Design, synthesis, and evaluation of antigenic peptide conjugates containing Toll-like receptor agonists

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Chapter 1

Introduction

Pattern recognition receptors

Pattern recognition receptors (PRRs) belong to the first line of defense of vertebrates for the identification of pathogen invasions. PRRs are expressed by cells of the innate immune system, such as antigen presenting cells (APCs), and each type of receptor has been evolved to detect a specific type of highly conserved molecular moieties, that are termed pathogen associated molecular patterns (PAMPs) or damage associated molecular patterns (DAMPs). When these molecular patterns bind to an associated PRR on an APC many events are triggered, that initiate the host defense reactions and ultimately may result in an adaptive immune response. Several classes of PRRs are known such as, Nod-like receptors (NLRs),[1] C-type lectin receptors (CLRs),[2] and Toll-like receptors (TLRs).[3] With the objective to obtain structurally well-defined adjuvants and ultimately vaccines, a lot of research is devoted to the development of synthetic accessible ligands for PRRs, of which the TLRs are most pursued.

Toll-like receptors

Up until this day, ten different types of human TLRs have been discovered each recognizing a group of specific PAMPs and are mostly found on APCs, such as dendritic cells (DCs) and macrophages. All TLRs are single-pass transmembrane proteins, containing a horseshoe shaped extracellular domain responsible for ligand recognition, a transmembrane domain, and a conserved cytosolic Toll/IL-1 receptor (TIR) domain. The extracellular domain is made up of 16 to 28 leucine rich repeats (LRRs) each domain containing up to 20-29 amino acids with inside a LXXLXLXXN motif[4-5] which is responsible for the horseshoe shape. After recognition of a PAMP, TLRs form either a homo- or heterodimer which drives conformational adjustments in both receptors bringing both TIR-domains together. Afterwards, the TIR-domains initiate a signalling cascade, depending on the type of dimerization, either through the Myeloid differentiation primary response 88 (MyD88) dependent pathway or the TIR-domain-containing adapter-inducing interferon (TRIF) dependent pathway. The MyD88-dependent pathway results in the relocation of nuclear factor-kappa B (NF-κB) to the nucleus and starts the production of pro-inflammatory cytokines, whereas the TRIF-dependent pathway activates interferon regulatory factor (IRF) and starts transcribing Type 1 interferon genes. Increased production of interferons and cytokines maturate local APCs, that upregulate co-stimulatory factors, increase endocytosis, increase antigen loading on major-histocompatibility complexes (MHCs), and migrate to the lymph node. The APCs patrol the lymph node searching for a T-cell expressing a complementary T-cell receptor (TCR) to form a specific TCR-MHC complex that activates the T-cell and induces its proliferation, starting an adaptive immune response.[6]

TLRs can be divided into two classes depending on their cellular location and the type of PAMP they bind.[7-8] The first class of TLRs are situated on the cell membrane and consists out of TLR1, TLR2, TLR4, TLR5, and TLR6. Generally, these TLRs recognize extracellular bacterial PAMPs that primarily consists out of lipopeptides (TLR1, TLR2, and TLR6), liposaccharides (TLR4) and flagellin (TLR5). The second class comprises of TLR3, TLR7, TLR8, and TLR9, which are located on endosomal vesicles inside the cell. They bind PAMPs related to viral infections such as double-stranded RNA (TLR3), single-stranded RNA (TLR 7 and 8) and cytosine-phosphate-guanine (TLR9). TLR10 is not included in one of the two classes because the purpose of TLR10 has not been understood yet, but it has been suggested that TLR10 might function more as a regulatory receptor than an immune initiator.[9]

TLR2 receptor and its mode of action

Unique to TLR2 is its ability to heterodimerize with either TLR1 or TLR6 after ligand association.[10] Although TLR2 is primarily associated with the recognition of tri- or di-acylated lipopeptides, other naturally occurring lipophilic PAMPs such as lipoteichoic acid,[11] lipoarabinomannan,[12] and peptidoglycans[13] can bind TLR2 too. Based on the crystal structure of a TLR1-TLR2 complex loaded with Pam_3CSK_4, it is believed that TLR2 binds an agonist first to subsequently start recruiting either TLR1 or TLR6 to form a heterodimer (Figure 1).[14]

Furthermore, it is suspected that some costimulatory factors might be involved with agonist loading, since CD14 has been shown to enhance signaling[15] and CD36 is required for functional signalling for certain ligands.[16] Specificity for heterodimerization is determined by the degree of acylation of the ligand. Tri-acylated lipopeptides allow recruitment of TLR1, which is facilitated by an apolar binding pocket within TLR1s LRR repeats. Alternatively, a free polar moiety in di-acylated lipopeptides promotes dimerization with TLR6 by interacting with a hydrophilic region on the extracellular domain of TLR6. Both dimers trigger the MyD88-dependent signalling pathway inside APCs, resulting in the production of pro-inflammatory cytokines.

