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

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Dedicated to the memory of Professor Nikolay K. Kochetkov

**Abstract**—Ganglioside GM1 and its seven potential catabolic products: asialo-GM1, GM2, asialo-GM2, GM3, Lac-Cer, Glc-Cer and Cer, were labeled with tetramethylrhodamine (TMR) to permit ultra-sensitive analysis using laser-induced fluorescence (LIF) detection. The preparation involved acylation of the homogenous $C_{18}$ *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 labeled products using galactosidase, sialidase, and sialyltransferase enzymes. The TMR–glycolipid analogs produced are detectable on TLC down to the 1 ng level by the naked eye. All eight compounds could be separated within 4 min in capillary electrophoresis where they could be detected at the zeptomole (ca. 1000 molecule) level using LIF.


*Keywords:* Glycolipid metabolism; Ganglioside; GM1; Metabolomics; Fluorescence labeling; 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 (Chart 1) being a prominent member of this family.

Changes in cell-surface glycolipid structures accompany embryonic development and tumor progression,[6–9] 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

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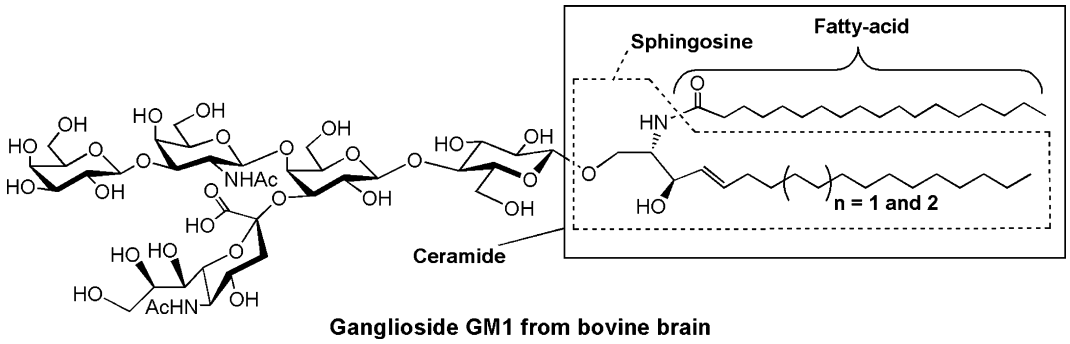



**Chart 1.** Structure and component-nomenclature of ganglioside GM1.

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 labeled natural glycolipids structures to deduce the rate and metabolic pathways accessed during the recycling (recently reviewed[21–23]). 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-labeled 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.[24–28] 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 (Chart 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 tumor 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.

