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Synthesis of reference standards to enable single cell metabolomic studies of tetramethylrhodamine-labelled ganglioside GM1

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Abstract

Ganglioside GM1 and its seven potential catabolic products: asialo-GM1, GM2, asialo-GM2, GM3, Lac-Cer, Glc-Cer and Cer, were labelled with tetramethylrhodamine (TMR) to permit ultra-sensitive analysis using laser-induced fluorescence (LIF) detection. The preparation involved acylation of the homogenous C18 lyso-forms of GM1, Lac-Cer, Glc-Cer and Cer with the N-hydroxysuccinimide ester of a β -alanine-tethered 6-TMR derivative, followed by conversion of these labelled products using galactosidase, sialidase and sialyltransferase enzymes. The TMR-glycolipid analogs produced are detectable on TLC down to the 1 ng level by naked eye. All 8 compounds could be separated in under 4 minutes in capillary electrophoresis where they could be detected at the zeptomole (ca 1000 molecule) level using LIF.

Keywords

glycolipid metabolism; ganglioside; GM1; metabolomics; fluorescence labelling; membrane recycling

1. Introduction

The surface of animal cells is covered with glycoproteins and glycolipids where the oligosaccharide chains face the outside of the cells and often act as recognition molecules. They are known to serve as receptors for the binding of other cells, proteins, bacteria, toxins and viruses and are postulated to have numerous other functions.¹⁻⁴ The brain is unique among the wide array of vertebrate cells and tissues in that >80% of the conjugated oligosaccharides are in the form of glycolipids.⁵ Among the glycolipids, glycosphingolipids are the most abundant and intriguing molecules. Their sugar chains range from 1 to over 20 residues and are attached to ceramide, a fatty-acyl sphingosine. Sialylated glycosphingolipids are often referred to as gangliosides, ganglioside GM1 (Fig. 1) being a prominent member of this family.

Dedicated to the memory of Professor N. K. Kochetkov

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Changes in cell-surface glycolipid structures accompany embryonic development and tumor progression⁶⁻⁹, and many inborn errors of metabolism involve enzymes in the metabolic pathways of glycolipid metabolism, including lysosomal storage diseases.^{10, 11} There has therefore been a long-standing interest in understanding the biosynthetic pathways for both glycolipid biosynthesis and catabolism.¹² Many studies have confirmed that the glycolipids on the cell surface are not static, but are continuously being internalized and recycled back to the cell surface, often on a time scale of hours.¹³⁻¹⁵ During this glycolipid recycling, the molecules can circulate through intracellular organelles and become distributed through the lysosomes, endoplasmic reticulum, Golgi apparatus and transport vesicles. This metabolism in healthy cells is thought to be tightly regulated, with lysosomal degradation closely matching the biosynthesis to produce a net stable cell-surface glycosylation pattern. This pattern is ultimately decided upon by the regulated distribution of glycolipids among the organelles and the relative expression, localization and activities of metabolic glycosidases and glycosyltransferases.^{12, 16-19} The glycolipid profile of a cell will therefore report on the relative abundance of these enzymes as well as on the integrity of the intracellular organelle system and filament network.²⁰

The earliest studies on glycolipid recycling used radioactively labelled natural glycolipids structures to deduce the rate and metabolic pathways accessed during the recycling (recently reviewed²⁴⁻²⁸). Superb data were obtained, but these studies necessarily required the extraction of glycolipids from large populations of cells thus masking any potential differences between individual cells. Later, fluorescently tagged glycolipids (e.g., synthetic BODIPY-labelled lactosylceramide analogs or GM1 analogs) were used and the rate of internalization and intracellular localization could be followed in single cells using confocal fluorescence microscopy.²²⁻²⁵ Metabolic products from single cells were, however, not analyzed.