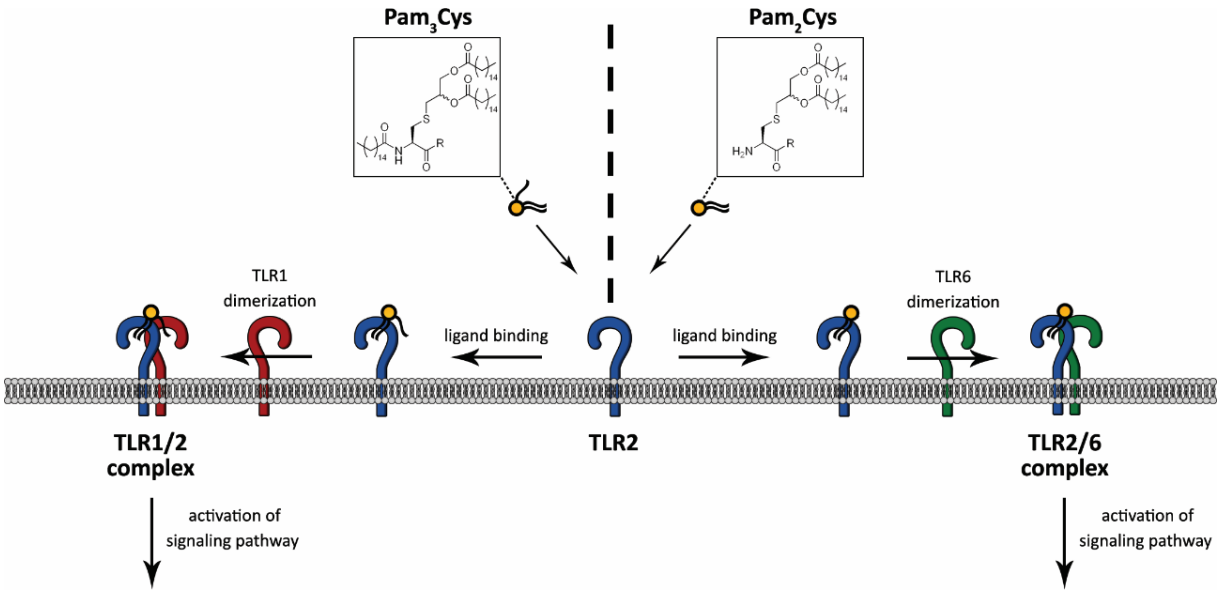


Figure 1: Schematic depiction of TLR1/TLR2 and TLR2/TLR6 dimerization upon association with either Pam_3Cys or Pam_2Cys, respectively.

Synthetic TLR2 ligands

Over the course of several decades, structure-activity relationship (SAR) and optimization studies accumulated in a range of relatively low weight molecules as an alternative to the larger natural agonists for TLR2[17-18]. First, there are the di- and triacylated lipopeptides Pam_2CSK_4 and Pam_3CSK_4 (Figure 2), originally derived from macrophage activating lipopeptide-2 (MALP-2), that induce TLR2-6 and TLR2-1 heterodimerization, respectively. Their structures are composed from a N-terminal lipidated cysteine, essential for agonistic activity, a serine, which is interchangeable with several smaller polar side chains such as di-amino butyric acid, and a tetralysyl spacer for solubility, that do not contribute to ligand binding. Even though it has been proven that only structures containing a R-configured glycerol moiety are agonistic, most research uses the Pam_2Cys and Pam_3Cys based lipopeptides as the mixtures of epimers at C-2 of the glycerol. A wide range of optimization have been performed on the palmitoyl tails, the second amino acid and the spacer (Figure 2). This resulted in two significant contributions, namely UPam[19] a next generation Pam_3CSK_4 agonist and the introduction of less lipophilic alternative, namely mono-acylated cysteines[20-22] as replacement for Pam_2CSK_4 and Pam_3CSK_4 . Though most research employs lipidated

cysteines as agonist of choice, other lipidated peptides have been used as a simpler substitute for Pam_2CSK_4 and Pam_3CSK_4. Different lipoamino acid-based synthetic peptides (lipid core peptides, LCP) were used in the agonist design such as lipidated lysine's in LLCP,[23] serine's in DPS, and lipoamino acids of various lengths as in LCP and LCPS2.[24-28] Although these type of agonists can activate TLR2, they are unable to compete with palmitoylated cysteines. Aside from these "natural" ligands, a group of aromatic small molecules have been found to be suitable agonists for TLR2.[29-31]

