Chemical Synthesis and Immunological Evaluation of Cancer Vaccines Based on Ganglioside Antigens and α-Galactosylceramide

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Supplementary Information

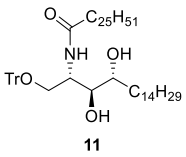
Chemistry

General methods

Unless noted, chemical reagents and solvents were used without further purification from commercial sources (Alfa Aesar, Carbosynth, Sigma-Aldrich or TCI chemicals) and all solvents were of HPLC quality. Anhydrous CH_2Cl_2, DMF, and CH_3CN were obtained using an Innovative Technology PS-MD-7 PureSolve solvent purification system. All reactions were monitored by thin-layer chromatography (TLC) and/or liquid-chromatography mass spectrometry (RP-UPLC-MS). TLC was performed on Merck aluminum sheets precoated with 0.25 mm silica gel, 60 F_{254} plates. TLC plates were visualized under UV-light (254 nm) fluorescence quenching, and/or by charring after dipping in either a solution of $Ce(SO_4)_2$ and $(NH_4)_6Mo_7O_{24}$ in 10% aq. H_2SO_4 or 8% H_2SO_4. Eluent systems are specified for each R_f value, and ratios are given as volume ratios. Silica gel chromatography was performed using Merck Genduran® Si 60 (40–63 µm) silica gel. The eluent system for flash column chromatography is specified under each protocol, and the eluent ratio is given as volume ratio. Analytical RP-UPLC-MS (ESI) analysis was performed on a Waters AQUITY RP-UPLC system equipped with a diode array detector using a Thermo accucore C18 column (d 2.6 µm, 2.1 × 50 mm; column temp: 50 °C; flow: 0.6 mL/min). Four different methods were used. Method A: eluents A (0.1% HCO_2H in milli-Q water) and B (0.1% HCO_2H in CH_3CN) were used in a linear gradient (5% B to 100% B) in a total run time of 2.6 min. Method B: eluents A (0.1% HCO_2H in H_2O) and B (0.1% HCO_2H in CH_3CN) were used in a linear gradient (5% B to 100% B) in a total run time of 5.0 min. Method C: eluents A (10 mM NH_4OAc in milli-Q water) and B (0.1% NH_4OAc in milli-Q water/CH_3CN, 90/10, v/v) were used in a linear gradient (5% B to 100% B) in a total run time of 2.6 min. Method D: eluents A (0.1% NH_4OAc in H_2O) and B (0.1% NH_4OAc in CH_3CN) were used in a linear gradient (5% B to 100% B) in a total run time of 5.0 min. The LC system was coupled to a SQD mass spectrometer operating in both positive and negative electrospray modes. All new compounds were characterized by ^1H NMR, ^{13}C NMR, HRMS (ESI), and optical rotation. ^1H and ^{13}C NMR spectra were recorded with a Bruker Ascend 400 or a Bruker Ascend 800 spectrometer at 25 °C. The chemical shifts are reported in ppm relative to the residual solvent peak from $CDCl_3$ (δ_H = 7.26 ppm, δ_C = 77.16 ppm), MeOD (δ_H = 3.35 ppm, δ_C = 49.0 ppm), D_2O (δ_H = 4.79 ppm, δ_C reported relative to TMS, d = 0.0 ppm, using the lock signal as secondary reference). Coupling constants (*J*) are reported in Hz and the field is reported in each case. Multiplicities are reported as: singlet (s), apparent singlet (*a*s), doublet (d), apparent doublet (*a*d), triplet (t), doublet of doublets (dd), doublet of doublet of doublets (ddd), doublet of triplets (dt), doublet of doublet of triplets (ddt), triplet of doublets (td), quartet (q), and multiplet (m). Assignment of ^1H and ^{13}C resonances was aided by COSY, HSQC, H2BC, HMBC, TOCSY, and HSQC-TOCSY experiments. Melting points were measured on a Stuart melting point SMP30 and reported in °C uncorrected. Optical rotations were recorded on a Perkin-Elmer polarimeter (Model 241) at the sodium D-line (589 nm) at 20 °C using a 1 dm cell. LC-HRMS analysis was performed on an UHPLC-QTOF system (Dionex ultimate 3000 and Bruker MaXis) with an electrospray ionization (ESI) source and controlled using DataAnalysis v4.2 software. MALDI-HRMS was performed on a Bruker TIMS TOF Flex with DHB with 0.1% TFA in 70% CH_3CN and 30% H_2O as the matrix. Deprotected sugars were lyophilised using a ScanVac CoolSafe freeze-dryer (T = –103 °C; 29.7 mbar).

Experimental procedures

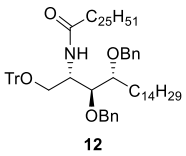
(*2S*,*3S*,*4R*)-2-Hexacosanoylamido-1-triphenylmethoxy-octadecane-3,4-diol (11)



11

To a solution of hexacosanoic acid (2.0 g, 5.04 mmol) in CH_2Cl_2 (60 mL) were added EDC hydrochloride (1.5 g, 8.07 mmol) and NHS (1.0 g, 8.6 mmol) and the reaction was stirred at 40 °C for 3 hours. Then the mixture was cooled to room temperature, diluted with water, and transferred into a separatory funnel. The organic layer was separated, while the aqueous layer was extracted with Et_2O (×3). The combined organic layers were then dried over Na_2SO_4, filtered, and concentrated *in vacuo*. The crude product was dissolved in THF (72 mL) and phytosphingosine (1.4 g, 4.03 mmol) was added followed by Et_3N (0.7 mL, 15.1 mmol). The reaction was stirred at 50 °C overnight, then it was cooled to room temperature and concentrated *in vacuo*. Finally, the crude residue was dissolved in pyridine (60 mL) and treated with trityl chloride (2.5 g, 9.07 mmol) and DMAP (25 mg, 0.2 mmol) at 80 °C overnight. The crude residue was obtained after solvent evaporation *in vacuo*. Purification by flash column chromatography (toluene:EtOAc, 95:5, v/v) afforded **11** (2.0 g, 2.3 mmol, 46% over three steps) as a white foam. R_f = 0.36 (toluene/EtOAc, 8:2); ^{1}H NMR (400 MHz, chloroform-*d*) δ 7.44 (d, *J* = 7.3 Hz, 5H, H_{Ar}), 7.39 – 7.22 (m, 15H, H_{Ar}), 6.07 (d, *J* = 8.3 Hz, 1H, NH), 4.29 (dq, *J* = 8.4, 4.2 Hz, 1H, H-2), 3.60 (q, *J* = 5.9 Hz, 1H, H-4), 3.53 (dd, *J* = 9.8, 3.6 Hz, 1H, H-1), 3.47 – 3.26 (m, 2H, H-1, H-3), 3.17 (d, *J* = 8.3 Hz, 1H, OH), 2.17 (t, *J* = 7.6 Hz, 2H, CH_2), 1.77 – 1.58 (m, 4H, 2 CH_2), 1.54 – 1.39 (m, 1H, C*H*H), 1.29 (s, 69H, CH_2), 0.91 (t, *J* = 6.7 Hz, 6H, 2 CH_3); ^{13}C NMR (101 MHz, chloroform-*d*) δ 173.3 (CO), 143.3 (3 C_{Ar}), 129.8, 129.2, 128.6, 128.4, 128.2, 128.1, 127.9, 127.5, 127.4, 125.4 (C_{Ar}), 87.8 (C_q), 75.8 (C-4), 73.4 (C-3), 63.1 (C-1), 50.5 (C-2), 37.0 (CH_2), 33.5, 32.1, 29.9, 29.9, 29.8, 29.7, 29.6, 29.5, 26.0, 25.9, 22.8 (CH_2), 14.3 (2 CH_3). All analytical data were consistent with literature values.[1]

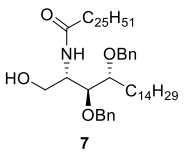
(*2S*,*3S*,*4R*)-3,4-Di-*O*-benzyl-2-hexacosanoylamido-1-triphenylmethoxy-octadecane-3,4-diol (12)



12

Compound **11** (2.2 g, 2.3 mmol) was dissolved in anhydrous DMF (20 mL) together with BnBr (3.1 mL, 26.3 mmol), then the solution was cooled to 0 °C and NaH (0.7 g of a 60% oil dispersion, 17.6 mmol) and TBAI (0.16 g, 0.44 mmol) were added. The reaction was stirred at room temperature for 2 hours, then it was neutralized with MeOH, and concentrated *in vacuo*. The crude was purified by flash column chromatography (toluene:EtOAc, 98:2, v/v) to afford **12** (2.3 g, 2 mmol, 87%) as a clear to yellowish oil. R_f = 0.7 (toluene/EtOAc, 9:1); ^{1}H NMR (400 MHz, chloroform-*d*) δ 7.44 – 7.38 (m, 5H, H_{Ar}), 7.38 – 7.20 (m, 20H, H_{Ar}), 5.62 (d, *J* = 8.9 Hz, 1H, NH), 4.84 (d, *J* = 11.5 Hz, 1H, C*H*HPh), 4.56 – 4.49 (m, 3H, CH*H*Ph, CH_2Ph), 4.34 (ddd, *J* = 9.0, 6.6, 4.4 Hz, 1H, H-2), 3.87 (dd, *J* = 6.7, 2.9 Hz, 1H, H-3), 3.59 – 3.46 (m, 2H, H-4, H-1a), 3.34 (dd, *J* = 9.5, 4.1 Hz, 1H, H-1b), 1.96 (t, *J* = 7.6 Hz, 2H, CH_2), 1.68 – 1.57 (m, 2H, CH_2), 1.56 – 1.42 (m, 2H, CH_2), 1.33 – 1.25 (m, 68H, CH_2), 0.94 – 0.87 (m, 6H, 2 CH_3); 13C NMR (101 MHz, chloroform-d) δ 172.6 (CO), 143.9 (3 C_{Ar}), 138.8 (C_{Ar}), 138.7 (C_{Ar}), 128.8, 128.5, 128.4, 128.0, 127.9, 127.7, 127.6, 127.2 (C_{Ar}), 86.9 (C_q), 80.5 (C-4), 79.6 (C-3), 73.4 (CH_2Ph), 72.1 (CH_2Ph), 62.5 (C-1), 50.2 (C-2), 37.0, 32.1, 30.1, 30.0, 29.9, 29.8, 29.7, 29.6, 29.5, 29.5, 26.1, 25.8, 22.8 (CH_2), 14.3 (2 CH_3). All analytical data were consistent with literature values.[2]

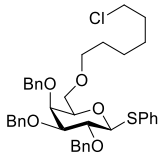
(*2S*,*3S*,*4R*)-3,4-Di-*O*-benzyl-2-hexacosanoylamido-octadecane-1,3,4-triol (7)



7

Compound **12** (310 mg, 0.277 mmol) was dissolved in CH_2Cl_2 (8 mL) and MeOH (4 mL), followed by addition of *p*-toluenesulfonic acid monohydrate (24 mg, 0.126 mmol). The reaction mixture was stirred for 3 hours at room temperature and then was neutralized by Et_3N. The solvent was removed *in vacuo* and the resulting crude was purified by flash column chromatography (toluene/EtOAc, 75:25, v/v) to yield compound **7** (203 mg, 0.232 mmol, 84%) as a white solid. R_f = 0.48 (toluene/EtOAc, 8:2); ^{1}H NMR (400 MHz, chloroform-*d*) δ 7.42 – 7.27 (m, 10H, H_{Ar}), 6.06 (d, *J* = 8.2 Hz, 1H, NH), 4.72 (d, *J* = 11.7 Hz, 1H, C*H*HPh), 4.67 (d, *J* = 11.4 Hz, 1H, C*H*HPh), 4.61 (d, *J* = 11.4 Hz, 1H , CH*H*Ph), 4.46 (d, *J* = 11.7 Hz, 1H, CH*H*Ph), 4.15 (dq, *J* = 7.7, 3.7 Hz, 1H, H-2), 4.00 (dd, *J* = 11.5, 3.1 Hz, 1H, H-1a), 3.75 – 3.66 (m, 2H, H-3, H-4), 3.61 (dd, *J* = 11.5, 4.4 Hz, 1H, H-1b), 2.08 – 1.92 (m, 2H, CH_2), 1.76 – 1.66 (m, 1H, C*H*H), 1.65 – 1.57 (m, 1H, CH*H*), 1.57 – 1.39 (m, 2H, CH_2), 1.32 – 1.20 (m, 68H, CH_2), 0.93 – 0.84 (m, 6H, 2 CH_3); ^{13}C NMR (101 MHz, chloroform-*d*) δ 172.9 (CO), 138.3 (C_{Ar}), 137.9 (C_{Ar}), 128.9, 128.6, 128.3, 128.2, 128.1, 127.9 (C_{Ar}), 82.4 (C-3), 79.2 (C-4), 73.2 (CH_2Ph), 73.0 (CH_2Ph), 63.1 (C-1), 50.7 (C-2), 36.8, 32.1, 30.9, 29.9, 29.8, 29.8, 29.7, 29.7, 29.5, 29.5, 26.2, 25.8, 22.8 (CH_2), 14.3 (2 CH_3). All analytical data were consistent with literature values.[2]

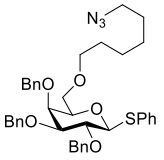
Phenyl 2,3,4-tri-*O*-benzyl 6-(6'-chlorohexyl)-1-thio-β-D-galactopyranoside (14)



14

Compound **13**[3] (1.99 g, 3.67 mmol) was dissolved in anhydrous DMF (9.1 mL) and 6-chlorohexyl 4-methylbenzenesulfonate[4] (3.2 g, 11 mmol) was added to the solution. The mixture was cooled to 0 °C and then NaH (0.66 g of 60% oil dispersion, 16.5 mmol) was added. The reaction was stirred for 15 minutes at 0 °C then for 3.5 hours at room temperature, before neutralizing with MeOH. The solvent was removed *in vacuo*, then the crude residue was dissolved in CH_2Cl_2 and washed with sat. aq. NH_4Cl. The aqueous phase was then re-extracted with CH_2Cl_2 (×3). Combined organic layers were dried over Na_2SO_4, filtered, and concentrated *in vacuo*. The crude residue was purified by flash column chromatography (toluene:EtOAc, 95:5, v/v) to afford **14** (1.8 g, 2.7 mmol, 74%) as a colorless oil. R_f = 0.64 (toluene/EtOAc, 9:1); $[\alpha]_D^{20}$ = +2.2 (*c* 1.0, $CHCl_3$); ^{1}H NMR (400 MHz, chloroform-d) δ 7.57 (dd, J = 6.5, 3.1 Hz, 2H, H_{Ar}), 7.42 – 7.14 (m, 18H, H_{Ar}), 4.99 (d, J = 11.6 Hz, 1H, C*H*HPh), 4.82 – 4.72 (m, 4H, CH_2Ph, CH_2Ph), 4.67 – 4.62 (m, 2H, H-1, CH*H*Ph), 4.50 (s, 1H, CH*H*Ph), 3.99 – 3.91 (m, 2H, H-2, H-4), 3.62 (dd, J = 9.3, 2.8 Hz, 1H, H-3), 3.58 (s, 3H, H-6a, H-6b, H-5), 3.52 (t, J = 6.7 Hz, 2H, OCH_2Cl), 3.41 (td, J = 6.5, 3.0 Hz, 1H, OC*H*H), 3.33 (dt, J = 9.4, 6.6 Hz, 1H, OCH*H*), 1.76 (p, J = 6.8 Hz, 2H, CH_2), 1.63 (dd, J = 8.3, 5.1 Hz, 1H, CH*H*), 1.55 – 1.49 (m, 1H, C*H*H), 1.48 – 1.29 (m, 4H, 2CH_2); ^{13}C NMR (101 MHz, chloroform-*d*) δ 138.9 (C_{Ar}), 138.5 (C_{Ar}), 138.4 (C_{Ar}), 134.4 (C_{Ar}), 131.6, 129.2, 128.9, 128.6, 128.5, 128.4, 128.3, 127.9, 127.8, 127.7, 127.6, 127.5, 127.1 (20 C_{Ar}), 87.8 (C-1), 84.3 (C-3), 77.4 (C-2, C-5), 75.8 (CH_2Ph), 74.6 (CH_2Ph), 73.8 (C-4), 72.9 (CH_2Ph), 71.5 (OCH_2), 69.2 (C-6), 45.2 (CH_2Cl), 32.7 (CH_2), 29.7 (CH_2), 26.8 (CH_2), 25.6 (CH_2). HRMS (ESI-TOF) m/z: calcd. for $C_{39}H_{45}ClO_5S$: 661.2756 $[M+H]^+$; found 661.2744.

Phenyl 6-(6'-azidohexyl)-2,3,4-tri-*O*-benzyl 1-thio-β-D-galactopyranoside (15)

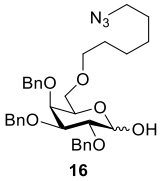


15

Compound **14** (2.1 g, 3.2 mmol) was dissolved in DMF (24 mL). While stirring, NaN_3 (1 g, 15.9 mmol) was added and the reaction was heated to 80 °C overnight. The mixture was then diluted with H_2O and EtOAc, transferred into a separating funnel, and the organic layer was collected. The aqueous layer was then re-extracted with EtOAc (×3). The combined organic layers were dried over Na_2SO_4, filtered, and concentrated *in vacuo*. The crude residue was purified by flash column chromatography (toluene:EtOAc, 95:5, v/v) to yield compound **15** (1.8 g, 2.7 mmol, 84%) as a transparent to yellow oil. R_f = 0.63 (toluene/EtOAc, 9:1);

$[\alpha]_D^{20}$ = +2.0 (*c* 1.0, $CHCl_3$); ^{1}H NMR (400 MHz, chloroform-*d*) δ 7.57 (dd, *J* = 6.6, 3.1 Hz, 2H, H_{Ar}), 7.41 – 7.28 (m, 18H, H_{Ar}), 5.00 (d, *J* = 11.5 Hz, 1H, C*H*HPh), 4.80 (d, *J* = 10.2 Hz, 1H, C*H*HPh), 4.77 – 4.72 (m, 3H, CH*H*Ph, CH_2Ph), 4.68 – 4.61 (m, 2H, H-1, CH*H*Ph), 3.99 – 3.90 (m, 2H, H-2, H-4), 3.62 (dd, *J* = 9.3, 2.8 Hz, 1H, H-3), 3.58 (s, 2H, H-5, H-6a, H-6b), 3.45 – 3.38 (m, 1H, OCH*H*), 3.33 (dt, *J* = 9.4, 6.6 Hz, 1H, OC*H*H), 3.24 (t, *J* = 6.9 Hz, 2H, CH_2N_3), 1.66 – 1.47 (m, 4H, $2CH_2$), 1.43 – 1.25 (m, 4H, $2CH_2$); ^{13}C NMR (101 MHz, chloroform-*d*) δ 138.9 (C_{Ar}), 138.4 (C_{Ar}), 138.4 (C_{Ar}), 134.4 (C_{Ar}), 131.6, 128.9, 128.6, 128.5, 128.3, 127.9, 127.8, 127.7, 127.6, 127.5, 127.2 (20 C_{Ar}), 87.8 (C-1), 84.3 (C-3), 77.4 (C-2, C-5), 75.8 (CH_2Ph), 74.6 (CH_2Ph), 73.8 (C-4), 72.9 (CH_2Ph), 71.5 (OCH_2), 69.3 (C-6), 51.5 (CH_2N_3), 29.7 (CH_2), 28.9 (CH_2), 26.7 (CH_2), 25.9 (CH_2). HRMS (ESI-TOF) m/z: calcd. for $C_{39}H_{45}N_3O_5S$: 668.3160 $[M+H]^+$; found: 668.3161.

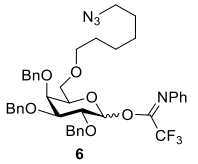
6-(6'-Azidohexyl)-2,3,4-tri-*O*-benzyl-D-galactopyranose (16)



16

Compound **15** (2.0 g, 3.0 mmol) was dissolved in acetone (20 mL) and water (2 mL), followed by addition of *N*-bromosuccinimide (1.0 g, 5.6 mmol) at 0 °C. The reaction was stirred for 1 hour at room temperature in absence of light. After removal of the solvent, the residue was diluted with EtOAc, washed with 10% aq. $Na_2S_2O_3$ solution, and brine. The organic layer was collected, dried over $MgSO_4$, concentrated, and purified by flash column chromatography (toluene/EtOAc, 8:2, v/v) to afford compound **16** (1.45 g, 2.52 mmol, 84%) as a colorless oil. R_f = 0.42 (toluene/EtOAc, 8:2); ^{1}H NMR (400 MHz, chloroform-d) δ 7.46 – 7.22 (m, 30H, H_{Ar}), 5.29 (d, *J* = 3.6 Hz, 1H, H-1$_\alpha$), 5.01 – 4.88 (m, 3H, 3 C*H*HPh), 4.88 – 4.67 (m, 7H, 7 C*H*HPh), 4.68 (d, J = 7.4 Hz, 1H, H-1$_\beta$), 4.68 – 4.58 (m, 2H, 2 C*H*HPh), 4.12 (t, *J* = 6.4 Hz, 1H, H-5$_\alpha$), 4.04 (dd, *J* = 9.8, 3.6 Hz, 1H, H-2$_\alpha$), 3.99 – 3.85 (m, 3H, H-3$_\alpha$, H-4$_\alpha$,H-5$_\beta$), 3.77 (dd, *J* = 9.6, 7.4 Hz, 1H, H-2$_\beta$), 3.62 – 3.50 (m, 3H, H-3$_\beta$, H-4$_\beta$, H-6a$_\beta$), 3.53 – 3.35 (m, 5H, H-6b$_\beta$, H-6a$_\alpha$, H-6b$_\alpha$, 2 OC*H*H), 3.37 – 3.27 (m, 2H, 2 OC*H*H), 3.25 (*t*, J = 6.9 Hz, 4H, 2 CH_2N_3), 1.66 – 1.47 (m, 8H, 4 CH_2), 1.43 – 1.23 (m, 8H, 4 CH_2); ^{13}C NMR (101 MHz, chloroform-*d*) *δ* 138.7, 138.7, 138.5, 138.3, 128.5, 128.5, 128.4, 128.4, 128.3, 128.1, 128.0, 127.8, 127.8, 127.7, 127.7, 127.6 (C_{Ar}), 97.9(C-1$_\beta$), 92.1(C-1$_\alpha$), 82.3 (C-3$_\beta$), 80.9(C-2$_\beta$), 78.9 (C-3$_\alpha$), 76.8 (C-2 $_\alpha$), 75.2 (CH_2Ph), 74.9 (C-4$_\alpha$), 74.8 (CH_2Ph), 74.7 (CH_2Ph), 73.8 (C-4$_\beta$), 73.7 (C-5$_\beta$), 73.8 (CH_2Ph), 73.1 (CH_2Ph), 73.1 (CH_2Ph), 71.5 ($OCH_{2\beta}$), 71.5($OCH_{2\alpha}$), 69.7 (C-5$_\alpha$), 69.6 (C-6$_\alpha$), 69.6 (C-6$_\beta$), 51.5 (CH_2N_3), 29.5, 28.9, 26.7, 25.8 (CH_2). All analytical data were consistent with literature values.[5]

6-(6'-Azidohexyl)-2,3,4-*tri*-*O*-benzyl-*β*-D-galactopyranosyl *N*-phenyl trifluoroacetimidate (6)

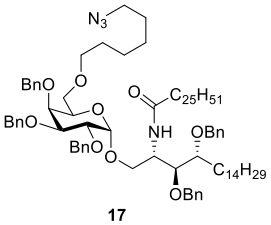


6

Compound **16** (1.45 g, 2.53 mmol) was dissolved in anhydrous CH_2Cl_2 (25 mL) and 2,2,2-trifluoro-*N*-phenylacetimidoyl chloride (0.8 mL, 5.0 mmol and Cs_2CO_3 (1.63 g, 5.0 mmol) were added to the solution. The reaction mixture was stirred at room temperature for 2 hours, after which it was filtered through a Celite pad. The solvent was removed *in vacuo* and the resulting crude was purified by flash column chromatography (toluene/EtOAc, 9:1, v/v) to yield compound **6** (1.56 g, 2.16 mmol, 85%) as a white solid. R_f = 0.58 (toluene/EtOAc, 9:1, v/v); ^{1}H NMR (400 MHz, chloroform-*d*) *δ* 7.35 – 7.13 (m, 20H, H_{Ar}), 7.00 (t, *J* = 7.4 Hz, 1H, H_{Ar}), 6.70 (d, *J* = 7.8 Hz, 2H, H_{Ar}), 5.56 (s, 1H, H-1), 4.89 (d, *J* = 11.5 Hz, 1H, C*H*HPh), 4.75 (d, *J* = 1.8 Hz, 2H, C*H*$_2$Ph), 4.67 (s, 2H, C*H*$_2$Ph), 4.58 (d, *J* = 11.6 Hz, 1H, C*H*HPh), 3.99 (t, *J* = 8.9 Hz, 1H, H-5), 3.84 (d, *J* = 2.9 Hz, 1H, H-2), 3.65 – 3.37 (m, 4H, H-3, H-4, H-6a, H-6b), 3.38 – 3.30 (m, 1H, OC*H*H), 3.23 (dt, *J* = 9.3, 6.5 Hz, 1H, OC*H*H), 3.14 (t, *J* = 6.9 Hz, 2H, CH_2N_3), 1.53 – 1.36 (m, 4H, 2 CH_2), 1.26 (dq, *J* = 12.4, 4.2, 3.6 Hz, 4 CH_2); ^{13}C NMR (101 MHz, chloroform-*d*) *δ* 143.7, 138.6, 138.3, 138.1, 128.8, 128.6, 128.5, 128.4, 128.4, 128.3, 128.0, 127.9, 127.8, 127.7, 124.3, 119.4 (C_{Ar}), 97.5 (C-1), 82.2 (C-3, C-4), 78.3 (C-5), 75.7 (CH_2Ph),

74.9 (CH_2Ph), 73.4 (C-2), 73.1 (CH_2Ph), 71.4 (OCH_2), 68.7 (C-6), 51.5 (CH_2N_3), 29.7 (CH_2), 28.9 (CH_2), 26.7 (CH_2), 25.8 (CH_2). All analytical data were consistent with literature values.[5]

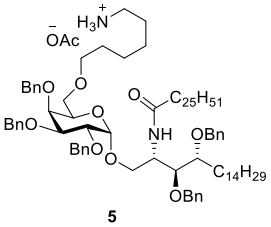
(*2S,3S,4R*)-3,4-Bis-benzyloxyl-2-hexacosanoylamino-1-(6-(6'-azidohexyl)-2,3,4-tri-*O*-benzyl)-D-galactopyranosyl)octadecane (17)



17

Acceptor **7** (180 mg, 0.205 mmol) and donor **6** (229 mg, 0.307 mmol) were dissolved in anhydrous THF (3 mL) and Et_2O (18 mL) and stirred with 4 Å MS for 30 minutes at –20 °C. TMSOTf (10.5 µL, 0.061 mmol) was added to the mixture at –20 °C. After 1 hour the complete consumption of the acceptor was observed by TLC analysis (toluene/EtOAc, 9:1), the reaction mixture was neutralized with Et_3N and filtered through a Celite pad. The solution was concentrated *in vacuo* and the crude was purified by flash column chromatography (toluene/EtOAc, 95:5 → 9:1, v/v) to yield product **17** (214 mg, 0.146 mmol, 72%) as a white amorphous solid. R_f = 0.38 (toluene/EtOAc, 9:1, v/v); $[\alpha]_D^{20}$ = +24.6 (*c* 1.0, $CHCl_3$); ^{1}H NMR (400 MHz, chloroform-*d*) δ 7.40 – 7.22 (m, 25H, H_{Ar}), 6.16 (d, *J* = 8.5 Hz, 1H, NH), 4.96 (d, *J* = 11.5 Hz, 1H, C*H*HPh), 4.85 (d, *J* = 3.7 Hz, 1H, H-1), 4.84 – 4.73 (m, 4H, 2 CH_2Ph), 4.69 – 4.57 (m, 3H, CH*H*Ph, C*H*HPh, C*H*HPh), 4.52 (d, *J* = 11.5 Hz, 1H, CH*H*Ph), 4.44 (d, *J* = 11.6 Hz, 1H, CH*H*Ph), 4.15 (tq, *J* = 8.0, 3.9, 3.3 Hz, 1H, H-2_{Sph}), 4.06 (dd, *J* = 9.9, 3.6 Hz, 1H, H-2), 3.99 (dd, *J* = 10.9, 5.4 Hz, 1H, H-1_{Sph}), 3.95 – 3.91 (m, 2H, H-4, H-3), 3.90 – 3.85 (m, 2H, H-5, H-3_{Sph}), 3.75 (dd, *J* = 10.9, 3.9 Hz, 1H, H-1_{Sph}), 3.50 (dt, *J* = 8.6, 3.1 Hz, 1H, H-4_{Sph}), 3.40 (dtd, *J* = 13.5, 9.5, 6.5 Hz, 3H, H-6a, H-6b, OC*H*H), 3.33 – 3.27 (m, 1H, OCH*H*), 3.22 (t, *J* = 6.9 Hz, 2H; CH_2N_3), 2.04 – 1.88 (m, 2H, CH_2), 1.69 – 1.41 (m, 10H, CH_2), 1.37 – 1.18 (m, 70H, CH_2), 0.89 (t, *J* = 6.7 Hz, 6H, 2 CH_3); ^{13}C NMR (101 MHz, chloroform-*d*) δ 172.9 (CO), 138.8, 138.8, 138.7, 138.5 (C_{Ar}), 128.5, 128.4, 128.3, 128.0, 127.9, 127.8, 127.7, 127.6 (C_{Ar}), 99.6 (C-1), 80.3 (C-4_{Sph}), 79.1 (C-3), 78.7 (C-3_{Sph}), 76.7 (C-2), 74.9, 74.9 (CH_2Ph, C-4), 73.7 (CH_2Ph), 73.6 (CH_2Ph), 73.0 (CH_2Ph), 71.9 (CH_2Ph), 71.6 (OCH_2), 69.9 (C-5), 69.6 (C-6), 69.2 (C-1_{Sph}), 51.5 (CH_2N_3), 50.5 (C-2_{Sph}), 36.8, 32.1, 30.0, 29.9, 29.8, 29.7, 29.6, 29.5, 28.92, 26.7, 26.23, 25.9, 25.8, 22.8 (CH_2), 14.3 (2 CH_3). HRMS MALDI-TOF m/z: calcd. for $C_{91}H_{140}N_4O_9$: 1472.0252 $[M+K]^+$; found: 1472.0262.

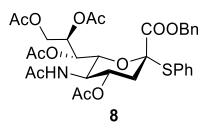
(*2S,3S,4R*)-3,4-Bis-benzyloxyl-2-hexacosanoylamino-1-(6-(6'-aminohexyl)-2,3,4-tri-*O*-benzyl)-D-galactopyranosyl)octadecane (5)



5

Compound **17** (155 mg, 0.108 mmol) was dissolved in CH_2Cl_2 (7.7 mL) and AcOH (77 µL) was added followed by Zn powder (424 mg, 6.5 mmol). The reaction was stirred at room temperature for 45 minutes, filtered over Celite, and concentrated *in vacuo* to give **5** (101 mg, 0.072 mmol, 67%) as a white powder. The compound was used directly in the coupling with either **3** or **4**.

