# Synthesis of Chiral Sphingolipids and their Stereochemical Effects on Induction of Neurite Outgrowth and Sphingomyelin Synthase Activity

（キラルスフィンゴ脂質の合成とその立体化学による神経突起伸長及びスフィンゴミエリン合成酵素活性への影響について）

**A Thesis Submitted for the Degree of**

**Doctor of Life Science**

**by**

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*To my parents,*

*and to my wife and daughter*

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## Abbreviations

$AC_2O$: acetic anhydride

AcOH: acetic acid

AD: Alzheimer's disease

$BF_3.OEt_2$: boron trifluoride diethyl etherate

Boc: *t*-butoxycarbonyl

BuLi: butyl lithium

BzCl: benzoyl chloride

CBB: coomassie brilliant blue

CCK-8: cell counting kit-8

Cdase: ceramidase

CD: circular dichroism

CERK: ceramide kinase

CerS: ceramide synthase

C1P: ceramide-1-phosphate

$CHCl_3$: chloroform

$CH_2Cl_2$: dichloromethane

$Cl_3CN$: trichloro acetonitrile

CNS: central nervous system

CPP: ceramide-1-phosphate phosphatase

$Cs_2CO_3$: cesium carbonate

d: doublet

DAG: diacylglycerol

DBU: 1,8-diazabicyclo[5.4.0]undec-7-ene

dd: doublet of doublet

dt: doublet of triplet

DE: D-erythro

DIC: diisopropyl carbodiimide

DIPEA: *N,N*-Diisopropylethylamine

DMAP: 4-Dimethylaminopyridine

DMEM: dulbecco's modified eagle's medium

DMF: *N,N*-dimethylformamide

DMMP: dimethyl methyl phosphonate

DMP:2,2-dimethoxy propane

DSS: dextran sodium sulfate

DT: D-threo

ESI-MS: electrospray ionization mass spectrometry

EtOAc: ethylacetate

FBS: fetal bovine serum

GalCS: galactosyl ceramide synthase

GalC: galactosyl ceramidase

GCS: glucosyl ceramide synthase

GSL: glycosphingolipid

HBTU: 3-[Bis(dimethylamino)methyliumyl]-3*H*-benzotriazol-1-oxide hexafluorophosphate

HOBt: hydroxy benzotriazole

HRMS: high resolution mass spectrometry

HIV: human immunodeficiency virus

HS: horse serum

$H_2SO_4$: sulfuric acid

IRS-1: insulin receptor substrate-1

$IC_{50}$: concentration for 50% inhibition of enzyme activity

$K_2CO_3$: potassium carbonate

LE: L-erythro

LCS: lactosyl ceramide synthase

LT: L-threo

m: multiplet

MeOH: methanol

MeI: iodomethane

$NaHCO_3$: sodium bicarbonate

NaOMe: sodium methoxide

NGF: nerve growth factor

$NH_2.NH_2.H_2O$: hydrazine monohydrate

NMR: nuclear magnetic resonance

nSMase2: neutral sphingomyelinase 2

o.n.: overnight

PBS: phosphate-buffered saline

PC: phosphatidylcholine

PD: Parkinson's disease

PDMP: 1-phenyl-2-decanoylamino-3-morpholino-1-propanol

r.t: room temperature

q: quartet

quint: quintet

s: singlet

SD: standard deviation

SM: sphingomyelin

SMase: sphingomyelinase

$SOCl_2$: thionyl chloride

SK: sphingosine kinase

SMS: sphingomyelin synthase

SMSr: sphingomyelin synthase related protein

SPHK: sphingosine kinase

SPP: sphingosine 1-phosphate phosphatase

SPT: serine palmitoyl transferase

S1P: sphingosine 1-phosphate

t: triplet

THF: tetrahydro furan

TLC: thin layer chromatography

TNF: tumor necrosis factor

TEA: triethylamine

TrtCl: trityl chloride

UV: ultra violet

VCD: vibrational circular dichroism

$Zn(BH_4)_2$: zinc borohydride

TBLAH: Lithium tri *tert*-butoxy aluminum hydride

# Thesis abstract

Sphingolipids are class of lipids having a backbone of sphingoid base with amide linked fatty acids and having different polar head groups. They are first discovered from the brain extract in the 1870s. Sphingolipids are named after the mythological Sphinx because of their enigmatic nature. They are known to protect cell surface by forming plasma membrane lipid bilayer. These sphingolipids play an important role in signal transmission and cell recognition, involved in many cellular processes such as apoptosis, senescence, differentiation, autophagy etc. Sphingolipids are important biomolecules and they are chiral. Chirality of sphingolipids and understanding their stereochemical effects is very important because they play a very crucial role in the biological system. Most of the drugs are chiral. Naturally the stereochemistry of most common sphingolipid, ceramide has D-*erythro* stereochemistry. So, the Chirality of sphingolipids plays key role in the drug discovery because one enantiomer of the drug may be useful medicine for a disease, but another isomer may be inactive or may be toxic to that disease.

The second chapter of the thesis consists of the study of stereochemical effects of chiral GM3 on induction of neurite outgrowth and their comprehensive synthesis. The synthesis of different isomers of GM3 was performed from possible stereoisomers of sphingosine and study their stereochemical effects on induction of neurite outgrowth with or without NGF in PC12 cells. Neurite outgrowth activity was measured by staining with Coomassie brilliant blue (CBB) and fluorescence imaging with an inverted microscope and fluorescence microscope. All four isomers are enhancing the neurite outgrowth in the presence of NGF and without NGF. The result of the

neurite outgrowth assay suggests that L-*erythro* GM3 inducing more neurite outgrowth as compared to the other three isomers.

Chapter 3 mainly focusing the importance of stereochemistry of ceramides and it was confirmed by creating the chiral ceramide library. To create the ceramide library, four different isomers of sphingosine was used and derivatize with different types of carboxylic acids by using solid-phase synthesis. Total of 128 chiral ceramides was synthesized and performed the sphingomyelin synthase (SMS) assay to understand the inhibitory activity towards SMS1 and SMS2. The cell-based assay of sphingomyelin synthase in the presence of unique chiral ceramides suggested that L-*threo* type ceramides showing higher inhibitory activities towards SMS1 and SMS2.

In chapter 4, novel ceramide library was created by using different types of sphingoid bases with variety of acyl chains. By using solid-phase synthesis, 132 ceramides were created to investigate their inhibitory activities against sphingomyelin synthase1 and 2 (SMS1 &2) using a cell-based assay. We have screened 95 ceramides so far and found two selective and potent inhibitors of SMS2 (DM-16 and DS-8) shows more than 100-fold selectivity. The libraries of this sort will be a rich source of biologically active synthetic molecules.

# Chapter 1

## General Introduction

## 1.1 Sphingolipids

Sphingolipids are major class of lipids that are preserved in all eukaryotic organism and having important role in cell architecture[1]. They were first discovered by J. L. W. Thudichum in 1876 in brain extracts and were named after the mythological sphinx because of their enigmatic nature[2]. They contain backbone of sphingoid bases, derivative of amino alcohol sphingosine, sphinganine or phytosphingosine. Sphingolipids help to define the structural properties of membranes and lipoproteins and also have important roles in cell signaling, cell–cell interaction, cell recognition, and help to regulate cell growth and differentiation[3].

Sphingolipids are amphipathic nature because they have hydrophobic and hydrophilic properties. In the hydrophobic region there is a sphingoid long chain base with a fatty acid chain attached by amide bond at carbon 2. In the hydrophilic region, there are phosphate groups, sugar residues, and hydroxyl groups[4]. This amphipathic nature of sphingoid base allowed for the diffusion of sphingolipids between membranes and for the flipping of sphingolipids between membrane leaflets. There are several classes of sphingolipids differ in the hydrophilic head attached at C-1 as shown in **Figure 1**.

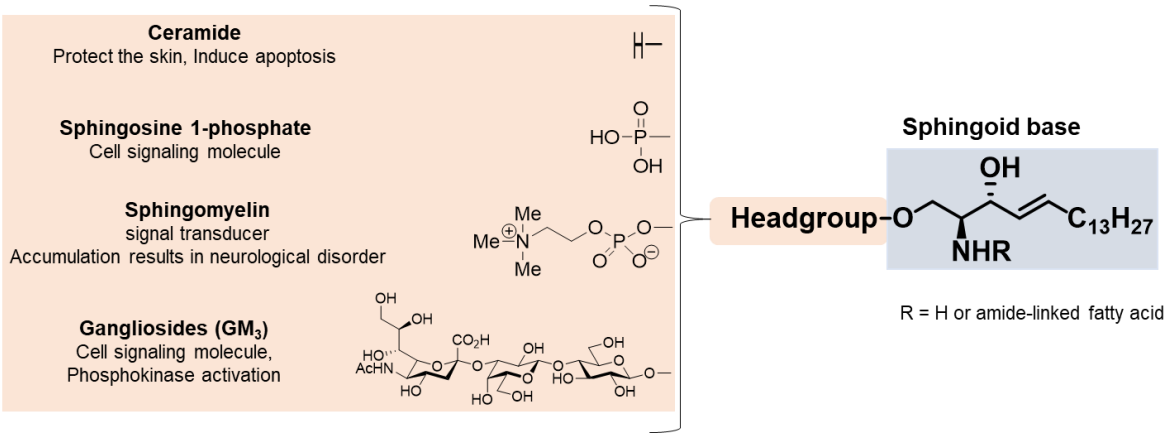


**Figure 1**: General structure of sphingolipids and their important functions

## 1.2 Sphingolipids metabolism

Sphingolipids metabolism has been studied extensively and most of the biochemical pathways of synthesis and degradation, including all the enzymes involved, have been determined successfully[5]. This pathway is an important cellular pathway that represents a highly coordinated system linking together various pathways, where ceramide plays central role in both biosynthesis and catabolism[6]. The sphingolipid metabolism involved in the formation of ceramide and other sphingolipids are represented in **Figure 2**.

The first step of de novo synthesis of sphingolipids involves the production of ceramide by decarboxylation of a serine residue and condensation of fatty acyl-CoA catalyzed by serine palmitoyl transferase (SPT)[7,8]. After the formation ceramide, it converted to various complex sphingolipids by modifying at the 1-hydroxyl group to obtain sphingosine-1-phosphate (S1P), ceramide-1-phospahate (C1P), sphingomyelin, gangliosides (glycosphingolipids) etc[9,10].

In the sphingolipid catabolic pathway, ceramide is generated by the hydrolysis of sphingomyelin, C1P and glycosphingolipids and the constitutive catabolism occurs in the lysosome. The ceramide is then deacylated to sphingosine and converted to sphingosine-1-phosphate by phosphorylation. Finally, S1P cleaves to a fatty aldehyde and ethanolamine phosphate by the action of S1P lyase[11,12].

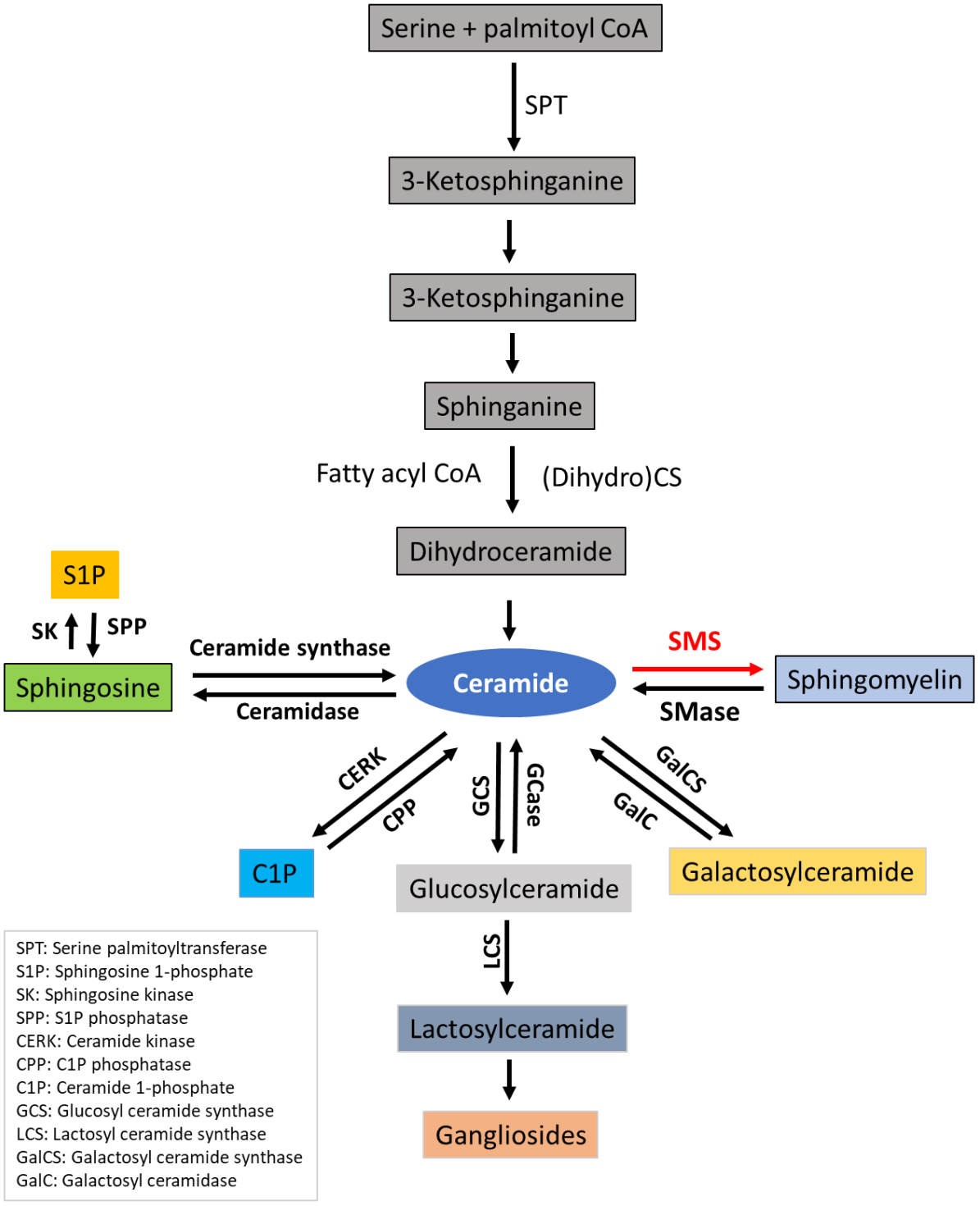


**Figure 2**. Important reactions involved in the sphingolipid metabolic pathway. Ceramide is the central molecule in sphingolipid metabolism giving rise to all the major forms of sphingolipids in the cell.

## 1.3 Types of sphingolipids and their importance

Sphingolipids act as both structural and functional lipids. They have important role as a skin barrier. Their physical properties affect the properties of membranes and lipoproteins. Sphingolipids play important biological functions including the promotion of cell survival through proliferation, the regulation of gene expression related to pluripotency, differentiation, inflammation, apoptosis, cell cycle regulation, cell polarity and migration[13,14, 15.] The simplest sphingolipid, ceramide play a central role in the sphingolipid metabolism having important physiological functions including apoptosis, cell growth arrest, differentiation, cell senescence, cell migration and adhesion[16]. Important types of sphingolipids involve, ceramide, sphingomyelin and gangliosides. (**Figure 3)**

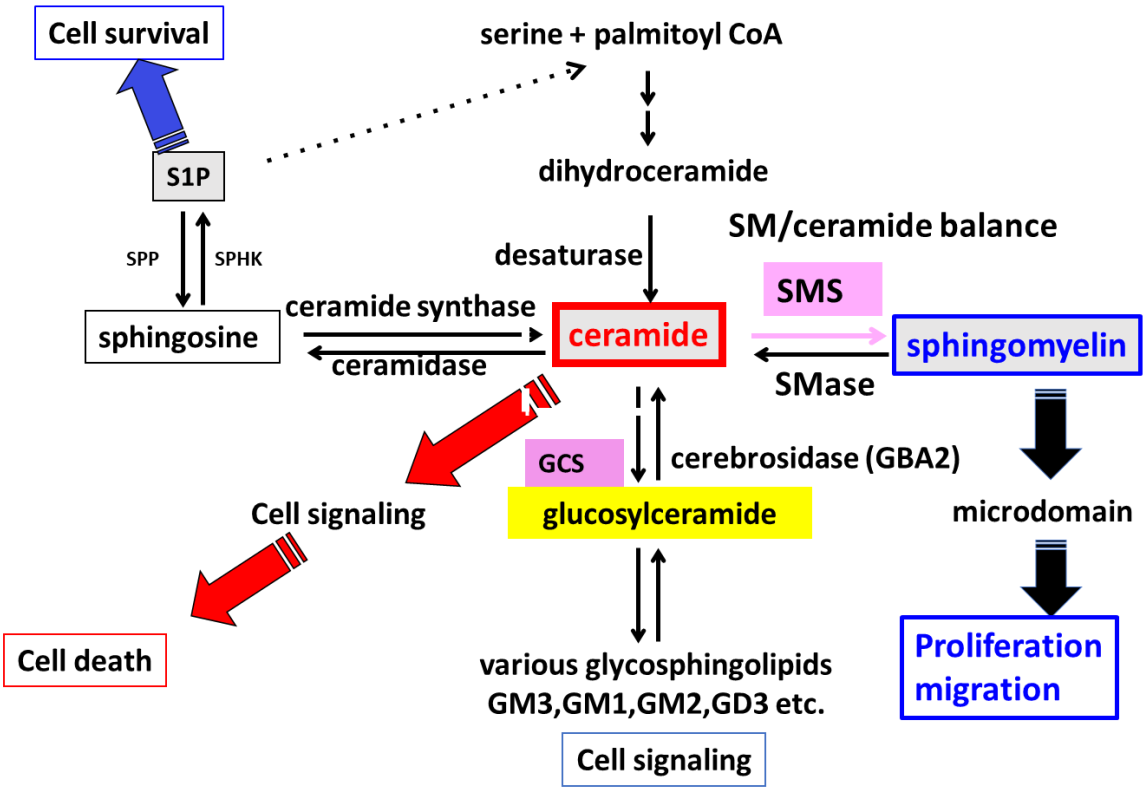


**Figure 3**. Important functions of sphingolipids

## Ceramide

Ceramide is a family of highly hydrophobic molecule composed of sphingosine and a fatty acid and they are synthesized in the endoplasmic reticulum[4]. They are found high in concentrations within the cell membrane of eukaryotic cells Ceramide play a central role in the sphingolipid metabolism for the generation of complex sphingolipids. It has number of important physiological functions that regulate cellular homeostasis including induction of cell differentiation, regulation of cell cycle arrest, apoptosis and regulation of stress response. Roles for ceramide and its downstream metabolites have also been suggested in a number of pathological states including cancer, diabetes, microbial pathogenesis, obesity, and inflammation neurodegeneration. Ceramides can only exist in membranes, but they have a rapid flip rate between leaflets, means that, they cannot leave the organelle they are created in but will have access to binding proteins and enzymes on both sides of the lipid bilayer. So, ceramides can usually only modify by the enzymes that present in the membrane compartment where the ceramides were produced in[17]. It has been reported that ceramide accumulation is found during the treatment of cells with chemotherapeutic agents and UV light and it is signifying that ceramide has an important role as apoptosis inducing effect on cancer cells. Because of this effect ceramide is termed as "**tumor suppressor lipid"**[18].

## Sphingomyelin

Sphingomyelin (SM) is an abundant sphingolipids and important component of cell plasma membrane[19]. It is enriched in the central nervous system (CNS) particularly abundant in the myelin sheath that surrounds neuronal axons. SM hydrolysis releases ceramide and phosphocholine and several stimuli are known to activate

sphingomyelin hydrolysis. It regulates membrane fluidity and microdomain structure and represents about 85% of all sphingolipids in human and it is a major component of plasma membrane lipids (10 to 20%).

SM has very important role along with other sphingolipids in the formation of lipid raft to get more structural rigidity to the plasma membrane as compared to other part of the plasma membrane lipid bilayer. Excess expression of SM in the lipid raft to leads to insulin resistance[20]. It has been reported that SM play an important role in cell processes, in the regulation of inflammatory responses and signal transduction. SM deficiency in the nerve cells leads to multiple sclerosis that affect the signal transmission process.

**Gangliosides**

Gangliosides are glycosphingolipids (ceramide and oligosaccharide) containing one or more sialic acid attached to sugar chain and provide a significant part of cell surface glycans on neuronal cells. The name ganglioside was first applied by the German scientist Ernst Klenk in 1942 to lipids newly isolated from ganglion cells of the brain[21]. They are concentrated on cell surfaces, with the two hydrocarbon chains of the ceramide moiety embedded in the plasma membrane and the carbohydrate part placed on the extracellular surface of the cell and it helps to the cell recognition and cell-cell interaction. Gangliosides are mainly found in the nervous system where they constitute 6% of all phospholipids[22].

The carbohydrate part of the gangliosides is located on the extra cellular membrane and act as distinctive surface markers that can serve as specific determinant factor in cell recognition and cell-cell interaction and act as specific receptors for certain glycoprotein hormones and certain bacterial protein toxins. Gangliosides have

important functions in cancer, especially in the regulation of signal transduction induced by growth-factor receptors in a specific microdomain termed a 'glycosynapse' in the cancer cell membranes, and in interactions with glycan recognition molecules involved in cell adhesion and immune regulation[23]. Common gangliosides are GM1, GM2, GM3 (only one sialic acid), GD1, GD2, GD3 (two sialic acids) and GT1b, GT3 (three sialic acids).

## 1.4 GM3 (monosialodihexosyl ganglioside) and its importance

GM3 is a simplest type of ganglioside. The letter G refers to ganglioside and M refers to monosialic acid. GM3 is the first product in the biosynthetic pathway of ganglio-series gangliosides and common precursor of complex gangliosides.GM3 is located on the outer leaflet of the cell membranes and interact with various molecules on the plasma membrane. GM3 has various important role, help to regulate many important biological processes, including cell proliferation, signal transduction, and differentiation, via formation of functional microdomains in plasma membranes.

GM3 involved in various diseases. It has important pathophysiological functions in disease like cancer, diabetes and neurodegenerative disorders[24]. Recently, it has been reported that the expression of ganglioside GM3, which is synthesized by GM3 synthase enzyme, is increased in obese condition. Regulated GM3 level leads to metabolic disorders such as insulin resistance and type 2 diabetes[25]. Interaction of adipocytes and macrophage leads to increase the GM3 level. The TNF-α secreted from macrophage binds to its receptor in adipocytes enhances the activity of GM3 synthase enzyme and it leads to the accumulation of GM3 level[26]. So, the over expression of GM3 level leads to various diseases like obesity, type 2 diabetes, autoimmune diseases etc. **Figure 4.**

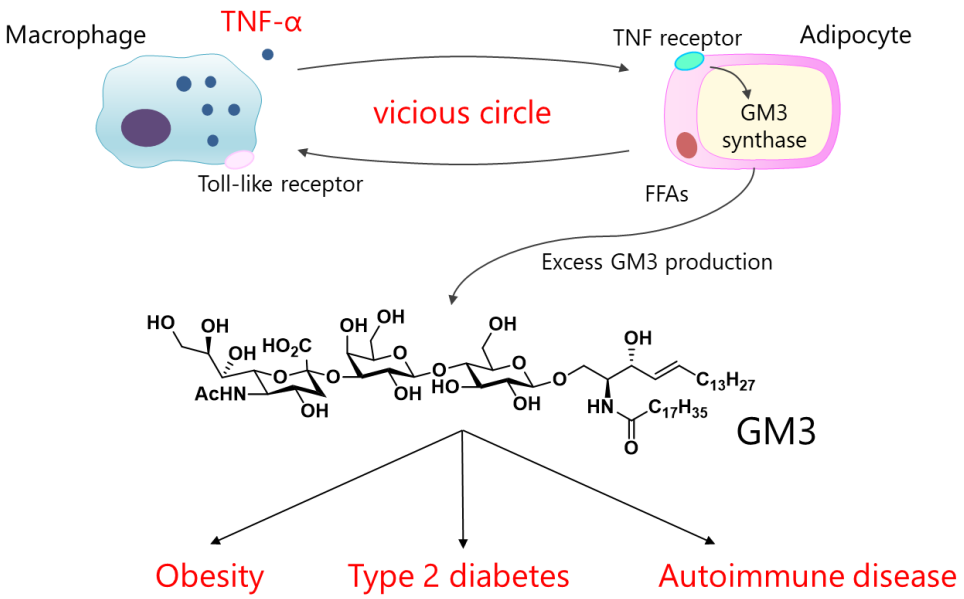


**Figure.4**. Interaction between macrophage and adipocyte upon regulation of GM3 level.

## 1.5 Sphingomyelin synthase and related disorders

Sphingomyelin synthase (SMS) is an enzyme which catalyzes the conversion of ceramide and phosphatidylcholine to sphingomyelin and diacylglycerol. **Figure 5.** Sphingomyelin synthase has three isoforms SMS1, SMS2 and SMSr. SMS1 and SMSr control the ceramide homeostasis in Golgi apparatus and endoplasmic reticulum respectively. The membrane protein, SMS2 associated with metabolic disorder such as obesity, diabetes, atherosclerosis etc[27,28]. SMS2 activity also responsible for the generation of amyloid-beta peptide[29], HIV-1 envelop-mediated membrane fusion[30] and induction of colitis-associated colon cancer[31]. Thus, SMS2 could be a potential therapeutic target for these diseases.

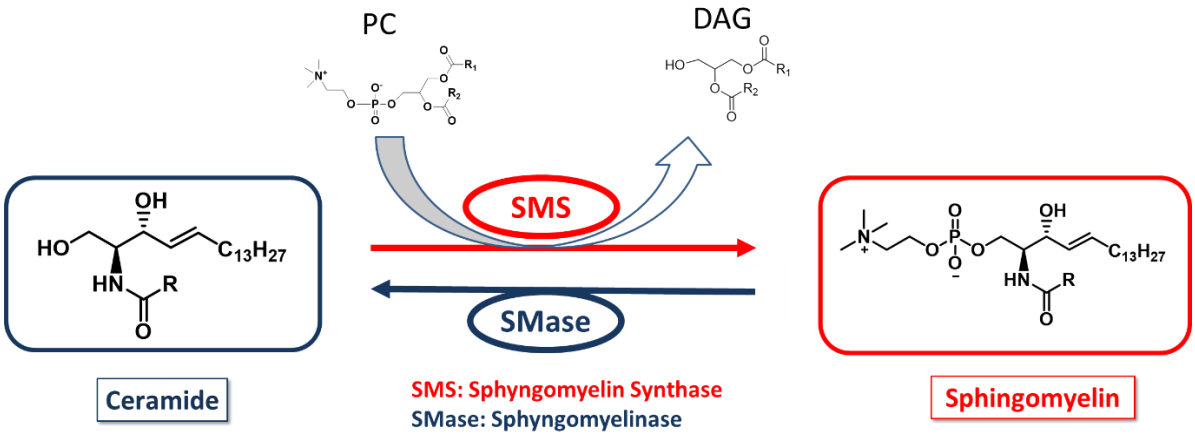


**Figure 5**. Conversion of ceramide to sphingomyelin and SMS act as a catalyst

**SMS and related disorders**

SMS has been involved in metabolic disorders such as insulin resistance, fatty liver, obesity and type2 diabetes[27,28]. The deficiency of SMS2 reduce the formation of fatty liver[32]. SMS2 knockout (KO) experiments in high fat diet induced mice proved that there is drastic decrease in formation of fat around the liver as compared to wild type. **Figure 6a.** SMS2 also involved in Alzheimer's disease, the secretion of neuronal exosomes is modulated by the activities of sphingolipid metabolizing enzymes, neutral sphingomyelinase 2 (nSMase2) and SMS2. Up-regulation of exosome secretion from neuronal cells by treatment of SMS2 siRNA enhanced Aβ (pathogenic agent of Alzheimer disease) uptake into microglial cells and significantly decreased extracellular levels of Aβ. **Figure 6b**. Recent report showed that SMS2 inhibits DSS-(dextran sodium sulfate)-induced colitis and it leads to the decrease of subsequent colitis-associated colon cancer via inhibition of colon epithelial cell–mediated inflammation.[33]

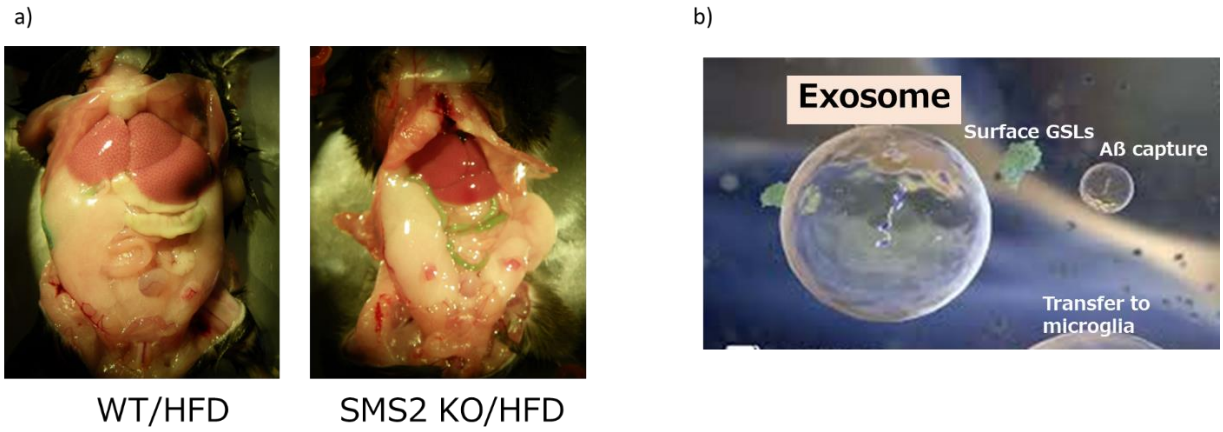


**Figure 6:** (a)SMS2 KO studies of high fat induced mouse liver prevents fatty liver (b) Exosome secretion leads to Aβ uptake.

## 1.6 Sphingolipids and neurite outgrowth

Neurite outgrowth is a process of developing neurons that produce new projections from the cell surface and helps to recover the nervous system after injury. **Figure 7**. Neurite loss is one of the cardinal features of neuronal disease. It has been suggested that the reconstruction of the neuronal and synaptic networks in the injured brain is important for the recovery of the nervous system after injury[34].

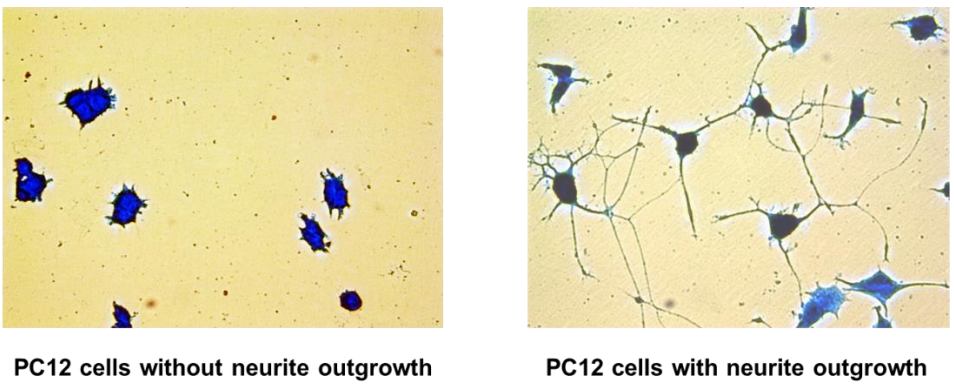


**Figure 7**. Neurite outgrowth in PC12 cells

Previous studies suggest that sphingolipids are induce neurite outgrowth. Gangliosides are enhancing the neurite outgrowth with the presence of NGF and without NGF in PC12 cells[35]. Gangliosides may be incorporated at the level of cell

surface, there by affecting and facilitating membrane phenomena involved neurite outgrowth. Also, GM3 and its analogues enhance neuritogenesis towards PC12 cells[36,37]. All these studies proved that glycosphingolipids are capable of inducing neurite outgrowth may be useful in the treatment of neurological disorders such as Parkinson's disease (PD) and Alzheimer's disease (AD)[38,39]. Hence these compounds can be used for neuroregeneration in various forms of neuronal injury.

## 1.7 Sphingolipids chirality and biological system

Chirality is an important geometric property of some molecules has become major role in the development of drugs[40]. Most of the drugs discovered are chiral. The biological activity of a drug mainly depends on their interaction with biological molecule such proteins, nucleic acid, and bio membrane. Chirality play very crucial role in the drugs because one enantiomer of drug may be a useful medicine for a disease, but another isomer may be inactive or may be toxic to that disease.

Sphingolipids are important biomolecules and they are chiral. Chirality of sphingolipids are very important because they play very crucial role in the biological system. The most common ceramide in mammals consists of the amino alcohol sphingosine as a backbone moiety, which has two chiral centers at the C-2 and C-3 positions. Theoretically, there are four stereoisomers, (D-*erythro*), (L-*erythro*), (D-*threo*) and (L-*threo*), in sphingosine, but only D-*erythro* type [(2*S*, 3*R*, 4*E*)-2-aminooctadec-4-ene-1,3-diol] has been found in mammals. **Figure 8**.

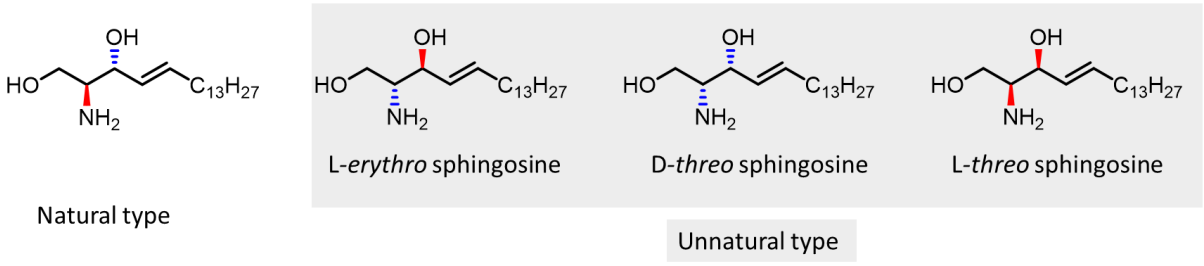


**Figure 8**. Stereoisomers of sphingosine

As per the previous report, eight stereoisomers of C2-ceramide and dihydroceramide were showing different cellular activity[41]. The four stereoisomers of C2-ceramides were active in inhibition of cell growth and induction of apoptosis but in the case of C2-dihydroceramide only the *threo* type compounds were showing activity in these assays whereas the *erythro* compounds completely inactive. In addition to this, the important inhibitor of GCS (glucosyl ceramide synthase), PDMP showed an interesting stereochemical effect on GCS. Out of four isomers of PDMP, only D-*threo* PDMP showing inhibition of GCS[42] and L-*threo* showing enhancement of GCS activity[43]. **Figure 9**. So, the inhibitor binds stereospecifically to the ceramide-binding part of the active site and at some modifying site on the enzyme molecule.

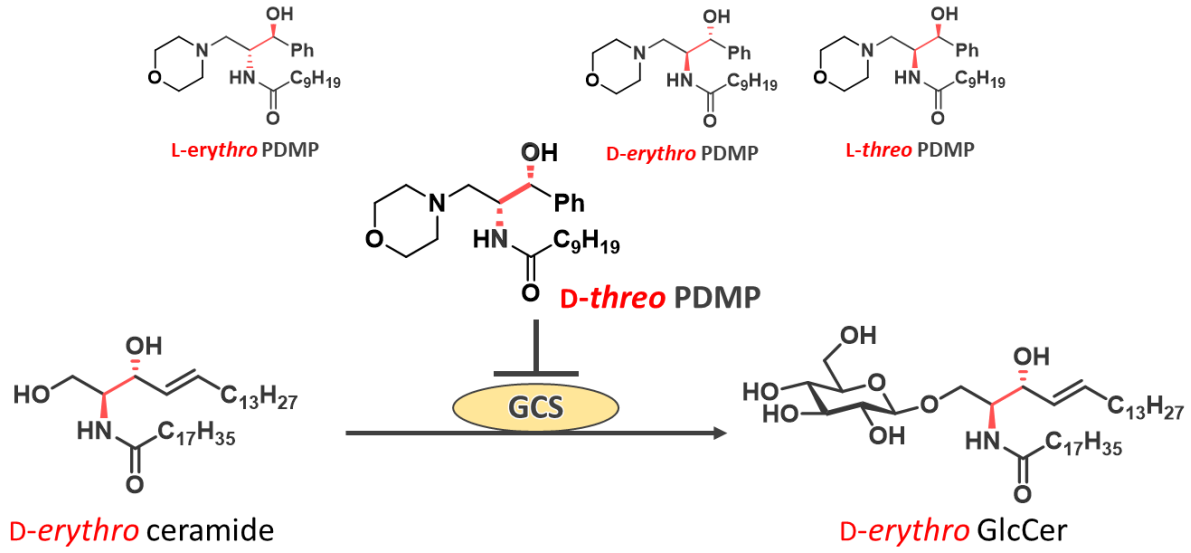


**Figure 9**. Inhibition of GCS by D-*threo* PDMP. Out of four isomers of PDMP only D-*threo* type showing inhibition of GCS

All these studies revealed that chirality of sphingolipids is very important aspects for the development of drugs. There are very few studies reported regarding the chirality of sphingolipids. Sphingolipids chirality and their stereochemical effects could be an emerging field for the development of drug discovery. Chiral molecules behave differently to the biological system depends on their stereochemistry.

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# Chapter 2

## Stereochemical effects of chiral ganglioside GM3 on induction of neurite outgrowth

## 2.1 Abstract

GM3 is one of simple mono-sialylated ganglioside (NeuAca2-3Galb1-4Glcb1-1'-ceramide) and its aberrant expression in adipocytes is involved in a variety of physiological and pathological processes of diabetes mellitus and obesity. Due to GM3 exposes on the outer surface of cell membranes, which deeply associates with type 2 diabetes and insulin resistance. Neurite outgrowth is a key process in the development of functional neuronal circuits and neuroregeneration after nerve injury. Therefore, regulation of GM3 level of nerve tissues may lead to treatment of these disorders. Here, we demonstrated comprehensive synthesis of chiral GM3 isomers and confirmed their biological activities inducing neurite outgrowth, which provides the valuable sights for potential neuroregenerative treatment.

## 2.2 Introduction

Gangliosides, which are a family of one or more sialic acid-containing glycosphingolipids, have numerous crucial functions within fundamental processes of human physiology. Gangliosides are very important in biochemical signaling and appear to play a crucial role in virtually everywhere, including cell growth, proliferation, differentiation, adhesion, senescence, and apoptosis[1]. Recently, GM3 is one of simple mono-sialylated ganglioside (NeuAca2-3Galb1-4Glcb1-1'-ceramide) has been reported that it could help relieve symptoms of cancer[2], diabetes and Alzheimer disease[3]. Particularly, it has been reported GM3 suppressed LPS-induced inflammatory response in RAW264.7 macrophages by suppressing the TNF-$\alpha$, NF-kB and MAPKs signaling[4]. Also, GM3 and its analogue have regulated neurite outgrowth which is a key process in the development of functional neuronal circuits and regeneration of the nervous system after injury[5]. For example, in mouse neuroblastoma Neuro2a cells, previous studies showed that GM3 enriched microdomains were involved in signal transduction during neurite formation[6]. In addition to those facts, several reports have also reported evaluating the potential effect of GM3 in neurite outgrowth[7]. Therefore, the development of novel diagnosis of metabolic syndrome by identifying the specific ganglioside species is expected for a therapeutic strategy "membrane microdomain ortho-signaling therapy".

Research so far in gangliosides were almost focused on a significance of the carbohydrate chain, because carbohydrate modification not only affects protein structure, function and stability, but also leads to the phenomena causing a variety of diseases[8]. By the way, no study up to now has focused on the stereochemistry of sphingosine moiety in GM3 to possess its biological activities. In addition, there is only a study that an inhibitor (D)-*threo*-1-phenyl-2-decanoylamino-3-morpholino-

1propanol (D-PDMP) of glucosylceramide synthase to suppress GM3 in adipocytes showed the TNF-α induced defect in insulin-dependent tyrosine phosphorylation of insulin receptor substrate-1 (IRS-1) reported by Inokuchi and Radin[9]. Notably, the stereochemistry of active D-PDMP is different from that of endogenous ceramide. Based on this information, the different types of stereoisomers in GM3 also could behave as the potential therapeutic agents to recover nervous functionality after injury. In this paper, to address these unclear points, we report the first synthesis chiral GM3 isomers and application of them to the biological evaluation for the effects of the neuritogenesis. (**Figure 1**).

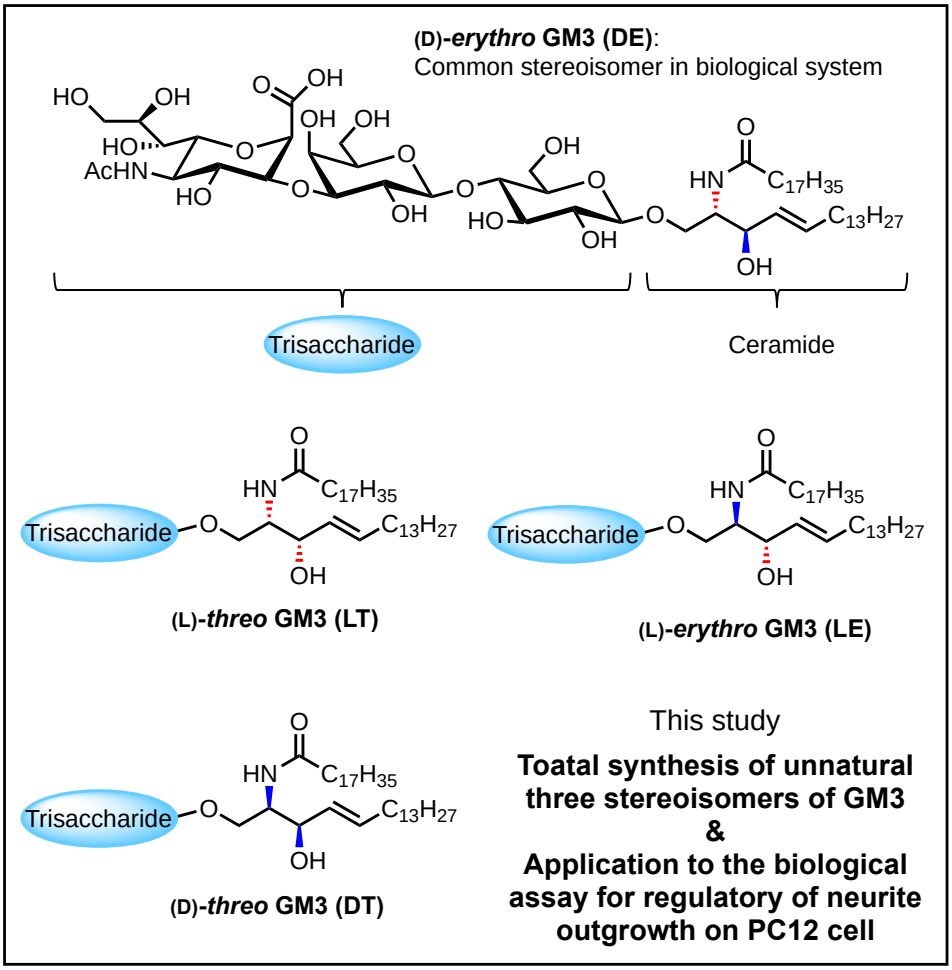
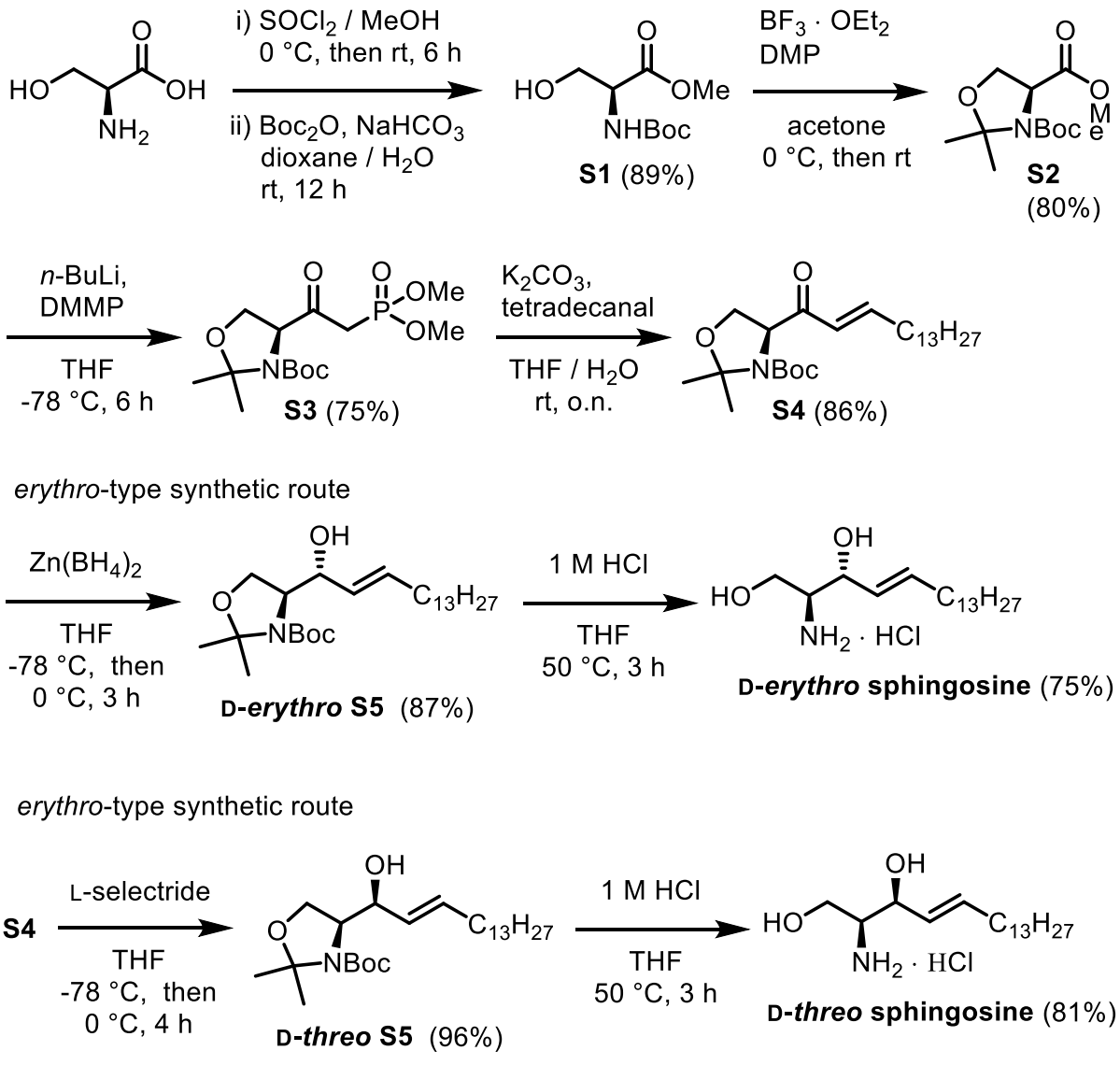



**Figure 1**. Structures of common GM3 structure in biological system and unnatural three stereoisomers of GM3.

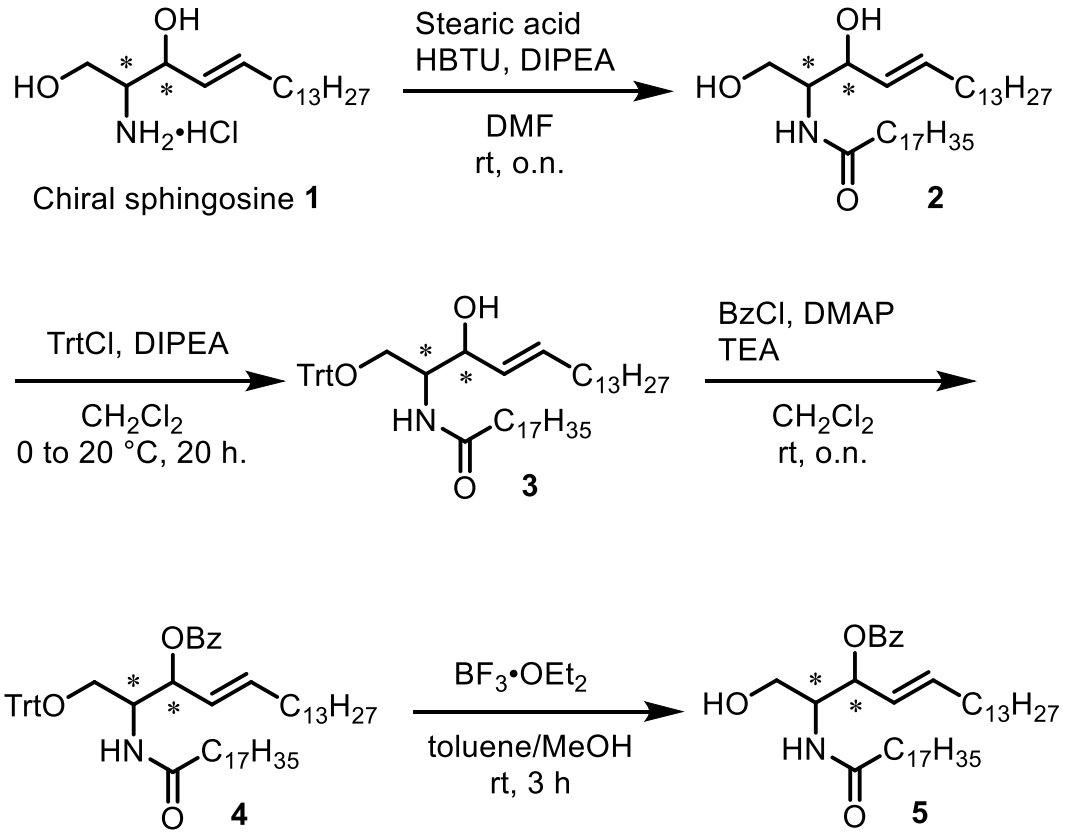
## 2.3 Results and discussion

Previously, Nishimura and Yamada reported a chemo-enzymatic synthesis of GM3 using water soluble polymer[10]. In this case, common D-*erythro* sphingolipid in biological system was used as a substrate because of substrate specificity for ceramide glycanase. Therefore, other three stereoisomers of sphingolipid were expected not to apply to chemo-enzaymatic method, we prepared three stereoisomers of GM3 by chemical synthesis. Initially, other three stereoisomers of sphingosine were prepared according to slightly modified well established methods from (L)- or (D)-serine[11] (**Scheme 1**).



**Scheme 1**: Synthesis of D-*erythro* and -*threo* sphingoid bases. L-*erythro* and -*threo* sphingoid bases were prepared from D-serine in the same manner as shown above scheme.

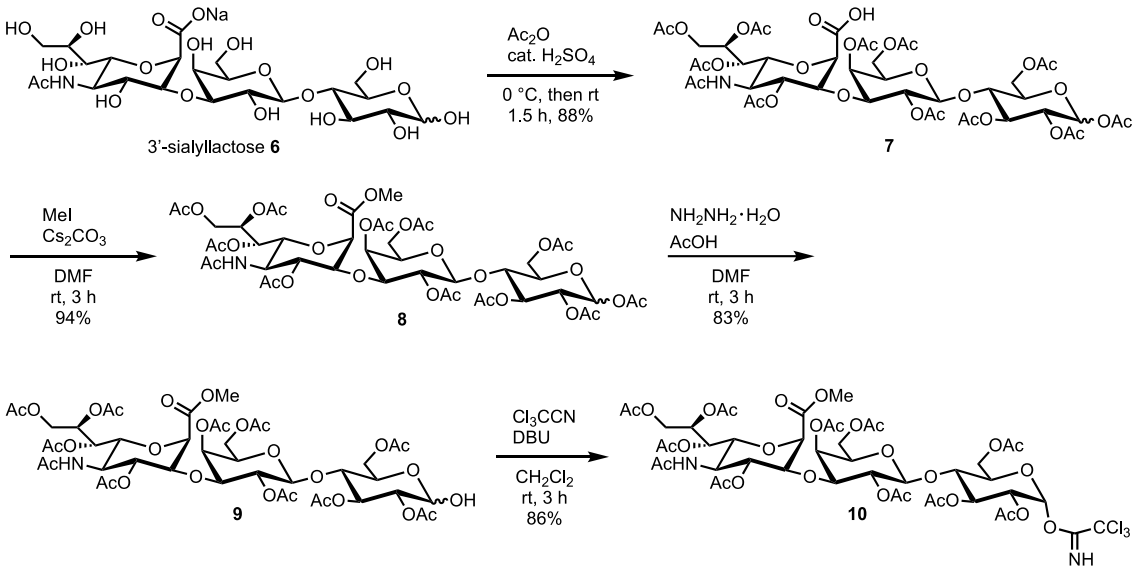
Chiral sphingosines **1** were subjected to acylation with stearic acid by a standard manner to afford chiral ceramides **2**. Subsequently, the primary alcohol in **2** was selectively protected with trityl group **3**, followed by the secondary alcohol protection with benzoyl group **4** and detritylation to afford chiral ceramide derivatives **5** in moderate yield (**Scheme 2**).




**Scheme 2**. Synthesis of benzoyl-protected ceramide derivative **5** for glycosylation acceptor.

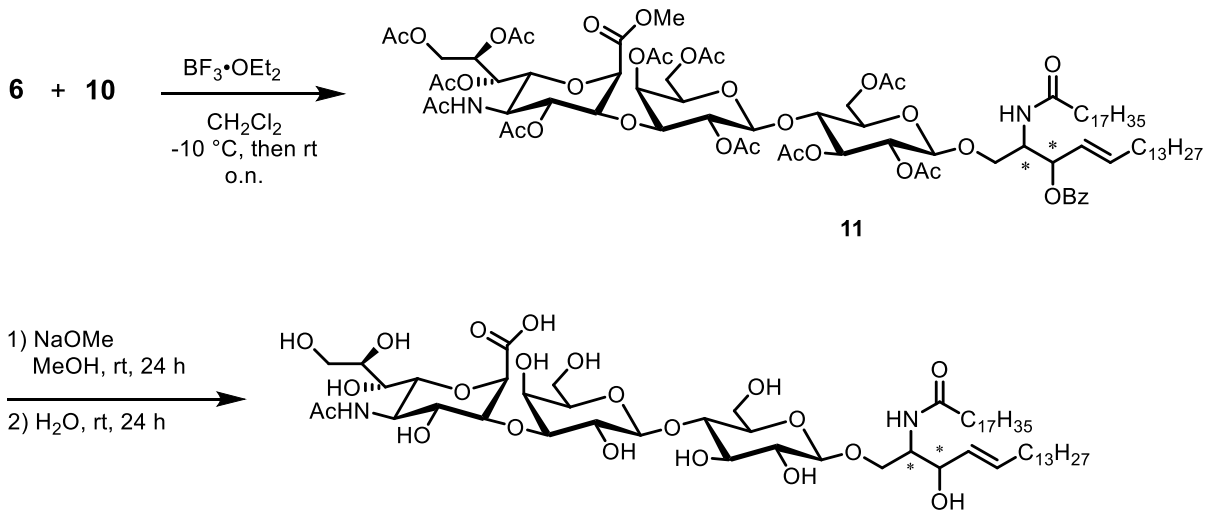
On the other hand, one side partner for glycosylation, trichloroacetimidate trisaccharide **10** was prepared from commercial 3'-sialyllactose **6**. Compound **6** was subjected to O-acetylation of hydroxy groups with $Ac_2O$ in catalytic amount of $H_2SO_4$

to afford acid **7** in high 88% yield without lactone formation. Subsequently, the carboxyl group in **7** was protected with methyl ester **8**, then 1'-OAc group in **8** was hydrolyzed with hydrazine to afford alcohol **9** in good yield through two step reactions. Compound **9** was efficiently transformed into the glycosyl donor, trichloroacetimidate trisaccharide **10** (**Scheme 3**).




**Scheme 3**. Synthesis of trichloroacetimidate trisaccharide derivative (**10**) for glycosylation donor.

Finally, the glycosylation with chiral ceramide (**6**) and **10** was employed in the presence of $BF_3 \cdot OEt_2$ to obtain the desired β-linked O-acylated chiral GM3 derivatives **11** in major product. The each of anomeric configurations were determined by $^1$H-NMR *J* value of 7.40-7.82 Hz. Finally, O-protected chiral GM3 **11** was subjected to deprotection with sodium methoxide and quenched by Amberlite IR-120 ($H^+$ form) to obtain three stereoisomers of GM3 in quantitative yield (**Scheme 4**).




**Scheme 4**. Synthesis of chiral GM3 isomers through the glycosylation between chiral ceramides **6** and activated trisaccharide **10**.

Next, we evaluated the cytotoxicity of chiral GM3 isomers towards PC12 cell. Cell viability was measured by using a cell counting kit-8 (CCK-8). As shown in **Figure. 2**, all the stereoisomers exhibited comparable cytotoxicity towards the cells as what normal (D)-*erythro* GM3 did at the same dose (positive controls). These results indicate that three stereoisomers of GM3 were expected to be useful as metabolic substrates and can assure their potential for further biological studies.

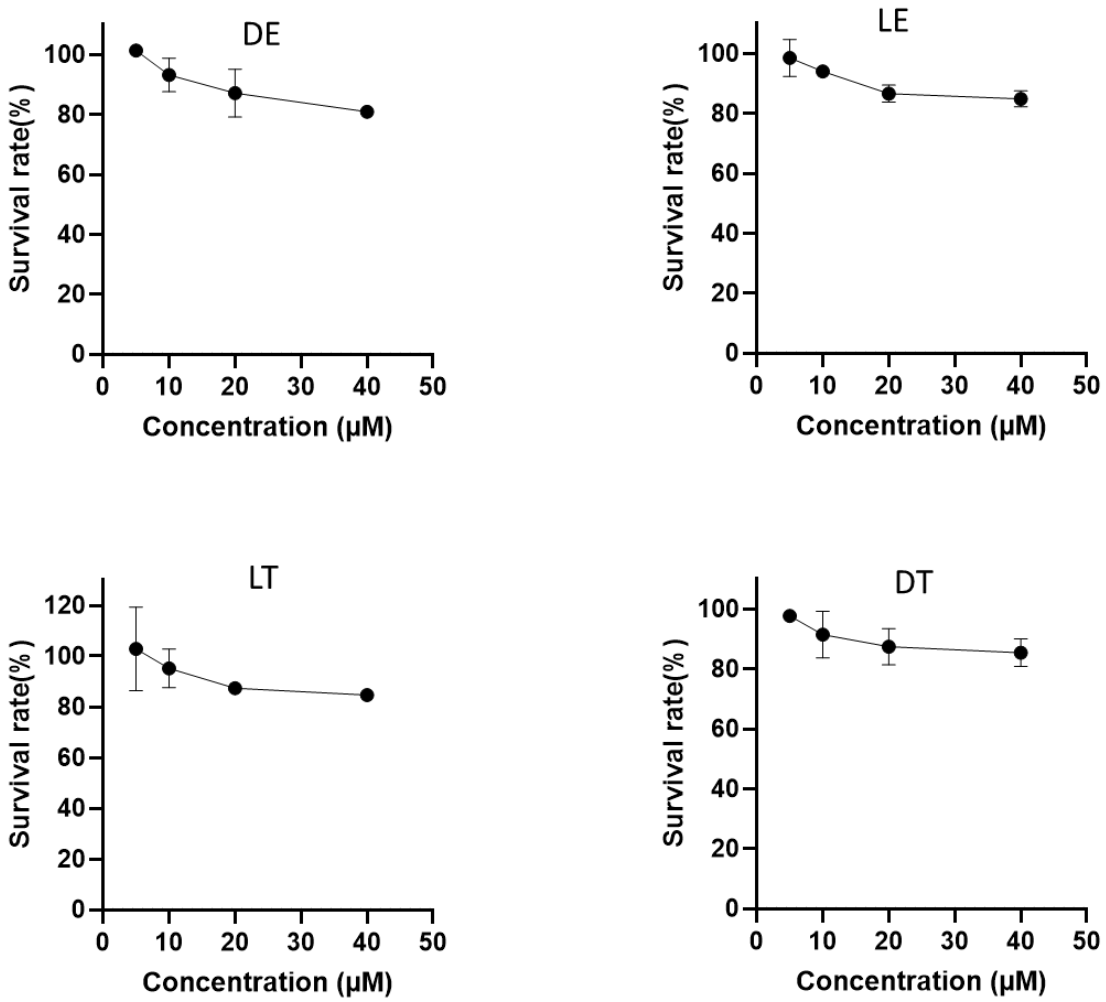
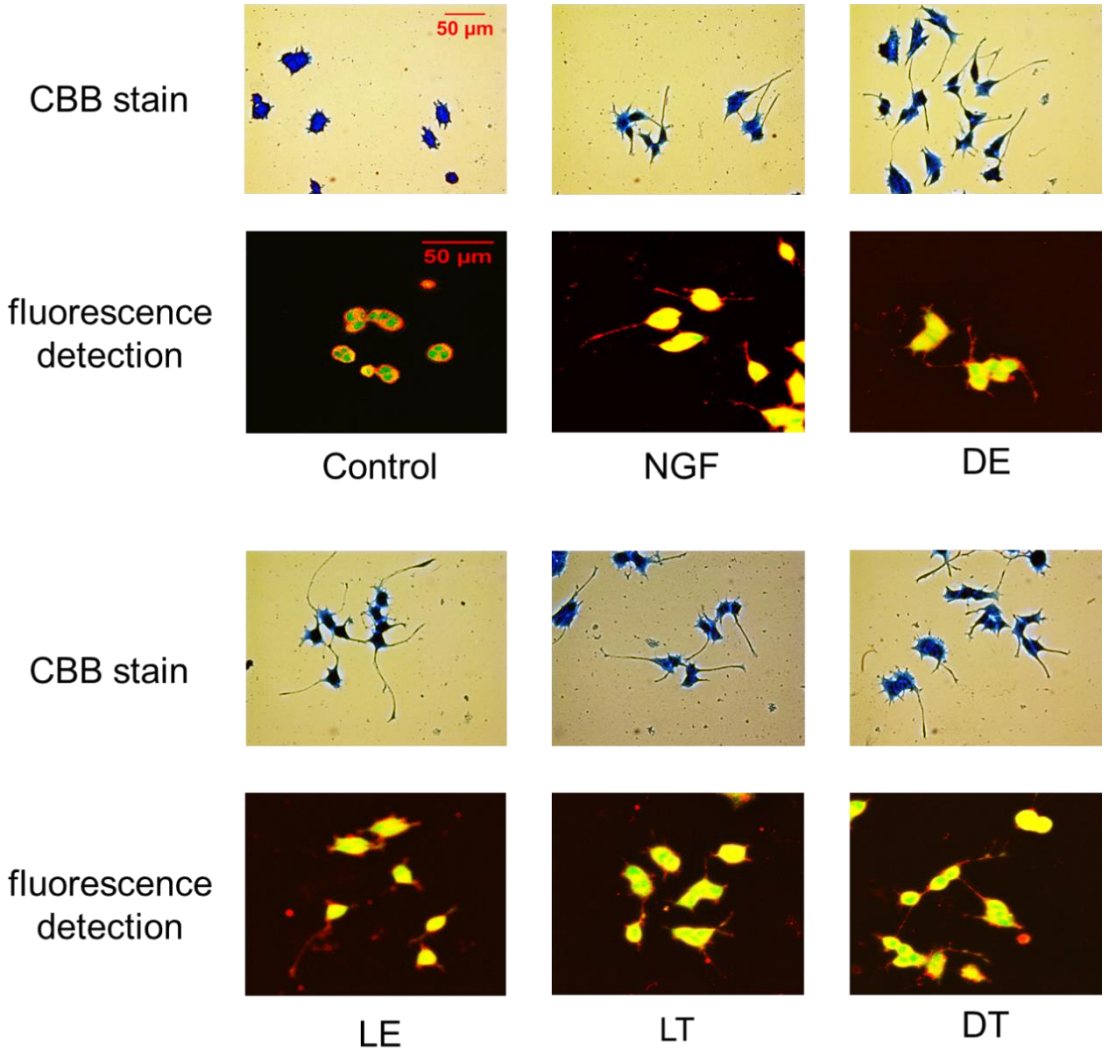



**Figure 2**. Viability assays of PC12 cells exposed to three stereoisomers of GM3 and control normal (D)-*erythro* GM3. Cell viability were tested after 24 hours of treatment with four compounds at concentration levels of 5-40 μM. Data are presented as the mean ± standard deviation (SD).

Subsequently to confirm physical phenomenon and evaluation of three chiral GM3s, initially we performed acceleration of neurite outgrowth assay triggered by chiral GM3s without NGF induced model. The activities were measured by staining with coomassie brilliant blue and fluorescence imaging with an inverted microscope and fluorescence microscope. Then, in this assay, all chiral GM3s were confirmed to enhance neurite outgrowth higher than only NGF addition (**Figure 3**). Interestingly, in

adding (L)-*erythro*-GM3, the neuritogenesis was augmented most strongly comparing to other chiral GM3s. The percentage of neurite length with L-*erythro*-GM3 was 37% higher beyond the reach of NGF treatment.



