CSIRO PUBLISHING

www.publish.csiro.au/journals/ajc

Aust. J. Chem. **2009**, *62*, 993–999

Design and Synthesis of Lipopeptide–Carbohydrate Assembled Multivalent Vaccine Candidates Using Native Chemical Ligation

Wei Zhong,[A] *Mariusz Skwarczynski*,[A] *Yoshio Fujita*,[A] *Pavla Simerska*,[A] *Michael F. Good*,[B] *and Istvan Toth*[A,C]

[A]The University of Queensland, School of Chemistry and Molecular Biosciences, St. Lucia, Qld 4072, Australia.

[B]Queensland Institute of Medical Research, Herston, Qld 4029, Australia.

[C]Corresponding author. Email: i.toth@uq.edu.au

Development of a synthetic vaccine against group A streptococcal infection is increasingly paramount due to the induction of autoimmunity by the main virulent factor – M protein. Peptide vaccines, however, are generally poorly immunogenic, necessitating administration with carriers and adjuvants. One of the promising approaches to deliver antigenic peptides is to assemble peptides on a suitable template which directs the attached peptides to form a well defined tertiary structure. For self-adjuvanting human vaccines, the conjugation of immunostimulatory lipids has been demonstrated as a potentially safe method. This study describes the design and optimized synthesis of two lipopeptide conjugated carbohydrate templates and the assembling of peptide antigens. These lipopeptide–carbohydrate assembled multivalent vaccine candidates were obtained in high yield and purity when native chemical ligation was applied. Circular dichroism studies indicated that the template-assembled peptides form four α-helix bundles. The developed technique extends the use of carbohydrate templates and lipopeptide conjugates for producing self-adjuvanting and topology-controlled vaccine candidates.

Manuscript received: 2 February 2009.
Manuscript accepted: Accepted without change.

Introduction

Group A streptococcal (GAS) infection is associated with a broad range of diseases including common pharyngitis and life-treating post-infectious diseases such as rheumatic fever (RF).[1] The cell surface M protein, an α-helical coiled-coil protein, is a major virulence factor in GAS infection.[2] Protective immunity to GAS is mediated by serotype-specific opsonic antibodies against the hypervariable *N*-terminal regions of M protein, as well as antibodies specific to the conserved *C*-terminal regions.[1,3] Development of vaccines against GAS face substantial obstacles. There are more than 180 identified serotypes that require serotype-specific opsonising antibodies for protection.[4] Furthermore, the conserved *C*-terminal region contains epitopes which are capable of inducing autoimmunity.[5,6] These problems may be addressed by the development of subunit vaccines which contain non-cross-reactive B-cell epitopes. However, subunit vaccines containing short peptides are generally poorly immunogenic due to lack of either the correct three-dimensional structure or an appropriate helper T-cell epitope.[7] The immunogenicity can be enhanced by incorporating an appropriate adjuvant, which facilitates the uptake and presentation of antigen by antigen-presenting cells.[7] Because humans are the only host of the GAS pathogen, an effective and non-toxic adjuvant is required. Current approved adjuvants for human use mainly contain alum-based adjuvants.[8] This limited choice has spawned a need to develop new adjuvants. The covalent conjugation of lipids (self-adjuvanting moiety) to synthetic peptides has been demonstrated as a potentially safe method to adjuvant otherwise poorly immunogenic peptides.[9–12]

Over the past decade, our laboratory has performed extensive studies of subunit vaccine candidates against GAS.[13–16] We have also developed a unique lipid core peptide (LCP) system[17] featuring a lipopeptide moiety (inbuilt adjuvant), a polylysine branching core[18] (carrier), and multiple peptides (antigen) in one molecular entity. The LCP system gives greater flexibility in the physico-chemical properties, such as lipophilicity and multiplicity of the incorporated peptides, which can improve their biological activities.[19] The aim of this study was to accommodate peptide epitopes in a spatial arrangement on a suitable template to afford new LCPs with well-defined antigen secondary structure (Fig. 1). We have chosen carbohydrates as the templates for assembling the antigenic peptides as the distinct conformations and the rigid ring make them promising scaffolds to control the topology of the assembled peptides.[20–23] Although such branched multivalent peptides could be synthesized using stepwise solid phase peptide synthesis (SPPS),[24] synthetic errors relating to SPPS itself and the purification of high molecular weight multivalent peptides present a clear challenge.[18] Therefore, we examine here the potential of chemoselective ligation to provide highly pure vaccine candidates that allow accurate conformational studies and provide potential vaccines suitable for use in humans. Native chemical ligation (NCL) previously enabled the synthesis of several lipopeptide-polylysine-antigen constructs and the technique,

 10.1071/CH09065 0004-9425/09/090993

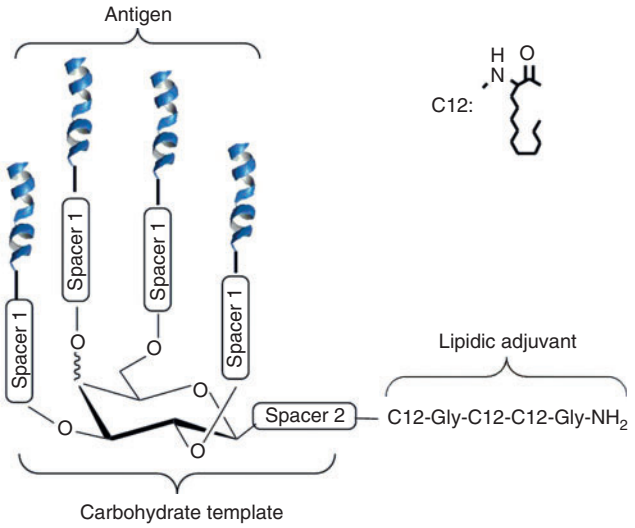


Fig. 1. Carbohydrate-based lipid core peptide system.

using sodium dodecyl sulfate (SDS) as a solubilizing agent, enabled the ligation of the highly hydrophobic lipid core to the highly hydrophilic peptide.[25,26] Therefore, this method was examined for the synthesis of lipopeptide–carbohydrate assembled multivalent peptides.

This paper describes the design and optimized synthesis of two lipopeptide conjugated, carbohydrate templates and the assembling of peptide antigens onto the carbohydrate templates using NCL. This work also investigated the secondary structure of the obtained vaccine candidates by circular dichroism (CD) measurements. Important comparisons were drawn between the conformational characteristics of the tetravalent peptides, based on different carbohydrate templates, and the free antigenic peptide.

