Electronic Supplementary Information

Fully synthetic invariant NKT cell-dependent self-adjuvanting antitumor vaccines eliciting potent immune response in mice

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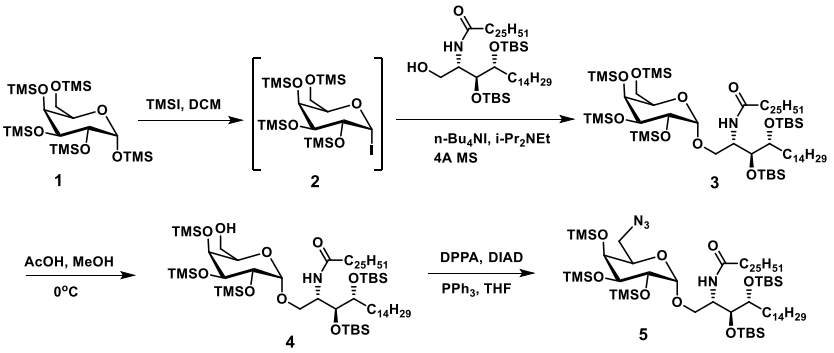
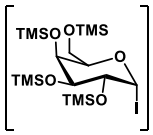
General Information.

All chemical reagents were obtained from TCI, Alfa-Aesar, Sigma companies. Fmoc protected amino acid building blocks, coupling regents and resins were purchased from GL BioChem. The synthesis of peptides and glycopeptides were followed by standard Fmoc solid phased peptide synthesis strategy. All glycopeptides and glycopeptide conjugates were purified by Shimadzu LC-6AD with preparative C18 column (YMC, Japan, 5 μm, 20×250 mm) and analyzed by Shimadzu LC-2010A with analytic C18 column (YMC, Japan, 5μm, 4.6×150 mm). The eluents of RP-HPLC were solution A (80% MeCN/H_2O with 0.06% TFA), solution B (100% H_2O with 0.06% TFA) and solution C (100% MeOH with 0.06% TFA). The deprotection of acetyl groups on glycopeptides was catalyzed by 1% MeONa in MeOH for 20 hours at room temperature. After RP-HPLC purification, the glycopeptides and glycopeptide conjugates were obtained by lyophilization. The glycopeptides were identified by Thermo Scientific UltiMate 3000 (ESI-MS). The glycopeptide conjugates were identified by an Applied Bio-systems 4700 Proteomics Analyzer 283 with the matrix of 2, 5-dihydroxybenzoic acid (DHB) or α-cyano-4-hydroxycinnamic acid (CHCA). The synthesis of Fmoc-Thr(α-Ac_3GalNAc)-OH and alkynyl spacer building blocks were according to reported protocols. All ^{1}H-NMR spectra were measured on a 400MHz Jeol-ECA-300 spectrometer. The immunization and immunological evaluations of vaccine candidates were processed as reported methods.[1-4]



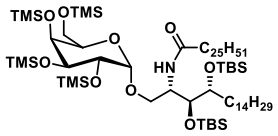
Scheme S1 The synthesis of azide-modified α-GalCer **5**.

Trimethylsilyl 2,3,4,6-tetrakis-O- iodo-α-D-galactopyranoside (2)



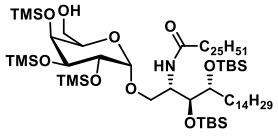
To a solution of per-trimethylsilyl galactose **1** (812 mg, 1.5 mmol) in 6 ml DCM was added 203 μL trimethylsilyl iodide (1.5 mmol) at 0°C. After stirring under Ar atmosphere for 15 mins, the reaction mixture was added 7ml benzene and then removed the solvent under reduced pressure to afford the glycosyl iodide intermediate **2** without further purification.

(2S, 3S, 4R)-2-[(N- hexacosanoyl) amino]-3,4-di-tert-butyldimethylsilyloxyoctadecan- 1-O-(2,3,4,6 -tetrakis-O-trimethylsilyl-α-D-galactopyranosyl)octadecane (3)



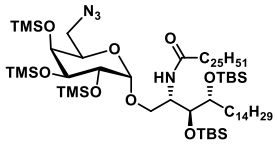
The glycosyl acceptor alcohol was synthesized according to reported protocols. To a solution of 462 mg di-tert-butyldimethylsilyl protected alcohol (0.5 mmol) in 6 ml dry DCM was added 1.11 g tetrabutylammonium iodide (3 mmol), 1ml N, N-diisopropylethylamine (2.25 mmol) and1.03 g activated 4Å molecular sieves. The reaction mixture was stirred at room temperature for 15min under Ar atmosphere and further dropwise added with the prepared glycosyl iodide intermediate **2** in 3 ml DCM over 5 mins. The resulting mixture was further stirred overnight at room temperature. After filtered by diatomite, the solvent was removed under reduced pressure and the mixture was added with 20 mL Et_2O and 20 ml H_2O. The organic phase was separated and dried by $MgSO_4$. After concentrated under reduced pressure, the resulting solid was purified by column chromatography (1.7% EtOAc in hexane) to give 303 mg per-protected α-galactosylceramide **3** with only α-anomer as colorless oil. (0.22mmol, 44.1% yield) ^{1}H NMR (400MHz, $CDCl_3$): δ = 6.03 (d, 1H, J = 4 Hz), 4.65 (d, 1H, J = 4 Hz), 3.98 (dd, 1H, J_1 = 4Hz, J_2 = 4 Hz), 3.91 (m, 4H), 3.78-3.72 (m, 4H), 3.63 (dd, 2H, J_1 = 12Hz, J_2 = 4 Hz), 3.51 (dd, 1H, J_1 = 12 Hz, J_2 = 4 Hz), 2.13 (dt, 2H J_1 = 8 Hz, J_2 = 4 Hz), 1.50 (s, 1H), 1.25 (s, 68H), 0.90 (s, 18H), 0.87 (d, 6H, J = 8 Hz), 0.12 (d, 48H, J =12 Hz). ^{13}C NMR (100MHz, $CDCl_3$): δ =174.2, 101.1, 76.7, 71.6, 71.4, 71.3, 70.7, 70.0, 52.0, 37.8, 34.3, 32.6, 30.6, 30.4, 30.2, 30.0, 27.9, 26.8, 26.7, 26.4, 23.4, 19.0, 18.9, 14.8, 1.3, 1.1, 1.0, -3.0, -3.3, -3.8, -4.1. HR ESI Calcd for $C_{74}H_{159}NO_9Si_6$ $[M+H]^+$:1376.6; ESI MS found: $[M+H]^+$:1376.2.