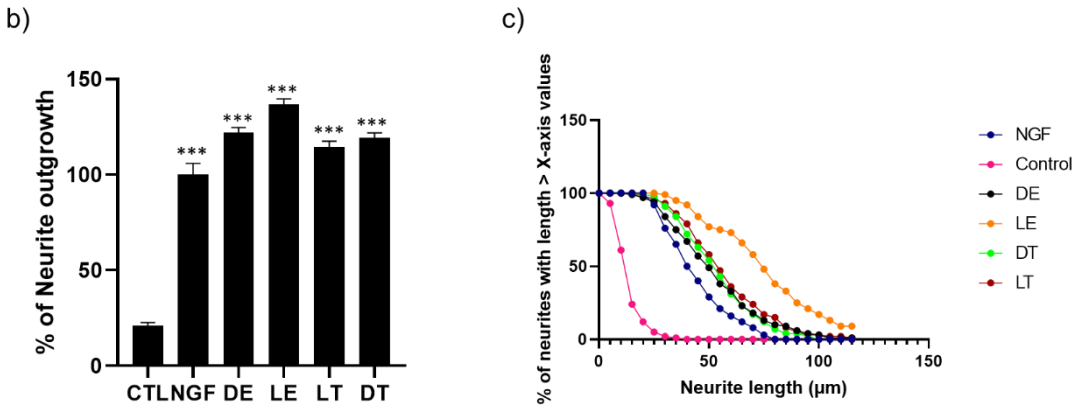



**Figure 3**: PC12 cells exhibit an enhanced neurite outgrowth by three chiral GM3s. a) Neurite outgrowth activity was measured by staining with CBB and fluorescence detection. The length of each neurite was measured, and the mean neurite length was calculated as the percentage of neurite outgrowth activity by red scale-bar, 50 μm. b) Quantification of the percentage of neurite outgrowth on PC12 in each group were shown. Results are presented as means ± S.D., and experiments were repeated at least 5 times. ***, $p < 0.001$. c) Distribution of the neurite lengths in cells following differentiation in the presence of each chiral GM3, NGF or no treatment. The data represent the means of five individual experiments with standard deviations.

Furthermore, we then investigated the effect of chiral GM3s on neurite outgrowth in the NGF-induced experiment at the same manner. The results were shown in f**igure 4**. All chiral GM3s could also assist neuritogenesis in the presence of NGF. It seemed that (L)-*erythro*-GM3 showed the highest neuritogenesis effect comparing to other chiral isomers, the percentage of it reached 50% more than that of only NGF treatment. These data indicated that chiral GM3s potently enhance neurite outgrowth in PC12 cells both in NGF free and NGF-induced conditions.

In the present study, we firstly demonstrated that chiral chemistry on GM3 (gangliosides) could affect the enhancement of neurite outgrowth. The ability of chiral chemistry on gangliosides to affect nerve systems can provide a valuable sight

for developing potential therapeutic agents to recover nervous functionality after injury.

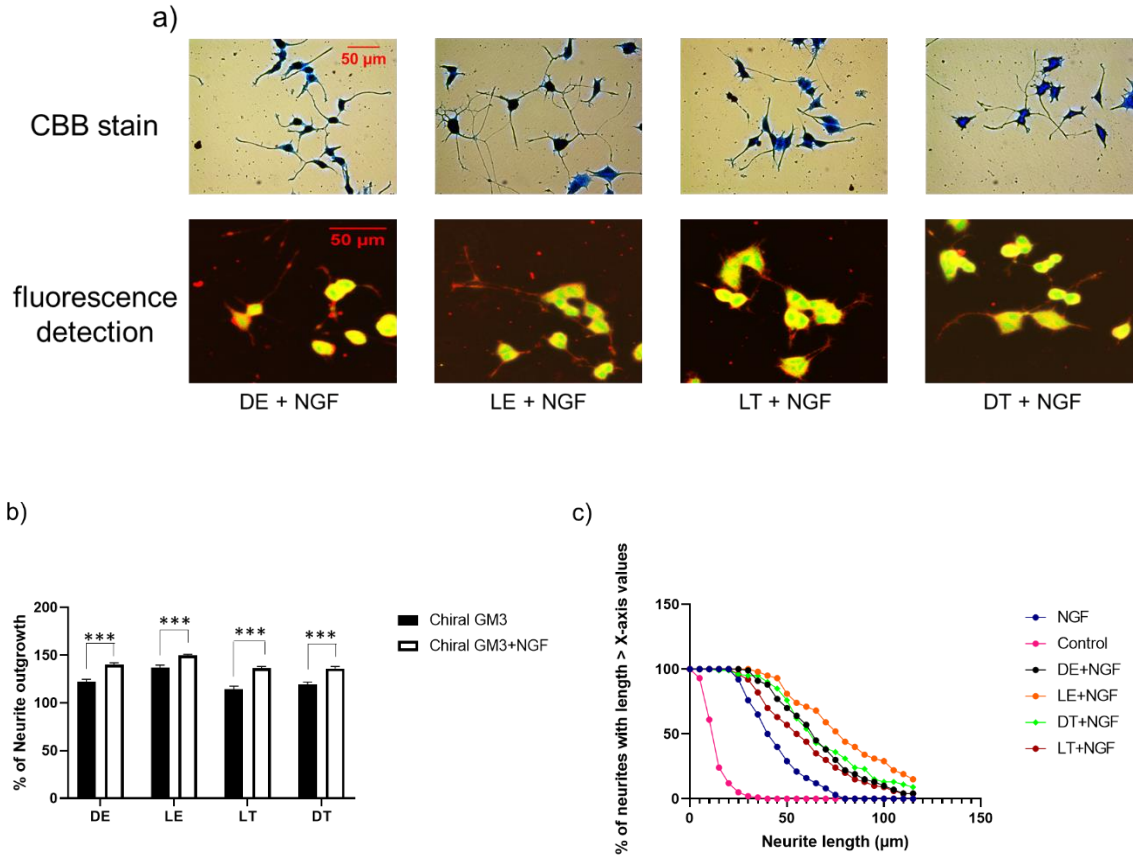



**Figure 4**: PC12 cells promoted NGF-induced exhibit an enhanced neurite outgrowth by three chiral GM3s. a) Neurite outgrowth activity was measured by staining with CBB and fluorescence detection. The length of each neurite was measured, and the mean neurite length was calculated as the percentage of neurite outgrowth activity by red scale-bar, 50 μm. b) Quantification of the percentage of neurite outgrowth on PC12 in each group were shown. Results are presented as means ± S.D., and experiments were repeated at least 5 times. ***, $p < 0.001$. c) Distribution of the neurite lengths in cells following differentiation in the presence of each chiral GM3, NGF or no treatment. The data represents the means of five individual experiments with standard deviations.

## 2.4 Conclusion

We have described an efficient pathway for the synthesis of three stereoisomers of GM3. A cytotoxicity activity test by the CCK-8 assay showed that the prepared compounds scarcely exhibited cytotoxicity towards the cells as did normal (D)-*erythro* GM3. In addition, they showed as a promising induction of neuritogenesis on PC12 cells. In the future Chiral GM3s and gangliosides are expected as a beneficial molecular agent for the treatment of recovery nerve functionality after injury because neurite outgrowth is a key process in the development of functional neuronal circuits and regeneration of the nervous system after injury.

## 2.5 Experimental section

### *Cell culture*

Rat adrenal pheochromocytoma PC12 cells were obtained from JCRB Cell Bank (Osaka, Japan). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 50 mL 10% fetal bovine serum (FBS), 50 mL 10% horse serum (HS), and 5mL penicillin–streptomycin solution (Wako. Co., Osaka, Japan), maintained at 37 °C in 5% $CO_2$.

### *Cytotoxicity assay for PC12*

The cytotoxicity of chiral GM3 for PC12 cells was estimated by using Cell Counting Kit-8 (CCK-8; Dojindo Laboratories). After trypsinization, 5.0 $\times 10^5$ cells/mL (100 μL; $5.0 \times 10^4$ cells/well) of PC12 cells were added to wells in 96-well plate and this well plate was incubated at 37 °C, 5% $CO_2$ overnight. After incubation removed the medium and added 100 μL of media containing each sample with different concentrations like 40 μM, 20 μM, 10 μM and 5 μM. The microplate kept for Incubation for 24 h and added 10μL CCK-8 reagent to each well on the 96-well microplate. Placed in a CO2 incubator for 2 h to react and measured an absorbance at 450 nm by using microplate reader. The graph plotted with concentrations of sample on the X-axis and absorbance on the Y-axis.

### *Neurite outgrowth assay (CBB stain)*

PC12 cells were cultured in poly-L-lysine precoated 12-well plates with 400 mL Dulbecco's modified Eagle's medium (DMEM) supplemented with 50 mL 10% fetal bovine serum (FBS), 50 mL 10% horse serum (HS), and 5mL penicillin–streptomycin solution (Wako. Co., Osaka, Japan), maintained at 37 °C in 5% $CO_2$. Cells were washed with fresh serum-free DMEM and incubated for 72 h with serum free DMEM

containing 10 μM of chiral GM3s in the presence of NGF (100ng/mL) or not. After removing the supernatant by suction, phosphate-buffered saline (PBS) solution containing 2 % glutaraldehyde was gently overlaid to fix cells at room temperature for 20 min. The glutaraldehyde solution was changed to 1 % Coomassie Brilliant Blue (CBB) G-250 solution (1 % CBB in 50 % methanol/PBS), and cells were incubated for 2 h at room temperature. Then, cells were destained with 50 % methanol/PBS, and washed with PBS. Bright-field cell images were obtained using light microscopy at 20-fold magnification (BZ-X700All-in-one Fluorescence Microscope, KEYENCE, Osaka, Japan), and the neurite length was measured by ImageJ as percentage of neurite outgrowth activity.

***Neurite outgrowth assay (fluorescent stain)***

The PC12 cells were stimulated in the Poly-L-lysine pre-coated 35mm dishes at $7.5\times10^3$ cells/dish and incubated in DMEM with 10% Horse Serum, 10% Fetal Bovine Serum and 1% Penicillin/streptomycin for overnight. The cells were washed with serum-free DMEM and incubated for 72 hours with 10 μM of chiral GM3s in serum-free DMEM containing NGF (100 ng/mL) and without NGF. After incubation, remove the medium by suction from each dish. The staining assay was performed by using the neurite outgrowth staining assay kit. Cell viability indicator and cell membrane stain in PBS containing calcium and magnesium (1.5 μl of each cell viability indicator and cell membrane stain in 1.5 mL PBS) was applied to the dishes. Incubated for 20 minutes and remove the stain solution. Background suppression dye (15 μL in 15 mL PBS containing magnesium and calcium) was applied to each dish to be assayed. Visualize the cells and obtained the images by using FLUO VIEW FV 10i automated confocal laser scanning microscope.

**Experimental procedure**

***N*-((2*S*,3*S*,*E*)-1,3-dihydroxyoctadec-4-en-2-yl)stearamide (2a)**

A solution of stearic acid (129 mg, 0.45 mmol) in DMF was cooled to 0 °C. HBTU (172 mg, 0.45 mmol) and DIPEA (79 µL, 0.45 mmol) were added to the solution. After stirring at this temperature for 5 min, a solution of L-*threo* sphingosine (113 mg, 0.38 mmol) in DMF (5 mL) was added drop wise at 0 °C. The resulting mixture was stirred for overnight at room temperature under $N_2$ atmosphere. The reaction mixture was diluted with EtOAc and washed successively with 10% citric acid (2 x 20 mL), 1M NaOH (2 x 20 mL) and brine. The organic layer was dried over $MgSO_4$ and evaporated, then the residue was chromatographed on silica gel (*n*-hexane/EtOAc = 1:1). Compound 2a (153 mg, 72%) was obtained as a white solid. $^1$H NMR (500 MHz, $CDCl_3$) $\delta$ ppm 6.12 (d, *J*=7.58 Hz, 1 H), 5.72 - 5.80 (m, 1 H), 5.48 (dd, *J*=15.27, 6.23 Hz, 1 H), 4.40 (m, 1 H), 3.93 (m, 1 H), 3.82 (m, 2 H), 2.23 (t, *J*=6.96 Hz, 2 H), 2.04 (q, *J*=6.60 Hz, 2 H), 1.63 (m, 2 H), 1.36 (m, 2 H), 1.22 - 1.33 (m, 48 H), 0.89 (t, *J*=5.99 Hz, 6 H). $^{13}$C NMR (126 MHz,$CDCl_3$) $\delta$ 174.2, 134.0, 129.2, 73.1, 64.4, 54.9, 36.9, 32.3, 31.9, 29.7, 29.6, 29.5, 29.4, 29.3, 29.2, 25.8, 22.7, 14.0. HRMS (*m/z*): $[M+H]^+$ calculated for $C_{36}H_{72}NO_3$: 566.5506, found 566.5490.

***N*-((2*R*,3*S*,*E*)-1,3-dihydroxyoctadec-4-en-2-yl)stearamide (2b)**

In tha same manner as described for 2a, 2b was synthesized from L-*erythro* sphingosine. Compound 2b (183 mg, 70%) was obtained as a white solid.

$^1$H NMR (500 MHz, $CDCl_3$) $\delta$ 6.24 (d, *J*=7.33 Hz, 1H), 5.75 - 5.83 (m, 1H), 5.54 (dd, *J*=6.48, 15.52 Hz, 1H), 4.29 - 4.33 (m, 1H), 3.94 (dd, *J*=3.91, 11.00 Hz, 1H), 3.89 – 3.92 (m, 1H), 3.71 (dd, *J*=3.18, 11.00 Hz, 1H), 2.85 (br. s, 1H), 2.23 (t, *J*=7.58 Hz, 2H), 2.06 (q, *J*=6.93 Hz, 2H), 1.60 - 1.68 (m, 2H), 1.36 - 1.42 (m, 2H), 1.22 - 1.35 (m,

48H), 0.89 (t, *J*=6.97 Hz, 6H). $^{13}$C NMR (126 MHz, $CDCl_3$) δ 174.2, 134.5, 129.1, 74.8, 62.7, 54.9, 37.1, 32.5, 32.2, 30.0, 29.9, 29.8, 29.7, 29.6, 29.5, 29.4, 26.0, 22.9, 14.3. HRMS (*m/z*): $[M+H]^+$ calculated for $C_{36}H_{72}NO_3$: 566.5506, found 566.5492.

***N*-((2*R*,3*R*,*E*)-1,3-dihydroxyoctadec-4-en-2-yl)stearamide (2c)**

In tha same manner as described for 2a, 2c was synthesized from D-*threo* sphingosine. Compound 2c (221 mg, 69%) was obtained as a white solid.

$^1$H NMR (500 MHz,$CDCl_3$) δ 6.15 (d, 1H), 5.70 - 5.80 (m, 1H), 5.47 (dd, *J*=6.35 Hz, *J*=15.64 Hz, 1H), 4.39 (dd, *J*=3.54, 5.74 Hz, 1H), 3.88 - 3.95 (m, 1H), 3.77 - 3.83 (m, 2H), 2.22 (t, *J*=7.82 Hz, 2H), 2.04 (q, *J*=6.84 Hz, 2H), 1.57 - 1.67 (m, 2H), 1.20 - 1.40 (m, 50H), 0.85 - 0.92 (m, 6H). $^{13}$C NMR (126 MHz, $CDCl_3$) δ 174.3, 133.8, 129.1, 72.9, 64.2, 54.8, 36.9, 32.3, 31.9, 29.7, 29.6, 29.5, 29.4, 29.3, 29.2, 25.8, 22.7, 14.1. HRMS (*m/z*): $[M+H]^+$ calculated for $C_{36}H_{72}NO_3$: 566.5506, found 566.5497.

***N*-((2*S*,3*S*,*E*)-3-hydroxy-1-(trityloxy)octadec-4-en-2-yl)stearamide (3a)**

To a solution of L-*threo* ceramide 2 (140 mg, 0.25 mmol) and DIPEA (65 µL, 0.37 mmol) in dry $CH_2Cl_2$ (5 mL) was added TrtCl (76 mg, 0.27 mmol) in $CH_2Cl_2$ (2 mL) slowly over 15 min. The reaction mixture was maintained below 20 °C and stirred for 20 h. The mixture was evaporated and purified by silica column chromatography (*n*-hexane/EtOAc = 4:1) to obtain compound 3 (160 mg, 80%) as pale-yellow sticky oil. $^1$H NMR (500 MHz, $CDCl_3$) δ 7.42 (d, *J*=7.82 Hz, 6H), 7.32 (t, *J*=7.33 Hz, 6H), 7.23 - 7.27 (m, 3H), 5.98 (d, *J*=8.31 Hz, 1H), 5.68 - 5.76 (m, 1H), 5.38 (dd, *J*=5.86, 15.40 Hz, 1H), 4.37 (m, 1H), 3.96 - 4.05 (m, 1H), 3.36 - 3.42 (m, 1H), 3.29 - 3.36 (m, 1H), 3.06 (br. s., 1H), 2.17 (t, *J*=7.58 Hz, 2H), 2.00 (q, *J*=7.09 Hz, 2H), 1.62 (m, 2H), 1.22 - 1.36 (m, 50H), 0.89 (t, *J*=6.60 Hz, 6H). $^{13}$C NMR (126 MHz, $CDCl_3$) δ 173.6, 143.4, 133.5, 128.8, 128.5, 128.4, 128.0, 127.3, 87.2, 73.1, 64.2, 53.1, 36.9, 32.3, 31.9,

29.7, 29.6, 29.5, 29.4, 29.3, 29.1, 25.8, 22.7, 14.1. HRMS (*m/z*): $[M+H]^+$ calculated for $C_{55}H_{86}NO_3$: 808.6602, found 808.6619.

***N*-((2*R*,3*S*,*E*)-3-hydroxy-1-(trityloxy)octadec-4-en-2-yl)stearamide (3b)**

In tha same manner as described for 3a, 3b was synthesized from2b. Compound 3b (148 mg, 73%) was obtained as a colorless sticky oil.

$^1$H NMR (500 MHz, $CDCl_3$) δ 7.86 (d, *J*=7.09 Hz, 6H), 7.31 (t, *J*=7.36, 6H), 7.23 - 7.28 (m, 3H), 6.10 (d, *J*=8.07 Hz, 1H), 5.61 - 5.69 (m, 1H), 5.28 (dd, *J*=6.23, 15.52 Hz, 1H), 4.20 (m, 1H), 4.06 – 4.10 (m, 1H), 3.40 (dd, *J*=3.79, 9.66 Hz, 1H), 3.32 (dd, *J*=4.16, 9.78 Hz, 1H), 2.19 - 2.23 (t, *J*=7.33 Hz 2H), 1.93 (q, *J*=6.11 Hz, 2H), 1.65 (q, *J*=7.39 Hz, 2H), 1.19 - 1.30 (m, 50H), 0.90 (t, *J*=6.84 Hz, 6H). $^{13}$C NMR (126 MHz, $CDCl_3$) δ 173.3, 143.3, 133.3, 128.7, 128.5, 128.4, 128.4, 128.0, 127.9, 127.2, 87.3, 74.2, 63.0, 53.3, 36.8, 32.2, 31.9, 31.5, 29.7, 29.6, 29.5, 29.4, 29.3, 29.2, 29.0, 25.8, 22.6, 22.6, 14.1. HRMS (*m/z*): $[M+Na]^+$ calculated for $C_{55}H_{85}NO_3Na$: 830.6421, found 830.6485.

***N*-((2*R*,3*R*,*E*)-3-hydroxy-1-(trityloxy)octadec-4-en-2-yl)stearamide (3c)**

In tha same manner as described for 3a, 3c was synthesized from 2c. Compound 3c (86 mg, 74%) was obtained as a colorless sticky oil.

$^1$H NMR (500 MHz, $CDCl_3$) δ 7.42 (d, *J*=7.58 Hz, 6H), 7.33 (t, *J*=7.82 Hz, 6H), 7.25 - 7.28 (m, 3H), 6.00 (d, *J*=8.31 Hz, 1H), 5.70 - 5.76 (m, 1H), 5.39 (dd, *J*=6.11, 15.40 Hz, 1H), 4.39 (m, 1H), 4.03 (dq, *J*=3.97, 8.13 Hz, 1H), 3.40 (dd, *J*=4.52, 9.65 Hz, 1H), 3.34 (dd, *J*=3.54, 9.65 Hz, 1H), 3.10 (br. s., 1H), 2.19 (t, *J*=7.58 Hz, 2H), 2.01 (q, *J*=6.92 Hz, 2H), 1.60 - 1.68 (m, 2H), 1.28 - 1.33 (m, 50H), 0.91 (t, *J*=6.84 Hz, 6H). $^{13}$C NMR (126 MHz, $CDCl_3$) δ 173.6, 143.4, 133.5, 128.8, 128.5, 128.4, 128.0,

127.2, 87.2, 73.1, 64.2, 53.1, 36.8, 32.3, 31.9, 29.7, 29.7, 29.6, 29.5, 29.4, 29.3, 29.1, 25.8, 22.7, 14.1. HRMS (*m/z*): $[M+Na]^+$ calculated for $C_{55}H_{85}NO_3Na$: 830.6421, found 830.6407.

**(2*S*,3*S*,*E*)-2-stearamido-1-(trityloxy)octadec-4-en-3-yl benzoate (4a)**

To a mixture of L-*threo* ceramide 3 (155 mg, 0.19 mmol) and DMAP (47 mg, 0.38 mmol) and triethylamine (80 µL, 0.57 mmol) in $CH_2Cl_2$ (3 mL) was added benzoyl chloride (45 µL, 0.38 mmol). The reaction was stirred for overnight at room temperature. The mixture was diluted with $CH_2Cl_2$ and washed successively with 1M HCl (2 x 10 mL), $NaHCO_3$ (2 x 10 mL), then dried over $MgSO_4$. The organic layer was evaporated under reduced pressure and the crude product was purified by silica gel chromatography (*n*-hexane/EtOAc = 5:1) to obtain compound 4 (150 mg, 85%) as colorless oil. $^1$H NMR (500 MHz, $CDCl_3$) $\delta$ 7.97 (d, *J*=8.06 Hz, 2H), 7.54 (t, *J*=6.96 Hz, 1H), 7.37 - 7.45 (m, 9H), 7.29 (m, 2H), 7.25 (m, 2H), 7.17 - 7.24 (m, 4H), 5.78 - 5.88 (m, 3H), 5.42 (dd, *J*=7.70, 15.27 Hz, 1H), 4.36 - 4.43 (m, 1H), 3.29 ( m, 2H), 1.97 - 2.12 (m, 2H), 1.90 - 1.97 (m, 2H), 1.40 - 1.49 (m, 2H), 1.18 - 1.34 (m, 50H), 0.88 (t, *J*=6.60 Hz, 6H). $^{13}$C NMR (126 MHz, $CDCl_3$) $\delta$ 172.6, 166.1, 143.5, 137.4, 132.9, 130.2, 129.7, 128.6, 128.5, 128.3, 127.8, 127.1, 124.6, 86.7, 74.6, 62.2, 52.0, 37.0, 32.3, 31.9, 29.7, 29.6, 29.4, 29.3, 29.2, 28.7, 25.7, 22.7, 14.1. HRMS (*m/z*): $[M+H]^+$ calculated for $C_{62}H_{90}NO_4$: 912.6864, found 912.6876.

**(2*R*,3*S*,*E*)-2-stearamido-1-(trityloxy)octadec-4-en-3-yl benzoate (4b)**

In the same manner as described for 4a, 4b was synthesized from 3b. Compound 4b (126 mg, 81%) was obtained as a colorless oil.

$^1$H NMR (500 MHz, $CDCl_3$) $\delta$ 7.95 (d, *J*=7.33 Hz, 2H), 7.57 (t, *J*=7.33 Hz 1H), 7.38 - 7.44 (m, 8H), 7.17 - 7.33 (m, 9H), 5.83 - 5.93 (m, 1H), 5.72 (t, *J*=7.33 Hz, 1H), 5.67

(d, *J*=9.29 Hz, 1H), 5.46 (dd, *J*=7.58, 15.40 Hz, 1H), 4.47 - 4.53 (m, 1H), 3.46 (dd, *J*=3.67, 9.53 Hz, 1H), 3.21 (dd, *J*=4.16, 9.53 Hz, 1H), 2.11 (t, *J*=7.58 Hz, 2H), 2.01 (q, *J*=7.09 Hz, 2H), 1.54 - 1.62 (m, 2H), 1.21 - 1.35 (m, 50H), 0.89 (t, *J*=6.97 Hz, 6H). $^{13}$C NMR (126 MHz, $CDCl_3$) δ 172.4, 165.3, 143.5, 137.2, 132.9, 130.3, 129.7, 128.6, 128.5, 128.3, 127.9, 127.8, 127.7, 127.1, 125.0, 86.8, 74.4, 61.6, 51.1, 37.0, 32.3, 31.9, 29.7, 29.6, 29.5, 29.4, 29.3, 29.2, 28.9, 25.8, 22.7, 14.1. HRMS (*m/z*): $[M+H]^+$ calculated for $C_{62}H_{90}NO_4$: 912.6864, found 912.6893.

**(2*R*,3*R*,*E*)-2-stearamido-1-(trityloxy)octadec-4-en-3-yl benzoate (4c)**

In the same manner as described for 4a, 4c was synthesized from 3c. Compound 4c (85 mg, 88%) was obtained as a colorless oil.

$^1$H NMR (500 MHz, $CDCl_3$) δ 7.86 (d, *J*=7.09 Hz, 2H), 7.43 (t, *J*=7.58 Hz, 1H), 7.27 - 7.34 (m, 8H), 7.14 - 7.19 (m, 6H), 7.08 - 7.14 (m, 3H), 5.69 - 5.77 (m, 3H), 5.31 (dd, *J*=7.70, 15.52 Hz, 1H), 4.24 - 4.32 (m, 1H), 3.19 (d, *J*=3.42 Hz, 2H), 1.88 - 1.99 (m, 2H), 1.83 (q, *J*=6.76 Hz, 2H), 1.34 (quin, *J*=7.39 Hz, 2H), 1.00 - 1.22 (m, 50H), 0.77 (t, *J*=6.72 Hz, 6H). $^{13}$C NMR (126 MHz, $CDCl_3$) δ 172.7, 162.3, 143.5, 137.3, 134.5, 130.5, 130.5, 129.7, 128.8, 128.8, 128.6, 128.3, 127.8, 127.1, 86.7, 74.6, 62.6, 52.0, 36.9, 32.2, 31.9, 29.7, 29.7, 29.6, 29.6, 29.6, 29.6, 29.6, 29.4, 29.3, 29.3, 29.3, 29.2, 29.2, 28.7, 25.7, 22.7, 14.1. HRMS (*m/z*): $[M+Na]^+$ calculated for $C_{62}H_{89}NO_4Na$: 934.6683, found 934.6669.

**(2*S*,3*S*,*E*)-1-hydroxy-2-stearamidooctadec-4-en-3-yl benzoate (5a)**

L-*threo* Ceramide 4 (120 mg, 0.13 mmol) in a mixture toluene (2 mL) and methanol (160 µL) was cooled to 0 °C. $BF_3.OEt_2$ (50 µL, 0.39 mmol) was added to the reaction mixture and stirred for 3 h at room temperature. The mixture was diluted with $CH_2Cl_2$ and washed with saturated $NaHCO_3$, then the organic phase was dried over $MgSO_4$.

The organic layer was removed, and the crude product was purified by silica gel column chromatography (*n*-hexane/EtOAc = 1:1) to obtain 5 (72 mg, 82%) as white solid. $^{1}$H NMR (500 MHz, $CDCl_3$) $\delta$ 8.03 (d, *J*=7.82 Hz, 2H), 7.57 (t, *J*=7.21 Hz, 1H), 7.45 (t, *J*=7.09 Hz, 2H), 6.06 (d, *J*=8.55 Hz, 1H), 5.88 - 5.96 (m, 1H), 5.72 (t, *J*=6.96 Hz, 1H), 5.55 (dd, *J*=7.21, 15.27 Hz, 1H), 4.28 - 4.36 (m, 1H), 3.72 (m, 2H), 2.09 – 2.25 (m, 2H), 2.06 (q, *J*=6.68 Hz, 2H), 1.47 - 1.56 (m, 2H), 1.17 - 1.37 (m, 50H) , 0.88 (t, *J*=6.60 Hz, 6H). $^{13}$C NMR (126 MHz, $CDCl_3$) $\delta$ 173.9, 166.3, 137.4, 133.2, 129.8, 129.7, 128.4, 124.7, 74.1, 62.5, 54.1, 36.9, 32.3, 31.9, 29.7, 29.6, 29.4, 29.3, 29.2, 28.8, 25.7, 22.7, 14.1. HRMS (*m/z*): $[M+H]^+$ calculated for $C_{43}H_{76}NO_4$: 670.5768, found 670.5776.

**(2*R*,3*S*,*E*)-1-hydroxy-2-stearamidooctadec-4-en-3-yl benzoate (5b)**

In the same manner as described for 5a, 5b was synthesized from 4b. Compound 5b (112 mg, 80%) was obtained as a white solid.

$^{1}$H NMR (500 MHz,$CDCl_3$) $\delta$ 8.04 (d, *J* = 7.09 Hz, 2H), 7.60 (t, *J*=7.45 Hz, 1H), 7.46 (t, *J*=7.82 Hz, 2H), 6.09 (d, *J*=8.55 Hz, 1H), 5.83 - 5.90 (m, 1H), 5.62 (dd, *J*=7.82, 15.15 Hz, 1H), 4.25 - 4.31 (m, 1H), 3.68 - 3.75 (m, 2H), 2.16 – 2.23 (m, 2H), 2.05 (q, *J* = 6.92 Hz, 2H), 1.62 (quin, *J*=7.33 Hz, 2H), 1.21 - 1.39 (m, 50H), 0.89 (t, *J*=6.97 Hz, 6H). $^{13}$C NMR (126 MHz, $CDCl_3$) $\delta$ 173.4, 166.5, 137.5, 133.1, 129.8, 129.6, 128.5, 124.8, 74.7, 61.8, 53.4, 36.9, 32.3, 31.9, 29.7, 29.5, 29.3, 29.3, 29.2, 28.9, 25.7, 22.7, 14.1. HRMS (*m/z*): $[M+H]^+$ calculated for $C_{43}H_{76}NO_4$: 670.5768, found 670.5762.

**(2*R*,3*R*,*E*)-1-hydroxy-2-stearamidooctadec-4-en-3-yl benzoate (5c)**

In the same manner as described for 5a, 5c was synthesized from 4c. Compound 5c (30 mg, 88%) was obtained as a white solid

$^{1}$H NMR (500 MHz, $CDCl_3$) $\delta$ 8.03 (d, *J*=7.09 Hz, 2H), 7.58 (t, *J*=7.45 Hz, 1H), 7.45 (t, *J*=7.82 Hz, 2H), 5.98 (d, *J*=8.55 Hz, 1H), 5.88 - 5.96 (m, 1H), 5.72 (t, *J*=6.96 Hz, 1H), 5.55 (dd, *J*=7.45, 15.52 Hz, 1H), 4.29 - 4.36 (m, 1H), 3.67 - 3.77 (m, 2H), 2.10 - 2.21 (m, 2H), 2.06 (q, *J*=6.92 Hz, 2H), 1.52 (quin, *J*=7.45 Hz, 2H), 1.34 - 1.40 (m, 2H), 1.33 – 1.17 (m, 48H), 0.88 (t, *J*=6.84 Hz, 6H). $^{13}$C NMR (126 MHz, $CDCl_3$) $\delta$ 173.9, 166.3, 137.4, 133.3, 129.8, 129.7, 128.4, 124.7, 74.1, 62.6, 54.1, 36.9, 32.3, 31.9, 29.7, 29.6, 29.4, 29.3, 29.2, 28.8, 25.7, 22.7, 14.1. HRMS (*m/z*): $[M+H]^+$ calculated for $C_{43}H_{76}NO_4$: 670.5768, found 670.5743.

**D-Glucopyranose, *O*-(*N*-acetyl-4,7,8,9-tetra-*O*-acetyl-α-neuraminosyl)-(2→3)-*O*-2,4,6-tri-*O*-acetyl-β-D-galactopyranosyl-(1→4)-, 1,2,3,6-tetraacetate (7)**

To a solution of 3'-sialyl lactose **6** (217 mg, 0.331 mmol) in $Ac_2O$ (5 mL) was added $H_2SO_4$ (500 µL in 5 mL $Ac_2O$) dropwise at 0 °C. Then the temperature increased to 40 °C and stirred for 1.5 h. The reaction was quenched with the solution ($CHCl_3$/ice water). The organic phase was dried over $MgSO_4$ and evaporated. The crude product was purified by silica column chromatography ($CHCl_3$/MeOH = 10:1) to obtain compound **7** (319 mg, 88%) as white solid. $^{1}$H NMR (500 MHz, $CDCl_3$) $\delta$ 2.24, 2.18, 2.16, 2.09, 2.08 (2 $CH_3$), 2.06 (3 $CH_3$), 2.02, 2.01, 1.85 [(s, 3H, $CH_3$) × 12]. **Glc**: 6.25 (d, $J_{1,2}$=3.67 Hz, 1H, H-1), 5.47 (m, 1H, H-3), 5.02 (dd, *J*=3.91, 10.26 Hz,1H, H-2), 4.40 (m, 1H, H-6b), 4.19 (dd, *J*=5.38, 11.98 Hz, 1H, H-6a), 3.94 – 4.05 (m, 1H, H-5), 3.90 (m, 1H, H-4). **Gal:** 4.92 (dd, *J*=8.31, 9.78 Hz, 1H, H-2), 4.69 (br d, 1H, H-4), 4.73 (d, $J_{1,2}$=7.58 Hz, 1H, H-1), 4.13 – 4.22 (m, 1H, H-3), 3.93 – 4.05 (m, 2H, H-6a,

6b), 3.88 (m, 1H, H-5), **Neu:** 5.49 (d, *J*=9.53 Hz, 1H, 5-NH), 5.34 – 5.40 (m, 1H, H-8), 5.26 (m, 1H, H-7), 4.22 – 4.30 (m, 1H, H-9b), 4.02 (ddd, *J*=10.26, 10.02 Hz, 1H, H-5), 3.87 – 4.00 (m, 1H, H-9a), 3.84 – 4.01 (m, 1H, H-6), 2.52 (m, 1H, H-3eq), 1.47 (m, 1H, H-3ax). HRMS (*m/z*): $[M+Na]^+$ calculated for $C_{45}H_{61}NO_{30}Na$: 1118.3170, found 1118.3141.

**D-Glucopyranose, *O*-(*N*-acetyl-4,7,8,9-tetra-*O*-acetyl-1-methyl-α-neuraminosyl)-(2→3)-*O*-2,4,6-tri-*O*-acetyl-β-D-galactopyranosyl-(1→4)-, 1,2,3,6-tetraacetate (8)**

To a solution of 7 (324 mg, 0.296 mmol) in DMF (5 mL) was added $CH_3I$ (55 µL, 0.888 mmol) and $Cs_2CO_3$ (97 mg, 0.296 mmol). The reaction mixture was stirred for 4 h at room temperature. The reaction mixture was extracted with EtOAc. The organic phase was dried by $MgSO_4$ and evaporated by reduced pressure. The crude product was purified by silica gel column chromatography (EtOAc only) to obtain **8** (309 mg, 94%) as white solid. $^1$H NMR (500 MHz, $CDCl_3$) δ 2.25, 2.18, 2.16, 2.10 (2 $CH_3$), 2.08, 2.07 (2 $CH_3$), 2.05, 2.01 (2 $CH_3$), 1.86 [(s, 3H, $CH_3$) × 12]. **Glc**: 6.25 (d, $J_{1,2}$=3.67 Hz, 1H, H-1), 5.42 (dd, *J*=9.29 Hz,1H, H-3), 5.02 (dd, *J*=3.67, 10.26 Hz, H-2), 4.42 (dd, J=2.69, 12.71 Hz, 1H, H-6b), 4.22 (dd, *J*=4.40, 12.22 Hz, 1H, H-6a), 3.96 – 4.07 (m, 1H, H-5), 3.91 (m, 1H, H-4). **Gal:** 4.93 (m, 1H, H-2), 4.89 (br d, 1H, H-4), 4.65 (d, $J_{1,2}$=7.82 Hz, 1H, H-1), 4.52 (dd, *J*=3.42, 10.26 Hz, 1H, H-3), 3.98 – 4.04 (m, 2H, H-6a, 6b), 3.87 (m, 1H, H-5). **Neu:** 5.50 – 5.53 (m, 1H, H-8), 5.47 (m, 1H, H-7), 5.08 (d, *J*=10.02 Hz, 1H, 5-NH), 4.88 (m, 1H, H-4), 4.39 (dd, *J*=12.21, 1.71 Hz,1H, H-9b), 4.02 (m, 1H, H-5), 3.96 – 4.06 (m, 1H, H-9a), 3.85 (s, 3H), 3.63 (dd, *J*=2.69, 10.75 Hz, 1H, H-6), 2.57 (dd, J=4.64, 12.71 Hz, 1H, H-3eq), 1.68 (m, 1H, H-3ax). HRMS (*m/z*): $[M+Na]^+$ calculated for $C_{46}H_{63}NO_{30}Na$: 1132.3327, found 1132.3306.

**D-Glucose, *O*-(*N*-acetyl-4,7,8,9-tetra-*O*-acetyl-1-methyl-α-neuraminosyl)-(2→3)-*O*-2,4,6-tri-*O*-acetyl-β-D-galactopyranosyl-(1→4)-, 2,3,6-triacetate (9)**

To a solution of **8** (185 mg, 0.167 mmol) in DMF (5 mL) and cooled to 0 °C. Hydrazine monohydrate (8.0 mg, 0.167 mmol) and acetic acid (9.5 µL, 0.167 mmol) were added to the mixture and stirred for 3 h at room temperature. The mixture was diluted with EtOAc and washed successively with brine (3 times). The organic phase was dried with $MgSO_4$ and evaporated under reduced pressure. The crude product was purified by silica column chromatography ($CHCl_3$/MeOH = 10:1) to obtain **9** (148 mg, 83%) as white solid. $^1$H NMR (500 MHz, $CDCl_3$) δ 2.24, 2.16, 2.11, 2.10, 2.09 (2 $CH_3$), 2.07 (2 $CH_3$), 2.05, 2.01, 1.86 [(s, 3H, $CH_3$) × 11]. **Glc**: 5.38 (d, $J_{1,2}$=3.42 Hz, 1H, H-1), 5.52 (m, 1H, H-3), 4.86 (dd, *J*=3.42, 10.26 Hz, H-2), 4.42 (d, *J*=11.25 Hz, 1H, H-6b), 4.17 – 4.20 (m, 1H, H-6a), 3.87 (m, 1H, H-4), 3.63 – 3.65 (m, 1H, H-5). **Gal:** 4.94 – 4.98 (m, 1H, H-2), 4.88 (m, 1H, H-4), 4.66 (d, $J_{1,2}$=8.06 Hz, 1H, H-1), 4.51( dd, *J*=3.42, 10.26 Hz, 1H, H-3), 3.97 – 4.07 (m, 2H, H-6a, 6b), 3.86 (m, 1H, H-5. **Neu:** 5.49 – 5.55 (m, 1H, H-8), 5.41 (m, 1H, H-7), 5.10 (d, *J*=10.02 Hz, 1H, 5-NH), 4.89 (m, 1H, H-4), 4.42 (m, 1H, H-9b), 3.97 – 4.07 (m, 2H, H-5, 9a), 3.85 (s, 3H), 3.63 (m, 1H, H-6), 2.57 (dd, *J*=4.64, 12.95 Hz, 1H, H-3eq), 1.68 (m, 1H, H-3ax). HRMS (*m/z*): $[M+Na]^+$ calculated for $C_{44}H_{61}NO_{29}Na$: 1090.3221, found 1090.3205.

**D-Glucopyranose, *O*-(*N*-acetyl-4,7,8,9-tetra-*O*-acetyl-1-methyl-α-neuraminosyl)-(2→3)-*O*-2,4,6-tri-*O*-acetyl-β-D-galactopyranosyl-(1→4)-, 2,3,6-triacetate 1-(2,2, 2-trichloroethanimidate) (10)**

Sialyllactose derivative **9** (148 mg, 0.139 mmol) was dissolved in dry DCM and cooled to 0°C. Trichloroacetonitrile (292 µL, 2.9 mmol) and DBU (23 µL, 0.153 mmol) were added and the resulting mixture was stirred for 3 h at room temperature. The reaction mixture was concentrated and then purified by flash chromatography (*n*-

hexane/EtOAc = 1:50) to yield **10** (145 mg, 86%) as yellow color solid. $^1$H NMR (500 MHz, $CDCl_3$) δ 2.25, 2.16, 2.09, 2.08 (2 $CH_3$), 2.07 (2 $CH_3$), 2.05, 2.01 (2 Me), 1.86 [(s, 3H, $CH_3$) × 11]. **Glc**: 6.49 (d, $J_{1,2}$=3.59 Hz, 1H, H-1), 5.55 (dd, *J*=10.02 Hz,1H, H-3), 5.08 (m, 1H, H-2), 4.43 (m, 1H, H-6b), 4.24 (dd, *J*=4.64, 11.97 Hz, 1H, H-6a), 4.12 (m, 1H, H-5), 3.93 – 3.98 (m, 1H, H-4). **Gal:** 4.96 (dd, *J*=8.06 Hz, 1H, H-2), 4.90 (br d, 1H, H-4), 4.67 (d, $J_{1,2}$=8.06 Hz, 1H, H-1), 4.52 ( dd, *J*=3.18, 10.26 Hz, 1H, H-3), 4.03 (m, 2H, H-6a, 6b), 3.86 (m, 1H, H-5). **Neu:** 5.48 – 5.52 (m, 1H, H-8), 5.41 (dd, *J*=2.69, 9.29 Hz, 1H, H-7), 5.09 (d, *J*=10.26, 1H, 5-NH), 4.92 – 4.94 (m, 1H, H-4), 4.42 (m, 1H, H-9b), 3.99 – 4.02 (m, 1H, H-5), 3.99 – 4.05 (m, 1H, H-9a), 3.85 (s, 3H, OMe), 3.64 (dd, *J*=2.69, 10.75 Hz, 1H, H-6), 2.58 (dd, *J*=4.64, 12.71 Hz, 1H, H-3eq), 1.68 (m, 1H, H-3ax). HRMS (*m/z*): $[M+Na]^+$ calculated for $C_{46}H_{61}Cl_3N_2O_{29}Na$: 1233.2478, found 1233.2451.

**_N_-[(1_S_,2_S_,3_E_)-2-(Benzoyloxy)-1-[[[O-4,7,8,9-tetra-O-acetyl-5-(acetylamino)-3,5-dideoxy-1-methyl-L-glycero-α-D-galacto-2-nonulopyranosonosyl-(2→3)-O-2,4,6-tri-O-acetyl-β-D-galactopyranosyl-(1→4)-2,3,6-tri-O-acetyl-β-D-glucopyranosyl]oxy]methyl]-3-heptadecen-1-yl]octadecanamide (11a)**

A solution of activated sialyllactose (38 mg, 0.031 mmol) and **5a** (32 mg, 0.047 mmol), and dried 4Å molecular sieves (200 mg) in dry DCM were stirred for 2 h at room temperature. Then the mixture was cooled to 0 °C and $BF_3{\cdot}OEt_2$ (12 µL, 0.093 mmol) was added to the mixture. The reaction mixture was stirred for 2 h at 0 °C, then the reaction mixture was neutralized with $Et_3N$ and solvent was removed by under reduced pressure. The resulting crude product was purified by silica gel chromatography (EtOAc/MeOH = 24:1) to yield **11** (18 mg, 33%) as white solid. $^1$H NMR (500 MHz, $CDCl_3$) δ 8.02 (d, *J*=7.09 Hz, 2H), 7.57 (t, *J*=7.58 Hz, 1H), 7.42 - 7.48 (m, 2H), 2.25, 2.17, 2.09 (2 $CH_3$), 2.08 (2 $CH_3$), 2.09, 2.05, 2.03, 1.86 [(s, 3H,

$CH_3$) × 11]. **Glc**: 5.18 (m, 1H, H-3), 4.85 – 4.90 (m, 1H,H-2), 4.44 (d, $J_{1,2}$=7.58 Hz, 1H,H-1), 3.84 (m, 1H, H-4), 3.55 (m, 1H, H-5), 4.05 (m, 1H, H-6a), 4.38 – 4.42 (m, 1H, H-6b). **Gal:** 4.91-4.95 (m, 1H, H-2), 4.87 – 4.90 (m, 1H, H-4), 4.66 (d, $J_{1,2}$=7.82 Hz, 1H, H-1), 4.52 (dd, *J*=3.18, 10.26 Hz, 1H, H-3), 3.95 – 4.07 (m, 2H, H-6a, 6b), 3.83 – 3.86 (m,1H, H-5). **Neu**: 5.52 - 5.57 (m, 1H, H-8), 5.41 (dd, *J*=2.69, 9.29 Hz, 1H, H-7), 5.04 (d, *J*=10.26 Hz, 1H, 5-NH), 4.85 – 4.90 (m, 1H, H-4), 4.16 (dd, *J*=5.25, 12.10 Hz, 1H, H-9b), 3.95 – 4.07 (m, 2H, H-5, H-9a), 3.85 (s, 3H, OMe), 3.64 (dd, *J*=2.69, 10.75 Hz, 1H, H-6), 2.58 (dd, *J*=4.52, 12.83 Hz, 1H, H-3b), 1.66 – 1.71 (m, 1H, H-3a). **Cer:** 5.84 – 5.90 (m, 1H), 5.80 (d, *J*=9.29 Hz, 1H), 5.62 (t, *J*=7.21 Hz, 1H), 5.48 (dd, *J*=7.3. 15.4 Hz, 1H), 4.39 – 4.42 (m, 1H), 3.54 - 3.60 (m, 2H), 2.11 – 2.15 (m, 2H), 2.03-2.05 (m, 2H)1.49 (quin, *J*=7.3 Hz, 2H), 1.12 - 1.36 (m, 50H), 0.89 (t, *J*=6.35 Hz, 6H). HRMS (*m/z*): $[M+Na]^+$ calculated for $C_{87}H_{134}N_2O_{32}Na$: 1741.8811, found 1741.8866.

**_N_-[(1_R_,2_S_,3_E_)-2-(Benzoyloxy)-1-[[[O-4,7,8,9-tetra-O-acetyl-5-(acetylamino)-3,5-dideoxy-1-methyl-L-glycero-α-D-galacto-2-nonulopyranosonosyl-(2→3)-O-2,4,6-tri-O-acetyl-β-D-galactopyranosyl-(1→4)-2,3,6-tri-O-acetyl-β-D-glucopyranosyl]oxy]methyl]-3-heptadecen-1-yl]octadecanamide (11b)**

In the same manner as described for 11a, 11b was synthesized from 5b. Compound 11b (20 mg, 30%) was obtained as a white solid.

$^1$H NMR (500 MHz, $CDCl_3$) δ 8.01 (d, *J*=7.14 Hz, 2H), 7.58 (t, *J*=7.45 Hz 1H), 7.46 (t, *J*=7.76 Hz, 2H), 2.23, 2.16, 2.08 (2 $CH_3$), 2.08 (2 $CH_3$), 2.09, 2.07, 2.06, 2.04, 2.00, 1.85 [(s, 3H, $CH_3$) × 11]. **Glc**: 5.14 (m, 1H, H-3), 4.86 – 4.90 (m, 1H,H-2), 4.38 (d, $J_{1,2}$=7.76 Hz, 1H,H-1), 3.83 (m, 1H, H-4), 3.56 (ddd, *J*=2.17, 5.59, 9.94 Hz, 1H, H-5), 4.02 – 4.06 (m, 1H, H-6a), 4.40 – 4.44 (m, 1H, H-6b). **Gal:** 4.91-4.94 (m, 1H, H-2), 4.87 – 4.90 (m, 1H, H-4), 4.66 (d, $J_{1,2}$=8.07 Hz, 1H, H-1), 4.52 (dd, *J*=3.42, 10.25

Hz, 1H, H-3), 3.95 – 4.07 (m, 2H, H-6a, 6b), 3.85 – 3.87 (m,1H, H-5). **Neu**: 5.52 - 5.56 (m, 1H, H-8), 5.40 (dd, *J*=2.79, 9.62 Hz, 1H, H-7), 5.04 (d, *J*=10.25 Hz, 1H, 5-NH), 4.85 – 4.90 (m, 1H, H-4), 4.16 (dd, *J*=5.90, 12.11 Hz, 1H, H-9b), 3.95 – 4.07 (m, 2H, H-5, H-9a), 3.84 (s, 3H, OMe), 3.64 (dd, *J*=2.79, 10.87 Hz, 1H, H-6), 2.58 (dd, *J*=4.66, 12.73 Hz, 1H, H-3b), 1.65 – 1.71 (m, 1H, H-3a). **Cer:** 5.80 – 5.87 (m, 1H), 5.78 (d, *J*=9.62 Hz, 1H), 5.47 (dd, *J*=6.83, 15.29 1H), 5.41 (t, *J*=8.69 1H), 4.48 (m, 1H), ), 3.71 (dd, *J*=2.48, 9.94 Hz, 1H), 3.95 (m, 1H), 2.17 – 2.20 (m, 2H), 2.00-2.03 (m, 2H), 1.58 – 1.62 (m, 2H), 1.22 - 1.34 (m, 50H), 0.88 (t, *J*=6.99 Hz, 6H). HRMS (*m/z*): $[M+Na]^+$ calculated for $C_{87}H_{134}N_2O_{32}Na$: 1741.8811, found 1741.8741.

**_N_-[(1_R_,2_R_,3_E_)-2-(Benzoyloxy)-1-[[[O-4,7,8,9-tetra-O-acetyl-5-(acetylamino)-3,5-dideoxy-1-methyl-L-glycero-α-D-galacto-2-nonulopyranosonosyl-(2→3)-O-2,4,6-tri-O-acetyl-β-D-galactopyranosyl-(1→4)-2,3,6-tri-O-acetyl-β-D-glucopyranosyl]oxy]methyl]-3-heptadecen-1-yl]octadecanamide (11c)**

In the same manner as described for 11a, 11c was synthesized from 5c. Compound 11c (17 mg, 31%) was obtained as a white solid.

$^1$H NMR (500 MHz, $CDCl_3$) δ 8.00 (d, *J*=7.82 Hz, 2H), 7.54 (t, *J*=7.21 Hz, 1H), 7.38 - 7.48 (m, 2H), 2.25, 2.17, 2.08 (2 $CH_3$), 2.09 (2 $CH_3$), 2.09, 2.07, 2.05, 2.03, 2.01, 1.86 [(s, 3H, $CH_3$) × 11]. **Glc**: 5.20 (m, 1H, H-3), 4.85 – 4.90 (m, 1H,H-2), 4.42 (d, $J_{1,2}$=8.80 Hz, 1H,H-1), 4.40 – 4.44 (m, 1H, H-6b), 3.99 – 4.06 (m, 1H, H-6a), 3.88 – 3.93 (m, 1H, H-4), 3.56 – 3.61 (m, 1H, H-5). **Gal:** 4.91-4.96 (m, 1H, H-2), 4.89 – 4.92 (m, 1H, H-4), 4.66 (d, $J_{1,2}$=8.06 Hz, 1H, H-1), 4.53 (dd, *J*=2.93, 10.26 Hz, 1H, H-3), 3.95 – 4.07 (m, 2H, H-6a, 6b), 3.86 – 3.89 (m,1H, H-5). **Neu**: 5.53 (m, 1H, H-8), 5.40 (dd, *J*=2.76, 9.14 Hz, 1H, H-7), 5.04 (d, *J*=10.02 Hz, 1H, 5-NH), 4.85 – 4.96 (m, 1H, H-4), 4.17 (dd, *J*=5.38, 11.97 Hz, 1H, H-9b), 3.95 – 4.07 (m, 2H, H-5, H-9a), 3.85 (s, 3H, OMe), 3.65 (dd, *J*=2.20, 10.75 Hz, 1H, H-6), 2.58 (dd, *J*=4.40, 12.95 Hz, 1H, H-

3b), 1.65 – 1.71 (m, 1H, H-3a). ). **Cer:** 5.87 – 5.93 (m, 1H), 5.88 (d, *J*=9.77 Hz, 1H), 5.48 (dd, *J*=7.82, 15.14 1H), 5.51 (m, 1H), 4.48 (m, 1H), ), 3.72 (dd, *J*=1.95, 10.26 Hz, 1H), 3.97 (m, 1H), 2.11 – 2.20 (m, 2H), 2.04-2.08 (m, 2H), 1.40 – 1.46 (m, 2H), 1.34 - 1.39 (m, 2H), 1.39 – 1.09 (m, 48H), 0.89 (t, *J*=6.11 Hz, 6H). HRMS (*m/z*): $[M+Na]^+$ calculated for $C_{87}H_{134}N_2O_{32}Na$: 1741.8811, found 1741.8757.

**_N_-[(1_S_,2_S_,3_E_)-1-[[[O-(N-Acetyl-α-neuraminosyl)-(2→3)-O-β-D-galactopyranosyl-(1→4)-β-D-glucopyranosyl]oxy]methyl]-2-hydroxy-3-heptadecen-1-yl]octadecanamide (L-*threo* GM3)**

To a solution of **11a** (10 mg, 5.82 µM) in methanol (1 mL) was added 1.3 M NaOMe (49 µL in methanol). After stirring for 24 h at room temperature, $H_2O$ (100 µL) was added and the stirring continued for another 24 h. The reaction mixture was cooled to 0 ºC and neutralized by using Amberlite IRA 120. The resin was removed by filtration and washed with methanol. The combined filtrate was evaporated, the crude product was purified by silica gel chromatography ($CHCl_3$/MeOH/$H_2O$ = 60:35:8) to yield **L-*threo* GM3** (5.5 mg, 80%) as white solid. $^1$H NMR (500 MHz, DMSO-$d_6$) δ 6.18 (br s, 1H, OH)**,** 5.09 (d, *J*=4.56 Hz, 1H, OH), 4.87 (d, *J*=5.21 Hz, H, OH), 4.79 (d, *J*=6.18 Hz, 1H, OH), 4.64 (t, *J*=5.53 Hz, 1H, OH), 4.58 (d, *J*=3.58 Hz, 1H, OH), 4.51 (s, 1H, OH), 4.45 (d, *J*=4.88 Hz 1H, OH), 4.39 (s, 1H, OH), 4.22 (br. s., 1H, OH), **Glc**: 4.17 (d, $J_{1,2}$=7.48 Hz, 1H,H-1), 3.87 – 3.92 (m, 1H, H-6b), 3.59 – 3.63 (m, 1H, H-6a), 3.30 – 3.40 (m, 2H, H-3,4), 3.27 – 3.29 (m, 1H, H-5), 2.99 – 3.04 (m, 1H, H-2). **Gal:** 4.19 (d, $J_{1,2}$=8.13 Hz, 1H, H-1), 3.97 (dd, *J*=2.93, 9.76 Hz, 1H, H-3), 3.73 (m, 1H, H-4), 3.41 – 3.50 (m, 2H, H-6a,6b), 3.30 – 3.40 (m,2H, H-2,5). **Neu**:8.08 (s, 1H, 5-NH), 3.55 – 3.65 (m, 1H, H-4), 3.54 – 3.62 (m, 1H, H-8), 3.54 – 3.65 (m, 1H, H-9b), 3.30 – 3.40 (m, 3H, H-5, 6, 9a), 3.20 (br d, *J*=8.48 Hz, 1H, H-7), 2.75 (dd, *J*=4.9, 12.0 Hz, 1H, H-3eq),1.89 (s, 3H, 5-NAc), 1.32 – 1.39 (m, 1H, H-3ax). **Cer:**

7.36 (d, *J*=8.78 Hz, 1H),5.54 - 5.59 (m, 1H), 5.37 (dd, *J*=5.21, 15.29 Hz, 1H), 4.47 (m, 1H), 3.69 (m, 1H) 3.44 – 3.56 (m, 2H), 1.90 – 2.14 (m, 2H), 1.91 – 1.97 (m, 2H), 1.40 – 1.50 (m, 2H), 1.23 – 1.30 (m, 50H), 0.85 (t, *J*=6.51, 6H). HRMS (*m/z*): $[M+2Na]^+$ calculated for $C_{59}H_{108}N_2O_{21}Na_2$: 1226.7234, found 1226.7232.

**_N_-[(1_R_,2_S_,3_E_)-1-[[[O-(N-Acetyl-α-neuraminosyl)-(2→3)-O-β-D-galactopyranosyl-(1→4)-β-D-glucopyranosyl]oxy]methyl]-2-hydroxy-3-heptadecen-1-yl]octadecanamide (L-*erythro* GM3)**

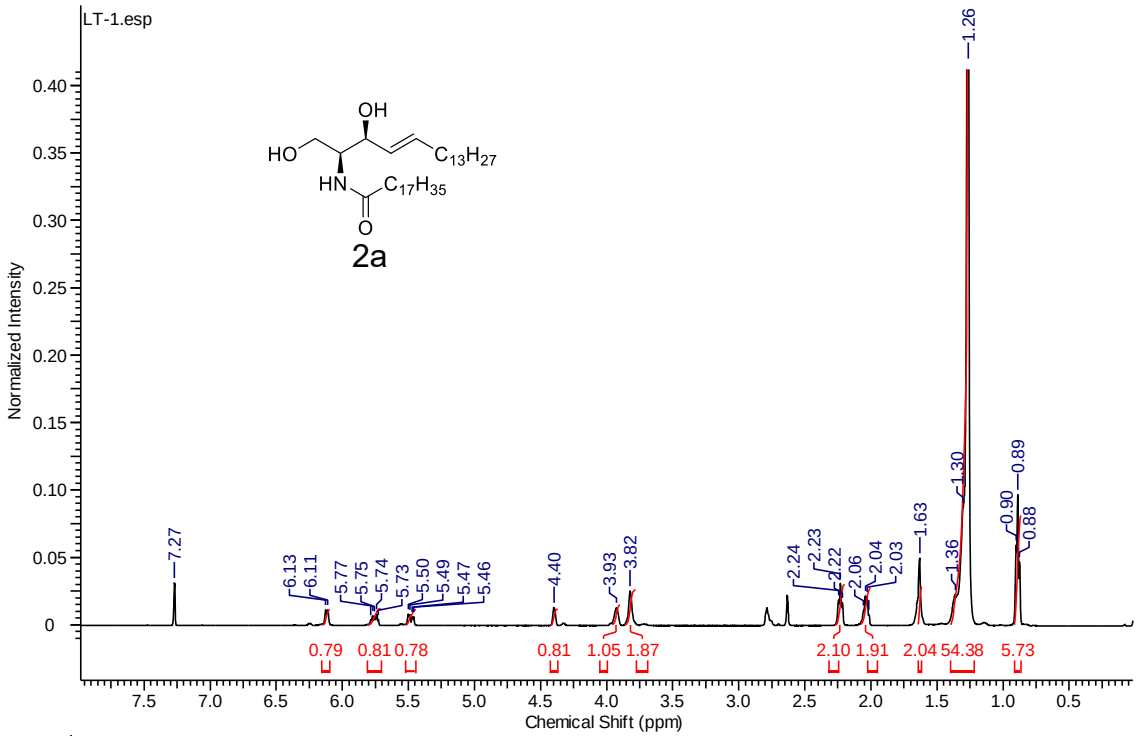
In the same manner as described for L-*threo* GM3, L-*erythro* GM3 was synthesized from 11b. L-*erythro* GM3 (10 mg, 95%) was obtained as a white solid.

$^1$H NMR (500 MHz, DMSO-$d_6$) δ 4.97 (d, *J*=4.50 Hz, 1H, OH), 4.86 (d, *J*=5.47 Hz, 1H, OH), 4.78 (d, *J*=5.79 Hz, 1H, OH), 4.64 (t, *J*=5.31 Hz, 1H, OH), 4.58 (d, *J*=2.89 Hz, 1H, OH), 4.53 (s, 1H, OH), 4.44 (d, *J*=4.18 Hz, 1H, OH), 4.38 (br. s., 1H, OH). **Glc**: 4.17 (d, $J_{1,2}$=7.40 Hz, 1H,H-1), 3.87 – 3.93 (m, 1H, H-6b), 3.59 – 3.63 (m, 1H, H-6a), 3.35 – 3.40 (m, 2H, H-3,4), 3.23 – 3.27 (m, 1H, H-5), 2.99 – 3.06 (m, 1H, H-2). **Gal:** 4.19 (d, $J_{1,2}$=8.04 Hz, 1H, H-1), 3.97 (dd, *J*=2.25, 9.65 Hz, 1H, H-3), 3.73 – 3.81 (m, 1H, H-4), 3.41 – 3.51 (m, 2H, H-6a,6b), 3.35 – 3.41 (m,2H, H-2,5). **Neu**: 8.08 (s, 1H, 5-NH), 3.53 – 3.63 (m, 1H, H-4), 3.53 – 3.63 (m, 1H, H-8), 3.53 – 3.63 (m, 1H, H-9b), ), 3.40 – 3.52 (m, 3H, H-5, 6, 9a), 3.20 (br d, *J*=8.36 Hz, 1H, H-7), 2.75 (dd, *J*=4.50, 11.58 Hz, 1H, H-3eq),1.89 (s, 3H, 5-NAc), 1.32 – 1.38 (m, 1H, H-3ax). **Cer:** 7.45 (d, *J*=8.68 Hz, 1H),5.48 - 5.56 (m, 1H), 5.34 (dd, *J*=6.75, 15.11 Hz, 1H), 4.50 – 4.54 (m, 1H), 3.67 – 3.71 (m, 1H) 3.65 – 3.73 (m, 2H), 1.99 – 2.06 (m, 2H), 1.91 – 1.96 (m, 2H), 1.40 – 1.49 (m, 2H), 1.23 – 1.290 (m, 50H), 0.85 (t, *J*=6.43, 6H). HRMS (*m/z*): $[M+2Na]^+$ calculated for $C_{59}H_{108}N_2O_{21}Na_2$: 1226.7234, found 1226.7291.

**_N_-[(1_R_,2_R_,3_E_)-1-[[[O-(N-Acetyl-α-neuraminosyl)-(2→3)-O-β-D-galactopyranosyl-(1→4)-β-D-glucopyranosyl]oxy]methyl]-2-hydroxy-3-heptadecen-1-yl]octadecanamide (D-*threo* GM3)**

In the same manner as described for L-*threo* GM3, D-*threo* GM3 was synthesized from 11c. D-*threo* GM3 (12 mg, 93%) was obtained as a white solid.