Encouraged by observations that fluorescently tagged lactosylceramide and GM1 analogs were indeed internalized and metabolized in a variety of cells, and that recently developed technology has permitted the assay of glycosidase activity in single cells, we decided to prepare GM1 and all of its possible catabolites (Fig. 2) tagged with a fluorescent dye that would afford sufficient sensitivity to analyze the metabolites in single cells^{29, 30}. The dye chosen was the brilliant red tetramethylrhodamine, previously shown to offer a sensitivity of detection in the zeptomol (10^{-21} mol) range when assayed in capillary electrophoresis (CE) using laser-induced fluorescence (LIF) detection in an instrument equipped with a sheath-flow cuvette.³¹ The ultimate objective of the present work is to see if there are significant differences in glycolipid recycling pathways between individual cells, such as tumour cells or stem cells, in culture. Such a large-scale metabolomic study will ultimately require the development of high-throughput automated cell picking coupled to rapid high-sensitivity analysis. These are the long-term goals of the present research program.

2. Results and discussion

2.1 Synthetic strategy

The potential flux through the catabolic and biosynthetic pathways for GM1 are shown in Fig. 2. The “natural” pathway in humans is the vertical one, with sequential glycosidase-mediated removal of single sugar residues in the sequence GM1→GM2→GM3→Lac→Glc→Cer. The alternate pathways going from GM1→asialo-GM1 and/or GM2→asialo-GM2 are not active in human ganglioside catabolism, although they have been reported in the mouse.^{32,33} Since all 8 compounds may in principle be formed, the decision was made to prepare all of them as reference standards. The biosynthesis of GM1 proceeds in the reverse direction beginning with ceramide and using glycosyltransferases to add monosaccharides one at a time.

We chose a well-precedented³⁴ approach for installing the required fluorescent tag on the glycolipid structures. The fatty-acid moiety of glycosphingolipids can be cleaved either chemically or enzymatically, producing the so-called “*lyso*” derivatives (Fig. 3) with a free amino group that can be readily acylated by a reactive dye derivative. The starting material used for the preparation of GM1-TMR, asialo-GM1-TMR, GM2-TMR and asialo-GM2-TMR was GM1 isolated from bovine brain³⁵ (Fig. 3). We used the commercially available sphingolipid ceramide N-deacylase (Takara) to cleave the fatty acid chain to give the *lyso*-GM1 structure(s) that contained a free amino group to permit installation of the TMR label. Mass-spectrometry confirmed that the *lyso*-GM1 produced was clearly a ca. 1:1 mixture of compounds differing in the chain length of the sphingosine residue, one with C18- and the other with C20-sphingosine. Since single pure compounds would be required as reference standards, these had to be separated.

Separation of GM1 chain-length isomers by reverse-phase chromatography has previously been reported,^{36,37} and it proved especially convenient to do so here at the *lyso*-stage, as the enzyme reaction mixture contained the C18 *lyso* form, the C20 *lyso*-form along with some unreacted GM1. The C18-form cleanly eluted first from a reverse-phase (C18) reverse-phase column, using a stepwise methanol/water gradient, and could easily be isolated in pure form. Later fractions contained the C20 form followed by the much more hydrophobic GM1. The conversion of GM1 to the *lyso*-GM1 having a homogenous C18 sphingosine was routinely carried out on a 20 mg scale of GM1, yielding >5 mg of homogenous C18 product each time (Fig. 3).

2.2 Preparation of tetramethylrhodamine-acylating agent 4

Since the structure of both the dye, and any linker to the dye, are expected to importantly influence the recycling properties of fluorescently-tagged GM1 and its metabolites³⁸, we elected a flexible procedure for labelling where the dye is first coupled to a spacer whose length and structure may be varied. Attachment of the dye to the very valuable *lyso*-GM1 would then occur in a single last step. Fig. 4 shows the preparation of the TMR-labelling reagent **4**, starting with isomerically-pure 6-tetramethylrhodamine N-hydroxysuccinimide ester³⁹ (**1**) which was coupled to a short spacer, a Boc-protected β -alanine in the present instance, yielding compound **2** from which the Boc-group was removed under standard conditions to give **3** which was then converted to the active-ester **4**.

