Synthesis of a Highly Pure Lipid Core Peptide Based Self-Adjuvanting Triepitopic Group A Streptococcal Vaccine, and Subsequent Immunological Evaluation

Peter M. Moyle,[†] Colleen Olive,[‡] Mei-Fong Ho,[‡] Michael F. Good,[‡] and Istvan Toth[†,§,*]

School of Pharmacy, The University of Queensland, St. Lucia 4072, Queensland, Australia, School of Molecular and Microbial Sciences (SMMS), The University of Queensland, St. Lucia 4072, Queensland, Australia, and The Queensland Institute of Medical Research (QIMR), Herston QLD 4029, Australia

Received April 23, 2006

We have developed a highly pure, self-adjuvanting, triepitopic Group A Streptococcal vaccine based on the lipid core peptide system, a vaccine delivery system incorporating lipidic adjuvant, carrier, and peptide epitopes into a single molecular entity. Vaccine synthesis was performed using native chemical ligation. Due to the attachment of a highly lipophilic adjuvant, addition of 1% (w/v) sodium dodecyl sulfate was necessary to enhance peptide solubility in order to enable ligation. The vaccine was synthesized in three steps to yield a highly pure product (97.7% purity) with an excellent overall yield. Subcutaneous immunization of B10.BR (H-2^k) mice with the synthesized vaccine, with or without the addition of complete Freund's adjuvant, elicited high serum IgG antibody titers against each of the incorporated peptide epitopes.

Introduction

Streptococcus pyogenes (group A streptococcus; GAS) is a common human pathogen, associated with a broad spectrum of diseases including impetigo (an infection of the skin) and pharyngitis (strep throat).[1] Following GAS infection, particularly where patients are not treated adequately, immune-mediated post-streptococcal sequelae (e.g. glomerulonephritis or rheumatic fever) may develop. Rheumatic fever (RF) is associated with the production of antibodies and T cells against GAS, which cross-react with human tissues of the heart, joints, and brain.[1] The associated inflammation of the heart valves and pericardium may lead to rheumatic heart disease (RHD) and eventually heart failure. Because RF and RHD only occur following GAS infection, the development of a prophylactic GAS vaccine holds the potential to greatly reduce the incidence of these diseases.

Numerous research groups[2−4] are working toward the development of prophylactic GAS vaccines, with the most advanced vaccine development strategies focusing on the GAS M protein. The M protein is an α-helical coiled-coil cell surface protein, which is associated with resistance to complement-mediated phagocytosis.[5] The M protein amino (N)-terminal domain is highly variable between GAS strains.[1] Antibodies elicited to N-terminal epitopes are type-specific, opsonic, and form the basis for GAS serotyping.[6] As over 100 different GAS serotypes have been characterized, vaccines based upon GAS M protein N-terminal peptides need to be multivalent, incorporating appropriate N-terminal epitopes to prevent infection with common circulating strains.[1] In comparison, the carboxyl (C)-terminal M protein (C-repeat) region is highly conserved among GAS strains,[2] offering the potential to develop vaccines capable of protecting against infection caused by many different GAS serotypes. However, the C-terminal region has been demonstrated to contain epitopes which are capable of eliciting autoreactive antibodies and T cells.[7,8] Thus, the use of whole M protein or M protein components for vaccine development may result in RF. Investigations have therefore focused on identifying C-terminal sequences devoid of potentially deleterious T and B cell epitopes. An example is the J8 peptide[9] (QAEDKVKQ**SREAKKQVEKAL**KQLEDKVQ), which contains an M protein C-terminal minimal B cell epitope (in bold) enclosed within peptide sequences designed to maintain the α-helical conformation of the native M protein.

Previously we have described the development of experimental M protein based GAS vaccines utilizing the J8 peptide, and N-terminal epitopes from GAS strains common to the Australian aboriginal populations of the Northern Territory and northern Queensland.[10−17] These vaccines were developed using the lipid core peptide (LCP) system,[18] a vaccine delivery system incorporating peptide epitopes, a lipidic adjuvant (the LCP lipid core; Figure 1), and a carrier into a single molecular entity. These systems were synthesized using solid-phase peptide synthesis (SPPS) and incorporated in up to four different GAS M protein epitopes. Subcutaneous immunization of mice in general elicited high-titer systemic IgG antibodies against each of the incorporated epitopes without the need for additional adjuvants.[10−16] Furthermore, the elicited antibodies have been demonstrated to protect mice against intraperitoneal challenge with GAS serotypes for which N-terminal M protein epitopes were included in the vaccine.[13] Despite these promising results, LCP systems synthesized using SPPS are generally not suitable for use in human clinical trials due to difficulties obtaining highly pure LCP systems. Attempts have therefore been made to synthesize analogues of the LCP system using native chemical ligation in order to provide highly pure LCP analogues suitable for use in humans.

Previously we have published the synthesis, using native chemical ligation, of a branched peptide containing three GAS M protein derived peptides.[10] Two of these peptides, 88/30 (DNGKAIYERARERALQELGP) and PL1 (EVLTRRQSQDPKYVTQRIS), are N-terminal peptides from GAS strains common to the Australian aboriginal populations of the Northern Territory and northern Queensland.[19] The other peptide was the previously described J8 peptide.[9] As the vaccine did not feature an inbuilt adjuvant, immunization of B10.BR mice failed to elicit systemic IgG antibodies unless the vaccine was administered with an adjuvant (e.g. complete Freund's adjuvant (CFA)). Methods for incorporating the LCP lipid core into this vaccine using native chemical ligation were therefore investigated. This

* To whom correspondence should be addressed. Phone: +61 (7) 3346 9892. Fax: +61 (7) 3365 1688. E-mail: i.toth@uq.edu.au.
[†] School of Pharmacy, The University of Queensland.
[‡] The Queensland Institute of Medical Research.
[§] SMMS, The University of Queensland.

10.1021/jm060475m CCC: $33.50
Published on Web 09/26/2006

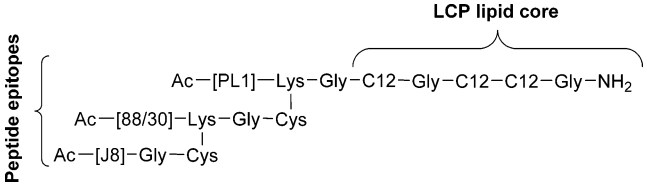
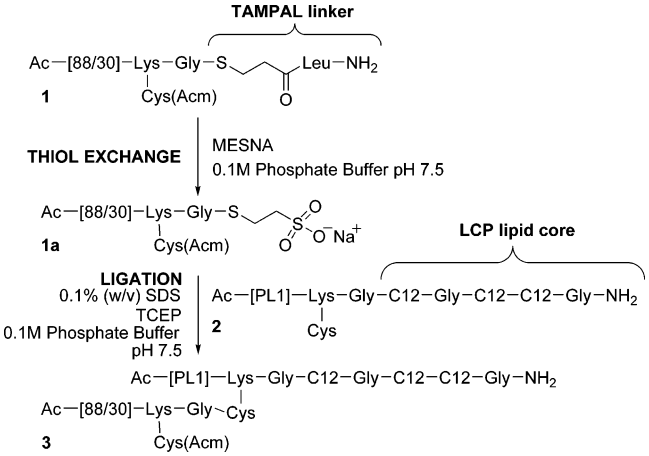


Figure 1. Structure of the synthesized triepitopic lipopeptide vaccine. The vaccine incorporates N-terminal serotype-specific epitopes from PL1 and 88/30 GAS strains, as well as the J8 peptide, and a built-in adjuvant (the LCP lipid core) synthesized using multiple copies of the synthetic lipidic amino acid 2-amino-D,L-dodecanoic acid (C12) with glycine used as a spacer.

