A modular approach to sphingolipid analogs mediated by aziridines: Synthesis and biological studies

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"A MODULAR APPROACH TO SPHINGOLIPID ANALOGS MEDIATED BY AZIRIDINES: SYNTHESIS AND BIOLOGICAL STUDIES"

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A mis abuelos

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Abbreviations

Ac	Acetyl or acyl
Ac_2O	Acetic anhydride
AcOEt	Ethyl acetate
aGC	α-Galactosylceramide
aq	Aqueous
app.	Aparent
ASMase	Lysosomal acid sphingomyelinase
ATP	Adenosine triphosphate
βGlcCer	β-Glucosylceramide
Bn	Benzyl
BMDC	Bone marrow dendritic cells
BMT	Bone marrow transplantation
BnBr	Benzyl bromide
BSA	Bovine serum albumin
Bu	Butyl
Bu_2BOTf	Boron trifluoride dibutyl etherate
Bz	Benzoyl
cat.	Catalytic
Cdase	Ceramidase
Cdases	Ceramidases
Cer	Ceramide
CERK	Ceramide kinase
CerS	(Dihydro)ceramide synthase
CERT	Ceramide transfer protein
CGT	Galactosyltransferase
CHAPS	3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate hydrate
C1P	Ceramide-1-phosphate
C1PP	Ceramide-1-phosphate phosphatase
C6-NBD	*N*-[6-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]hexanoyl]
CMT	Cell-mediated therapy
CoA	Coenzyme A
CSA	(1*S*)-(+)-10-Camphorsulfonic acid
d	Day(s)
DAG	Diacylglicerol
DBU	1,8-Diazabicyclo[5.4.0]undec-7-ene
DCM	Dichloromethane
DES	Dihydroceramide desaturase
dhCer	Dihydroceramide
dhSph	Dihydrosphingosine
DIPEA	*N*,*N*-Diisopropylethylamine
DMAP	4-(Dimethylamino)pyridine
DMEM	Dulbecco's modified eagle's medium
DMF	*N*,*N*-Dimethylformamide
DMP	2,2-Dimethoxypropane
DMSO	Dimethyl sulfoxide
EDC	*N*-(3-Dimethylaminopropyl)-*N*'-ethylcarbodiimide
EET	Enzyme-enhancement therapy
ELISA	Enzyme-linked immunosorbent assay

ELS	Evaporative light scattering
eq	Equivalent
ER	Endoplasmic reticulum
ERT	Enzyme replacement therapy
Et	Ethyl
EtOH	Ethanol
Et_2O	Diethyl ether
GBA	β-Glucocerebrosidase
α-GalCer	α-Galactosylceramide
GCH	Glucosylceramide hydrolase
GCS	Glucosylceramide synthase
gDQCOSY	gradient Double Quantum Correlation Spectroscopy
gHSQC	gradient Heteronuclear Single Quantum Correlation
gHMBC	gradient Heteronuclear Multiple-Bond Correlation
GlcCer	Glucosylceramide
β-GlcCer	β-Glucosylceramide
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GSL	Glycosphingolipid
h	Hour(s)
HPLC	High-performance liquid chromatography
HRMS	High-ressolution mass spectrometry
HRP	Horseradish peroxidase
IFN	Interferon
IL	Interleukin
iNKT	Invariant natural killer T
IPC	Inositol phosphorylceramide
IPCS	Inositolphosphoryl ceramide synthase
IR	Infrared
KDHR	3-Ketodihydrosphingosine reductase
3KdhSph	3-Ketodihydrosphingosine
liq.	Liquid
LPP	Lipid phosphate phosphatase
LPPs	Lipid phosphate phosphatases
LSD	Lysosomal storage disorders
LTA	Lead(IV) tetraacetate
M	Molarity
MAMs	Mitochondria associated membranes
Me	Methyl
MeCN	Acetonitrile
MeOH	Methanol
MHC	Major histocompatibility complex
min	Minute(s)
MIPC	Mannosyl inositol phosphoceramide
$[M(IP)_2C]$	Mannosyl diinositol diphosphoceramide
MOM	Methoxymethyl ether
MOMBr	Bromomethyl methyl ether
MOMCl	Chloromethyl methyl ether
mp	Melting point
Ms	Mesyl
MsCl	Methanesulfonyl chloride
MW	Microwave
N	Normality

NADPH	Nicotinamide adenine dinucleotide phosphate
NKT	Natural killer T
NaOMe	Sodium methoxide
NMR	Nuclear magnetic resonance
NOE	Nuclear Overhauser effect
NSMase	Neutral magnesium-dependent sphingomyelinase
Nu	Nucleophile
PBS	Phosphate buffer saline
PDA	Photodiode array
PDMP	1*R*-Phenyl-2*R*-decanoylamino-3-morpholino-1-propanol D-*threo*
PG	Protecting group
Ph	Phosphine
PPh_3	Triphenylphosphine
PhSH	Thiophenol
PIDA	Diacetoxy(phenyl) iodane
$PthNH_2$	*N*-aminophthalimide
pTSA	*p*-Toluenesulfonic acid
py	Pyridine
rt	Room temperatrure
sat	Saturated
Ser	L-serine
SK	Sphingosine kinase
SKases	Sphingosine kinases
SM	Sphingomyelin
SMase	Sphingomyelinase
SMases	Sphingomyelinases
SMS	Sphingomyelin synthase
SMSs	Sphingomyelin synthases
SN2	Bimolecular nucleophilic substitution
S1P	Sphingosine 1-phosphate
Sph	Sphingosine
SPT	Serine palmitoyltransferase
SPL	Sphingosine-1-phosphate lyase
SRT	Substrate reduction therapy
TBAF	Tetrabutylammonium fluoride
TBAHS	tetra-*n*-Butylammonium hydrogensulfate
TBAI	Tetrabutylammonium iodide
TBDPSCl	t-Butyldiphenylsilyl chloride
TCR	T cell receptor
TEA	Triethylamine
Tf	Triflate
TfN_3	Trifluoromethanesulfonyl azide
TFA	Trifluoroacetic acid
Th	T helper
THF	Tetrahydrofuran
TLC	Thin layer chromatography
TrCl	Trityl chloride
TRIS/HCl	Tris(hydroxymethyl)aminomethane hydrochloride
UDP	Uridine diphosphate

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1.1. Sphingolipids: structure and functions

Sphingolipids are a class of natural compounds first characterized by the German-born and clinician Johann L. W. Thudichum in 1884.[1] He isolated several compounds from ethanolic brain extracts and these molecules when subjected to acid hydrolysis gave sugar residues, fatty acids and an aminoalcohol which was called "sphingosine" referring to the Greek Sphinx to indicate its enigmatic structure and properties.

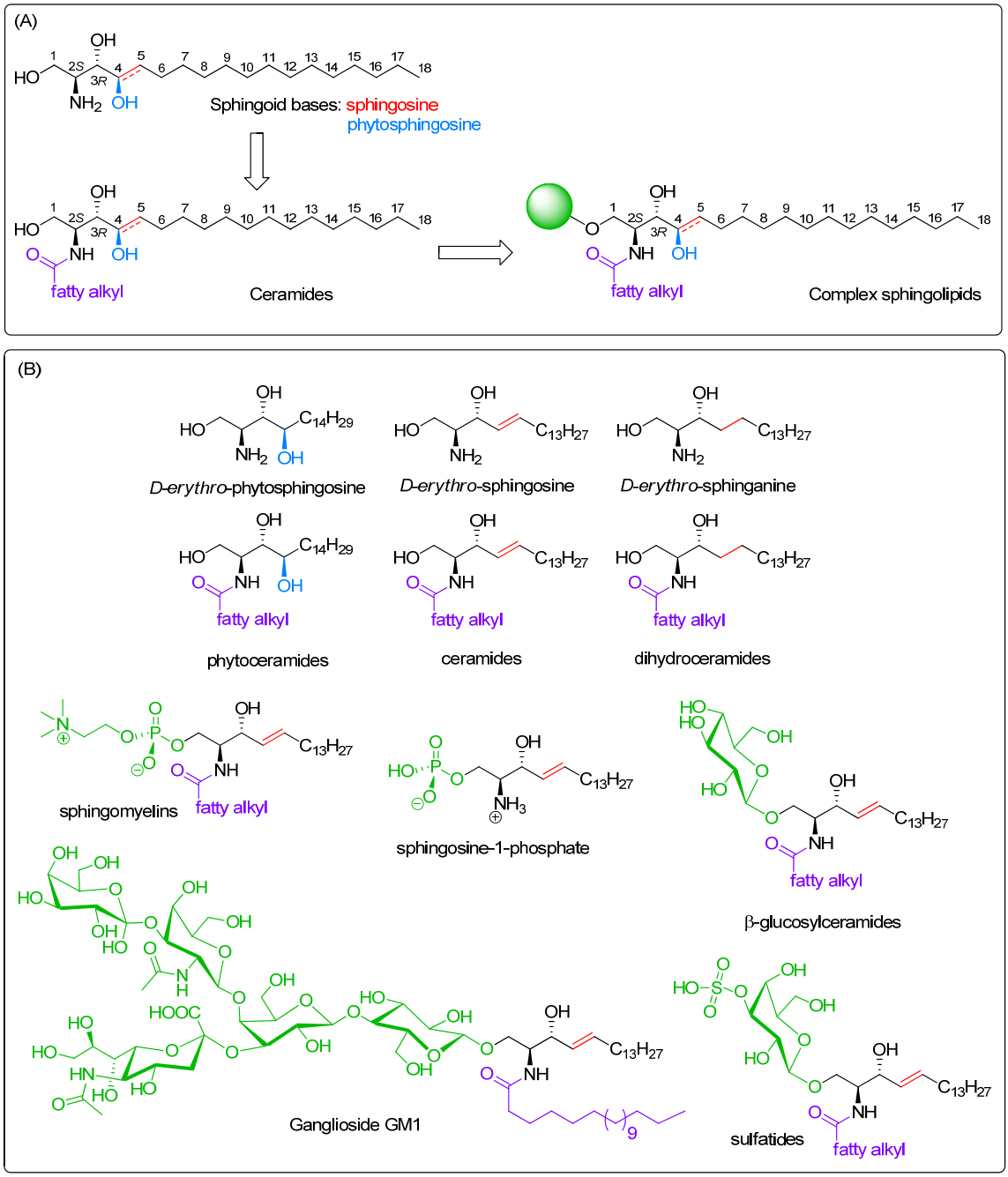

Figure 1.1. (A) General structure of sphingolipids. **(B)** Chemical structures of naturally occurring sphingolipids.

