A Lucia Chiara

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THESIS ABSTRACT

Glycosphingolipids (GSL) are anphiphilic membrane components consisting in a hydrophobic moiety, ceramide, and a hydrophilic oligosaccharide headgroup. Ceramide is responsible for their insertion into the outer layer of plasma membranes, with the oligosaccharide chain protruding in the extracellular environment. GSLs participate to the signaling processes across the membrane[1] determining the lateral organization of cellular membranes and modulating the function of several classes of membrane proteins.[2] Their function rely to the ability they have to form clusters with sphingomyelin, ceramide, cholesterol and signal trasduction proteins such as GPI-anchored and acylated proteins (including Src family-kinases), to form specialized membrane domains called "lipid-rafts".[3]

In mature neutrophils, which play the first line of defense against invading microorganisms and have an important role in acute inflammatory reaction, more than 70% of GSLs are LacCers, which are aberrantly expressed at high levels on mature neutrophils. It is possible that LacCer activates NADPH oxidase, thereby affecting the functions of superoxide-producing cells. However, the mechanisms by which LacCer activates NADPH oxidase in neutrophils have not yet been well characterized.

It is known that lactosylceramide (LacCer) is specifically coupled with Src family kinase Lyn in plasma membrane microdomains of human neutrophils. Ligand binding to LacCer activates Lyn, resulting in neutrophils functions, such as superoxide generation and migration.[4] The β-D-Gal-(1→4)-β-D-Glc structure is necessary but it is not sufficient for LacCer-mediated Lyn activation. For this

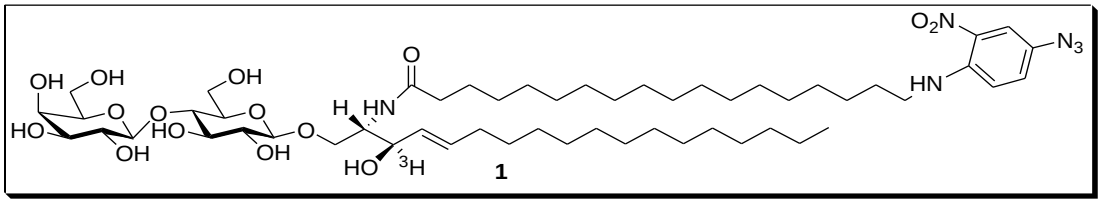
function, the presence of a LacCer molecular species with a ceramide containing a very long fatty acid is also required.

GSLs containing very long fatty acids might participate in reducing the membrane thickness through interdigitation of the two membrane leaflets. The interdigitation is proposed to be the switch for the transduction of information throughout the membrane perhaps by allowing contact and interaction between proteins belonging to the two layers of the plasma membrane.[5] Anyway the molecular mechanism by which the interactions between GSLs and protein influence the cell functions has still to be elucidated.

The aim of this thesis is to give a contribute in the comprehension of the intermediate steps of the signaling process mediated by Lyn proteins. In particular, the purpose is to explore the role of long chain LacCer in this process, with a particular interest in the identification of the proteins associated with LacCer in the immune response to several microorganisms in human neutrophils.

The cross-linkage between gangliosides and proteins can be investigated by cell photolabeling using radioactive photoactivable gangliosides carrying the reactive group at different positions of the molecule. In fact the photoactivable group, when illuminated, yield a very reactive intermediate that covalently binds to the molecules in the environment.[6,7]

With the final aim to identify LacCer associated proteins, we have developed probes **1** and **2**, containing one or two photoactivable groups, located at specific points of the molecule and, in principle, capable to interact with proteins belonging to the cytoplasmic and/or to the extracellular membrane layer.



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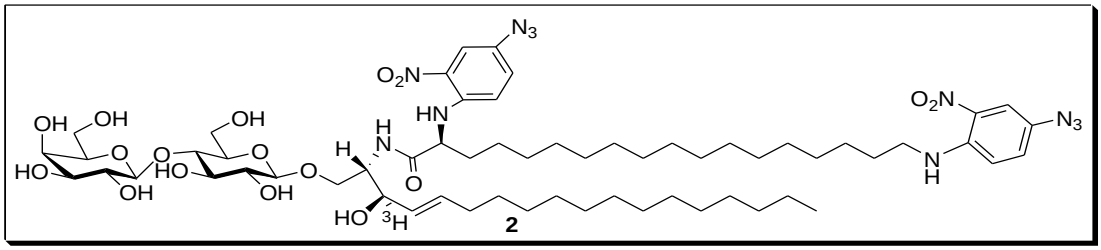

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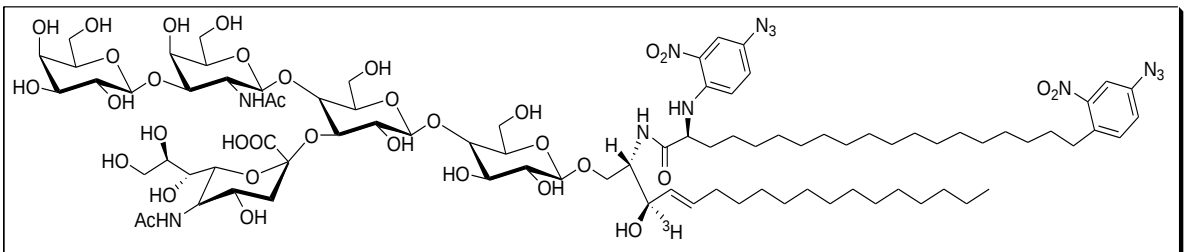
Probes **1** e **2** display an acyl chain long enough for LacCer-mediated Lyn activation, as preliminary experiments have shown.[8] The initial task of the thesis has been the preparation of the acyl derivatives, suitable for the construction of probes **1** and **2**. The synthetic efforts were mainly devoted to prepare the proper aminoacid derivatives for conjugation to lactosylsphingosine and subsequent derivatization with the photoactivable probe. Both the C-18 ω-aminoacid and the C-18 α,ω-diaminoacid were prepared by chemical synthesis, since they are not commercially available. In addition, the synthesis of the long chain α,ω-diamino acid can be very interesting for the possibility of simultaneous capture of the proteins belonging to the two leaflets.

The C-18 ω-aminoacid was derived from commercially available octadecandioic acid through a eight steps synthetic pathway. On the contrary, the α,ω-diamino homologue was constructed in seven steps by subsequent condensations of shorter building blocks, starting from suitable protected aspartic

acid, through a synthetic route that showed several tricky points.

Both aminoacids, activated as pentafluorophenolates, have been coupled to lactosylsphingosine, which has been previously tritiated in order to follow the sphingolipid biological pathway. The obtained proper LacCers were finally derivatized with nitrophenilazide to give the target probes **1** and **2**.

To assess the capability of an α,ω-diamino fatty acid functionalized probe, *i.e.* **2**, to be internalized by the cells, we have designed probe **3** in which the hydrophilic moiety is ganglioside GM1.



probe 3

Previous studies have in fact demonstrated the capability of GM1, with one photoactivable group at the end of the fatty chain, to be inserted in the membrane lipid core in a way that closely resembles that of endogenous gangliosides.[9]

Photolabelling experiments are now in progress both in Milan (probe **3**, GM1) and in Japan (probe **1** and **2,** experiments on human neutrophils in collaboration with Professor Iwabuchi).

1. INTRODUCTION

1.1 Glycosphingolipids

The discovery of sphingolipids is generally attributed to Johan L.W. Thudicum, which in his study on the chemical composition of the brain, isolated several compounds from ethanolic brain extracts, called by him cerebroside. He carried out an acidic hydrolysis on one of them, phrenosin (now known as galactosylceramide) and produced three distinct components, one identified as a fatty acid and another as an isomer of D-glucose, which is now known to be a D-galactose. The third component, an alkaloid, presented many enigma and was named by Thudicum sphingosine, related to the mythological riddle of the Sphinx.

Glycosphingolipids (GSLs) (figura 1.1) are heterogeneous components of eukaryotes and few bacteria and consist of a ceramide hydrophobic backbone and a sugar headgroup. As already mentioned above, the hydrophobic ceramide part consists of a sphingoid base acylated by a fatty acid and is inserted in the cellular membrane, whereas the hydrophilic headgroup protudes in the extracellular environment.

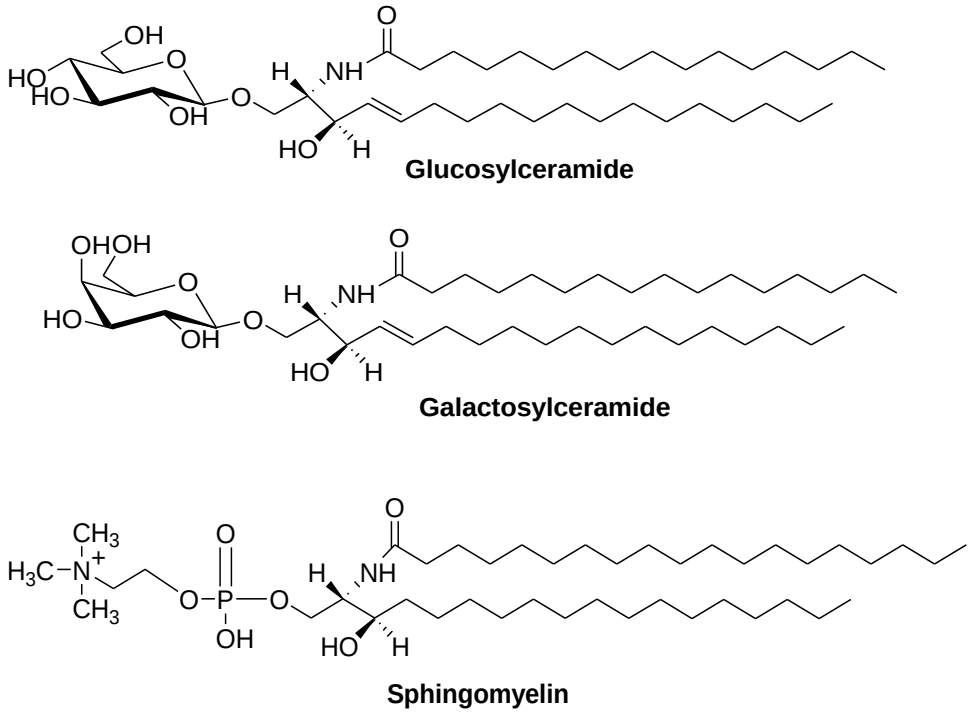


Figure 1.1 Different chemical structures of glycosphingolipids

GLSs exibit a huge heterogeneity of structure, both in the ceramide backbone and headgroup. The hydrophilic part is linked to the primary hydroxyl group of the sphingosine moiety through a glycosidic bond. A glucose or galactose linked through a β-linkage give rise to the simplest glycosphingolipids: glucosylceramide and galactosylceramide. Linkage of the sugar to phosphorilcoline results in sphingomyelin. Further addition of oligosaccharides and sulphate groups to glycosphingolipids gives rise to a broad range of complex glycosphingolipids. Those containing N-acetylneuraminic acid (sialic acid) are known as gangliosides, which can be classified as neutral, acidic (anionic) and basic (cationic) (Figure 1.2).

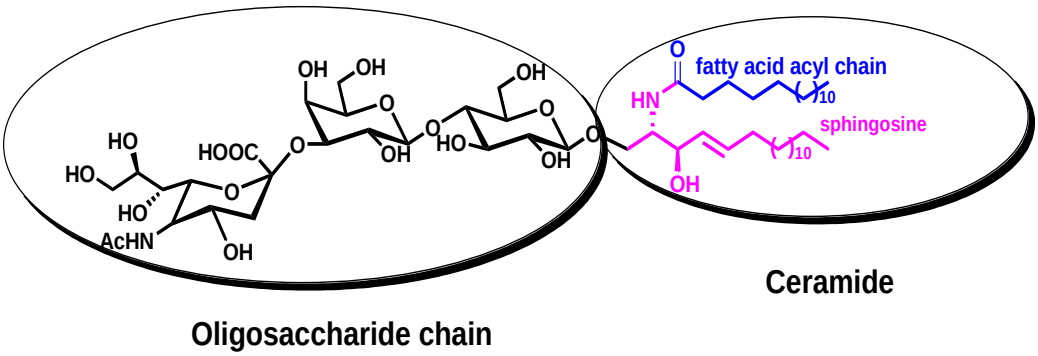


Figure 1.2 Structure of the glycosphingolipid ganglioside GM3

The sphingoid base can differ in length, saturation, hydroxylation and branching The main sphingoid base in mammal is D-*erythro*- sphingosine or 2S,3R,4E-1,3-dihydroxy-2-aminooctadecene or *trans*-4-sphingenine (d18:1). Sphinganine, which corresponds to sphingosine without the *trans* C4-C5 double bond, and phytosphingosine (C4-OH sphinganine) are also common sphingoid bases.[10,11] (Figure 1.3).[12]

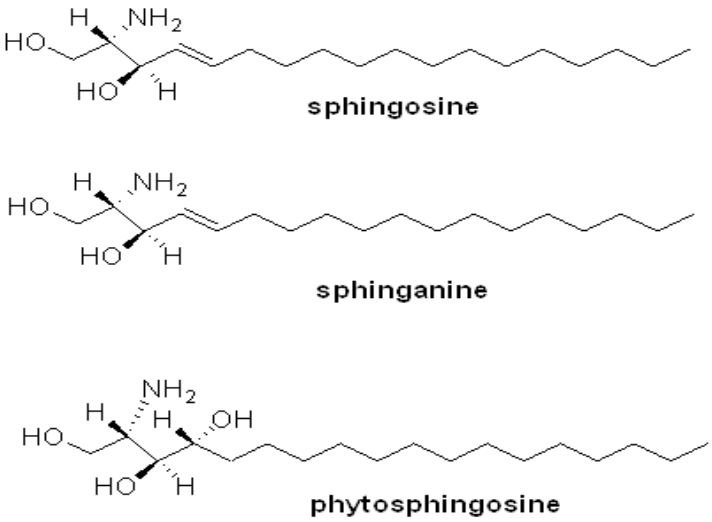


Figura 1.3 Chemical structures of the most common sphingoid base

The fatty acid is linked to the amine group of the sphingoid base through an amide bond. Depending on the tissue, the length and saturation of the N-acyl tail in SLs and GSLs can vary significantly, but in mammal the fatty acid are generally long (C≥16) and saturated, although sometimes they are unsaturated at C15 and hydroxylated at C2.

1.1.1 Synthesis of glycosphingolipids

The biosynthesis of sphingolipids (SLs) starts at the cytosolic leaflet of the endoplasmic reticulum membrane(ER).

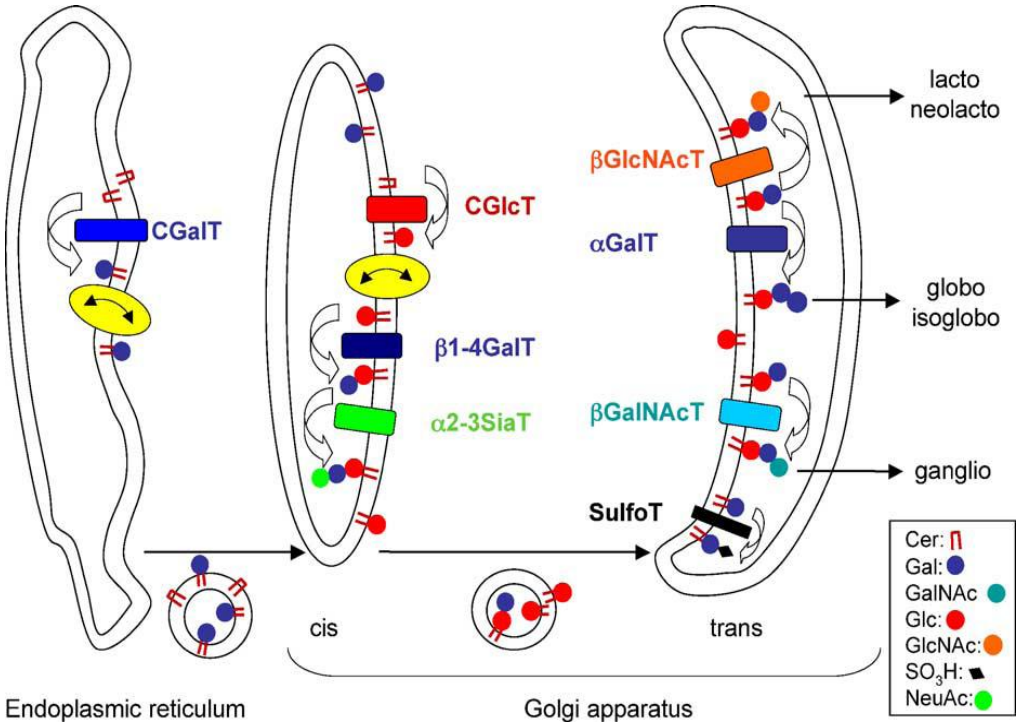


Figure 1.4 adopted from *Seminars in Cell & Developmental Biology* **2004**, *15*, 375-387

Mammalian GSL synthesis. Ceramide is produced in the endoplasmic Reticulum, where the sphingoid base can be desaturated or hydroxylated. In specialized cells, ceramide can be converted to GalCer in the ER lumen by the Gal transferase CGalT. The glucosyltransferase CGcT, active on the cytosolic side of the Golgi apparatus, synthesizes GlcCer which must be translocated to the Golgi lumen to be converted to LacCer by the β1,4-galactosyltransferase GalT-2. LacCer is the precursor of different series of GSLs, mostly only one in any specific cell type. GalCer and LacCer can be sulfated in the late Golgi.

The first step is the condensation between serine and palmitoyl-CoA to 3-ketosphinganine, the precursor of all sphingoid bases. Subsequent acylation produces ceramide,[13,14] which can spontaneously cross the ER membrane and be converted to GalCer, in specialized cell types. Ceramide can also follow the vesicular pathway to early Golgi compartments, where it is converted to GlcCer. Ceramide can also reaches the *trans*-Golgi via CERT, a ceramide transfer protein that is able to extract ceramide from ER and deliver it to the Golgi after docking to phosphatidylinositol-4-phosphate.[15-17] Ceramide here equilibrates between the cytosolic and luminal side of the trans Golgi membrane. On the luminal inside, sphingomyelin synthase 1 (SMS1) converts ceramide into sphingomyelin (SM) by transfer of a phosphorylcholine headgroup from phosphoglicerolipids. A second enzyme, SMS2, is located at the plasma membrane, its role is to convert ceramide there into sphingomyelin, since zwitterionic SM can't pass appreciably through the membrane. Ceramide can be regenerate from SM by a neutral or acidic form of the enzyme sphyngomyelinase.

In the ER a Gal residue is transferred from UDP-Gal to ceramide through a UDP-Gal:ceramide galactosyltransferase or GalCer synthase CGalT.[18-21] CGalT is a class I integral membrane protein. On its way to the plasma membrane GalCer passes the lumen of the Golgi apparatus, where it can be sulphated to HO_3S-3GalCer (sulfatide).

In contrast to GalCer, GlcCer is present in most eukayotic cells and a few bacteria and it represents the major precursor for complex GSLs. It is synthesized by the UDP-Glc:ceramide glucosyltransferases or GlcCer synthase (CGlcT), which is a type III protein. GlcCer synthesis occurs on the cytosolic side of the

Golgi membrane.[22-24] CGlcT activity has been detected not only in the early Golgi compartments, but also in a pre-Golgi compartment or the late Golgi.[24] This can explain the existence of the two pools of GlcCer in cells, possibly having two different functions.

Complex GSLs are made by the stepwise addition of individual sugar from their activated nucleotide precursor onto GlcCer. In mammals the first reaction is the conversion of GlcCer to lactosylceramide (β-D-Gal-(1→4)-β-D-Glc, or LacCer) by the LacCer synthase,[25-26] which acts in the Golgi lumen.[27-28] Several galactosyl-, N-acetylgalactosaminyl- N-acetylglucosaminyl, sialyl- and fucosyltransferases can elongate the oligosaccharide chain of mammalian GSLs.[29] These enzymes are involved in the formation of the oligosaccharide backbone, the structure of which defines the different series of GSLs.

Sialoglycosphingolipids are also called "gangliosides" and abbreviations have been assigned to them according to the number of sialic acids (see Table 1). Except for ganglioside GM4, which corresponds to NeuAcα2-3GalCer, all gangliosides have LacCer as the precursor. Gangliosides are abundant in brain and nervous tissues.

The type of sugars present in the oligossacharide backbone of ganglioside depends on the glycosyltransferases present and active in the Golgi apparatus of the cell, which may be vary depending on the cell type, the developmental stage or the disease stage.[30-32]

Dramatic changes in glycolipid glycosylation and metabolism have been described in many cancer cells, which often result in aberrant sialylation and/or fucosylation.[33]

GSLs are degradeted in the lysosomes, where they are hydrolyzed by specific hydrolases. Defects in the degradation of GSLs result in GSL storage in lysosomes which can give rise to very serious pathologies. Treatment of these disease includes replacement of the enzyme, gene therapy and inhibition of synthesis.[34]

Table 1.1

Gangliosides abbreviations according to the Svennerholm system

Abbreviations	**Structure**
GM4	NeuAcα2-3Galβ1-Cer
GM3	NeuAcα2-3Galβ1-4Glcβ1-4Glcβ1-C
GM2	GalNacβ1-4 (NeuAcα2-3)Galβ1-4Glcβ1-Cer
GM1a	Galβ1-3GalNacβ1-4 (NeuAcα2-3)Galβ1-4Glcβ1 Cer
GM1b	NeuAcα2-3Galβ1-3GalNAcβ1-4Galβ1-4Glcβ1-Cer
GD3	(NeuAcα2-8NeuAcα2-3)Galβ1-4Glcβ1-Cer
GD2	GalNAcβ1-4 (NeuAcα2-8NeuAcα2-3)Galβ1-4Glcβ1-Cer
GD1a	NeuAcα2-3Galβ1-3GalNAcβ1-4 (NeuAcα2-3)Galβ1-4Glcβ1-Cer
GD1b	Galβ1-3GalNAcβ1-4 (NeuAcα2-8NeuAcα2-3)Galβ1-4Glcβ1-Cer
GT1a	NeuAcα2-8NeuAcα2-3Galβ1-3GalNAcβ1-4 (NeuAcα2-3)Galβ1-4Glcβ1-Cer
GT1b	NeuAcα2-3Galβ1-3GalNAcβ1-4 (NeuAcα2-8NeuAcα2-3)Galβ1-4Glcβ1-Cer
GT1c	Galβ1-3GalNAcβ1-4 (NeuAcα2-8NeuAcα2-8NeuAcα2-3) Galβ1-4Glcβ1-Cer

1.1.2 GSLs physical properties[35,36]

GSLs, in contrast to glycerolipids, have chemical groups in the region between the polar headgroup and the hydrophobic backbone that can function both as hydrogen bond donor and hydrogen bond acceptor. GSLs also differ from glycerolipids for the saturation of the lipid chains, which corresponds to a denser packing that, in combination with the higher hydrogen bonding, involve an increased melting temperature (T_m), which represents the temperature above which a bilayer of a single lipid switches from a frozen state, the gel or solid-ordered (s_0) phase, to a fluid state, the liquid-crystalline or liquid-disordered (l_d) phase. GSLs have a much higher T_m than glycerolipids.

The network of hydrogen bonds segregate GSL and cholesterol within the surrounding glycerophospholipid-rich environment, the interaction between GSLs and cholesterol are preferentially due to Van der Waals forces.

Studies performed on model membranes showed the possibility of co-existence of a s_o and l_d phase. In model membranes, containing mixtures of a high T_m lipid and cholesterol, has also been observed a fluid-fluid phase separation between l_d and a liquid ordered l_0 phase, the sterols have definitively the ability to modulate the phase separation of lipids. We will discuss physical features of sphingolipids in a comprehensive way in the next sections.

1.1.3 Glycosphingolipids in biological membrane organization

It is widely accepted that different levels of order exist in biological membranes, the first level of being is the creation of a lipid bilayer, as a consequence of the aggregational properties of complex amphipathic membrane lipids. The lipid bilayer provides a physical boundary between the cellular and extracellular environments and it is characterized by several basic properties that are relevant to its biological functions. The bilayer as a whole is a very stable structure, however it allows its components a certain degree of lateral motility. As a consequence of this fluidity, components of biological membranes can be arranged following a non-homogenous lateral distribution, leading to the creation of membrane areas ("domains") with a highly differentiated molecular composition and supermolecular architecture. The non-homogenous lateral distribution of membrane components is made possible by the existence of lateral interactions stabilizing different membrane domains and creating a second level of order in the organization of biological membranes.[37]

Membrane domains are in general characterized by the presence of specific subsets of proteins, and differential sorting and trafficking of proteins has been understood as one of the mechanisms responsible for the creation of polarized domains.