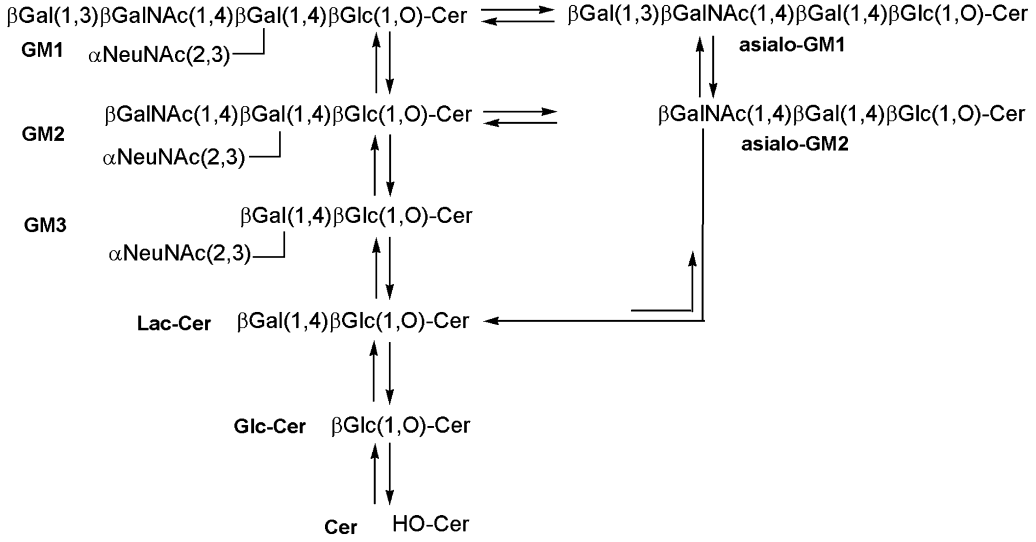


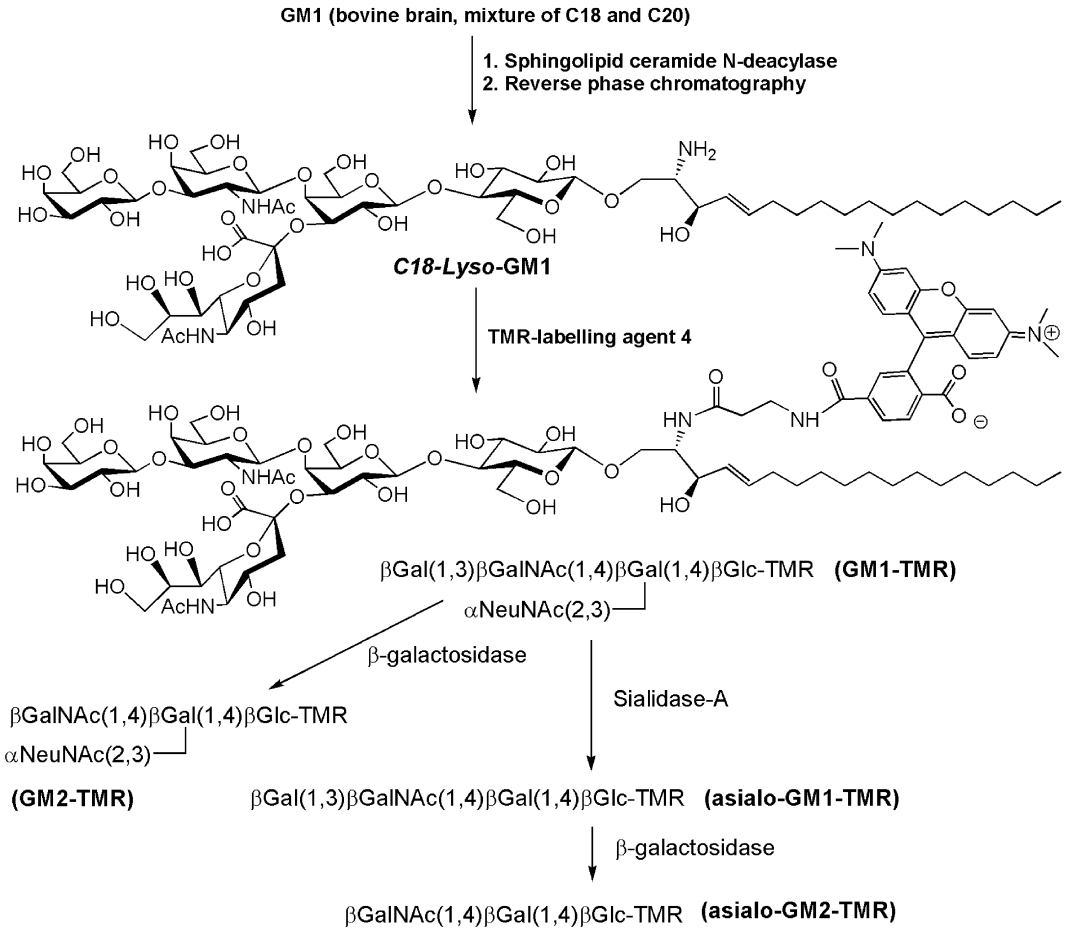

**Chart 2.** Potential catabolic and biosynthetic pathways for GM1.

## 2. Results and discussion

### 2.1. Synthetic strategy

The potential flux through the catabolic and biosynthetic pathways for GM1 is shown in Chart 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 eight 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-precedented[34] 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 (Scheme 1) 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 brain[35] (Scheme 1). 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 $C_{18}$- and the other with $C_{20}$-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 $C_{18}$ *lyso*-form, the $C_{20}$ *lyso*-form along with some unreacted GM1. The $C_{18}$-form cleanly eluted first from a reverse-phase ($C_{18}$) reverse-phase column, using a stepwise methanol/water gradient, and could easily be isolated in pure form. Later fractions contained the $C_{20}$ form followed by the much more hydrophobic GM1. The conversion of GM1 to the *lyso*-GM1 having

