Support information

Targeting STING with cyclic di-GMP greatly augmented immune responses of glycopeptide cancer vaccines

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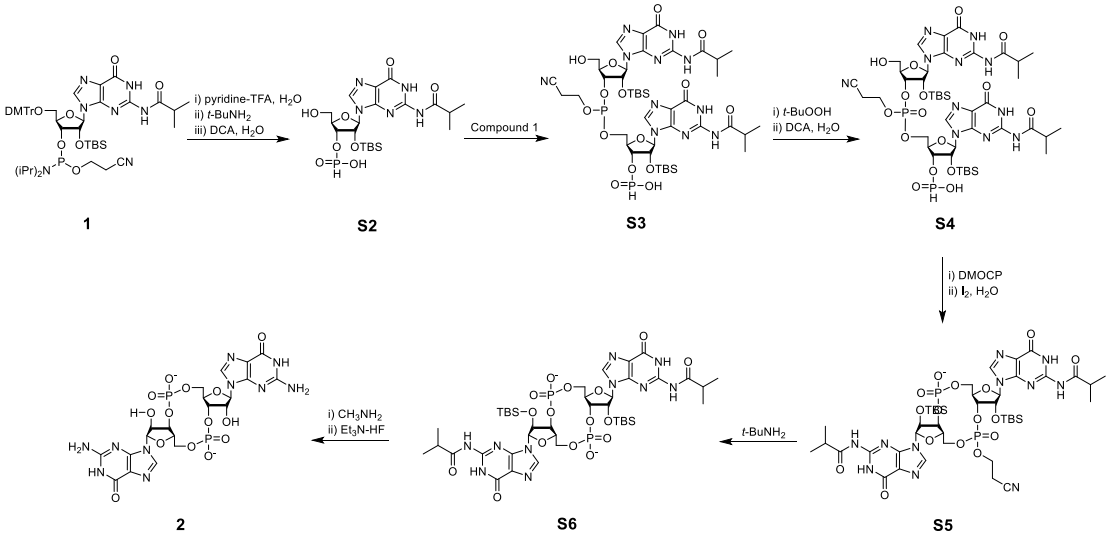
General information.

All reagents used in experiments were obtained from commercial companies and used without further purification. All solvents were reagent/HPLC grade. Anhydrous solvents were commercially available or dehydrated and distilled. The purifications of peptides, small molecules and conjugate were carried on Shimadzu LC-6AD reversed-phase HPLC (YMC C18 column, 5 μm, 20×250mm, Japan) at a flow rate of 10 mL/min. The analyses of compounds above were performed on Shimadzu LC-2010A HPLC (YMC analytic C18 column, Japan, 5μm, 4.6×150 mm) at a flow rate of 0.8 mL/min. Thermo Scientific UltiMate 3000 (ESI-MS) and Shimadzu Biotech AXIMA Performance instrument (MALDI-TOF MS) were used to identify compounds above. NMR spectrum were recorded on Jeol-ECA-400 spectrometer (400MHz) and Jeol-ECA-600 spectrometer (600MHz).

Synthesis of cyclic diguanosine monophosphate/CDG (2)

The CDG (**2**) was synthesized according to the one-flask synthesis strategy by Jones et al. And detailed protocol has been described in their published papers.[1] The compound was obtained as triethylammonium salt with crude yield of 46%. The crystals were then purified with semi-preparative HPLC (solution A: 0.05M triethylamine acetate, solution B: acetonitrile, pH=7.0, gradient is 2% to 40% of solution B in solution A in 30 min on the C18 column, 12mL/min). To record NMR spectra, triethylammonium group was further exchanged with ammonium through using 0.05mM NH_4HCO_3 as solution A. ^{1}H NMR (400M, D_2O) δ 7.91 (s, 2H), 5.86 (s, 2H), 4.98 (dd, *J*=13.0, 7.9Hz, 2H), 4.44 – 4.33 (m, 4H), 4.06 (dd, *J*=11.7, 4.3Hz, 2H). ^{31}P NMR (600M, D_2O) δ -1.12. ESI-HRMS (negative mode): $C_{20}H_{23}N_{10}O_{14}P_2^-$ [M-H]$^-$ calculated 689.0876; found 689.0862. The NMR spectra data were consistent with the published results.[2]

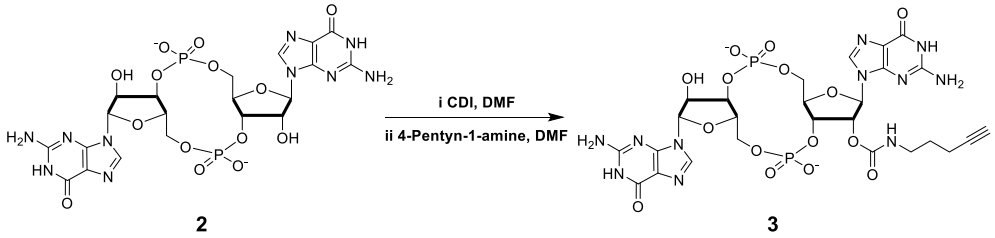


Scheme S1 Synthesis of cyclic diguanosine monophosphate/CDG (2).[1]

Synthesis of 2'-O-(4-pentyncarbamoyl)-CDG (3)

20mg (0.03mmol) CDG (**2**) was dissolved into 6mL anhydrous DMF. 6.4mg (0.04mmol, 1.37equiv) carbonyldiimidazole (CDI) was dissolved in 35μL anhydrous DMF. At first, 25μL CDI solution was added slowly to CDG solution. The reaction was stirred at RT for 13h and another 10μL CDI solution was then added to the mixture and stirred for 4h.[3] Without purification, 7mg (0.06mmol, 2equiv) 4-pentyn-1-amine and 50μL Et_3N were added to the solution and stirred for 23h. The mixture was then purified with semi-preparative HPLC (solution A: 0.05M triethylamine

acetate, solution B: acetonitrile, pH=7.0, gradient is 2% to 40% of solution B in solution A in 30 min on the C18 column, 12mL/min) to obtain compound **3** with crude yield of 54%. To record NMR spectra, triethylammonium group was further exchanged with ammonium through using 0.05mM NH_4HCO_3 as solution A. ^{1}H NMR (400 MHz, D_2O) δ 7.95 (s, 1H), 7.91 (s, 1H), 5.93 (s, 1H), 5.87 (s, 1H), 5.14 (dd, *J* = 13.4, 8.2 Hz, 1H), 5.02 (dd, *J* = 12.9, 7.9 Hz, 1H), 4.44 – 4.33 (m, 4H), 4.07 (td, *J* = 11.8, 4.0 Hz, 2H), 3.33 – 3.21 (m, 2H), 2.26 (t, *J* = 7.0 Hz, 2H), 1.72 (p, *J* = 6.9 Hz, 2H). ^{31}P NMR (600M, D_2O) δ -1.23, -1.86. ESI-HRMS (negative mode): $C_{26}H_{30}N_{11}O_{15}P_2^-$ $[M-H]^-$ calculated 798.1404; found 798.1408.

