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Development of highly pure α-helical lipoglycopeptides as self-adjuvanting vaccines

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ABSTRACT

The incorporation of lipid moieties into synthetic peptide vaccines has been demonstrated to self-adjuvant otherwise poorly immunogenic peptides, whereas carbohydrates have emerged to be advantageous carriers for assembling these peptides. With the advent of an efficient native chemical ligation method, which is compatible with both peptides and carbohydrates, we have developed highly pure self-adjuvanting tetravalent group A streptococcal vaccine candidates assembled on carbohydrate templates. The utility of chemoselective ligation has overcome difficulties in the synthesis and purification of branched high molecular weight peptides. Circular dichroism measurements provided the evidence of α-helix formation of the assembled peptide epitopes, which may have impact on their immunogenicity.

1. Introduction

Vaccination, which has the potential to eradicate diseases, has proved to be one of the most cost-effective medical interventions. Problems associated with conventional vaccines, such as instability, risks of infection, and autoimmunity, as well as manufacturing difficulties, have called for the development of safe and effective synthetic vaccines that contain minimal and optimal antigenic determinant(s) rather than the whole pathogen. The major limitation to the development of such vaccines has been the poor immunogenicity and the consequent necessity of co-administration with effective adjuvants.[1,2] Many powerful experimental adjuvants, such as complete Freund's adjuvant, are toxic and not suitable for human use. Furthermore, in most cases, antigen fragments need to be conjugated to or mixed with protein carriers to adopt T-helper epitopes, which are necessary for stimulating immune responses in outbreed human populations.[1,3] Commonly used protein carriers (e.g., bovine serum albumin, keyhole limpet hemocyanin, and tetanus toxoid) present significant problems including autoreactivity and epitopic suppression.[4–6] These problems have resulted in significant interest in the development of potent and safe adjuvants to overcome the very limited choice of adjuvant for human vaccination (mainly aluminum-based), and inert small molecular carriers to minimize unfavorable effects on epitopic specificity.

A promising 'one entity' strategy, lipid core peptide (LCP) system,[7] has been developed based on the principle of lipopeptide vaccines, which have emerged as a potential approach for human vaccination.[2,3] The LCP delivery system features a non-microbial lipopeptide adjuvant (synthetic lipidic amino acids and glycine spacers) and a polylysine-based multiple antigenic peptide (MAP)[8] dendrimer. The physiochemical properties of the LCP system may be readily altered to improve vaccine efficacy. This includes changing alkyl side-chain length or the number of lipoamino acids, conjugation of same or different epitopes, or the use of different carriers. One of the de novo designs of peptide immunogens involves the application of a topological template predetermining tertiary structure of the conjugated peptide epitopes. Carbohydrates have emerged as suitable templates[9–12] due to (i) the multiple hydroxyl groups allowing for the attachment of several copies of an epitope or possibly different epitopes, (ii) the rigid ring directing antigens in a three-dimensional space, and (iii) the ready availability of different carbohydrate entities facilitating an optimal orientation for antigen recognition. Thus, adaptation of the carbohydrate core in the LCP system, to produce conformation-defined constructs, may have important implications for the development of synthetic vaccines.

The LCP system was employed to develop numerous experimental vaccine candidates against group A streptococcus (GAS), which is believed to be responsible for a range of common health problems (e.g., sore throat) and even fatal diseases (e.g., rheumatic fever).[13] Previous studies have shown that LCP vaccine candidates synthesized using stepwise solid phase peptide synthesis (SPPS) generally elicited high serum IgG antibody titers against the incorporated epitope(s) without the need for additional adjuvants.[14–17] To produce highly pure polylysine-based LCP vaccines, native chemical ligation (NCL) has been successfully used.[18–21]

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In the current study, NCL was utilized to prepare highly pure carbohydrate-based LCP vaccine candidates. To possibly optimize the vaccine efficacy by altering the carbohydrate types, three different monosaccharides (D-glucose, D-galactose, and D-mannose) were functionalized, incorporated to a lipopeptide, and conjugated with four copies of B-cell peptide epitopes. In this project, a GAS peptide epitope derived from the N-terminal region of the major virulence factor (M protein) of GAS 8830 strain was selected as a model epitope. However, other antigens can be incorporated into this carbohydrate LCP system to produce vaccine candidates against other diseases. Comparison of conformational properties of these carbohydrate-based LCP vaccines was carried out by means of circular dichroism (CD) spectroscopy.

