Lipopeptides

DOI: 10.1002/ange.201305620

Direct Peptide Lipidation through Thiol–Ene Coupling Enables Rapid Synthesis and Evaluation of Self-Adjuvanting Vaccine Candidates

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Synthetic peptide vaccines generally consist of the minimal region of a protein antigen capable of eliciting an immune response.[1] This approach to vaccine development has a number of advantages, including ease of synthesis, avoidance of potentially toxic biological by-products, and straightforward characterization. However, a key issue for the development of peptide vaccines is the lack of immunogenicity displayed by peptides as sole vaccine components. Thus, the inclusion of an adjuvant, designed to activate components of the innate immune system, is required.[2] An attractive strategy in peptide vaccine design is the creation of self-adjuvanting vaccines in which the epitope of interest is covalently linked to an appropriate adjuvant, with the intention of enhancing antigen uptake, presentation, and dendritic cell maturation when compared to simple coformulation of the vaccine with an external adjuvant.

Many currently available adjuvants exert their activity through stimulation of Toll-like receptors (TLRs), highly-conserved pattern-recognition receptors responsible for activation of the innate immune system.[3] Certain lipopeptide motifs are known to signal through Toll-like receptor 2 heterodimers[4] expressed on the cell surface of a range of immune cells, including monocytes, dendritic cells, and macrophages.[2]

In 1975, Braun reported that a lipoprotein component of *E. coli* cell walls was potently immunogenic.[5] Since then, synthetic analogues such as Pam_2Cys (Pam = palmitoyl) and its N-palmitoylated analogue Pam_3Cys have become widely exploited in research.[6] The molecular basis for the adjuvant activity of these lipopeptides has been well established and involves signaling through the MyD88-dependent pathway, resulting in activation of NFκB and cytokine production.[4]

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Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201305620.

A number of groups have turned their attention to the development of efficient methods for synthesis of self-adjuvanting vaccine constructs. Copper-catalyzed azide–alkyne cycloaddition (CuAAC) has been utilized by us and others for vaccine–adjuvant conjugation.[7a,b] Thioether and oxime conjugations have also been explored.[8,9] These studies have demonstrated that the biological activity of vaccine constructs may exhibit a marked dependence on the conjugation chemistry employed.[8]

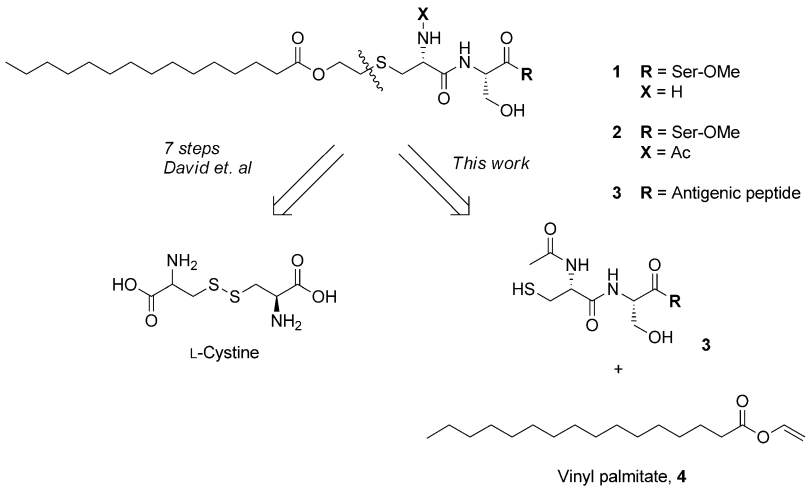
In 2012, David and co-workers reported unexpected, human-specific TLR2 agonist activity for monoacyl lipopeptides that are structurally simpler analogues of Pam_2Cys.[10] Previously, the monoacyl, cysteine-derived lipopeptide **1** had been found to have weak TLR2 agonist activity in both humans and mice. David and co-workers, however, established that acetylation of the N-terminal amino group afforded **2**, a human-specific TLR2 agonist with potency comparable to Pam_2Cys (Scheme 1).