2.3 Preparation of GM1-TMR, Lac-TMR, Glc-TMR and Cer-TMR

Acylation of *lyso*-GM1 with **4** proved highly effective and produced the target **GM1-TMR** which was isolated in 87% yield on product scales of over 5 mg. The *lyso*-forms of Lac-Cer, Glc-Cer and Cer are fortunately commercially available with homogenous C18-sphingosine chains (Avanti Polar Lipids). These could be readily functionalized, as described for the preparation of **GM1-TMR**, to produce **Lac-TMR** (90%), **Glc-TMR** (95%) and **Cer-TMR** (94%) (Fig 5).

2.4 Enzymatic preparation of asialo-GM1-TMR, GM2-TMR, asialo-GM2-TMR and GM3-TMR

The glycosidases acting in the natural biosynthetic pathway for GM1 catabolism are complex, requiring chaperone proteins and operating in a defined order. For example, most sialidases do not act on GM1 or GM2, but only on GM3.⁴⁰ The preparation of the missing metabolic standards therefore used commercial glycosidase preparations with sufficient cross-reactivity to produce generally small but isolable quantities of the desired products.

Conversion of **GM1-TMR** to **GM2-TMR** was carried out on mg scale using β -galactosidase from bovine testes (Fig. 3). This, and the other, enzyme reactions were very easy to monitor by silica-gel TLC where monosaccharide removal resulted in faster moving spots (or bands)

that could be seen by naked eye at high sensitivity. Younger researchers (ca. 30 years old) can readily detect about 1 ng by naked eye, while more experienced researchers (in their 50's) commonly require about 10 ng. **GM2-TMR** was thus readily separated from unreacted **GM1-TMR** using preparative TLC with simple visual detection of the bands.

Incubation of **GM1-TMR** with commercial sialidase A (*Arthrobacter ureafaciens*) for 6 days yielded **asialo-GM1-TMR** which could be isolated in 65% yield. Further incubation of **asialo-GM1-TMR** with β -galactosidase yielded **asialo-GM2-TMR** in 58% yield on small (0.1 mg) scale (Fig. 3). The missing **GM3-TMR** was readily produced on a >5 mg scale from **Lac-TMR** in 96% yield using CMP-sialic acid and a recombinant α 2,3-sialyltransferase.⁴¹

Representative mass-spectra for **GM1-TMR** and its expected natural catabolic products are shown in Fig. 6, confirming their expected structures. The level of purity of each of the compounds was assessed by routine CE analysis, where their retention times spanned from 20 - 40 minutes. In each case, the major peak integrated for over 95% of the fluorescence signal. The ¹H-NMR spectra displayed very broad lines in a variety of solvents and were thus not useful for product characterization.

3. Separation of the 8 standards by capillary electrophoresis

As stated in the introduction, a major challenge of the present project is to achieve rapid separation of the 8 metabolites synthesized in the present work, in combination with the efficient selection of single cells for analysis. Preliminary conditions were found that achieved the required separation in under 4 minutes (Fig 7) using a running buffer consisting of 10 mM sodium tetraborate, 35 mM sodium deoxycholate and 5 mM methyl- β -cyclodextrin in 20 μ M i.d. capillaries. Details of the custom-designed and built instrumentation will be reported elsewhere. The limit of detection was found to be in the range 2-5 zeptomoles.

4. Conclusion

In summary, we have prepared highly fluorescent TMR-derivatives of GM1 and seven of its potential catabolites using a combined chemical-enzymatic strategy. This approach was greatly simplified by performing the enzymatic reactions on compounds that were already labelled, permitting the monitoring of the progress of the reactions by tlc using the naked eye and, similarly, aiding in the isolation and quantitation of the products. The synthetic approach chosen will easily permit the installation of different spacer molecules and different fluorescent dyes, which may have important consequences on the uptake and recycling of these artificial glycosphingolipid analogs. Finally, a rapid separation of all 8 compounds was achieved in under 4 minutes by CE, with detection sensitivities in the fmol range. Preliminary results indicate that **GM1-TMR** is indeed taken up by PC12-cells, yielding several metabolites co-eluting with the reference standards prepared in this work, suggesting the utility of these compounds in single-cell metabolic studies. These results will be reported in detail elsewhere.