Sphingolipid structures are defined by their eighteen carbon backbones with a 2-amino-1,3-diol functionality (usually 2*S*, 3*R*), which are called sphingoid bases. These organic bases can be *N*-acylated by fatty acids of different length giving ceramides. Modification of this general structure gives rise to a family of sphingolipids depending on a variety of charged, neutral, phosphorylated and/or glycosylated moieties attached at position 1 (Figure 1.1).

A high variety of complex sphingolipids is known ranking from the simplest sphingosine-1-phosphate to more complex structures such as cerebrosides β-glucosylceramides and galactosylceramides, or higher glycosilated ceramide species called glycosphingolipids.
When galactosylceramide adds a sulphate group at 3-position of the sugar residue gives rise to sulfatides, while the addition of a sialic acid in the carbohydrate head group of glycosphingolipids, results in a new subclass of glycolipids known as gangliosides.[2]

Sphingolipids are essential structural components of eukaryotic membranes with amphipathic character that tend to aggregate into membranous structures, where they are mostly present in the plasma membrane and related cell membranes, such as Golgi membranes and lysosomes.
In the plasma membrane the distribution of lipids is not uniform, particularly, sphingolipids and cholesterol form platforms or rafts that float in the liquid phase.[3] Furthermore, these lipid rafts are important in signal transduction processes and some key components of signal transduction are located on rafts.[4]

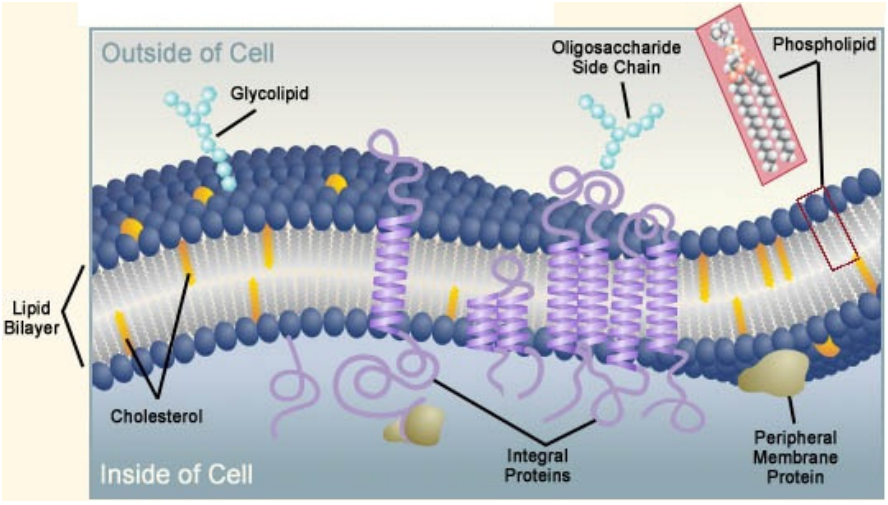

Figure 1.2. The fluid mosaic model for cell membranes.

As it has been mentioned, in addition to the structural role of sphingolipids in membranes, these lipids are also bioactive signalling molecules that have crucial functions in signal transduction, cell growth, cell regulation, death, differentiation, senescence, adhesion, migration, inflammation, angiogenesis, apoptosis and intracellular trafficking, so they provide essential biomolecules for the physiological cell function, exemplified by sphingolipids, such as ceramide, sphingosine, sphingosine-1-phosphate, ceramide-1-phosphate and lyso-sphingomyelin.[2,5]

Specifically, ceramide and sphingosine induce cell cycle arrest, differentiation, or cell death in most transformed cell lines, in contrast, sphingosine-1-phosphate mostly stimulates cell growth and suppresses apoptosis, modulates adhesion and cell motility, and affects cell differentiation.[6]

In mammals, the most common sphingoid base is *D-erytho*-(2*S*,3*R*)-sphingosine, although smaller amounts of *D-erythro*-sphinganine and *D-erythro*-phytosphingosine may also be present. For example, in human skin, 40% of the total epidermal ceramides contain phytosphingosine as a sphingoid base.[2]

D-erythro-phytosphingosine predominates in plants, and yeast are also abundant in this base.[7] More structural variations in the sphingolipid backbone can be found in plants, such as additional double bonds or double bond on C8-C9. Changes in the amido-bound fatty acids can also be found in their carbon length (C14 to C24), saturation grade, and α-hydroxilation.[6]

In yeasts, such as *Saccharomyces cerevisiae*, sphingolipids constitute approximately 10% of total membrane lipids and approximately 40% of total inositol containing lipids and phytosphingosine is the main long chain amino base component in this yeast. The three main sphingolipid groups in *Saccharomyces cerevisiae* include inositol phosphorylceramide (IPC), mannosyl inositol phosphoceramide (MIPC) and mannosyl diinositol diphosphoceramide [$M(IP)_2C$], mostly consisting of phytosphingosine with a long chain fatty acid (usually C26-hydroxy fatty acid) bound by amide bond, and a polar head group consisting of myoinositol, phosphate and carbohydrate.[7]

1.1.1. Sphingolipid metabolism in mammals

Sphingolipid metabolism in mammals,[2,5,8-10] is a cell process based on a highly complex network of interconnected pathways, in which ceramide occupies a central position in both biosynthesis and catabolism.

1.1.1.1. *De novo* synthesis

Sphingolipids are synthesized by *de novo* synthesis which begins at the cytosolic leaflet of the endoplasmic reticulum (ER) from nonsphingolipid precursors (Figure 1.3 and 1.4). The first reaction in sphingolipid synthesis requires the pyridoxal phosphate-dependent enzyme serine palmitoyltransferase[11] (SPT) and this condensation reaction takes place through cytosolic L-serine and a fatty acyl coenzyme A (CoA) which is usually palmitoyl CoA. This leads to 3-ketosphinganine (3-ketodihydrosphingosine), which is reduced at its ketone group to a hydroxyl group by the enzyme 3-ketodihydrosphingosine reductase (KDHR) in a NADPH dependent manner.

In next step, dihydrosphingosine (sphinganine) is further acylated by the action of six distinct (dihydro)ceramide synthases, which in mammals are abbreviated as CerS1-6 (Figure 1.3 and 1.4). It is important to highlight that there is a significant amount of evidence that each CerS has a distinct, but overlapping acyl CoA preference that can provide different dihydroceramide or ceramide species profiles.[8]
All known CerS have been localized to the ER with their catalytic sites facing the cytosol, which are in position to acylate newly generated dihydrosphingosine molecules at their 2-amino group in the presence of available fatty acyl CoAs.

After the *N*-acylation step, dihydroceramide desaturase (DES) is responsible of a dehydrogenation process of dihydroceramide that generates a 4,5-*trans*-double bond to give ceramide (Figure 1.3 and 1.4). This enzyme can also exhibit C4 hydroxylase activity from a common initial C-H activation step to form a very short-lived radical intermediate or its organoiron equivalent which can collapse to give either alcohol or olefin.

Ceramide is a membrane bound molecule generated in the ER and this molecule is transported to the Golgi apparatus, where it is modified to complex sphingolipids, such as sphingomyelin and glycosphingolipids.

