Bioorganic & Medicinal Chemistry Letters 19 (2009) 821–824

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Synthesis of a *Streptococcus pyogenes* vaccine candidate based on the M protein PL1 epitope

Pavla Simerska *, Hao Lu, Istvan Toth

The University of Queensland, School of Molecular and Microbial Sciences (SMMS), Chemistry Building 68, St. Lucia, Brisbane, Qld 4072, Australia

ARTICLE INFO

Article history:
Received 7 November 2008
Revised 28 November 2008
Accepted 3 December 2008
Available online 7 December 2008

Keywords:
Streptococcus pyogenes
Self-adjuvanting vaccine
PL1 peptide epitope
Carbohydrate carrier

ABSTRACT

Group A streptococcus is a Gram-positive bacteria that causes a range of infectious diseases. Targeting the bacteria, a new self-adjuvanting vaccine candidate, incorporating a carbohydrate carrier and an amino acid-based adjuvant, was synthesised utilising carbohydrate chemistry and solid-phase peptide synthesis procedures. Characterisation of the candidate was achieved using reverse-phase HPLC and electrospray ionisation mass spectrometry. The successful synthesis and characterisation of the vaccine candidate may contribute to the discovery of a therapeutically and clinically viable vaccine against group A streptococcus.

Group A streptococcus (GAS, *Streptococcus pyogenes*) is responsible for a range of infectious conditions, ranging from mild tonsillitis to more severe form of necrotizing fasciitis. Despite successful treatment of these infections with adequate doses of antibiotics, sequential post-infectious autoimmune responses, including rheumatic heart disease (RHD) and rheumatic fever (RF), still affect millions of lives each year.[1] To avoid pandemic situations and to reduce the fatality rate of both GAS infections and post-streptococcal diseases, initial preventative measures to impede primary infections caused by GAS bacteria need to be considered. Vaccination, remains one of the most effective and cost-efficient methods in infectious disease prevention and control, and thus is the preferred approach to managing GAS infections.[1,2]

The aim of this project was to synthesise a peptide subunit vaccine candidate against GAS; utilising peptide, carbohydrate and lipid moieties. Four copies of an antigenic peptide epitope known as PL1 were incorporated into the carbohydrate core of the vaccine construct. PL1 is a peptide epitope derived from the variable N-terminus region of the antigenic M-protein of a virulent GAS serovar, this α-helical surface protein present in most GAS strains.[1,3,4] PL1 epitope was shown[1,3] to instigate high levels of immune response against specified serotypes of the GAS family and it was reported[4–6] that multiple copies of peptide epitope elicited higher antibody titres than a single peptide unit. The carbohydrate core acts as a carrier of peptide epitopes and the lipoamino acid-based unit acts as an adjuvant (Fig. 1).

Antigenic epitopes attached to a peptide-based carrier and a lipid moiety were shown by previous studies to be highly immunogenic and generate comparable titres of antibodies against incorporated epitopes when compared to the same epitopes administered with an adjuvant (e.g., complete Freund's adjuvant, CFA).[7–10] The presence of lipoamino acids (LAAs) resulted in an increased lipophilic character of the construct which contributed to enhancing the uptake of the vaccine candidate and also acted as a built in adjuvant. The co-administration of alternative and often toxic adjuvants (e.g., CFA or cholera toxins) was not necessary. This novel adjuvant system also opens the way towards alternative vaccination routes such as the intranasal or oral routes.[1,8,10]

