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Method for the synthesis of highly pure vaccines using the lipid core peptide system

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Abstract: Traditional vaccines consisting of whole attenuated microorganisms, killed microorganisms, or microbial components, administered with an adjuvant (e.g. alum), have been proved to be extremely successful. However, to develop new vaccines, or to improve upon current vaccines, new vaccine development techniques are required. Peptide vaccines offer the capacity to administer only the minimal microbial components necessary to elicit appropriate immune responses, minimizing the risk of vaccination associated adverse effects, and focusing the immune response toward important antigens. Peptide vaccines, however, are generally poorly immunogenic, necessitating administration with powerful, and potentially toxic adjuvants. The attachment of lipids to peptide antigens has been demonstrated as a potentially safe method for adjuvanting peptide epitopes. The lipid core peptide (LCP) system, which incorporates a lipidic adjuvant, carrier, and peptide epitopes into a single molecular entity, has been demonstrated to boost immunogenicity of attached peptide epitopes without the need for additional adjuvants. The synthesis of LCP systems normally yields a product that cannot be purified to homogeneity. The current study describes the development of methods for the synthesis of highly pure LCP analogs using native chemical ligation. Because of the highly lipophilic nature of the LCP lipid adjuvant, difficulties (e.g. poor solubility) were experienced with the ligation reactions. The addition of organic solvents to the ligation buffer solubilized lipidic species, but did not result in successful ligation reactions. In comparison, the addition of approximately 1% (w/v) sodium dodecyl sulfate (SDS) proved successful, enabling the synthesis of two highly pure, tri-epitopic *Streptococcus pyogenes* LCP analogs. Subcutaneous immunization of B10.BR (H-2^k) mice with one of these vaccines, without the addition of any adjuvant, elicited high levels of systemic IgG antibodies against each of the incorporated peptides. Copyright © 2006 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: adjuvants; group A streptococcus; lipid core peptide; lipoamino acids; lipopeptides; native chemical ligation; sodium dodecyl sulfate; vaccine development

INTRODUCTION

Vaccination has been proved to be one of the most successful and cost-effective public health interventions [1]. Traditionally, vaccines have been produced using live attenuated microorganisms, killed microorganisms, or microbial components (e.g. diphtheria toxoid), with alum as an adjuvant. The capacity to develop vaccines against many diseases for which vaccines are currently unavailable necessitates new vaccine development strategies. Subunit vaccines represent such a technology, where individuals are immunized with only the minimal microbial components necessary to elicit protective immune responses, bypassing the administration of unnecessary bacterial components which may cause adverse effects [2].

Peptide vaccines represent a subunit vaccine approach whereby B- and T-cell epitopes are administered to elicit protective immune responses [3]. They offer many advantages over traditional vaccines including lot-to-lot consistency, high purity, the capacity to be chemically defined, and stability in lyophilized form, negating any requirement for refrigerated storage and transportation [2–4]. Peptides, however, are poorly immunogenic and lack the T-helper epitopes needed to elicit immune responses in an outbred population, and therefore may require conjugation to a carrier protein (e.g. bovine serum albumin) to provide T-cell help, as well as administration with powerful adjuvants to render them immunogenic [3].

The conjugation of synthetic or bacterial lipids to peptide epitopes has been demonstrated to provide adjuvant activity for the production of cellular or antibody mediated immune responses [4]. Examples include the conjugation of single chain lipids such as palmitic acid [5], or clustered lipids such as tripalmitoyl-*S*-glyceryl cysteine (Pam_3Cys) [6]. Clinical trials of lipopeptide vaccines have demonstrated a high degree of safety with few or no side effects reported [5,7], and furthermore, peptide lipidation may allow for immunization via mucosal routes (e.g. nasally or orally) [8].

Previously we have described the synthesis of experimental *Streptococcus pyogenes* lipopeptide vaccines [9–16] using the lipid core peptide (LCP) system [17] (Figure 1), a vaccine development system that incorporates a lipidic adjuvant, carrier, and peptide epitopes into a single molecular entity. This system