Results and Discussion

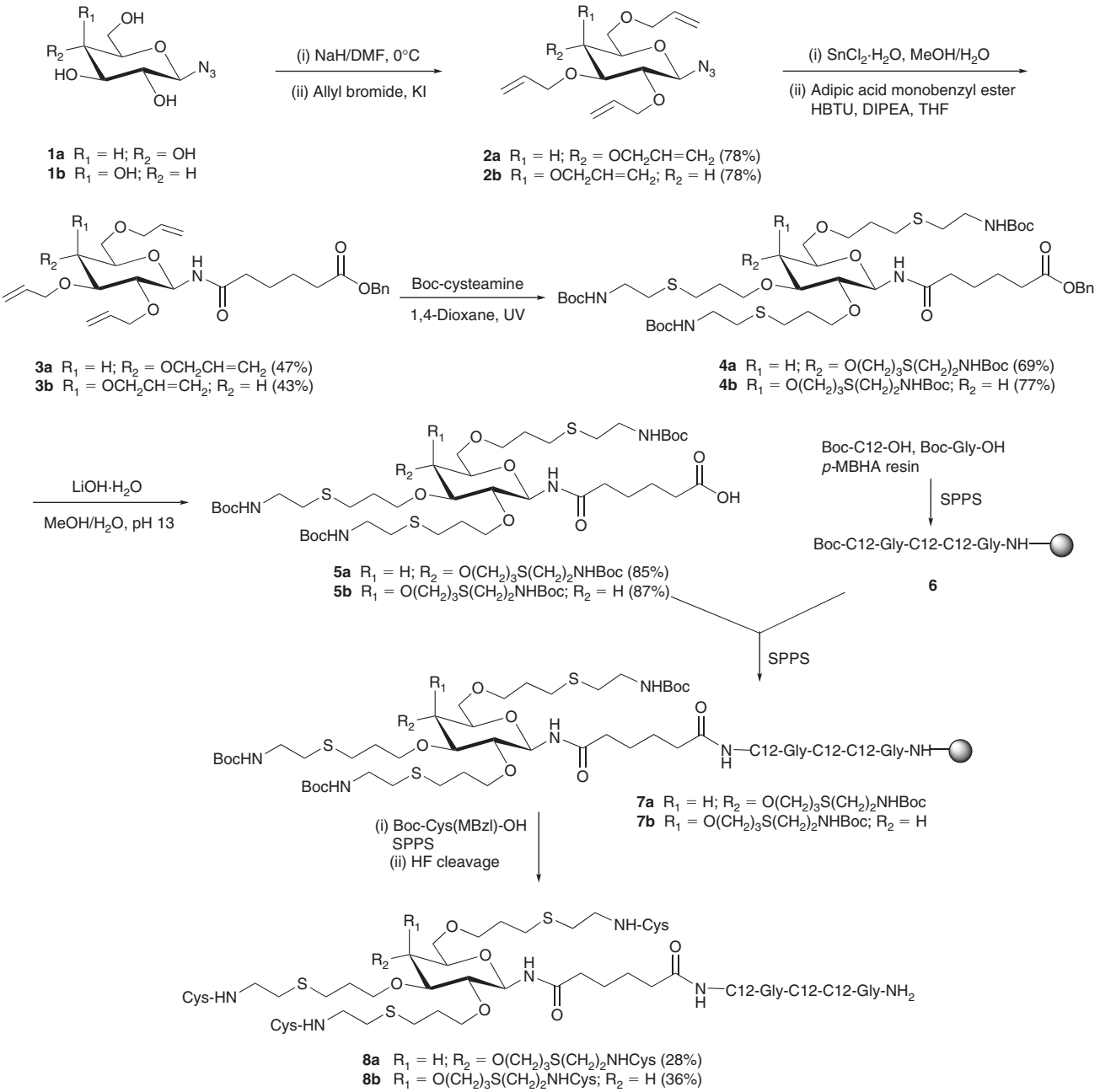
Each vaccine candidate is composed of (i) multiple copies of an antibody-inducing peptide epitope, (ii) a self-adjuvanting lipopeptide moiety (C12-Gly-C12-C12-Gly; C12: 2-(*R*/*S*)-aminododecanoic acid[27]), and (iii) a functionalized monosaccharide template acting as a branching core and a topology controlling scaffold. Two different monosaccharide (D-glucose and D-galactose) derivatives were developed as templates. In all cases the antigenic peptides are conjugated to the four functionalized non-anomeric positions of the sugar ring and the lipopeptide moiety is conjugated to the functionalized anomeric position. The convergent synthetic approach to vaccine candidates was designed based on chemoselective ligation of the peptide thioester and the Cys-containing lipopeptide–carbohydrate templates. The covalent linkage of lipopeptide and carbohydrate was achieved using Boc-based SPPS. To afford this, hydroxyl groups of β-D-glycosyl azides (**1a** and **1b**)[28] were suitably modified to **5a** and **5b** which possess four Boc-protected amine groups at the non-anomeric positions and a free carboxylic acid group at the anomeric position of the sugar ring (Scheme 1).

The glucose derivative **5a** was synthesized from readily available azide **1a**. However, initial allylation of **1a** with NaH/allyl bromide in THF according to the literature[29] was not successful, resulting in recovery of the starting material. Allylation was then carried out using the procedure described by McGeary et al.[30] The reaction led to a complex mixture of di-, tri-, and tetra-allylated derivatives even when the reaction time was extended to overnight with excess of reagents. However, by in situ converting allyl bromide to allyl iodide with potassium iodide and performing the reaction at room temperature for 5 h, **2a** was obtained in 78% yield. Reduction of **2a** was achieved using stannous chloride.[31] The following coupling with adipic acid monobenzyl ester[32] using HBTU/DIPEA method afforded **3a** in 47% overall yield (two steps). Free-radical reaction of **3a** with *tert*-butyl-*N*-(2-mercaptoethyl) carbamate (Boc-cysteamine)[33] in 1,4-dioxane under UV irradiation at 254 nm[34] proceeded efficiently and gave **4a** in 69% yield. Subsequent benzyl-deprotection was achieved by saponification instead of standard hydrogenation due to the risk of hydrogenation catalysts deactivation by sulfur-containing compound **4a**.

The preparation of galactose derivatives **2b**–**5b** was previously reported.[31] Modified procedures for the synthesis of **2b** and **3b** were used. Instead of performing the allylation using allyl bromide at 50°C for 4 h, the reaction was carried out at room temperature for 5 h and the allyl bromide was converted to allyl iodide in situ, leading to an increased yield (78% instead of 60%[31]). The synthesis of compound **3b** was also more efficient than previously reported (43% instead of 32%[31]) when the crude corresponding amine was reacted directly with adipic acid monobenzyl ester.

