Supporting information

Synthesis and functional studies of self-adjuvanting multicomponent anti-HER2 cancer vaccines

Qi Feng[*acde1*], Xiaoyue Yu[be1], Yixue ~~Wang[1]~~Wang[acde], Shiyang Li[acde], Yang Yang[*f2*]

[a]*Department of Nephrology, The First Affiliated Hospital of Zhengzhou University, Zhengzhou 450052, P. R. China. E-mail: fengqi2019@zzu.edu.cn*

[b]*Research Institute of Nephrology, Zhengzhou University, Zhengzhou 450052, P. R. China*

[c]*Henan Province Research Center For Kidney Disease, Zhengzhou 450052, P. R. China*

[d]*Key Laboratory of Precision Diagnosis and Treatment for Chronic Kidney Disease in Henan Province, Zhengzhou 450052, P. R. China*

[e]*Department of Cardiovascular Medicine, The First Affiliated Hospital of Zhengzhou University, Zhengzhou 450052, P. R. China*

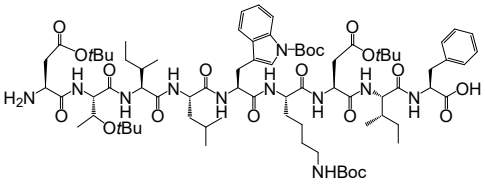
[f]*Clinical Systems Biology Laboratories, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, 450052, P. R. China. E-mail: yangyangbio@163.com*

~~[1]Research Institute of Nephrology, The First Affiliated Hospital of Zhengzhou University, Zhengzhou 450052, P. R. China~~

~~[2]Clinical Systems Biology Laboratories, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, 450052, P. R. China~~

~~* Correspondence: fengqi2019@zzu.edu.cn; yangyangbio@163.com~~

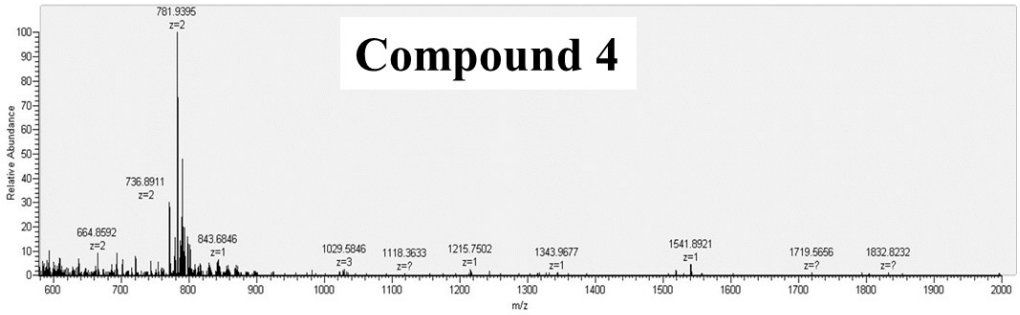
1. General synthetic procedures of compounds



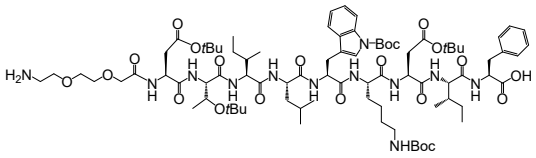
Compound **4**

2-Chlorotrityl chloride resin preloaded with phenylalanine (47.0 mg, 0.04 mmol; 0.85 mmol/g, 100-200 mesh, Watanabe Chemical Industries, Ltd) was swollen in dry

DCM (1 mL) for 1 h at room temperature. After filtration by DMF (4 mL) for 3 times, the resin was treated with Fmoc-Ile-OH (0.2 mmol, 5 eq.), HOBt (0.2 mmol, 5 eq.), HBTU (0.2 mmol, 5 eq.) and NMM (0.4 mmol, 10 eq.) in DMF (2 mL) with shaking for 3 h at room temperature. The resin was filtered and washed with DMF (3 × 2 mL), DCM (3 × 2 mL) and DMF (3 × 2 mL). After the removal of Fmoc group with 20% piperidine in DMF (2 mL) for 30 min, the resin was filtered and washed by DMF (3 × 2 mL), DCM (3 × 2 mL) and DMF (3 × 2 mL), followed by elongating Fmoc protected amino acids (Fmoc-AA-OH) for **4**. The elongation of peptide chains was carried out with same the protocol. After all coupling steps were finished, side chain protected peptide fragments **4** was cleaved from the resin by using AcOH/TFE/DCM (1:1:8, 4 mL) for 2 h. After the filtration, the collected solution was added to cold diethyl ether (30 mL) to give white precipitate. The precipitate was collected by centrifugation and concentrated *in vacuo*. Purification of crude **4** was carried out by silica-gel column chromatography (methanol/chloroform, 1:8 v/v) to give **4** (38.4 mg, 63%) (R_f 0.33: methanol/chloroform, 1:8 v/v)) as white solid.



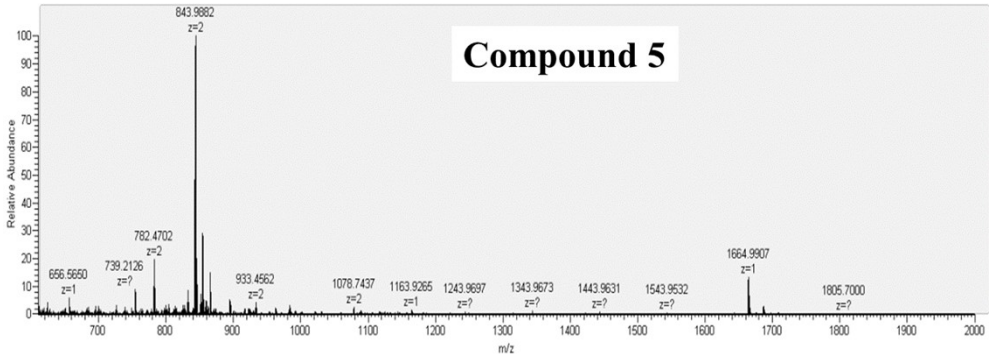
Compound **4**: HRMS (ESI-LIT-orbitrap): *m/z* for $C_{78}H_{123}N_{11}O_{19}$ $[M+2Na]^{2+}$ calcd 781.9391, found 781.9395; $[M+Na]^{+}$ calcd 1541.8922, found 1541.8921.



