Supporting Information

A totally synthetic, self-assembling, adjuvant-free, MUC1 glycopeptide vaccine for cancer therapy

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General information. All reagents used in experiments were purchased from Aldrich, Sigma and Acros, and used without further purification if not specified. Tn antigen building block was synthesized by chemical protocol as reported.[1] ^{1}H NMR spectra was recorded by a jeol-ECA-300 spectrometer with the frequency of 300M. Peptides were synthesized on Liberty CEM microwave peptide synthesizer. MALDI-TOF MS spectra were recorded on an Applied Bio-systems 4700 Proteomics Analyzer 283 with the matrix of α-cyano-4-hydroxycinnamic acid (CHCA) or 2, 5-dihydroxybenzoic acid (DHB). RP-HPLC separation and analysis were processed on a Waters-600-2487 with solution A (80% acetonitrile in water with 0.06% trifluoroacetic acid) and solution B (pure water with 0.06% trifluoroacetic acid). The absorption was detected by a UV detector of 215nm.

General protocol of peptide sysnthesis. Peptides were synthesized by standard solid phase peptide synthesis method of Fmoc chemistry with Nα-Fmoc-protected amino acids (GL Biochem(Shanghai) Ltd.). For coupling of natural amino acid, 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethylhexafluorophosphate(HBTU, 6.0 equiv) plus N-hydroxybenzotriazole (HOBt, 6.0 equiv) (GL Biochem(Shanghai) Ltd.) were used as activator and N,N-Diisopropylethylamine (DIEA, 12.0 equiv)(Alfa-aesar) as activator base. For coupling of Tn antigen building block, 2-(7-Aza-1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU, 2.0 equiv) plus 1-Hydroxy-7-azabenzotriazole (HOAt, 2.0 equiv)(GL Biochem(Shanghai) Ltd.) were used as activator and N-methylmorpholine(NMM, 5.0 equiv)(Sigma) as activator base. Peptide then was cleaved from resin by TFA/TIS/H_2O=10/0.6/0.6 for 2h. After removing TFA, peptide was precipitated three times by diethyl ether and centrifuged with 10000r/min for 15mins.Then the peptides were dried overnight and purified by HPLC. The product was identified by MALDI-TOF. For **H2**, **H3**, **H4**, **M2**, **M3**, **M4** acetyl protected peptides, designated as **p-H2**, **p-H3**, **p-H4**, **p-M2**, **p-M3**, **p-M4** were first synthesized. After purification, acetyl groups of Tn antigens on **p-H2**, **p-H3**, **p-H4**, **p-M2**, **p-M3**, **p-M4** were removed.

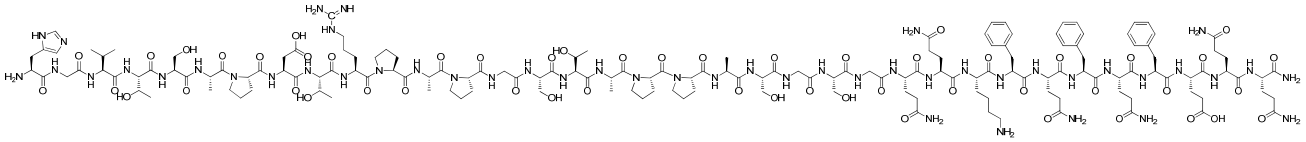
General protocol of RP-HPLC purification. Separation of peptides was processed with a preparative C18 column (YMC, Japan, 5 μm, 20×250 mm) with a flow rate of 6.0 ml/min. The pure products were lyophilized and stored at -20°C. Before use, all peptides were analyzed by RP-HPLC with an analytic C18 column (YMC, Japan, 10 μm, 4.6×150 mm) with a flow rate of 0.8 ml/min.

General protocol of deprotection of carbohydrate.[2] **p-H2**, **p-H3**, **p-H4**, **p-M2**, **p-M3** or **p-M4** were dissolved in MeOH, and 1%NaOMe/MeOH solution was added to adjust pH to 9.5. After stirred overnight at room temperature, acetic acid was added to the solution to neutralize. After removing the solvent, glycopeptides were dissolved in solution of water and acetonitrile, and separated by RP-HPLC. The product was identified by MALDI-TOF.

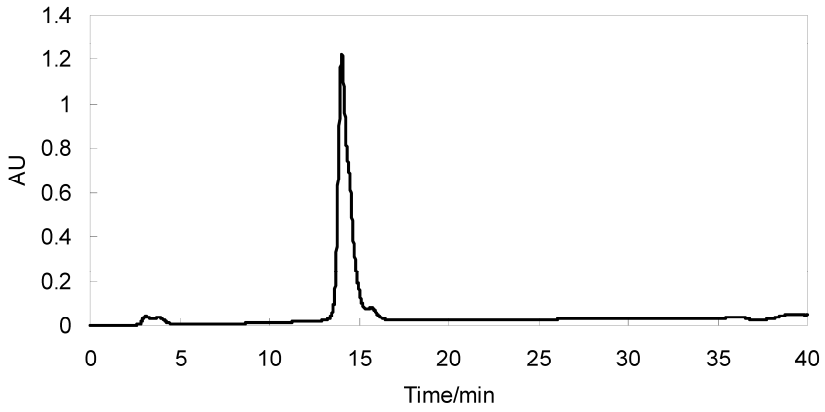
Analytical data

Synthesis of Tn building block. Fmoc-Thr(αAc_3GalNAc)-OH was synthesized by chemical protocol as reported.[1] MS(MALDI-TOF): Calcd. for $C_{33}H_{39}N_2O_{13}$ $[M+H]^+$ 671.2452, found 671.1810; $C_{33}H_{38}N_2NaO_{13}$ $[M+Na]^+$ 693.2272, found 693.1597; $C_{33}H_{38}N_2KO_{13}$ $[M+K]^+$ 709.2011, found 709.1316. ^{1}H-NMR (300 MHz, $CDCl_3$), δ (ppm): 7.75 (d, 2H, H4-, H5-Fmoc, $J_{H4,H3} = J_{H5,H6}$ = 7.5 Hz), 7.60 (d, 2H, H1-, H8-Fmoc, $J_{H1,H2} = J_{H8,H7}$ = 7.2 Hz), 7.38-7.26 (m, 4H, H2-, H3-, H6-,H7-Fmoc), 6.14 (d, 1H, NH-Fmoc, $J_{NH,T\alpha}$ = 8.9 Hz), 5.91 (d, 1H, NH-GalNAc, $J_{NH,H2}$ =8.3 Hz), 5.38 (d, 1H, H4, $J_{H4,H5}$ = 2.4 Hz), 5.12 (dd, 1H, H3, $J_{H3,H4}$ = 2.4 Hz, $J_{H3,H2}$ =11.3 Hz), 5.05 (d, 1H, H1, $J_{H1,H2}$ = 3.1 Hz), 4.64-4.40 (m, 3H, H2, CH2-Fmoc), 4.34-4.00 (m, 6H, H9-Fmoc, H5, H6a, H6b, Tα, Tβ), 2.17, 2.04, 2.03, 1.96(s, 12H,CH3), 1.29 (d, 3H, Tγ)

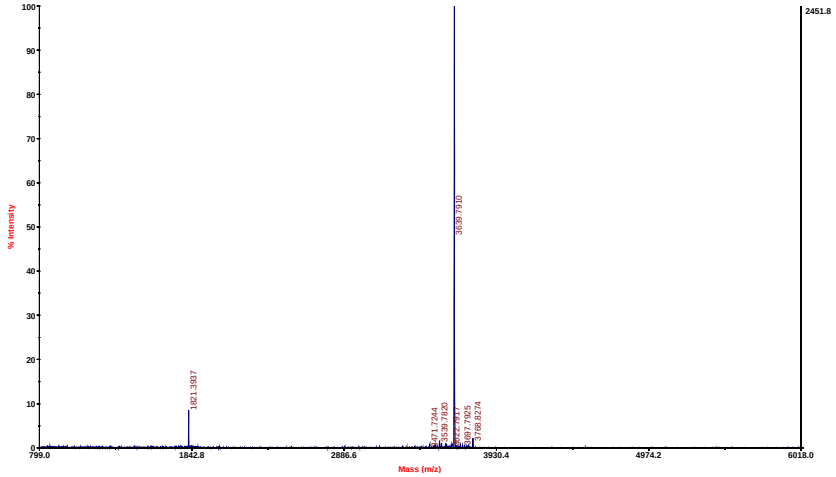
H1. H-His-Gly-Val-Thr-Ser-Ala-Pro-Asp-Thr-Arg-Pro-Ala-Pro-Gly-Ser-Thr-Ala-Pro-Pro-Ala-Ser-Gly-Ser-Gly-Gln-Gln-Lys-Phe-Gln-Phe-Gln-Phe-Glu-Gln-Gln-NH_2. 625mg Rink-amide AM Resin of 0.15mmol scale was used. The elution gradient of separation is from 20% to 60% of Solvent A in 30min. Retention time was 19.1min. 28.0mg product was available after purification. MS (MALDI-TOF): Calcd. for $C_{158}H_{239}N_{48}O_{52}$ $[M+H]^+$ 3640.7527; found 3639.7910.



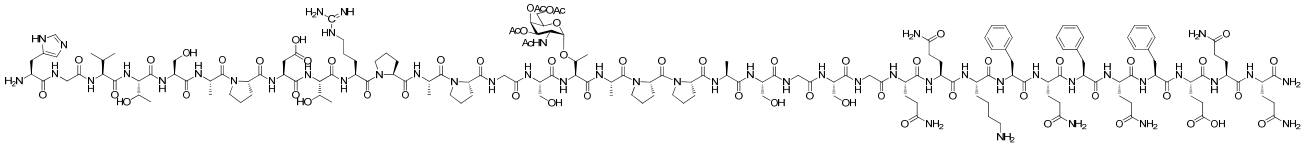
Analytic HPLC trace of **H1.** Analytic gradient is 20% to 60% of solution A in 30min. Retention time is 14.0min.



