Supporting Information

Increasing the Antigenicity of Synthetic Tumor-Associated Carbohydrate Antigens by Targeting Toll-like Receptors.

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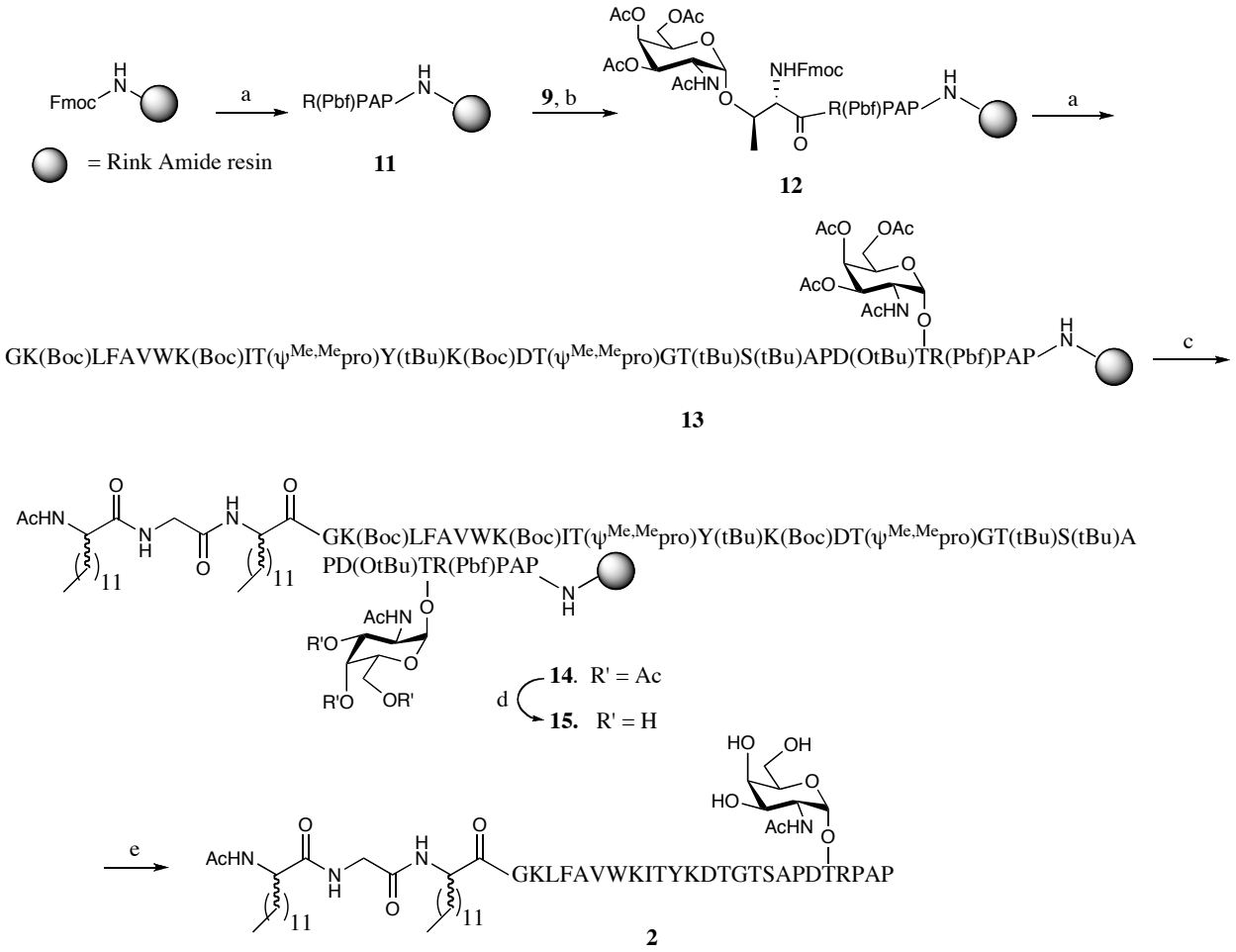
EXPERIMENTAL

General methods: Fmoc-L-amino acid derivatives and resins were purchased from NovaBioChem and Applied Biosystems; peptide synthesis grade *N, N*-dimethylformamide (DMF) from EM Science; and *N*-methylpyrrolidone (NMP) from Applied Biosystems. Egg phosphatidylcholine (PC), phosphatidylglycerol (PG), cholesterol (Chol), and monoposphoryl lipid A (MPL-A) were obtained from Avanti Polar Lipids. EZ-Link® NHS-Biotin reagent (succinimidyl-6-(biotinamido)hexanoate) was obtained from Pierce. All other chemical reagents were purchased form Aldrich, Acros, Alfa Aesar, and Fisher Scientific and used without further purification. All solvents employed were reagent grade. Reversed phase high performance liquid chromatography (RP-HPLC) was performed on an Agilent 1100 series system equipped with an auto-injector, fraction-collector, and UV-detector (detecting at 214 nm) using an Agilent Zorbax Eclipse^{TM} C8 analytical column (5 μm, 4.6 x 150 mm) at a flow rate of 1 mL/min, Agilent Zorbax Eclipse^{TM} C8 semi preparative column (5 μm, 10 x 250 mm) at a flow rate of 3 mL min^{-1} or Phenomenex Jupiter^{TM} C4 semi preparative column (5 μm, 10 x 250 mm) at a flow rate of 2 mL min^{-1}. All runs were performed using a linear gradient of 0-100% solvent B over 40 min (solvent A = 5% acetonitrile, 0.1% trifluoroacetic acid (TFA) in water, solvent B = 5% water, 0.1% TFA in acetonitrile). Matrix assisted laser desorption ionization time of flight mass spectrometry (MALDI-ToF) mass spectra were recorded on a ABI 4700 proteomic analyzer.