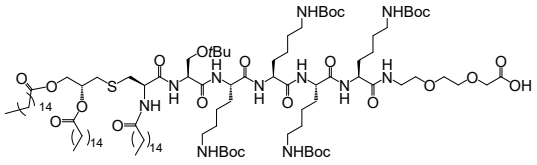
Compound **5**

2-Chlorotrityl chloride resin preloaded with phenylalanine (23.5 mg, 0.02 mmol; 0.85 mmol/g, 100-200 mesh, Watanabe Chemical Industries, Ltd) was swollen in dry DCM (1 mL) for 1h. After washing by DMF (2 mL) for 3 times, then the resin was treated with Fmoc-Ile-OH (0.2 mmol, 5 eq.), HOBt (0.1 mmol, 5.0 eq.), HBTU (0.1 mmol, 5.0 eq.) and NMM (0.2 mmol, 10.0 eq.) in DMF (1 mL) with shaking for 3 h.

Subsequently, the resin was filtered and washed with DMF (3 × 2 mL), DCM (3 × 2 mL) and DMF (3 × 2 mL). After removing Fmoc group with 20% piperidine in DMF (2 mL) for 30 min, the resin was filtered and washed by DMF (3 × 2 mL), DCM (3 × 2 mL) and DMF (3 × 2 mL), followed by elongating Fmoc protected amino acids (Fmoc-AA-OH). The elongation of peptide chains was carried out with same the protocol. After all coupling steps were finished, side chain protected peptide fragments **5** was cleaved from the resin by using AcOH / TFE / DCM (1:1:8, 2 mL) for 2 h. After the filtration, the collected solution was added to cold diethyl ether (20 mL) to give crude precipitate. The crude precipitate was collected by centrifugation and evaporated *in vacuo*. Purification of crude **5** was carried out by silica-gel column chromatography (methanol/chloroform, 1:8 v/v) to give **5** (17.9 mg, 54%) as white solid.



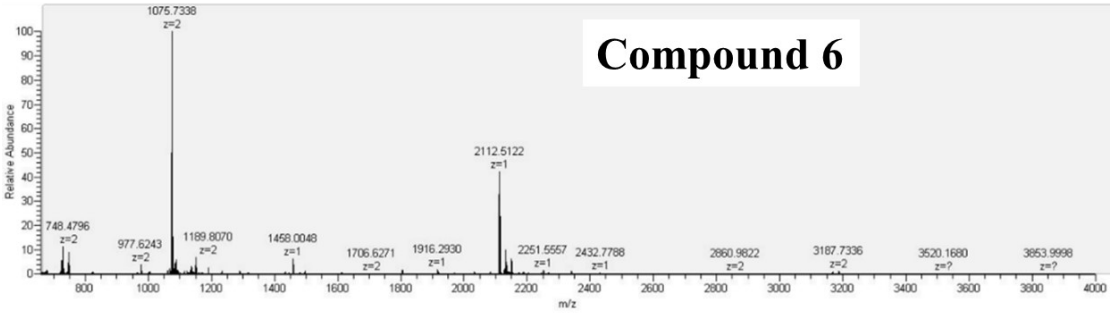
Compound **5**: HRMS (LTQ-Orbitrap): *m/z* for $C_{84}H_{134}N_{12}O_{22}$ $[M+H]^+$ calcd 1664.9842, found 1664.9907; $[M+ H+Na]^{2+}$ calcd 843.9867, found 843.9882.



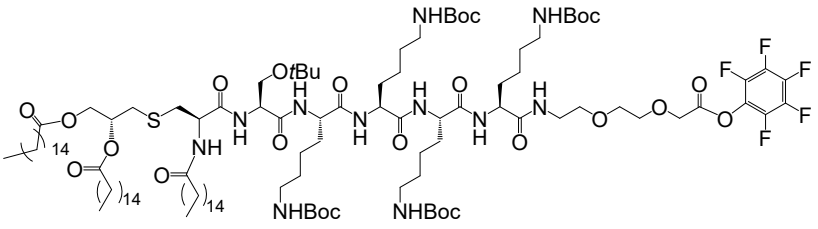
Compound **6**: This compound was characterized in our previous report[1].

2-chlorotrityl chloride resin (0.02 mmol, 200-400 mesh, GL Biochem) was immersed in dry DCM (1 mL) for 1 h. After filtration by DMF (2 mL) for 3 times, the resin was treated with Fmoc-NH-PEG_2-CH_2-COOH (0.04 mmol, 2.0 eq.) and DIPEA (0.08 mmol, 4.0 eq.) in DMF/DCM (500 μL / 500 μL) with shaking overnight. After the removal of Fmoc group by 20% piperidine in DMF (2 mL) for 30 min, the resin

was filtered and washed by DMF (3 × 2 mL), DCM (3 × 2 mL) and DMF (3 × 2 mL). The resin was treated with Fmoc-Lys (Boc)-OH (0.1 mmol, 5.0 eq.), PyBOP (0.1 mmol, 5.0 eq.) and NMM (0.2 mmol, 10.0 eq.) in dry DMF (2 mL) with shaking for 3 h at room temperature. The resin was filtered and washed by DMF (3 × 2 mL), DCM (3 × 2 mL) and DMF (3 × 2 mL). After Fmoc deprotection with 20% piperidine in DMF (2 mL) for 30 min, the resin was filtered and washed by DMF (3 × 2 mL), DCM (3 × 2 mL) and DMF (3 × 2 mL). The elongation of peptide chain (4 × Lys, 1 × Ser) was carried out with the same protocol. Then, the resultant resin was added Pam_3Cys-OH (0.06 mmol, 3.0 eq.), PyBOP (0.2 mmol, 10.0 eq.) and DIPEA (0.4 mmol, 10.0 eq.) in dry DCM/NMP (1 mL / 1 mL) with shaking for 16 h at room temperature. The resin was filtered and washed by NMP (3 × 2 mL), DCM (3 × 2 mL) and NMP (3 × 2 mL) to obtain resin bound lipopeptide. After all coupling steps were finished, the peptide with protected groups was cleaved from the resin by using AcOH / TFE / DCM (1:1:8, 2 mL) solution for 2 h. After the filtration, the collected solution was added to cold diethyl ether (25 mL) to give white crude precipitate. The precipitate was collected by centrifugation and concentrated *in vacuo*. Purification was carried out by silica-gel column chromatography (methanol/chloroform, 1:10 v/v) to give **6** (26.4 mg, 62%) as white solid: R_f 0.25 (methanol/chloroform, 1:10 v/v).