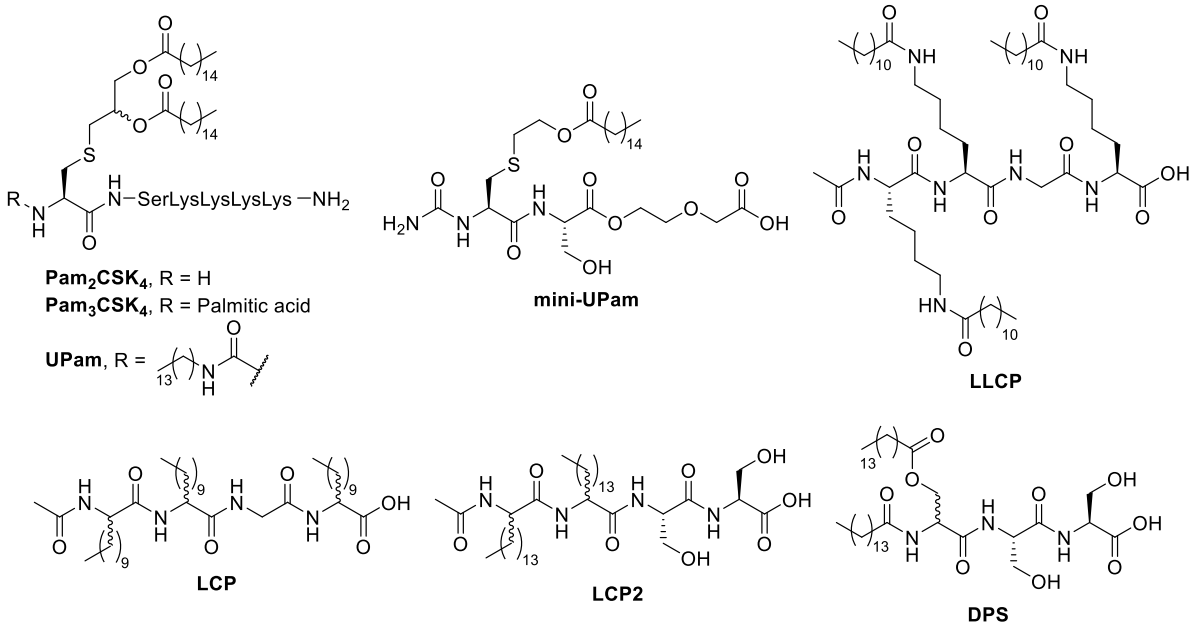


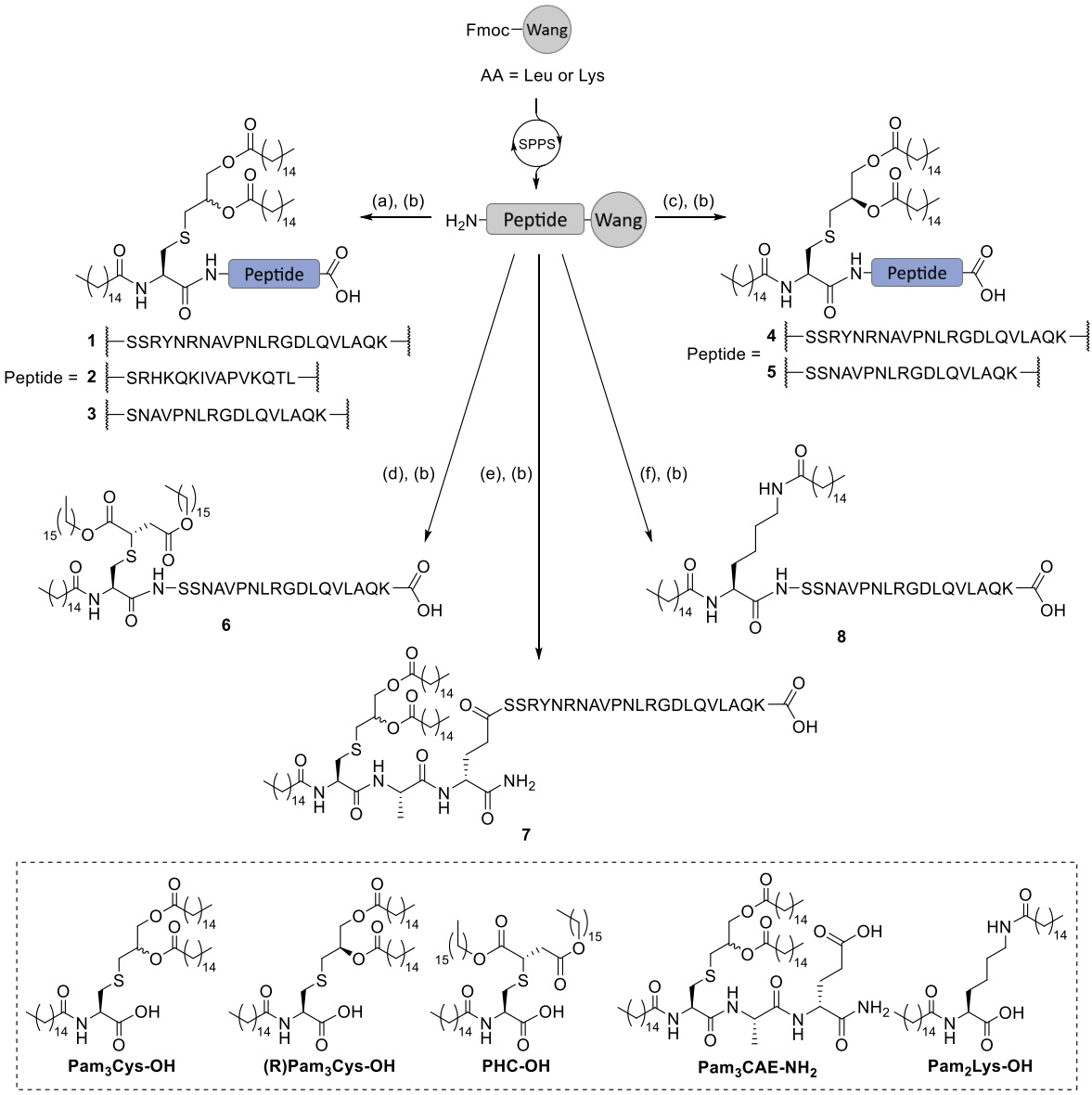
Figure 2: Structures of various lipopeptides determined to be TLR2 agonists and incorporated in future conjugates, namely Pam_2CSK_4, Pam_3CSK_4, LCP, LCP2, LLCP, and DPS.

