A Paradigm Case for the Merging of Glycal and Enzymatic Assembly Methods in Glycoconjugate Synthesis: A Highly Efficient Chemo-Enzymatic Synthesis of GM_3

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Abstract: A concise, regio- and stereoselective synthesis of the ganglioside GM_3 has been achieved. Use of an α-1,2-oxirane derived from lactal facilitated installation of the anomeric β-ceramide side chain. Introduction of the sialic acid residue at C_3 of the lactose was accomplished regiospecifically by enzymatically mediated sialyl transfer from CMP-Neu-5-Ac.

Keywords
anhydrosugars · enzymatic catalysis · gangliosides · glycoconjugates · glycosidations

Introduction

Gangliosides are cell-surface sialic acids containing glycolipids. They are encountered in the central nervous system where they may play an important role in the process of transmembrane signal transduction.[1, 2] The lipid-like ceramide backbone anchors the gangliosides to the cell membrane. Gangliosides have also been identified as tumor-associated antigens[3] and as cell differentiation markers.[4] They are implicated in other significant biological settings such as cell–cell adhesion[5, 6] and cell growth regulation.[7] The diversity of gangliosides in either moderating or masking biological events arises mainly from their complex oligosaccharide domains. Additional diversity is contributed by homologous series variations in the N-acyl substituent. While there are no ganglioside drugs, GM_1 has been suggested as a promising therapeutic agent for the treatment of Parkinson's disease.[8]

In 1952, Yamakawa's group first discovered and isolated ganglioside GM_3 from equine erythrocytes.[9] It has been shown to serve as a biosynthetic precursor for other complex gangliosides.[10] It is known to modulate the epidermal growth factor (EGF) and the platelet-derived growth factor (PDGF) receptors.[11] Abnormal supression of GM_3 (with concurrent buildup of GD_3) has been observed in tumor cells.[12] It has also been demonstrated that cytotoxic T cells and suppressor T cells in antimelanoma immune responses recognize GM_3.[13] Given the extreme difficulty of isolating homogeneous gangliosides from natural sources,[14] effective routes for their preparation are much to be desired.

The major challenges in a synthesis of GM_3 are those of overall conciseness as well an efficient, regiospecific sialylation reaction. It is also necessary to incorporate the ceramide moiety with high positional control and stereoselectively. For the latter goal, the C-2 hydroxyl group of a proposed donor has to be differentiated from the remaining masked hydroxyl groups. Placement of a participatory protecting and directing group on this α-"hydroxyl" center promotes the formation of the β-ceramide-like glycosidation product. It was in this respect that α-1,2-anhydrosugars, derived from direct epoxidation of glycals, might be attractive as glycosyl donors for the formation of β-glycosides.[15] This methodology could avoid the need for fashioning a unique β-directing group at C-2.

Another major advance in the field of ganglioside synthesis could be occasioned by application of glycosyl transferases in the construction of oligosaccharides.[16] Enzymatic methods can provide several advantages over classic chemical methods. High regio- and stereoselectivity may be achieved by biologically based catalysis, thus obviating the need for much of the protection and deprotection of classical oligosaccharide assembly. The prospect of introducing the synthetic equivalent of ceramide through reaction with a glycal epoxide before the sialic acid is already in place seemed attractive in that it might then not be necessary to protect this sialic acid functionality. However, success of the enzymatic sialylation requires acceptance of the substrate by the sialyl transferase for delivery of the sialic acid from the cytidine monophosphate (CMP) sialic acid donor. Thus, the challenge to a combined chemoenzymatic synthesis of GM_3 became that of identifying an intermediate where enzymatic sialylation could be conducted at a stage where the introduction of the ceramide residue was already well advanced. Our solution to this problem is described and documented below, wherein we describe a highly expeditious route to GM_3.[17, 18]