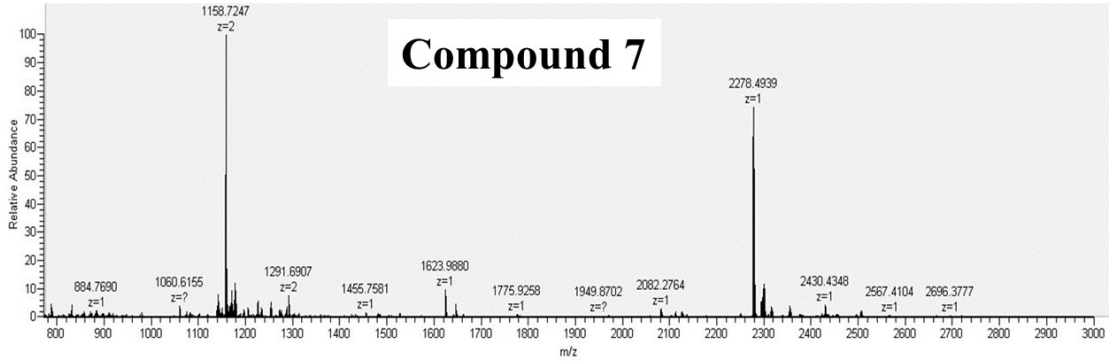


Compound **6**: HRMS (LTQ Orbitrap): *m/z* for $C_{111}H_{207}N_{11}O_{24}S$ $[M+H+K]^{2+}$ calcd 1075.7387, found 1075.7338; $[M+H]^+$ calcd 2112.5142, found 2112.5122.

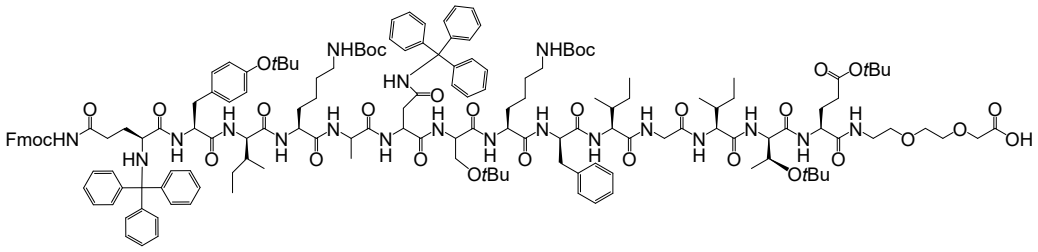


Compound **7**: This compound was characterized in our previous report[1].

To a solution of lipopeptide fragment **6** (2.12 mg, 1 μmol) in dry DMF was added pyridine (4.25 μmol, 5.0 eq.), followed by drop wisely adding pentafluorophenol trifluoroacetate (4.25 μmol, 5.0 eq.). The coupling reaction was stirred for 5 h under Argon at room temperature. After quantitative conversion of **6** to **7** was confirmed by MS, the solution was concentrated *in vacuo* to give crude **7** as white solid. Crude **7** was used for next coupling reaction without further purification.



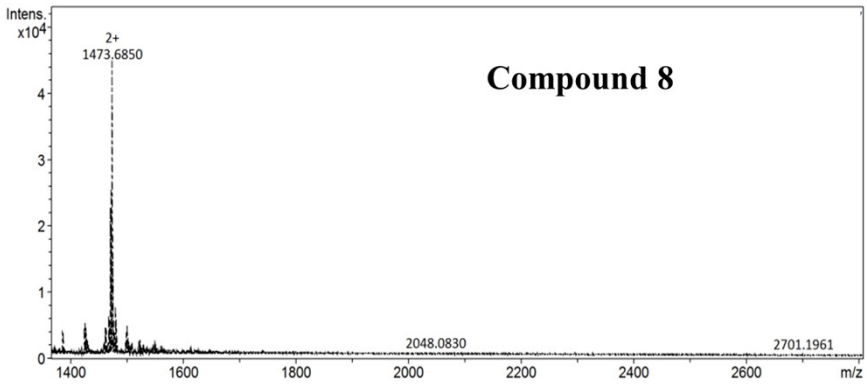
Compound **7**: HRMS (ESI-LIT-orbitrap): *m/z* for $C_{117}H_{206}F_5N_{11}O_{24}S$ $[M+H+K]^{2+}$ calcd 1158.7308, found 1158.7247; $[M+H]^+$ calcd 2278.4945, found 2278.4939.



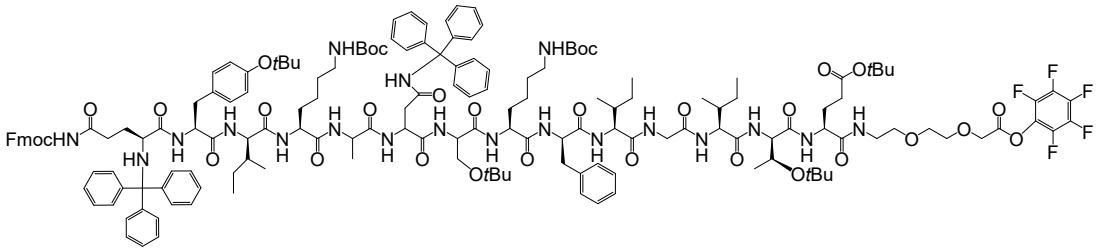
Compound **8**

The synthesis of compound **8** was started from loading Fmoc-protected triethylene glycolic acid (0.04 mmol, 2.0 eq.) on 2-chlorotrityl chloride resin (0.02 mmol, 200-400 mesh, GL Biochem) in the presence of DIPEA in DMF/DCM (500 μL / 500 μL) with shaking for 16h at rt., followed by deprotection with 20% piperidine in DMF. Then, coupling the Fmoc protected amino acids (0.1 mmol, 5.0 eq.) was carried out one by one with the coupling reagents of HOBT (0.1 mmol, 5.0 eq.) and HBTU (0.1 mmol, 5.0 eq.) with NMM (0.2 mmol, 10.0 eq.) in DMF (1 mL) according to the standard Fmoc solid-phase peptide synthesis method. After Fmoc-Gln (Trt)-OH coupled as the final amino acid, side-chain protected peptide was released from the