The lipopeptide **2** was originally synthesized from L-cystine (Scheme 1), however, this "building block" approach to adjuvant incorporation could be construed as inefficient owing to poor step economy and because an excess of the lipidated amino acid is usually required for solid-phase peptide synthesis (SPPS). We envisioned a more expedient ("posttranslational") route to the monoacyl functionality through the late-stage thiol–ene reaction of a fully formed peptide **3** with cheap, commercially available vinyl palmitate (**4**; Scheme 1), affording the desired chemotype in a single step after cleavage of the peptide from resin.

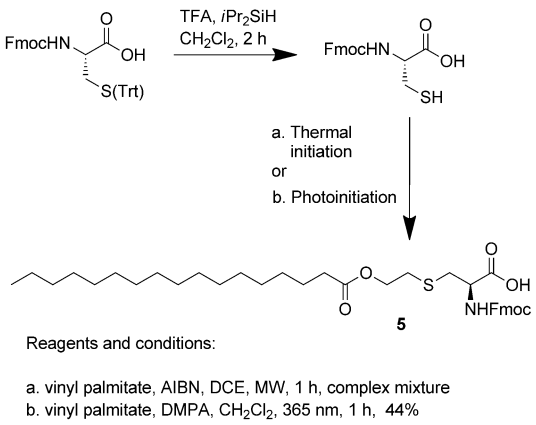
The radical hydrothiolation of an alkene, known as the thiol–ene reaction, has been widely applied in polymer science and demonstrates promise as a chemoselective ligation reaction in peptide chemistry.[11] Syntheses of neoglycopeptides and neoglycoproteins utilizing the thiol–ene reaction have been reported,[12,13] including the photoinduced conjugation of MUC1 glycopeptide antigens to the bovine serum albumin (BSA) carrier protein.[14] Of particular relevance to the current work, Waldmann and co-workers established the use of thermally initiated thiol–ene chemistry for the S-alkylation of cysteine.[15]

We herein report the synthesis of monoacyl lipopeptides prepared by thiol–ene coupling and their immunological evaluation as TLR2 agonists.

The viability of the S-alkylation was first examined using 9-fluorenylmethyloxycarbonyl (Fmoc)-protected cysteine and vinyl palmitate (Scheme 2). Conditions used to effect the desired thiol–ene reaction with vinyl palmitate were initially based on literature reports,[12,15] and both thermal and photoinitiation methods were explored. Firstly, refluxing



Scheme 1. Synthetic routes to monoacyl lipopeptides.



Scheme 2. Model reaction of Fmoc-Cys-OH with vinyl palmitate. AIBN = azobisisobutyronitrile, MW = microwave.

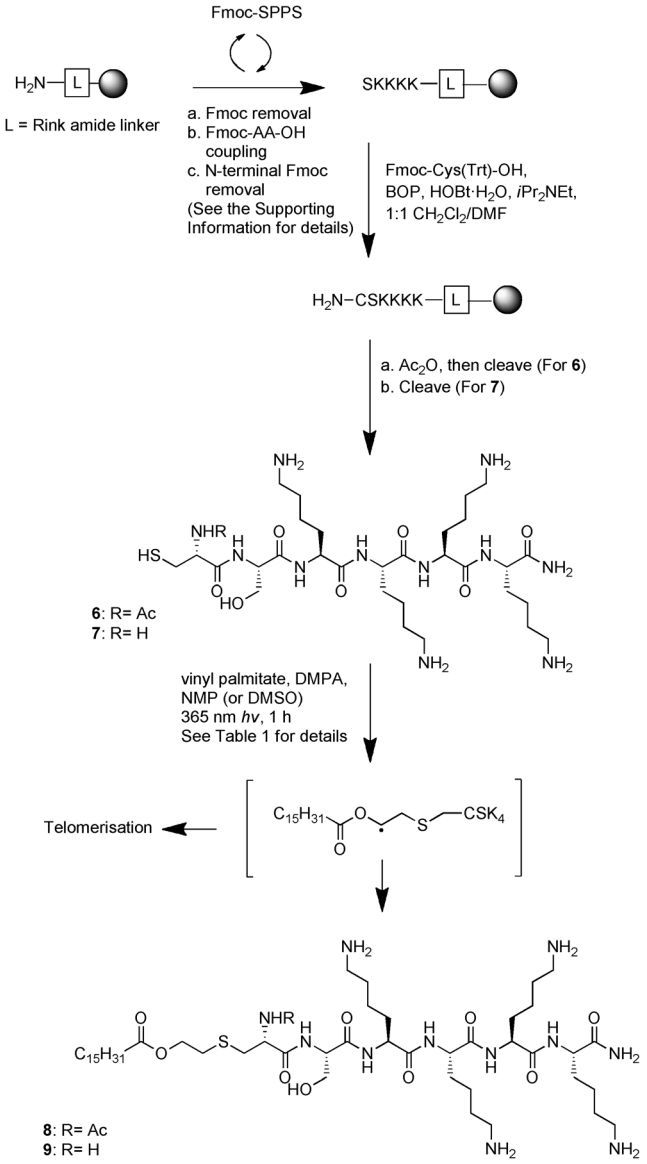
Fmoc-cysteine with vinyl palmitate (5 equiv) in 1,2-dichloroethane (DCE), using AIBN (0.2 equiv) as radical initiator afforded the desired thioether, **5**, albeit as part of a complex mixture of products.

