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Synthesis of ganglioside GM1 containing a thioglycosidic bond to its labeled ceramide(s). A facile synthesis starting from natural gangliosides [1]

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Abstract

Capitalizing on the readily available ganglioside, GM1, we have devised a simple synthesis of labeled GM1 analogues with sulfur in place of oxygen in their linkage to the ceramide residue (SGM1). The sugar moiety of ganglioside GM1 was released by ozonolysis and subsequent alkaline fragmentation in good yield. During acetylation of the ganglioside sugar, the carboxyl group of the sialic acid residue lactonized with the 2-hydroxyl group of the inner galactose moiety (galactose II). The resulting sialoyl-II^2-lactone of pentadeca-*O*-acetyl-monosialogangliotetraose could be readily transformed into the α-glycosyl bromide. Subsequent treatment of this glycosyl bromide with potassium thioacetate afforded the sialoyl-II^2-lactone of tetradeca-*O*-acetyl-1-*S*-acetyl-1-thio-β-monosialogangliotetraose. The latter could be condensed with (2*R*,3*R*,4*E*)-3-*O*-benzoyl-2-dichloroacetamido-1-iodo-4-octadecen-3-ol in methanolic sodium acetate to afford a protected lyso-SGM1 derivative. One-step removal of the protecting groups under alkaline conditions gave β-monosialogangliotetraosyl thiosphingosine. This lyso-SGM1 was converted into labeled analogues of SGM1 using the *N*-succinimidoyl derivative of radiocarbon-labeled octanoic and octadecanoic acid, respectively. Subsequent actions of GM1-β-galactosidase, β-hexosaminidase A, sialidase and again GM1-β-galactosidase on these labeled analogues of SGM1 in the presence of taurodeoxycholate produced the respective analogues of GM2, GM3, lactosylceramide and glucosylceramide, respectively. © 1997 Elsevier Science Ltd.

Keywords: Thiogangliosides; Labeled glycolipids; Thioglycosides; Thio-GM1; Sialogangliotetraose; Labeled thio-GM1

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[1] Dedicated to Professor Roger W. Jeanloz on the occasion of his 80th birthday.

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1. Introduction

Gangliosides are ubiquitous in vertebrate cells and are notably abundant in the brain [1]. The gangliosides comprise a family of amphiphatic molecules that contain a ceramide moiety, as a lipophilic anchor embedded in the outer leaflet of plasma membranes, and a sialooligosaccharide residue exposed towards the extracellular space [2].

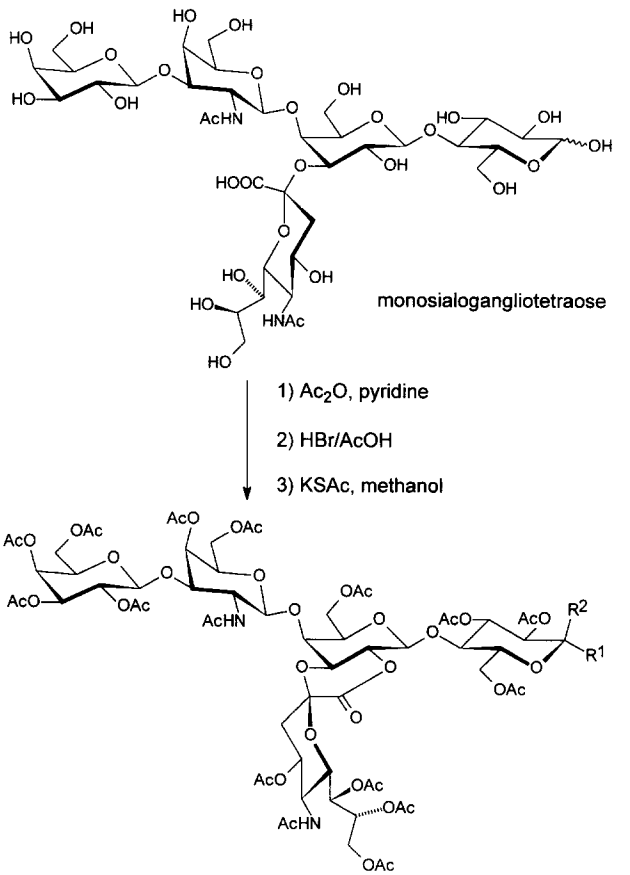
Gangliosides were first discovered more than 50 years ago [3]. During the years since, gangliosides have been adequately studied for their chemical structure, physicochemical behaviour and cellular distribution. However, there is only scarce information on their biological function and structural organization in membranes. Increasing evidence demonstrates that gangliosides play a role in cell-surface phenomena relevant to signal transduction [4,5]. Though most of the biological functions of gangliosides occur in the plasma membrane, it is likely that most, if not all, steps in ganglioside metabolism take place in intracellular compartments. Therefore, besides regulation of the individual metabolic steps, their localization and relation to intracellular lipid transport has become a matter of increasing interest [6,7]. As was shown for glucosylceramide [8], it is to be expected that the study of lipid transport and ganglioside metabolism would be greatly facilitated by use of the respective labeled, and partially nondegradable, gangliosides.

We have, therefore, synthesized two analogues of ganglioside GM1 containing a β-thioglycosidic bond to the ceramide moiety. These analogues contain either a long radioactive fatty acyl residue (C_{18}) or a radioactive acyl chain of medium chain length (C_8) in lieu of the natural fatty acid residue.

For the total synthesis of gangliosides, efficient regio- and stereoselective routes affording various gangliosides and their analogues have been worked out in recent years in the laboratories of Hasegawa [9] and Ogawa [10]. The focal point in these syntheses has been the stereoselective α-sialylation of the respective ganglio series sugar backbone. In addition to the synthesis of gangliosides containing exclusively *O*-glycosidic bonds, a variety of ganglioside analogues containing the thioglycosidic linkage(s) have also been synthesized by Hasegawa's group [9] in Japan. We have previously shown that various labeled analogues of ganglioside GM1 can be conveniently prepared from its sialooligosaccharide unit [11,12]. The latter is derived from GM1 in good yields by ozonolysis and subsequent alkaline fragmentation [11,13]. Gangliosides, and especially ganglioside GM1, are available in huge quantities from cattle brains. The exploitation of the readily available ganglioside GM1 and the ease of obtaining its respective ganglioside sugar facilitated the synthesis of labeled GM1 analogues. This approach may also be very useful for the study of metabolism and transport of other glycosphingolipids.

