Synthetic carbohydrate-based vaccines: synthesis of an L-*glycero*-D-*manno*-heptose antigen–T-epitope–lipopeptide conjugate

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A facile and highly efficient synthesis of a glycopeptidolipid containing a heptose sugar, a synthetic T-epitope and a synthetic immunoadjuvant is presented which represents an important step towards the development of a fully synthetic vaccine against *Neisseria meningitidis*.

Neisseria meningitidis can cause a severe form of meningitis and bacteriamia and is the only bacterium that is capable of generating epidemics of meningitis.[1] Although it is more than a century since meningococcus was discovered, no vaccine has been developed that matches requirements such as safety, long-lasting immunity to all age groups and cross-protection against meningococcal serotypes and sub-serotypes.

Saccharides representing partial structures of the inner-core oligosaccharide of meningococcal lipopolysaccharides (LPS) have been synthesised.[2] These structures were equipped with an artificial spacer which enabled controlled conjugation to tetanus toxoid (TT). The conjugates were used to immunise mice and evoked an L-2 or L-3,7,9 specific immune response.[3] Thus, small synthetic saccharides derived from meningococcal LPS functioned as effective B-epitopes. Furthermore, tetanus toxoid acted as a carrier protein as well as a source of T-epitopes to give immunological memory.

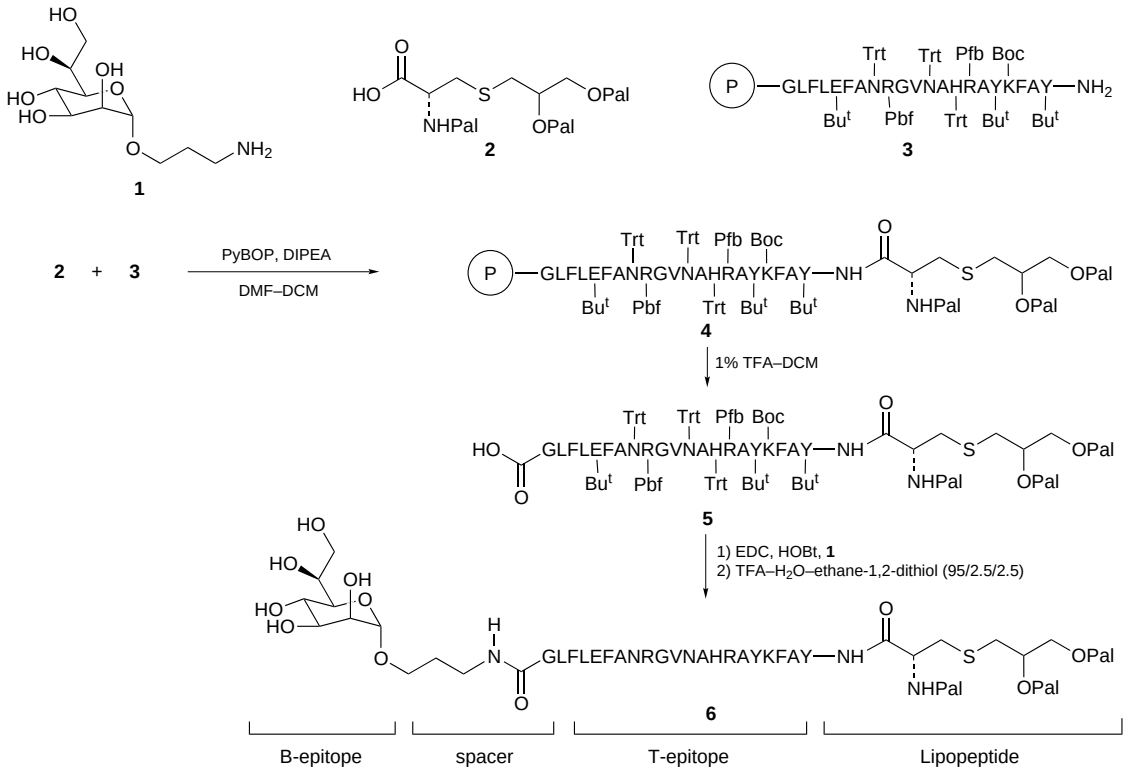
The use of TT as a carrier protein is problematic in several respects. For example, the antibody response to a haptan which is coupled to a carrier protein can be ineffective when vaccines have previously been immunised with the carrier protein.[4] Furthermore, heterologous memory T-cells (for example induced with TT conjugates) may not be reactivated during infection. Therefore, incorporation of homogeneous (*i.e.* meningococcal-derived) T-cell epitopes into a vaccine will be advantageous as the memory response will be more effective.[5] In addition, the advantages of the use of synthetic peptides derived from immunodominant T-cell epitopes compared to bacterial protein carriers are obvious: the immune response is directed selectively towards effective determinants, whereas sequences homologous to human self-protein can be excluded from the vaccine. The feasibility of the use of synthetic T-epitopes as vaccine components has recently been shown[6] by a conjugate composed of a *Heamophilus influenzae* capsular polysaccharide and functional T-helper cell peptide epitope, that elicited protective levels of antibodies in rabbits.