The synthesis of the carbohydrate carrier (Scheme 1) involved the cyanoethylation[11,12] of β-D-glucopyranosyl azide (**1**) giving tetra-*O*-(cyanoethyl)-D-glucopyranosyl azide (**2**) followed by selective hydrogenation of the azido group of compound **2** in dry tetrahydrofuran (THF) in the presence of palladium. The resulting amino group was then coupled to monobenzyl adipate using *O*-benzotriazole-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HBTU) and *N*,*N*-diisopropylethylamine (DIPEA) in THF.[13] The four cyanoethyl groups of the benzyl ester derivative **4** were immediately reduced to primary amines via the addition of sodium borohydride ($NaBH_4$) and a catalytic amount of cobalt chloride ($CoCl_2$) and then Boc-protected to yield ester **5**. The benzyl group of compound **5** was cleaved by reductive hydrogenation (H_2 and Pd/C), resulting in a carboxylic acid at the anomeric position of β-D-glucosyl derivative **6**. Yields were found to be much higher when performing the reaction with a hydrogenator (Thales H-Cube Hydrogenator, Thales Nanotechnology, Hungary) rather than under standard procedure (H_2 atmosphere).[14]

* Corresponding author. Tel.: +61 7 33469892; fax: +61 7 33654273.
E-mail address: p.simerska@uq.edu.au (P. Simerska).

0960-894X/$ - see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmcl.2008.12.013

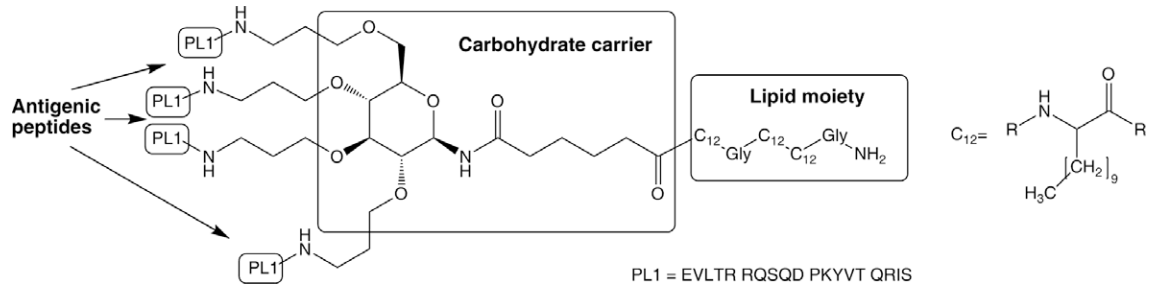
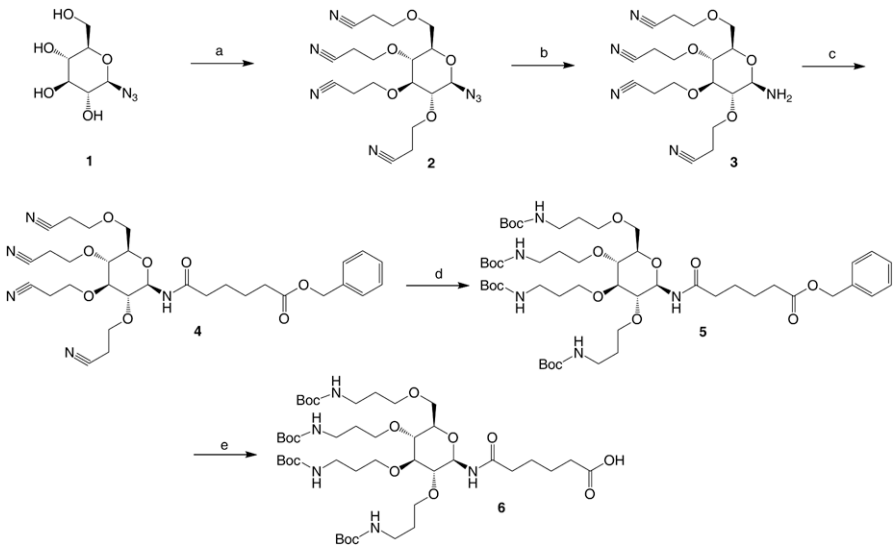
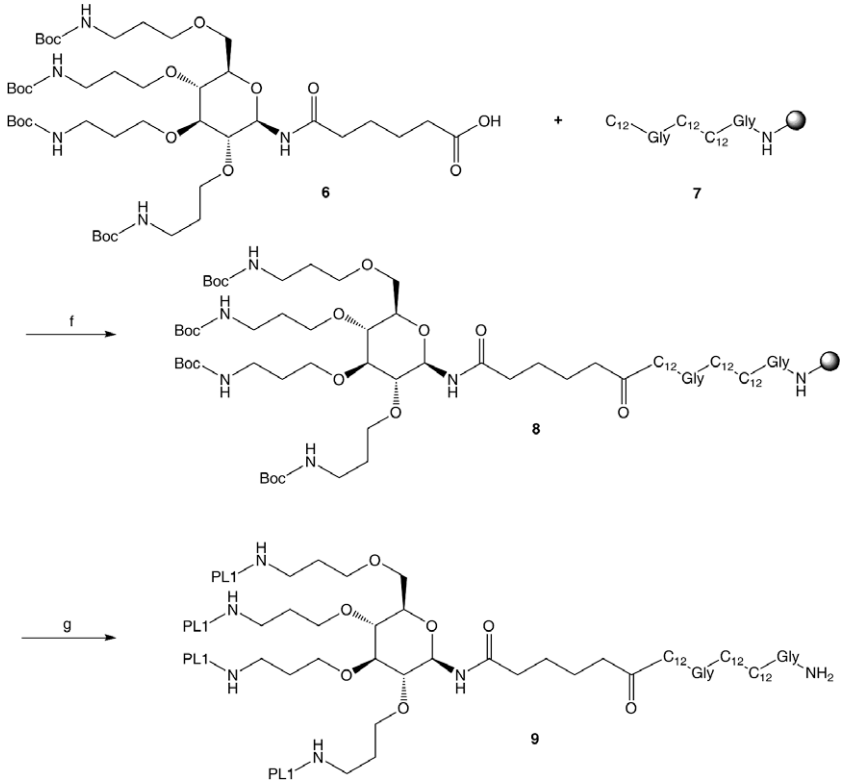