Scheme 1. Synthesis of the Branched Diepitopic Lipopeptide **3** Containing Serotype-Specific N-Terminal M Protein Epitopes from PL1 and 88/30 GAS Serotypes Using Native Chemical Ligation



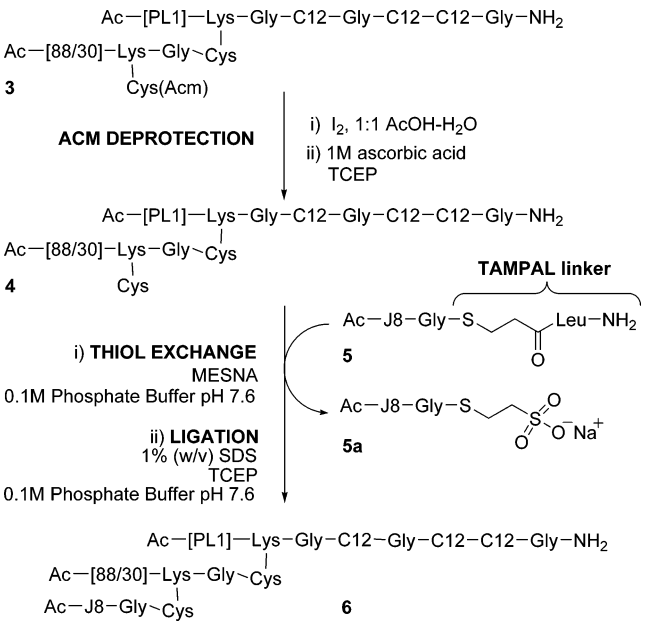
lead to the development of a technique utilizing sodium dodecyl sulfate (SDS) to enable ligation of LCP lipid core conjugated peptide epitopes onto other GAS epitopes.

In the present study, the synthesis of a highly pure, highly characterized LCP analogue (Figure 1) using native chemical ligation in the presence of SDS and incorporating the LCP lipid core at the C-terminus is described. The aim of this study was to synthesize a triepitopic LCP analogue using native chemical ligation, incorporating the J8, 88/30, and PL1 epitopes. Herein describes the synthesis of appropriate peptide building blocks, and the ligation conditions utilized for vaccine synthesis. Assessment of the vaccine's α-helical content using circular dichroism (CD) is then reported. This was performed as the J8 peptide contains a conformational B cell epitope, which needs to adopt an α-helical conformation in order to elicit antibodies. Subcutaneous immunization of B10.BR (H-2^k) mice and assessment of the vaccine's capacity to elicit high-titer antigen-specific systemic IgG antibodies against each of the attached peptide epitopes are then described.

Results and Discussion

Peptide building blocks **1**, **2**, and **5** (Schemes 1 and 2) were synthesized using in situ neutralization *t*-butoxycarbonyl (Boc)-chemistry[20] on *p*-methylbenzhydrylamine (*p*MBHA) resin. The trityl-associated mercaptopropionic acid leucine (TAMPAL) linker of Hackeng et al.[21] was incorporated at the C-terminus of **1** and **5** (Schemes 1 and 2) to enable their participation in native chemical ligation. In comparison, the LCP lipid core [C12-Gly-$(C12)_2$-Gly] was incorporated at the C-terminus of **2** (Scheme 1) to act as an inbuilt adjuvant, and it was synthesized using multiple copies of the synthetic lipidic amino acid

Scheme 2. Conjugation of the J8 Antigen, Containing a Conserved C-Terminal GAS M Protein B Cell Epitope, onto the Diepitopic Lipopeptide **3** Using Native Chemical Ligation



2-amino-D,L-dodecanoic acid[22] (C12) and glycine spacers. For **1**, **2**, and **5**, a glycine residue was coupled following TAMPAL linker or LCP lipid core synthesis. For **1** and **2**, this was followed by the coupling of an N^{ϵ}-fluorenylmethoxycarbonyl (Fmoc) protected lysine residue to enable branching. Peptide epitopes were then synthesized on the lysine (**1**, **2**) or glycine α-amine (**5**), followed by acetylation of the terminal amine. For **1** and **2**, the lysine N^{ϵ}-Fmoc protecting group was then removed. This was achieved using 20% piperidine in *N*,*N*-dimethylformamide (DMF) for **2**, or the thioester sparing cocktail of Li et al.[23] for **1**. Following Fmoc-deprotection of **2**, Boc-Cys(pMeBzl)-OH was coupled to the lysine ϵ-amine to provide a cysteine residue onto which **1** could be ligated. In comparison, Boc-Cys(Acm)-OH was coupled to the lysine ϵ-amine of **1**. This was to prevent cyclization due to intramolecular native chemical ligation, and to provide a cysteine residue onto which **5** could be ligated, following the ligation of **1** and **2** and deprotection of the cysteine S^{β}-acetamidomethyl (Acm) protecting group. Peptides **1**, **2**, and **5** were liberated from *p*MBHA resin using the high HF method, followed by preparative reversed-phase high-performance liquid chromatography (RP-HPLC) to yield **1**, **2**, and **5** in 10.2, 29.8, and 24.8% yields, respectively.

Native chemical ligation was performed at room temperature in 0.1 M phosphate buffer (pH 7.6), containing sodium 2-mercaptoethanesulfonate (MESNA) as a thiol additive and tris-2-carboxyethylphosphine hydrochloride (TCEP) to prevent disulfide bond formation. Ligation of **1** and **2** (Scheme 1) was attempted using 50% (v/v) 2,2,2-trifluoroethanol (TFE) or 1% (w/v) SDS to enhance the aqueous solubility of **2**. While 50% (v/v) TFE in 0.1 M phosphate buffer (pH 7.6) dissolved **2**, no ligation of **1** and **2** was observed by 24 h (unpublished data, Peter Moyle, UQ). In comparison, 1% (w/v) SDS in 0.1 M phosphate buffer also dissolved **2**. However, in this case ligation was successful, giving **3** in 83.9% yield following purification. Monitoring by analytical RP-HPLC demonstrated that ligation was rapid and essentially complete by 2 h.

To enable ligation of **3** and **5** (Scheme 2), cleavage of the cysteine Acm protecting group of **3** was required. Standard Acm-deprotection conditions utilizing mercury(II) acetate (20 equiv) in acidified double-distilled water (ddH_2O; pH 4, acetic

acid (AcOH)) were tried. Acm-deprotection completed within an hour to give the mercury complex of **4** (identified by ESI-MS). The peptide was then treated with a large excess of β-mercaptoethanol (BME; 240 equiv) for 5 h to precipitate the mercury. However, despite the long treatment time and large excess of BME utilized, **4** could only be obtained as the mercury salt. Previously similar peptides, albeit lacking the LCP lipid core, have been synthesized using the described technique, with successful Acm-deprotection using mercury(II) acetate, followed by mercury precipitation with BME (unpublished data, Peter Moyle, UQ). These results suggest that incorporation of lipids may prevent mercury precipitation following Acm-deprotection.

