Supplementary Information for:

6''-Modifed α-GalCer-peptide conjugate vaccine candidates protect against liver-stage malaria.

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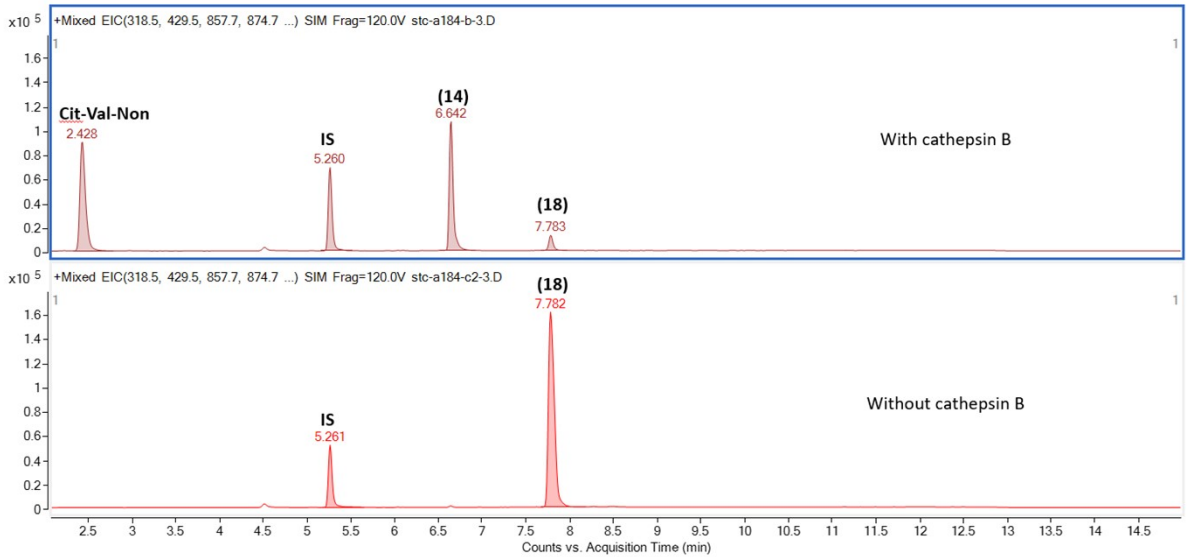
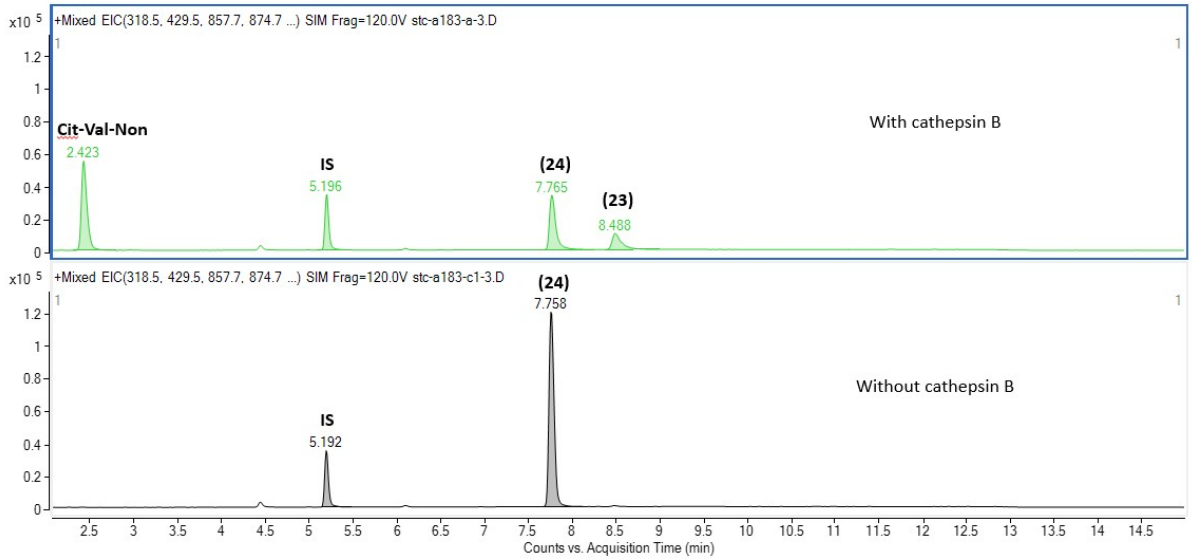
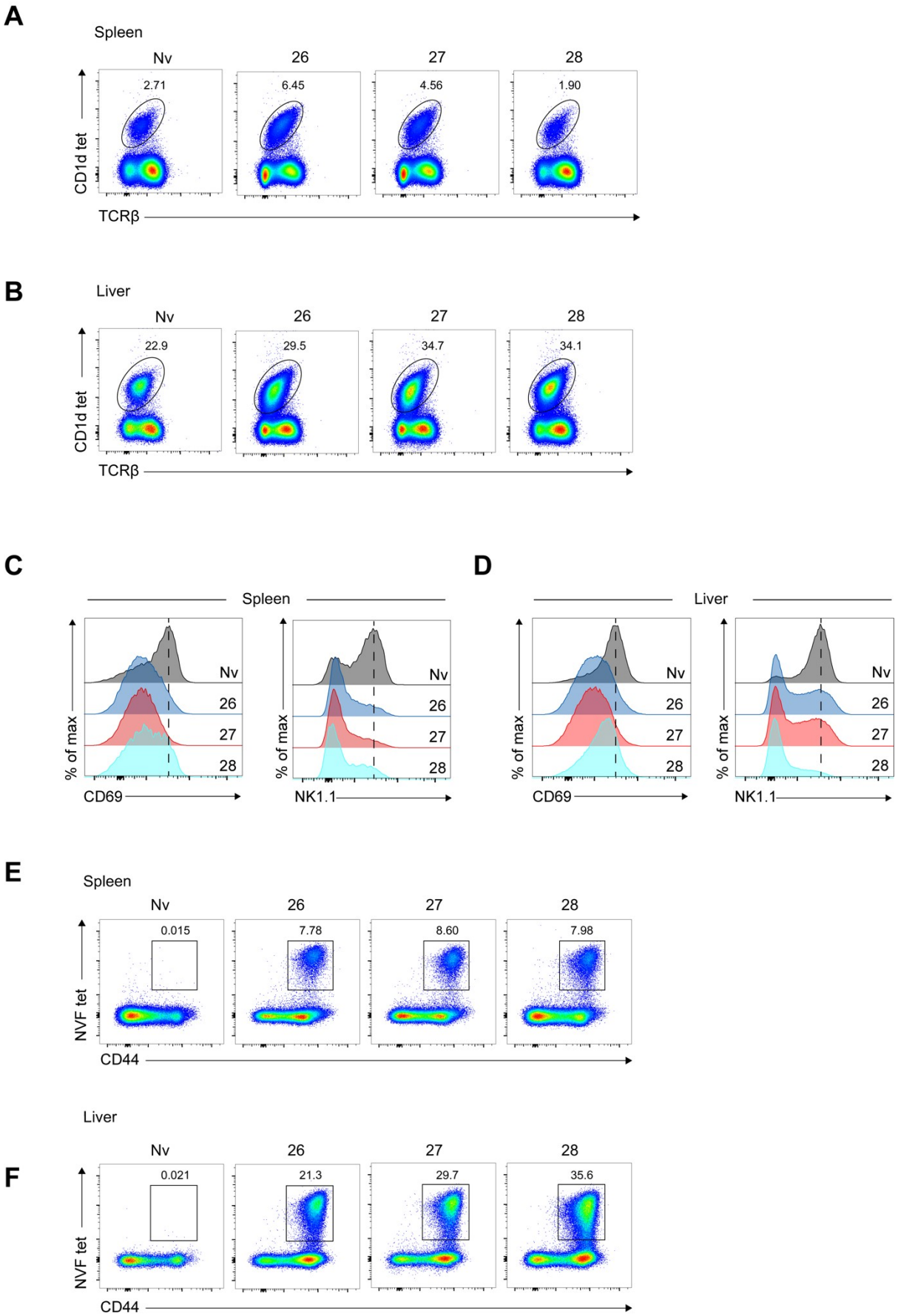


SI Figure 1. **Processing of pro-adjuvant 18 by cathepsin B.** Selected ion monitoring (SIM) data for **18** at 24 hr time point with and without cathepsin B (internal standard (IS) is phytosphingosine)

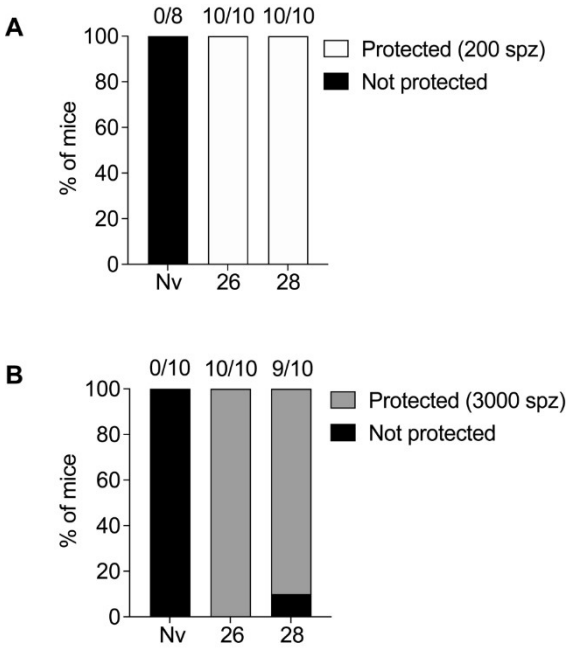


SI Figure 2. **Processing of pro-adjuvant 24 by cathepsin B.** Selected ion monitoring (SIM) data for **24** at 24 hr time point with and without cathepsin B (internal standard (IS) is phytosphingosine)



SI Figure 3. **Representative FACS plots and histograms induced by vaccine candidates 26, 27 and 28**. B6 mice were immunised intravenously with **26**, **27** or **28** and then examined for acute NKT and NVF-specific T cell

responses at day 7. (**A** and **B**) Representative FACS plots of NKT cells in the spleen (**A**) and liver (**B**). (**C** and **D**) Representative histograms of CD69 expression (left) and NK1.1 expression (right) in the spleen (**C**) and liver (**D**). (**E** and **F**) Representative FACS plots of NVF-tet$^+$ CD44$^+$ CD8$^+$ T cells in the spleen (**E**) and liver (**F**). Nv, naïve.



SI Figure 4. Sterile protection induced by vaccine candidates 26 and 28. Individual graphs of Figure 5 showing the percentage of mice that were protected and unprotected from (**A**) 200 sporozoite challenge and subsequent (**B**) rechallenge with 3000 sporozoites 21 days later. Numbers above bars indicate the number of mice that were protected against sporozoite challenge over total number of mice challenged. Nv, naïve.

Supplementary Methods:

Mice:

Female C57BL/6 (B6) were purchased from the Animal Resources Centre (Canning Vale, Australia) and maintained at the Department of Microbiology and Immunology, The University of Melbourne. Mice were used at the age of 6-12 weeks. Animals used for the generation of the sporozoites were 4-5-week-old male Swiss Webster mice purchased from the Monash Animal Services (Melbourne, Victoria, Australia) and maintained at the School of Biosciences, The University of Melbourne, Australia.

Vaccination:

Solubilization of vaccine candidates **26-28** was achieved by freeze-drying the samples in the presence of sucrose, L-histidine and Tween 20 as previously described for the solubilization of α-GalCer.[1] The solubilized compounds were diluted with phosphate-buffered saline (PBS) and mice were intravenously injected with 0.135 nmol of conjugate vaccines in 200µL PBS.

Lymphocyte isolation:

Livers and spleens were harvested and processed to generate single-cell suspensions as previously described.[2]

Flow cytometry:

Single-cell suspensions of the spleen and liver were stained with PE-conjugated H2-K^b NVF tetramers (produced in-house) for 1 hour at room temperature before staining with monoclonal antibodies

specific for the following markers: CD69-PeCy5 (H1.2F3) from ThermoFisher Scientific (Waltham, MA, USA); CD44-APC-R700 (IM7) from BD (Franklin Lakes, NJ, USA); CD19-FITC (1D3), CX3CR1-APC (SA011F11), CXCR6-BV421 (SA051D1), KLRG1-BV605 (2F1), CD8α-BV711 (53-6.7), and CD62L-PeCy7 (MEL-14) from BioLegend (CA, USA), at 4°C for 30 min. For NKT cell analysis, cells were co-stained with PE-conjugated α-GalCer (PBS57)-loaded CD1d tetramers (produced in-house) and monoclonal antibodies specific for NK1.1-BV605 (PK136) and CD19-FITC (1D3) from BioLegend (CA, USA); TCRβ-APCCy7 (H57-597) from BD (Franklin Lakes, NJ, USA); and CD69-PeCy5 (H1.2F3) from ThermoFisher Scientific (Waltham, MA, USA), at 4°C for 30 min. Propidium iodide was used to stain dead cells. Cells were acquired by flow cytometry on a LSRFortessa (BD Biosciences) and data were analysed using Flowjo software (Tree Star Inc.).

Plasmodium infection:

Generation, maintenance, and isolation of *Plasmodium berghei* ANKA (PbA; BEI Resources, MRA-871) parasites were performed as previously described.[2] For challenge experiments, mice were intravenously infected with 200 (or 3000 for re-challenge) PbA sporozoites, and blood-stage parasitemia was measured at various time points post-infection by flow cytometry as described,[2] with absence of parasitemia on day 12 indicating sterile protection.

Quantification and statistical analysis:

Figures were generated using GraphPad Prism 9 (GraphPad Software). Data are shown as mean values ± SEM. Data were log-transformed (unless otherwise stated) and assessed for normality, and then a one-way ANOVA with a Kruskal-Wallis test and Dunn's or Tukey's multiple comparison test was performed. The statistical tests used and P values are indicated in the figure legends.

Cathepsin B enzymatic degradation assays:

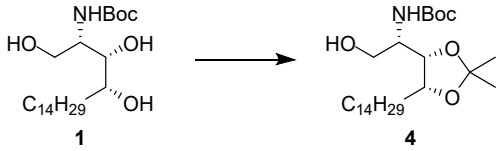
A stock solution of phytosphingosine (190 µM) in DMSO (as the internal standard, IS) was pre-mixed with ammonium acetate buffer (50 mM, pH 5.3) containing EDTA (2.5 mM) and dithiothreitol (2.5 mM) to a final phytosphingosine concentration of 6.3 µM. Compound **18** (190 µM in 0.2% HFIP in DMSO) was added to the pre-mixed buffer solution to give a final substrate concentration of 12.7 µM. Cathepsin B from human liver (Sigma) dissolved in ammonium acetate buffer (50 mM, pH 5.3, EDTA (2.5 mM), dithiothreitol (2.5 mM)) was added to the reaction mixture to give a final cathepsin B concentration of 47.4 units/mL. After 4 h, more cathepsin B was added to give a final concentration of 94.9 units/mL. For the control reaction (without enzyme) the same volume of buffer was added. The reaction mixtures were then incubated at 37 °C. Aliquots of 10 µL were taken from the reactions and analysed by LCMS at 1, 4 and 24 hours after start of reaction.

A stock solution of phytosphingosine (190 µM) in DMSO (as the internal standard, IS) was pre-mixed with ammonium acetate buffer (50 mM, pH 5.3) containing EDTA (2.5 mM) and dithiothreitol (2.5 mM) to a final phytosphingosine concentration of 6.3 µM. Compound **24** (190 µM in 0.2% HFIP in DMSO) was added to the pre-mixed buffer solution to give a final substrate concentration of 12.7 µM. Cathepsin B from human liver (Sigma) dissolved in ammonium acetate buffer (50 mM, pH 5.3, EDTA (2.5 mM), dithiothreitol (2.5 mM)) was added to the reaction mixture to give a final cathepsin B concentration of 19.6 units/mL. After 4 hours, more cathepsin B was added to give a final concentration of 39.2 units/mL. For the control reaction (without enzyme) the same volume of buffer was added. The reaction mixtures were then incubated at 37 °C. Aliquots of 10 µL were taken from the reactions and analysed by LCMS at 1, 4 and 24 hours after start of reaction.

General Experimental:

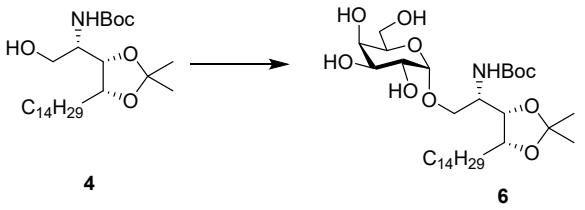
Anhydrous solvents were obtained commercially. Air-sensitive reactions were carried out under Ar. Thin layer chromatography (TLC) was performed on aluminium sheets coated with 60 F254 silica. Flash column chromatography was performed on a Buchi Pure or Reveleris X2 automated chromatography instrument using 40 or 25 µm silica or RP-C18 silica with the stated eluents. NMR spectra were recorded on a Bruker 500 MHz spectrometer. ^{1}H NMR spectra were referenced to tetramethylsilane at 0 ppm (internal standard) or to the residual solvent peak ($CHCl_3$ 7.26 ppm, CHD_2OD 3.31 ppm, $CHD_2(SO)CD_3$ 2.50 ppm). ^{13}C NMR spectra were referenced to tetramethylsilane at 0 ppm (internal standard) or to the deuterated solvent peak ($CDCl_3$ 77.0 ppm, CD_3OD 49.0 ppm, $(CD_3)_2SO$ 39.5 ppm). $CDCl_3$-CD_3OD solvent mixtures were always referenced to the methanol peak, $(CD_3)_2SO$-$CDCl_3$-CD_3OD solvent mixtures were referenced to the dimethyl sulfoxide peak. High resolution electrospray ionization (ESI) mass spectra were obtained on a Waters Q-TOF Premier™ Tandem Mass spectrometer fitted with a Waters 2795 HPLC. Analytical HPLC analysis was obtained on an Agilent 1260 Infinity Quaternary HPLC equipped with both an Agilent 1260 Diode Array Detector and either an Agilent 6130 single quadrupole mass spectroscopic detector using ESI/APCI multimode or a Dionex Corona Ultra RS charged aerosol detector (CAD) employing a Phenomenex Kinetex 100 Å C18 (2.6 µm, 3.0 x 50 mm) functionalized silica column. Method: mobile phase A = 100:0.02 water/TFA, mobile phase B = MeOH; 0-4 min: 60-100% B; 4-10 min: 100% B; 10-11 min: 100-60% B; 11-12 min: 60% B; flow rate = 0.50 mL min^{-1}; temperature = 40 °C. Preparative HPLC was performed on an Agilent 160 Infinity II system employing a Phenomenex Luna C18(2) (5 µm, 250 x 21.2 mm) functionalized silica column. Method: mobile phase A = 85:15:0.05 MeOH/water/TFA; mobile phase B = 100:0.05 MeOH/TFA; 0-6 min: 0-100% B; 6-11 min: 100% B; 11-12 min: 100-0% B; 12-14 min: 0% B; flow rate = 20 mL min^{-1}; temperature = 40 °C. Aminooxyacetyl-FFRKAAASTNVFDFNNLS (**25**) was obtained from commercial manufacturer Peptides International.