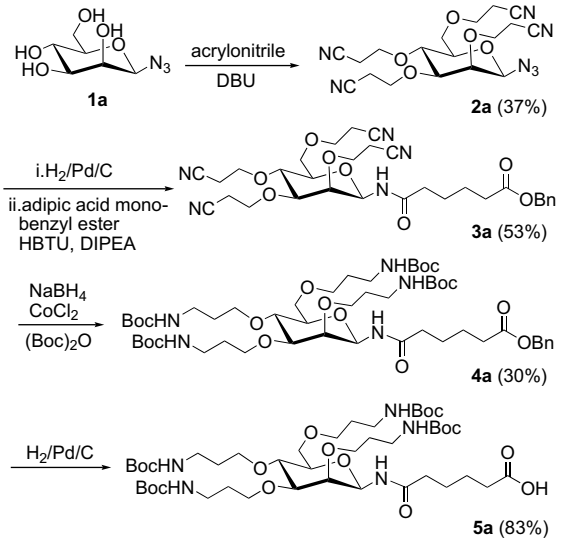
2. Results and discussion

2.1. Synthetic strategy

The designed vaccine candidates, which individually incorporate a lipopeptide (C12–G–C12–C12–G; C12: 2-(*R*/*S*)-aminododecanoic acid[22]), a carbohydrate template, and four copies of 8830 peptide antigen (DNGKAIYERARERALQELGP),[23] were synthesized using NCL, which allows the branched lipopeptide–carbohydrate–antigen to be generated from two building blocks by a single ligation reaction. NCL is a chemoselective technique of coupling a C-terminal peptide thioester and an N-terminal cysteine residue at physiological pH in the presence of an added thiol catalyst. As there is no cysteine residue in the 8830 sequence, the carbohydrate templates were individually equipped with four N-terminal cysteine residues and the 8830 peptide was synthesized as a thioester.

2.2. Synthesis of Cys-containing lipopeptide–carbohydrate templates

β-D-Mannopyranosyl azide **1a**[24] was cyanoethylated with acrylonitrile in the presence of 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) to give tetranitrile **2a** in 37% yield. Reduction of the azido group of **2a** was achieved by catalytic hydrogenation, and the corresponding amine was reacted directly with adipic acid monobenzyl ester[25] using *O*-benzotriazole-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HBTU)/*N*,*N*-diisopropylethylamine (DIPEA) activation method to afford **3a** in 53% overall yield. The nitrile groups of **3a** were reduced to primary amines using sodium borohydride and cobalt(II) chloride[26] and in situ converted to Boc-protected amines in the presence of di-*tert*-butyl dicarbonate (Boc_2O), giving compound **4a** in 30% yield. Following removal of the benzyl group by catalytic hydrogenation, compound **5a** was obtained in 83% yield (Scheme 1). The glucose derivative **5b** and the galactose derivative **5c** were synthesized according to reported procedures.[13,27]

