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**Development of novel methods for rapid and efficient
extraction of naturally occurring bioactive sphingoid bases**

(生理活性を有する天然由来スフィンゴイド塩基類の迅速かつ効率的な新規抽出法の開発)

A Thesis Submitted for the Degree of
Doctor of Life Science

By

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Dedication

This dissertation is dedicated to my family; father Lt. Bomme Gowda, mother Gowramma, elder sisters Mangala, Neela, sweet wife Divyavani and my little angel Tanvi S. Gowda without their supports and encouragements, the study described herein would not have been successful.

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Abbreviations

LMSD	lipid maps structure database
FA	fatty acyls
GL	glycerolipids
GP	glycerophospholipids
SP	sphingolipids
ST	sterol lipids
PR	prenol lipids
SL	saccharolipids
PK	polyketides
S1P	sphingosine-1-phosphate
C1P	ceramide-1-phosphate
ESI	electrospray ionization
TBME	<i>tert</i> -butyl methyl ether
SPE	solid phase extraction
HPLC	high-performance liquid chromatography
GR	glutaraldehyde resin
OPA	<i>o</i> -phthaldehyde
FT/IR	fourier transform/Infrared
TLC	thin layer chromatography
NMR	nuclear magnetic resonance
GlcCer	glucosylceramide
DCM	dichloro methane
EtOAc	ethyl acetate
CHCl ₃	chloroform
DMF	<i>N,N</i> -dimethylformamide
THF	tetrahydro furan
TBAF	tetra- <i>n</i> -butylammonium fluoride
UV	ultraviolet
TBDPSCI	<i>tert</i> -butyldiphenylsilyl chloride

DDQ	2,3-dichloro-dicyanobenzoquinone
HAD	hetero-diels-alder
DMAP	<i>N,N</i> -dimethyl-4-aminopyridine
LiAlH ₄	lithium aluminum hydride
BnCl	benzyl chloride
SMS	sphingomyelin synthase
GCS	glucosylceramide synthase
CDase	ceramidases
CerS	ceramide synthase
SPT	serine palmitoyltransferase
GCase	glucosylceramidase
SK	sphingosine kinase
PC	phosphatidylcholine
DAG	diacylglycerol
DES	dihydroceramide desaturase
CK	ceramide kinase
KDS	3-keto-dihydrosphingosine reductase
SPPase	sphingosine phosphate phosphatase
COSY	correlated spectroscopy
d	doublet
s	singlet
t	triplet
MS	mass spectrometry
r.t	room temperature
h	hour(s)

Thesis abstract

Sphingoid bases are the functional and structural component of sphingolipids with more than 60 structural varieties occurs in nature. They are majorly found in plasma membranes of all eukaryotic and prokaryotic cells. Studies focusing on the efficient extraction/preparation of sphingoid bases of various natural resources were limited due to their low abundance in free form and difficulty in purification. Also, these types of molecules are commercially less seen and hence their biological activities are less studied. In the present study, the author focused on developing a new techniques/methods to obtain free sphingoid bases from natural resources in a most economical way. The first chapter of this thesis is general introduction to sphingolipids with their recent scientific updates. A brief literature survey on existing methods for extraction and analysis of sphingoid bases, followed by future prospective of sphingolipid research were discussed in detail.

The second chapter of this thesis will focus on the novel resin-based rapid extraction method for sphingoid bases. A common mammalian sphingoid base called “sphingosine” is hard to extract from biological samples, even by using complex extraction protocols such as Bligh-Dyer method. Currently, the author succeeded in extracting sphingosine from human serum by a simple and rapid extraction method using a novel glutaraldehyde resin. Also, the auhor demonstrated the chemoselective extraction of other sphingoid bases from golden oyster mushroom. The established method as wider applications in terms of its rapidity and selectivity.

The third chapter of this thesis will focus on the highly efficient preparation of sphingoid bases by a chemoenzymatic method. In this method a high-yield, two-step protocol has been established to prepare sphingoid bases from naturally abundant glucosylceramides (glucocerebroside) varying from small-scale to large-scale. Expansion of this strategy to various glucosylceramides of rice, wheat, soy, konjac and porcine brain were discussed.

The fourth chapter describes the first application of prepared sphingoid bases and their derivatives such as ceramides, N,N dimethylated sphingoid bases, as multidrug resistance inhibitors (MDR) in breast cancer. The author established adriamycin resistant MCF-7 (MCF-7/ADR) by a sequential selection of adriamycin treated MCF-7 and the preliminary results from the cell viability assay suggests that, prepared natural sphingoid bases and their derivatives are cytotoxic to MCF-7/ADR.

Chapter 1

General Introduction

1.1 Life and lipids

Lipids are a class of biological molecules defined by low solubility in water and high-solubility in non-polar solvents. They constitute about 50% of the mass of most animal cell membranes and that there are 10^9 lipid molecules in the plasma membrane of a small animal cell.¹ Lipids are integral to our body and also one of the major constituents of various products encountered on daily basis such as foods, cosmetics and medicines. Lipids have several roles in the body; these include acting as chemical messenger, storage and provision of energy and so forth. Other than energy storage, lipids perform a range of vital biological functions including structuring and compartmentalization of cellular organelles in the form of biomembranes, cell differentiation, signal transmission, protection and insulation of important organs and synthesis of essential biomolecules such as hormones and bile acids (**Figure 1**).² Cell membranes and lipids indeed know to be crucial to the life of the cell.

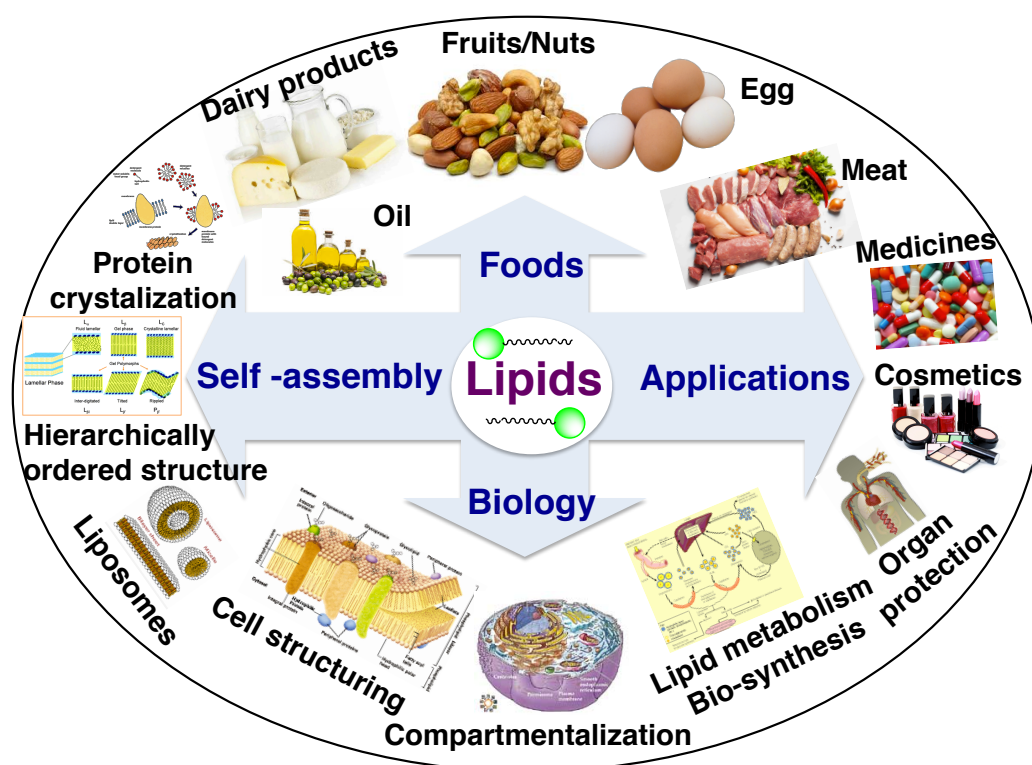


Figure 1. Significance and diverse applications of lipid

1.2 Classification of lipids

There are different ways to categorize lipids, based on source, structure, functions *etc.*, Lipid Maps Structure Database (LMSD) as reported more than 40,000 lipid structures, which were classified into eight major categories such as fatty acyls (FA), glycerolipids (GL), glycerophospholipids (GP), sphingolipids (SP), sterol lipids (ST), prenol lipids (PR), saccharolipids (SL), and polyketides (PK) as shown in **Figure 2**. And still new lipids continue to be found, and no doubt many remain to be discovered.³

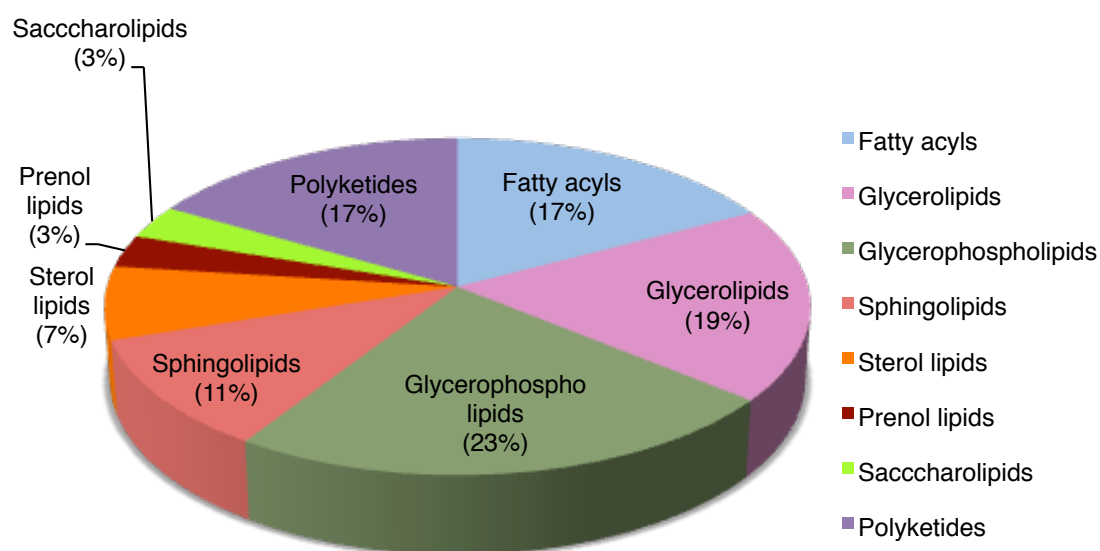


Figure 2. Lipid Maps Structure Database has listed 40,360 unique lipid structures, which are classified as shown here. It is the largest public lipid-only database in the world (Original data obtained from www.lipidmaps.org, as on 4/2/15)

Biological lipids generally adopt fairly simple chemical structures consisting of one or two C₁₀–C₂₀ chains and one or more functional groups like ester, phosphate, hydroxyl or amino. In general, the non-polar nature of lipids makes them soluble in organic solvents and insoluble or sparingly soluble in water. The representative structures of each type were shown below.

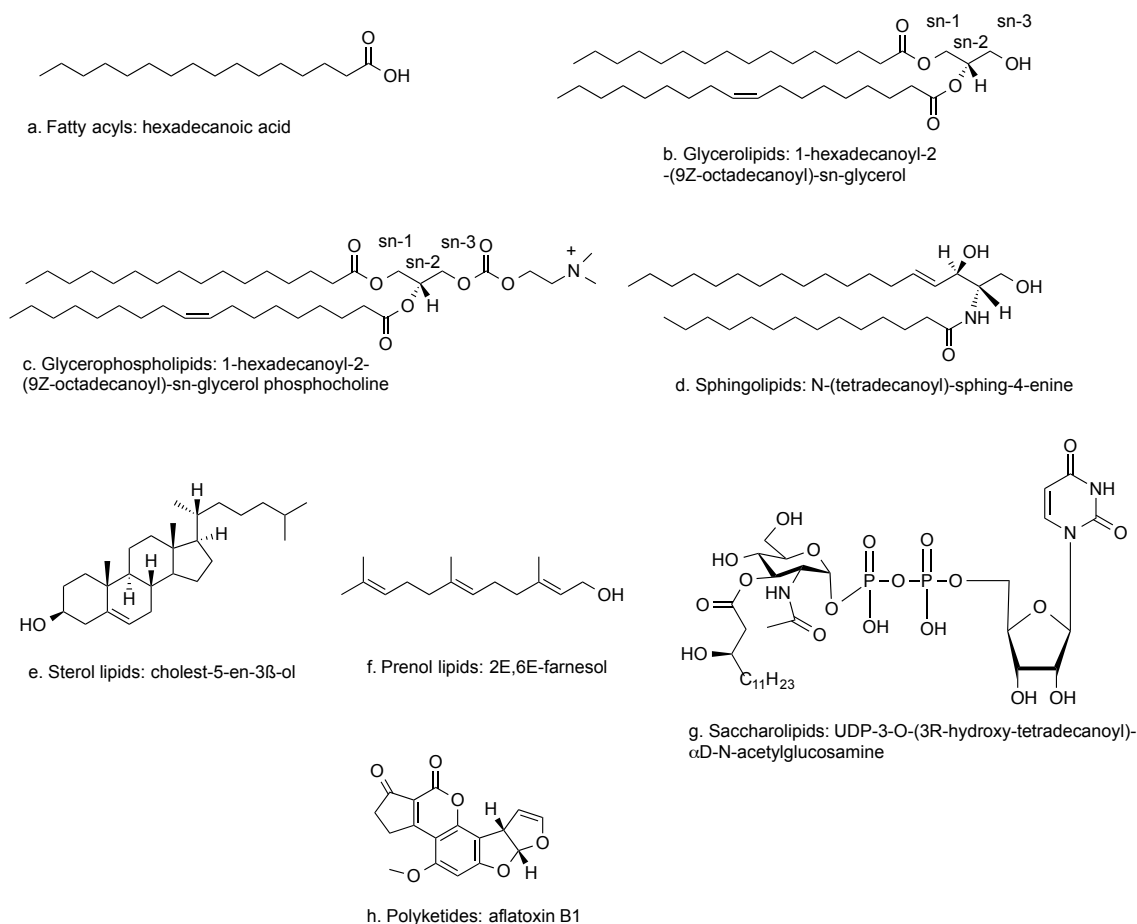


Figure 3. Representative structure of each class of lipids

1.3 Sphingolipids and their importance

Sphingolipids (SPs), are a major class of lipids and are the principle constituents of the eukaryotic cell membranes, enriched in lipid rafts, having a role in the cell architecture and also in protecting the cell from mechanical and chemical cues. The sphingolipids were named in 1884 after the Greek mythological creature, the “Sphinx” by J.L.W. Thudichum, owing to their enigmatic nature.⁴ Later, the term “sphingolipide” was introduced by Herbert Carter and colleagues in 1947. While they are perhaps less enigmatic than they once were, SPs are extremely versatile molecules and surprises are certainly expected as new knowledge is gained of their functions in healthy and diseased animal and plant tissues.

The cellular membranes like endoplasmic reticulum, golgi complex, plasma membrane, inner and outer mitochondrial membrane have a wide distribution of

sphingolipids. They are abundantly generated in the endoplasmic reticulum and golgi complex and are translocated to the cell membrane by the cytoplasm.⁵ Sphingolipids are amphipathic molecules that have both hydrophobic and hydrophilic properties, albeit to varying extents. The hydrophobic region consists of a sphingoid base backbone (normally sphingosine, sphinganine or phytosphingosine) to which a fatty acid tail attached by an amide bond to C-2.⁶ And different hydrophilic head attached at C-1 as shown in **Figure 4**.

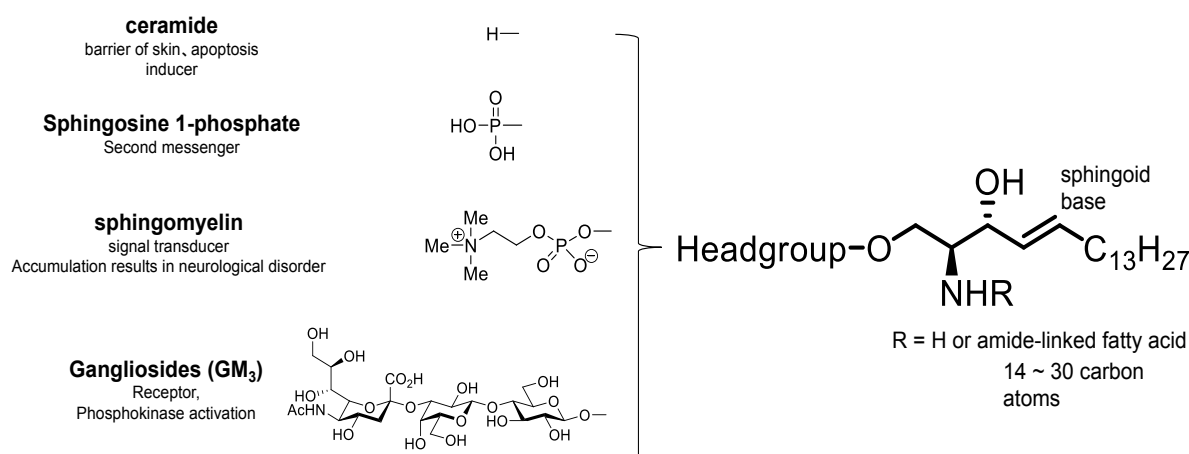


Figure 4. General structure of sphingolipids

Sphingolipids encompass hundreds of sphingoid backbones, which vary in chain length, number, position, and stereochemistry of double bonds, hydroxyl groups, and other functionalities. Some have especially intriguing features, such as the tail-to-tail combination of two sphingoid base in the α,ω -sphingoids produced by sponges.⁷ Also there are at least 400 head group variants of sphingolipids, out of which sphingolipids, free sphingoid bases and sphingolipid degradation products such as sphingomyelin, sphingosylphosphorylcholine, ceramide, sphingosine, and their phosphorylated forms ceramide 1- phosphate (C1P) and sphingosine 1-phosphate (S1P) are the major sphingolipids that have a prominent role in cell signal transduction, adhesion, angiogenesis, apoptosis, cell proliferation, growth arrest, differentiation, migration, senescence, or intracellular trafficking.^{8,9} The simplest sphingolipid, ceramide, functions as a key player in cell signaling and as the precursors of more complex sphingolipids. In contrast to complex sphingolipids, which contain a hydrophilic region at C-1 ceramide, has a minimal hydrophilic region

comprising two OH groups.

Other common variations are for the alkyl chain to be fully saturated (which have the name “sphinganines”) or have an additional hydroxyl at position 4 (which is named (2S, 3S, 4R)-2-amino-1,3,4-octadecanetriol, or phytosphingosine). Mammals have small amounts of sphingoid bases with hydroxyl groups at other positions, such as carbon 6 (a species found in skin) and plants and fungi have sphingoid bases with double bonds at other positions (for examples, sphing-4-enine sphing-8-enine and 4-hydroxy-8-sphingenine) and methyl-branched species. An abbreviated nomenclature for sphingoid bases is for the number of hydroxyls to be designated by “d” (di-) or “t” (tri-) followed by the number of carbons, then the number and position of the double bonds; for examples: d18:1^{Δ4} for sphingosine, d18:2^{Δ4,8} for 4,8-sphingadiene, and t18:0 for 4-D-hydroxysphinganine.

The sphingolipid metabolic pathway displays an intricate network of reactions resulting in the formation of a multitude of SPs, with Ceramide (Cer) as the center of SP biosynthesis, catabolism, and as precursors of complex SPs. Cer can be produced in at least two distinct ways. First, it can be synthesized through the de novo pathway, and second, through the hydrolysis of complex lipids, especially sphingomyelin (SM) (**Figure 5**).⁹ The de novo pathway commences with the condensation of serine and palmitoyl-CoA catalyzed by serine palmitoyl transferase to generate 3-ketodihydrosphingosine. 3-Keto-dihydrosphingosine is subsequently reduced to form dihydrosphingosine (sphinganine), which is then N-acylated by (dh) Cer synthases to produce dhCer or Cer. And Cer is metabolized by ceramidases (CDases), removing the amide-linked FA to form sphingosine. Finally, sphingosine is available for recycling into SP pathways or for phosphorylation by one of two sphingosine kinases, SK1 and SK2, to form S1P.

Sphingolipids are distinguished from the more abundant phospholipids by the use of serine rather than glycerol as the head group to which the hydrophobic lipid tails are attached. They are found in essentially all animal, plants and fungi, as well as some prokaryotic organisms and viruses. SPs are also constituents of lipoproteins, the multi-lamellar water barrier of skin, and as extracellular signals. They tend to associate more tightly with each other in cell membranes than phospholipids, thereby modulating the fluidity of membranes and forming the basis, together with cholesterol, for the densely packed regions of the membrane referred to as lipid rafts.¹⁰⁻¹¹ In addition to this fundamental membrane role, glycosphingolipids pattern

the surface of the cell with a diverse array of oligosaccharide structures that dictate cell-cell interactions as well as modulating intracellular signalling.

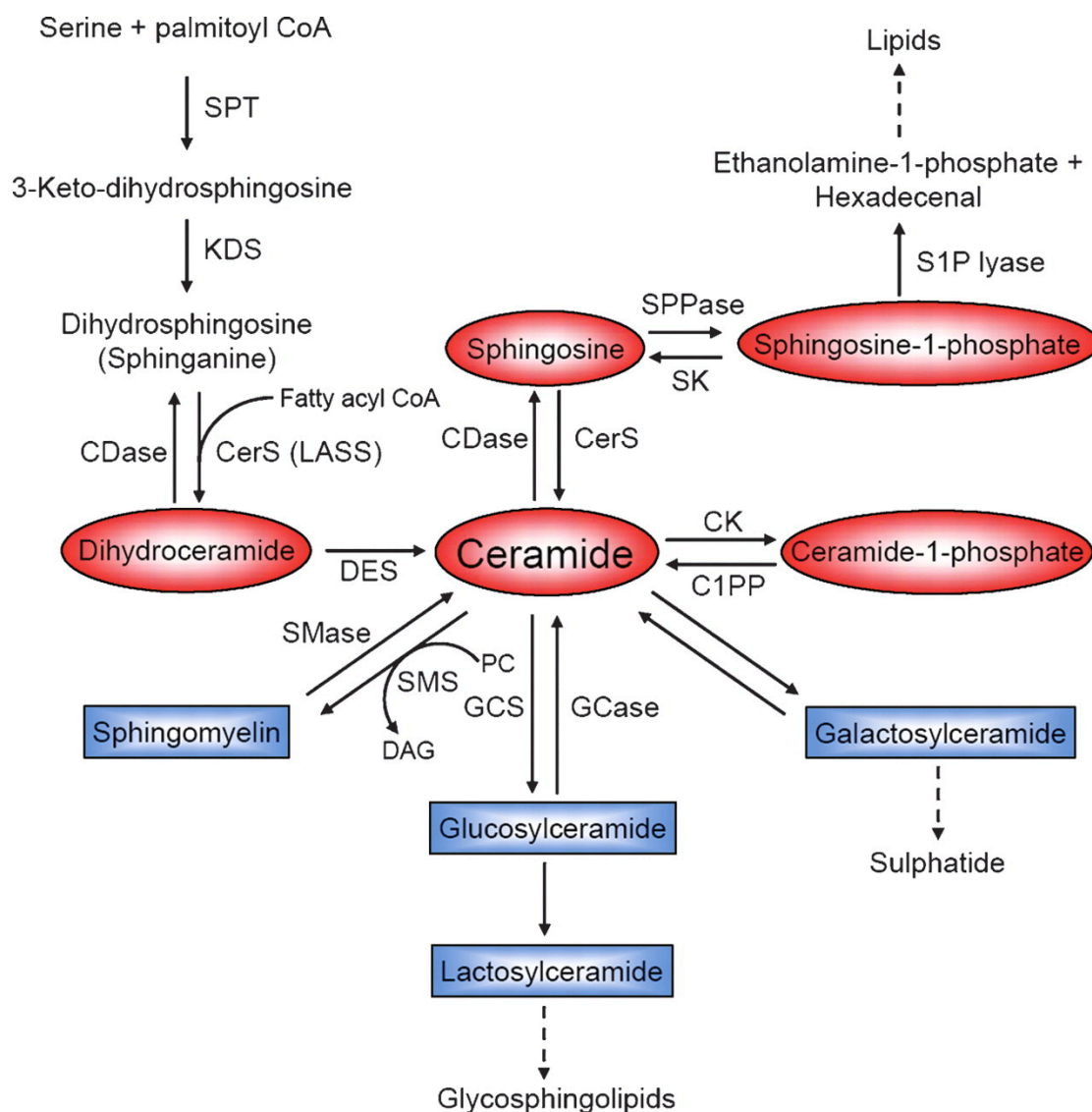


Figure 5. Schematic representation of sphingolipid metabolism pathways

In recent years, the mystery of the many hues of the sphingolipid metabolites has been unraveled with the advent of research techniques in the field. Valuable insights into interpreting the physiological and pathophysiological role of sphingolipid metabolites are being made possible by the generation of knockout mice by the ability to clone the regulatory proteins and enzymes involved in the sphingolipid metabolism. The therapeutic potential has been unleashed by the development of inhibitors of signaling enzymes and also specific agonists and

antagonists of the S1P receptors. Also, the sphingosine analogue FTY720¹², which is already in the phase III clinical trials for the treatment of multiple sclerosis, has shown to be a novel therapeutic agent in diseases caused by abnormal sphingolipid metabolism. The concurrent analysis and quantification of sphingolipid species by positive ion electrospray ionization (ESI) liquid chromatography tandem mass spectrometry (LC-MS/MS) has played an indispensable role in elucidating their role in disease.¹³

1.4 Sphingoid bases

Sphingoid bases are the structural units of sphingolipids, with various structural varieties (**Figure 6**). There was evidence for more than 60 structural variations in sphingoid backbone.¹⁴ Most common chain length variants of sphingosine (d18:1) 1 are d16:1, which has been found, for examples, in plasma sphingolipids¹⁵ and bovine milk¹⁶ and d20:1 (eicosasphingosine), which is present in substantial amounts in brain gangliosides, especially with advanced age. Little is known about the biological significance of this seemingly subtle backbone chain-length variation; however, the alkyl-chain length mismatch has substantial biophysical consequences.¹⁷ The very powerful technique of tissue-imaging mass spectrometry has established that d20:1 gangliosides are selectively localized along the entorhinal-hippocampus projections, especially in the molecular layer of the dentate gyrus, whereas those with the 18-carbon sphingoid base backbone are widely distributed throughout the frontal brain.¹⁸

Many of the naturally occurring and synthetic sphingoid bases are cytotoxic for cancer cells and pathogenic microorganisms or have other potentially useful bioactivities; hence, they offer promise as pharmaceutical leads. Recently, considerable attention has been given to other types of sphingoid base derivatives, namely the 1-phosphates and other “lyso” (devoid of fatty acid) types of compounds (sphingosyl- phosphocholine, galactosylsphingosine, etc.) because these are highly bioactive. N-methyl derivatives (N-methylsphingosine, N,N-dimethylsphingosine and N,N,N-trimethylsphingosine) have also been found in biological systems, but little is known about their origin or function(s).¹⁹

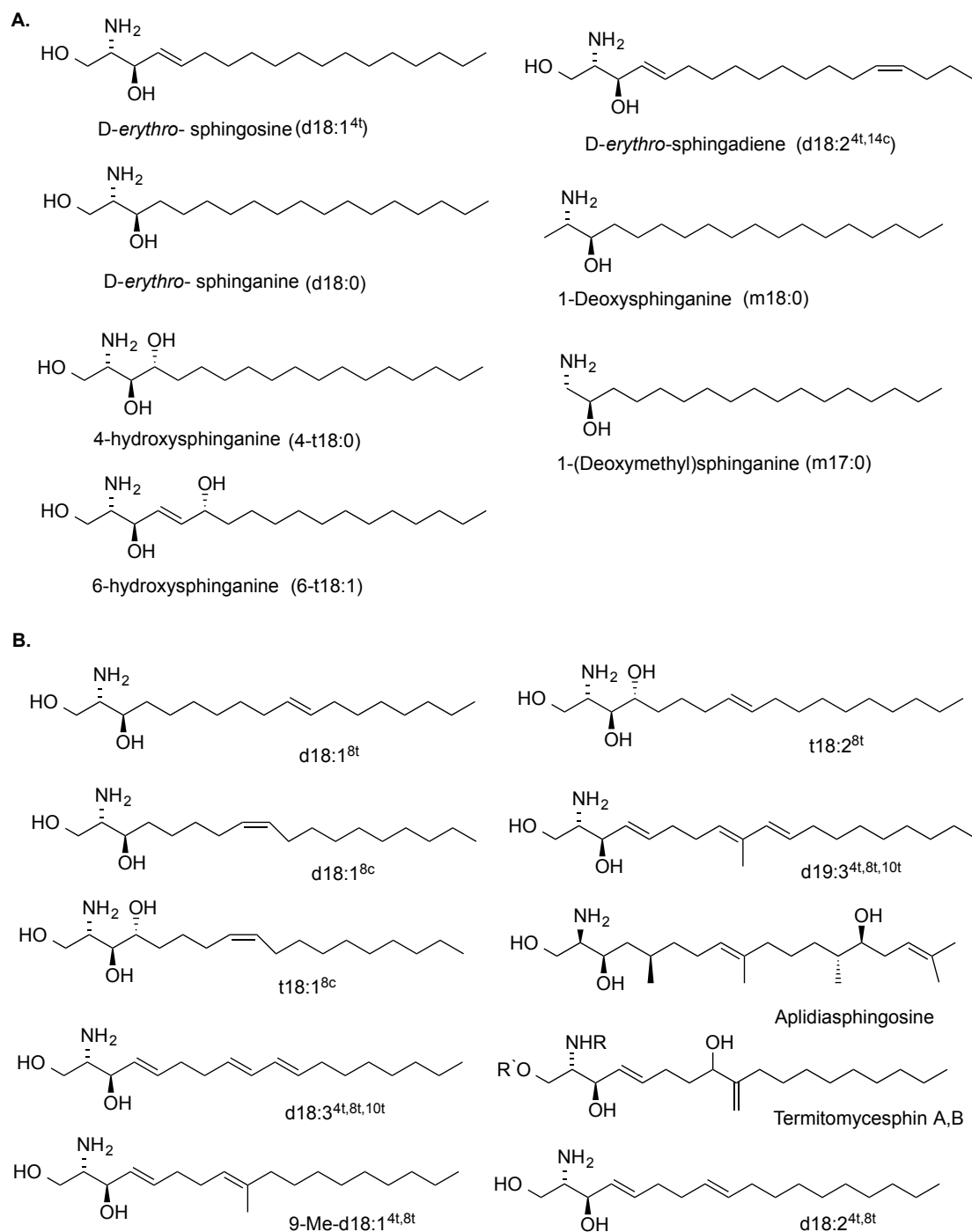


Figure 6. A. Mammalian sphingoid bases **B.** Sphingoid bases from other organisms like plants, fungi etc.,

1.5 Extraction and analysis of sphingoid bases

Free sphingoid bases are usually present in cells in very small amounts, with most being derivatized on the 2-amino group with a long-chain fatty acid and conjugated at the 1-hydroxyl with a polar headgroup to produce “complex” sphingolipids. The most common sphingoid base, sphingosine levels in human serum was found to be 81 ± 41 ng/ml (male), 85 ± 33 ng/ml (female).²⁰ Whereas in case plants, sphingoid bases are present in trace amounts.²¹ Quantitative isolation of lipids from tissues in their native state and free of non-lipid contaminants must be accomplished before analysis is attempted.

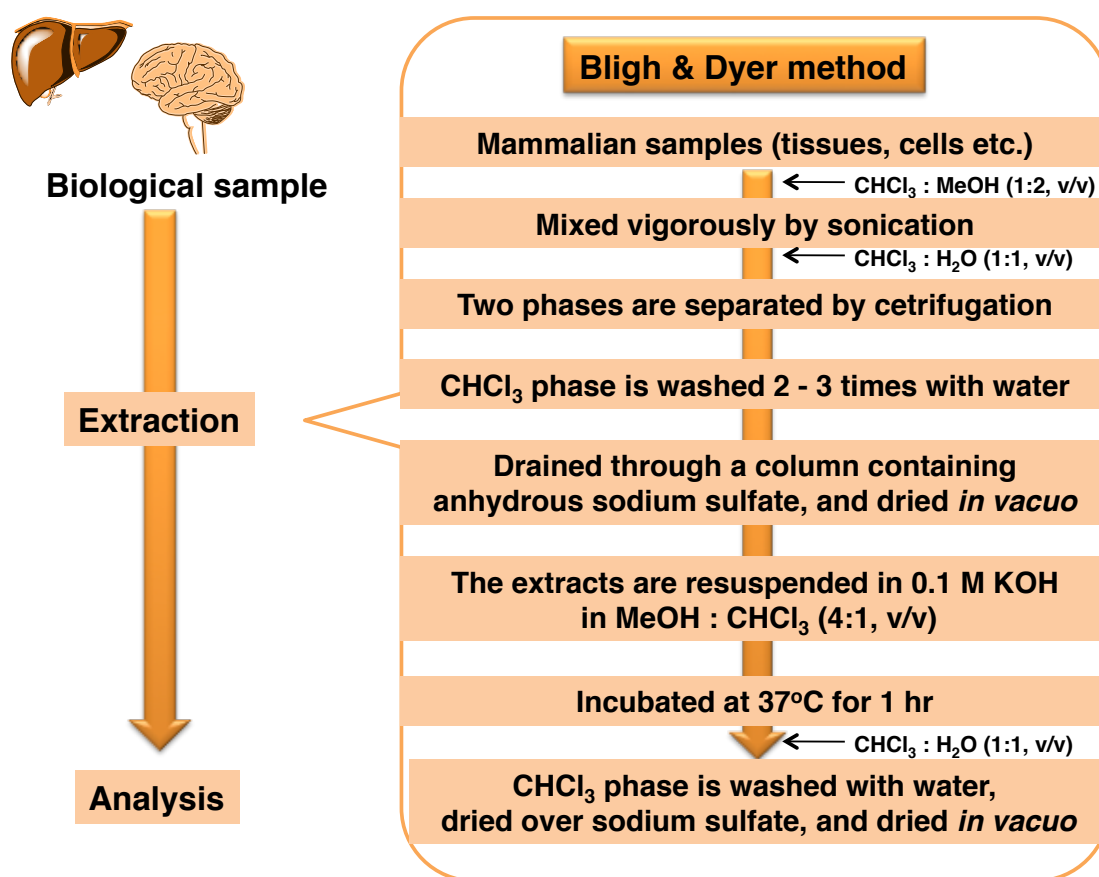


Figure 7. Bligh and Dyer extraction of sphingolipids

A large number of different extraction procedures, varying in the nature of the solvents, the method of homogenizing, removal of contaminants and many other aspects, have been described in the literature. Unfortunately, a high proportion of

these have been tried in only one laboratory or with a limited range of tissues or organisms. The extraction of free sphingoid bases from many tissues, serum, cells in culture, were carried out by Bligh and Dyer using chloroform-methanol solvent systems, hence their method is well known as Bligh-Dyer method (Figure 7).²² Many modifications to this method such as acidified Bligh and Dyer were done to improve the extraction efficiency. It is one of the widely used method for lipid extraction, therefore suited to many routine applications. However, incomplete recovery of the metabolically important but quantitatively minor acidic lipids is inevitable. When it has apparently been found wanting, the author has sometimes noted that the strict protocol laid down by Bligh and Dyer has not always been followed.

Other solvent extraction methods like Folch method, methanol-*tert*-butyl methyl ether (TBME), solid phase extraction (SPE), and hexane-isopropanol method were also used for the extraction of lipids including sphingoid bases.²³ The choice of extraction procedure will depend on the nature of the tissue matrix, and for example, whether the sample is of animal, plant or microbial origin and type of the lipid. All these methods are tedious, time-consuming and relatively uninteresting, any slackness will almost certainly result in the loss of specific components and in the production of artifacts. While using many solvents or solvent combinations care must be taken about non-lipid contaminants and recovery. Moreover extraction of tiny sphingoid bases by these complex methods is very difficult, leads to poor extraction efficiency and hence these methods are limited only for major membrane lipids.

The sphingoid base contents were analyzed by various methods, including gas-liquid chromatography,²⁴ high-performance liquid chromatography (HPLC)-fluorescence detection,²⁵ HPLC-UV²⁶ and enzymatic assay.²⁷ HPLC-fluorescence method for detection of sphingoid bases by solid-liquid extraction was also developed.²⁸ The first step in the analysis of the sphingoid bases of sphingolipids is hydrolysis of any glycosidic linkage or phosphate bonds as well as the amide bond to the fatty acyl group. This should be accomplished by a procedure in which the minimum degradation or rearrangement of the bases occurs, such as *O*- or *N*-methylation. While many analysts claim that base-catalysed hydrolysis causes least disruption, rapid acid-catalyzed methods are often preferred for convenience. Subsequently, the bases are best analyzed by gas chromatography after derivatization to reduce their polarity. Analysis of long-chain bases in intact sphingolipids by

modern mass spectrometry methods now appears to be an alternative.²⁹ However, all these methods were time-consuming and labor-intensive purification as well as hydrolysis step. In addition, the large scale of solvents used to measure sphingosine and sphinganine in liver and other tissues after base hydrolysis by HPLC–fluorescence technique. Nonetheless, much more sophisticated technologies will be needed to analyze a sphingolipidome of this complexity.

1.6 Prospective on sphingolipid research

The study of bioactive sphingolipids in inflammation, cancer and various other diseases is only now coming to the foreground. As always in science, answering one question leads to many more, and this is especially true of these enigmatic sphingolipids. Clearly, more work is required to understand how inflammation regulates bioactive sphingolipid levels, the activities of the enzymes that control their levels and the proteins they signal through, and how this shapes the immune response. Although around a dozen extracellular and intracellular targets of signalling sphingolipids have been identified, it is unlikely that we have found them all, based on their pleiotropic effects.

The complexity of sphingolipid metabolism and the 'sphingolipidome', and the fast interconversion of bioactive sphingolipids make this task even more daunting. This endeavour goes hand-in-hand with current systems-biology research, and it will be greatly aided by advanced sphingolipidomics—which can quantitatively measure changes in sphingolipids bound to their protein targets—combined with the development of conditional and tissue-specific sphingolipid-metabolic-enzyme knockout mice. Moreover, current sphingolipidomic technology for simultaneous quantification during inflammation of multiple sphingolipid species, for example, ceramides with different fatty acids, will be particularly helpful as these signalling molecules could have distinct and, sometimes, even opposing functions. In addition, sphingolipids can serve as biomarkers for disease and even as a biological signature, as illustrated by a study of the physiological status and bacterial diversity of estuarine microbial mats, which used the presence of sphingoid bases (d18:0, d19:0, and d21:1) and hydroxy fatty acids to predict the presence of organisms in the *Bacteroides* genus. The biodiversity of the sphingoid bases and sphingoid base-like compounds will continue to amaze, challenge, and amuse scientists for many years.

