Electronic Supplementary Material (ESI) for ChemComm.


# Electronic Supplementary Information

# Synthesis and Immunological Evaluation of Self-Adjuvanting MUC1-Macrophage Activating Lipopeptide 2 Conjugate Vaccine Candidates

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# Contents

# 1 Materials and Methods

Commercial materials were used as received unless otherwise noted. Amino acids, coupling reagents and resins were obtained from Novabiochem and GL Biochem. Dichloromethane was distilled from calcium hydride and stored on activated 4 Å molecular sieves. Dimethylformamide was obtained as peptide synthesis grade from Merck or Labscan and stored on activated 4 Å molecular sieves.

## 1.1 HRMS

High resolution ESI-FTICR mass spectra of compounds **7** and **8** were measured on a Bruker–Daltonics Apex Ultra 7.0T Fourier transform mass spectrometer (FTICR) operating in positive ion mode.

Vaccine constructs **1-3** were analyzed using a MicroFlex MALDI-TOF MS (Bruker Daltonics) and a 6538 Ultra High Definition Accurate-Mass Q-TOF (Agilent). For MALDI-MS, (glyco)lipopeptides were desalted in 0.1 % (v/v) aqueous trifluoroacetic acid (TFA) on custom-made $C_{18}$ SPE micro-columns packed with Poros R2 resin (Applied Biosystems). Desalted peptides were eluted in 1 µL 0.1 % (v/v) TFA in acetonitrile and deposited directly onto the MALDI target plate. The eluate was mixed with 0.5 µL matrix consisting of 10 mg/ml 2,5-dihydroxybenzoic acid (Sigma) in 0.1 % (v/v) aqueous TFA/acetonitrile (1:1 v/v) and air-dried. The mass spectrometer was operated in positive polarity ion mode with the reflectron enabled. A minimum of 400 shots were accumulated for the individual compounds. The TOF was calibrated prior to acquisition using a four-point calibration of known masses of tryptic peptides derived from bovine serum albumin. MALDI-MS data was viewed and handled with DataAnalysis v4.0 (Bruker Daltonics). For LC-ESI MS, peptides were individually injected onto an equilibrated Jupiter $C_4$ column (dimension: 150 x 1 mm, particle size: 5 µm, pore size: 300 Å) operated at a constant flow rate of 20 µL/min by a cap-pump using a 1260 Agilent HPLC connected directly to the mass spectrometer. The loading condition was 98 % solvent A (0.1 % aqueous formic acid) and 2 % solvent B (0.1 % formic acid in acetonitrile) and a linear gradient up to 40 % solvent B over 30 min was applied to elute the compounds. The column was then washed in 80 % solvent B for 10 min before bringing the column back to loading conditions. MS spectra were recorded in the Q-TOF in positive polarity ion mode. The Q-TOF was

calibrated prior to data acquisition using a five-point calibration of known masses of tune mix compounds (Agilent). Q-TOF data was viewed and handled with MassHunter vB.04.01 (Agilent).

### 1.2 HPLC

Analytical reverse-phase HPLC was performed on a Waters System 2695 separations module with an Alliance series column heater at 30 °C and 2996 photodiode array detector. Vaccine candidates **1-3** were analysed using a Waters Symmetry 300 5 µm, 2.1 x 150 mm column (C4) at a flow rate of 0.2 mL $min^{-1}$ using a mobile phase of 0.1 % (v/v) TFA in water/isopropanol/acetonitrile (8:1:1 v/v/v; Solvent A) and 0.1 % (v/v) TFA in acetonitrile/isopropanol (1:1 v/v; Solvent B) (***Eluent A***). Protected peptide pentafluorophenyl esters **7** and **8** were analysed using an Agilent Zorbax 5 µm, Rx-SIL analytical column (Silica) at a flow rate of 1.0 mL $min^{-1}$ using a mobile phase of 1.0 % (v/v) acetic acid in DCM (Solvent A) and 1.0 % (v/v) acetic acid in methanol (Solvent B) (***Eluent B***). (Glyco)peptides **10-12** were analysed using a Waters Symmetry 300 5 µm, 2.1 x 150 mm column (C4) at a flow rate of 0.2 mL $min^{-1}$ using a mobile phase of 0.1 % (v/v) TFA in water (Solvent A) and 0.1 % (v/v) TFA in acetonitrile (Solvent B) (***Eluent C***).

Preparative reverse-phase HPLC was performed using a Waters 600 Multisolvent Delivery System and Waters 500 pump with 2996 photodiode array detector or Waters 490E Programmable wavelength detector operating at 230 and 214 nm. (Glyco)peptides **1-3, 10-12** were purified on a Vydac semi-preparative column (C4) operating at a flow rate of 5 mL $min^{-1}$ using a linear gradient of ***Eluent A***. Pentafluorophenyl esters **7** and **8** were purified on a Zorbax Rx-SIL preparative column (silica) using a linear gradient of ***Eluent B***.

### 1.3 LC-MS

LC-MS was performed on a Shimadzu LC-MS 2020 instrument consisting of a LC-M20A pump, a SPD-M20A Photodiode array detector coupled to a Shimadzu 2020 mass spectrometer (ESI) operating in positive mode. Separations were performed on a Waters Sunfire 5 µm, 2.1 x 150 mm column (C18) or a Waters Symmetry 300 5 µm, 2.1 x 150 mm (C4) column, operating at a flow rate of 0.2 mL $min^{-1}$. Separations

were performed using a linear gradient of 0.1 % (v/v) formic acid in water (Solvent A) and 0.1 % (v/v) formic acid in acetonitrile (Solvent B) as the mobile phase.

# 2 General procedures

## 2.1 Fmoc-strategy solid phase peptide synthesis (Fmoc-SPPS)

Manual Fmoc-SPPS was performed in Torviq fritted plastic syringes. Automated, microwave-assisted Fmoc-SPPS was performed using a CEM Liberty 1 microwave synthesiser. Deprotection and coupling methods were chosen to suit the appropriate resin (50 $^{o}$C, 28 W for 2-chlorotrityl chloride resin-supported peptides and default microwave power and temperature settings for Rink amide resin).

***Loading 2-chlorotrityl chloride resin (250 µmol scale)***

2-chlorotrityl chloride resin (1.22 mmol $g^{-1}$, Novabiochem) was swollen in dry dichloromethane (DCM) (5 mL) for 30 min. A solution of the Fmoc-protected amino acid (2.0 eq.) and *N,N*-diisopropylethylamine (DIEA, Hünig's base) (4.0 eq.) in dimethylformamide (DMF)/DCM (1:1 v/v, 2.5 mL) was added and the resin shaken at rt for 16 h. The resin was filtered and washed with DMF (5 × 5 mL), DCM (5 × 5 mL) and DMF (5 × 5 mL). The resin was treated with a solution of DCM/$CH_3OH$/DIEA (17:2:1 v/v/v, 5 mL) for 1 h, filtered and washed with DMF (5 × 5 mL), DCM (5 × 5 mL) and DMF (5 × 5 mL).

***Iterative Fmoc-SPPS (250 µmol scale)***

*Fmoc deprotection:* Pre-loaded resin was initially swollen in DMF (5 mL) for 15 min. A solution of piperidine/DMF (1:9 v/v, 5 mL) was added to the resin which was shaken for 3 min and the procedure repeated. The two filtrates were retained in a 10 mL volumetric flask and the resin washed with fresh piperidine/DMF (1:9 v/v) such that the total volume did not exceed 10 mL. The efficiency of the initial loading was quantified by measurement of the piperidine-fulvene adduct using UV-VIS spectrophotometry ($\lambda$ = 301 nm, $\epsilon$ = 7800 $M^{-1}$ $cm^{-1}$). The resin was subsequently washed with DMF (5 × 5 mL), DCM (5 × 5 mL), and DMF (5 × 5 mL).

*Unglycosylated Amino Acid Coupling (250 µmol):* A solution of Fmoc- and side-chain-protected amino acid (1 mmol, 4 eq.), benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate (PyBOP) (1 mmol, 4 eq.) and

*N*-methylmorpholine (NMM) (2 mmol, 8 eq.) in DMF (2.5 mL) was added to the resin and shaken. After 1 h the resin was washed with DMF (5 × 5 mL), DCM (5 × 5 mL), and DMF (5 × 5 mL).

*Coupling of Glycosylamino Acids:* The general protocol for coupling glycosylamino acids (1.2 eq.) using 1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxide hexafluorophosphate (HATU) (1.2 eq.) and DIEA (2.4 eq.) has been modified to include 1.5 equivalents of 1-hydroxy-7-azabenzotriazole (HOAt) within the reaction mixture to suppress epimerisation.[1] The mixture needs to be pre-activated for at least 15 min prior to addition to resin.

*Capping:* Acetic anhydride/pyridine (1:9 v/v, 2 mL) was added to the resin and shaken. After 3 min the resin was washed with DMF (5 × 5 mL), DCM (5 × 5 mL) and DMF (5 × 5 mL).

The above steps were repeated in an iterative fashion to assemble the desired peptide/glycopeptide on the resin.

*Resin cleavage and ether precipitation:* The resin was washed thoroughly with DCM (20 × 3 mL) and treated with a solution of trifluoroacetic acid (TFA)/triisopropylsilane (TIS)/water (90:5:5 v/v/v, 2 mL) and shaken for 2 h at rt. For peptides bearing pseudoproline residues, a mixture of TFA/TIS/thioanisole/water (85:5:5:5 v/v/v/v) was used. The resin was filtered and the filtrate was evaporated to dryness. Cold diethyl ether (*ca.* 10 mL) was then added the precipitate suspended and cooled on ice. The suspension was transferred to a 50 mL conical tube and centrifuged at 3000 rpm for 5 min. The supernatant was carefully removed and the precipitate was dried *in vacuo*.

*Resin cleavage (for side-chain protected peptides):* The 2-chlorotrityl chloride resin-bound peptide was washed thoroughly with DCM (20 × 3 mL) and treated with a solution of 30 % (v/v) hexafluoroisopropanol (HFIP) in DCM. The resin was agitated for 2 h and then filtered and washed with DCM (2 × 3 mL). The filtrate was evaporated to dryness and co-evaporated with toluene (2 × 3 mL). The crude peptide

was dried under high-vacuum and used in the next step (derivatisation as the pentafluorophenyl ester) without additional purification.

*De-O-acetylation of glycopeptides*: Acetylated glycopeptides were suspended in Milli-Q water (approx. 6 mL) and filtered through a 5 μm membrane filter. The filtrate was treated with hydrazine monohydrate (300 μL) and gently agitated at rt for 2 h. Glycopeptides **5** and **6** were then purified by reverse-phase preparative HPLC.

### 2.2 Pentafluorophenyl ester activation

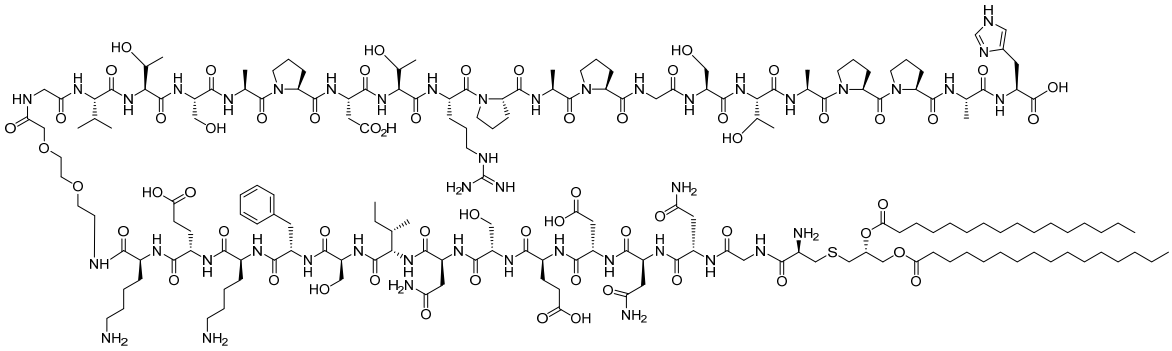
The crude peptide acid was dissolved in dry DMF (0.1 M) under argon. Pyridine (5.0 eq.) was added followed by drop-wise addition of pentafluorophenyl trifluoroacetate (5.0 eq.). The reaction was stirred at rt for 4 h and the solvent was removed *in vacuo*. The crude residue was dissolved in DCM containing 1.0 % (v/v) AcOH and purified by preparative, normal-phase HPLC to afford pentafluorophenyl esters **7** and **8** as colourless solids following evaporation to dryness [compound **8** was subsequently lyophilised from *tert*-butanol/acetonitrile (4:1 v/v)].

### 2.3 Pentafluorophenyl ester-mediated fragment condensation (Synthesis of (glyco)peptides 10-12, 1-3)

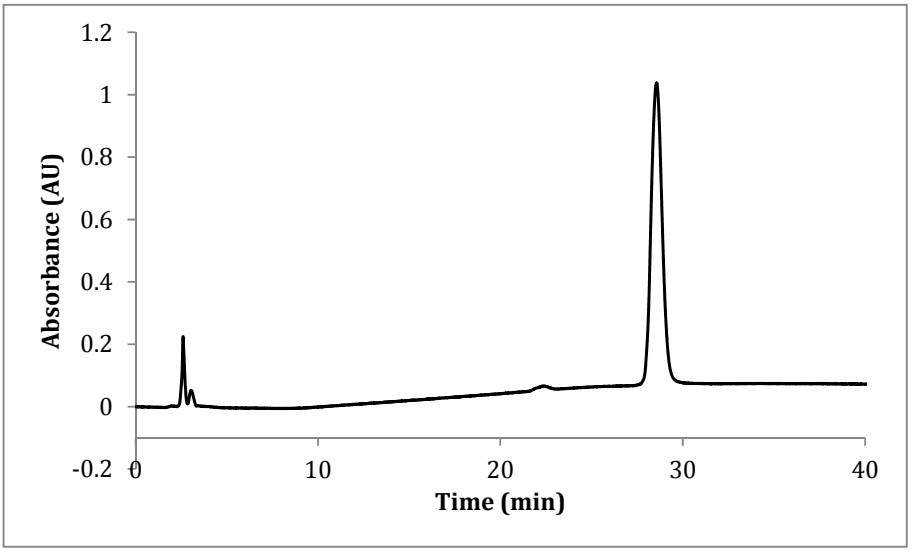
*2.0 μmol scale:* To a solution of MUC1 (glyco)peptide (2.0 μmol) in dry DMF (80 μL) was added DIEA (10 μL of a 48 μL $mL^{-1}$ solution in DMF, 4.8 μmol, 2.4 eq.) and 1-hydroxybenzotriazole (10 μL of a 24 mg $mL^{-1}$ solution in DMF, 2.4 μmol, 1.2 eq.). This solution was then added to the peptide pentafluorophenyl ester (3.0 μmol, 1.5 eq.) and the mixture gently agitated at rt for 16 h. Piperidine (20 μL) was added and the mixture agitated for a further 30 min at rt. The solvent was removed *in vacuo* and the residue purified by preparative, reverse-phase HPLC followed by lyophilisation to afford the desired (glyco)peptides **10-12**. For glycopeptides **1-3**, treatment of the residue with an acidic cocktail of TFA/TIS/thioanisole/water (85:5:5:5 v/v/v) afforded vaccine candidates **1-3** following purification by semi-preparative C4 HPLC and lyophilisation.