Protein-protein interactions have been regarded for several years as the main factor responsible for the stabilization of membrane macro- and microdomains. Anyway, in the last years, a role for membrane lipids in determining the lateral organization of the membrane has emerged and the concept that certain membrane lipids, in particular GSLs, possess a high potential for the creation of order in

biological membranes, is now well established. Lipid membrane microdomains, or **lipid raft,** which are membrane areas different in lipid composition for a higher percentage of GSLs from other membrane regions, have been involved in a large number of biological events. Lipid rafts, in addition to GSLs and cholesterol, contain GPI-anchored proteins and membrane-anchored signaling molecules. Because of their physicochemical characteristics, GSLs tend to form clusters with cholesterol on plasma membranes.[1] However, the ability of glycosphingolipids to influence the organization and function of other components of biological membranes is still only partially understood and it might only in part depend on their tendency to segregate, forming GSL-rich domains within the membrane. Lipid bilayers at physiological temperature usually exist in a liquid-disordered (l_d) phase characterized by high fluidity, in which the lipid acyl chains are disordered and highly mobile. Lowering the temperature below the melting point freezes the lipid acyl chains in an ordered gel phase with very limited freedom of movement. Membrane lipids can also exist in a third physical phase, the liquid-ordered (l_0) phase. In the l_0 phase, the acyl chains of lipids are extended and ordered, as in the gel phase, but have higher lateral mobility in the bilayer. The l_0 phase is stabilized by the presence of cholesterol, that fill the hydrophobic gaps between the phospholipid or glycolipid acyl chains.[3] The coesistence of lipids in different phases within the same model membrane was probably the first evidence leading to the concept of lipid domains. GSL-rich domains are likely more ordered than the l_d phase, being in this regard similar to a l_0 or a metastable gel phase.[37]

The earliest evidence supporting the existence of lipid domains was obtained studying artificial membrane models. SLs, and in particular GSLs,

strongly differ from glycerolipids for their molecular structure and conformational properties thus leading to a strong tendency to segregate within phospholipid bilayers. They form laterally separated phases characterized by reduced fluidity and hydrocarbon chain mobility. Segregation of membrane sphingolipids is responsible for the creation of less fluid membrane regions, where membrane associated-proteins can be confined, favouring lateral interactions between proteins that are segregated in the same lipid domain or preventing interactions between proteins that are associated with different domains. Moreover, proteins residing in SLs or GLSs-rich areas have a higher probability to laterally interact with the confining lipids. The great interest for lipid-rich membrane domains, that gave rise to the complex and controversial discipline of raftology in the last two decades, derived from the observation that some membrane associated proteins are highly concentrated in these domains, even if the overall protein content of these membrane areas is very low. It has been assumed that the trapping of certain proteins in lipid rafts might be somehow functional to their biological role.[38] Studies on model membranes, on detergent-resistent membrane fractions (DRM), which are membrane fragments isolated biochemically at low temperature from cellular membranes using non-ionic detergents, such as Triton X-100, and in intact cells indicated without doubt that several classes of membrane-associated proteins display a strong preference for the association of lipid-rich membrane domains.

Anyway, as mentioned above, it has been suggested, and in some cases proven, that the association with lipid-rich membrane domains can influence the function of a membrane protein but what are the mechanisms through which the

association with lipid domains, or within lipid domains can affect the functional properties of a membrane protein?

Several working hypotheses can be done in this regard. 1) The association with lipid rafts could represent a mechanism to facilitate the co-clustering of different membrane proteins. Against this simplistic view it can be argued that the surface density of proteins in lipid rafts is very low, and that in this case the limitations in lateral motility could indeed hamper protein-protein interactions. On the other hand, trapping of a protein within lipid rafts could prevent it from interacting with other proteins preferentially localized in fluid membrane regions: fats association might thus have an inhibitory significance for biological events requiring protein-protein interactions. 2) The association of a protein with a rigid membrane area could induce conformational changes in the polypeptide chain affecting its functional activity, independently from the formation of specific high affinity lateral interactions with other raft components. 3) Proteins concentrated in lipid rafts are favoured in their interactions with lipids component of the rafts.[37]

GSLs, due to their complex oligosaccharide groups, are good candidates for specific lipid-proteins lateral interactions. Apart from association with lipid rafts, the ability of GSLs, and gangliosides, in particular, to modulate the activity of membrane-associated proteins, such as receptor tyrosine kinases, has been widely documented. Still to be elucidated in most cases remain the molecular aspects of GSL-protein interactions underlying the modulator effect of GSLs. The oligosaccharide chain of a GSL inserted in the plasma membrane could interact with a membrane protein via a) aminoacidic residues belonging to the extracellular loops of the protein, if the conformation of the polipeptyde chain

allows them to be sufficiently close to the membrane surface; b) sugar residues in the glycans of a glycosylated protein, if the dynamics of the protein oligosaccharide chain allows the correct orientation toward the cell surface; c) the hydrophilic portion to the anchor in the case of GPI-anchored proteins (that is surely located in proximity of the extracellular surface of the membrane).[37]

Anyway many biological effects of gangliosides can at least in part be due to the modulation of several protein kinase systems. The latter observation that receptor protein kinases are highly enriched in lipid rafts suggested novel models for the interpretation of gangliosides-mediated signal transduction.

We are interested in a particular system in which protein functions are influenced by the association with GSL-rich microenvironment in the plasma membrane: lactosylceramide and Lyn proteins, about which it will be exhaustively discuss in one of the next section.

1.2 Neutrophils properties [39]

Neutrophil is a type of white blood cell (leukocyte) that is characterized histologically by its ability to be stained by neutral dyes and functionally by its role in mediating immune responses against infectious microorganisms. Neutrophils, along with eosinophils and basophils, constitute a group of white blood cells known as granulocytes. The granules of neutrophils typically stain pink or purple-blue following treatment with a dye. About 50 to 80 percent of all the white bloods cells occurring in the human body are neutrophils. The

neutrophils are fairly uniform in size with a diameter between 12 and 15 micrometres. The nucleus consists of two to five lobes joined together by hairlike filaments. Neutrophils move with amoeboid motion. They extend long projections called pseudopodium into which their granules flow; this action is followed by contraction of filaments based in the cytoplasm which draws the nucleus and rear of the cell forward. In this way neutrophils rapidly advance along a surface. The bone marrow of a normal adult produces about 100 billion neutrophils daily. It takes about one week to form a mature neutrophil from a precursor cell in the marrow; yet, once in the blood, the mature cells live only a few hours or perhaps a little longer after migration to the tissues. To guard against rapid depletion of the short-lived neutrophil (for example, during infection), the bone marrow holds a large number of them in reserve to be mobilized in response to inflammation or infection. Within the body the neutrophils migrate to areas of infection or tissue injury. The force of attraction that determines the direction in which neutrophils will move is known as chemotaxis and is attributed to substances liberated at sites of tissue damage. Of the 100 billion neutrophils circulating outside the bone marrow, half are in the tissues and half are in the blood vessels; of those in the blood vessels, half are within the mainstream of rapidly circulating blood and the other half move slowly along the inner walls of the blood vessels (marginal pool), ready to enter tissues on receiving a chemotactic sign. Neutrophils are actively phagocytic; they engulf bacteria and other microorganisms and microscopic particles. The granules of neutrophil are microscopic packets of potent enzymes capable of digesting many types of cellular materials. When a bacterium is engulfed by a neutrophil, it is encased in a vacuole lined by the invaginated

membrane. The granules discharge their contents into the vacuole containing the organism. As this occurs, the granules of the neutrophil are depleted (degranulation). A metabolic process within the granules produces a highly active form of oxygen (superoxide), which destroy the ingested bacteria. Final digestion of the invading organism is accomplished by enzymes. An abnormally high number of neutrophils circulating in the blood is called neutrophilia. This condition is typically associated with acute inflammation, though it may result from chronic myelogenous leukemia, a cancer of the blood-forming tissues. An abnormally low number of neutrophils is called neutropenia. This condition can be caused by various inherited disorders that affect the immune system as well as by a number of acquired diseases, including certain disorders that arise from exposure to harmful chemicals. Neutropenia significantly increases the risk of life-threatening bacterial infection.

1.3 Lyn protein

Lyn represents a non-receptor tyrosine kinase that transmits signal from cell surface receptors and plays an important role in the regulation of innate and adaptive immune responses, hematopoiesis, responses to growth factors and cytokines, integrin, signaling, but also responses to DNA damage and genotoxic agents. It functions primarily as a negative regulator but can also function as an activator, depending on the context. In particular it negatively regulates signalling

pathways through phosphorylation of inhibitory receptors, enzymes and adaptors but it is also a key mediator in several pathways of B cell activation.

Lyn exists in two isoforms, p53 and p56, arising from alternate splicing of exon 2 (Stanley et al 1991), but no functional differences between isoforms are currently known. It belongs to rafts microdomains of plasma membrane and is inserted at the cytoplasmic leaflet of rafts by virtue of its dual acyl chain modification.[40] Lipid rafts, as already mentioned, act as signalling platform by segregating activated components of signalling pathways into small membrane microdomains where they are in close juxtaposition to each other. In lipids rafts Lyn has predominantly been found in phosphorilated form confirming its role in the immune response but regarding the specific role of Lyn related to the immune response in human neutrophils we will more extensively discuss in several next sections.

1.3.1 Role of long fatty acid chains in the connection of Lyn with LacCer-enriched microdomains

Lactosylceramide (LacCer), a neutral glycosphingolipid, is the most abundant GSL in human neutrophil[41] and it has a relevant role in neutrophil physiological functions.

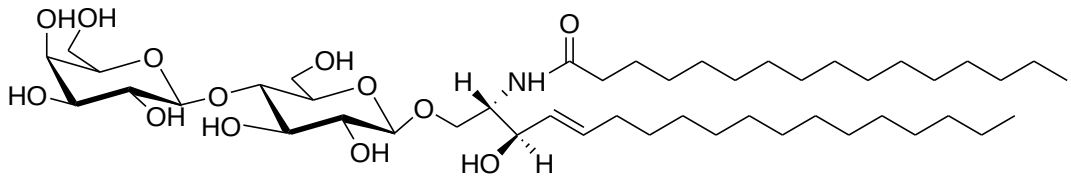


Figure 1.5 Lactosylceramide chemical structure

LacCer on neutrophil cell surface binds several pathogenic microorganism and play roles in the interaction between these microorganism and host cells. On neutrophil plasma membranes, LacCer forms lipid rafts with Src family kinase Lyn, these rafts are also called LacCer-enriched glycosignaling domains.[4]

Dimethyl sulfoxide (DMSO)-treated neutrophilic differentiated human promyelocitic leukemia HL-60 cells (D-HL-60 cells), an experimental model for the study of neutrophil functions, posses chemotactic and superoxide activities induced by formyl peptide fMLP. Interestingly, D-HL-60 cells do not show superoxide-generating or chemotactic activity induced by anti-LacCer antibody, as in neutrophils, although these cells express almost the same amount of LacCer on the plasma membrane as neutrophils. LacCer is localized mainly in the detergent resistant membrane fractions (DRMs).[4,42,43] It is thought to be a close relationship between rafts and DRMs, and isolation of DRMs is one of the most widely used biochemical methods for studying lipid rafts.[44] The Src family kinase Lyn has been immunoprecipitated by anti-LacCer antibody in neutrophils but not in D-HL-60 cells.[8] These observation has suggested that some essential molecules linking LacCer with Lyn may be lacking in the LacCer-enriched lipid-rafts of D-HL-60 cells. In fact, C24:0 and C24:1-fatty acid-containing LacCers are the major

species of plasma membrane DRM of neutrophils, whereas are minor species of D-HL-60 cells, which prevalently consisted of C16:0 LacCer. Although all LacCer species are incorporated into the plasma membrane DRM of D-HL-60 cells, C24:0- and C24:1-LacCer, but not C16:0- or C22:0-LacCer-loaded cells, showed LacCer dependent functions.[8] Without stimulation of the cells, Lyn has been co-immunoprecipitated with anti-LacCer antibody from the plasma membrane DRM of neutrophils that naturally have C24:0- and C24:1-LacCer contents, and from C24:0- and C24:1-LacCer-loaded D-HL-60 cells that originally contain only small amounts of C24:0 and C24:1-LacCer. In contrast, Lyn has not been co-immunoprecipitated with anti-LacCer antibody in C16- or C22-LacCer loaded D-HL-60 cells. Furthermore, immunoelectron microscopic observations indicated that loading with C24:1-LacCer, but not C16:0-LacCer, resulted in association of LacCer clusters with Lyn. These observation suggested that the presence of Lyn-coupled LacCer-enriched lipid rafts is indispensable for LacCer-mediated neutrophil superoxide generation and migration, and that the presence of a C24-carbon fatty acid chain in the LacCer molecule is necessary for the functional connetion with Lyn in LacCer-enriched lipid rafts. Loading with LacCer containing fatty acid chains with shorter chain lengths, such as C16 and C22, did not confer LacCer-mediated biological functions on D-HL-60 cells.[8]

Sphingolipids-enriched lipid rafts are more tightly packed than the surrounding non rafts phase of the bilayer containing complex lipid species carrying 16-18 carbon acyl chains,[45,46] which due to their high transition temperature, reduce membrane fluidity and favor cholesterol segregation within the same membrane domains.[3] The assembly of cholesterol could induce vacant

pockets in the central part of the membrane, thus allowing interdigitation of longer alkyl chains.(**Figure1.6**)

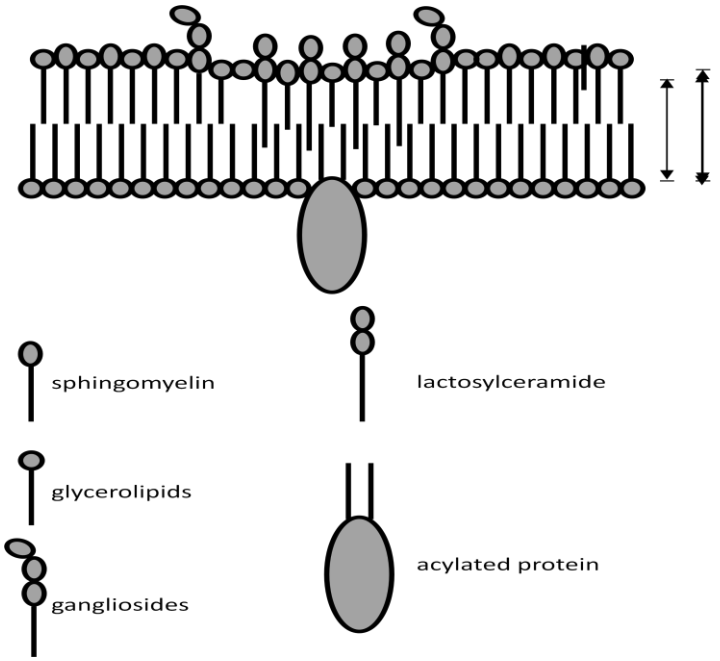


Figure 1.6 Schematic representation of the membrane with partially intergitated layers

Minor intedigitation of the longer alkyl chains usually occurs at the center of the membrane core, and its occurrence increases markedly when the maximum length of hydrophobic chain exceeds the half thickness of the membrane. The thickness of lipid rafts has been estimated to be about 5nm.[47] According to previous calculations,[48] C24:0- and C24:1-LacCer species have a hydrophobic maximum length of about 3.2 nm, about 44% longer than the half hydrophobic thickness of the membrane. NMR studies on artificial lipid bilayers indicated that C24 fatty acid chains of LacCer interdigitated with fatty acids of the opposing

monolayer.[49] It is clear that the C24:0- and C24:1- LacCer but not C16:0- or C22:0 LacCer hydrophobic chains reconstruct the LacCer-mediated Lyn phosphorilation, proper of the immune response, in D-HL-60 cells, which have only trace amounts of C24-LacCers.[8] Thus, one possibility is that the protusion into the cytoplasmic membrane leaflet of the hydrophobic chains of LacCer could be so pronounced as to allow direct Van der Waals interactions between the acyl chains of Lyn and those of LacCer. Actually, direct connetion between gangliosides present in the external leaflet of the plasma membrane and proteins of the cytoplasmic plasma membrane face, such as Src-family protein kinases, caveolin and tubulin, have been demonstrated in cells by loading with radioactive photoactivable group at the end of the acyl chain; radioactive proteins cross-linked with the ganglioside could be isolated from the DRM and characterized.[50-52]

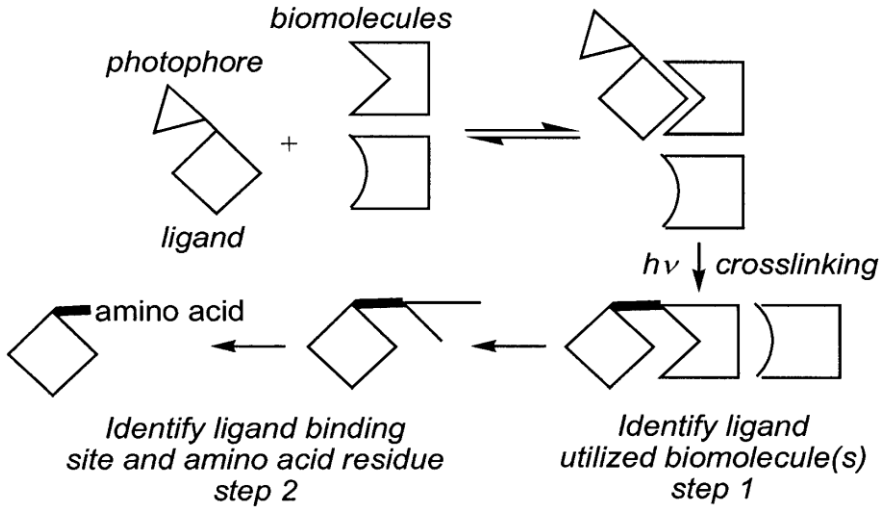
Moreover several pathogenic microorganism, such as *C. albicans*, bind selectively to LacCer among the GSLs,[53,54] indicating that the oligosaccharide chain of LacCer is also required for LacCer-mediated biological functions.

Taken together, it seems that the oligosaccharide chains of GSLs play a role in specific ligand interactions on the cell surface, whereas very long fatty acid chains of GSLs function as key elements for the functional organization of GSL-enriched lipid rafts, which may be formed by lipid interdigitation. Further studies are required to elucidate the molecular machinery responsible for the association between fatty acid chains of LacCer and Lyn in LacCer-enriched lipid rafts.

1.4 Photoaffinity labeling for the study of interactions between glycosphingolipids and proteins

Photoaffinity labeling is a useful biochemical method to reveal structural and functional relationships between low molecular weight bioactive compounds and biomolecules.[55] The method is suitable for analyzing biological interactions because it is based on the affinity of bioactive compounds for biomolecules.

Photochemically generated highly reactive species introduce covalent bonds between ligand and protein in a nonselective manner (Figure 1.7).[56]



Scheme 1.7 Schematic representation of photoaffinity labeling.

Succesfull applications of photoaffinity labeling include the identification of target molecules in crude extracts with the aid of radioisotope-labeled probes as highly sensitive detection tags (step 1 in Figure 2). The covalent bond fixes the

tag to the contact point even thought affinity has been destroyed by the denaturation conditions.

There are many options in photoaffinity labeling experiments: choice and synthesis of the photophore, photolysis conditions, and choice of tags for the identification of biocomponents.

Both organic chemistry (the preparation of the photophore and ligand modification) and biochemistry (handling of labeled components) are needed in order to perform photoaffinity labeling experiments.

The photophore I am going to discuss are photoactivable glycosphingolipids, exogenously administered GSLs are able to become components of the cell membrane, virtually indistinguishable from cell membrane gangliosides. Thus, the administration of photoactivable GSLs to cultered cells followed by illumination allows to obtain membrane proteins cross-linked to GSLs molecules located in the protein microenvironment, reflecting the interactions occurring between ganglioside and protein molecules within the membrane.[57-59]

Various photoactivable group, such as phenyldiazirine, arylazide, and benzophenone, can be used,[60] in our group of work, as photoactivable group, we use the nitrophenylazide. Linked to a nitrophenyl, the azide group becomes very sensitive to light in solution and it must be handled with care in dark conditions (i.e. under red safe light). Illumination yields a nitrene group, whose reactivity leads to three main reactions: addition, insertion, and nucleophilic reactions (figure 1.8). When the photoactivable ganglioside, inserted at the membrane level, is illuminated, the major part of it cross-links to membrane lipid molecules which

are more abundant and close to the photoactivable group,[61] a minor part links to proteins.[62] Administration of photoactivable ganglioside to cell, when followed by a sufficiently long chase time, allows the ganglioside to be internalized and to enter the general metabolic and trafficking cellular processes. Thus, when illumination reaches the photoactivable ganglioside inside the cells, the activated derivatives cross-link to intracellular proteins that are in the ganglioside environment. To allow the detection of membrane or soluble proteins cross-linked to gangliosides, a radioactive tracer within the photoactivable ganglioside is needed.

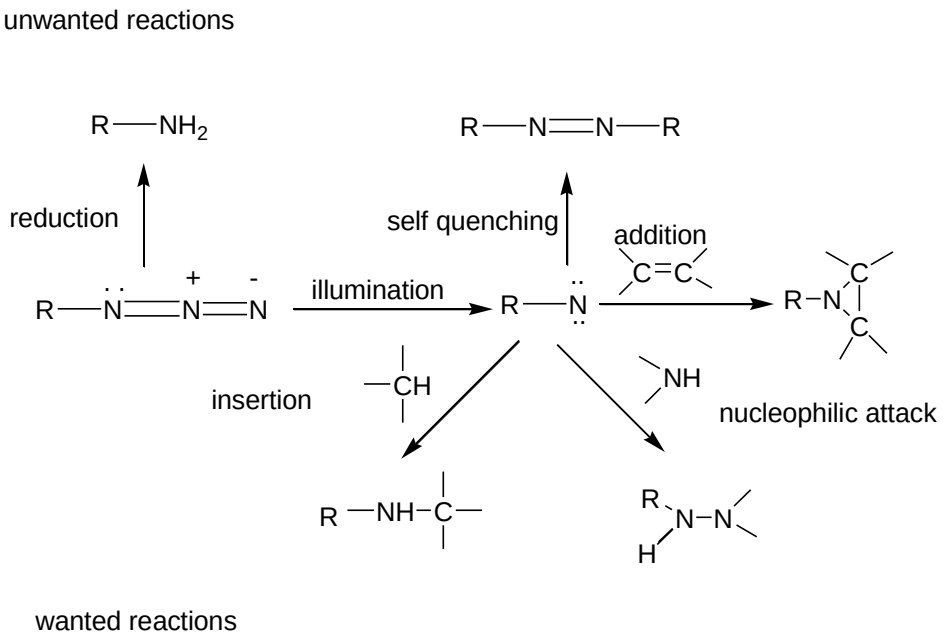


Figure 1.8 Scheme of the reactions involving the azide before and after illumination

2. AIM OF THE WORK

Human neutrophils play the first line of defense against invading microorganism and an important role in acute inflammatory reaction.[63] They exert their bactericidal activities through a mechanisms that lead to the destruction of a microorganism, involving the generation of toxic oxygen derivatives by reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and releasing of microbicidal molecules from the granules.