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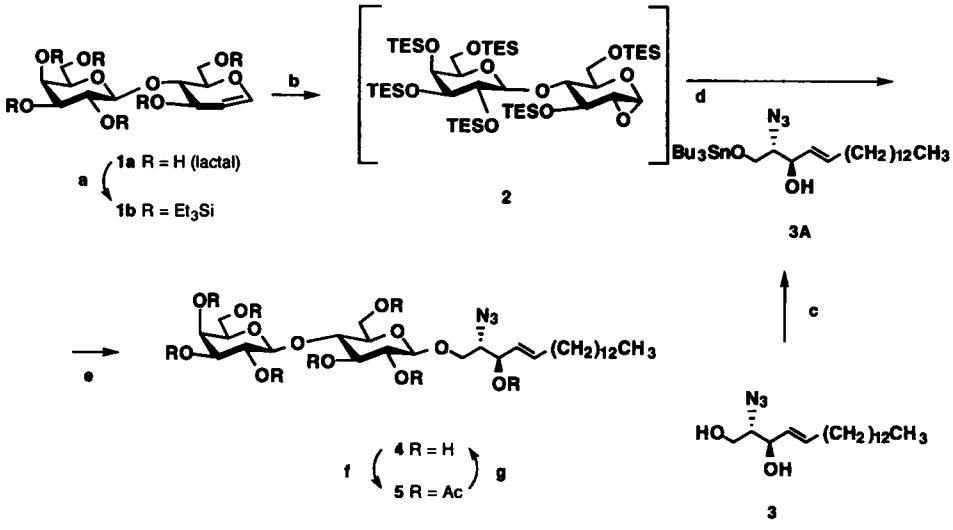
Results and Discussion

Lactal **1a**[19] was treated with excess triethylsilyl triflate in pyridine to give the pertriethylsilylated product **1b** in 81% yield (Scheme 1). Exposure of this uniformly per-protected glycal to



Scheme 1. a) 9 equiv TESOTf, cat. DMAP, pyridine (81%). b) 2,2-dimethyldioxirane, acetone, 0°C. c) 1.1 equiv **3**, 0.5 equiv (nBu$_3$Sn)$_2$O, toluene, reflux. d) 2 equiv **3**, 2 equiv Zn(OTf)$_2$, THF, O°C → RT. e) TBAF, THF. f) Acetic anhydride, cat. DMAP, pyridine (44%, **1b** → **5**). g) NaOMe, MeOH (95%).

2,2-dimethyldioxirane in acetone[20, 15] gave rise to per-TES 1,2-anhydrolactal derivative **2**. To confirm that the 1,2-anhydrosugar had the α-configuration, compound **2** was treated with excess methanol thereby affording, after deprotection, the readily characterizable β-methyl lactoside.

Confident of the α-configuration of the 1,2-anhydrosugar linkage, we turned to the use of **2** as a glycosyl donor with **3**.[21] We hoped for a straightforward extension of our earlier methodology (i.e. anhydrous zinc chloride as the promoter), as practiced in our synthesis of GM_4.[22] Application of the zinc chloride protocol gave disappointing results. Perhaps, in this case, the presence of a diol linkage in the ceramide precursor **3** interferes with the ability of zinc chloride to orchestrate the glycosidation. Furthermore, in the GM_4 synthesis, we made use of the α-1,2-anhydrosugar derived from galactal cyclic 3,4-carbonate. In general, this glycal epoxide is among our most effective and high-yielding donors. Thus, success with this donor is not necessarily extendable to other glycosylating combinations.[23]

Fortunately, a new procedure, which strengthens the nucleophility of the glycosyl acceptor and obviates the need for differential protection of the diol acceptor, was developed. The stannyl alkoxide (cf. **3A**) was generated in situ from the reaction of **3** with bis(tributyltin) oxide in refluxing toluene under a Dean–Stark trap.[24] After being dried on a pump, **3A** was treated with **2** in the presence of zinc triflate. Following exhaustive desilylation, β-glycoside **4** was generated. The product in crude form was acetylated (purely for purposes of purification and spectral analysis) to provide the octaacetate **5** (44% yield from **1b**). A minor contaminant (ca. 5%) derived from glycosidation of the C3 hydroxyl of **3** was also found. Deacetylation of **5** with sodium methoxide in methanol provided homogeneous **4** in 95% yield. The generality of the stannyl alkoxide glycosidation was further applied to our syntheses of digitalis saponin[25] as well as of the human breast tumor associated antigen.[26]

