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**ORIGINAL PAPER**

# Synthesis and Characterization of ß-*D*-(2,3,4-tri-O-acetyl)Galactopyranosyl - and ß-*D*-(2,4,6-tri-O-acetyl)Galactopyranosyl -1'(3'-O-acetyl)ceramide, two Versatile Biochemical Intermediates

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## Abstract

*Galactocerebroside has been purified from swine brain and tritylated on C-6 by stirring with trityl chloride in pyridine. Tritylated derivative has been peracetylated by stirring overnight with a mixture of acetic anhydride-pyridine. Then trityl group was removed by solvolysis with p-toluenesulfonic acid in a mixture of methanol-dichloromethane. Partially acetylated galactocerebroside was separated from uncleaved ß-D-(2,3,4-tri-O-acetyl-6-O-trityl)galactopyranosyl-1'(3'-O-acetyl)ceramide by column chromatography on silica gel. Sulfatide (galactocerebroside 3-sulfate) has been separated from the same material and peracetylated. Sulfate ester was cleaved by stirring with p-toluenesulfonic acid, reaction product being separated by column chromatography on silica gel. Both isomers, ß-D-(2,3,4-tri-O-acetyl)galactopyranosyl - and ß-D-(2,4,6-tri-O-acetyl)galactopyranosyl-1'(3'-O-acetyl)ceramide, were characterized by spectral – NMR, IR – chemical and chromatographical means. Considerations about chemical and biochemical versatility of the two compounds have been made.*

Keywords: : ß-D-(2,3,4-tri-O-acetyl)galactopyranosyl -1'(3'-O-acetyl)ceramide, ß-D-(2,4,6-tri-O-acetyl)galactopyranosyl -1'(3'-O-acetyl)ceramide, cerebroside, sulfatide, $^{1}H$ and $^{13}C$ NMR spectra.

## Introduction

There are numerous reasons for chemical modifications of natural glycosphingolipids or synthesis of neoglycosphingolipids. Double bond of sphingosine from glycosphingolipids has been hydrogenated with tritium in order to accomplish tritium label of these compounds [1]. Although the method has been used by many researchers, some authors considered it doubtable, due to the fact that the trans double bond is essential for some biological activities of glycosphingolipids [2]. Labeling on C-6 of D-galactose from cerebroside has been made by its oxidation with galactose oxidase to an aldehyde and then back reducing the resultant aldehyde group to the original carbinol with radioactive borohydride [3]. This method was applied also to the labeling of galactosylsphingosine and served to identification of galactosylsphingosine and the corresponding galactosidase in living matter [4]. A fluorescent derivative of cerebroside sulfate ((12-1-pyrene)dodecanoyl-sphingosylgalactosyl-O-3-sulfate (P12-sulfatide)) has been synthesized, *via* lyso-sulfatide, as a potential substrate for the determination of cerebroside sulfatidase (arylsulfatase A) activity, and implicitly of metachromatic leukodystrophy [5]. Stearoyl[1-$^{14}$C]sulfogalactosylsphingosine ([$^{14}$C]sulfatide) has been synthesized, as substrate for cerebroside sulfatase assay, also *via* lysosulphatide [6].

[$^3$H]-Labeling of lysosulphatide was performed by stirring its solution in water-tetrahydrofuran with NaB[$^3$H]$_4$ at alkaline pH determined by potassium hydroxide in the presence of palladium acetate; the reaction was stopped by swerving the pH in acid range with acetic acid [7]. Sulphotransferase, the enzyme transforming cerebroside to sulfatide, was purified by affinity chromatography on a Sepharose column having Gal-Cer tethered by an amino group of an ω-amino fatty acid that had replaced the native fatty acid of the respective cerebroside. Similarly modified cerebrosides served for substrate specificity investigations of this important enzyme [8]. Perbenzoylation of glycosphingolipids by heating has had two practical and important consequences: on one hand the peracylated compounds absorbed cumulatively by 230 nm, and this has constituted an important analytical tool; on the other hand, two neutral glycosphingolipids are obtained by debenzoylation: one having the initial fatty acid and the other having N-benzoyl instead of N-fatty acyl [9]. The activity of sphingolipid ceramide deacylase was measured using C12-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-GalCer and [$^{14}$C]GM1a labeled at the stearic acid, as substrates [10]. Fluorescent derivatives of cerebroside sulfate (sulfogalactosyl ceramide) containing long-wavelength-emission fluorophores were synthesized by using cerebroside 3-sulfate derivative with an amino group on the terminal carbon atom of its fatty acyl residue. To the amino group, alternatively lissamine-rhodamine, fluoresceine, eosine or 7-nitrobenz-2-oxa-1,3-diazol-4-yl were linked [11]. Two other fluorescent derivatives of cerebroside sulfate have been synthesized and used as substrates for determining arylsulfatase A activity, i. e., 12-(1-pyrene)dodecanoyl cerebroside sulfate (P12-sulfatide) and 12(1-pyrenesulfonylamido)dodecanoyl cerebroside sulfate (PSA12-sulfatide) [12].

