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Design and Synthesis of Thiol-Reactive Lipopeptides

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Abstract: Lipopeptides are potent adjuvants that trigger an immune response against covalently conjugated low molecular mass antigens. We report here the design and synthesis of thiol-reactive lipopeptides (**6**, **7**) which can be incorporated into liposomes and react, under mild conditions, with synthetic peptides carrying a thiol function.

Synthetic lipopeptide analogs of *Escherichia coli* lipoprotein, such as $Pam_3CysAlaGly$ (**Scheme 3**) or $Pam_3CysSerSer$, are potent immunoadjuvants able to induce an immune response against low molecular mass antigens[1] when injected either as an admixture[2] or as covalent conjugates.[3] Importantly, strong humoral and cellular immune responses could be obtained against peptide antigens covalently coupled to such lipopeptides.[4] Our aim is the design of synthetic peptide-based vaccines using liposomes as carriers.[5] We have previously shown that intense and long lasting immune responses could be triggered against peptide antigens conjugated, via a phospholipid anchor, to the surface of small unilamellar vesicles that incorporated in their bilayer amphiphilic adjuvants such as monophosphoryl lipid A[6] or lipopeptides.[7] We have now developed a second generation of liposomal constructs in which a single vesicle carries two different peptides, such as B-cell and T-helper cell epitopes, one of the epitopes being specifically conjugated to a lipopeptide.[8] This strategy required the design of lipopeptide derivatives which, after incorporation into preformed liposomes, could be conjugated to the peptides.

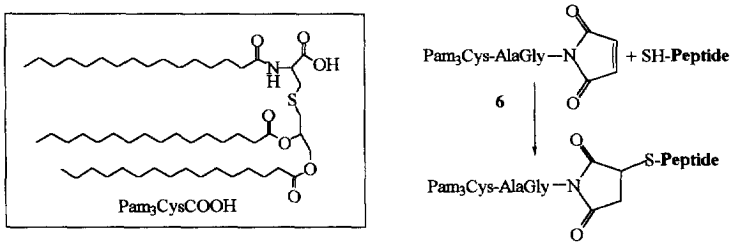

Figure 1: Coupling of a peptide-SH to a thiol-reactive lipopeptide.

The classical approach for the design of lipopeptide-peptide antigen conjugates is based on solid phase synthesis for each desired structure.[9] To improve the flexibility of this strategy, the C-terminal functions of lipopeptides were modified with e.g. *N*-hydroxysuccinimido esters[10] to react with the N-terminal function of a free peptide. But this procedure is not exempt of limitations, at least for our purpose. For example these esters,

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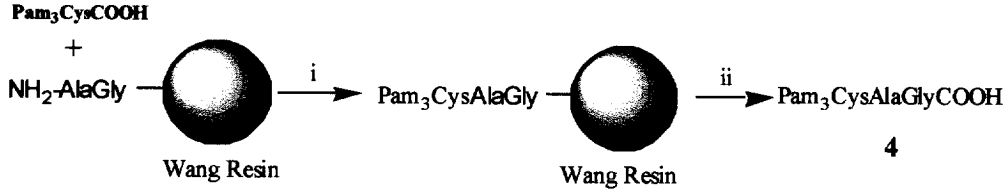
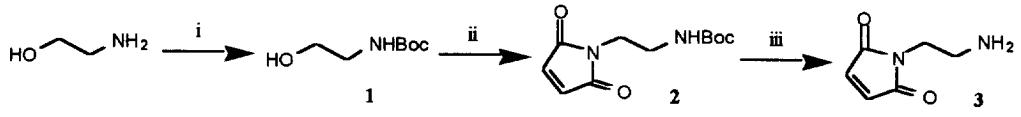
besides their lability in aqueous media, may also react with the ε-NH_2 of lysine residues present in peptides and yield a mixture of different products. We adopted a different strategy and designed a "universal" thiol-reactive lipopeptide (TRLP), whose C-terminal amino acid was modified with a maleimide group (**Figure 1**), that could be incorporated into liposomes. This approach allows the specific coupling under mild conditions, and in aqueous environments, of peptide epitopes carrying free thiol functions either at their N- or C-terminus, to the surface of preformed vesicles via a lipopeptide anchor (**Figure 1**). Moreover, because of the high reactivity of the maleimide group of such TRLP with peptides-SH, one can envisage the incorporation into the same liposomes of another, but lesser reactive, thiol-reactive amphiphilic anchor (e.g. bromoacetyl derivative of phosphatidylethanolamine) and thus perform a controlled sequential coupling of a second epitope[11] giving acess to diepitope constructs.[8]