We report here a synthetic methodology that makes it possible to construct fully synthetic glycopeptidolipids that contain a carbohydrate-based B-epitope, a peptide T-epitope and a lipopeptide. Such a conjugate constitutes the minimal structural features required for an effective immunological response. We have synthesised a compound (**6**) that contains a L-*glycero*-D-*manno*-heptose sugar that will act as a B-epitope, the peptide sequence YAFKYARHANVGRNAFELFL that has been identified as a MHC class II restricted site for human T-cells and is derived from an outer-membrane protein of *N. meningitidis*[7] and the lipopetide *S*-[(*R*/*S*)-2,3-dipalmitoyloxypropyl]-*N*-palmitoyl-(*R*)-cysteine (Pam_3Cys). The lipopeptide Pam_3Cys is a highly potent B-cell and macrophage activator derived from the immunologically active N-terminal sequence of the principal lipoprotein of *Escherichia coli*[8] and has been used in synthetic peptide-based vaccine and cancer vaccine development.[9] The lipid moiety will also serve as a membrane anchor in the lipid layer of cell membranes which will ensure efficient and long-term delivery of the antigen.

The synthesis of the complex target compound **6** requires a highly convergent strategy and the synthetic methods selected should be compatible with the requirements of carbohydrate, peptide and lipid chemistry. It was envisaged that the artificial spacer containing saccharide building block **1**, the lipopeptide **2** and solid supported peptide **3** would be suitable starting materials. As can be seen in Scheme 1, they will be coupled by well-established amide-bond forming reactions. Thus, the carboxylic acid of the glycolipid **2** will be coupled to the amino terminus of a peptide (**3**) that has been assembled on a solid support. Next, cleavage of the lipopeptide from the solid support followed by coupling of the released C-terminus to the amino containing spacer of saccharide **1** will afford a fully protected conjugate. An additional glycine residue has been incorporated at the C-terminus of the peptide to avoid epimerisation during the latter coupling step. The proposed assembly and deprotection requires only four synthetic steps and extensive purification procedures are avoided since most of the reactions have been performed on a solid support.

The preparation of the spacer containing saccharide unit **1** is rather straightforward and was performed as described previously.[2] Lipopeptide **2** was prepared from cysteine in five steps according to the procedure of Wiesmüller *et al.*[10] The peptide **3** was prepared by automated continuous flow solid-phase peptide synthesis on a commerically available HMPB–MBHA-resin using N^{α}-fluorenylmethoxycarbonyl (Fmoc) amino acid derivatives, according to standard procedures.[11] The hyper acid sensitive linker allows the cleavage of the lipopeptide from the solid support without removal of the side-chain protecting groups. This feature is important since the unprotected side chains of aspartic acid, glutamic acid and lysine will interfere with the incorporation of the saccharide unit. The following side chain protecting groups were selected: Trt for Asn and His, Pbf for Arg, Boc for Lys and Bu^t for Tyr and Glu. Deprotection can be performed under mild acid conditions without affecting the glycosidic linkages and palmitoyl ester moieties.

The N-terminus of the solid supported peptide **3** was linked to the carboxylic acid unit of the Pam_3Cys **2** to give lipopeptide **4**. Model studies had established that the benzotriazol-1-yloxytris-(pyrrolidino)phosphonium hexafluorophosphate–Pr^i_2EtN coupling reagents allowed highly efficient and fast coupling. Furthermore, this method required less of the rather precious Pam_3Cys **3** compared to the use of a carbodiimide–1-hydroxybenzotriazole (HOBT) coupling system. Next, the conjugate was released from the solid support by treatment with 1% TFA in CH_2Cl_2 followed by immediate neutralisation with 10% pyridine–MeOH. The crude product was purified by size-exclusion column chromatography (Sephadex LH-20, CH_2Cl_2–MeOH 1 : 1, v/v) and 2D NMR and mass spectrometric analysis of the product obtained showed that the side-chain protecting groups had not been effected. Compound **5** was isolated in a yield of 75% based on a resin loading of 30 μmol. The revealed carboxylic acid at the C-terminus of the lipopeptide **5** was