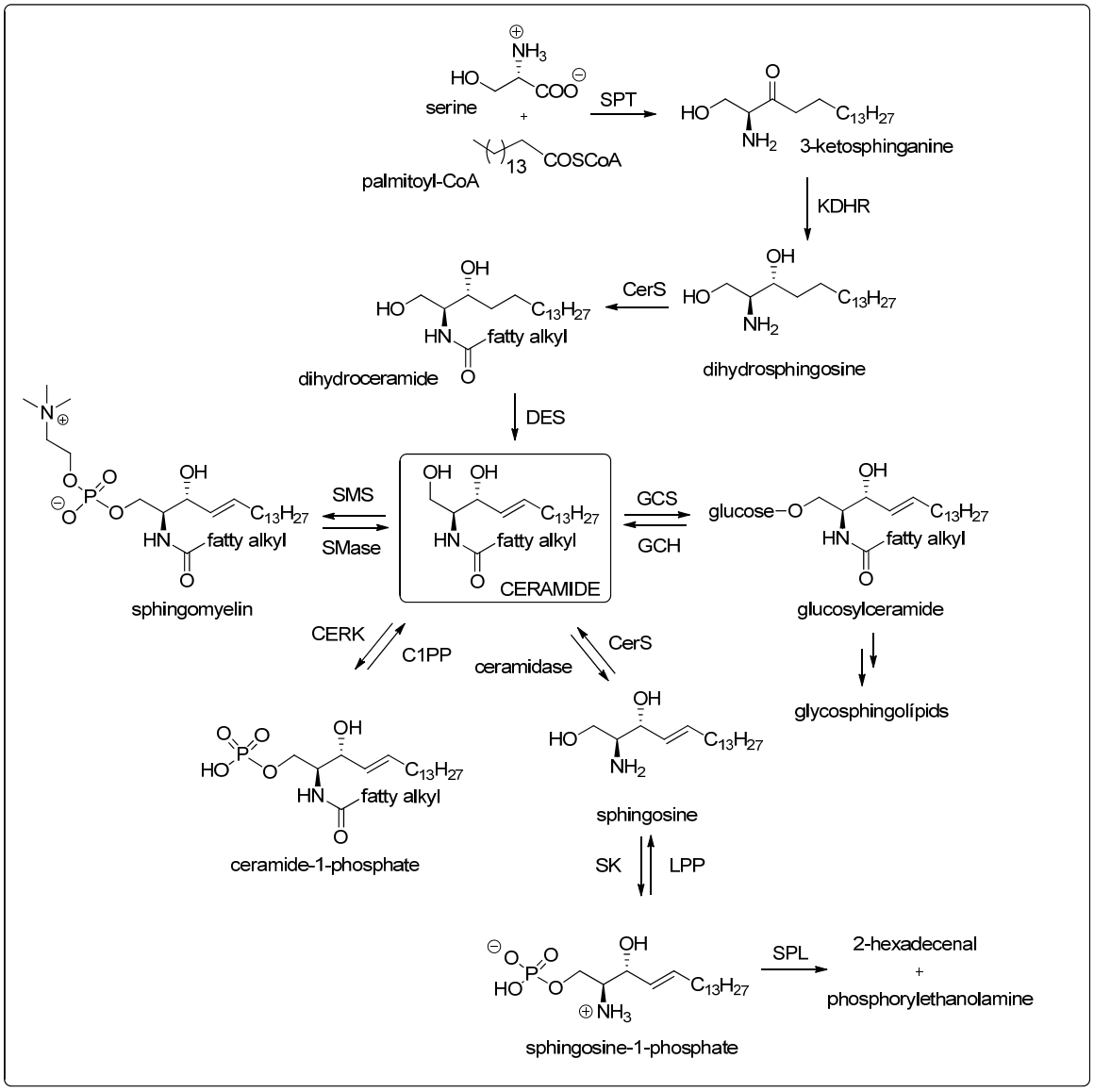

Figure 1.3. Sphingolipid metabolism in mammals. SPT: serine palmitoyltransferase; KDHR: 3-ketodihydrosphingosine reductase; CerS: (dihydro)ceramide synthase; DES: dihydroceramide desaturase; SMS: sphingomyelin synthases; GCS: glucosylceramide synthase; GCH: glucosylceramide hydrolase; CERK: ceramide kinase; C1PP: ceramide-1-phosphate phosphatase; SMase: sphingomyelinase ; SK: sphingosine kinase; LPP: lipid phosphate phosphatases. SPL: sphingosine-1-phosphate lyase.

As ceramide has a low solubility in aqueous environment, the cell transports it from the ER to the Golgi apparatus by employing two major mechanisms. The first mechanism mobilizes ceramide through vesicular transport and the second through a cytosolic protein called ceramide transfer protein (CERT) (Figure 1.4).

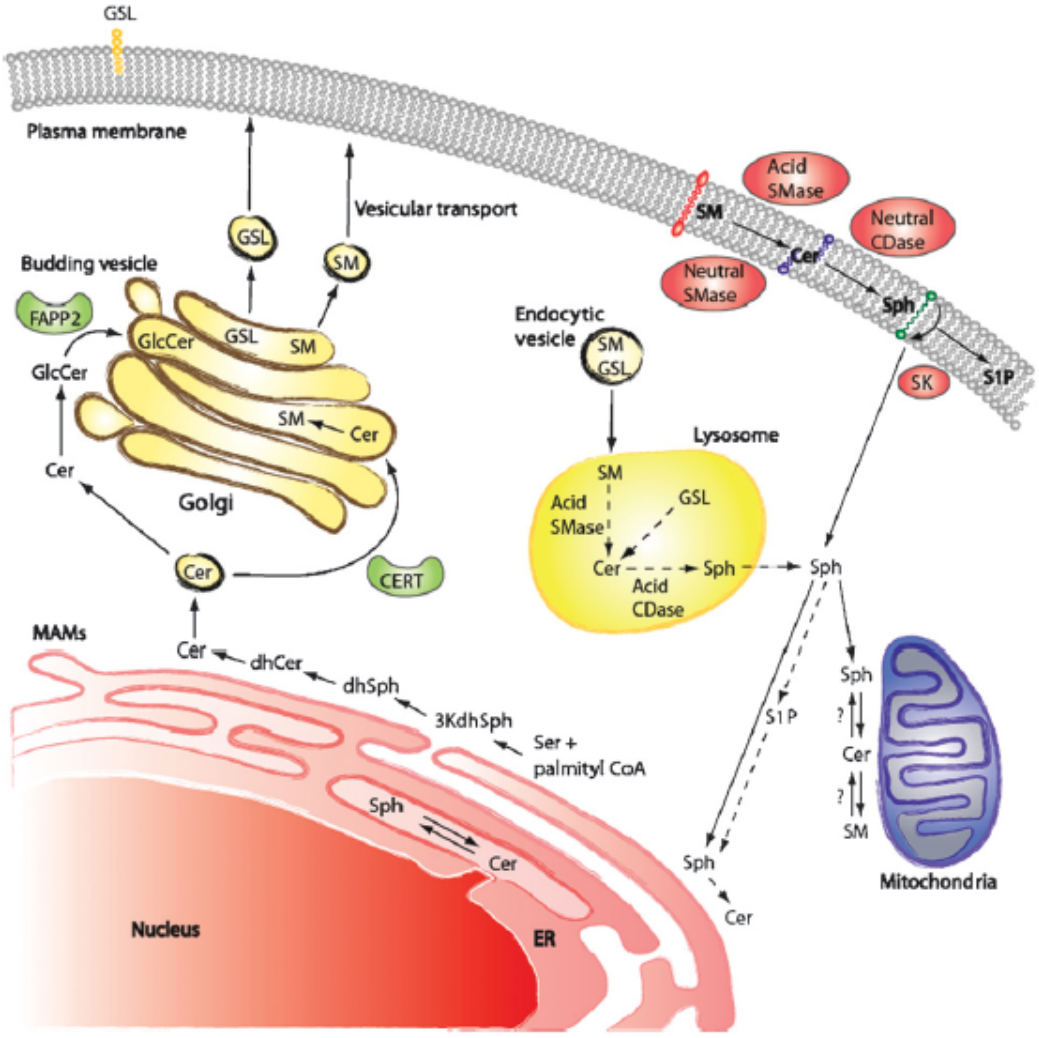

Figure 1.4. Localization of sphingolipid and enzymes of the sphingolipid pathway.[9]
ASMase: lysosomal acid sphingomyelinase; Cdase: ceramidase; Cer: ceramide; CERT: ceramide transfer protein; C1P: ceramide-1-phosphate; dhCer: dihydroceramide; ER: endoplasmic reticulum; GlcCer: glucosylceramide; GSL: glycosphingolipid; NSMase: neutral magnesium-dependent sphingomyelinase; S1P: sphingosine-1-phosphate; SK: sphingosine kinase; SM: sphingomyelin; SMase: sphingomyelinase; Sph: sphingosine: 3KdhSph: 3-ketodihydrosphingosine; dhSph: dihydrosphingosine; MAMs: mitochondria associated membranes. Ser: L-serine.

Ceramide is the central molecule in the biosynthesis of sphingolipids and glycosphingolipids, because this molecule is the substrate for the formation of sphingomyelins by the action of sphingomyelin synthases (SMSs) or formation of gluco- or galacto- glycosphingolipids by the action of the enzymes glucosylceramide synthase (GCS) and ceramide galactosyltransferase (CGT), respectively.

Glucosylceramide and galactosylceramide are essential sphingolipids for mammalian development and tissue specific functions, but they are not required for the viability of cells in culture. Glucosylceramide is synthesized in the cis-Golgi from ceramide and UDP-glucose by

the enzyme GCS, which has its catalytic site facing the cytosol (Figure 1.4). In contrast, CGT is an ER transmembrane protein that has its catalytic site facing the lumen of the ER and it utilizes UDP-galactose and ceramide to create galactosylceramide. Both products can also be hydrolyzed by specific β-glucosidases and galactosidases to release ceramide.

An important sphingolipid with an essential role in eukaryotic cell viability is sphingomyelin and that is displayed by the inability of mammalian or yeast cells to survive in culture when they are unable to produce this molecule through CERT mutation or defects in *de novo* sphingolipid synthesis.
Sphingomyelin, predominantly located in the outer leaflet of the plasma membrane is produced by the action of sphingomyelin synthases (localized in the Golgi and the plasma membrane) through the transfer of a phosphocoline group from phosphatidylcholine to ceramide giving diacylglycerol (DAG) and sphingomyelin. Both products are considered bioactive lipids with opposing effects on cellular proliferation and survival, and SMS has been proposed to play an essential role in regulating cellular fate.

Although ceramide is the substrate for the synthesis of complex sphingolipids, this central molecule can also be phosphorylated to give ceramide-1-phosphate, which is produced in the trans-Golgi and potentially the plasma membrane by the action of the enzyme ceramide kinase (CERK). This enzyme has preference for the phosphorylation of species with acyl chain lengths greater than 12 carbons long, but it has no preference for the degree of saturation.