Synthetic Procedures:



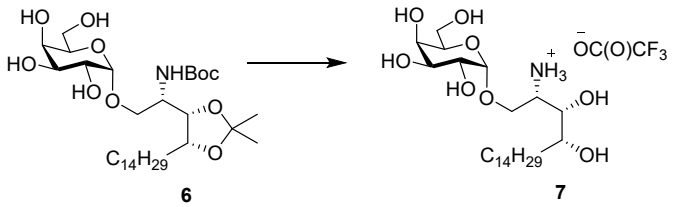
(2*S*,3*S*,4*R*)-2-(*N*-*tert*-Butoxycarbonyl) amino-3,4-*O*-isopropylidene-octadecan-1,3,4-triol (**4**).[3] To a stirred solution of *N*-Boc-phytosphingosine (**1**) (10.2 g, 24.4 mmol) in dry CH_2Cl_2/DMF (1:1 v/v, 56 mL) at 0 °C under an Ar atmosphere was added Et_3N (5.1 mL, 36.6 mmol, 1.5 equiv.), DMAP (256 mg, 2.10 mmol, 8.6 mol%) and TBDPSCl (8.8 mL, 33.9 mmol, 1.4 equiv.) and the reaction was stirred at room temperature (19 h). The reaction was diluted with EtOAc (210 mL) and washed with brine (2 x 100 mL). The organic layers were dried ($MgSO_4$), filtered and concentrated *in vacuo*. The crude material was dissolved in 2,2-dimethoxypropane/THF (1:1 v/v, 340 mL), then added *p*-TsOH·H_2O (910 mg, 4.78 mmol, 0.2 equiv.) and the reaction mixture was stirred at room temperature (1.5 h). The reaction was quenched with $NaHCO_3$ (satd. aq. 100 mL) and concentrated *in vacuo*. The crude material was taken up in EtOAc (200 mL), washed with brine (2 x 100 mL), dried ($MgSO_4$), filtered and concentrated *in vacuo*. The crude material was dissolved in dry THF (200 mL), cooled to 0 °C then TBAF (1.0 M in THF, 33 mL, 33 mmol, 1.1 equiv.) was added dropwise and the reaction mixture was stirred at room temperature (64 h). The reaction was concentrated *in vacuo* and the crude material was taken up in EtOAc (300 mL), washed with H_2O (3 x 150 mL), dried ($MgSO_4$), filtered and concentrated *in vacuo.* The

resulting orange oil was purified by automated flash column chromatography (EtOAc/petroleum ether, 1.5:8.5 to 6:4) to give **4** as an amorphous white solid (6.14 g, 55% over three steps). NMR data was consistent with literature reports.



4 **6**

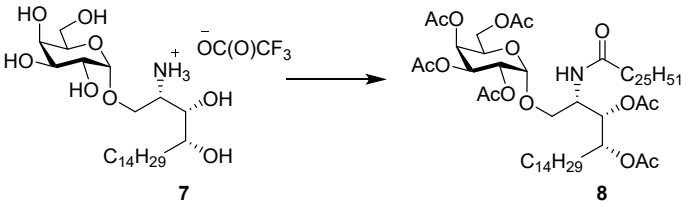
(2*S*,3*S*,4*R*)-2-(*N*-*tert*-Butoxycarbonyl) amino-1-*O*-(α-D-galactopyranosyl)-3,4-*O*-isopropylidene-octadecan-1,3,4-triol (**6**). To a solution of 1,2,3,4,6-penta-*O*-trimethylsilyl-α-D-galactopyranose (**5**)[4] (24.1 g, 44.5 mmol) in dry CH_2Cl_2 (125 mL) over 4 Å molecular sieves at 0 °C under argon was added dropwise neat iodotrimethylsilane[5] (6.4 mL, 45 mmol) and the reaction was allowed warm to room temperature (30 min). To a solution of **4** (5.10 g, 11 mmol) in dry CH_2Cl_2 (125 mL) over 4 Å molecular sieves at 0 °C under argon was added tetrabutylammonium iodide (8.4 g, 22 mmol) and the reaction was stirred at room temperature (15 min) before the addition of *N,N*-diisopropylethylamine (11. 7 mL, 67 mmol) followed by the previously prepared dichloromethane solution of glycosyl iodide. The mixture was stirred at room temperature (2 d), filtered through Celite and the filtrate was concentrated *in vacuo*. The resulting residue was suspended in dry THF (200 mL) and tetrabutylammonium fluoride (77 mL, 77 mmol, 1 mol/L in THF) was added and the reaction was stirred at room temperature (18 h). The reaction was filtered through Celite, the filtrate was concentrated *in vacuo*, and the resulting residue was purified by automated flash column chromatography (EtOAc/petroleum ether, 2:8 to 6:4) to give **6** as a white solid contaminated with tetrabutylammonium salts. This material was re-purified by automated RP-C18 flash column chromatography (MeOH/water, 6:4 to 10:0) to give **6** as a white solid (5.27 g, 64%). ^{1}H NMR (500 MHz, 1:1 $CDCl_3$/CD_3OD) δ 6.62 (d, *J* = 9.1 Hz, 1H), 4.90 (d, *J* = 2.4 Hz, 1H), 4.14 – 4.06 (m, 2H), 3.93 (br s, 1H), 3.81 (dd, *J* = 6.8 Hz, 1H), 3.77 – 3.69 (m, 7H), 1.53 – 1.47 (m, 2H), 1.42 (s, 9H), 1.39 (s, 3H), 1.30 (s, 3H), 1.30 – 1.20 (m, 24H), 0.86 (t, *J* = 6.8 Hz, 3H); ^{13}C NMR (126 MHz, 1:1 $CDCl_3$/ CD_3OD) δ 156.67, 108.68, 100.37, 80.03, 78.74, 76.02, 71.29, 71.03, 70.46, 69.99, 69.01, 62.24, 50.83, 50.74, 32.48, 30.22, 30.19, 30.14, 30.06, 29.90, 29.47, 28.70, 28.52, 26.94, 25.99, 23.20, 14.30; HRMS(ESI) *m/z* calcd. for $C_{32}H_{62}NO_{10}$ $[M+H]^+$: 620.4368, found 620.4374.



6 **7**

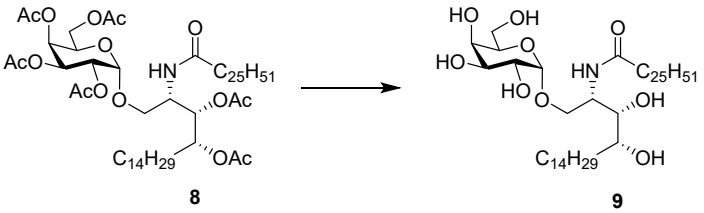
(2*S*,3*S*,4*R*)-2-Amino-1-*O*-(α-D-galactopyranosyl)-octadecan-1,3,4-triol (**7**).[6] To a solution of **6** (660 mg, 1.1 mmol) in trifluoroacetic acid/CH_2Cl_2 (1:1 v/v, 8 mL) was added anisole (1.2 mL, 11 mmol, 10 equiv.) and the reaction was stirred at room temperature for 90 min. MeOH (90 µL, 2.2 mmol, 2.0 equiv.) was added and the reaction was stirred for a further 30 min before being concentrated in vacuo. The

residue was purified by automated reversed phase flash column chromatography (MeOH/H_2O, 2:8 to 10:0) to give **7** as a white solid (600 mg, 95%). NMR data was consistent with literature reports.



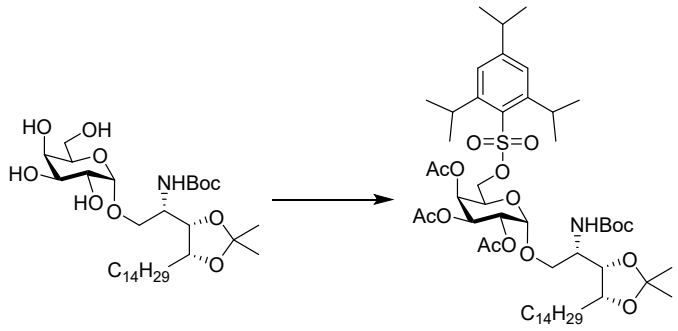
7 **8**

(2*S*,3*S*,4*R*)-3,4-Di-O-acetyl-1-O-(2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl)-2-(hexacosanoylamino)octadecane-1,3,4-triol (**8**).[7] To a stirred suspension of hexacosanoic acid (836 mg, 2.00 mmol, 1.1 equiv.) in dry CH_2Cl_2 (80 mL) under argon was added Et_3N (4.8 mL, 34 mmol) then isobutyl chloroformate (0.29 mL, 2.2 mmol, 1.2 equiv.) and the reaction was stirred at room temperature (50 min). To a solution of **7** (TFA salt, 1.05 g, 1.77 mmol) in dry DMF (30 mL) at 0 °C under argon was added the mixed carbonic anhydride solution and the stirred reaction was allowed to warm to room temperature (90 min). Ac_2O (10 mL, 104 mmol) followed by DMAP (40 mg, 0.32 mmol, 0.2 equiv.) was then added and the reaction was stirred at room temperature (18 h). The reaction was diluted with CH_2Cl_2 (120 mL), washed with H_2O (3 x 200 mL), brine (200 mL), dried ($MgSO_4$), filtered and concentrated *in vacuo*. The residue was purified by automated flash column chromatography (EtOAc/petroleum ether, 1:9 to 4:6) to give **8** as a white foam (1.75 g, 89%). NMR data was consistent with literature reports.



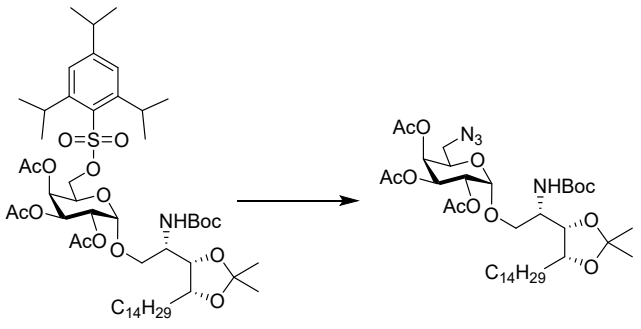
8 **9**

(2*S*,3*S*,4*R*)-1-*O*-α-D-galactopyranosyl-2-(hexacosanoylamino)octadecane-1,3,4-triol (**α-GalCer**) (**9**).[7, 8] To a solution of **8** (1.74 g, 1.6 mmol) in dry CH_2Cl_2/MeOH (1:2 v/v, 30 mL) under argon was added NaOMe (5.4 M in MeOH, 350 µL, 1.9 mmol, 1.2 equiv.) and the reaction was stirred at room temperature (2 h). The reaction was diluted with CH_2Cl_2/MeOH (1:1 v/v, 50 mL) and concentrated *in vacuo.* Precipitation from hot EtOH gave α-GalCer (**9**) as a white amorphous solid (1.16 g, 87%). NMR data was consistent with literature reports.



6 **10**

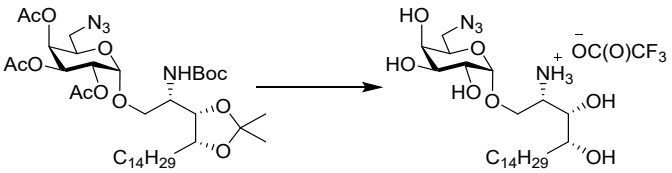
(2*S*,3*S*,4*R*)-2-(*N*-*tert*-butoxycarbonyl)amino-1-*O*-(6-*O*-(2,4,6-triisopropylbenzenesulfonyl)-2,3,4-*O*-acetyl-α-D-galacto-pyranosyl)-3,4-*O*-isopropylidene-octadecan-1,3,4-triol (**10**). To a solution of **6** (1.00 g, 1.61 mmol) in dry pyridine (35 mL) under Ar at -30 °C was added 2,4,6-triisopropylbenzenesulfonyl chloride (1.72 g, 5.56 mmol, 3.5 equiv.) and the reaction was stirred and maintained at -30 °C (3 d). The reaction was then diluted with EtOAc (200 mL), washed with H_2O (3 x 150 mL) and brine (150 mL), dried ($MgSO_4$), filtered and concentrated *in vacuo.* The residue was purified by automated flash column chromatography (EtOAc/petroleum ether, 0:10 to 4:6) to give **10** as a thick colourless oil (770 mg, 47%). R*f* = 0.67 (30:70 EtOAc/ petroleum ether); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.17 (s, 2H), 5.46 (d, *J* = 3.5 Hz, 1H), 5.35 (dd, *J* = 10.5, 3.4 Hz, 1H), 5.15-5.08 (m, 2H), 4.71 (d, *J* = 9.8 Hz, 1H, NH), 4.29 (t, *J* = 6.2 Hz, 1H), 4.12 – 4.03 (m, 4H), 4.00 (dd, *J* = 10.3, 6.9 Hz, 1H), 3.96-3.89 (m, 1H), 3.82 (t, *J* = 9.8 Hz, 1H), 3.77 – 3.65 (m, 2H), 2.91 (dt, *J* = 14.3, 7.1 Hz, 1H), 2.09 (s, 3H), 2.04 (s, 3H), 1.98 (s, 3H), 1.59-1.49 (m, 3H), 1.44 (s, 9H), 1.36 (s, 3H), 1.33 – 1.19 (m, 44H), 0.88 (t, *J* = 6.8 Hz, 3H); ^{13}C-NMR (126 MHz, $CDCl_3$) δ 170.3-170.0, 154.8, 154.1, 151.1, 129.1, 124.0, 108.0, 97.5, 80.0, 78.2, 76.0, 70.2, 68.3, 68.1, 67.5, 66.8, 66.1, 50.0, 34.4, 32.1, 30.0-29.72, 29.70, 29.5, 28.7, 28.5, 28.3, 26.9, 25.9, 25.0-24.7, 23.7, 23.6, 22.8, 20.82, 20.80, 20.6, 14.2; HRMS(ESI) *m/z* calcd. for $C_{53}H_{89}NO_{15}SNa$ $[M+Na]^+$: 1034.5851, found 1034.5833.



10 **11**

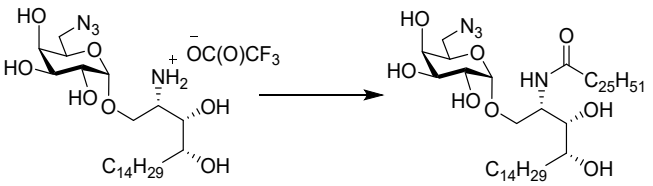
(2*S*,3*S*,4*R*)-2-(*N*-*tert*-Butoxycarbonyl)amino-1-*O*-(6-azido-2,3,4-*O*-acetyl-α-D-galactopyranosyl)-3,4-*O*-isopropylidene-octadecan-1,3,4-triol (**11**). To a solution of **10** (780 mg, 0.77 mmol) in dry DMF (30 mL) under Ar was added sodium azide (1.76 g, 27.1 mmol, 35 equiv.) and 15-crown-5 (155 µL, 0.78 mmol, 1 equiv.) and the reaction was stirred at 100 °C (2 d). The reaction was diluted with EtOAc (120 mL) and washed with brine (120 mL). The aqueous layer was extracted with EtOAc (120 mL) and the combined organic layers were dried ($MgSO_4$), filtered and concentrated *in vacuo.* The resulting yellow solid was purified by automated flash column chromatography (EtOAc/petroleum ether, 15:85 to

30:70) to give **11** as a thick colourless oil (517 mg, 0.67 mmol, 87%). R*f* = 0.59 (30:70 EtOAc/petroleum ether); ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.41 (d, *J* = 3.00 Hz, 1H), 5.36-5.30 (m, 1H), 5.17-5.11 (m, 2H), 4.71 (d, *J* = 9.34 Hz, NH), 4.17-4.07 (m, 2H), 4.00-3.93 (m, 1H), 3.90-3.79 (m, 2H), 3.76-3.69 (m, 1H), 3.43 (dd, *J* = 8.1, 12.8 Hz, 1H), 3.16 (dd, *J* = 4.7, 12.8 Hz, 1H), 2.15 (s, 3H), 2.04 (s, 3H), 1.99 (s, 3H), 1.60-1.50 (m, 3H), 1.46 (s, 9H), 1.39 (s, 3H), 1.34-1.22 (m, 26H), 0.88 (t, *J* = 7.1 Hz, 3H); ^{13}C-NMR (126 MHz, $CDCl_3$) δ 170.3, 170.25, 170.22, 154.9, 108.0, 97.3, 80.1, 78.1, 76.1, 70.0, 69.0, 68.2, 67.7, 50.8, 50.1, 20.83, 20.79, 20.76, 32.1, 29.9-29.72, 29.7, 29.5, 28.9, 28.5, 28.2, 26.8, 25.9, 22.8, 14.2; HRMS(ESI) *m/z* calcd. for $C_{38}H_{66}N_4O_{12}Na$ $[M+Na]^+$: 793.4575, found 793.4561.



11 **12**

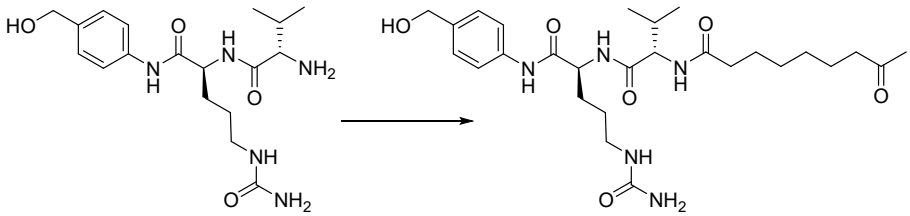
(2*S*,3*S*,4*R*)-2-Amino-1-*O*-(6-azido-α-D-galactopyranosyl)-octadecan-1,3,4-triol (**12**).[9] To a solution of **11** (450 mg, 0.58 mmol) in CH_2Cl_2/MeOH (1:1 v/v, 20 mL) was added NaOMe (5.4 M in MeOH, 320 µL, 1.7 mmol, 3.0 equiv.) and the reaction was stirred at room temperature (30 min). The reaction was concentrated to near dryness then quenched with NH_4Cl (satd. aq. 40 mL). The mixture was extracted with EtOAc (50 mL), and the organic layer was washed with brine (40 mL), dried ($MgSO_4$), filtered and concentrated *in vacuo*. The crude product was dissolved in CH_2Cl_2 (10 mL), then anisole (640 µL, 5.83 mmol, 10 equiv.) and trifluoroacetic acid (10 mL) were added and the reaction was stirred at room temperature (5 min) before addition of MeOH (50 µL, 1.2 mmol, 2.1 equiv.) and stirring was continued (1 h). The reaction was concentrated *in vacuo* and the residue taken up in hydrazine hydrate (5% w/w in H_2O, 1 mL). After 30 min, the reaction was concentrated *in vacuo* and the residue was purified by automated RP C18 flash column chromatography (MeOH/H_2O, 2:8 to 10:0) to give **12** as an amorphous white solid (287 mg, 82%). R*f* = 0.23 (15:85 MeOH/$CHCl_3$); ^{1}H NMR (500 MHz, CD_3OD) δ 4.90 (d, *J* = 4.0 Hz, 1H), 4.19 (dd, *J* = 10.6, 3.0 Hz, 1H), 3.92 (dd, *J* = 8.6, 4.2 Hz, 1H), 3.87 (dd, *J* = 10.1, 4.0 Hz, 1H), 3.83 (dd, *J* = 3.4, 0.8 Hz, 1H), 3.75 (dd, *J* = 10.1, 3.3 Hz, 1H), 3.68 (dt, *J* = 9.2, 3.2 Hz, 1H), 3.64-3.57 (m, 2H), 3.54 (dd, *J* = 8.8, 3.5 Hz, 1H), 3.52-3.47 (m, 1H), 3.31 (1H obscured by CD_3OH peak), 1.84-1.76 (m, 1H), 1.60-1.51 (m, 1H), 1.44-1.25 (m, 24H), 0.90 (t, *J* = 6.9 Hz, 3H); ^{13}C-NMR (126 MHz, CD_3OD) δ 101.0, 73.7, 73.2, 71.9, 71.2, 71.0, 69.9, 65.5, 55.0, 52.7, 35.3, 33.0, 30.8-30.6, 30.3, 26.2, 23.7, 14.4; HRMS(ESI) *m/z* calcd. for $C_{24}H_{49}N_4O_7$ $[M+H]^+$: 505.3596, found 505.3598.