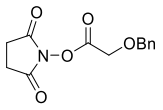
Benzyl (phenyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-2-thio-β-D-glycero-D-galacto-2-nonulopyranosid)onate (8)



8

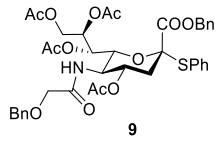
N-Acetylneuraminic acid (5 g, 16.2 mmol) was suspended in H_2O and Cs_2CO_3 (2.6 g, 8.1 mmol) was added until pH = 7. The solvent was removed *in vacuo* and the crude product was dried under vacuum before dissolving in DMF (6 mL). BnBr (1.2 mL, 10.1 mmol) was then added and the reaction was stirred overnight at room temperature. The mixture was filtered under suction and the filtrate was poured into 200 mL of CH_2Cl_2 to precipitate a white solid, which was filtered and dried under vacuum. To a suspension of the crude residue in pyridine (20 mL) was slowly added acetic anhydride (10 mL) and the reaction was stirred overnight at room temperature. The solvents were removed *in vacuo* and the crude residue was dissolved in EtOAc and washed with 1 M aq. HCl, then sat. aq. $NaHCO_3$, and finally with brine. The combined organic layers were dried over Na_2SO_4, filtered, and concentrated *in vacuo*. The crude residue was purified by flash column chromatography (toluene:acetone, 1:1, v/v) to afford benzyl (5-acetamido-2,4,7,8,9-penta-*O*-acetyl-3,5-dideoxy-2-thio-β-D-glycero-D-galacto-2-nonulopyranoside)onate (4.5 g, 7.4 mmol, 46% over three steps) as a white foam; ^{1}H NMR (400 MHz, chloroform-*d*) δ 7.41 – 7.29 (m, 5H, H_{Ar}), 5.37 (d, *J* = 5.2 Hz, 1H, H-7), 5.33 – 5.19 (m, 2H, H-4, C*H*HPh), 5.16 (d, *J* = 12.2 Hz, 1H, CH*H*Ph), 5.12 – 5.02 (m, 1H, H-8), 4.44 (dd, *J* = 12.5, 2.6 Hz, 1H, H-9a), 4.18 – 4.06 (m, 3H, H-9b, H-5. H-6), 2.55 (dd, *J* = 13.5, 4.5 Hz, 1H, H-3a), 2.14 – 2.05 (m, 7H, H-3b, 2 CH_3), 2.04 – 1.99 (m, 9H, 3 CH_3), 1.89 (s, 3H, CH_3); ^{13}C NMR (101 MHz, chloroform-*d*) δ 171.1, 170.7, 170.4 (2C), 170.3, 168.4, 165.6 (7 CO), 135.0 (C_{Ar}), 129.2, 128.8, 128.7, 128.6, 128.5, 128.4, 128.4 (C_{Ar}), 97.8 (C-2), 73.0 (C-6), 71.3 (C-8), 68.4 (C-4), 68.1 (CH_2Ph), 67.9 (C-7), 62.1 (C-9), 49.5 (C-5), 35.9 (C-3), 23.4 (NHCO*C*H_3), 21.6, 21.0, 20.9, 20.9, 20.9 (5 CH_3). All analytical data were consistent with literature values.[6] Benzyl (5-acetamido-2,4,7,8,9-penta-*O*-acetyl-3,5-dideoxy-2-thio-β-D-glycero-D-galacto-2-nonulopyranoside)onate (5.4 g, 8.8 mmol) was dissolved in acetyl chloride (17 mL), then the solution was cooled to 0 °C and MeOH anhydrous (3.6 mL, 88.3 mmol) was slowly added. The mixture was stirred at room temperature overnight, then the solvent was removed *in vacuo*. The crude residue was purified by flash column chromatography (toluene:acetone, 7:3, v/v) to yield the corresponding sialyl chloride (4.9 g, 8.3 mmol, 94%) as a white foam. The sialyl chloride (4.9 g, 8.3 mmol) was dissolved in CH_2Cl_2 (55 mL) and reacted with thiophenol (1.3 mL, 12.4 mmol) in the presence of DIPEA (2.2 mL, 12.4 mmol). The reaction was stirred overnight at room temperature, then the solvent was removed *in vacuo*. The obtained crude oil was dissolved in EtOAc and washed with sat. NaHCO3 solution and then brine. The combined organic layers were dried over Na_2SO_4, filtered, and concentrated *in vacuo*. Purification by flash column chromatography (toluene:acetone, 7:3, v/v) afforded **8** (4.7 g, 7.2 mmol, 87%) as a white foam. R_f = 0.53 (toluene/acetone, 1:1); $[\alpha]_D^{20}$ = +11.3 (*c* 1.0, $CHCl_3$); ^{1}H NMR (400 MHz, chloroform-*d*) δ 7.50 – 7.32 (m, 5H, H_{Ar}), 5.31 (dd, *J* = 6.6, 2.0 Hz, 1H, H-7), 5.19 (td, *J* = 6.2, 2.7 Hz, 1H, H-8), 5.02 (s, 2H, CH_2Ph), 4.84 (ddd, *J* = 11.7, 10.1, 4.7 Hz, 1H, H-4), 4.39 (dd, *J* = 12.4, 2.7 Hz, 1H, H-9a), 4.22 (dd, *J* = 12.4, 5.9 Hz, 1H, H-9b), 4.08 – 3.97 (m, 1H, H-5), 3.93 (dd, *J* = 10.7, 1.9 Hz, 1H, H-6), 2.87 (dd, *J* = 12.9, 4.6 Hz, 1H, H-3), 2.14 (s, 3H, CH_3), 2.10 – 2.03 (m, 7H, 2 CH_3, H-3b), 2.02 (d, *J* = 1.7 Hz, 3H, CH_3), 1.85 (s, 3H, NHCO*C*H_3); ^{13}C NMR (101 MHz, chloroform-*d*) δ 170.8, 170.7, 170.3, 170.2, 170.1, 167.3 (6 CO), 136.5, 134.8, 129.8, 129.1, 128.9, 128.7, 128.6, 128.5, 128.3 (C_{Ar}), 87.3 (C-2), 74.9 (C-6), 70.4 (C-8), 69.8 (C-4), 67.9, 67.8 (CH_2Ph, C-7), 62.1 (C-9), 49.2 (C-5), 38.3 (C-3), 23.2 (NHCO*C*H_3), 21.0, 20.9, 20.8 (4 CH_3). HRMS (ESI-TOF) m/z: calcd. for $C_{32}H_{37}NO_{12}S$: 660.2109 $[M+H]^+$; found: 660.2117.

_O_-Benzylglycolic acid succinimidyl ester



2-Benzyloxy acetic acid (1.0 g, 6.0 mmol) was dissolved in anhydrous THF (23.4 mL) and reacted with *N*-hydroxysuccinimide (0.83 g, 7.2 mmol) and DCC (1.48 g, 7.2 mmol) at 0 °C for 1 hour. The suspension was refrigerated for 16 hours and the precipitated solid was filtered off. The filtrate was concentrated *in vacuo* to yield the crude product (1.5 g, 5.7 mmol, 95%), which was used without further purification.

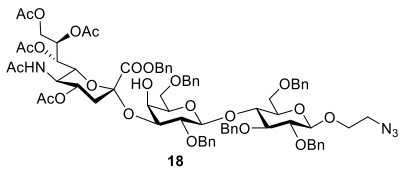
Benzyl (phenyl 4,7,8,9-tetra-*O*-acetyl-5-(2-benzyloxy)acetamido-3,5-dideoxy-2-thio-β-D-glycero-D-galacto-2-nonulopyranosid)onate (9)



9

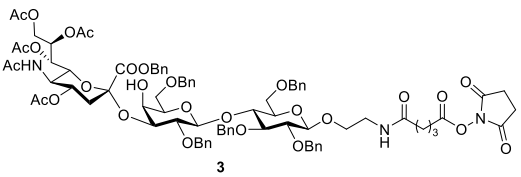
To a solution of compound **8** (3 g, 4.5 mmol) in anhydrous MeOH (90 mL) was added methanesulfonic acid (1.5 mL, 23 mmol) and the reaction was stirred overnight at 75 °C in the darkness. The reaction was neutralized by the addition of Et_3N until pH = 7, then it was concentrated *in vacuo*. The crude residue was dissolved in CH_3CN:H_2O (50 mL, 15:1, v/v) and reacted with *O*-benzylglycolic acid succinimidyl ester (1.6 g, 6.0 mmol) and Et_3N (1.3 mL, 9.5 mmol) at room temperature for 2 hours. The solvent was removed *in vacuo* and the crude residue was dried *in vacuo* before using directly in the next step. The crude compound was dissolved in pyridine (20 mL) and acetic anhydride (10 mL) and the reaction was stirred overnight at room temperature. Solvents were then removed *in vacuo* and the crude residue was purified by flash column chromatography (toluene:acetone, 7:3, v/v) to afford **9** (1.9 g, 2.5 mmol, 56% over three steps) as a white foam. R_f = 0.46 (toluene/acetone, 7:3); $[\alpha]_D^{20}$ = –6.0 (*c* 1.0, $CHCl_3$); ^{1}H NMR (400 MHz, chloroform-*d*) δ 7.45 – 7.26 (m, 15H, H_{Ar}), 6.30 (d, *J* = 10.2 Hz, 1H, NH), 5.32 (dd, *J* = 7.5, 1.9 Hz, 1H, H-7), 5.21 (ddd, *J* = 7.7, 5.2, 2.7 Hz, 1H, H-8), 5.02 (d, *J* = 1.9 Hz, 2H, CH_2Ph), 4.86 (ddd, *J* = 11.6, 10.1, 4.6 Hz, 1H, H-4), 4.59 (d, *J* = 11.8 Hz, 1H, C*H*HPh), 4.52 (d, *J* = 11.7 Hz, 1H, CH*H*Ph), 4.36 (dd, *J* = 12.5, 2.7 Hz, 1H, H-9a), 4.19 (dd, *J* = 12.5, 5.2 Hz, 1H, H-9b), 4.05 (*a*q, *J* = 10.3 Hz, 1H, H-5), 3.96 (dd, *J* = 10.7, 1.9 Hz, 1H, H-6), 3.90 (d, *J* = 15.4 Hz, 1H, COC*H*H), 3.83 (d, *J* = 15.5 Hz, 1H, COCH*H*), 2.90 (dd, *J* = 12.9, 4.7 Hz, 1H, H-3a), 2.13 (s, 3H, CH_3), 2.04 (s, 3H, CH_3), 2.01 (s, 4H, H-3b, CH_3), 1.99 (s, 3H, CH_3); ^{13}C NMR (101 MHz, chloroform-*d*) δ 170.8 (CO), 170.4 (CO), 170.3 (CO), 170.2 (CO), 170.1 (CO), 167.3 (CO), 136.9, 136.5, 134.9, 129.9, 129.2, 129.0, 128.8, 128.7, 128.6, 128.5, 128.4, 128.2, 125.4, 87.3 (C-2), 74.7 (C-6), 73.6 ($COCH_2OCH_2$Ph), 69.8 (C-8), 69.7 (C-4), 69.3 (CH_2Ph), 68.0 ($COCH_2OCH_2$Ph), 67.6 (C-7), 62.0 (C-9), 48.4 (C-5), 38.5 (C-3), 21.1, 21.0, 20.9 (4 CH_3). HRMS (ESI-TOF) m/z: calcd. for $C_{39}H_{43}NO_{13}S$: 766.2535 $[M+H]^+$; found: 766.2497.

2-Azidoethyl (benzyl 5-acetamido-7,8,9-tri-*O*-acetyl-3,5-dideoxy-D-glycero-α-D-galacto-non-2-ulopyranosylonate)-(2→3)-(2,6-di-*O*-benzyl-β-D-galactopyranosyl)-(1→4)-2,3,6-tri-*O*-benzyl-β-D-glucopyranoside (18)


18

Acceptor **10** (200 mg, 0.232 mmol) and donor **8** (459 mg, 0.697 mmol) were dissolved in anhydrous CH_3CN (20 mL) and CH_2Cl_2 (5 mL), and stirred with 3 Å MS for 30 minutes at room temperature. After cooling down to −78 °C, a solution AgOTf (239 mg, 0.930 mmol) in anhydrous CH_3CN (5 mL) was added. The mixture was stirred for 1 hour at −78 °C. Then IBr (0.697 mL, 1 M in CH_2Cl_2, 0.697 mmol) was added dropwise. The reaction mixture was stirred for another 2 hours and slowly warmed up to −50 °C over this period. After complete consumption of acceptor was observed by TLC analysis (toluene/acetone, 7:3), the reaction was neutralized with DIPEA, filtered through a Celite pad, and concentrated *in vacuo*. The crude was dissolved in CH_2Cl_2, washed with 10% $Na_2S_2O_3$, and brine, dried over $MgSO_4$, filtered, and concentrated. The resulting crude was purified by flash column chromatography (toluene/ acetone, 95:5 → 7:3, v/v) to afford **18** (280 mg, 0.198 mmol, 85%) as a white foam. R_f = 0.44 (toluene/acetone, 7:3); $[\alpha]_D^{20}$ = +5.3 (*c* 1.0, $CHCl_3$); ^{1}H NMR (400 MHz, chloroform-*d*) *δ* 7.50 – 7.24 (m, 30H, H_{Ar}), 5.42 (ddd, *J* = 8.4, 6.1, 2.6 Hz, 1H, H-8''), 5.35 (dd, *J* = 7.8, 2.2 Hz, 1H, H-7''), 5.26 (d, *J* = 12.1 Hz, 1H, C*H*HPh), 5.19 (d, *J* = 10.0 Hz, 1H, NH), 5.14 (d, *J* = 12.1 Hz, 1H, C*H*HPh), 5.03 (d, *J* = 10.8 Hz, 1H, C*H*HPh), 4.94 (d, *J* = 11.0 Hz, 1H, C*H*HPh), 4.86 (ddd, *J* = 12.1, 10.2, 4.6 Hz, 1H, H-4''), 4.82 – 4.74 (m, 3H, 3 C*H*HPh), 4.67 (d, *J* = 11.8 Hz, 1H, C*H*HPh), 4.61 (d, *J* = 7.7 Hz, 1H, H-1'), 4.55 – 4.46 (m, 3H, 3 C*H*HPh), 4.43 (d, *J* = 7.8 Hz, 1H, H-1), 4.40 – 4.33 (m, 2H, C*H*HPh, H-9''a), 4.18 – 3.96 (m, 6H, H-5'', H-3, H-6a, H-6'', H-4, H-9''b), 3.85 (d, *J* = 3.4 Hz, 1H, H-4'), 3.79 – 3.65 (m, 4H, OCH_2, H-6b, H-6'a), 3.63 – 3.38 (m, 8H, H-3, H-2', H-5, H-6'b, CH_2N_3, H-2, H-5), 2.59 (dd, *J* = 13.0, 4.6 Hz, 1H, H-3''a), 2.14 (s, 3H, CH_3), 2.12 – 2.03 (m, 4H, CH_3, H-3''b), 2.01 (s, 3H, CH_3), 1.92 (s, 3H, CH_3), 1.90 (s, 3H, CH_3); ^{13}C NMR (101 MHz, chloroform-*d*) *δ* 170.7, 170.7, 170.4, 170.2, 170.0, 167.8 (CO), 139.2, 139.0, 138.7, 138.5, 138.5, 134.5 (C_{Ar}), 128.9, 128.6, 128.4, 128.3, 128.2, 128.2, 128.1, 127.7, 127.6, 127.6, 127.5, 127.3, 127.3 (C_{Ar}), 103.6 (C-1), 102.4 (C-1'), 98.6 (C-2''), 82.9 (C-3), 81.9 (C-2), 78.5 (C-2'), 76.5 (C-3'), 76.5 (C-4), 75.4 (CH_2Ph), 75.2 (C-5), 75.1 (CH_2Ph), 75.0 (CH_2Ph), 73.4 (CH_2Ph), 73.1 (CH_2Ph), 72.9 (C-6''), 72.6 (C-5'), 69.1 (C-4''), 69.0 (C-8')', 68.7 (C-6'), 68.6 (OCH_2), 68.2 (C-6), 68.2 (CH_2Ph), 68.0 (C-4'), 67.3 (C-7''), 62.4 (C-9''), 51.1 (CH_2N_3), 49.2 (C-5''), 36.5 (C-3''), 23.2, 21.3, 20.9, 20.8, 20.6 (CH_3). HRMS (ESI-TOF) m/z: calcd. for $C_{75}H_{86}N_4O_{23}$: 1433.5575 $[M+Na]^+$; found: 1433.5599.

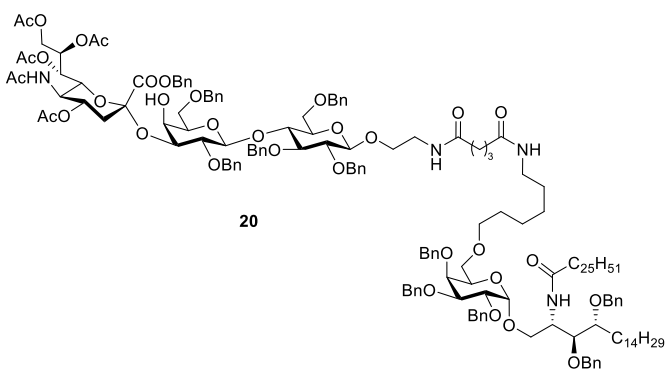
2'-*N*-[4-(Succinimidooxycarbonyl)butan-1-oyl]-aminoethyl-(benzyl 5-acetamido-7,8,9-tri-*O*-acetyl-3,5-dideoxy-D-glycero-α-D-galacto-non-2-ulopyranosylonate)-(2→3)-(2,6-di-*O*-benzyl-β-D-galactopyranosyl)-(1→4)-2,3,6-tri-*O*-benzyl-β-D-glucopyranoside (3)


3

Compound **18** (110 mg, 78.0 µmmol) was dissolved in anhydrous CH_2Cl_2 (7 mL), followed by addition of Zn powder (304 mg, 4.67 mmol) an AcOH (70 µL). The reaction mixture was stirred at room temperature for 1 hour, then it was filtered through a Celite pad. The filtrate was concentrated, dried under vacuum for 2 hours and dissolved in anhydrous DMF (7 mL). Disuccinimidyl glutarate (27.9 mg, 85.6 µmmol) was added to the solution and the reaction mixture was stirred for 1 hour at room temperature. After removal of the solvents, the crude residue was purified by flash column chromatography (toluene/acetone, 9:1 → 6:4, v/v) to afford compound **3** (98 mg, 61.4 µmmol, 79% over 2 steps) as a white amorphous solid. R_f = 0.35 (toluene/acetone, 6:4); $[\alpha]_D^{20}$ = +4.0 (*c* 1.0, $CHCl_3$); ^{1}H NMR

(400 MHz, chloroform-*d*) δ 7.43 – 7.09 (m, 30H, H_{Ar}), 6.41 (t, *J* = 5.6 Hz, 1H, CH_2NHCO), 5.31 (ddd, *J* = 8.5, 6.1, 2.6 Hz, 1H, H-8''), 5.22 (dd, *J* = 7.9, 2.1 Hz, 1H, H-7''), 5.18 – 5.07 (m, 2H, C*H*HPh, NH), 5.02 (d, *J* = 12.1 Hz, 1H, C*H*HPh), 4.92 (d, *J* = 10.9 Hz, 1H, C*H*HPh), 4.80 – 4.61 (m, 5H, H-4'', 4 C*H*HPh), 4.54 (d, *J* = 11.8 Hz, 1H, C*H*HPh), 4.49 (d, *J* = 7.7 Hz, 1H, H-1'), 4.40 – 4.29 (m, 3H, 3×C*H*HPh), 4.30 – 4.19 (m, 3H, H-1, C*H*HPh, H-9''a), 4.07 – 3.87 (m, 3H, H-5'', H-3', H-6''), 3.90 – 3.78 (m, 2H, H-9''b, H-4), 3.81 – 3.63 (m, 4H, H-6a, H-4', H-6b, OC*H*H), 3.63 – 3.51 (m, 2H, OC*H*H, H-6'a), 3.54 – 3.26 (m, 8H, H-3, H-2', H-5', CH_2NH, H-6'b, H-5, H-2), 2.68 – 2.58 (m, 4H, 2 CH_2), 2.54 – 2.39 (m, 3H, H-3''a, CH_2), 2.02 (s, 3H, CH_3), 2.00 – 1.82 (m, 11H, H-3''b, CH_2, 2CH_3, CH_2), 1.78 (s, 3H, CH_3), 1.78 (s, 3H, CH_3); ^{13}C NMR (101 MHz, chloroform-*d*) *δ* 171.9, 170.7×2, 170.4, 170.2×2, 170.0, 169.3, 168.4, 167.8 (CO), 139.1, 139.0, 138.6, 138.4, 138.1, 134.5 (C_{Ar}), 128.9, 128.9, 128.6, 128.5, 128.4, 128.4, 128.2, 128.2, 128.1, 128.0, 127.9, 127.8, 127.8, 127.6, 127.6, 127.6, 127.4, 127.4, 127.3 (C_{Ar}), 103.9 (C-1), 102.5 (C-1'), 98.5 (C-2''), 83.0 (C-3), 82.0 (C-2), 78.6 (C-2'), 76.8 (C-4), 76.5 (C-3'), 75.4 (CH_2Ph), 75.1 (CH_2Ph), 75.0 (CH_2Ph), 74.7 (C-5), 73.4 (CH_2Ph), 73.2 (CH_2Ph), 72.9 (C-6''), 72.7 (C-5'), 70.0 (C-6), 69.1 (C-4''), 68.9 (OCH_2), 68.9 (C-8''), 68.8 (C-6'), 68.2 (CH_2Ph), 68.1 (C-4'), 67.3 (C-7''), 62.4 (C-9''), 49.2 (C-5''), 40.1 (CH_2N_3), 36.6 (C-3''), 34.1(CH_2), 30.1 (CH_2), 25.6 (2 CH_2), 23.3 (CH_3), 21.3 (CH_3), 20.9 (CH_3), 20.8(CH_3), 20.6(CH_2), 20.6(CH_3). HRMS (ESI-TOF) m/z: calcd. for $C_{84}H_{97}N_3O_{28}$: 1618.6151 $[M+Na]^+$; found: 1618.6170.

Protected GM3–αGalCer conjugate (20)

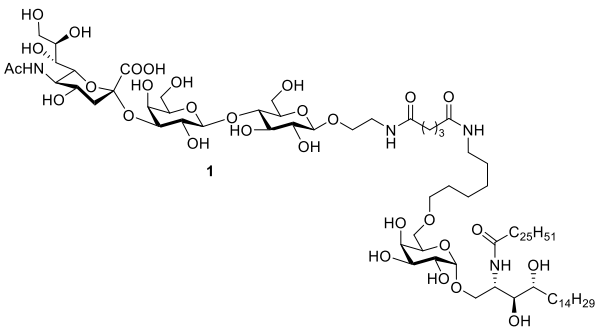


20

The spacer-equipped GM3 glycoside **3** (51 mg, 0.032 mmol) and αGalCer amine **5** (54 mg, 0.038 mmol) were dissolved in anhydrous DMF (4 mL), followed by addition of 2 drops of Et_3N. The reaction mixture was stirred for 4 hours at room temperature. After removal of solvent, the resulting crude was purified by flash column chromatography (toluene/acetone, 6:4, v/v) to afford compound **20** (68 mg, 23.8 μmmol, 74%) as white amorphous solid. R_f = 0.48 (toluene/acetone, 6:4); $[\alpha]_D^{20}$ = +10.4 (*c* 1.0, $CHCl_3$); ^{1}H NMR (800 MHz, chloroform-*d*) *δ* 7.34 – 7.10 (m, 55H, H_{Ar}), 6.14 (d, *J* = 8.5 Hz, 1H, NH_{Sph}), 5.32 (ddd, *J* = 8.5, 6.1, 2.7 Hz, 1H, H-8''), 5.23 (dd, *J* = 7.9, 2.3 Hz, 1H, H-7''), 5.14 (dd, *J* = 12.0, 3.9 Hz, 1H, CH*H*Ph), 5.08 – 5.00 (m, 2H, N*H*Ac, CH*H*Ph), 4.93 (d, *J* = 10.8 Hz, 1H, CH*H*Ph), 4.87 (d, *J* = 11.5 Hz, 1H, CH*H*Ph), 4.78 (d, *J* = 3.7 Hz, 1H, H-$1_{αGal}$), 4.77 – 4.73 (m, 1H, H-4''), 4.73 – 4.66 (m, 9H, 8 CH*H*Ph, NH), 4.58 – 4.50 (m, 5H, 5 CH*H*Ph), 4.46 (d, *J* = 7.7 Hz, 1H, H-1'), 4.44 (d, *J* = 11.5 Hz, 1H, CH*H*Ph), 4.36 (d, *J* = 12.0 Hz, 2H, 2 CH*H*Ph), 4.31 – 4.25 (m, 2H, 2 CH*H*Ph), 4.25 – 4.21 (m, 2H, H-9''a, H-1), 4.08 (tt, *J* = 8.6, 4.6 Hz, 1H, H-2_{Sph}), 4.03 – 3.96 (m, 3H, H-5'', H-3', H-$2_{αGal}$), 3.92 (dd, *J* = 10.7, 2.4 Hz, 1H, H-6''), 3.90 – 3.77 (m, 7H, H-1_{Sph}a, H-$4_{αGal}$, H-9''b, H-$3_{αGal}$, H-4, H-$5_{αGal}$, H-3_{Sph}), 3.74 – 3.64 (m, 4H, OCH_2, H-6, H-1_{Sph}b), 3.58 (dd, *J* = 10.6, 5.9 Hz, 1H, H-6b), 3.55 (dd, *J* = 9.4, 6.3 Hz, 1H, H-6'b), 3.49 (t, *J* = 8.9 Hz, 1H, H-3), 3.46 (dd, *J* = 9.4, 7.8 Hz, 1H, H-2'), 3.43 – 3.36 (m, 4H, H-4_{Sph}, H-5', CH*H*NH, H-6'b), 3.35 – 3.25 (m, 6H, H-6a, H-$6_{αGal}$, H-5, H-2, OCH*H*, CH*H*NH), 3.19 (dt, *J* = 9.5, 6.8 Hz, 1H, OCH*H*), 3.17 – 3.08 (m, 2H, CH_2NH), 2.49 (dd, *J* = 13.0, 4.6 Hz, 1H, H-3''a), 2.02 (s, 3H, CH_3), 1.96 (t, *J* = 12.5 Hz, 1H, H-3''b), 1.93 (s, 3H, CH_3), 1.94 – 1.89 (m, 9H, 2 CH_3, CH_2, CH*H*), 1.89 – 1.83 (m, 1H, CH*H*), 1.79 (s, 3H, CH_3), 1.78 (s, 3H, CH_3), 1.67 (td, *J* = 12.4, 10.8, 5.4 Hz, 2H, CH_2), 1.59 (dtd, *J* = 14.2, 9.4, 4.8 Hz, 1H, CH*H*), 1.51 (tdt, *J* = 9.5, 5.9, 3.3 Hz, 1H, CH*H*), 1.48 – 1.34 (m, 7H, CH_2, CH*H*), 1.26 – 1.07 (m, 74H, CH_2), 0.81 (t, *J* = 7.1 Hz, 6H, 2 CH_3); ^{13}C NMR (201 MHz, chloroform-*d*) *δ* 173.0, 170.7 (2C), 170.7 (2C), 170.4, 170.2, 170.0, 167.8 (CO),

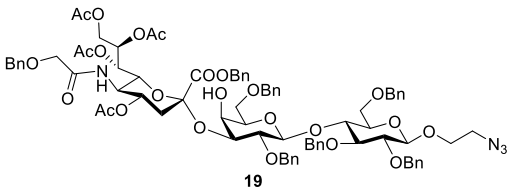
139.1, 139.0, 138.8, 138.8, 138.8, 138.7, 138.6, 138.6, 138.4, 137.9, 134.5 (C_{Ar}), 129.0, 128.9, 128.7, 128.6, 128.5, 128.5, 128.5, 128.5, 128.4, 128.4, 128.3, 128.3, 128.3, 128.3, 128.2, 128.2, 128.1, 128.0, 128.0, 127.9, 127.9, 127.9, 127.8, 127.8, 127.7, 127.7, 127.6, 127.6, 127.6, 127.5, 127.5, 127.4 (C_{Ar}), 103.9 (C-1), 102.6 (C-1'), 99.5 (C-$1_{\alpha Gal}$), 98.5 (C-2''), 82.9 (C-3), 81.9 (C-2), 80.3 (C-4_{Sph}), 79.1 (C-$3_{\alpha Gal}$), 78.7 (C-3_{Sph}), 78.6 (C-2'), 77.0 (C-4), 76.8 (C-$2_{\alpha Gal}$), 76.5 (C-3'), 75.5 (CH_2Ph), 75.2 (CH_2Ph), 75.1 (CH_2Ph), 75.1 (CH_2Ph), 74.9 (C-$4_{\alpha Gal}$), 74.9 (C-5), 74.8 (CH_2Ph), 73.7 (CH_2Ph), 73.7 (CH_2Ph), 73.5 (CH_2Ph), 73.3 (CH_2Ph), 73.2 (CH_2Ph), 72.9 (2C, C-6'', CH_2Ph), 72.7 (C-5'), 71.8 (CH_2Ph), 71.6 (OCH_2), 70.0 (C-$5_{\alpha Gal}$), 69.8 (OCH_2), 69.5 (C-$6_{\alpha Gal}$), 69.1 (C-4''), 69.1 (C-6), 69.0 (C-1_{Sph}), 68.9 (C-8''), 68.8 (C-6'), 68.3 (CH_2Ph), 68.1 (C-4'), 67.3 (C-7''), 62.4 (C-9''), 50.4 (C-2_{Sph}), 49.3 (C-5''), 40.4 (CH_2NH), 40.2 (CH_2NH), 36.8 (CH_2), 36.7 (C-3), 34.2, 32.1, 31.6, 30.3, 30.0, 29.9, 29.9, 29.8, 29.8, 29.8, 29.6, 29.6, 29.6, 29.5, 29.5, 29.3, 26.9, 26.2, 25.9, 25.8 (CH_2), 23.3 (CH_3), 22.8, 21.7 (CH_2), 21.3, 20.9, 20.9, 20.6, 14.3, 14.3 (CH_3). HRMS (MALDI-TOF) m/z: calcd. for $C_{171}H_{234}N_4O_{34}$: 2888.6778 $[M+H]^+$; found: 2888.6812.

GM3–αGalCer conjugate (1)



The protected conjugate **20** (40 mg, 13.8 µmmol) was mixed with Pd/C (5% wt, 30 mg) in EtOH (2 mL) and AcOH (0.2 mL). The reaction mixture was stirred under a H_2 atmosphere at room temperature overnight. The mixture was then filtered over a Celite pad and concentrated *in vacuo*. The resulting crude was dissolved in anhydrous MeOH, followed by addition of MeONa until pH reached 9–10. The reaction mixture was then kept stirring at room temperature overnight, then H_2O (0.2 mL) was added. After stirring for another 15 minutes, the mixture was neutralized with Dowex 50WX8 H^+ resin, filtered, and concentrated *in vacuo*. The crude was purified by flash column chromatography ($CHCl_3$/MeOH, 1:1, v/v) to afford compound **1** (21 mg, 12.1 µmmol, 88% over 2 steps) as a white powder. R_f = 0.52 (EtOAc/MeOH/AcOH, 40:60:0.5); $[\alpha]_D^{20}$ = +67.7 (*c* 1.0, $CHCl_3$/MeOH, 1:1); ^{1}H NMR (800 MHz, methanol-d_4/chloroform-*d* 1:1) δ 5.14 (d, *J* = 3.7 Hz, 1H, H-$1_{\alpha Gal}$), 4.67 (d, *J* = 7.8 Hz, 1H, H-1'), 4.55 (d, *J* = 7.8 Hz, 1H, H-1), 4.42 (q, *J* = 5.2, 4.6 Hz, 1H, H-2_{Sph}), 4.25 (dd, *J* = 9.7, 2.9 Hz, 1H, H-3'), 4.21 – 4.07 (m, 9H, H-6a,b, H-$6_{\alpha Gal}$a, H-1_{Sph}a, H-8'', H-7'', H-4', H-$3_{\alpha Gal}$, OC*H*H), 4.07 – 4.02 (m, 2H, H-$2_{\alpha Gal}$, C-6'a), 4.02 – 3.80 (m, 16H, H-6'b, H-$6_{\alpha Gal}$b, H-5'', H-4'', H-1_{Sph}b, OCH*H*, H-2', H-3, H-5', H-4, H-3_{Sph}, H-6'', H-4_{Sph}, H-$4_{\alpha Gal}$, H-9''a,b), 3.75 (ddt, *J* = 22.0, 15.1, 6.3 Hz, 4H, C*H*HNH, H-$5_{\alpha Gal}$, OCH_2), 3.69 – 3.65 (m, 1H, H-5), 3.57 – 3.54 (m, 2H, OCH*H*NH, H-2), 3.41 (q, *J* = 6.6 Hz, 2H, CH_2NH), 3.06 – 3.01 (m, 1H, H-3''a), 2.52 – 2.43 (m, 7H, CH_2), 2.27 (s, 3H, NHCOCH_3), 2.18 – 2.12 (m, 2H, CH_2), 2.12 – 2.07 (m, 1H, H-3''b), 1.91 – 1.70 (m, 8H, CH_2), 1.51 (s, 75H, CH_2), 1.15 – 1.09 (m, 6H, $2CH_3$); ^{13}C NMR (201 MHz, methanol-d_4/chloroform-*d* 1:1) δ 173.97, 173.87, 173.83, 173.55, 171.36 (5 CO), 103.47 (C-1'), 102.59 (C-1), 99.37 (C-$1_{\alpha Gal}$), 98.40 (C-2''), 79.51 (C-4), 76.11 (C-3'), 75.04 (C-5'), 74.62 (C-5), 74.33 (C-3), 73.72, 73.61 (C-3_{Sph}, C-6''), 72.96 (C-2), 71.52 (C-4_{Sph}), 71.12 (OCH_2), 70.93 (C-8''), 69.83, 69.75 (C-9'', C-$4_{\alpha Gal}$), 69.50 (C-4'), 68.98, 68.88 (C-$3_{\alpha Gal}$, C-2'), 68.55, 68.46, 68.29 (C-$2_{\alpha Gal}$, OCH_2, C-$5_{\alpha Gal}$), 67.54 (C-7''), 67.06, 66.97 (C-1_{Sph}, C-4''), 63.34 (C-$6_{\alpha Gal}$), 61.00 (C-6'), 60.33 (C-6), 52.15 (C-5''), 49.95 (C-2_{Sph}), 39.74 (C-3''), 38.94, 38.86 (CH_2NH, CH_2NH), 35.96, 34.80, 34.77, 31.52, 31.48, 31.47, 29.35, 29.33, 29.29, 29.28, 29.26, 29.24, 29.23, 29.21, 29.20, 29.17, 29.13, 29.03, 28.94, 28.91, 28.88, 28.70, 26.15, 25.50, 25.47, 25.22, 22.19, 22.18 (CH_2), 21.74 (NHCO*C*H_3), 21.63 (2 CH_3). HRMS (MALDI-TOF) m/z: calcd. for $C_{86}H_{160}N_4O_{30}$: 1752.1009 $[M+Na]^+$; found: 1752.0970.

