Deoxy-6-[4-(4,4-difluoro-1,3,5,7-tetramethyl-3a,4a-diaza-4-bora-*s*-indacen-8-yl)benzylamido])- α -D-galactopyranosyloxy)-2-hexacosanoylamido-octadecane-3,4-diol (3) ^1H NMR, pyridine- d_5 , 600 MHz



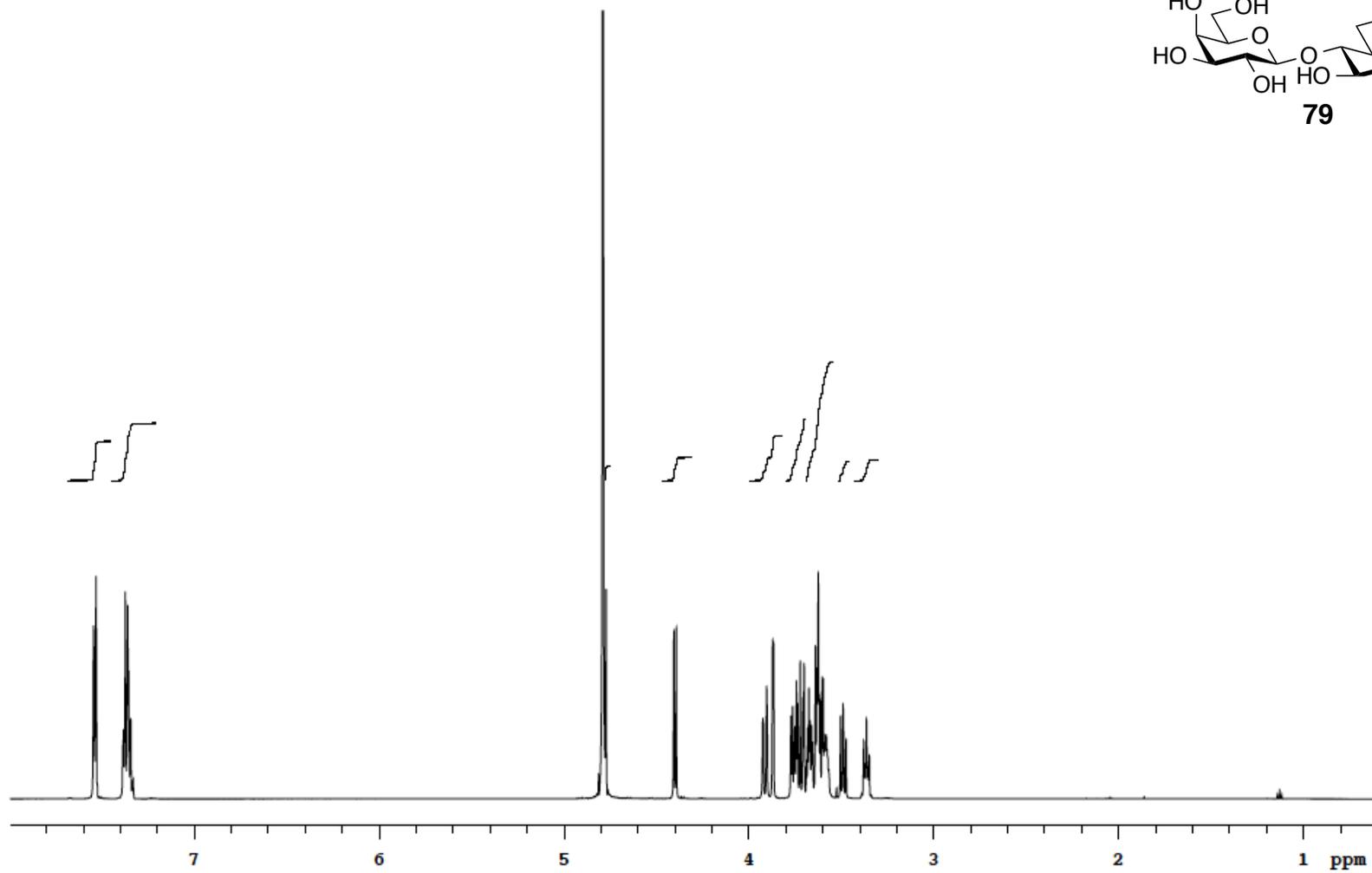
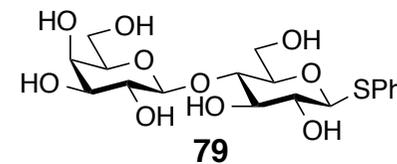
(2*S*,3*S*,4*R*)-1-(6-Deoxy-6-[4-(4,4-difluoro-1,3,5,7-tetramethyl-3a,4a-diaza-4-bora-*s*-indacen-8-yl)benzylamido])- α -D-galactopyranosyloxy)-2-hexacosanoylamido-octadecane-3,4-diol (**3**) ^{13}C NMR, pyridine- d_5 , 150 MHz



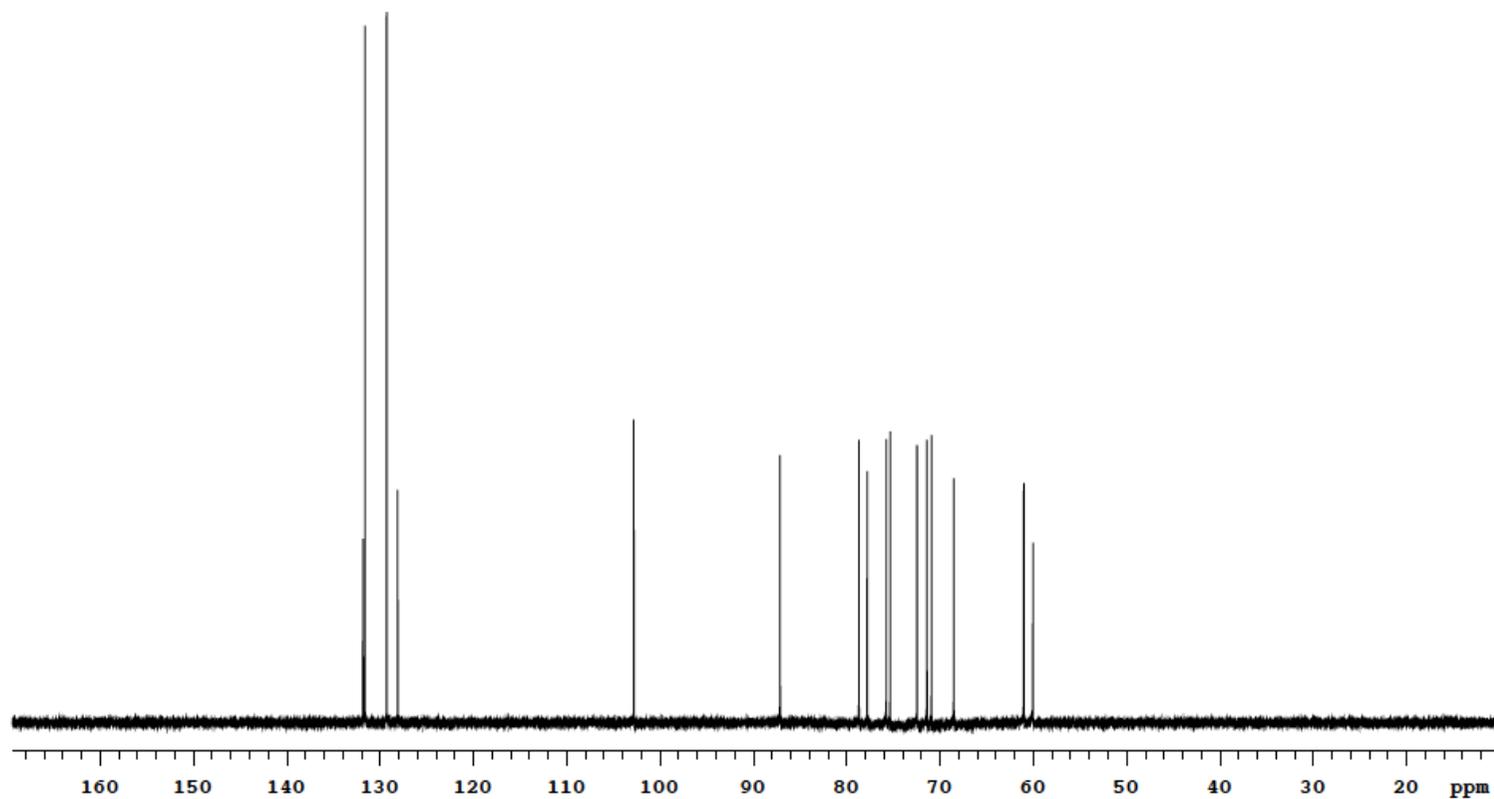
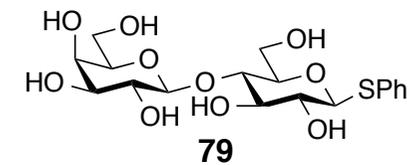
^1H & ^{13}C NMR Spectra

Chapter 4

Phenyl 4-*O*-(β -D-galactopyranosyl)-1-thio- β -D-glucopyranoside (**79**) ^1H NMR, D_2O , 600 MHz

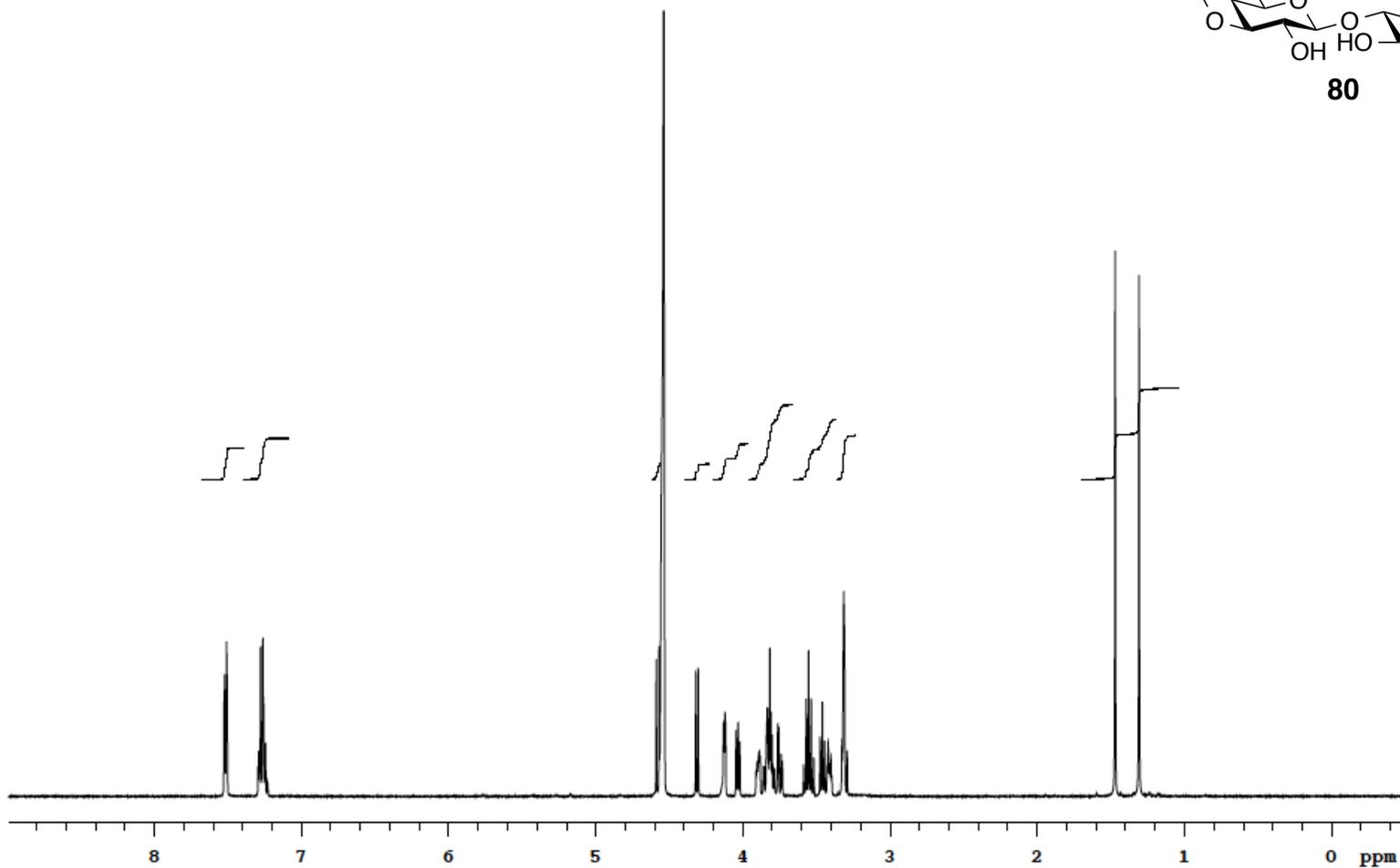
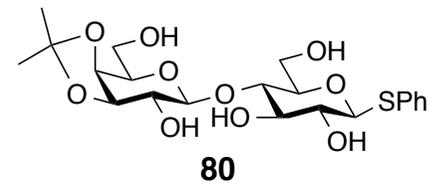


Phenyl 4-*O*-(β -D-galactopyranosyl)-1-thio- β -D-glucopyranoside (**79**) ^{13}C NMR, D_2O , 150 MHz



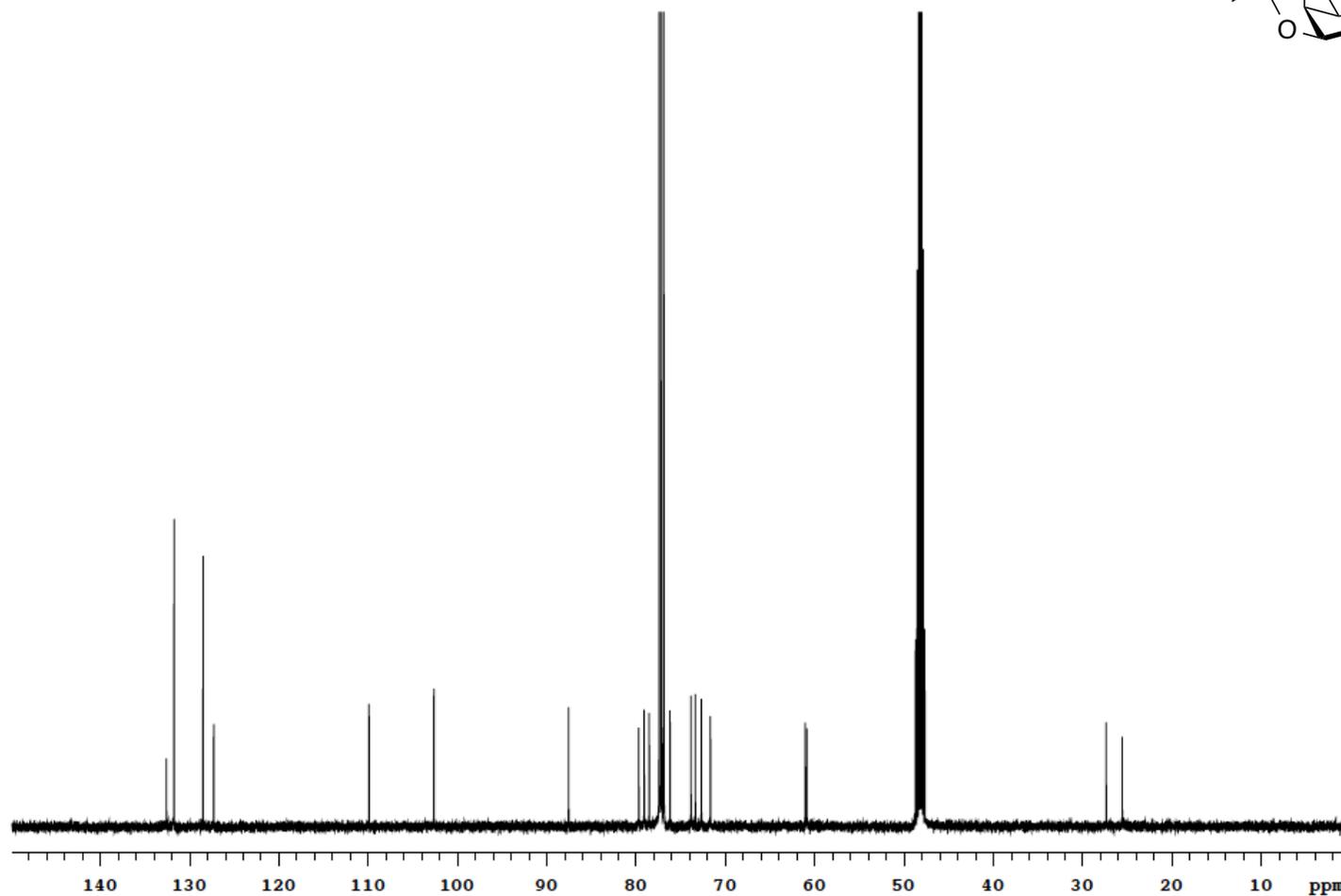
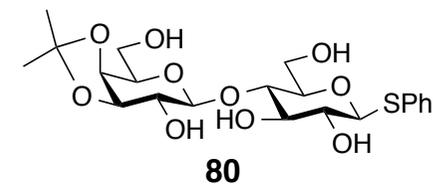
Phenyl 4-*O*-(3,4-*O*-isopropylidene- β -D-galactopyranosyl)-1-thio- β -D-glucopyranoside (80)

^1H NMR, $\text{CDCl}_3/\text{CD}_3\text{OD}$, 1/1, v/v, 500 MHz



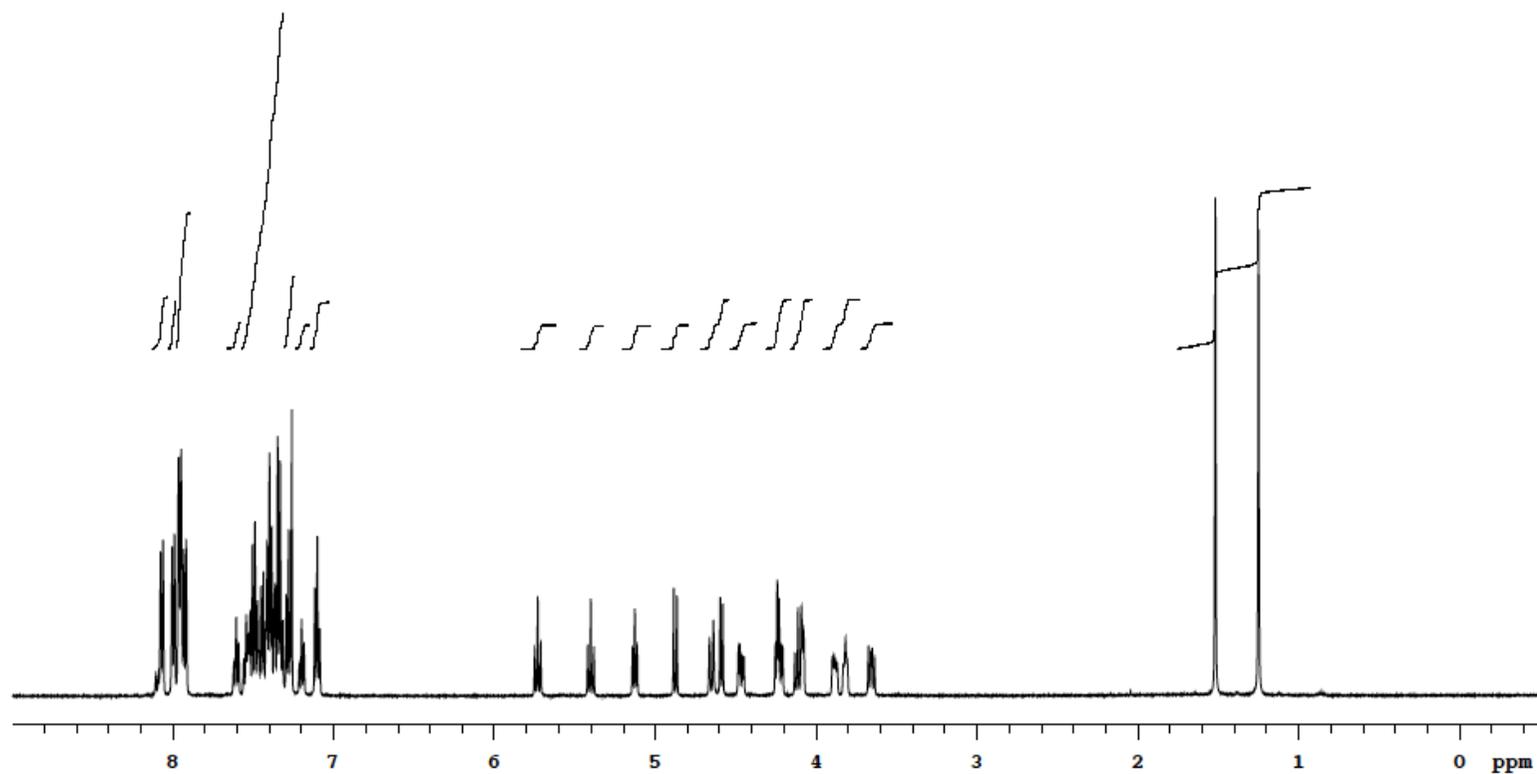
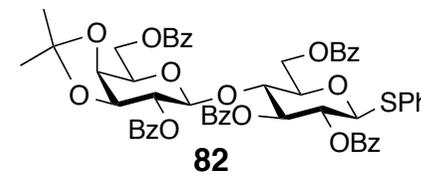
Phenyl 4-*O*-(3,4-*O*-isopropylidene- β -D-galactopyranosyl)-1-thio- β -D-glucopyranoside (80)

^{13}C NMR, $\text{CDCl}_3/\text{CD}_3\text{OD}$, 1/1, v/v, 125 MHz



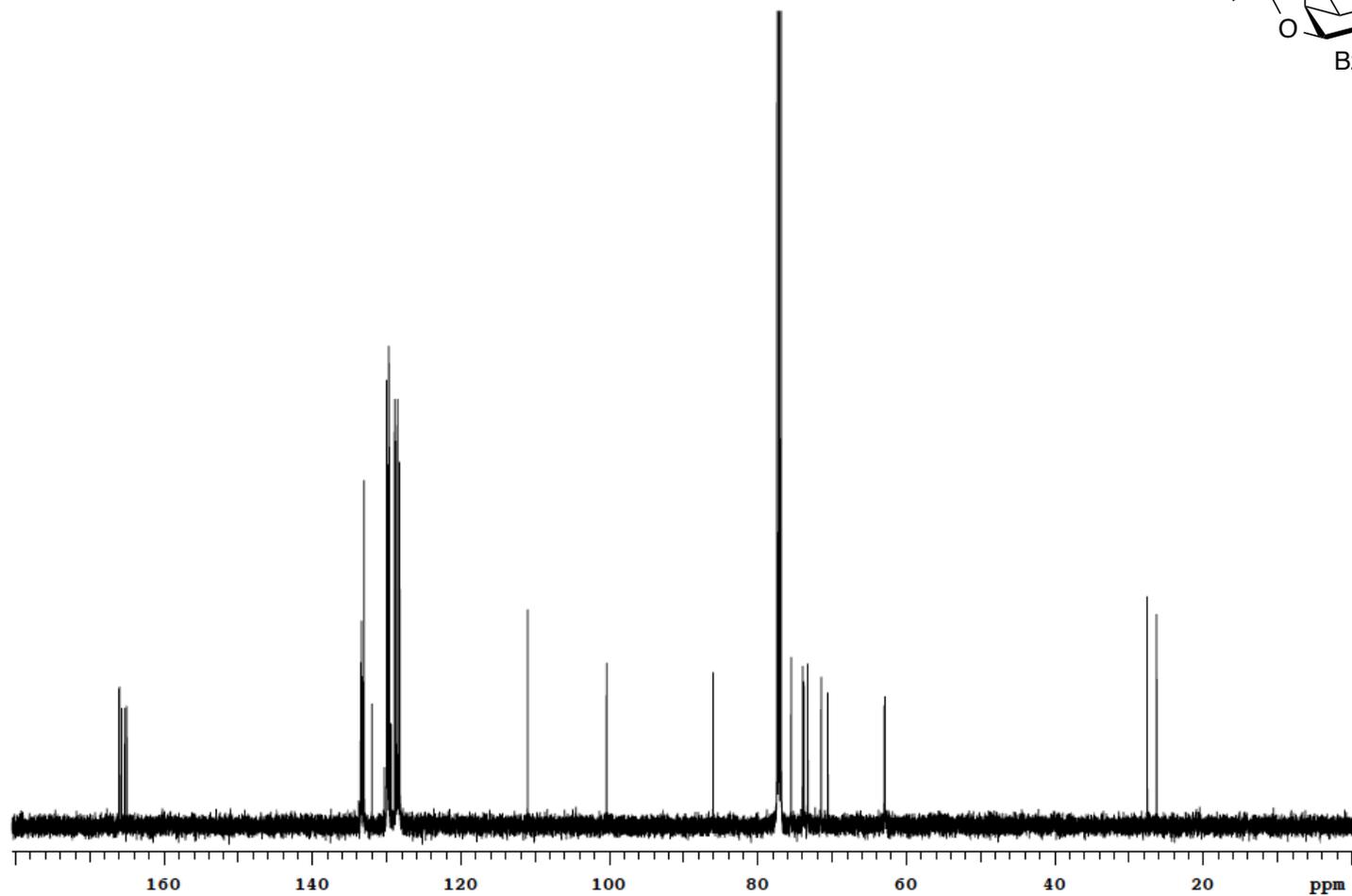
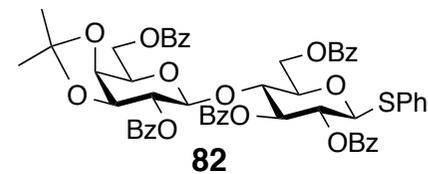
Phenyl 4-*O*-(2,6-di-*O*-benzoyl-3,4-*O*-isopropylidene- β -D-galactopyranosyl)-2,3,6-tri-*O*-benzoyl-1-thio- β -D-glucopyranoside (**82**)

