Electronic Supplementary Material (ESI) for Organic & Biomolecular Chemistry.

Supporting Information

Enhancing T cell responses to vaccination through targeted enzymatic release of a weak NKT cell agonist

Benjamin J. Compton, Kathryn J. Farrand, Ching-wen Tang, Taryn L. Osmond, Mary Speir, Astrid Authier-Hall, Jing Wang, Peter M. Ferguson, Susanna T. S. Chan, Regan J. Anderson, Taylor R. Cooney, Colin M. Hayman, Geoffrey M. Williams, Margaret A Brimble, Collin R. Brooks, Lin-Kin Yong, Leonid S. Metelitsa, Dirk M. Zajonc, Dale I. Godfrey, Olivier Gasser, Robert Weinkove, Gavin F. Painter, Ian F. Hermans

Table S1. Crystallography data refinement and statistics

Data collection statistics	
PDB ID	
Space group	$C222_1$
Cell dimension	
a, b, c, (Å)	79.3, 191.6, 151.2
α, β, γ (°)	90, 90, 90
Resolution range (Å)	40.0-3.15
[outer shell]	[3.26-3.15]
No. of unique reflections	19,762 [1,906]
R_{meas} (%)	19.2 [50.5]
R_{pim} (%)	8.3 [23.8]
Multiplicity	5.0 [4.2]
Average I/σI	6.6 [1.7]
Completeness (%)	98.4 [96.4]
Refinement statistics	
No. atoms	6,511
Protein	6,359
Ligand/spacer	33/18
Carbohydrate	101
Ramachandran plot (%)	
Favored	96.3
Allowed	100
R.m.s. deviations	
Bonds (Å)	0.007
Angles (°)	1.15
B-factors (Å2)	
Protein	51.9
Ligand/spacer	52.9/68.2
Carbohydrate	47.3
R factor (%)	18.9
Rfree (%)	24.8

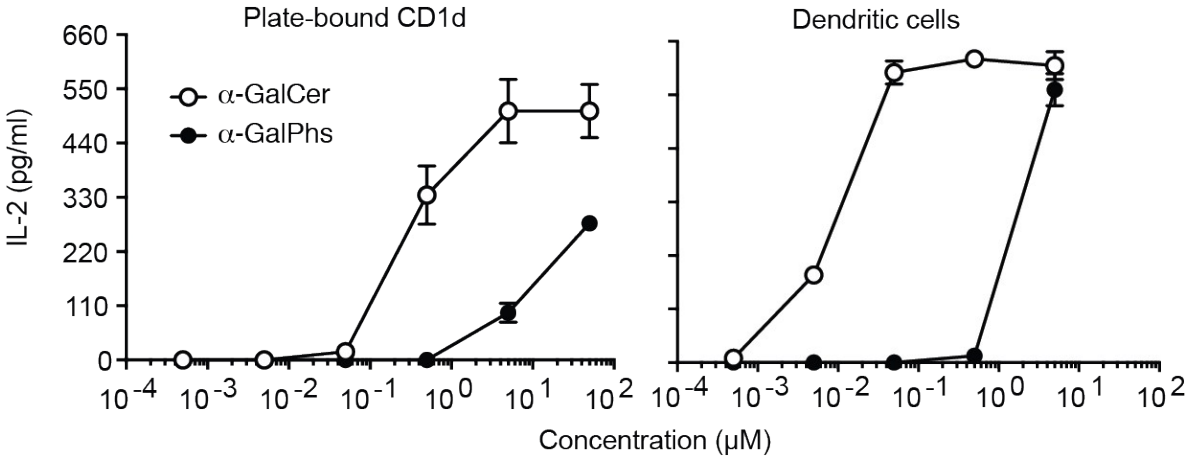


Figure S1. α-GalPhs has weak activity in vitro.

In vitro NKT cell proliferation assay. Plate-bound monomeric CD1d molecules were incubated with the indicated glycolipid compounds for 1 h at 37 °C, and then DN32-D3 NKT cell hybridoma cells were added for 18 h at 37 °C. Supernatants from the cultures were then assayed for IL-2 levels by ELISA as an indication of NKT cell activation (left). Using the same readout, DN32-D3 cell were incubated with cells of a mouse DC line that had been incubated with the glycolipids for 24 h (right). Error bars represent SEM of technical replicates in triplicate

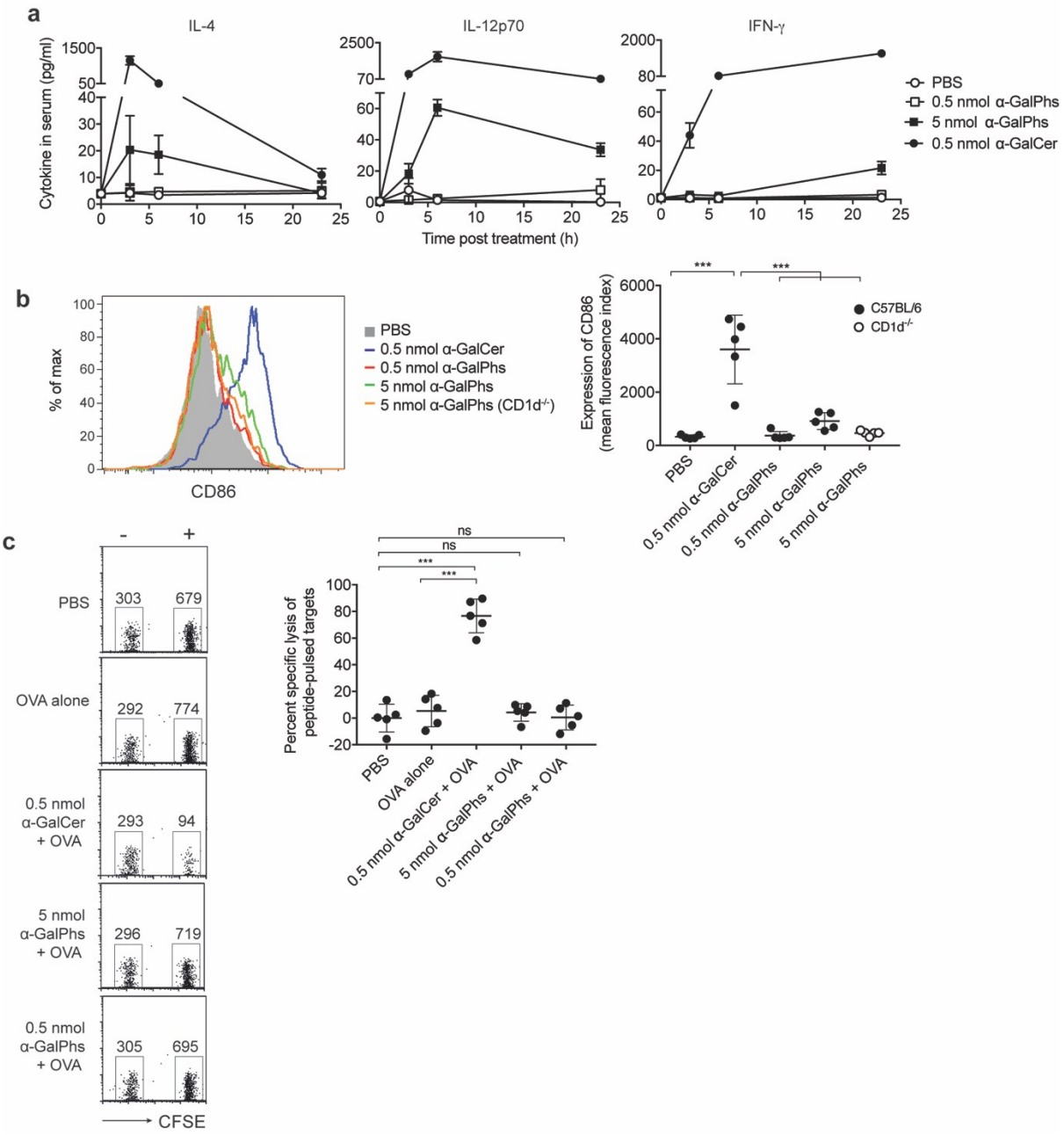


Figure S2. α-GalPhs has limited activity in vivo.

(a) Analysis of cytokines released into serum in response to NKT cell agonists in vivo. Levels of IL-4, IFN-γ and IL-12p70 and were assessed in serum at the indicated times after i.v. administration of the glycolipids at 5 nmol or 0.5 nmol. Means ± SEM for each treatment group are shown (*n* = 5 per group). (b) Analysis of DC activation in response to NKT cell agonists in vivo. The expression of activation marker CD86 was assessed by flow cytometry on splenic CD8α$^+$ DCs 24 h after i.v. administration of 5 nmol of the indicated glycolipids. Representative histograms are shown on left. Data in graph on right are expressed as mean fluorescence index of fluorescent antibody staining, with each dot graphed representing

analysis from a separate mouse; means ± SEM are shown for each treatment group. ***P<0.001; one way ANOVA with Tukey's multiple comparison test. (c) Assessment of $CD8^+$ T cell-mediated cytotoxic activity against fluorscent peptide-loaded targets in vivo one week after administration of the indicated compounds. Analysis was conducted by flow cytometry on blood 24 h after the fluorescent cells were injected. Representative flow plots are shown on left, gated on fluorescent cells showing population without (-) or with (+) peptide; number of events in each population are shown. On right, graph shows survival of injected peptide-pulsed fluorescent targets based on ratio relative to population without peptide, with each dot representing analysis from an individual mouse; means ± SEM shown for each treatment group are shown. *** P<0.001; one way ANOVA with Tukey's multiple comparison test.

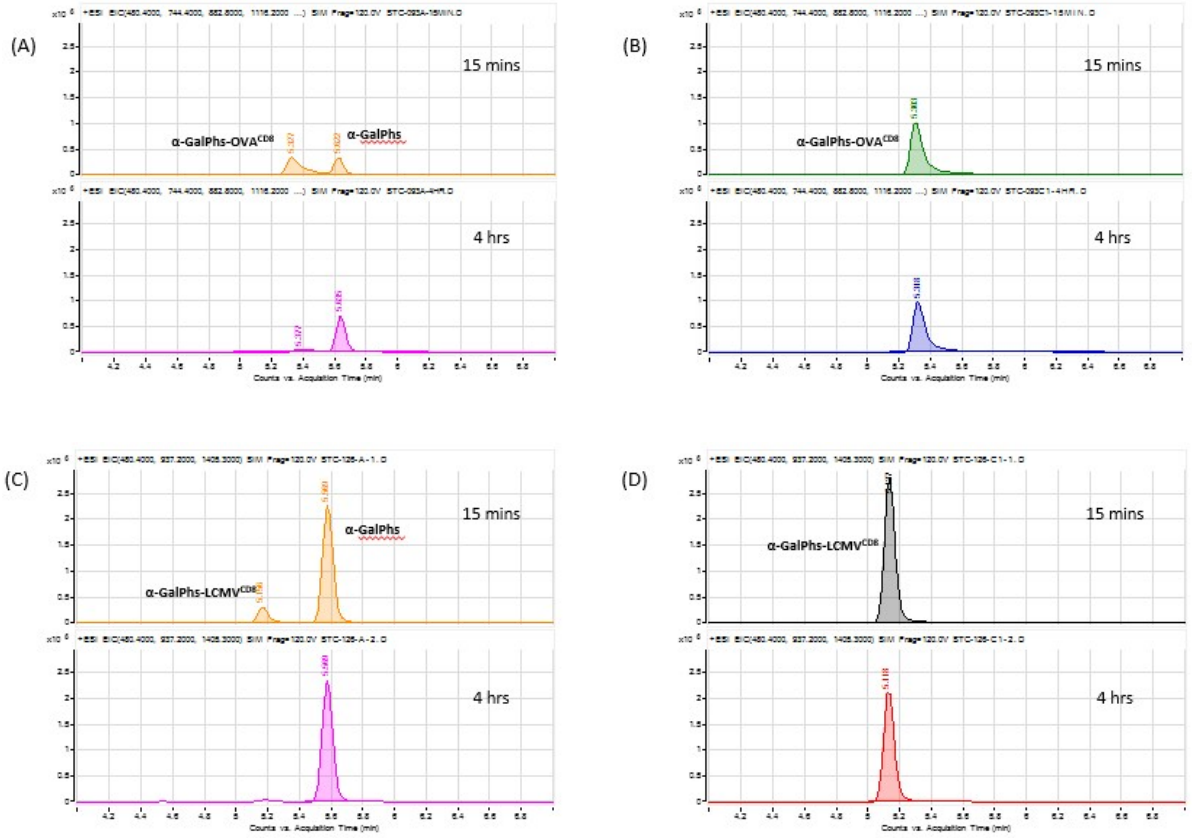


Figure S3. α-GalPhs-peptide conjugates undergo linker cleavage with cathepsin B

HPLC-MS analysis employing selected ion monitoring (SIM) detection for conjugates **α-GalPhs-OVA^{CD8} 6** and **α-GalPhs-LCMV^{CD8} 8** with and without the addition of cathepsin B enzyme: (A) Conjugate **6** treated with cathepsin B releases >98% α-GalPhs **1** within 4h; (B) Conjugate **6** remains intact when no cathepsin B was added; (C) Conjugate **8** treated with cathepsin B releases >92% α-GalPhs **1** with 4h, (D) Conjugate **6** remains intact when no cathepsin B was added

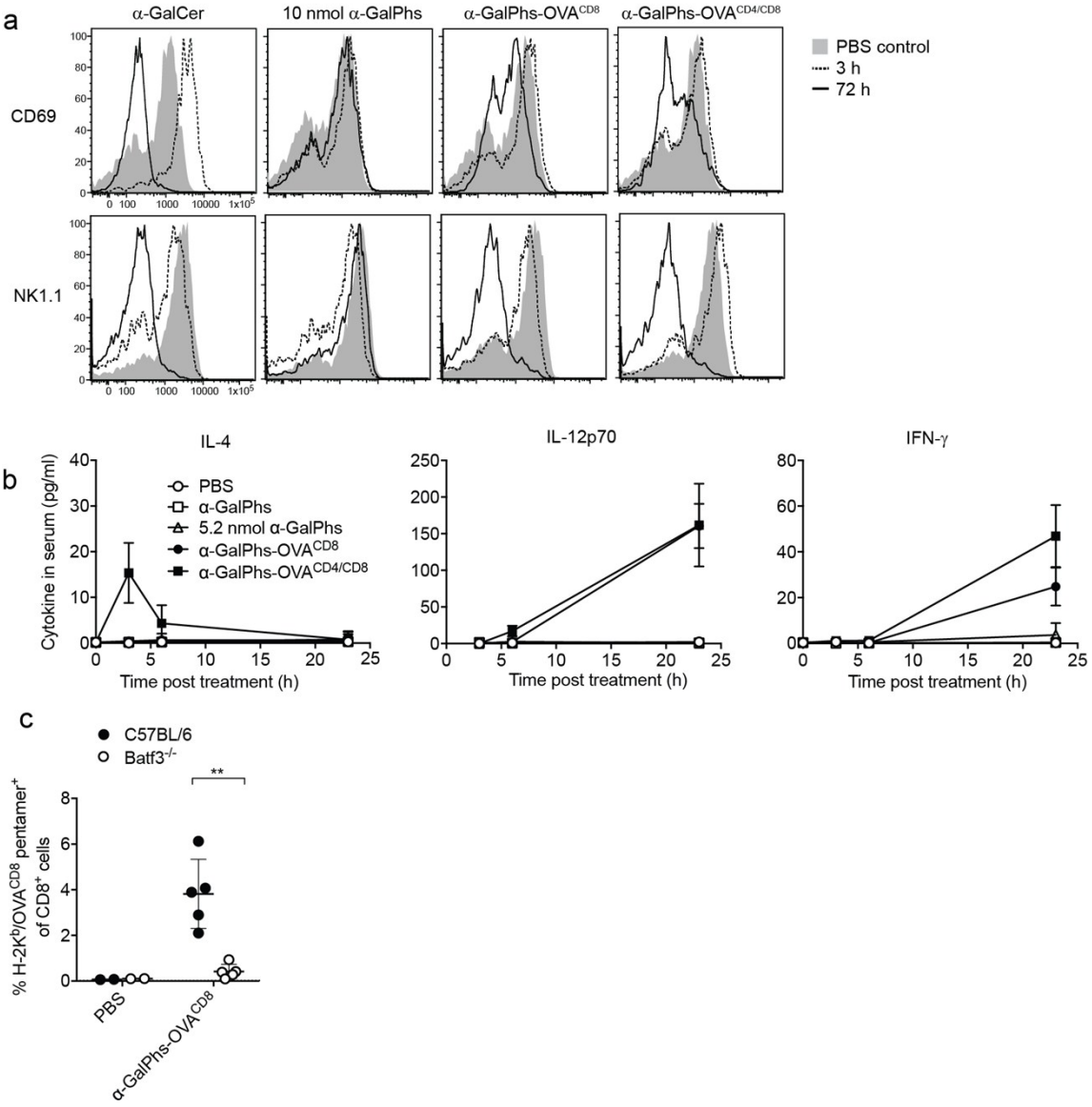


Figure S4. Peptide conjugated α-GalPhs activates NKT cells and has adjuvant activity in vivo. (a) Expression of CD69 and NK1.1 on splenic $CD3^+CD1d/\alpha$-GalCer tetramer$^+$ cells 3 and 72 h after i.v. administration. Unless otherwise indicated, 0.5 nmol was injected. (b) Cytokines released into serum at the indicated times after i.v. administration of the conjugates or α-GalPhs. Means ± SEM for each treatment group are shown (n = 5 per group). (c) Assessment of frequency of OVA_{257-64} peptide-specific $CD8^+$ T cells in blood of C57BL/6 or Batf3-deficient mice one week after i.v. administration.

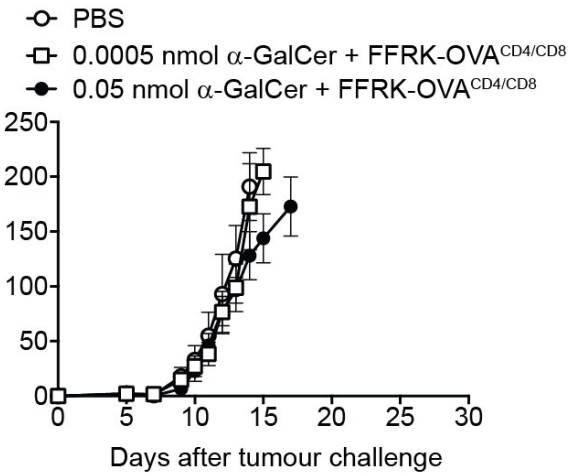
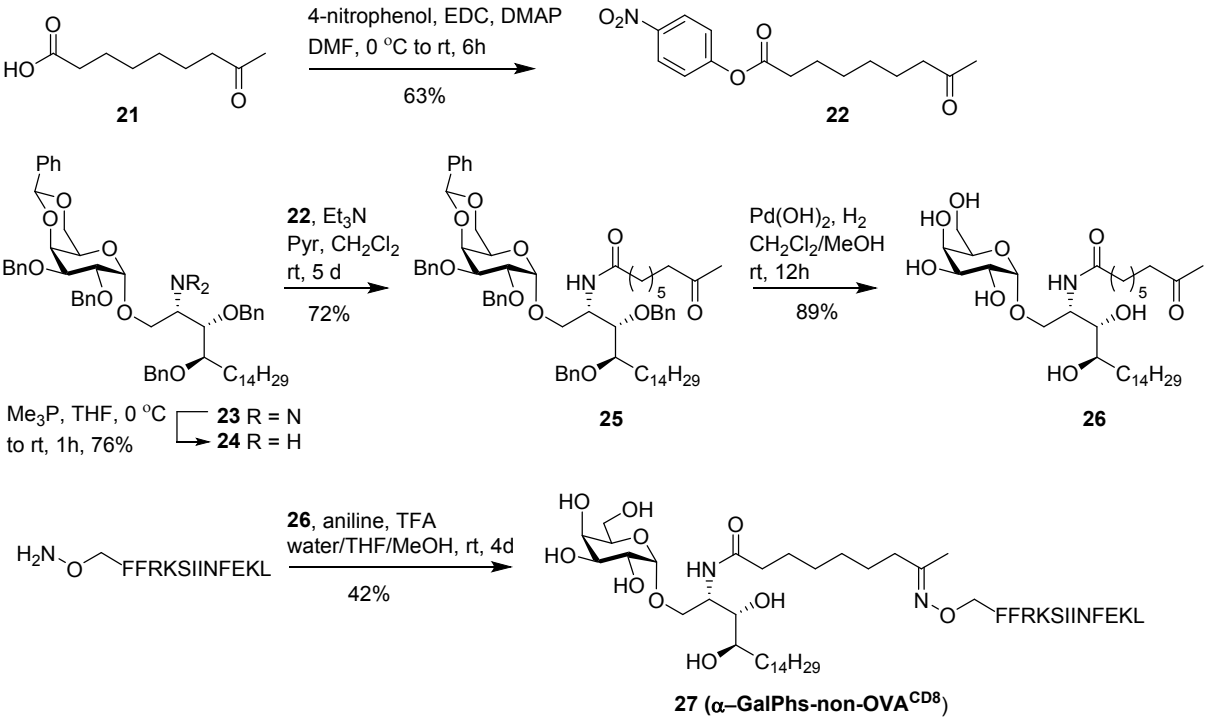


Figure S5. Assessment of antitumour activity. Groups of animals (*n* = 5) were challenged s.c. with B16.OVA, and then treated 5 d later with α-GalCer mixed with peptide at the indicated dose. The mean tumour size per treatment group ± SEM is shown.



Scheme S1. Synthetic route to a peptide conjugate of α-GalPhs lacking the protease-cleavable linker.

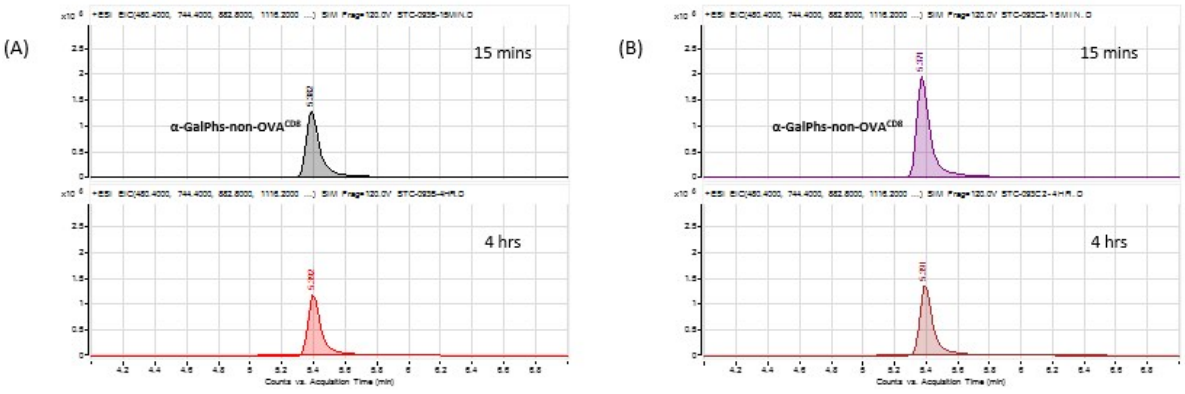


Figure S6. α-GalPhs-non-OVA^{CD8} does not undergo linker cleavage with cathepsin B

HPLC-MS analysis employing selected ion monitoring (SIM) detection for **α-GalPhs-non-OVA^{CD8} 27** with and without the addition of cathepsin B enzyme: (A) Conjugate **27** remains intact when treated with cathepsin B. (B) No cathepsin

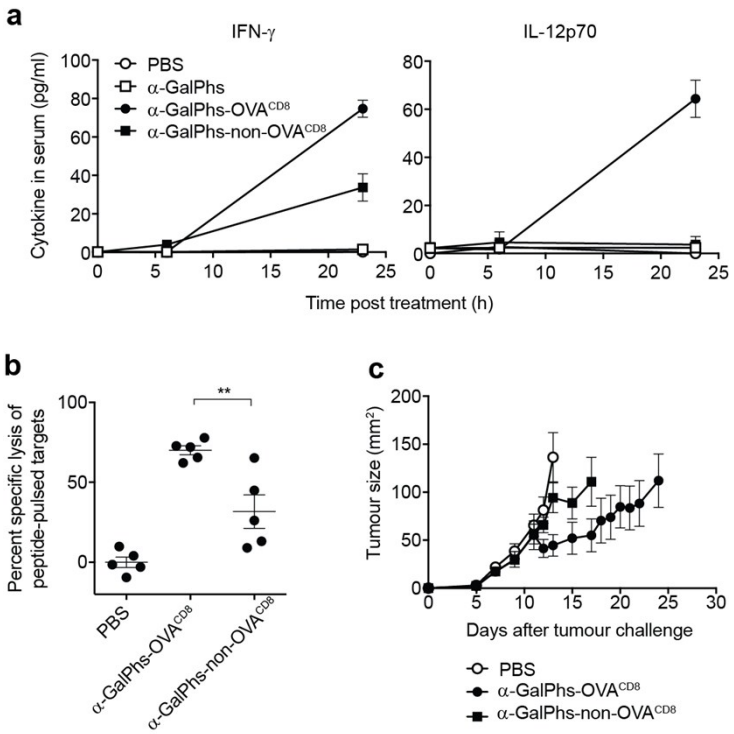


Figure S7. Assessment of conjugates with or without protease-cleavable linker.

(a) Cytokines released into serum at the indicated times after i.v. administration of the conjugates, or α-GalPhs alone. Means ± SEM for each treatment group are shown (*n* = 5 per group). (b) Cytotoxic activity against OVA_{257-64} peptide-loaded targets in vivo. Each dot represents analysis from an individual mouse; means ± SEM for each treatment group are shown. **P<0.01; one way ANOVA with Tukey's multiple comparison test. (c) Groups of animals (*n* = 5) were challenged s.c. with B16.OVA, and then treated 5 d later with 0.5 nmol of conjugates with or without cathepsin-cleavable linker. The mean tumor size per treatment group ± SEM is shown.

Supporting material and methods

General synthetic methods

Dry solvents were obtained commercially. Air-sensitive reactions were carried out under Ar. Thin layer chromatography (TLC) was performed on aluminium sheets coated with 60 F_{254} silica. Flash column chromatography was performed on Reveleris® silica cartridges (38.6 μm) or SiliCycle® silica gel (40-63 μm). 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 or CHD_2OD 3.31 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 or CD_3OD 49.0 ppm). $CDCl_3$-CD_3OD solvent mixtures were always referenced to the methanol peak. High resolution electrospray ionization (ESI) mass spectra analyses were undertaken on a Waters Q-TOF Premier™ Tandem Mass spectrometer fitted with a Waters 2795 HPLC. Preparative HPLC was conducted on an Agilent 1260. Semi-preparative HPLC was conducted on an Agilent 1100. Analytical HPLC data were obtained on an Agilent 1260 either coupled to a Dionex Corona Ultra RS charged aerosol detector (CAD) or an Agilent 6130 single quadrupole mass spectroscopic detector using ESI.

Peptide synthesis

The following peptides were purchased from Peptides & Elephants GmbH (Potsdam, Germany); 4-pentynoyl-FFRKSIINFEKL, 4-pentynoyl-FFRKNLVPMVATV, 5-azidopentanoyl-FFRKSVYDFFVWLKFFHRTCKCTGNFA. 5-Azidopentanoyl-FFRKKISQAVHAAHAEINEAGRESIINFEKLTEWT was synthesized as previuosly reported[1] and the peptides 5-azidopentanoyl-FFRKKAVYNFATM and 2-(aminooxy)acetyl-FFRKSIINFEKL were chemically synthesized by us as detailed below.

Materials. 9-Fluorenylmethoxycarbonyl (Fmoc) protected L-α-amino acids, 2-(6-chloro-1H-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate (HCTU) and benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (PyBOP) were purchased from Bachem (Switzerland). Dimethyloxazolidine dipeptides were purchased from either Bachem (Switzerland) or Aapptec (Louisville, Ky). Dimethylformamide (DMF) and acetonitrile were purchased from Global Science (Auckland, NZ). 3,6-Dioxa-1,8-octanedithiol (DODT), triisopropylsilane (TIPS), 4-methylmorpholine (NMM), 2,4,6-trimethylpyridine and piperidine were purchased from Sigma Aldrich. Trifluoroacetic acid (TFA) was obtained from Oakwood Products, Inc (West Colombia, SC). Preloaded (Wang-type linker) Tentagel resin was obtained from Rapp Polymere GmbH (Tübingen).

General methods. Analytical HPLC employed a Dionex Ultimate 3000 HPLC system fitted with a Phenomenex Gemini C18 3μm 110Å 4.6x150mm column, with water/0.1% TFA as eluent A and MeCN/0.1% TFA as eluent B. Mass spectra were recorded using an Agilent 1100MSD spectrometer.

SPPS: Fmoc synthesis was carried out at room temperature on Tentagel resin using a Tribute peptide synthesiser (Protein Technologies International, Tucson, Az) and conducted on a 0.1 mmol scale. Generally, individual peptide couplings employed a 5-fold molar excess (relative to resin) of the protected amino acid in DMF activated by a 4.8-fold molar excess of HCTU in the presence of a 10-fold molar excess of 4-methylmorpholine in DMF, with a coupling time of 60 minutes. For the coupling of Fmoc-histidine residues, PyBOP was used as the coupling

agent and 2,4,6 trimethylpyridine (5-fold excess) was used in place of 4-methylmorpholine. The Fmoc protecting group was removed using two separate treatments of 20% piperidine in DMF (3 min, then 7 min). The side-chains of the amino acids were protected where necessary with TFA-labile groups. Cleavage from resin with concomitant deprotection of the peptide was achieved by incubating the resin in 5 mL/mmol (resin) of 94% TFA, 2.5% water, 2.5% DODT for 1.5 h. The crude peptide was recovered by draining the TFA solution into chilled diethyl ether (5 vol) to induce precipitation. The resin was washed once more with TFA (1-2 mL/mmol) and this was added to the ethereal mixture. After centrifugation the pellet was washed twice with ether, allowed to air-dry, dissolved in 1:1 water/MeCN (approx. 10 mL) and heated at 65 °C for 20 minutes to degrade residual carboxylated Trp. The material was then freeze-dried and analyzed by LCMS.

Purification: Semi-preparative HPLC purification employed a Dionex Ultimate 3000 HPLC system fitted with a Phenomenex Gemini C18 5 µm 110Å 10x250mm column, with water/0.1% TFA as eluent A and MeCN/0.1% TFA as eluent B. Approximately 90 mg of crude peptide was dissolved to a concentration of 8 mg/mL in 20% MeCN in water and loaded in its entirety onto the column. A shallow gradient from 25% B to 40% B over 216 min (eluting at 4 mL/min) was generated and fractions collected at 1 minute intervals. The fractions were analysed by HPLC, with those containing the desired peptide at sufficient purity being pooled and lyophilized.

Study design and ethics

All in vitro and in vivo results were confirmed by two or more independent experiments. All studies in mice were approved by the Victoria University Animal Ethics Committee and performed according to institutional guidelines. Power calculations from past studies were used to calculate the number of mice needed for statistical power. Typically, groups of 5-7 animals were used. All mice were bred at the Malaghan Institute of Medical Research and used at 6-12 wk of age. Male and female animals from the following strains were used: C57BL/6J (Jackson Laboratories, Bar Harbor, ME, USA); $CD1d^{-/-}$ mice, which are devoid of CD1d-restricted NKT cells [2]; and $Batf3^{-/-}$ mice [3] crossed onto a C57BL/6 background. Human blood cells for in vitro studies were donated with written informed consent, and with approval of the Victoria University Human Ethics Committee, or were derived from buffy coats purchased from Gulf Coast Regional Blood Center. Results were confirmed in two or more independent experiments and key experiments conducted using cells from multiple human donors.

Solublisation of compounds for biological studies

Where required, solubilisation of all synthetic compounds was achieved by freeze-drying the samples in the presence of sucrose, L-histidine and TWEEN 20 as previously described for the solubilisation of α-GalCer[4]. All solubilised compounds were diluted in PBS for i.v. administration. Equivalent molar doses were used in all groups in a given experiment, as indicated in text.