Synthesis of glycolipopeptide 2: The synthesis of **2** was carried out on a Rink amide resin (0.1 mmol) as described under peptide synthesis in the experimental. The first four amino acids, Arg-Pro-Ala-Pro were coupled on the peptide synthesizer using a standard protocol to obtain **11**. After the completion of the synthesis, a manual coupling of N^{α}-Fmoc-Thr-(AcO_3-α-D-GalNAc) **8** (0.2 mmol, 134 mg) was carried out. **8**[1] was dissolved in NMP (5 mL) and *O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N'*,*N'*-tetramethyl-uronium hexafluorophosphate (HATU; 0.2 mmol, 76 mg), 1-hydroxy-7-azabenzotriazole (HOAt; 0.2 mmol, 27 mg), and diisopropylethylamine (DIPEA; 0.4 mmol, 70 μL) were added to the solution and the resulting mixture was added to the resin. The coupling reaction was

monitored by standard Kaiser test. After 12 h, the resin was washed with NMP (6 mL) and methylene chloride (DCM; 6 mL), and resubjected to the same coupling conditions to ensure complete coupling. The glycopeptide **12**. was then elongated on the peptide synthesizer. After the completion of the synthesis, the resin was thoroughly washed with NMP (6 mL), DCM (6 mL) and methanol (MeOH; 6 mL) and dried *in vacuo*. The resin was then swelled in DCM (5 mL) for 1 h and the rest of the couplings were carried out manually. Next, N^{α}-Fmoc-lipophilic amino acid (N^{α}-Fmoc-D,L-tetradeconic acid) **9**[2,3] (0.3 mmol, 139 mg) dissolved in NMP (5 mL), benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate (PyBOP; 0.3 mmol, 156 mg), 1-hydroxy-benzotriazole (HOBt; 0.3 mmol, 40 mg) and DIPEA (0.4 mmol, 67 μL) were premixed for 2 min, and then added to the resin. The coupling reaction was monitored by the Kaiser test and was complete after standing for 8 h. The N^{α}-Fmoc group was cleaved using piperidine (20%) in DMF (6 mL). N^{α}-Fmoc-Gly-OH (0.3 mmol, 90 mg) dissolved in NMP (5 mL), PyBOP (0.3 mmol, 156 mg), HOBt (0.3 mmol, 40 mg), and DIPEA (0.4 mmol, 67 μL) were premixed for 2 min, and were then added to the resin. The coupling reaction was monitored by Kaiser test and was complete after standing for 4 h. The N^{α}-Fmoc group was cleaved using piperidine (20%) in DMF (6 mL). One more cycle of coupling of **2S** (0.3 mmol, 139 mg) was carried out as described above using PyBOP (0.3 mmol, 156 mg), HOBt (0.3 mmol, 40 mg), and DIPEA (0.4 mmol, 67 μL) in NMP (5 mL). Finally, the N^{α}-Fmoc group was cleaved using piperidine (20%) in DMF (6 mL) and the resulting free amino group was acetylated by treatment of the resin with Ac_2O (10%) and DIPEA (5%) in NMP (5mL) for 10 min. The resin was washed thoroughly with NMP (5 mL x 2), DCM (5 mL x 2), and MeOH (5 mL x 2), and dried *in vacuo*. The resin was swelled in DCM (5 mL) for 1 h, treated with hydrazine (60%) in MeOH[4,5] (10 mL) for 2 h, thoroughly washed with NMP (5 mL x 2), DCM (5 mL x 2), and MeOH (5 mL x 2), and dried in *vacuo*. The resin was swelled in DCM (5 mL) for 1 h and then treated with reagent B (TFA (88%), water (5%), phenol (5%), and TIS (2%), 10 mL) for 2 h. The resin was filtered, washed with neat TFA (2 mL), and the filtrate was then concentrated *in vacuo* to approximately 1/3 of its original volume. The glycolipopeptide was precipitated using diethyl ether (0°C, 40 mL) and recovered by centrifugation at 3,000 rpm for 15 min. The crude glycolipopeptide was purified by RP-HPLC on a semi

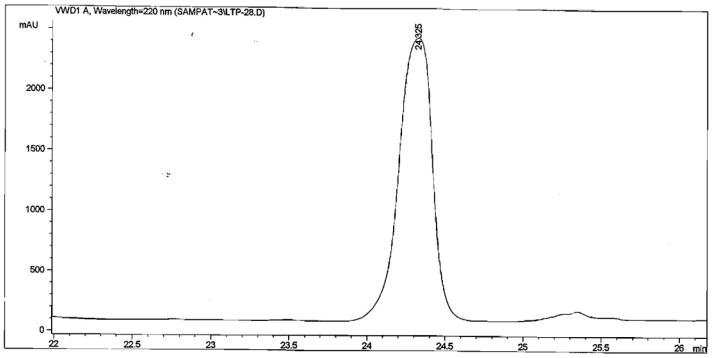
preparative C-4 column using a linear gradient of 0-95% solvent B in A over 40 min, and the appropriate fractions were lyophilized to afford **2** (57 mg, 16%). $C_{165}H_{267}N_{37}O_{44}$, MALDI-ToF MS: observed, [M+] 3473.4900Da; calculated, [M+] 3473.1070Da.



Scheme S1. Reagents and conditions: a) SPPS using Fmoc-chemistry, coupling with HBTU/HOBt in the presence of DIPEA in NMP; b) **8**, HATU/HOAt, DIPEA, NMP, overnight; c) i. manual coupling of **9** with PyBOP/HOBt in the presence of DIPEA in NMP; ii. 20% piperidine in DMF; iii. manual coupling of **8** with PyBOP/HOBt in the presence of DIPEA in NMP; iv. 20% piperidine in DMF; v. manual coupling of **9** with PyBOP/HOBt in the presence of DIPEA in NMP; vi. 20% piperidine in DMF; vii. 10% Ac_2O, 5% DIPEA in NMP for 10 min; (d) 60% hydrazine in MeOH, 2 h; e) reagent B, TFA (88%), phenol (5%), water (5%), TIS (2%), 2 h.