^1H NMR, CDCl_3 , 500 MHz



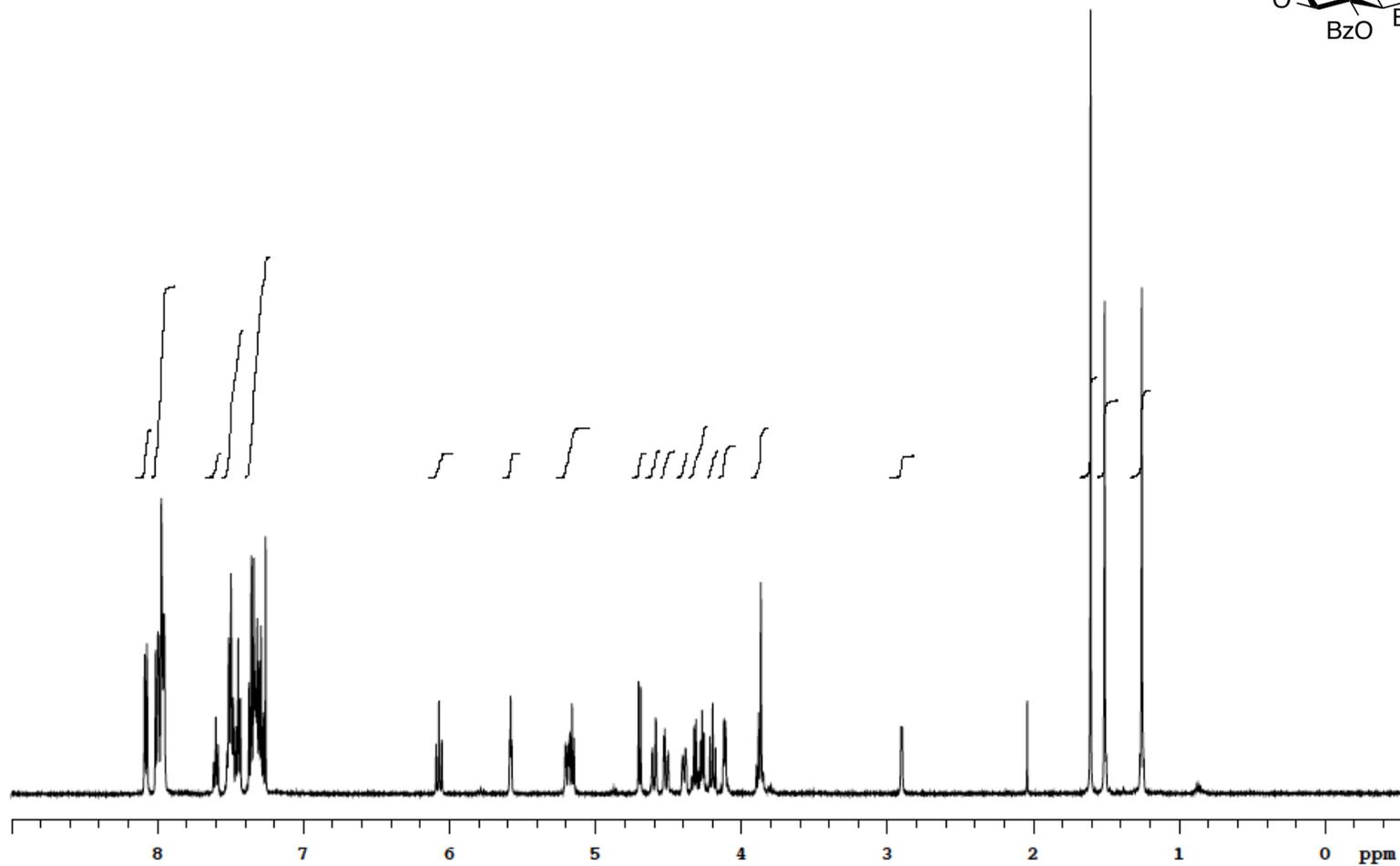
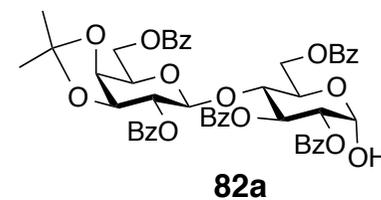
Phenyl 4-*O*-(2,6-di-*O*-benzoyl-3,4-*O*-isopropylidene- β -D-galactopyranosyl)-2,3,6-tri-*O*-benzoyl-1-thio- β -D-glucopyranoside (**82**)

^{13}C NMR, CDCl_3 , 125 MHz



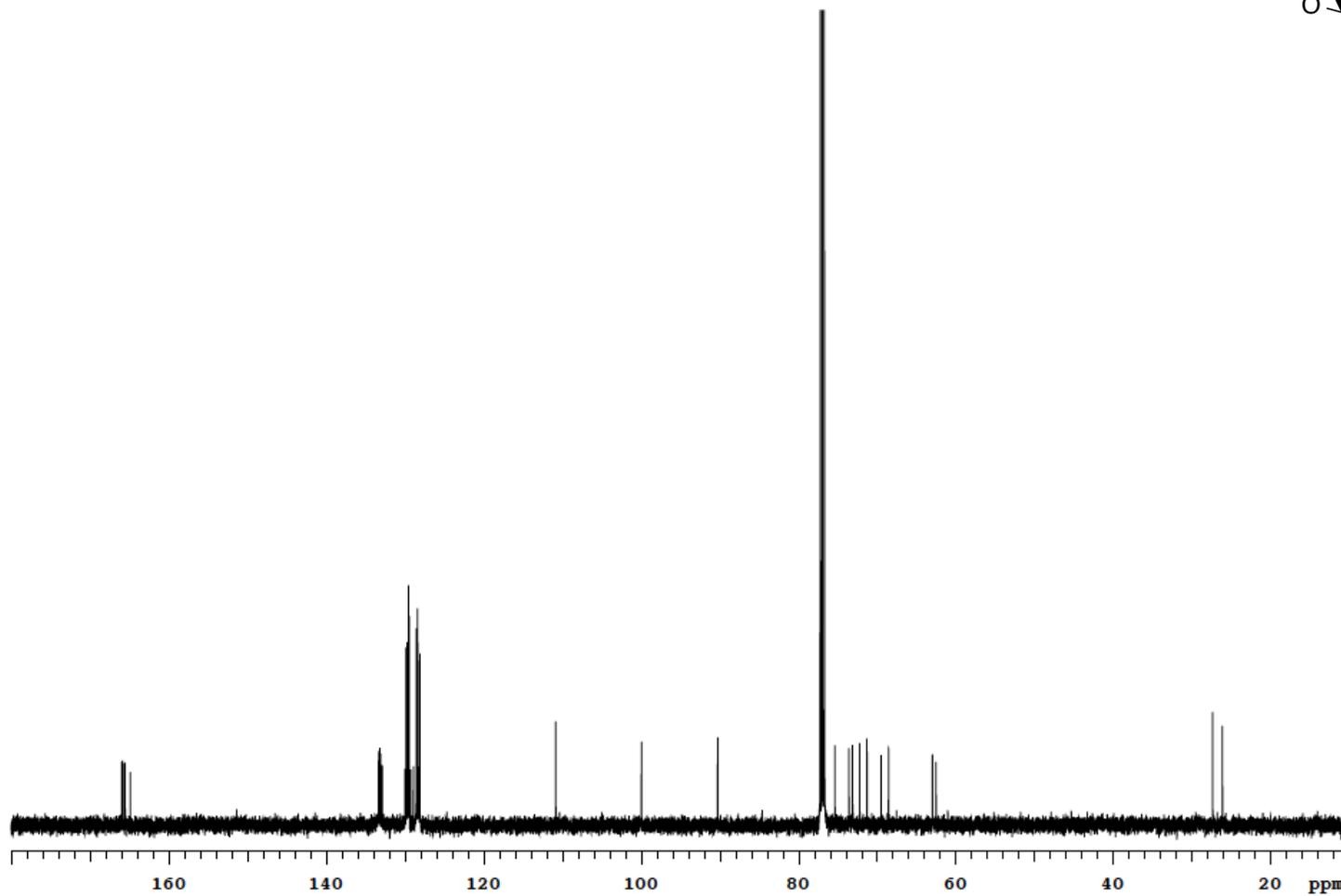
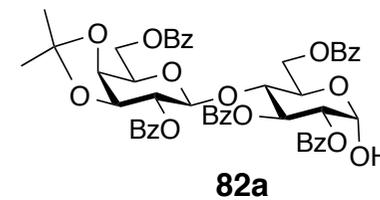
4-O-(2,6-Di-O-benzoyl-3,4-O-isopropylidene-β-D-galactopyranosyl-2,3,6-tri-O-benzoyl-α-D-glucopyranose (82a)

¹H NMR, CDCl₃, 500 MHz

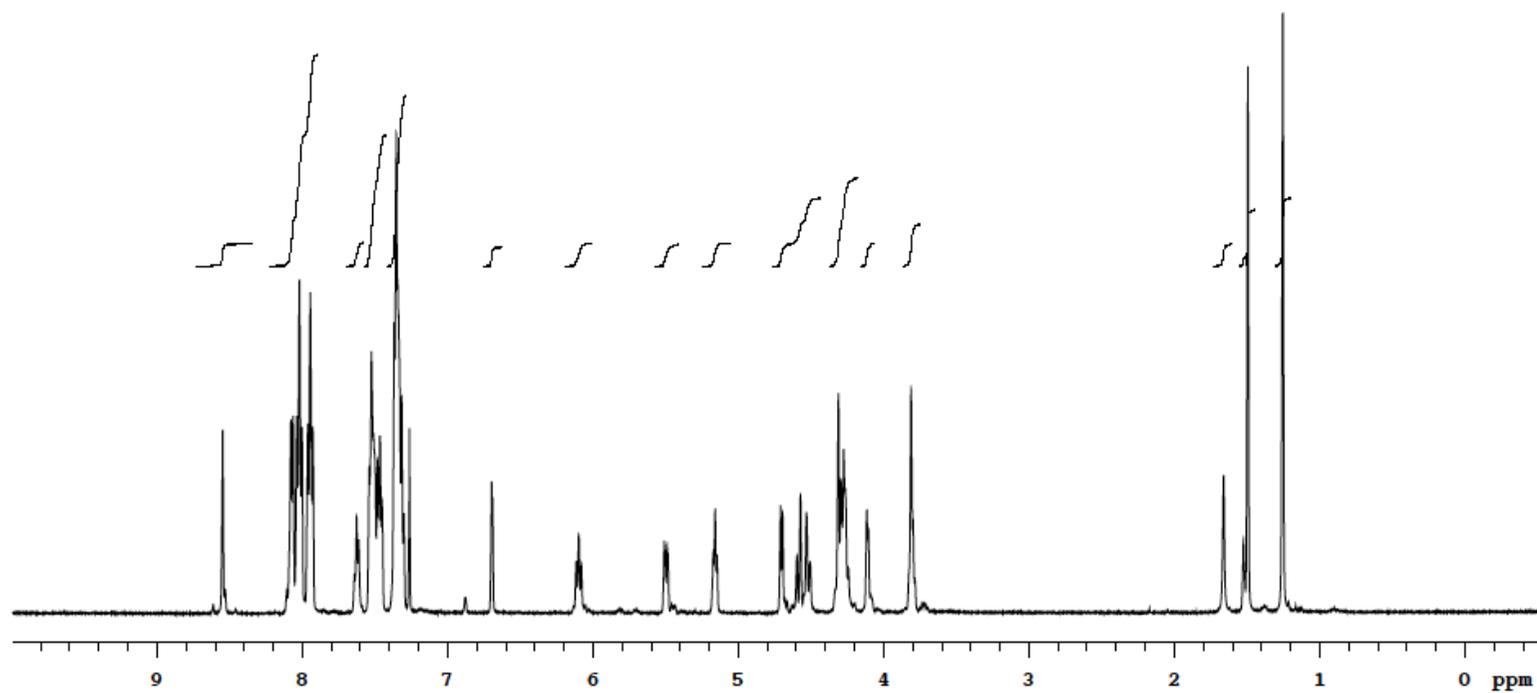
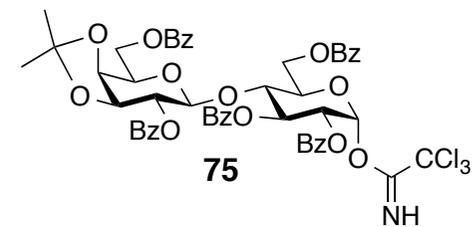


4-*O*-(2,6-Di-*O*-benzoyl-3,4-*O*-isopropylidene- β -D-galactopyranosyl-2,3,6-tri-*O*-benzoyl- α -D-glucopyranose (82a)

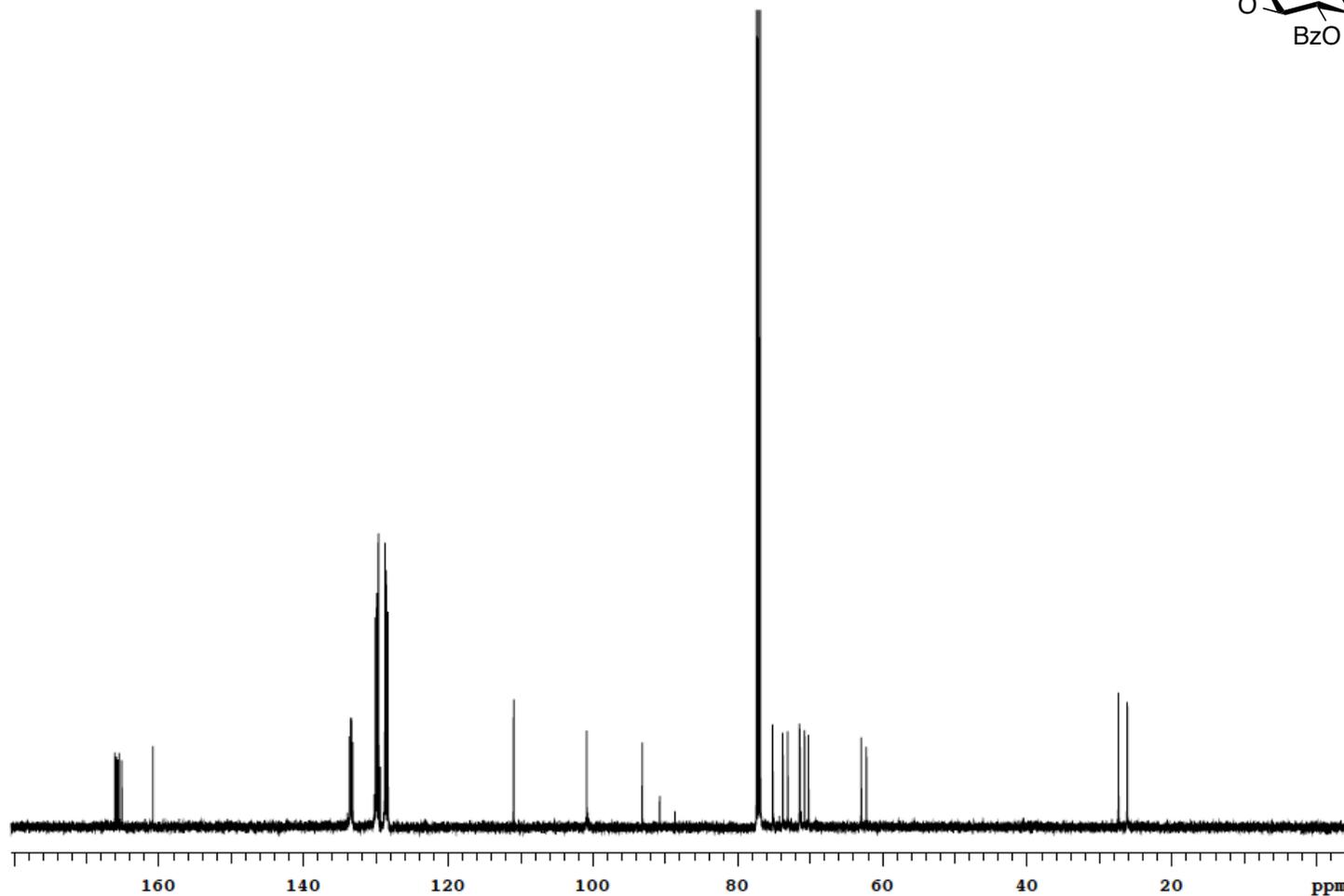
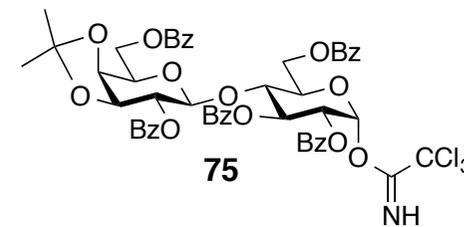
^{13}C NMR, CDCl_3 , 125 MHz



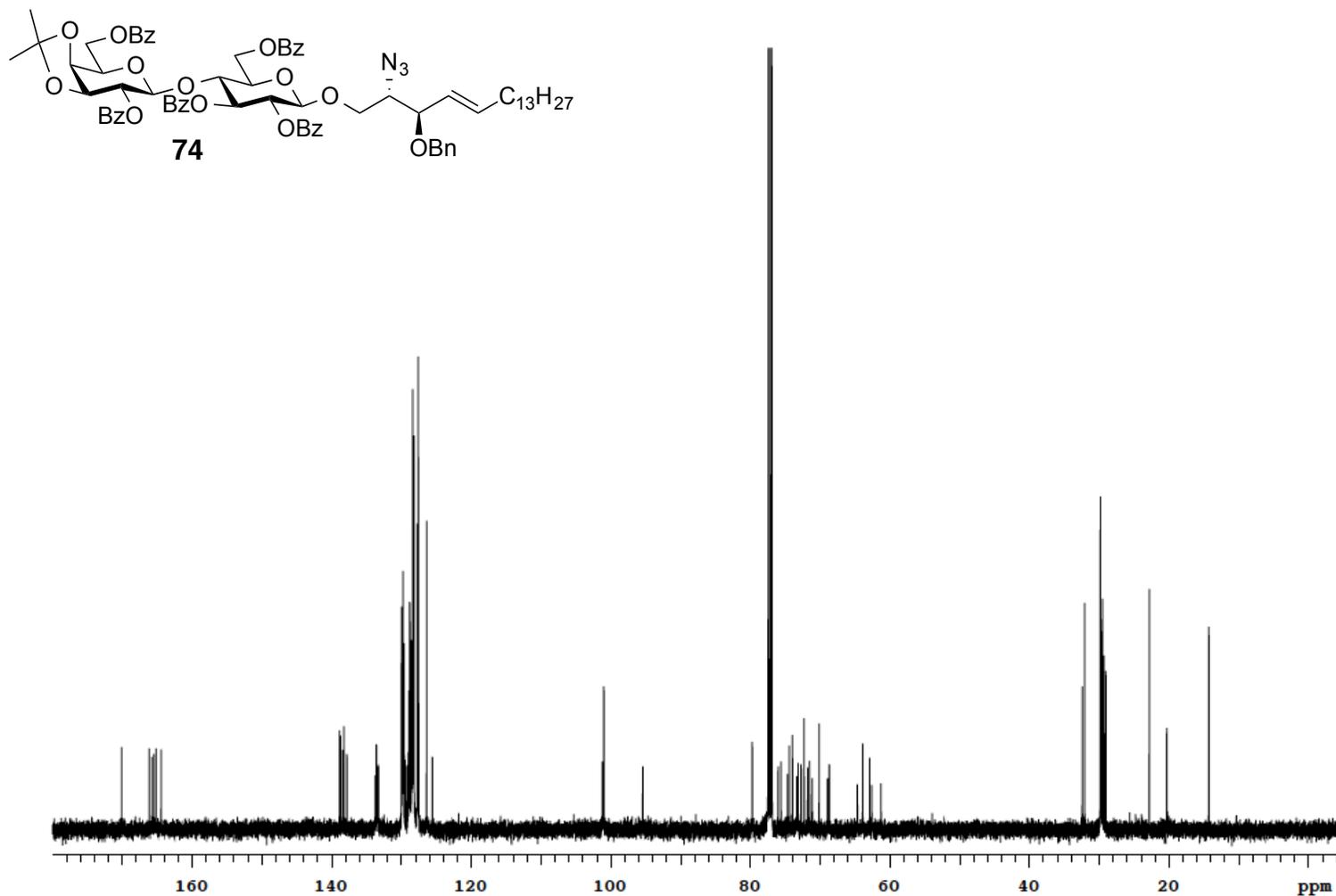
O-(4-*O*-(2,6-Di-*O*-benzoyl-3,4-*O*-isopropylidene- β -D-galactopyranosyl)-2,3,6-tri-*O*-benzoyl- α -D-glucopyranosyl) trichloroacetimidate (**75**) ^1H NMR, CDCl_3 , 500 MHz