Several reports of difficulties relating to mercury precipitation following Acm-removal have been published.[24,25] In many of these cases, replacement of mercury(II) acetate with silver(I) salts has proven effective for Acm-removal due to silver's lower affinity toward thiols compared with mercury, which as a consequence reduces the difficulties associated with disruption of peptide−metal adducts by the addition of excess thiols. Based on this research, various methods[24,26,27] using silver(I) salts were investigated for Acm-deprotection of **3** without success. As both silver(I) and mercury(II) salts were unsuitable for Acm-deprotection, iodine was investigated (Scheme 2) despite its capacity to promote disulfide bond formation. Acm-deprotection was performed in 50% (v/v) aqueous AcOH, as this solvent is recommended to reduce iodination of tyrosine, histidine, and tryptophan residues and promotes rapid Acm-deprotection.[28] Monitoring of the deprotection reaction by ESI-MS demonstrated complete Acm-deprotection within 1.5 h, with ESI-MS and analytical RP-HPLC demonstrating that no disulfide formation occurred under these conditions. The Acm-deprotected product (**4**) was then obtained in 84.5% yield following preparative RP-HPLC.

The ligation of **4** and **5** (Scheme 2) was attempted using 50% (v/v) TFE or 1% (w/v) SDS in 0.1 M phosphate buffer (pH 7.6). When TFE was utilized, the ligation rate was slow, leading to hydrolysis of thioester peptide **5**, conversion of **4** into a higher molecular weight species (+29), and the formation of very little product (**6**) (not quantified). In comparison, ligation of **4** and **5** was successful in the presence of 1% (w/v) SDS, although the rate of ligation was much slower than observed between **1** and **2**, taking 10 h to complete. This was most likely due to steric hindrance of the peptide **4** cysteine residue due to branching, as well as the close proximity of the cysteine residue to the LCP lipid core alkyl chains. In addition, the amphiphilic nature of **4**, as well as the use of SDS, would likely result in the incorporation of **4** into micelles. It is likely that the LCP lipid core would be located on the inside of micelles, while the peptide chains would face toward the aqueous solvent. This would result in the peptide **4** cysteine residue being buried within micelles, hindering its capacity to react with **5** in the aqueous buffer. Following ligation of **4** and **5** and purification, lipopeptide vaccine **6** was obtained in 61.1% yield, with an overall yield of 44.1% for the synthesis.

The concentration of SDS utilized during native chemical ligation has been previously reported to significantly affect ligation efficiency. Sato et al.[29] reported that reducing SDS concentrations from 35 mM [equivalent to 1% (w/v) SDS] to 7 mM, a concentration less than the 8 mM critical micelle concentration (CMC), greatly improved ligation efficiency. An experiment was therefore performed to compare ligation rates between **4** and **5** in the presence of 7 mM or 35 mM SDS, with ligation rates monitored by analytical RP-HPLC. Faster ligation rates were demonstrated when 35 mM SDS was utilized, with

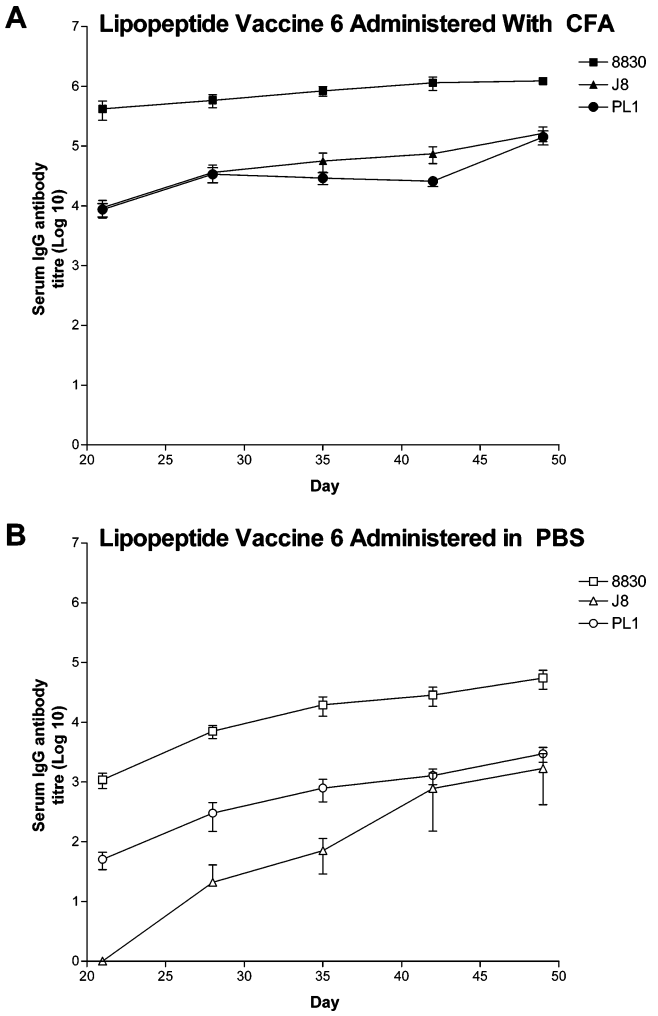


Figure 2. Time-course for elicitation of antigen-specific serum IgG antibodies in response to immunization with lipopeptide vaccine **6** emulsified in CFA (graph A) or in PBS (graph B), as determined by ELISA. Mean antigen-specific IgG antibody titers are shown with the standard error of the mean.

4 observed to form a higher molecular weight side product (+328) when 7 mM SDS was utilized (unpublished data, Peter Moyle, UQ). These results suggested that SDS concentrations greater than the CMC are beneficial for this ligation.

The J8 peptide's secondary structure, when incorporated into **6**, is important for vaccine efficacy. This is due to the presence of a conformational B cell epitope within the J8 sequence, which needs to adopt an α-helical secondary structure for antibody elicitation.[9] To assess the secondary structure of the J8 peptide incorporated into **6**, a CD study was performed. Peptides **3**, **5**, and **6** were each dissolved in 10 mM phosphate buffer pH 7 to mimic biological conditions in the blood. Following the acquisition of CD data, the data was analyzed for α-helical content. This was achieved using the *k2d*[30,31] neural network, an algorithm which estimates protein secondary structure from CD spectra. The J8 peptide **5** was found to have approximately 99% α-helical character. In comparison, peptide **3**, which contained the LCP lipid core, as well as the PL1 and 88/30 peptides demonstrated approximately 59% α-helical character. On the basis of these results, it was expected that **6** should demonstrate approximately 73% α-helical character if the J8 peptide remained α-helical, following conjugation to **3**. The *k2d* analysis suggested that **6** had approximately 93% α-helical character. As this was greater than expected, it was assumed the J8 peptide