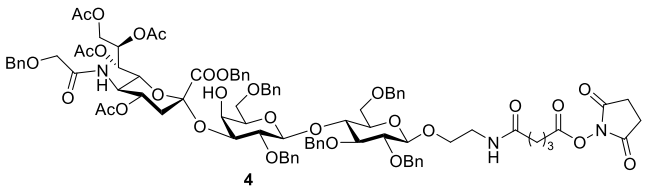
2-Azidoethyl (benzyl 4,7,8,9-tetra-*O*-acetyl-5-(2-benzyloxy)acetamido-dideoxy-D-glycero-α-D-galacto-non-2-ulopyranosylonate)-(2→3)-(2,6-di-*O*-benzyl-β-D-galactopyranosyl)-(1→4)-2,3,6-tri-*O*-benzyl-β-D-glucopyranoside (19)



19

Glycosyl acceptor **10** (300 mg, 0.35 mmol) and donor **9** (370 mg, 0.49 mmol) were dissolved in anhydrous CH_2Cl_2 (4.5 mL) and stirred together with 3 Å molecular sieves (670 mg) at room temperature for 1 hour. The mixture was cooled to –40 °C and a solution of AgOTf (268 ,g. 1.04 mmol) in CH_3CN (6.8 mL) was slowly added. Stirring was continued for 15 minutes, then 1M IBr in CH_2Cl_2 (0.7 mL, 0.7 mmol) was added to the mixture and the reaction was kept at the same temperature and in the darkness for another 2 hours. The reaction was finally neutralized with DIPEA, filtered over Celite, and concentrated *in vacuo*. The crude residue was purified by flash column chromatography (toluene:CH_3CN, 8:2, v/v) to afford **19** (380 mg, 0.25 mmol, 71%) as a white foam. R_f = 0.4 (toluene:CH_3CN, 3:1, v/v); $[\alpha]_D^{20}$ = +8.6 (*c* 1.0, $CHCl_3$); ^{1}H NMR (400 MHz, chloroform-*d*) δ 7.46 – 7.19 (m, 35H, H_{Ar}), 6.30 (d, *J* = 10.3 Hz, 1H, NH), 5.46 – 5.40 (m, 1H, H-8''), 5.33 (dd, *J* = 8.3, 2.3 Hz, 1H, H-7''), 5.25 (d, *J* = 12.0 Hz, 1H, C*H*HPh), 5.13 (d, *J* = 12.1 Hz, 1H, CH*H*Ph), 5.01 (d, *J* = 10.8 Hz, 1H, C*H*HPh), 4.93 – 4.82 (m, 2H, C*H*HPh, H-4''), 4.80 – 4.70 (m, 3H, CH*H*Ph, CH*H*Ph, C*H*HPh), 4.67 – 4.52 (m, 4H, H-1', 3 CH*H*Ph), 4.50 – 4.33 (m, 5H, H-1, 4 CH*H*Ph), 4.28 (dd, *J* = 12.5, 2.7 Hz, 1H, H-9''a), 4.16 (d, *J* = 10.5 Hz, 1H, H-5''), 4.12 – 3.94 (m, 5H, H-9''b, H-6a, H-6'', H-3', H-4), 3.89 (d, *J* = 10.6 Hz, 2H, CH_2OBn), 3.81 (d, *J* = 3.3 Hz, 1H, H-4'), 3.77 – 3.63 (m, 4H, OCH_2, H-6b, H-6'a), 3.60 – 3.35 (m, 8H, CH2N3, H-6'b, H-5, H-5', H-2', H-2, H-3), 2.61 (dd, *J* = 13.0, 4.6 Hz, 1H, H-3''a), 2.13 (s, 3H, CH_3), 2.11 – 2.05 (m, 1H, H-3''b), 2.00 (s, 3H, CH_3), 1.98 (s, 3H, CH_3), 1.89 (s, 3H, CH_3); ^{13}C NMR (101 MHz, chloroform-*d*) δ 170.7, 170.34, 170.2, 170.1, 170.0, 167.8 (6 CO), 139.2, 139.1, 138.8, 138.5, 136.9, 134.5 (7 C_{Ar}), 128.9, 128.8, 128.7, 128.6, 128.4, 128.3, 128.2, 127.7, 127.6, 127.4, 127.3 (C_{Ar}), 103.7 (C-1), 102.4 (C-1'), 98.5 (C-2''), 82.9 (C-3), 82.0 (C-2), 78.6 (C-2'), 76.5 (2C, H-3', H-4), 75.5 (CH_2Ph), 75.2 (C-5), 75.1 (CH_2Ph), 75.0 (CH_2Ph), 73.6 (CH_2Ph), 73.5 (CH_2Ph), 73.2 (CH_2Ph), 72.7 (2C, C-6'', C-5'), 69.3 (CH_2OBn), 69.0 (C-4''), 68.7 (H-6'), 68.6 (H-6), 68.4 (H-8''), 68.2 (CH_2Ph), 68.1 (H-4'), 67.2 (H-7''), 62.4 (H-9''), 51.1 (CH_2N_3), 48.3 (H-5''), 36.7 (H-3''), 21.3, 20.9, 20.9, 20.7 (4 CH_3). HRMS (ESI-TOF) m/z: calcd. for $C_{82}H_{92}N_4O_{24}$: 1539.5994 $[M+Na]^+$; found: 1539.6002.

2'-*N*-[4-(Succinimidooxycarbonyl)butan-1-oyl]-aminoethyl-(benzyl 4,7,8,9-tetra-*O*-acetyl-5-(2-benzyloxy)acetamido-dideoxy-D-glycero-α-D-galacto-non-2-ulopyranosylonate)-(2→3)-(2,6-di-*O*-benzyl-β-D-galactopyranosyl)-(1→4)-2,3,6-tri-*O*-benzyl-β-D-glucopyranoside (4)

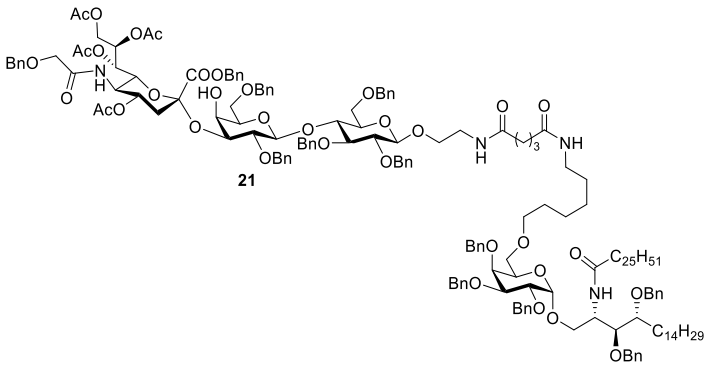


4

To a solution of trisaccharide **19** (170 mg, 112 µmol) in CH_2Cl_2 (8.6 mL) and AcOH (86 µL) was added Zn powder (440 mg, 6.7 mmol) and the reaction was stirred at room temperature for 1 hour. The reaction was then filtered, diluted with toluene, and concentrated *in vacuo*. The crude residue was dissolved in anhydrous DMF (10 mL) and reacted with disuccinimidyl glutarate (51 mg, 156 µmol) in the presence of Et_3N (36 µL, 260 µmol) at room temperature. After 2 hours the reaction was diluted with EtOAc, washed with sat. $NaHCO_3$, and brine. The combined organic layers were dried over Na_2SO_4, filtered, and concentrated *in vacuo*. The crude residue was purified by flash column chromatography (toluene:acetone, 8:2→7:3, v/v) to afford **4** (114 mg, 67 µmol, 60% over two steps) as a white foam. R_f = 0.6 (toluene:acetone, 1:1, v/v); $[\alpha]_D^{20}$ = +8.2 (*c* 1.0, $CHCl_3$); ^{1}H NMR (400 MHz, chloroform-

d) δ 7.43 – 7.15 (m, 35H, H_{Ar}), 6.49 (t, *J* = 5.7 Hz, 1H, CH_2NHCO), 6.27 (d, *J* = 10.3 Hz, 1H, NH), 5.42 (ddt, *J* = 9.7, 6.6, 3.3 Hz, 1H, H-8''), 5.34 – 5.28 (m, 1H, H-7''), 5.22 (d, *J* = 12.1 Hz, 1H, C*H*HPh), 5.11 (d, *J* = 12.1 Hz, 1H, CH*H*Ph), 5.00 (d, *J* = 10.9 Hz, 1H, C*H*HPh), 4.88 – 4.71 (m, 5H, H-4'', CH*H*Ph, C*H*HPh; CH_2Ph), 4.65 – 4.50 (m, 4H, H-1', CH*H*Ph, C*H*HPh, C*H*HPh), 4.46 – 4.37 (m, 3H, CH*H*Ph, CH*H*Ph, C*H*HPh), 4.36 – 4.29 (m, 2H, CH*H*Ph, H-1), 4.26 (dd, *J* = 12.5, 2.7 Hz, 1H, H-9''a), 4.18 – 4.01 (m, 3H, H-5'', H-6'', H-3'), 3.98 – 3.84 (m, 4H, CH_2OBn, H-9''b, H-4), 3.85 – 3.71 (m, 4H, OCH_2, H-4', H-6a), 3.69 – 3.33 (m, 10H, H-6b, H-6'a, CH_2NH, H-5', H-5, H-2', H-3, H-2, H-6'b), 2.73 – 2.67 (m, 4H, 2 CH_2), 2.65 – 2.58 (m, 1H, H-3''a), 2.56 – 2.50 (m, 2H, CH_2), 2.36 (s, 3H, CH_3), 2.11 (s, 3H, CH_3), 2.06 – 1.88 (m, 12H, CH_3, H-3''b, 4 CH_2), 1.84 (s, 3H, CH_3); ^{13}C NMR (101 MHz, chloroform-*d*) δ 171.9, 170.7, 170.4, 170.2, 170.1, 170.0, 169.3 (2C), 168.4, 167.7 (CO), 139.2, 139.1, 138.7, 138.5, 138.0, 136.9, 134.5, 129.2, 128.9, 128.9, 128.8, 128.7, 128.5, 128.4, 128.4, 128.3, 128.2, 128.0, 127.9, 127.8, 127.7, 127.6, 127.5, 127.4, 127.3, 125.4 (C_{Ar}), 104.0 (C-1), 102.5 (C-1'), 98.5 (C-2''), 83.0 (C-3), 82.1 (C-2), 78.7 (C-2'), 76.6 (C-4, C-3'), 75.4 (CH_2Ph), 75.1 (CH_2Ph), 75.0 (CH_2Ph), 74.8 (C-5), 73.7 (CH_2Ph), 73.5 (CH_2Ph), 73.2 (CH_2Ph), 72.7 (CH_2Ph), 72.6 (C-6'', C-5'), 70.0 (CH_2OBn), 69.3 (OCH_2), 69.0 (C-4'', C-6), 68.8 (C-6'), 68.5 (C-8''), 68.4 (CH_2Ph), 68.1 (C-4'), 67.2 (C-7''), 62.4 (C-9''), 48.3, 40.1 (C-5''), 36.8 (CH_2N_3), 34.1 (C-3''), 30.2 (3 CH_2), 29.8 (CH_2), 25.6 (2 CH_2), 21.6, 21.3, 20.9, 20.8, 20.7 (4 CH_3, CH_2). HRMS (ESI-TOF) m/z: calcd. for $C_{91}H_{103}N_3O_{29}$: 860.3544 $[M+NH_4+H]^{2+}$; found: 860.3509.

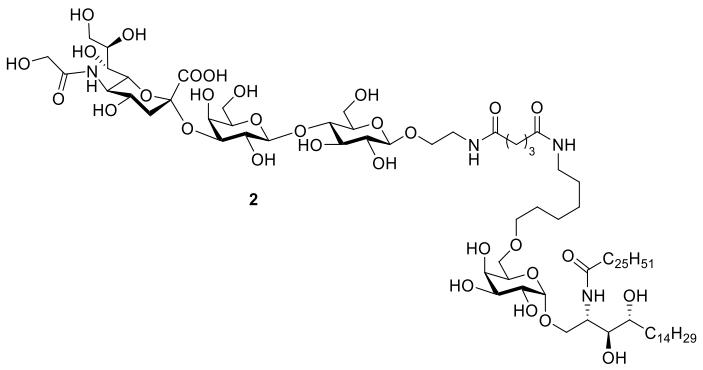
Protected (Neu5Gc)GM3–αGalCer conjugate (21)



Linker-equipped Neu5Gc(GM3) **4** (47 mg, 27 µmol) and αGalCer amine **5** (53 mg, 37 µmol) were dissolved in anhydrous DMF (2 mL) and then treated with Et_3N (7.5 µL, 54 µmol). The reaction was stirred at room temperature for 6 hours, then the solvent was removed *in vacuo*. The crude residue was purified by flash column chromatography (toluene:acetone, 7:3, v/v) to give **21** (65 mg, 22 µmol, 80%) as a white amorphous solid. R_f = 0.7 (toluene:acetone, 1:1, v/v); $[\alpha]_D^{20}$ = +13.7 (*c* 1.0, $CHCl_3$); ^{1}H NMR (400 MHz, chloroform-*d*) δ 7.44 – 7.20 (m, 60H, H_{Ar}), 6.29 (d, *J* = 10.3 Hz, 1H, NH), 6.19 (d, *J* = 8.5 Hz, 1H, NH), 5.45 (ddd, *J* = 8.4, 5.6, 2.6 Hz, 1H, H-8''), 5.33 (dd, *J* = 8.4, 2.3 Hz, 1H, H-7''), 5.24 (d, *J* = 12.1 Hz, 1H, C*H*HPh), 5.13 (d, *J* = 12.1 Hz, 1H, CH*H*Ph), 5.03 (d, *J* = 10.8 Hz, 1H, C*H*HPh), 4.97 (d, *J* = 11.5 Hz, 1H, C*H*HPh), 4.92 – 4.72 (m, 11H, H-4'', H-$1_{\alpha Gal}$, 7 C*H*HPh, 2 NH), 4.69 – 4.51 (m, 9H, H-1', 8 C*H*HPh), 4.50 – 4.30 (m, 6H, H-1, 5 C*H*HPh), 4.27 (dd, *J* = 12.5, 2.6 Hz, 1H, H-9''a), 4.22 – 4.02 (m, 5H, H-2_{Sph}, H-5'', H-6'', H-3', H-$2_{\alpha Gal}$), 4.01 – 3.86 (m, 9H, H-3_{Sph}, H-$3_{\alpha Gal}$, H-4, H-$4_{\alpha Gal}$, H-$5_{\alpha Gal}$, H-$6_{\alpha Gal}$, H-9''b, H-1_{Sph}a,b), 3.86 – 3.74 (m, 5H, OCH_2, CH_2OBn, H-4'), 3.71 – 3.63 (m, 2H, H-6a, H-6'a), 3.62 – 3.53 (m, 2H, H-3, H-2'), 3.53 – 3.34 (m, 10H, CH_2NH, H-5, H-5', H-4_{Sph}, H-2, H-6b, H-6´b, OC*H*H, H-$6_{\alpha Gal}$b), 3.28 (dt, *J* = 9.4, 6.9 Hz, 1H, OCH*H*), 3.20 (q, *J* = 6.6 Hz, 2H, CH_2NH), 2.69 – 2.59 (m, 1H, H-3''a), 2.13 (s, 3H, CH_3), 2.07 – 1.93 (m, 11H, H-3''b, 2 CH_3, 2 CH_2), 1.86 (s, 3H, CH_3), 1.78 (p, *J* = 7.4 Hz, 2H, CH_2), 1.65 (ddt, *J* = 22.7, 9.3, 4.5 Hz, 1H, C*H*H), 1.58 – 1.38 (m, 7H, CH*H*, 3 CH_2), 1.39 – 1.16 (m, 71H, CH_2), 0.90 (t, *J* = 6.7 Hz, 6H, 2 CH_3); ^{13}C NMR (101 MHz, chloroform-*d*) δ 172.9, 170.7, 170.4, 170.2 (2C), 170.2 (2C), 170.0, 167.8 (9 CO), 139.1, 139.1, 138.8, 138.8, 138.7, 138.6, 138.5, 138.0, 136.9, 134.5, 128.9, 128.8, 128.7, 128.5, 128.4, 128.3, 128.2, 128.0, 127.9, 127.8, 127.7, 127.6, 127.5, 127.4 (C_{Ar}), 103.9 (C-1), 102.5 (C-1'), 99.5 (C-$1_{\alpha Gal}$), 98.5 (C-2''), 82.9 (C-3), 81.9 (C-2), 80.3 (C-4_{Sph}), 79.1, 78.7 (C-$3_{\alpha Gal}$, C-3_{Sph}, C-2'), 77.4 (C-4), 76.6 (C-3', C-$2_{\alpha Gal}$), 75.5, 75.2, 75.1, 74.9 (C-$4_{\alpha Gal}$, C-5, 4 CH_2Ph),

73.7, 73.6, 73.5, 73.3, 72.9, 72.7 (C-6'', C-5', 6 CH_2Ph), 71.8 (CH_2Ph), 71.64 (OCH_2), 70.0, 69.9, 69.5, 69.3, 69.0, 68.8, 68.5, 68.4, 68.0 (C-8'', C-4'', C-4', C-$5_{\alpha Gal}$, C-6, C-6', C-$6_{\alpha Gal}$, C-1_{Sph}, OCH_2OBn), 67.2 (C-7''), 62.4 (C-9''), 50.4 (C-5''), 48.3 (C-2_{Sph}), 40.3 (CH_2NH), 39.9 (CH_2NH), 36.8 (C-3''), 34.4 (CH_2), 32.1, 30.0, 29.9, 29.8, 29.7, 29.6, 29.5, 26.9, 26.2, 25.9, 22.8, 21.9 (CH_2), 21.4, 20.9, 20.9, 20.7 (4 CO*C*H_3), 14.3 (2C, 2 CH_3). HRMS (MALDI-TOF) m/z: calcd. for $C_{178}H_{240}N_4O_{35}$: 2994.7196 $[M+H]^+$; found: 2994.7250.

(Neu5Gc)GM3–αGalCer conjugate (2)



Protected conjugate **21** (60 mg, 20 µmol) was dissolved in EtOH (2 mL). Acetic acid (0.2 mL) was added followed by Pd/C (5% wt, 60 mg). The reaction was then placed under a H_2 atmosphere overnight at room temperature. Afterwards, the mixture was filtered over a Celite pad and concentrated *in vacuo*. The crude material was then dissolved in anhydrous MeOH and treated with freshly prepared MeONa in MeOH solution until pH = 9. The mixture was stirred at room temperature for 5 hours and then H_2O (0.3 mL) was added. The reaction was stirred overnight at room temperature. Afterwards the mixture was neutralized with Dowex 50WX8 H^+ resin, filtered, and concentrated *in vacuo*. The crude residue was purified by flash column chromatography ($CHCl_3$:MeOH, 1:1, v/v) to afford **2** (30 mg, 17 µmol, 85% over 2 steps) as a white powder. R_f = 0.19 ($CHCl_3$:MeOH, 1:1, v/v); $[\alpha]_D^{20}$ = +16.5 (*c* 1.0, $CHCl_3$); ^{1}H NMR (800 MHz, Methanol-d4/$CDCl_3$ 1:1) δ 5.24 (d, J = 3.8 Hz, 1H, H-$1_{\alpha Gal}$), 4.77 (under D_2O peak, H-1'), 4.65 (d, J = 7.8 Hz, 1H, H-1), 4.50 (q, J = 4.6 Hz, 1H, H-2_{Sph}), 4.38 (d, J = 46.9 Hz, 3H, CH_2OH, H-3'), 4.29 – 4.23 (m, 5H, H-6a, H-7'', OC*H*H, H-4', H-$3_{\alpha Gal}$), 4.23 – 3.89 (m, 21H, H-6b, H-6'a, H-6'b, H-6 $_{\alpha Gal}$a, H-6 $_{\alpha Gal}$b, H-1_{Sph}a,b, H-4'', H-$2_{\alpha Gal}$, H-8'', H-$4_{\alpha Gal}$, OCH*H*, H-9''a,b, H-2', H-6'', H-4Sph, H-3Sph, H-5', H-3, H-6''), 3.88 – 3.75 (m, 5H, OCH2, H-5, H-$5_{\alpha Gal}$, C*H*HNH), 3.71 – 3.63 (m, 2H, CH*H*NH, H-2), 3.50 (hept, J = 6.9 Hz, 2H, CH_2NH), 3.22 – 3.15 (m, 1H, H-3''a), 2.60 – 2.50 (m, 6H, CH_2), 2.24 (p, J = 7.5 Hz, 2H, CH_2), 2.14 (t, J = 12.0 Hz, 1H, H-3''b), 2.01 – 1.80 (m, 9H, CH_2), 1.60 (s, 84H, CH_2), 1.24 – 1.19 (m, 6H, 2CH_3); ^{13}C NMR (201 MHz, Methanol-d4/$CDCl_3$ 1:1) δ 175.9, 174.2, 174.1, 173.8 (4 CO), 103.8 (C-1'), 102.9 (C-1), 99.7 (C-$1_{\alpha Gal}$), 99.2 (C-2''), 79.8 (C-4), 76.3 (C-3'), 75.4 (C-5'), 74.9, 74.6 (C-5, C-3), 74.0 (C-3_{Sph}), 73.5 (C-6''), 73.2 (C-2), 71.9 (C-4_{Sph}), 71.5 (OCH_2, H-8''), 70.1 (C-9'', C-$4_{\alpha Gal}$), 69.9 (C-$3_{\alpha Gal}$), 69.2 (C-2', C-4'), 68.7 (C-$5_{\alpha Gal}$, OCH_2, C-$2_{\alpha Gal}$), 67.5, 67.4 (C-1_{Sph}, C-7''), 63.5 (C-$6_{\alpha Gal}$), 61.5, 61.4, 60.7 (C-6, C-6', CH_2OH), 52.1 (C-5''), 50.2 (C-2_{Sph}), 39.3, 39.2 (CH_2NH, CH_2NH, C-3''), 36.3 (CH_2), 35.1 (CH_2), 31.8, 29.7, 29.6, 29.5, 29.4, 29.3, 29.3, 29.2, 29.2, 29.0, 26.4, 25.8, 25.8, 25.5, 22.5, 22.1 (CH_2), 13.8 (2 CH_3). HRMS (MALDI-TOF) m/z: calcd. for $C_{86}H_{160}N_4O_{31}$: 1768.0959 $[M+Na]^+$; found: 1768.0968.

Preparation of HSA–conjugates 22 and 23

General procedure for NHS-activated glycans. To a solution of **18** or **19** (0.045 mmol) in MeOH (2 mL) was added MeONa until pH = 8–9. The reaction was stirred at room temperature for 4 hours, then H_2O (500 µL) was added and the mixture was left stirring overnight. The reaction was then neutralized with Dowex 50WX8 H^+ resin, filtered, and concentrated *in vacuo*. The crude residue was dissolved in EtOH (2 mL), then Pd/C (5% wt) was added followed by 2 drops of AcOH. The reaction was left stirring overnight under H2 atmosphere, then it was filtered over a Celide pad and concentrated *in vacuo*. The product was not further purified and used directly in the next step. The crude residue (0.01 mmol) was dissolved in DMF (600 µL) and PBS (150 µL, 0.1 M, pH = 8), then disuccinimidyl glutarate (0.05 mmol) was added to the solution and the reaction was stirred for 4 hours (TLC EtOAc/MeOH/H_2O/AcOH, 4:3:3:1). The mixture was then concentrated *in vacuo* and the crude residue was washed with EtOAc (×5) to remove the excess of the linker. The residue was then dried under vacuum and purified by reverse phase chromatography (C18, H_2O:MeOH, 9:1, v/v) to afford the corresponding linker equipped derivatives over three steps.

*General procedure for the preparation of HSA–conjugates **22** and **23**.* NHS-linker equipped compound (2.4 µmol) and human serum albumin (0.06 µmol) were dissolved in PBS (0.1 M, pH = 8, 1.2 mL) and the reaction was stirred at room temperature for 4 days. The mixture was then filtered with Amicon 10 KDa filters (Amicon-15) and the total volume was reduced to 500 µL and freeze dried to yield a white foam. The products were analyzed by MALDI-TOF (matrix: SDHB, matrix/analyte 1:1).

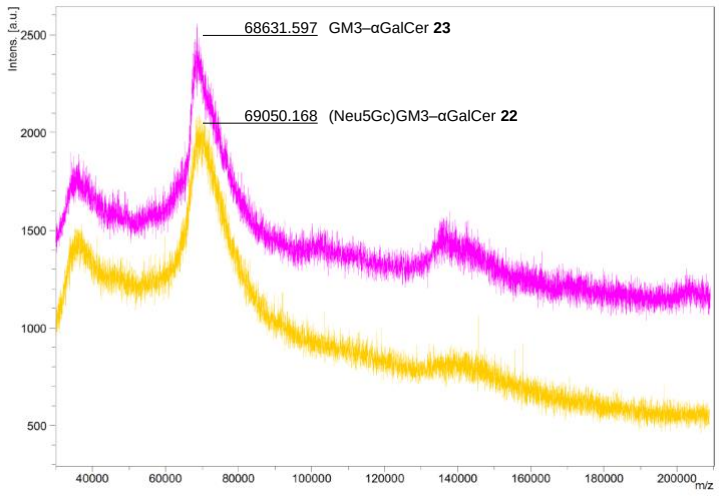
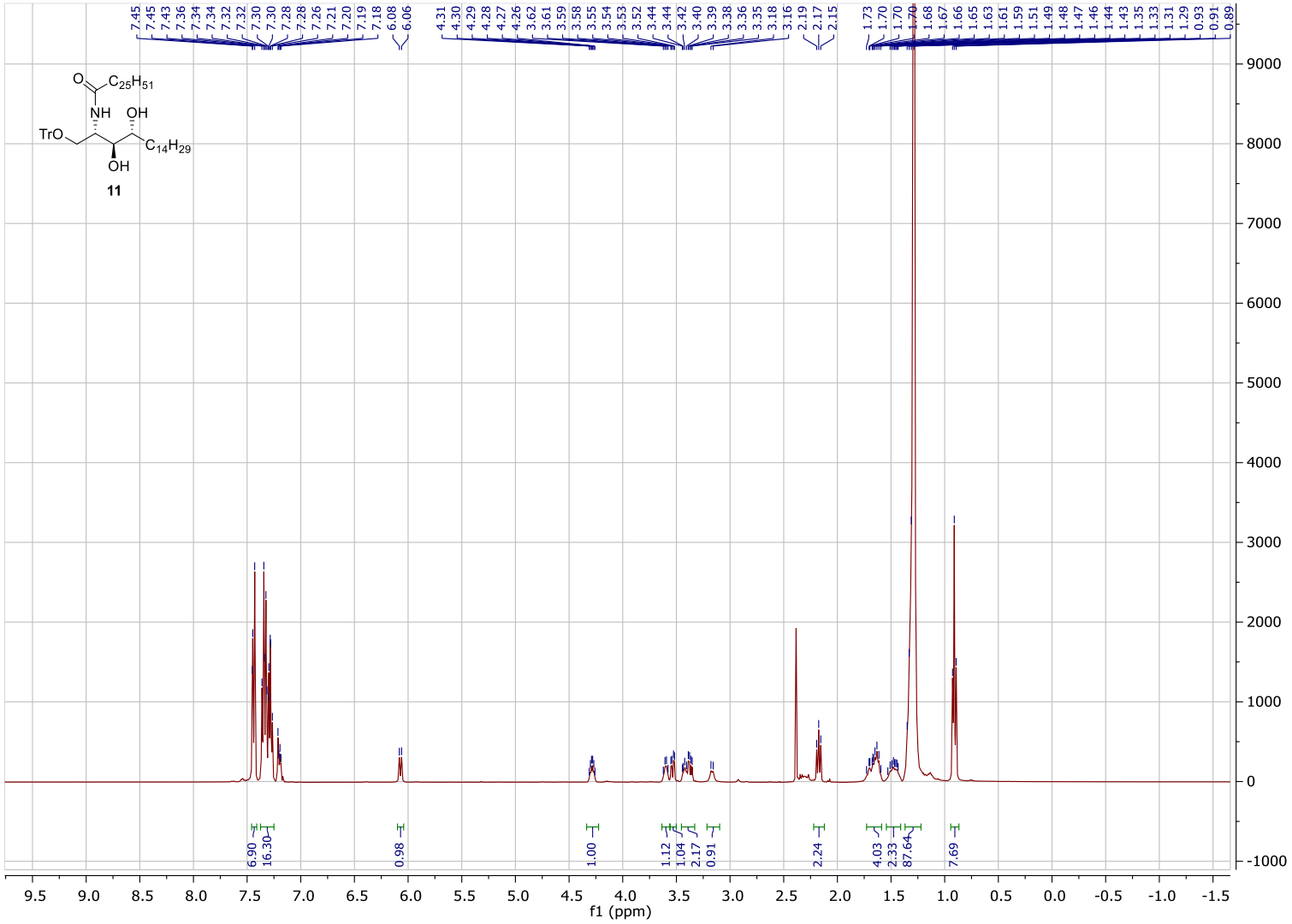
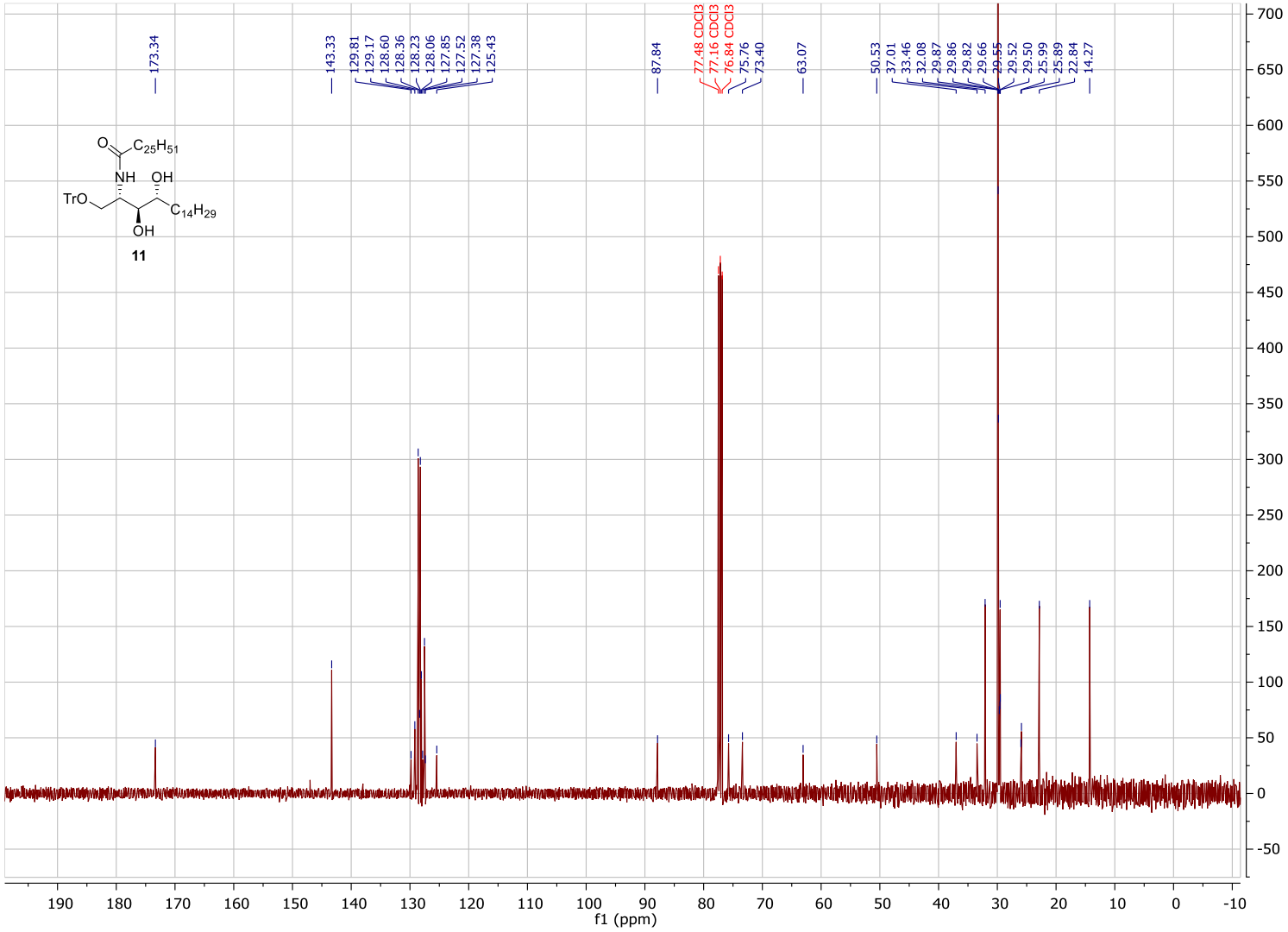


Figure S1. MALDI-TOF analysis of conjugates **22** and **23**. HSA (non-conjugated) was found at 67597.994. The loading was quantified using the formula: $loading\ of\ glycans\ per\ carrier = \frac{MW_{HSAconjugate} - MW_{HSA}}{MW_{glycan}}$ and resulted in 3.3 copies of (Neu5Gc)GM3 in **22** and 4 copies of GM3 in **23**.