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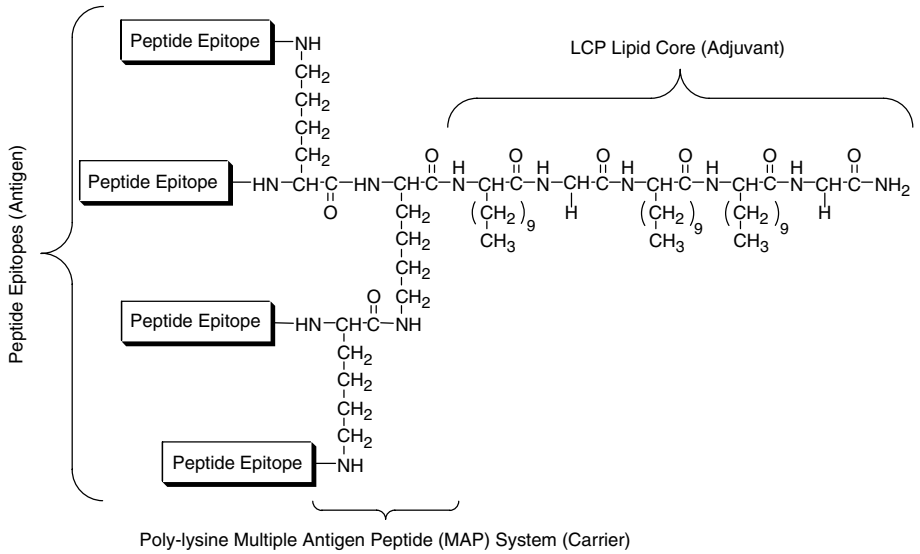

Figure 1 Schematic representation of the lipid core peptide (LCP) system.

uses synthetic lipoamino acids [18] (usually 2-amino-D,L-dodecanoic acid (C12)) to mimic the structure of Pam_3Cys and to provide adjuvant activity, while a multiple antigen peptide (MAP) system [19] is used to allow the conjugation of multiple copies of a single peptide epitope, or several different peptide epitopes. In many studies [9–16], vaccines synthesized using the LCP system have been demonstrated to be immunogenic without the need for additional adjuvants.

Peptide vaccine development normally requires the incorporation of multiple B-cell, T-helper, and cytotoxic lymphocyte epitopes [20], particularly where high sequence variability exists between strains (e.g. human immunodeficiency virus, hepatitis B or C viruses, and malaria) [21]. Many techniques have been tried for the development of multiepitopic vaccines including the polymerization of purified peptide epitopes [22,23], the synthesis of linear peptides incorporating multiple peptide epitopes (polyepitopes/oligoepitopes) [21,24], DNA vaccines encoding for multiple peptide epitopes [25], or the expression of proteins containing multiple peptide epitopes [21,26]. The LCP system offers several advantages over these techniques. For example, the LCP system enables the incorporation of unnatural components (e.g. lipids and sugars) or unnatural structural features (e.g. branching) into the vaccine structure. Furthermore, compared with polymerization, the order of antigen inclusion and the vaccine size can be precisely controlled. However, a major problem that has prevented the use of LCP-based vaccines in human clinical trials is the difficulty in purification to homogeneity. This is associated with their synthesis by stepwise SPPS and the incorporation of a MAP system. Therefore, an aim of this research was to develop methods for the synthesis of highly pure LCP analogs that could be used to develop multiepitopic lipopeptide vaccines for possible use in human clinical trials.

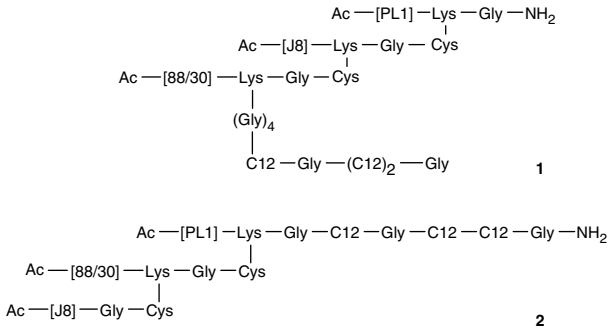

Figure 2 Structures of tri-epitopic lipopeptide vaccines synthesized using native chemical ligation.

The current paper describes techniques that were developed to synthesize highly pure LCP analogs. The synthesis of two LCP analogs (**1** and **2**; Figure 2) using native chemical ligation is described. These analogs were designed to incorporate a highly conserved *S. pyogenes* antigen (J8) [27], as well as two species-specific *S. pyogenes* antigens (PL1 [28] and 88/30 [23]). To demonstrate the self-adjuvanting activity of these systems, analog **2** was administered subcutaneously to B10.BR mice without additional adjuvants, with systemic IgG antibody titers against each of the incorporated peptide epitopes reported.