## 3 Analytical data

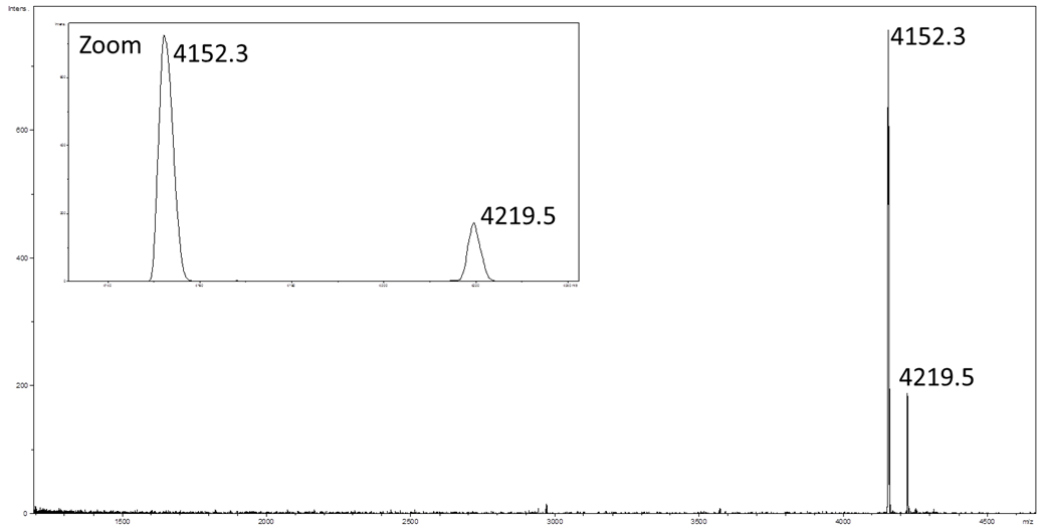
**1: H-*S*-[2,3-Bis(palmitoyloxy)-(2*R*)-propyl]-(*R*)-cysteine-Gly-Asn-Asn-Asp-Glu-Ser-Asn-Ile-Ser-Phe-Lys-Glu-LysNH($CH_2CH_2O)_2CH_2$-Gly-Val-Thr-Ser-Ala-Pro-Asp-Thr-Arg-Pro-Ala-Pro-Gly-Ser-Thr-Ala-Pro-Pro-Ala-His-OH.**



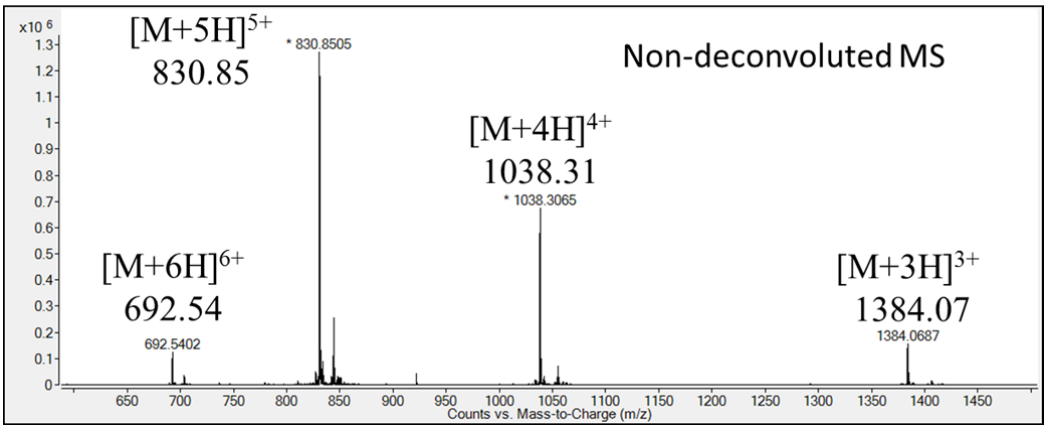
Vaccine candidate **1** was prepared by pentafluorophenyl ester-mediated fragment condensation of **10** (3.0 mg) and **8** (1.1 mg, 1.5 eq.) as outlined in the general procedures and purified by semi-preparative C4 HPLC (20 % B for 10 min to 100 % B over 40 min; *Eluent A*, $R_t$ 37 min) and isolated in 87 % yield (2.3 mg).

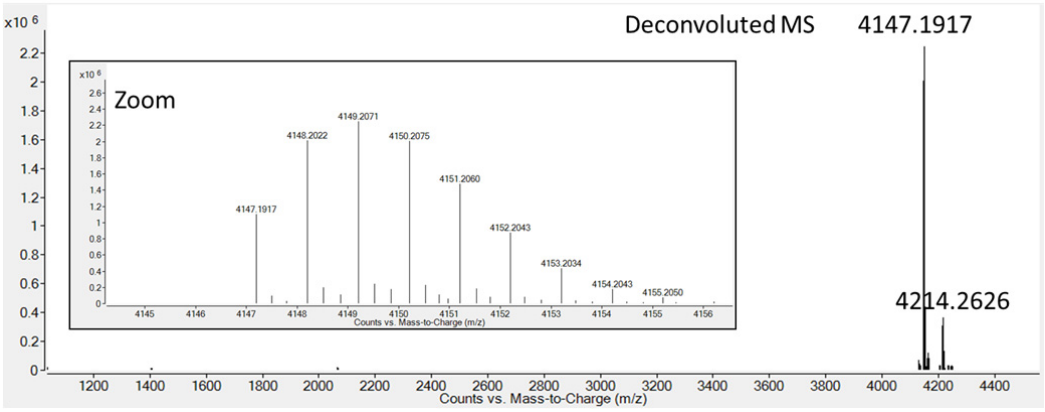



Analytical HPLC: $R_t$ 28.5 min (0-100 % B over 40 min, λ = 230 nm; *Eluent A*)



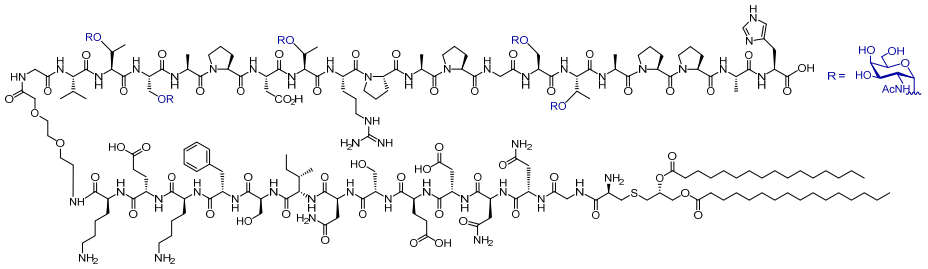

MALDI-MS: Calcd for $C_{185}H_{303}N_{45}O_{60}S$: $[M_{Av}+H]$ = 4150.8 Da, found 4152.3 Da



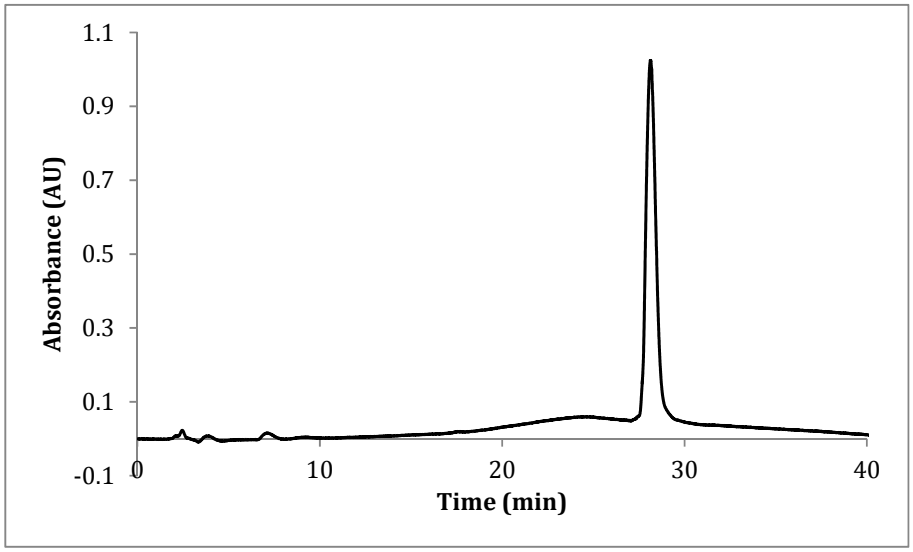




ESI-Q-ToF-MS: Calcd for $C_{185}H_{303}N_{45}O_{60}S$: $[M_{monoisotopic,\ deconvoluted}]$ = 4147.1763 Da, found 4147.1917 Da.

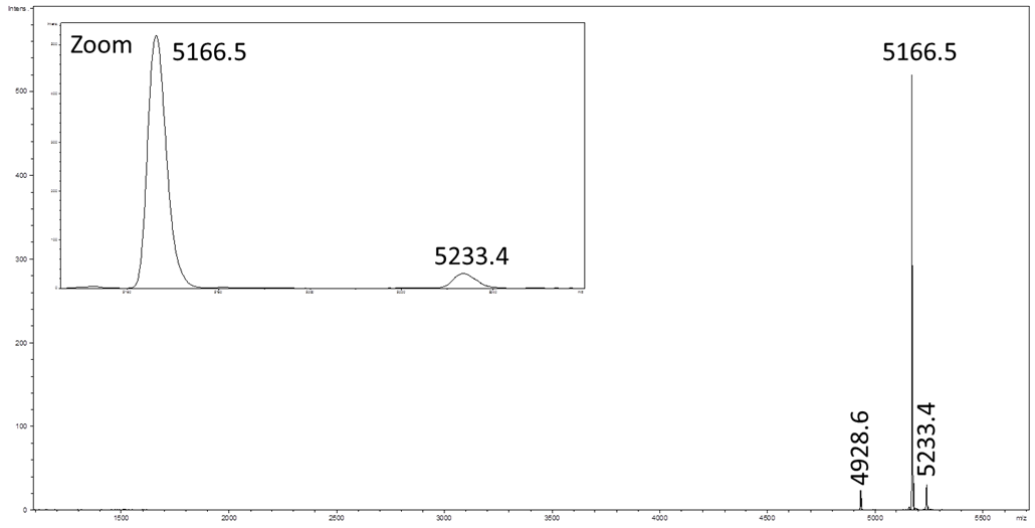
**2: H-*S*-[2,3-Bis(palmitoyloxy)-(2*R*)-propyl]-(*R*)-cysteine-Gly-Asn-Asn-Asp-Glu-Ser-Asn-Ile-Ser-Phe-Lys-Glu-LysNH($CH_2CH_2O)_2CH_2$-Gly-Val-Thr(α-D-GalNAc)-Ser(α-D-GalNAc)-Ala-Pro-Asp-Thr(α-D-GalNAc)-Arg-Pro-Ala-Pro-Gly-Ser(α-D-GalNAc)-Thr(α-D-GalNAc)-Ala-Pro-Pro-Ala-His-OH**



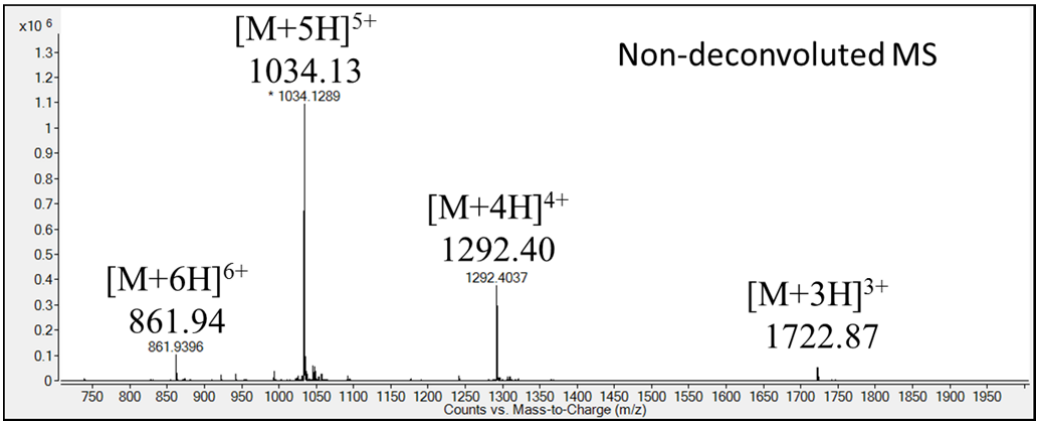
Vaccine candidate **2** was prepared by pentafluorophenyl ester-mediated fragment condensation of **11** (4.5 mg) and **8** (1.7 mg, 1.5 eq.) as outlined in the general procedures and purified by semi-preparative C4 HPLC (20 % B for 10 min to 100 % B over 40 min; *Eluent A*, $R_t$ 36 min) and isolated in 86 % yield (4.4 mg).