(2S, 3S, 4R)-2-[(N- hexacosanoyl)amino]-3,4-di-tert-butyldimethylsilyloxy-1-O- (2,3,4-tri-O-trimethylsilyl-α-D-galactopyranosyl)octadecane (4)



To a solution of 100 mg per-protected α-galactosylceramide **3** (73 μmol) in 4 mL THF was added with 50 μL AcOH at 0°C. Then the reaction mixture was stirred at room temperature and detected by TLC. When the reaction was completed, the mixture was neutralized by adding with 80 mg $NaHCO_3$. After that, the mixture was filtered and concentrated. After purified by column chromatography (2.5% EtOAc in hexane), 53 mg alcohol **4** was obtained colorless oil. (41μmol, 56%) ^{1}H NMR (400MHz, $CDCl_3$): δ = 6.01 (d, 1H, J = 8 Hz), 4.77 (d, 1H, J = 4 Hz), 4.29 (m,1H), 4.06 (dd, 1H, J_1 =12Hz, J_2 =4Hz), 3.91(m, 2H), 3.84 (m, 2H), 3.66 (m, 2H), 2.14 (dd, 2H, J_1 =16Hz, J_2 =8Hz), 1.61 (m, 2H), 1.25 (s, 70H), 0.91 (s,18H), 0.88 (s, 6H), 0.14 (s, 39H). ^{13}C NMR (100MHz, $CDCl_3$): δ =173.7, 102.9, 76.3, 75.0, 73.5, 71.1, 70.9, 70.3, 69.9, 52.1, 37.6, 33.8, 32.6, 30.6, 30.4, 30.2, 30.1, 26.8, 26.7, 26.3, 23.4, 22.3, 19.0, 18.8, 14.8, 1.3, 1.1, 1.0, -3.0, -3.3, -3.9, -4.2. HR ESI Calcd for $C_{71}H_{151}NO_9Si_5$ $[M+H]^+$:1304.4; ESI MS found: $[M+H]^+$:1304.1.

(2S, 3S, 4R)-2-[(N- hexacosanoyl)amino]-3,4-di-tert-butyldimethylsilyloxy-1-O-(6-deoxy -6-azido-2,3,4-tri-O-trimethylsilyl-α-D-galactopyranosyl)octadecane (5)



To a solution of 90mg alcohol **4** (69 μmol) in 1.5 mL THF was sequentially added with 42 mg triphenylphosphine (138 μmol), 32 μl diisopropyl azodicarboxylate (138 μmol) and 35 μL diphenylphoryl azide (138 μmol) at 0°C. The reaction mixture was stirred at room temperature overnight. After that, the mixture was concentrated by reduced pressure and purified by column chromatography (1.7% EtOAc in hexane) to afford 63 mg azide-modified α-galactosylceramide **5** (48 μmol, 69%) as colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 6.01 (d, 1H, *J* = 8 Hz), 4.67 (d, 1H, *J* = 8 Hz), 4.14 (m, 1H), 3.83 (m, 8H), 3.66 (t, 1H, *J* =4 Hz), 3.49 (dd, 1H, J_1 = 8 Hz, J_2 = 4 Hz), 3.31 (dd, 1H, J_1 = 8 Hz, J_2 = 4 Hz), 2.15 (t, 2H, *J* = 8 Hz), 1.61 (t, 2H, *J* = 4 Hz), 1.25 (m, 70H), 0.90 (s, 18H), 0.87 (d, 6H, *J* = 8 Hz), 0.15-0.05(m, 46H). ^{13}C NMR (100MHz, $CDCl_3$): δ = 173.7, 103.0, 76.3, 73.6, 71.2, 71.0, 70.4, 70.0, 52.1, 37.7, 33.9, 32.6, 30.6, 30.4, 30.2, 30.1, 26.8, 26.7, 26.3, 23.4, 19.0, 18.9, 14.8, 1.3, 1.1, 1.0, -2.8, -3.1, -3.8, -4.0. HR ESI Calcd for $C_{71}H_{150}N_4O_8Si_5$ $[M+H]^+$:1329.4; ESI MS found: $[M+H]^+$:1329.3.

Alkynyl linker-His-Gly-Val-Thr-Ser-Ala-Pro-Asp-Thr-Arg-Pro-Ala-Pro-Gly-Ser-Thr-Ala-Pro-Pro -Ala-OH. (6)

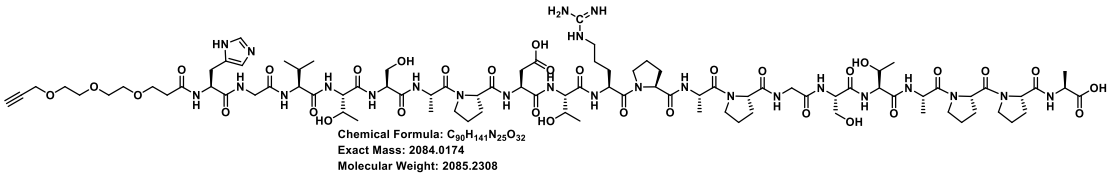


Figure S1 The chemical structure of compound **6**.

681 mg Fmoc-Ala-Wang resin of 0.15 mmol scale was used for coupling. Rt= 19.1 min (A/B: (5/95)→(40/60), 30 min). 52 mg product was obtained after deprotection and purification. Overall yield was 17%.

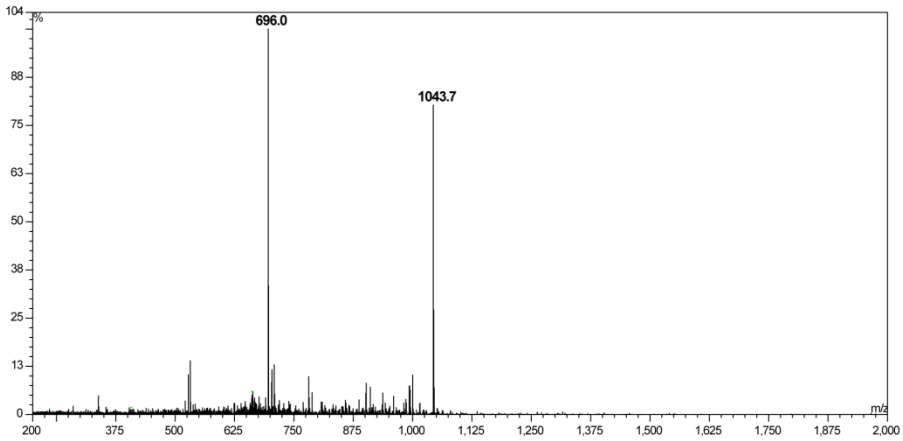


Figure S2 The ESI-MS spectrum of compound **6**. Calcd $[M+H]^+$: 2086.2; ESI MS found: $[M+2H]^{2+}$ 1043.7, $[M+3H]^{3+}$ 696.0.

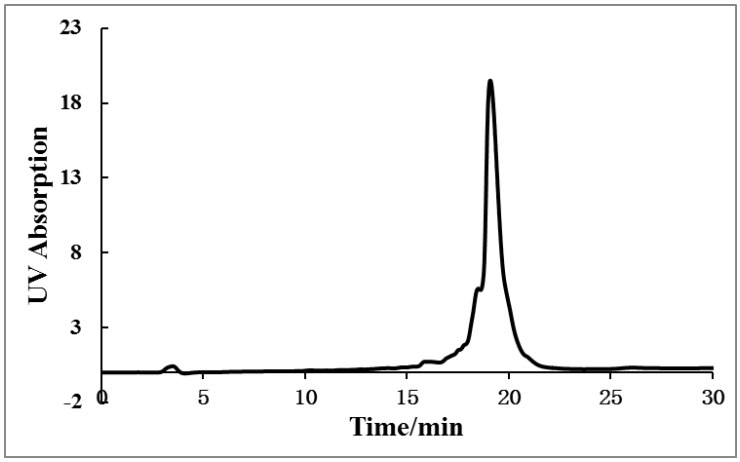


Figure S3 The analytic RP-HPLC spectrum of compound **6.** Analytic gradient is 5% to 40% of solution A in 30min. Retention time is 19.1 min.