The synthesis of the TRLP **6** and **7** first required the production of lipopeptides **4** and **5** (**Scheme 3**). This was achieved by solid-phase synthesis on a Wang resin. N^{α}-palmitoyl-*S*-(2,3-*bis*-palmitoyloxy-(2*RS*)-propyl)-(*R*)-cysteine ($Pam_3CysCOOH$; **Figure 1**), obtained in 6 steps following the procedure of Wiesmüller et al,[12] was coupled in the last step to the dipeptides AlaGly (**Scheme 1**) and SerSer previously prepared as resin conjugates according to conventional *N*-α-Fmoc amino acid methodologies. Compounds **4** and **5** were finally isolated, after cleavage from the resin by a simple treatment with TFA (**Scheme 1**; step ii), by precipitation in a mixture of $CHCl_3/CH_3OH$ (1:5) at -20°C (total yield : 40 to 60%). Compared to the liquid phase synthesis, this strategy gave much better yields of pure compounds mostly because it allowed a straightforward removal of reagents and $Pam_3CysCOOH$ which were used in a 3-fold excess in step (i) (**Scheme 1**).



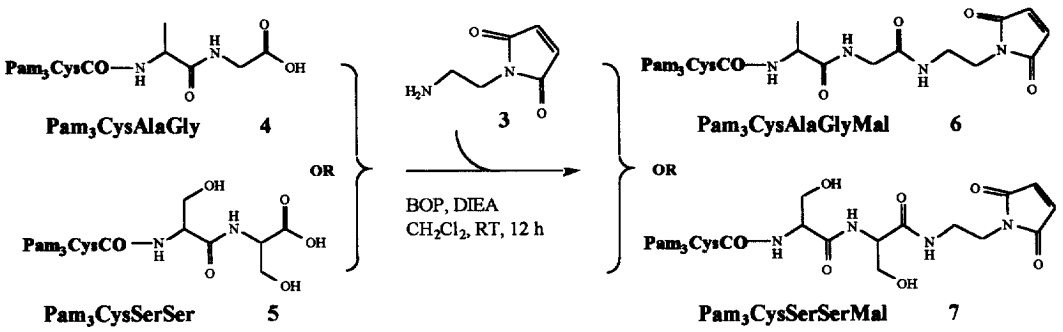
Scheme 1: i) BOP, DIEA, CH_2Cl_2, RT, 12 h; ii) TFA (100%), RT, 1h, (yield: 40 to 60 %).

The last step consisted in the introduction of the maleimido group on the lipopeptides (**Scheme 3**). This was accomplished by coupling to the C-terminal carboxylic groups of **4** and **5** the bifunctional molecule 1-(2-aminoethyl)-pyrrole-2,5-dione (**3**) that contains a free amino group and a maleimide group (**Scheme 2**). The synthesis of compound **3** is given in **Scheme 2**. In a first step, ethanolamine was protected with Boc. Then a Mitsunobu reaction was carried out in the presence of 1.2 eq. of maleimide; this reaction allows the convenient transformation of the alcohol into a maleimide group in a single step, and in good yields, under particularly mild conditions (neutral and at room temperature). The same approach has been described recently for the preparation of bifunctional maleimide cross-linkers.[13] After deprotection of the amino group, product **3** was isolated. Other attempts to obtain **3**, such as via the classical introduction of the maleimide group on monoprotected diaminoethane by reaction of the free amine with maleic anhydride, either under acidic conditions or by dehydration,[14] gave comparatively poor yields.



Scheme 2: i) Boc_2O, Dioxane/H_2O/NaOH 1M, 0°C to RT, 30 min.; ii) Ph_3P, DIAD, Maleimide, THF, RT, 1h; iii) TFA (40% in CH_2Cl_2), 0°C, 1h.

To achieve the synthesis of molecules **6** and **7** (**Scheme 3**), the carboxylic acid functions of the lipopeptides **4** and **5** were activated with benzotriazol-*N*-oxytrisdimethylaminophosphonium hexafluorophosphate (BOP) at room temperature in the presence of diisopropylethylamine. Compound **3** was then added and the reaction was carried out for 12 hours. The final products **6**,**7** were obtained in about 70 to 80% yield.[15]



Scheme 3: Synthesis of the thiol-reactive lipopeptides $Pam_3CysAlaGlyMal$ **6** and $Pam_3CysSerSerMal$ **7**.

The amphiphilic properties of the TRLPs allowed their easy incorporation into liposomes,[16] which were then reacted with peptides-SH to form peptide-based vaccines (**Figure 3**).