12 **13**

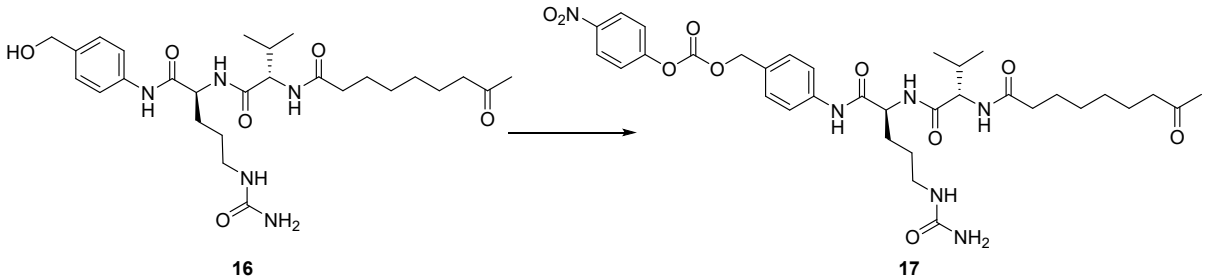
(2*S*,3*S*,4*R*)-1-*O*-(6-Deoxy-6-azido-α-D-galactopyranosyl)-2-hexacosanoylamino-octadecan-1,3,4-triol (**13**).[9] To a suspension of hexacosanoic acid (230 mg, 0.55 mmol, 1.12 equiv.) in dry CH_2Cl_2 (20 mL) under an argon atmosphere was added Et_3N (1.3 mL, 9.1 mmol, 20 equiv.) then isobutyl

chloroformate (75 µL, 0.56 mmol, 1.2 equiv.) and the mixture was stirred at room temperature (1 h). To a solution of **12** (287 mg, 0.46 mmol) in dry DMF (12 mL) under argon was added the mixed carbonic anhydride mixture and the reaction was stirred at room temperature (18 h). The reaction was diluted with MeOH (10 mL) and diethyl amine (60 µL) was added, stirred at room temperature (10 min.) before concentrating *in vacuo*. The residue was purified by automated flash column chromatography (MeOH/$CHCl_3$, 0:10 to 2:8) to give **13** as an amorphous white solid (351 mg, 86%). NMR data was consistent with literature reports.



15 **16**

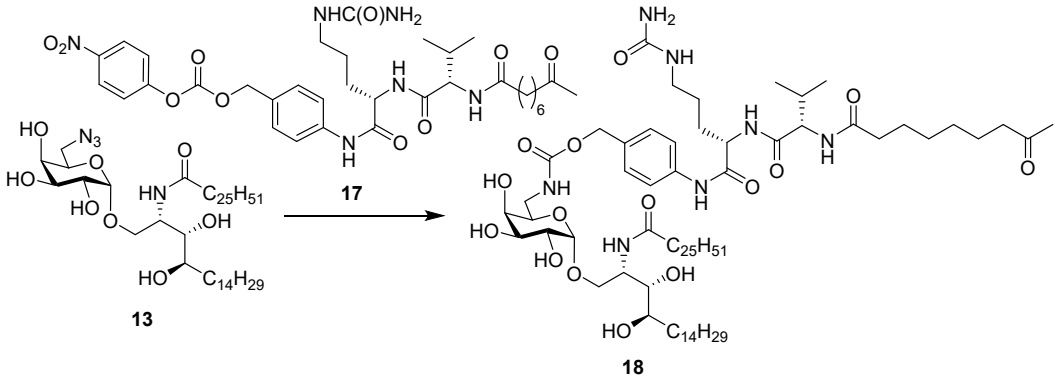
4-(*N*-(8-Oxononanoyl)-L-valinyl-L-citrullinamido)benzyl alcohol (**16**).[10] To a solution of 8-oxononanoic acid (280 mg, 1.63 mmol) in dry CH_2Cl_2 (8 mL) at 0 °C was added *N*-methylmorpholine (0.17 mL, 1.5 mmol) followed by isobutyl chloroformate (0.19 mL, 1.5 mmol). The ice bath was removed and the mixture was stirred at room temperature (40 min), before adding a suspension of **15**[11] (500 mg, 1.32 mmol) in MeOH (8 mL). The mixture was stirred at room temperature (18 h) and the solvents were concentrated *in vacuo*. The crude material was triturated with MeOH and the solid residue kept aside. The MeOH-soluble fraction was concentrated and triturated again using 3:2 EtOH/diethyl ether. The combined solids were dried to give **16** as a light tan solid (619 mg, 88%). ^{1}H NMR (500 MHz, d_6-DMSO) δ 9.86 (s, 1H), 8.01 (d, *J* = 7.5 Hz, 1H), 7.79 (d, *J* = 8.5 Hz, 1H), 7.53 (d, *J* = 8.3 Hz, 2H), 7.23 (d, *J* = 8.3 Hz, 2H), 5.97 (br t, *J* = 5.5 Hz, 1H), 5.39 (br s, 2H), 5.10 (t, *J* = 5.6 Hz, 1H), 4.42 (d, *J* = 5.6 Hz, 2H), 4.39-4.35 (m, 1H), 4.17 (dd, *J* = 7.0, 8.3 Hz, 1H), 3.05-2.98 (m, 1H), 2.97-2.90 (m, 1H), 2.38 (t, *J* = 7.3 Hz, 2H), 2.21-2.10 (m, 2H), 2.05 (s, 3H), 2.01-1.94 (m, 1H), 1.74-1.67 (m, 1H), 1.62-1.55 (m, 1H), 1.51-1.31 (m, 6H), 1.26-1.17 (m, 4H), 0.86 (d, *J* = 6.8 Hz, 3H), 0.83 (d, *J* = 6.8 Hz, 3H); ^{13}C NMR (126 MHz, d_6-DMS0) δ 208.7, 172.6, 171.3, 170.4, 159.0, 137.50, 137.46, 127.0, 118.9, 62.6, 57.8, 53.1, 42.7, 38.7, 35.2, 30.3, 29.7, 29.4, 28.4, 28.3, 26.8, 25.3, 23.2, 19.3, 18.2; HRMS(ESI) *m/z* calcd. for $C_{27}H_{44}N_5O_6$ $[M+H]^+$: 534.3286, found 534.3294.



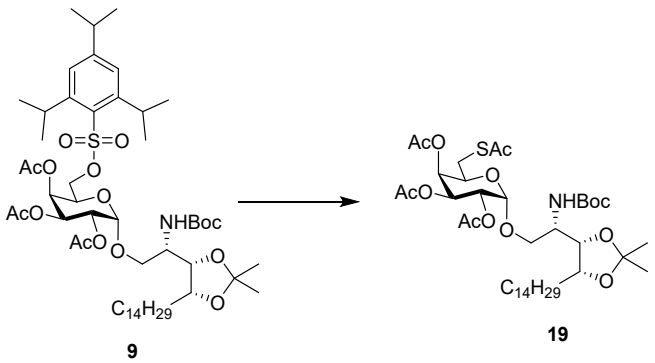
16 **17**

4-Nitrophenyl 4-(*N*-(8-oxononanoyl)-L-valinyl-L-citrullinamido)benzyl carbonate (**17**).[10] To a mixture of **16** (265 mg, 0.496 mmol) in DMF (2.5 mL) was added bis(4-nitrophenyl) carbonate (165 mg, 0.537 mmol) and Et_3N (0.08 mL, 0.6 mmol). After stirring under Ar at room temperature (24 h), the reaction mixture was concentrated *in vacuo* onto silica gel and purified by flash chromatography on silica gel (MeOH/CH_2Cl_2 = 5:95 to 13:87) to give **17** as an off-white solid (271 mg, 78%). ^{1}H NMR (500 MHz, d_6-

DMSO) δ 10.02 (s, 1H), 8.32-8.29 (m, 2H), 8.05 (d, *J* = 7.5 Hz, 1H), 7.79 (d, *J* = 8.6 Hz, 1H), 7.64 (d, *J* = 8.6 Hz, 2H), 7.57-7.54 (m, 2H), 7.40 (d, *J* = 8.6 Hz, 2H), 5.98 (br t, *J* = 5.7 Hz, 1H), 5.40 (br s, 2H), 5.24 (s, 2H), 4.40-4.36 (m, 1H), 4.18 (dd, *J* = 6.9, 8.5 Hz, 1H), 3.06-2.99 (m, 1H), 2.98-2.91 (m, 1H), 2.38 (t, *J* = 7.3 Hz, 2H), 2.21-2.10 (m, 2H), 2.05 (s, 3H), 2.01-1.94 (m, 1H), 1.75-1.68 (m, 1H), 1.64-1.56 (m, 1H), 1.51-1.32 (m, 6H), 1.26-1.17 (m, 4H), 0.86 (d, *J* = 6.8 Hz, 3H), 0.84 (d, *J* = 6.8 Hz, 3H); ^{13}C NMR (126 MHz, d_6-DMSO) δ 208.4, 172.4, 171.3, 170.7, 158.8, 155.3, 151.9, 145.1, 139.3, 129.4, 129.3, 125.3, 122.5, 119.0, 70.2, 57.6, 53.1, 42.7, 38.5, 35.1, 30.3, 29.6, 29.2, 28.4, 28.2, 26.8, 25.2, 23.1, 19.2, 18.2; HRMS(ESI) *m/z* calcd. for $C_{34}H_{47}N_6O_{10}$ $[M+H]^+$: 699.3348, found 699.3360.

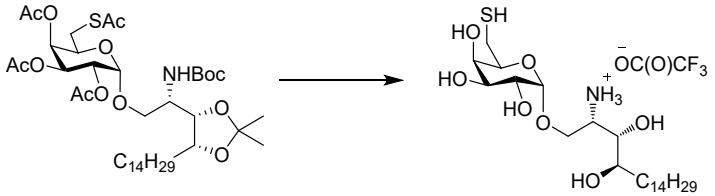


(2*S*,3*S*,4*R*)-1-*O*-[6-Deoxy-6-(*N*-(8-oxononanoyl)-Val-Cit-4-aminobenzyloxycarbonyl)amino]-α-D-galactopyranosyl-2-hexacosanoylamino-octadecan-1,3,4-triol (**18**). To a solution of **13** (40 mg, 0.045 mmol) in CH_2Cl_2/MeOH (1:1, 12 mL) as was added Pd/C (10%, 30 mg, 0.038 mmol) then HCl (100 µL, 1 M). The atmosphere was replaced with H_2 and the reaction was stirred at room temperature (18 h). The catalyst was filtered through glass fibre, washed with hot EtOH (3 x 50 mL) and the filtrate was concentrated to give 6''-amino-6''-deoxy-α-GalCer (**14**) as an off-white solid. To crude amine (**14)** (40 mg) was added **17**[10] (45 mg, 0.064 mmol) and the two solids were thoroughly dried under high vacuum then placed under Ar before the addition of dry pyridine (5 mL) then dry Et_3N (100 µL). The reaction mixture was briefly sonicated before stirring at room temperature (18 h). MeOH (10 mL) was added, the reaction mixture was concentrated *in vacuo* and the residue was purified by automated flash column chromatography (MeOH/$CHCl_3$, 0:100 to 30:70) to give **18** as a white solid (44 mg, 69%, 97.8% pure by HPLC-CAD); HRMS(ESI) *m/z* calcd. for $C_{78}H_{142}N_7O_{15}$ $[M+H]^+$: 1417.0564, found 1417.0570.

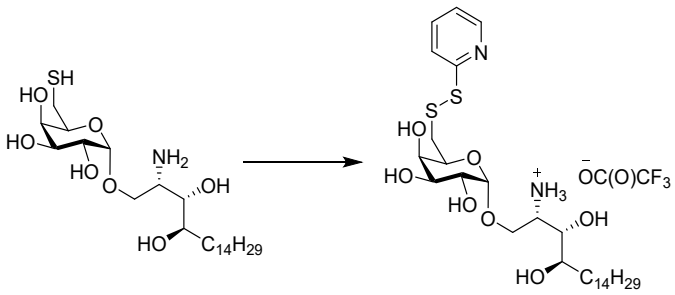


(2*S*,3*S*,4*R*)-2-(*N*-*tert*-Butoxycarbonyl) thio-1-*O*-(6-azido-2,3,4-*O*-acetyl-α-D-galactopyranosyl)-3,4-*O*-isopropylidene-octadecan-1,3,4-triol (**19**). To a solution of **9** (735 mg, 0.73 mmol) in dry DMF (15 mL)

under Ar was added potassium thioacetate (0.83 g, 7.2 mmol) and the reaction was stirred at 80 °C (18 h). The reaction was then diluted with EtOAc (150 mL) and washed with water (100 mL). The aqueous layer was extracted with EtOAc (100 mL) and the combined organic layers were dried ($MgSO_4$), filtered and concentrated *in vacuo.* The resulting light-yellow solid was purified by automated flash column chromatography (EtOAc/petroleum ether, 0:100 to 40:60) to give **19** as a pale yellow oil (510 mg, 87%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.47 (dd, *J* = 3.5, 1.2 Hz, 1H), 5.32 (dd, *J* = 10.6, 3.4 Hz, 1H), 5.15 – 5.07 (m, 2H), 4.74 (d, *J* = 9.7 Hz, 1H), 4.16 – 4.07 (m, 1H), 4.04 – 3.94 (m, 2H), 3.85 (d, *J* = 9.4 Hz, 1H), 3.77 (d, *J* = 10.4 Hz, 1H), 3.70 (d, *J* = 10.5 Hz, 1H), 3.10 (dd, *J* = 13.8, 6.3 Hz, 1H), 2.93 (dd, *J* = 13.9, 7.8 Hz, 1H), 2.32 (s, 3H), 2.16 (s, 3H), 2.04 (s, 3H), 1.99 (s, 3H), 1.56 – 1.50 (m, 2H), 1.46 (s, 9H), 1.39 (s, 3H), 1.28 (s, 3H), 1.34 – 1.21 (m, 24H), 0.88 (t, *J* = 6.9 Hz, 3H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 194.31, 170.33, 170.11, 154.76, 107.86, 97.23, 79.85, 77.94, 75.90, 69.69, 68.97, 68.06, 67.86, 67.75, 49.91, 31.92, 30.42, 29.68, 29.64, 29.55, 29.35, 28.68, 28.38, 28.12, 26.70, 25.72, 22.68, 20.68, 14.09; HRMS(ESI) *m/z* calcd. for $C_{40}H_{69}NO_{13}NaS$ $[M+Na]^+$: 826.4387, found 826.4402.

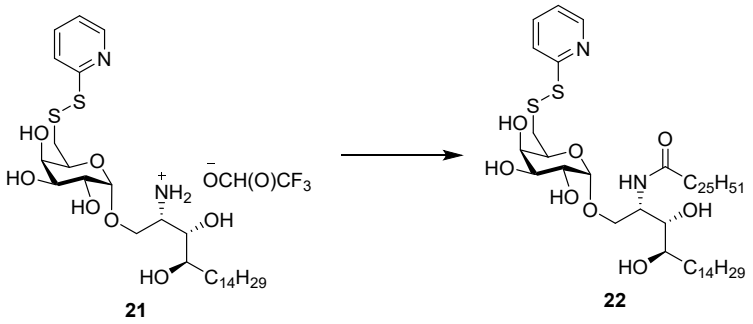


(2*S*,3*S*,4*R*)-2-Amino-1-*O*-(6-thio-α-D-galactopyranosyl)-octadecan-1,3-4-triol (**20**)**.** To a solution of **19** (270 mg, 0.34 mmol) in dry MeOH (4 mL) was added NaOMe/MeOH (30 µL, 30% solution) and the reaction was stirred at room temperature (1 h). The reaction was concentrated *in vacuo* and the crude product (as an 85:15 mixture of thiol/disulfide) was dissolved in CH_2Cl_2 (4 mL) then added water (800 µL), anisole (400 µL) and TFA (4 mL). The reaction mixture was stirred at room temperature (1 h) then concentrated *in vacuo.* The crude residue was dissolved in MeOH (3 mL) and treated with an aqueous solution of TCEP-HCl (100 mg/mL, 1 mL). After stirring at room temperature (2 h) the reaction mixture was concentrated *in vacuo* and the residue was purified by automated RP C18 flash column chromatography (MeOH/H_2O + 0.05% TFA, 30:70 to 100:0) to give **20** as a white solid (113 mg, 68%). ^{1}H NMR (500 MHz, CD_3OD) δ 4.88 (d, *J* = 3.9 Hz, 1H), 4.27 (dd, *J* = 10.8, 3.2 Hz, 1H), 3.99 (d, *J* = 3.9 Hz, 1H), 3.86 (dd, *J* = 10.1, 3.9 Hz, 1H), 3.79 – 3.73 (m, 2H), 3.70 (dt, *J* = 9.3, 3.3 Hz, 1H), 3.62 – 3.54 (m, 2H), 3.54 – 3.49 (m, 1H), 2.80 (dd, *J* = 13.7, 8.0 Hz, 1H), 2.67 (dd, *J* = 13.7, 5.7 Hz, 1H), 1.81 (ddd, *J* = 10.5, 8.5, 2.8 Hz, 1H), 1.64 – 1.50 (m, 1H), 1.31 (s, 24H), 0.92 (t, *J* = 6.9 Hz, 3H); ^{13}C NMR (126 MHz, CD_3OD) δ 99.45, 72.96, 72.41, 71.63, 69.98, 69.75, 68.60, 63.87, 53.64, 33.92, 31.65, 29.37, 29.34, 29.04, 24.80, 24.08, 22.31, 13.00; HRMS(ESI) *m/z* calcd. for $C_{24}H_{49}NO_7NaS$ $[M+Na]^+$: 518.3127, found 518.3134.