$^1$H NMR (500 MHz, DMSO-$d_6$) δ 5.03 (d, *J*=4.85 Hz, 1H, OH), 4.86 (d, *J*=4.85 Hz, 1H, OH), 4.83 (d, *J*=5.82 Hz, 1H, OH), 4.66 (t, *J*=4.85 Hz, 1H, OH), 4.59 (d, *J*=3.23 Hz, 1H, OH), 4.53 (s, 1H, OH), 4.46 (d, *J*=4.85 Hz, 1H, OH), 4.38 (br. s., 1H, OH). **Glc**: 4.17 (d, $J_{1,2}$=7.76 Hz, 1H,H-1), 3.87 – 3.94 (m, 1H, H-6b), 3.58 – 3.63 (m, 1H, H-6a), 3.23 – 3.40 (m, 2H, H-3,4), 3.23 – 3.28 (m, 1H, H-5), 2.97 – 3.03 (m, 1H, H-2). **Gal:** 4.19 (d, $J_{1,2}$=7.76 Hz, 1H, H-1), 3.98 (dd, *J*=2.43, 9.86 Hz, 1H, H-3), 3.72 – 3.83 (m, 1H, H-4), 3.41 – 3.52 (m, 2H, H-6a,6b), 3.35 – 3.41 (m,2H, H-2,5). **Neu**: 8.13 (s, 1H, 5-NH), 3.52 – 3.63 (m, 1H, H-4), 3.52 – 3.63 (m, 1H, H-8), 3.52 – 3.63 (m, 1H, H-9b), ), 3.24 – 3.41 (m, 3H, H-5, 6, 9a), 3.20 (br d, *J*=9.06 Hz, 1H, H-7), 2.75 (dd, *J*=4.85, 11.32 Hz, 1H, H-3eq),1.89 (s, 3H, 5-NAc), 1.32 – 1.38 (m, 1H, H-3ax). **Cer:** 7.34 (d, *J*=8.41 Hz, 1H),5.52 - 5.60 (m, 1H), 5.35 (dd, *J*=5.50, 15.20 Hz, 1H), 4.50 – 4.54 (m, 1H), 3.71 (m, 1H), 3.63 – 3.83 (m, 2H), 1.99 – 2.14 (m, 2H), 1.91 – 1.97 (m, 2H), 1.39 – 1.52 (m, 2H), 1.12 – 1.29 (m, 50H), 0.85 (t, *J*=6.79, 6H). HRMS (*m/z*): $[M+2Na]^+$ calculated for $C_{59}H_{108}N_2O_{21}Na_2$: 1226.7234, found 1226.7268.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HO
HN
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$C_{17}H_{35}$
O
2a
Normalized Intensity
Chemical Shift (ppm)
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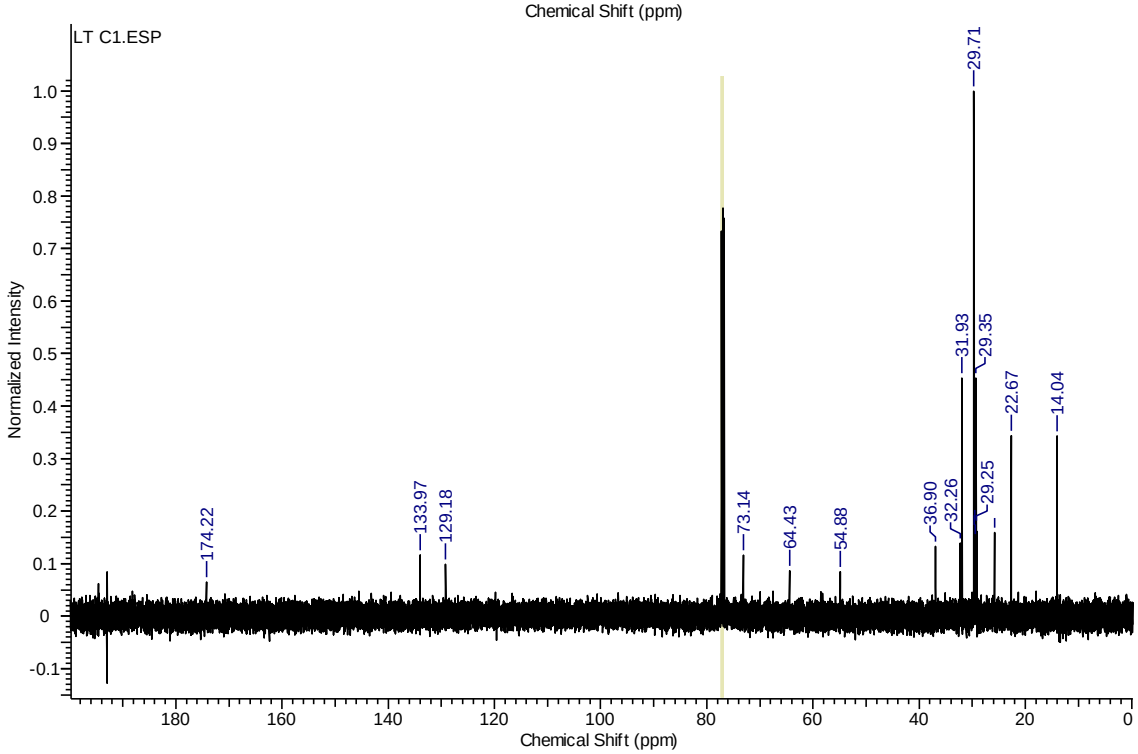
7.27
6.13
6.11
5.77
5.75
5.74
5.73
5.50
5.49
5.47
5.46
4.40
3.93
3.82
2.24
2.23
2.22
2.06
2.04
2.03
1.63
1.36
1.30
0.90
0.89
0.88
0.79
0.81
0.78
0.81
1.05
1.87
2.10
1.91
2.04
54.38
5.73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Normalized Intensity
Chemical Shift (ppm)
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29.35
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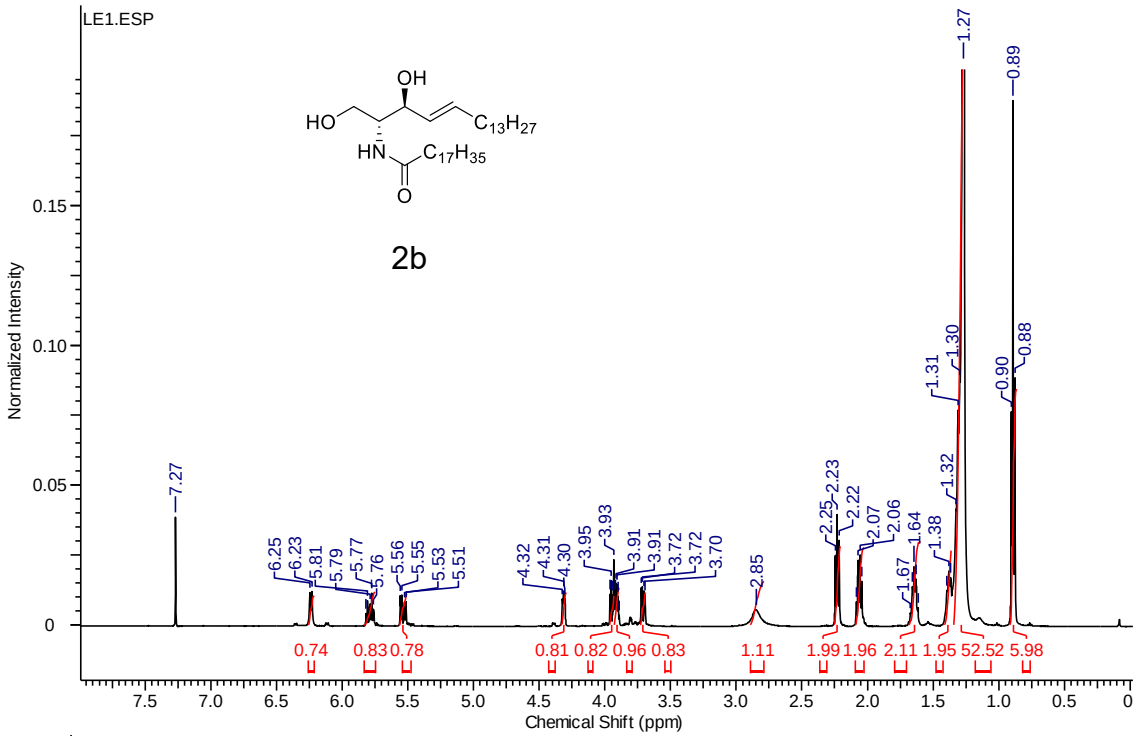
174.22
133.97
129.18
73.14
64.43
54.88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HO
$C_{13}H_{27}$
HN
$C_{17}H_{35}$
O
2b
Normalized Intensity
Chemical Shift (ppm)
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6.25
6.23
5.81
5.79
5.77
5.76
5.56
5.55
5.53
5.51
4.32
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4.30
3.95
3.93
3.91
3.91
3.72
3.72
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2.25
2.23
2.22
2.07
2.06
1.67
1.64
1.38
1.32
1.31
1.30
1.27
0.90
0.89
0.88
0.74
0.83
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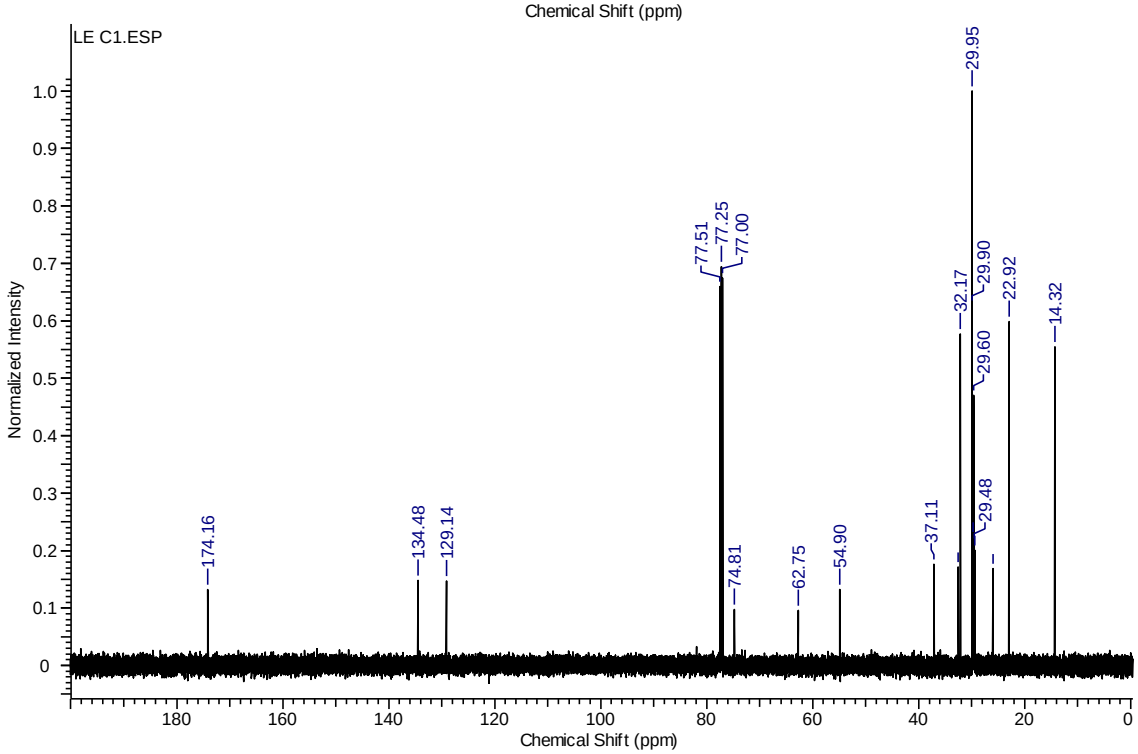
1.96
2.11
1.95
52.52
5.98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Chemical Shift (ppm)
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29.90
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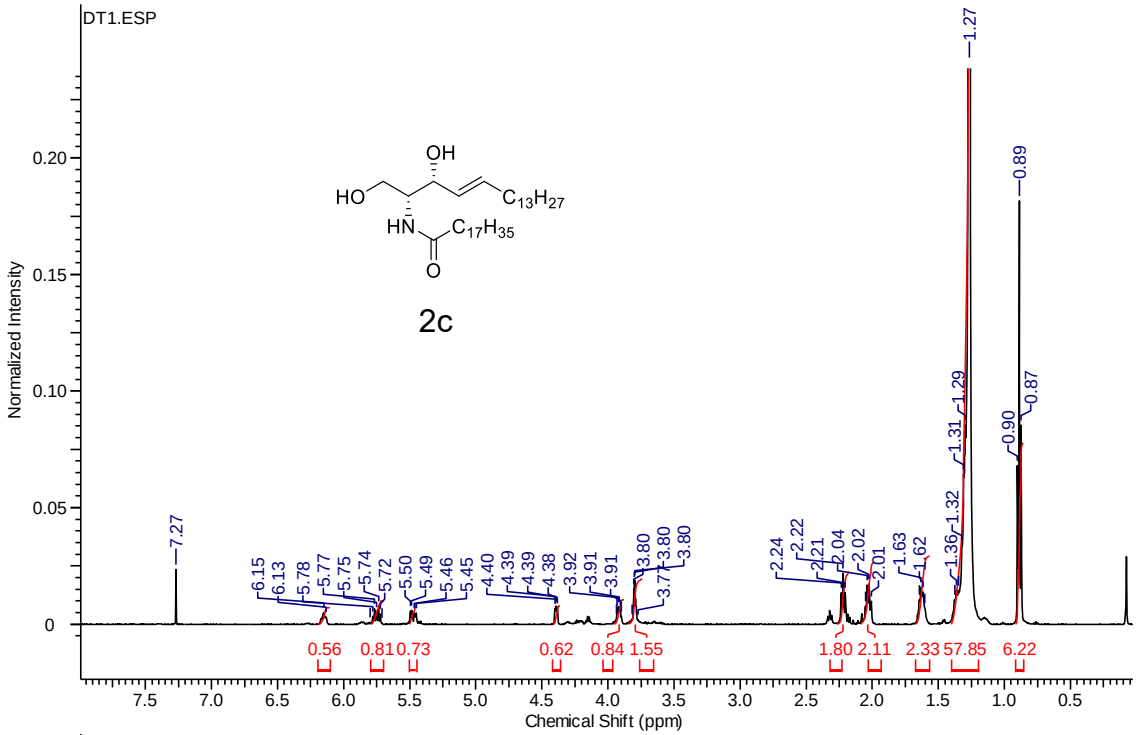
29.48
22.92
14.32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Normalized Intensity
Chemical Shift (ppm)
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2.22
2.21
2.04
2.02
2.01
1.63
1.62
1.36
1.32
1.31
1.29
1.27
0.90
0.89
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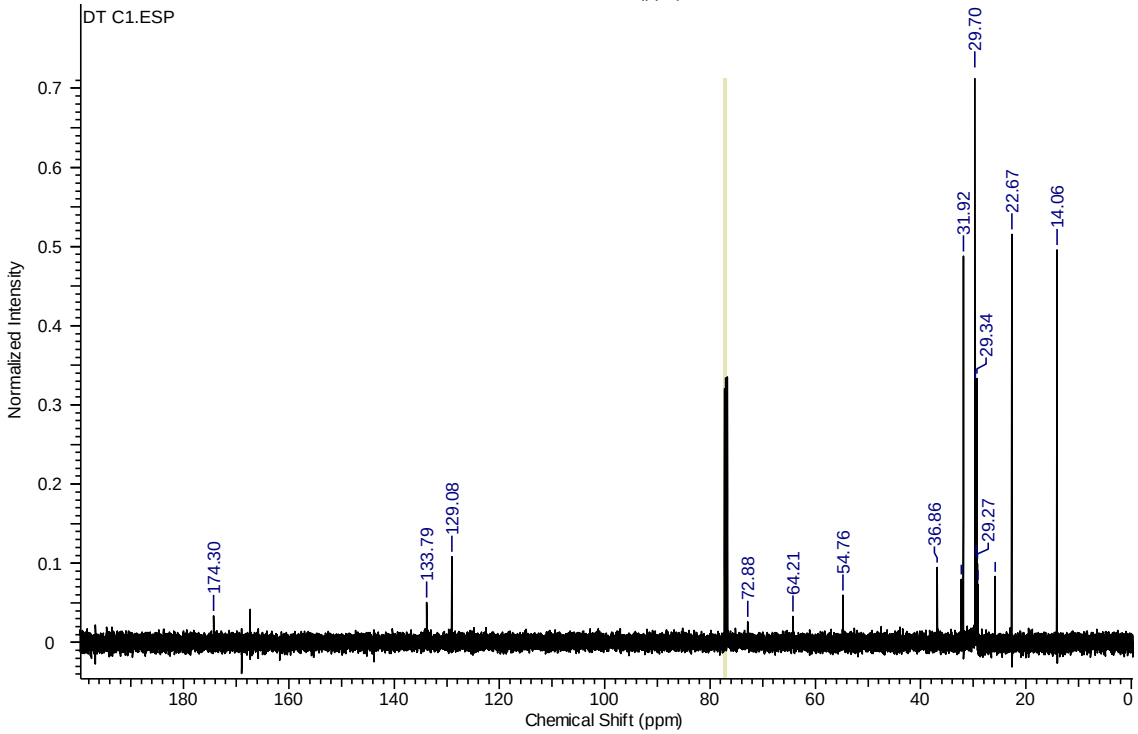
6.22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Chemical Shift (ppm)
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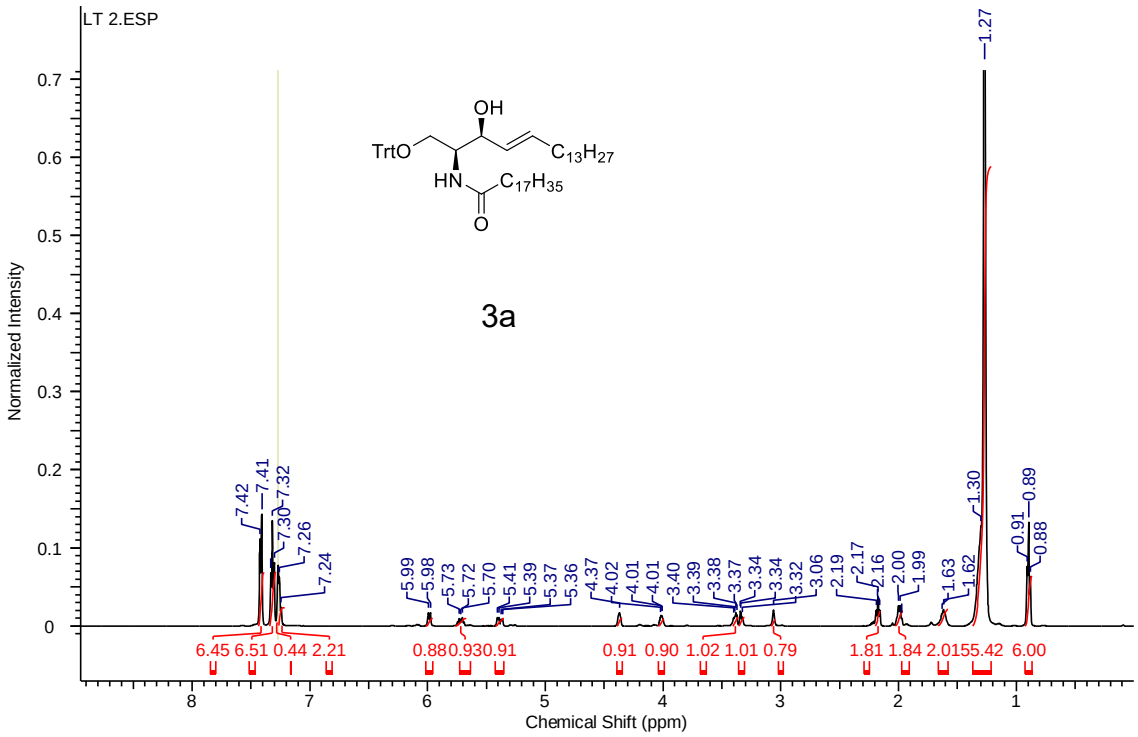
29.27
22.67
14.06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HN
$C_{17}H_{35}$
O
3a
Normalized Intensity
Chemical Shift (ppm)
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7.42
7.41
7.32
7.30
7.26
7.24
5.99
5.98
5.73
5.72
5.70
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5.39
5.37
5.36
4.37
4.02
4.01
4.01
3.40
3.39
3.38
3.37
3.34
3.34
3.32
3.06
2.19
2.17
2.16
2.00
1.99
1.63
1.62
1.30
0.91
0.89
0.88
6.45 6.51 0.44 2.21
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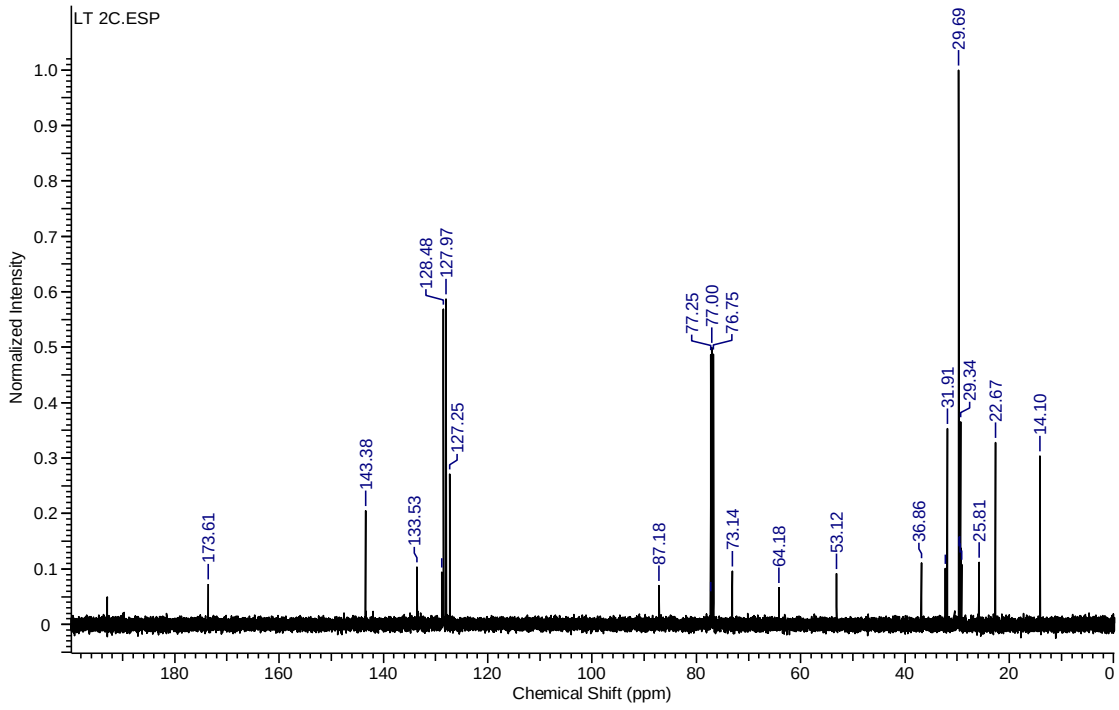
1.81 1.84 2.01 55.42 6.00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Normalized Intensity
Chemical Shift (ppm)
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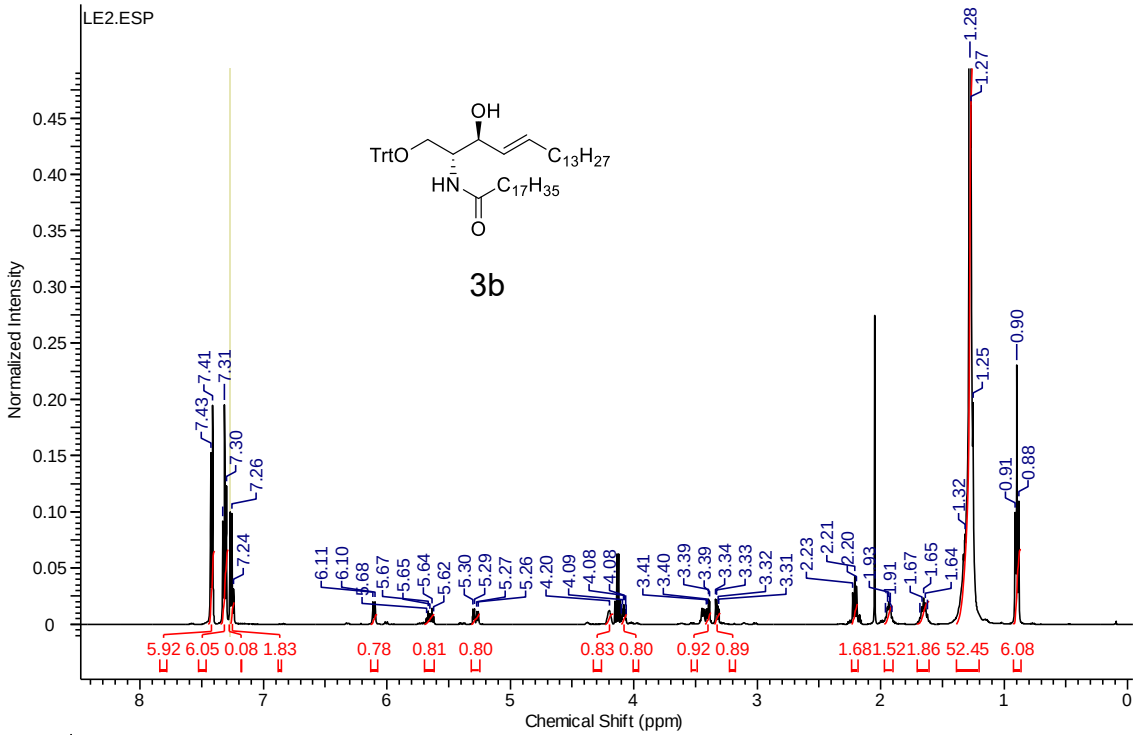
64.18
53.12
36.86
25.81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Chemical Shift (ppm)
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HN
$C_{17}H_{35}$
O
3b
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6.11 6.10 5.68 5.67 5.65 5.64 5.62 5.30 5.29 5.27 5.26 4.20 4.09 4.08 4.08 3.41 3.40 3.39 3.39 3.34 3.33 3.32 3.31
2.23 2.21 2.20 1.93 1.91 1.67 1.65 1.64 1.32
1.28 1.27 1.25 0.90 0.91 0.88
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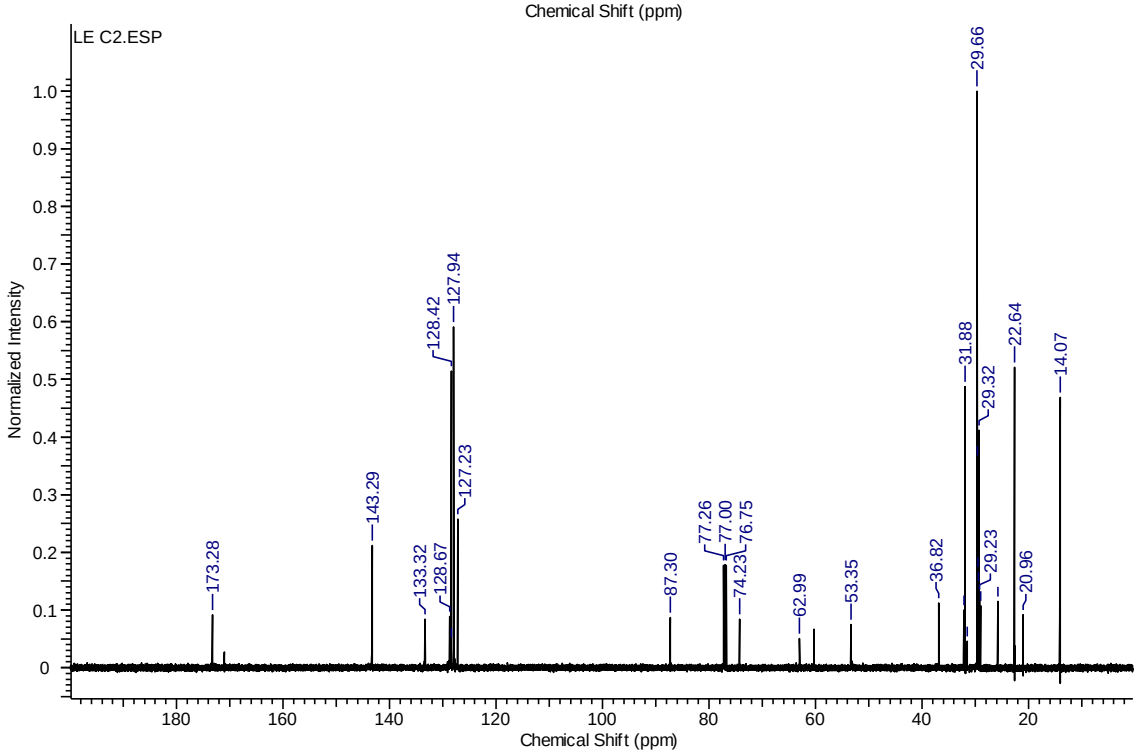
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Normalized Intensity
Chemical Shift (ppm)
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127.94
127.23
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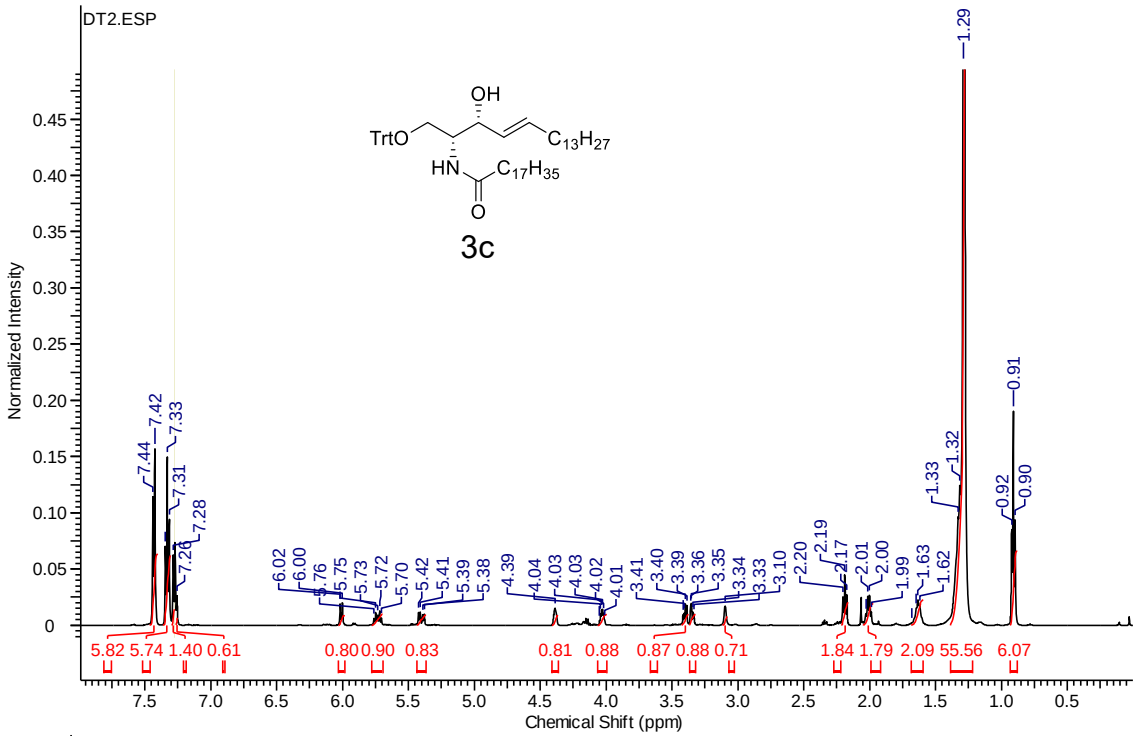
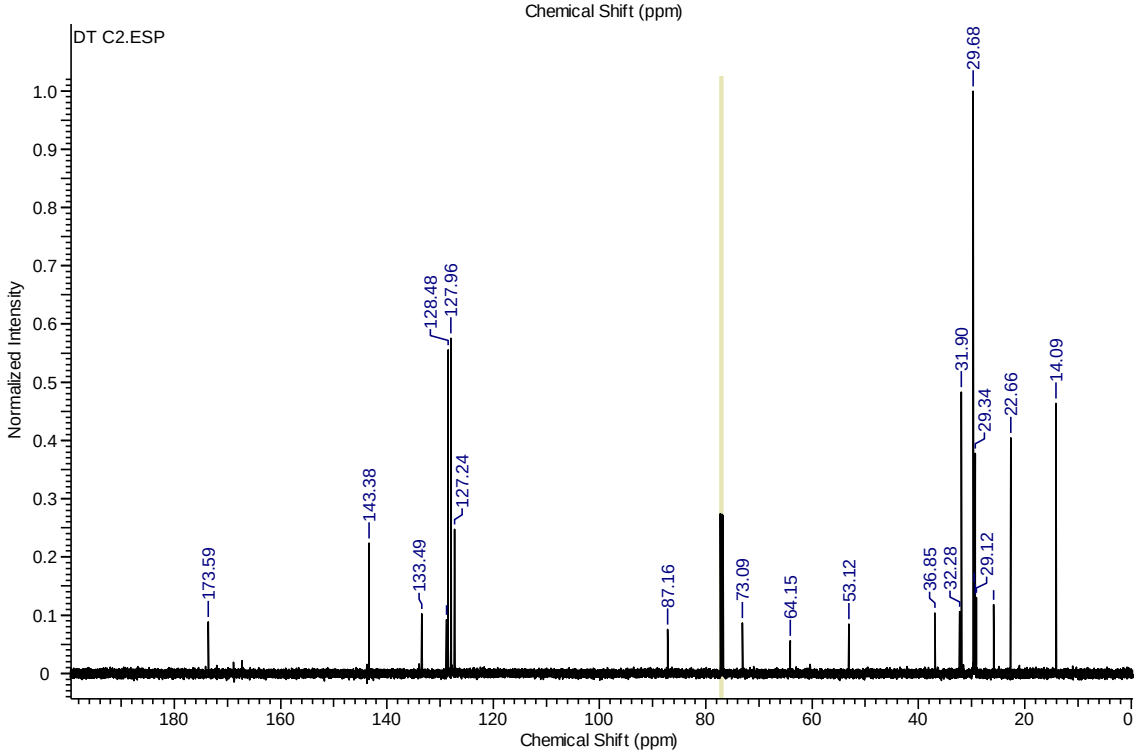
29.66
29.32
29.23
22.64
20.96
14.07


DT2.ESP
OH
TrtO
$C_{13}H_{27}$
HN
$C_{17}H_{35}$
O
3c
Normalized Intensity
Chemical Shift (ppm)
1.29
0.91
7.42
7.33
7.44
7.31
7.28
1.32
1.33
0.92
0.90
5.82 5.74 1.40 0.61
0.80 0.90 0.83
0.81 0.88
0.87 0.88 0.71
1.84 1.79 2.09 55.56 6.07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Normalized Intensity
Chemical Shift (ppm)
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127.96
31.90
14.09
29.34
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127.24
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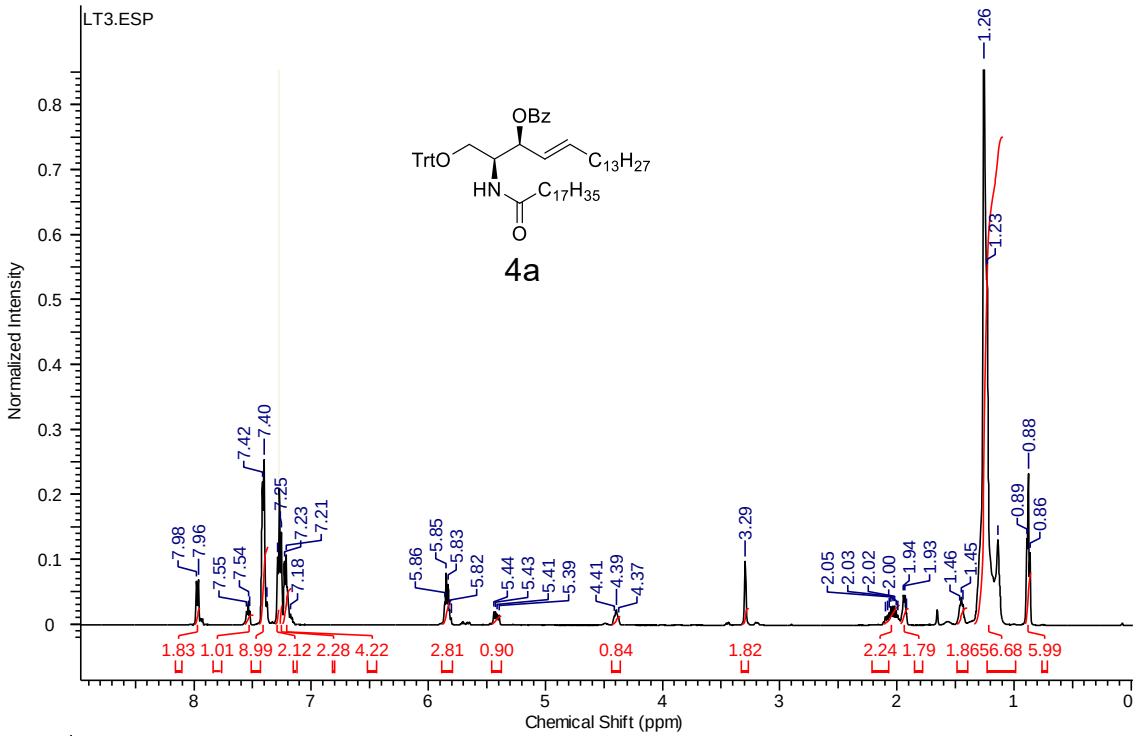
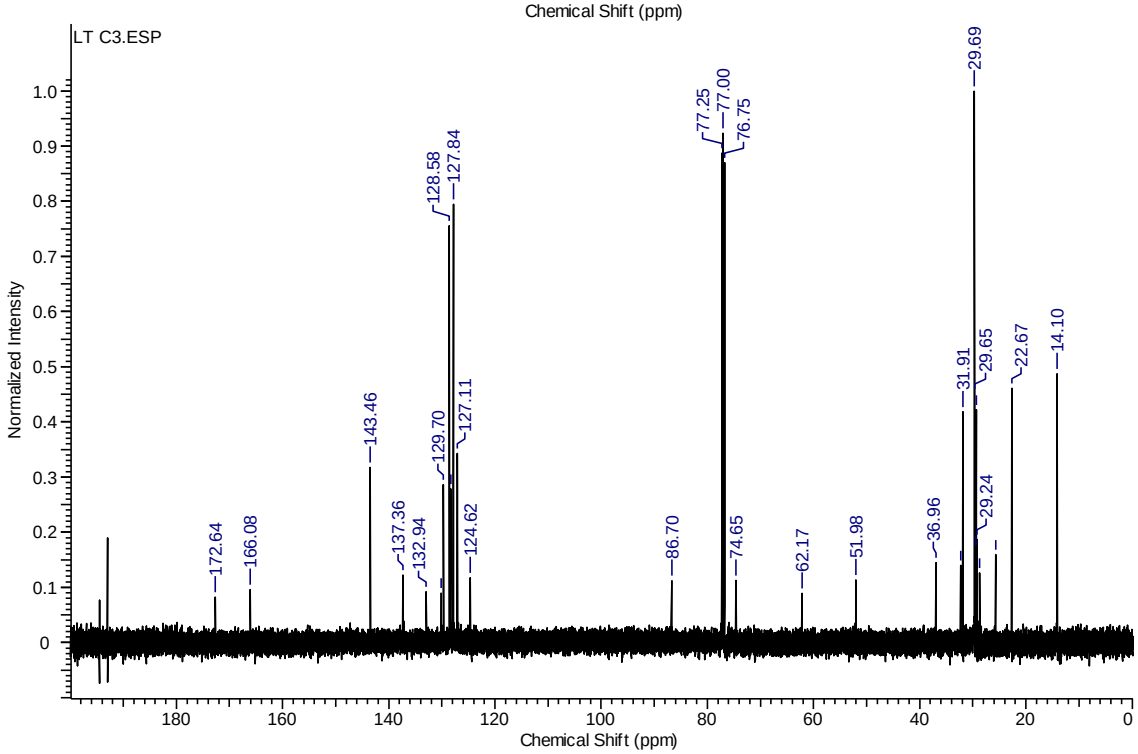
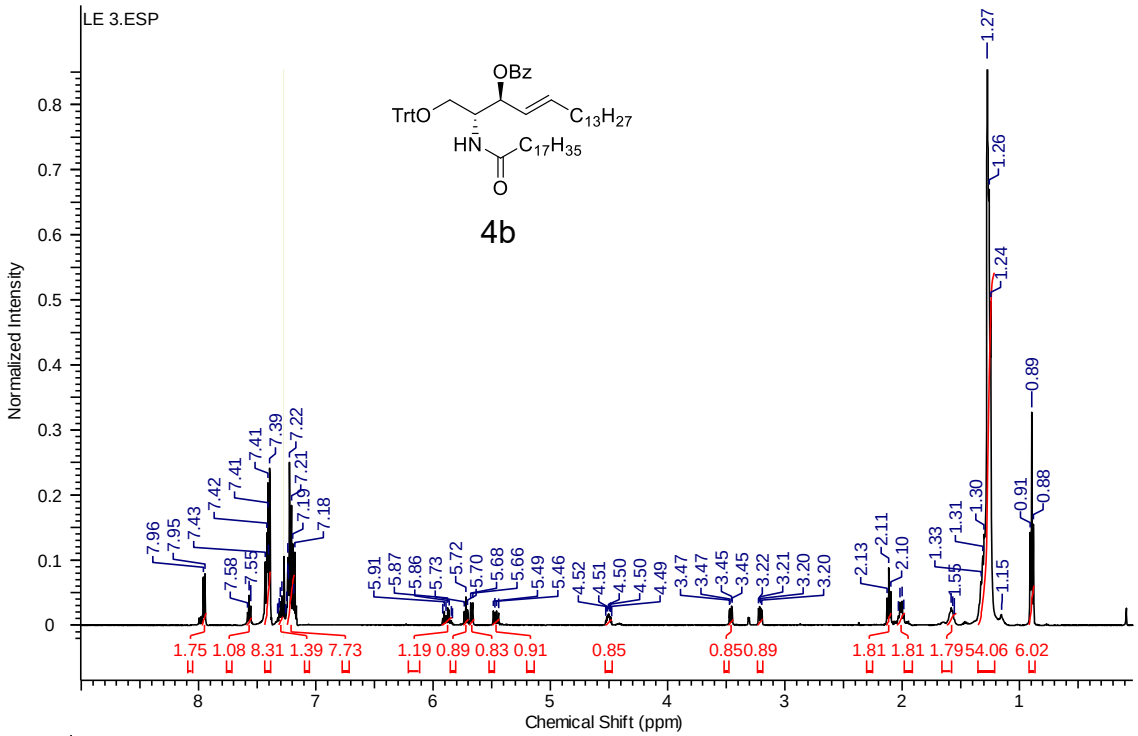
133.49
87.16
73.09
64.15
53.12
36.85
32.28
29.12


LT3.ESP
OBz
TrtO
$C_{13}H_{27}$
HN
$C_{17}H_{35}$
O
4a
Normalized Intensity
Chemical Shift (ppm)
1.83 1.01 8.99 2.12 2.28 4.22 2.81 0.90 0.84 1.82 2.24 1.79 1.86 56.68 5.99


LT C3.ESP
Normalized Intensity
Chemical Shift (ppm)


LE 3.ESP
OBz
TrtO
$C_{13}H_{27}$
HN
$C_{17}H_{35}$
O
4b
Normalized Intensity
Chemical Shift (ppm)
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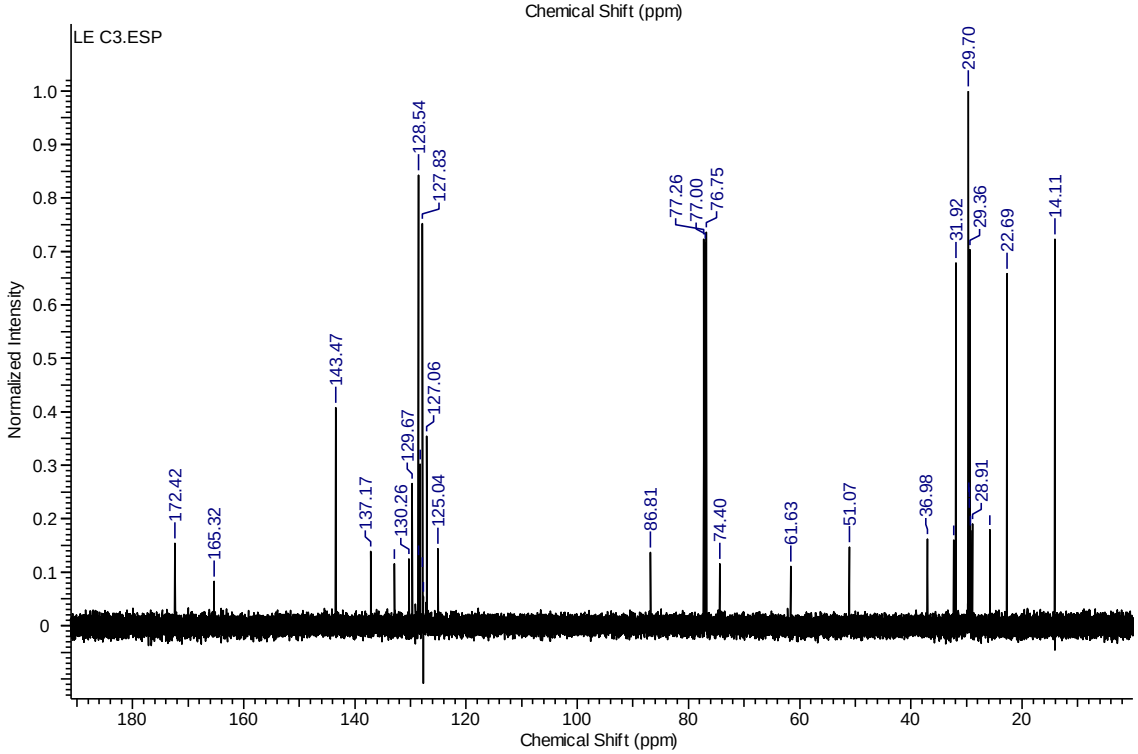
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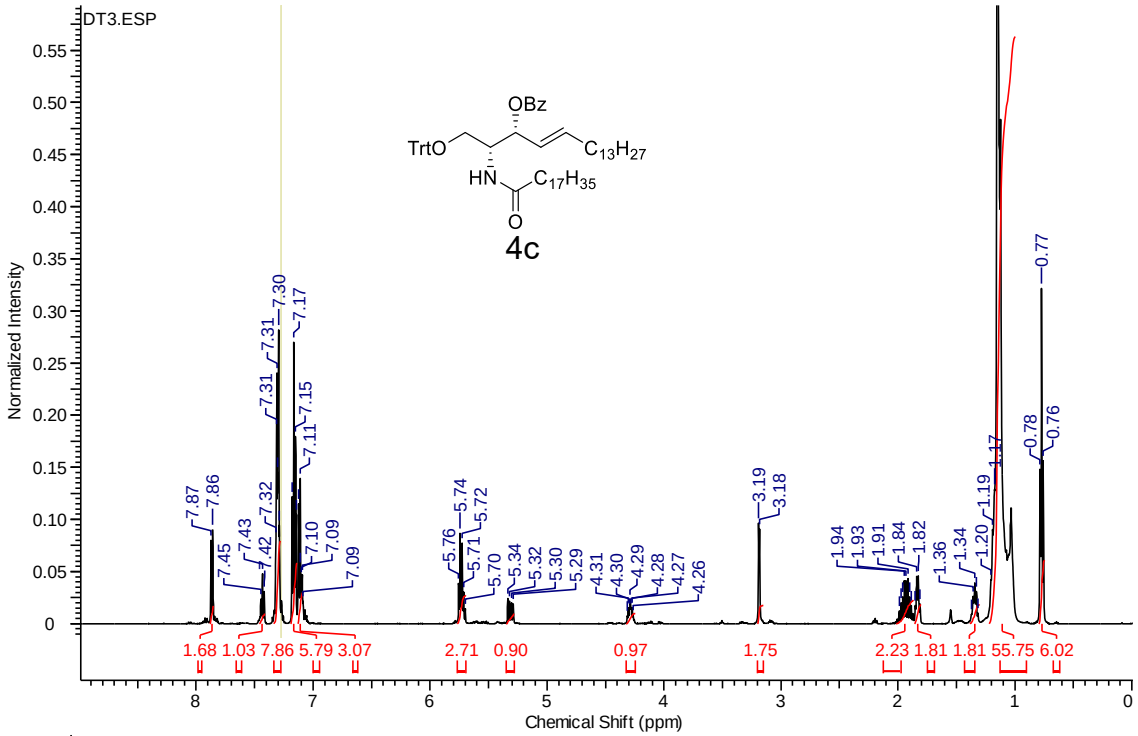
Normalized Intensity
Chemical Shift (ppm)


DT3.ESP
Normalized Intensity
Chemical Shift (ppm)
OBz
TrtO
$C_{13}H_{27}$
HN
$C_{17}H_{35}$
O
4c
7.87
7.86
7.45
7.43
7.42
7.32
7.31
7.31
7.30
7.17
7.15
7.11
7.10
7.09
7.09
5.76
5.74
5.71
5.72
5.70
5.34
5.32
5.30
5.29
4.31
4.30
4.29
4.28
4.27
4.26
3.19
3.18
1.94
1.93
1.91
1.84
1.82
1.36
1.34
1.20
1.19
1.17
0.78
0.77
0.76
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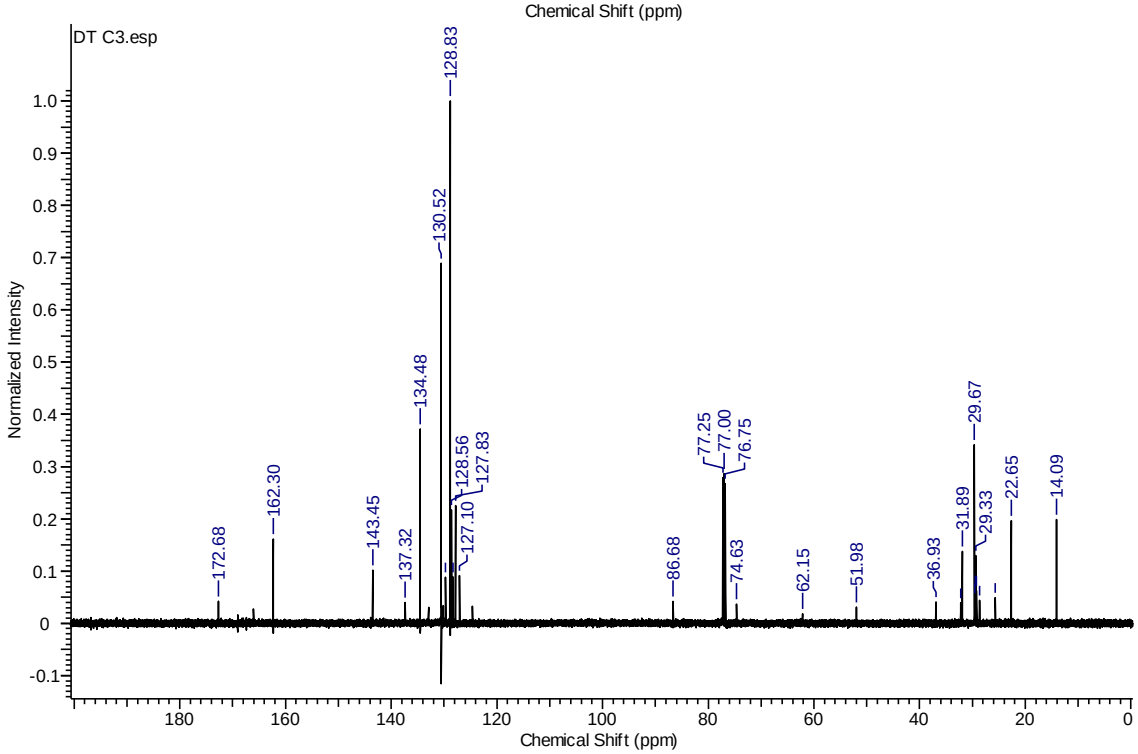
1.75
2.23 1.81 1.81 55.75 6.02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Normalized Intensity
Chemical Shift (ppm)
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128.83
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127.83
127.10
86.68
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36.93
31.89
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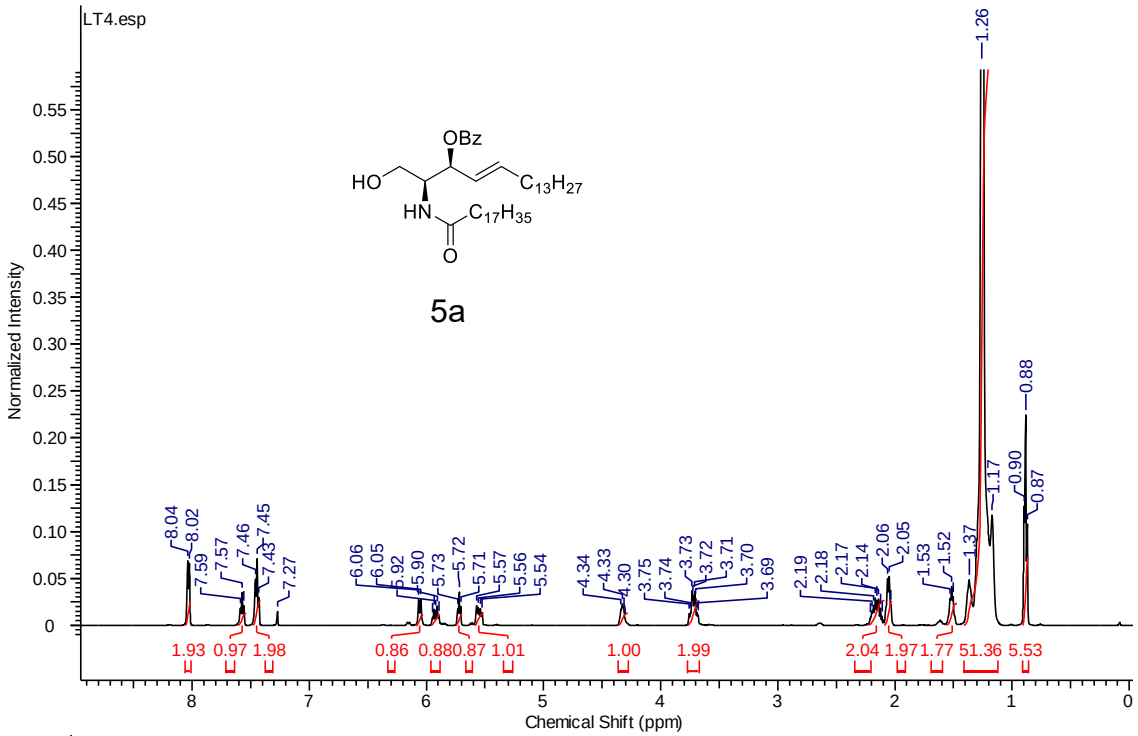
29.33
22.65
14.09


LT4.esp
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HO
HN
$C_{13}H_{27}$
$C_{17}H_{35}$
O
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Normalized Intensity
Chemical Shift (ppm)
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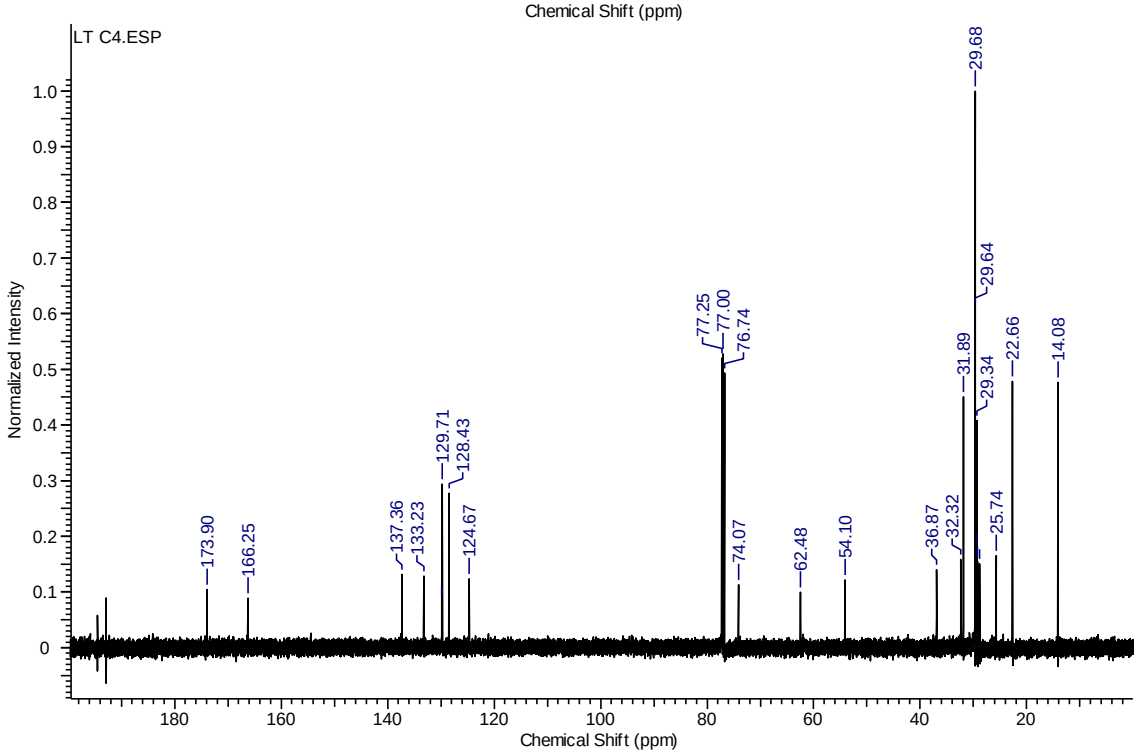
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Normalized Intensity
Chemical Shift (ppm)
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133.23
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77.00
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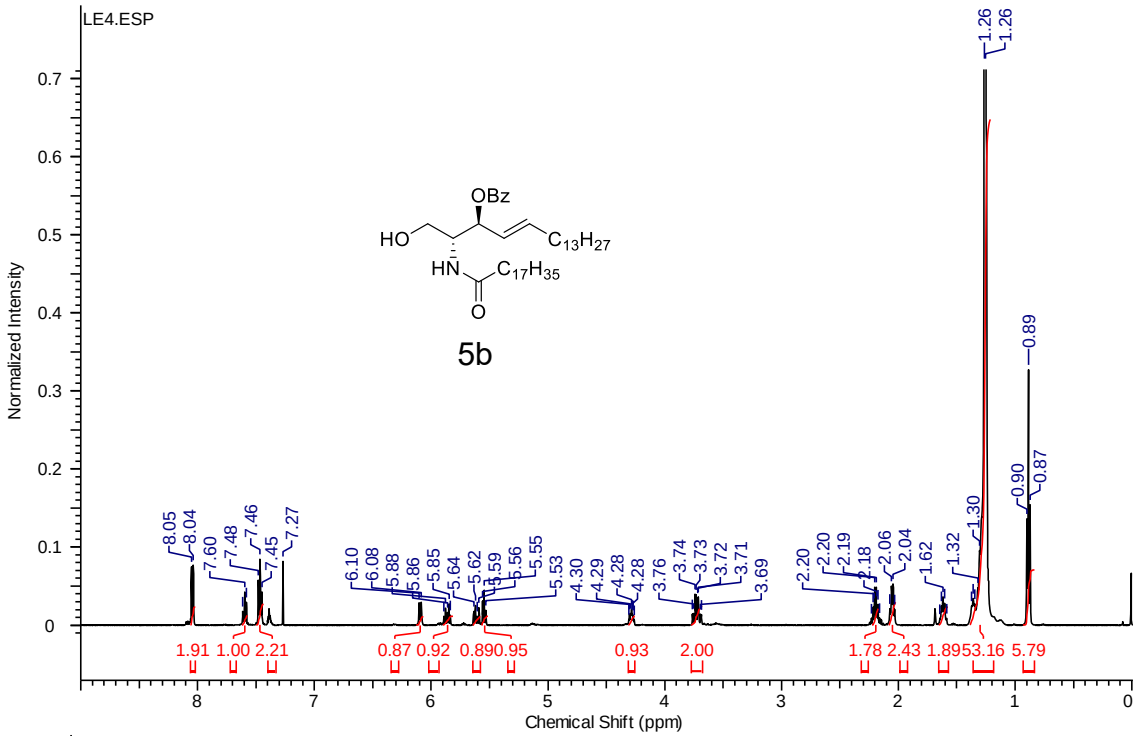
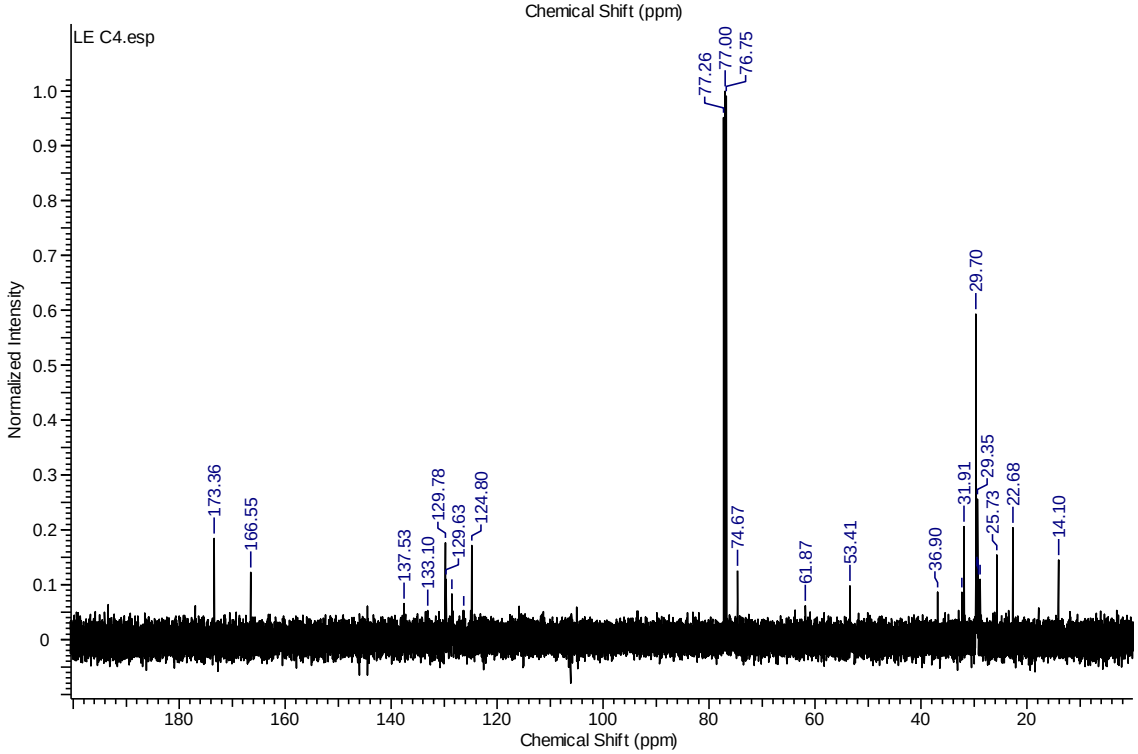
29.64
29.34
25.74
22.66
14.08


LE4.ESP
Normalized Intensity
Chemical Shift (ppm)
OBz
HO
HN
$C_{13}H_{27}$
$C_{17}H_{35}$
O
5b
8.05 8.04 7.60 7.48 7.46 7.45 7.27
6.10 6.08 5.88 5.86 5.85 5.64 5.62 5.59 5.56 5.55 5.53
4.30 4.29 4.28 4.28
3.76 3.74 3.73 3.72 3.71 3.69
2.20 2.20 2.19 2.18 2.06 2.04 1.62 1.32 1.30
1.26 1.26 0.90 0.89 0.87
1.91 1.00 2.21 0.87 0.92 0.89 0.95 0.93 2.00 1.78 2.43 1.89 53.16 5.79