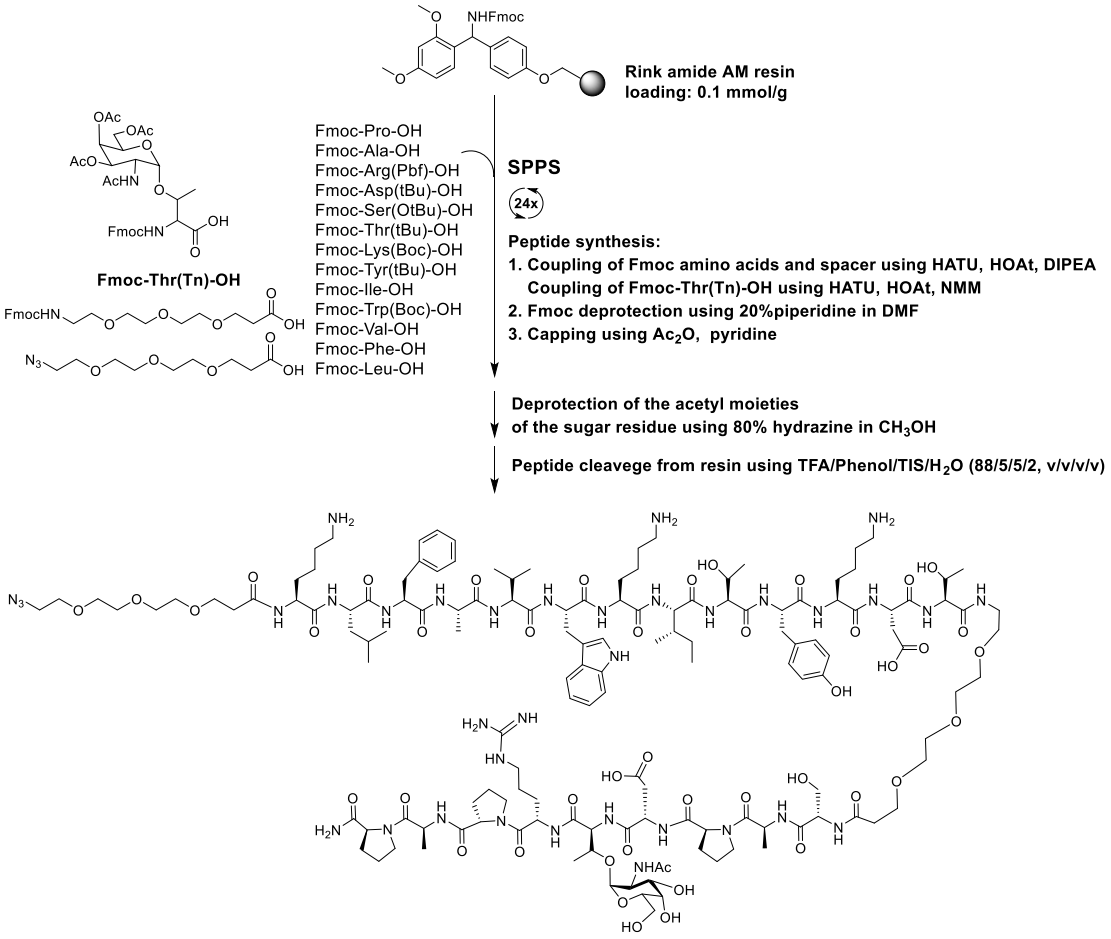


2 **3**

Scheme S2 Synthesis of 2'-O-(4-pentyncarbamoyl)-CDG (3).

Peptide synthesis and purification

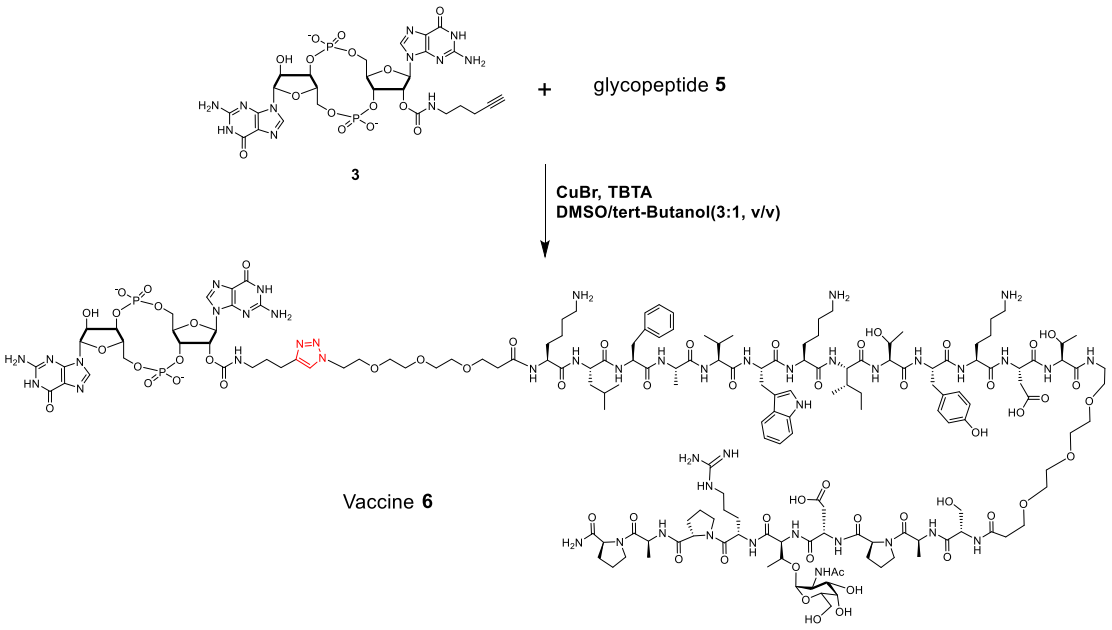
Glycopeptides were synthesized through Solid Phase Peptide Synthesis (SPPS) using Fmoc protocol. The synthesis was performed on Rink amide AM resin (loading 0.1mmol/g). Natural amino acids (4 equiv), triethyleneglycol derived amino acid (4 equiv) and azido-functionalized spacer carboxylic acid (4 equiv) were coupled with 2-(7-Aza-1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU, 4equiv), 1-hydroxy-7-azabenzotriazole (HOAt, 4equiv) and diisopropylethylamine (DIPEA, 8equiv) in DMF for 1h. α-GalNAc glycosylated threonine (2equiv) was synthesized according to our previous works and coupled with HATU (4equiv), HOAt (4equiv) and N-methylmorpholine (NMM, 4equiv) in N-methyl-2-pyrrolidone (NMP) overnight.[4] Fmoc group was deprotected using 20% piperidine in DMF for 15min. Before detaching from resin, the acetyl groups of the Tn antigen were deprotected using 80% hydrazine in CH_3OH for 1h. Glycopeptide 8 (Linker-SAPDT(Tn)RPAP) was cleaved from the resin with TFA/H_2O/TIS (95/2.5/2.5, v/v/v) reagent. And other peptides were cleaved using TFA/Phenol/TIS/H_2O (88/5/5/2, v/v/v/v). After precipitating with diethyl ether, the peptides were purified with semi-preparative HPLC using C18 column and identified with ESI-MS or MALDI-TOF MS. The conjugate **BSA-8** was prepared by coupling glycopeptide 8 to BSA through diethyl squarate based on the described procedures.[4] And the coupling enficiency was 7 copies of glycopeptide per BSA analyzed with MALDI-TOF mass spectrometry (see Analytic data of **BSA-8**).



Scheme S3 Solid-phase synthesis of glycopeptide **5**

Chemical synthesis of vaccine 6

The click reaction was performed according to the work of Jones et al and Sharpless et al.[5] Azido-functionalized glycopeptide **5** (6mg, 1.93μmol, 1equiv) and compound **3** (3mg, 3.68μmol, 2equiv) were dissolved in 0.8mL solvent mixture of DMSO/*tert*-BuOH (3/1, v/v). CuBr (1.1mg, 7.72μmol, 4equiv) and stabilizing ligand tris ((1-benzyl-1H-1, 2, 3-triazol-4-yl) methyl) amine (TBTA, 5.96mg, 11.3μmol) were chosen to catalyze reaction at Ar atmosphere. The mixture system was shaken at 200rpm for 21h at RT. After that, the conjugate product **6** was purified by semi-preparative HPLC using C18 column with the gradients of 20% to 60% of solution B (80% acetonitrile/water with 0.06% trifluoroacetic acid) in solution A (water with 0.06% trifluoroacetic acid) in 40 min (λ=215nm). Retention time is 22.9 min. After lyophilization, vaccine candidate **6** was obtained with yield of 66%.



Scheme S4 Synthesis of vaccine **6** through click reaction.

Evaluation of macrophage activation in vitro using RAW264.7 cell line

RAW264.7 cells (mouse leukaemic monocyte macrophage cell line) were cultured in DMEM medium containing 10% fetal bovine serum (FBS) at 37℃, 5% CO_2. After harvesting, the cells were planted on 24-well culture plates with a density of 5×10^5 cells/well and cultured overnight. For the transfected group, Lipofectamine® 3000 was adopted as transfection reagent and performed according to manufacturer's protocol. Succinctly, samples (**4**, **CDG**, **6** and **7**) were mixed with DMEM as A solution (150μL). Lipofectamine® 3000 (1.5μL for **4**, **CDG** and **6**, 4μL for **7**) was mixed with DMEM as B solution (150μL). After 5mins, A solution was added to B solution. Waiting for 15mins, the mixture was added dropwise to 24-well plate at a final concentration of 12μM and incubated for 18h. 0.1μg/mL of LPS was used as positive control. Then, cells were harvested and incubated with mouse anti-CD86- phycoerythrin antibodies (BD Pharmingen, dilution 1/200) at 0℃ for 1h. After washing, the cells were analyzed on BD Calibur flow cytometry.