Figure 1. Structure of the vaccine candidate against group A streptococcus.



Scheme 1. Synthesis of the D-glucose-based carrier. Reagents and conditions: (a) MeCN, acrylonitrile, DBU (b) THF, H_2, 10% Pd/C, rt; (c) HBTU, DIPEA, THF, monobenzyl adipate, rt; (d) $NaBH_4$, $CoCl_2$, Boc_2O, rt; (e) hydrogenation (Thales H-Cube Hydrogenator).



Scheme 2. Synthesis of the vaccine construct-utilising SPPS procedures. Reagents and conditions: (f) coupling of Gly, C_{12}, C_{12} Gly and C_{12} and carbohydrate core **6** onto *p*MBHA resin in the presence of HBTU (1.5 equiv) and DIPEA (1.6 equiv) in DMF, Boc-protecting groups were removed with TFA; (g) coupling of four copies of the PL1 using HBTU, DIPEA in DMF; Boc-deprotection by TFA; cleavage of crude product from the *p*MBHA resin using HF and *p*-cresol scavenger.

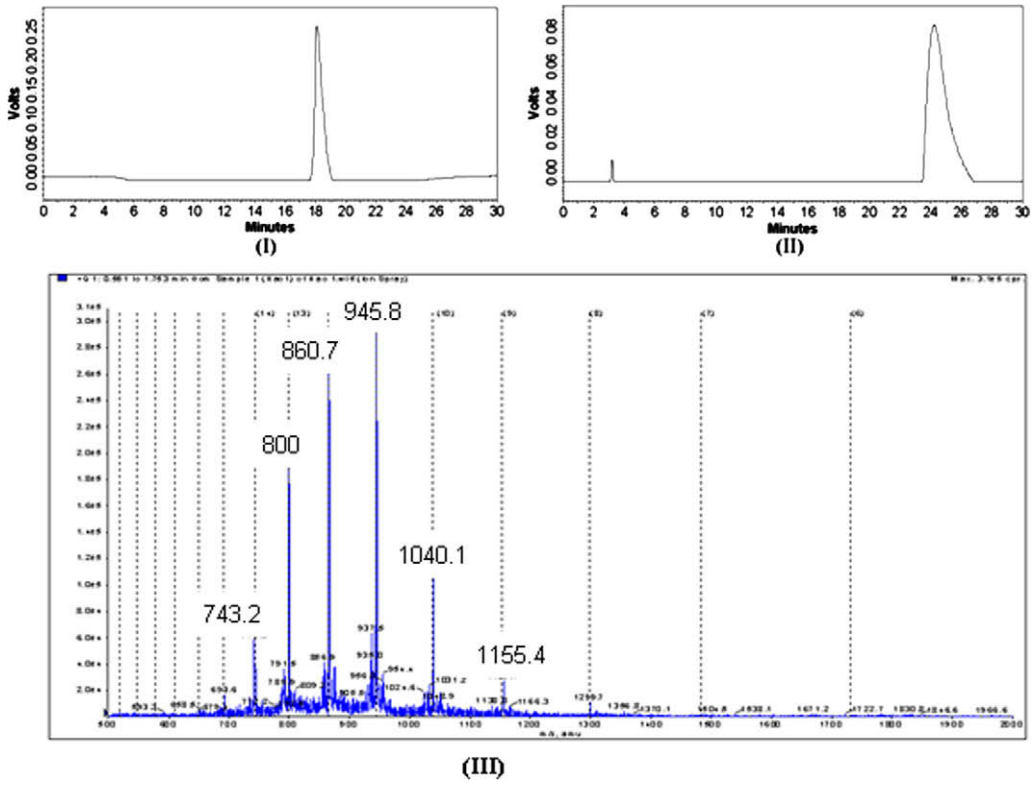


Figure 2. Characterisation of the pure GAS vaccine candidate (**9**) by analytical RP-HPLC and ESI-MS results. Reagents and conditions: (I) RP-HPLC eluent A—0.1% TFA/H_2O; eluent B—90% MeCN/10% H_2O/0.1% TFA; 0–100% gradient of the eluent B over 30 min, 1 mL/min flow rate, 214 nm; Vydac C4 column (214TP54, 5 μm, 4.6 × 250 mm); (II) RP-HPLC eluent A—0.1% TFA/H_2O; eluent B—90% MeOH/10% H_2O/0.1% TFA; 0–100% gradient of the eluent B over 30 min, 1 mL/min flow rate, 214 nm; Vydac C4 column (214TP54, 5 μm, 4.6 × 250 mm); (III) ESI-MS—multiple charged ions from the vaccine molecule (Mw = 10,316.8 g/mol).

The lipid moiety **7** was generated using stepwise solid-phase peptide synthesis (SPPS) (Scheme 2), where three C_{12} LAAs and two glycine spacers were coupled to *p*-methylbenzhydrylamine (*p*MBHA) resin. The same methods were applied for further coupling of the D-glucose carrier **6** to lipid moiety **7** to form liposaccharide **8**. Upon removal of the Boc-protecting groups, four copies of PL1 epitope (EVLTR RQSQD PKYVT QRIS)[15] were coupled onto the liposaccharide **8**. Coupling efficiencies during the SPPS procedures were checked after coupling steps by ninhydrin test, ensuring that a minimal 99.6% coupling efficiency was achieved (if necessary double coupling was employed).[16,17] Upon completion of the synthesis (Scheme 2), the crude vaccine candidate **9** was cleaved from the *p*MBHA resin using anhydrous HF.[18]

The crude product **9** was purified by preparative reversed-phase HPLC (RP-HPLC) and its purity was confirmed by analytical RP-HPLC and electrospray ionisation mass spectrometry (ESI-MS). The retention times of the pure compound **9** were detected to be 18.1 and 24.2 min using conditions A (Fig. 2, I) and B (Fig. 2, II), respectively, and the structure of the purified product was confirmed by ESI-MS (Fig. 2, III).[19]

The physiological efficacy of the synthesised compound **9** will be evaluated in vivo in mice and reported elsewhere.

Acknowledgements

This work was financially supported by the Australian National Health and Medical Research Council (NHMRC). The author acknowledges the encouragements and supports provided by all parties.