Conjugation of TLR2 ligands to antigenic peptides

As a DC requires both activation of a pattern recognition receptor (PRR) and an antigen loaded MHC to launch an adaptive immune response, covalently linking PAMPs to a peptide containing an epitope has been investigated as an approach to the initiation of epitope specific immune responses.[7, 32-53] It has been reported that these conjugated constructs outperform their non-conjugated counterparts in terms of antigen presentation and T-cell activation, whilst retaining most PAMP agonists activity. This proves that PRR ligand-antigen conjugation is a promising methodology for future immunotherapy, and for this many distinct conjugation designs have been employed throughout the last decades. In the next section, the work of nine different articles will be discussed to demonstrated design strategies for conjugation.

All conjugates were synthesized using Fmoc-based peptide chemistry utilizing an Applied Biosystems 430A peptide synthesizer with Wang-resin as solid support. Elongation of the oligopeptide was performed by repeating the following three-step cycle: (i) treatment with 55% piperidine in NMP for 1 x 2 min and 1 x 5 min to deprotect the Fmoc group; (ii) double coupling of the protected amino acids by pre-activation of Fmoc-AA-OH (1.5 eq.) in NMP with

DIC (1.5 eq.) and HOBt (1.5 eq.), followed by coupling for 1.5 h at RT; (iii) masking the remaining free amino functions by treatment with a solution of Ac_2O with D*i*PEA in NMP. The obtained immobilized peptides were then manually condensed with the appropriate lipidated cysteine building blocks (Scheme 1) in the presence of DIC and HOBt as activation reagents and D*i*PEA as base. Afterwards, the conjugates were released from the resin and deprotected with 5% thioanisole in TFA for 4.5 h. Products **6** and **7** were purified by the precipitation from AcOH induced by the addition of diethyl ether and reprecipitation from a chloroform-trifluoroethanol mixture by addition of aceton and water. No chromatographic purification has been attempted.