Crystal structure determination

Mouse CD1d/β_2M heterodimeric protein was expressed and purified from SF9 insect cells using the baculovirus expression system as described [5]. Briefly, SF9 cells were infected for 3 days with an MOI of 3 with baculovirus encoding the CD1d heavy chain, as well as β_2M. Supernatant was collected by centrifugation, concentrated to 500 ml and buffer exchanged against PBS using a tangential flow through filtration system (Pall). His-tagged CD1d/β_2M was purified with Ni-NTA resin and dialysed against 10mM Tris pH8.0. Further purification included anion-exchange chromatography using MonoQ GL5/50, followed by size exclusion chromatography (SEC) on a Superdex S200 GL 10/300 using 50mM Hepes pH 7.5, 150 mM NaCl as running buffer. α-GalPhs was loaded into CD1d using a 6 molar excess of lipid (5 mg/ mL in DMSO) overnight at room temperature under slight agitation. Vα14Vβ8.2 TCR was expressed as separate TCRα and TCRβ chains in *E. coli* inclusion bodies and refolded as reported previously [5]. TCR was added to α-GalPhs loaded mCD1d with 2-fold molar excess of mCD1d and CD1d-ligand-TCR complexes were purified from individual unbound components by SEC as described above. The protein complex was concentrated to 6-8 mg/ml and subjected to crystallisation using the sitting drop vapor diffusion method. Quality diffracting crystals were obtained at room temperature by mixing 0.5 µl of protein with 0.5 µl of precipitant (20 % polyethylene glycol 3350, 200 mM sodium malonate, pH 5.0). Diffraction data was remotely collected at the Stanford Synchrotron Radiation Lightsource (SSRL) beamline 9-2 and integrated and merged using HKL2000 [6]. The ternary complex structure was determined by molecular replacement in PHASER [7], first using the coordinates PDB ID 2Q7Y for CD1d, followed by PDB ID 3QUZ as the search model for the TCR. The model was built and refined iteratively using COOT [8] and REFMAC5 [9], and monitored by a continuous drop in R_{free} values and improvement in electron density. Refinement was carried out to a final R_{cryst} and R_{free} of 18.9 % and 24.9 % Data collection and refinement statistics are presented in Table S1. The crystal structure has been deposited in the Protein Data Bank (http://www.rcsb.org/) under accession code 5VCJ.

CD1d presentation assays

The capacity of glycolipids to bind CD1d and stimulate NKT cells was initially tested by cell-free assay. Flat-bottomed 96 well tissue culture plates were coated with 5 µg/ml hCD1d monomers (NIH Tetramer Core Facility) in PBS for 1 h at 37 °C, washed, and incubated with 50 ng/well of glycolipid for 1 h at 37 °C. The plates were then washed with complete medium consisting of Iscove's Minimum Essential Medium supplemented with 5 % FCS (Sigma Aldrich, Auckland, NZ), 2 mM glutamax, 100 U/ml penicillin, 100 mg/ml streptomycin and 50 mM 2-mercaptoethanol (all Invitrogen, Auckland, New Zealand). Each well was provided 3 x 10^4 DN32-D3 NKT cell hybridoma cells [10] in complete medium and incubated for 16 h at 37 °C. Supernatants were collected and tested for IL-2 levels by ELISA (Biolegend, San Diego, CA). Presentation via cell-expressed CD1d was examined by incubating the mouse DC cell line DC2114 [11] with glycolipid (10^4 cells per well) for 20 h and then each well was provided 3 x 10^4 DN32-D3 NKT cell hybridoma cells and incubated for 16 h at 37 °C. Supernatants were collected and tested for IL-2 levels.

Flow cytometry

All antibody staining steps were performed on ice. Nonspecific FcR-mediated Ab staining was blocked by incubation for 5 min with anti-CD16/32 antibody (24G2, prepared in-house from hybridoma supernatant). Flow cytometry was performed on a BD Biosciences FACSCalibur or BD Biosciences LSRII SORP with data analysis using FlowJo software (Tree Star, Inc., OR, USA).

Assessment of DC activation in vivo

The experimental compounds were administered i.v. into groups of C57BL/6 mice (*n* = 5), and the spleens were removed 20 h later for analysis. The spleens were teased through a cell strainer and the red blood cells (RBCs) lysed with RBC lysis buffer, and then the remaining cells were labeled with antibodies for CD11c (HL3, BD Pharmingen), B220 (RA3-6B2, eBioscience, CA, USA), CD8 (53-6.7, BD Bioscience), CD86 (GL1, eBioscience) and propidium iodide (BD Pharmingen) and analyzed by flow cytometry.

In vivo cytotoxicity assay

As targets, syngeneic splenocytes were loaded with 50 nM peptide, and then labeled with 2.5 μM carboxyfluorescein succinimidyl ester (Life Technologies). A control population without antigen was labeled with 10 μM chloro-methyl-benzoyl-aminotetramethyl-rhodamine (Life Technologies). A mixture of the two populations was injected i.v. into immunised mice, and specific lysis of the peptide-loaded targets was monitored by flow cytometry analysis of PBL. Mean percentage of survival of peptide-pulsed targets was calculated relative to that of the control population, and cytotoxic activity was expressed as percent specific lysis (100 - mean percentage of survival of peptide-pulsed targets).

Analysis of cytokine release into serum

Blood was collected from the lateral tail vein at different time intervals after administration of the experimental compounds. Serum was collected after blood had clotted, and levels of cytokines IL-12p70, IL-4, and IFN-γ were assessed by bioplex cytokine bead arrays (Bio-Rad, CA, USA) according to the manufacturer's instructions.

Intracellular cytokine analysis of NKT cells

Splenocytes were collected 2 h after administration of experimental compounds (as indicated in the text), stained with α-GalCer-loaded mouse CD1d tetramer (kindly provided by the NIH, MD, USA) and anti-CD3 (145-2C11, Biolegend), fixed and permeabilised using 0.1 % Saponin, and then stained with antibodies to murine TNF (MP6-XT22, BD Pharmingen) for analysis by flow cytometry.

Assessment of biodistribution of glycolipids in vivo

Fluorescent compounds α-GalCer-BODIPY, α-GalPhs-BODIPY and α-GalPhs-OVA$^{CD4/CD8}$-BODIPY (0.5 nmol) were administered i.v. into groups of C57BL/6 mice (*n* = 4), and the spleens and livers were removed 20 h later to detect BODIPY^{hi} cells by flow cytometry, together with antibodies for with CD11c (N418, Biolegend) and CD19 (1D3, eBioscience).

Assessment of uptake and presentation of compounds by DCs in vitro

Cells of the JAWS II cell line [12] were pulsed for 4 h in αMEM (In vitro Technologies) with α-GalCer, α-GalPhs or α-GalPhs-OVA$^{CD4/CD8}$, or with OVA_{257-64} peptide (all 12 μM). The cells were

then washed and incubated in complete medium for a further 72 h. At 24 h and 72 h samples were collected for flow cytometry to assess expression of CD1d/α-galactosyl glycolipid complexes using the L363 monoclonal antibody [13], or H-2K^b/OVA$_{257-64}$ complexes using the 25-D1.16 monoclonal antibody [14] (both Biolegend).

Assessment of antitumour response

The murine melanoma cell line B16.GP33 (kindly provided by Hanspeter Pircher, University of Freiburg, Freiburg, Germany) which expresses LCMV glycoprotein aa's 33-44 [15], or B16.OVA (generated by Drs. Edith Lord and John G. Frelinger, University of Rochester, Rochester, NY and kindly provided by Drs Dick Dutton, Trudeau Institute, NY, USA), which expresses OVA protein [16], were maintained in complete medium. The cells were free of mycoplasma, as determined using MycoAlert detection kit (Lonza, Rockland, USA). For i.v. challenge, the cells were harvested and strained through a 70-mm filter and resuspended in incomplete medium for injection. Each mouse received 3 x 10^5 cells via the tail vein one week after vaccination, and then all animals were culled and lung weights measured on day 16. For s.c. challenge 5 x 10^5 cells were injected into the flank, with treatment initiated on day 5, when tumours were palpable. Tumour growth was monitored every 2–3 d, with tumour size calculated as the product of the two bisecting diameters. Measurements were stopped for each group when the first mouse developed a tumour >200 mm^2.

In vitro analysis of human cells

Human peripheral blood mononuclear cells (PBMCs) were obtained by density centrifugation (Lymphoprep; Axis-Shield, Oslo, Norway) of heparinised venous blood diluted 1:1 in PBS, and were washed twice then either used fresh or cryopreserved in 10 % DMSO (Sigma Aldrich) and 90 % FBS (Gibco). HLA-A2 status was determined by flow cytometry (clone BB7.2, Biolegend). To assess NKT cell proliferation, 3 x 10^5 PBMCs were cultured in IMDM supplemented with 5% human AB serum (Sigma Aldrich), 2 mM Glutamax, penicillin, streptomycin and 2-mercaptoethanol (cIMDM); glycolipids or conjugates were added at 1 μM unless otherwise stated; 50 U/mL recombinant human IL-2 (Peprotech, Rocky Hill, NJ, USA) was added after 48 h. For some experiments, 50 μg/mL LEAF-purified anti-CD1d (clone 51.1; BioLegend) or matched isotype control antibody was added. Frequency of NKT cells was determined by flow cytometry using anti-CD3 and anti-Vα24Jα18 antibodies (clones UCHT1 and 6B11; Biolegend); CMV pp65$_{495-503}$ peptide-specific T cells were assessed using anti-CD3, anti-CD8 (clone RPA-T8; Biolegend) and PE-labelled HLA-A2 dextramers loaded with NLVPMVATV peptide (Immudex, Copenhagen, Denmark); proliferation was determined using anti-Ki67 (clone B56; BD Pharmingen). For all flow cytometric experiments, doublets, dead cells and non-specific staining were excluded using doublet gating, Zombie NIR Fixable Viability (Biolegend) and anti-CD19 (HIB19; Biolegend), respectively. A human IFN-γ ELISpot kit (Mabtech, Nacka Strand, Sweden) was used according to manufacturer's instructions to quantify IFN-γ spot forming units (SFUs) 24 h after incubation of 2 x10^5 PBMCs/well in cIMDM containing the relevant compounds and 50 μg/mL anti-CD1d or isotype control. Developed plates were counted using an automated ELISpot reader (Autoimmun Diagnostika, Strasberg, Germany). In some experiments, expansion of NKT cells was examined after incubation with autologous APCs pulsed with agonists or conjugates. Briefly, PBMCs from healthy donors were isolated by gradient centrifugation from buffy coats purchased from Gulf Coast Regional Blood Center (Houston, TX), and then NKT cells were isolated by positive selection with anti-

NKT microbeads (Miltenyi Biotec) according to the manufacturer's instructions. The negative fraction was irradiated, pulsed with 50 nM ligand, and added to NKT cells at the ratio of 5 to 1 followed by culture with 200 IU/ml recombinant human IL-2 (NIH/NCI, Fredrick, MD) added every other day in complete RPMI-1640. Fold-expansion of NKT cells was determined by flow cytometry at day 14.

Histopathological assessment of hepatotoxicity

Mice were euthanised 72 h after administration of α-GalCer or α-GalPhs-OVA$^{CD4/CD8}$. The livers were removed and fixed in neutral buffered formalin. Following fixation, the livers were cut into 2 mm coronal slices that were entirely embedded in paraffin, cut into 4 μm thick sections, and stained with H&E. Liver histology was assessed by a histopathologist blinded to treatment. Any necrosis evident was scored as follows: 1, focal necrosis; 2, necroinflammatory foci averaging 1-2 per lobule; 3, greater than two necroinflammatory foci per lobule; 4, confluent necrosis.

Statistical analysis

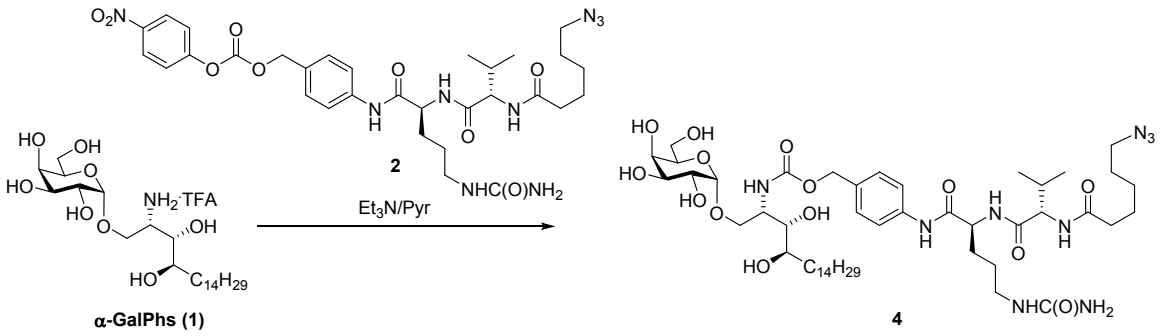
Data presented in graphs were analysed by 1-way ANOVA with multiple comparisons by Tukey test using Prism software (GraphPad Software Inc., La Jolla, CA). **P*<0.05, **P*<0.01, ****P*<0.001.

Assessment of linker cleavage in conjugates by cathespsin B

A stock solution of phytosphingosine (190 μM) in DMSO 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 5.9 uM. The substrate conjugate (190 μM in DMSO) was added to the pre-mixed buffer solution to give a final substrate concentration of 23.8 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 18.4 units/mL. The control reaction (without enzyme) was added the same amount of volume in buffer. The reaction mixtures was then incubated at 37 °C. Aliquots of 10 uL were taken from the reactions and analysed by LCMS at 30 mins and 4 hours after start of reaction.

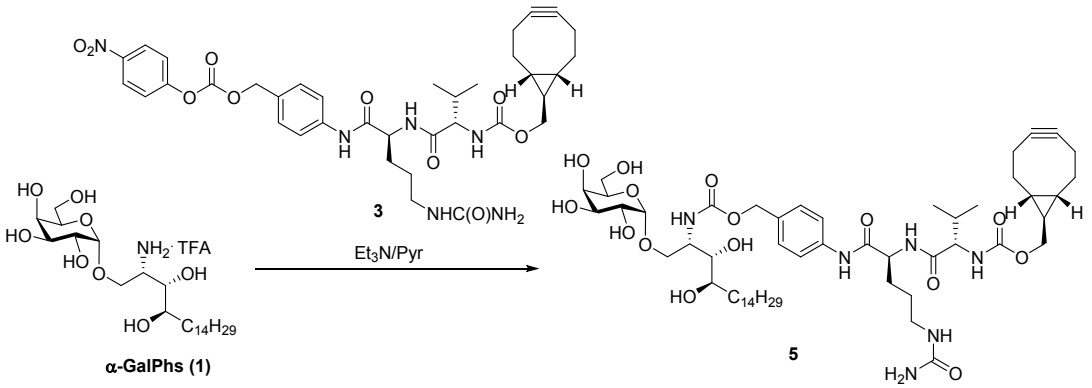
Preparation of synthetic compounds

Preparation of (2*S*,3*S*,4*R*)-1-(α-D-galactopyranosyloxy)-2-(N-(6-azidohexanoyl)-L-valinyl-L-citrullinyl-4-aminobenzyloxycarbonyl)amino-3,4-octadecandiol (**4**)



To a mixture of α-GalPhs (**1**)[17] (28 mg, 0.047 mmol) and **2**[18] (38 mg, 0.056 mmol) in dry pyridine (2.0 mL) under Ar was added Et_3N (100 µL, 0.72 mmol) and the mixture stirred at rt (14 h). The mixture was concentrated and the crude residue purified by column chromatography on silica gel ($MeOH/CH_2Cl_2$ = 0:10 to 3:7), to afford the title compound **4** as a white solid (25 mg, 0.024 mmol, 51%). ^1H NMR (500 MHz, 1:1 $CDCl_3/CD_3OD$) δ 7.63 – 7.53 (m, 2H), 7.32 (d, *J* = 8.3 Hz, 2H), 5.04 (s, 2H), 4.89 (d, *J* = 3.8 Hz, 1H), 4.53 (dd, *J* = 8.8, 5.1 Hz, 1H), 4.19 (d, *J* = 7.4 Hz, 1H), 4.06 – 3.97 (m, 1H), 3.94 (dd, *J* = 10.7, 4.7 Hz, 1H), 3.90 – 3.86 (m, 1H), 3.83 – 3.76 (m, 2H), 3.76 – 3.64 (m, 4H), 3.56 (d, *J* = 4.6, 2.1 Hz, 2H), 3.28 (t, *J* = 6.9 Hz, 2H), 3.25 – 3.18 (m, 1H), 3.17 – 3.07 (m, 1H), 2.34 – 2.27 (m, 2H), 2.09 (h, *J* = 6.9 Hz, 1H), 1.99 – 1.86 (m, 1H), 1.83 – 1.51 (m, 8H), 1.50 – 1.38 (m, 2H), 1.28 (s, 24H), 0.97 (dd, *J* = 6.8, 5.3 Hz, 6H), 0.93 – 0.86 (m, 3H); ^{13}C NMR (126 MHz, 1:1 $CDCl_3/CD_3OD$) δ 174.76, 172.49, 170.71, 160.80, 156.83, 137.90, 132.59, 128.44, 119.90, 99.70, 74.90, 71.66, 70.92, 70.20, 69.71, 68.85, 67.10, 66.09, 61.61, 59.10, 53.38, 52.00, 51.02, 38.90, 35.43, 32.40, 31.73, 30.38, 29.48, 29.15, 28.34, 26.31, 26.10, 25.57, 25.11, 22.42, 18.73, 17.78, 13.40 ; HRMS-ESI $[M+Na]^+$ calcd for $C_{49}H_{85}N_9NaO_{14}$: 1046.6114; found 1046.6104.

Preparation of (2*S*,3*S*,4*R*)-1-(α-D-galactopyranosyloxy)-2-N-((bicyclo[6.1.0]non-4-yn-9-yl)-methoxycarbonyl)-L-valinyl-L-citrullinyl-4-aminobenzyloxycarbonyl)amino-3,4-octadecandiol (**5**)

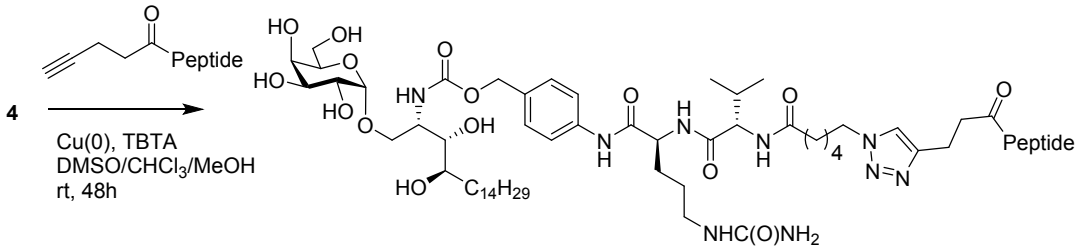


To a solution of α-GalPhs (10 mg, 16 µmol) and **3**[1] (15 mg, 21 µmol) in dry pyridine (1 mL, 12 mmol) was added dry triethylamine (35 µL, 251 µmol) and stirred under Ar at rt (22 h). The reaction was diluted with $CHCl_3$ (5 mL) and MeOH (5 mL), concentrated and the crude product was purified by column chromatography on silica gel ($MeOH/CH_2Cl_2$ = 0:10 to 2:8) to afford the title compound contaminated with triethylammonium salts. These salts were removed by precipitating the product-salt mixture in Et_2O/CH_2Cl_2 (1:2, 1.5 mL), filtered, and the filtrate washed with CH_2Cl_2/Et_2O (2:1) (to give the triethylammonium salts) then CH_2Cl_2/MeOH (1:1)

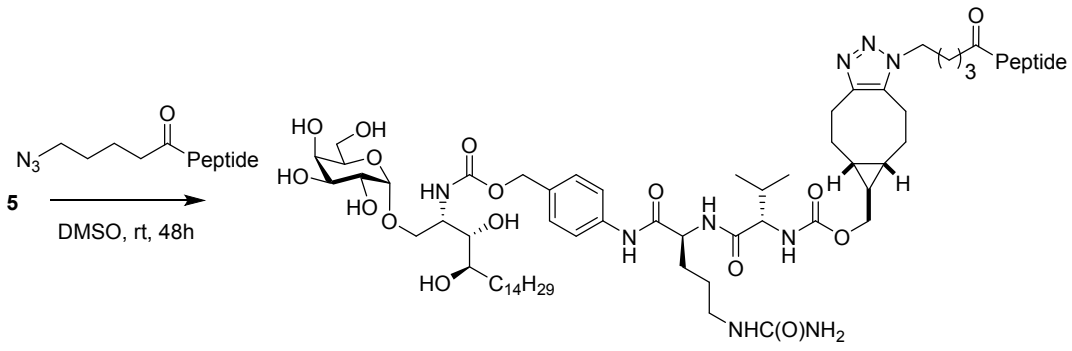
to afford the title compound **5** as a white solid (14 mg, 13 µmol, 78%). ^{1}H NMR (500 MHz, 1:1 $CDCl_3/CD_3OD$) δ 7.48 (d, *J* = 8.2 Hz, 2H), 7.22 (d, *J* = 8.2 Hz, 2H), 5.06 – 4.89 (m, 2H), 4.80 (d, *J* = 3.8 Hz, 1H), 4.49 – 4.43 (m, 1H), 4.14 (s, 1H), 3.96 – 3.81 (m, 4H), 3.79 (d, *J* = 3.3 Hz, 1H), 3.73 – 3.56 (m, 6H), 3.52 – 3.42 (m, 2H), 3.20 – 3.09 (m, 1H), 3.10 – 2.97 (m, 1H), 2.30 (d, *J* = 13.7 Hz, 2H), 2.24 – 2.10 (m, 2H), 2.10 – 1.96 (m, 3H), 1.91 – 1.76 (m, 1H), 1.70 – 1.57 (m, 1H), 1.57 – 1.40 (m, 3H), 1.36 – 1.11 (m, 26H), 0.90 (d, *J* = 6.8 Hz, 3H), 0.86 (d, *J* = 6.8 Hz, 3H), 0.79 (t, *J* = 6.9 Hz, 3H), 0.70 – 0.54 (m, 4H); ^{13}C NMR (126 MHz, 1:1 $CDCl_3/CD_3OD$) δ 174.26, 172.05, 162.15, 159.02, 158.19, 139.26, 133.97, 129.95, 121.38, 101.10, 99.92, 79.17, 78.91, 78.65, 76.40, 73.15, 72.22, 71.64, 71.14, 70.83, 70.26, 68.63, 67.60, 64.55, 63.10, 62.03, 54.76, 53.33, 50.19, 50.02, 49.85, 49.68, 49.51, 49.33, 49.16, 40.37, 34.50, 33.96, 33.18, 32.19, 30.94, 30.60, 27.74, 27.03, 24.99, 24.32, 23.88, 22.35, 20.26, 18.88, 14.98; HRMS-ESI: *m/z* calcd for $C_{54}H_{89}N_6O_{15}$ $[M+H]^+$ 1061.6395, found 1061.6386.

Scheme for synthesis of glycolipid-peptide conjugates employing CuAAC and SPAAC chemistry.

The synthetic route to the peptide conjugates α-GalPhs-OVA^{CD8}, α-GalPhs-CMV^{CD8}, α-GalPhs-OVA$^{CD4/CD8}$, α-GalPhs-LCMV^{CD8} and α-GalPhs-TRP2$^{CD4/CD8}$ is shown in supplementary scheme 3. The synthesis of intermediate compounds (labelled numerically in bold in the scheme) is described in detail below.



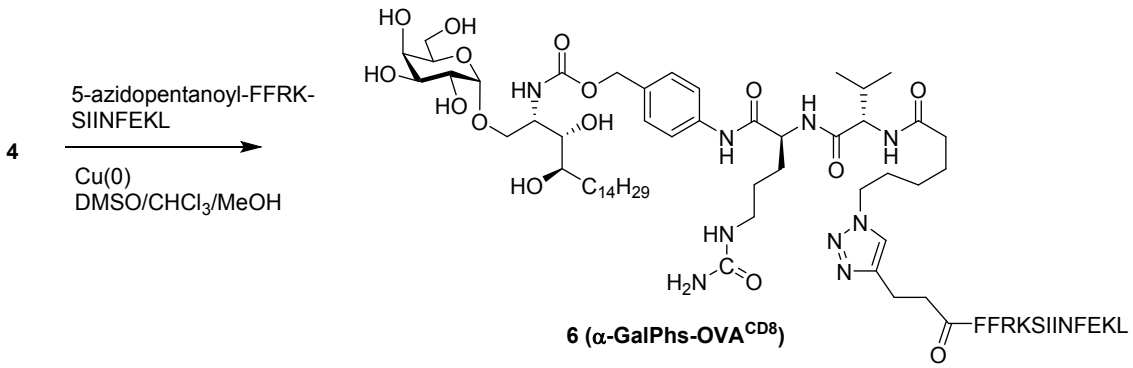
6 (α-GalPhs-OVA^{CD8}) Peptide = FFRK-SIINFEKL (37%)
7 (α-GalPhs-CMV^{CD8}) Peptide = FFRK-NLVPMVATV (72%)



8 (**α-GalPhs-LCMV^{CD8}**) Peptide = FFRK-KAVYNFATM (35%)
9 (**α-GalPhs-TRP2$^{CD4/CD8}$**) Peptide = FFRK-SVYDFFVWLKFFHRTCKCTGNFA (48%)
10 (**α-GalPhs-OVA$^{CD4/CD8}$**) Peptide = FFRK-KISQAVHAAHAEINEAGRESIINFEKLTEWT (44%)

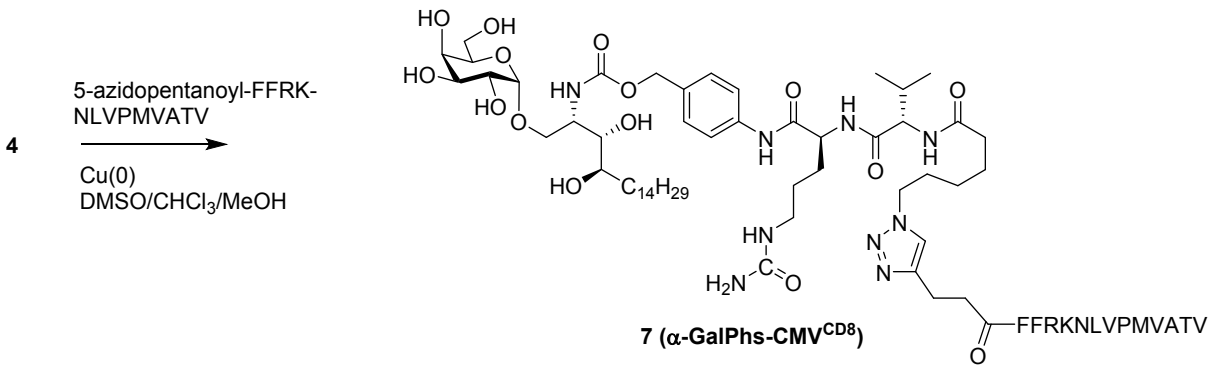
Supplementary scheme 3. Synthetic route to peptide conjugates of α-GalPhs utilizing CuAAC and SPAAC chemistry.

Preparation of α-GalPhs-OVA^{CD8} (**6**)



To a stirred solution of 4-pentynoyl-FFRKSIINFEKL (7.0 mg, 4.3 µmol), **4** (3.0 mg, 2.9 µmol) and TBTA[19] (1.0 mg, 1.9 µmol) in DMSO (280 µL) was added $CHCl_3$ (280 µL) and MeOH (280 µL) was added a small piece of copper foil (5 mm x 2 mm) and the reaction mixture was stirred at 20 °C (48 h). After evaporation of the volatiles under an Ar stream, the product was precipitated by the addition of aq 0.05 M EDTA (pH 7.7, 10 mL) and separated by centrifugation. The pellet was spun again with further EDTA (10 mL) and water (2 x 10 mL), and dried under vacuum. The crude product was purified by preparative HPLC (Phenomenex Luna C18(2), 5 µm, 250 x 30 mm, 30 °C, 40 mL/min; Mobile phase A = 100:0.1 water/ TFA; Mobile phase B = 100:0.1 MeOH/TFA; 0-12 min: 60-100% B; to give compound **6** (**α-GalPhs-OVA^{CD8}**) (2.90 mg, 37%, 96% pure by HPLC-CAD); HRMS-ESI *m/z* calcd for $C_{129}H_{206}N_{27}O_{32}$ $[M+H]^+$ 2645.5322, found 2645.5313.

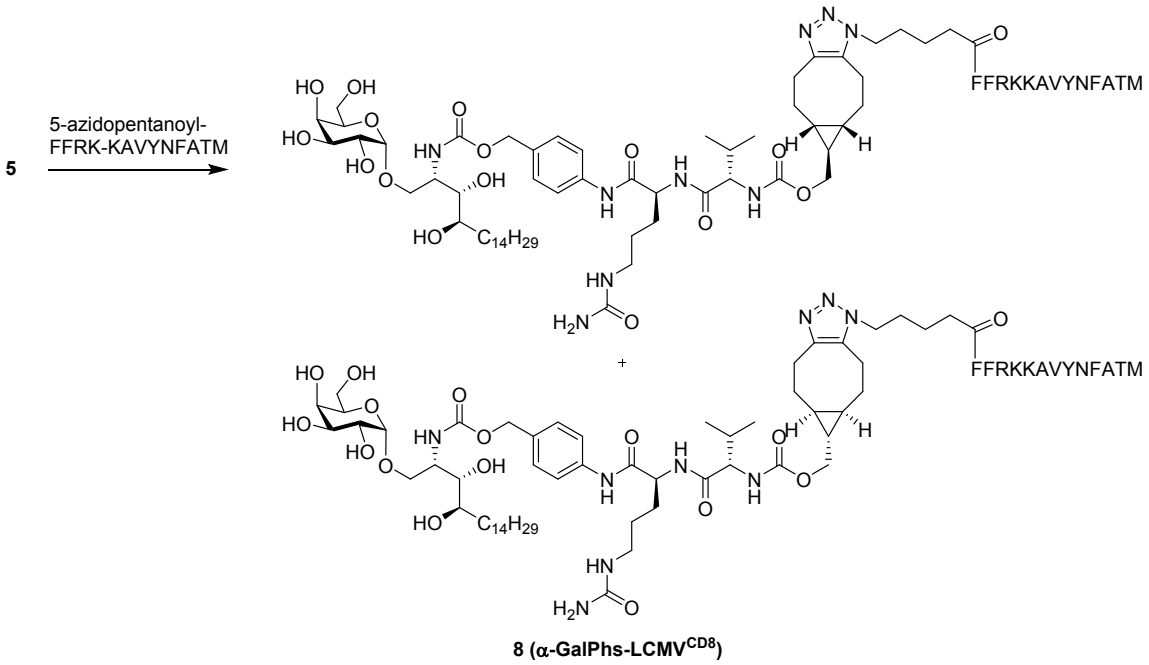
Preparation of α-GalPhs-CMV^{CD8} (**7**)



To a stirred solution of 4-pentynoyl-FFRKNLVPMVATV (7.4 mg, 4.6 µmol), **4** (2.0 mg, 2.0 µmol) and TBTA (0.7 mg, 1.3 µmol) in DMSO (155 µL) was added $CHCl_3$ (155 µL) and MeOH (155 µL) followed by a small piece of copper foil (5 mm x 2 mm). The reaction mixture was stirred at rt (48 h). After evaporation of the volatiles under an Ar stream, the product was precipitated by the addition of aq 0.05 M EDTA (pH 7.7, 10 mL) and separated by centrifugation. The pellet was spun again with further EDTA (10 mL) and water (2 x 10 mL), and dried under vacuum.

The crude product was purified by preparative HPLC (Phenomenex Luna C18(2), 5 µm, 250 x 30 mm, 30 °C, 40 mL/min; Mobile phase A = 100:0.1 water/ TFA; Mobile phase B = 100:0.1 MeOH/TFA; 0-12 min: 60-100% B; to give compound **7** (**α-GalPhs-CMV^{CD8}**) (3.7 mg, 72%, 94% pure by HPLC-CAD); HRMS-ESI *m/z* calcd for $C_{126}H_{207}N_{27}O_{31}$ $[M+2H]^{2+}$ 1313.2508, found 1313.2582.