The introduction of the lipopeptide moiety (C12-G-C12-C12-G) and free thiol functionalities (cysteine) to the carbohydrate templates **5a** and **5b** were achieved by stepwise SPPS. In order to improve the efficiency of synthesis of resin-bound lipopeptide **6**,[35] the lipo-amino acid was pre-activated (HBTU/DIPEA) for 5 min or *N*-methyl pyrrolidinone was used as a co-solvent. By pre-activating the template **5a** or **5b** (2 mol. equiv.) for 5 min and coupling to **6** for 6 h, the coupling efficiency reached 99.8% according to the quantitative ninhydrin test[36] for both **7a** and **7b**. Four copies of Boc-Cys(MBzl)-OH were then coupled to **7a** or **7b**. The lipopeptide–carbohydrate templates **8a** and **8b** were cleaved from the resin with anhydrous hydrofluoric acid (HF). During and after HF cleavage, to prevent thiazolidine formation of the *N*-terminal free thiols, the use of acetone for washing glassware or lyophilization was avoided.[37] Following purification by preparative reverse phase (RP)-HPLC, **8a** and **8b** were obtained with a total yield of 28% and 36%, respectively.

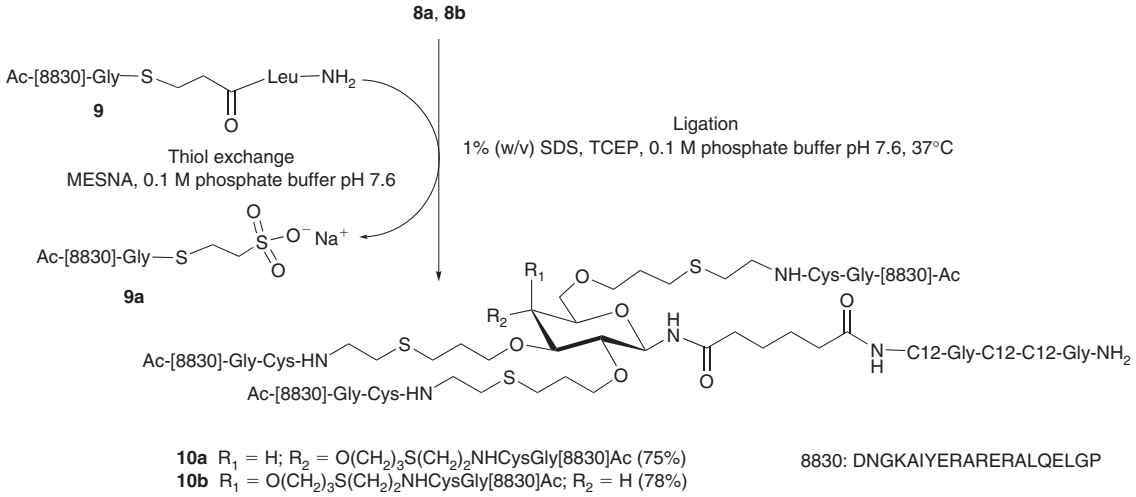
The examined epitope (DNGKAIYERARERALQELGP),[3] derived from the *N*-terminal of M protein of commonly circulated GAS 8830 strain, was chemically synthesized as a thioester **9**[25] using Boc-based SPPS. Both standard and microwave-assisted SPPS were investigated. Microwave-assisted SPPS increased the yield by 1.5-fold. To perform ligation between building blocks **8a** or **8b** and **9**, the selection of solvents was carefully assessed due to poor solubility of **8a** and **8b** in aqueous ligation buffer (phosphate buffer). It was previously reported[16,26] that attempts to solubilize Cys-lipopeptide constructs by the addition of organic solvent such as 10% DMF and 50% trifluoroethanol resulted in slow and incomplete ligations, although these co-solvents were used to improve NCL yields.[38–40] Nevertheless, the ligation between a Cys-containing lipopeptide-polylysine core and a thioester was successful in the presence of SDS, with a higher yield and a shorter reaction time when performing the ligation at 37°C.[25] Co-lyophilization of the poorly water soluble peptide with SDS significantly improved solubility of the peptide in aqueous ligation buffer. Thus, the hydrophobic peptides **8a** and **8b** were dissolved in SDS solution, lyophilized, and ligated to peptide thioester **9** in 0.1 M phosphate buffer, pH 7.6, containing sodium 2-mercaptoethanesulfonate (MESNA) as a thiol additive and



Scheme 1. Synthesis of lipopeptide–carbohydrate templates **8a** and **8b**.

tris-2-carboxyethylphosphine hydrochloride (TCEP) to prevent disulfide bond formation (Scheme 2). It was reported that the use of MESNA, instead of commonly used thiol additive thiophenol, may facilitate the ligation when SDS was used to solubilize hydrophobic peptides.[41] The reaction proceeded efficiently and completed within 24 h. SDS kept the peptides in ligation buffer throughout the course of reaction. Following purification by preparative RP-HPLC, tetravalent peptides **10a** and **10b** were obtained in 75% and 78% yield respectively. The HPLC profiles and the electrospray ionization-mass spectrometry (ESI-MS) spectra (Fig. 2) showed the high purity of **10a** and **10b** and verified the correct primary structure of the synthetic vaccine candidates.

The free antigenic peptide **11** (DNGKAIYERARERALQ ELGP), synthesized using standard SPPS, was tested for helical potential and it was found that the free peptide exists in phosphate buffer (pH 7.3) in an almost random structure (Fig. 3). In contrast, the CD spectra of **10a** and **10b** recorded in phosphate buffer (pH 7.3) showed that the constructs contained an α-helices, with the template itself (**8a** or **8b**) showing very low mean residue ellipticity values (Fig. 3). The CD spectra of **10a** and **10b** are characterized by two negative minima at 207 nm and 222 nm which are typical for the onset of a helical structure. Because the accuracy of the calculated α-helical content relies on the accurate determination of tetravalent peptide concentration, the UV absorbance of stock solutions of **10a** and **10b** (both containing four Tyr residues) was measured at 280 nm.[42] The peptide α-helical content was calculated to be 40% and 36% for **10a** and **10b**, respectively from the mean residue ellipticity at 222 nm (MRE, $[\theta]_{222}$) according to the



Scheme 2. Synthesis of tetravalent vaccine candidates **10a** and **10b**.