Alkynyl linker-His-Gly-Val-Thr-Ser-Ala-Pro-Asp-Thr-Arg-Pro-Ala-Pro-Gly-Ser-Thr (α-GalNAc)- Ala-Pro-Pro-Ala-OH. (7)

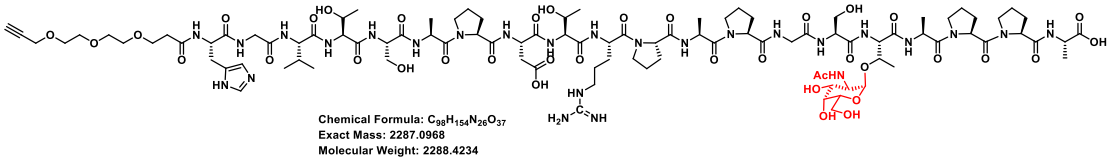


Figure S4 The chemical structure of compound **7**.

681 mg Fmoc-Ala-Wang resin of 0 .15 mmol scale was used for coupling. Rt= 18.4 min (A/B: (5/95)→(40/60), 30 min). 42 mg product was obtained after deprotection and purification. Overall yield was 12%.

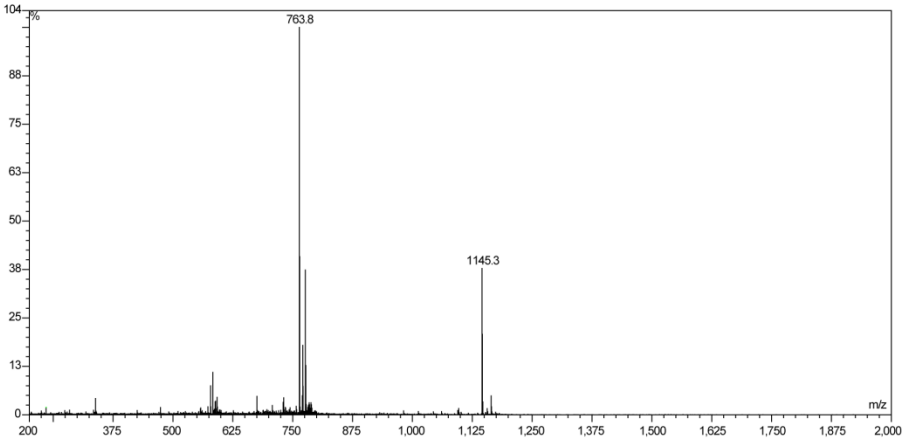


Figure S5 The ESI-MS spectrum of compound **7**. Calcd $[M+H]^+$: 2289.4; ESI MS found: $[M+2H]^{2+}$ 1145.3, $[M+3H]^{3+}$ 763.8.

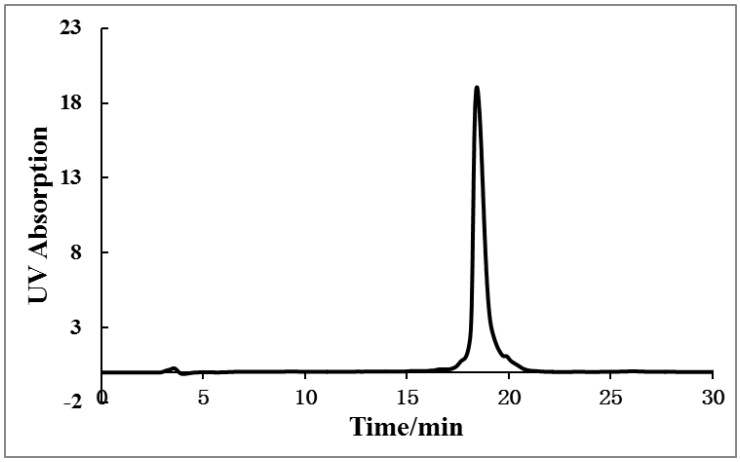


Figure S6 The analytic RP-HPLC spectrum of compound **7**. Analytic gradient is 5% to 40% of solution A in 30 min. Retention time is 18.4 min.

Alkynyl linker-His-Gly-Val-Thr-Ser-Ala-Pro-Asp-Thr(α-GalNAc)-Arg-Pro-Ala-Pro-Gly-Ser-Thr -Ala-Pro-Pro-Ala-OH. (8)

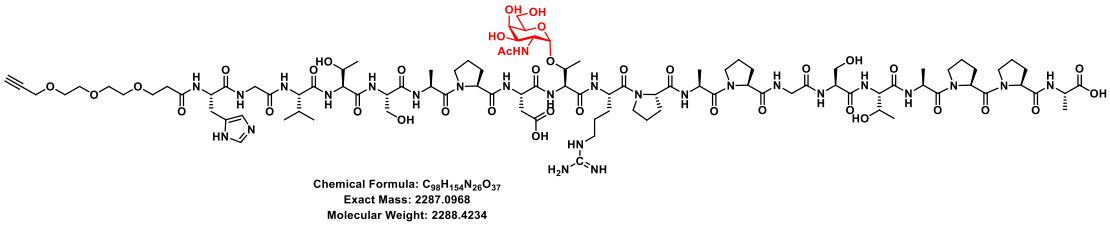


Figure S7 The chemical structure of compound **8**.

681 mg Fmoc-Ala-Wang resin of 0.15 mmol scale was used for coupling. Rt= 18.3min (A/B: (5/95)→(40/60), 30min). 38 mg product was obtained after deprotection and purification. Overall yield was 11%.

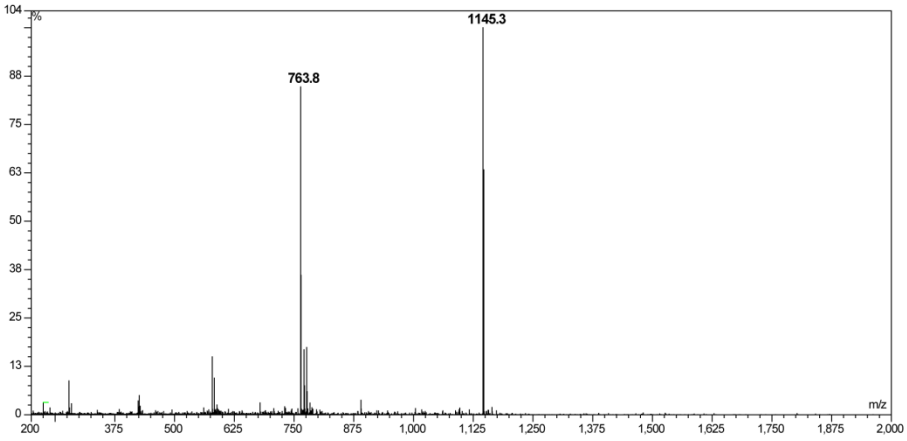


Figure S8 The ESI-MS spectrum of compound **8**. Calcd $[M+H]^+$: 2289.4; ESI MS found: $[M+2H]^{2+}$ 1145.3, $[M+3H]^{3+}$ 763.8.