resin by treating 30% HFIP in DCM (1 mL) for 2h. After the filtration, the collected solution was added to cold diethyl ether (20 mL) to give white precipitate. The precipitate was collected by centrifugation and concentrated *in vacuo*. Finally, the crude compound was purified by silica-gel column chromatograph (MeOH: $CHCl_3$ = 1:5) to afford purified compound **8** (24.9 mg, 43%) as white solid.

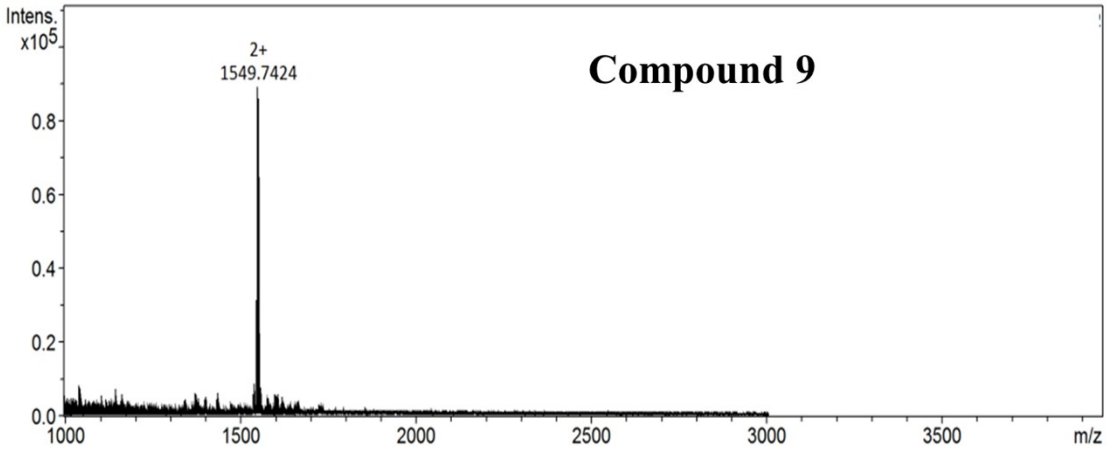


Compound **8**: HRMS (ESI-LIT-orbitrap): *m/z* for $C_{159}H_{215}N_{19}O_{31}$ $[M+2Na]^{2+}$ calcd 1474.2678, found 1473.6850.

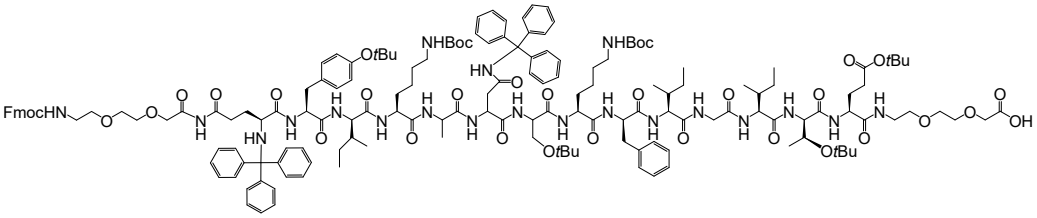


Compound **9**

To a solution of compound **8** (2.47 mg, 0.85 μmol) in co-solvent of DMF/DCM (100 μL / 100 μL) was added pentafluorophenol trifluoroacetate (0.73 μL, 4.25 μmol) together with pyridine (0.35 μL, 4.25 μmol) under Ar at room temperature. After the mixture was stirred for 5h, the solution was removed by evaporation to give **9** as white solid and the compound **9** was used without further purification in the next reaction. By LC-MS analysis, the start material **8** was completely consumed and the main peak is **9**.

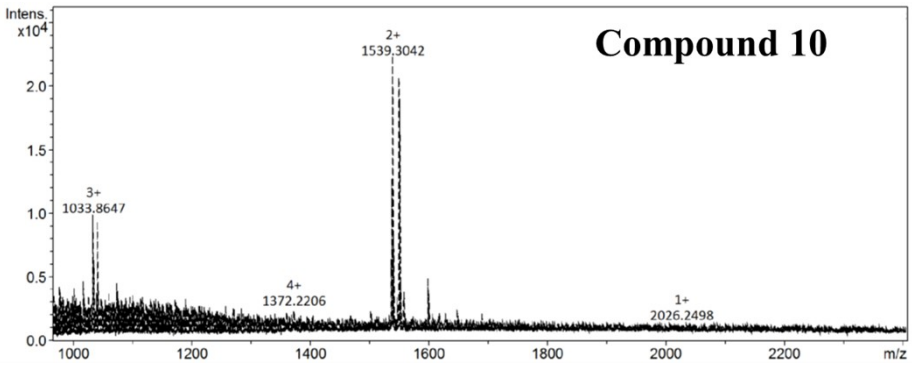


Compound **9**: HRMS (ESI-LIT-orbitrap): m/z for $C_{165}H_{214}F_5N_{19}O_{31}$ $[M+2Na]^{2+}$ calcd 1549.7746, found 1549.7424