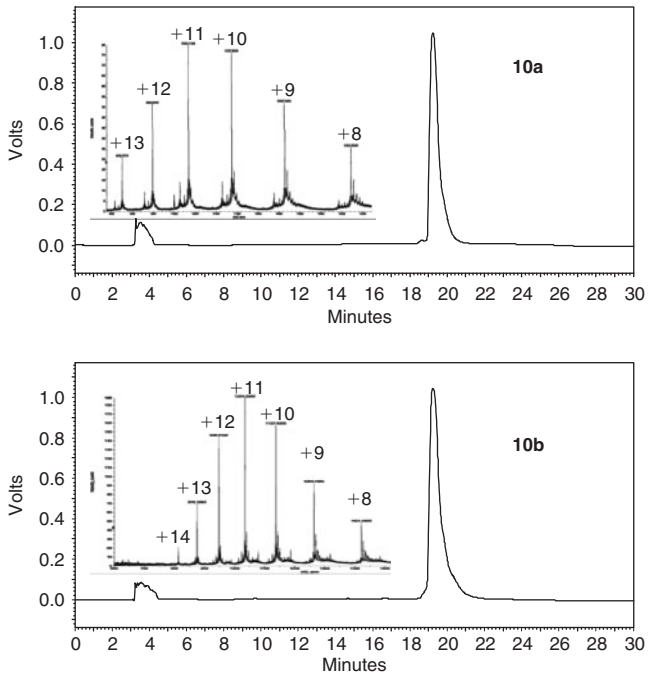


Fig. 2. Reverse phase-HPLC and electrospray ionization-mass spectrometry profiles of **10a** and **10b**.

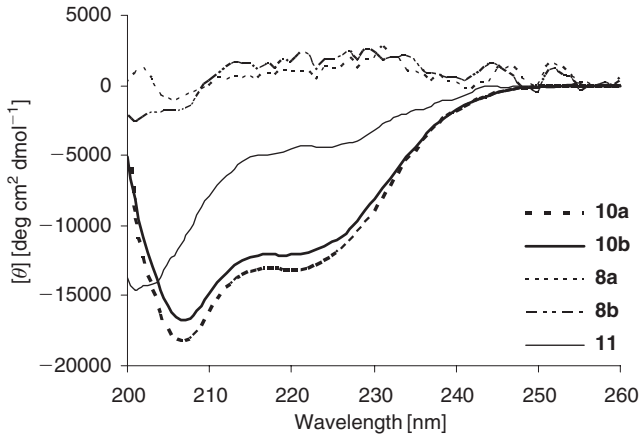


Fig. 3. Circular dichroism spectra of **8a**, **8b**, **10a**, **10b**, and **11**.

published formula.[43] The glucose-based and galactose-based tetravalent peptides showed comparable helicity. Concentration dependences of helicity of peptide **10a** and **10b** over a range of concentrations from 20 to 33 μM were investigated. It was found that the CD spectra of **10a** and **10b** were concentration-independent within the range studied, suggesting that there was no oligomerization.

Conclusions

In conclusion, we have demonstrated the utility of chemoselective ligation for the synthesis of two carbohydrate template assembled vaccine candidates, featuring four copies of epitopes and a self-adjuvanting lipopeptide moiety. Significantly enhanced α-helicity of the attached peptide antigens over the free peptide was observed. This result suggests that the two monosaccharide molecules are promising templates for constructing α-helix bundles that may be useful as mimics of conformational epitopes for vaccine development. The use of appropriate ligation techniques provided multivalent immunogens in highly pure state suitable for use in humans. The design and synthesis extend the use of carbohydrate templates and LCP system for producing self-adjuvanting and topology-controlled vaccine candidates.

Experimental

General Procedures

N-α-Boc-L-amino acids were purchased from Novabiochem (Laufelfingen, Switzerland) or GL Biochem (Shanghai, China). *p*-MBHA resin was purchased from Peptides International (Louisville, Kentucky, USA). Peptide synthesis grade TFA, DIEA, DMF, and HBTU were purchased from Auspep (Melbourne, Australia) or GL Biochem. HPLC grade ACN and IPA were purchased from Honeywell-Burdick & Jackson (Morristown, NJ, USA) or Labscan (Dublin, Ireland). Microwave-assisted SPPS was performed in a SPS mode CEM Discovery reactor (CME Corporation, Matthews, NC, USA) equipped with an external FO temperature sensor. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker Avance 500 MHz

spectrometer (Bruker Biospin, Germany). ESI-MS was performed on either a Perkin–Elmer-Sciex API3000 instrument or a LC ESI MS (Agilent 1200 HPLC (Waldbronn, Germany) and API QSTAR pulsar i ESI-MS). 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 tuneable 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 in gradient mode using 0.1% TFA/H_2O as solvent A, and 90% ACN/0.1% TFA/H_2O (B1) or 90% IPA/0.1% TFA/H_2O (B2) or 90% MeOH/0.1% TFA/H_2O (B3) as solvent B. Separation was achieved on either a Vydac analytical C18 column or a Vydac analytical C4 column using a gradient of 0–100% solvent B over 30 min unless otherwise specified. CD spectra were measured on a JASCO-810 (CD-ORD) spectropolarimeter using quartz cuvette of 1 mm path length at 23°C.

*2,3,4,6-Tetra-*O*-allyl-β-*D*-glucopyranosyl Azide* ***2a***

NaH (60% in dispersion oil, 1.17 g, 29.3 mmol) was added portion wise to a solution of β-D-glucopyranosyl azide **1a** (1.0 g, 4.9 mmol) at 0°C in DMF (80 mL). After stirring for 45 min, KI (3.4 g, 19.5 mmol) was added, followed by drop wise addition of a solution of allyl bromide (3.25 mL, 39 mmol) in DMF (8 mL). The mixture was stirred at room temperature under an inert atmosphere. After 5 h, the excess NaH was quenched by addition of MeOH (15 mL). The solvent was evaporated and the residue was dissolved in DCM (120 mL), washed with water (3 × 100 mL), dried ($MgSO_4$), filtered, and concentrated under reduced pressure. Purification of the crude residue by flash chromatography (EtOAc:hexane 1:7) gave **2a** (1.39 g, 78%) as a colourless oil. δ_H (500 MHz, $CDCl_3$) 3.10 (t, 1H, *J* 8.8, H2), 3.33–3.41 (m, 3H, H3, H4, H5), 3.61 (dd, 1H, *J* 4.0, 11.0, H6a), 3.68 (dd, 1H, *J* 1.4, 11.0, H6b), 3.97–4.31 (m, 8H, 4 × C*H*$_2$CH=CH$_2$), 4.47 (d, 1H, *J* 8.6, H1), 5.12–5.28 (m, 8H, 4 × CH=C*H*$_2$), 5.83–5.96 (m, 4H, 4 × C*H*=CH$_2$). δ_C (125 MHz, $CDCl_3$) 68.3, 76.8, 74.3, 73.8, 73.8, 72.5, 77.0, 80.9, 84.3, 90.0, 117.4, 117.2, 117.0, 116.7, 134.9, 134.6, 134.4. *m/z* (ESI) 338.3 [MH-CH=CH$_2$]$^+$, 383.3 [M + NH$_4$]$^+$.