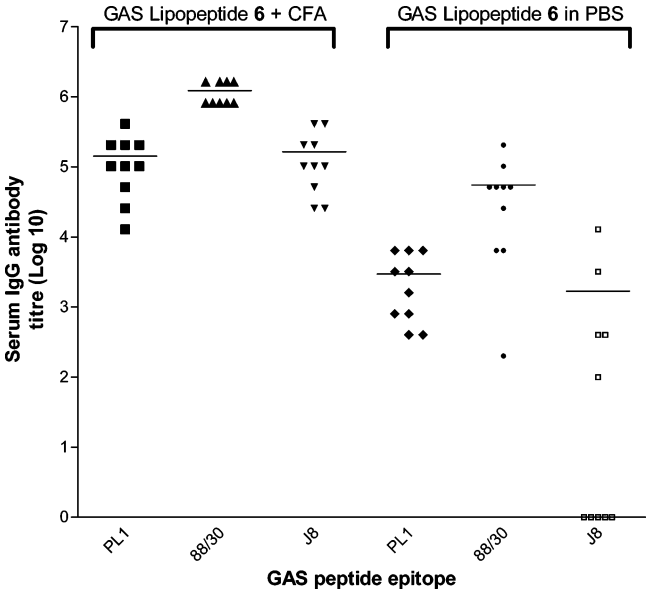


Figure 3. Antigen-specific serum IgG antibody titers (day 49) elicited in response to immunization of B10.BR mice at the tail base with lipopeptide vaccine **6**, as determined by ELISA. Primary immunizations were performed in the presence or absence of CFA. Antibody titers are shown for individual mice to the J8, 88/30, and PL1 GAS peptide epitopes. Mean antigen-specific serum IgG antibody titers are represented as a bar.

retained its α-helical character when conjugated to **3** and may have promoted the other peptide epitopes to adopt an α-helical character.

Immunological evaluation of **6** was performed in B10.BR (H-2^k) mice (10/group). Mice received a primary immunization with **6** (30 μg) at the tail base either in phosphate buffered saline (50 μL total volume) or emulsified 1:1 with CFA. The PL1, 88/30, and J8 peptides were not administered as controls in this experiment as previous studies have demonstrated that these peptides are not capable of eliciting antigen-specific systemic IgG antibodies without the addition of adjuvants.[10,12,16] Three weeks later, the mice received five boosts of **6** (3 μg in PBS) at weekly intervals. Sera was collected from the mice prior to each boost and one week after the last boost to assess the levels of serum antigen-specific IgG antibodies elicited in response to immunization. IgG antibody levels were determined using an enzyme-linked immunosorbent assay (ELISA). Mice that received primary immunization with **6** plus CFA demonstrated a rapid increase in antigen-specific serum IgG antibody levels against the 88/30, J8, and PL1 peptides (Figure 2A), with all mice showing high-titer IgG antibodies against each of the incorporated epitopes at first bleed (day 21). In comparison, mice that received primary immunization with **6** in PBS demonstrated a slow increase in antigen-specific serum IgG antibodies over time (Figure 2B). Average serum IgG antibody titers against the J8 (1.0 versus 9.3×10^3, $p = 0.014$), 88/30 (1.1×10^3 versus 4.2×10^5, $p = 0.021$), and PL1 (5.1×10^1 versus 8.7×10^3, $p = 0.003$) epitopes were significantly lower in mice immunized with **6** in PBS than in mice immunized with **6** in CFA at first bleed (day 21). At the final bleed (day 49) all mice administered **6** emulsified in CFA demonstrated high-titer antigen-specific serum IgG antibodies against each of the included peptide epitopes (Figure 3). In comparison, mice administered **6** in PBS also demonstrated high-titer mean serum IgG antibodies against each of the included peptide epitopes at the final bleed (day 49) (Figure 3). However, mean IgG antibody levels against the J8 (1.7×10^3 versus 1.6×10^5, $p = 0.006$), 88/30 (5.5×10^4 versus 1.2×10^6, $p = 0.00001$), and PL1 (2.9×10^3 versus 1.4×10^5, $p = 0.004$) epitopes were significantly lower compared to mice administered **6** emulsified in CFA. Furthermore, while all mice administered **6** emulsified in CFA responded to the J8 peptide, only 50% (5 out of 10 mice) responded to J8 when **6** was administered in PBS. Similar results have previously been observed with the J8 peptide incorporated into an LCP system.[11,13] While the levels of antigen-specific serum IgG antibodies were lower in mice immunized with **6** in PBS compared to mice immunized with **6** in CFA, this does not necessarily indicate that protection against GAS challenge would not be induced. Mice which failed to elicit high-titer antigen-specific IgG antibodies against the J8 peptide would likely elicit high-titer antibodies against the serotype specific 88/30 and PL1 epitopes. In a previous study, mice administered an LCP system incorporating two copies of the J8 peptide and two copies of the 88/30 epitope elicited average J8 (6.91×10^3 and 1.80×10^3) and 88/30 (1.04×10^5 and 1.39×10^6) antigen-specific systemic IgG antibody titers[13] similar to those observed in the current study (Figure 3). Intraperitoneal challenge of these mice with 88/30 strain GAS demonstrated 100% survival against GAS challenge.[13] The data therefore suggests that mice which fail to elicit J8-specific systemic IgG antibodies may be protected against challenge with GAS strains for which serotype-specific N-terminal epitopes are included in the vaccine.

Conclusion

In conclusion, the current study has demonstrated the capacity to utilize native chemical ligation for the synthesis of highly pure, multi-epitopic, LCP-based lipopeptide vaccines. As per our previous findings, the only successful method to date for the ligation of highly lipophilic LCP lipid core containing peptides has involved the use of surfactants (e.g. SDS). This has enabled the synthesis of a self-adjuvanting, triepitopic GAS vaccine in high purity (97.7%) and in excellent yield (44.1%). As the J8 peptide contains a conformational B cell epitope, it was important to ensure that the J8 epitope maintained its α-helical secondary structure following incorporation into the vaccine. Assessment of the percentage α-helical content using CD, and the *k2d* neural network,[30,31] suggested J8 α-helical character was preserved. Finally, immunological assessment of the vaccine demonstrated its capacity to elicit high-titer systemic IgG antibodies against each of the incorporated peptide epitopes. This research has provided a novel method for the synthesis of highly pure, multi-epitopic lipopeptide vaccines that could be potentially utilized in human clinical trials.