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Chapter 2

Development of resin-based rapid and facile extraction of sphingoid bases

2.1 Abstract:

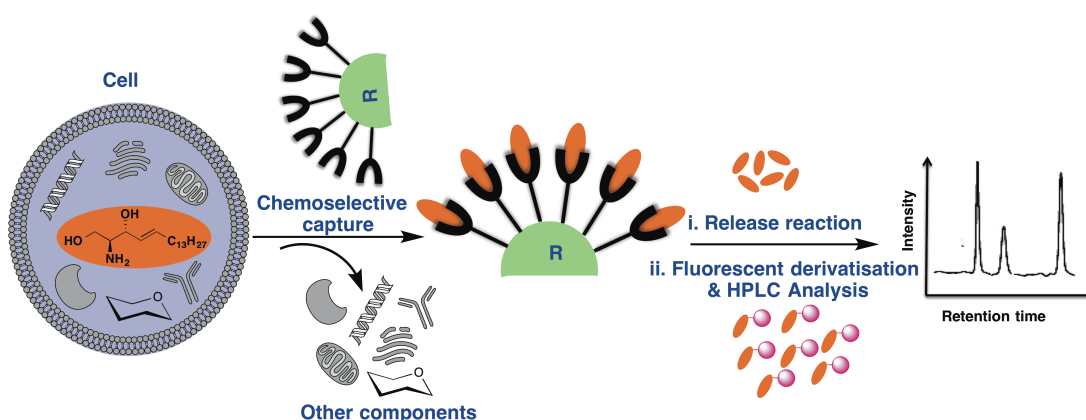
Extraction of sphingoid bases from biological samples remains a greater challenge for biochemists as well as chemists, due to their low abundance and difficulty in purification by their amphiphilic properties. These molecules are found to be most promising chemotherapeutic agents and of greater industrial importance. So far, there is no practically efficient extraction method for sphingoid bases, unless conventional solvent extraction for total lipids, which is more complex and often limited to major membrane lipids and the type of sample. Herein, for the first time, synthesis of a novel glutaraldehyde resin (GR), which is highly efficient for the rapid extraction of long-chain sphingoid bases is reported. In the present findings, GR was enforced to extract *D-erythro*-sphingosine from human serum that is found to be in nano molar levels as well as 9-methylsphingadienine, glucosyl 9-methylsphingadienine from golden oyster fungi has been demonstrated.

2.2 Introduction

Sphingolipids (SPs) are ubiquitous structural and functional constituents of the plasma membranes of eukaryotic and prokaryotic cells. SPs and their metabolites play crucial roles in many physiological processes, including immune response, cell proliferation, differentiation, and apoptosis.¹⁻³ They are composed of a structurally related family of backbones having 2-amino-1,3-diols termed sphingoid bases. These sphingoid bases are highly bioactive compounds that appear as intermediates of sphingolipid metabolism and cell signaling. Recent studies implicate sphingoid bases inhibit proliferation and growth of cells of various cancer cell lines *in vitro* and *in vivo*.⁴⁻⁷ The most common mammalian sphingoid base is *D-erythro*-sphingosine, and its cellular levels was controlled by the action of ceramide synthase and sphingosine kinase. Plants and fungus sphingolipids also have diverse sphingoid base structures and sphingoid bases found in aquatic plants are widely used for medicinal purposes in traditional oriental medicine.⁸⁻¹⁰ Structurally modified sphingoid bases have attracted more attention because some of their analogues have been observed to introduce morphological changes in neuronal cells and behave as enzyme inhibitors.^{11,12}

Since natural abundance of sphingoid bases and its derivatives are limited and because of purity requirement for biological testing, many methods have been developed for sphingoid base extraction and analysis, most of which are modified Bligh-dyer method coupled with reversed phase high-performance liquid chromatography (HPLC) as *o*-phthalaldehyde (OPA) derivatives.^{13,14} Although sphingoid bases can be extracted by this method, fluorescent contaminants that elute (on reversed-phase HPLC) along with the naturally occurring sphingoid bases cause a serious problem with the analysis and purification.¹⁵ Moreover, this protocol is tedious and often couldn't give concurrent extraction results and not efficient enough to extract lysosphingolipids (Psychosine), more polar sphingoid bases and their derivatives. However, these polar bases can be extracted by solid phase extraction, but it is limited due to the decomposition of the samples while drying.^{16,17} Due to wide variety of biological activities, and unique structure with an array of functionalities, sphingoid bases had a greater attention in recent years. And there is a growing interest in developing efficient extraction methods.

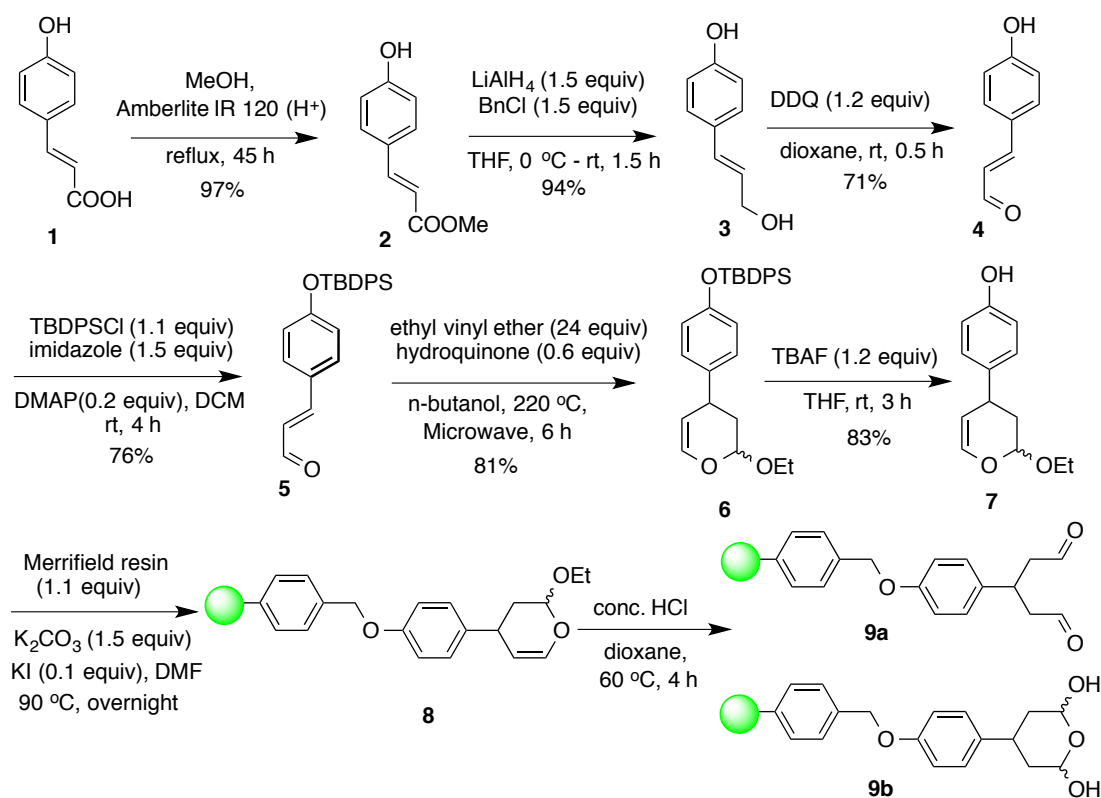
Herein, authors describe the synthesis of a novel glutaraldehyde resin (GR) and its use for the enrichment of sphingoid bases, psychosine from serum and fungi extract. The expected high efficiency of this method for sphingoid base enrichment is due to the chemoselective reaction of glutaraldehyde with 2-amino-1,3-diols,¹⁸ which are distinctive of sphingoid bases as well as formation of a highly stable adduct. The procedure for extraction of endogenous sphingoid bases using GR is illustrated in **Scheme 1**.



Scheme 1. Extraction and analysis methodology for sphingoid bases using GR

2.3 Results and discussion

The synthesis of GR (**Scheme 2**) was carried out by the reaction of *trans-p*-coumaric acid (**1**) to its methyl ester (**2**), which was reduced to coumaric alcohol (**3**) by alane reduction.¹⁹ Further oxidation with 2,3-dichloro-5,6-dicyanobenzoquinone (DDQ) afforded *trans-p*-coumaraldehyde (**4**)²⁰ and its phenolic hydroxyl group was protected as *tert*-butyldiphenyl silyl ether (**5**). Novel microwave assisted inverse-electron-demand hetero-Diels-Alder (HDA) reaction of ethyl vinyl ether²¹ was performed in the presence of catalytic amount of hydroquinone to give **6** as a diastereomixture. Deprotection of the silyl ether with a tetrabutylammonium fluoride produced **7**, which was immobilized on the Merrifield resin.



Scheme 2. Synthesis of glutaraldehyde resin (GR)

Novel microwave assisted inverse-electron-demand hetero-Diels-Alder (HDA) reaction of ethyl vinyl ether²¹ was performed in the presence of catalytic amount of hydroquinone to give **6** as a diastereomixture. Deprotection of the silyl ether with a tetrabutylammonium fluoride produced **7**, which was immobilized on the Merrifield resin. The structure of **8** was confirmed by comparing its IR spectrum with that of the model compound **8m** (**Figure 1**). The ion chromatographic elemental analysis (ICS1600 (Dionex), Dionex IonPac AG12A-AS12A (4×200mm), standard: 0.2/2.8mM NaHCO₃/Na₂CO₃, flow rate: 1.5mL/min) was performed for both modified resin **8** and commercially available Merrifield resin, to confirm the amount of chloride reacted and it was found that 88% of chloride reacted with compound **7**.

The following hydrolysis with hot hydrochloric acid in dioxane successfully afforded the desired glutaraldehyde resin (**9**). When it was characterized by FT-IR, IR spectrum indicates a mixture of dialdehyde (**9a**) and cyclic dihemiacetal (**9b**) (**Figure 2**). And it is notable that the resin is quite stable in spite of the presence of highly labile dialdehyde.

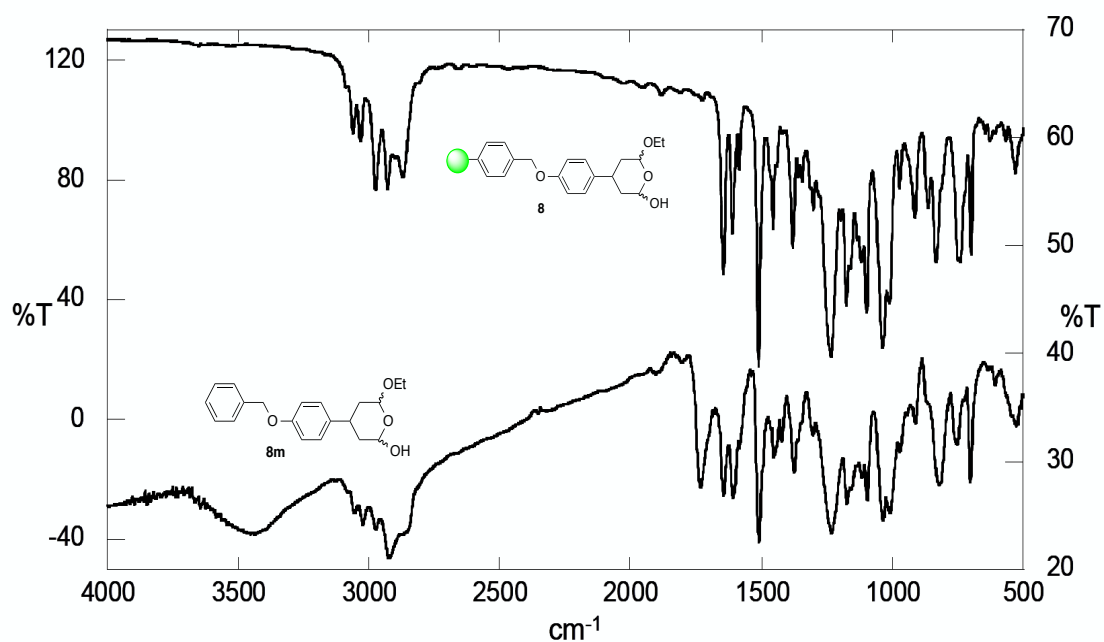


Figure 1. IR spectrum of model compound **8m** and **8**

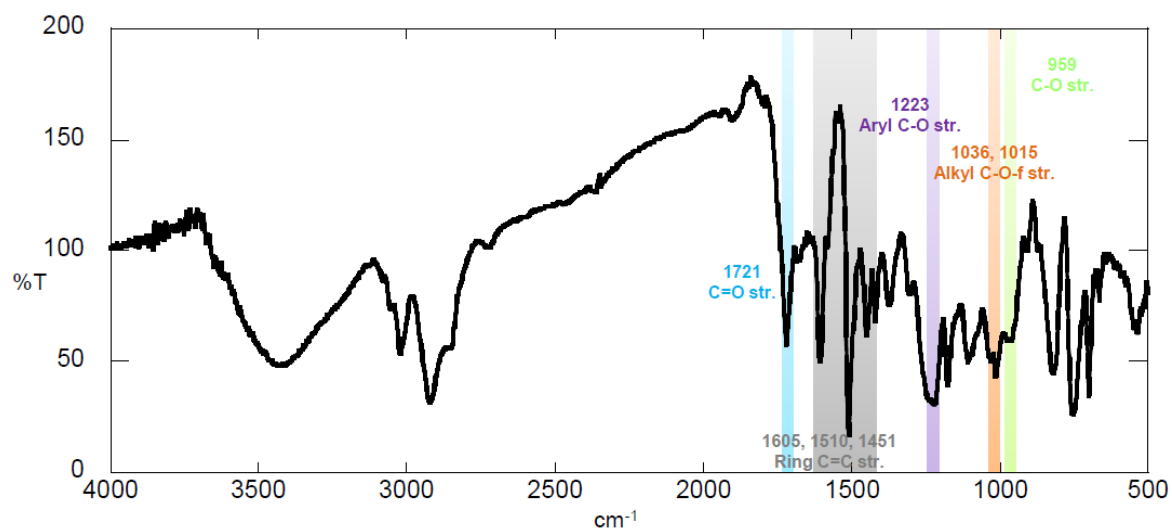


Figure 2. IR spectrum of compound **9a** and **9b**

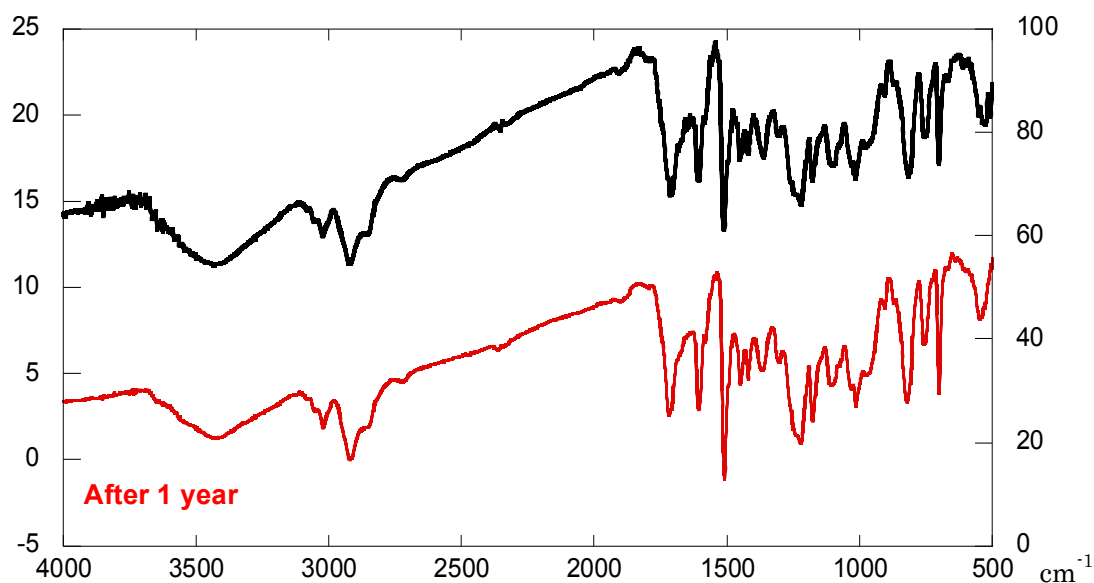
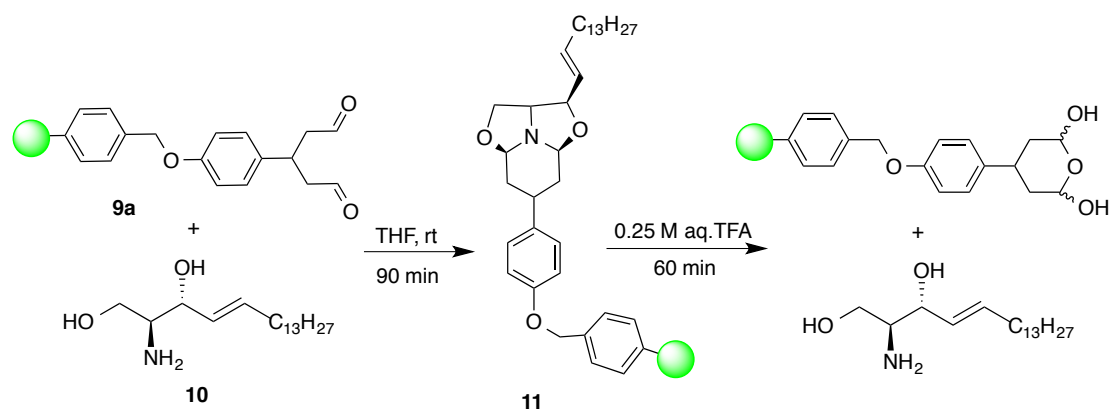


Figure 3. Long-term stability of resin at room temperature.

The IR spectrum of resin did not show any spectral changes even after the one-year incubation at room temperature (**Figure 3**). This unusual stability could be ascribed to the distance among each dialdehyde group immobilized on the resin, which is long enough to hamper their self-condensation. In addition, cyclic dihemiacetal structure might contribute to its stability, like cyclic hemiacetal structures of carbohydrates.



Scheme 3. Chemoselective reaction of *D-erythro*-sphingosine with GR, followed by hydrolysis.

A model reaction was carried out using standard *D-erythro*-sphingosine as shown in **Scheme 3**. GR reacted chemoselectively with sphingosine to form a stable tricyclic adduct, which was further hydrolyzed under mild acidic conditions to liberate free sphingosine. The structure of adduct **11** was confirmed by carrying out a model reaction between *D-erythro*-sphingosine and glutaraldehyde to give (**12**). Several set of experiments were carried out to stabilize releasing conditions using various acids and 0.25 M aq. trifluoroacetic acid (TFA) was found to be the best. The total extraction efficiency of the resin was calculated from the calibration curve using, standard *D-erythro*-sphingosine, in coupling with reverse phase HPLC and it was to be about 75-80%. GR shows high selectivity towards sphingoid base at pH 5 in the presence of alanine (**Figure 4**). To the best of our knowledge, this is the first novel resin for the rapid and efficient extraction of free sphingoid bases by a chemoselective reaction, which are purporting to be minor components of total lipids.

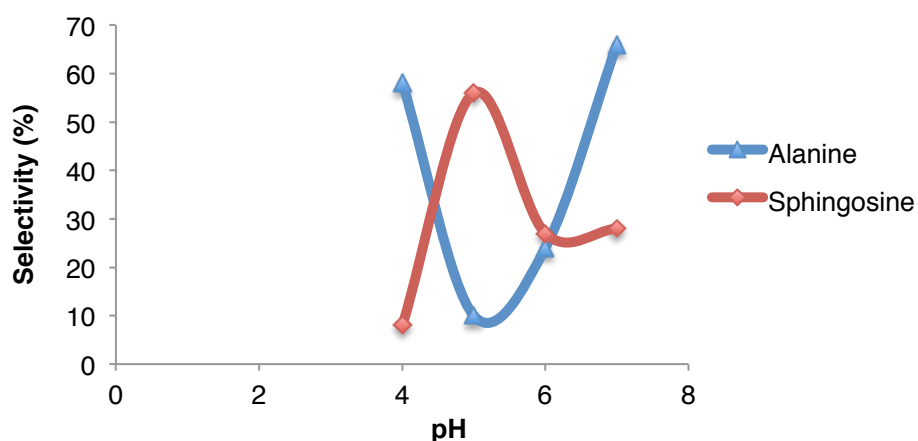
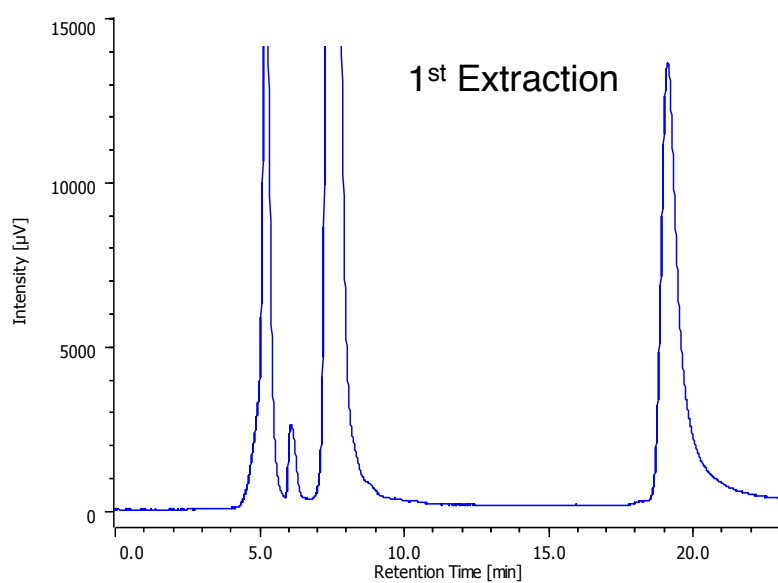


Figure 4. Effect of pH on selectivity of resin

Further to check the re-usability of the resin, repeated extraction of the spiked sphingosine was carried out and analyzed by reverse phase HPLC (C_8 column, flow rate 0.5 ml/min, CH_3CN : Buffer (85:15)) after *o*-phthaldehyde derivatization. Obtained results from the fluorescence chromatograms (**Figure 5**) clearly show that, resin is still efficient in extracting sphingosine even after three repeated extractions. Hence, this novel GR can be re-usable like a catalyst in a chemical reaction. Also, the IR spectrum of the used resin shows the significant intensity of dialdehyde peak, which is similar as that of the IR spectrum of resin before its use for extraction.



R.T 17.5 min,
Sph-OPA

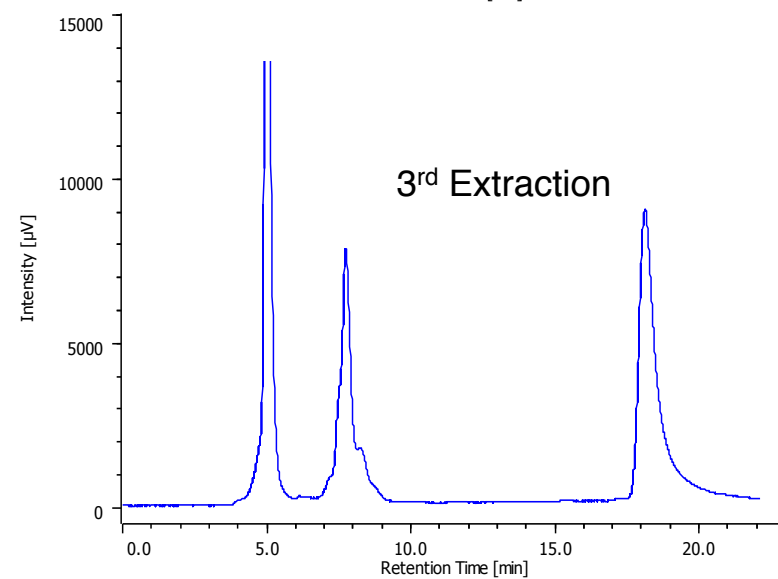
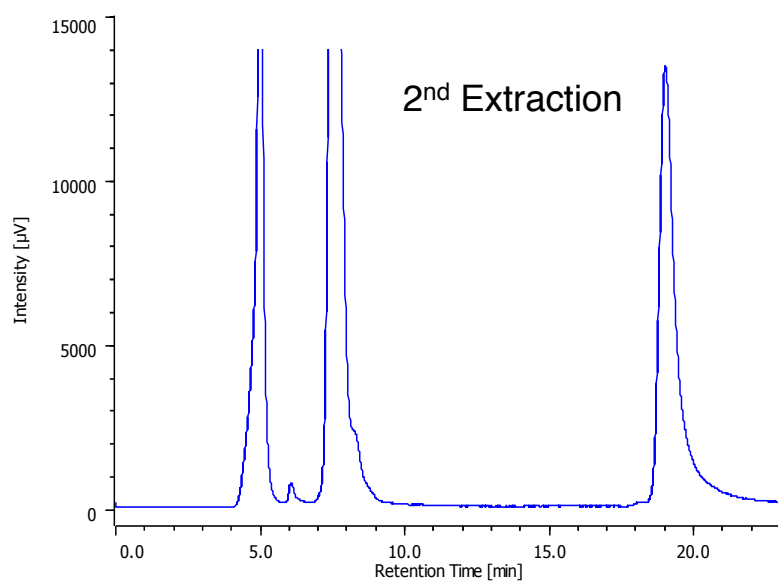


Figure 5. Re-usability of GR after repeated extractions (R.T: retention time)

The aim of this work is to develop a technology for the efficient extraction of sphingoid bases; such technology is a crucial requirement for the discovery of novel sphingoid bases from biological samples and natural resources, to evaluate their biological activity. Based on the above strategy GR was applied to extract free sphingosine from human serum. To the 100 μ L of human pooled serum 400 μ L of methanol was added, centrifuged, centrifugate was added to GR as a human serum extract. After chemoselective capture and releasing, the release filtrate was analyzed for free sphingoid bases using reverse-phase fluorescent HPLC with OPA derivatization technique. Comparison of chromatograms from methanolic extract of human serum before extraction with resin (**Figure 6, A**) and after extraction with resin (**Figure 6, B**), clearly shows the selective capture of sphingosine, excluding all other components from serum. The serum sphingosine was confirmed by comparing retention time with that of standard sphingosine and from ESI-mass spectrum. This data demonstrate that, GR is highly chemo selective towards sphingosine and hence highly sensitive HPLC method in coupling with capture resin should enable an extremely sensitive analysis of biological samples.

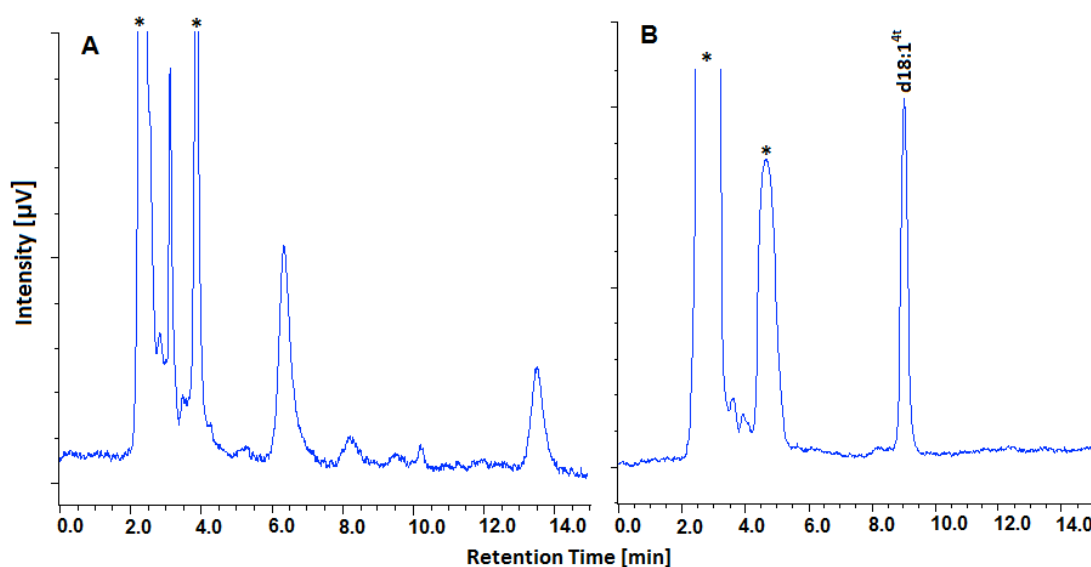


Figure 6. HPLC profiles of *O*-phthalaldehyde derivatives of methanolic extract of human serum. A. Before extraction with GR. B. After extraction with GR (d18:1^{dt}: sphingosine, *OPA contamination)

To demonstrate the general versatility of GR, fungi called golden oyster mushroom (*Pleurotus citrinopileatus*) was used. It is an edible mushroom and found to be rich in glucosyl ceramide²². The sphingolipids in methanolic extract of the mushroom was hydrolyzed to liberate free sphingoid bases using modified Cahoon and Lynch method²³. The liberated bases were analyzed by OPA derivatization technique.

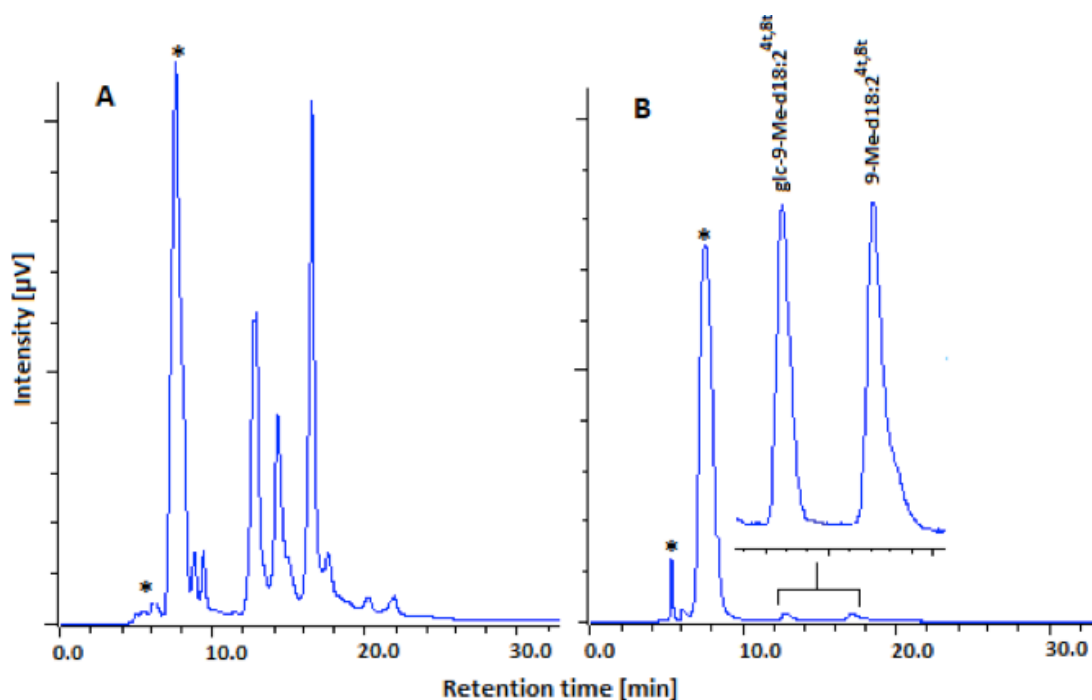


Figure 7. Elution profiles of *O*-phthalaldehyde derivatives of hydrolyzed methanolic extract of golden oyster. A. Before extraction with GR. B. After extraction with GR (d18:1^{4t,8t}: sphingosine, glc-9-Me-d18^{4t,8t}: glucosyl 9-methylsphingadienine *OPA contamination)

There are many peaks in the chromatogram (**Figure 7, A**), shows it as some other components along with free sphingoid bases. Upon extracting with our resin, it showed good selectivity towards 9-methylsphingadienine (9-Me-d18:2^{4t,8t}) and glucosyl 9-methylsphingadienine (glc-9-Me-d18:2^{4t,8t}), (**Figure 7, B**). These are produced by hydrolysis of glucosyl ceramides from fruiting bodies of golden oyster mushroom. Both sphingoid base and glucosylsphingoid were confirmed by their standard retention time and from ESI-Mass spectra. The reaction of glc-9-Me-d18:2^{4t,8t} with glutaraldehyde forms a stable bicyclic adduct (**13**), which further evident the reactivity of resin towards it.

In summary, a novel glutaraldehyde resin (GR) was synthesized and applied for the extraction of endogenous sphingoid bases from serum sample as well as fungi as been demonstrated. GR methodology is highly efficient and rapid compared to classical solvent extraction, which is limited to major membrane lipid and type of sample. Within a period of 6 h extraction will be completed with 75-80% yield and applicable for any kind of sample. This will provide the opportunity to establish an analytical method for rapid analysis of sphingoid bases in nanoscale with ultra purity. Authors believe that the highly efficient extraction of sphingoid bases from biological samples and other natural resources by GR is a very promising technology for the discovery of new sphingoid bases, based chemotherapeutics.

2.4 Experimental section

General procedures

^1H NMR (500 MHz) and ^{13}C NMR (125 MHz) spectra were recorded on a Varian Inova instrument at 25 °C in CDCl_3 . Chemical shifts (δ) are reported in ppm and coupling constant values (J) are in Hertz (Hz) relative to CDCl_3 (^1H , δ 7.26; ^{13}C , δ 77.00) or tetramethylsilane. The following abbreviations were used for signal multiplicities: s = singlet; d = doublet; t = triplet; q = quadruplet; m = multiplet. Electrospray ionization mass spectra (ESI-MS) were obtained by a JEOL JMS-T100LP spectrometer. Analytical TLC was performed on 0.2 mm silica gel plates (Merck 60 F-254). SiO_2 gel column chromatography was carried out using silica gel (Kanto Silica Gel 60, spherical, 40-50 μm) with air flashing. Microwave reaction was performed by using Biotage® Initiator+. HPLC analyses were conducted on a JASCO FP-2020 HPLC system equipped with a JASCO PU-2089 FL detector and YMC-Pack C8 column (0.46 cm ϕ x 15 cm).

Human pooled serum was purchased from Cosmo Bio Co. Ltd., (Tokyo, Japan), *trans-p*-Coumaric acid, 2,3-Dichloro-5,6-dicyano-1,4-benzoquinone, Ethyl vinyl ether, Tetrabutyl ammonium fluoride, Lithium Aluminum Hydride, Benzyl Chloride, *tert*-Butyldiphenylchl -orosilane, Trifluoro acetic acid, were purchased from Tokyo Chemical Industries, Ltd., (Tokyo, Japan), Imidazole, 4-Dimethylaminopyridine, Hydroquinone, Potassium Carbonate, Potassium Iodide,

Boric acid, *O*-phthalaldehyde (OPA), *D*-erythro-Sphingosine and all other solvents are of HPLC grade, were purchased from Wako Pure Chemical Industries, Ltd., (Tokyo, Japan), Amberlite IR 120 from MP Biomedicals, Merrifield resin from Sigma Aldrich (Tokyo, Japan), Bariumhydroxide octahydrate from Nacalai Tesque (Kyoto, Japan). Dried fruiting bodies of golden oyster mushroom were obtained from Nisse Bio Co. Ltd., (Tokyo, Japan) CDCl₃ and CD₃OD was purchased from Cambridge Isotope Laboratories (Tewksbury, USA). Libra tubes were purchased from Hipec laboratories (Kyoto, Japan).

Synthesis of glutaraldehyde resin (GR)

1) Synthesis of compound 2

To a stirred solution of a *p*-*trans*-coumaric acid **1** (5 g, 30.45 mmol) in excess of methanol, catalytic amount of Amberlite IR 120 (H⁺) was added and refluxed at 75°C for 45 h. The catalyst was filtered off and reaction mixture was concentrated under reduced pressure, the crude mixture was purified by column chromatography (hexane/EtOAc: 4:1) to give **2** as amorphous white solid in 97% yield.

¹H NMR (500 MHz, CDCl₃): δ 7.63 (1H, d, *J* = 15.9Hz), 7.43-7.45 (2H, m), 6.84-6.87 (2H, m), 6.30 (1H, d, *J* = 15.85Hz), 5.19 (1H, s), 3.80 (3H, s); HRMS (ESI) Calcd for C₁₀H₁₀NaO₃ (M+Na⁺) 201.0528, found 201.0523.

2) Synthesis of compound 3

To a stirred solution of LiAlH₄ (5.1 g, 28.6 mmol, 1.5 equiv) in dry THF, N₂ atmosphere, benzyl chloride (4.9 mL, 42.9 mmol, 1.5 equiv) was added drop wise. After 15 min the ester (**2**) was pre-dissolved in dry THF was added slowly through a dropping funnel, reaction was continued to stirred for 90 min, and the reaction mixture was quenched with water, filtered over celite, washed with aqueous sodium potassium tartrate and extracted with EtOAc, dried over Na₂SO₄, filtered, and concentrated. The residue was purified by flash column chromatography (hexane/EtOAc: 7:3) to give allyl alcohol (**3**) as an amorphous white solid in 94% yield.

^1H NMR (500 MHz, CD_3OD): δ 7.24 (2H, d, J = 8.8Hz), 6.73 (2H, d, J = 8.4Hz), 6.50 (1H, d, J = 15.8Hz), 6.16 (1H, td, J = 5.9, 15.8Hz), 4.18 (2H, dd, J = 1.1, 5.9Hz), HRMS (ESI) Calcd for $\text{C}_9\text{H}_{10}\text{NaO}_2$ ($\text{M}+\text{Na}^+$) 173.0578, found 173.0574.

3) Synthesis of compound 4

To a stirred solution of compound **3** (3.5 g, 23 mmol) in 400 mL of dioxane, DDQ (5.82 g, 25.3 mmol, 1.1 equiv) was added; reaction was stirred at room temperature for 30 min, filtered over celite and concentrated. The solid residue was purified by flash column chromatography (hexane/EtOAc: 9:1) to give *trans-p*-coumaraldehyde (**4**) as an amorphous light yellow solid in 71% yield.

^1H NMR (500 MHz, CD_3OD): δ 9.55 (1H, d, J = 7.8 Hz), 7.57 (1H, d, J = 15.85Hz), 7.53-7.56 (2H, m), 6.83-6.86 (2H, m), 6.59 (1H, dd, J = 8.05, 15.9Hz), HRMS (ESI) Calcd for $\text{C}_9\text{H}_8\text{NaO}_2$ ($\text{M}+\text{Na}^+$) 171.0422, found 171.0416.

4) Synthesis of Compound 5

To a stirred solution of *trans-p*-coumaraldehyde (**4**) (2.1 g, 14 mmol) in dry DCM (60 mL) were added imidazole (1.9 g, 28 mmol, 2 equiv) and *tert*-butyldiphenylsilyl chloride (4.3 mL, 15.4 mmol, 1.1 equiv) at room temperature. The reaction mixture was stirred for 4 h, evaporate off DCM and add water, extracted with EtOAc (3 x 150 mL). The combined organic extracts were dried over MgSO_4 , filtered and concentrated. The residue was purified by flash column chromatography (hexane: ethyl acetate = 9.5:0.5) to give **5** as an amorphous white solid in 76% yield.