*6-[2,3,4,6-Tetra-*O*-(2-propenyl)-β-*D*-glucopyranosylamino]-6-oxohexanoic Acid, Benzyl Ester* ***3a***

$SnCl_2 \cdot H_2O$ (620 mg, 2.7 mmol) in MeOH (5 mL) with a few drops of deionized water was added to a solution of **2a** (100 mg, 270 μmol) and the mixture was stirred overnight at room temperature under an inert atmosphere. The solvent was then evaporated under reduced pressure and the residue was diluted with EtOAc (30 mL). The solution was basified by saturated $NaHCO_3$ solution (30 mL) and filtered. The organic phase of the filtrate was separated and basified by saturated $NaHCO_3$ solution (30 mL) and filtered. The organic phase was separated, dried ($MgSO_4$), filtered, and evaporated. The residue was dissolved in dry THF (10 mL) to which adipic acid monobenzyl ester (62 mg, 270 μmol), HBTU (100 mg, 270 μmol), and DIPEA (46 μL, 270 μmol) in dry THF (5 mL) were added with stirring. The reaction mixture was stirred overnight under an inert atmosphere. The solvent was then evaporated under reduced pressure and the residue was dissolved in EtOAc (50 mL). The organic phase was washed with 5% HCl solution (3 × 30 mL), saturated $NaHCO_3$ (2 × 30 mL), and brine (50 mL), dried ($MgSO_4$), filtered, and concentrated. Purification by flash chromatography (EtOAc/hexane 2:3) gave **3a** (71.3 mg, 47%) as a white solid. δ_H (500 MHz, $CDCl_3$) 1.61–1.67 (m, 4H, 2 × CH$_2$C*H*$_2$CH$_2$), 2.15–2.19 (m, 2H, CH$_2$CO), 2.33–2.36 (m, 2H, CH$_2$CO), 3.11 (t, 1H, *J* 9.0, H2), 3.36–3.49 (m, 3H, H3, H4, H5), 3.58 (dd, 1H, *J* 3.1, H6a), 3.62 (dd, 1H, *J* 2.1, H-6b), 3.91–4.28 (m, 8H, 4 × C*H*$_2$CH=CH$_2$), 5.02 (t, 1H, *J* 9.3, H-1), 5.08 (s, 2H, CH$_2$OPh), 5.08–5.28 (m, 8H, 4 × CH=C*H*$_2$), 5.77–5.97 (m, 4H, 4 × C*H*=CH$_2$), 7.27–7.35 (m, 5H, 5 × ArH). δ_C (125 MHz, $CDCl_3$) 24.2, 24.5, 33.8, 36.2, 66.2, 67.9, 72.4, 73.5, 73.7, 74.2, 76.2, 78.7, 80.4, 85.1, 116.8, 116.8, 117.4, 117.4, 128.1, 128.5, 134.4, 134.7, 134.8, 134.9, 135.9, 172.4, 173.1. *m/z* (ESI) 558.3 [M + H]$^+$, 575.4 [M + NH$_4$]$^+$, 580.4 [M + Na]$^+$. HRMS (ESI, [M + Na]$^+$) Found: 580.2861. Calc. for $C_{31}H_{43}NO_8Na$: 580.2881.

*6-[2,3,4,6-Tetra-*O*-[3-(2-*tert*-butoxycarbonylaminoethylthio)-propyl]-β-*D*-glucopyranosylamino]-6-oxohexanoic Acid Benzyl Ester* ***4a***

Compound **3a** (78.9 mg, 140 μmol) and Boc-cysteamine (400 mg, 2.26 mmol) were dissolved in dried and degassed 1,4-dioxane (1.2 mL) and the mixture was irradiated at 254 nm for 4 h at 0°C. The solvent was then evaporated under reduced pressure and the residue was purified by flash chromatography (EtOAc:hexane 3:2, 5% Et_3N) to give **4a** (123 mg, 69%) as a colourless oil. δ_H (500 MHz, $CDCl_3$) 1.41 (s, 36H, 4 × C(CH$_3$)$_3$), 1.64–1.87 (m, 12H, 6 × CH$_2$C*H*$_2$CH$_2$), 2.22 (br t, 2H, *J* 7.6, CH$_2$CO), 2.35 (br t, 2H, *J* 6.9, CH$_2$CO), 2.52–2.60 (m, 16H, 8 × CH$_2$S), 3.01 (t, 1H, *J* 8.5, H2), 3.23–3.29 and 3.45–3.79 (2m, 21H, 4 × CH$_2$N, 5 × CH$_2$O, 3 × CHO), 5.00 (t, 1H, *J* 9.3, H1), 5.07 (s, 2H, CH$_2$OPh), 7.27–7.35 (m, 5H, ArH). δ_C (125 MHz, $CDCl_3$) 24.4, 24.6, 27.6, 28.1, 28.4, 28.4, 29.9, 30.1, 30.2, 30.5, 31.9, 32.0, 32.1, 33.8, 36.1, 39.9, 66.1, 68.9, 69.2, 70.7, 70.8, 71.7, 76.2, 77.4, 78.3, 78.8, 79.4, 79.5, 81.6, 85.4, 128.1, 128.5, 135.9, 155.7, 155.8, 156.0, 172.9, 173.2. *m/z* (ESI) 1266.9 [M + H]$^+$, 1283.8 [M + NH$_4$]$^+$. HRMS (ESI, [M + Na]$^+$) Found: 1288.6138. Calc. for $C_{59}H_{103}N_5O_{16}S_4Na$: 1288.6175.