Experimental Section

Materials and Methods. Boc-L-amino acids and *p*MBHA resin were purchased from Novabiochem (Läufelfingen, Switzerland) or Reanal (Budapest, Hungary). Peptide synthesis grade DMF, TFA, dichloromethane (DCM), and 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) were purchased from Auspep (Melbourne, VIC, Australia). HPLC solvents [MeOH, acetonitrile (ACN), isopropyl alcohol (IPA)] were purchased from Honeywell-Burdick & Jackson (Morristown, NJ) or Labscan (Dublin, Ireland). All other reagents were purchased from Sigma-Aldrich (Castle Hill, NSW, Australia) at the highest available purity. ESI-MS was performed on a Perkin-Elmer-Sciex API3000 instrument using ACN−water mobile phases containing 0.1% (v/v) formic acid. ESI-MS data was acquired using Analyst 1.4 (Applied Biosystems/MDS Sciex, Toronto, Canada) software. Elemental analysis was performed using a Carlo Erba elemental analyzer model 1106. CD was performed on a Jasco J-710 spectropolarimeter. 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 either 90% ACN/0.1% TFA/H_2O (solvent B1), 90% IPA/ 0.1% TFA/H_2O (solvent B2), or 90% MeOH/0.1% TFA/H_2O (solvent B3) 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; 5 μm; 4.6 × 250 mm) or a Vydac analytical C4 column (214TP54; 5 μm; 4.6 × 250 mm). The following methods were run over 30 min: method 1, 0−100% solvent B1; method 2, 0−100% solvent B2; method 3, 0−100% solvent B3; method 4, 10−60% solvent B1; method 5, 20−80% solvent B1; method 6, 20−100% solvent B3; method 7, 30−100% solvent B3; method 8, 0−85% solvent B1. Preparative RP-HPLC was performed on a Waters Delta 600 system in gradient mode using 0.1% TFA/H_2O as solvent A and 90% ACN/0.1% TFA/H_2O as solvent B. Separations were performed on either a Vydac preparative C18 column (218TP1022; 10 μm; 22 × 250 mm) or a Vydac preparative C4 column (214TP1022; 10 μm; 22 × 250 mm) with a 10 mL/min flow-rate and detection at 230 nm. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS−PAGE) was performed on 1 mm NuPAGE Novex 12% Bis-Tris gels (Invitrogen), with Multimark Colored Standards (Invitrogen) as molecular weight markers. Gels were run in NuPAGE MES buffer (Invitrogen) at a constant 200 V for 45 min and then stained with Simplyblue Safestain (Invitrogen).

Synthesis of Peptide Building Blocks. Peptides **1**, **2**, and **5** (Schemes 1 and 2) were synthesized by manual SPPS on *p*MBHA resin (0.54 mmol NH_2/g; 0.5 mmol scale) using HBTU/*N*,*N*-diisopropylethylamine (DIPEA) in situ neutralization[20] and Boc-chemistry. Each coupling cycle consisted of Boc-deprotection by 2 × 1 min treatments with neat TFA, a 1 min DMF flow wash, followed by 15−60 min couplings with 4 equiv of preactivated amino acid. Amino acid activation was achieved by dissolving amino acids (2.2 mmol; 4.4 equiv) in 0.5 M HBTU/DMF solution (4 mL; 2.0 mmol; 4 equiv) to which DIPEA (500 μL; 2.87 mmol) was added. Amino acids were preactivated for 1 min prior to their addition to the resin. Coupling yields were determined using the quantitative ninhydrin test.[32] Where necessary, couplings were repeated to give coupling yields greater than 99.7%. Boc-amino acids with the following side-chain protection were utilized: Arg(Tos), Asn(Xan), Asp(OcHx), Cys(Acm), Cys(pMeBzl), Gln(Xan), Glu(OcHx), Lys(2-Cl-Z), Lys(Fmoc), Ser(Bzl), Thr(Bl), Tyr(2-Br-Z). The synthetic lipidic amino acid 2-(*t*-butoxycarbonylamino)-D,L-dodecanoic acid (Boc-C12-OH) was synthesized as previously described.[22] After the coupling of glutamine residues, the resin was washed with DCM before and after Boc-deprotection to prevent high-temperature catalyzed pyrrolidone carboxylic acid (Pca) formation.[20] N-Terminal acetylation of peptide epitopes was achieved 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. Thioester peptides were synthesized using a TAMPAL linker as previously described.[21] Following TAMPAL synthesis, the trityl-protecting group was removed by 2 × 2-minute treatments with 2.5% (v/v) triisopropylsilane, 2.5% (v/v) H_2O, and 95% (v/v) TFA, followed by a 1 min DMF flow-wash. The thioester bond was then formed by 2 × 1 h couplings of Boc-Gly-OH (5 mmol; 10 equiv) using standard HBTU/DIPEA in situ neutralization[20] to give Boc-Gly-TAMPAL resin. The sequences of the peptide epitopes utilized in this study were J8: QAEDKVKQSREAKKQVEKALKQLEDKVQ; 88/30: DNGKAIYERARERALQELGP; PL1: EVLTRRQSQDP-KYVTQRIS. Lysine N$^\epsilon$-Fmoc deprotection was achieved using 20% piperidine in DMF for nonthioester peptides, with the cleavage monitored by observing the UV absorbance of the dibenzofulvene-piperidine adduct at 304 nm. Lysine N$^\epsilon$-Fmoc deprotection for thioester peptides was achieved by treating the resin for 5 min and then 1 h with 1-methylpyrrolidine (25% v/v), hexamethylene imine (2% v/v), and 1-hydroxybenzotriazole monohydrate (HOBt; 2% w/v), in 1:1 1-methyl-2-pyrrolidinone-dimethyl sulfoxide.[23] Following synthesis, peptidyl resins were washed with DMF, DCM, and MeOH and dried under vacuum prior to cleavage with HF. HF cleavage (10 mL HF/g resin) was performed for 2 h at 0 °C. Following HF cleavage, HF was removed under reduced pressure, with peptides precipitated in ice-cold ether, filtered, and dissolved in 40% aqueous ACN containing 0.1% TFA and lyophilized.

Stepwise Synthesis of Thioester Peptide Ac-88/30-K[C(Acm)]-G-MPAL (1). Boc-Lys(Fmoc)-OH was coupled to Boc-Gly-TAMPAL resin, followed by synthesis of the 88/30 epitope on the lysine α-amine. The N-terminal Boc-protecting group was then removed, and the N-terminal amine acetylated. The lysine N$^\epsilon$-Fmoc group was then cleaved, followed by coupling Boc-Cys(Acm)-OH to the N$^\epsilon$-amine and cleavage of the cysteine N$^\alpha$-Boc protecting group. The product (**1**) was then cleaved from the resin using HF in the presence of 7% (v/v) *p*-cresol and purified by preparative RP-HPLC on a C18 column using a gradient of 10% solvent B to 60% solvent B over 60 min. The fractions were analyzed by ESI-MS and where appropriate combined to give **1** (147 mg; 10.2% yield) following lyophilization.

HPLC: t_R = 15.642 min (method 1, C18 column), t_R = 14.233 min (method 2, C18 column), t_R = 15.817 min (method 3, C18 column), t_R = 18.457 (method 4, C18 column); HPLC purity = 98.1%; ESI-MS: $[M + 2H^+]^{2+}$ *m*/*z* 1445.5 (calc 1445.1), $[M + 3H^+]^{3+}$ *m*/*z* 963.7 (calc 963.8), $[M + 4H^+]^{4+}$ *m*/*z* 723.1 (calc 723.1), $[M + 5H^+]^{5+}$ *m*/*z* 579.1 (calc 578.7); MW 2888.29 g/mol; Anal. ($C_{122}H_{203}N_{39}O_{38}S_2$·6TFA·19$H_2O$) C, H, N.