MATERIALS AND METHODS

Amino acids and *p*-methylbenzhydrylamine (*p*MBHA) resin were purchased from Novabiochem (Läufelfingen, Switzerland) or Reanal (Budapest, Hungary). Peptide synthesis grade DMF, TFA, DCM, and HBTU were purchased from Auspep (Melbourne, Australia). HPLC grade acetonitrile (MeCN) was purchased from Labscan (Dublin, Ireland). All other reagents were purchased from Sigma-Aldrich (Castle Hill, Australia) at the highest available purity. The synthetic lipoamino acid

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2-(*t*-butoxycarbonylamino)-D,L-dodecanoic acid (Boc-C12-OH) was synthesized as previously described [18]. ESI-MS was performed on a Perkin Elmer-Sciex API3000 instrument using MeCN-water mobile phases containing 0.1% (v/v) formic acid. ESI-MS data was acquired using Analyst 1.4 (Applied Biosystems/MDS Sciex, Canada) software. Analytical RP-HPLC was performed using Shimadzu instrumentation (Class Vp 6.12 software, SCL-10AVp controller, SIL-10A auto-injector, LC-10AT pump, LC-10AD pump, Waters 486 tunable absorbance detector). Analytical RP-HPLC was performed in gradient mode using 0.1% TFA/H_2O as solvent A, and 90% MeCN/0.1% TFA/H_2O as solvent B. Analysis was run at 1 ml/min with detection at 214 nm. Separation was achieved on either a Vydac analytical C18 column (218TP54; 4.6 × 250 mm) or a Vydac analytical C4 column (214TP54; 4.6 × 250 mm) using a gradient of 0–100% solvent B over 30 min.

Peptide Synthesis

Peptide building blocks (3–8; Table 1) were synthesized by manual stepwise SPPS on *p*MBHA resin using HBTU/DIPEA *in situ* neutralization [29] and Boc-chemistry. The sequences of the peptide epitopes used in this study are described in Table 1. Thioester peptides (**5**–**8**) were synthesized using the trityl-associated mercaptopropionic acid leucine (TAMPAL) linker [30]. Coupling yields were monitored using the quantitative ninhydrin test [31] or the chloranil test [32,33]. Where necessary, couplings were repeated to give coupling yields greater than 99.7%. The following Boc-protected amino acids were used: Arg(Tos), Asn(Xan), Asp(OcHx), Cys(Acm), Cys[Bzl(Me)], Gln(Xan), Glu(OcHx), Lys[Z(2Cl)], Lys(Fmoc), Ser(Bzl), Thr(Bzl), Tyr[Z(2Br)]. Acetylation was performed by treating the resin with a mixture of acetic anhydride (0.5 ml; 5.29 mmol), DIPEA (0.47 ml; 2.70 mmol) and DMF (14 ml) for 5 min, and repeating for 30 min. Following TAMPAL linker synthesis, the Trt-group was removed as previously described [30], with the thioester bond formed by two 1 h couplings of Boc-Gly-OH (10 eq) using standard HBTU/DIPEA *in situ* neutralization [29] to give Boc-Gly-TAMPAL resin. The orthogonally protected amino acid Boc-Lys(Fmoc)-OH was used (for peptides **3**–**7**) to enable branching. Following the synthesis of the peptide epitope of interest on the α-amine, and acetylation of the amino-terminus, the Lys ε-amine Fmoc

Table 1 Lipopeptide vaccine building block structures and analytical RP-HPLC data

Ac—[PL1]—Lys—Gly—X
Cys

Compound no.	Substituent X	Purification (RP-HPLC)	Yield (%)	RP-HPLC column	RP-HPLC t_R (min)	RP-HPLC purity (%)
3[a]	-NH_2	10–60% B 60 min[d]	12.1	C18	13.5	97.9
4[a]	-C12-Gly-$(C12)_2$-Gly-NH_2	35–95%B 60 min[e]	29.8	C4	23.6	99.5

Ac—[Epitope]—Lys—Gly—S—Leu—NH_2
X

Compound no.	Epitope	Substituent X	Purification (RP-HPLC)	Yield (%)	RP-HPLC Column	RP-HPLC t_R (min)	RP-HPLC purity (%)
5[b]	J8	-Cys(Acm)	10–60% B 60 min[d]	7.0	C18	16.2	98.3
6[c]	88/30	-Cys(Acm)	10–60% B 60 min[d]	10.2	C18	15.6	98.1
7[c]	88/30	-$(Gly)_4$-C12-Gly-$(C12)_2$-Gly	30–90% B 45 min[e]	11.7	C4	21.5	97.5

