Online Supplementary Information for

Robust immune responses elicited by a fully synthetic three-component vaccine

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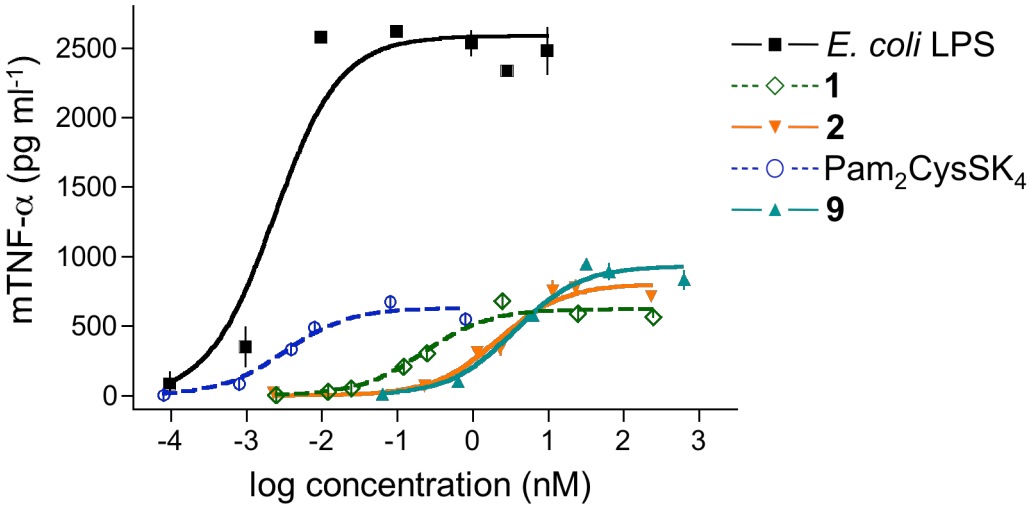
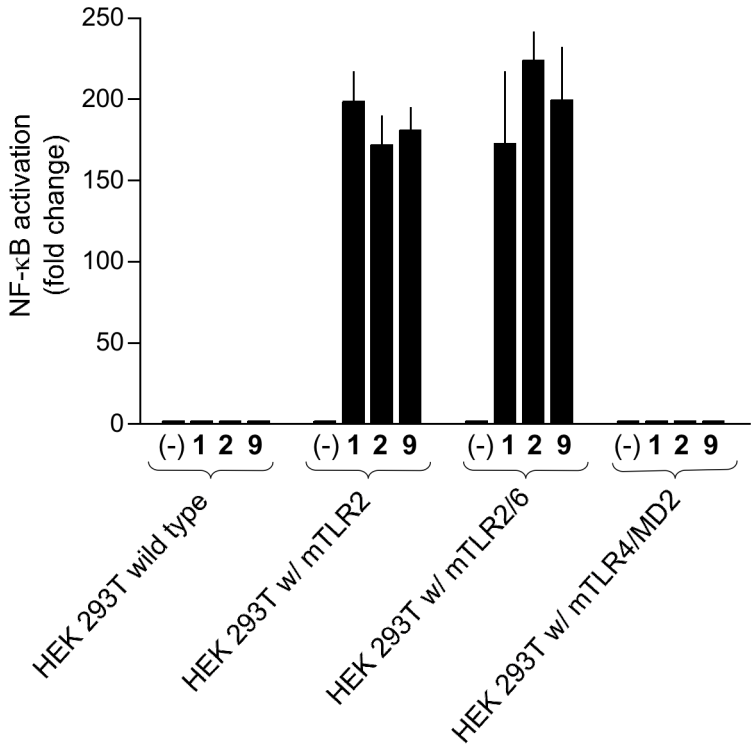
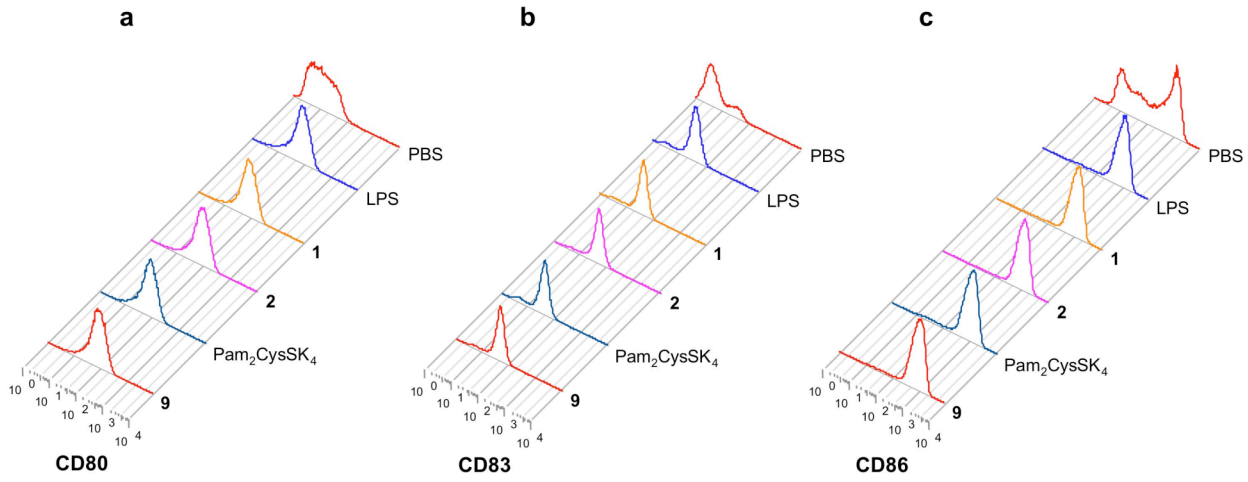


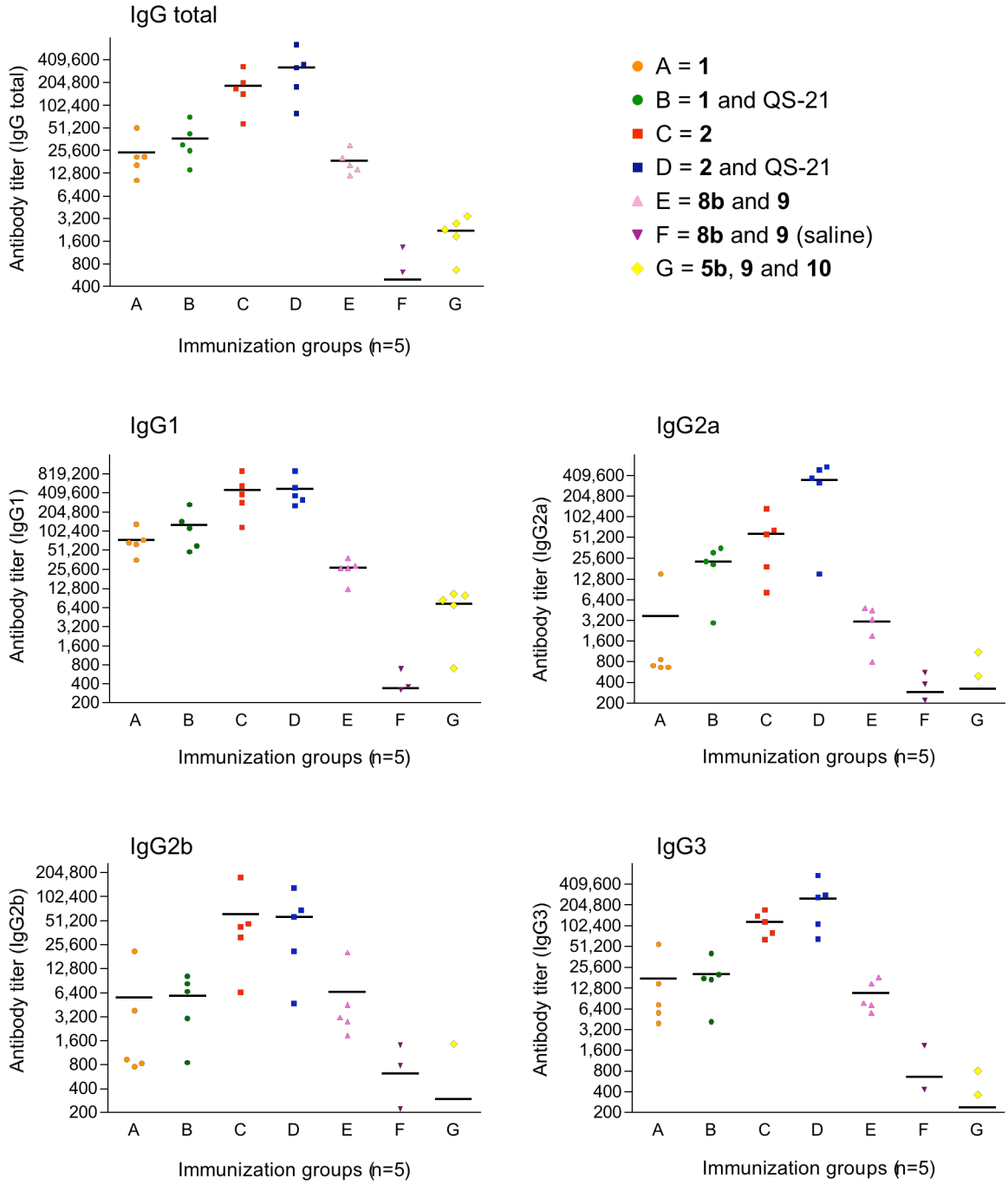
Supplementary Figure 1 TNF-α production by murine macrophages after stimulation with LPS and synthetic compounds **1**, **2**, Pam_2CysSK_4 and **9**. Murine 264.7 RAW γNO(-) cells were incubated for 5.5 h with increasing concentrations of *E. coli* LPS or synthetic compounds **1**, **2**, Pam_2CysSK_4 and **9** as indicated. Data represent mean values ± s.d. (n=3).



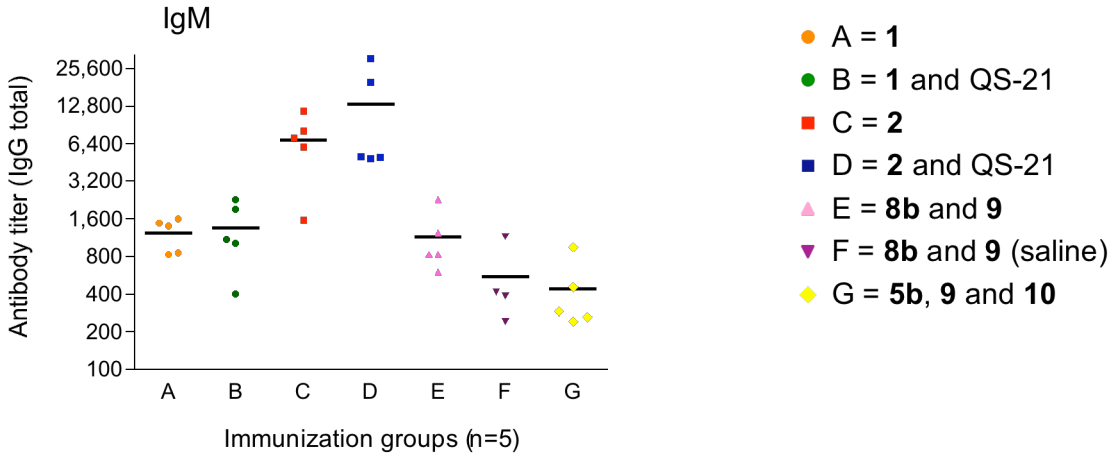
Supplementary Figure 2 Involvement of TLR2 in NF-κB activation by compounds **1**, **2** and **9**. Induction of NF-κB activation was determined in triplicate cultures of HEK293T cells (wild type and stable transfected with murine TLR2, TLR2/TLR6 or TLR4/MD2) transiently transfected with the expression vectors pELAM-Luc and pRL-TK. Forty-four h post-transfection, cells were treated with the synthetic compounds **1**, **2** and **9** (6.1 μM each) or were left untreated (control; (-)). Forty-eight h post-transfection, NF-κB activation was determined by firefly luciferase activity relative to *Renilla* luciferase activity. Data represent mean values ± s.d. (n=3). In the transfection experiment shown, human TNF-α (10 ng ml^{-1}) induced 145 ± 11, 82 ± 25, 141 ± 10 and 42 ± 2 fold activation of NF-κB in wild type and stable transfected murine TLR2, TLR2/TLR6 and TLR4/MD2 cells, respectively.



Supplementary Figure 3 Induction of dendritic cell maturation. After exposure of MDDCs to PBS (negative control), LPS (1 μg ml^{-1}; positive control) and compounds **1**, **2**, Pam_2CysSK_4 and **9** (1 μg ml^{-1} each) for 16-24 h, expression of CD80 (**a**), CD83 (**b**) and CD86 (**c**) was determined by flow cytometry analysis.



Supplementary Figure 4 ELISA anti-MUC1 IgG antibody titers after 4 immunizations with **1**, **1**/QS-21, **2**, **2**/QS-21, **8b**/**9**, **8b**/**9** (saline) and **5b**/**9**/**10**. ELISA plates were coated with BSA-BrAc-MUC1 conjugate and titers were determined by linear regression analysis, plotting dilution *vs.* absorbance. Titers were defined as the highest dilution yielding an optical density of 0.1 or greater over that of normal control mouse sera. Each data point represents the titer for an individual mouse after 4 immunizations and the horizontal lines indicate the mean for the group of five mice.



Supplementary Figure 5 ELISA anti-MUC1 IgM antibody titers after 4 immunizations with **1**, **1**/QS-21, **2**, **2**/QS-21, **8b**/**9**, **8b**/**9** (saline) and **5b**/**9**/**10**. ELISA plates were coated with BSA-BrAc-MUC1 conjugate and titers were determined by linear regression analysis, plotting dilution *vs.* absorbance. Titers were defined as the highest dilution yielding an optical density of 0.1 or greater over that of normal control mouse sera. Each data point represents the titer for an individual mouse after 4 immunizations and the horizontal lines indicate the mean for the group of five mice.

Supplementary Table 1 ELISA anti-MUC1 antibody titers* one week after the third, fourth and fifth immunization.

Immunization**	IgG total	IgG1	IgG2a	IgG2b	IgG3
2 (3rd imm.)	18,900	31,700	3,200	5,700	22,900
2 (4th imm.)	169,600	389,300	56,500	42,700	116,800
2 (5th imm.)	252,900	726,700	64,900	92,400	163,100
2 and QS-21 (3rd imm.)	50,800	71,100	37,700	16,600	83,400
2 and QS-21 (4th imm.)	322,800	371.300	378,900	56,800	263,500
2 and QS-21 (5th imm.)	140,400	292,500	274,000	32,300	259,900

* Anti-MUC1 antibody titers are presented as the median for groups of five mice. ELISA plates were coated with BSA-BrAc-MUC1 conjugate and titers were determined by linear regression analysis, plotting dilution vs. absorbance. Titers are defined as the highest dilution yielding an optical density of 0.1 or greater over that of normal control mouse sera.