Compound (2)

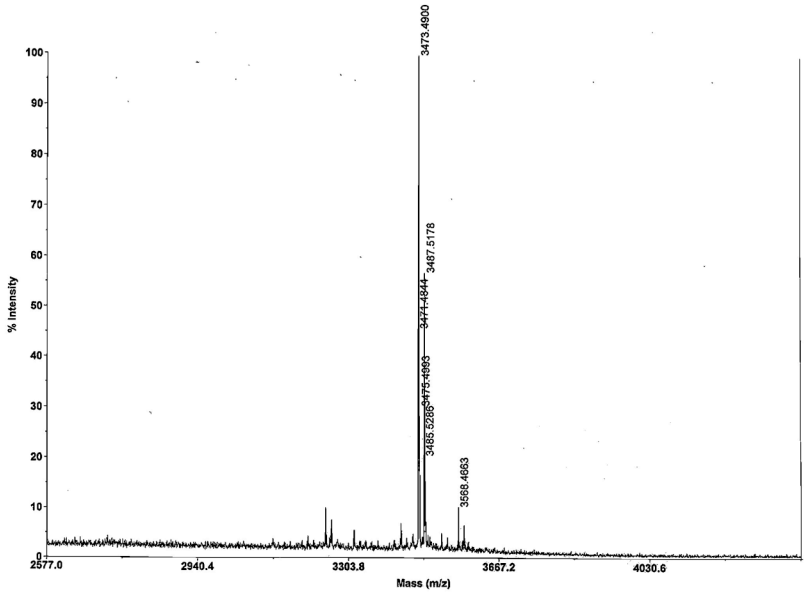
HPLC chromatogram:



Column: Semi prep. C4 Reversed phase

Eluent: 0-95% of Solvent B in A over period of 40 min

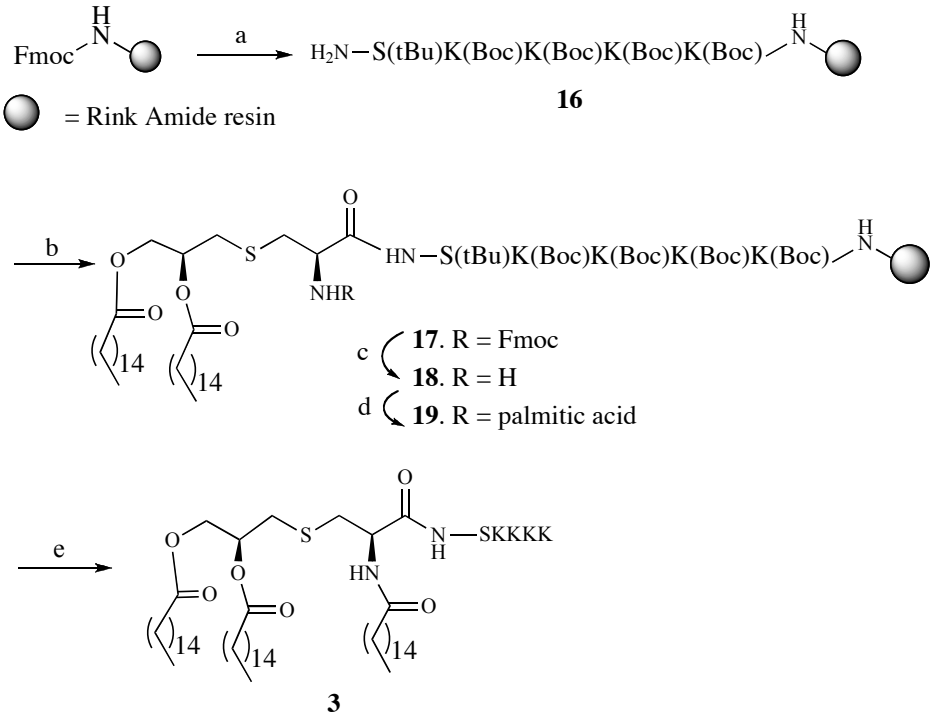
MALDI-ToF spectra:



Observed, [M+], 3473.4900Da

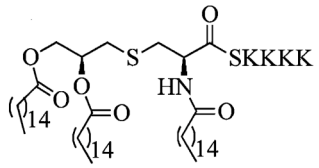
Calculated, [M+], 3473.1070Da

Synthesis of lipopeptide 3: The synthesis of **3** was carried out on a Rink amide resin (0.1 mmol) as described under peptide synthesis in the experimental. After coupling of the first five amino acids, the lipid portion of the molecule was coupled manually. N^{α}-Fmoc-*S*-(2,3-bis(palmitoyloxy)-(2*R*-propyl)-(*R*)-cysteine, **10**[6,7] (0.3 mmol, 267 mg) was dissolved in DMF (5 mL) and PyBOP (0.3 mmol, 156 mg), HOBt (0.3 mmol, 40 mg), and DIPEA (0.4 mmol, 67 μL) were added to the solution. After 2 min the reaction mixture was added to the resin. The coupling reaction was monitored by the Kaiser test and was complete after standing for 12 h. Next, the N^{α}-Fmoc group was cleaved using piperidine (20%) in DMF (6 mL) to obtain **18**. Palmitic acid (0.3 mmol, 77 mg) was coupled to the free amine of **18** as described above using PyBOP (0.3 mmol, 156 mg), HOBt (0.3 mmol, 40 mg), and DIPEA (0.4 mmol, 67 μL) in DMF. The resin was washed thoroughly with DMF (5 mL x 2), DCM (5 mL x 2), and MeOH (5 mL x 2) and then dried *in vacuo.* The resin was swelled in DCM (5 mL) for 1 h and then treated with TFA (95%), water (2.5%), and TIS (2.5%) (10 mL) for 2 h at room temperature. The resin was filtered and washed with neat TFA (2 mL). The filtrate was then concentrated *in vacuo* to approximately 1/3 of its original volume. The lipopeptide was precipitated using diethyl ether (0 ^{o}C; 30 mL) and recovered by centrifugation at 3000 rpm for 15 min. The crude lipopeptide was purified by RP-HPLC on a semi preparative C-4 column using a linear gradient of 0 to 95% solvent B in solvent A over a 40 min period and the appropriate fractions were lyophilized to afford **3** (40 mg, 26%). $C_{81}H_{156}N_{11}O_{12}S$, MALDI-ToF MS: observed [M+Na],1531.2240Da; calculated [M+Na], 1531.1734Da.

