# Supporting Information

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**Bacterial strains, plasmids, materials and general methods**

*E. coli* electrocompetent DH5α and chemically competent BL21 (DE3) cells were purchased from Invitrogen (Carlsbad, CA). Synthetic gene for *E. coli* O126 WbgL with codon optimized for *E. coli* system was synthesized by Invitrogen GeneArt Gene Synthesis of ThermoFisher Scientific (Waltham, MA, USA). Vector plasmids pET22b (+) was from Novagen (EMD Biosciences Inc. Madison, WI, USA). Restriction enzymes NdeI and XhoI were purchased from New England Biolabs, Inc. (Beverly, MA, USA). Nickel-nitrilotriacetic acid agarose ($Ni^{2+}$-NTA agarose) was from Qiagen (Valencia, CA, USA). GeneJET Plasmid Miniprep kit, and GeneJET PCR Purification kit were from Thermo scientific (San Diego, CA, USA). Herculase-enhanced DNA polymerase was from Stratagene (La Jolla, CA, USA). T4 DNA ligase and 1 kb DNA ladder were from Promega (Madison, WI, USA). Chemicals were purchased and used without further purification. $^{1}$H NMR and $^{13}$C NMR spectra were recorded on 800 MHz Bruker Avance III spectrometers. High resolution electrospray ionization (ESI) mass spectra were obtained using Thermo Electron LTQ-Orbitrap Hybrid MS at the Mass Spectrometry Facility in the University of California, Davis. Silica gel 60 Å (230–400 mesh, Sorbent Technologies) was used for flash column chromatography. Discover® C18 cartridges were bought from Sigma. Thin-layer chromatography (TLC, Sorbent Technologies) was performed on silica gel plates using anisaldehyde sugar staining or 5% sulfuric acid in ethanol staining for detection. Phytoshingosine was purchased from TCI America. Trifluoromethanesulfonic anhydride, thionly chloride, Hydrogen fluoride pyridine**,** 1,3-propane dithiol, 1,8-diazabicyclo(5.4.0)undec-7-ene(DBU) were bought from Sigma Aldrich. Ruthenium(III) chloride hydrate and ammonia solution (7 N in methanol) were bought from Acros. Boron trifluoride diethyl etherate**,** trichloroacetonitrile and benzoyl chloride were bought from Alfa Aesar. D-Galactose (Gal) and D-lactose were purchased from Fisher Scientific (Pittsburgh, Pennsylvania, USA. D-GalNAc was from Carbosynth US. L-Fucose was from V-LABS (Cov-ington, USA). Neu5Ac were bought from NingBo Hongxiang bio-chem (Ningbo, China). UTP, CTP, and GTP were bought from Hangzhou Meiya Phar-macy (Hangzhou, China). Adenosine 5'-triphosphate (ATP) was from Beta Pharma Scientific, Inc. (Branford, USA). Recombinant enzymes *Neisseria meningitidis* CMP-sialic acid synthetase (NmCSS),[1] *Pasteurella multocida* multifunctional α2–3-sialyltransferase 3 (PmST3),[2] *Campylobacter jejuni* multifunctional α2–3/8-sialyltransferase (CjCstII),[3] *Bifidobacterium longum* strain ATCC55813 *N*-acetylhexosamine-1-kinase (BLNahK),[4] *Pasteurella multocida N*-acetylglucosamine uridyltransferase (PmGlmU),[5] *Pasteurella multocida* inorganic pyrophosphatase (PmPpA),[6] *Campylobacter jejuni* β1–4GalNAcT (CjCgtA),[7] *Escherichia coli* galactokinase (EcGalK),[8] *Bifidobacterium longum* UDP-sugar pyrophosphorylase (BLUSP),[9] *Campylobacter jejuni* β1–3-galactosyltransferase (CjCgtB),[10] and *Bacteroides fragilis* strain NCTC9343 bifunctional L-fucokinase/GDP-fucose pyrophosphorylase (BfFKP)[11] were expressed and purified as described previously. Purified enzymes without dialysis can be directly used in the enzymatic reactions and the presence of imidazole didn't affect the enzymatic reactions.

**Cloning, expression, and purification of EcWbgL**

*E. coli* O126 WbgL (NCBI Reference Sequence: ABE98421.1) was cloned as a C-$His_6$-tagged fusion protein in pET22b(+) vector using a synthetic gene with codon optimization in *E. coli* system (ThermoFisher Scientific) as the template for polymerase chain reactions (PCR). The primers used for EcWbgL were: forward primer 5'-GATC*CATATG*AGCATTATTCGTCTGCAGG -3' (*Nde*I restriction site is italicized) and reverse primer 5'- CGC*CTCGAG*ACAGCTGCTATGTTTATCCACG -3' (*Xho*I restriction site is

italicized). PCR was performed in a 50 μL reaction mixture containing genomic DNA (1 μg), forward and reverse primers (1 μM each), 10× Herculase buffer (5 μL), dNTP mixture (1 mM), and 5 U (1 μL) of Herculase-enhanced DNA polymerase. The reaction mixture was subjected to 35 cycles of amplification with an annealing temperature of 52 °C. The resulting PCR product was purified and digested with corresponding restriction enzymes. The purified and digested PCR product was ligated with predigested pET22b(+) vector and transformed into electrocompetent *E. coli* DH5α cells. Selected clones were grown for minipreps and characterization by restriction mapping and DNA sequencing performed by Davis Sequencing Facility at the University of California-Davis.

*E. coli* strains were cultured in LB rich medium (10 g/L tryptone, 5 g/L yeast extract, and 10 g/L NaCl) supplemented with ampicillin (100 μg/mL). Over-expression of EcWbgL was achieved by inducing the *E. coli* BL21 (DE3) cell culture with 0.1 mM of isopropyl-1-thio-β-D-galactopyranoside (IPTG) when the $OD_{600}$ nm of the culture reached 0.8–1.0 followed by incubation at 20 °C for 20 h.

Bacterial cells were harvested by centrifugation at 4 °C in a Sorvall Legend RT centrifuge at 10,000 × rpm for 15 min. Harvested cells were resuspended in lysis buffer (Tris-HCl buffer, 100 mM, pH 8.0 containing 0.1% Triton X-100) (2 mL for cells collected from 50 mililiter cell culture). Lysozyme (100 μg/mL) and DNaseI (5 μg/mL) were added to the cell resuspension. The resulting mixture was incubated at 37 °C for 50 min with shaking at 200 rpm. Cell lysate (supernatant) was obtained by centrifugation at 12,000 × rpm for 15 min.

Purification was carried out by loading the supernatant onto a $Ni^{2+}$-NTA column pre-equilibrated with 10 column volumes of binding buffer (5 mM imidazole, 0.5 M NaCl, 50 mM Tris-HCl, pH 7.5). The column was washed with 10 column volumes of binding buffer and 10 column volumes of washing buffer (16 mM imidazole, 0.5 M NaCl, 50 mM Tris-HCl, pH 7.5). The target protein was eluted with Tris-HCl buffer (50 mM, pH 7.5) containing imidazole (200 mM) and NaCl (0.5 M). The fractions containing the active enzymes were collected, and stored at 4 °C.

**Synthetic gene and protein sequences of EcWbgL:**

```
  1 ATGAGCATTATTCGTCTGCAGGGTGGTCTGGGTAATCAGCTGTTTCAGTTTAGCTTTGGT
  1 M  S  I  I  R  L  Q  G  G  L  G  N  Q  L  F  Q  F  S  F  G

 61 TATGCCCTGAGCAAAATTAACGGTACACCGCTGTATTTTGATATCAGCCATTATGCCGAA
 21 Y  A  L  S  K  I  N  G  T  P  L  Y  F  D  I  S  H  Y  A  E

121 AACGATGATCATGGTGGTTATCGTCTGAATAACCTGCAGATTCCGGAAGAATATCTGCAG
 41 N  D  D  H  G  G  Y  R  L  N  N  L  Q  I  P  E  E  Y  L  Q

181 TATTATACCCCGAAAATCAACAACATCTACAAACTGCTGGTTCGTGGTAGCCGTCTGTAT
 61 Y  Y  T  P  K  I  N  N  I  Y  K  L  L  V  R  G  S  R  L  Y

241 CCTGATATTTTTCTGTTTCTGGGCTTTTGCAACGAATTTCATGCCTATGGCTATGACTTT
 81 P  D  I  F  L  F  L  G  F  C  N  E  F  H  A  Y  G  Y  D  F

301 GAATATATCGCCCAGAAATGGAAAAGCAAAAAATACATTGGTTACTGGCAGAGCGAACAC
101 E  Y  I  A  Q  K  W  K  S  K  K  Y  I  G  Y  W  Q  S  E  H

361 TTTTTTCATAAACATATCCTGGATCTGAAAGAGTTTTTTATCCCGAAAAATGTTAGCGAA
121 F  F  H  K  H  I  L  D  L  K  E  F  F  I  P  K  N  V  S  E

421 CAGGCAAATCTGCTGGCAGCAAAAATTCTGGAAAGCCAGAGCAGCCTGAGCATTCATATT
141 Q  A  N  L  L  A  A  K  I  L  E  S  Q  S  S  L  S  I  H  I

481 CGTCGTGGTGATTATATCAAAAACAAAACCGCAACCCTGACCCATGGTGTTTGTAGCCTG
161 R  R  G  D  Y  I  K  N  K  T  A  T  L  T  H  G  V  C  S  L

541 GAATACTACAAAAAAGCGCTGAACAAAATTCGTGATCTGGCCATGATTCGTGATGTGTTT
181 E  Y  Y  K  K  A  L  N  K  I  R  D  L  A  M  I  R  D  V  F

601 ATCTTTAGCGACGATATCTTTTGGTGCAAAGAGAACATTGAAACCCTGCTGTCCAAAAAA
201 I  F  S  D  D  I  F  W  C  K  E  N  I  E  T  L  L  S  K  K

661 TACAACATCTATTATAGCGAGGACCTGAGCCAAGAAGAGGATCTTTGGCTGATGAGCCTG
221 Y  N  I  Y  Y  S  E  D  L  S  Q  E  E  D  L  W  L  M  S  L

721 GCAAATCATCATATTATTGCCAACAGCAGCTTTAGTTGGTGGGGTGCATATCTGGGTAGC
241 A  N  H  H  I  I  A  N  S  S  F  S  W  W  G  A  Y  L  G  S

781 AGCGCAAGCCAGATTGTTATTTATCCGACCCCGTGGTATGATATTACACCGAAAAACACC
261 S  A  S  Q  I  V  I  Y  P  T  P  W  Y  D  I  T  P  K  N  T

841 TATATCCCGATTGTGAACCATTGGATCAACGTGGATAAACATAGCAGCTGTCTCGAGCAC
281 Y  I  P  I  V  N  H  W  I  N  V  D  K  H  S  S  C  L  E  H

901 CACCACCACCACCACTGA
301 H  H  H  H  H  Stop
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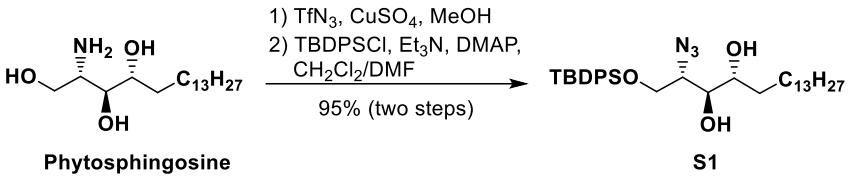
***Large scale synthesis of Lacβ Sph (5) in 13 grams scale***

***(2S,3S,4R)-2-Azido-1-(tert-butyldiphenylsilyoxy)octadecane-3,4-diol (S1)[12]***



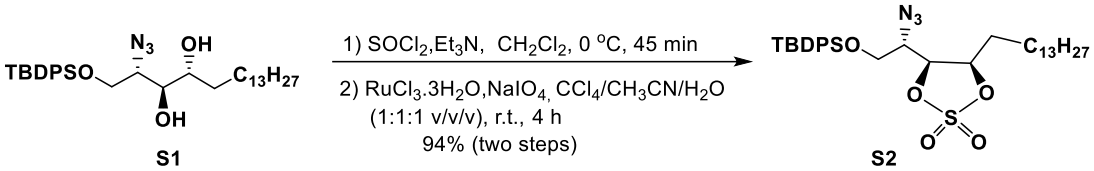

**Phytosphingosine** **S1**

*Preparation of $TfN_3$:* To a solution of $NaN_3$ (18.2 g, 280 mmol) in water (42 mL), $CH_2Cl_2$ (20 mL) was added. The reaction mixture was cooled down to 0 °C in an ice-water bath. To the vigorously stirred mixture, $Tf_2O$ (7.8 mL, 46.3 mmol) was added drop-wisely. After stirring the mixture at 0 °C for an additional 2 h, the organic phase was separated and the aqueous phase was extracted with $CH_2Cl_2$ (20 mL). Because of the potential explosive nature of the $TfN_3$,[13] the same reaction was carried out in triplicates in three different flasks to minimize the danger from possible accidents. After extraction, the dichloromethane layers containing $TfN_3$ were combined. The combined mixture was washed with saturated aqueous $NaHCO_3$ (150 mL) and used immediately for the next step.

Phytosphingosine (15 g, 47.24 mmol) was dissolved in MeOH (100 mL) in a 500 mL round-bottom flask. $Et_3N$ (20 mL, 143.5 mmol) and a solution of $CuSO_4$ (382 mg, 2.4 mmol in 20 mL $H_2O$) were added to the flask. Freshly prepared $TfN_3$ in dichloromethane solution prepared from the process described above was then added.[14] Additional amount of MeOH was added as needed to produce the reaction mixture miscible. The resulting solution was stirred at room temperature for 10 h. The volume of the reaction mixture was then reduced to 100 mL using a rotary evaporator. Water (100 mL) was added and the solution was cooled down by putting the container in ice-water. The mixture was filtered through a Büchner funnel and the residue was washed with ice cold water for 3 times and dried in a vacuum desiccator for 3 to 4 days before being proceeded to the next step without further purification.

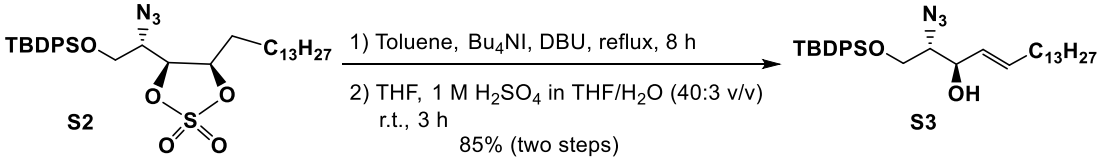
To a solution of 2-azido-phytosphingosine (16 g, 46.6 mmol) in $CH_2Cl_2$ (100 mL) and DMF (25 mL) at 0 °C, triethylamine (19.5 mL, 140 mmol), 4-dimethyl amino pyridine (DMAP) (285 mg, 2.33 mmol) and TBDPS-Cl (15 mL, 57.7 mmol) were added. The reaction mixture was stirred at room temperature for 24 h, after the reaction showed complete consumption of the starting material, the volume of the reaction mixture was reduced to around one-fourth and then diluted with EtOAc. The organic layer was washed with brine (10% NaCl solution), and was dried using $Na_2SO_4$. After filtration, the solvent was removed under reduced pressure and the product was purified by silica gel chromatography using hexane:EtOAc = 4:1 (by volume) as an eluent to produce compound **S1** (26.1 g, 95%) as a colorless oil. $^1$H NMR (400 MHz, $CDCl_3$) δ 7.75–7.73 (m, 4H), 7.53–7.19 (m, 6H), 4.08 (dd, *J* = 10.9, 3.9 Hz, 1H), 3.95 (dd, *J* = 10.9, 6.0 Hz, 1H), 3.78–3.64 (m, 2H), 3.66–3.52 (m, 1H), 2.88 (s, 1H), 2.35 (s, 1H), 1.32 (s, 24H), 1.13 (s, 9H), 0.93 (t, *J* = 6.6 Hz, 3H). $^{13}$C NMR (100 MHz, $CDCl_3$) δ 135.72, 135.67, 132.70, 132.62, 130.08, 127.97, 74.12, 72.46, 64.38, 63.63, 32.04, 31.78, 29.82, 29.78, 29.76, 29.71, 29.48, 26.85, 25.82, 22.80, 19.19, 14.24.

