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## Self adjuvanting immunopeptides : design and synthesis

Gential, G.P.P.

### Citation

Gential, G. P. P. (2018, December 17). *Self adjuvanting immunopeptides : design and synthesis*. Retrieved from <https://hdl.handle.net/1887/67530>

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**Author:** Gential, G.P.P.

**Title:** Self adjuvanting immunopeptides : design and synthesis

**Issue Date:** 2018-12-17

# Chapter 1: Introduction

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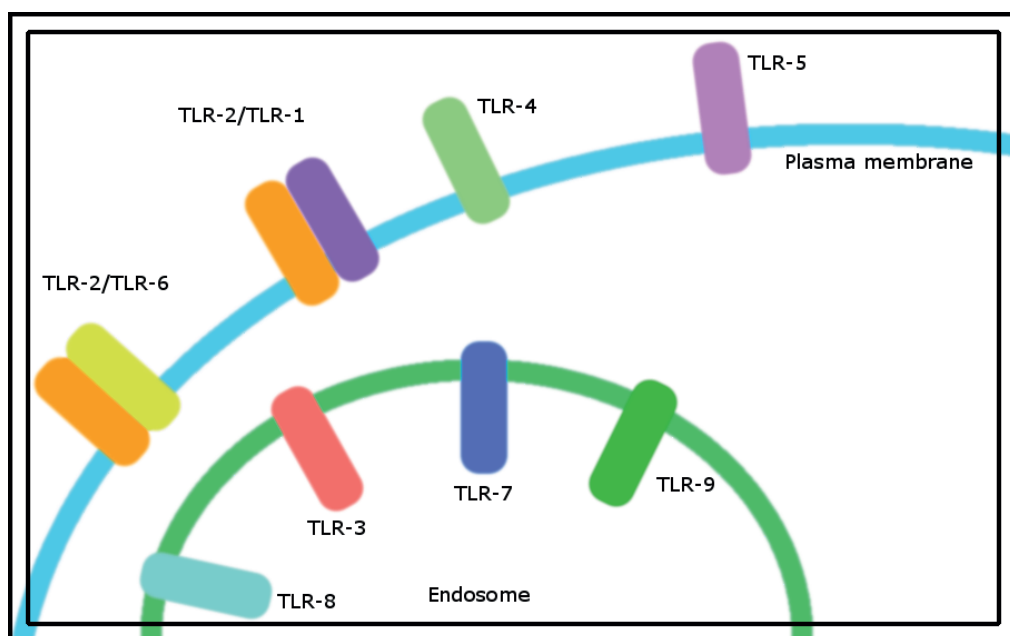
The mammalian immune system consists of two interdependent parts, namely the innate and adaptive immune system.<sup>1-3</sup> Adaptive immune responses can be divided in humoral (antibody) and cytotoxic (cellular) responses. B cells, T cells, and dendritic cells (DCs) are involved in generating these immune responses that ultimately can lead to the ability of the host to identify and memorize specific pathogens. Whilst cytotoxic T cells (CTLs) are key players in cellular responses and B cells mediate humoral responses each of these responses require T-helper (Th) cells. Th cells release cytokines, soluble proteins that can induce activation and proliferation of CTLs as well as B cell antibody class switching. Two major subtypes of T-helper cells are Th1 cells and Th2 cells. The cells of Th1-type produce the cytokine interferon-gamma and are involved in combatting intracellular pathogens. Th2-cells produce interleukin-4, -5, and -13 and help combatting extracellular pathogens. Antigen presenting cells (APCs) such as DCs and macrophages present peptides derived from pathogens within the cell on major histocompatibility complex class I (MHC class I) molecules.<sup>4, 5</sup> Recognition of peptides, derived from viral proteins and that are presented by MHC class I molecules, by CTLs initiates a cellular cytotoxic response which can eradicate for instance virus-infected cells. Peptides derived from extracellular pathogens are presented by APCs on major histocompatibility complex class II (MHC class II) molecules. Upon recognition by T-helper cells an activation process is initiated through which these specific T-helpers respond to B cells that have taken up the same antigen and therefore display the same MHC II-peptide complex. This interaction result in differentiation of B cells into plasma cells that secrete antigen-specific antibodies which can neutralize for instance bacterial pathogens.<sup>6</sup>

The above brief impression of immune responses indicates the importance of peptide dependent recognition processes for controlling extracellular and intracellular infection. However, single pathogen-derived peptides epitopes are by themselves not effective in inducing an immune response.<sup>7, 8</sup> Peptides are poorly immunogenic because peptides do not function as danger signals that activate the innate immune system, which is required for inducing adequate adaptive immune responses. Induction of an effective adaptive immune response therefore requires signals of the innate immune system to activate antigen presenting cells which can strongly

stimulate peptide-specific T cells. In modern vaccination technologies combinations of defined molecules stimulating both the innate and adaptive responses are used.<sup>9, 10</sup>