In this paper, ß-*D*-(2,3,4-tri-O-acetyl)galactopyranosyl-1'(3'-O-acetyl)ceramide was prepared by using galactocerebroside and ß-*D*-(2,4,6-tri-O-acetyl)galactopyranosyl-1'(3'-O-acetyl)ceramide from sulfatide. They and the corresponding intermediates were characterized by spectral – NMR, IR – chemical and chromatographical means. Moreover, considerations about chemical and biochemical versatility of the two compounds have been made.

## Materials and methods

**Materials**. D-Galactose, toluene, sodium metal, acetic anhydride, pyridine, ninhydrin, ethanol, thin layer plates ready-to-use, silicagel for column chromatography, methanol, chloroform, concd. sulfuric acid, ammonium molibdate, $Ce(SO_4)_2$, were either from Merck or from Fluka. Swine brain was purchased from a slaughter house and processed immediately. Galactocerebroside and sulfatide have been purified as described by [13].

**Methods used**

**NMR Spectra registration**. $^1$H and $^{13}$C NMR spectra of initial precursors, intermediates, and final products were registered in peracylated form in $CDCl_3$ containing TMS. The spectra of intermediates and final products have been referred to the spectra of peracetylated initial precursors.

**One-dimensional NMR studies**.

NMR experiments were performed on a Bruker Avance DRX 400 spectrometer using 400 and 100 MHz for $^1$H and $^{13}$C frequencies, respectively.

**Two-dimensional NMR experiments**.

The 1H-1H correlation spectroscopy (COSY) and $^1$H-$^{13}$C heteronuclear multiple quantum coherence (HMQC) experiments were carried out with an inverse probe.

**IR spectra** were recorded as KBr pellets on a Bruker Equinox 55 FT-IR spectrometer.

**Thin-layer and column chromatography**.

The following solvent systems (SS) were used for TLC: chloroform-methanol-water, 50:10:1 (v/v, SS I), toluene-methanol, 7:1 (v/v, SS II), chloroform-methanol-water-concd. ammonia, 70:30:4:1 (v/v, SS III). Visualization was made by dipping the plates in a solution of amonium molybdate, sulfuric acid and cerium(IV) sulfate, or in a 0.1% solution of ninhydrin in water saturated n-butanol for amino group containing compounds, followed by heating in both cases.

Column chromatography was made on Florisil or silica gel in a gradient of methanol in chloroform (0-40%).

**Acidic hydrolysis**.

Total acidic hydrolysis was accomplished in a mixture of chloroform-ethanol-conc. HCl as indicated [13]. Solvents were evaporated to dryness and the residue partitioned between chloroform and water. The two phases were separated, in water phase *D*-galactose was determined by anthrone reaction [13], while in chloroformic phase sphingosine (ninhydrin reaction) was determined [13]. In all cases, hydrolysis was monitored by TLC in SS I.

**Other chemical methods**.

Acetylation was made by stirring the compound overnight in an excess of pyridine-acetic anhydride 2/1 (v/v) mixture. Tritylation was made as [14] and detritylation as well as desulfation by stirring the respective compound with p-toluenesulfonic acid in a mixture of dichloromethane-methanol [15].