** Liposomal preparations were employed.

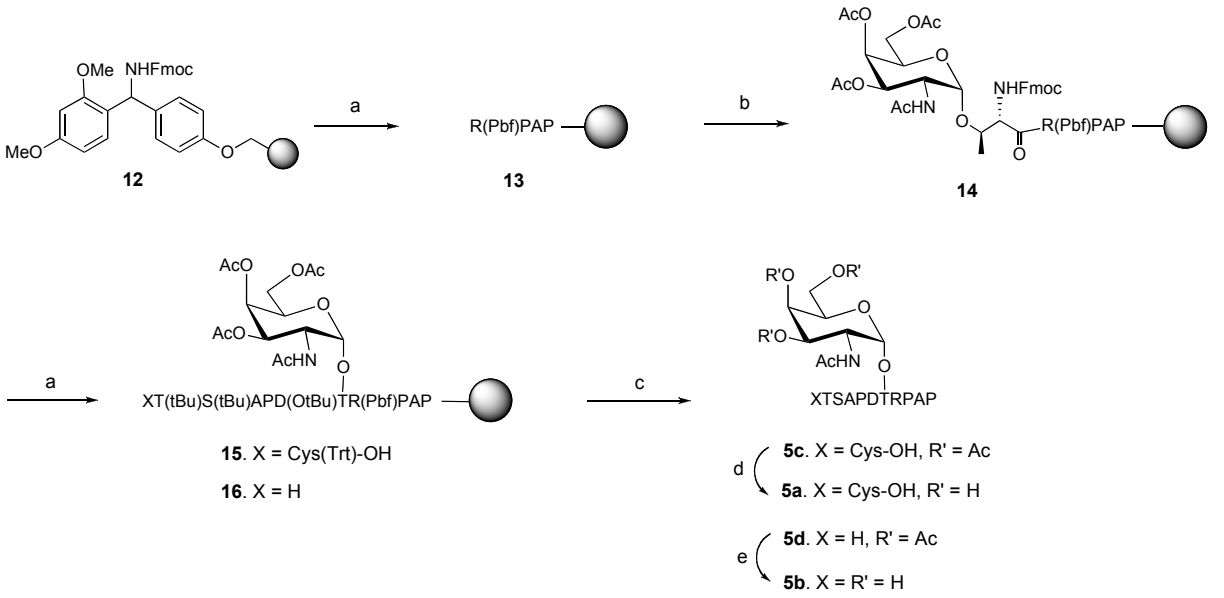
METHODS

Reagents and general experimental procedures for synthesis. Amino acid derivatives and resins were purchased from NovaBioChem and Applied Biosystems. *N*,*N*-dimethylformamide (DMF) was obtained from EM Science, *N*-methylpyrrolidone (NMP) was from Applied Biosystems and egg phosphatidylcholine, phosphatidylglycerol, cholesterol and dodecyl phosphocholine (DPC) were from Avanti Polar Lipids. All other chemical reagents were purchased form Aldrich, Acros, Alfa Aesar and Fischer 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 autosampler, UV-detector and fraction-collector using a Zorbax Eclipse C8 analytical column (5 μm, 4.6 x 150 mm) at a flow rate of 1 ml min^{-1}, a semi-preparative C8 column (5 μm, 25 x 250 mm) at a flow rate of 4 ml min^{-1}, a Synchropak C4 analytical column (5 μm, 4.6 x 100 mm) at a flow rate of 1 ml min^{-1} or a Vydac C4 semi-preparative column (5 μm, 4.6 x 250 mm) at a flow rate of 2 ml min^{-1}. All runs used linear gradients of 0 to 95% solvent B (0.1% trifluoroacetic acid (TFA) in acetonitrile) in solvent A (0.1% TFA in water) over a 40 min period unless otherwise specified. MALDI-ToF mass spectra were recorded on a ABI 4700 proteomic analyzer.

Chemical synthesis of compound 5a. SPPS was performed on Rink amide AM resin (**12**; 0.1 mmol) according to the general methods for SPPS. The first four amino acids were coupled on the peptide synthesizer using a standard protocol to obtain **13** followed by manual coupling of **3** (0.4 mmol, 268 mg) using *O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N'*,*N'*-tetramethyl-uronium hexafluorophosphate (HATU; 0.4 mmol, 152 mg), 1-hydroxy-7-azabenzotriazole (HOAt; 0.4 mmol, 55 mg) and diisopropylethylamine (DIPEA; 0.4 mmol, 70 μl) in DMF for 12 h. The coupling reaction was monitored by standard Kaiser test. The resin was washed with DMF (6 ml) and methylene chloride (DCM; 6 ml) and resubjected to the same coupling conditions to ensure complete coupling. The glycopeptide **14** was then elongated on the peptide synthesizer to give **15**. The resin was thoroughly washed with DMF (6 ml), DCM (6 ml) and methanol (MeOH; 6 ml) and dried *in vacuo* to constant weight. The resin was then swelled in DCM (5 ml) for 1 h after which it was treated with TFA (94%), water (2.5%), 1,2-ethanedithiol (EDT; 2.5%) and triisopropylsilane (TIS; 1%) (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

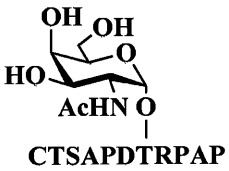
of its original volume. The glycopeptide was then precipitated using diethyl ether (0 ^{o}C) and recovered by centrifugation at 3000 rpm for 15 min. The crude glycopeptide was purified by RP-HPLC on a semi-preparative C-18 reversed phase column using a linear gradient of 0 to 95% solvent B in solvent A over a period of 40 min and lyophilization of the appropriate fractions afforded **5c** (90% based on resin loading capacity). $C_{59}H_{94}N_{16}O_{24}S$, MALDI-ToF MS: observed [M+], 1443.8918Da; calculated [M+], 1443.5371Da. The glycopeptide **5c** (5 mg, 3.4 μmol) was treated with aqueous hydrazine (5%; 2 ml) containing excess of dithiothreitol (DTT; 12 mg), the reaction was monitored by MALDI-ToF MS. After standing for 1 h at room temperature, the crude product was purified by RP-HPLC on a semi-preparative C-18 reversed phase column using a linear gradient of 0 to 95% solvent B in solvent A over a period of 40 min to afford after lyophilization compound **5a** (4.0 mg, 88%). $C_{53}H_{88}N_{16}O_{21}S$, MALDI-ToF MS: observed [M+], 1317.9580Da; calculated [M+], 1317.4271Da.

Chemical synthesis of compound 5b. The synthesis of glycopeptide **5d** was carried out by using a similar method as described for **5c** without coupling of N^{α}-Fmoc-Cys(Trt)-OH. The crude glycopeptide was purified by RP-HPLC on a semi-preparative C-18 reversed phase column using a linear gradient of 0 to 95% solvent B in solvent A over a period of 40 min and lyophilization of the appropriate fractions afforded **5d** (83% based on resin loading capacity). $C_{56}H_{89}N_{15}O_{23}$, MALDI-ToF MS: observed [M+], 1340.7689Da; calculated [M+], 1340.3926Da. The glycopeptide **5d** (5.8 mg, 4.3 μmol) was treated with aqueous hydrazine (5%; 2 ml) and the reaction was monitored by MALDI-ToF MS. After standing for 1 h at room temperature, the crude product was neutralized with acetic acid (1.5 ml) and purified by RP-HPLC on a semi-preparative C-18 reversed phase column using a linear gradient of 0 to 95% solvent B in solvent A over a period of 40 min to afford after lyophilization compound **5b** (5.0 mg, 86%). $C_{50}H_{83}N_{15}O_{20}$, MALDI-ToF MS: observed [M+], 1214.7357Da; calculated [M+], 1214.2825Da.



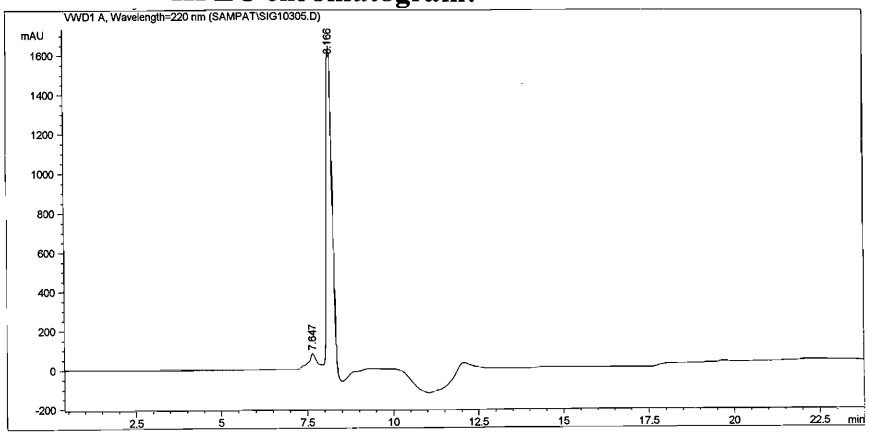
Supplementary Scheme 1 Reagents and conditions: a) SPPS using N^{α}-Fmoc-chemistry, coupling with 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyl hexafluorophosphate (HBTU)/*N*-hydroxybenzotriazole (HOBt)[S1] in the presence of DIPEA in NMP followed by Fmoc cleavage with 20% piperidine in DMF; b) **3**, HATU/HOAt, DIPEA, DMF, 12 h; c) TFA (94.0%), water (2.5%), EDT (2.5%), TIS (1%), 2 h; d) aqueous hydrazine (5%), excess of DTT; e) aqueous hydrazine (5%).

S1. Knorr, R., Trzeciak, A., Bannwarth, W. & Gillessen, D. New coupling reagents in peptide chemistry. *Tetrahedron. Lett*. **30**, 1927-1930 (1989).



5a

HPLC chromatogram:



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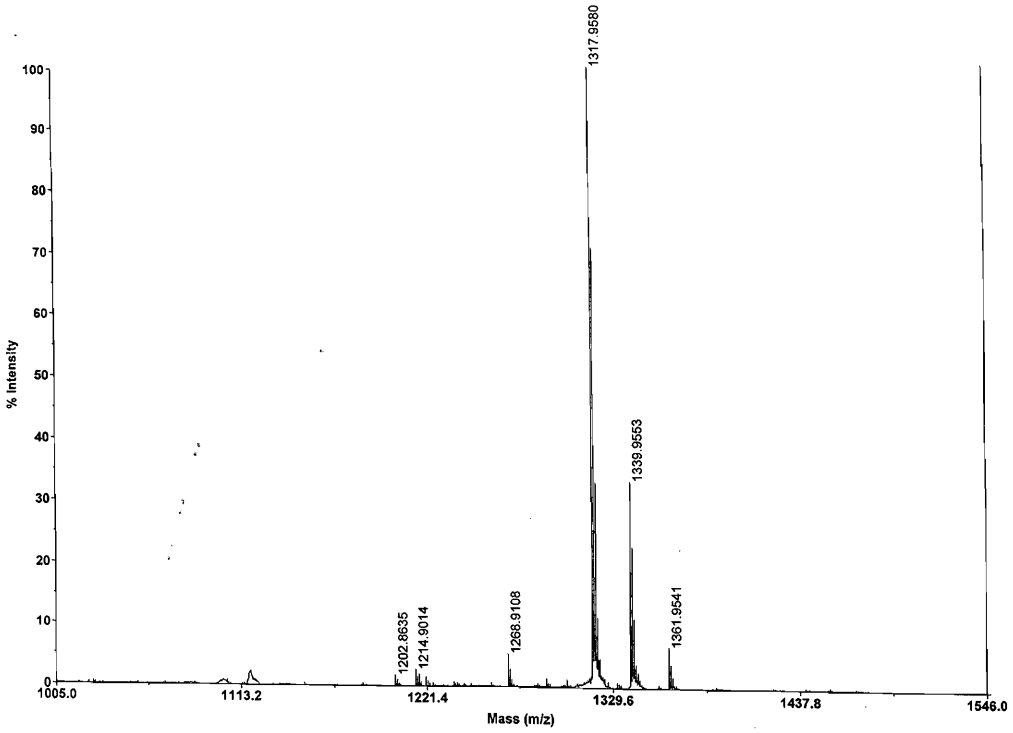
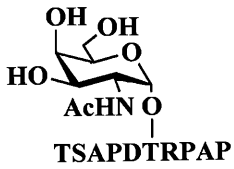
Eluent: 0-95% of Solvent B in A over period of 40 min

MALDI-ToF spectra:



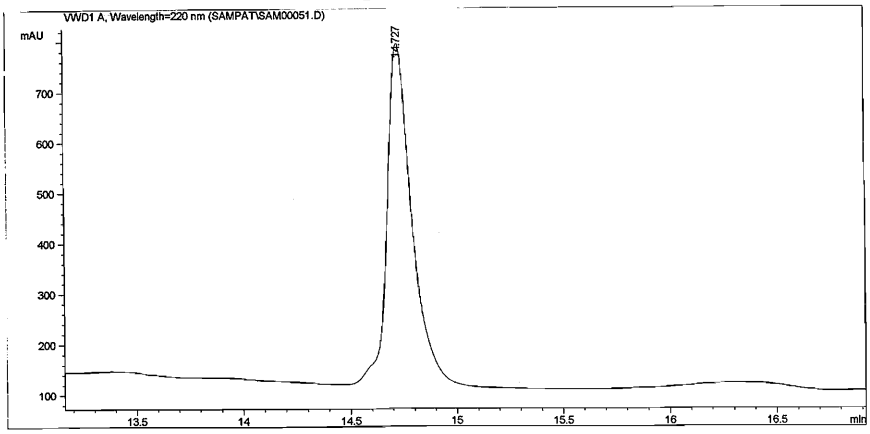
Observed, [M+], 1317.9580Da

Calculated, [M+], 1317.4217Da



5b

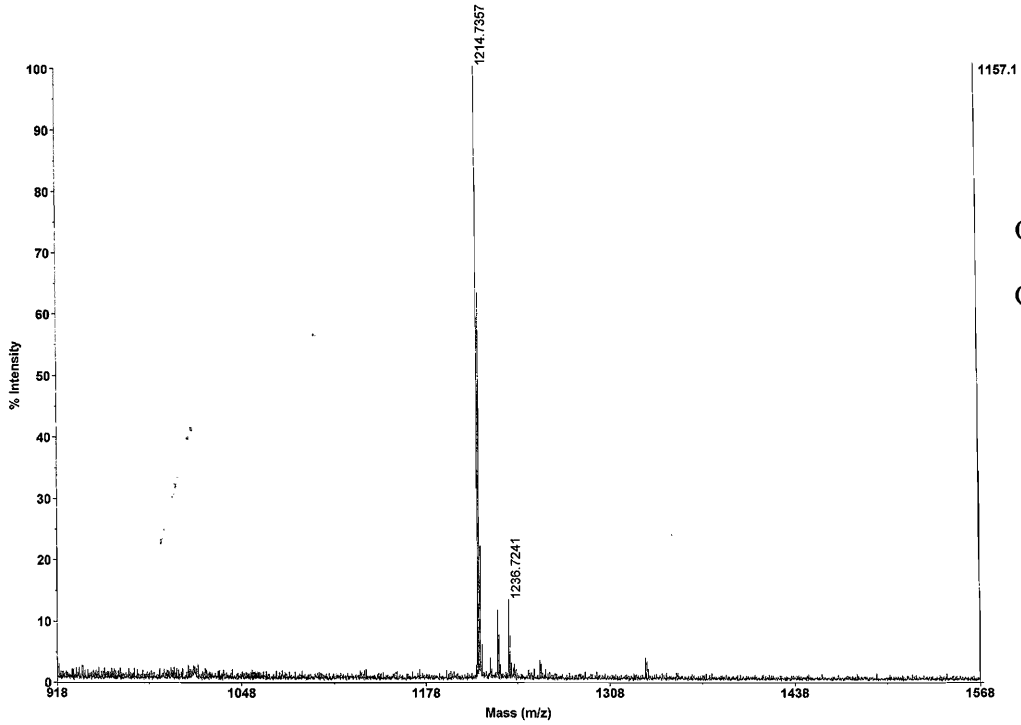
HPLC chromatogram:



Column: Semi-prep. C18 Reversed phase

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

MALDI-ToF spectra:



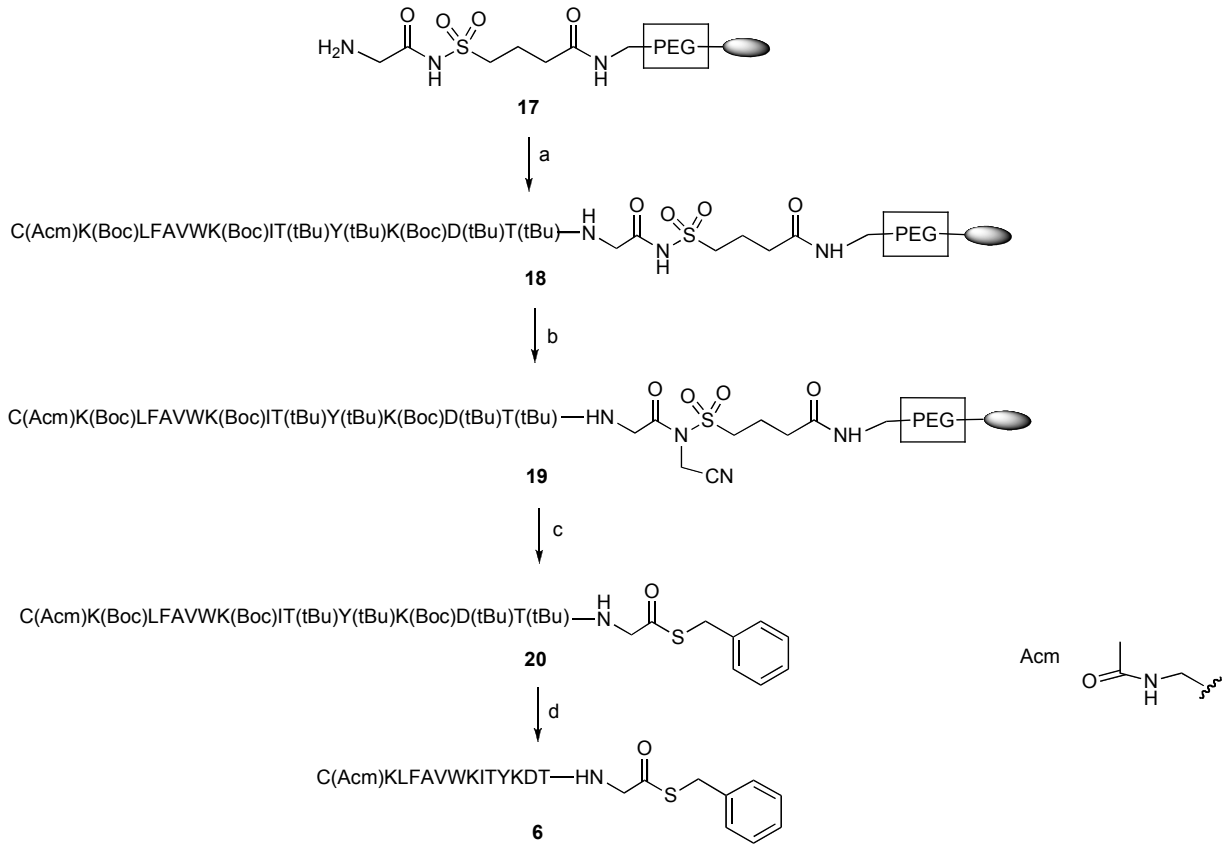
Observed, [M+], 1214.7357Da

Calculated, [M+], 1214.2825Da

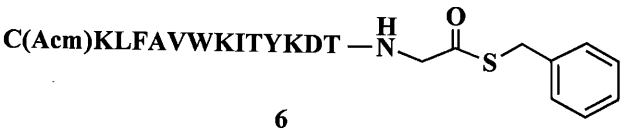
Chemical synthesis of compound 6. The synthesis of Acm protected peptide thioester was carried out on preloaded H-Gly-sulfamylbutyryl Novasyn TG resin[S2,S3] (**17**; 0.1 mmol) as described in the general methods section for peptide synthesis. The resin-bound peptide **18** was washed thoroughly with DCM (10 ml) and *N*-methyl-2-pyrrolidone (NMP; 10 ml) Next the resin was swelled in DCM (5 ml) for 1 h. The resin was then treated with DIPEA (0.5 ml, 3 mmol) and iodoacetonitrile (0.36 ml, 5 mmol) in NMP (6 ml). Before addition, iodoacetonitrile (0.36 ml) was filtered through a plug of basic alumina. The resin was then agitated under the exclusion of light for 24 h, filtered and then washed with NMP (4 x 5 ml), DCM (4 x 5 ml) and tetrahydrofuran (THF; 4 x 5 ml). The activated *N*-acyl sulfonamide resin **19** was swollen in DCM (5 ml), drained and then transferred to a 50 ml round bottom flask. To the resin-containing flask was added THF (4 ml), benzyl mercaptan (0.64 ml, 5 mmol) and sodium thiophenate (27 mg, 0.2 mmol). After agitation for 24 h, the resin was filtered and washed with DMF (3 ml). The combined filtrate and washings were concentrated *in vacuo*. The crude peptide was triturated with *tert*-butyl methyl ether (0 ^{o}C; 60 ml) and the precipitate was obtained by centrifugation at 3000 rpm for 15 min followed by decanting of the solvent. The peptide precipitate was purified by LH-20 column chromatography using MeOH/DCM (1/1, v/v) as the eluent to give the fully protected peptide thioester **20**. Peptide **20** was treated with reagent B (TFA (88%), phenol (5%), H_2O (5%), TIS (2%); 5 ml) for 6 h at room temperature. The TFA solution was then added drop wise to a screw cap centrifuge tube containing ice cold *tert*-butyl methyl ether (40 ml) and the resulting suspension was left overnight at 4 ^{o}C after which the precipitate was collected by centrifugation at 3000 rpm (20 min). The peptide precipitate was resuspended in ice cold *tert*-butyl methyl ether (40 ml) and recovered by centrifugation and decanting of the ether layer. This process of washing was repeated twice. The crude peptide was purified by semi-preparative C-8 reversed phase column using a linear gradient of 0 to 95% solvent B in solvent A over a period of 40 min and lyophilization of the appropriate fractions afforded **6** in good yield (74% based on resin loading capacity). $C_{94}H_{140}N_{20}O_{21}S_2$, MALDI-ToF MS: observed [M+Na],1972.1240Da; calculated [M+Na], 1973.3716Da.

S2. Shin, Y. *et al.* Fmoc-based synthesis of peptide-(alpha)thioesters: application to the total chemical synthesis of a glycoprotein by native chemical ligation. *J. Am. Chem. Soc.* **121**, 11684-11689 (1999).

S3. Ingenito, R., Bianchi, E., Fattori, D. & Pessi, A. Solid phase synthesis of peptide C-terminal thioesters by Fmoc/t-Bu chemistry. *J. Am. Chem. Soc.* **121**, 11369-11374 (1999).

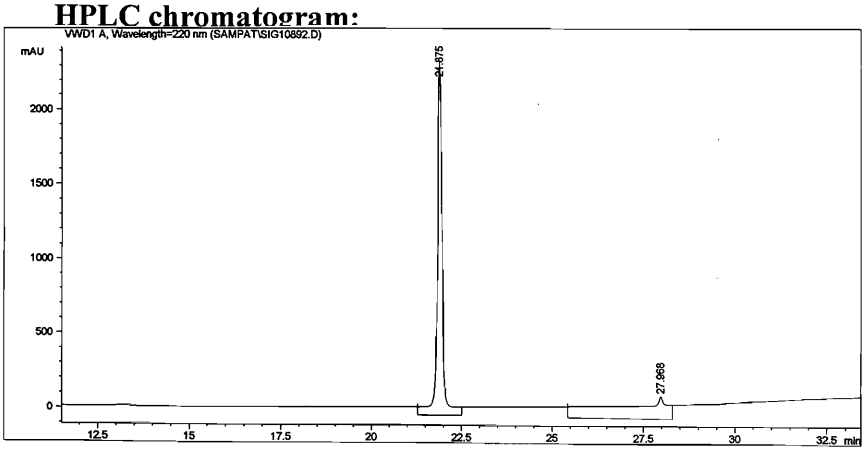


Supplementary Scheme 2 Reagents and conditions: a) SPPS using N^{α}-Fmoc-chemistry, coupling with HBTU/HOBt in the presence of DIPEA in NMP followed by Fmoc cleavage with 20% piperidine in DMF; b) ICH_2CN, DIEA, NMP, 24 h; c) BnSH, Na-thiophenate, THF, 24 h; d) reagent B (TFA (88%), phenol (5%), H_2O (5%), TIS (2%)), 6 h.



6

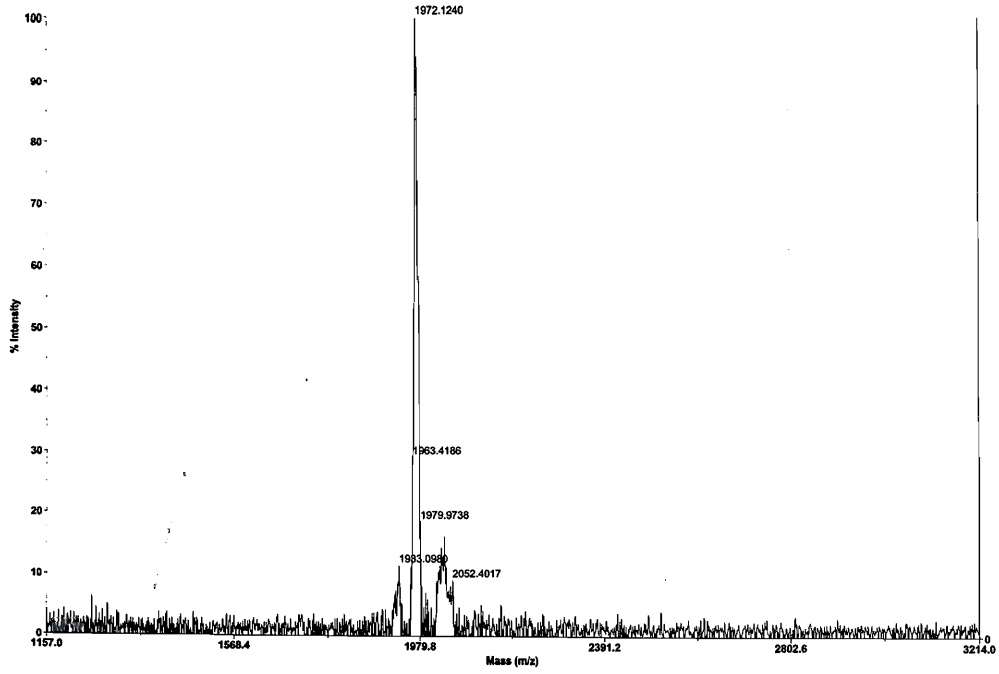
HPLC chromatogram:



Column: Semi-prep. C8 Reversed phase

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

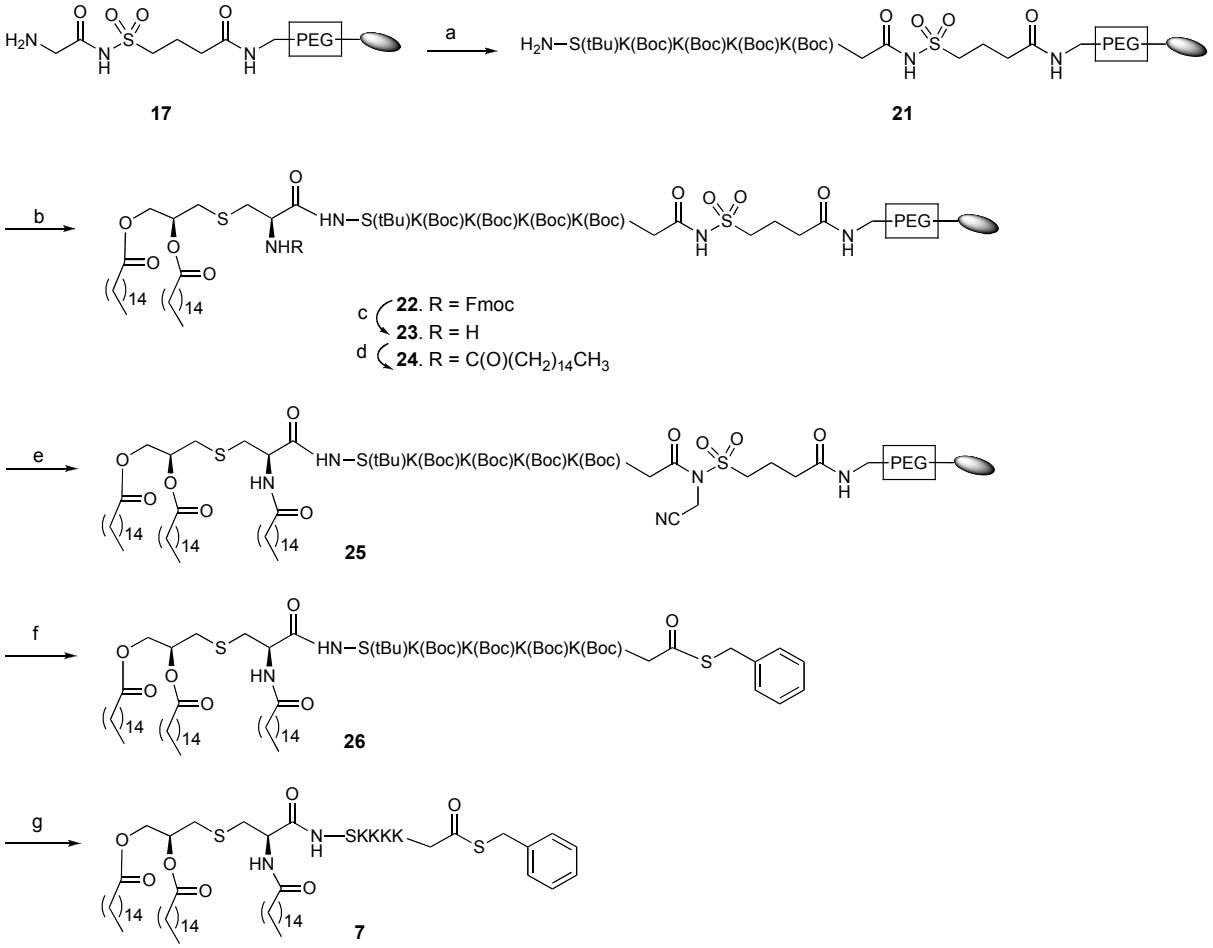
MALDI-ToF spectra:



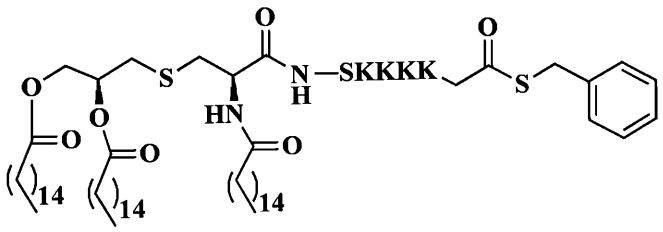
Observed, [M+Na], 1972.1240Da

Calculated, [M+Na], 1973.3716Da

Chemical synthesis of compound 7. The synthesis of **7** was carried out on a H-Gly-sulfamylbutyryl Novasyn TG resin (**17**; 0.1 mmol) according to the general methods for SPPS. The first five amino acids were coupled using an automated synthesizer to give resin-bound peptide **21** and the remaining steps were performed manually. Compound **4** (267 mg, 0.3 mmol) was dissolved in DMF (5 ml) and benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium (PyBOP; 156 mg, 0.3 mmol), HOBt (40 mg, 0.3 mmol) and DIPEA (67 µl, 0.4 mmol) were added. After premixing for 2 min, the mixture was added to the resin. The coupling reaction was monitored by the Kaiser test. Upon completion of the coupling, the N^{α}-Fmoc group of **22** was cleaved using piperidine (20%) in DMF (6 ml) to give **23**. Palmitic acid (77 mg, 0.3 mmol) was coupled to the free amine of **23** as described above using PyBOP (156 mg, 0.3 mmol), HOBt (40 mg, 0.3 mmol) and DIPEA (67 µl, 0.4 mmol) in DMF to afford **24**. The resin was thoroughly washed with DMF (10 ml), DCM (10 ml) and MeOH (10 ml) and then dried *in vacuo*. Activation of **24** to give **25** followed by cleavage from the resin to provide **26** and finally side chain deprotection were carried out according to the methods described for peptide **6**. After purification by HPLC on a semi-preparative C-4 reversed phase column using a linear gradient of 0 to 95% solvent B in solvent A over a 40 min period and lyophilization of the appropriate fractions afforded **7** (65% based on resin loading capacity). $C_{90}H_{165}N_{11}O_{13}S_2$, MALDI-ToF MS: observed [M+Na], 1695.2335Da; calculated [M+Na], 1695.4714Da.