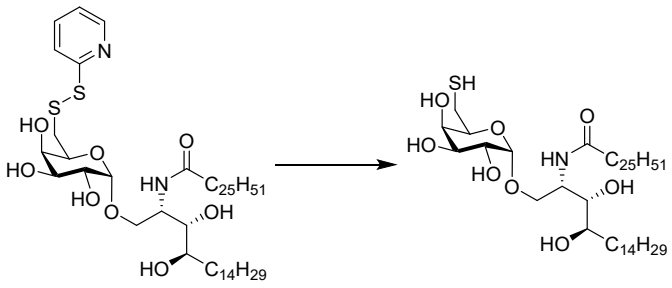
20 **21**

(2*S*,3*S*,4*R*)-2-Amino-1-*O*-(6-deoxy-6(pyridin-2-yldisulfaneyl)-α-D-galactopyranosyl)-octadecan-1,3,4-triol (**21**). To a solution of 2,2'-dipyridyl disulfide (250 mg, 1.12 mmol) in MeOH (5 mL) was added dropwise a solution of **20** (113 mg, 0.2280 mmol) in MeOH (2 mL). The reaction mixture was stirred at room temperature (30 min), concentrated *in vacuo*, and the crude product was purified by RP C18 flash column chromatography (MeOH/H_2O + 0.05% TFA, 5:5 to 8:2) to afford **21** (110 mg, 80%) as a white amorphous solid. ^{1}H NMR (500 MHz, 3:1 $CD_3OD/CDCl_3$) δ 8.42 (d, *J* = 4.8 Hz, 1H), 7.88 (d, *J* = 8.1 Hz, 1H), 7.84-7.80 (m, 1H), 7.24 (ddd, *J* = 7.4, 4.8, 1.0 Hz, 1H), 4.89 (d, *J* = 3.8 Hz, 1H), 4.24 (dd, *J* = 10.7, 3.2 Hz, 1H), 4.06 (dd, *J* = 8.4, 5.2 Hz, 1H), 3.89 (d, *J* = 3.2 Hz, 1H), 3.86 (dd, *J* = 10.0, 3.6 Hz, 1H), 3.74 (dd, *J* = 10.1, 3.3 Hz, 1H), 3.72-3.70 (m, 1H), 3.63-3.57 (m, 3H), 3.11 (dd, 13.7, 8.1 Hz, 1H), 3.01 (dd, *J* = 13.7, 5.2 Hz, 1H), 1.85-1.76 (m, 1H), 1.60-1.50 (m, 1H), 1.45-1.35 (m, 2H), 1.35-1.21 (m, 22H) 0.89 (t, *J* = 7.0 Hz, 3H); ^{13}C NMR (126 MHz, 3:1 $CD_3OD/CDCl_3$) δ 160.5, 150.0, 139.0, 122.2, 121.4, 100.6, 73.3, 72.5, 71.1, 70.8, 70.1, 69.5, 65.2, 54.8, 40.0, 35.1, 32.7, 30.4, 30.1, 25.9, 23.4, 14.3; HRMS(ESI) *m/z* calcd for $C_{29}H_{53}N_2O_7S_2$ $[M+H]^+$: 605.3294, found 605.3289.



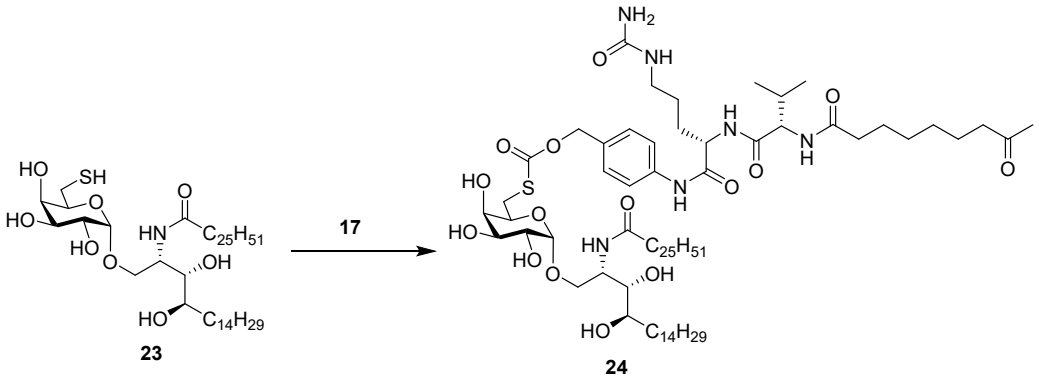
21 **22**

(2*S*,3*S*,4*R*)-1-O-[6-Deoxy-6-(2-pyridyl)disulfanyl]-α-D-galactopyranosyl)-2-hexacosanoylamino-1,3,4-octadecantriol (**22**).[12] To a suspension of hexacosanoic acid (80 mg, 0.20 mmol) in dry CH_2Cl_2 (10 mL) under Ar was added Et_3N (0.500 mL, 3.59 mmol) followed by isobutyl chloroformate (26 µL, 0.20 mmol. The reaction mixture was stirred at room temperature (1 h) before adding it to a solution of **21** (100 mg, 0.17 mmol) in dry DMF (5 mL). The reaction was stirred at room temperature (18h), diluted with MeOH (6 mL) then concentrated *in vacuo*. The residue was purified by automated flash column chromatography (MeOH/$CHCl_3$, 0:100 to 20:80) to give **22** as a white solid (130 mg, 79%). NMR data was consistent with literature reports.



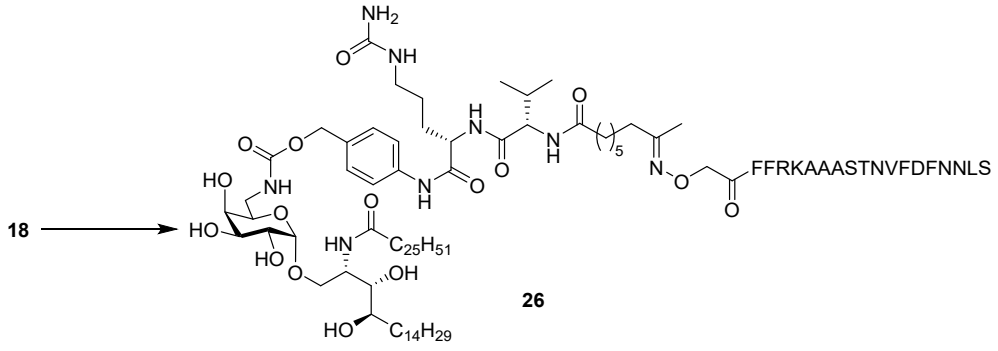
22 23

(2*S*,3*S*,4*R*)-1-*O*-(6-Deoxy-6-mercapto)-α-D-galactopyranosyl-2-hexacosanoylamino-1,3,4-octadecantriol (**23**).[12] To a solution of **22** (130 mg, 0.13 mmol) in anhydrous CH_2Cl_2/MeOH (1:1, 10 mL) was added a solution of tris(2-carboxyethyl)phosphine hydrochloride (100 mg/mL in H_2O, 0.75 mL, 0.26 mmol) and the reaction stirred at room temperature (1 h). The reaction mixture was concentrated *in vacuo*, and the crude product purified by automated flash column chromatography (MeOH/$CHCl_3$, 0:100 to 20:80) to afford **23** (100 mg, 87%) as a white amorphous solid. NMR data was consistent with literature reports.



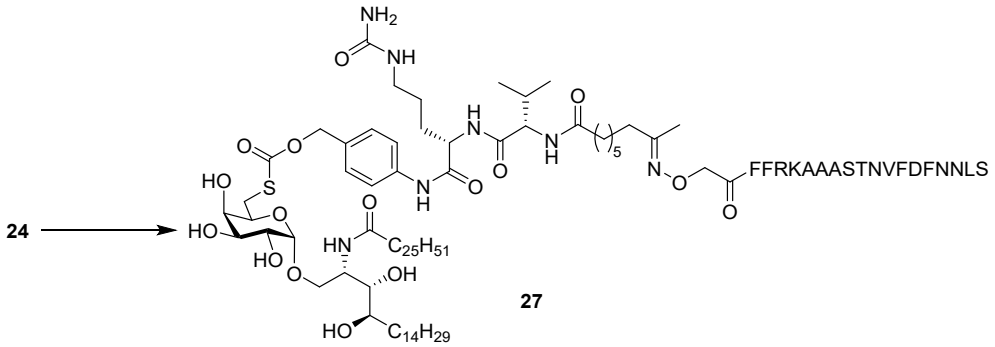
23 **17** 24

(2*S*,3*S*,4*R*)-1-*O*-[6-Deoxy-6-(*N*-(8-oxononanoyl)-Val-Cit-4-aminobenzyloxycarbonyl)thio]-α-D-galactopyranosyl-2-hexacosanoylamino-1,3,4-octadecantriol (**24**). To a mixture of **23** (18 mg, 0.021 mmol) and **17**[10] (22 mg, 0.031 mmol) thoroughly dried under high vacuum (1 h) then placed under Ar, was added dry pyridine (2 mL) and the reaction mixture was stirred at room temperature (18 h). MeOH (10 mL) was added and the reaction mixture was concentrated *in vacuo*. The residue was purified by automated flash column chromatography (MeOH/$CHCl_3$, 0:100 to 30:70) to give **24** as a white solid (20 mg, 0.014 mmol, 68%, 96.3% pure by HPLC-CAD); HRMS(ESI) *m/z* calcd. for $C_{78}H_{142}N_6O_{15}S$ $[M+H]^+$: 1434.0176; found 1434.0181.

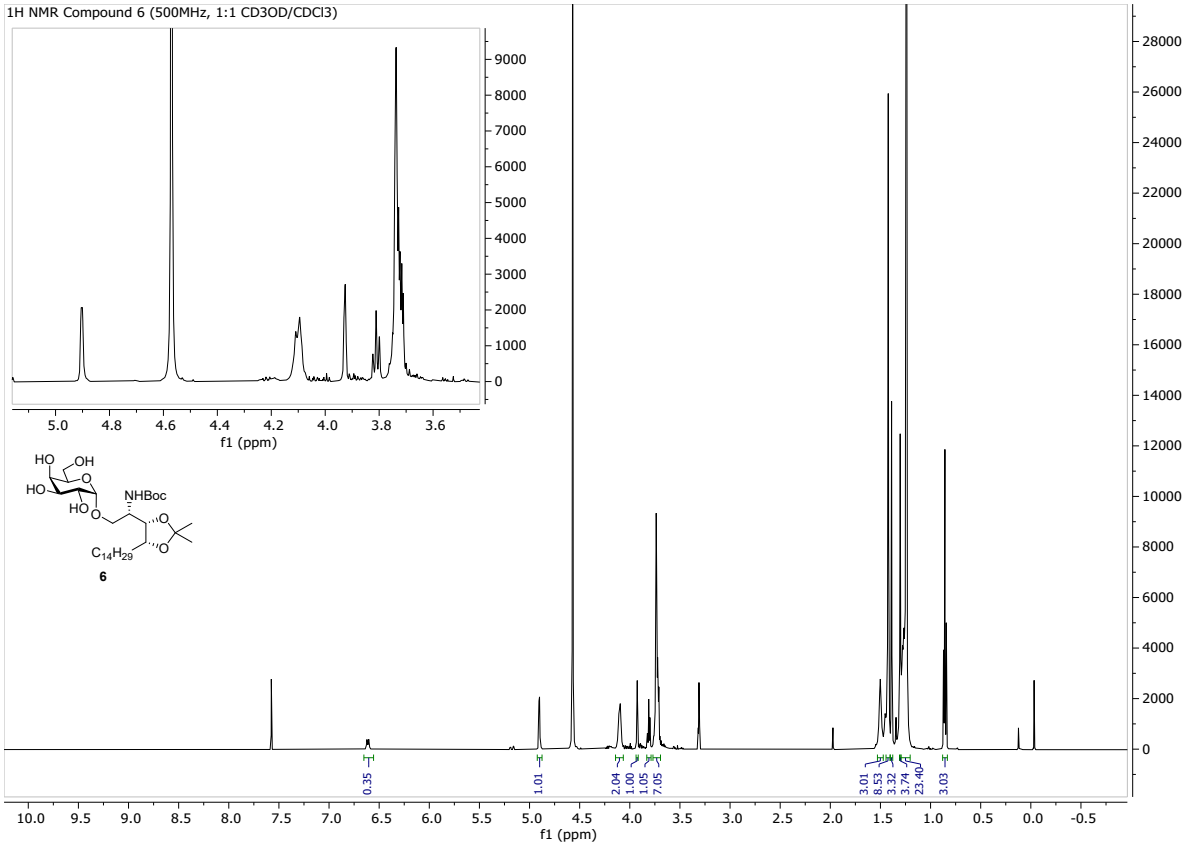


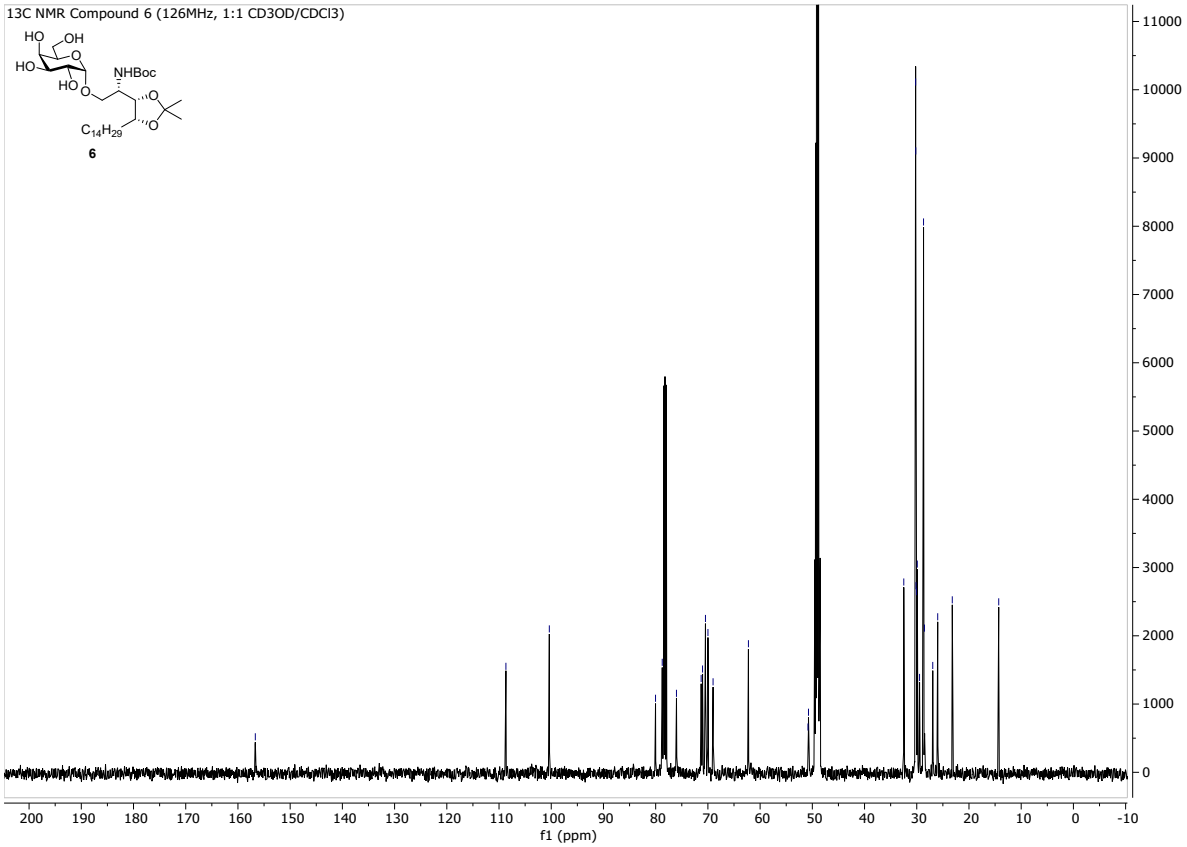
18 26

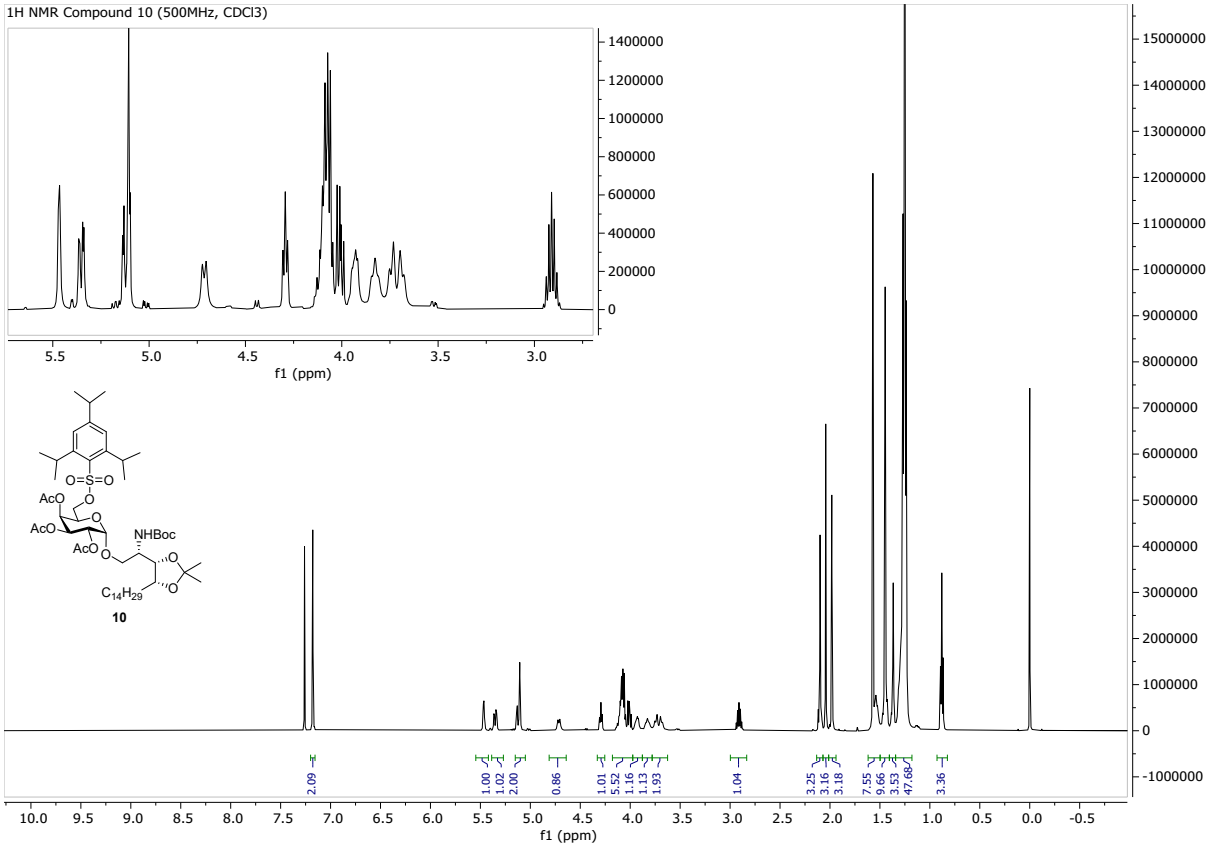
GLP-conjugate vaccine candidate (**26**). Aniline/HFIP solution was prepared by adding freshly distilled aniline (45.5 µL) to HPLC grade HFIP (1 mL, 99.9% pure) followed by TFA (10 µL). To a mixture of **18** (1.5 mg, 1.1 µmol) and aminooxyacetyl-FFRKAAASTNVFDFNNLS (**25**) (4 mg, 1.6 µmol) under Ar was added a solution of aniline in HFIP (200 µL) and the reaction was stirred at 35 °C (18 h). The reaction mixture was diluted with DMSO (600ul) and purified by preparative HPLC to give **26** (2.24 mg, 60%, 98.9% pure by HPLC-CAD) as a white solid. HRMS(ESI) *m/z* calcd. for $C_{174}H_{282}N_{33}O_{43}$ $[M+3H]^{3+}$: 1174.0298; found 1174.0331.

