Journal of Peptide Science, Vol. 2, 66–72 (1996)

# Synthesis of a New Template with a Built-in Adjuvant and Its Use in Constructing Peptide Vaccine Candidates Through Polyoxime Chemistry

**WEIGUANG ZENG[1,3], DAVID C. JACKSON[2] and KEITH ROSE[1]**

[1] Biochimie Médicale, CMU, CH-1211 Geneva 4, Switzerland
[2] Department of Microbiology, University of Melbourne, Parkville 3052, Victoria, Australia
[3] Gryphon Sciences, 250E Grand Ave, South San Francisco, CA 94080, USA

*Received 20 September 1995*
*Accepted 12 October 1995*

Synthetic lipopeptides are showing promise as vaccine candidates, but until now it has been very difficult to prepare them in homogeneous form. We describe the synthesis and characterization of a new water-soluble, four-branched template with a built-in lipophilic adjuvant ($Pam_3Cys$). Through the use of oxime chemistry, we attached four copies of an unprotected influenza virus peptide and characterized the product (13 kDa) by reversed-phase HPLC and electrospray ionization mass spectrometry. Several other such constructions were made using the new template and different peptides. We seem to have a general method for making synthetic lipopeptides in homogeneous form.

Keywords: MAPS; $Pam_3Cys$; polyoxime; polypeptide vaccine

## INTRODUCTION

In recent years there have been many reports on the use, and prospective use, of synthetic peptides as vaccines. Short peptides usually need to be coupled to carrier proteins to induce an antibody response against haptenic determinants, but the hapten-carrier system is not always a well-defined entity: it usually contains many irrelevant determinants, which may cause undesirable side effects [1–3]. There have been several attempts to circumvent the problems of using carrier molecules. They generally involve incorporating covalently several copies of the same or different peptide antigens on a polyfunctional core, e.g. multiple antigen peptide (MAP) system from Tam [4, 5], using an oligomeric branching lysine as the core; multiple attachment of antigenic peptides to the human IgG1 hinge region from Wünsch *et al.* [6], and the design of TASP (template assembled synthetic protein) from Mutter *et al.* [7]. All of these methods have been used with some success. However, the application of these approaches to synthesize large, complex molecules does not always lead to a homogeneous product.

Chemoselective ligation has been used to synthesize such large, complex molecules with defined structure [8]. There are several methods currently in use, e.g. oxime formation [9], thioester formation [10, 11], thioether formation [5], disulphide formation [12], segment ligation [13], domain ligation [14–16], hydrazone formation [16] and mostly recently native chemical ligation [17]. With these methods, homogeneous products are now much more easily accessible. The polyoxime chemistry, developed in our laboratory and studied and exploited in conjunction with Gryphon Sciences, has the advantages of very mild conditions, good yield and a very pure product [9]. With this method unprotected peptides, functionalized to carry either aldehyde or aminooxy groups, react with an appropriate template through

Abbreviations: AoA, aminooxyacetyl; CTL, cytotoxic T lymphocytes; DIEA, ethyldiisopropylamine; FCA, Freund's complete adjuvant; HBTU, 2-(1H-benzotriazole-1-yl-)-1,1,3,3,-tetramethyluronium hexafluorophosphate; MAPS, multiple antigen peptide system; $Pam_3Cys$, *N*-palmitoyl-S-(2,3-bis-(palmitoyloxy)-propyl)-cysteine; TASP, template assembled synthetic protein; TBTU, 2-(1H-benzotriazole-1-yl-)-1,1,3,3-tetramethyluronium tetrafluoroborate.

Address for correspondence: Dr Keith Rose, Biochimie Médicale, CMU, 1 rue Michel-Servet, CH-1211 Geneva 4, Switzerland; Phone (0041) 22 702 55 23, Fax (0041) 22 346 87 58.


CCC 1075-2617/96/010066-07

the formation of oxime bonds to yield homogeneous artificial proteins. We have used this approach to construct several polypeptide vaccine candidates, one of which has already given very promising results in assays of antibody titre and T-cell proliferation [18, 19].