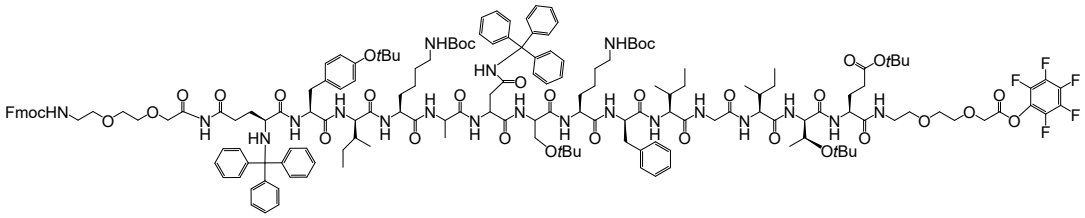


Compound **10**

The synthesis of compound **10** was started from loading Fmoc-protected triethylene glycolic acid (0.04 mmol, 2.0 eq.) on 2-chlorotrityl chloride resin (0.02 mmol, 200-400 mesh, GL Biochem) in the presence of DIPEA in DMF/DCM (500 μL / 500 μL) with shaking for 16h at rt., followed by deprotection with 20% piperidine in DMF. Then, coupling the Fmoc protected amino acids (0.1 mmol, 5.0 eq.) was carried out one by one with the coupling reagents of HOBT (0.1 mmol, 5.0 eq.) and HBTU (0.1 mmol, 5.0 eq.) with NMM (0.2 mmol, 10.0 eq.) in DMF (1 mL) according to the standard Fmoc solid-phase peptide synthesis method. After Fmoc-protected triethylene glycolic acid coupled as the final coupling residue, side-chain protected peptide was released from the resin by treating 30% HFIP in DCM (1 mL) for 2h. After the filtration, the collected solution was added to cold diethyl ether (20 mL) to give white precipitate. The precipitate was collected by centrifugation and concentrated *in vacuo*. Finally, the crude compound was purified by silica-gel column chromatograph (MeOH: $CHCl_3$ = 1:8) to afford purified compound **10** (25.1 mg, 41%) as white solid.

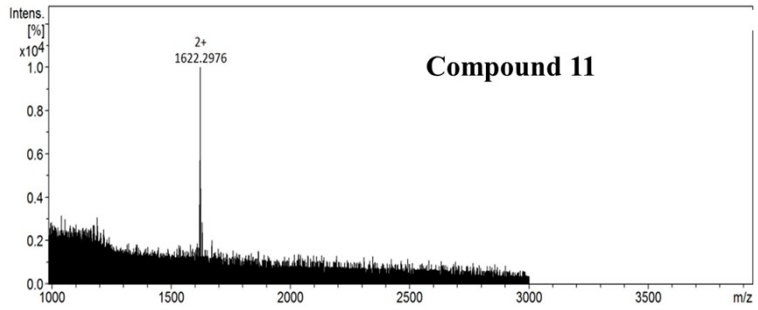


Compound **10**: HRMS (ESI-LIT-orbitrap): m/z for $C_{165}H_{226}N_{20}O_{34}$ $[M+3Na]^{3+}$ calcd 1033.8660, found 1033.8647; $[M+3Na]^{3+}$ calcd 1539.3194, found 1539.3042.

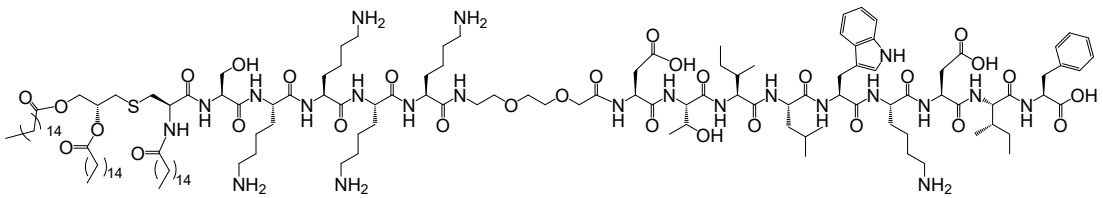


Compound **11**

To a solution of compound **10** (2.58 mg, 0.85 μmol) in co-solvent of DMF/DCM (100 μL / 100 μL) was added pentafluorophenol trifluoroacetate (0.73 μL, 4.25 μmol) together with pyridine (0.35 μL, 4.25 μmol) under Ar at room temperature. After the mixture was stirred for 5h at room temperature. The solution was removed by evaporation to give **11** as white solid and **11** was used without further purification in the next reaction. By LC-MS analysis, the start material **7** was completely consumed and the main peak is **11**.



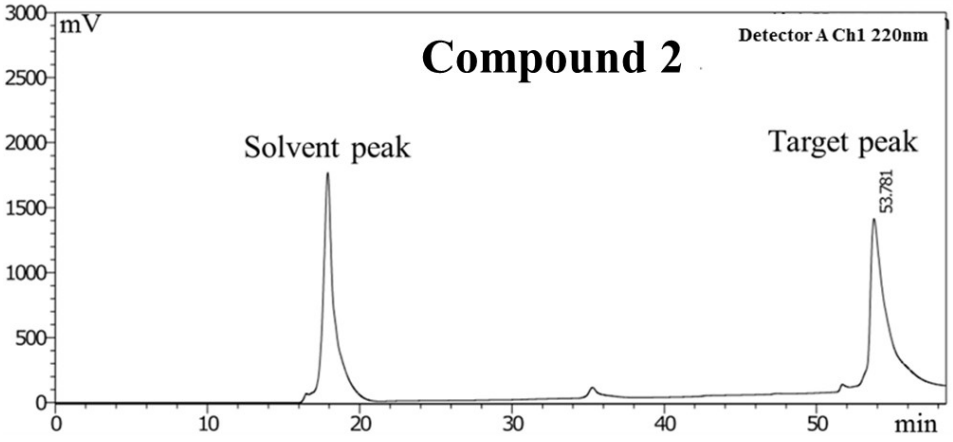
Compound **11**: HRMS (ESI-LIT-orbitrap): m/z for $C_{171}H_{225}F_5N_{20}O_{34}$ $[M+2Na]^{2+}$ calcd 1622.3115, found 1622.2976.