2. Results and discussion

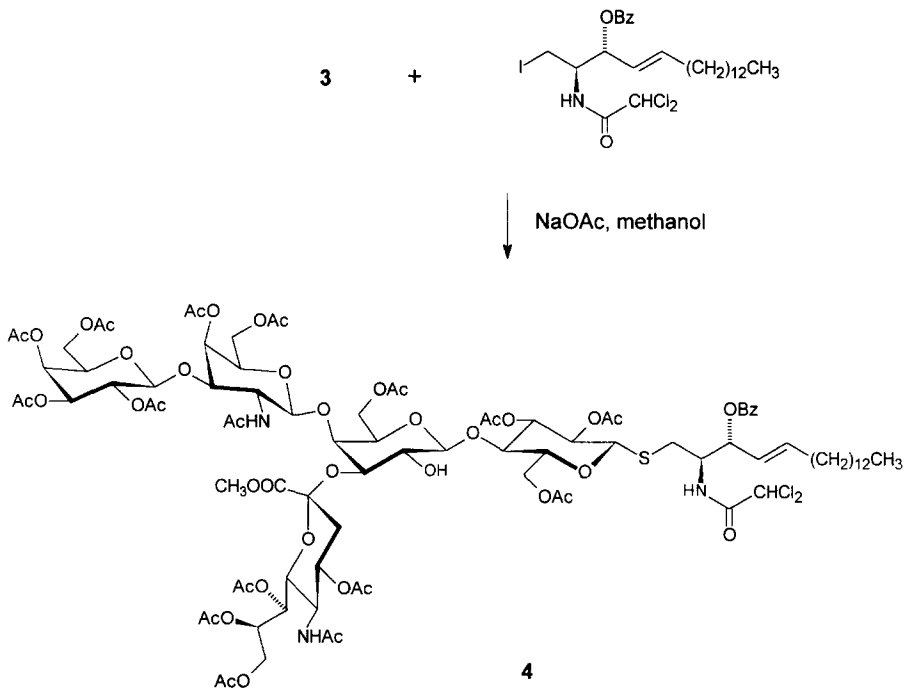
The release of the intact sugar portion from its ceramide residue in gangliosides by ozonolysis and subsequent alkaline fragmentation was first accomplished more than 30 years ago [13] and has been utilized as a pivotal step in the preparation of spin-labeled ganglioside analogues [12] in which the glucose moiety was converted, by reductive amination, into a 1-amino-1-deoxysorbitol residue. The use of ganglioside sugars in the synthesis of labeled gan-



1a R^1 = OAc, R^2 = H **2** R^1 = H, R^2 = Br

1b R^1 = H, R^2 = OAc **3** R^1 = SAc, R^2 = H

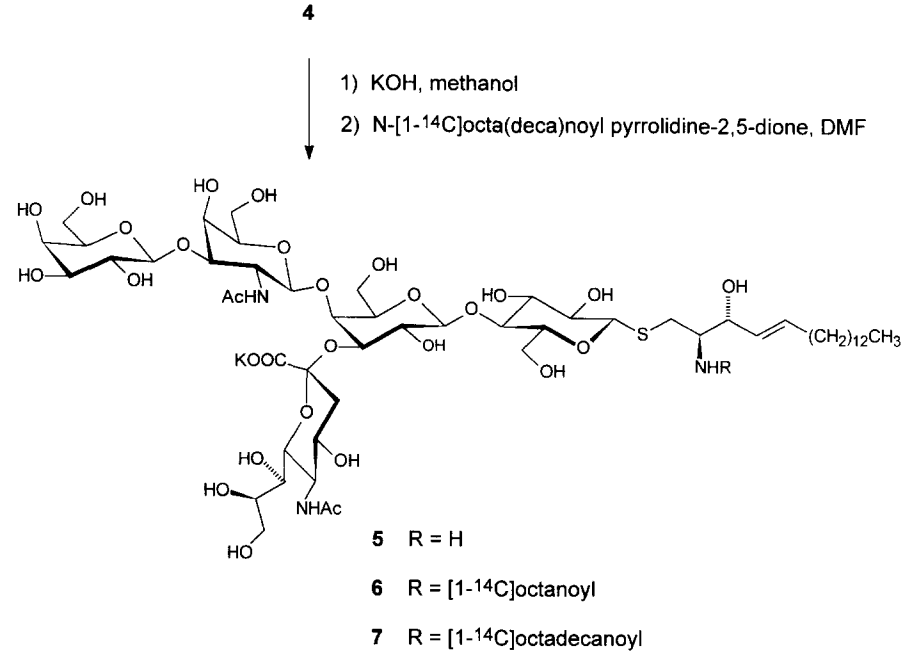
Scheme 1.



Scheme 2.

gliosides with an intact glucose residue that is linked *O*-glycosidically to a defined and labeled ceramide residue has been limited so far to the synthesis of gangliosides GM3 and GM1 containing either a spin label or a fluorescent tag in its ceramide residue [11]. This approach, however, takes advantage of already existing interglycosidic bonds, thus facilitating the synthesis of labeled gangliosides.

Gangliosides from bovine brain were isolated and treated with sialidase to convert oligosialogangliosides GD1a, GD1b, GT1b and GQ1b into GM1. During this hydrolysis all sialyl residues are split but for the one that is linked to position-3 of the inner galactose moiety [14]. Ganglioside GM1 was then treated with ozone and fragmented to furnish the intact GM1 sugar in 60% yield after purification by



Scheme 3.

anion-exchange and gel-permeation chromatography [11].

Treatment of the ganglioside GM1 sugar, monosialogangliotetraose [11], with acetic anhydride in pyridine afforded a mixture of the α and β anomer of pentadeca-*O*-acetyl-monosialogangliotetraose sialoyl-II^2-lactone (**1a** and **1b**) in 72% yield (Scheme 1). This lactone was characterized by FABMS as well as by ^{1}H NMR spectroscopy. The abundance of the signal at 5.70 ppm indicated that during acetylation of monosialogangliotetraose the β-acetate was predominantly formed ($\beta:\alpha = 1.7:1.0$). The formation of a methyl ester for protection of the carboxyl function was expendable since lactonization occurred spontaneously and simultaneously in the acetylation step. Bromination of the mixture of **1a** and **1b** in ice-cold 33% hydrogen bromide in acetic acid gave the α-glycosyl bromide **2** in 94% yield after freeze-drying from benzene as an almost pure product that was used without further purification. Nearly complete conversion of **2** into the β thioacetate **3** was accomplished using potassium thioacetate in methanol (Scheme 1).

The ^{1}H NMR spectra of **1** and **3** were compared: Resonances for H-1 of the glucose residue of **1a** and **1b** were observed at δ 6.26 and 5.70 ppm for the α and β anomers of the acetates, respectively. The resonances for H-1 of the glucose residue of the thioacetyl compound **3** were observed at δ 5.24 ppm clearly indicating the shielding effect of the sulfur atom on the anomeric center. The β-anomeric configuration of the thioacetyl residue of tetradeca-*O*-acetyl-1-*S*-acetyl-1-thio-β- monosialogangliotetraose sialoyl-II^2-lactone **3** is clearly shown by the spin–spin coupling of protons H-1 and H-2 of the glucose moiety ($J_{1,2}$ 10.4 Hz).