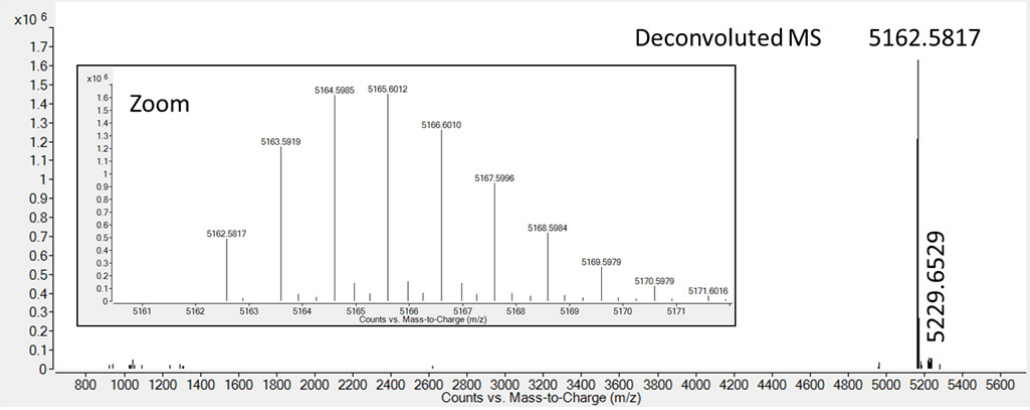



Analytical HPLC: $R_t$ 28.1 min (0-100 % B over 40 min, λ = 230 nm; *Eluent A*)



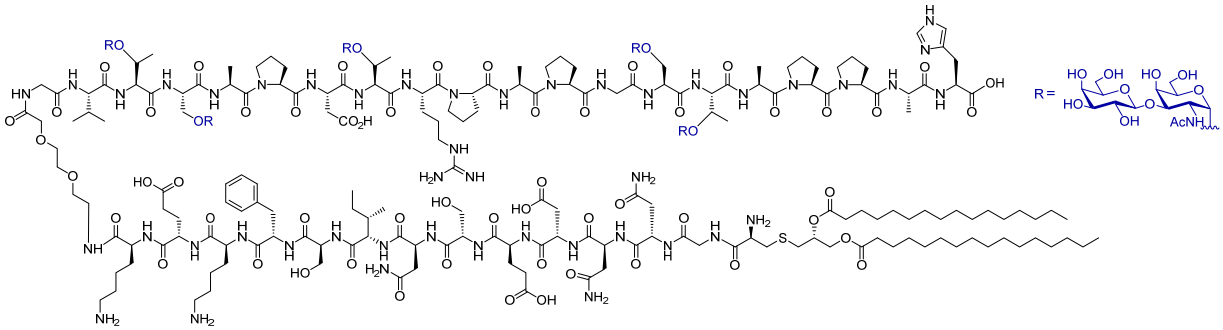

MALDI-MS: Calcd for $C_{225}H_{368}N_{50}O_{85}S$: $[M_{Av}+H]$ = 5166.8, found 5166.5



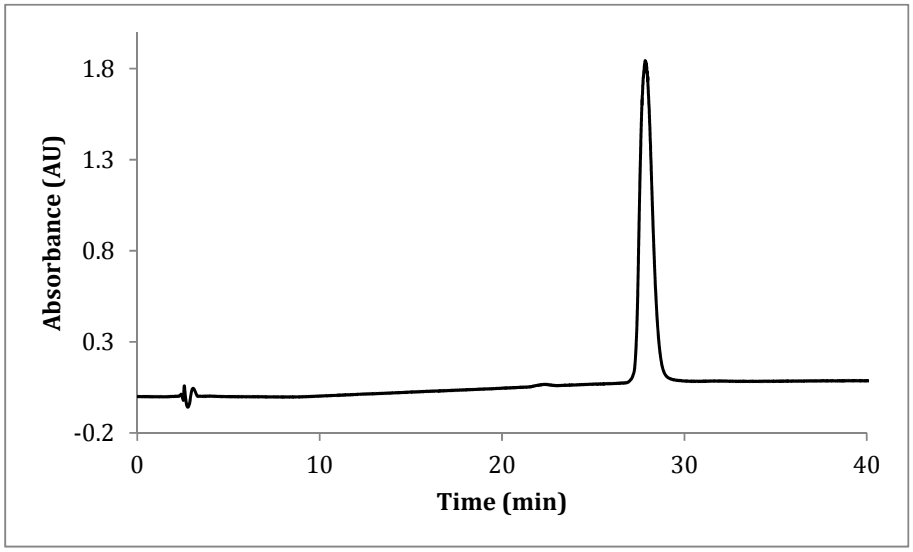




ESI-Q-ToF-MS: Calcd for $C_{225}H_{378}N_{50}O_{85}S$: $[M_{monoisotopic,\ deconvoluted}]$ = 5162.5731 Da, found 5162.5817 Da.

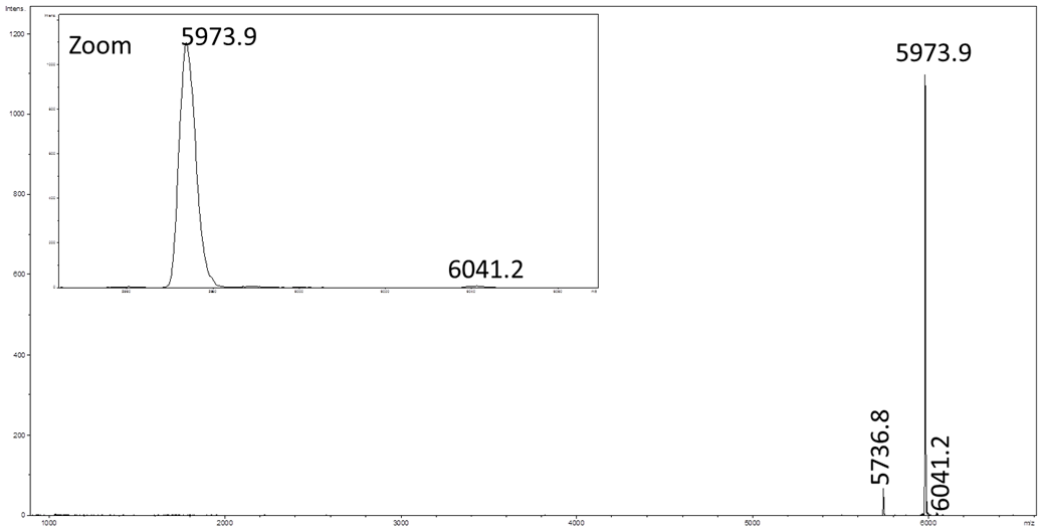
**3: H-*S*-[2,3-Bis(palmitoyloxy)-(2*R*)-propyl]-(*R*)-cysteine-Gly-Asn-Asn-Asp-Glu-Ser-Asn-Ile-Ser-Phe-Lys-Glu-LysNH($CH_2CH_2O)_2CH_2$-Gly-Val-Thr(β-D-Gal-(1→3)-α-D-GalNAc)-Ser(β-D-Gal-(1→3)-α-D-GalNAc)-Ala-Pro-Asp-Thr(β-D-Gal-(1→3)-α-D-GalNAc)-Arg-Pro-Ala-Pro-Gly-Ser(β-D-Gal-(1→3)-α-D-GalNAc)-Thr(α-D-GalNAc)-Ala-Pro-Pro-Ala-His-OH.**



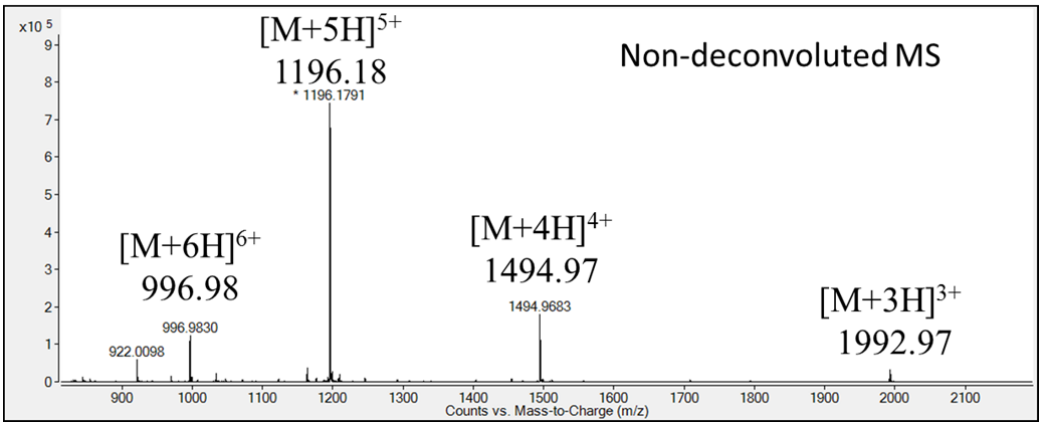
Vaccine candidate **3** was prepared by pentafluorophenyl ester-mediated fragment condensation of **12** (5.3 mg) and **8** (1.7 mg, 1.5 eq.) as outlined in the general procedures and purified by semi-preparative C4 HPLC (20 % B for 10 min to 100 % B over 40 min; *Eluent A*, $R_t$ 37 min) and isolated in 82 % yield (4.9 mg).

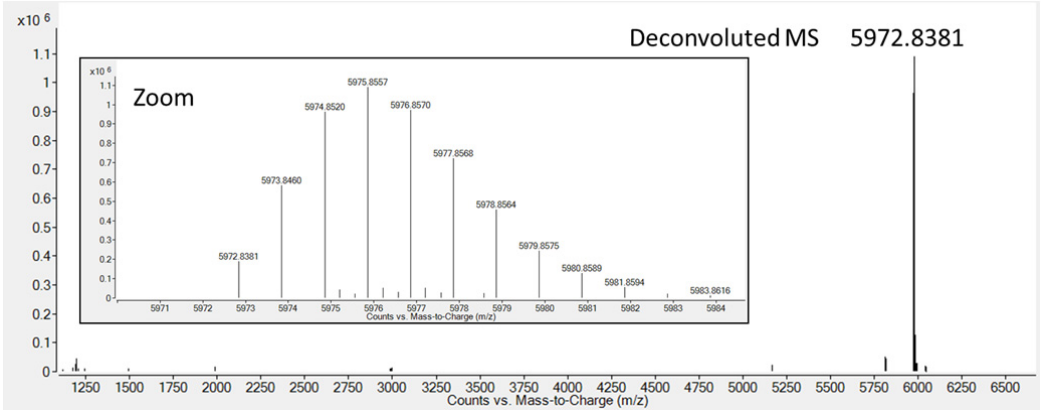



Analytical HPLC: $R_t$ 27.8 min (0-100 % B over 40 min, λ = 230 nm; *Eluent A*)



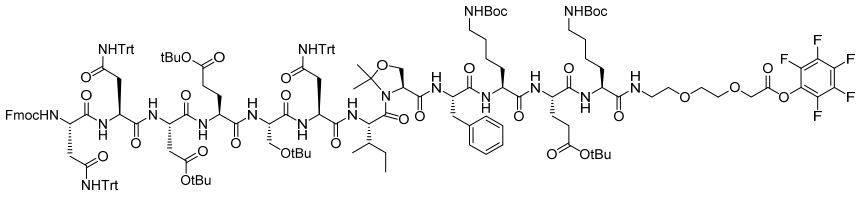

MALDI-MS: Calcd for $C_{255}H_{418}N_{50}O_{110}S$: $[M_{Av}+H]$ = 5977.5 Da, found 5973.9 Da



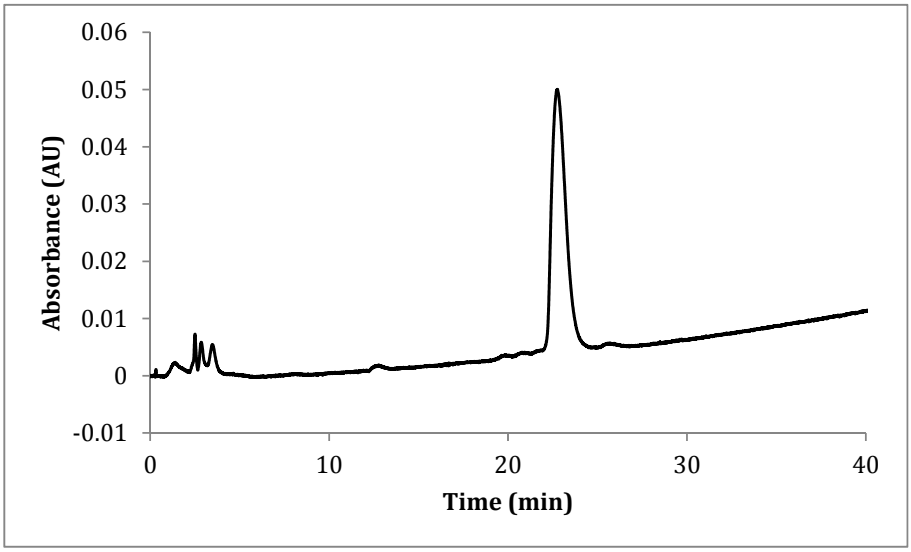




ESI-Q-ToF-MS: Calcd for $C_{255}H_{425}N_{50}O_{110}S$: $[M_{monoisotopic,\ deconvoluted}]$ = 5972.8372 Da, found 5972.8381 Da.