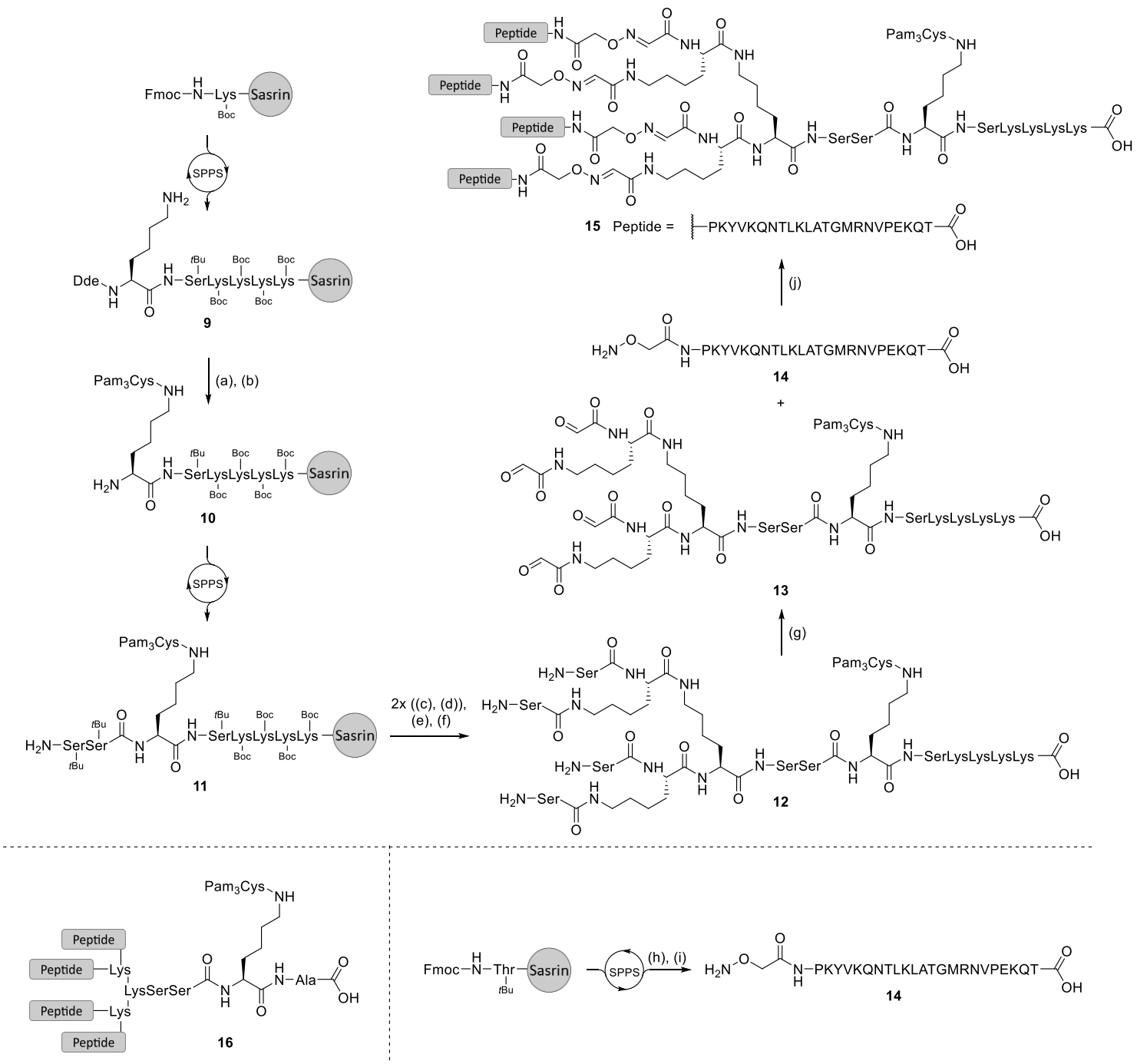


Scheme 1: Synthetic work published by Wiesmüller et al. Reagents and Conditions. **SPPS:** deprotection cycle) 55% piperidine in NMP, 1x 2 min, 1x 5 min; coupling cycle) Fmoc-AA-OH, DIC, HOBt, NMP, 2x 1.5 h; capping cycle) Ac_2O, DiPEA, NMP. **(a)** i) Pam_3Cys-OH, DIC, HOBt, 2:1 DCM:DMF, 4h; ii) NMM, 1h; **(b)** 95:5 TFA:thioanisole, 4.5 h **(c)** i) (R)Pam_3Cys-OH, DIC, HOBt, 2:1 DCM:DMF, 4h; ii) NMM, 1h; **(d)** i) PHC-OH, DIC, HOBt, 2:1 DCM:DMF, 4h; ii) NMM, 1h; **(e)** i) Pam_3CAE-NH_2, DIC, HOBt, 2:1 DCM:DMF, 4h; ii) NMM, 1h; **(f)** i) Pam_2Lys-OH, DIC, HOBt, 2:7:1 DCM:DMF:THF, 14h; ii) NMM, 1h.

The conjugates were tested for their ability to elicit an antibody response, quantified by the neutralization titre and an anti-peptide ELISA assay, and their protection rate against FMD in guinea pigs. The criteria set for a protected animal was the absence of secondary lesions for ten days. In all tests, guinea pigs were immunized with 0.5 mg of pure conjugate except for conjugates **2** and **3**, which were administered as a mixture in a 1:1 weight ratio. Of all conjugates only conjugates **4**, **7** and the combination of **2** and **3** were able to induce both substantial protection and a stronger antibody response. Conjugates **3**, **5**, and **8** all protected one out of four immunized animals as the conjugate was outfitted with a functional TLR2 ligand however lacked a functional epitope in its peptide sequence. Conjugates **6**, **7** and **8** displayed higher absorption values for the anti-peptide assay values but lack any indication of an increased antibody response in the neutralization titre. Overall, Wiesmüller and Jung have proven that conjugates in which synthetic peptide antigens are decorated with a TLR2 ligand can induce functional protection, therefor establishing the foundation for self-adjuvanting vaccines. In the following years they publish two more articles describing a Pam_3Cys linked epitope, one derived from the influenza nucleoprotein[56] and the other from the circumsporozoite protein from malaria[57].

To increase the load of antigenic material connected to a single Pam_3Cys ligand and thus potentiate the immunogenicity of the construct, *Defoort et al.*[58] designed a peptide scaffold (structure **16**, Scheme 2) ending with a so-called multiple antigen peptide system (MAPS)[59]. MAPSs are branched amino acid cores that allow incorporation of an exponentially increasing number of antigens, based on the degree of repeated branching. A triple chloroacetylated lysyl branched structure was chosen as MAPS which was connected by thiolalkylation to four peptides with three HIV-1 epitopes. When BALB/c mice were immunized with the construct, an increased level of epitope specific antibodies was observed in the sera harvested from the animals and capable to elicit epitope specific CTLs responses. *Zeng et al.* continued this research by developing an alternative MAPS provided with four influenza epitope containing antigenic peptides (PKYVKQNTLKLATGMRNVPEKQT), a single Pam_3Cys ligand and a solubilizing pentapeptide, resulting in conjugate **15** (scheme 2).[60] The conjugate features the same triple lysyl branched as used by *Defoort et al.* while the C-terminus is elongated with the pentapeptide SK_4, similar to the TLR2 ligand Pam_3CysSK_4, as solubility handle.[61-62] *Zeng at al*. applied an aldehyde-oxime ligation, they developed[63] to introduce the unprotected antigenic peptides claiming the production of water soluble and more homogenous vaccine candidates.