This thioacetate **3** could successfully be condensed with (2*R*,3*R*,4*E*)-3-*O*-benzoyl-2-dichloroacetamido-1-iodo-4-octadecen-3-ol [15] in methanolic sodium acetate to produce the desired β thioglycoside **4**, i.e., a protected lyso-SGM1 [*S*-(tetradeca-*O*-acetyl-β-monosialogangliotetraosyl)-(1 → 1)-(2*R*,3*R*,4*E*)-3-benzoyloxy-2-dichloroacetamido-4-octadecen-1-thiol methyl ester], in 52% yield after purification (Scheme 2). During this condensation in methanol, under mild alkaline conditions, the lactone ring opened, yielding the methyl ester of the sialic acid residue and a free hydroxyl group at position-2 of the inner galactose moiety of the GM1 sugar. The structure of compound **4** could clearly be demonstrated by ^{1}H NMR spectroscopy. The β-anomeric configuration of the thioglycosidic linkage is evidenced by the spin–spin

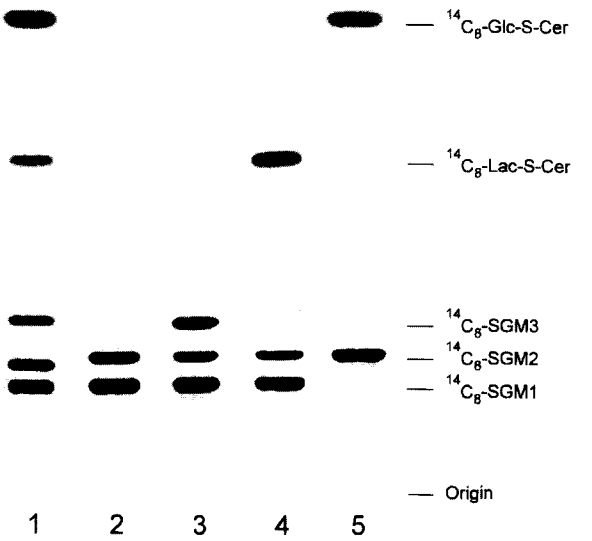


Fig. 1. TLC separation of the products obtained from $^{14}C_8$-SGM1 (**6**) by treatment with various glycohydrolases. As described in Experimental, in four assay mixtures 0.5 nmol of **6** was treated with GM1-β-galactosidase alone or sequentially with GM1-β-galactosidase and β-hexosaminidase A, or with GM1-β-galactosidase, β-hexosaminidase A and sialidase, or with GM1-β-galactosidase, β-hexosaminidase A, sialidase and again with GM1-β-galactosidase. All assay mixtures were then freed of salts as described in Experimental. The degradation products were separated by TLC with 60:35:8 $CHCl_3$–MeOH–15 mM $CaCl_2$ as the mobile phase and visualized by exposure to X-ray film. Lane 1: Reference lipids from top to bottom are as follows: $^{14}C_8$-Glc-S-Cer [14], i.e., *S*-(β-D-glucopyranosyl)-(1 → 1)-(2*R*,3*R*,4*E*)-2-[1-^{14}C]octanamido-3-hydroxy-4-octadecen-1-thiol, $^{14}C_8$-Lac-S-Cer [14], i.e., β-D-galactopyranosyl-(1 → 4)-*S*-(β-D-glucopyranosyl)-(1 → 1)-(2*R*,3*R*,4*E*)-2-[1-^{14}C]octanamido-3-hydroxy-4-octadecen-1-thiol, $^{14}C_8$-SGM3, i.e., α-D-*N*-acetylneuraminosyl-(2 → 3)-β-D-galactopyranosyl-(1 → 4)-*S*-(β-D-glucopyranosyl)-(1 → 1)-(2*R*,3*R*,4*E*)-2-[1-^{14}C]-octanamido-3-hydroxy-4-octadecen-1-thiol (this analog of ganglioside GM3 was prepared as described here for the synthesis of $^{14}C_8$-SGM1 but using sialyllactose from cow colostrum as the starting oligosaccharide [11], $^{14}C_6$-GM2 [8], i.e., ganglioside GM2 containing a [1-^{14}C]hexanoyl residue in place of the native fatty acyl residue, $^{14}C_8$-SGM1. Lane 2: $^{14}C_8$-SGM1 treated with β-galactosidase yielding $^{14}C_8$-SGM2 which migrates a little further than the reference lipid $^{14}C_6$-GM2 due to the longer acyl chain and sulfur in place of oxygen [8]. Lane 3: $^{14}C_8$-SGM1 treated with β-galactosidase and β-hexosaminidase A yielding $^{14}C_8$-SGM2 and $^{14}C_8$-SGM3. Lane 4: Same as for Lane 3 with additional sialidase treatment yielding $^{14}C_8$-Lac-S-Cer at the expense of $^{14}C_8$-SGM3. Lane 5: Same as for lane 4 with a second β-galactosidase treatment yielding $^{14}C_8$-Glc-S-Cer at the expense of $^{14}C_8$-Lac-S-Cer. During this treatment the still remaining $^{14}C_8$-SGM1 (see lanes 2 to 4) is almost completely hydrolyzed to $^{14}C_8$-SGM2.

coupling of protons H-1 and H-2 of the glucose moiety ($J_{1,2}$ 9.9 Hz). This coupling is in the same range as was found for the thioacetate **3**. The fact that during the condensation reaction the hydroxyl group at position-2 of the inner galactose moiety became free is also evidenced by the resonances for H-2 of galactose II, the inner galactose moiety of the GM1 sugar, at δ 3.43 ppm. In contrast, the corresponding fully acetylated terminal galactose showed a resonance for H-2 at δ 5.02 ppm. The structure of compound **4** was also proved by FAB mass spectrometry. Peaks observed for $[M + H^+]$ at *m/z* 2112, 2114 and 2116 as well as for $[M + Na^+]$ at *m/z* 2134, 2136 and 2138 indicated the presence of two chlorine atoms as expected for the dichloroacetamido residue. In addition, a similar triplet occurred at *m/z* 2012, 2014 and 2016 for $[(M + Na^+)-PhCO_2H]$, which is indicative for the presence of the benzoyloxy residue.