***(2S,4S,5R)-[2-Azido-2-(2,2-dioxo-5-tetradecyl-2λ⁶-[1,3,2]dioxathiolan-4-yl)ethoxy]-tert-butyldiphenylsilane (S2)[15]***




To a solution of diol **S1** (26 g, 44.7 mmol) in $CH_2Cl_2$ (100 mL), triethylamine (18.7 mL, 134.1 mmol) was added. Thionyl chloride (3.9 mL, 53.65 mmol) was then added drop by drop over a period of 15 min at 0 °C. As a large amount of gas was generated during the reaction, a gas outlet (nitrogen bubbler) was connected. After 45 min, this reaction mixture was poured into brine and extracted with $CH_2Cl_2$. The organic layer was dried over $Na_2SO_4$ and concentrated. This crude cyclic sulfite was dried in vacuo for 5 h and dissolved in a mixed solution of $CCl_4$:$CH_3CN$:$H_2O$ = 1:1:1 (by volume, total 90 mL). To this solution, $RuCl_3 \cdot 3H_2O$ (467 mg, 2.25 mmol) and $NaIO_4$ (28.7 g, 134.6 mmol) were added. After the reaction mixture was stirred at room temperature for 4 h, it was diluted with EtOAc and washed with saturated $NaHSO_3$ solution. The organic layer was dried using $Na_2SO_4$. After filtration, the solvent was removed under reduced pressure and the product was purified by silica gel chromatography using hexane:EtOAc = 12:1 (by volume) as an eluent to produce cyclic sulfate **S2** (27 g, 94%) as a colorless oil. $^1$H NMR (400 MHz, $CDCl_3$) δ 7.73–7.65 (m, 4H), 7.51–7.40 (m, 6H), 5.02–4.96 (m, 1H), 4.93 (dd, *J* = 10.0, 5.2 Hz, 1H), 4.05 (dd, *J* = 11.6, 2.4 Hz, 1H), 3.91 (dd, *J* = 11.6, 5.2 Hz, 1H), 3.70 (ddd, *J* = 10.1, 5.3, 2.4 Hz, 1H), 2.03–1.86 (m, 1H), 1.84–1.70 (m, 1H), 1.70–1.54 (m, 1H), 1.28 (s, 23H), 1.11 (s, 9H), 0.90 (t, *J* = 6.6 Hz, 3H). $^{13}$C NMR (100 MHz, $CDCl_3$) δ 135.72, 135.69, 132.30, 132.06, 130.29, 130.28, 128.12, 128.10, 86.60, 79.98, 63.69, 59.31, 32.07, 29.83, 29.81, 29.80, 29.78, 29.72, 29.60, 29.50, 29.44, 29.09, 28.26, 26.87, 25.32, 22.84, 19.31, 14.27.

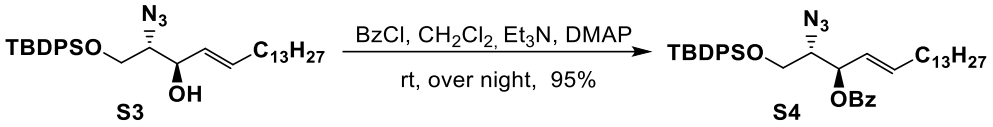
***(2S,3R)-(E)-2-Azido-1-(tert-butyldiphenylsilyoxy)octadec-4-en-3-ol (S3)[16]***




To a solution of cyclic sulfate **S2** (27 g, 41.96 mmol) in anhydrous toluene (100 mL), $Bu_4NI$ (18.6 g, 50.35 mmol) and DBU (9.4 mL, 63 mmol) were added. The reaction mixture was refluxed for 8 h. The reaction was cooled down to r.t. and evaporated to dryness and dissolved in 75 mL of THF. A solution of 1 M $H_2SO_4$ in THF:$H_2O$ = 40:3 (by volume) was then added drop-wisely until the pH of the reaction mixture reached <2. The mixture was stirred for 3 h before it was diluted with EtOAc. It was then washed with saturated aqueous $NaHCO_3$ solution and brine (10% NaCl solution), and dried using $Na_2SO_4$. After filtration, the solvent was removed under reduced pressure and the product was purified by silica gel chromatography using hexane:EtOAc = 10:1 (by volume) as an eluent to produce compound **S3** (20 g, 85%) as a colorless oil. $^1$H NMR (800 MHz, $CDCl_3$) δ 7.68–7.67 (m, 4H), 7.50–7.37 (m, 6H), 5.73 (dt, *J* = 15.2, 6.4 Hz, 1H), 5.43 (ddt, *J* = 15.2, 7.2, 1.2 Hz, 1H), 4.22 (q, *J* = 5.6 Hz, 1H), 3.84–3.73 (m, 2H), 3.51 (dt, *J* = 6.4, 4.8 Hz, 1H), 2.11 (d, *J* = 5.0 Hz, 1H), 2.04–1.96 (m, 2H), 1.35–1.20 (m, 2H), 1.07 (s, 8H), 0.88 (t, *J* = 7.2 Hz, 3H). $^{13}$C NMR (200 MHz, $CDCl_3$) δ 135.73, 135.72, 135.60, 132.88, 130.05, 127.96, 72.99,

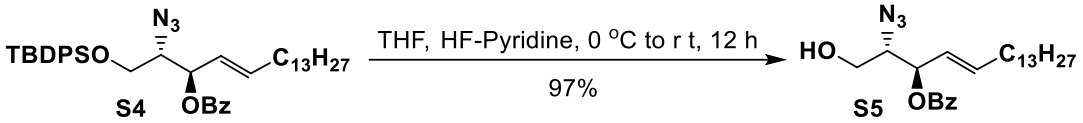
67.01, 64.28, 32.44, 32.08, 29.85, 29.84, 29.82, 29.75, 29.63, 29.52, 29.33, 29.10, 26.86, 22.85, 19.27, 14.28.

***(2R,3R,4E)-2-Azido-3-O-benzoyloxy-1-O-tertbutyldiphenylsilyloxy-octadec-4-ene*** **(S4)**[17]



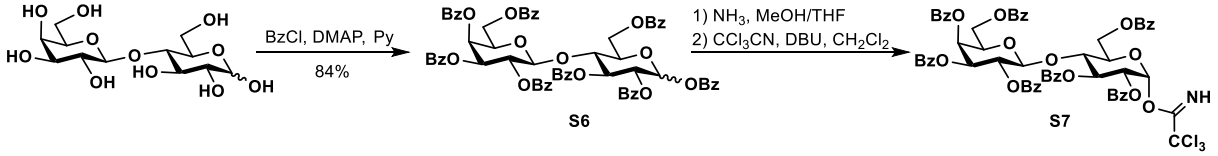

To a solution of compound **S3** (20 g, 35.49 mmol) in dry $CH_2Cl_2$ (100 mL), $Et_3N$ (35 mL, 250 mmol) and 4-dimethyl amino pyridine (DMAP) (433.6 mg, 3.5 mmol) were added and the mixture was stirring at 0 °C. Benzoyl chloride (8.25 mL, 71 mmol) was then added to the stirring reaction mixture drop-wisely. The reaction was allowed to warm up to r.t. and was stirred for overnight. The reaction was then diluted with $CH_2Cl_2$, washed with HCl (1 N), saturated $NaHCO_3$ solution, and brine (10% NaCl solution), and was dried using $Na_2SO_4$. After filtration, the solvent was removed under reduced pressure and the product was purified by silica gel chromatography using hexane:EtOAc = 20:1 (by volume) as an eluent to produce compound **S4** (22.5 g, 95%) as a colorless oil. $^1$H NMR (800 MHz, $CDCl_3$) δ 8.02–8.01 (m, 2H), 7.69–7.65 (m, 4H), 7.59–7.56 (m, 1H), 7.45–7.39 (m, 6H), 7.3 (t, *J* = 7.5 Hz, 2H), 5.90 (dt, *J* = 15.2, 6.4 Hz, 1H), 5.69–5.67 (m, 1H), 5.53–5.49 (m, 1H), 3.85–3.82 (m, 1H), 3.76–3.74 (m, 2H), 2.05–2.00 (m, 2H), 1.35–1.24 (m, 22H), 1.08 (br s, 9H), 0.89 (t, *J* = 7.2 Hz, 3H). $^{13}$C NMR (200 MHz, $CDCl_3$) δ 165.35, 138.68, 135.74, 135.73, 133.26, 133.00, 132.87, 130.24, 130.04, 130.00, 129.91, 129.85, 128.58, 127.99, 127.98, 127.94, 123.38, 77.36, 77.20, 77.04, 74.49, 65.94, 63.53, 32.53, 32.12, 29.88, 29.86, 29.85, 29.76, 29.61, 29.55, 29.31, 28.89, 26.87, 22.89, 19.30, 14.32. ESI HRMS (m/z) calculated for $C_{41}H_{57}N_3O_3Si$ (M+H) 668.4241, found 668.4278.

***(2S,3R,4E)-2-Azido-3-O-benzoyloxy-octadec-4-ene-1-ol*** **(S5)**[17]




To a solution of compound **S4** (22.5 g, 5.61 mmol) in dry THF (75 mL) in a plastic flask, 65–70% HF·pyridine solution (15 mL) was added drop-wisely using a plastic pipette to the stirred mixture at 0 °C. The reaction mixture was stirred at r.t. for 12 h until compound **S4** was completely consumed**.** The reaction mixture was then quenched by adding solid $NaHCO_3$ very slowly (pinch by pinch at a time). EtOAc (250 mL) and $H_2O$ (250 mL) were then added. The aqueous phase was extracted thrice with EtOAc. The combined organic phase was washed with a saturated aqueous solution of $NaHCO_3$ and dried using $Na_2SO_4$. After filtration, the solvent was removed under reduced pressure and the product was purified by silica gel chromatography using hexane:EtOAc = 6:1 (by volume) as an eluent to produce compound **S5** (14 g, 97%) as a white residue. $^1$H NMR (800 MHz, $CDCl_3$) δ 8.07–8.02 (m, 2H), 7.60–7.56 (m, 1H), 7.45 (t, *J* = 7.2 Hz, 2H), 5.96 (dt, *J* = 15.2, 6.4 Hz, 1H), 5.65–5.58 (m, 2H), 3.83–3.79 (m, 1H), 3.77–3.73 (m, 1H), 3.65–3.61 (m, 1H), 2.18–2.16 (m, 1H), 2.11–2.03 (m, 2H), 1.43–1.34 (m, 2H), 1.31–1.20 (m, 22H), 0.88 (t, *J* = 7.2 Hz, 3H). $^{13}$C NMR (200 MHz, $CDCl_3$) δ 165.68, 138.99, 133.50, 129.96, 129.89, 128.65, 123.40, 74.81, 66.37, 62.14, 32.54, 32.09, 29.85, 29.84, 29.82, 29.81, 29.74, 29.58, 29.53, 29.30, 28.85, 22.86, 14.30. ESI HRMS (m/z) calculated for $C_{25}H_{39}N_3O_3$ (M+Na) 452.2883, found 452.2835.

***1,2,3,6-Tetra-O-benzoyl-4-O-(2,3,4,6-tetra-O-benzoyl-β-D-galactopyranosyl)-α/β-D-glucopyranosyl trichloroacetimidate (S7)***

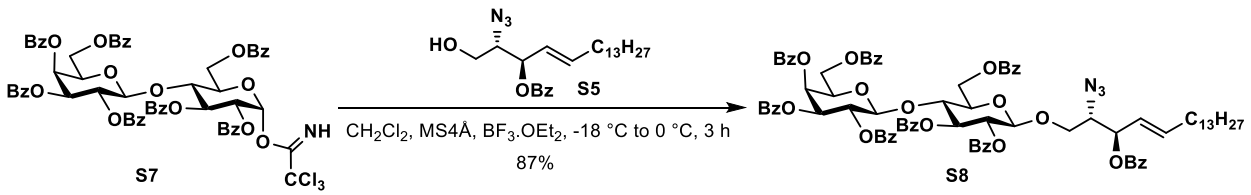



To a solution of lactose monohydrate (25 g, 69.38 mmol) in dry pyridine (125 mL), 4-dimethyl amino pyridine (DMAP) (84 mg, 6.9 mmol) was added and the mixture was stirring at 50 °C for 30 min under an inert atmosphere ($N_2$). Benzoyl chloride (80.6 mL, 693.8 mmol) was then added to the stirring reaction mixture drop-wisely. The reaction was stirred for 8 h at the same temperature. The solvent was then removed completely under reduced pressure and 250 mL of ethylacetate was added. The mixture was cooled down to 4 °C and filtered through a Celite® bed and washed with ice cold ethylacetate (50 mL) for 3 times. If some precipitation was observed in the filtrate portion, the procedure described above should be repeated. After filtration, the solvent was removed under reduced pressure and the product was purified by silica gel chromatography using hexane:EtOAc = 2:1 (by volume) as an eluent to produce perbenzoylated lactose (**S6**) (68.4 g, 84%) as an amorphous solid.

To a solution of perbenzoylated lactose (**S6**) (30 g, 25.54) in dry THF (100 mL), commercially available ammonia solution (50 mL, 7 N in methanol) was added and the mixture was stirred at r.t. under an inert atmosphere ($N_2$) for 18 h before the mixture was diluted with EtOAc (100 mL). The organic layer was washed sequentially with HCl (1 N) and a saturated aqueous solution of $NaHCO_3$, and dried using $Na_2SO_4$. The solvents were removed under reduced pressure. The residue was dried *in vacuo* and used in the next step without further purification.

To a solution of benzoylated lactose hemiacetal in dry $CH_2Cl_2$ (100 mL), trichloroacetonitrile (15.4 mL, 153.58 mmol) was added and the reaction mixture was stirred under an inert atmosphere at 0 °C for 15 min. 1,8-Diazabicyclo[5.4.0]undec-7-ene (DBU) (1.3 mL, 7.6 mmol) was added to the stirred reaction mixture and the mixture was stirred it at room temperature for overnight. The solvents were then removed under reduced pressure and the product was purified by silica gel chromatography using hexane:EtOAc = 1.5:1 (by volume) as an eluent to produce benzoylated lactose trichloroacetimadate derivative (**S7**) (27.3 g, 88%) as an amorphous solid. The residue was dried *in vacuo* and used in the next step without further characterization.

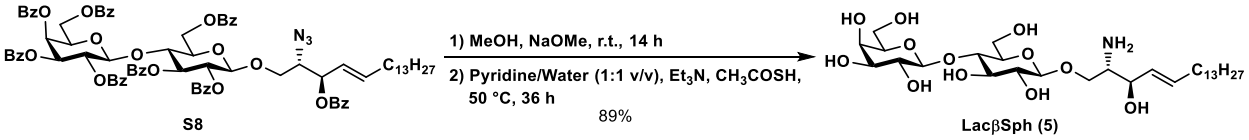
***O-(2,3,4,6-Tetra-O-benzoyl-β-D-galactopyranosyl)-(1→4)-(2,3,6-tri-O-benzoyl-α-D-glucopyranosyl)-(1→1)-(2S, 3R, 4E)-2-azido-3-O-benzoyloxy-octadec-4-ene*** **(S8)**[17]




To a solution of perbenzoylated lactosyl trichloroacetimidate **S7** (24 g, 19.78 mmol)[16] and acceptor **S5** (6.5 g, 15.14 mmol) in 25 mL of dry $CH_2Cl_2$, powdered molecular sieves (4 Å, 7.5 g)

were added. The mixture was stirred under argon at r.t. for 30 min. The reaction mixture was cooled down to -18 °C and $BF_3{\cdot}OEt_2$ (6.2 mL, 50 mmol) was added. The reaction mixture was then stirred at -18 °C for 30 min and the temperature was slowly increased to 0 °C. The stirring was continues at 0 °C until TLC analysis (hexane:ethyl acetate = 3:1 by volume and detected with *p*-anisaldehyde sugar stain) showed complete consumption of the acceptor (2–3 h). The reaction was quenched with $Et_3N$, and the solid was filtered off. The filtrate was concentrated under vacuum, and the residue was purified by silica gel chromatography using hexane:EtOAc = 3:1 (by volume) as an eluent to produce compound **S8** (22.4 g, 87%) as a colorless oil. $^1$H NMR (800 MHz, $CHCl_3$) δ 8.01–7.15 (40 H, Ar-H), 5.82 (t, *J* = 9.6 Hz, 1H), 5.73–5.70 (m, 2H), 5.67 (dt, *J* = 16.0, 6.4 Hz, 1H), 5.51–5.48 (m, 2H),5.43–5.37 (m, 2H), 4.87 (d, *J* = 8.0 Hz, 1H), 4.73 (d, *J* = 8.0 Hz, 1H), 4.57 (dd, *J* = 12.0, 1.6 Hz, 1H), 4.48 (dd, *J* = 12.0, 4.8 Hz, 1H), 4.28 (t, *J* = 9.6 Hz,1H), 3.91–3.88 (m, 2H), 3.86–3.84 (m, 2H), 3.74–3.69 (m, 2H), 3.55–3.53 (m, 1H), 1.88(q, *J* = 7.4 Hz, 2H), 1.30–1.2 (m, 2H), 1.25–1.20(m, 20H), 0.88 (t, *J*= 7.2 Hz, 3H). $^{13}$C NMR (200 MHz, $CHCl_3$): δ 165.82, 165.57, 165.42, 165.24, 165.03, 164.94, 164.83, 138.98, 133.56, 133.44 (2C), 133.40, 133.36, 133.28, 133.25, 133.22, 133.07, 130.01, 129.92–122.39 (Ar-C), 101.03, 100.83, 75.87, 74.79, 73.11, 72.86, 71.77, 71.62, 71.41, 69.88, 68.29, 67.52, 63.41, 62.26, 61.06, 32.28, 31.95, 29.72, 29.69, 29.67, 29.60, 29.39, 29.38, 29.15, 28.60, 22.72, 14.16. ESI HRMS (m/z) calculated for $C_{86}H_{87}N_3O_{20}$ (M+H) 1482.5955, found 1482.5921.