Ac—[Epitope]—Gly—S—Leu—NH_2

Compound no.	Epitope	Purification (RP-HPLC)	Yield (%)	RP-HPLC Column	RP-HPLC t_R (min)	RP-HPLC purity (%)
8[b]	J8	10–60% B 60 min[d]	24.8	C18	16.8	99.9

[a] The PL1 peptide sequence is EVLTRRQSQDPKYVTQRIS.
[b] The J8 peptide sequence is QAEDKVKQSREAKKQVEKALKQLEDKVQ.
[c] The 88/30 peptide sequence is DNGKAIYERARERALQELGP.
[d] Product purified by preparative RP-HPLC on a Vydac C18 column (218TP1022; 10 μm; 250 × 10 mm) at a 10 ml/min flow-rate.
[e] Product purified by preparative RP-HPLC on a Vydac C4 column (214TP1022; 10 μm; 250 × 10 mm) at a 10 ml/min flow-rate.

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protecting group was removed (where applicable) using 20% piperidine in DMF for nonthioester peptides (**3** and **4**), or the thioester sparing cocktail of Li *et al.* [34] for thioester peptides (**5**–**7**). Prior to hydrogen fluoride (HF) cleavage, peptidyl-resins were washed with DMF, DCM, and methanol, and then dried under vacuum. HF cleavage (10 ml HF/g resin) was performed for 2 h at 0 °C. For thioester peptides **5**–**8**, 7% (v/v) *p*-cresol was used as a scavenger. For cysteinyl peptides **3** and **4**, 5% (v/v) *p*-thiocresol and 5% (v/v) *p*-cresol were used as scavengers. Following HF cleavage, the HF was removed under reduced pressure, and the peptides precipitated with ice-cold diethyl ether, filtered, and dissolved in 40% (v/v) aqueous MeCN containing 0.1% TFA and lyophilized.

Native Chemical Ligation

Prior to ligation, lipoamino acid containing peptides (**4**, **7**, **12**; Tables 1 and 2) were dissolved in double distilled water (ddH_2O) containing sodium dodecyl sulfate (SDS), frozen, and lyophilized. The quantity of SDS utilized was sufficient to ensure an SDS concentration of approximately 1% (w/v) during the ligation reaction. Native chemical ligation was performed in 0.1 M phosphate buffer pH 7.6 at room temperature with peptide concentrations between 0.8 mM and 1.9 mM (Table 2). Prior to ligation, thioester peptides (**5**–**8**) were incubated with ten equivalents of sodium 2-mercaptoethanesulfonate (MESNA) in 0.1 M phosphate buffer pH 7.6 for 1 h. The cysteinyl peptide (**3**, **4**, **10**, or **12**) was then added to this solution along with three equivalents of tris(2-carboxyethyl)phosphine hydrochloride (TCEP), and sufficient 0.2 M sodium phosphate dibasic to adjust the pH to 7.5. Ligation reactions were monitored by RP-HPLC for consumption of the thioester and cysteinyl peptides. The time taken for each ligation reaction to complete is described in Table 2.

Acm Deprotection Using $Hg(II)(OAc)_2$

$Hg(II)(OAc)_2$ (10 eq/Acm) was added to a 1.7 mM solution of **9** in acidified ddH_2O [pH 4; acetic acid (AcOH)]. After 1 h, β-mercaptoethanol (BME; 665 eq/Acm) was added and the reaction left for 5 h. The product **10** was then purified by gel filtration on a Sephadex G-25 column eluting with acidified ddH_2O (pH 4; AcOH).

Acm Deprotection Using I_2

Peptide **11** was dissolved in 15 : 6 : 4 : 15 (v/v/v) AcOH/MeCN/1 M HCl/ddH_2O to give a 0.17 mM solution. To this solution, 0.1 M I_2 in 50% (v/v) aqueous AcOH (10eq/Acm) was added. The reaction was then left to proceed for 1.5 h, followed by the dropwise addition of 1 M aqueous ascorbic acid until the solution was colorless. TCEP (5eq/Cys) was then added to the solution prior to purification of the product **12** by preparative RP-HPLC (Table 2).