Photoinitiation of the above reaction was next attempted. A solution of vinyl palmitate (2 equiv) and Fmoc-cysteine in dichloromethane, using 2,2-dimethoxy-2-phenylacetophenone (DMPA, 0.2 equiv) as photoinitiator, was irradiated for 1 h with UV light. A wavelength of 365 nm was chosen for compatibility with all naturally occurring amino acid side chains.[16] In contrast to the thermally initiated reaction, complete consumption of the Fmoc-Cys starting material was observed, with **5** being formed with a much improved product profile. Purification afforded **5** in a satisfactory 44% yield.

Encouraged by the results of these preliminary experiments, the thiol–ene reaction was then applied to more complex peptide substrates. It was decided to attempt the reaction of Ac-CSK_4 (**6**) and its nonacetylated congener CSK_4 (**7**), since the desired products **8** and **9** would be directly comparable to the commercially available Pam_2Cys-SK_4, enabling evaluation of the effect of N-acetylation on the immune response.

Synthesis of the required peptide substrates was carried out using automated Fmoc SPPS on Rink Amide PS resin (Scheme 3, see the Supporting Information for details of peptide synthesis). Fmoc-Cys(Trt)-OH was coupled manually using conditions optimized to minimize epimerization. After generation of the CSK_4 sequence, a portion of resin was removed and N-acetylated to give **6** after cleavage; the remainder was cleaved directly, to afford **7**. Peptides **6** and **7** were obtained in high purity and were used without further purification.

Again, both thermal and photochemical initiation was explored. Intriguingly, thermal



Scheme 3. Synthesis of monoacyl-containing CSK_4 peptides by using thiol–ene coupling of vinyl palmitate. BOP = benzotriazol-1-yloxy-tris-(dimethylamino)phosphonium hexafluorophosphate, NMP = *N*-methylpyrrolidone.

Table 1: Thiol–ene reaction of NAc-CSK_4 and vinyl palmitate.

Entry	Peptide concentration [mM]	Additive	Conversion [%]	Purity [%]
1[a]	10[c]		0	–
2[b]	10[c]		>90	60
3[b]	5[d]		0	–
4[b]	25[c]		>90	80
5[b]	5[e]	GSH[g]	50	75
6[b]	5[c]	DODT[g]	80	c.m.
7[b]	10[c]	DTT[g]	>90	85
8[b]	25[f]	DTT[g]	90	>95

[a] Δ; [b] *hν*, 365 nm; [c] NMP; [d] 1:1 CH_2Cl_2/DMF; [e] NMP/H_2O/DMSO (4:2:1); [f] DMSO; [g] 3 equiv. c.m. = complex mixture.

initiation resulted in no traces of the desired alkylated product, despite consumption of the starting material (Table 1, entry 1). Photochemical initiation proved more successful. When using crude peptide (10 mM) and an excess of vinyl palmitate (5 equiv relative to peptide) in NMP with DMPA (0.4 equiv) as photoinitiator, the reaction proceeded to completion after irradiation for 1 h (Table 1, entry 2). The desired alkylated products **8** and **9** were confirmed by ESI-MS. The choice of NMP as solvent was crucial for a successful reaction, with reactions carried out in CH_2Cl_2/DMF mixtures producing no product (Table 1, entry 3).