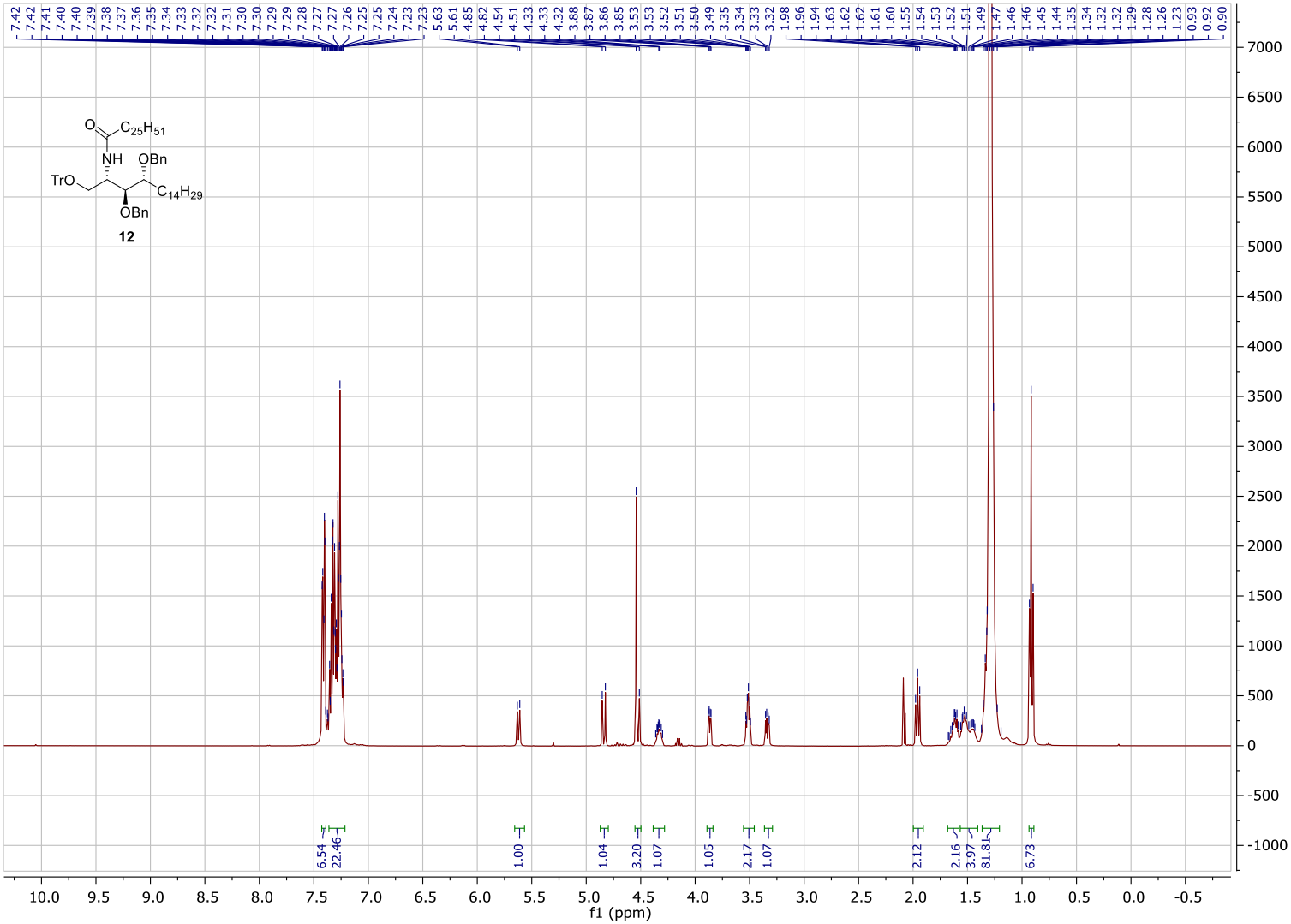
(2S,3S,4R)-2-Hexacosanoylamido-1-triphenylmethoxy-octadecane-3,4-diol (11)



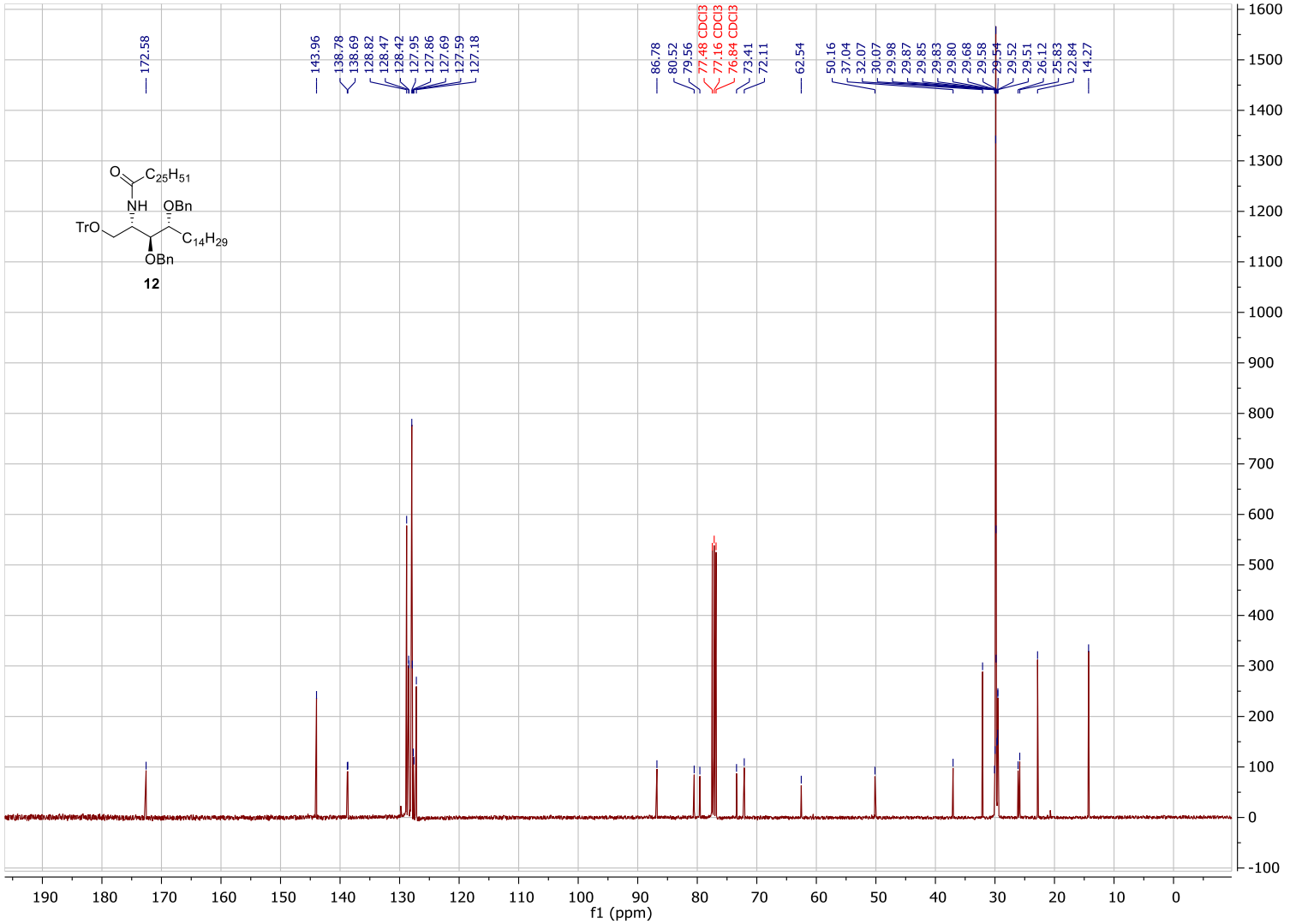
(2S,3S,4R)-2-Hexacosanoylamido-1-triphenylmethoxy-octadecane-3,4-diol (11)



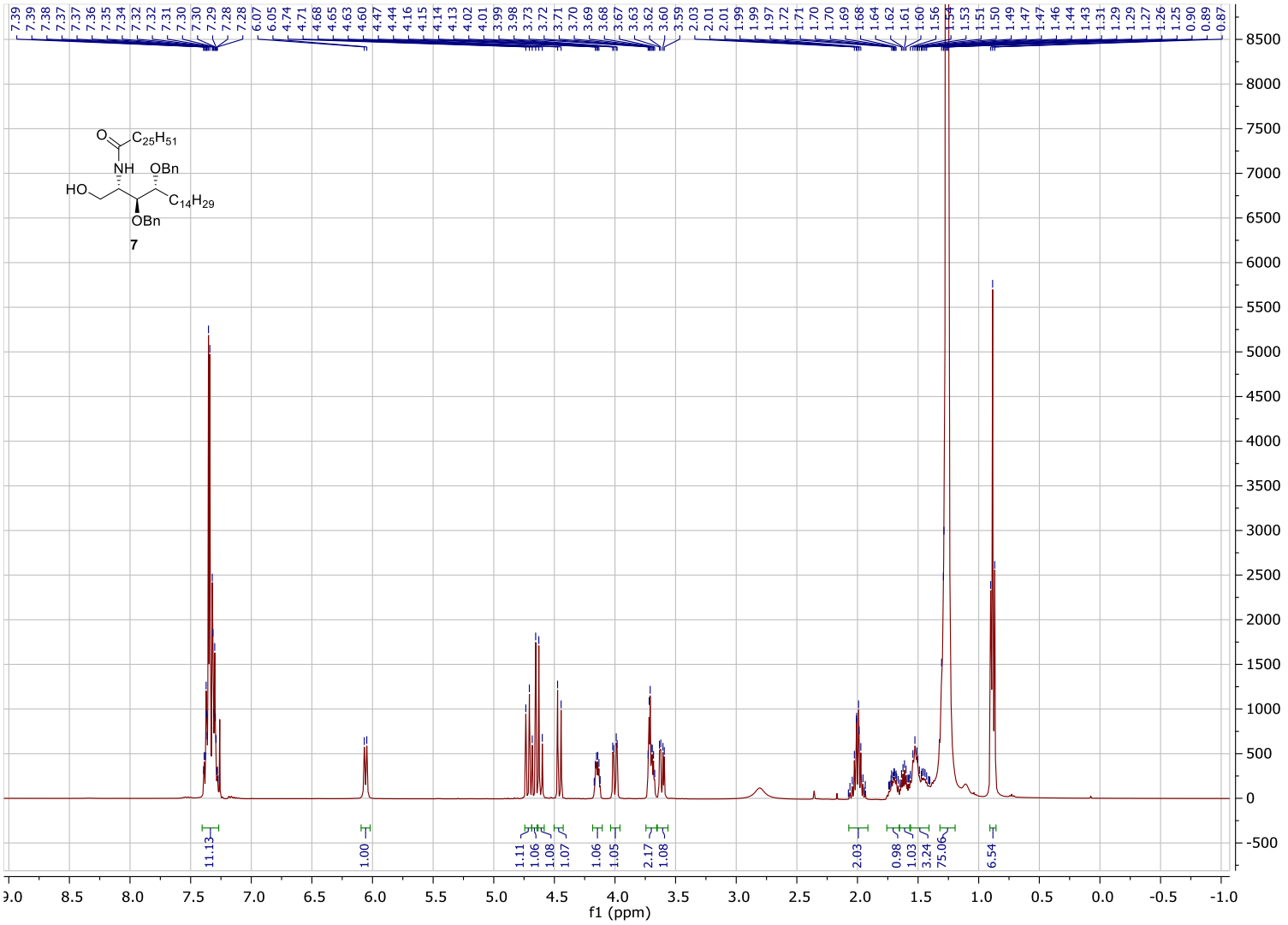
(2S,3S,4R)-3,4-Di-*O*-benzyl-2-hexacosanoylamido-1-triphenylmethoxy-octadecane-3,4-diol (12)



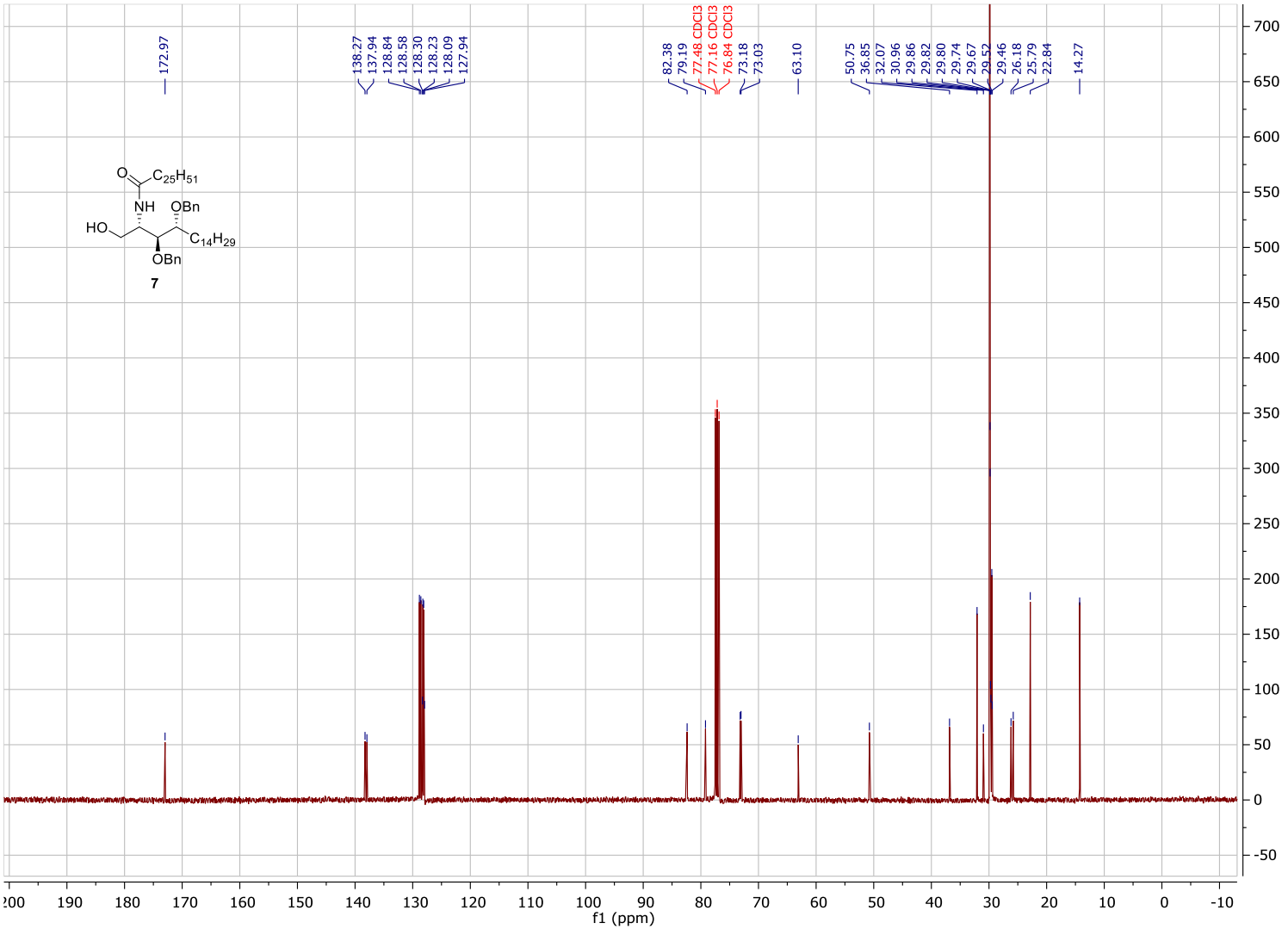
(2S,3S,4R)-3,4-Di-*O*-benzyl-2-hexacosanoylamido-1-triphenylmethoxy-octadecane-3,4-diol (12)



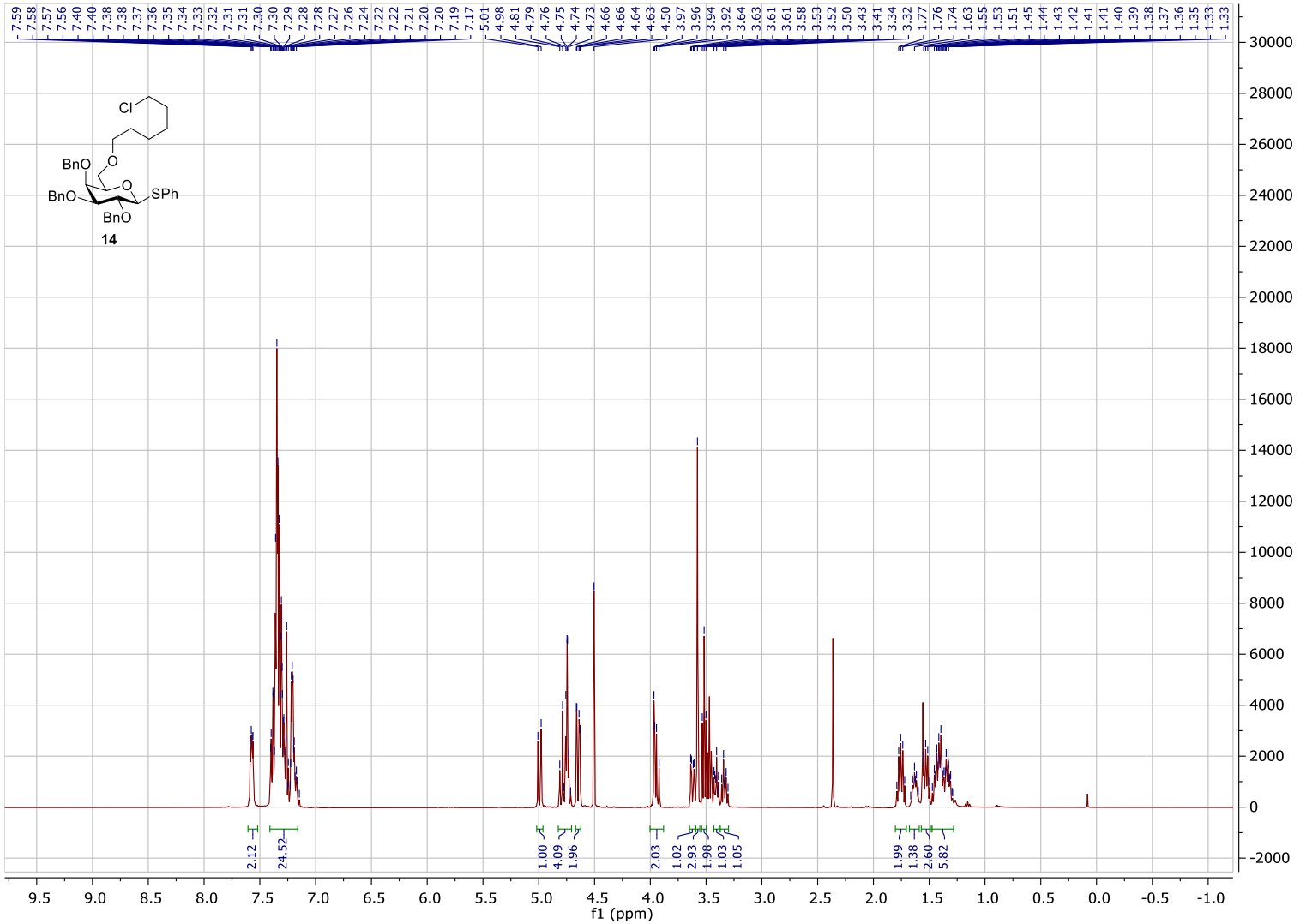
(2S,3S,4R)-3,4-Di-*O*-benzyl-2-hexacosanoylamido-octadecane-1,3,4-triol (7)



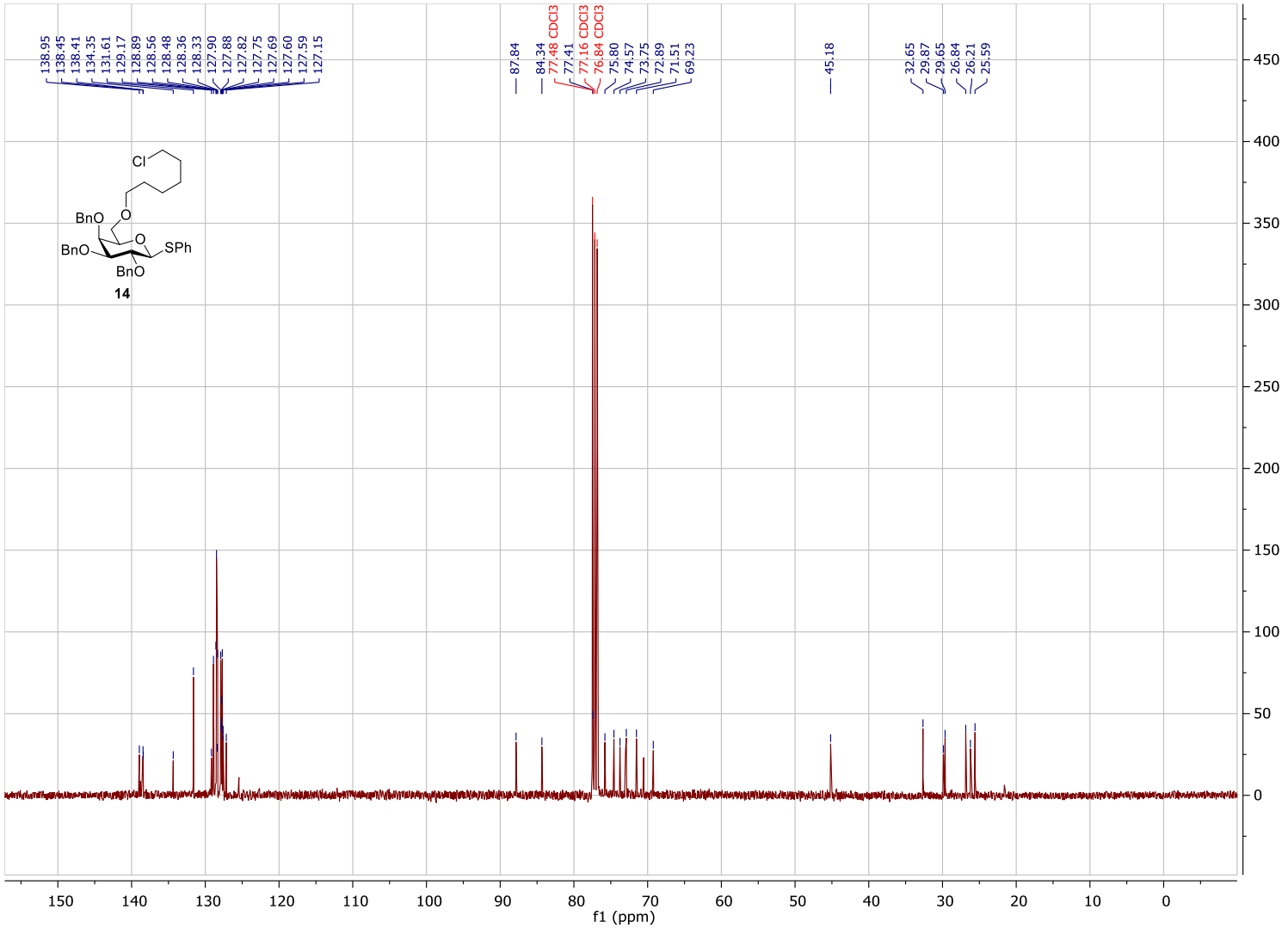
(2S,3S,4R)-3,4-Di-*O*-benzyl-2-hexacosanoylamido-octadecane-1,3,4-triol (7)



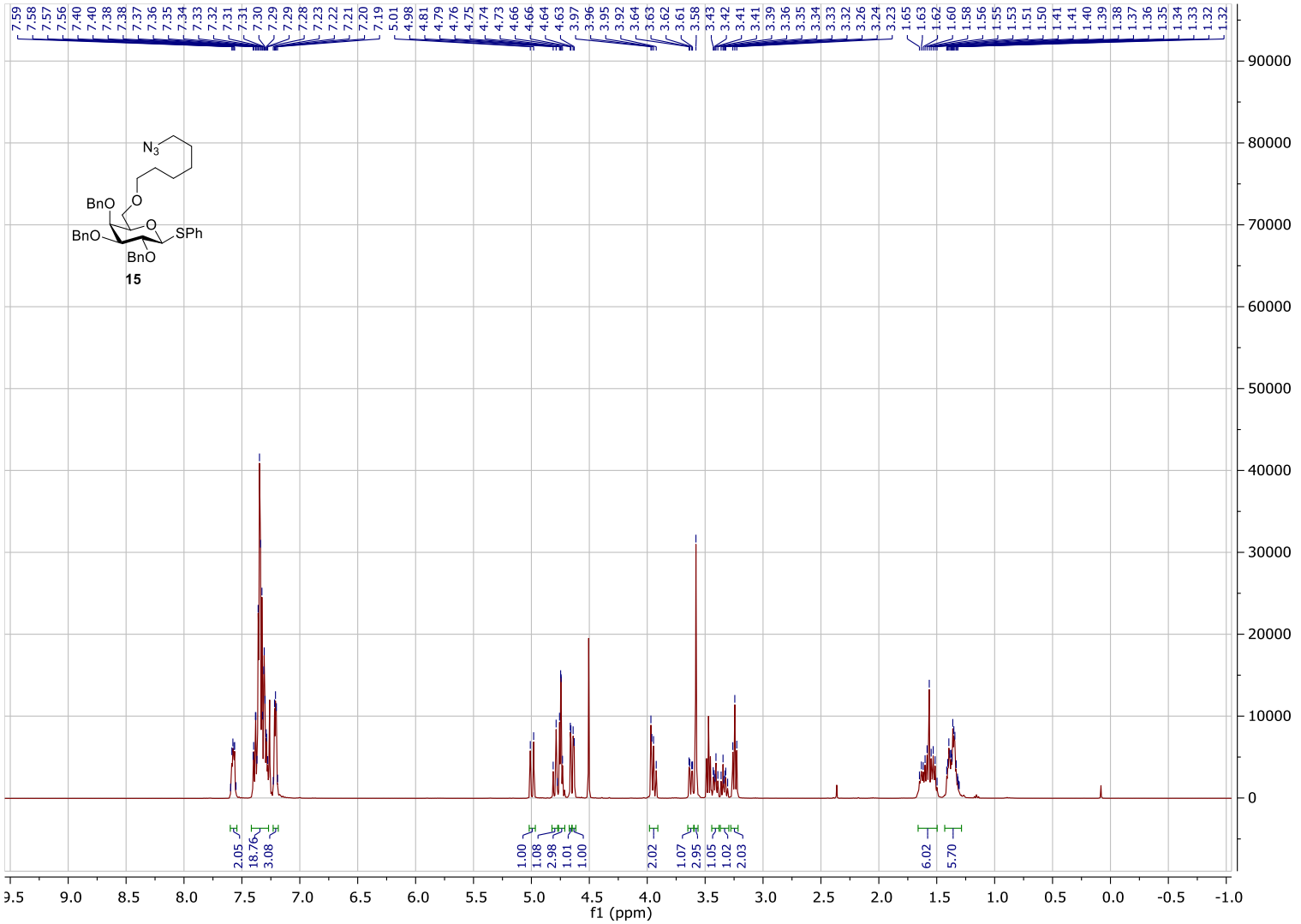
Phenyl 2,3,4-tri-*O*-benzyl 6-(6'-chlorohexyl)-1-thio-β-D-galactopyranoside (14)



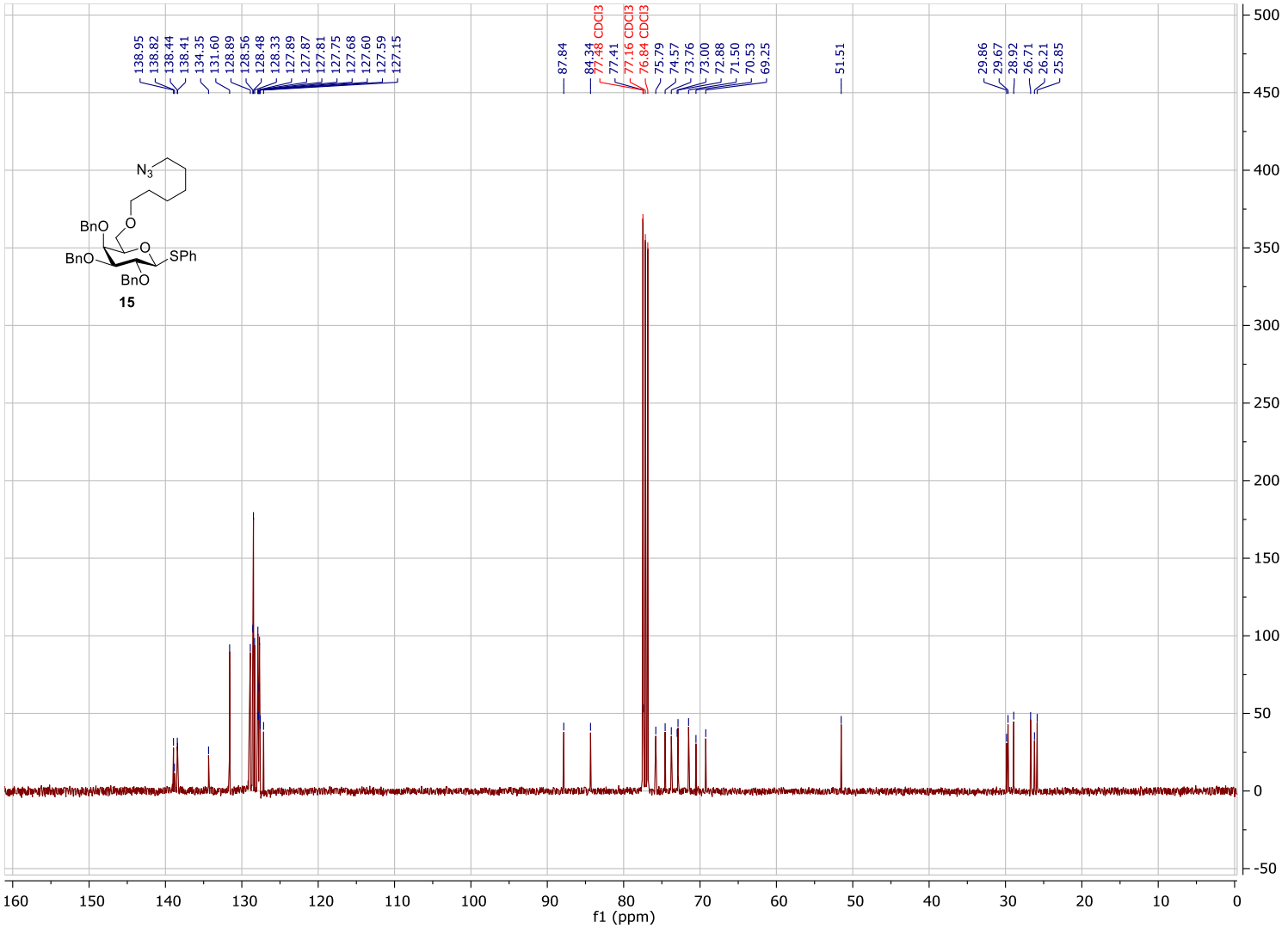
Phenyl 2,3,4-tri-*O*-benzyl 6-(6'-chlorohexyl)-1-thio-β-D-galactopyranoside (14)