Tripalmitoyl-*S*-glyceryl cysteine ($Pam_3Cys$) was derived from a lipoprotein of *Escherichia coli* and has been found to act as a potent adjuvant for vaccines [5, 20, 21]. It has been shown that $Pam_3Cys$ increases immunogenicity significantly when linked to a peptide antigen. Synthetic viral peptides covalently linked to $Pam_3Cys$-Ser can efficiently prime influenza virus-specific CTL (cytotoxic T lymphocytes) *in vivo* [21]. CTLs are part of the cellular immune response that can provide effective protection against a viral infection. These desirable properties make $Pam_3Cys$ suitable as a built-in adjuvant.

Wishing to combine the advantages offered by oxime-based chemospecific ligation and by the presence of $Pam_3Cys$, we synthesized a new water-soluble template with a built-in adjuvant $Pam_3Cys$ and attached unprotected peptide fragments to this template using polyoxime chemistry.

## MATERIALS AND METHODS

### General Procedures

Unless otherwise specified, all solvent and reagents were obtained from commercial sources, were of analytical or higher grade, and were used without further purification.

Boc-Ser(tBu)-OH was prepared according to Wünsch and Jentsch [22]. Dde-Lys(Fmoc)-OH was purchased from Nova, Switzerland, and repurified by RP-HPLC. $Pam_3Cys$-OH was prepared according to Wiesmüller *et al.* [23] with a minor modification of the step of esterification of the diol. Instead of a 1.25-fold excess of palmitic acid as used in the original literature, a 4-fold excess of palmitic acid was used here. In this way there was no $Pam_2Cys$-OH contaminant. The excess palmitic acid was easily removed by column chromatography using silica gel as adsorbent and solvent system ($CHCl_3$/$CH_3COOC_2H_5$/37%$NH_4OH$, 100 : 4 : 0.5, by volume). The palmitic acid eluted much later than $Pam_3Cys$ *tert*-butylester under these chromatographic conditions.

Analytical reversed-phase HPLC was performed using a 250 × 4 mm i.d. Nucleosil 300-A 5 μm $C_4$ column; solvent A was 0.1% TFA; solvent B was 0.1% TFA in 90% acetonitrile (1 g TFA/100 ml HPLC-grade water, made up to 1 litre with HPLC-grade acetonitrile), and monitoring at 214 nm. Except where otherwise noted, the conditions were as follows: flow rate, 0.6 ml/min; 5 min isocratic at 100% A followed by a linear gradient of 2% solvent B/min to 100% B. Semi-preparative HPLC was carried out using a column 250 × 10 mm i.d. packed with Nucleosil 300-A 5 μm $C_8$ particles eluted at a flow rate of 4 ml/min. Components were collected manually at the detector outlet, frozen and lyophilized. ESI-MS was performed in positive ion mode on a Trio 2000 machine (VG BioTech, Altrincham, England) equipped with a 3000 a.m.u. RF generator. Samples were introduced at 2 μl/min in solution in water/acetonitrile/AcOH (49.5 : 49.5 : 1, by volume).

### Synthesis of the Linear Peptide

The antigenic peptide of sequence P K Y V K Q N T - L K L A T G M R N V P E K Q T [24] was synthesized using an automated solid-phase synthesizer (model ABI 430A, Applied Biosystems Inc.) with 0.5 mmol of preloaded Sasrin resin (Bachem, Switzerland). Side-chain protection of the Fmoc-protected amino acids was as follows: Glu(tBu), Lys(Boc), Gln(Trt), Asn(Trt), Arg(Pmc), Thr(tBu) and Tyr(tBu). 2.2 mmol of each Fmoc-protected amino acid (4.4-fold excess over the substitution of the resin) were weighed in the cartridges. HBTU was prepared as a 0.5 M solution in DMF. During the synthesis, the Fmoc-protected amino acids were dissolved by automated addition of 4 ml HBTU solution (2 mmol) and 1 ml of DIEA and activated for 8 min. The coupling time was 20 min. Single couplings were performed throughout the synthesis. Fmoc group was removed by 2 × 2 min and 1 × 10 min treatment with 20% piperidine in DMF. After assembly of the peptide on the resin and machine-assisted removal of the N-terminal Fmoc group, the resin was removed and the aminooxyacetyl group was attached manually using Boc-aminooxyacetyl *N*-hydroxysuccinimide ester (0.1 M in DMF, 2.5 equivalents over the resin-bound amino groups, apparent pH 8–9 with *N*-methyl morpholine, 2 h). The peptide with N-terminal aminooxyacetyl modification was deprotected and cleaved from the resin using a mixture consisting of TFA (90%), thioanisole (5%), ethanedithiol (3%) and anisole (2%). After stirring at room temperature for 2 h, the mixture was filtered through a porous Teflon filter, partially evaporated under a stream of nitrogen, and then precipitated with cold diethyl ether. The precipitate was washed three times with ether, dried, taken up in water and then lyophilised. The crude