*6-[2,3,4,6-Tetra-*O*-[3-(2-*tert*-butoxycarbonylaminoethylthio)-propyl]-β-*D*-glucopyranosylamino]-6-oxohexanoic Acid* ***5a***

Compound **4a** (1.0 g, 790 μmol) was dissolved in H_2O/MeOH mixture (1:4) (200 mL) in which the pH had been adjusted to 13 with $LiOH \cdot H_2O$ and the reaction mixture was stirred at room temperature overnight. The solvent was then evaporated under reduced pressure and the residue was dissolved in EtOAc (100 mL). The organic layer was washed with cold 0.1 M citric acid solution (2 × 100 mL) and brine (3 × 50 mL), dried ($MgSO_4$), filtered, and concentrated. The residue was washed via a plug of silica gel with $CHCl_3$/MeOH (9:1) to give **5a** (791 mg, 85%) as a colourless oil. δ_H (500 MHz, $CDCl_3$) 1.41 (s, 36H, 4 × C(CH$_3$)$_3$), 1.59–1.86 (m, 12H, 6 × CH$_2$C*H*$_2$CH$_2$), 2.25 (br t, 2H, *J* 7.0, CH$_2$CO), 2.33 (br t, 2H, *J* 6.6, CH$_2$CO), 2.50–2.66 (m, 16H, 8 × CH$_2$S), 3.01 (t, 1H, *J* 8.9, H2), 3.24–3.28 and 3.45–3.79 (2m, 21H, 4 × CH$_2$N, 5 × CH$_2$O, 3 × CHO), 5.00 (t, 1H, *J* 9.3, H1). δ_C (125 MHz, $CDCl_3$) 24.2, 24.6, 27.6, 28.0, 28.2, 28.3, 29.7, 30.1, 30.2, 30.5, 31.8, 31.8, 32.1, 33.4, 36.2, 39.5, 39.7, 68.8, 69.2, 70.8, 71.7, 76.2, 77.4, 78.8, 79.3, 79.5, 79.7, 81.5, 85.4, 155.7, 156.0, 173.0; *m/z* (ESI) 1176.7 [M + H]$^+$, 1193.9 [M + NH$_4$]$^+$. HRMS (ESI, [M + Na]$^+$) Found: 1198.5661. Calc. for $C_{52}H_{97}N_5O_{16}S_4Na$: 1198.5705.

*Synthesis of **6**, **7a**, **7b**, **8a**, and **8b***

Stepwise solid peptide synthesis of resin-bound lipopeptide **6** was carried out on *p*-MBHA resin (0.45 mmol NH_2/g, 250 μmol scale) using HBTU/DIPEA in situ neutralization procedure.[44] 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 pre-activated amino acid. Amino acid activation was achieved by dissolving amino acids (1.1 mmol, 4.4 equiv.) in 0.5 M HBTU/DMF solution (2 mL, 1 mmol) to which DIPEA (350 μL, 2 mmol) was added and activation proceeded for 5 min. Coupling efficiency was monitored by quantitative ninhydrin test.[42] Where necessary, couplings were repeated to give coupling efficiency greater than 99.7%. Following cleavage of the *N*-terminal Boc group, carbohydrate template **5a** or **5b** was *C*-terminally coupled to **6** (100 μmol scale). Activation of **5a**, **5b** was achieved by dissolving the compound (200 μmol, 2 equiv.) in 0.5 M HBTU/DMF solution (380 μL, 190 μmol) to which DIPEA (70 μL, 400 μmol) was added and activation proceeded for 5 min. Coupling proceeded for 6 h at which time coupling efficiency was greater than 99.8% to give **7a** and **7b**, respectively. Following cleavage of the *N*-terminal Boc group, four copies of Boc-Cys(MBzl)-OH were then coupled to the carbohydrate template residue. Boc-Cys(MBzl)-OH activation was achieved by dissolving the amino acid (1.76 mmol, 17.6 equiv.) in 0.5 M HBTU/DMF solution (3.16 mL, 1.58 mmol) to which DIPEA (522 μL, 3 mmol) was added and activation proceeded for 1 min. Three couplings were performed to give coupling efficiency greater than 99.9%. Following removal of the *N*-terminal Boc groups, the peptidyl resin was washed with DMF (3 × 10 mL), DCM (3 × 10 mL) and MeOH (3 × 10 mL), and dried under vacuum before 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. Following HF cleavage, peptides were precipitated by washing with ice-cold ether and dissolved in 50% aqueous ACN containing 0.1% TFA and lyophilized. The crude peptides were purified by preparative RP-HPLC on a C4 column using a gradient of 40–70% solvent B1 over 45 min. Fractions were analyzed by ESI-MS and combined where appropriate to give **8a** (52.3 mg, 28%) and **8b** (68.5 mg, 36%) following lyophilization. The characteristic data for the Cys-containing lipopeptide–carbohydrate templates **8a** and **8b** are shown below.

8a: HPLC: $t_R = 21.3$ min (solvent B1, C4 column), $t_R = 23.5$ min (solvent B3, 30–100% B over 30 min, C4 column); *m/z* (ESI) 1893.2 $[M + H]^+$ (calc. 1894.8), 948.1 $[M + 2H]^{2+}$ (calc. 947.9); MW 1893.79 g mol^{-1}.

8b: HPLC: $t_R = 20.8$ min (solvent B1, C4 column), $t_R = 18.3$ min (solvent B2, C4 column); *m/z* (ESI) 1894.4 $[M + H]^+$ (calc. 1894.8), 948.1 $[M + 2H]^{2+}$ (calc. 947.9); MW 1893.79 g mol^{-1}.

*Microwave-Assisted Synthesis of 8830 Thioester **9***

Microwave-assisted synthesis of peptide **9** was carried out using a trityl-associated mercaptopropionic acid-leucine (TAMPAL) linker.[45] The TAMPAL linker was synthesised on *p*-MBHA resin by coupling of Boc-Leu-OH (600 μmol, 3 equiv.) (2 × 10 min) and *S*-trityl-mercaptopropionic acid (800 μmol, 4 equiv.) (1 × 10 min) using the standard HBTU/DIPEA in situ neutralization procedure.[44] Following TAMPAL synthesis, the trityl protecting group was removed by three 2 min treatments with 2.5% (v/v) triisopropylsilane, 2.5% (v/v) H_2O, 95% (v/v) TFA, followed by a 1 min DMF flow wash. The thioester bond was then formed by the 2 × 10 min coupling of Boc-Gly-OH (800 μmol, 4 equiv.). The sequence of the peptide epitope (8830: DNGKAIYERARERALQELGP) was then *C*-terminally coupled by stepwise SPPS to the Boc-Gly-TAMPAL resin. Each amino acid coupling cycle consisted of Boc-deprotection with neat TFA (2 × 1 min), a 1 min DMF flow wash, followed by a 2 × 10 min coupling with pre-activated amino acid. Amino acid activation was achieved by dissolving amino acids (600 μmol, 3 equiv.) in 0.5 M HBTU/DMF solution (1.1 mL, 550 μmol) to which DIPEA (210 μL, 1.2 mmol) was added and activation proceeded for 1 min. All couplings were carried out under microwave irradiation (SPS mode, power 20 W, temperature 60°C, $\Delta T = 1$°C). After coupling glutamine residue, the resin was washed with DCM before and after Boc-deprotection to prevent pyrrolidone carboxylic acid formation.[44] Spectroscopic data and purity of **9** are identical to previously reported.[25]