Chromatography and Characterization

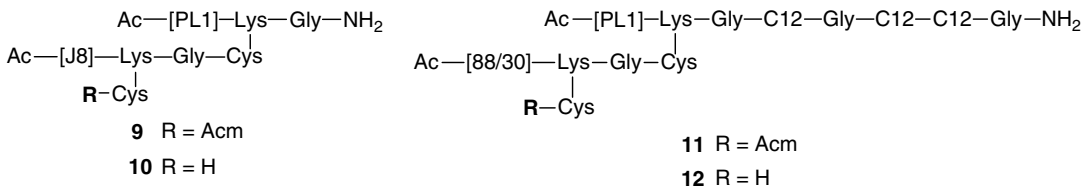
Peptides were purified by RP-HPLC using a Waters Delta 600 system with detection at 230 nm. Purification was performed in gradient mode using 0.1% TFA/H_2O as solvent A, and 90% MeCN/0.1% TFA/H_2O as solvent B. The columns and flow-rates used are described in Tables 1 and 2. Following purification, peptides were characterized by analytical RP-HPLC (see Section on Material and Methods; Tables 1 and 2) and ESI-MS (Table 3). The expected multiply charged species were observed for each peptide by ESI-MS, with data for vaccines **1** and **2** reported in Table 3, as well as Figures 3 and 4.

Immunization and Collection of Sera

Immunization was performed as previously described [35]. Four-to-six week old female B10.BR mice ($H\text{-}2^k$) (Animal

Table 2 Native chemical ligation and Acm deprotection data

Reaction (conc. mM)	Product	Reaction time (h)	SDS	Purification (RP-HPLC)	Yield (%)	RP-HPLC column	RP-HPLC t_R (min)	RP-HPLC purity (%)
3 (1.5) + **5** (1.4)	**9**	5	No	10–60% B 45 min[a]	65.3	C18	15.7	98.6
9 + $Hg(II)(Oac)_2$	**10**	1	No	N/A	63.3	C18	15.9	97.6
10 (0.9) + **7** (1.0)	**1**	48	Yes	0–70% B 60 min[a]	41.3	C4	18.7	97.8
4 (1.9) + **6** (1.3)	**11**	2	Yes	15–70% B 60 min[b]	83.9	C4	22.6	99.2
11 + I_2	**12**	1.5	No	0–100% B 30 min[b]	84.5	C4	21.8	99.1
12 (0.8) + **8** (1.2)	**2**	20	Yes	10–85% B 60 min[b]	61.1	C4	18.7	97.7



[a] Product purified by semipreparative RP-HPLC on a Vydac C4 column (214TP1010; 10 μm; 250 × 10 mm) at a 4 ml/min flow-rate.
[b] Product purified by preparative RP-HPLC on a Vydac C4 column (214TP1022; 10 μm; 250 × 10 mm) at a 10 ml/min flow-rate.

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Table 3 ESI-MS data for lipopeptide vaccines **1** and **2**

Compound no.	ESI-MS	Molecular weight (g/mol)
1	$[M + 5H^+]^{5+}$ m/z 1933.7 (calc 1932.8), $[M + 6H^+]^{6+}$ m/z 1611.4 (calc 1610.8), $[M + 7H^+]^{7+}$ m/z 1381.6 (calc 1380.9), $[M + 8H^+]^{8+}$ m/z 1208.7 (calc 1208.4), $[M + 9H^+]^{9+}$ m/z 1074.8 (calc 1074.2).	9659.1
2	$[M + 5H^+]^{5+}$ m/z 1862.8 (calc 1861.5), $[M + 6H^+]^{6+}$ m/z 1552.7 (calc 1551.5), $[M + 7H^+]^{7+}$ m/z 1330.6 (calc 1330.0), $[M + 8H^+]^{8+}$ m/z 1164.5 (calc 1163.8), $[M + 9H^+]^{9+}$ m/z 1035.5 (calc 1034.6), $[M + 10H^+]^{10+}$ m/z 932.4 (calc 931.3).	9302.7