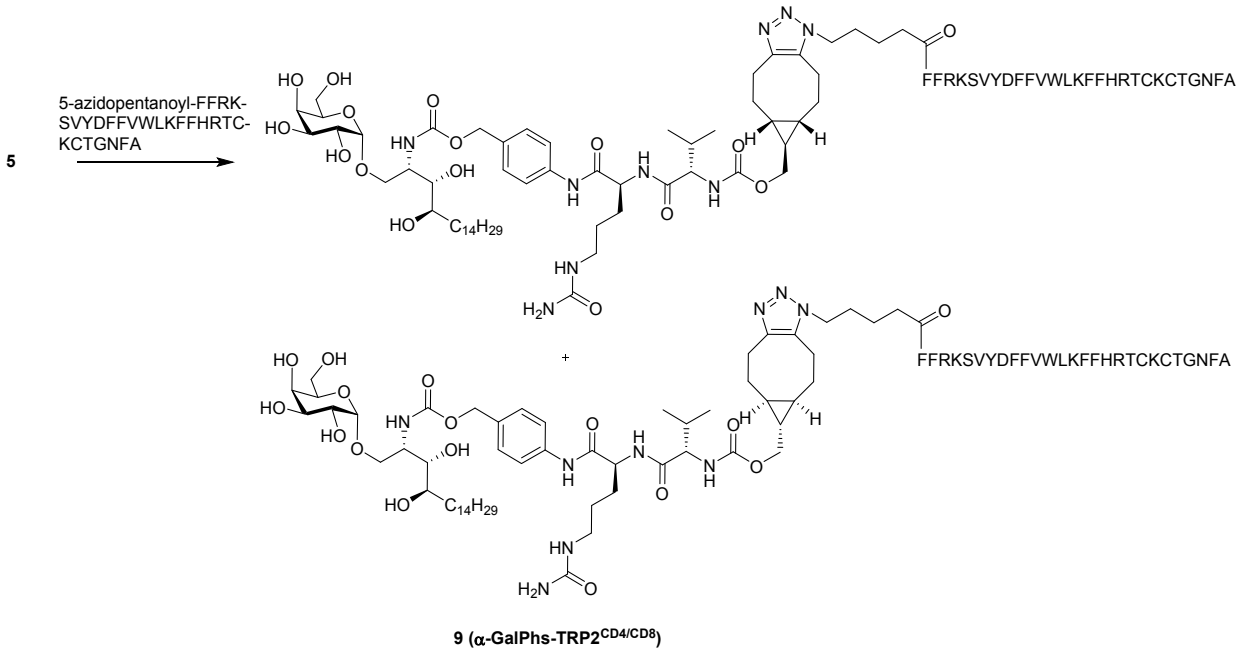
Preparation of α-GalPhs-LCMV^{CD8} (**8**)



8 (α-GalPhs-LCMV^{CD8})

A solution of 5-azidopentanoyl-FFRKKAVYNFATM (3.9 mg, 2.20 µmol) and **5** (2.2 mg, 2.0 µmol) in DMSO (100 µL) was stirred at rt (18 h). The crude product was purified by preparative HPLC (Phenomenex Luna C18(2), 5 µm, 250 x 21.2 mm, 40 °C, 20 mL/min; Mobile phase A = 100:0.05 water/TFA; Mobile phase B = 100:0.05 MeOH/TFA; 0-10 min: 60-100% B; 10-12 min: 100% B; 12-13 min: 100-60% B; 13-15 min: 60% B) to give compound **8** (**α-GalPhs-LCMV^{CD8}**) (1.96 mg, 35%, 99% pure by HPLC-CAD). HRMS-ESI m/z calcd for $C_{137}H_{212}N_{28}O_{33}S$ $[M+2H]^{2+}$ 1404.7668, found 1404.7760.

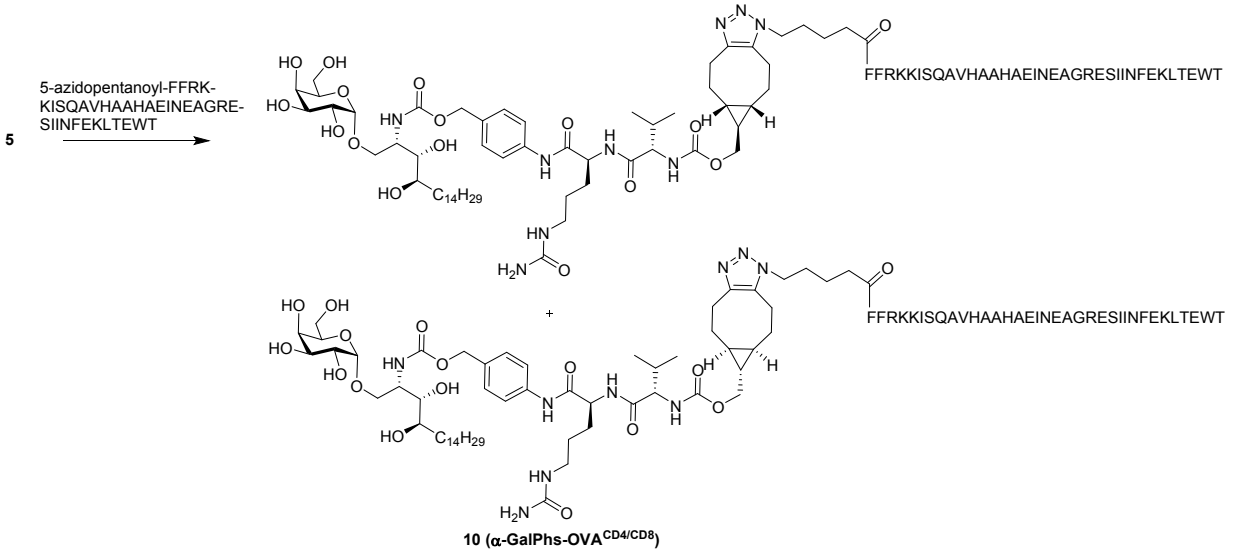
Preparation of α-GalPhs-TRP2$^{CD4/CD8}$ (**9**)



9 (α-GalPhs-TRP2$^{CD4/CD8}$)

A solution of 5-azidopentanoyl-FFRKSVYDFFVWLKFFHRTCKCTGNFA (4.4 mg, 1.2 µmol) and **5** (1.1 mg, 1.0 µmol) in DMSO (200 µL) was stirred at rt (18 h). The crude product was purified by preparative HPLC (Phenomenex Luna C18(2), 5 µm, 250 x 21.2 mm, 40 °C, 20 mL/min; Mobile phase A = 100:0.05 water/TFA; Mobile phase B = 100:0.05 MeOH/TFA; 0-10 min: 60-100% B; 10-12 min: 100% B; 12-13 min: 100-60% B; 13-15 min: 60% B) to give compound **9** (**α-GalPhs-TRP2$^{CD4/CD8}$**) (2.26 mg, 48%, 95% pure by HPLC-CAD). HRMS-ESI m/z calcd for $C_{224}H_{325}N_{49}O_{51}S_2Na$ $[M+2H+Na]^{3+}$ 1534.7876, found 1534.7805.

Preparation of α-GalPhs-OVA$^{CD4/CD8}$ (**10**)



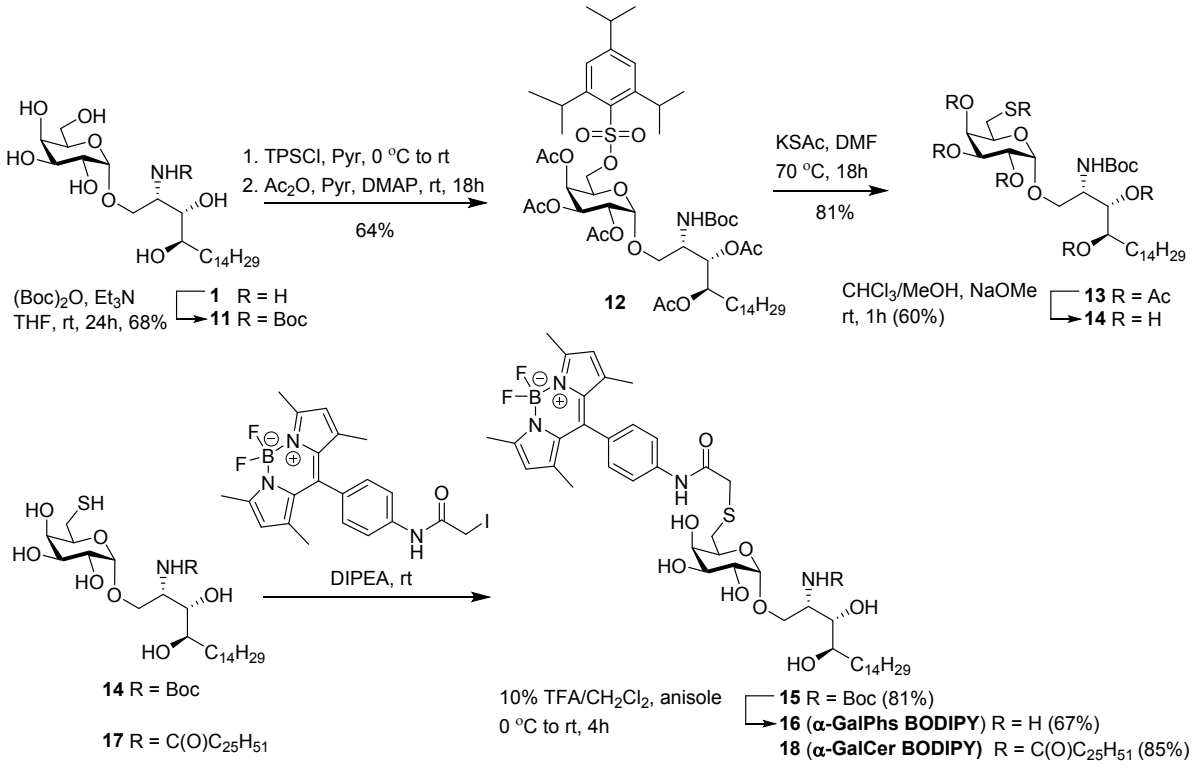
10 (α-GalPhs-OVA$^{CD4/CD8}$)

A solution of 5-azidopentanoyl-FFRKKISQAVHAAHAEINEAGRESIINFEKLTEWT (4.4 mg, 1.1

µmol) and **5** (1.1 mg, 1.0 µmol) in DMSO (100 µL) was stirred at rt (18 h). The crude product was purified by preparative HPLC (Phenomenex Luna C18(2), 5 µm, 250 x 21.2 mm, 40 °C, 20 mL/min; Mobile phase A = 100:0.05 water/TFA; Mobile phase B = 100:0.05 MeOH/TFA; 0-10 min: 60-100% B; 10-12 min: 100% B; 12-13 min: 100-60% B; 13-15 min: 60% B) to give compound **10** (**α-GalPhs-OVA$^{CD4/CD8}$**) (2.37 mg, 44%, 98% pure by HPLC-CAD). HRMS-ESI m/z calcd for $C_{243}H_{382}N_{61}O_{69}$ $[M+3H]^{3+}$ 1752.9465, found 1752.9341.

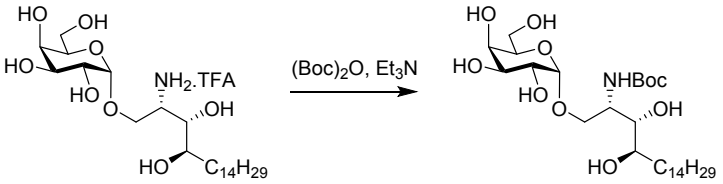
Scheme for synthesis of BODIPY-labelled glycolipids

The synthetic route to BODIPY-labelled α-GalPhs and α-GalCer is shown in supplementary scheme 4. The synthesis of intermediate compounds (labelled numerically in bold in the scheme) is described in detail below.



Supplementary scheme 4. Synthetic route to BODIPY-labelled α-GalPhs and α-GalCer.

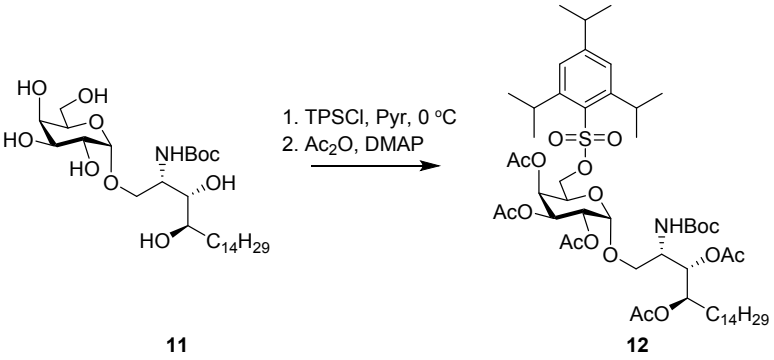
Preparation of (2*S*,3*S*,4*R*)-1-(α-D-galactopyranosyloxy)-2-(tert-butoxycarbonylamino)-3,4-octadecandiol (**11**)



α-GalPhs (1) **11**

To a stirred suspension of α-GalPhs (**1**)[17] (102 mg, 0.172 mmol) in THF (11 mL) was added di-*tert*-butyl dicarbonate (40 mg, 0.181 mmol) and trimethylamine (29 µL) and the reaction was stirred at ambient temperature (24 h). The reaction mixture was diluted with $CHCl_3$ (20 mL), the volatiles removed *in vacuo* and the crude residue purified by column chromatography on silica gel (MeOH/$CHCl_3$ = 0:10 to 3:7) to afford the crude product contaminated with triethylamonium salt. The sample was repurified by RP-C18 chromatography (MeOH/water = 2:8 to 10:0) to afford the title compound **11** (68 mg, 0.117 mmol, 68%) as a white solid. ^{1}H NMR (500 MHz, CD_3OD) δ 4.87 (d, *J* = 3.6 Hz, 1H), 3.93 – 3.86 (m, 3H), 3.84 (td, *J* = 6.0, 1.2 Hz, 1H), 3.81 – 3.67 (m, 4H), 3.64 (q, *J* = 5.9 Hz, 1H), 3.60 – 3.52 (m, 2H), 1.74 – 1.63 (m, 1H), 1.60 – 1.51 (m, 1H), 1.44 (s, 9H), 1.41 – 1.23 (m, 24H), 0.90 (t, *J* = 6.9 Hz, 3H); ^{13}C NMR (126 MHz, CD_3OD) δ 157.82, 101.14, 80.35, 76.17, 73.00, 72.54, 71.63, 71.11, 70.29, 68.58, 62.81, 52.94, 33.23, 33.09, 30.82, 30.78, 30.48, 28.85, 27.04, 23.74, 14.46; HRMS-ESI $[M+H]^+$ calcd for $C_{29}H_{58}NO_{10}$: 580.4055; found 580.4059.

Preparation of (2*S*,3*S*,4*R*)-2-(tert-butoxycarbonylamino)-1-(2,3,4-tri-O-acetyl-6-O-(2,4,6-triisopropylphenylsulfonyl)-α-D-galactopyranosyloxy)-3,4-di(acetyloxy)octadecane (**12**)

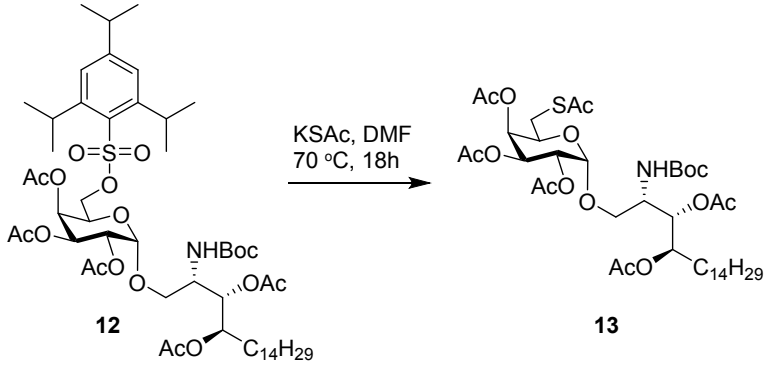


11 **12**

To a solution of **11** (160 mg, 0.276 mmol) in dry pyridine (1.5 mL) under Ar at 0 °C was added 2,4,6-triisopropylbenzenesulfonyl chloride (TPSCl) (220 mg, 0.73 mmol) and the reaction mixture was allowed to warm to rt (18 h). Acetic anhydride (1.5 mL, 16 mmol) and DMAP (2 mg, 16 µmol) was added to the reaction mixture and allowed to stir at rt (18 h). The reaction mixture was diluted with CH_2Cl_2 (20 mL), washed with sat. NaCl (2 x 30 mL), dried ($MgSO_4$) and volatiles removed *in vacuo*. The crude residue purified by column chromatography on silica gel (EtOAc/petroleum ether = 0:10 to 4:6), to afford the title compound **12** as a white foam (187 mg, 0.177 mmol, 64%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.18 (s, 2H), 5.47 (d, *J* = 3.9 Hz, 1H), 5.29 (dd, *J* = 10.9, 3.4 Hz, 1H), 5.20 (dd, *J* = 9.7, 2.5 Hz, 1H), 5.14 (dd, *J* = 10.9, 3.7 Hz, 1H), 5.03 (d, *J* = 10.2 Hz, 1H), 5.01 – 4.96 (m, 1H), 4.86 (d, *J* = 3.6 Hz, 1H), 4.23 (t, *J* = 6.1 Hz, 1H), 4.13 – 4.00 (m, 2H), 4.03 (d, *J* = 4.9 Hz, 2H), 3.97 (t, *J* = 10.0 Hz, 1H), 3.67 (dd, *J* = 10.7, 2.9 Hz, 1H), 3.30 (dd, *J* = 10.7, 2.9 Hz, 1H), 2.92 (hept, *J* = 6.9 Hz, 1H), 2.09 (s, 3H), 2.08 (s, 3H), 2.05 (s, 3H), 1.98 (s, 3H), 1.97 (s, 3H), 1.82 – 1.69 (m, 1H), 1.68 – 1.55 (m, 1H), 1.47 (s, 10H), 1.41 – 1.13 (m, 41H), 0.88 (t, *J* = 6.9 Hz, 3H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 170.67, 170.57, 169.90,

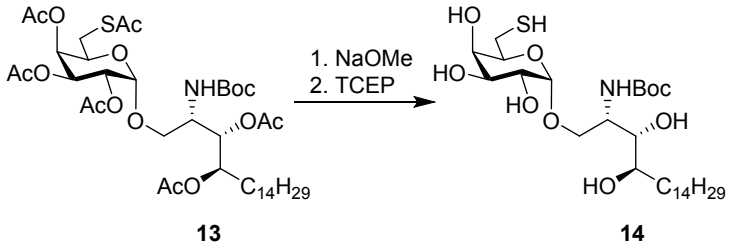
169.85, 169.69, 154.91, 153.94, 150.85, 128.97, 123.83, 97.19, 80.16, 71.13, 68.04, 67.45, 67.13, 66.83, 66.26, 49.52, 34.20, 31.87, 29.64, 29.60, 29.58, 29.53, 29.51, 29.30, 29.19, 28.28, 27.50, 25.65, 24.67, 24.64, 23.52, 23.42, 22.63, 20.88, 20.67, 20.54, 20.52, 20.42, 14.05; HRMS-ESI $[M+H]^+$ calcd for $C_{54}H_{89}NO_{17}S$: 1056.5931; found 1056.5929.

Preparation of (2*S*,3*S*,4*R*)-2-(tert-butoxycarbonylamino)-1-(2,3,4-tri-O-acetyl-6-deoxy-acetylthio-α-D-galactopyranosyloxy)-3,4-di(acetyloxy)octadecane (**13**)



To a solution of **12** (160 mg, 0.15 mmol) in dry DMF (3 mL) was added potassium thioacetate (180 mg, 1.54 mmol) and the reaction mixture was stirred at 70 °C (18 h). The reaction mixture was diluted with EtOAc (40 mL), washed with sat. NaCl (40 mL), dried ($MgSO_4$) and volatiles removed *in vacuo*. The crude residue purified by column chromatography on silica gel (EtOAc/petroleum ether = 0:10 to 4:6), to afford the title compound **13** as a white foam (104 mg, 0.12 mmol, 81%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.41 (d, *J* = 3.1 Hz, 1H), 5.19 (dd, *J* = 10.9, 3.4 Hz, 1H), 5.13 (dd, *J* = 9.6, 2.7 Hz, 1H), 5.04 (dd, *J* = 10.9, 3.7 Hz, 1H), 4.95 (dd, *J* = 18.1, 10.4 Hz, 2H), 4.81 (d, *J* = 3.7 Hz, 1H), 3.98 – 3.83 (m, 2H), 3.65 (d, *J* = 10.8 Hz, 1H), 3.31 (d, *J* = 10.7 Hz, 1H), 3.02 (dd, *J* = 13.8, 6.4 Hz, 1H), 2.85 (dd, *J* = 13.8, 7.7 Hz, 1H), 2.25 (s, 3H), 2.08 (s, 3H), 2.02 (s, 3H), 1.99 (s, 3H), 1.92 (s, 3H), 1.91 (s, 3H), 1.71 – 1.59 (m, 1H), 1.58 – 1.51 (m, 1H), 1.41 (s, 9H), 1.33 – 1.08 (m, 24H), 0.81 (t, *J* = 6.9 Hz, 3H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 193.14, 169.68, 169.23, 168.93, 168.62, 154.07, 96.21, 79.20, 76.27, 72.23, 70.15, 67.82, 66.87, 66.59, 66.43, 48.62, 30.91, 29.40, 28.67, 28.63, 28.61, 28.55, 28.33, 28.24, 27.63, 27.34, 26.64, 24.66, 21.67, 19.93, 19.73, 19.65, 19.60, 13.09. HRMS-ESI $[M+H]^+$ calcd for $C_{41}H_{69}NNaO_{15}S$: 870.4286; found 870.4290.

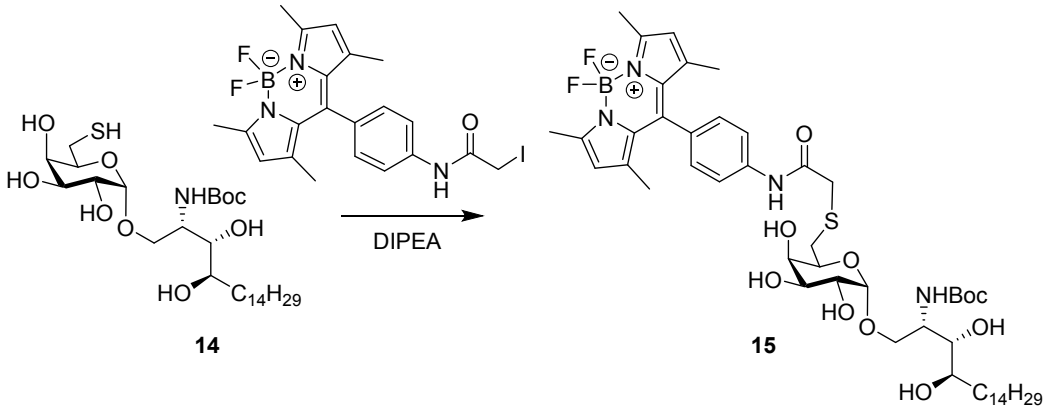
Preparation of (2*S*,3*S*,4*R*)-1-(6-deoxy-6-mercapto-α-D-galactopyranosyloxy)-2-(tert-butoxycarbonylamino)-3,4-octadecandiol (**14**)



To a solution of **13** (180 mg, 0.212 mmol) in $CHCl_3$/MeOH (2:1, 21 mL) was added a solution sodium methoxide in MeOH (0.5 M, 1 mL, 0.50 mmol) and the reaction mixture was stirred at

rt (1 h). The volatiles were removed *in vacuo* and the crude residue was passed through a pad of silica gel, washed with MeOH/$CHCl_3$ (3:7; 50 mL) and concentrated. To the resulting product in MeOH/$CHCl_3$ (1:1, 4 mL) was added TCEP (50 mg, 0.17 mmol) and the reaction mixture was stirred at rt (18 h). The volatiles were removed *in vacuo* and the crude residue purified by column chromatography on silica gel (MeOH/$CHCl_3$ = 0:10 to 3:7) to afford the title compound **14** as a white solid (76 mg, 0.13 mmol, 60%). ^{1}H NMR (500 MHz, 3:1 $CDCl_3$/CD_3OD) δ 5.93 (d, *J* = 9.2 Hz, 1H), 4.89 (s, 1H), 4.01 (s, 1H), 3.94 (dd, *J* = 10.4, 4.2 Hz, 1H), 3.89 (s, 1H), 3.84 – 3.69 (m, 4H), 3.63 – 3.52 (m, 2H), 2.79 (dd, *J* = 13.6, 7.4 Hz, 1H), 2.67 (dd, *J* = 13.6, 6.6 Hz, 1H), 1.74 – 1.61 (m, 1H), 1.60 – 1.52 (m, 1H), 1.46 (s, 9H), 1.39 – 1.20 (m, 24H), 0.89 (t, *J* = 7.0, 1.8 Hz, 3H); ^{13}C NMR (126 MHz, 3:1 $CDCl_3$/CD_3OD) δ 156.05, 99.50, 79.67, 74.80, 72.49, 72.12, 70.32, 69.29, 68.76, 67.96, 58.64, 50.96, 31.81, 29.58, 29.53, 29.23, 28.16, 25.80, 24.33, 23.71, 22.54, 19.56, 13.81; HRMS-ESI $[M+H]^+$ calcd for $C_{29}H_{58}NO_9S$: 596.3832; found 596.3826.

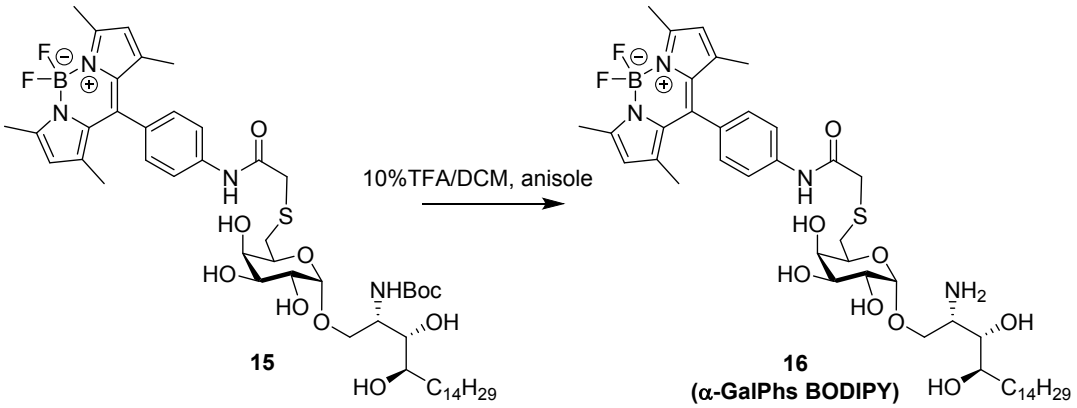
Preparation of (2*S*,3*S*,4*R*)-1-(6-deoxy-6-thio(1,3,5,7-tetramethyl-difluoroboradiaza-s-indacenyl-8-phenyl(4-acetamido)-α-D-galactopyranosyloxy)-2-(tert-butoxycarbonylamino)-3,4-octadecandiol (**15**)



To solid **14** (25 mg, 42 µmol) was added a solution of 1,3,5,7-tetramethyl-8-phenyl(4-iodoacetamido)difluoroboradiaza-*s*-indacene [20] (30 mg, 59 µmol) in CH_2Cl_2 (7 mL) followed by *N,N*-diisopropylethylamine (25 µL, 0.14 mmol) and stirred at rt (30 min). The reaction mixture was filtered, concentrated and the crude residue purified by column chromatography on silica gel (MeOH/$CHCl_3$ = 0:10 to 2:8), to afford the title compound **15** as a red film (33 mg, 33.9 µmol, 81%). ^{1}H NMR (500 MHz, CD_3OD) δ 7.80 (dd, *J* = 8.5, 1.4 Hz, 2H), 7.26 (dd, *J* = 8.5, 2.9 Hz, 2H), 6.54 (d, *J* = 9.1 Hz, 1H), 6.05 (s, 2H), 4.84 (d, *J* = 3.4 Hz, 1H), 4.04 (t, *J* = 7.0 Hz, 1H), 3.96 (dd, *J* = 10.3, 4.5 Hz, 1H), 3.94 – 3.89 (m, 2H), 3.82 – 3.70 (m, 2H), 3.66 (dd, *J* = 10.3, 4.3 Hz, 1H), 3.64 – 3.56 (m, 2H), 3.48 (d, *J* = 14.2 Hz, 1H), 3.40 (d, *J* = 14.2 Hz, 1H), 2.96 (dd, *J* = 13.9, 7.8 Hz, 1H), 2.87 (dd, *J* = 13.9, 6.1 Hz, 1H), 2.48 (s, 6H), 1.75 – 1.63 (m, 1H), 1.62 – 1.51 (m, 1H), 1.46 (s, 6H), 1.43 (s, 9H), 1.38 – 1.23 (m, 24H), 0.89 (t, *J* = 6.9 Hz, 3H); ^{13}C NMR (126 MHz, CD_3OD) δ 169.72, 156.38, 155.19, 143.17, 141.83, 139.52, 131.40, 130.29, 128.51, 120.81, 120.13, 99.51, 78.93, 78.05, 74.59, 71.62, 70.28, 70.23, 68.72, 67.12, 51.50, 36.45,

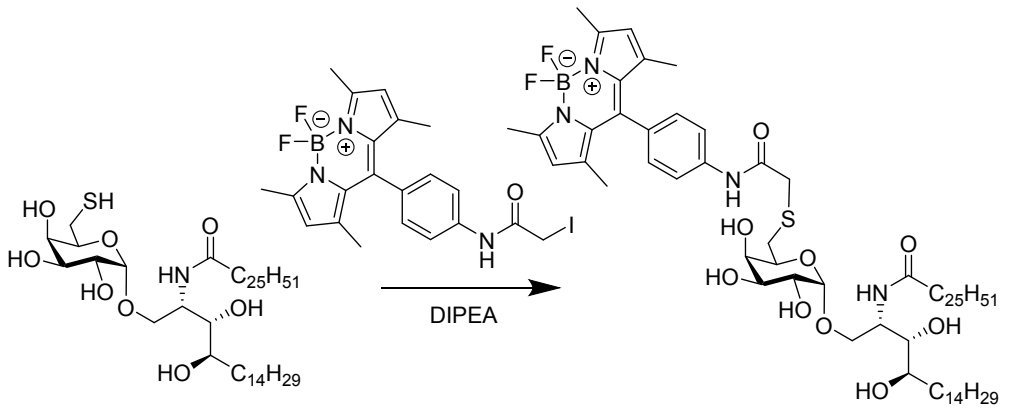
32.63, 31.81, 31.66, 29.40, 29.06, 27.47, 25.66, 22.32, 13.51, 13.18, 13.04; HRMS-ESI $[M+H]^+$ calcd for $C_{50}H_{77}N_4NaO_{10}SF_2B$: 997.5319; found 997.5312.

Preparation of (2*S*,3*S*,4*R*)-1-(6-deoxy-6-thio(1,3,5,7-tetramethyl-difluoroboradiaza-s-indacenyl-8-phenyl(4-acetamido)-α-D-galactopyranosyloxy)-2-amino-3,4-octadecandiol (α-GalPhs-BODIPY, **16**)



To a solution of **15** (25 mg, 0.026 mmol) in CH_2Cl_2 (3 mL) was added anisole (300 µL, 2.76 mmol) followed by trifluoroacetic acid (300 µL, 3.91 mmol) and the reaction mixture was stirred at 0 °C (2 h) and at rt (2 h). MeOH (10 mL) was added, concentrated and the crude residue purified by column chromatography on silica gel (MeOH/$CHCl_3$ = 0:10 to 5:5), to afford the title compound **16** (**α-GalPhs-BODIPY**) as a red film (15 mg, 0.017 mmol, 67%). ^{1}H NMR (500 MHz, CD_3OD) δ 7.88 – 7.74 (m, 2H), 7.33 – 7.21 (m, 2H), 6.06 (s, 2H), 4.84 (d, *J* = 3.9 Hz, 1H), 4.30 (dd, *J* = 10.7, 3.2 Hz, 1H), 4.01 – 3.93 (m, 2H), 3.84 (dd, *J* = 10.1, 3.8 Hz, 1H), 3.78 – 3.71 (m, 2H), 3.60 – 3.55 (m, 3H), 3.49 (d, *J* = 14.0 Hz, 1H), 3.42 (d, *J* = 14.1 Hz, 1H), 2.98 (dd, *J* = 14.1, 7.8 Hz, 1H), 2.85 (dd, *J* = 14.1, 5.9 Hz, 1H), 2.48 (s, 6H), 1.86 – 1.74 (m, 1H), 1.63 – 1.50 (m, 1H), 1.47 (s, 6H), 1.42 – 1.18 (m, 24H), 0.89 (t, *J* = 7.0 Hz, 3H); ^{13}C NMR (126 MHz, CD_3OD) δ 169.84, 161.56, 155.26, 143.11, 139.45, 131.37, 128.55, 120.81, 120.17, 117.96, 115.63, 99.36, 72.52, 71.59, 70.52, 69.98, 68.58, 63.74, 53.62, 36.59, 34.09, 32.54, 31.65, 29.45, 29.36, 29.04, 25.03, 22.31, 13.49, 13.15, 13.01; HRMS-ESI $[M+H]^+$ calcd for $C_{45}H_{70}N_4O_8SF_2B$: 875.4975; found 875.4983.