References and notes

1. Olive, C.; Batzloff, M. R.; Toth, I. *Expert Rev. Vaccine* **2004**, *3*, 43.
2. Olive, C.; Toth, I.; Jackson, D. *Mini-Rev. Med. Chem* **2001**, *1*, 429.
3. Brandt, E. R.; Hayman, W. A.; Currie, B.; Pruksakorn, S.; Good, M. F. *Vaccine* **1997**, *15*, 1805.
4. Brandt, E. R.; Teh, T.; Relf, W. A.; Hobb, R. I.; Good, M. F. *Infect. Immun.* **2000**, *68*, 6587.
5. Brandt, E. R.; Good, M. F. *Immunol. Res.* **1999**, *19*, 9.
6. Brandt, E. R.; Sriprakash, K. S.; Hobb, R. I.; Hayman, W. A.; Zeng, W.; Batzloff, M. R.; Jackson, D. C.; Good, M. F. *Nat. Med.* **2000**, *6*, 455.
7. McGeary, R. P.; Jablonkai, I.; Toth, I. *Tetrahedron* **2001**, *57*, 8733.
8. McGeary, R. P.; Olive, C.; Toth, I. *J. Pept. Sci.* **2003**, *9*, 405.
9. Horvath, A.; Olive, C.; Karpati, L.; Sun, H. K.; Good, M.; Toth, I. *J. Med. Chem.* **2004**, *47*, 4100.
10. Horvath, A.; Olive, C.; Wong, A.; Clair, T.; Yarwood, P.; Good, M.; Toth, I. *J. Med. Chem.* **2002**, *45*, 1387.
11. *Materials and reagents:* All materials and chemicals used in the experiments were of analytical grade or equivalent. Boc-protected-L-amino acids and *p*MBHA resin were purchased from Novabiochem (Laufelfingen, Switzerland), Renanal (Budapest, Hungary), and Peptides International (Louisville, Kentucky). Peptide synthesis reagents such as *N*,*N*′-dimethylformamide (DMF), dichloromethane (DCM), HBTU, TFA, and di-*tert*-butyldicarbonate were purchased from Auspep (Melbourne, Vic., Australia). Acetonitrile (ACN), isopropyl alcohol (IPA), and MeOH, used during RP-HPLC, were purchased from Labscan (Dublin, Ireland). Hydrogen bromide utilised during the ninhydrins assay were supplied by Merck (Kilsyth, Vic., Australia). Hydrofluoric acid (HF), used for peptide–resin cleavage, was purchased from BOC gases (Sydney, NSW, Australia). All other chemicals were supplied by Sigma–Aldrich (Castle Hill, NSW, Australia).
12. Simerska, P.; Abdel-Aal, A. M.; Fujita, Y.; Moyle, P. M.; McGeary, R. P.; Batzloff, M. R.; Olive, C.; Good, M. F.; Toth, I. *J. Med. Chem.* **2008**, *51*, 1447.
13. *6-(2,3,4,6-Tetra-O-(cyanoethyl)-β-D-glucopyranosylamino)-6-oxohexanoic acid benzyl ester (**3**):* Cyanoethylated azide (**2**) (1.40 g, 3.33 mmol) was hydrogenated under atmospheric pressure over 10% Pd/C (0.14 g) in THF (88 mL) for 2 h. Thin layer chromatography (TLC, aluminium-backed silica gel 60 F254 plates) was performed to determine the degree of completion of the reaction using ethyl acetate (EtOAc) as eluent. For detection, mixture of 20% (v/v) H_2SO_4 in EtOH was used. Adipic acid monobenzyl ester (0.83 g; 3.51 mmol), HBTU (1.34 g, 3.56 mmol), and DIPEA (0.62 g, 4.76 mmol) were added to the reaction mixture and stirred at room temperature under argon atmosphere for 24 h. The crude mixture was filtered through Celite and washed with MeOH. After condensation (rotary evaporation), the filtrate was dissolved in EtOAc (200 mL) and washed with HCl (5% aq, 3× 100 mL), $NaHCO_3$ (aq, 2× 100 mL), and saturated NaCl (aq, 50 mL). The mixture was dried over $MgSO_4$ and condensed. The product **3** was obtained (1.51 g, 2.54 mmol) in 76.13% yield.