In mature neutrophils, more than 70% of GSLs are LacCers, which are the most abundant neutral glycosphingolipids and are expressed on plasma and granular membrane of human neutrophils. LacCer binds specifically to several microorganisms, such as *Escherichia coli, Bordetella pertussis, Bacillus dysenteriae, Helicobacter pylori,* and *C. albicans.*[64-66] Moreover, anti-LacCer antibodies induce superoxide generation and migration in neutrophils.[4,54] In more details, on neutrophil plasma membranes, LacCer forms lipid rafts with different kind of proteins like Src family kinase Lyn.[4] These LacCer-enriched lipid rafts are thought to act as a signal transduction unit responsible for superoxide generation and migration of neutrophils.[4,54] However the mechanisms by which LacCer activates NADPH oxidase in neutrophils have not yet been well characterized. To elucidate the mechanism involved studies on LacCer-enriched DIM (detergent-insoluble membrane) have been carried out providing the evidence that LacCer forms GSDs (glycosphingolipids signaling domains) coupled with Src family kinase Lyn in neutrophils and that LacCer-mediated Lyn activation causes the activation of phosphatidylinositol-3 kinase (PI-3K), p38 mitogen-activated protein

kinase (MAPK), and protein kinase C, leading to NADPH oxidase activation (Figure 2.1).[4]

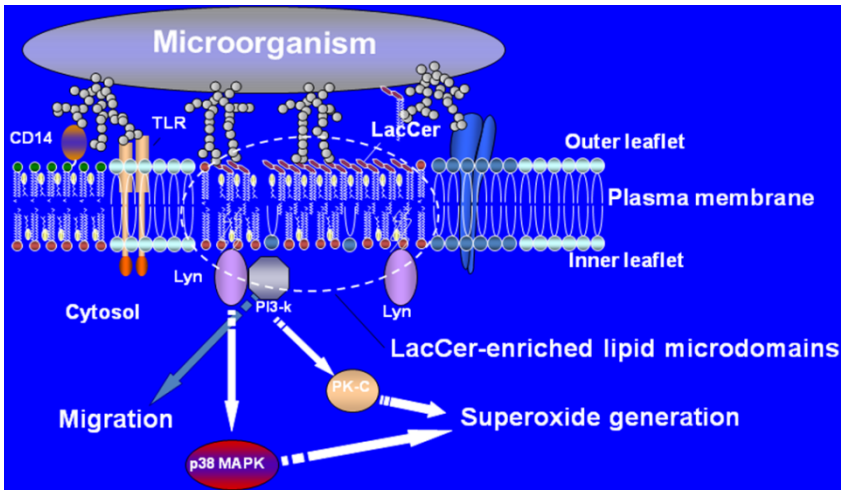


Figure 2.1 Role of neutrophils in innnate immunity. The binding of microorganism to LacCer induces clustering of LacCer-enriched lipid domains, leading to superoxide generation and migration through Lyn-, PI3-k-, p38 MAPK-, and protein kinase C-dependent signal transduction pathways.

These studies have demonstrated that the association of Lyn with LacCer seems to be a key for LacCer-mediated activation of Lyn, leading to superoxide generation by neutrophils. LacCer, like other GSLs, is not located in the cytoplasmic leaflet of membrane bilayer. The connection of LacCer, located in the external leaflet of the bilayer, with Lyn, located in a cytoplasmic site of membrane, is unclear at this time.

Several protein kinases including Lyn have been found in DIM and associated with the cytoplasmic leaflet through double acylation.[67] So connection of GSLs to signal transducers may be mediated by such proteins. However, a

study performed with reconstituted membrane containing GM3 ganglioside, cSrc, sphingomyelin and cholesterol indicated that stimulation of GM3 induces activation of cSrc without any protein.[69]

Another possibility is that cholesterol may play a functional role in connecting LacCer in exoplasmic to cytoplasmic leaflets, because cholesterol exists in both exoplasmic and cytoplasmic leaflets, and forms dimers between the leaflets under certain conditions.[68] Because cholesterol functions as a spacer in the leaflets by filling voids created by interdigitating fatty acid chains,[70] cholesterol depletion from LacCer-enriched GSDs may cause narrowing of the spaces in exoplasmic and cytoplasmic leaflets, resulting in the packing of LacCer and Lyn and enhancement of LacCer-Lyn interaction.

Furthermore, it is possible that interdigitation of long-chain LacCer into the opposing membrane leaflet could lead to the preferential binding of LacCer to acyl chains of Lyn.[68] LacCer molecules of neutrophils have been reported to possess C-24 acyl chains,[71] which could interdigitate with the cytoplasmic leaflet of the bilayer.[72] Several studies have demonstrated that cross-linking of LacCer by anti LacCer antibody induces LacCer-mediated signaling. Thus the clustering of LacCer at exoplasmic leaflet may activate the cytoplasmic Lyn molecules via the interaction between acyl chains of these molecules. It is clear that sphingolipid-enriched domains could provide a microenvironment within the plasma membrane for reciprocal interactions between lipid and protein molecules involved in the control of signal transduction but the mechanism underlying these interactions are still poorly understood.

In order to give a small contribute in this complex issue I started this PhD project focusing on the observation that C-24 fatty acid chain of LacCer is indispensable for connecting Lyn with LacCer-enriched microdomains, which are responsible for LacCer-mediated functions. In fact, very long fatty acids might participate in reducing the membrane thickness through interdigitation of the two membrane leaflets. The interdigitation is proposed to be the switch for the transduction of information throughout the membrane perhaps by allowing contact and interaction between proteins belonging to the two layers of the plasma membrane.[5] The purpose is to explore the role of long chain LacCer in this process, with a particular interest in the identification of the proteins associated with LacCer in the immune response to several microorganisms in human neutrophils.

A possible approach to this problem relies on the administration of photoactivable lactosylceramide molecules, which are able to become components of the cell membrane, virtually indistinguishable from cell membrane lactosylceramide, to human neutrophils followed by illumination. This cell photolabelling experiment allows to discriminate membrane protein cross-linked to lactosylceramide molecules located in the protein microenvironment, reflecting the interactions occurring between lactosylceramide and protein molecules within the membrane. In this way the cross-linkage between LacCer and proteins can be investigated by using the proper radioactive photoactivable probes, carrying the photoactivable group at specific points of the molecules and in principle capable to interact with proteins belonging to the cytoplasmic and/or to extracellular leaflet. With the final aim to identify LacCer associated proteins, I have projected

and prepared **probe 1** and **probe 2** (Figure 2.2), in which the photoactivable group, a nitrophenilazide, is located at the end or both at position 2 and at the end of the acyl chain. A C-18 alkyl chain has been inserted on probe 1 and 2, with the purpose to have, after the reaction with nitrophenilazide, a length very similar to that of a C-24 fatty acid, which is required for LacCer-mediated Lyn activation.

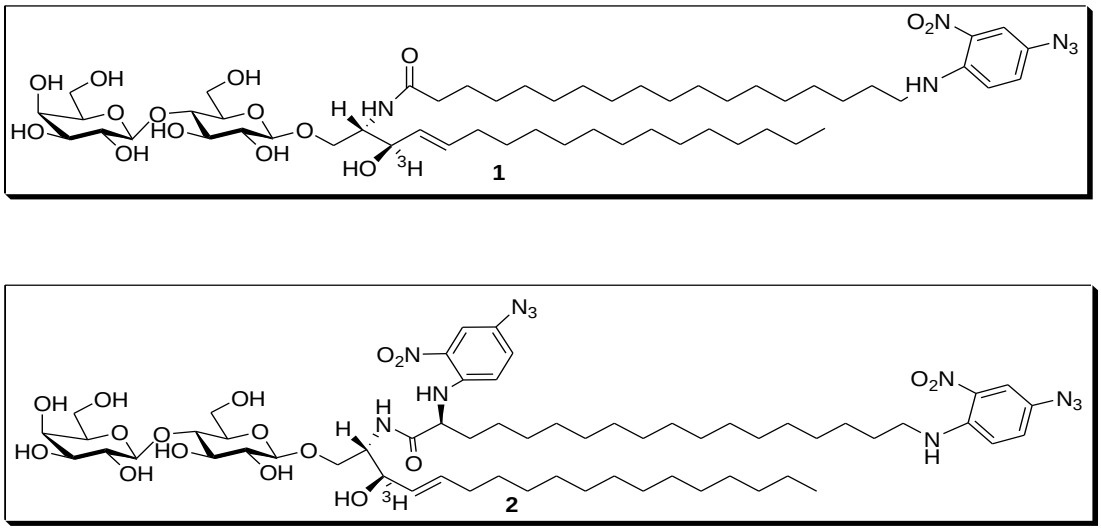


Figure 2.2 Probe 1 and 2

The photoactivable group, when illuminated, yield a very reactive nitrene intermediate that covalently binds to the molecules in the environment.[6,7] In order to follow the biological pathway of the probes it is necessary to introduce a radioactive tracer in the molecules. For this reason the glycosphingolipid has to be tritiated.

In parallel, I have also prepared **probe 3**, similar to probe 1 but with a C-12 acyl chain instead of a C-18. This compound would be useful to demonstrate that the interaction between LacCer and proteins is strongly dependent on the length of

LacCer acyl chain. We should observe interaction in the case of probe 1 and not with probe 3.

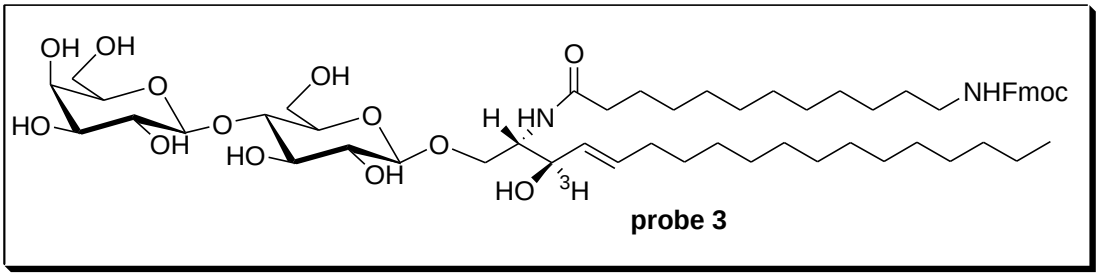


Figure 2.3 Probe 3

Finally, to assess the capability of an α,ω-diamino fatty acid functionalized GSL, *i.e.* **2**, to be internalized by the cells, I have designed **probe 4** in which the hydrophilic moiety is ganglioside GM1, instead of lactose.

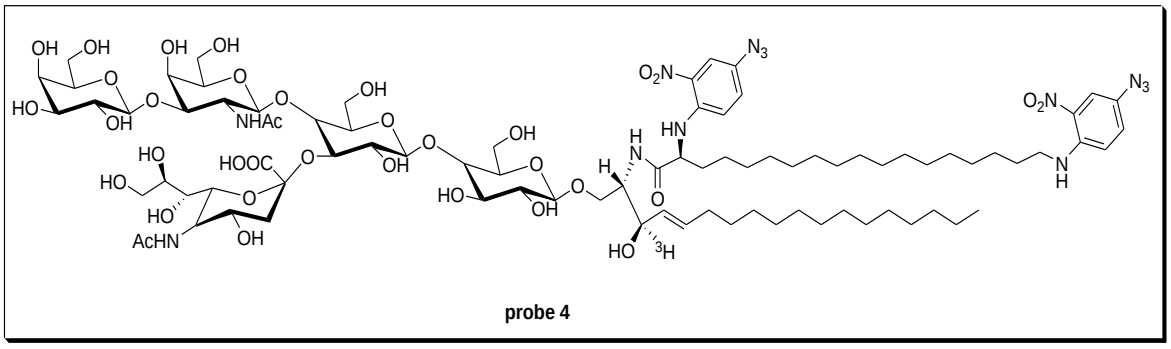


Figure 2.4 Probe 4

Previous studies have in fact demonstrated the capability of GM1, with one photoactivable group at the ω-position of the fatty chain, to be inserted in the membrane lipid core in a way that closely resembles that of endogenous gangliosides.[9] We aim to observe the same behaviour also in the case of probe 4,

which means that the second photoactivable group at position 2 does not influence the biological behaviour of GM1.

3. RESULTS AND DISCUSSION

The preparation of the target probes 1-3 requires a common synthetic strategy, which consists in the conjugation of a lactosylsphingosine moiety to the properly functionalized fatty acid.

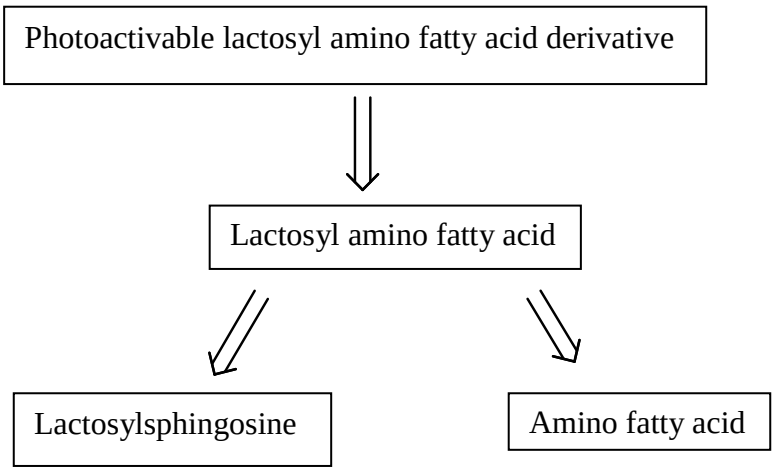


Figure 3.1 General retrosynthetic scheme of required photoactivable probes

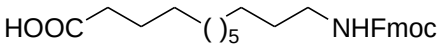
To this aim the following items describe the synthetic sequence that I have followed for the obtainment of the projected compounds:

1) LACTOSYLSPHINGOSINE: lactosylsphingosine is the common precursor of probes 1-3. It has been previously obtained by alkaline hydrolysis of lactosyl ceramide, resulting from natural sources and available in our laboratory.

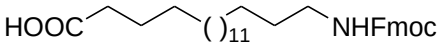
2) PREPARATION OF THE AMINO FATTY ACIDS: the synthesis of the lactosyl ceramide requires the conjugation of lactosylsphingosine with the fatty acid, containing amino groups at the position where it has been planned to introduce the photoactivable groups. In fact, the nitrophenilazide group can be easily introduced

at the final stage of the synthesis by a nucleophilic substitution of an amino group of the acyl chain on the fluoro derivative of the nitrophenyl azide.

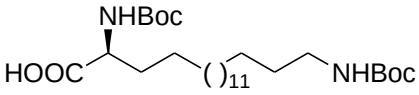
While the ω-amino lauric acid (figure 3.2), necessary for the synthesis of probe 3, was commercially available, the C-18 ω-amino stearic acid and the C-18 α,ω-diamino stearic acid, necessary for probe 1 and 2, were not. In the following I will describe the synthetic strategy adopted for their preparation.



N-Fmoc-ω–amino lauric acid



N-Fmoc-ω–amino stearic acid



N,N'-Boc-α,ω–di-amino stearic acid

Figure 3.2 The required amino fatty acid

3) CONJUGATION BETWEEN LACTOSYLSPHINGOSINE AND THE AMINO PROTECTED FATTY ACID.

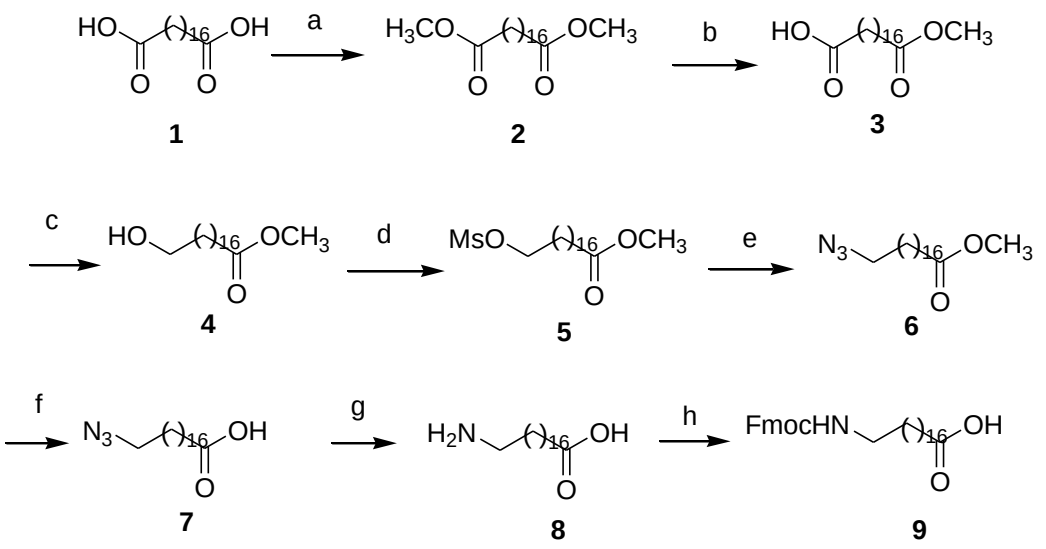
4) DEPROTECTION OF THE AMINO GROUP AND INTRODUCTION OF THE PHOTOACTIVABLE GROUP.

I will describe in the next chapter points 2 to 4 for each single probe.

3.1 Synthesis of probe 1

3.1.1 Preparation of Fmoc protected ω-amino stearic acid

To the aim of obtaining the not commercially available long chain ω-amino fatty acids we moved the first steps in the synthesis of compound **8**, 18-aminooctadecanoic acid, starting from octadecanedioic acid, which is commercially available, according to the scheme reported below:



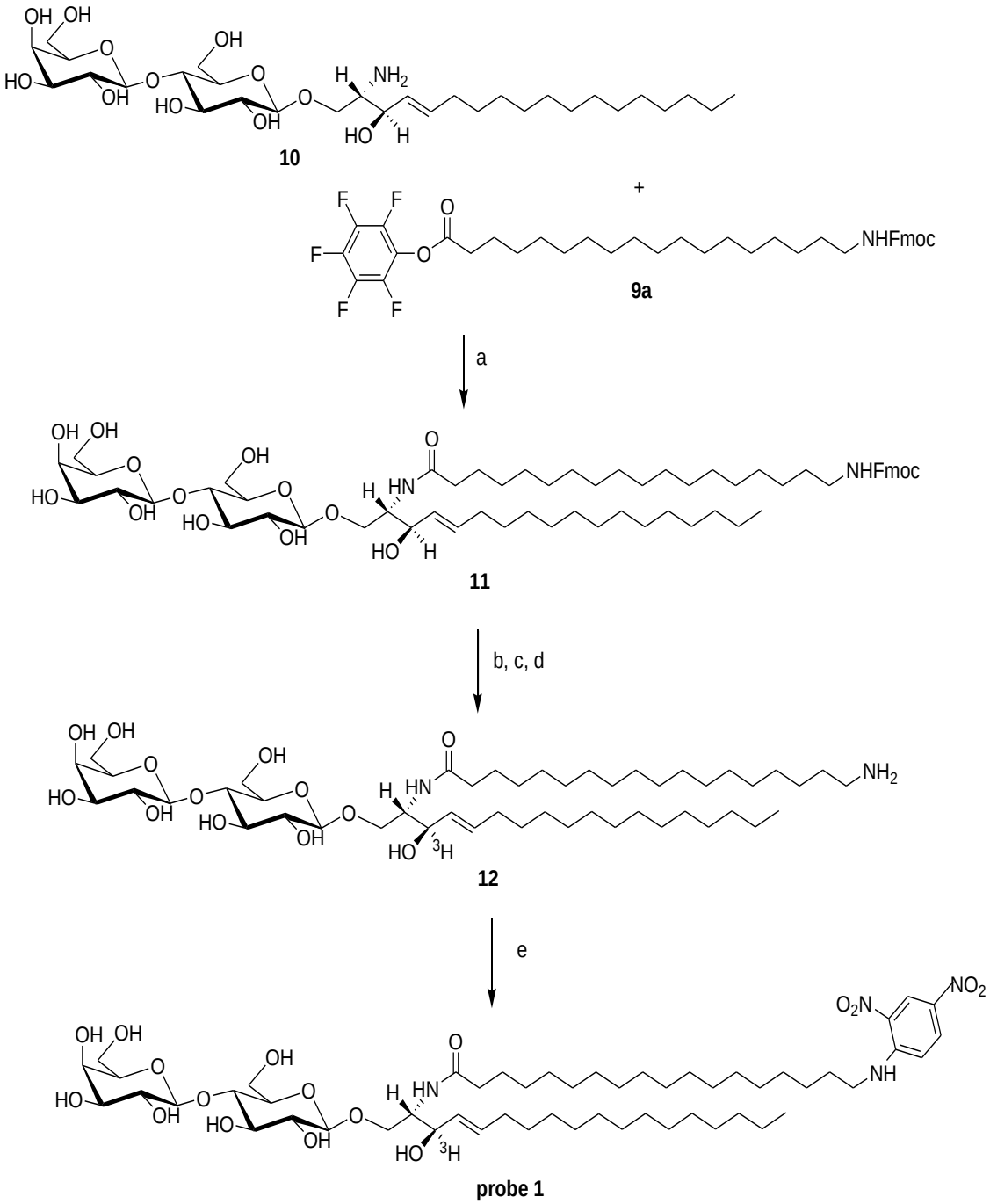
Scheme 3.1 a) CH_3OH, H_2SO_4, reflux[73]; b) $Ba(OH)_2$, MeOH, 40^0C[73]; c) BH_3/THF, -20^0C to RT;[74] d) MsCl, TEA, DCM, 0^0C to RT;[74] e) NaN_3, DMF, 45^0C[75]; f) KOH, MeOH; g) Pd/C, 5% CH_3COOH in MeOH; h) FmocCl, Na_2CO_3 sol 10%.

In the first step the acid groups of dicarboxylic acid **1** were metoxylated to give dimethyl octadecanedioate, which gave, in the second step, by saponification

with barium hydroxide, the mono methyl ester of octandecandioic acid **3**. By reduction of the carboxylic group of compound **3,** alcohol **4** was obtained. With the purpose to have a better leaving group for the nucleophilic substitution with NaN_3, the alcoholic moiety was activated as a mesylate. Azide **6** was then obtained in the next step and, after saponification of the methylester, reduced to yield the final ω-amino acid **8**. Compound **8** was at the end protected as fluorenylmethyloxycarbonyl derivative (Fmoc) in order to mask the amino functionality to yield **9**.

The reported synthetic scheme presented several critical points, all of them related to the amphiphilic feature of the molecules, thus to the solubility. My efforts were directed to find the proper solvents for the reaction work-up to improve the yields, which at the beginning of the synthetic work were very low but finally more than satisfactory.

Compound **9**, after activation of the carboxylic functionality as pentafluorophenolate, was conjugated to lactosylsphingosine through a condensation reaction according to the next scheme.



Schema 3.2 a) HOBt, Bu_3N, DCM/MeOH[7]; b) DDQ, dioxane, 50^0C; c) NaB^3H_4, MeOH;[7] d) aqueous NH_3[7]; e) TEA, 4-F-3-NO_2-phenilazide, DMF.[7]

After this reaction, I first tried to radiolabel compound **11** with tritium at position 6 of the terminal galactose by the galactose oxidase-NaB^3H_4 method (Hajra et al., 1969). The reaction was unsuccessful. So, I decided to label the position 3 of sphingosine. To this purpose, the hydroxy group of ceramide was oxidized by 2,3-dichloro-5,6-dicyanobenzoquinone (DDQ), a widely employed oxidizing agent, which results in the selective oxidation of the α,β-unsaturated alcohol. Then the 3-keto group was reduced with NaB^3H_4.[73]

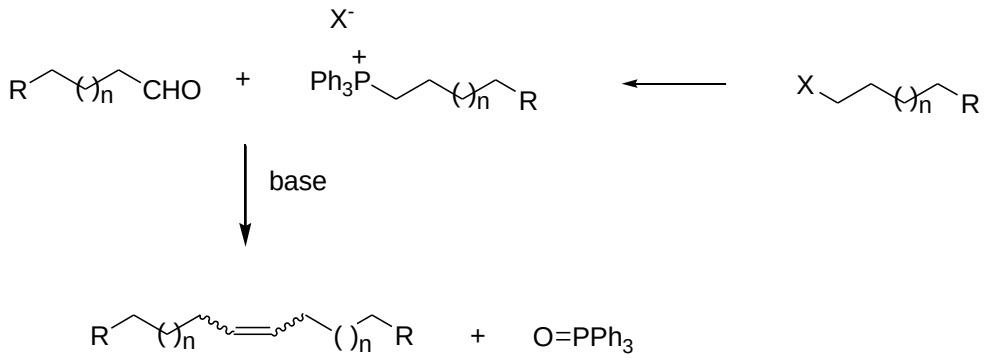
After deprotection of the amino group, the substitution with the photoactivable 4-F-3-NO_2-phenylazide group was performed. I have already discussed in section **1.4** that this group has been inserted in the molecule with the purpose to follow the metabolic pathway of **probe 1**. At this moment, we are carrying out photolabeling experiments on human neutrophils with probe 1 in the laboratory of Professor Iwabuchi in Japan.

3.2 Synthesis of probe 2

3.2.1 Preparation of Boc protected α,ω-diamino stearic acid

I have devoted a lot of efforts during my PhD thesis to the synthesis of the C-18 α,ω-diamino fatty acid, which is not commercially available and about which the literature is quite poor. For the preparation of this compound the synthetic strategy I decided to follow involves the construction of the C-18 skeleton through a series of Wittig condensation.