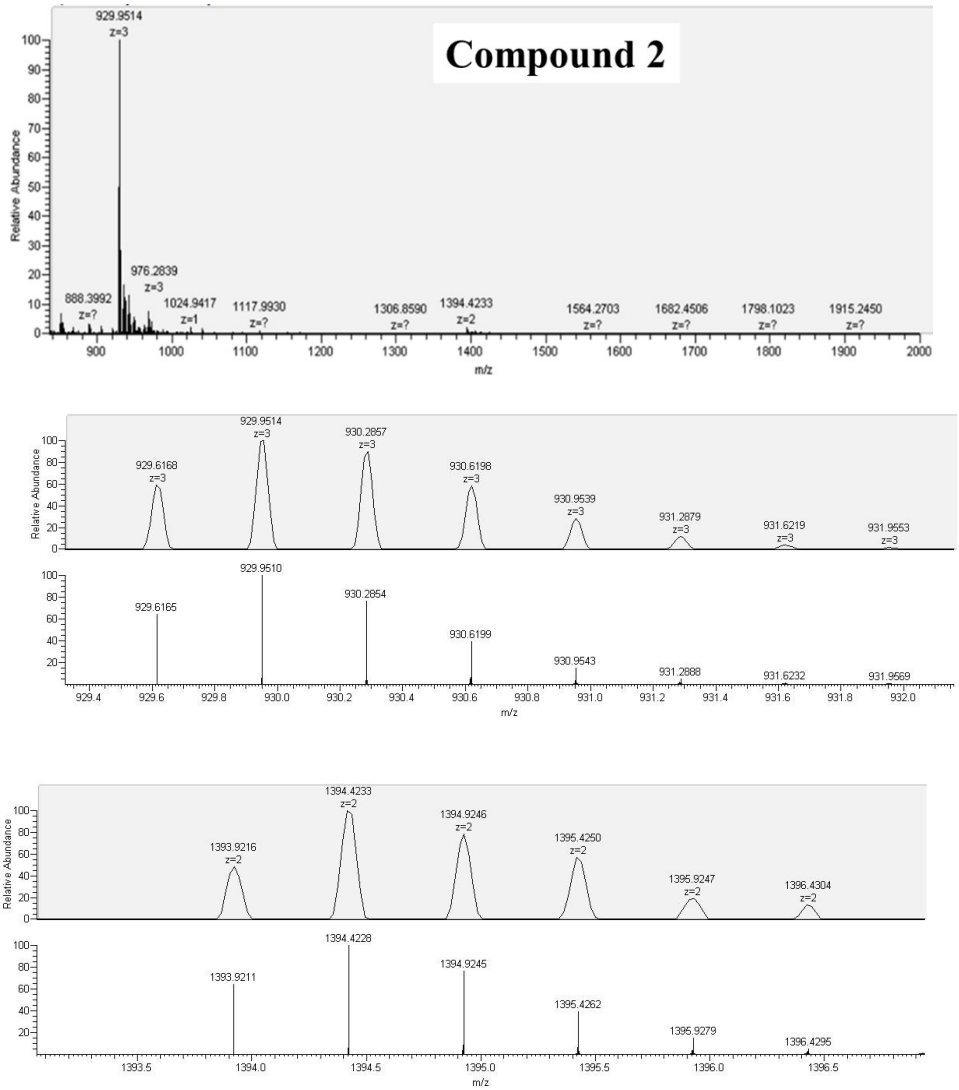


Compound **2**

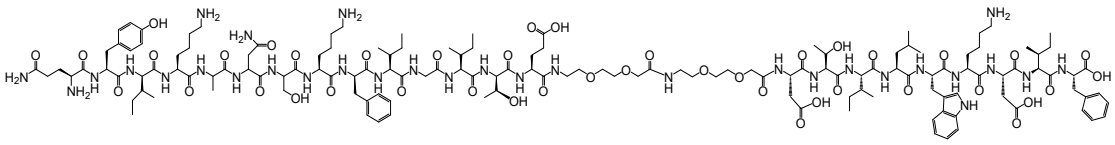
To a solution of peptide fragments **4** (2.43mg, 1.6 μmol) in dry NMP/DCM (150 μL/100 μL) were added DIPEA (0.58μL，3.2μmol) and HOBT (0.45mg, 3.2μmol) with stirring for 5 min, respectively. Then the above solution was dropwisely added to lipopeptide pentafluorophenol active ester **7** (1.82 mg, 0.8 μmol) in dry NMP/DCM (150 μL/100 μL) with stirring for 16h at room temperature, respectively. After finishing the coupling reaction, the solution was concentrated *in vacuo* to give crude yellow solid. The residual crude yellow solid was treated with 1 mL cleavage solution (95% TFA, 2.5% TIPS and 2.5% H_2O) at room temperature for 2 h, respectively. To the resultant solution was added cold diethyl ether (10 mL) to give crude precipitates. After the precipitates were collected by centrifugation, HPLC using C4 column was carried out to give **2** (1.78 mg, 80%).

HPLC conditions: C4 column (COSMOSIL, C4-AR-300, ϕ10 × 250 mm) was used with 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). Analytical HPLC: Rt (retention time) =53.78 min (20%-100% solvent B over 40 min, and then 100%-60% solvent B over 20 min, 1 mL/min, λ=220 nm).





Compound **2**: HRMS (ESI-LIT-orbitrap): *m/z* for $C_{143}H_{248}N_{22}O_{30}S$ $[M+3H]^{3+}$ calcd 929.6165, found 929.6168; $[M+2H]^{2+}$ calcd 1393.9211, found 1393.9216.