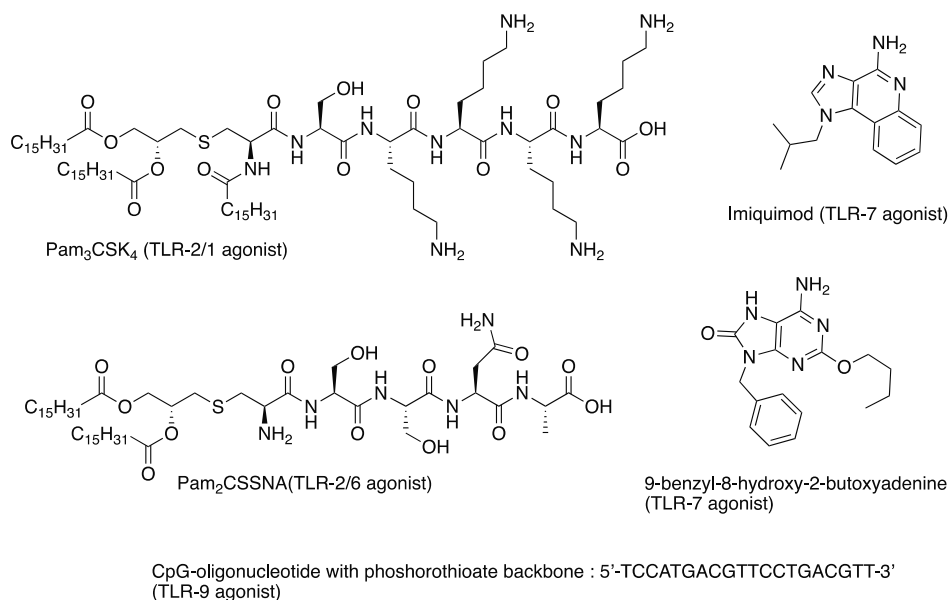
The innate immune system forms the first line of defence against pathogenic invaders by recognising pathogen associated molecular patterns (PAMPs) or microbe-associated-molecular-pattern (MAMPs) with the aid of pathogen recognizing receptors (PRRs). Toll-like receptors (TLRs) and NOD-like receptors (NLRs), RIG-like receptors (RLRs) and C-type lectins are part of the innate immune system.

Toll-like receptors (TLRs)<sup>11</sup> are a family of membrane bound glycoproteins that have been studied most among the PRRs.<sup>12</sup> Upon recognition of a specific PAMP by the corresponding TLR, a signal transduction pathway is started that activates specialized cells of both the innate and adaptive immune system, leading eventually to eradication of the pathogen. Ten different human TLRs can be discerned that are expressed in different ratios by immune and epithelial cells, while mice has 12 receptors (TLR1-9+TLR11-TLR13). To detect pathogens that are present in the extracellular environment as well as the internalized ones, both human and murine TLRs are situated at the cell surface (TLR1, TLR2, TLR4, TLR5 and TLR6) or in the various intracellular (TLR3, TLR7, TLR8 and TLR9) compartments (Figure 1). Each TLR recognizes PAMPs with certain structural identity.<sup>13</sup> TLR2 along with TLR1 or TLR6 binds a wide variety of microbial membrane components such as peptidoglycans. Bacterial lipopolysaccharide is the ligand for TLR4 while TLR5 recognizes bacterial flagellin. TLRs inside the cell are specialized in recognizing nucleic acids of different origin. TLR3 recognizes viral double-stranded RNA and also small self-RNAs derived from damaged cells. TLR8 binds to viral and bacterial single-stranded RNA. TLR9 recognizes bacterial and viral single stranded unmethylated CpG-DNA. Single-stranded RNA from viruses is the ligand of TLR7. The finding, that TLR agonists are capable of linking the innate and adaptive immune system, make the study toward TLRs and the corresponding ligands important for the development of prophylactic and therapeutic vaccines. This has stimulated structure and activity studies of TLR ligands, leading to several well-defined small molecular modulators (mostly agonists but also antagonists). In particular, structurally defined ligands for TLR2, TLR4, TLR7 and TLR9 have been developed.<sup>14, 15</sup>



**Figure 1.** Schematic view of the location of the various TLRs

Lipopeptides and lipoteichoic acids originating from gram positive bacteria are the naturally occurring agonists for TLR2. Heterodimerization of TLR2 with TLR1 or TLR6 is a prerequisite for recognition of bacterial lipoproteins or lipopeptides. Structure-activity studies have revealed that synthetic Pam<sub>3</sub>CSK<sub>4</sub>, (Figure 2) the structure of which is based on triacylated lipopeptide derived from *Escherichia coli* membrane protein<sup>16,17</sup>, targets specifically heterodimeric TLR1/TLR2.<sup>18</sup> Pam<sub>2</sub>CSSNA (Figure 2) and macrophage-activating lipopeptide (MALP-2) are examples of synthetic accessible agonists for the TLR2/TLR6 combination.<sup>19</sup> Further studies have led to, amongst others, water soluble and structurally less complex TLR2 agonists.<sup>20-22</sup> Double-stranded viral RNA, the natural ligand for TLR3, can be replaced by polyinosinic-polycytidylic acid (poly I:C).<sup>23</sup> Homodimerisation of TLR3 occurs and it appears that an (I:C) oligomer of at least 100 base pairs is needed for a sufficient immune response. Also, double-stranded RNA mimics are explored as adjuvants.<sup>23</sup> Lipopolysaccharides (LPS) originating from Gram-negative bacteria are the naturally occurring agonists of TLR4.<sup>24</sup> Lipid A is an important part of LPS and a lot of structure and activity studies have resulted in synthetic compounds which can serve as either antagonist or agonist for TLR4.<sup>25</sup> Mono-phosphoryl lipid A (MPLA), a lipid A derivative from *Salmonella enterica* that has stimulatory properties but lacks endotoxicity and pyrogenicity, is approved as a human vaccine adjuvant.<sup>26</sup> While the natural agonists for TLR7 is single-stranded RNA from viruses<sup>27</sup>, several structural defined small molecules have been discovered that can function as ligands for TLR7/8, such as dimidazoquinolines and adenine derivatives (Figure 2).<sup>15, 28-30</sup> TLR9 is the only receptor that recognizes synthetic ssDNA fragments. Specific oligodeoxynucleotides with CpG motifs and a nuclease-resistant phosphorothioate backbone function as agonist of TLR9 (Figure 2).<sup>31, 32</sup> Up to now TLR10 is the only receptor without a known ligand or signalling function.<sup>33</sup> TLR agonists are used in the development of new immune therapeutics<sup>14, 34, 35</sup> while TLR antagonists are explored for the treatment of autoimmune diseases such as rheumatoid arthritis.<sup>36</sup>