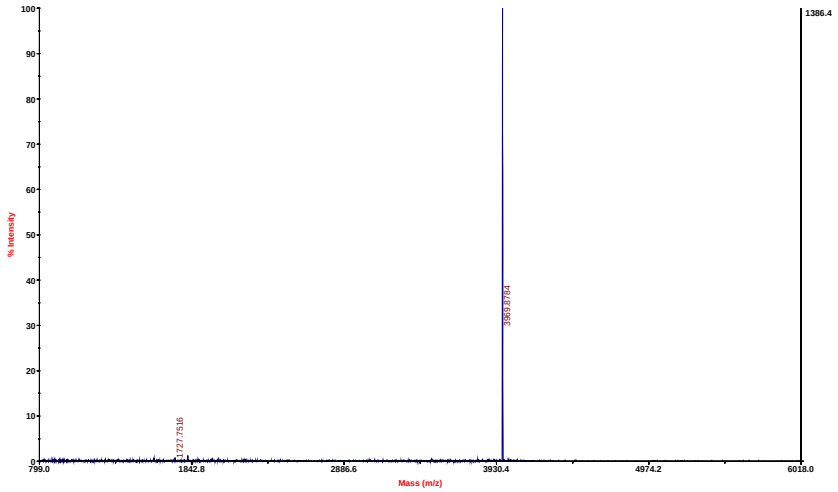
MALDI-TOF of **H1**



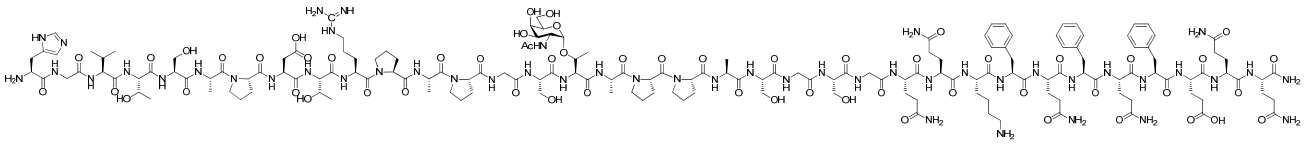
p-H2. H-His-Gly-Val-Thr-Ser-Ala-Pro-Asp-Thr-Arg-Pro-Ala-Pro-Gly-Ser-Thr(α-Ac_3GalNAc)-Ala-Pro-Pro-Ala-Ser-Gly-Ser-Gly-Gln-Gln-Lys-Phe-Gln-Phe-Gln-Phe-Glu-Gln-Gln-NH_2. 625mg Rink-amide AM Resin of 0.15mmol scale was used. The elution gradient of separation is from 20% to 60% of Solvent A in 30min. Retention time was 24.6min. 47.8mg product was available after purification. MS (MALDI-TOF): Calcd. for $C_{172}H_{258}N_{49}O_{60}$ $[M+H]^+$ 3969.8638; found m/z= 3969.8784.



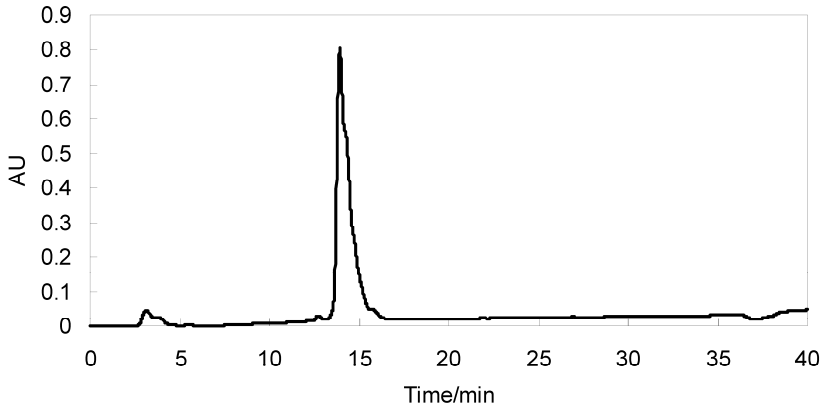
MALDI-TOF of **p-H2**



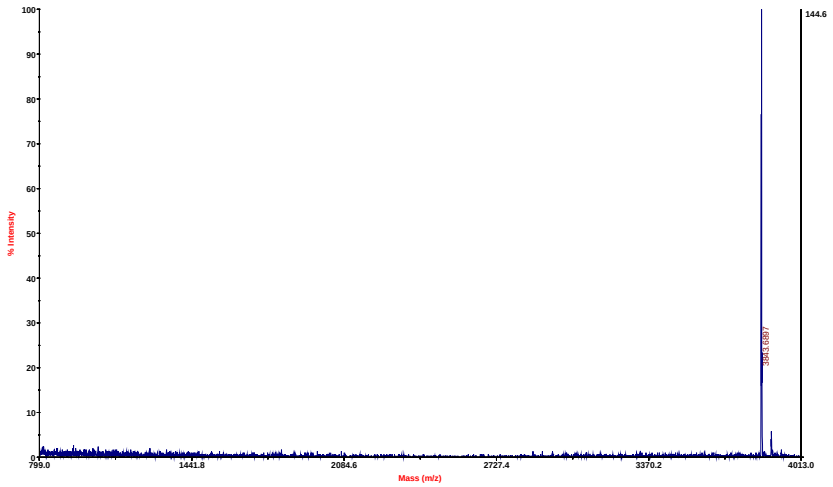
H2. H-His-Gly-Val-Thr-Ser-Ala-Pro-Asp-Thr-Arg-Pro-Ala-Pro-Gly-Ser-Thr(α-GalNAc)-Ala-Pro-Pro-Ala-Ser-Gly-Ser-Gly-Gln-Gln-Lys-Phe-Gln-Phe-Gln-Phe-Glu-Gln-Gln-NH_2. 47.8 mg p-H2 was dissolved in 60ml MeOH (HPLC grade), and add 1% Na-OMe/MeOH solution to adjust pH to 9.5. After stirred for 16h at room temperature, acetic acid was added to the solution to adjust pH to 4. After removing the solvent, sample was dissolved in 15ml acetonitril/water (1/1) and purified by HPLC. The elution gradient of separation is 20% to 60% of Solvent A in 30min. Retention time was 19.9min. 26.8mg product was available after purification. MS (MALDI-TOF): Calcd. for $C_{166}H_{252}N_{49}O_{57}$ $[M+H]^+$ 3843.8321; found 3843.6897.



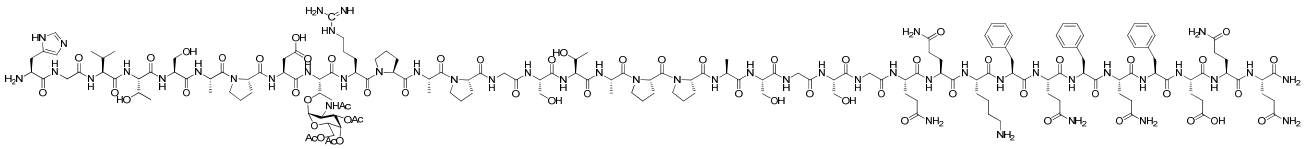
Analytic HPLC trace of **H2.** Analytic gradient is 20% to 60% of solution A in 30min. Retention time is 13.9min.



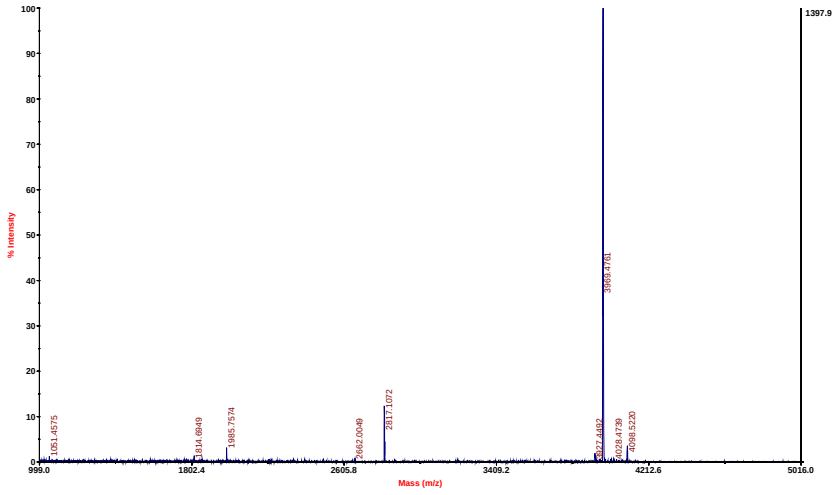
MALDI-TOF of **H2**



p-H3. H-His-Gly-Val-Thr-Ser-Ala-Pro-Asp-Thr(α-Ac_3GalNAc)-Arg-Pro-Ala-Pro-Gly-Ser-Thr-Ala-Pro-Pro-Ala-Ser-Gly-Ser-Gly-Gln-Gln-Lys-Phe-Gln-Phe-Gln-Phe-Glu-Gln-Gln-NH_2. 650mg Rink-amide MBHA Resin of 0.15mmol scale was used. The elution gradient of separation is from 20% to 60% of Solvent A in 30min. Retention time was 21.1min. 89.0mg product was available after purification. MS (MALDI-TOF): Calcd. for $C_{172}H_{258}N_{49}O_{60}$ $[M+H]^+$ 3969.8638; found 3969.4761.

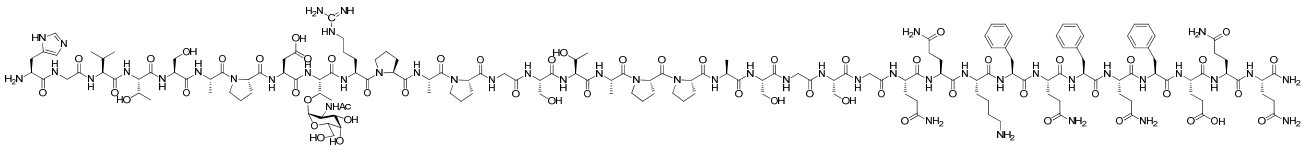


MALDI-TOF of **p-H3**

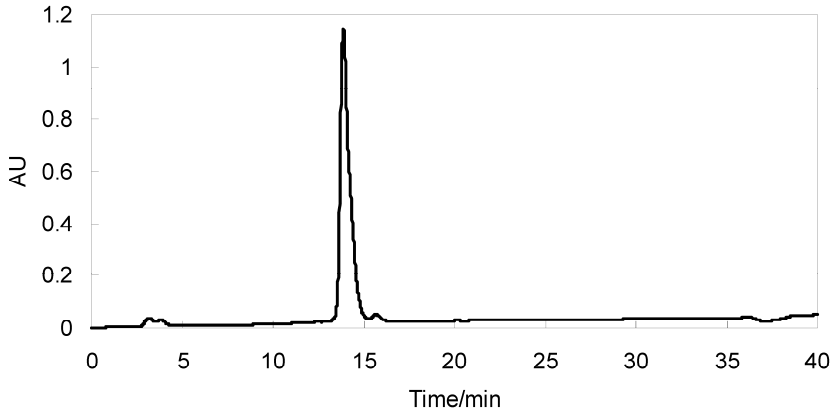


H3. H-His-Gly-Val-Thr-Ser-Ala-Pro-Asp-Thr(α-GalNAc)-Arg-Pro-Ala-Pro-Gly-Ser-Thr-Ala-Pro-Pro-Ala-Ser-Gly-Ser-Gly-Gln-Gln-Lys-Phe-Gln-Phe-Gln-Phe-Glu-Gln-Gln-NH_2. 89.0 mg **p-H3** was dissolved in 60ml MeOH (HPLC grade), and 1% NaOMe/MeOH

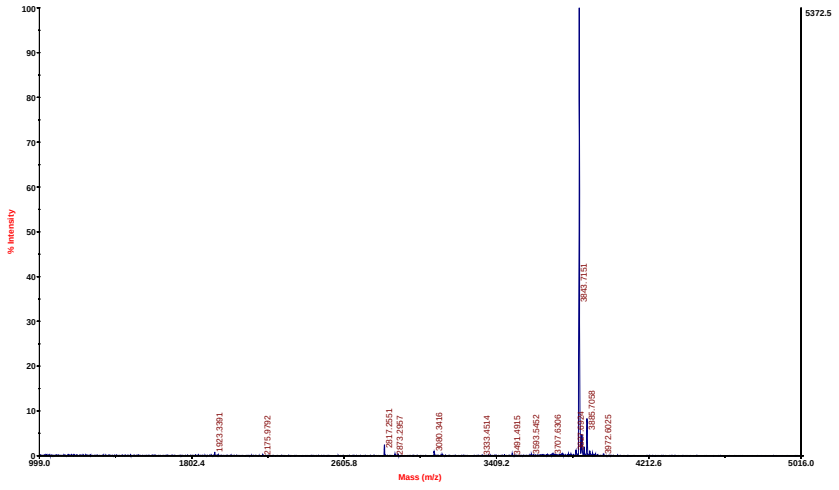
solution was added to adjust pH to 9.5. After stirred for 16h at room temperature, acetic acid was added to the solution to adjust pH to 4. After removing the solvent, sample was dissolved in 15ml acetonitril/water (1/1) and purified by HPLC. The elution gradient of separation is from 20% to 60% of Solvent A in 30min. Retention time was 19.2min. 30.0mg product was available after purification. MS (MALDI-TOF): Calcd. for $C_{166}H_{252}N_{49}O_{57}$ $[M+H]^+$ 3843.8321; found m/z= 3843.7151.