^1H NMR (500 MHz, CDCl_3): δ 9.62 (1H, d, J = 8.0 Hz), 7.74-7.70 (4H, m), 7.48-7.32 (9H, m), 6.81 (2H, dt), 6.54 (1H, dd), HRMS (ESI) Calcd for $\text{C}_{25}\text{H}_{26}\text{NaO}_2\text{Si}$ ($\text{M}+\text{Na}^+$) 409.1594, found 409.1590.

5) Microwave assisted synthesis of compound 6

To a microwave reaction vial (2-5 mL) of compound **5** (500 mg, 1.29 mmol) in ethyl vinyl ether (1.5 mL, 15.48 mmol, 12 equiv) were added hydroquinone (80 mg, 0.774

mmol, 0.6 equiv), n-butanol as a co-solvent to reduce vapor pressure (1 mL). The vial is tightly teflon capped and placed inside the microwave and temperature was set to 220 °C, for 6 h, the resulting reaction mixture was concentrated and purified by flash chromatography (hexane: ethyl acetate = 9.7:0.3) to give **6** as a pale yellow oil in 81% yield. Obtained diastereomeric mixture product was confirmed by ¹H NMR (500 MHz, CDCl₃), ¹³C NMR (125 MHz, CDCl₃). HRMS (ESI) Calcd for C₂₉H₃₄NaO₃Si (M+Na⁺) 481.2169, found 481.2167.

6) Synthesis of compound 7

To a stirred solution of compound **6** (3.387 g, 7.38 mmol) in 40 mL of THF, TBAF (8.85 mL, 8.86 mmol, 1.2 equiv) was added; reaction was stirred at room temperature for 3 h, concentrated and extracted with Et₂O, dried over MgSO₄. The residue was purified by flash column chromatography (hexane/EtOAc: 9.5:0.5) to give *trans-p*-coumaraldehyde (**4**) as an amorphous light yellow solid in 83 % yield.

¹H NMR (500 MHz, CDCl₃): δ 7.09-7.14 (4H, m), 6.75-6.78 (4H, m), 6.41-6.42 (1H, dd, J = 2.2, 6.1 Hz), 6.36-6.38 (1H, dd, J = 2.4, 6.3), 5.02-5.04 (2H, m), 4.82-4.84 (1H, m), 4.70-4.72 (1H, m), 3.85-3.98 (2H, m), 3.53-3.63 (4H, m), 2.07-2.24 (2H, m), 1.77-1.87 (2H, m), 1.22-1.27 (6H, m) HRMS(ESI) Calcd for C₂₅H₂₆NaO₂Si (M+Na⁺) 243.0997, found 243.0987.

7) Synthesis of compound 8

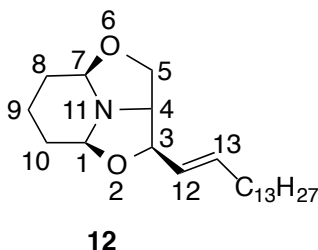
To a stirred solution of compound **7** (1.29 g, 5.89 mmol) in 40 mL of DMF, K₂CO₃ (0.814 g, 8.835 mmol, 1.5 equiv), KI (97.8 mg, 0.589 mmol, 0.1 equiv), Merrifield resin (1.427 g, 6.479 mmol, 1.1 equiv) was added; reaction was stirred at 90 °C for 30 min, filtered the resin using glass crucible, washed with water, acetone, hexane, EtOAc, MeOH repeatedly. The structure of product **8** was confirmed from its IR spectrum by comparing with that of model compound **8m**.

Almost all of the IR absorptions showed good agreement between **8** and **8m** except for the characteristic band of **8** around 1720 cm⁻¹ which is attributable to the C=O stretch of the aldehyde produced from the partial hydrolysis.

8) Synthesis of compound 9

The resin **8** (1.42 g, 5.89 mmol) in 40 mL of dioxane, was swelled for an hour at room temperature, followed by addition of conc. HCl (11.5 mL), refluxed at 60 °C for 4 h. The hydrolyzed resin was filtered and washed with water, acetone, hexane, EtOAc, MeOH repeatedly. The structure of product **9** was confirmed by IR spectrum and indicates its a mixture of **9a** and **9b**.

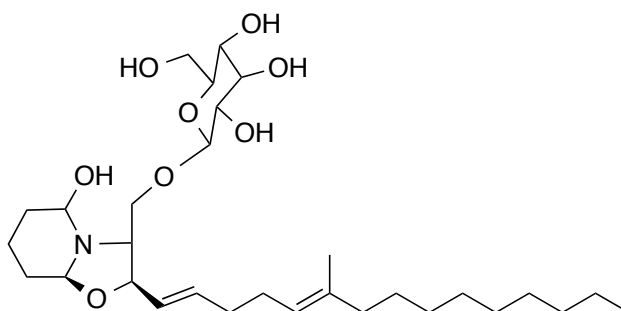
Synthesis of model compound 12



To a stirred solution of *D-erythro* sphingosine (**1**) (17 mg, 56.8 μmol) in toluene (1 mL) was added glutaraldehyde (5.6 M in water, 11 mL, 61.6 μmol, 1.1 eq) at room temperature, and the reaction mixture was stirred overnight. After evaporation of toluene, the aqueous layer was extracted with ethyl acetate (3 x 10 mL). The combined organic extracts were dried over MgSO₄, filtered and concentrated. The residue was purified by flash column chromatography (hexane:ethyl acetate = 3:1) to give **15** as a colorless oil in 52% yield.

¹H NMR (500 MHz, CDCl₃): δ 5.82 (1H, dtd, *J* = 15.5, 6.6, 0.8 Hz, H-13), 5.52 (1H, ddt, *J* = 15.5, 7.5, 1.6 Hz, H-12), 4.51 (1H, brt, H-1), 4.39 (1H, brt, H-7), 4.30 (1H, brddd, *J* = 7.5, 6.8, 0.8 Hz, H-3), 4.10 (1H, dd, *J* = 9.4, 1.7 Hz, H-5a), 3.64 (1H, td, *J* = 6.8, 1.7 Hz, H-4), 3.49 (1H, dd, *J* = 9.4, 6.8 Hz, H-5b), 2.17-2.05 (4H, m, H-14, H-10a and H-8a), 1.85-1.74 (3H, m, H-10b, H-8b and H-9a), 1.44-1.35 (2H, m, H-15), 1.36-1.31 (1H, m, H-9b), 1.31-1.20 (20H, m, H-16, H-17, H-18, H-19, H-20, H-21, H-22, H-23, H-24 and H-25), 0.88 (3H, t, *J* = 7.0 Hz, H-26). HRMS (APCI) Calcd for C₂₃H₄₂NO₂ (M+H⁺) 364.3210, found 364.3207.

Synthesis of model compound 13



13

To a stirred solution of glucosyl 9-methyl sphingadienine (10 mg, 22.2 mmol) in toluene (1 mL) was added glutaraldehyde (5.6 M in water, ~3 mL, 24.42 mmol, 1.1 eq) at room temperature, and the reaction mixture was stirred overnight. After evaporation of toluene, the aqueous layer was extracted with ethyl acetate (3 x 10 mL). The combined organic extracts were dried over MgSO_4 , filtered and concentrated. The residue was redissolved in methanol and measured ESI-mass spectrum to confirm the structure. Measured mass: 537.98 ($\text{M}+\text{H}-\text{H}_2\text{O}$).

Extraction of free sphingosine from human serum

To 100 μL of serum, 400 μL of MeOH was added and the mixture was centrifuged at 20 $^\circ\text{C}$, 3000 rpm, for 15 min. After centrifugation, upper layer (350 μL) was taken out and that solution was added to resin as human serum extract.

Human serum extract was added to 5 mg of resin in Libra tube, swollen with 100 μL THF for an hour. After 1.5 h capturing by vortex stirring at room temperature, the solution was filtered and the resin was washed off with water, chloroform, and methanol. Followed by swollen the resin again with 100 μL of THF for 1h, and 200 μL of 0.25 M TFA was added as a releasing reaction. After 1 h vortex stirring, solution was filtered. The filtrate was analyzed for sphingosine with OPA derivatisation (**Figure 7**).

The pH of the filtrate was adjusted to around 5 with boric acid buffer to make OPA reagent react to sphingosine effectively. To 50 μL of filtrate, same amount of OPA reagent was added, and the mixture was vortex-mixed vigorously for 15 min at

room temperature. Following that, 100 μL of mobile phase (85:15 acetonitrile/phosphate buffer pH 7.0) was added and 20 μL of solution was injected to the HPLC on YMC-Pack C8 column (0.46 cm ϕ x 15 cm) using acetonitrile-phosphate buffer pH 7.0 (85:15). The fluorescence detector was set at 340 nm and 450 nm as an excitation and an emission wavelength respectively. The flow rate was 1.0 mL/min.

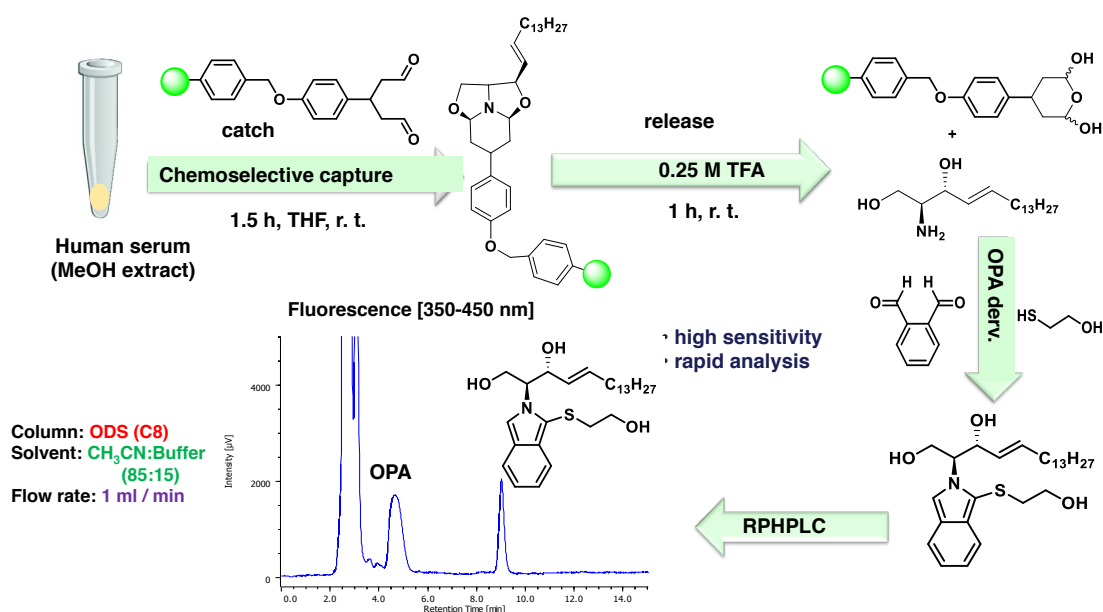


Figure 7. Schematic representation extraction of sphingosine from human serum

Extraction of fungal sphingoids from golden oyster

About 1g of dried fruiting body of golden oyster was extracted with methanol, concentrated and 20 mL of dioxane and 20 mL of 10% (w/v) Ba(OH)₂ were added and refluxed at 110 °C for 24 h. After hydrolysis 40 mL of 2%(w/v) (NH₄)₂SO₄ was added to precipitate barium ions and vortexed, centrifuged, the centrifugate was extracted with diethyl ether (50 mL x 3). Ether layer was concentrated, dried and redissolved in MeOH (3 mL). Capturing and releasing of free sphingoids were carried out as described above by adding 200 μL of hydrolyzed mushroom extract as a sample. And analyzed under same condition as described above, except the flow rate was maintained as 0.5 mL/min.

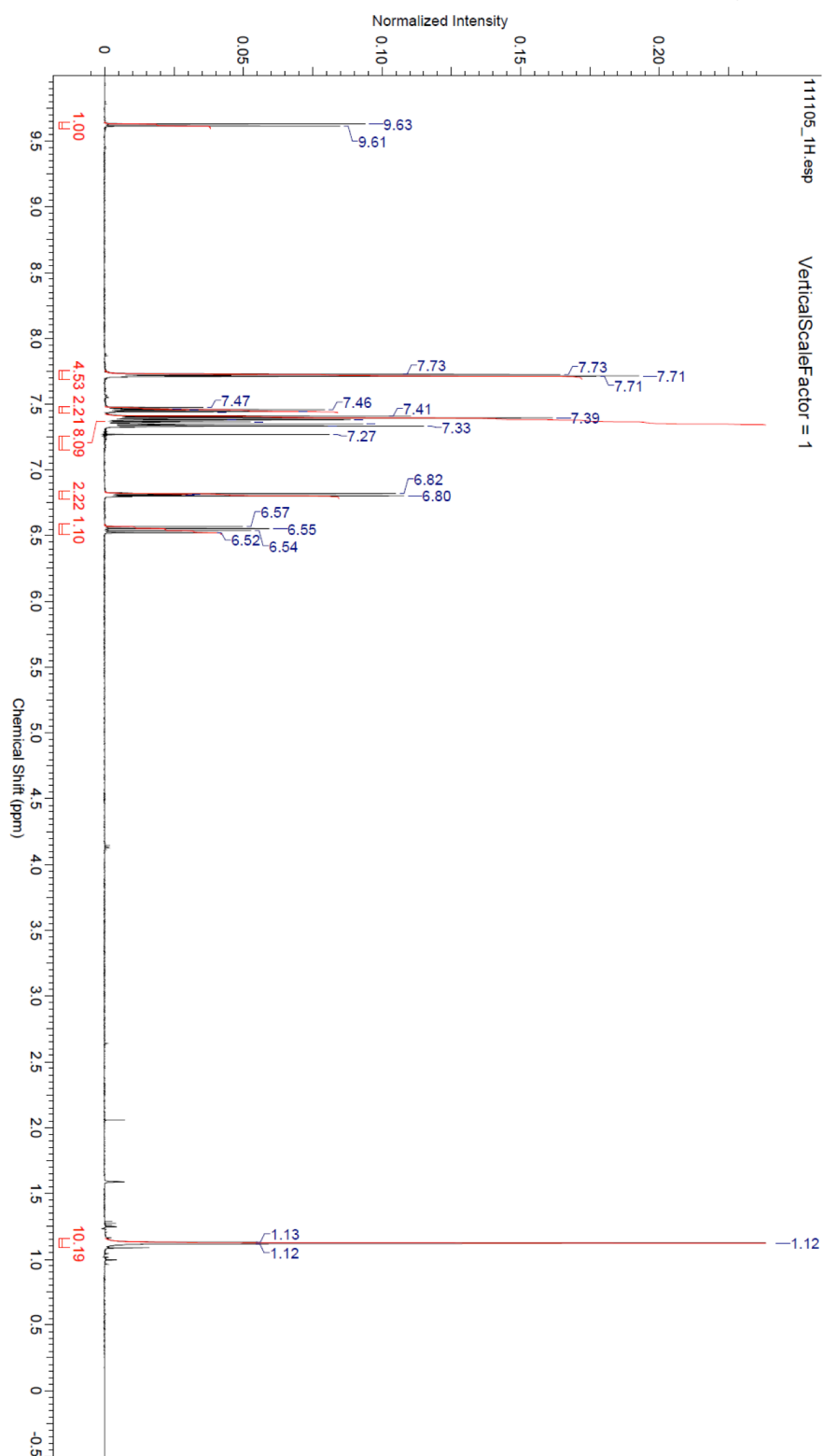
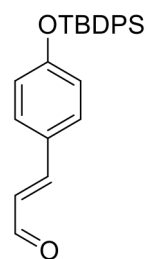
Extraction of free sphingosine from human serum

To 100 μL of serum, 400 μL of MeOH was added and the mixture was centrifuged at 20 $^{\circ}\text{C}$, 3000 rpm, for 15 min. After centrifugation, upper layer (350 μL) was taken out and that solution was added to resin as human serum extract.

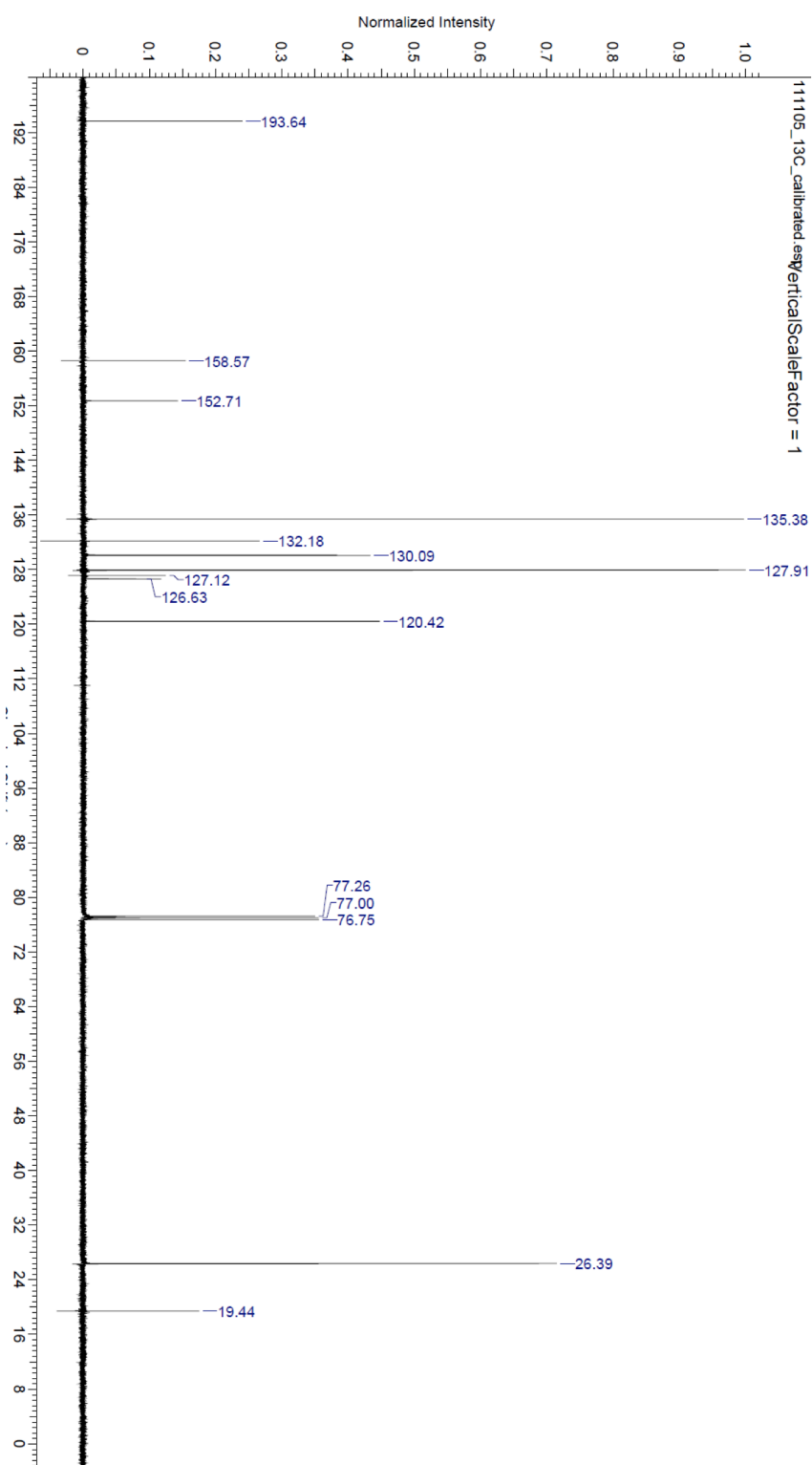
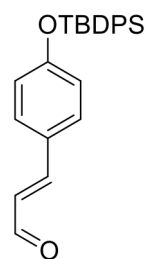
Human serum extract was added to 5 mg of resin in Libra tube, swollen with 100 μL THF for an hour. After 1.5 h capturing by vortex stirring at room temperature, the solution was filtered and the resin was washed off with water, chloroform, and methanol. Followed by swollen the resin again with 100 μL of THF for 1h, and 200 μL of 0.25 M TFA was added as a releasing reaction. After 1 h vortex stirring, solution was filtered. The filtrate was analyzed for sphingosine with OPA derivatisation.

The pH of the filtrate was adjusted to around 5 with boric acid buffer to make OPA reagent react to sphingosine effectively. To 50 μL of filtrate, same amount of OPA reagent was added, and the mixture was vortex-mixed vigorously for 15 min at room temperature. Following that, 100 μL of mobile phase (85:15 acetonitrile /phosphate buffer pH 7.0) was added and 20 μL of solution was injected to the HPLC on YMC-Pack C8 column (0.46 cm ϕ x 15 cm) using acetonitrile-phosphate buffer pH7.0 (85:15). The fluorescence detector was set at 340 nm and 450 nm as an excitation and an emission wavelength respectively. The flow rate was 1.0 mL/min.

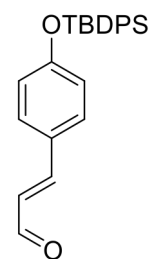
¹H-NMR spectrum of **5**



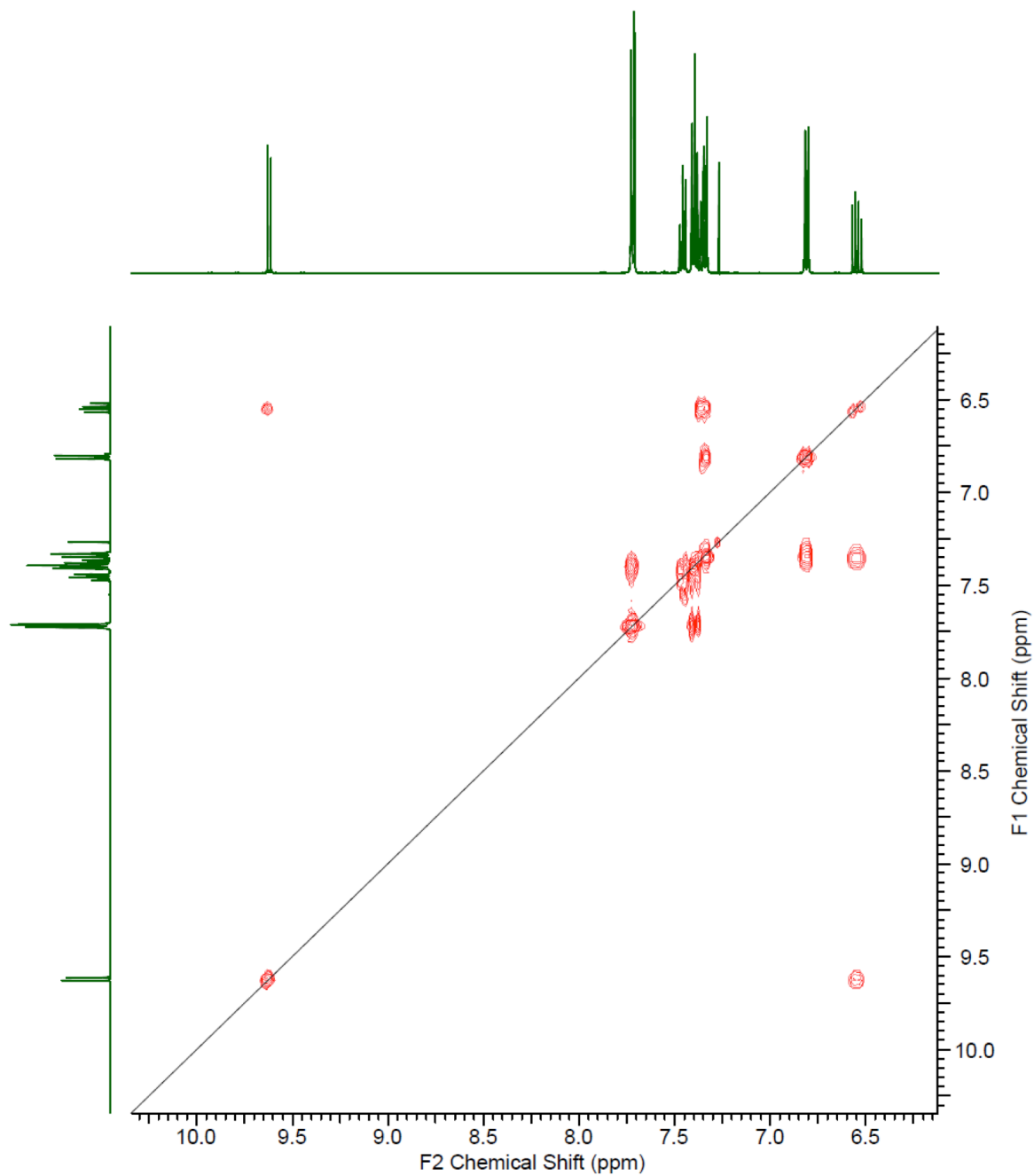
^{13}C -NMR spectrum of **5**



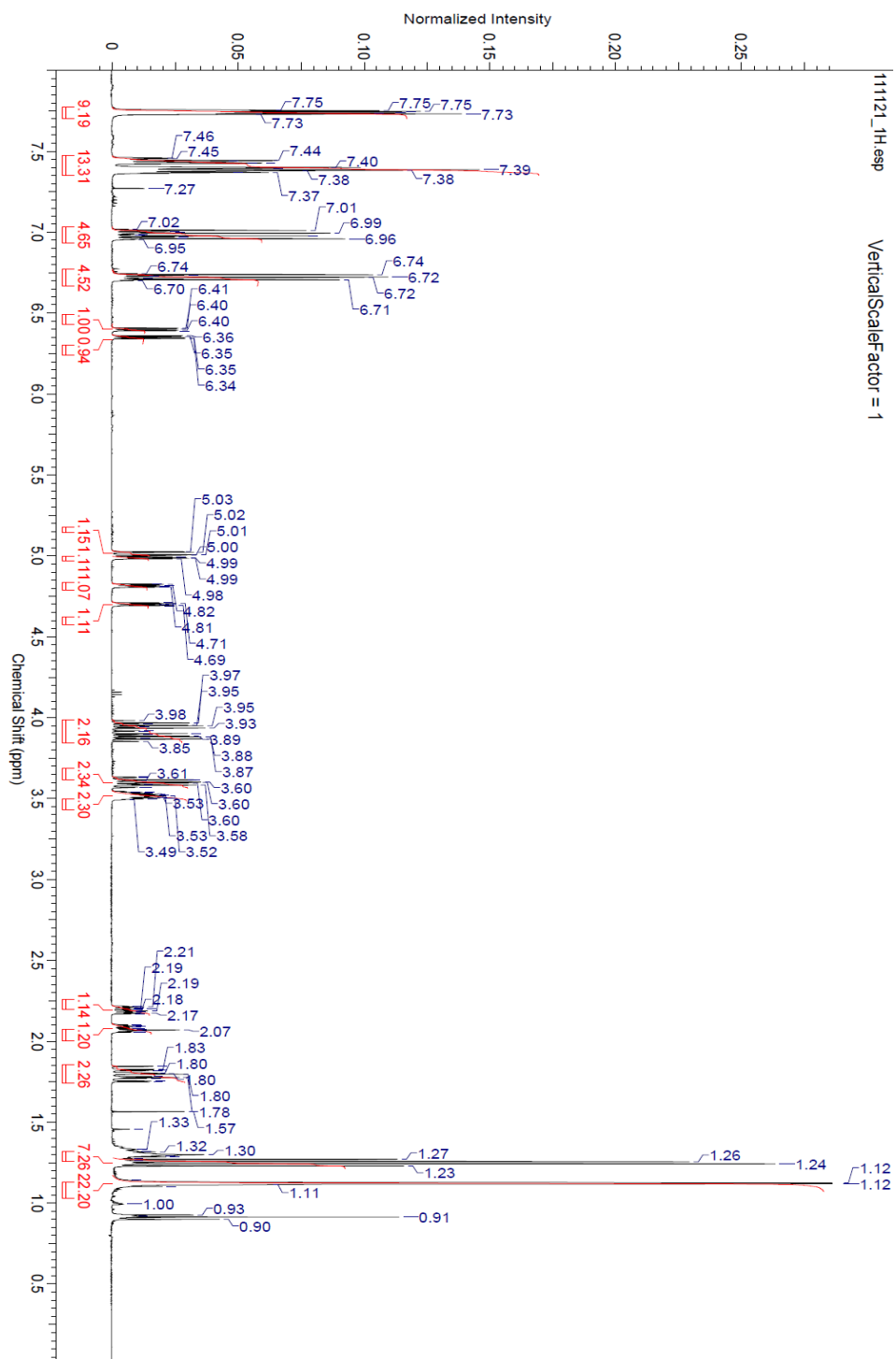
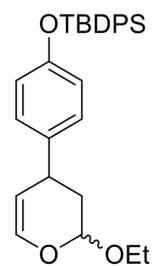
$^1\text{H}, ^1\text{H}$ -COSY spectrum of **5**



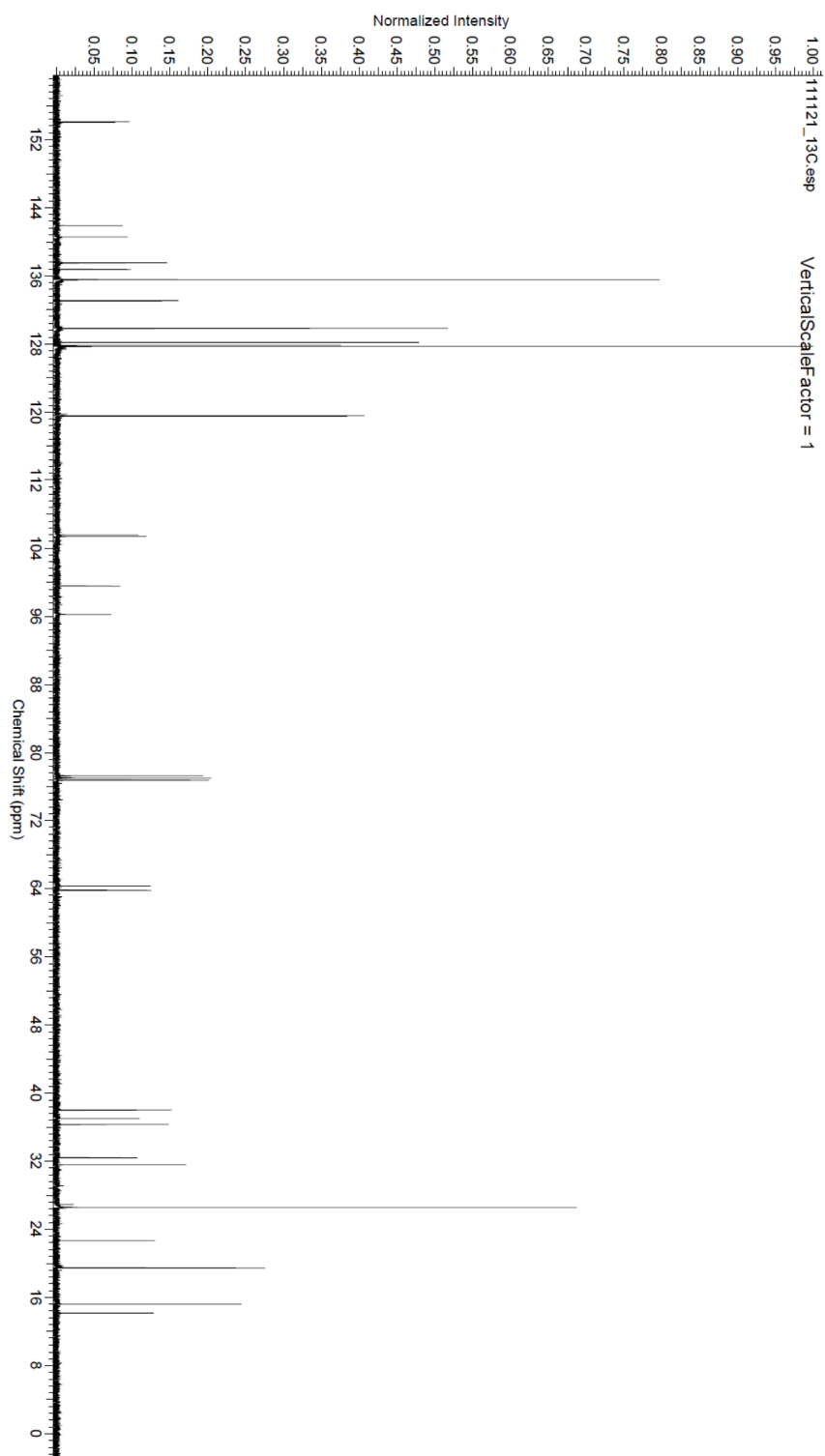
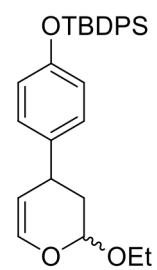
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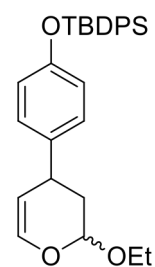
¹H-NMR spectrum of **6**



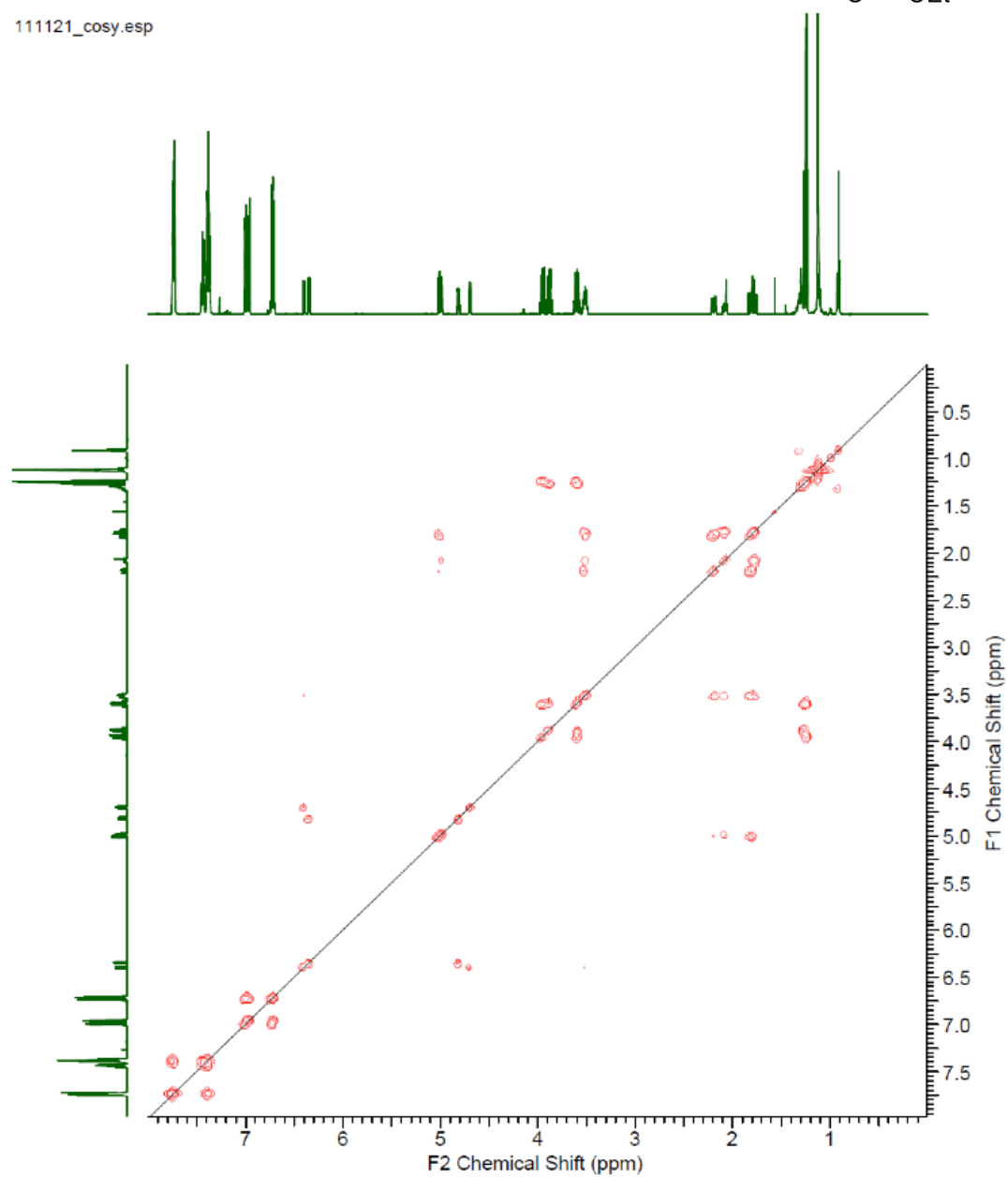
^{13}C -NMR spectrum of **6**



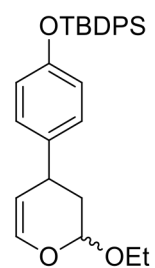
^1H , ^1H -COSY spectrum of **6**



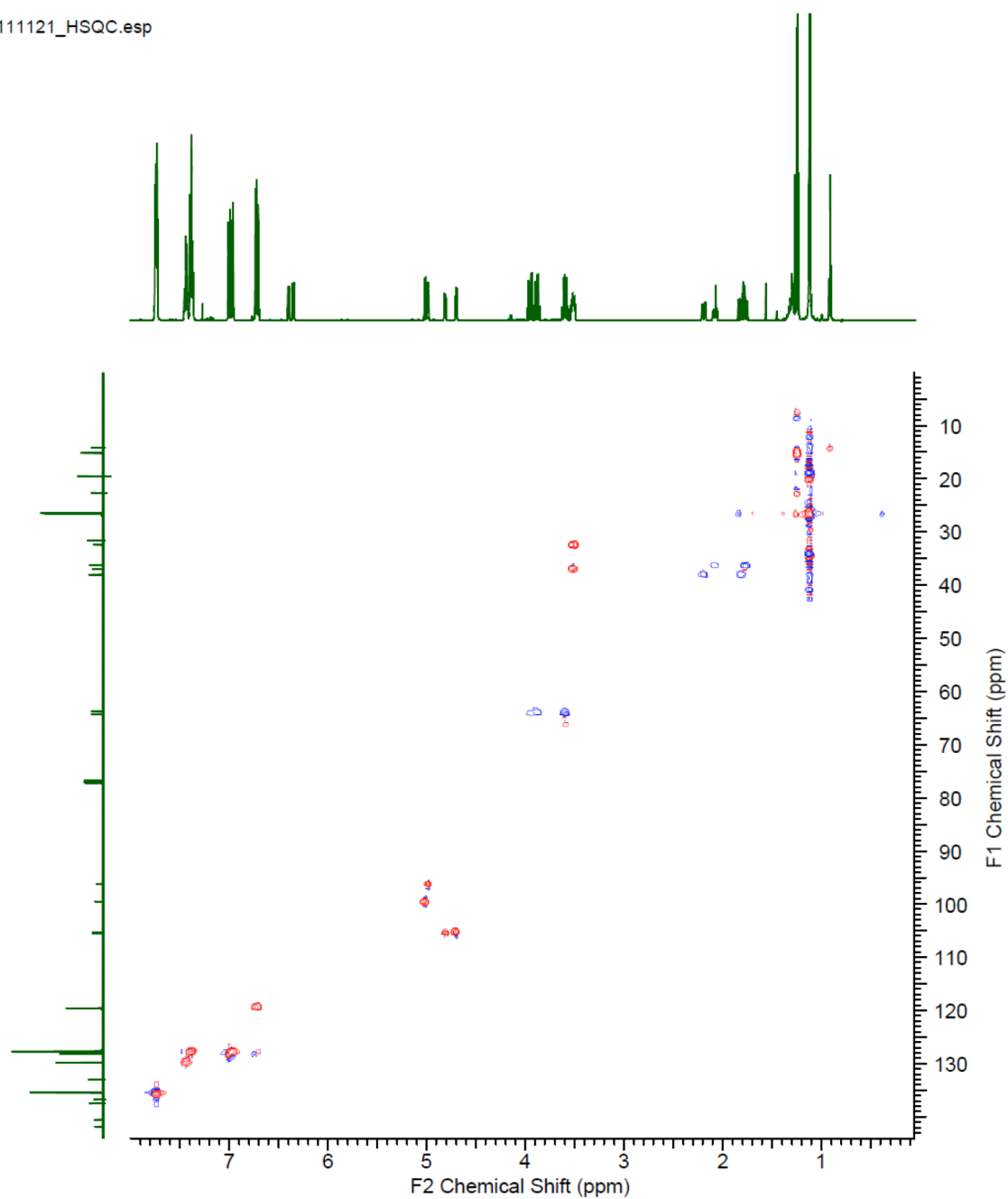
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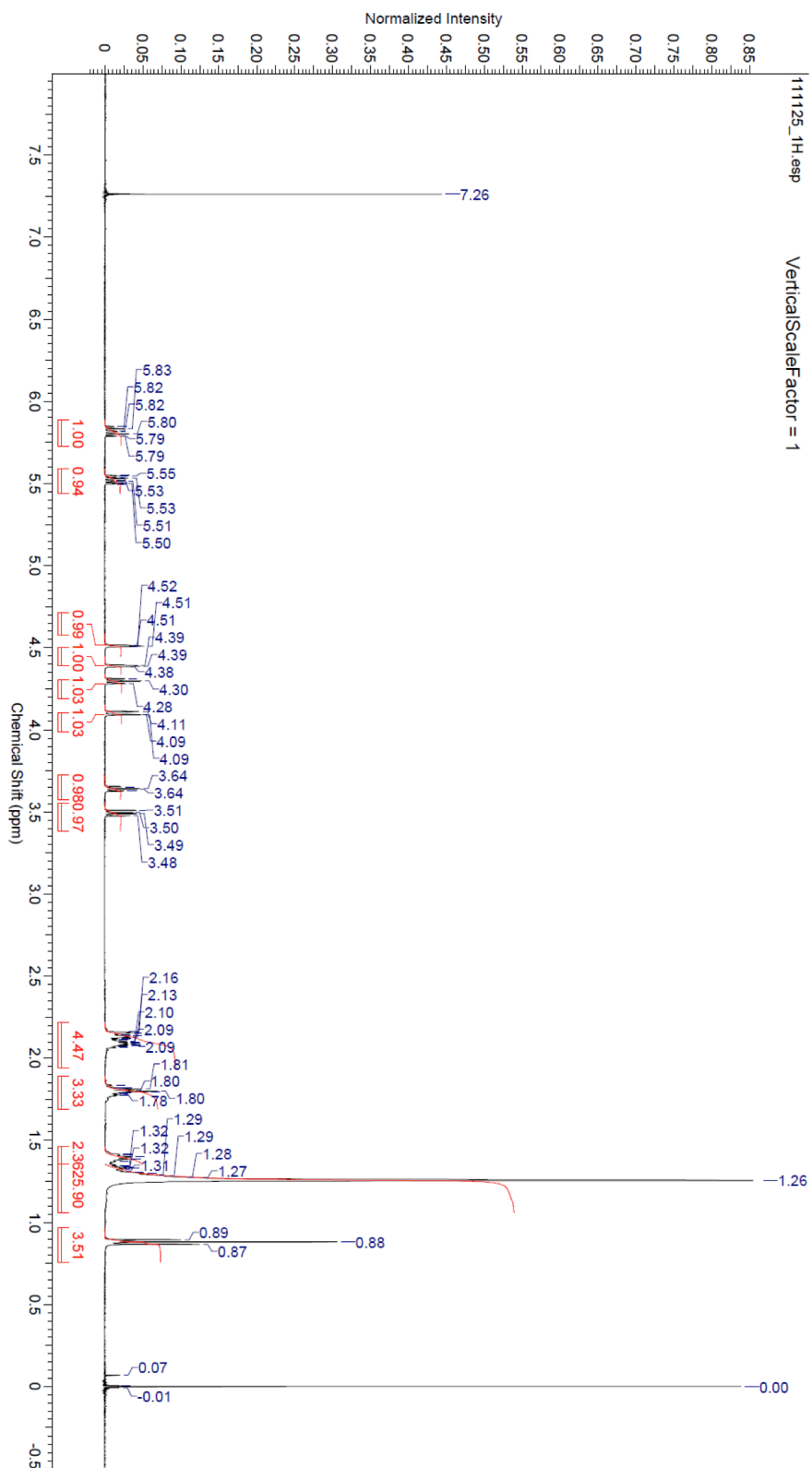
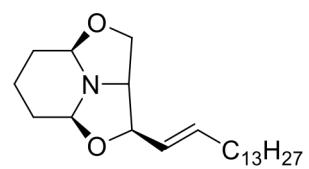
HSQC spectrum of **6**