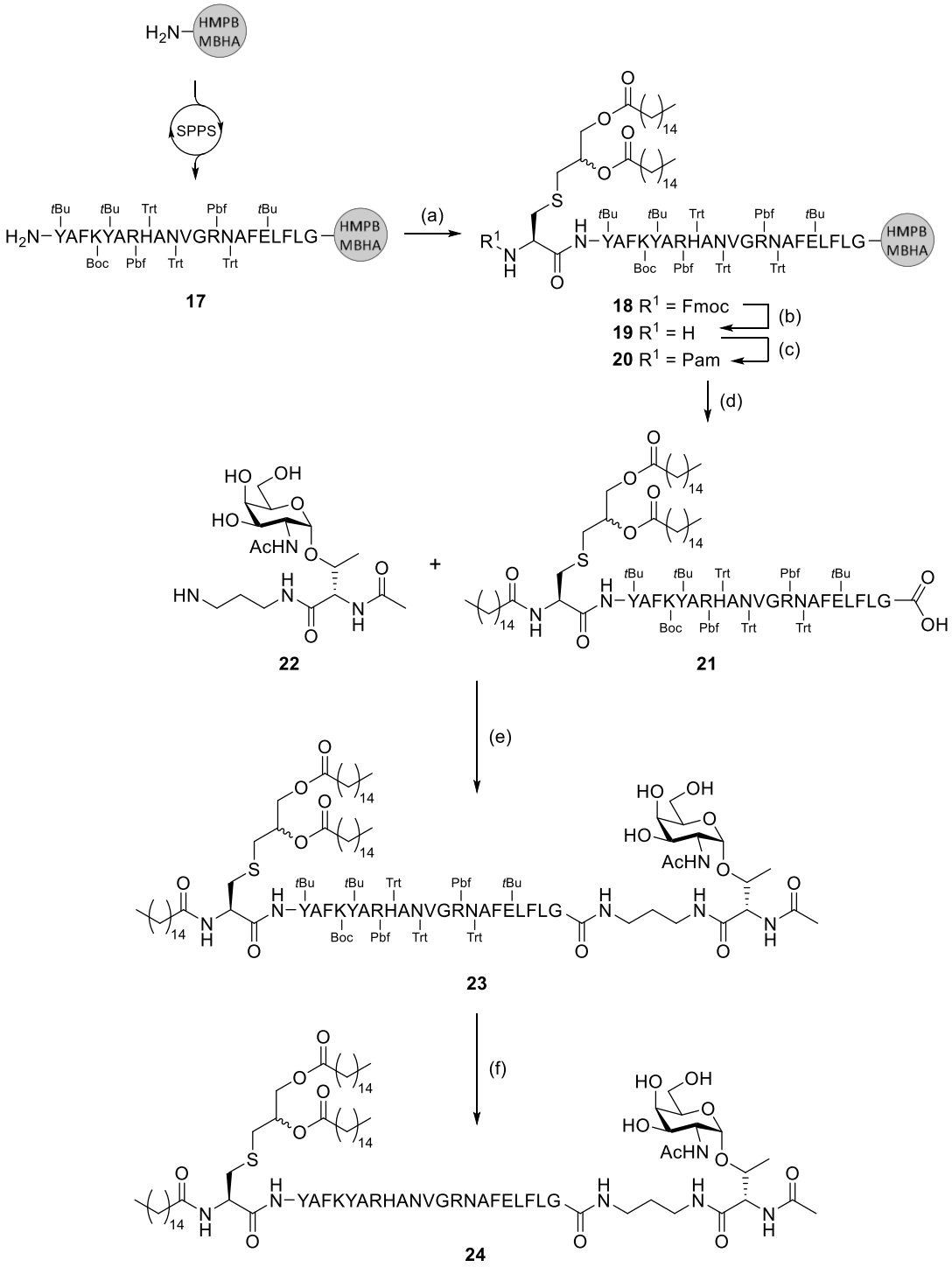
The synthesis of conjugate **15** is outlined in Scheme 2 and started with the manual SPPS of oligopeptide **13** utilizing Fmoc-based chemistry and Sasrin resin as solid support. The following two-step cycle was used for the elongation of the oligopeptide: (i) treatment with 50% piperidine in DMF for 20 min to remove Fmoc group; (ii) coupling of the protected amino acids by treating the resin with a solution of Fmoc-AA-OH (3 eq.) in DMF with TBTU (3 eq.), HOBt (3 eq.) and D*i*PEA (4.2 eq.) for either 120 min (for Fmoc-Lys(Fmoc)-OH, Pam_3Cys-OH and Boc-Ser(*t*Bu)-OH) or 45 min (all other building blocks) at RT. Sasrin resin with preloaded with lysine was elongated with SK_4 and subsequently with Dde-Lys(Fmoc)-OH followed by Fmoc removal