peptide was purified using semi-preparative HPLC. The product was a single peak on analytical HPLC and had the expected mass: calculated 2718; found, 2718.

### Synthesis of Template I

Synthesis of template **I** (Scheme 1) was performed manually with 0.30 g preloaded Sasrin resin (substitution 0.6 mmol/g) using TBTU/HOBt/DIEA activation. An excess of three equivalents of amino acid derivative over resin-bound amino groups was used. The three equivalents of amino acid derivatives and the same equivalents of TBTU were mixed as powers. Solutions of 1 M HOBt and 1.4 M DIEA in DMF were prepared. During the synthesis, amino acid derivatives and TBTU were dissolved by adding an equivalent (with respect to amino acid derivatives) of HOBt (1 M in DMF) and 1.4 equivalents of DIEA (1.4 M in DMF), with respect of the amino acid derivatives, and adding immediately to the free amino peptide resin with vortexing. The coupling efficiency was controlled by the Kaiser test. The coupling times were 2 h for Fmoc-Lys(Fmoc)-OH, $Pam_3Cys$-OH and Boc-Ser(t-Bu)-OH, and 30–45 min for the other amino acid derivatives. For the introduction of $Pam_3Cys$ we used Dde-Lys(Fmoc)-OH [25] as the sixth amino acid coupled on the resin. Following Fmoc removal the $\varepsilon$-amino group was acylated with $Pam_3Cys$-OH under the same coupling conditions as with the other amino acids except for addition of some dichloromethane to solubilize the $Pam_3Cys$-OH. The Dde group was removed with 2% hydrazine in DMF for 10 min. After further extension (Scheme 1), Boc-Ser(tBu)-OH was coupled for the later generation of aldehyde functions. Peptide–resin was cleaved with methyl sulphide/water/triisopropylsilane/TFA (0.5 : 0.5 : 0.2 : 10) for 2 h and the products precipitated by addition of cold ether. The crude material was purified by semi-preparative HPLC and characterized by ESI-MS.

### Generation of Aldehyde Functions on the Template [26]

An amount weighing 1.15 mg of template **I** was dissolved in 800 $\mu$l of imidazole buffer (50 mM, pH 6.95, chloride counter ion) and 500 $\mu$l of acetonitrile, and then 180 $\mu$l of a solution of methionine (200 mM) was added. Finally 72 $\mu$l of a solution of $NaIO_4$ (100 mM in water) was added to start the oxidation. After 2 min the oxidation was quenched by adding 144 $\mu$l of a solution of ethyleneglycol (100 mM in water). The product was isolated on the analytical column and lyophilized.

### Oximation [9]

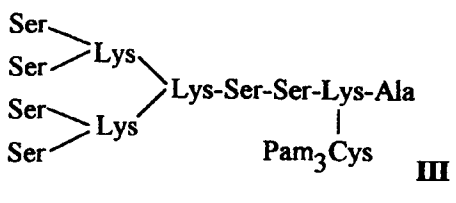
Solutions of the oxidized template (2.5 mM in water) and the aminooxyacetyl peptide (10 mM in 0.5 M acetate buffer, counter-ion sodium, pH 3.7) were prepared. Oxime formation was initiated by mixing 200 $\mu$l (0.50 $\mu$mol) template with 400 $\mu$l (4.0 $\mu$mol) peptide derivative (Scheme 2). After 24 h at room temperature, the tetraoxime was isolated by semi-preparative RP-HPLC.