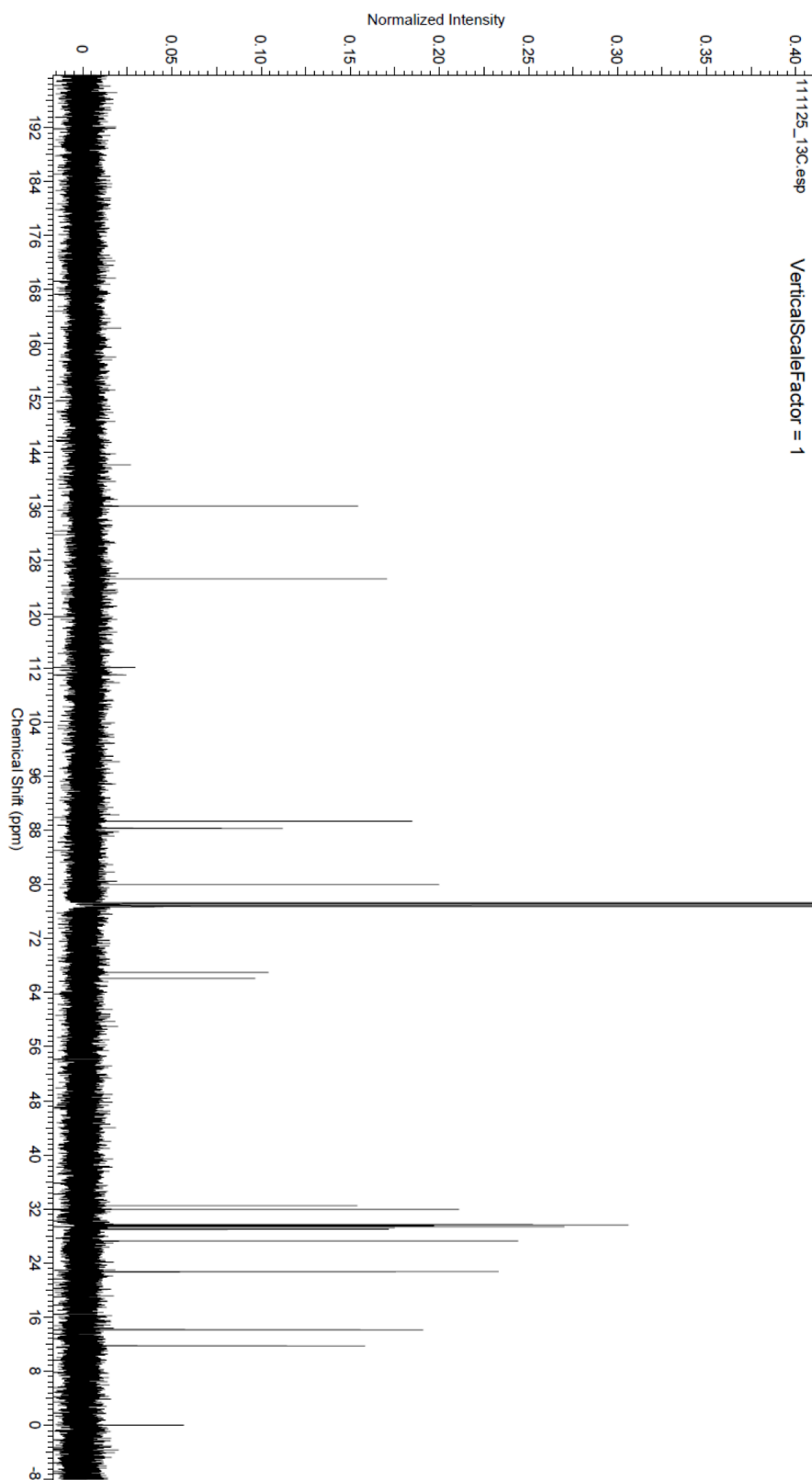
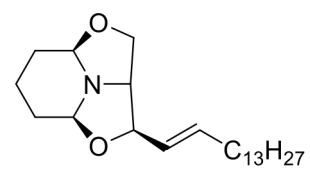
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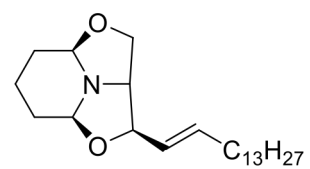
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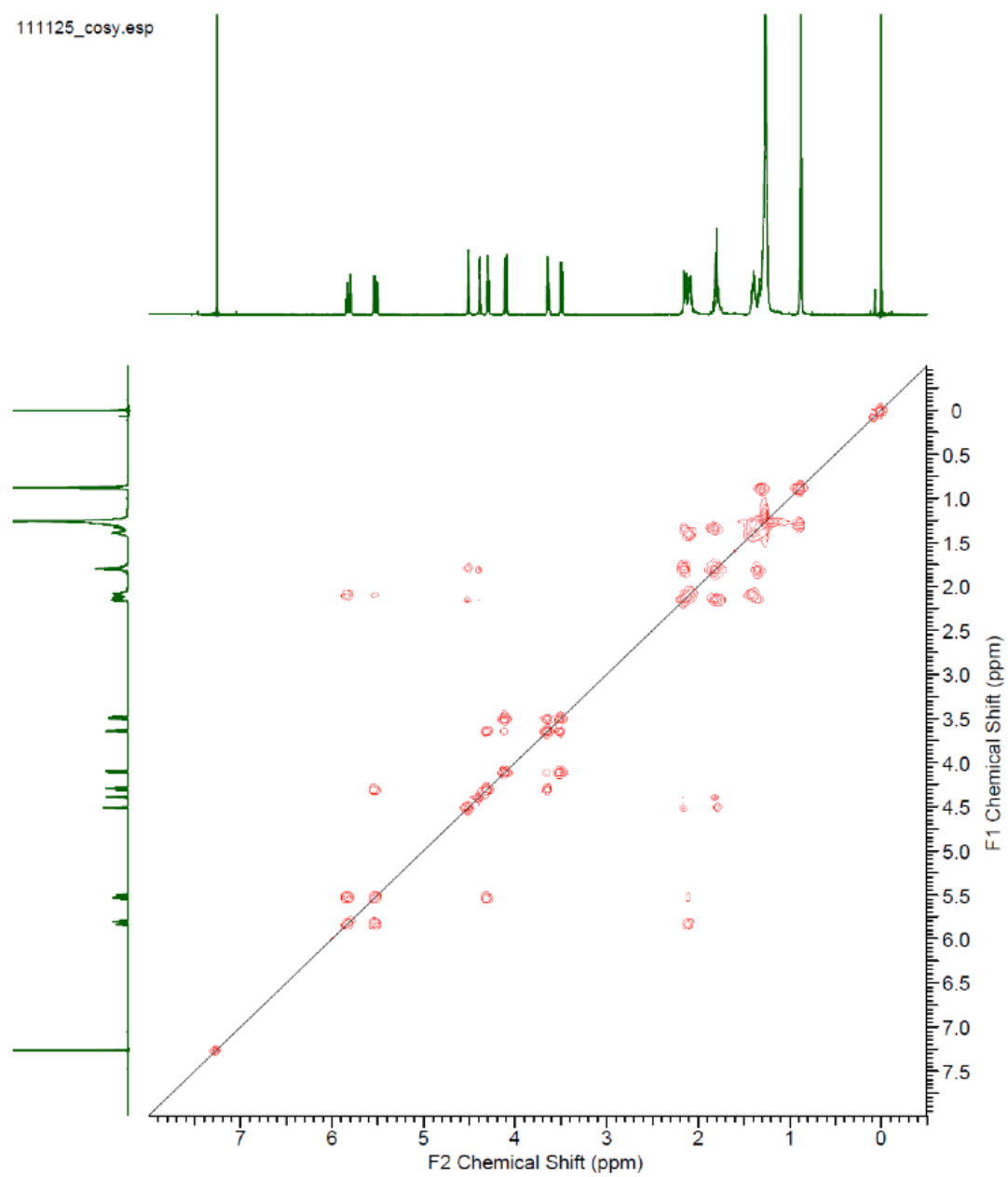
^{13}C -NMR spectrum of **12**



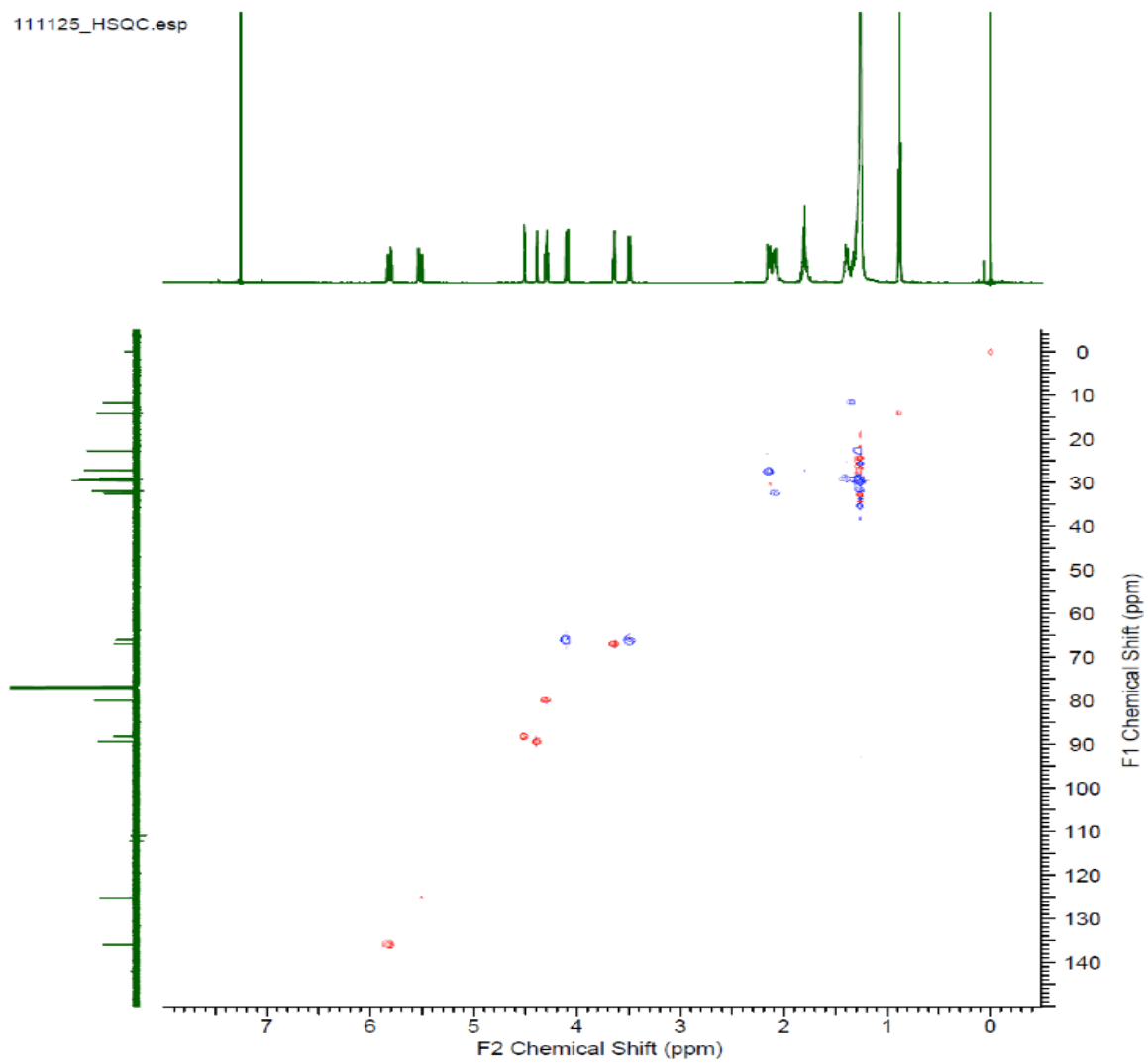
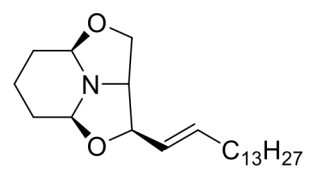
^1H , ^1H -COSY spectrum of **12**



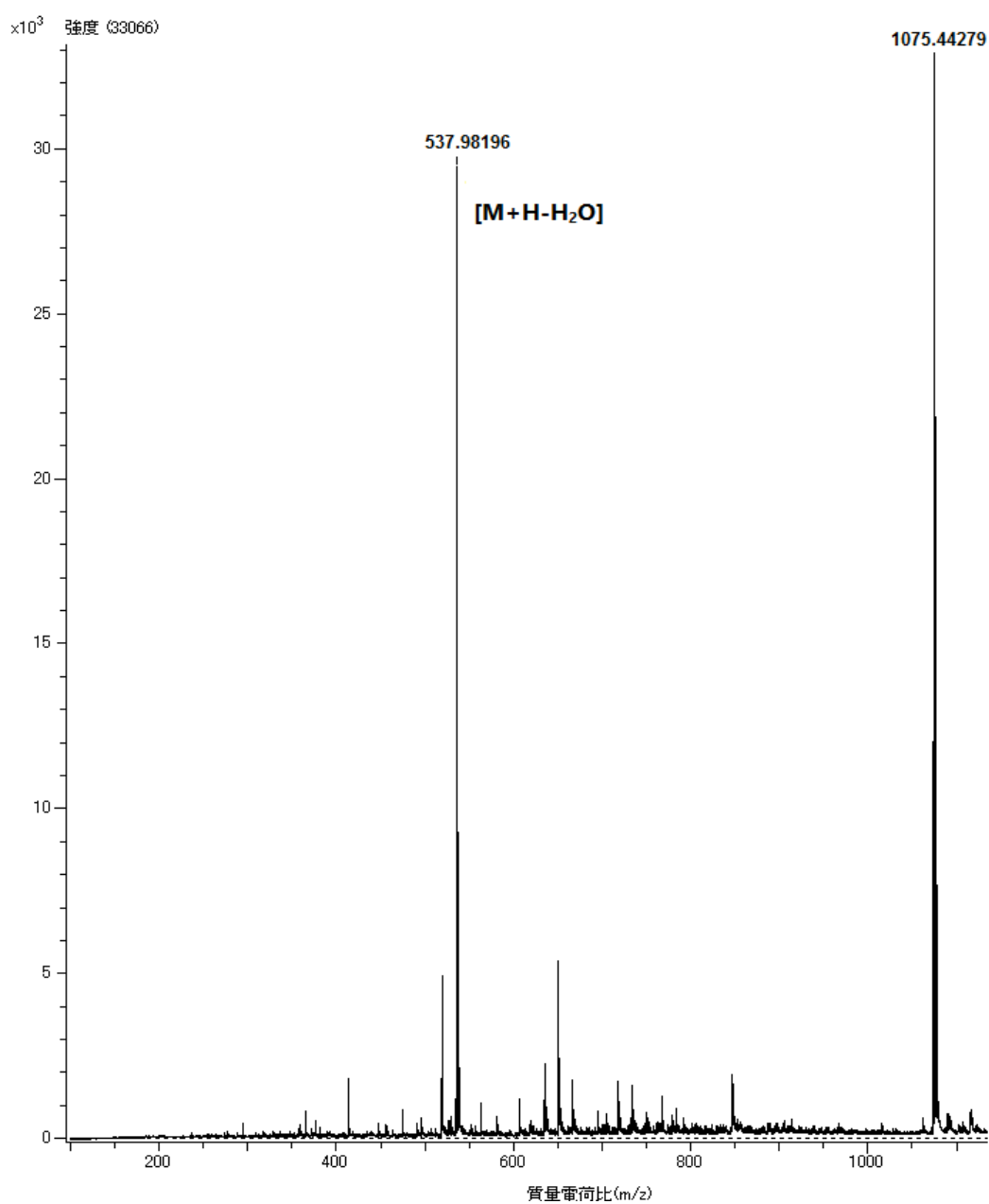
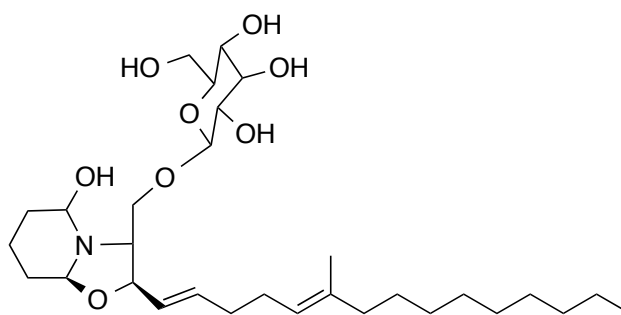
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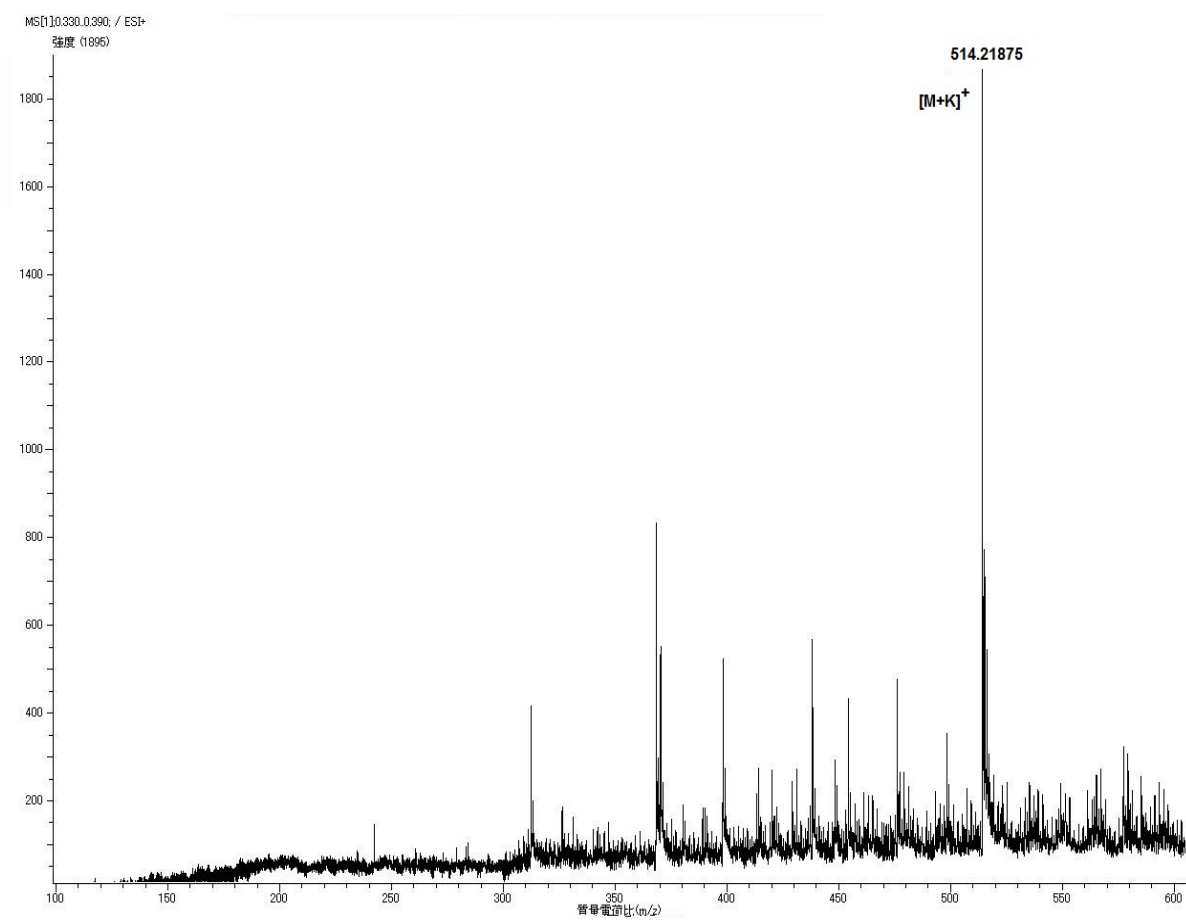
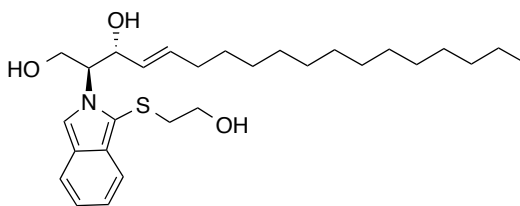
HSQC spectrum of **12**



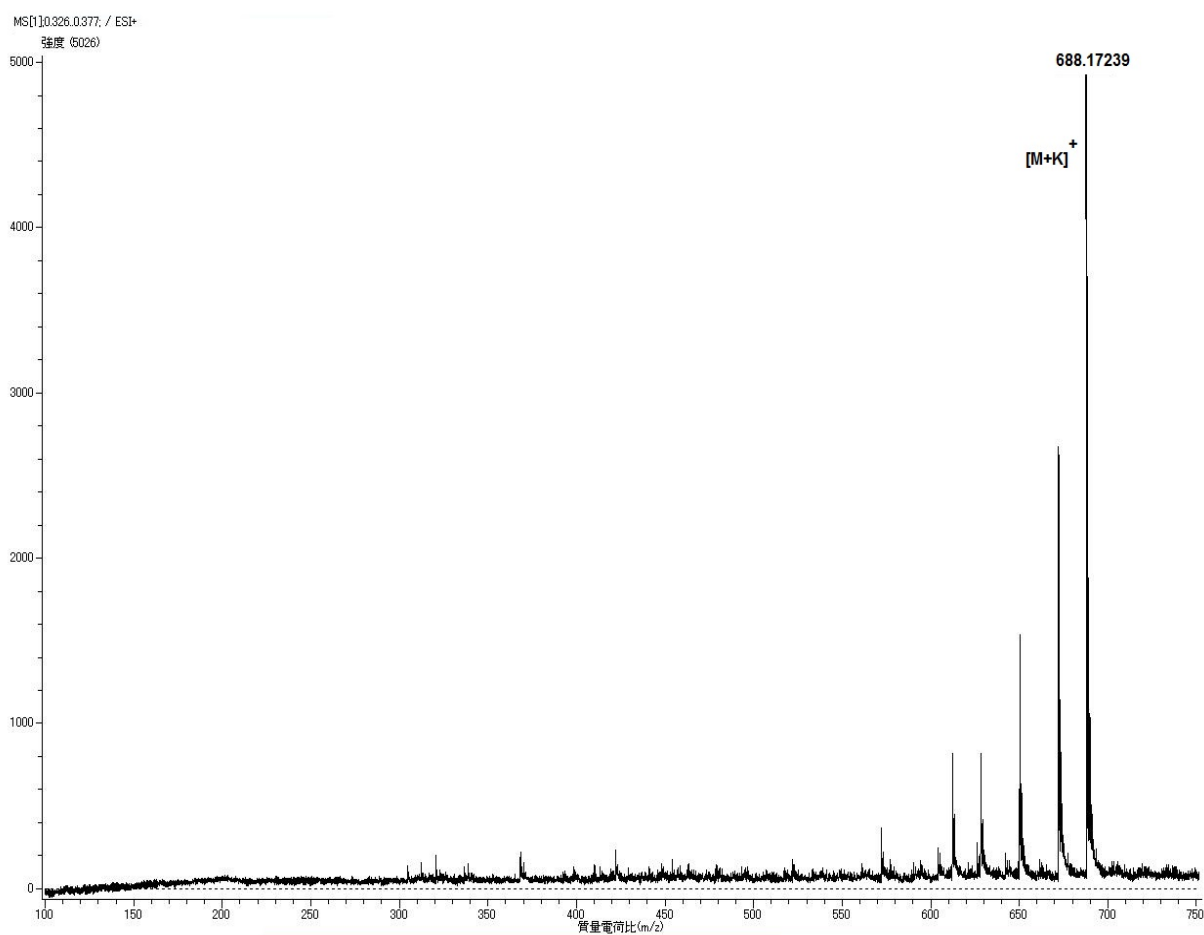
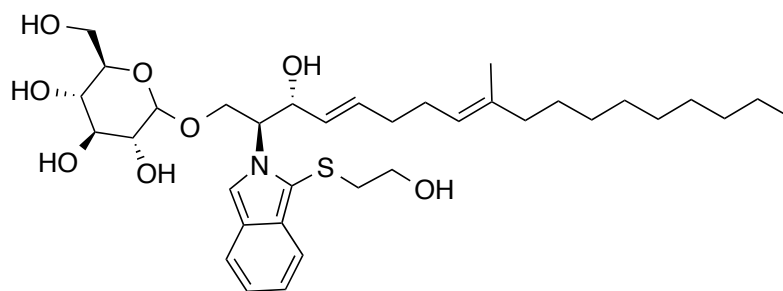
ESI-Mass Spectra of **13**



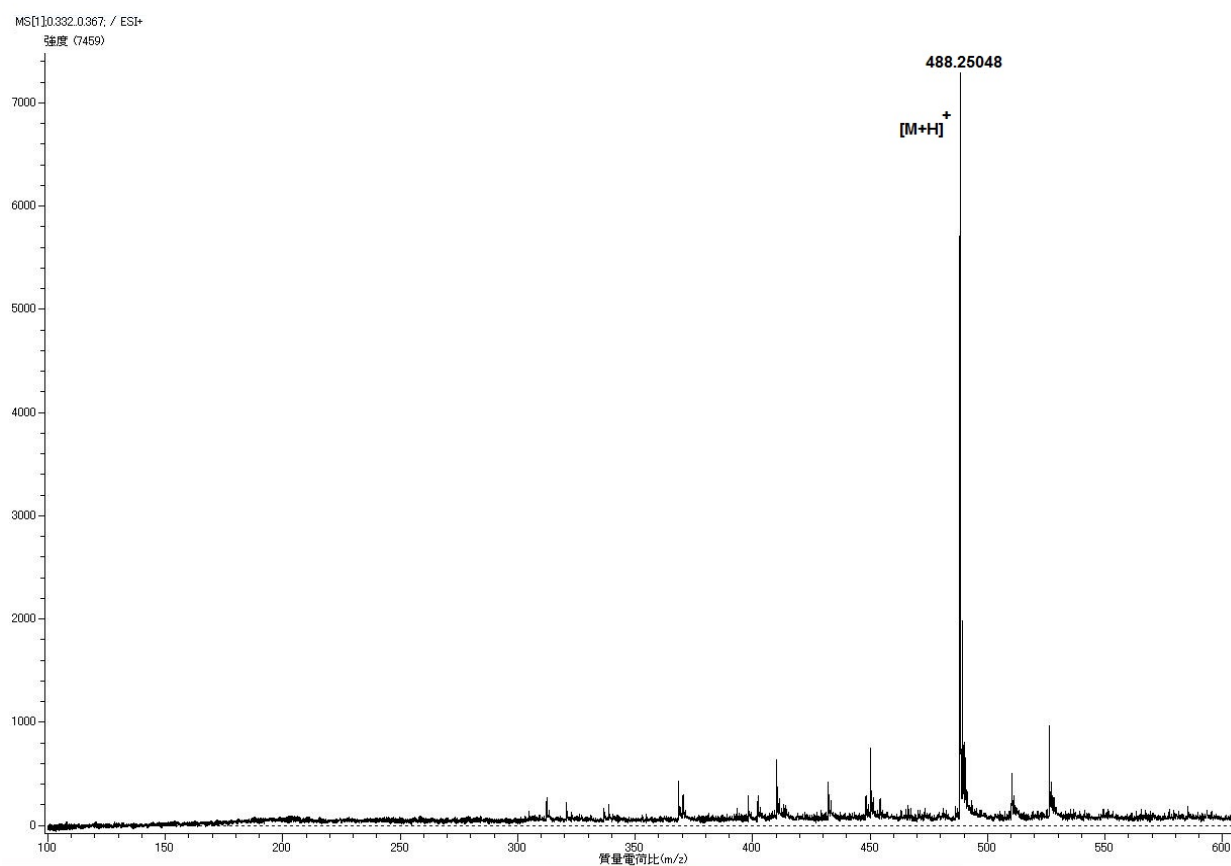
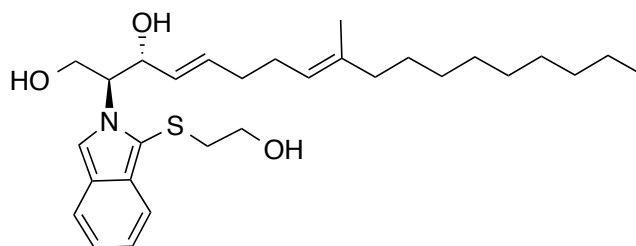
ESI-Mass Spectra of **sphingosine - OPA** complex



ESI-Mass Spectra of **glc-9-Me-d18^{4t,8t}** - **OPA** complex



ESI-Mass Spectra of **9-Me-d18^{4t,8t}** - OPA complex



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Chapter 3

Highly efficient preparation of sphingoid bases from glucosylceramides by chemoenzymatic method

3.1 Abstract

Sphingoid base derivatives have attracted increasing attention as promising chemotherapeutic candidates against lifestyle diseases such as diabetes and cancer. Natural sphingoid bases can be a potential resource instead of those by time-consuming total organic synthesis. In particular, glucosylceramides (GlcCers) in food plants are enriched sources of sphingoid bases, differing from ones of animals. Several chemical transformation methodologies from glucosylceramides to sphingoid bases have already investigated, however, these conventional methods using acid or alkaline hydrolysis are not efficient ones due to poor reaction yield, producing complex by-products and resulting separation problems. In this study, an extremely efficient and practical chemoenzymatic transformation method has been developed using microwave-enhanced butanolysis of GlcCers and large amount suppliable almond β -glucosidase for its deglycosylation reaction of lysoGlcCers. The method is superior to conventional acid/base hydrolysis methods in its rapidity, its reaction cleanness (no isomerization, no rearrangement) with excellent over-all yield.

3.2 Introduction

Glucosylceramides (GlcCers) are found in plants, animals, enriched in human epidermis, and fungi as one of the major lipid components¹⁻². GlcCer is composed of a sphingoid base whose amino group at C-2 position is N-acylated with a fatty acid and hydroxyl group at C-1 position is glycosylated with a glucose. LysoGlcCers are defined as GlcCers lacking of fatty acids, and found rarely with relatively small amounts in their plasma membranes. Further lacking of its glucose part lead to sphingoid bases, which are basic and characteristic structural units of GlcCers with more than 60 structural variations in nature.³

Although sphingoid bases possess basic structures of sphingolipids family, few research have been investigated due to small abundance in nature. It has been reported that the sphingoid bases have a wide range of biological activity, such as anticoagulant, mycostatic and bacteriostatic properties as well as changes in circulation dynamics.⁴ Recently, considerable attention has been devoted towards their properties against human colon cancer, intestinal cancer, breast cancer and various other pathogens.⁵⁻⁷ To investigate detailed biological activities, supply of diverse and enough amounts of sphingoid bases is essential. Natural sphingoid bases can be expected as resource instead of those by time-consuming total organic synthesis. In particular, glucosylceramides (GlcCers) in food plants are enriched sources of sphingoid bases, differing from ones of animals. The conventional transformation from GlcCers to sphingoid base is accompanied by acid or alkali hydrolysis reaction with heating. However, these methods have practical disadvantages as a purification problem and its low efficiency.

In the case of acid-catalyzed hydrolysis reaction, isomerization and degradation were frequently observed. This undesired reaction is caused by its allylic alcohol group, which readily react with solvents such as methanol leading to introduction a methoxy group at C-3 position, also which reacts as an ester via *N*-acyl migration, and more allylic rearrangements.^{3,8} These complex reactions caused a separation problem as well as a low yield. While in the case of base-catalyzed hydrolysis, the β -glycosidic bond is much more resistant than N-acylated bond against alkali hydrolysis and thus, the reaction does not produce sphingoid bases but lysoGlcCers as a major product.⁹⁻¹⁰ Besides acid or base-catalyzed hydrolysis, oxidative-reductive-hydrolysis method was also used to prepare ceramides from

GlcCers and further to synthesize sphingosine by alkali hydrolysis ¹¹, however the method is relatively laborious and N-acyl linkages of the GlcCer must be protected during the hydrolysis.

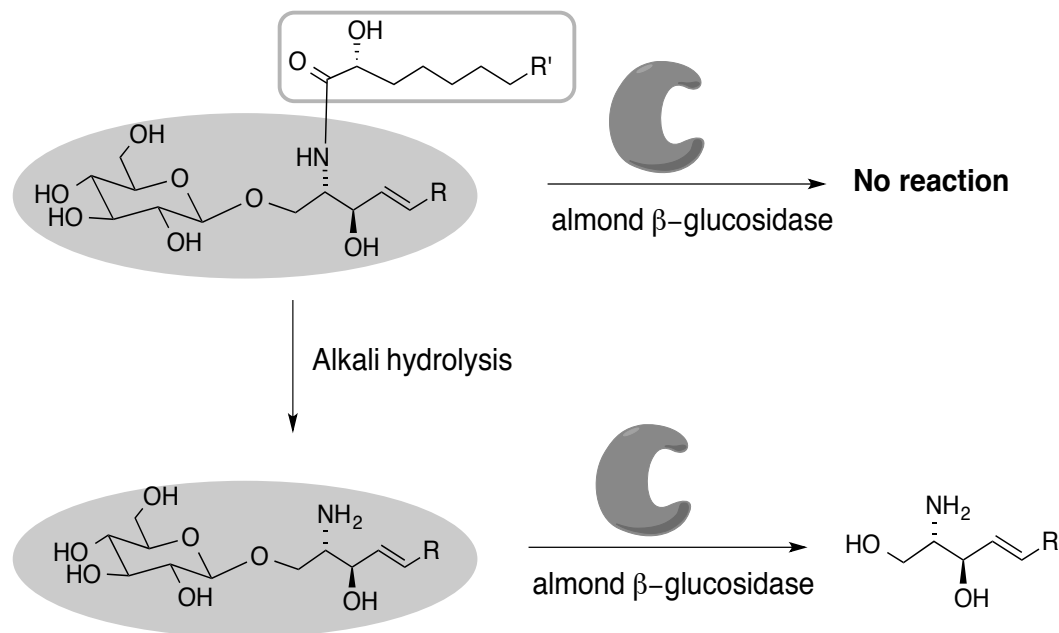


Figure 1. Chemoenzymatic methodology for the preparation of sphingoid bases

In this study, a novel chemoenzymatic method was developed to prepare sphingoid bases from various kinds of GlcCers with high efficiency. The first step is performed by an alkali-catalyzed hydrolysis reaction accelerated by microwave irradiation leading selective and efficient cleave of the amide bond to obtain lysoGlcCers. The second step is accomplished by an enzymatic hydrolysis of the β -glycosidic bond in GlcCers by almond β -glucosidase for industrial use (**Figure 1**). The removal of the acyl chain will increase the hydrophilic character to increase accessibility toward its enzymatic active center of β -glucosidase. In fact, hydrolysis of original GlcCer from golden oyster mushroom (*P. citrinopileatus*) by almond β -glucosidase didn't work even after adding Triton X-100. Our strategy to increase accessibility toward the enzyme by removing the hydrophobic fatty acid chain by microwave-assisted efficient basic hydrolysis is quite unique and practical. Several sphingoid bases were successfully prepared in high yield by employing the designed method from a wide variety of GlcCers. This novel method provides a new way to

prepare various kinds of sphingoid bases from GlcCers of dietary natural resources such as rice, wheat, and soy suitable for practical use.