Evaluation of macrophage activation *in vivo*

6-8 weeks old female Balb/c mice (3 mice/group) were intraperitoneally injected with samples **4**, **CDG**, **6**, **7** (12nmol in 100μL PBS solution per mouse) and PBS as negative control respectively. LPS (3μg/mouse) was used as positive control. After 20h, the spleens were harvested for analysis. The spleens were grinded and filtered through a 40μm cell strainer. And red blood cells (RBCs) were lysed using lysis buffer for RBC. The left cells were first blocked with anti-CD16/32 antibody (BD Pharmingen, dilution 1/200) and then labeled with mouse antibodies for CD11b (anti-CD11b-FITC, BD Pharmingen, dilution 1/200) and CD86 (anti-CD86- phycoerythrin, BD Pharmingen, dilution 1/200) at 0℃ for 1h. After washing, the cells were analyzed on BD LSRFortessa flow cytometry. Mice used in the experiments were raised in Animal Facility of Center of Biomedical Analysis in Tsinghua University and treated in compliance with the animal ethics guidelines. The animal protocol (approval number: 16-LYM2) was approved by Institutional Animal Care and Use Committee (IACUC) of Tsinghua University. Animal Facility of Center of Biomedical Analysis in Tsinghua University has been authenticated by Association for Assessment and Accreditation of

Laboratory Animal Care (AAALAC).

Vaccine immunization

6-8 week old Babl/c mice (5 mice per group, female) were separately intraperitoneally vaccinated with 12nmol vaccines (**4**, **6** and **7** in 100μL PBS solution per mouse) without the aid of any other adjuvant or liposomes encapsulation. Booster immunizations were performed for five times biweekly. Antisera was collected one week after the last administration. Mice used in the experiments were raised in Animal Facility of Center of Biomedical Analysis in Tsinghua University and treated in compliance with the animal ethics guidelines. The animal protocol (approval number: 16-LYM2) was approved by Institutional Animal Care and Use Committee (IACUC) of Tsinghua University. Animal Facility of Center of Biomedical Analysis in Tsinghua University has been authenticated by Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC).

Antibody titers

116μg/mL BSA-8 in coating buffer (0.1M $NaHCO_3$ solution, pH=9.6) was added to high-binding 96-well ELISA plate (Costar 3590, 100μL/well). After incubation for 12h at 4℃ and washing with PBST solution (0.05% Tween in PBS buffer), the wells were blocked by 0.25% gelatin PBS solution for 3h at room temperature (RT). After washing with PBS and PBST, the diluted antisera (1:200) was added to each well (100μL per well) and incubated for 1.5h at 37℃. After washing again, diluted rabbit anti-mouse IgG-Peroxidase antibodies (1/2000 dilution, Sigma) were added to each well (100μL per well) and incubated for 1h at 37℃. After washing and spin-drying, 1, 2-phenylenediamine substrate (1.5μl/ml 30% H_2O_2 and 1mg/ml 1,2-phenylenediamine) was added to plate (100μL per well) and incubated for 20min. Optical absorption was measured at wavelength of 450nm and antibody titer was defined as highest dilution yielding an optical absorption of 0.1 or greater over that of negative control antisera.[6]

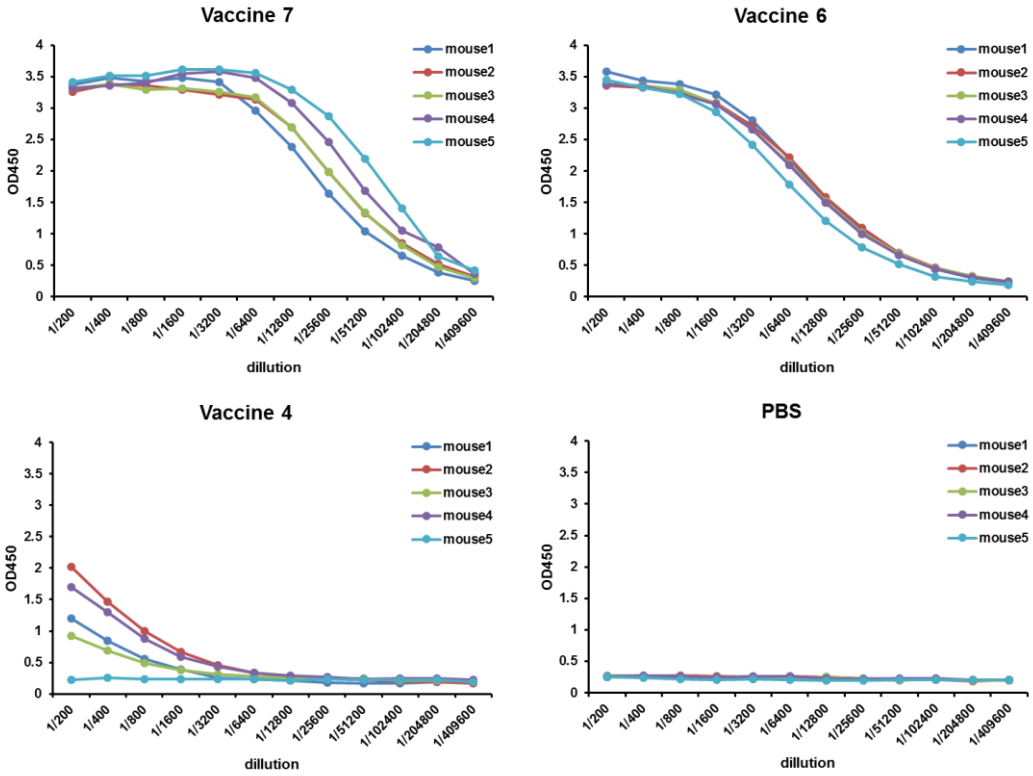


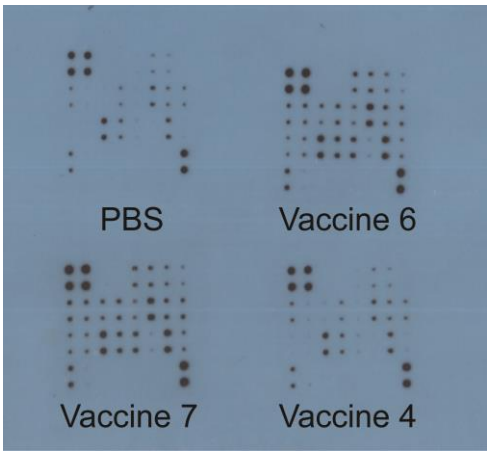
Fig. S1 ELISA for total IgG antibody from individual mice sera of vaccine 7, 6, 4 and PBS group.

Antibody isotypes

96-well ELISA plate was coated with BSA-8 according to the procedure described above. The antisera were diluted to 1:12800 and added to each well, incubating for 1.5h at 37°C. After washing with PBS and PBST, isotype antibodies IgG1, IgG2a, IgG2b, IgG3 and IgM (anti-mouse antibodies from goat, Sigma) were diluted to 1:1000 and added to each well (100μL per well). After incubation for 1h at 37°C and washing, 100μL 1, 2-phenylenediamine substrate described above was added to plate and incubated for 20min. Optical absorption was also measured at wavelength of 450nm.

Cytokine profile of antisera

Mouse Cytokine Antibody Array (22 targets, Abcam) was used to screen the cytokine profile of antisera from immunized mice. The assay was performed according to the manufacturer's protocol. Before incubation, the cytokine antibody array membranes were blocked with blocking buffer at RT for 0.5h. The antisera from immunized mice was then diluted to 1:10 with blocking buffer and incubated with cytokine antibody array membranes at RT for 2h. After washing, the membranes were incubated with biotin-conjugated anti-cytokine antibodies at RT for 2h. After washing again, HRP-conjugated streptavidin was added to incubate with membranes at RT for 2h. As for chemiluminescence detection, the membranes were covered with detection buffers mixture and sent to exposure on X-ray film. To obtain the densitometry data, the digitized images scanning from X-ray film with resolution of 1200 dpi were processed with ImageJ software. The raw densitometry data was then subtracted with background and normalized to the positive control signals, following by proceeding to analysis.