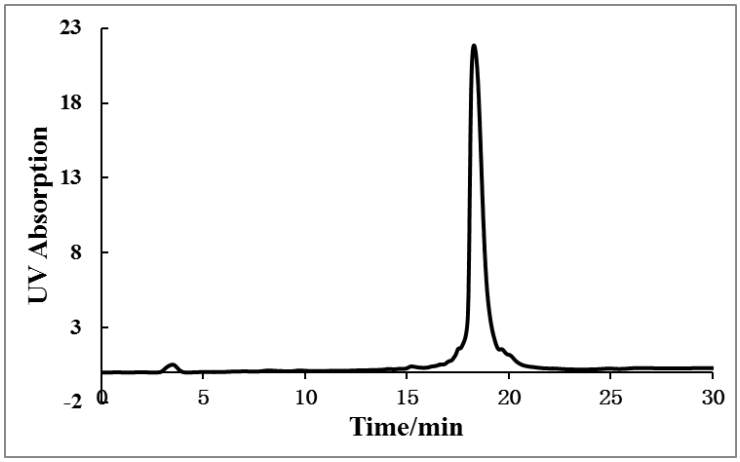


Figure S9 The analytic RP-HPLC spectrum of compound **8**. Analytic gradient is 5% to 40% of solution A in 30min. Retention time is 18.3 min.

Alkynyl linker-His-Gly-Val-Thr-Ser-Ala-Pro-Asp-Thr(α-GalNAc)-Arg-Pro-Ala-Pro-Gly-Ser-Thr (α-GalNAc)-Ala-Pro-Pro-Ala-OH. (9)

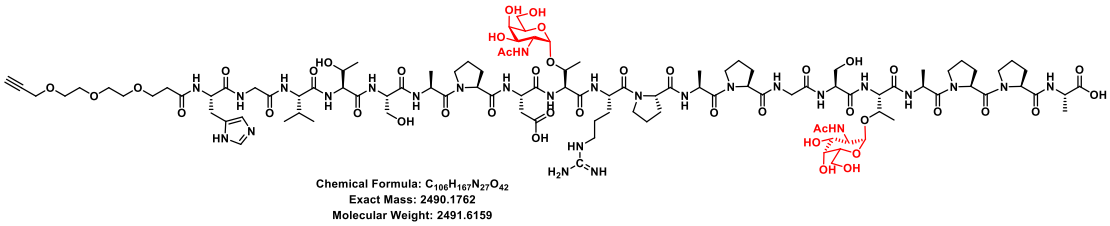


Figure S10 The chemical structure of compound **9**.

681 mg Fmoc-Ala-Wang resin of 0 .15 mmol scale was used for coupling. Rt= 17.4min (A/B: (5/95)→(40/60), 30min). 35 mg product was obtained after deprotection and purification. Overall yield was 9%.

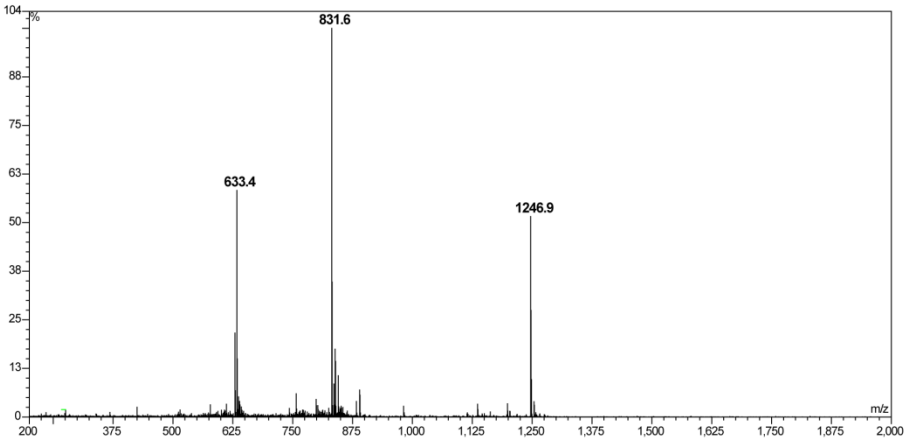


Figure S11 The ESI-MS spectrum of compound **9**. Calcd $[M+H]^+$: 2492.6; ESI MS found: $[M+2H]^{2+}$ 1246.9, $[M+3H]^{3+}$ 831.6, $[M+4H]^{4+}$ 633.4.

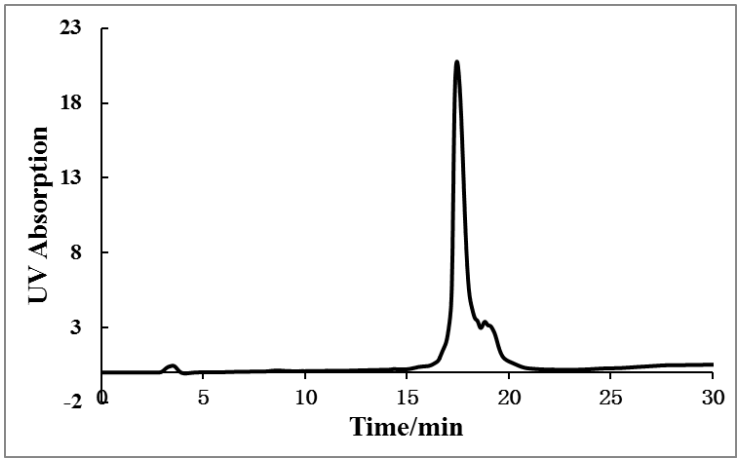
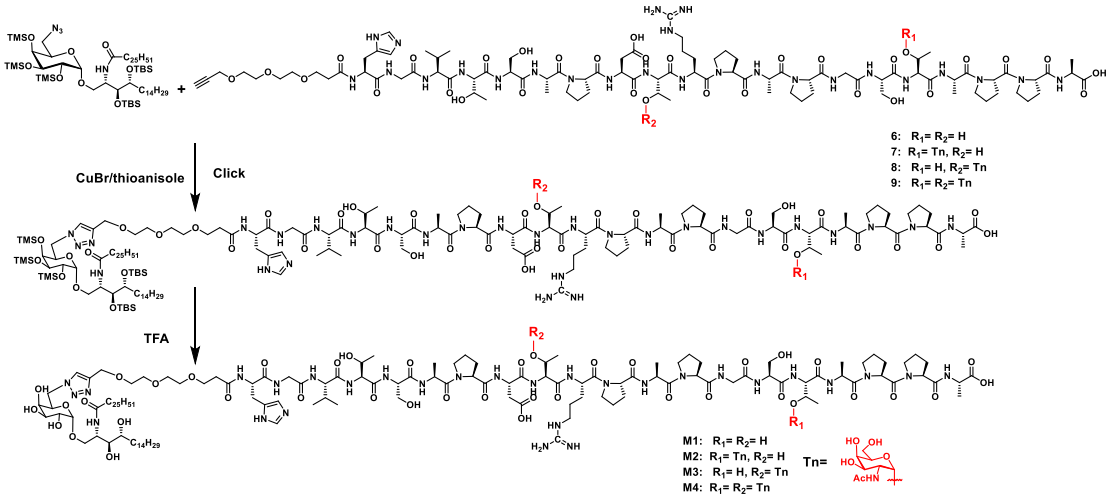


Figure S12 The analytic RP-HPLC spectrum of compound **9**. Analytic gradient is 5% to 40% of solution A in 30min. Retention time is 17.4 min.



Scheme S2 The synthesis of vaccine candidates **M1-M4**.

Vaccine candidate M1

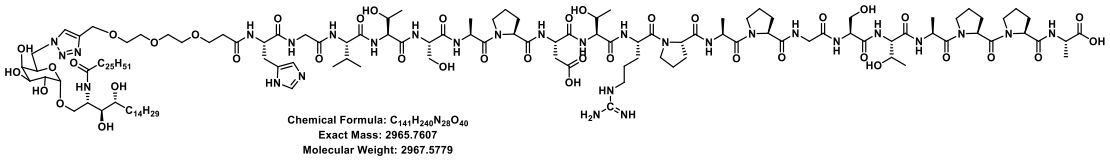


Figure S13 The chemical structure of compound **M1**.