Stepwise Synthesis of Cysteinyl Peptide Ac-PL1-K(C)-G-C12-G-C12-C12-G-NH_2 (2). Ac-PL1-K(C)-G-C12-G-C12-C12-G-NH_2 (**2**) was synthesized by coupling Boc-Gly-OH to *p*MBHA resin, followed by two cycles of Boc-C12-OH, Boc-Gly-OH, Boc-C12-OH, Boc-Gly-OH, and Boc-Lys(Fmoc)-OH. The PL1 epitope was then synthesized on the lysine N$^\alpha$-amine. Following synthesis of the PL1 epitope, the N-terminal Boc protecting group was removed and the N-terminal amine acetylated. The lysine N$^\epsilon$-Fmoc group was then cleaved, Boc-Cys(pMeBzl)-OH coupled to the lysine N$^\epsilon$-amine, and the cysteine N$^\alpha$-Boc-group removed. The product (**2**) was then cleaved from the resin using HF in the presence of 5% (v/v) *p*-cresol and 5% (v/v) *p*-thiocresol and purified by preparative RP-HPLC on a C4 column using a gradient of 35% solvent B to 95% solvent B over 60 min. The fractions were analyzed by ESI-MS and where appropriate combined to give **2** (498 mg; 29.8% yield) following lyophilization.

HPLC: t_R = 23.608 min (method 1, C4 column), t_R = 22.417 min (method 2, C4 column), t_R = 23.708 min (method 3, C4 column), t_R = 24.283 min (method 5, C4 column); HPLC purity = 99.5%; ESI-MS: $[M + 2H^+]^{2+}$ *m*/*z* 1672.5 (calc 1671.0), $[M + 3H^+]^{3+}$ *m*/*z* 1115.3 (calc 1114.3), $[M + 4H^+]^{4+}$ *m*/*z* 837.1 (calc 836.0); MW 3340.04 g/mol; Anal. ($C_{151}H_{264}N_{42}O_{40}S$·7TFA·8$H_2O$) C, H, N.

Stepwise Synthesis of Thioester Peptide Ac-J8-G-MPAL (5). Thioester peptide **5** was synthesized on Boc-Gly-TAMPAL resin. Following cleavage of the N-terminal Boc-protecting group, the J8-epitope was synthesized, with acetylation performed after each coupling step. The N-terminal Boc-protecting group was then removed, and the N-terminal amine acetylated. The product (**5**) was then cleaved from the resin using HF in the presence of 7% (v/v) *p*-cresol and purified by preparative RP-HPLC on a C18 column using a gradient of 10% solvent B to 60% solvent B over 60 min. The fractions were analyzed by ESI-MS and where appropriate combined to give **5** (444 mg; 24.8% yield) following lyophilization.

HPLC: t_R = 16.825 min (method 1, C4 column), t_R = 16.792 min (method 1, C18 column), t_R = 16.000 min (method 2, C18 column), t_R = 27.008 min (method 3, C18 column), t_R = 20.833 min (method 4, C18 column); HPLC purity = 99.9%; ESI-MS: $[M + 2H^+]^{2+}$ *m*/*z* 1793.4 (calc 1792.1), $[M + 3H^+]^{3+}$ *m*/*z* 1195.8 (calc 1195.0), $[M + 4H^+]^{4+}$ *m*/*z* 897.0 (calc 896.5), $[M + 5H^+]^{5+}$ *m*/*z* 718.3 (calc 717.4); MW 3582.10 g/mol; Anal. ($C_{153}H_{266}N_{46}O_{50}S$·9TFA·14$H_2O$) C, H, N; circular dichroism (*k2d*): 99% α-helical, 0% β-sheet, 0.01% random.

Native Chemical Ligation of 1a and 2. Compound **2** (80.0 mg; 23.95 μmol) was dissolved in ddH_2O (10 mL) containing SDS (120 mg), frozen, and lyophilized. The powder was then rehydrated with

0.1 M phosphate buffer pH 7.6 (7.5 mL) to which TCEP (20.6 mg; 71.86 μmol) was added. The pH was then adjusted to 7.5 with 0.2 M sodium phosphate dibasic aqueous solution (300 μL). Peptide **1** (46.4 mg; 16.06 μmol) was then dissolved in 0.1 M phosphate buffer pH 7.6 (3 mL) to which MESNA (32 mg; 194.91 μmol) was added. The thiol exchange was left to proceed for 1 h to form peptide **1a** (HPLC: t_R = 14.950 min (method 1, C4 column); ESI-MS: $[M - Na^+ + 2H^+]^{2+}$ *m/z* 1407.7 (calc 1406.6), $[M - Na^+ + 3H^+]^{3+}$ *m/z* 938.9 (calc 938.1); MW 2834.15 g/mol). The solution containing lipopeptide **2** was then transferred into the vessel containing peptide **1a**, with an extra 1.5 mL of 0.1 M phosphate buffer pH 7.6 utilized in the transfer. The ligation was monitored by analytical RP-HPLC, with ligation appearing complete by 2 h. The product **3** was then purified by preparative RP-HPLC on a C4 column using a gradient of 15% solvent B to 70% solvent B over 60 min. The fractions were analyzed by ESI-MS, and where appropriate they were combined to give **3** (81.0 mg; 83.9% yield) following lyophilization.

HPLC: t_R = 22.617 min (method 1, C4 column), t_R = 19.892 min (method 2, C4 column), t_R = 26.492 min and 27.067 min (method 7, C4 column); HPLC purity = 99.2%; ESI-MS: $[M + 3H^+]^{3+}$ *m/z* 2005.2 (calc 2004.3), $[M + 4H^+]^{4+}$ *m/z* 1504.2 (calc 1503.5), $[M + 5H^+]^{5+}$ *m/z* 1203.9 (calc 1203.0), $[M + 7H^+]^{7+}$ *m/z* 860.1 (calc 859.6); MW 6010.01 g/mol; circular dichroism (*k2d*): 59% α-helical, 8% β-sheet, 33% random.

***S*$^{\beta}$-Acm Deprotection of 3.** Compound **3** (20 mg; 3.33μmol) was dissolved in 1:1 AcOH−ddH_2O (15 mL) to which ACN (3 mL) was added. To this solution was added 1 M aqueous HCl (2 mL; 2 mmol) and 0.1 M iodine in 1:1 AcOH−ddH_2O (0.333 mL; 33.30 μmol). The reaction was left to proceed for 1.5 h. Aqueous ascorbic acid (1 M) was then added dropwise until the solution was colorless. TCEP (10 mg; 34.89 μmol) was then added, and the reaction was left for 30 min. The product **4** was then purified by preparative RP-HPLC on a C4 column using a gradient of 0% solvent B to 100% solvent B over 30 min followed by 100% solvent B for 20 min. The fractions were analyzed by ESI-MS and where appropriate combined to give **4** (16.7 mg; 84.5% yield) following lyophilization.

HPLC: t_R = 21.842 min (method 1, C4 column), t_R = 19.642 min (method 2, C4 column), t_R = 25.733 and 26.325 min (method 7, C4 column); HPLC purity = 99.1%; ESI-MS: $[M + 3H^+]^{3+}$ *m/z* 1981.1 (calc 1980.6), $[M + 4H^+]^{4+}$ *m/z* 1486.5 (calc 1485.7), $[M + 5H^+]^{5+}$ *m/z* 1189.4 (calc 1188.8); MW 5938.93 g/mol.