LE C4.esp
Normalized Intensity
Chemical Shift (ppm)
77.26 77.00 76.75
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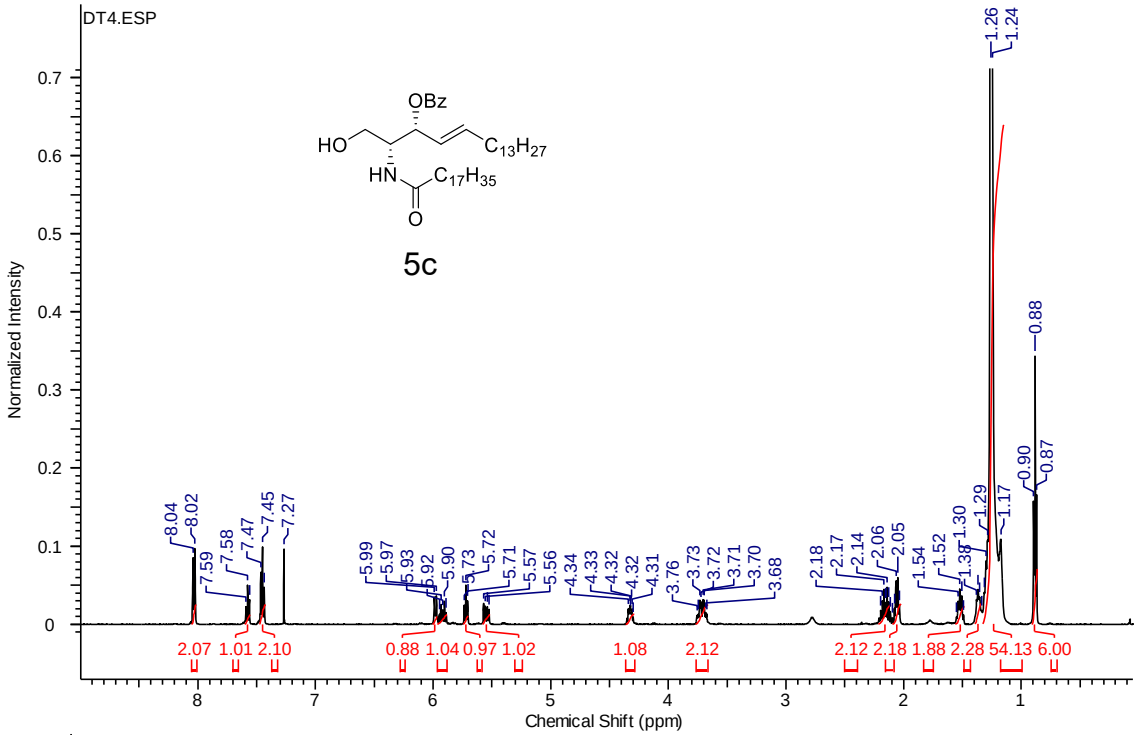
DT4.ESP
OBz
HO
$C_{13}H_{27}$
HN
$C_{17}H_{35}$
O
5c
Normalized Intensity
Chemical Shift (ppm)
8.04 8.02 7.59 7.58 7.47 7.45 7.27
5.99 5.97 5.93 5.92 5.90 5.73 5.72 5.71 5.57 5.56
4.34 4.33 4.32 4.31 3.76 3.73 3.72 3.71 3.70 3.68
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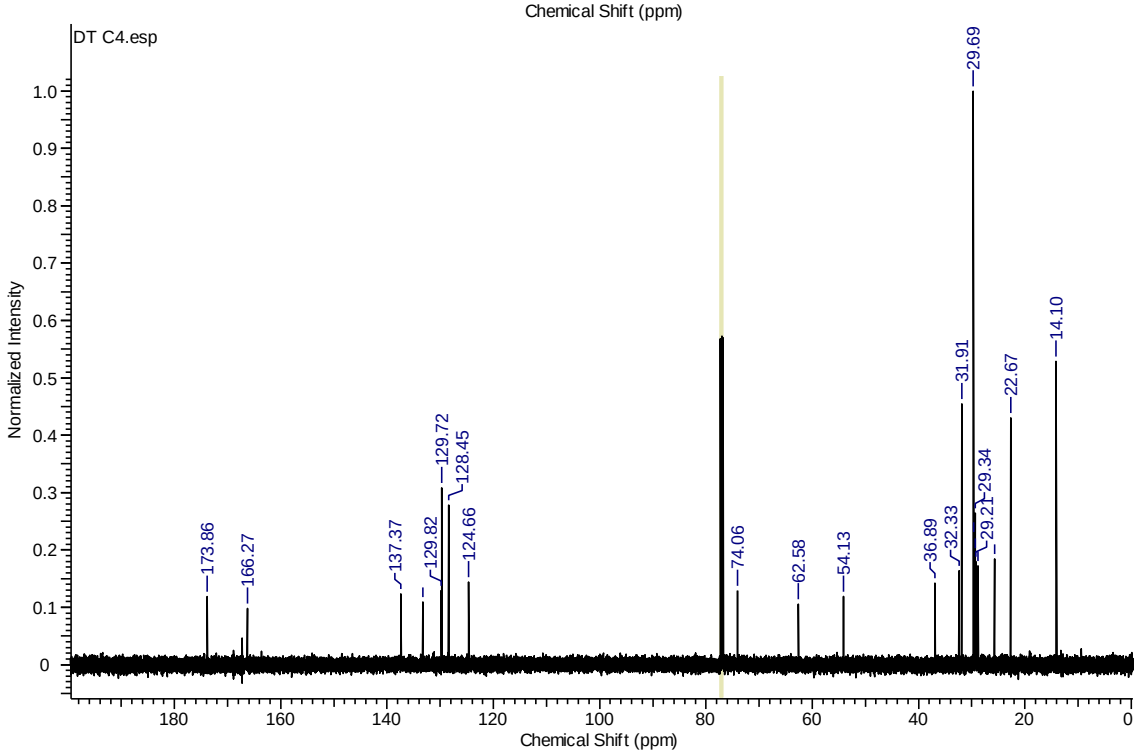
DT C4.esp
Normalized Intensity
Chemical Shift (ppm)
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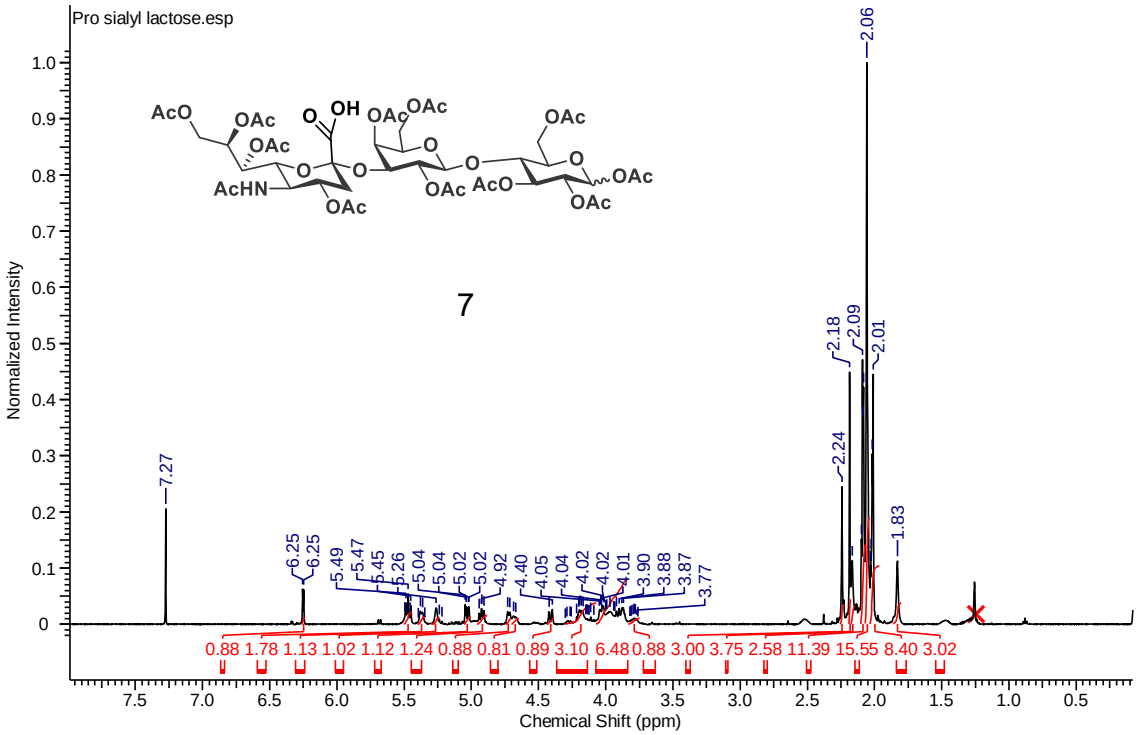
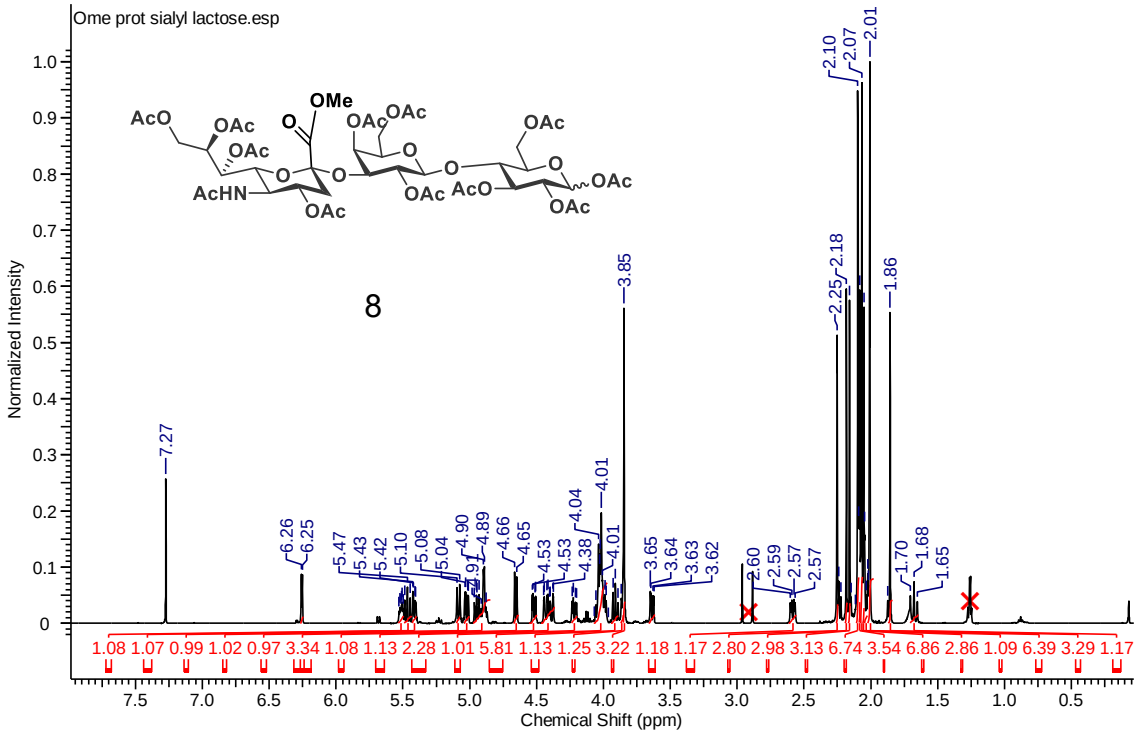
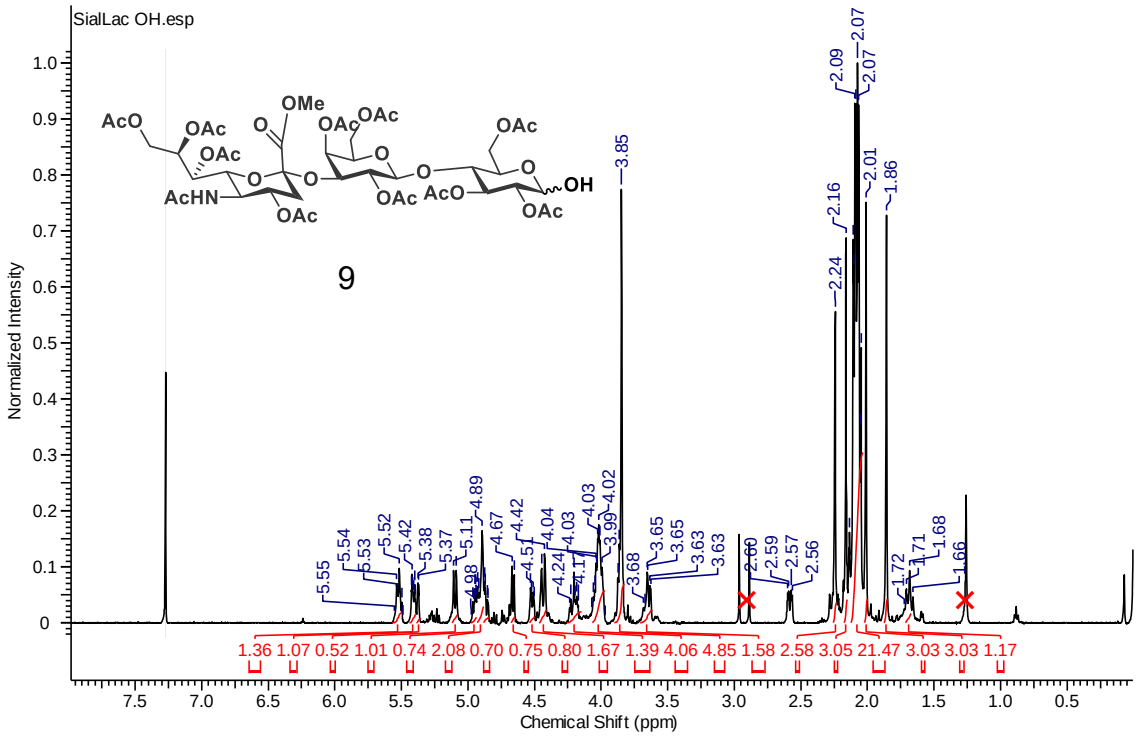
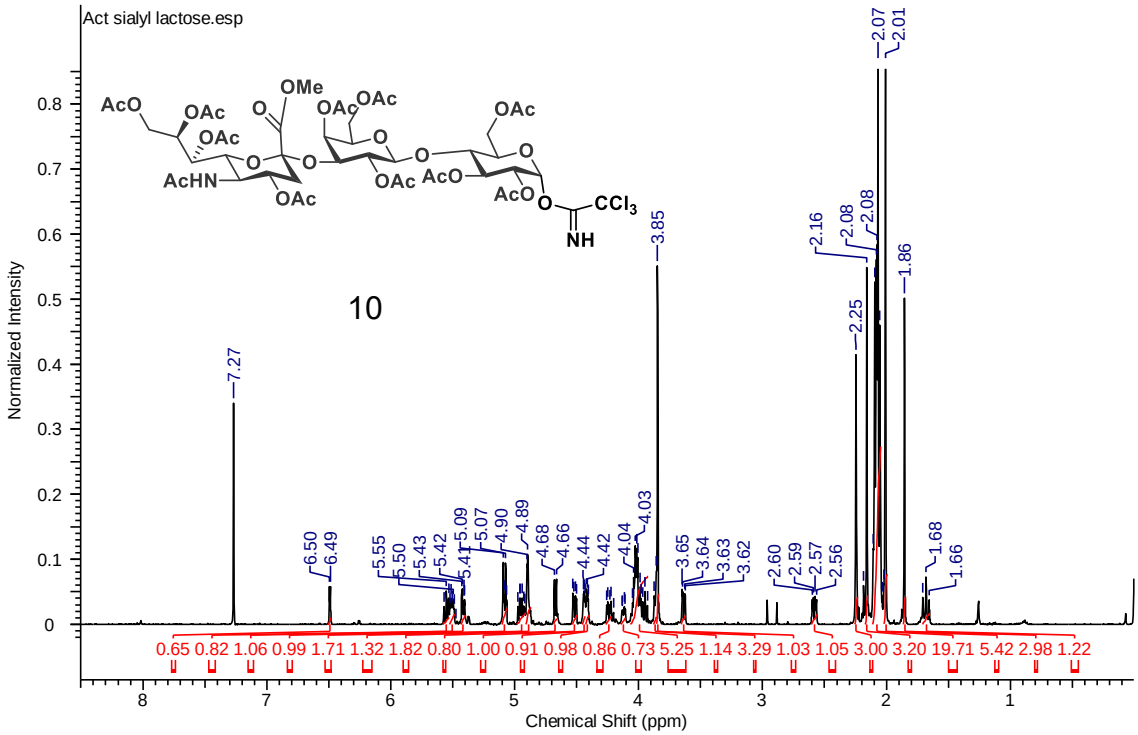
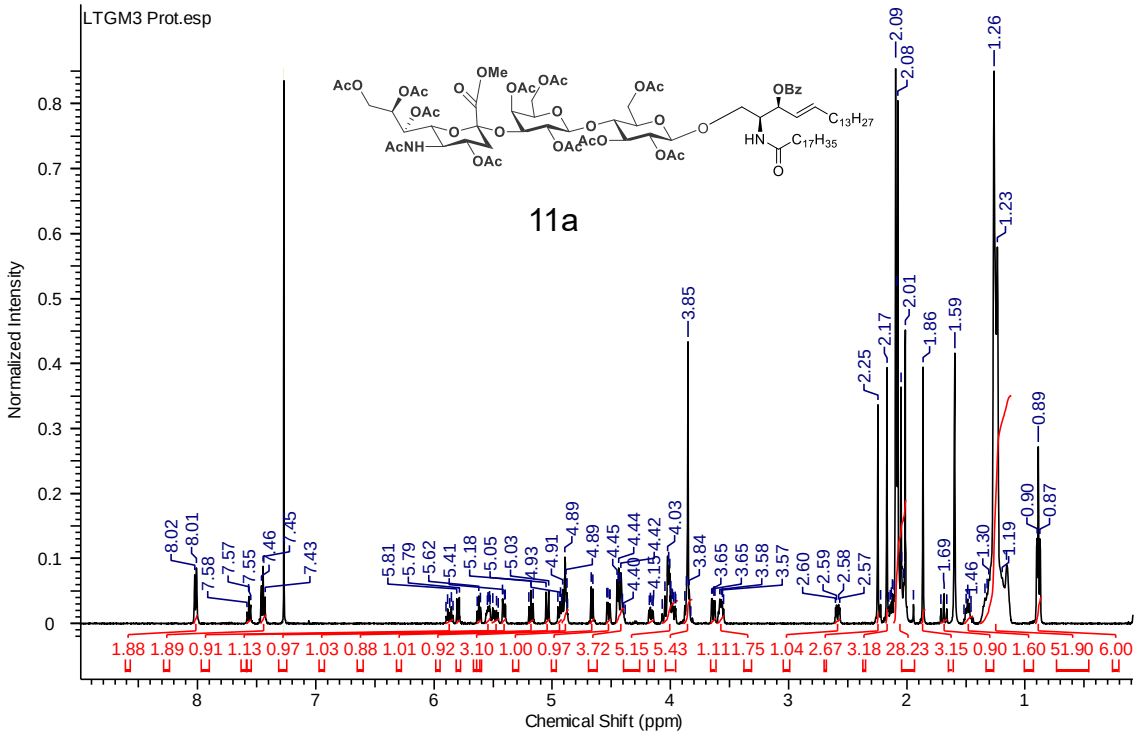
74.06 62.58 54.13 36.89 32.33 31.91 29.69 29.34 29.21 22.67 14.10

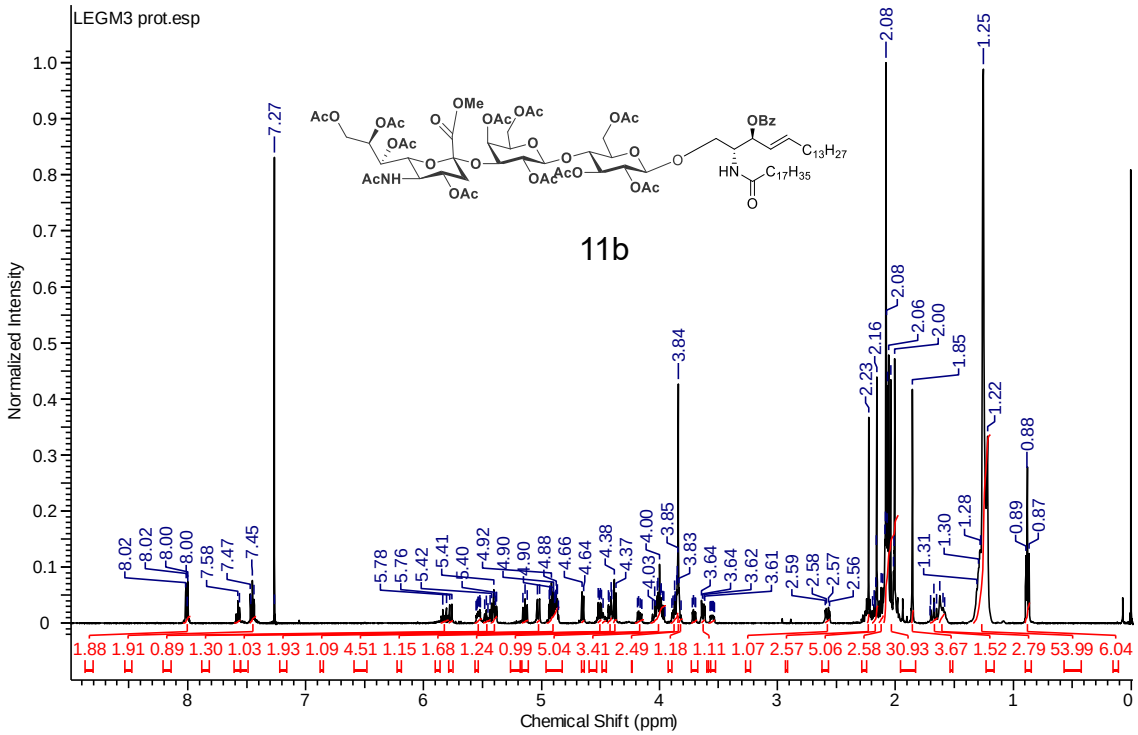

Pro sialyl lactose.esp
7
Normalized Intensity
Chemical Shift (ppm)


Ome prot sialyl lactose.esp
8
Normalized Intensity
Chemical Shift (ppm)


SialLac OH.esp
9
Normalized Intensity
Chemical Shift (ppm)


Act sialyl lactose.esp
10
Normalized Intensity
Chemical Shift (ppm)


LTGM3 Prot.esp
11a
Normalized Intensity
Chemical Shift (ppm)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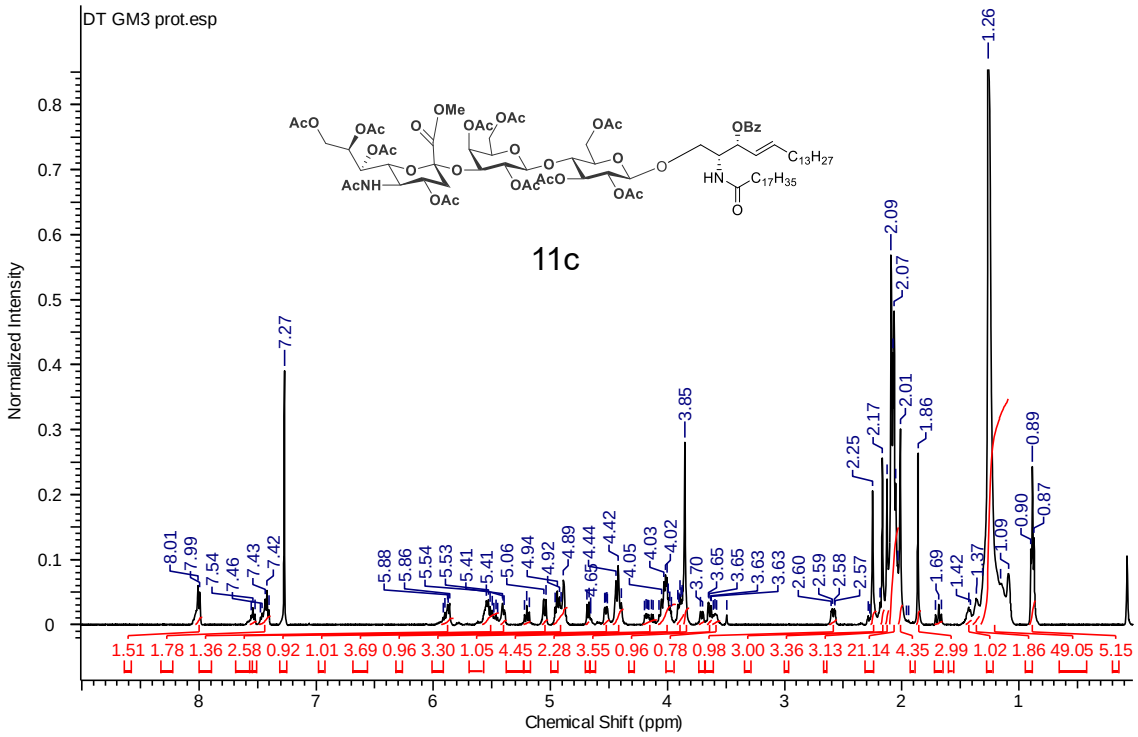
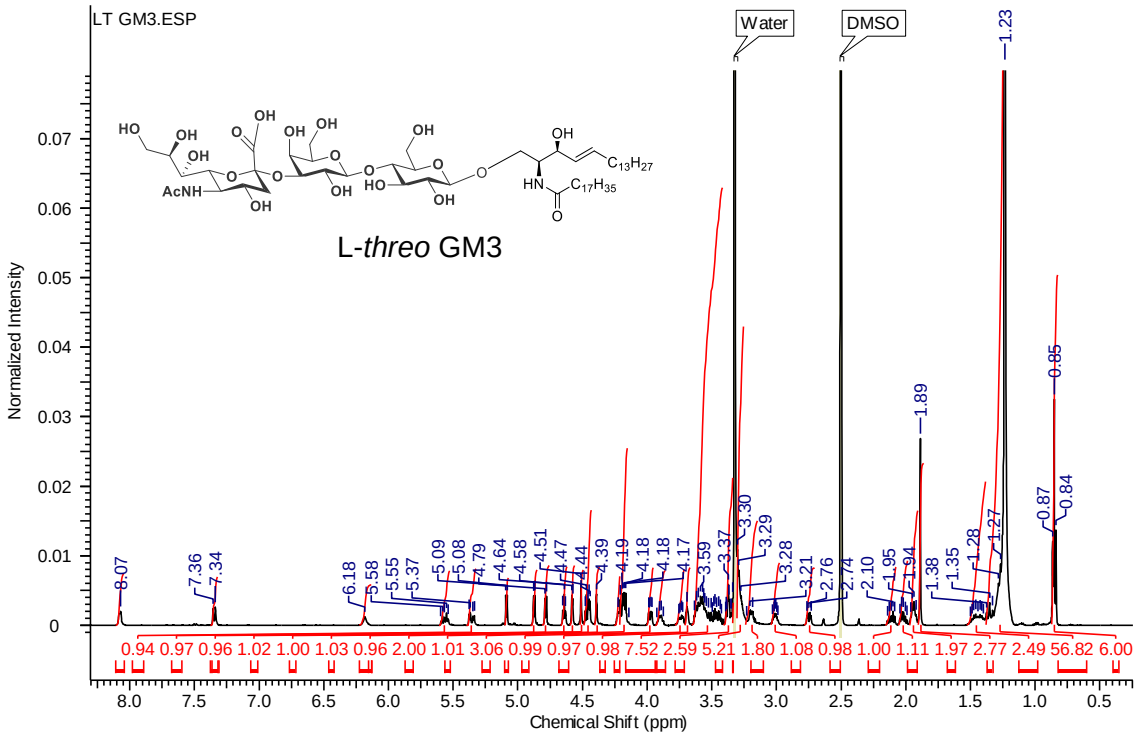
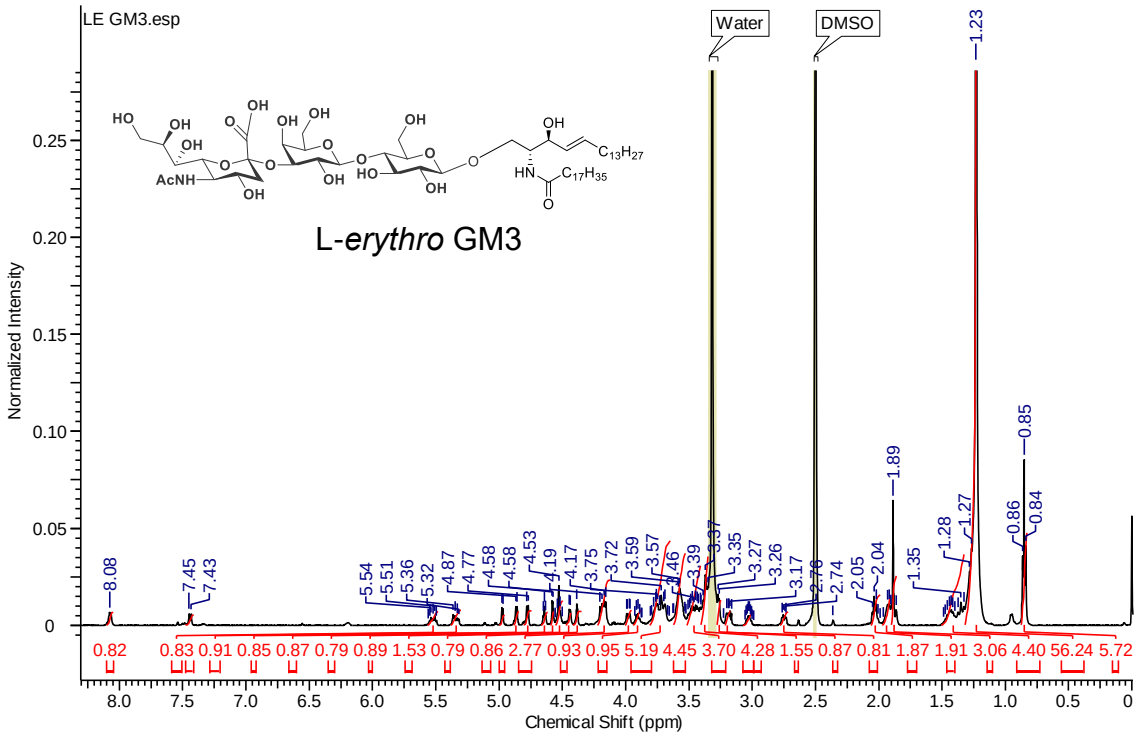
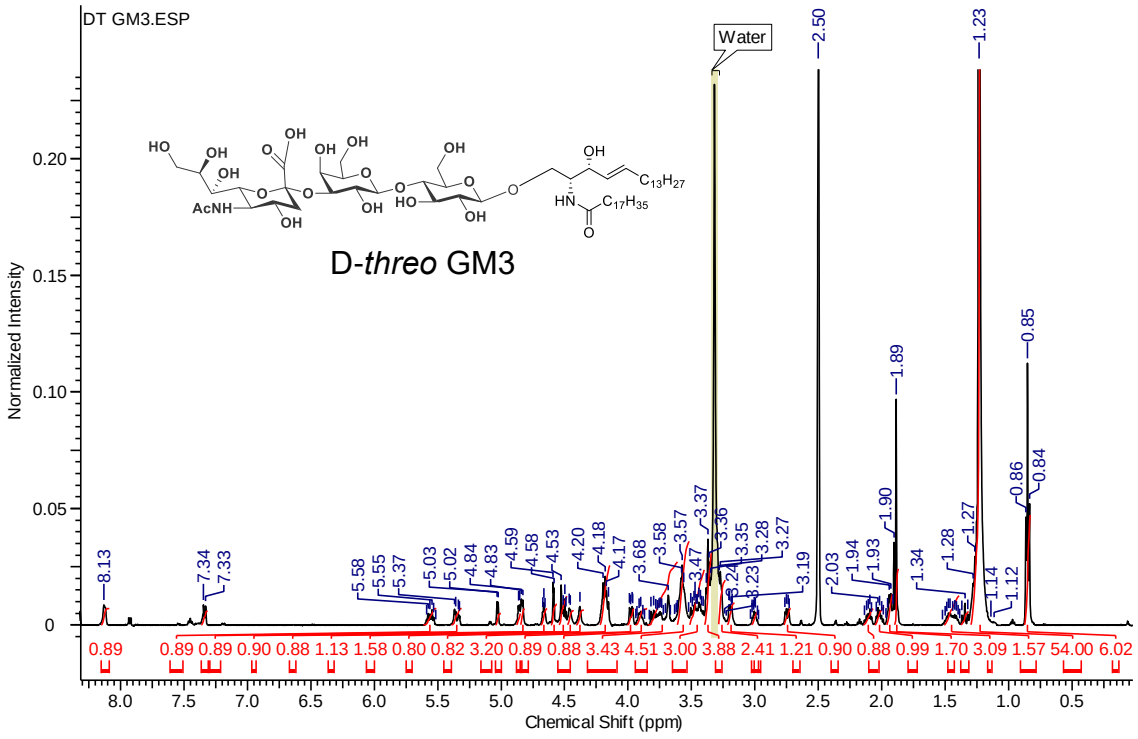
11b
Normalized Intensity
Chemical Shift (ppm)


DT GM3 prot.esp
11c
Normalized Intensity
Chemical Shift (ppm)


LT GM3.ESP
Water
DMSO
L-threo GM3
Normalized Intensity
Chemical Shift (ppm)


LE GM3.esp
Water
DMSO
L-erythro GM3
Normalized Intensity
Chemical Shift (ppm)


DT GM3.ESP
Water
D-threo GM3
Normalized Intensity
Chemical Shift (ppm)

## 2.6 References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# Chapter-3

## Creation of chiral ceramide library and their stereochemical effects on sphingomyelin synthase activity

## 3.1 Abstract

Enantiomers or diastereomers of chiral bioactive compounds often exhibit different biological and toxicological properties. Here, we report the efficient synthesis of four stereoisomers of sphingosine and derivatization of unique chiral ceramides through a combinatorial chemistry by solid phase activated resin ester. In addition, to test the effectivity of stereochemistry of ceramide, we demonstrated a cell-based assay of sphingomyelin synthase inhibition in the presence of chiral unique ceramides, which suggested libraries of this sort will be a rich source of biologically active synthetic molecules.

## 3.2 Introduction

Chirality is a property of matter found throughout biological systems, from basic building blocks of biomolecules such as amino acids, carbohydrates, and lipids. In the field of new drug discovery and pharmacotherapeutics, information on the stereoisomerism of a bioactive compound is very important because living systems are themselves chiral and each of the stereoisomers of chiral drugs show very different effects *in vivo*. In fact, over 50% of commercially available drugs are chiral substances[1], and it is therefore also important to realize the safety and efficacy of chiral drugs in order to avoid drug-induced incidents such as the thalidomide disaster in Japan in the late 1950s. Over the past several decades, protein structure-based drug design in silico using structural information from solution-state NMR[2] and X-ray crystallography[3] have often provided detailed structural information on target biomolecules and novel chiral drug candidates. In the case of membrane proteins, such as a membrane receptor and enzyme, it is often more efficient to design drug candidates from original substrates due to difficulty in using NMR and X-ray methods.

In this study, we established new chiral inhibitors based on the ceramide backbone for the membrane protein sphingomyelin synthase (SMS), and evaluated their inhibitory efficacy. SMS catalyzes ceramide and phosphatidylcholine (PC) as substrates to produce sphingomyelin (SM) and diacylglycerol (DAG)[4]. It has been shown that SMS modulates the levels of SM and other sphingolipids levels, thereby regulating membrane fluidity, ceramide-dependent apoptosis, lipid metabolism, and signal transduction[5]. In addition, it has reported that mice with SMS knockout or knockdown showed a decrease in plasma inflammatory cytokines[6] and showed resistance to the development of high-fat diet-induced obesity[7], insulin resistance[8],

Alzheimer's disease[9], and tumorigenesis[10]. Therefore, SMS inhibition is a novel therapeutic approach for these diseases.

Our strategy for creating SMS inhibitors is to design the structure of SMS inhibitors from ceramide as an original substrate. The most common ceramide in mammals consists of the amino alcohol sphingosine as a backbone moiety, which has two chiral centers at the C-2 and C-3 positions. Theoretically, there are four stereoisomers, (D-*erythro*: DE), (L-*erythro*: LE), (D-*threo*: DT) and (L-*threo*: LT), in sphingosine, but only D-*erythro* type [(2*S*, 3*R*, 4*E*)-2-aminooctadec-4-ene-1,3-diol] has been found in mammals (Figure 1). Nakanishi and Berova *et al*. determined their absolute configurations by sensitive and reliable stereochemical analysis utilizing HPLC[11] and circular dichroism (CD) [12] techniques. We have also established an efficient methodology for those of sphingosine by using the vibrational circular dichroism (VCD) technique[13], which measures the differential absorption of left versus right circularly polarized infrared radiation. For investigating the relationship between stereochemistry and biological activity, the four isomers were synthesized in some stidies and their biological activities were evaluated[14]. In addition to this, Inokuchi and Norman reported that an inhibitor, (D)-*threo*-1-phenyl-2-decanoylamino-3-morpholino-1propanol (D-PDMP), of glucosylceramide synthase suppressing GM3 in adipocytes showed a TNF-$\alpha$-induced defect in insulin-dependent tyrosine phosphorylation of insulin receptor substrate-1 (IRS-1)[15]. Notably, the stereochemistry of active D-PDMP is different from that of endogenous ceramide. Therefore, stereospecific activity is a common feature for sphingolipids, suggesting that their targets also have specific spatial configurations, and study on the stereochemistry of sphingolipids for revealing the structure-activity relationships of these pharmacologically active derivatives. However, a deeper understanding of the

biological role of ceramide stereochemistry in human pathophysiology and the potential therapeutic use of SMS has still been veiled. In this study, to address these unclear points, we synthesized several unique ceramides of all of four stereoisomers by solid-phase activated resin ester and evaluated the importance of their stereochemistry efficacy towards lipid-metabolizing enzymes.

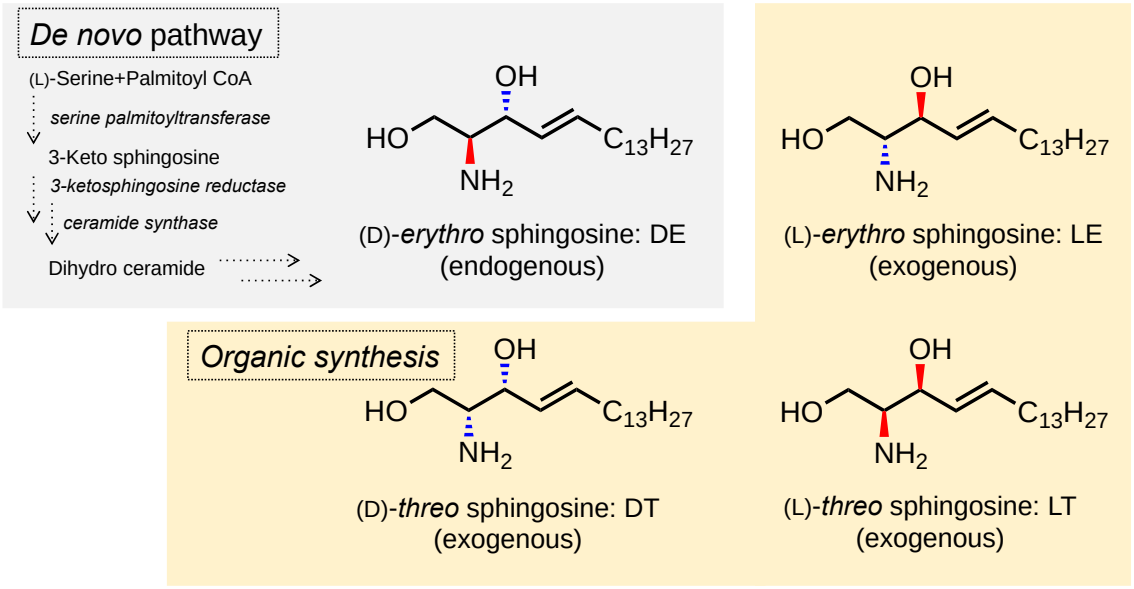
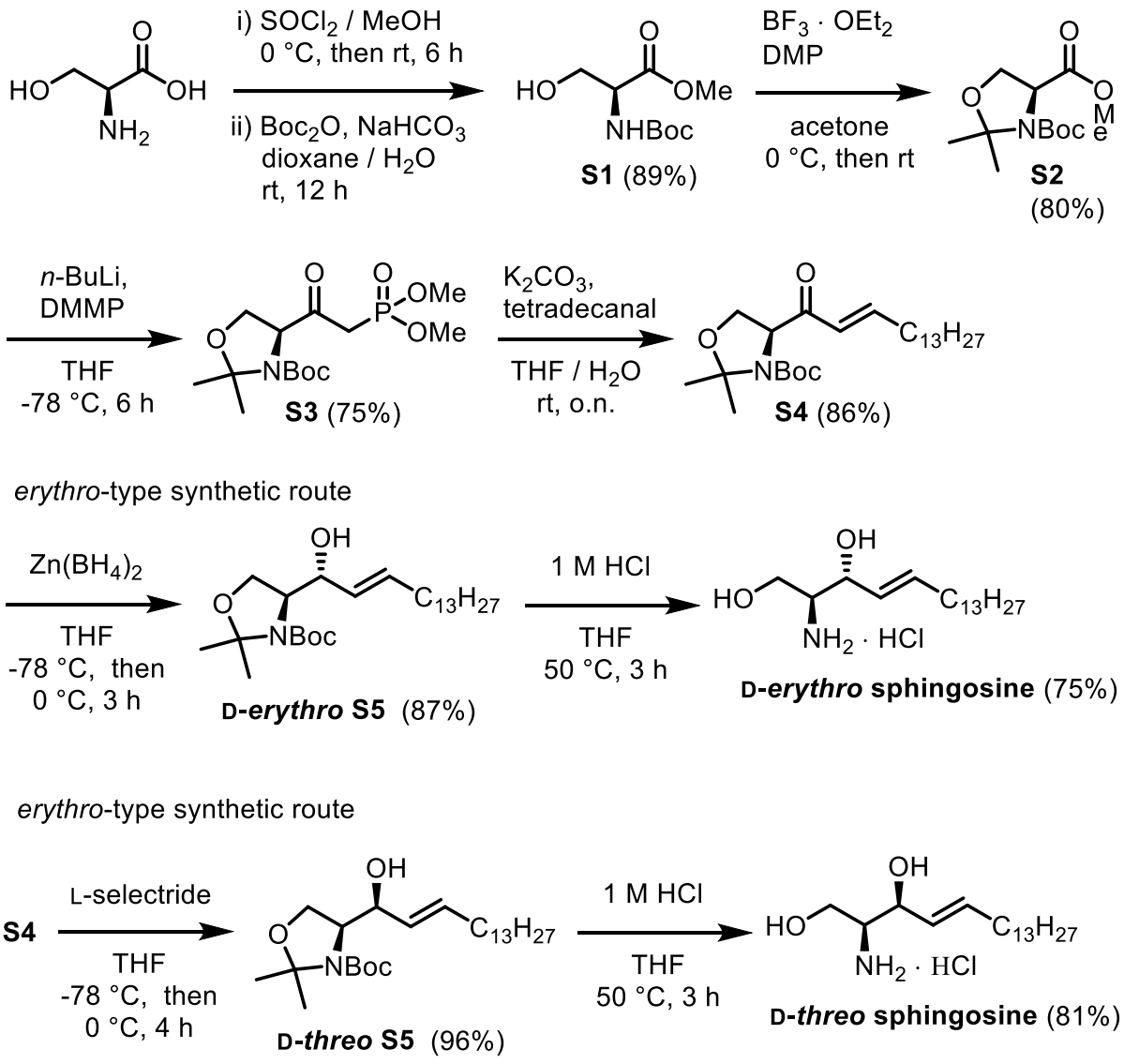



**Figure 1.** *De novo* pathway of endogenous (D)-*erythro* sphingosine and exogenous stereoisomers of sphingosine, (L)-*erythro*, (D)-*threo*, and (L)-*threo* sphingosines.

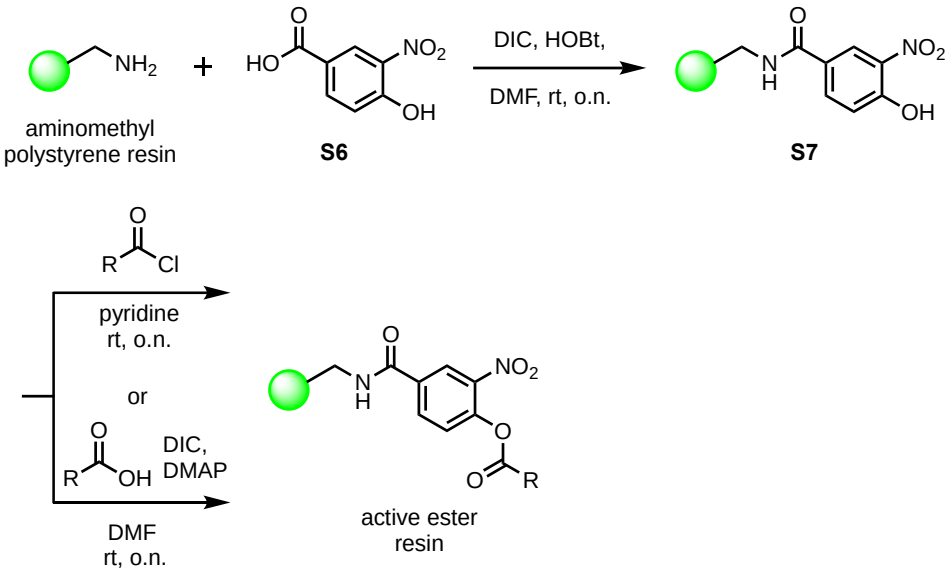
## 3.3 Result and discussion

First, we started to synthesize all four stereoisomers of sphingosine according to established methods[16] from (L)- or (D)-serine with slight modification **(Scheme 1**). The specific rotations of the stereoisomers were measured, DE: $[\alpha]_D$ -4.4, LE: +4.6, DT: -2.9, and LT: +2.2 (c 1.0; $CHCl_3$). Next, to prepare unique ceramides efficiently through a combinatorial chemistry, we prepared several activated esters coupling with 32 commercially available carboxylic acids or acid chlorides and nitrophenol on polystyrene as reported by Chang et al.[17] (**Scheme 2**). Subsequently, each of the four chiral shingosines was subjected to acylation with 32 kinds of activated ester

resin, followed by filtration to generate 128 unique ceramides without starting material sphingosine and byproducts (**Figure 2**). The products and their purity were characterized by measuring ESI-MS and $^{1}$H-NMR of 40 randomly selected ceramides among the 128 ceramides.




**Scheme 1.** Synthetic route for all four strereoisomers of sphingoid base. L-*erythro* and -*threo* sphingoid bases were prepared from D-serine in the same manner as shown above scheme.




**Scheme 2.** Synthetic route of active ester resin from an amino methyl polystyrene resin.

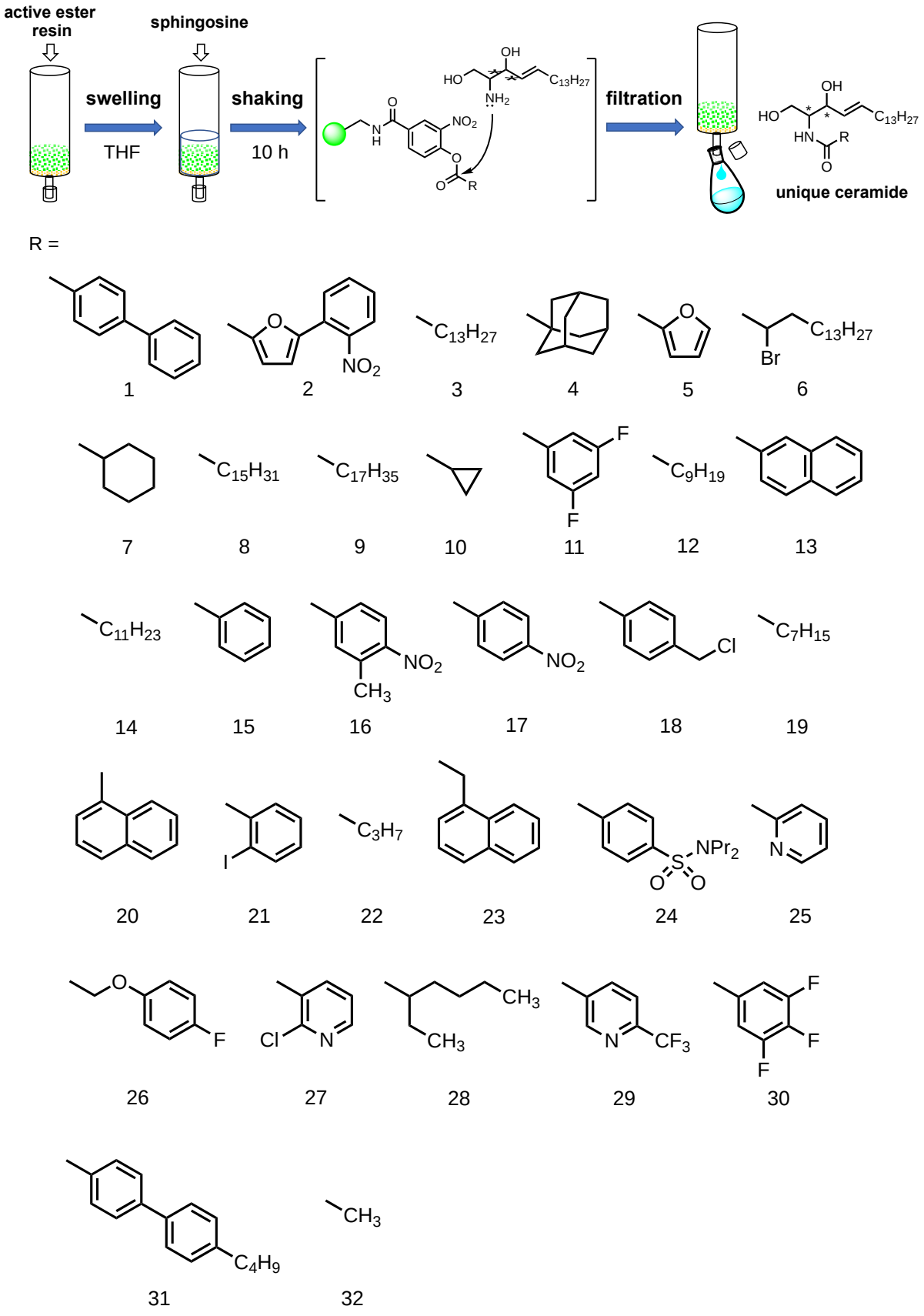



**Figure 2**. Efficient preparation of unique chiral ceramides by active ester resin.

Finally, to evaluate the inhibitory efficacy of the stereochemistry of each of the chiral four unique ceramides, we carried out cell-based assays for both isozymes SMS1 and SMS2[18] in the presence of the 128 unique ceramides. As shown in Table 1, several ceramides showed relatively moderate inhibitory activities ($IC_{50}$ 0.2 ~ 1 μM, **Table 1**) compared with those of our previously reported natural compounds[19]. Furthermore, to determine the differences in the inhibitory activities of the four stereoisomers, we represented the $IC_{50}$ values as a heatmap graphical representation in which individual values contained in a matrix are displayed as colors. Although the natural stereoisomer is (D)-*erythro* type, the unnatural (L)-*threo* stereoisomer indicated that the ratio of ceramides showing strong inhibitory activities towards SMS1 and SMS2 is high (**Figure 3**). In the present study, we demonstrated that the chiral chemistry of ceramide affected its inhibitory activity towards the lipid-metabolizing enzymes.

| | SMS1 ($IC_{50}$) μM | | | | SMS2 ($IC_{50}$) μM | | | |
|---|---|---|---|---|---|---|---|---|
| **entry (# of R)** | **DE** | **LE** | **LT** | **DT** | **DE** | **LE** | **LT** | **DT** |
| 1 | 20 | >100 | 70 | >100 | 7 | 15 | 20 | 30 |
| 2 | 3 | 15 | 7 | 30 | 0.2 | 1 | 0.3 | 0.4 |
| 3 | 50 | >100 | >100 | >100 | >100 | >100 | 80 | >100 |
| 4 | 4 | 50 | 25 | >100 | 10 | 9 | 9 | 4 |
| 5 | 5 | 20 | 20 | 20 | 3 | 6 | 3 | 4 |
| 6 | 30 | 100 | 70 | 70 | 30 | 60 | 60 | 40 |
| 7 | 3 | 30 | 25 | 70 | 8 | 7 | 15 | 20 |
| 8 | >100 | >100 | >100 | >100 | 70 | 100 | 40 | >100 |
| 9 | >100 | >100 | >100 | >100 | 70 | 80 | 90 | >100 |
| 10 | 0.8 | 10 | 3.5 | 20 | 20 | 10 | 5 | 3 |
| 11 | 100 | 90 | 30 | >100 | 30 | 25 | 10 | 10 |
| 12 | 50 | 60 | 25 | >100 | 30 | 30 | 7 | 40 |
| 13 | 20 | 20 | 6 | 50 | 9 | 10 | 2 | 6 |
| 14 | >100 | >100 | 60 | >100 | >100 | >100 | 50 | >100 |
| 15 | 15 | 20 | 9 | 30 | 15 | 10 | 3 | 5 |
| 16 | 30 | 40 | 20 | 70 | 10 | 20 | 4 | 7 |
| 17 | 20 | 50 | 15 | 20 | 4 | 10 | 2 | 5 |
| 18 | 4 | 25 | 15 | 20 | 1.5 | 6 | 1.5 | 3 |
| 19 | 1 | 15 | 7 | 30 | 1 | 5 | 2 | 7 |
| 20 | 15 | 50 | 7 | 20 | 9 | 10 | 4 | 5 |
| 21 | 30 | 40 | 8 | 15 | 10 | 15 | 3 | 5 |
| 22 | 1 | 9 | 6 | 20 | 3 | 7 | 3 | 5 |
| 23 | 15 | 20 | 15 | 25 | 10 | 7 | 4 | 7 |
| 24 | 8 | 30 | 15 | 30 | 2 | 5 | 1.5 | 4 |
| 25 | 5 | 8 | 10 | 10 | 3 | 5 | 4 | 6 |
| 26 | 50 | 50 | 30 | 50 | 30 | 15 | 9 | 7 |
| 27 | 20 | 20 | 20 | 20 | 10 | 15 | 7 | 7 |
| 28 | 2 | 10 | 3 | 15 | 2 | 5 | 2 | 4 |
| 29 | 10 | >100 | 40 | 80 | 3 | 50 | 15 | 40 |
| 30 | 50 | 100 | 50 | 100 | 30 | 30 | 20 | 30 |
| 31 | 30 | 100 | 40 | 100 | 30 | 50 | 40 | 70 |
| 32 | 16 | 30 | 20 | 30 | 12 | 20 | 10 | 20 |

**Table 1.** $IC_{50}$ values are the means of each of the four unique chiral ceramides in separate determinations for SMS1 or SMS2 expressed in SMS1/2 double knockout mouse fibroblast cell lysate and were determined by using more than four concentrations of each inhibitor.

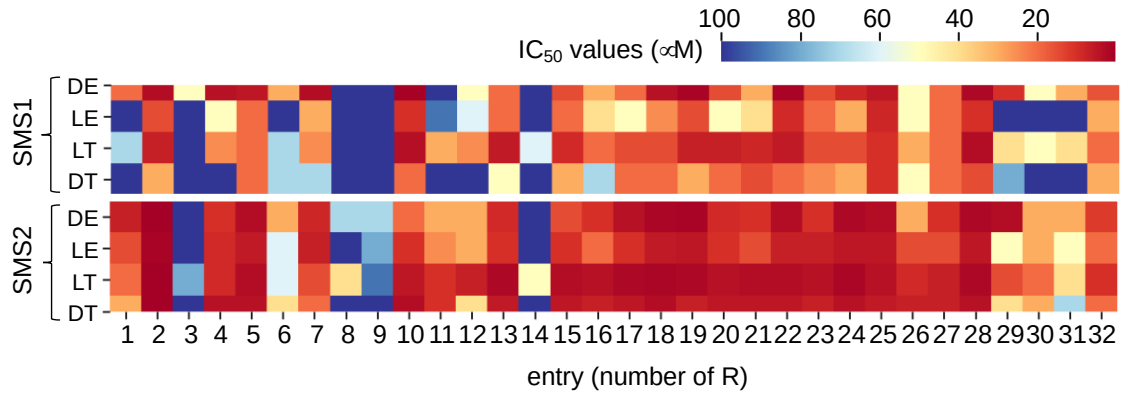



**Figure 3**. Heatmap analysis of $IC_{50}$ values of each of the four unique chiral ceramides for SMS1 and SMS2.

## 3.4 Conclusion

We successfully synthesized 128 unique chiral ceramides through combinatorial chemistry by solid-phase activated ester without starting material sphingosine and byproducts. Additionally, we proved that these ceramides have good inhibitory activities ($IC_{50}$ = 0.2 ~ 1 μM) for sphingomyelin synthases 1 and 2. Furthermore, according to heatmap analysis of $IC_{50}$ values, we confirmed that most of the unnatural (L)-*threo* stereoisomer ceramide derivatives showed strong inhibitory activities towards SMS1 and SMS2 respectively compared to the inhibitory activities of other stereoisomers. Therefore, to create competent bioactive compounds based on the sphingolipid moiety, it is important to thoroughly consider the information on the stereoisomerism of them.

## 3.5 Experimental section

**Synthetic route of active ester resin from an amino methyl polystyrene resin.**

To a mixture of aminomethyl polystyrene (1.5 g, 1.3 mmol) in DMF (15 mL), 4-hydroxy-3-nitrobenzoic acid S6 (1 g, 5.4 mmol), HOBt (972 mg, 7.2 mmol) and Diisopropyl carbodiimide (962 µL, 6.6 mmol) were added. The mixture was shaking for overnight at room temperature. After overnight shaking, the reaction mixture was filtered and washed with DMF (5×20 mL), MeOH (5×20 mL) and dichloromethane (5×20 mL). DMF (10 mL) and piperidine (1 mL) were added to the resin and shake for another 1.5 h to remove undesired side products. The resin was filtered and washed with 10% HCl in DMF, MeOH (5×20 mL) and dichloromethane (5×20 mL), then dried to obtain S7. Next, S7 (500 mg, 0.43 mmol) was suspended in DMF (10 mL) and the corresponding acid (3.0 mmol), *N*,*N'*-diisopropylcarbodiimide (471 µL, 3.0 mmol) and DMAP (63 mg, 0.52 mmol) were added to the suspension. The mixture was agitated on a shaker for overnight at room temperature. The resin was filtered and washed with DMF (5×10 mL), MeOH (5×10 mL) and dichloromethane (5×10 mL) and dried to obtain the active ester resin.

*In case of using acid chlorides;*

S7 (500 mg, 0.43 mmol) was suspended in DMF (10 mL). Pyridine (173 µL, 2.15 mmol) and an acid chloride (3.0 mmol) were added to the mixture. The mixture was agitated on a shaker for overnight at room temperature. The resin was filtered and washed with DMF (5×10 mL), MeOH (5×10 mL) and dichloromethane (5×10 mL) and dried to obtain the active ester resin.

**General procedure for construction of chiral ceramide library**

Solid phase active ester resin (80 mg, 68 µmol), which was prepared by a slightly modified literature procedure in THF (1 mL) was added sphingosine (1 mg, 3.3 µm) and the mixture was agitated on a shaker for 10 h at room temperature. The reaction mixture was filtered and washed with 1mL THF. The combined filtrate was evaporated and dried. The reaction was monitored by TLC and confirmed the completion of reaction by negative ninhydrin staining. The purity of the all ceramides confirmed by TLC profile, each gave a single spot. Further confirmed the identity of all ceramides by ESI-MS and randomly selected ceramides were confirmed by $^{1}$H NMR.

***N*-((2*S*,3*R*,*E*)-1,3-dihydroxyoctadec-4-en-2-yl)-[1,1'-biphenyl]-4-carboxamide (DE 1)**

$^{1}$H NMR (500 MHz, $CDCl_3$): δ = 7.87 - 7.93 (m, 2 H), 7.68 (d, *J*=8.3 Hz, 2 H), 7.63 (d, *J*=7.1 Hz, 2 H), 7.48 (t, *J*=7.6 Hz, 2 H), 7.38 - 7.43 (m, 1 H), 7.03 (d, *J*=7.3 Hz, 1 H), 5.82 - 5.90 (m, 1 H), 5.63 (dd, *J*=15.4, 6.4 Hz, 1 H), 4.47 - 4.52 (m, 1 H), 4.14 - 4.17 (m, 1 H), 4.11 (dd, *J*=11.2, 3.4 Hz, 1H), 3.86 (dd, *J*=11.2, 3.2 Hz, 1 H), 2.09 (q, *J*=7.0 Hz, 2 H), 1.34 - 1.42 (m, 2 H), 1.21 - 1.33 (m, 20 H), 0.89 (t, *J*=7.0 Hz, 3 H). ESI-MS(m/z): 502.3796 $[M+Na]^+$.

***N*-((2s,3*S*,*E*)-1,3-dihydroxyoctadec-4-en-2-yl)-[1,1'-biphenyl]-4-carboxamide (LT 1)**

$^{1}$H NMR (500 MHz, $CDCl_3$): δ = 7.88 (d, *J*=8.3 Hz, 2 H), 7.68 (d, *J*=8.3 Hz, 2 H), 7.62 (d, *J*=7.3 Hz, 2 H), 7.48 (t, *J*=7.6 Hz, 2 H), 7.37 - 7.43 (m, 1 H), 6.88 (d, *J*=7.8 Hz, 1 H), 5.78 - 5.87 (m, 1 H), 5.58 (dd, *J*=15.5, 6.2 Hz, 1 H), 4.54 (dd, *J*=6.0, 3.1 Hz, 1 H),

4.14 - 4.21 (m, 1 H), 3.98 (d, *J*=4.2 Hz, 2 H), 2.05 (q, *J*=7.3 Hz, 2 H), 1.32 - 1.41 (m, 2 H), 1.20 - 1.31 (m, 20 H), 0.89 (t, *J*=7.0 Hz, 3 H). ESI-MS(m/z): 502.3796 $[M+Na]^+$.

**_N_-((2_R_,3_S_,_E_)-1,3-dihydroxyoctadec-4-en-2-yl)-[1,1'-biphenyl]-4-carboxamide (LE 1)**

$^1$H NMR (500 MHz, $CDCl_3$): δ = 7.88 - 7.92 (d, *J*=8.3 Hz, 2 H), 7.66 - 7.70 (d, *J*=8.3 Hz, 2 H), 7.63 (d, *J*=7.3 Hz, 2 H), 7.48 (t, *J*=7.6 Hz, 2 H), 7.38 - 7.43 (m, 1 H), 7.04 (d, *J*=7.3 Hz, 1 H), 5.82 - 5.90 (m, 1 H), 5.63 (dd, *J*=15.5, 6.5 Hz, 1 H), 4.48 - 4.52 (m, 1 H), 4.14 - 4.18 (m, 1 H), 4.10 - 4.14 (m, 1 H), 3.86 (dd, *J*=11.1, 3.1 Hz, 1 H), 2.09 (q, *J*=7.1 Hz, 2 H), 1.35 - 1.42 (m, 2 H), 1.20 - 1.32 (m, 20 H), 0.89 (t, *J*=7.0 Hz, 3 H). ESI-MS(m/z): 502.3796 $[M+Na]^+$.

**_N_-((2_R_,3_R_,_E_)-1,3-dihydroxyoctadec-4-en-2-yl)-[1,1'-biphenyl]-4-carboxamide (DT 1)**

$^1$H NMR (500 MHz, $CDCl_3$): δ = 7.88 (d, *J*=8.3 Hz, 2 H), 7.67 (d, *J*=8.3 Hz, 2 H), 7.62 (d, *J*=7.1 Hz, 2 H), 7.48 (t, *J*=7.6 Hz, 2 H), 7.38 - 7.43 (m, 1 H), 6.89 (d, *J*=7.8 Hz, 1 H), 5.78 - 5.87 (m, 1 H), 5.58 (dd, *J*=15.5, 6.2 Hz, 1 H), 4.51 - 4.57 (m, 1 H), 4.17 (dq, *J*=7.5, 3.6 Hz, 1 H), 3.97 (d, *J*=3.9 Hz, 2 H), 2.05 (q, *J*=7.1 Hz, 2 H), 1.19 - 1.39 (m, 22 H), 0.88 (t, *J*=7.0 Hz, 3 H). ESI-MS(m/z): 502.3796 $[M+Na]^+$.

**_N_-((2_S_,3_R_,_E_)-1,3-dihydroxyoctadec-4-en-2-yl)-5-(2-nitrophenyl)furan-2-carboxamide (DE 2)**

$^1$H NMR (500 MHz, $CDCl_3$): δ = 7.80 – 7.85 (m, 1H), 7.75 (t, *J*=7.2 Hz, 1 H), 7.65 (m, 1 H), 7.49 - 7.57 (m, 1 H), 7.21 (d, *J*=3.7 Hz, 1 H), 7.03 (d, *J*=7.6 Hz, 1 H), 6.77 (d, *J*=3.4 Hz, 1 H), 5.81 - 5.90 (m, 1 H), 5.60 (dd, *J*=15.5, 6.5 Hz, 1 H), 4.44 (m, 1 H), 4.06 - 4.10 (m, 1 H), 4.02 - 4.06 (m, 1 H), 3.92 (s, 1 H), 3.82 (m, 1 H), 2.03 - 2.12 (m,

2 H), 1.34 – 1.41 (m, 2 H), 1.18 - 1.32 (m, 20 H), 0.89 (t, *J*=6.7 Hz, 3 H). ESI-MS(m/z): 537.2929 $[M+Na]^+$.

**_N_-((2_S_,3_S_,_E_)-1,3-dihydroxyoctadec-4-en-2-yl)-5-(2-nitrophenyl)furan-2-carboxamide (LT 2)**

$^1$H NMR (500 MHz, $CDCl_3$): δ = 7.82 (ddd, *J*=13.7, 7.9, 1.1 Hz, 1 H), 7.73 - 7.76 (m, 1 H), 7.62 - 7.69 (m, 1 H), 7.50 - 7.56 (m, 1 H), 7.20 (d, *J*=3.7 Hz, 1 H), 6.93 (d, *J*=7.8 Hz, 1 H), 6.77 (d, *J*=3.7 Hz, 1 H), 5.80 - 5.88 (m, 1 H), 5.56 (dd, *J*=15.4, 6.6 Hz, 1 H), 4.49 (dd, *J*=6.4, 3.4 Hz, 1 H), 4.08 (dq, *J*=8.0, 4.1 Hz, 1 H), 3.89 - 3.95 (m, 3 H), 2.01 - 2.08 (m, 2 H), 1.22 - 1.40 (m, 22 H), 0.89 (t, *J*=6.8 Hz, 3 H). ESI-MS(m/z): 537.3526 $[M+Na]^+$.

**_N_-((2_R_,3_S_,_E_)-1,3-dihydroxyoctadec-4-en-2-yl)-5-(2-nitrophenyl)furan-2-carboxamide (LE 2)**

$^1$H NMR (500 MHz, $CDCl_3$): δ = 7.80 - 7.86 (m, 1 H), 7.73 - 7.77 (m, 1 H), 7.63 - 7.68 (m, 1 H), 7.50 - 7.56 (m, 1 H), 7.22 (d, *J*=3.7 Hz, 1 H), 7.04 (d, *J*=7.3 Hz, 1 H), 6.77 (d, *J*=3.7 Hz, 1 H), 5.80 - 5.89 (m, 1 H), 5.59 (dd, *J*=15.4, 6.35 Hz, 1 H), 4.42 - 4.47 (m, 1 H), 4.07 - 4.10 (m, 1 H), 4.05 (m, 1 H), 3.78 - 3.84 (m, 1 H), 2.04 - 2.11 (m, 2 H), 1.33 - 1.40 (m, 2 H), 1.20 - 1.32 (m, 20 H), 0.89 (t, *J*=7.0 Hz, 3 H). ESI-MS(m/z): 537.3952 $[M+Na]^+$.

**_N_-((2_R_,3_R_,_E_)-1,3-dihydroxyoctadec-4-en-2-yl)-5-(2-nitrophenyl)furan-2-carboxamide (DT 2)**

$^1$H NMR (500 MHz, $CDCl_3$): δ = 7.82 (ddd, *J*=13.4, 8.0, 1.1 Hz, 1 H), 7.72 - 7.76 (m, 1 H), 7.62 - 7.68 (m, 1 H), 7.50 - 7.56 (m, 1 H), 7.20 (d, *J*=3.7 Hz, 1 H), 6.93 (d, *J*=7.8 Hz, 1 H), 6.77 (d, *J*=3.4 Hz, 1 H), 5.80 - 5.87 (m, 1 H), 5.56 (dd, *J*=15.4, 6.6 Hz, 1 H), 4.49 (dd, *J*=6.1, 3.4 Hz, 1 H), 4.06 - 4.11 (m, 1 H), 3.91 - 3.94 (m, 2 H), 2.01 - 2.09

(m, 2 H), 1.22 - 1.36 (m, 22 H), 0.89 (t, *J*=7.0 Hz, 3 H). ESI-MS(m/z): 537.3526 $[M+Na]^+$.

**_N_-((2_S_,3_R_,_E_)-1,3-dihydroxyoctadec-4-en-2-yl)tetradeca-2,4,6,8,10,12-hexaynamide compound with dihydrogen (DE 3)**

$^1$H NMR (500 MHz, $CDCl_3$): $\delta$ = 6.25 (d, *J*=7.1 Hz, 1 H), 5.75 - 5.84 (m, 1 H), 5.54 (dd, *J*=15.3, 6.0 Hz, 1 H), 4.31 - 4.35 (m, 1 H), 3.95 - 4.00 (m, 1 H), 3.89 – 3.94 (m, 1 H), 3.72 (dd, *J*=11.2, 2.7 Hz, 1 H), 2.24 (t, *J*=7.7 Hz, 2 H), 2.06 (q, *J*=7.1 Hz, 2 H), 1.65 (quin, *J*=6.7 Hz, 2 H), 1.23 - 1.38 (m, 42 H), 0.89 ppm (t, *J*=6.6 Hz, 6 H). ESI-MS(m/z): 532.5146 $[M+Na]^+$.