A variety of Lewis acids were evaluated for this stannyl alkoxide glycosidation reaction before we selected zinc triflate as the preferred glycosidation promoter. Among the other promoters surveyed, tin triflate also resulted in a seemingly clean reaction at the TLC level, but the isolated yield was lower than with zinc triflate. Interestingly, when silver tetrafluoroborate was used, we observed that α-glycosidation took place. While not useful for the case at hand, this capability enhanced the value of α-1,2-anhydrosugars because they can be used to introduce either α- or β-glycosides with silver tetrafluoroborate or zinc triflate, respectively.[27]

Compound **4** was subjected to chemical sialylation with various sialyl donors, including sialyl phosphite, sialyl chloride, sialyl xanthate and sialyl methanethiolate. However, these attempts proved to be fruitless. Chemically selective sialylation of the lactosyl ceramide had once more been shown to be difficult, when the pre-ceramide side chain was already in place. The wisdom of the previous "all-chemical" syntheses, where the reversed order is practiced, was thus confirmed.[18, 28]

At this pont, our interests were directed to enzymatic sialylation. For this goal, it would be crucial to find a compound which would be tolerated by α-2,3-sialyltransferase.[16, 18c] In this way, introduction of the sialic acid residue by enzymatically mediated transfer from cytidine 5′-monophospho-*N*-acetylneurminic acid (CMP-Neu-5-Ac, **6**)[29] could be achieved. We were mindful that the enzyme α-2,3-sialyltransferase has been reported to reject lactosylceramide as a substrate.[30] Whether this failure is a consequence of innate topological incompatibility between substrate and enzyme, or arises from the poor solubility of the lactosylceramide in aqueous solution is not clear.

In seeking an appropriate substrate, we decided to try the less hydrophobic "preceramide lactoside" **4**, hoping that it would be recognized and accepted by the transferase. Happily, compound **4** was indeed sialylated by the transferase in the presence of CMP-Neu-5-Ac (**6**) in pH = 7.4 buffer solution, to produce **7** in good yield (75%) (Scheme 2). *This key finding, in principle, provides many new avenues in the field of ganglioside synthesis.*

In the case at hand, sialylation was followed by reduction of the azide group of **7** (saturated H_2S in pyridine/H_2O solution). The crude amine was stearoylated in a biphasic system (THF/50% NaOAc) according to the Shapiro method[31] without affecting the hydroxyl and carboxylic groups. The crude amide was purified on LiChroprep RP-18 resin and Bio-gel P-2 column to give GM_3 in 40% yield from **7**.

When our program was in progress, another chemoenzymatic synthesis of GM_3 was reported.[18c] However, extensive manipulations were necessary to prepare the substrate for enzymatic sialylation and to achieve selective glycosidation for late-stage 1,2-transglycosidation.

Conclusion

In summary, the α-1,2-anhydrosugar **2** derived from readily available lactal **1a** functioned as an accessible, stereoselective lactosyl donor without the need for multistep hydroxyl group