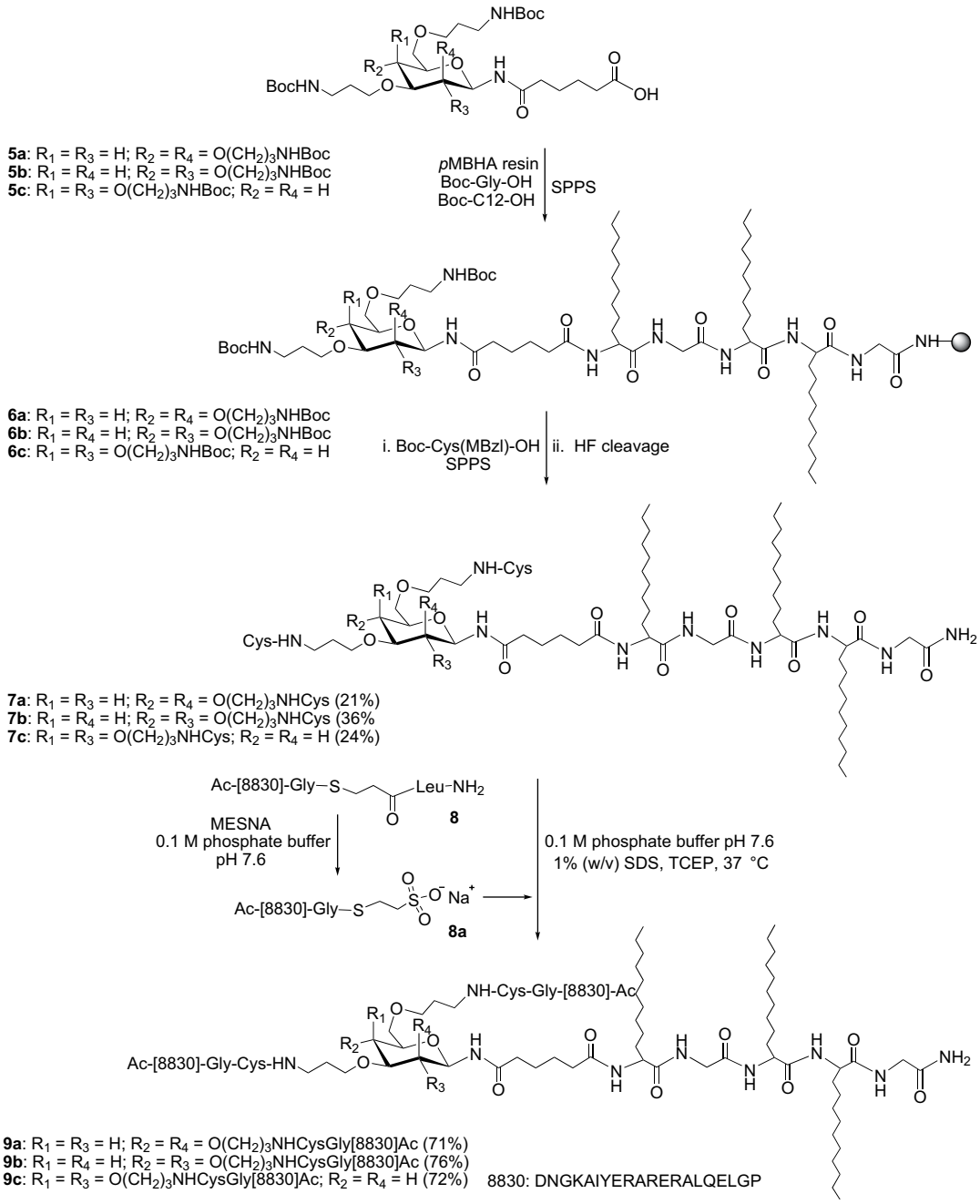


Scheme 1. Synthesis of carbohydrate template **5a**.

The conjugation of lipopeptide and cysteines to compound **5a**, **5b** or **5c** is shown in Scheme 2. Glycine and aminododecanoic acid (C12) residues were sequentially (C12–G–C12–C12–G) attached to *p*-methylbenzhydrylamine (*p*-MBHA) resin using Boc-based SPPS according to previously reported procedures.[28] To improve the coupling efficiency, either the bulky lipoamino acids were pre-activated for 5 min or the reaction was performed in the presence of *N*-methyl pyrrolidinone. To minimize the consumption of the synthesized carbohydrate templates, a modified version of in situ neutralization protocol[29] was utilized in the coupling of **5a**, **5b** or **5c** to the C12–G–C12–C12–G–resin. This modification involved using 2 equiv (instead of 4 equiv) of the acid (**5a**, **5b** or **5c**) and a longer coupling time (6 h instead of 10 min). The obtained peptidyl resin **6a**, **6b** or **6c** was Boc-deprotected using trifluoroacetic acid (TFA) and four copies of the Cys residue were coupled to the amine moieties of the compound to give resin-bound **7a**, **7b**, and **7c**. Cleavage of peptide **7a**, **7b**, and **7c** from the resin was accomplished using standard hydrofluoric acid (HF)/scavenger conditions. Following purification by preparative RP-HPLC, **7a**, **7b**, and **7c** were obtained in 21%, 36%, and 24% yield, respectively.

2.3. Chemical ligation

Given the high hydrophobicity of the Cys-containing lipopeptide–carbohydrate templates and the high hydrophilicity of the peptide thioester, reaction conditions needed to be identified under which both building blocks could be maintained in the ligation buffer throughout the course of reaction, thus allowing an efficient NCL. Published successful conditions involve the addition of organic co-solvents,[30–32] liposomes,[33] detergents,[34] and lipid bilayers.[35] However, little success was reported for the ligations of hydrophobic lipopeptide constructs and hydrophilic peptide thioesters when organic co-solvents such as isopropyl alcohol, acetonitrile (ACN), dimethyl sulfoxide (DMSO), *N*,*N*-dimethylformamide (DMF), trifluoroethanol or dioxane were used.[18,19,21] The failure of those reactions was most likely due to the very poor water solubility and long lipid chains of the lipopeptide, which may have hampered the Cys residue from reacting with the thioester. In contrast, the use of sodium dodecyl sulfate (SDS) was demonstrated to be a successful alternative,[18,19,21] presumably due to the lipid part entering SDS micelles, and leaving the Cys residue faced toward the aqueous solution. It was envisaged that the presence of SDS would facilitate ligation of the lipopeptide–carbohydrate templates and the thioester. Thus, peptide **7a**, **7b** or **7c** in an SDS aqueous solution was lyophilized and redissolved in 0.1 M phosphate buffer (pH 7.6), with tris(2-carboxyethyl)phosphine (TCEP) added to suppress disulfide formation. Peptide thioester **8**[20] was assembled by microwave-assisted SPPS using Boc-protected amino acids on *p*-MBHA resin. Thioester **8** was dissolved in 0.1 M phosphate buffer (pH 7.6) and pre-activated for 1 h using 2-mercaptoethane sulfonate (MESNA) to give **8a**. This solution was added to the phosphate buffer containing **7a**, **7b** or **7c** and the mixture was incubated at 37 °C (Scheme 2). The ligation was monitored by HPLC and initially peaks corresponding to partially substituted lipopeptide–carbohydrates were observed, followed by the appearance of a peak with a lower retention time corresponding to the tetra-substituted compound. The three vaccine candidates **9a**, **9b**, and **9c** were obtained in high yields (71%, 76%, and 72%, respectively), with purity and identity confirmed by RP-HPLC and ESI-MS (see Fig. 1 and Experimental section).