**_N_-((2_S_,3_S_,_E_)-1,3-dihydroxyoctadec-4-en-2-yl)tetradeca-2,4,6,8,10,12-hexaynamide compound with dihydrogen (LT 3)**

$^1$H NMR (500 MHz, $CDCl_3$): $\delta$ = 6.10 (d, *J*=7.3 Hz, 1 H), 5.71 - 5.81 (m, 1 H), 5.49 (dd, *J*=15.4, 6.4 Hz, 1 H), 4.40 (dd, *J*=6.4, 3.4 Hz, 1 H), 3.90 - 3.95 (m, 1 H), 3.80 - 3.87 (m, 2 H), 2.24 (t, *J*=7.6 Hz, 2 H), 2.04 (q, *J*=7.1 Hz, 2 H), 1.60 - 1.68 (m, 2 H), 1.26 - 1.38 (m, 42 H), 0.89 (t, *J*=6.8 Hz 6 H). ESI-MS(m/z): 532.5489 $[M+Na]^+$.

**_N_-((2_R_,3_S_,_E_)-1,3-dihydroxyoctadec-4-en-2-yl)tetradeca-2,4,6,8,10,12-hexaynamide compound with dihydrogen (LE 3)**

$^1$H NMR (500 MHz, $CDCl_3$): $\delta$ = 6.26 (d, *J*=7.6 Hz, 1 H), 5.76 - 5.84 (m, 1 H), 5.54 (dd, *J*=15.4, 6.6 Hz, 1 H), 4.31 - 4.36 (m, 1 H), 3.97 (dd, *J*=11.2, 3.7 Hz, 1 H), 3.90 – 3.94 (m, 1 H), 3.72 (dd, *J*=11.2, 3.4 Hz, 1 H), 2.24 (t, *J*=7.6 Hz, 2 H), 2.06 (q, *J*=7.3 Hz, 2 H), 1.60 - 1.68 (m, 2 H), 1.21 - 1.39 (m, 42 H), 0.89 (t, *J*=6.8 Hz, 6 H). ESI-MS(m/z): 532.5489 $[M+Na]^+$.

***N*-((2*R*,3*R*,*E*)-1,3-dihydroxyoctadec-4-en-2-yl)tetradeca-2,4,6,8,10,12-hexaynamide compound with dihydrogen (DT 3)**

$^1$H NMR (500 MHz, $CDCl_3$): δ = 6.11 (d, *J*=7.6 Hz, 1 H), 5.71 - 5.80 (m, 1 H), 5.48 (dd, *J*=15.4, 6.4 Hz, 1 H), 4.40 (dd, *J*=6.0, 3.5 Hz, 1 H), 3.93 (m,1H), 3.78 - 3.86 (m, 2 H), 2.23 (t, *J*=7.6 Hz, 2 H), 2.04 (q, *J*=7.1 Hz, 2 H), 1.64 (quin, *J*=7.2 Hz, 2 H), 1.25 - 1.39 (m, 42 H), 0.89 (t, *J*=7.0 Hz, 6 H). ESI-MS(m/z): 532.5489 $[M+Na]^+$.

***N*-((2*S*,3*R*,*E*)-1,3-dihydroxyoctadec-4-en-2-yl)adamantane-1-carboxamide (DE 4)**

$^1$H NMR (500 MHz, $CDCl_3$): δ = 6.38 (d, *J*=5.4 Hz, 1 H), 5.72 - 5.81 (m, 1 H), 5.53 (dd, *J*=15.3, 6.0 Hz, 1 H), 4.31 (m, 1 H), 3.93 – 3.96 (m, 1 H), 3.89 (m, 1 H), 3.72 (m, 1 H), 3.67 (br. s., 1 H), 2.03 – 2.10 (m, 5 H), 1.89 (m, 6 H), 1.68 – 1.77 (m, 6 H), 1.38 – 1.41 (m, 2 H), 1.27 (m., 20 H), 0.89 (t, *J*=6.0 Hz, 3 H). ESI-MS(m/z): 484.4509 $[M+Na]^+$.

***N*-((2*S*,3*S*,*E*)-1,3-dihydroxyoctadec-4-en-2-yl)adamantane-1-carboxamide (LT 4)**

$^1$H NMR (500 MHz, $CDCl_3$): δ = 6.30 (d, *J*=7.1 Hz, 1 H), 5.71 - 5.81 (m, 1 H), 5.47 (dd, *J*=15.4, 6.4 Hz, 1 H), 4.40 (dd, *J*=5.7, 3.8 Hz, 1 H), 3.91 (td, *J*=7.9, 4.3 Hz, 1 H), 3.82 (dd, *J*=4.4, 1.5 Hz, 2 H), 2.01 - 2.09 (m, 5 H), 1.88 (d, *J*=2.7 Hz, 6 H), 1.74 (q, *J*=12.3 Hz, 6 H), 1.33 - 1.42 (m, 2 H), 1.22 - 1.33 (m, 20 H), 0.89 (t, *J*=7.0 Hz, 3 H). ESI-MS(m/z): 484.4509 $[M+Na]^+$.

***N*-((2*R*,3*S*,*E*)-1,3-dihydroxyoctadec-4-en-2-yl)adamantane-1-carboxamide (LE 4)**

$^1$H NMR (500 MHz, $CDCl_3$): δ = 6.40 (d, *J*=7.1 Hz, 1 H), 5.74 - 5.83 (m, 1 H), 5.52 (dd, *J*=15.4, 6.6 Hz, 1 H), 4.28 - 4.34 (m, 1 H), 3.94 (dd, *J*=11.2, 4.2 Hz, 1 H), 3.87 – 3.91 (m, 1 H), 3.71 (dd, *J*=11.2, 3.4 Hz, 1 H), 2.02 - 2.10 (m, 5 H), 1.88 (d, *J*=2.7 Hz, 6 H), 1.68 – 1.79 (m, 6 H), 1.34 - 1.41 (m, 2 H), 1.25 - 1.31 (m, 20 H), 0.89 (t, *J*=7.0 Hz, 3 H). ESI-MS(m/z): 484.4509 $[M+Na]^+$.

***N*-((2*R*,3*R*,*E*)-1,3-dihydroxyoctadec-4-en-2-yl)adamantane-1-carboxamide (DT 4)**

$^1$H NMR (500 MHz, $CDCl_3$): δ = 6.30 (d, *J*=7.3 Hz, 1 H), 5.70 - 5.80 (m, 1 H), 5.47 (dd, *J*=15.4, 6.4 Hz, 1 H), 4.40 (dd, *J*=5.7, 3.8 Hz, 1 H), 3.91 (m, 1 H), 3.81 (dd, *J*=4.5, 1.3 Hz, 2 H), 1.99 - 2.09 (m, 5 H), 1.88 (d, *J*=2.4 Hz, 6 H), 1.69 - 1.79 (m, 6 H), 1.32 - 1.42 (m, 2 H), 1.21 - 1.32 (m, 20 H), 0.89 (t, *J*=7.0 Hz, 3 H). ESI-MS(m/z): 484.4509 $[M+Na]^+$.

***N*-((2*S*,3*R*,*E*)-1,3-dihydroxyoctadec-4-en-2-yl)furan-2-carboxamide (DE 5)**

$^1$H NMR (500 MHz, $CDCl_3$): δ = 7.45 - 7.48 (m, 1 H), 7.14 (d, *J*=2.7 Hz, 1 H), 7.10 - 7.13 (m, 1 H), 6.52 (dd, *J*=3.4, 1.7 Hz, 1 H), 5.80 - 5.87 (m, 1 H), 5.60 (dd, *J*=15.5, 6.7 Hz, 1 H), 4.42 - 4.46 (m, 1 H), 4.07 - 4.10 (m, 1 H), 4.07 (m, 1 H), 3.79 - 3.85 (m, 1 H), 2.08 (q, *J*=7.3 Hz, 2 H), 1.34 - 1.40 (m, 2 H), 1.25 - 1.33 (m, 20 H), 0.89 (t, *J*=6.8 Hz, 3 H). ESI-MS(m/z): 416.3063 $[M+Na]^+$.

***N*-((2*S*,3*S*,*E*)-1,3-dihydroxyoctadec-4-en-2-yl)furan-2-carboxamide (LT 5)**

$^1$H NMR (500 MHz, $CDCl_3$): δ = 7.43 - 7.47 (m, 1 H), 7.12 (d, *J*=3.4 Hz, 1 H), 6.99 (d, *J*=8.1 Hz, 1 H), 6.51 (dd, *J*=3.4, 1.7 Hz, 1 H), 5.76 - 5.83 (m, 1 H), 5.54 (dd, *J*=15.4, 6.6 Hz, 1 H), 4.48 (dd, *J*=6.4, 3.4 Hz, 1 H), 4.04 - 4.12 (m, 1 H), 3.90 - 3.94 (m, 2 H), 2.02 (q, *J*=7.1 Hz, 2 H), 1.22 - 1.36 (m, 22 H), 0.88 (t, *J*=7.0 Hz, 3 H). ESI-MS(m/z): 416.3551 $[M+Na]^+$.

***N*-((2*R*,3*S*,*E*)-1,3-dihydroxyoctadec-4-en-2-yl)furan-2-carboxamide (LE 5)**

$^1$H NMR (500 MHz, $CDCl_3$): δ = 7.47 (dd, *J*=1.7, 1.0 Hz, 1 H), 7.15 - 7.18 (m, 1 H), 7.13 - 7.15 (m, 1 H), 6.52 (dd, *J*=3.4, 1.7 Hz, 1 H), 5.79 - 5.87 (m, 1 H), 5.59 (dd, *J*=15.5, 6.5 Hz, 1 H), 4.42 - 4.47 (m, 1 H), 4.09 (m, 1 H), 4.05 – 4.08 (m, 1 H), 3.78 -

3.83 (m, 1 H), 2.07 (q, *J*=7.1 Hz, 2 H), 1.33 - 1.41 (m, 2 H), 1.25 - 1.33 (m, 20 H), 0.89 (t, *J*=6.6 Hz, 3 H). ESI-MS(m/z): 416.3779 $[M+Na]^+$.

**_N_-((2_R_,3_R_,_E_)-1,3-dihydroxyoctadec-4-en-2-yl)furan-2-carboxamide (DT 5)**

$^1$H NMR (500 MHz, CDCl3): δ = 7.47 (dd, *J*=1.7, 0.7 Hz, 1 H), 7.13 (dd, *J*=3.4, 0.7 Hz, 1 H), 7.00 (d, *J*=8.1 Hz, 1 H), 6.52 (dd, *J*=3.4, 1.7 Hz, 1 H), 5.76 - 5.85 (m, 1 H), 5.55 (dd, *J*=15.4, 6.6 Hz, 1 H), 4.49 (dd, *J*=6.2, 3.3 Hz, 1 H), 4.06 - 4.13 (m, 1 H), 3.89 - 3.95 (m, 2 H), 2.03 (q, *J*=7.1 Hz, 2 H), 1.22 - 1.37 (m, 22 H), 0.89 (t, *J*=7.0 Hz, 3 H). ESI-MS(m/z): 416.3544 $[M+Na]^+$.

**2-bromo-_N_-((2_S_,3_R_,_E_)-1,3-dihydroxyoctadec-4-en-2-yl)hexadecanamide (DE 6)**

$^1$H NMR (500 MHz, $CDCl_3$): δ = 5.72 - 5.91 (m, 1 H), 5.5 (dd, *J*=15.4, 6.6 Hz, 1 H), 4.32 - 4.40 (m, 1 H), 4.25 (m, 1 H), 3.95 - 4.07 (m, 1 H), 3.84 - 3.94 (m, 1 H), 1.99 - 2.16 (m, 3 H), 1.26 - 1.42 (m, 48 H), 0.89 (t, *J*=7.0 Hz, 6 H). ESI-MS(m/z): 638.5202 $[M+Na]^+$.

**2-bromo-_N_-((2_S_,3_S_,_E_)-1,3-dihydroxyoctadec-4-en-2-yl)hexadecanamide (LT 6)**

$^1$H NMR (500 MHz, $CDCl_3$): δ = 5.72 - 5.82 (m, 1 H), 5.48 (dd, *J*=15.6, 7.6 Hz, 1 H), 4.45 (m, 1 H), 4.28 - 4.36 (m, 1 H), 3.92 (br. s., 1 H), 3.79 - 3.88 (m, 2 H), 2.09 – 2.14 (m, 1 H), 1.96 - 2.06 (m, 2 H), 1.39 - 1.51 (m, 2 H), 1.25 - 1.34 (m, 44 H), 0.88 (t, *J*=7.0 Hz, 6 H). ESI-MS(m/z): 638.4904 $[M+Na]^+$.

**2-bromo-_N_-((2_R_,3_S_,_E_)-1,3-dihydroxyoctadec-4-en-2-yl)hexadecanamide (LE-6)**

$^1$H NMR (500 MHz, $CDCl_3$): δ = 5.76 - 5.86 (m, 1 H), 5.54 (dd, *J*=15.4, 6.6 Hz, 1 H), 4.35 - 4.40 (m, 1 H), 4.29 - 4.34 (m, 1 H), 4.02 (m, 1 H), 3.89 (m, 1 H), 3.73 - 3.77 (m, 1 H), 2.13 (m, 1 H), 1.94 - 2.09 (m, 2 H), 1.25 - 1.41 (m, 48 H), 0.89 (t, *J*=6.9 Hz, 6 H). ESI-MS(m/z): 638.4904 $[M+Na]^+$.

**2-bromo-*N*-((2*R*,3*R*,*E*)-1,3-dihydroxyoctadec-4-en-2-yl)hexadecanamide (DT 6)**

$^1$H NMR (500 MHz, $CDCl_3$): $\delta$ = 5.72 - 5.83 (m, 1 H), 5.49 (dd, *J*=15.4, 6.4 Hz, 1 H), 4.43 - 4.49 (m, 1 H), 4.33 (m, 1 H), 3.89 - 3.96 (m, 1 H), 3.80 - 3.89 (m, 2 H), 2.07 - 2.17 (m, 1 H), 1.96 - 2.06 (m, 2 H), 1.41 - 1.53 (m, 2 H), 1.25 - 1.37 (m, 46 H), 0.89 (t, *J*=7.09 Hz, 6 H). ESI-MS(m/z): 638.5202 $[M+Na]^+$.

***N*-((2*S*,3*R*,*E*)-1,3-dihydroxyoctadec-4-en-2-yl)cyclohexanecarboxamide (DE 7)**

$^1$H NMR (500 MHz, $CDCl_3$): $\delta$ = 6.24 (d, *J*=7.1 Hz, 1 H), 5.73 - 5.84 (m, 1 H), 5.53 (dd, *J*=15.5, 5.6 Hz, 1 H), 4.28 - 4.34 (m, 1 H), 3.92 - 3.98 (m, 1 H), 3.90 (m, 1 H), 3.71 (dd, *J*=11.1, 3.3 Hz, 1 H), 3.66 (br. s., 1 H), 2.10 - 2.19 (m, 1 H), 1.99 - 2.10 (m, 4 H), 1.73 - 1.89 (m, 6 H), 1.41 - 1.52 (m, 2 H), 1.34 - 1.40 (m, 2 H), 1.26 (m, 22 H), 0.87 - 0.91 (m, 3 H). ESI-MS(m/z): 432.3736 $[M+Na]^+$.

***N*-((2*S*,3*S*,*E*)-1,3-dihydroxyoctadec-4-en-2-yl)cyclohexanecarboxamide (LT 7)**

$^1$H NMR (500 MHz, $CDCl_3$): $\delta$ = 6.12 (d, *J*=7.3 Hz, 1 H), 5.71 - 5.79 (m, 1 H), 5.48 (dd, *J*=15.4, 6.4 Hz, 1 H), 4.40 (dd, *J*=5.9, 3.7 Hz, 1 H), 3.88 - 3.95 (m, 1 H), 3.78 - 3.86 (m, 2 H), 2.15 (tt, *J*=11.6, 3.3 Hz, 1 H), 2.01 - 2.08 (m, 2 H), 1.85 - 1.92 (m, 2 H), 1.77 - 1.84 (m, 2 H), 1.68 (m, 1 H), 1.42 - 1.50 (m, 2 H), 1.33 - 1.41 (m, 2 H), 1.26 - 1.31 (m, 22 H), 0.89 ppm (t, *J*=7.0 Hz, 3 H). ESI-MS(m/z): 432.3982 $[M+Na]^+$.

**N-((2*R*,3*S*,*E*)-1,3-dihydroxyoctadec-4-en-2-yl)cyclohexanecarboxamide (LE 7)**

$^1$H NMR (500 MHz, $CDCl_3$): $\delta$ = 6.26 (d, *J*=7.3 Hz, 1 H), 5.75 - 5.83 (m, 1 H), 5.53 (dd, *J*=15.4, 6.6 Hz, 1 H), 4.32 (dd, *J*=5.7, 4.5 Hz, 1 H), 3.96 (dd, *J*=11.2, 3.9 Hz, 1 H), 3.87 - 3.93 (m, 1 H), 3.71 (dd, *J*=11.4, 3.3 Hz, 1 H), 2.11 - 2.19 (m, 1 H), 2.06 (q, *J*=7.2 Hz, 2 H), 1.85 - 1.92 (m, 2 H), 1.77 - 1.83 (m, 2 H), 1.69 (m, 1 H), 1.45 (q,

*J*=12.3 Hz, 2 H), 1.33 - 1.41 (m, 2 H), 1.25 - 1.31 (m, 22 H), 0.89 (t, *J*=7.0 Hz, 3 H). ESI-MS(m/z): 432.3982 $[M+Na]^+$.

**_N_-((2_R_,3_R_,_E_)-1,3-dihydroxyoctadec-4-en-2-yl)cyclohexanecarboxamide (DT 7)**

$^1$H NMR (500 MHz, $CDCl_3$): δ = 6.12 (d, *J*=7.8 Hz, 1 H), 5.71 - 5.80 (m, 1 H), 5.47 (dd, *J*=15.4, 6.4 Hz, 1 H), 4.40 (dd, *J*=5.9, 3.7 Hz, 1 H), 3.88 - 3.95 (m, 1 H), 3.78 - 3.86 (m, 2 H), 2.15 (tt, *J*=11.7, 3.5 Hz, 1 H), 2.03 (q, *J*=6.8 Hz, 2 H), 1.85 - 1.91 (m, 2 H), 1.76 - 1.84 (m, 2 H), 1.65 - 1.72 (m, 1 H), 1.40 - 1.49 (m, 2 H), 1.33 - 1.39 (m, 2 H), 1.24 - 1.32 (m, 22 H), 0.89 (t, *J*=7.0 Hz, 3 H). ESI-MS(m/z): 432.4227 $[M+Na]^+$.

**_N_-((2_S_,3_R_,_E_)-1,3-dihydroxyoctadec-4-en-2-yl)tetradeca-2,4,6,8,10,12-hexaynamide compound with dihydrogen (DE 8)**

$^1$H NMR (500 MHz, $CDCl_3$): δ = 6.23 (d, *J*=7.6 Hz, 1 H), 5.76 - 5.84 (m, 1 H), 5.54 (dd, *J*=15.8, 6.2 Hz, 1 H), 4.31 - 4.36 (m, 1 H), 3.97 (dd, *J*=11.4, 3.8 Hz, 1 H), 3.92 (m, 1 H), 3.72 (dd, *J*=11.4, 3.3 Hz, 1 H), 2.24 (t, *J*=7.6 Hz, 2 H), 2.03 - 2.10 (m, 2 H), 1.62 - 1.70 (m, 2 H), 1.22 - 1.38 (m, 46 H), 0.89 (t, *J*=6.7 Hz, 6 H). ESI-MS(m/z): 560.5482 $[M+Na]^+$.

**_N_-((2_S_,3_S_,_E_)-1,3-dihydroxyoctadec-4-en-2-yl)tetradeca-2,4,6,8,10,12-hexaynamide compound with dihydrogen (LT 8)**

$^1$H NMR (500 MHz, $CDCl_3$): δ = 6.10 (d, *J*=7.8 Hz, 1 H), 5.72 - 5.81 (m, 1 H), 5.49 (dd, *J*=15.4, 6.4 Hz, 1 H), 4.40 (dd, *J*=6.1, 3.7 Hz, 1 H), 3.93 (m, 1 H), 3.80 – 3.86 (m, 2 H), 2.24 (t, *J*=7.6 Hz, 2 H), 2.01 - 2.08 (m, 2 H), 1.60 - 1.68 (m, 2 H), 1.19 - 1.41 (m, 46 H), 0.89 (t, *J*=6.8 Hz, 6 H). ESI-MS(m/z): 560.6041 $[M+Na]^+$.

**_N_-((2_R_,3_S_,_E_)-1,3-dihydroxyoctadec-4-en-2-yl)tetradeca-2,4,6,8,10,12-hexaynamide compound with dihydrogen (LE 8)**

$^1$H NMR (500 MHz, $CDCl_3$): $\delta$ = 6.23 (d, *J*=7.6 Hz, 1 H), 5.75 - 5.83 (m, 1 H), 5.54 (dd, *J*=15.4, 6.6 Hz, 1 H), 4.31 - 4.35 (m, 1 H), 3.97 (dd, *J*=11.2, 3.7 Hz, 1 H), 3.90 – 3.94 (m, 1 H), 3.72 (dd, *J*=11.2, 3.2 Hz, 1 H), 2.24 (t, *J*=7.7 Hz, 2 H), 2.07 (q, *J*=7.1 Hz, 2 H), 1.60 - 1.69 (m, 2 H), 1.35 - 1.41 (m, 2 H), 1.26 - 1.35 (m, 44 H), 0.89 (t, *J*=7.0 Hz, 6 H). ESI-MS(m/z): 560.6041 $[M+Na]^+$.

**_N_-((2_R_,3_R_,_E_)-1,3-dihydroxyoctadec-4-en-2-yl)tetradeca-2,4,6,8,10,12-hexaynamide compound with dihydrogen (DT 8)**

$^1$H NMR (500 MHz, $CDCl_3$): $\delta$ = 6.10 (d, *J*=7.6 Hz, 1 H), 5.71 - 5.81 (m, 1 H), 5.48 (dd, *J*=15.4, 6.6 Hz, 1 H), 4.40 (dd, *J*=5.9, 3.4 Hz, 1 H), 3.91 – 3.95 (m, 1 H), 3.78 - 3.87 (m, 2 H), 2.23 (t, *J*=7.6 Hz, 2 H), 2.04 (q, *J*=7.2 Hz, 2 H), 1.60 - 1.68 (m, 2 H), 1.34 - 1.40 (m, 2 H), 1.24 - 1.33 (m, 44 H), 0.89 (t, *J*=7.0 Hz, 6 H). ESI-MS(m/z): 560.6320 $[M+Na]^+$.

**_N_-((2_S_,3_R_,_E_)-1,3-dihydroxyoctadec-4-en-2-yl)stearamide (DE 9)**

$^1$H NMR (500 MHz, $CDCl_3$): $\delta$ = 6.23 (d, *J*=7.3 Hz, 1 H), 5.75 - 5.85 (m, 1 H), 5.54 (dd, *J*=15.4, 6.4 Hz, 1 H), 4.33 (dd, *J*=5.5, 4.3 Hz, 1 H), 3.97 (dd, *J*=11.2, 3.9 Hz,1 H), 3..90 – 3.94 (m, 1H), 3.72 (dd, *J*=11.2, 3.3 Hz, 1 H), 2.24 (t, *J*=7.3 Hz, 2 H), 2.07 (q, *J*=7.2 Hz, 2 H), 1.59 - 1.70 (m, 2 H), 1.36 - 1.40 (m, 2 H), 1.24 - 1.35 (m, 48 H), 0.89 (t, *J*=7.0 Hz, 6 H). ESI-MS(m/z): 588.6060 $[M+Na]^+$.

**_N_-((2_S_,3_S_,_E_)-1,3-dihydroxyoctadec-4-en-2-yl)stearamide (LT 9)**

$^1$H NMR (500 MHz, $CDCl_3$): $\delta$ = 6.10 (d, *J*=8.1 Hz, 1 H), 5.72 - 5.79 (m, 1 H), 5.49 (dd, *J*=15.4, 6.4 Hz, 1 H), 4.40 (dd, *J*=6.2, 3.5 Hz, 1 H), 3.91 – 3.95 (m, 1 H), 3.80 -

3.86 (m, 2 H), 2.24 (t, *J*=7.6 Hz, 2 H), 2.04 (q, *J*=6.8 Hz, 2 H), 1.60 - 1.68 (m, 2 H), 1.20 - 1.42 (m, 50 H), 0.89 (t, *J*=7.0 Hz, 6 H). ESI-MS(m/z): 588.6060 $[M+Na]^+$.

***N*-((2*R*,3*S*,*E*)-1,3-dihydroxyoctadec-4-en-2-yl)stearamide (LE 9)**

$^1$H NMR (500 MHz, $CDCl_3$): $\delta$ = 6.22 (d, *J*=7.3 Hz, 1 H), 5.74 - 5.84 (m, 1 H), 5.54 (dd, *J*=15.4, 6.4 Hz, 1 H), 4.31 - 4.36 (m, 1 H), 3.97 (dd, *J*=11.2, 3.6 Hz, 1 H), 3.92 (m, 1 H), 3.72 (dd, *J*=11.1, 3.3 Hz, 1 H), 2.24 (t, *J*=7.6 Hz, 2 H), 2.07 (q, *J*=6.8 Hz, 2 H), 1.61 - 1.69 (m, 2 H), 1.35 - 1.41 (m, 2 H), 1.26 - 1.35 (m, 48 H), 0.89 (t, *J*=7.0 Hz, 6 H). ESI-MS(m/z): 588.6346 $[M+Na]^+$.

***N*-((2*R*,3*R*,*E*)-1,3-dihydroxyoctadec-4-en-2-yl)stearamide (DT 9)**

$^1$H NMR (500 MHz, $CDCl_3$): $\delta$ = 6.10 (d, *J*=7.6 Hz, 1 H), 5.71 - 5.80 (m, 1 H), 5.49 (dd, *J*=15.5, 6.5 Hz, 1 H), 4.40 (dd, *J*=5.7, 3.5 Hz, 1 H), 3.93 (m, 1 H), 3.79 - 3.87 (m, 2 H), 2.23 (t, *J*=7.6 Hz, 2 H), 2.04 (q, *J*=7.1 Hz, 2 H), 1.61 - 1.68 (m, 2 H), 1.26 - 1.38 (m, 50 H), 0.89 (t, *J*=7.0 Hz, 6 H). ESI-MS(m/z): 588.6919 $[M+Na]^+$.

***N*-((2*S*,3*R*,*E*)-1,3-dihydroxyoctadec-4-en-2-yl)cyclopropanecarboxamide (DE 10)**

$^1$H NMR (500 MHz, $CDCl_3$): $\delta$ = 6.45 (d, *J*=6.84 Hz, 1 H), 5.75 - 5.84 (m, 1 H), 5.55 (dd, *J*=15.4, 6.6 Hz, 1 H), 4.33 - 4.37 (m, 1 H), 3.95 - 3.99 (m, 1 H), 3.91 – 3.94 (m, 1 H), 3.73 – 3.76 (m, 1 H), 2.02 - 2.10 (m, 2 H), 1.41 – 1.46 (m, 1 H), 1.38 (m, 2 H), 1.25 - 1.32 (m, 20 H), 0.95 - 1.03 (m, 2 H), 0.89 (t, *J*=6.8 Hz, 3 H), 0.76 – 0.81 (m, 2 H). ESI-MS(m/z): 390.3356 $[M+Na]^+$.

***N*-((2*S*,3*S*,*E*)-1,3-dihydroxyoctadec-4-en-2-yl)cyclopropanecarboxamide (LT 10)**

$^1$H NMR (500 MHz, $CDCl_3$): $\delta$ = 6.29 (d, *J*=7.3 Hz, 1 H), 5.72 - 5.81 (m, 1 H), 5.51 (dd, *J*=15.4, 6.6 Hz, 1 H), 4.39 (dd, *J*=6.1, 4.2 Hz, 1 H), 3.93 (dq, *J*=7.8, 4.1 Hz, 1 H), 3.84 (m, 2 H), 2.05 (q, *J*=7.0 Hz, 2 H), 1.40 – 1.45 (m, 1 H), 1.34 - 1.41 (m, 2 H),

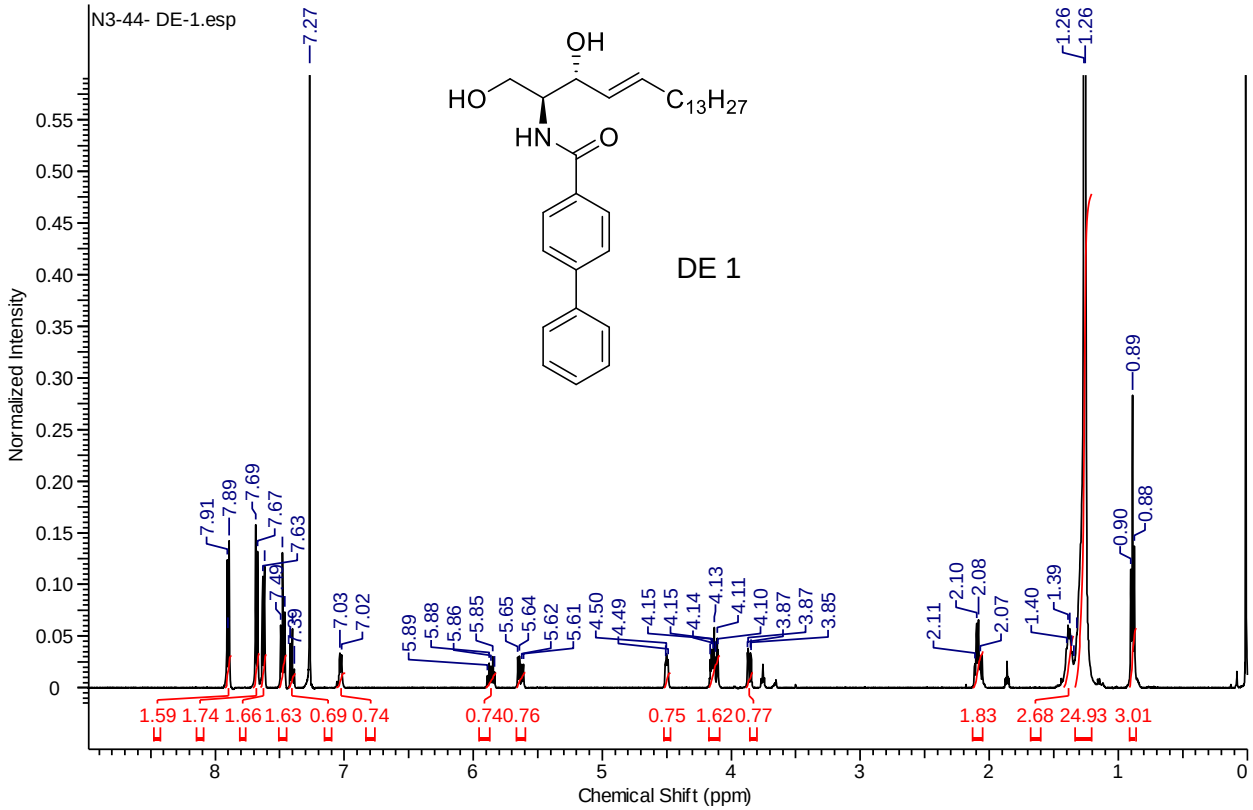
1.26 - 1.33 (m, 20 H), 0.99 (quin, *J*=3.5 Hz, 2 H), 0.89 (t, *J*=6.8 Hz, 3 H), 0.76 - 0.81 (m, 2 H). ESI-MS(m/z): 390.3590 $[M+Na]^+$.

**_N_-((2_R_,3_S_,_E_)-1,3-dihydroxyoctadec-4-en-2-yl)cyclopropanecarboxamide (LE 10)**

$^1$H NMR (500 MHz, $CDCl_3$): $\delta$ = 6.45 (d, *J*=6.6 Hz, 1 H), 5.75 - 5.85 (m, 1 H), 5.55 (dd, *J*=15.4, 6.6 Hz, 1 H), 4.33 - 4.38 (m, 1 H), 3.96 - 4.00 (m, 1 H), 3.93 (m, 1 H), 3.73 (dd, *J*=11.1, 3.3 Hz, 1 H), 2.07 (q, *J*=6.8 Hz, 2 H), 1.41 - 1.46 (m, 1 H), 1.33 - 1.40 (m, 2 H), 1.21 - 1.33 (m, 20 H), 0.97 - 1.02 (m, 2 H), 0.89 (t, *J*=7.0 Hz, 3 H), 0.75 - 0.81 (m, 2 H). ESI-MS(m/z): 390.3590 $[M+Na]^+$.

**_N_-((2_R_,3_R_,_E_)-1,3-dihydroxyoctadec-4-en-2-yl)cyclopropanecarboxamide (DT 10)**

$^1$H NMR (500 MHz, $CDCl_3$): $\delta$ = 6.30 (d, *J*=7.3 Hz, 1 H), 5.71 - 5.84 (m, 1 H), 5.51 (dd, *J*=15.4, 6.6 Hz, 1 H), 4.38 (dd, *J*=6.1, 3.9 Hz, 1 H), 3.93 (m, 1 H), 3.83 (d, *J*=3.9 Hz, 2 H), 2.05 (q, *J*=6.9 Hz, 2 H), 1.43 (m, 1 H), 1.34 - 1.40 (m, 2 H), 1.22 - 1.33 (m, 20 H), 0.99 (m, 2 H), 0.89 (t, *J*=6.8 Hz, 3 H), 0.74 - 0.81 (m, 2 H). ESI-MS(m/z): 390.3823 $[M+Na]^+$.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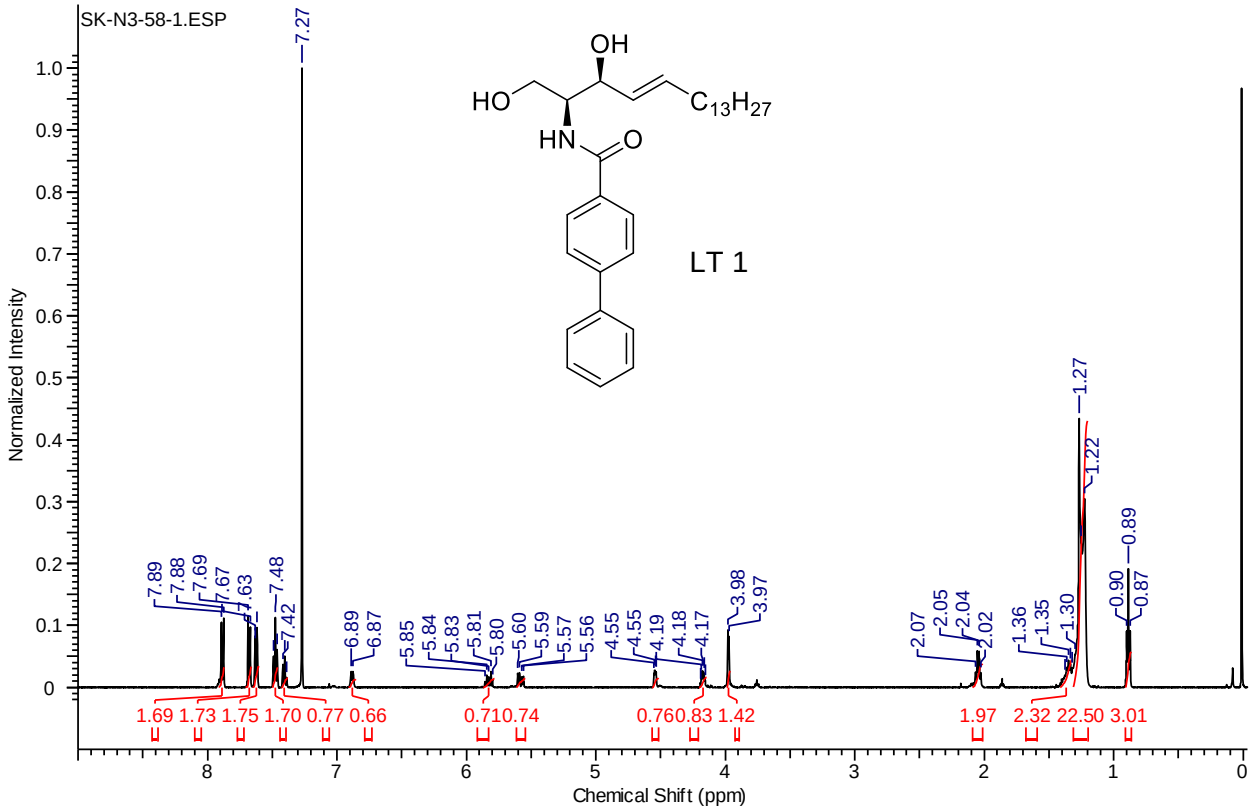
Normalized Intensity
Chemical Shift (ppm)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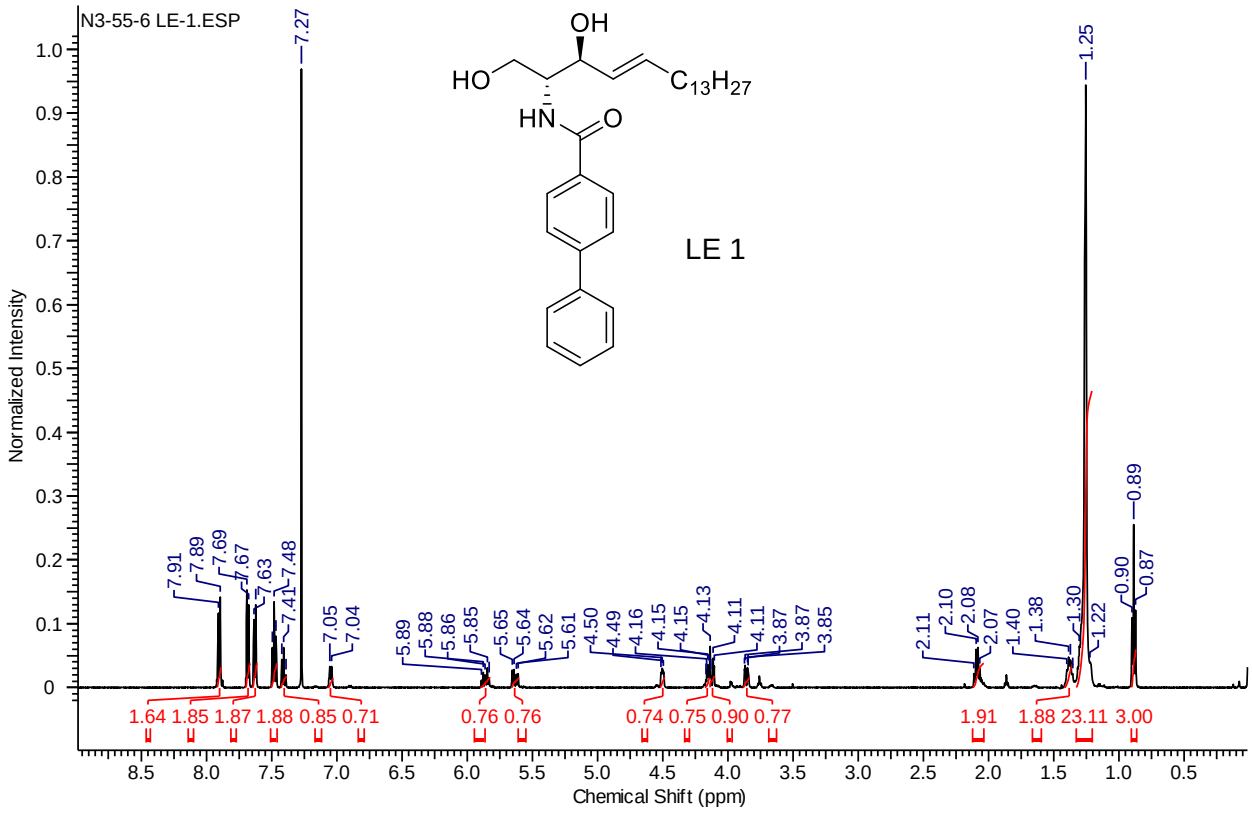
Chemical Shift (ppm)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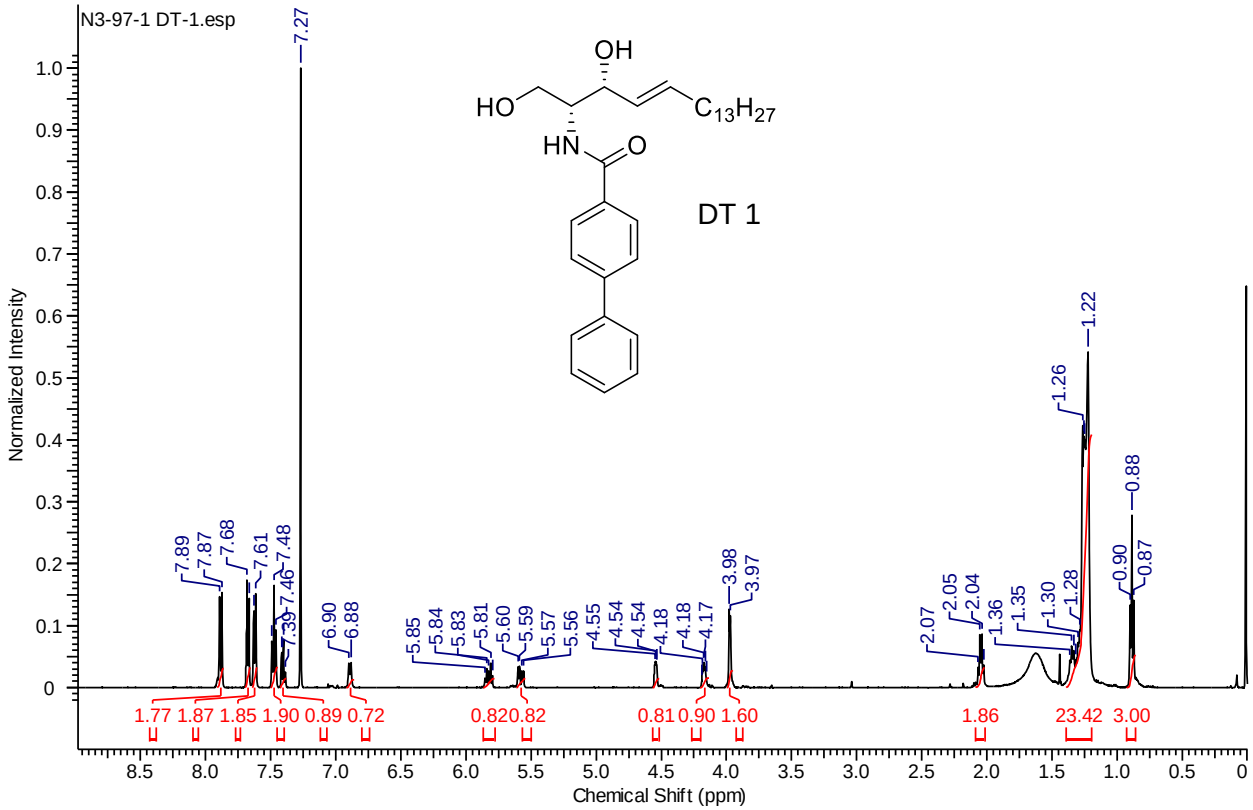
Chemical Shift (ppm)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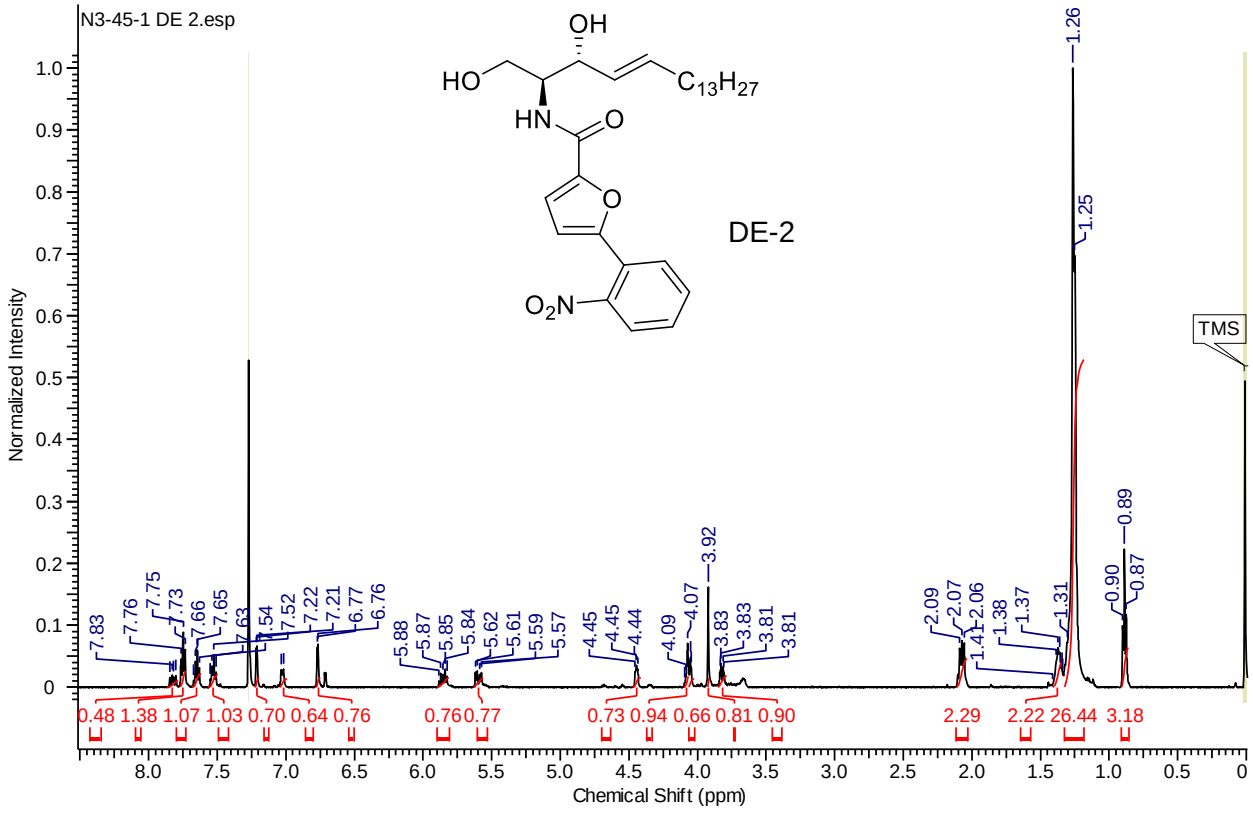
Chemical Shift (ppm)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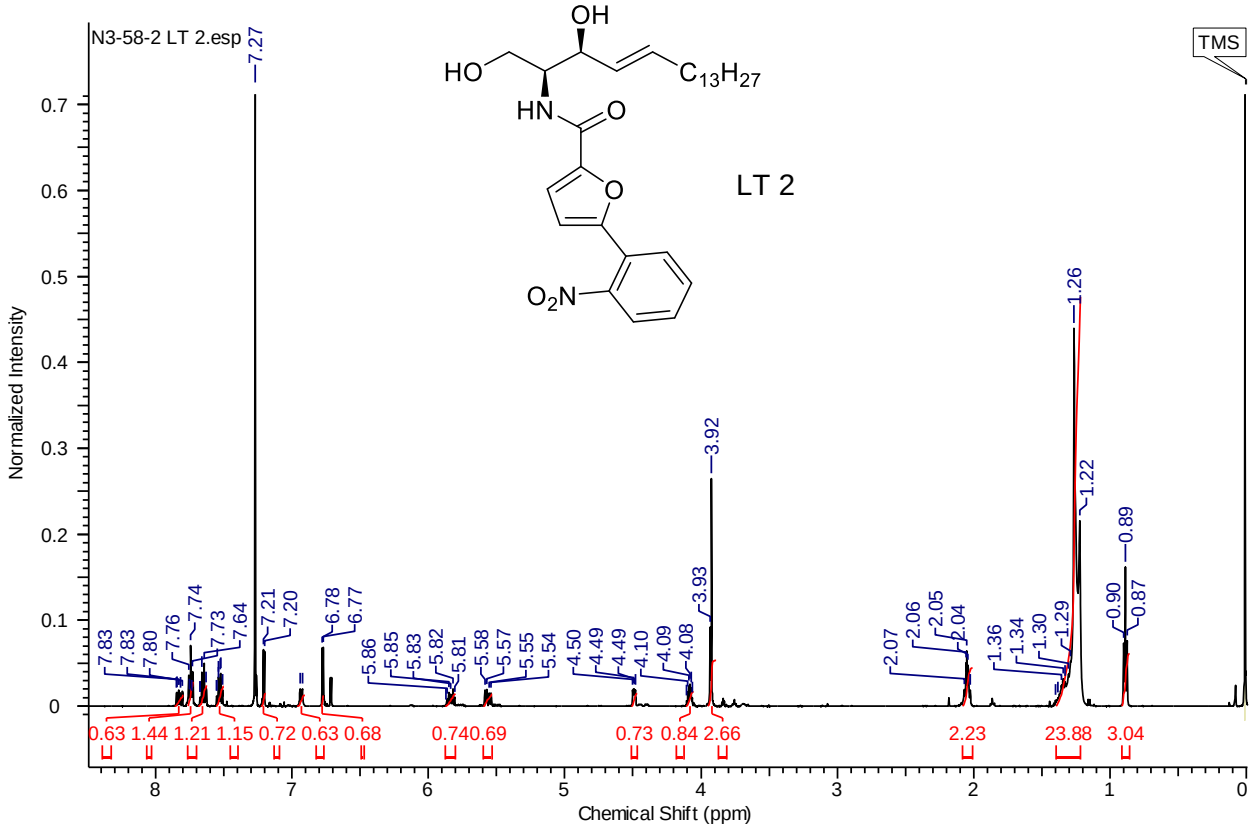
Normalized Intensity
Chemical Shift (ppm)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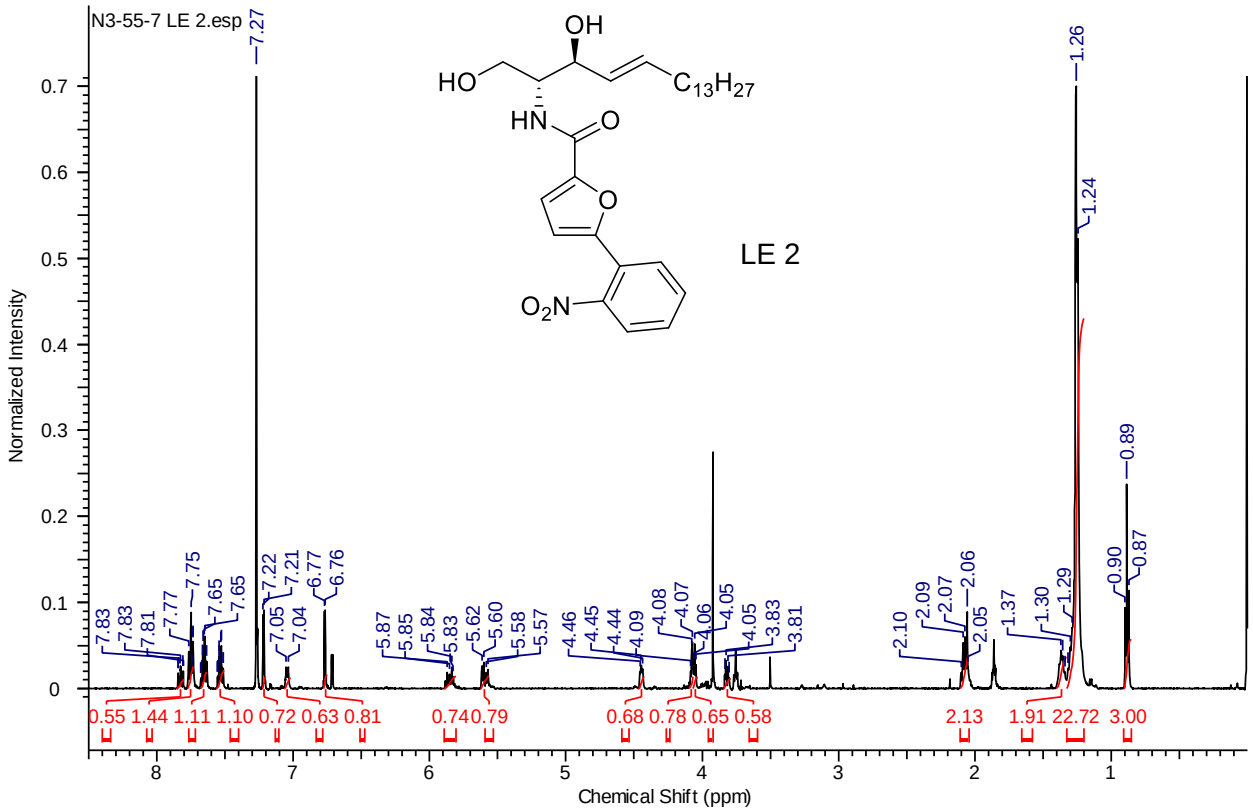
Normalized Intensity
Chemical Shift (ppm)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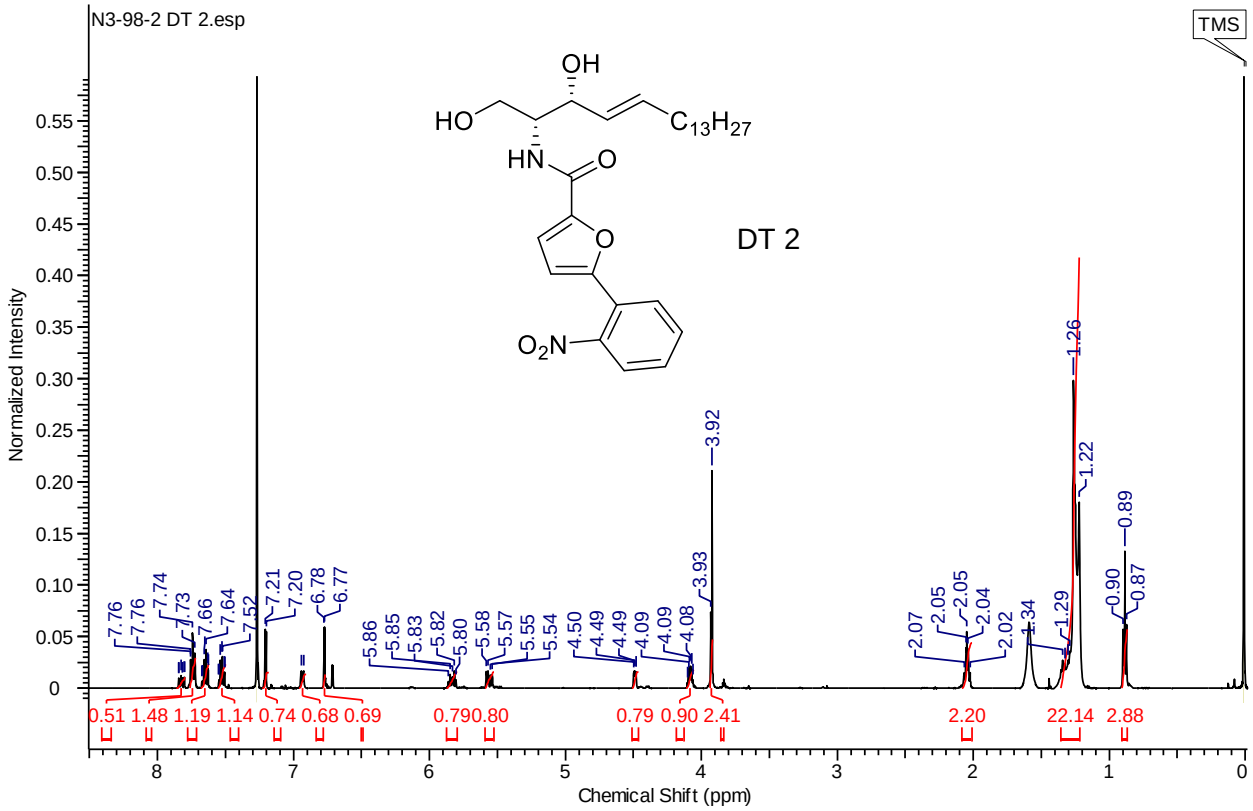
Normalized Intensity
Chemical Shift (ppm)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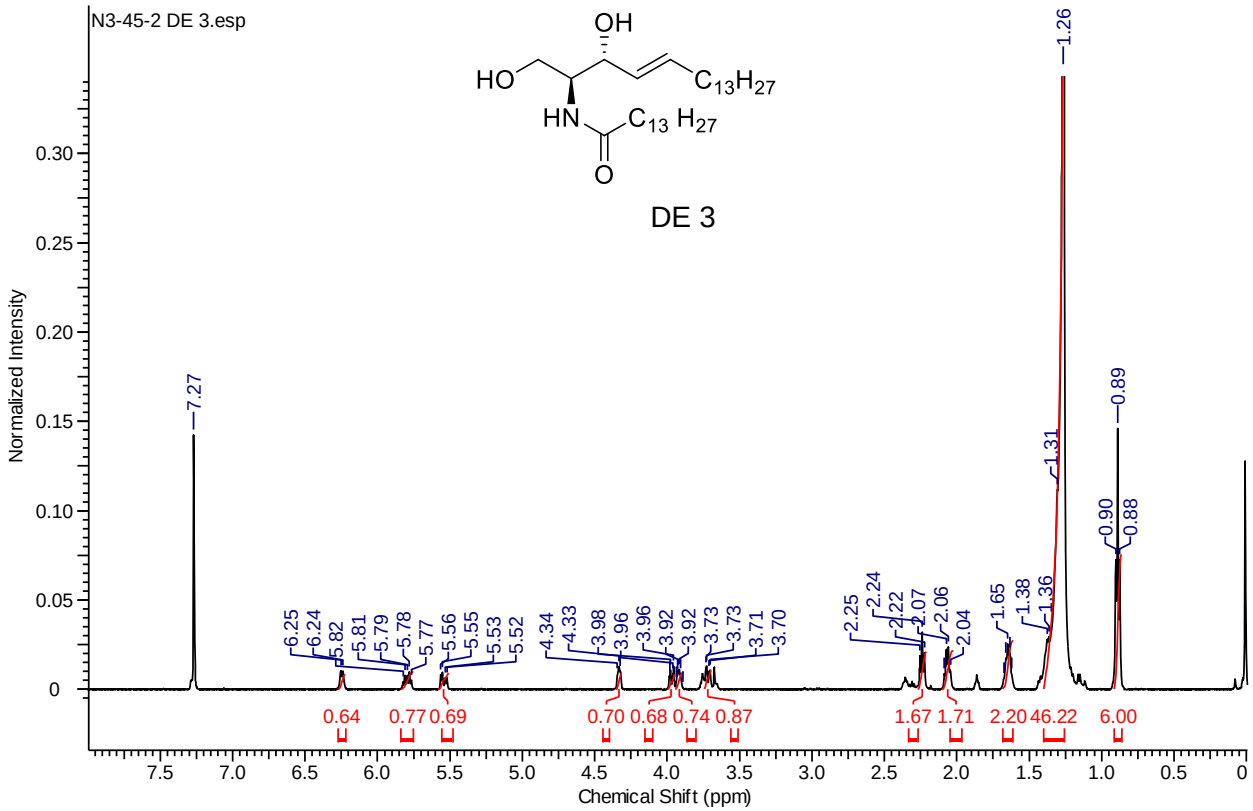
Normalized Intensity
Chemical Shift (ppm)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Normalized Intensity
Chemical Shift (ppm)
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6.24
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5.79
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5.77
5.56
5.55
5.53
5.52
4.34
4.33
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3.70
2.25
2.24
2.22
2.07
2.06
2.04
1.65
1.38
1.36
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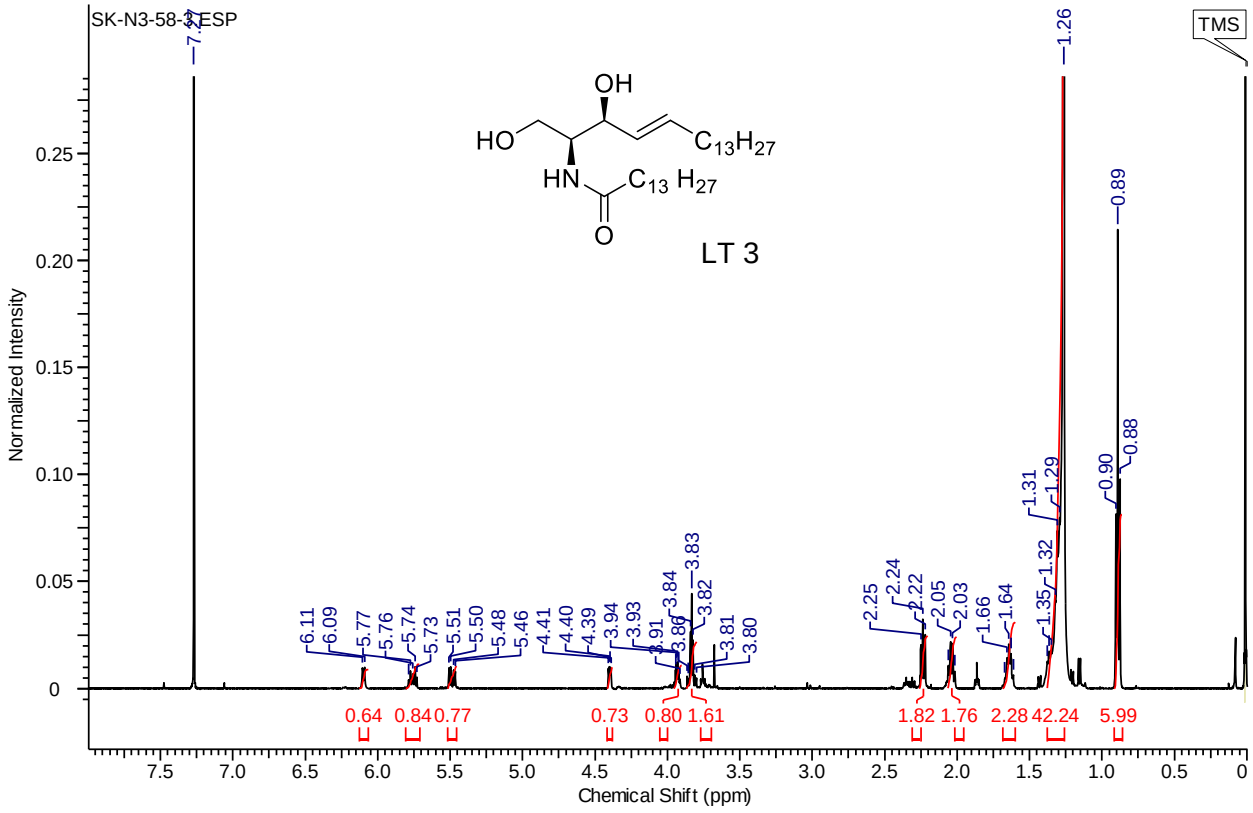
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$C_{13}H_{27}$
HN
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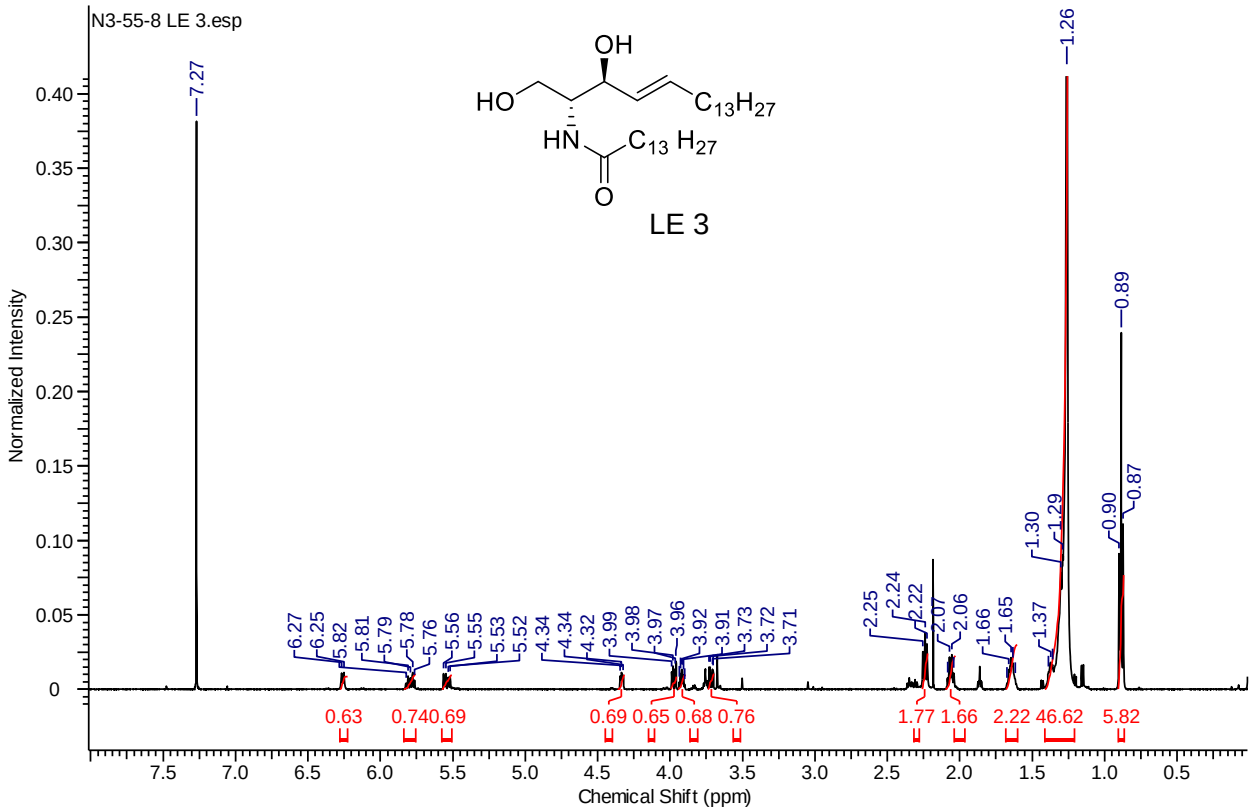
O
LT 3
TMS
Normalized Intensity
Chemical Shift (ppm)
1.26
0.89
1.31
1.29
0.90
0.88
6.11
6.09
5.77
5.76
5.74
5.73
5.51
5.50
5.48
5.46
4.41
4.40
4.39
3.94
3.93
3.91
3.86
3.84
3.83
3.82
3.81
3.80
2.25
2.24
2.22
2.05
2.03
1.66
1.64
1.35
1.32
0.64
0.84
0.77
0.73
0.80
1.61
1.82
1.76
2.28
42.24
5.99