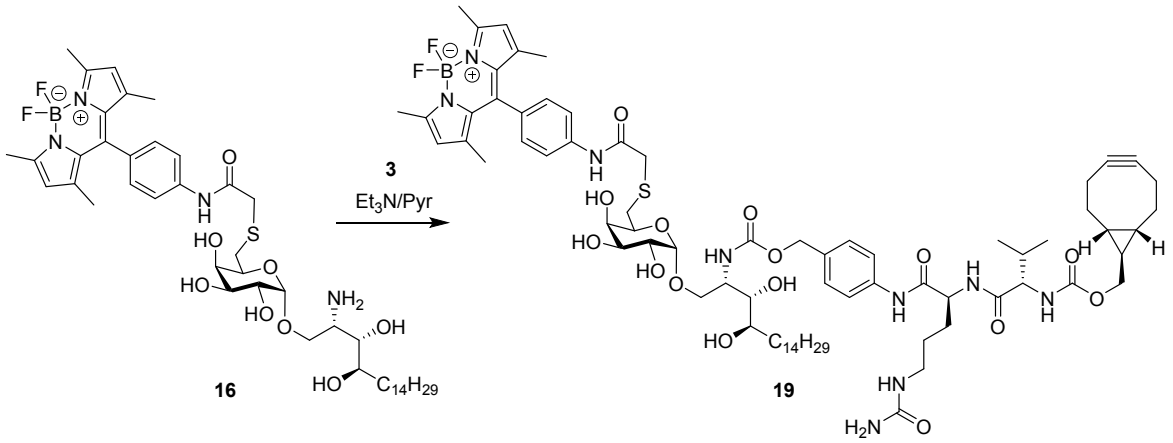
Preparation of (2*S*,3*S*,4*R*)-1-(6-deoxy-6-thio(1,3,5,7-tetramethyl-difluoroboradiaza-s-indacenyl-8-phenyl(4-acetamido)-α-D-galactopyranosyloxy)-2-hexacosanoylamino-3,4-octadecandiol (α-GalCer-BODIPY, **17**)



17 **18 (α-GalCer BODIPY)**

To a solution of **17**[21] (50 mg, 0.057 mmol) and 1,3,5,7-tetramethyl-8-phenyl(4-iodoacetamido)-difluoroboradiaza-*s*-indacene (32.1 mg, 0.063 mmol) in dry DMF/$CHCl_3$ (1:1, 5 mL) was added *N,N*-diisopropylethylamine (18 µL, 0.10 mmol) and stirred at rt (3.5 h). The reaction mixture was concentrated and the crude residue purified by column chromatography on silica gel (EtOAc/CH_2Cl_2 0:10 to 1:9 then MeOH/CH_2Cl_2 = 0:10 to 1:9) to afford the title compound **18** (**α-GalCer-BODIPY**) as a red film (60.8 mg, 0.049 mmol, 85%). ^{1}H NMR (500 MHz, 1:2 $CDCl_3$/CD_3OD) δ 7.85 – 7.78 (m, 2H), 7.36 – 7.24 (m, 2H), 6.03 (s, 2H), 4.87 (d, *J* = 3.6 Hz, 1H), 4.29 – 4.20 (m, 1H), 4.05 – 3.95 (m, 2H), 3.94 (d, *J* = 2.6 Hz, 1H), 3.77 (qd, *J* = 10.0, 3.3 Hz, 2H), 3.71 (dd, *J* = 10.5, 4.5 Hz, 1H), 3.63 (t, *J* = 6.0 Hz, 1H), 3.59 (ddd, *J* = 8.9, 6.1, 2.4 Hz, 1H), 3.49 (d, *J* = 14.4 Hz, 1H), 3.40 (d, *J* = 14.4 Hz, 1H), 2.98 (dd, *J* = 13.9, 8.0 Hz, 1H), 2.87 (dd, *J* = 13.9, 5.8 Hz, 1H), 2.51 (s, 6H), 2.24 (t, *J* = 7.5 Hz, 2H), 1.75 – 1.52 (m, 2H), 1.47 (s, 6H), 1.44 – 1.21 (m, 70H), 0.89 (t, *J* = 6.9 Hz, 6H). ^{13}C NMR (126 MHz, 1:2 $CDCl_3$/CD_3OD) δ 175.40, 170.33, 156.16, 144.18, 142.63, 140.33, 132.44, 131.27, 129.44, 121.95, 121.08, 100.42, 75.05, 72.69, 71.47, 71.22, 71.12, 69.64, 67.88, 51.35, 37.63, 37.17, 33.69, 32.83, 32.68, 30.54, 30.51, 30.46, 30.43, 30.38, 30.31, 30.21, 30.11, 30.07, 26.74, 26.66, 23.37, 14.93, 14.61, 14.34; HRMS-ESI $[M+Na]^+$ calcd for $C_{71}H_{119}N_4NaO_9SF_2B$: 1275.8657; found 1275.8673.

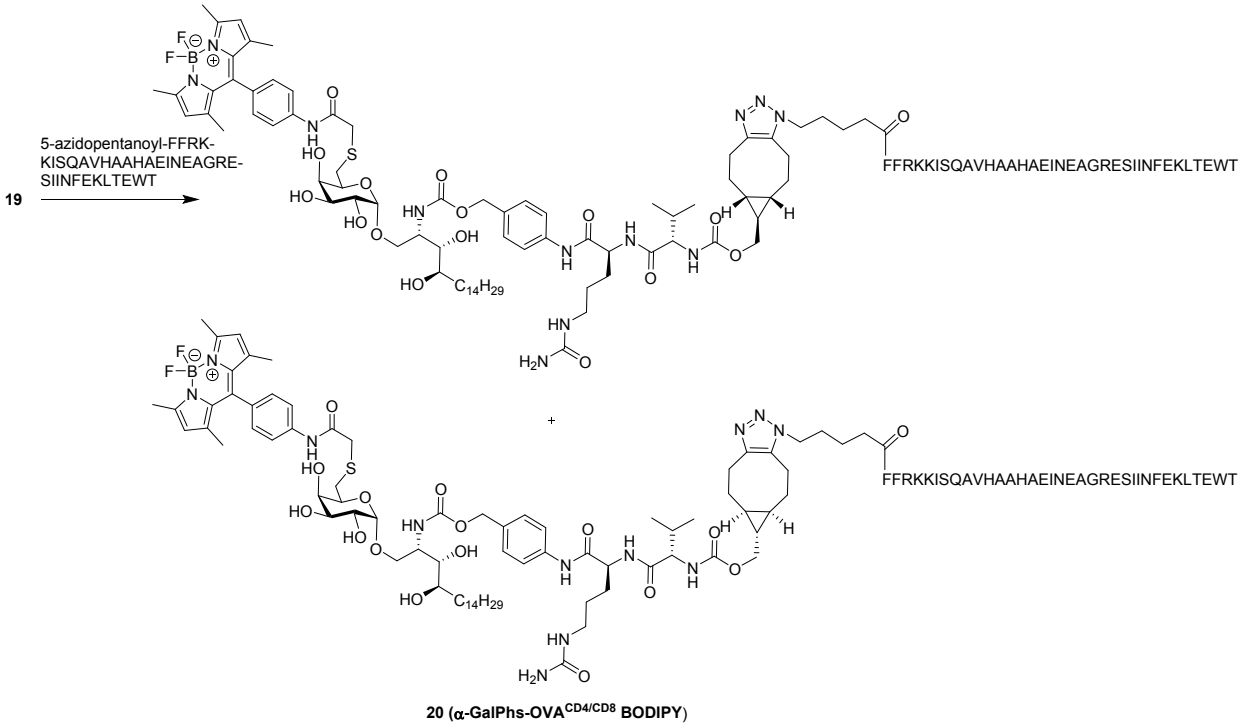
Preparation of (2*S*,3*S*,4*R*)-1-(6-deoxy-6-thio(1,3,5,7-tetramethyl-difluoroboradiaza-s-indacenyl-8-phenyl(4-acetamido)-α-D-galactopyranosyloxy)-2-(N-((bicyclo[6.1.0]non-4-yn-9-yl)-methoxycarbonyl)-L-valinyl-L-citrullinyl-4-aminobenzyloxycarbonyl)amino-3,4-octadecandiol (**19**)



16 **19**

To a solution of **16** (**α-GalPhs-BODIPY**) (8.0 mg, 9.1 µmol) and **3** (12 mg, 0.017 mmol) in dry pyridine (1.5 mL) was added triethylamine (25 µL, 0.18 mmol) and stirred at rt (18 h). A mixture of MeOH/CH_2Cl_2 (1:1, 10 mL) was added, concentrated and the crude residue purified by column chromatography on silica gel (MeOH/$CHCl_3$ = 0:10 to 3:7), to afford the title compound **19** as a red film (8.0 mg, 5.5 mmol, 60%). ^{1}H NMR (500 MHz, 3:1 $CDCl_3$/CD_3OD) δ 7.88 – 7.72 (m, 2H), 7.56 (d, *J* = 8.1 Hz, 2H), 7.30 (d, *J* = 8.2 Hz, 2H), 7.27 – 7.22 (m, 2H), 6.01 (s, 2H), 5.09 (d, *J* = 12.2 Hz, 1H), 5.02 (d, *J* = 12.3 Hz, 1H), 4.88 (d, *J* = 3.8 Hz, 1H), 4.55 (dd, *J* = 9.0, 5.2 Hz, 1H), 4.11 – 3.85 (m, 8H), 3.77 (dd, *J* = 10.0, 4.2 Hz, 1H), 3.73 – 3.67 (m, 2H), 3.63 – 3.57 (m, 2H), 3.43 (d, *J* = 14.9 Hz, 1H), 3.29 – 3.17 (m, 1H), 3.15 – 3.08 (m, 1H), 3.00 – 2.90 (m, 1H), 2.81 (dd, *J* = 13.9, 5.8 Hz, 1H), 2.54 (s, 6H), 2.40 (d, *J* = 13.2 Hz, 2H), 2.32 – 2.21 (m, 1H), 2.20 – 2.07 (m, 3H), 1.96 – 1.87 (m, 1H), 1.80 – 1.63 (m, 2H), 1.61 – 1.51 (m, 4H), 1.45 (s, 8H), 1.26 (d, *J* = 3.2 Hz, 28H), 1.18 (d, *J* = 6.1 Hz, 5H), 0.98 (d, *J* = 6.8 Hz, 3H), 0.94 (d, *J* = 6.8 Hz, 3H), 0.88 (t, *J* = 7.0 Hz, 3H); ^{13}C NMR (126 MHz, 3:1 $CDCl_3$/CD_3OD) δ 174.11, 171.95, 170.42, 161.99, 158.85, 158.07, 156.74, 144.71, 142.94, 140.48, 139.20, 133.89, 132.96, 131.86, 130.00, 122.61, 121.55, 121.44, 100.74, 100.10, 76.20, 73.35, 71.93, 71.63, 71.50, 70.97, 70.11, 68.64, 67.71, 65.15, 61.90, 60.04, 54.66, 53.12, 40.35, 38.36, 34.54, 34.20, 34.02, 33.23, 32.32, 31.11, 31.01, 30.95, 30.65, 29.51, 27.70, 27.12, 25.98, 25.09, 24.96, 24.34, 24.28, 23.96, 22.53, 20.98, 20.43, 19.02, 15.79, 15.61, 15.21, 14.66; HRMS-ESI $[M+H]^+$ calcd for $C_{75}H_{108}N_9NaO_{15}SF_2B$: 1478.7644; found 1478.7644.

Preparation of α-GalPhs-OVA$^{CD4/CD8}$-BODIPY (**20**)

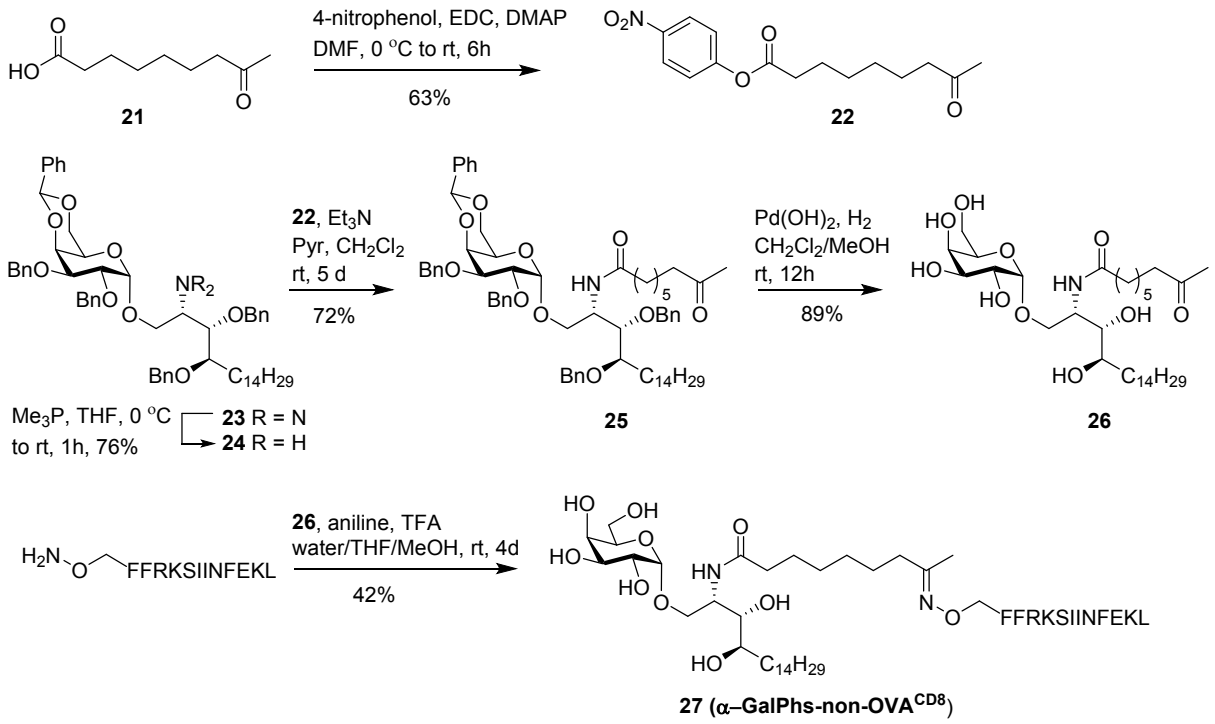


20 (α-GalPhs-OVA$^{CD4/CD8}$ BODIPY)

A solution of 5-azidopentanoyl-FFRKKISQAVHAAHAEINEAGRESIINFEKLTEWT (8.5 mg, 2.0 µmol) and **19** (2.0 mg, 1.4 µmol) in DMSO (300 µL) was stirred at rt (18 h). The crude product was purified by preparative HPLC (Phenomenex Luna C18(2), 5 µm, 250 x 21.2 mm, 40 °C, 20 mL/min; Mobile phase A = 100:0.05 water/TFA; Mobile phase B = 100:0.05 MeOH/TFA; 0-11 min: 60-100% B; 11-13 min: 100% B; 13-14 min: 100-60% B; 14-16 min: 60% B) to give compound **20** (**α-GalPhs-OVA$^{CD4/CD8}$-BODIPY**) (1.37 mg, 18%, 93% pure by HPLC-CAD). HRMS-ESI m/z calcd for $C_{264}H_{403}BF_2N_{64}O_{69}S$ $[M+4H]^{4+}$ 1413.7366, found 1413.7456

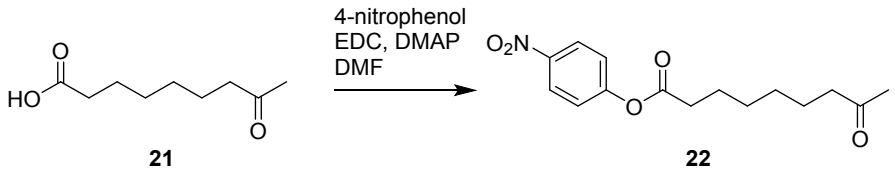
Scheme for synthesis of the glycolipid-peptide conjugate without a protease-cleavable linker

The synthetic route to α-GalPhs-non-OVA^{CD8} is shown in supplementary scheme 5. The synthesis of intermediate compounds (labelled numerically in bold in the scheme) is described in detail below.



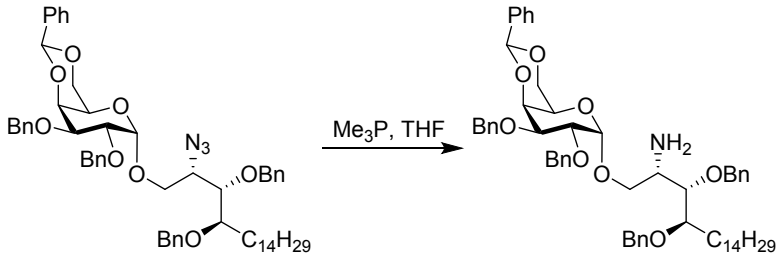
Supplementary scheme 5. Synthetic route to a peptide conjugate of α-GalPhs lacking the protease-cleavable linker.

Preparation of 4-nitrophenyl 8-oxononanoate (**22**)



To a solution of **21**[22] (30 mg, 0.174 mmol) in dry DMF was added 4-nitrophenol (26 mg, 0.19 mmol) and DMAP (2 mg, 15 µmol) under Ar at rt. After 10 min, the reaction mixture was cooled to 0 °C before then addition of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (HCl salt, 43 mg, 0.224 mmol) and the reaction mixture was allowed to warm to rt (6 h). The reaction mixture was diluted with EtOAc (15 mL), washed with water (30 mL), dried ($MgSO_4$), concentrated and the crude product was purified by column chromatography on silica gel (EtOAc/toluene = 0:100 to 6:94) to afford the title compound **22** as a thin film (32 mg, 0.109 mmol, 63%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.31 – 8.23 (m, 2H), 7.31 – 7.24 (m, 2H), 2.60 (t, *J* = 7.5 Hz, 2H), 2.44 (t, *J* = 7.3 Hz, 2H), 2.14 (s, 3H), 1.77 (p, *J* = 7.4 Hz, 2H), 1.66 – 1.55 (m, 2H), 1.50 – 1.40 (m, 2H), 1.40 – 1.32 (m, 2H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 208.85, 171.12, 155.48, 145.29, 125.17, 122.39, 43.53, 34.20, 29.88, 28.79, 28.71, 24.50, 23.50; HRMS-ESI m/z calcd for $C_{15}H_{19}NO_5Na$ $[M+Na]^+$ 316.1161, found 316.1161.

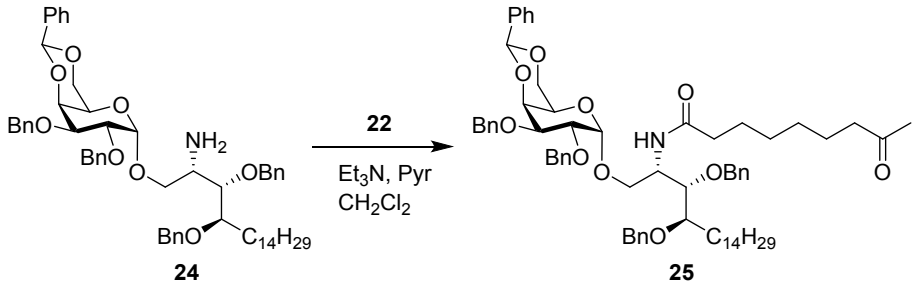
Preparation of (2*S*,3*S*,4*R*)-2-amino-1-(2,3-di-O-benzyl-4,6-O-benzylidene)-α-D-galactopyranosyloxy)-3,4-di-O-benzyl-octadecane (**15**)



23 **24**

To a solution of **23**[23] (170 mg, 0.178 mmol) in dry THF at 0 °C under Ar was added a solution of trimethylphosphine (1.0 M in THF, 800 µL, 0.8 mmol) and the solution was allowed to warm to rt (1 h). A solution of NaOH (1 M, 1 mL) was added and the reaction mixture was stirred at rt (1h) before being diluted with EtOAc (100 mL), washed with water (100 mL), dried ($MgSO_4$), concentrated and the crude product was purified by column chromatography on silica gel (MeOH/$CHCl_3$ = 0:100 to 15:85) to afford the title compound **24** as a pale yellow oil (125 mg, 0.135 mmol, 76%). ^{1}H NMR (500 MHz, $CDCl_3$) δ ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.54 – 7.48 (m, 2H), 7.42 – 7.21 (m, 23H), 5.45 (s, 1H), 4.96 (d, *J* = 3.5 Hz, 1H), 4.84 (d, *J* = 11.7 Hz, 1H), 4.79 (d, *J* = 12.1 Hz, 1H), 4.76 – 4.68 (m, 2H), 4.67 – 4.59 (m, 2H), 4.53 (dd, *J* = 11.2 Hz, 2H), 4.17 (dd, *J* = 3.6, 1.1 Hz, 1H), 4.12 – 4.05 (m, 2H), 4.03 – 3.96 (m, 2H), 3.90 (dd, *J* = 12.5, 1.8 Hz, 1H), 3.70 (dt, *J* = 7.7, 3.8 Hz, 1H), 3.58 – 3.51 (m, 2H), 3.34 (dd, *J* = 9.8, 8.2 Hz, 1H), 3.21 – 3.13 (m, 1H), 1.83 – 1.52 (m, 2H), 1.51 – 1.40 (m, 2H), 1.40 – 1.16 (m, 22H), 0.88 (t, *J* = 6.9 Hz, 3H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 138.78, 138.64, 138.47, 137.88, 128.83, 128.37, 128.33, ^{23}128.30, 128.28, 128.09, 127.86, 127.75, 127.66, 127.61, 127.57, 127.54, 126.33, 101.06, 99.31, 82.00, 79.92, 76.17, 75.81, 74.55, 73.65, 73.56, 72.09, 71.83, 71.57, 69.41, 62.71, 52.80, 31.92, 30.65, 29.86, 29.71, 29.67, 29.36, 25.80, 22.68, 14.10; HRMS-ESI m/z calcd for $C_{59}H_{77}NO_8Na$ $[M+Na]^+$ 950.5547, found 950.5549.

Preparation of Preparation of (2*S*,3*S*,4*R*)-2-N-(8-oxononanoyl)amino-1-(2,3-di-O-benzyl-4,6-O-benzylidene)-α-D-galactopyranosyloxy)-3,4-di-O-benzyl-octadecane (**25**)

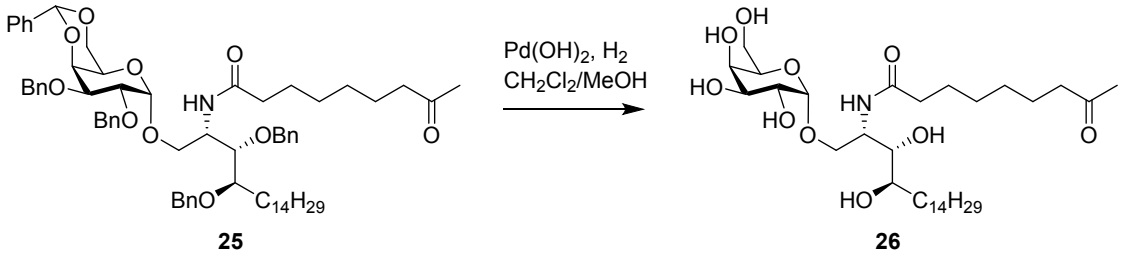


24 **25**

To a solution of **24** (30 mg, 32 µmol) and **22** (32 mg, 0.109 mmol) in a mixture of dry CH_2Cl_2 (2 mL) and pyridine (0.2 mL) was added triethylamine (10 µL) and stirred under Ar at rt (5 d). The reaction was concentrated and the crude product was purified by column chromatography on silica gel (EtOAc/petroleum ether = 0:10 to 6:4) to afford the title compound **25** as a white solid (25 mg, 23 µmol, 72%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.53 – 7.48 (m, 2H), 7.41 – 7.19 (m, 23H), 5.86 (d, *J* = 8.3 Hz, 1H), 5.46 (s, 1H), 4.95 (d, *J* = 3.5 Hz, 1H), 4.84

(d, *J* = 11.6 Hz, 1H), 4.74 (d, *J* = 2.0 Hz, 2H), 4.71 (d, *J* = 11.6 Hz, 1H), 4.63 (d, *J* = 11.5 Hz, 1H), 4.57 (d, *J* = 11.6 Hz, 1H), 4.49 (dd, *J* = 11.6, 3.5 Hz, 2H), 4.33 – 4.25 (m, 1H), 4.19 (dd, *J* = 3.5, 1.2 Hz, 1H), 4.10 (dd, *J* = 12.5, 1.5 Hz, 1H), 4.06 (dd, *J* = 10.1, 3.6 Hz, 1H), 3.96 – 3.88 (m, 3H), 3.80 – 3.72 (m, 2H), 3.57 (s, 1H), 3.53 (dt, *J* = 7.7, 3.9 Hz, 1H), 2.36 (t, *J* = 7.4 Hz, 2H), 2.10 (s, 3H), 1.95 – 1.79 (m, 2H), 1.70 – 1.57 (m, 2H), 1.57 – 1.37 (m, 6H), 1.35 – 1.15 (m, 26H), 0.88 (t, *J* = 6.9 Hz, 3H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 208.87, 172.73, 138.69, 138.56, 138.43, 137.87, 128.83, 128.43, 128.36, 128.33, 128.30, 128.08, 127.89, 127.82, 127.70, 127.60, 127.56, 126.31, 101.00, 99.60, 71.91, 71.65, 69.41, 68.13, 62.98, 50.36, 43.59, 36.54, 31.92, 30.30, 29.84, 29.82, 29.71, 29.69, 29.67, 29.36, 29.07, 28.87, 25.84, 25.43, 23.62, 22.68, 14.10; HRMS-ESI m/z calcd for $C_{68}H_{91}NO_{10}Na$ $[M+Na]^+$ 1104.6541, found 1104.6538.

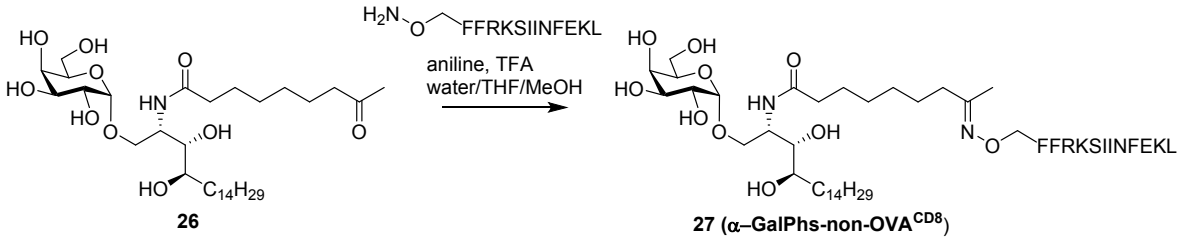
Preparation of (2*S*,3*S*,4*R*)-1-(α-D-galactopyranosyloxy)-2-N-(8-oxononanoyl)amino-3,4-octadecandiol (**26**)



25 **26**

To a solution of **25** (25 mg, 23 µmol) in a mixture of CH_2Cl_2/MeOH (1:1, 10 mL) was added $Pd(OH)_2$/C (20%, 10 mg) and the atmosphere was exchanged for H_2. The reaction mixture was stirred at rt for 12 h before being passed through glass-fibre filter paper, concentrated and the crude residue purified by column chromatography on silica gel (MeOH/$CHCl_3$ = 0:100 to 25:75), to afford the title compound **26** as a white solid (13 mg, 20 µmol, 89%). ^{1}H NMR (500 MHz, 1:1 $CDCl_3$/CD_3OD) δ δ 4.87 (d, *J* = 3.9 Hz, 1H), 4.22 – 4.14 (m, 1H), 3.90 (d, *J* = 3.3 Hz, 1H), 3.86 (dd, *J* = 10.8, 4.7 Hz, 1H), 3.81 – 3.74 (m, 2H), 3.74 – 3.63 (m, 4H), 3.54 – 3.47 (m, 2H), 2.44 (t, *J* = 7.4 Hz, 2H), 2.18 (t, *J* = 7.6 Hz, 2H), 2.12 (s, 3H), 1.72 – 1.45 (m, 6H), 1.42 – 1.19 (m, 28H), 0.85 (t, *J* = 6.7 Hz, 3H); ^{13}C NMR (126 MHz, 1:1 $CDCl_3$/CD_3OD) δ 211.59, 174.93, 100.36, 75.31, 72.50, 71.53, 70.87, 70.31, 69.51, 67.81, 62.31, 44.01, 36.73, 33.12, 32.41, 30.28, 30.21, 30.18, 30.12, 29.94, 29.82, 29.48, 29.28, 26.33, 26.11, 24.08, 23.12, 14.26; HRMS-ESI m/z calcd for $C_{33}H_{63}NO_{10}Na$ $[M+Na]^+$ 656.4350, found 656.4346.

Preparation of α-GalPhs-non-OVA^{CD8} (**27**)

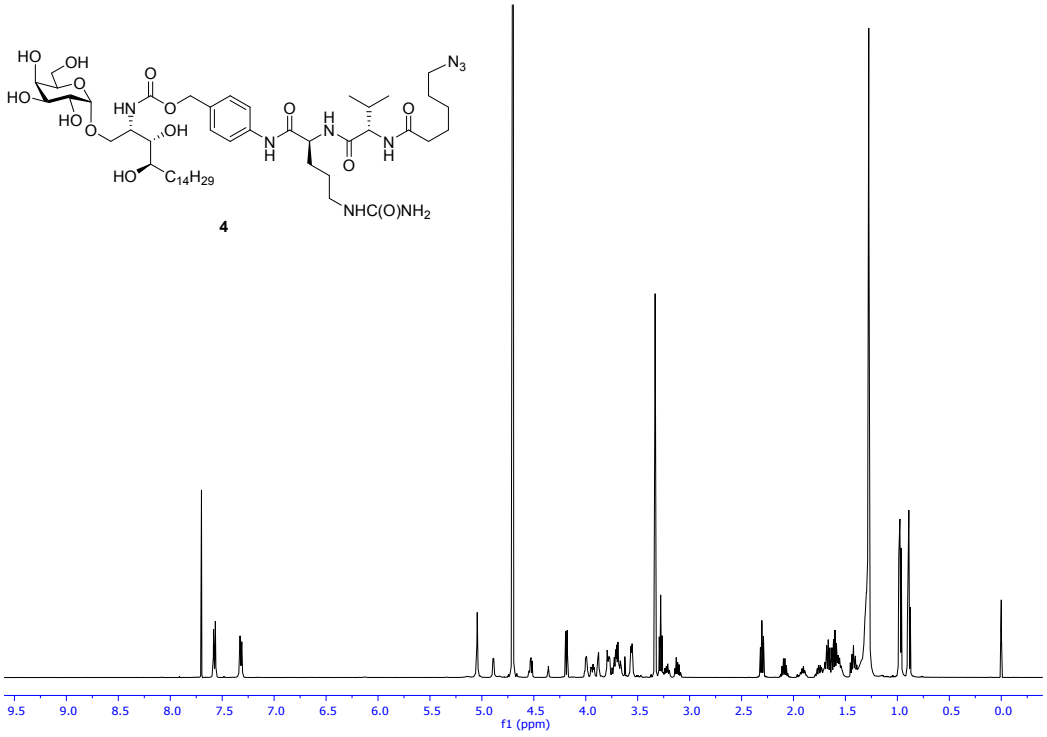


26 **27 (α–GalPhs-non-OVA^{CD8})**

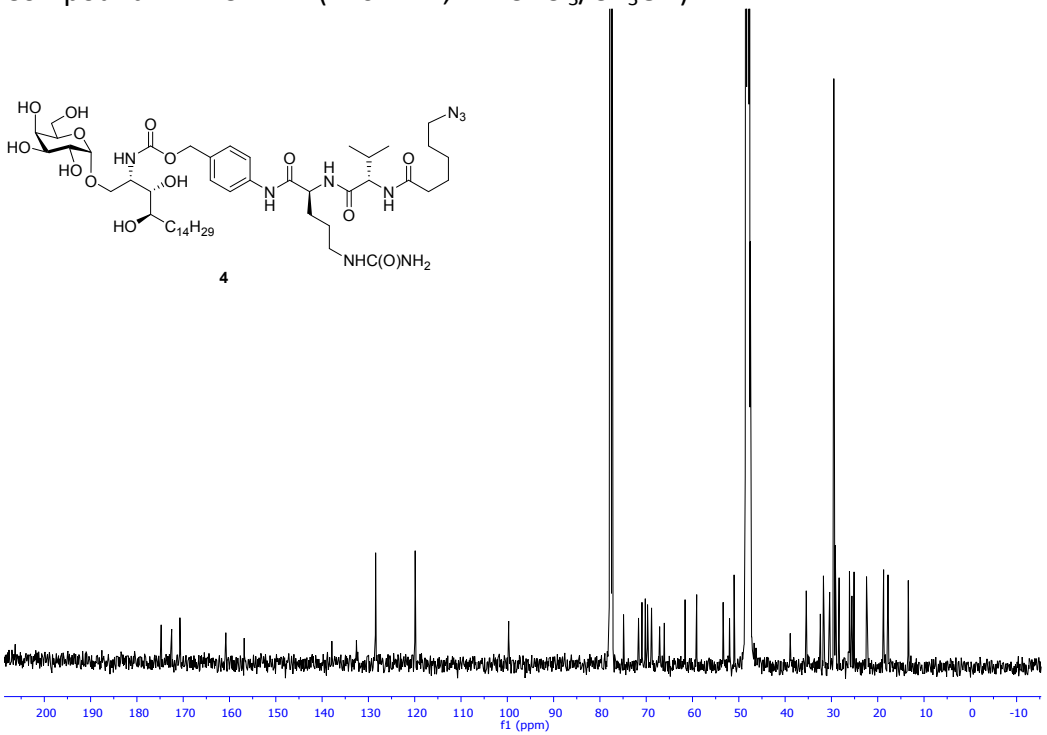
To a stirred suspension of 2-(aminooxy)acetyl-FFRKSIINFEKL (8.7 mg, 5.4 µmol) in THF/MeOH (2:1, 600 µl) was added an mixture of water/aniline/TFA (200:6:4, 300 µL). Once dissolved, a solution of **26** (2.3 mg, 3.6 µmol), dissolved in THF/MeOH (1:1, 400 µl) was added and thereaction mixture was stirred at rt for 4 d. The solvent was removed and the crude product

purified by semi-preparative HPLC (Phenomenex Luna C18, 5 µm, 250 x 10 mm, 40 °C, 2.6 mL/min; Mobile phase A = 100:0.05 water/ TFA; Mobile phase B = 100:0.05 MeOH/TFA; 0-9 min: 70-100% B; 9-12 min: 100% B; 12-13 min: 100-70% B; 13-15 min: 70% B) to afford compound **27** (**α-GalPhs-non-OVA^{CD8}**) (5 mg, 42%, 92% pure by HPLC). HRMS-ESI m/z calcd for $C_{110}H_{182}N_{20}O_{28}$ $[M+2H]^{2+}$ 1115.6638, found 1115.6707.