Finally, removal of all protecting groups of **4** with 0.2 M potassium hydroxide in methanol under argon afforded the lyso-SGM1 **5**, i.e., β-monosialoganglio-tetraosyl thiosphingosine, in 81% yield (Scheme 3). This 'lyso' compound could easily be converted into the desired radioactive GM1 analogues $^{14}C_8$-SGM1 **6** and $^{14}C_{18}$-SGM1 **7** by selective *N*-acylation [11] with the *N*-succinimidoyl derivative of [1-^{14}C]octanoic acid and [1-^{14}C]octadecanoic acid, respectively (Scheme 3). The structures of these analogues were proved by FABMS and by sequential enzymatic degradation using ganglioside specific glycohydrolases in the presence of detergent. The results, as demonstrated in Fig. 1 and Fig. 2, show that $^{14}C_8$-SGM1 **6** and $^{14}C_{18}$-SGM1 **7** were sequentially hydrolyzed to the respective analogues of GM2, GM3, lactosylceramide and glucosylceramide. The latter compound could not be further deglycosylated by mammalian glucosylceramidase, i.e., by β-D-glucosyl-*N*-acylsphingosine glucohydrolase (EC 3.2.1.45), due to the thioglycosidic linkage [15]. It is interesting to note that in general the glycohydrolases used here seem to have acted more on the shorter acyl chain analogue $^{14}C_8$-SGM1 **6** (Fig. 1, lanes 2 to 5) than on the longer acyl chain analogue $^{14}C_{18}$-SGM1 **7** (Fig. 2, lanes 2 to 5). This is especially the case for the β-hexosaminidase A, i.e., β-*N*-acetyl-D-hexosaminide *N*-acetylhexosaminohydrolase (EC 3.2.1.52), which converted much more of $^{14}C_8$-SGM2 into $^{14}C_8$-SGM3 (Fig. 1, lane 3) than of $^{14}C_{18}$-SGM2 into the respective ganglioside analogue GM3 (Fig. 2, lane 3). Furthermore, the action of sialidase (*N*-acetylneuraminylhydrolase; EC 3.2.1.18) did not completely hydrolyze $^{14}C_{18}$-SGM3 into the respective lactosylceramide analogue (Fig. 2, lane 4). One possible reason may be that the long acyl chain derivatives form more stable mixed micelles with the detergent than do the short chain analogues and are thus less susceptible to enzyme action. Similar observations have been made in [16]

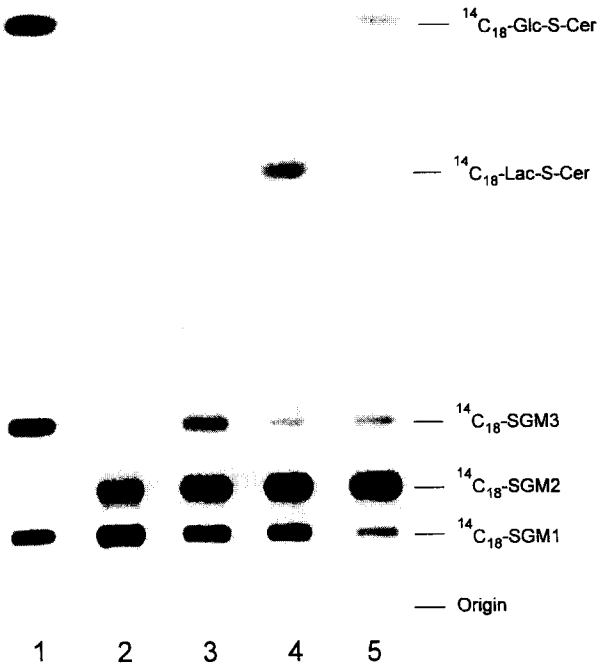


Fig. 2. TLC separation of the products obtained from $^{14}C_{18}$-SGM1 (**7**) by treatment with various glycohydrolases. The long acyl chain analog **7** was processed as described for $^{14}C_8$-SGM1 (**6**) in Fig. 1. In this case the degradation products were separated by TLC with 60:35:6:2 $CHCl_3$–MeOH–water–acetic acid as the mobile phase to improve separation of the long acyl chain derivatives from the detergent. Radioactive products were visualized by exposure to X-ray film. Lane 1: Reference lipids from top to bottom are as follows: $^{14}C_{18}$-Glc-S-Cer, i.e., *S*-(β-D-glucopyranosyl)-(1 → 1)-(2*R*,3*R*,4*E*)-2-[1-^{14}C]octadecanamido-3-hydroxy-4-octadecen-1-thiol (prepared as described in [14] for $^{14}C_8$-Glc-S-Cer using [1-^{14}C]stearic acid, $^{14}C_{18}$-SGM3, i.e., α-D-*N*-acetylneuraminosyl-(2 → 3)-β-D-galactopyranosyl-(1 → 4)-*S*-(β-D-glucopyranosyl)-(1 → 1)-(2*R*,3*R*,4*E*)-2-[1-^{14}C]octadecanamido-3-hydroxy-4-octadecen-1-thiol (prepared as described here for the synthesis of $^{14}C_{18}$-SGM1 using sialyllactose as starting oligosaccharide), $^{14}C_{18}$-SGM1. Lane 2: $^{14}C_{18}$-SGM1 treated with β-galactosidase yielding $^{14}C_{18}$-SGM2. Lane 3: $^{14}C_8$-SGM1 treated with β-galactosidase and β-hexosaminidase A yielding $^{14}C_{18}$-SGM2 and $^{14}C_{18}$-SGM3. Lane 4: Same as for lane 3 with additional sialidase treatment yielding $^{14}C_{18}$-Lac-S-Cer. Lane 5: Same as for lane 4 with a second β-galactosidase treatment yielding $^{14}C_{18}$-Glc-S-Cer. During this treatment some of the remaining $^{14}C_{18}$-SGM1 (see lanes 2 to 4) is further hydrolyzed to $^{14}C_{18}$-SGM2.

where analogues of ganglioside GM1 with varying acyl chain lengths have been subjected to the action of GM1-β-galactosidase (β-D-galactoside galactohydrolase; EC 3.2.1.23) in the presence of sodium taurodeoxycholate.

In this paper we have shown that, starting from the readily available GM1 sugar, it is possible to prepare, with relatively few, simple synthetic steps, labeled derivatives of ganglioside GM1 that contain a thioglycosidic linkage between the oligosaccharide and the ceramide residue. This procedure is applicable to monosialogangliosides GM3 and GM2 as well (data not shown). The suitability of these labeled derivatives for the study of intracellular lipid transport and ganglioside metabolism is currently under investigation and will be published elsewhere.

3. Experimental

General methods.—^{1}H NMR spectra were recorded in $CDCl_3$ or in 10:1 $CDCl_3$–CD_3OD at 500 MHz on a Bruker AMX 500 NMR spectrometer (Karlsruhe, Germany). Chemical shifts (δ) are expressed in ppm downfield from tetramethylsilane. Fast-atom bombardment mass spectra (FABMS) were recorded either in the negative- or positive-ion mode on a ZAB HF instrument (VG Analytical, Manchester, UK) [17]. Elemental analyses were carried out with a CHN-O-Rapid analyzer (Heraeus, Osterode, Germany). The progress of reactions and column chromatographic elution profiles were routinely followed by thin-layer chromatography (TLC) on glass plates precoated with Silica Gel 60 (E. Merck, Darmstadt, Germany) in tanks with vapor saturation. Detection was effected by spraying the plates with a mixture of 500:10:2 acetic acid–sulfuric acid–anisaldehyde, followed by heating for 10 min at 120 °C [18], or by dipping the chromatograms into a solution of ceric ammonium nitrate $[Ce(NH_4)_2(NO_3)_6]$ in 20% sulfuric acid and charring them with a heat gun. Lyso-SGM1 was also detected by spraying with ninhydrin. Radioactivity was determined using a Tri-Carb 1900 CA liquid scintillation counter (Canberra Packard, Frankfurt, Germany). Radioactive spots were localized and quantified by the use of a Fuji BAS 1000 Bio Imaging analyzer (Raytest, Pforzheim, Germany). In addition, TLC plates were exposed to X-ray film (Kodak X-Omat XAR-5). Column chromatography was performed using Silica Gel Si-60 (0.015–0.040 mm, E. Merck). Amphiphilic reaction products were freed of salts and other polar and water-soluble materials by reversed-phase chromatography using LiChroprep RP-18 (0.063–0.200 mm, E. Merck) similar to the procedure described [19].