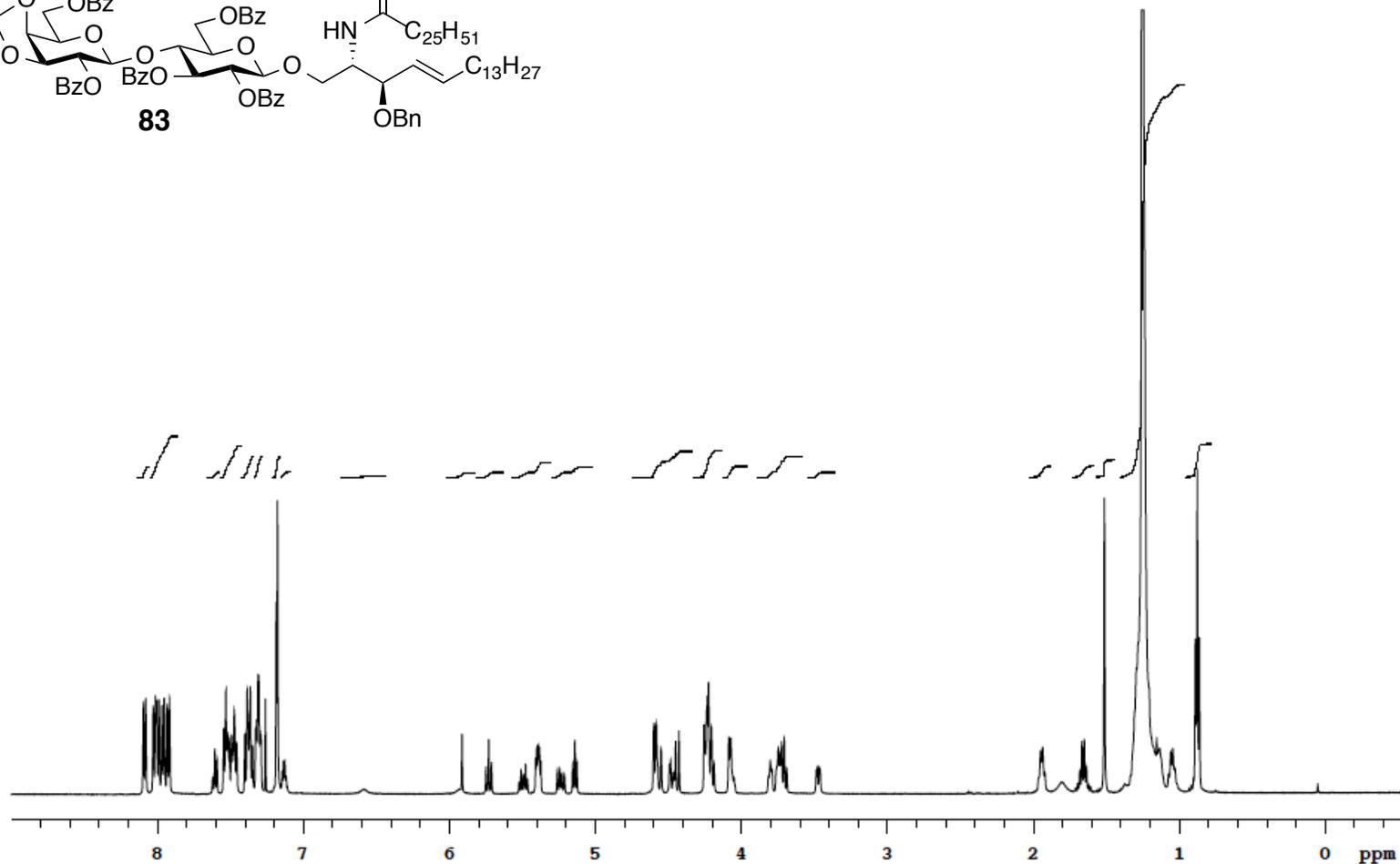
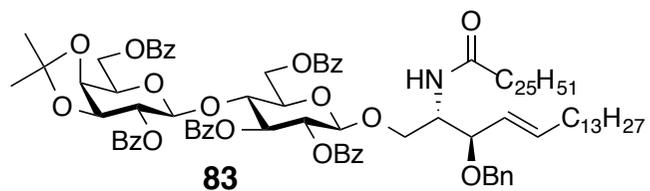
O-(4-*O*-(2,6-Di-*O*-benzoyl-3,4-*O*-isopropylidene- β -D-galactopyranosyl)-2,3,6-tri-*O*-benzoyl- α -D-glucopyranosyl) trichloroacetimidate (**75**) ^{13}C NMR, CDCl_3 , 125 MHz



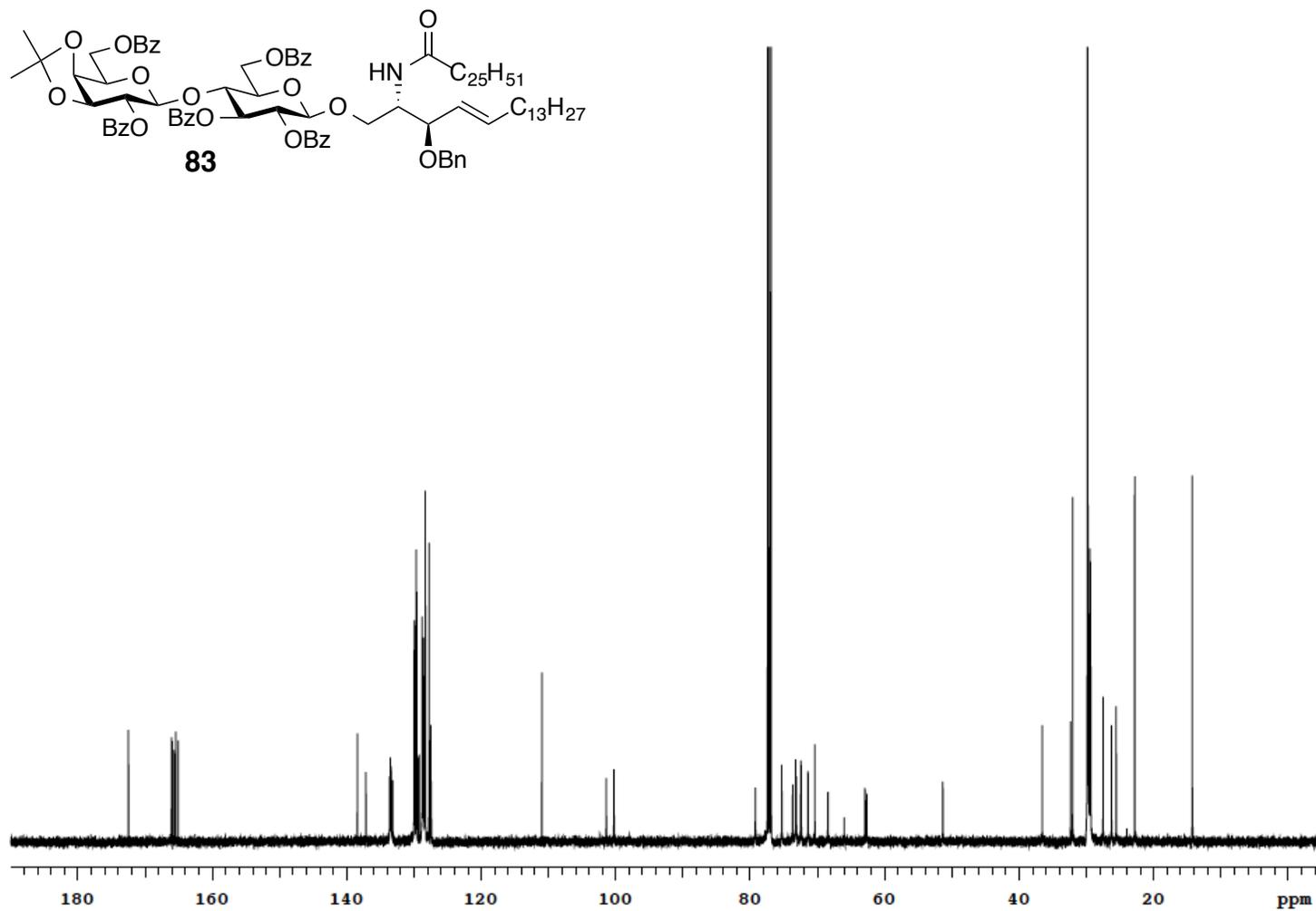
(2*S*,3*R*,4*E*)-2-Azido-1-(4-*O*-(2,6-di-*O*-benzoyl-3,4-*O*-isopropylidene- β -D-galactopyranosyl)-2,3,6-tri-*O*-benzoyl- β -D-glucopyranosyloxy)-3-benzyloxy-octadec-4-ene (**74**) ^{13}C NMR, CDCl_3 , 125 MHz



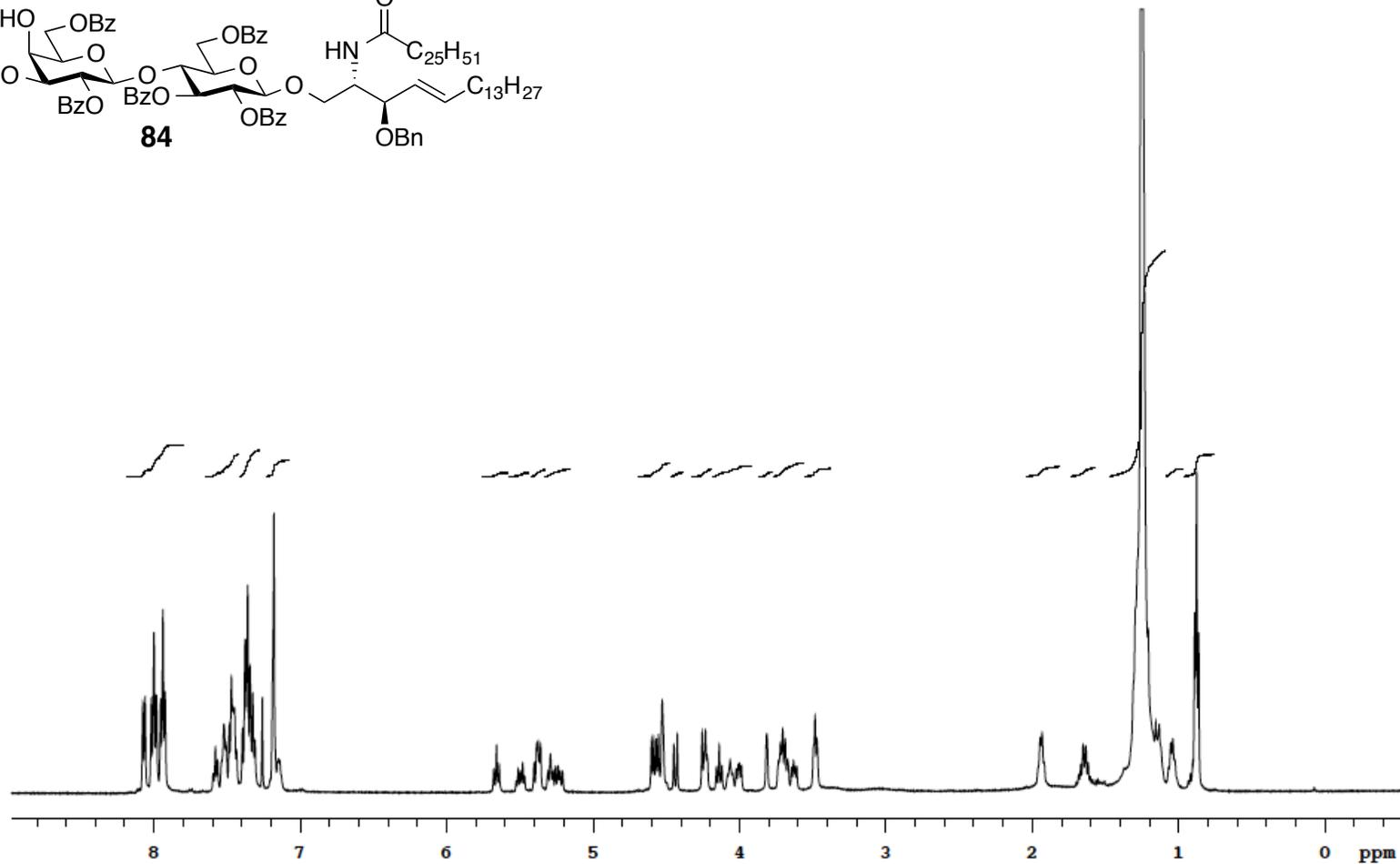
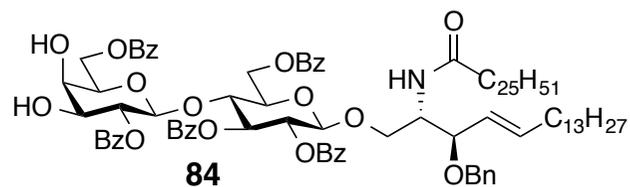
(2*S*,3*R*,4*E*)-1-(4-*O*-(2,6-Di-*O*-benzoyl-3,4-*O*-isopropylidene- β -D-galactopyranosyl)-2,3,6-tri-*O*-benzoyl- β -D-glucopyranosyloxy)-3-benzyloxy-2-hexacosanoylamido-octadecen-4-ene (**83**) ^1H NMR, CDCl_3 , 500 MHz



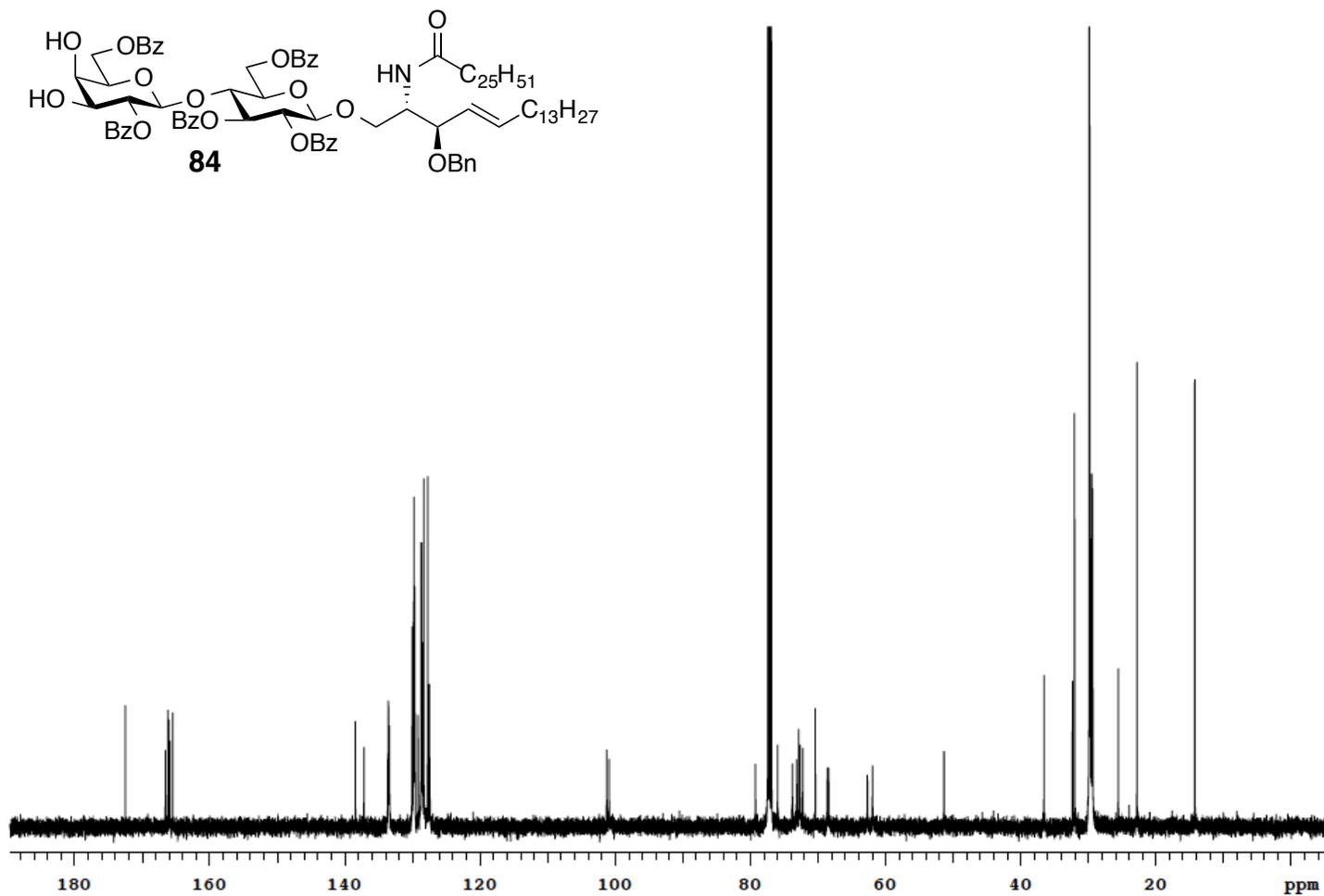
(2*S*,3*R*,4*E*)-1-(4-*O*-(2,6-Di-*O*-benzoyl-3,4-*O*-isopropylidene- β -D-galactopyranosyl)-2,3,6-tri-*O*-benzoyl- β -D-glucopyranosyloxy)-3-benzyloxy-2-hexacosanoylamido-octadecen-4-ene (**83**) ^{13}C NMR, CDCl_3 , 125 MHz



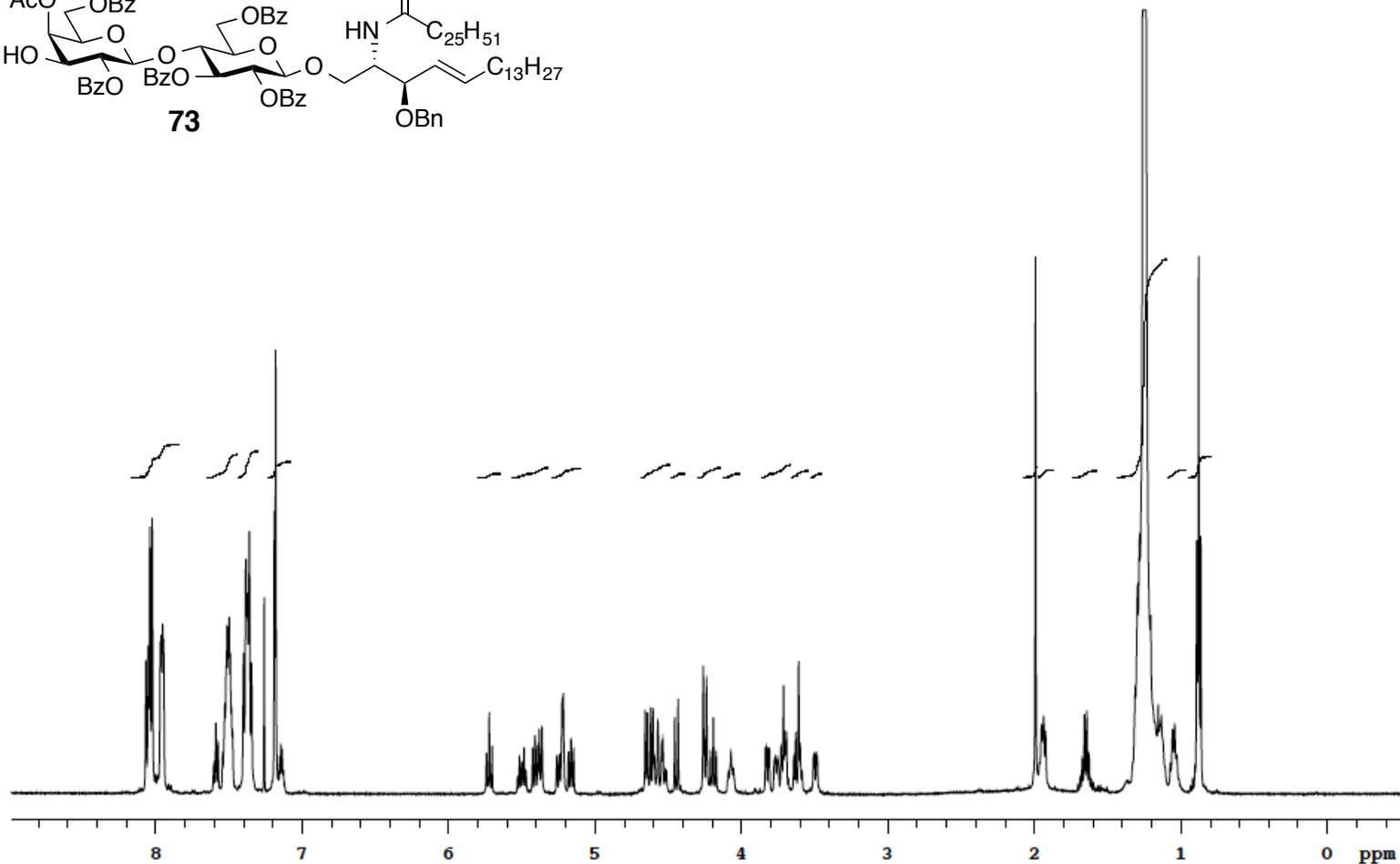
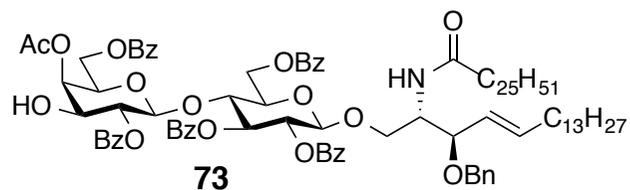
(2*S*,3*R*,4*E*)-1-(4-*O*-(2,6-Di-*O*-benzoyl- β -D-galactopyranosyl)-2,3,6-tri-*O*-benzoyl- β -D-glucopyranosyloxy)-3-benzyloxy-2-hexacosanoylamido-octadec-4-ene (**84**) ^1H NMR, CDCl_3 , 500 MHz



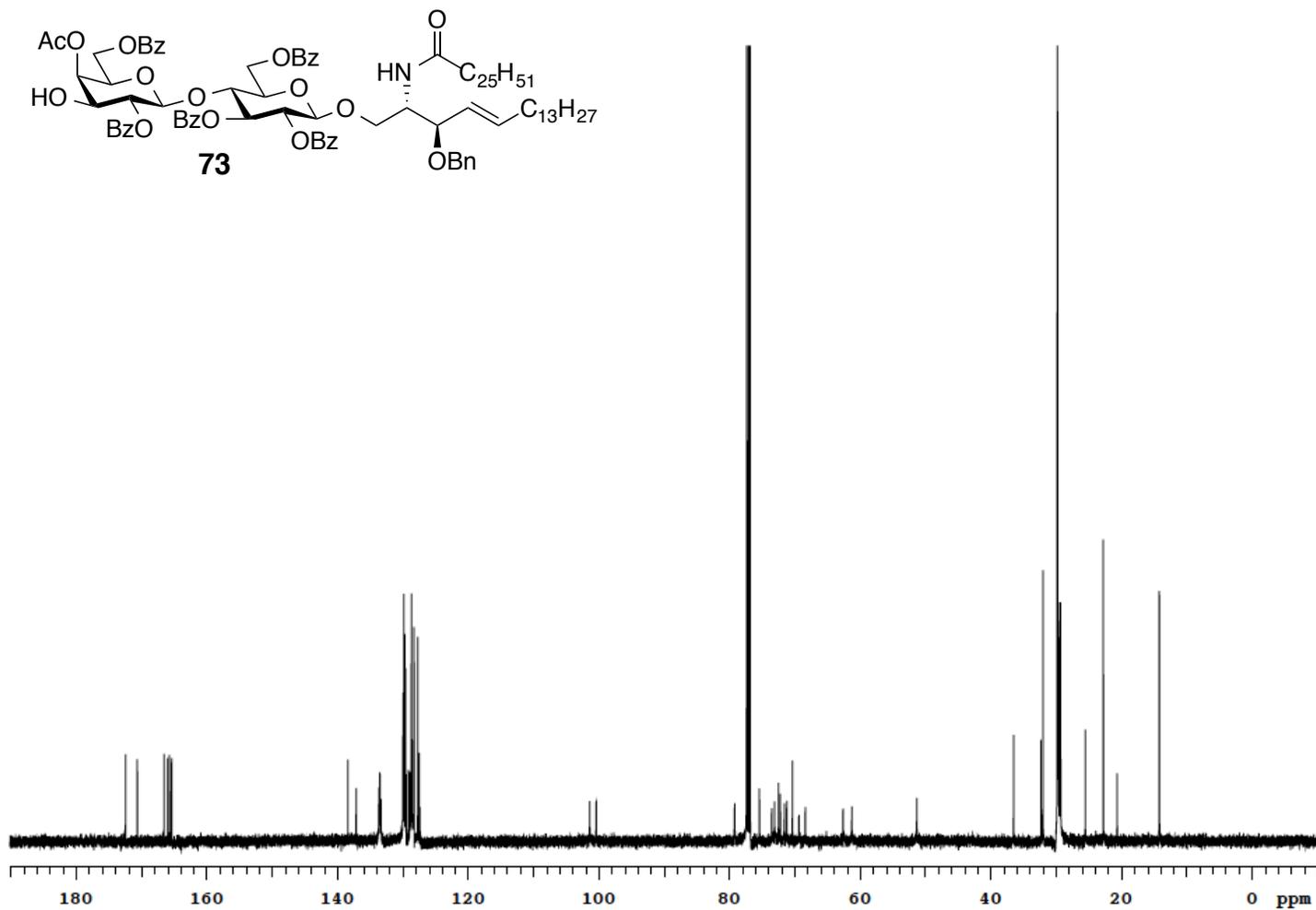
(2*S*,3*R*,4*E*)-1-(4-*O*-(2,6-Di-*O*-benzoyl- β -D-galactopyranosyl)-2,3,6-tri-*O*-benzoyl- β -D-glucopyranosyloxy)-3-benzyloxy-2-hexacosanoylamido-octadec-4-ene (**84**) ^{13}C NMR, CDCl_3 , 125 MHz