Pos	Pos	Neg	Neg	GCSF	GCSF	IL-2	IL-3
Pos	Pos	Neg	Neg	GCSF	GCSF	IL-2	IL-3
IL-4	IL-5	IL-6	IL-9	IL-10	IL-12 p40p70	IL-12 p70	IL-13
IL-4	IL-5	IL-6	IL-9	IL-10	IL-12 p40p70	IL-12 p70	IL-13
IL-17	IFN-γ	MCP-1	MCP-5	RANTES	SCF	sTNFRI	TNF-α
IL-17	IFN-γ	MCP-1	MCP-5	RANTES	SCF	sTNFRI	TNF-α
Thrombo poietin	VEGF	BLANK	BLANK	BLANK	BLANK	BLANK	Pos
Thrombo poietin	VEGF	BLANK	BLANK	BLANK	BLANK	BLANK	Pos

Fig. S2 Representative image of cytokine antibody array membranes (left) and cytokine array maps (right).

The binding of antisera to tumor cells

MCF-7 cells (human breast cancer cell line) were cultured in DMEM medium containing 10% fetal bovine serum (FBS) at 37℃, 5% CO_2. The cells were digested with Trypsin solution (0.25%, w/v) and then washed with PBS solution containing 1% FBS. 4×10^5 MCF-7 cells suspensions were incubated with 200μL antisera (1/50 dilution in PBS) on ice for 1h. After washing three times with PBS, cells were incubated with FITC-conjugated rabbit antimouse IgG antibody (1/200 dilution, GeneTech) for 1h on ice. After washing three times, the cells were suspended in 700μL PBS and analyzed on a BD Calibur flow cytometry.

Complement dependent cytotoxicity (CDC)

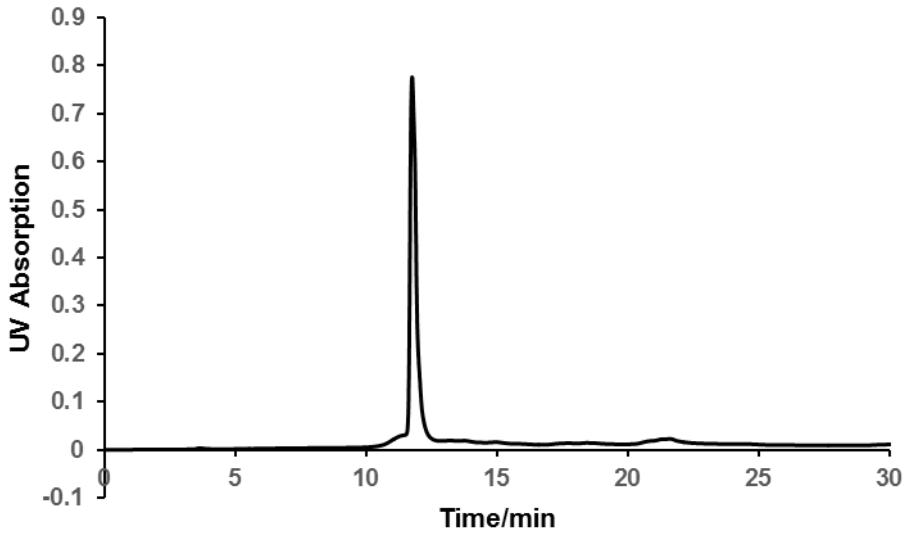
The CDC analysis was based on the MTT assay (MTT: 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2-H-tetrazolium bromide). MCF-7 cells were incubated with antisera (1/20 diluted with PBS) on the ice for 1h. After centrifugation and washing, MCF-7 cells were suspended with culture medium (DMEM containing 10% FBS) and planted on into 96-well cell plate with 8000 cells in 50μL medium per well. 100μL rabbit complement (Bio-Rad) diluted to 1:10 was added to each well and incubated for 4h. 20μL/well MTT solution (0.5% MTT in PBS) was then added and incubated for 3h. After removing the culture medium, 100μL DMSO was added to each well and the absorption was measured at 490nm wavelength. Each sample was performed in four repeats. The complement dependent cytotoxicity was measured with following formula.

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

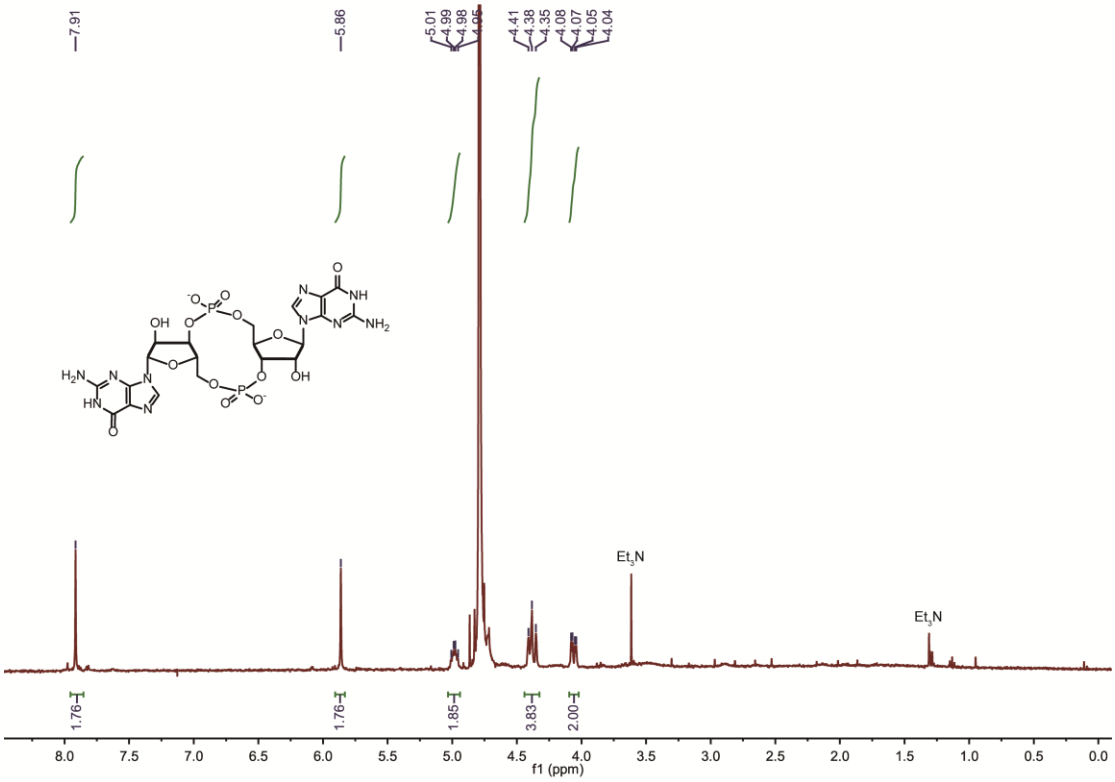
Analytic data of compounds and peptides

Compound 2 (CDG). Analytic gradient is 2% to 40% of solution B (acetonitrile) in solution A (0.05M triethylamine acetate, pH=7.0) in 30 min on the analytic C18 column (λ=260nm). Retention time is 11.7 min.