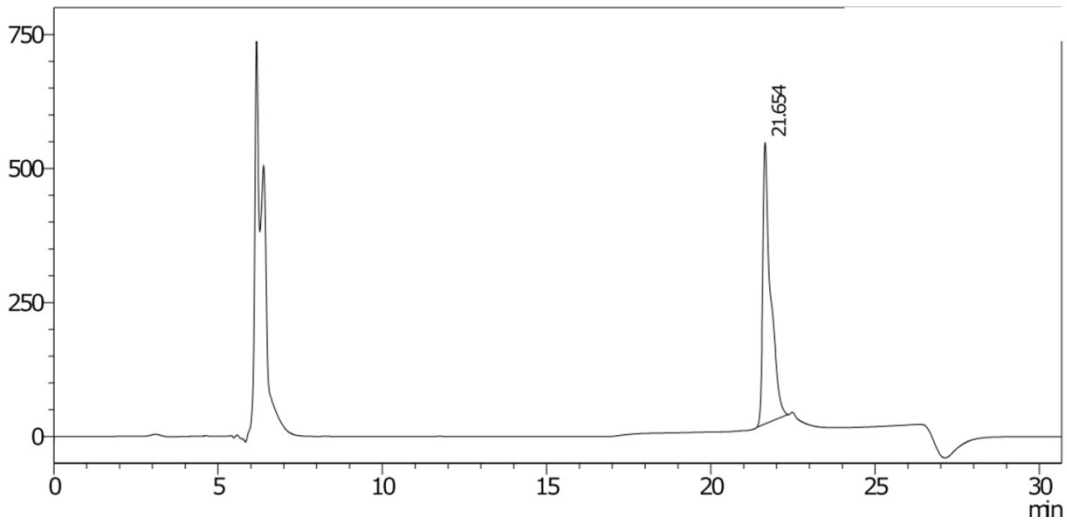


Compound **3**

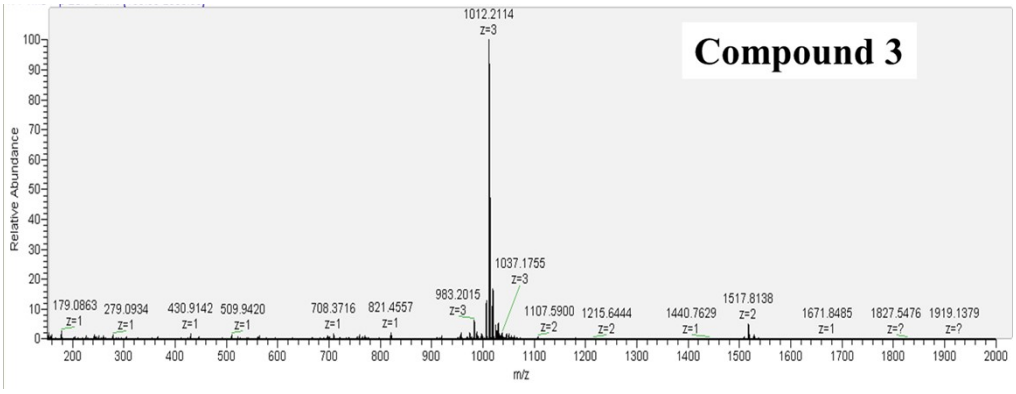
To a solution of compound **9** (2.44mg, 0.8 μmol) in dry co-solvent of NMP/DCM (120 μL / 80 μL) was added compound **5** (2.66 mg, 1.6 μmol) along with HOBt (0.45mg，3.2μmol) and DIPEA (0.58μL, 3.2 μmol) at room temperature. After the mixture was stirred for 16h, the solution was removed by evaporation to give a crude yellow solid. Subsequently, the crude compound was treated with 20% piperidine in DMF for 30min to remove Fmoc group, and followed by treating with

TFA/TIPS/H_2O (95:2.5:2.5) for 2h to remove side chain protecting group. Then cold diethyl ether (20 mL) was added to the solution to give a crude precipitate. Then, the crude precipitate was collected by centrifugation. Finally, purification was carried out by RP-HPLC on a C4 column to achieve the final compound **3** (36%) as white solid.

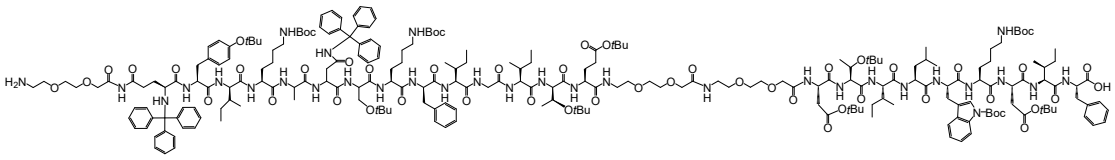
HPLC conditions: C4 column (Agilent, 300Å, 10 µm, 10 x 250 mm) was used with a mobile phase of 0.1 % (v/v) TFA in water/isopropanol/acetonitrile (8:1:1 v/v/v; Solvent A) and 0.1 % (v/v) TFA in acetonitrile/isopropanol (1:1 v/v; Solvent B) (**Eluent A**).



Analytical HPLC: Rt (retention time) = 21.65 min (20% **Eluent A** over 10 min and 20%-100% **Eluent A** over 40min on a C4 column, 2mL/min, λ=220 nm). Yield 34% (0.5mg).

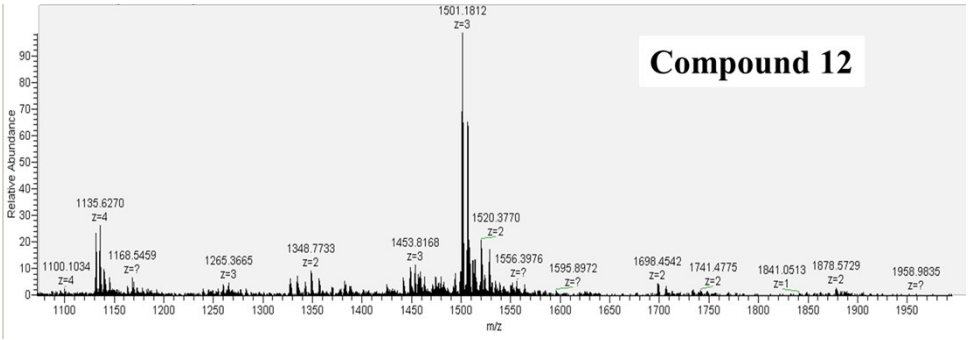


Compound **3**: HRMS (ESI-LIT-orbitrap): m/z for $C_{142}H_{221}N_{31}O_{42}$ $[M+3H]^{3+}$ calcd 1012.2121, found 1012.2114; $[M+2H]^{2+}$ calcd 1517.8145, found 1517.8138.