*Ligation of Thioester **9** to Lipopeptide–Carbohydrate Templates **8a** and **8b***

Peptide **8b** (8.35 mg; 4.41 μmol) was dissolved in 1% (w/v) aqueous SDS (10 mL), frozen, and lyophilized. The obtained powder was then dissolved in 0.1 M phosphate buffer pH 7.6 (5 mL), followed by addition of TCEP (15.2 mg; 52.9 μmol) and the pH adjustment to 7.5 with 0.1 M sodium phosphate dibasic (0.8 mL). Peptide **9** (91.2 mg; 35.3 μmol) was dissolved in 0.1 M phosphate buffer pH 7.6 (4.2 mL) to which MESNA (115 mg; 710 μmol) was added. The thioester exchange was proceeded for 1 h, and then analyzed by ESI-MS (thiol exchange product **9a**: *m/z* 1256.6 $[M + 2H]^{2+}$ (calc. 1255.4), 837.5 $[M + 3H]^{3+}$ (calc. 837.3); MW 2508.77 g mol^{-1}). This solution was then transferred into the solution of lipopeptide **8b**. The ligation was performed at 37°C under an inert atmosphere and monitored by analytical RP-HPLC (solvent B1, C4 column). The ligation appeared complete within 24 h. The ligation mixture was purified by preparative RP-HPLC (30–70% solvent B1 over 40 min, C4 column). The appropriate fractions were combined and lyophilized to give **10b** (39 mg, 78%). Peptide **10a** (9.8 mg, 75%) was synthesized and purified according to the procedure as for **10b**. The characteristic data for **10a** and **10b** are shown below.

10a: HPLC: $t_R = 19.2$ min (solvent B1, C4 column), $t_R = 16.3$ min (solvent B2, C4 column); *m/z* (ESI) 1421.5 $[M + 8H]^{8+}$ (calc. 1421.5), 1263.7 $[M + 9H]^{9+}$ (calc. 1263.7), 1137.4 $[M + 10H]^{10+}$ (calc. 1137.4), 1034.1 $[M + 11H]^{11+}$ (calc. 1034.1), 948.0 $[M + 12H]^{12+}$ (calc. 948.0), 875.2 $[M + 13H]^{13+}$ (calc. 875.1); MW 11364.09 g mol^{-1}.

10b: HPLC: $t_R = 19.2$ min (solvent B1, C4 column), $t_R = 16.5$ min (solvent B2, C4 column); *m/z* 1421.5 $[M + 8H]^{8+}$ (calc. 1421.5), 1263.6 $[M + 9H]^{9+}$ (calc. 1263.7), 1137.4 $[M + 10H]^{10+}$ (calc. 1137.4), 1034.1 $[M + 11H]^{11+}$ (calc. 1034.1), 948.0 $[M + 12H]^{12+}$ (calc. 948.0), 875.1 $[M + 13H]^{13+}$ (calc. 875.1), 812.7 $[M + 14H]^{14+}$ (calc. 812.7); MW 11364.09 g mol^{-1}.

CD Measurements

CD measurements were performed on a Jasco J-710 spectropolarimeter at 23°C using a circular 0.1 cm path length quartz cell. Stock solutions of **10a**, **10b**, and **11** were prepared by dissolving the peptide in a phosphate buffer (10 mM, pH 7.3). The actual concentration of the peptide was measured by UV absorption and calculated according to the published formula based on

Tyr absorption at 280 nm.[42] Spectral measurements of the peptides **10a** and **10b** were obtained over a range of concentrations (20–33 μM). The samples **8a** and **8b** were dissolved in phosphate buffer (10 mM, pH 7.3). Blank was subtracted from the CD spectra. Content of helicity was calculated according to the published formula.[43]

Acknowledgements

This work was supported by the National Health and Medical Research Council (NHMRC) of Australia and the Australian National Heart Foundation (NHF). The author would like to acknowledge Chris Wood (University of Queensland, SCMB) for LC-ESI-MS analysis.

References

[1] M. R. Batzloff, K. S. Sriprakash, M. F. Good, *Curr. Drug Targets* **2004**, *5*, 57. doi:10.2174/1389450043490721

[2] V. A. Fischetti, *Clin. Microbiol. Rev.* **1989**, *2*, 285.

[3] E. R. Brandt, K. S. Sriprakash, R. I. Hobb, W. A. Hayman, W. G. Zeng, M. R. Batzloff, D. C. Jackson, M. F. Good, *Nat. Med.* **2000**, *6*, 455. doi:10.1038/74719

[4] P. R. Smeesters, P. Mardulyn, A. Vergison, R. Leplae, L. Van Melderen, *Vaccine* **2008**, *26*, 5835. doi:10.1016/J.VACCINE.2008.08.037

[5] E. H. Beachey, M. Bronze, J. B. Dale, W. Kraus, T. Poirier, S. Sargent, *Vaccine* **1988**, *6*, 192. doi:10.1016/S0264-410X(88)80027-6

[6] S. Pruksakorn, B. Currie, E. Brandt, C. Phornphutkul, S. Hunsakunachai, A. Manmontri, J. H. Robinson, M. A. Kehoe, A. Galbraith, M. F. Good, *Int. Immunol.* **1994**, *6*, 1235. doi:10.1093/INTIMM/6.8.1235

[7] C. Olive, I. Toth, D. Jackson, *Mini Rev. Med. Chem.* **2001**, *1*, 429. doi:10.2174/1389557013406666

[8] C. Olive, M. Batzloff, A. Horváth, T. Clair, P. Yarwood, I. Toth, M. F. Good, *Infect. Immun.* **2003**, *71*, 2373. doi:10.1128/IAI.71.5.2373-2383.2003

[9] P. M. Moyle, C. Olive, M. F. Good, I. Toth, *J. Pept. Sci.* **2006**, *12*, 800. doi:10.1002/PSC.815

[10] B. Y. Chua, E. M. Eriksson, L. E. Brown, W. Zeng, E. J. Gowans, J. Torresi, D. C. Jackson, *Vaccine* **2008**, *26*, 4866. doi:10.1016/J.VACCINE.2008.03.032

[11] A. B. M. Abdel-Aal, M. R. Batzloff, Y. Fujita, N. Barozzi, A. Faria, P. Simerska, P. M. Moyle, M. F. Good, I. Toth, *J. Med. Chem.* **2008**, *51*, 167. doi:10.1021/JM701091D

[12] Y. Fujita, A. B. Abdel-Aal, N. Wimmer, M. R. Batzloff, M. F. Good, I. Toth, *Bioorg. Med. Chem.* **2008**, *26*, 4866.