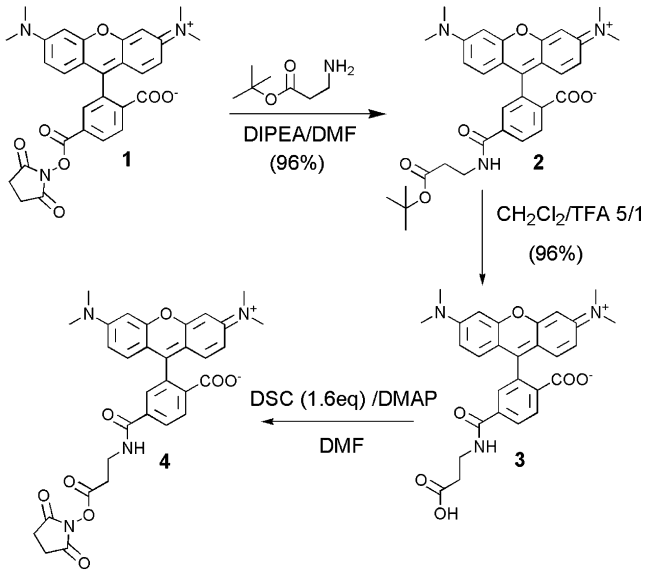



**Scheme 1.** Labeling and further enzymatic conversions of GM1.

a homogenous $C_{18}$ sphingosine was routinely carried out on a 20 mg scale of GM1, yielding >5 mg of homogenous $C_{18}$ product each time (Scheme 1).

### 2.2. Preparation of tetramethylrhodamine-acylating agent 4

Since the structures of both the dyes, and any linkers to the dyes, are expected to importantly influence the recycling properties of fluorescently tagged GM1 and its metabolites,[38] we elected a flexible procedure for labeling 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. Scheme 2 shows the preparation of the TMR-labeling reagent **4**, starting with isomerically pure 6-tetramethylrhodamine *N*-hydroxysuccinimide ester[39] (**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**.




**Scheme 2.** Synthesis of TMR-labeling agent **4**.

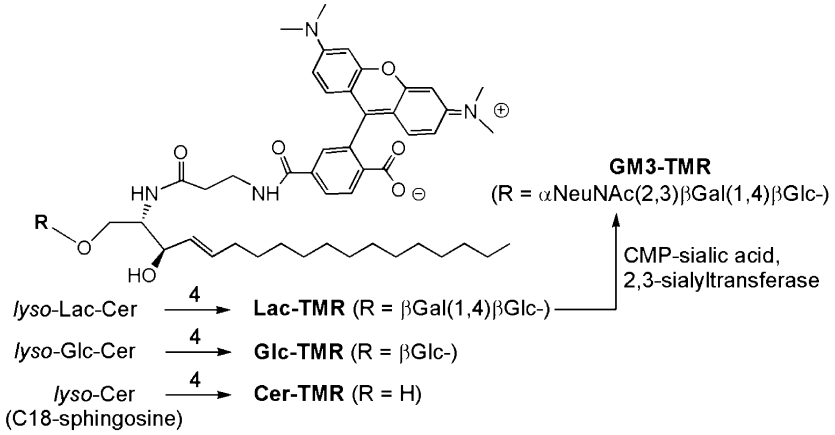
### 2.3. Preparation of GM1-TMR, Lac-TMR, Glc-TMR and Cer-TMR

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 $C_{18}$-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%) (Scheme 3).

### 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 milligram scale using β-galactosidase from bovine testes (Scheme 1). 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 the 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