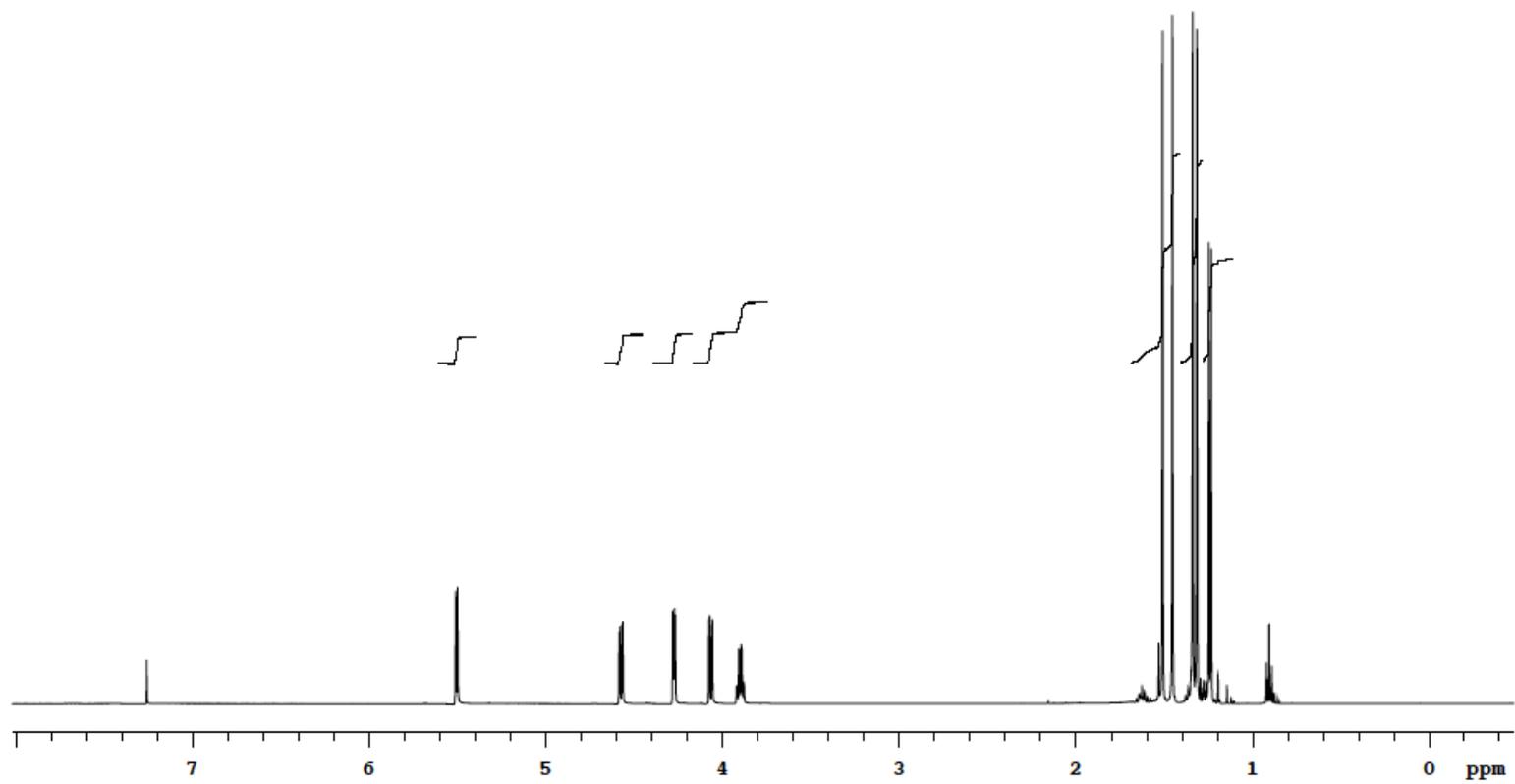
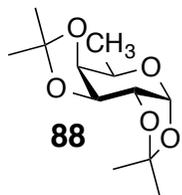
(2*S*,3*R*,4*E*)-1-(4-*O*-(4-*O*-Acetyl-2,6-di-*O*-benzoyl- β -D-galactopyranosyl)-2,3,6-tri-*O*-benzoyl- β -D-glucopyranosyloxy)-3-benzyloxy-2-hexacosanoylamido-octadec-4-ene (73) ^1H NMR, CDCl_3 , 500 MHz



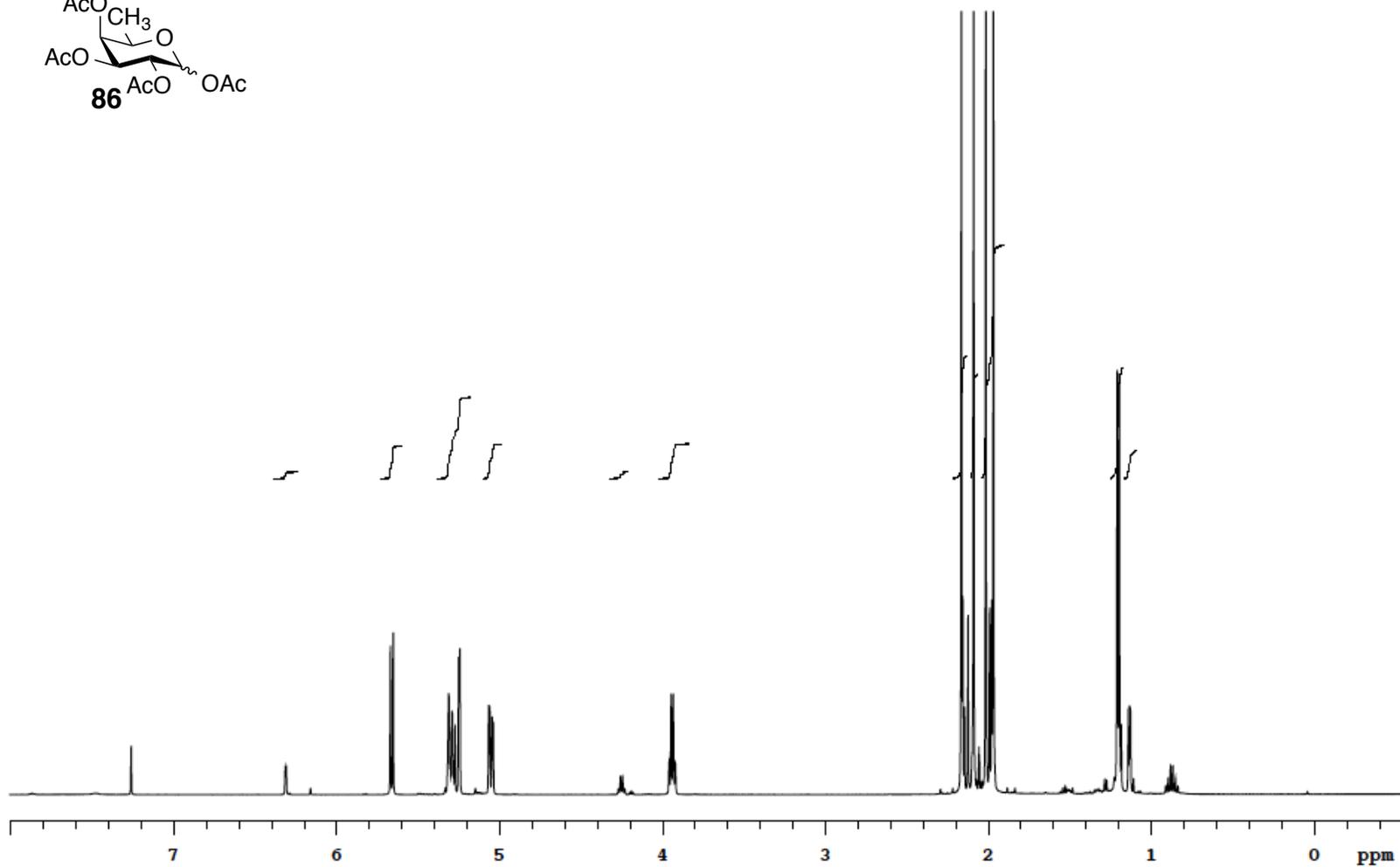
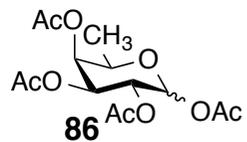
(2*S*,3*R*,4*E*)-1-(4-*O*-(4-*O*-Acetyl-2,6-di-*O*-benzoyl- β -D-galactopyranosyl)-2,3,6-tri-*O*-benzoyl- β -D-glucopyranosyloxy)-3-benzyloxy-2-hexacosanoylamido-octadec-4-ene (**73**) ^{13}C NMR, CDCl_3 , 125 MHz



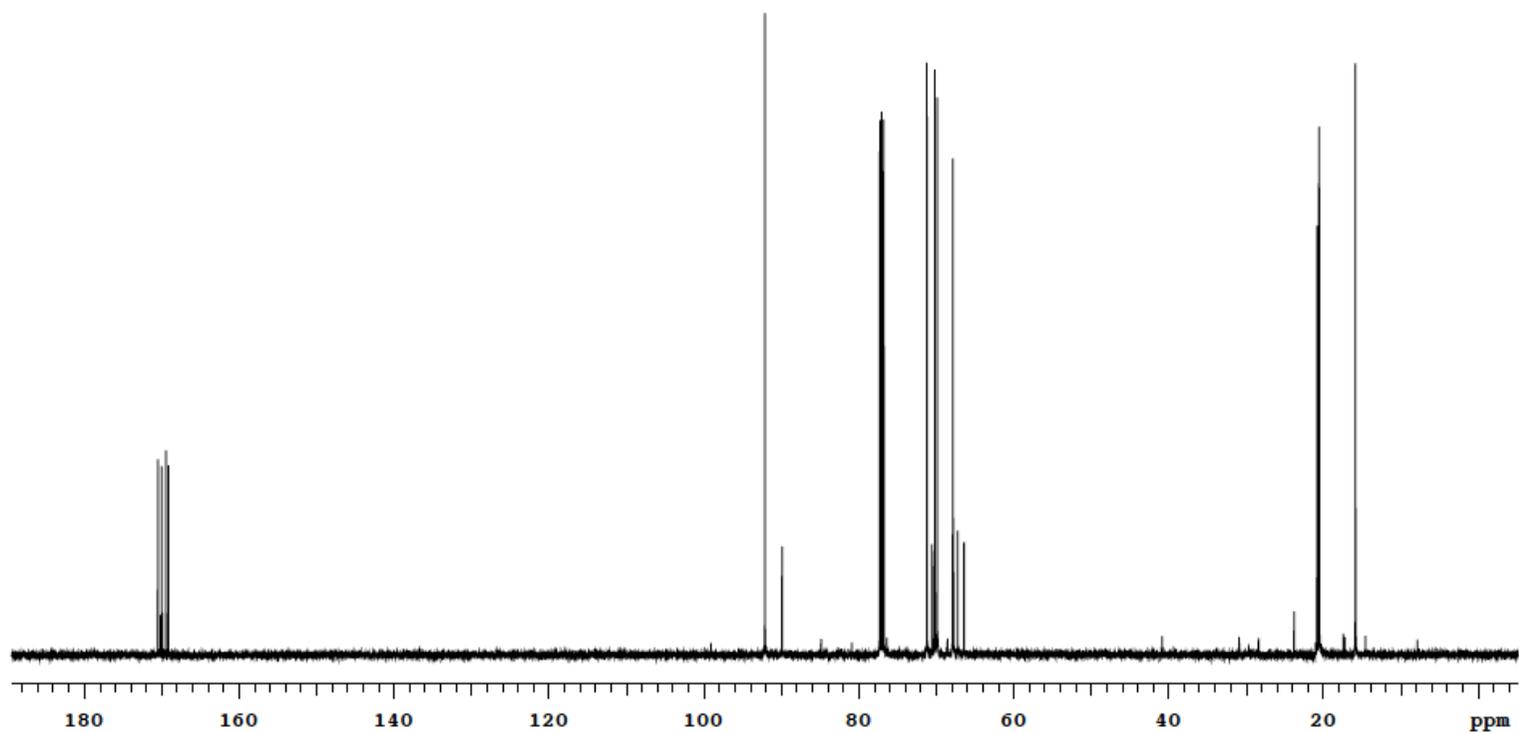
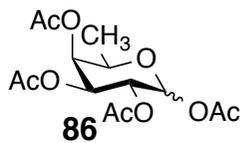
6-Deoxy-1,2:3,4-di-*O*-isopropylidene- α -D-galactopyranose (**88**) ^1H NMR, CDCl_3 , 500 MHz



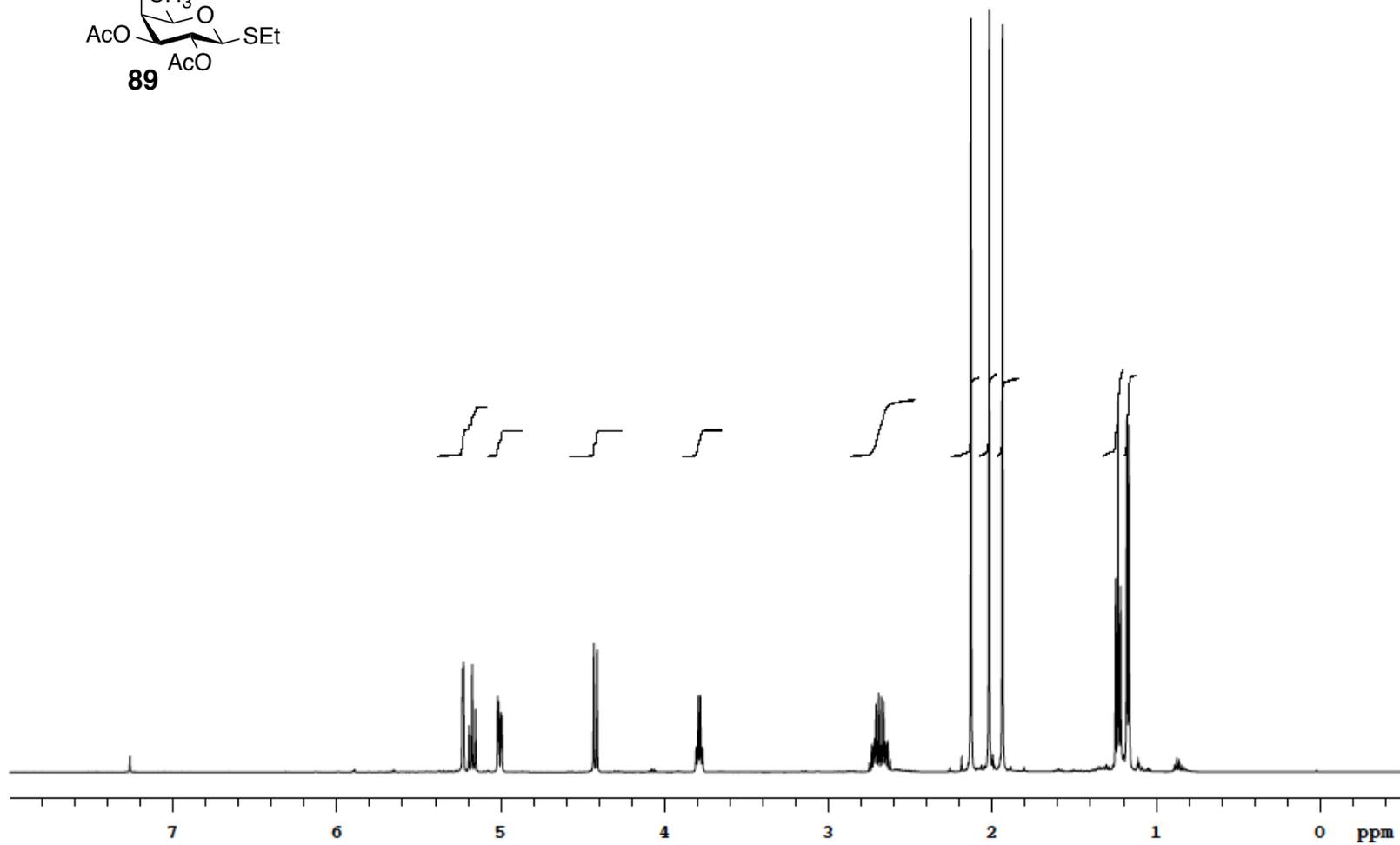
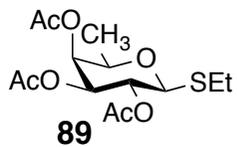
1,2,3,4-Tetra-*O*-acetyl-6-deoxy- α/β -D-galactopyranose (**86**) ^1H NMR, CDCl_3 , 500 MHz



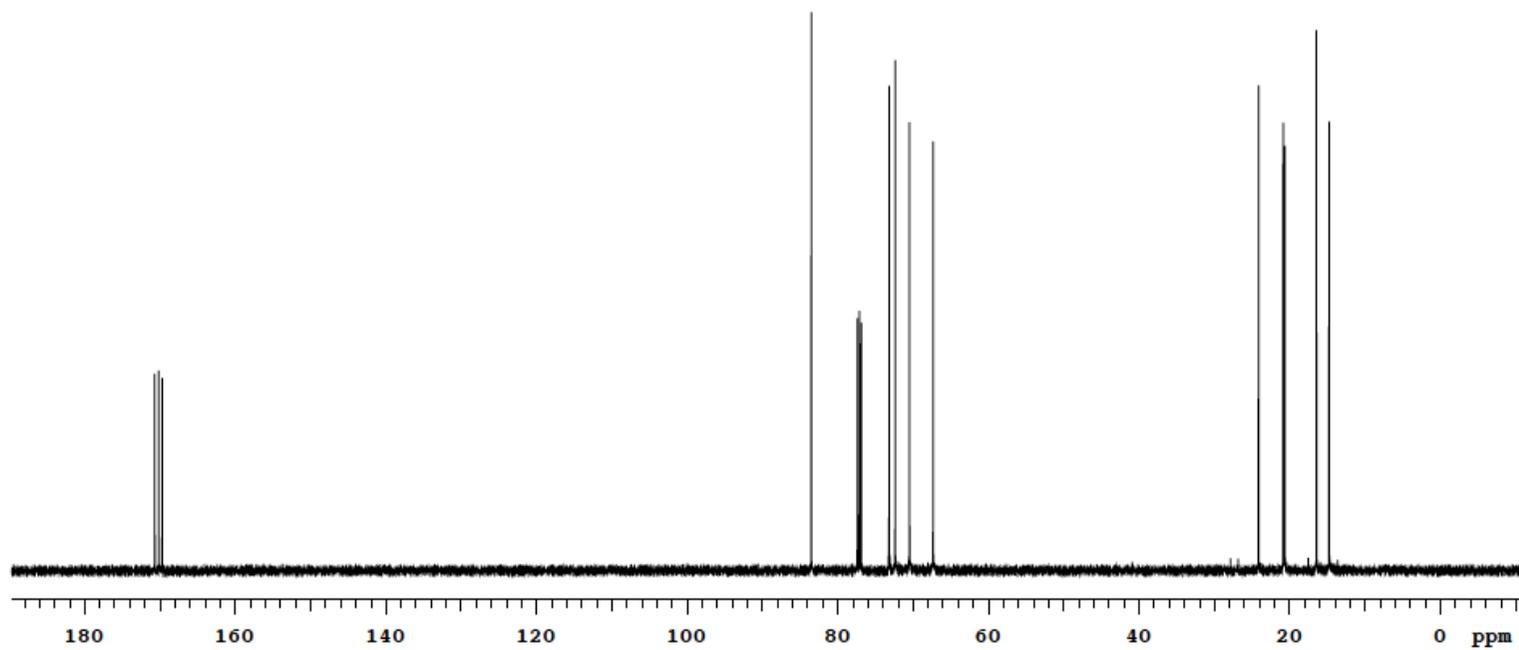
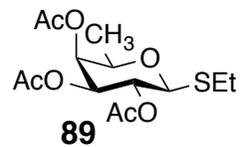
1,2,3,4-Tetra-*O*-acetyl-6-deoxy- α/β -D-galactopyranose (**86**) ^{13}C NMR, CDCl_3 , 125 MHz



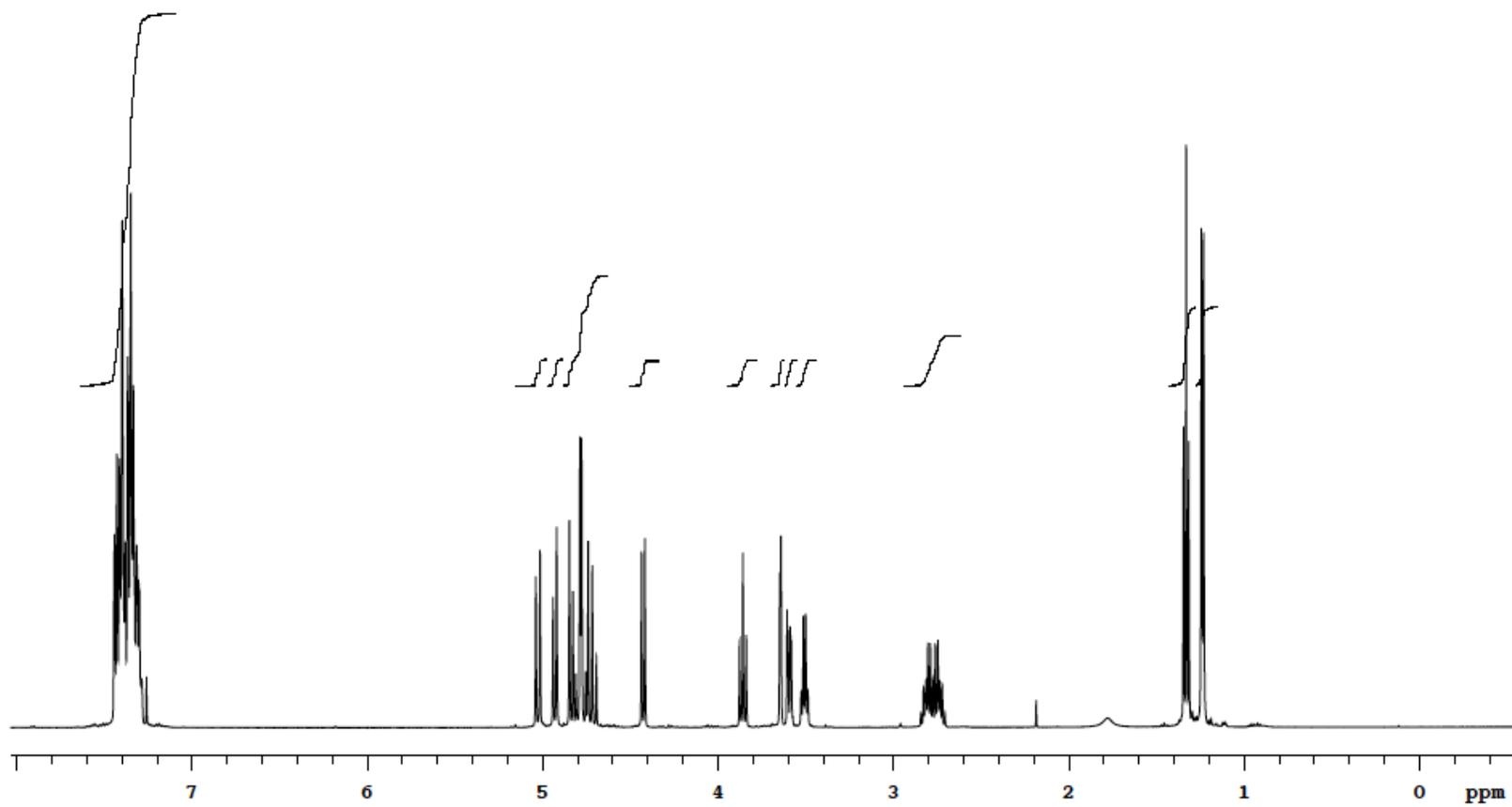
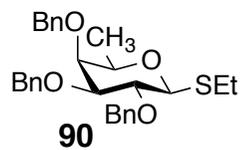
Ethyl 2,3,4-tri-*O*-acetyl-6-deoxy-1-thio- β -D-galactopyranoside (**89**) ^1H NMR, CDCl_3 , 500 MHz