[13] C. Olive, K. Hsien, A. Horváth, T. Clair, P. Yarwood, I. Toth, M. F. Good, *Vaccine* **2005**, *23*, 2298. doi:10.1016/J.VACCINE.2005.01.041

[14] C. Olive, M. F. Ho, J. Dyer, D. Lincoln, N. Barozzi, I. Toth, M. F. Good, *J. Infect. Dis.* **2006**, *193*, 1666. doi:10.1086/504266

[15] C. Olive, S. Hsien Kuo, H. Mei-Fong, J. Dyer, A. Horváth, I. Toth, M. F. Good, *J. Infect. Dis.* **2006**, *194*, 316. doi:10.1086/505580

[16] P. M. Moyle, C. Olive, M. F. Ho, M. F. Good, I. Toth, *J. Med. Chem.* **2006**, *49*, 6364. doi:10.1021/JM060475M

[17] I. Toth, M. Danton, N. Flinn, W. A. Gibbons, *Tetrahedron Lett.* **1993**, *34*, 3925. doi:10.1016/S0040-4039(00)79265-3

[18] J. P. Tam, *Proc. Natl. Acad. Sci. USA* **1988**, *85*, 5409. doi:10.1073/PNAS.85.15.5409

[19] C. Olive, M. R. Batzloff, I. Toth, *Expert Rev. Vaccines* **2004**, *3*, 43. doi:10.1586/14760584.3.1.43

[20] L. X. Wang, J. Ni, S. Singh, *Bioorg. Med. Chem.* **2003**, *11*, 159. doi:10.1016/S0968-0896(02)00339-5

[21] J. H. Ni, R. Powell, I. V. Baskakov, A. DeVico, G. K. Lewis, L. X. Wang, *Bioorg. Med. Chem.* **2004**, *12*, 3141. doi:10.1016/J.BMC.2004.04.008

[22] J. Brask, K. J. Jensen, *Bioorg. Med. Chem. Lett.* **2001**, *11*, 697. doi:10.1016/S0960-894X(01)00035-X

[23] J. Brask, J. M. Dideriksen, J. Nielsen, K. J. Jensen, *Org. Biomol. Chem.* **2003**, *1*, 2247. doi:10.1039/B301948A

[24] P. Simerska, A. B. M. Abdel-Aal, Y. Fujita, P. M. Moyle, R. P. McGeary, M. R. Batzloff, C. Olive, M. F. Good, I. Toth, *J. Med. Chem.* **2008**, *51*, 1447. doi:10.1021/JM701410P

[25] P. M. Moyle, Y. Hari, N. Huang, C. Olive, M. F. Good, I. Toth, *Tetrahedron Lett.* **2007**, *48*, 4965. doi:10.1016/J.TETLET.2007.05.129

[26] Y. Fujita, P. M. Moyle, S. Hieu, P. Simerska, I. Toth, *Biopolymers* **2008**, *90*, 624. doi:10.1002/BIP.21002

[27] W. A. Gibbons, R. A. Hughes, M. Charalambous, M. Christodoulou, A. Szeto, A. E. Aulabaugh, P. Mascagni, I. Toth, *Liebigs Ann. Chem.* **1990**, 1175. doi:10.1002/JLAC.1990199001215

[28] Z. Györgydeak, L. Szilágyi, H. Paulsen, *J. Carbohydr. Chem.* **1993**, *12*, 139. doi:10.1080/07328309308021266

[29] C. Li, A. S. Peters, E. L. Meredith, G. W. Allman, P. B. Savage, *J. Am. Chem. Soc.* **1998**, *120*, 2961. doi:10.1021/JA973881R

[30] R. P. McGeary, I. Jablonkai, I. Toth, *Tetrahedron* **2001**, *57*, 8733. doi:10.1016/S0040-4020(01)00852-3

[31] P. Y. Hayes, B. P. Ross, B. G. Thomas, I. Toth, *Bioorg. Med. Chem.* **2006**, *14*, 143. doi:10.1016/J.BMC.2005.08.004

[32] A. R. English, D. Girard, V. J. Jasys, R. J. Martingano, M. S. Kellogg, *J. Med. Chem.* **1990**, *33*, 344. doi:10.1021/JM00163A055

[33] S. Lopez, S. S. Simons Jr, *J. Med. Chem.* **1991**, *34*, 1762. doi:10.1021/JM00110A002

[34] C. Kieburg, M. Dubber, T. K. Lindhorst, *Synlett* **1997**, 1447.

[35] A. Horváth, C. Olive, A. Wong, T. Clair, P. Yarwood, M. Good, I. Toth, *Lett. Pept. Sci.* **2002**, *8*, 285.

[36] V. K. Sarin, S. B. H. Kent, J. P. Tam, R. B. Merrifield, *Anal. Biochem.* **1981**, *117*, 147. doi:10.1016/0003-2697(81)90704-1

[37] P. E. Dawson, *Methods Enzymol.* **1997**, *287*, 34. doi:10.1016/S0076-6879(97)87005-X

[38] B. Wu, J. H. Chen, J. D. Warren, G. Chen, Z. H. Hua, S. J. Danishefsky, *Angew. Chem. Int. Ed.* **2006**, *45*, 4116. doi:10.1002/ANIE.200600538

[39] R. Balambika, T. Inui, H. Sargsyan, B. Arshava, L. S. Cohen, F. X. Ding, J. M. Becker, F. Naider, *Int. J. Pept. Res. Ther.* **2007**, *13*, 251. doi:10.1007/S10989-006-9076-6

[40] F. I. Valiyaveetil, R. MacKinnon, T. W. Muir, *J. Am. Chem. Soc.* **2002**, *124*, 9113. doi:10.1021/JA0266722

[41] T. Sato, Y. Saito, S. Aimoto, *J. Pept. Sci.* **2005**, *11*, 410. doi:10.1002/PSC.634

[42] C. N. Pace, F. Vajdos, L. Fee, G. Grimsley, T. Gray, *Protein Sci.* **1995**, *4*, 2411. doi:10.1002/PRO.5560041120

[43] P. C. Lyu, J. C. Sherman, A. Chen, N. R. Kallenbach, *Proc. Natl. Acad. Sci. USA* **1991**, *88*, 5317. doi:10.1073/PNAS.88.12.5317

[44] M. Schnolzer, P. Alewood, A. Jones, D. Alewood, S. B. Kent, *Int. J. Pept. Protein Res.* **1992**, *40*, 180.

[45] T. M. Hackeng, J. H. Griffin, P. E. Dawson, *Proc. Natl. Acad. Sci. USA* **1999**, *96*, 10068. doi:10.1073/PNAS.96.18.10068