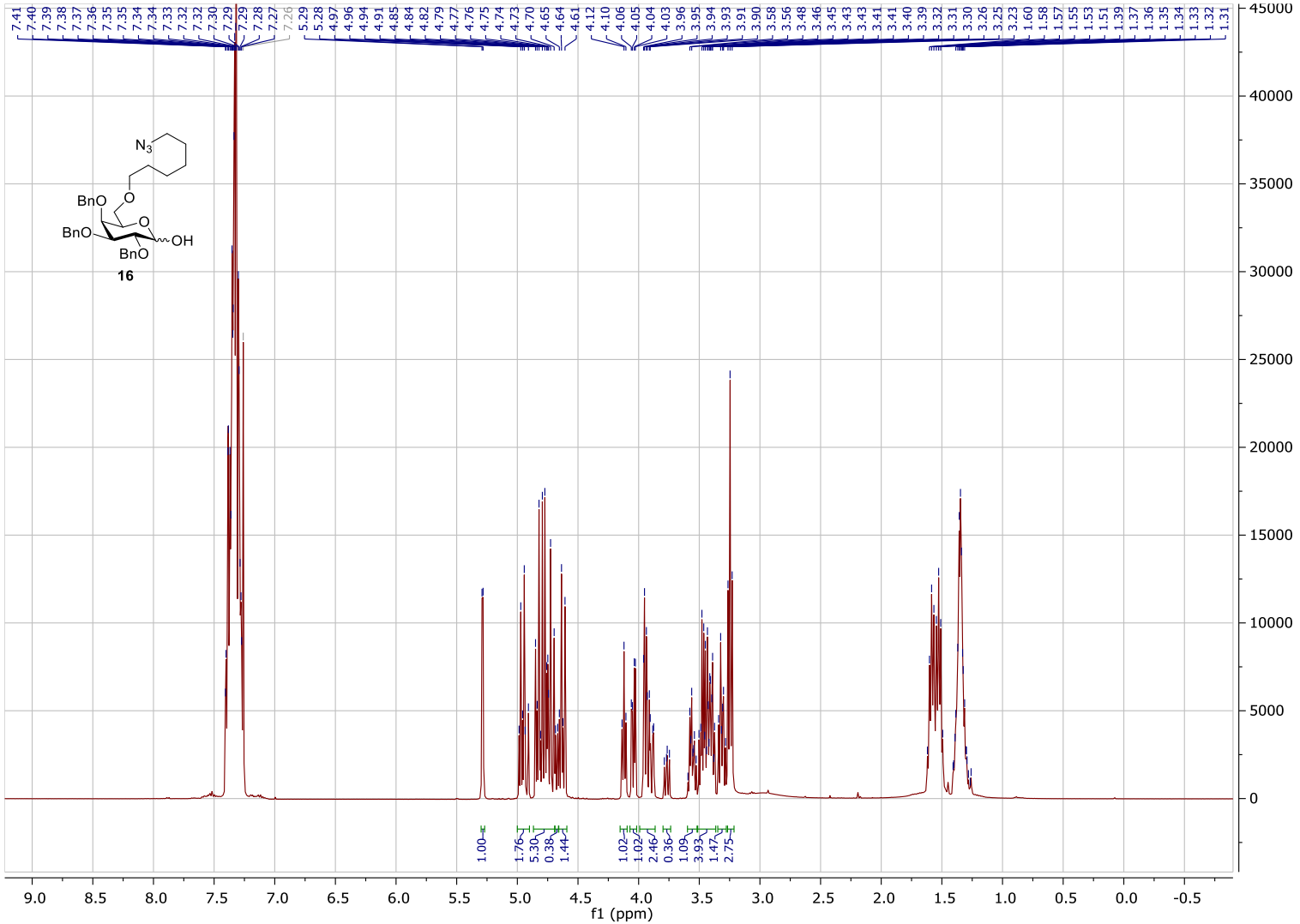
Phenyl 6-(6'-azidohexyl)-2,3,4-tri-*O*-benzyl 1-thio-β-D-galactopyranoside (15)



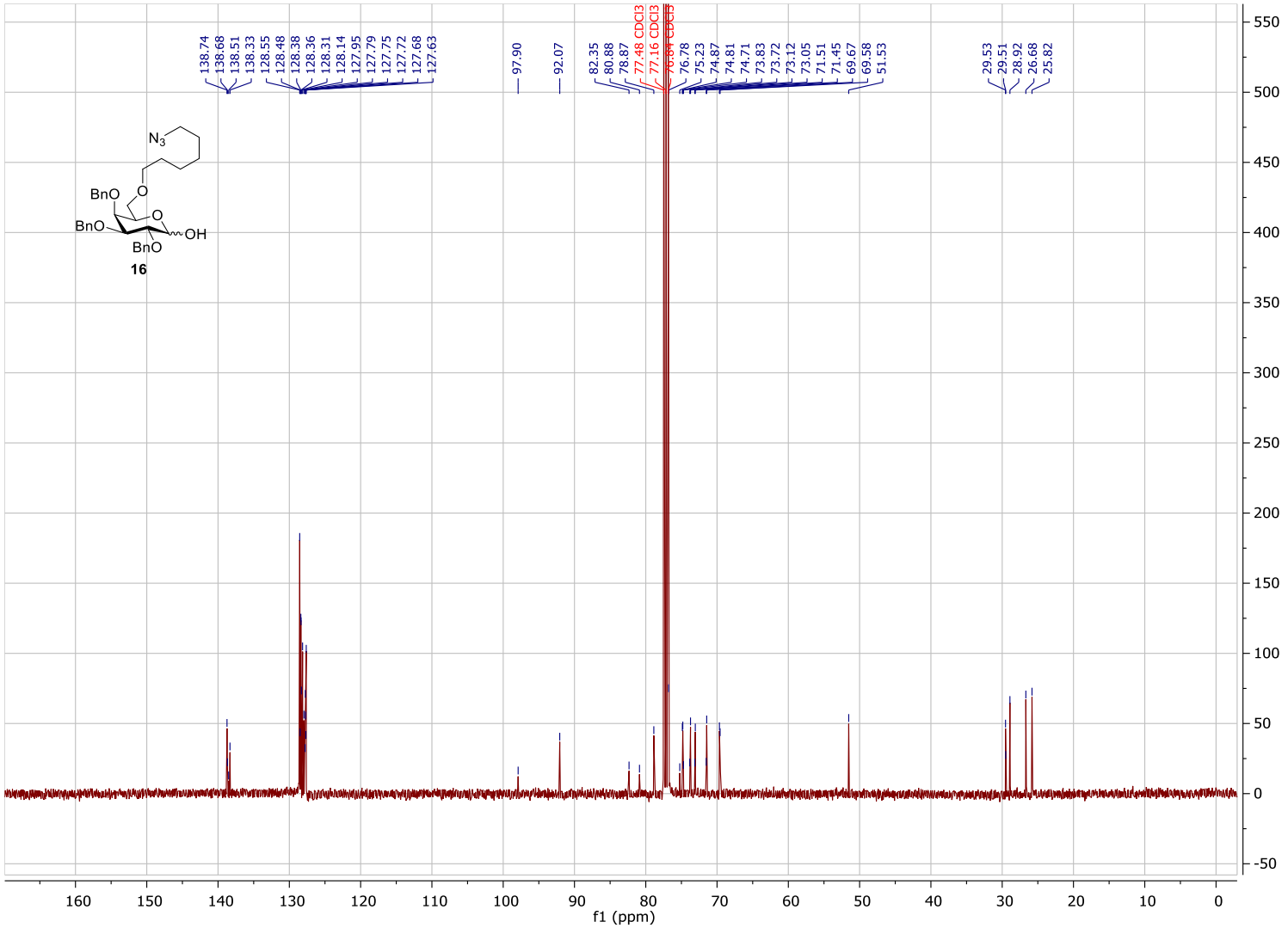
Phenyl 6-(6'-azidohexyl)-2,3,4-tri-*O*-benzyl 1-thio-β-D-galactopyranoside (15)



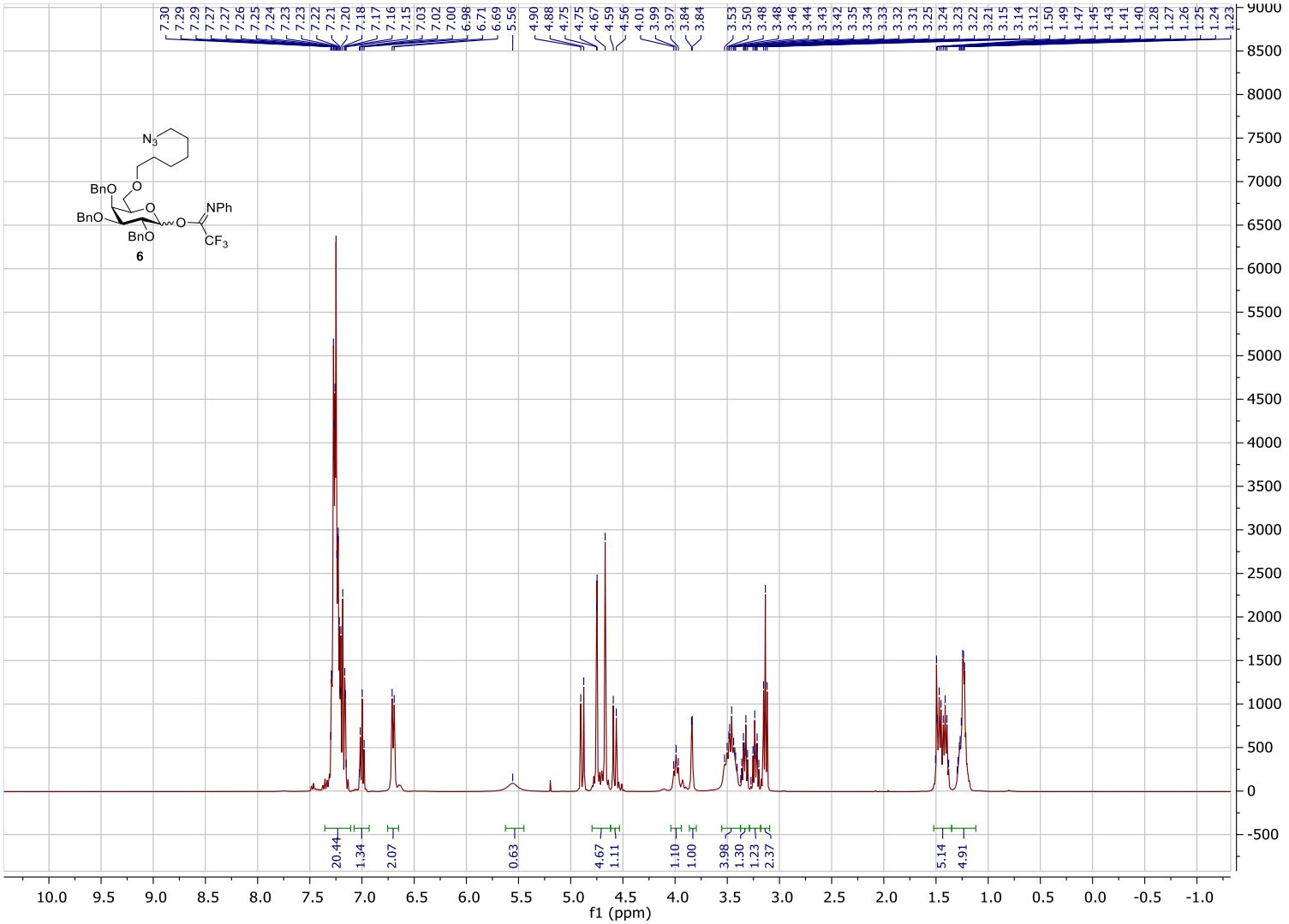
6-(6'-Azidohexyl)-2,3,4-tri-*O*-benzyl-D-galactopyranose (16)



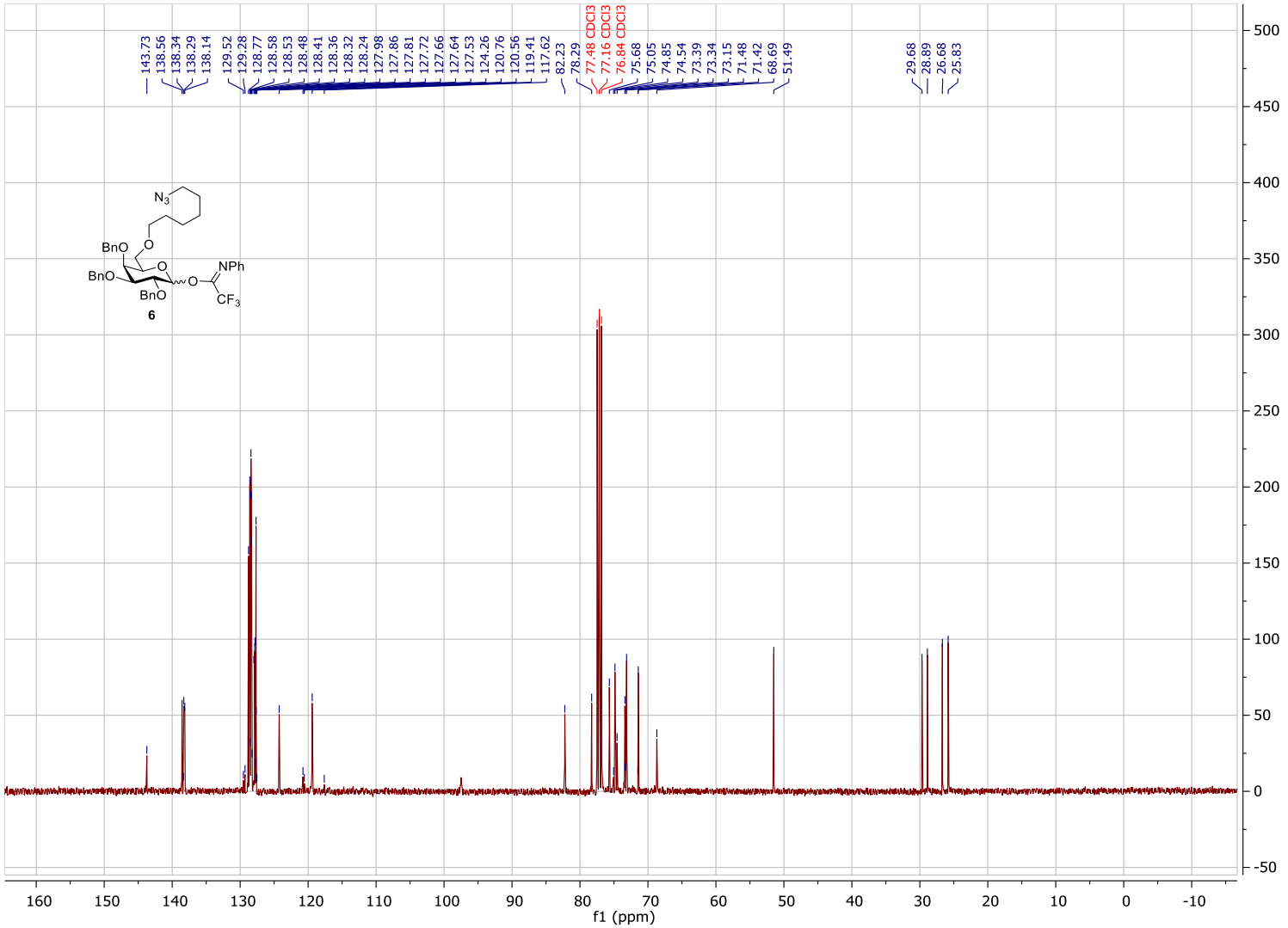
6-(6'-Azidohexyl)-2,3,4-tri-*O*-benzyl-D-galactopyranose (16)



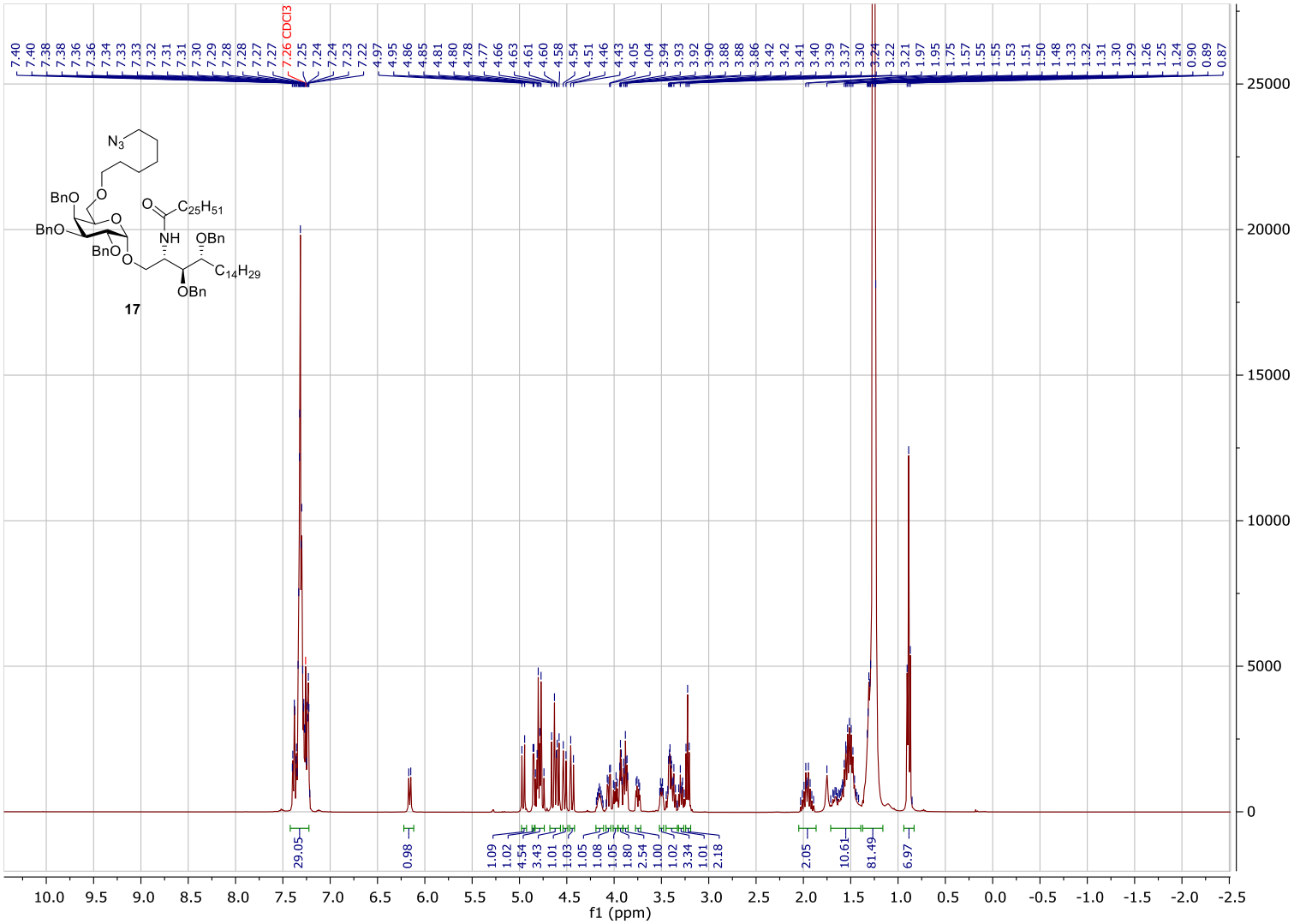
6-(6'-Azidohexyl)-2,3,4-*tri*-*O*-benzyl-*β*-D-galactopyranosyl *N*-phenyl trifluoroacetimidate (6)



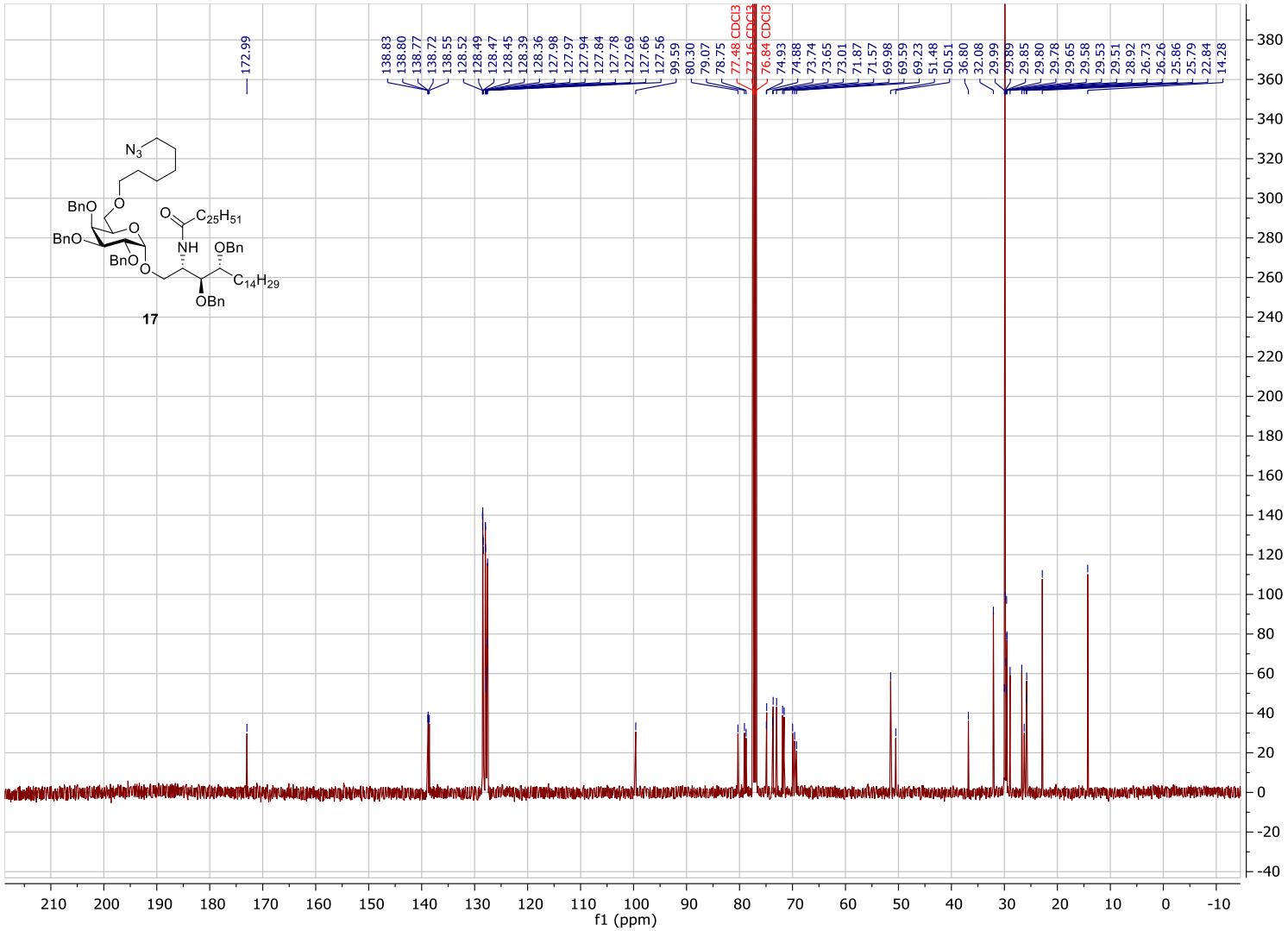
6-(6'-Azidohexyl)-2,3,4-*tri*-*O*-benzyl-*β*-D-galactopyranosyl *N*-phenyl trifluoroacetimidate (6)



(*2S,3S,4R*)-3,4-Bis-benzyloxyl-2-hexacosanoylamino-1-(6-(6'-azidohexyl)-2,3,4-tri-*O*-benzyl)-D-galactopyranosyl)octadecane (17)

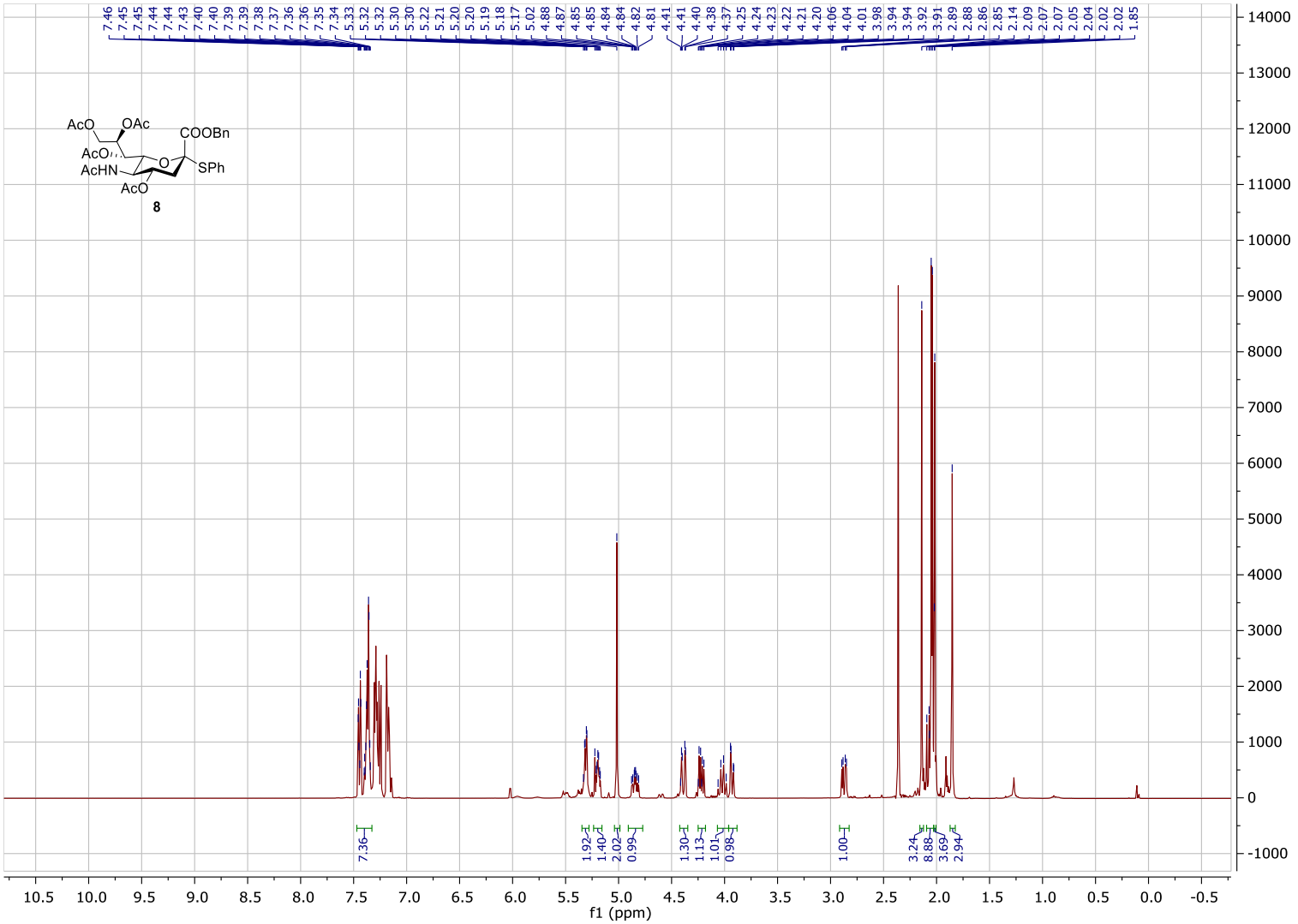


(*2S,3S,4R*)-3,4-Bis-benzyloxyl-2-hexacosanoylamino-1-(6-(6'-azidohexyl)-2,3,4-tri-*O*-benzyl)-D-galactopyranosyl)octadecane (17)



17

Benzyl (phenyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-2-thio-β-D-glycero-D-galacto-2-nonulopyranosid)onate (8)



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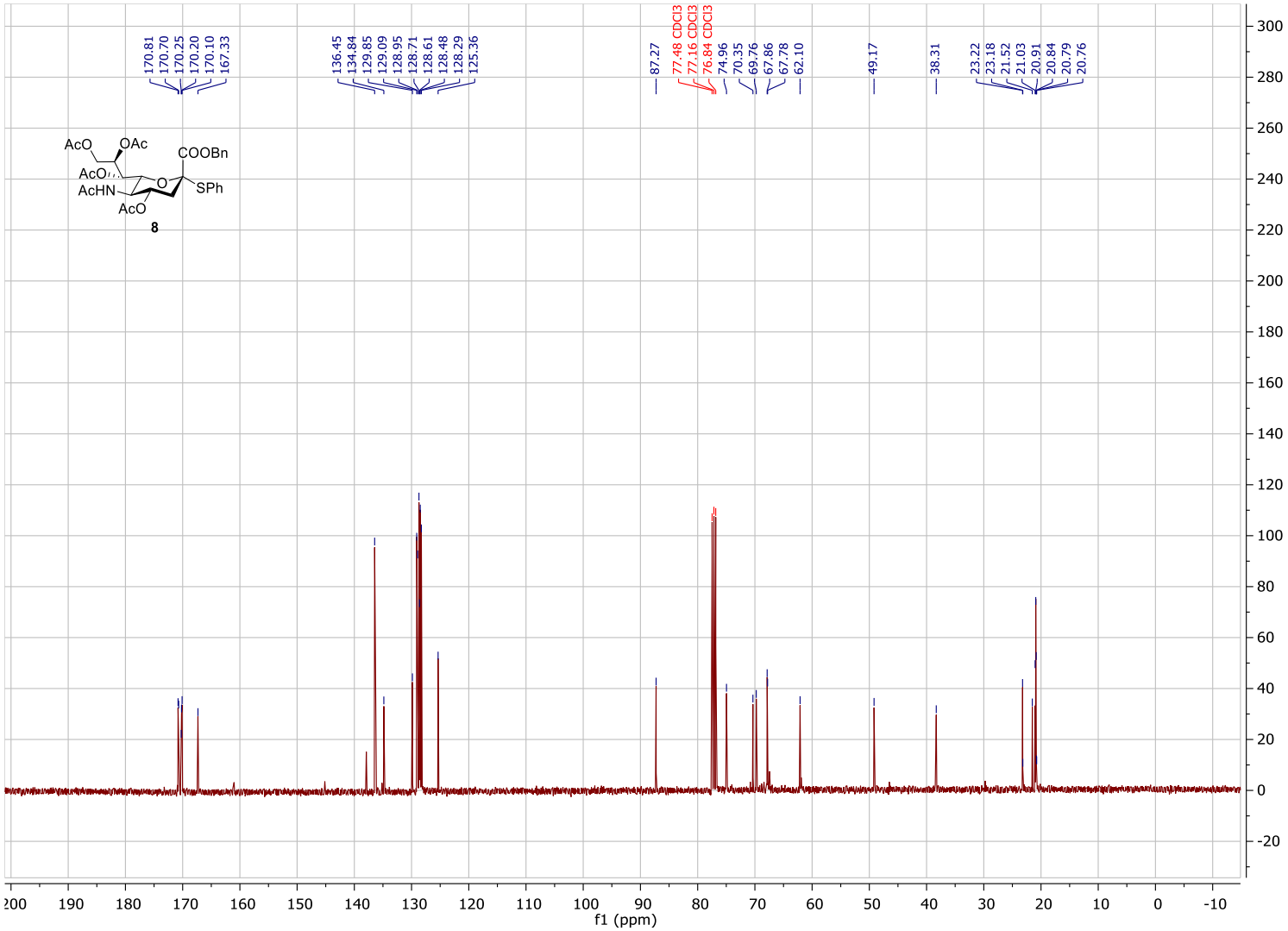