To a solution of 3.8 mg azide-modified α-galactosylceramide **5** (2.9 μmol) in 200 μl DMF was added with 6.6 mg compound **6** (3.2 μmol), 0.8mg CuBr (5.8 μmol) and 20 μl thioanisole. The reaction mixture was stirred at room temperature for 5 hours. After that, 200 μl TFA was added into the mixture and stirred for another 30 mins to remove all protection groups in azide-modified α-galactosylceramide **5**. Then the solvent was removed under reduced pressure and the residue was purified by RP-HPLC in C18 column. After lyophilization, 2.2 mg white powder (7.4 μmol, 26%) to give vaccine candidate **M1**. The purified product was analyzed by analytic RP-HPLC and MALDI-TOF MS.

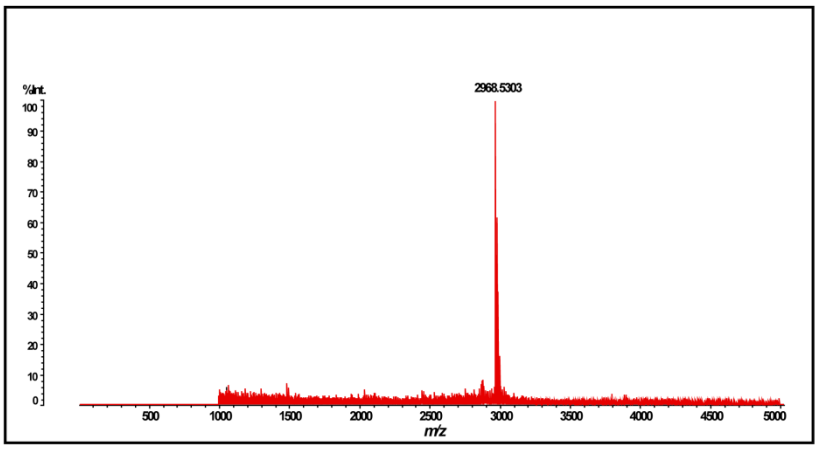


Figure S14 The MALDI-TOF MS spectrum of compound **M1**. Calcd for $C_{141}H_{240}N_{28}O_{40}$ $[M+H]^+$: 2968.5779; MALDI-TOF MS found: 2968.5303

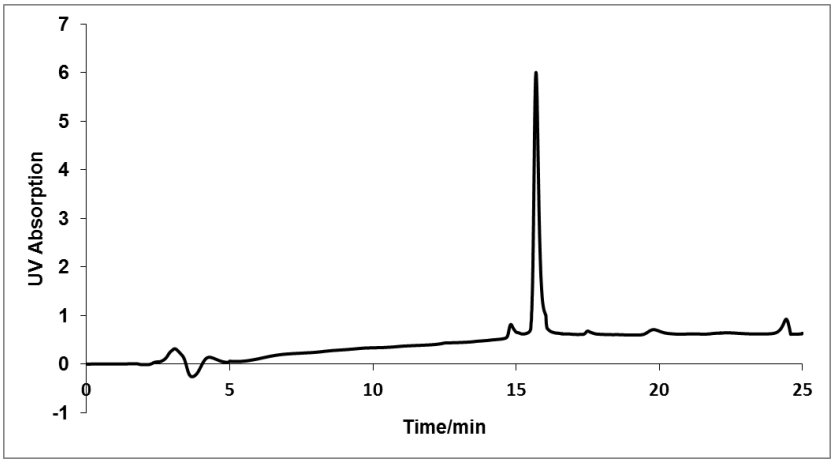


Figure S15 The analytic RP-HPLC spectrum of compound **M1**. Analytic gradient is 80% to 100% of solution C in 10min and 100% solution C for further 9min. Retention time is 15.7 min.

Vaccine candidate M2

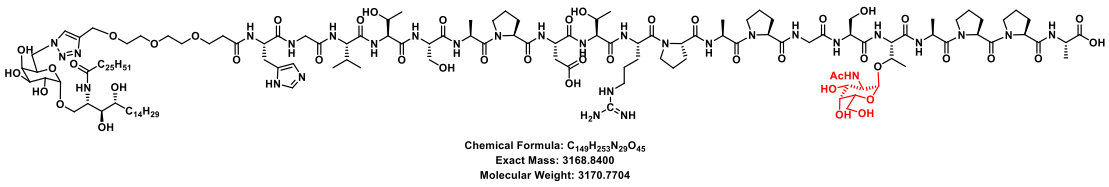


Figure S16 The chemical structure of compound **M2**.

The synthesis of vaccine candidate **M2** was according to protocol of **M1**. After lyophilization, 1.5 mg white powder (4.7 μmol, 25%) to give vaccine candidate **M2**. The purified product was analyzed by analytic RP-HPLC and MALDI-TOF MS.

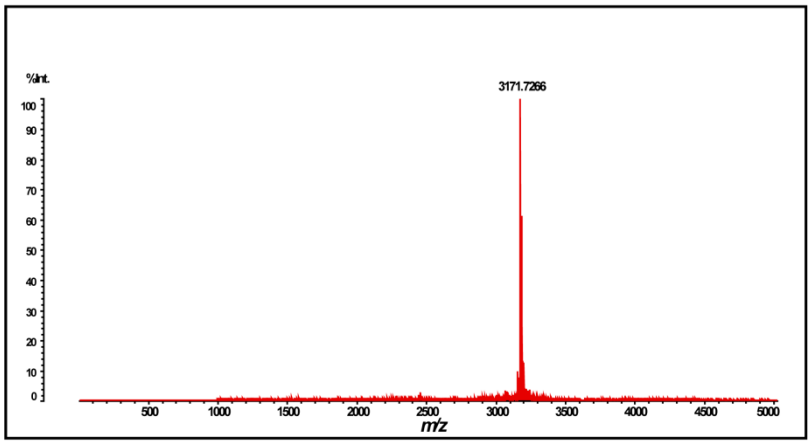


Figure S17 The MALDI-TOF MS spectrum of compound **M2**. Calcd for $C_{149}H_{253}N_{29}O_{45}$ $[M+H]^+$: 3171.7704; MALDI-TOF MS found: 3171.7266

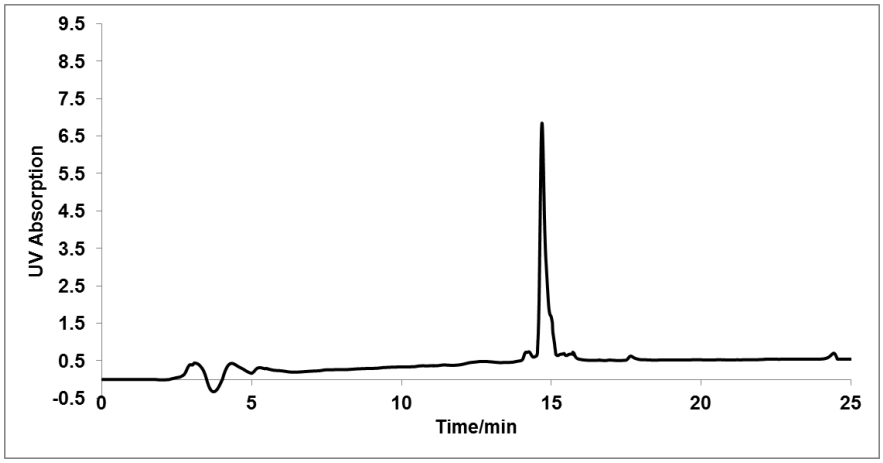


Figure S18 The analytic RP-HPLC spectrum of compound **M2**. Analytic gradient is 80% to 100% of solution C in 10min and 100% solution C for further 9min. Retention time is 14.7 min.