3.3 Results and discussion

Characterization of GlcCer from golden oyster mushroom

GlcCer **1** was isolated from golden oyster mushroom by the method as previously reported ¹⁴. The residue of chloroform extract was yellow oil of 64 g, which upon purification yield 2.5 g of GlcCer **1**, which has the same R_f value of 0.25 (chloroform/methanol/water = 65/16/2, v/v), as the authentic standard. The detailed analysis of GlcCer **1** by mass spectra and NMR (^1H , ^{13}C , ^1H - ^1H COSY, HMBC, and HMQC) spectra, and in comparison with the reported spectra, help us confirm it as *N*-(2-hydroxy-hexadecanoyl)-1- β -glucosyl-4*E*,8*E*-9-methyl-sphingadienine.

Comparison of acid and alkali hydrolysis products of GlcCer **1**

To know, the extent of byproduct formation, GlcCer **1** was subjected to acid and alkali hydrolysis, independently. About 140 μg of GlcCer **1**, was hydrolyzed with aqueous methanolic HCl, under reflux conditions at 90 $^\circ\text{C}$.⁹ Similarly same amount of GlcCer **1** was hydrolyzed using aqueous barium hydroxide, under reflux conditions at 110 $^\circ\text{C}$.¹²

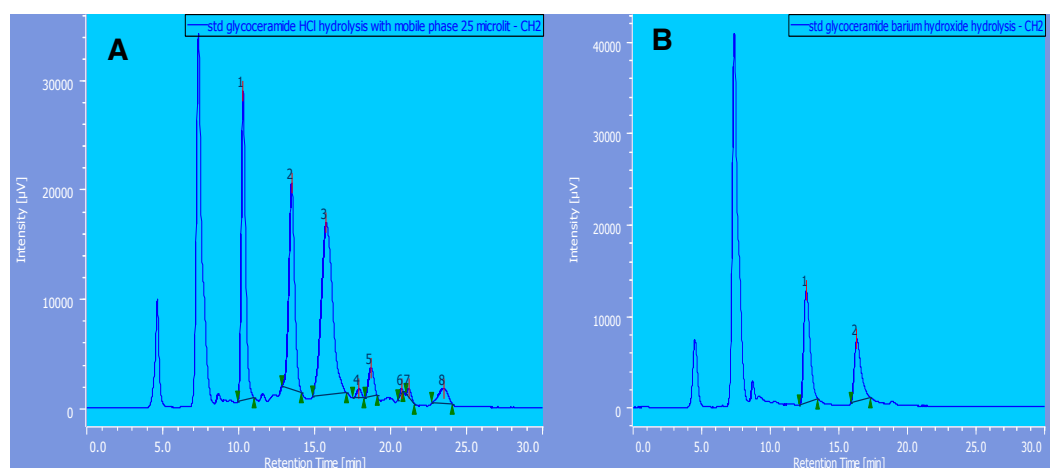


Figure 2. A. Products of acid hydrolysis, B. Products of alkali hydrolysis (HPLC conditions, Column: YMC-pack-C₈, UV 254 nm, Fluorescence 340-450 nm, solvent: methanol:buffer (pH 7) (80:20), flow rate: 0.5 mL/min)

The obtained products were extracted by bligh-dyer method, concentrated in vacuum and redissolved in methanol. The methanolic samples were analyzed by reverse-phase HPLC after *o*-phthaldehyde (OPA) derivatization. The chromatograms of OPA-derivatized products of acid (**A**) and alkali (**B**) hydrolysis are shown in **Figure 2**. Comparison of both the chromatogram indicates, acid hydrolysis produces many byproducts over alkali hydrolysis. Upon confirmation of products of alkali hydrolysis showed lysoGlcCer as a major product instead of respective sphingoid base. Hence in the present work alkali hydrolysis was preferred over acid hydrolysis and alkali hydrolysis of GlcCer **1** under various classical conditions were discussed below.

Hydrolysis GlcCer by classical methods

Two classical methods **a** and **b**, were used for the preparation lysoGlcCer starting from GlcCer. In the method **a**, 10% aq. Ba(OH)₂ was used for hydrolysis of GlcCer **1** resulting in lysoGlcCer **2** (1-β-glucosyl-4*E*,8*E*-9-methyl-sphingadienine), with 50% yield and in this method sphingoid base **3** was also produced as a minor component with a qualitative yield.¹² Another method **b** using aq. KOH, hydrolysis of GlcCer **1** resulting in lysoGlcCer **2** with 65% yield. However, these methods have major limitations in purification process and requires large amount of samples. And hence, these methods are practically invalid for small-scale preparations.

Microwave enhanced butanolysis of GlcCer

Preparation of 4*E*,8*E*-9-methyl-sphingadienine from GlcCer **1** was performed by the two-step chemoenzymatic reaction (**Figure 3**) as discussed below. Base-catalyzed hydrolysis of GlcCers by classical methods (**a** and **b**) was limited due to poor yield. In terms of the barium hydroxide hydrolysis, removal of barium ion is quite challenging, which often causes purification problems by chelating with the product, and the yield of the desired product is low. The use of KOH water solution under reflux conditions didn't push the reaction towards completion and by-products increased when the reaction was conducted for a long time under the same condition.⁹ To solve these problems, the microwave method was used instead of classical reflux method to enhance the rate of reaction in a short period of time. GlcCer **1** was subjected to microwave assisted butanolysis under a different set of experimental conditions (**Table 1**); It was found the high concentration of aqueous KOH and high

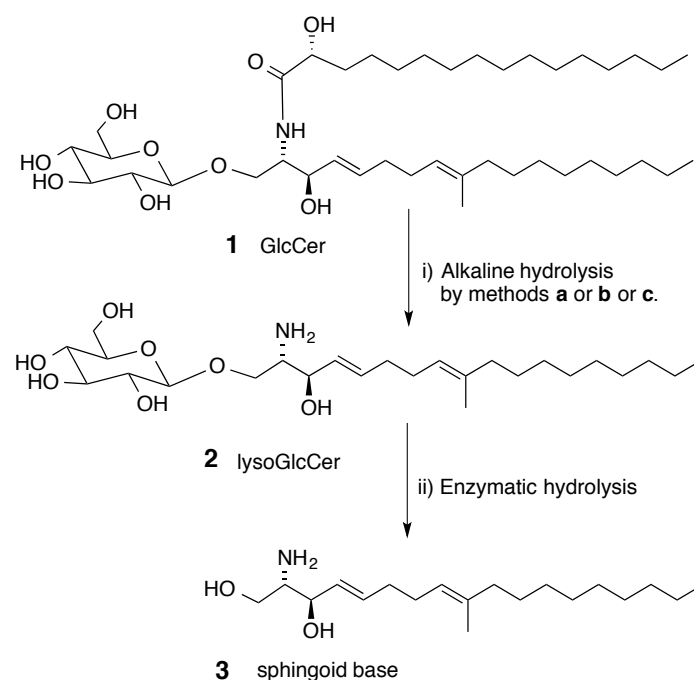
temperature favor the hydrolysis reaction to yield lysoGlcCer **2** with 92% yield. Obtained product was characterized by NMR and ESI-MS, respectively.

Table 1: Optimization of microwave assisted alkali hydrolysis GlcCer **1**^a

Entry	Aq. KOH ^b (M)	Temp (°C)	Time (min)	Yield (%)
1	1	125	180	66
2	3	125	120	60
3	6	125	90	85
4	6	110	150	72
5	12	125	60	88
6	12	125	45	92

^a In all the experiments 5 mg of GlcCer **1** was used and w.r.t it yields were calculated.

^b Different conc. of KOH were made by dissolving in distilled water and in each experiments, 1 mL aq. KOH and 1 mL n-butanol was used in each experiment.



- i) **a.** 10 % aq. Ba(OH)₂, 110 °C, 22 h, dioxane, 50 % **b.** 12 M aq. KOH, 125 °C, 4 h, reflux, butanol, 65 % **c.** 12 M aq. KOH, 125 °C, 45 min, microwave, butanol, 92 %
- ii) β-glucosidase from almonds, buffer (0.1M NaAc pH 5), incubation at 37 °C, 16 h, 90 %

Figure 3. Preparation of 4*E*,8*E*-9-methyl-sphingadienine

Hydrolysis of lysoGlcCer by β -glucosidase

The idea behind the enzymatic reaction is the decrease of hydrophobicity will enhance the solubility of lysoGlcCer and thus accelerate the reaction. Also, it was thought that lysoGlcCer molecule could fit into the active site of the enzyme more rigidly than GlcCer. Enzymatic preparation of sphingoid base from lysoGlcCer was demonstrated here for the first time. The lysoGlcCer **2** obtained from alkali hydrolysis was subjected to enzymatic hydrolysis using almond β -glucosidase to give sphingoid base **3** (4*E*,8*E*-9-methyl-sphingadienine) as a white amorphous powder in 90% yield and it was characterized by NMR and ESI-MS.

The positions of double bonds in sphingoid base **3** at C-4 and C-8 were unambiguously assigned from ^1H - ^1H COSY by the observed correlations between H-3 and H-4, H-4 and H-5, H-5 and H-6, H-6 and H-7, H-7 and H-8. Sphingoid base **3** was acetylated and measured the ^1H -NMR to obtain the coupling constant between H4 and H5. The large vicinal coupling constant ($J = 15.6$ Hz) between H-4 and H-5 was observed, which confirms the geometry around its C4/C5 double bond is trans (*E*). ^{13}C NMR chemical shift of C-19 methyl group (δ_{C} 14.6) in turn supports C-8 is trans, as demonstrated by comparison of the chemical shifts of the C-3 methyl groups in the *E* (δ_{C} 15.4) and *Z* (δ_{C} 22.7) isomers of 3-methyl-3-hexene¹⁴. The presence of a C-19 allylic methyl group in sphingoid base is confirmed by the HMBC spectrum, in which correlation between H-8 and C-19 was observed and also in ^1H NMR indicates a singlet at δ_{H} 1.61.

Effect of pH and enzyme concentration on enzymatic hydrolysis

The best pH value for β -glucosidase hydrolysis of lyso-glucosylceramide to sphingoid base was found to be 5.0 (with 0.1M sodium acetate buffer). As the pH increases, enzyme activity was substantially decreased, leading to the decrease of reaction yield. The reaction was performed by keeping temperature, concentration of enzyme and substrate constant. Optimal activity of almond β -glucosidase with respect to pH showed identical results as previously reported¹⁵. The influence of enzyme concentration on the rate of hydrolysis, which is directly proportional to the reaction yield, was studied. At low concentration of the enzyme, hydrolytic rate was limited. As the concentration of enzyme increases, reaction yield increases and reaches its maximum level (**Figure 4**). When the ratio of enzyme and substrate is 1:1, the

hydrolytic activity was found to be maximum, which was tending to the formation of products.

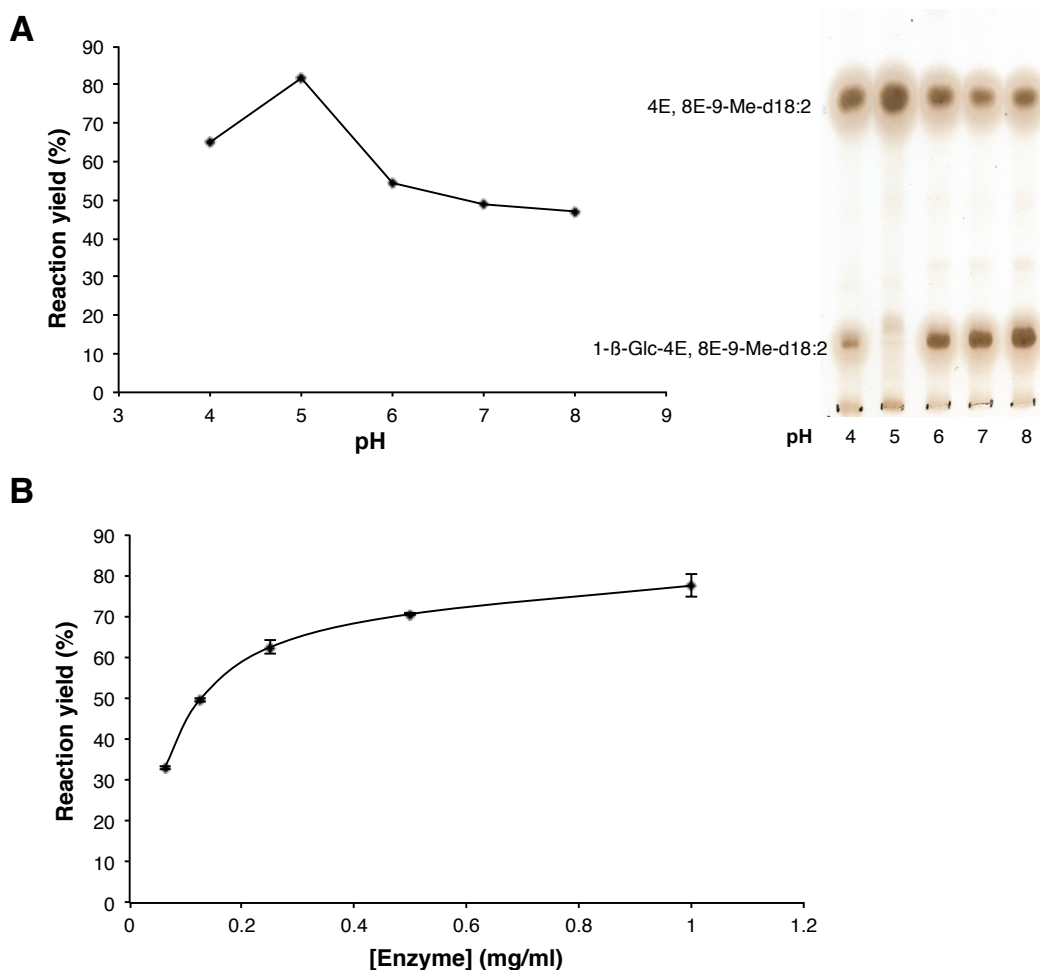


Figure 4. Influence of pH and enzyme concentration on hydrolysis

A. Effect of pH on hydrolysis of lysoGlcCer. Incubation mixtures, having 1mg of lysoGlcCer, 1mg of enzyme in 0.5 mL of buffer at various pH values were used and the mixture were incubated for 16 h at 37 °C. After Bligh-Dyer extraction, residue was re-dissolved in 100 μ L of $\text{CHCl}_3/\text{MeOH}$ (2/1) and 5 μ L was used for each lane. TLC solvent system: $\text{CHCl}_3/\text{MeOH}/\text{NH}_3$ (40/10/1), stained by cupric sulfate in phosphoric acid.

B. Effect of enzyme concentration on hydrolysis of lysoGlcCer. Incubation mixtures, having 1mg of lysoGlcCer, various concentrations of enzyme in 0.5 mL of buffer at pH 5 were used and the mixture were incubated for 16 h at 37 °C.

Application for preparation of various sphingoid bases

The accuracy and validity of the present chemoenzymatic method, employing microwave facilitated alkali hydrolysis followed by β -glucosidase treatment, were confirmed with a variety of GlcCers from alimentary natural resources. The various commercially available glucosylceramide mixtures from rice, wheat and soy were purchased and hydrolyzed by this method to prepare their respective sphingoid bases (**Figure 5A**). All GlcCers hydrolyzed with more than 70% of reaction yield was calculated from their TLC data (**Figure 5B**) and the sphingoid bases obtained were characterized by ESI-MS.

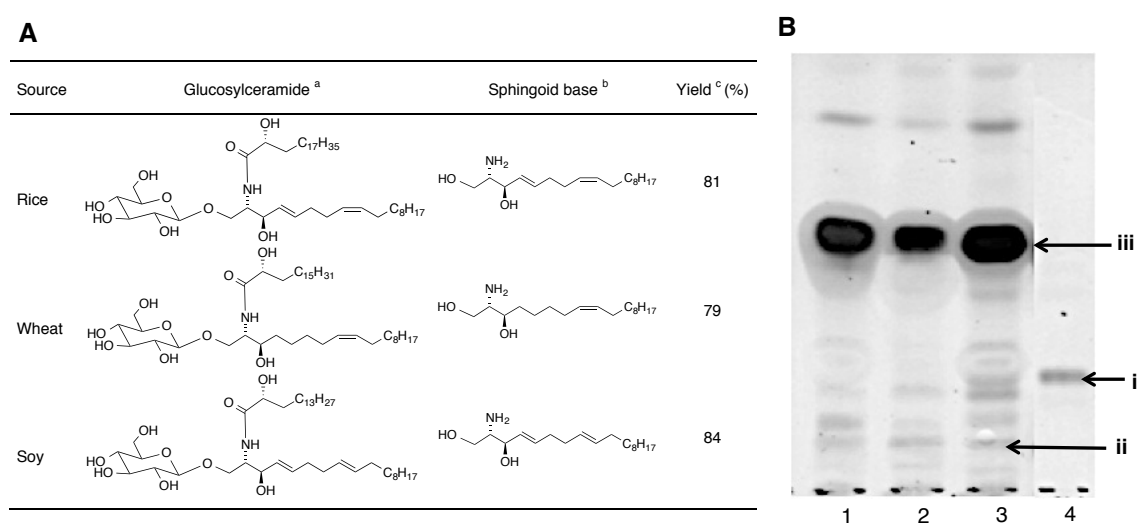


Figure 5. Preparation of different kinds of dietary sphingoid bases

A. Glucosylceramides from different sources and their respective sphingoid bases.

^a Glucosylceramide mixture was used for hydrolysis and the structure shown here is major component of it. ^b Sphingoid bases obtained are also mixture. ^c Overall yield was calculated w.r.t representative sphingoid bases.

B: TLC profiles of hydrolysed lysoGlcCer. Lane 1, rice lysoGlcCer; lane 2, wheat lysoGlcCer; lane 3, soy lysoGlcCer; lane 4, a standard GlcCer (*golden oyster mushroom*). TLC plate was developed with chloroform/methanol/aqueous NH_3 (40/10/1, v/v), stained by cupric sulfate in phosphoric acid. i. Standard GlcCer, ii. lysoGlcCers from rice, wheat, soy, iii. Sphingoid bases

To show the substrate specificity of used enzyme in our experiments, galactosyl-sphingosine prepared from galactosylceramide from porcine brain was used as a substrate for almond β -glucosidase. Surprisingly, the reaction successfully gives sphingosine. In order to understand the extent of contamination of β -galactosidase in this commercial enzyme product, further β -glucosidase assay was performed using *p*-nitrophenyl (*p*NP) glucoside and *p*-nitrophenyl (*p*NP) galactoside substrates and found the enzyme used is a mixture having β -galactosidase activity too. Results of this assay showed β -glucosidase was 6 folds more reactive than β -galactosidase (**Figure 6A**) in the commercially available almond β -glucosidase (total amount of protein: 363 μ g/mL). This result suggests that the purification of the enzyme is not essential to do β -glucosidase hydrolysis of lysoGlcCer. To improve cost-effectiveness of our method, sodium acetate buffer (pH = 5) extract of partially defatted sweet-almond flour was used as an enzyme stock solution, which prepared as crude enzyme¹⁵. The total amount of protein present in the stock solution determined by bicinchoninic acid assay was 260 μ g/mL. This stock solution was used to carry out the hydrolysis of lyso-GlcCer **2** at various concentrations (**Figure 6B**). The β -glucosidase activity of our crude preparation was found to be 10% lesser than the commercial β -glucosidase, whereas giving same result as commercial one with respect to conversion of lyso-GlcCer into sphingoid bases when reaction was conducted under same experimental conditions. As for now, to the best of our knowledge, this is the most economical and efficient way to prepare sphingoid bases and hence this methodology will have wide industrial applications.

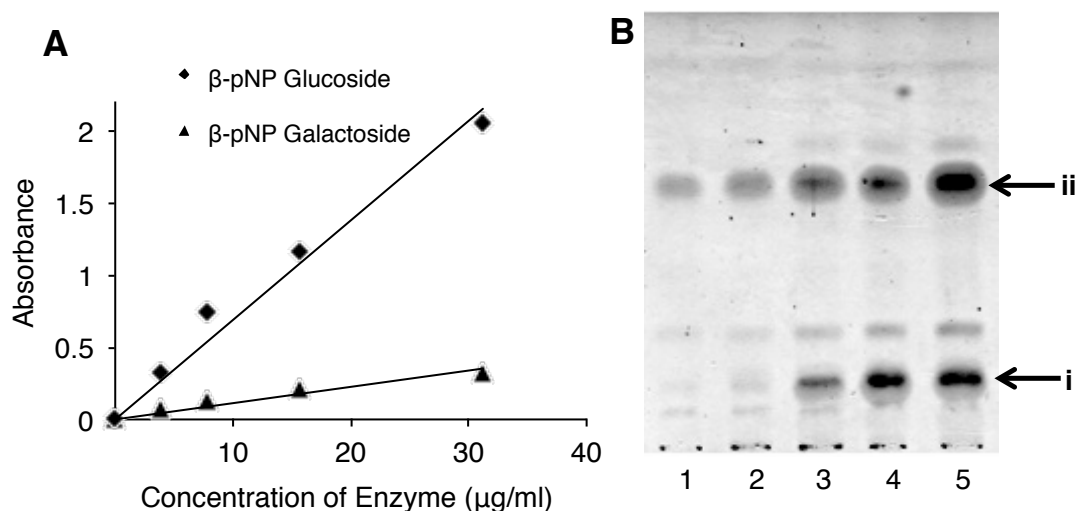


Figure 6. A. β -glucosidase assay using standard β -pNP glucoside and β -pNP galactoside substrates, absorbance was recorded using Bio-Rad micro plate reader at 405 nm. **B.** Hydrolysis of lysoGlcCer **2** by crude enzyme from almonds. The Lanes 1, 2, 3, 4, 5 are reactions at different concentrations of lysoGlcCer as 100 μ g/mL, 250 μ g/mL, 500 μ g/mL, 1000 μ g/mL. In each reaction, 1 mL of enzyme stock solution was used. i. lysoGlcCer **2** ii. Sphingoid base (4*E*,8*E*-9Me-d18:2).

To show further expandable applications of current strategy, ganglioside mixture (GM) from bovine brain was alkali hydrolyzed under microwave, to obtain lysoGM. The obtained lysoGM was subjected to β -glucosidase hydrolysis under similar conditions as described above. The preliminary TLC data (**Figure 7**) suggests that, enzyme can hydrolyses lysoGM and produce sphingosine. However, further studies are needed for complete characterization of the products and to do hydrolysis with a specific type of ganglioside.

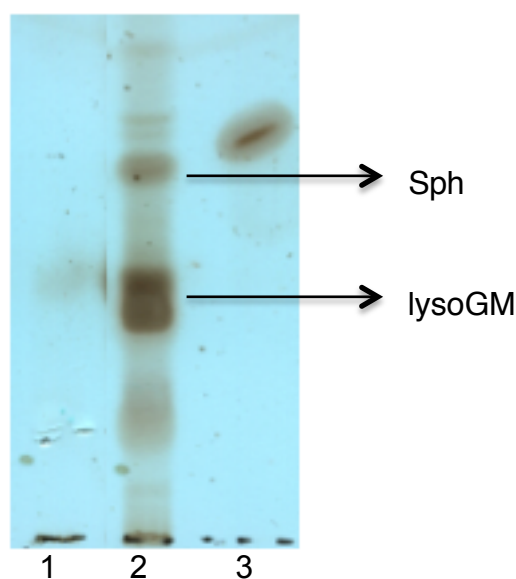


Figure 7: Hydrolysis of lysoganglioside mixture from bovine brain

1. lysoGM 2. lysoGM+ β -glucosidase 3. Sph (sphingosine)

GlcCers could be acid/base hydrolyzed to generate sphingoid bases, however its application has been limited as result of the low yield by the reaction byproducts. In this study, we present a highly efficient chemoenzymatic method to produce sphingoid bases from GlcCers, conveniently. This method is applicable to many GlcCers and is easily scalable even for large-scale preparation of lysoGlcCers and sphingoid bases.

The chemoenzymatic method described in this work has several distinguishing features, which makes it a satisfactory method for production of a high-yield sphingoid bases from GlcCers, in contrast to other previously reported methods. As discussed in earlier section, acid catalyzed hydrolysis of GlcCers could produce many kinds of by-products via rearrangements and isomerization reactions.⁸ An intermediate product of base-catalytic hydrolysis reaction, a lysosphingolipid could be reduced its by-products formation in reaction condition of methanolic HCl, however; the isomerization reactions cannot be completely prevented.¹⁶ Another chemical transformation has been examined by using an oxidative-reductive-hydrolysis method. Preparation of sphingoid bases by oxidative-reduction-hydrolysis method has two drawbacks. First, the essential three continuous reaction steps are not efficient. Second, phytosphingosine type of ceramides, which possess a vicinal diol group, is tend to be oxidized by a oxidation reagent, sodium periodate leading to complex reaction mixture.¹⁷

On the other hand, an enzymatic hydrolysis of GlcCer could be expected a mild, selective, and efficient method. In fact, a hydrolysis by β -glucosidase from ox brain has been reported as a sole enzymatic method.¹⁸ However, this method requires conversion of ceramides to sphingoid bases by either strong alkali hydrolysis or N-deacylase treatment. We have also carried out hydrolysis of GlcCer **1** using β -glucosidase in presence of triton X-100, unfortunately reactions doesn't proceeds even after 48 hr incubation at 37 °C, pH = 5. It might be due to its low solubility, and its high hydrophobicity of GlcCer **1**. This limitation make previously reported methods are inefficient for the preparation of sphingoid bases especially on large-scale. As it showed in examples, sphingoid bases were prepared only in qualitative yield under drastic conditions.^{5, 11} In contrast, extremely high yield (83%) was achieved following simple two-step protocol for the preparations of sphingoid bases from GlcCer **1** in this report.

To the best of our knowledge, no efficient methods, which can practically prepare sphingoid bases from GlcCers, have been reported. In contrast, our two step protocol has many advantages compared with all the other methods as shown below. Since, deacylation was carried out under microwave assisted conditions in yield higher than 90%, all types of acyl chains were hydrolyzed without leaving any starting material for short period. Microwave is an emerging technique to accelerate reaction, this is the first example to apply to GlcCers hydrolysis reaction. The

specific cleavage of the glycosidic bond between glucose and sphingoid base portion in lysoGlcCer with a mild reaction conditions was employed using β -glucosidase instead of drastic acid hydrolysis. The elimination of the acyl chains will increase accessibility toward its enzymatic active center of the β -glucosidase as well as enhancement polarity of the lysoGlcCer compare to GlcCer to dissolve in the aqueous phase. Furthermore, this method could be expanded for more complex glycosphingolipids such as lactosylceramides and gangliosides.

In summary, analysis and functional studies of naturally occurring sphingoid bases are an emerging area of lipid research and are promising drug leads.¹⁹ A practically efficient method for the preparation of sphingoid bases starting from naturally abundant glucosylceramides was provided. In this study, the two-stage cleavage procedure for sphingoid bases preparation was performed on a medium and small-scale. However, this procedure has a potential to be expanded to large-scale experiments. This method is a simple, economical, and reliable, which is not required a limited amount of starting material and providing a suitable tool for convenient preparation and the proper analysis of sphingoids without interference of any artifacts. Furthermore, this method may also be applicable for the preparation of sphingoid bases from other glycolipids, which helps in their structural characterization. Application of this method towards construction of natural sphingoid base chemical library and their biological studies is in progress and it will be published in near future.

3.4 Experimental section

Materials

Standard glucosylceramides mixture from golden oyster mushroom (tamogitake in Japanese), rice, wheat, and soy were purchased from Funakoshi (Tokyo, Japan). Cerebrosides from porcine brain (C18 galactosylceramide, purity >99%) were purchased from Avanti Polar Lipids (Alabaster, AL). β -Glucosidase from almonds (Amygdalase from almonds), *p*-nitrophenyl glucoside, and *p*-nitrophenyl galactoside were obtained from Tokyo Chemical Industry (Tokyo, Japan). Defatted sweet-almond flour was purchased at Asahi Food Industries (Hyogo, Japan). Ammonia solution (28% in water), n-butanol (>98%), TritonTM X-100 and ninhydrin spray (0.5% in n-butanol) were purchased from Wako Pure Chemical Industries

(Tokyo, Japan), Potassium hydroxide and barium hydroxide were purchased from Nacalai Tesque (Tokyo, Japan). All the other solvents used of reagent grade were purchased from Kanto Chemical (Tokyo, Japan). NMR solvents, C₅D₅N, CDCl₃ and CD₃OD were purchased from Cambridge Isotope Laboratories (Tewksbury, MA). A methanol extract of dried fruiting bodies of golden oyster mushroom was kindly supplied by Nissei Bio (Sapporo, Japan). Ganglioside mixture from bovine brain was kindly donated by Prof. Seigo usuki, Hokkaido University.

¹H-NMR, ¹³C, ¹H-¹H correlation spectroscopy (COSY), heteronuclear multiple quantum correlation (HMQC), and heteronuclear multiple bond correlation (HMBC), were recorded on a Varian Inova instrument (500 MHz) at 25°C in pyridine-*d*₅, CDCl₃, and CD₃OD. Chemical shifts (δ) are reported in ppm and coupling constant values (*J*) are reported in Hertz (Hz). Electrospray ionization mass spectra (ESI-MS) were obtained by a JEOL JMS-T100LP spectrometer. Optical rotation measurements were carried out by JASCO-P-1020 polarimeter. Analytical TLC was performed on 0.2 mm silica gel plates (Merck 60 F-254). Silica gel column chromatography was carried out by using silica gel (Kanto Silica Gel 60, spherical, 40-50 μm) with air flashing. Microwave reaction was performed by using Biotage® Initiator+ (as maximum temperature 300 °C and pressure 30 bar) with Biotage® microwave reaction kits (2 mL). Shaking incubator (EYELA FMC-100) was used to carry out an enzymatic reaction.

Preparation of crude enzyme from almonds

2 grams of defatted sweet-almond flour was shaken with 15 mL of 0.1M sodium acetate buffer (pH = 5), for 15 min., kept for 2 h at 4°C and filtered by using Buchner funnel to give the clear stock enzyme solution. And it was used for experiments as crude enzyme preparation.

Extraction and GlcCer Purification

Dried methanol extract of golden oyster mushroom (100 g) was extracted between chloroform/water (3/1, v/v). The chloroform layer obtained was washed with chloroform/methanol/water (3/4847, v/v, 250 mL x 3) and the combined chloroform extract was concentrated under vacuum to afford yellow oil. The yellow oil was dissolved in hexane/isopropanol (7/3, v/v, 150 mL) and kept at room temperature for

24 h. The mixture was dried under vacuum, re-dissolved in chloroform and purified by silica gel chromatography [400 g, 90 cm i.d x 16 cm] with gradient elution (from chloroform to chloroform:methanol <95/5, v/v> to chloroform:methanol <90/10, v/v>) to obtain GlcCer **1**.

¹H-NMR (500 MHz, C₅D₅N): δ 8.35-8.37 (1H, d, *J* = 8.4 Hz), δ 6.0-6.04 (1H, dd, *J* = 5.7, 15.5 Hz), 5.93-5.96 (1H, m), 5.27 (1H, br.s) 4.92-4.93 (1H, d, *J* = 7.7 Hz), 4.77-4.80 (1H, m), 4.70-4.75 (1H, m), 4.57-4.62 (1H, m), 4.50-4.55 (1H, dd, *J* = 5.6, 11.7 Hz), 4.35-4.40 (1H, dd, *J* = 5.6, 11.8 Hz), 4.20-4.28 (3H, m), 4.02-4.07 (1H, m), 3.91-3.95 (1H, br.s), 2.15-2.24 (5H, m), 2.0-2.05 (3H, m), 1.72-1.73 (2H, m), 1.63 (3H, s), 1.38-1.43 (4H, m), 1.27-1.32 (36H, m), 0.86-0.90 (6H, t, *J* = 6.4 Hz). ESI-MS *m/z*: 750.5490 [M+Na]⁺

Alkaline hydrolysis: Preparation of lysoGlcCer (2)

GlcCer **1** was deacylated by the well-known classical alkali hydrolysis methods as shown by method **a** and **b** in Figure 1, although method **c** was the most efficient method established by employing microwave. Obtained products were detected on the TLC plate by spraying with 5% H₂SO₄/methanol or ninhydrin and purified by silica gel chromatography eluting with ethyl acetate/methanol/water (4/1/1, v/v).

*Method a*¹²: *hydrolysis using Ba(OH)₂/dioxane*. GlcCer **1** (200 mg) were dissolved in 10 mL dioxane in a round bottom flask at room temperature and the solution was heated up to 110°C by oil bath. Subsequently, 10 mL of warm 10% aqueous Ba(OH)₂ were added and the reaction mixture was refluxed for 22 h at 110°C. After the reaction mixture was cooled to room temperature, 20 mL of 2% aqueous ammonium sulfate was added to precipitate barium ions, followed by addition of 20 mL of diethyl ether. The samples were shaken, vortexed and centrifuged to separate the phases. The upper ethereal layer was concentrated under vacuum, purified by silica gel chromatography by using chloroform/methanol/water (70/27/3, v/v. By using this method, sphingoid base **3** was also produced as a minor component in qualitative yield.

*Method b*¹³: *butanolysis*. GlcCer **1** (100 mg) was dissolved in 4 mL of butanol and 4 mL of KOH (12.5M, aq.) was added. The reaction mixture was refluxed at 125°C for 4 h and then cooled to room temperature. 5.3 mL chloroform/methanol/water (2/2/1.3, v/v) mixture solvent was added, followed by vortex mixing, centrifuging. Chloroform

phase was collected and the aqueous phase was extracted twice with 2 mL chloroform. Combined chloroform extract was concentrated under vacuum and purified by silica gel column chromatography by using chloroform/methanol/water (70/27/3, v/v).

Method c. Microwave- assisted butanolysis. GlcCer (50 mg) was suspended in 1 mL of butanol in a 2 mL screw capped microwave vial. 1 mL KOH (12M, aq.) was added and the reaction mixture was stirred under the microwave with high absorption at 125°C for 60 min. After the microwave treatment, lysoGlcCer **2** (1- β -glucosyl-4*E*,8*E*-9-methyl-sphingadienine) was extracted from the reaction mixture and purified by the same procedure as described in method **b**.