***O-(β-D-Galactopyranosyl)-(1→4)-(β-D-glucopyranosyl)-(1→1)-(2S, 3R, 4E)-2-aminooctadec-4-ene-1,3-diol* (LacβSph, 5)[17]**




To a solution of **S8** (35 g, 23.6 mmol) in dry MeOH (150 mL), NaOMe (3 g) was added. After being stirred at r.t. for 14 h, the reaction mixture was neutralized with Dowex 50W ($H^+$), filtered and concentrated under reduced pressure. This intermediate was used in the next step without further purification. To the dry intermediate in pyridine-water (1:1v/v, 30 mL), 1,3-propanedithiol (25.5 mL, 236 mmol) and $Et_3N$ (25 mL) were added and the mixture was stirred at 50 °C for 36 h. The reaction mixture was concentrated and purified by silica gel chromatography using chloroform:methanol:water = 5:4:1 (by volume) as an eluent to produce **5** (LacβSph, 13.1 g, 89%) as a white amorphous powder. $^1$H NMR (800 MHz, MeOD) δ 5.81–5.74 (m, 1H), 5.49 (dd, *J* = 12.4, 7.2 Hz, 1H), 4.36 (d, *J* = 8.0 Hz, 1H), 4.31 (d, *J* = 8.0 Hz, 1H), 4.06 (t, *J* = 6.4 Hz, 1H), 3.91 (dd, *J* = 12.0, 2.4 Hz, 1H), 3.89–3.82 (m, 2H), 3.82 (dd, *J* = 3.2, .8 Hz, 1H), 3.80–3.76 (m, 2H), 3.70 (dd, *J* = 12.0, 4.8 Hz, 1H), 3.61–3.51 (m, 4H), 3.48 (dd, *J* = 9.6, 3.2 Hz, 1H), 3.44–3.40 (m, 1H), 3.28 (dd, *J* = 8.8, 7.2 Hz, 1H), 3.02–2.97 (m, 1H), 2.11–2.07 (m, 2H), 1.45–1.39 (m, 2H), 1.36–1.25 (m, 20H), 0.90 (t, *J* = 7.2 Hz, 3H). $^{13}$C NMR (200 MHz, MeOD) δ 136.02, 130.54, 105.31, 104.37, 80.70, 77.30, 76.73, 76.47, 75.02, 74.88, 74.04, 72.74, 70.92, 70.50, 62.70, 61.99, 56.50, 33.63, 33.28, 31.00, 30.96, 30.84, 30.68, 30.58, 30.54, 23.94, 14.64. ESI HRMS (m/z) calculated for $C_{30}H_{57}NO_{12}$ (M+H) 624.3954, found: 624.3945.

***One-pot two-enzyme synthesis of GM3Sph (6): (5-Acetamido-3,5-dideoxy-D-*glycero*-α-D-galacto-2-nonulopyranosylonic acid)-(2→3)-β-D-galactopyranosyl-(1→4)-β-D-glucopyranosyl-(1→1)-(2S, 3R, 4E)-2-amino-4-octadecene-1,3-diol***

Lactosyl sphingosine (LacβSph, 1.0 g, 1.60 mmol), Neu5Ac (0.643 g, 2.08 mmol), and CTP (1.44 g, 2.55 mmol) were incubated at 37 °C in a Tris-HCl buffer (160 mL, 100 mM, pH 8.5) containing $MgCl_2$ (20 mM), NmCSS (30 mg), PmST3 (20 mg). The reaction was incubated in an incubator shaker at 37 °C for 15 h with agitation at 100 rpm. The product formation was monitored by mass spectrometry. Upon completion, the same volume (160 mL) of cold ethanol was added and the mixture was incubated at 4 °C for 30 min before it was centrifuged to remove precipitates. The supernatant was concentrated and the residue was dissolved in 20 mL water. Half of the sample was purified using a 51 g ODS-SM column (50 μM, 120 Å, Yamazen) on a CombiFlash® Rf 200i system. After loading the sample, the column was washed with water for 5 min. The product was eluted with 60% acetonitrile in water (v/v) and remaining LacβSph (**5**) was recovered by eluting with pure acetonitrile. The whole process took about 25 minutes. The same purification process was repeated to purified the product from the other half of the sample. The fractions containing the product were collected, and the target glycolipid GM3βSph (**6**) (1.44 g, 96% yield) was obtained as a white powder after lyophilization. $^1$H NMR (800 MHz, $CD_3OD$) δ 5.89 (dt, *J* = 14.4, 6.4 Hz, 1H), 5.53 (dd, *J* = 15.2, 7.2 Hz, 1H), 4.46 (d, *J* = 8.0 Hz, 1H), 4.39 (d, *J* = 8.0 Hz, 1H), 4.30 (t, *J* = 6.4 Hz, 1H), 4.10 (dd, *J* = 9.6, 3.2 Hz, 1H), 4.01–3.32 (m, 19H), 2.91 (dd, *J* = 12.0, 4.0 Hz, 1H), 2.15 (m, 2H), 2.06 (s, 3H, $CH_3$), 1.76 (t, *J* = 12.0 Hz, 1H), 1.50–1.31 (m, 22 H, 11×$CH_2$), 0.95 (t, *J* = 7.2 Hz, 3H, $CH_3$); $^{13}$C NMR (200 MHz, $CD_3OD$) δ 174.06, 173.38, 135.05, 127.22, 103.62, 102.36, 99.57, 79.12, 76.24, 75.67, 75.10, 74.76, 73.48, 73.04, 71.49, 70.06, 69.34, 68.65, 67.82, 67.51, 66.50, 63.22, 61.27, 60.16, 55.20, 52.48, 40.66, 31.91, 31.59, 29.32, 29.31, 29.28, 29.27, 29.16, 29.00, 28.92, 28.73, 22.26, 21.09, 12.97. ESI HRMS (m/z) calculated for $C_{41}H_{73}N_2O_{20}$ (M - H) 913.4762, found 913.4722.

***One-pot four-enzyme synthesis of GM2βSph (7): 2-Acetamido-2-deoxy-β-D-galactopyranosyl-(1→4)-(5-acetamido-3,5-dideoxy-D-*glycero*-α-D-galacto-2-nonulopyranosylonic acid)-(2→3)-β-D-galactopyranosyl-(1→4)-β-D-glucopyranosyl-(1→1)-(2S, 3R, 4E)-2-amino-4-octadecene-1,3-diol***

GM3βSph (**6**) (100 mg, 0.11 mmol), GalNAc (47 mg, 0.21 mmol), ATP (118 mg, 0.21 mmol), and UTP (112 mg, 0.21 mmol) were incubated in 10 mL of Tris-HCl buffer (100 mM, pH 7.5) containing BLNahK (5 mg), PmGlmU (4.6 mg), CjCgtA (4.5 mg), and PmPpA (2 mg). The reaction was carried out by incubating the solution in an incubator shaker at 30 °C for 48 h with agitation at 100 rpm. The product formation was monitored by LC-MS. When an optimal yield was achieved, the reaction was quenched by adding the same volume (10 mL) of ice-cold ethanol. The mixture was incubated at 4 °C for 30 min and centrifuged to remove precipitates. The supernatant was concentrated and the residue was purified using a 51 g ODS-SM column (50 μM, 120 Å, Yamazen) on a CombiFlash® Rf 200i system. After loading the sample, the column was washed with water for 5 min and the product was eluted with 45% acetonitrile in water (v/v). The whole process took about 25 minutes. The fractions containing pure product were collected and lyophilized to obtain GM2βSph (**7**) as a white powder (120 mg, 98% yield). $^1$H NMR (800 MHz, $CD_3OD$) δ 5.90 (dt, *J* = 13.6, 7.2 Hz, 1H), 5.53 (dd, *J* = 16.0, 7.2 Hz, 1H), 4.88 (d, *J* = 8.0 Hz, 1H), 4.46 (d, *J* = 8.0 Hz, 1H), 4.40 (d, *J* = 8.0 Hz, 1H), 4.32 (t, *J* = 6.4 Hz, 1H), 4.18 (d, *J* = 3.2 Hz, 1H), 4.07–3.32 (m, 25H), 2.78 (dd, *J* = 12.0, 4.8 Hz, 1H), 2.14 (m, 2H), 2.06 (s, 3H, $CH_3$), 1.94 (t,

*J* = 12.0 Hz, 1H), 1.48–1.24 (m, 22 H, 11×$CH_2$), 0.94 (t, *J* = 6.4 Hz, 3H, $CH_3$); $^{13}$C NMR (200 MHz, $CD_3OD$) δ 174.20, 173.69, 173.23, 135.05, 127.18, 103.40, 102.79, 102.31, 101.93, 79.64, 77.53, 75.08, 74.93, 74.82, 74.67, 74.20, 73.61, 73.05, 72.59, 71.95, 69.95, 69.55, 68.93, 68.31, 68.13, 66.29, 63.90, 61.56, 60.32, 60.27, 55.14, 52.78, 52.32, 37.11, 31.92, 31.59, 29.32, 29.32, 29.28, 29.28, 29.17, 29.00, 28.93, 28.74, 22.26, 22.18, 21.16, 12.98. ESI HRMS (m/z) calculated for $C_{49}H_{86}N_3O_{35}$ (M - H) 1116.5556, found 1116.5508.

***One-pot four-enzyme synthesis of GM1βSph (8): β-D-Galactopyranosyl-(1→3)-(2-acetamido-2-deoxy-β-D-galactopyranosyl)-(1→4)-(5-acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonic acid)-(2→3)-β-D-galactopyranosyl-(1→4)-β-D-glucopyranosyl-(1→1)-(2S, 3R, 4E)-2-amino-4-octadecene-1,3-diol***

A reaction mixture in Tris-HCl buffer (100 mM, pH 7.5) in a total volume of 5 mL containing GM2βSph (**7**) (50 mg, 0.044 mmol), galactose (16 mg, 0.089 mmol), ATP (49 mg, 0.089 mmol), and UTP (47 g, 0.089 mmol), $MgCl_2$ (20 mM), *E. coli* GalK (1.5 mg), BLUSP (1.5 mg), CjCgtB (6.5 mg), and PmPpA (1.0 mg) was incubated in a shaker at 30 °C by agitating at 100 rpm. The reaction was monitored by mass spectrometry. When an optimal yield was achieved (after 2 day), 5 mL of prechilled EtOH was added and the mixture was incubated at 4 °C for 30 min. The precipitates were removed by centrifugation and the supernatant was concentrated and the residue was purified using a 14 g ODS-SM column (50 μM, 120 Å, Yamazen) on a CombiFlash® Rf 200i system. After loading the sample, the column was washed with water for 5 min and the product was eluted with 40% acetonitrile in water (v/v). The whole process took about 20 minutes. The fractions containing the pure product were collected and concentrated to obtain the final pure GM1βSph (**8**) as a white powder (57 mg, 99% yield). $^1$H NMR (800 MHz, $CD_3OD$) δ 5.85 (dt, *J* = 14.4, 7.2 Hz, 1H), 5.39 (dd, *J* = 16.0, 7.2 Hz, 1H), 4.95 (d, *J* = 8.8 Hz, 1H), 4.49 (d, *J* = 8.0 Hz, 1H), 4.45 (d, *J* = 8.0 Hz, 1H), 4.37 (d, *J* = 8.0 Hz, 1H), 4.22–4.18 (m, 2H), 4.06–4.04 (m, 2H), 3.98–3.87 (m, 8H), 3.82–3.21(m, 21H), 2.77 (dd, *J* = 12.0, 4.8 Hz, 1H), 2.14 (m, 2H), 2.06 (s, 3H, $CH_3$), 2.04 (s, 3H, $CH_3$), 1.94 (t, *J* = 12.0 Hz, 1H), 1.48–1.31 (m, 22 H, 11x$CH_2$), 0.94 (t, *J* = 7.2 Hz, 3H, $CH_3$); $^{13}$C NMR (200 MHz, $CD_3OD$) δ 174.16, 173.75, 173.42, 173.33, 134.74, 127.90, 105.18, 103.44, 102.67, 102.65, 102.44, 101.97, 81.59, 81.56, 79.71, 77.53, 75.05, 75.00, 74.88, 74.67, 74.43, 74.18, 73.62, 73.10, 73.10, 71.91, 71.03, 69.53, 68.97, 68.73, 68.25, 68.20, 67.59, 63.93, 61.52, 60.90, 60.31, 60.28, 55.00, 52.29, 51.35, 51.27, 37.09, 31.93, 31.59, 29.32, 29.31, 29.28, 29.27, 29.16, 29.00, 28.91, 28.79, 22.36, 22.31, 22.26, 21.12, 12.97. ESI HRMS (m/z) calculated for $C_{55}H_{96}N_3O_{30}$ (M - H) 1278.6084, found 1278.6025.

***One-pot four-enzyme synthesis of Fuc-GM1βSph (9): α-L-fucopyranosyl-(1→2)-β-D-galactopyranosyl-(1→3)-(2-acetamido-2-deoxy-β-D-galactopyranosyl)-(1→4)-(5-Acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonic acid)-(2→3)-β-D-galactopyranosyl-(1→4)-β-D-glucopyranosyl-(1→1)-(2S, 3R, 4E)-2-amino-4-octadecene-1,3-diol***

A reaction mixture in Tris-HCl buffer (100 mM, pH 7.5) in a total volume of 5 mL containing GM1βSph (**8**) (50 mg, 0.038 mmol), L-fucose (13 mg, 0.079 mmol), ATP (44 mg, 0.080 mmol), and GTP (45 g, 0.080 mmol), $MgCl_2$ (20 mM), BfFKP (1.5 mg), PmPpA (1.0 mg), and EcWbgL (0.5 mg) was incubated in a shaker at 30 °C with agitating at 100 rpm. The reaction was monitored by mass spectrometry. When an optimal yield was achieved (after 2 day), 5 mL of prechilled EtOH was added and the mixture was incubated at 4 °C for 30 min. The precipitates were removed by centrifugation and the supernatant was concentrated. The residue was purified using a 14 g ODS-

SM column (50 µM, 120 Å, Yamazen) on a CombiFlash® Rf 200i system. After loading the sample, the column was washed with water for 5 min and the product was eluted with 40% acetonitrile in water (v/v). The fractions containing the pure product were collected and concentrated to obtain the final pure Fuc-GM1βSph (**9**) as a white powder (55 mg, quant. yield). $^1$H NMR (800 MHz, $CD_3OD$) δ 5.90 (dt, *J* = 14.4, 7.2 Hz, 1H), 5.53 (dd, *J* = 16.0, 7.2 Hz, 1H), 5.31 (d, *J* = 3.2 Hz, 1H), 4.97 (m, 1H), 4.78 (d, *J* = 8.8 Hz, 1H), 4.68 (d, *J* = 8.0 Hz, 1H), 4.43 (d, *J* = 8.0 Hz, 1H), 4.40 (d, *J* = 8.0 Hz, 1H), 4.35–4.30 (m, 2H), 4.20–4.14 (m, 2H), 4.06–3.35(m, 32H), 2.80 (dd, *J* = 12.0, 4.8 Hz, 1H), 2.14 (m, 2H), 2.06 (s, 6H, $CH_3$), 1.91 (t, *J* = 12.0 Hz, 1H), 1.48–1.30 (m, 22 H, 11×$CH_2$), 1.27 (d, *J* = 6.4 Hz, 3H), 0.94 (t, *J* = 7.2 Hz, 3H, $CH_3$); $^{13}$C NMR (200 MHz, $CD_3OD$) δ 174.19, 173.75, 172.84, 135.11, 127.06, 103.94, 103.55, 102.52, 102.31, 101.70, 99.17, 79.48, 78.38, 77.40, 75.95, 75.13, 75.03, 74.86, 74.68, 74.48, 74.18, 73.58, 73.01, 72.13, 71.94, 70.02, 69.78, 69.37, 69.28, 68.99, 68.96, 68.61, 68.15, 66.45, 66.06, 63.89, 61.55, 60.96, 60.26, 60.12, 55.17, 52.38, 50.99, 37.47, 31.92, 31.59, 29.32, 29.31, 29.28, 29.27, 29.17, 29.00, 28.94, 28.84, 28.73, 22.79, 22.74, 22.26, 21.18, 15.24, 12.98. ESI HRMS (m/z) calculated for $C_{61}H_{106}N_3O_{34}$ (M - H) 1424.6663, found 1424.6608.