Vaccine candidate M3

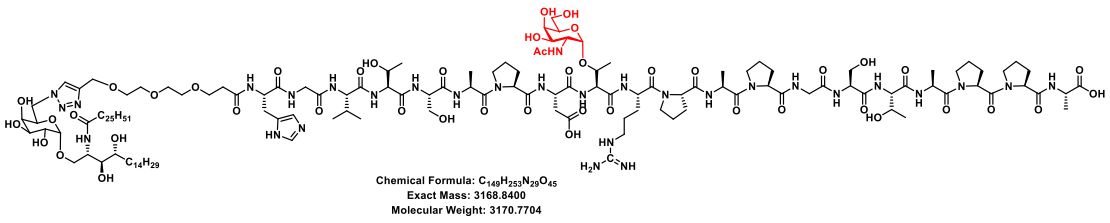


Figure S19 The chemical structure of compound **M3**.

The synthesis of vaccine candidate **M3** was according to protocol of **M1**. After lyophilization, 1.8 mg white powder (5.7 μmol, 23%) to give vaccine candidate **M3**. The purified product was analyzed by analytic RP-HPLC and MALDI-TOF MS.

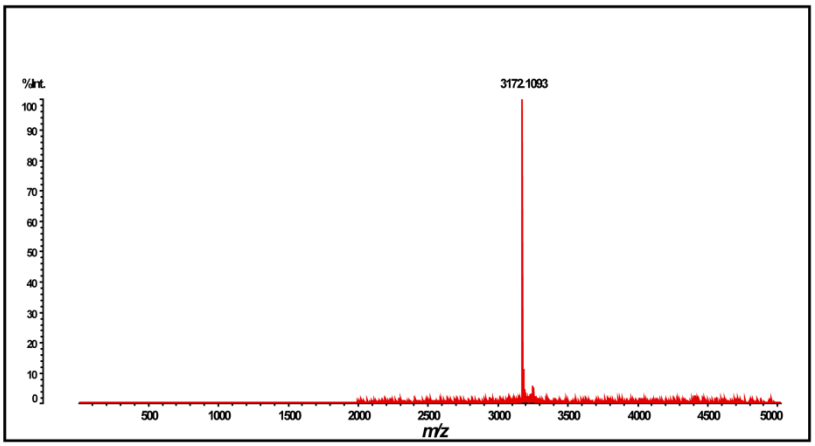


Figure S20 The MALDI-TOF MS spectrum of compound **M3**. Calcd for $C_{149}H_{253}N_{29}O_{45}$ $[M+H]^+$: 3171.7704; MALDI-TOF MS found: 3172.1093.

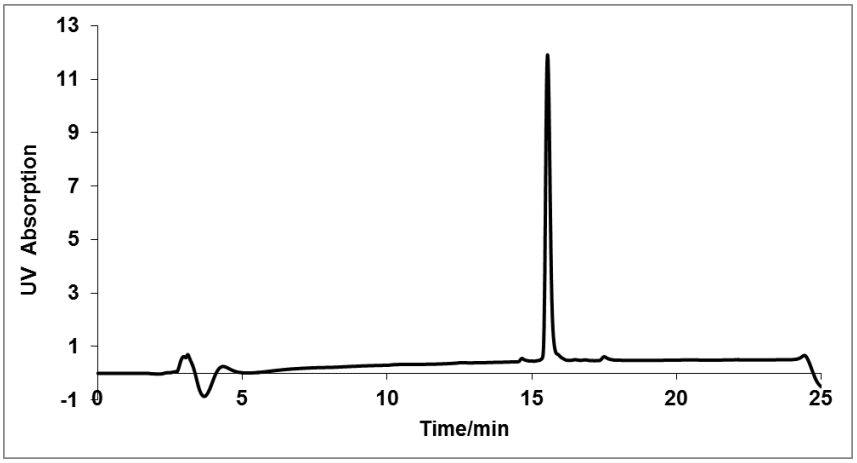


Figure S21 The analytic RP-HPLC spectrum of compound **M3**. Analytic gradient is 80% to 100% of solution C in 10min and 100% solution C for further 9min. Retention time is 15.5 min.

Vaccine candidate M4

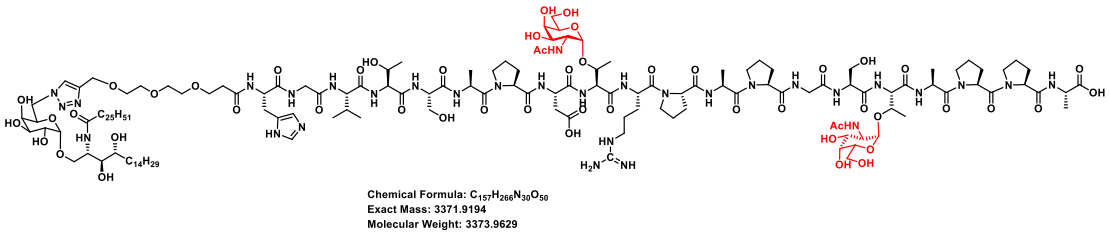


Figure S22 The chemical structure of compound **M4**.

The synthesis of vaccine candidate **M4** was according to protocol of **M1**. After lyophilization, 3.2 mg white powder (9.5 μmol, 36%) to give vaccine candidate **M4**. The purified product was analyzed by analytic RP-HPLC and MALDI-TOF MS.

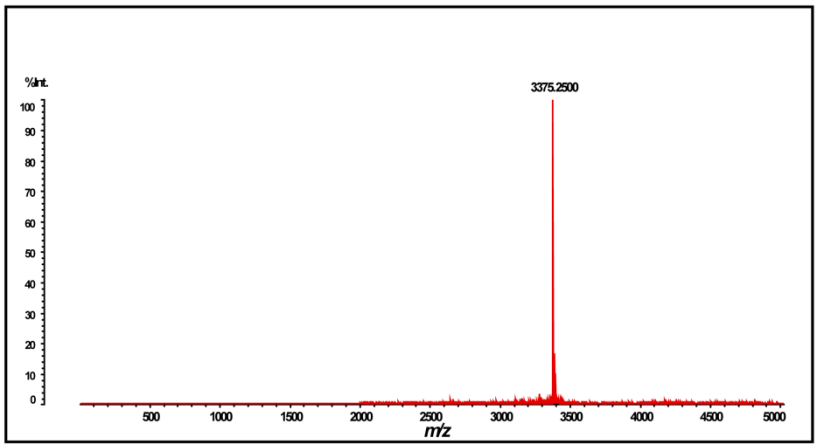


Figure S23 The MALDI-TOF MS spectrum of compound **M4**. Calcd for $C_{157}H_{266}N_{30}O_{50}$ $[M+H]^+$: 3374.9629; MALDI-TOF MS found: 3375.2500.