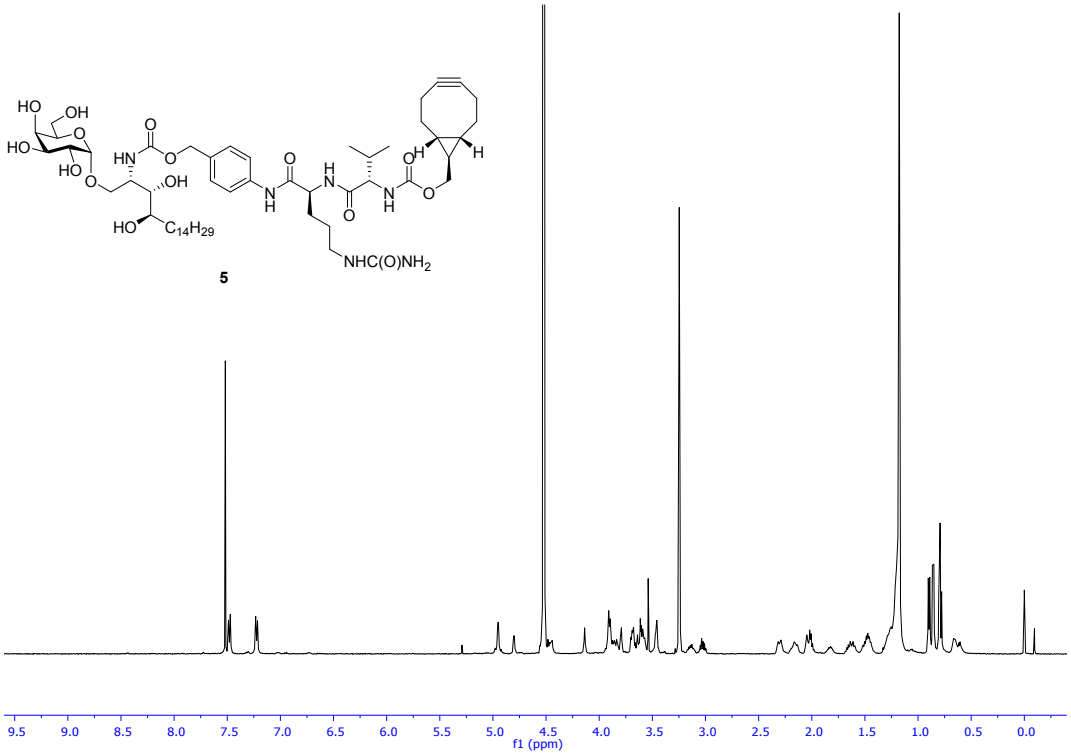
Compound **4** – ^{1}H NMR (500 MHz, 1:1 $CDCl_3/CD_3OD$)



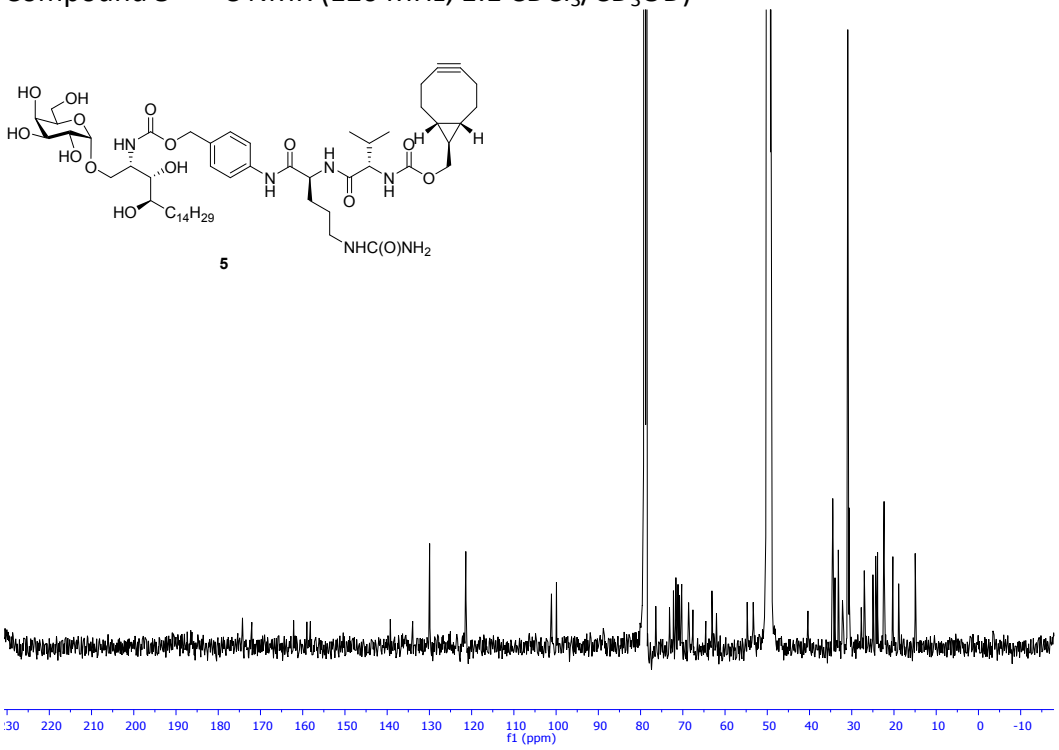
Compound **4** – ^{13}C NMR (126 MHz, 1:1 $CDCl_3/CD_3OD$)



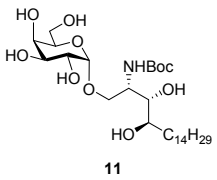
Compound **5** – ^{1}H NMR (500 MHz, 1:1 $CDCl_3/CD_3OD$)



Compound **5** – ^{13}C NMR (126 MHz, 1:1 $CDCl_3/CD_3OD$)

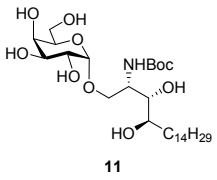


Compound **11** - ^{1}H NMR (500 MHz, $CDCl_3$)



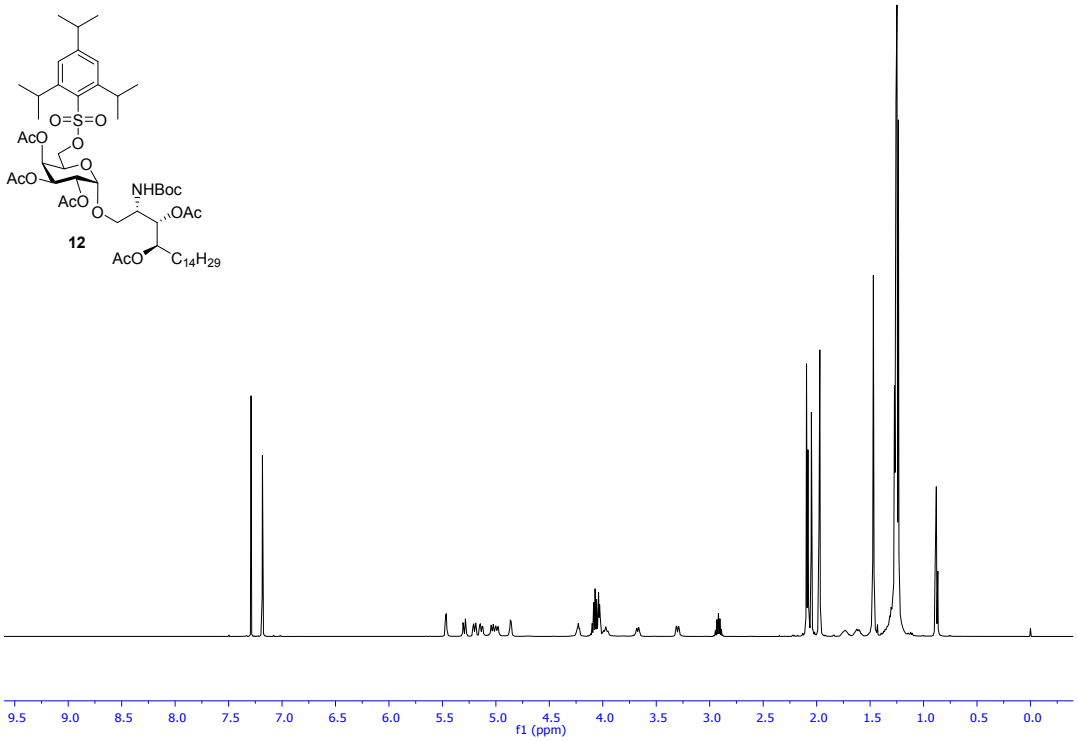
11

Compound **11** – ^{13}C NMR (126 MHz, $CDCl_3$)

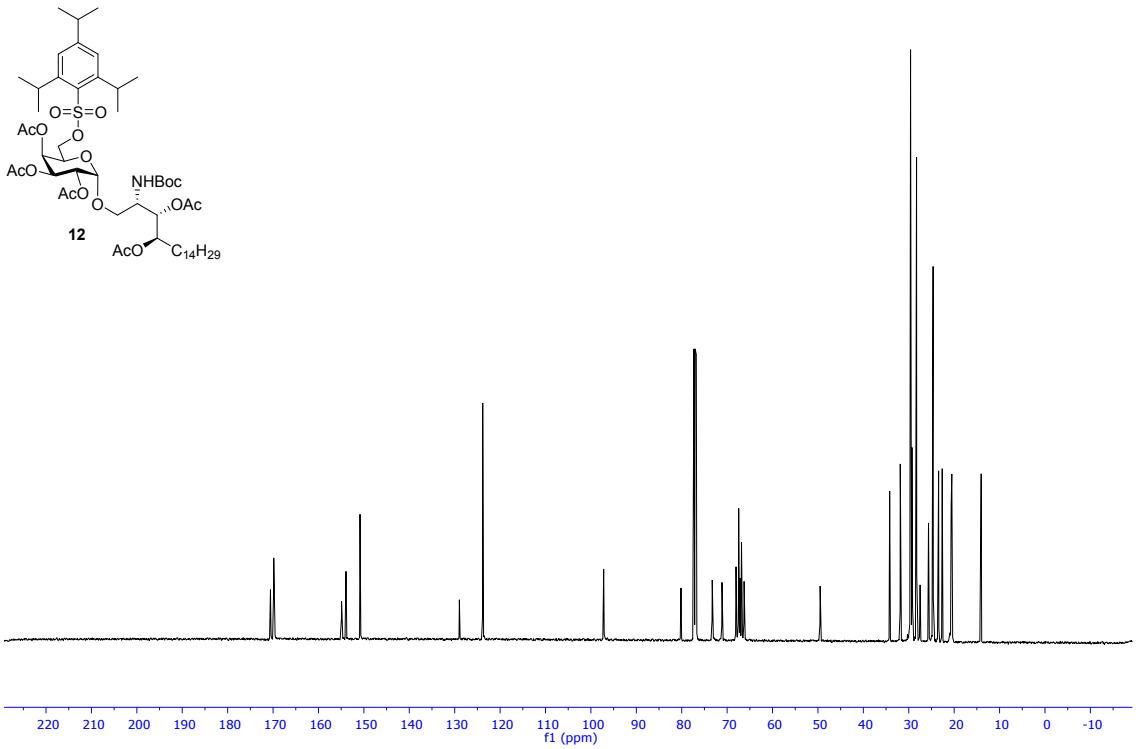


11

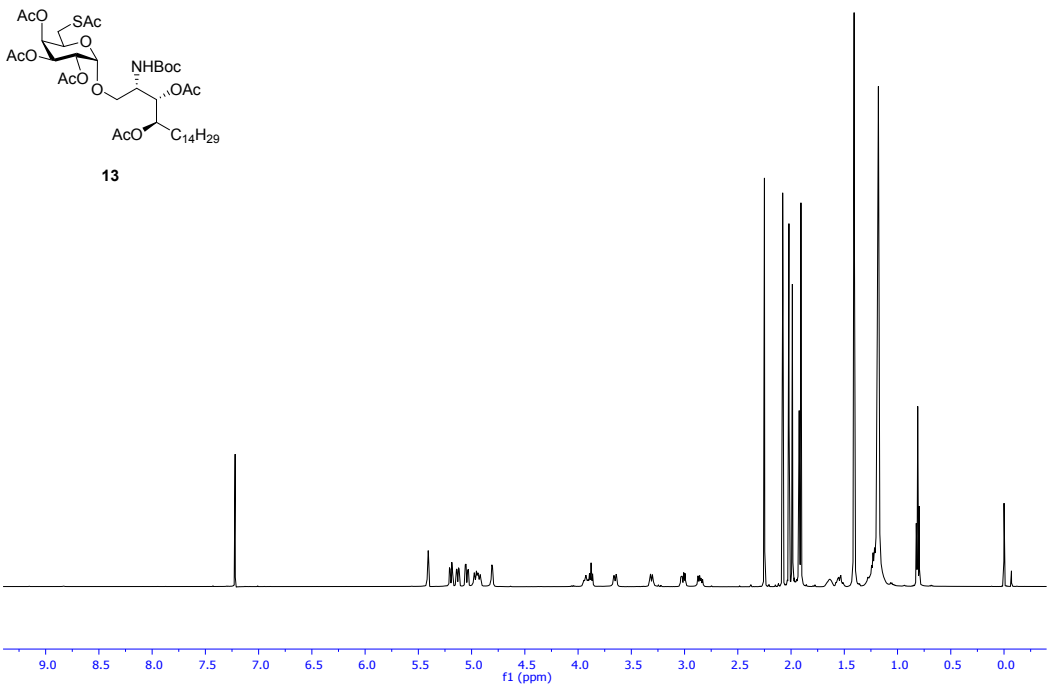
Compound **12** - ^{1}H NMR (500 MHz, $CDCl_3$)



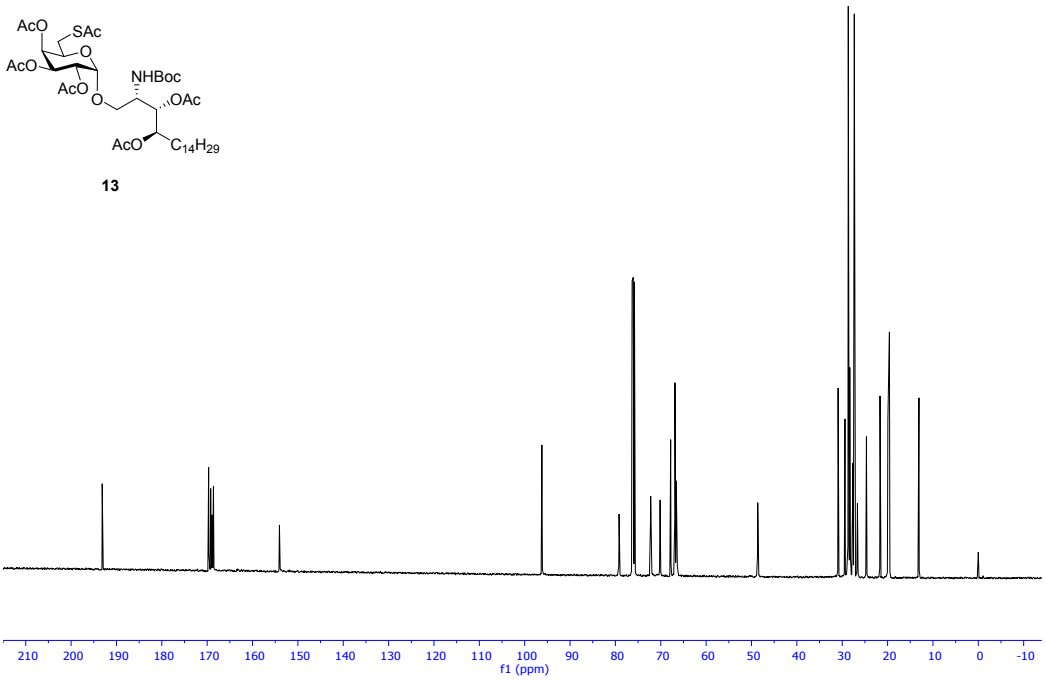
Compound **12** – ^{13}C NMR (126 MHz, $CDCl_3$)



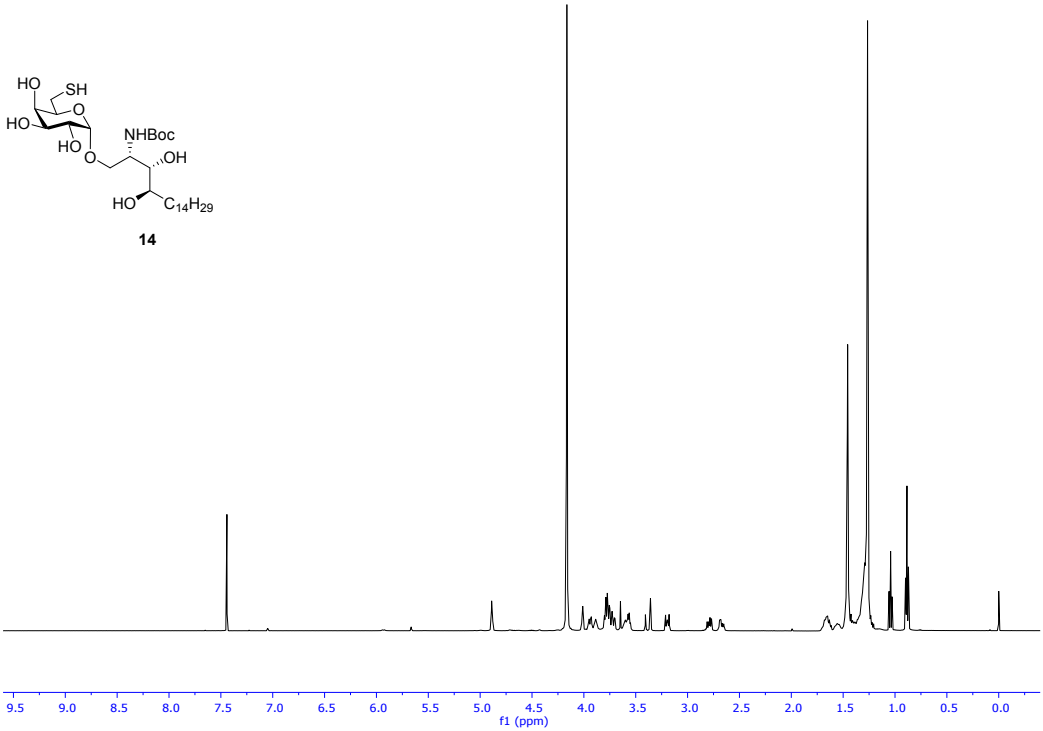
Compound **13** - ^{1}H NMR (500 MHz, $CDCl_3$)



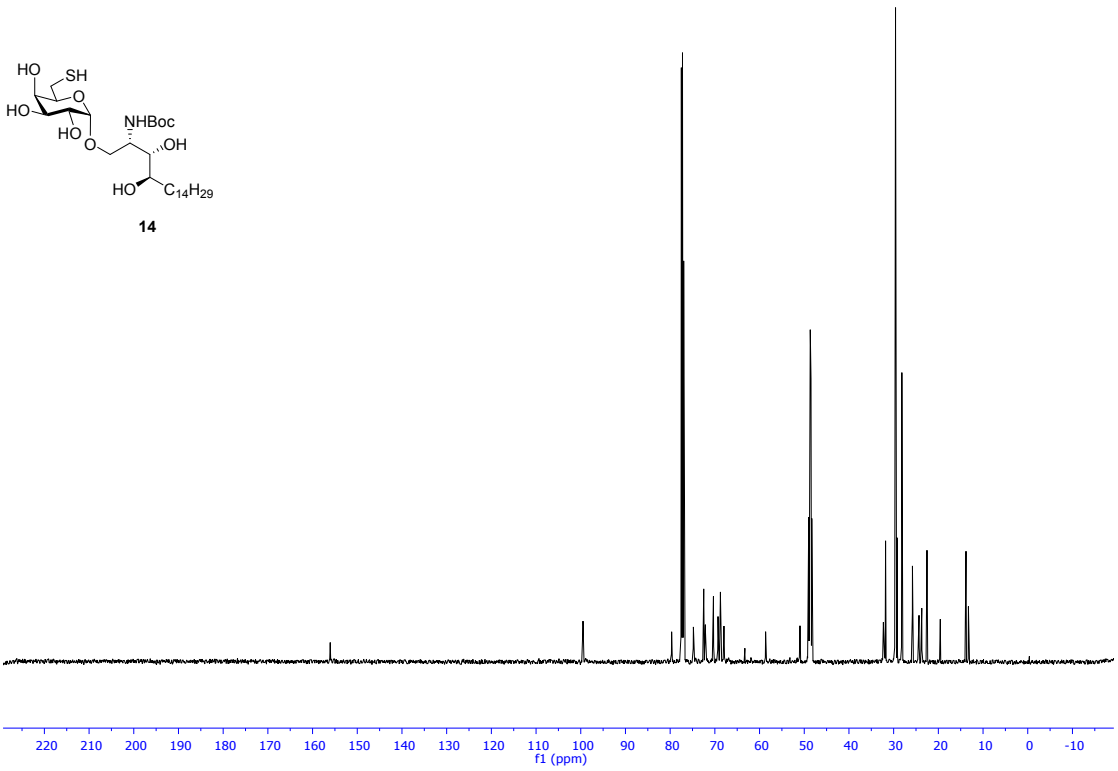
Compound **13** – ^{13}C NMR (126 MHz, $CDCl_3$)



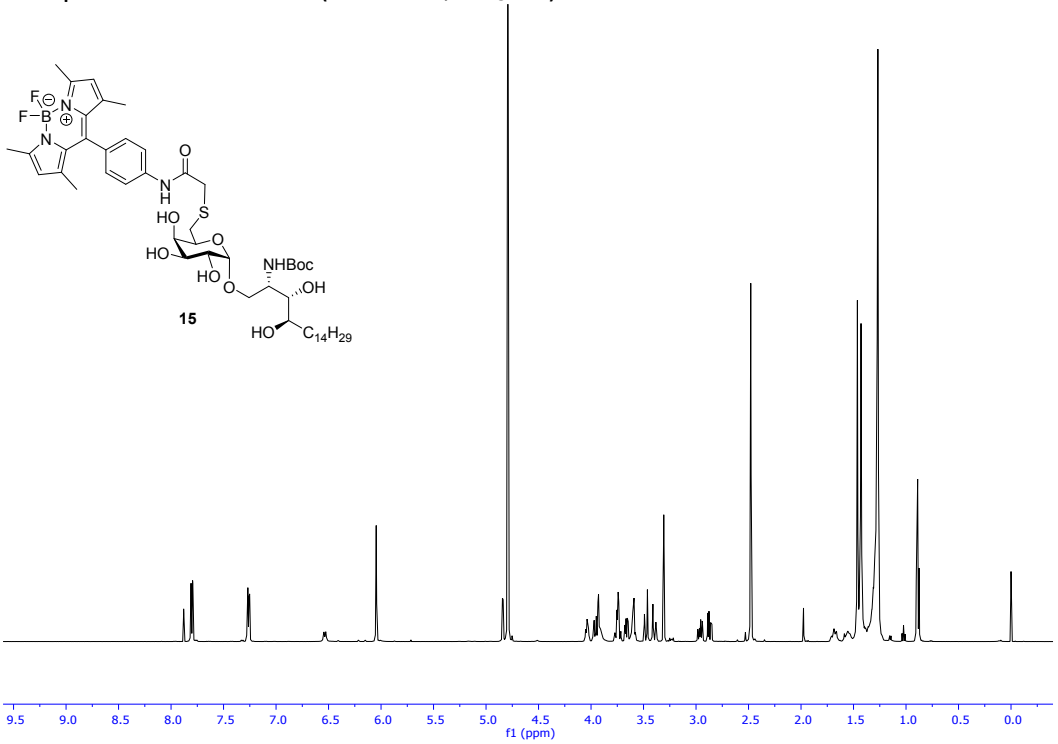
Compound **14** - ^{1}H NMR (500 MHz, 3:1 $CDCl_3/CD_3OD$)



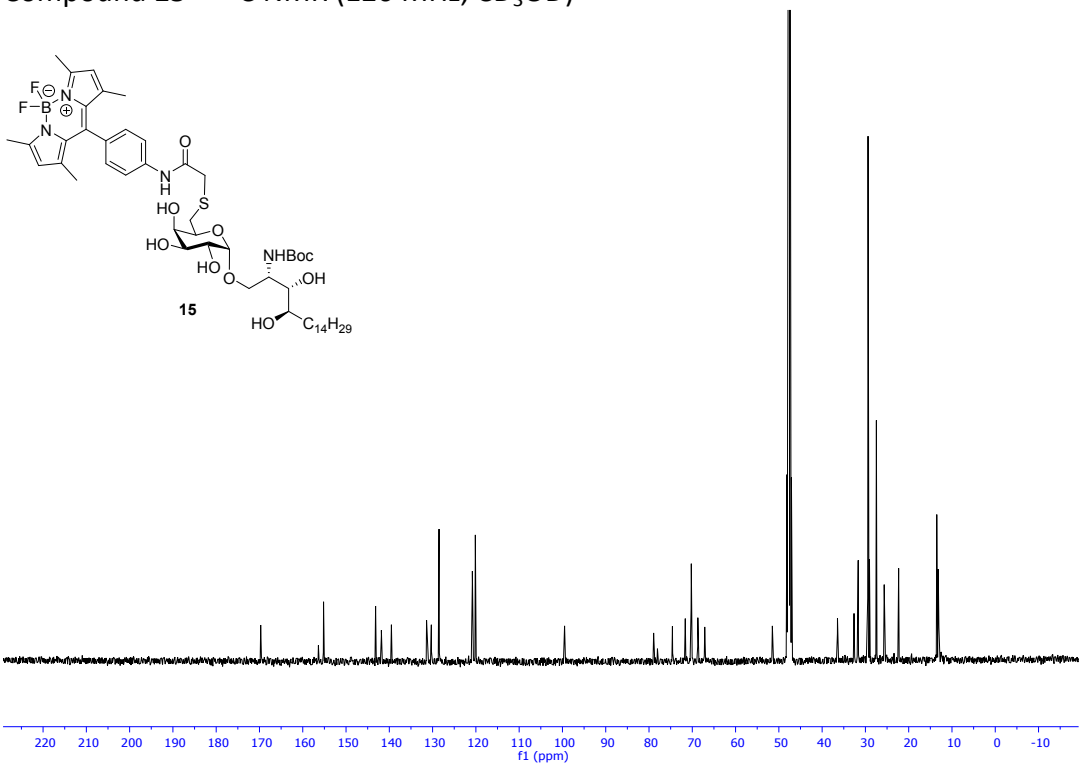
Compound **14** – ^{13}C NMR (126 MHz, 3:1 $CDCl_3/CD_3OD$)



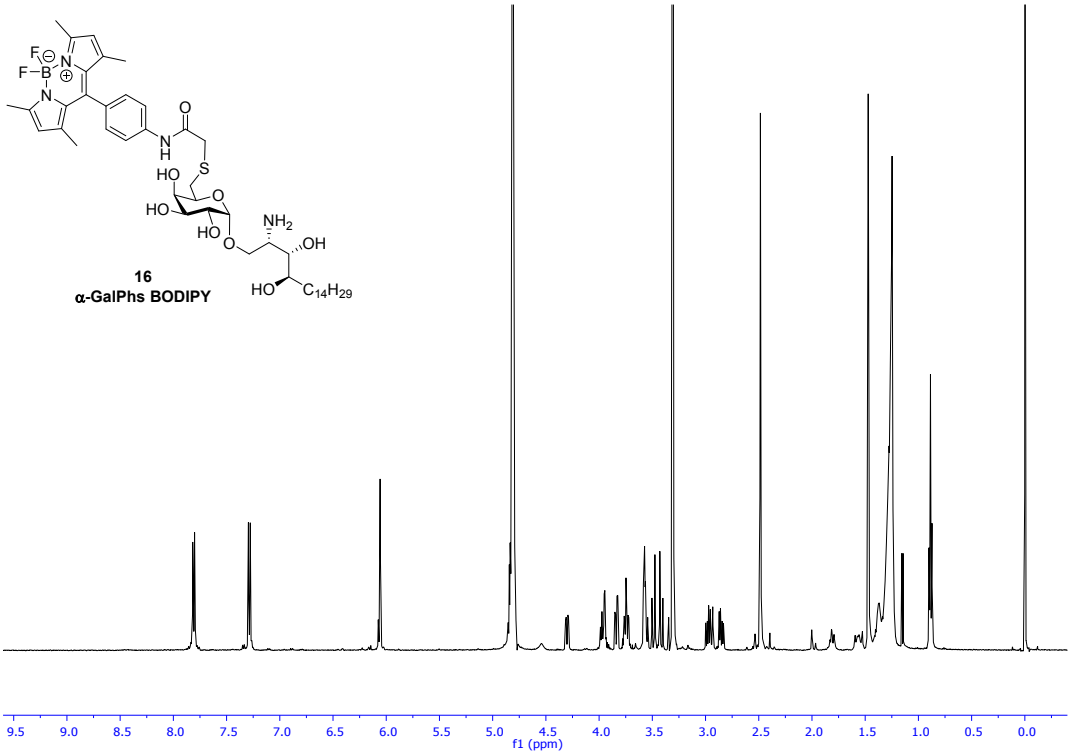
Compound **15** - ^{1}H NMR (500 MHz, CD_3OD)