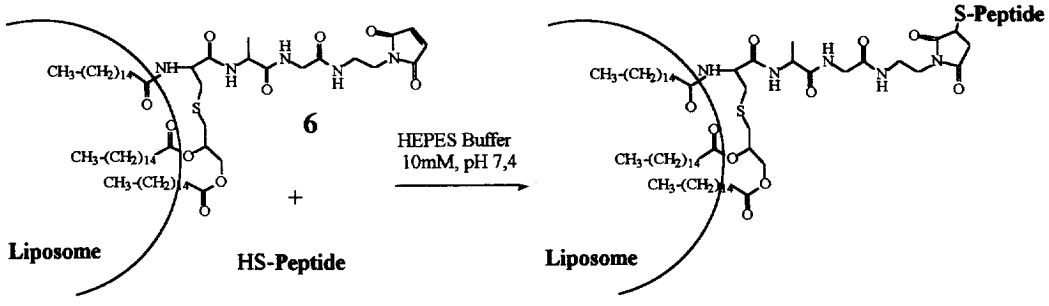


Figure 3: Coupling of Peptide-SH on preformed liposomes incorporating $Pam_3CysAlaGlyMal$ **6**.

In conclusion, the synthesis described here introduces the notion of "universal" lipopeptides. Molecules such as $Pam_3CysAlaGlyMal$ **6** and $Pam_3CysSerSerMal$ **7** react readily, under chemically defined conditions, with peptides-SH to provide lipopeptide-antigen conjugates. These novel thiol-reactive lipopeptides, when incorporated into liposomes, provide very versatile tools for the coupling of many different peptides that can be used for the preparation of synthetic vaccines. For example, constructs in which a B-cell epitope (from *Streptococcus mutans* adhesin I/II) and a "universal" Th-epitope (from tetanus toxin) were conjugated respectively to a functionalized phospholipid[6a] and to **6** gave, in BALB/c mice, remarkably potent and long lasting (over a year) humoral immune responses.[8]

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15. All new compounds gave spectroscopic data in agreement with the assigned structures. Compound **3** had ^{1}H NMR δ (200 MHz, CD_3OD) 6,93 (s, 2H, Mal), 3,86 (t, J=5,5 Hz, 2H, CH_2Mal), 3,20 (t, J=6,0 Hz, 2H, CH_2NH_2). Compound **6** had ^{1}H NMR δ (300 MHz, $CDCl_3/CD_3OD$) 6,73 (s, 2H, Mal), 5,19-5,13 (m, 1H, OC*H*CH_2S), 4,56-4,51 (m, 1H, NHC*H*CO), 4,48-4,45 (m, 1H, CH_3CH), 4,39-4,12 (m, 2H, OCH_2CH), 4,01-3,83 (m, 2H, NHCH_2CO), 3,95 (br, 2H, CH_2Mal), 3,05-2,82 (m, 2H, CH_2SCH_2CHNH), 2,97 (t, J=4,5 Hz, 2H, NHCH_2CH_2Mal), 2,79-2,73 (m, 2H, OCHCH_2S), 2,35-2,29 (m, 4H, CH_2COO), 2,24 (t, J=7,5 Hz, 2H, CH_2CONH), 1,70-1,63 (m, 6H, CH_2CH_2CO), 1,39 (d, J=7,1 Hz, 3H, CH_3CH), 1,29-1,22 (m, 72H, CH_2), 0,87 (t, J=6,8 Hz, 9H, CH_3CH_2); FAB MS (4-nitrobenzylic alcohol matrix): mass calculated for $C_{65}H_{117}O_{10}N_5S$: *m* 1160; *m/z* 1161 [M+H$^+$]. Compound **7** had ^{1}H NMR δ (300 MHz, $CDCl_3/CD_3OD$) 6,73 (s, 2H, Mal), 5,19-5,13 (m, 1H, OC*H*CH_2S), 4,56-4,51 (m, 1H, NHC*H*CO), 4,48-4,45 (m, 2H, C*H* CH_2OH), 4,39-4,12 (m, 2H, OCH_2CH), 4,00-3,79 (m, 4H, $CHCH_2$OH), 3,95 (br, 2H, CH_2Mal), 3,05-2,82 (m, 2H, CH_2SCH_2CHNH), 2,97 (t, J=4,5 Hz, 2H, NHCH_2CH_2Mal), 2,79-2,73 (m, 2H, OCHCH_2S), 2,35-2,29 (m, 4H, CH_2COO), 2,24 (t, J=7,5 Hz, 2H, CH_2CONH), 1,70-1,63 (m, 6H, CH_2CH_2CO), 1,29-1,22 (m, 72H, CH_2), 0,87 (t, J=6,8 Hz, 9H, CH_3CH_2); FAB MS (4-nitrobenzylic alcohol matrix): mass calculated for $C_{65}H_{119}O_{12}N_5S$: *m* 1206; *m/z* 1207 [M+H$^+$].

16. In brief, **6** was mixed with egg phosphatidylcholine, phosphatidylglycerol and cholesterol (10/55/25/50 molar ratio) in chloroform. The solvent was evaporated to dryness under high vacuum and the lipid film was rehydrated in a 10 mM HEPES buffer containing 145 mM NaCl, pH 7.4, and sonicated. The liposomes obtained were reacted with the free peptide-SH for 12 hours at 4°C (100% yield as measured by the disappearance of maleimide group on the surface of the vesicles).