The pure ganglioside GM1 sugar, monosialogangliotetraose, $II^3NeuAcGgOse_4$, β-D-galactopyranosyl-(1 → 3)-2-acetamido-2-deoxy-β-D-galactopyranosyl-(1 → 4)-[α-D-N-acetylneuraminosyl-(2 → 3)]-β-D-galactopyranosyl-(1 → 4)-β-D-glucopyranose, was prepared according to a modified procedure [11] developed by Wiegandt and Baschang [13] starting from bovine brain ganglioside GM1.

Pentadeca - O - acetyl - monosialogangliotetraose sialoyl-II^2-lactone, i.e., *2,3,4,6-tetra-O-acetyl-β-D-galactopyranosyl-(1 → 3)-2-acetamido-2-deoxy-4,6-di-O-acetyl-β-D-galactopyranosyl-(1 → 4)-[4,7,8,9-tetra-O-acetyl-α-D-N-acetylneuraminosyl-(2 → 3)]-6-O-acetyl-β-D-galactopyranosyl-(1 → 4)-1,2,3,6-tetra-O-acetyl-α,β-D-glucopyranose sialoyl-II^2-lactone* (**1a**, **1b**).—A solution of monosialogangliotetraose, $II^3NeuAcGgOse_4$, (100 mg, 100 μmol) in a mixture of water (0.2 mL) and pyridine (4.0 mL) was treated with acetic anhydride (2 mL). The resulting solution was briefly cooled in ice until no more heat evolved and then kept for 3 h at 50 °C under argon before it was azeotropically dried in vacuo with several additions of toluene. The residue was now fully acetylated with acetic anhydride (4 mL) in pyridine (8 mL) at 50 °C for 24 h under argon. After complete removal of pyridine, acetic acid, and acetic anhydride by azeotropic distillation with toluene under reduced pressure, the brownish residue was purified by chromatography under medium pressure on a column of silica gel with 1:1 acetone–toluene. The elution profile was monitored by TLC with 1:1 acetone–toluene as the mobile phase. All fractions containing pure **1a** and **1b** ($R_f = 0.19$) were dried and freeze-dried from benzene to give a pure mixture of α- and β-pentadeca-*O*-acetyl-monosialogangliotetraose sialoyl-II^2-lactone (120 mg, 72% yield). ^{1}H NMR ($CDCl_3$): glucose (I-α) unit δ 6.27 (d, $J_{1,2}$ 3.7 Hz, H-1), 5.53 (dd, 1 H, $J_{2,3}$ 9.9 Hz, $J_{3,4}$ 9.6 Hz, H-3), 5.03 (dd, 1 H, H-2), 4.25 (dd, 1 H, $J_{5,6}$ 4.5 Hz, $J_{6a,6b}$ 12 Hz, H-6b), 4.14 (m, 1 H, H-6a), 3.91 (m, 1 H, H-4), 3.64 (m, 1 H, H-5); glucose (I-β) unit δ 5.70 (d, $J_{1,2}$ 8.3 Hz, H-1), 5.32 (dd, 1 H, $J_{2,3}$ 9.3 Hz, $J_{3,4}$ 9.3 Hz, H-3), 5.06 (dd, 1 H, Hz, H-2), 4.41 (m, 1 H, H-6a), 4.06 (m, 1 H, H-6b), 3.96 (m, 1 H, H-4), 3.82 (m, 1 H, H-5); galactose (II) unit δ 4.59 (m, 1 H, H-2), 4.43 (d, 1 H, $J_{1,2}$ 7.3 Hz, H-1), 4.19 (bs, 1 H, H-4), 3.79 (ddd, 1 H, $J_{4,5}$ 2.4 Hz, $J_{5,6a}$ 6.7 Hz, $J_{5,6b}$ 10.5 Hz, H-5), 3.77 (dd, 1 H, $J_{2,3}$ 6.7 Hz, $J_{3,4}$ 2.4 Hz,

H-3); GalNAc (III) unit δ 6.51 (d, 1 H, $J_{NH,2}$ 7.7 Hz, NH), 5.40 (bs, 1 H, H-4), 5.09 (d, 1 H, $J_{1,2}$ 11.9 Hz, H-1), 4.57 (m, 1 H, H-3), 3.89 (m, 1 H, H-5), 3.57 (m, 1 H, H-2); galactose (IV) unit δ 5.30 (bs, 1 H, H-4), 5.07 (dd, 1 H, $J_{1,2}$ 7.9 Hz, $J_{2,3}$ 6.6 Hz, H-2), 4.94 (dd, 1 H, $J_{3,4}$ 3.5 Hz, H-3), 4.75 (d, 1 H, H-1); NeuAc unit δ 5.59 (m, 1 H, H-4), 5.47 (m, 1 H, NH), 5.28 (m, 1 H, H-8), 5.25 (dd, 1 H, $J_{6,7}$ 2.0 Hz, $J_{7,8}$ 9.7 Hz, H-7), 4.47 (dd, 1 H, $J_{8,9a}$ 2.0 Hz, $J_{9a,9b}$ 12.5 Hz, H-9a), 4.12 (dd, 1 H, $J_{4,5}$ 5.5 Hz, $J_{5,6}$ 6.0 Hz, H-5), 3.98 (dd, 1 H, $J_{8,9b}$ 6.9 Hz, H-9b), 3.74 (m, 1 H, H-6), 2.48 (dd, 1 H, $J_{3ax,3eq}$ 13.3 Hz, $J_{3,4}$ 5.7 Hz, H-3eq), 1.84 (dd, 1 H, H-3ax); acetyl groups δ 2.20–2.00, 1.96, 1.89, 1.88, 1.69 (15 s, 51 H, 17 $COCH_3$). FABMS: ($C_{67}H_{90}N_2O_{43}$, MW 1611.43) $[M + H^+]$ at *m/z* 1611, and $[M + Na^+]$ at *m/z* 1633. Anal. Calcd for $C_{67}H_{90}N_2O_{43} \cdot H_2O$ (1629.44): C, 49.39; H, 5.69; N, 1.72. Found: C, 49.41; H, 5.73; N, 1.55.