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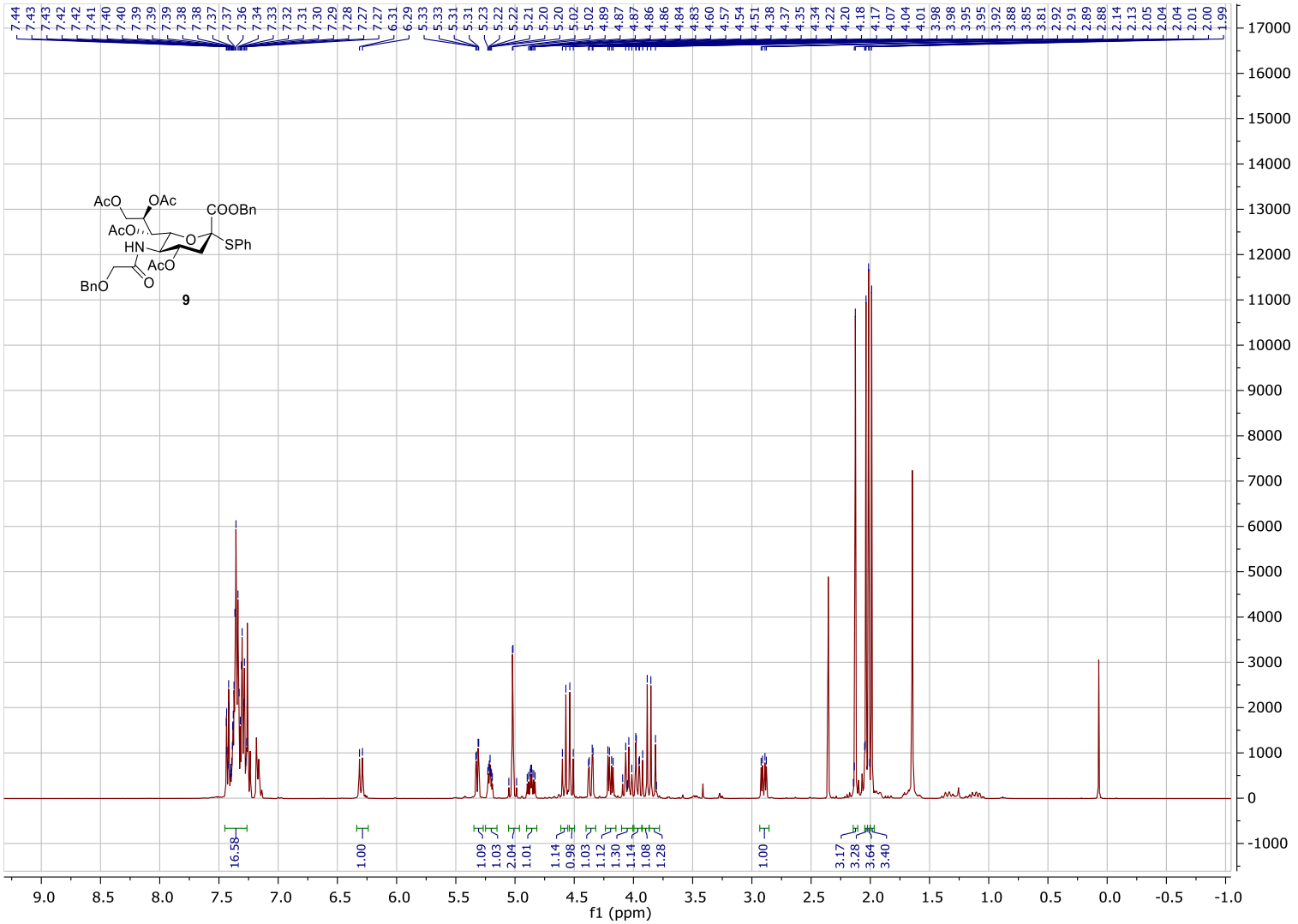

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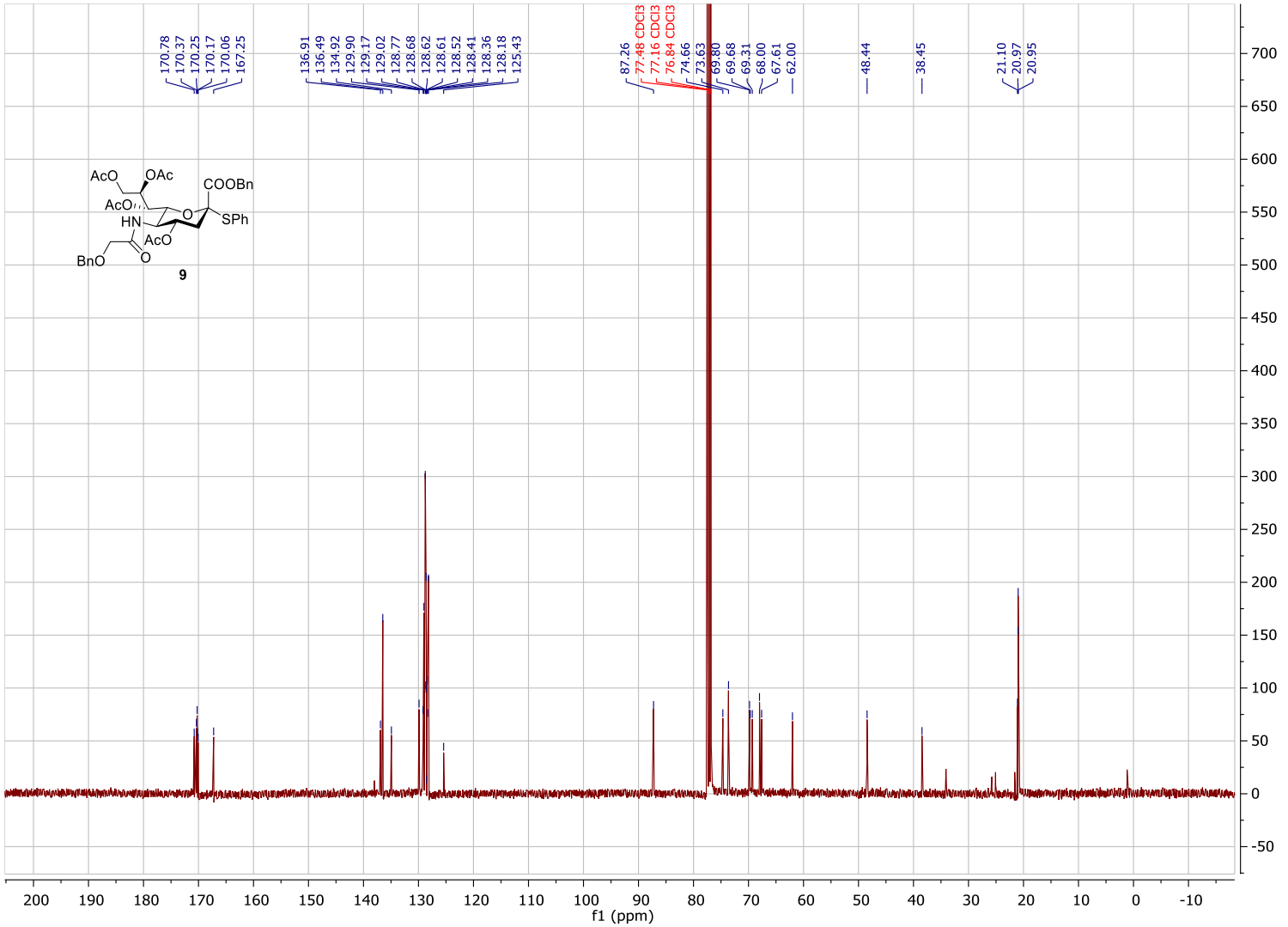

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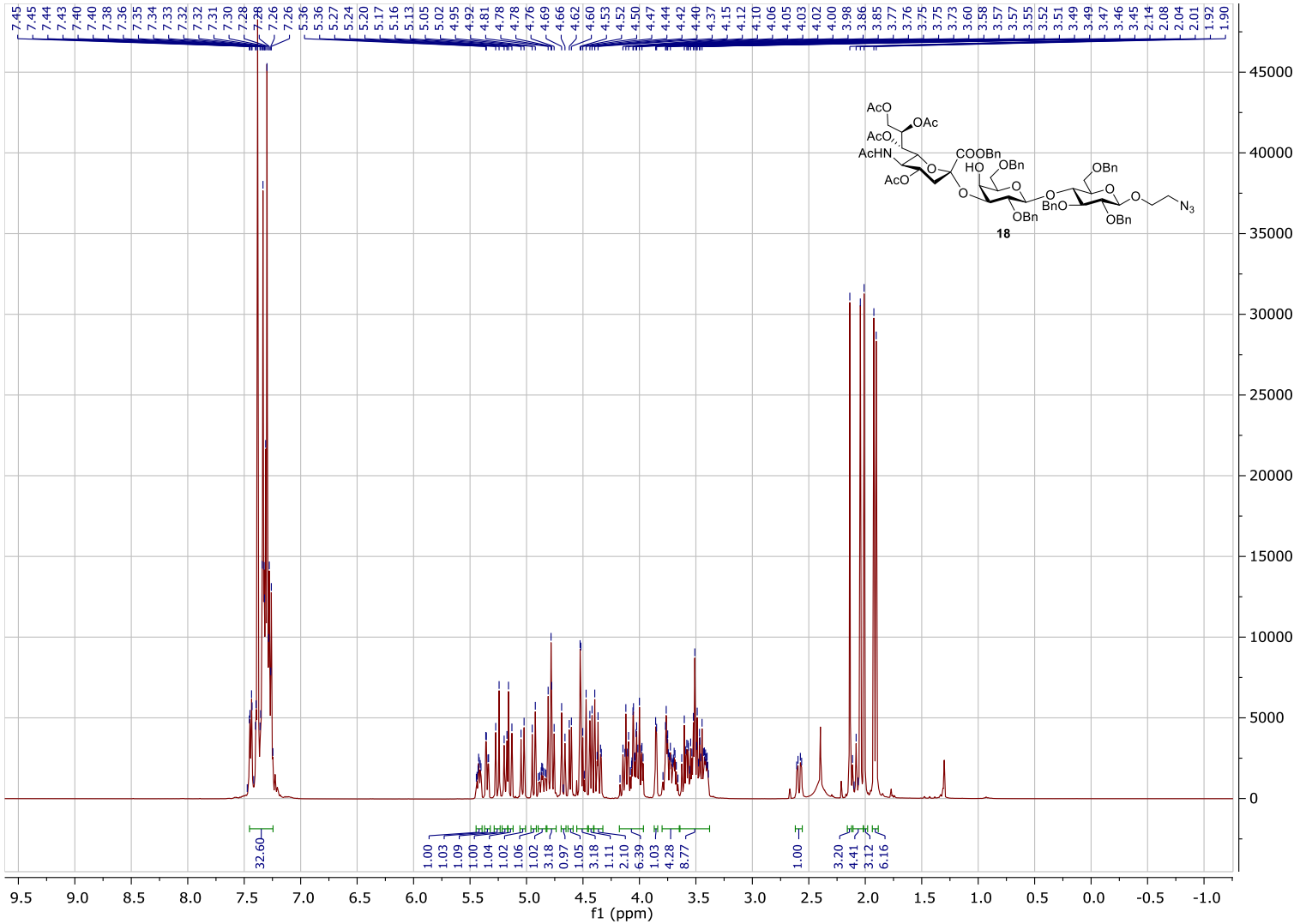

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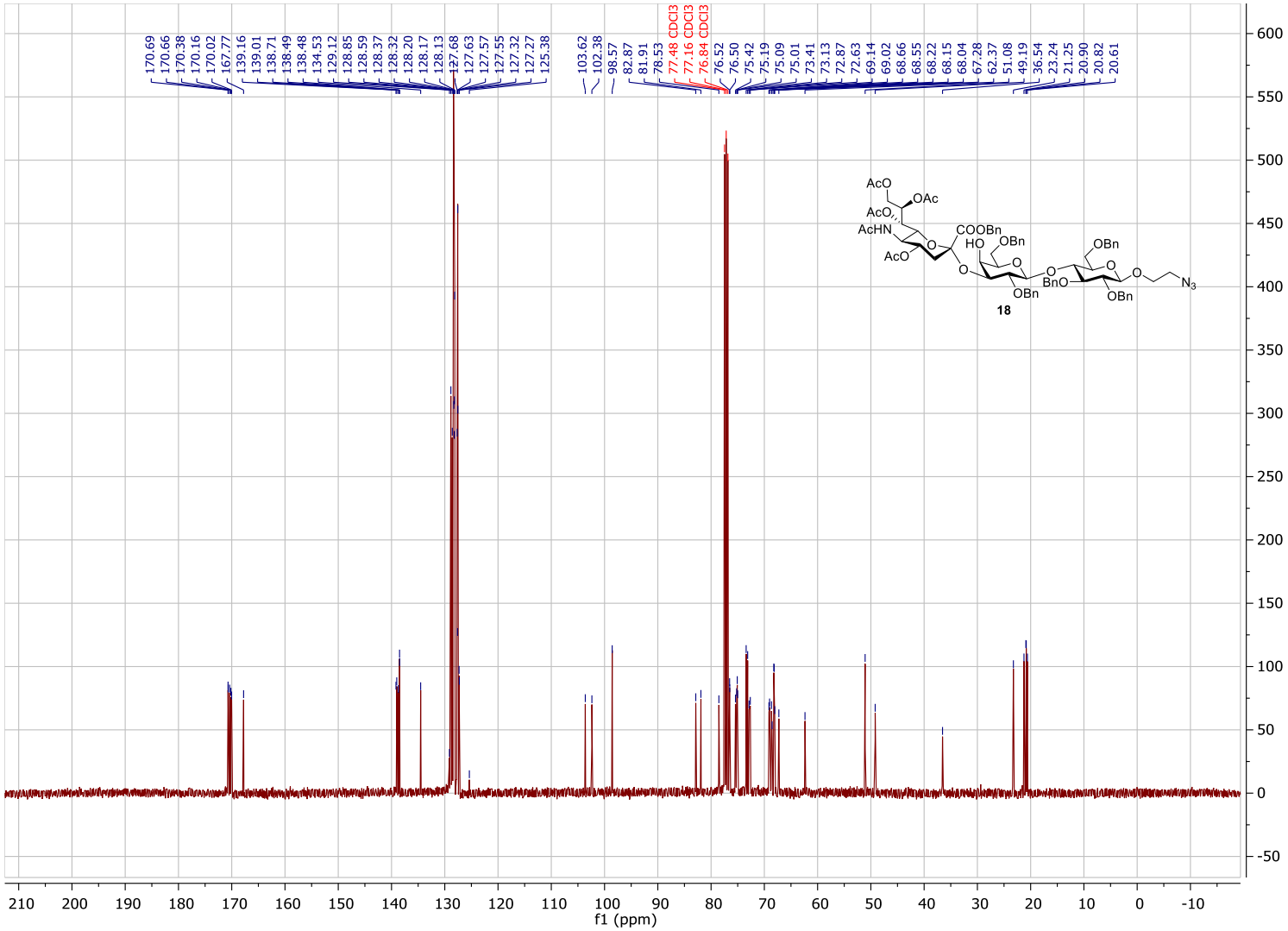

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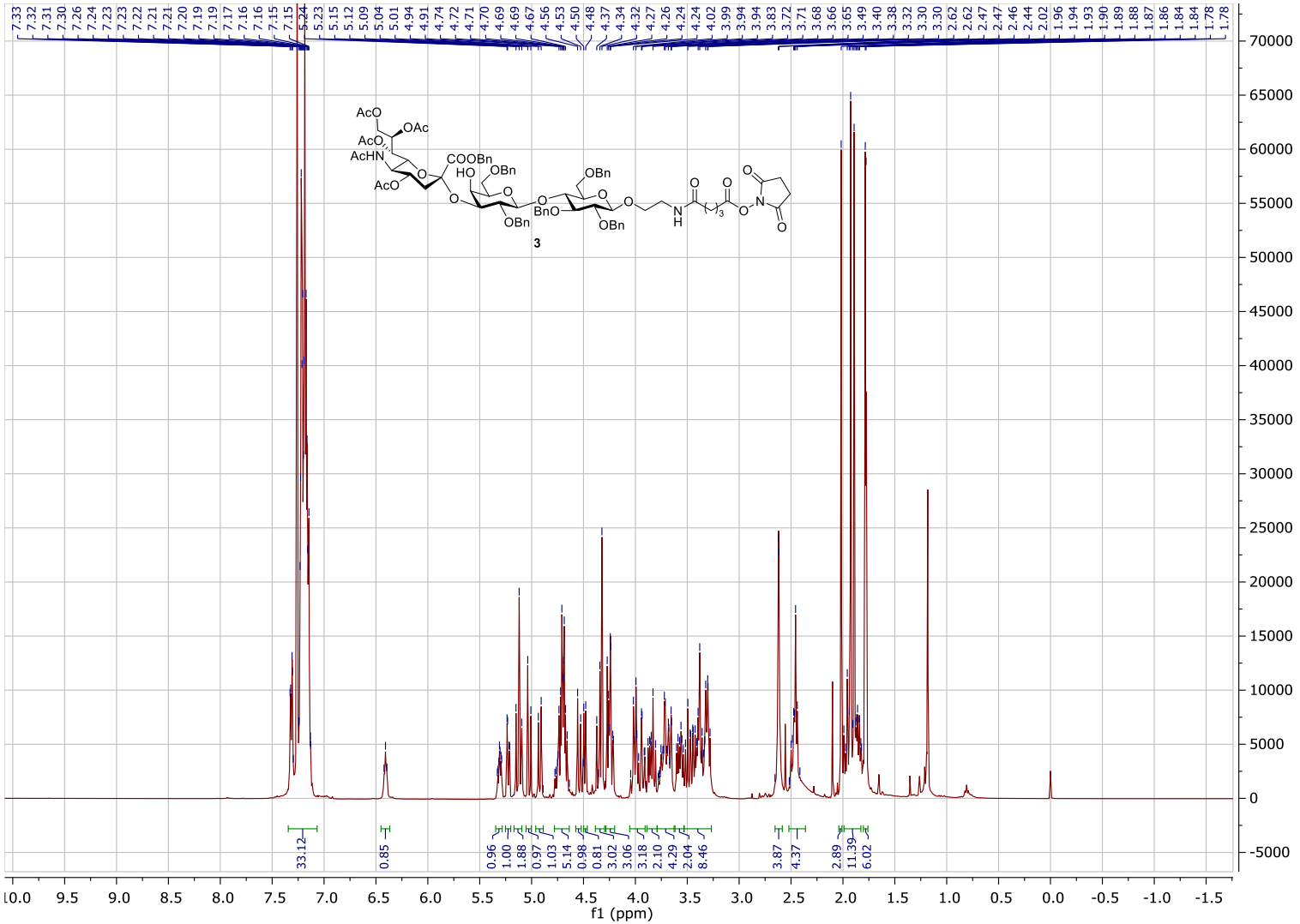

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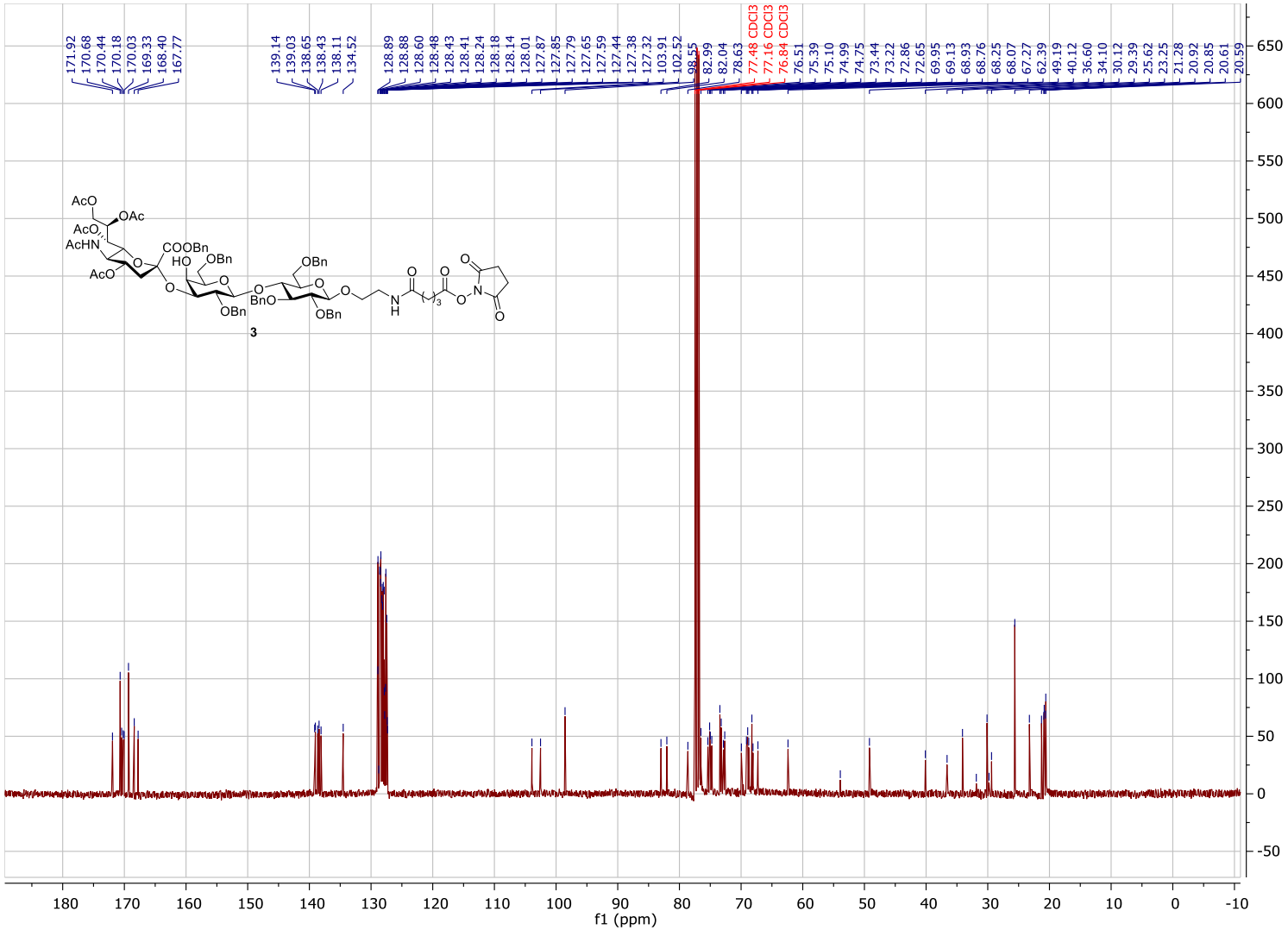

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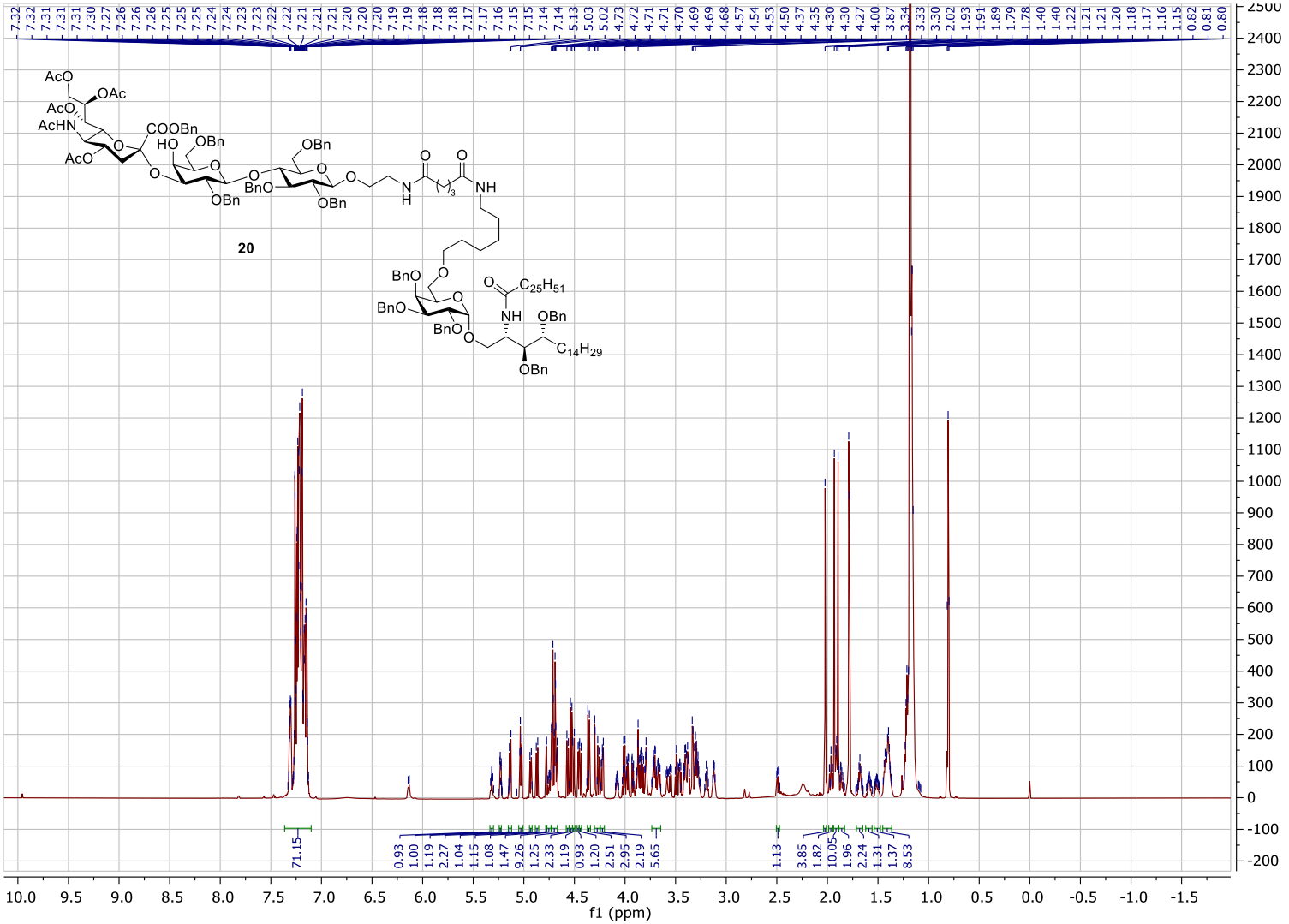

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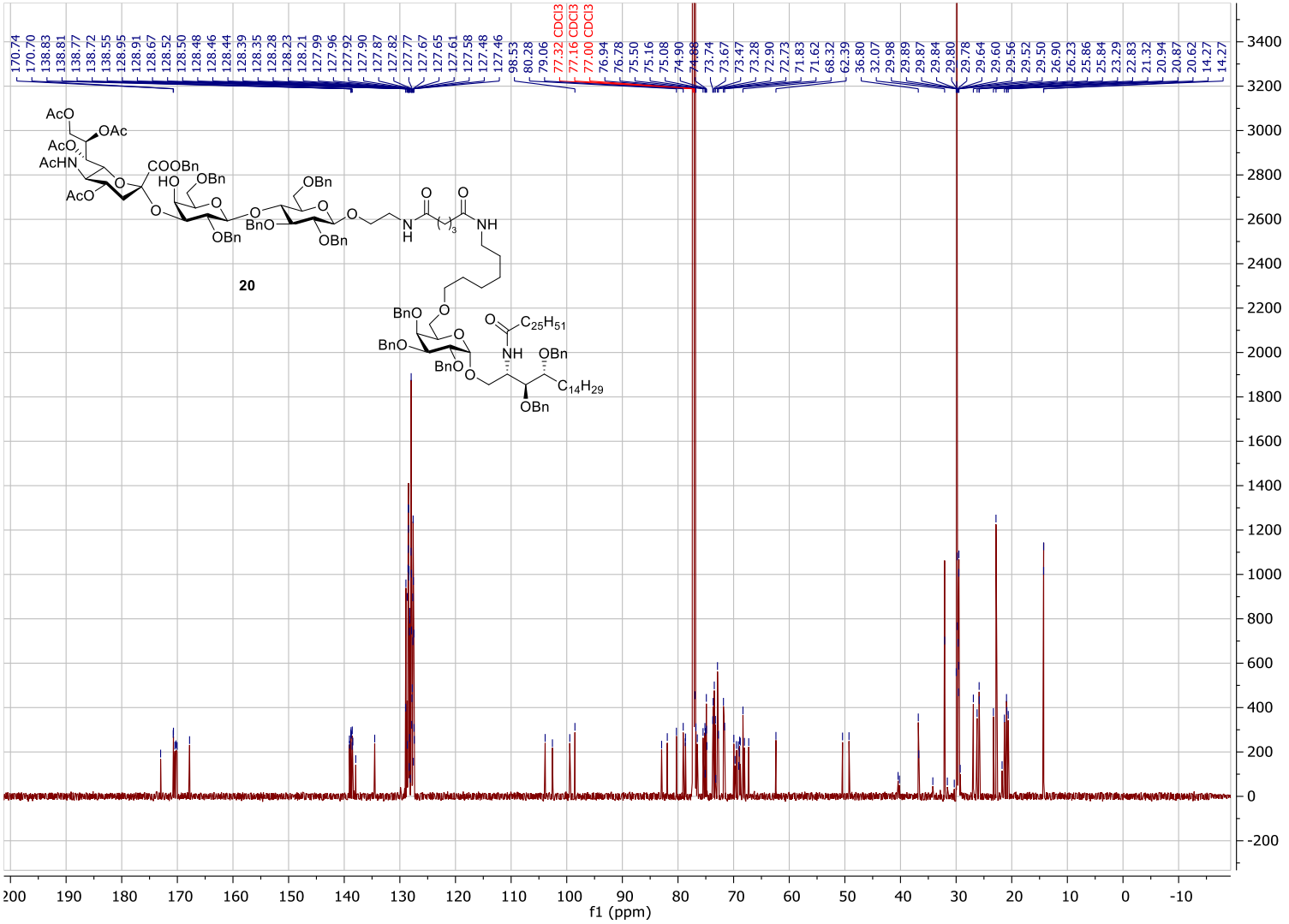
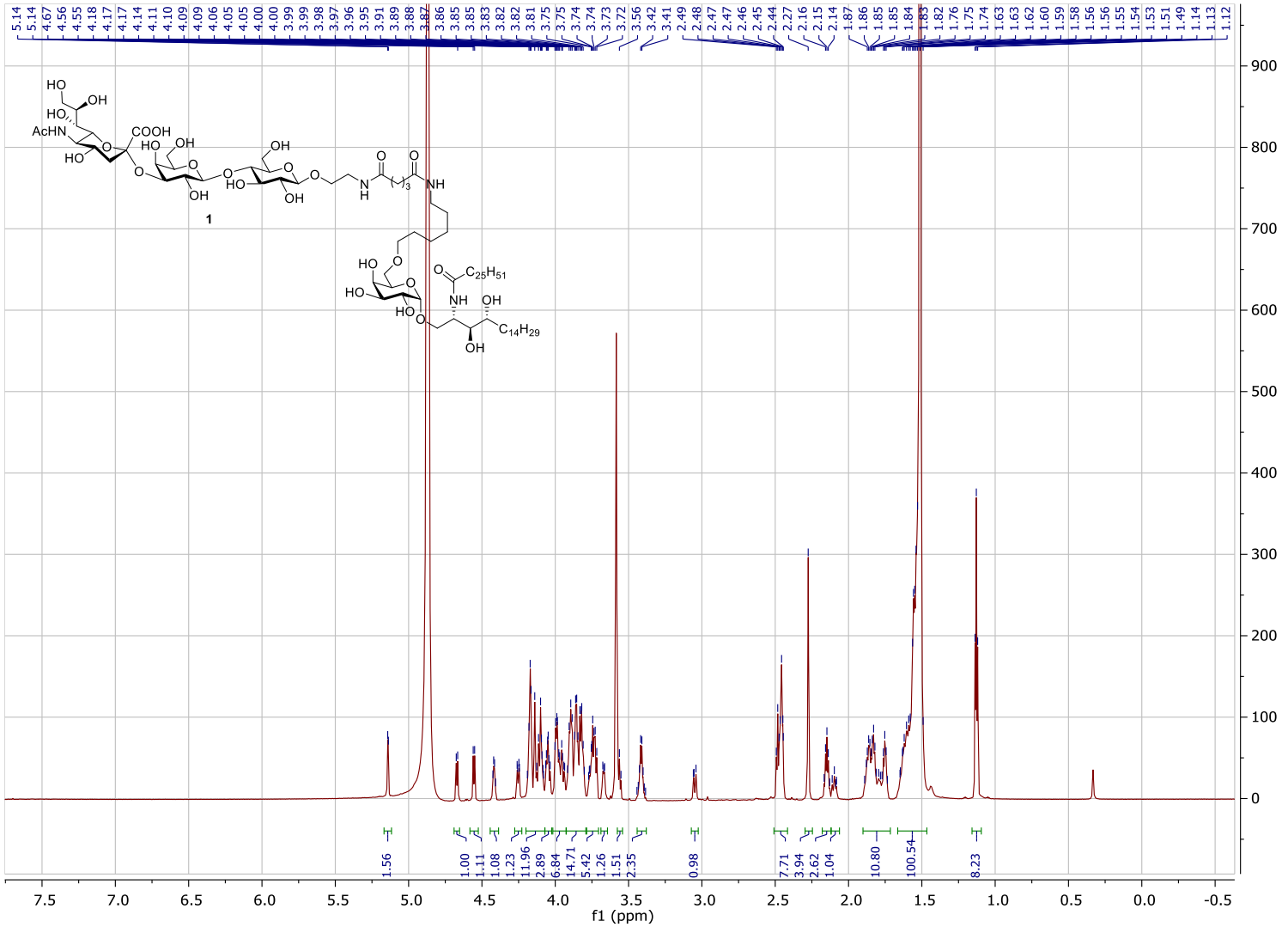
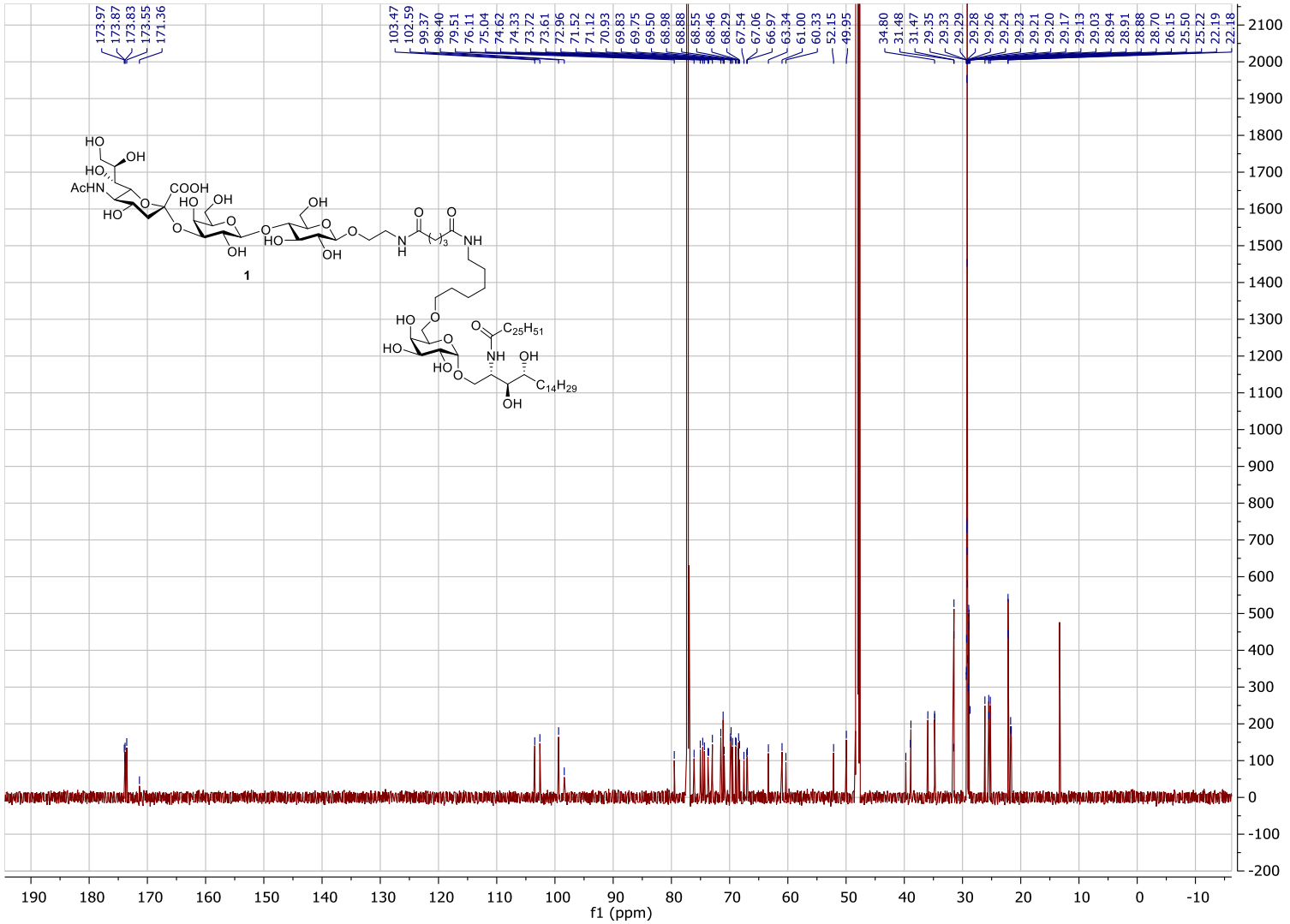