**Figure 2.** Examples of TLR-2/1, TLR2/6, TLR7 and TLR9 agonists

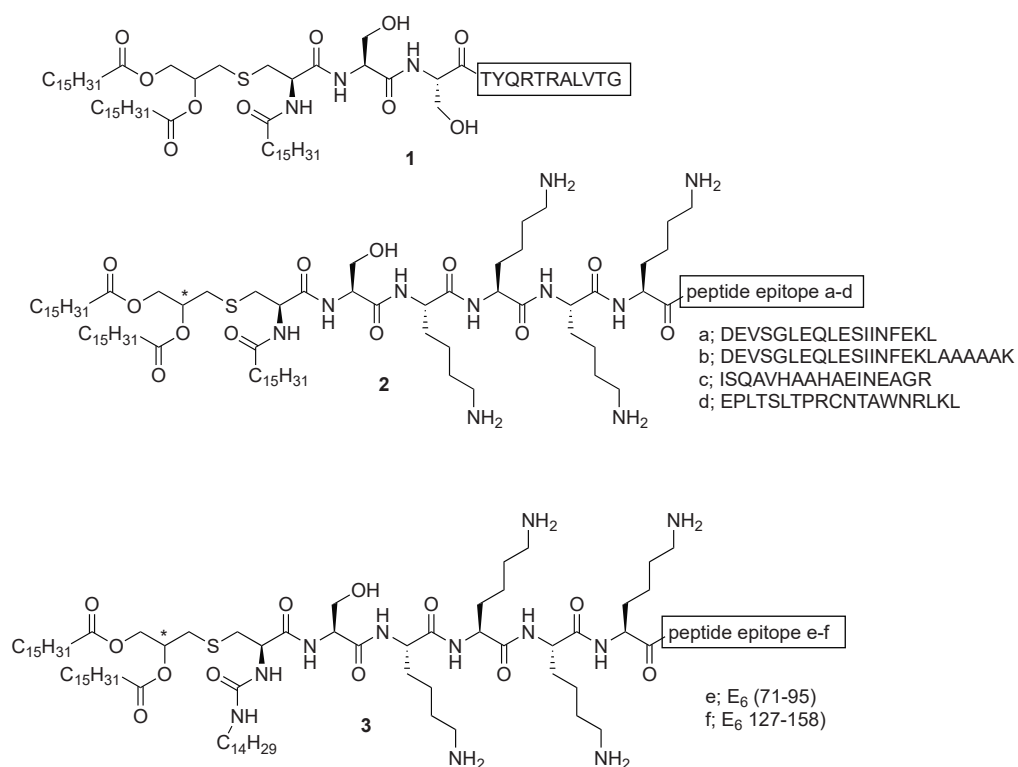
## Self adjuvanting TLR peptide conjugates

With the objective to develop new classes of vaccines with more precise characteristics and new applications, considerable research is devoted to the use of agonistic ligands of PRRs and in particular TLRs.<sup>37</sup> For instance, individual TLR ligands have been investigated as adjuvants with improved properties.<sup>15</sup> TLR agonists have also been used in the quest toward fully synthetic vaccines.<sup>38, 39</sup> The role of oligopeptides in recognition processes inherent to immune responses makes peptide epitopes essential components of these types of vaccines.<sup>9</sup> The inability of oligopeptides to induce sufficient immune responses requires the presence of either an adjuvant or a suitable TLR agonist.<sup>9</sup> Both antigenic proteins and epitopes embedded in synthetic long peptides (SLP) in combination with specific TLR agonists have been evaluated for their immunological properties.<sup>40, 41</sup> In particular ligands of TLR1/2<sup>35, 42</sup>, TLR2/6<sup>43</sup>, TLR3<sup>44</sup>, TLR4<sup>45-47</sup>, TLR7<sup>48</sup> and TLR9<sup>49, 50</sup> were evaluated. In the course of these studies it was discovered that conjugates in which a peptide epitope is covalently attached to a specific TLR agonist proved to be more potent than just a mixture of the same TLR agonist and the epitope.<sup>8, 31, 35</sup> Several examples of these potential vaccines, termed “self adjuvanting peptide conjugates” have been reported and this chapter presents a selected number of examples of peptide conjugates that target different TLRs.