*Tetradeca-*O*-acetyl-α-monosialogangliotetraosyl bromide sialoyl-II²-lactone* (**2**), i.e., *2,3,4,6-tetra-*O*-acetyl-β-*D*-galactopyranosyl-(1 → 3)-2-acetamido-2-deoxy-4,6-di-*O*-acetyl-β-*D*-galactopyrano-syl-(1 → 4)-[4,7,8,9-tetra-*O*-acetyl-α-*D*-N-acetylneuraminosyl-(2 → 3)]-6-*O*-acetyl-β-*D*-galactopyranosyl-(1 → 4)-2,3,6-tri-*O*-acetyl-α-*D*-glucopyranosyl bromide sialoyl-II²-lactone.*—Pentadeca-*O*-acetyl-monosialoganglio-tetraose sialoyl-II²-lactone (**1a, 1b**) (100 mg, 62 μmol) was dissolved in 33% hydrogen bromide in acetic acid (2 mL) in a screw capped vial and kept for 30 min at 0 °C and then for further 30 min at 20 °C. The reaction mixture was then quickly transferred to a separatory funnel containing ice-cold $CHCl_3$ (15 mL). Hydrogen bromide and acetic acid were extracted into ice water. The organic layer was washed several times with ice water, 0.02 M $NaHCO_3$, and again with ice water. After brief drying over anhydrous Na_2SO_4, the $CHCl_3$ layer was dried in a nitrogen stream with no heating. The residue was freeze-dried from benzene. TLC (1:1 acetone–toluene) revealed one single spot ($R_f = 0.21$) with no residual starting material. The crude α-glycosyl bromide **2** (96 mg, 94%) was used without further purification. We have observed that the addition of 2% acetic anhydride to the solution of hydrogen bromide in acetic acid 3 h before its use was beneficial for the formation of the α-glycosyl bromide.

*Tetradeca-*O*-acetyl-1-*S*-acetyl-1-thio-β-monosialogangliotetraose sialoyl-II²-lactone* (**3**), i.e., *2,3,4,6-tetra-*O*-acetyl-β-*D*-galactopyranosyl-(1 → 3)-2-acetamido-2-deoxy-4,6-di-*O*-acetyl-β-*D*-galactopyranosyl-(1 → 4)-[4,7,8,9-tetra-*O*-acetyl-α-*D*-N-acetylneuraminosyl-(2 → 3)]-6-*O*-acetyl-β-*D*-galactopyranosyl-(1 → 4)-2,3,6-tri-*O*-acetyl-1-*S*-acetyl-1-thio-β-*D*-glucopyranose sialoyl-II²-lactone.*—The α-glycosyl bromide **2** (90 mg, 55 μmol) was treated with potassium thioacetate (9.1 mg, 80 μmol) in MeOH (0.5 mL) for 12 h at 20 °C. Thereafter, the mixture was diluted with EtOAc (9 mL). After the addition of water (3 mL) and 4 M sodium hydrogen sulfite (0.2 mL), the product was extracted into EtOAc. The organic layer was washed several times with water (5 mL), dried over Na_2SO_4 and concentrated. The residue was purified on a column of silica gel with 1:1 acetone–toluene to afford tetradeca-*O*-acetyl-1-*S*-acetyl-1-thio-β-monosialogangliotetraose sialoyl-II²-lactone (**3**) (82.2 mg, 92%) after freeze-drying from benzene. TLC of **3** in 1:1 acetone–toluene as the mobile phase revealed one single spot ($R_f = 0.20$) upon detection with either anisaldehyde spray or ceric ammonium nitrate dipping reaction. ^{1}H NMR ($CDCl_3$): glucose (I) unit δ 5.34 (m, 1 H, H-3), 5.24 (d, $J_{1,2}$ 10.4 Hz, H-1), 5.02 (dd, 1 H, $J_{2,3}$ 9.3 Hz, H-2), 4.06 (m, 1 H, H-6b), 3.80 (m, 1 H, H-5), 3.39 (m, 1 H, H-4); galactose (II) unit δ 4.55 (dd, 1 H, $J_{1,2}$ 7.3 Hz, $J_{2,3}$ 10.7 Hz, H-2), 4.40 (d, 1 H, H-1), 4.20 (bs, 1 H, H-4), 4.07 (m, 1 H, H-6a), 3.78 (m, 1 H, H-5), 3.74 (dd, 1 H, $J_{3,4}$ 2.4 Hz, H-3); GalNAc (III) unit δ 6.49 (d, 1 H, $J_{NH,2}$ 8.1 Hz, NH), 5.39 (d, 1 H, $J_{3,4}$ 3.5 Hz, H-4), 5.07 (d, 1 H, $J_{1,2}$ 11.8 Hz, H-1), 4.53 (m, 1 H, H-3), 3.87 (m, 1 H, H-5), 3.58 (m, 1 H, H-2); galactose (IV) unit δ 5.31 (bs, 1 H, H-4), 5.06 (dd, 1 H, $J_{1,2}$ 7.8 Hz, $J_{2,3}$ 10.5 Hz, H-2), 4.95 (dd, 1 H, $J_{3,4}$ 3.0 Hz, H-3), 4.77 (d, 1 H, H-1), 4.43 (m, 1 H, H-6b), 4.38 (m, 1 H, H-6a); NeuAc unit δ 5.49 (m, 1 H, H-4), 5.43 (m, 1 H, $J_{NH,2}$ 10.2 Hz, NH), 5.27 (m, 1 H, H-8), 5.23 (m, 1 H, H-7), 4.46 (dd, 1 H, $J_{8,9a}$ 2.0 Hz, $J_{9a,9b}$ 12.0 Hz, H-9a), 4.12 (dd, 1 H, $J_{4,5}$ 5.5 Hz, $J_{5,6}$ 6.0 Hz, H-5), 3.94 (m, 1 H, H-9b), 3.67 (m, 1 H, H-6), 2.48 (dd, 1 H, $J_{3ax,3eq}$ 13.4 Hz, $J_{3eq,4}$ 5.5 Hz, H-3eq), 1.84 (dd, 1 H, $J_{3ax,4}$ 2.0 Hz, H-3ax); acetyl groups δ 2.20–2.00, 1.96, 1.89 (11 s, 51 H, 17 $COCH_3$). FABMS: ($C_{67}H_{90}N_2O_{42}S$, MW 1627.49) $[M + H^+]$ at *m/z* 1627, and $[M + Na^+]$ at *m/z* 1649. Anal. Calcd for $C_{67}H_{90}N_2O_{42}S \cdot H_2O$ (1645.50): C, 48.91; H, 5.64; N, 1.70; S, 1.95. Found: C, 49.14; H, 5.83; N, 1.44; S, 2.09.