## RESULTS

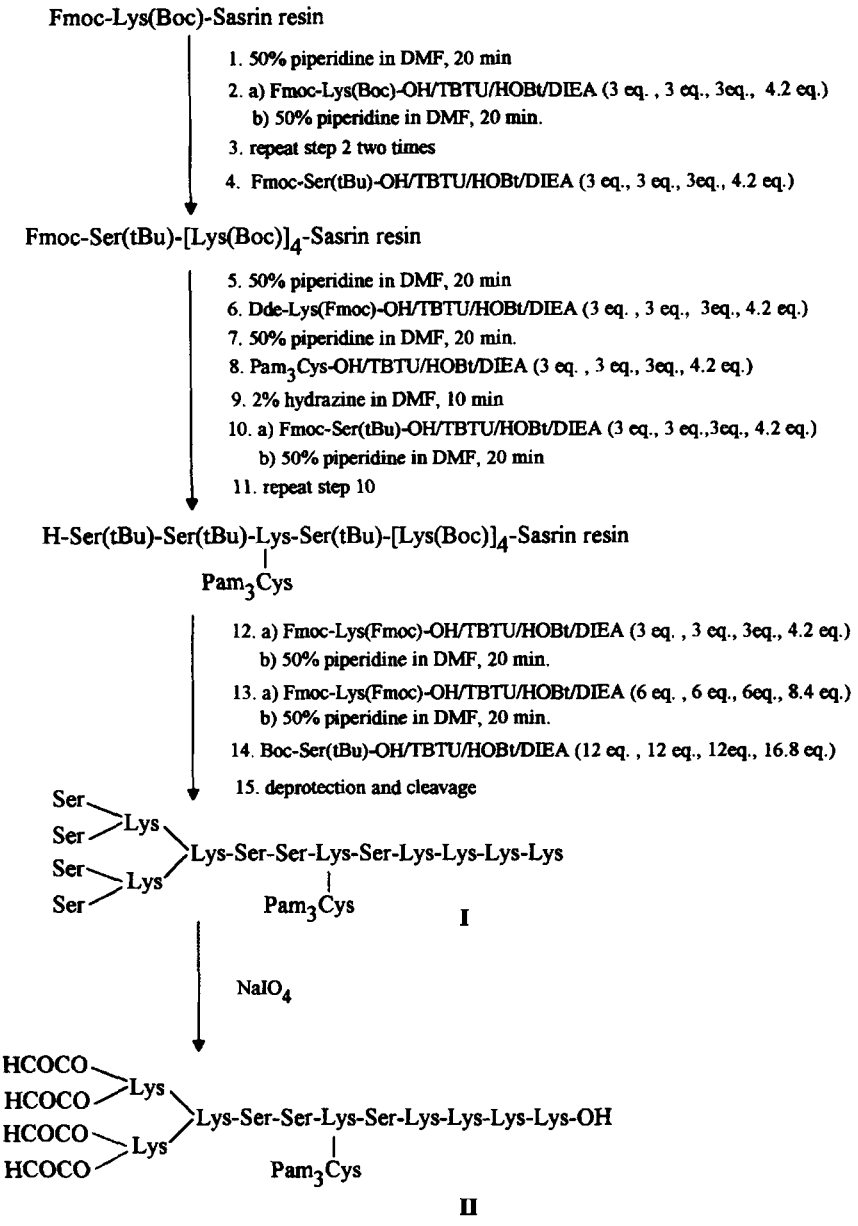
The first template we synthesized has the structure **III**. It is similar to the template described by Tam, with two Ser residues incorporated as spacers and Ala at the C-terminus [5]. The synthesis was completed successfully and the crude material was easily purified to yield product with the expected mass spectrum (not shown). However, template **III** was very hydrophobic and had poor water-solubility due to the highly hydrophobic $Pam_3Cys$. For further handling a more hydrophilic template is desirable.

An amphiphilic and water-soluble lipohexapeptide $Pam_3CysSer(Lys)_4$-OH has been described by Metzger *et al.* [27]. This $Pam_3Cys$ peptide has the same functions as $Pam_3Cys$, acting as a non-toxic, non-pyrogenic immune adjuvant whether mixed with or covalently linked to antigens and haptens. So we decided to incorporate this peptide on our template **I** to increase its solubility in water.