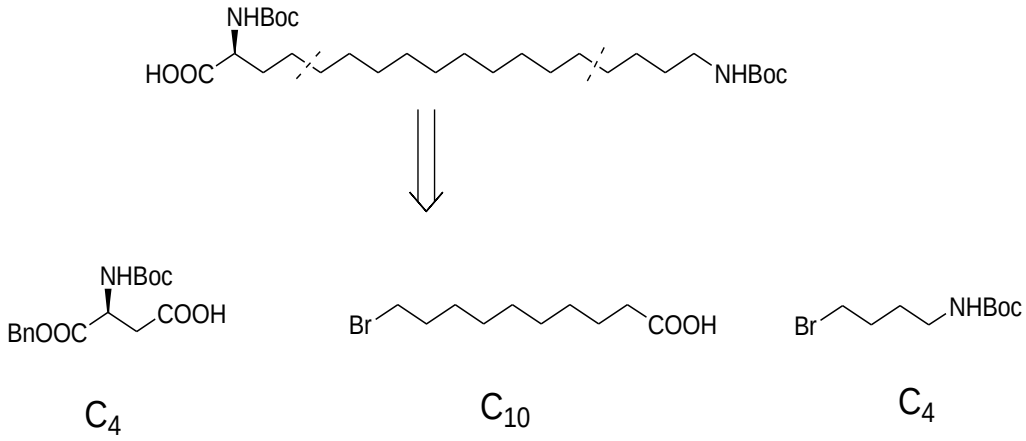
The Wittig reaction is a synthetic procedure commonly used for the preparation of alkenes by coupling of aldehydes or ketones to alkyl triphenyl phosphonium ylides to give alkenes and triphenylphosphine oxide.



Scheme 3.3 General scheme of a Wittig reaction

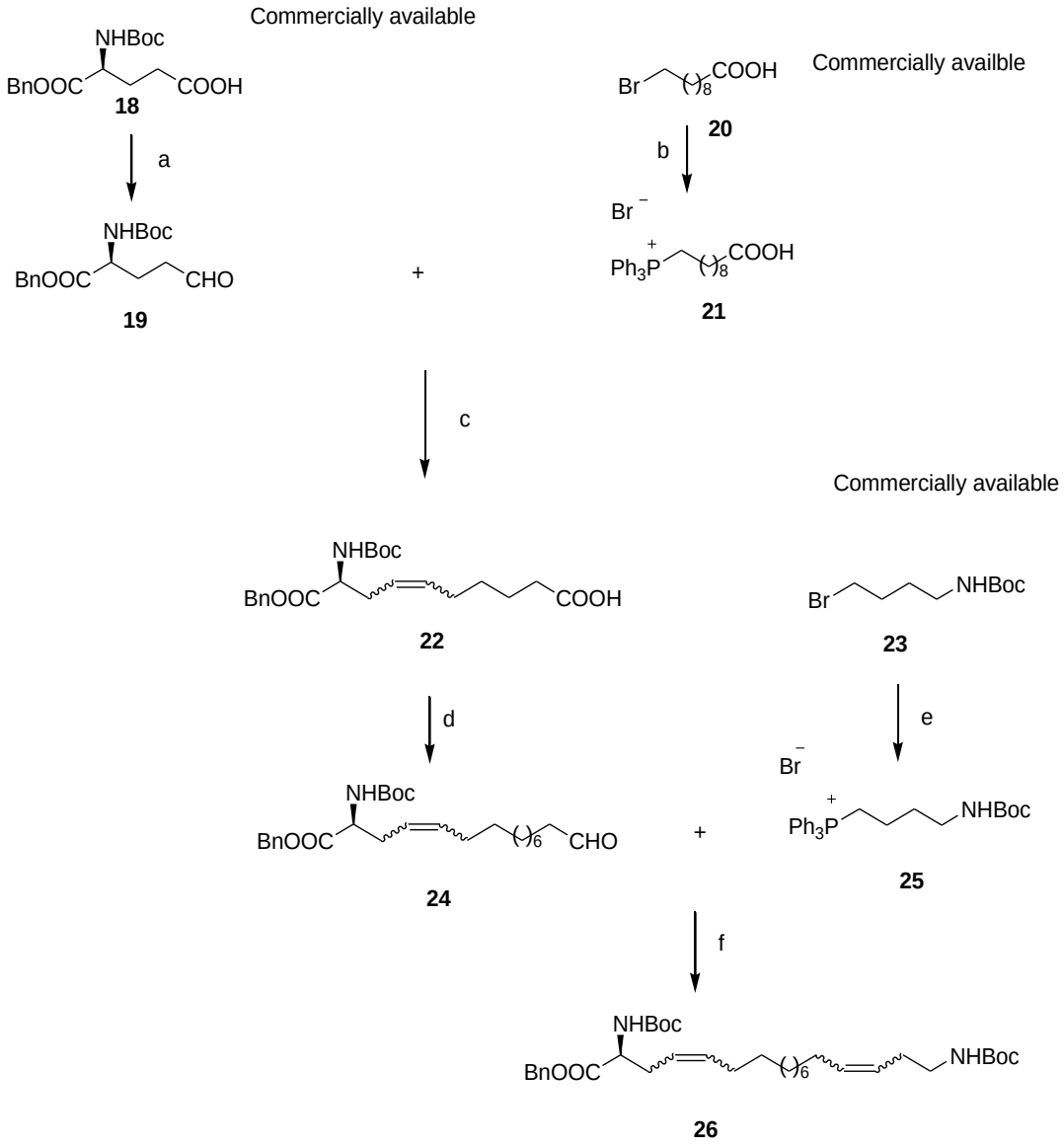
This strategy implies that it is necessary to chose *a priori* where to disconnect the molecule in order to obtain the final alkyl chain through condensation of smaller pieces.

At the beginning, we started with the approach showed in the scheme below, which consists in an initial condensation between a C4 and a C10 fragment followed by a subsequent C4 homologation.



Scheme 3.4 Retrosynthetic scheme **approach 1**

This disconnection was planned because it allows to obtain the desired alkyl chain already functionalized in ω with an amine group by using three commercially available synthons. In addition, the expected terminal protected amino group is already installed on the chain. To this aim, I decided to start from N-Boc–aspartic aminoacid.

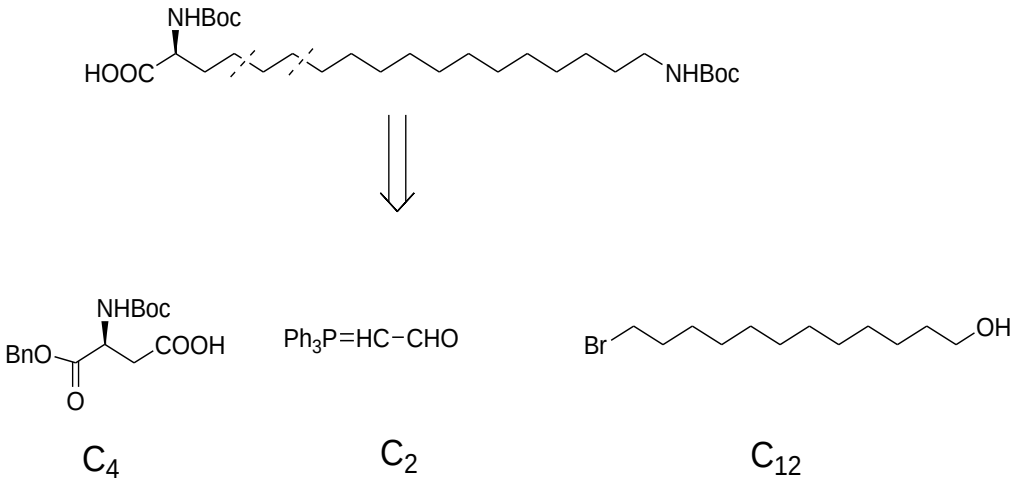


Scheme 3.5 Synthetic **approach 1**

Compound **18**, was chemoselectively converted to aldehyde **19** using N,N-dimethylchloromethyleniminium chloride, prepared *in situ* from freshly distilled oxalyl chloride and dimethylformamide,[77,78] and LiAlH(OBu^t) with a satisfactory yield. The obtained aldehyde **19** was used in the Wittig reaction with the phosphonium salt **21**, derived from commercially avilable 10-Br-aminodecanoic

acid, the C10 synthon, but unsuccessfully, although many different reaction conditions and several bases (LiHMDS, NaHMDS, NaH, and BuLi) were tried. This reaction was not the only tricky point of the designed synthetic route. In fact, unfortunately, we could never recover the phosphonium salt **25**, derived from Boc-amino butyl bromide, although the easiness of the reaction, most probably for the lability of the t-butoxycarbonyl (Boc) protective group to the reaction conditions, which need refluxing temperature.

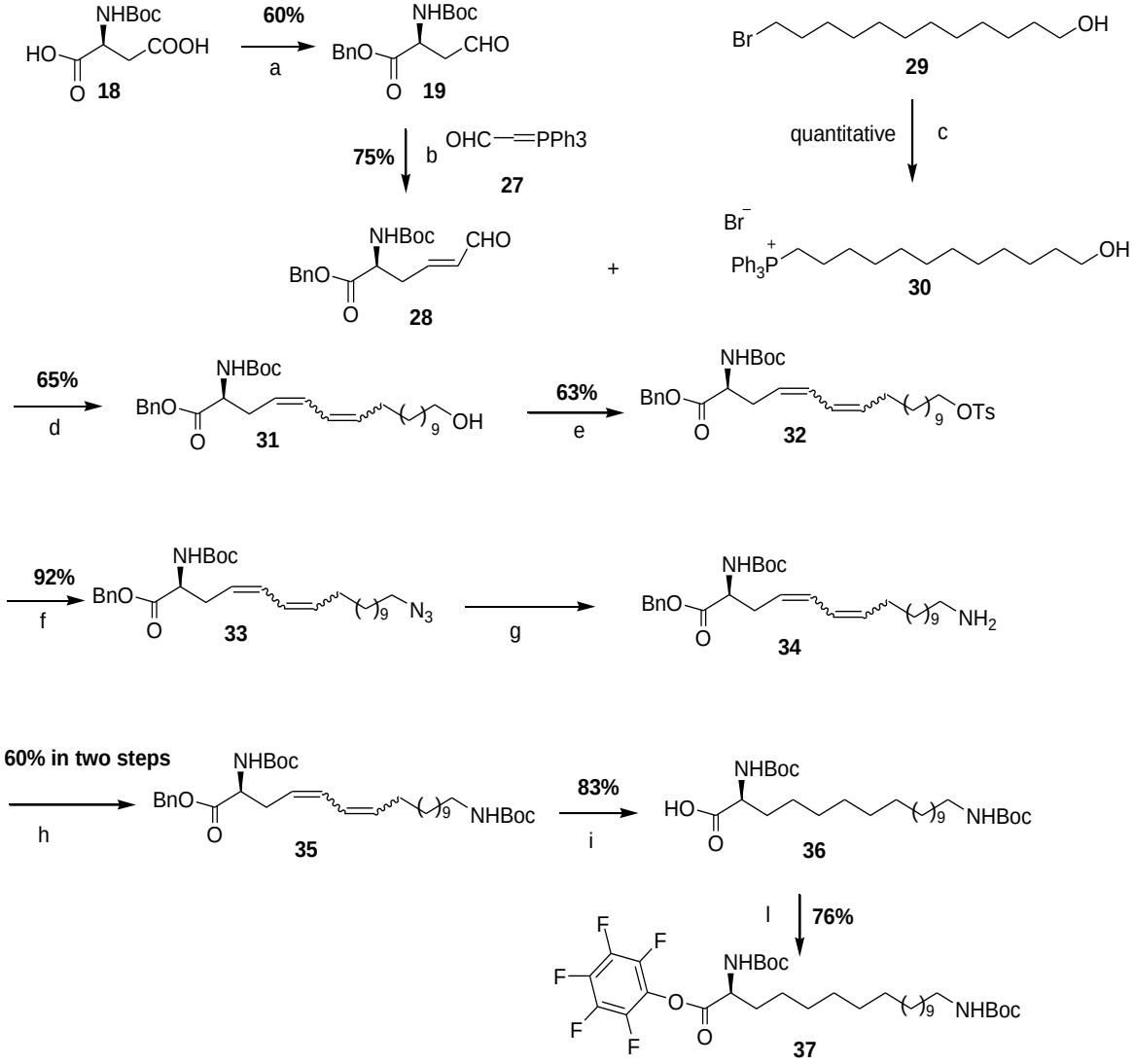
A different protecting group on the amino functionality could not be considered at this stage, since a base stable protecting group is necessary for the basic conditions of the Wittig reaction. So, I decided to plane a different synthetic pathway which exploits the experience I have acquired with the previous route.



Scheme 3.6 Retrosynthetic scheme **approach 2**

In this new approach the C-4 aspartic synthon is the same as in **approach 1** but the disconnections are different and the introduction of an amino group is

planned after the construction of the C-18 alkyl skeleton. In particular, the first condensation is between the C4 synthon **19** and the commercially available aldehyde-ylide **27**, in order to obtain the elongated alkyl chain already installed with an aldehyde function for the next Wittig reaction.



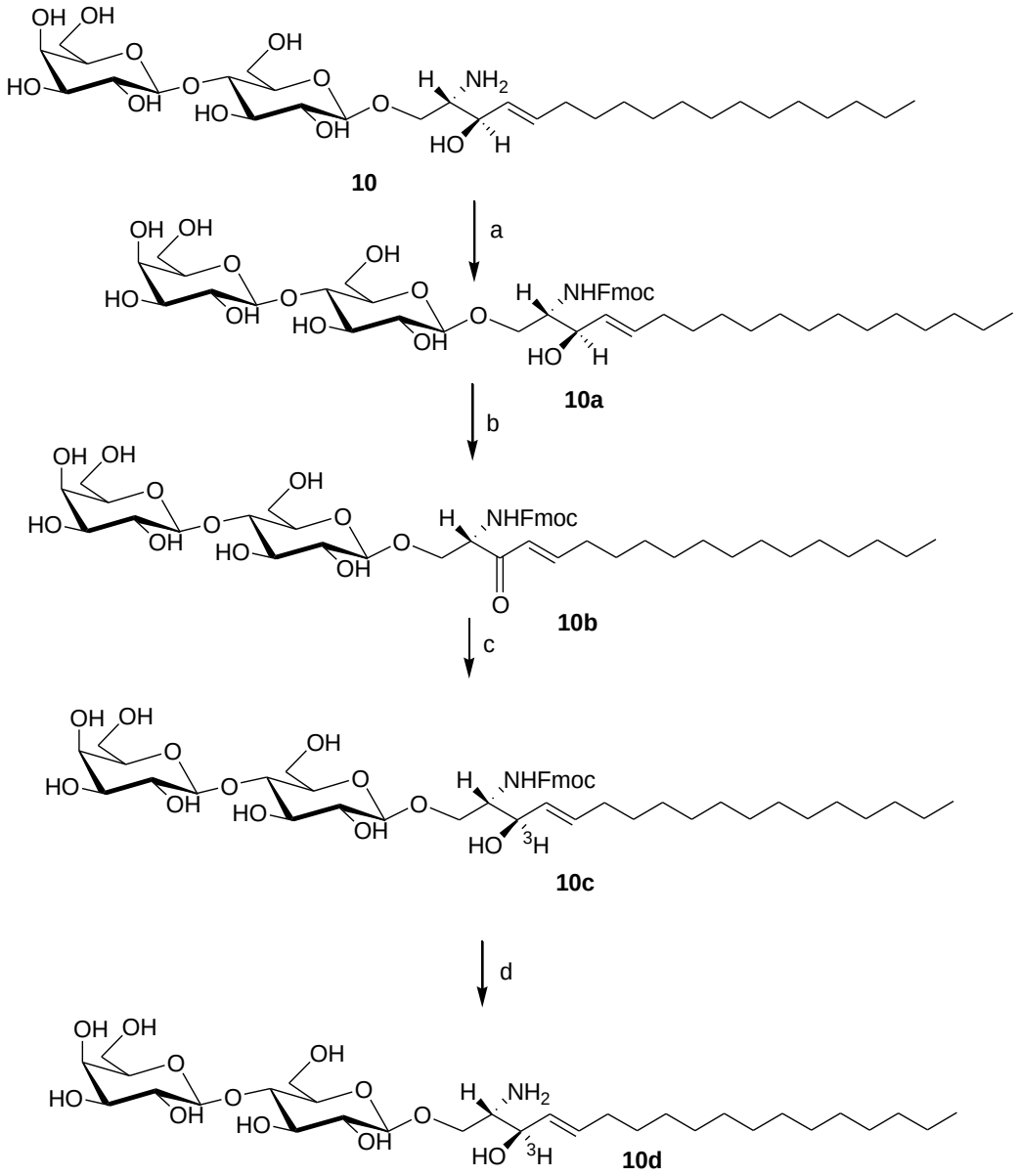
Scheme 3.7 Synthetic scheme **approach 2** a) oxalyl chloride, DMF, $LiAlH(t\text{-}OBu)_3$, THF/CH_3CN -78^0C[77,78]; b) PPh_3, EtOH, reflux;[70,80] c) toluene, 70^0C; d) LiHMDS, THF, -60^0C[79,81,82]; e) TsCl, DMAP, DIPEA, DCM, 0^0C to RT, f) NaN_3, TBAI, DMF; g) PPh_3, THF; h) Boc_2O, Et_3N, DCM; i) Pd/C, MeOH; l) Pentafluorophenol, EDCI, CH_2Cl_2.

So, as reported in **scheme 3.7**, the first reaction was the chemoselective reduction of Boc-L-aspartic acid **18,** which was already optimized in **approach 1**. The second step was again a Wittig condensation between aldehyde **19** and the commercially available ylide **27,** yielding aldehyde **28** in very good yield. Although the aldehyde instability we could even purify it choosing the right eluents to make the purification faster. On the contrary, the Wittig reaction to make compound **31** required an appreciable effort to find the right reaction conditions and the right base. Small differences in the reaction temperature changed considerably the yield, which was also improved by varying the base for the formation of ylide and moving from KHMDS and NaHMDS to LiHMDS. This confirm the importance of the base cation on the outcome of the reaction.

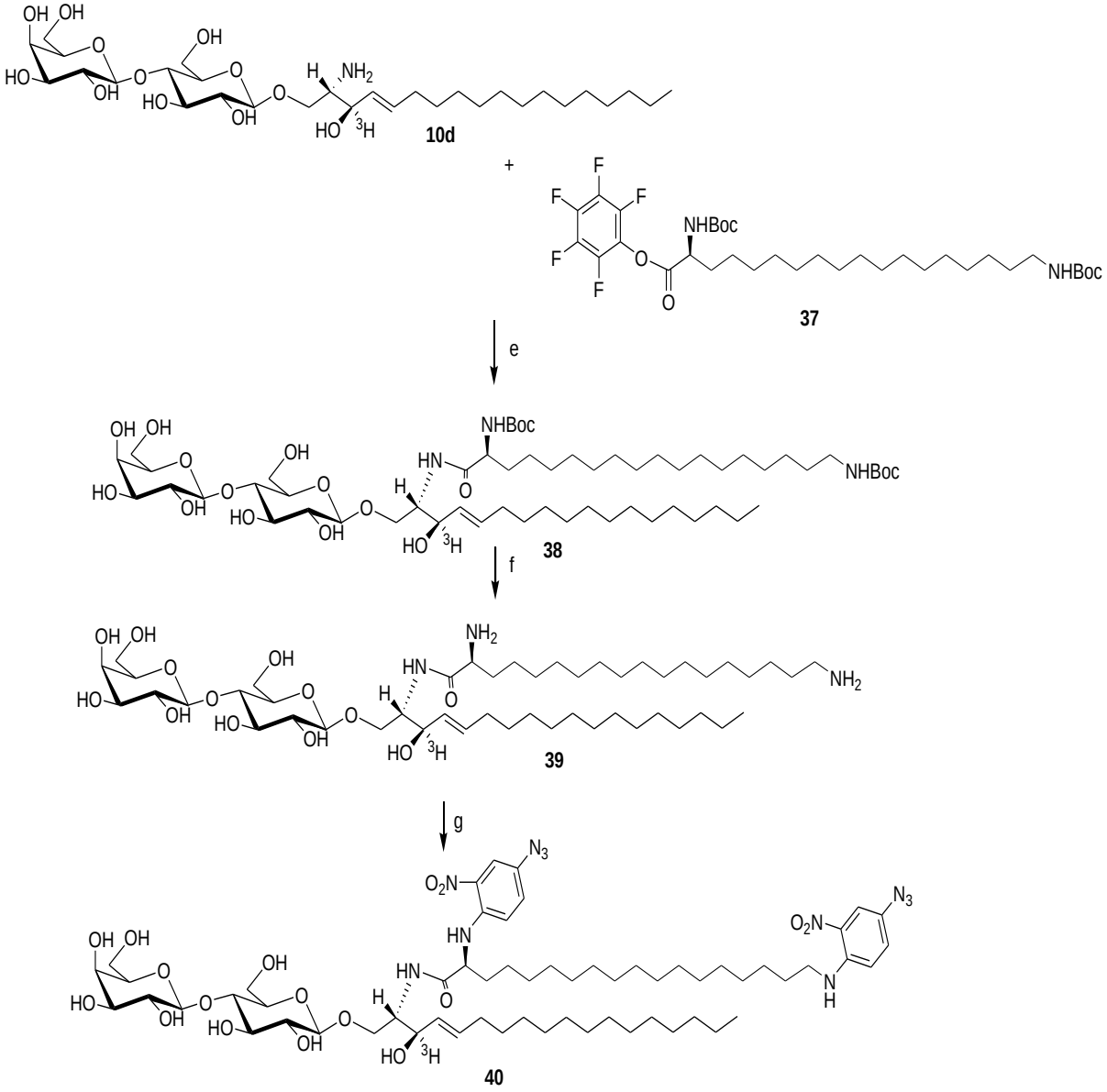
Compound **31** was recovered as a mixture of isomers but I had not care about it because in the last step of the α,ω-diaminoacid synthesis the double bonds will be reduced by catalytic hydrogenation. The alcoholic moiety in compound **31** was then replaced by an amino functionality passing through an azide, resulting from the corresponding tosylate. The amine was obtained by Staudinger reaction, a mild method of reducing an azide to an amine. In our case we could even obtain directly the Boc-protected amine by a one-pot conversion of azide. Finally the double bonds were reduced by catalytic hydrogenation using Pd/C which provided also the free carboxylic acid. The required protected α,ω-diaminoacid **36** furnishes the double amino functionalities useful for the substitution with the photoactivable groups, as required to our biological purpose.

The condensation of the fatty acid was performed on tritiated lactosylshingosine in order to reduce the synthetic steps after the introduction of the di-amino fatty acid **37**, which is a synthetic previous intermediate. Tritium was introduced on lactosylsphingosine as described in scheme **3.8a**, by exploiting the oxidation of the hydroxyl group of sphingosine followed by reduction with NaB^3H_4.

Differently from **probe 1**, in **probe 2** we used, as protective group of the amino functionalities, the tert-butoxycarbonyl (Boc) instead of fluorenylmethyloxycarbonyl (Fmoc). This group requires different deprotection conditions, being Boc an acid labile protective group whereas Fmoc is a base unstable protective group. However, we could carry out the deprotection reaction without particular problems by treatment with trifluoroacetic acid.