Protected lyso-SGM1, S*-(tetradeca-*O*-acetyl-β-monosialogangliotetraosyl)-(1 → 1)-(2*R*, 3*R*,4*E*)-3-benzoyloxy-2-dichloroacetamido-4-octadecen-1-thiol methyl ester* (**4**), i.e., *2,3,4,6-tetra-*O*-acetyl-β-*D*-galactopyranosyl-(1 → 3)-2-acetamido-2-deoxy-4,6-di-*O*-acetyl-β-*D*-galactopyranosyl-(1 → 4)-[4,7,8,9-tetra-*

O-*acetyl*-α-D-N-*acetylneuraminosyl*-(2 → 3)]-6-O-*acetyl*-β-D-*galactopyranosyl*-(1 → 4)-2,3,6-*tri*-O-*acetyl*-β-D-*glucopyranosyl*-(1 → 1)-(2R,3R,4E)-3-*benzoyloxy*-2-*dichloroacetamido*-4-*octadecen*-1-*thiol methyl ester*.—Under an argon atmosphere tetradeca-*O*-acetyl-1-*S*-acetyl-1-thio-β-monosialogangliotetraose sialoyl-II^2-lactone (**3**) (70 mg, 43 μmol) and (2*R*,3*R*,4*E*)-3-*O*-benzoyl-2-dichloroacetamido-1-iodo-4-octadecen-3-ol [15] (30 mg, 48 μmol) were dissolved in a 1:2 mixture (0.40 mL) of acetone and 1 M NaOAc in MeOH; the mixture was stirred at 20 °C for 8 h. The reaction mixture, after buffering NaOAc with acetic acid (0.005 mL), was dried in a nitrogen jet. The resulting residue of crude **4** was purified by chromatography on a column of silica gel (50 g) using a linear gradient from 2:8 acetone–toluene (300 mL) to 8:2 acetone–toluene (300 mL) to afford, after freeze-drying from benzene, solid **4** (47.3 mg, 52% with respect to **3**) as an amorphous mass. TLC of **4** in 1:1 acetone–toluene as the mobile phase revealed one single spot ($R_f = 0.35$) upon detection with either anisaldehyde spray or ceric ammonium nitrate dipping reaction. ^{1}H NMR (10:1 $CDCl_3$–CD_3OD): sphingosine unit δ 7.25 (d, 1 H, $J_{NH,2}$ 9.0 Hz, NH), 5.82 (td, 1 H, $J_{4,5}$ 15.4 Hz, $J_{5,6}$ 6.8 Hz, H-5), 5.54 (dd, 1 H, $J_{2,3}$ 7.3 Hz, $J_{3,4}$ 7.3 Hz, H-3), 5.42 (dd, 1 H, H-4), 4.38 (m, 1 H, H-2), 2.96 (m, 1 H, H-1b), 2.84 (dd, 1 H, $J_{1a,1b}$ 13.6 Hz, $J_{1a,2}$ 5.5 Hz, H-1a), 1.96 (m, 2 H, H-6), 1.31–1.14 (m, 22 H, 11 CH_2), 0.82 (t, 3 H, $J_{17,18}$ 7.0 Hz, CH_3); glucose (I) unit δ 5.12 (dd, 1 H, $J_{2,3}$ 9.4 Hz, $J_{3,4}$ 9.4 Hz, H-3), 4.90 (dd, 1 H, $J_{1,2}$ 9.9 Hz, H-2), 4.61 (dd, 1 H, $J_{5,6a}$ 1.5 Hz, $J_{6a,6b}$ 11.6 Hz, H-6a), 4.44 (d,1 H, H-1), 4.19 (m, 1 H, $J_{5,6b}$ 6.6 Hz, H-6b), 3.78 (m, 1 H, H-4), 3.65 (m, 1 H, H-5); galactose (II) unit δ 4.31 (d, 1 H, $J_{1,2}$ 7.6 Hz, H-1), 3.92 (m, 1 H, H-3), 3.56 (d, 1 H, $J_{3,4}$ 2.7 Hz, H-4), 3.43 (dd, 1 H, $J_{2,3}$ 9.7 Hz, H-2); galactose (IV) unit δ 5.30 (d, 1 H, $J_{3,4}$ 3.3 Hz, H-4), 5.02 (dd, 1 H, $J_{1,2}$ 7.8 Hz, $J_{2,3}$ 10.4 Hz, H-2), 4.88 (dd, 1 H, H-3), 4.54 (d, 1 H, H-1), 4.18 (dd, 1 H, $J_{5,6a} < 1.5$ Hz, $J_{6a,6b}$ 13.6 Hz, H-6a), 3.95 (m, 1 H, H-5); NeuAc unit δ 4.81 (m, 1 H, H-4), 3.91 (m, 1 H, H-5), 2.73 (dd, 1 H, $J_{3ax,3eq}$ 13.3 Hz, $J_{3eq,4}$ 4.6 Hz, H-3eq), 1.84 (m, 1 H, H-3ax); acetyl groups δ 2.10–1.96, 1.92, 1.91, 1.88, 1.81 (12 s, 48 H, 16 $COCH_3$); protecting group unit δ 7.96 (m, 2 H, H-$Bz_{2,6}$), 7.52 (m, 1 H, H-Bz_4), 7.39 (m, 2 H, H-$Bz_{3,5}$), 5.94 (s, 1 H, $CHCl_2$), 3.79 (s, 3 H, CH_3OCO). FABMS: ($C_{93}H_{131}Cl_2N_3O_{45}S$, MW 2114.02) [M + H$^+$] at *m/z* 2112, 2114, 2116, [M + Na$^+$] at *m/z* 2134, 2136, 2138, and [(M + Na)$^+$–$PhCO_2H$] at *m/z* 2012, 2014, 2016.

Lyso-*SGM1*, S-β-*monosialogangliotetraosyl*-(1 → 1)-(2R,3R,4E)-2-*amino*-3-*hydroxy*-4-*octadecen*-1-*thiol* (**5**), i.e., β-D-*galactopyranosyl*-(1 → 3)-*acetamido*-2-*deoxy*-β-D-*galactopyranosyl*-(1 → 4)-(α-D-N-*acetylneuraminosyl*-(2 → 3))-β-D-*galactopyranosyl*-(1 → 4)-S-β-D-*glucopyranosyl*-(1 → 1)-(2R,3R,4E)-2-*amino*-3-*hydroxy*-4-*octadecen*-1-*thiol*.—Protected lyso-SGM1 **4** (30 mg, 14 μmol) was hydrolyzed with 0.2 M KOH in deaerated and argon-saturated MeOH (6 mL) for 3 h at 65 °C. Following cooling to room temperature, the solution was buffered with acetic acid (0.12 mL) and diluted with water (24 mL). The mixture thus obtained was passed over LiChroprep RP-18 (4 mL) to adsorb **5**. Salts and other water-soluble material were washed out with water, and the retained **5** was subsequently eluted with 0.20 M methanolic ammonia (15 mL). Lyso-SGM1 was finally purified by chromatography on a column of silica gel (100 g) with 60:35:8 $CHCl_3$–MeOH–2.5 M ammonia to afford, after lyophilizing, pure **5** (14.7 mg, 81%). TLC of **5** in 60:40:9 $CHCl_3$–MeOH–2.5 M ammonia as the mobile phase revealed one single spot ($R_f = 0.09$) upon detection with either ninhydrin spray or ceric ammonium nitrate dipping reaction. FABMS: ($C_{55}H_{97}N_3O_{29}S$, MW 1296.43) [M + H$^+$] at *m/z* 1296, and [M + Na$^+$] at *m/z* 1318.