Unfortunately, despite showing the presence of the desired products **8** and **9**, ESI-MS analysis of the crude reaction mixtures also revealed extensive by-product formation. This was attributed to polymerization ("telomerization") of vinyl palmitate and NMP-derived products on the peptide substrate.[17] The radical-mediated thiol–ene reaction proceeds through three distinct reaction processes: initiation, polymerization or coupling, and termination.[11] Radical generation gives rise to an electrophilic thiyl radical, which propagates across the ene group, forming a carbon-centered radical, which is then quenched by chain-transfer from an additional thiol molecule to give the final product. However, if the rate of chain transfer relative to propagation is slow, telomerization may be a significant side reaction.[17] (Scheme 3).

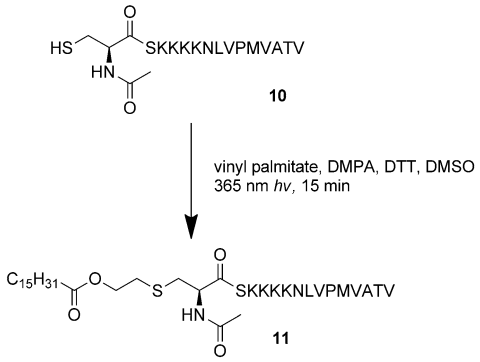
It has been shown that extraneous thiols can reduce telomerization products, presumably by facilitating chain transfer.[17] Recent work concluded that reduced glutathione (GSH) was the most suitable thiol for this purpose,[17] however, in our hands it persistently led to mixed disulfide formation with the substrate (Table 1, entry 5). Addition of 2,2′-(ethylenedioxy)diethanethiol (DODT) also led to a complex mixture of products (Table 1, entry 6).

We postulated that the use of 1,4-dithiothreitol (DTT) could obviate the issue of mixed disulfide formation while still acting as an additional chain-transfer agent, since DTT has a strong propensity to form a stable six-membered ring upon oxidation. Gratifyingly, upon addition of DTT (3 equiv) to the reaction mixture (Table 1, entry 7) no by-products resulting from vinyl palmitate telomerization or mixed disulfides were observed.

With these optimized conditions, the reaction of Ac-CSK_4 with vinyl palmitate proceeded with high conversion (>90%). We also speculated that inclusion of DTT would enable us to conduct the reaction in DMSO, a more benign and versatile solvent. This was indeed the case (Table 1, entry 8).

We next sought to extend the strategy beyond the model system by introducing the monoacyl, adjuvant moiety to an antigenic peptide, to demonstrate the strategic advantages of our approach for vaccine design. A truncated peptide epitope derived from the cytomegalovirus (CMV) ppUL83 protein (sequence NLVPMVATV) was chosen for a model system.[18] This peptide is able to stimulate $CD8^+$ cytotoxic T-cells, and has been utilized in our group for previous studies of click chemistry approaches to vaccine–adjuvant conjugation.

The desired peptide sequence **10** was constructed by automated Fmoc SPPS using standard conditions. In addition, the solubilizing polylysine (K_4) tag and the serine residue necessary for TLR 2/6 binding were coupled to the N terminus of the sequence in a linear fashion. Finally, the key cysteine residue was coupled manually and the N terminus acetylated. The peptide was then cleaved from the resin and lyophilized in good yield, ready for thiol–ene coupling. The thiol–ene reaction of the unprotected peptide **10** and vinyl palmitate was accomplished in DMSO by using the optimized conditions described above (Scheme 4, Figure S1 in the Supporting Information). ESI-MS and HPLC analysis indicated good conversion to the palmitoylated product **11**. Purification was accomplished by semipreparative RP-HPLC, giving the desired product in >95% purity.