## TLR-2 targeting peptide conjugate

Already in 1989, the group of Rammensee reported the synthesis and immunological evaluation of a conjugate (**1**, Figure 3) consisting of Pam<sub>3</sub>CSS and the peptide epitope TYQRTALVTG, derived

from the nucleoprotein of influenza virus.<sup>51</sup> Conjugate **1** was assembled with the aid of an automated SPPS procedure, using Fmoc-chemistry. This group of Rammensee showed for the first time that priming of virus-specific cytotoxic T cells, which is an important event in the immune response against viral infections, can be induced *in vivo* with conjugate **1**. With the objective to attain TLR-2 ligands with improved properties several groups designed and synthesized analogues of Pam<sub>3</sub>C.<sup>16, 52-54</sup> Evaluation of their immunological properties resulted in Pam<sub>3</sub>CSK<sub>4</sub> as a potent TLR-2 agonist with increased solubility by virtue of the hydrophilic lysine residues.<sup>54</sup> Khan *et al.* prepared conjugates (e.g. **2**, Figure 3) composed of CD8<sup>+</sup> cytotoxic T-lymphocyte SIINFEKL epitope (a model MHC I epitope derived from ovalbumin and often used in immunology studies in mice or murine-derived tissue) covalently linked to the ligand Pam<sub>3</sub>CSK<sub>4</sub>.<sup>31</sup> Immunological evaluation showed that this conjugate was able to induce DC maturation to the same amount as the single Pam<sub>3</sub>CSK<sub>4</sub> ligand. Importantly, in comparison with a mixture of the free ligand and the peptide epitope, conjugate **2** showed not only enhanced MHC class I antigen presentation but also enhanced antigen uptake resulting in a robust and systemic response of specific T-cells. Interestingly, the enhanced uptake was found to be independent of the expression of cell-surface TLR2.<sup>31</sup> These studies were expanded with the synthesis and evaluation of three different conjugates containing the ovalbumin derived CTL epitope DEVSGLEQLESIINFEKLAAAAAK, the ovalbumin derived Th epitope ISQAVHAAHAEINEAGR and the Moloney virus envelope derived Th epitope.<sup>35</sup> The outcome of the *in vivo* studies shows that the conjugates of type **2** have superior capacity to prime both CTL (CD8<sup>+</sup>) and T-helper (CD4<sup>+</sup>) cells in mice as compared to a mixture of the corresponding free epitope and the free Pam<sub>3</sub>CSK<sub>4</sub> ligand. In addition, vaccination with these conjugates leads to efficient induction of antitumor immunity in mice challenged with aggressive transplantable melanoma or lymphoma.<sup>35</sup> The same group investigated the influence of the chiral centre in the glycerol moiety of the Pam<sub>3</sub>CSK<sub>4</sub> ligand on the immunological properties of conjugates of type **2**.<sup>55</sup> Although both the R- and S-stereoisomers were internalized into cells to similar extent in a clathrin- and caveolin-dependent manner the R-stereoisomer was not only superior in facilitating activation and maturation of dendritic cells but also in induction of specific CTLs (CD8<sup>+</sup> T-cells).<sup>55</sup> All these conjugates were accessible via an automated on-line solid phase peptide synthesis (SPPS) approach using Fmoc-chemistry.



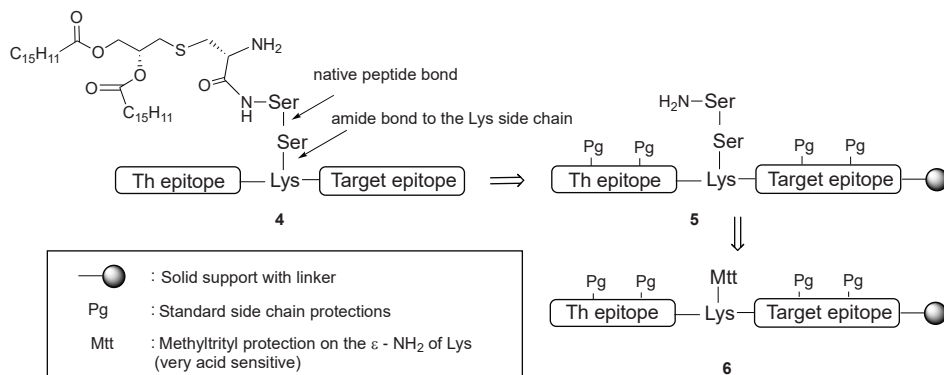
**Figure 3.** Examples of conjugates comprising a TLR-2/1 ligand and a synthetic long peptide epitope. Synthetic Pam<sub>3</sub>Cys-lipopetides are mixtures of epimers at the glycerol residue (indicated by asterisk).