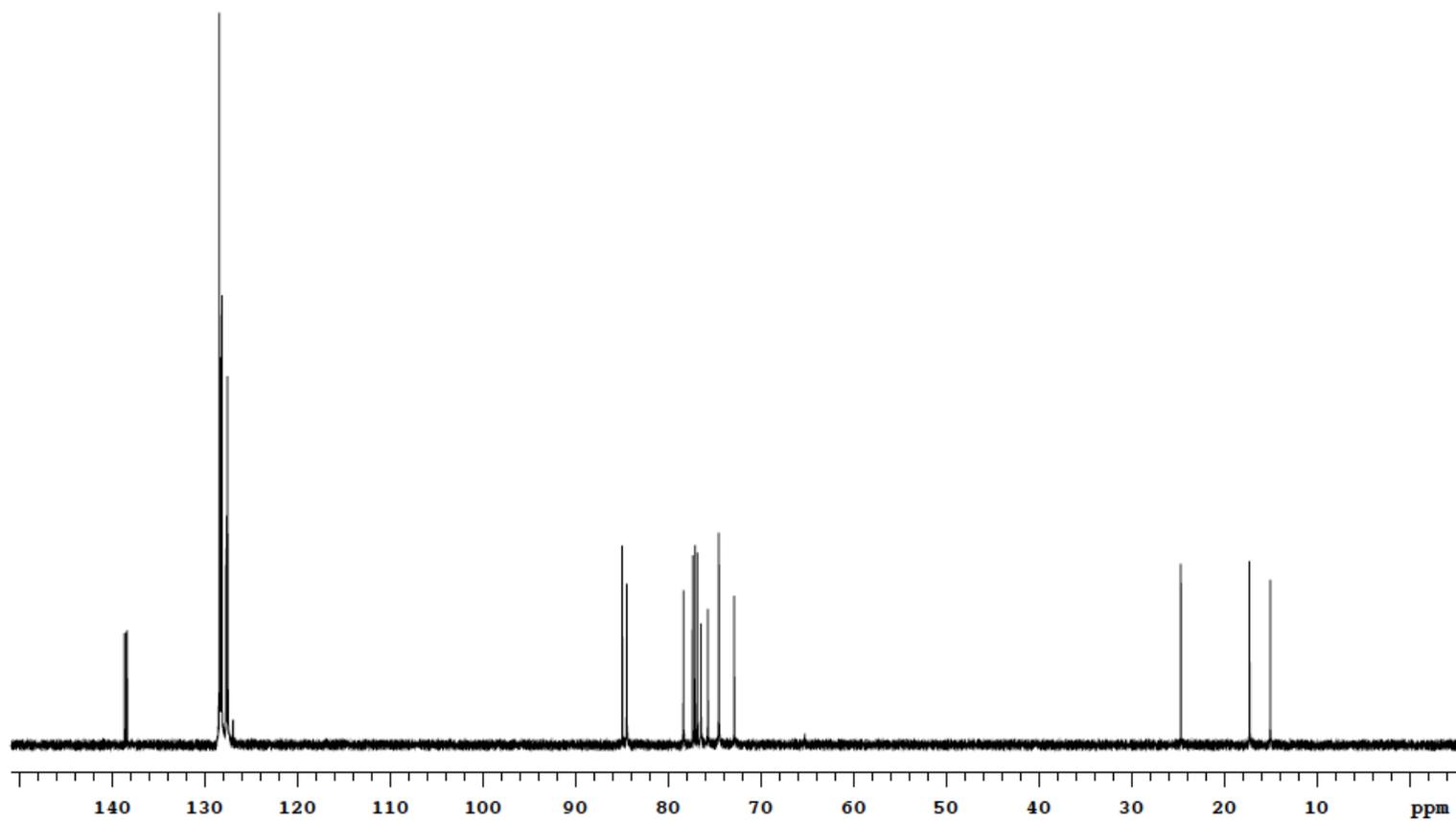
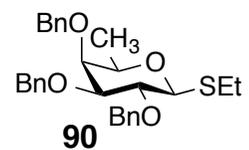
Ethyl 2,3,4-tri-*O*-acetyl-6-deoxy-1-thio- β -D-galactopyranoside (**89**) ^{13}C NMR, CDCl_3 , 125 MHz



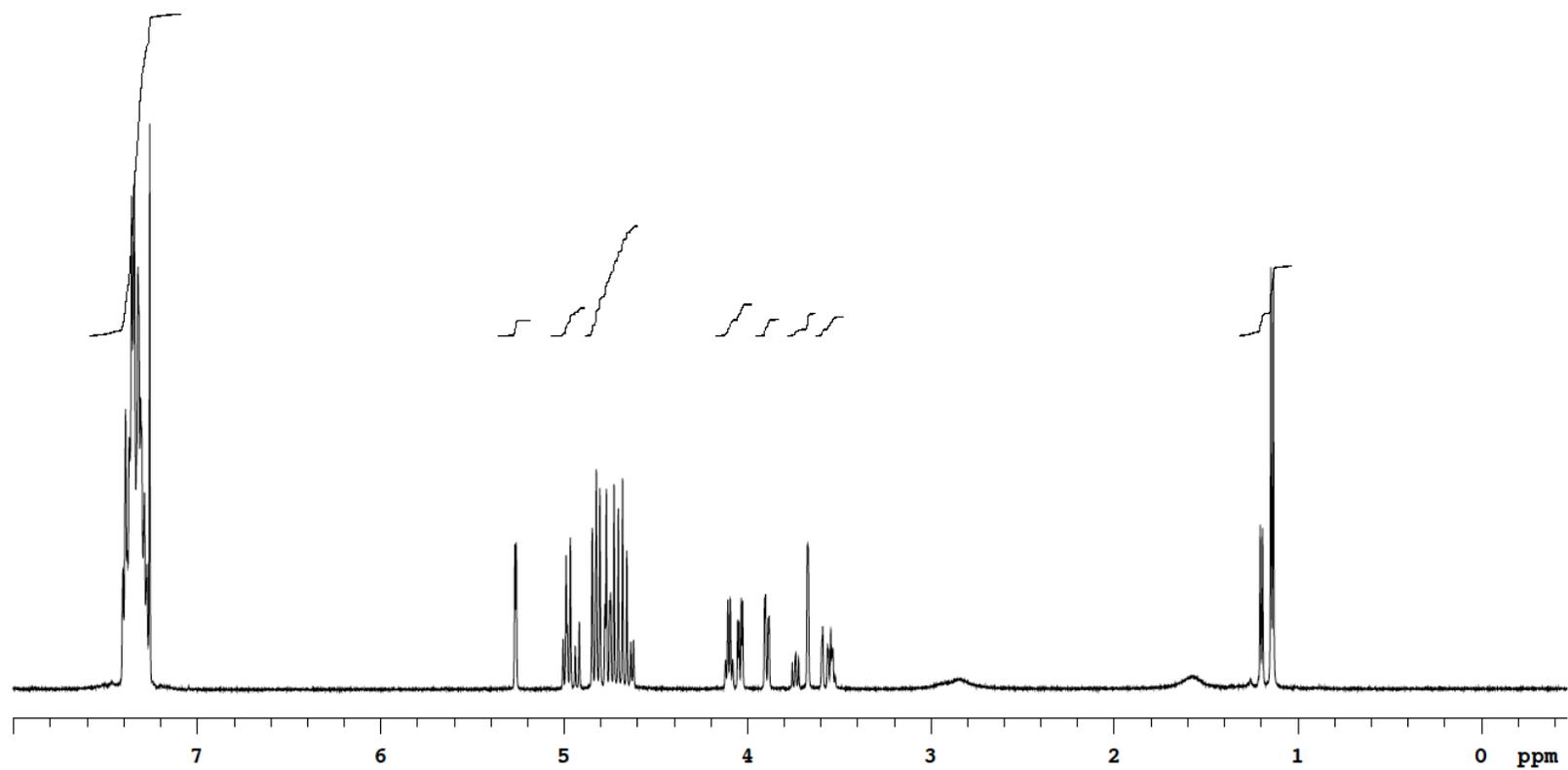
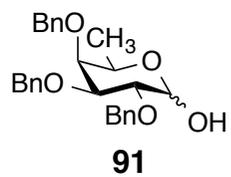
Ethyl 2,3,4-tri-*O*-benzyl-6-deoxy-1-thio- β -D-galactopyranoside (**90**) ^1H NMR, CDCl_3 , 500 MHz



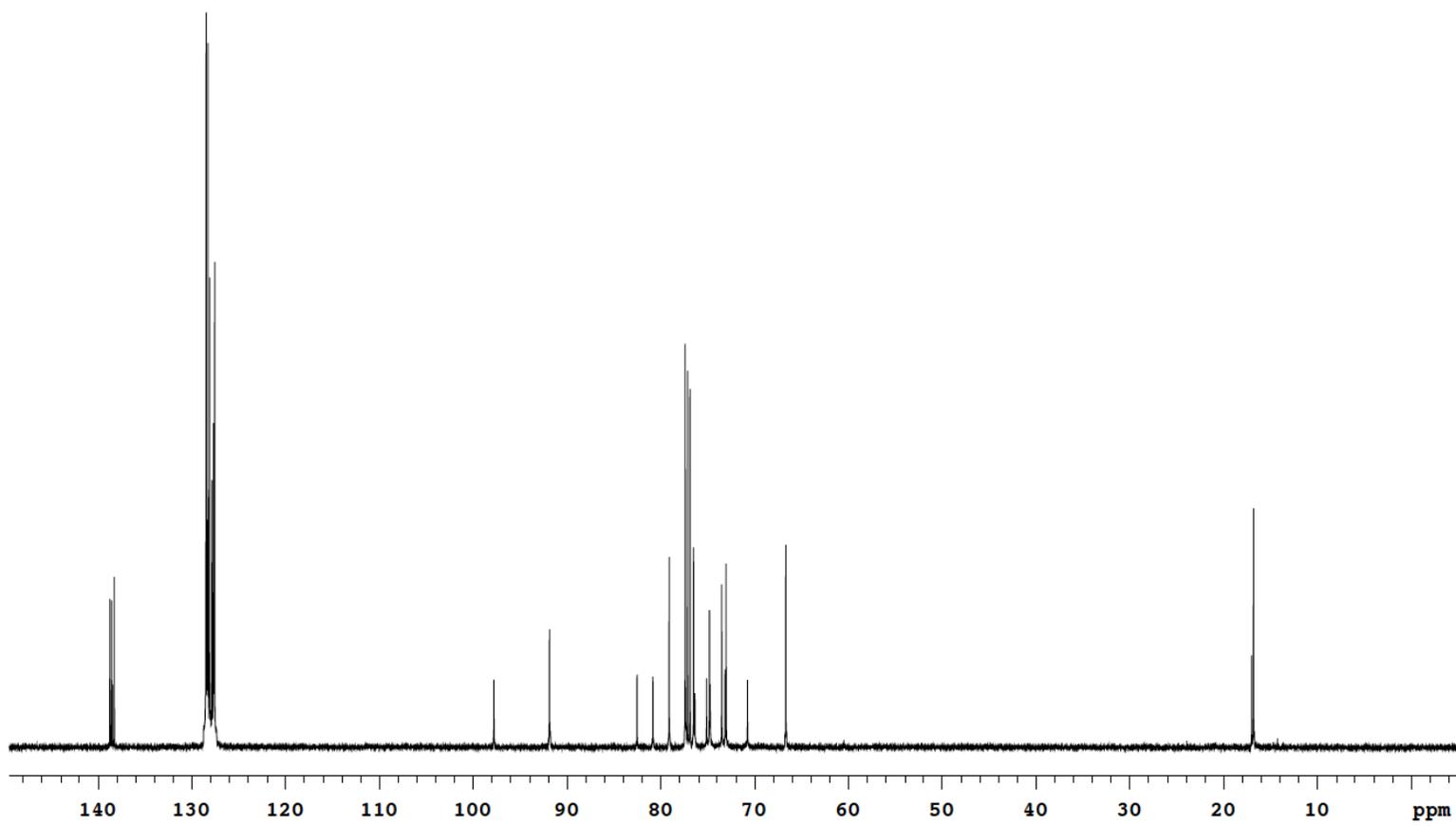
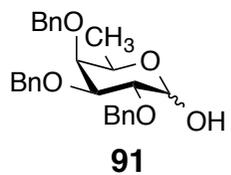
Ethyl 2,3,4-tri-*O*-benzyl-6-deoxy-1-thio- β -D-galactopyranoside (**90**) ^{13}C NMR, CDCl_3 , 125 MHz



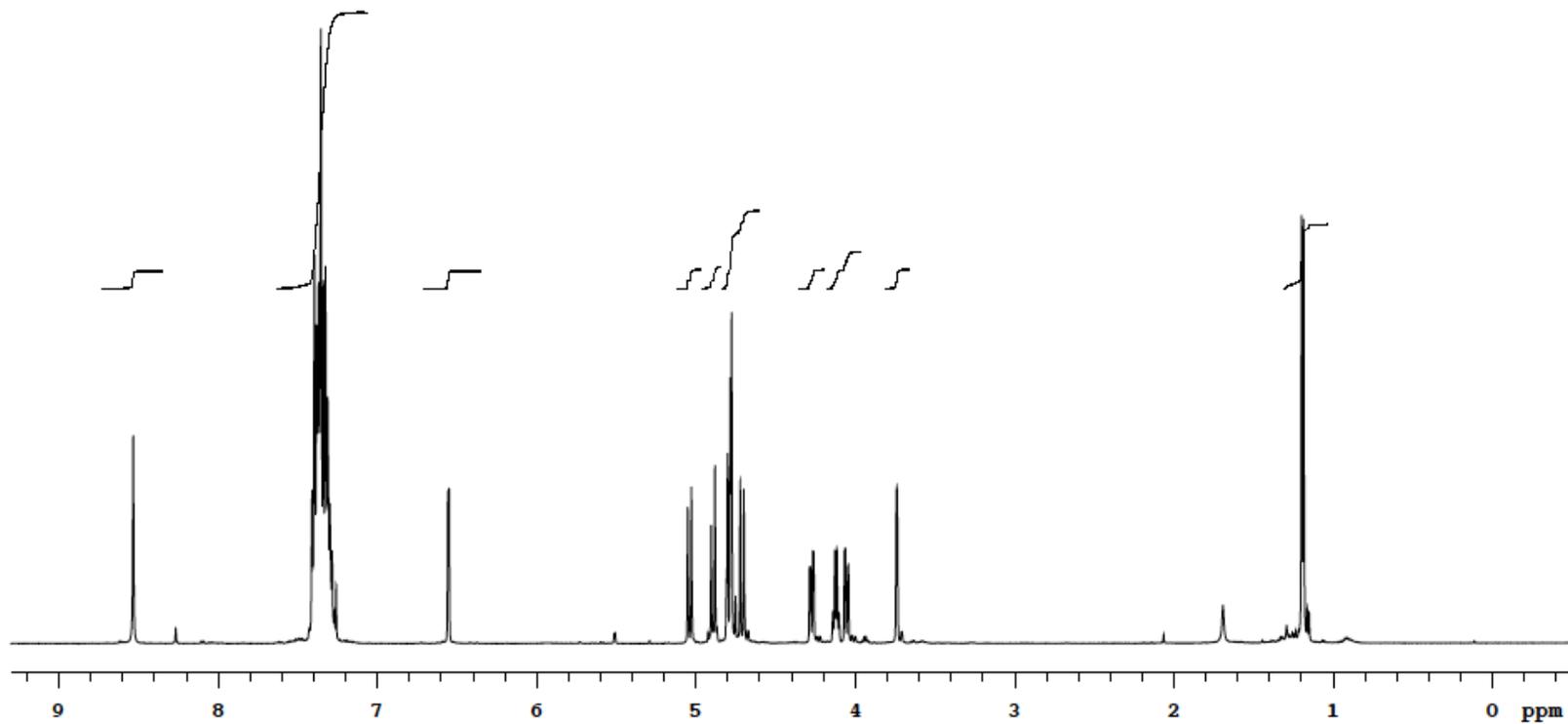
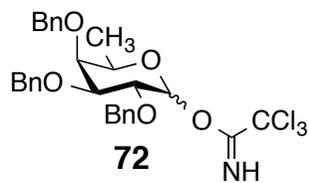
2,3,4-Tri-*O*-benzyl-6-deoxy- α/β -D-galactopyranose (**91**) ^1H NMR, CDCl_3 , 500 MHz



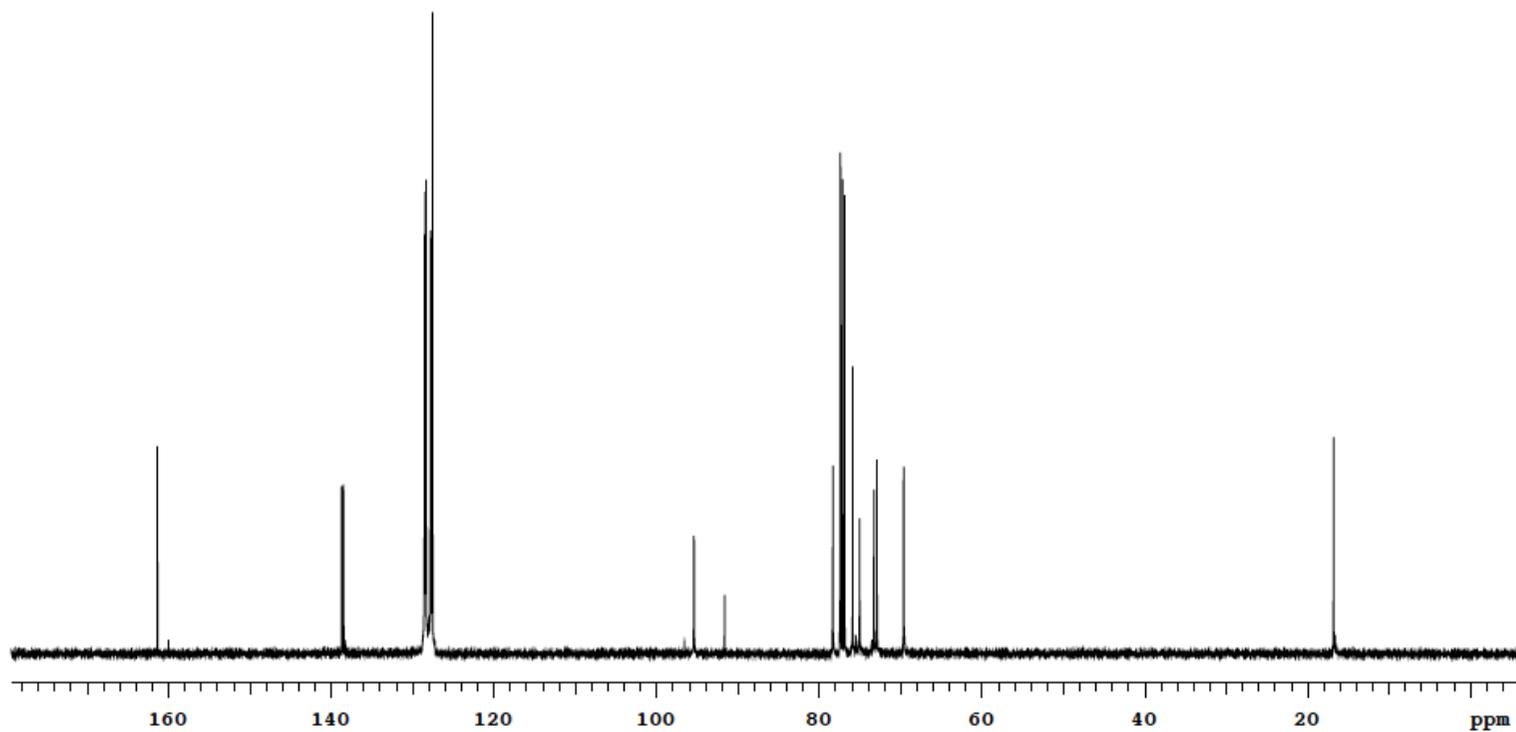
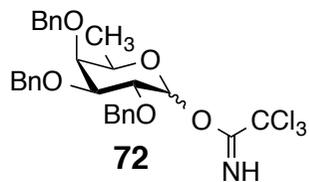
2,3,4-Tri-*O*-benzyl-6-deoxy- α/β -D-galactopyranose (**91**) ^{13}C NMR, CDCl_3 , 125 MHz



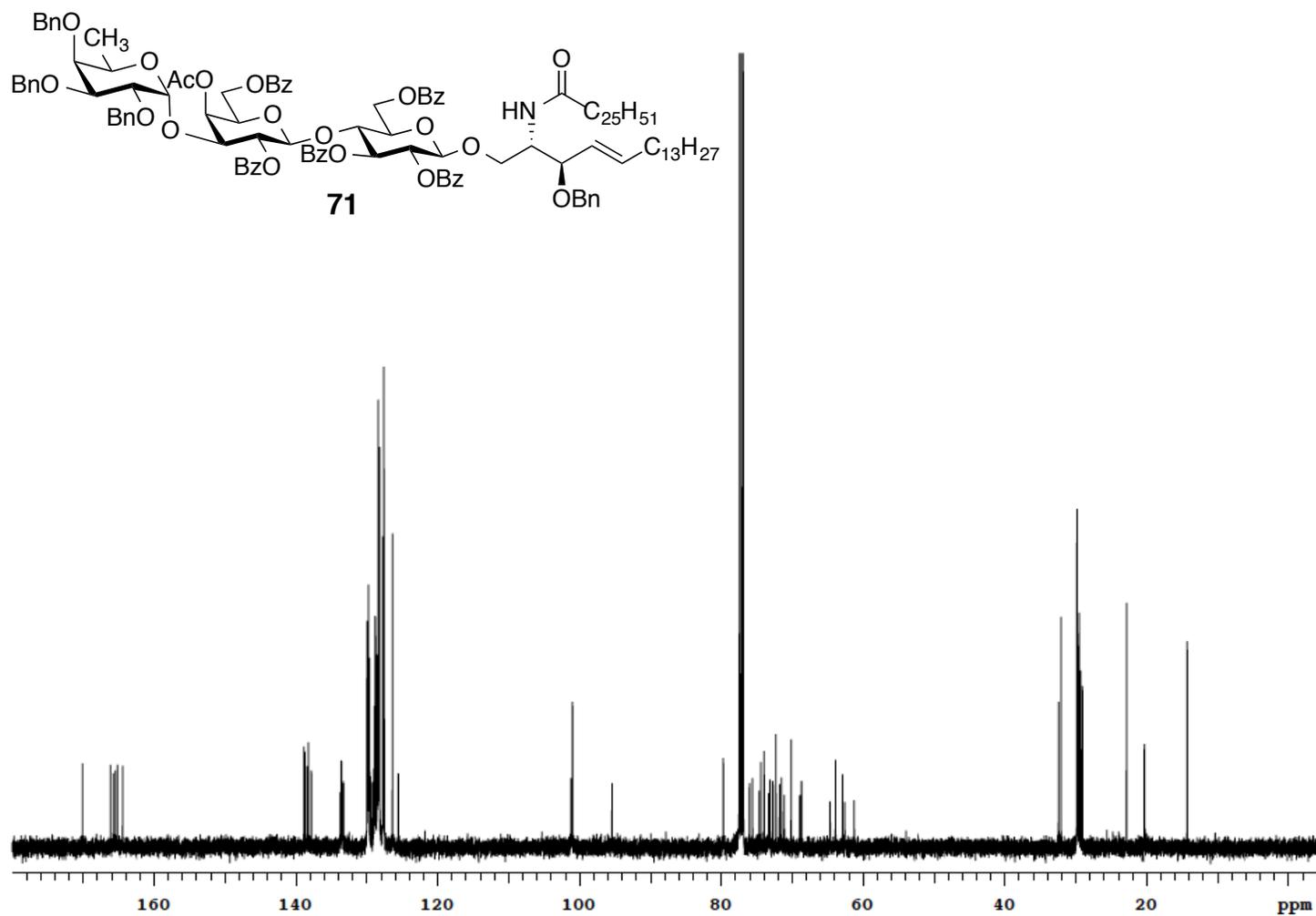
O-(2,3,4-Tri-*O*-benzyl-6-deoxy- α/β -D-galactopyranosyl) trichloroacetimidate (**72**) ^1H NMR, CDCl_3 , 500 MHz