Ceramide-1-Phosphate levels are controlled both by its synthesis through CERK and its dephosphorylation back into ceramide by ceramide-1-phosphate phosphatase (C1PP). Not only can C1PP generate ceramide from complex sphingolipids, but also a family of sphingomyelinases (SMases) can give ceramide from sphingomyelin, the most abundant complex sphingolipid in human cells. The SMase family catalyze the sphingomyelin hydrolysis to afford ceramide and free phospholine. Depending on their pH optimum, the mammalian SMases fall into three major categories which are: acid SMase (displaying a pH optimum of 4.5 and localized in acidic compartments of the cell), alkaline SMase (pH optimum of 9) and neutral SMase (neutral pH optimum having different localizations within the cell).

Finally, ceramide can be deacylated through a family of enzymes known as ceramidases (Cdases), which can also be biochemically classified according to their pH optimum as acid, neutral and alkaline ceramidases. Deacylation of ceramide affords sphingosine, which is then available for recycling into sphingolipid pathways or it can be phosphorylated by sphingosine kinases (SKases) to give sphingosine-1-phosphate and be subsequently degraded.

1.1.1.2. Ceramide catabolism and final breakdown

In general terms, all sphingolipids are eventually catabolyzed to ceramide, sphingosine and finally, sphingosine-1-phosphate, which is then degraded to produce hexadecenal and phosphorylethanolamine. However, the released ceramide can be either recycled into sphingolipid synthesis.

As pointed out before, the deacylation of ceramide species to give sphingosine is achieved through the family of enzymes known as Cdases. Sphingosine, as well, is either recycled into sphingolipid biosynthesis or phosphorylated by a cytosolic sphingosine kinase (SK), yielding sphingosine-1-phosphate. These sphingosine kinases are also classified as SK1 and SK2 and need ATP to phosphorylate the hydroxyl group at position 1 of free sphingosine, dihydrosphingosine or, in the case of SK2, also phytosphingosine.[8]

Sphingosine-1-phosphate can be then regenerated to sphingosine by the action of the lipid phosphate phosphatases (LPPs), or it can metabolize to release phosphorylethanolamine and 2-hexadecenal. This step is catalyzed by sphingosine-1-phosphate lyase (SPL) in pyridoxal 5'-phosphate dependent manner, serving as the final step in sphingolipid degradation.

It should be highlighted that several intermediates occurring in this degradation pathway are hypothesized to be signalling molecules, specially ceramide, sphingosine and their corresponding 1-phosphates.[5]

Interestingly, sphingolipids play an essential role in mammalian systems and that is the reason why it is important to understand how sphingolipids are synthesized and degraded to maintain their functional levels and this, can be induced by several factors.

1.1.1.3. Lysosomal Storage Disorders: sphingolipidoses

Lysosomal storage disorders (LSD)[12] are defined as rare inherited methabolic defects of the lysosomal degradation of macromolecules or the delibery of catabolic products into the cytosol. This results in the accumulation of large amounts of different methabolic products in the lysosomes and in certain tissues and/or organs.

In the LSD one can differentiate between sphingolipidoses, mucopolysaccharidoses, mucolipidoses, glycoprotein storage diseases and a glycogen storage disease, known as Pompe disease.[2] These diseases are considered rare disorders because there is a frequency of 1 in 7000-8000 live births.[13,14]

Although sphingolipids are minor components in some cells, their accumulation in certain cells and tissues form the basis of many human diseases, such as sphingolipidoses, which are a group of inherited diseases caused by defects in genes encoding proteins involved in the lysosomal degradation of sphingolipids.
The majority of human diseases associated with sphingolipid metabolism are degradation disorders and only a few of them are cause by alteration of biosynthetic enzymes.[14]

The degradation of sphingolipids occurs in the acidic compartments of the cell, for example, in the late endosomes or lysosomes. When some lysosomal cleaving enzymes are deficient, the corresponding lipid substrate accumulates in cell types and organs in which the lipid is predominantly synthesized and it is stored in the lysosomal compartment, giving rise to the sphingolipid storage diseases. Defects in one or more degradation steps leads to the accumulation of a non-degradable sphingolipid and to a lysosomal storage disease classified by the accumulated substance.[2] Moreover, lipid intermediates formed in these degradation processes are trapped within the endosomal/lysosomal compartment and are not available for signalling processes inside or outside the cell. This give rise to biochemical changes with important implications for therapy.

The majority of LSD become manifest in infants and children, thus some LSD were confined to children, but recently adult forms of these diseases have been recognized. Some of them, present severe forms in childhood and milder forms in adults, but the natal and neonatal forms are usually fatal.

LSD in adults are clinically classified into three major categories. The first shows predominantly early onset patients while late onset are minority. The second category comprises disorders showing some disease manifestations already in childhood, but the full blown picture is seen in adolescents or young adults and the disease is compatible with prolonged life. The third group is characterised by disorders which usually become clinically manifest in adults, but some cases are already found in adults.[15]

Some of the known LSD classified as sphingolipidoses are: Gaucher disease, Fabry disease, Farber disease, Tay-Sachs disease, Sandhoff disease, Krabbe disease, Niemann-Pick and GM1 gangliosidoses.[16]

Gaucher disease[17] is the most common LSD and it is an autosomal recessive disorder caused by a deficiency in glucosylceramide-β-glucosidase (β-glucocerebrosidase) leading to accumulation of glucosylceramide mainly in macrophages but also in tissues. Three different types of Gaucher disease are known: type I, type II and type III.[14]

The most frequent form of Gaucher disease is type I with a frequency of 1:50000-200000 births, but higher among the Ashkenazi Jewish population[18] with 1:1000 and these patients range between 6-80 years.[14]
Gaucher disease type II is characterized by the involvement of the nervous system with a life expectancy of less than two years. Type III is mainly found in the Northern Swedish population and it is an intermediate variant of the other two types. The neurological symptoms have a later onset and a slower development than in type II and patients can have a survival date between a few years and forty years.

Gaucher disease is an heterogeneous disease because a large number of mutations within the glucocerebrosidase gene are known. Approximately 200 mutations at the β-glucocerebrosidase (GBA) have been found in patients with Gaucher disease.
Interestingly, recent evidences establish an association between Gaucher disease and the development of parkinsonism.[19,20] Clinical, genetic and pathological studies all demonstrate that mutations in GBA are an important and common risk factor for Parkinson disease and related disorders.[21] These studies show that some patients with Gaucher disease and Gaucher carriers develop parkinsonism and subjects with Parkinson disease have a greatly increased frequency of GBA mutations, this opening an important breakthrough for understanding the origin of this devastating neurological disorder.

Farber disease[22] is characterized by the inherited deficiency of lysosomal acid ceramidase and storage of ceramide in the lysosomes. The symptoms of Farber disease appear several months after birth and death occurs within the first year of life. However, patients with milder forms can reach adulthood. The most usual clinical manifestation of this disease is the development of painful and progressive joint deformations, subcutaneous nodules and progressive hoarseness.

In humans, genetic deficiency of acid SMase results in autosomal recessive Niemann-Pick disease.[23] More than 50 mutations in the SMase gene have been identified in patients affected with this disease. Niemann-Pick patients can be divided in four types, known as A, B, C and D, and they are characterized by the accumulation of sphingomyelin due to a deficiency of acid SMase.

Finally, other LSD are Fabry disease,[24] which is caused by deficient activity of α-galactosidase A; Krabbe disease[25] that is caused by an inherited deficiency of galactosylceramide-β-galactosidase (β-galactocerebrosidase); Sandhoff disease, characterized by storage of negatively charged glycolipids and elevation of uncharged glycolipids, and Tay-Sachs (or the B1-variant of GM2-gangliosidosis), which is deficient in β-hexosaminidase A, leading to an accumulation of ganglioside GM2 in neuronal cells, the main sites for ganglioside synthesis.

1.1.1.4. Treatment of sphingolipidoses

The most current therapies that are in use or under evaluation for the treatment of sphingolipidoses are: the enzyme replacement therapy (ERT), cell-mediated therapy (CMT) (including bone marrow transplantation[26] (BMT)), gene therapy, enzyme-enhancement therapy (EET) and substrate reduction therapy (SRT).[14,27]

ERT consist of diminishing the substrate storage by the exogenous supply of the defective lysosomal enzyme. This therapy is successfully applied to patients with type I Gaucher disease and Fabry disease.[28]

In CMT, cells are used to replace or compensate the defective cell population with normal equivalents to restore the tissue or organ function or to release enzymes for uptake by deficient cells.

In reference to the gene therapy, it should be noted that it is based on the insertion of a functional copy of the mutated gene into cells to produce the deficient protein. In this case, the deficient enzyme should be over-expressed by a few cells, secreted in high levels, and thus correct the phenotype of adjacent cells.

The enzyme-enhancement therapy consist of the use of chemical chaperones,[29,30] which can bind to not completely defective enzymes by certain mutations that have an intact catalytic center. Specifically, chemical chaperones stabilize the residual functional conformation and prevent the premature degradation of these enzymes.

Finally, SRT is based on the administration of inhibitors of sphingolipid enzymes to reduce the substrate in the lysosomes. This therapy is expected to be helpful in the treatment of sphingolipidoses of patients with some residual enzymatic activity or combined with methods which restore this activity. For that reason, substrate analogs that can inhibit sphingolipid enzymes are useful compounds for the treatment of sphingolipidoses based on SRT.