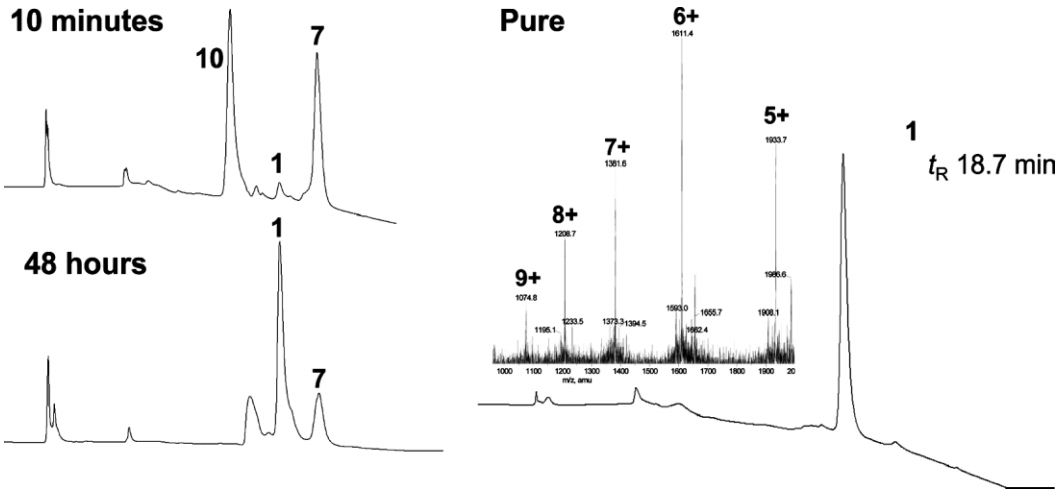

Figure 3 Native chemical ligation of peptides **7** and **10** to give lipopeptide vaccine **1**. Analytical RP-HPLC of the ligation reaction at 10 min and 48 h is depicted, along with ESI-MS spectra and analytical RP-HPLC of **1** following purification. Separation was achieved on a Vydac analytical C4 column (214TP54; 4.6 × 250 mm).

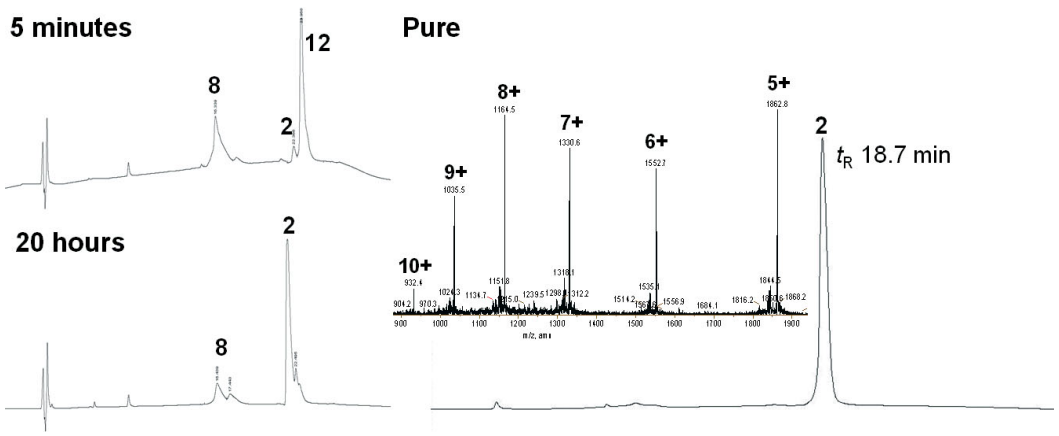

Figure 4 Native chemical ligation of peptides **8** and **12** to give lipopeptide vaccine **2**. Analytical RP-HPLC of the ligation reaction at 5 min and 20 h is depicted, along with ESI-MS spectra and analytical RP-HPLC of **2** following purification. Separation was achieved on a Vydac analytical C4 column (214TP54; 4.6 × 250 mm).

Resource Centre, Australia) were immunized at the tail base on day 0 with 30 µg of **2** emulsified 1 : 1 with Freund's complete adjuvant (CFA) or administered in 50 µl of sterile-filtered phosphate buffered saline (PBS). The mice received 3 µg boosts of **2** in PBS on days 21, 28, 35, 42, and 49. One week after the last immunization, blood was collected from the tail artery of each mouse. The blood was then left to clot at 37 °C for 1 h, and then centrifuged for 10 min at 3000 RPM to remove clots. The sera were then stored at −20 °C.

Enzyme-Linked Immunosorbent Assay (ELISA)

Enzyme-linked immunosorbent assays (ELISAs) were performed as previously described [27,35]. Antigens (J8, 88/30,

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and PL1) were diluted with carbonate coating buffer pH 9.6 to give 5 μg/ml solutions. Each antigen (100 μl/well) was then coated onto polyvinyl chloride microplates (ICN, Irvine, CA) at 4 °C overnight. The antigen solution was then removed and the plates blocked with 5% skim milk PBS for 90 min at 37 °C. The plates were then washed five times with 0.05% Tween 20-PBS. Serial dilutions of sera were produced in 0.5% skim milk PBS-Tween 20 buffer, starting at 1 : 100 concentration with twofold dilutions. Antibody titers were assessed following the addition of peroxidase-conjugated goat anti-mouse IgG, and *O*-phenylenediamine. Optical density was read at 450 nm in a microplate reader. The antibody titer was defined as the lowest dilution with an optical density more than three standard deviations greater than the mean absorbance of control wells containing normal mouse serum.