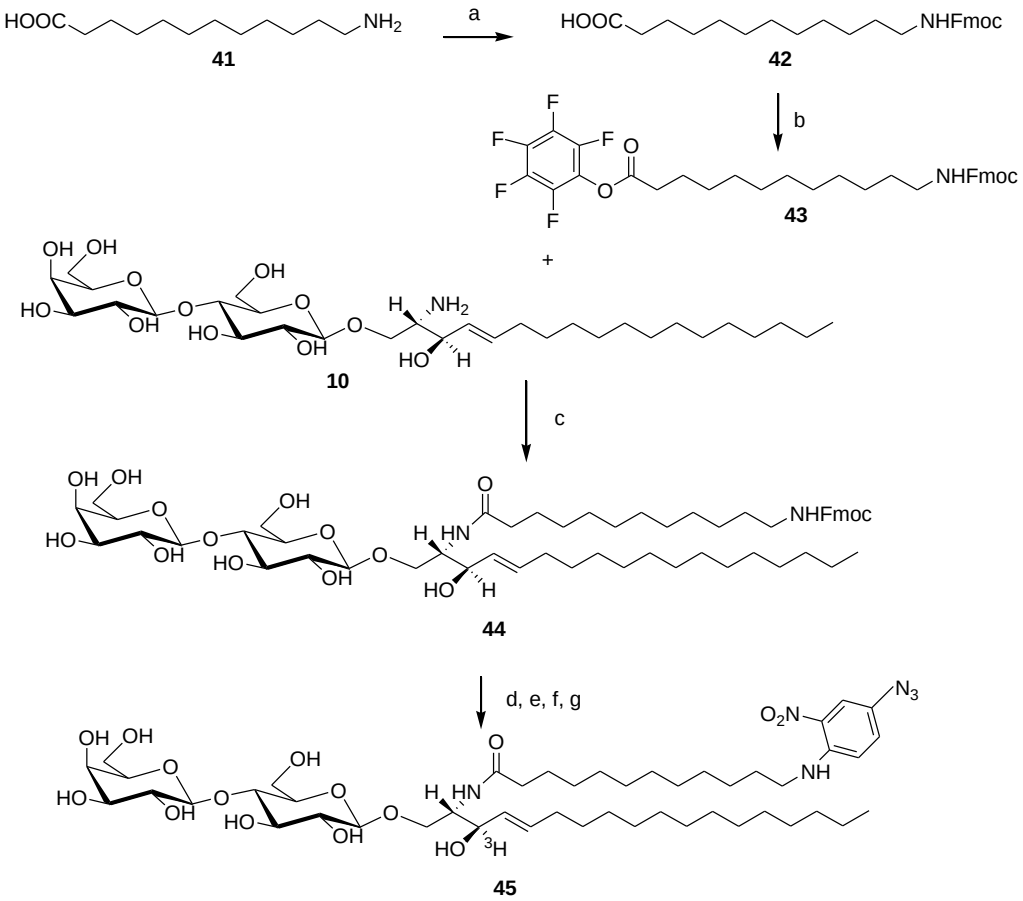
Schema 3.8a a) FmocCl, THF, Na_2CO_3 10% sol; b) DDQ, dioxane, 50^0C; c) NaB^3H_4, MeOH[7]; d) aqueous NH_3.[7]



Scheme 3.8b e) HOBt, Bu_3N, DCM/MeOH[7]; f) CF_3COOH/MeOH, 50% v/v; g) TEA, 4-F-3-NO_2-phenilazide, DMF.[7]

3.3 Synthesis of probe 3

The commercially available 12-aminododecanoic acid, protected as fluorenylmethyloxycarbonyl and activated as pentafluorophenolate, was conjugated to lactosylsphingosine according to the scheme reported below:



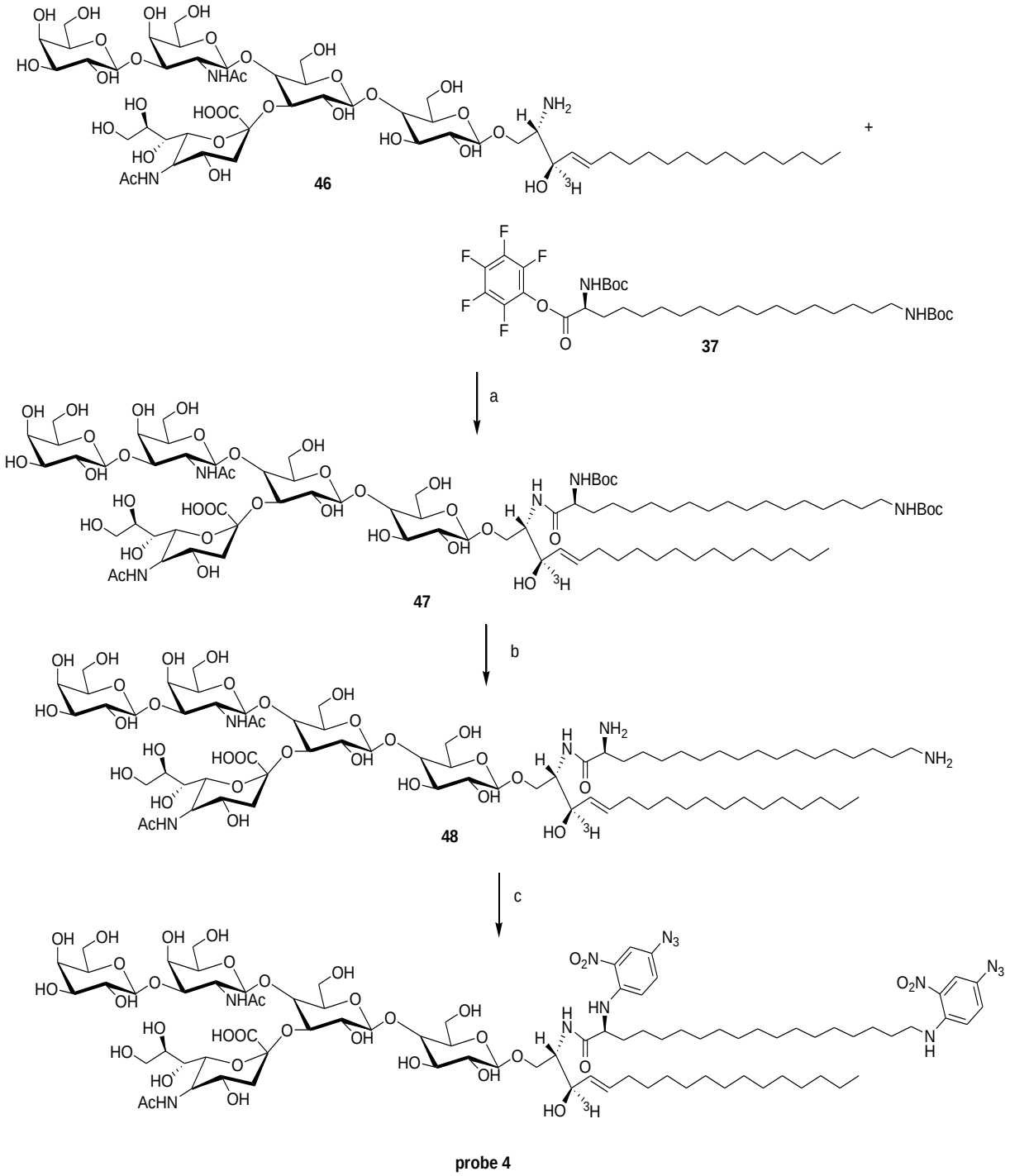
Schema 3.9 a) FmocCl, Na_2CO_3 sol 10%; b) pentafluorophenol, Bu_3N, 2-chloro-1-methylpyridinium iodide, DCM; c) HOBt, Bu_3N, DCM/MeOH[7]; d) DDQ, dioxane, 50^0C; e) NaB^3H_4, MeOH;[7] f) NH_3 aqueous; g) TEA, 4-F-3-NO_2-phenilazide, DMF.

The synthetic procedure has been already developed and optimized along the past experience of the Professor Sonnino group on the preparation of photoactivable probes.

3.4 Synthesis of probe 4

The activated α,ω-diaminoacid **37** was also conjugated to the lyso derivative of ganglioside GM1, which was obtained by alkaline hydrolysis of natural GM1, available by extraction in our laboratory, with the purpose to assess the capability of an α,ω-diamino fatty acid to be internalized by the cells. In fact, previous studies have demonstrated that GM1 ganglioside with one photoactivable group at the end of the fatty acid chain can be inserted in the membrane lipid core in a way that closely resembles that of endogenous gangliosides.[9]

All the reactions already developed for **probe 2** were repeated for **probe 4**, with the exception for the solvent used in step b, due to the different solubility of the substrate.



Scheme 3.10 a) HOBt, Bu_3N, DCM/MeOH.[7]; b) CF_3COOH/H_2O, 50% v/v; c) TEA, 4-F-3-NO_2-phenilazide, DMF.

CONCLUSIONS AND FUTURE PERSPECTIVES

During my PhD, with the purpose to study the interactions between lactosyl ceramide species containing long fatty acid chains and proteins in the transduction process occurring on plasma membrane of human neutrophils, I have prepared lactosylceramide photoactivable probes. In particular an important part of the work has been devoted to the development of new synthetic strategies to obtain the proper long chain amino acids for the construction of the required photoactivable compounds.

An ω-amino and an α,ω-diamino stearic acid were planned for our purposes, with the amino group as the anchor of the photoactivable nitrophenylazide residue. The C-18 ω-aminoacid was derived from commercially available octadecandioic acid through an eight steps synthetic pathway. On the contrary, the α,ω-diamino homologue was constructed in seven steps by subsequent condensations of shorter building blocks, starting from suitable protected aspartic acid, through a synthetic route that showed several tricky points. Although the remarkable efforts, the synthesis of the long chain α,ω-diamino acid can be very useful for the possibility, after derivatization with the photoactivable group, of simultaneous capture of the proteins belonging to the two leaflets of the plasma membrane. In fact (with the photolabeling experiments, in progress at this moment), we hope to catch both the proteins, of the cytoplasmic layer by the nitrophenilazide located at the end of the acyl chain, and of the extracellular layer interacting with the C2 located in the aqueous–lipid interface as a mirror of the protein-lactose interaction.

In addition, the synthetic route developed for the C18 α,ω-diamino fatty acid represents a versatile method, usable for the construction of long chain α,ω-diamino acids of different length. In fact I have already planned to use the made experience for the preparation of several probes for different biological studies.

The yields as a whole were satisfactory, and in some cases very good. It is noteworthy the yield (65%) of the Wittig reaction between synthon C-12 and the aldehyde obtained by the first Wittig homologation. Literature data shows, in fact, yields substantially lower for the Wittig homologation, performed with such substrates.

The biological results, obtained by the first experiments, in progress at this moment, with probe 1 and probe 3, confirm our hypothesis about the necessity to have a long fatty acid chain for LacCer signalling.

4. EXPERIMENTAL SECTION

4.1 General methods

^{1}H and ^{13}C NMR spectra were recorded with a Bruker AVANCE-500 spectrometer at a sample temperature of 298 K. NMR spectra were recorded in $CDCl_3$ or CD_3OD and calibrated by using the TMS signal as internal reference.

Optical rotations were measured with a Perkin-Elmer 241 polarimeter at 20°C.

The mass spectrometric analyses were performed in positive or negative electrospray (ESI-MS). MS spectra were recorded on a Hewlett-Packard HP-5988-A or a Thermo Quest Finnigan LCQ™DECA ion trap mass spectrometer; the mass spectrometer was equipped with a Finnigan ESI interface; data were processed by Finnigan Xcalibur software system.

All reactions were monitored by TLC on silica gel 60 F-254 plates (Merck), spots being developed with Pancaldi, Anysaldehyde and Ninidrine solutions and subsequently heated at 110 °C.
Flash column chromatography was performed on silica gel 60 (230-400 mesh, Merck).

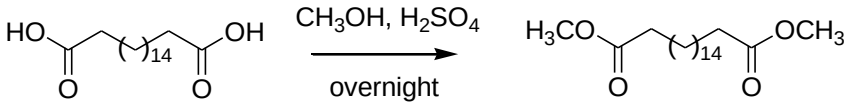
Reagents and dry solvents were added with oven-dried syringes through septa. Dry solvents and liquid reagents were distilled prior to use. Tetrahydrofuran (THF) and toluene were distilled from sodium; dichloromethane (CH_2Cl_2 or DCM) and pyridine (Py) were distilled from calcium hydride; *N*,*N*-dimethylformamide (DMF), acetonitrile (CH_3CN), methanol (MeOH) and dimethylsulfoxide (DMSO) were dried on activated molecular sieves; triethylamine (TEA) was distilled from KOH.

Compounds **1**, **13**, **18**, **20** and **21** are commercially available while compounds **8** and **36** were prepared by elaborations of literature procedures.

List of abbreviations : MsCl= methane sulfonyl chloride; TEA=triethylamine; DMF=dimethylformamide; HOBt=Hydrocybenzotriazole hydrate; DDQ = 2,3-dichloro-5,6-dicyano-1,4-benzoquinone; FmocCl= 9-fluorenylmethyl chloroformate; Bu_3N= tributhylamine; LiHMDS= lithium hexamethyldisilazide; TsCl=P-toluenesulfonyl chloride; DMAP = 4-dimethylamino pyridine; DIPEA=N,N-Diisopropylethylamine; TBAI = tetrabutylammonium iodide; PPh_3=triphenylphosphine; Boc_2O=Di-t-butyl-dicarbonate; EDCI=1-ethyl-3-(3-dimethylaminopropyl) carbodiimide); PFP=pentafluorophenol

4.2 Synthesis of probe 1

4.2.1 Synthesis of dimethyl octadecanedioate

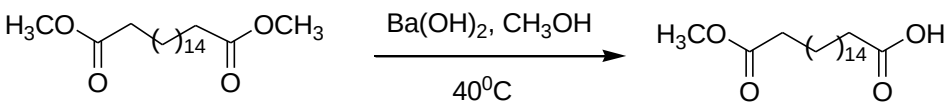


1 **2**

Compound	M.W.	D(g/ml)	eq.	mmol	mg	ml
1	**314.46**		**1**	**6.04**	**1900**	
H_2SO_4 conc.						**0.104**
CH_3OH						**8**

To a suspension of octadecanedioic acid in CH_3OH was added H_2SO_4 conc. and the reaction was stirred overnight under reflux. The reaction mixture was diluted with Et_2O and washed with sat. Na_2CO_3. The organic layer was dried over Na_2SO_4 and evaporated, obtaining 2.0 g of compound **2** (5.8mmol, 97%)

4.2.2 Synthesis of 18-metoxy-18-oxooctadecanoic acid



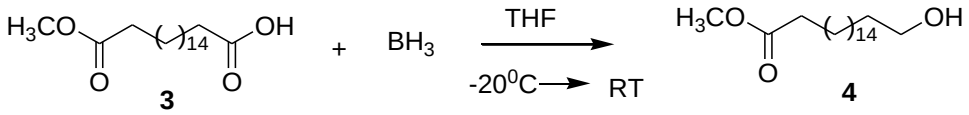
2 **3**

Compound	M.W.	D(g/ml)	eq.	mmol	mg	ml
2	**342.51**		**1**	**5.8**	**2000**	
$Ba(OH)_2$	**171.34**		**1**	**5.8**	**994**	
CH_3OH						**85**

Compound **2** and $Ba(OH)_2$ were suspended in dry CH_3OH, the mixture was warmed to 40^0C and stirred for 24 hours. The reaction mixture was filtered under reduced pressure and the precipitate was washed with Et_2O and suspended in water. A solution of HCl 1M was added until reaching the acidic pH and extracted with DCM. 770 mg (2.3 mmol, 52%) of compound **3** were obtained after flash chromatography with petroleum ether:ethyl acetate 8:2.

The mater liqueur contained the starting material. Longer reaction time did not improve the yield.

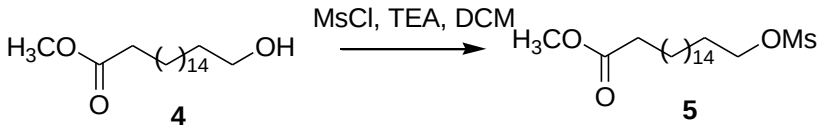
4.2.3 Synthesis of methyl 18-hydroxyoctadecanoate



Compound	M.W.	D(g/ml)	eq.	mmol	mg	ml
3	**328.49**		**1**	**3.7**	**1200**	
BH_3/THF(sol. 1M)						**24**
THF						**20**

To a solution of compound **3** in THF was added the solution of BH_3 (in THF) at -20^0C, the reaction reached spontaneously room temperature and was stirred for 24 hours. At 0^0C was added water (50 ml) and after 10 min K_2CO_3 (1 g) and extracted with Et_2O (3x50ml), the combined organic layers were dried over Na_2SO_4 and evaporated under reduced pressure to yield an already pure compound **4** (920 mg, 2.9 mmol, 81%).

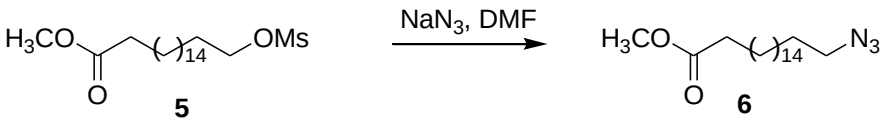
4.2.4 Synthesis of methyl 18-(methylsulfonyloxy)-octadecanoate



Compound	M.W.	D(g/ml)	eq.	mmol	mg	ml
4	**314.50**		**1**	**3**	**920**	
MsCl	**114.55**	**1.480**	**1.4**	**4.2**	**483**	**0.326**
TEA	**101.19**	**0.726**	**2**	**6**	**607**	**0.836**
DCM						**15**

Compound **4** was dissolved in DCM, Et_3N was added and at 0^0C MsCl. After 30 min at 0^0C the reaction reached spontaneously room temperature and was stirred for 4 hours. The reaction mixture was diluted in DCM (50 ml) and washed with water (3x50 ml). The organic layer was dried over Na_2SO_4 and evaporated under reduced pressure, obtaining compound **5** (1.09 g, 2.8 mmol, 93%).

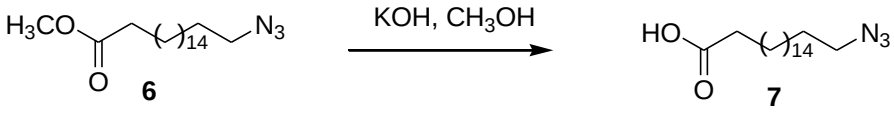
4.2.5 Synthesis of methyl 18-azidooctadecanoate



Compound	M.W.	D(g/ml)	eq.	mmol	mg	ml
6	**392.59**		**1**	**4.8**	**1900**	
NaN_3	**65.01**		**3.1**	**15**	**975**	
DMF						**15**

Compound **5** was dissolved in DMF and at 45^0C, NaN_3 was added and the obtained solution stirred overnight. The reaction mixture at RT was diluted with DCM (50 ml) and washed with water (3x50ml). The organic layer was dried over Na_2SO_4 and evaporated under reduced pressure using as azeotrope a mixture of toluene:eptane 7:3 yielding compound **6** (940 mg, 2.8 mmol, 58%.)

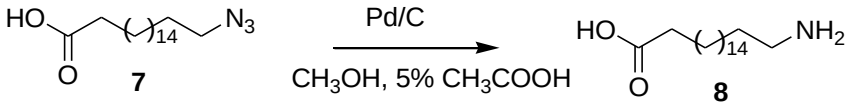
4.2.6 Synthesis of methyl 18-azidooctadecanoic acid



Compound	M.W.	D(g/ml)	eq.	mmol	mg	ml
6	**339.52**		**1**	**2.8**	**940**	
KOH (sol in MeOH 0.5 M, 1% H_2O						**45**

Compound **6** was dissolved in the solution of KOH and stirred over the weekend. A solution of aqueous HCl (1 M) was added until reaching of the acidic pH, CH_3OH was evaporated under reduced pressure and the mixture extracted with Et_2O (3x50 ml), the combined organic layers were dried over Na_2SO_4 and concentrated obtaining compound **7** (662 mg, 2 mmol, 71%).

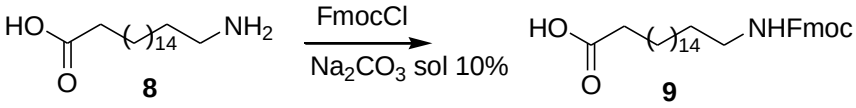
4.2.7 Synthesis of 18-aminooctadecanoic acid



Compound	M.W.	D(g/ml)	eq.	mmol	mg	ml
7	**325.49**		**1**	**1**	**330**	
Pd/C					**6**	
CH_3OH/CH_3COOH						**5**

Compound **7** was dissolved in the solution of CH_3OH 5% CH_3COOH and a catalytic amount of palladium on charcoal was added in argon atmosphere, the mixture was hydrogenated at atmospheric pressure for 20h, the mixture was filtered through a MILLIPORE filter and the catalyst washed with the solution of CH_3OH 5% CH_3COOH, the solvent was evaporated to dryness giving compound **8** (245 mg, 1.8 mmol, 80%).

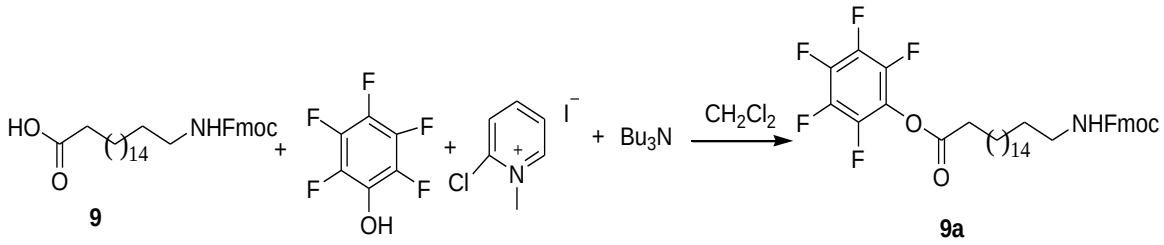
4.2.8 Synthesis of N-Fmoc-18-aminooctadecanoic acid



Compound	M.W.	D(g/ml)	eq.	mmol	mg	ml
8	**299.49**		**1**	**0.70**	**215**	
FmocCl			**1.1**	**0.78**	**200**	
THF						**22**
Na_2CO_3 sol. 10%						**27**

Compound **8** was suspended in the solution of Na_2CO_3 and the solution of FmocCl in THF was added. The reaction mixture was stirred for 5 hours, acidified with HCl 6N and extracted with Et_2O (3x50 ml) and with $CHCl_3$ (3x50 ml), the combined organic layers were dried over Na_2SO_4 and evaporated under reduced pressure. The product was purified by flash chromatography Esane:EtOAc 6:4 yielding compound **9** (146 mg, 0.28 mg, 40%).

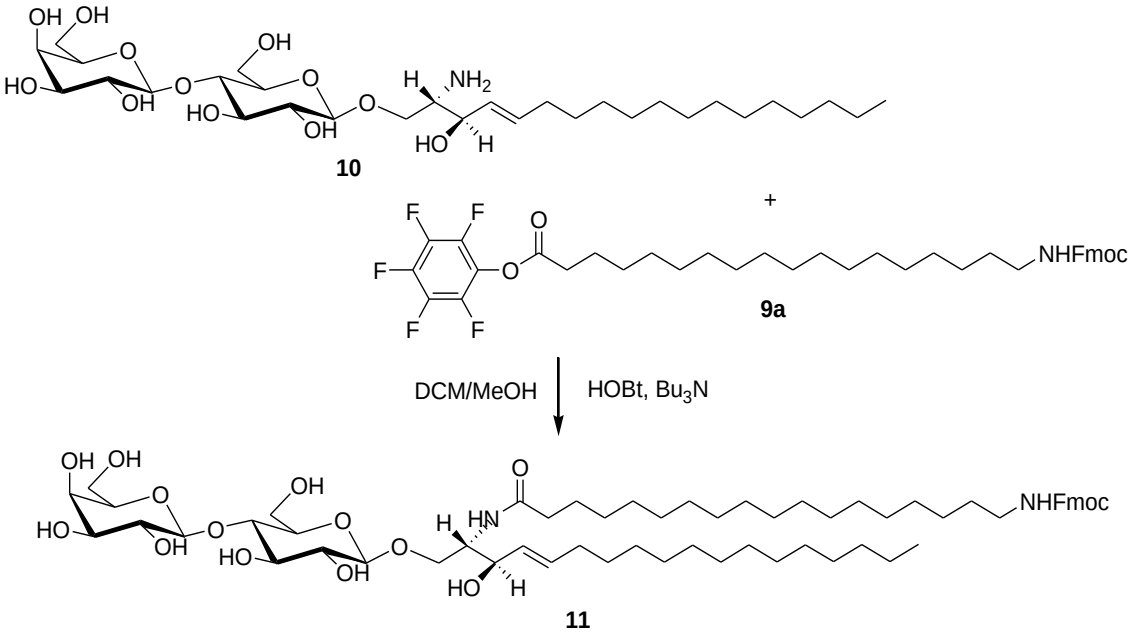
4.2.9 Synthesis of perfluorophenyl N-Fmoc-18-aminooctadecanoate



Compound	M.W.	D(g/ml)	eq.	mmol	mg	ml
9	**521.73**		**1**	**0.15**	**80**	
PFP	**183.99**		**1**	**0.15**	**28**	
2-chloro-1-methylpyridinium iodide	**255.49**		**1.2**	**0.18**	**46**	
Bu_3N	**185.21**	**0.778**	**2.4**	**0.36**	**67**	**0.086**
CH_2Cl_2						**2**

2-chloro-1-methylpyridinium iodide, fluorenyl protected 18-aminododecanoic acid and dry tributylamine were dissolved in dry DCM. The reaction mixture was refluxed for 3 h under continuous stirring, then cooled and evaporated under vacuum. The product was purified by flash chromatography with Esane:EtOAc 5:1 obtaining compound **10** (82 mg, 0.12 mmol, 80%).