Scheme 2: Assembly of a four synthetic peptide carrying TLR2 functionalized construct by utilizing an oxime coupling. Reagents and conditions: **SPPS**: conditions for compound **9**, deprotection cycle) 50% piperidine in DMF, 20 min; coupling cycle) Fmoc-AA-OH, TBTU, HOBt, DiPEA, DMF, 45 or 120 min (for Fmoc-Lys(Fmoc)-OH, Pam_3Cys-OH and Boc-Ser(tBu)-OH); **SPPS** conditions for compound **14**: deprotection cycle) 20% piperidine in DMF, 2x 2 min 1x 10 min; coupling cycle) Fmoc-AA-OH, HBTU, DiPEA, DMF, 8 min for the preactivation and 20 min for the coupling; **(a)** Pam_3Cys-OH, TBTU, HOBt, DiPEA, DMF; **(b)** 2% hydrazine in DMF, 10 min; **(c)** Fmoc-Lys(Fmoc)-OH, TBTU, HOBt, DiPEA, DMF; **(d)** 50% piperidine in DMF, 20 min; **(e)** Boc-Ser(tBu)-OH, TBTU, HOBt, DiPEA, DMF, 2h; **(f)** 88:5:5:2 TFA:MeSH:H_2O:TIS, 2h; **(g)** $NaIO_4$; **(h)** Boc-aminooxyacetyl N-hydroxysuccinimide ester, DMF, NMM, 2h; **(i)** 90:5:3:2 TFA:thioanisole:EDT:anisole, 2h; **(j)** pH 3.7 acetate buffer.

providing immobilized peptide **9** with a liberated Lys side chain. Now, Pam_3Cys was selectively installed at the free amine of the side chain and subsequent cleavage of the Dde protecting group with 2% hydrazine in DMF for 10 min yielded lipopeptide **10**. This immobilized peptide functionalized with Pam_3Cys was elongated with two serine residues, giving resin **11** with a free amine. The construction of MAPS started by repeating the elongation cycle two more times with Fmoc-Lys(Fmoc)-OH, delivering four free amines that were condensed with Boc-Ser(*t*Bu)-OH to give the immobilized fully protected precursor of the MAPS template, that was deprotected and cleaved from the resin using 88:5:5:2 TFA:MeSH:H_2O:TIS as deprotection cocktail. The crude product was precipitated by adding the deprotection cocktail to cooled diethyl ether, the precipitate was purified by HPLC giving pure lipopeptide **12**. Using a periodate cleavage reaction, the N-terminal serines of **12** were oxidated into aldehydes and MAPS template **13** was isolated with analytical HPLC. Parallel to the synthesis of tetraoxime **13**, hydroxyamino **14** was assembled using SPPS, SASRIN resin preloaded with threonine, and a single manual condensation to install the hydroxylamine moiety on the N-terminal end of the peptide. The following two-step cycle was used for the elongation of the oligopeptide: (i) treatment with 20% piperidine in DMF for 2 x 2 min and 1 x 10 min to remove Fmoc group; (ii) coupling of the protected amino acids by treating the resin with an 8 min long preactivated solution of Fmoc-AA-OH (4.4 eq.) in DMF with HBTU (4 eq.), and an excess of D*i*PEA for 20 min at RT. After the final deprotection cycle, the liberated amine was reacted with Boc-aminooxyacetyl N-hydroxysuccinimide ester in DMF with N-methylmorpholine (NMM) as a base. The modified peptide was deprotected and cleaved of the resin using a mixture of 90:5:3:2 TFA:thioanisole:EDT:anisole, the crude peptide was subsequently precipitated with Et_2O and purified by HPLC. With epitope **14** functionalized with an aminooxylacetyl moiety in hand, the final oxime ligation with MAPS template **13** was undertaken by addition of **13** to epitope **14**, stirring for 24 h at RT and purification by HPLC to provide pure tetraoxime target **15**. Sadly, nothing was published regarding the immunogenicity of this MAPS candidate vaccine **15**. On the other hand, the template was implemented in the development of a malaria vaccine candidate by *Nardin et al.*[64-65] However, based on a literature search the self-adjuvanting peptide carrier was not continued as vaccine strategy.

A frequently observed phenomenon of cancer pathogenesis is the overexpression of oligosaccharides on the cell surface of malignant cells. As a result, researchers have exploited tumor-associated carbohydrate antigens for the development of therapeutic tumor vaccines. A well-known example is the Tn antigen, made up from 2-deoxy-2-acetamido-D-galactose (GalNAc) α-O-linked to either serine or threonine. However, carbohydrates are different from peptides/protein or lipid antigens as carbohydrates are T cell independent. To overcome this limitation *Buskas et al.*[66] designed the first three component vaccine, namely lipidated glycopeptide **24**, featuring a N-terminal Pam_3Cys TLR2 ligand as PAMP to activate DCs, a T-cell epitope containing peptide (YAFKYARHANVGRNAFELFLG) and at C-terminal end connected with a small spacer a Tn antigen as T- and B-cell epitope respectively (Scheme 3).