Guided by an X-ray structure of the TLR1/TLR2 dimer co-crystallized with the Pam<sub>3</sub>C-ligand, Willems *et al.* designed a new and improved Pam<sub>3</sub>CSK<sub>4</sub> ligand termed UPam, in which the cysteine amide bond was replaced by an urea linkage.<sup>42</sup> With the aid of an automated SPPS and using Fmoc-chemistry the new TLR2 ligand was incorporated into a conjugate, containing human papillomavirus type 16 (HPV16)-encoded synthetic long peptide epitopes to give conjugates **3** (Figure 3).<sup>56</sup> It was shown that these conjugates can activate both circulating and lymph node derived tumor specific T-cells.<sup>56</sup>

While gram-negative bacterial lipoproteins are provided with three fatty acid residues, gram-positive bacterial lipoproteins contain two fatty acid chains.<sup>57</sup> It was established that Pam<sub>2</sub>Cys functions as a TLR-2/6 ligand.<sup>58</sup> Jackson *et al.* have prepared and evaluated a number of fully synthetic conjugates, composed of a helper (Th) T cell epitope, a target epitope and S-[2,3-bis(palmitoyloxy)propyl]cysteine as (Pam<sub>2</sub>Cys) ligand (**4** in Figure 4).<sup>59</sup> In conjugates of type **4** two different Th peptide sequences were combined with sequences of various MHC-class I restricted target epitopes, such as the TYQRTRALV sequence derived from influenza virus and the SIINFEKL model epitope. In conjugates of type **4**, the Th epitope is situated at the N-terminal end and the target epitope is positioned at the C-terminal end.<sup>59</sup> In the first stage of the on-line SPPS toward conjugates **4** immobilized peptide **6** is assembled having the epitopes separated by a single lysine (K) residue, of which the amino group in the side chain was protected with the orthogonal Mtt group. The TLR ligand was next installed by selective removal of the mild acid labile Mtt group in immobilized peptide **6**. To improve the immunogenicity of the conjugate the released amino function in the lysine side chain was first elongated with two serine residues and subsequently with Pam<sub>2</sub>Cys.<sup>59</sup> Removal of the protecting groups and cleavage of the conjugate from the solid

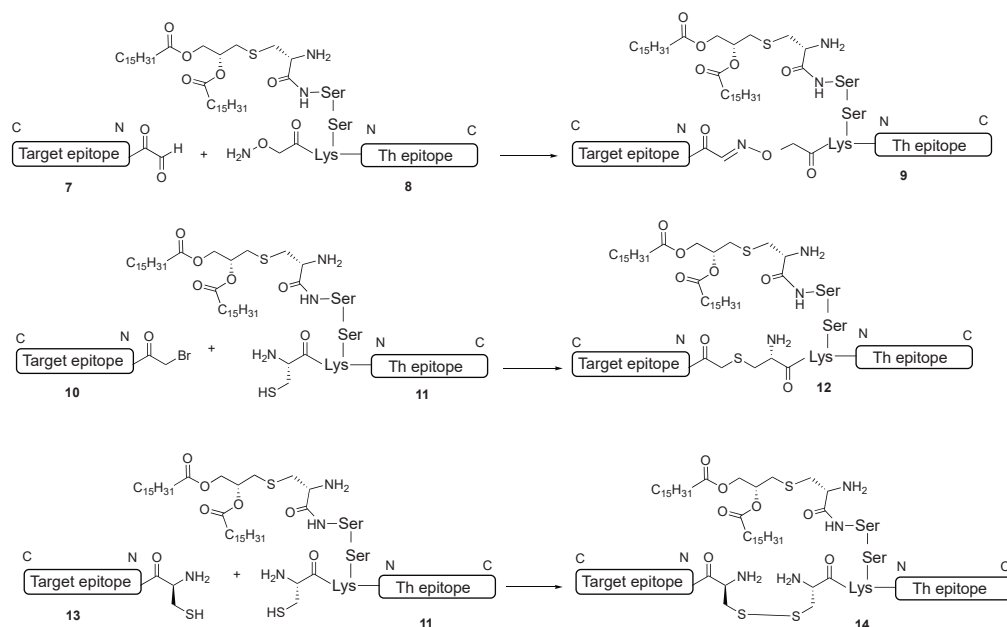


support gave conjugates of type **4**. Immunological evaluation indicate that these conjugates were able to induce both humoral and cellular immunity, thereby potentially provide protection against viral or bacterial infection.<sup>59</sup>



**Figure 4.** Retro synthesis and the generic structure of branched lipopeptide conjugates that contain TLR-2/TLR6 ligand, as developed by Jackson *et al.*<sup>59</sup>