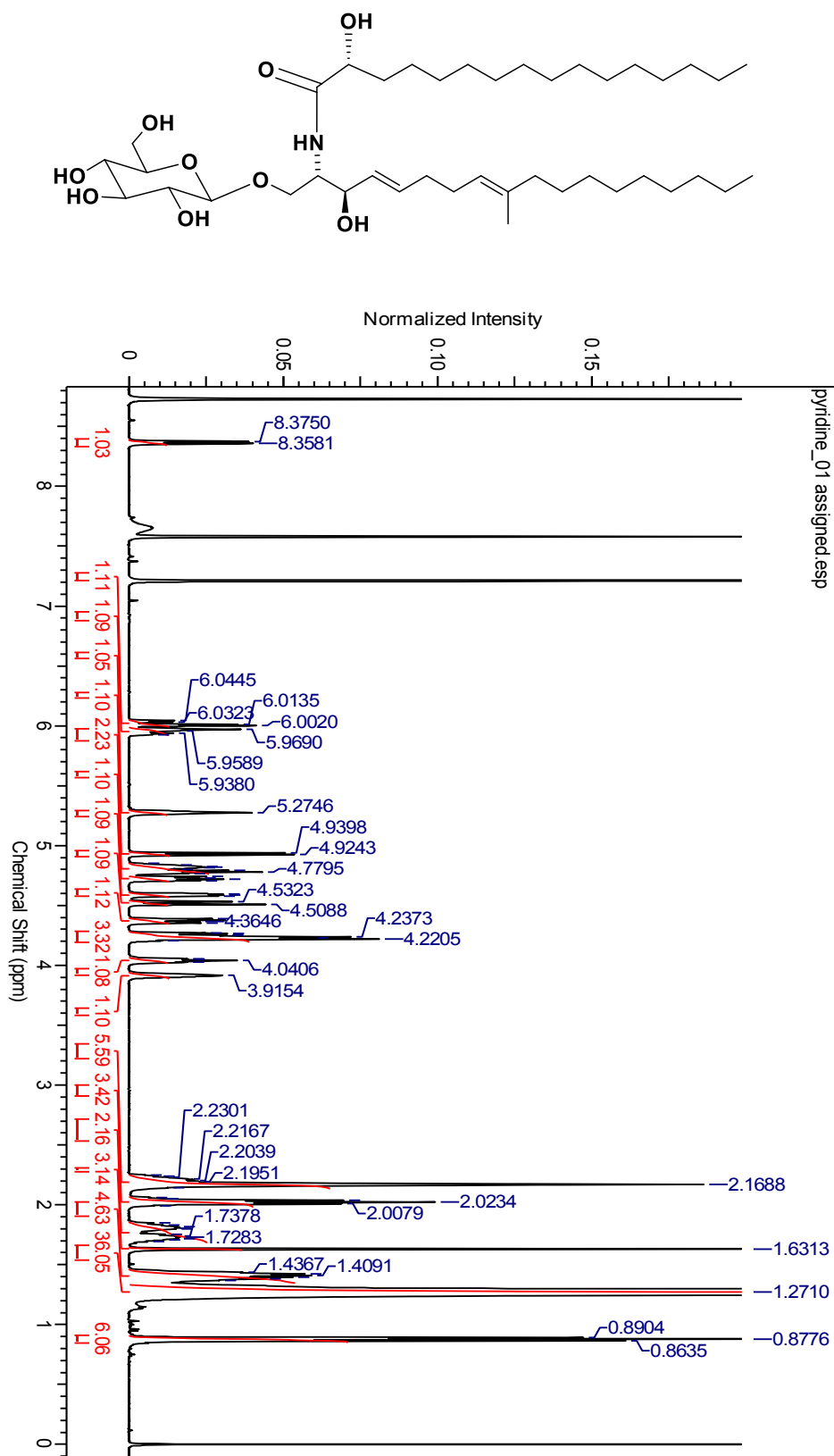
¹H NMR (500 MHz, C₅D₅N): δ 5.90-6.00 (2H, m), 5.24-5.27 (1H, t, J = 5.6, 12.2 Hz), 4.98-5.00 (1H, d, J = 7.8 Hz), 4.53-4.57 (2H, m), 4.38-4.46 (2H, m), 4.24-4.31 (3H, m), 4.08-4.11 (1H, m), 3.93-3.99 (1H, m), 4.25-4.28 (1H, dd, J = 4.3, 10.3 Hz), 4.13-4.16 (2H, dd, J = 6.8, 10.6 Hz), 3.51-3.55 (1H, m), 2.11-2.18 (4H, m), 1.99-2.02 (2H, t, J = 7.5, 15.1 Hz), 1.60 (3H, s), 1.37-1.45 (2H, m), 1.18-1.32 (12H, m), 0.85-0.88 (3H, t, J = 7.1, 13.7Hz), ESI-MS m/z : 474.343 [M+H]⁺

Enzymatic hydrolysis: Preparation of sphingoid base (3)

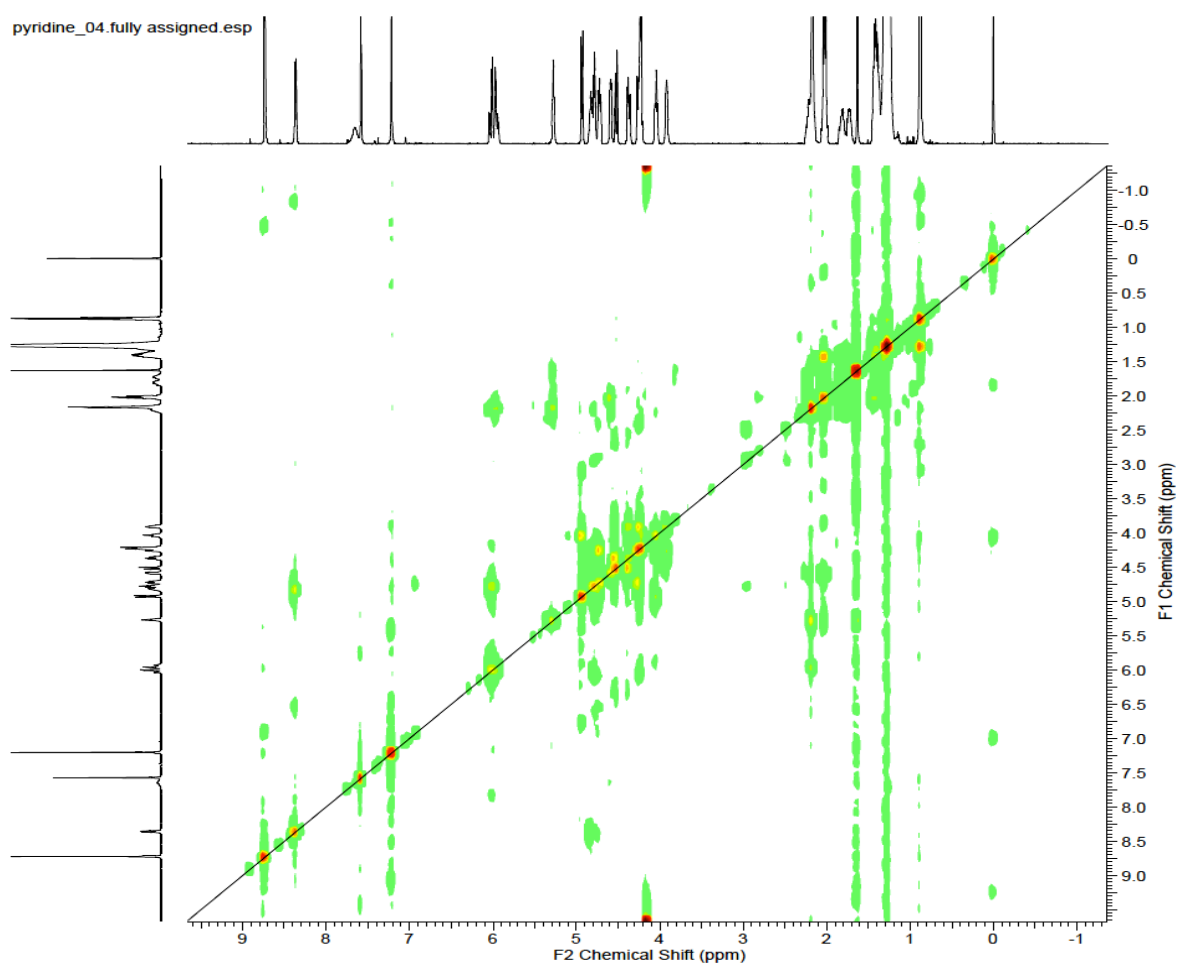
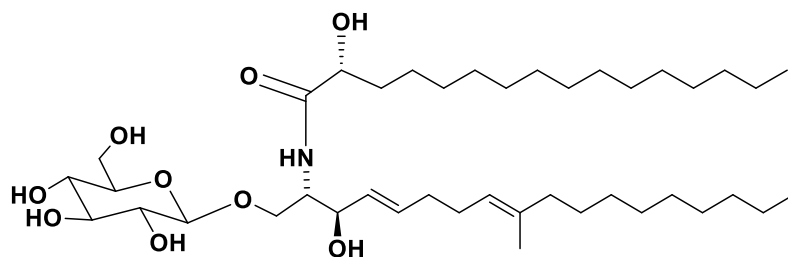
3 mL of β -glucosidase solution from almond (2 mg/mL in 0.1M sodium acetate buffer, pH = 5) was added to 6 mg lysoGlcCer (**2**) and the reaction mixture was vortexed and incubated at 37°C for 16 h. After the starting material completely disappeared, 5.3 mL chloroform/methanol/water (2/2/1.3, v/v) was added. The mixture was vortexed and centrifuged. The lower chloroform phase was collected after repeated extraction of aqueous phases with chloroform. The combined chloroform extract was concentrated under vacuum and subsequently purified by silica gel flash column chromatography by using chloroform/methanol/aqueous NH₃ (40/10/1, v/v).

¹H NMR (500 MHz, C₅D₅N): δ 5.99-6.01 (2H, m), 5.26-5.29 (1H, t, J = 6.2, 12.7 Hz), 4.57-4.59 (1H, t, J = 5.0, 10.1 Hz), 4.25-4.28 (1H, dd, J = 4.3, 10.3 Hz), 4.13-4.16 (2H, dd, J = 6.8, 10.6 Hz), 3.38-3.42 (1H, q, J = 6.2, 10.9 Hz), 2.14-2.22 (4H, m), 1.99-2.02 (2H, t, J = 7.8, 15.2 Hz), 1.61 (3H, s), 1.39-1.44 (2H, m), 1.20-1.30 (12H, m), 0.85-0.88 (3H, t, J = 7.2, 14.0Hz), ESI-MS 312.2895 [M+H]⁺. $[\alpha]_D^{20} + 4.2^\circ$, c = 1, CHCl₃.

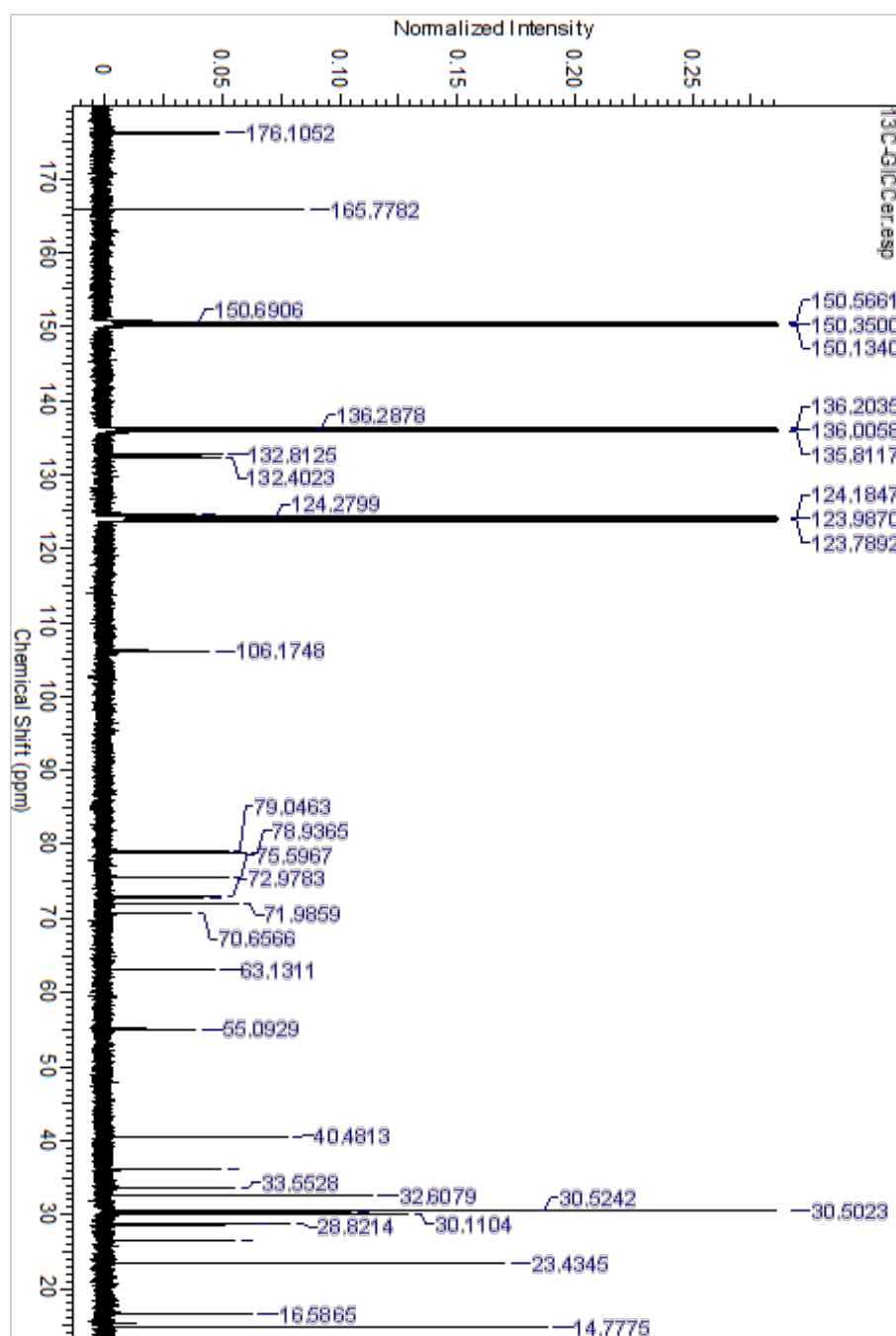
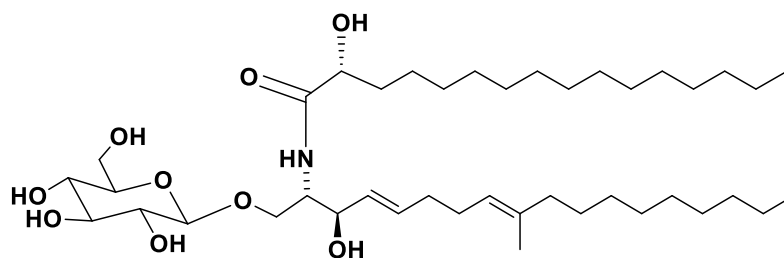
¹H-NMR spectrum of GlcCer 1



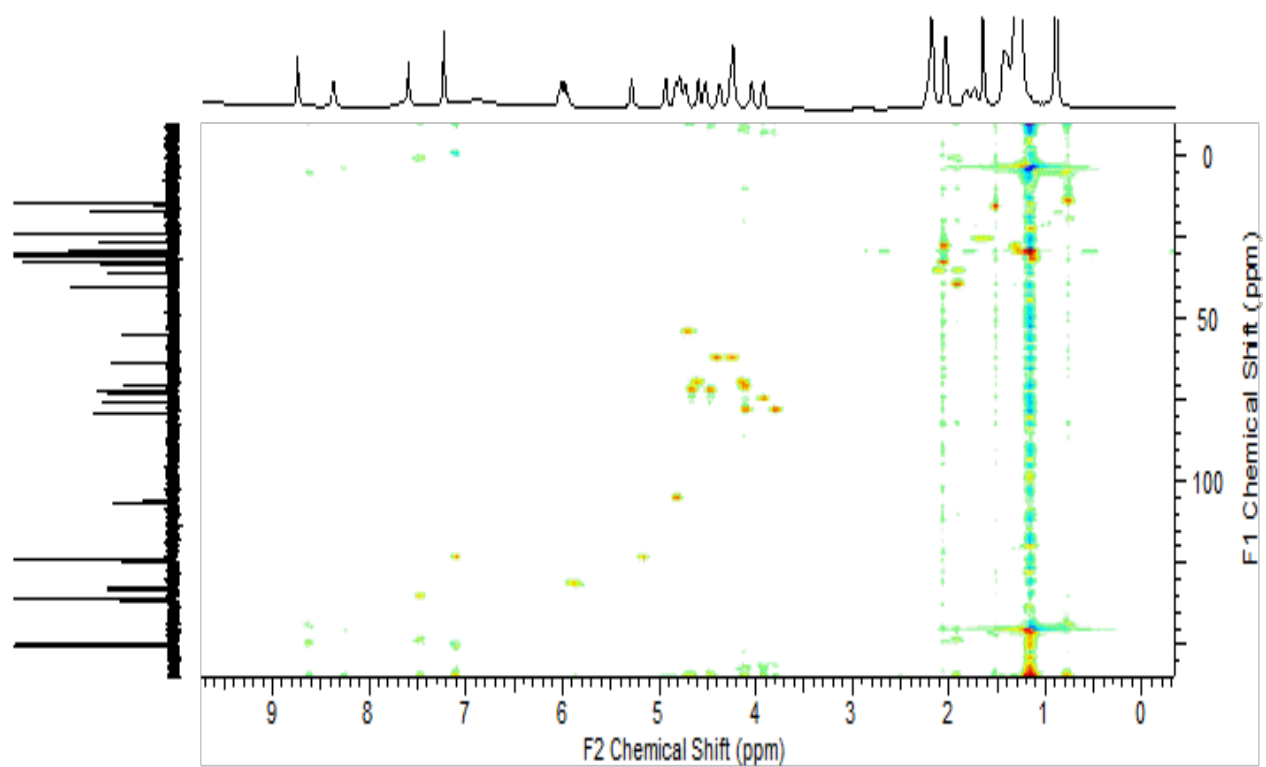
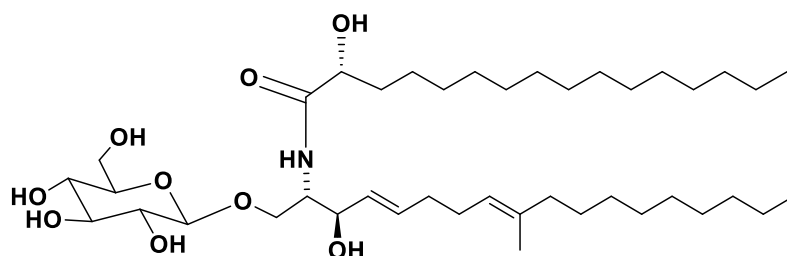
^1H - ^1H COSY spectrum of GlcCer 1



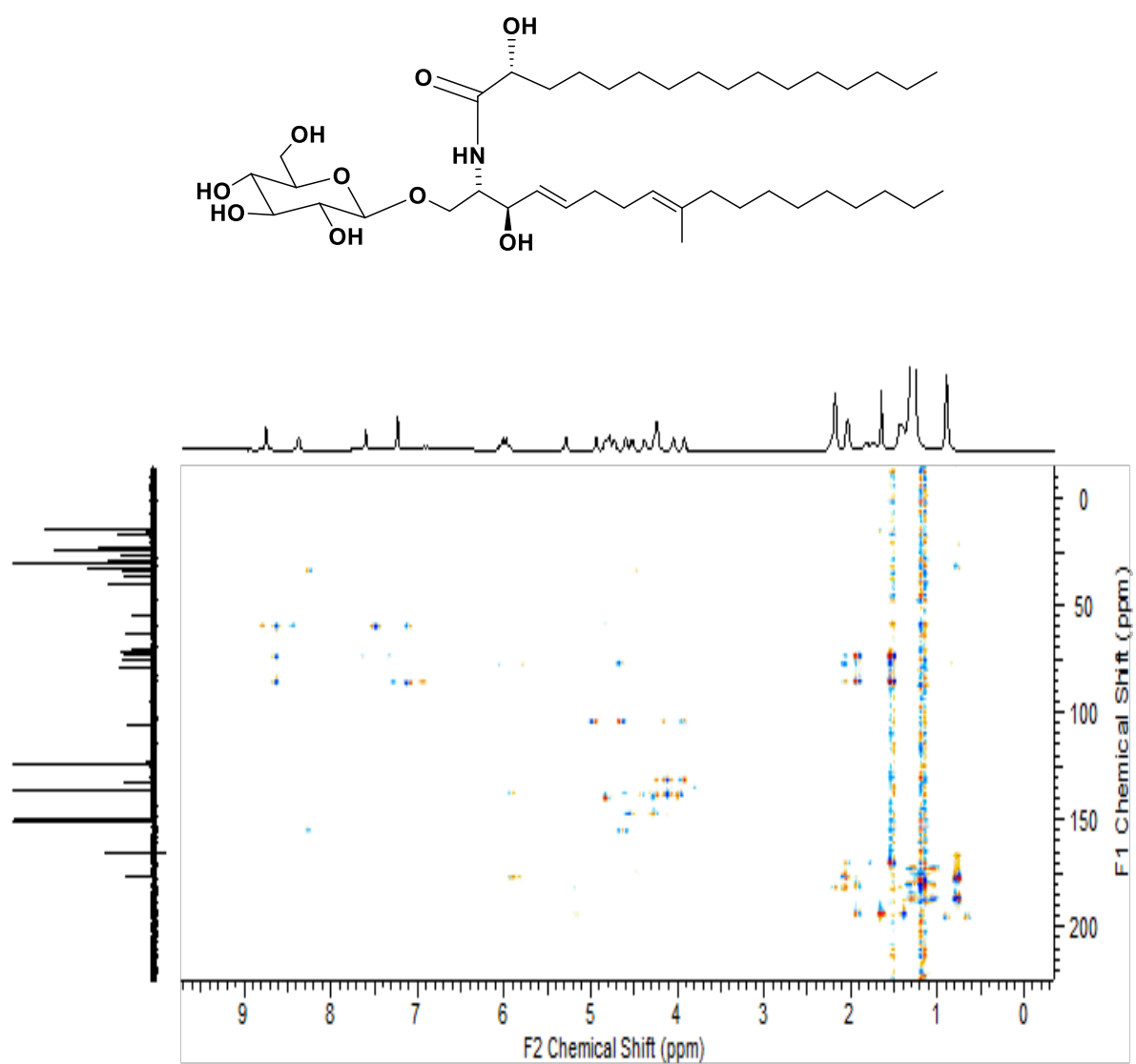
^{13}C - spectrum of GlcCer 1



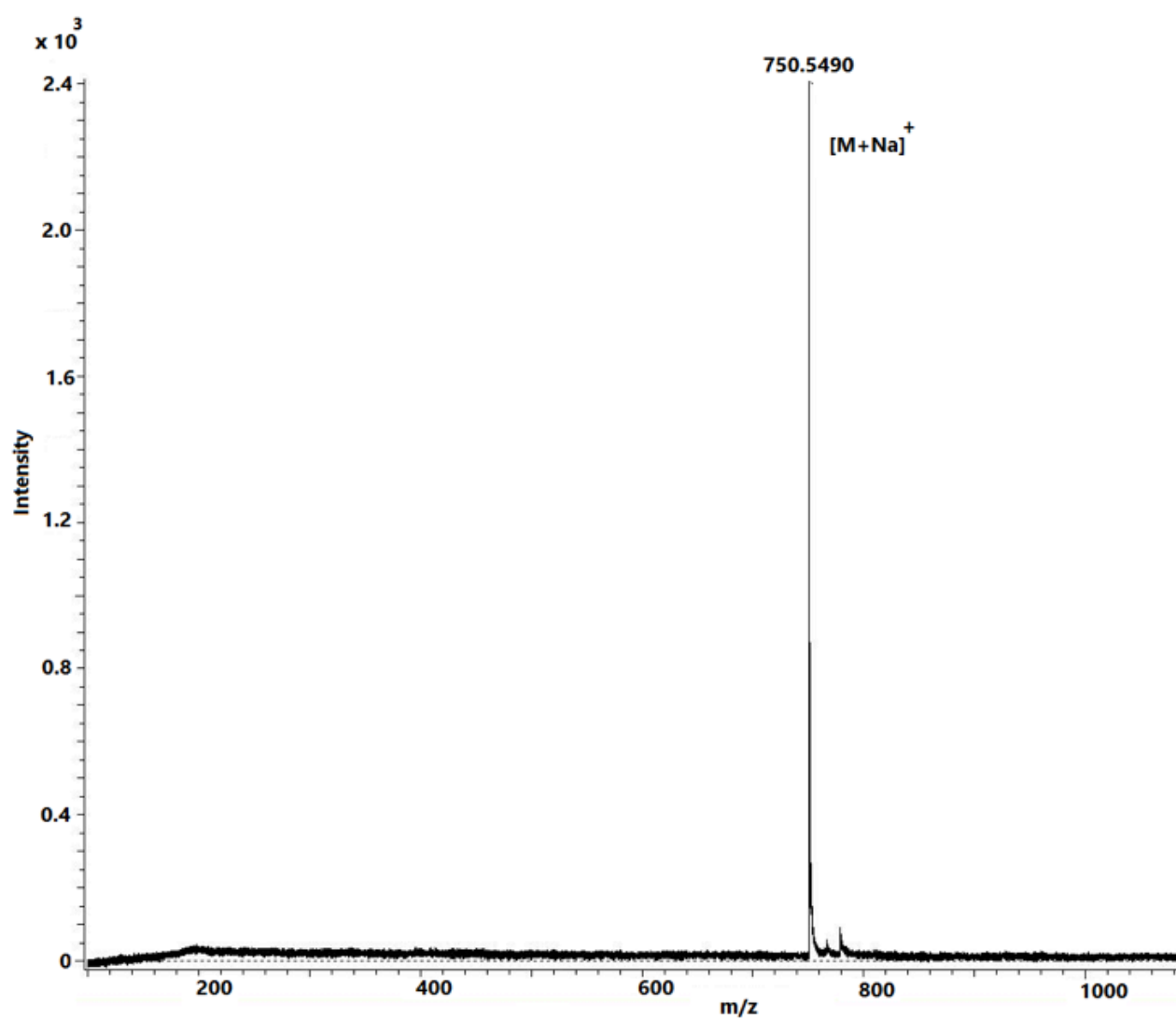
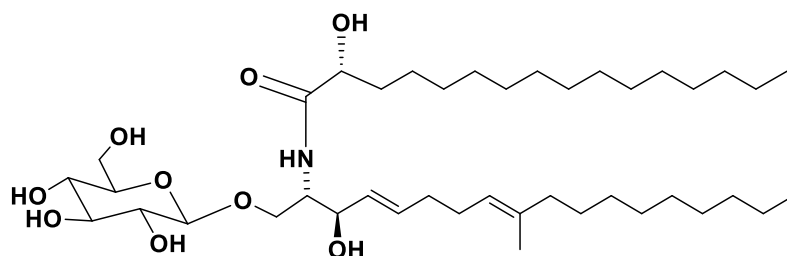
HMQC spectrum of **GlcCer 1**



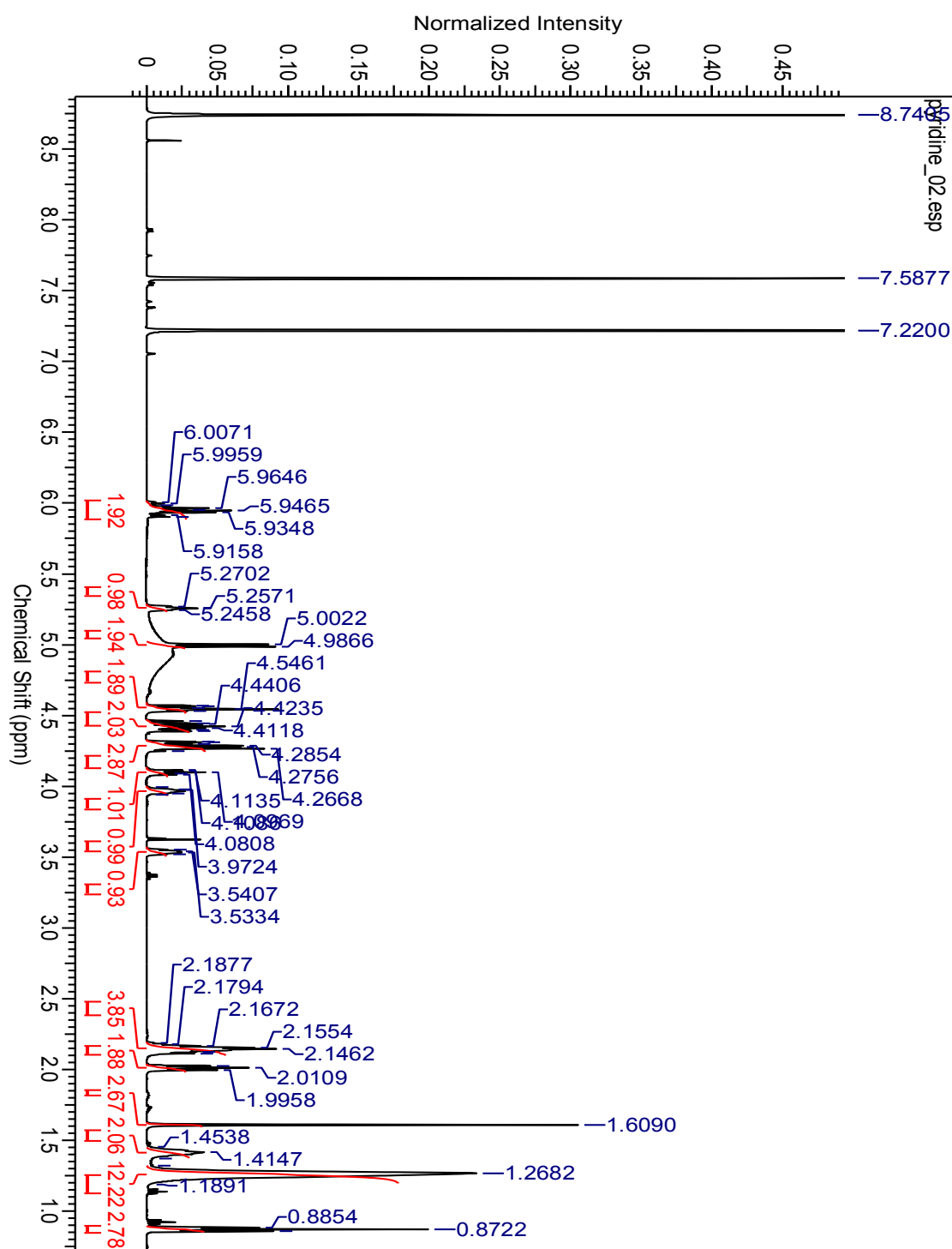
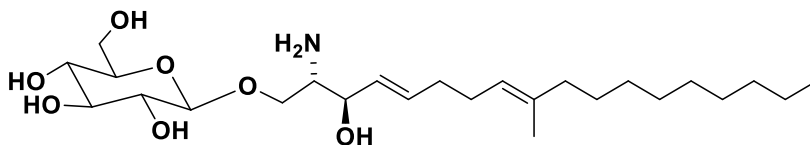
HMBC spectrum of **GlcCer 1**



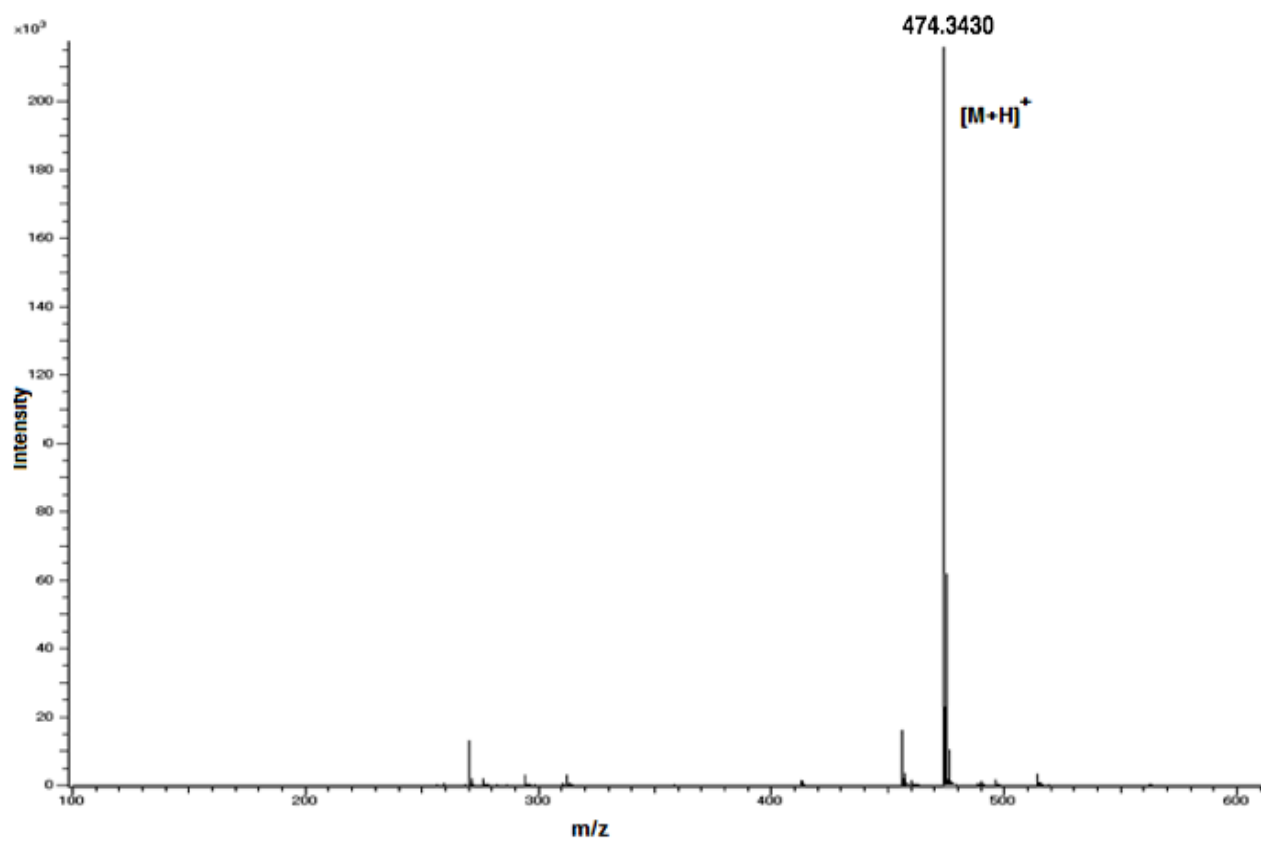
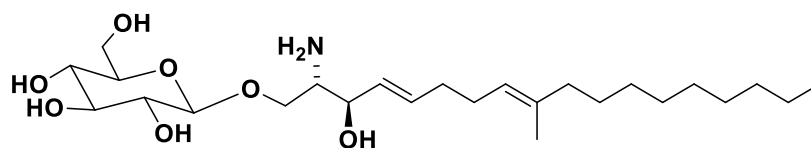
ESI-mass spectrum of GlcCer 1



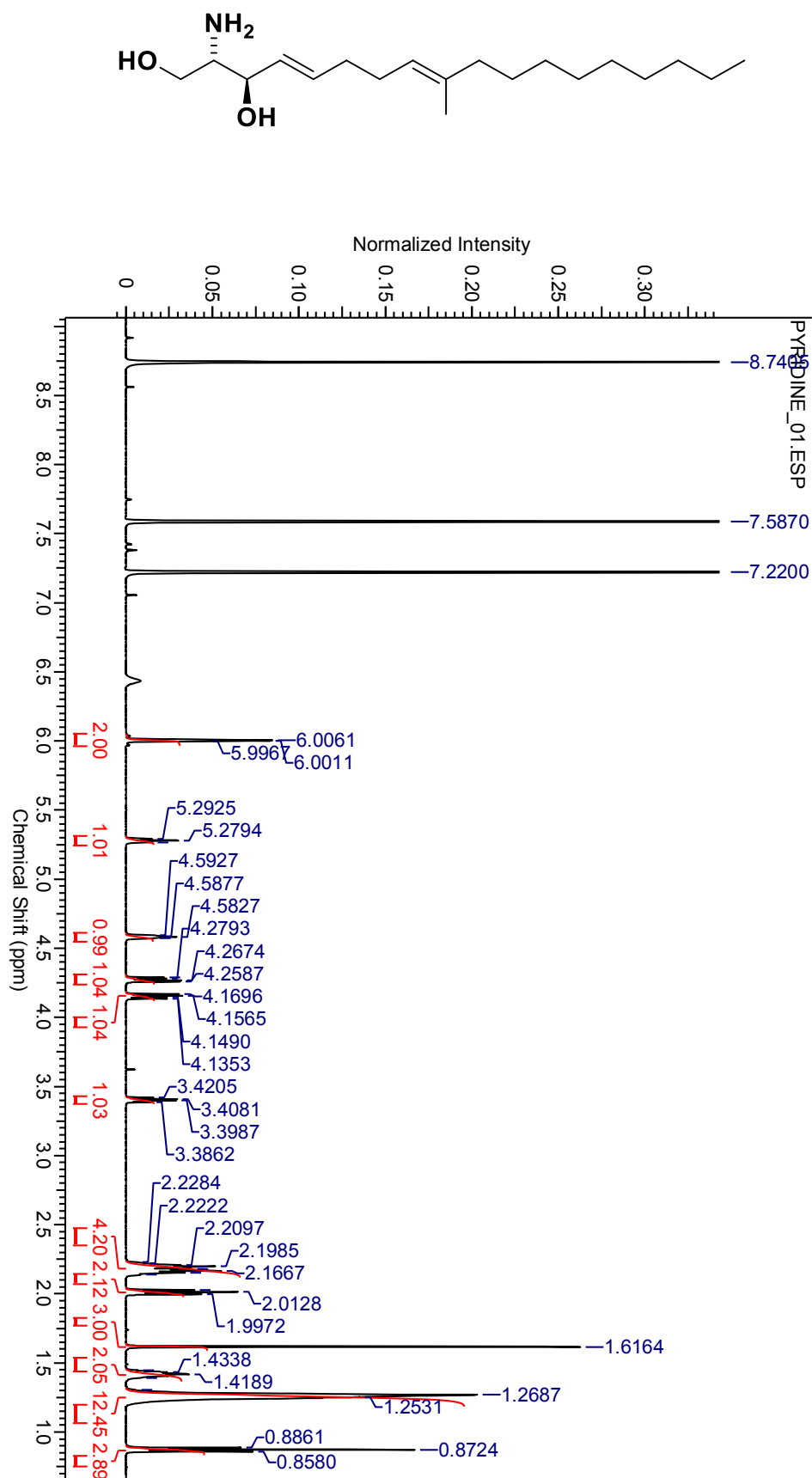
¹H-NMR spectrum of lysoGlcCer 2

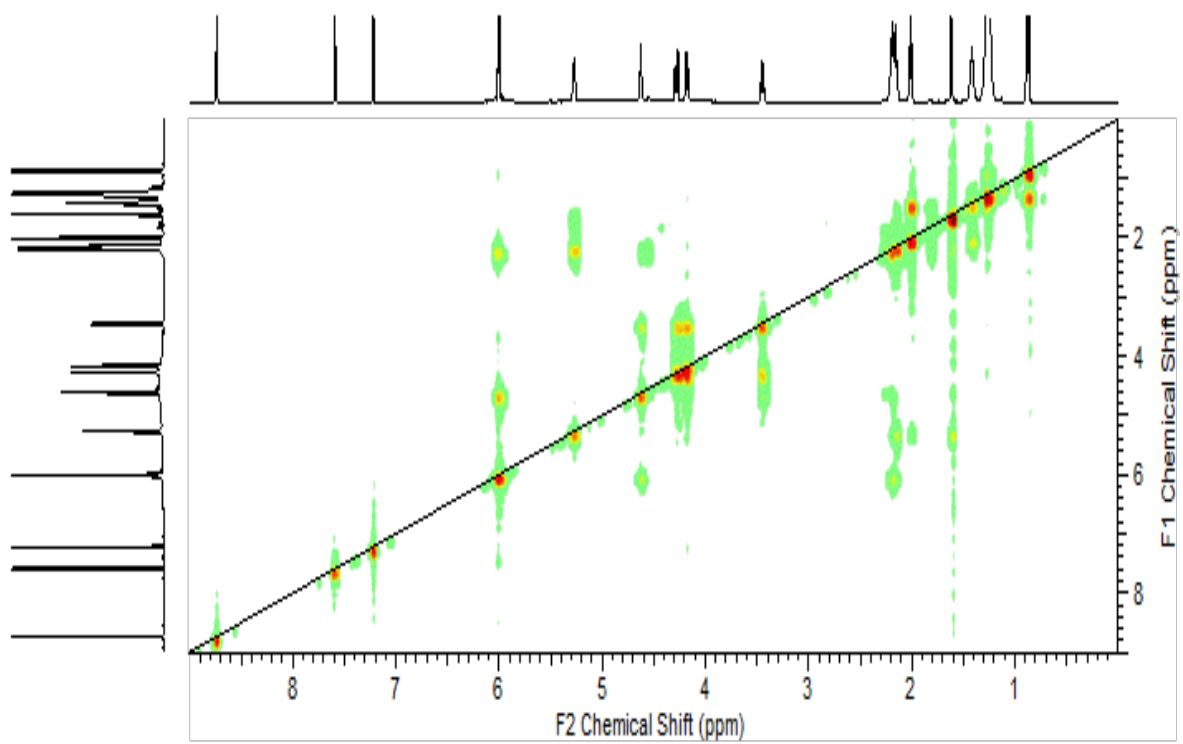
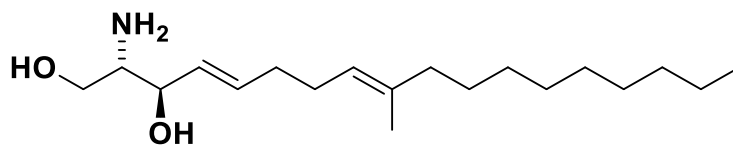