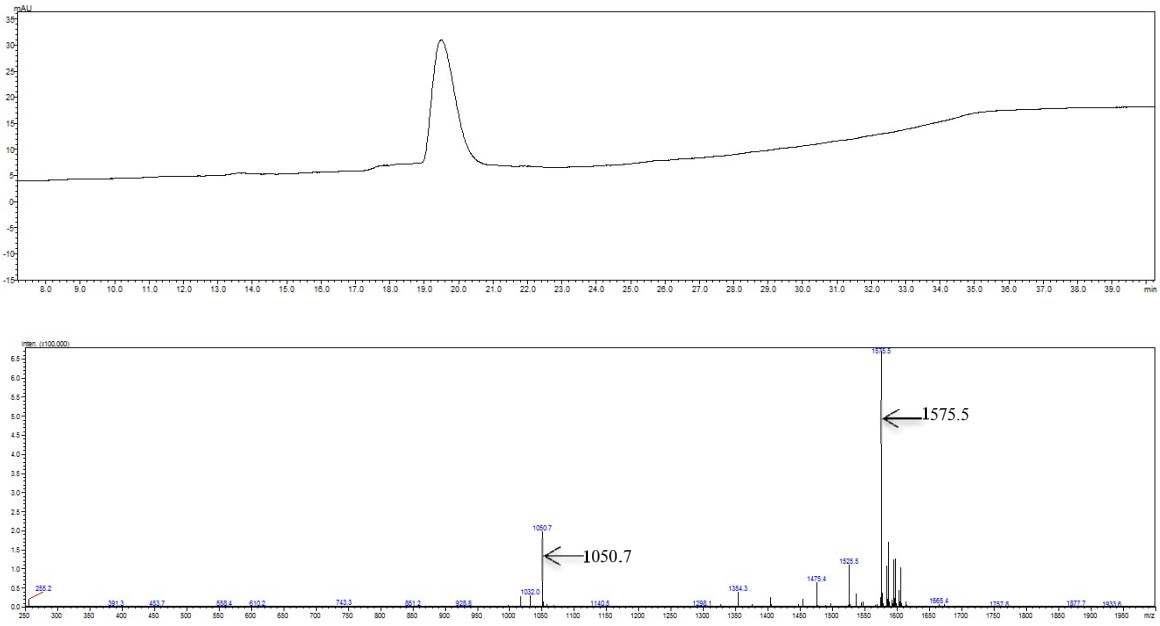
**7: Fmoc-Asn(Trt)-Asn(Trt)-Asp(*t*Bu)-Glu(*t*Bu)-Ser(*t*Bu)-Asn(Trt)-Ile-Ser($\psi^{Me,Me}$Pro)-Phe-Lys(Boc)-Glu(*t*Bu)-Lys(Boc)-NH($CH_2CH_2O)_2CH_2$-O*Pfp***




The synthesis of the protected peptide pentafluorophenyl ester **7** was achieved using manual Fmoc-SPPS, commencing from 2-chlorotrityl chloride resin pre-loaded with commercially available Fmoc-PEG(9 atoms)-OH (250 μmol, see general procedures for details). The peptide included the insertion of the pseudoproline dipeptide Fmoc-Ile-Ser($\psi^{Me,Me}$Pro)-OH (0.144 g, 1.2 eq.) to suppress aggregation on-resin. Following elongation, the peptide was cleaved using 30 % (v/v) HFIP in DCM. After rigorous drying, the crude, protected peptide was derivatised as the C-terminal pentafluorophenyl ester using excess pentafluorophenyl trifluoroacetate (0.35 g, 5 eq.) and pyridine (100 μL, 5 eq.) in dry DMF. After 1 h, the mixture was concentrated by rotary evaporation and re-dissolved in dry DCM containing 1 % (v/v) acetic acid and subjected to normal-phase HPLC purification (0 to 15 % B over 60 min; *Eluent B*, $R_t$ 22.6 min). The solvent was concentrated by rotary evaporation, azeotropically dried with toluene and dried under high vacuum overnight to afford pure **7** as a colourless solid (96.3 mg, 12 % based on the original resin loading).



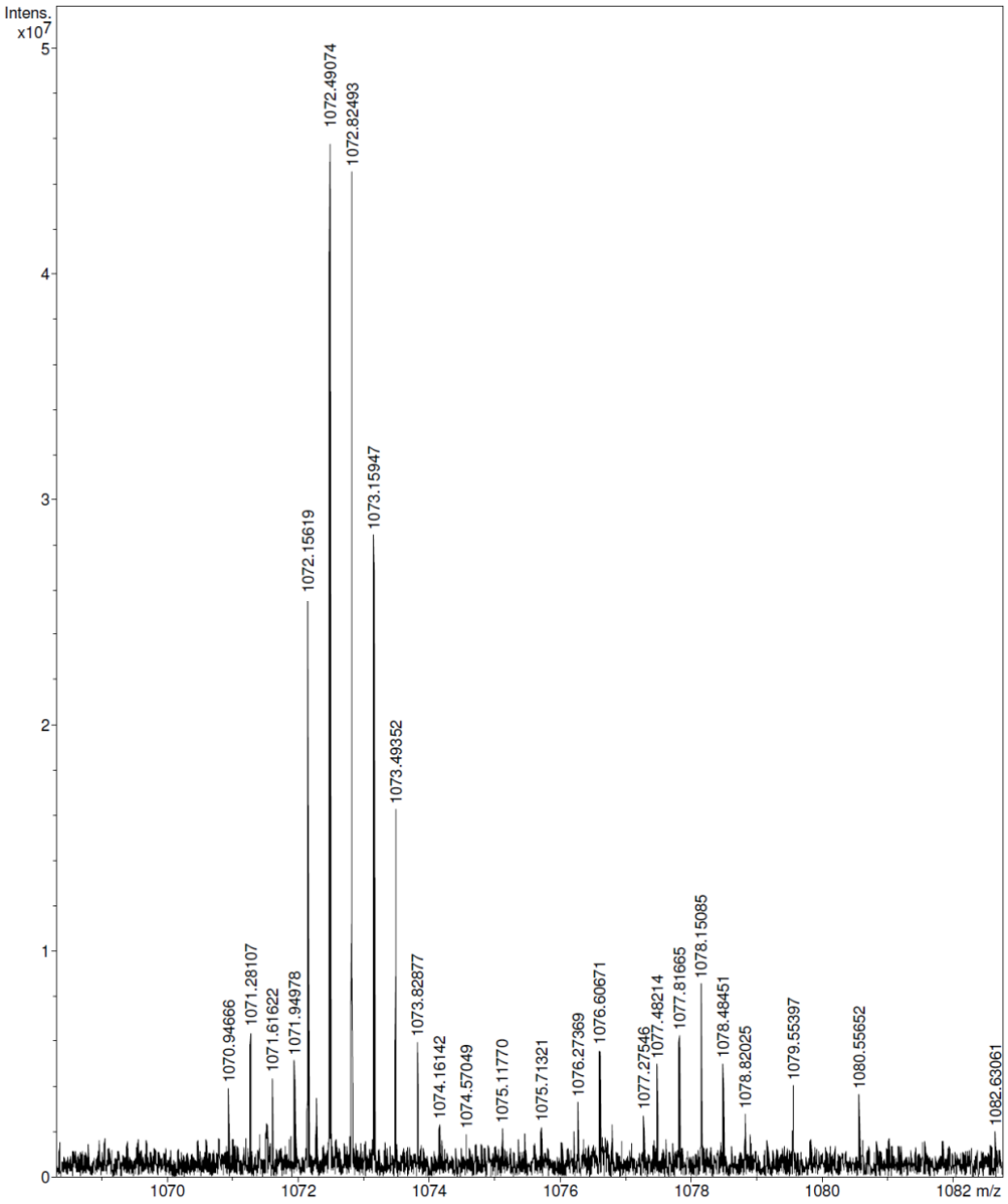

Analytical HPLC: $R_t$ 22.6 min (0-15 % B over 60 min, λ = 280 nm; *Eluent B*)




LC-MS ($ESI^+$): $R_t$ 19.5 min (20-100 % B over 30 min, λ = 254 nm; 0.1 % (v/v) formic acid in water (Solvent A) and 0.1 % (v/v) formic acid in acetonitrile (Solvent B))

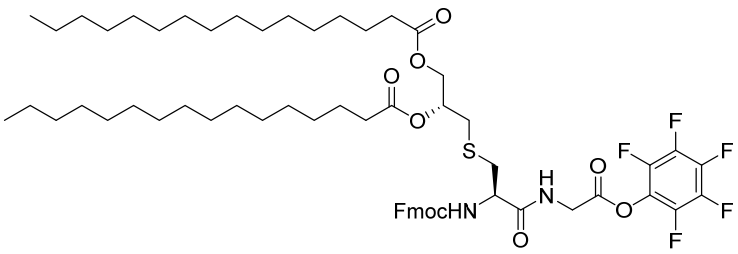
Calculated for $C_{172}H_{207}F_5N_{18}O_{33}$, $[M_{Av}]$ = 3149.50 Da

Found: *m/z* 1575.5 $[M+2H]^{2+}$, 1050.7 $[M+3H]^{3+}$.

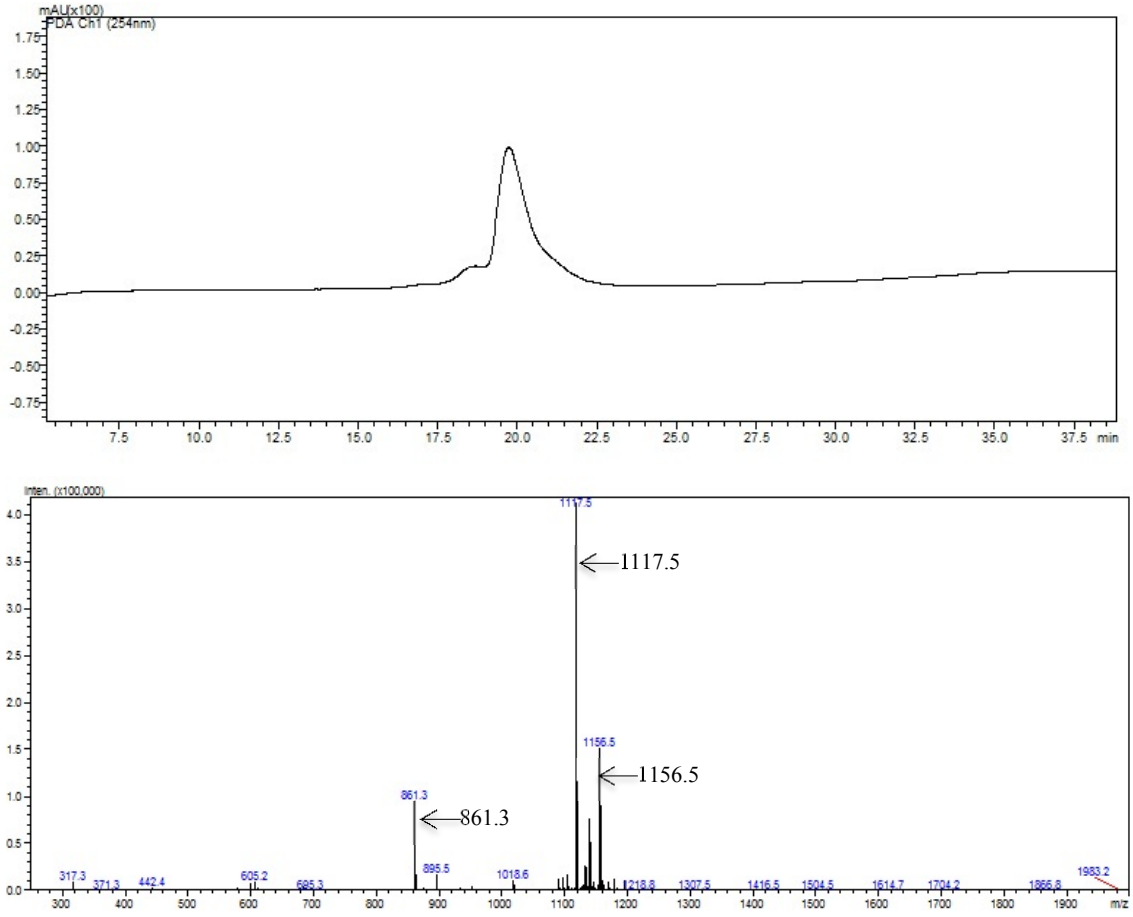



HRMS (ESI$^+$): Calcd for $C_{172}H_{207}F_5N_{18}O_{33}Na_3$: *m/z* 1072.4901 $[M+3Na]^{3+}$, found 1072.4907 $[M+3Na]^{3+}$.

**8: Fmoc-Pam$_2$Cys-Gly-O*Pfp***



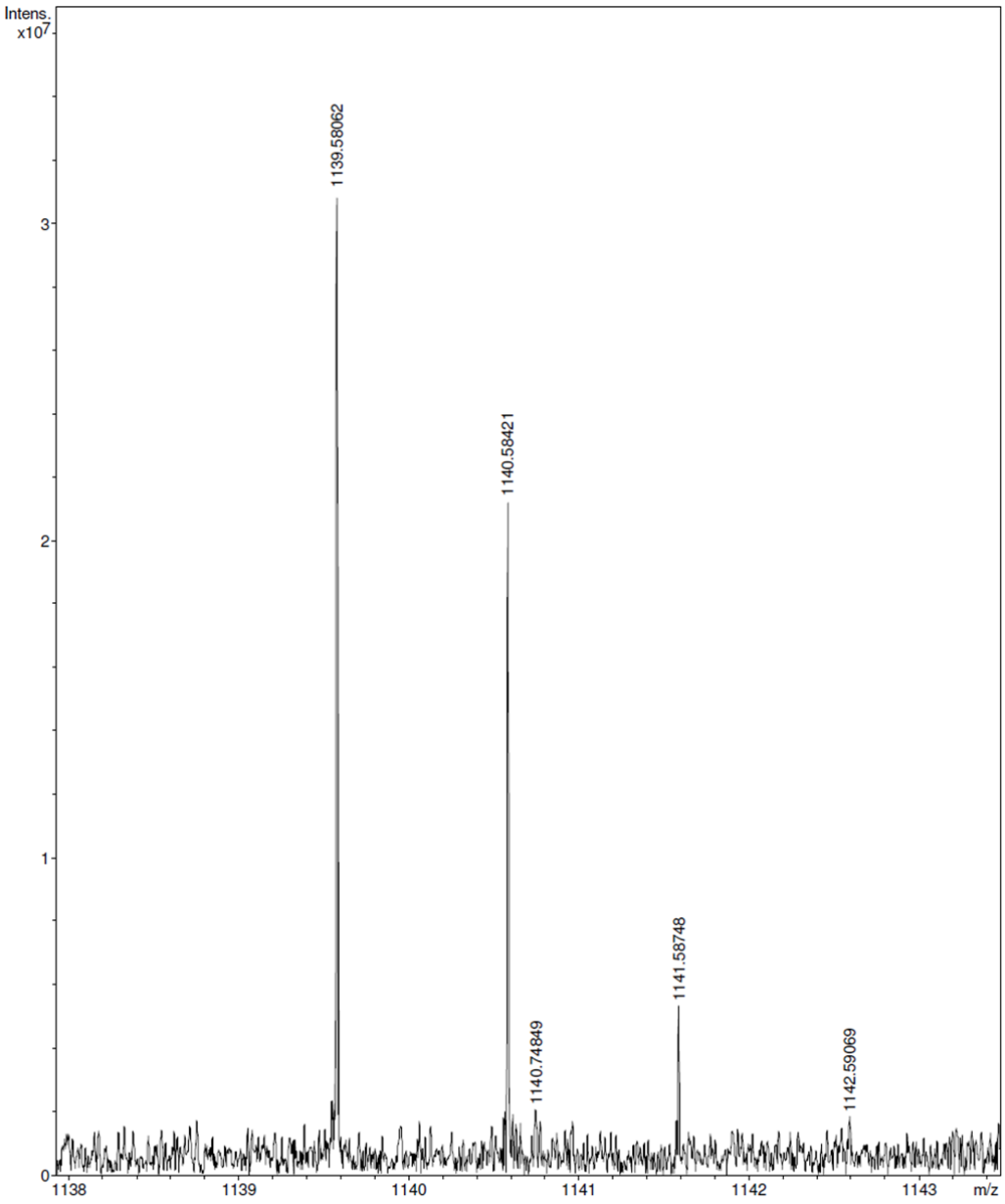

Synthesis of lipopeptide pentafluorophenyl ester **8** was also achieved using Fmoc-SPPS commencing from 2-chlorotrityl chloride resin pre-loaded with Fmoc-Gly-OH (100 μmol). Installation of the synthetically derived, optically pure Fmoc-Pam$_2$Cys-OH[2] (0.101 g, 1.2 eq.) with HATU (0.046 g, 1.2 eq.) and DIEA (2.4 eq.) for 16 h (15 min pre-activation) afforded the resin bound lipopeptide. Mild acidic cleavage using 30 % (v/v) HFIP in DCM then gave crude lipopeptide **8** which was used in the next step without further purification. The resin was filtered, washed with dry DCM and the combined filtrates concentrated to dryness. The crude lipopeptide was then derivatised as the pentafluorophenyl ester using identical conditions described for **7** and isolated in high purity and excellent yield (70.4 mg, 63 % based on the initial resin loading) following normal-phase HPLC purification (0 to 15 % D over 60 min; *Eluent B*, $R_t$ 27 min) and lyophilisation from *tert*-butanol. The structure and purity was confirmed by C4 analytical HPLC and HRMS.