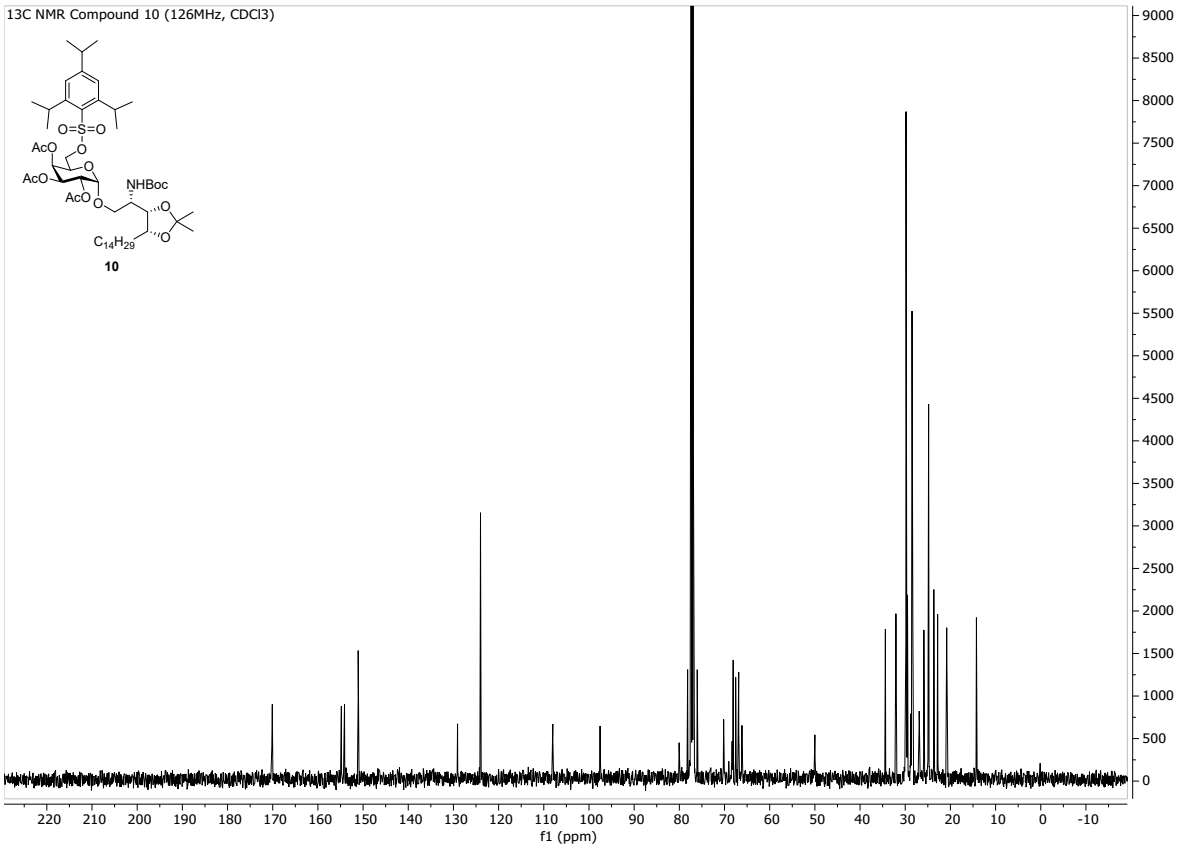


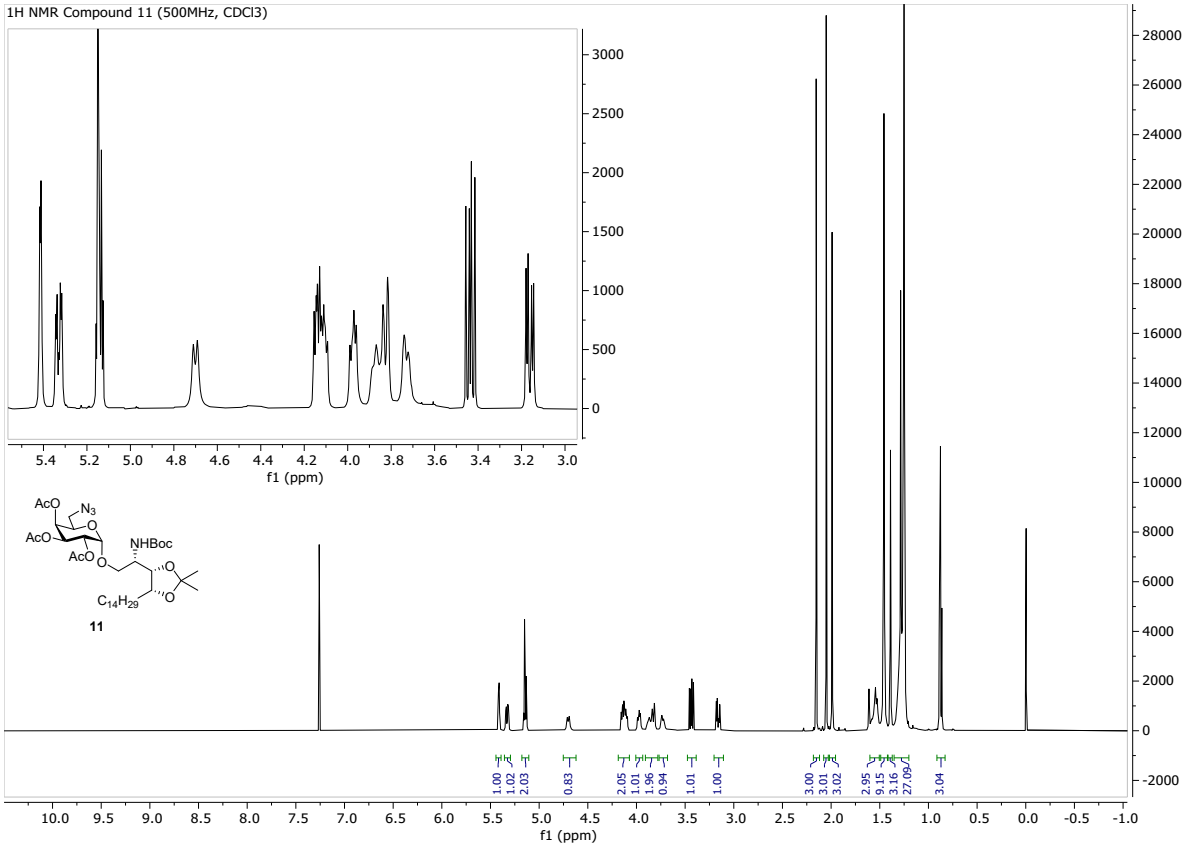
GLP-conjugate vaccine candidate (**27**). Aniline/HFIP solution was prepared by adding freshly distilled aniline (45.5 µL) to HPLC grade HFIP (1 mL, 99.9% pure) followed by TFA (10 µL). To a mixture of **23** (1.5 mg, 1.0 µmol) and aminooxyacetyl-FFRKAAASTNVFDFNNLS (**25**) (4 mg, 1.6 µmol) under Ar was added the aniline/HFIP solution (200 µL) and the reaction was stirred at 35 °C (18 h). The reaction mixture was diluted with DMSO (600 µL) and purified by preparative HPLC to give **27** (1.85 mg, 50%, 99.3% pure by HPLC-CAD) as a white solid. HRMS(ESI) *m/z* calcd. for $C_{174}H_{280}N_{32}O_{43}S$ $[M+2H]^{2+}$: 1769.0214; found 1769.0220.

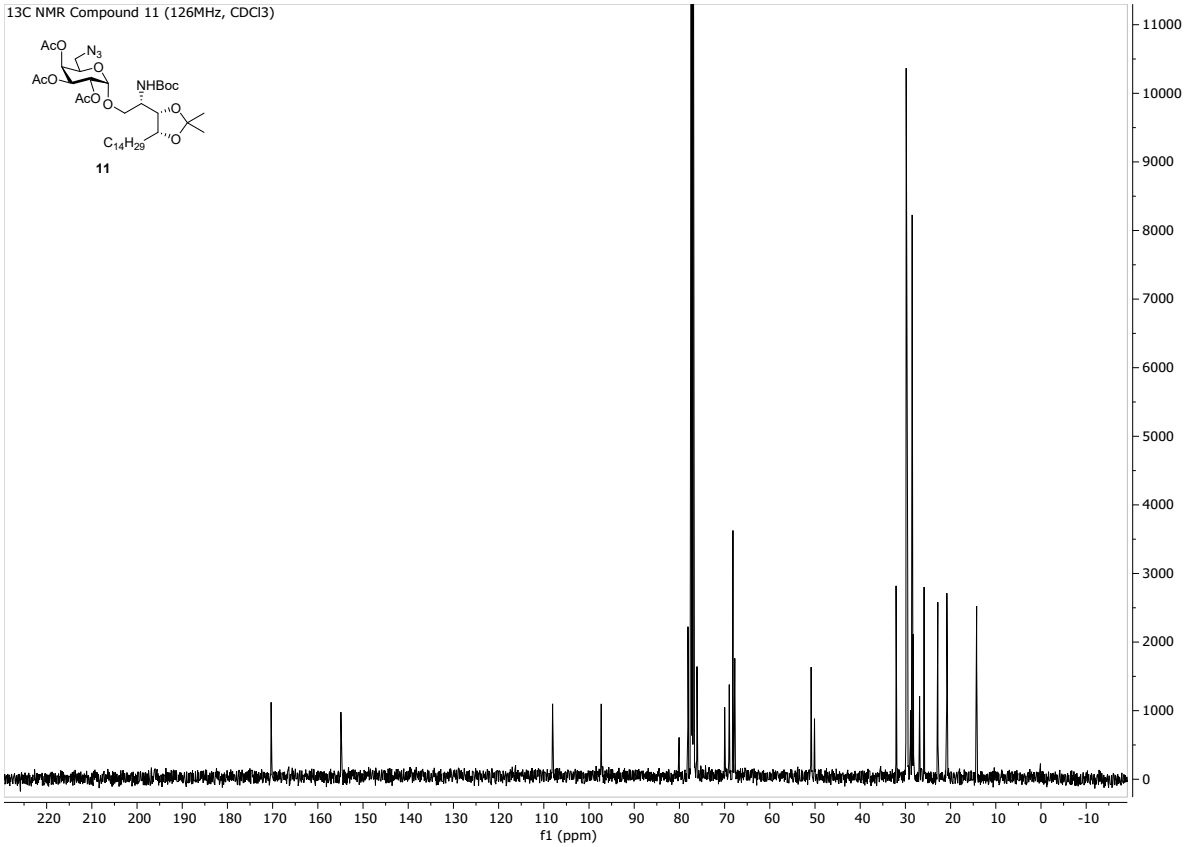

1H NMR Compound 6 (500MHz, 1:1 CD3OD/CDCl3)
f1 (ppm)
HO
OH
HO
HO
O
NHBoc
O
O
C14H29
6
0.35
1.01
2.04
1.00
1.05
7.05
3.01
8.53
3.32
3.74
23.40
3.03

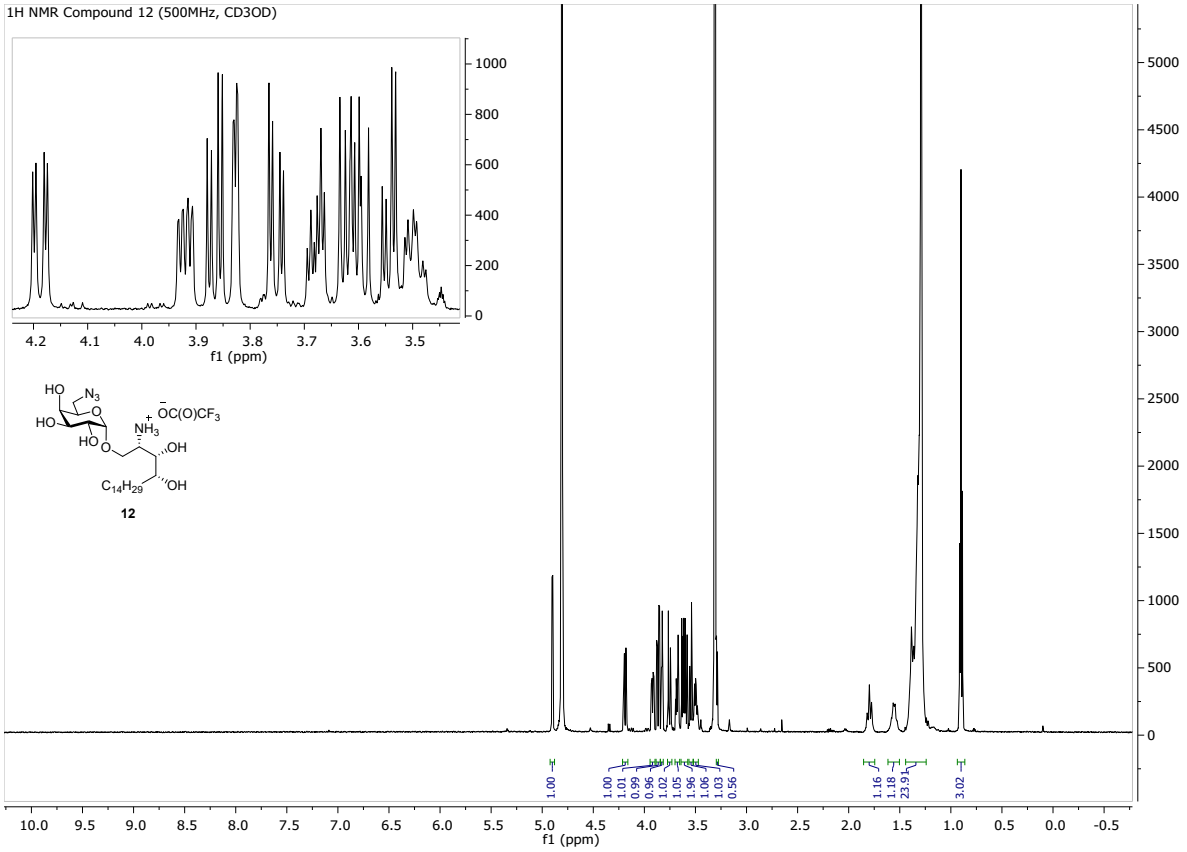

13C NMR Compound 6 (126MHz, 1:1 CD3OD/CDCl3)
f1 (ppm)
HO
OH
HO
HO
O
NHBoc
O
O
C14H29
6