Scheme 2. Synthesis of lipopeptide–carbohydrate template assembled peptides **9a**, **9b**, and **9c**.

2.4. Conformational studies

Our interest was in investigating whether the formation of 4-α-helix bundles of the 8830 antigen could have impact on its immunogenicity. Therefore, the helical characteristics of vaccine candidates **9a**, **9b**, and **9c** were studied by CD measurements. Helix-inducing features of carbohydrate template assembled peptides have been previously reported,[9,10,12] as such, similar carbohydrate influence on peptide topology was envisaged in our study. Indeed, spectra typical for α-helices were observed for **9a**, **9b**, and **9c** (in phosphate buffer, pH 7.3), showing double minima at 208 nm and 222 nm (Fig. 2). The free 8830 peptide **10**[20] was found to exist in phosphate buffer (pH 7.3) in an almost random structure (Fig. 2), demonstrating that the helical character of the attached peptides is

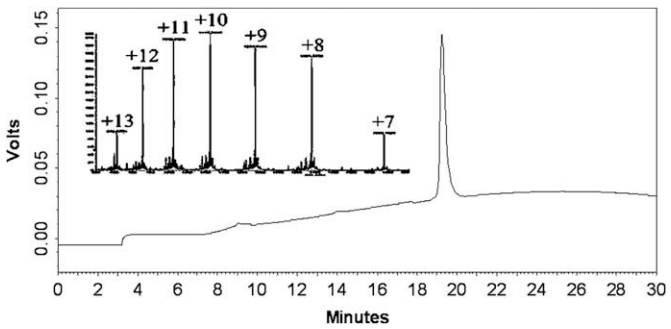


Figure 1. RP-HPLC and ESI-MS spectra of **9b**.

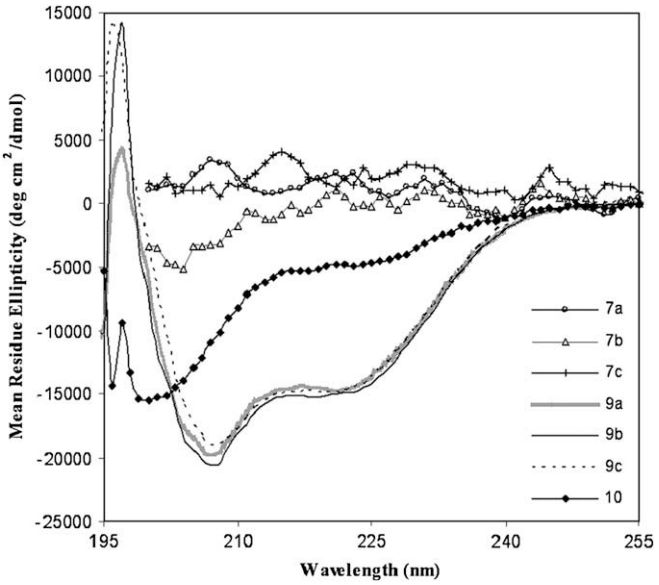


Figure 2. CD spectra of peptides **7a**, **7b**, **7c**, **9a**, **9b**, **9c**, and **10**.

not intrinsic to the sequence itself, but due to the template topology-controlling nature. α-Helical content was calculated based on the mean residue ellipticity (MRE, $[\theta]_{222}$) according to the published equation.[36] The contents of α-helix for **9a**, **9b**, and **9c** were found to be 44%, 45%, and 45%, respectively. The accurate determination of the content relied on the quantification by UV absorption at 280 nm as each tetravalent peptide contains four tyrosine residues.[37] Under the same conditions, the lipopeptide–carbohydrate templates were recorded with very low MRE values, suggesting that the templates have an insignificant contribution to the CD spectra of the tetravalent peptides. It was also found that the CD spectra of **9a**, **9b**, and **9c** were concentration independent in the range of 14–32 μM (see Supplementary data), implying there was no oligomerization or aggregation of the peptide within the concentration range.