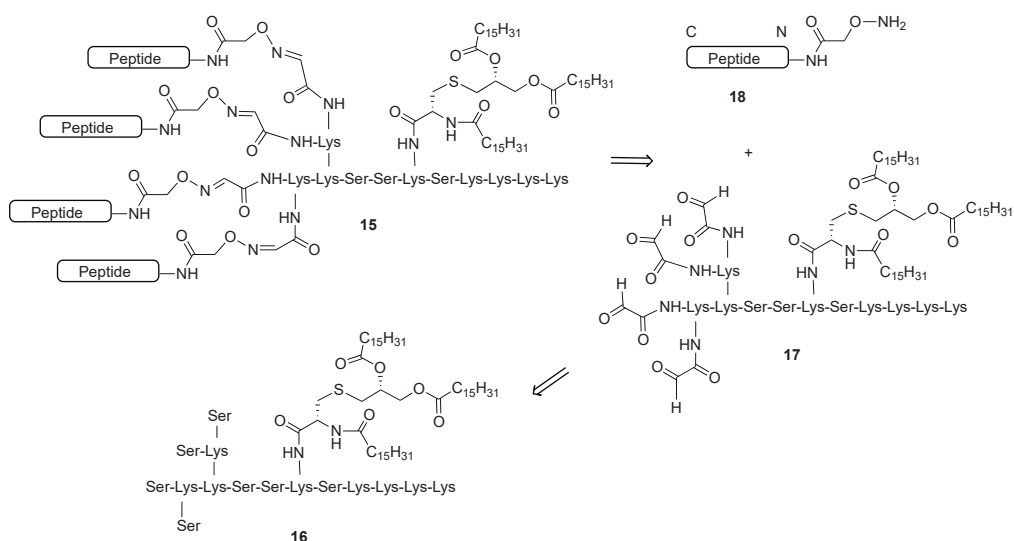
Although the synthetic method fulfilled well for several conjugates, the overall yield and quality of the final conjugate was inadequate for conjugates in which the peptide epitope probably could adopt a specific tertiary/quarternary structure. The construction of a new class of conjugates, composed of a Th epitope, a CTL epitope and Pam<sub>2</sub>Cys, was investigated by a modular approach that is terminated by a block coupling.<sup>60</sup> As branched conjugates showed more favorable immunological properties than their linear counterparts, the Pam<sub>2</sub>Cys ligand was appended to the N terminal end of the Th epitope to give a lipopeptide that was coupled to a separately prepared target epitope.<sup>60</sup> Three different reactions for the final block coupling were explored (Figure 5). The participating reactive functional groups were installed at the N-terminal end of both the target epitope and the lipopeptide composed of the Th epitope and the Pam<sub>2</sub>Cys. An oxime linkage was introduced by the reaction of the aldehyde in target epitope **7** with hydroxyl amine of lipopeptide **8** to give conjugate **9** (Figure 5). In the second conjugation strategy the bromo acetyl at the N-terminus of target epitope **10** reacts with the terminal cysteine in lipopeptide **11** to furnish conjugate **12** with a thioether linkage.<sup>60</sup>



**Figure 5.** Three coupling strategies towards branched TLR2/TLR6-ligand peptide conjugates

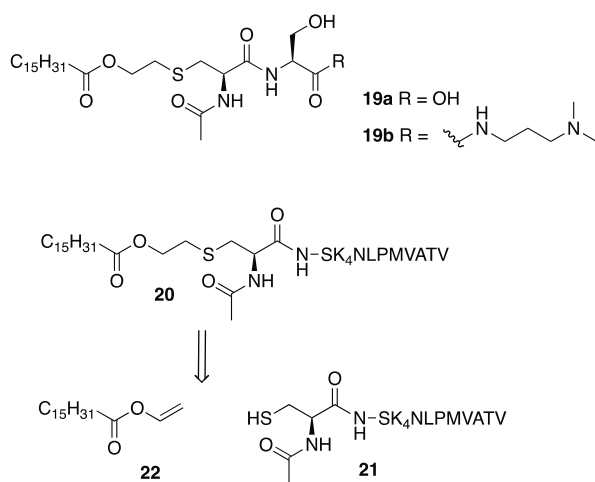
Lipopeptide **11** was also used in the third strategy, in which a disulfide linkage was introduced by a reaction with the terminal cysteine in **13** to provide conjugate **14**. All three reactions proceeded successfully to provide the final lipidated peptide in sufficient quality while stepwise solid phase synthesis as previously described failed. It appears that alkylation of the bromoacetylated peptide with cysteine, leading to conjugates of type **12** is the most efficient out of the three strategies. Although, the non-natural thioether bond formed between the target epitope and the rest of the construct tends to decrease the processability of the conjugate by the proteasome, all constructs could induce significant immune response.<sup>60</sup>

Prior to the above described modular approach, the oxime ligation approach was also used in the synthesis of self adjuvanting immunopeptides **15** by Rose *et al.* as depicted in Figure 6. An important aspect of these conjugates is the presence of several copies of the peptide antigen on a multifunctional core.<sup>61</sup> Conjugates with multivalent epitopes often showed an increase in immunogenicity. The multiple antigen peptide system **16** that uses an oligomeric branching lysine was selected as a core. Construct **16** was prepared by SPPS using SASRIN resin and Fmoc-chemistry. After six coupling cycles the TLR2/1 ligand, Pam<sub>3</sub>Cys-OH, could be condensed to the side chain of the terminal lysine after selective cleavage of the orthogonal Dde group with hydrazine.<sup>61</sup>



**Figure 6.** TLR2/TLR1 ligand peptide conjugate bearing multiple peptides

Next, the synthesis was continued by elongation with two serine residues and one lysine residue. The lysine at the N terminus was fully deprotected and the released alpha amine and the epsilon amine were simultaneously condensed with two protected lysines. Subsequent deprotection of both amines in the lysines allowed the coupling of four serine residues. Finally, removal of the protecting groups, cleavage from the solid support and purification furnished core **16**. The aldehyde functions were produced by reaction of the 1,2-amino alcohols in the N-terminal serine residues with sodium periodate to give construct **17**. Peptide **18** was separately assembled by standard SPPS, in which the final coupling entails the introduction of the hydroxyl amine moiety by reaction with Boc-aminoxyacetyl N-hydroxysuccinimide ester. Zeng *et al.* completed the synthesis by condensation of aminoxyacetyl peptide **18** and template **17** provided with four aldehydes to give immunopeptides **15**.<sup>61</sup>