***One-pot two-enzyme synthesis of GD3βSph (10): (5-Acetamido-3,5-dideoxy-*D*-glycero-α-D-galacto-2-nonulopyranosylonic acid)-(2→8)-(5-acetamido-3,5-dideoxy-D-glycero-α-D-galacto-2-nonulopyranosylonic acid)-(2→3)-β-D-galactopyranosyl-(1→4)-β-D-glucopyranosyl-(1→1)-(2S, 3R, 4E)-2-amino-4-octadecene-1,3-diol***

GM3βSph (**6**) (1.1 g, 1.17 mmol), Neu5Ac (1.09 g, 3.52 mmol), and CTP (1.98 g, 3.52 mmol) were in 40 mL of Tris-HCl buffer (100 mM, pH 8.5) containing $MgCl_2$ (20 mM), NmCSS (10 mg), CjCstII (12 mg). The reaction was carried out by incubating the solution in an incubator shaker at room temperature for overnight with agitation at 100 rpm. The reaction was quenched by adding the same volume (40 mL) of pre-chilled ethanol and the solution was incubated at 4 °C for 30 min. The mixture was centrifuged and the precipitates were removed. The supernatant was concentrated and the residue was purified using a 51 g ODS-SM column (50 µM, 120 Å, Yamazen) on a CombiFlash® Rf 200i system. After loading the sample, the column was washed with water for 5 min, and the byproduct $GT_3$βSph was eluted from the C18 cartridge with 30% acetonitrile in water (v/v) (it contains some $GD_3$βSph). The product GD3βSph (**10**) was then eluted with 35% acetonitrile in water (v/v). The unreacted $GM_3$βSph was eluted with 60% acetonitrile in water (v/v). The whole process took about 30 minutes. The fractions containing the pure product were collected and concentrated to obtain the desired pure GD3βSph (**10**) (0.91 g, 62% yield). $^1$H NMR (800 MHz, $CD_3OD$) δ 5.81 (dt, *J* = 15.2, 7.2 Hz, 1H), 5.53 (dd, *J* = 15.2, 7.2 Hz, 1H), 4.53 (d, *J* = 8.0 Hz, 1H), 4.36 (d, *J* = 8.0 Hz, 1H), 4.14–3.47 (m, 26H), 3.27 (dd, *J* = 9.6, 8.0 Hz, 1H), 2.98 (m, 2H), 2.76 (dd, *J* = 12.0, 4.0 Hz, 1H), 2.12 (m, 2H), 2.07 (s, 3H, $CH_3$), 2.05 (s, 3H, $CH_3$), 1.74 (m, 2H), 1.47–1.28 (m, 22 H, 11x$CH_2$), 0.93 (t, *J* = 7.2 Hz, 3H, $CH_3$); $^{13}$C NMR (200 MHz, $CD_3OD$) δ 174.01, 173.53, 173.30, 173.10, 134.29, 129.08, 103.32, 102.64, 100.83, 99.91, 79.19, 77.06, 75.33, 75.31, 74.96, 74.59, 74.09, 73.18, 73.13, 72.73, 71.51, 69.73, 69.35, 68.82, 68.09, 67.09, 63.13, 62.00, 61.33, 60.33, 54.79, 52.68, 52.45, 41.34, 40.84, 31.96, 31.59, 29.31, 29.28, 29.16, 29.00, 28.91, 28.88, 22.26, 21.57, 21.15, 12.98. ESI HRMS (m/z) calculated for $C_{52}H_{90}N_3O_{28}$ (M - H) 1204.5716, found 1204.5669.

***One-pot two-enzyme synthesis of GD2βSph (11): 2-Acetamido-2-deoxy-β-D-galactopyranosyl-(1→4)-(5-Acetamido-3,5-dideoxy-D-glycero-α-D-galacto-2-nonulopyranosylonic acid)-(2→8)-***

***(5-acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonic acid)-(2→3)-β-D-galactopyranosyl-(1→4)-β-D-glucopyranosyl-(1→1)-(2*S*, 3*R*, 4*E*)-2-amino-4-octadecene-1,3-diol***

GD3βSph (**10**) (30 mg, 0.024 mmol), GalNAc (8 mg, 0.036 mmol), ATP (20 mg, 0.036 mmol), and UTP (192 mg, 0.024 mmol) were incubated in 3 mL of Tris-HCl buffer (100 mM, pH 7.5) containing BLNahK (2 mg), PmGlmU (2 mg), CjCgtA (4 mg), and PmPpA (1.5 mg).The reaction was carried out by incubating the solution in an incubator shaker at 30 °C for 48 h with agitation at 100 rpm. The product formation was monitored by LC-MS. When an optimal yield was achieved, the reaction was quenched by adding the same volume (3 mL) of pre-chilled ethanol. The mixture was then incubated at 4 °C for 30 min, centrifuged to remove precipitates. The supernatant was concentrated and the residue was purified using a 25 g ODS-SM column (50 µM, 120 Å, Yamazen) on a CombiFlash® Rf 200i system. After loading the sample, the column was washed with water for 5 min and the product was eluted with 35% acetonitrile in water (v/v). The whole process took about 25 minutes. The fractions containing the pure product were collected and concentrated to obtain the desired pure GD2βSph (**11**) (34 mg, 98% yield). $^{1}$H NMR (800 MHz, $CD_3OD$) δ 5.87 (dt, *J* = 15.2, 7.2 Hz, 1H), 5.50 (dd, *J* = 15.2, 7.2 Hz, 1H), 4.83 (d, *J* = 8.0 Hz, 1H), 4.47 (d, *J* = 8.0 Hz, 1H), 4.36 (d, *J* = 8.0 Hz, 1H), 4.34 (t, *J* = 5.6 Hz, 1H), 4.25–3.40 (m, 34H), 2.88 (dd, *J* = 12.0, 3.2 Hz, 1H), 2.75 (dd, *J* = 12.8, 4.8 Hz, 1H), 2.12–2.09 (m, 2H), 2.05 (s, 3H, $CH_3$), 2.04 (s, 3H, $CH_3$), 2.02 (s, 3H, $CH_3$), 1.81–1.74 (m, 2H), 1.46–1.29 (m, 22 H, 11x$CH_2$), 0.93 (t, *J* = 7.2 Hz, 3H, $CH_3$); $^{13}$C NMR (201 MHz, $CD_3OD$) δ 174.00, 173.37, 173.30, 172.97, 172.69, 135.12, 126.81, 103.46, 102.89, 102.27, 100.92, 100.39, 78.97, 76.87, 76.64, 75.10, 74.85, 74.80, 74.54, 74.30, 73.99, 73.22, 73.06, 72.56, 71.37, 69.37, 68.77, 68.51, 68.13, 65.65, 63.15, 61.77, 61.14, 60.21, 59.92, 59.60, 55.30, 53.15, 52.66, 52.56, 48.01, 40.65, 39.83, 31.90, 31.59, 29.32, 29.31, 29.28, 29.27, 29.16, 28.99, 28.97, 28.93, 28.83, 28.71, 22.25, 22.09, 21.61, 21.13, 12.96. ESI HRMS (m/z) calculated for $C_{60}H_{103}N_4O_{33}$ (M - H) 1407.6510, found 1407.6495.

**General procedures for converting glycosylsphingosines to gangliosides:** To a solution of glycosphingosines (15–30 mg) in sat. $NaHCO_3$-THF (3 mL, 2:1), palmitoyl chloride in 1 mL of THF was added. The resulting mixture was stirred vigorously at room temperature for 2 h. The solution was then concentrated and dissolved in 2 mL of water. The sample was loaded to a preconditioned Discovery® C18 cartridge (Sigma) through a 10 mL plastic syringe and washed with water (10 mL). The ganglioside products were eluted from the C18 cartridge using a solution of 50–80% acetonitrile in water and fractions of 1–1.5 mL each were collected. The whole process took about 15–20 minutes.

***GM3 (1): (5-Acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonic acid)-(2→3)-β-D-galactopyranosyl-(1→4)-β-D-glucopyranosyl-(1→1)-(2*S*, 3*R*, 4*E*)-2-hexadecanamino-4-octadecene-1,3-diol***

(eluted using 80% $CH_3CN$ in water), white powder, 25 mg, 99% yield. $^{1}$H NMR (800 MHz, $CD_3OD$) δ 5.72 (dt, *J* = 14.4, 7.2 Hz, 1H), 5.48 (dd, *J* = 15.2, 7.2 Hz, 1H), 4.46 (d, *J* = 8.0 Hz, 1H), 4.35 (d, *J* = 8.0 Hz, 1H), 4.10 (dd, *J* = 9.6, 4.0 Hz, 1H), 4.12–3.45 (m, 19H), 3.32 (t, *J* = 8.0 Hz, 1H), 2.90 (dd, *J* = 12.0, 4.0 Hz, 1H), 2.22 (t, *J* = 8.0 Hz, 2H), 2.09–2.04 (m, 2H), 2.05 (s, 3H, $CH_3$), 1.79–1.75 (m, 1H), 1.63–1.60 (m, 2H), 1.44–1.25 (m, 46 H, 23×$CH_2$), 0.94 (t, *J* = 7.2 Hz, 6H, 2×$CH_3$); $^{13}$C NMR (200 MHz, $CD_3OD$) δ 174.46, 174.00, 173.44, 133.54, 129.90, 103.59, 102.99, 99.60, 79.31, 76.16, 75.58, 74.97, 74.69, 73.44, 73.34, 71.48, 69.34, 68.60, 68.44, 67.88, 67.48, 63.10, 61.24, 60.29, 53.23, 52.45, 40.61, 35.90, 32.00, 31.63, 31.62, 29.41, 29.38, 29.36, 29.35,

29.34, 29.32, 29.30, 29.28, 29.24, 29.17, 29.04, 29.03, 29.00, 28.99, 28.96, 25.70, 22.28, 21.10, 12.99. ESI HRMS (m/z) calculated for $C_{57}H_{103}N_2O_{21}$ (M - H) 1151.7059, found 1151.7006.

***Fucosyl GM1 (2): α-L-fucopyranosyl-(1→2)-β-D-galactopyranosyl-(1→3)-(2-acetamido-2-deoxy-β-D-galactopyranosyl)-(1→4)-(5-Acetamido-3,5-dideoxy-D-*glycero*-α-D-galacto-2-nonulopyranosylonic acid)-(2→3)-β-D-galactopyranosyl-(1→4)-β-D-glucopyranosyl-(1→1)-(2S, 3R, 4E)-2-hexadecanamino-4-octadecene-1,3-diol***

(eluted using 50% $CH_3CN$ in water), white powder, 35 mg, 99% yield. $^1$H NMR (800 MHz, $CD_3OD$) δ 5.72 (dt, *J* = 14.4, 7.2 Hz, 1H), 5.48 (dd, *J* = 16.0, 7.2 Hz, 1H), 5.30 (d, *J* = 3.2 Hz, 1H), 4.97 (m, 1H), 4.79 (d, *J* = 8.8 Hz, 1H), 4.68 (d, *J* = 8.0 Hz, 1H), 4.44 (d, *J* = 8.0 Hz, 1H), 4.34–4.30 (m, 2H), 4.22–3.31(m, 34H), 4.32 (d, *J* = 8.0 Hz, 1H), 2.80 (dd, *J* = 12.0, 4.8 Hz, 1H), 2.21 (t, *J* = 8.0 Hz, 2H), 2.09–2.06 (m, 2H), 2.05 (s, 6H, 2x$CH_3$), 1.92 (t, *J* = 12.0 Hz, 1H), 1.65–1.59 (m, 2H), 1.43–1.30 (m, 46 H, 23×$CH_2$), 1.27 (d, *J* = 6.4 Hz, 3H), 0.94 (t, *J* = 7.2 Hz, 6H, 2×$CH_3$); $^{13}$C NMR (200 MHz, $CD_3OD$) δ 174.47, 174.17, 173.73, 172.84, 133.59, 129.88, 103.90, 103.54, 102.98, 102.50, 101.77, 99.21, 79.58, 78.30, 77.40, 76.02, 75.03, 74.99, 74.80, 74.62, 74.49, 74.18, 74.09, 73.59, 73.34, 72.11, 71.92, 71.49, 70.01, 69.47, 69.30, 68.97, 68.95, 68.63, 68.44, 68.22, 66.49, 63.83, 61.52, 60.97, 60.29, 60.06, 53.22, 52.35, 50.94, 37.38, 35.90, 32.00, 31.62, 31.61, 29.40, 29.37, 29.35, 29.34, 29.33, 29.31, 29.29, 29.28, 29.23, 29.16, 29.03, 29.02, 29.00, 28.98, 28.95, 25.70, 22.68, 22.27, 21.12, 15.18, 12.99. ESI HRMS (m/z) calculated for $C_{77}H_{136}N_3O_{35}$ (M - H) 1662.8960, found 1662.8927.

***GD3 (3): (5-Acetamido-3,5-dideoxy-D-*glycero*-α-D-galacto-2-nonulopyranosylonic acid)-(2→8)-(5-acetamido-3,5-dideoxy-D-*glycero*-α-D-galacto-2-nonulopyranosylonic acid)-(2→3)-β-D-galactopyranosyl-(1→4)-β-D-glucopyranosyl-(1→1)-(2S, 3R, 4E)-2-hexadecanamino-4-octadecene-1,3-diol***

(eluted using 50% $CH_3CN$ in water), white powder, 36 mg, 99% yield. $^1$H NMR (800 MHz, $CD_3OD$) δ 5.73 (dt, *J* = 15.2, 7.2 Hz, 1H), 5.49 (dd, *J* = 15.2, 7.2 Hz, 1H), 4.54 (d, *J* = 8.0 Hz, 1H), 4.35 (d, *J* = 8.0 Hz, 1H), 4.26–3.47 (m, 26H), 3.33 (t, *J* = 8.0 Hz, 1H), 2.96 (dd, *J* = 12.0, 4.0 Hz, 1H), 2.76 (dd, *J* = 12.0, 4.0 Hz, 1H), 2.21 (t, *J* = 7.2 Hz, 2H), 2.08–2.05 (m, 2H), 2.07 (s, 3H, $CH_3$), 2.05 (s, 3H, $CH_3$), 1.76–1.71 (m, 2H), 1.64–1.60 (m, 2H), 1.45–1.26 (m, 46 H, 23×$CH_2$), 0.94 (t, *J* = 7.2 Hz, 6H, 2×$CH_3$); $^{13}$C NMR (200 MHz, $CD_3OD$) δ 174.47, 174.02, 173.56, 173.33, 173.11, 133.62, 129.89, 103.35, 102.94, 100.73, 99.93, 79.13, 76.98, 75.29, 75.23, 74.92, 74.59, 74.15, 73.33, 73.11, 71.53, 71.48, 69.33, 68.84, 68.56, 68.05, 67.08, 63.10, 62.01, 61.32, 60.17, 53.19, 52.69, 52.45, 41.37, 40.77, 37.82, 35.92, 32.02, 31.63, 31.63, 31.60, 29.43, 29.41, 29.39, 29.38, 29.37, 29.35, 29.33, 29.32, 29.31, 29.29, 29.26, 29.24, 29.20, 29.05, 29.04, 29.03, 29.02, 29.00, 28.97, 25.72, 22.29, 22.26, 21.57, 21.16, 13.02, 12.98. ESI HRMS (m/z) calculated for $C_{68}H_{120}N_3O_{29}$ (M - H) 1442.8013, found 1442.8037.