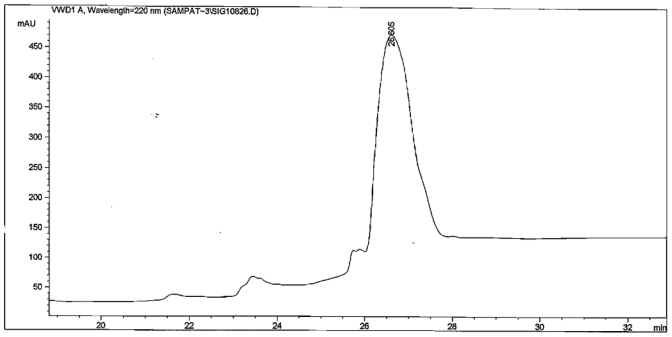


Scheme S2. Reagents and conditions: a) SPPS using Fmoc-chemistry, coupling with HBTU/HOBt in the presence of DIPEA in NMP; b) manual coupling of **10** by PyBOP/HOBt activation in the presence of DIPEA in DMF; c) piperidine (20%) in DMF; d) coupling of palmitic acid by PyBOP/HOBt activation in the presence of DIPEA in DMF; e) TFA (95%), water (2.5%), TIS (2.5%), 2 h.

Compound (3)



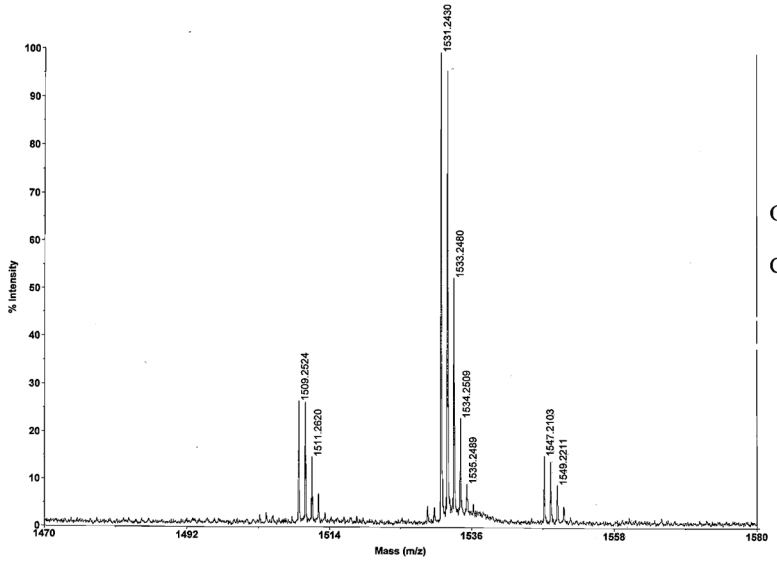
HPLC chromatogram:



Column: Semi prep. C4
Reversed phase

Eluent: 0-95% of Solvent B in A over period of 40 min

MALDI-ToF spectra:

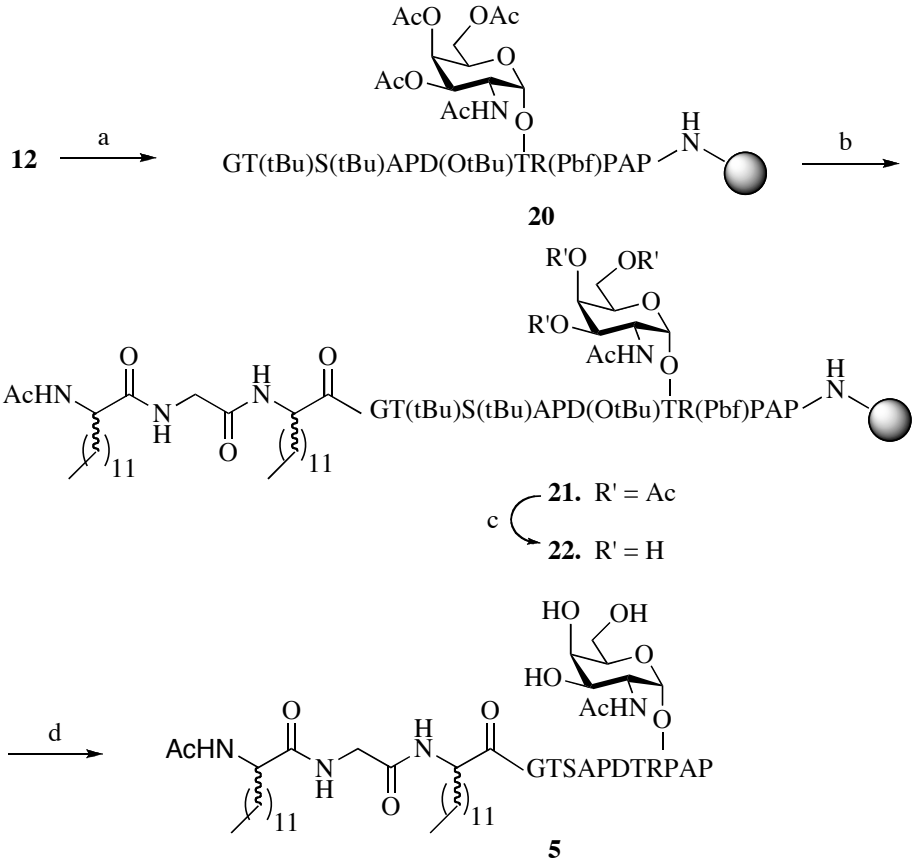


Observed, [M+Na], 1531.2240Da

Calculated, [M+Na], 1531.2386Da

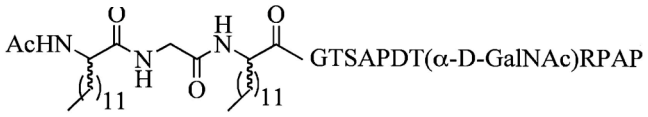
Synthesis of glycolipopeptide 5: The synthesis **5** was carried out on a Rink amide resin (0.1 mmol) as described under peptide synthesis in the experimental. The first four amino acids, Arg-Pro-Ala-Pro were coupled on the peptide synthesizer using a standard protocol to obtain **11**. After the completion of the synthesis, a manual coupling was carried out using **8** (0.2 mmol, 134 mg). **8** was dissolved in NMP (5 mL) and HATU (0.2 mmol, 76 mg), HOAt (0.2 mmol, 27 mg), and DIPEA (0.4 mmol, 70 μL) were added and the resulting mixture was added to the resin. The coupling reaction was monitored by standard Kaiser test. After 12 h, the resin was washed with NMP (6 mL) and DCM (6 mL), and re-subjected to the same coupling conditions to ensure complete coupling. Glycopeptide **12** was then elongated on the peptide synthesizer. After the completion of the synthesis, the resin was thoroughly washed with NMP (6 mL), DCM (6 mL), and MeOH (6 mL) and dried *in vacuo*. The resin was then swelled in DCM (5 mL) for 1 h and the rest of the peptide sequence was completed manually. **9** (0.3 mmol, 139 mg) was dissolved in NMP (5 mL) and PyBOP (0.3 mmol, 156 mg), HOBt (0.3 mmol, 40 mg), and DIPEA (0.4 mmol, 67 μL) were added to the solution. After 2 min, the mixture was added to the resin. The coupling reaction was monitored by standard Kaiser test and was complete after standing for 8 h. Next, the N^{α}-Fmoc group was cleaved using piperidine (20%) in DMF (6 mL). N^{α}-Fmoc-L-glycine (0.3 mmol, 90 mg) was dissolved in NMP (5 mL) and premixed with PyBOP (0.3 mmol, 156 mg), HOBt (0.3 mmol, 40 mg), and DIPEA (0.4 mmol, 67 μL) for 2 min before the reaction mixture was added to the resin. The coupling reaction was monitored by Kaiser test and was complete after standing for 4 h. The N^{α}-Fmoc group was cleaved using piperidine (20%) in DMF (6 mL). One more cycle of coupling of **9** (0.3 mmol, 139 mg) was carried out as described above using PyBOP (0.3 mmol, 156 mg), HOBt (0.3 mmol, 40 mg), and DIPEA (0.4 mmol, 67 μL) in NMP (5 mL). Finally, the N^{α}-Fmoc group was cleaved using piperidine (20%) in DMF (6 mL) and the resulting free amino group was acetylated using Ac_2O (10%) and DIPEA (5%) in NMP (5 mL) for 10 min. The resin was washed thoroughly with NMP (5 mL x 2), DCM (5 mL x 2), and MeOH (5 mL x 2), and dried *in vacuo*. The resin was swelled in DCM (5 mL) for 1 h, treated with hydrazine (60%) in MeOH (10 mL) for 2 h, washed thoroughly with NMP (5 mL x 2), DCM (5 mL x 2) and MeOH (5 mL x 2)

and dried *in vacuo*. The resin was swelled in DCM (5 mL) for 1 h after which it was treated with reagent B (TFA (88%), water (5%), phenol (5%), and TIS (2%), 10 mL) for 2 h. The resin was filtered, washed with neat TFA (2 mL) and the filtrate was then concentrated *in vacuo* to approximately 1/3 of its original volume. The glycolipopeptide was precipitated using diethyl ether (0°C; 40 mL) and recovered by centrifugation at 3,000 rpm for 15 min. The crude glycolipopeptide was purified by RP-HPLC on a semi preparative C-4 column using a linear gradient of 0-95% solvent B in A over 40 min, and the appropriate fractions were lyophilized to afford **5** (35 mg, 19%). $C_{84}H_{145}N_{19}O_{25}$, MALDI-ToF MS: observed, [M+] 1821.1991Da; calculated, [M+] 1821.1624Da.

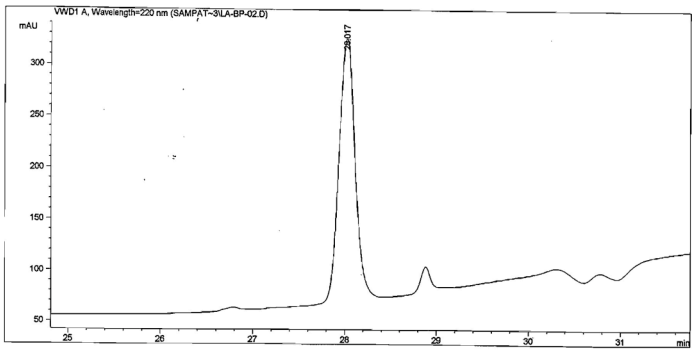


Scheme S3. Reagents and conditions: a) SPPS using Fmoc-chemistry, coupling with HBTU/HOBt in the presence of DIPEA in NMP; b) i. manual coupling of **9** with PyBOP/HOBt in the presence of DIPEA in NMP; ii. 20% piperidine in DMF; iii. manual coupling of N^{α}-Fmoc-Gly-OH with PyBOP/HOBt in the presence of DIPEA in NMP; iv. 20% piperidine in DMF; v. manual coupling of **9** with PyBOP/HOBt in the presence of DIPEA in NMP; vi. 20% piperidine in DMF; vii. 10% Ac_2O, 5% DIPEA in NMP for 10 min; c) 60% hydrazine in MeOH, 2 h; d) reagent B, TFA (88%), phenol (5%), water (5%), TIS (2%), 2 h.