LC-MS (ESI$^+$): $R_t$ 19.8 min (20-100 % B over 30 min, λ = 254 nm; 0.1 % (v/v) formic acid in water (Solvent A) and 0.1 % (v/v) formic acid in acetonitrile (Solvent B))

Calculated for $C_{61}H_{85}F_5N_2O_9S$, $[M_{Av}]$ = 1116.59 Da

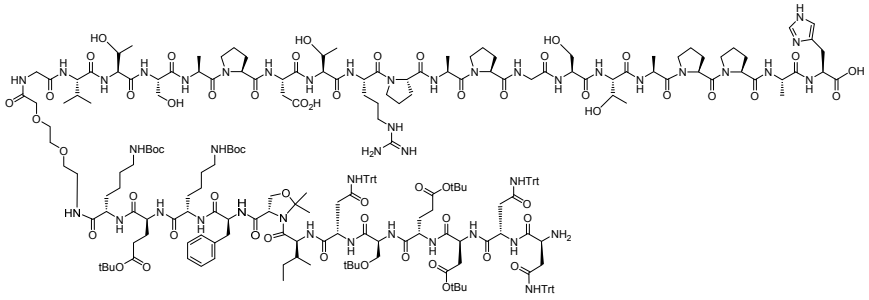
Found: *m/z* 1117.5 $[M+H]^+$, 1156.5 $[M+K]^+$.

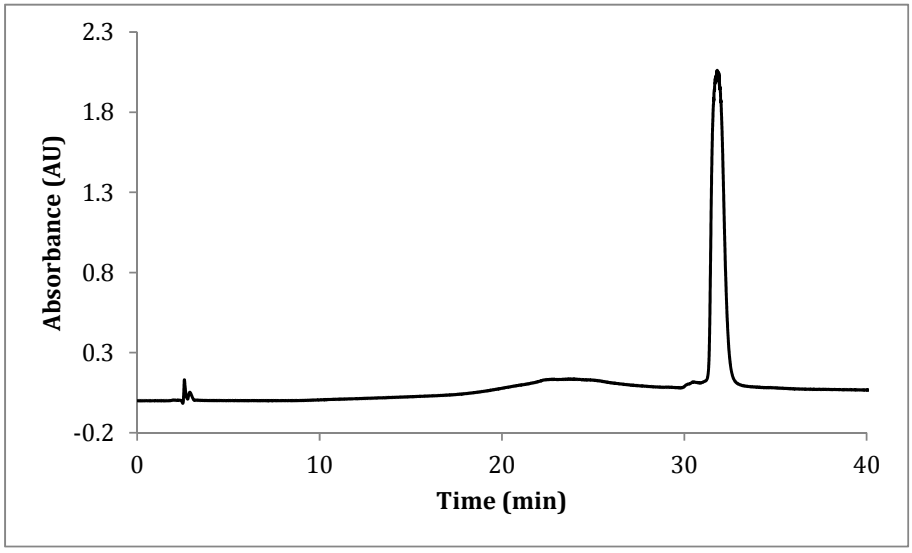



HRMS (ESI$^+$): Calcd for $C_{61}H_{85}F_5N_2O_9SNa$: *m/z* 1139.5788 $[M+Na]^+$, found 1139.5806 $[M+Na]^+$.

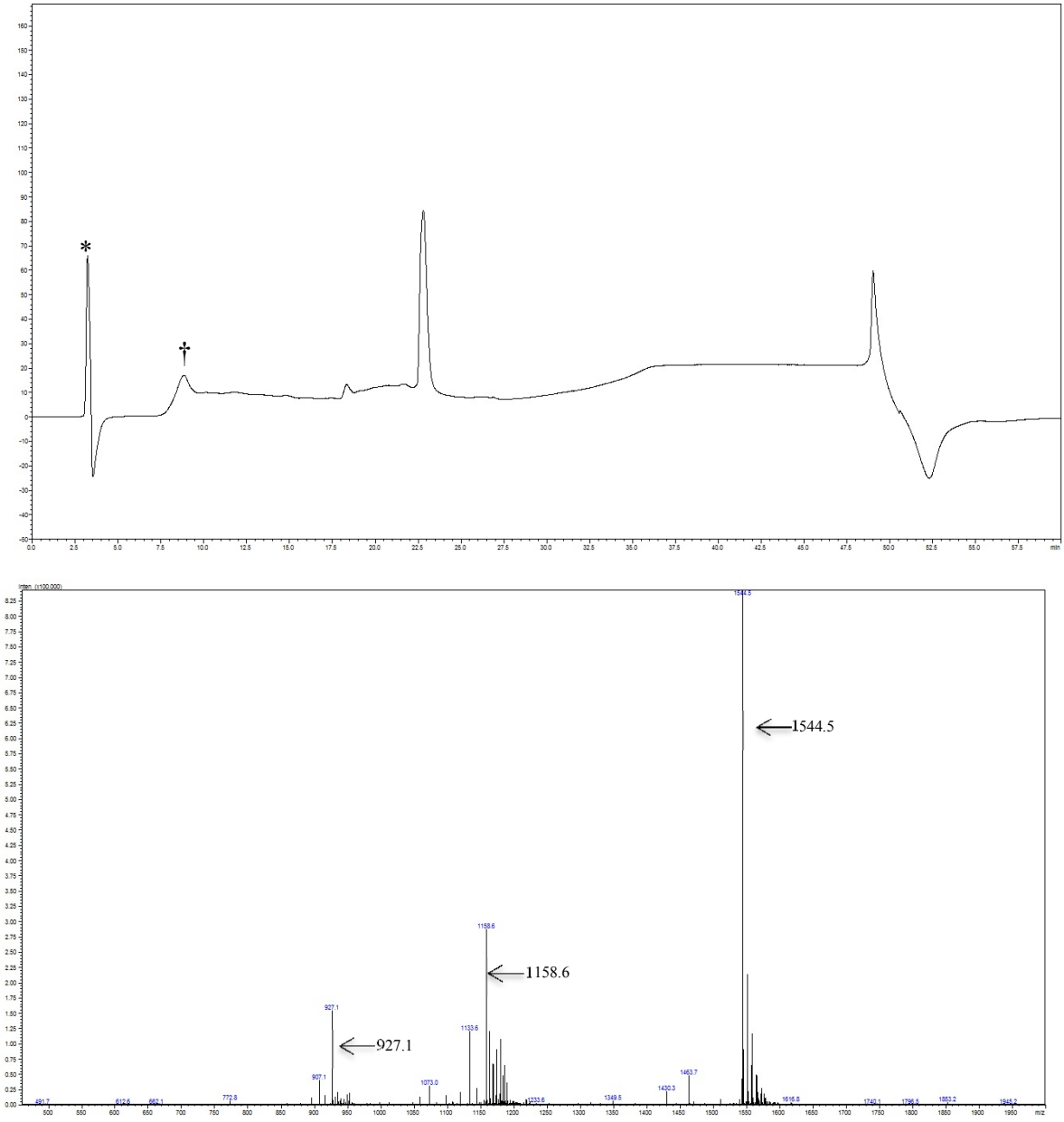
**10: H-Asn(Trt)-Asn(Trt)-Asp(*t*Bu)-Glu(*t*Bu)-Ser(*t*Bu)-Asn(Trt)-Ile-Ser($\psi^{Me,Me}$Pro)-Phe-Lys(Boc)-Glu(*t*Bu)-Lys(Boc)-NH($CH_2CH_2O)_2CH_2$-Gly-Val-Thr-Ser-Ala-Pro-Asp-Thr-Arg-Pro-Ala-Pro-Gly-Ser-Thr-Ala-Pro-Pro-Ala-His-OH.**

The partially protected peptide **10** was prepared by pentafluorophenyl ester-mediated fragment condensation of **4** (3.7 mg) and **7** (12.5 mg, 1.5 eq.) as outlined in the general procedures and purified by semi-preparative C4 HPLC (20 % B for 10 min to 100 % B over 60 min; *Eluent C*, $R_t$ 42 min) and isolated in 40 % yield (3.71 mg).






Analytical HPLC: $R_t$ 31.8 min (0-100 % B over 40 min, λ = 230 nm; *Eluent C*)




LC-MS ($ESI^+$): $R_t$ 22.5 min (0-100 % B over 30 min, λ = 230 nm; 0.1 % (v/v) formic acid in water (Solvent A) and 0.1 % (v/v) formic acid in acetonitrile (Solvent B))

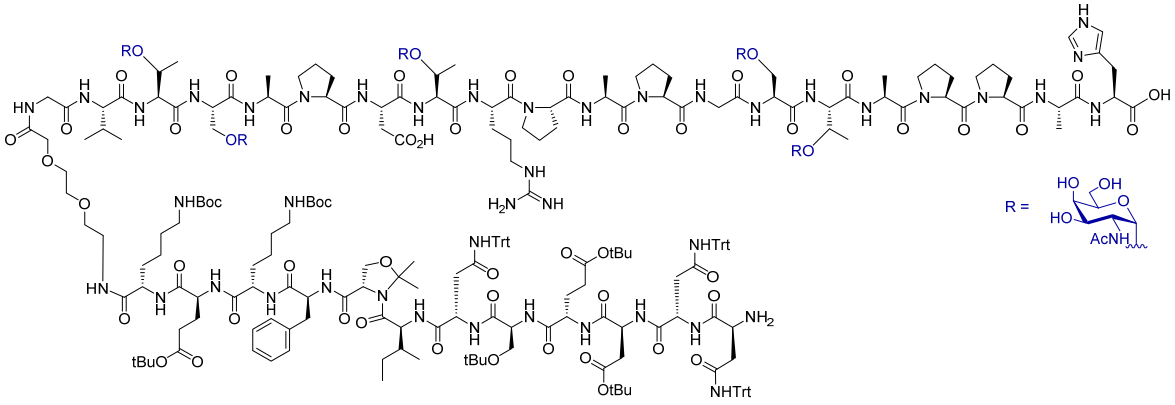
*Injection peak, † Injection artefact with no associated mass.

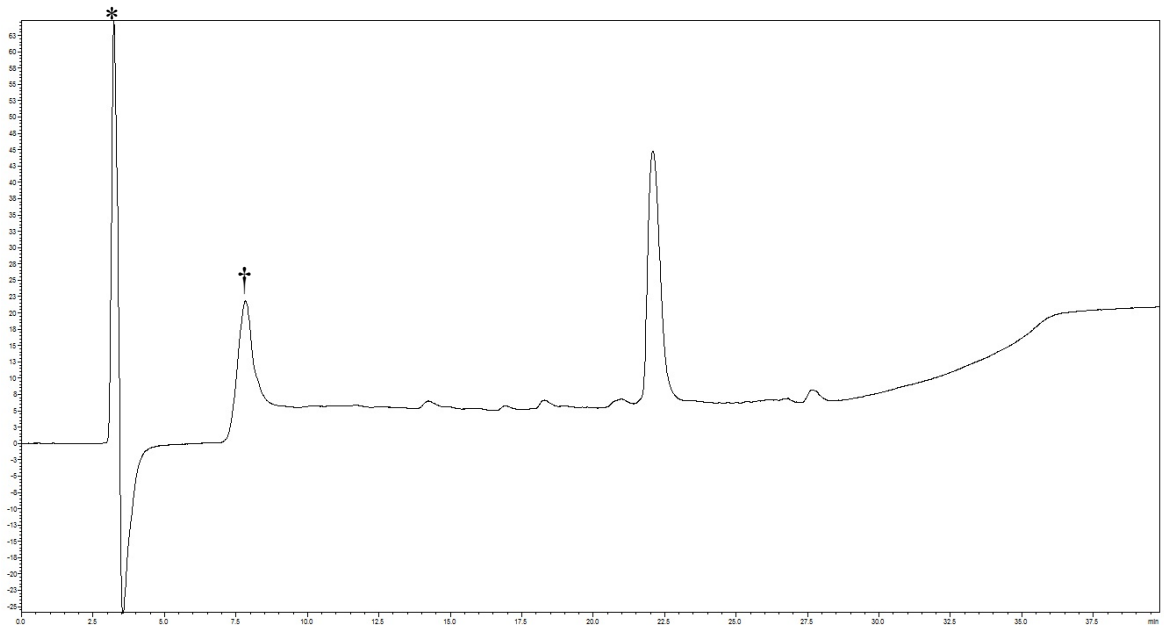
Calculated for $C_{231}H_{323}N_{43}O_{58}$, $[M_{Av}]$ = 4629.36 Da