Analytic HPLC trace of **H3.** Analytic gradient is from 20% to 60% of solution A in 30 min. Retention time is 13.9 min.

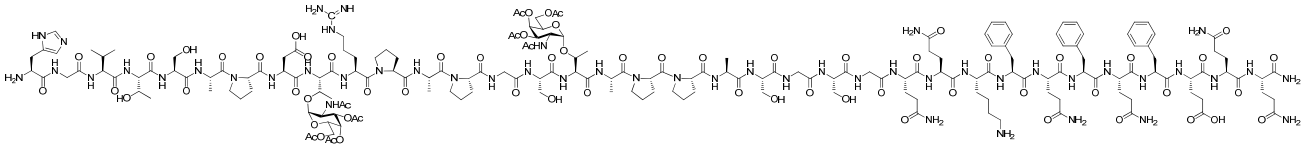


MALDI-TOF of **H3**

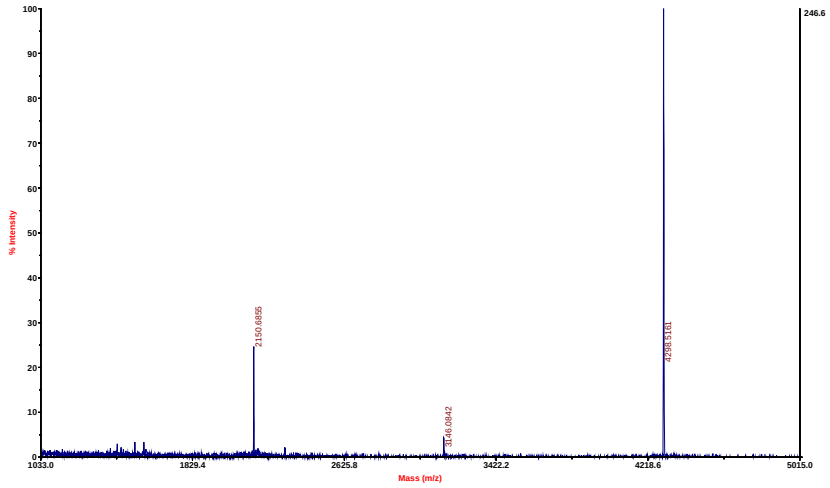


p-H4. H-His-Gly-Val-Thr-Ser-Ala-Pro-Asp-Thr(α-Ac_3GalNAc)-Arg-Pro-Ala-Pro-Gly-Ser-Thr(α-Ac_3GalNAc)-Ala-Pro-Pro-Ala-Ser-Gly-Ser-Gly-Gln-Gln-Lys-Phe-Gln-Phe-Gln-Phe-Glu-Gln-Gln-NH_2. 650mg Rink-amide MBHA Resin of 0.15mmol scale was used.

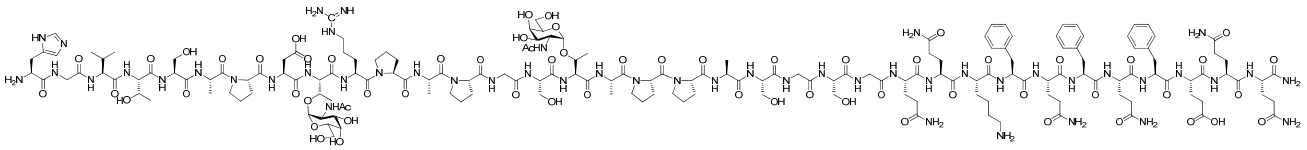
The elution gradient of separation is from 20% to 60% of Solvent A in 30min. Retention time was 22.5min. 110.0mg product was available after purification. MS (MALDI-TOF): Calcd. for $C_{186}H_{277}N_{50}O_{68}$ $[M+H]^+$ 4298.9754; found 4298.5161.



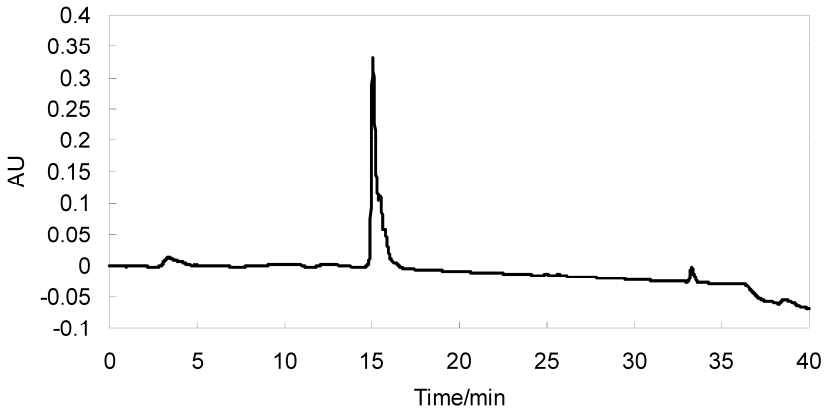
MALDI-TOF of **p-H4**



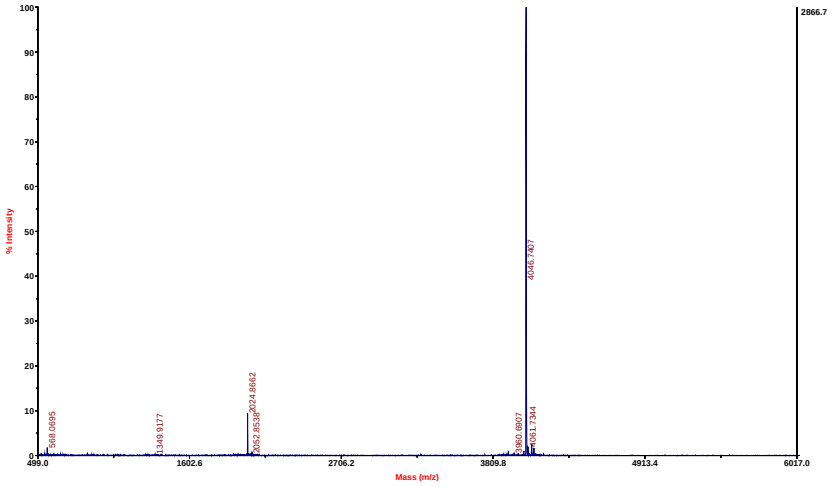
H4. H-His-Gly-Val-Thr-Ser-Ala-Pro-Asp-Thr(α-GalNAc)-Arg-Pro-Ala-Pro-Gly-Ser-Thr(α-GalNAc)-Ala-Pro-Pro-Ala-Ser-Gly-Ser-Gly-Gln-Gln-Lys-Phe-Gln-Phe-Gln-Phe-Glu-Gln-Gln-NH_2. 110.0 mg **p-H4** was dissolved in 60ml MeOH (HPLC grade), and 1% NaOMe/MeOH solution was added to adjust pH to 9.5. After stirred for 16h at room temperature, acetic acid was added to the solution to adjust pH to 4. After removing the solvent, sample was dissolved in 15ml acetonitril/water (1/1) and purified by HPLC. The elution gradient of separation is from 20% to 60% of Solvent A in 30min. Retention time was 19.3min. 40.0mg product was available after purification. MS (MALDI-TOF): Calcd. for $C_{166}H_{252}N_{49}O_{57}$ $[M+H]^+$ 4046.9120; found m/z= 4046.7407.



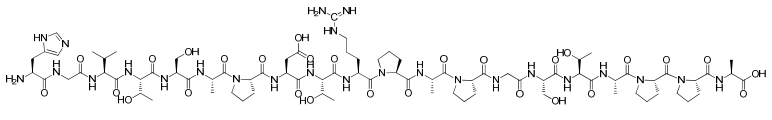
Analytic HPLC trace of **H4.** Analytic gradient is from 20% to 60% of solution A in 30 min. Retention time is 15.1 min.



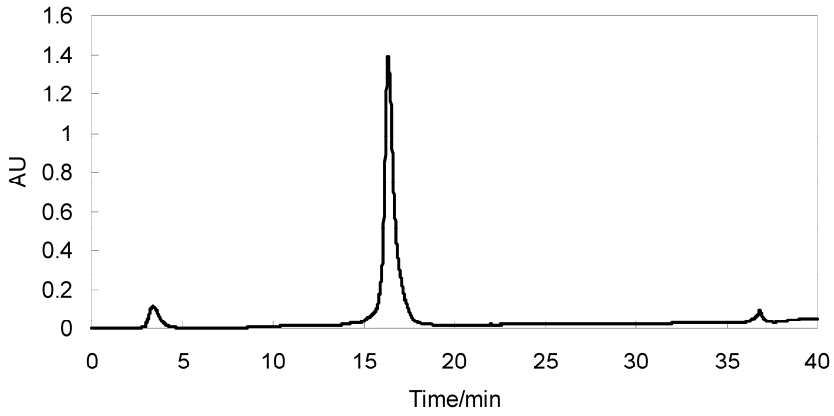
MALDI-TOF of **H4**



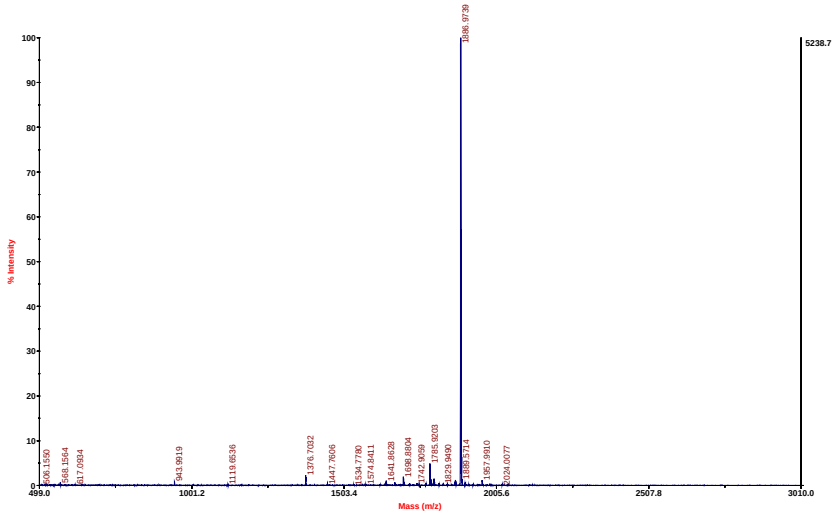
M1. H-His-Gly-Val-Thr-Ser-Ala-Pro-Asp-Thr-Arg-Pro-Ala-Pro-Gly-Ser-Thr-Ala-Pro-Pro-Ala-OH. 227mg Fmoc-Ala-Wang Resin of 0.05mmol scale was used. The elution gradient of separation is from 10% to 40% of Solvent A in 30min. Retention time was 17.6min. 36.4mg product was available after purification. $C_{80}H_{127}N_{25}O_{28}$. MS (MALDI-TOF): Calcd. for $C_{80}H_{128}N_{25}O_{28}$ $[M+H]^+$ 1886.9355; found 1886.9739.