**Scheme 3.** Synthesis of **Lac-TMR**, **Glc-TMR**, **Cer-TMR** and **GM3-TMR**.

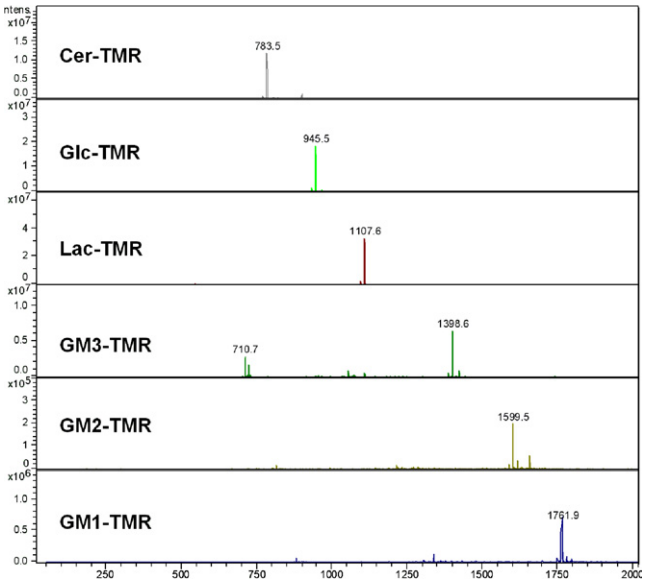



**Figure 1.** Electrospray mass spectra for **GM1-TMR** and its expected natural catabolites. The mass spectra were acquired in the positive-ion mode except for **GM1-TMR** and **GM2-TMR**. **GM3-TMR** exhibits a peak at 710.7 corresponding to the doubly charged ion.

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 a small (0.1 mg) scale (Scheme 1). 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 α2,3-sialyltransferase.[41]

Representative mass-spectra for **GM1-TMR** and its expected natural catabolitic products are shown in Figure 1, 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 to 40 min. In each case, the major peak integrated for over 95% of the fluorescence signal. The $^{1}$H NMR spectra displayed very broad lines in a variety of solvents and were thus not useful for product characterization.

## 3. Separation of the eight standards by capillary electrophoresis

As stated in the introduction, a major challenge of the present project is to achieve rapid separation of the eight 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 within 4 min (Fig. 2) using a running buffer consisting of 10 mM sodium tetraborate,

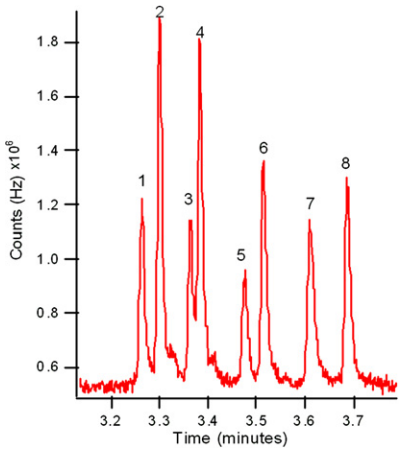



**Figure 2.** Separation of standards by CE: (1) **asialo-GM1-TMR**; (2) **GM1-TMR**; (3) **asialo-GM2-TMR**; (4) **GM2-TMR**; (5) **GM3-TMR**; (6) **Lac-TMR**; (7) **Glc-TMR**; (8) **Cer-TMR**.

35 mM sodium deoxycholate and 5 mM methyl-β-cyclodextrin in 20 μM 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 labeled, 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 eight compounds was achieved within 4 min 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

### 5.1. General experimental

Methyl-β-cyclodextrin was product 66292 from Fluka, with a methylation extent of 1.7–1.9 per glucose residue.

ESIMS was recorded on a Bruker Esquire 3000-Plus Ion Trap instrument with samples injected as solutions in either MeOH or MeOH–$CH_3CN$–$H_2O$ (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 μm i.d. with a length between 50 and 80 cm using 50 mM borate, pH 9.3 and 25 mM sodium dodecyl sulfate (SDS) as running buffer. TMR-labeled 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, that is, inlet anodic. Preparative TLC was performed on silica plates (E. Merck Prod. No.1.05745, 20 × 20 cm, 2 mm thickness). *lyso*-Lac-Cer, *lyso*-Glc-Cer and $C_{18}$-sphingosine were from Avanti Polar Lipids (AL, USA). β-Galactosidase and Sialidase A were from Prozyme (WA, USA).

**5.1.1. 6-Tetramethylrhodamine-(*tert*-butyl β-alanine)-amide 2.** 6-Tetramethylrhodamine *N*-hydroxysuccinimide ester (**1**, 28.5 mg, 53.8 μmol) and β-alanine *t*-butyl ester hydrochloride (16 mg, 88.1 μmol) were dissolved in 500 μL dry DMF in a 1.5 mL Eppendorf tube. The sample was vortexed for 1 min, 15 μL diisopropylethylamine (DIPEA) was added and the sample was covered with aluminum foil and placed on a shaker. TLC (1:1 $CH_2Cl_2$–MeOH) and ESIMS indicated almost complete conversion to the product after 10 min. After 1 h, the reaction mixture was transferred to a round bottom flask and concentrated to near dryness. Water (25 mL) was added and the solution was applied to two tandem reverse-phase $C_{18}$ Sep-Pak cartridges. The cartridges were washed with water (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 ESIMS 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 μmol, 96%). ESIMS: *m*/*z* 558.25 $[M+H]^+$.