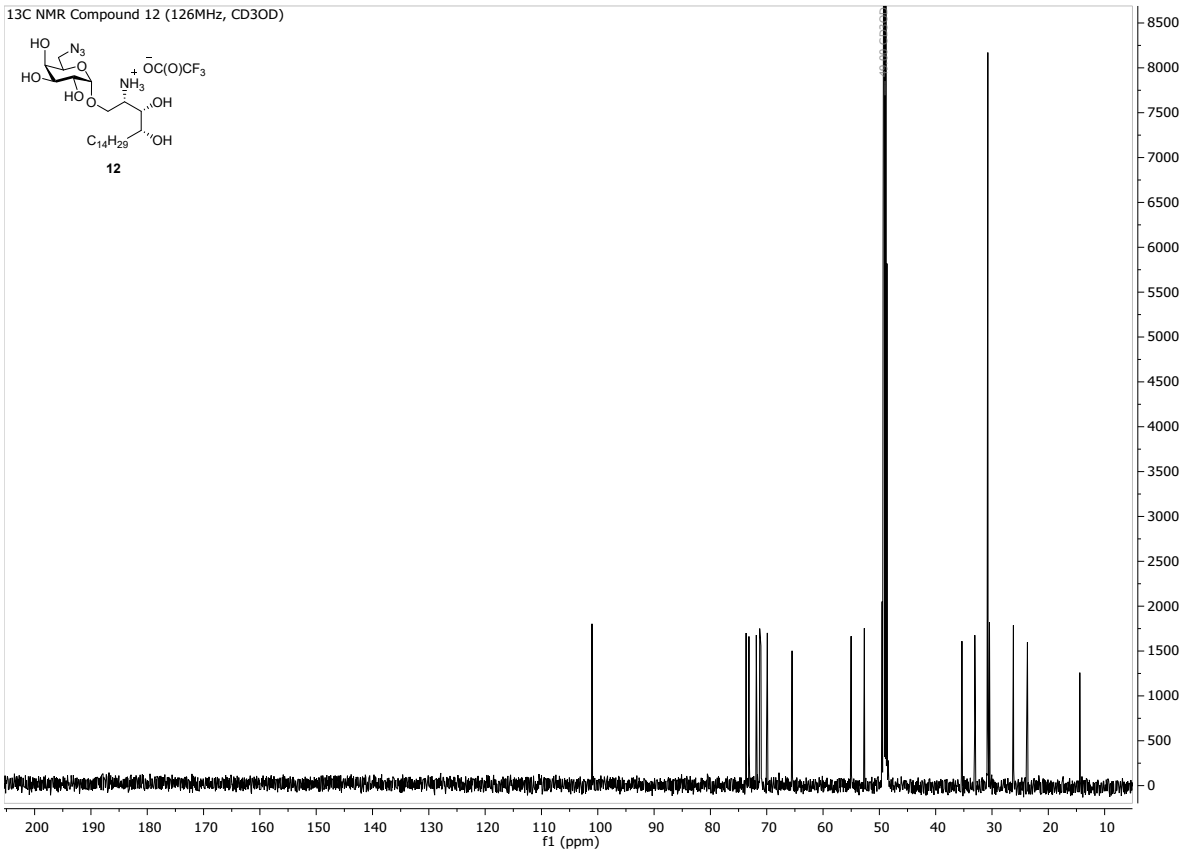

1H NMR Compound 10 (500MHz, CDCl3)
f1 (ppm)
10

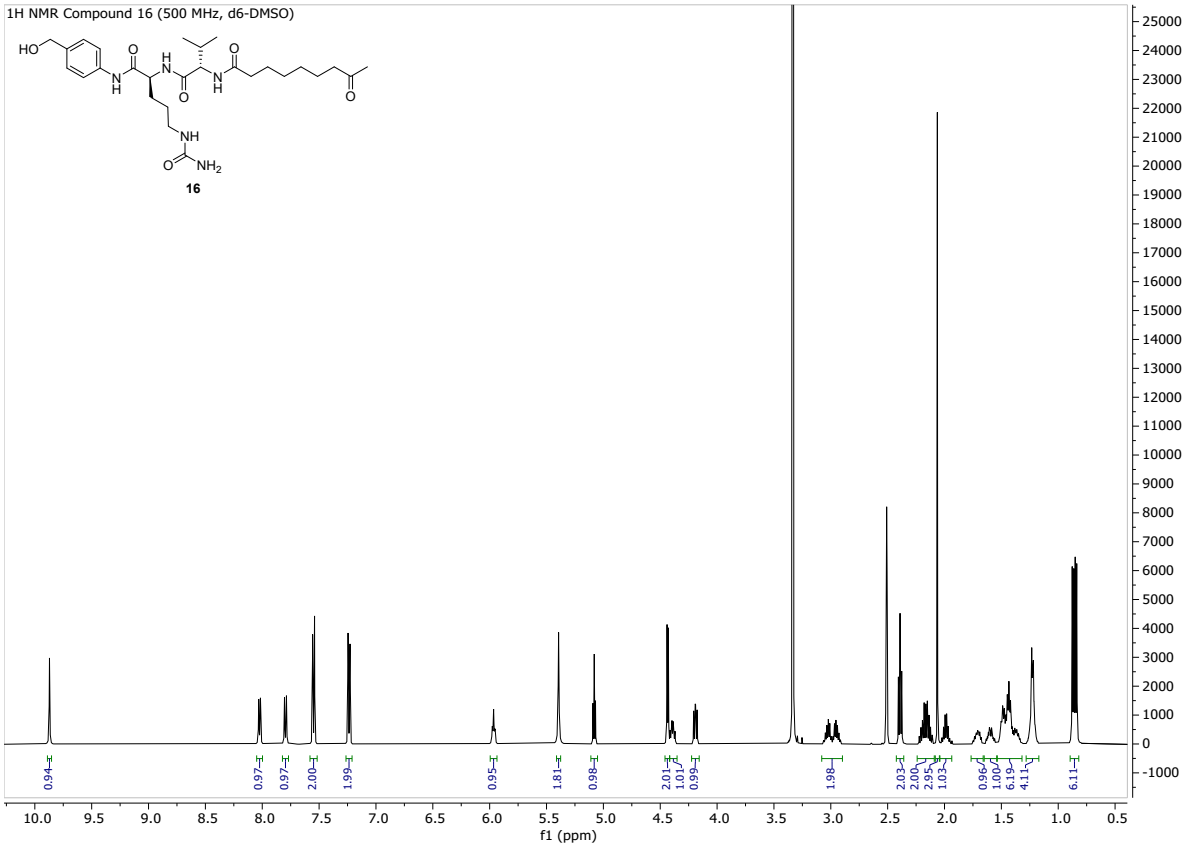

13C NMR Compound 10 (126MHz, CDCl3)
f1 (ppm)
10

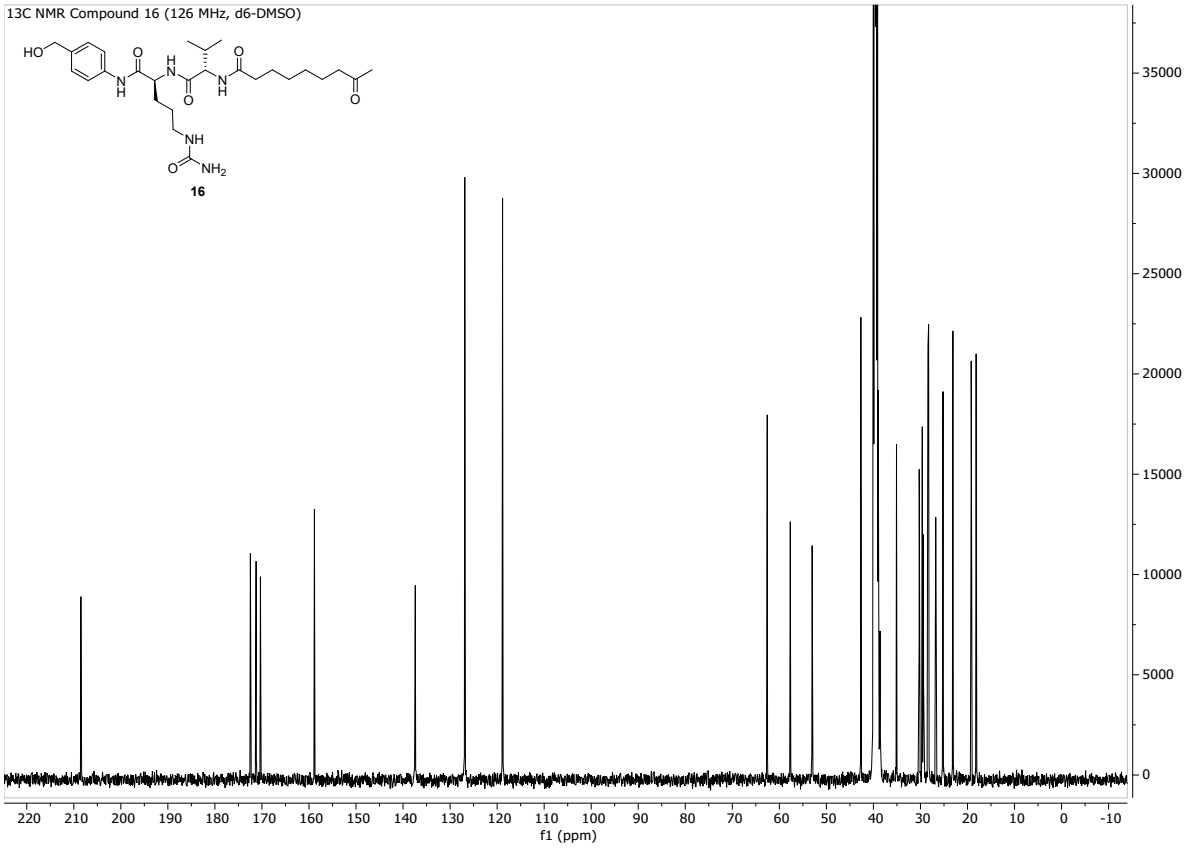

1H NMR Compound 11 (500MHz, CDCl3)
f1 (ppm)
11

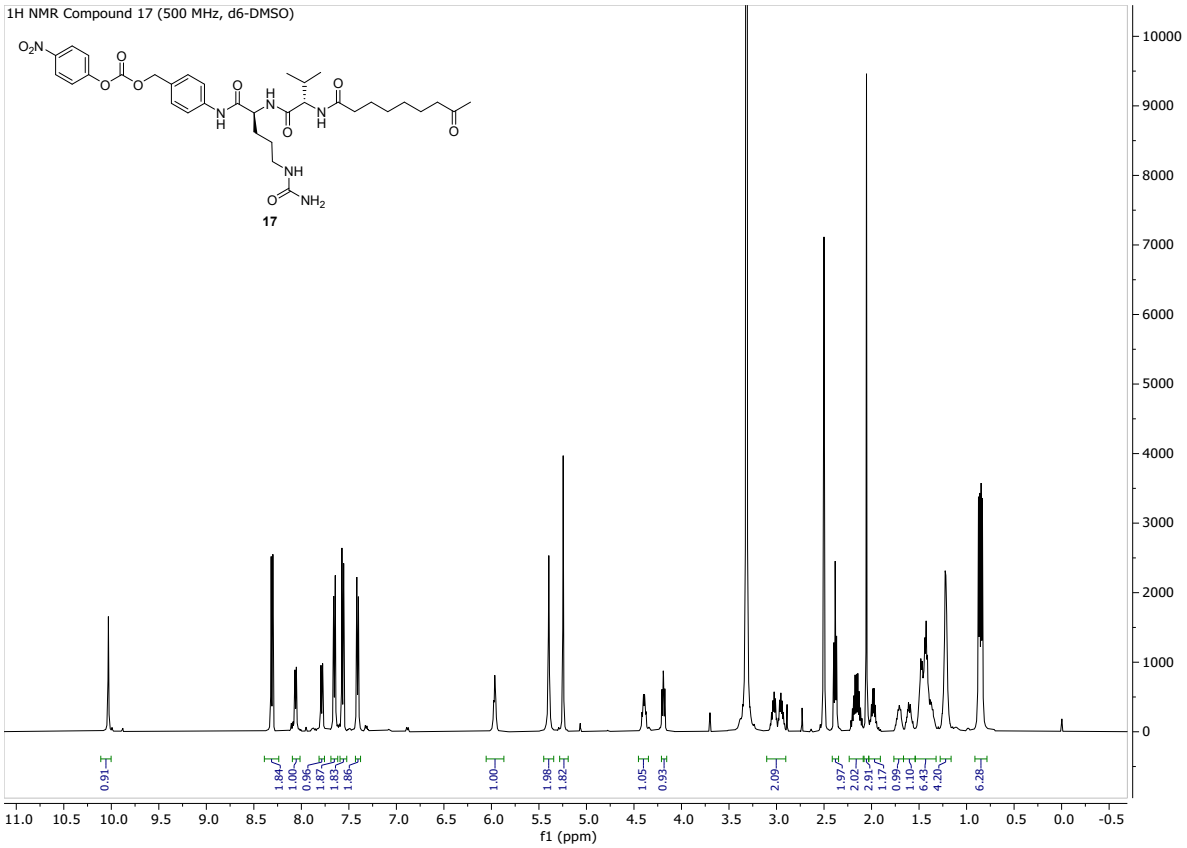

13C NMR Compound 11 (126MHz, CDCl3)
f1 (ppm)
11

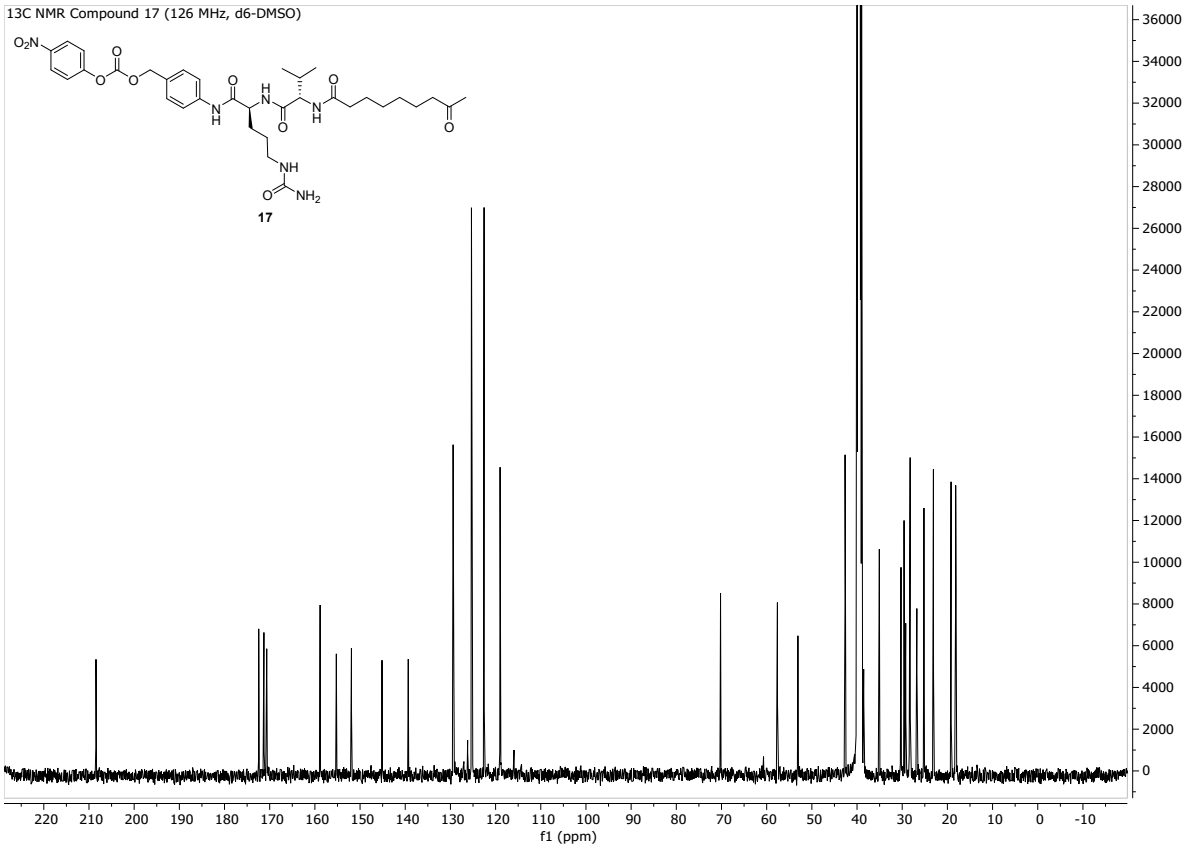

1H NMR Compound 12 (500MHz, CD3OD)
f1 (ppm)
12

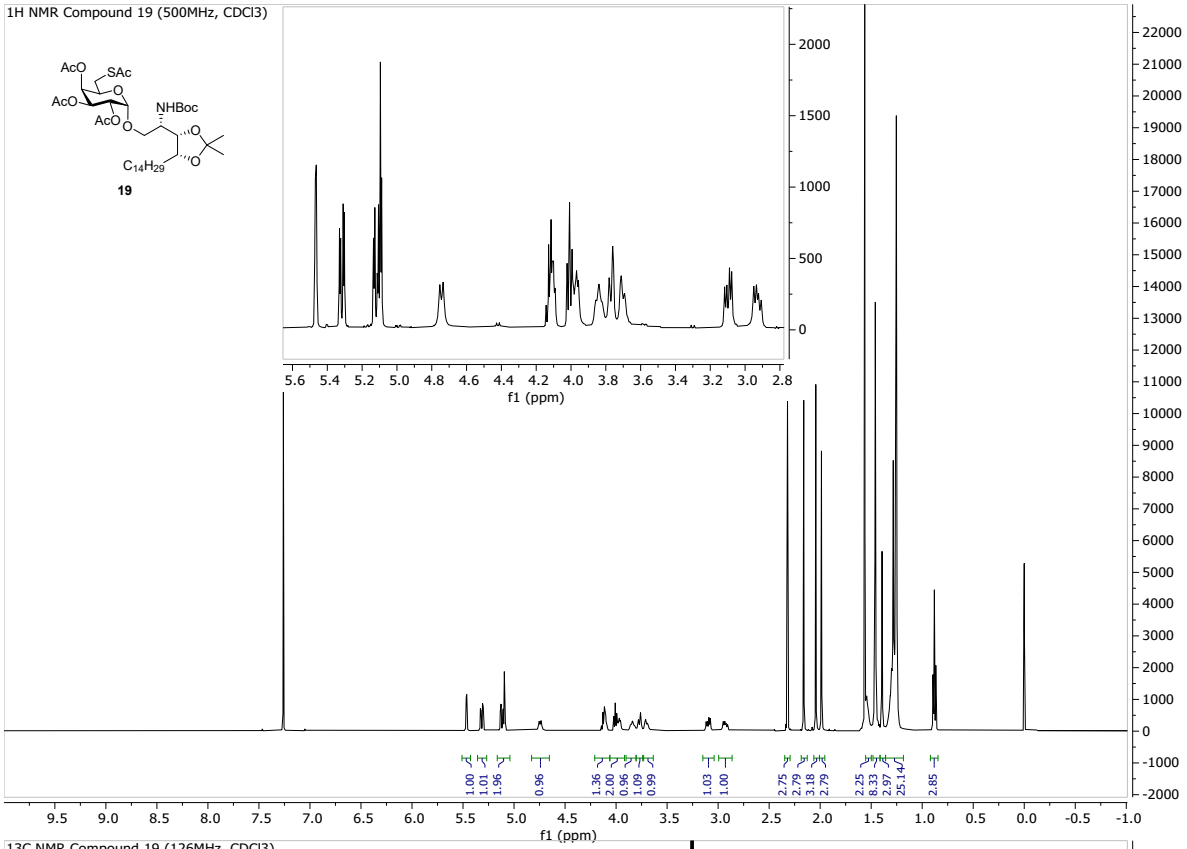

13C NMR Compound 12 (126MHz, CD3OD)
f1 (ppm)
12

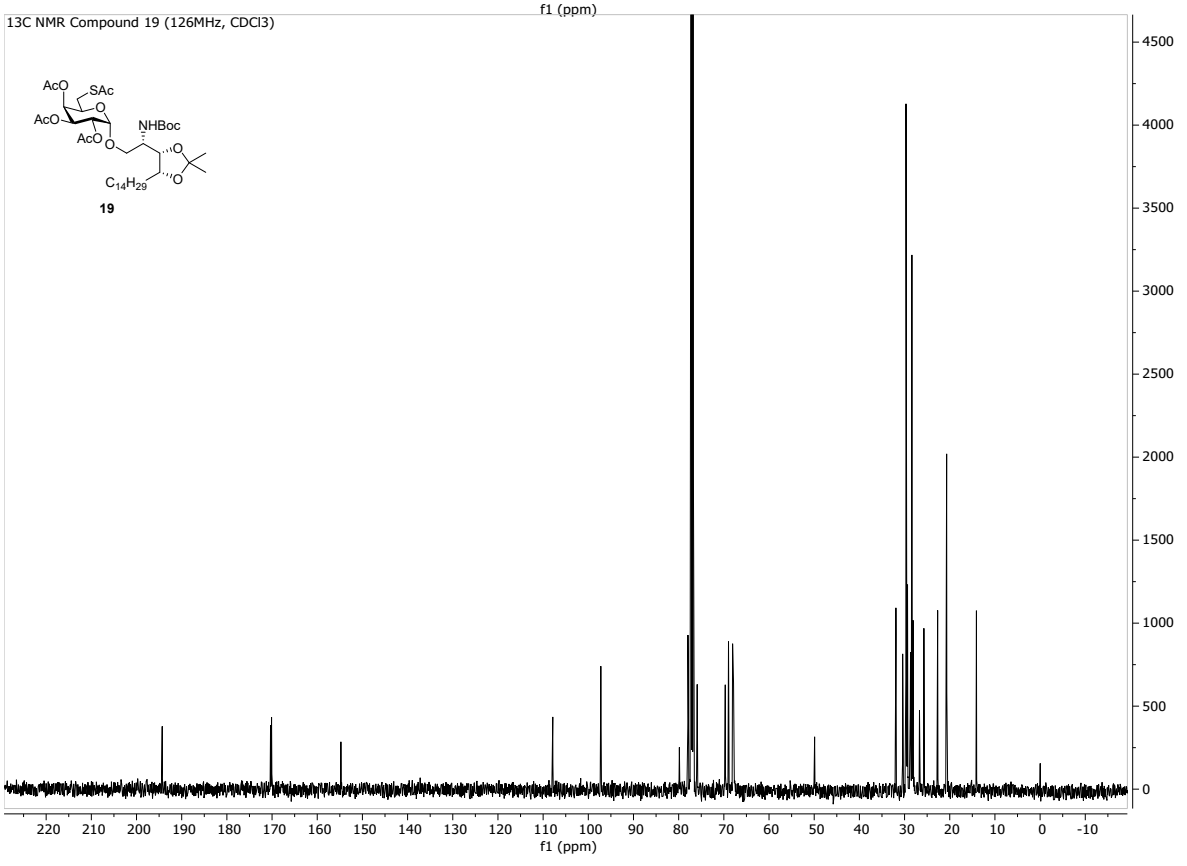

1H NMR Compound 16 (500 MHz, d6-DMSO)
16
f1 (ppm)