GM3–αGalCer conjugate (1)



GM3–αGalCer conjugate (1)



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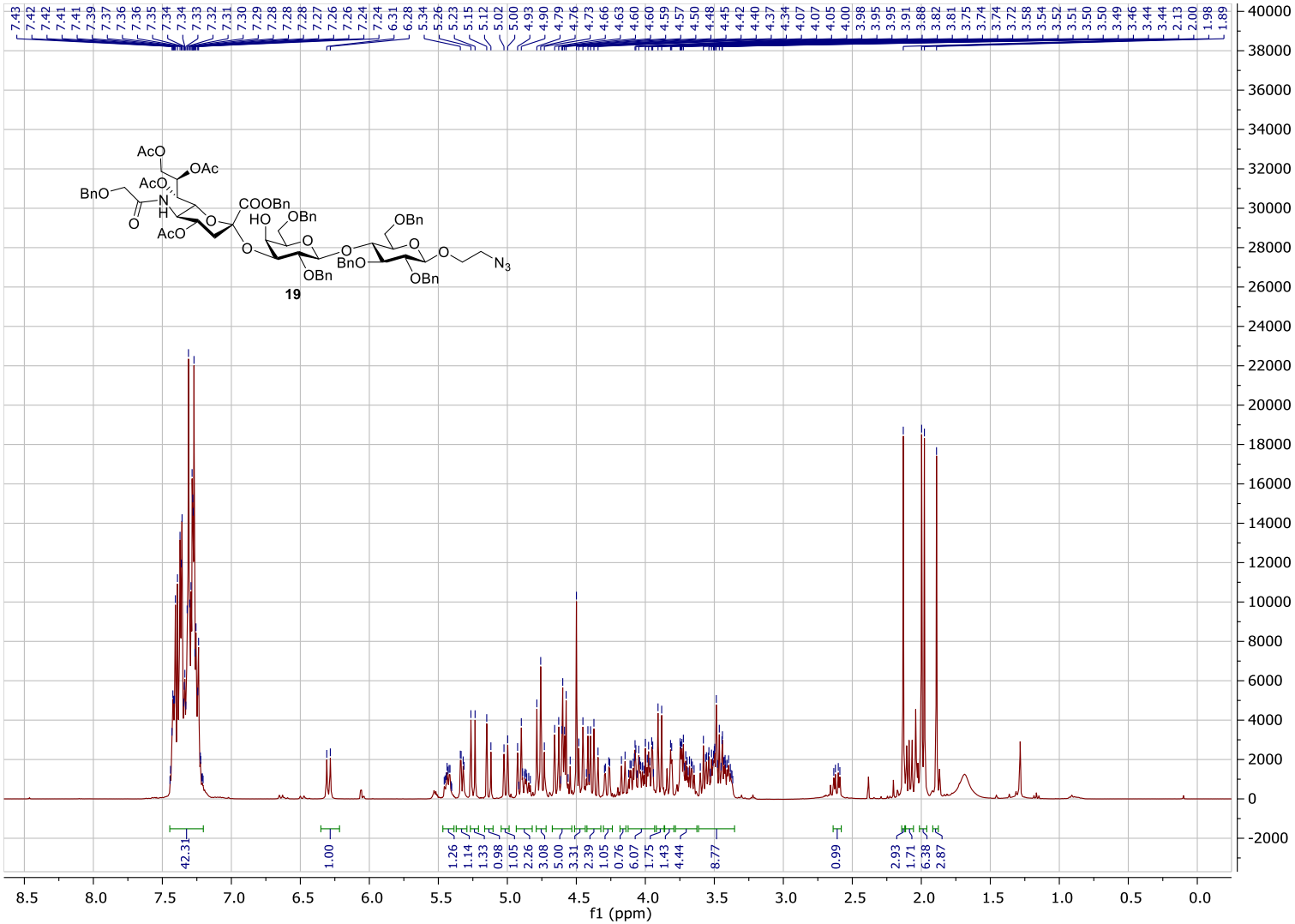

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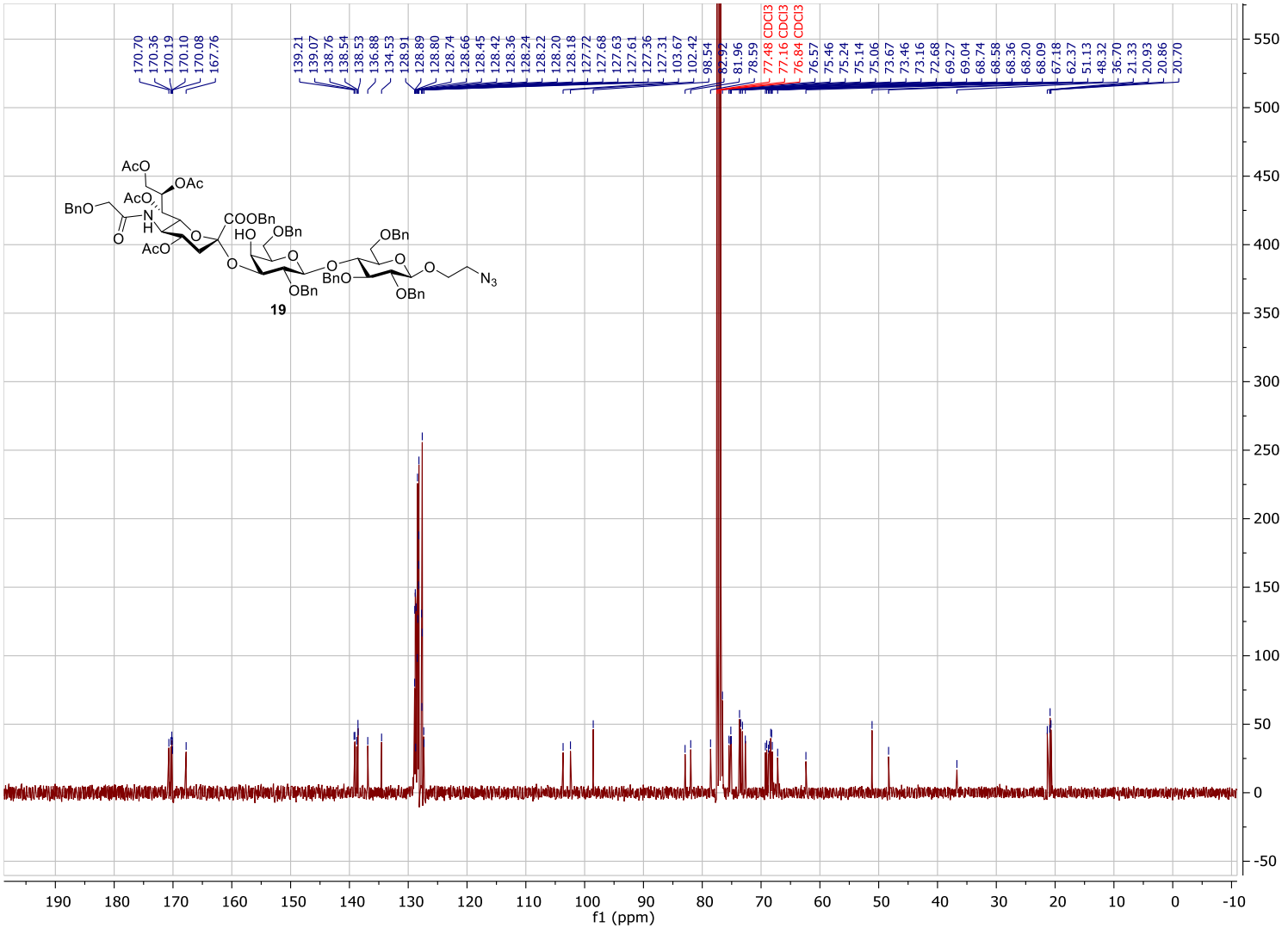

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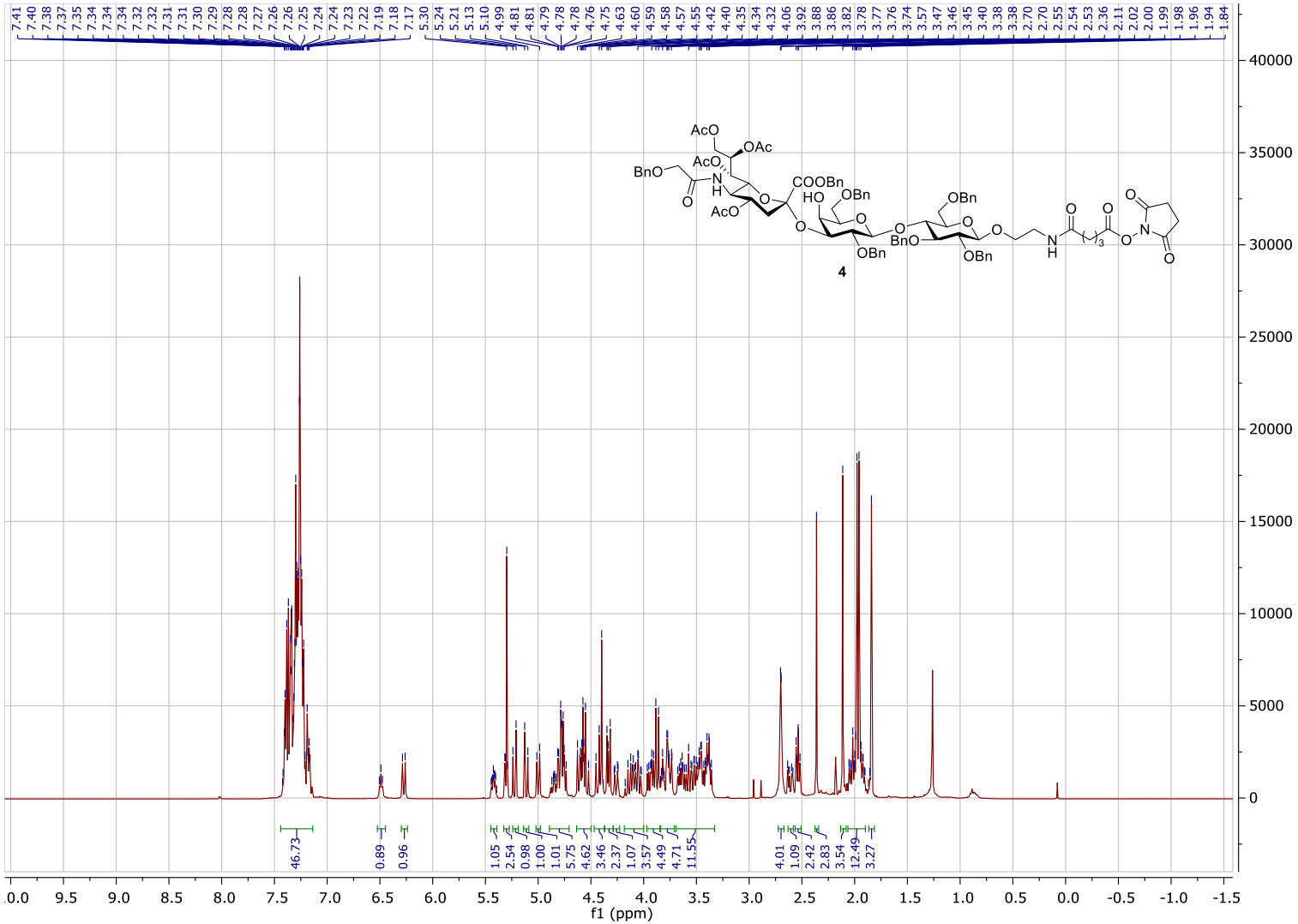

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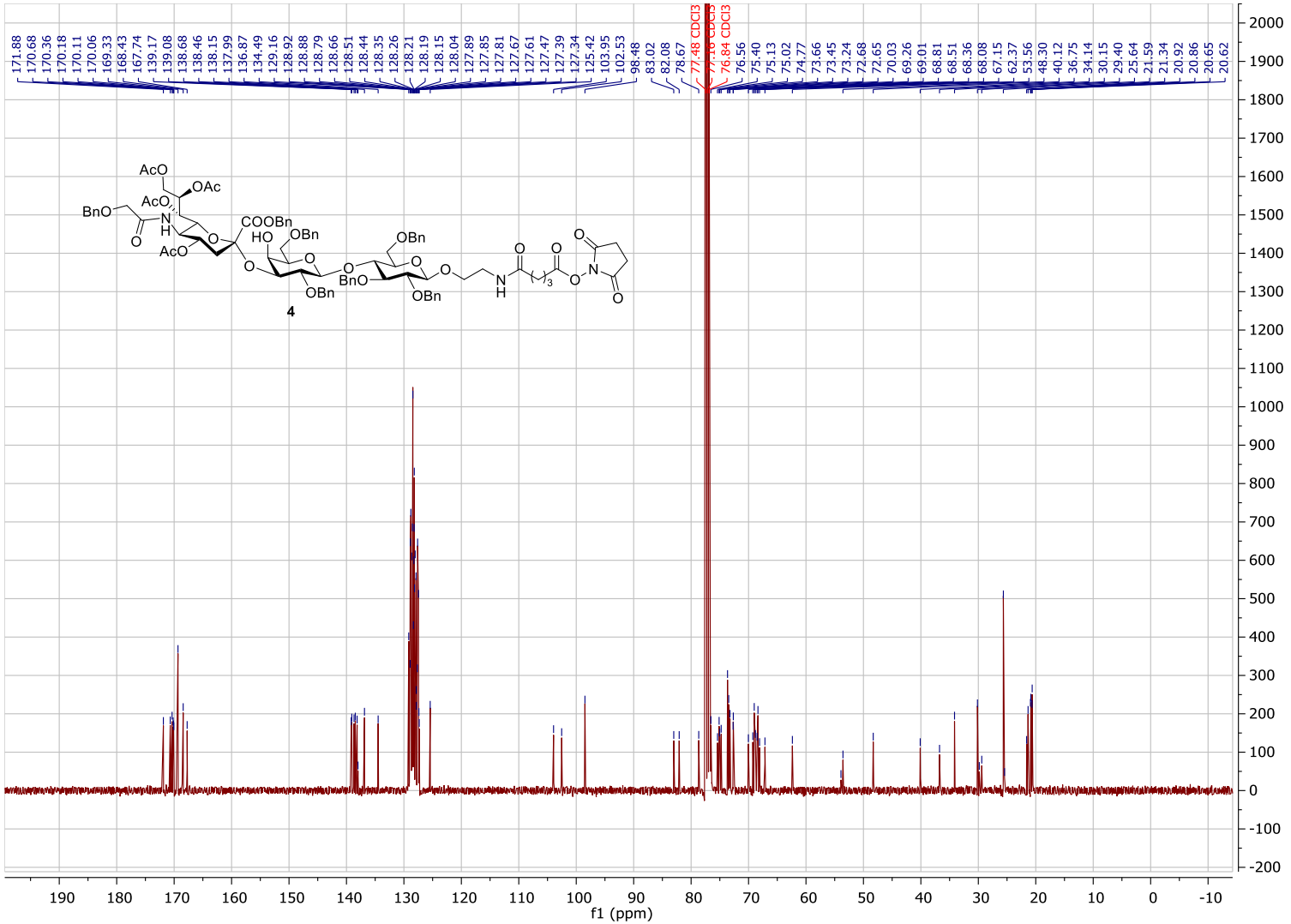

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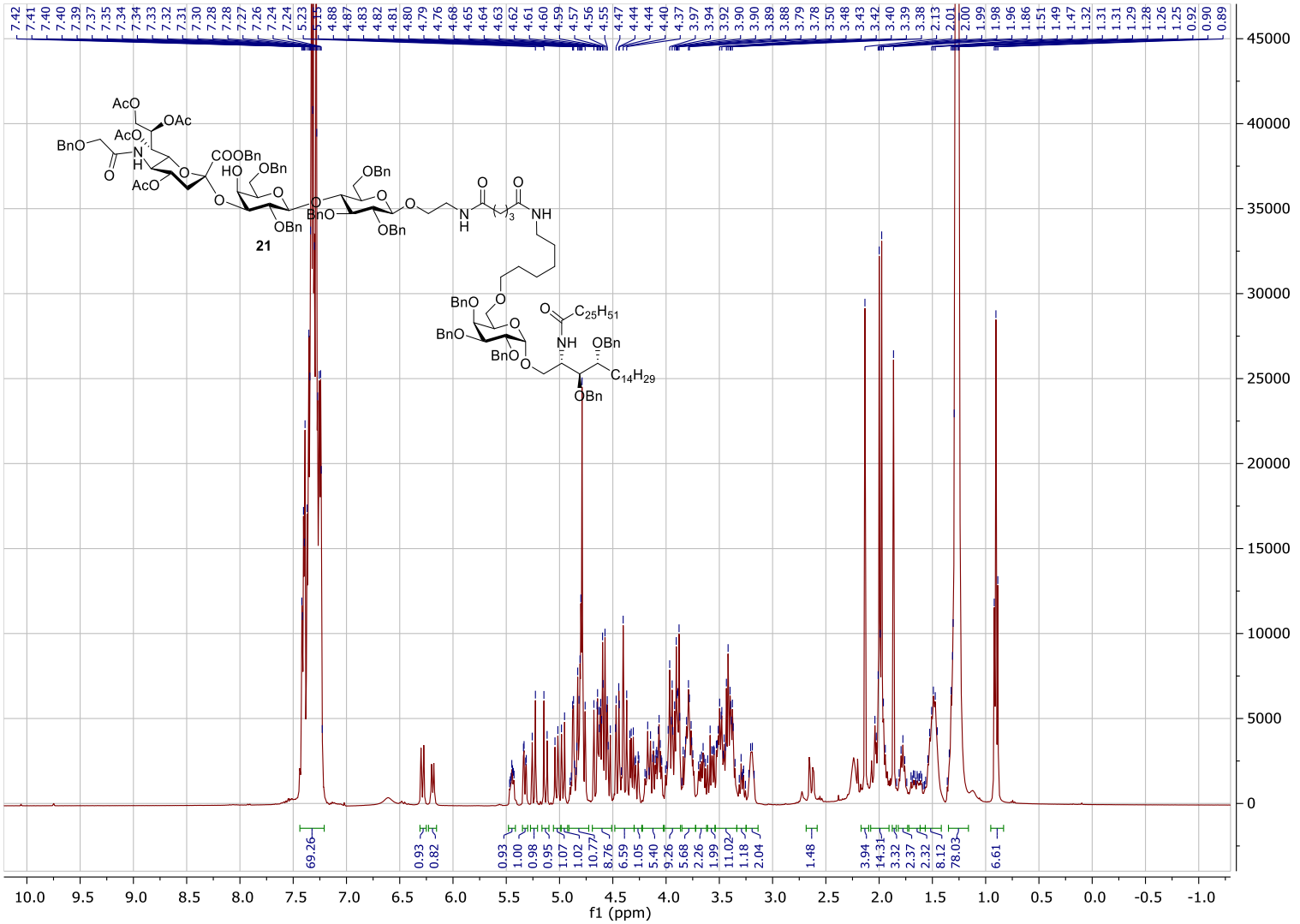

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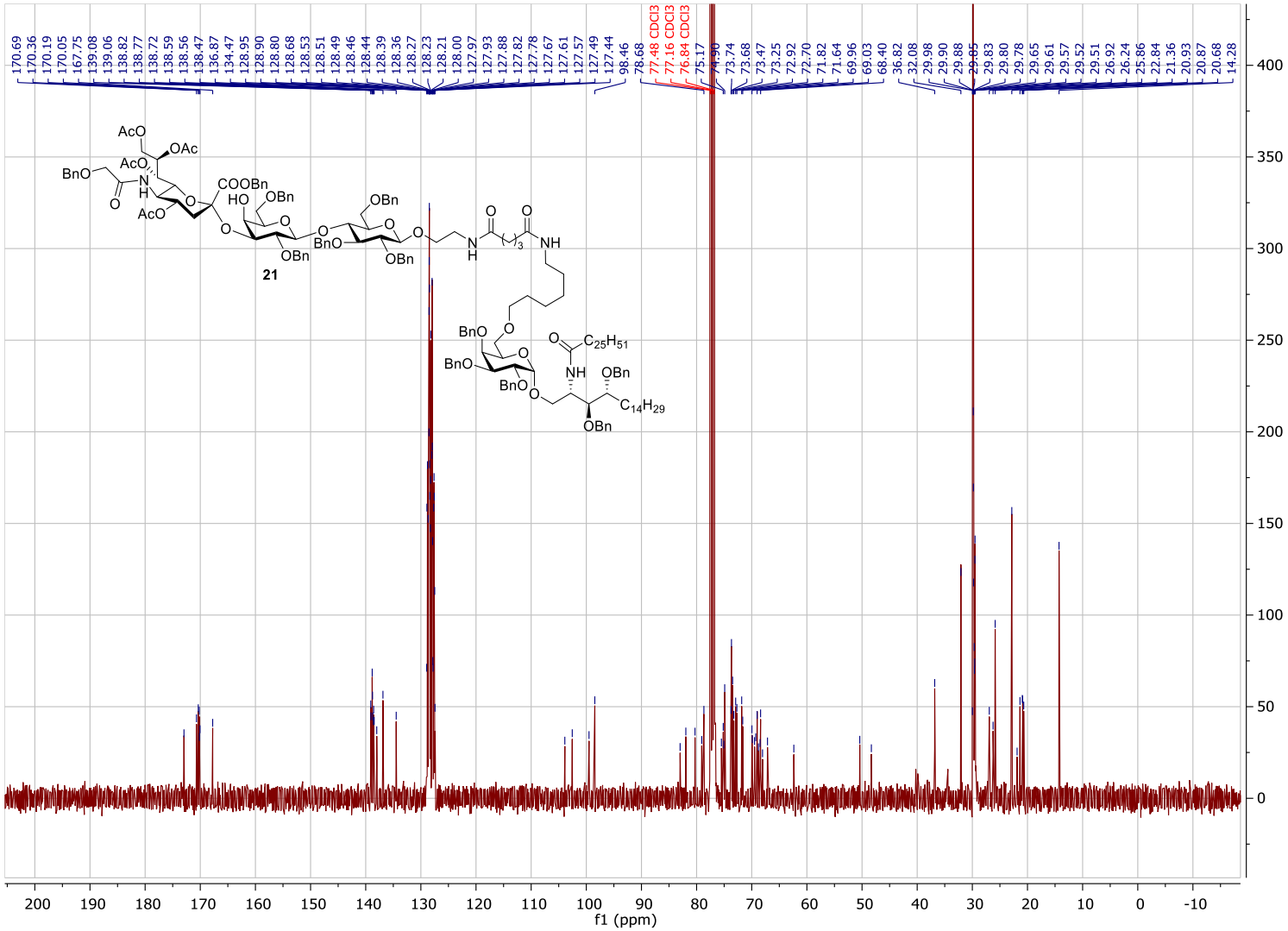

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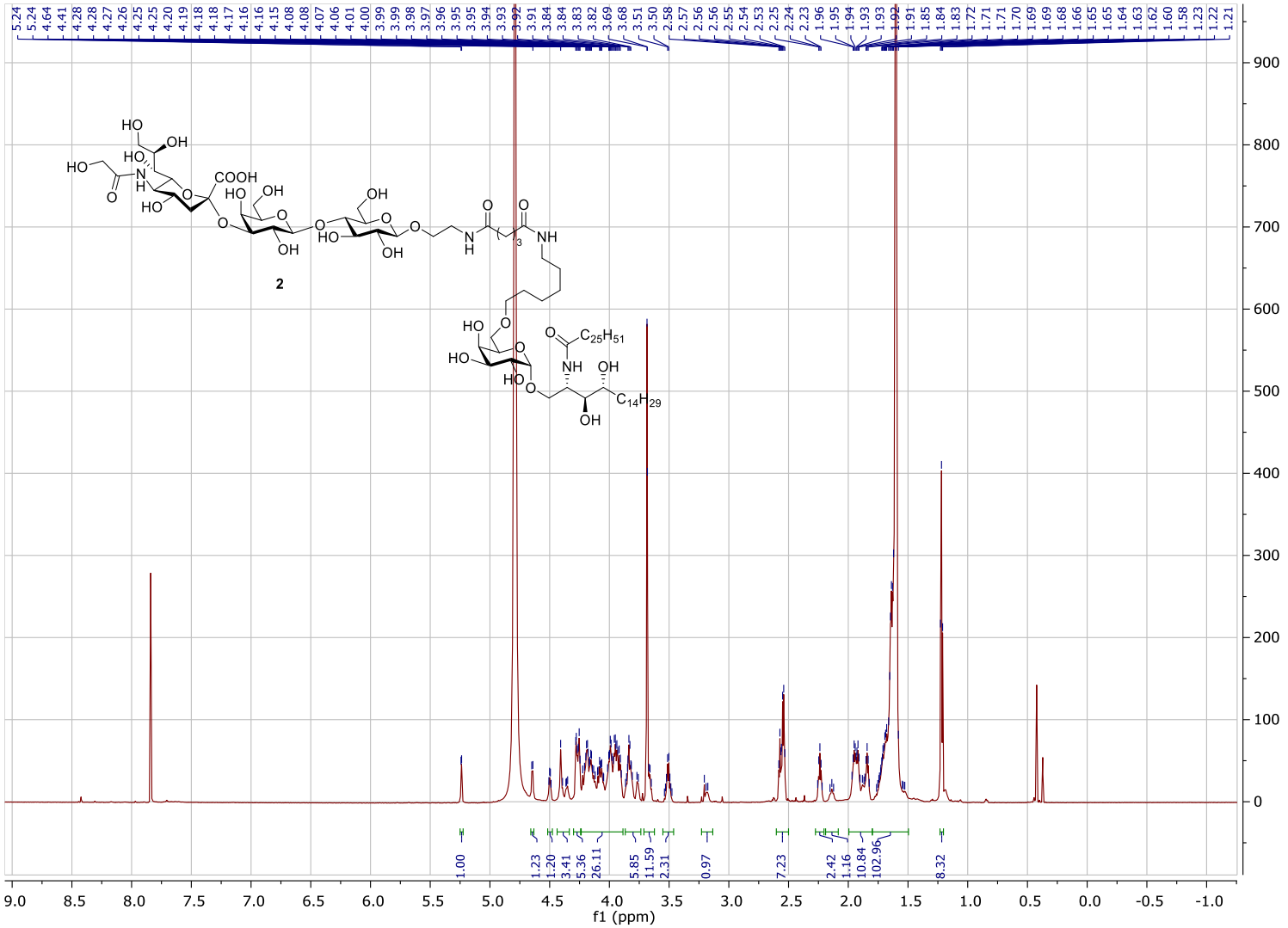
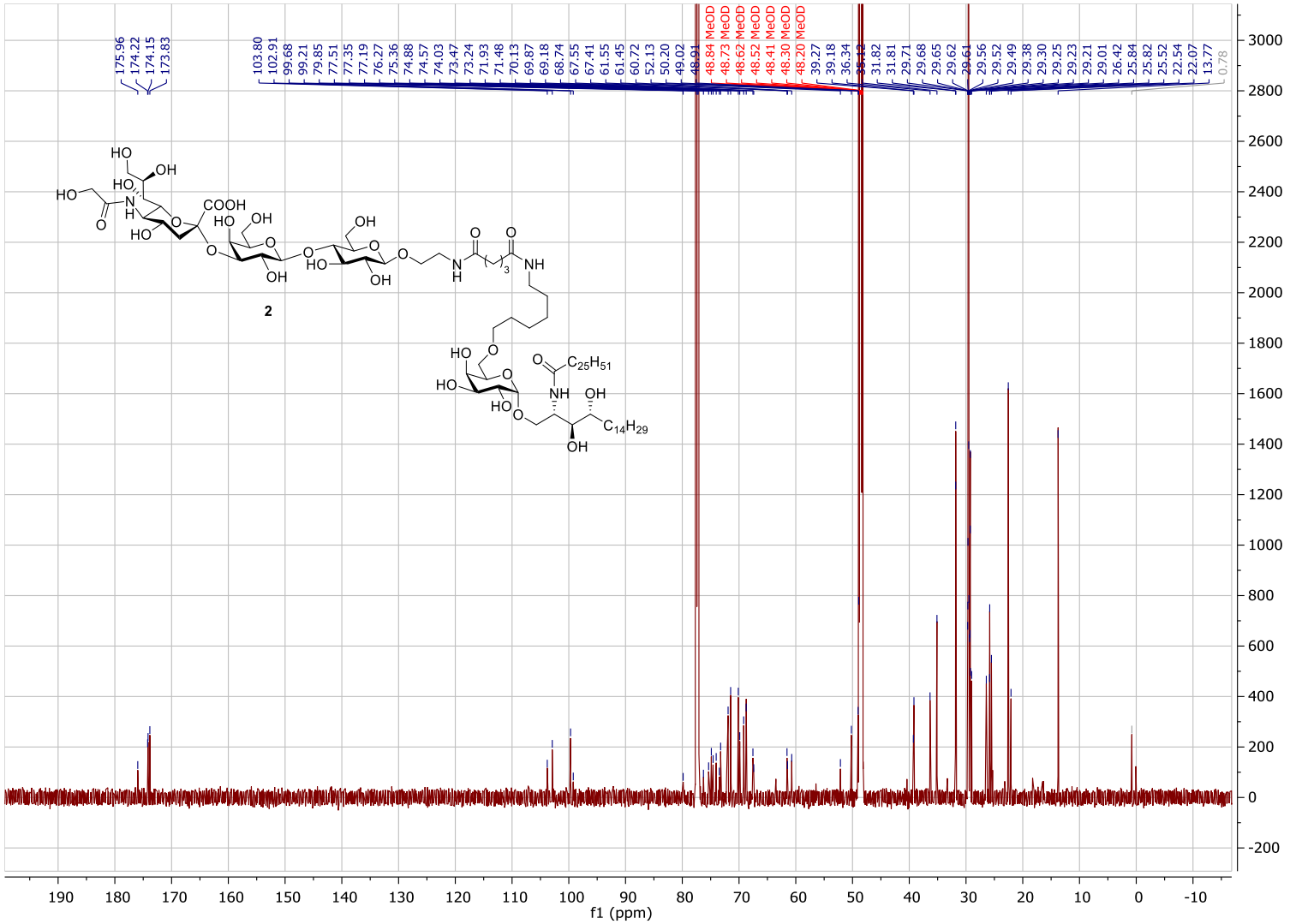


(Neu5Gc)GM3–αGalCer conjugate (2)



Formulations

Preparation and Characterization of Liposomes. Liposome precursor solutions were prepared by mixing either commercial GM3 (CarboSynth Ltd., UK) and αGalCer (CarboSynth Ltd., UK) or **1**, or **2**, or equimolar amounts of **1** and **2** with DSPC (TCI, USA) and cholesterol (Sigma-Aldrich, USA) in *tert*-butanol:H_2O (9:1) at 70 °C for 30 minutes. The solvent was removed by freeze-drying overnight to yield a thin film on the glass vial. The lipid film was re-hydrated by adding 1 mL of HEPES buffer (pH= 7.4) under stirring at 70 °C for 1 hour. The suspension was then extruded, using a preheated (70 °C) extruder (Avanti Polar Lipids), through a polycarbonate membrane of 200 nm pore size (Avanti Polar Lipids, USA). Each lipid suspension was passed a minimum number of 21 times through the membrane to ensure the formation of liposomes with desired sizes. Liposomes were then characterized by Zetasizer μV device (Malvern Instruments Ltd.) and the characteristics are shown in Table S1.