3. Conclusion

Subunit peptide vaccines display significant advantages over the conventional whole pathogen vaccines. Research on subunit peptide vaccines for human vaccination has led to the development of self-adjuvanting lipopeptide vaccines as a potentially effective and safe approach. Covalent attachment of antigenic units of the virulent protein on carbohydrate molecules has produced multivalent immunogens with predetermined tertiary structure. Herein, we have provided techniques, using chemoselective ligation, for the synthesis of lipopeptide–carbohydrate assembled multivalent peptides in a highly pure state. The synthesized monosaccharide molecules have induced the formation of 4-α-helix bundles of the attached peptides, which may induce better immune responses. The presented carbohydrate-based LCP system, together with the synthetic strategy, opens the way for the development of highly pure, self-adjuvanting, and topologically defined synthetic vaccines.

4. Experimental

4.1. General experimental procedures

Microwave-assisted SPPS was performed using a CEM (Matthews, NC, USA) Discovery reactor equipped with an external FO temperature sensor. ^{1}H NMR and ^{13}C NMR spectra were acquired using a Bruker Avance 400 Hz or 500 MHz spectrometer (Bruker Biospin, Germany). NMR spectra were obtained in $CDCl_3$ or DMSO-d_6 at room temperature and were internally referenced to $CHCl_3$ (δ_H 7.24 and δ_C 77.0) or DMSO (δ_H 2.49 and δ_C 39.5). Positive low resolution ES-MS (LRESMS) was performed on either a Perkin Elmer-Sciex API 3000 instrument (Applied Biosystems, Foster city, CA, USA) or an API QSTAR pulsar i ES-MS (Applied Biosystems, Foster city, CA, USA) equipped with Agilent 1200 HPLC (Waldbronn, Germany). High resolution ES-MS (HRESMS) was performed on a MicroTof Q instrument (Bruker Daltonics, Bremen, Germany). Analytical RP-HPLC was performed using Shimadzu (Tokyo, Japan) instrumentation (Class Vp 6.12 software, SCL-10AVp controller, SIL-10A autoinjector, LC-10AT pump, LC-10AD pump, Waters 486 tunable absorbance detector or SPD-6A UV detector). Peptide purification was performed on either a Vydac preparative C18 column or a Vydac preparative C4 column. Analytical RP-HPLC was performed on either a Vydac analytical C18 column or a Vydac analytical C4 column. Separation was achieved in a gradient mode using 0.1% TFA/H_2O as solvent A and 90% ACN/0.1% TFA/H_2O (B1) or 90% MeOH/0.1% TFA/H_2O (B2) as solvent B. CD spectra were measured on a JASCO (Tokyo, Japan) J-710 spectropolarimeter using quartz cuvette of 1 mm path length at 23 °C.

4.2. Synthesis of mannose derivatives 2a–5a

*4.2.1. 2,3,4,6-Tetra-O-(cyanoethyl)-β-D-mannopyranosyl azide (**2a**)*

Acrylonitrile (6.4 mL, 5.2 g, 97 mmol) and DBU (0.6 mL, 0.6 g, 3.9 mmol) were added to a suspension of compound **1a** (1.7 g, 8.28 mmol) in ACN (50 mL) with stirring. After 3 h, the next portion of acrylonitrile (6.4 mL, 5.2 g, 97 mmol) and DBU (0.6 mL, 0.6 g, 3.9 mmol) was added, and the mixture was stirred overnight. The solution was evaporated under reduced pressure and the residue was purified by flash column chromatography on silica (EtOAc/hexane 3:1) to give **2a** as a colorless oil (1.26 g, 37%). ν_{max} (ATR) 2252, 2116, 1108 cm^{-1}; ^{1}H NMR ($CDCl_3$, 500 MHz) δ 4.41 (1H, d, J=0.9 Hz, H-1), 4.07–3.82 (10H, m, 4×CH_2O, H-2, H-4), 3.73–3.69 (2H, m, CH_2O), 3.41–3.37 (2H, m, H-3, H-5), 2.76–2.54 (8H, m, 4×CH_2CN); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 118.6, 118.3, 118.0, 117.9, 86.2, 82.7, 77.8, 77.7, 73.4, 69.2, 67.8, 67.3, 66.5, 65.8, 19.4, 19.3, 19.3, 19.1; LRESMS m/z 440.2 $[M+Na]^+$; HRESMS m/z 440.1654 $[M+Na]^+$ (calcd for $C_{18}H_{23}N_7O_5Na$, 440.1653).