5. Experimental

General experimental

Electrospray mass spectra (ESI-MS) were recorded on a Bruker Esquire 3000-Plus Ion Trap instrument with samples injected as solutions in either MeOH or MeOH-CH₃CN/H₂O (1/1) mixtures. Routine CE was performed using an automated PrinCE 2-lift, model 560 CE system (Prince Technologies, The Netherlands). Separations were carried out in uncoated fused-silica capillary of 75 μ m i.d. with a length between 50-80 cm using 50 mM borate, pH 9.3 and 25 mM sodium dodecyl sulfate (SDS) as running buffer. TMR-labelled compounds were detected and quantitated using an Argos 250B fluorescence detector (Flux Instruments, Switzerland)

equipped with an excitation filter of 546.1/10 nm and an emission filter of 570 nm. All experiments were carried out at a normal polarity, i.e. inlet anodic. Preparative TLC was performed on silica plates (Merck Prod. No.1.05745, 20 × 20 cm, 2 mm thickness). *Lyso-Lac-Cer*, *lyso-Glc-Cer* and C18-sphingosine were from Avanti Polar Lipids (AL, USA). β -Galactosidase and Sialidase A were from Prozyme (WA, USA)

6-Tetramethylrhodamine-(tert-butyl β -alanine)-amide 2—6-Tetramethylrhodamine N-hydroxysuccinimide ester (**1**, 28.5 mg, 53.8 μ mole) and β -alanine t-butyl ester hydrochloride (16 mg, 88.1 μ mole) were dissolved in 500 μ L dry DMF in a 1.5 mL Eppendorf tube. The sample was vortexed for 1 min, 15 μ L diisopropylethylamine (DIPEA) was added and the sample was covered with aluminium foil and placed on a shaker. TLC ($\text{CH}_2\text{Cl}_2/\text{MeOH}$ 1/1) and ESI-MS indicated almost complete conversion to product after 10 min. After 1h, the reaction mixture was transferred to a round bottom flask and concentrated to near dryness. H_2O (25 mL) was added and the solution was applied to two tandem reverse-phase C_{18} Sep-Pak cartridges. The cartridges were washed with H_2O (30 mL), followed by elution of product with 18 mL fractions of 10% aq. MeOH, 20% MeOH, 3 × 18 mL 30% MeOH, 50% MeOH and finally 50 mL of 100% MeOH. TLC and ESI-MS analysis showed that the 50 and 100% fractions contained pure product and these fractions were pooled and concentrated to give the 6-TMR-derivative **2** (28.95 mg, 51.9 μ mole, 96%). ESI-MS; expected: $[\text{M}+\text{H}]^+$ 558.25 found: 558.2

6-Tetramethylrhodamine-(β -alanine)-amide 3—Compound **2** (28.95 mg, 51.9 μ mole) was dissolved in CH_2Cl_2 (5 mL) followed by addition of 1 mL of trifluoroacetic acid. After 3h, TLC ($\text{CHCl}_3/\text{MeOH}$ 1/1) and ESI-MS indicated that all starting-material was consumed. The reaction mixture was concentrated, dried over high vacuum overnight and the crude residue was purified on a 10 × 2.5 cm Iatrobead column ($\text{CHCl}_3/\text{MeOH}$ gradient from 10/1 to 1/3) to give **3** (25.1 mg, 50.0 μ mole, 96%). ESI-MS; expected: $[\text{M}+\text{H}]^+$ 502.19 found: 502.1