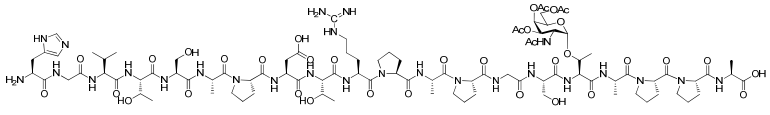
Analytic HPLC trace of **M1.** Analytic gradient is from 10% to 40% of solution A in 30 min. Retention time is 16.4 min.



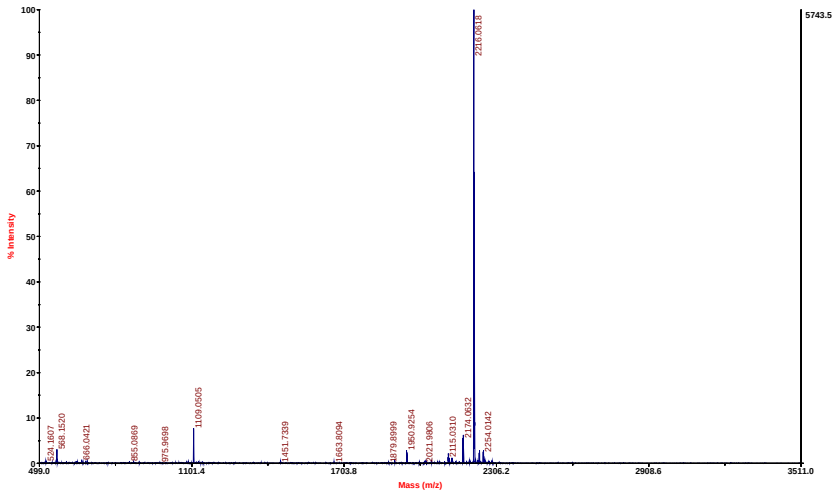
MALDI-TOF of **M1**



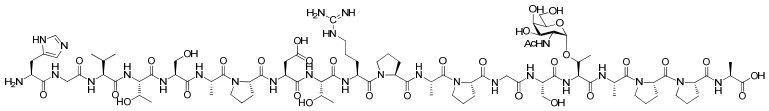
p-M2. H-His-Gly-Val-Thr-Ser-Ala-Pro-Asp-Thr-Arg-Pro-Ala-Pro-Gly-Ser-Thr(α-Ac_3GalNAc)-Ala-Pro-Pro-Ala-OH. 227mg Fmoc-Ala-Wang Resin of 0.05mmol scale was used. The elution gradient of separation is from 15% to 45% of Solvent A in 30min. Retention time was 24.2min. 37.6mg product was available after purification. MS (MALDI-TOF): Calcd. for $C_{94}H_{147}N_{26}O_{36}$ $[M+H]^+$ 2216.0466; found 2216.0618.



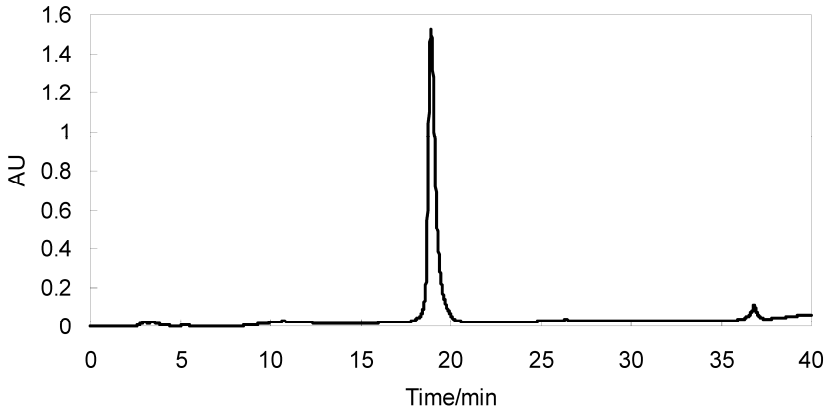
MALDI-TOF of **p-M2**



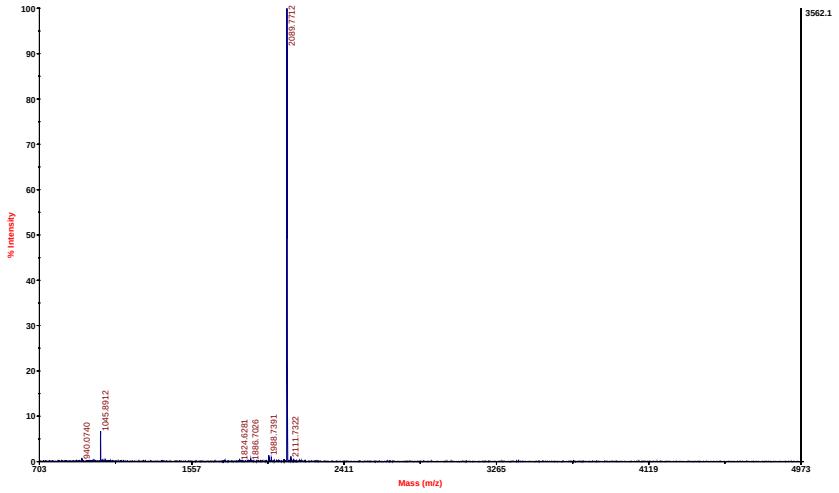
M2. H-His-Gly-Val-Thr-Ser-Ala-Pro-Asp-Thr-Arg-Pro-Ala-Pro-Gly-Ser-Thr(α-GalNAc)-Ala-Pro-Pro-Ala-OH. 37.6 mg **p-M2** was dissolved in 60ml MeOH (HPLC grade), and 1% NaOMe/MeOH solution was added to adjust pH to 9.5. After stirred for 16h at room temperature, acetic acid was added to the solution to adjust pH to 4. Sample was dissolved in 15ml acetonitril/water (1/1) and purified by HPLC. The elution gradient of separation is 5% to 40% of Solvent A in 30min. Retention time was 26.5min. 25.4mg product was available after purification. MS (MALDI-TOF): Calcd. for $C_{88}H_{141}N_{26}O_{33}$ $[M+H]^+$ 2090.0149; found 2089.7712.



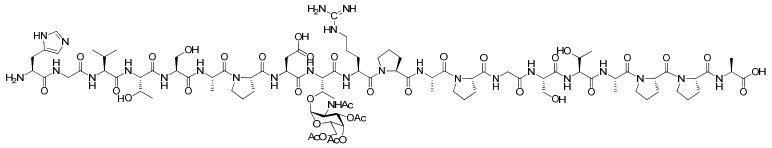
Analytic HPLC trace of **M2.** Analytic gradient is 5% to 40% of solution A in 30min. Retention time is 18.9min.



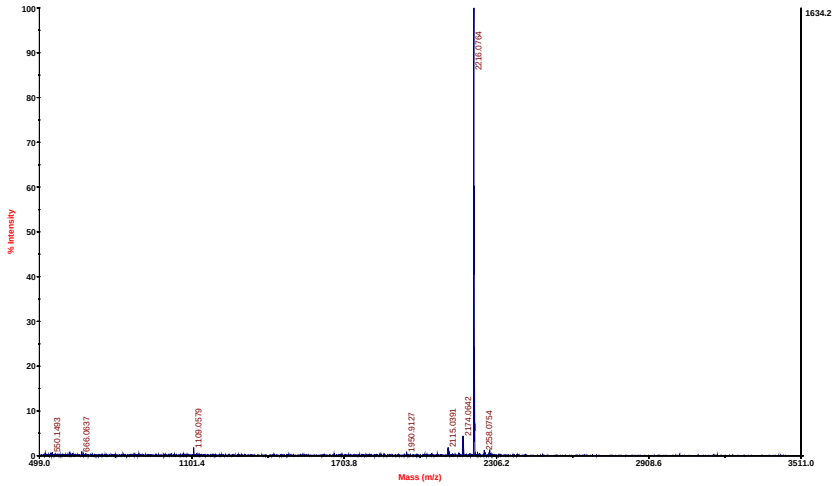
MALDI-TOF of **M2**



p-M3. H-His-Gly-Val-Thr-Ser-Ala-Pro-Asp-Thr(α-Ac_3GalNAc)-Arg-Pro-Ala-Pro-Gly-Ser-Thr-Ala-Pro-Pro-Ala-OH. 227mg Fmoc-Ala-Wang Resin of 0.05mmol scale was used. The elution gradient of separation is 15% to 45% of Solvent A in 30min. Retention time was 23.6min. 24.2mg product was available after purification. MS (MALDI-TOF): Calcd. for $C_{94}H_{147}N_{26}O_{36}$ $[M+H]^+$ 2216.0466; found 2216.0764.

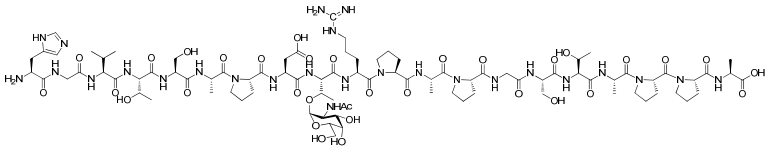


MALDI-TOF of **p-M3**

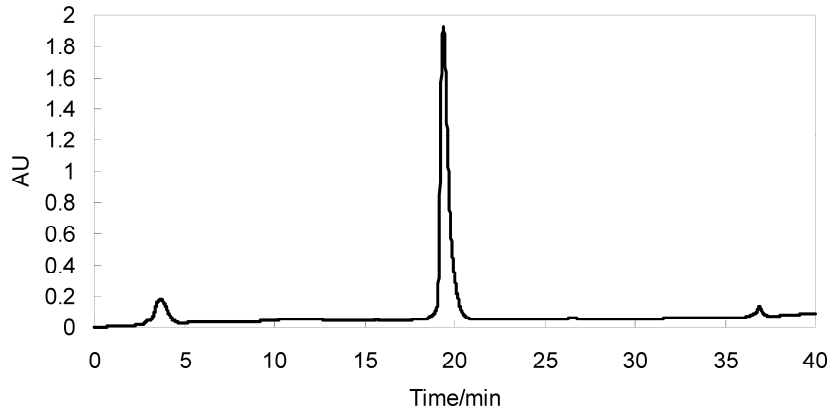


M3. H-His-Gly-Val-Thr-Ser-Ala-Pro-Asp-Thr(α-GalNAc)-Arg-Pro-Ala-Pro-Gly-Ser-Thr-Ala-Pro-Pro-Ala-OH. 24.2mg **p-M3** was dissolved in 60ml MeOH (HPLC grade), and 1% NaOMe/MeOH solution was added to adjust pH to 9.5. After stirred for 16h at room