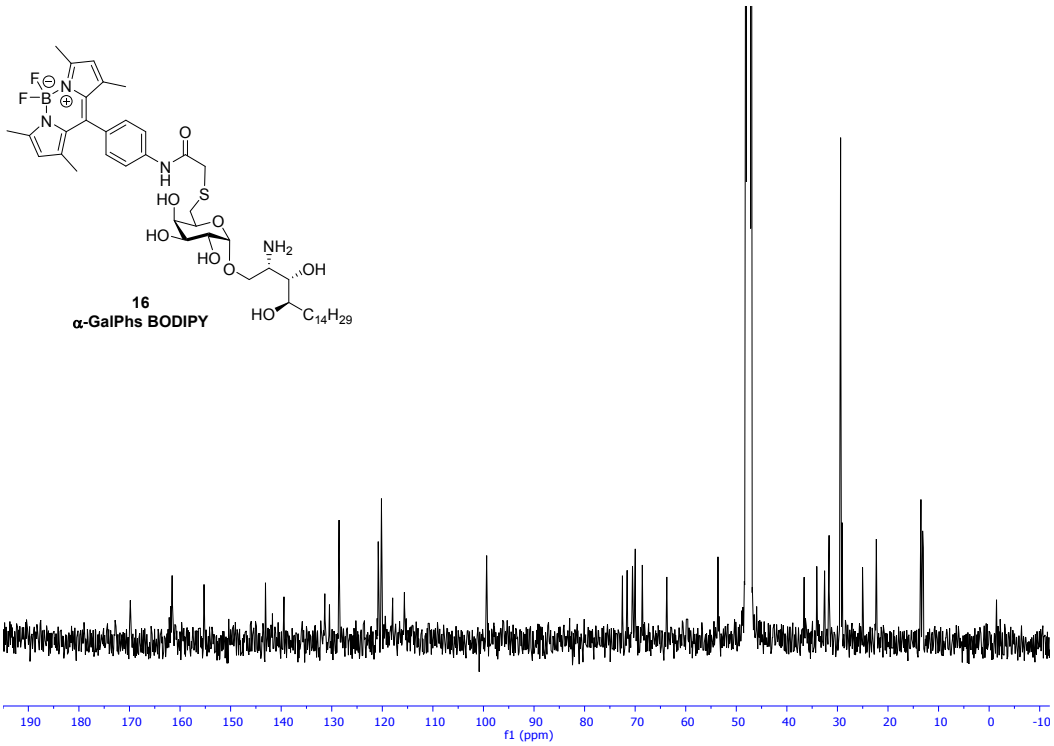
Compound **15** – ^{13}C NMR (126 MHz, CD_3OD)



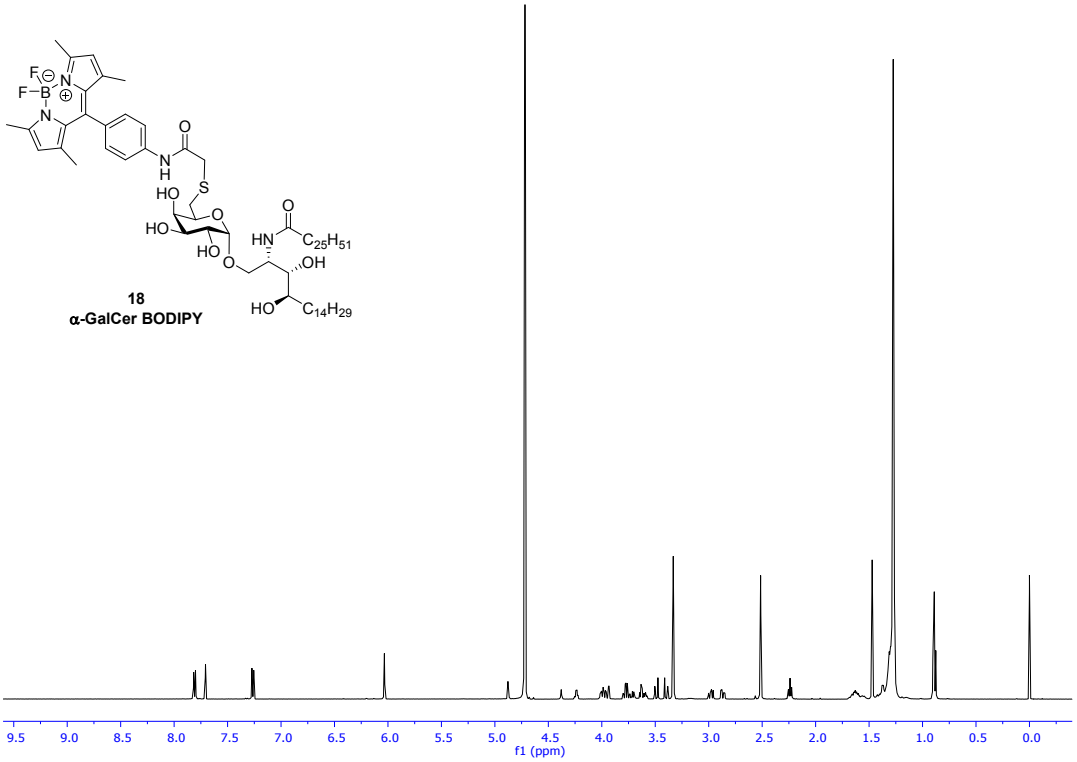
Compound **16** (α-GalPhs BODIPY) - ^{1}H NMR (500 MHz, CD_3OD)



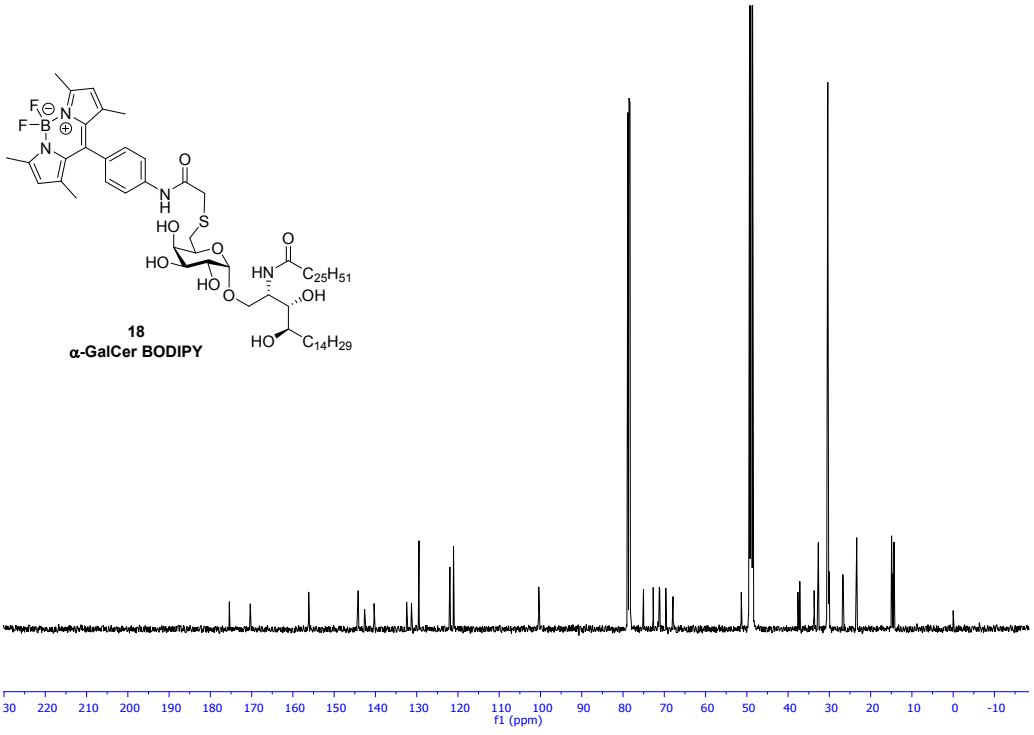
Compound **16** (α-GalPhs BODIPY) – ^{13}C NMR (126 MHz, CD_3OD)



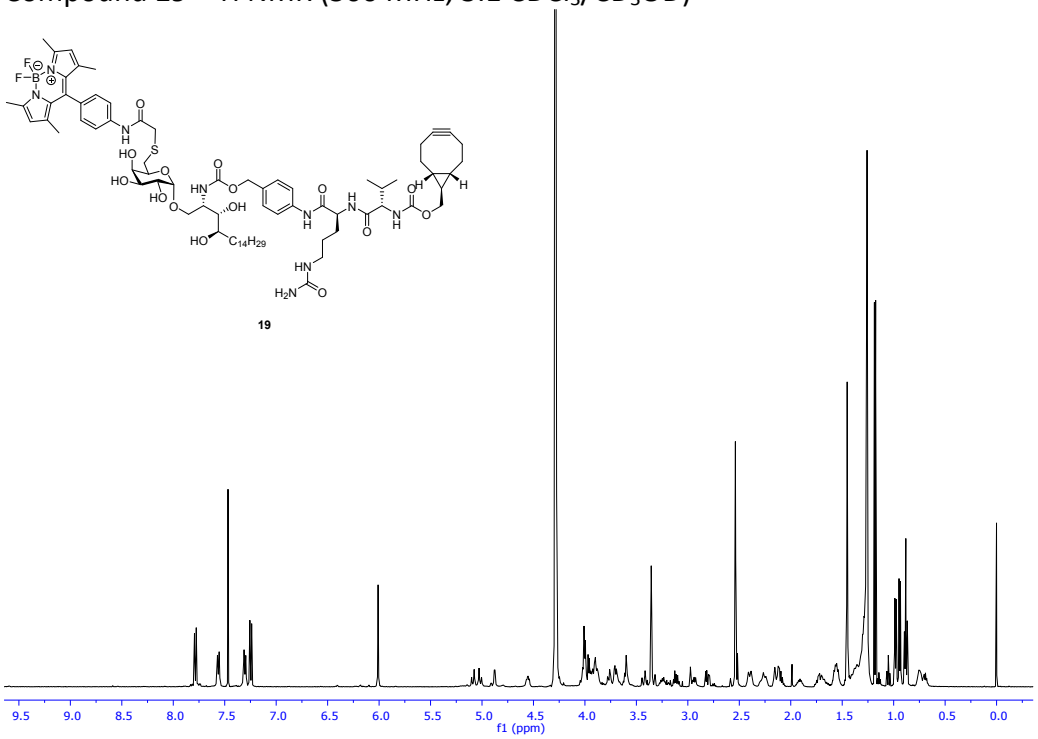
Compound **18** (α-GalCer BODIPY) - ^{1}H NMR (500 MHz, 1:2 $CDCl_3/CD_3OD$)



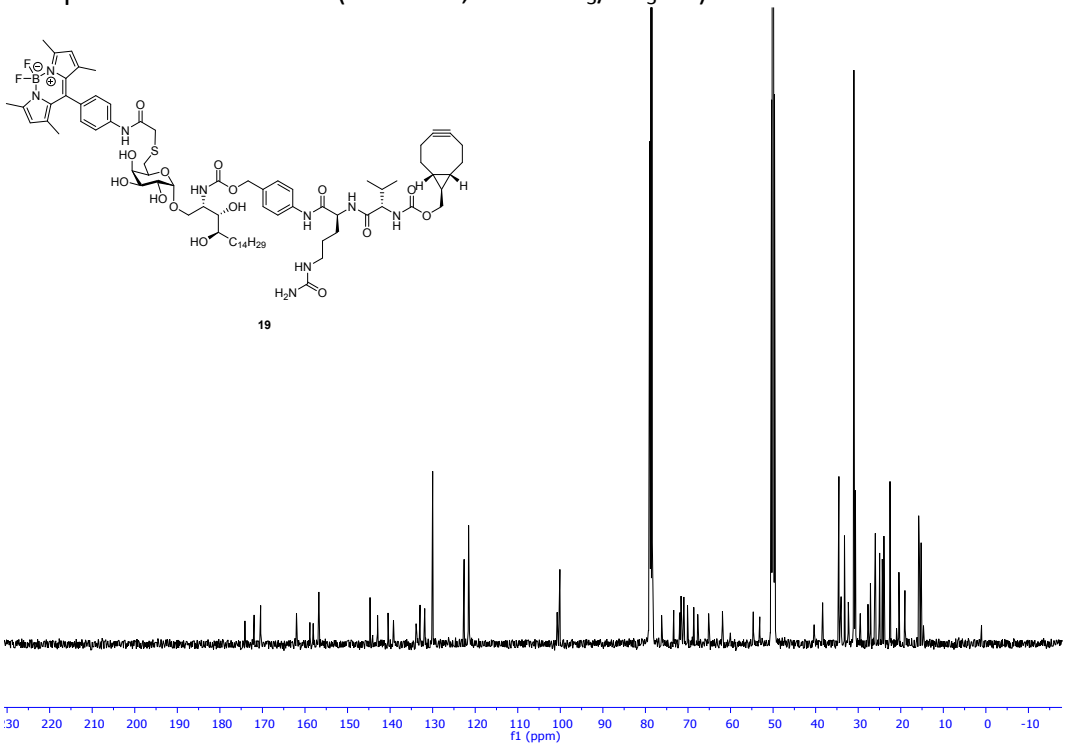
Compound **18** (α-GalCer BODIPY) – ^{13}C NMR (126 MHz, 1:2 $CDCl_3/CD_3OD$)



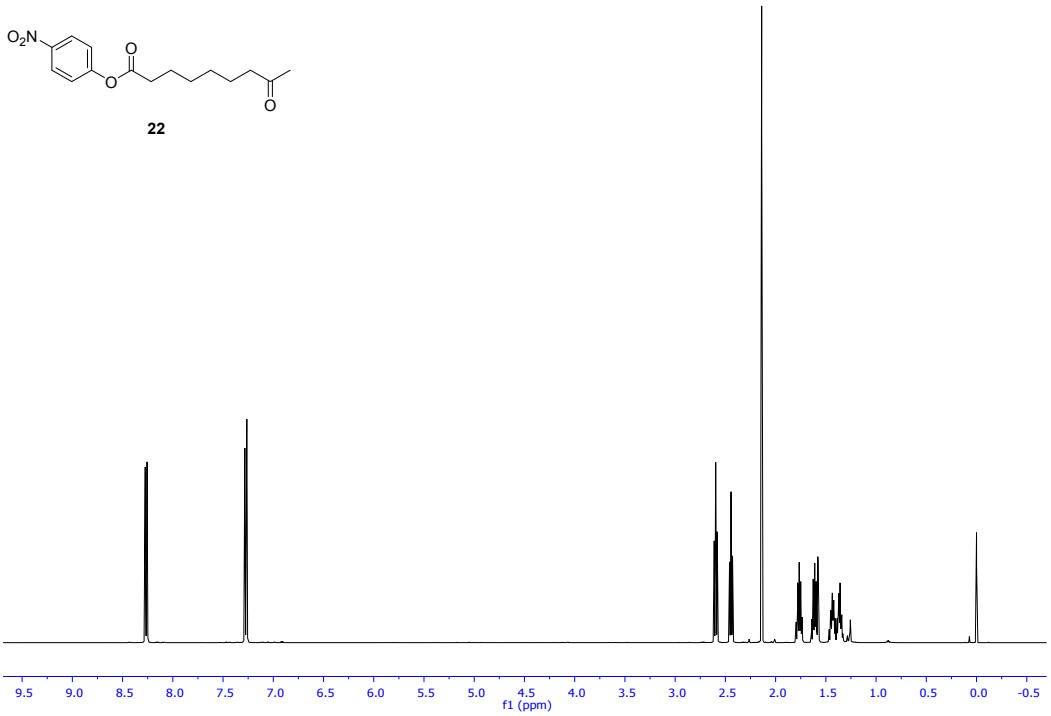
Compound **19** - ^{1}H NMR (500 MHz, 3:1 $CDCl_3/CD_3OD$)



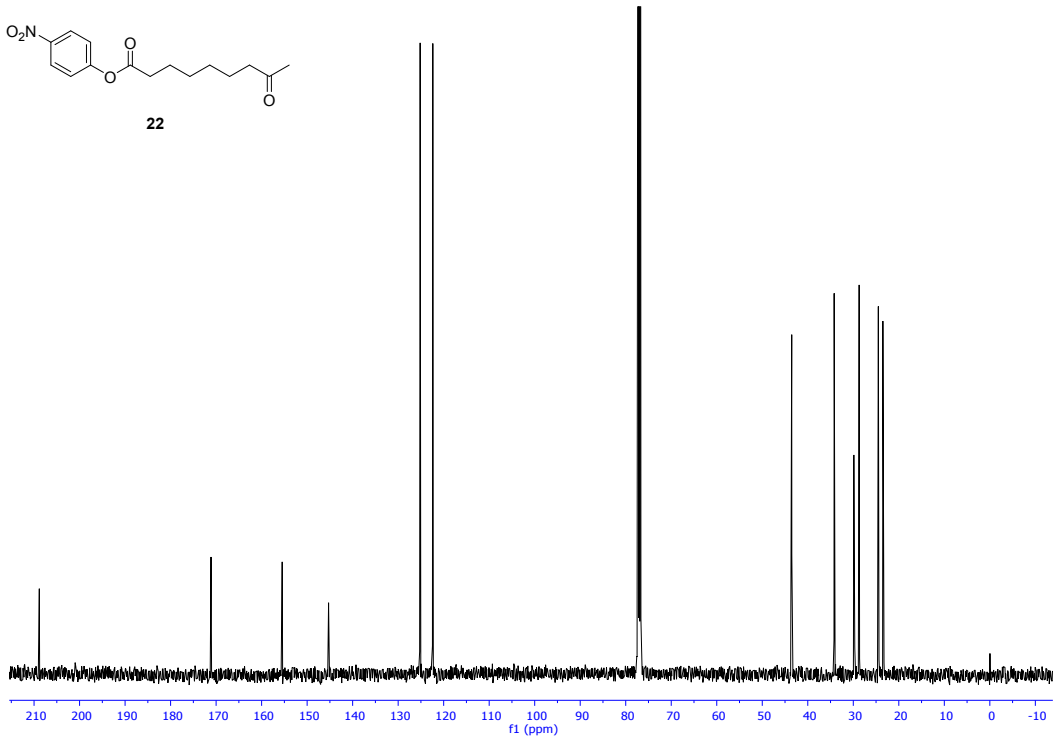
Compound **19** – ^{13}C NMR (126 MHz, 3:1 $CDCl_3/CD_3OD$)



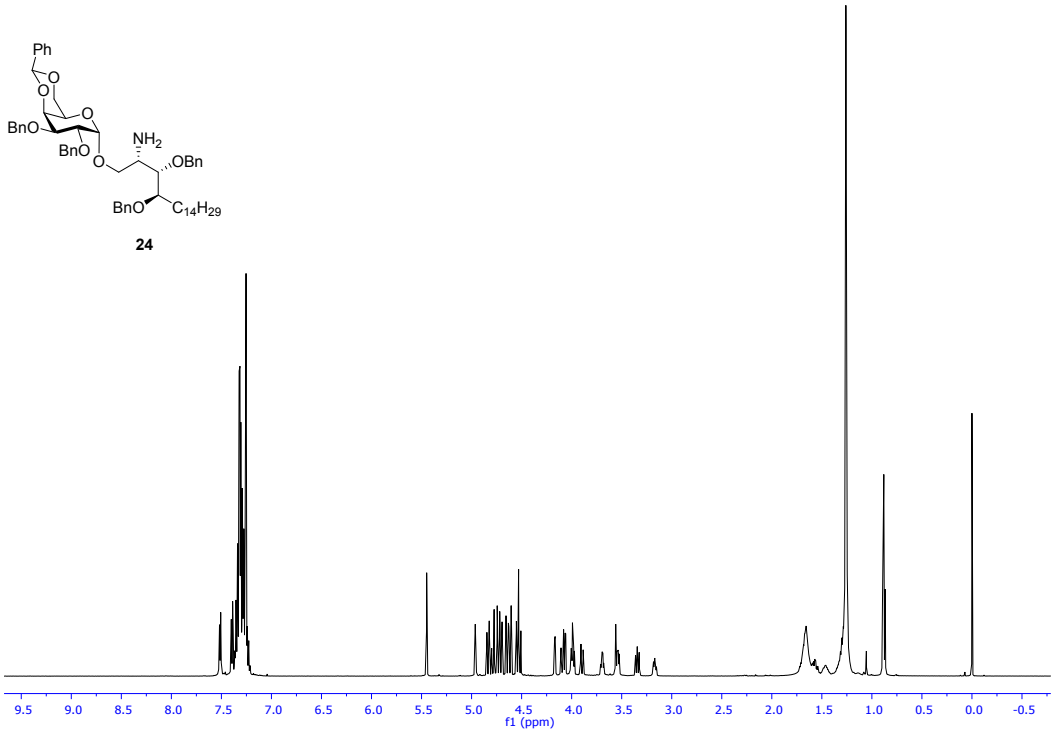
Compound **22** – ^{1}H NMR (500 MHz, $CDCl_3$)



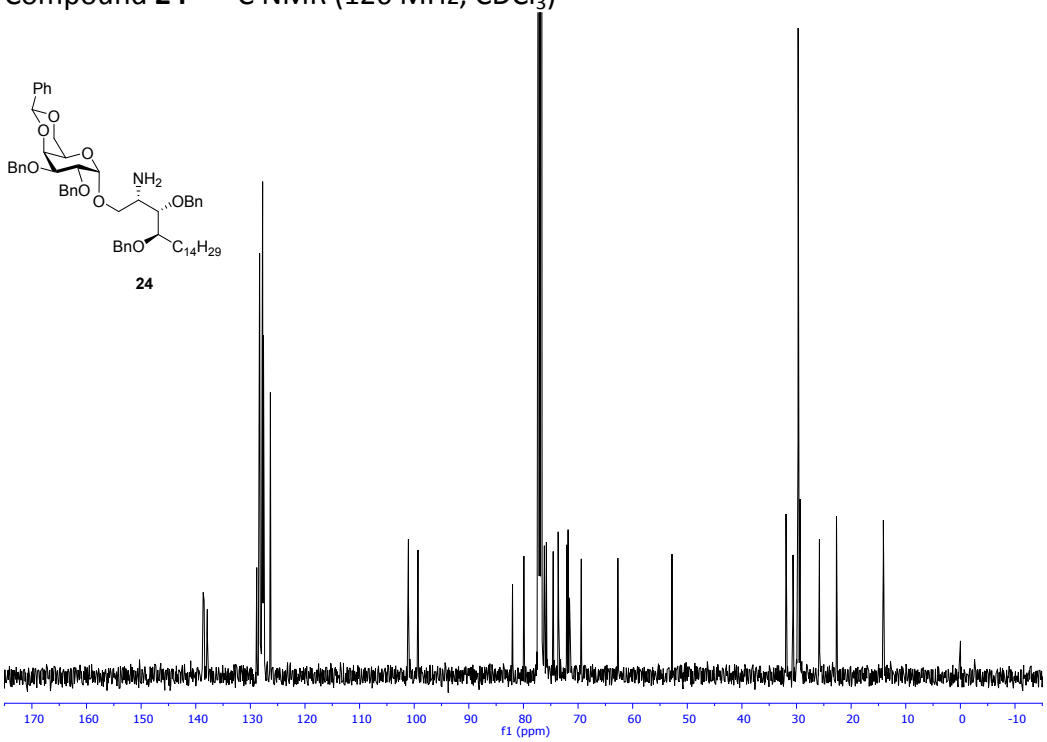
Compound **22** – ^{13}C NMR (126 MHz, $CDCl_3$)



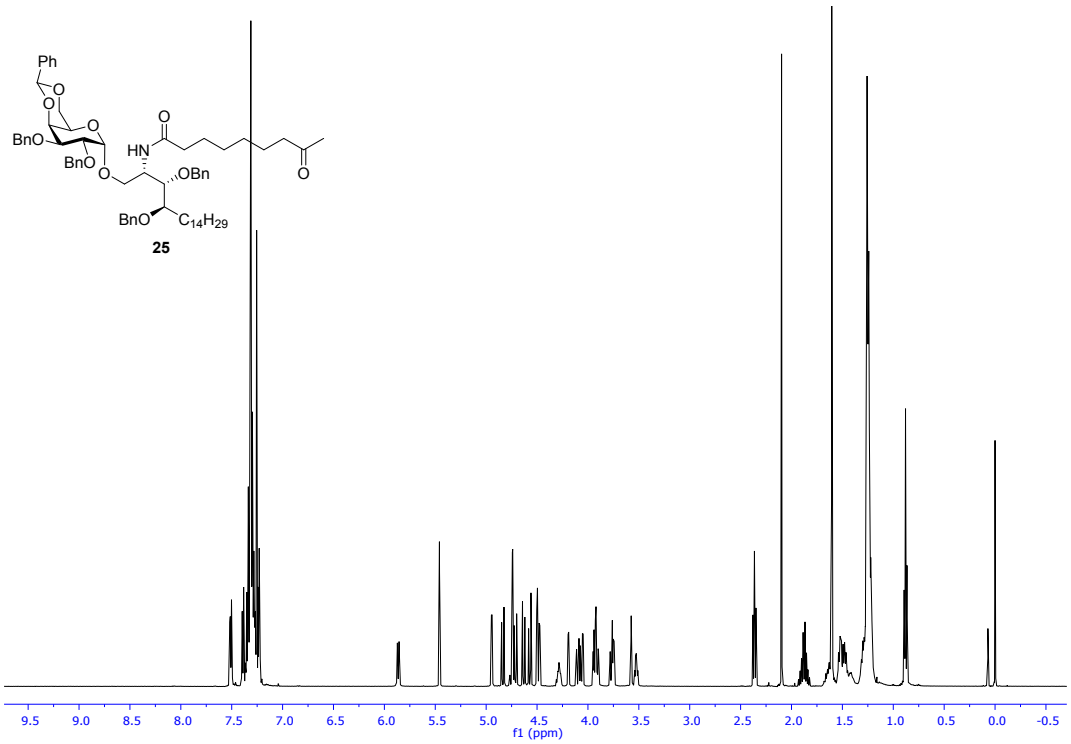
Compound **24** – ^{1}H NMR (500 MHz, $CDCl_3$)



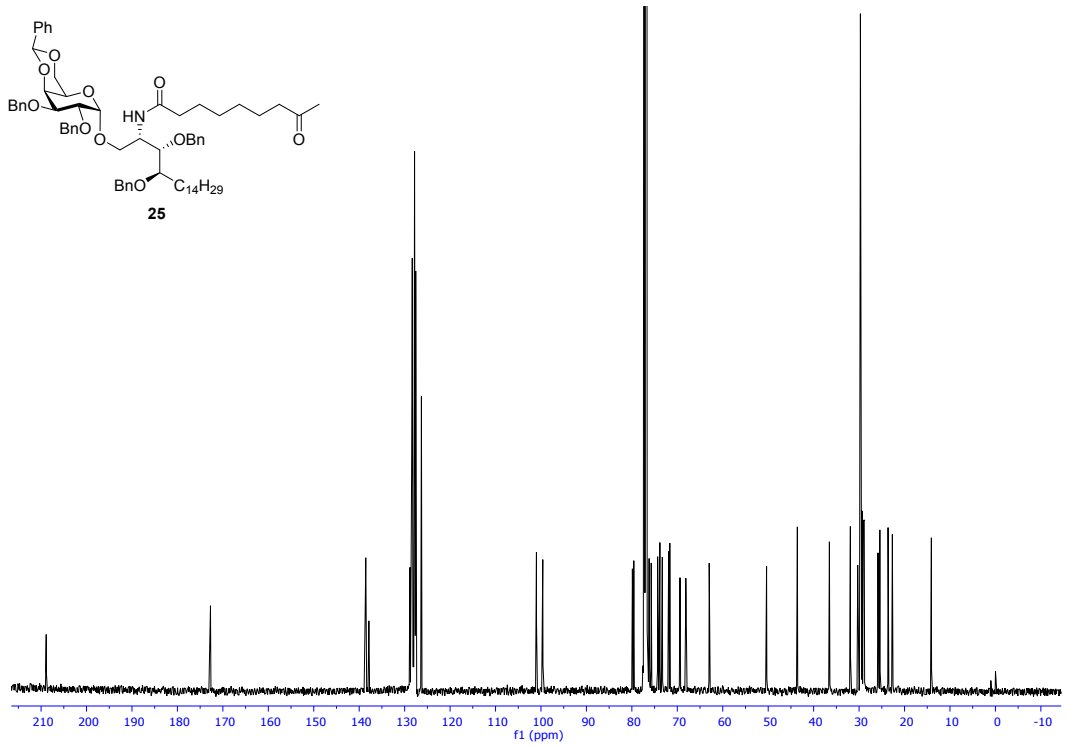
Compound **24** – ^{13}C NMR (126 MHz, $CDCl_3$)



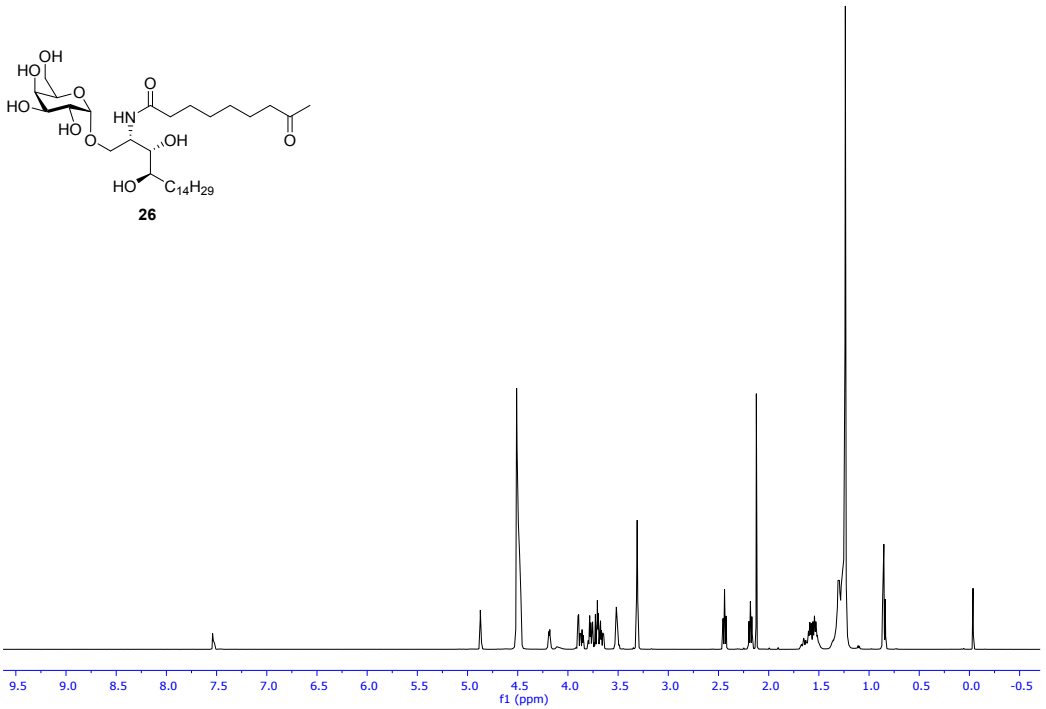
Compound **25** – ^{1}H NMR (500 MHz, $CDCl_3$)



Compound **25** – ^{13}C NMR (126 MHz, $CDCl_3$)

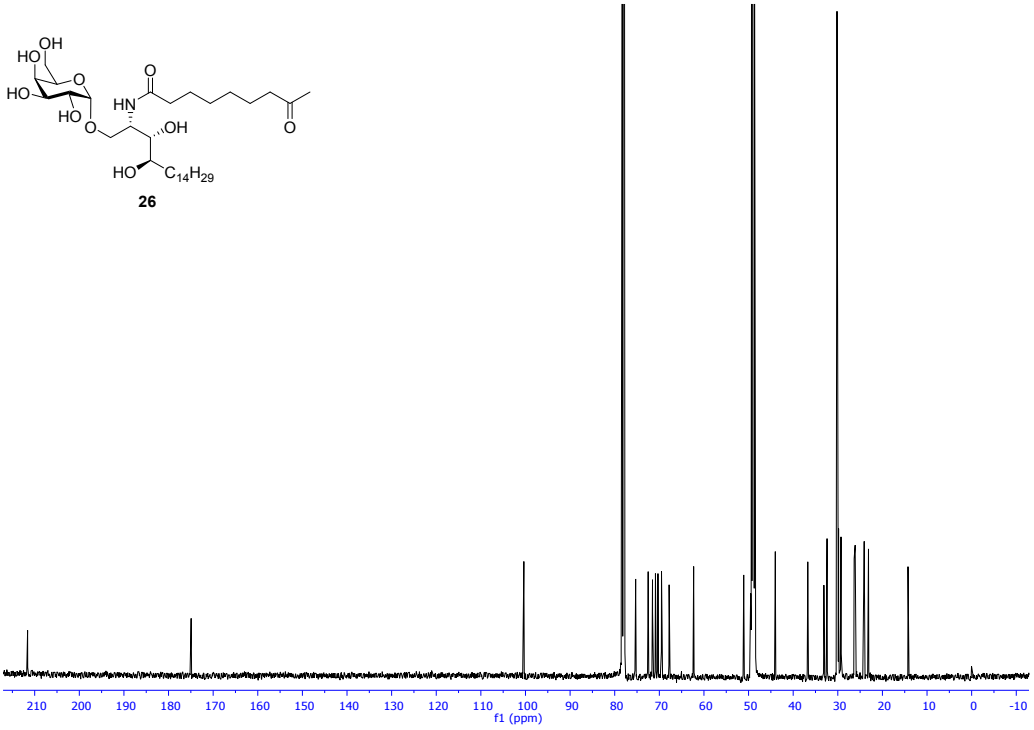


Compound **26** – ^{1}H NMR (500 MHz, 1:1 $CDCl_3/CD_3OD$)