## Results and discussions

Reactions accomplished on galactocerebroside are indicated in Fig. 1. Trityl group being

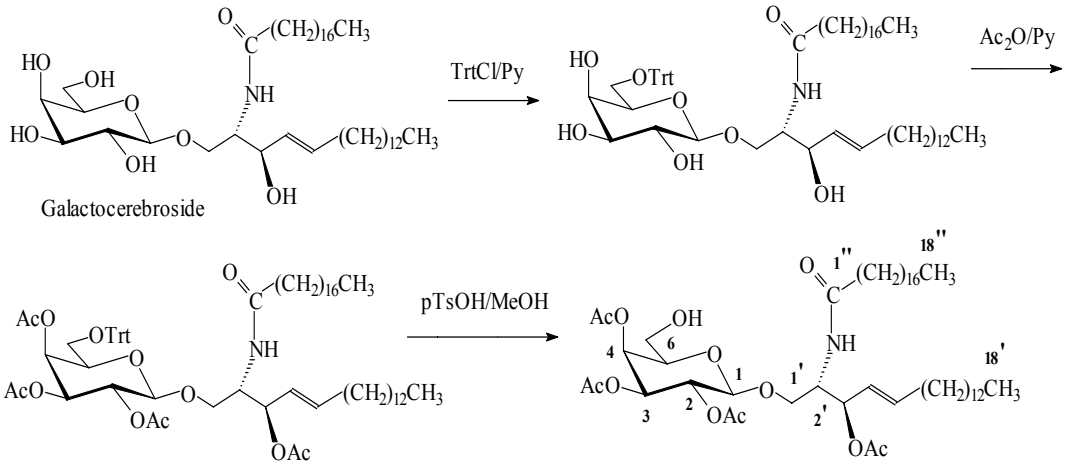


**Figure 1.** Chemical reactions describing preparation of ß-*D*-(2,3,4-tri-O-acetyl)galactopyranosyl -1'(3'-O-acetyl)ceramide from galactocerebroside.

unpolar, it increases mobility of glycosphingolipid and tritylation (Fig. 2) and column separation (Fig. 3) were easily followed by TLC.

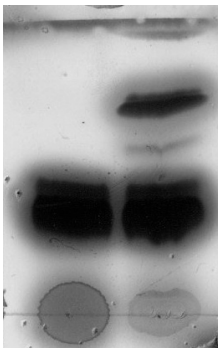

**Figure 2.** Tritylation of galactocerebroside. Start 1, galactocerebroside; start 2, ß-*D*-(6-O-trityl)galactopyranosyl -1'ceramide (tritylated galactocerebroside). Migration, SS I; visualisation, mostain

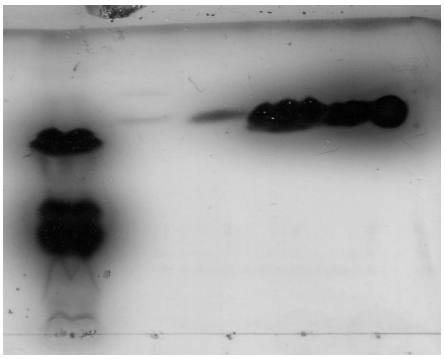

**Figure 3.** Separation of tritylated galactocerebroside from unreacted compound. Start 1, initial mixture; start 3, 4, 5, tritylated galactocerebroside. Migration, SS I; visualisation, mostain.

Acetylation of tritylated galactocerebroside as well as detritylation of ß-*D*-(2,3,4-tri-O-acetyl 6-O-trityl)galactopyranosyl -1'(3'-O-acetyl)ceramide to ß-*D*-(2,3,4-tri-O-acetyl)galactopyranosyl -1'(3'-O-acetyl)ceramide were also followed by TLC, the reaction products being separated by column chromatography on silicagel and characterized chemically, chromatographically and spectrometrically. The following arguments can be brought in favour of the fact that trityl group selectively entered at C-6 of D-Gal in cerebroside: the signal 61.19 (C-6) in peracetylated cerebroside disappeared in ß-*D*-(2,3,4-tri-O-acetyl 6-O-trityl)galactopyranosyl-1'(3'-O-acetyl)ceramide and two signals, alternatively attributable to C-6, appeared at 58.27 or 67.10. After removing of trityl group, a signal at 61.81 (C-6) appeared in ß-*D*-(2,3,4-tri-O-acetyl)galactopyranosyl-1'(3'-O-acetyl)ceramide. Moreover, in a high resolution chromatographic system, tritylation product appeared homogenously *en bloc*. At the same time, as chromatographical systems revealed, the same tritylation product was formed at room temperature or at $100^0$C, although the yields differed.