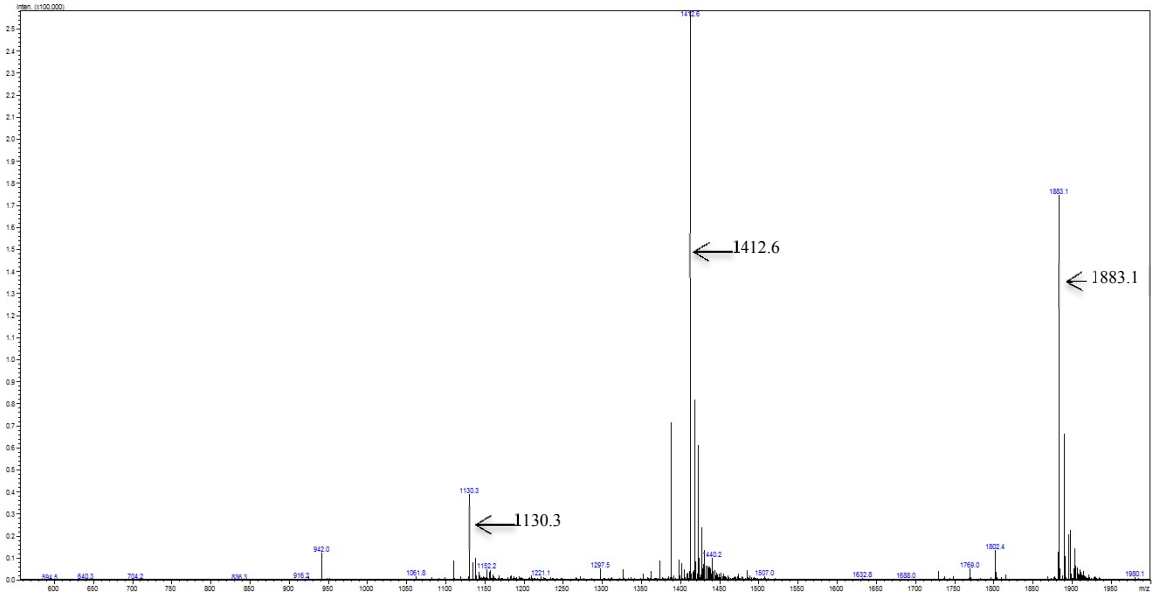
Found: *m/z* 1544.5 $[M+3H]^{3+}$, 1158.6 $[M+4H]^{4+}$, 927.1 $[M+5H]^{5+}$.

**11: H-Asn(Trt)-Asn(Trt)-Asp(*t*Bu)-Glu(*t*Bu)-Ser(*t*Bu)-Asn(Trt)-Ile-Ser($\psi^{Me,Me}$Pro)-Phe-Lys(Boc)-Glu(*t*Bu)-Lys(Boc)-NH($CH_2CH_2O)_2CH_2$-Gly-Val-Thr(α-D-GalNAc)-Ser(α-D-GalNAc)-Ala-Pro-Asp-Thr(α-D-GalNAc)-Arg-Pro-Ala-Pro-Gly-Ser(α-D-GalNAc)-Thr(α-D-GalNAc)-Ala-Pro-Pro-Ala-His-OH.**

The partially protected glycopeptide **11** was prepared by pentafluorophenyl ester-mediated fragment condensation of **5** (5.8 mg) and **7** (12.5 mg, 1.5 eq.) as outlined in the general procedures and purified by semi-preparative C4 HPLC (20 % B for 10 min to 100 % B over 60 min; *Eluent C*, $R_t$ 43 min) and isolated in 63 % yield (7.1 mg).






LC-MS ($ESI^+$) $R_t$ 22.5 min (0-100 % B over 30 min, λ = 230 nm; 0.1 % (v/v) formic acid in water (Solvent A) and 0.1 % (v/v) formic acid in acetonitrile (Solvent B))

*Injection peak, † Injection artefact with no associated mass.

Calculated for $C_{271}H_{388}N_{48}O_{83}$, $[M_{Av}]$ = 5645.76

Found: *m/z* 1883.1 $[M+3H]^{3+}$, 1412.6 $[M+4H]^{4+}$, 1130.3 $[M+5H]^{5+}$.

# 4 Immunological studies

## 4.1 Mice

Female C57BL/6 mice were obtained from the Animal Research Centre in WA, Australia. Mice were six weeks of age at the time of initial vaccination. All experiments were approved by the University of Sydney Animal Ethics Committee (approval number K14/9-2012/3/5840)

## 4.2 Vaccinations

Mice were vaccinated *via* two sub-cutaneous injections (one in each flank) of 50 μL PBS alone or PBS containing 1.75 nmol vaccine candidates **1-3** (a total of 3.5 nmol per vaccination).

## 4.3 Serum

Blood (100 μL) was collected *via* sub-mandibular bleed from mice one day prior to each vaccination.

## 4.4 MUC1 Antibody ELISA

MUC1-specific serum antibody titres were elucidated by standard indirect ELISA. Briefly, ELISA plates (BD Falcon) were coated with MUC1 VNTR (glyco)peptides (1 μg/mL) diluted in carbonate/bicarbonate coating buffer (0.05 M, pH 9.6) for 2 h at 37 °C. After washing 4 times with PBS + 0.05 % (v/v) Tween20 (PBST), plates were blocked with PBS containing BSA (1 % w/v) for 1 h at 37 °C and washed 4 times with PBST. Serum samples diluted in blocking buffer were added to each well and plates were incubated for 1 h at 37 °C and washed 4 times with PBST. Unless otherwise stated, sera were incubated with the immobilised MUC1 (glyco)peptides against which they were raised, and sera from PBS-treated mice were incubated with unglycosylated MUC1 peptides. Horseradish peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch and Life Technologies), diluted in blocking buffer according to the supplier's instructions, were added and plates were incubated for 30 min at 37 °C. Plates were then soaked in PBST and washed 4 times. TMB substrate (Life Technologies) was added, and after 20 min, an equal volume of aqueous hydrochloric acid (2 M) was added to stop the colourimetric reaction.

Absorbance was read at 450 nm on an ELISA plate reader, and endpoints were determined as $A_{450} < 0.100$.

## 4.5 MUC1-specific IgG cross-reactivity studies

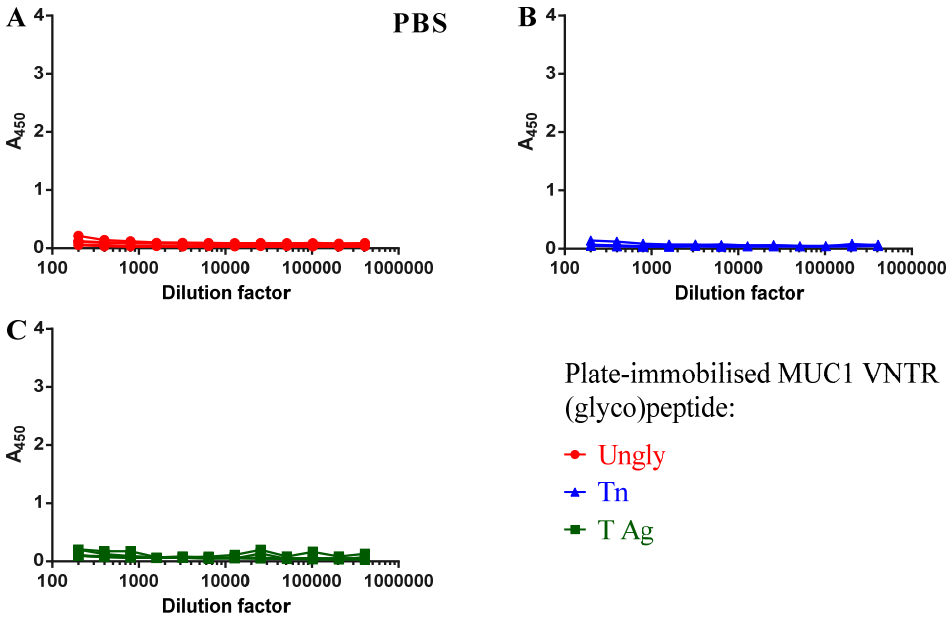



**Figure 4.5-1:** ELISA of Total IgG specific for **A)** unglycosylated MUC1, or MUC1 containing the **B)** Tn and **C)** T antigens in sera collected from PBS-treated mice on day 27 of the immunisation schedule.

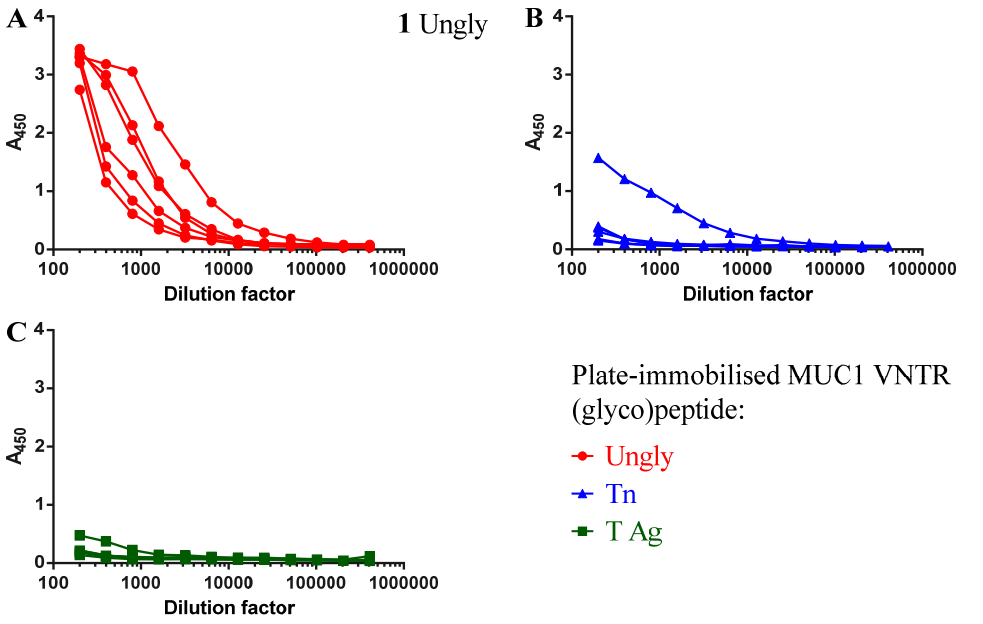



**Figure 4.5-2:** ELISA of Total IgG specific for **A)** unglycosylated MUC1, or MUC1 containing the **B)** Tn and **C)** T antigens in sera collected from mice immunised with unglycosylated vaccine candidate **1** on day 27 of the immunisation schedule.

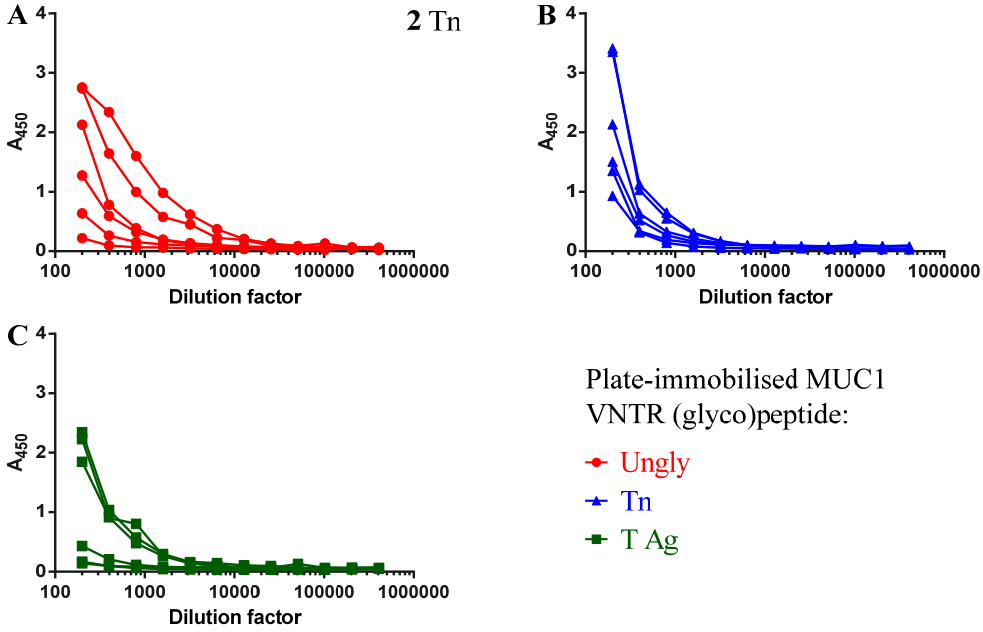



**Figure 4.5-3:** ELISA of Total IgG specific for **A)** unglycosylated MUC1, or MUC1 containing the **B)** Tn and **C)** T antigens in sera collected from mice immunised with Tn-containing vaccine candidate **2** on day 27 of the immunisation schedule.

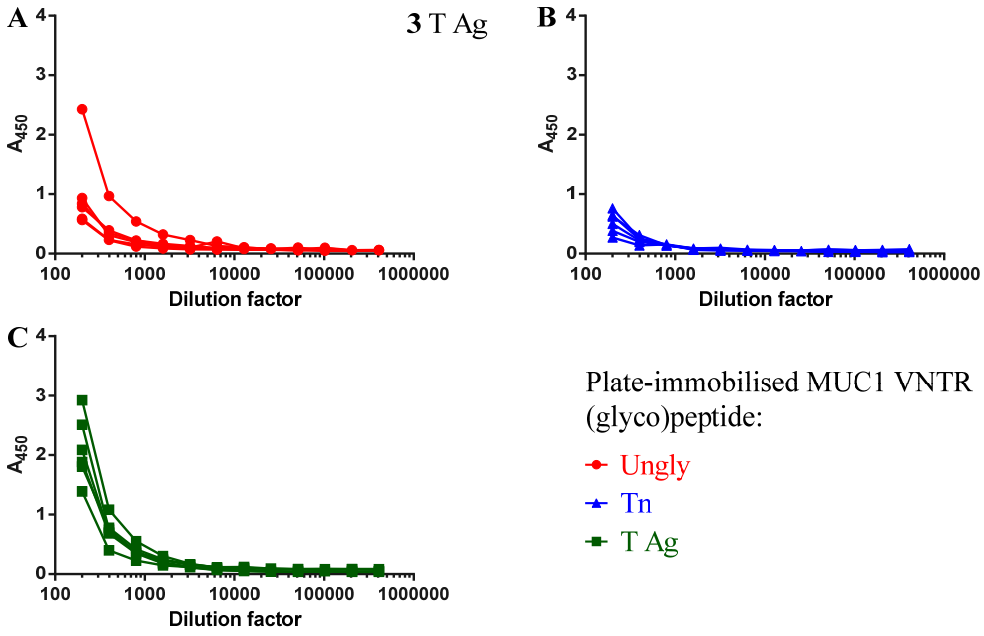



**Figure 4.5-4:** ELISA of Total IgG specific for **A)** unglycosylated MUC1, or MUC1 containing the **B)** Tn and **C)** T antigens in sera collected from mice immunised with T Ag-containing vaccine candidate **3** on day 27 of the immunisation schedule.