***GD2 (4): 2-Acetamido-2-deoxy-β-D-galactopyranosyl-(1→4)-(5-acetamido-3,5-dideoxy-D-*glycero*-α-D-galacto-2-nonulopyranosylonic acid)-(2→8)-(5-acetamido-3,5-dideoxy-D-*glycero*-α-D-galacto-2-nonulopyranosylonic acid)-(2→3)-β-D-galactopyranosyl-(1→4)-β-D-glucopyranosyl-(1→1)-(2S, 3R, 4E)-2-hexadecanamino-4-octadecene-1,3-diol***

(eluted using 40% $CH_3CN$ in water), white powder, 17 mg, 98% yield. $^1$H NMR (800 MHz, $CD_3OD$) δ 5.69 (dt, *J* = 15.2, 7.2 Hz, 1H), 5.45 (dd, *J* = 15.2, 7.2 Hz, 1H), 4.47 (d, *J* = 8.0 Hz, 1H), 4.30 (d, *J* = 8.0 Hz, 1H), 4.24–3.39 (m, 33H), 3.29 (t, *J* = 8.8 Hz, 1H), 2.86 (dd, *J* = 12.0, 4.0 Hz, 1H), 2.74 (dd, *J* = 12.0, 4.0 Hz, 1H), 2.18 (t, *J* = 8.0 Hz, 2H), 2.04–2.02 (m, 2H), 2.04 (s, 3H, $CH_3$),

2.03 (s, 3H, $CH_3$), 2.01 (s, 3H, $CH_3$), 1.78–1.72 (m, 2H), 1.62–1.56 (m, 2H), 1.41–1.22 (m, 46 H, 23×$CH_2$), 0.90 (t, *J* = 8.0 Hz, 6H, 2×$CH_3$); $^{13}$C NMR (200 MHz, $CD_3OD$) δ 174.46, 173.98, 173.36, 173.34, 173.32, 172.70, 133.57, 129.90, 103.45, 102.99, 102.89, 100.58, 100.44, 79.08, 77.00, 76.42, 74.98, 74.76, 74.57, 74.46, 74.16, 74.00, 73.38, 73.12, 72.47, 71.48, 71.39, 69.56, 69.34, 68.87, 68.70, 68.47, 68.14, 63.14, 62.14, 61.14, 60.22, 60.05, 53.21, 53.06, 52.71, 52.52, 40.83, 39.98, 35.89, 35.05, 32.00, 31.62, 31.61, 29.41, 29.40, 29.37, 29.35, 29.34, 29.33, 29.32, 29.31, 29.30, 29.29, 29.28, 29.23, 29.20, 29.16, 29.12, 29.03, 29.02, 29.00, 28.99, 28.98, 28.97, 28.95, 28.87, 28.84, 28.75, 25.70, 22.27, 22.25, 22.10, 21.64, 21.10, 12.98, 12.95. ESI HRMS (m/z) calculated for $C_{76}H_{133}N_4O_{34}$ (M - H) 1645.8807, found 1645.8802.