Galactocerebroside (ß-D-galactopyranosyl-1'ceramide).The galactocerebroside, purified from swine brain as indicated by [13], gave *D*-galactose, sphingosine and fatty acid in the molar ratio 1:1:1, after total acidic hydrolysis. NMR spectra of peracetylated galactocerebroside gave signals disclosing the presence of sugar, sphingosine and fatty acid [13]:

$^1$H-NMR ($CDCl_3$; δ, ppm; *J*, Hz): 4.46 (d, 7.6, H-1), 5.38 (H-4), 5.31 (H-2), 5.02 (H-3), 4.14 (d, 6.8, H-6a and H-6b), 3.92 (dt, 6.4, H-5), 0.88 (methy groups of ceramide), 1.25

(methylene groups of ceramide), 1.97, 2.03, 2.04, 2.15, 2.18 (methyl groups of acetate esters), 5.29 (H-4'), 5.83 (H-5'), 6.34 (NH of sphingosine).

$^{13}$C-NMR ($CDCl_3$): 100.90 (C-1), 68.68 (C-2), 70.82 (C-3), 66.96 (C-4), 70.79 (C-5), 61.19 (C-6), 14.12 (methyl groups of ceramide, 20.95 (methylene groups of ceramide), 28.95-29.72 (methylene groups of ceramide), 129.91 (C-4'), 137.35 (C-5'), 169.44, 169.74, 170.73, 170.38 (C=O group of acetate ester).

ß-D-(6-O-Trityl)galactopyranosyl-1'ceramide and ß-D-(2,3,4-tri-O-acetyl-6-O-trityl)galactopyranosyl-1'(3'-O-acetyl)ceramide. NMR spectra of peracetylated trityl derivative:

$^{1}$H-NMR ($CDCl_3$; δ, ppm; *J*, Hz): 4.40 (d, 7.6, H-1), 5.38 (H-4), 5.31 (H-2), 5.02 (H-3), 3.91 (d, 6.8, H-6a and H-6b), 3.82 (dt, 6.4, H-5), 0.88 (methyl groups of ceramide), 1.25 (methylene groups of ceramide), 1.91, 1.94, 2.02, 2.10 (methyl groups of acetate esters), 5.80 (H-5'), 7.23, 7.25, 7.35, 7.37 (phenyl groups of trityl).

$^{13}$C-NMR ($CDCl_3$): 100.76 (C-1), 68.86 (C-2), 72.07 (C-3), 67.10 (C-4), 70.97 (C-5), 58.27 (C-6), 14.05 (methyl groups of ceramide), 20.46 (methylene groups of ceramide), 28.84-29.65 (methylene groups of ceramide), 129.83 (C-4'), 137.22 (C-5'), 169.39, 169.62, 170.10 (C=O groups of acetate esters).

NMR Spectra of ß-D-(2,3,4-tri-O-acetyl)galactopyranosyl-1'(3'-O-acetyl)ceramide:

$^{1}$H-NMR ($CDCl_3$; δ, ppm; *J*, Hz): 4.46 (d, 7.6, H-1), 5.38 (H-4), 5.31 (H-2), 5.02 (H-3), 4.14 (d, 6.8, H-6a and H-6b), 3.92 (dt, 6.4, H-5), 0.88 (methyl groups of ceramide), 1.25 (methylene groups of ceramide), 1.99, 2.03, 2.05, 2.12, 2.18 (methyl groups of acetate esters), 5.81 (m, 15.2, H-5').

$^{13}$C-NMR ($CDCl_3$): 101.32 (C-1), 69.20 (C-2), 71.11 (C-3), 67.13 (C-4), 70.79 (C-5), 61.81 (C-6), 14.09 (methyl groups of ceramide), 20.96 (methylene groups of ceramide), 28.96-29.75 (methylene groups of ceramide), 129.87 (C-4'), 137.42 (C-5'), 169.64, 169.99, 170.37, 170.90 (C=O groups of acetate esters).