6-Tetramethylrhodamine-(N-hydroxysuccinimidyl- β -alanine)-amide 4—Compound **3** (7.34 mg, 14.6 μ mole), 1.6 eq. *N,N'*-disuccinimidyl carbonate (DSC, 5.90 mg, 23.0 μ mole) and 1.7 eq. 4-dimethylaminopyridine (DMAP, 3.01 mg, 24.6 μ mole) were weighed into three separate Eppendorf tubes. Dry DMF (200 μ L) was added to each tube and they were vortexed vigorously to assist dissolution. The DSC and DMAP solutions were added to the solution of **3** in succession with rapid vortexing between each addition. The reaction tube was sealed with Parafilm, covered with aluminum-foil and placed on a shaker. The reaction was monitored by TLC (CHCl_3/DMF 2/1) and ESI-MS that showed that ester **4** had formed after 2.5 hr. This DMF solution was then used directly for labelling of the lyso-glycosphingolipids. ESI-MS; expected: $[\text{M}+\text{H}]^+$ 599.21 found: 599.3

C18-Lyso-GM1—GM1 (20 mg, 12.8 μ mol, an approximately equimolar mixture of C18 and C20 sphingosine isomers) was dissolved in 2 mL of 100 mM sodium acetate buffer (pH 5.8) followed by the addition of 5.15 mL H_2O and 0.8 mL taurodeoxycholate (TDC, 8 mg/mL, Sigma).

Sphingolipid ceramide N-deacylase (SCDase), 50 μ L of a 5 mU/ μ L solution (*Pseudomonas* sp. Takara) was added, the reaction was covered with 40 mL of decane and incubated at 37°C for 2 weeks. Aliquots were periodically analyzed by TLC ($\text{CHCl}_3/\text{MeOH}/0.25\% \text{ KCl}$ 60/39/10), visualized by spraying with orcinol and heating. When the reaction was nearly complete, the mixture was frozen at -20°C and the decane was removed. The thawed mixture was applied to a column (1.2 × 12 cm) packed with Bondapak C_{18} resin (125 Å 37-55 μ m, Waters). The column eluent was monitored at 206 nm using a UV-detector (LKB). The column was washed with H_2O until absorbance reached baseline levels, followed by development with 60% aqueous methanol to remove the TDC and 75 % aqueous methanol to elute the lyso-GM1.

The lyso-GM1 came out as two peaks (C18-*lyso*-GM1 followed by C20-*lyso*-GM1). Methanol (100%) was used to elute any unreacted GM1 from the column. The fractions containing the separated *lyso*-GM1 isomers were pooled, concentrated to dryness and the products were analysed by ESI-MS. **C18-*Lyso*-GM1** (5.45 mg, 4.26 μ mol, 33%) expected $[M+H]^+$ 1280.62 found 1280.6; C20-*Lyso*-GM1 (4.80 mg, 3.67 μ mol, 29%) expected $[M+H]^+$ 1308.65 found 1308.6.

GM1-TMR—C-18-*Lyso*-GM1 (12.0 mg, 9.37 μ mole) was dissolved in of 1.33 mL DMF/ $CHCl_3/H_2O$ (100/12/21). To this solution, 1.55 eq. of NHS-ester **4** in DMF (600 μ L) was added, followed by DIPEA (30 μ L). The reaction tube was sealed with Parafilm, covered with aluminum foil and placed on a shaker. Analysis by TLC ($CHCl_3/MeOH/0.25\%$ KCl 60/35/8) and ESI-MS after 1 h showed a significant amount of product together with excess unreacted **4**. The reaction was therefore allowed to stand overnight (15h) to go to completion and to hydrolyse the reactive dye to facilitate product purification. The reaction mixture was diluted with 30 ml H_2O and then applied onto 2 tandem C_{18} Sep-Pak cartridges. The cartridges were washed with 30 mL H_2O , then 20 mL fractions of each of 25% aqueous MeOH, 33% MeOH, 40% MeOH, 50% MeOH, 75% MeOH and MeOH. The fractions were analysed by TLC ($CHCl_3/MeOH/0.25\%$ KCl 60/35/8) and ESI-MS. The 25-40% fractions contained hydrolysed **4** and the 50-100% fractions contained labelled GM1. The glycolipid containing fractions were pooled and evaporated to give **GM1-TMR** (14.45 mg, 8.19 μ mole, 87%). ESI-MS; Expected: $[M+H]^+$ 1763.80 found: 1763.8; $[M-H]^-$ 1762.0.