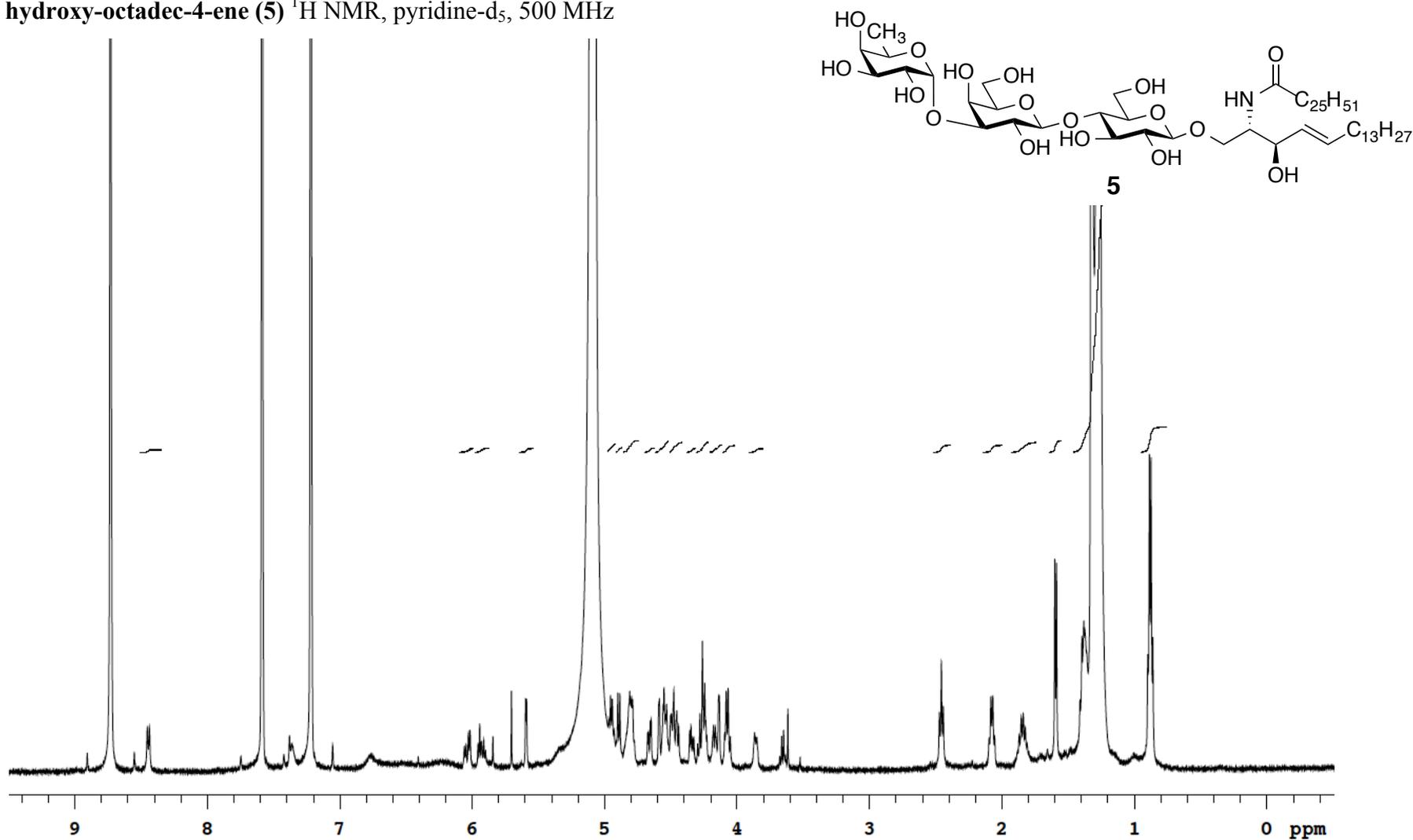
O-(2,3,4-Tri-*O*-benzyl-6-deoxy- α/β -D-galactopyranosyl) trichloroacetimidate (**72**) ^{13}C NMR, CDCl_3 , 125 MHz



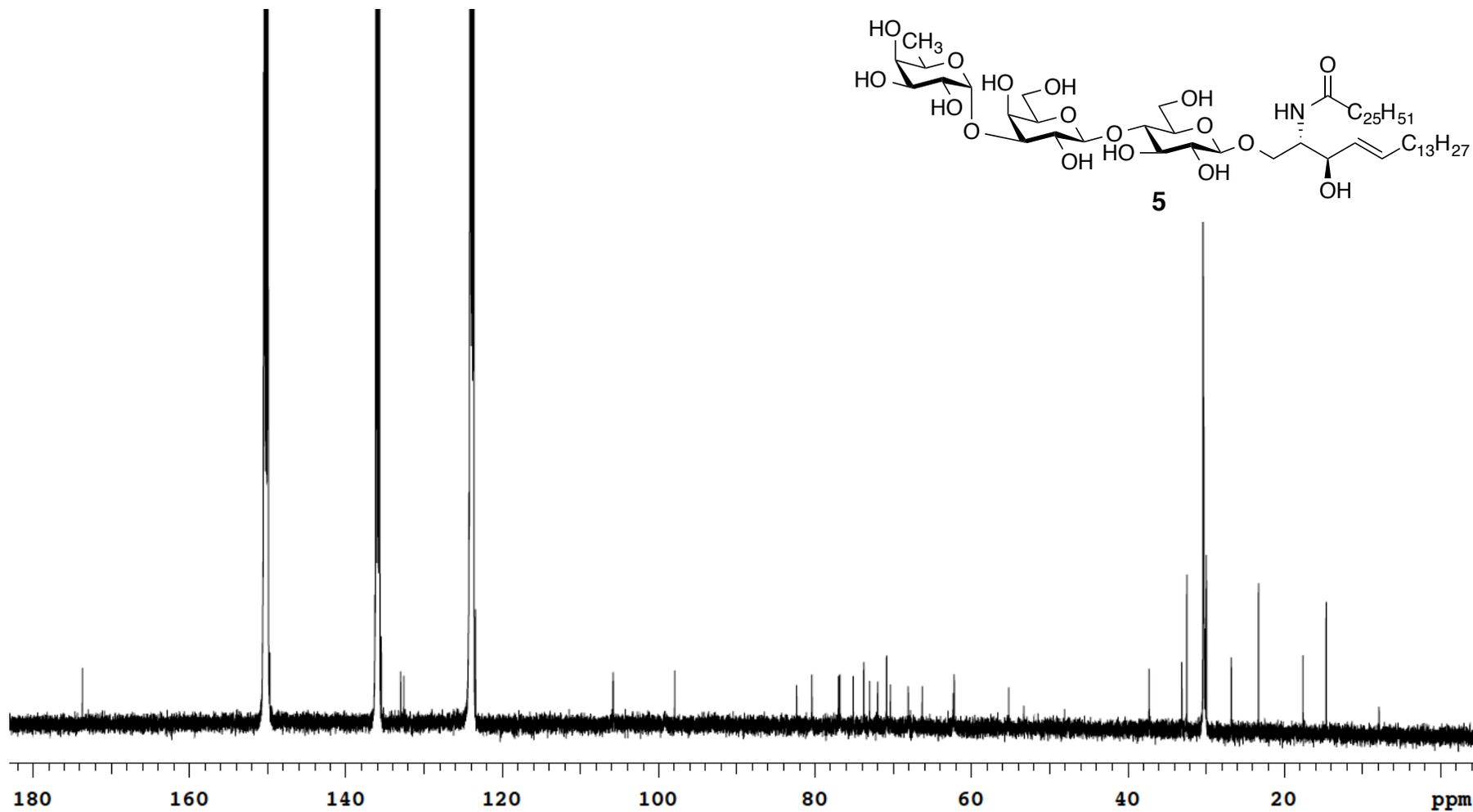
(2*S*,3*R*,4*E*)-1-(4-*O*-(4-*O*-Acetyl-2,6-di-*O*-benzoyl-3-*O*-(2,3,4-tri-*O*-benzyl-6-deoxy- α -D-galactopyranosyl)- β -D-galactopyranosyl)-2,3,6-tri-*O*-benzoyl- β -D-glucopyranosyloxy)-3-benzyloxy-2-hexacosanoylamido-octadec-4-ene (71) ^{13}C NMR, CDCl_3 , 125 MHz



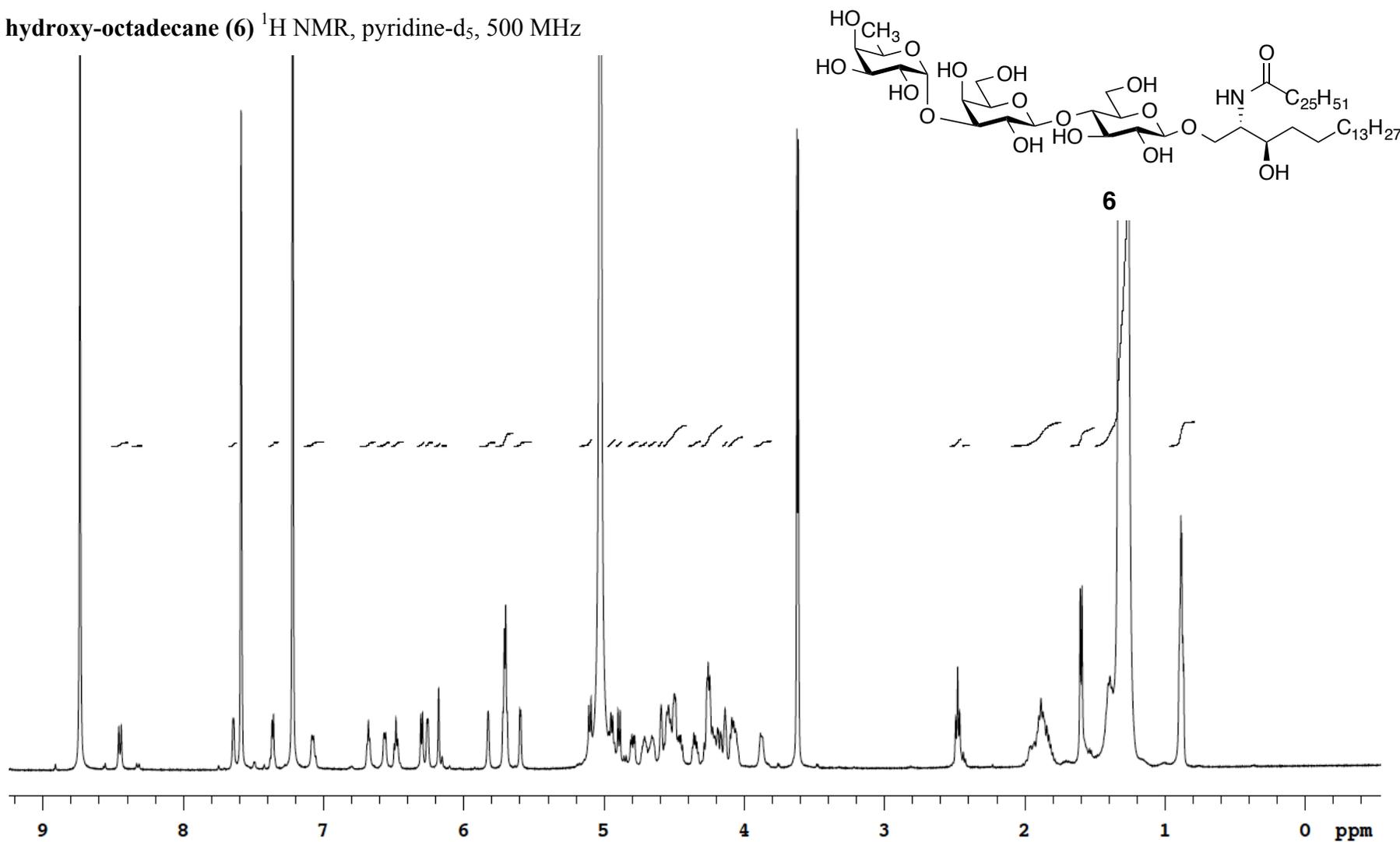
(2*S*,3*R*,4*E*)-1-(4-*O*-(3-*O*-(6-Deoxy- α -D-galactopyranosyl)- β -D-galactopyranosyl)- β -D-glucopyranosyloxy)-2-hexacosanoylamido-3-hydroxy-octadec-4-ene (**5**) ^1H NMR, pyridine- d_5 , 500 MHz



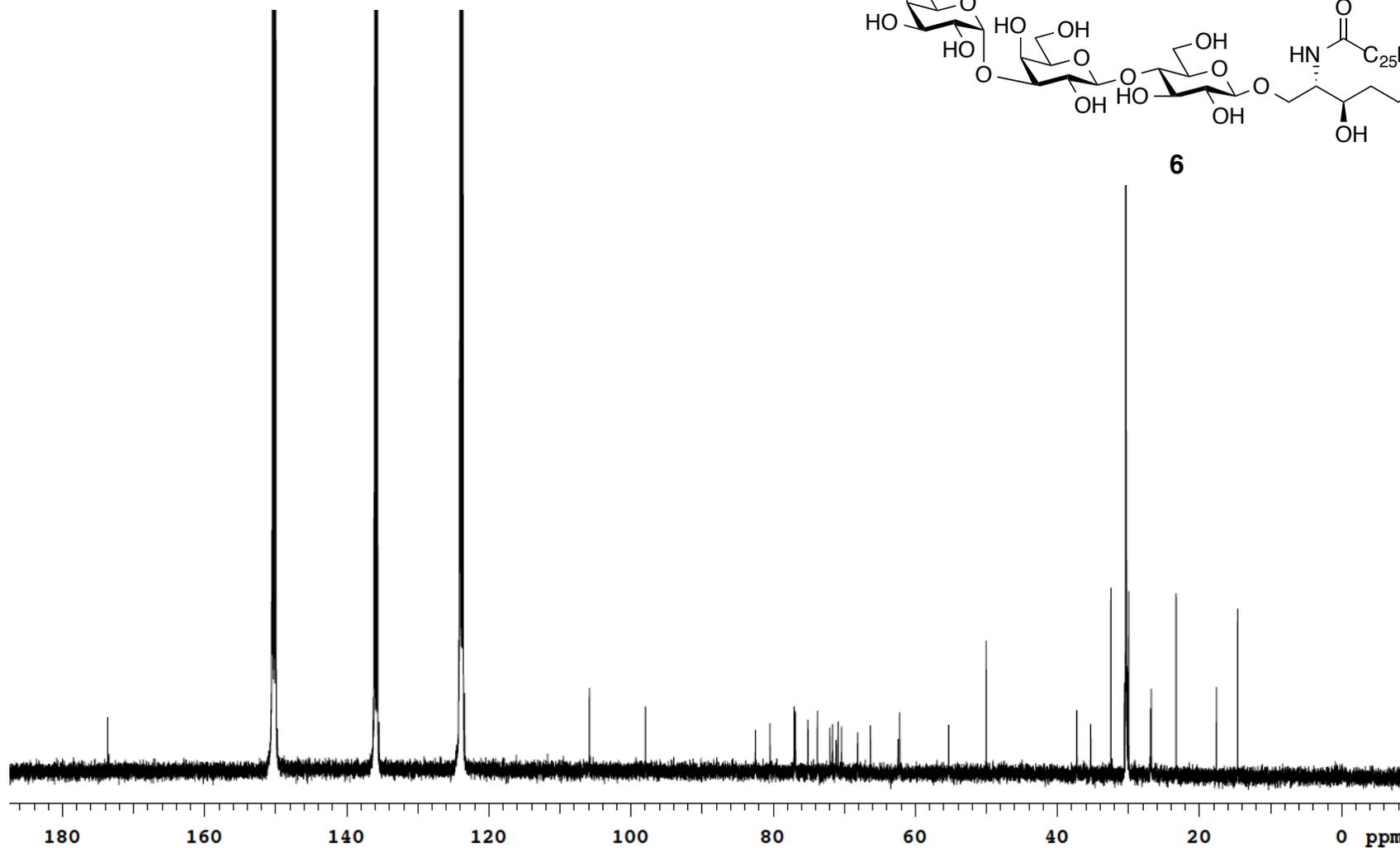
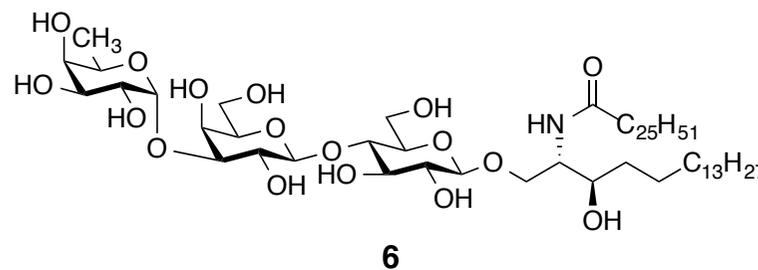
(2*S*,3*R*,4*E*)-1-(4-*O*-(3-*O*-(6-Deoxy- α -D-galactopyranosyl)- β -D-galactopyranosyl)- β -D-glucopyranosyloxy)-2-hexacosanoylamido-3-hydroxy-octadec-4-ene (**5**) ^{13}C NMR, pyridine- d_5 , 125 MHz



(2*S*,3*R*,4*E*)-1-(4-*O*-(3-*O*-(6-Deoxy- α -*D*-galactopyranosyl)- β -*D*-galactopyranosyl)- β -*D*-glucopyranosyloxy)-2-hexacosanoylamido-3-hydroxy-octadecane (6) ^1H NMR, pyridine- d_5 , 500 MHz



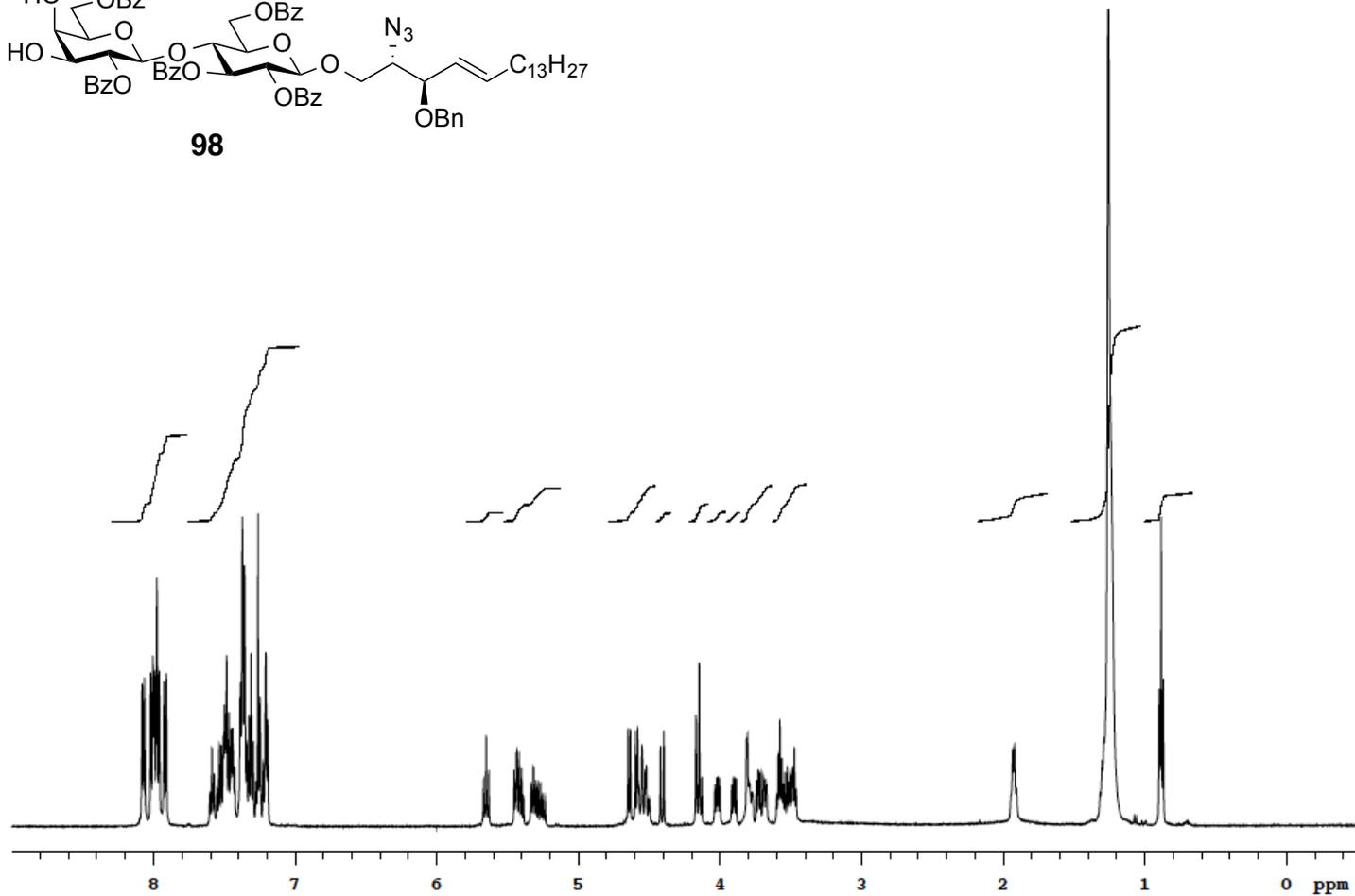
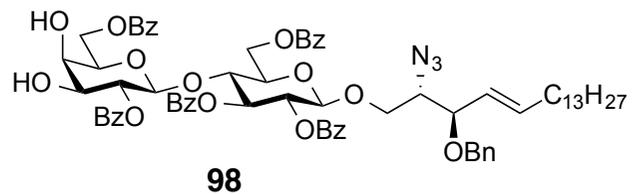
(2*S*,3*R*,4*E*)-1-(4-*O*-(3-*O*-(6-Deoxy- α -*D*-galactopyranosyl)- β -*D*-galactopyranosyl)- β -*D*-glucopyranosyloxy)-2-hexacosanoylamido-3-hydroxy-octadecane (6) ^{13}C NMR, pyridine- d_5 , 125 MHz



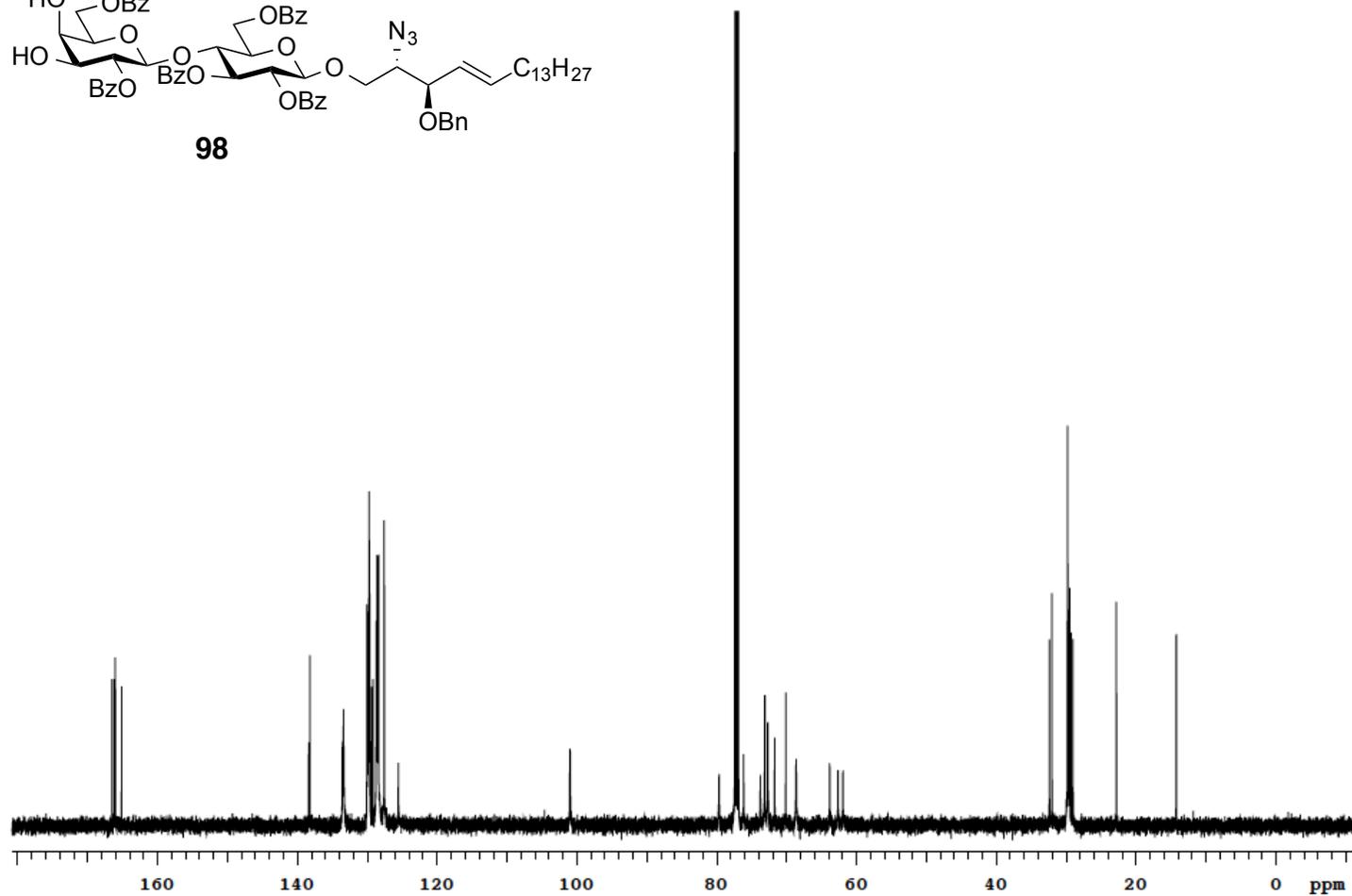
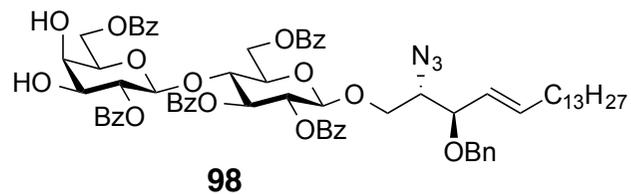
^1H & ^{13}C NMR Spectra