*4.2.2. 6-[2,3,4,6-Tetra-O-(cyanoethyl)-β-D-mannopyranosylamino]-6-oxohexanoic acid, benzyl ester (**3a**)*

Compound **2a** (650 mg, 1.55 mmol) was dissolved in THF (40 mL) and hydrogenated over 10% Pd/C (50 mg) overnight. Upon completion, adipic acid monobenzyl ester (400 mg, 1.7 mmol), HBTU (640 mg, 1.7 mmol), and DIPEA (324 μL, 239 mg, 1.86 mmol) were added, and the mixture was stirred under an inert atmosphere overnight. The solution was filtered through Celite® and the filtrate was evaporated under reduced pressure. The residue was dissolved in EtOAc (100 mL) and washed with 5% HCl (3×50 mL), saturated $NaHCO_3$ (2×50 mL), and saturated NaCl (50 mL). The organic layer was dried ($MgSO_4$), filtered, and concentrated. The obtained residue was purified by flash column chromatography on silica (EtOAc) to yield **3a** as a colorless oil (499 mg, 53%). ν_{max} (ATR) 2251, 1730, 1687, 1503, 1107 cm^{-1}; ^{1}H NMR ($CDCl_3$, 500 MHz) δ 7.35–7.29 (5H, m, 5×Ar–H), 6.88 (1H, d, J=9.5 Hz, NH), 5.24 (1H, dd, J=9.5, 1.2 Hz, H-1), 5.08 (2H, s, CH_2Ph), 4.42–4.38 (1H, m, 1H of CH_2O), 4.01–3.79 (8H, m, 7H of CH_2O, H-4), 3.77 (1H, dd, J=2.6, 1.2 Hz, H-2), 3.73–3.62 (2H, m, CH_2O), 3.46 (1H, dd, J=9.7, 2.7 Hz, H-3), 3.36 (1H, m, J=1.4 Hz, H-5), 2.84–2.52 (8H, m, 4×CH_2CN), 2.37–2.33 (2H, m, CH_2CON), 2.26–2.23 (2H, m, CH_2COO), 1.68–1.62 (4H, m, $CH_2CH_2CH_2CH_2$); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 173.2, 172.7, 136.0, 128.5, 128.1, 128.1, 119.3, 118.6, 118.5, 118.0, 84.2, 76.8, 76.5, 73.8, 69.3, 68.0, 67.3, 66.4, 66.2, 66.1, 35.7, 33.8, 24.6, 24.2, 19.8, 19.4, 19.3, 19.0; LRESMS m/z 632.3 $[M+Na]^+$, 648.3 $[M+K]^+$; HRESMS m/z 632.2701 $[M+Na]^+$ (calcd for $C_{31}H_{39}N_5O_8Na$, 632.2691).

*4.2.3. 6-[2,3,4,6-Tetra-O-(3-tert-butoxycarbonylaminopropyl)-β-D-mannopyranosylamino]-6-oxohexanoic acid, benzyl ester (**4a**)*

Sodium borohydride (3.84 g, 101 mmol) was added portionwise at 0 °C to a solution of compound **3a** (3.09 g, 5.07 mmol), $CoCl_2 \cdot 6H_2O$ (9.67 g, 40.6 mmol), and Boc_2O (6.65 g, 30.4 mmol) in MeOH (170 mL) with stirring. After 4 h, MeOH was evaporated under reduced pressure and $CHCl_3$ (100 mL) was added. The mixture was filtered through Celite® and the filtrate was concentrated under reduced pressure. The crude residue was purified by flash column chromatography on silica (EtOAc/hexane 4:1) to give **4a** as a colorless oil (1.54 g, 30%). ν_{max} (ATR) 3347, 1688, 1515, 1112 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 7.35–7.26 (5H, m, 5×Ar-H), 5.22 (1H, br s, H-1), 5.06 (2H, s, CH_2Ph), 3.82–3.12 (22H, m, 4×CH_2N, 5×CH_2O, 4×CHO), 2.36–2.27 (m, 4H, 2×CH_2CO), 1.82–1.58 (12H, m, 4×$CH_2CH_2CH_2$, $CH_2CH_2CH_2CH_2$), 1.40 (36H, s, 4×$C(CH_3)_3$); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 173.2, 156.1, 156.0, 146.7, 136.0, 128.5, 128.1, 85.1, 79.1, 79.0, 78.9, 77.2, 77.1, 74.1, 71.1, 69.7, 68.4, 66.0, 38.3, 38.0, 37.3, 35.5, 33.9, 30.3, 30.2, 29.6, 28.4, 28.4, 28.1, 27.3, 24.8, 24.4; LRESMS *m*/*z* 1048.6 $[M+Na]^+$; HRESMS *m*/*z* 1048.6003 $[M+Na]^+$ (calcd for $C_{51}H_{87}N_5O_{16}Na$, 1048.6040).