Lac-TMR—D-Lactosyl- β 1-1'-D-erythro-sphingosine (C18 isomer, 0.50 mg, 0.80 μ mole) was treated as described for lyso-GM1, giving **Lac-TMR** (0.80 mg, 0.72 μ mole, 90%). ESI-MS; Expected: $[M+H]^+$ 1107.57 found: 1107.6.

Glc-TMR—D-Glucosyl- β 1-1'-D-erythro-sphingosine (4.83 mg, 10.46 μ mole) was treated as described for lyso-GM1, giving **Glc-TMR** (9.43 mg, 9.98 μ mole, 95%). ESI-MS; Expected: $[M+H]^+$ 945.51 found: 945.5.

Cer-TMR—D-erythro-sphingosine (3.11 mg, 10.38 μ mole) was treated as described for lyso-GM1, giving **Cer-TMR** (7.69 mg, 9.82 μ mole, 95%). ESI-MS; Expected: $[M+H]^+$ 783.46 found: 783.5.

Enzymatic conversions TMR-labelled sphingolipid analogs

General procedures—The progress of enzyme incubations was monitored by TLC with visual detection of the red starting materials and products. After reaction progress was judged to be adequate, the products were purified as follows. Water (typically 10 mL) was added and the reaction mixture was applied to reverse-phase C_{18} Sep-Pak cartridges that had been conditioned by washing with HPLC-grade MeOH (10 mL) followed by H_2O (50 mL). After sample application, the cartridges were washed with 100 mL H_2O then TMR-labelled products were eluted with MeOH (10-20 mL) which was evaporated. When more than one fluorescent compound was present, they were separated by preparative TLC using $CHCl_3/MeOH/0.25\%$ KCl (60/39/10) as eluent. The red bands were scraped off the plates and extracted with MeOH until the silica became colourless. After evaporation of the MeOH, the residue was dissolved in water (5 mL) passed through a 0.45 μ m Millex-GV PVDF filter to remove particulates and then lyophilized.

GM2-TMR GM1-TMR (1.33 mg) was dissolved in 40 μ L of 5X galactosidase-reaction buffer (Prozyme) and 140 μ L H_2O , then 20 μ L of β -galactosidase (5mU/ μ L, bovine testes, Prozyme) was added and the sample was mixed gently. The tube was covered with aluminium foil and

the reaction was incubated at 37°C for 4 day. **GM2-TMR** (0.65 mg, 0.41 μ mol, 54%) was obtained after preparative TLC, ESI-MS expected $[M-H]^-$ 1599.74 found 1599.5.

Asialo-GM1-TMR GM1-TMR (1.31 mg) was dissolved in 40 μ L of 5X sialidase reaction buffer (Prozyme) and 140 μ L H₂O. When the GM1 was dissolved, 20 μ L of sialidase A (5U/mL) was added. The tube was shaken, then covered with aluminium foil and the reaction was incubated at 37°C for 6 days. Preparative TLC gave **Asialo-GM1-TMR** (0.75 mg, 0.58 μ mol, 65%), ESI-MS expected $[M+H]^+$ 1472.7 found 1472.2.

Asialo-GM2-TMR Asialo-GM1-TMR (0.1 mg) was dissolved in 20 μ L of 5X reaction buffer (Prozyme) and 70 μ L H₂O and 10 μ L of β -galactosidase (5mU/ μ L) was added and the sample was gently mixed. The tube was covered in aluminium foil and the reaction was incubated at 37°C for 4 days. The TLC suggested ca 50% conversion, and the product was isolated by preparative TLC. CE quantitation of the purified product indicated a 58% yield, ESI-MS expected $[M+H]^+$ 1310.65 found 1310.6.