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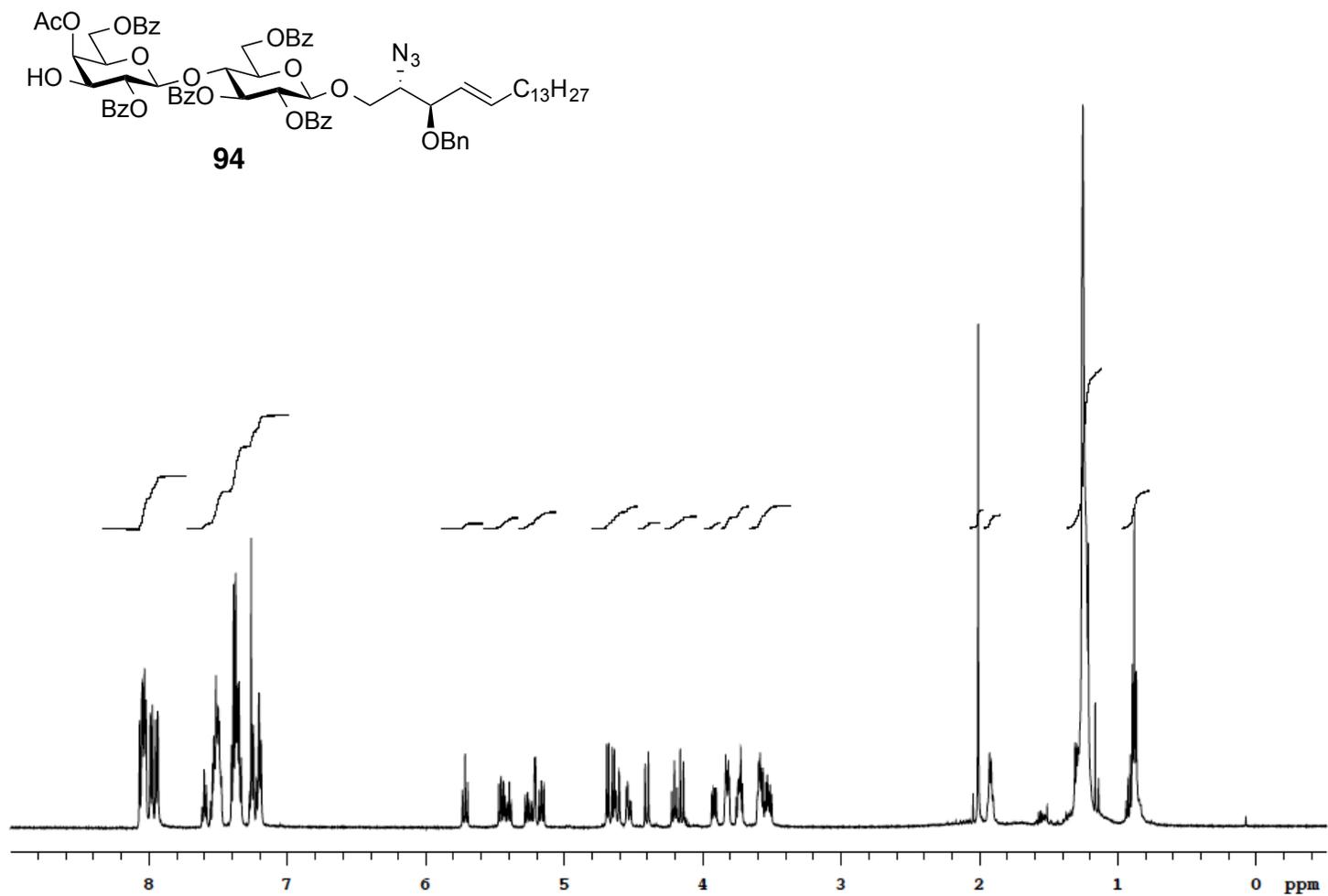
(2*S*,3*R*,4*E*)-2-Azido-1-(4-*O*-(2,6-di-*O*-benzoyl- β -D-galactopyranosyl)-2,3,6-tri-*O*-benzoyl- β -D-glucopyranosyloxy)-3-benzyloxy-octadec-4-ene (**98**) ^1H NMR, CDCl_3 , 500 MHz



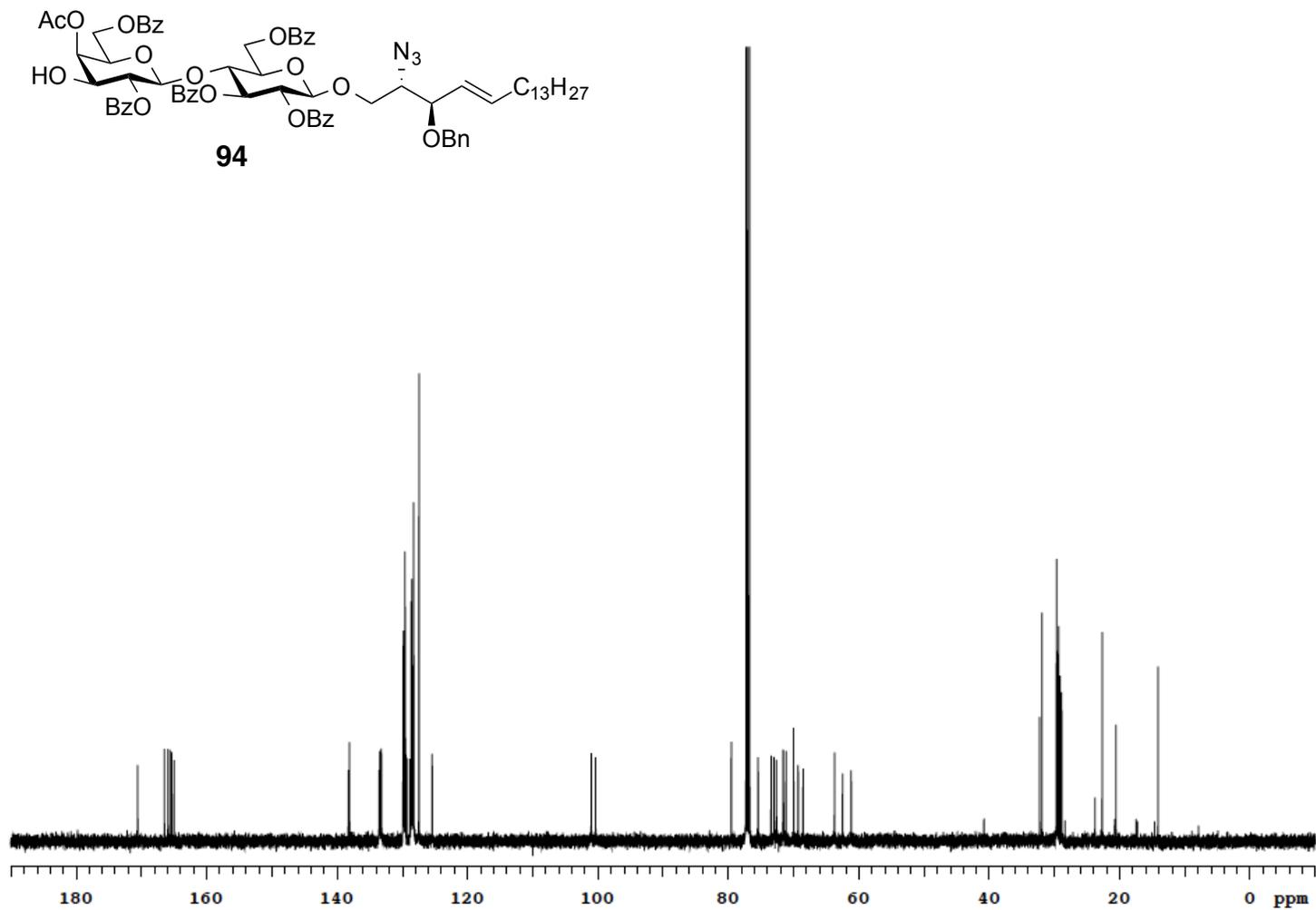
(2*S*,3*R*,4*E*)-2-Azido-1-(4-*O*-(2,6-di-*O*-benzoyl- β -D-galactopyranosyl)-2,3,6-tri-*O*-benzoyl- β -D-glucopyranosyloxy)-3-benzyloxy-octadec-4-ene (**98**) ^{13}C NMR, CDCl_3 , 125 MHz



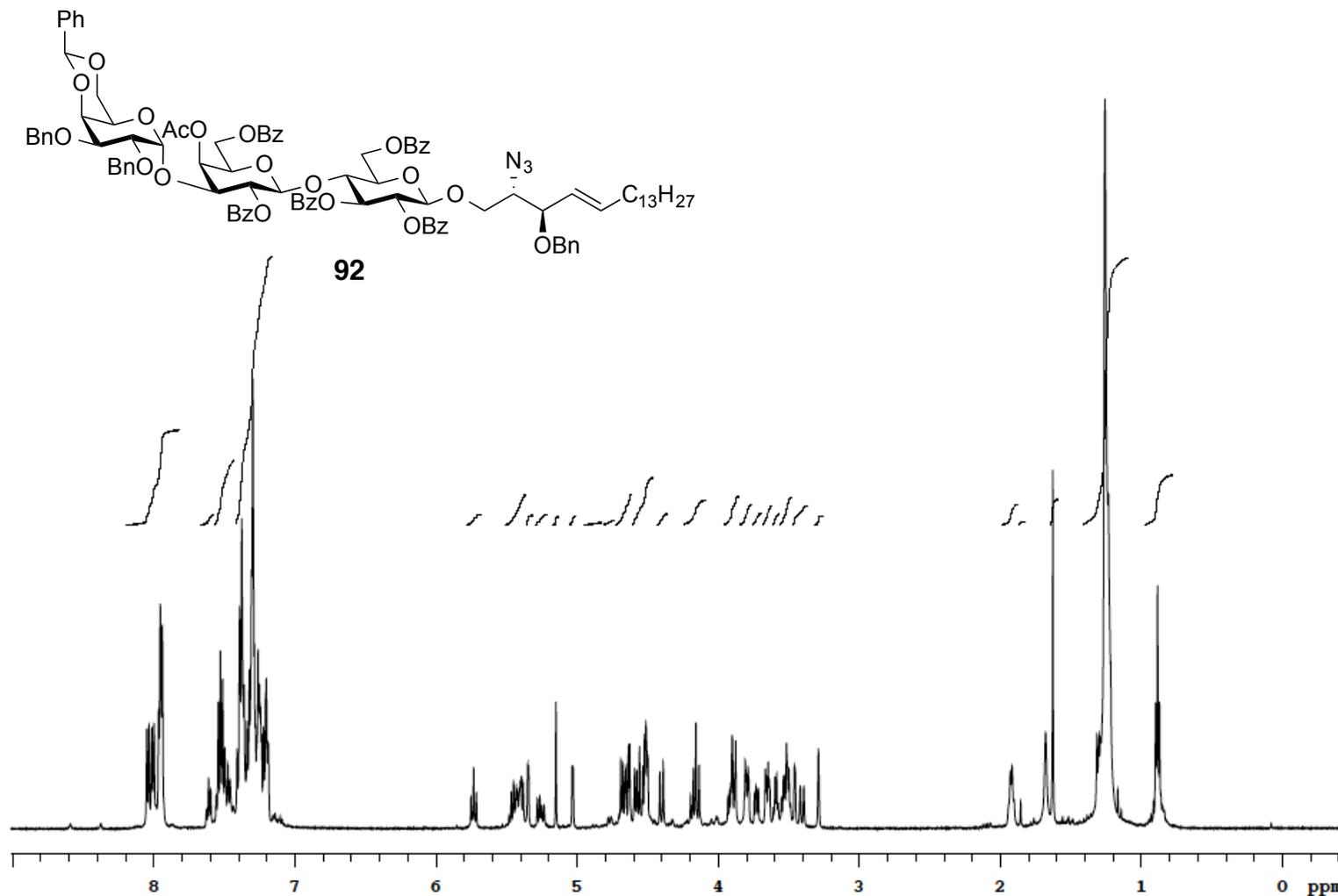
(2*S*,3*R*,4*E*)-1-(4-*O*-(4-*O*-Acetyl-2,6-di-*O*-benzoyl- β -D-galactopyranosyl)-2,3,6-tri-*O*-benzoyl- β -D-glucopyranosyloxy)-2-azido-3-benzyloxy-octadec-4-ene (**94**) ^1H NMR, CDCl_3 , 500 MHz



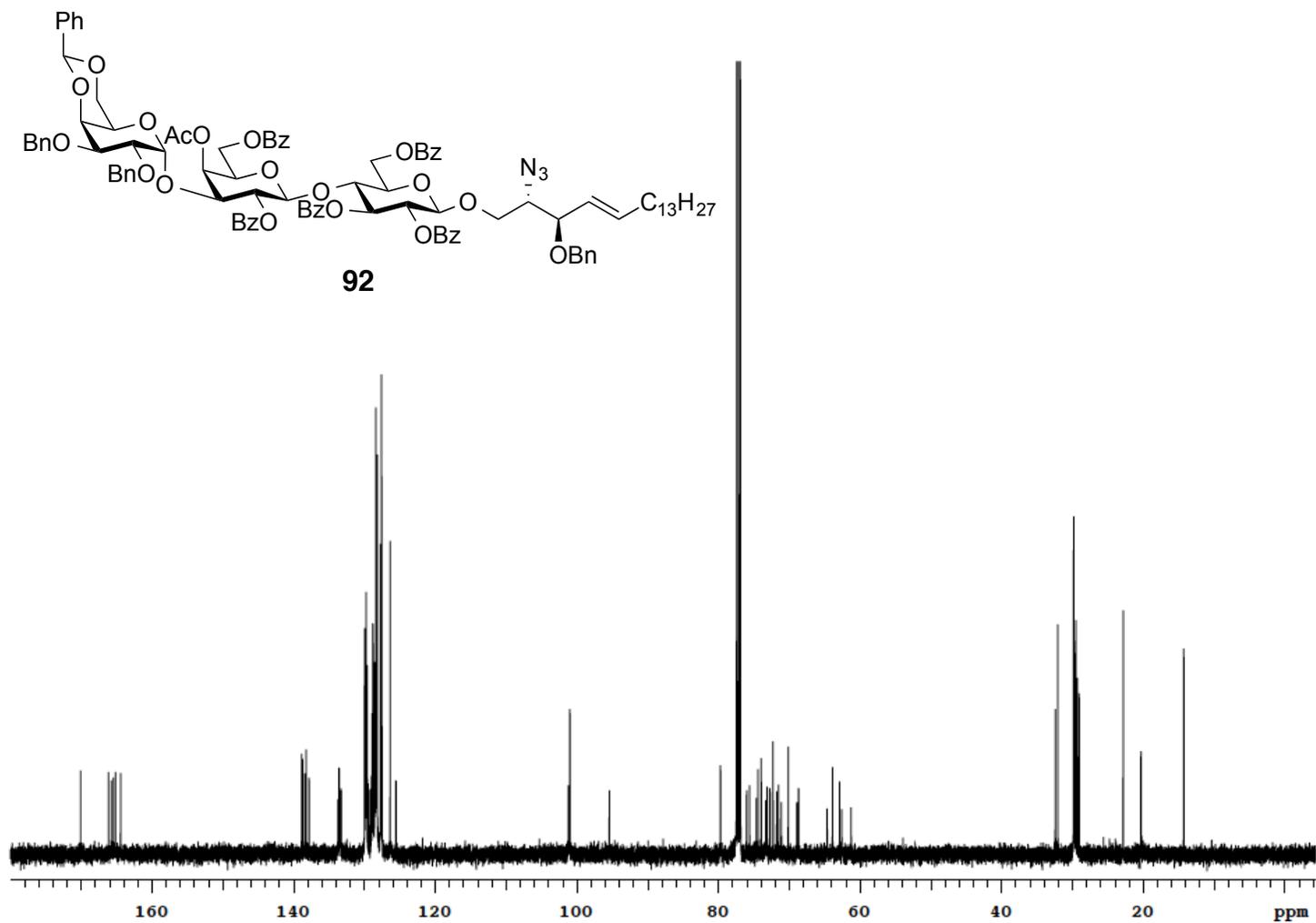
(2*S*,3*R*,4*E*)-1-(4-*O*-(4-*O*-Acetyl-2,6-di-*O*-benzoyl- β -D-galactopyranosyl)-2,3,6-tri-*O*-benzoyl- β -D-glucopyranosyloxy)-2-azido-3-benzyloxy-octadec-4-ene (**94**) ^{13}C NMR, CDCl_3 , 125 MHz



(2*S*,3*R*,4*E*)-1-(4-*O*-(4-*O*-Acetyl-2,6-di-*O*-benzoyl-3-*O*-(2,3-di-*O*-benzyl-4,6-*O*-benzylidene- α -D-galactopyranosyl)- β -D-galactopyranosyl)-2,3,6-tri-*O*-benzoyl- β -D-glucopyranosyloxy)-2-azido-3-benzyloxy-octadec-4-ene (**92**) ^1H NMR, CDCl_3 , 500 MHz

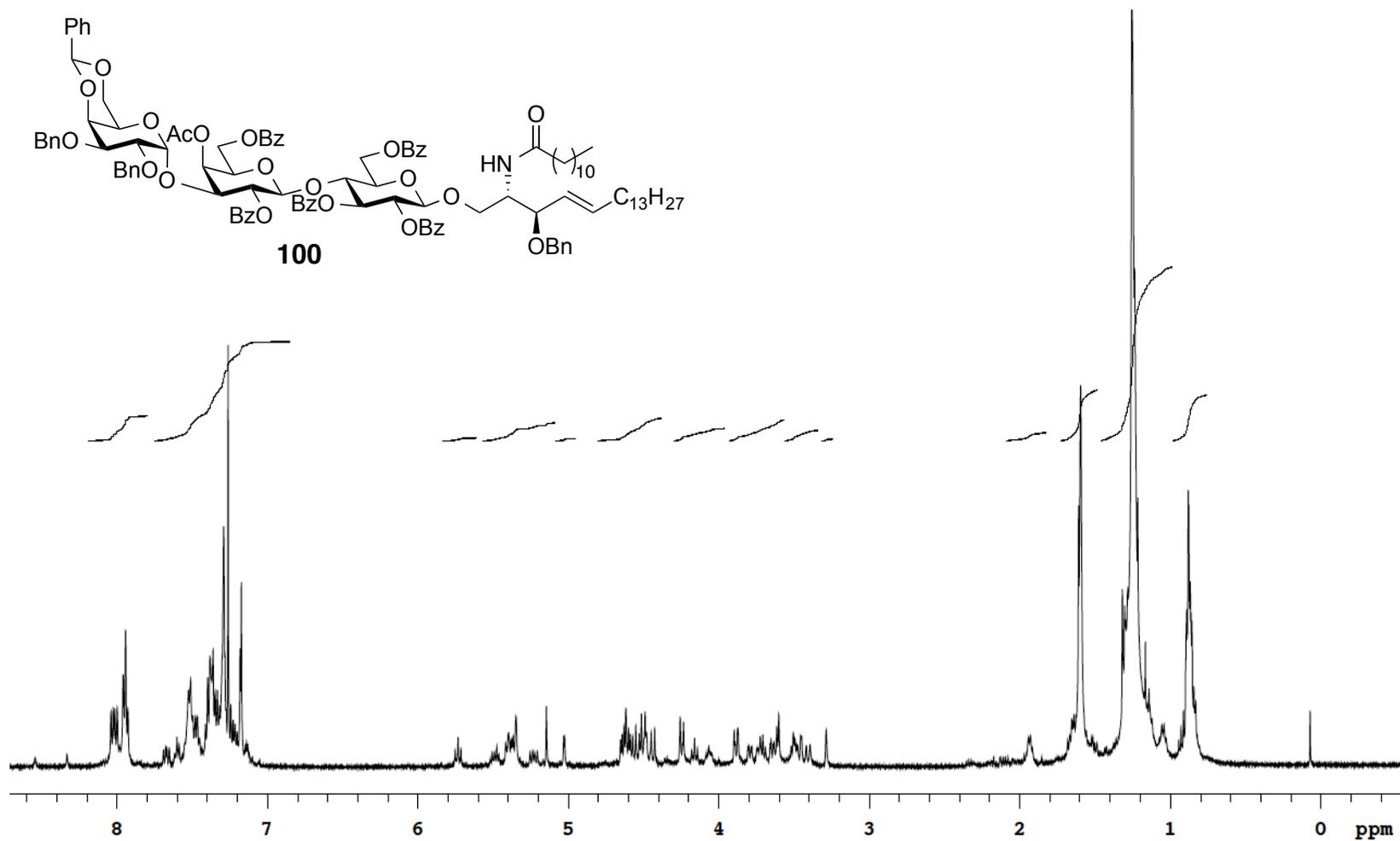


(2*S*,3*R*,4*E*)-1-(4-*O*-(4-*O*-Acetyl-2,6-di-*O*-benzoyl-3-*O*-(2,3-di-*O*-benzyl-4,6-*O*-benzylidene- α -D-galactopyranosyl)- β -D-galactopyranosyl)-2,3,6-tri-*O*-benzoyl- β -D-glucopyranosyloxy)-2-azido-3-benzyloxy-octadec-4-ene (**92**) ^{13}C NMR, CDCl_3 , 125 MHz



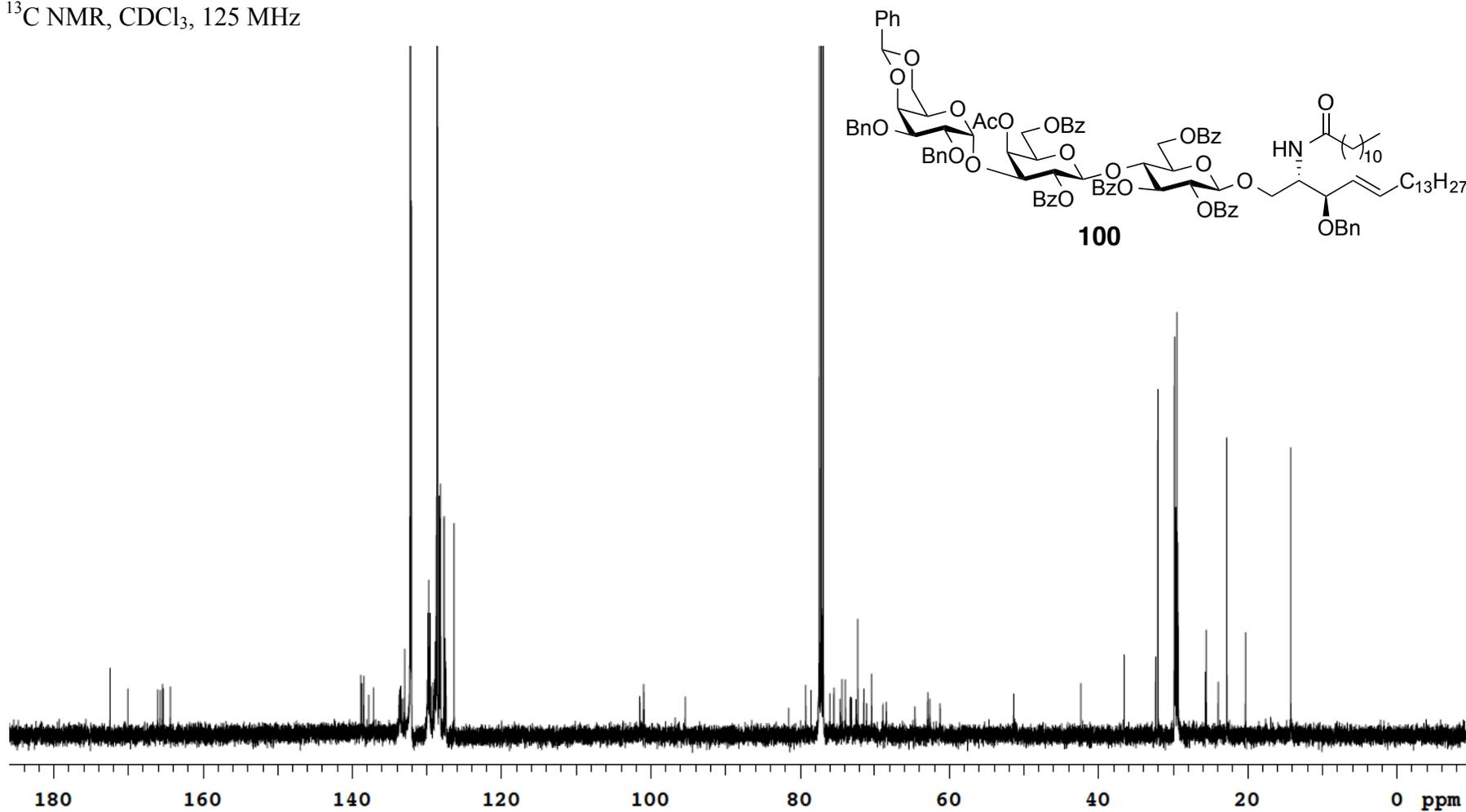
(2*S*,3*R*,4*E*)-1-(4-*O*-(4-*O*-Acetyl-2,6-di-*O*-benzoyl-3-*O*-(2,3-di-*O*-benzyl-4,6-*O*-benzylidene- α -D-galactopyranosyl)- β -D-galactopyranosyl)-2,3,6-tri-*O*-benzoyl- β -D-glucopyranosyloxy)-3-benzyloxy-2-dodecanoylamido-octadec-4-ene (100)