1.1.1.5. Inhibitors of sphingolipid metabolism enzymes

Because of the existence of LSD, the search for natural or synthetic sphingolipid enzyme inhibitors is a subject of constant interest and it should be noted that the design and synthesis of inhibitors of sphingolipid enzymes is mainly addressed to treat sphingolipidoses mediated by SRT. However, the inhibition of these enzymes can also contribute to the treatment of other diseases such as cancer,[31] or Alzheimer's disease,[32] in which sphingolipid levels and the expression of sphingolipid metabolizing enzymes are altered.

Examples of substrate analogs as inhibitors of sphingolipid enzymes are nojirimycin and its derivatives, which are glucose analogs with potent inhibitory activity to treat diseases that are caused by accumulation of substances derived from glucosylceramide. *N*-butyl-1-deoxynojirimycin (Zavesca®) is a good inhibitor of glucosylceramide synthase and its efficacy was demonstrated in clinical trials for the treatment of human patients of Gaucher disease, type I (Figure 1.5).[33]

In addition, the synthesis of iminosugar-based inhibitors of glucosylceramide synthase, has attracted considerable attention in the search for new therapeutic agents against Gaucher disease.[34]

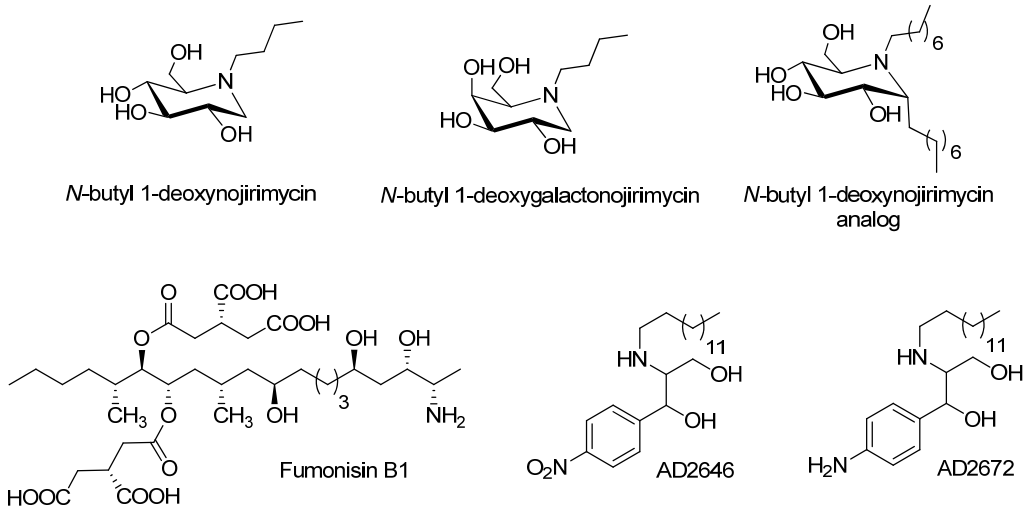

Figure 1.5. Chemical structures of natural an synthetic inhibitors of sphingolypid metabolism enzymes. Stereochemistry for compounds AD2646 and AD2672 is not described in the literature reference.[35]

Fumonisin B1 and fumonisin B2, are two mycotoxins with structural similarities to sphingosine, which are produced by *Fusarium moniliforme*, a fungi on maize and other grains. These naturally occurring substances are potent competitive inhibitors of ceramide synthase.[2,36]

Other non-natural sphingolipid analogs reported[35] in the literature are AD2646 or AD2672, which can inhibit the synthesis of sphingomyelin and glycosphingolipids in HL60 human myeloid leukemic cells, inducing apoptosis that led to cell death.

1.1.2. Sphingolipid metabolism in fungi

1.1.2.1. Biosynthesis of sphingolipids in fungi

The sphingolipid synthesis in fungi[7,37] has some similarities with that on mammals. It starts with the condensation of L-serine and palmitoyl-CoA, catalyzed by SPT, a piridoxal phosphate-containing enzyme that is the target of several potent natural inhibitors[38,39] (Figure 1.6). In analogy with mammalian sphingolipid metabolism, this biosynthesis continues with the reduction of 3-ketosphinganine by the action of an enzyme that has not been identified and characterized.

Then, carbon 4 of dihydrosphingosine is hydroxylated to afford phytosphingosine, the main long-chain base found in fungal and plant ceramides, and this reaction is catalyzed by the enzyme dihydrosphingosine C4-hydroxylase.[40]

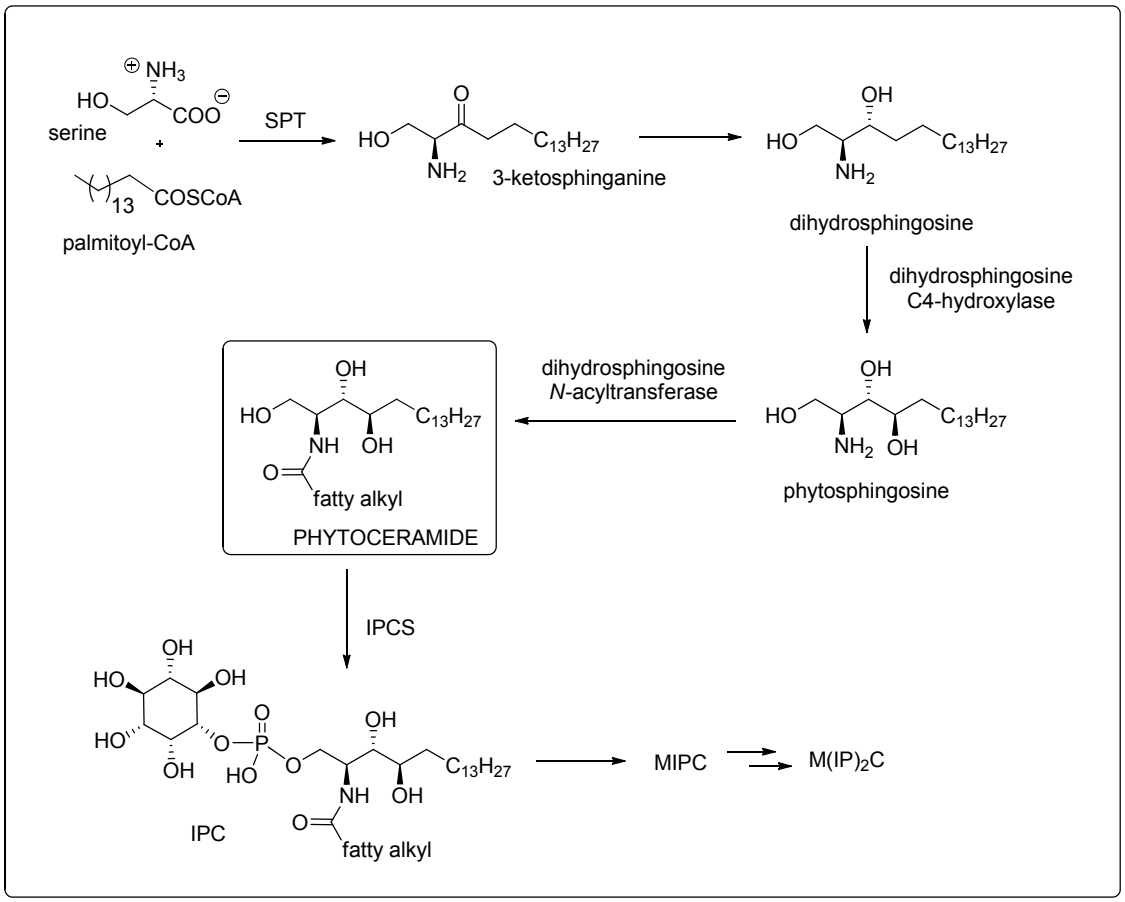

Figure 1.6. Sphingolipid metabolism in fungi. SPT: serine palmitoyltransferase; IPCS: inositol phosphorylceramide; MIPC: mannosyl inositol phosphoceramide; [$M(IP)_2C$]: mannosyl diinositol diphosphoceramide.

After that, phytosphingosine is subsequently acylated by the enzyme dihydrosphingosine *N*-acyltransferase that usually link a fatty acid of 24 or 26 carbons,[37] and phytoceramide is obtained. Then, this ceramide is converted to IPC by transfering inositol phosphate from phosphatidylinositol by the action of inositol phosphorylceramide synthase (IPCS), which is an essential enzyme for fungi, but it is not present in mammals where sphingomyelin synthase is the equivalent enzyme. Finally, the addition of mannose and other modifications can lead to more complex metabolites such as MIPC or [$M(IP)_2C$].

When trying to compare the sphingolipid metabolism of mammals and fungi, we can observe that they have in common part of their synthesis. However, as it has been highlighted, there are important differences in the ceramide generation step. Specifically, mammals generate

ceramide, which is transformed to complex sphingolipids or sphingosine, in contrast, fungi generate phytoceramide, a precursor of inositol phosphoceramide. Therefore, the search for inhibitors of the enzyme inositol phosphoceramide synthase has become an attractive option to find novel antifungal agents with the major interest of being specific inhibitors for fungi, because they do not inhibit mammalian sphingolipid synthesis.

Additionally it should be noted that potent natural inhibitors of the enzyme SPT have been described. Among them, we can find sphingophungins, lipoxamycin,[39] myriocin[38] and viridofungins. However, the inhibition of SPT enzyme is not as attractive as IPCS in the search of specific antifungal agents.

1.1.2.2. IPCS inhibitors as antifungal drugs

Fungal pathogens present an increasing threat to human health and the current developed drugs are not efficacious, since they cause serious side effects and become less useful because of increased resistance to them.