Scheme 4. Thiol–ene reaction of an antigenic peptide.

To test the bioactivity of lipopeptides **8**, **9**, and **11**, we utilized flow cytometry to measure up-regulation of the costimulatory molecule CD80 on human monocytes in fresh blood samples (Figure 1). As described in the Supporting Information, monocytes were identified in five donor samples by characteristic cell surface markers, and the expression of CD80 determined before and after exposure to each compound at three dosages, with commercially available Pam_3CSK_4 (10 μM) serving as a positive control. Pleasingly, **8** and **11** both strongly upregulated expression of CD80 at all doses tested. An equivalent potency to Pam_3CSK_4 at 10 μM was observed in most donors. In three donors, **9** showed lower potency than **8** and **11**, consistent with the observation that acetylation of the cysteine amino group improves potency of

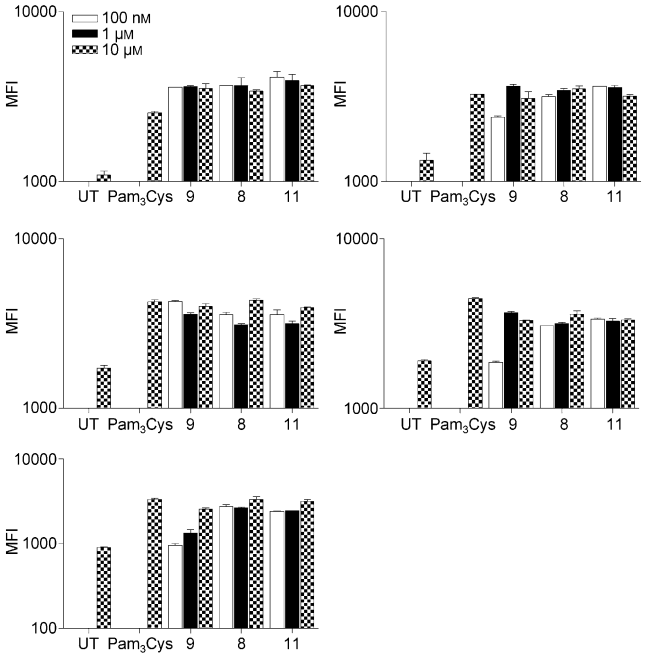


Figure 1. CD80 response across 5 donors. MFI = Mean fluorescence intensity, UT = untreated.

TLR2 agonism in human cells.[10] Significantly, the similar activity of **11** demonstrates that conjugation of antigenic peptides to the CSK_4 system does not appear to affect TLR2 agonism. This strongly suggests that the procedure can be generally applied for the purposes of antigen–adjuvant conjugation. It is particularly encouraging to note the similar potency of our constructs to the commercially available Pam_3CSK_4.

In conclusion, we have developed a novel method for the one-step synthesis of self-adjuvanting antigenic peptides through thiol–ene lipidation. Notably, this method eliminates the need for tedious solution-phase building block synthesis. In addition, no non-natural amino acids are required as tags for chemical modification, meaning only natural bonds are presented to the cellular antigen-processing machinery. To our knowledge, this study provides the first example of lipidation of unprotected peptide substrates using the thiol–ene reaction. The operational simplicity of the procedure gives it broad scope for application in vaccine design.

Experimental Section

General method for thiol–ene reaction on unprotected peptides: Vinyl palmitate (50 mM) was added to a solution of crude or purified peptide (10 mM), DTT (30 mM), and DMPA (4 mM) in DMSO. The resultant mixture was irradiated, with agitation, at 365 nm for 15 min in a standard UV photochemical apparatus. The desired product was detected by ESI mass analysis. To achieve full conversion, further addition of DMPA photoinitiator was sometimes required. The crude product was purified by using semipreparative RP-HPLC on a Phenomonex Gemini C18 column by running a gradient of 1–65% $MeCN/H_2O + 0.1\%$ TFA (3% MeCN per min). Pooled fractions were lyophilized to afford the pure products as white powders.

Received: June 30, 2013
Published online: August 12, 2013

Keywords: adjuvants · immunology · peptides · thiol–ene reaction · vaccines

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