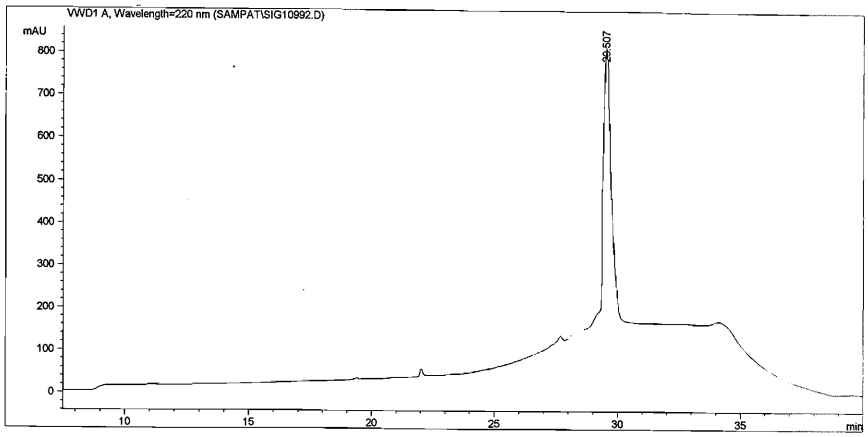


Supplementary Scheme 3 Reagents and conditions: a) SPPS using N^{α}-Fmoc-chemistry, coupling with HBTU/HOBt in the presence of DIPEA in NMP followed by Fmoc cleavage with 20% piperidine in DMF; b) manual coupling of **4**, PyBOP, HOBt in the presence of DIPEA in DMF; c) piperidine (20%) in DMF; d) coupling of palmitic acid, PyBOP, HOBt in the presence of DIPEA in DMF; e) ICH_2CN, DIEA, NMP, 24 h; f) BnSH, Na-thiophenate, THF, 24 h; g) reagent B (TFA (88%), phenol (5%), H_2O (5%), TIS (2%)), 4 h.



7

HPLC chromatogram:



Column: Analytical C4 Reversed phase

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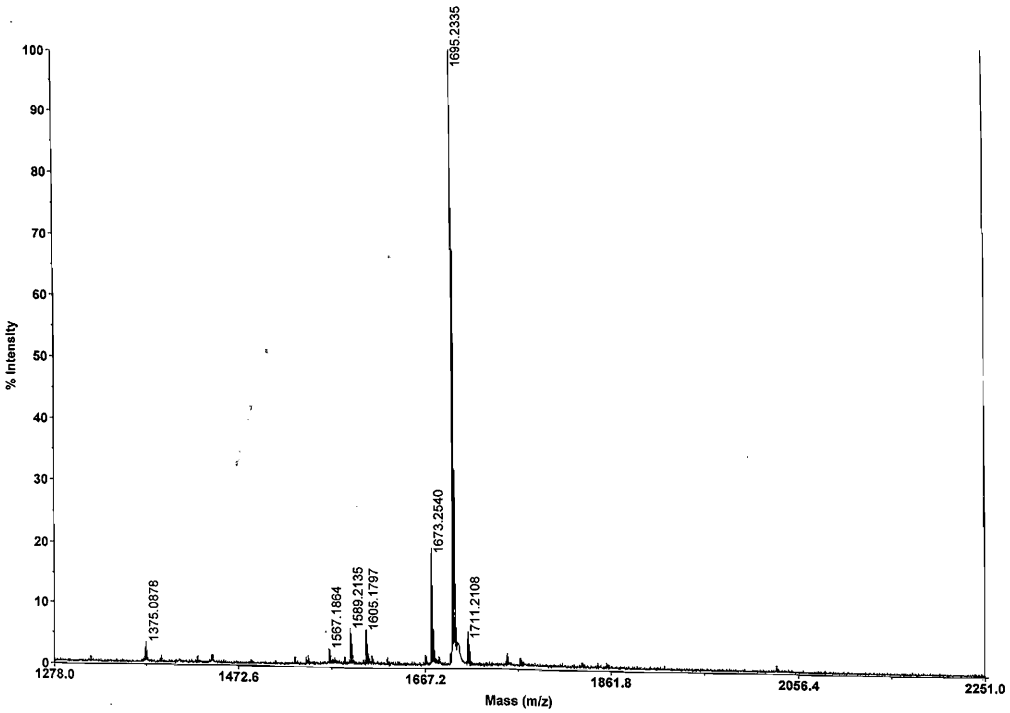


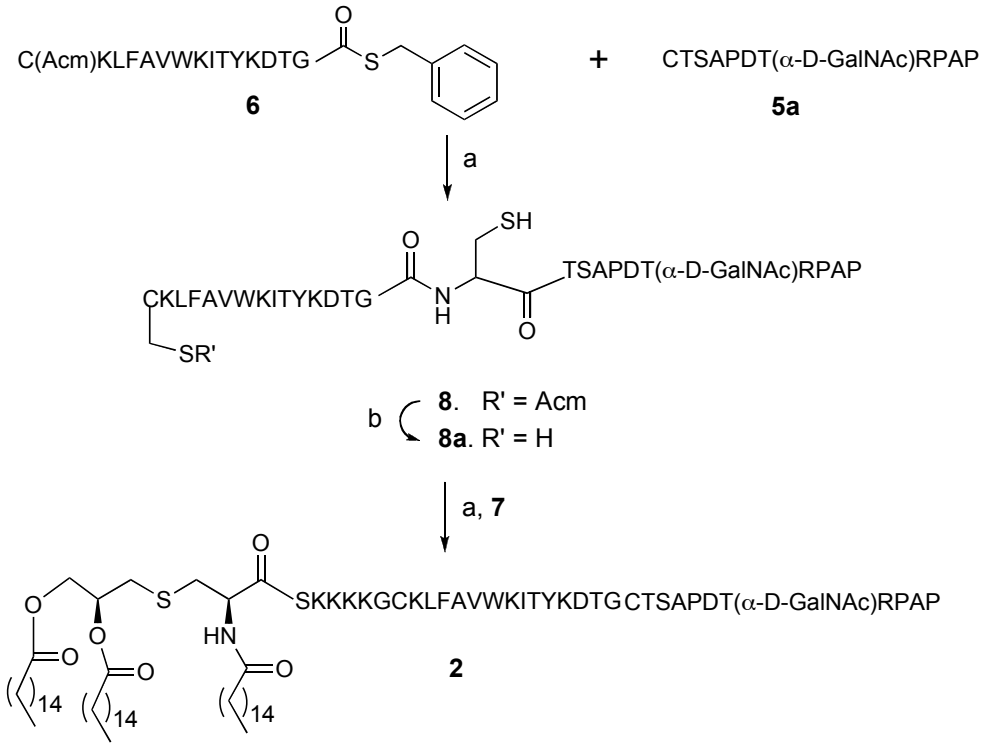
Observed, [M+Na], 1695.2355Da

Calculated, [M+Na], 1695.4714Da

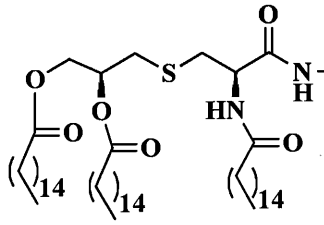
Chemical synthesis of compound 8a. Peptide **5a** (5.6 mg, 4.3 μmol), peptide thioester **6** (6.0 mg, 3.0 μmol) and DPC (8.8 mg, 25.8 μmol) were dissolved in a mixture of trifluoroethanol (TFE) and $CHCl_3$ (2.5 ml / 2.5 ml). The solvents were removed under reduced pressure to give a lipid/peptide film on the surface of the round bottom flask. The lipid/peptide film was hydrated for 4 h at 37 ^oC using a phosphate buffer (200 mM, pH 7.5; 2 ml) in the presence of tris-(2-carboxyethyl)phosphine (TCEP; 2% w/v) and ethylenediaminetetraacetic acid (EDTA; 0.1% w/v). The mixture was ultrasonicated for 1 min. The peptide/lipid suspension was extruded through 1.0 μm polycarbonate membranes (Whatman, Nucleopore, Track-Etch Membrane) at 50 ^oC to obtain uniformly sized vesicles. Sodium 2-mercaptoethane sulfonate (2% w/v) was added to the vesicle suspension to initiate the ligation reaction. The reaction was agitated for 2 h at 37 ^oC after which it was diluted with 2-mercaptoethanol (20%) in ligation buffer (2 ml). The resulting mixture was purified by RP-HPLC on a semi-preparative C-8 reversed phase column using a linear gradient of 0 to 95% solvent B in solvent A over a 40 min period. The fractions possessing the expected mass were collected and lyophilized to give **8** (7.4 mg, 79%). The Acm protecting group of the ligated product was removed by dissolving the glycopeptide in aqueous AcOH (10%, pH 4.0; 2 ml) followed by the treatment of Hg(II)acetate (11.5 mg, 30 μmol) for 30 min, after which the reaction was quenched by addition of DTT (7.4 mg, 40 μmol). The Acm deprotected product was purified by semi-preparative C-8 reversed phase column using a water/acetonitrile gradient to yield **8a** (5.6 mg, 77%). MALDI-ToF MS: observed [M+], 3072.4436Da; calculated [M+], 3072.5129Da.

Chemical synthesis of compound 2. Peptide **8a** (1.5 mg, 0.48 μmol), lipopeptide thioester **7** (0.98 mg, 0.58 μmol) and DPC (0.9 mg, 2.88 μmol) were dissolved in a mixture of TFE and $CHCl_3$ (5 ml, 1/1 , v/v). The solvents were removed under reduced pressure to give a lipid/peptide film on the surface of a round bottom flask, which was hydrated for 4 h at 37 ^oC using a 200 mM phosphate buffer (pH 7.5, 2 ml) in the presence of TCEP (2% w/v, 40.0 μg) and EDTA (0.1% w/v, 20.0 μg). After the mixture was ultrasonicated for 1 min, the suspension was extruded through 1.0 μm polycarbonate membranes (Whatman, Nucleopore, Track-Etch Membrane) at 50 ^oC to obtain uniformed sized vesicles. Sodium 2-mercaptoethane sulfonate (2% w/v, 40.0 μg) was added to the vesicle suspension to initiate the ligation reaction. The reaction mixture was incubator at 37 ^oC and the progress of the reaction was periodically monitored by

MALDI-ToF, which indicated that it was completed within 2 h. The crude peptide was purified by HPLC using a semi-preparative C-4 reversed phase column and a linear gradient of 0 to 95% solvent B in solvent A over a 40 min period. Lyophilization of the appropriate fractions afforded **2** (1.8 mg, 85%). $C_{220}H_{371}N_{46}O_{54}S_3$, MALDI-ToF MS: observed [M+], 4622.3549Da; calculated [M+], 4621.7785Da.

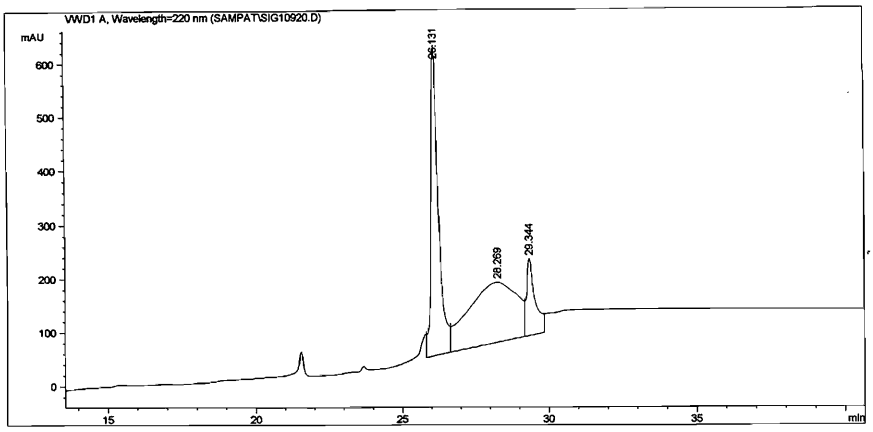


Supplementary Scheme 4 Reagents and conditions: a) DPC, sodium phosphate buffer (200 mM), pH 7.5, TCEP (2% w/v), EDTA (0.1% w/v), sodium 2-mercapto-ethanesulfonate (2% w/v); b) $Hg(OAc)_2$, 10% aq HOAc, DTT.



2

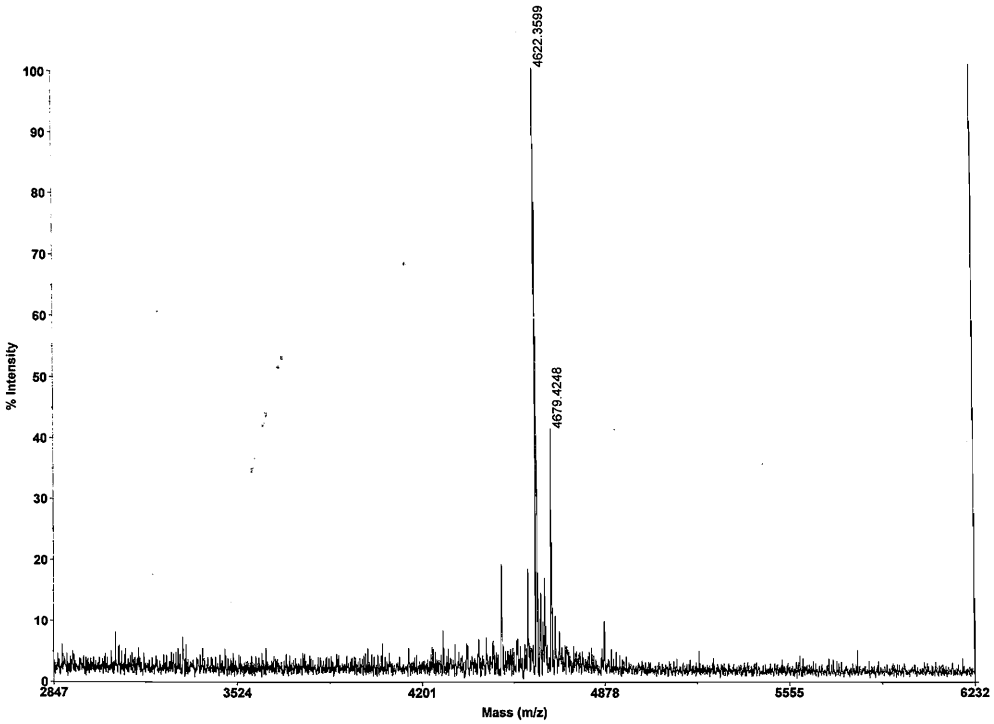
HPLC chromatogram:



Column: Semi-prep. C4 Reversed phase

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

MALDI-ToF spectra:



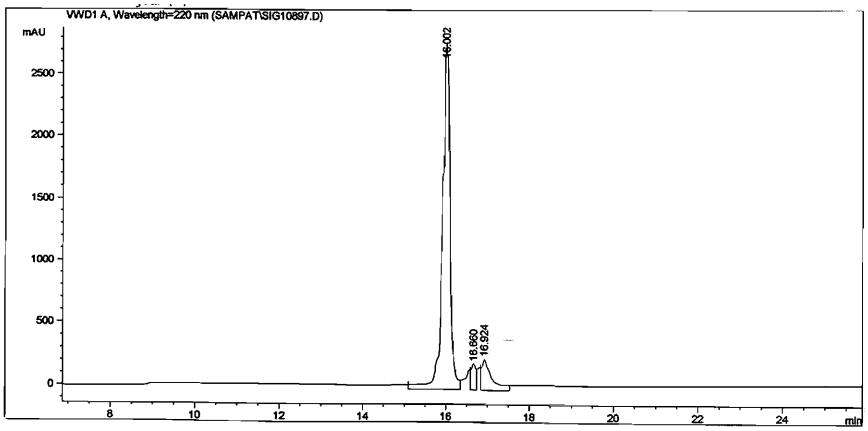
Observed, [M+], 4622.3549Da

Calculated, [M+], 4621.7785Da

CKLFAVWKITYKDTGCTSAPDT(α-D-GalNAc)RPAP

8a

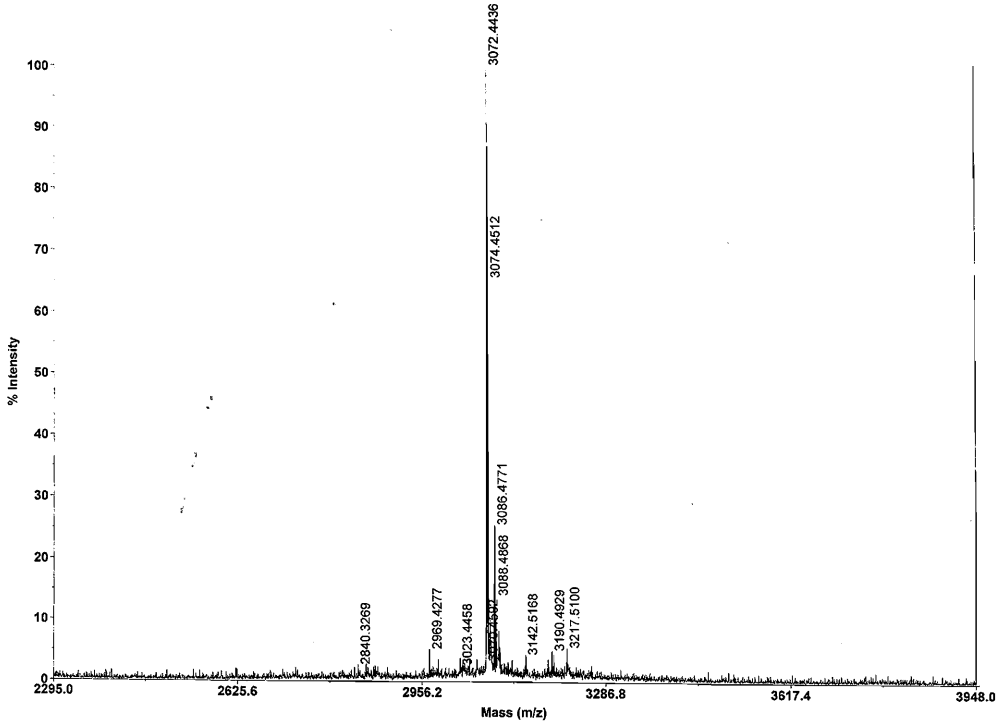
HPLC chromatogram:



Column: Semi-prep. C8 Reversed phase

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

MALDI-ToF spectra:



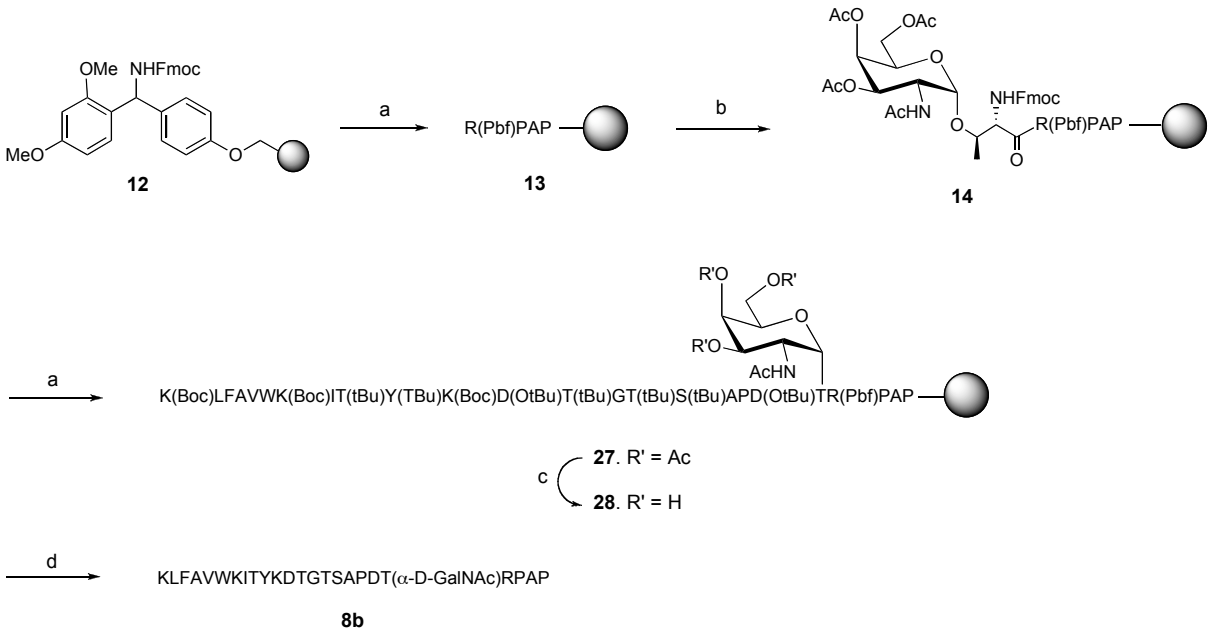
Observed, [M+], 3072.4436Da

Calculated, [M+], 3072.5129Da

Chemical synthesis of compound 8b. The synthesis of **8b** was carried out on a Rink amide AM resin (**12**; 0.1 mmol) according to the general methods for SPPS. The first four amino acids were coupled on the peptide synthesizer using a standard protocol to obtain **13** followed by manual coupling of **3** (0.4 mmol, 268 mg) using HATU (0.4 mmol, 152 mg), HOAt (0.4 mmol, 55 mg) and DIPEA (0.4 mmol, 70 μl) in DMF for 12 h. The coupling reaction was monitored by standard Kaiser test. The resin was washed with DMF (6 ml) and DCM (6 ml) and resubjected to the same coupling conditions to ensure complete coupling. The glycopeptide **14** was then elongated on the peptide synthesizer to afford **27**. The resin was thoroughly washed with DMF (6 ml), DCM (6 ml) and MeOH (6 ml) and dried *in vacuo* to constant weight. After completion of the synthesis, resin **27** was treated with hydrazine (80%) in MeOH[S4,S5] (10 ml) for 1 h. This procedure was repeated twice for 30 min to ensure complete deprotection of the acetyl moieties of the sugar residue to obtain **28**. The resin was then thoroughly washed with DMF (4 x 5 ml), DCM (4 x 5 ml) and MeOH (4 x 5 ml) and then dried *in vacuo* to constant weight. The resin was swelled in DCM (5 ml) for 1 h, after which it was treated with TFA (88%), water (5%), phenol (5%) and TIS (2%) (10 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 then precipitated using diethyl ether (0 ^{o}C) and recovered by centrifugation at 3000 rpm for 15 min. The crude glycopeptide was purified by RP-HPLC on a semi-preparative C-18 reversed phase 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 **8b** (65% based on resin loading capacity). $C_{131}H_{205}N_{33}O_{39}$, MALDI-ToF MS: observed [M+], 2866.6770Da; calculated [M+Na], 2866.2271Da.

S4. Schultheiss-Reimann, P. & Kunz, H. *O*-Glycopeptide synthesis using 9-fluorenylmethoxycarbonyl (Fmoc)-protected synthetic units. *Angew. Chem. Int. Ed.* **1**, 62-63 (1983).

S5. Mitchell, S. A., Pratt, M. R., Hruby, V.J. & Polt, R. Solid-phase synthesis of *O*-linked glycopeptide analogues of enkephalin. *J. Org. Chem*. **66**, 2327-2342 (2001).

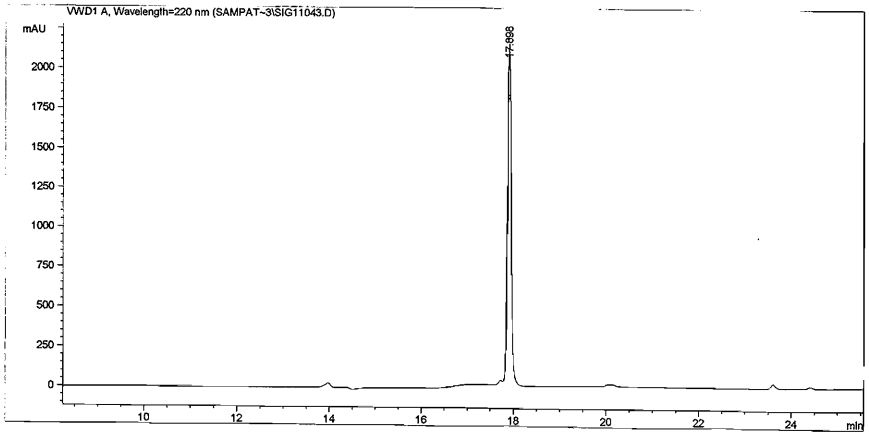


Supplementary Scheme 5 Reagents and conditions: a) SPPS using N^{α}-Fmoc-chemistry, coupling with HBTU/HOBt in the presence of DIPEA in NMP followed by Fmoc cleavage with 20% piperidine in DMF; b) **3**, HATU/HOAt, DIPEA, DMF, 12 h; c) hydrazine (80%) in MeOH, 2 h; d) reagent B (TFA (88%), phenol (5%), H_2O (5%), TIS (2%)), 2 h.

KLFAVWKITYKDTGTSAPDT(α-D-GalNAc)RPAP

8b

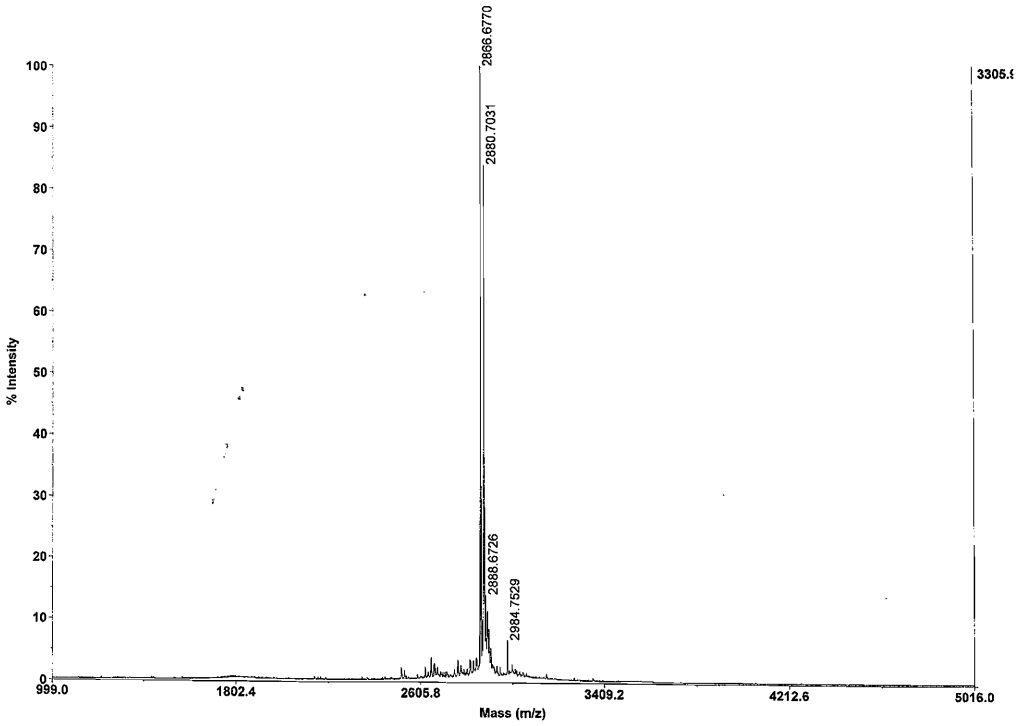
HPLC chromatogram:



Column: Semi-prep. C8 Reversed phase

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

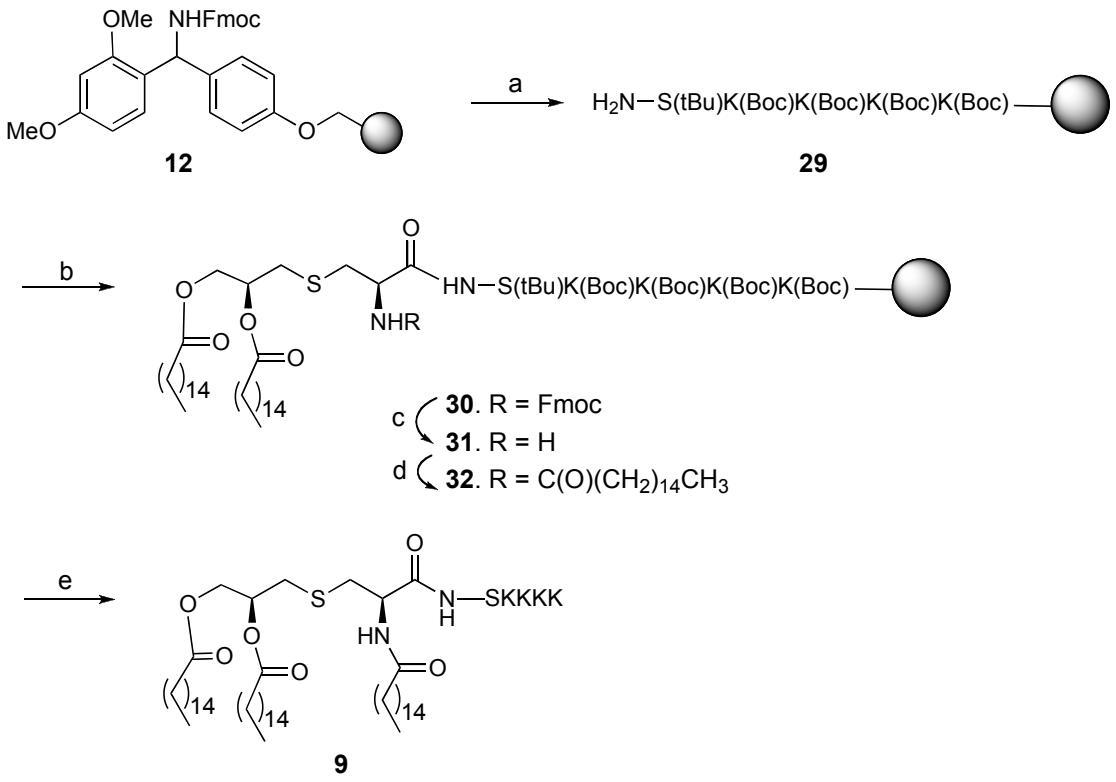
MALDI-ToF spectra:



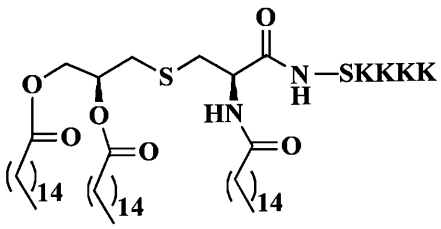
Observed, [M+], 2866.6770Da

Calculated, [M+], 2866.2271Da

Chemical synthesis of compound 9. The synthesis of **9** was carried out on a Rink amide AM resin (**12**; 0.1 mmol) according to the general methods for SPPS. After coupling the first five amino acids on an automated synthesizer to obtain **29**, the remaining steps were performed manually. Compound **4** (267 mg, 0.3 mmol) was dissolved in DMF (5 ml) and PyBOP (156 mg, 0.3 mmol), HOBt (40 mg, 0.3 mmol) and DIPEA (67 µl, 0.4 mmol) were added. After premixing for 2 min, the mixture was added to the resin. The coupling reaction was monitored by the Kaiser test. Upon completion of the coupling, the N^{α}-Fmoc group of **30** was cleaved using piperidine (20%) in DMF (6 ml) to obtain **31**. Palmitic acid (77 mg, 0.3 mmol) was coupled with the free amine as described above using PyBOP (156 mg, 0.3 mmol), HOBt (40 mg, 0.3 mmol) and DIPEA (67 µl, 0.4 mmol) in DMF to afford **32**. The resin was thoroughly washed with DMF (2 x 5 ml), DCM (2 x 5 ml) and MeOH (2 x 5 ml) and then dried *in vacuo* to constant weight. The resin was then swelled in DCM (5 ml) for 1 h, after which it was 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 peptide was then precipitated using diethyl ether (0 ^{o}C) and recovered by centrifugation at 3000 rpm for 15 min. The crude lipopeptide was purified by RP-HPLC on a semi-preparative C-4 reversed phase 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 **9** (65% based on resin loading capacity). $C_{81}H_{156}N_{11}O_{12}S$, MALDI-ToF MS: observed [M+Na],1531.2430Da; calculated [M+Na], 1531.1734Da.