Liposome	Size (nm)	PDI	ζ potential (mV)
I: GM3–αGalCer conjugate **1**	177.9±51.1	0.051±0.03	-21.8±5.0
II: (Neu5Gc)GM3–αGalCer conjugate **2**	185.2±63.1	0.066±0.01	-28.5±4.8
III: Equimolar amounts of conjugate **1** and **2**	167.4±43.9	0.073±0.06	-29.2±3.5
IV: GM3 and αGalCer	180.6±33.4	0.045±0.03	-27.8±4.4

Table S1. Liposomal size, PDI (polydispersity index) and zeta potential. The data are the mean ± SEM of three measurements

The contents of the liposomes were analyzed by RP-HPLC-MS/MS and ICP-MS in order to evaluate the recovery of each individual component. The concentration of all antigens and adjuvants as well as cholesterol, used as a proxy for liposome concentration, was determined using reverse phase liquid chromatography tandem mass spectrometry (RP-HPLC-MS/MS) using a Shimadzu LCMS 8054 equipped with Phenomex C8 column (d 2.6 μm, 100 x 2.1 mm). Eluents: A (50% CH_3CN in water with 1% HCOOH) and B (iPrOH with 1% HCOOH). The LC program was setup to get separation between relevant compounds to prevent ion suppression (see Table S2 for details). Setup of precursor and product ions for all compounds was performed prior to analysis (see Table S3 for details). 5 μL of sample were injected for each analysis and all liposomes were analyzed in duplicate. The stock liposomes were diluted 1:200 in water:CH_3CN (1:1). To ensure semi-quantitative results external standards were prepared of all components in a blank matrix at relevant concentrations (Cholesterol: 3.13–100 μM; GM3, αGalCer, and all conjugates: 0.38–12 μM).

Time	% B
1	30
5	100
8	100
10	30
12	30

Table S2. Full LC program used for the analysis of the liposomal formulations

Compound	Precursor m/z	Product m/z	CE	Q1 Bias	Q3 Bias	RT (min)	Mode
Cholesterol[a]	369.5	109	-35			5.99	Pos
αGalCer[a]	859	679	-35			6.70	Pos
GM3	1179.5	290.2	52	44	19	6.30	Neg
GM3–αGalCer 1	1728	290.3	70	40	19	6.58	Neg
(Neu5Gc)GM3–αGalCer 2	1744.1	306.3	55	40	21	6.61	Neg

Table S3. A list of the MRM events, CE, Q1/Q3 bias and retention time for all compounds in the liposomes. The tQ MS used the following general parameters: Nebulizing gas flow 3 L/min; drying gas flow 10 L/min; heating gas flow 10 L/min; interface temperature 300 °C; DL temperature 250 °C; heat block temperature 400 °C. [a] MRM event setup without the need to optimizing Q1 and Q3 bias.

Immunological evaluation

Animals and Immunization Schedule. Female C57BL/6 mice of 6-8 weeks age used for the immunological studies were purchased from Janvier Labs. Mice were divided randomly into IVC-cages with six mice in each cage to serve as nontreated group or immunized with liposomal formulations **I**–**IV**, while four mice were dedicated to immunization with **22** emulsified in IFA. The animal work was approved by the Danish Committee for Animal Research using internationally accepted principles for the use of laboratory animals. Mice were immunized by *sc* injection on day 1, 15, and 29. The mice were bled on day 0, 14, 28, and 42 for serum preparation (Figure S2). Moreover, in order to analyze the secretion of IFN-γ, IL-4, and IL-12p70, sera were collected 24 hours after the first injection. Blood was withdrawn from the tongue and collected in Microvette® 200 capillary tube (Sarstedt, Germany). The blood was allowed to clot at room temperature for 30 minutes and centrifuged at 5000g for 5 minutes to obtain sera, which were stored at –80 °C before use.

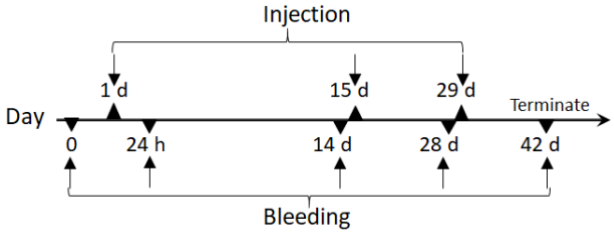


Figure S2. Mice Immunization and bleeding schedule

Isolation of Mononuclear cells, induction of Bone marrow-derived Dendritic Cells and iNKT Cells Activation Assay. In vitro antigen presentation by living APCs was performed in order to evaluate the secretion of cytokines as a result of activation of the cells. Dendritic cells were induced from mononuclear cells in bone marrow of C57BL/6 mice. Briefly, mice were sacrificed by cervical dislocation and disinfected in 75% ethanol. Spleens were cut and smashed through 70 µm cell strainers with PBS + 2 % FBS. $NK1.1^+$ iNKT cells were isolated by a magnetic-activated cell sorting kit (Miltenyi Biotec 130-096-513) according to the manufacturer's protocol. Femurs and tibias were soaked in RPMI-1640 medium supplemented with 1%

FBS for 5 minutes. The ends of bones were cut off and, with a help of 1 mL syringe filled with RPMI-1640, the contents of the bone cavity were flushed into a sterile petri dish with RPMI-1640. The cell suspension was centrifuged at 1200 rpm for 5 min. The supernatant was discarded, and pellet was re-suspended in RPMI-1640 supplemented with 10% FBS, 1% penicillin/streptomycin, 20 ng/mL GM-CSF at a density of 2×10^6 cell/mL. The culture medium was replaced 72 hours later with fresh RPMI media supplemented with 10% FBS, 1% penicillin/streptomycin, 20 ng/mL GM-CSF, and unattached cells were removed. On day 7, the semi-suspended as well as loosely attached cells were collected and plated at 2.5×10^4 cells/well in 96-well plate and incubated at 37 °C with either media, LPS, or liposomal formulations **I–IV.** After 2 hours, iNKT cells were added at the density of 0.5×10^5 cells/well. Cell culture supernatants were harvested 48 hours later and cytokine secretion was measured by BD™ cytokine bead array (CBA) kit (see below).

Cytokine Assay. Levels of IFN-γ, IL-4, and IL-12p70 cytokines in the supernatant and serum samples were measured with BD™ CBA Mouse Cytokine kit which allows for the simultaneous measurement of several analytes from a single sample. The procedures were performed according to the manufacturer's instruction. Samples were measured on Canto II Flow Cytometer (BD FACS Calibur) and concentrations of the cytokines were calculated based on the standard curve using FlowJo (software version 10.7.1).

Flowcytometry Analysis. To investigate the immune responses following liposome introduction, mice were injected *sc* with liposomal formulations and spleens were collected 48 hours post injection. The spleens were placed in incomplete RPMI medium on ice. Splenocytes were prepared by homogenizing the spleen through a 70 µm cell strainer with a 3 mL syringe plunger. Red blood cells were lysed and removed with 1 mL VersaLyse lysis buffer (Beckman Coulter, IN, USA). The remaining cells were incubated with anti-CD16/32-purified mAb (Clone 2.4G2, BD Biosciences) and then stained with the following antibodies: PBS57- or αGC-loaded mouse CD1d tetramer-phycoerythrin (PE; Tetramer Shop, Denmark), anti-TCRβ-BV711 (H57-597, BD Biosciences), anti-CD69-BUV395 (H1.2F3, BD Biosciences), anti-CD25-BB515 (PC61, BD Biosciences), anti-IFN-γ-BV650 (XMG1.2, BD Biosciences). All antibodies were titrated to yield the best concentration for staining the cells. Cells were acquired on LSRFortessa flow-cytometer (BD FACS Calibur). All events were gated based on light scattering, staining with viability reagent (FVS780, BD Biosciences) and pulse width of the forward scatter signal to include only individual viable cells. Samples were analyzed using FlowJo (software version 10.7.1). The gating strategies and staining examples are provided in **Figure S3**.

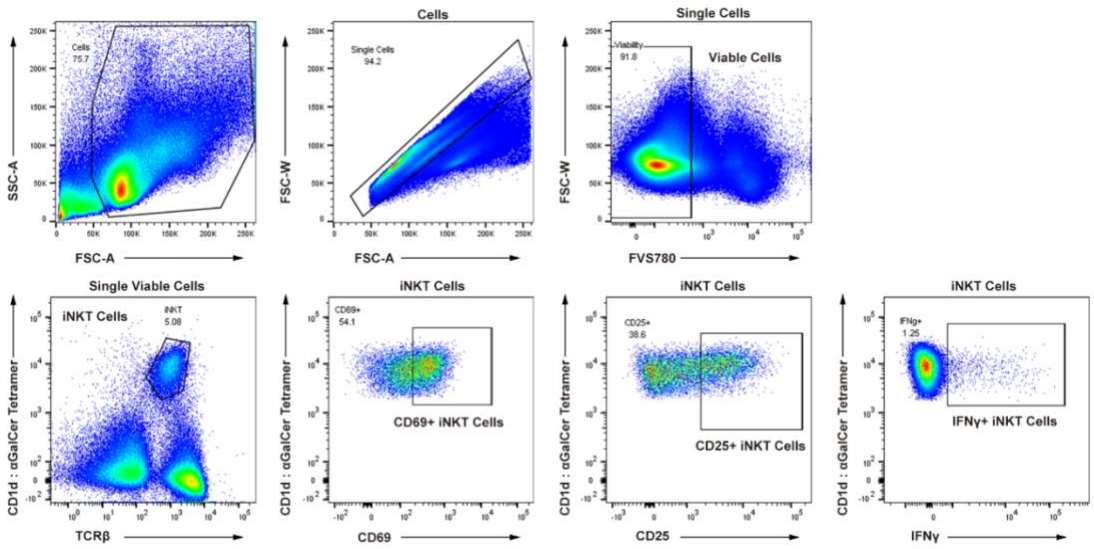


Figure S3. Gating strategy used to identify iNKT cells (CD1d:αGalCer TCRβ^{int}) as well as the subpopulations which presented CD69 and CD25. IFN-γ producing iNKT cells are also shown

ELISA Procedure. For detection of antibodies, 96-well microtiter plates (MAXISORP, NUNC-IMMUNO PLATE, Thermo Fisher Scientific, Denmark) were first coated with either GM3–HSA or (Neu5Gc)GM3–HSA (1 µg/plate) at 4 °C overnight. The HSA-conjugated gangliosides were dissolved in a 0.1 M bicarbonate buffer (pH = 9.6). To reduce the background signal, plates were washed three times with PBS containing Tween 20 (0.05%, v/v), thereafter blocked with 1% bovine serum albumin in PBS for 1 hour at 37 °C. Serially diluted serum samples, HRP-conjugated goat anti-IgM (Bethyl Laboratories, TX, United States), IgG (Abcam, Cambridge, United Kingdom), biotinylated rat anti-IgG1, IgG2a, IgG2b, IgG3 (all from Biolegend, United Kingdom) and streptavidin-conjugated alkaline phosphatase were all diluted in blocking buffer and sequentially added to microwells (100 µL/well) and incubated for 1 hour at 37 °C. Plates were extensively washed between each incubation. Sera were also added on wells which were not coated with ganglioside to serve as the background. After the final wash, the enzymatic reaction was visualized with 1-Step™ Ultra TMB-ELISA Substrate Solution (Thermo Fisher Scientific, Massachusetts, United States) and the absorbance was measured at 450 nm with Infinite® microplate reader (Tecan, Switzerland) and expressed as optical density or OD_{450} value. Six identical 4-fold serial dilutions starting with 1:100 with no missing data points were performed for all samples and data reported as the absorbance summation. The absorbance at the non-coated wells was subtracted from that of the ganglioside-coated wells.

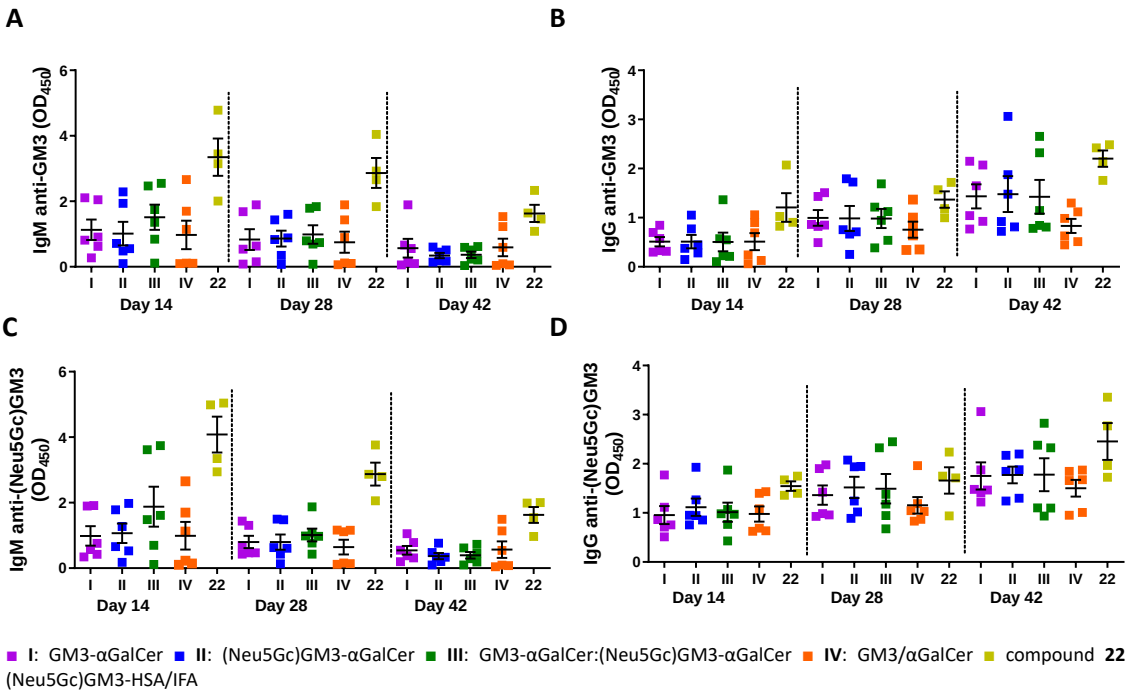


Figure S4. Anti-GM3 IgM (A) and IgG (B) and anti-(Neu5Gc)GM3 IgM (C) and IgG (D) responses on day 14, 28 and 42 following immunizations of female C57BL/6 mice. The data are indicated as the average value ± SEM, n=6 for **I–IV** and 4 for **22**.

Complement dependent cytotoxicity assay (CDC). B16F10 or THP-1 cells were seeded in 96-well plate (1 × 10^4 cell/well) and let to grow overnight at 37 °C. Wells were washed with medium without FBS, thereafter incubated for 1h with antisera prepared on day 42 (diluted 1:10, 50 µL/well). After washing with PBS, the rabbit complement (Cedarlane Laboratories Ltd., Ontario, Canada) in 1% BSA/PBS was added according to manufacturer's instructions. As a control for background lysis, inactivated complement by treatment at 65 °C for 30 minutes was used. After incubation at 37 °C for 2 hours, Alamar Blue (Thermo Fisher Scientific, USA) was added (20 µL/well) and incubated for additional 2 hours at 37 °C protected from light. Fluorescence was recorded at 560/590nm. Percentage of Cytotoxicity of cells was measured according to this formula: [1-(experimental OD/control OD)] × 100. Reported data is representative of four independent experiments.

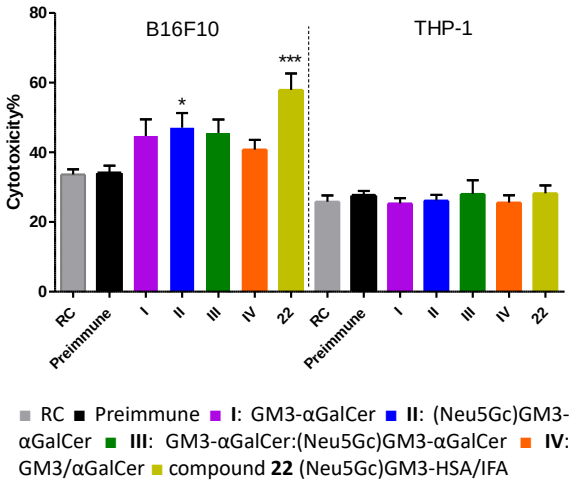


Figure S5. Complement dependent cytotoxicity (CDC) assay on B16F10 and THP-1 cells using sera. GM3-expressing B16F10 and not GM3-expressing THP-1 cells were incubated with antisera followed by addition of the complement proteins. RC: Viability of B16F10 and THP-1 cells incubated with just complement proteins, and no antisera. Data are represented as the average value ± SEM in each group (n = 6). Asterisks show significant differences to RC using one-way ANOVA followed by Dunnett's multiple comparison test. *P ≤ 0.05, ***P* ≤ 0.01, ****P* ≤ 0.001. Results are indicative of four independent experiments.

Serum binding assay. The ability of antisera prepared on day 42 to bind to the B16F10 cell line, presenting the GM3 antigen, was determined by flow cytometry. Cells were cultured in RPMI-1640 containing 10% FBS and 1% penicillin/streptomycin. Cells were harvested and washed with FACS buffer (PBS containing 5% FBS). Thereafter, 5×10^5 cells were incubated with antisera prepared on day 42 at 4 °C for 1 hour (1:10 dilution in FACS buffer with 0.1% NaN_3). Cells were washed three times with wash buffer (FACS buffer with 0.1% NaN_3) and incubated with PE-labeled goat anti-mouse IgG (Biolegend, San Diego, USA) at 4 °C for 30 minutes. Thereafter, cells were washed three times with wash buffer and analyzed by LSR Fortessa flow-cytometer (BD FACS Calibur). Cells were gated by FSC-A/H for singlets, viability stain for live cells, and PE-conjugated goat anti-mouse IgG for the positive

Statistical analysis. All analyses were performed using GraphPad Prism version 5.1 (GraphPad Software, La Jolla, CA, USA). Differences were considered significant at **P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0. 001.* All error bars are mean ± SEM. Significance between two groups was evaluated by unpaired two-tailed Student's *t*-test. Significance of multiple groups in comparison to the control group was determined by one-way ANOVA followed by a Dunnett's multiple comparison test.

Stimulation of human iNKT cells by MUTZ-3 derived DCs matured in presence of liposomes I–V

MUTZ-3 culture and generation of immature MUTZ-3 DCs. The human acute myelomonocytic leukemia cell line MUTZ-3 (ACC 295, DSMZ) was maintained every 3-4 days in MEM-α medium (Gibco) supplemented with 20% FBS, 1% penicillin/streptomycin and 20% conditioned medium from the human cell line 5637 (ACC 34, DSMZ). Immature MUTZ-3-derived DCs (iDCs) were obtained as previously

described.[7–9] In brief, the MUTZ-3 cells were seeded in a density of $1x10^5$ cells/mL and differentiated by the addition of 100 ng/mL GM-CSF (Peprotech), 10 ng/mL IL-4 (Peprotech) and 2.5 ng/mL TNFα (Peprotech) and incubated for 3 days. On day 3 new differentiation medium was added (cell dilution 1:2) and cells were further differentiated for 4 days.[8] Afterwards, the MUTZ-3 iDCs were evaluated by flow cytometry to confirm differentiation status (described in details in the *Flow cytometry analysis of in vitro study* section*)*.

*Priming of MUTZ-3 iDCs in the presence of liposomal formulations **I**–**V**.* The priming of the MUTZ-3-derived iDCs, was performed by seeding $2×10^5$ cells/mL MUTZ-3 iDCs in MEM-α medium (supplemented with 20% FBS, 1% penicillin/streptomycin) containing 75 ng/mL TNFα and 100 ng/mL of liposomal formulation **I**–**V** for 3 days in a 6-well plate. The matured MUTZ-3-derived DCs (mDC) were analyzed by flow cytometry (described in details in section *Flow cytometry analysis of in vitro study*) or used for T-cell co-culture.

*Isolation of primary cells and co-culture of MUTZ3 derived DCs primed in the presence of **I**–**V** and T-cells.* The buffy coat was obtained from an anonymized healthy donor obtained from the State Hospital, Copenhagen, Denmark. Informed written consent was obtained in accordance with legislation and guidelines from the local ethics committee (Region Hovedstaden, Denmark). PBMCs were isolated by a two-step density gradient centrifugation (200xg and 460xg) using Histopaque-1077 (Sigma Aldrich). Human PBMCs were isolated from the interphase and washed several times with Dulbecco's PBS (DPBS). After, PBMCs were incubated with red blood cell lysis buffer (eBioscience) and washed several times in DBPS prior T cell purification. T cells were isolated from the PBMCs using Dynabeads untouched human T-cells kit following manufactures protocol (Invitrogen) as previously described. [7,10] The purified T-cells were resuspended in RPMI 1640 (Sigma Aldrich) , 10% FBS, 1% penicillin/streptomycin, 1% Glutamine 2mM, 0.01 mM mercaptoethanol and 10 ng/mL IL-15 (Peprotech) at a density of $1.5x10^6$ cells/mL. Primed MUTZ-3 DCs were resuspended in the same culture medium as the T-cells but at a cell density of $1.5x10^5$ cells/mL. Primed MUTZ-3 DCs and T-cells were co-cultured in a round bottom 96-well plate by mixing 100µL of each cell solution. The co-culture was incubated for seven days and the expansion of iNKT cells were evaluated by flow cytometry (described in details in section *Flow cytometry analysis of the human in vitro study).*

Flow cytometry analysis of the human in vitro study. To evaluate the differentiation from naïve MUTZ-3 cells into MUTZ-3 iDCs and primed MUTZ-3 iDCs in presence of liposomal formulations **I–V**, the expression of specific DC maturation antigens were analyzed by flow cytometry. Cells were incubated with LIVE/DEAD™ Fixable Yellow Dead Cell Stain (ThermoFisher Scientific, L34967) and FcR blocking reagent (Miltenyi, 130059901) prior to staining with the following antibodies: anti-CD14-Vioblue (clone REA599, Miltenyi), anti-CD34-FITC (AC136, Miltenyi), anti-CD83-PerCPeFluor710 (HB15E, eBioscience), anti-CD1d-APC (51.1, Miltenyi), anti-CD1a-APC-Vio770 (REA736, Miltenyi), anti-CD209-PE (9E9A8, BioLegend), anti-HLA-DR-BV650 (G46-6, BD). All antibodies were used in a final dilution of 1:100 in the cell suspension. Cells were acquired on MACSQuant Analyzer16 (Miltenyi) and analyzed using software MACSQuantify (Miltenyi). The gating strategy for MUTZ-3 DCs are provided in **Figure S6** (A).

To investigate the ability of MUTZ-3 derived DCs primed in the presence of the liposomal formulations **I–V** to stimulate iNKT cells, T-cells were analyzed by flow cytometry after the seven days of co-culture. Cells were incubated with LIVE/DEAD™ Fixable Yellow Dead Cell Stain (ThermoFisher Scientific, L34967) and FcR blocking reagent (Miltenyi, 130059901) prior staining with the following antibodies: anti-CD3-VioBlue (REA614, Miltenyi), anti-CD4-VB667 (REA623, Miltenyi), anti-CD8-APC-Vio770 (REA734, Miltenyi), anti-

CD69-PE-Vio770 (REA824, Miltenyi), CD1d-tetramer-B515 (Tetramer Shop, Denmark) or aGalCer loaded CD1d dextramer (XD8002-FITC). All antibodies were used in final dilution of 1:100 in the cell suspension. CD1d-tetramer was diluted to obtain a final dilution of 1:50 in the cell suspension while the CD1d-dextramer was diluted 1:10. Cells were acquired on MACSQuant Analyzer16 (Miltenyi) and analyzed using software MACSQuantify (Miltenyi). The gating strategy and staining examples for iNKT cells are provided in **Figure S6** (B). **Figure S7** summarizes the percentage of positive cells or mean fluorescence intensity (MFI) values from three independent experiments using T-cells from different donors.

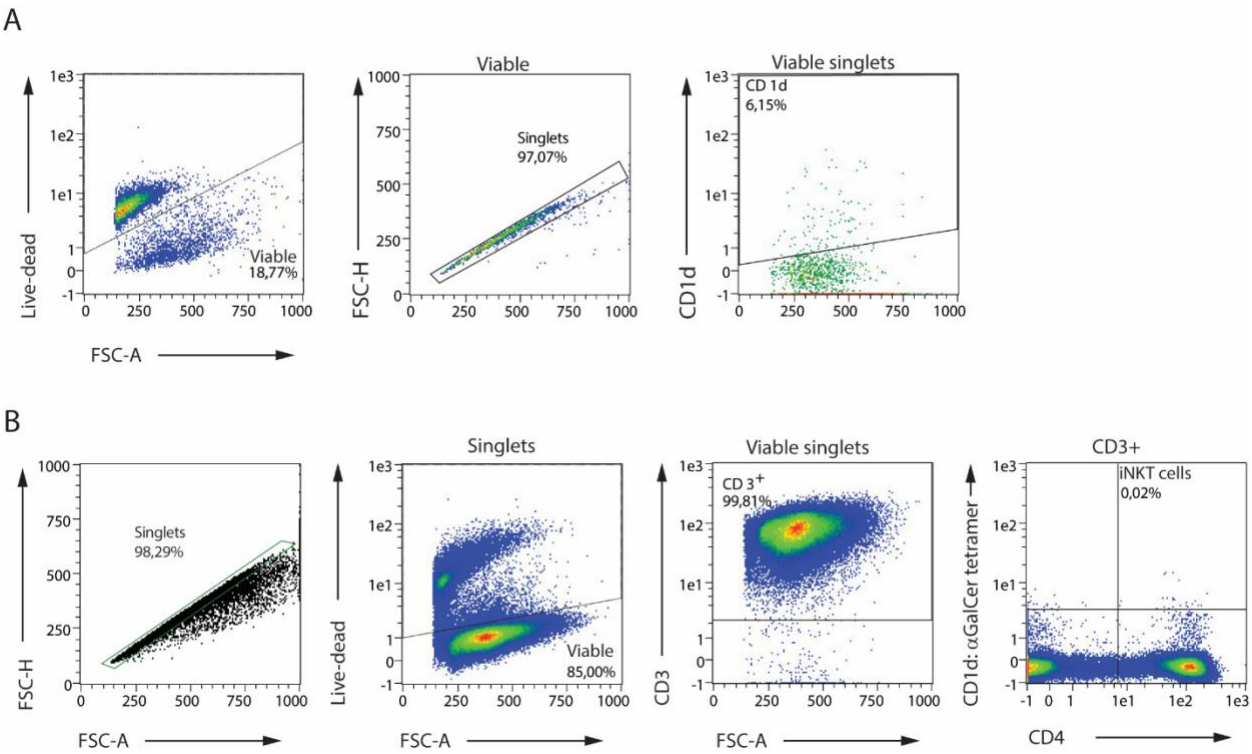


Figure S6

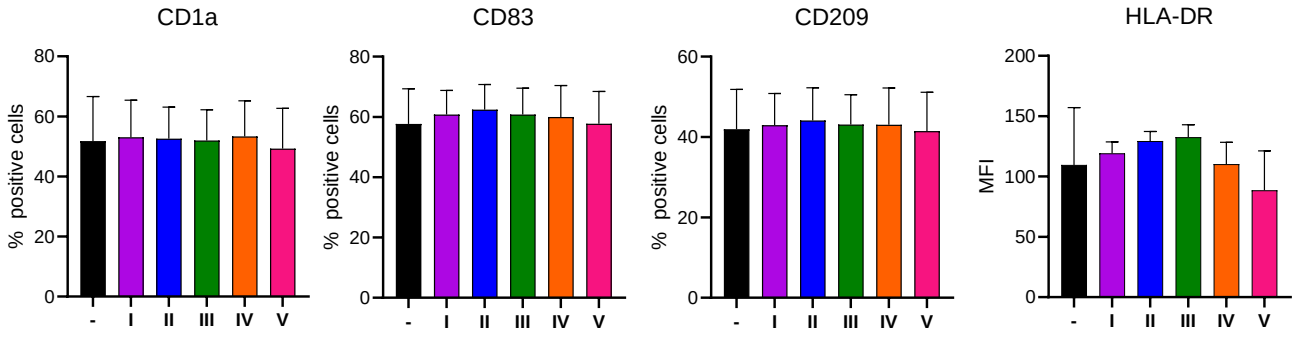


Figure S7

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