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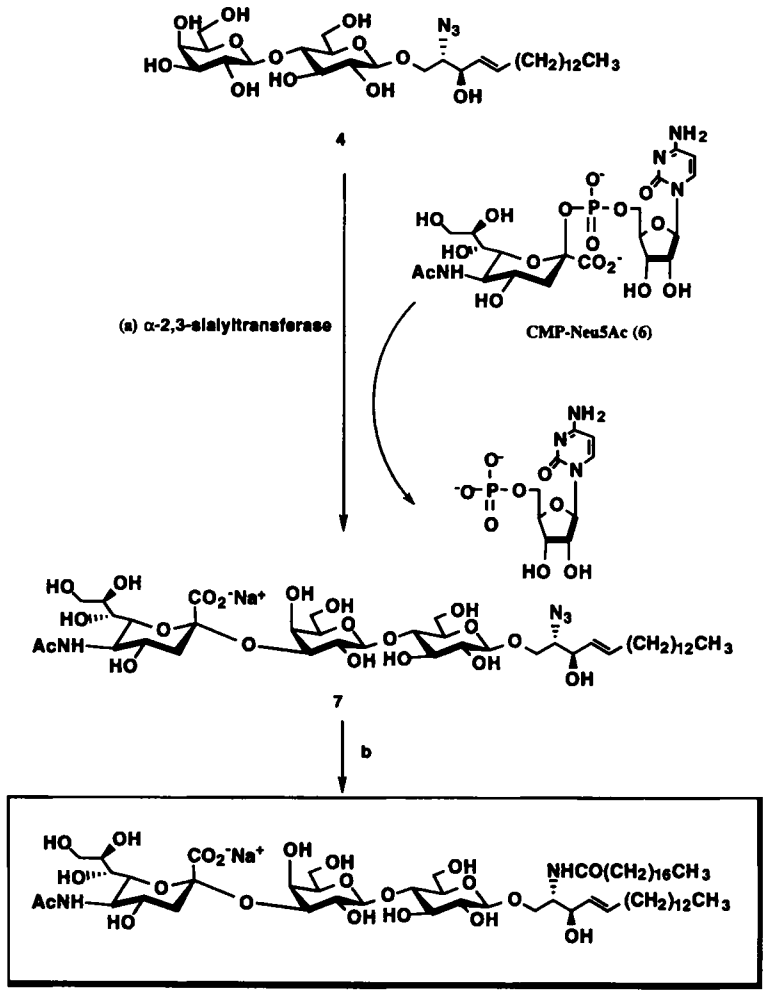

Scheme 2. a) α-2,3 sialyltransferase ([E. C.2.4.99.4]), Triton CF-54 (0.5%), bovine serum albumin, calf intestine alkaline phosphatase, HEPES buffer (pH 7.4), 5 d, RT (75%). b) i) H_2S, pyridine/water = 1:1. ii) stearoyl chloride, 50% NaOAc/THF = 1:2 (40%, two steps).

differentiation. Based on our chemistry and that of other laboratories,[32] glycals have been demonstrated to be convertible to a number of derivatives. They have emerged as attractive starting materials for the construction of biologically important glycoconjugates such as adhesion molecules[33] and glycopeptides.[34] The combination of the powers of glycal chemistry and enzymatic glycosidation holds considerable promise for simplifications in the synthesis of other complex glycoconjugates.[35] Further extensions of these findings will be the subject of future reports.

Experimental Procedure

General Methods: Infrared spectra were recorded on a Perkin Elmer 1600 Series FTIR. ^{1}H NMR spectra were obtained on a General Electric QE Plus NMR (300 MHz) or Bruker WM-250 and are reported in parts in million (δ) relative to $SiMe_4$ (0.00 ppm) or to [D_4]methanol (3.65 ppm) as internal reference, with coupling constants (J) report in hertz. High-resolution mass spectra were recorded on a Kratos MS-80RFA mass spectrometer. Optical rotations were recorded on a Jasco DIP-370 polarimeter with a 1 dm cell at the reported temperatures and concentrations. Sialyltransferase was kindly provided by Cytel Corp. (La Jolla, California). Chemicals used were reagent grade and used as supplied except where noted. Pyridine, toluene, benzene, and dichloromethane were distilled from calcium hydride under N_2. Tetrahydrofuran was distilled from sodium/benzophenone ketyl under N_2. Analytical thin-layer chromatography was performed on E. Merck silica gel 60 F_{254} plates (0.25 mm). Compounds were visualized by dipping the plates in a cerium sulfate/ammonium molybdate solution followed by heating. Liquid column chromatography was performed using forced flow of the indicated solvents on E. Merck silica gel 60 (40–63 μm).