Native Chemical Ligation of 5a and 4. Compound **4** (16.4 mg; 2.76 μmol) was dissolved in ddH_2O (2 mL) containing SDS (33 mg), frozen, and lyophilized. The power was then rehydrated with 0.1 M phosphate buffer pH 7.6 (1 mL) to which TCEP (5.6 mg; 19.54 μmol) was added. The pH was then adjusted to 7.5 with 0.2 M sodium phosphate dibasic aqueous solution (400 μL). Peptide **5** (14.8 mg; 4.14μmol) was then dissolved in 0.1 M phosphate buffer pH 7.6 (1 mL) to which MESNA (15 mg; 91.36 μmol) was added. The thiol exchange was left to proceed for 1 h to form peptide **5a** (HPLC: t_R = 15.517 min (method 1, C4 column); ESI-MS: $[M - Na^+ + 2H^+]^{2+}$ *m/z* 1755.2 (calc 1753.5), $[M - Na^+ + 3H^+]^{3+}$ *m/z* 1170.9 (calc 1169.3), $[M - Na^+ + 4H^+]^{4+}$ *m/z* 878.7 (calc 877.2), $[M - Na^+ + 5H^+]^{5+}$ *m/z* 703.4 (calc 701.9); MW 3527.96 g/mol). This solution was then transferred into the vessel containing lipopeptide **4**, with an extra 1 mL of 0.1 M phosphate buffer, pH 7.6, utilized in the transfer. The ligation was monitored by analytical RP-HPLC, with ligation appearing complete by 20 h. The product **6** was then purified by preparative RP-HPLC on a C4 column using a gradient of 10% solvent B to 85% solvent B over 60 min. The fractions were analyzed by ESI-MS and where appropriate combined to give **6** (15.7 mg; 61.1% yield) following lyophilization.

HPLC: t_R = 18.733 min (method 1, C4 column), t_R = 22.833 min (method 1, C18 column), t_R = 17.142 min (method 2, C4 column), t_R = 27.683 min (method 4, C4 column), t_R = 27.292 min (method 6, C4 column); HPLC purity = 97.7%; SDS−PAGE: 9 kDa (expected 9.3 kDa); ESI-MS: $[M + 5H^+]^{5+}$ *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); MW 9302.71 g/mol; circular dichroism (*k2d*): 93% α-helical, 0% β-sheet, 7% random.

Circular Dichroism. Peptides **3**, **5**, and **6** were dissolved individually in 10 mM phosphate buffer pH 7 to a concentration of 0.33 mg/mL and filtered through a 0.45 μm nylon syringe filter prior to use. All measurements were made at 22 °C using a circular 0.1 cm path length quartz cell. Background measurements on the buffer were performed, and subtracted from the final data. Data were acquired between 200 and 241 nm with 1 nm steps as required by the *k2d* neural network.[30,31] The acquired data was then converted to mean residue ellipticity [θ] (deg cm^2 dmol^{-1}) prior to submission to the *k2d* server (http://www.embl-heidelberg.de/~andrade/k2d/). This provided semiquantitative estimates of peptide α-helical, β-sheet, and random coil content.

Mice and Subcutaneous Immunization. All protocols were approved by the Bancroft Centre Research Animal Ethics Committee (approval number P415). Immunization was performed as previously described.[13] Briefly, immunizations were conducted in four-to-six week old female B10.BR mice (H-2^k) (Animal Resource Centre, Perth, WA, Australia). On day 0, mice (n = 10/group) were immunized at the tail base with 30 μg of **6** either emulsified 1:1 with CFA (Sigma, Castle Hill, NSW, Australia) or administered in 50 μL total volume of sterile-filtered phosphate buffered saline (PBS). Three weeks later, mice received five boosts at weekly intervals (days 21, 28, 35, 42, and 49) with 3 μg of **6** in PBS prior to collection of blood.

Collection of Sera. Blood was collected from the tail artery of each mouse one week after the last immunization. The blood was left to clot at 37 °C for 1 h, and then centrifuged for 10 min at 3000 rpm to remove clots. Sera was then stored at − 20 °C.

Detection of Systemic IgG Antibodies by ELISA. ELISA for determination of serum IgG antibodies against the PL1, 88/30, and J8 epitopes included in the vaccine was performed as previously described.[33] Briefly, serial dilutions of sera were produced in 0.5% skim milk PBS-tween 20 buffer, starting at 1:100 concentration with 2-fold 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.

Statistics. Statistical analysis was performed using a two-sided, paired students *t*-test for comparison of antibody titers between groups. GraphPad Prism 4 was used for statistical analysis, with $p < 0.05$ taken as statistically significant.

Acknowledgment. This work was supported by the National Health and Medical Research Council (Australia), the National Heart Foundation (Australia), and the Prince Charles Hospital Foundation. The author acknowledges the Queensland Government for their financial support through the award of a Growing the Smart State Ph.D. funding scholarship.

Supporting Information Available: HPLC chromatograms, ESI-MS, SDS−PAGE, reaction monitoring, and circular dichroism data. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Batzloff, M. R.; Sriprakash, K. S.; Good, M. F. Vaccine development for group A streptococcus infections and associated diseases. *Curr. Drug Targets* **2004**, *5* (1), 57−69.
(2) Bisno, A. L.; Rubin, F. A.; Cleary, P. P.; Dale, J. B. Prospects for a group A streptococcal vaccine: rationale, feasibility, and obstacles − report of a National Institute of Allergy and Infectious Diseases workshop. *Clin. Infect. Dis.* **2005**, *41* (8), 1150−1156.
(3) Dale, J. B. Group A streptococcal vaccines. *Infectious Disease Clinics of North America* **1999**, *13* (1), 227−243.
(4) McMillan, D. J.; Chhatwal, G. S. Prospects for a group A streptococcal vaccine. *Curr. Opin. Mol. Ther.* **2005**, *7* (1), 11−16.