**5.1.2. 6-Tetramethylrhodamine-(β-alanine)-amide 3.** Compound **2** (28.95 mg, 51.9 μmol) was dissolved in $CH_2Cl_2$ (5 mL) followed by addition of 1 mL of trifluoroacetic acid . After 3 h, TLC (1:1 $CHCl_3$–MeOH) and ESIMS indicated that all the 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 ($CHCl_3$–MeOH gradient from 10/1 to 1/3) to give **3** (25.1 mg, 50.0 μmol, 96%). ESIMS: *m*/*z* 502.19 $[M+H]^+$.

**5.1.3. 6-Tetramethylrhodamine-(*N*-hydroxysuccinimidyl-β-alanine)-amide 4.** Compound **3** (7.34 mg, 14.6 μmol), 1.6 equiv, *N*,*N*′-disuccinimidyl carbonate (DSC, 5.90 mg, 23.0 μmol), and 1.7 equiv 4-dimethylaminopyridine (DMAP, 3.01 mg, 24.6 μmol) were weighed into three separate Eppendorf tubes. Dry DMF (200 μL) 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 (2:1 $CHCl_3$–DMF) and ESIMS showed that ester **4** had formed after 2.5 h. This DMF solution was then used directly for labeling of the *lyso*-glycosphingolipids. ESIMS: *m*/*z* 599.21 $[M+H]^+$.

**5.1.4. $C_{18}$-*lyso*-GM1.** GM1 (20 mg, 12.8 μmol, an approximately equimolar mixture of $C_{18}$ and $C_{20}$ sphingosine isomers) was dissolved in 2 mL of 100 mM sodium acetate buffer (pH 5.8) followed by the addition of 5.15 mL water and 0.8 mL taurodeoxycholate (TDC, 8 mg/mL, Sigma). Sphingolipid ceramide *N*-deacylase (SCDase), 50 μL of a 5 mU/μL 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 (60:39:10 $CHCl_3$–MeOH–0.25% KCl), 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 μm, Waters). The column eluent was monitored at 206 nm using a UV-detector (LKB). The column was washed with water 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 (**$C_{18}$-*lyso*-GM1** followed by **$C_{20}$-*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 analyzed by ESIMS. **$C_{18}$-*lyso*-GM1** (5.45 mg, 4.26 μmol, 33%) expected: $[M+H]^+$ 1280.62, found: 1280.6; **$C_{20}$-*lyso*-GM1** (4.80 mg, 3.67 μmol, 29%) expected: $[M+H]^+$ 1308.65, found: 1308.6.

**5.1.5. GM1-TMR.** **$C_{18}$-*lyso*-GM1** (12.0 mg, 9.37 μmol) was dissolved in 10:12:21 DMF–$CHCl_3$–water (1.33 mL). To this solution, 1.55 equiv of NHS-ester **4** in DMF (600 μL) was added, followed by DIPEA (30 μL). The reaction tube was sealed with Parafilm, covered with aluminum foil and placed on a shaker. Analysis by TLC (60:35:8 $CHCl_3$–MeOH–0.25% KCl) and ESIMS after 1 h showed a significant amount of

product together with excess unreacted **4**. The reaction was therefore allowed to stand overnight (15 h) to go to completion and to hydrolyze the reactive dye to facilitate product purification. The reaction mixture was diluted with water (30 mL) and then applied onto two tandem $C_{18}$ Sep-Pak cartridges. The cartridges were washed with water (30 mL), then 20 mL fractions of each of 25% aqueous MeOH, 33% MeOH, 40% MeOH, 50% MeOH, 75% MeOH and MeOH. The fractions were analyzed by TLC (60:35:8 $CHCl_3$–MeOH–0.25% KCl) and ESIMS. The 25–40% fractions contained hydrolyzed **4** and the 50–100% fractions contained labeled GM1. The glycolipid containing fractions were pooled and evaporated to give **GM1-TMR** (14.45 mg, 8.19 μmol, 87%). ESIMS: *m*/*z* 1763.80 $[M+H]^+$.