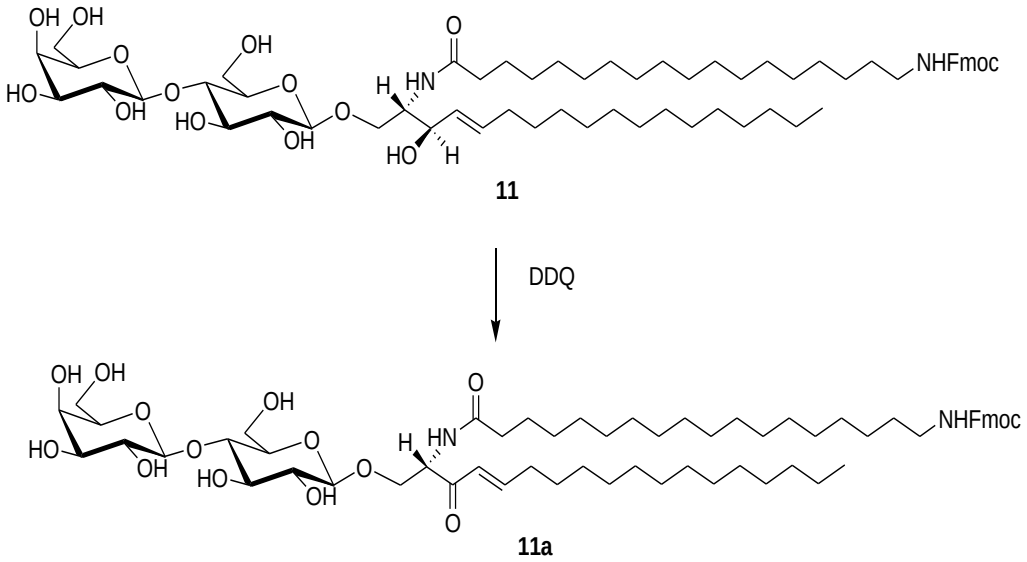
4.2.10 N-acylation of lactosylsphingosine with N-Fmoc 18-aminooctadecanoic acid



Compound	M.W.	D(g/ml)	eq.	mmol	mg	ml
9	**687.33**		**1**	**0.11**	**80**	
10	**623.39**		**1.2**	**0.14**	**84**	
HOBt	**135.04**		**1.2**	**0.14**	**19**	
Bu_3N	**185.21**	**0.778**	**2.4**	**0.29**	**53**	**0.07**
MeOH						**1**
DCM						**1**

To compound **9a**, dissolved in DCM, were added compound **10** and HOBt, dissolved in MeOH, and Bu_3N. After stirring at room temperature overnight, the solvents were evaporated and the residue was purified by column chromatography with $CHCl_3$:MeOH:NH_3 (2N), 60:25:4 to yield 100 mg of compound **11** (0.088 mmol, 80%).

4.2.11 Oxydation of the position 3 of sphingosine

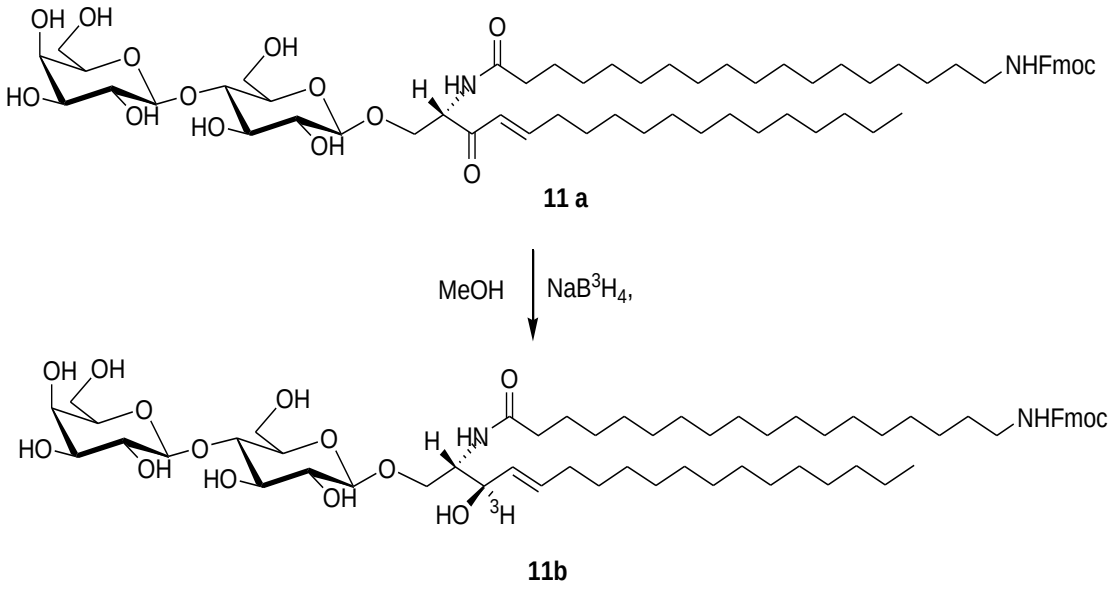


Compound	M.W.	D(g/ml)	eq.	mmol	mg	ml
11	**1141.75**			**0.018**	**10**	
DDQ in dioxane						**20**
Triton X-100						**10**

A solution of compound **11** in chloroform/methanol 2:1 (by vol.) (10 ml) was mixed with 10 ml of Triton X-100 solution (60 mg/ml) in the same solvent. The solvent was evaporated (at 37^0C) to dryness under vacuum. The residue was carefully dissolved in solution of DDQ (36 mg/ml). The mixture was allow to react at 37^0C for 40 hours under continuous stirring in a screw-capped tube; the contents were then evaporated (at 37^0C) to dryness under vacuum. The dark brown residue was suspended in acetone (10 ml) (in which the compound is not soluble), briefly (15-20 sec) sonicated in an ultrasonic bath; centrifugated at 300 rpm for 10 min, and the supernatant, containing Triton X-100 and DDQ, was

discarded. This treatment was repeated until a clear precipitate was obtained. The oxidized compound (**11a**), contained in the final precipitate, was further purified by chromatography with CH_2Cl_2:MeOH:H_2O 60:35:7.

4.2.12 Tritiation with NaB^3H_4

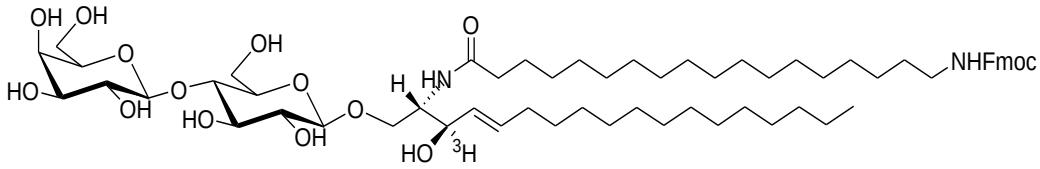


Compound	M.W.	D(g/ml)	eq.	mmol	mg	ml
11a	**1139.74**				**2 mg**	
NaB^3H_4					**1 mg**	
MeOH						**3**

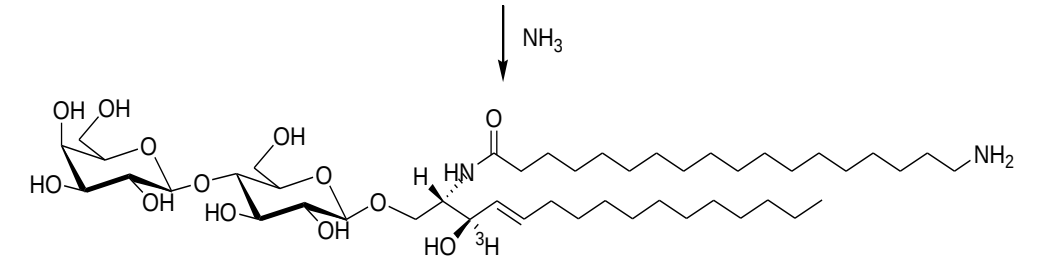
Compound **11a** was dissolved in MeOH and a 0.1 M NaOH was added until reaching pH 7.5, 100 mCi NaB^3H_4 was added too and the reaction was allowed to proceed at room temperature for three days. The reaction mixture was dried and

the tritiated derivative was purified by flash chromatography with CH_2Cl_2:MeOH:NH_3 (2N), 60:25:4.

4.2.13 Fmoc deprotection



11b

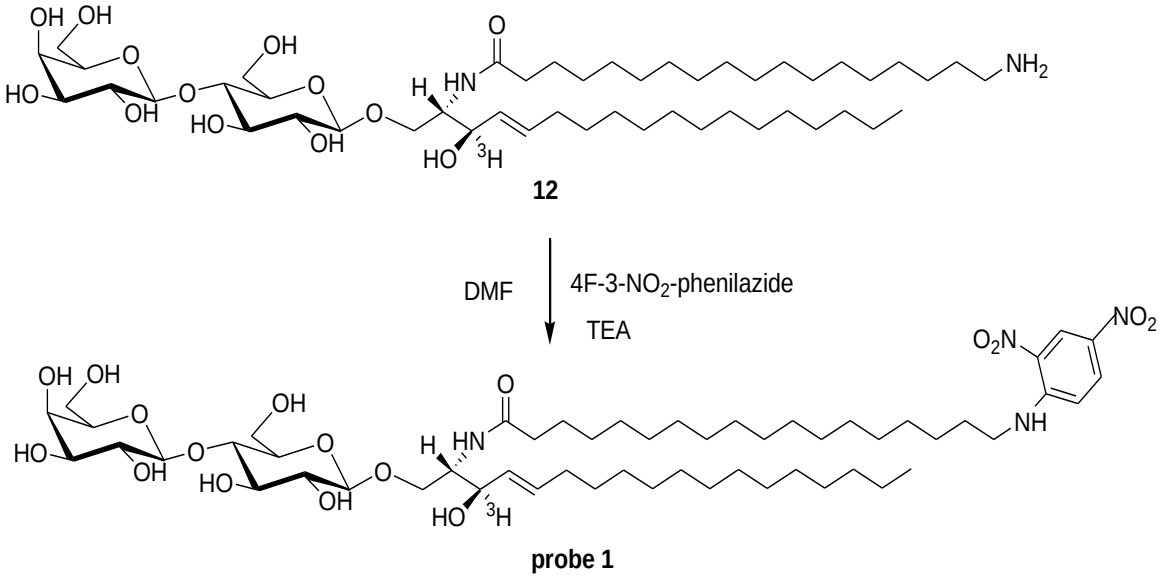


12

Compound	M.W.	D(g/ml)	eq.	mmol	mg	ml
11b	**1143.76**			**0.017**	**2**	
NH_3 (32% sol)						**2**

Compound **11b** was dissolved in 32% aqueous ammonia in a screw-cap flask and the reaction was left under vigorous stirring for 24 h at room temperature. The solvent was evaporated and the residue was purified by flash chromatography with CH_2Cl_2:MeOH:H_2O, 60:35:4.

4.2.14 Introduction of photoactivable group



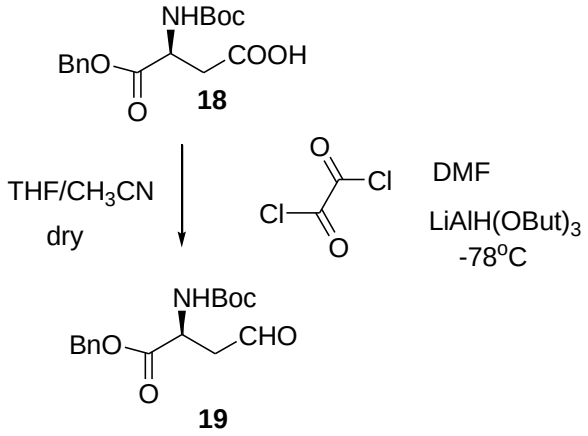
12

probe 1

The radioactive compound **12** was dissolved in dry DMF (10μmoles/ml). An equimolar quantity of triethylamine and a two-fold molar quantity of 4-F-3-NO_2-phenylazide, dissolved in ethanol (25 μmoles of triethylamine/ml), was added, and the mixture was stirred overnight at 80^0C. The reaction mixture was dried and the radioactive and photoactivable probe was purified by chromatography with CH_2Cl_2:MeOH:H_2O, 30:50:13. Fractions containing the homogeneous product were dried and the residue immediately solubilized in methanol and stored at 4^0C.

4.3 Synthesis of probe 2

4.3.1 Synthesis of aldehyde 19



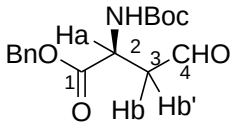
Compound	M.W.	D(g/ml)	eq.	mmol	mg	ml
18	**323.14**		**1.0**	**6.20**	**2000**	
Oxalyl chloride	**123.93**	**1.33**	**3.8**	**23.6**	**3000**	**1.1**
DMF	**73.05**	**0.944**	**1.3**	**8**	**588**	**0.624**
pyridine	**79.04**	**0.982**	**1.2**	**7.4**	**588**	**0.598**
THF						**40**
DCM						**40**
CH_3CN						**12**
LiAlH(t-OBu)$_3$			**1.1**	**6.2**		**6.8**

To a mixture of DCM and DMF, freshly distilled oxalyl chloride was added slowly at 0^0C. A white solid formed, part of which was slowly dissolved in the mixture, which, at the end of the addition, appeared white opalescent. The reaction mixture was stirred at 0^0C for 1 hour, DCM was evaporated and the resulting white solid was suspended in THF and CH_3CN.

1-Benzyl-Boc-L-aspartaat was dissolved in THF and pyridine was added. The obtained solution was added slowly by pipe at -30^{0}C to the mixture of formed white solid and was stirred for 1 hour.

The mixture was cooled to -78^{0}C and $LiAlH(OBu)_3$ (sol 1M in THF) was added slowly by syringe. When the reaction was complete it was quenched by addition of a HCl 1N solution (20 ml) and the product was extracted with EtOAc (3x20ml). The combined organic layers were washed with a saturated $NaHCO_3$ solution, dried over Na_2SO_4 and evaporated under reduced pressure.

The residue was purified by flash chromatography using as eluent a mixture of Esane:EtOAc 7:3, 1% Et_3N, yielding compound **2** (1124 mg, 3.66 mmol, 60%) as an colourless oil with 60% yield.

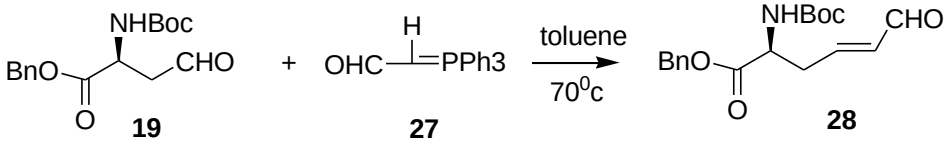


^{1}H NMR (500.13 MHz, $CDCl_3$, ppm) 1.45 (s, 9H, -tBu); 3.02-3.15 (ddd, 2H, Hb, Hb'); 4.72-4.73 (m, 1H, Ha);5.20 (s, 2H, CH_2Ph) 5.43-5.44 (d, 1H, -NH) 7.34-7-40 (m, 5H, benzene) 9.74 (s, 1H, -CHO).

^{13}C NMR (125.76 MHz, $CDCl_3$, ppm) 28.2 (3C t-Bu), 45.9 (1C, C3), 48.8 (1C, C2), 67.6 (2C,-CH_2Ph), 80.2 (1C, quaternary C, Boc), 128.3-128.6 (4C, arom), 135.1 (1C, quaternary arom), 155.3 (1C, C1), 170.8 (1C, -CONH), 199.3 (1C, CHO)

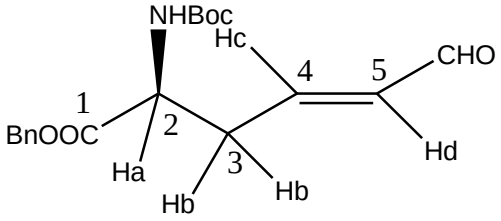
α_D ($CHCl_3$; C=1): +15.5

4.3.2 Synthesis of aldehyde 28



Compound	M.W.	eq.	mmol	mg	ml
19	**307.34**	**1**	**3.02**	**928**	
20	**304.32**	**1.5**	**4.53**	**1370**	
toluene					**30**

To compound **19** (obtained carrying out twice the first reaction), kept under argon and dissolved in toluene, was added triphenylphosphoranylidene and the orange reaction mixture was warmed to 70^0C and stirred for 3.5 h, the mixture was cooled to room temperature, the solvent was removed under reduced pressure and the crude purified by flash chromatography (petroleum ether/EtOAc, 8:2, 1% Et_3N) afforded pure **28** (750 mg, 2,16 mmol, 75%) as a pale yellow oil.

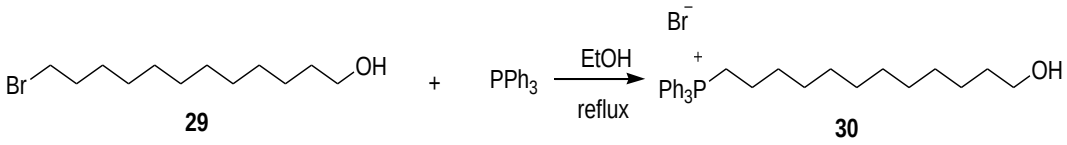


^{1}H NMR (500.13 MHz, $CDCl_3$, ppm) 1.45 (s, 9H, -tBu); 2.69-2.93 (m, 2H, 2Hb); 4.54 (1H, Ha), 5.15-5.30 (m, 3H, benzyl CH_2, NH), 6.06-6.11 (m, 1H, Hd), 6.63-6.70 (m, 1H, Hc), 7.35-7.39 (m, 5H, arom.), 9.42-9.43 (d, 1H, CHO)

^{13}C NMR (125.76 MHz, $CDCl_3$, ppm) 28.3 (3C, t-Bu); 36 (1C, C3); 52.4 (1C, C2); 67.6 (2C, benzyl); 136 (1C, C5); 151.1 (1C, C4); , 155 (1C, carbonyl carbamate); 171 (1C, carbonyl C1); 193.2 (1C, carbonyl, CHO).

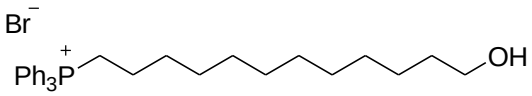
α_D ($CHCl_3$; C=1): +10

4.3.3 Synthesis of phosphonium salt 30



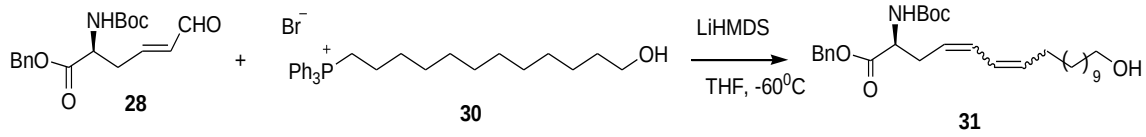
Compound	M.W.	eq.	mmol	mg	ml
21	**265.24**	**1**	**7.5**	**2000**	
PPh_3	**262.09**	**1.3**	**9.8**	**1000**	
ethanol					**20**

Compound **29** was dissolved in EtOH and PPh_3 was added, the reaction mixture was heated to reflux and stirred for 48 h. The solvent was removed under reduced pressure and the obtained phosphonium salt was purified by flash chromatography (CH_2Cl_2:MeOH, 10:1) yielding a colourless oil, which by adding and evaporation of THF becomes a white solid (3.6g, 6.8mmol, 90%).



^{1}H NMR (500.13 MHz, $CDCl_3$, ppm) 1.19-1.62 (m, 20H, alkyl chain); 2.55-2.62 (m, 2H, C11) 3.61-3.64 (t, 2H, CH_2OH); 3.57-3.73 (broad m, 1H, OH); 7.76-7.85 (m, 15H, arom.)

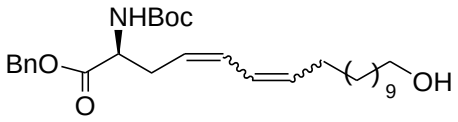
4.3.4 Synthesis of diene 31



Compound	M.W.	eq.	mmol	mg	ml
28	**527.52**	**3**	**6.6**	**3481**	
30	**333.38**	**1**	**2.2**	**750**	
LiHMDS	**183.37**	**6**	**2.7**	**496**	**2.7**
THF					**12**

Phosphonium salt (white solid) was suspended in THF (12 ml) and LiHMDS was added slowly at -60^{0}C. The orange reaction mixture was stirred for 40', after that compound **28** was added by pipe within 30', at the end of the adding the reaction was left stirred for 1 h and then was quenched by adding of HCl 1N(20 ml). The product was extracted with EtOAc (3x40 ml), the combined organic layers were washed with saturated solution of $NaHCO_3$, dried over Na_2SO_4 and evaporated.

The crude compound was purified by flash chromatography with DCM:EtOAc, 9:1, 1% Et_3N, obtaining a pale yellow oil (738mg, 1.5mmol, 67%).



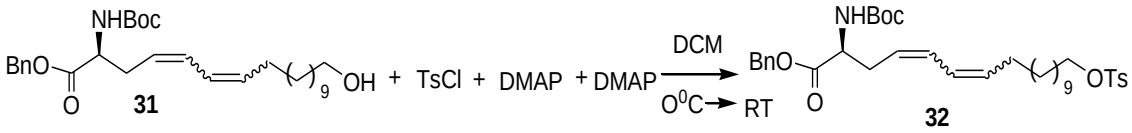
^{1}H NMR (500.13 MHz, $CDCl_3$, ppm) 1.26-1.39 (m, 18H, alkyl chain); 1.45 (s, 9H, t-Bu); 1.54-1.59 (m, 2H C-16); 2.03-2.16 (m, 2H, C17); 2.41-2.72 (m, 2H, C3); 3.62-3.64 (t, 2H, C18); 4.40-4.46 (m, 1H, OH); 5.19-6.39 (m, 6H, double bonds, benzyl CH_2) 7,34-7.39 (m, 5H, arom.)

^{13}C NMR (125.76 MHz, $CDCl_3$, ppm) 25.7 (3C, t-Bu); 27.7-29.6 (7C, alkyl chain); 32.8 (1C, C17); 35.8 (1C, C9); 43.8 (1C, C8); 45.7 (1C, C3); 53.3 (1C, C2); 62.9 (1C, C18); 67.0 (1C, quaternary arom); 80.0 (1C, quaternary t-Bu); 124-135 (10 C, 5 arom, 4 alkene); 155 (1C, carbonyl, carbamate); 172 (1C, carbonyl, ester).

Mol. Wt. = 501

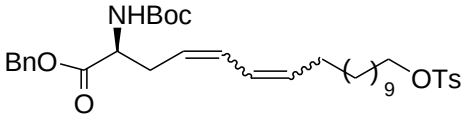
ESI-MS (positive-ion mode) : *m/z* = 524 $[M+Na]^+$

4.3.5 Synthesis of tosylate 32



Compound	M.W.	D(g/ml)	eq.	mmol	mg	ml
31	**501**		**1**	**1.4**	**700**	
TsCl	**190.65**		**2**	**2.8**	**533**	
DMAP	**122.17**		**1**	**1.4**	**171**	
DIPEA	**129.25**	**0.742**	**4**	**5.6**	**724**	**0.980**
DCM						**14**

Compound **31** was dissolved in DCM and freshly distilled DIPEA, ricrystallized TsCl and DMAP were added at 0^0C. The reaction was warmed spontaneously to room temperature. After 5 h the reaction was complete, water (10 ml) was added to reaction mixture, which was extracted with EtOAc (3x10 ml), the combined organic layers were dried over Na_2SO_4 and evaporated. The crude compound was purified by flash chromatography with Esane:EtOAc, 85:15, 1% Et_3N, to furnish 578 mg of compound **32** (63%).