IR Spectra of partially acetylated galactocerebroside, in comparison with partially acetylated tritylated galactocerebroside, indicated a significant absorption at 3250-3500 $cm^{-1}$ due to hydroxy group on C-6 of sugar galactocerebroside. On the other hand, partially acetylated galactocerebroside migrated slower than peracetylated galactocerebroside.

Sulfatide, purified according to [13], gave *D*-galactose, sphingosine, fatty acid and inorganic sulfate in the molar ratio 1:1:1:1. Contrary to galactocerebroside, sulfatide was retained by DEAE-Sephadex A-25, being eluted with 0.25 M sodium acetate methanolic solution [13]. IR Spectra indicated the presence of sulfate ester (1240 $cm^{-1}$). Chemical transformations leading from sulfatide to ß-D-(2,4,6-tri-O-acetyl)galactopyranosyl-1'(3'-O-acetyl)ceramide are described in Fig. 4. Acetylation of sulfatide increases mobility as hydroxyl

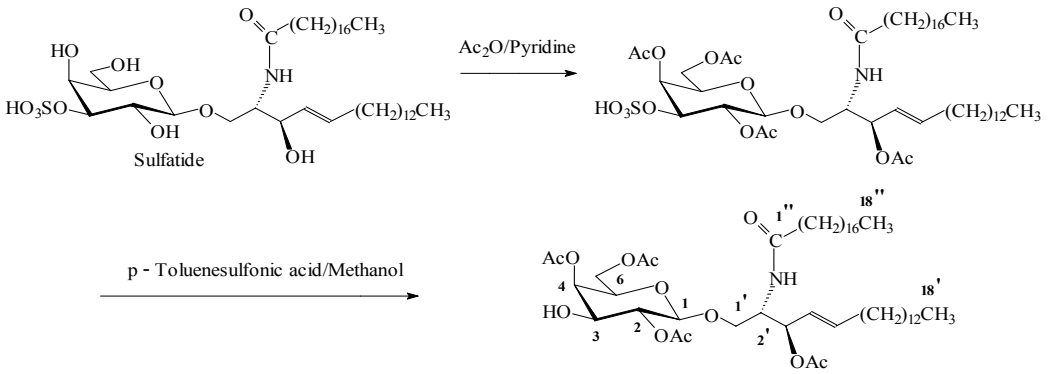


**Figure 4.** Stepwise transformation of sulfatide to ß-*D*-(2,4,6-tri-O-acetyl)galactopyranosyl-1'(3'-O-acetyl)ceramide

groups are blocked and the former hydrogen bonds with chromatographic material are replaced by Van der Waals associations. At its turn, desulfation of ß-*D*-(2,4,6-tri-O-acetyl 3-O-sulfo)galactopyranosyl -1'(3'-O-acetyl)ceramide to ß-*D*-(2,4,6-tri-O-acetyl)galactopyranosyl -1'(3'-O-acetyl)ceramide replaces a strongly ionized polar group (sulfate) by a less polar function, hydroxyl; as a consequence, a faster compound is produced (Fig. 5 and Fig. 6). All compounds have been characterized by chemical, chromatographical and physical means.

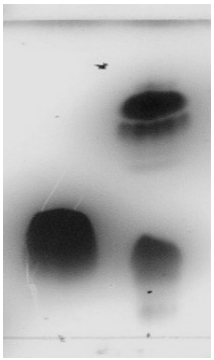

**Figure 5.** TLC Indicating desulfation of ß-*D*-(2,4,6-tri-O-acetyl 3-sulfo)galactopyranosyl -1'(3'-O-acetyl)ceramide (start 1) to ß-*D*-(2,4,6-tri-O-acetyl)galactopyranosyl-1'(3'-O-acetyl)ceramide (start 2, upper spot). Migration, SS II; visualisation, mostain.

**NMR spectra of peracetylated sulfatide**:

$^{1}$H-NMR ($CDCl_3$; δ, ppm; *J*, Hz): 4.57 (H-1), 5.38 (H-4), 5.00 (H-2), 5.29 (H-3), 4.07 (H-6a), 4.21 (H-6b), 3.85 (H-5), 0.88 (methyl groups of ceramide), 1.25 (methylene groups of ceramide), 1.99, 2.03, 2.06, 2.09 (methyl groups of acetate esters), 5.29 (H-4'), 5.83 (H-5'), 6.33 (NH of sphingosine).