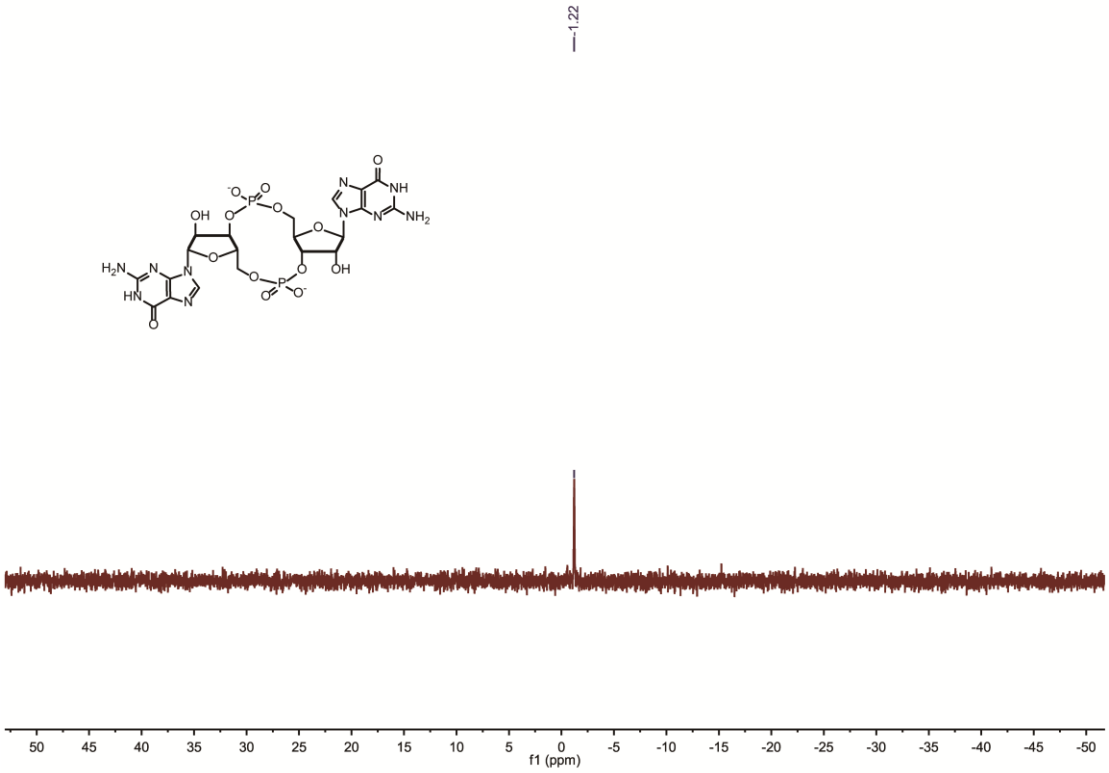
Analytic HPLC trace of **CDG**:



^{1}H NMR of **CDG**:

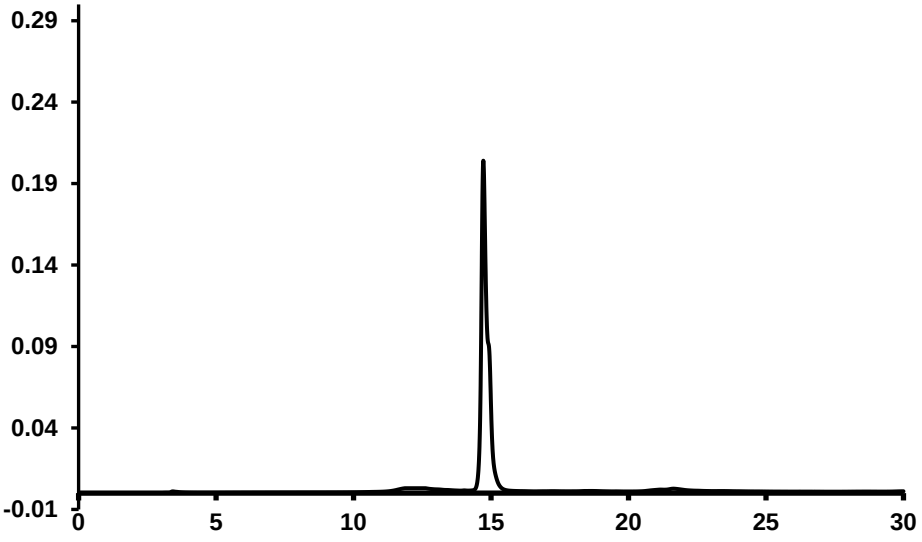


^{31}P NMR of **CDG**:

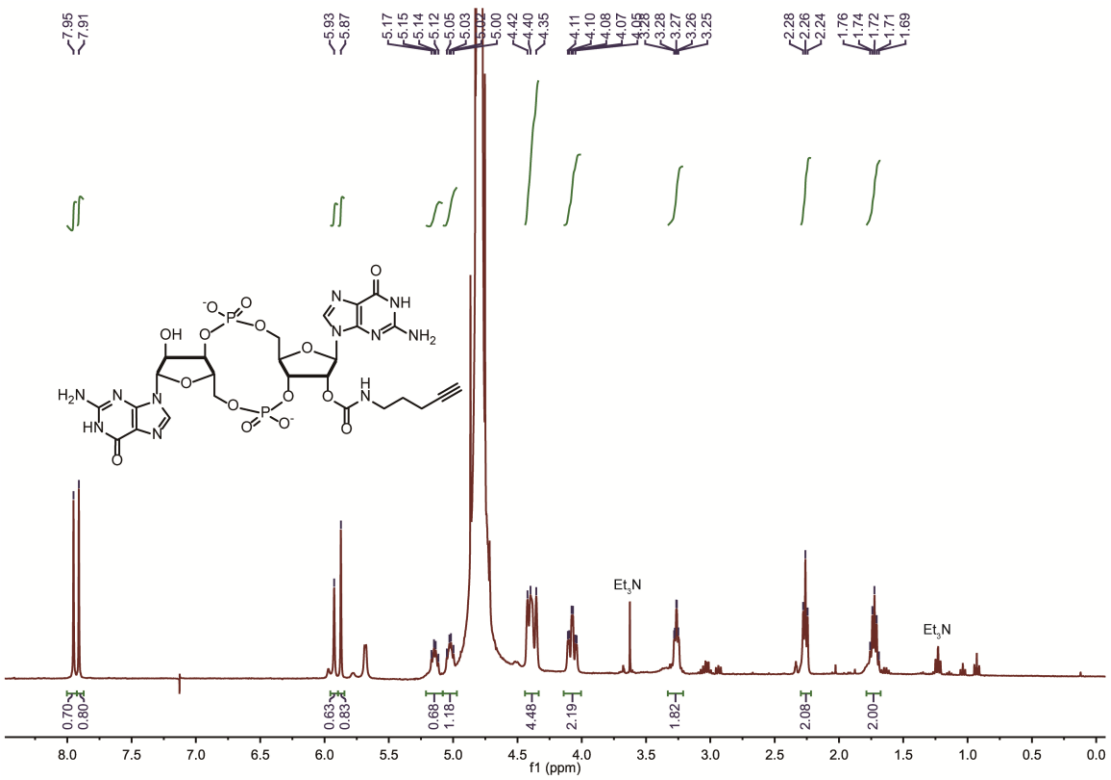


Compound 3. Analytic gradient is 2% to 40% of solution B (acetonitrile) in solution A (0.05M triethylamine acetate, pH=7.0) in 30 min on the analytic C18 column (λ=260nm). Retention time is 14.7 min.

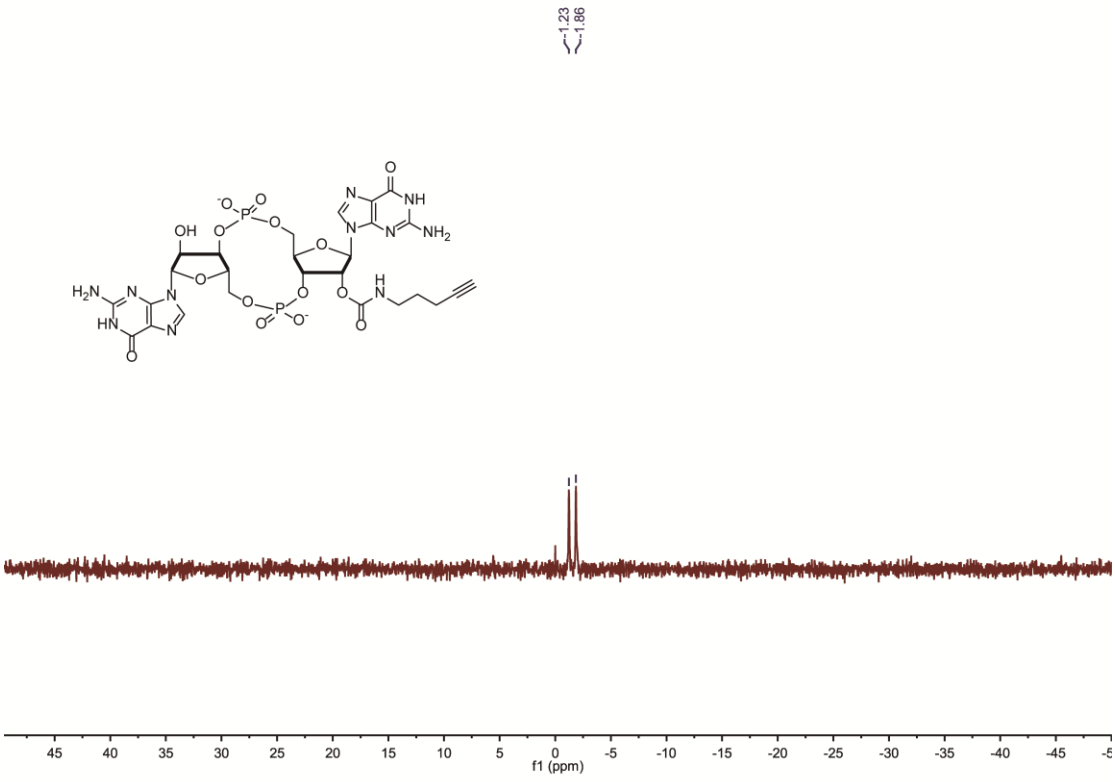
Analytic HPLC trace of **compound 3**:



^{1}H NMR of **compound 3**:

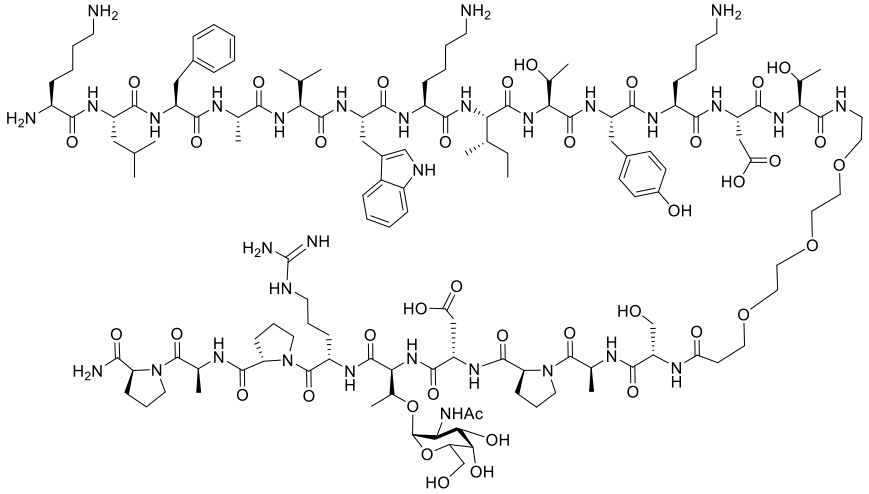


^{31}P NMR of **compound 3**:

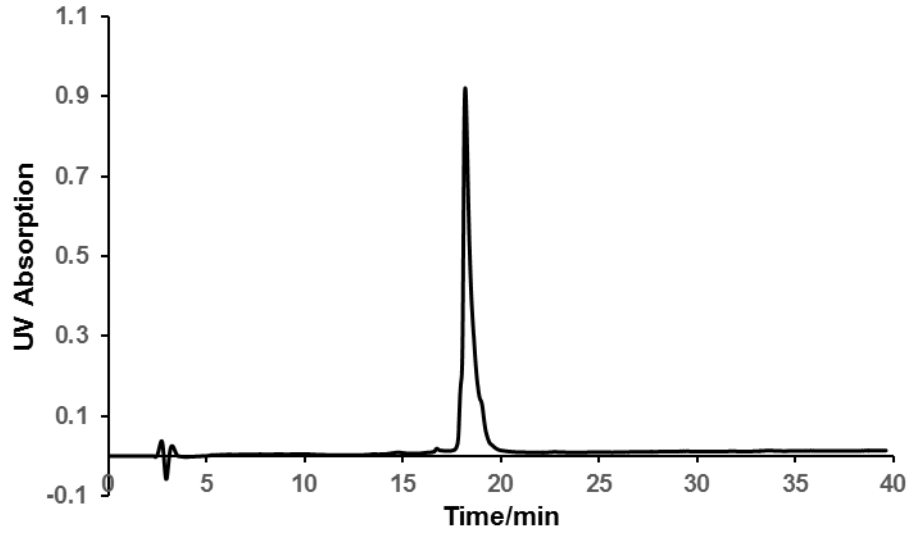


Glycopeptide 4. Analytic gradient is 20% to 60% of solution B (80% acetonitrile/water with 0.06% trifluoroacetic acid) in solution A (water with 0.06% trifluoroacetic acid) in 40 min on the analytic C18 column (λ=215nm). Retention time is 18.1 min. ESI-MS: $C_{134}H_{212}N_{32}O_{40}$. Calculated: 2912.5,

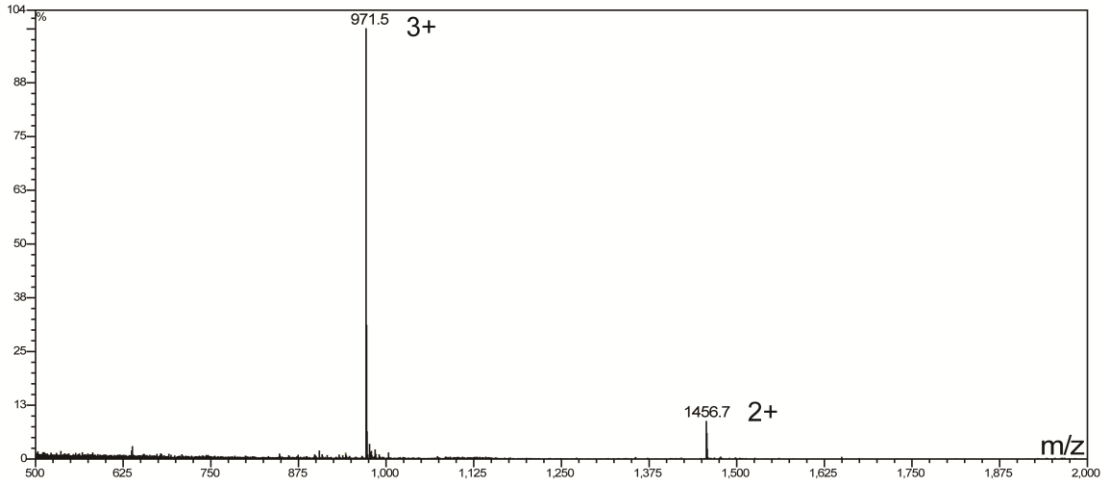
$[M+H]^+$. Found: 1456.7, $[M+2H]^{2+}$; 971.5, $[M+3H]^{3+}$.



Analytic HPLC trace of **glycopeptide 4**:

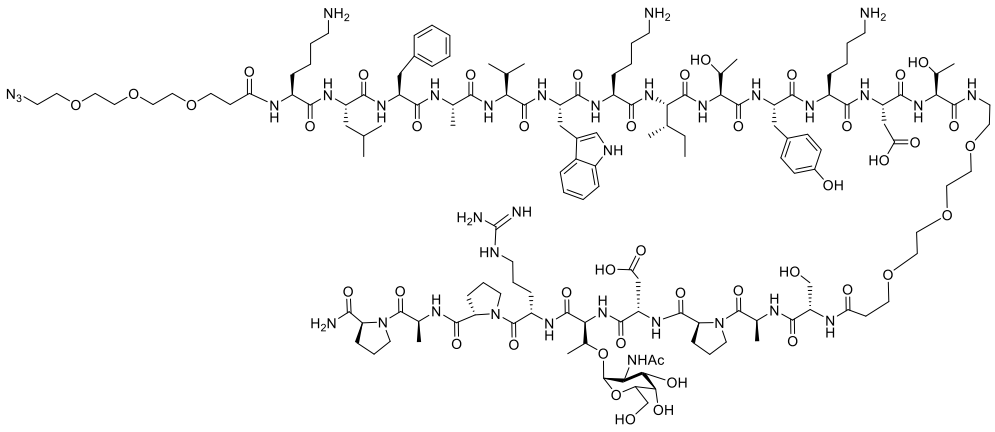


ESI-MS of **glycopeptide 4**:

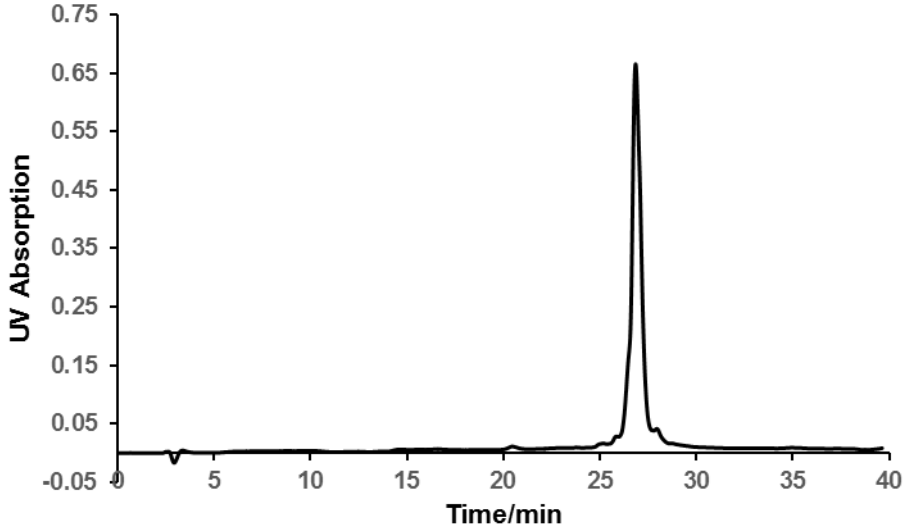


Glycopeptide 5. Analytic gradient is 20% to 60% of solution B (80% acetonitrile/water with 0.06% trifluoroacetic acid) in solution A (water with 0.06% trifluoroacetic acid) in 40 min on the analytic

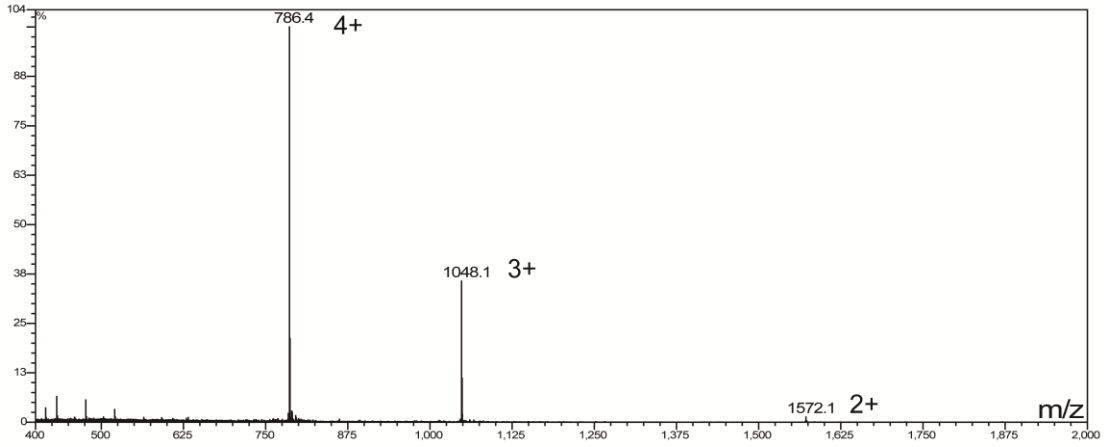
C18 column (λ=215nm). Retention time is 26.8 min. ESI-MS: $C_{143}H_{227}N_{35}O_{44}$. Calculated: 3142.7, $[M+H]^+$. Found: 1572.1, $[M+2H]^{2+}$;1048.1, $[M+3H]^{3+}$; 786.4, $[M+4H]^{4+}$.



Analytic HPLC trace of **glycopeptide 5**:

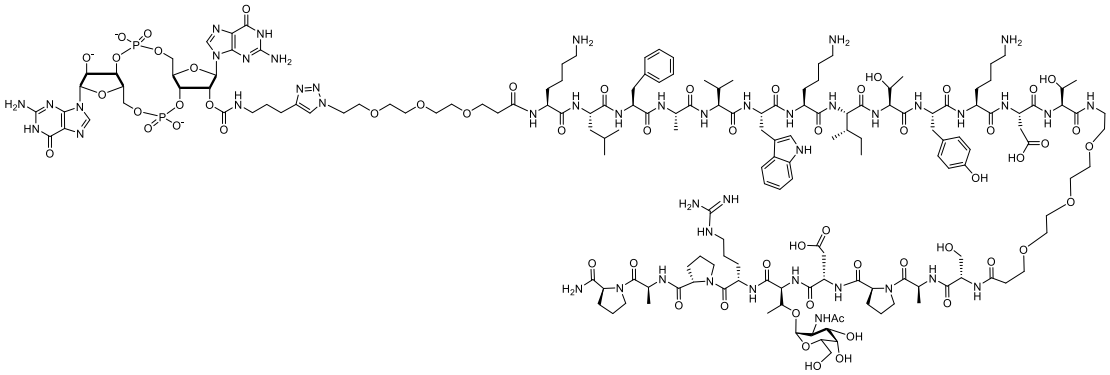


ESI-MS of **glycopeptide 5**:

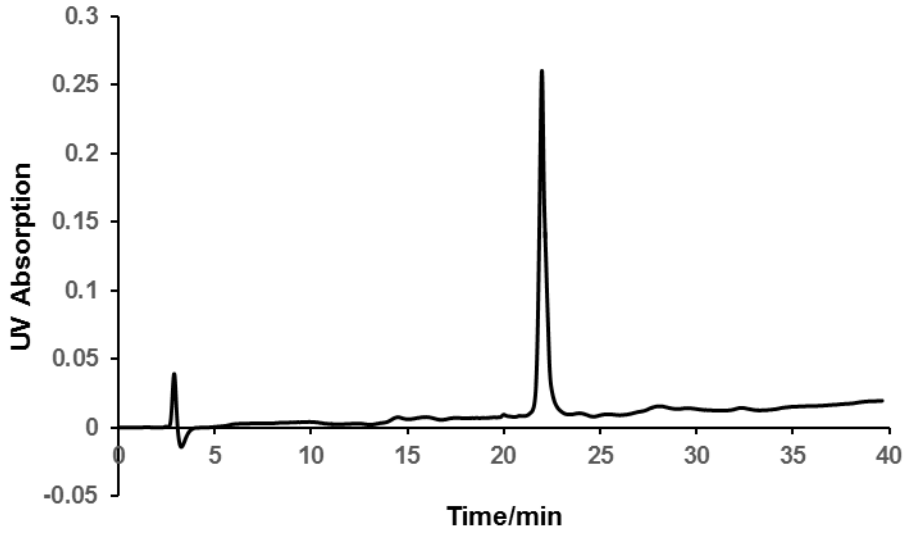


Vaccine candidate 6. Analytic gradient is 20% to 60% of solution B (80% acetonitrile/water with 0.06% trifluoroacetic acid) in solution A (water with 0.06% trifluoroacetic acid) in 40 min on the analytic C18 column (λ=215nm). Retention time is 22.9 min. ESI-MS: $C_{169}H_{258}N_{46}O_{59}P_2$.

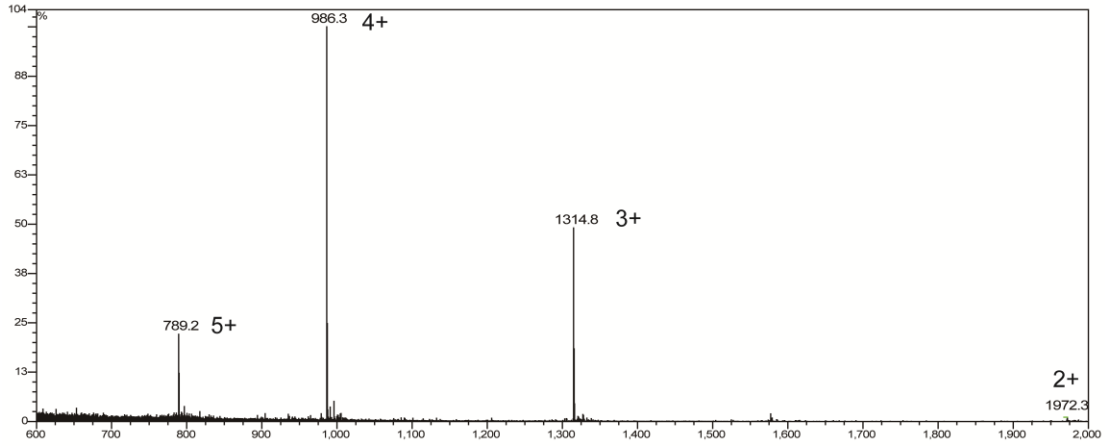
Calculated: 3941.8, $[M+H]^+$. Found: 1972.3, $[M+2H]^{2+}$;1314.8, $[M+3H]^{3+}$; 986.3, $[M+4H]^{4+}$; 789.2, $[M+5H]^{5+}$. MALDI-TOF-MS: $[M+H]^+$, calculated 3941.8258, found 3941.4810.