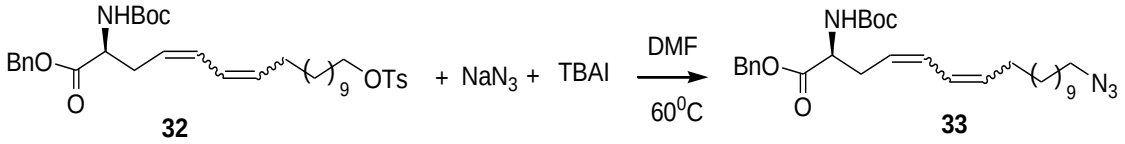
^{1}H NMR (500.13 MHz, $CDCl_3$, ppm) 1.23-1.37 (m, 16 H, alkyl chain); 1.45 (s, 9H, -tBu); 1.60-1.68 (m, 4H, C17, C9); 2.05-2.16 (m, 2H, C8); 2.47 (s, 3H, CH_3, Ts); 2.52-2.67 (m, 2H, C3); 4.02-4,05 (t, 2H, C18); 4.40-4.46 (broad m, 1H, C2);

5.04-6.44 (m, 6H, CH_2 benzyl, double bonds); 7.36-7.40 (m, 5H, arom); 7.81 (d, 4H, Ts)

Mol. Wt. =655

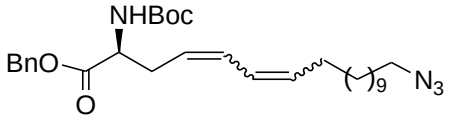
ESI-MS (positive-ion mode) : *m/z* = 678 $[M+Na]^+$

4.3.6 Synthesis of azide 33



Compound	M.W.	eq.	mmol	mg	ml
32	**655**	**1**	**0.85**	**560**	
NaN_3	**65.01**	**3**	**2.6**	**169**	
TBAI	**369.38**	**0.06**	**0.051**	**18**	
DMF					**18**

Tosylate was dissolved in DMF, NaN_3 and TBAI were added, the reaction mixture was warmed to 60^0C and stirred for two hours. The solution was diluted with EtOAc and washed with water, the organic layer was dried over Na_2SO_4 and evaporated, obtaining 500 mg of crude compound, which was purified by flash chromatography, Esane:EtOAc, 85:15, 1% Et_3N to furnish 411 mg of compound **33** (92%).

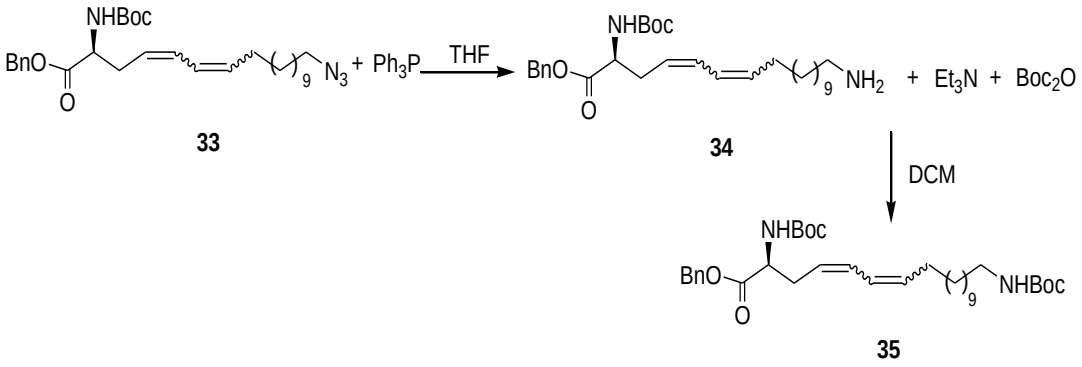


^{1}H NMR (500.13 MHz, $CDCl_3$, ppm) 1.29-1.38 (m, 18H, alkyl chain); 1.46 (s, 9H, t-Bu); 1.59-1.63 (m, 4H, C17, C9); 2.04-2,17 (m, m, 2H, C8); 2.52-2.63 (m, m, 2H, C3); 3.26-3.29 (t, 2H, C18); 4.41-4.46 (broad m, 1H, C2); 5.06-6.4 (m, 6H, CH_2 benzyl, double bonds); 7.35-7.40 (m, 5H, benzene).

Mol. Wt.= 526

ESI-MS (positive-ion mode) : *m/z* = 548.9 $[M+Na]^+$

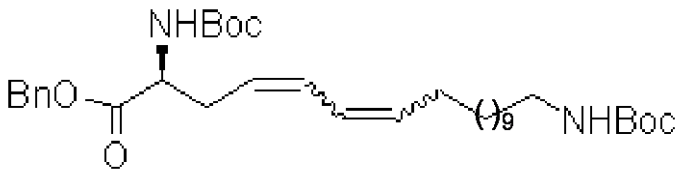
4.3.7 Synthesis of N,N'-Boc-2,18-di-amine 35



Compound	M.W.	D(g/ml)	eq.	mmol	mg	ml
33	**526.35**		**1**	**0.76**	**400**	
PPh_3	**262.09**		**2.4**	**1.8**	**478**	
THF						**13**
Et_3N	**101.12**	**0.727**	**4**	**3.0**	**307**	**0.42**
Boc_2O	**218**		**2.5**	**1.9**	**414**	
DCM						**13**

Compound **33** and PPh_3 were dissolved in THF and after 1 h water (1 ml) was added. The reaction mixture was left stirring overnight. The day after it was diluted with water and extracted with EtOAc (3x10 ml), the combined organic layers were dried over Na_2SO_4 and evaporated under reduced pressure.

The obtained crude compound was dissolved in dry DCM, and Et_3N was added, after 10 minutes stirring, Boc_2O, dissolved in DCM was added as well. The reaction was quenched with MeOH, diluted in water and extracted with EtOAc (3x10 ml). The combined organic layers were dried over Na_2SO_4 and evaporated under reduced pressure. The crude compound was purified by flash chromatography Esane:EtOAc, 85:15, 1% Et_3N to yield 296 mg of compound **35** (65% over two steps).

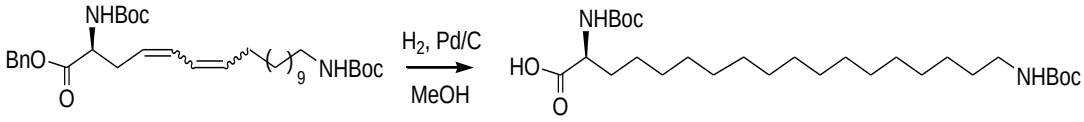


^{1}H NMR (500.13 MHz, $CDCl_3$, ppm) 1.28-1.38 (m, 18H, alkyl chain); 1.45 (s, 9H, t-Bu); 1.46 (s, 9H, t-Bu); 2.04-2.17 (m, 2H, C17); 2.54-2.65 (m, 2H, C3); 3.12-3.13 (t, 2H, C18); 4.44-4.59 (m, 1H, C2); 5.05-6.40 (m, 6H, benzyl CH_2, double bonds); 7.31-7.40 (m, 5H, arom.).

Mol. Wt.=601

ESI-MS (positive-ion mode) : *m/z* = 523.2 $[M+Na]^+$; 1223 $[2M+Na]^+$

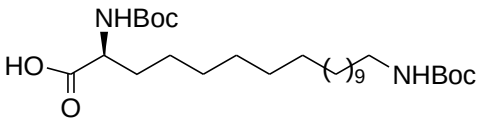
4.3.8 Synthesis of (S)-N,N-Boc-2,18-di-amino octadecanoic acid



35 **36**

Compound	M.W.	D(g/ml)	eq.	mmol	mg	ml
35	**601**		**1**	**0.42**	**250**	
Pd/C			**cat**			
MeOH						**11**

Compound **35** was dissolved in MeOH and a catalytic amount of palladium on charcoal was added in argon atmosphere. The mixture was hydrogenated at atmospheric pressure for 24h, then diluted with MeOH, filtered through a MILLIPORE filter, the solvent evaporated to dryness giving compund **36** (183mg, 85%), which was purified by column chromatography with DCM:MeOH 9:1.



^{1}H NMR (500.13 MHz, $CDCl_3$:MeOD, ppm) 1.26-1.29 (m, 26H, alkyl chain); 1.43 (s, 9H, t-Bu); 1.44 (s, 9H, t-Bu); 1.55-1.80 (m, 4H, C17, C3); 3.02-3.05 (t, 2H, C18); 3.98-4.10 (broad m, 1H, C2).

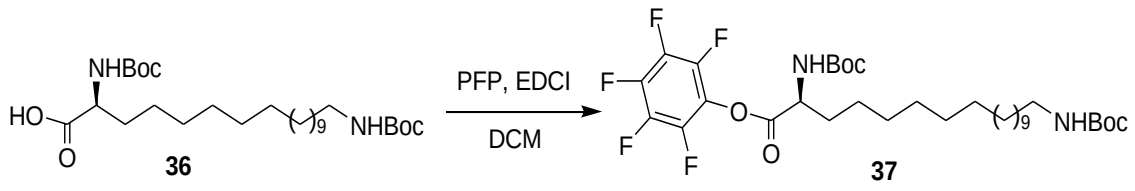
^{13}C NMR (125.76 MHz, $CDCl_3$:MeOD 1:1, ppm) 25.5-29.7 (14 C. Alkyl chain); 32.7 (1C, C3); 40.3 (1H, C18); 55.2 (1C, C2); 156 (1C; carbonyl carbamate); 157 (1C, carbonyl carbamate); 179 (1C; C1).

Mol. Wt.=514

ESI-MS (negative-ion mode) : *m/z* = 513 $[M-H]^-$; 1027 $[2M-H]^-$

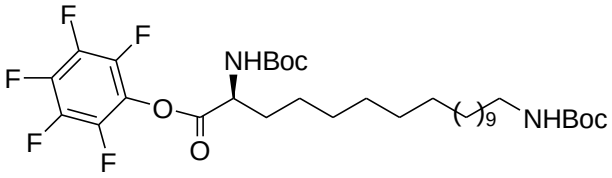
α_D ($CHCl_3$:MeOH 1:1; c=0.5)=+4.6

4.3.9 Activation of (S)-N,N-Boc-2,18-di-amino octadecanoic acid



Compound	M.W.	D(g/ml)	eq.	mmol	mg	ml
36	**514**		**1**	**0.12**	**63**	
EDCI	**192**		**1.5**	**0.18**	**35**	
PFP	**185**		**1.5**	**0.18**	**33**	
DCM						**6**

Compound **36** and EDCI were dissolved in dry DCM (5 ml) and pentafluorophenol, dissolved in dry DCM (1 ml) was added. The reaction was stirred at room temperature overnight. The reaction mixture was diluted in EtOAc and washed with water, the organic layer was washed with EtOAc (3x 10 ml), the combined organic layers were dried over Na_2SO_4 and evaporated under vacuum. The residue was purified by flash chromatography with Esane: Ethylacetate 8:2. 62 mg of compound **37** were obtained (76%).



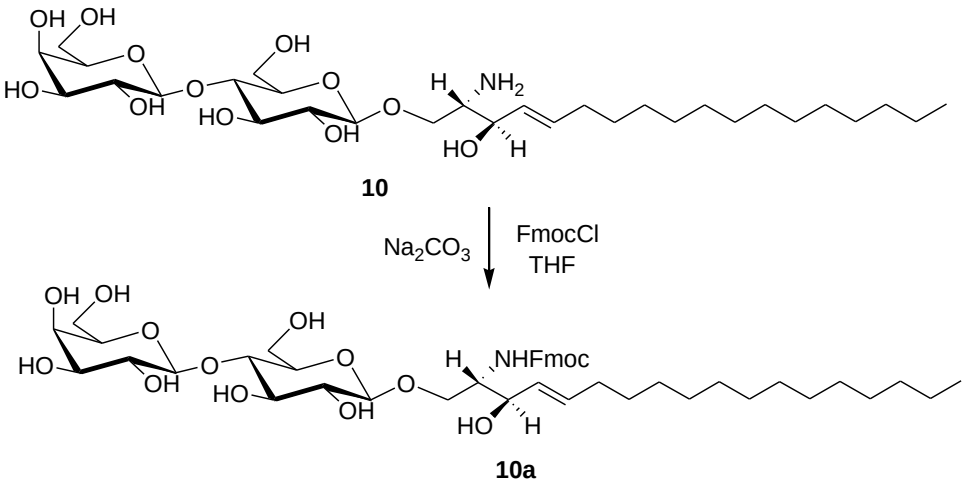
^{1}H NMR (500.13 MHz, $CDCl_3$:MeOD, ppm) 1.28-1.31 (m, 26H, alkyl chain); 1.46 (s, 9H, t-Bu); 1.49 (s, 9H, t-Bu); 1.80-2.02 (m, 2H; C3); 3.12-3.14 (t, 2H; C18); 4.50-4.54 (broad m, 1H, C2);

^{13}C NMR (125.76 MHz, $CDCl_3$:MeOD 1:1, ppm)) 25.1-30.0 (14 C. Alkyl chain); 32.3 (1C, C3); 40.6 (1H, C18); 53.5 (1C, C2); 136-142 (6C, benzene); 155 (1C; carbonyl carbamate); 156 (1C, carbonyl carbamate); 169 (1C; C1).

Mol. Wt.=680

ESI-MS (positive-ion mode) : *m/z* = 703.3 $[M+Na]^+$; 1383 $[2M+Na]^+$

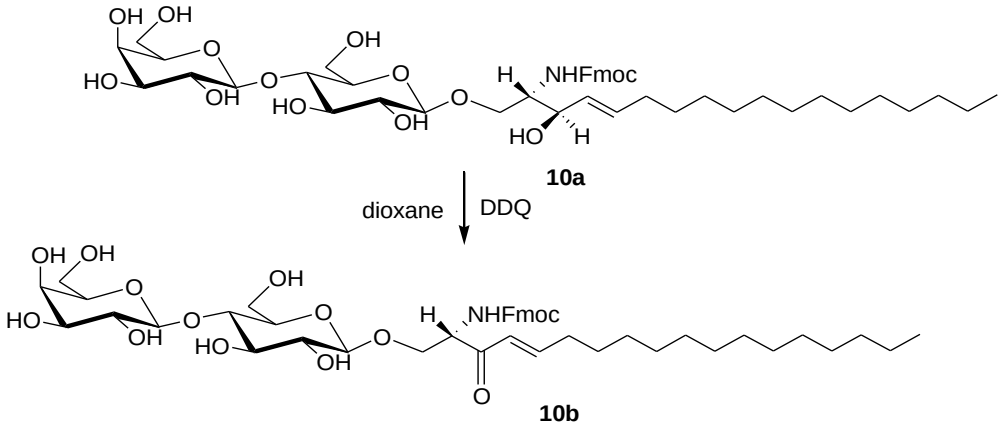
4.3.10 Fmoc-protection of lactosylsphingosine



Compound	M.W.	D(g/ml)	eq.	mmol	mg	ml
10	**624**		**1**	**0.064**	**40**	
FmocCl	**258**		**1.1**	**0.071**	**18**	
THF						**2**
Na_2CO_3 10% sol						**2**

FmocCl, dissolved in THF, was added to the suspension of compound **10** in the solution of Na_2CO_3 at 0^0C. The reaction mixture was left to reach spontaneously room temperature and was stirred overnight. The layers were centrifugated at 3000 rpm and separated, the organic layer was evaporated and the residue purified by gradient flash chromatography starting from $CHCl_3$:MeOH 9:1 to $CHCl_3$:MeOH 7:3 and $CHCl_3$:MeOH 1:1 obtaining 50 mg of compound **10a** in almost quantitative yield.

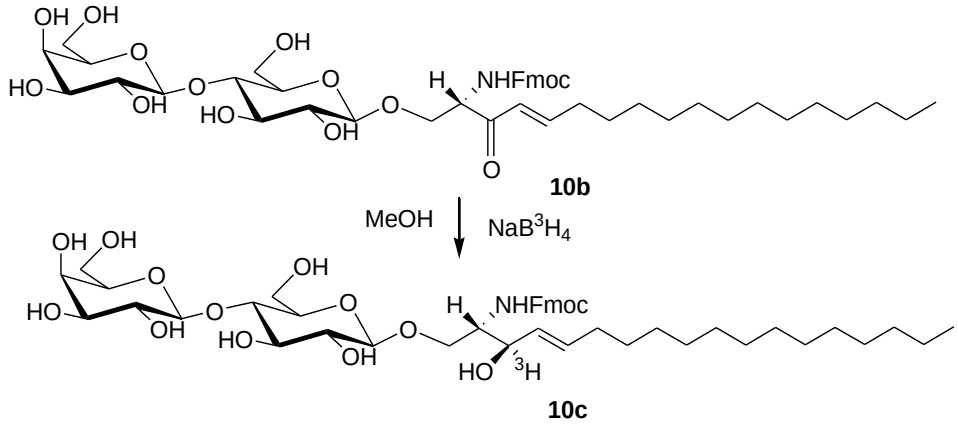
4.3.11 Oxydation of the position 3 of sphingosine



Compound	M.W.	D(g/ml)	eq.	mmol	mg	ml
10a	**846**		**1**	**0.059**	**50**	
DDQ in dioxane (3g in 100ml)	**227**					**50**

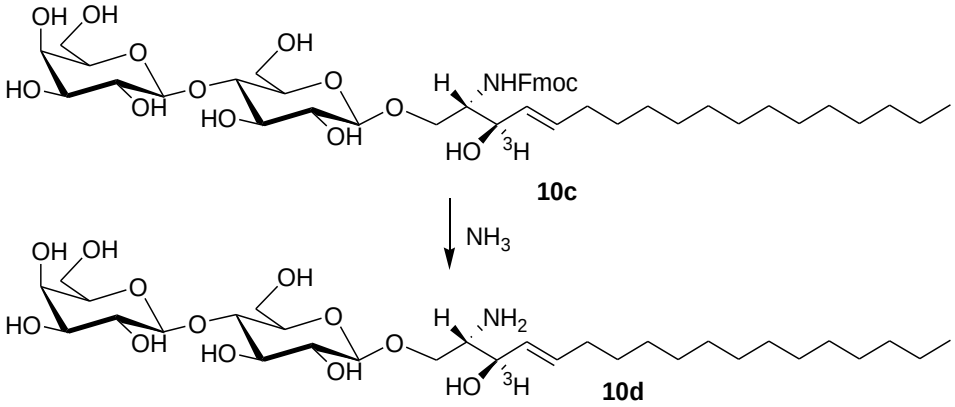
Compound **10a** was suspended in the solution of DDQ and left under vigorous stirring at 50^0C overnight. The day after the heating was stopped and the reaction mixture evaporated. The residue was purified by flash chromatography, the column was equilibrated with acetone and the first fractions were eluted with acetone as well, then was used a mixture of $CHCl_3$:MeOH 47:3 but was necessary a further purification with $CHCl_3$:MeOH:2-Propanol:H_2O 70:5:25:3. 25 mg of compound **10b** were obtained. Unfortunately yield was only 50% but more than enough for the next steps.

4.3.12 Tritiation of the position 3 of sphingosine



Compound **10c** (2mg) was dissolved in MeOH and a 0.1 M NaOH until reaching pH 7.5, 100 mCi NaB^3H_4 was added and the reaction was allowed to proceed at room temperature for three days. The reaction mixture was evaporated and the tritiated derivative was purified by flash chromatography with CH_2Cl_2:MeOH:2-propanol:H_2O, 70:5:25:3.

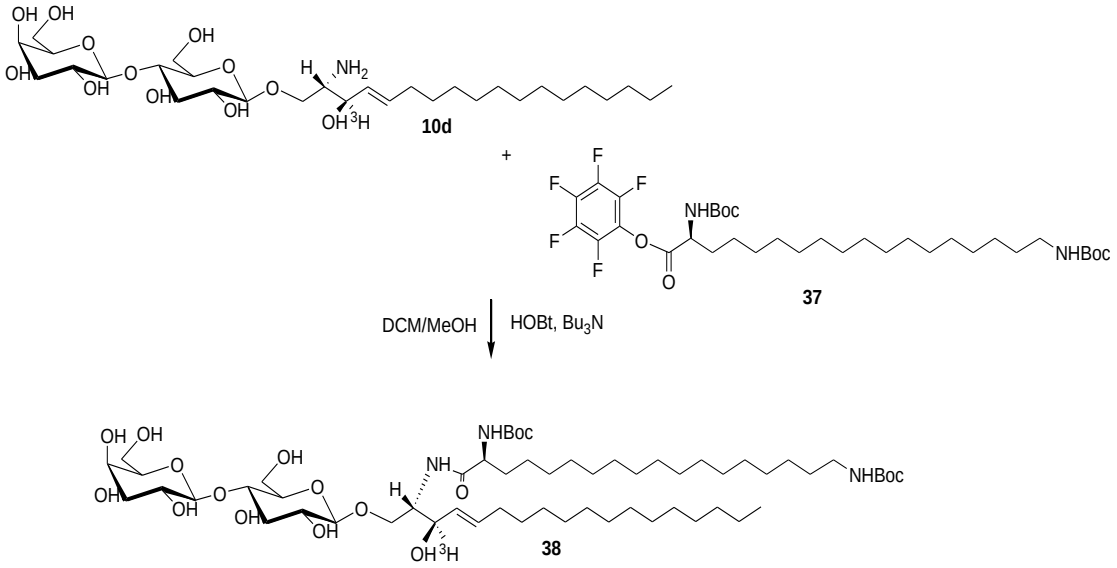
4.3.13 Fmoc-deprotection



Compound	M.W.	D(g/ml)	eq.	mmol	mg	ml
10c	**848**			**0.017**	**4**	
NH_3 (32% sol)						**2**

Compound **10c** was dissolved in 32% aqueous ammonia in a screw-cap flask and the reaction was left under vigorous stirring for 24 h at room temperature. The reaction mixture was evaporated.

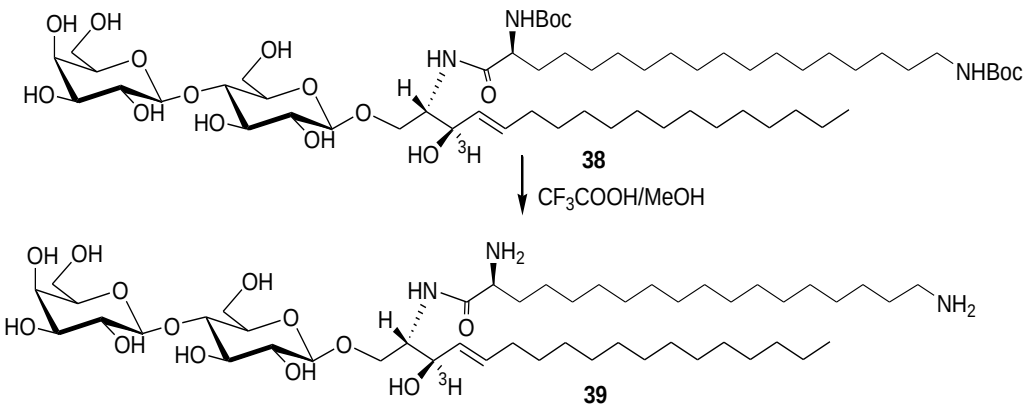
4.3.14 N-acylation of lactosylsphingosine with N,N'-Boc-2,18- di-amino octadecanoic acid



Compound	M.W.	D(g/ml)	eq.	mmol	mg	ml
10d	**626**		**1**	**0.0064**	**4**	
37	**665**		**2**	**0.012**	**8**	
HOBt	**135.04**		**1.2**	**0.0077**	**1**	
Bu_3N	**185.21**	**0.778**	**2.4**	**0.015**	**2.8**	**0.004**
MeOH						**1**
DCM						**1**

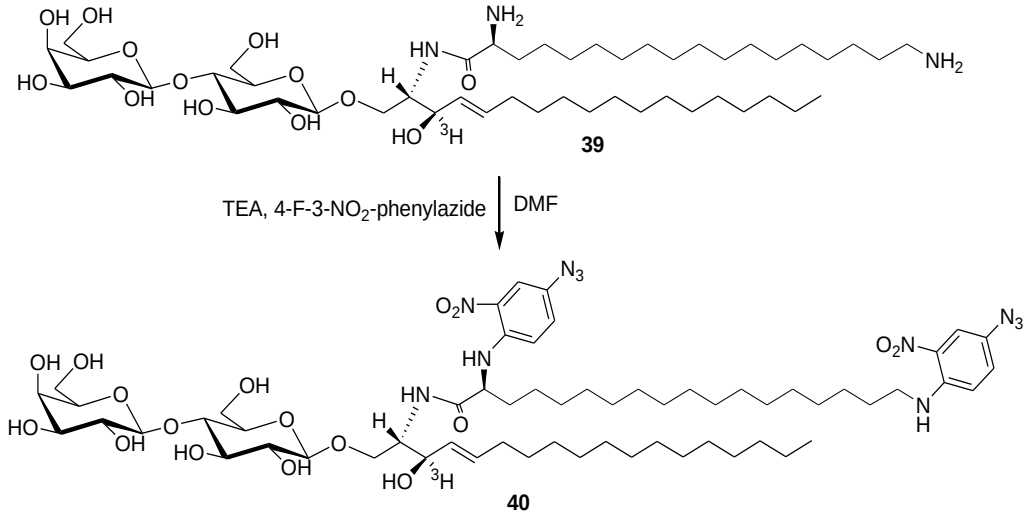
Compound **10d** was dissolved in DCM and compound **37**, HOBt, dissolved in MeOH, and Bu_3N were added. After stirring at room temperature overnight, the reaction mixture was evaporated and the residue was purified by column chromatography with CH_2Cl_2:MeOH:NH_3 (2N), 60:25:4. The obtained compound was immediately dissolved in MeOH.