***GM2 (12): 2-Acetamido-2-deoxy-β-D-galactopyranosyl-(1→4)-(5-acetamido-3,5-dideoxy-D-*** **glycero-*α-D*-galacto-*2-nonulopyranosylonic acid)-(2→3)-β-D-galactopyranosyl-(1→4)-β-D-glucopyranosyl-(1→1)-(2S, 3R, 4E)-2-hexadecanamino-4-octadecene-1,3-diol**

(eluted using 50% $CH_3CN$ in water), white powder, 24 mg, 98% yield. $^1$H NMR (800 MHz, $CD_3OD$) δ 5.71 (dt, *J* = 13.6, 7.2 Hz, 1H), 5.49 (dd, *J* = 16.0, 7.2 Hz, 1H), 4.45 (d, *J* = 8.0 Hz, 1H), 4.34 (d, *J* = 8.0 Hz, 1H), 4.22 (dd, *J* = 9.6, 4.0 Hz, 1H), 4.19 (d, *J* = 4.0 Hz, 1H), 4.11 (t, *J* = 8.0 Hz, 1H), 4.06–3.30 (m, 25H), 2.78 (dd, *J* = 12.0, 4.8 Hz, 1H), 2.20 (t, *J* = 8.0 Hz, 2H), 2.09–2.04 (m, 2H), 2.05 (s, 3H, $CH_3$), 1.94 (t, *J* = 12.0 Hz, 1H), 1.65–1.59 (m, 2H), 1.44–1.25 (m, 46 H, 23×$CH_2$), 0.94 (t, *J* = 7.2 Hz, 6H, 2×$CH_3$); $^{13}$C NMR (200 MHz, $CD_3OD$) δ 174.46, 174.15, 173.64, 173.22, 133.56, 129.89, 103.44, 102.97, 102.79, 101.97, 79.82, 77.54, 74.95, 74.91, 74.81, 74.64, 74.12, 73.62, 73.36, 72.67, 71.92, 71.48, 69.57, 68.94, 68.42, 68.37, 68.20, 63.89, 61.55, 60.33, 60.22, 53.22, 52.79, 52.29, 37.09, 35.89, 31.99, 31.62, 31.61, 31.59, 29.40, 29.37, 29.35, 29.34, 29.32, 29.30, 29.29, 29.28, 29.23, 29.19, 29.15, 29.03, 29.02, 28.99, 28.97, 28.95, 25.70, 22.27, 22.12, 21.08, 12.98. ESI HRMS (m/z) calculated for $C_{65}H_{116}N_3O_{26}$ (M - H) 1354.7853, found 1354.7795.