26

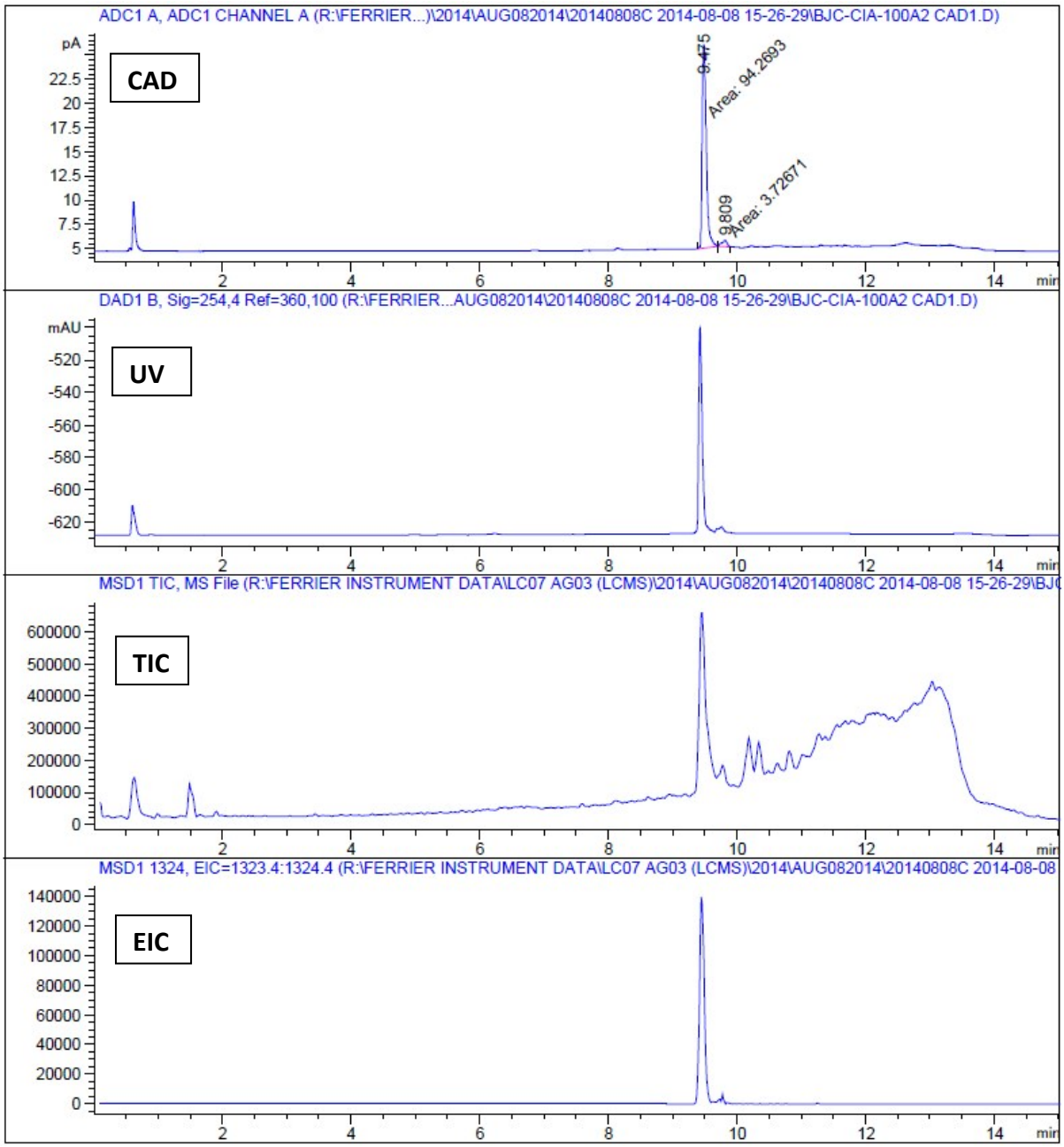
Compound **26** – ^{13}C NMR (126 MHz, 1:1 $CDCl_3/CD_3OD$)



26

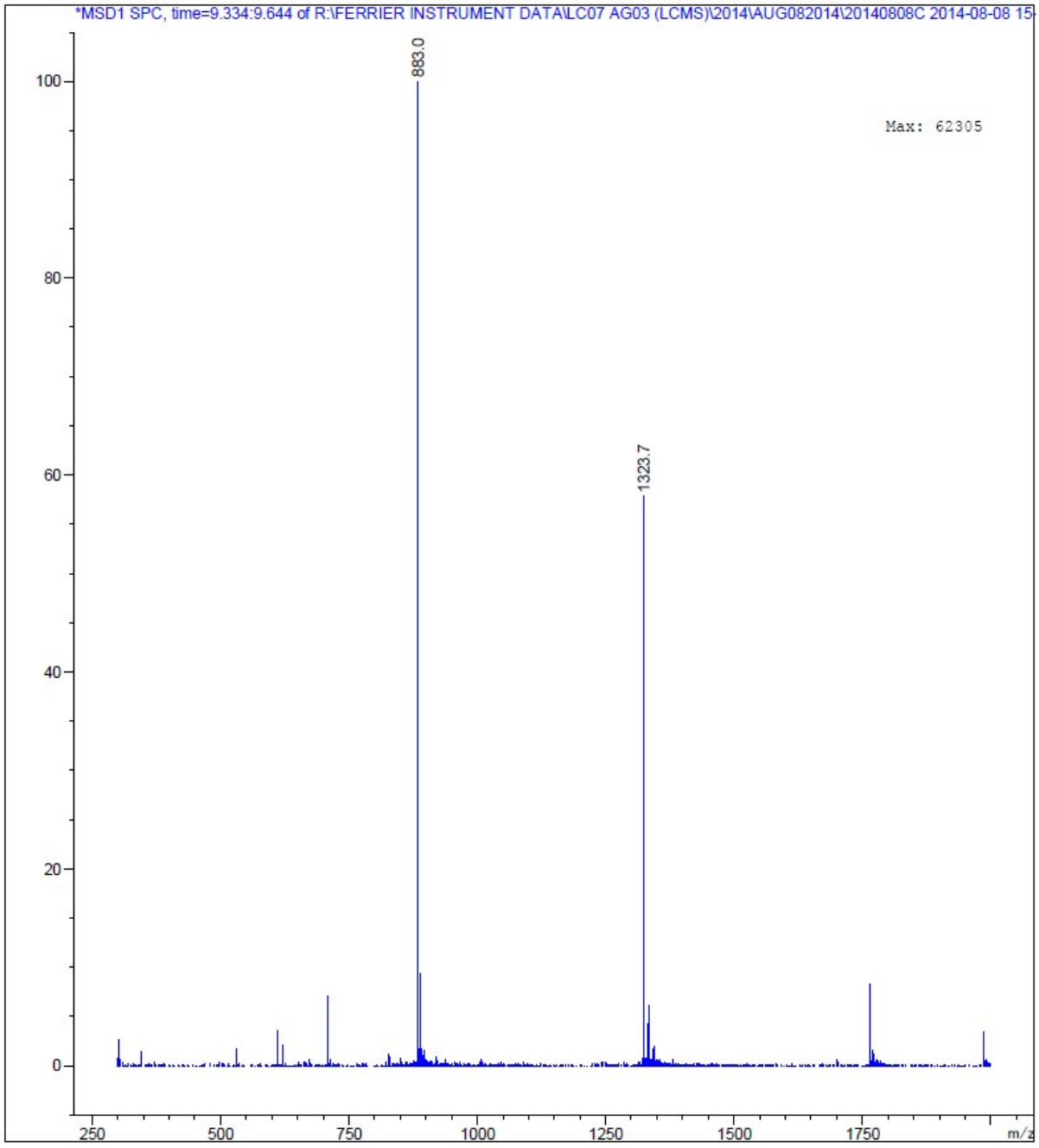
Compound **6** (α-GalPhs-OVA^{CD8}) – HPLC-CAD/LCMS

Column: Agilent Poroshell 120 EC-C18 2.7 µm, 3 x 50 mm.
Mobile Phase A: Water
Mobile Phase B: Methanol + 0.05% TFA
Gradient (A:B): T0 = 95:5, T10 = 0:100, T12 = 0:100, T13 = 95:5, T15 = 95:5
Flow: 0.5 mL/min
Temperature: 40 °C



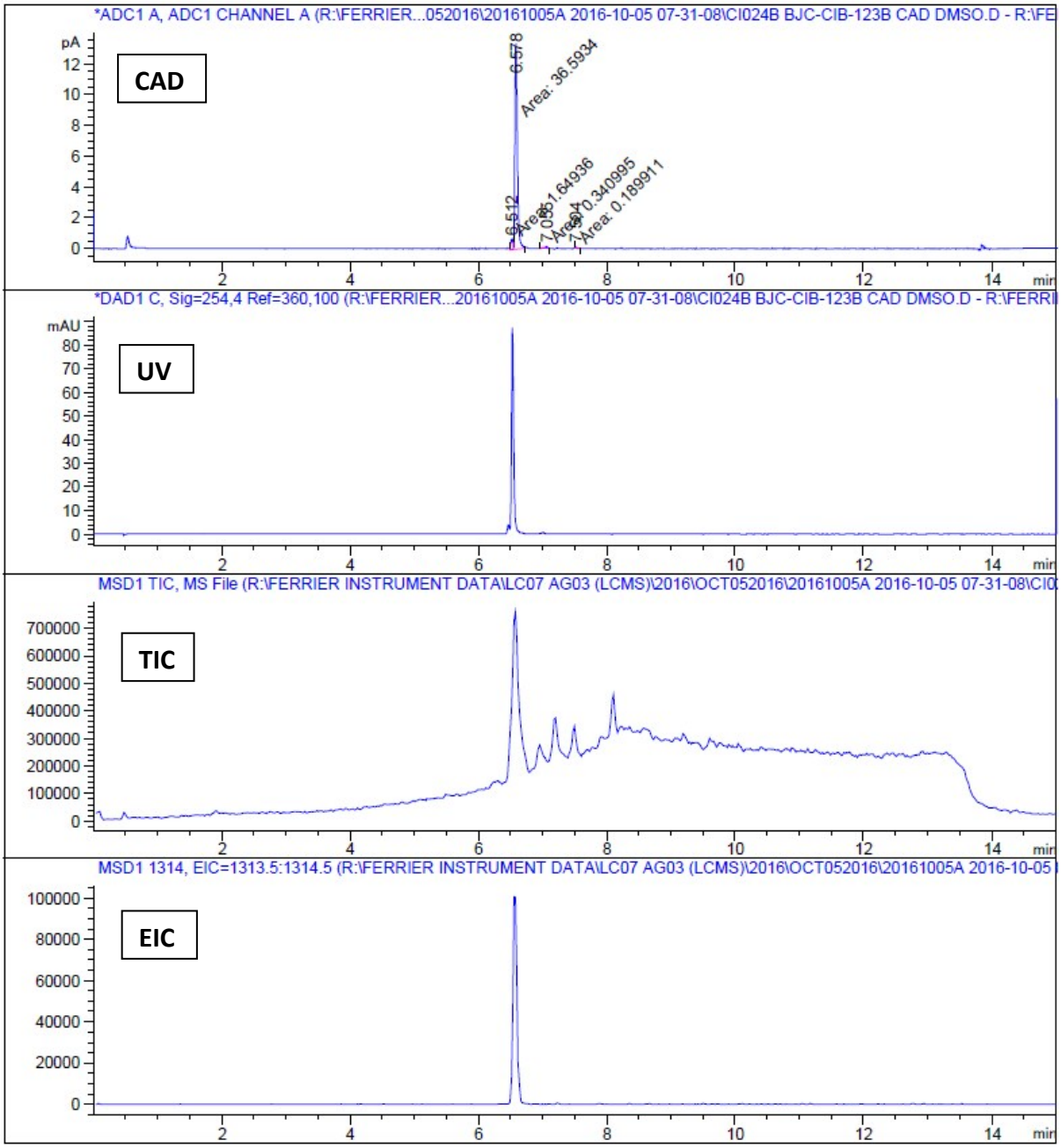
Extracted MS from TIC

	$[M+2H]^{2+}$	$[M+3H]^{3+}$
Calcd avg Mw	1323.8	882.8



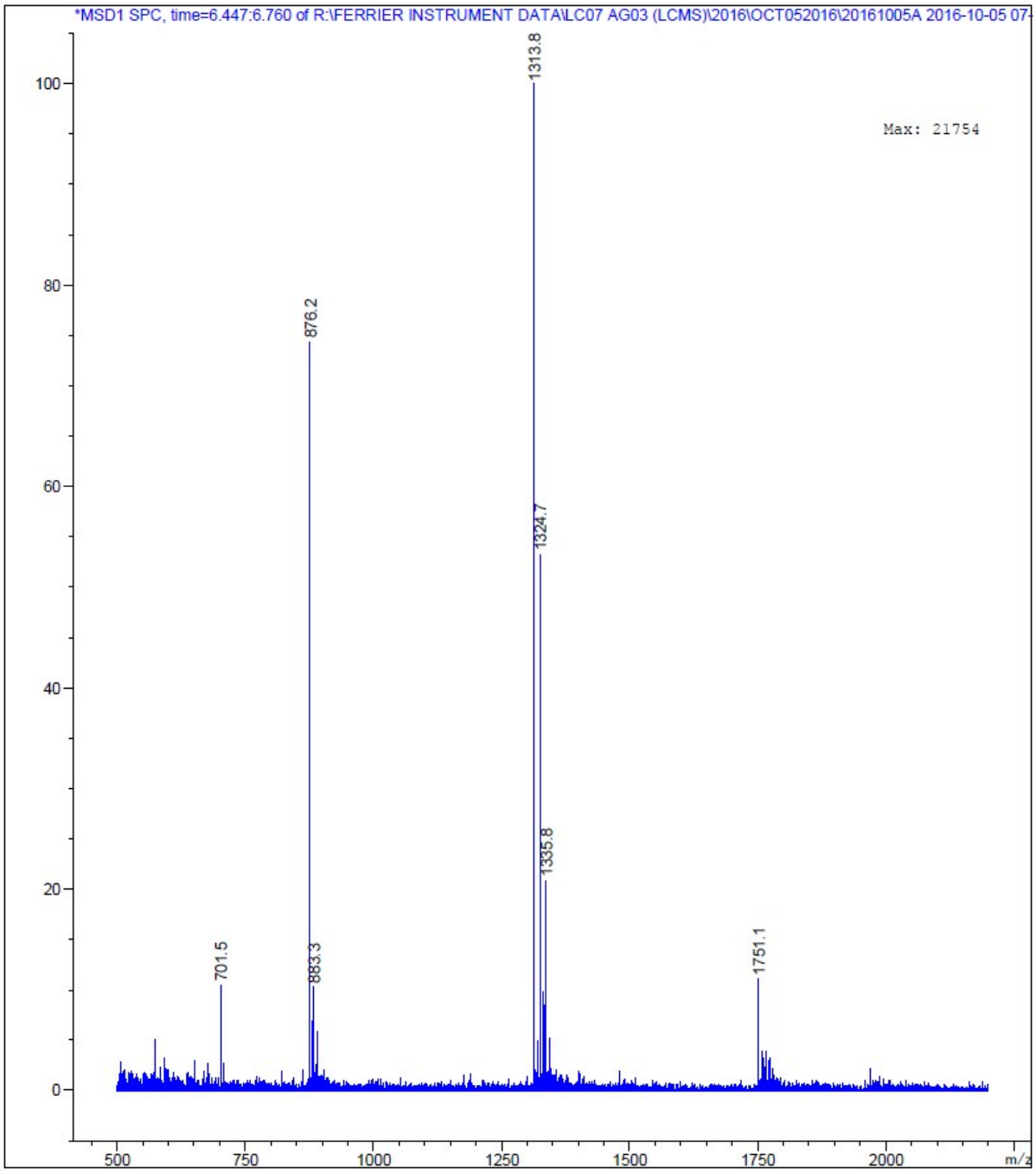
Compound **7** (α-GalPhs-CMV^{CD8}) – HPLC-CAD/LCMS

Column: Phenomenex Kinetex 2.6 µm, 100 Å, 3 x 50 mm.
Mobile Phase A: Water + 0.05% TFA
Mobile Phase B: Methanol + 0.05% TFA
Gradient (A:B): T0 = 70:30, T7 = 0:100, T12 = 0:100, T13 = 70:30, T15 = 70:30
Flow: 0.5 mL/min
Temperature: 40 °C



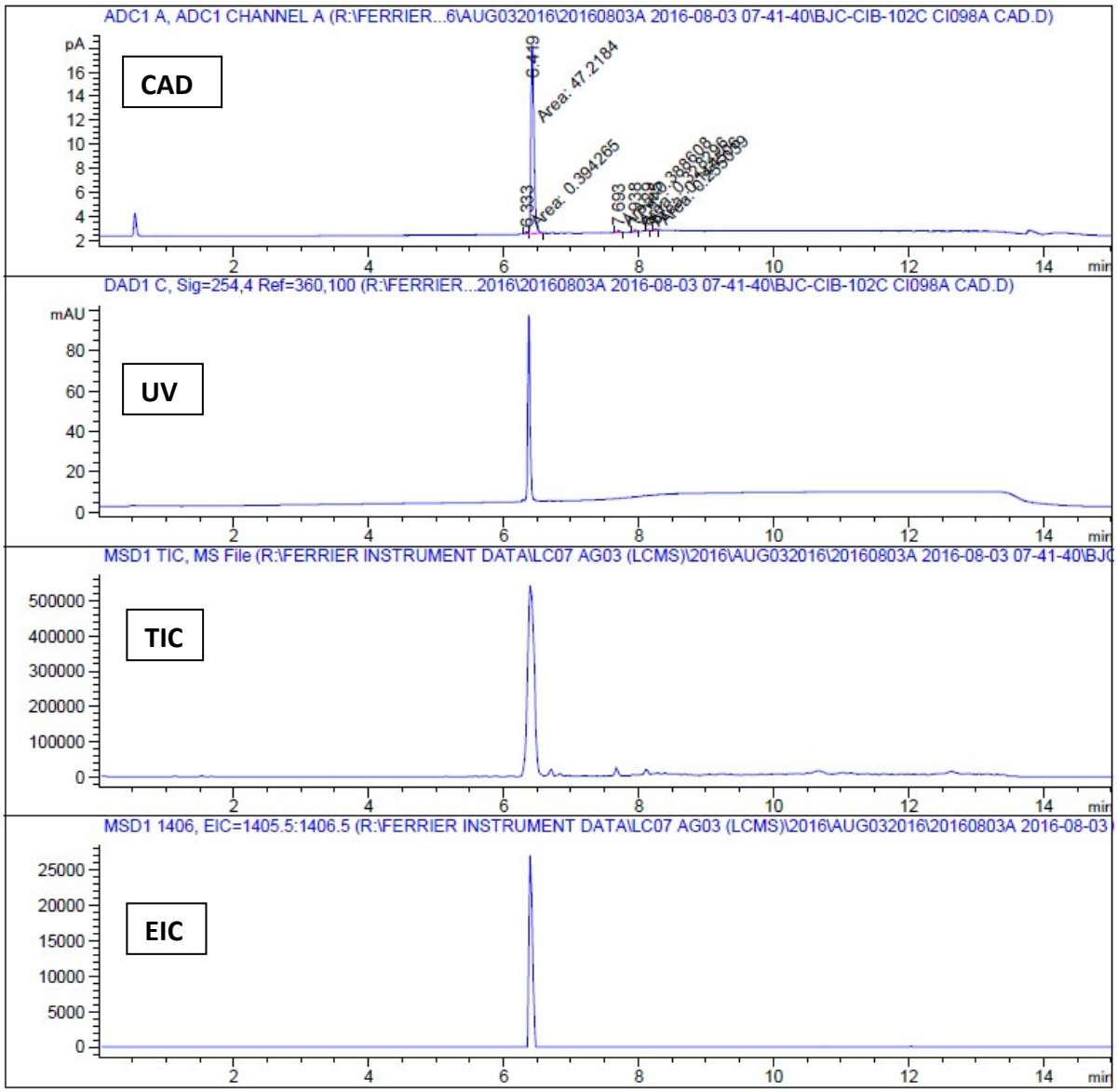
Extracted MS from TIC

	$[2M+3H]^{3+}$	$[M+2Na]^{2+}$	$[M+H+Na]^{2+}$	$[M+2H]^{2+}$	$[M+3H]^{3+}$
Calcd avg Mw	1751.3	1335.8	1324.8	1313.8	876.2



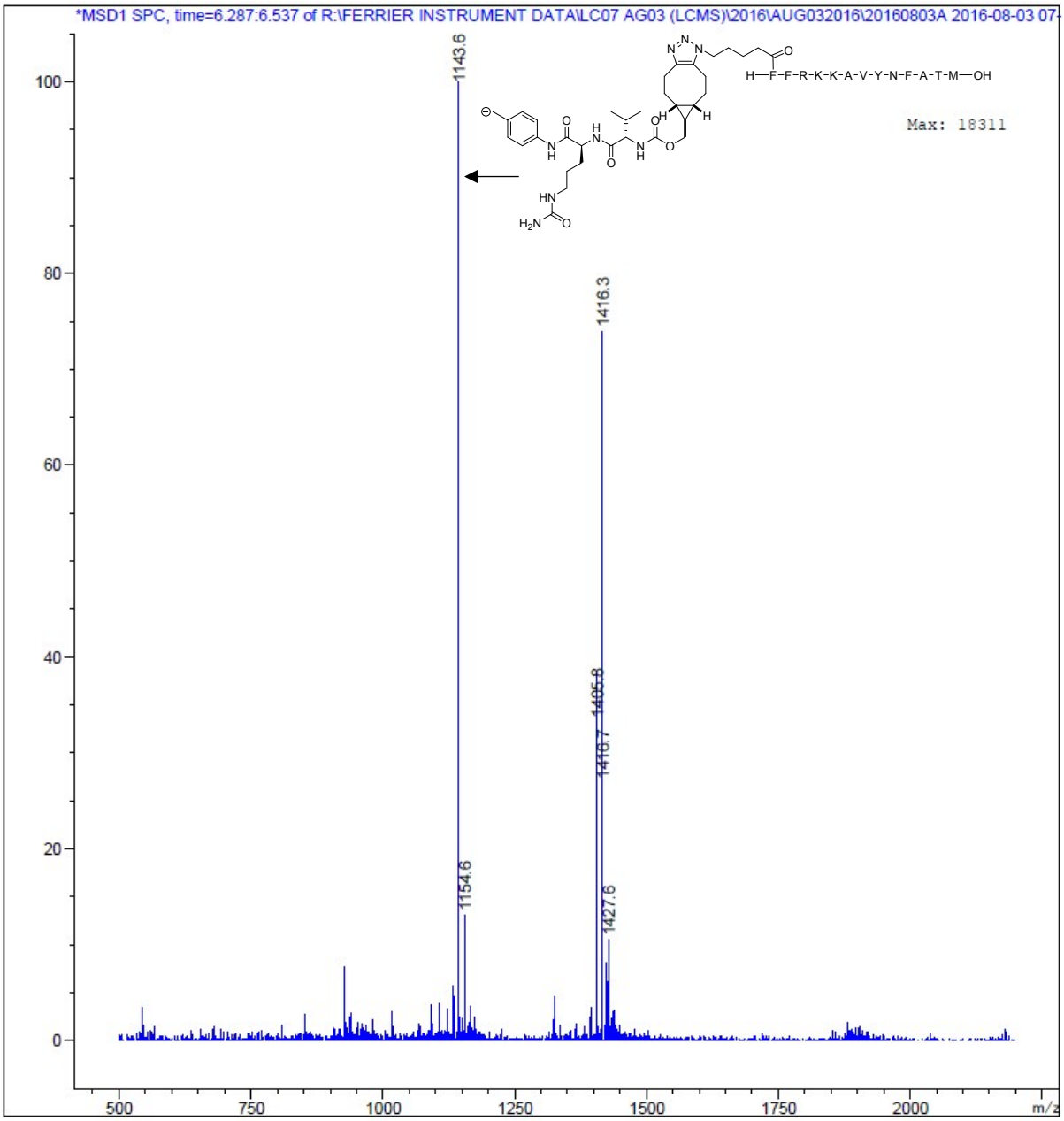
Compound **8** (α-GalPhs-LCMV^{CD8}) – HPLC-CAD/LCMS

Column: Phenomenex Kinetex 2.6 µm, 100 Å, 3 x 50 mm.
Mobile Phase A: Water + 0.05% TFA
Mobile Phase B: Methanol + 0.05% TFA
Gradient (A:B): T0 = 70:30, T7 = 0:100, T12 = 0:100, T13 = 70:30, T15 = 70:30
Flow: 0.5 mL/min
Temperature: 40 °C



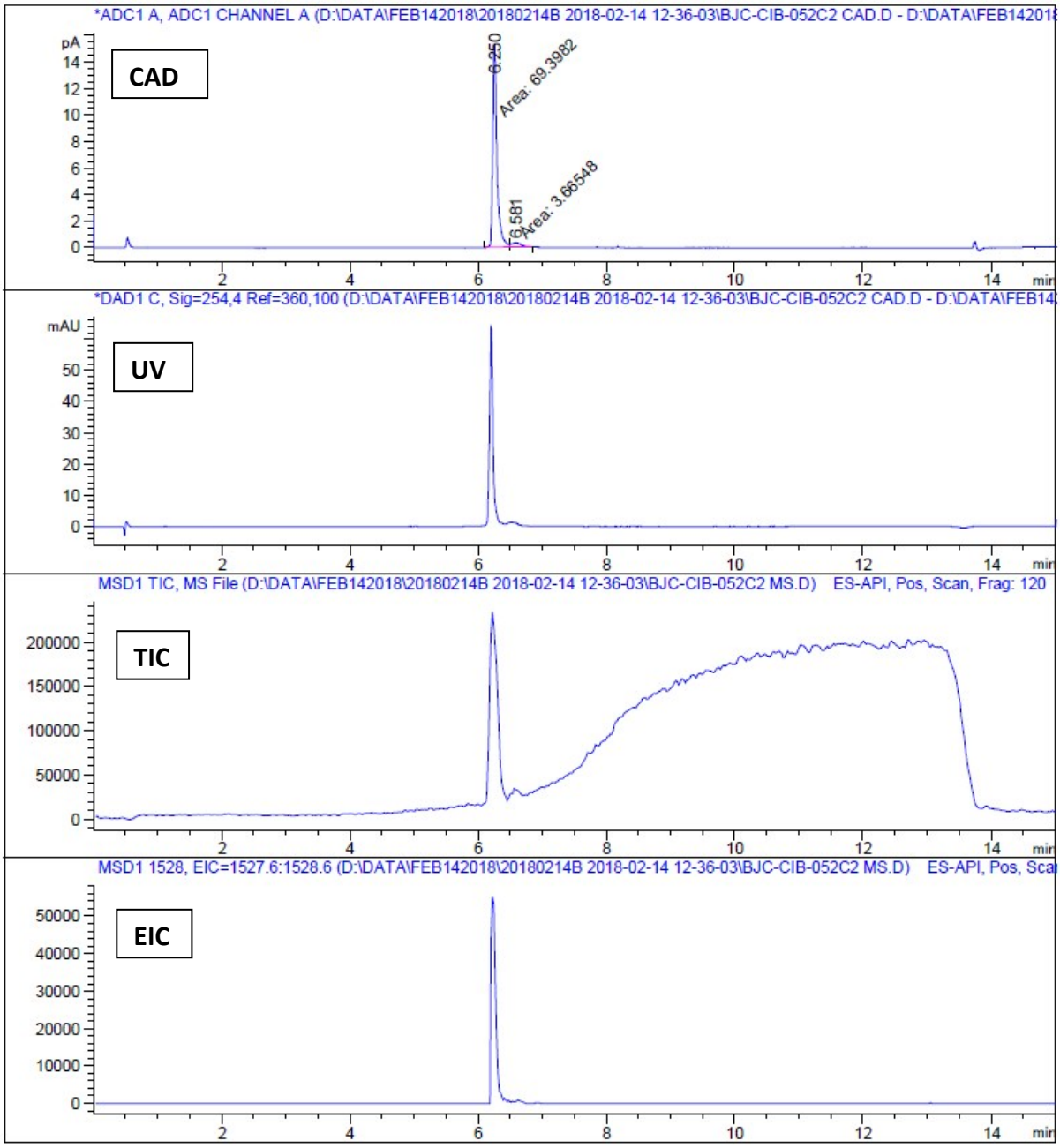
Extracted MS from TIC

	$[M+H+Na]^{2+}$	$[M+2H]^{2+}$	$[M–(linker+peptide)+H]^{2+}$
Calcd avg Mw	1416.3	1405.8	1143.6



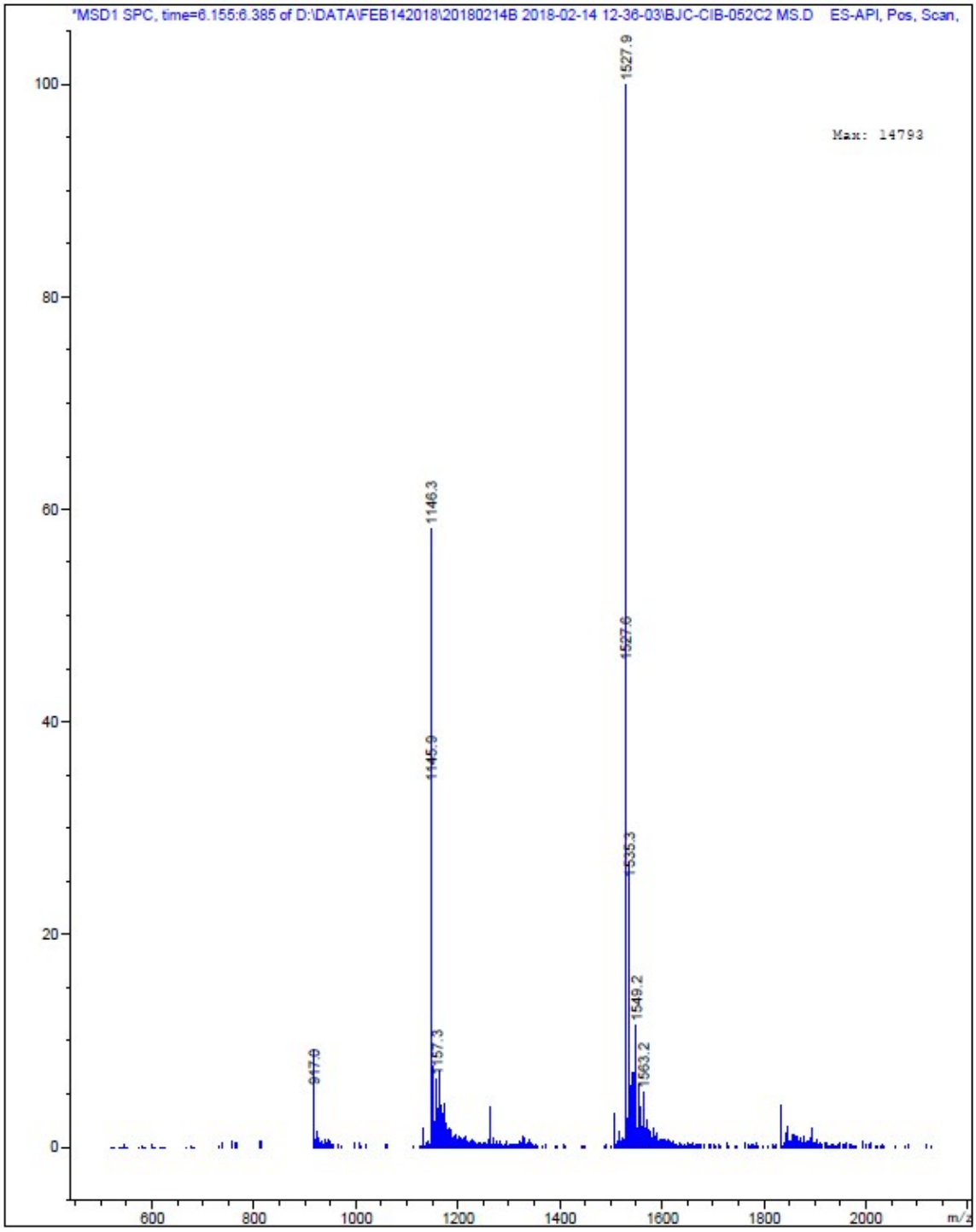
Compound **9** (α-GalPhs-TRP2$^{CD4/CD8}$) – HPLC-CAD/LCMS

Column: Phenomenex Kinetex 2.6 µm, 100 Å, 3 x 50 mm.
Mobile Phase A: Water + 0.05% TFA
Mobile Phase B: Methanol + 0.05% TFA
Gradient (A:B): T0 = 70:30, T7 = 0:100, T12 = 0:100, T13 = 70:30, T15 = 70:30
Flow: 0.5 mL/min
Temperature: 40 °C