4.3.15 Boc-deprotection



The solution obtained from the previous reaction was evaporated and a solution of CF_3COOH in MeOH 50% v/v (2 ml) was added at room temperature. The reaction was stirred for 2 h. The reaction mixture was evaporated and the residue was purified with CH_2Cl_2:MeOH:NH_3 (2N), 60:35:5.

4.3.16 Introduction of photoactivable group



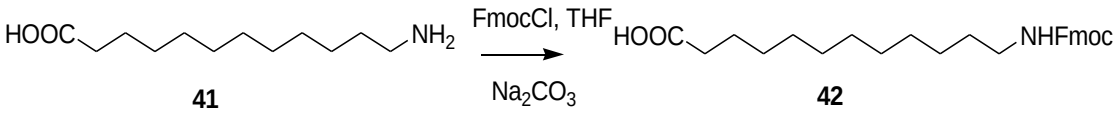
39

40

The radioactive compound **39** was dissolved in dry DMF (10μmoles/ml). An equimolar quantity of triethylamine and a two-fold molar quantity of 4-F-3-NO_2-phenylazide, dissolved in ethanol (25 μmoles of triethylamine/ml), was added, and the mixture was stirred overnight at 80^0C. The reaction mixture was evaporated and the radioactive and photoactivable probe was purified by chromatography with CH_2Cl_2:MeOH:H_2O, 30:50:13. Fractions containing the homogeneous product were dried and the residue immediately solubilized in methanol and stored at 4^0C.

4.4 Synthesis of probe 3

4.4.1 Synthesis of N-Fmoc-12-amino-dodecanoic acid

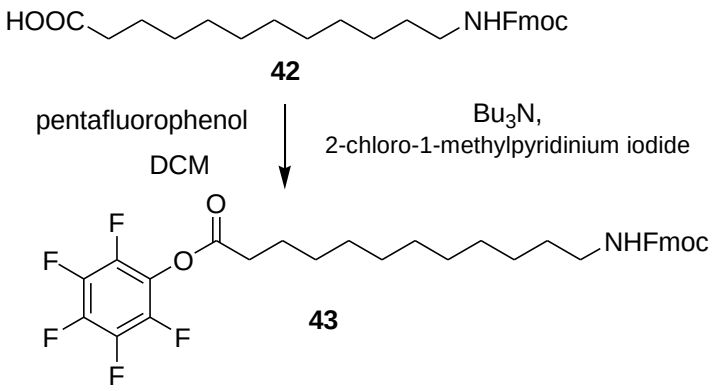


41 **42**

Compound	M.W.	D(g/ml)	eq.	mmol	mg	ml
41	**215.18**		**1**	**0.11**	**24**	
FmocCl	**258.04**		**1**	**0.11**	**29**	
Na_2CO_3 sol 10%						**2.4**
THF						**3**

A solution of 9-fluorenylmethylchloroformate in THF was added under vigorous magnetic stirring to an equimolar quantity of 12 aminododecanoic acid in 10% aqueous Na_2CO_3. The mixture was stirred for a further 30 min at room temperature and then dried to eliminate THF. The water layer was acidified and extracted with $CHCl_3$ (3x10 ml), the combined organic layers were dried over Na_2SO_4 and evaporated under vacuum. The residue was purified by flash chromatography with $CHCl_3$:MeOH, 98:2. 43 mg of compound **42** were obtained (90%).

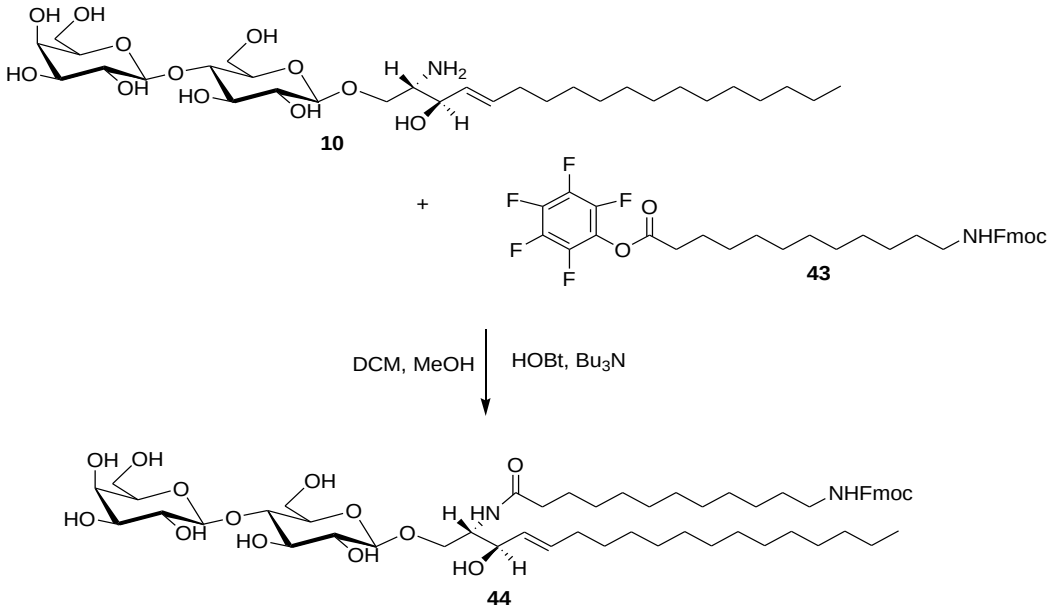
4.4.2 Synthesis of activated N-Fmoc-12-amino-dodecanoic acid



Compound	M.W.	D(g/ml)	eq.	mmol	mg	ml
42	**437.26**		**1**	**0.10**	**45**	
pentafluorophenol	**184**		**1**	**0.10**	**19**	
2-chloro-1-methylpyridinium iodide	**255.49**		**1.2**	**0.12**	**31**	
Bu_3N	**185.21**	**0.778**	**2.4**	**0.24**	**44**	**0.057**
DCM						**5**

2-Chloro-1-methyl piridinium iodide, pentafluorophenol, fluorenyl protected 12-aminododecanoic acid and dry tributylamine were solubilized in dry dichloromethane and heated to reflux. The reaction mixture was refluxed for 3 h under continuous stirring, then cooled and evaporated under vacuum. The product was purified by column chromatography with Esane:EtOAc 8:2.The yield was about 90%.

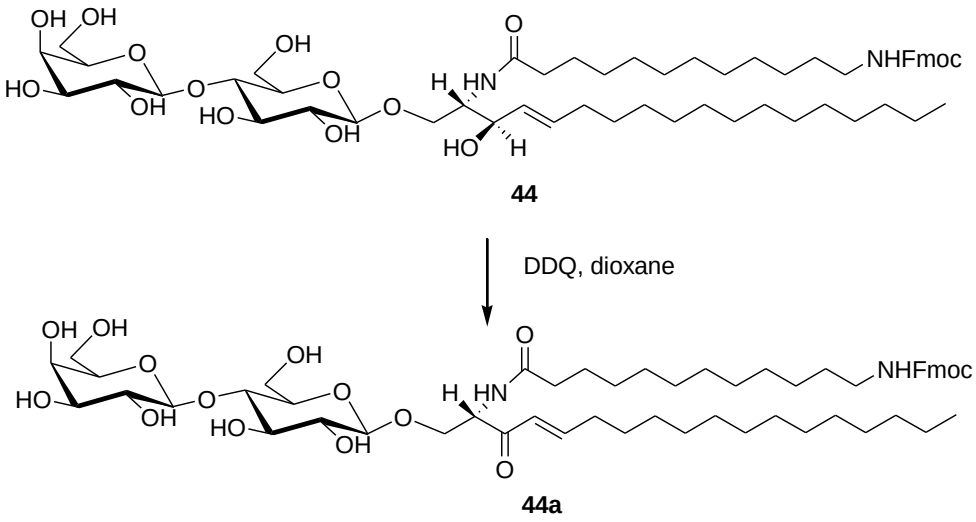
4.4.3 N-acylation of lactosylsphingosine with 12 amino-dodecanoic acid



Compound	M.W.	D(g/ml)	eq.	mmol	mg	ml
43	**603.24**		**1**	**0.016**	**10**	
10	**623.39**		**1.2**	**0.019**	**12**	
HOBt	**135.04**		**1.2**	**0.019**	**2.6**	
Bu_3N	**185.21**	**0.778**	**2.4**	**0.038**	**7**	**0.009**
MeOH						**1**
DCM						**0.5**

N-fluorenylmethylformate-aminolauric acid pentafluorophenylester, 1-hydroxybenzotryazole and tributylamine, dissolved in DCM, were added to a solution of lactosylsphingosine in MeOH. The reaction mixture was stirred for 75 min at room temperature, then was dried and the residue was purified by flash chromatography with $CHCl_3$:MeOH:H_2O, 60:25:4. 13 mg of compound **44** were obtained (80%).

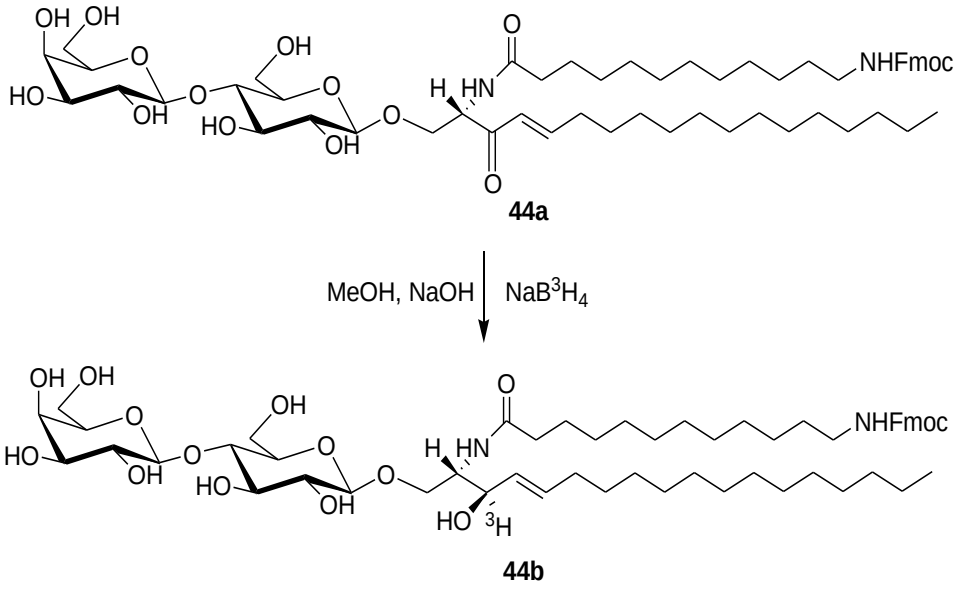
4.4.4 Oxidation of the position 3 of sphingosine



Compound	M.W.	D(g/ml)	eq.	mmol	mg	ml
44	**1042.63**			**0.012**	**13**	
DDQ in dioxane (3g in 100 ml)	**225.93**					**10**

Compound **44** was suspended in the solution of DDQ and left under vigorous stirring at 50^0C overnight. The day after the heating was stopped and the reaction mixture evaporated. The residue was purified by flash chromatography, the column was equilibrated with acetone and the first fractions were eluted with acetone as well, then was used a mixture of $CHCl_3$:MeOH 47:3 but was necessary a further purification with $CHCl_3$:MeOH:2-Propanol:H_2O, 70:5:25:3. 10 mg of compound **44a** were obtained (80%).

4.4.5 Tritiation with NaB^3H_4

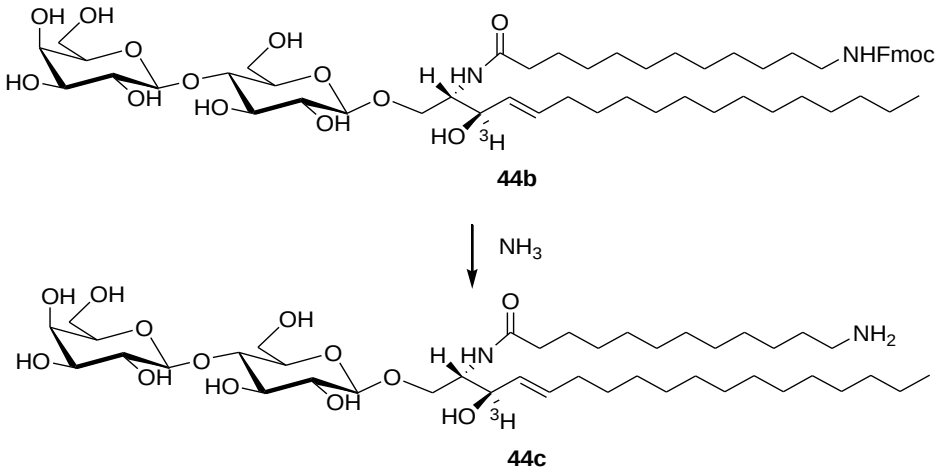


44a

44b

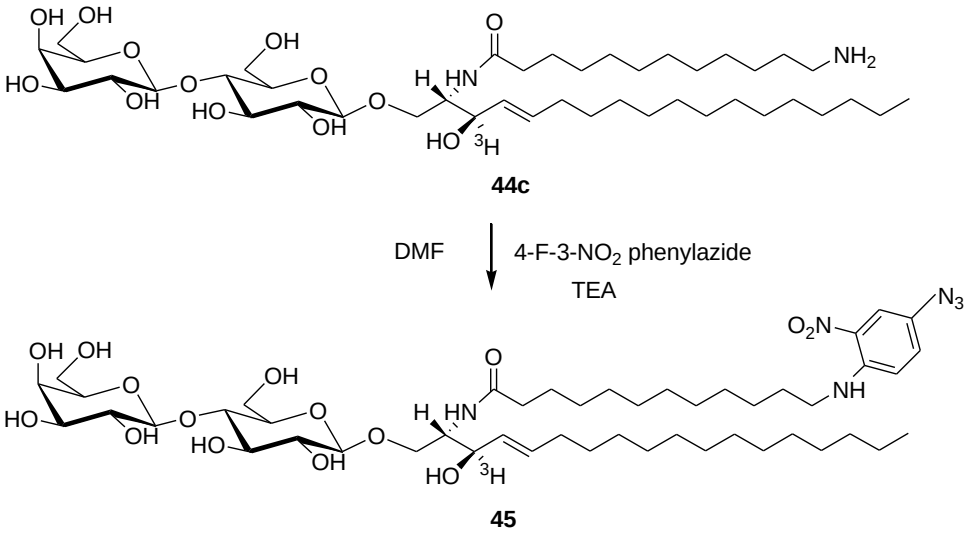
Compound **44a** (2 mg) was dissolved in MeOH and a 0.1 M NaOH until reaching pH 7.5. 100 mCi NaB^3H_4 was added and the reaction was allowed to proceed at room temperature for three days. The reaction mixture was dried and the tritiated derivative was purified by flash chromatography with CH_2Cl_2:MeOH:H_2O, 60:25:4.

4.4.6 Fmoc-deprotection



Compound **44b** was dissolved in the solution of NH_3 in a screw-cap flask for 24 h under vigorous stirring at room temperature. The reaction mixture was dried and the residue was purified by column chromatography with CH_2Cl_2:MeOH:NH_3 (2N), 60:35:5.

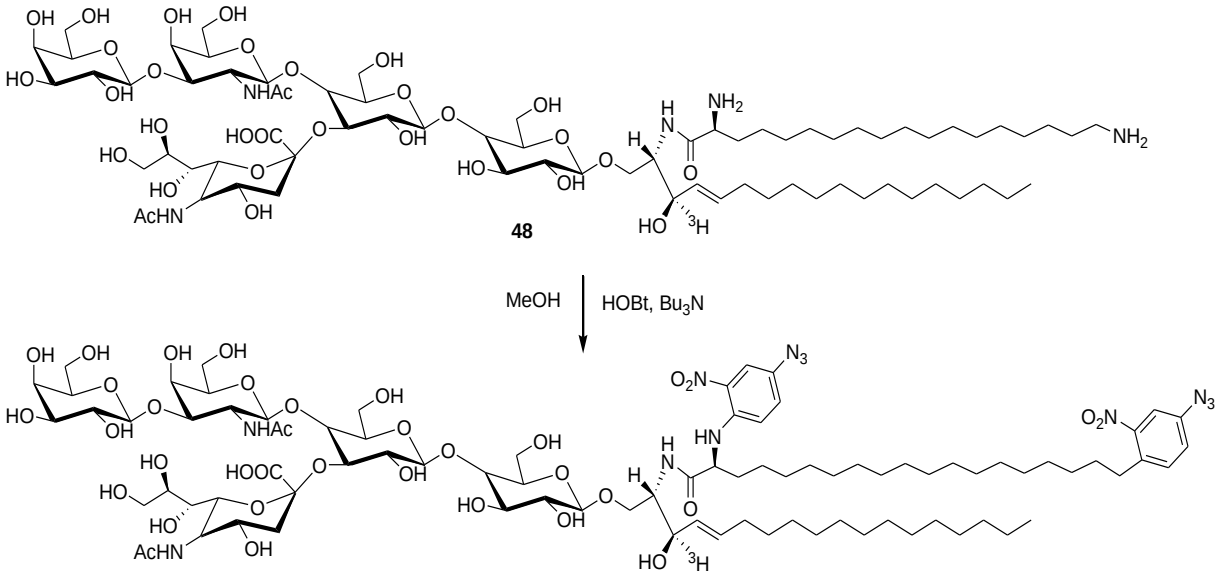
4.4.7 Introduction of photoactivable group



The radioactive compound was dissolved in anhydrous DMF, Et_3N and 4-F-3-NO_2-phenylazide, dissolved in EtOH, were added and the reaction mixture was stirred overnight at 80^0C. The reaction mixture was concentrated and the radioactive and photoactivable derivative was purified by flash chromatography with CH_2Cl_2:MeOH:H_2O, 30:50:13. Fractions containing the homogeneous product were evaporated and the residue immediately solubilized in methanol and stored at $+4^0$.

4.5 Synthesis of probe 4

4.5.1 N-acylation of lyso GM1 with N,N'-Boc-2,18-α,ω-di-amino stearic acid

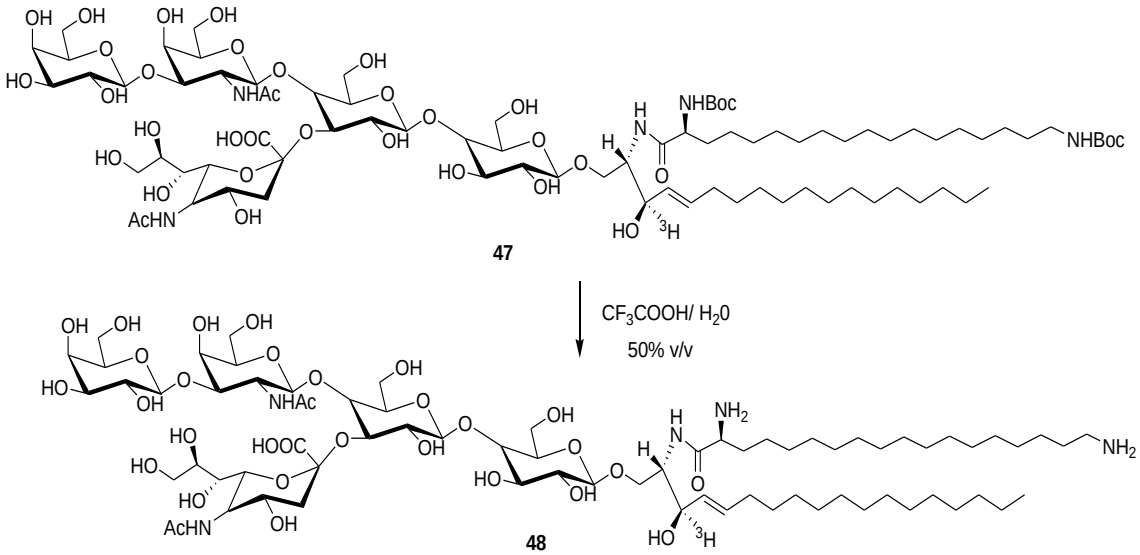


Compound	M.W.	D(g/ml)	eq.	mmol	mg	ml
46	**1282**		**1**	**0.0031**	**4**	
37	**681**		**1.2**	**0.0037**	**2.5**	
HOBt	**135.04**		**1.2**	**0.0037**	**0.5**	
Bu_3N	**185.21**	**0.778**	**2.4**	**0.0074**	**1.3**	**0.002**
MeOH						**1**

To the radioactive compound **46**, dissolved in MeOH, were added compound **37**, HOBt and Bu_3N. The reaction mixture was stirred overnight at room temperature,

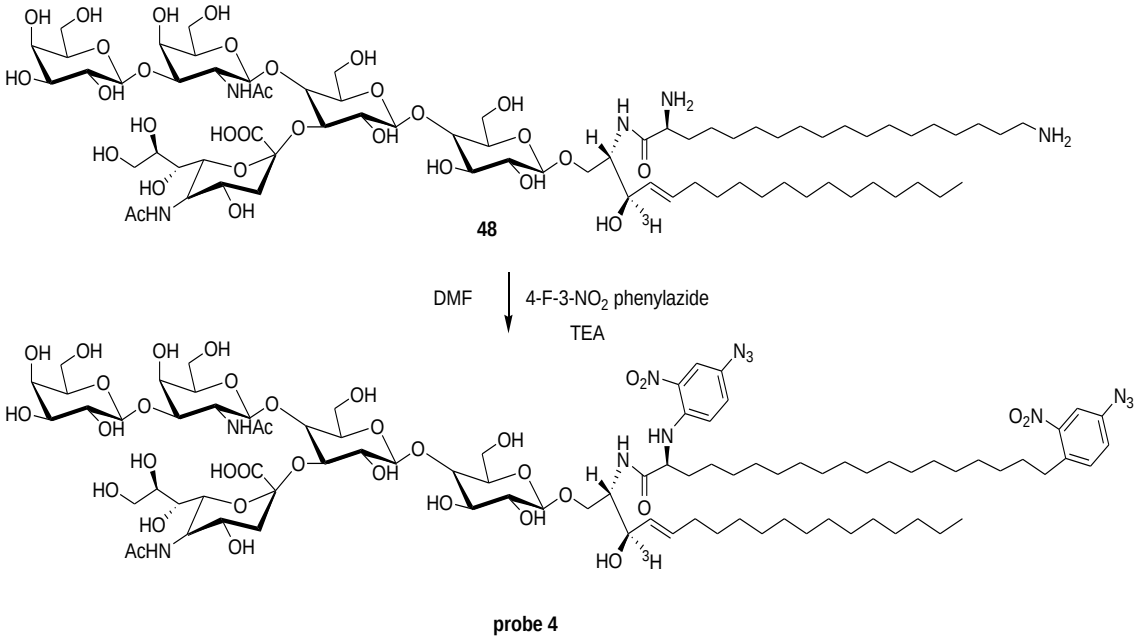
when the reaction was finished, monitoring by TLC, the reaction mixture was evaporated. The residue was purified by flash chromatography with 60:30:5:4 CH_2Cl_2:MeOH:2-propanol:H_2O.

4.5.2 Boc-deprotection



To the compound **47** was added a solution of CF_3COOH in H_20 50% v/v (200µl) and the reaction was stirred for 2 h at room temperature. The reaction mixture was evaporated and the residue purified with 50:42:11 CH_2Cl_2:MeOH:NH_3 (2N) and 60:35:5 Chloroform:MeOH:NH_3 (2N).

4.5.2 Introduction of photoactivable group



The radioactive compound was dissolved in anhydrous DMF, Et_3N and 4-F-3-NO_2-phenylazide, dissolved in EtOH, were added and the reaction mixture was stirred overnight at 80^0C. The reaction mixture was dried and the radioactive and photoactivable derivative was purified by flash chromatography with CH_2Cl_2:MeOH:H_2O, 30:50:13. Fractions containing the homogeneous product were evaporated and the residue immediately solubilized in methanol and stored at $+4^0$.

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