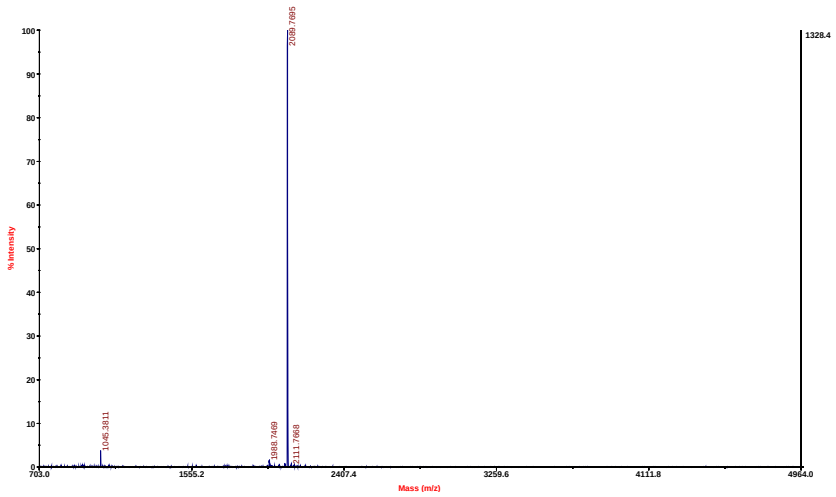
temperature, acetic acid was added to the solution to adjust pH to 4. Sample was dissolved in 15ml acetonitril/water (1/1) and purified by HPLC. The elution gradient of separation is 5% to 40% of Solvent A in 30min. Retention time was 25.1min. 16.0mg product was available after purification. MS (MALDI-TOF): Calcd. for $C_{88}H_{141}N_{26}O_{33}$ $[M+H]^+$ 2090.0149; found 2089.7696.



Analytic HPLC trace of **M3.** Analytic gradient is 5% to 40% of solution A in 30min. Retention time is 19.4min.

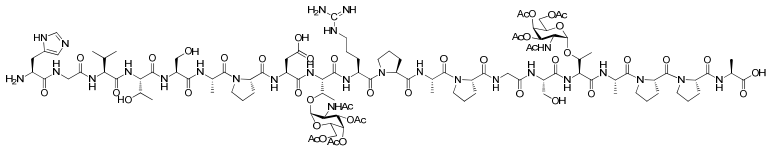


MALDI-TOF of **M3**

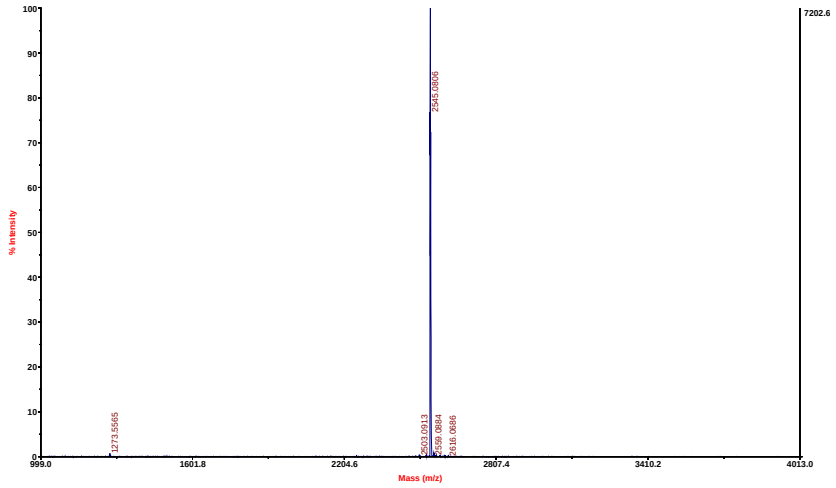


p-M4. H-His-Gly-Val-Thr-Ser-Ala-Pro-Asp-Thr(α-Ac_3GalNAc)-Arg-Pro-Ala-Pro-Gly-Ser-Thr(α-Ac_3GalNAc)-Ala-Pro-Pro-Ala-OH. 227mg Fmoc-Ala-Wang Resin of 0.05mmol scale was used. The elution gradient of separation is 20% to 50% of Solvent A in 30min.

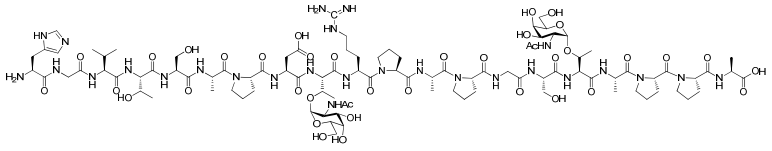
Retention time was 23.0min. 40.0mg product was available after purification. MS (MALDI-TOF): Calcd. for $C_{108}H_{166}N_{27}O_{44}$ $[M+H]^+$ 2545.1582; found 2545.0806.



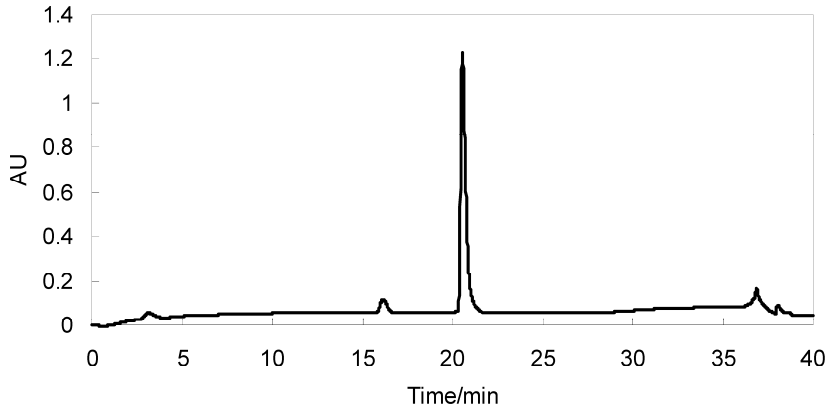
MALDI-TOF of **p-M4**



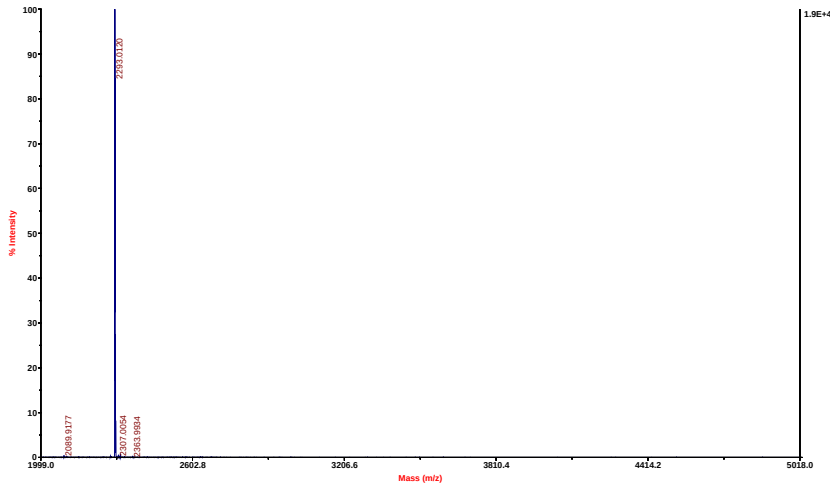
M4. H-His-Gly-Val-Thr-Ser-Ala-Pro-Asp-Thr(α-GalNAc)-Arg-Pro-Ala-Pro-Gly-Ser-Thr-Ala-Pro-Pro-Ala-OH. 40.0mg **p-M4** was dissolved in 60ml MeOH (HPLC grade), and 1% NaOMe/MeOH solution was added to adjust pH to 9.5. After stirred for 16h at room temperature, acetic acid was added to the solution to adjust pH to 4. Sample was dissolved in 15ml acetonitril/water (1/1) and purified by HPLC. The elution gradient of separation is 5% to 40% of Solvent A in 30min. Retention time was 23.3min. 32.3mg product was available after purification. MS (MALDI-TOF): Calcd. for $C_{96}H_{154}N_{27}O_{38}$ $[M+H]^+$ 2293.0948; found 2293.0120.



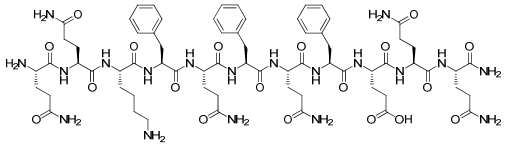
Analytic HPLC trace of **M4.** Analytic gradient is 5% to 40% of solution A in 30min. Retention time is 20.6min.



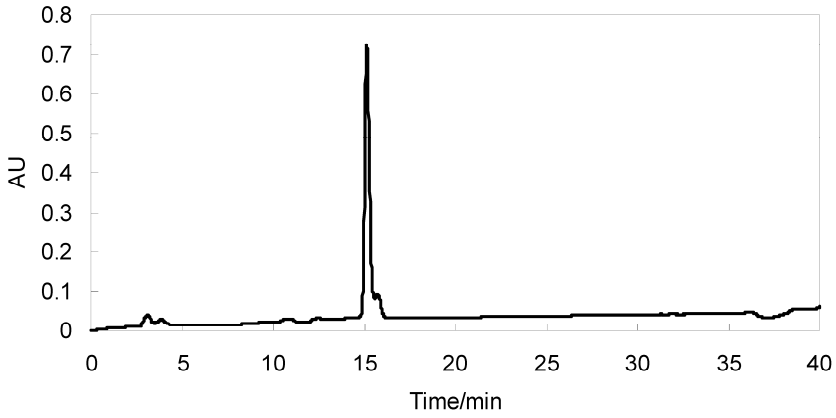
MALDI-TOF of **M4**



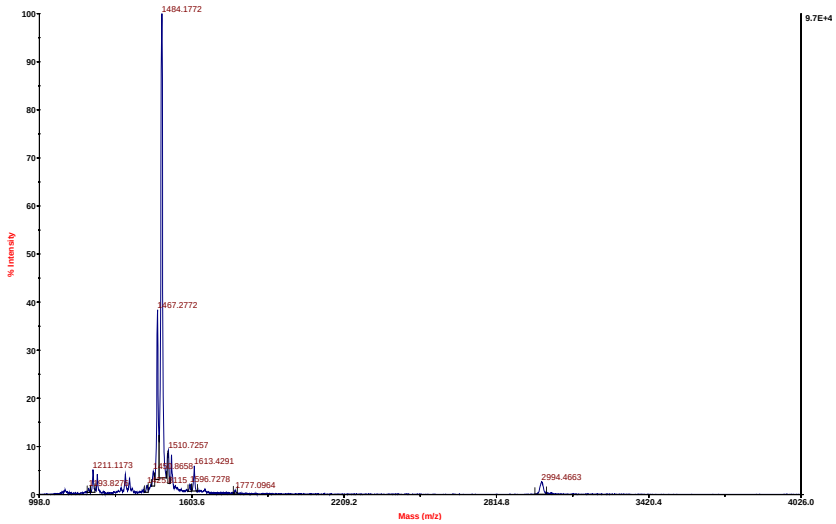
Q11. H-Gln-Gln-Lys-Phe-Gln-Phe-Gln-Phe-Glu-Gln-Gln-NH_2. 227mg Rink-amide MBHA Resin of 0.05mmol scale was used. The elution gradient of separation is 15% to 45% of Solvent A in 30min. Retention time was 21.4min. 19.0mg product was available after purification. Calcd. for $C_{68}H_{98}N_{19}O_{19}$ $[M+H]^+$ 1484.7281; found 1484.1772.