14. *6-(2,3,4,6-Tetra-O-(butoxycarbonylaminopropyl)-β-D-glucopyranosylamino)-6-oxohexanoic acid* (***5***)*:* Compound **3** (1.51 g, 2.54 mmol), cobalt chloride hexahydrate (2.84 g; 11.90 mmol), and di-*tert*-butyl dicarbonate (1.63 g, 7.98 mmol) were dissolved in MeOH (40 mL, 0 °C). Sodium borohydride (2.26 g, 59.70 mmol) was added to the reaction mixture and stirred for 2 h at rt. TLC (eluent EtOAc) was used to determine the degree of completion and the crude mixture was filtered to elude crude compound **4**, which was then dissolved in THF, the benzyl group was cleaved via hydrogenation. Upon completion (checked with TCL, eluent EtOAc), crude product **5** (1.10 g, 1.18 mmol, 46.40% yield) was condensed via rotary evaporation.
15. Moyle, P. M.; Olive, C.; Good, M.; Toth, I. *J. Pept. Sci.* **2006**, *12*, 800.
16. *Solid-phase peptide synthesis (SPPS):* The vaccine construct **9** was synthesised using SPPS procedures and Boc-chemistry. *p*MBHA resin (0.40 mmol, 0.12 mmol scale), was swelled in DMF for 2 h followed by in situ neutralisation with DIPEA (10% v/v, 3× 15 min) in DMF. SPPS procedures involved a cycle of Boc-deprotection with TFA (2× 1-min treatments), flow-wash with DMF, and addition of pre-activated amino acids (4.4 equiv, 60–100 min). Amino acids were activated by mixing with 0.5 M HBTU–DMF solution (2.56 mL, 1.28 mmol, 4 equiv) and DIPEA (0.33 mL; 1.92 mmol). The coupling efficiency was checked with ninhydrin test to ensure 99.6% coupling efficiency. SPPS procedures were repeated until coupling efficiency was achieved. Lipid moiety was synthesised in the sequence of Boc-Gly-OH, 2× Boc-C12-OH, Boc-Gly-OH and Boc-C12-OH. The activated carbohydrate core **6** was then coupled to lipid moiety **7**. Following successful coupling, four copies of PL1 (EVLTR RQSQD PKYVT QRIS) peptide epitope, were coupled by stepwise SPPS to the liposaccharide **8**.
17. Sarin, V. K.; Kent, S. B. H.; Tam, J. P.; Merrifield, R. B. *Anal. Biochem.* **1981**, *177*, 147.
18. *Hydrofluoric acid (HF) cleavage procedures:* Completed vaccine construct was cleaved from *p*MBHA resin using anhydrous HF (Kel-F HF apparatus, Peptide Institute, Osaka, Japan). *Caution:* Highly hazardous to health. The procedure was performed at 0 °C for 3 h. *p*-Cresol (5%, v/v) was also present during the cleaving process as a scavenger. Upon completion, the product **9** was precipitated (diethyl ether), filtered, dissolved (40% ACN/H_2O) and lyophilised to elicit the crude vaccine candidate (**9**).
19. *Vaccine Construct* (***9***)*:* LC–ESI-MS, $[M+9H^+]^{9+}$ *m*/*z* 1155.4 (calcd, 1147.3), $[M+10H^+]^{10+}$ *m*/*z* 1040.1 (calcd, 1032.7), $[M+11H^+]^{11+}$ *m*/*z* 945.8 (calcd, 938.9), $[M+12^+]^{12+}$ *m*/*z* 860.7 (calcd, 866.8), $[M+13H^+]^{13+}$ *m*/*z* 800 (calcd, 794.6), $[M+14H^+]^{14+}$ *m*/*z* 743.2 (calcd, 737.9); Mw 10,316.8 g/mol.