(5) Lancefield, R. C. Current knowledge of type-specific M antigens of group A streptococci. *J. Immunol.* **1962**, *89*, 307−313.
(6) Fischetti, V. A. Streptococcal M protein: molecular design and biological behavior. *Clin. Microbiol. Rev.* **1989**, *2* (3), 285−314.
(7) Beachey, E. H.; Bronze, M. S.; Dale, J. B.; Kraus, W.; Poirier, T.; Sargent, S. Protective and autoimmune epitopes of streptococcal M proteins. *Vaccine* **1988**, *6* (2), 192−196.
(8) Pruksakorn, S.; Currie, B.; Brandt, E.; Phornphutkul, C.; Hunsakunachai, S.; Manmontri, A.; Robinson, J. H.; Kehoe, M. A.; Galbraith, A.; Good, M. F. Identification of T cell autoepitopes that cross-react with the C-terminal segment of the M protein of group A streptococci. *Int. Immunol.* **1994**, *6* (8), 1235−1244.
(9) Hayman, W. A.; Brandt, E. R.; Relf, W. A.; Cooper, J.; Saul, A.; Good, M. F. Mapping the minimal murine T cell and B cell epitopes within a peptide vaccine candidate from the conserved region of the M protein of group A streptococcus. *Int. Immunol.* **1997**, *9* (11), 1723−1733.
(10) Horváth, A.; Olive, C.; Karpati, L.; Sun, H. K.; Good, M.; Toth, I. Toward the development of a synthetic group A streptococcal vaccine of high purity and broad protective coverage. *J. Med. Chem.* **2004**, *47* (16), 4100−4104.
(11) Horváth, A.; Olive, C.; Wong, A.; Clair, T.; Yarwood, P.; Good, M.; Toth, I. Lipoamino acid-based adjuvant carrier system: enhanced immunogenicity of group A streptococcal peptide epitopes. *J. Med. Chem.* **2002**, *45* (6), 1387−1390.
(12) Horváth, A.; Olive, C.; Wong, A.; Clair, T.; Yarwood, P.; Good, M.; Toth, I. A lipophilic adjuvant carrier system for antigenic peptides. *Lett. Pept. Sci.* **2002**, *8* (3−5), 285−288.
(13) Olive, C.; Batzloff, M.; Horváth, A.; Clair, T.; Yarwood, P.; Toth, I.; Good, M. F. Potential of lipid core peptide technology as a novel self-adjuvanting vaccine delivery system for multiple different synthetic peptide immunogens. *Infect. Immun.* **2003**, *71* (5), 2373−2383.
(14) Olive, C.; Batzloff, M.; Horváth, A.; Clair, T.; Yarwood, P.; Toth, I.; Good, M. F. Group A streptococcal vaccine delivery by immunization with a self-adjuvanting M protein-based lipid core peptide construct. *Indian J. Med. Res.* **2004**, *119* (Suppl), 88−94.
(15) Olive, C.; Batzloff, M. R.; Horváth, A.; Wong, A.; Clair, T.; Yarwood, P.; Toth, I.; Good, M. F. A lipid core peptide construct containing a conserved region determinant of the group A streptococcal M protein elicits heterologous opsonic antibodies. *Infect. Immun.* **2002**, *70* (5), 2734−2738.
(16) Olive, C.; Hsien, K.; Horvath, A.; Clair, T.; Yarwood, P.; Toth, I.; Good, M. F. Protection against group A streptococcal infection by vaccination with self-adjuvanting lipid core M protein peptides. *Vaccine* **2005**, *23* (17−18), 2298−2303.
(17) Moyle, P. M.; Horváth, A.; Olive, C.; Good, M. F.; Toth, I. Development of lipid-core-peptide (LCP) based vaccines for the prevention of group A streptococcal (GAS) infection. *Lett. Pept. Sci.* **2004**, *10* (5−6), 605−613.
(18) Toth, I.; Danton, M.; Flinn, N.; Gibbons, W. A. A combined adjuvant and carrier system for enhancing synthetic peptides immunogenicity utilizing lipidic amino acids. *Tetrahedron Lett.* **1993**, *34* (24), 3925−3928.
(19) Brandt, E. R.; Sriprakash, K. S.; Hobb, R. I.; Hayman, W. A.; Zeng, W.; Batzloff, M. R.; Jackson, D. C.; Good, M. F. New multideterminant strategy for a group A streptococcal vaccine designed for the Australian Aboriginal population. *Nat. Med.* **2000**, *6* (4), 455−459.
(20) Schnölzer, M.; Alewood, P.; Jones, A.; Alewood, D.; Kent, S. B. H., In situ neutralization in Boc-chemistry solid-phase peptide synthesis. *Int. J. Pept. Protein Res.* **1992**, *40* (3−4), 180−193.
(21) Hackeng, T. M.; Griffin, J. H.; Dawson, P. E. Protein synthesis by native chemical ligation: expanded scope by using straightforward methodology. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96* (18), 10068−10073.
(22) Gibbons, W. A.; Hughes, R. A.; Charalambous, M.; Christodoulou, M.; Szeto, A.; Aulabaugh, A. E.; Mascagni, P.; Toth, I. Lipidic peptides, I. Synthesis, resolution and structural elucidation of lipidic amino acids and their homo- and hetero-oligomers. *Liebigs Ann. Chem.* **1990** (12), 1175−1183.
(23) Li, X.; Kawakami, T.; Aimoto, S. Direct preparation of peptide thioesters using a Fmoc solid-phase method. *Tetrahedron Lett.* **1998**, *39* (47), 8669−8672.
(24) Bang, D.; Chopra, N.; Kent, S. B. H. Total chemical synthesis of crambin. *J. Am. Chem. Soc.* **2004**, *126* (5), 1377−1383.
(25) Boysen, R. I.; Hearn, M. T. The metal binding properties of the CCCH motif of the 50S ribosomal protein L36 from Thermus thermophilus. *J. Pept. Res.* **2001**, *57* (1), 19−28.
(26) Kawakami, T.; Toda, C.; Akaji, K.; Nishimura, T.; Nakatsuji, T.; Ueno, K.; Sonobe, M.; Sonobe, H.; Aimoto, S. Synthesis of a molt-inhibiting hormone of the American crayfish, *Procambarus clarkia*, and determination of the location of its disulfide bridges. *J. Biochem.* **2000**, *128* (3), 455−461.
(27) Fujii, N.; Otaka, A.; Watanabe, T.; Okamachi, A.; Tamamura, H.; Yajima, H.; Inagaki, Y.; Nomizu, M.; Asano, K. Silver trifluoromethanesulphonate as an S-deprotecting reagent for the synthesis of cystine peptides. *J. Chem. Soc., Chem. Commun.* **1989** (5), 283−284.
(28) Kudryavtseva, E. V.; Sidorova, M. V.; Evstigneeva, R. P. Some peculiarities of synthesis of cysteine-containing peptides. *Russ. Chem. Rev.* **1998**, *67* (7), 545−562.
(29) Sato, T.; Saito, Y.; Aimoto, S. Synthesis of the C-terminal region of opioid receptor like 1 in an SDS micelle by the native chemical ligation: effect of thiol additive and SDS concentration on ligation efficiency. *J. Pept. Sci.* **2005**, *11* (7), 410−416.
(30) Andrade, M. A.; Chacon, P.; Merelo, J. J.; Moran, F. Evaluation of secondary structure of proteins from UV circular dichroism spectra using an unsupervised learning neural network. *Protein Eng.* **1993**, *6* (4), 383−390.
(31) Merelo, J. J.; Andrade, M. A.; Prieto, A.; Moran, F. Proteinotopic feature maps. *Neurocomputing* **1994**, *6* (4), 443−454.
(32) Sarin, V.; Kent, S. B. H.; Tam, J. P.; Merrifield, B. Quantitative monitoring of solid-phase peptide synthesis by the ninhydrin reaction. *Anal. Biochem.* **1981**, *117* (1), 147−157.
(33) Pruksakorn, S.; Galbraith, A.; Houghten, R. A.; Good, M. F., Conserved T and B cell epitopes on the M protein of group A streptococci. Induction of bactericidal antibodies. *J. Immunol.* **1992**, *149* (8), 2729−35.

JM060475M