GM3-TMR CMP-sialic acid (10.4 mg) was dissolved in 500 μ L 40 mM Tris-HCl buffer (pH 7.5). This was transferred to a tube containing **Lac-TMR** (6.16 mg, 5.56 μ mol). After dissolving the **Lac-TMR**, 100 μ L 100mM MgCl₂, 3 μ L alkaline phosphatase (Roche No. 108 138, 1500 21U/ μ L), 197 μ L H₂O and 200 mU (1U/ml) of α 2,3-sialyltransferase (MalE fusion-protein from *Campylobacter jejuni*⁴¹) were added and the mixture was mixed gently. The tube was covered with aluminium foil and the reaction was incubated at room temperature overnight. TLC showed complete conversion, and the product was isolated without need for preparative HPTLC. **GM3-TMR** (7.5 mg, 5.36 μ mol, 96%), ESI-MS expected $[M+H]^+$ 1398.66 found 1398.7.

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References

1. Karlsson K-A. Ann. Rev. Biochem 1989;58:309–350. [PubMed: 2673013]
2. Varki A. Biological roles of oligosaccharides: all of the theories are correct. Glycobiology 1993;97–130. [PubMed: 8490246]
3. Lowe JB, Marth JD. Annu. Rev. Biochem 2003;72:643–691. [PubMed: 12676797]
4. Taylor, ME.; Drickamer, K. Introduction to Glycobiology. Oxford University Press; Oxford, UK: 2003. p. 207
5. Schnaar RL. Arch. Biochem. Biophys 2004;426:163–172. [PubMed: 15158667]
6. Hakomori S-I. Cancer. Res 1996;5309–5318. [PubMed: 8968075]
7. Dennis JW, Granovsky M, Warren CE. BioEssays 1999;412–421. [PubMed: 10376012]
8. Hakomori S-I. Adv. Exp. Med. Biol 2001;491:369–402. [PubMed: 14533809]
9. Birkle SZ, Zeng G, Gao L, Yu RK, Aubry J. Biochimie 2003;85:455–463. [PubMed: 12770784]
10. Giesleemann V. Biochim. Biophys. Acta 1995;1270:103–136. [PubMed: 7727535]
11. Marquart T, Denecke J. Eur. J. Pediatr 2003;162:359–379. [PubMed: 12756558]
12. Kolter T, Sandhoff K. Angew. Chemie Int. Ed 1999;38:1532–1568.
13. Gillard BK, Thurmon LT, Marcus DM. Glycobiology 1993;3:57–67. [PubMed: 8448386]
14. Maccioni HJ, Giraudo CG, Daniotti JL. Neurochem. Res 2002;27:629–636. [PubMed: 12374198]
15. Sandhoff K, Kolter T. Philos. Trans. Royal. Soc. Lond. B Biol. Sci 2003;358:847–861.
16. Sandhoff K, van Echten G. Prog. Brain Res 1994;101:17–29. [PubMed: 8029449]
17. Maccioni HJF, Daniotti JL, Martina JA. Biochim. Biophys. Acta 1999;1437:101–118. [PubMed: 10064894]