ESI-mass spectrum of lysoGlcCer 2

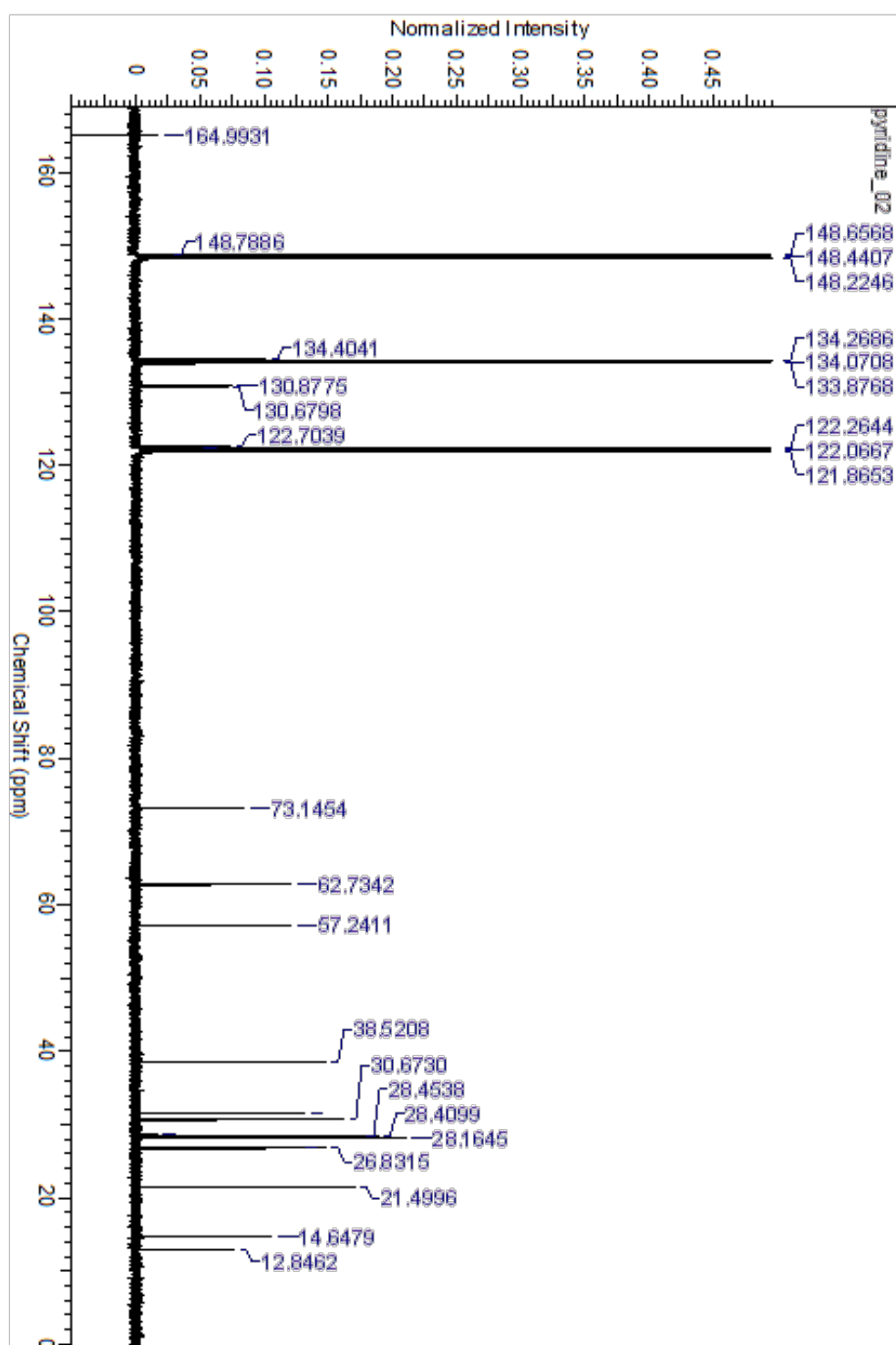
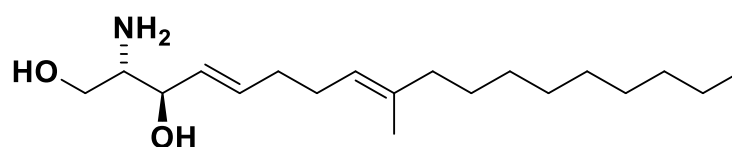


¹H-NMR spectrum of sphingoid base 3

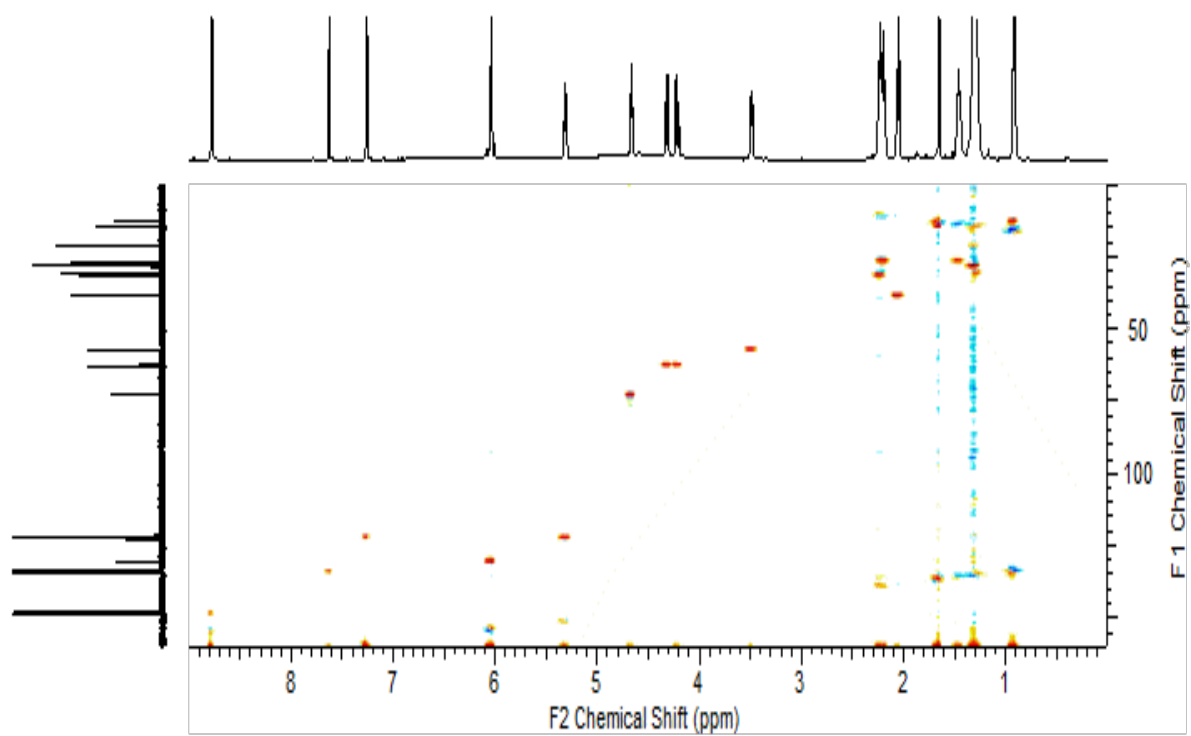
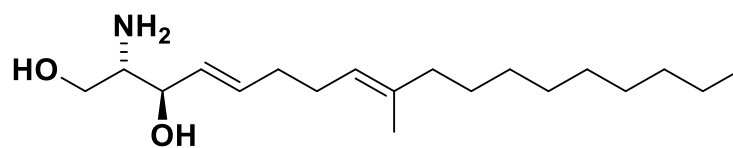


^1H - ^1H COSY spectrum of **sphingoid base 3**

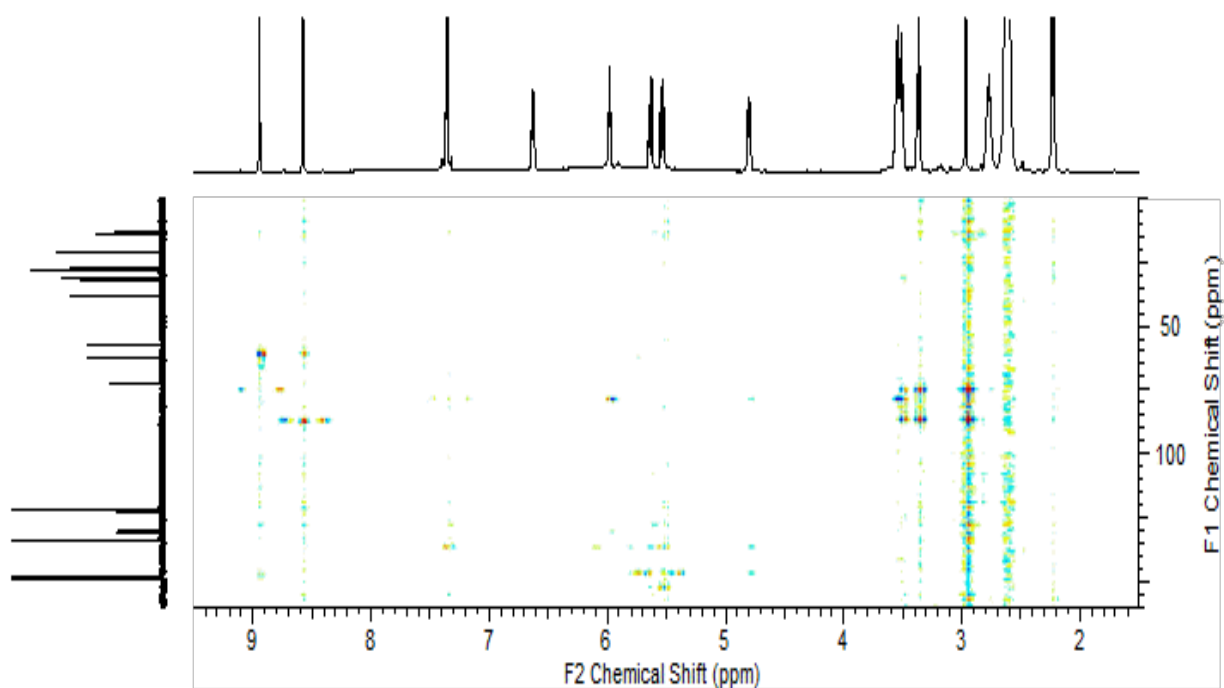
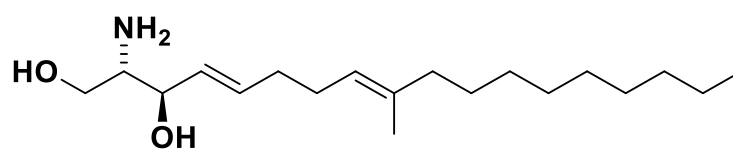
^{13}C - spectrum of **sphingoid base 3**



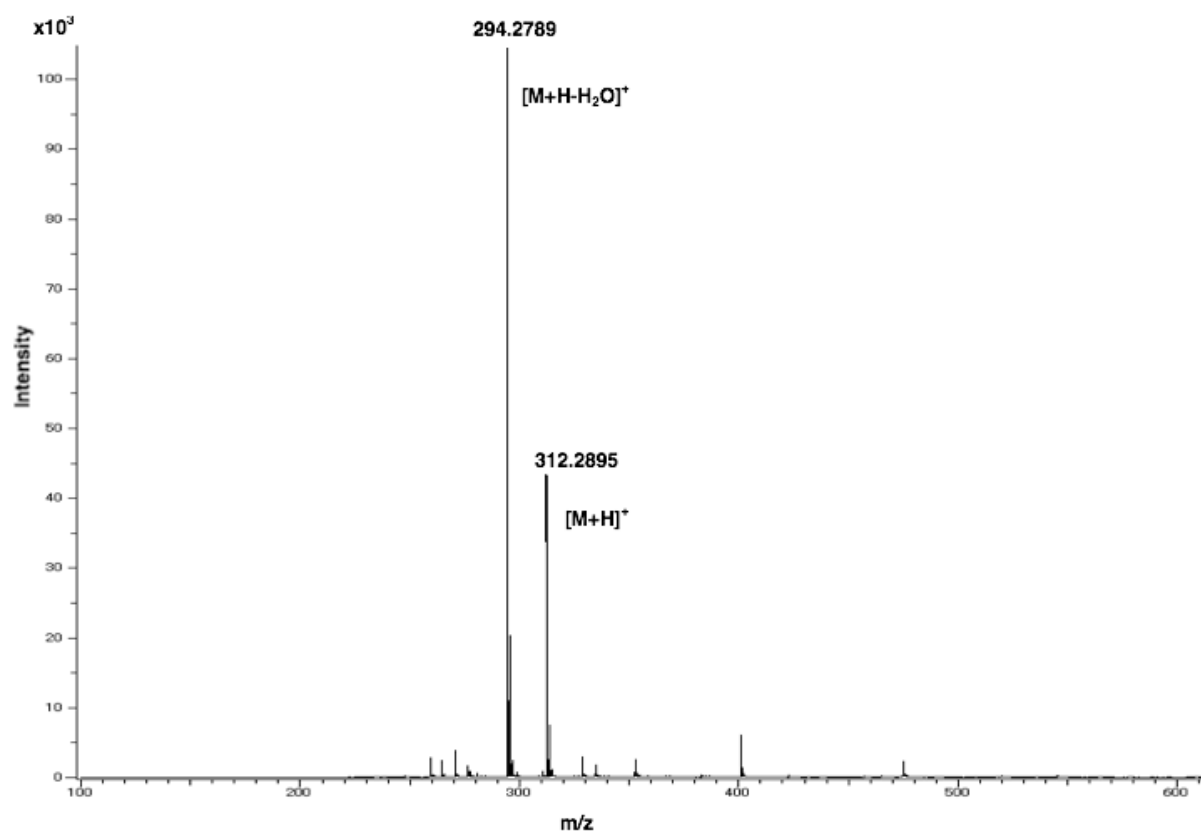
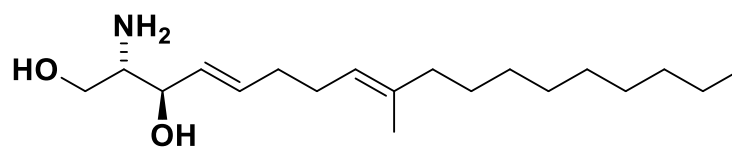
HMQC spectrum of **spingoid base 3**



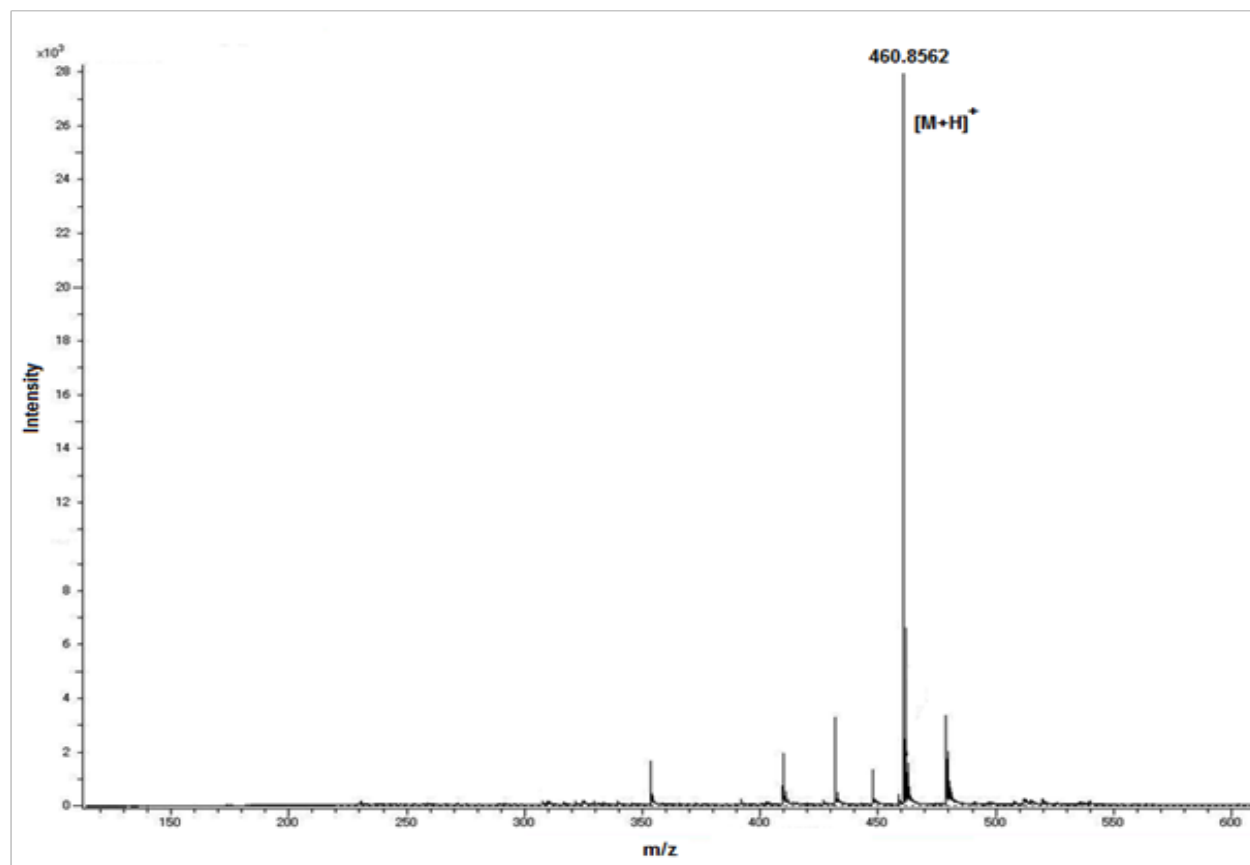
HMBC spectrum of **spingoid base 3**



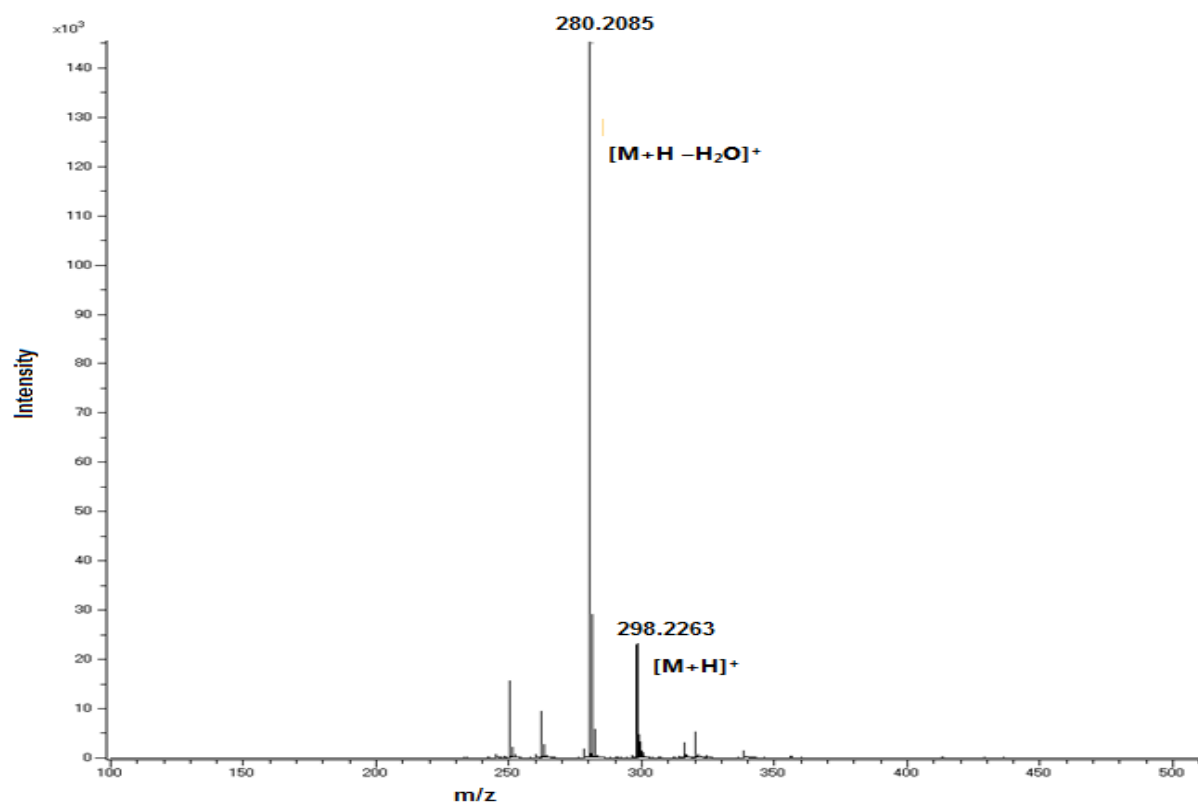
ESI-mass spectrum of **sphingoid base 3**



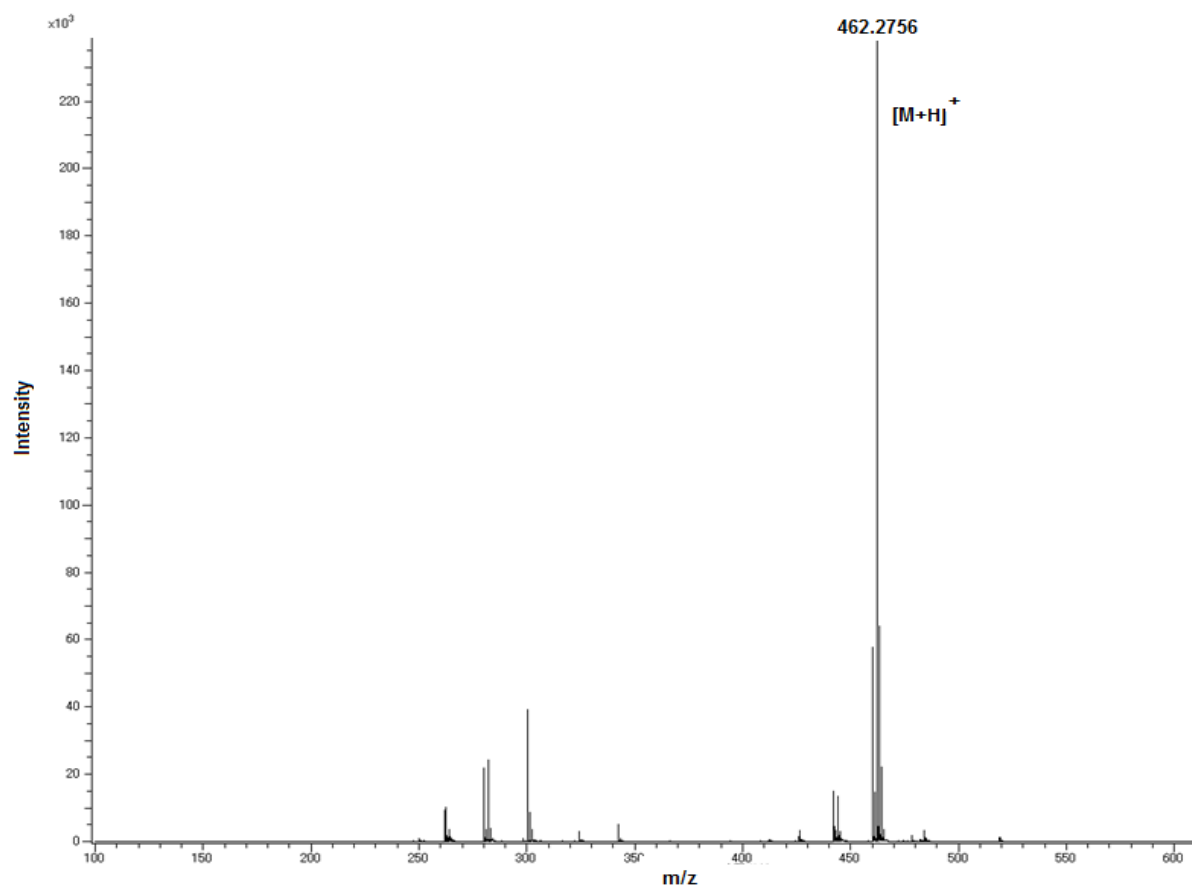
ESI-mass spectrum of rice lysoGlcCers



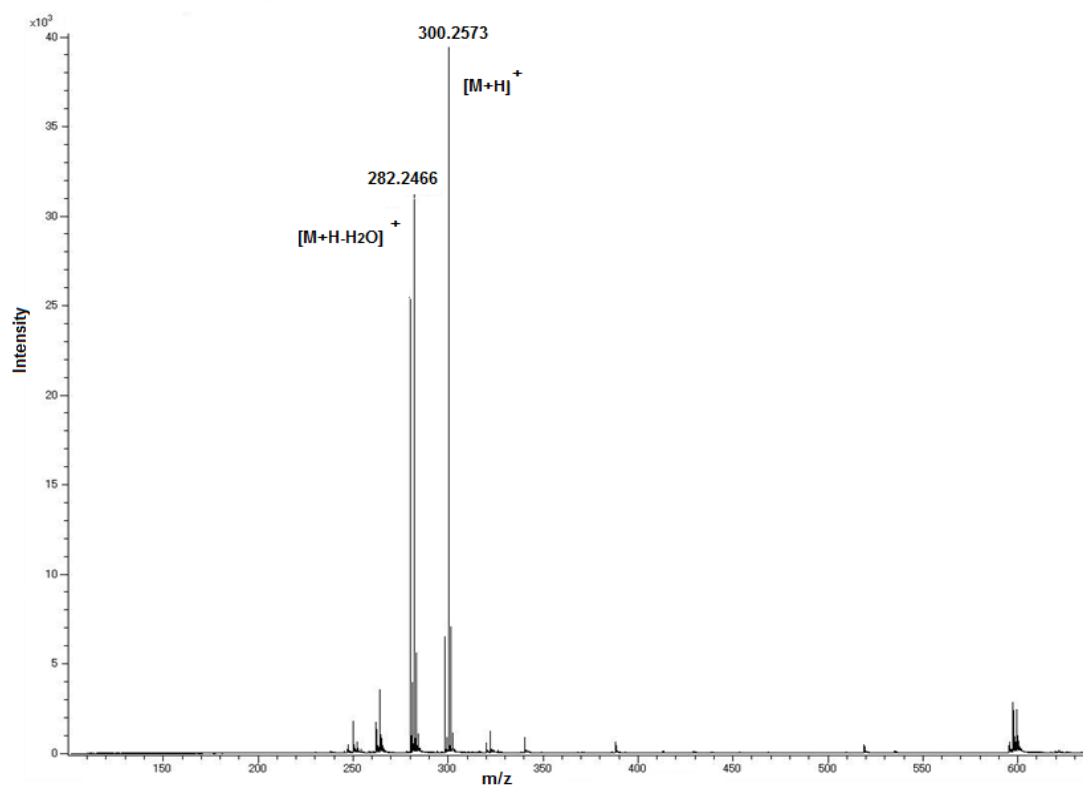
ESI-mass spectrum of rice sphingoid bases



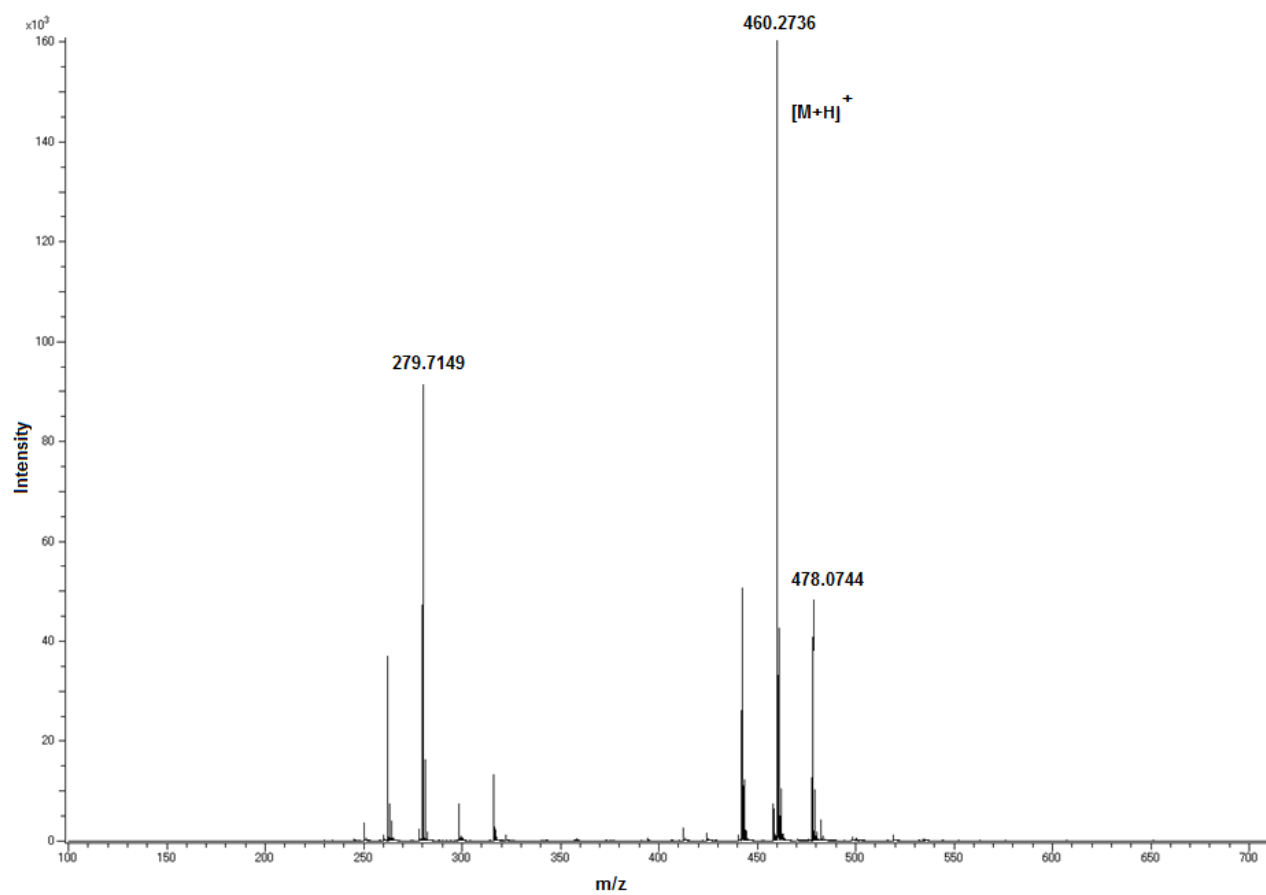
ESI-Mass spectrum of wheat lysoGlcCers



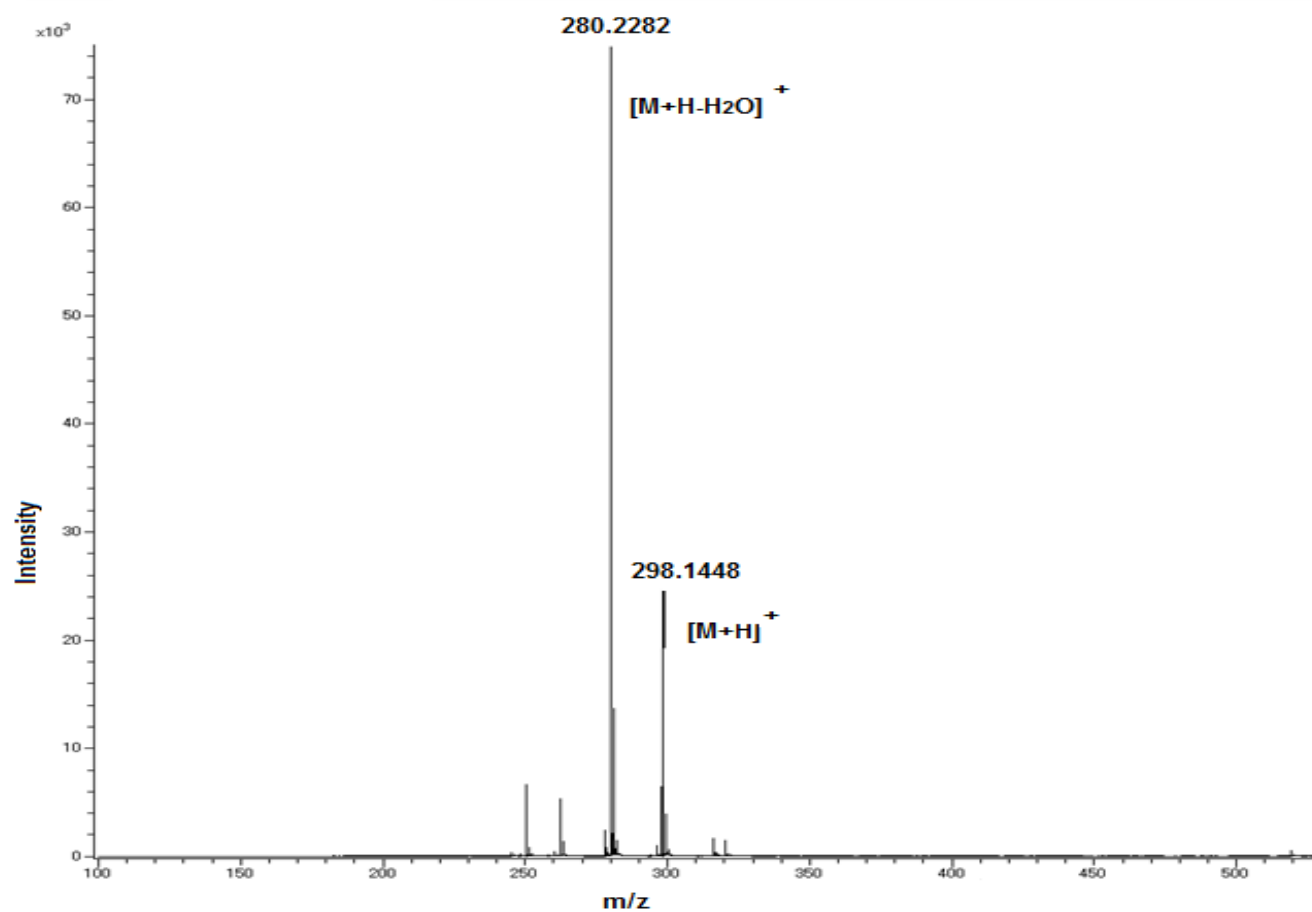
ESI-mass spectrum of wheat sphingoid bases



ESI-mass spectrum of soy lysoGlcCer



ESI-mass spectrum of soy sphingoid bases



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Chapter 4

Development of sphingoid bases and their derivatives as multidrug resistance inhibitors in cancer

4.1 Abstract

Chemotherapy is the main strategy for the treatment of cancer. However, the main problem limiting the success of chemotherapy is the development of multidrug resistance (MDR). MDR is often associated with the over-expression of permeability-glycoprotein (P-gp), the most extensively characterized of the drug efflux pumps. The development of P-gp inhibitors is acknowledged as a viable means of reversing this MDR phenotype. However, most P-gp inhibitors identified to date have demonstrated limited clinical success due to limitations in potency and specificity. On the other hand, sphingolipids incorporating a diverse array of structural variants that exert a powerful influence over cell fate and physiology. Metabolic alterations that reduce levels of the pro-apoptotic lipid ceramide, have frequently associated with cancer drug resistance.

The aim of this study is to develop sphingoid base based inhibitors for MDR. Henceforth, we prepared various sphingoid bases and their derivatives from dietary natural resources. Also, we made an effort towards establishment of adriamycin resistant breast cancer cell line (MCF-7/ADR) with MDR1 overexpression by stepwise selection of MCF-7 cells. The preliminary results of this study suggest that these molecules show same cytotoxic effect to both MCF-7 and MCF-7/ADR cell lines. Therefore natural sphingoid bases may serve as better MDR inhibitors to reverse cancer drug resistance.

4.2 Introduction

Many cancers are resistant to or ultimately develop resistance to chemotherapeutic agents. One molecular mechanism of resistance is up-regulation of the membrane transporter multidrug resistance protein1 (MDR1) also known as P-glycoprotein (permeability glycoprotein, P-gp), ATP-binding cassette subfamily B member 1 (ABCB1), or cluster of differentiation 243 (CD243).¹ During normal development, MDR1 plays a critical role in exporting xenobiotics from human tissues, particularly in the gut, liver, kidneys, and blood-brain barrier.^{2,3} MDR-1 is most crucial for chemotherapy, because P-gp is mostly distributed in intestine and determines intestinal absorption and export of anti-cancer drugs after oral administration. MDR1-induced multidrug resistance is a major cause of treatment failure in metastatic lung, breast, ovarian, cervical, and kidney cancers.⁴⁻⁶

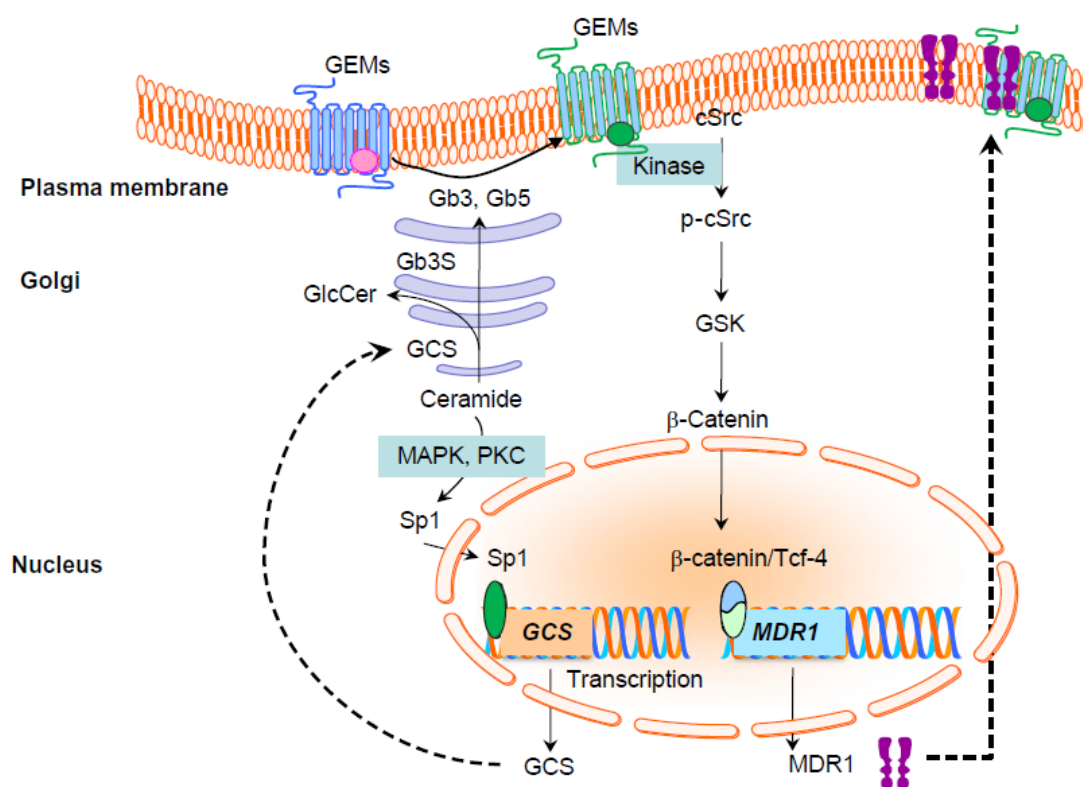


Figure 1. Cells exposed to drugs upregulate drug-resistant genes via actions of ceramide and glycosphingolipids.