N3-55-8 LE 3.esp
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HO
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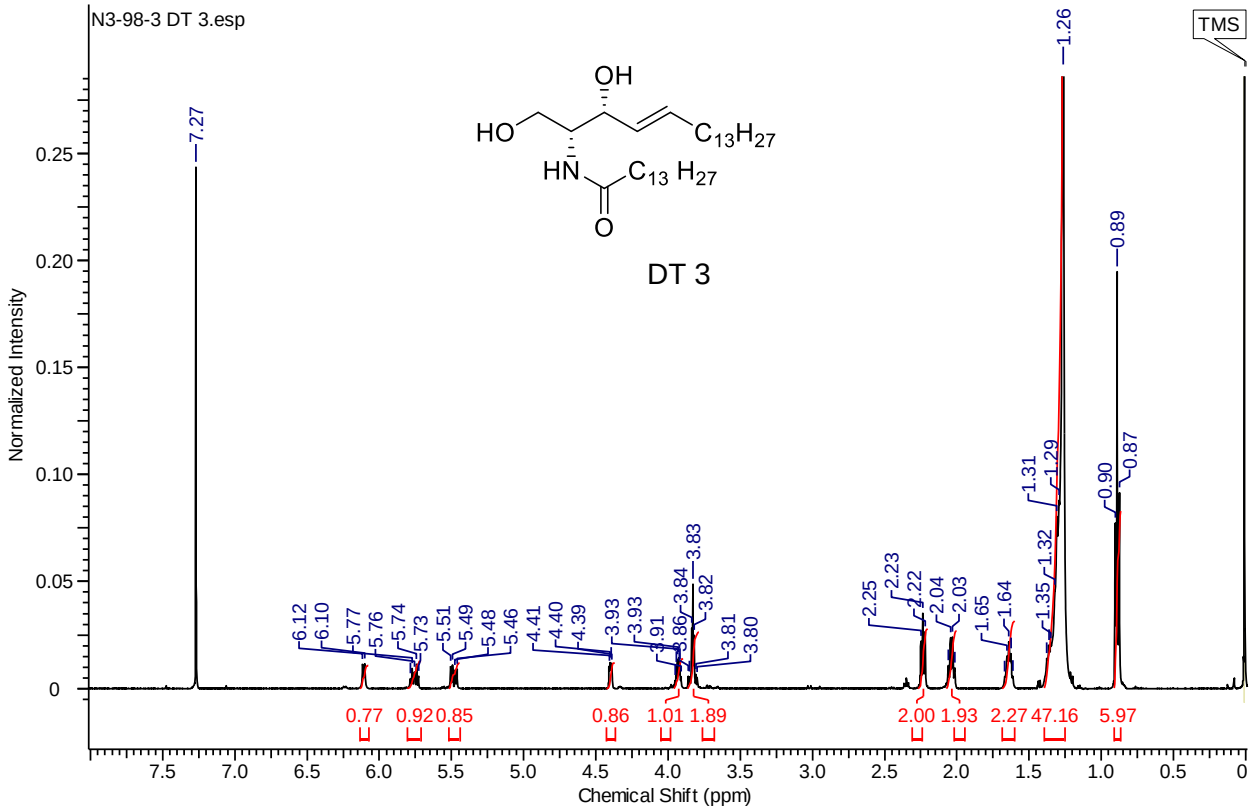
HN
$C_{13}H_{27}$
O
LE 3
Normalized Intensity
Chemical Shift (ppm)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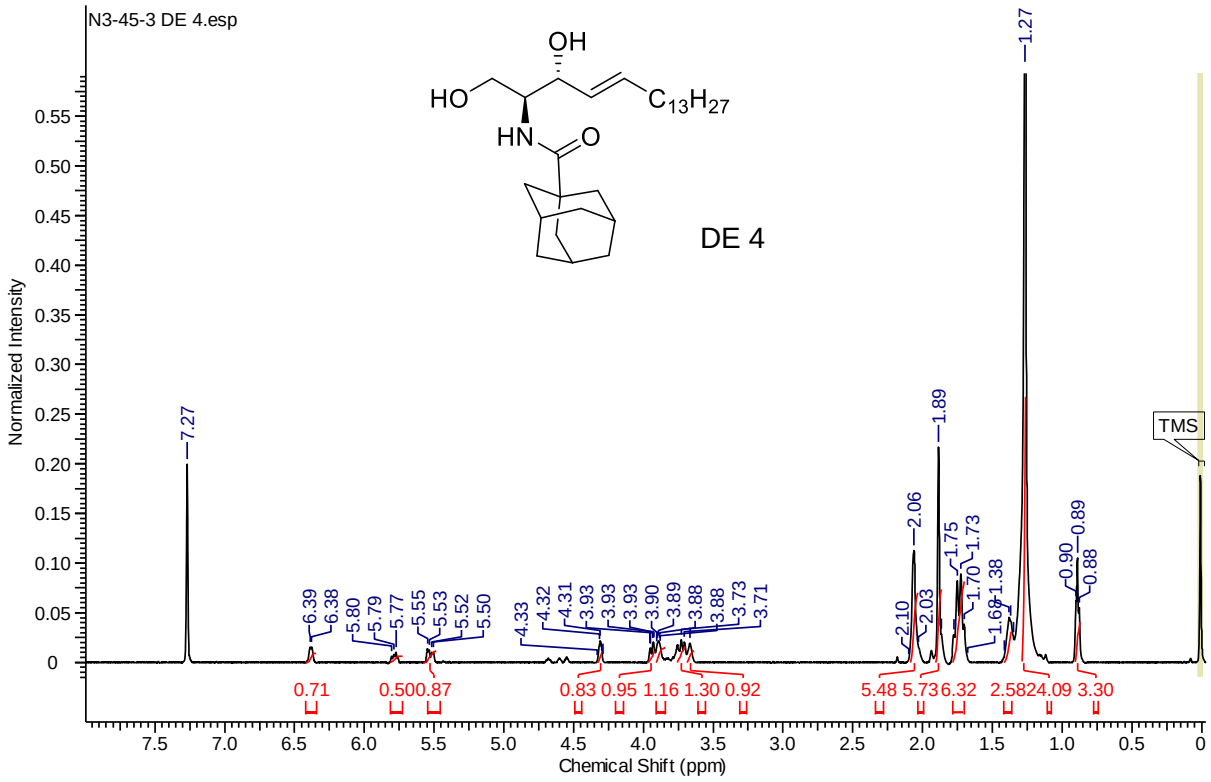
TMS
OH
HO
$C_{13}H_{27}$
HN
$C_{13}H_{27}$
O
DT 3
Normalized Intensity
Chemical Shift (ppm)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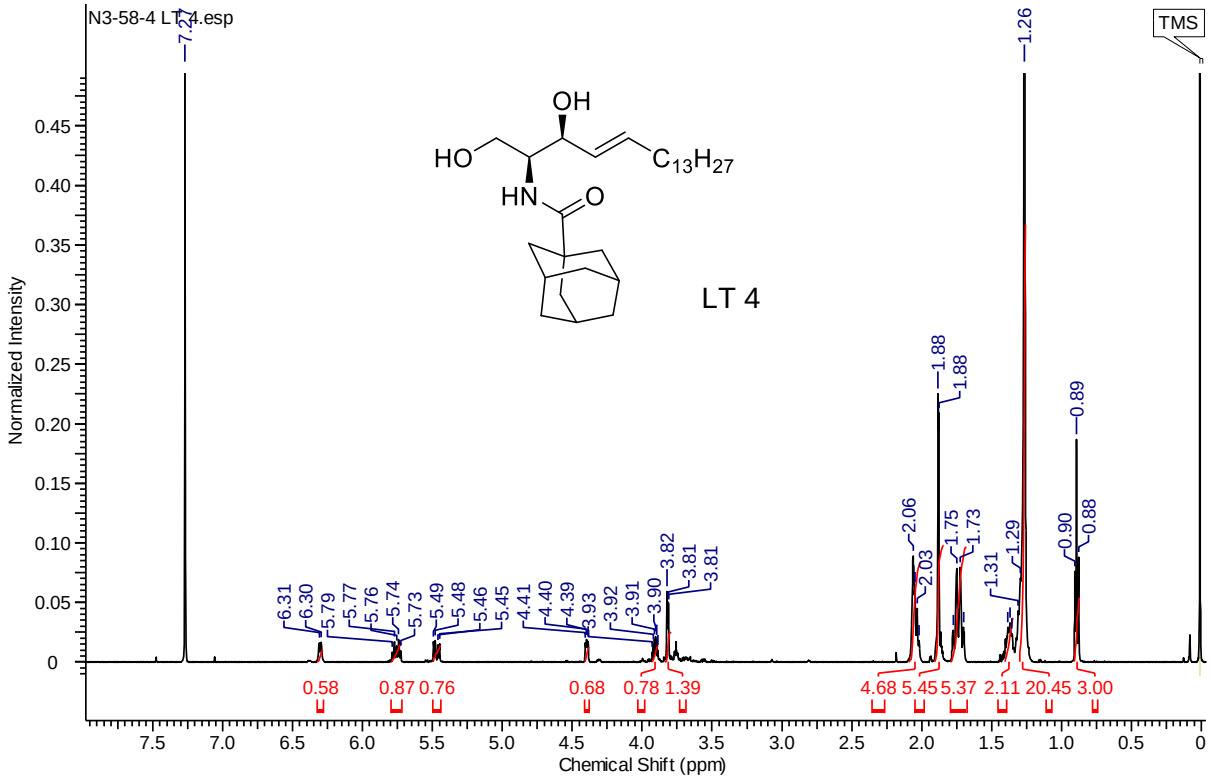
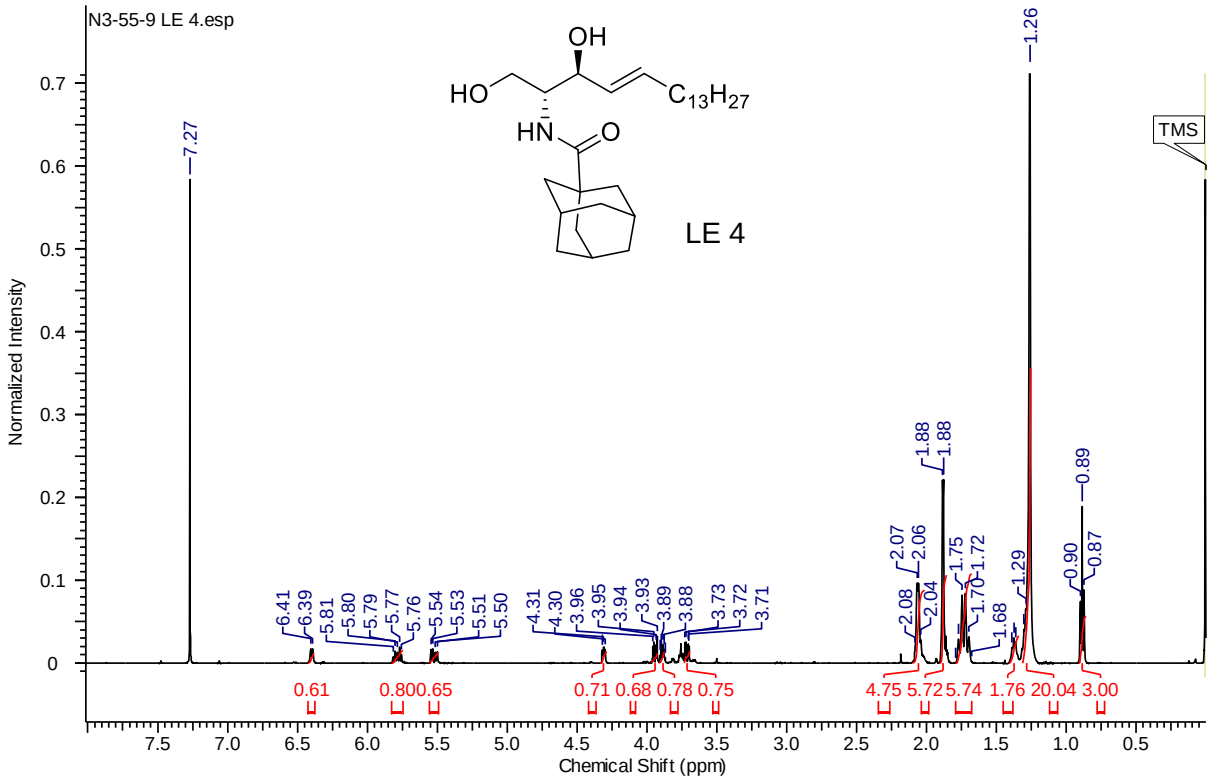
OH
HO
HN
O
$C_{13}H_{27}$
DE 4
Normalized Intensity
Chemical Shift (ppm)
TMS
7.27
1.27
1.89
2.06
1.75
1.73
1.70
1.38
0.90
0.89
0.88
2.10
2.03
6.39
6.38
5.80
5.79
5.77
5.55
5.53
5.52
5.50
4.33
4.32
4.31
3.93
3.93
3.93
3.90
3.89
3.88
3.88
3.73
3.71
0.71
0.50
0.87
0.83
0.95
1.16
1.30
0.92
5.48
5.73
6.32
2.58
4.09
3.30


N3-58-4 LT 4.esp
OH
HO
HN
O
$C_{13}H_{27}$
LT 4
Normalized Intensity
Chemical Shift (ppm)
TMS
7.27
1.26
1.88
1.88
0.89
2.06
2.03
1.75
1.73
1.31
1.29
0.90
0.88
6.31
6.30
5.79
5.77
5.76
5.74
5.73
5.49
5.48
5.46
5.45
4.41
4.40
4.39
3.93
3.92
3.91
3.90
3.82
3.81
3.81
0.58
0.87
0.76
0.68
0.78
1.39
4.68
5.45
5.37
2.11
20.45
3.00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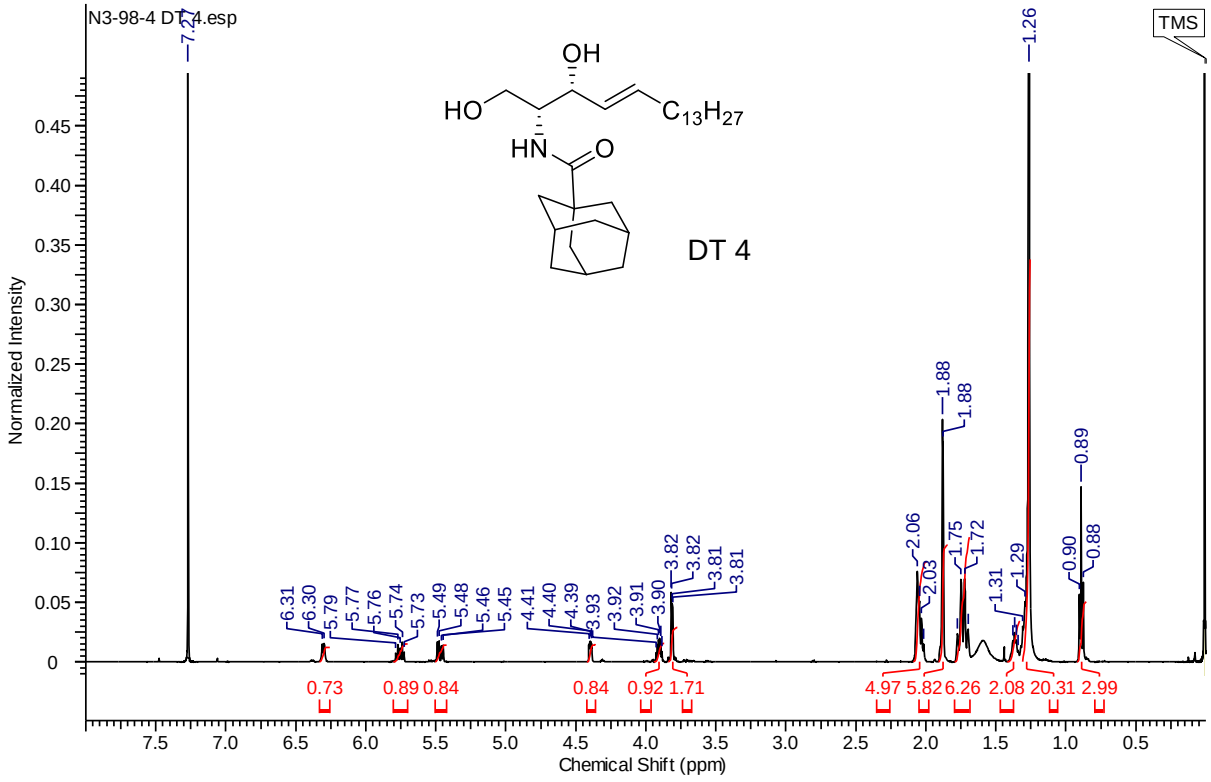
OH
HO
$C_{13}H_{27}$
HN
O
LE 4
TMS
Normalized Intensity
Chemical Shift (ppm)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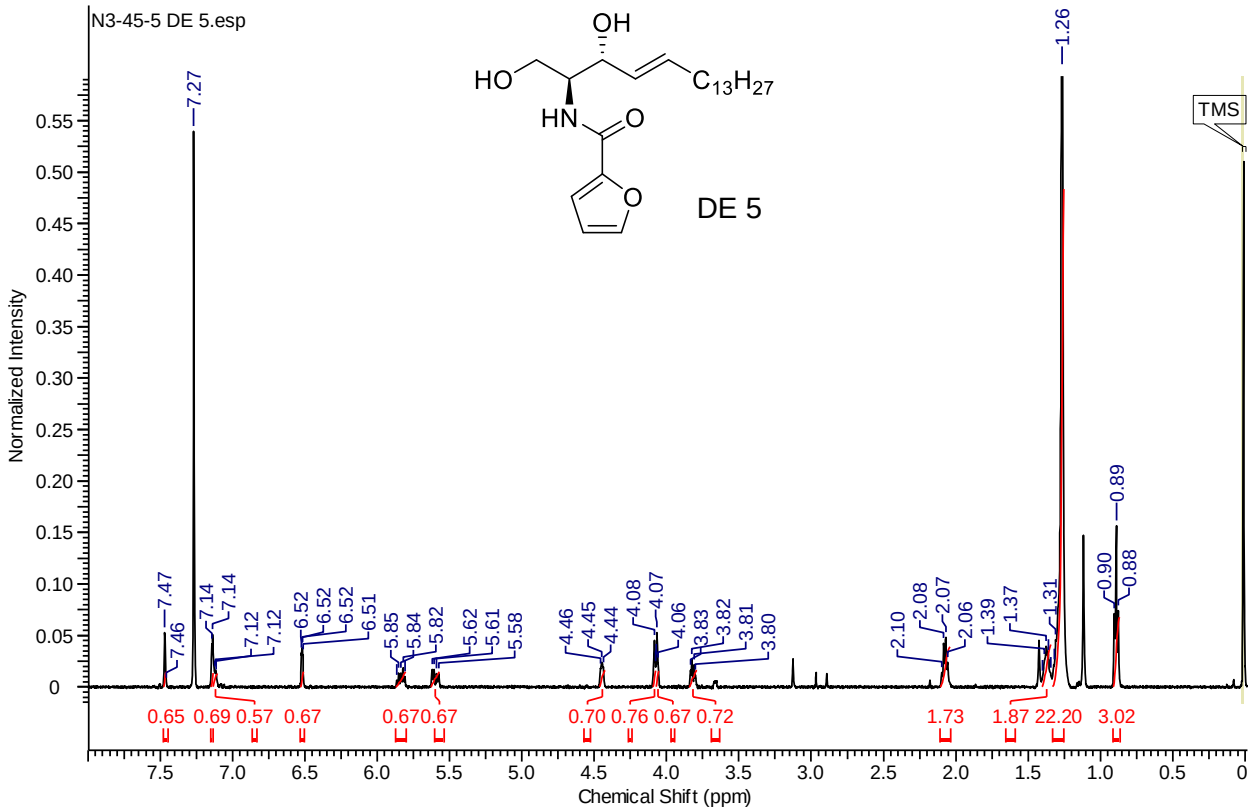
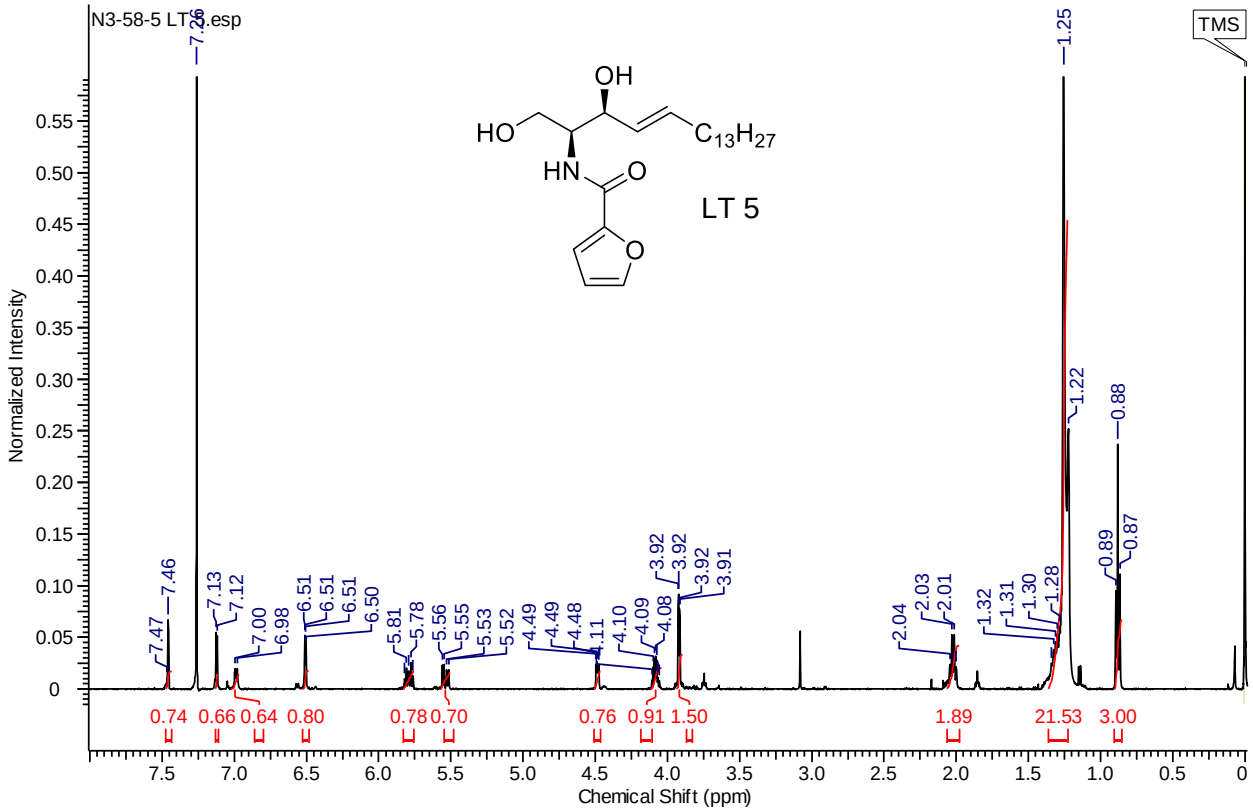
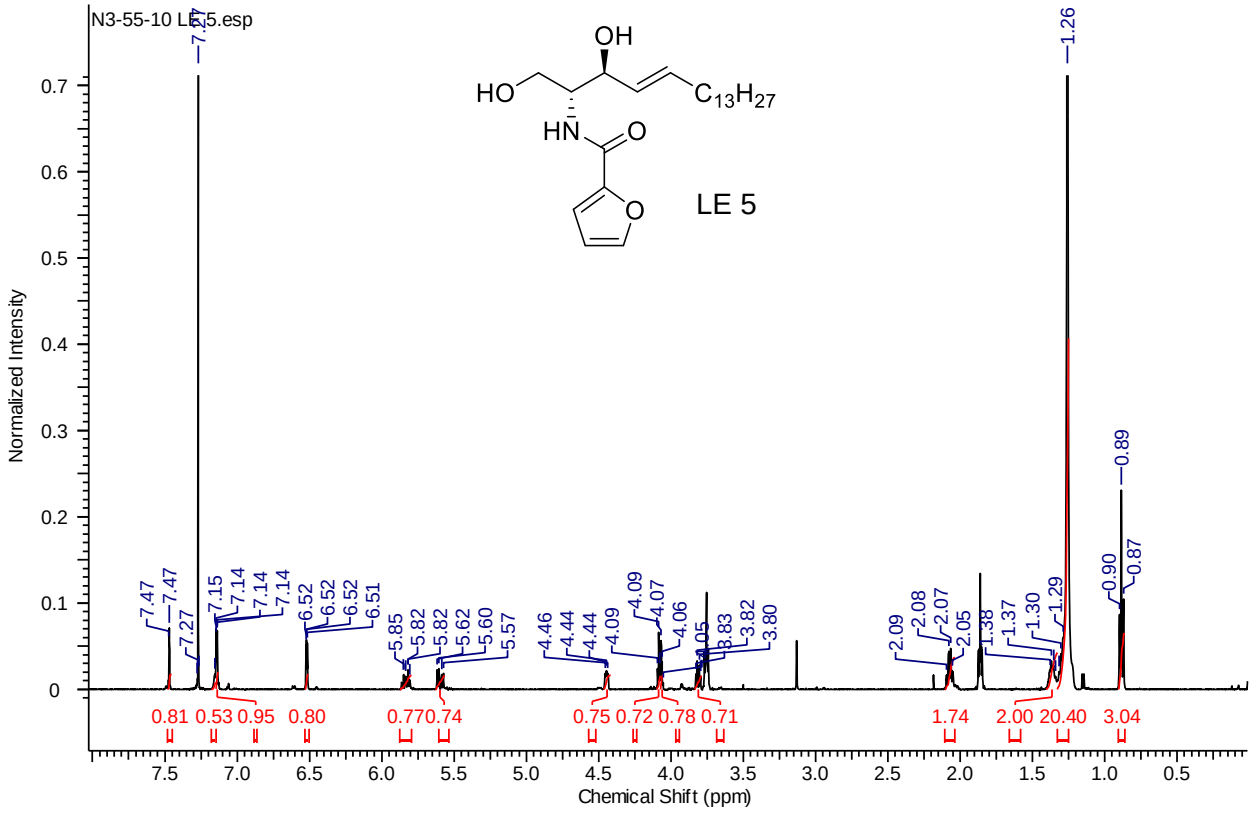
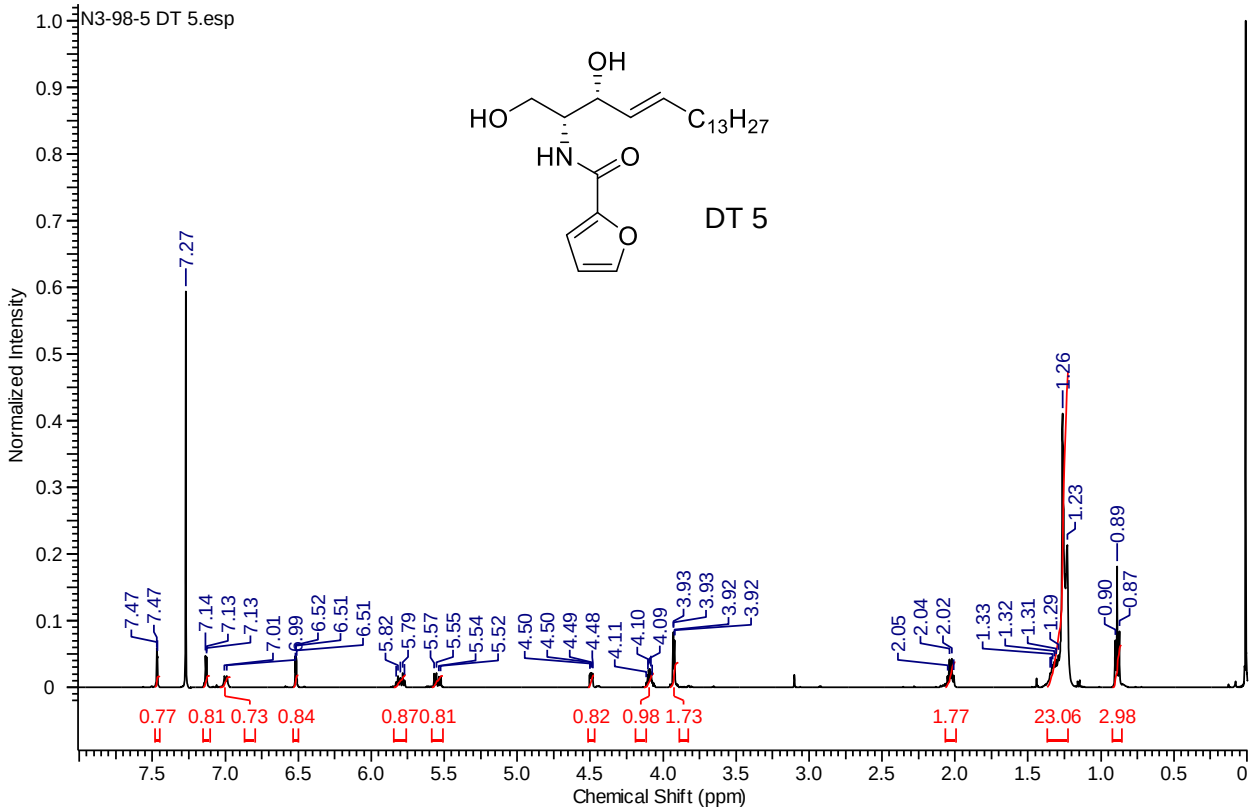
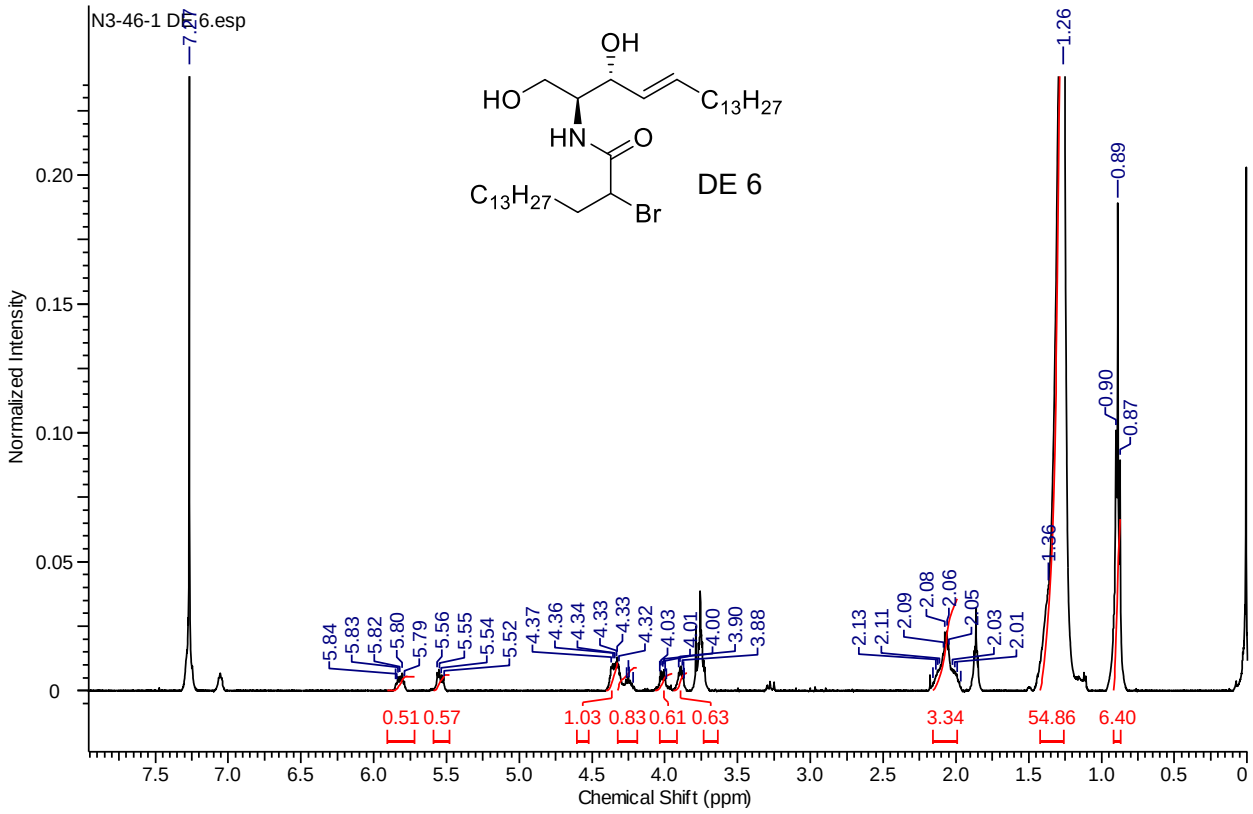
HO
$C_{13}H_{27}$
HN
O
DT 4
TMS
Normalized Intensity
Chemical Shift (ppm)

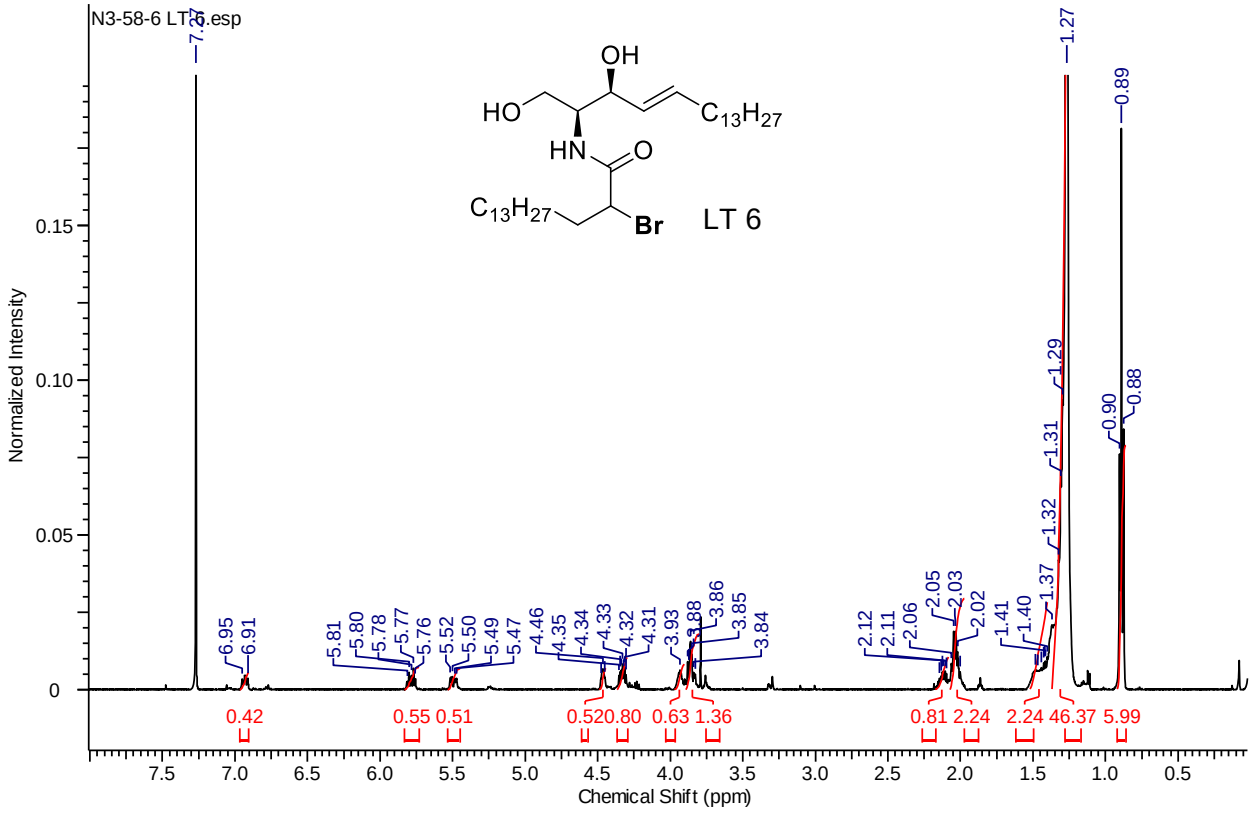

N3-45-5 DE 5.esp
OH
HO
HN
O
O
$C_{13}H_{27}$
DE 5
TMS
Normalized Intensity
Chemical Shift (ppm)

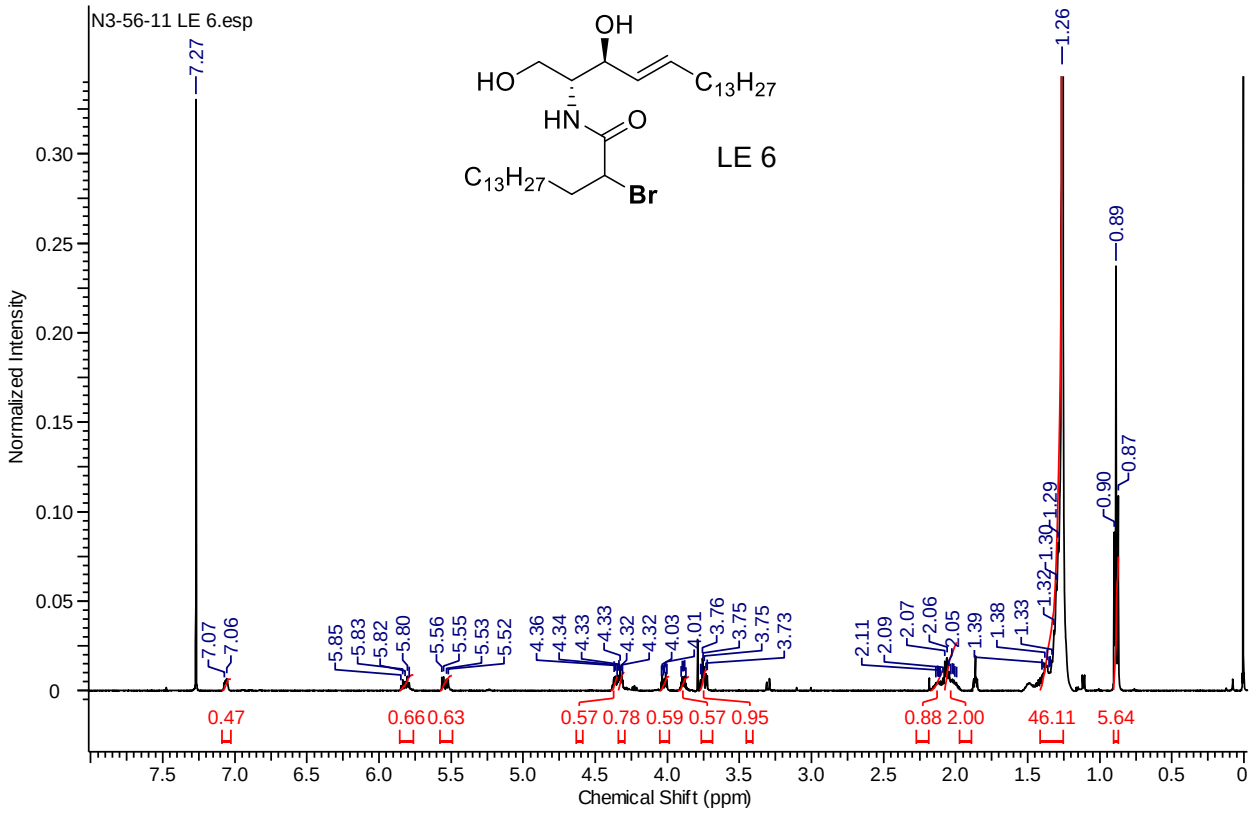

N3-58-5 LT5.esp
OH
HO
HN
O
O
$C_{13}H_{27}$
LT 5
TMS
Normalized Intensity
Chemical Shift (ppm)

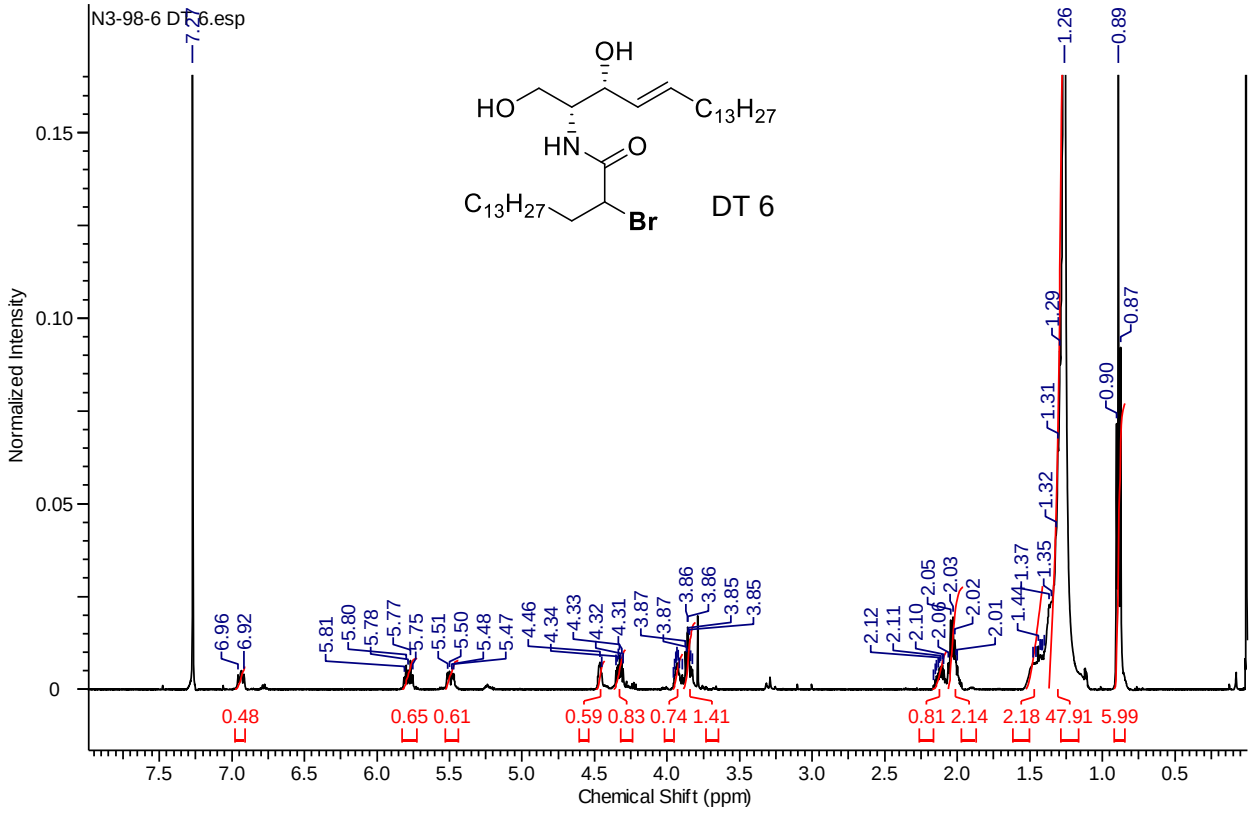

N3-55-10 LE 5.esp
OH
HO
HN
O
O
$C_{13}H_{27}$
LE 5
Normalized Intensity
Chemical Shift (ppm)
7.27
1.26
0.89
0.90
0.87
0.81 0.53 0.95 0.80 0.77 0.74 0.75 0.72 0.78 0.71 1.74 2.00 20.40 3.04

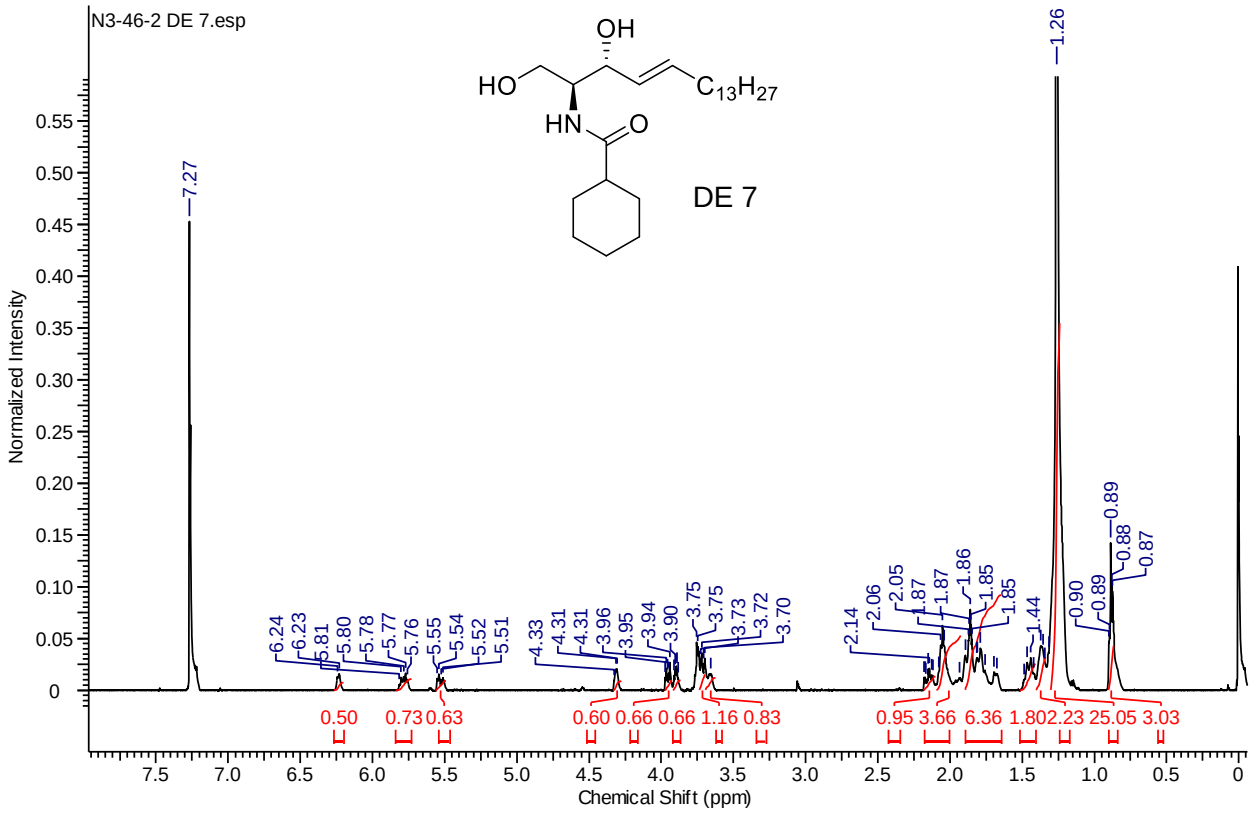

N3-98-5 DT 5.esp
OH
HO
HN
O
O
$C_{13}H_{27}$
DT 5
Normalized Intensity
Chemical Shift (ppm)
7.27
1.26
1.23
0.89
0.90
0.87
0.77 0.81 0.73 0.84 0.87 0.81 0.82 0.98 1.73 1.77 23.06 2.98

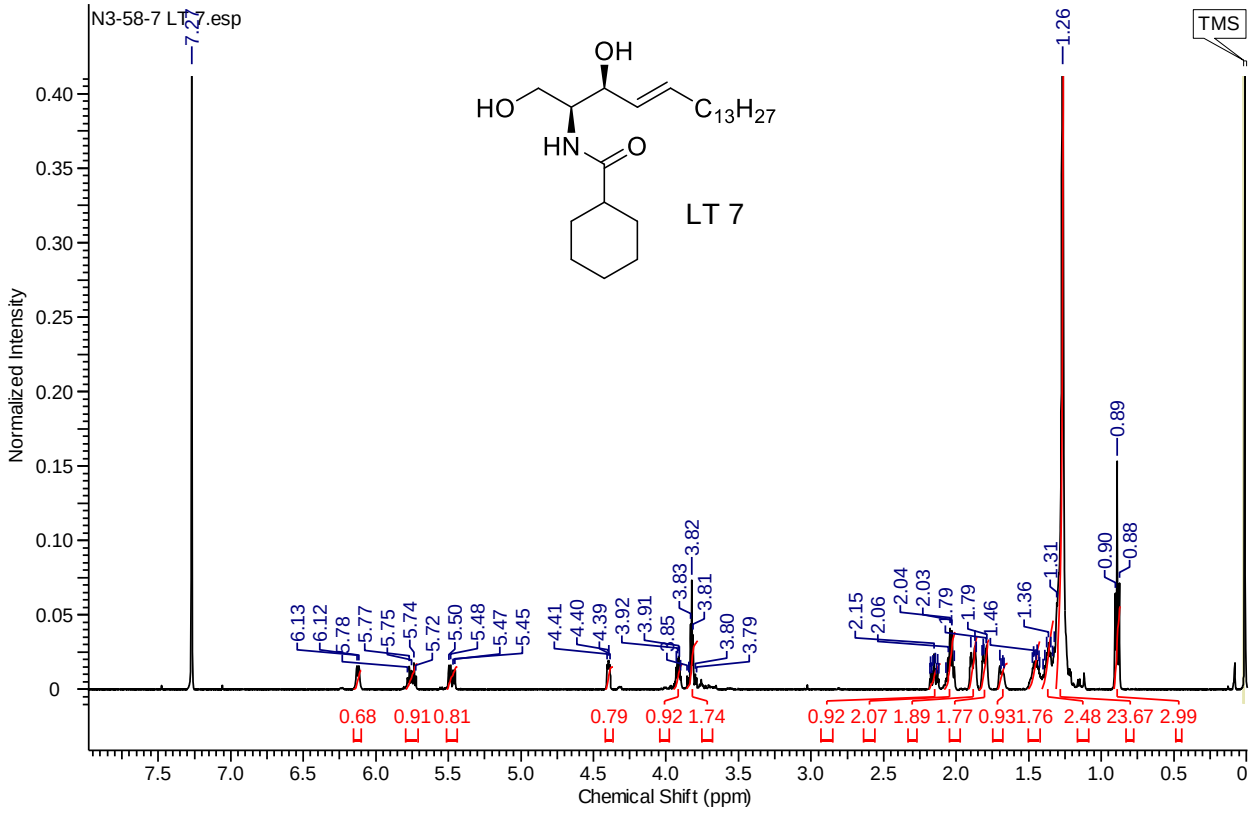

N3-46-1 DE 6.esp
OH
HO
$C_{13}H_{27}$
HN
O
$C_{13}H_{27}$
Br
DE 6
Normalized Intensity
Chemical Shift (ppm)

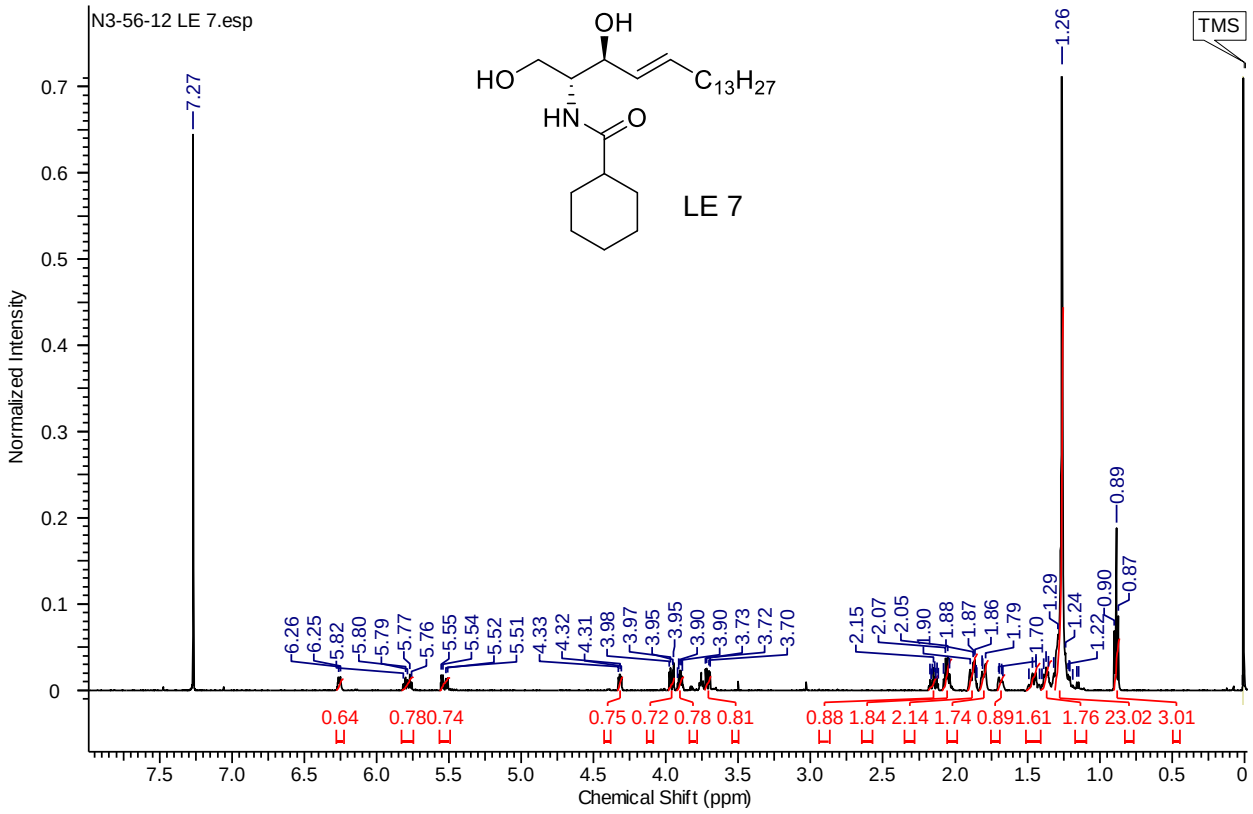

N3-58-6 LT6.esp
OH
HO
$C_{13}H_{27}$
HN
O
$C_{13}H_{27}$
Br
LT 6
Normalized Intensity
Chemical Shift (ppm)


N3-56-11 LE 6.esp
OH
HO
$C_{13}H_{27}$
HN
O
$C_{13}H_{27}$
Br
LE 6
Normalized Intensity
Chemical Shift (ppm)


N3-98-6 DT 6.esp
OH
HO
$C_{13}H_{27}$
HN
O
$C_{13}H_{27}$
Br
DT 6
Normalized Intensity
Chemical Shift (ppm)


N3-46-2 DE 7.esp
OH
HO
HN
O
$C_{13}H_{27}$
DE 7
Normalized Intensity
Chemical Shift (ppm)


N3-58-7 LT 7.esp
OH
HO
HN
O
$C_{13}H_{27}$
LT 7
TMS
Normalized Intensity
Chemical Shift (ppm)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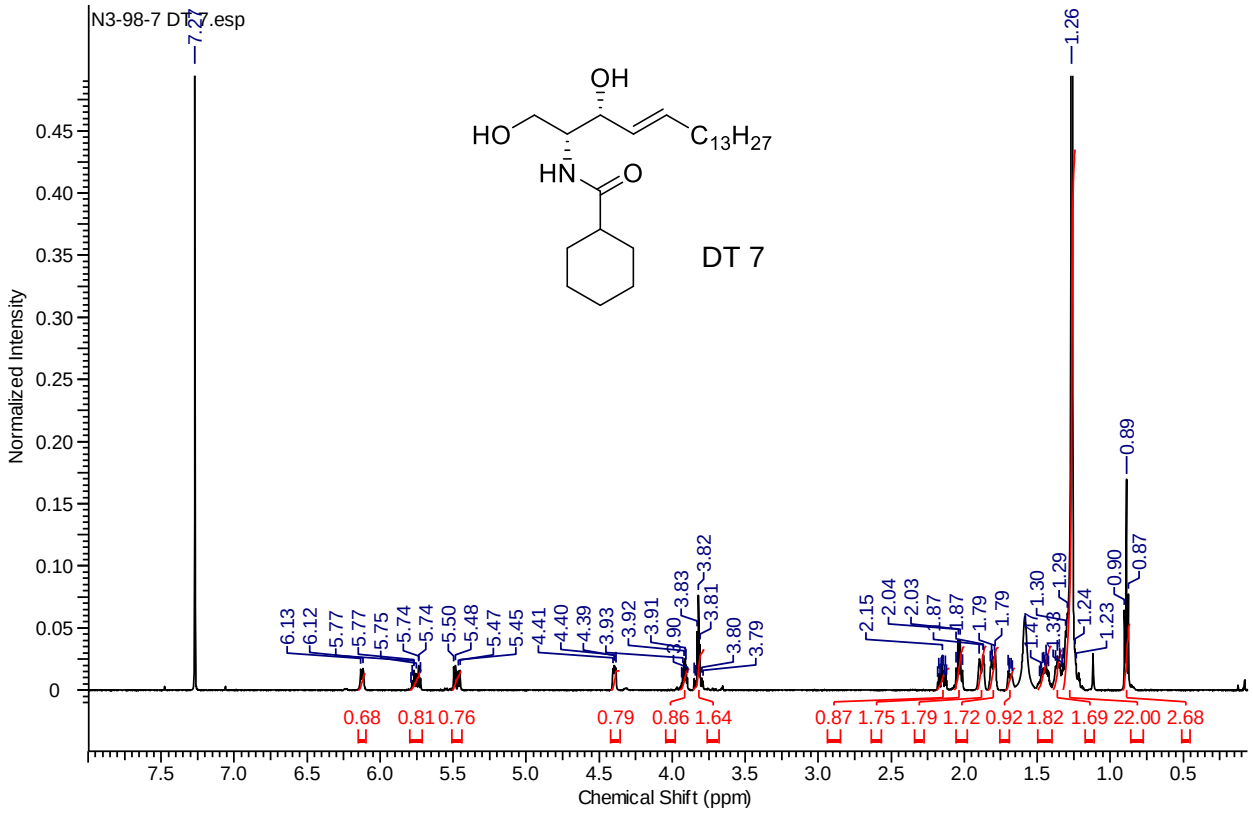
OH
HO
HN
O
$C_{13}H_{27}$
LE 7
TMS
Normalized Intensity
Chemical Shift (ppm)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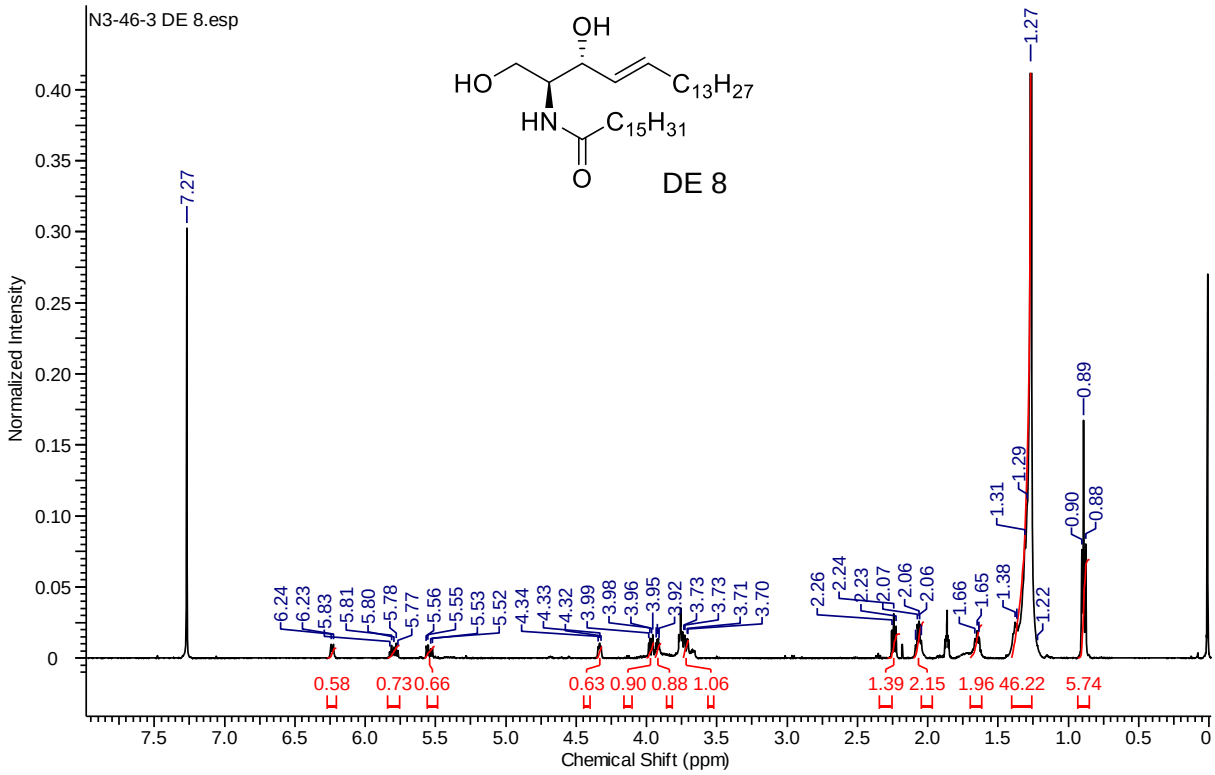
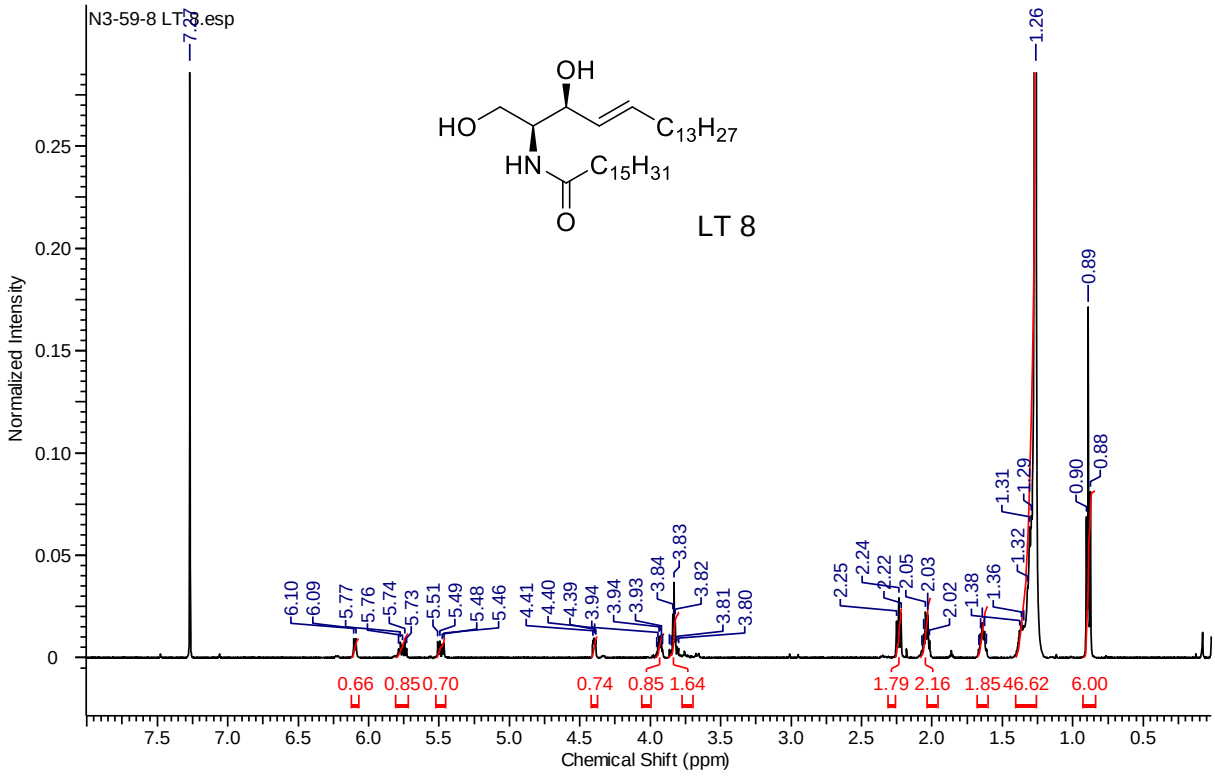
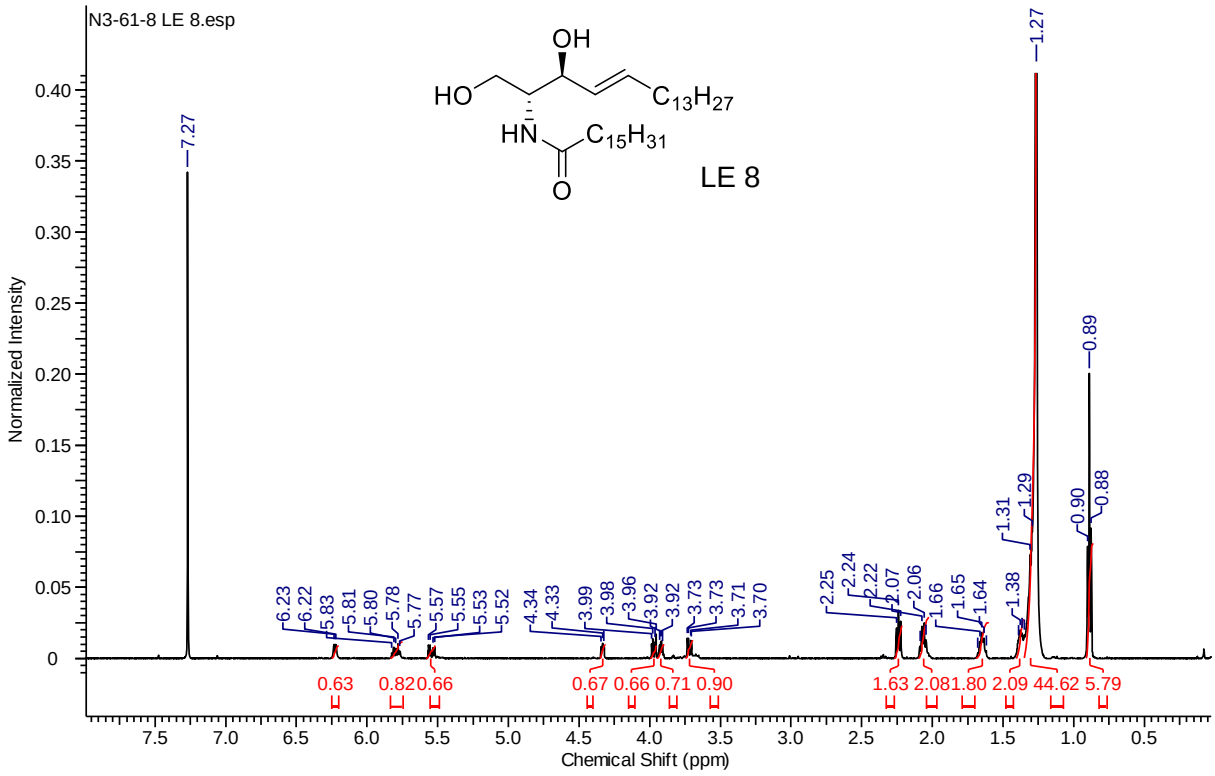
HO
HN
O
$C_{13}H_{27}$
DT 7
Normalized Intensity
Chemical Shift (ppm)