Analytic HPLC trace of **Q11**. Analytic gradient is 15% to 45% of solution A in 30min. Retention time is 15.2min.



MALDI-TOF of Q11



Transmission Electron Microscopy analysis

Peptide was dissolved in pure water, mixed 6:1 with PBS solution to working concentration of 400 μM and fibrillized for 8 h at room temperature.[3] 6 μL of each simple was applied to copper grids with carbon support films, negative-stained with 6 μL tungstophosphoric acid (15mg/ml, pH6-7), and imaged on Hitachi H-7650B transmission electron microscopy after dried overnight.

ThT (ThioflavinT) fluorescence analysis

H3 and **H4** were dissolved in hexafluoroisopropanol at the concentration of 1mg/ml and disassembled overnight. The solvent were evaporated by nitrogen. After further dried in vacuum over one hour, the samples were dissolved in pure water and mixed 6:1 with PBS solution.[3] 20 μL of sample and 180 μL ThT test solution were added into 96-well plate and fluorescence intensity at 485nm was measured with the excitation of 440nm. All samples were performed in five repeats.

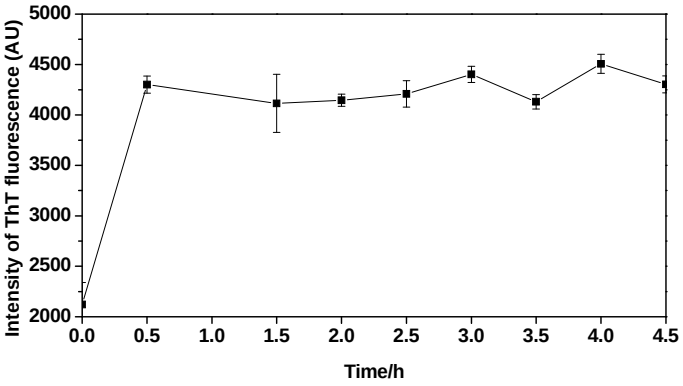


Figure S1. ThT fluorescence analysis of **H3** at the concentration of 400 μM

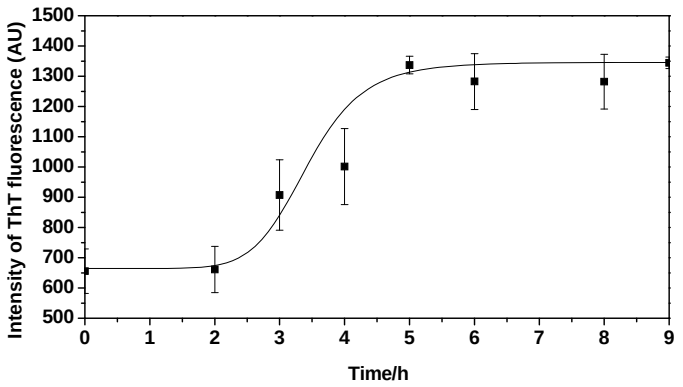


Figure S2. ThT fluorescence analysis of **H3** at the concentration of 200 μM

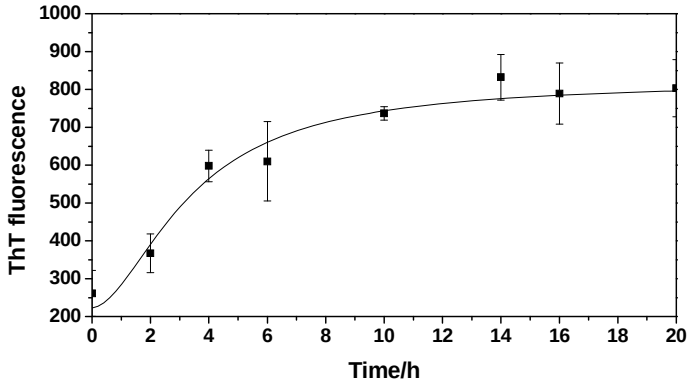


Figure S3. ThT fluorescence analysis of **H3** at the concentration of 100 μM

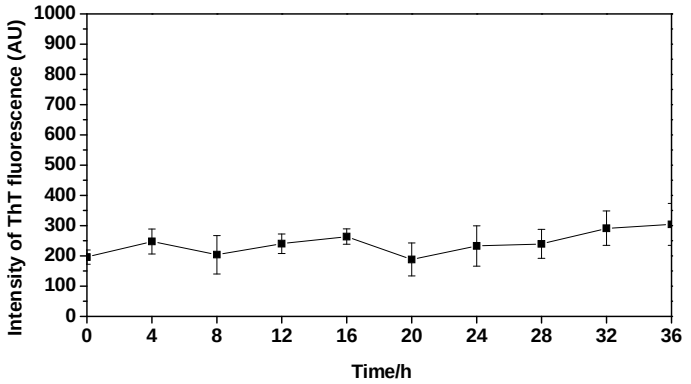


Figure S4. ThT fluorescence analysis of **H3** at the concentration of 50 μM

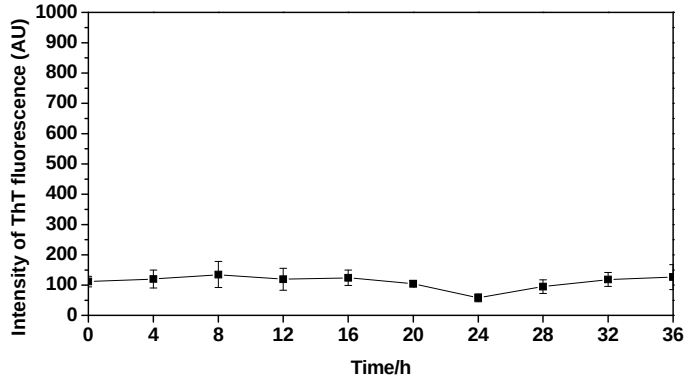


Figure S5. ThT fluorescence analysis of **H3** at the concentration of 25 μM

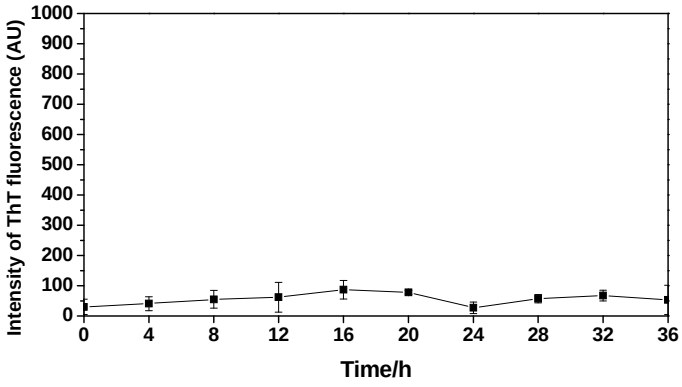


Figure S6. ThT fluorescence analysis of **H3** at the concentration of 10 μM

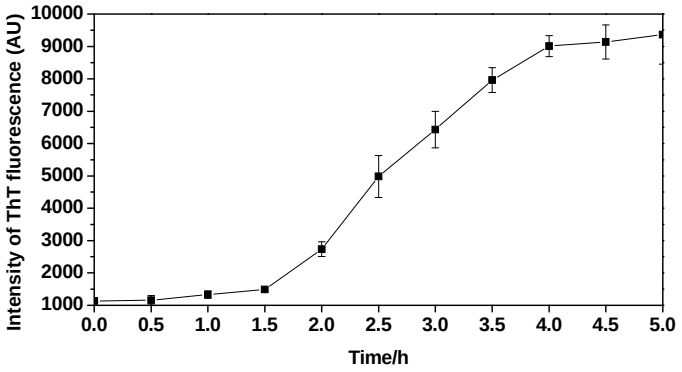


Figure S7. ThT fluorescence analysis of **H4** at the concentration of 400 μM

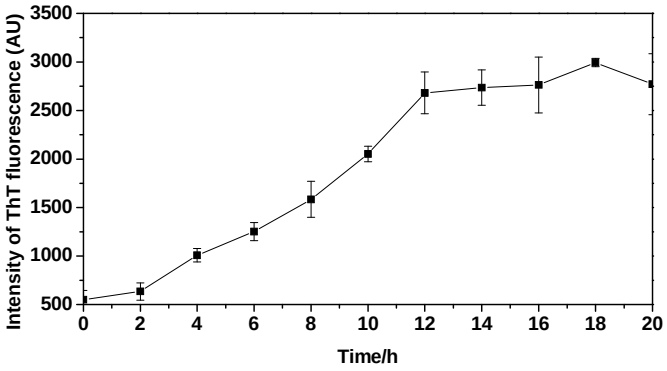


Figure S8. ThT fluorescence analysis of **H4** at the concentration of 200 μM

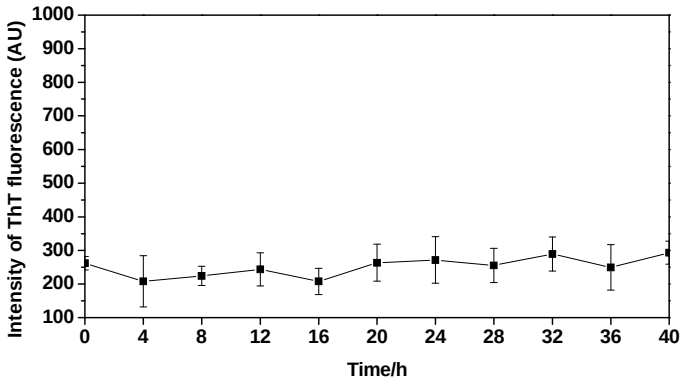


Figure S9. ThT fluorescence analysis of **H4** at the concentration of 100 μM

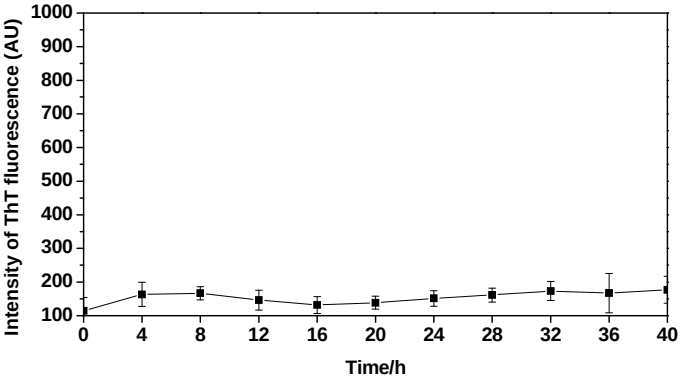


Figure S10. ThT fluorescence analysis of **H4** at the concentration of 50 μM

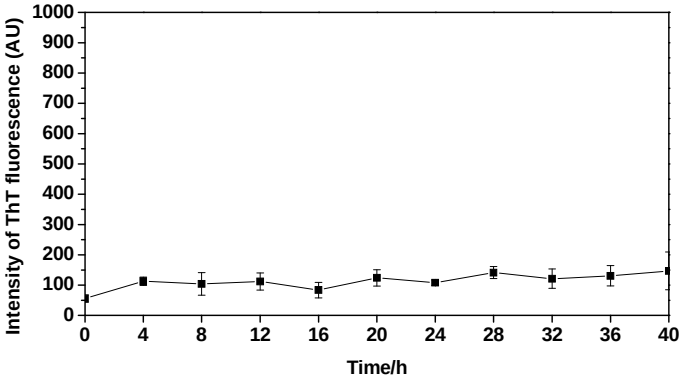


Figure S11. ThT fluorescence analysis of **H4** at the concentration of 25 μM

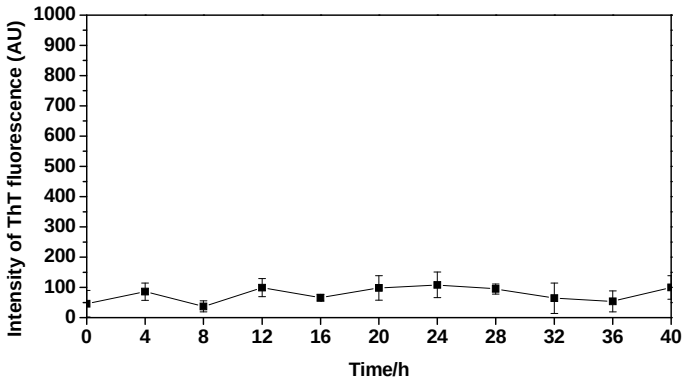


Figure S12. ThT fluorescence analysis of **H4** at the concentration of 10 μM

Immunization

Balb/C mice of 4-6 weeks were purchased, fed in Animal Facility of Center of Biomedical Analysis, Tsinghua Universiy. **H1**, **H2**, **H3** and **H4** were dissolved in pure water with the concentration of 8 mM and incubated at 4°C overnight. Then the solution was diluted with PBS solution to work concentration. For **H1** to **H4** group, four mice of Balb/C per group were immunized with 100 μL solution by intraperitoneal injection, which contained 100 nmol peptide aggregate. For **H5**, **H6**, **H7** and **H8** group, four mice of Balb/C per group were immunized with 100 μL solution mixed with 100 μL Freund's adjuvant (Sigma, CFA for the first injection, IFA for the rest). **H5** group was immunized by the mixture of **H1** and adjuvant, **H6** group was immunized by the mixture of **H2** and adjuvant, **H7** group was immunized by the mixture of **H3** and adjuvant, **H8** group was immunized by the mixture of **H4** and adjuvant. Mice were injected on days 0, 14, 28, 42, 56, and sera were collected on day 63. Sera were also collected before immunization as negative control.