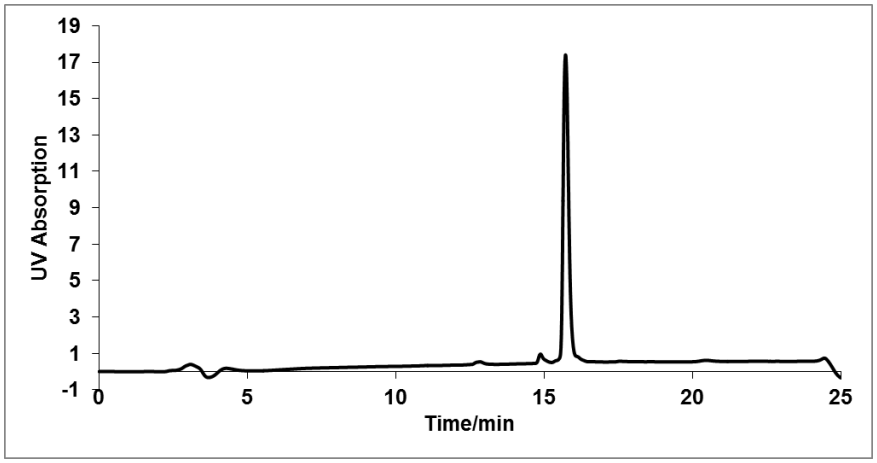


Figure S24 The analytic RP-HPLC spectrum of compound **M4**. Analytic gradient is 80% to 100% of solution C in 10min and 100% solution C for further 9min. Retention time is 15.7 min.

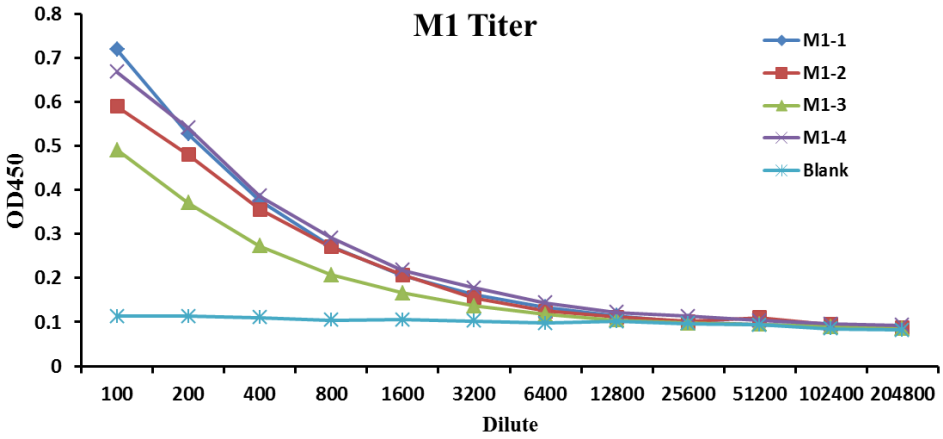


Figure S25 The antibody titer of vaccine **M1** candidate.

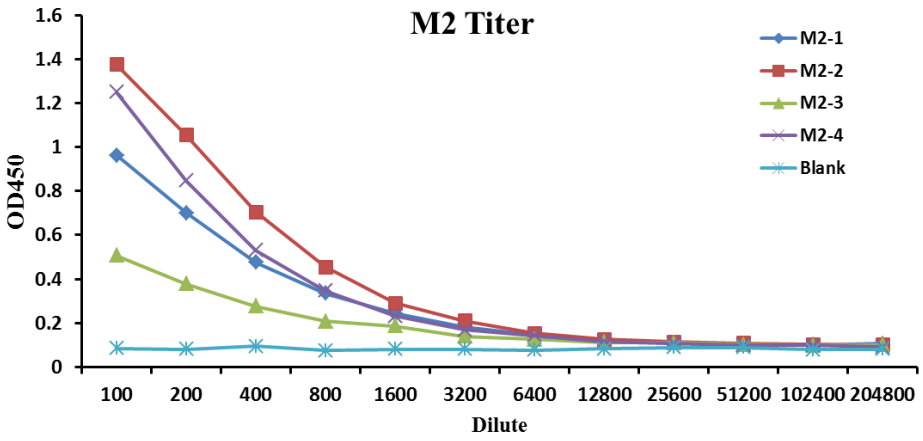


Figure S26 The antibody titer of vaccine **M2** candidate.

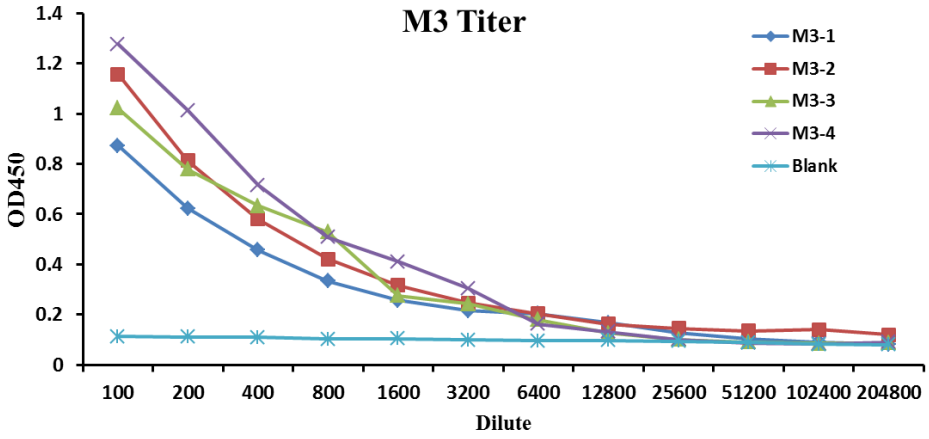


Figure S27 The antibody titer of vaccine **M3** candidate.

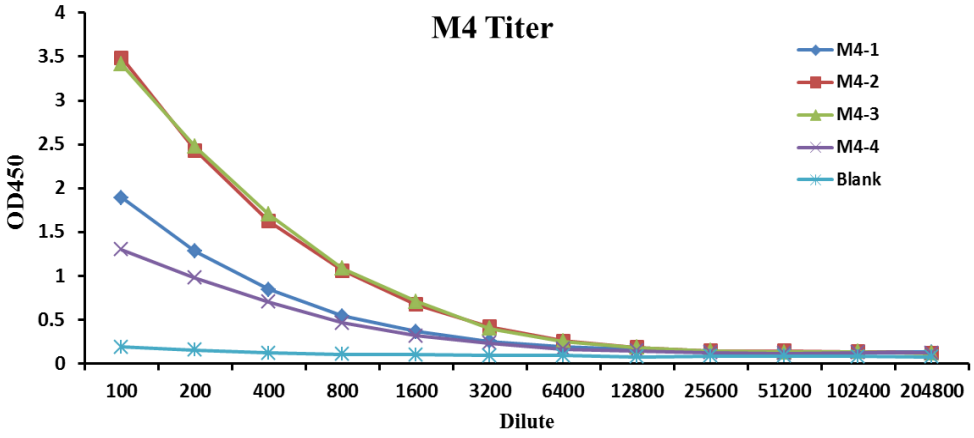


Figure S28 The antibody titer of vaccine **M4** candidate.