*4.2.4. 6-[2,3,4,6-Tetra-O-(3-tert-butoxycarbonylaminopropyl)-β-D-mannopyranosylamino]-6-oxohexanoic acid (**5a**)*

Compound **4a** (1.54 g, 1.5 mmol) was dissolved in THF (100 mL) and hydrogenated over 10% Pd/C (200 mg) overnight. After completion, the mixture was filtered through Celite®. Evaporation of the filtrate under reduced pressure without further purification gave **5a** as a colorless foam (1.17 g, 83%). ν_{max} (ATR) 3345, 1688, 1515, 1103 cm^{-1}; ^1H NMR (DMSO-d_6, 500 MHz) δ 8.10 (1H, d, *J*=8.6 Hz, NHCO), 6.75–6.65 (4H, m, 4×NHCOO), 5.04 (1H, br d, *J*=8.9 Hz, H-1), 3.67–2.93 (22H, m, 4×CH_2N, 5×CH_2O, 4×CHO), 2.17–2.16 (4H, m, 2×CH_2CO), 1.63–1.45 (12H, m, 4×$CH_2CH_2CH_2$, $CH_2CH_2CH_2CH_2$), 1.35 (36H, s, 4×$C(CH_3)_3$); ^{13}C NMR (DMSO-d_6, 125 MHz) δ 174.3, 171.6, 155.6, 155.5, 82.8, 77.4, 77.3, 76.2, 76.1, 74.3, 70.1, 69.9, 69.6, 69.4, 67.3, 59.7, 37.3, 37.2, 37.1, 36.8, 34.5, 33.4, 30.2, 30.1, 30.0, 29.6, 28.2, 24.5, 24.0; LRESMS *m*/*z* 958.6 $[M+Na]^+$. HRESMS *m*/*z* 958.5534 $[M+Na]^+$ (calcd for $C_{44}H_{81}N_5O_{16}Na$, 958.5571).

4.3. Synthesis of Cys-containing lipopeptide–carbohydrate templates 7a, 7b, and 7c

Peptides **7a**, **7b**, and **7c** were synthesized on a *p*-MBHA resin using the standard Boc-based in situ neutralization SPPS.[29] Boc–C12–G–C12–C12–G–resin was synthesized (0.45 mmol NH_2/g, 0.5 mmol scale) according to the reported procedure.[28] Each amino acid coupling cycle consisted of Boc-deprotection with neat TFA (2×1 min), a 1 min DMF flow wash, followed by a 1–4 h coupling with the pre-activated amino acid. Amino acid activation was achieved by dissolving the amino acid (2.2 mmol) in 0.5 M HBTU/DMF solution (4 mL, 2 mmol) to which DIPEA (700 μL, 4 mmol) was added, and the activation proceeded for 1 min (Boc–Gly–OH) or 5 min (Boc–C12–OH[22]). Coupling efficiency was monitored by quantitative ninhydrin test.[38] Where necessary, couplings were repeated to give coupling efficiency greater than 99.7%. Following removal of the N-terminal Boc group, carbohydrate template **5a**, **5b** or **5c** was C-terminally coupled to the Boc–C12–G–C12–C12–G–resin (0.1 mmol scale) to give **6a**, **6b** or **6c**. Carbohydrate template activation was achieved by dissolving the compound (**5a**, **5b** or **5c**) (0.2 mmol) in 0.5 M HBTU/DMF solution (0.38 mL, 0.19 mmol) to which DIPEA (70 μL, 0.4 mmol) was added and the activation proceeded for 5 min. Coupling proceeded for 6 h with coupling efficiency greater than 99.7%. Following removal of the N-terminal Boc groups, four Boc–Cys(MBzl) residues were then coupled to **6a**, **6b** or **6c**. Boc–Cys(MBzl)–OH activation was achieved by dissolving the amino acid (1.76 mmol) in 0.5 M HBTU/DMF solution (3.16 mL, 1.58 mmol) to which DIPEA (522 μL, 3 mmol) was added and the activation proceeded for 1 min. Following removal of the N-terminal Boc groups, the peptidyl resin was then washed with DMF (3×10 mL), DCM (3×10 mL), and MeOH (3×10 mL) and then dried under vacuum prior to cleavage with HF. HF cleavage (10 mL HF/g resin) was performed for 2 h at 0 °C in the presence of 5% (v/v) *p*-cresol and 5% (v/v) thiocresol as scavengers. The crude peptides were purified by preparative RP-HPLC (40–70% B1 over 40 min, C4 column). The yields and characteristic data for the Cys-containing lipopeptide–carbohydrate templates **7a**, **7b** and **7c** are shown in Table 1.