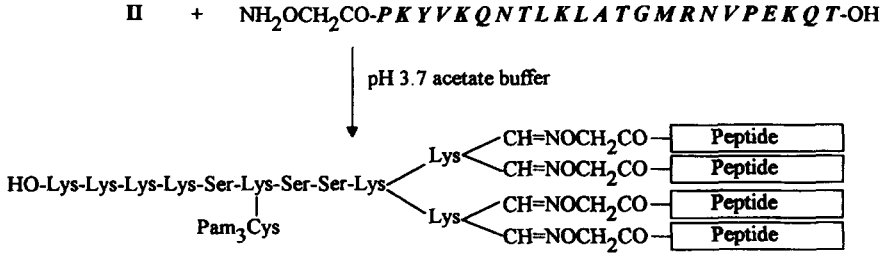
For the introduction of $Pam_3Cys$ in MAPS templates Fmoc-Lys($Pam_3Cys$)-OH has been used [5]. But the preparation of this Lys derivative is a three-step synthesis and the yield was low. We used a more direct method and coupled $Pam_3Cys$ directly to the peptide resin. For this purpose Dde-Lys(Fmoc)-OH [25] was coupled as the sixth amino acid to the peptide resin. The Fmoc protecting group was removed selectively with 50% piperidine. $Pam_3Cys$ was then coupled to this $\varepsilon$-amino group. After the removal of the Dde group with 2% hydrazine, the




**III**




Scheme 1 Synthesis of the new template **I** and its oxidation to give **II**.




Scheme 2 Synthesis of the tetraoxime. Note that for convenience template **II** is drawn unconventionally with its C-terminus on the left. It is shown conventionally in Scheme 1.

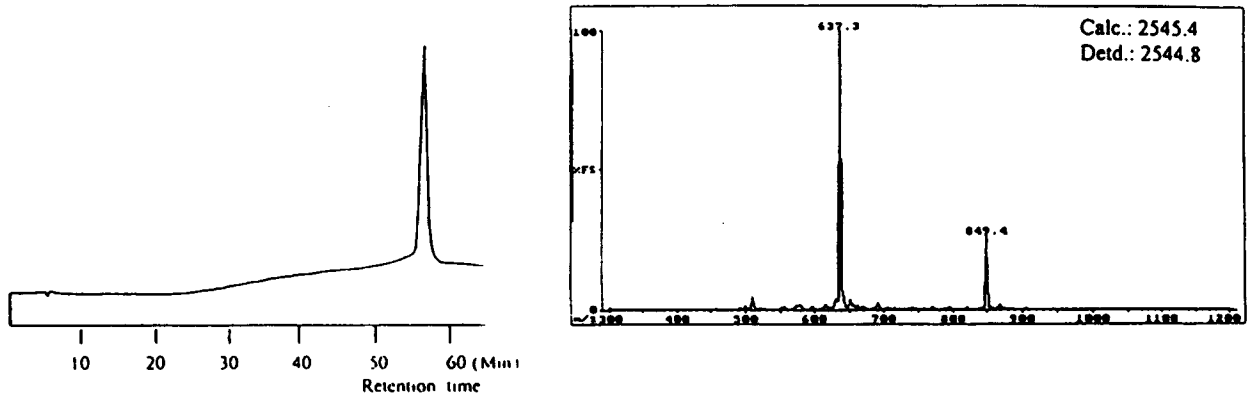



Figure 1 Characterization of template **I** by analytical RP-HPLC and ESI-MS.

assembly of the template was carried on in the normal way on the $N^{\alpha}$-amino group. The two ester bonds of the $Pam_3Cys$ were stable under these conditions as controlled by ESI-MS of the cleaved crude products.

Peptide–resin was cleaved with TFA in the presence of scavengers and the products precipitated by addition of cold ether. HPLC and ESI-MS were used to control the quality of the crude product. The analysis showed that the main component was the expected material and that products of side reactions, probably formed by acylation of Ser residues by the trifluoroacetyl cation generated in the cleavage mixture, exhibited masses which were 96 and 192 Da greater than expected. Several different scavengers with water, phenol, thioanisole, methyl sulphide, ethanedithiol and their combinations were tested to suppress this side reaction. The best result we achieved was the combination: methyl sulphide/water/triisopropylsilane/TFA (0.5 : 0.5 : 0.2 : 10). This side reaction was observed also by us in the synthesis of a eight-branched template with eight N-terminal Ser residues, and this side reaction has been recently reported by others [28]. The crude material was purified by semi-preparative HPLC and characterized by ESI-MS (Figure 1).