Titer measurement

High-binding ELISA plates (Costar 3590) were coated with 20 μg/ml **M1** or **M2** or **M3** or **M4** peptides in PBS (100 μL per well) overnight at 4°C. Wells were blocked with 0.25% gelatin in PBS for 3hours. Wash steps were processed by 0.5% Tween-20 in PBS solution. After washed three times, the antiserum was diluted and incubated in each well for 1.5 hours at 37°C. After washed three times, the second antibody of Anti-Mouse IgG (whole molecule)-Peroxidase antibody produced in rabbit (Sigma) was diluted to 1/2000 and incubated (100 μL per well) for 1h at 37°C. After washed four times, 1mg/ml o-Phenylenediamine (Sigma) in 0.1M critic acid buffer (pH=5) with 1.5 μL/mL 30%H_2O_2 was added to wells (100 μL per well) as substrate. After 30 min incubation at room temperature, Optical absorption was measured at 450nm. Titers are defined as the highest dilution yielding an optical absorption of 0.1 or greater over that of negative control sera[4].

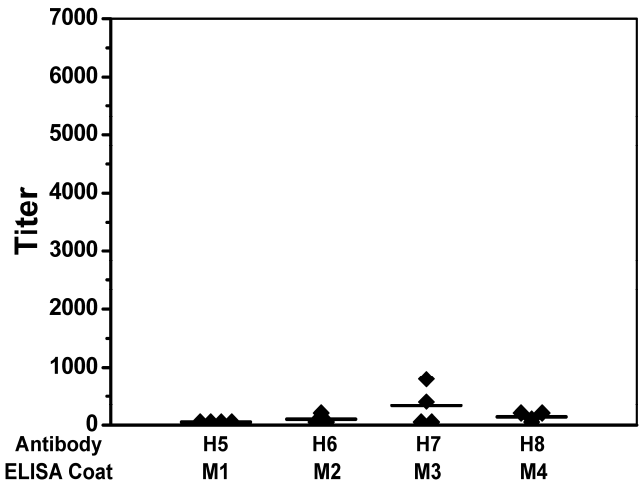


Figure S13. Anti-MUC1 IgG Titers of **H5**, **H6**, **H7** and **H8** by ELISA. **M1**, **M2**, **M3** and **M4** were used for ELISA coat. Every spot represented serum of one mouse after fifth immunization. Black line represented the average value of each group. Titers are defined as the highest dilution yielding an optical absorption of 0.1 or greater over that of negative control sera[4].

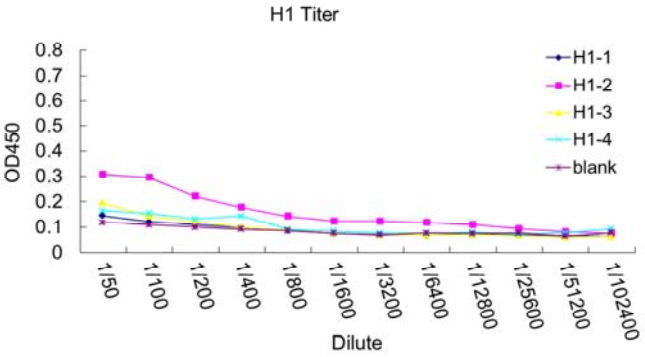


Figure S14. Determination of **H1** titer. **M1** peptide was coated on the 96-well plate. Each curve represented serum of one mouse after fifth immunization

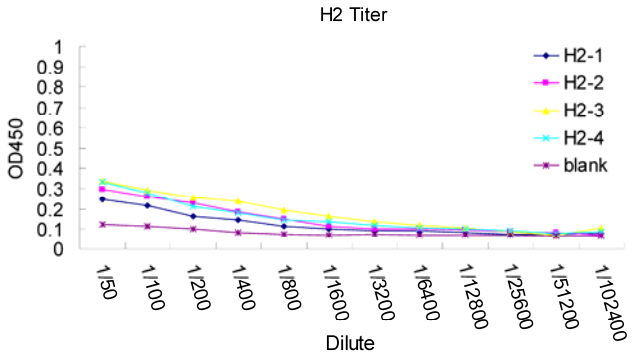


Figure S15. Determination of **H2** titer. **M2** peptide was coated on the 96-well plate. Each curve represented serum of one mouse after fifth immunization.

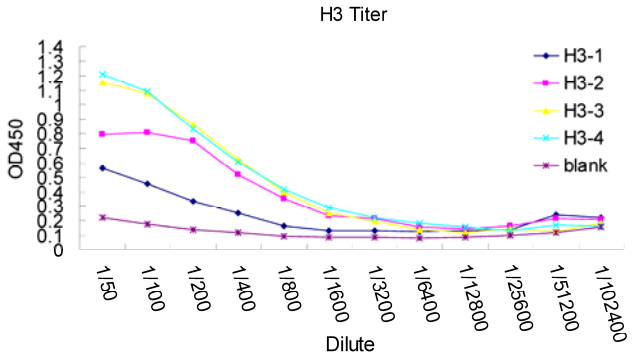


Figure S16. Determination of **H3** titer. **M3** peptide was coated on the 96-well plate. Each curve represented serum of one mouse after fifth immunization.

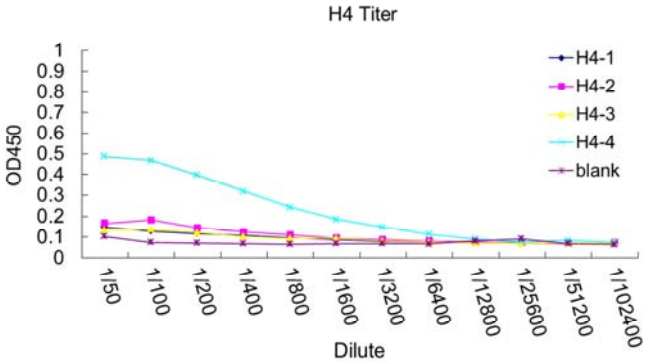


Figure S17. Determination of **H4** titer. **M4** peptide was coated on the 96-well plate. Each curve represented serum of one mouse after fifth immunization

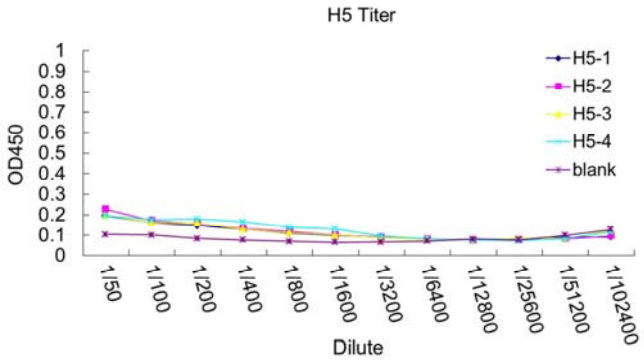


Figure S18. Determination of ti **H5** ter. **M1** peptide was coated on the 96-well plate. Each curve represented serum of one mouse after fifth immunization

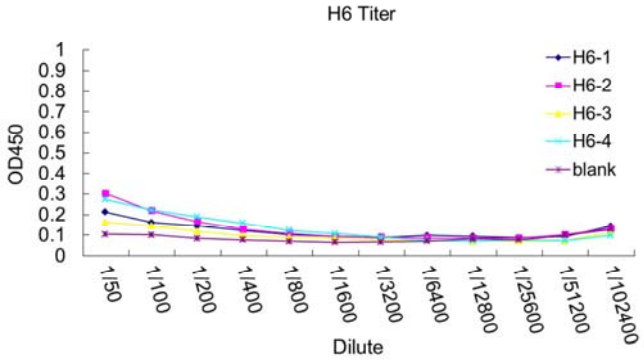


Figure S19. Determination of **H6** titer. **M2** peptide was coated on the 96-well plate. Each curve represented serum of one mouse after fifth immunization.

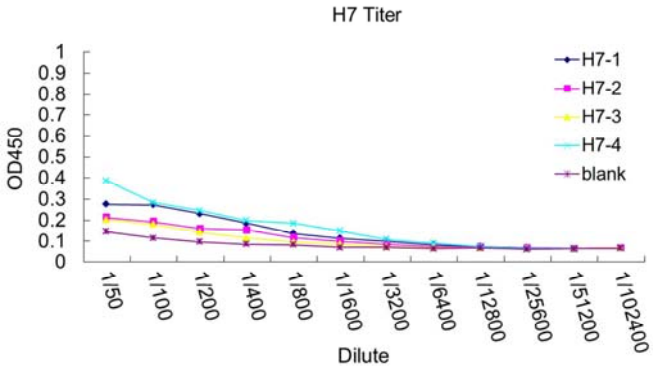


Figure S20. Determination of **H7** titer. **M3** peptide was coated on the 96-well plate. Each curve represented serum of one mouse after fifth immunization.