***O*-(2,3,4,6-Tetra-*O*-triethylsilyl-β-D-galactopyranosyl)-(1→4)-1,5-anhydro-3,6-di-*O*-triethylsilyl-2-deoxy-D-arabinohex-1-enitol (1b)**: A solution of lactal **1a** (310 mg, 1 mmol) in dry pyridine (10 mL) was treated with triethylsilyl trifluoromethanesulfonate (2 mL, 9 mmol) and DMAP (24 mg, 0.2 mmol) at 0 °C. The solution was allowed to warm up to RT and was stirred for 36 h. The residue, after concentration, was purified by SiO_2 column chromatography (ethyl acetate:hexane = 1:7) to give **1b** (804 mg, 81% yield) as a viscous liquid. $[\alpha]_D^{23} = -5.06$ (c = 2.49, $CHCl_3$); IR ($CHCl_3$): $\tilde{\nu}$ = 2953, 2876, 1651, 1241, 1103, 1008 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 6.26 (dd, J = 6.0, 1.1 Hz, 1H, H-1), 4.60 (dd, J = 6.1, 2.9 Hz, 1H), 4.35 (d, J = 7.5 Hz, 1H), 4.20 (m, 1H), 4.06 (dd, J = 11.4, 3.7 Hz), 3.92–3.90 (m, 2H), 3.85–3.82 (m, 1H), 3.75 (dd, J = 11.5, 2.5 Hz, 1H), 3.71 (dd, J = 7.4, 2.2 Hz, 1H), 3.64–3.68 (m, 1H), 3.55 (dd, J = 9.6, 4.9 Hz, 1H), 3.35 (dd, J = 9.3, 2.3 Hz, 1H), 3.18 (dd, J = 9.2, 5 Hz, 1H), 0.97–0.88 (m, 36H), 0.66–0.50 (m, 54H); HRMS (FAB) calcd for (M + Na$^+$): 1015.6193, found: 1015.5530.

(2*S*,3*R*,4*E*)-3-(Acetyloxy)-2-(azido)-1-[(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-(1 → 4)-(2,3,6-tri-*O*-acetyl-β-D-glucopyranosyloxy)]-4-octadecene (5): Compound **1b** (121 mg, 0.12 mmol) was dissolved in dry CH_2Cl_2 (2 mL) and cooled to 0 °C under a nitrogen atmosphere. Dimethyldioxirane (4 mL of a 0.07 M solution, 0.28 mmol) was added dropwise, and stirring was continued at 0 °C for 30 min. The solution containing the resultant epoxide **2** was concentrated to dryness under a stream of nitrogen followed by vacuum drying for 1 h. Zinc triflate (87 mg, 0.24 mmol) was added to the resultant product under a nitrogen atmosphere. A solution of (2*S*,3*R*,4*E*)-2-azido-3-hydroxy-1-tributylstanyloxy-4-octadecene (**3**) [21] (0.24 mmol, 2 equiv) in dry THF (5 mL) was added to **2**, and the reaction mixture was cooled to 0 °C. The reaction was allowed to slowly warm up to RT and stirred overnight. The reaction mixture was passed through silica gel plug and concentrated under reduced pressure. The residue was treated with tetrabutylammonium fluoride (TBAF) (1 M, 1 mL, 1 mmol) in THF (5 mL) at RT for 24 h. Solvent was removed under reduced pressure, and the residue dissolved in dry pyridine (5 mL). Acetic anhydride (1.5 mL) and 4-dimethylaminopyridine (DMAP) (14 mg, 0.12 mmol) were added to the pyridine solution. After 24 h, the solvent was concentrated under reduced pressure, and the residue purified by SiO_2 column chromatography (ethyl acetate:hexane = 1:1) to give 55 mg of compound **5** as a viscous liquid (44% overall yield from **1b**). $[\alpha]_D^{23} = -20.86$ (c = 0.7, $CHCl_3$); IR ($CHCl_3$): $\tilde{\nu}$ = 2924, 2853, 2101, 1750, 1369, 1229, 1057 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 5.81 (dt, J = 6.6, 15.3 Hz, 1H), 5.40 (dd, J = 6.1, 15.4 Hz, 1H), 5.33 (d, J = 3.3 Hz, 1H), 5.30 (dd, J = 4.4, 8.2 Hz, 1H), 5.17 (t, J = 9.2 Hz, 1H), 5.10 (dd, J = 7.9, 10.3 Hz, 1H), 4.94 (dd, J = 3.4, 10.4 Hz, 1H), 4.90 (t, J = 8.8 Hz, 1H), 4.51–4.47 (m, 3H), 4.14–4.05 (m, 3H), 3.87–3.71 (m, 4H), 3.59 (ddd, J = 2, 4.9, 9.8 Hz, 1H), 3.49 (dd, J = 5.2, 9.9 Hz, 1H), 2.15 (s, 3H), 2.12 (s, 3H), 2.06 (s, 3H), 2.05 (s, 3H), 2.05–2.02 (m, 2H), 2.04 (s, 3H), 2.02 (s, 3H), 2.01 (s, 3H), 1.96 (s, 3H), 1.35–1.25 (m, 22H), 0.87 (t, J = 6.6 Hz, 3H); HRMS (FAB) calcd for (M + Na$^+$): 1008.4528, found: 1008.4596.