For the generation of the aldehyde functions the 1,2-amino alcohol moiety of the N-terminal Ser was oxidized with sodium periodate at pH 6.95 [26]. Methionine was added to preclude the oxidation of the thioether bond of $Pam_3Cys$ [29]. Because of the higher hydrophobic property of the oxidized product addition of acetonitrile was necessary to get a homogeneous product. Analytical HPLC of the oxidation reaction at various times (2–10 min) showed that the oxidation was completed in 2 min.

Condensation of the unprotected peptide derivative to the template molecule through oxime formation was achieved under mild conditions in sodium acetate buffer monitored by analytical HPLC. It was

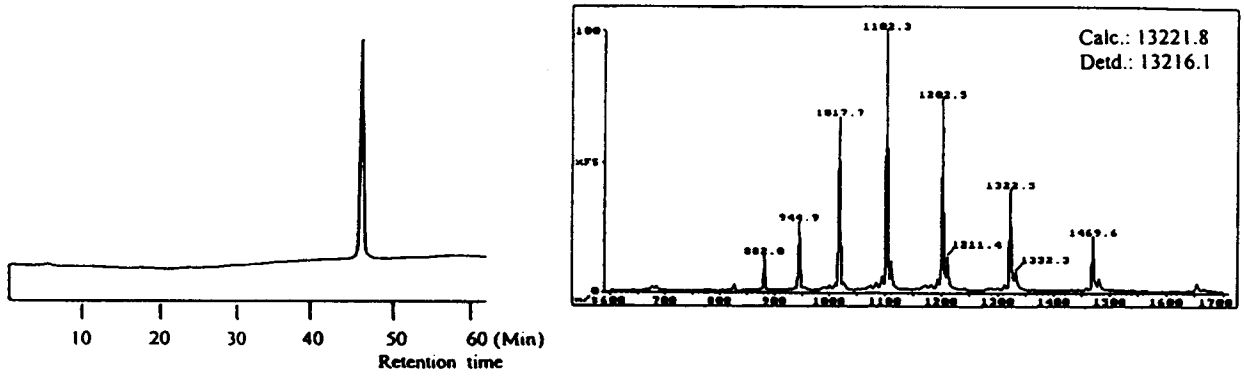



Figure 2 Characterization of the tetraoxime by analytical RP-HPLC and ESI-MS. The small series of signals at $m/z$ 1211.4, 1332.3 is due to non-covalent association with phosphoric acid, a common feature of the ESI-MS spectra of proteins. The product is homogeneous as judged by HPLC and ESI-MS.

observed that oxime formation was much slower than similar reactions without the $Pam_3Cys$ moiety on the template and had to be performed under more acidic condition (pH 3.7) to compensate. The reaction proceeded essentially to completion over 18 h. The tetraoxime product was isolated with shallow gradient HPLC and characterized by ESI-MS (Figure 2).

## DISCUSSION

Our earlier reports have already shown that macromolecules synthesized by the polyoxime approach are not only easy to make and to purify but that they performed well in a variety of biological assays used to test candidate vaccines [18]. The experiments described here represent an extension of the previous work by showing the synthesis of a new template with a built-in adjuvant $Pam_3Cys$ and the addition of unprotected peptides through polyoxime chemistry.