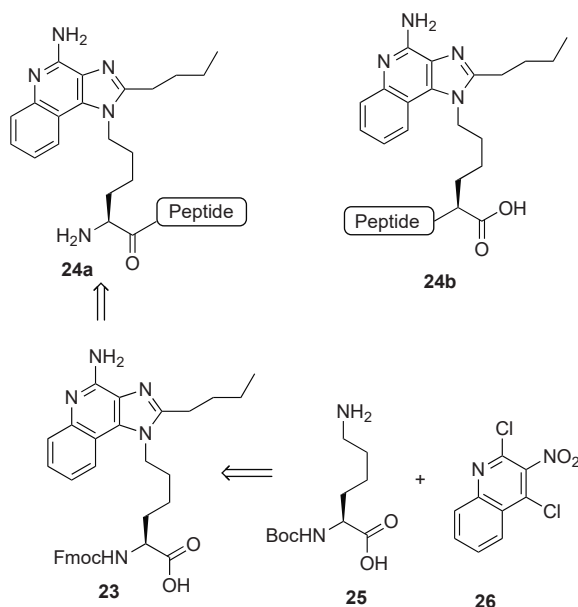


**Figure 7.** Simplified monoacyl lipopeptide **19** and retro synthesis of the incorporation of this ligand in antigenic peptide conjugate **20**.

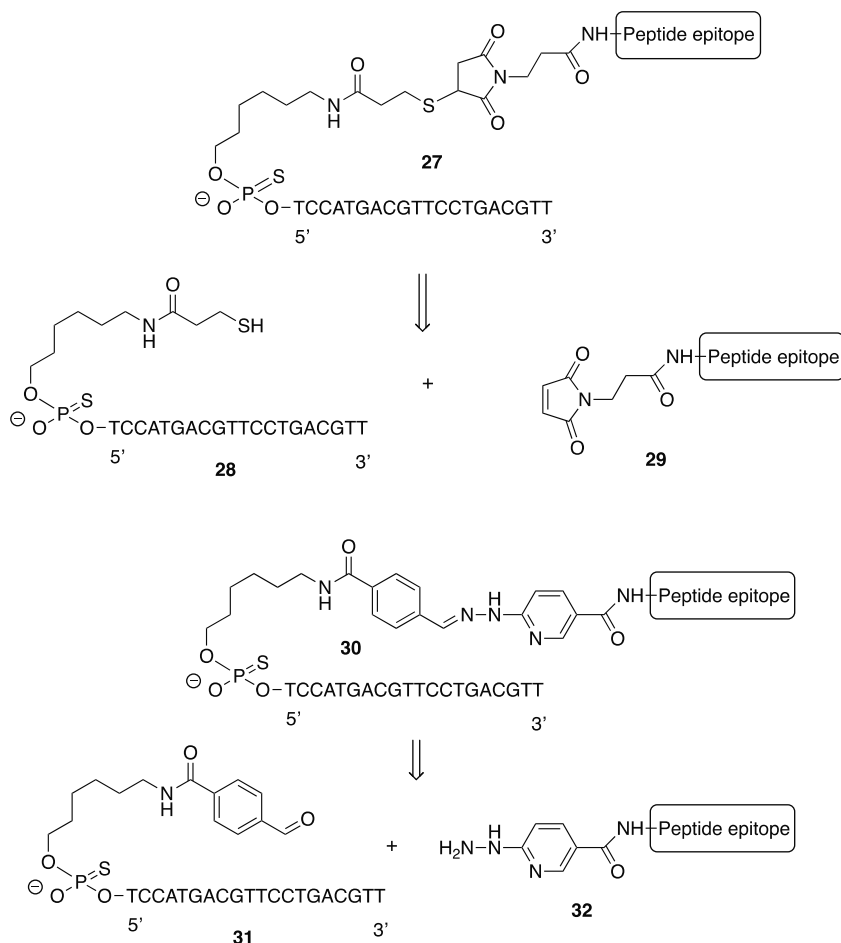
The group of David explored structure-activity relationships of several immunostimulatory TLR agonists, including TLR2 ligands.<sup>20-22</sup> These studies led, among other findings, to the interesting discovery of monoacyl lipopeptide **19a**, a simplified TLR2 ligand which unexpectedly showed exclusive human TLR2 agonistic activity (Figure 7). With the objective to increase the water solubility of this ligand compound **19b** was found as a stable, water soluble, highly potent, human specific TLR agonist. Brimble *et al.* applied ligand **19a** in the construction of conjugate **20**, via an innovative synthetic approach.<sup>62</sup> Most of the reported preparations to these type of molecules use a convergent synthesis, in which a specific building block was pre-synthesized and then coupled to an amino acid or an oligopeptide. The group of Brimble developed a thiol-ene coupling procedure which does not require any separately prepared building block.<sup>62</sup> The thioylated peptide **21** and vinyl palmitate **22** were irradiated with UV light in presence of 2,2-dimethoxy-2-phenylacetophenone (DMPA) as photo-initiator, leading to over 90% conversion. This new self adjuvanting peptide conjugate **20** prove to be remarkably potent, but its exact target, either TLR1/2 or TLR2/6 heterodimer, and also the reason for the specificity of **20** for human TLR2 remain unclear.<sup>22</sup>