(2*S*,3*R*,4*E*)-1-[(β-D-Galactopyranosyl)-(1→4)-β-D-glucopyranosyloxy]-2-azido-4-octadecene-3-ol (4): A solution of compound **5** (100 mg, 0.1 mmol) and 25% sodium methoxide (0.1 mL) in dry MeOH (5 mL) was stirred at RT for 12 h. The mixture was neutralized with Amberlite, IR 120 (H$^+$). Filtration, concentration of the filtrate, and purification with reverse-phase column chromatography (LiChroprep. RP-18, MeOH) gave 62 mg of **4** (95% yield) as a viscous liquid. $[\alpha]_D^{23} = -8.57$ (c = 0.7, CH_3OH); IR (neat): $\tilde{\nu}$ = 3354, 2959, 2923, 2853, 2096 cm^{-1}; ^{1}H NMR (400 MHz, CD_3OD): δ = 5.75 (dt, J = 6.8, 15.4 Hz, 1H), 5.47 (dd, J = 6.3, 15.3 Hz, 1H), 4.31 (d, J = 7.6 Hz, 1H), 4.26 (d, J = 8 Hz, 1H), 4.13–4.10 (m, 1H), 3.05–3.87 (m, 15H), 2.02 (q, J = 7.2 Hz, 2H), 1.23–1.33 (m, 22H), 0.84 (t, J = 7.1 Hz, 3H); HRMS (FAB) calcd for (M + Na$^+$): 672.3683, found: 672.3946.

***O*-(Sodium 5-acetamido-3,5-dideoxy-D-glycerol-α-D-galacto-2-nonulopyranosylonate)-(2→3)-D-(β-D-galactopyranosyl)-(1→4)-*O*-(β-D-glucopyranosyl)-(1→1)-(2*S*,3*R*,4*E*)-2-azido-4-octadecene-1,3-diol (7)**: The acceptor **4** (20 mg, 0.031 mmol) was sonicated for 10 min in HEPES buffer (2 mL, pH = 7.4) containing Triton CF-54 (30 uL) and NaN_3 (1%). The suspension was incubated at 25 °C in the presence of bovine serum albumin (2 mg), α-2,3-sialyl transferase (0.4 Unit), alkaline phosphatase (from calf intestinal mucosa, 4 Unit), and CMP-Neu-5-Ac (40 mg, 0.064 mmol) for 5 d. The reaction mixture was lyophilized to remove water. The residue was purified with reverse-phase C_{18} column (LiChroprep. RP-18, EM Science, MeOH:H_2O = 4:1) and Bio-gel P-2 column to give 7, 23 mg, 75% yield, as a fluffy compound. $[\alpha]_D^{23} = -10.87$ (c = 0.8, MeOH); IR (neat): $\tilde{\nu}$ = 3350, 2922, 2852, 2098, 1657, 1640, 1616, 1071, 1031 cm^{-1}; ^{1}H NMR (400 MHz, CD_3OD):