RESULTS AND DISCUSSION

Vaccines **1** and **2** were synthesized using native chemical ligation [36]. This technique involves a chemoselective reaction between two unprotected peptides, one containing an amino (*N*)-terminal Cys residue, and the other containing a carboxyl (C)-terminal thioester. The reaction is performed at approximately neutral pH in aqueous buffers, and results in the formation of a native peptide bond. To synthesize these vaccines, appropriate building blocks (**3**–**8**) were produced using stepwise SPPS (Table 1). The first building blocks (**3**, **4**) needed to incorporate an *N*-terminal Cys residue, to enable the ligation of further peptide epitopes. As we wanted to mimic the branched structure of the LCP system, the *N*-terminal Cys residue was incorporated near the *C*-terminus off a Lys ε-amine. This was achieved by incorporating an ε-Fmoc protected Lys residue into the peptide, which was subsequently deprotected using 20% (v/v) piperidine in DMF after synthesizing the PL1 peptide and acetylating the *N*-terminus. This provided an amine onto which Boc-Cys[Bzl(Me)]-OH could be coupled, to provide a deprotected *N*-terminal Cys residue following HF cleavage. For peptides **5**–**7** the same technique was used to provide branching; however, as these peptides were synthesized on the TAMPAL thioester linker [30], piperidine could not be used for Fmoc deprotection as it would result in aminolysis of the thioester. The thioester sparing cocktail of Li *et al.* [34] was therefore used for Fmoc deprotection of **5**–**7** following peptide epitope synthesis and acetylation of the *N*-terminal amine. While Li *et al.* [34] state that a 2.9 min treatment, followed by a 18 min treatment with the Fmoc deprotection cocktail was sufficient for Fmoc deprotection, we found that the second treatment needed to be extended to 1 h for complete Fmoc deprotection of our peptides.

Chemical ligation of building blocks **3**–**8** (Table 2) was performed in 0.1 M phosphate buffer pH 7.6 with MESNA used as a thiol additive and TCEP added to keep Cys residues in the reduced form. Using these conditions, peptides **3** and **5** were successfully ligated to give di-peptide **9** (Table 2). However, these conditions were not suitable for the ligation of peptides **4** and **6**. This was because of the poor solubility of lipopeptide **4** in 0.1 M phosphate buffer. Initially the addition of organic solvents (e.g. TFE, MeCN, DMF, and dioxane) to 0.1 M phosphate buffer was tried for improving the solubility of the lipidic peptides (**4**, **7**, **12**). While this improved the solubility of these peptides in the aqueous buffer, little to no ligation was observed to occur over a 24-h period. This was most likely because of effects of organic solvents on protein hydration. Organic solvents are known to strip water molecules from proteins as well as promoting a more compact structure [37–39]. These effects would likely combine to increase steric hindrance, preventing the reaction from succeeding. As organic solvents were not suitable for these reactions, detergents were tried for solubilizing the lipophilic peptides. Mixed micelles were formed by dissolving the lipophilic peptides in water containing approximately 1% (w/v) SDS [40]. This solution was then freeze dried, and the resulting powder rehydrated with 0.1 M phosphate buffer pH 7.6. Using these conditions we were able to successfully ligate lipopeptide **4** and thioester peptide **6** to give di-peptide **11** in 83.9% yield following purification.

To ligate peptides **7** and **8** to the di-epitopic peptides **9** and **11**, respectively, removal of the Acm-protecting groups on peptides **9** and **11** was required. For peptide **9**, mercury (II) acetate was used for Acm deprotection. This yielded an Acm deprotected mercury salt of peptide **9**. A large excess of BME was then added to the solution to precipitate the mercury. Despite the large excess of BME used, precipitation of the mercury was slow, taking 5 h. The Acm deprotected peptide **10** was then purified by gel filtration on a Sephadex G-25 column. In comparison, attempts to remove the Acm-protecting group of **11** with mercury (II) acetate proved difficult. While Acm deprotection was rapid, taking less than 1 h, the formed mercury salt could not be disrupted by the addition of large amounts of thiol. Silver (I) salts were therefore tried for Acm deprotection as silver has a lower affinity for thiols than mercury, and thus disruption of the silver salt using thiols would be expected to be easier. However, silver salts proved incapable of removing the Acm-protecting group in our hands. Iodine was then tried for removing the Acm group of **11**. Iodine is normally used for simultaneous Acm removal and oxidation to give disulfide bonds. However, in this case iodine proved capable of rapidly cleaving the Acm-protecting groups (<1.5 h), without any oxidation observed by ESI-MS or RP-HPLC.