***GM1 (13): β-D-Galactopyranosyl-(1→3)-(2-acetamido-2-deoxy-β-D-galactopyranosyl)-(1→4)-(5-acetamido-3,5-dideoxy-D-*** **glycero-*α-D*-galacto-*2-nonulopyranosylonic acid)-(2→3)-β-D-galactopyranosyl-(1→4)-β-D-glucopyranosyl-(1→1)-(2S, 3R, 4E)-2-hexadecanamino-4-octadecene-1,3-diol**

(eluted using 50% $CH_3CN$ in water), white powder, 24 mg, 99% yield. $^1$H NMR (800 MHz, $CD_3OD$) δ 5.79 (dt, *J* = 14.4, 7.2 Hz, 1H), 5.55 (dd, *J* = 16.0, 7.2 Hz, 1H), 5.02 (d, *J* = 8.8 Hz, 1H), 4.56 (d, *J* = 8.0 Hz, 1H), 4.53 (d, *J* = 8.0 Hz, 1H), 4.40 (d, *J* = 8.0 Hz, 1H), 4.28–4.24 (m, 2H), 4.17 (t, *J* = 8.0 Hz, 1H), 4.14–3.37 (m, 30H), 2.84 (dd, *J* = 12.0, 4.8 Hz, 1H), 2.22 (t, *J* = 8.0 Hz, 2H), 2.13–2.11 (m, 2H), 2.12 (s, 3H, $CH_3$), 2.10 (s, 3H, $CH_3$), 2.01 (t, *J* = 12.0 Hz, 1H), 1.71–1.68 (m, 2H), 1.52–1.30 (m, 46 H, 23×$CH_2$), 1.00 (t, *J* = 7.2 Hz, 6H, 2×$CH_3$); $^{13}$C NMR (200 MHz, $CD_3OD$) δ 174.49, 174.18, 173.74, 173.36, 133.59, 129.88, 105.09, 103.43, 102.97, 102.62, 101.98, 81.46, 79.60, 77.56, 75.02, 74.97, 74.85, 74.58, 74.42, 74.10, 73.62, 73.35, 73.10, 71.90, 71.51, 71.00, 69.57, 68.94, 68.77, 68.53, 68.22, 68.17, 63.84, 61.48, 60.93, 60.30, 60.24, 53.22, 52.32, 51.22, 37.09, 35.90, 32.00, 31.63, 31.62, 31.60, 29.41, 29.38, 29.36, 29.35, 29.34, 29.32, 29.32, 29.31, 29.30, 29.28, 29.24, 29.20, 29.17, 29.04, 29.03, 29.00, 28.96, 25.71, 22.29, 22.28, 22.26, 21.12, 13.00, 12.97. ESI HRMS (m/z) calculated for $C_{71}H_{126}N_3O_{31}$ (M - H) 1516.8381, found 1516.8350.