## 4.6 MUC1-specific antibody isotype ELISAs

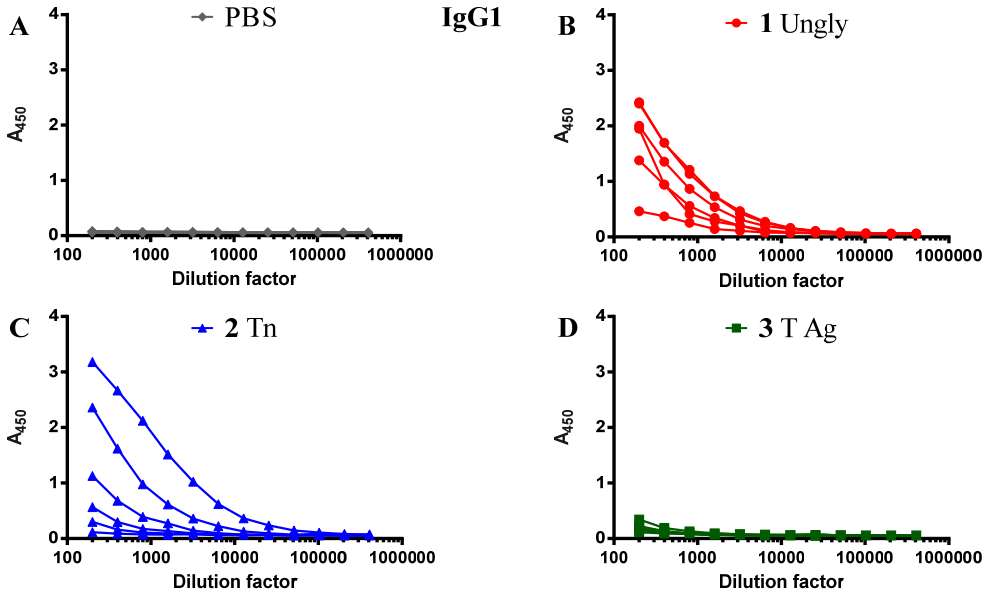



**Figure 4.6-1:** ELISA of MUC1-specific IgG1 in sera collected from mice immunised with **A)** PBS or **B-D)** Vaccine candidates **1-3** on day 27 of the immunisation schedule.

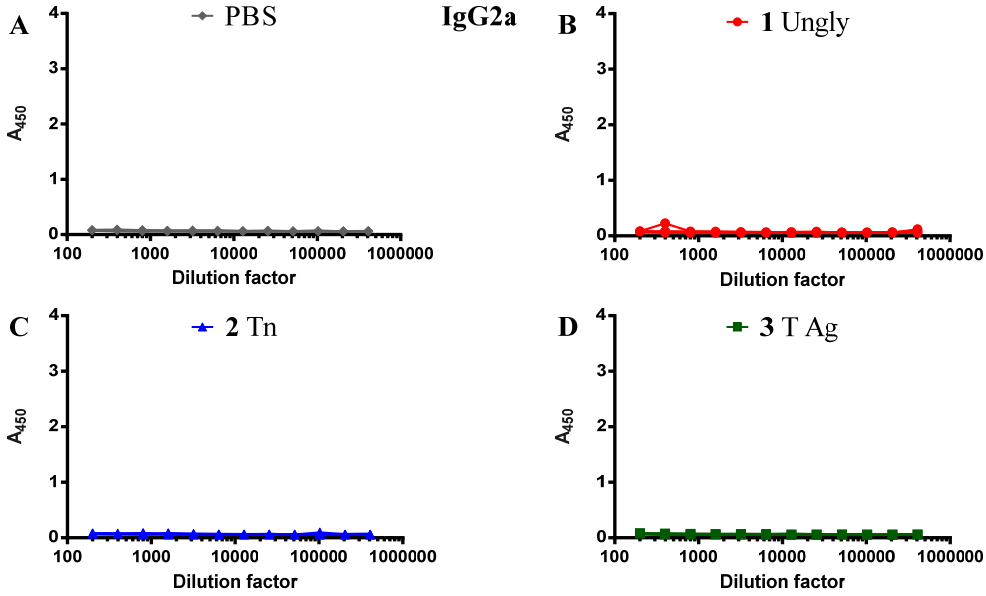



**Figure 4.6-2:** ELISA of MUC1-specific IgG2a in sera collected from mice immunised with **A)** PBS or **B-D)** Vaccine candidates **1-3** on day 27 of the immunisation schedule..

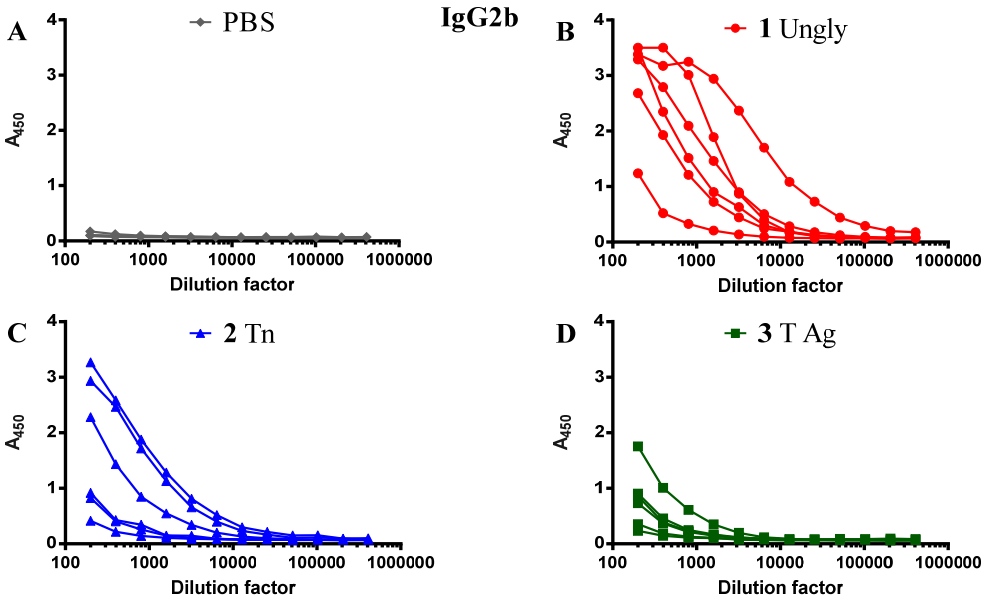



**Figure 4.6-3:** ELISA of MUC1-specific IgG2b in sera collected from mice immunised with **A)** PBS or **B-D)** Vaccine candidates **1-3** on day 27 of the immunisation schedule.

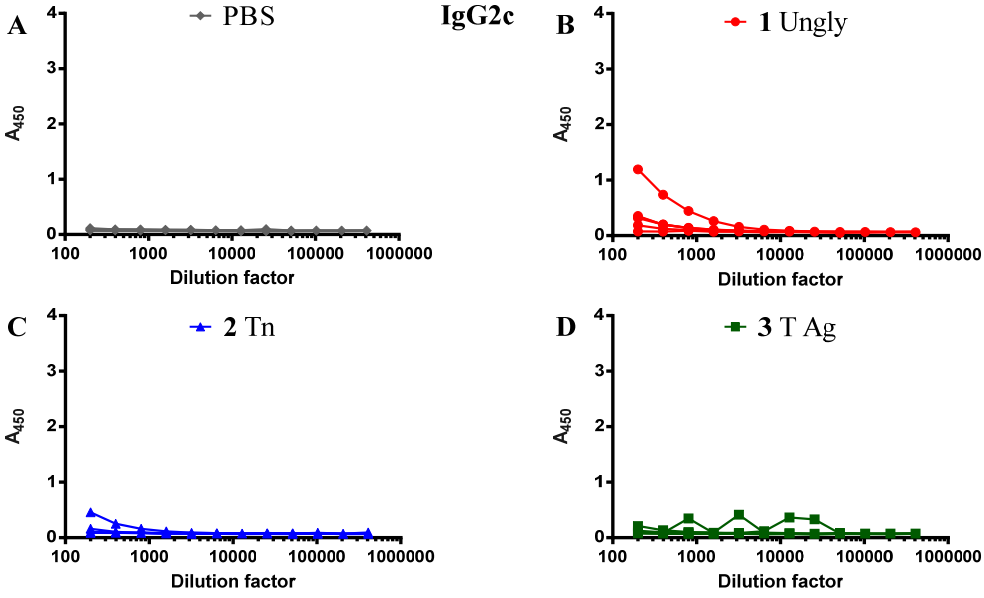



**Figure 4.6-4:** ELISA of MUC1-specific IgG2c in sera collected from mice immunised with **A)** PBS or **B-D)** Vaccine candidates **1-3** on day 27 of the immunisation schedule.

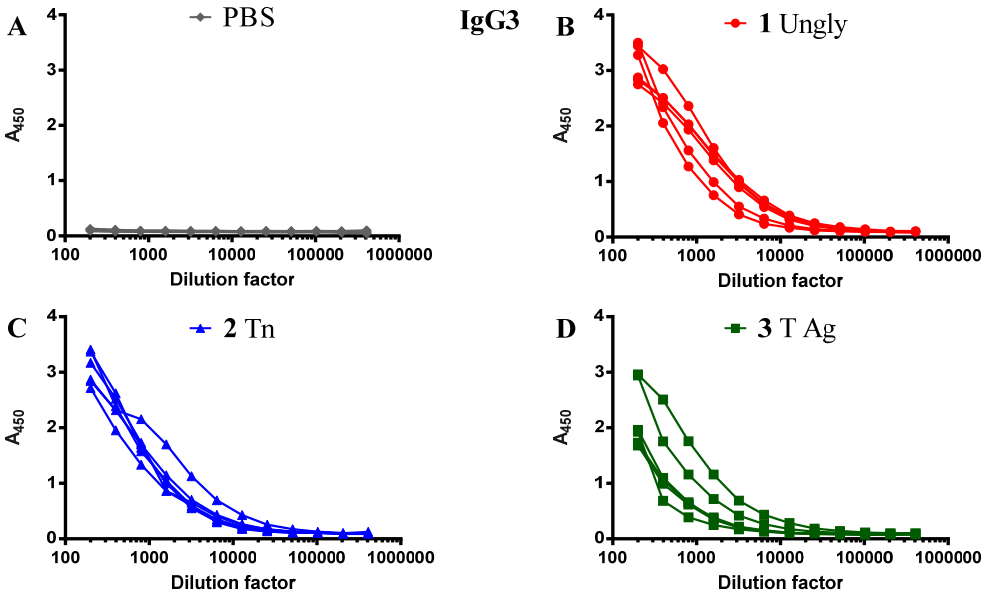



**Figure 4.6-5:** ELISA of MUC1-specific IgG3 in sera collected from mice immunised with **A)** PBS or **B-D)** Vaccine candidates **1-3** on day 27 of the immunisation schedule.

## 4.7 Intracellular cytokine staining

***Isolation of splenocytes***

Splenic single cell suspensions were generated by pressing spleens through an 80 µm stainless steel mesh sieve. Cells were washed twice with RPMI 1640 (Life Technologies) + 2 % foetal calf serum (FCS, Sigma Aldrich), counted and resuspended in RPMI 1640 + 10 % FCS.

**In vitro *activation and intracellular cytokine staining***

5 x $10^6$ splenocytes from mice immunised with PBS or vaccine candidates **1-3** were incubated at 37 °C overnight in 24-well plates (Corning), and then phorbol-12-myrsitate-13-acetate (PMA), ionomycin and Brefeldin A (Sigma Aldrich) were added to induce activation and prevent cytokines from being transported outside of the cell. After 4 h, cells were stained for flow cytometry in 96-well, round-bottom microtitre plates (Becton Dickinson, Corning). 1-2 x $10^6$ leukocytes suspended in PBS were added to each well. Cells were resuspended in 40 µL PBS containing 10 % normal mouse serum and fluorophore-conjugated antibodies (CD4-AlexaFluor700, CD8-APC_Cy7, CD25-APC, BioLegend, Becton Dickinson) and incubated for 30 min at 4 °C. Cells were washed twice with PBS and then with PBS containing Live/Dead UV (Becton Dickinson). Cells were washed twice then fixed for 30 min in fixation and permeabilisation buffer (eBioscience) at 4 °C. Cells were washed twice with permeabilisation buffer then resuspended in 40 µL staining buffer (eBioscience) containing fluorophore-conjugated antibodies specific for intracellular targets (IL-4-PerCP, IFN-γ-PE_Cy7, BioLegend) and incubated at 4 °C for a further 20 min. After two final washes in permeabilisation buffer, cells were resuspended in staining buffer, filtered through 70 µm nylon mesh and acquired on a flow cytometer.

***Flow cytomtry***

Flow cytometry was carried out using an LSR-II or LSR-Fortessa (Becton Dickinson), and data was acquired using BD FACSDiva software (Becton Dickinson). Flow cytometry data was analysed using FlowJo software (TreeStar Inc.) version 10 for Macintosh OSX.