Table 1
Yields and analytical data of synthesized peptides

Cys, Cys, carbohydrate template, -C12-Gly-C12-C12-Gly-NH_2, Cys, Cys

Compound	Yield (%)	t_R (min)	ES-MS (MW: 1653.31)
7a	21	22.1[a]	1653.9 (*z*=1), 827.2 (*z*=2)
7b	36	22.1[a]	1653.7 (*z*=1), 827.3 (*z*=2)
7c	24	22.0[a]	1653.2 (*z*=1), 827.3 (*z*=2)

Ac-[8830]-Gly—S, Leu–NH_2, O

Compound	Yield (%)	t_R (min)	ES-MS (MW: 2585.89)
8	14	17.0[b]	1294.4 (*z*=2), 863.3 (*z*=3)

Ac-[8830]-Gly-Cys, Ac-[8830]-Gly-Cys, carbohydrate template, -C12-Gly-C12-C12-Gly-NH_2, Ac-[8830]-Gly-Cys, Ac-[8830]-Gly-Cys

Compound	Yield (%)	t_R (min)	ES-MS (MW: 11 124.23)
9a	71	19.1,[a] 27.8[c]	1391.5 (*z*=8), 1237.0 (*z*=9), 1113.4 (*z*=10), 1012.3 (*z*=11), 928.0 (*z*=12), 856.7 (*z*=13)
9b	76	19.2,[a] 26.9[c]	1590.1 (*z*=7), 1391.4 (*z*=8), 1236.9 (*z*=9), 1113.3 (*z*=10), 1012.2 (*z*=11), 927.9 (*z*=12), 856.6 (*z*=13)
9c	72	19.0,[a] 27.1[c]	1590.1(z=7), 1391.5 (z=8), 1237.0 (z=9), 1113.4 (z=10), 1012.3 (*z*=11), 928.0 (*z*=12)

[a] Column: C4; Gradient: 0–100% B1 over 30 min.
[b] Column: C18; Gradient: 0–100% B1 over 30 min.
[c] Column: C4; Gradient: 0–100% B2 over 30 min.

4.4. Chemical ligation

Peptide **7a** (2.2 mg, 1.33 μmol) was dissolved in 1% (w/v) aqueous SDS (3 mL), frozen, and lyophilized. The powder was then dissolved in 0.1 M phosphate buffer pH 7.6 (1.5 mL), to which TCEP (4.57 mg; 15.9 μmol) was added and the pH was adjusted to 7.3 with 0.1 M sodium phosphate dibasic (300 μL). Peptide **8** (27.5 mg; 10.6 μmol) was dissolved in 0.1 M phosphate buffer pH 7.6 (1 mL) with addition of MESNA (26.2 mg; 160 μmol). The thioester exchange proceeded for 1 h to give **8a** (ES-MS: $[M+2H]^{2+}$ m/z 1256.6 (calcd 1255.4), $[M+3H]^{3+}$ m/z 837.5 (calcd 837.3), MW 2508.77 g/mol). This solution was then transferred into the vessel containing lipopeptide **7a** with extra 0.1 M phosphate buffer pH 7.6 (200 μL) and the reaction mixture was incubated at 37 °C. The ligation was monitored by analytical RP-HPLC (0–100% B1 over 30 min, C4 column) and initially, peaks corresponding to partially substituted lipopeptide–carbohydrates were observed (19.7 min and 20.3 min for the ligation of **7a** and **8a**, 19.4 min and 20.0 min for the ligation of **7b** and **8a**, and 19.7 min and 20.1 min for the ligation of **7c** and **8a**). The ligation appeared to be completed within 24 h. The reaction mixture was separated by preparative RP-HPLC on a C4 column using a gradient of 30–70% B1 over 40 min. The fractions were analyzed by analytical RP-HPLC, combined where appropriate and lyophilized to give **9a**. Compound **9b** was synthesized by coupling **7b** and **8** according to the procedure as for compound **9a**. Compound **9c** was synthesized by coupling **7c** and **8** according to the procedure as for compound **9a**. The yields and characteristic data for **9a**, **9b**, and **9c** are shown in Table 1.

4.5. CD measurements

CD measurements were performed using a Jasco J-710 spectropolarimeter. The CD spectra were measured at 23 °C under continuous nitrogen purging of the sample chamber using a quartz cuvette of 0.1 cm path length with an average of three scans were taken. Blanks were subtracted from the CD spectra. Sample solutions of **9a**, **9b**, **9c**, and **10** were prepared at a range of concentrations of 14–32 μM in 10 mM phosphate buffer, pH 7.3 (concentrations of stock solutions determined from the Tyr absorption at 280). The samples of **7a**, **7b**, and **7c** were dissolved individually in phosphate buffer, pH 7.3. Content of helicity was calculated according to the published formula.[36]

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Supplementary data

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.tet.2009.02.060.

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