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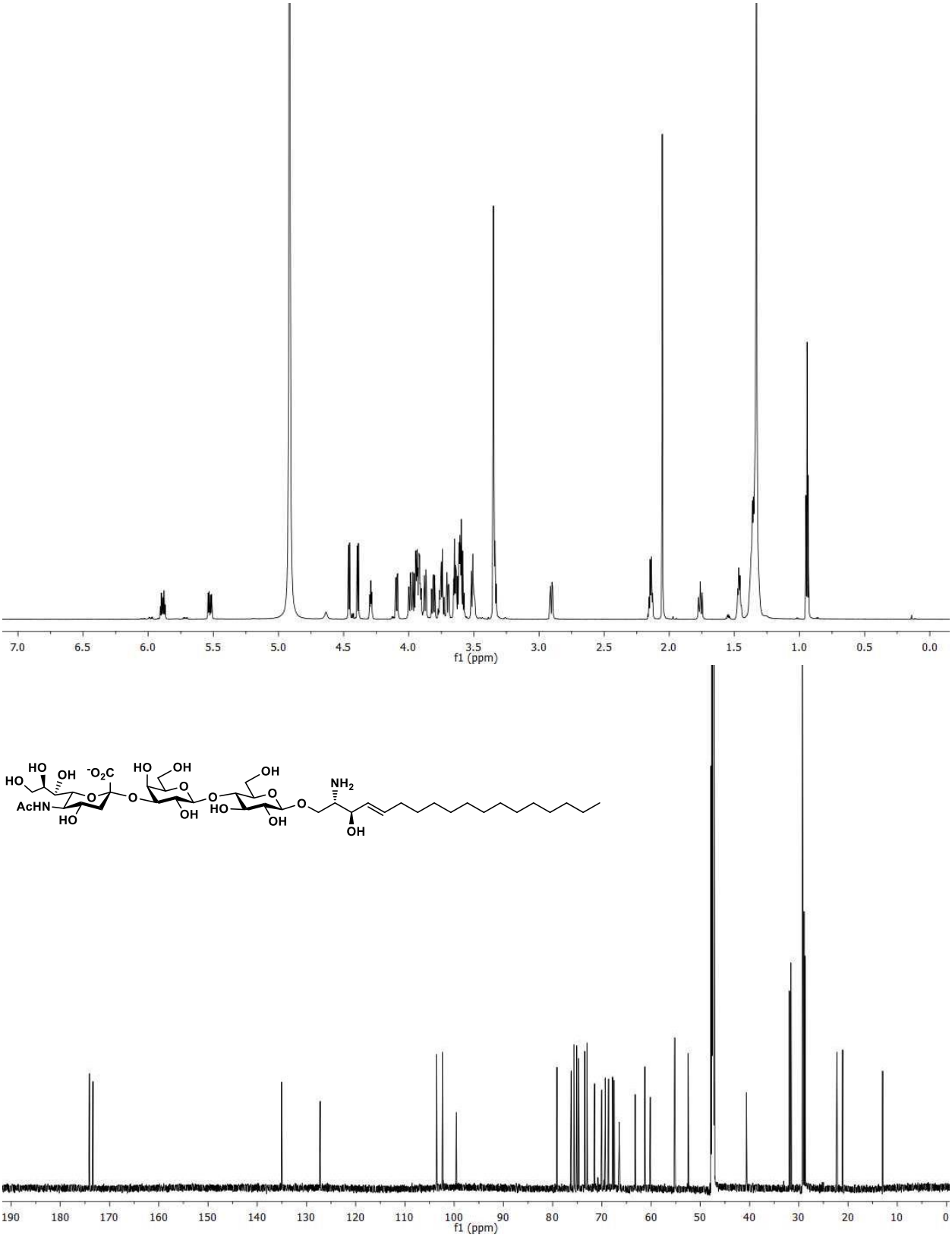
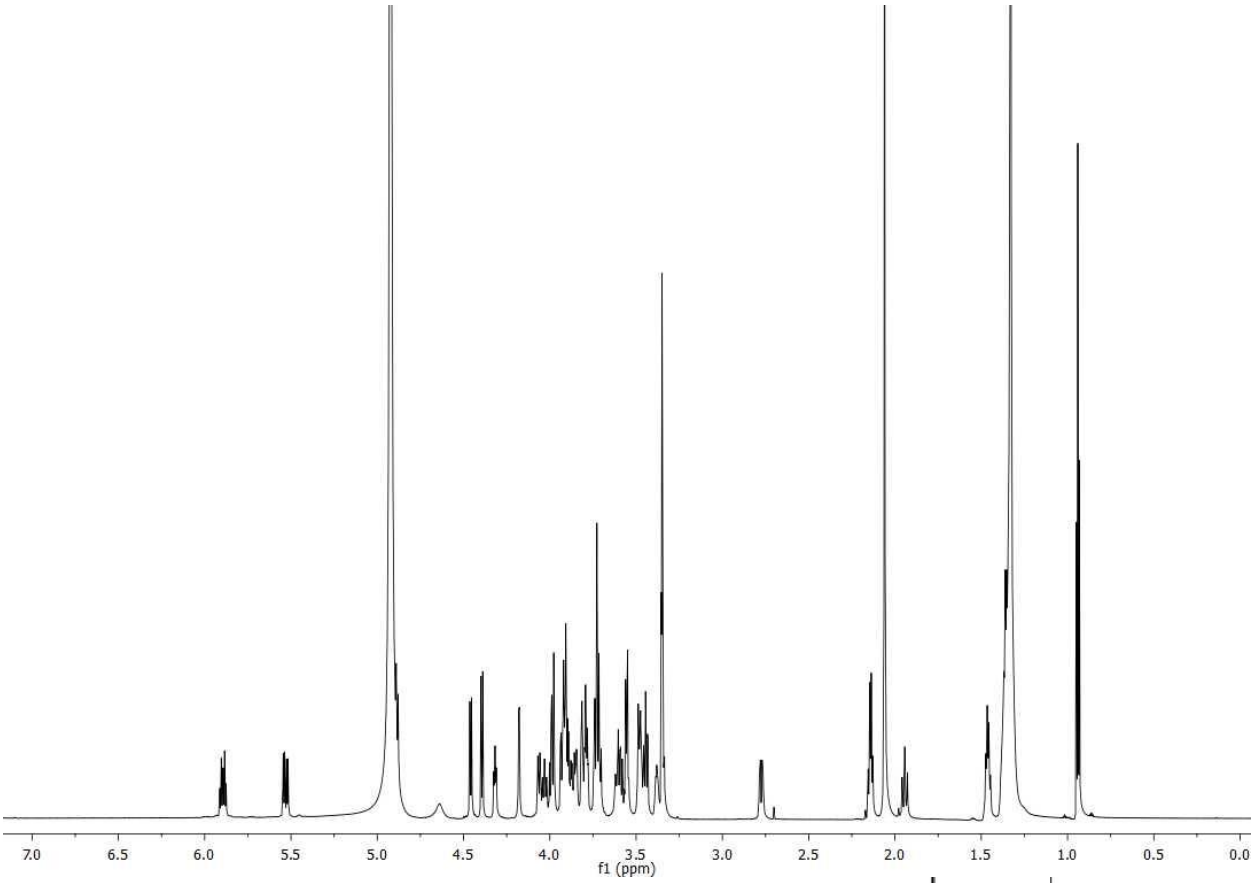
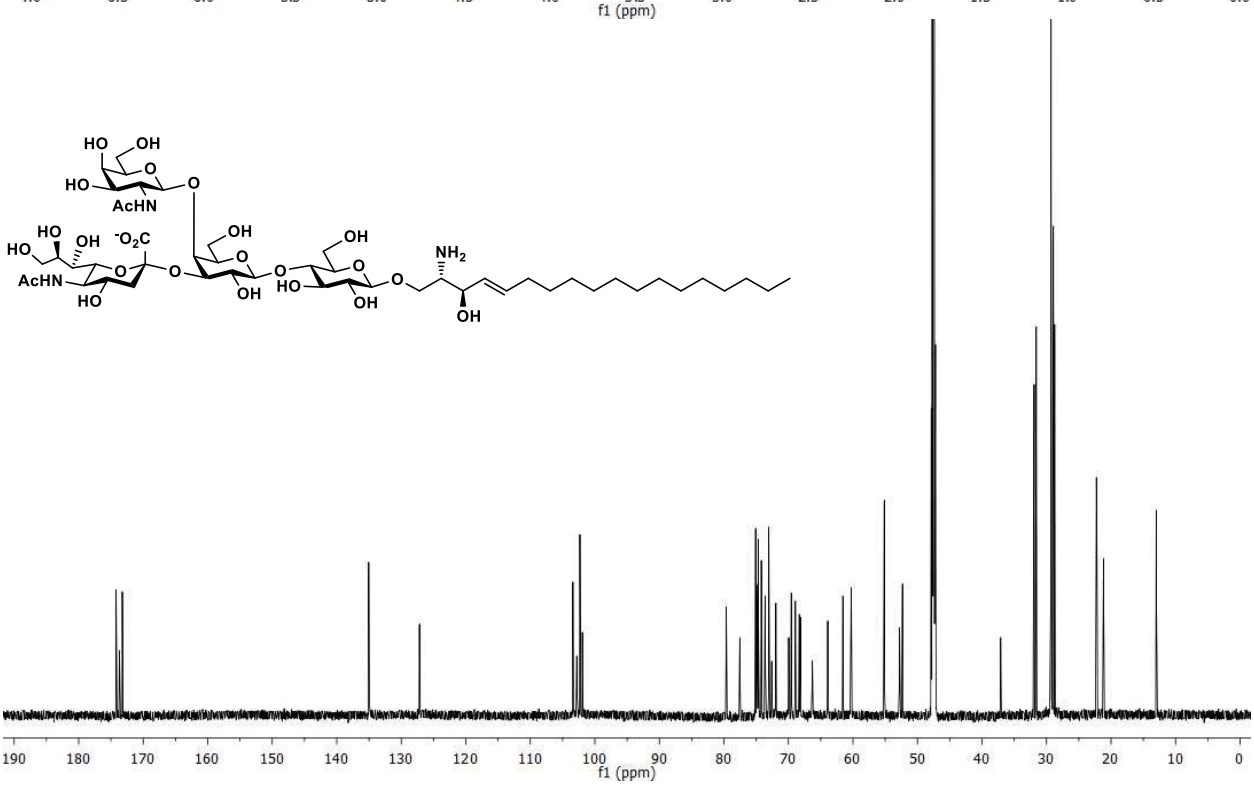
$^{1}H$ and $^{13}C$ NMR spectra of GM3βSph (**6**)

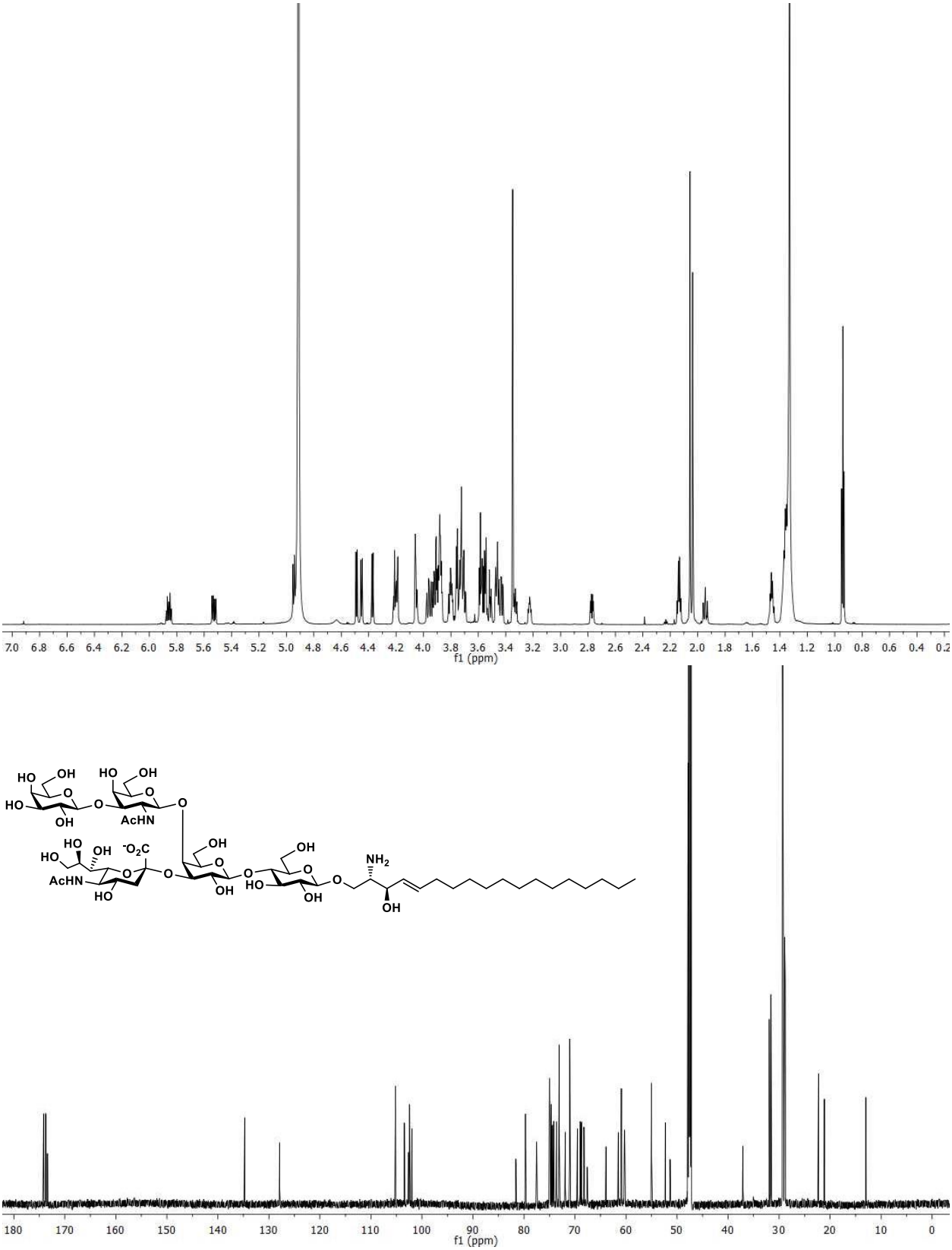


$^{1}H$ and $^{13}C$ NMR spectra of GM2βSph (**7**)

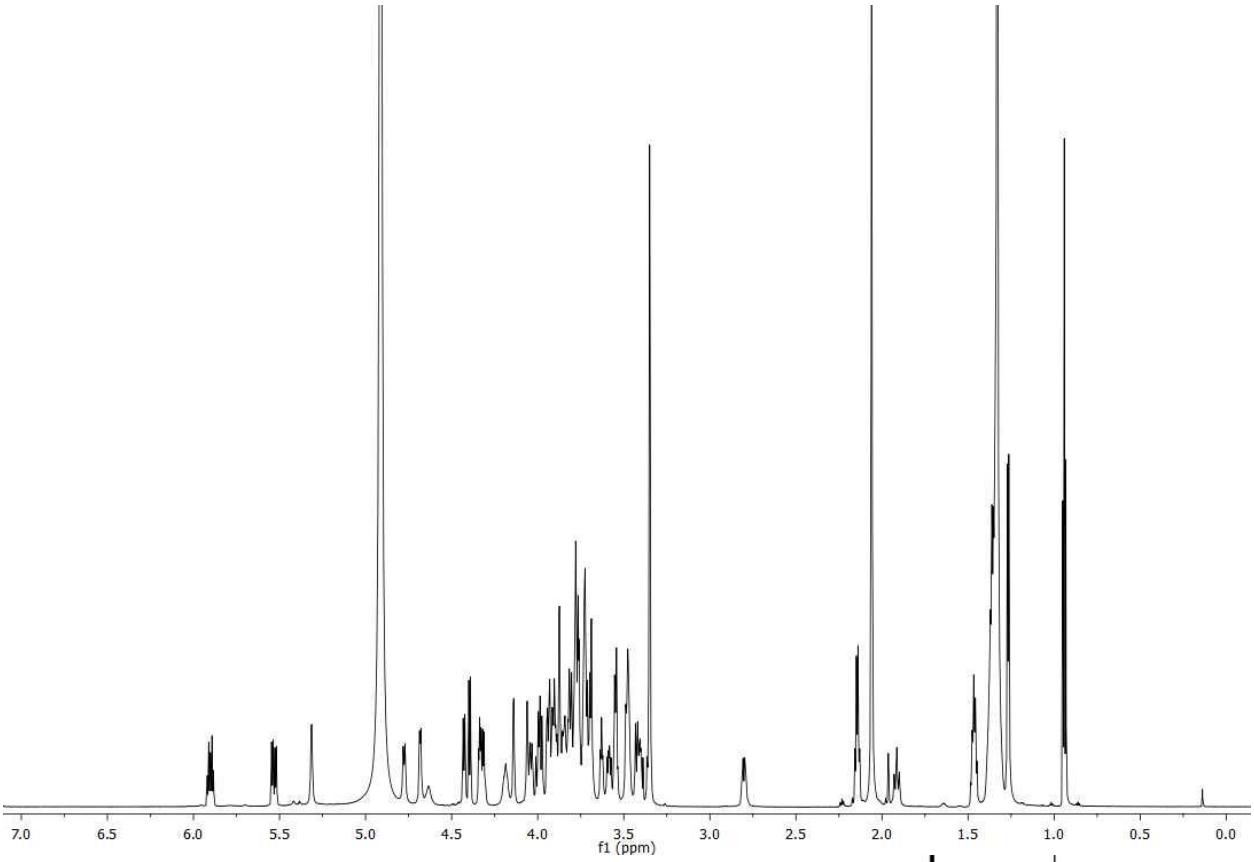


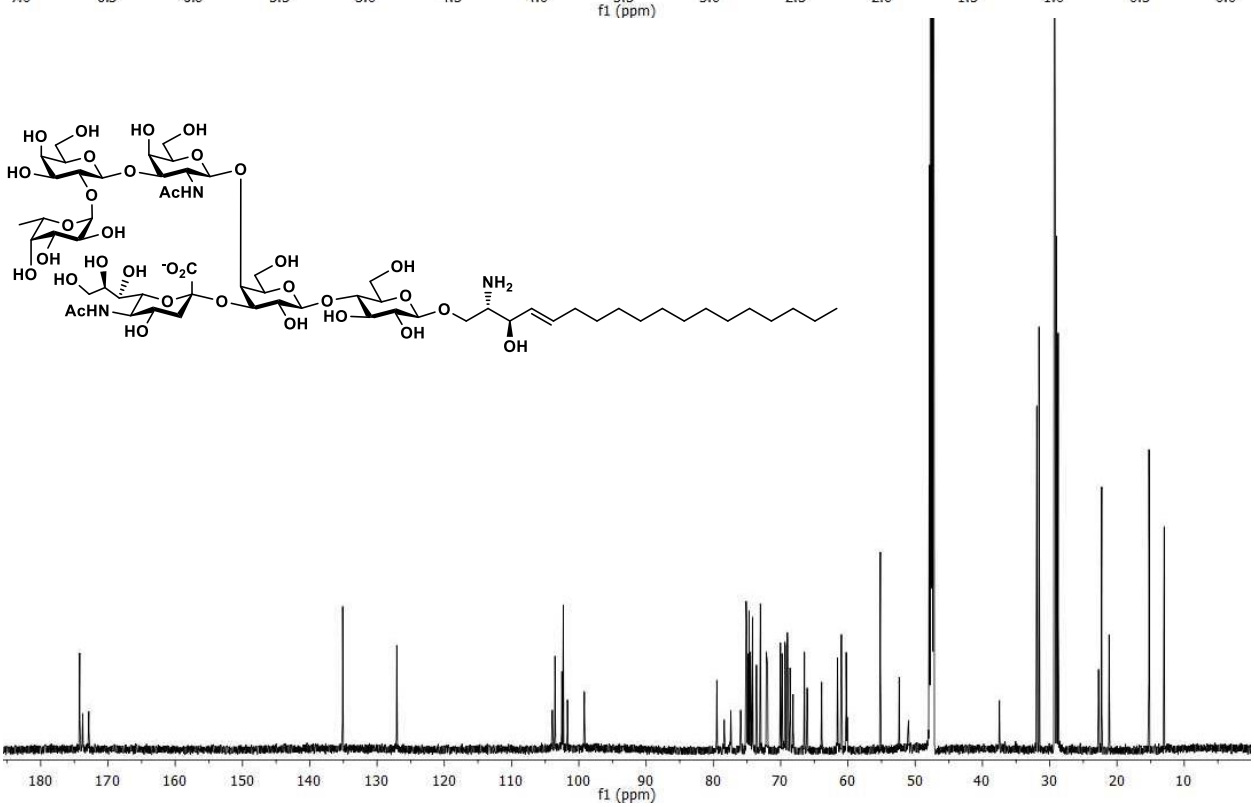



$^{1}H$ and $^{13}C$ NMR spectra of GM1βSph (**8**)

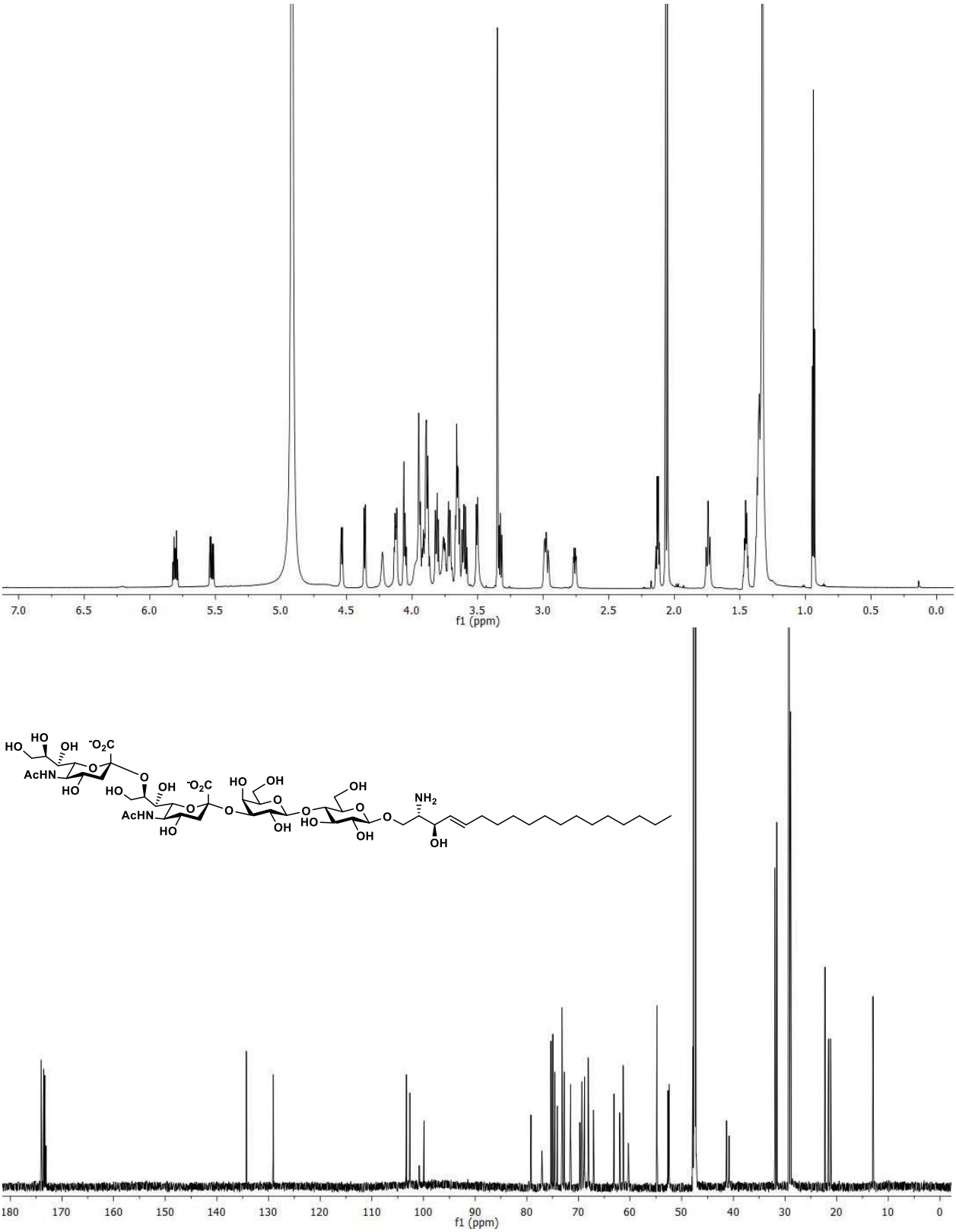


$^{1}H$ and $^{13}C$ NMR spectra of Fuc-GM1βSph (**9**)

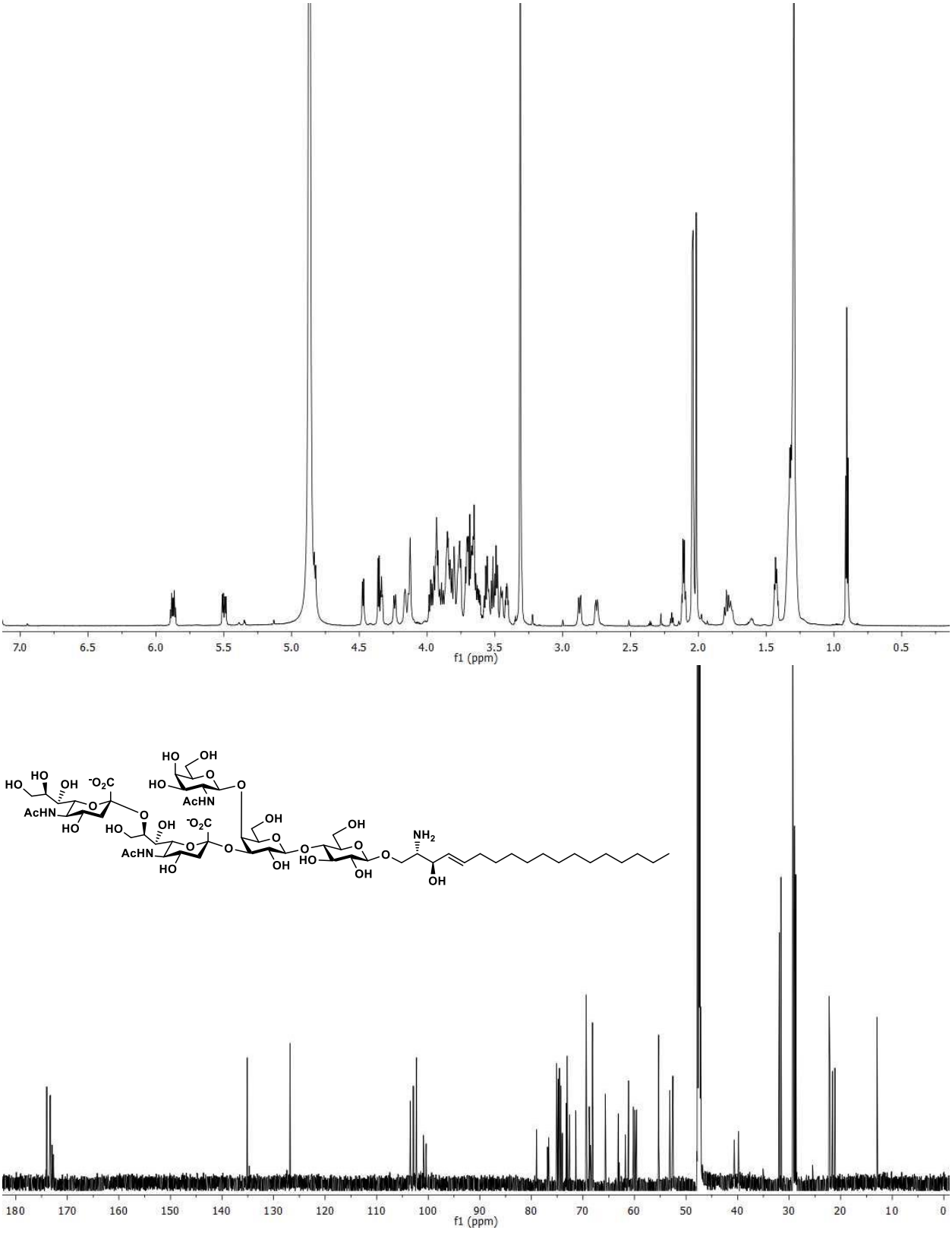


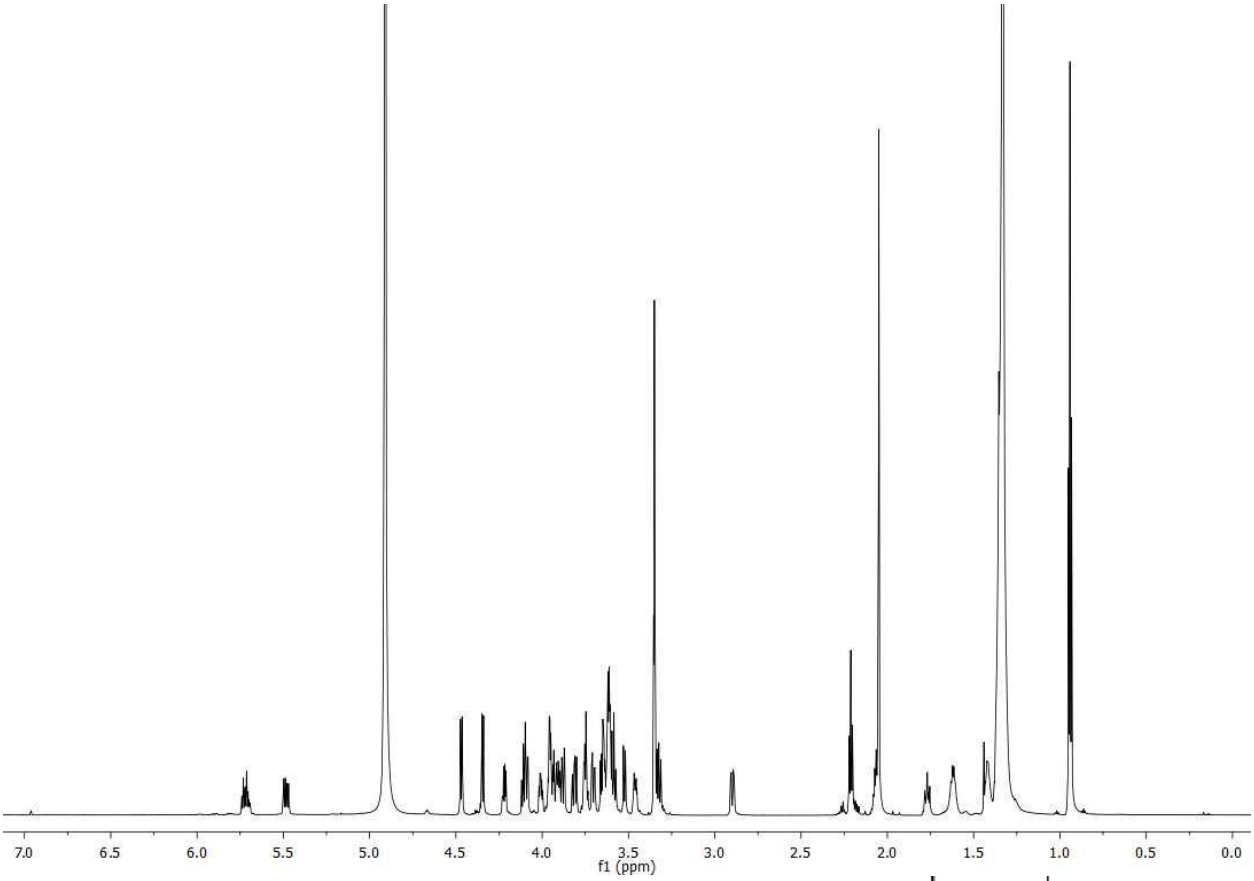
$^{1}H$ and $^{13}C$ NMR spectra of GD3βSph (**10**)

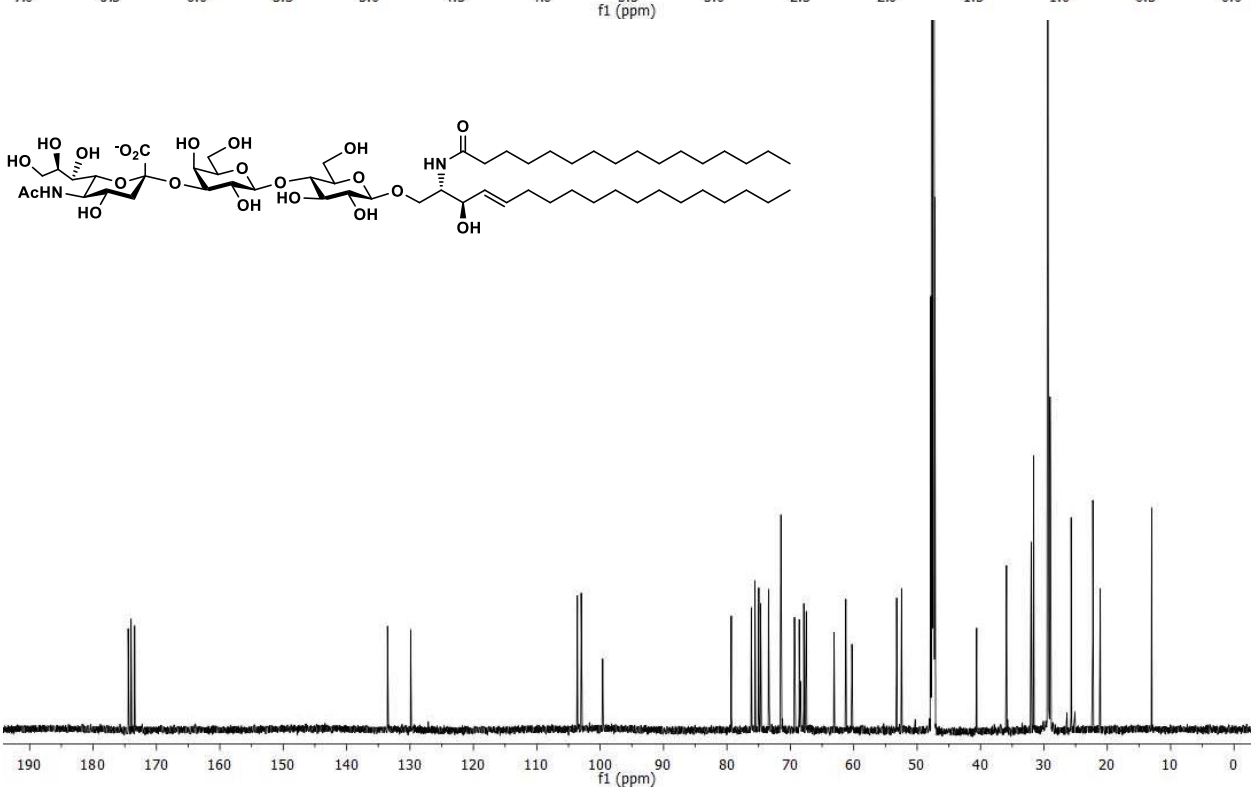


$^{1}H$ and $^{13}C$ NMR spectra of GD2βSph (**11**)

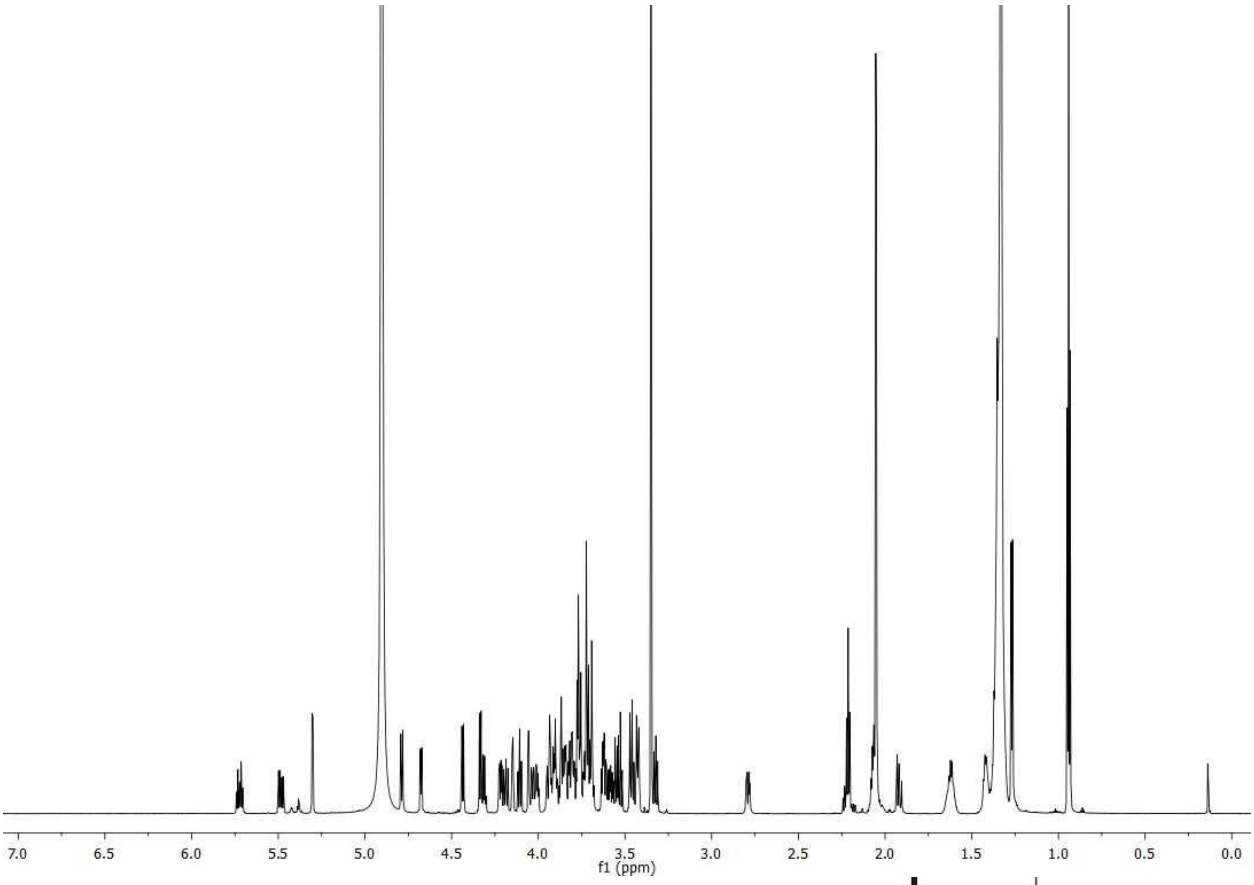


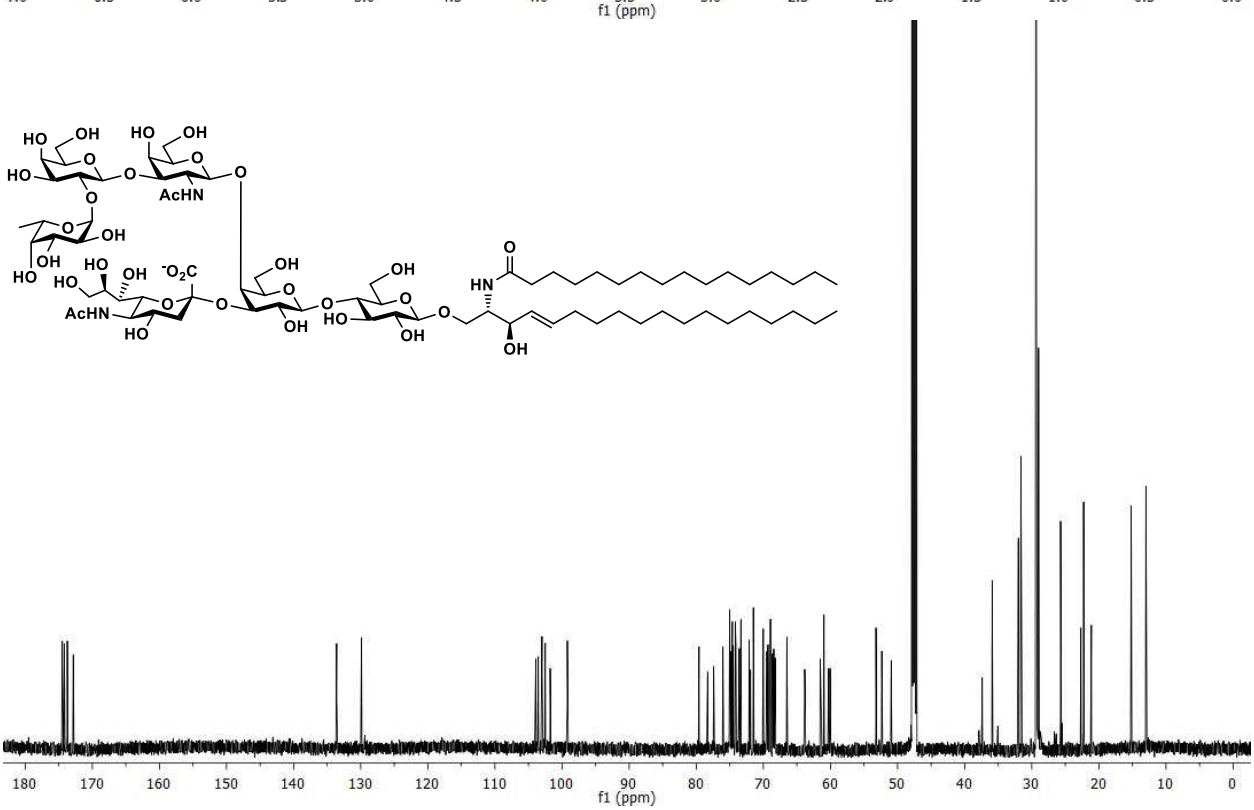
$^{1}H$ and $^{13}C$ NMR spectra of GM3 (**1**)



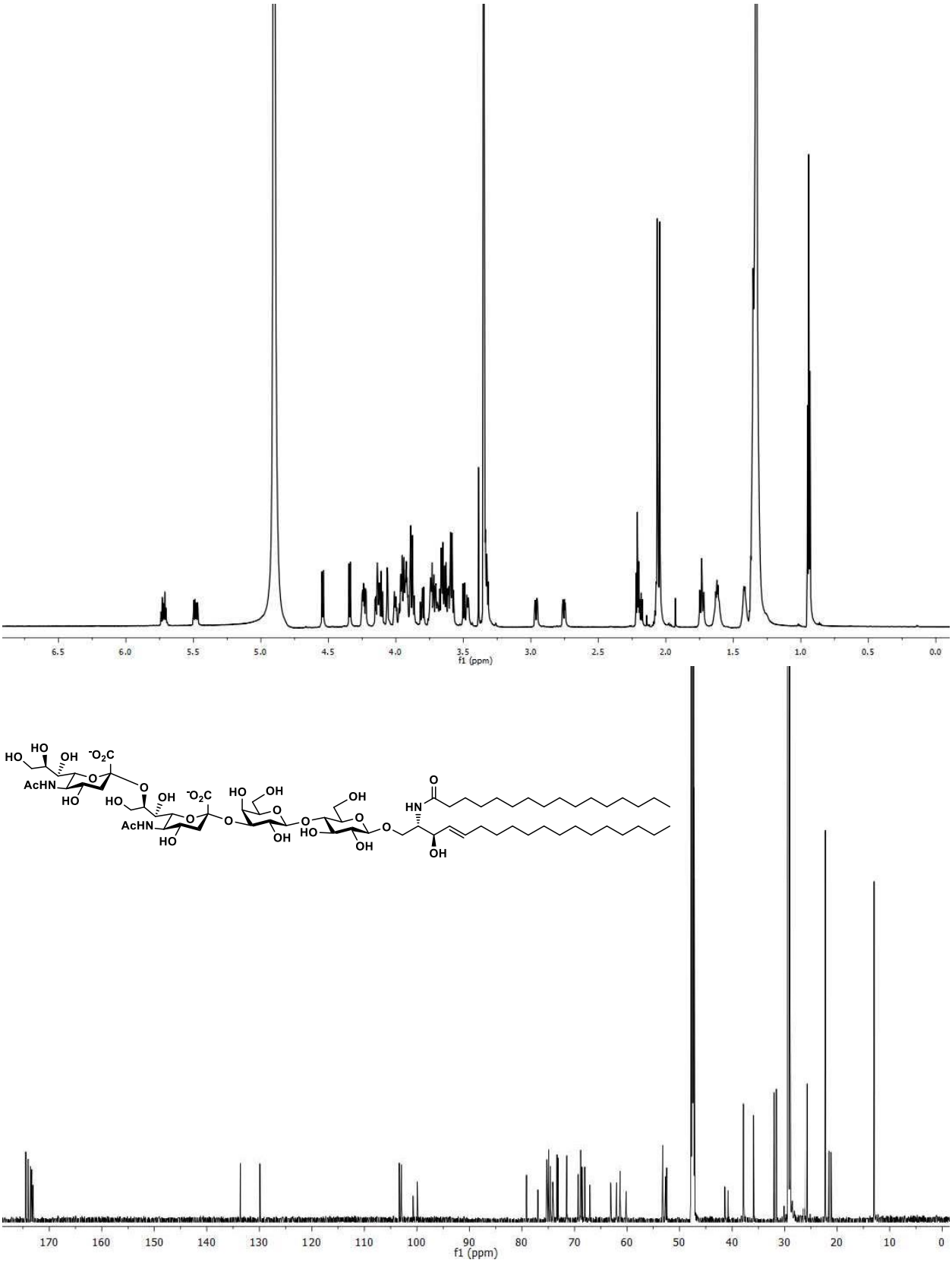



$^{1}H$ and $^{13}C$ NMR spectra of fucosyl GM1 (**2**)

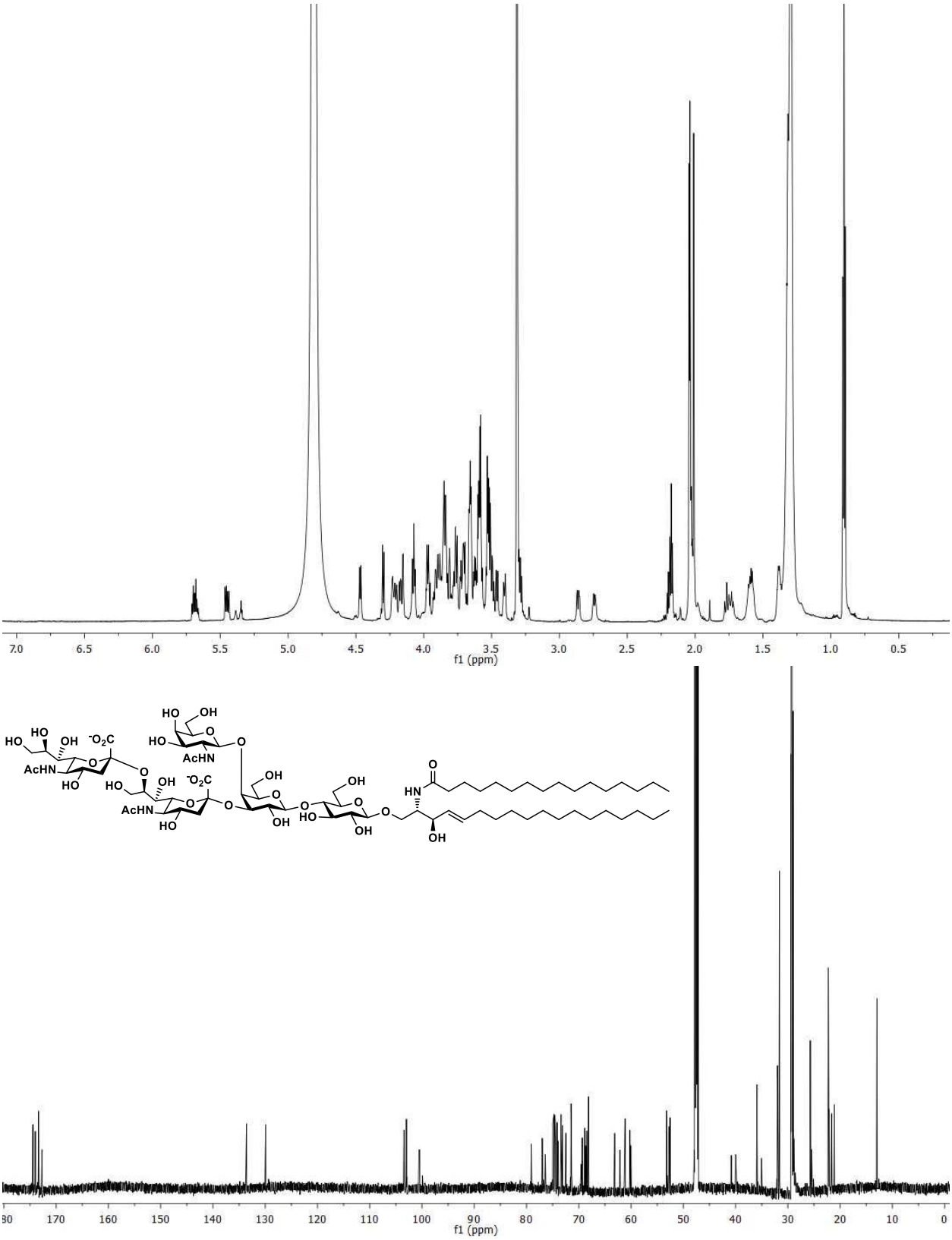


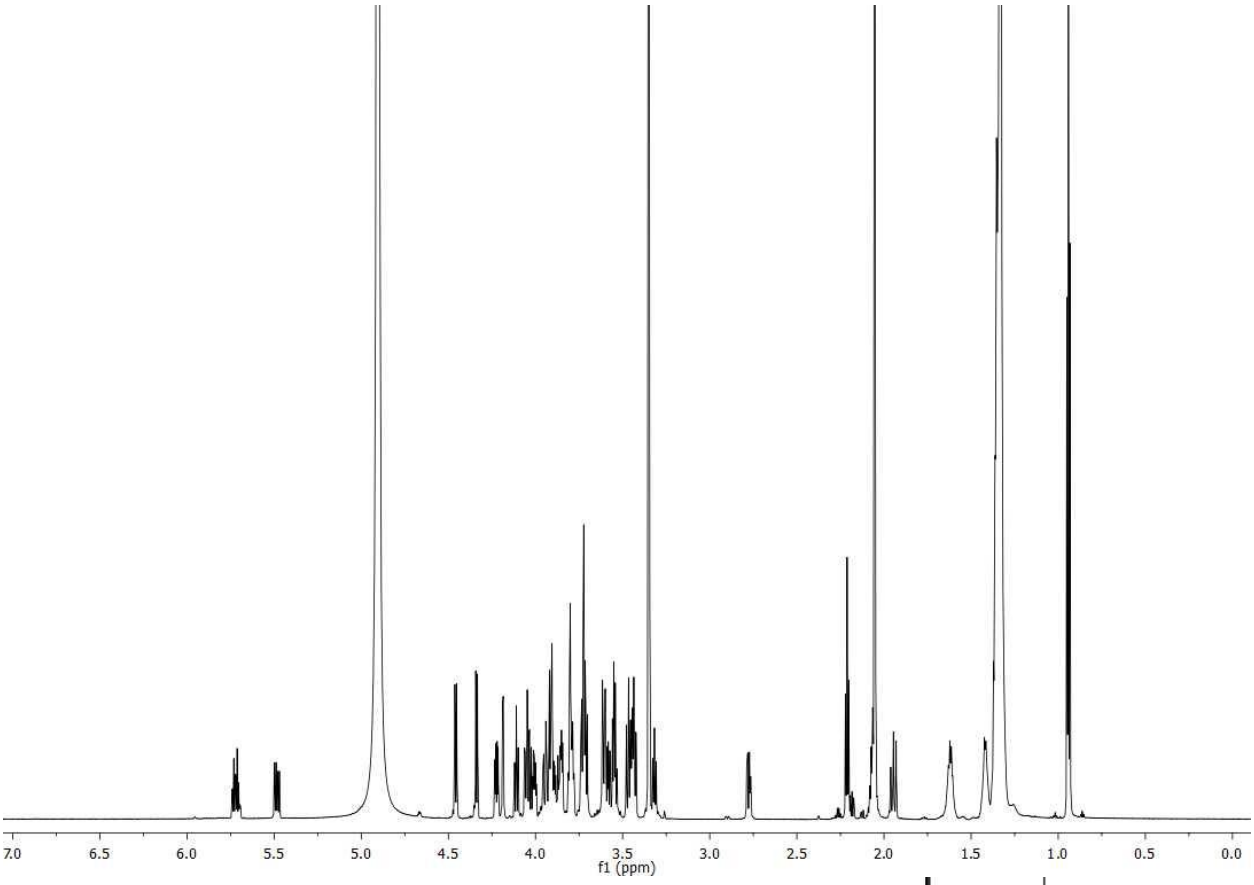
$^{1}H$ and $^{13}C$ NMR spectra of GD3 (**3**)

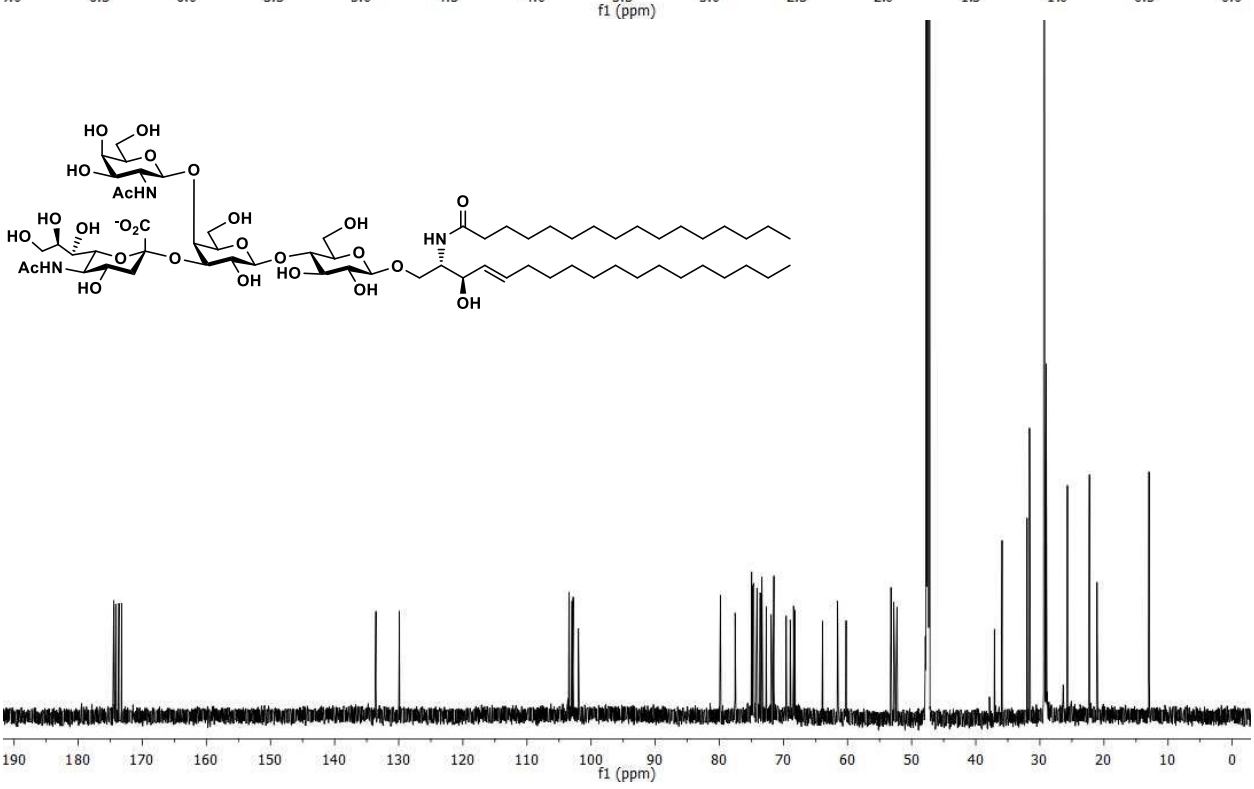


$^{1}H$ and $^{13}C$ NMR spectra of GD2 (**4**)



$^{1}H$ and $^{13}C$ NMR spectra of GM2 (**12**)



$^{1}H$ and $^{13}C$ NMR spectra of GM1 (**13**)