δ = 5.86 (m, 1H), 5.58 (m, 1H), 4.56 (d, J = 7.6 Hz), 4.48 (d, J = 7.8 Hz, 1H), 4.20–4.14 (m, 2H), 3.99–3.52 (m, 18H), 3.40–3.37 (m, 1H), 2.79 (broad d, J = 7.2 Hz, 1H, sialyl-H_{3eq}), 2.16–2.10 (m, 2H), 2.04 (s, 3H), 1.86 (m, 1H, sialyl-H_{3ax}), 1.46–1.26 (m, 22H), 0.93 (t, J = 7.2 Hz, 3H). HRMS (FAB) calcd for (M + Na^+): 985.4457, found: 985.4503.

***O*-(Sodium 5-acetamido-3,5-dideoxy-D-glycero-α-D-galacto-2-nonulopyranosylnate)-(2→3)-*O*-β-D-galactopyranosyl-(1→4)-*O*-β-D-glucopyranosyl(1→1)-(2*S*,3*R*,4*E*)-2-octadecanamido-4-octadecene-1,3-diol (GM_3)**: Compound **7** (10 mg, 0.01 mmol) in pyridine/water (1:1, 5 mL) was saturated at RT with hydrogen sulfide. After one day, TLC analysis indicated that the reaction was finished, and the reaction mixture was concentrated to give crude amine (TLC: *i*PrOH:EtOAc:H_2O = 2:2:1, R_f = 0.35, detection with 1% solution of ninhydrin in ethanol). The crude amine was used directly in the next step. To a rigorously stirred suspension of the crude amine in THF (2 mL) and 50% sodium acetate in water (1 mL) was added stearoyl chloride (10 uL, 0.028 mmol). After 21 h, the mixture was concentrated and purified with a reverse-phase chromatographic column (LiChroprep RP-18; MeOH:H_2O = 9:1) and a gel filtration column (Lipophilic Sephadex, LH-20, 100% MeOH) to give GM_3 (5 mg, 40% yield from **7**) as a viscous liquid. The spectroscopic data for this synthetic GM_3, which were consistent with the previously reported data [18], were as follows. $[\alpha]_D^{23}$ = 1.65 (c = 0.1, MeOH:$CHCl_3$ = 1:1); IR (neat): $\tilde{\nu}$ = 3750–2810, 2930, 2834, 1675, 1643, 1550; ^{1}H NMR (400 MHz, CD_3OD): lactose unit δ = 4.45 (d, 1H, J = 8 Hz), 4.32 (d, 1H, J = 7.9 Hz); Neu_5Ac unit δ = 1.95 (5.5H, AcN), 2.897 (dd, 1H, J = 11.8 Hz, 2.5 Hz, H-3C); ceramide unit δ = 0.92 (t, 6H, J = 6.7 Hz, 2 × CH_3), 2.20 (t, 2H, J = 6.6 Hz), 4.19 (dd, 1H, J = 9.8, 3.9 Hz), 5.48 (dd, 1H, J = 7.5, 15.4 Hz), 5.69 (dt, 1H, J = 15.3, 7.6 Hz). HRMS (FAB) calcd for (M + Na^+): 1203.7342, found: 1203.7285.

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