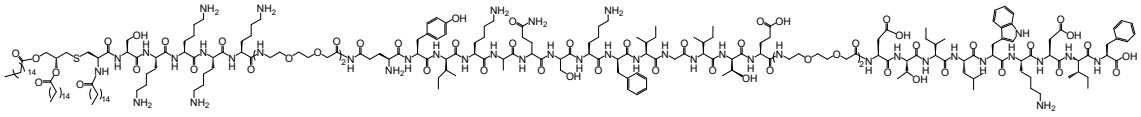


Compound **12**

To a solution of compound **11** (2.56 mg, 0.8 μmol) in dry co-solvent of NMP/DCM (120 μL / 80 μL) was added compound **5** (2.66 mg, 1.6 μmol) along with HOBt (0.45 mg, 3.2μmol) and DIPEA (0.58 μL, 3.2μmol) at room temperature. After the mixture was stirred for 16h, the solution was removed by evaporation to give a crude yellow solid, followed by removing Fmoc group in the presence of 20% piperidine in DMF for 30min. Then the solution was filtered and cold diethyl ether (20 mL) was added to the filtrate to give a crude precipitate. Then, the precipitate was collected by centrifugation. Finally, purification of **12** was carried out by silica-gel column chromatography (MeOH: $CHCl_3$ =1:8) to give **12** (27%) as white solid.



Compound **12**: HRMS (ESI-LIT-orbitrap): m/z for $C_{234}H_{348}N_{32}O_{53}$ $[M+H+2Na+K]^{4+}$ calcd 1135.626, found 1135.6270; $[M+H+2Na]^{3+}$ calcd 1501.1815, found 1501.1812.

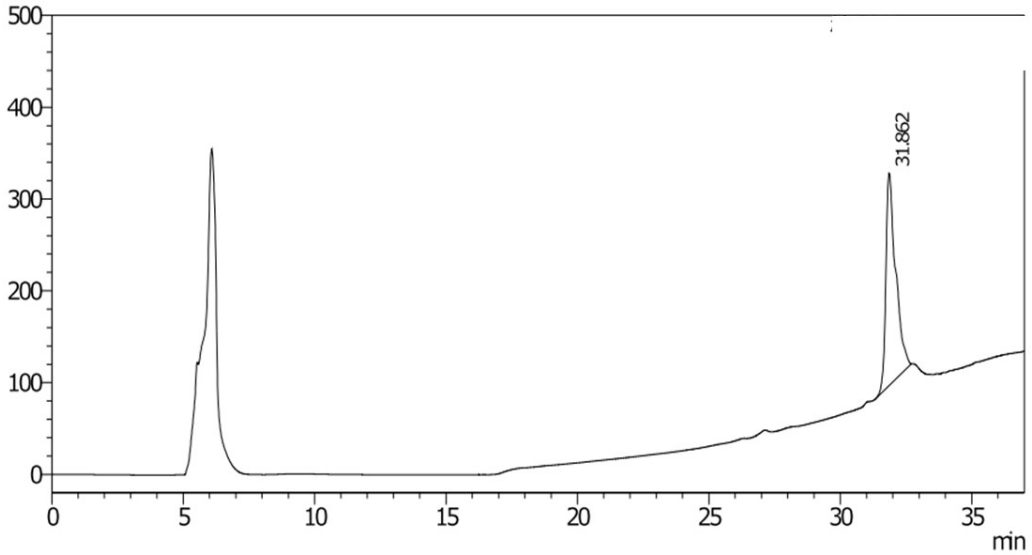


Compound **1**

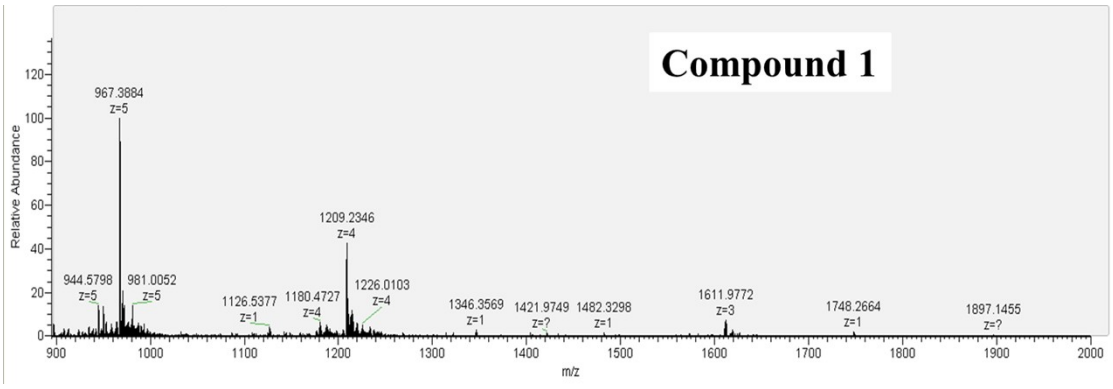
To a solution of compound **12** (3.57 mg, 0.8 μmol) in dry co-solvent of NMP/DCM (120 μL / 80 μL) was added compound **7** (3.64mg, 1.6 μmol) along with HOBt (0.45mg, 3.2μmol) and DIPEA (0.58 μL, 3.2μmol) at room temperature. After the mixture was stirred for 16h, the solution was removed by evaporation to give a crude yellow solid, followed by removing all side chains in the presence of 2 mL cleavage

solution (95%TFA, 2.5%TIPS and 2.5% H_2O) for 2h. Then the solution was filtered and cold diethyl ether (20 mL) was added to the filtrate to give a crude precipitate. Then, the precipitate was collected by centrifugation. Finally, purification of **1** was carried out by RP-HPLC on a C4 column to achieve the final compound **1** as white solid (24%).

HPLC conditions: C4 column (Agilent, 300Å, 10 µm, 10 x 250 mm) was used with 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**).



Analytical HPLC: Rt (retention time) = 31.8 min (20% **Eluent A** for 10 min to 100 % **Eluent A** over 30 min on a C4 column, 3 mL/min, λ=220 nm). Yield 24% (1.0 mg).



Compound **1**: HRMS (LTQ-Orbitrap): *m/z* for $C_{235}H_{397}N_{43}O_{60}S$ $[M+ H_2O+5H]^{5+}$ calcd 967.3905, found 967.3884; $[M+H_2O+4H]^{4+}$ calcd 1209.2372, found 1209.2346; $[M+H_2O+3H]^{3+}$ calcd 1611.9805, found 1611.9772.

1. Feng Q, Manabe Y, Kabayama K, Aiga T, Miyamoto A, Ohshima S, Kametani Y, Fukase K: **Syntheses and Functional Studies of Self-Adjuvanting Anti-HER2 Cancer Vaccines**. *Chem Asian J* 2019, **14**(23):4268-4273.