N3-46-3 DE 8.esp
OH
HO
$C_{13}H_{27}$
HN
$C_{15}H_{31}$
O
DE 8
Normalized Intensity
Chemical Shift (ppm)


N3-59-8 LT 8.esp
OH
HO
$C_{13}H_{27}$
HN
$C_{15}H_{31}$
O
LT 8
Normalized Intensity
Chemical Shift (ppm)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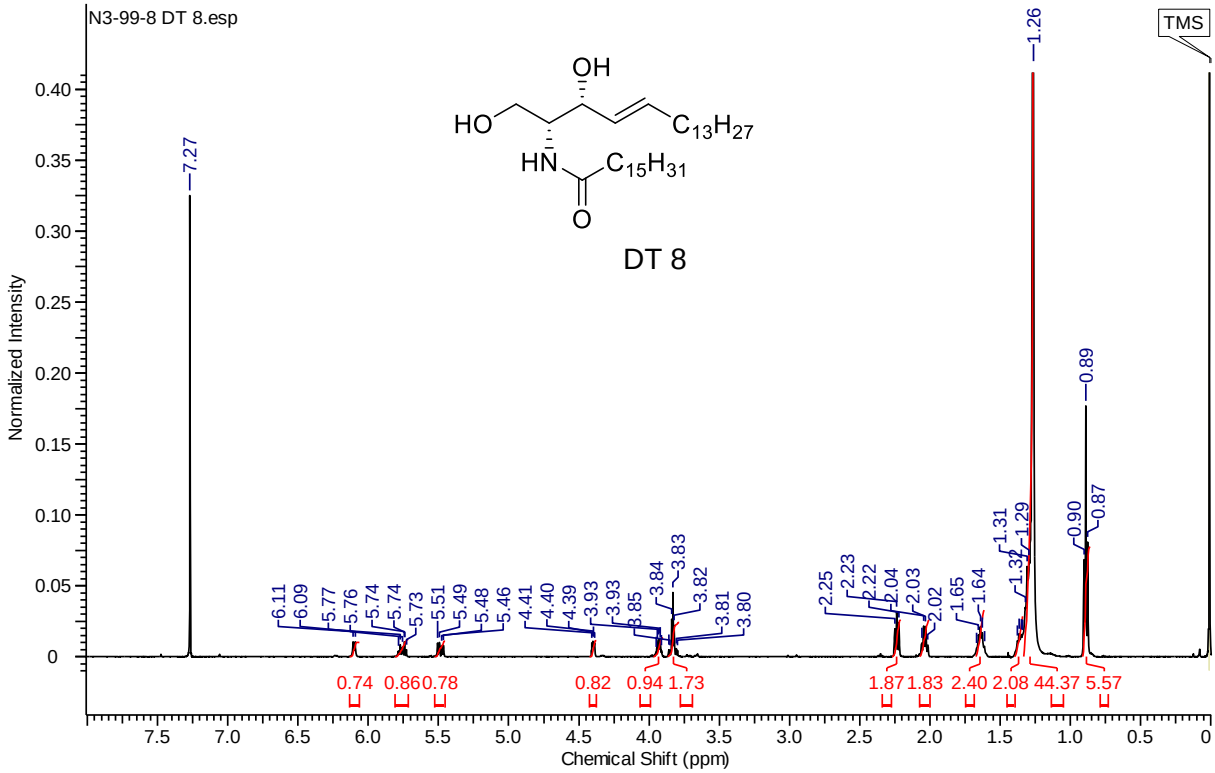
HO
$C_{13}H_{27}$
HN
$C_{15}H_{31}$
O
LE 8
Normalized Intensity
Chemical Shift (ppm)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HO
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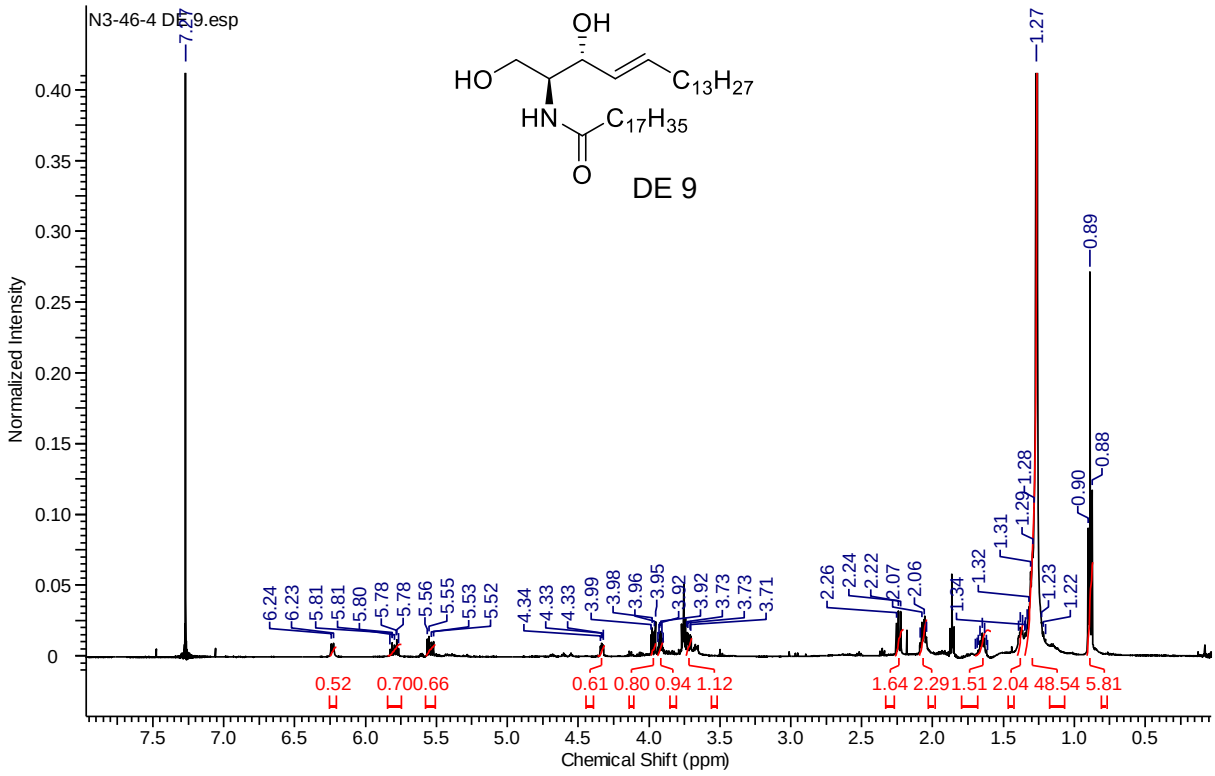
HN
$C_{15}H_{31}$
O
DT 8
TMS
Normalized Intensity
Chemical Shift (ppm)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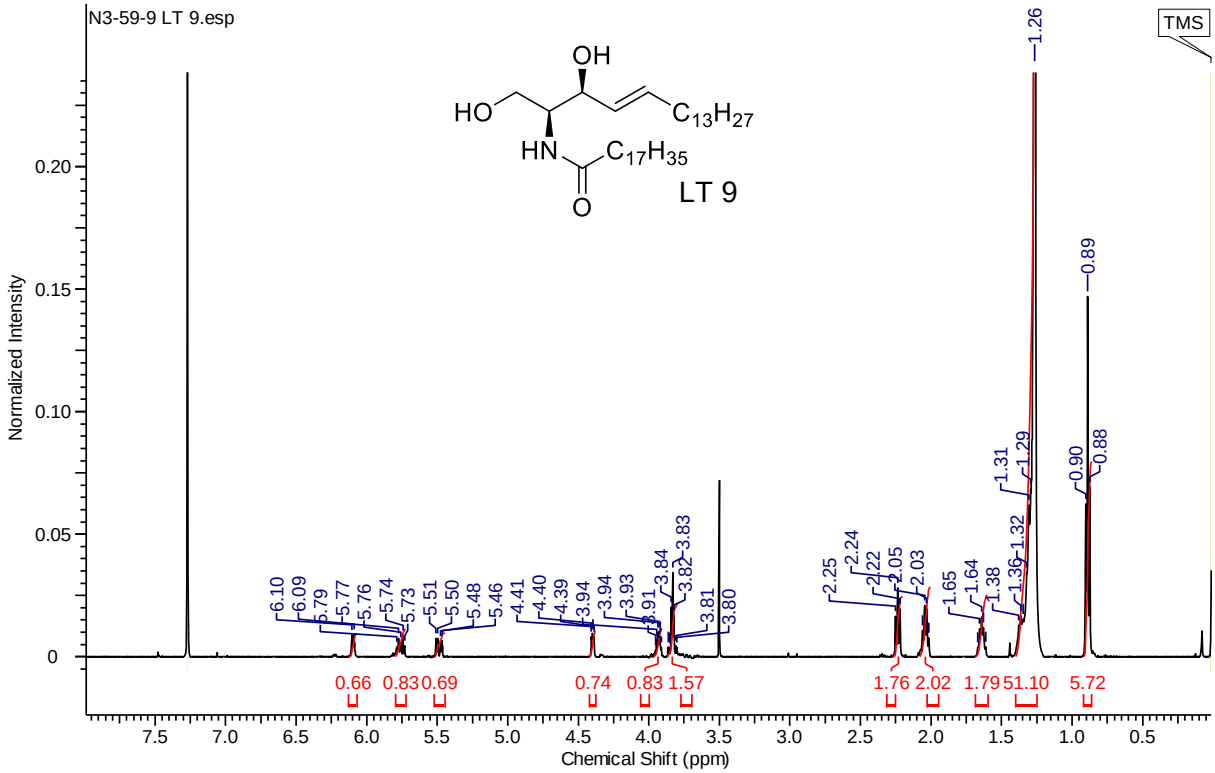
OH
HO
$C_{13}H_{27}$
HN
$C_{17}H_{35}$
O
DE 9
Normalized Intensity
Chemical Shift (ppm)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HO
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HN
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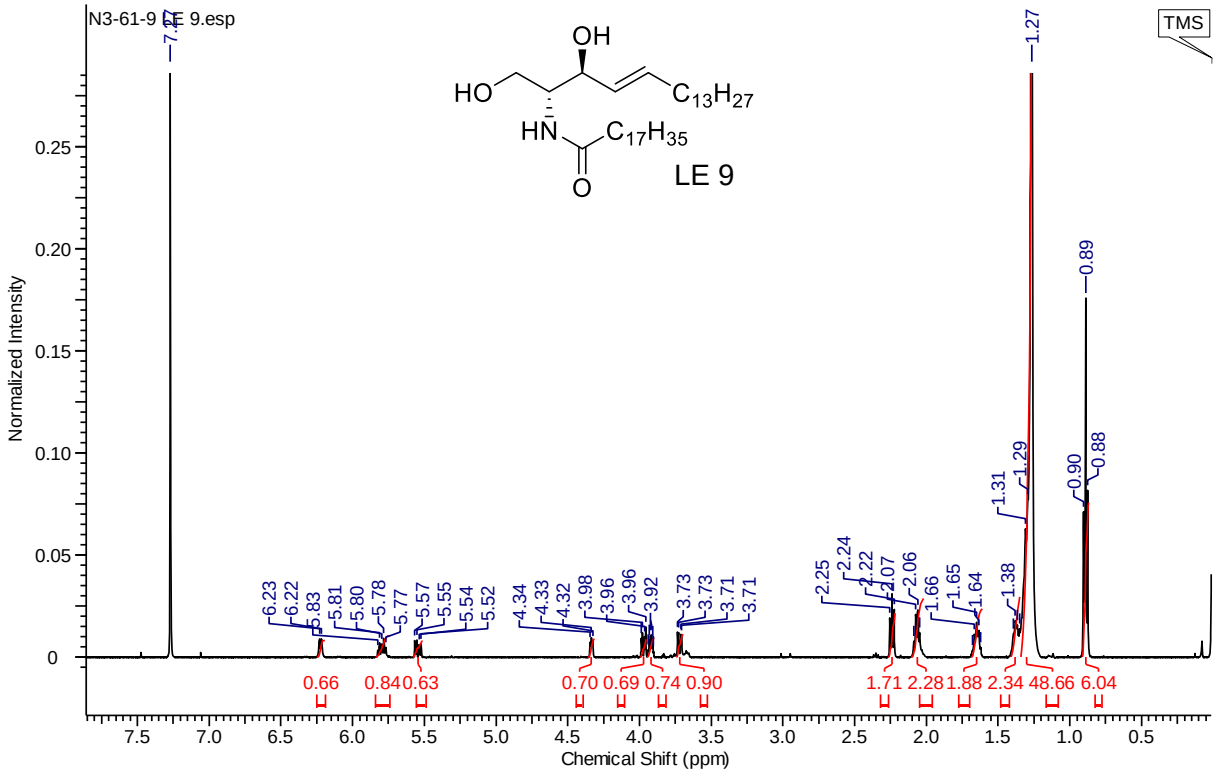
O
LT 9
TMS
Normalized Intensity
Chemical Shift (ppm)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TMS
OH
HO
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HN
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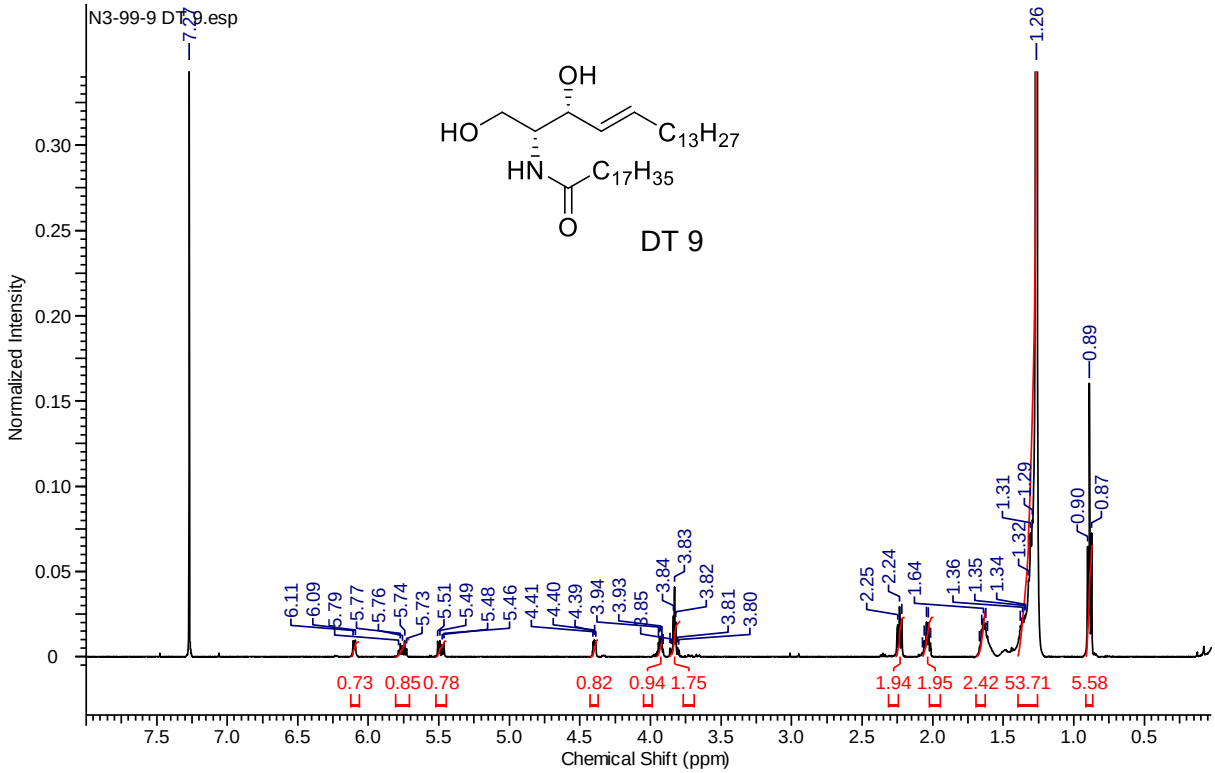
O
LE 9
Normalized Intensity
Chemical Shift (ppm)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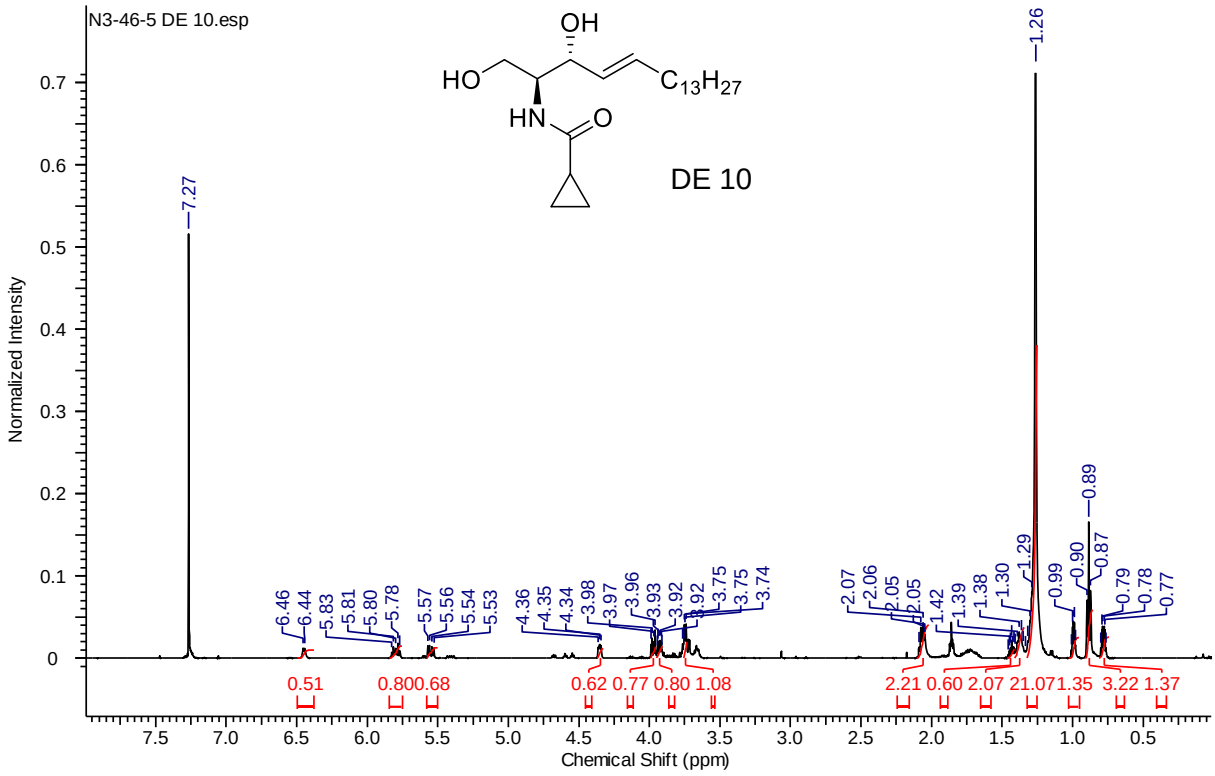
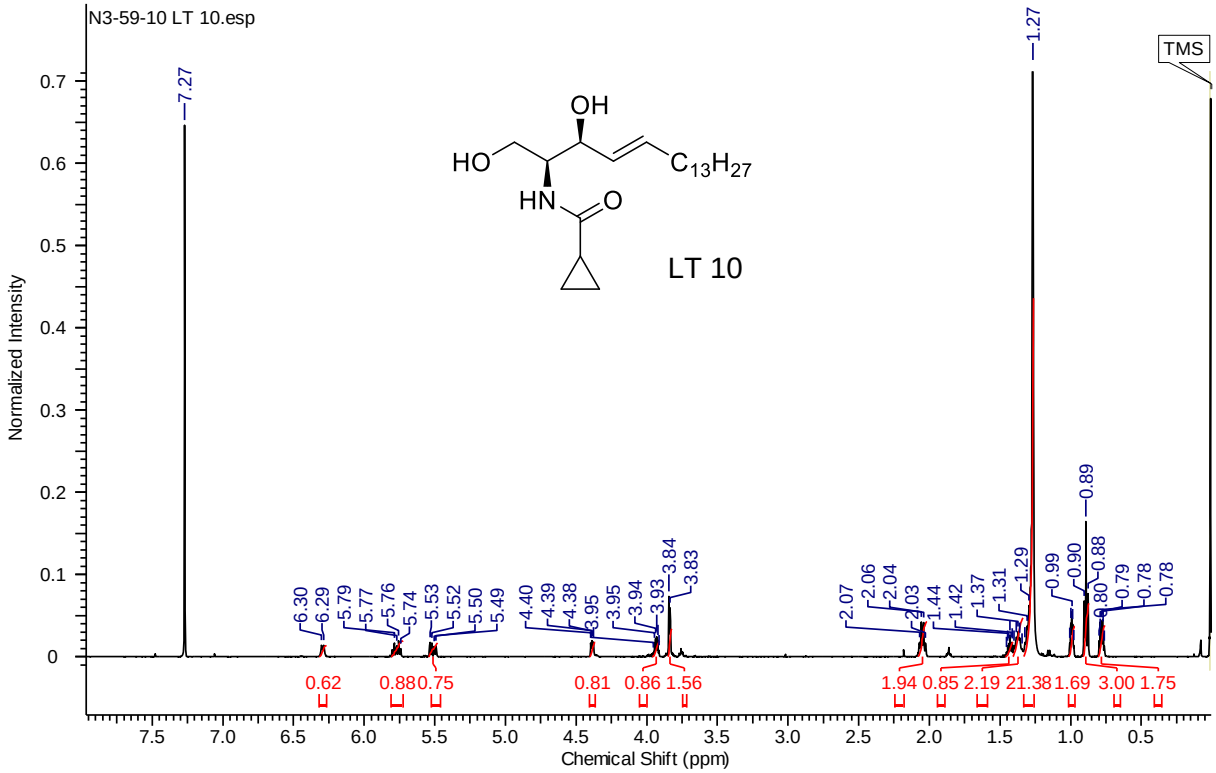
OH
HO
$C_{13}H_{27}$
HN
$C_{17}H_{35}$
O
DT 9
Normalized Intensity
Chemical Shift (ppm)


N3-46-5 DE 10.esp
OH
HO
HN
O
$C_{13}H_{27}$
DE 10
Normalized Intensity
Chemical Shift (ppm)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HO
HN
O
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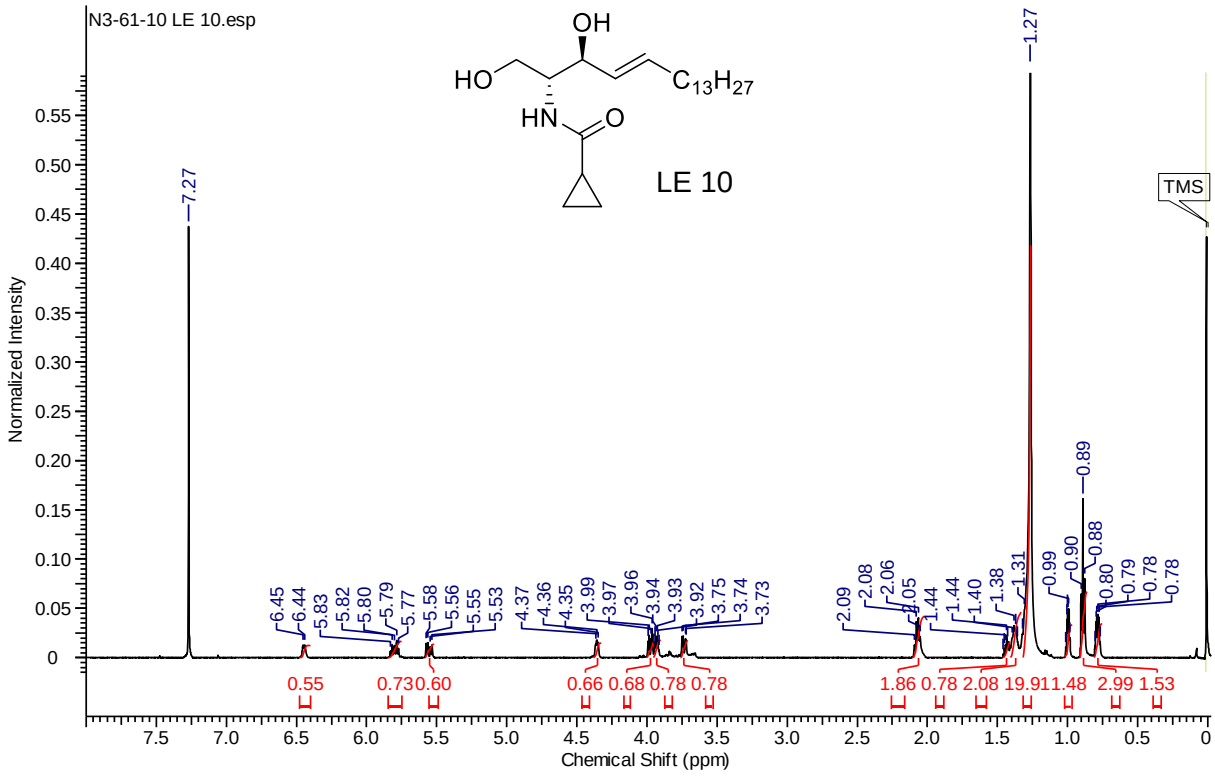
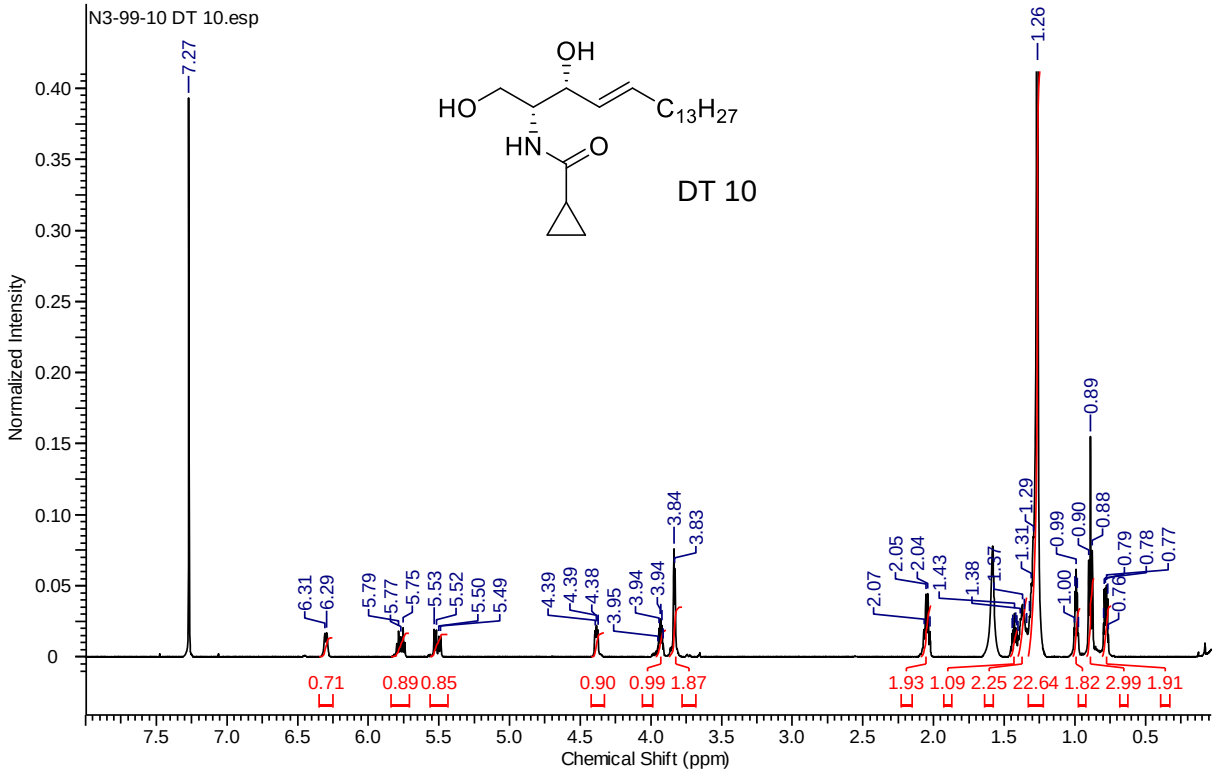
LT 10
TMS
Normalized Intensity
Chemical Shift (ppm)


N3-61-10 LE 10.esp
OH
HO
HN
O
$C_{13}H_{27}$
LE 10
TMS
Normalized Intensity
Chemical Shift (ppm)


N3-99-10 DT 10.esp
OH
HO
HN
O
$C_{13}H_{27}$
DT 10
Normalized Intensity
Chemical Shift (ppm)

## 3.6 References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# Chapter 4

## Construction of novel ceramide library towards discovery of selective and potent inhibitors for sphingomyelin synthase

## 4.1 Abstract

Sphingolipids are class of lipids containing a backbone of sphingoid base. Sphingomyelin synthase is an enzyme which catalyzes the conversion of ceramide and phosphatidylcholine to sphingomyelin and diacylglycerol. Sphingolipids, such as ceramide and sphingomyelin play an important role in signal transmission and cell recognition. Recently, ceramides have attracted intense research interests because of their functional roles as signaling molecules in many important physiological processes. Their well-defined modular structures are ideally agreeable to library formation for medicinal chemistry investigations. Sphingomyelin synthase has three isoforms SMS1, SMS2 and SMSr. SMS1 and SMSr control the ceramide homeostasis in Golgi apparatus and endoplasmic reticulum respectively. The membrane protein, SMS2 associated with metabolic disorder such as obesity, diabetes, atherosclerosis etc. To address the SMS biology in a diseased condition, a selective and potent SMS2 is required. Herein, we report the construction of novel ceramide library by using different sphingoid bases to investigate their inhibitory activities against sphingomyelin synthase2 (SMS2) using a cell-based assay. We have screened 95 ceramides so far and found two selective and potent inhibitors of SMS2 (DM-16 and DS-8) shows more than 100-fold selectivity. The library of these sort will be used for the development of therapeutic targets for various diseases.

## 4.2 Introduction

Sphingolipids and their metabolites play important roles as second messengers in cellular signal transduction and involved in many biological processes ranging from transcription of the genetic code to regulation of vital metabolic pathways and physiological responses. For example, ceramides are sphingosine-based signaling molecules that regulate cell cycle arrest, proliferation, differentiation, and apoptosis[1,2]. Sphingolipids are particularly abundant in nervous tissue and they tend to concentrate in the outer leaflet of the plasma membrane. Ceramide has long been recognized as a signaling molecule in the inflammatory response and it play a central role in sphingolipid metabolism[3]. It acts as a second messenger in the signal transduction pathway triggered by several agents of stress, including oxidative stress and ionizing radiation, and extracellular stimuli such as proinflammatory cytokines and lipopolysaccharide. Recently, ceramides have attracted intense research interests because of their functional roles as signaling molecules in many important physiological processes. Their well-defined modular structures are ideally agreeable to library formation for medicinal chemistry investigations. Understanding the role of ceramides in the sphingolipid metabolism is essential for the development therapeutic target for various diseases. In this study, we report the construction of novel ceramide library by solid phase synthesis and performed the cell-based screening of the library to discover the selective and potent inhibitors for sphingomyelin synthase. These results suggested that libraries of this sort will be a rich source of biologically active molecules.

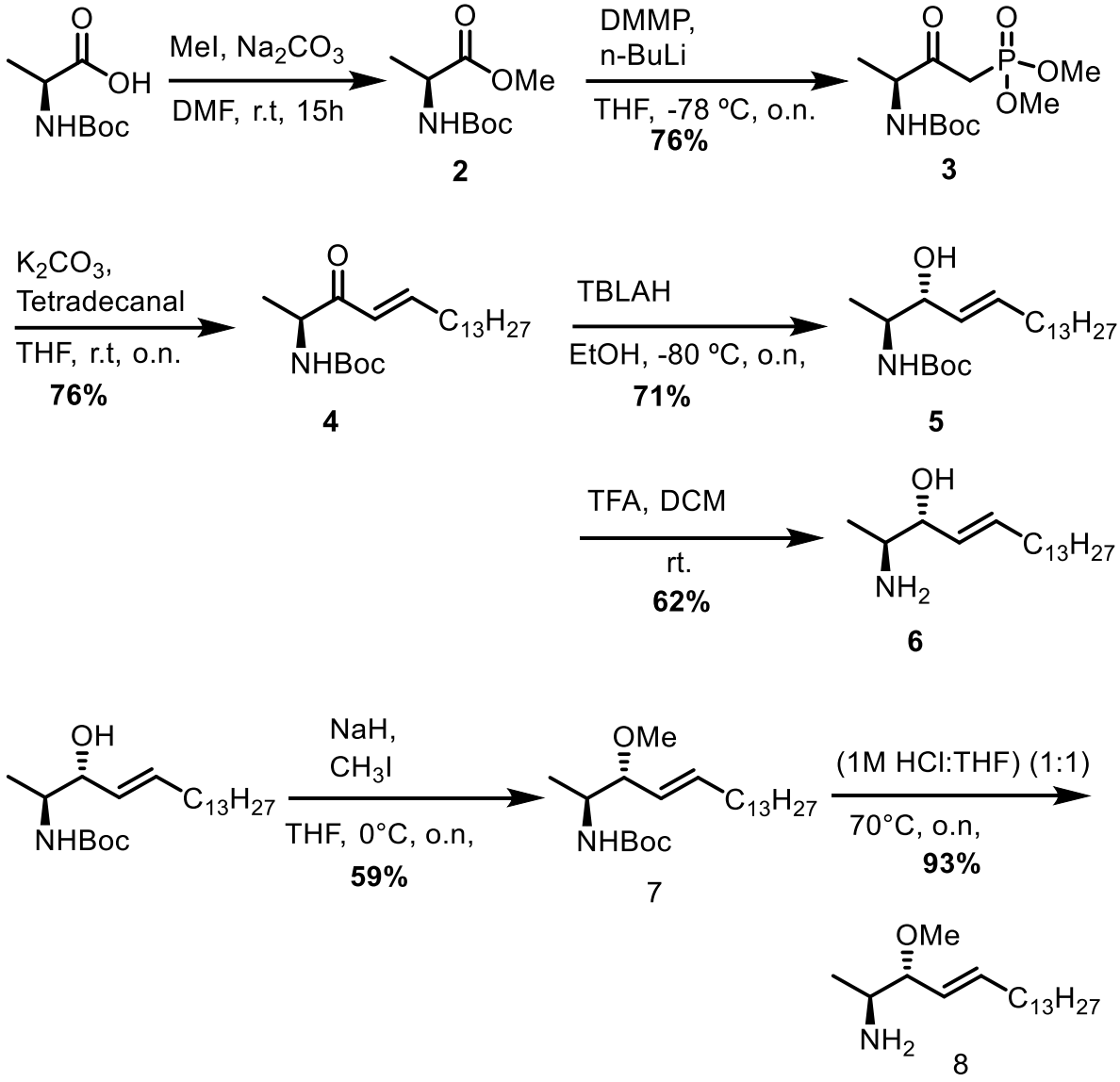
Sphingomyelin synthase is an enzyme that catalyze the formation of sphingomyelin from ceramide. It has three isoforms SMS1, SMS2 and SMSr. SMS1 and SMSr control the ceramide homeostasis in Golgi apparatus and endoplasmic

reticulum respectively[4]. The membrane protein, SMS2 associated with metabolic disorder such as obesity, diabetes, atherosclerosis etc[5,6]. To address the SMS biology in a diseased condition, a selective and potent SMS2 is required. Herein, we report the construction of novel ceramide library by using different sphingoid bases to investigate their inhibitory activities against sphingomyelin synthase2 (SMS2) using a cell-based assay and it could be a better therapeutic target for metabolic syndrome.

It is thus much desired to develop new small-molecule SMS inhibitors with higher potency and better stability. Such compounds may be developed into successful drug candidates or applied as effective chemical tools for probing the biological functions of SMS. In our study, we applied structure-based virtual screening to discover small-molecule compounds targeting at SMS.

## 4.3 Results and discussion

First, we started to synthesize different types of sphingoid bases as a core structure for the creation of ceramide library. Initially we synthesized 1-deoxy sphingosine by using Boc protected Alanine as a starting material. L-Boc Alanine was protected by using MeI to obtain compound 2 and followed by reacting with dimethyl methyl phosphonate to yield compound 3. Compound 3 was then proceeding with Horner-Wadsworth-Emmons reaction to produce compound 4, followed by stereoselective reduction of compound 4 to obtain the compound 5 and the deprotection of Boc group produced 1-deoxy sphingosine (6), and the hydroxyl group was protected to obtain compound 8 in good yield. (**Scheme 1**)




**Scheme 1**: Synthetic scheme of 1-deoxy sphingosine and 3-methoxy-1-deoxy sphingosine

Compound 6 and 8 obtained by synthetic route, used as a core structure to create the ceramide library and used another two types of sphingoid bases, such as phytosphingosine and psychosine. Phytosphingosine obtained from commercial source and psychosine was extracted from golden oyster mushroom[7].

In order to synthesize the ceramide library, the core structure was derivatized in solution with a series of solid phase acylating agents[8]. A library of 132 ceramides were synthesized by using 4 different types of sphingosine-like core structures and

33 acyl groups **(Figure 1**), followed by filtration to generate 132 ceramides without starting material sphingosine and byproducts (**Figure 2**). The products and their purity were characterized by measuring ESI-MS and $^{1}$H-NMR of randomly selected ceramides among the 132 ceramides.

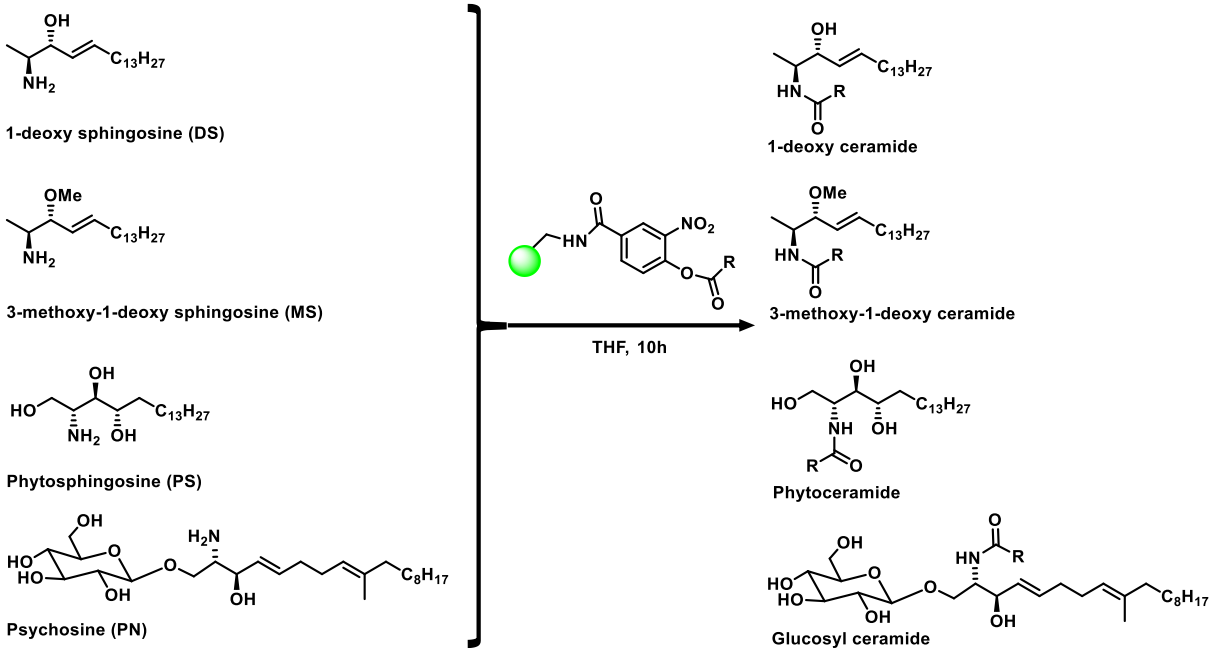



**Figure 2:** Synthesis of ceramide library by using 4 different types of sphingoid bases by acylating with activated ester resin.

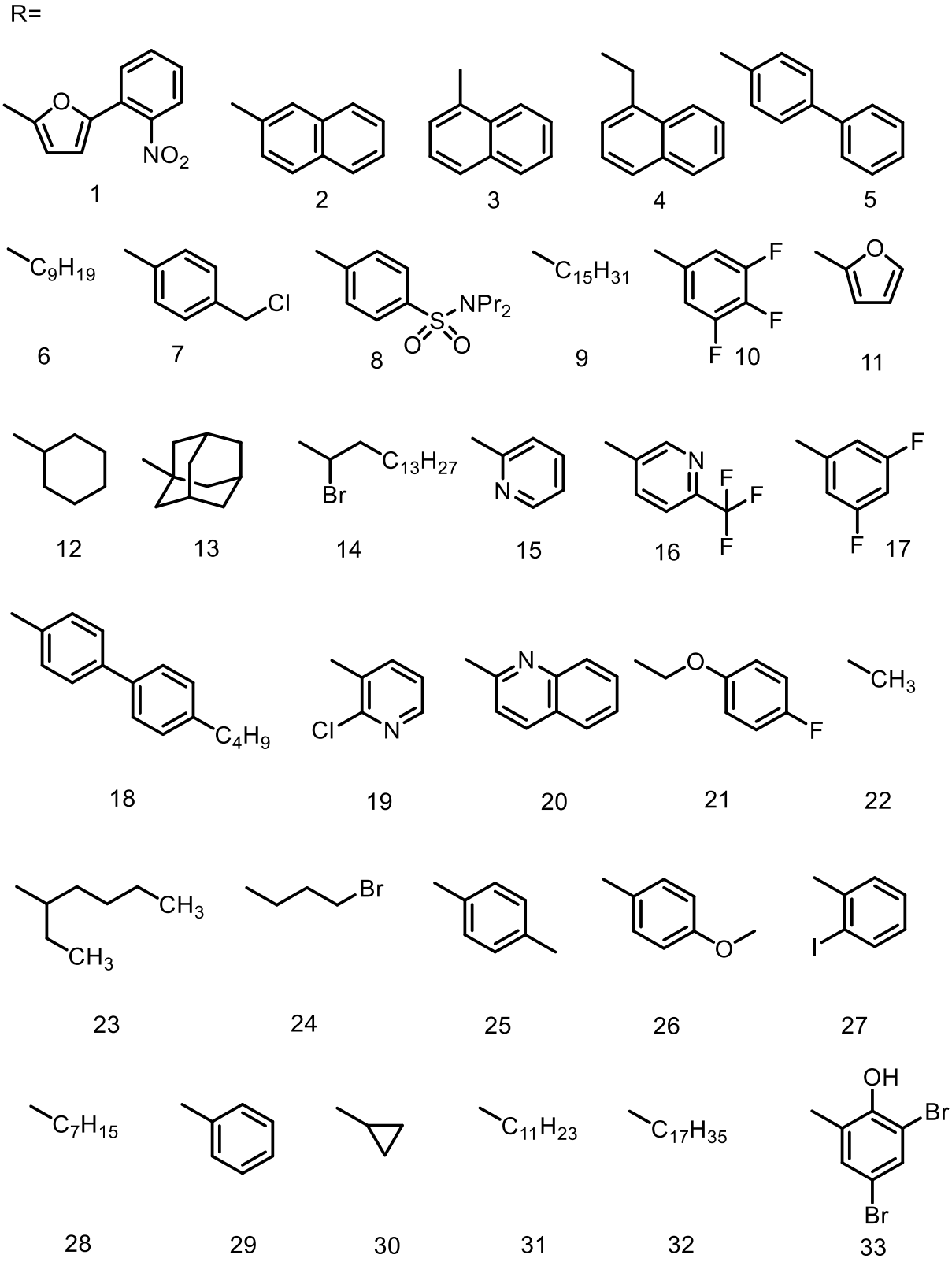



**Figure 1:** Different types of acyl chain used for the creation of ceramide library

In order to understand the biological importance of the ceramide library, initially performed the cell-based assay to discover the most selective and potent inhibitor for sphingomyelin synthase. We carried out cell-based assays for both isozymes SMS1 and SMS2 in the presence of the unique ceramides[9]. To find out the selective inhibitor for SMS, we screened 95 ceramides except glucosyl ceramides. As shown in Table 1, several ceramides showed relatively moderate inhibitory activities ($IC_{50}$ 0.3 ~ 2 μM, Table 1) compared with those of our previously reported natural compounds[10,11].

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| Entry (# of R) | SMS1 ($IC_{50}$) μM | | | SMS2 ($IC_{50}$) μM | | |
|---|---|---|---|---|---|---|
| | DS | MS | PS | DS | DM | PS |
| 1 | 10 | 100 | 1 | 1.5 | 3 | 0.3 |
| 2 | >100 | >100 | 15 | 15 | 80 | 7 |
| 3 | 30 | >100 | 40 | 20 | 100 | 50 |
| 4 | 70 | >100 | >100 | 10 | 30 | >100 |
| 5 | >100 | >100 | 40 | >100 | >100 | >100 |
| 6 | >100 | >100 | >100 | 30 | >100 | >100 |
| 7 | 80 | >100 | 10 | 2 | 10 | 3 |
| 8 | >100 | >100 | 3 | 2 | 10 | 2 |
| 9 | 50 | >100 | >100 | 20 | >100 | >100 |
| 10 | >100 | >100 | 60 | 20 | >100 | 20 |
| 11 | 20 | 30 | 40 | 10 | 20 | 5 |
| 12 | 40 | >100 | 20 | >100 | >100 | >100 |
| 13 | 100 | >100 | 80 | 40 | >100 | 30 |
| 14 | 30 | 40 | 70 | 2 | 70 | >100 |
| 15 | 9 | 50 | 20 | 2 | >100 | 6 |
| 16 | 90 | >100 | 10 | 20 | 0.8 | 3 |
| 17 | 100 | >100 | 40 | 2 | >100 | 20 |
| 18 | 100 | >100 | >100 | 20 | >100 | >100 |
| 19 | 20 | 40 | 20 | 20 | 30 | 40 |
| 20 | 20 | >100 | | 40 | >100 | |
| 21 | 50 | >100 | 40 | 5 | 4 | 4 |
| 22 | 40 | 100 | 10 | 10 | 20 | 25 |
| 23 | | >100 | >100 | | 50 | 8 |
| 24 | | 40 | | | 30 | |
| 25 | 60 | >100 | 30 | 20 | 15 | 4 |
| 26 | 60 | >100 | >100 | 20 | 5 | 5 |
| 27 | 40 | >100 | 40 | 20 | 100 | 20 |
| 28 | >100 | >100 | 5 | 10 | >100 | 5 |
| 29 | 40 | >100 | 30 | 15 | 50 | 15 |
| 30 | 30 | 100 | 5 | 7 | 40 | 15 |
| 31 | >100 | >100 | >100 | >100 | >100 | >100 |
| 32 | >100 | 100 | >100 | >100 | >100 | >100 |
| 33 | >100 | | 25 | >100 | | 15 |

**Table 1.** $IC_{50}$ values are the means of each of the ceramides in separate determinations for SMS1 or SMS2 expressed in SMS1/2 double knockout mouse fibroblast cell lysate and were determined by using more than four concentrations of each inhibitor.

## 4.4 Conclusion

We reported that the construction of novel ceramide library by using different sphingoid bases and variety of acyl chain. By using solid-phase synthesis, 132 ceramides were created to investigate their inhibitory activities against sphingomyelin synthase1 and 2 (SMS1 &2) using a cell-based assay. We have screened 95 ceramides so far and found two selective and potent inhibitors of SMS2 (DM-16 and DS-8) shows more than 100-fold selectivity. The ceramide library of these sort will be used for the development of novel drug candidates or application as an effective chemical tool for probing the biological functions of SMS. In the future, these ceramide libraries will be used for the identification of their biological functions in the sphingolipid metabolism.

## 4.5 Experimental procedure

### Synthesis of methyl (tert-butoxycarbonyl)-L-alaninate (2)

To a stirred solution of L-Boc-Alanine (500 mg, 2.64 mmol) and $Na_2CO_3$ (560 mg, 2.64 mmol) in DMF was added $CH_3I$ (700 µL, 10.56 mmol) drop wise to the reaction mixture. The reaction mixture was stirred for 20h at room temperature. Next the reaction mixture was filtered. The resulting filtrate were extracted by EtOAc and washed with brine. The combined organic layers were dried with MgSO4 before being concentrated under reduced pressure. This material was used in next step without further purification.

### Synthesis of tert-butyl (S)-(4-(dimethoxyphosphoryl)-3-oxobutan-2-yl) carbamate (3)

To a stirred solution of dimethyl methyl phosphonate (795 µL, 7.43 mmol) and THF, n-BuLi (700 µL, 7.43 mmol) was added drop wise at -78ºC. After 30 minutes stirring, compound 2 (503 mg, 2.48 mmol) in 10 mL THF was added to the reaction mixture dropwise and stirred for overnight at -78ºC. Then the reaction mixture was quenched with 10% citric acid. Next, the solvent was removed under reduced pressure and the resulting residue was taken up in ethyl acetate. The organic layer was separated, and the aqueous layer was further extracted with ethyl acetate. The combined organic layers were dried with MgSO4 before being concentrated under reduced pressure. Then the residue was chromatographed on silica gel (*n*-hexane/EtOAc = 1:1). Compound 3 (183 mg, 76%) was obtained. $^1$H NMR (501 MHz, DMSO-$d_6$) $\delta$ 7.76 (d, *J* = 7.09 Hz, 1H), 4.60 (t, *J* = 7.21 Hz, 1H), 4.15 (s, 3H), 4.18 (s, 3H), 3.74 - 3.91 (m, 2H), 1.85 - 1.94 (m, 9H), 1.68 (d, *J* = 7.34 Hz, 3H). ESI-MS(m/z): 295.14427 $[M]^+$

**Synthesis of tert-butyl (S, E) -(3-oxooctadec-4-en-2-yl) carbamate (4)**

To a stirred solution of Tetradecanal (3.7 g, 17.3 mmol), β-ketophosphonate (5.1 g, 17.3 mmol) and LiCl (2.2 g, 51.9 mmol) in THF, triethyl amine (7.2 mL, 51.9 mmol) was added dropwise at 0ºC. The reaction mixture was stirred for overnight at room temperature. The reaction mixture was acidified with citric acid solution and extracted with Et2O. The organic phase was washed with NaHCO3 and brine, and dried with MgSO4, and the solvents were evaporated. The crude product was purified by SiO2 column chromatography (*n*-hexane/ EtOAc, 10:1) to give **4** (5 g, 76 %). $^{1}$H NMR (501 MHz, CHLOROFORM-d) δ 6.94 - 7.03 (m, 1H), 6.16 (d, *J* = 15.65 Hz, 1H), 5.42 (d, *J* = 6.36 Hz, 1H), 4.56 (quin, *J* = 6.72 Hz, 1H), 2.22 (q, *J* = 7.09 Hz, 2H), 1.44 - 1.49 (m, 2H), 1.40 - 1.43 (m, 9H), 1.24 - 1.32 (m, 23H), 0.86 (t, *J* = 6.72 Hz, 3H). ESI-MS(m/z): 404.35467 $[M+Na]^{+}$

**Synthesis of tert-butyl ((2S,3R, E)-3-hydroxyoctadec-4-en-2-yl) carbamate (5)**

TBLAH (2.8 g, 11.13 mmol) in ethanol was cooled to -78ºC and compound 4 (1.7 g, 4.45 mmol) in 10 mL ethanol was added drop wise to the reaction mixture. Slowly raised the temperature of the reaction mixture to -30ºC and stirred for overnight. The reaction mixture was quenched with 1N HCl and the mixture was extracted with EtOAc. The organic layer was dried over $MgSO_4$ and the solvent was removed. The crude product was purified by SiO2 column chromatography (*n*-hexane/ EtOAc, 5:1) to give **5** (1.2 g, 71 %). $^{1}$H NMR (501 MHz, CHLOROFORM-d) δ 5.66 - 5.75 (m, 1H), 5.43 (dd, *J* = 6.60, 15.40 Hz, 1H), 4.71 (br. s., 1H), 4.11 (d, *J* = 3.42 Hz, 1H), 3.78 (br. s., 1H), 2.04 (q, *J* = 7.09 Hz, 2H), 1.45 (s, 9H), 1.32 - 1.40 (m, 2H), 1.07 (d, *J*=6.85 Hz, 3H), 1.26 (s, 20H), 0.88 (t, *J* = 6.60 Hz, 3H). $^{13}$C NMR (126 MHz,

CHLOROFORM-d) δ 156.1, 134.0, 128.4, 79.6, 51.1, 32.4, 31.9, 29.7, 29.7, 29.6, 29.6, 29.5, 29.3, 29.2, 28.4, 22.7, 15.5, 14.1. ESI-MS(m/z): 406.39793 $[M+Na]^+$

**Synthesis of 1-deoxy sphingosine (6)**

Compound 5 (3.2 g, 8.34 mmol) was dissolved in 10 mL dichloromethane and cooled to 0ºC. TFA (5 mL) was added to the reaction mixture slowly and stirred for 1h. TFA was removed by evaporation. The crude product was dissolved in DCM and adjusted the pH to 12 by using $NaHCO_3$. The mixture was extracted with DCM and wash with brine. The combined organic layer was dried over $MgSO_4$ and the solvent was removed. The crude product was purified by SiO2 column chromatography (CHCl3/MeOH/TEA, 90:5:2) to give **6** (1.4 g, 62 %). $^1$H NMR (501 MHz, METHANOL-$d_4$) δ 5.68 - 5.76 (m, 1H), 5.47 (dd, *J* = 7.09, 15.40 Hz, 1H), 3.83 (t, *J* = 6.11 Hz, 1H), 2.78 - 2.84 (m, 1H), 2.08 (q, *J* = 7.01 Hz, 2H), 1.41 (m, 2H), 1.29 (s, 20H), 1.05 (d, *J* = 6.60 Hz, 3H), 0.90 (t, *J* = 6.72 Hz, 3H). ESI-MS(m/z): 284.34998 $[M+H]^+$

**Synthesis of tert-butyl ((2S,3R, E)-3-methoxyoctadec-4-en-2-yl) carbamate (7)**

Compound 5 (556 mg, 1.45 mmol) was dissolved in THF and cooled to 0ºC, NaH (116 mg, 2.89 mmol) was added to the reaction mixture. The reaction mixture was allowed to stir for 30 minutes .$CH_3I$ (108 µL, 1.73 mmol) was added slowly and stirred for overnight at room temperature. The reaction mixture was quenched with 10% citric acid and the mixture was extracted with EtOAc. The organic layer was dried over $MgSO_4$ and the solvent was removed. The crude product was purified by SiO2 column chromatography (*n*-hexane/ EtOAc, 10:1) to give **7** (178 mg, 59 %). $^1$H NMR (501 MHz, CHLOROFORM-d) δ 5.64 - 5.72 (m, 1H), 5.27 (dd, *J* = 7.94, 15.52

Hz, 1H), 4.83 (br. s., 1H), 3.68 (br. s., 1H), 3.56 (br. s., 1H), 3.25 (s, 3H), 2.05 (q, *J* = 7.01 Hz, 2H), 1.44 (s, 9H), 1.33 - 1.41 (m, 2H), 1.22 - 1.33 (m, 20H), 1.08 (d, *J* = 6.85 Hz, 3H), 0.87 (t, *J* = 6.97 Hz, 3H). $^{13}$C NMR (126 MHz, CHLOROFORM-d) δ 155.3, 136.0, 126.9, 84.7, 75.5, 56.5, 32.3, 31.9, 29.7, 29.7, 29.6, 29.6, 29.6, 29.4, 29.4, 29.3, 29.1, 29.1, 28.4, 22.7, 14.1. ESI-MS(m/z): 420.43376 $[M+Na]^+$

**Synthesis of (2S,3R, E)-3-methoxyoctadec-4-en-2-amine (8)**

Compound 7 (178 mg, 0.45 mmol) was dissolved in (5mL THF: 5mL 1N HCl) and temperature was raised to 70ºC. The reaction mixture was stirred for overnight at 70ºC. The reaction mixture was cooled to room temperature and the pH was adjusted to 12. The mixture was extracted with EtOAc and wash with brine. The combined organic layer was dried over $MgSO_4$ and the solvent was removed. The crude product was purified by SiO2 column chromatography (CHCl3/MeOH/TEA, 93:2:5) to give **8** (133 mg, 93%). $^1$H NMR (501 MHz, CHLOROFORM-d) d 5.65 (td, *J* = 6.88, 15.34 Hz, 1H), 5.27 (dd, *J* = 8.31, 15.40 Hz, 1H), 3.26 (dd, *J* = 4.89, 8.31 Hz, 1H), 3.23 (s, 3H), 2.93 (dd, *J* = 5.01, 6.48 Hz, 1H), 2.06 (q, *J* = 7.09 Hz, 2H), 1.32 - 1.40 (m, 2H), 1.20 - 1.30 (m, 20H), 1.01 (d, *J* = 6.60 Hz, 3H), 0.85 (t, *J* = 6.85 Hz, 3H). $^{13}$C NMR (126 MHz, CHLOROFORM-d) δ 136.9, 126.5, 87.4, 56.1, 50.0, 32.4, 31.9, 30.8, 29.7, 29.6, 29.6, 29.6, 29.6, 29.4, 29.3, 29.2, 29.1, 22.6, 19.0, 14.1. ESI-MS(m/z): 298.33612 $[M+H]^+$

**General procedure for construction of chiral ceramide library**

Solid phase active ester resin (80 mg, 68 µmol), which was prepared by a slightly modified literature procedure in THF (1 mL) was added sphingosine (1 mg, 3.3 µm) and the mixture was agitated on a shaker for 10 h at room temperature. The reaction

mixture was filtered and washed with 1mL THF. The combined filtrate was evaporated and dried. The reaction was monitored by TLC and confirmed the completion of reaction by negative ninhydrin staining. The purity of the all ceramides confirmed by TLC profile, each gave a single spot. Further confirmed the identity of all ceramides by ESI-MS and randomly selected ceramides were confirmed by $^{1}$H NMR.