Compound (5)



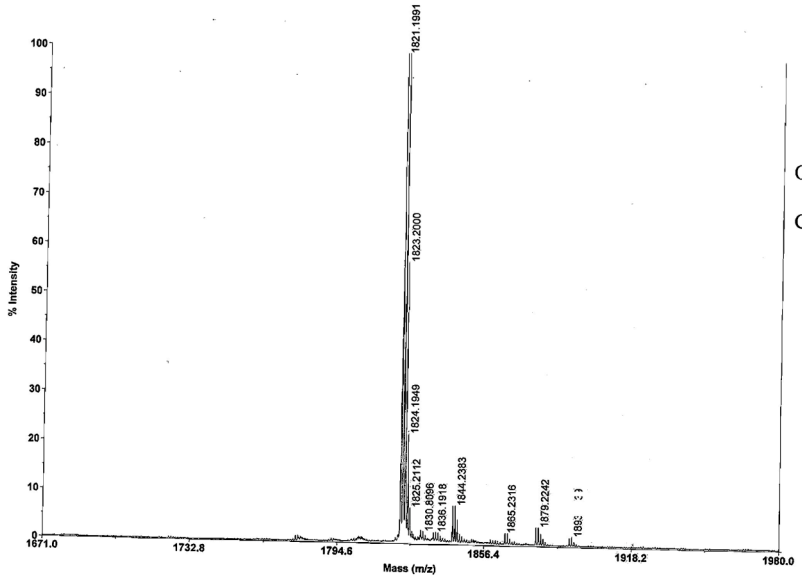
HPLC chromatogram:



Column: Semi prep. C4 Reversed phase

Eluent: 0-95% of Solvent B in A over period of 40 min

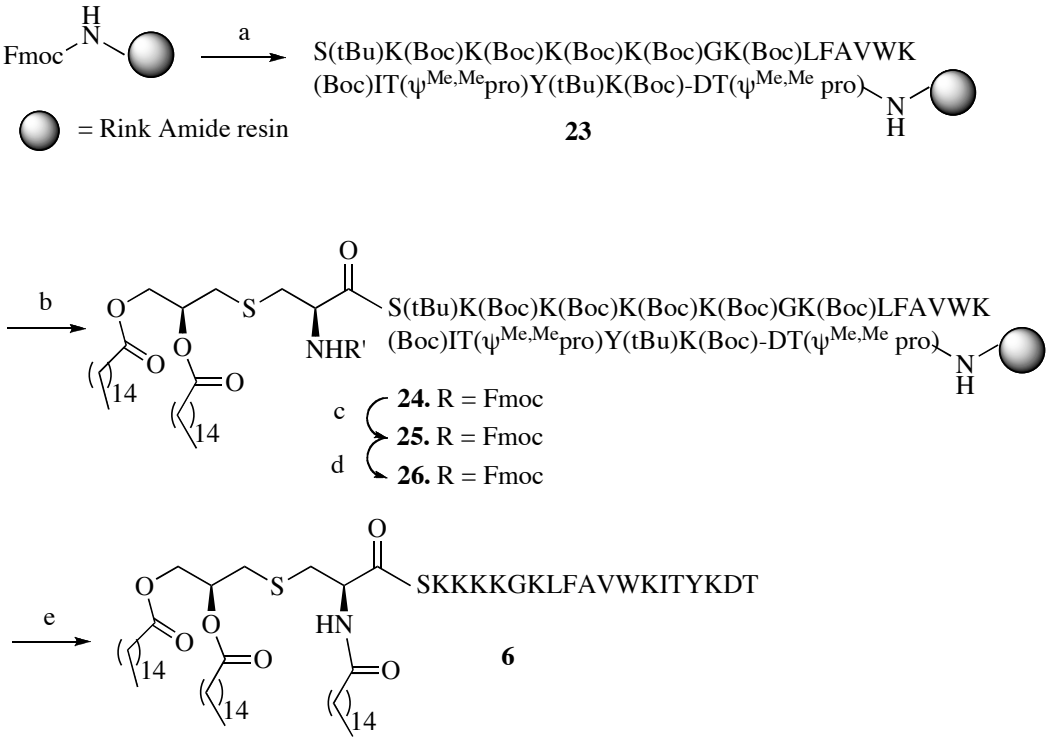
MALDI-ToF spectra:



Observed, [M+], 1821.1991Da

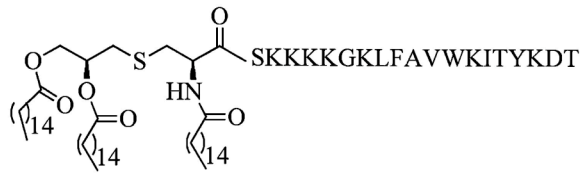
Calculated, [M+], 1821.1624Da

Synthesis of lipopeptide 6: The synthesis of **6** was carried out on a Rink amide resin (0.1 mmol). After the assembly of the peptide by using standard SPPS, the lipid portion of the molecule was coupled manually. **10** (0.3 mmol, 267 mg) was dissolved in DMF (5 mL) and PyBOP (0.3 mmol, 156 mg), HOBt (0.3 mmol, 40 mg), and DIPEA (0.4 mmol, 67 μL) were added to the solution. After activation of **10** for 2 min the reaction mixture was added to the resin. The coupling reaction was monitored by the Kaiser test and was complete after standing for 12 h. The N^{α}-Fmoc group was cleaved using piperidine (20%) in DMF (6 mL) to obtain **25**. Palmitic acid (77 mg, 0.3 mmol) was coupled to the free amine of **25** as described above using PyBOP (0.3 mmol, 156 mg,), HOBt (0.3 mmol, 40 mg), and DIPEA (0.4 mmol, 67 μL) in DMF. The resin was washed thoroughly with DMF (5 mL x 2), DCM (5 mL x 2), and MeOH (5 mL x 2) and then dried in *vacuo*. The resin was swelled in DCM (5 mL) for 1 h, treated with reagent B (TFA (88%), water (5%), phenol (5%), and TIS (2%), 10 mL) for 2 h, filtered and washed with neat TFA (2 mL). The filtrate was then concentrated *in vacuo* to approximately 1/3 of its original volume, and the lipopeptide was precipitated using diethyl ether (0°C; 30 mL) and recovered by centrifugation at 3000 rpm for 15 min. The crude lipopeptide was purified by RP-HPLC on a semi preparative C-4 column using a linear gradient of 0-95% solvent B in A over a 40 min., and the appropriate fractions were lyophilized to afford **6** (57 mg, 18%). $C_{162}H_{278}N_{29}O_{31}S$, MALDI-ToF MS: observed, [M+] 3160.9423Da; calculated, [M+] 3160.1814Da.

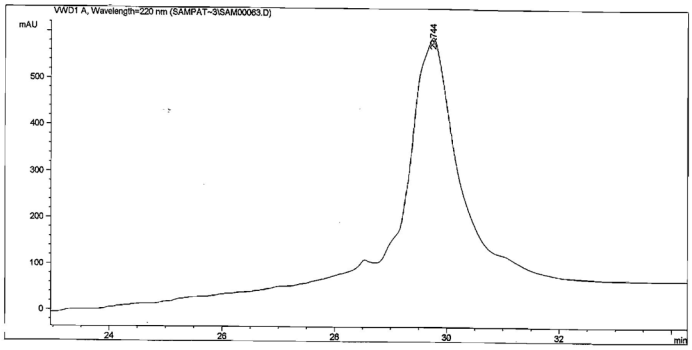


Scheme S4. Reagents and conditions: a) SPPS using Fmoc-chemistry, coupling with HBTU/HOBt in the presence of DIPEA in NMP; b) manual coupling of **10**, PyBOP, HOBt in the presence of DIPEA in DMF; c) 20% piperidine in DMF; d) manual coupling of palmitic acid, PyBOP, HOBt in the presence of DIPEA in DMF; e) reagent B, TFA (88%), phenol (5%), water (5%), TIS (2%), 2 h.

Compound (6)



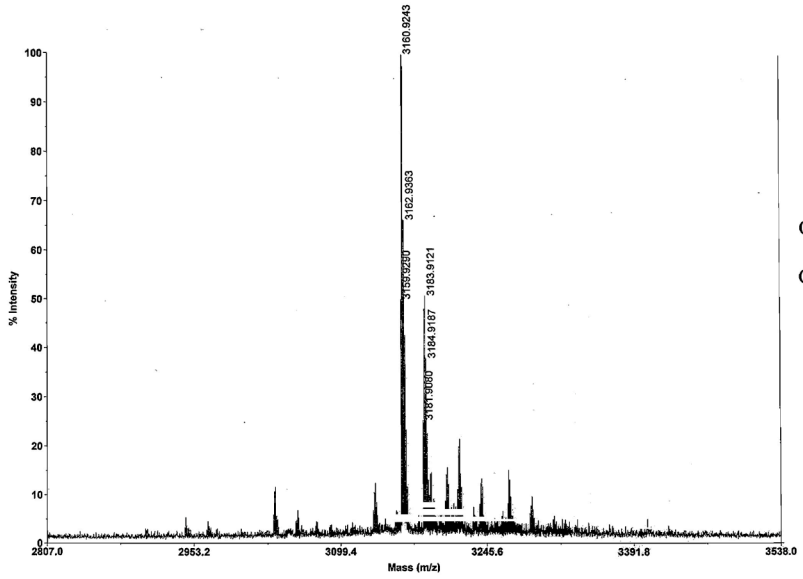
HPLC chromatogram:



Column: Semi prep. C4 Reversed phase

Eluent: 0-95% of Solvent B in A over period of 40 min

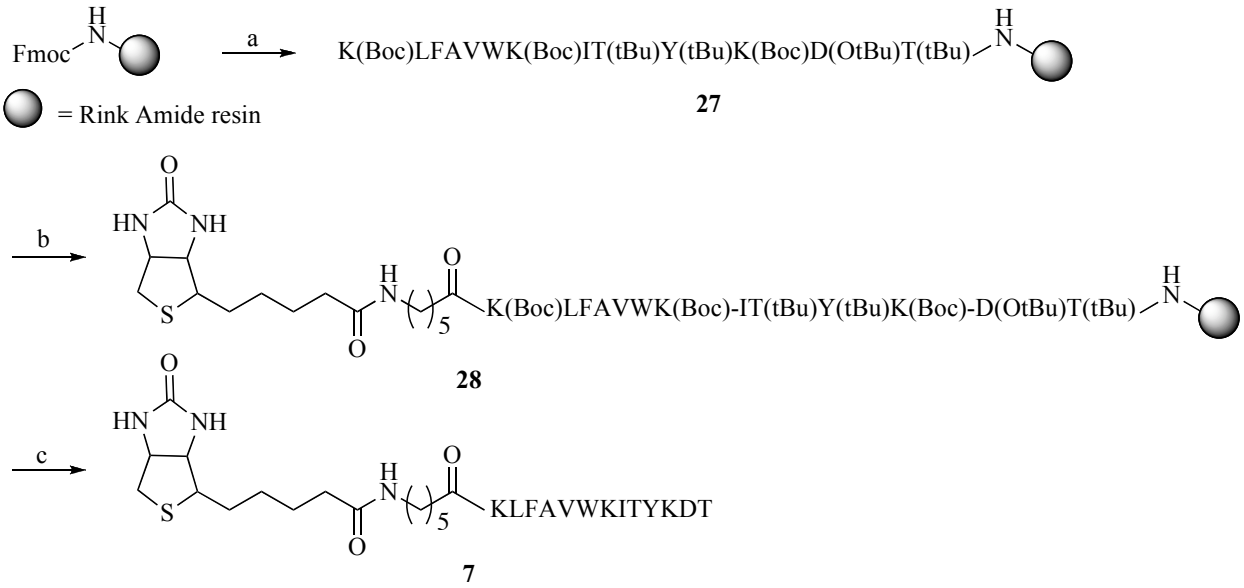
MALDI-ToF spectra:



Observed, [M+], 3160.9243Da

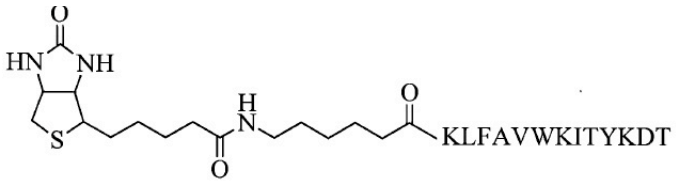
Calculated, [M+], 3160.1814Da

Synthesis of biotin-T-epitope peptide 7: The synthesis of **7** was carried out on a Rink amide resin (0.1 mmol) as described in the general method. After the completion of synthesis the resin was washed thoroughly with DMF (5 mL x 2), DCM (5 mL x 2), and MeOH (5 mL x 2) and then dried *in vacuo*. The resin was swelled in DCM (5 mL) for 1 h. Next, a mixture of EZ-Link® NHS-Biotin reagent (succinimidyl-6-(biotinamido)hexanoate) (0.2 mmol, 90 mg) and DIPEA (0.2 mmol, 36 μL) in DMF (5 mL) was added to the resin. The coupling was monitored by standard Kaiser test and was complete within 8 h. The resin was washed thoroughly with DMF (5 mL x 2), DCM (5 mL x 2), and MeOH (5 mL x 2) and then dried *in vacuo*. The resin was swelled in DCM (5 mL) for 1 h and treated with reagent B (TFA (88%), water (5%), phenol (5%), and TIS (2%), 15 mL) for 2 h at room temperature. The resin was filtered and washed with neat TFA (2 mL). The filtrate was concentrated *in vacuo* to approximately 1/3 of its original volume. The peptide was precipitated using diethyl ether (0 ^{o}C; 30 mL) and recovered by centrifugation at 3,000 rpm for 15 min. The crude peptide was purified by RP-HPLC on a semi preparative C-8 column using a linear gradient of 0 to 95% solvent B in solvent A over a 40 min period and the appropriate fractions were lyophilized to afford **7** (60% based on resin loading capacity). $C_{95}H_{147}N_{21}O_{21}S$, MALDI-ToF MS: observed [M+], 1951.2966Da; calculated [M+], 1951.3768Da.

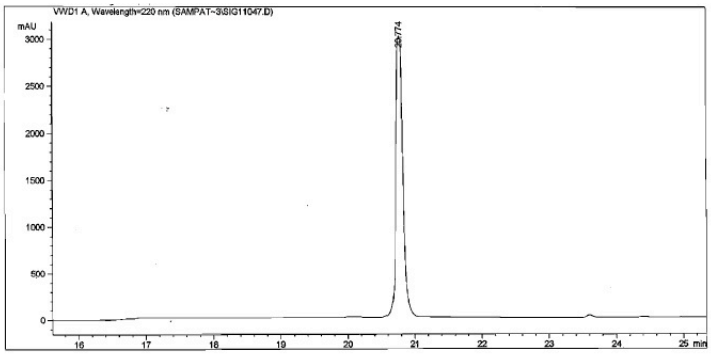


Scheme S5. Reagents and conditions: a) SPPS using Fmoc-chemistry, coupling with HBTU/HOBt in the presence of DIPEA in NMP; b) manual coupling of succinimidyl-6-(biotinamido)hexonate in the presence of DIPEA in DMF; c) reagent B, TFA (88%), Phenol (5%), water (5%), TIS (2%), 2 h.

Compound (7)



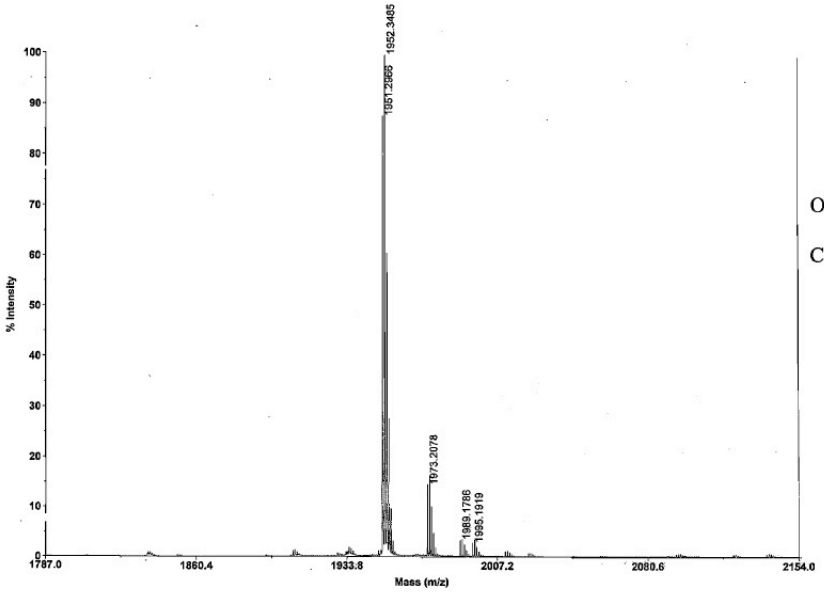
HPLC chromatogram:



Column: Semi prep. C8 Reversed phase

Eluent: 0-95% of Solvent B in A over period of 40 min

MALDI-ToF spectra:



Observed, [M+Na], 1951.2966Da

Calculated, [M+Na], 1951.3768Da

References

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