^1H NMR, CDCl_3 , 500 MHz

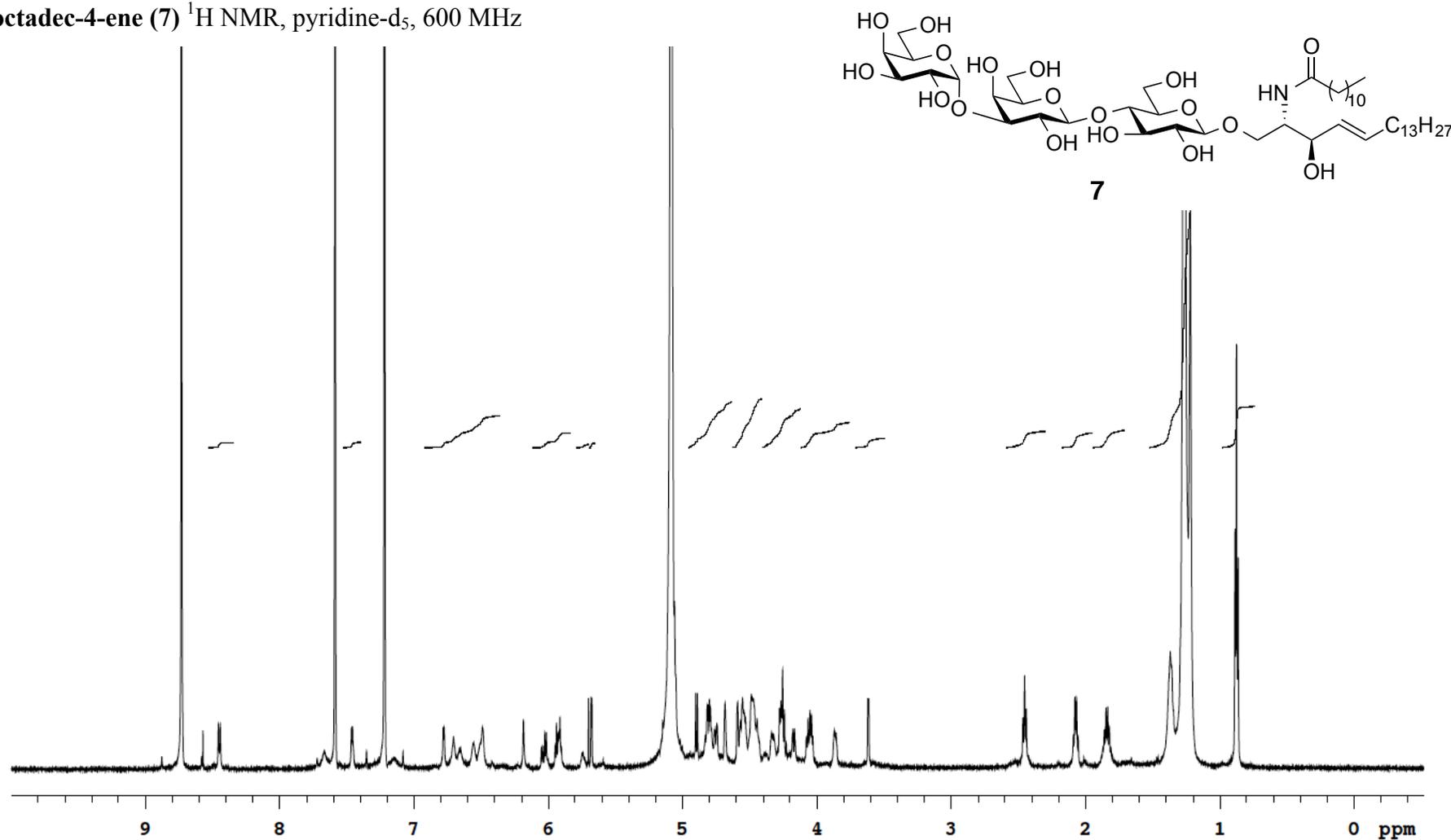


(2*S*,3*R*,4*E*)-1-(4-*O*-(4-*O*-Acetyl-2,6-di-*O*-benzoyl-3-*O*-(2,3-di-*O*-benzyl-4,6-*O*-benzylidene- α -D-galactopyranosyl)- β -D-galactopyranosyl)-2,3,6-tri-*O*-benzoyl- β -D-glucopyranosyloxy)-3-benzyloxy-2-dodecanoylamido-octadec-4-ene (100)

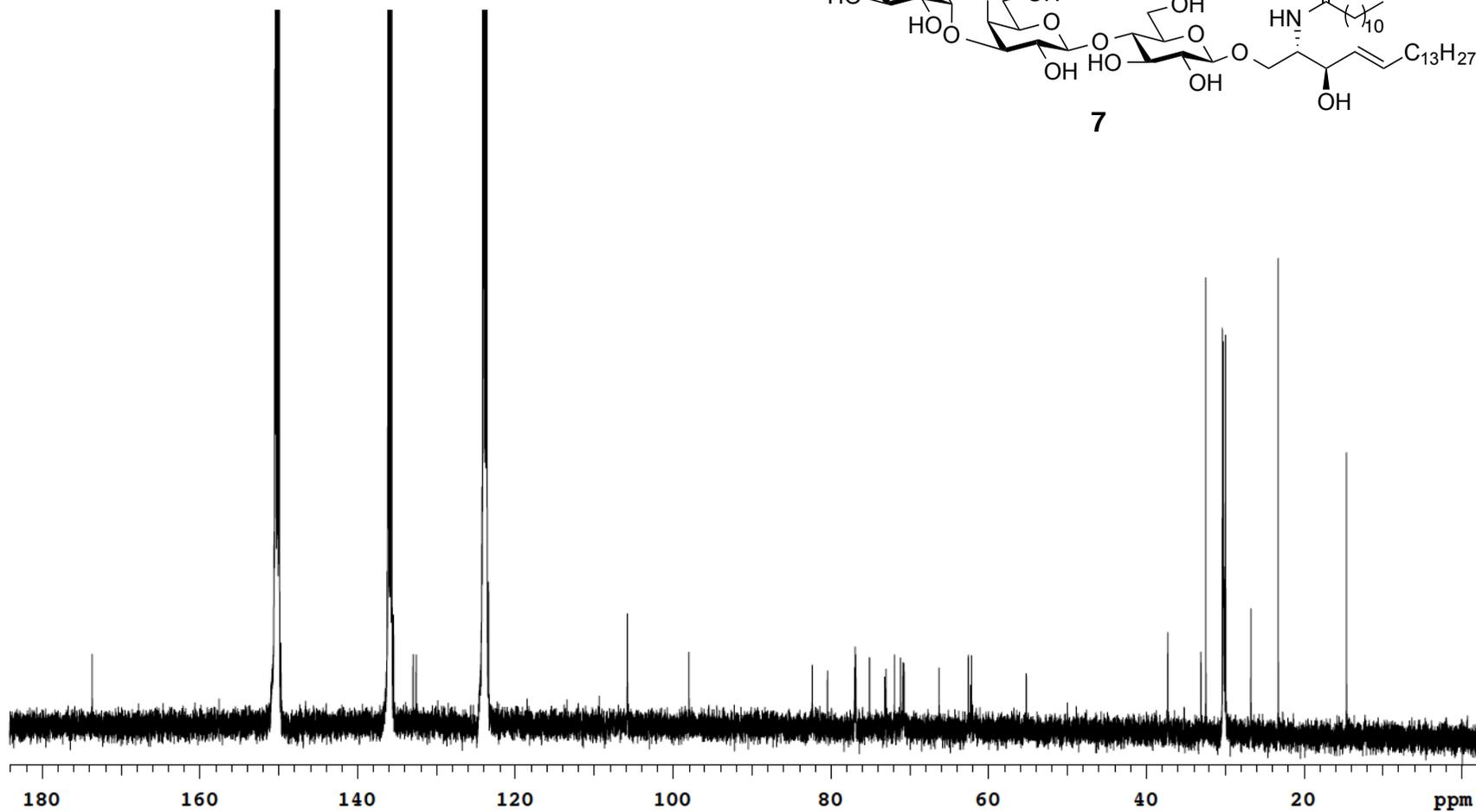
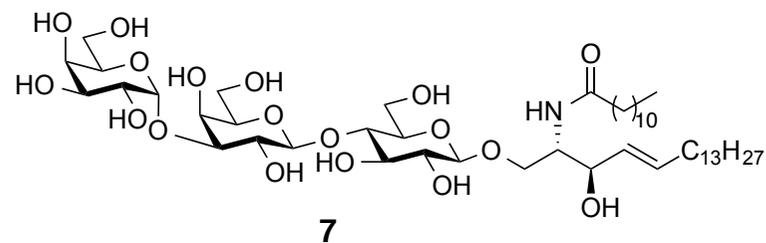
^{13}C NMR, CDCl_3 , 125 MHz



(2*S*,3*R*,4*E*)-2-Dodecanoylamido-1-(4-*O*-(3-*O*- α -D-galactopyranosyl)- β -D-galactopyranosyl)- β -D-glucopyranosyloxy)-3-hydroxy-octadec-4-ene (7) ^1H NMR, pyridine- d_5 , 600 MHz

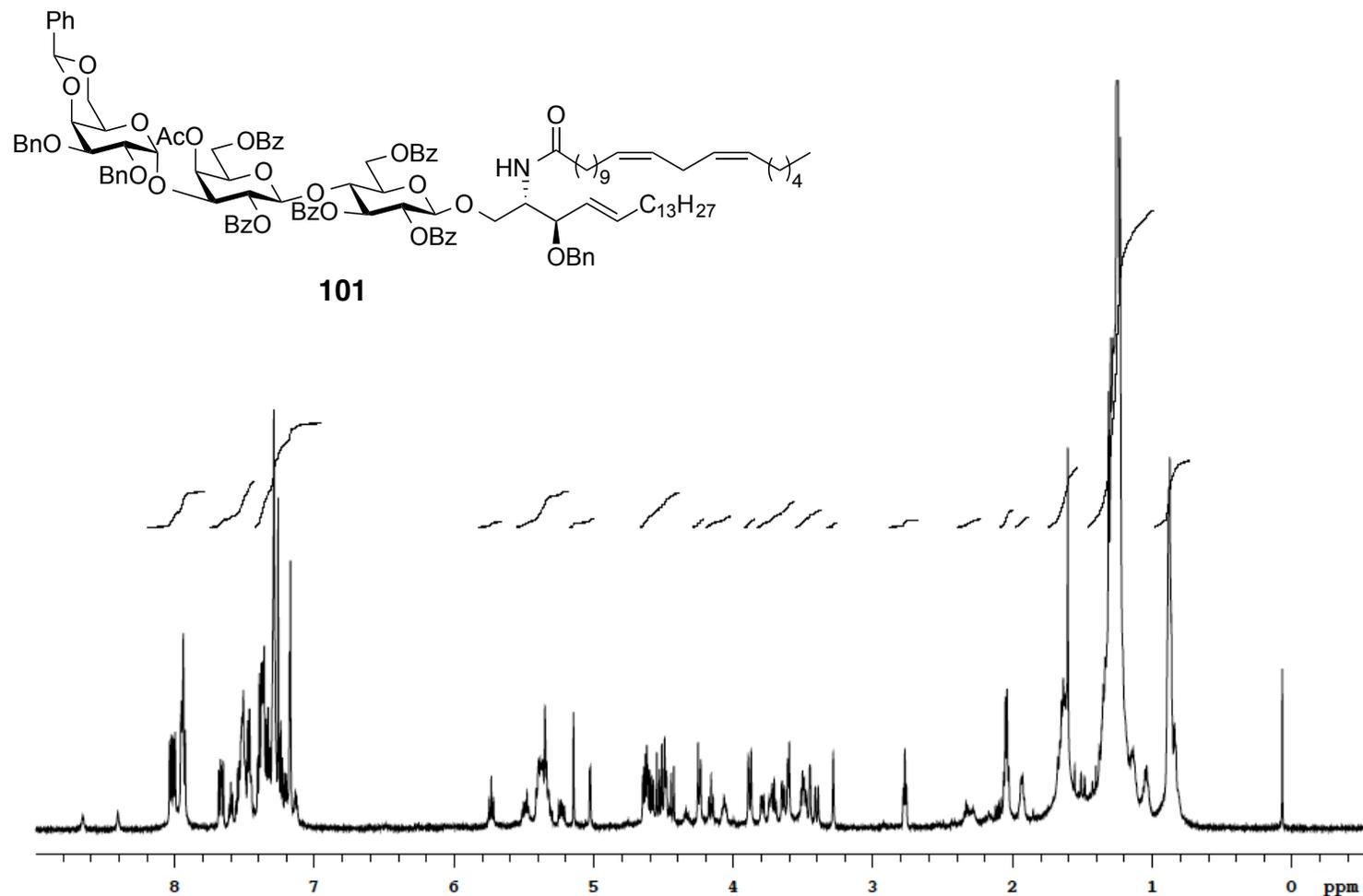


(2*S*,3*R*,4*E*)-2-Dodecanoylamido-1-(4-*O*-(3-*O*- α -D-galactopyranosyl)- β -D-galactopyranosyl)- β -D-glucopyranosyloxy)-3-hydroxy-octadec-4-ene (7) ^{13}C NMR, pyridine- d_5 , 125 MHz



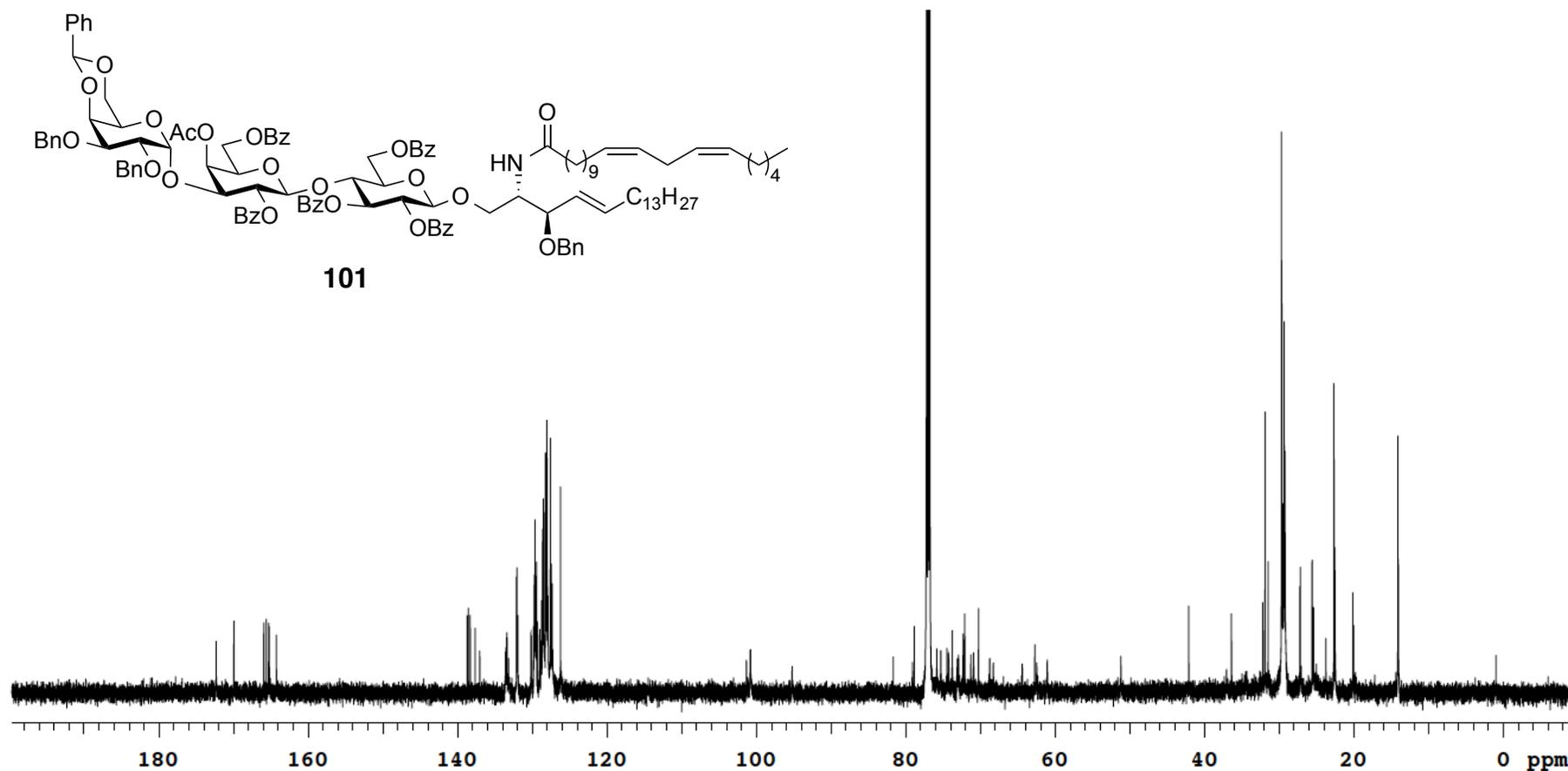
(2*S*,3*R*,4*E*)-1-(4-*O*-(4-*O*-Acetyl-2,6-di-*O*-benzoyl-3-*O*-(2,3-di-*O*-benzyl-4,6-*O*-benzylidene- α -D-galactopyranosyl)- β -D-galactopyranosyl)-2,3,6-tri-*O*-benzoyl- β -D-glucopyranosyloxy)-3-benzyloxy-2-(11*Z*,14*Z*-eicosadienoylamido)-octadec-4-ene (101)

$^1\text{H NMR}$, CDCl_3 , 500 MHz

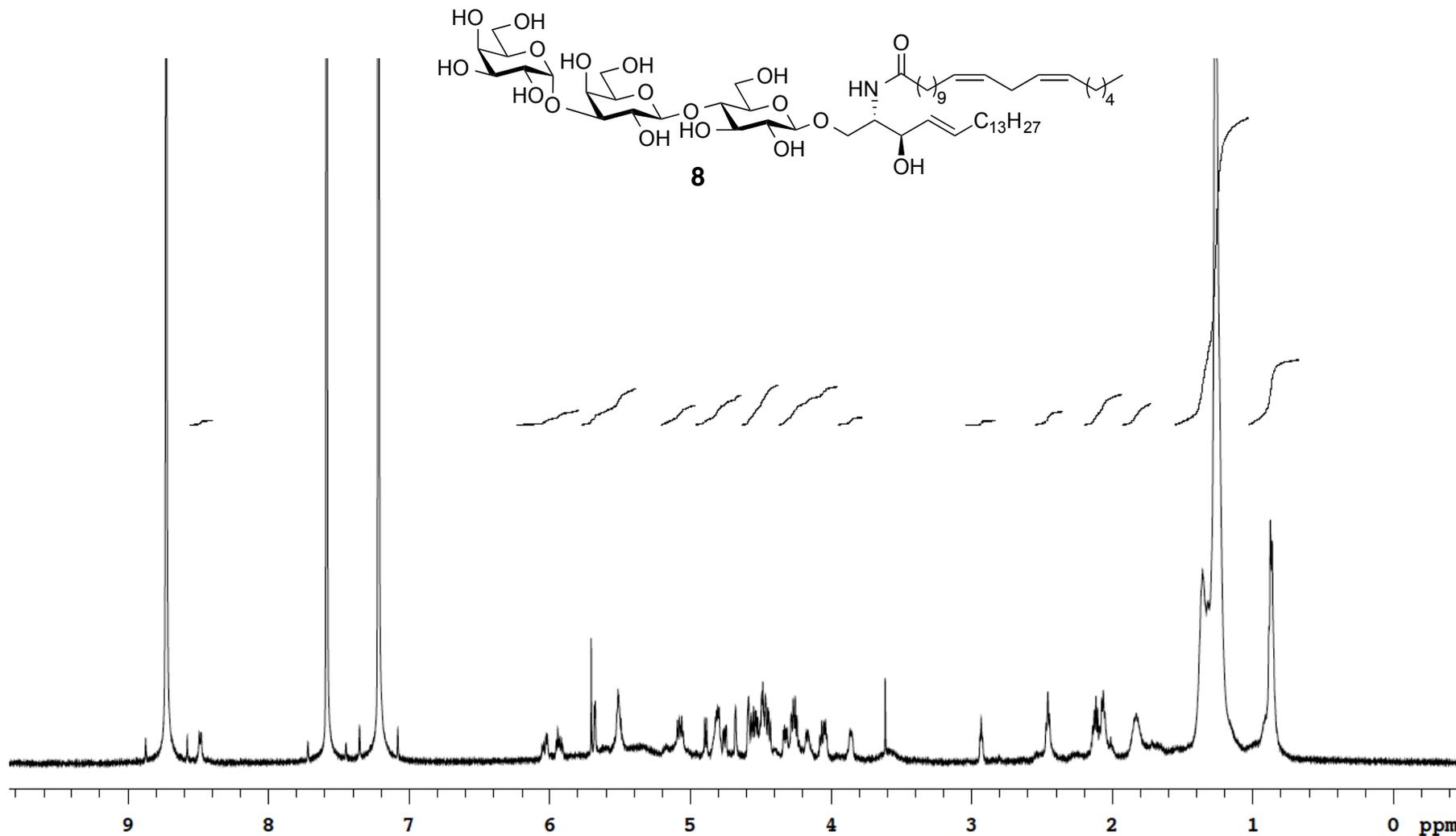


(2*S*,3*R*,4*E*)-1-(4-*O*-(4-*O*-Acetyl-2,6-di-*O*-benzoyl-3-*O*-(2,3-di-*O*-benzyl-4,6-*O*-benzylidene- α -D-galactopyranosyl)- β -D-galactopyranosyl)-2,3,6-tri-*O*-benzoyl- β -D-glucopyranosyloxy)-3-benzyloxy-2-(11*Z*,14*Z*-eicosadienoylamido)-octadec-4-ene (101)

^{13}C NMR, CDCl_3 , 125 MHz



(2*S*,3*R*,4*E*)-2-(11*Z*,14*Z*-Eicosadienoylamido)-1-(4-*O*-(3-*O*- α -D-galactopyranosyl)- β -D-galactopyranosyl)- β -D-glucopyranosyloxy)-3-hydroxy-octadec-4-ene (**8**) ^1H NMR, pyridine- d_5 , 600 MHz



(2*S*,3*R*,4*E*)-2-(11*Z*,14*Z*-Eicosadienoylamido)-1-(4-*O*-(3-*O*- α -D-galactopyranosyl)- β -D-galactopyranosyl)- β -D-glucofuranosyloxy)-3-hydroxy-octadec-4-ene (**8**) ^{13}C NMR, pyridine- d_5 , 150 MHz