5.22

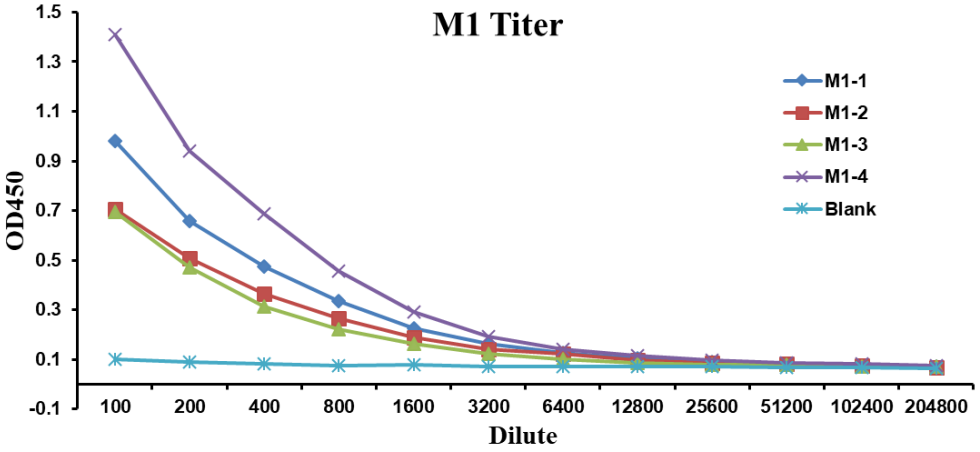


Figure S29 The antibody titer of vaccine **M1** candidate to nonglycosyalted MUC1 peptide.

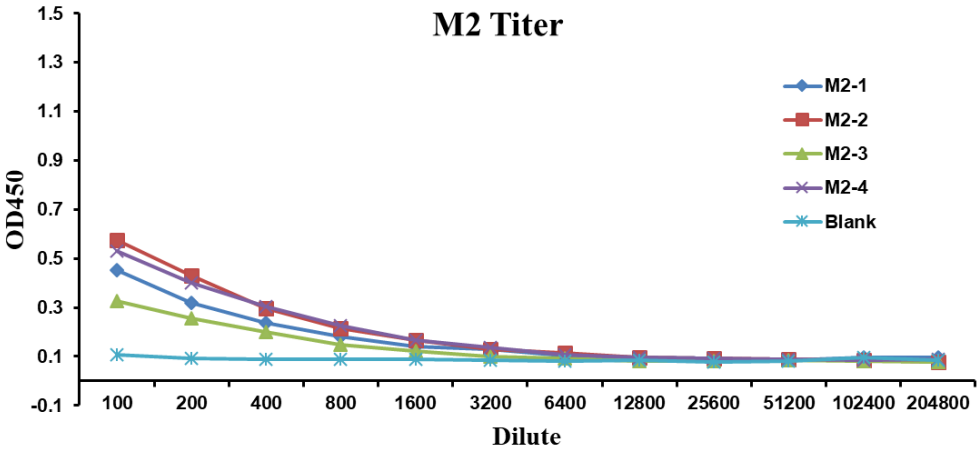


Figure S30 The antibody titer of vaccine **M2** candidate to nonglycosyalted MUC1 peptide.

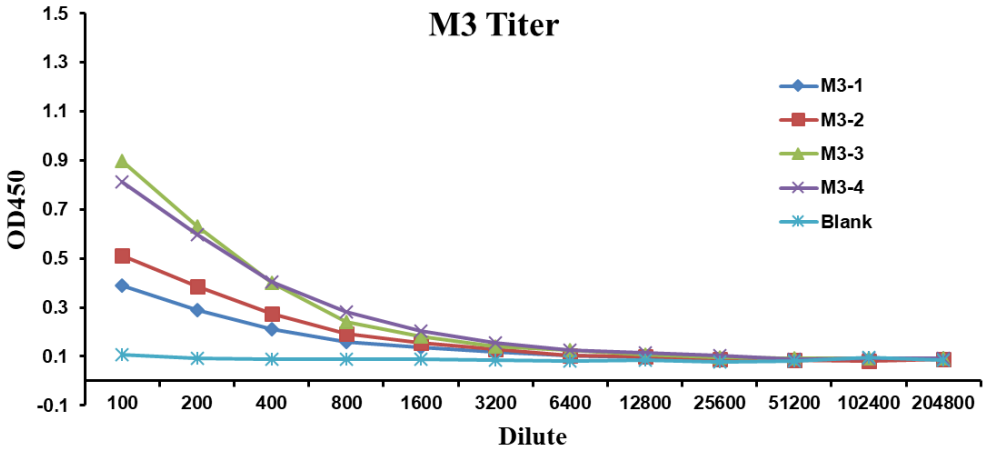


Figure S31 The antibody titer of vaccine **M3** candidate to nonglycosyalted MUC1 peptide.

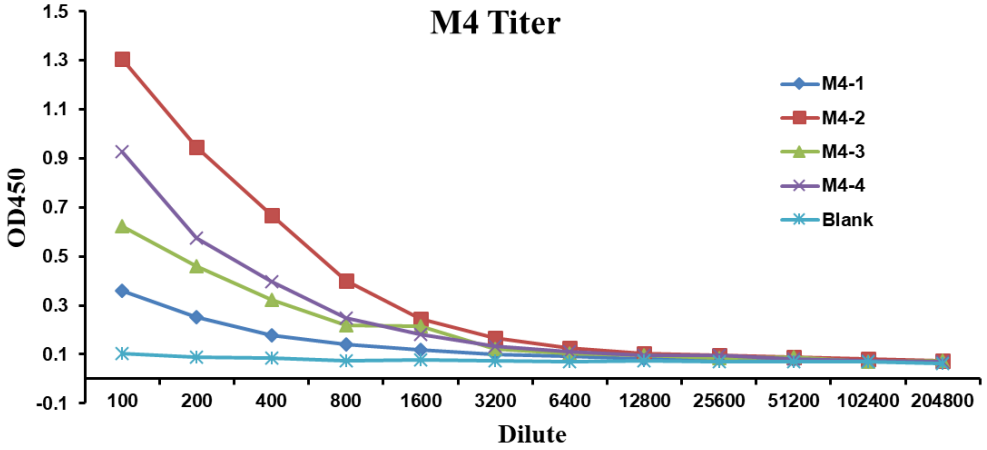


Figure S32 The antibody titer of vaccine **M4** candidate to nonglycosyalted MUC1 peptide.

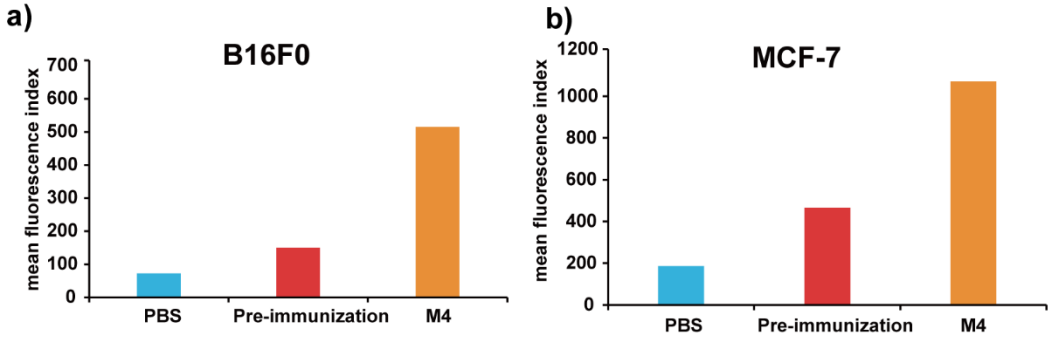


Figure S33 The quantification of the FCAS binding analysis.

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