### TLR-7 targeting peptide conjugate

Ligands of the TLR 7 and/or TLR-8 receptor are intensively investigated and several small molecule agonists<sup>15</sup> have been discovered and immunologically evaluated in a mixture with a protein or conjugated to a proteins<sup>63</sup>, antibodies<sup>64</sup>, lipids<sup>65</sup> or other entities.<sup>66</sup> Also a few conjugates in which a TLR7 ligand is covalently connected to an antigenic peptide are also reported.<sup>67</sup> Fujita *et al.* reported the synthesis of partially protected 6-(4-amino-2-butyl-imidazoquinolyl)-norleucine **23**, the structure of which was based on the TLR7/8 ligand imidazoquinoline (Figure 8).<sup>68</sup> This modified amino acid could be applied in SPPS, using Fmoc chemistry and Rink-amide PEG MBHA resin. This led to the assembly of peptide conjugates **24a** and **24b**, in which the TLR7/8 ligand was attached to the N- and C-terminal end of the peptide M2e antigen of influenza A virus. The produced conjugates led to a poorly antigenic peptide with self-adjuvanting properties.<sup>68</sup>



**Figure 8.** Retro synthesis of TLR-7 agonist peptide conjugate



**Figure 9.** Synthesis approaches to TLR9 peptide conjugates

### TLR-9 targeting peptide conjugate

CpG, an oligodeoxynucleotide fragment of specific sequence and length, is an agonist for TLR-9<sup>31, 69, 70</sup> In order to obtain a TLR9 peptide conjugate several convergent synthesis approaches are explored in which the CpG oligonucleotide with a reactive group at the 5'-end is coupled in solution with a selected peptide epitope provided at the N- or C-terminal end with a corresponding reactive group.<sup>50</sup> Both the functionalized CpG fragment and the functionalized peptide are prepared via a standard solid phase procedures and purified before conjugation. Diamond *et al.* successfully assembled self adjuvanting immuno peptides (29) using a peptide epitope bearing a maleimide moiety on the N terminus (27) and a CpG oligonucleotide, having a thiol function at the 5'-end (28 Figure 9).<sup>50</sup> This strategy was applied using various relevant peptide epitopes in order to synthesize a library of TLR-9 mediated self adjuvanting vaccine candidates. In another strategy a hydrazine reactive group in the peptide (32) was combined with an aldehyde at the 5'-end of the CpG oligonucleotide (31) to give a conjugate with a hydrazone

linkage (30, Figure 9) The obtained conjugate proved to be more potent than just a mixture of the CpG and the immunogenic peptide.<sup>50</sup>

## Conclusion

TLRs are very attractive drug targets that are intensively investigated not only for the development of new adjuvants for improved vaccines but also in the search for new classes of vaccines, such as cancer vaccines. In this respect multiple studies have been directed to design and optimize specific small molecule agonists for these PRRs. Besides, antagonists of TLRs may be applied for the treatment of autoimmune diseases. Furthermore structurally defined TLR ligands are explored in the search for fully synthetic vaccines. The first steps to the development of such vaccines are the here described conjugates comprising TLR ligand(s) and peptide epitope(s). From a synthesis point of view multiple challenges remain such as to overcome the low solubility of these conjugates and the development of improved functionalization methods (post synthetic labelling, introduction of multiple orthogonal handles).

## Outline of this thesis

**Chapter 2** describes a post-synthetic methodology to introduce a fluorescent label in highly lipophilic, Pam<sub>3</sub>Cys based conjugates, consisting of the TLR-2 ligand covalently connected to an immunogenic peptide. The fluorescent labels were appended to the peptide part of the conjugate with the aid of a strain promoted [3+2] azide-alkyne cycloaddition. The prepared fluorescent lipopeptides triggered DCs maturation in TLR-2-dependent way. Furthermore, the conjugates labelled with label Cy-5 could be successfully used in confocal microscopy studies and were taken up by dendritic cells in a TLR-independent manner. In **Chapter 3** a synthesis is discussed of a structurally simple human specific TLR-2 ligand with diminished lipophilicity, as compared to Pam<sub>3</sub>Cys. Conjugation of such moiety to peptide is studied and optimized to produce human specific analogues of the conjugates described in **Chapter 2** with higher solubility and an equal propensity to activate TLR-2. The synthesis of a newly designed TLR-7 agonist is demonstrated in **Chapter 4** as well as the synthesis of a selection of self-adjuvanting immunogenic peptides that contain a model MHC-I epitope (SIINFEKL). Such constructs are designed in a way similar to that described in **Chapter 2** and **Chapter 3**. A biocompatible methodology to reduce an azide in a side chains of peptides is described in **Chapter 5** with a particular focus on side reaction occurring during the reduction. A selection of phosphines is evaluated for their capacity to reduce the azide functionality in a peptide context and under biocompatible aqueous conditions. The pH dependency of the product ratio has been investigated as well. **Chapter 6** describes the development of a convergent synthesis of the naturally occurring conjugate between the 5'-terminal fragment of genomic RNA from Cocksackie virus and the full-length viral genome-linked protein (VPg). Towards this end, a novel solid-phase methodology has been developed, which is based on the 5'-O-levulinyl ester as the temporal protection in the synthesis of the target RNA-oligonucleotide attached to a pentapeptide fragment from the VPg.

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