In common with peptide antigens conjugated to protein carriers, artificial protein constructs without a built-in adjuvant usually require Freund's complete adjuvant (FCA) to elicit a high titred antibody response and a CTL response [5]. FCA, derived from the extraction of mycobacterial cell wall, is pyrogenic, induces many side effects and is only used in laboratory animals [30]. In contrast, $Pam_3Cys$ is synthetic and has been found non-toxic [20]. The advantages of using $Pam_3Cys$ as a built-in adjuvant in MAPS constructs have already been demonstrated by Tam's group [5]. They found that a peptide antigen in a tetravalent model with $Pam_3Cys$ as built-in adjuvant induced antibodies *in vitro* and primed cytotoxic T lymphocytes *in vivo* without any added adjuvants. They used two different routes to synthesize their constructs: a direct stepwise approach and an indirect modular approach. No HPLC chromatograms have been shown for the control of the qualities of their products, although the indirect modular approach using thioether formation produced products homogeneous enough to allow unambiguous identification by mass spectrometry. Lipopeptides are generally difficult to make and purify: a palmitoylated synthetic peptide construction was recently used in immunogenicity trials in humans but was only 85% pure [31]. It did, however, show the value of lipopeptides for adjuvant-free vaccines in humans. Our approach described here not only precludes the side reaction of oxidation of the sulphydryls, which otherwise accompanies thioether formation during the conjugation step, but yields products that are water-soluble and easy to make and to purify. We have since made several other tetraoximes using the same $Pam_3Cys$-containing template, so the approach seems to be general.

### Acknowledgements

We thank Mrs Irène Rossitto-Borlat and Mr Pierre-Olivier Regamey for expert technical assistance, Dr K.-H. Wiesmüller for a gift of a sample of $Pam_3Cys$-OH and Gryphon Sciences Inc. for financial support.

## REFERENCES

1. R. A. Lerner (1984). Antibodies of predetermined specificity in biology and medicine. *Adv. Immunol. 36*, 1–44.
2. M.-P. Schultze, C. Leclerc, M. Jolivet, F. Audibert and L. Chedid (1985). Carrier-induced epitopic suppression, a major issue for future synthetic vaccines. *J. Immunol. 135*, 2319–2322.
3. H. L. Niman, R. A. Houghten, L. E. Walker, R. A. Reisfeld, I. A. Wilson, J. M. Hogle and R. A. Lerner (1983). Generation of protein-reactive antibodies by short peptides is an event of high frequency. Implications for the structural basis of immune recognition. *Proc. Natl. Acad. Sci. USA 80*, 4949–4953.
4. J. P. Tam (1988). Synthetic peptide vaccine design: synthesis and properties of a high-density multiple antigenic peptide system. *Proc. Natl. Acad. Sci. USA 85*, 5409–5413.
5. J.-P. Defoort, B. Nardelli, W. Huang and J. P. Tam (1992). A rational design of synthetic peptide vaccine with a built-in adjuvant. *Int. J. Peptide Protein Res. 40*, 214–221.
6. E. Wünsch, L. Moroder, G. Hübener, H.-J. Musiol, R. Von Grünigen, W. Göhring, R. Scharf and C. H. Schneider (1991). Fully synthetic immunogens. *Int. J. Peptide Protein Res. 37*, 90–102.
7. M. Mutter, G. G. Tuchscherer, C. Miller, K.-H. Altman, R. I. Carey, D. F. Wyss, A. M. Labhardt and J. E. Rivier (1992). Template-assembled synthetic proteins with four-helix-bundle topology. Total synthesis and conformational studies. *J. Am. Chem. Soc. 114*, 1463–1470.
8. J. P. Tam and J. C. Spetzler (1995). Chemoselective approaches to the preparation of peptide dendrimers and branched artificial proteins using unprotected peptides as building blocks. *Biomed. Peptide Protein Nucleic Acids 1*, 123–132.
9. K. Rose (1994). Facile synthesis of artificial proteins. *J. Am. Chem. Soc. 116*, 30–33.
10. M. Schnölzer and S. B. H. Kent (1992). Constructing proteins by dovetailing unprotected synthetic peptides: back-bone engineering HIV protease. *Science 256*, 221–225.