$^{13}$C-NMR ($CDCl_3$): 100.01 (C-1), 69.74 (C-2), 74.06 (C-3), 73.42 (C-4), 67.03 (C-5), 62.32 (C-6), 14.09 (methyl groups of ceramide), 20.94 (methylene groups of ceramide), 28.98-29.72 (methylene groups of ceramide), 129.88 (C-4'), 137.14 (C-5'), 169.83, 171.02, 172.06 (C=O groups of acetate esters).

**NMR spectra of ß-D-(2,4,6-tri-O-acetyl)galactopyranosyl-1'(3'-O-acetyl)ceramide**:

$^{1}$H-NMR ($CDCl_3$; δ, ppm; *J*, Hz): 4.39 (d, 7.2, H-1), 5.27 (H-4), 4.93 (H-2), 3.58 (H-3), 3.90 (H-6a), 4.10 (H-6b), 3.83 (H-5), 0.88 (methyl groups of ceramide), 1.25 (methylene groups of ceramide), 2.03, 2.06, 2.11, 2.18 (methyl groups of acetate esters), 5.76 (H-5').

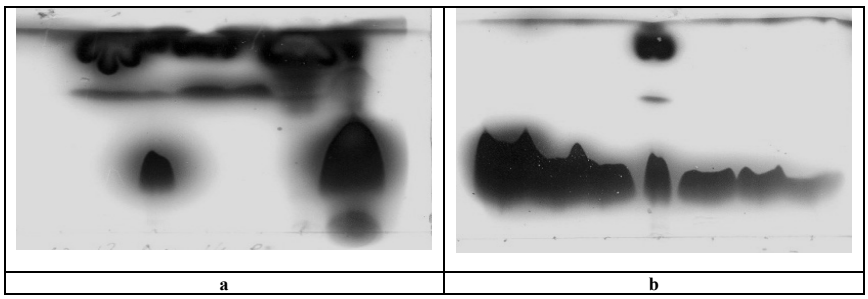


**Figure 6.** Separation of ß-*D*-(2,4,6-tri-O-acetyl)galactopyranosyl -1'(3'-O-acetyl)ceramide (plate a, upper spot) from ß-*D*-(2,4,6-tri-O-acetyl 3-sulfo)galactopyranosyl -1'(3'-O-acetyl)ceramide (plate 2); on both plates the initial mixture containing unseparated components is present as a reference. Migration, SS I; visualisation, mostain.

$^{13}$C-NMR ($CDCl_3$): 100.66 (C-1), 69.60 (C-2), 71.15 (C-3), 67.14 (C-4), 71.09 (C-5), 61.82 (C-6), 14.08 (methyl groups of ceramide), 20.67 (methylene groups of ceramide), 28.98-29.75 (methylene groups of ceramide), 129.88 (C-4'), 137.03 (C-5'), 169.64, 170.25, 170.91, 170.99 (C=O groups of acetate esters).

IR Spectra of partially acetylated galactocerebroside, in comparison with partially acetylated sulfated galactocerebroside, indicated a significant absorption at 3250-3500 $cm^{-1}$ due to hydroxy group on C-3 of sugar galactocerebroside, as well as the disappearance of absorption at 1250 $cm^{-1}$ due to sulfate ester. At the same time, partially acetylated galactocerebroside migrated slower than peracetylated galactocerebroside.