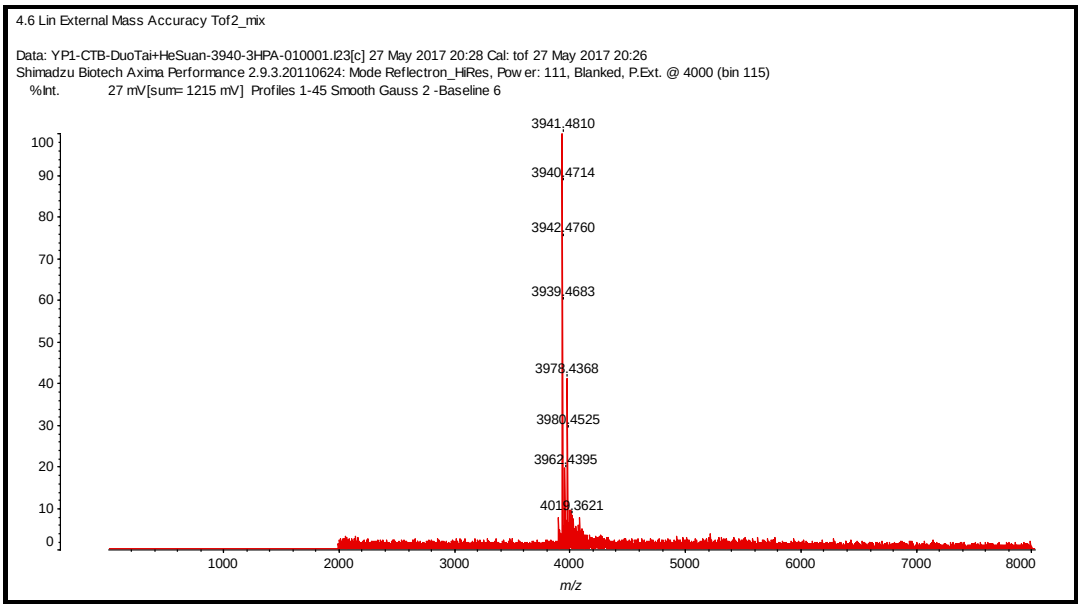
Analytic HPLC trace of **vaccine candidate 6**:

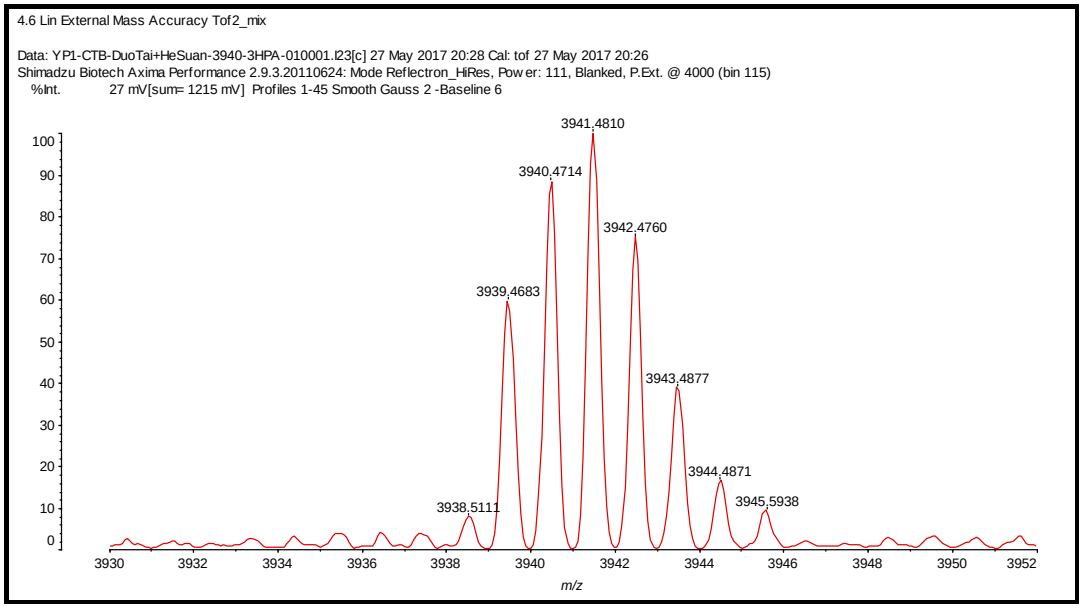


ESI-MS of **vaccine candidate 6**:

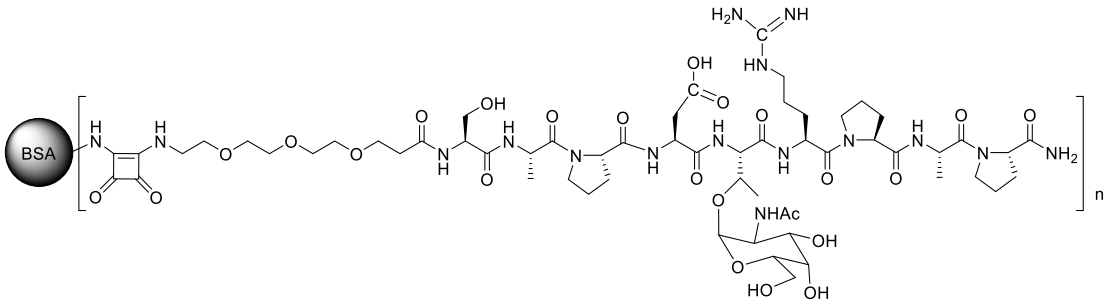


MALDI-TOF-MS of **vaccine candidate 6**:

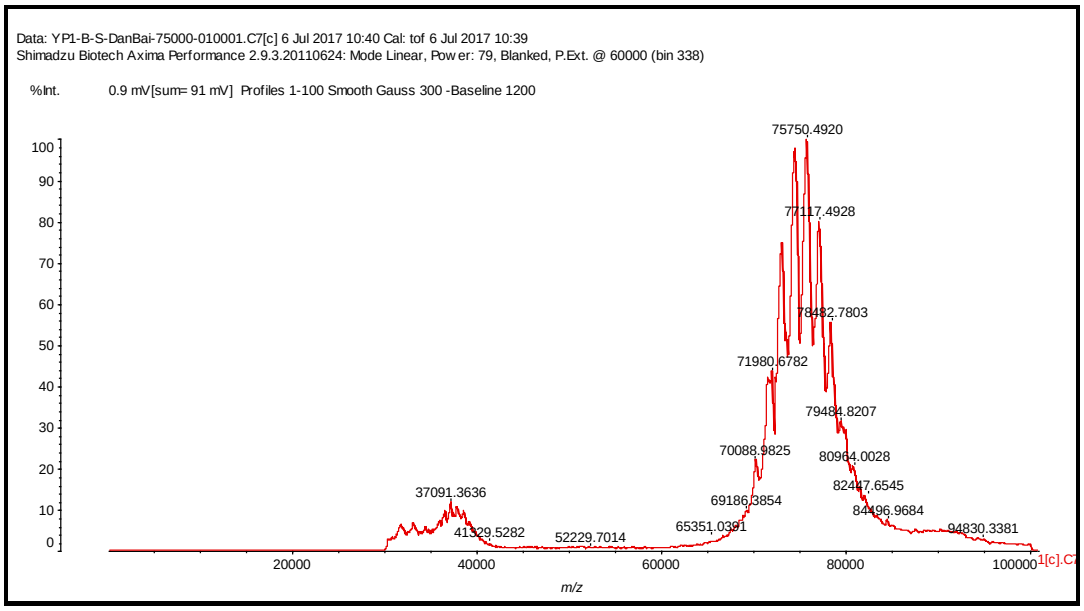




Conjugate **BSA-8**. The coupling efficiency was about 7 copies of glycopeptide/BSA determined by MALDI-TOF mass spectrometry.



MALDI-TOF-MS of **BSA-8**:



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