**5.1.6. Lac-TMR.** D-Lactosyl-β-1,1′-D-*erythro*-sphingosine ($C_{18}$ isomer, 0.50 mg, 0.80 μmol) was treated as described for *lyso*-GM1, giving **Lac-TMR** (0.80 mg, 0.72 μmol, 90%). ESIMS: *m*/*z* 1107.57 $[M+H]^+$.

**5.1.7. Glc-TMR.** D-Glucosyl-β-1,1′-D-*erythro*-sphingosine (4.83 mg, 10.46 μmol) was treated as described for *lyso*-GM1, giving **Glc-TMR** (9.43 mg, 9.98 μmol, 95%). ESIMS: *m*/*z* 945.5 $[M+H]^+$.

**5.1.8. Cer-TMR.** D-*erythro*-Sphingosine (3.11 mg, 10.38 μmol) was treated as described for *lyso*-GM1, giving **Cer-TMR** (7.69 mg, 9.82 μmol, 95%). ESIMS: *m*/*z* 783.5 $[M+H]^+$.

### 5.2. Enzymatic conversions of TMR-labeled sphingolipid analogs

**5.2.1. General procedures.** The progress of enzyme incubations was monitored by TLC with visual detection of the red starting materials and products. After the progress of the reaction 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 water (50 mL). After sample application, the cartridges were washed with 100 mL water then TMR-labeled 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 60:39:10 $CHCl_3$–MeOH–0.25% KCl as eluent. The red bands were scraped off the plates and extracted with MeOH until the silica became colorless. After evaporation of the MeOH, the residue was dissolved in water (5 mL) passed through a 0.45 μm Millex-GV PVDF filter to remove particulates and then lyophilized.

**5.2.2. GM2-TMR.** **GM1-TMR** (1.33 mg) was dissolved in 40 μL of 5× galactosidase-reaction buffer (Prozyme) and 140 μL water, then 20 μL of β-galactosidase (5 mU/μL, bovine testes, Prozyme) was added and the sample was mixed gently. The tube was covered with aluminum foil and the reaction was incubated at 37 °C for 4 day. **GM2-TMR** (0.65 mg, 0.41 μmol, 54%) was obtained after preparative TLC, ESIMS: *m*/*z* 1599.5 $[M-H]^-$.

**5.2.3. Asialo-GM1-TMR.** **GM1-TMR** (1.31 mg) was dissolved in 40 μL of 5× sialidase reaction buffer (Prozyme) and 140 μL water. When the GM1 was dissolved, 20 μL of sialidase A (5 U/mL) was added. The tube was shaken, then covered with aluminum foil and the reaction was incubated at 37 °C for 6 days. Preparative TLC gave **Asialo-GM1-TMR** (0.75 mg, 0.58 μmol, 65%), ESIMS: *m*/*z* 1472.2 $[M+H]^+$.

**5.2.4. Asialo-GM2-TMR.** **Asialo-GM1-TMR** (0.1 mg) was dissolved in 20 μL of 5× reaction buffer (Prozyme), and 70 μL water and 10 μL of β-galactosidase (5 mU/μL) were added and the sample was gently mixed. The tube was covered in aluminum 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, ESIMS: *m*/*z* 1310.6 $[M+H]^+$.

**5.2.5. GM3-TMR.** CMP–sialic acid (10.4 mg) was dissolved in 500 μL 40 mM Tris–HCl buffer (pH 7.5). This was transferred to a tube containing **Lac-TMR** (6.16 mg, 5.56 μmol). After dissolving the **Lac-TMR**, 100 μL 100 mM $MgCl_2$, 3 μL alkaline phosphatase (Roche No. 108 138, 1500 21 U/μL), 197 μL water and 200 mU (1 U/mL) of α2,3-sialyltransferase (MalE fusion-protein from *Campylobacter jejuni*[41]) were added and the mixture was mixed gently. The tube was covered with aluminum 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 μmol, 96%), ESIMS: *m*/*z* 1398.7 $[M+H]^+$.

## Acknowledgements

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