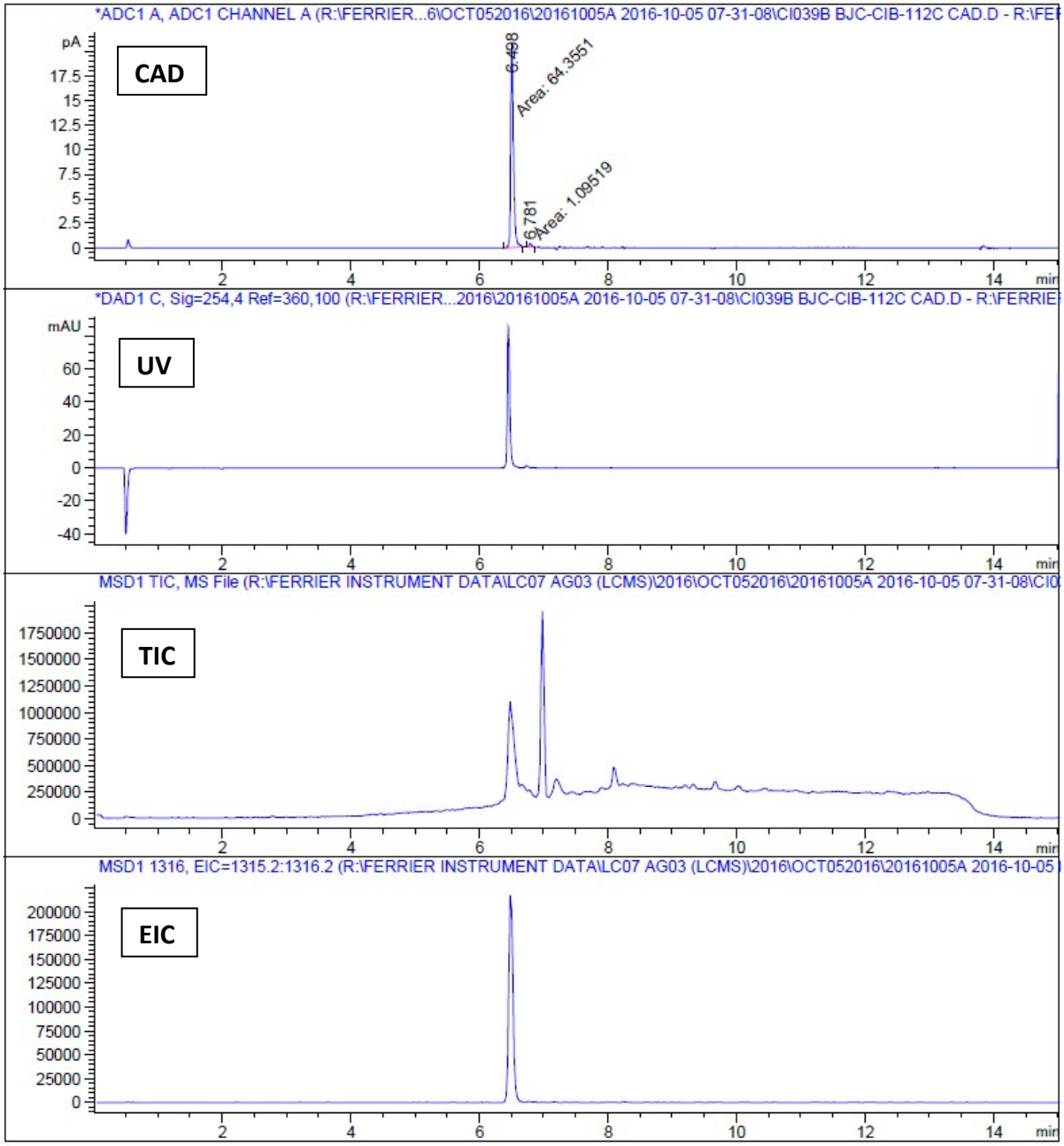
Extracted MS from TIC

	$[M+3H]^{3+}$	$[M+4H]^{4+}$
Calcd avg Mw	1528.1	1146.3



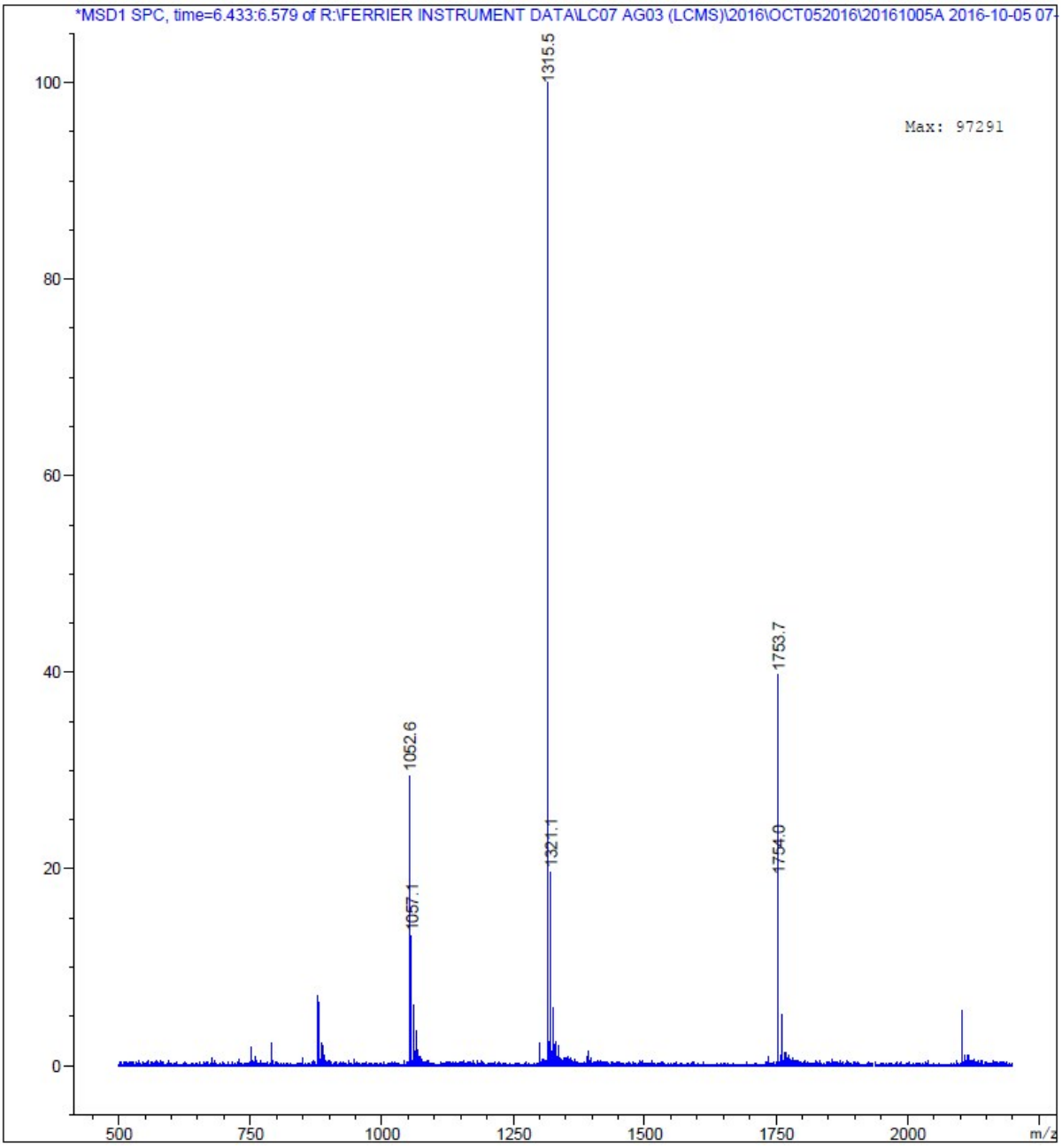
Compound **10** (α-GalPhs-OVA$^{CD4/CD8}$) – HPLC-CAD/LCMS

Column: Phenomenex Kinetex 2.6 µm, 100 Å, 3 x 50 mm.
Mobile Phase A: Water + 0.05% TFA
Mobile Phase B: Methanol + 0.05% TFA
Gradient (A:B): T0 = 70:30, T7 = 0:100, T12 = 0:100, T13 = 70:30, T15 = 70:30
Flow: 0.5 mL/min
Temperature: 40 °C



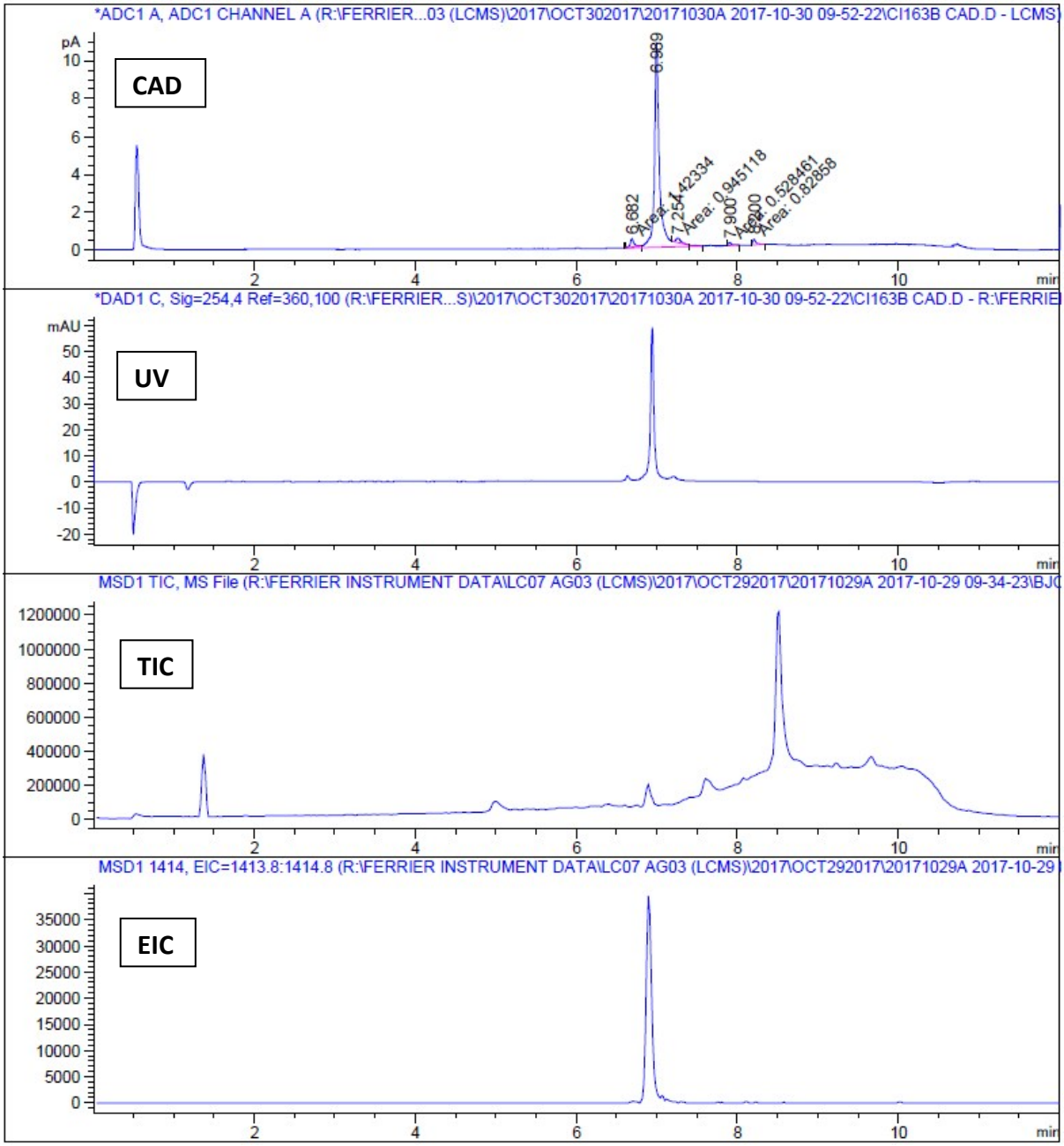
Extracted MS from TIC

	$[M+3H]^{3+}$	$[M+4H]^{4+}$	$[M+5H]^{5+}$
Calcd avg Mw	1753.6	1315.5	1052.6



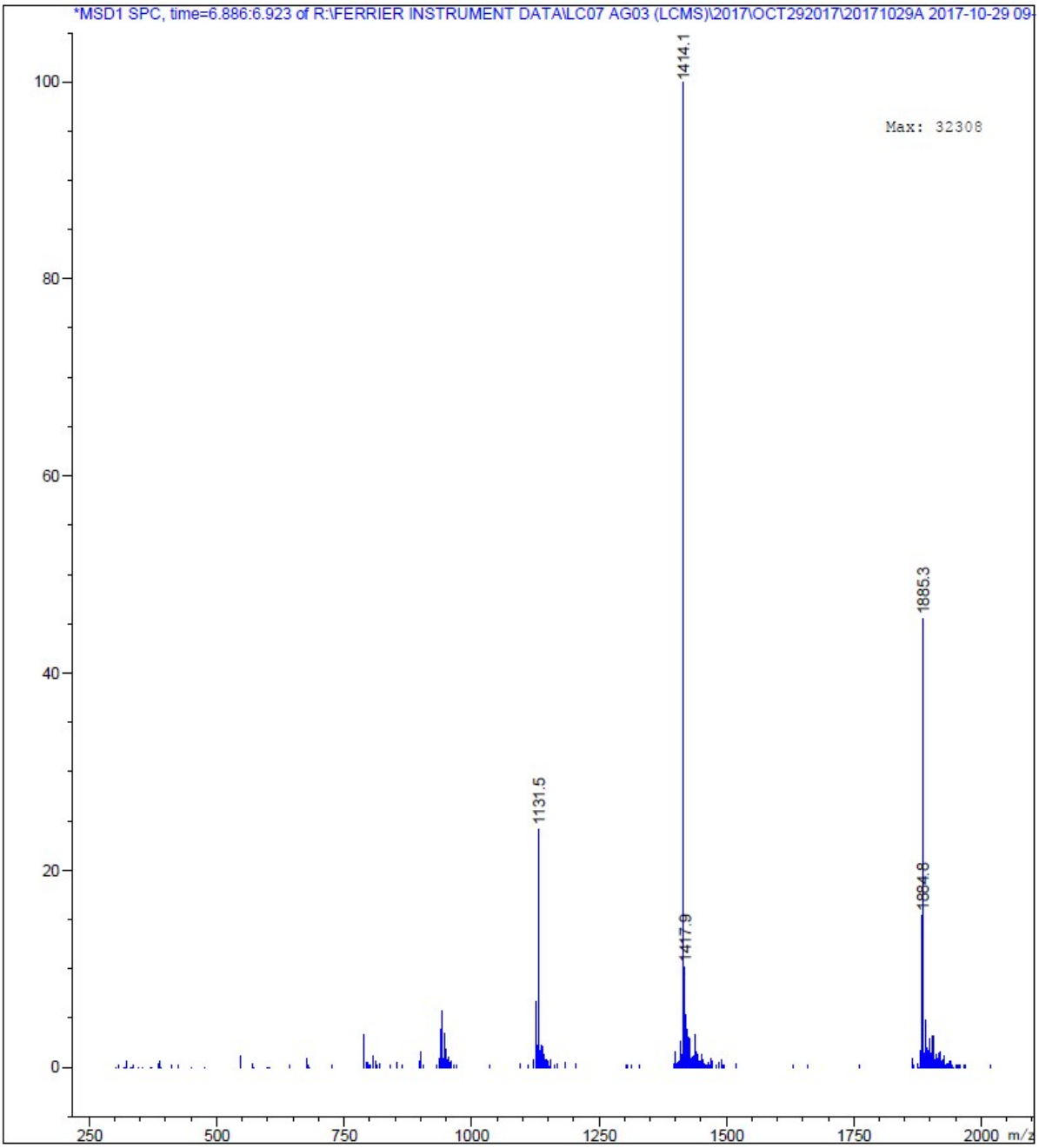
Compound **20** (α-GalPhs-OVA$^{CD4/CD8}$ BODIPY) – HPLC-CAD/LCMS

Column: Phenomenex Kinetex 2.6 µm, 100 Å, 3 x 50 mm.
Mobile Phase A: Water + 0.05% TFA
Mobile Phase B: Methanol + 0.05% TFA
Gradient (A:B): T0 = 70:30, T7 = 0:100, T12 = 0:100, T13 = 70:30, T15 = 70:30
Flow: 0.5 mL/min
Temperature: 40 °C



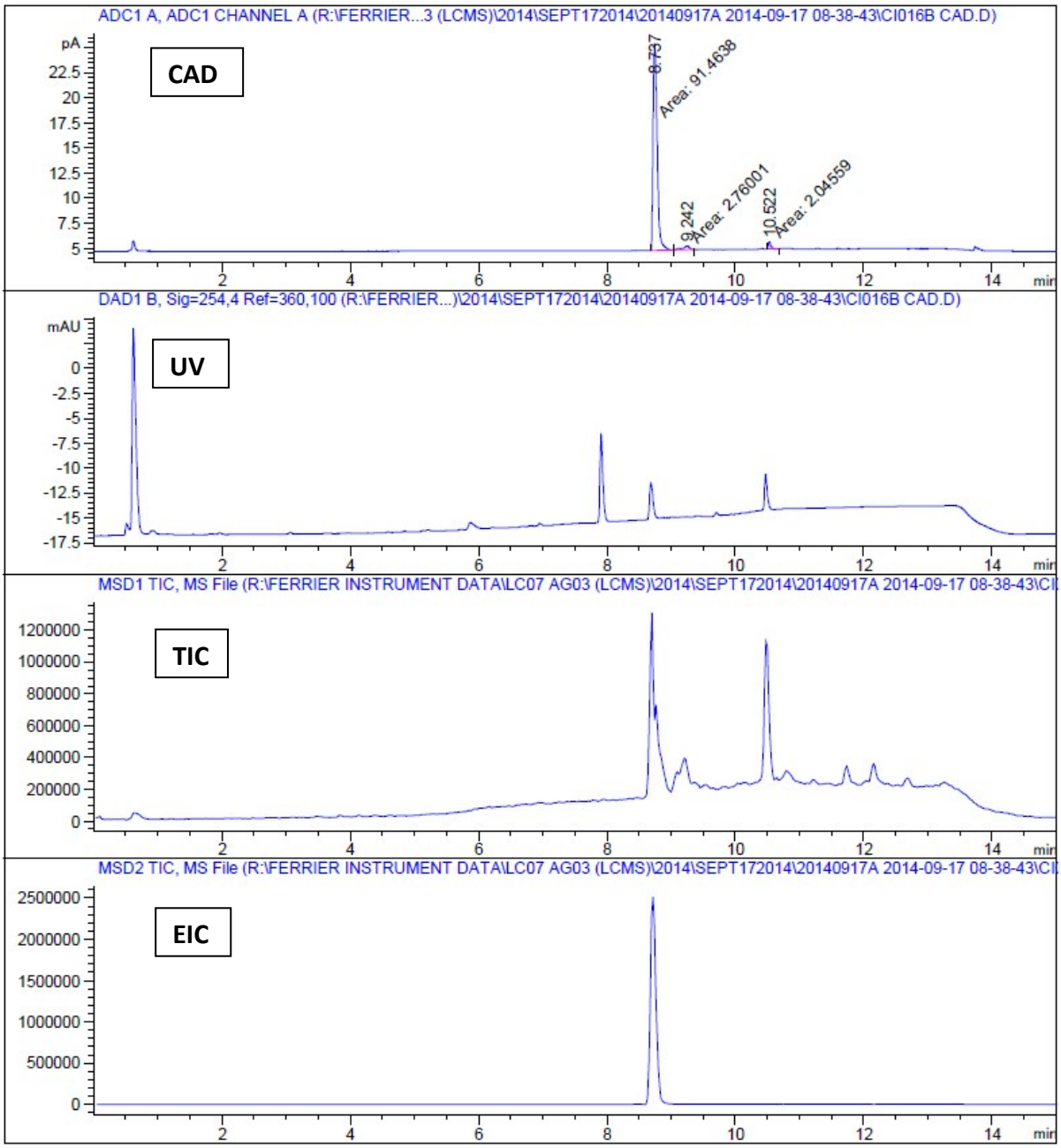
Extracted MS from TIC

	$[M+3H]^{3+}$	$[M+4H]^{4+}$	$[M+5H]^{5+}$
Calcd avg Mw	1888.5	1414.2	1131.5



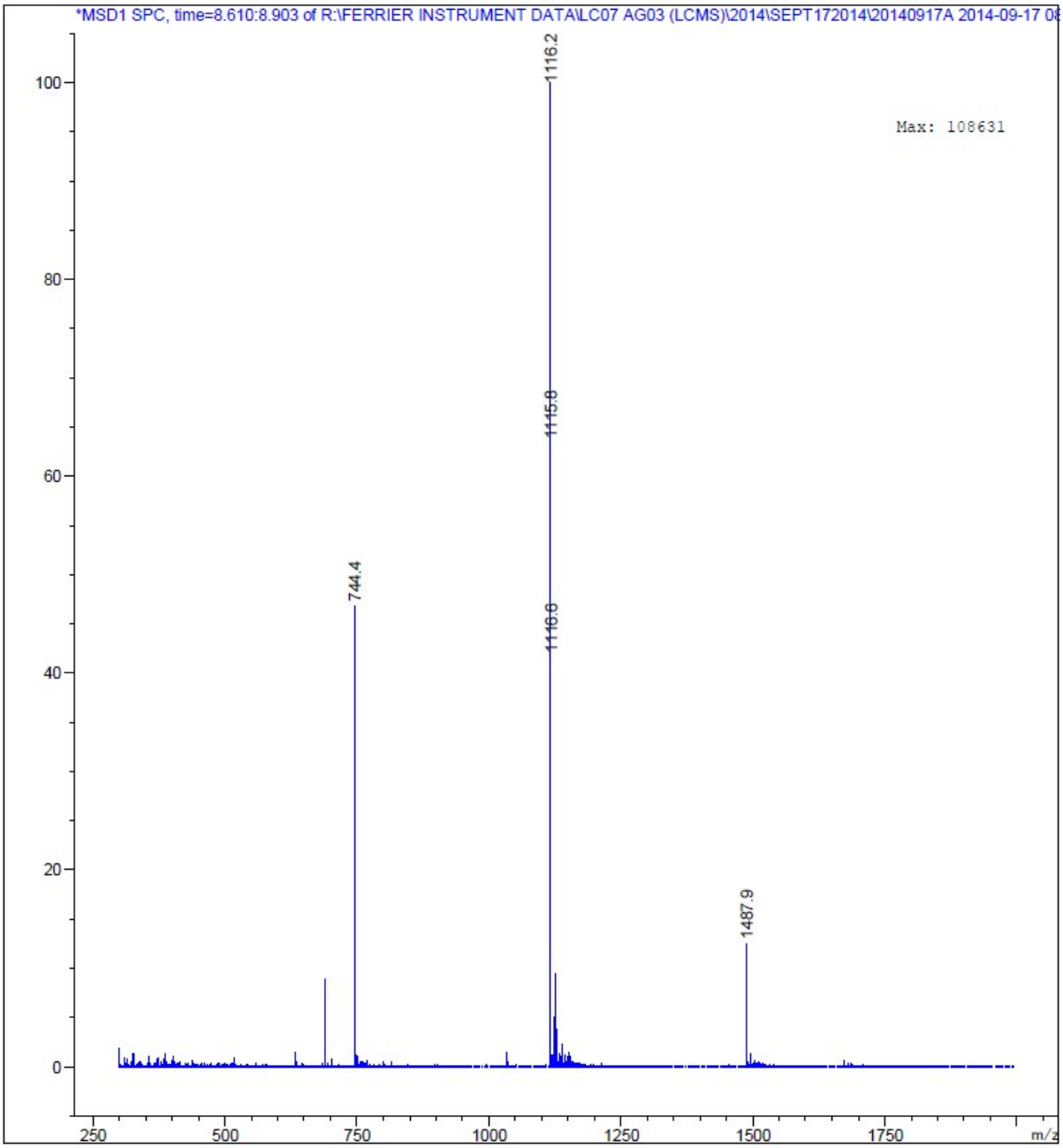
Compound **27** (α-GalPhs-non-OVA^{CD8}) – HPLC-CAD/LCMS

Column: Agilent Poroshell 120 EC-C18 2.7 µm, 3 x 50 mm.
Mobile Phase A: Water + 0.05% TFA
Mobile Phase B: Methanol + 0.05% TFA
Gradient (A:B): T0 = 95:5, T9 = 0:100, T12 = 0:100, T13 = 95:5, T15 = 95:5
Flow: 0.5 mL/min
Temperature: 40 °C



Extracted MS from TIC

	$[M+2H]^{2+}$	$[M+3H]^{3+}$	$[2M+3H]^{3+}$
Calcd avg Mw	1116.2	7444.4	1487.9



Supporting information references

1. Anderson, R. J.; Li, J.; Kedzierski, L.; Compton, B. J.; Hayman, C. M.; Osmond, T. L.; Tang, C. W.; Farrand, K. J.; Koay, H. F.; Almeida, C.; Holz, L. R.; Williams, G. M.; Brimble, M. A.; Wang, Z.; Koutsakos, M.; Kedzierska, K.; Godfrey, D. I.; Hermans, I. F.; Turner, S. J.; Painter, G. F., Augmenting Influenza-Specific T Cell Memory Generation with a Natural Killer T Cell-Dependent Glycolipid-Peptide Vaccine. *ACS Chem. Biol.* **2017,** *12* (11), 2898-2905.
2. Chen, Y. H.; Chiu, N. M.; Mandal, M.; Wang, N.; Wang, C. R., Impaired NK1+ T cell development and early IL-4 production in CD1-deficient mice. *Immunity* **1997,** *6* (4), 459-67.
3. Hildner, K.; Edelson, B. T.; Purtha, W. E.; Diamond, M.; Matsushita, H.; Kohyama, M.; Calderon, B.; Schraml, B. U.; Unanue, E. R.; Diamond, M. S.; Schreiber, R. D.; Murphy, T. L.; Murphy, K. M., Batf3 deficiency reveals a critical role for CD8alpha+ dendritic cells in cytotoxic T cell immunity. *Science* **2008,** *322* (5904), 1097-100.
4. Giaccone, G.; Punt, C. J.; Ando, Y.; Ruijter, R.; Nishi, N.; Peters, M.; von Blomberg, B. M.; Scheper, R. J.; van der Vliet, H. J.; van den Eertwegh, A. J.; Roelvink, M.; Beijnen, J.; Zwierzina, H.; Pinedo, H. M., A phase I study of the natural killer T-cell ligand alpha-galactosylceramide (KRN7000) in patients with solid tumors. *Clin Cancer Res* **2002,** *8* (12), 3702-9.
5. Wang, J.; Li, Y.; Kinjo, Y.; Mac, T. T.; Gibson, D.; Painter, G. F.; Kronenberg, M.; Zajonc, D. M., Lipid binding orientation within CD1d affects recognition of Borrelia burgorferi antigens by NKT cells. *Proc. Natl. Acad. Sci. U. S. A.* **2010,** *107* (4), 1535-40.
6. Otwinowski, Z.; Minor, W., Processing of X-ray diffraction data collected in oscillation mode. *Methods Enzymol.* **1997,** *276*, 307-26.
7. McCoy, A. J.; Grosse-Kunstleve, R. W.; Storoni, L. C.; Read, R. J., Likelihood-enhanced fast translation functions. *Acta Crystallogr D Biol Crystallogr* **2005,** *61* (Pt 4), 458-64.
8. Emsley, P.; Cowtan, K., Coot: model-building tools for molecular graphics. *Acta Crystallogr D Biol Crystallogr* **2004,** *60* (Pt 12 Pt 1), 2126-32.
9. Murshudov, G. N.; Vagin, A. A.; Dodson, E. J., Refinement of macromolecular structures by the maximum-likelihood method. *Acta Crystallogr D Biol Crystallogr* **1997,** *53* (Pt 3), 240-55.
10. Bendelac, A.; Lantz, O.; Quimby, M. E.; Yewdell, J. W.; Bennink, J. R.; Brutkiewicz, R. R., CD1 recognition by mouse NK1+ T lymphocytes. *Science* **1995,** *268* (5212), 863-5.
11. Steiner, Q. G.; Otten, L. A.; Hicks, M. J.; Kaya, G.; Grosjean, F.; Saeuberli, E.; Lavanchy, C.; Beermann, F.; McClain, K. L.; Acha-Orbea, H., In vivo transformation of mouse conventional CD8alpha+ dendritic cells leads to progressive multisystem histiocytosis. *Blood* **2008,** *111* (4), 2073-82.
12. MacKay, V. L. M.; Moore, E. E. Immortalized dendritic cells. July 2007, 1997.
13. Yu, K. O.; Im, J. S.; Molano, A.; Dutronc, Y.; Illarionov, P. A.; Forestier, C.; Fujiwara, N.; Arias, I.; Miyake, S.; Yamamura, T.; Chang, Y. T.; Besra, G. S.; Porcelli, S. A., Modulation of CD1d-restricted NKT cell responses by using N-acyl variants of alpha-galactosylceramides. *Proc. Natl. Acad. Sci. U. S. A.* **2005,** *102* (9), 3383-8.
14. Porgador, A.; Yewdell, J. W.; Deng, Y.; Bennink, J. R.; Germain, R. N., Localization, quantitation, and in situ detection of specific peptide-MHC class I complexes using a monoclonal antibody. *Immunity* **1997,** *6* (6), 715-26.
15. Prevost-Blondel, A.; Zimmermann, C.; Stemmer, C.; Kulmburg, P.; Rosenthal, F. M.; Pircher, H., Tumor-infiltrating lymphocytes exhibiting high ex vivo cytolytic activity fail to prevent murine melanoma tumor growth in vivo. *J. Immunol.* **1998,** *161* (5), 2187-94.

16. Bellone, M.; Cantarella, D.; Castiglioni, P.; Crosti, M. C.; Ronchetti, A.; Moro, M.; Garancini, M. P.; Casorati, G.; Dellabona, P., Relevance of the tumor antigen in the validation of three vaccination strategies for melanoma. *J. Immunol.* **2000,** *165* (5), 2651-6.
17. Veerapen, N.; Brigl, M.; Garg, S.; Cerundolo, V.; Cox, L. R.; Brenner, M. B.; Besra, G. S., Synthesis and biological activity of alpha-galactosyl ceramide KRN7000 and galactosyl (alpha1-->2) galactosyl ceramide. *Bioorg. Med. Chem. Lett.* **2009,** *19* (15), 4288-91.
18. Anderson, R. J.; Compton, B. J.; Tang, C. W.; Authier-Hall, A.; Hayman, C. M.; Swinerd, G. W.; Kowalczyk, R.; Harris, P.; Brimble, M. A.; Larsen, D. S.; Gasser, O.; Weinkove, R.; Hermans, I. F.; Painter, G. F., NKT cell-dependent glycolipid-peptide vaccines with potent anti-tumour activity. *Chem Sci* **2015,** *6* (9), 5120-5127.
19. Presolski, S. I.; Hong, V.; Cho, S. H.; Finn, M. G., Tailored ligand acceleration of the Cu-catalyzed azide-alkyne cycloaddition reaction: practical and mechanistic implications. *J. Am. Chem. Soc.* **2010,** *132* (41), 14570-6.
20. Guo, X. F.; Wang, H.; Guo, Y. H.; Zhang, Z. X.; Zhang, H. S., Simultaneous analysis of plasma thiols by high-performance liquid chromatography with fluorescence detection using a new probe, 1,3,5,7-tetramethyl-8-phenyl-(4-iodoacetamido)difluoroboradiaza-s-indacene. *J. Chromatogr. A* **2009,** *1216* (18), 3874-80.
21. Compton, B. J.; Tang, C. W.; Johnston, K. A.; Osmond, T. L.; Hayman, C. M.; Larsen, D. S.; Hermans, I. F.; Painter, G. F., Synthesis and Activity of 6''-Deoxy-6''-thio-alpha-GalCer and Peptide Conjugates. *Org. Lett.* **2015,** *17* (24), 5954-7.
22. Tsuji, J.; Kaito, M.; Yamada, T.; Mandai, T., Simple Synthetic Method for 2,15-Hexadecanedione from a Butadiene Telomer. *Bull. Chem. Soc. Jpn.* **1978,** *51* (6), 1915-1916.
23. Pauwels, N.; Aspeslagh, S.; Vanhoenacker, G.; Sandra, K.; Yu, E. D.; Zajonc, D. M.; Elewaut, D.; Linclau, B.; Van Calenbergh, S., Divergent synthetic approach to 6 ''-modified alpha-GalCer analogues. *Org. Biomol. Chem.* **2011,** *9* (24), 8413-8421.