In comparison with similar natural and synthesized compounds, the partially acetylated galactocerebrosides described in this paper, open interesting perspectives. Alternatively, on C-6 of D-Gal from natural compounds, ß-*D*-galactofuranose, fatty acid and sialic acid have been found. ß-*D*-Galactofuranosyl-6-αß-*D*-galactopyranose, as a structural motif, was obtained by partial hydrolysis of immunologically specific polysaccharide of *Mycoplasma mycoides* [16] or of *Mycobacterium tuberculosis* [17]. Subsequently, this disaccharide was also synthesized [18]. ß-*D*-Galactopyranoside 6-O-acylated with a fatty acid has been found as an important antigen in different strains of *Borrelia burgdorferi*, the causative agent of Lyme disease [19, 20]. The primary hydroxy group C-6 (Fig. 1) can be alternatively oxidized to carboxyl or to aldehyde. Oxidation to aldehyde has been made with *N*-chlorosuccinimide by using a catalytic amount of *N-tert*-butylbenzenesulfenamide in the co-existence of potassium carbonate and molecular sieves 4A [21]. A similar oxidation has been made with $I_2$-TEMPO, 10:1, in a solution of $NaHCO_3$ [22]. A series of unprotected β-D-glycosyl azides of mono- (including 2-acetamido-2-deoxy-D-glycopyranosyl) and disaccharides were oxidized by TEMPO to the corresponding uronic acids; in case of disaccharides (lactosyl- and cellobiosyl azides) diacid form were obtained. However, 4′,6′-*O*-benzylidene protection enabled selective oxidation of the C-6 hydroxyl [23]. Cellulose was oxidized in order to obtain polyanionic polymers mimicking nucleic acids or natural polyanionic macromolecules (hyaluronic acid, heparin, condroitin 4- and 6-sulfate, etc). A ratio of about 85% of primary hydroxy groups were oxidized to carboxyls and a significant number to aldehydes. Alternatively, these carbonyl functions were reduced with $NaBH_4$ or converted to amines (by reductive amination), the product being a macromolecular amfiionic structure similar to proteins, or cross-linked with a crosslinking agent [24]. Fatty acylated cerebroside (both as kerasin and cerebron) has been found for the first time by [25] and confirmed by [26, 27]. Subsequently, it was proved that fatty acyl group is linked on C-6 of D-galactose (6-acyl galactosyl ceramides) [28]. The same compound was found in the brain of Alaskan pollack (*Theragra chalcogramma*) [29, 30]. As a possible fetal antigen, a ganglioside containing the structural motif NeuAcα2-6Galp has been found in human meconium; in fact, D-galactose moiety linked to sialic acid is a constituent of neolactotetraosyl-ceramide [31]. Acidic oligosaccharides from the same material also contained the fragment NeuAcα2-6Galp [32]. All the above mentioned oxidations, as well as Jones oxidation and biological oxidations, can be accomplished on ß-D-(2,4,6-tri-O-acetyl)galactopyranosyl-1'(3'-O-acetyl)ceramide, prepared in this paper from sulfatide.

Longiside, ß-*D*-galactofuranosyl-3-α-*D*-galactopyranosyl-1'ceramide, was isolated from the Caribbean sponge *Agelas longissima* [33]. Ectyoceramide, ß-*D*-galactofuranosyl-1'ceramide, have been found in the marine sponge *Ectyoplasia ferox* [34]. It was proved unequivocally that agelagalastatin, α-*D*-Galf-2-ß-*D*-Galf-3-α-*D*-Galp-1'Cer, isolated from a marine sponge, *Agelas* sp., presented significant *in vitro* inhibitory activity against human cancer cell lines including brain, kidney, colon, lung, ovary, melanoma [35]. The partially acetylated galactocerebroside prepared in this paper from sulfatide can serve as chemical or biochemical precursor for at least another three compounds: ß-D-(3-O-fatty acyl)galactopyranosyl-1'ceramide, ß-D-(3-O-

[$^{35}$S]sulfo)galactopyranosyl-1'ceramide, ß-D-(3-O-sialosyl)galactopyranosyl-1'ceramide (gangliosides GM4).

## Conclusions

A suite of chromatographic methods produce galactocerebroside and sulfatide as pure and homogenous compounds.

By submitting galactocerebroside to tritylation, acetylation, detritylation, ß-*D*-(2,3,4-tri-O-acetyl)galactopyranosyl -1'(3'-O-acetyl)ceramide was prepared.

Protection of sulfatide by acetylation, followed by selective cleavage of sulfate ester, ß-*D*-(2,4,6-tri-O-acetyl)galactopyranosyl -1'(3'-O-acetyl)ceramide has been obtained.

$^{1}$H and $^{13}$C NMR proved extremely useful for characterization of precursors, intermediates and final products.

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