18. Bieberich E, MacKinnon S, Silva J, Li DD, Tencomnao T, Irwin L, Kapitonov D, Yu RK. *Biochemistry* 2002;41:11479–11487. [PubMed: 12234191]
19. Tettamanti G, Bassi R, Viani P, Riboni L. *Biochimie* 2003;85:423–437. [PubMed: 12770781]
20. Tettamanti G. *Glycoconjugate J* 2004;20:310–317.
21. Sonnino S, Chigorno V, Tettamanti G. *Methods Enzymol* 1999;311:639–656. [PubMed: 10563353]
22. Riboni L, Viani P, Tettamanti G. *Methods Enzymol* 1999;311:656–682. [PubMed: 10563354]
23. Sonnino S, Nicolini M, Chigorno V. *Glycobiology* 1996;6:479–487. [PubMed: 8877367]
24. Pagano, RE.; Martin., OC. Use of fluorescent analogs of ceramide to study the Golgi apparatus of animal cells. In: Celis, Julio E., editor. *Cell Biology: A Laboratory Handbook*. Academic Press; 1994. p. 387–393.
25. Pagano RE, Chen CS. *Ann. N. Y. Acad. Sci* 1998;845:152–160. [PubMed: 9668349]
26. Pagano RE. *Phil. Trans. R. Soc. Lond. B* 2003;358:885–891. [PubMed: 12803922]
27. Pagano RE, Watanabe R, Wheatley C, Dominguez M. *Methods Enzymol* 2000;312:523–534. [PubMed: 11070900]
28. Marks DL, Singh RD, Choudhury A, Wheatley CL, Pagano REJ. *Methods* 2005;36:186–195. [PubMed: 15905102]
29. Krylov SN, Arriaga EA, Chan NW, Dovichi NJ, Palcic MM. *Anal. Biochem* 2000;283:133–135. [PubMed: 10906232]
30. Shoemaker GK, Lorieau J, Lau LH, Gillmore CS, Palcic MM. *Anal. Chem* 2005;77:3132–3137. [PubMed: 15889901]
31. Zhao JY, Dovichi NJ, Hindsgaul O, Gosselin S, Palcic MM. *Glycobiology* 1994;4:239–242. [PubMed: 8054721]
32. Sango K, Yamanaka S, Hoffmann A, Okuda Y, Grinberg A, Westphal H, McDonald MP, Crawley JN, Sandhoff K, Suzuki K. *Nature Gent* 1995;11:170–176.
33. Li SC, Li YT, Moriya S, Miyagi T. *Biochem. J* 2001;360:233–237. [PubMed: 11696012]
34. Sonnino S, Acquotti D, Kirschner G, Uguaglianza A, Zecca L, Rubino F, Tettamanti G. *J. Lipid Res* 1992;33:1221–1226. [PubMed: 1431600]
35. Tettamanti G, Bonali F, Marchesini S, Zambotti V. *Biochim. Biophys. Acta* 1973;296:160–170. [PubMed: 4693502]
36. Sonnino S, Ghidoni R, Gazzotti G, Kirschner G, Galli G, Tettamanti G. *J. Lipid Res* 1984;25:620–629. [PubMed: 6747465]
37. Mauri L, Valsecchi M, Casellato R, Li SC, Li YT, Sonnino S. *Chromatogr. B. Analyt. Technol. Biomed. Life Sci* 2003;796:1–10.
38. Mukherjee S, Soe TT, Maxfield FR. *J. Cell Biol* 1999;144:1271–1284. [PubMed: 10087269]
39. Menchen, SM.; Fung, S. European Patent Application 0 272 007 “5- and 6- succinimidylcarboxylate isomers of rhodamine dyes” (published 22.06.1988)
40. Schauer R, Rudiger WV, Sander M, Corfield AP, Wiegandt H. *Adv. Exp. Med. Biol* 1980;125:283–294. [PubMed: 7361617]
41. Gilbert M, Brisson J-R, Karwaski M-F, Michniewicz J, Cunningham A-M, Wu Y, Young M, Wakarchuk WW. *J. Biol. Chem* 2000;275:3896–3906. [PubMed: 10660542]

Figure 1.
Structure and component-nomenclature of ganglioside GM1.

Figure 2.
Potential catabolic and biosynthetic pathways for GM1.

Figure 3.

Labelling and further enzymatic conversions of GM1.

Figure 4.
Synthesis of TMR-labelling agent **4**.

Figure 5.
Synthesis of **Lac-TMR**, **Glc-TMR**, **Cer-TMR** and **GM3-TMR**

Figure 6. Electrospray mass spectra for **GM1-TMR** and its expected natural catabolites. The mass spectra were acquired in the positive-ion mode except for **GM1-TMR** and **GM2-TMR**. **GM3-TMR** exhibits a peak at 710.7 corresponding to the doubly charged ion.

Figure 7. Separation of standards by CE. 1, **asialo-GM1-TMR**; 2, **GM1-TMR**; 3, **asialo-GM2-TMR**; 4, **GM2-TMR**; 5, **GM3-TMR**; 6, **Lac-TMR**; 7, **Glc-TMR**; 8, **Cer-TMR**.