Due to sphingolipid synthesis is vital for growth and viability of fungi, inhibition of the fungal enzyme IPCS and in turn the synthesis of sphingolipids, suppose an efficient and selective strategy to find antifungal drugs.

A 14-membered macrolide called rustimicin,[41] which was previously identified as an inhibitor of plant pathogenic fungi, is a potent antifungal agent by inhibition of IPCS (Figure 1.7). This molecule was isolated from fermentations of *Micromonospora chalcea* and it was called rustimicin because of its high activity against wheat stem rust fungus (*Puccinia graminis*). However, almost simultaneously, the same structural compound was reported as galbonolide A[42] from *Streptomyces galbus*, with potent activity agains *Botrytis cinerea* and other pathogens.

Khafrefungin[43] is a natural compound, isolated from an endophyritic fungus, composed of aldonic acid esterified to a linear polyketide. This molecule shows specificity for fungal sphingolipid enzymes, because it is a potent inhibitor of IPCS. Khafrefungin is active against IPCS of *Saccharomyces cerevisiae*, *Candida albicans*, and other pathogenic fungi, but it does not inhibit mammalian sphingolipid synthesis.

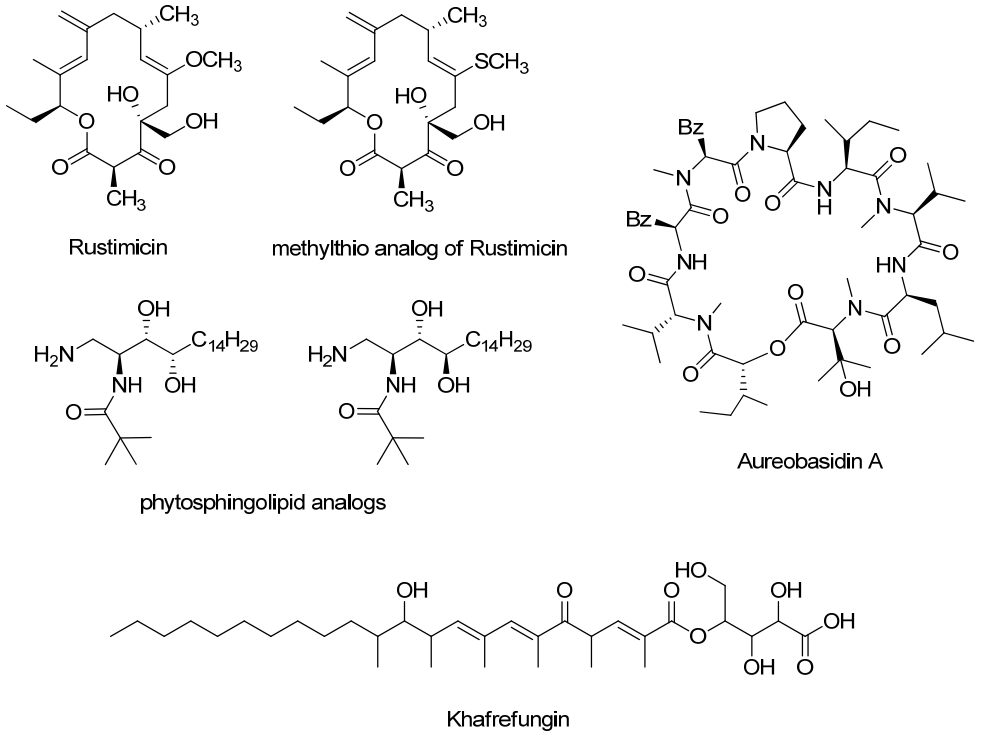

Figure 1.7. Chemical structure of natural and synthetic IPCS inhibitors.

Aureobasidin A[44,45] is a cyclic peptide isolated from the fungus *Aureobasidium pullulans*, which is highly active against many pathogenic fungi including *Candida albicans*, *Cryptococcus neoformans*, *Blastomyces dermatitidis* and *Histoplasma capsulatum*. Aureobasidin A is a large molecule with a number of side chains, several of which are believed to be important for activity.

In addition, it is reported in the literature the synthesis of analogs of IPCS inhibitors. A variety of novel rustimicin derivatives[46] with slightly modified structures of this natural compound have been reported, but they lacked the activity or retained a modest antifungal potency. Furthermore, the synthesis of phytosphingolipids can also be used for the design of new compounds to modulated sphingolipid metabolism and biosynthesis in fungi.[47,48]

1.2. Aziridines

Aziridines[49] are saturated three-membered heterocycles containing one nitrogen atom. These chemical species are considered the nitrogenous analogs of epoxides[50] and because of their highly strained three-membered ring, aziridines are susceptible to nucleophilic ring-opening reactions,[51,52] which make them useful as synthetic precursors of a variety of nitrogen-containing compounds (Figure 1.8).

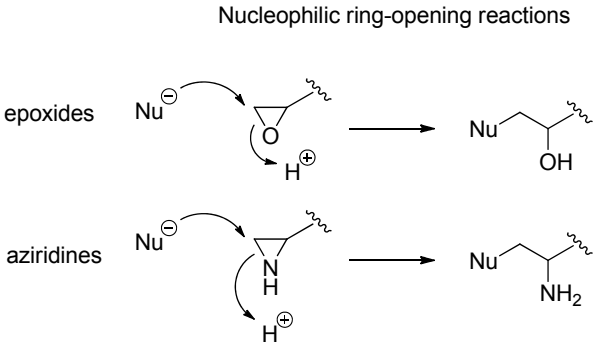

Figure 1.8. Nucleophilic ring-opening reaction of epoxides and aziridines.

Due to the diminished electronegativity of nitrogen in comparison with oxygen, neutral aziridines are less active than epoxides in ring-opening reactions and the nucleophilic attack at carbon proceed in an analogous manner to similar reactions with epoxides. However, basic aziridines have the possibility of being protonated leading to very reactive aziridinium salts. In addition several other groups can be attached to the aziridine nitrogen to increase its electrophilic character. In case of unsymmetrically-substituted aziridines the ring-cleavage reactions with a nucleophile can lead to two adducts, depending on the site of action of the aziridine.[53] Usually the nucleophiles direct their attack to the site of lesser substitution,[54-56] although some examples of the opposite reactivity are reported.[57-59]

1.2.1. Synthesis of aziridines

In 1999, Bob Atkinson[60] reported that aziridination reactions were "epoxidation's poor relation", when the scope of synthetic methods available to prepare aziridines had nothing to do with the diversity of procedures available for the preparation of epoxides. However, modern advances in the area of aziridine synthesis have enabled the discovery and research of new methodologies to their obtention.

A variety of methods are described for the synthesis of aziridines (Figure 1.9) and some of the most common are the addition of nitrenes to alkenes,[53,61,62] the addition of carbenes[63] or ylides[64] to imines, from 1,2-aminoalcohols[65], from 1,2-aminohalides[66,67] and from azidoalcohols.[68-70]

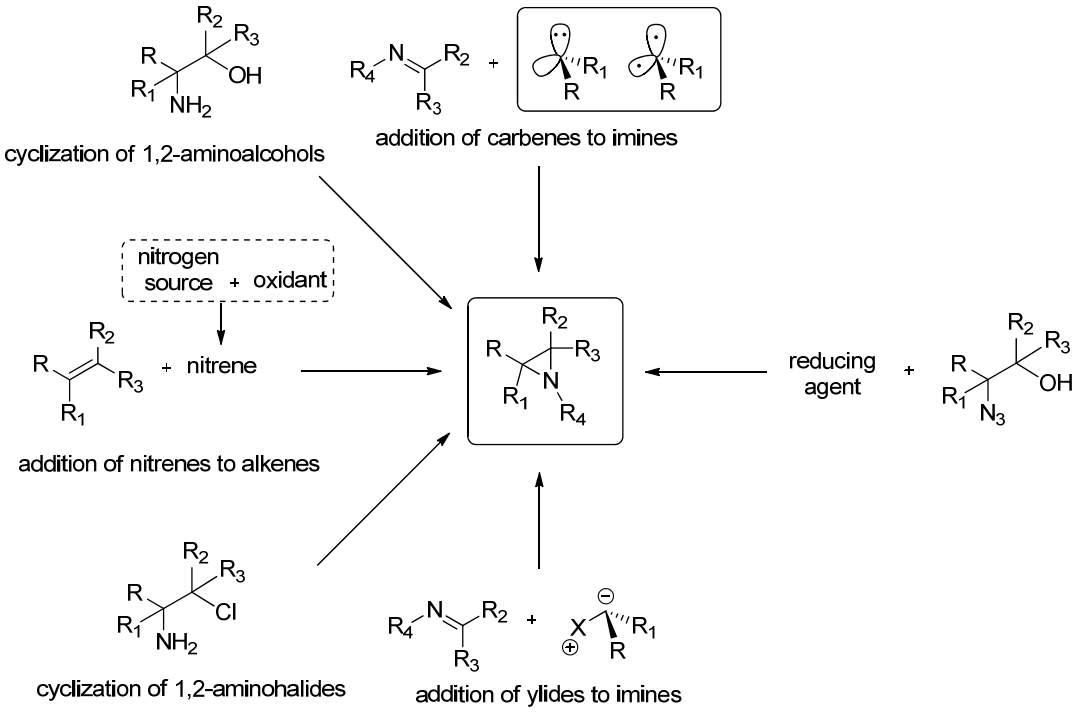

Figure 1.9. General scheme of the most common methods to obtain aziridines.