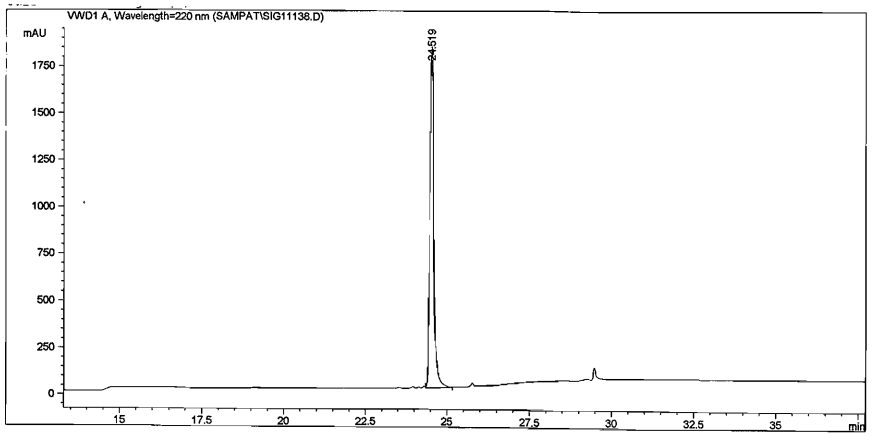


Supplementary Scheme 6 Reagents and conditions: a) SPPS using N^{α}-Fmoc-chemistry, coupling with HBTU/HOBt in the presence of DIPEA in NMP followed by Fmoc cleavage with 20% piperidine in DMF; b) manual coupling of **4**, PyBOP, HOBt in the presence of DIPEA in DMF; c) piperidine (20%) in DMF; d) coupling of palmitic acid, PyBOP, HOBt in the presence of DIPEA in DMF; e) TFA (95%), H_2O (2.5%), TIS (2.5%), 2 h.



9

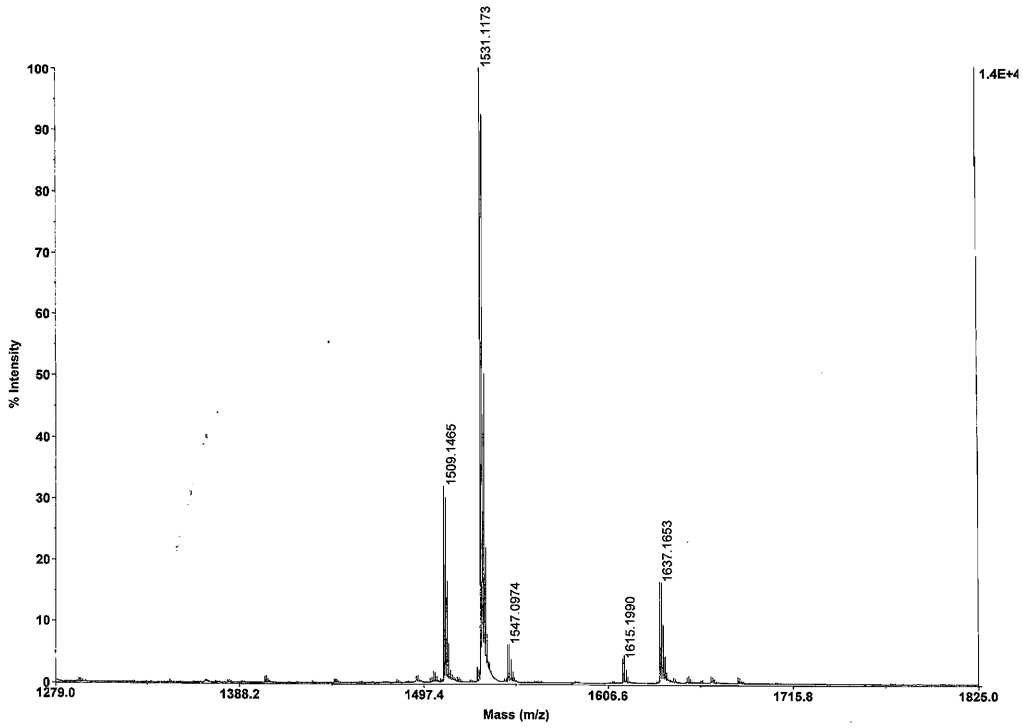
HPLC chromatogram:



Column: Semi-prep. C4 Reversed phase

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

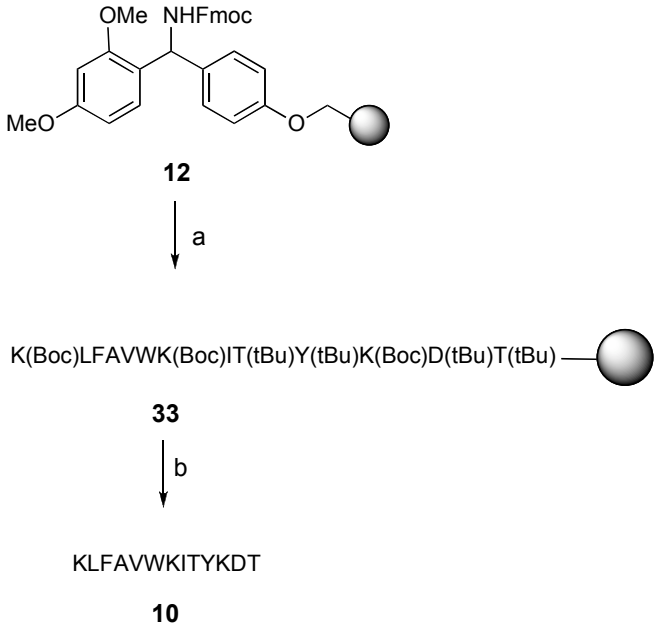
MALDI-ToF spectra:



Observed, [M+Na], 1531.1173Da

Calculated, [M+Na], 1531.2368Da

Chemical synthesis of compound 10. The synthesis of **10** was carried out on a Rink amide AM resin (**12**; 0.1 mmol) according to the general methods for SPPS. After completion of the synthesis, resin **33** was thoroughly washed with DMF (2 x 5 ml), DCM (2 x 5 ml) and MeOH (2 x 5 ml) and then dried *in vacuo* to constant weight. The resin was then swelled in DCM (5 ml) for 1 h, after which it was treated with TFA (88%), water (5%), phenol (5%) and TIS (2%) (30 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 peptide was then precipitated using diethyl ether (0 ^{o}C) and recovered by centrifugation at 3000 rpm for 15 min. The crude lipopeptide was purified by RP-HPLC on a semi-preparative C-8 reversed phase 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 **10** (60% based on resin loading capacity). $C_{79}H_{122}N_{18}O_8$, MALDI-ToF MS: observed [M+],1611.9182Da; calculated [M+], 1611.9238Da.

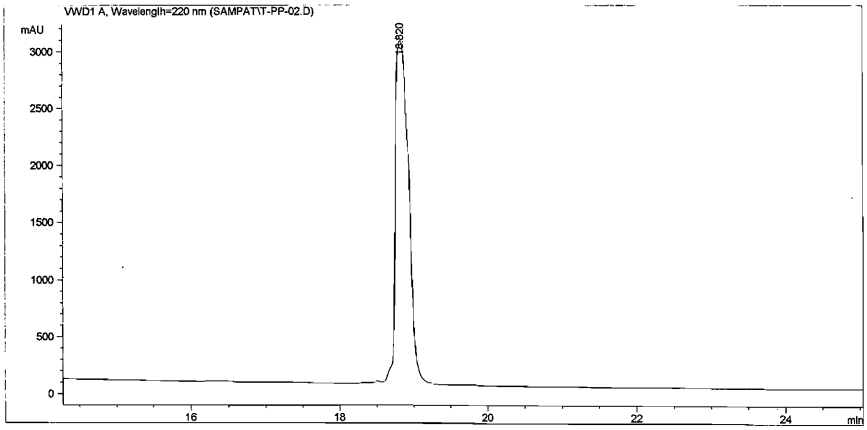


Supplementary Scheme 7 Reagents and conditions: a) SPPS using N^{α}-Fmoc-chemistry, coupling with HBTU/HOBt in the presence of DIPEA in NMP followed by Fmoc cleavage with 20% piperidine in DMF; b) reagent B (TFA (88%), phenol (5%), H_2O (5%), TIS (2%)), 2 h.

KLFAVWKITYKDT

10

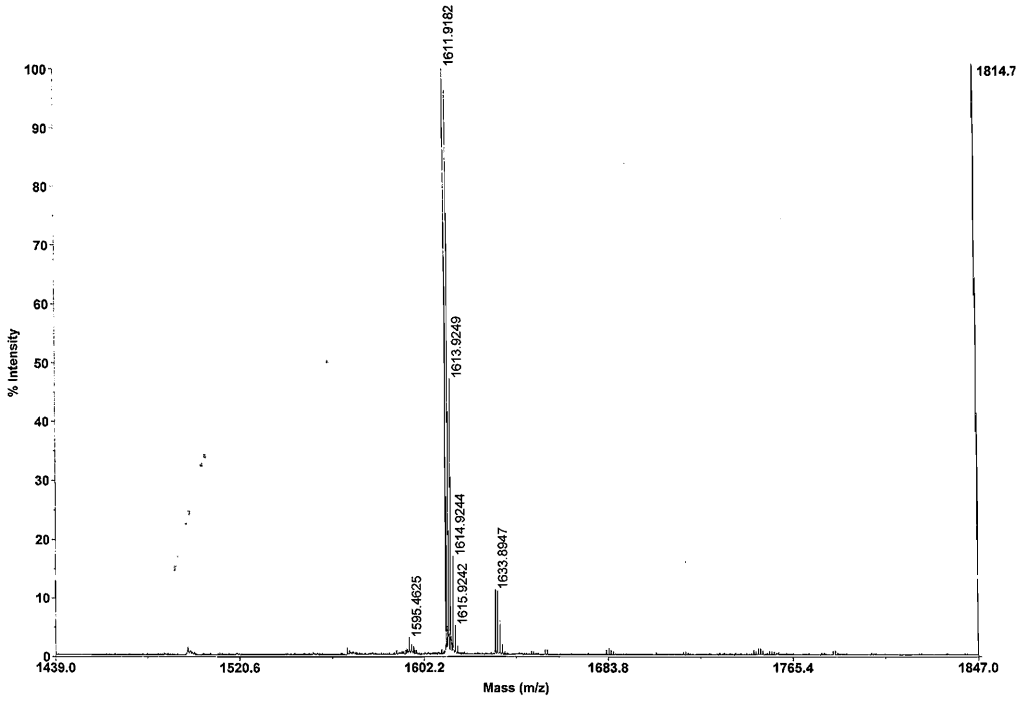
HPLC chromatogram:



Column: Semi-prep. C8 Reversed phase

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

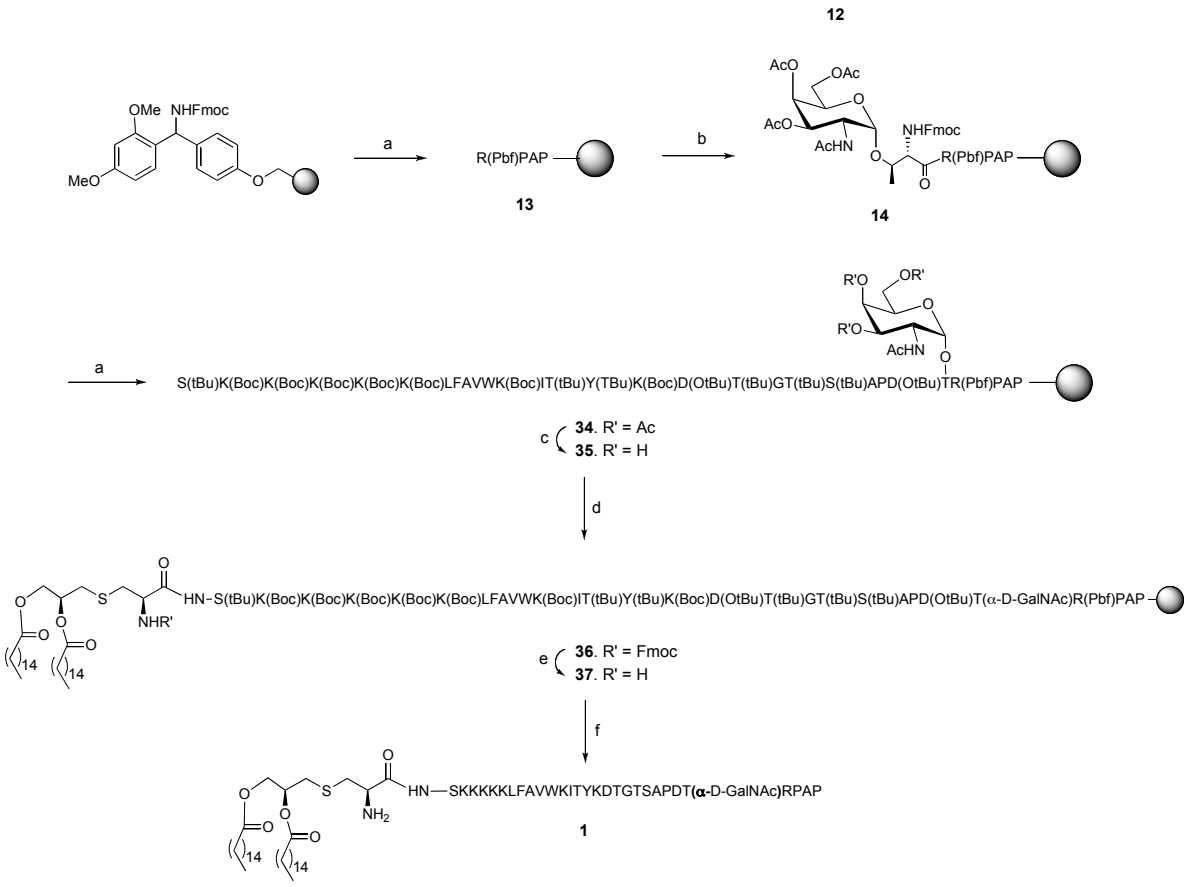
MALDI-ToF spectra:



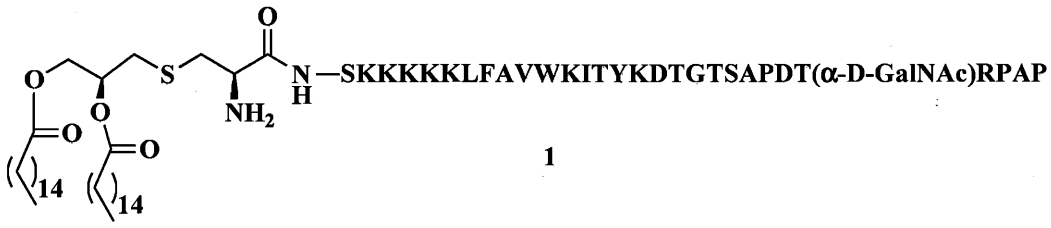
Observed, [M+], 1611.9182Da

Calculated, [M+], 1611.9238Da

Chemical synthesis of compound 1. The synthesis of **1** was carried out on a Rink amide AM resin (**12**; 0.1 mmol) according to the general methods for SPPS. The first four amino acids were coupled on the peptide synthesizer using a standard protocol to obtain **13** followed by manual coupling of **3** (0.4 mmol, 268 mg) using HATU (0.4 mmol, 152 mg), HOAt (0.4 mmol, 55 mg) and DIPEA (0.4 mmol, 70 μl) in DMF for 12 h. The coupling reaction was monitored by standard Kaiser test. The resin was washed with DMF (6 ml) and DCM (6 ml) and resubjected to the same coupling conditions to ensure complete coupling. The glycopeptide **14** was then elongated on the peptide synthesizer to obtain **34**. The resin was thoroughly washed with DMF (6 ml), DCM (6 ml) and MeOH (6 ml) and dried *in vacuo* to constant weight. The resin was then swelled in DCM (5 ml) for 1 h, after which **34** was treated with hydrazine (80%) in MeOH (10 ml) for 1 h. This procedure was repeated twice for 30 min to ensure complete deprotection of the acetyl moieties of the sugar residue to afford **35**. The resin was then thoroughly washed with DMF (4 x 5 ml), DCM (4 x 5 ml) and MeOH (4 x 5 ml) and then dried *in vacuo* to constant weight. The resin was swelled in DCM (5 ml) for 1 h and a mixture of **4** (267 mg, 0.3 mmol), PyBOP (156 mg, 0.3 mmol), HOBt (40 mg, 0.3 mmol) and DIPEA (67 μl, 0.4 mmol) in DMF (5 ml) was added. The coupling reaction was monitored by the Kaiser test. Upon completion of the coupling, the N^{α}-Fmoc group of **36** was cleaved by piperidine (20%) in DMF (6 ml) to obtain **37**. The resin was thoroughly washed with DMF (2 x 5 ml), DCM (2 x 5 ml) and MeOH (2 x 5 ml) and then dried *in vacuo* to constant weight. The resin was then swelled in DCM (5 ml) for 1 h, after which it was treated with TFA (88%), water (5%), phenol (5%) and TIS (2.5%) (10 ml) for 2 h at room temperature. The resin was filtered off and washed with neat TFA (2 ml). The combined filtrates were concentrated *in vacuo* to approximately 1/3 of its original volume. The peptide was then precipitated by the addition of diethyl ether (0 ^{o}C) and recovered by centrifugation at 3000 rpm for 15 min. The crude glycolipopeptide was purified by RP-HPLC on a semi-preparative C-4 reversed phase 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 **1** (30% based on resin loading capacity). $C_{196}H_{328}N_{43}O_{50}S$, MALDI-ToF MS: observed [M+], 4120.3638Da; calculated [M+], 4119.0246Da.

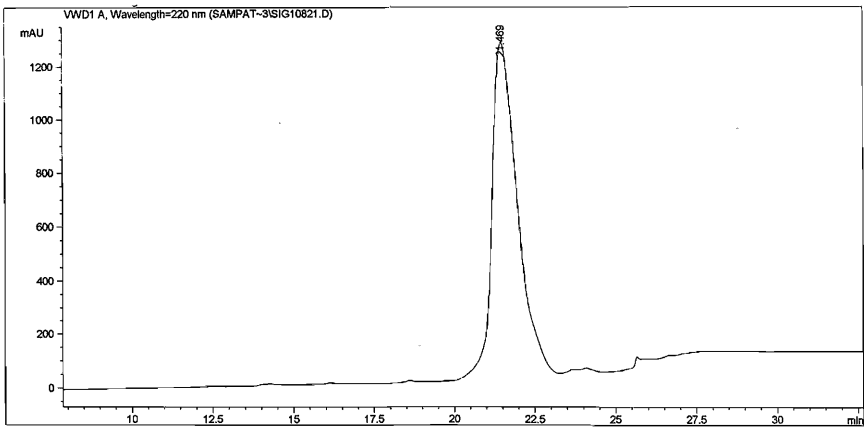


Supplementary Scheme 8 Reagents and conditions: a) SPPS using N^{α}-Fmoc-chemistry, coupling with HBTU/HOBt in the presence of DIPEA in NMP followed by Fmoc cleavage with 20% piperidine in DMF; b) **3**, HATU/HOAt, DIPEA, DMF, 12 h; c) hydrazine (80%) in MeOH, 2 h; d) manual coupling of **4**, PyBOP, HOBt in the presence of DIPEA in DMF; e) piperidine (20%) in DMF; f) reagent B (TFA (88%), phenol (5%), H_2O (5%), TIS (2%)), 2 h.



1

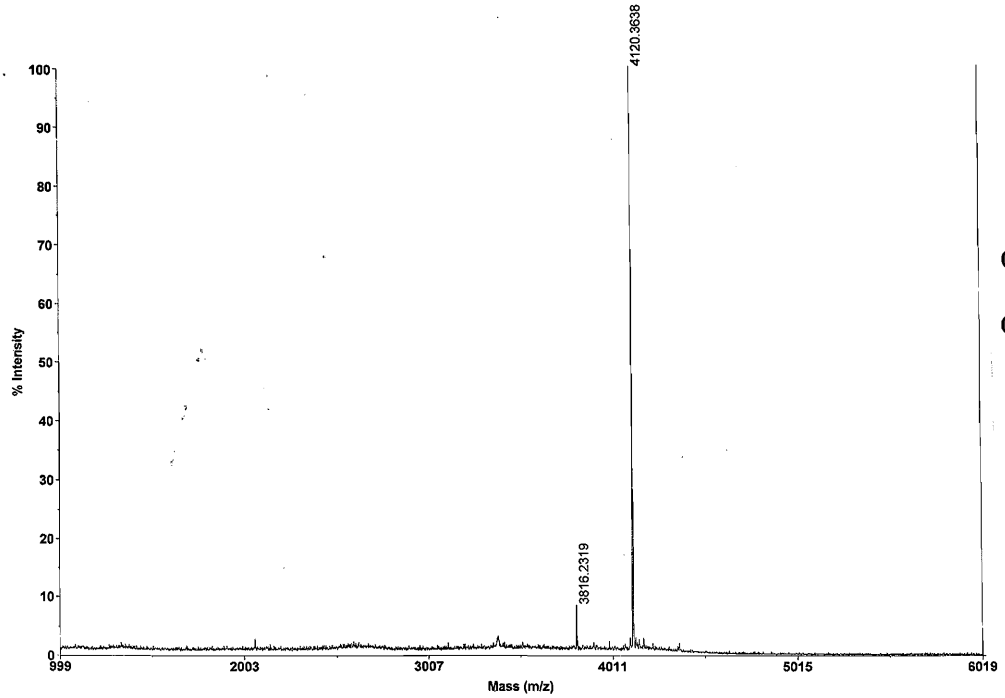
HPLC chromatogram:



Column: Semi-prep. C4 Reversed phase

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

MALDI-ToF spectra:

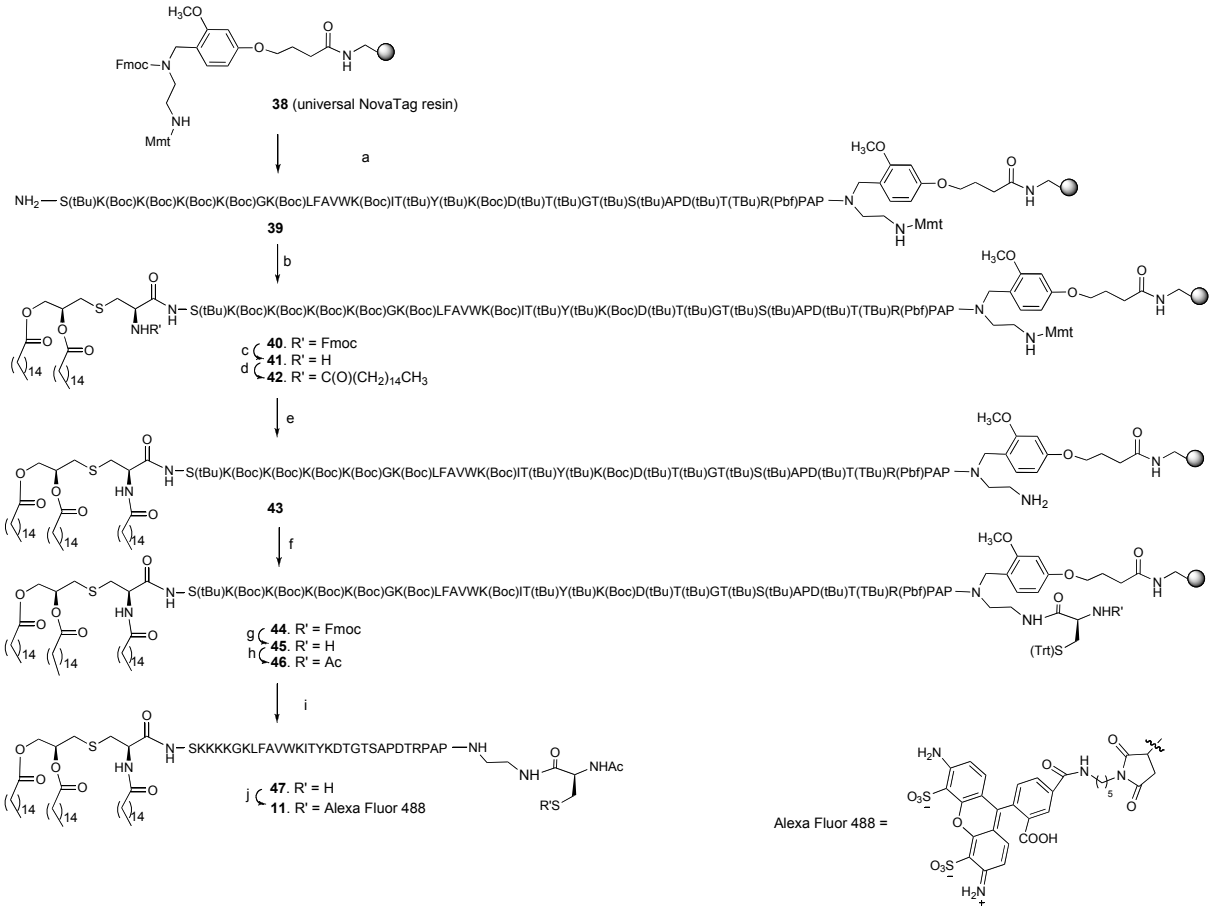


Observed, [M+], 4120.3638Da

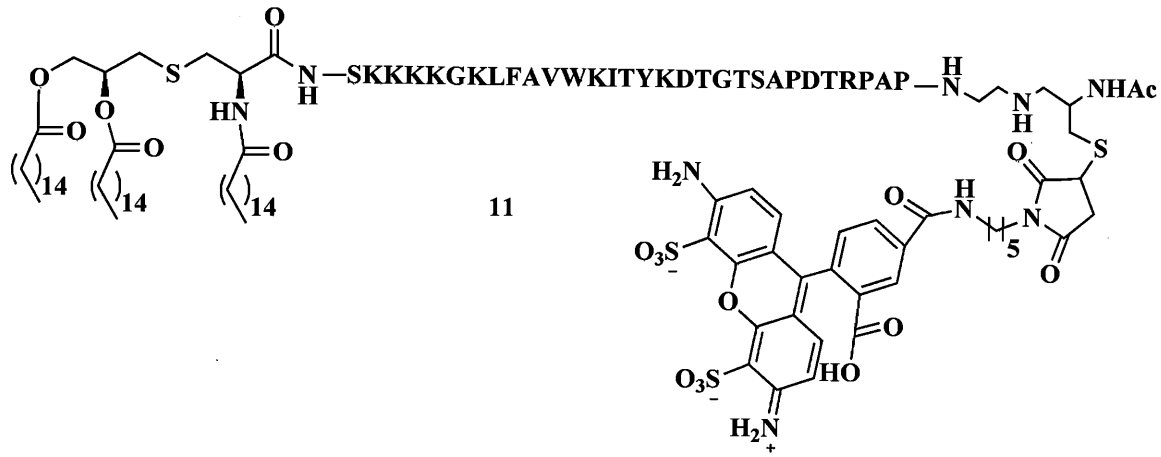
Calculated, [M+], 4119.0246Da

Chemical synthesis of compound 11. The synthesis of **11** was carried out on universal NovaTag resin (**38**; 0.1 mmol) as described in the general methods. After coupling of the amino acids to obtain **39**, the remaining steps were performed manually. Thus, compound **4** (267 mg, 0.3 mmol) was dissolved in DMF (5 ml) and PyBOP (156 mg, 0.3 mmol), HOBt (40 mg, 0.3 mmol) and DIPEA (67 μl, 0.4 mmol) were added. After premixing for 2 min, the mixture was added to the resin. The coupling reaction was monitored by the Kaiser test. Upon completion of the coupling, the N^{α}-Fmoc group of **40** was cleaved using piperidine (20%) in DMF (6 ml) to give **41**. Palmitic acid (77 mg, 0.3 mmol) was coupled to the free amine of **41** as described above using PyBOP (156 mg, 0.3 mmol), HOBt (40 mg, 0.3 mmol) and DIPEA (67 μl, 0.4 mmol) in DMF to afford **42**. The resin was thoroughly washed with DMF (10 ml) and DCM (10 ml). The pendant Mmt group of **42** was then removed by treatment of HOBt (1 M) in TFE/DCM (10 ml) for 2 h to obtain **43**. N^{α}-Fmoc-Cys(Trt)-OH (175.8 mg, 0.3 mmol) was coupled to the free amine of **43** using PyBOP (156 mg, 0.3 mmol), HOBt (40 mg, 0.3 mmol) and DIPEA (67 μl, 0.4 mmol) in DMF to give **44**. Upon completion of the coupling, the N^{α}-Fmoc group was cleaved using piperidine (20%) to give **45** and the free amino group was acelyated using acetic anhydride (Ac_2O; 10%), DPIEA (5%) in NMP (10 ml) for 30 min to obtain **46**. The lipopeptide was then cleaved from the resin by treatment of TFA (94%), EDT (2.5%), H_2O (2.5%), TIS (1%) (10 ml) for 4 h at room temperature. The resin was filtered and washed with neat TFA (2 ml). The filtrate was then concentrated *in vacuo* to approximately one third of its original volume. The peptide was then precipitated using diethyl ether (0 ^{o}C) and recovered by centrifugation at 3000 rpm for 15 min. The crude lipopeptide was purified by HPLC on a semi-preparative C-4 reversed phase column using a linear gradient of 0 to 95% solvent B (acetonitrile (95%), water (5%), TFA (0.01%)) in solvent A (water (95%), acetonitrile (5%), TFA (0.01%)) over a 40 min period and the appropriate fractions were lyophilized to give **47** (25% based on resin loading capacity). MALDI-ToF MS: observed [M+], 4341.4347Da; calculated [M+], 4342.6288Da. The lipopeptide **47** (0.5 mg, 0.11 μmol) was then treated with Alexa Fluor 488 C5 maleimide (0.4 mg, 0.55 μmol) in the presence of catalytic amount of TCEP and DIPEA in DMSO (1 ml) at room temperature for 12 h. The crude reaction mixture was then purified by RP-HPLC on a semi-preparative C-4 reversed phase column using a linear gradient of 0 to 95% solvent B (acetonitrile (95%), water (5%), TFA (0.01%)) in solvent A (water (95%), acetonitrile (5%),

TFA (0.01%)) over a 40 min period and the appropriate fractions were lyophilized to afford **11** (0.4 mg, 68%). MALDI-ToF MS: observed [M+], 5040.12Da; calculated [M+] 5039.76Da.