Following Acm deprotection, peptides **7** and **8** were ligated to dipeptides **10** and **12,** respectively (Table 2). In both cases the ligation reactions were performed in the presence of 1% (w/v) SDS to solubilize lipopeptides **7** and **12** as well as the ligation products (**1**, **2**). Overall, lipopeptide vaccines **1** and **2**, which were highly pure

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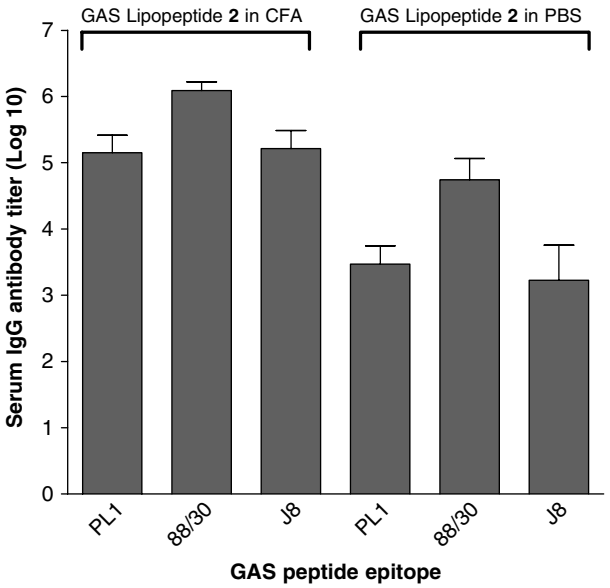

Figure 5 Systemic antigen-specific IgG antibody titers (day 49) elicited in response to immunization of mice with lipopeptide vaccine **2** in CFA or in PBS. The average systemic IgG antibody titers are reported with the standard deviation indicated.

(Table 2 and 3; Figures 3 and 4), were obtained in 17.1% and 44.1% overall yields, respectively.

Vaccine **2** was assessed for its capacity to elicit antibodies against each of the attached peptide epitopes following administration to B10.BR (H-2k) mice ($n = 10$). The mice were immunized at the tail base with 30 μg of **2** in PBS (50 μl total volume) or emulsified 1 : 1 with CFA as a positive control. The PL1, 88/30, and J8 peptides were not administered as negative controls, as previous studies have demonstrated that these peptides are not able to elicit antigen-specific IgG antibodies without the addition of adjuvants [11,15,16]. Three weeks after priming, the mice received five 3 μg boosts of **2** in PBS at weekly intervals. Prior to each boost, and 1 week after the last boost, sera were collected to assess the levels of serum antigen-specific IgG antibodies elicited in response to immunization using an ELISA. The study demonstrated that antigen-specific systemic IgG antibodies were elicited against each of the peptide epitopes in both the group administered **2** in CFA and the group administered **2** in PBS (Figure 5).

CONCLUSIONS

Previous studies have demonstrated that LCP systems synthesized using stepwise SPPS are capable of eliciting antigen-specific antibodies against attached peptide epitopes without the need for additional adjuvants [9–16]. The capacity to synthesize these vaccines in a highly pure, well-characterized state, however, would be required for their possible use in human trials. The current paper has described a technique for the synthesis of highly pure LCP analogs. Using native chemical ligation, two highly pure tri-epitope *S. pyogenes* LCP analogs were synthesized. The use of SDS to solubilize lipophilic species during ligation reactions was critical for the success of this technique. In comparison, the use of organic solvents to solubilize lipidic species in aqueous buffers was not successful. Immununological assessment of one of the tri-epitopic vaccines demonstrated its capacity to elicit high titer systemic IgG antibodies against each of the attached peptide epitopes without the need for additional adjuvants. Overall, this work has provided a technique that could be used for the development of highly pure, multiepitopic lipopeptide vaccines for the prevention or treatment of many diseases.

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