1.2.2. Activation of aziridines

Aziridines may be divided into activated and non-activated aziridines depending on their reactivity towards nucleophilic species and general properties.[71]

In one hand, activated aziridines[72,73] contain electron-withdrawing substituents which increase the electrophilicity of the aziridine and can stabilize the negative charge that is generated in the transition state for ring-opening by a nucleophile.

On the other hand, non-activated aziridines are *N*-unsubstituted aziridines[74,75] or *N*-substituted aziridines[76-78] that contain groups that increase the basicity of the nitrogen, but are not able to stabilize the anion resulting from the ring-opening reactions (Figure 1.10).

Figure 1.10. Classification of aziridines.

Due to aziridines are less active than epoxides, it is more usual the use of activated than non-activated aziridines, to increase their reactivity against nucleophilic ring-opening reactions.

1.2.3. Aziridines as important building blocks

Aziridines are one of the most valuable three membered rings in modern synthetic chemistry because of its widely versatility as a building block for chemical bond elaborations and functional group transformations. The high number of literature reports related to aziridines, highlights the powerful synthetic utility of these compounds as key synthetic precursors and they broad applications.

Although our interest for aziridines remains in their use as building blocks for chemical synthesis, it should be mentioned that they are also interesting heterocycles present in a wide variety of naturally occurring biologically active compounds.[79-81]

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2. RESEARCH OBJECTIVES

Sphingolipids are essential biomolecules for the physiological cell function. In addition to its structural role in cell membranes, these lipids have also crucial functions in signal transduction and cell regulation.[1-3] As a consequence of the important biological roles of sphingolipids, the synthesis of their analogs and the development of chemical inhibitors of sphingolipid enzymes are the object of current interest.[4-7] Furthermore, it has been reported that slight modifications on the natural C1 (phyto)sphingosine scaffold[8,9] or variations in its chain lengths can potentially alter the role and bioactivity of sphingolipid analogs.[10]

On the other hand, cyclitols are an important group of compounds due to their remarkable biological activities[11,12] as well as their synthetic usefulness in the synthesis of other natural compounds or pharmaceuticals. Specially, the development of carbohydrate mimetics prompted primarily by their properties as glycosidase inhibitors,[13] has led to a wide variety of novel structures by themselves or when considering them as key synthetic precursors of more complex molecules such as glycosphingolipids.

Bearing in mind the above considerations, the main goals of this thesis are:

(1) The development of a synthetic methodology to obtain (phyto)sphingolipid analog libraries by means of nucleophilic ring-opening reactions of an aziridine sphingolipid with different nucleophiles such as thiols or β-glycosil thiols, amines, phosphate derivatives or phosphorothioate derivatives (Figure 2.1).

This objective will involve the synthesis of two aziridine derivatives from commercial phytosphingosine hydrochloride to obtain phytosphingosine and sphingosine analogs (Figure 2.1).

In this way, it is intended to synthesize a variety of sphingolipid analogs with close structures, comprising modifications at C1 position as well as different acyl chains in the amide linkage.

The main benefit expected of this strategy is the synthetic versatility, allowing the obtention of different structural analogs from a variety of activated aziridine (phyto)sphingolipid precursors by using different nucleophiles.

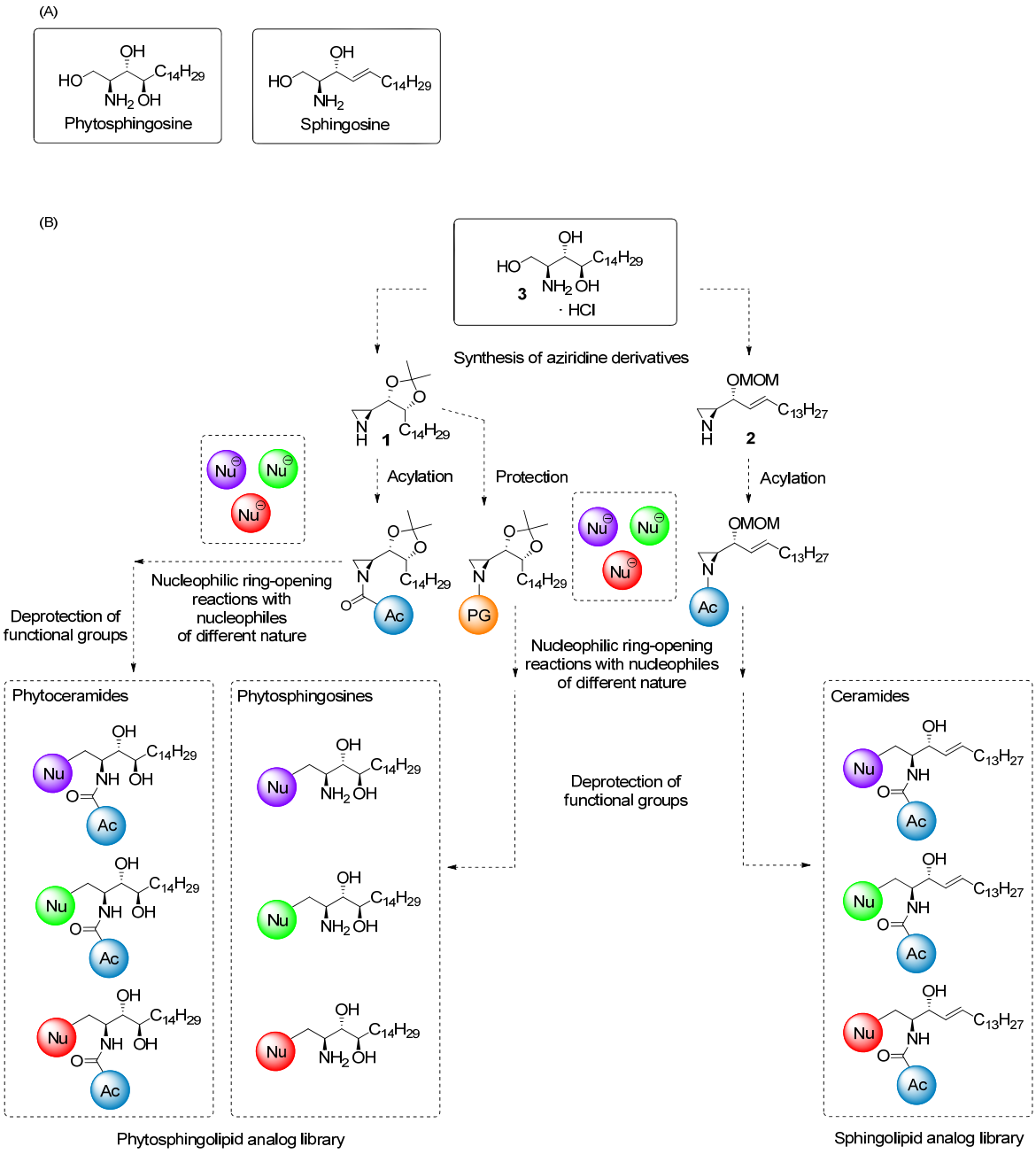

Figure 2.1. (A) Chemical structure of the sphingoid bases phytosphingosine and sphingosine. **(B)** General scheme of the synthetic methodology to obtain (phyto)sphingolipid analog libraries.

(2) The development of a synthetic strategy to obtain enantiomerically and diastereomerically pure galacto-configured aziridine derivatives (Figure 2.2) in order to synthesize glycolipid analogs by nucleophilic ring-opening reactions of these aziridines with lipid nucleophile derivatives or their precursors (Figure 2.3).

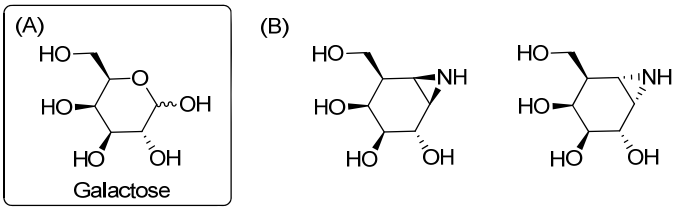

Figure 2.2. (A) Chemical structure of galactose. **(B)** Chemical structures of the galacto-configured aziridines of interest.

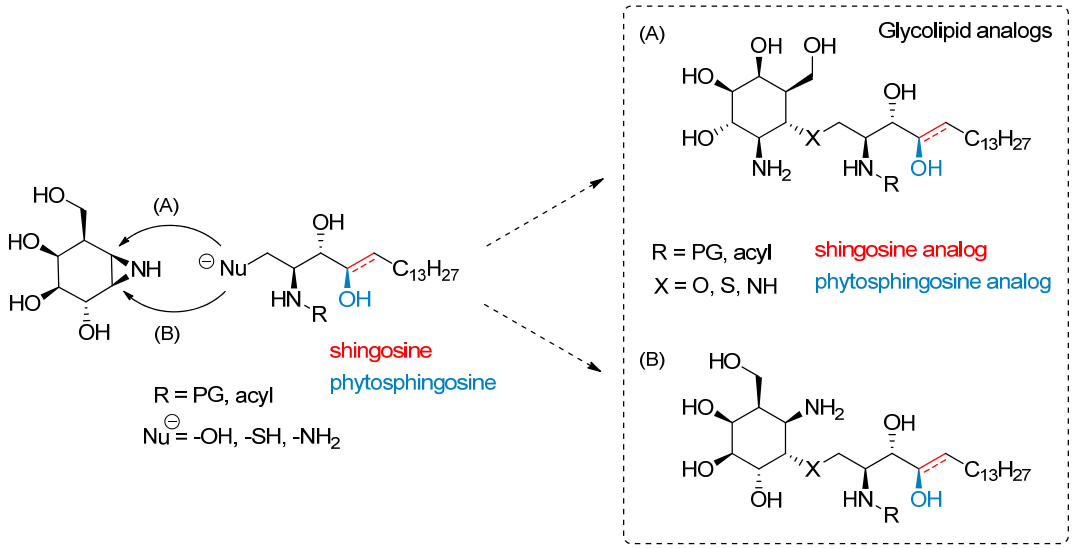

Figure 2.3. General scheme of a new strategy to obtain glycolipid analogs by ring-opening of galacto-configured aziridines with lipid derivatives.