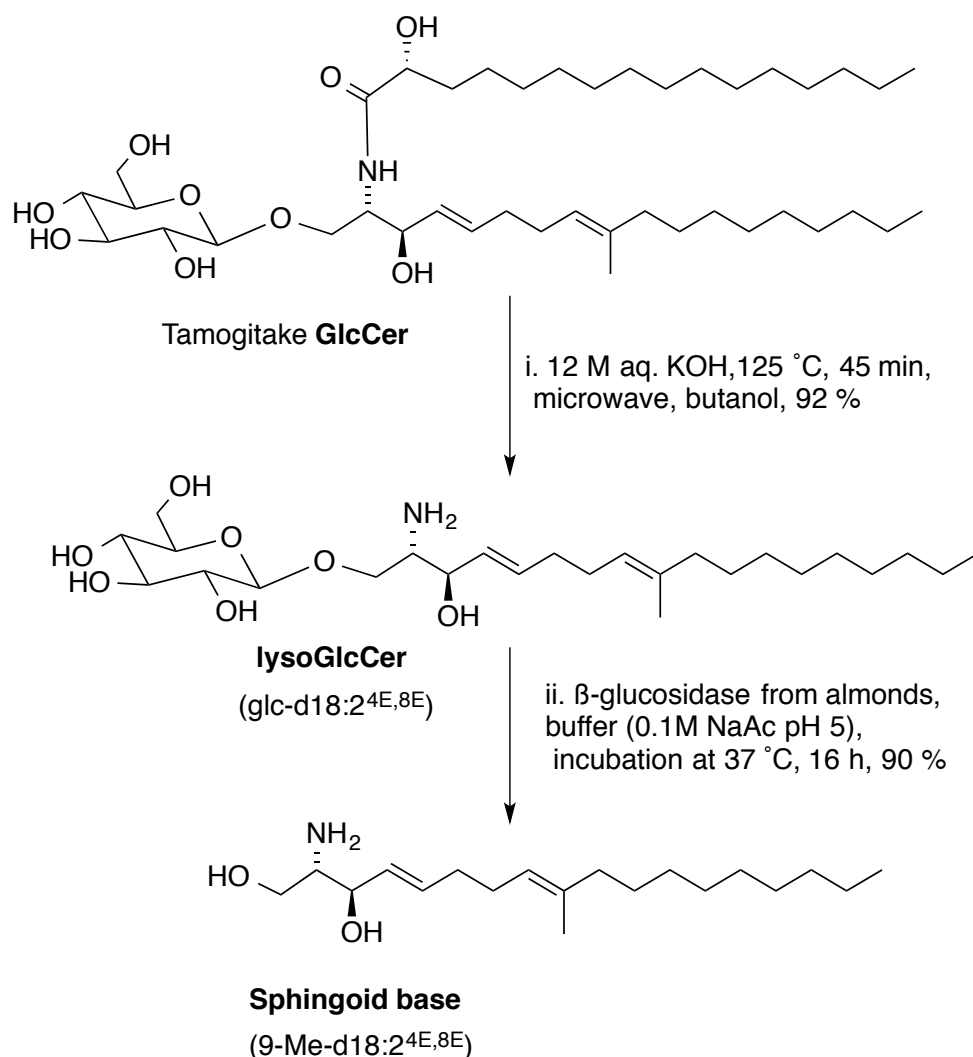
Understanding the mechanism of the multidrug transport is important for designing drugs of good bioavailability and efficient chemotherapy. Recent studies show MDR1 is closely related to sphingolipid metabolism, particularly, ceramides.⁷ Accumulation of glucosylceramide is a characteristic of MDR cancer cells of breast, ovarian, colon and epitheliod carcinomas.⁸ The plausible mechanistic pathway between GCS activity and MDR1 expression was already been described (**Figure 1**).⁹ Pathogenic alterations in endogenous ceramide levels contribute to breast cancer chemo- resistance.¹⁰⁻¹¹ Targeting the bioactive sphingolipid ceramide is a promising approach because of its regulation of cellular apoptosis and survival. Several chemotherapeutic agents, including paclitaxel and doxorubicin, induce apoptosis through the induction of ceramide signaling as a mechanism of action.¹²⁻¹³ In fact, decreased synthesis and increased metabolism of ceramide have been shown to be important components of chemoresistance in human breast cancer.¹⁴⁻¹⁵

Natural sphingoid bases are proven to be highly cytotoxic to various cancer cells.¹⁶ Sphingoids originated from dietary sources such as plants and fungi, are act as substrates for P-gp in differentiated Caco-2 cells, but mechanism of efflux of these bases is still remains unknown.¹⁷ In addition, natural sphingoid base derivatives and their effects on the functional activity and gene expression of P-gp have not been studied yet. Therefore, the present study aimed at preparation of sphingoid bases from the glucosylceramides of dietary natural resources like tamogitake, rice, wheat, soy and konjac, followed by their synthetic derivatives such as ceramides, N,N dimethyl sphingoids. To evaluate the effects of these compounds on functional activity of P-gp, an MCF-7/ADR cell line was established by stepwise selection of MCF-7 cells after adriamycin treatment.

4.3 Results and discussion

Glucosylceramide (GlcCer) mixture from various natural resources like tamogitake (golden oyster), rice, wheat, soy and konjac were isolated by the method as previously reported¹⁸ And further glucosyl sphingosine (lysoGlcCer) and sphingoid bases were prepared from the GlcCers by the previously established chemoenzymatic method as shown in **Scheme 1**. The same methodology was employed for all kinds of

GlcCers to prepare their respective sphingoid bases.



Scheme 1. Preparation of lysoGlcCer and sphingoid base from GlcCer

Thin layer chromatographic profiles shows obtained sphingoid bases are of isomeric mixture in case of rice, wheat, soy, and konjac, whereas sphingoid base obtained from tamogitake was highly pure with a single isomer (9-Me-d18:2^{4E,8E}). The purity of the isomeric sphingoid bases was analyzed by reverse phase HPLC after *o*-phthaldehyde derivatization and the result shows d18:2^{4E,8Z}, d18:2^{4E,8E}, d18:1^{8Z}, d18:2^{4E,8Z} are major isomers in rice, soy, wheat, konjac, respectively (**Figure 2**). Further their individual structural characterization was made by ESI-mass and NMR.

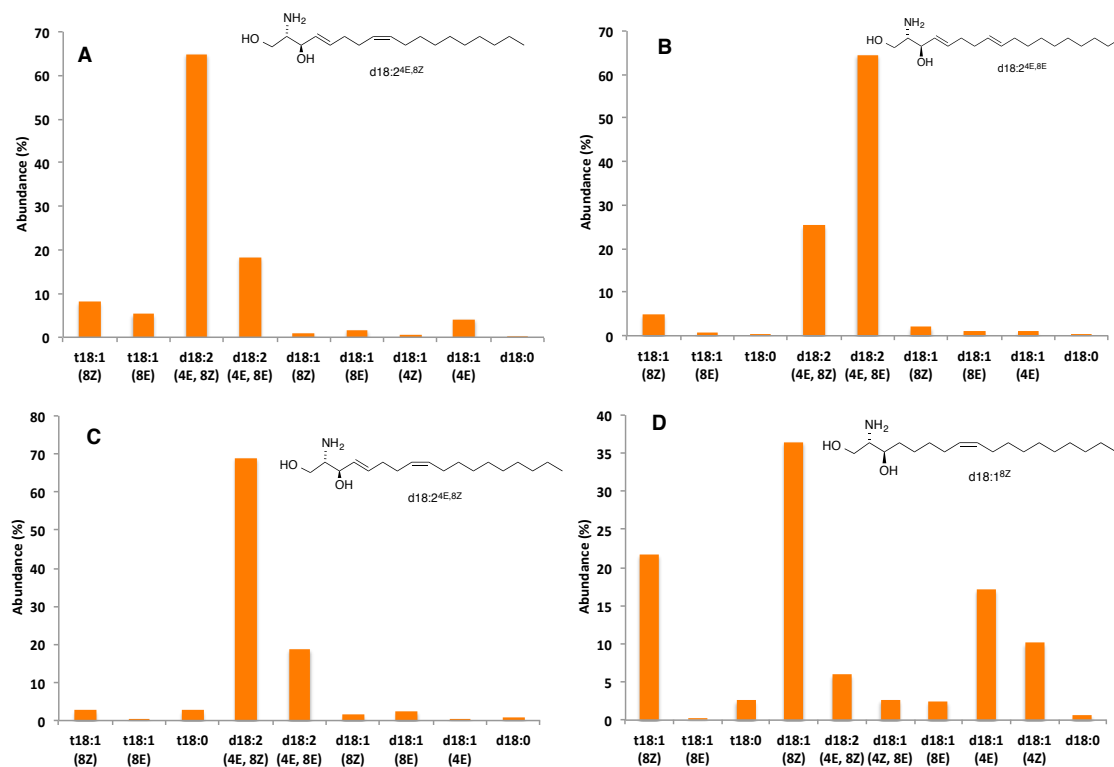
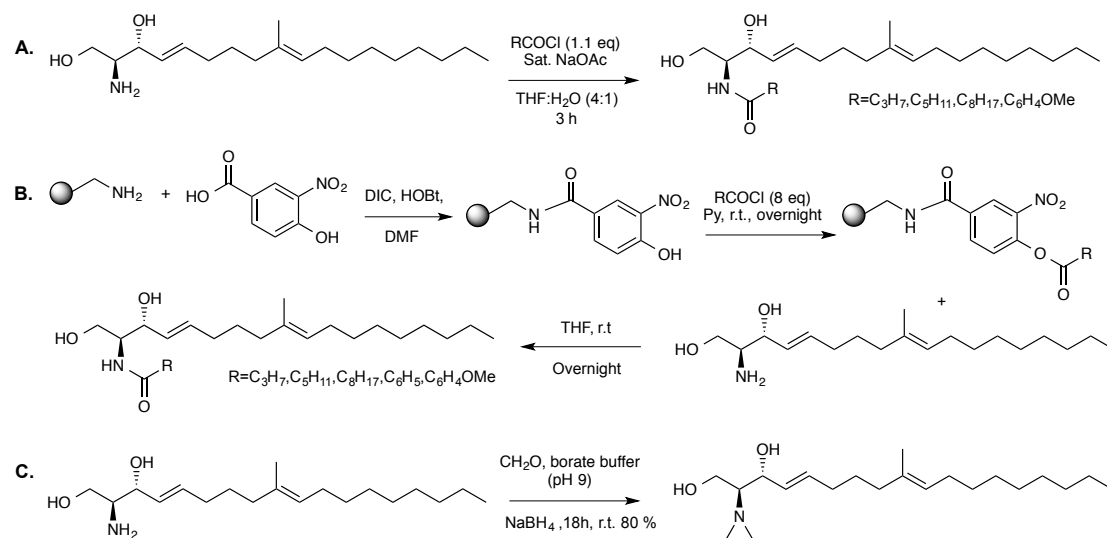


Figure 2. Purity analyses of sphingoid bases obtained by hydrolysis of GlcCers, **A.** Rice, **B.** Soy, **C.** Konjac, **D.** Wheat (Structures shown are major isomers)



Scheme 2. Preparation of sphingoid base derivatives

To prepare sphingoid base derivatives, tamogitake sphingoid base (d18:2^{4E,8E}) was used because of its high purity. And it was N-acylated using selected acid chlorides in presence of saturated sodium acetate, to produce ceramides (**Scheme 2A**).¹⁹ As an alternative pathway to produce ceramides with high purity 4-hydroxy-3-nitrobenzophenone resin was synthesized in the laboratory starting from aminomethyl-polystyrene (**Scheme 2B**) and its structure was confirmed by its IR spectrum (**Figure 3**).²⁰ Further dimethylation of sphingoid base was carried out by reductive methylation using excess of formaldehyde and sodium borohydride at ambient temperature to give N,N-dimethyl 9-methyl sphingadienine (**Scheme 2C**).²¹ All obtained sphingoid base derivatives were characterized by ESI-mass and NMR.

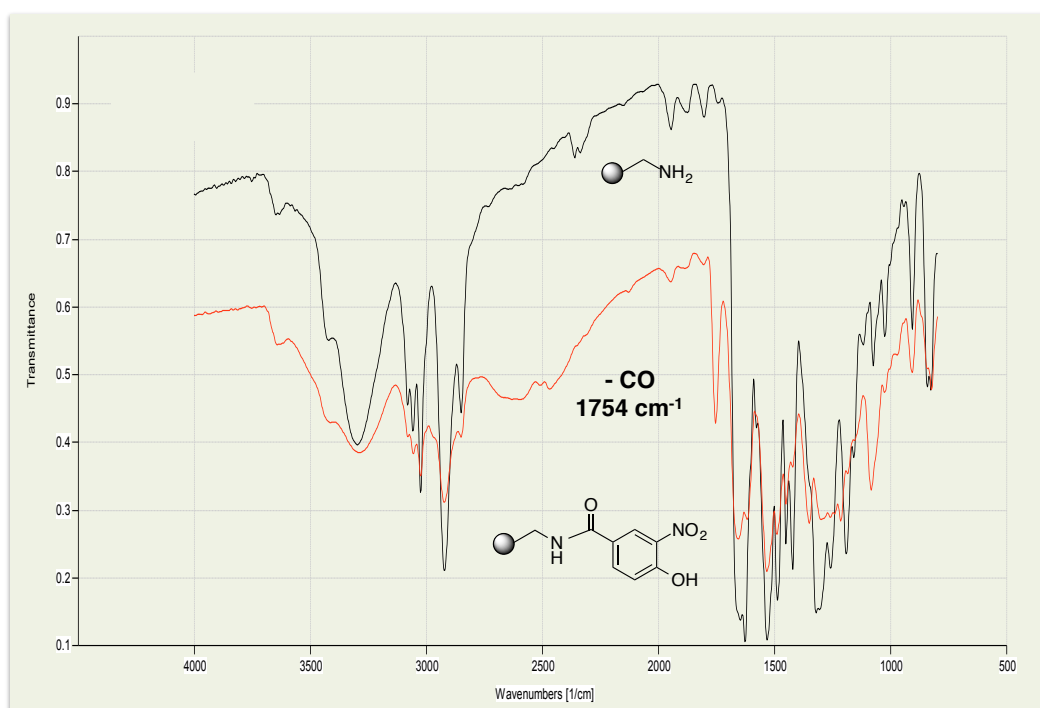


Figure 3. IR spectra of 4-hydroxy-3-nitrobenzophenone resin comparison with aminomethyl polystyrene.

Establishment of adriamycin resistant MCF-7

In this study, adriamycin resistant MCF-7 (MCF-7/ADR) cell lines were established from parental MCF7 by continuous exposure to increasing drug concentrations with a 320 nM final concentration of adriamycin.²² According to CCK-8 and MTT assay results, the MCF-7/ADR cells exhibited considerable resistance to

the corresponding drug. The relative resistance of MCF-7/ADR cells, as compared to the MCF-7, is demonstrated in **Figure 4**. The IC_{50} values of MCF-7 and MCF-7/ADR were 50 μ M and 112 μ M, respectively. The degree of resistance evaluated in terms of resistance index (R) which is calculated according to the relation: $R = IC_{50} \text{ resistant cells} / IC_{50} \text{ sensitive cells}$. Therefore, the MCF-7/ADR cells were approximately 2.3-fold more resistant to adriamycin than the parental cell line.

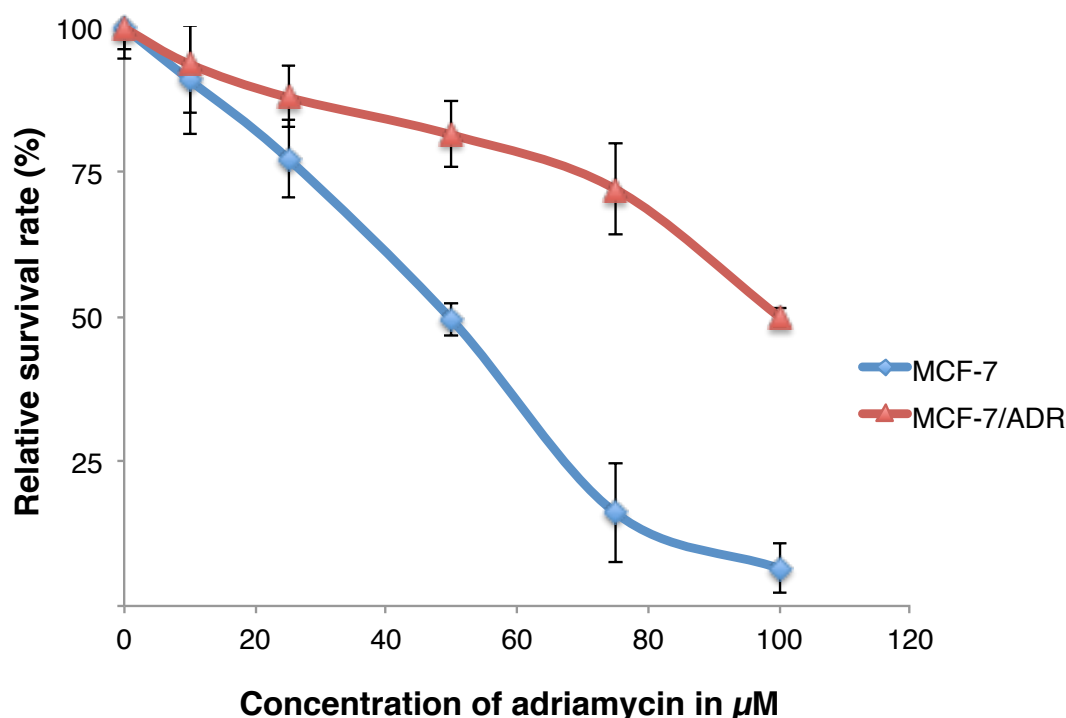
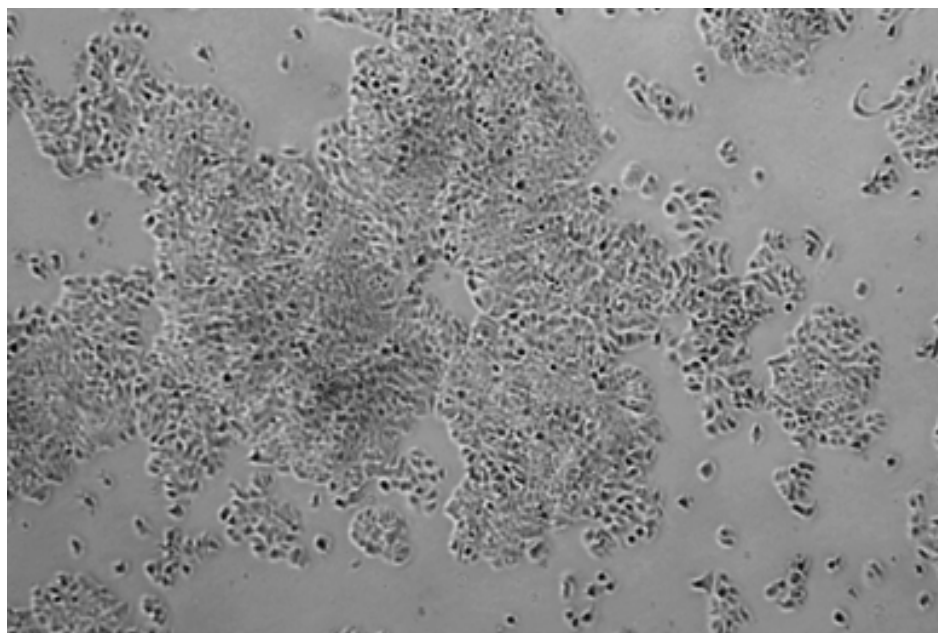


Figure 4. Cytotoxic response of MCF-7/ADR cells to adriamycin as compared to response of MCF-7 by CCK-8 assay.

The development of the drug resistance phenotype is accompanied by changes in different biological features of the malignant cells, including morphological ones. When compared to MCF-7/, the MCF-7/ADR cells were larger with less defined irregular, rounded shape and contain multiple nuclei in the cytoplasm. Multiple, relatively large vesicles were also observed in the cytoplasm. The shape of MCF-7 and MCF-7/ADR are shown in **Figure 5**. The MTT assay was performed to determine the cytotoxicity of prepared sphingoid bases and their derivatives against MCF-7 and MCF-7/ADR. The preliminary results are summarized in **Figure 6**. And all compounds show $IC_{50} \geq 50 \mu$ M. Sphingoid bases of tamogitake (IC_{50} of MCF-

7/(MCF-7/ADR)=100/60 μ M), wheat (100/45 μ M), soy (100/50 μ M), N,N dimethyl 9-methyl sphingadienine (100/52 μ M) and palmitoyl ceramide (100/40 μ M) are specifically cytotoxic towards MCF-7/ADR. The reason behind this specific cytotoxicity is remains unknown, but it is known that these bases may induce apoptosis before they efflux out of the cells.¹

MCF-7



MCF-7/ADR

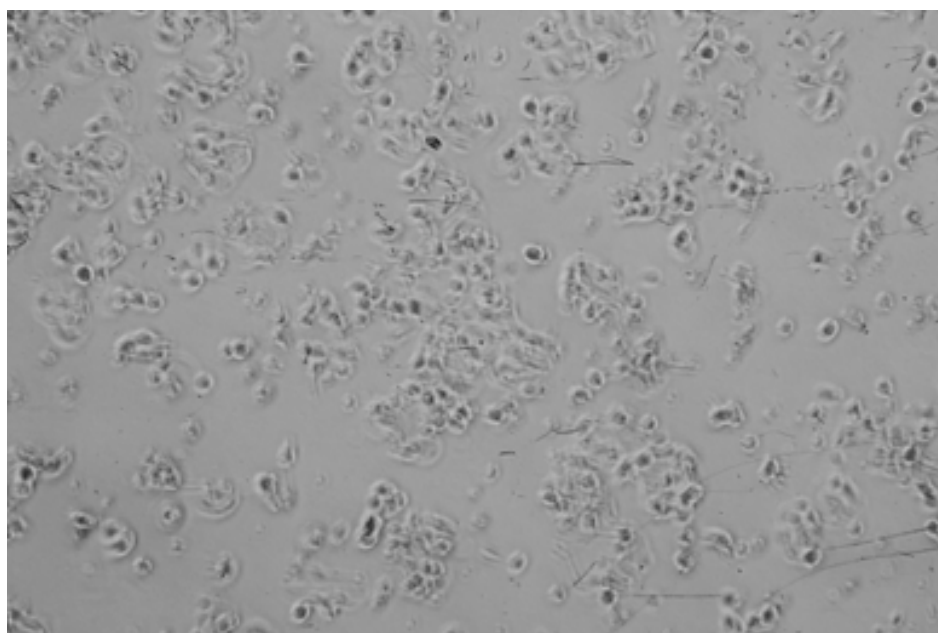


Figure 5. Comparison between morphology of MCF-7 and MCF-7/ADR.

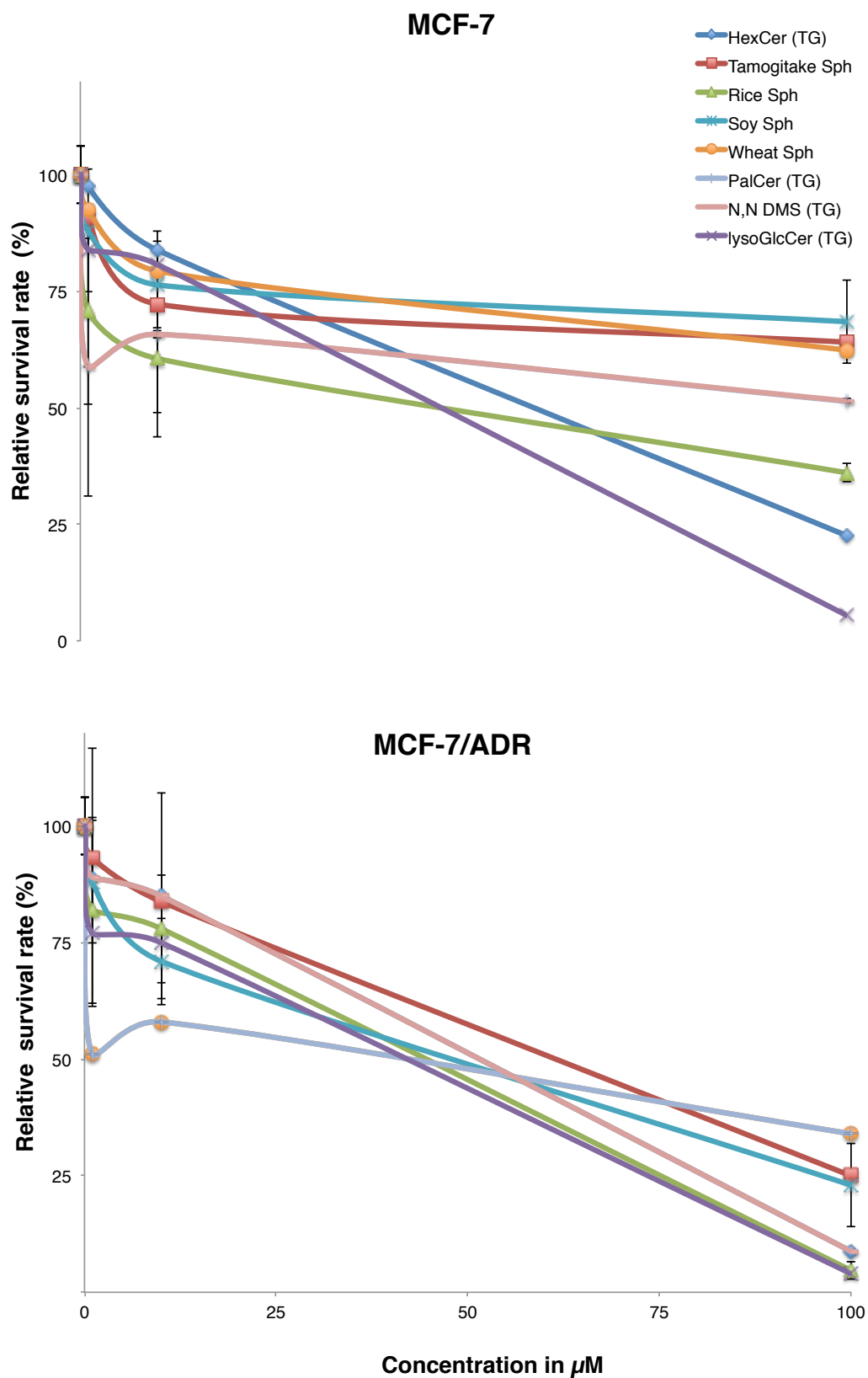


Figure 6. Cytotoxicity of sphingoid derivatives to MCF-7 and MCF-7/ADR by MTT assay (HexCer: hexanoyl ceramide (TG: tamogitake), Sph: sphingoid base, PalCer: palmitoyl ceramide, N,N DMS: N,N dimethyl sphingoid base).

In conclusion author prepared dietary sphingoid bases and synthetically modify them to ceramides and dimethyl sphingoids by different chemical methods. MCF-7/ADR as been established from the parental MCF-7 and two types of cell viability assays (CCK-8 and MTT) were performed to know antiproliferative effects of adriamycin and sphingoid bases. The morphological differences between MCF-7 and MCF-7/ADR were also, emphasized from the fluorescence microscopy. Preliminary results of the MTT assay suggest sphingoid bases and derivatives are cytotoxic to both the cell types, more specifically towards MCF-7/ADR. However, further studies were needed to know whether these molecules act as inhibitor for MDR1 or not.

4.4 Experimental section

Materials and methods

Sphingoid bases were prepared from their respective, glucosylceramides by an chemoenzymatic method described in the chapter 3.

Preparation of ceramides:

Method A

A mixture of sphingoide base (9-Me d18:2^{4E,8E}) (0.107 g, 0.357 mmol) in 4 mL of THF and NaOAc-3H₂O (1.94 g, 0.0142 mol) in 1.9 ml of H₂O was stirred vigorously during the addition of palmitoyl chloride (0.14 g, 0.51 mmol). The reaction mixture was stirred vigorously for an additional hour. The THF was separated, and the aqueous phase was given two additional extractions with 99.5:0.5 CH₂Cl₂/TEA. The organic extracts were combined, concentrated by rotatory evaporation, and filtered through a short column (94.5:5:0.5 CH₂Cl₂/MeOH/TEA). The crude product was purified by silicagel coulmcchromatography (95:5:0.1 CH₂Cl₂/MeOH/TEA,) to give palmitoyl ceramide (0.12 g, 63%).

Method B

i) Preparation of nitrophenol resin: In a 50-mL polystyrene cartridge, to an amino polystyrene resin (1 g, 1.2 mmol) in DMF (15 mL) were added 4-hydroxy-3-nitrobenzoic acid (1 g, 5.5 mmol), HOBt (1 g, 7.4 mmol), and DIC (1 mL, 6.4 mmol). After overnight shaking, the reaction mixture was washed with DMF (20 mL, 5 times), dichloromethane (DCM), and methanol (20 mL, 5 times alternatively). To remove any undesirable side product, DMF (5 mL) and piperidine (0.5 mL) were added to the cartridge and allowed to shake for 1.5 h. The resin was filtered and washed with DMF (20 mL, 5 times). The resulting piperidine salt was removed via the addition of a 10% HCl solution (in DMF, 20 mL) and was allowed to shake for 1.5 h. The resin was then filtered; washed with DMF, methanol, and DCM (20 mL, 5 times each); and dried by nitrogen gas flow.

ii) Preparation of activated esters: The nitrophenol resin (100 mg, 0.12 mmol) was suspended in 1-methyl-2-pyrrolidinone NMP (10 mL), and hexanoyl chloride (0.12 mL, 0.86 mmol) and pyridine (0.1 mL, 1.2 mmol) were added to the reaction mixture. The reaction mixture was allowed to shake overnight, and the resins were filtered and washed with DMF (10 mL, 3 times), methanol, and DCM (10 mL, 5 times, alternatively) and dried by nitrogen gas flow. All other activated esters were formed using the same procedure.

The protocol given above is generally applicable for the preparation of all other types of ceramides from their respective acyl chlorides.

iii) Preparation of ceramides: A mixture of hexanoate ester resin (40 mg, 0.048 mmol) in THF (1 mL) was treated with sphingoid base (9-Me d18:2^{4E,8E}) (7.4 μ L, 0.024 mmol) and stirred at room temperature overnight. The reaction mixture was filtered and washed with THF (1 mL, 3 times). The combined filtrate was evaporated and analyzed by ESI-mass. The peak at m/z 410.2848 (M+H) in the ESI mass spectrum confirms the product as hexanoyl ceramide.

Preparation of N,N dimethyl sphingoid base

Sphingoid base (9-Me d18:2^{4E,8E}) (20 mg) was dissolved in 0.2 M borate buffer (pH 9)/methanol (1:1) mixture by sonication. After addition of NaBH₄ (1 mg) to the mixture, 37% aqueous formaldehyde was added (60 μ L x 10 times, 5 min intervals). The reaction was continued for 18 h at room temperature with occasional

stirring with vortex mixer. And the reaction mixture was extracted by Bilgh-Dyer method using $\text{CHCl}_3\text{:MeOH:H}_2\text{O}$ (2:2:1.3), combined chloroform layer was concentrated in vacuum and purified by silicagel column chromatography using $\text{CHCl}_3\text{:MeOH:NH}_3$ (40:10:1). Obtained product of weight 17 mg (yield 80 %), the structural characterization of N,N dimethyl sphingoid base was confirmed by ESI-mass ($\text{M}+\text{H}$, m/z 340.2511) and ^1H -NMR.

Purity analysis of sphingoid bases

To 50 μL of sphingoid base solution in methanol (1 mg/mL), same amount of *o*-phthaldehyde (OPA) reagent was added, and the mixture was vortex-mixed vigorously for 15 min at room temperature. Following that, 100 μL of mobile phase (85:15 acetonitrile/phosphate buffer pH 7.0) was added and 20 μL of solution was injected to the HPLC on YMC-Pack C8 column (0.46 cm x 15 cm) using acetonitrile-phosphate buffer pH7.0 (85:15). The fluorescence detector was set at 340 nm and 450 nm as an excitation and an emission wavelength respectively. The flow rate was 0.5 mL/min.

Cell culture and growth conditions

The MCF-7 cell line, originally obtained from the American Type Culture Collection (ATCC; USA), was kindly provided by the Prof. Seigo Usuki, Hokkaido University. The MCF-7 cells were maintained as an attached monolayer culture in the commercially defined DMEM medium (Sigma Aldrich, Japan), supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) (Gibco, Japan), and penicillin–streptomycin (Sigma Aldrich, Japan). The cells were grown on 100 mm x 20 mm cell culture dish (Corning, Sigma Aldrich, Japan). The cells were then incubated at 37 °C in a 90% humidified atmosphere of 5% CO_2 .

Establishment of adriamycin resistant MCF-7

Adriamycin 2 mg/mL (TCI, Japan) was used to induce resistance in the MCF-7 cells. To resistant cells were selected by stepwise selection method²²; initially the cells were grown in a cell culture media containing a adriamycin concentration of 10 nM. When the cells were capable of growing and reaching appropriate confluency at a certain concentration, the cells were passaged and double the previous doxorubicin

concentration was used for stepwise selection of resistant cells; a final concentration of 320 nM adriamycin was applied. The developed sub-line is regarded as MCF-7ADR, and the wild type (untreated control) as MCF7 through this paper. To exclude the effects associated with long-term culture of MCF-7 cells, the MCF-7 cells were cultured under identical conditions and maintained in culture for the same period of time as the MCF-7/ADR cells but in the absence of doxorubicin. Before any further experiment, the MCF-7/ADR cells were maintained in doxorubicin free medium for at least 2 days. And morphological analysis of MCF-7 and MCF-7/ADR were done by the photographic image taken, using fluorescent microscope (Nikon, Japan).

Cell proliferation assay

CCK-8 assay:

The cells were seeded in 96-well plates (100 μ L/well at a density of 2×10^5 cells/mL). After incubating for 24 h, the culture medium was removed and the cells were cultured in a medium containing different doses of adriamycin. The adriamycin was dissolved in sterilized water with a original stock concentration 1mM. Each concentration was added in duplicates, and every plate contained a control of cells in plain medium and cells were then incubated at 37 °C for 72 h. The cell viability was determined by assaying the dehydrogenases activity of the treated cells with the highly sensitive CCK-8 (WST-8) assay. After incubation, the media were aspirated from the wells and replaced by fresh media (100 μ L/well); 10 μ L of the CCK-8 solution (Dojindo laboratories, Kumamoto, Japan) was added to each well. The plates were incubated at 37 °C for 2 h, Optical density (OD) at 450 nm wavelength was recorded using a 96-well plate reader (Bio-Rad, Japan). Measurements were performed in duplicates.

MTT assay:

This assay is a colorimetric test based on the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), a yellow tetrazole, to a purple formazan, a process that occurs in the mitochondria of viable cells. The cells were seeded in 96-well plates (100 μ L/well at a density of 2×10^5 cells/mL). After incubating for 24 h, the culture medium was removed and the cells were cultured in a medium containing different doses of sphingoid bases and their derivatives. The sphingoid bases were dissolved in dimethyl sulfoxide (DMSO) and diluted into the

culture medium (the final DMSO concentration was 0.1%). Control experiments were done with 0.1% DMSO as the vehicle. Each concentration was added in duplicates and cells were then incubated at 37 °C for 24 h.

After incubation, the media were aspirated from the wells and replaced by fresh media (100 µL/well); 20 µL of the MTT dye solution (5 mg/mL in sterilized water) was added to each well. The plates were incubated at 37 °C for 3 h, and then 100 µL of solubilisation/stop solution (DMSO) was added to each well. Optical density (OD) at 570 nm wavelength was recorded 1 h later using a 96-well plate reader (Bio-Rad, Japan). Measurements were performed in duplicates.

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Chapter 5

Concluding Remarks

In this thesis, the author has developed two novel methods for rapid and efficient extraction of naturally occurring bioactive sphingoid bases. In chapter 2, as a first method, a novel glutaraldehyde resin (GR) was synthesized, aiming at rapid extraction of sphingoid bases. After successful development of synthetic scheme for GR, the results revealed that, synthesized resin chemoselectively and efficiently captures the sphingosine from human serum sample, as well as sphingoid bases from fungal extract (tamogitake). Overall efficiency of the resin was found to be greater than 80%. Although further studies were needed, this preliminary results suggests that GR could be an efficient resin to extract sphingoid bases.

In the chapter 3, as second method, a highly efficient chemoenzymatic method for the hydrolysis of glucosylceramides to sphingoid bases was developed to prepare sphingoid bases from small-scale to large-scale. The proposed two-step protocol as many advantages in terms of its efficiency, purity, cost etc., The obtained results suggests that, established method was generally applicable to varieties of glucosylceramides, hence present strategy as a large industrial scope. Compared to the reported extraction/preparation methods these novel methods are more simple, efficient and practically applicable. Hence adopting these methodologies for extracting/preparing sphingoid bases by lipid chemists may solve the deficiency for sphingoid bases. Henceforth, it may open the door for exploring their useful bioactivities.

The first application of sphingoid bases as multidrug resistance inhibitors was discussed in chapter 4. Sphingoid base derivatives such as ceramides, N,N dimethyl sphingoids, were prepared, and MCF-7/ADR cell were established from parent MCF-7 by sequential treatment of adriamycin. The preliminary results from the cell viability assay suggest that, these natural sphingoid bases and their derivatives are cytotoxic more specifically towards MCF-7/ADR, than MCF-7.

Appendix (Publications)

- (1) **Siddabasave Gowda B. Gowda**, Seigo Usuki, Mostafa A. S. Hammam, Yuta Murai, Yasuyuki Igarashi, Kenji Monde, “Highly Efficient Preparation of Sphingoid Bases from Glucosylceramides by Chemoenzymatic Method” *J. Lipid Res.* **2016**, 57, 325-331.
- (2) **Siddabasave Gowda B. Gowda**, Keiko Yamane, Atsufumi Nakahashi, Ananda Kumar C.S., and Kenji Monde “Novel Resin-Based Efficient Extraction of Sphingoid Bases” *Angewandte Chemie International Edition* (**2016**). To be submitted
- (3) 2014/10/22(特許公開 2014-215478)糖スフィンゴシン及びスフィンゴ塩基の製造方法発明者:門出健次、五十嵐靖之、臼杵靖剛、ボメゴウダ シッダバサーブ ゴウダ
(Patent No: 2014-215478, Japan. “Development of a Method for Producing Glucosylsphingoids and Sphingoids”: Kenji Monde, Yasuyuki Igarashi, Seigo Usuki, **Bommegowda Siddabsavegowda**)