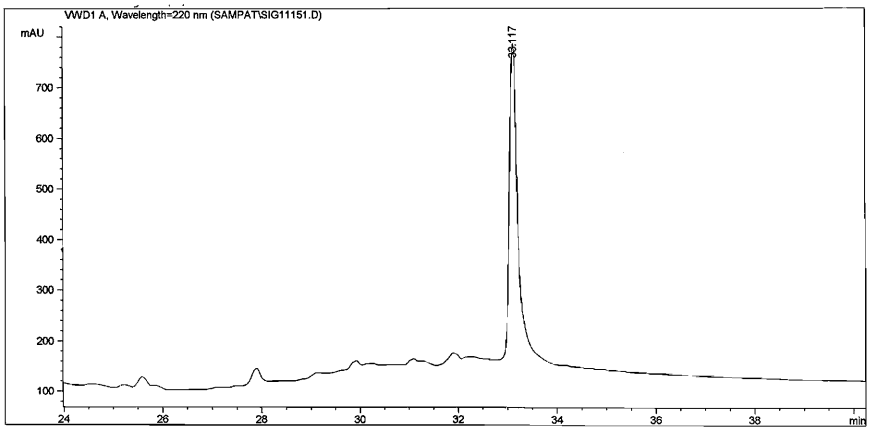


Supplementary Scheme 9 Reagents and conditions: a) SPPS using N^{α}-Fmoc-chemistry, coupling with HBTU/HOBt in the presence of DIPEA in NMP followed by Fmoc cleavage with 20% piperidine in DMF; b) manual coupling of **4**, PyBOP, HOBt in the presence of DIPEA in DMF; c) piperidine (20%) in DMF; d) coupling of palmitic acid, PyBOP, HOBt in the presence of DIPEA in DMF; e) HOBt (1M) in TFE/DCM, 2 h; f) N^{α}-Fmoc-Cys(Trt)-OH, PyBOP, HOBt in the presence of DIPEA in DMF, 4 h; g) piperidine (20%) in DMF; h) Ac_2O (10%), DIPEA (5%) in NMP, 30 min; i) TFA (94%), EDT (2.5%), H_2O (2.5%), TIS (1%), 4 h; j) Alexa Fluor® 488 C5 maleimide ester, TCEP, DIPEA in DMSO, RT, 12 h.



11

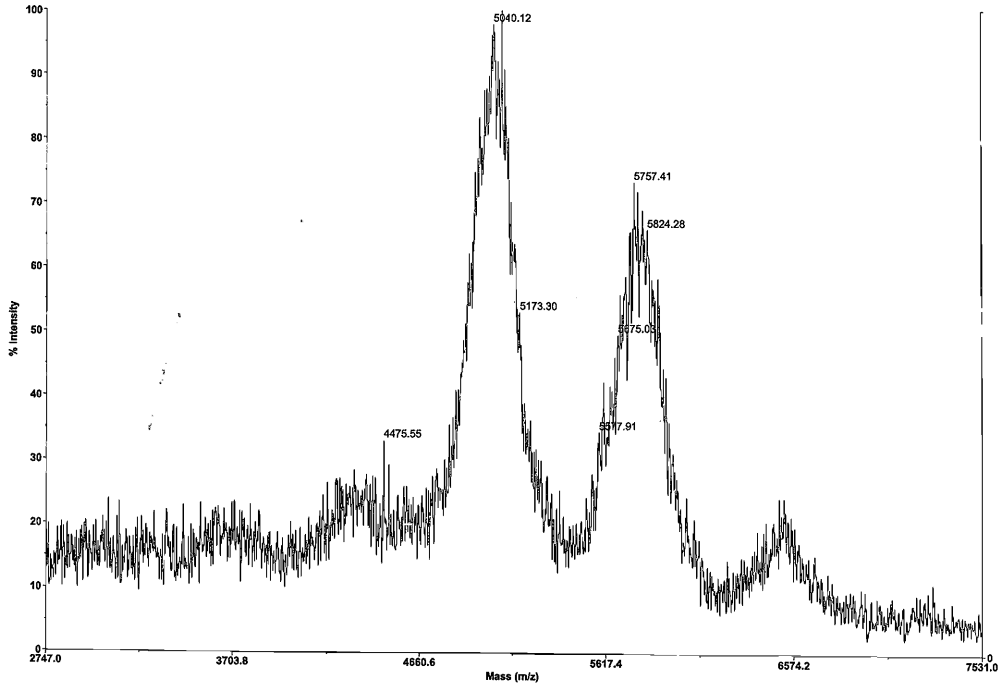
HPLC chromatogram:



Column: Analytical C8 Reversed phase

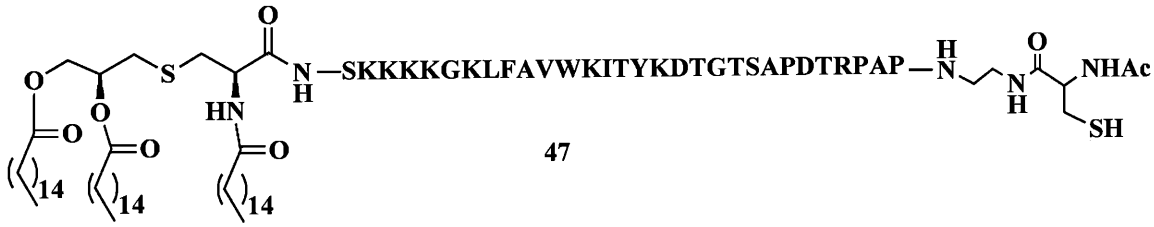
Eluent: 0-95% of Solvent B in A over period of 40 min

MALDI-ToF spectra:



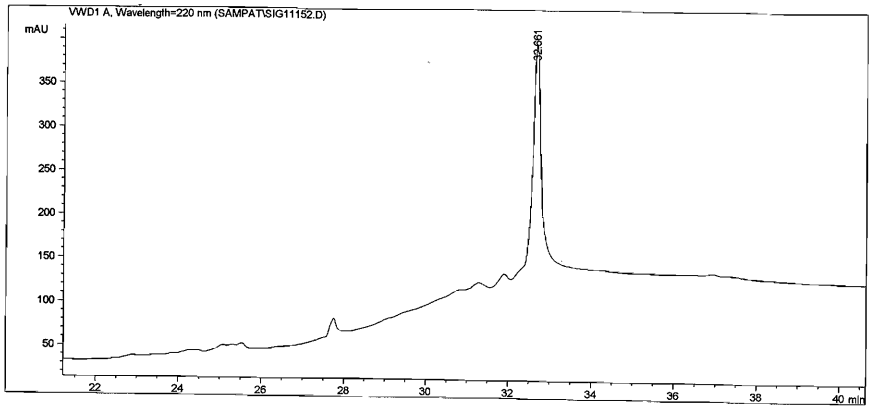
Observed, [M+], 5040.12Da

Calculated, [M+], 5041.1712Da



47

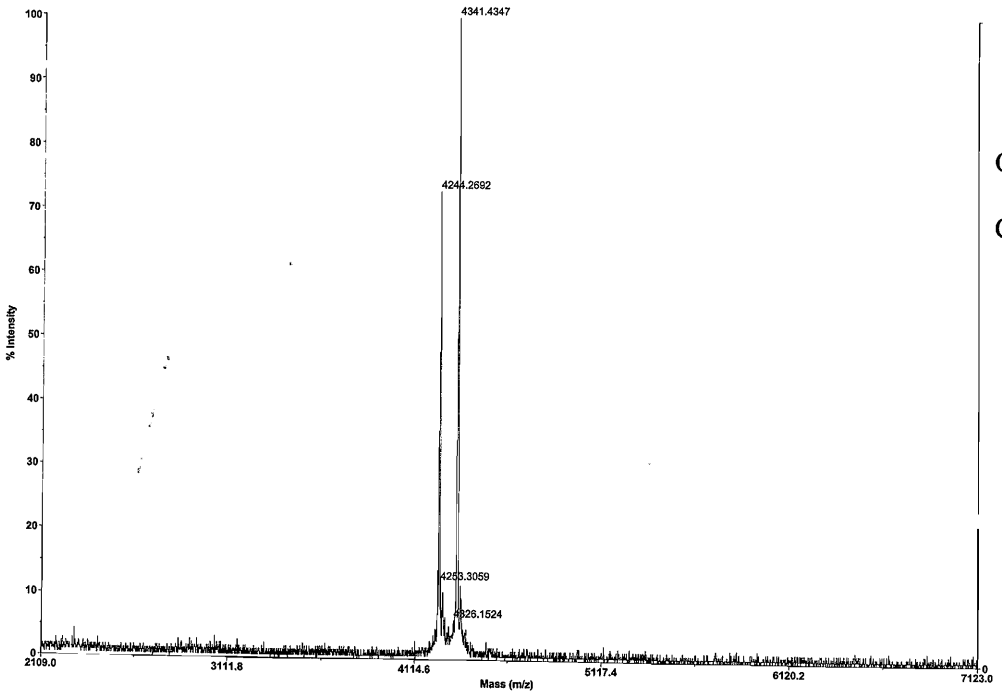
HPLC chromatogram:



Column: Analytical C4 Reversed phase

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

MALDI-ToF spectra:



Observed, [M+], 4341.4347Da

Calculated, [M+], 4342.4920Da

Cell culture. RAW 264.7 γNO(-) cells, derived from the RAW 264.7 mouse monocyte/macrophage cell line, were obtained from ATCC. The cells were maintained in RPMI 1640 medium with L-glutamine (2 mM), adjusted to contain sodium bicarbonate (1.5 g l^{-1}), glucose (4.5 g l^{-1}), HEPES (10 mM) and sodium pyruvate (1.0 mM) and supplemented with penicillin (100 u ml^{-1}) / streptomycin (100 μg ml^{-1}; Mediatech) and FBS (10%; Hyclone). The human embryonic kidney cell line (HEK 293T), obtained from ATCC, was grown in Dulbecco's modified Eagle's medium (ATCC) with L-glutamine (4 mM), glucose (4.5 g l^{-1}) and sodium bicarbonate (1.5 g l^{-1}) supplemented with penicillin (100 u ml^{-1}) / streptomycin (100 μg ml^{-1}; Mediatech), Normocin (100 μg ml^{-1}; InvivoGen) and FBS (10%). HEK293T cells, stable transfected with mouse TLR4/MD2/CD14, mouse TLR2 or mouse TLR2 and TLR6, were obtained from InvivoGen and grown in the same growth medium as for HEK 293T cells supplemented with blasticidin (10 μg ml^{-1}) and for the HEK w/ hTLR4/MD2/CD14 also with HygroGold (50 μg ml^{-1}). Human breast adenocarcinoma cells (MCF7)[S6], obtained from ATCC, were cultured in Eagle's minimum essential medium with L-glutamine (2 mM) and Earle's BSS, modified to contain sodium bicarbonate (1.5 g l^{-1}), non-essential amino acids (0.1 mM) and sodium pyruvate (1 mM) and supplemented with bovine insulin (0.01 mg ml^{-1}; Sigma) and FBS (10%). Human skin malignant melanoma cells (SK-MEL-28) were obtained from ATCC and grown in Eagle's minimum essential medium with L-glutamine (2 mM) and Earle's BSS, adjusted to contain sodium bicarbonate (1.5 g l^{-1}), non-essential amino acids (0.1 mM) and sodium pyruvate (1 mM) and supplemented with FBS (10%). Freshly isolated human monocytes were cultured in R-5-RPMI 1640 (Invitrogen) supplemented with heat-inactivated FBS (5%; Invitrogen), sodium pyruvate (1%; Sigma), 2-mercaptoethanol (50 μM; Invitrogen), HEPES buffer (1 M; Sigma) and gentamicin (50 μg ml^{-1}; Invitrogen). All cells were maintained in a humid 5% CO_2 atmosphere at 37 ^{o}C.

S6. Horwitz, K. B., Costlow, M. E. & McGuire, W. L. MCF-7; a human breast cancer cell line with estrogen, androgen, progesterone and glucocorticoid receptors. *Steroids* **26**, 785-795 (1975).

TNF-α assay. RAW 264.7 γNO(-) cells were plated on the day of the exposure assay as 2 x 10^5 cells/well in 96-well plates (Nunc) and incubated with different stimuli for 5.5 h in the presence or absence of polymyxin B. Culture supernatants were collected and stored frozen (–80 ^{o}C) until assayed for cytokine production. Concentrations of TNF-α were determined using the TNF-α DuoSet ELISA Development kit from R&D Systems. Concentration-response data were

analyzed using nonlinear least-squares curve fitting in Prism (GraphPad Software, Inc.). These data were fit with the following four parameter logistic equation: $Y = E_{max} / (1 + (EC_{50}/X)^{Hill\ slope})$, where Y is the TNF-α response, X is the concentration of the stimulus, E_{max} is the maximum response and EC_{50} is the concentration of the stimulus producing 50% stimulation. The Hill slope was set at 1 to be able to compare the EC_{50} values of the different inducers. All TNF-α values are presented as the means ± s.d. of triplicate cultures, with each experiment being repeated three times.

Evaluation of materials for contamination by LPS. To ensure that any increase in cytokine production was not caused by LPS contamination of the solutions containing the various stimuli, the experiments were performed in the absence and presence of polymyxin B, an antibiotic that avidly binds to the lipid A region of LPS, thereby preventing LPS-induced cytokine production[S7]. Cytokine concentrations in supernatants of cells preincubated with polymyxin B (30 μg ml^{-1}; Bedford Laboratories) for 30 minutes before incubation with *E. coli* O55:B5 LPS for 5.5 h showed complete inhibition of cytokine production, whereas preincubation with polymyxin B had no effect on cytokine synthesis by cells incubated with the synthetic compounds **1**, **2**, Pam_2CysSK_4 and **9**. Therefore, LPS contamination of the latter preparations was inconsequential.

S7. Tsubery, H., Ofek, I., Cohen, S. & Fridkin, M. The functional association of polymyxin B with bacterial lipopolysaccharide is stereospecific: studies on polymyxin B nonapeptide. *Biochemistry* **39**, 11837-11844 (2000).

Dendritic cell maturation. Monocytes were set-up on day 1 and were allowed to enrich for 3 days. On day 4 the monocyte-derived dendritic cells (MDDCs) were exposed to LPS (1 μg ml^{-1}), PBS and compounds **1**, **2**, Pam_2CysSK_4 and **9** (1 μg ml^{-1} each) for 16-24 h. Next, cells were washed and incubated with the following monoclonal antibodies: anti-CD80 conjugated to R-phycoerythrin (PE; BD BioSciences), anti-CD83 conjugated to fluorescein isothiocyanate (FITC; BD BioSciences) and anti-CD86 conjugated to PE (BD BioSciences) for 30 min. Cells were washed, fixed and analyzed by flow cytometry.

Transfection and NF-κB activation assay. The day before transfection, HEK293T cells and stable transfected HEK293T cells with murine TLR2, TLR2/TLR6 or TLR4/MD2 were plated in

96-well cell culture plates to reach the next day approximately 60% confluency. Cells were transiently transfected with expression plasmids using PolyFect Transfection Reagent (Qiagen). Briefly, HEK293T cells and stable transfected HEK293T cells with TLR2, TLR2/TLR6 or TLR4/MD2 were transfected with a NF-κB-dependent firefly luciferase reporter plasmid (pELAM-Luc; 50 ng/well)[S8] and a *Renilla* luciferase control reporter vector (pRL-TK; 1 ng/well; Promega) as an internal control to normalize results. The empty vector pcDNA3 (Invitrogen) was used as a control and to normalize the DNA concentration for all of the transfection reactions (total DNA 70 ng/well). Forty-four h post-transfection, cells were exposed to the stimuli at the indicated concentrations for 4 h, after which cell extracts were prepared. The luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega) and the Fluoroskan Accent FL combination luminometer/fluorometer (Thermo Electron Corporation). Expression of the firefly luciferase reporter gene was normalized for transfection efficiency with expression of *Renilla* luciferase. The data are reported as the means $\pm$ s.d. of triplicate treatments. The transfection experiments were repeated at least twice.

S8. Chow, J. C., Young, D. W., Golenbock, D. T., Christ, W. J. & Gusovsky, F. Toll-like receptor 4 mediates lipopolysaccharide-induced signal transduction. *J. Biol. Chem.* **274**, 10689-10692 (1999).