$^{14}C_8$-*SGM1*, S-β-*monosialogangliotetraosyl*-(1 → 1)-(2R,3R,4E)-2-[1-^{14}C]*octanamido*-3-*hydroxy*-4-*octadecen*-1-*thiol* (**6**), i.e., β-D-*galactopyranosyl*-(1 → 3)-2-*acetamido*-2-*deoxy*-β-D-*galactopyranosyl*-(1 → 4)-[α-D-N-*acetylneuraminosyl*-(2 → 3)]-β-D-*galactopyrano*-*syl*-(1 → 4)-S-β-D-*glucopyranosyl*-(1 → 1)-(2R,3R,4E)-[1-^{14}C]*octanamido*-3-*hydroxy*-4-*octadecen*-1-*thiol*.—A small amount of **5** (3.8 mg, 2.9 μmol) dissolved in DMF (0.2 mL) was, after the addition of *N*,*N*-diisopropylethylamine (0.01 mL), dried in a stream of nitrogen to remove any volatile amino and imino compounds that may otherwise interfere with the *N*-acylation reaction. The residue was then redissolved in DMF (0.2 mL) and *N*,*N*-diisopropylethylamine (0.01 mL) and treated at 40 °C for 48 h under argon with the *N*-succinimidoyl derivative of [1-^{14}C]octanoic acid prepared from [1-^{14}C]octanoic acid sodium salt (2.146 GBq/mmol, ICN Biomedicals, Eschwege, Germany) as described [15]. After evaporation of the solvents under reduced pressure, the crude product was purified by HPLC on ProSep C_{18} (1 × 25 cm) (Latek, Heidelberg, Germany) with 85:15 MeOH–water to yield **6** (2.5 mg, 60%). TLC of **6** in 60:35:8 $CHCl_3$–MeOH–15 mM $CaCl_2$ as the mobile phase revealed one single spot

($R_f = 0.16$) upon detection with either anisaldehyde spray or ceric ammonium nitrate dipping reaction. Exposure to X-ray-sensitive film, as well as analysis by the use of a Fuji BAS 1000 Bio Imaging analyzer, also showed one single spot, thus proving that the radiochemical purity of **6** was better than 99%. The specific radioactivity as determined by weight and sialic acid analysis according to Svennerholm [20] as modified by Miettinen and Takki-Luukkainen [21] was 2.14 ± 0.06 GBq/mmol. FABMS: ($C_{63}H_{111}N_3O_{30}S$, MW 1422.63) $[M + H^+]$ at *m/z* 1424, and $[M + Na^+]$ at *m/z* 1446. The shift of 2 *amu* in the observed ions is due to the high abundance of the isotope ^{14}C at this high specific radioactivity of this compound.

$^{14}C_{18}$-SGM1, S-β-monosialogangliotetraosyl-(1 → 1)-(2R,3R,4E)-2-[1-^{14}C]octadecanamido-3-hydroxy-4-octadecen-1-thiol (**7**), i.e., *β-D-galactopyranosyl-(1 → 3)-2-acetamido-2-deoxy-β-D-galactopyranosyl-(1 → 4)-[α-D-N-acetylneuraminosyl-(2 → 3)]-β-D-galactopyrano-syl-(1 → 4)-S-β-D-glucopyranosyl-(1 → 1)-(2R,3R,4E)-[1-^{14}C]octadecanamido-3-hydroxy-4-octadecen-1-thiol.*—Starting from **5** (3.8 mg, 2.9 μmol) this compound was prepared exactly as for **6** using the *N*-succinimidoyl derivative of [1-^{14}C]octadecanoic acid prepared from [1-^{14}C]octadecanoic acid (2.146 GBq/mmol, Amersham Life Science, Braunschweig, Germany) as described [15]. The yield of the purified compound **7** was 46%. TLC of **7** either in 60:40:9 $CHCl_3$–MeOH–2.5 M ammonia ($R_f = 0.24$) or in 60:35:6:2 $CHCl_3$–MeOH–water–acetic acid as the mobile phases revealed one single spot ($R_f = 0.11$) upon detection with either anisaldehyde spray or ceric ammonium nitrate dipping reaction. The radiochemical purity and the specific radioactivity was as determined for **6**. FABMS: ($C_{73}H_{131}N_3O_{30}S$, MW 1562.90) $[M + H^+]$ at *m/z* 1564, and $[M + Na^+]$ at *m/z* 1586. Again a shift of 2 *amu* is also observed for this compound owing to the abundance of radiocarbon at this high specific radioactivity.

Hydrolysis of $^{14}C_8$-SGM1 (**6**) *and $^{14}C_{18}$-SGM1* (**7**) *with various glycohydrolases.*—Briefly, in each of 4 Eppendorf vials, 0.5 nmol of **6** or **7** was dissolved in a total assay volume of 330 μL of 84 mM sodium citrate buffer pH 4.5, containing 2 mM taurodeoxycholate and BSA (0.015%, w/v) in addition to the respective glycohydrolases. Each solution contained GM1-β-galactosidase (β-D-galactoside galactohydrolase; EC 3.2.1.23) [22] (550 pKatal, from bovine testes, Boehringer Mannheim, Germany). The resulting mixtures were incubated for 10 h at 37 °C. Thereafter, one assay was stopped by adding MeOH (0.3 mL) to the reaction mixture. The remaining three reaction mixtures were supplemented with 5 μL of β-hexosaminidase A (β-*N*-acetyl-D-hexosaminide *N*-acetylhexosaminohydrolase; EC 3.2.1.52) [23] (8,500 pKatal) and kept for 10 h at 37 °C. One assay was stopped as above. Both the remaining mixtures were treated with a third enzyme, i.e., sialidase (*N*-acetylneuraminylhydrolase; EC 3.2.1.18) [24] (10 μL, 130 pKatal, from *Vibrio cholerae*, Boehringer Mannheim, Germany), for 10 h at 37 °C. After one assay was stopped as above, the remaining mixture was treated again with GM1-β-galactosidase as above and then mixed with MeOH to denature the enzymes. All assay mixtures were then diluted with water (0.4 mL) and freed of salts using LiChroprep RP-18. The degradation products were separated by TLC with 60:35:8 $CHCl_3$–MeOH–15 mM $CaCl_2$ and 60:35:6:2 $CHCl_3$–MeOH–water–acetic acid as the mobile phases for the short and long acyl chain derivatives, respectively, and visualized by exposure to X-ray film (see Figs. 1 and 2).

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