Scheme 1

coupled with the amino-containing spacer moiety of the sugar unit **1** and in this case the coupling was best performed with EDC and HOBT in the presence of $Pr^{i}_{2}EtN$. Purification was accomplished by gel filtration and the fully protected conjugate was isolated in a yield of 91%. The molecular structure was confirmed by 1D and 2D NMR spectroscopy and mass spectrometry. FAB mass analysis of **5** showed a peak at *m*/*z* 5108 corresponding to $[M + Na]^+$. Scanning was also performed over a narrow mass range between 4800 and 5200. Peaks were present at *m*/*z* 5086 and 5108 corresponding to $[M + H]^+$ and $[M + Na]^+$, respectively. Further peaks at *m*/*z* 4843 and 4865 correspond to a fragment lacking one trityl protecting group and its sodium adduct, respectively. It is noteworthy that the hydroxy groups of the sugar moiety were unprotected and did not interfere with the coupling reaction. This feature is important because the most commonly used permanent protecting groups in carbohydrate chemistry are benzyl ethers or acetyl or benzoyl esters. Removal of these protecting groups would cause problems as base-catalysed deacylation of a product may result in the loss of the palmitoyl moieties. Furthermore, catalytic hydrogenation of benzyl ethers would be difficult because of poisoning of the palladium catalyst by the sulfur residues in the compound. Finally, the side chain protecting groups of the peptide were removed by treatment with TFA–water in the presence of ethane-1,2-dithiol as a scavenger. The obtained compound was purified by HPLC on a phenogel 5 μ 100 Å column (eluent: DMSO) and after freeze drying the structural integrity of **6** was confirmed by FAB mass spectrometry and 1D and 2D NMR spectroscopy. FAB mass analysis of **6** showed a peak at *m*/*z* 3586 corresponding to $[M + H]^+$.

In conclusion, we have developed a highly convergent strategy for the assembly of compounds that contain a carbohydrate B-epitope, a peptide T-epitope and a lipopeptide adjuvant. The use of these glycoconjugates as vaccines will have several advantages compared to conventional polysaccharide-protein conjugate vaccines. They are well-defined and can be purified to homogeneity and are chemically stable. The incorporation of homogeneous T-epitopes will enhance the immunological response.

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Footnote and References

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1 D. A. A. Ala'Aldeen and K. A. C. Cartwright, *J. Infect.*, 1996, **33**, 153.
2 G. J. P. H. Boons, G. A. van der Marcel and J. H. van Boom, *Tetrahedron Lett.*, 1989, **30**, 229; G. J. P. H. Boons, M. Overhand, G. A. van der Marcel and J. H. van Boom, *Angew. Chem., Int. Ed. Engl.*, **28**, 1989, 1504; G. J. P. H. Boons, P. Hoogerhout, J. T. Poolman, G. A. van der Marel and J. H. van Boom, *Biomed. Chem. Lett.*, 1991, **1**, 303.
3 A. F. M. Verheul, G. J. P. H. Boons, G. A. van der Marcel, J. H. van Boom, H. J. Jennings, H. Snippe, J. Verhoef, P. Hoogerhout and J. T. Poolman, *Infect. Immun.*, 1991, **59**, 3566.
4 T. Barington, M. Skettrup, L. Jull and C. Heilmann, *Infect. Immun.*, 1993, **61**, 432.
5 L. M. Bradley, M. Croft and S. I. Swain, *Immunol. Today*, 1993, **14**, 197.
6 A. A. Kandil, N. Chan, M. Klein and P. Chong, *Glycoconjugate J.*, 1997, **14**, 13.
7 E. J. H. J. Wiertz, J. A. M. van Gaans-van den Brink, G. M. Th. Schreuder, A. A. M. Termijtelen, P. Hoogerhout and J. T. Poolman, *J. Immunol.*, 1991, **147**, 2012; E. J. H. J. Wiertz, J. A. M. van Gaans-van den Brink and H. Gausephol, *J. Exp. Med.*, 1992, 79.
8 P. Hoffmann, K. H. Weismüller, J. Metzger, G. Jung and W. G. Bessler, *Biol. Chem.*, 1989, **370**, 575; W. G. Bessler, M. Cox, A. Lex, B. Spur, K. Weismüller and G. Jung, *J. Immunol.*, 1985, **135**, 1900.
9 T. Toyakuni, B. Dean, S. Cai, D. Boivin, S. Hakomori and A. K. Singhal, *J. Am. Chem. Soc.*, 1994, **116**, 395; K. H. Wiesmüller, W. G. Bessler and G. Jung, *Int. J. Pept. Protein Res.*, 1992, **40**, 255; J. P. Defoort, B. Nardelli, W. Huang, D. D. Ho and J. P. Tam, *Proc. Natl. Acad. Sci. USA*, 1992, **89**, 3879.
10 K. H. Wiesmüller, W. G. Bessler and G. Jung, *Hoppe-Seyler's Z. Physiol. Chem.*, 1983, **364**, 593.
11 E. Atherton and R. C. Sheppard, *Solid phase synthesis: A practical approach*, IRL Press, Oxford University Press, Oxford, 1989.

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