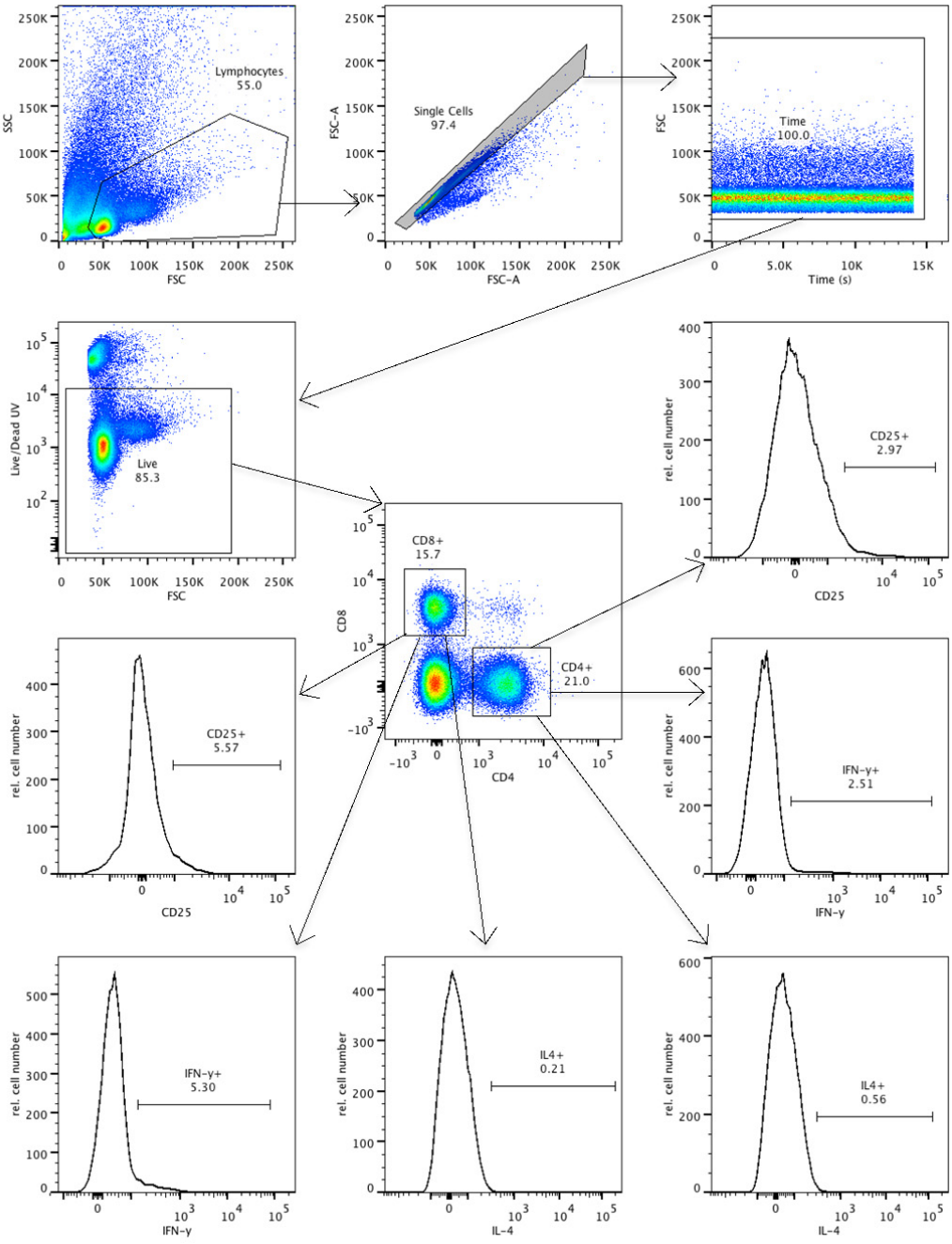



**Figure 4.7-1:** Representative gating strategy for the identification of $CD25^+$, IFN-$\gamma^+$ and IL-$4^+$ populations of $CD8^+$ and $CD4^+$ T cells by intracellular flow cytometry.

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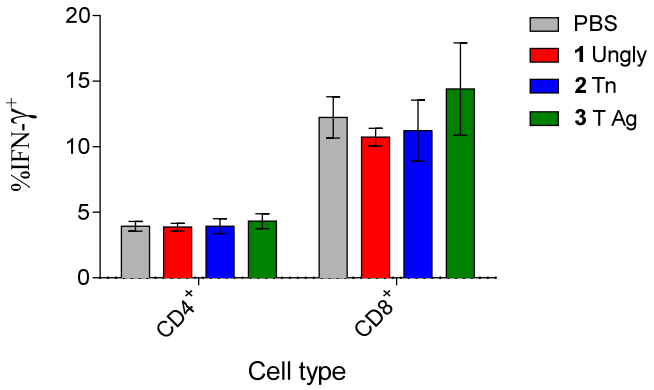


**Figure 4.7-2:** IFN-$\gamma^+$ splenocytes isolated from mice immunised with PBS or vaccine candidates **1-3** on day 28 of the immunisation schedule. Bars represent median ± interquartile range of n = 6 C57BL/6 mice.

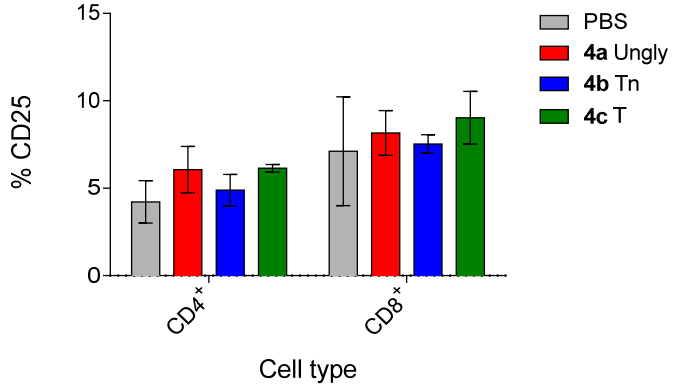


**Figure 4.7-3:** $CD25^+$ splenocytes isolated from mice immunised with PBS or vaccine candidates **1-3** on day 28 of the immunisation schedule. Bars represent median ± interquartile range of n = 6 C57BL/6 mice.

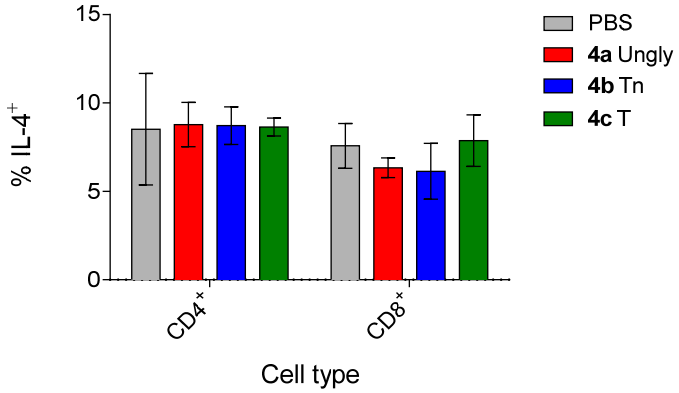


**Figure 4.7-4:** IL-$4^+$ splenocytes isolated from mice immunised with PBS or vaccine candidates **1-3** on day 28 of the immunisation schedule. Bars represent median ± interquartile range of n = 6 C57BL/6 mice.

## 4.8 *In vivo* CTL assays

### *CFSE and CTO labelling*

5-carboxyfluorescein di-acetate succinimidyl ester (CFSE) and cell tracker orange (CTO) labelling was performed as described previously.[3] Briefly, single cell suspensions were resuspended in RPMI 1640 (serum free) containing 1 μM or 10μM CFSE or CTO (Life Technologies). Cells were incubated at 37 $^{o}$C for 10 min in the dark with shaking, before staining was quenched with the addition of an equal volume of RPMI 1640 + 2 % FCS. resuspended in RPMI 1640 + 2 % FCS and incubated at 37 °C for 30 min. Cells were washed twice with RPMI 1640 + 2 % FCS and then counted and resuspended in the appropriate medium.

### *Adoptive transfer*

Single cell suspensions were resuspended in the appropriate volume of RPMI 1640 + 2 % FCS and 300 μL was injected into the tail vein of recipient mice.

### In vivo *CTL assay*

Single cell suspensions of naïve C57BL/6 splenocytes were resuspended in RPMI 1640 + 10 % FCS. Target cells were incubated for 45 min at 37 °C with 2 μg/mL MUC1 (glyco)peptide epitopes SAPDT*RPAP (* = unglycosylated, Tn or T antigens) and control cells were incubated with peptide with a scrambled sequence (PSAPRPDTA). Cells were then washed twice with RPMI 1640 + 2 % FCS and then stained with CFSE or CTO as described above. Target cells were stained with 10 μM CFSE (unglycosylated epitope), 10 μmol CTO (Tn-containing epitope) or 1 μmol CTO (T Ag-containing epitope), while control cells were stained with 1 μM CFSE. Equal numbers of target and control cells were adoptively transferred into recipient mice, and spleens were harvested 16 h later. Splenocytes were prepared for flow cytometry as described above, and CFSE/CTO intensity used to discriminate recovered target and control cells. % specific lysis was calculated as follows:

$$\left(1 - \frac{r^{naive}}{r^{sample}}\right) * 100, \text{ where } r = \frac{\#\ control\ cells}{\#\ target\ cells}$$

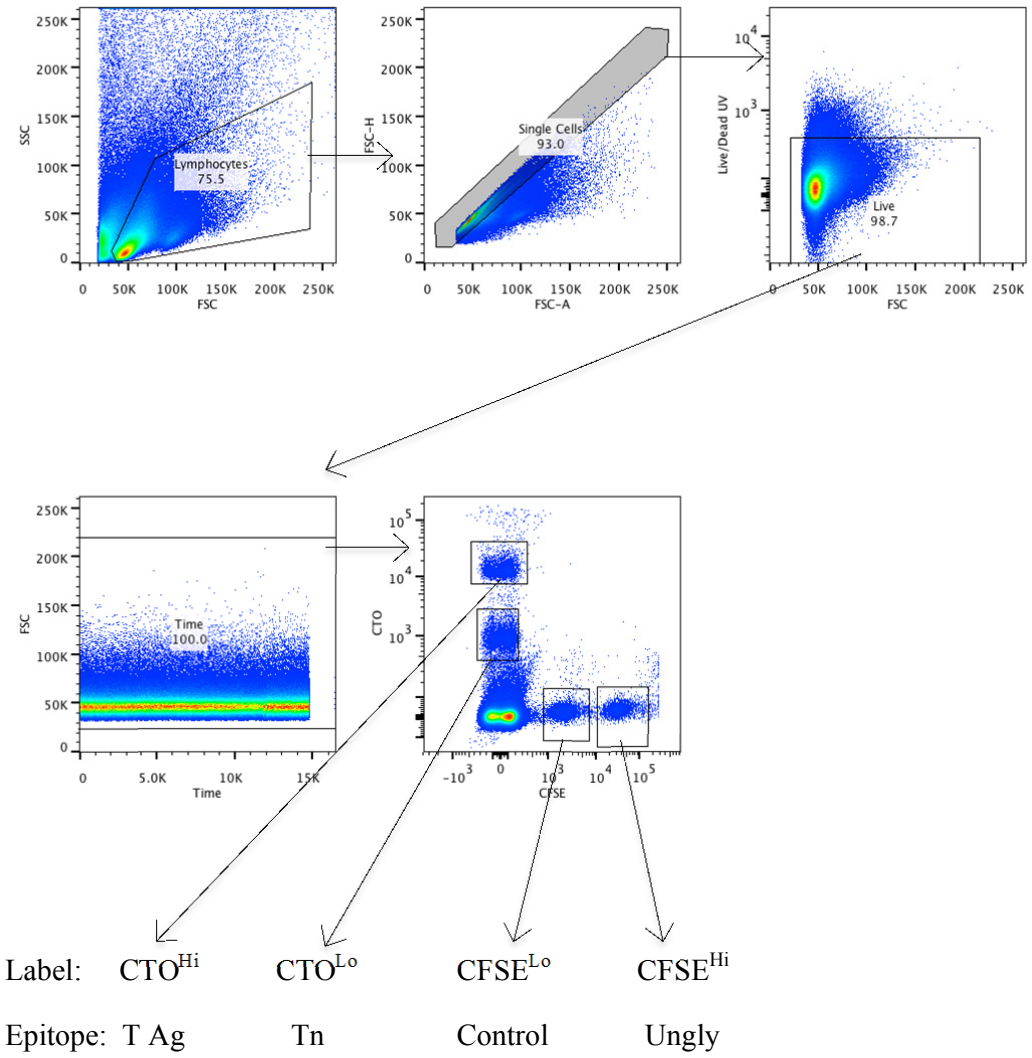


**Figure 4.8-1:** Representative gating strategy for the identification of CFSE- and CTO-labelled splenocytes by flow cytometry.

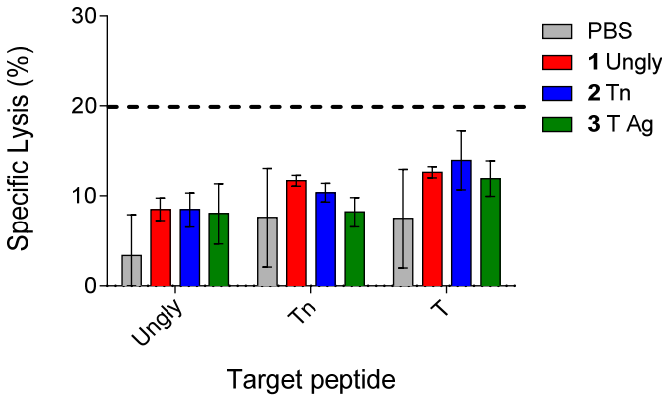


**Figure 4.8-2:** *in vivo* CTL function of mice immunised with PBS or vaccine candidates **1-3.** Mice were harvested on day 28 of the immunisation schedule. CTL activity was measured against unglycosylated, Tn and T antigen-containing MUC1 (glyco)peptide epitopes. Specific lysis values below 20 % are generally considered to be negative results. Bars represent median ± interquartile range of n = 6 C57BL/6 mice.

## References


1. Y. Zhang, S. M. Muthana, D. Farnsworth, O. Ludek, K. Adams, J. J. Barchi, Jr. and J. C. Gildersleeve, *J. Am. Chem. Soc.*, 2012, **134**, 6316-6325.
2. B. L. Wilkinson, L. R. Malins, C. K. Chun and R. J. Payne, *Chem. Commun.*, 2010, **46**, 6249-6251.
3. Lyons, A. B. & Parish, C. R. *J. Immunol. Methods,* 1994, **171,** 131-7.