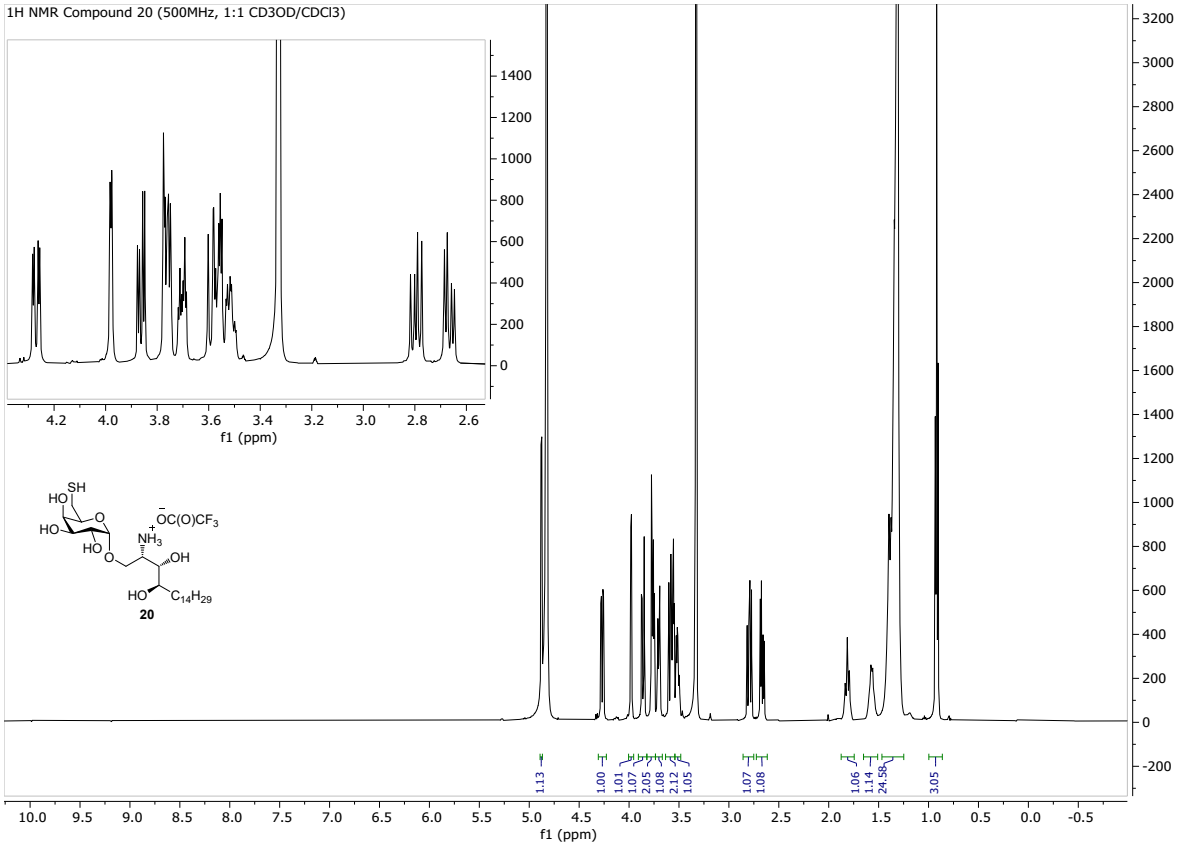

13C NMR Compound 16 (126 MHz, d6-DMSO)
16
f1 (ppm)

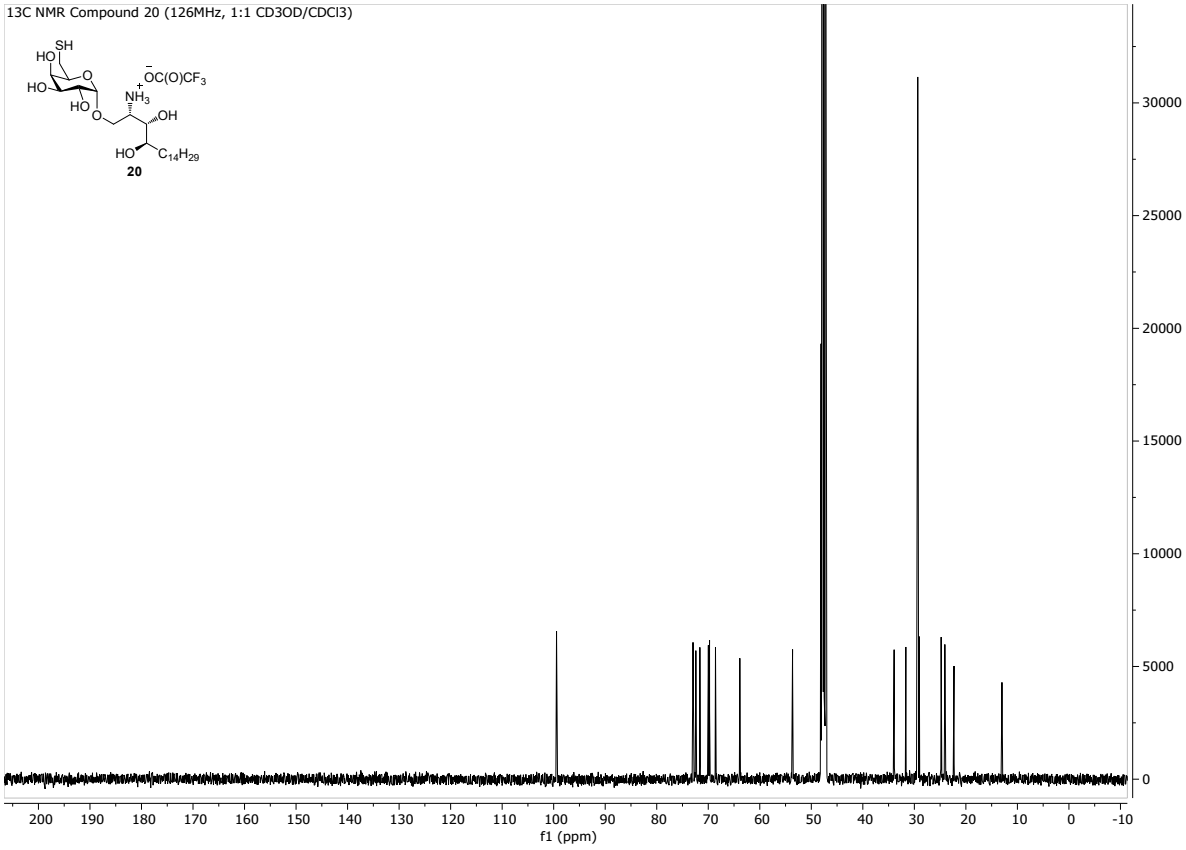

1H NMR Compound 17 (500 MHz, d6-DMSO)
17
f1 (ppm)

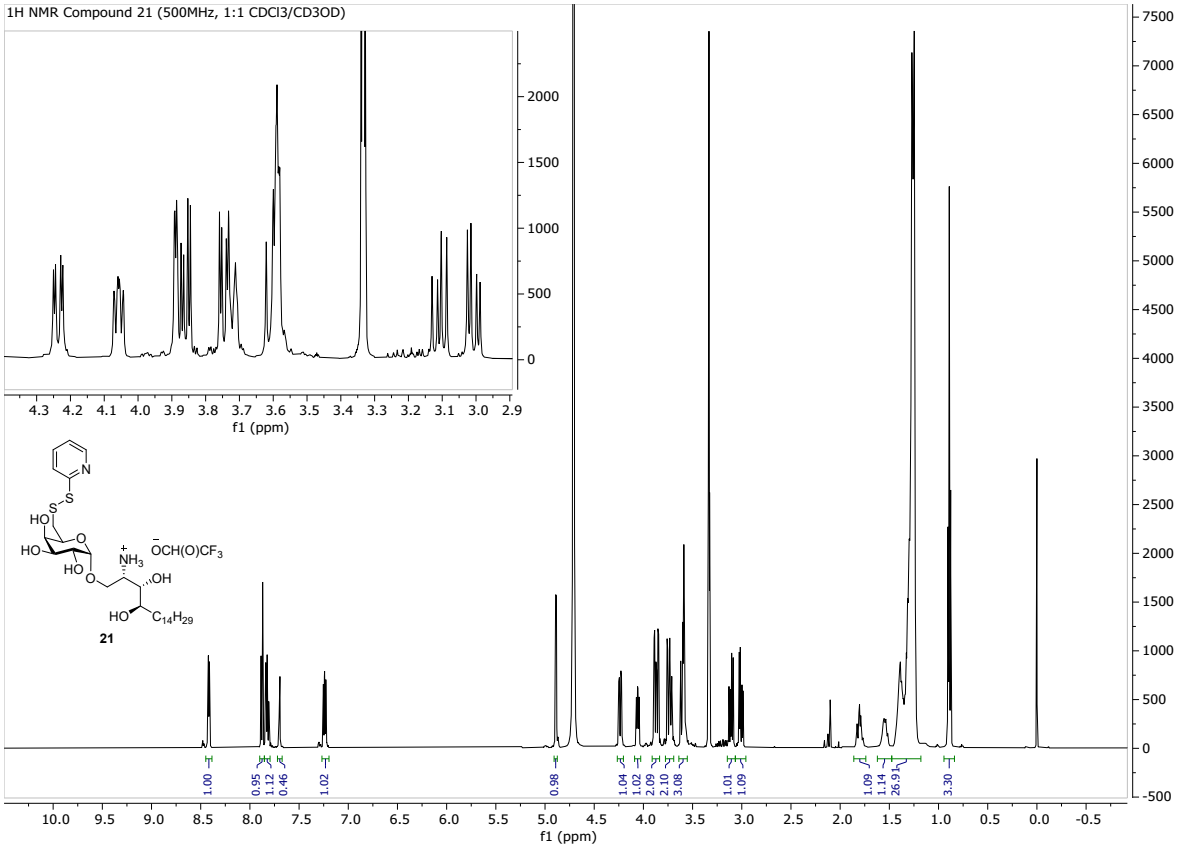

13C NMR Compound 17 (126 MHz, d6-DMSO)
17
f1 (ppm)

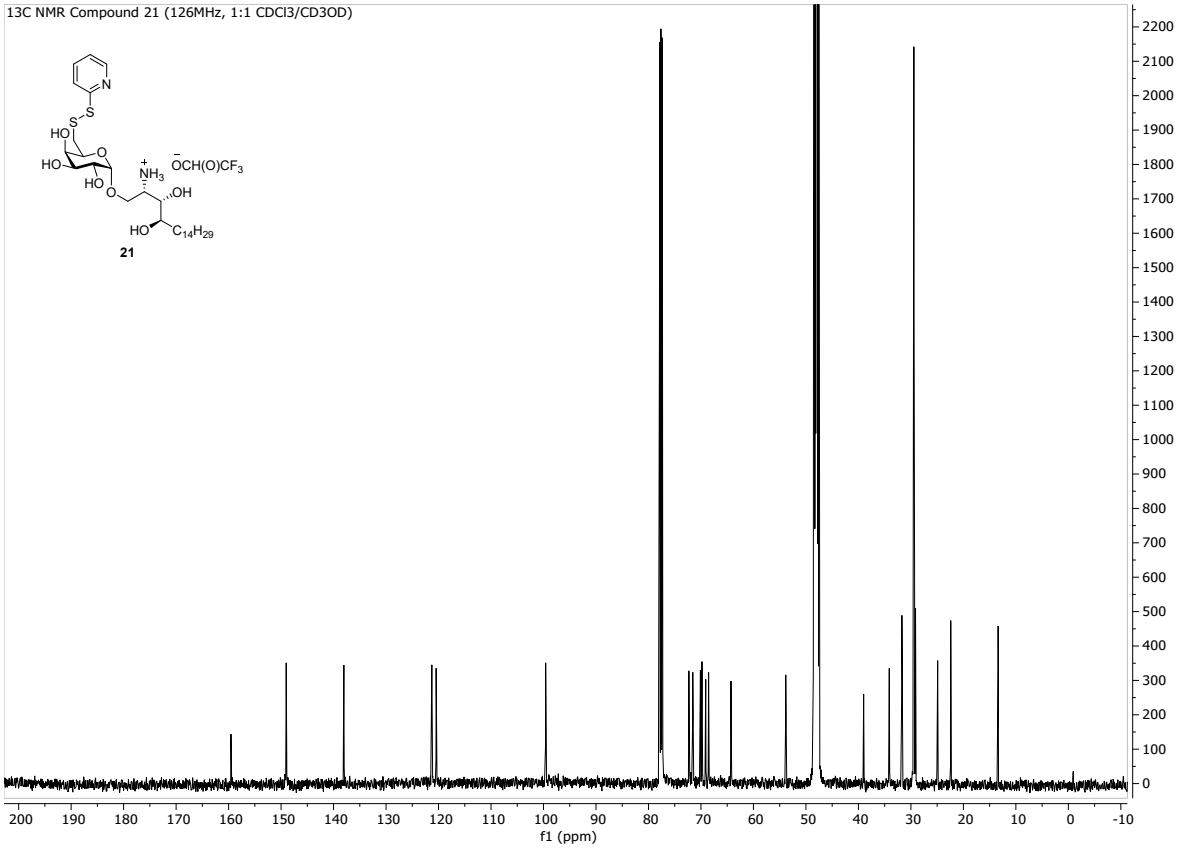

1H NMR Compound 19 (500MHz, CDCl3)
AcO
SAc
AcO
NHBoc
AcO
O
C14H29
19
f1 (ppm)


13C NMR Compound 19 (126MHz, CDCl3)
AcO
SAc
AcO
NHBoc
AcO
O
C14H29
19
f1 (ppm)