11. P. E. Dawson and S. B. H. Kent (1993). Convenient total synthesis of a 4-helix TASP molecule by chemoselective ligation. *J. Am. Chem. Soc. 115*, 7263–7266.
12. J. W. Drijfhout and W. Bloemhoff (1991). A new synthetic functionalized antigen carrier. *Int. J. Peptide Protein Res. 37*, 27–32.
13. C.-F. Liu and J. P. Tam (1994). Peptide segment ligation strategy without use of protecting groups. *Proc. Natl. Acad. Sci. USA 91*, 6584–6588.
14. C. Rao and J. P. Tam (1994). Synthesis of peptide dendrimer. *J. Am. Chem. Soc. 116*, 6975–6976.
15. J. Shao and J. P. Tam (1995). Unprotected peptides as building blocks for the synthesis of peptide dendrimers with oxime, hydrazone, and thiazolidine linkages. *J. Am. Chem. Soc. 117*, 3893–3899.
16. J. C. Spetzler and J. P. Tam (1995). Unprotected peptides as building blocks for branched peptides and peptide dentrimers. *Int. J. Peptide Protein Res. 45*, 78–83.
17. P. E. Dawson, T. W. Muir, I. Clark-Lewis and S. B .H. Kent (1994). Synthesis of proteins by native chemical ligation. *Science 266*, 776–779.
18. K. Rose, W. Zeng, L. E. Brown and D. C. Jackson. A synthetic peptide based polyoxime vaccine construct of high purity and activity. *Mol. Immunol.*, in press.
19. W. Zeng, K. Rose and D. C. Jackson in: *Peptides 1994*, H. L. S. Maia, Ed., pp. 855–856, ESCOM, Leiden, The Netherlands, 1995.
20. K.-H. Wiesmüller, G. Jung, D. Gillessen, C. Loffl, W. Bessler and T. Böltz (1991). The antibody response in BALB/c mice to the *Plasmodium falciparum* circumsporozoite repetitive epitope covalently coupled to synthetic lipopeptide adjuvant. *Immunology 72*, 109–113.
21. K. Deres, H. Schild, K.-H. Wiesmüller, G. Jung and H.-G. Rammensee (1989). *In vivo* priming of virus-specific cytotoxic T lymphocytes with synthetic lipopeptide vaccine. *Nature 342*, 561–564.
22. E. Wünsch and J. Jentsch (1964). Zur Darstellung von Hydroxyaminosäure-tert-butyläthern. *Ber. dtsch. chem. Ges. 97*, 2490–2496.
23. K.-H. Wiesmüller, W. Bessler and G. Jung (1983). Synthesis of the mitogenic *S*-[2,3-bis(palmitoyloxy)propyl]-*N*-palmitoylpentapeptide from *Escherichia coli* lipoprotein. *Hoppe-Seyler's Z. Physiol. Chem. 364*, 593–596.
24. D. C. Jackson and L. E. Brown (1991). A synthetic peptide of influenza virus hemagglutinin as a model antigen and immunogen. *Peptide Res. 4*, 114–124.
25. B. W. Bycroft, W. C. Chan, S. R. Chhabra and N. D. Hone (1993). A novel lysine-protection procedure for continuous flow solid phase synthesis of branched peptides. *J. Chem. Soc. Chem. Commun*, 778–779.
26. L. A. Vilaseca, K. Rose, R. Werlen, A. Meunier, R. E. Offord, C. L. Nichols and W. L. Scott (1993). Protein conjugates of defined structure. Synthesis and use of a new carrier molecule. *Bioconj. Chem. 4*, 515–520.
27. J. Metzger, K.-H. Wiesmüller, R. Schaude, W. G. Bessler and G. Jung (1991). Synthesis of novel immunologically active tripalmitoyl-*S*-glycerylcysteinyl lipopeptides as useful intermediates for immunogen preparations. *Int. J. Peptide Protein Res. 37*, 46–57.
28. P. W. Banda, L. Nuwaysir and L. Chen in: *Peptides: Chemistry, Structure and Biology*, P. T. P. Kaumaya, Ed., ESCOM, Leiden, The Netherlands, in press.
29. H. F. Gärtner, K. Rose, R. Cotton, D. Timms, R. Camble and R. E. Offord in *Peptides 1992*, C. H. Schneider and A. N. Eberle. Eds., pp. 239–240, ESCOM, Leiden, The Netherlands 1993.
30. J. R. Broderson (1989). A retrospective review of lesions associated with the use of Freund's adjuvant. *Lab. Anim. Sci. 39*, 400–405.
31. A. Vitiello, G. Ishioka, H. M. Grey, R. Rose, P. Farness, R. Lafond, L. Yuan, F. V. Chisari. J. Furze, R. Bartholomeuz and R. W. Chesnut (1995). Development of a lipopeptide-based therapeutic vaccine to treat chronic HBV infection. *J. Clin. Invest. 95*, 341–349.