**N-((2S,3R,E)-3-hydroxyoctadec-4-en-2-yl)-2-naphthamide (DS-2)**

$^{1}$H NMR (501 MHz, CHLOROFORM-d) $\delta$ 8.28 (s, 1H), 7.85 - 7.94 (m, 3H), 7.81 - 7.84 (m, 1H), 7.51 - 7.60 (m, 2H), 6.51 (d, *J* = 7.82 Hz, 1H), 5.72 - 5.84 (m, 1H), 5.53 (dd, *J* = 6.36, 15.40 Hz, 1H), 4.34 - 4.43 (m, 1H), 4.31 (d, *J* = 6.11 Hz, 1H), 2.07 (q, *J* = 7.01 Hz, 2H), 1.34 - 1.41 (m, 2H), 1.21 - 1.33 (m, 23H), 0.89 (t, *J* = 6.85 Hz, 3H). ESI-MS(m/z): 438.30751 $[M+H]^{+}$

**N-((2S,3R,E)-3-hydroxyoctadec-4-en-2-yl)-1-naphthamide (DS-3)**

$^{1}$H NMR (501 MHz, CHLOROFORM-d) $\delta$ 8.33 (d, *J* = 8.31 Hz, 1H), 7.92 (d, *J* = 8.07 Hz, 1H), 7.87 (d, *J* = 8.07 Hz, 1H), 7.61 (d, *J* = 7.09 Hz, 1H), 7.51 - 7.58 (m, 2H), 7.46 (t, *J* = 7.70 Hz, 1H), 6.19 (d, *J* = 7.82 Hz, 1H), 5.77 - 5.86 (m, 1H), 5.54 (dd, *J* = 6.36, 15.40 Hz, 1H), 4.39 - 4.48 (m, 1H), 4.28 - 4.33 (m, 1H), 2.07 (q, *J* = 7.09 Hz, 2H), 1.34 - 1.40 (m, 2H), 1.24 - 1.33 (m, 23H), 0.89 (t, *J* = 6.72 Hz, 3H). ESI-MS(m/z): 460.28009 $[M+Na]^{+}$

**4-(N,N-dipropylsulfamoyl)-N-((2S,3R,E)-3-hydroxyoctadec-4-en-2-yl)benzamide (DS-8)**

$^{1}$H NMR (501 MHz, CHLOROFORM-d) $\delta$ 7.84 - 7.90 (m, 4H), 6.45 (d, *J* = 8.07 Hz, 1H), 5.76 - 5.83 (m, 1H), 5.51 (dd, *J* = 6.36, 15.40 Hz, 1H), 4.27 - 4.34 (m, 2H), 3.07

- 3.12 (m, 4H), 2.07 (q, *J* = 7.01 Hz, 2H), 1.51 - 1.58 (m, 4H), 1.34 - 1.42 (m, 2H), 1.21 - 1.33 (m, 23H), 0.85 - 0.91 (m, 9H). ESI-MS(m/z): 574.15217 $[M+Na]^+$

**4-(chloromethyl)-N-((2S,3R,E)-3-hydroxyoctadec-4-en-2-yl)benzamide (DS-7)**

$^1$H NMR (501 MHz, CHLOROFORM-d) $\delta$ 7.77 (d, *J* = 8.07 Hz, 2H), 7.46 (d, *J* = 8.31 Hz, 2H), 6.99 (s, 1H), 6.37 (d, *J* = 8.07 Hz, 1H), 5.78 (m, 1H), 5.50 (dd, *J* = 6.36, 15.40 Hz, 1H), 4.62 (s, 2H), 4.29 - 4.35 (m, 1H), 4.27 (dd, *J* = 2.69, 6.36 Hz, 1H), 2.02 - 2.11 (m, 2H), 1.34 - 1.41 (m, 2H), 1.22 - 1.33 (m, 23H), 0.87 - 0.92 (m, 3H). ESI-MS(m/z): 458.87605 $[M+Na]^+$

**N-((2S,3R,E)-3-methoxyoctadec-4-en-2-yl)stearamide (DM-32)**

$^1$H NMR (501 MHz, CHLOROFORM-d) $\delta$ 5.68 - 5.77 (m, 2H), 5.29 (dd, *J* = 7.82, 15.40 Hz, 1H), 4.02 - 4.09 (m, 1H), 3.58 (dd, *J* = 3.18, 7.58 Hz, 1H), 3.27 (s, 3H), 2.16 (t, *J* = 7.58 Hz, 2H), 2.07 (q, *J* = 6.68 Hz, 2H), 1.62 (m, 2H), 1.35 - 1.42 (m, 2H), 1.21 - 1.35 (m, 48H), 1.09 (d, *J* = 6.85 Hz, 3H), 0.89 (t, *J* = 6.97 Hz, 6H). ESI-MS(m/z): 586.68944 $[M+Na]^+$

**N-((2S,3R,E)-3-methoxyoctadec-4-en-2-yl)-[1,1'-biphenyl]-4-carboxamide (DM-5)**

$^1$H NMR (501 MHz, CHLOROFORM-d) $\delta$ 7.83 - 7.87 (m, *J* = 8.56 Hz, 2H), 7.65 - 7.69 (m, *J* = 8.56 Hz, 2H), 7.60 - 7.64 (m, 2H), 7.48 (t, *J* = 7.58 Hz, 2H), 7.38 - 7.42 (m, 1H), 6.52 (d, *J* = 8.80 Hz, 1H), 5.74 - 5.82 (m, 1H), 5.37 (dd, *J* = 7.82, 15.65 Hz, 1H), 4.26 - 4.33 (m, 1H), 3.73 (dd, *J* = 3.42, 7.82 Hz, 1H), 3.32 (s, 3H), 2.10 (q, *J* = 7.17 Hz, 2H), 1.36 - 1.43 (m, 2H), 1.23 - 1.34 (m, 23H), 0.87 - 0.91 (m, 3H). ESI-MS(m/z): 500.55631 $[M+Na]^+$

**N-((2S,3R,E)-3-methoxyoctadec-4-en-2-yl)palmitamide (DM-10)**

$^1$H NMR (501 MHz, CHLOROFORM-d) δ 5.67 - 5.76 (m, 2H), 5.29 (dd, *J* = 7.82, 15.40 Hz, 1H), 4.05 (m, 1H), 3.58 (dd, *J* = 3.42, 7.82 Hz, 1H), 3.27 (s, 3H), 2.13 - 2.19 (m, 2H), 2.07 (q, *J* = 6.85 Hz, 2H), 1.58 - 1.67 (m, 2H), 1.35 - 1.42 (m, 2H), 1.17 - 1.35 (m, 46H), 1.09 (d, *J* = 6.85 Hz, 3H), 0.89 (t, *J* = 6.97 Hz, 6H). ESI-MS(m/z): 558.67792 $[M+Na]^+$

**N-((2R,3R,4S)-1,3,4-trihydroxyoctadecan-2-yl) cyclopropanecarboxamide (PS-30)**

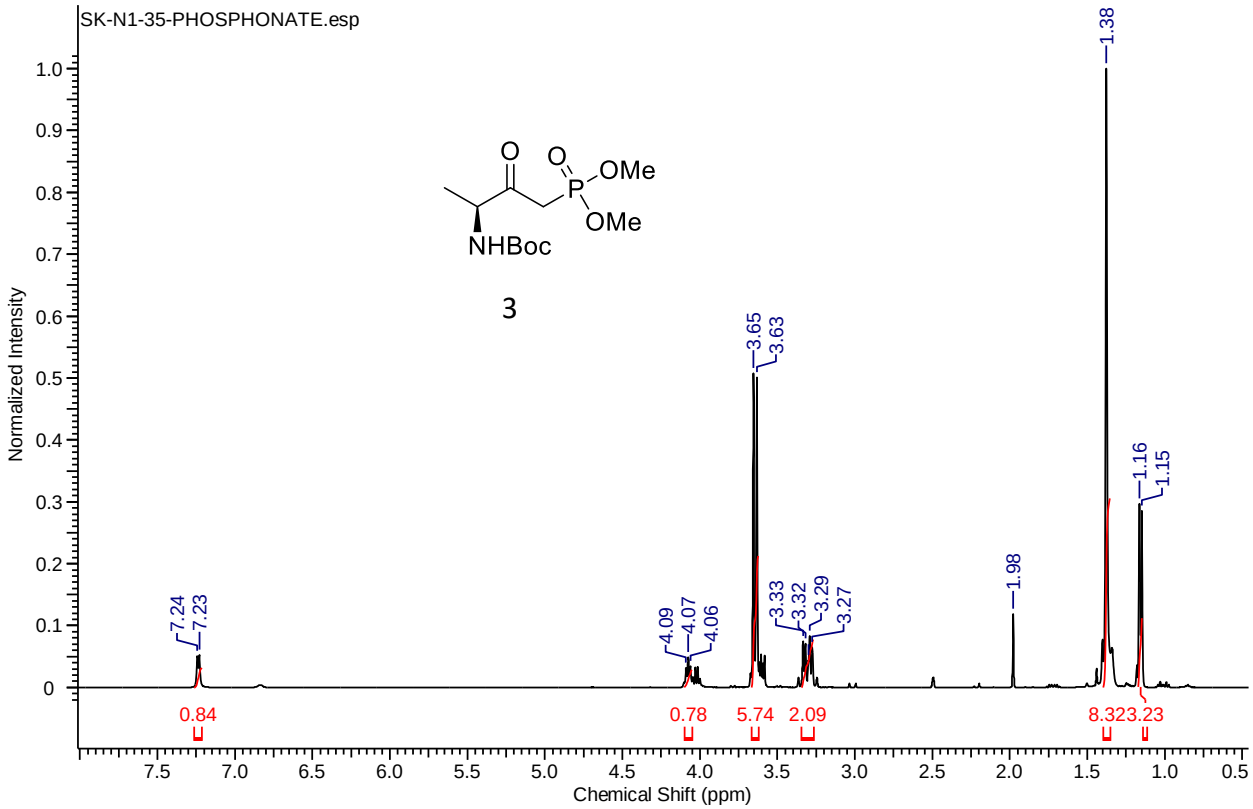
$^1$H NMR (501 MHz, CHLOROFORM-d) δ 6.50 (d, *J* = 6.85 Hz, 1H), 4.12 - 4.18 (m, 1H), 3.95 (d, *J* = 11.00 Hz, 1H), 3.73 - 3.86 (m, 2H), 3.56 - 3.68 (m, 2H), 1.41 - 1.53 (m, 3H), 1.23 - 1.40 (m, 24H), 1.00 (dd, *J* = 3.54, 7.46 Hz, 2H), 0.87 - 0.92 (m, 3H), 0.77 - 0.82 (m, 2H). ESI-MS(m/z): 386.38234 $[M+H]^+$

**2-iodo-N-((2R,3R,4S)-1,3,4-trihydroxyoctadecan-2-yl) benzamide (PS-27)**

$^1$H NMR (501 MHz, CHLOROFORM-d) δ 7.88 (dd, *J* = 0.73, 8.07 Hz, 1H), 7.37 - 7.45 (m, 2H), 7.11 - 7.15 (m, 1H), 6.64 (d, *J* = 7.82 Hz, 1H), 4.38 (m, 1H), 4.09 (dd, *J* = 2.69, 11.49 Hz, 1H), 3.94 (dd, *J* = 5.38, 11.49 Hz, 1H), 3.76 - 3.80 (m, 1H), 1.77 - 1.90 (m, 1H), 1.50 - 1.60 (m, 2H), 1.22 - 1.34 (m, 24H), 0.87 - 0.92 (m, 3H). ESI-MS(m/z): 548.23968 $[M+H]^+$

**N-((2R,3R,4S)-1,3,4-trihydroxyoctadecan-2-yl) furan-2-carboxamide (PS-11)**

$^1$H NMR (501 MHz, CHLOROFORM-d) δ 7.46 - 7.49 (m, 1H), 7.16 (d, *J* = 7.58 Hz, 1H), 7.15 (d, *J* = 3.67 Hz, 1H), 6.53 (dd, *J* = 1.71, 3.42 Hz, 1H), 4.29 - 4.34 (m, 1H), 4.04 (dd, *J* = 2.69, 11.49 Hz, 1H), 3.87 (dd, *J* = 5.50, 11.61 Hz, 1H), 3.72 (d, *J* = 2.69 Hz, 1H), 1.75 - 1.84 (m, 1H), 1.48 - 1.54 (m, 2H), 1.25 - 1.39 (m, 24H), 0.89 (t, *J* = 6.97 Hz, 3H). ESI-MS(m/z): 434.41271 $[M+Na]^+$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OMe
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4.07
4.06
3.65
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3.33
3.32
3.29
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1.16
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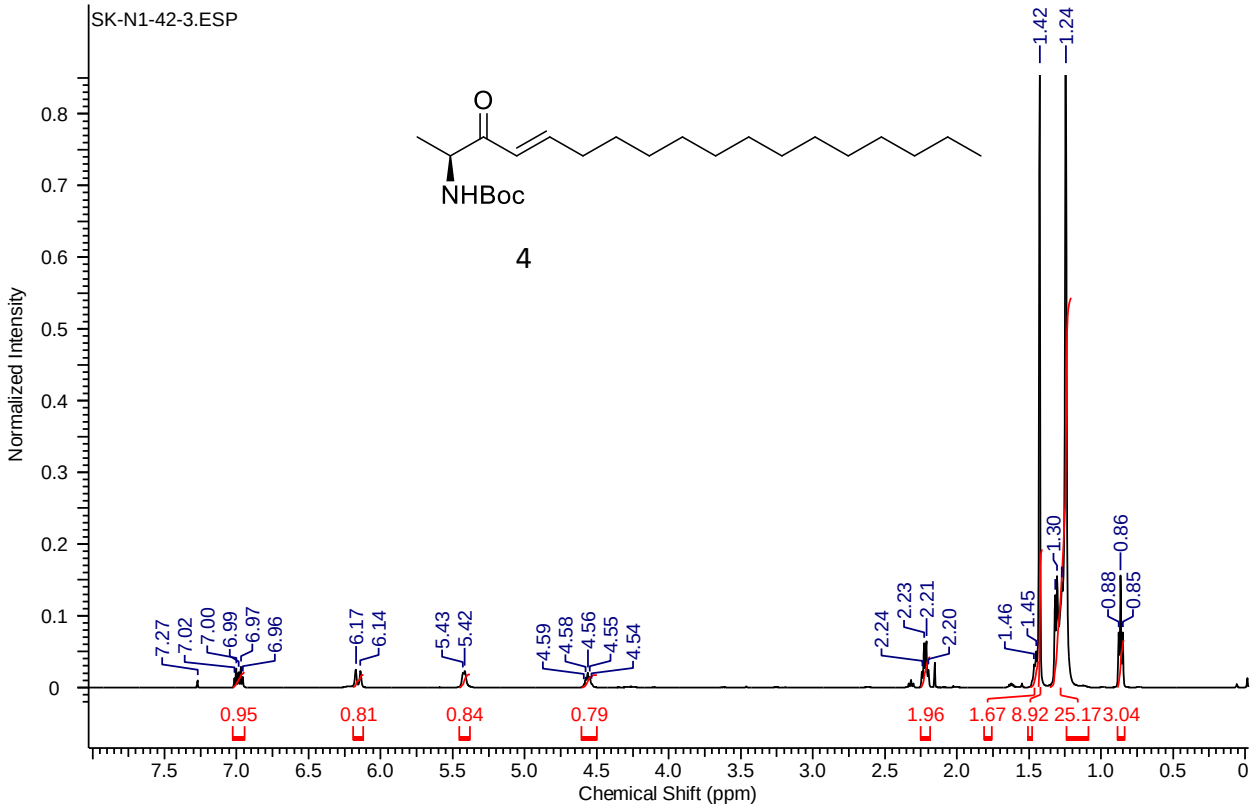
3.23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5.43
5.42
4.59
4.58
4.56
4.55
4.54
2.24
2.23
2.21
2.20
1.46
1.45
1.42
1.24
1.30
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0.84
0.79
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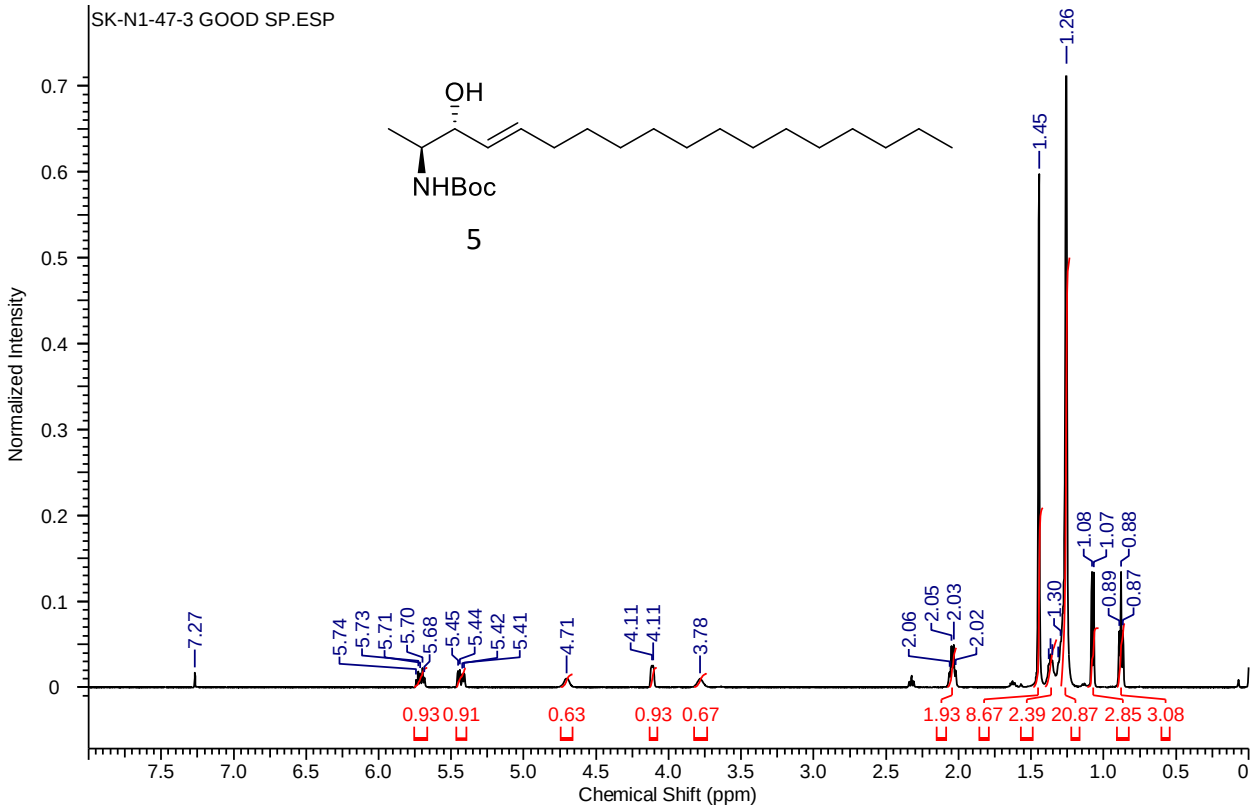
8.92
25.17
3.04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5.68
5.45
5.44
5.42
5.41
4.71
4.11
4.11
3.78
2.06
2.05
2.03
2.02
1.45
1.26
1.30
1.08
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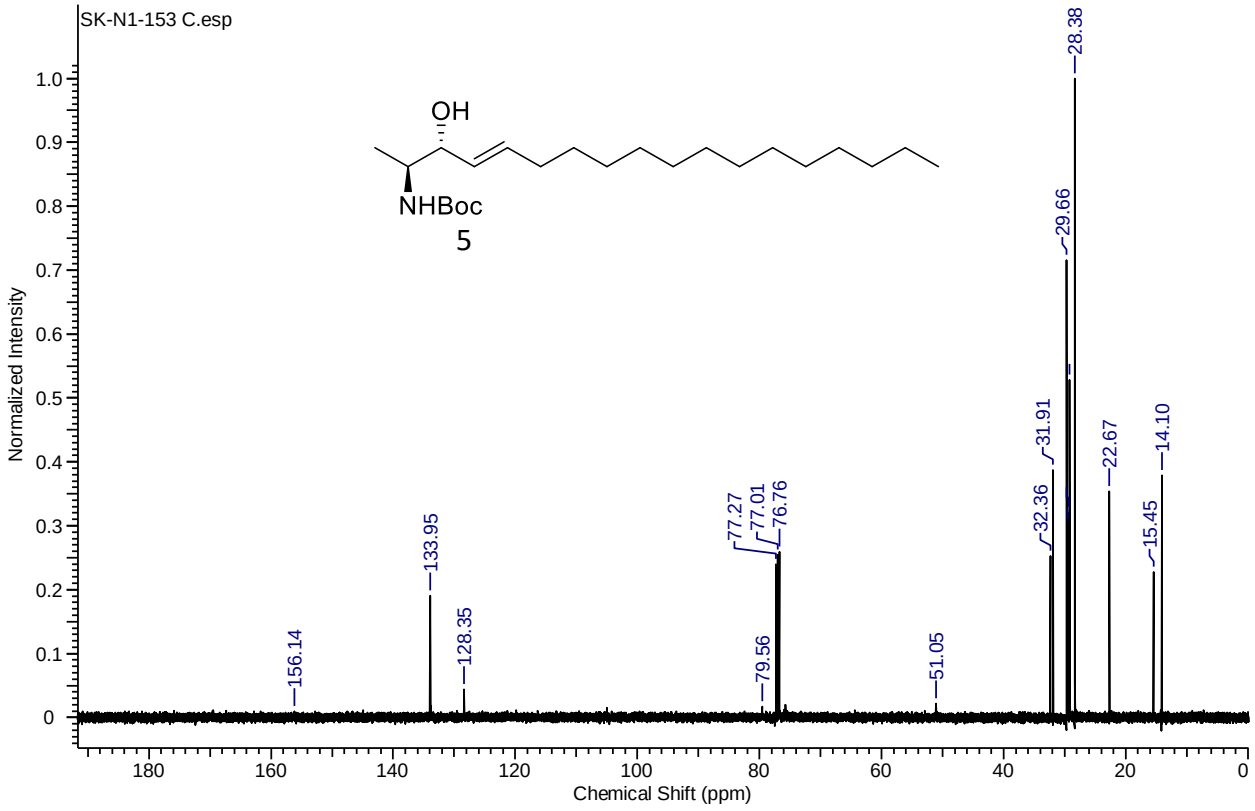
0.87
0.93 0.91 0.63 0.93 0.67
1.93 8.67 2.39 20.87 2.85 3.08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Chemical Shift (ppm)
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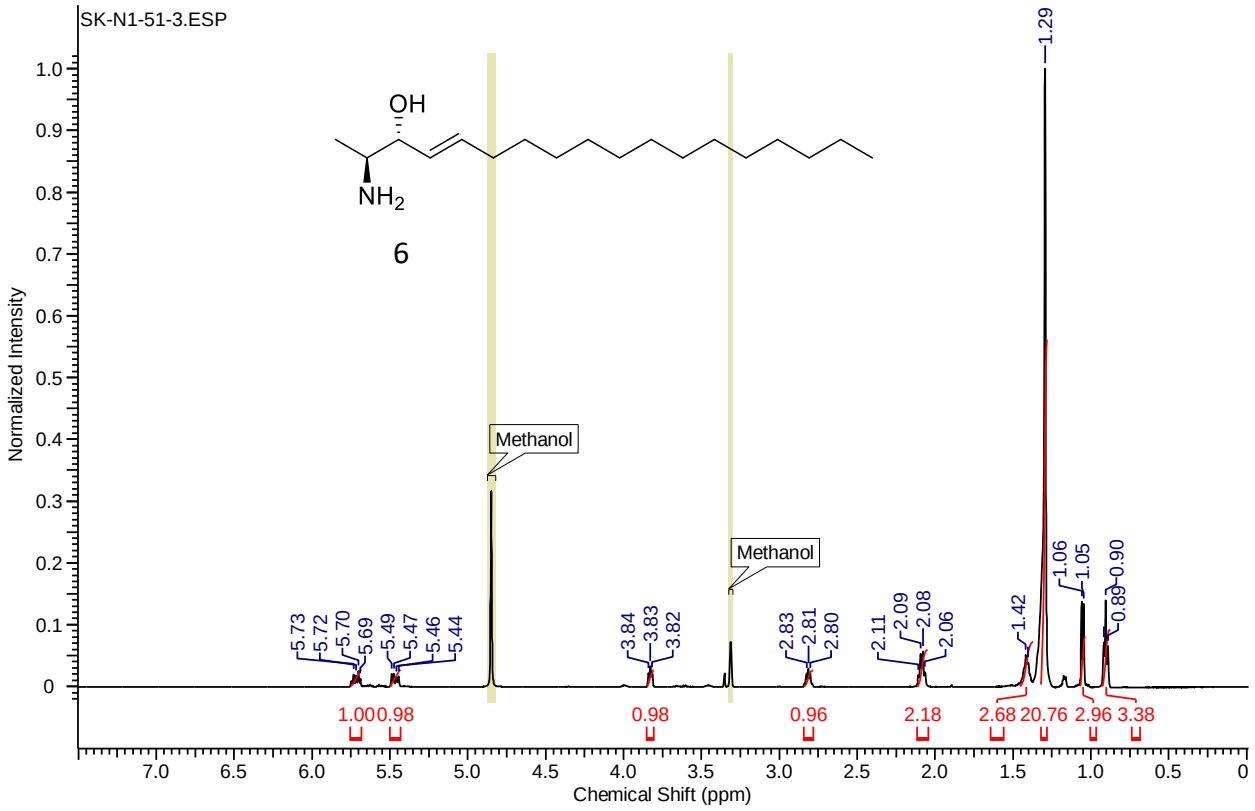
32.36
22.67
15.45
14.10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5.47
5.46
5.44
3.84
3.83
3.82
2.83
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2.80
2.11
2.09
2.08
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0.98
0.96
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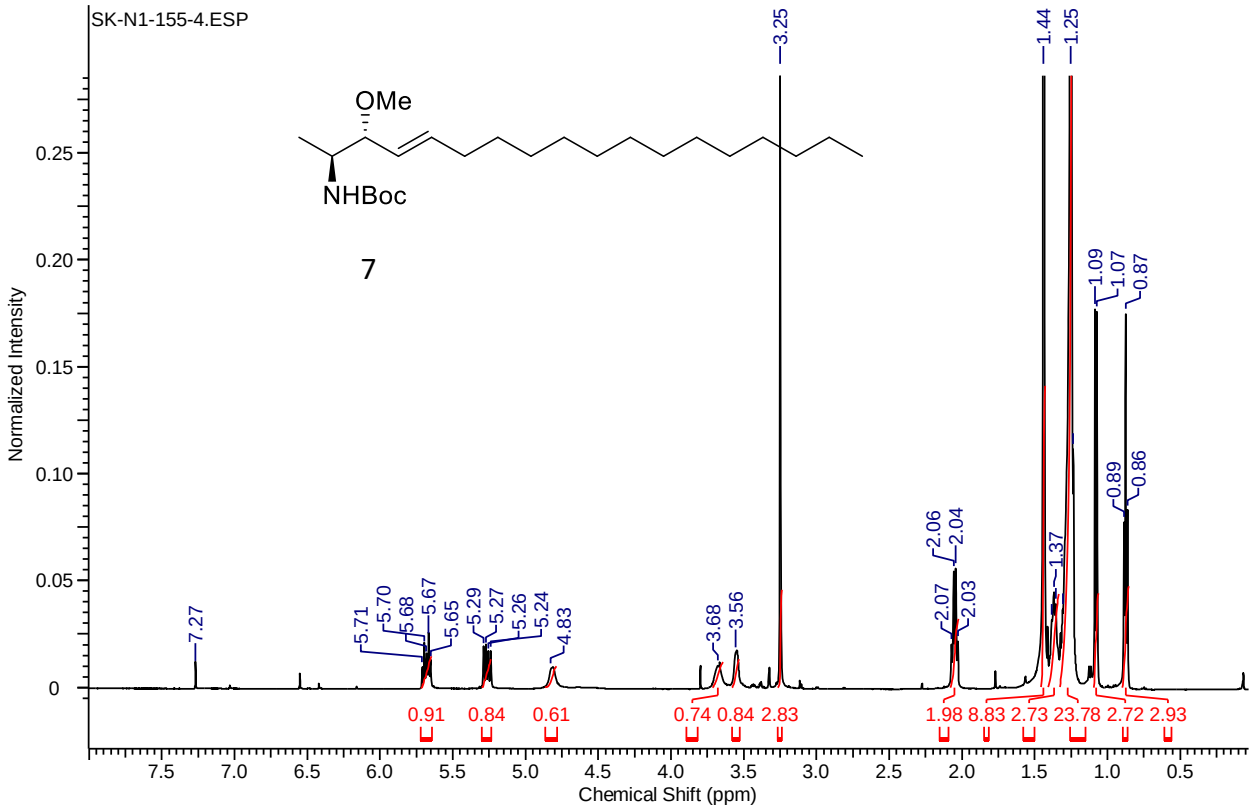
Normalized Intensity
Chemical Shift (ppm)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2.06
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2.07
2.03
7.27
5.71
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5.27
5.26
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4.83
3.68
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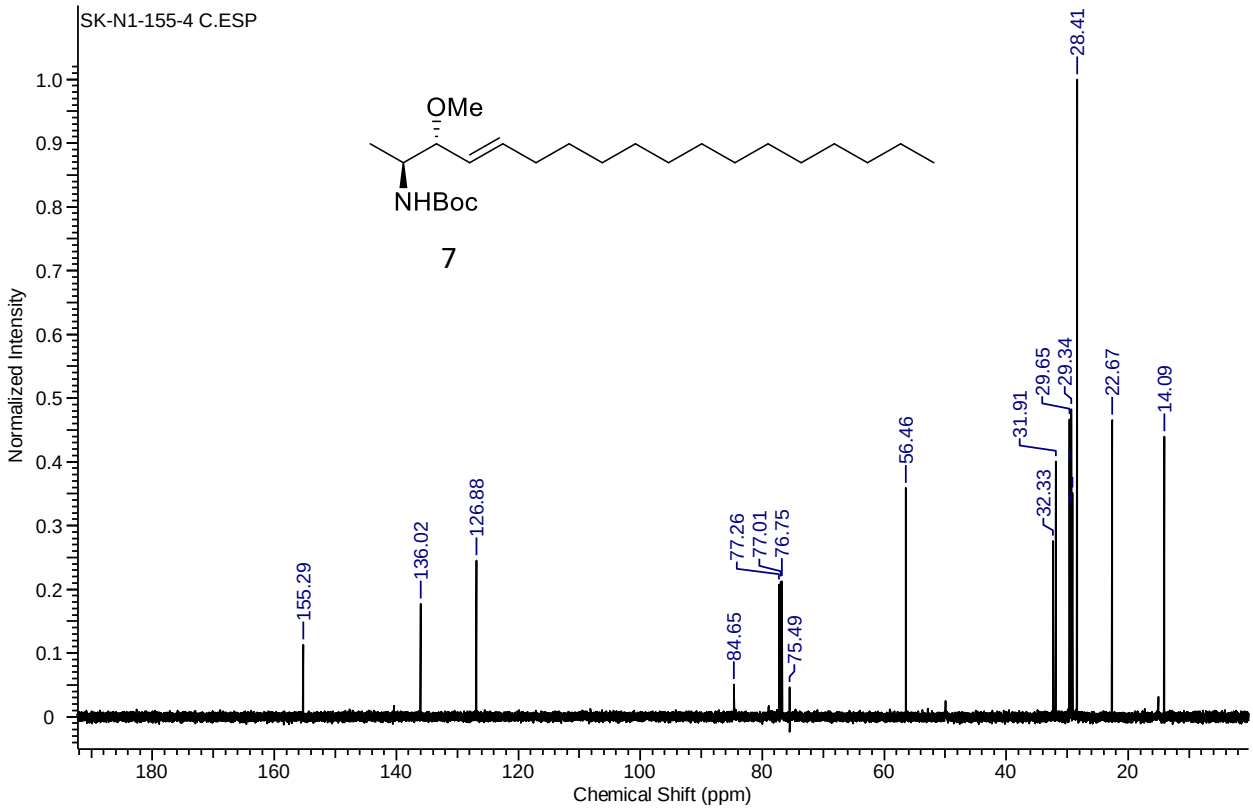
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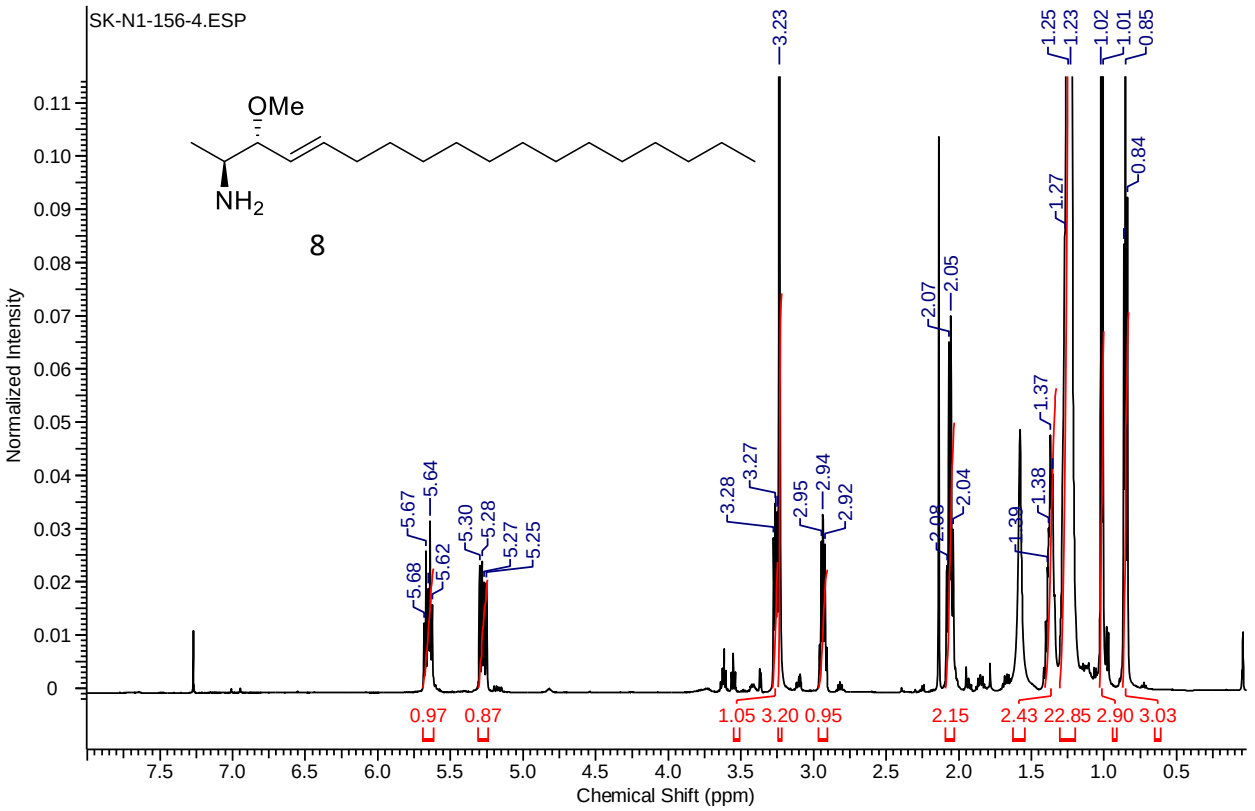
75.49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Normalized Intensity
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3.27
3.23
2.95
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2.07
2.05
2.08
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1.25
1.23
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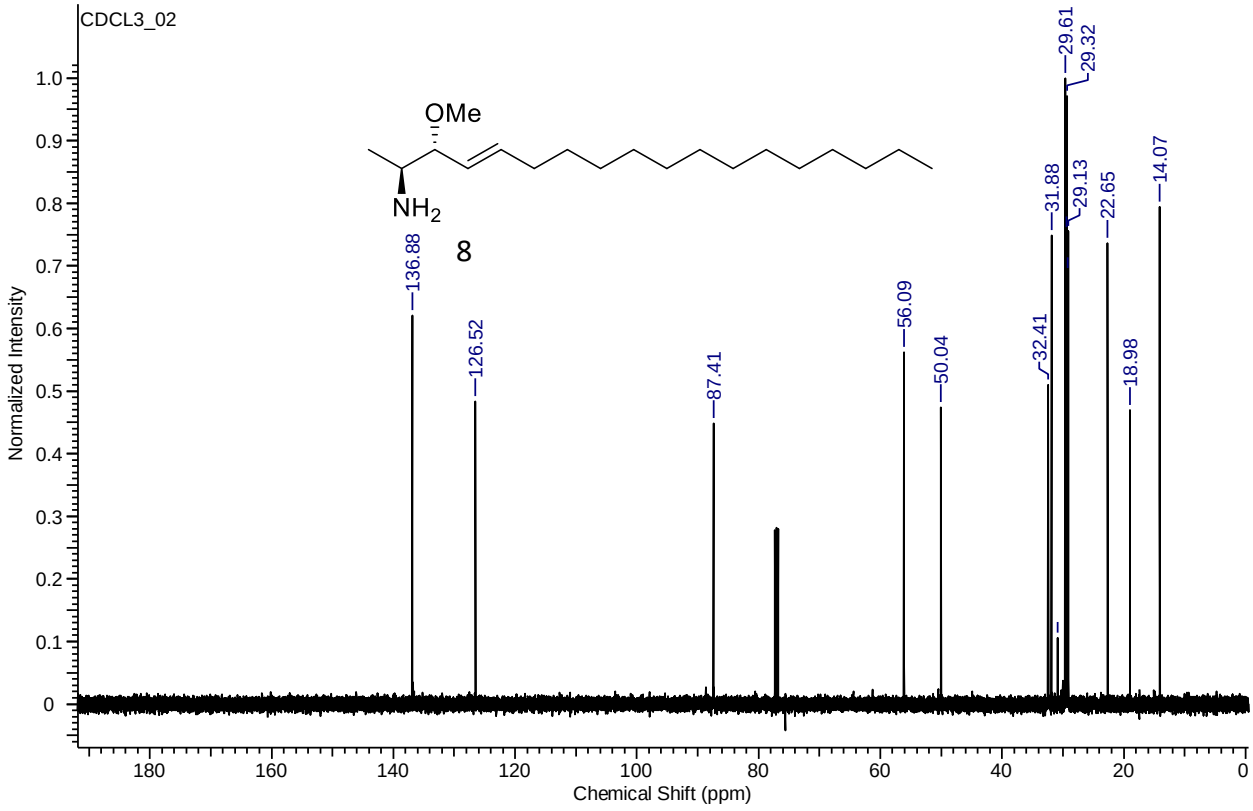
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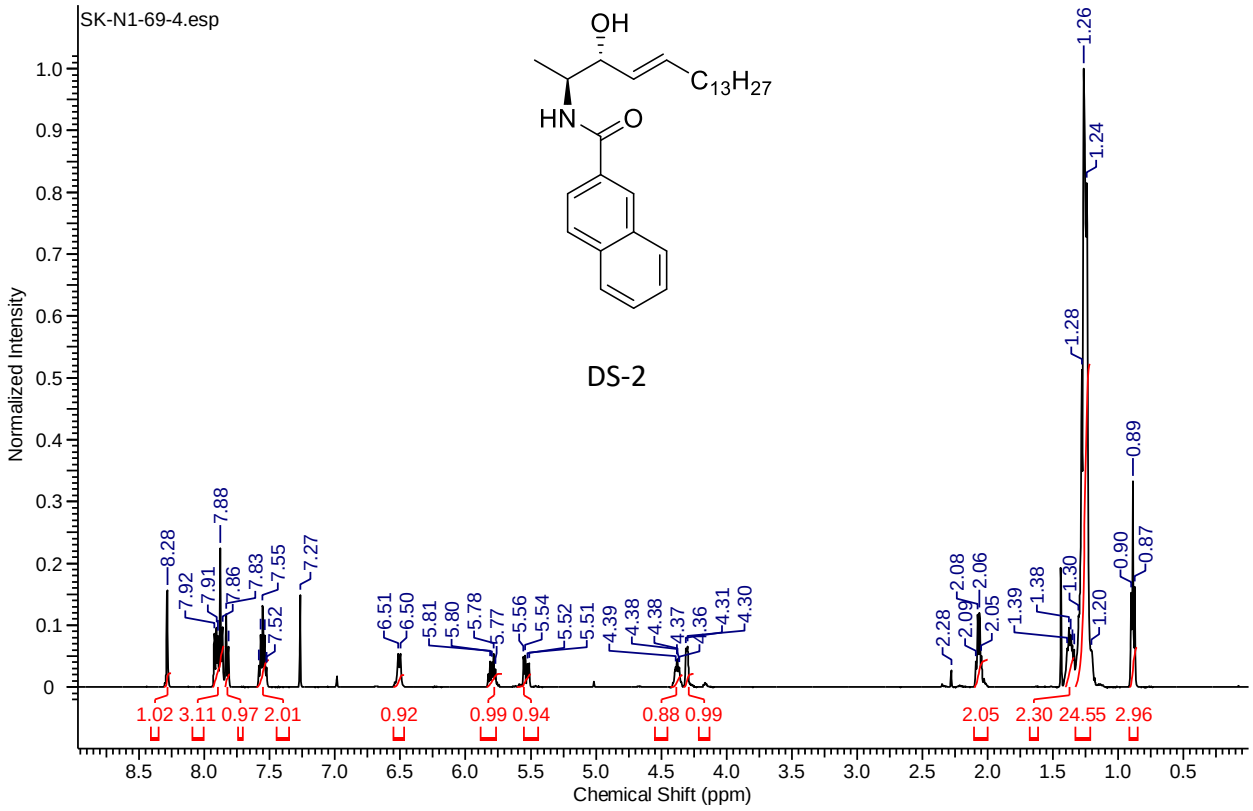
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2.05
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1.30
1.28
1.26
1.24
1.20
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0.88 0.99
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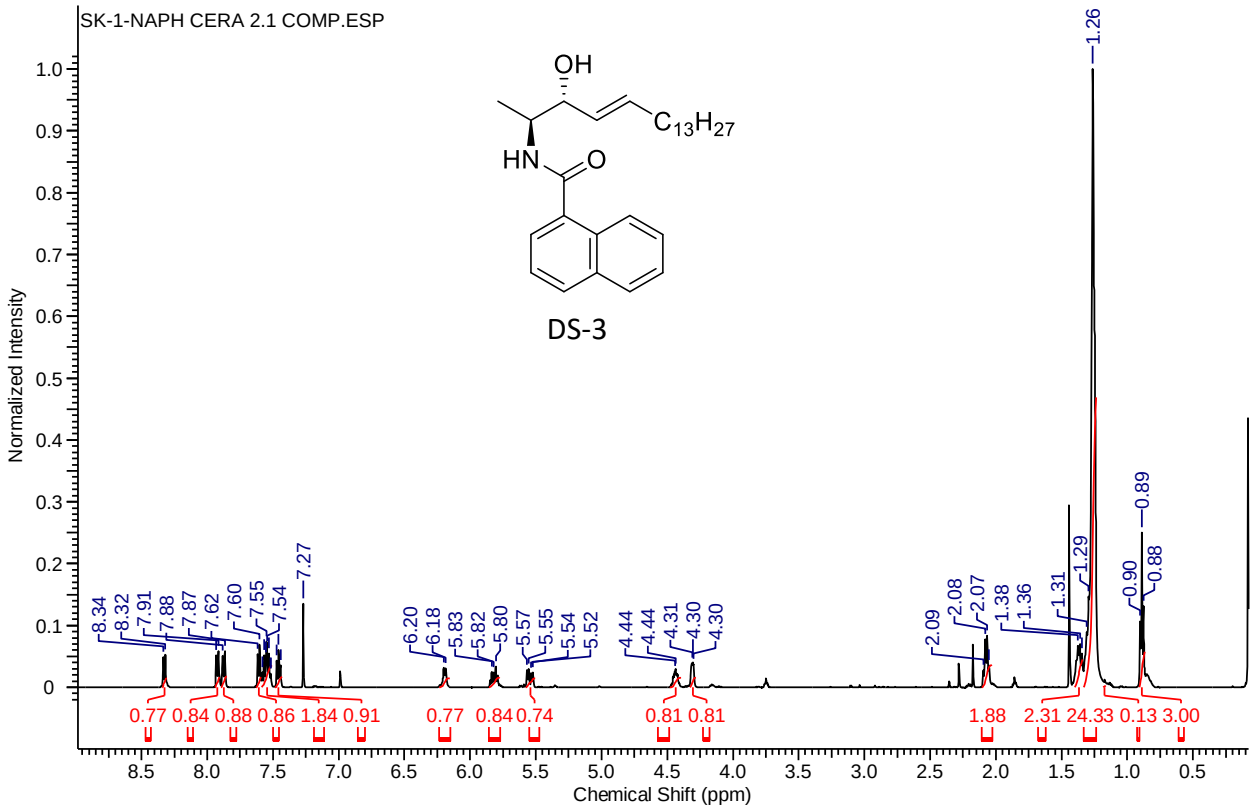
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HN
O
$C_{13}H_{27}$
DS-3
Normalized Intensity
Chemical Shift (ppm)
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7.87
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5.55
5.54
5.52
4.44
4.44
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2.07
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1.36
1.31
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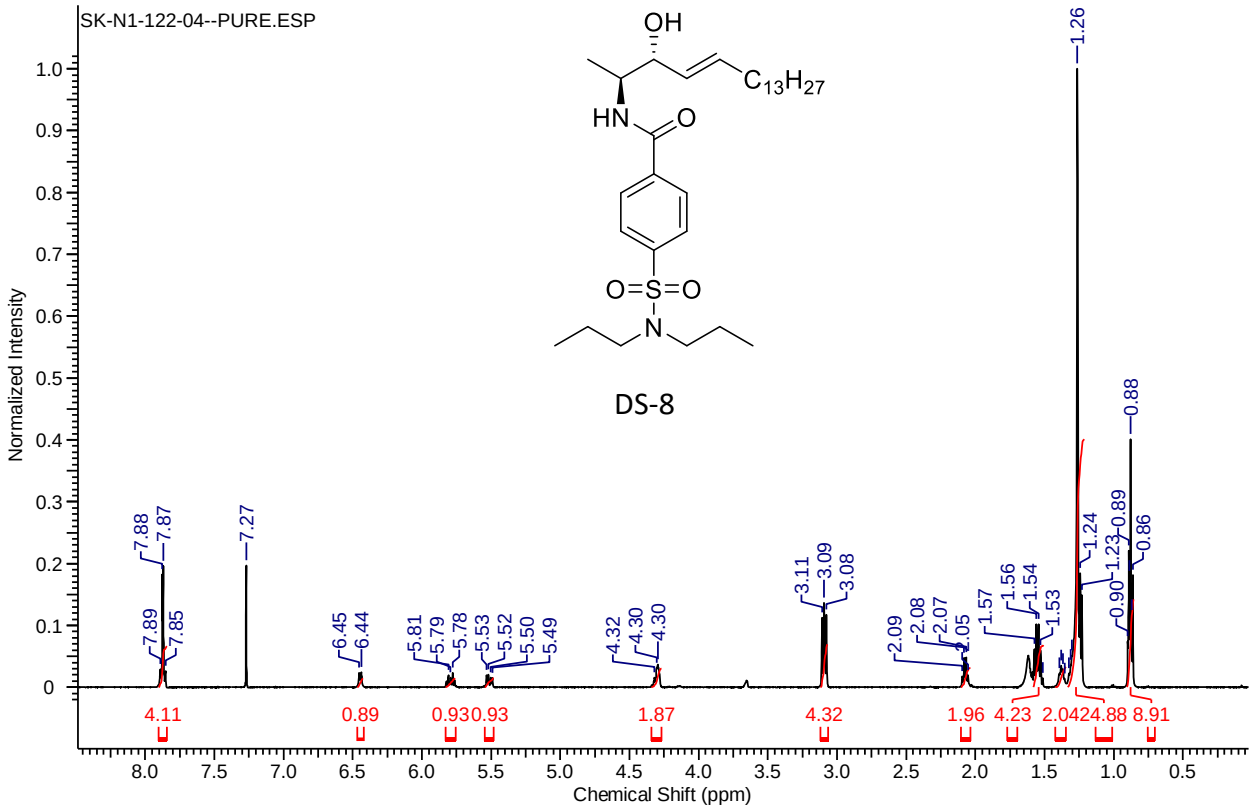
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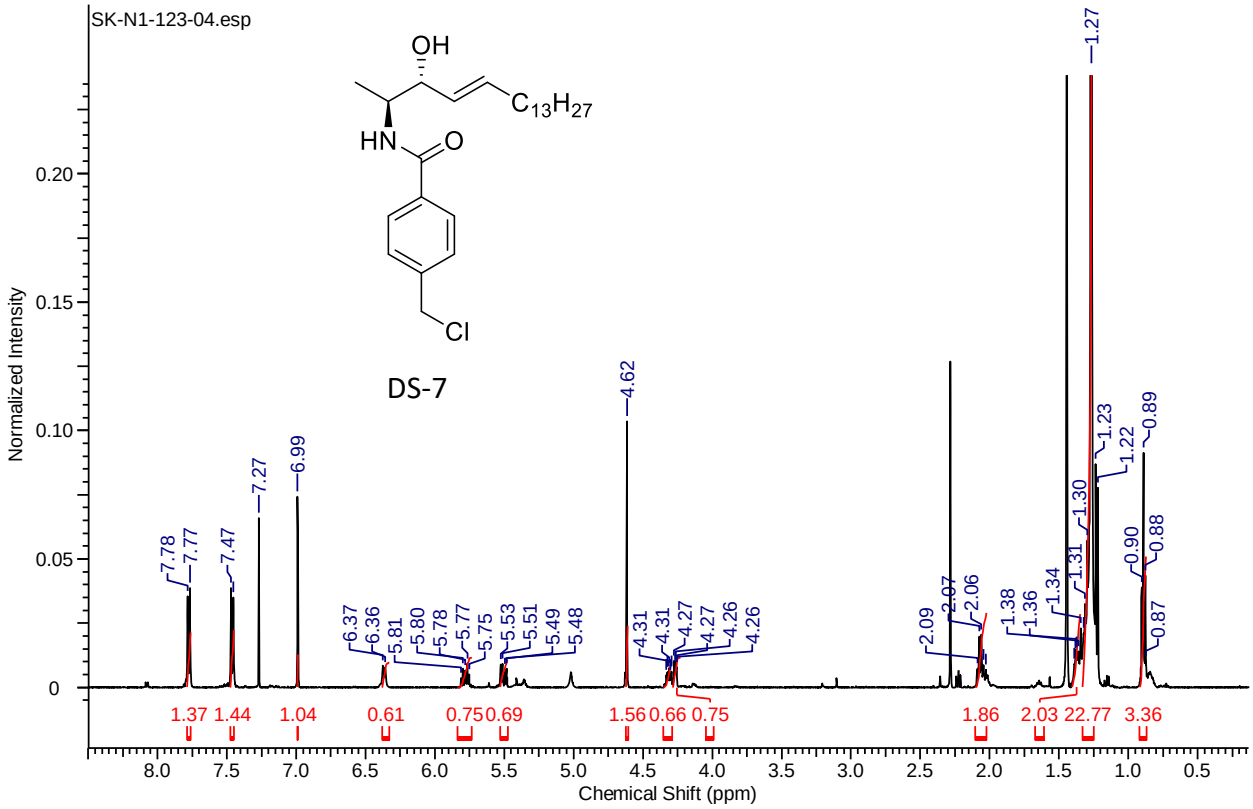
DS-8
Normalized Intensity
Chemical Shift (ppm)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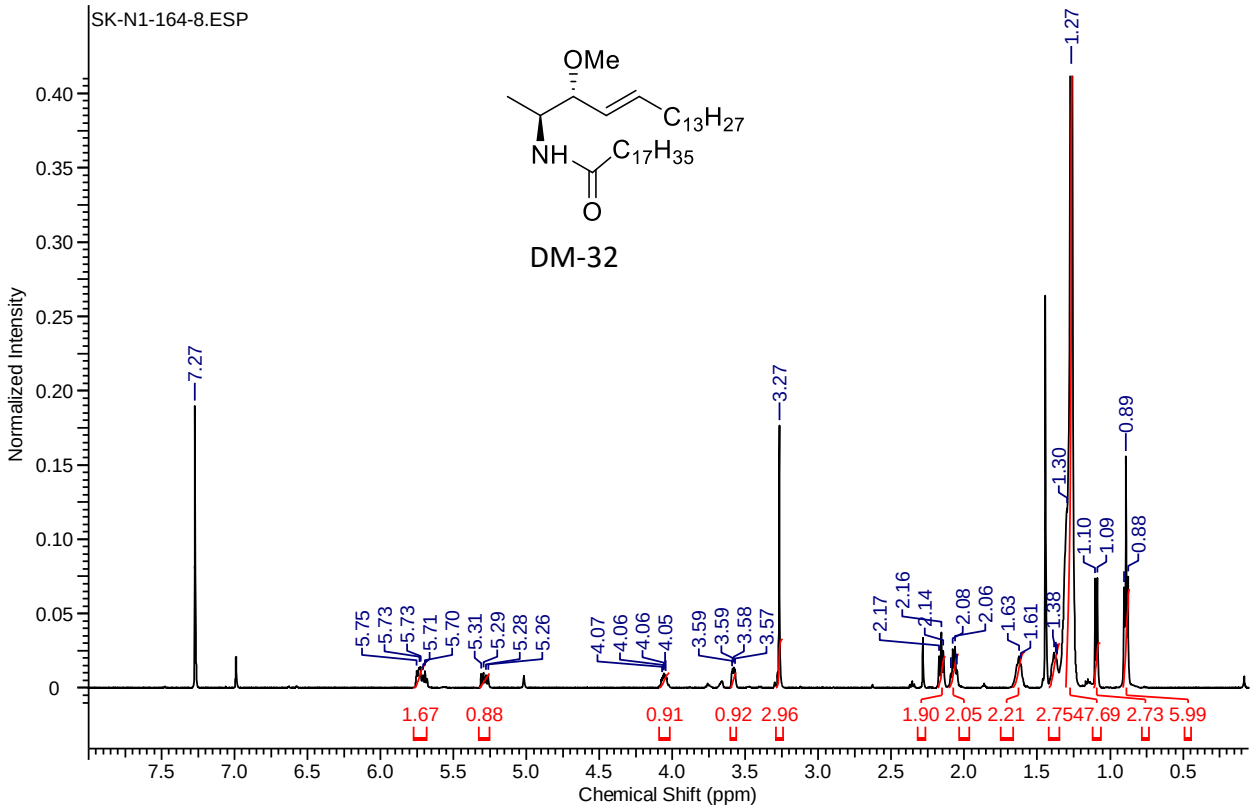
Normalized Intensity
Chemical Shift (ppm)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$C_{17}H_{35}$
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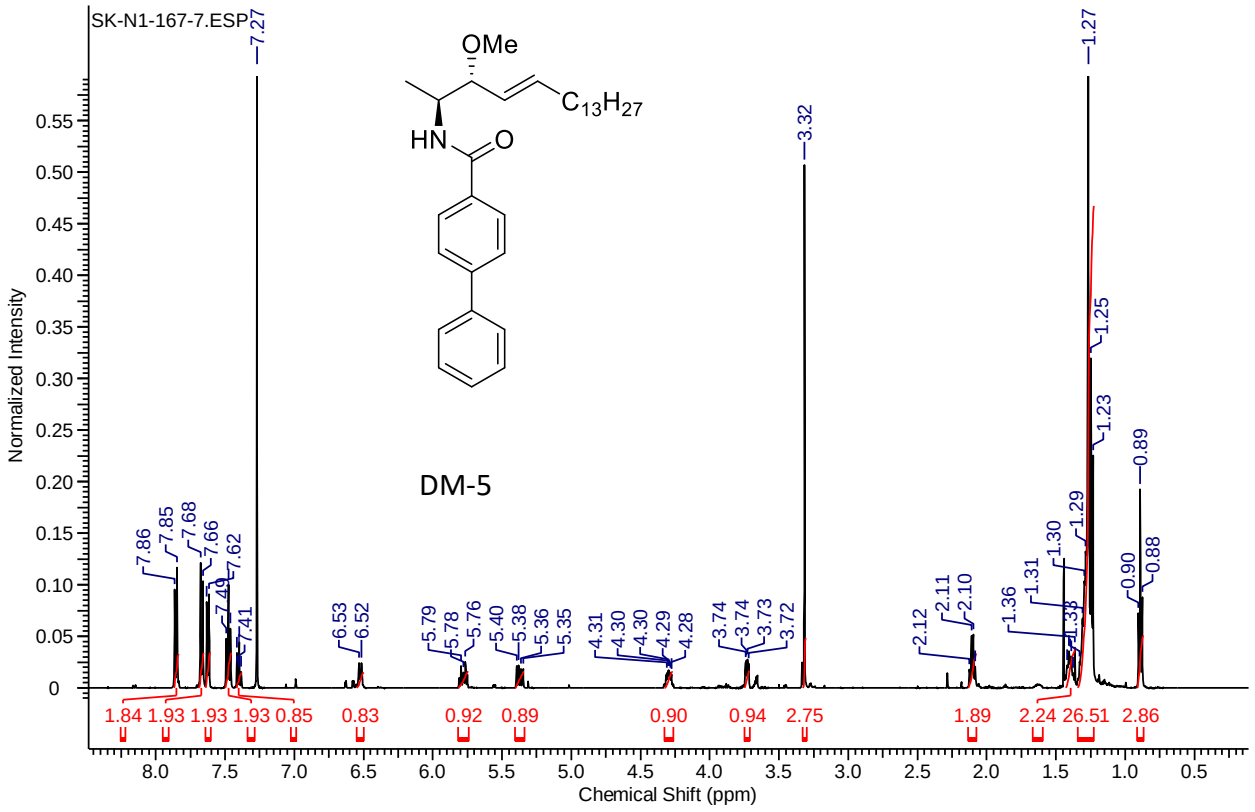
DM-32
Normalized Intensity
Chemical Shift (ppm)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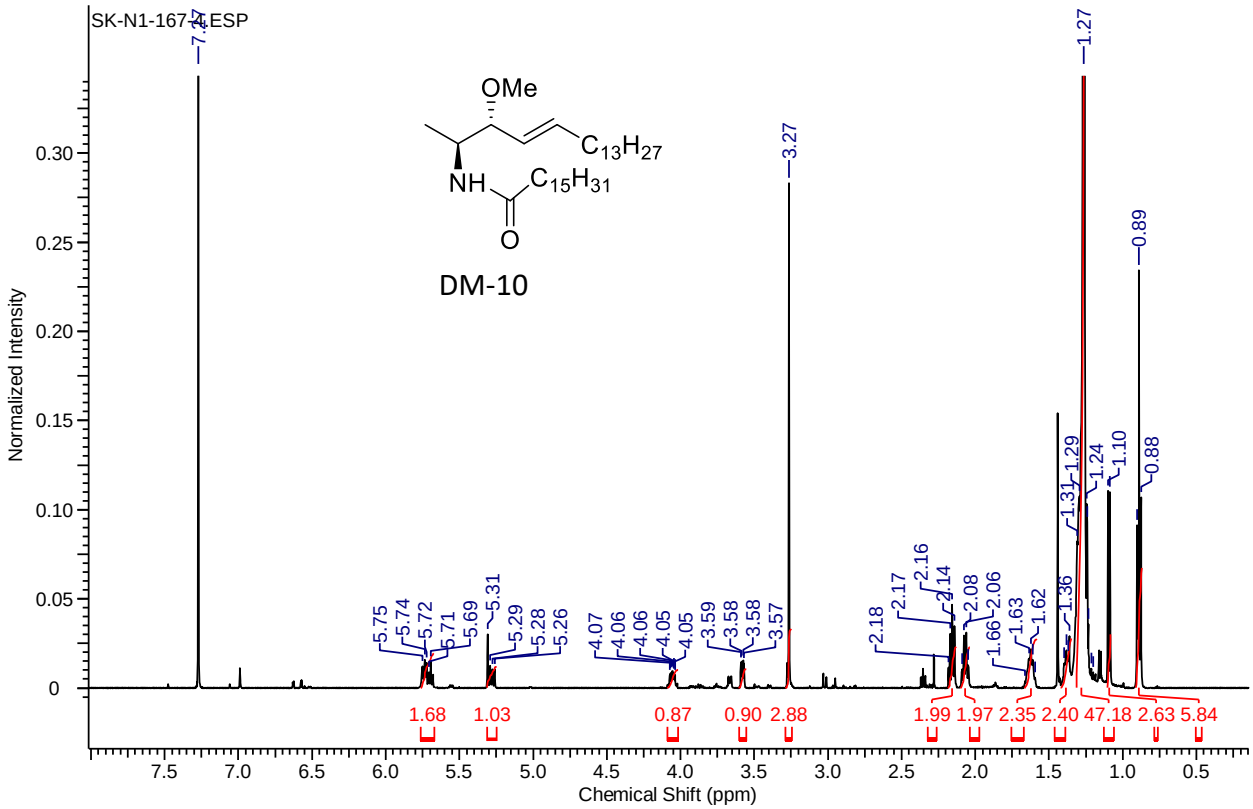
O
DM-5
Normalized Intensity
Chemical Shift (ppm)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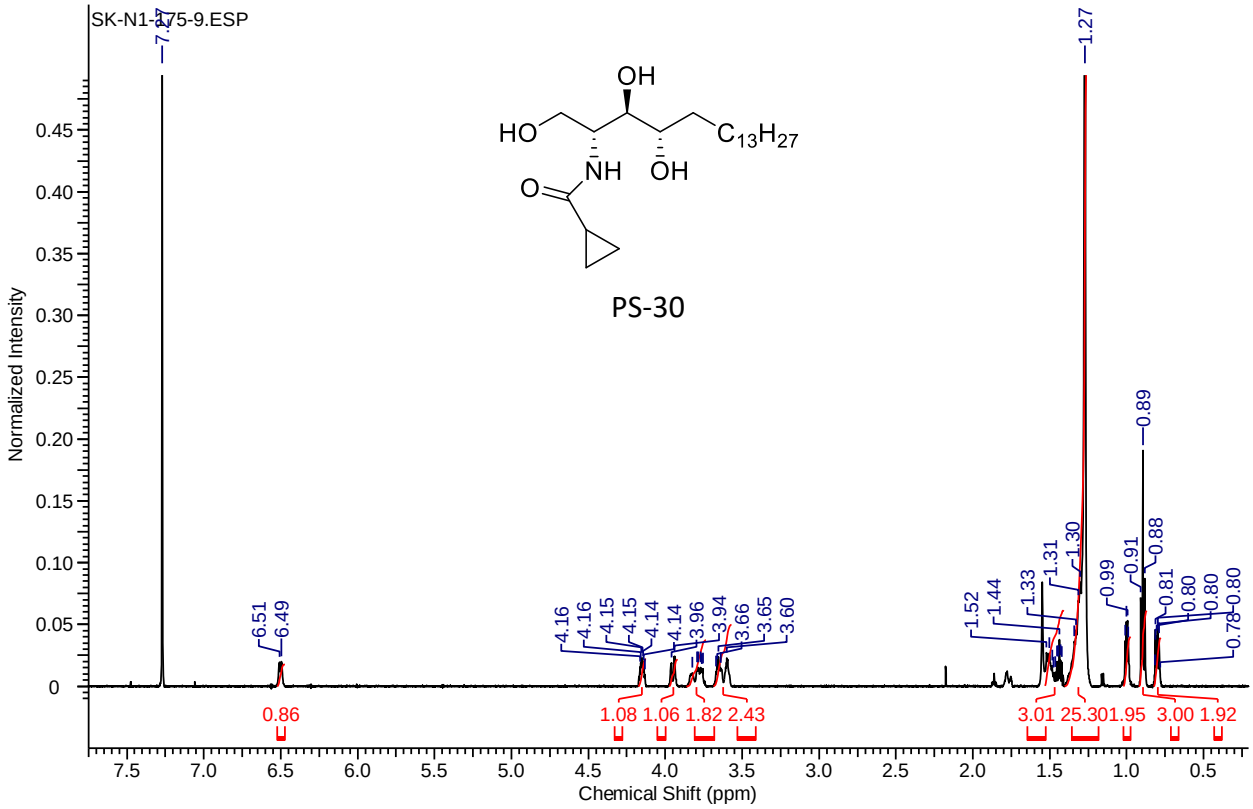
O
DM-10
Normalized Intensity
Chemical Shift (ppm)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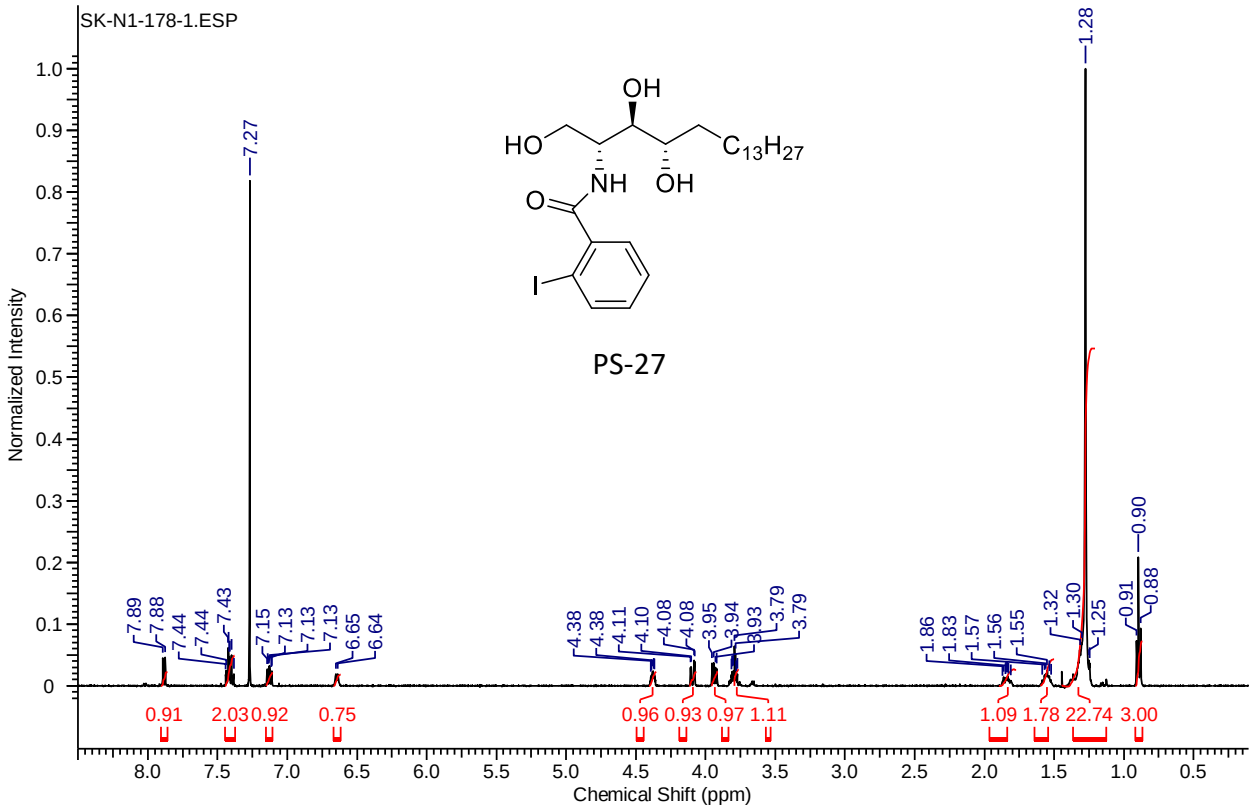
OH
O
PS-30
Normalized Intensity
Chemical Shift (ppm)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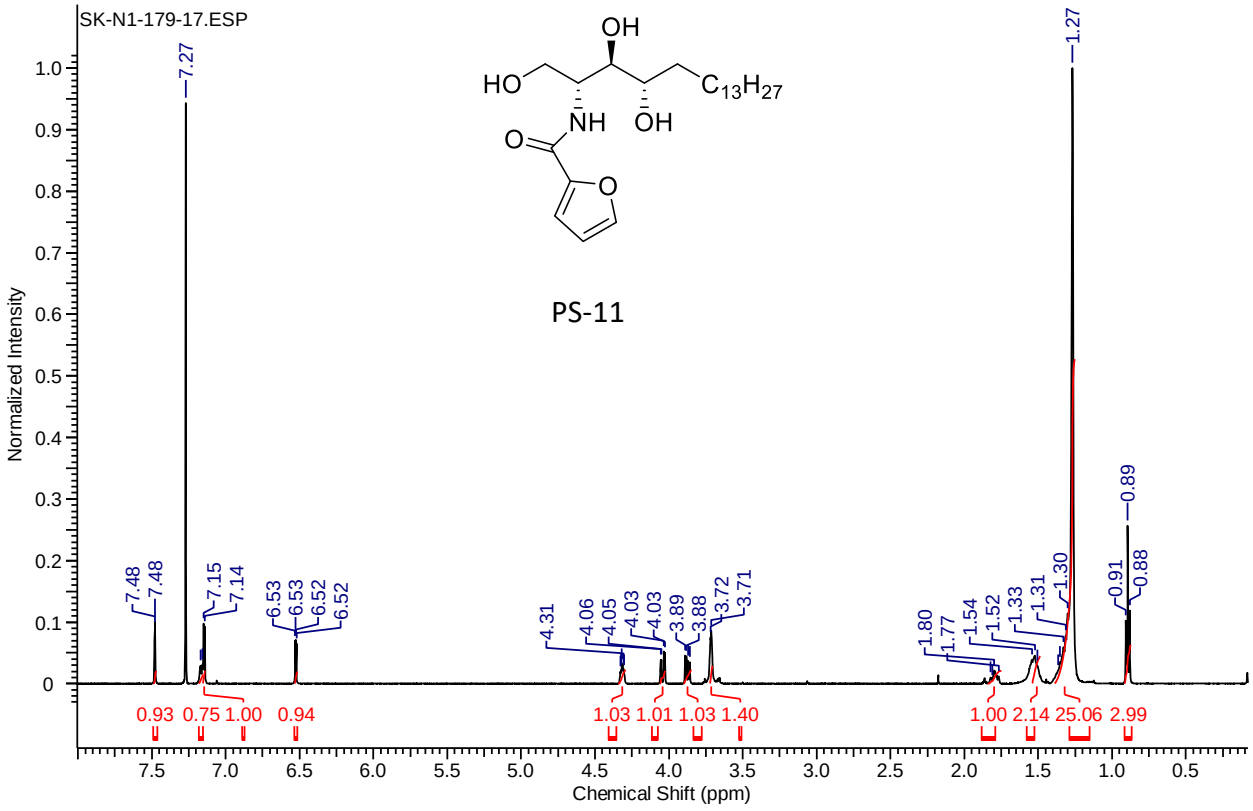
Chemical Shift (ppm)
PS-27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Chemical Shift (ppm)
PS-11

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## Publications

Papers (related to Doctoral Dissertation)

1. **Sajeer Koolath**, Yuta Murai, Yoshiko Suga and Kenji Monde. “Chiral combinatorial preparation and biological evaluation of unique ceramides for inhibition of sphingomyelin synthase” *Chirality.* **2020**, 32, 308-313.