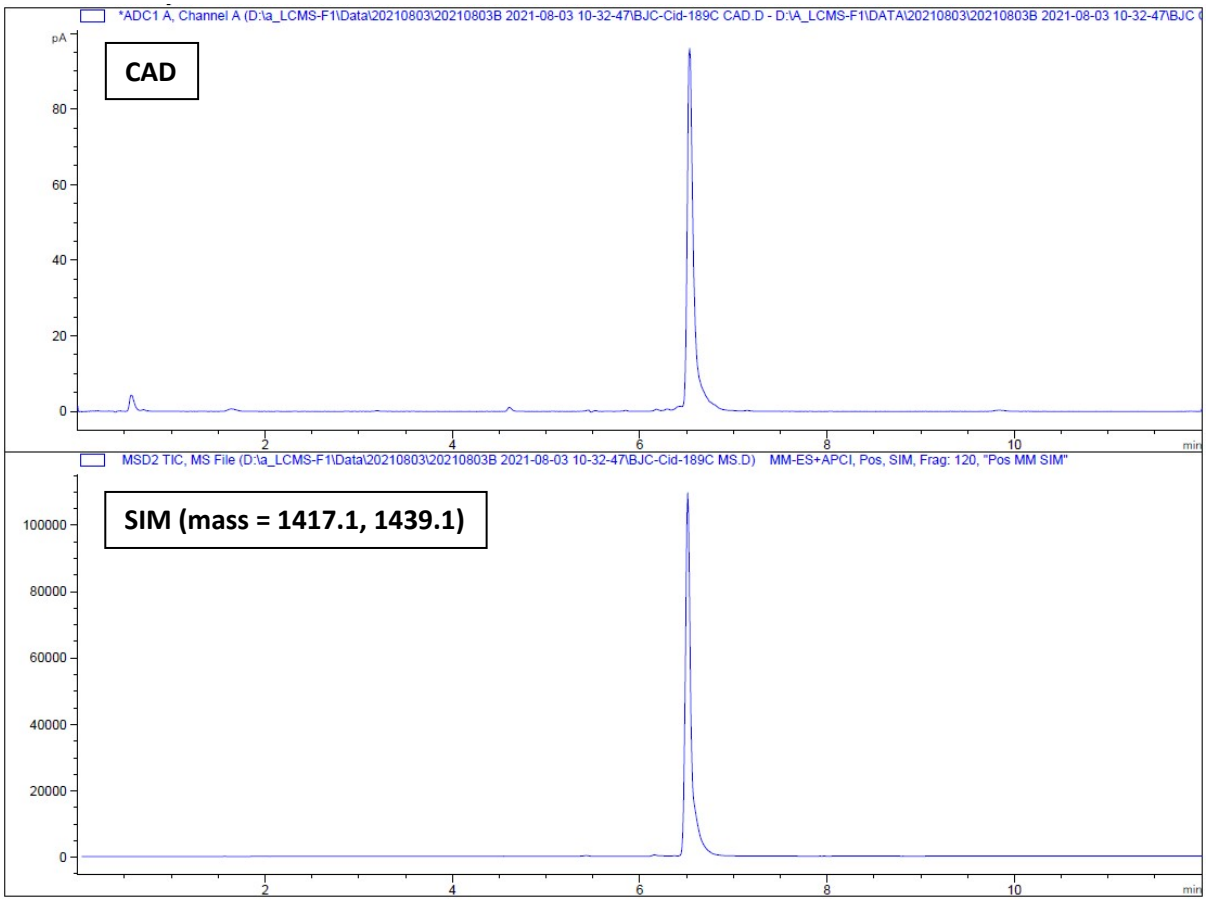

1H NMR Compound 20 (500MHz, 1:1 CD3OD/CDCl3)
f1 (ppm)
20

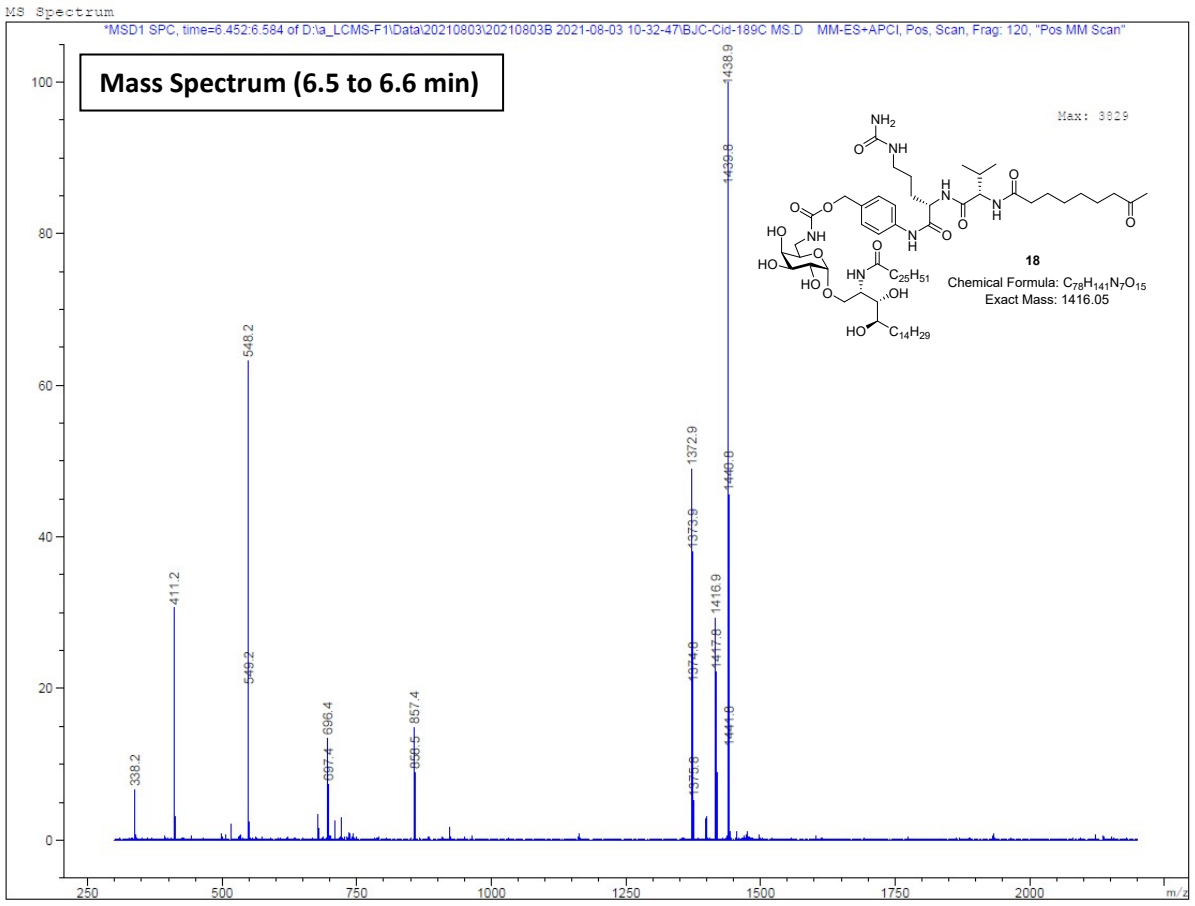

13C NMR Compound 20 (126MHz, 1:1 CD3OD/CDCl3)
f1 (ppm)
20


1H NMR Compound 21 (500MHz, 1:1 CDCl3/CD3OD)
f1 (ppm)
21

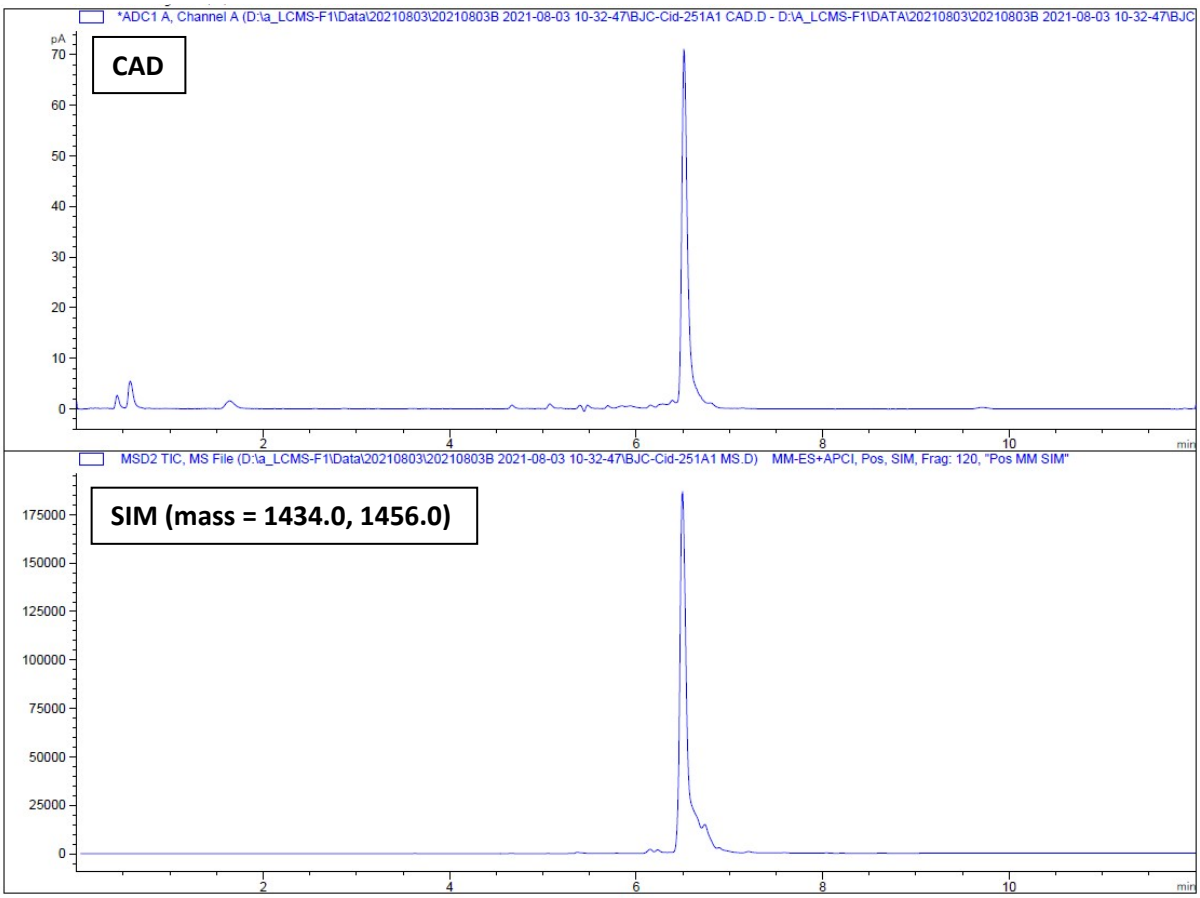

13C NMR Compound 21 (126MHz, 1:1 CDCl3/CD3OD)
f1 (ppm)
21

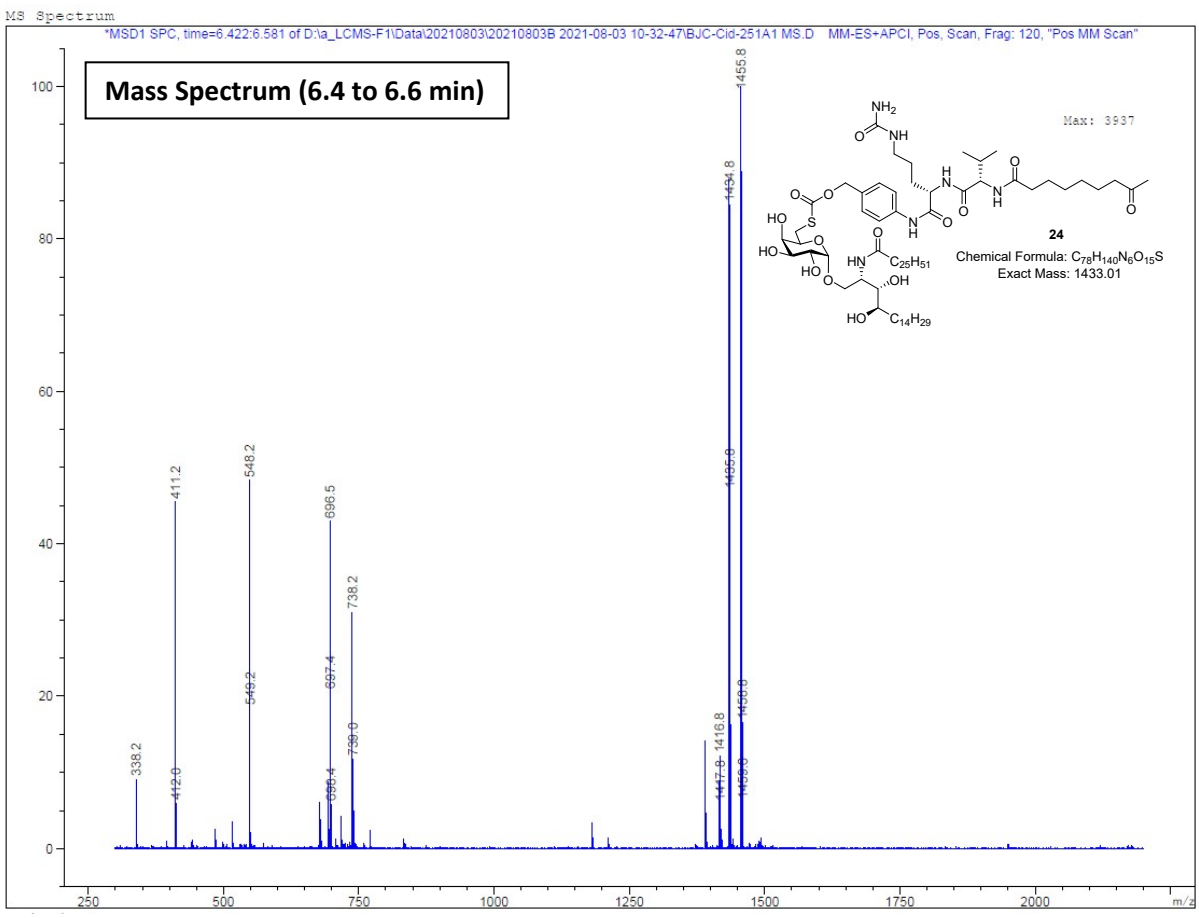
HPLC-MS-CAD analysis of pro-adjuvant **18** (97.8% pure)



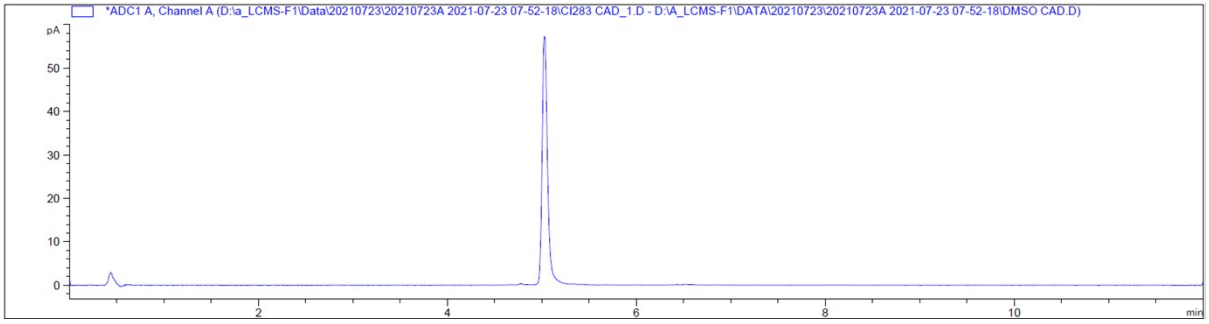


HPLC-MS-CAD analysis of pro-adjuvant **24** (96.3% pure)

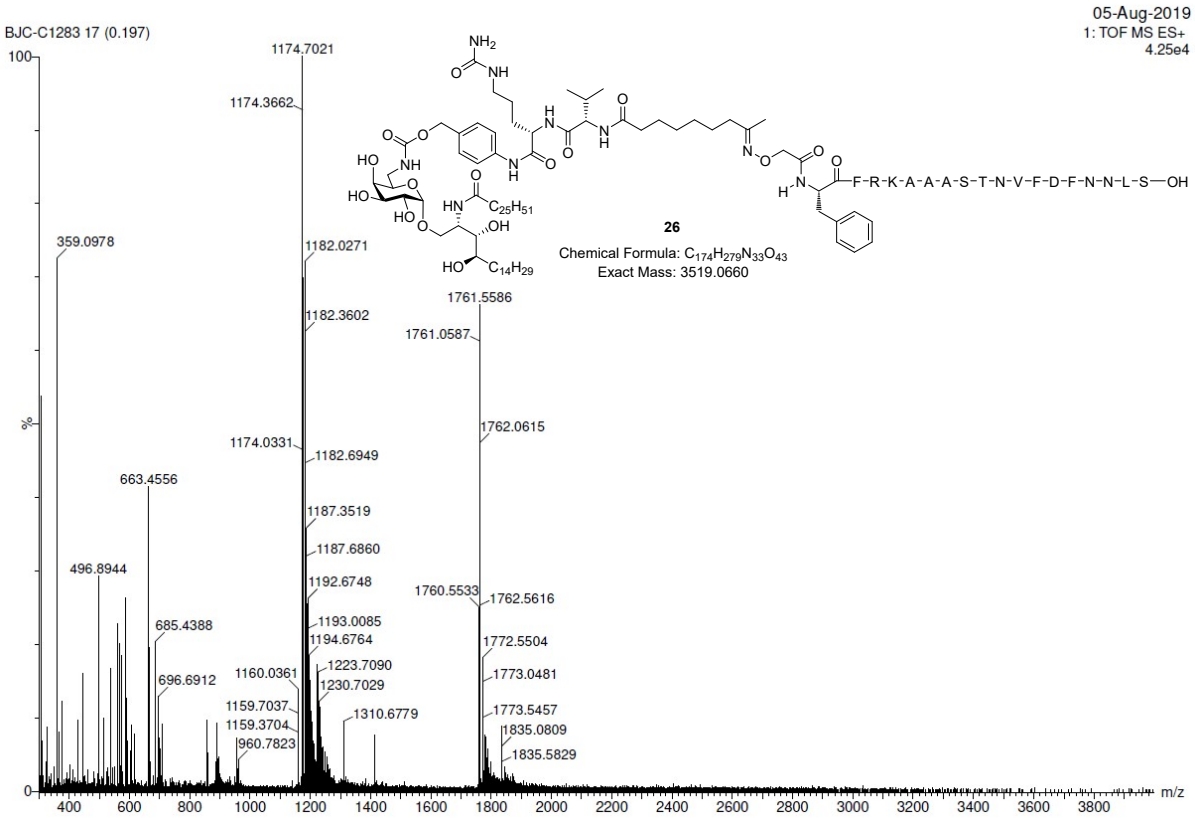




HPLC-CAD chromatogram of vaccine candidate **26** (98.9% pure)

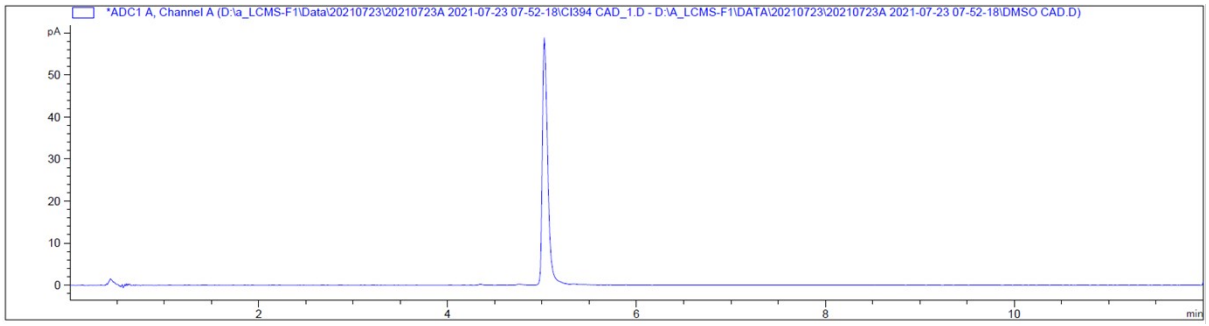


High resolution mass spectrum of vaccine candidate **26**

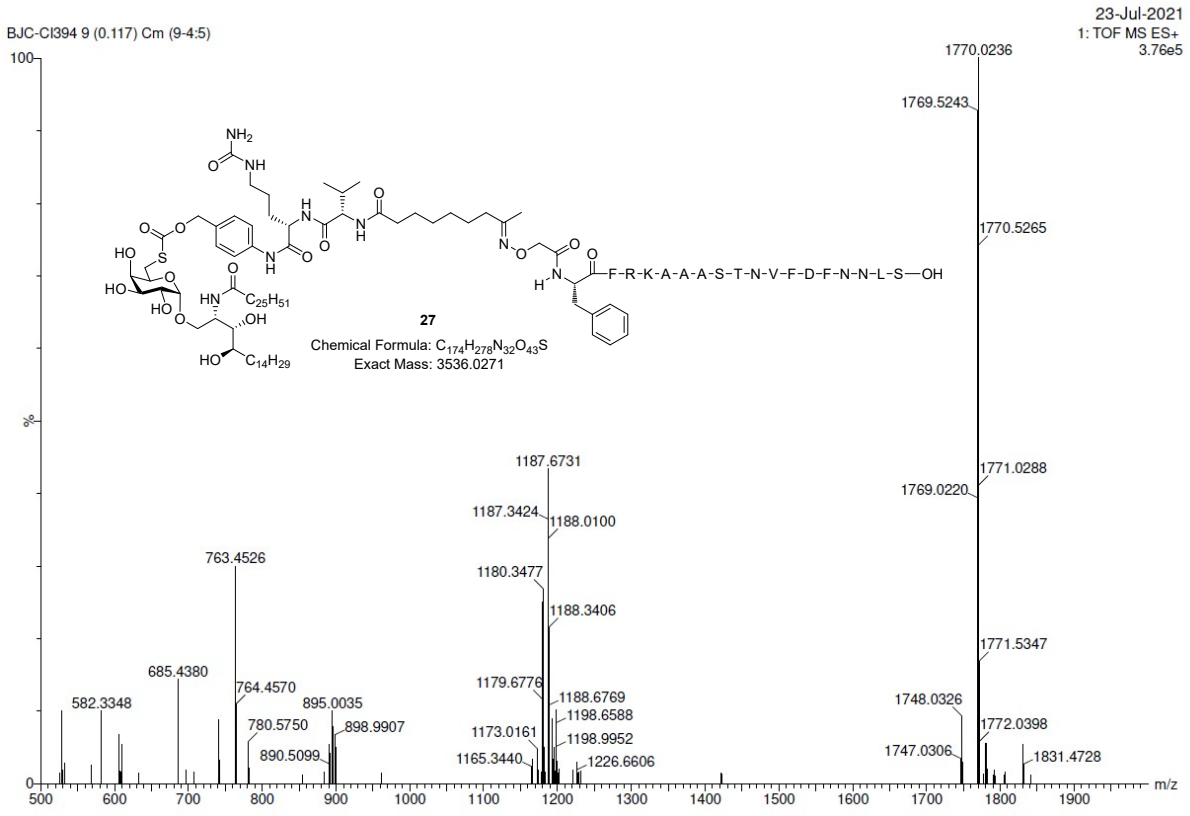


	$[M+2H]^{2+}$	$[M+3H]^{3+}$
Calcd. mass	1761.5330	1174.6887

HPLC-CAD chromatogram of vaccine candidate **27** (99.3% pure)



High resolution mass spectrum of vaccine candidate **27**



	$[M+2H]^{2+}$	$[M+2H+Na]^{3+}$	$[M+3H]^{3+}$
Calcd. mass	1770.0136	1187.6723	1180.3424

References:

1. G. Giaccone, C. J. A. Punt, Y. Ando, R. Ruijter, N. Nishi, M. Peters, B. M. E. von Blomberg, R. J. Scheper, H. J. J. van der Vliet, A. J. M. van den Eertwegh, M. Roelvink, J. Beijnen, H. Zwierzina and H. M. Pinedo, *Clin Cancer Res*, 2002, **8**, 3702-3709.
2. D. Fernandez-Ruiz, W. Y. Ng, L. E. Holz, J. Z. Ma, A. Zaid, Y. C. Wong, L. S. Lau, V. Mollard, A. Cozijnsen, N. Collins, J. Li, G. M. Davey, Y. Kato, S. Devi, R. Skandari, M. Pauley, J. H. Manton, D. I. Godfrey, A. Braun, S. S. Tay, P. S. Tan, D. G. Bowen, F. Koch-Nolte, B. Rissiek, F. R. Carbone, B. S. Crabb, M. Lahoud, I. A. Cockburn, S. N. Mueller, P. Bertolino, G. I. McFadden, I. Caminschi and W. R. Heath, *Immunity*, 2016, **45**, 889-902.
3. G. W. Chen, J. Schmieg, M. Tsuji and R. W. Franck, *Org Lett*, 2004, **6**, 4077-4080.
4. A. S. Bhat and J. Gervay-Hague, *Org Lett*, 2001, **3**, 2081-2084.
5. H. Sakurai, A. Shirahata, K. Sasaki and A. Hosomi, *Synthesis-Stuttgart*, 1979, 740-741.
6. A. J. Tyznik, E. Farber, E. Girardi, A. Birkholz, Y. L. Li, S. Chitale, R. So, P. Arora, A. Khurana, J. Wang, S. A. Porcelli, D. M. Zajonc, M. Kronenberg and A. R. Howell, *Chem Biol*, 2011, **18**, 1620-1630.
7. J. Wojno, J. P. Jukes, H. Ghadbane, D. Shepherd, G. S. Besra, V. Cerundolo and L. R. Cox, *Acs Chem Biol*, 2012, **7**, 847-855.
8. M. Michieletti, A. Bracci, F. Compostella, G. De Libero, L. Mori, S. Fallarini, G. Lombardi and L. Panza, *J Org Chem*, 2008, **73**, 9192-9195.
9. P. J. Jervis, L. R. Cox and G. S. Besra, *J Org Chem*, 2011, **76**, 320-323.
10. WO/2019/058289, 2018.
11. G. M. Dubowchik, R. A. Firestone, L. Padilla, D. Willner, S. J. Hofstead, K. Mosure, J. O. Knipe, S. J. Lasch and P. A. Trail, *Bioconjug Chem*, 2002, **13**, 855-869.
12. B. J. Compton, C. W. Tang, K. A. Johnston, T. L. Osmond, C. M. Hayman, D. S. Larsen, I. F. Hermans and G. F. Painter, *Org Lett*, 2015, **17**, 5954-5957.