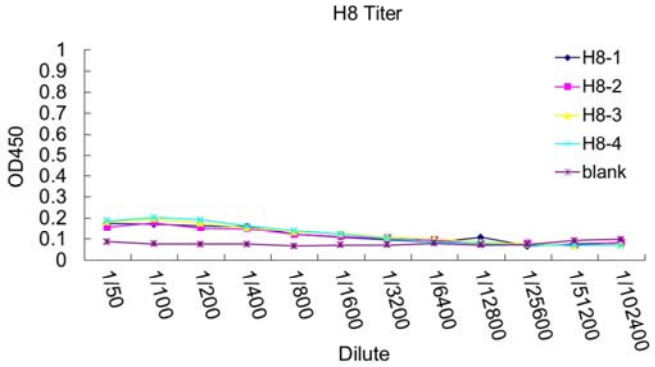


Figure S21. Determination of **H8** titer. **M4** peptide was coated on the 96-well plate. Each curve represented serum of one mouse after fifth immunization.

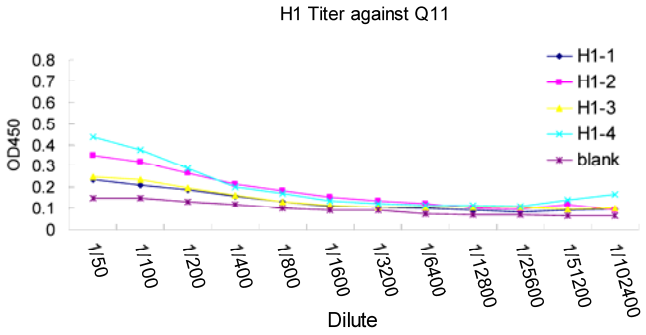


Figure S22. Detection of cross-activity of anti-**H1** antibody to **Q11** peptide. **Q11** peptide was coated on the 96-well plate. Each curve represented serum of one mouse after fifth immunization.

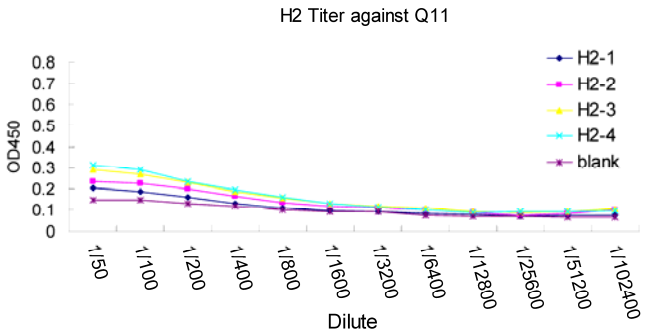


Figure S23. Detection of cross-activity of anti-**H2** antibody to **Q11** peptide. **Q11** peptide was coated on the 96-well plate. Each curve represented serum of one mouse after fifth immunization.

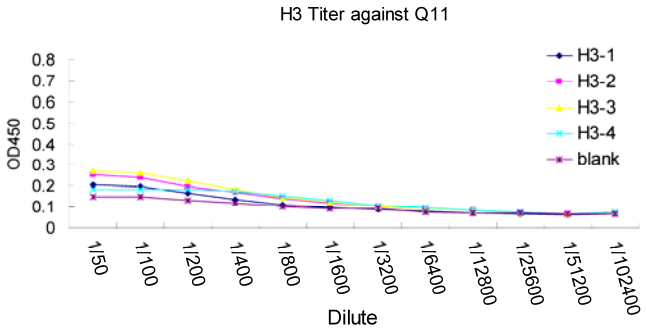


Figure S24. Detection of cross-activity of anti-**H3** antibody to **Q11** peptide. **Q11** peptide was coated on the 96-well plate. Each curve represented serum of one mouse after fifth immunization.

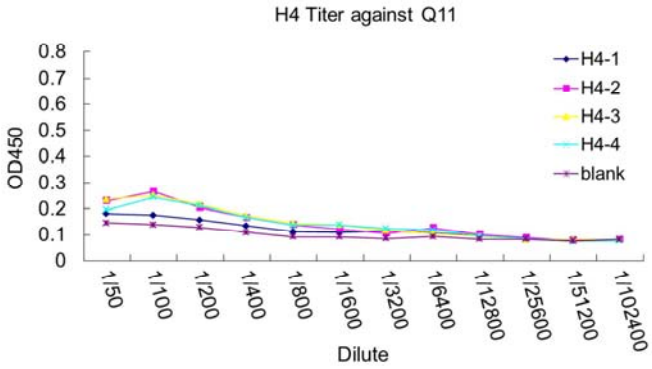


Figure S25. Detection of cross-activity of anti-**H4** antibody to **Q11** peptide. **Q11** peptide was coated on the 96-well plate. Each curve represented serum of one mouse after fifth immunization.

Isotype Determination

High-binding ELISA plates were coated with 20 μg/ml solution of **M1** or **M2** or **M3** or **M4** peptide in PBS by 100μL per well overnight at 4°C. Wells were blocked with 0.25% gelatin in PBS for 3h. Wash steps were processed by 0.5% Tween 20 in PBS solution. After washed three times, the antiserum was diluted to 1/50 and incubated in each well for 1.5 hours at 37°C. After washed three times, the second antibody of the kit of Mouse Monoclonal Antibody Isotyping Reagents (Sigma) including goat antimouse IgG1, IgG2a, IgG2b, IgG3, IgM was diluted to 1/1000 and incubated by 100 μL/well for 1h at 37°C. After washed three times, the third antibody of Anti-Goat IgG (whole molecule)-Peroxidase antibody (Sigma) produced in rabbit was diluted to 1/1000 and incubated by 100 μL/well for 1h at 37°C. After washed four times, 1mg/ml o-Phenylenediamine (Sigma) in 0.1M critic acid buffer (pH=5) with 1.5μL/mL 30%H_2O_2 was added by 100μL/well as substrate. After 30min, Optical absorption was measured at the wavelength of 450nm. All samples were performed in three repeats.

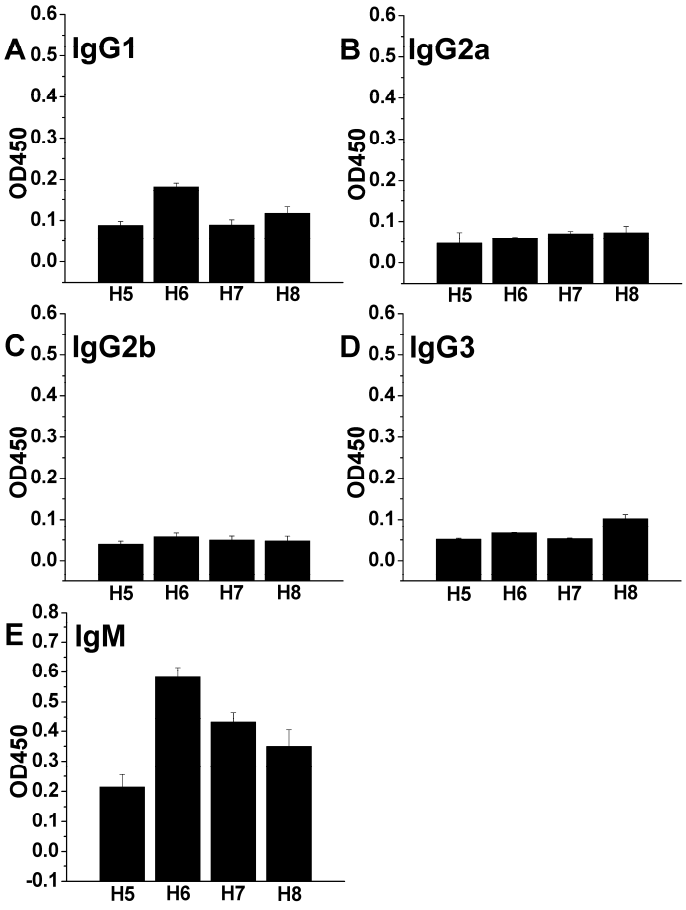


Figure S26. Isotype determination of vaccine **H5**, **H6**, **H7** and **H8.** (A) IgG1, (B) IgG2a, (C) IgG2b, (D) IgG3, (E) IgM after fifth immunization elicited by **H5**, **H6, H7** and **H8** were analyzed by ELISA. Negative control of antisera before immunization was cut off in the results.

Cell culture and Flow cytometry analysis

Materials were purchased from Biodee Inc, Beijing. MCF-7 human breast cancer cell line was cultured at 37°C in DMEM culture medium with 10% fetal bovine serum (FBS). Wash steps were processed by suspending with PBS solution containing 1% FBS and centrifugation for 5min at the speed of 1000r/min .Cells were digested by 0.25% (w/v) Trypsin solution and washed three times. Cells of 2.0×10^5 per simple were incubated with antiserum (1/10 dilution) from immunized mouse for 1h on ice. After washed three times, cells were incubated with FITC-conjugated rabbit antimouse IgG antibody (1/50 diluted, DAKO) for 1h on ice. After washed three times, FACS analysis was processed on BD Calibur Flow cytometry in Center of Biomedical Analysis, Tsinghua University.

Complement-dependent Cytotoxicity

MCF-7 cells were planted into 96-well cell culture plate with the density of 6000 cells per well. Cells were cultured for 12 h at 37°C, then antisera (1/10 diluted in culture medium) were added to the plate (50 μL per well). After cultured for 0.5 h at 37°C, rabbit complement (1/2 diluted in culture medium, AbD seroTec, 50 μL per well) were added. After cultured for 8 h, 20 μL per well MTT solution (0.5% MTT in PBS solution) was added, and the culture medium were removed carefully after culture of 4 h, and DMSO were added (150 μL per well) to dissolve the purpure crystal, then Optical absorption was measured at the wavelength of 490nm. All samples were performed in four repeats. Cells were cultured in culture medium as control. The cytotoxicity was calculated according to the formula below.

$$\text{Cytotoxicity (\%)} = [1-(\text{experimental OD}/\text{control OD})] \times 100$$

Reference

(1) Cai, H.; Huang, Z. H.; Shi, L.; Zou, P.; Zhao, Y. F.; Kunz, H.; Li, Y. M. *Eur. J. Org. Chem.* **2011**, 3685-3689.

(2) Westerlind, U.; Hobel, A.; Gaidzik, N.; Schmitt, E.; Kunz, H. *Angew. Chem. Int. Ed.* **2008**, *47*, 7551-7556.

(3) Rudra, J. S.; Tian, Y. F.; Jung, J. P.; Collier, J. H. *Proc. Natl. Acad. Sci. U. S. A.* **2010**, *107*, 622-627.

(4) Ingale, S.; Awolfert, M.; Gaekwad, J.; Buskas, T.; Boons, G. J. *Nat. Chem. Biol.* **2007**, *3*, 663-667.