(3) The biological evaluation of the synthesized (phyto)sphingolipid analog libraries as inhibitors of sphingolipid metabolism enzymes such as mammalian enzymes SMS and GCS, and fungal enzyme IPCS (Figure 2.4).

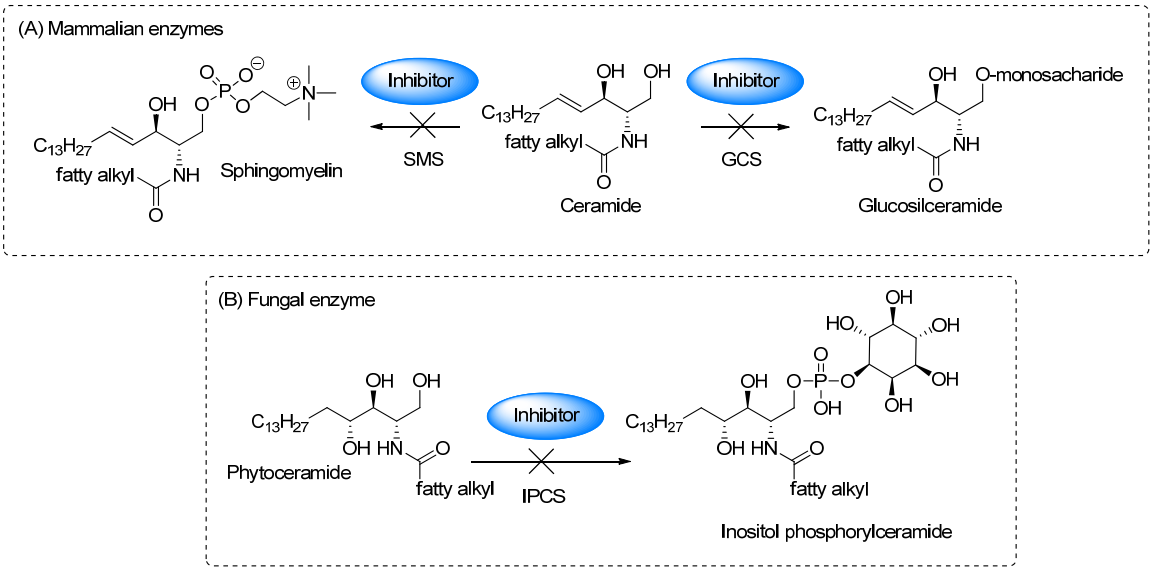

Figure 2.4. Inhibition of sphingolipid metabolism enzymes. **(A)** Inhibition of SMS and GCS. **(B)** Inhibition of IPCS.

(4) The biological study of the synthesized sphingolipid analogs in other biological models that resulted in their evaluation as antigens for CD1d-restricted iNKT cells.

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3. RESULTS AND DISCUSSION

3.1. Aziridine derivatives to synthesize (phyto)sphingosine analogs

3.1.1. Activation of aziridines for ring-opening reactions to obtain (phyto)sphingosine analogs

3.1.2. Reactivity of aziridines with thiols to obtain 1-thio-(phyto)sphingosine analogs

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3.1.3.2. Synthesis of 1-thio-glycolipid analogs

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3.1.4.1. Introduction

3.1.4.2. Synthesis of 1-amino-(phyto)ceramide analogs

3.1.5. Reactivity of aziridines with phosphates and phosphorothioates to obtain phyto-sphingosine analogs

3.1.5.1. Introduction

3.1.5.2. Synthesis of phosphorylated analogs

3.2. Galacto-configured aziridine derivatives

3.2.1. Introduction

3.2.2. Synthetic methodologies to obtain galacto-configured aziridine derivatives

3.2.3. Synthesis of galacto-configured aziridine derivatives by means of olefin aziridination reactions

3.3. Biological studies

3.3.1. Biological studies of (phyto)sphingosine analogs as inhibitors of sphingolipid enzymes

3.3.1.1. Biological studies of the analogs as inhibitors of mammalian SMS and GCS

3.3.1.2. Biological studies of the analogs as inhibitors of yeast IPCS

3.3.2. Biological studies of (phyto)sphingosine analogs as antigens for CD1d-restricted iNKT cells

3.3.2.1. Introduction

3.3.2.2. *In vitro* studies

3.3.2.3. *In vivo* studies

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4. SUMMARY AND CONCLUSIONS

Summary of results and conclusions

- A synthetic methodology towards sphingolipid analogs based on the regioselective ring-opening reactions of *N*-acylaziridines with different nucleophiles has been developed. This approach is a short and flexible route for the synthesis of a variety of sphingolipid analogs from aziridine **2** and phytosphingolipid analogs from aziridine **1**. This method leads to molecules with different groups bonded to the C-1 position of the sphingoid backbone from common intermediate aziridines by changing the nucleophile in the ring-opening reaction step. Moreover, the diversity of compounds obtained is extended by introducing different *N*-acyl groups at the nitrogen aziridine.

- The ring-opening aziridine methodology has been used for the preparation of a number of (phyto)sphingolipid analogs with thioeter, amine, and phosphorous functionalities having diverse structures at C1.

- A general and practical methodology by microwave promoted reactions of aziridine derivatives with thiols has been developed. This method has been used to obtain a collection of 18 1-thiosphingolipid analogs, including phytosphingosine or sphingosine backbones in moderate to good yields.

- The synthetic methodology explored to obtain 1-thio-β-glycolipid analogs resulted in low to moderate yields in the ring-opening reaction step. The lower yields with 1-thiosugars are originated from the instability of this thiol under the reaction conditions. This method has been used to prepare thioglycoside analogs and test them in biological systems.

- A library of 1-amino-(phyto)ceramide analogs has been synthesized by $LiClO_4$ promoted opening of *N*-acylaziridine derivatives with a variety of amines. This method led us to obtain a collection of 15 1-amino-(phyto)sphingolipid analogs. In addition, the catalytic hydrogenation of the double bond present in the ceramide analogs allowed us to obtain some of the dihydroceramide derivatives in good yields.

- A methodology to synthesize phosphosphingolipid analogs based on the ring-opening reactions of aziridine derivatives with phosphates or phosphorothioates was developed. This method led us to obtain three 1-phospho-phytosphingolipid analogs. This strategy is an alternative to the direct phosphorylation of the alcohol function in lipid compounds.

- A synthetic methodology to obtain enantio- and diastereomerically pure galacto-configured aziridines based on olefin aziridination reactions has been examined. This consists in the use of nitrene precursors and the galactose related cyclohexene **76**. The results obtained with this method show potential for the preparation of the aziridine compounds .

- A representative selection of (phyto)sphingolipid analogs has been tested in enzyme assays to identify inhibitors of mammalian sphingolipid metabolism enzymes, such as sphingomyelin synthase (SMS) and glucosylceramide synthase (GCS). Some of the tested analogs were potent inhibitors of GCS and none of them inhibited SMS at concentrations relevant for activity. Particularly, the best inhibitors contained pyrrolidino (**48d**, **48j**), morpholino (**48e**, **48k**) and diethanolamino (**48f**, **48l**) groups attached at position 1 of the lipid backbone. The inhibitory activity was higher for phytosphingosine analogs (**48d**, **48e**, **48f**) than their corresponding sphingosine analogs (**48j**, **48k**, **48l**). The presence of the double bond between C4 and C5 of the sphingosine backbone in analogs **48j**-**48k** is important for their bioactivity, since the dihydroceramide analogs **49j**-**49k** are less active.

- A collection of (phyto)sphingolipid analogs has been tested in an assay to identify inhibitors against yeast IPCS. In general, the inhibitory activities were lower than the inhibitions against GCS. Compound **48c** having a dibutilamino group at C1 of a phytoceramide depicted selectivity against baker's yeast IPCS since no inhibition of mammalian SMS and GCS was observed. For that reason, this analog shows potential properties as antifungal agent.

- The biological studies of the analogs as inhibitors of IPCS, led us to identify moderate inhibitors of this enzyme. The inhibitors with higher activity were 1-amino-phytosphingolipids that contained pyrrolidino, morpholino, diethanolamino and dibutylamino groups at C1 of phytosphingosine and 1-phospho-phytosphingolipid analogs with phosphate and *O*,*O*-dimethyl phosphorothioate groups at position 1 of the sphingoid base .

- Some (phyto)sphingolipid analogs tested as antigens for CD1d-restricted iNKT were active, although this activity was lower when comparing with the endogenous glycolipid β-glucosylceramide. The most active compound was the non-glycosidic analog **28d**, which contains a 2-hydroxymethylphenylthio group attached at position 1 of a phytoceramide. Furthermore, this compound shows a selecive Th1 polarization and *in vivo* activity in mice.

- Glycosphingolipid analogs **41a** and **41b** were also active in the activation of iNKT cells, showing a Th1 polarization profile, but these compounds were weaker antigens than **28d**.

5. EXPERIMENTAL SECTION

5.1. Synthesis and product characterization

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7. INDEX OF COMPOUNDS

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