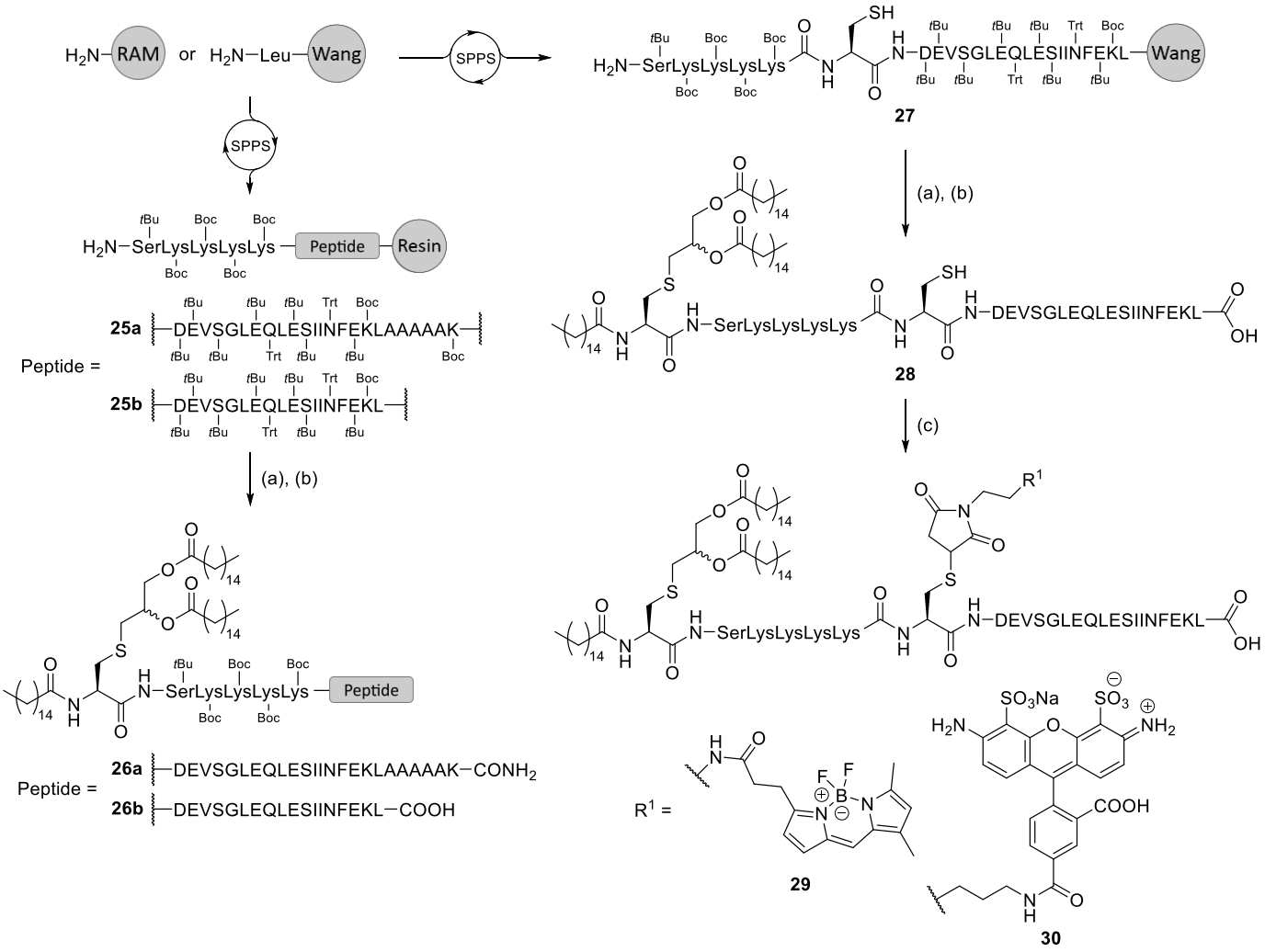
Scheme 3: Synthesis of Tn antigen carrying lipidated glycopeptide by *Buskas et al.* Reactions and conditions: **SPPS**: HBTU/HOBt as activator; **(a)** Pam_2Cys-OH, PyBOP, HOBt, DiPEA, 5:1 DMF:DCM; **(b)** 20% piperidine in DMF; **(c)** Palmitic acid, PyBOP, HOBt, 1:5 DMF:DCM; **(d)** 2% TFA in DCM; **(e)** DIC, HOAt, DiPEA, 2:1 DMF:DCM; **(f)** 95:2.5:2.5 TFA:H_2O:EDT.

Their synthetic approach to three component vaccine **24** entails a SPPS to afford protected peptide **17**, using an ABI 433A peptide synthesizer, Fmoc-based chemistry, HBTU and HOBt as activation reagents and HMPB-MBHA as solid support. HMPB-MBHA requires only mild acidic conditions for selective cleavage of the protected peptide from the resin. To prevent racemisation at the cysteine residue a three-step procedure was performed for the

installation of Pam_3Cys at the N-terminus of immobilized peptide **17**. First, lipopeptide **18** was produced by double coupling the free amine in **17** with Fmoc-Pam_2Cys-OH, using PyBOP/HOBt as activation reagents and D*i*PEA as base in a 5:1 solution of DMF:DCM, followed by cleavage of the Fmoc group with 20% piperidine in DMF. Finally, a standard condensation of the liberated amine in the obtained **19** with palmitic acid gave resin bound Pam_3Cys functionalized peptide **20**. Now, the immobilized peptide was released by treating the resin with 2% TFA in DCM and immediately quenching by filtering the deprotection solution into a solution of 5% piperidine in MeOH. The mixture was concentrated and pure protected lipopeptide **21** was isolated by size exclusion chromatography. Galactosamine building block **22** was coupled with the C-terminal carboxylic acid in protected peptide **21** in anhydrous DCM:DMF under an argon atmosphere in the presence of DIC, HOAt and DiPEA. Purification by size exclusion chromatography provided partially protected three-component vaccine **23**. Deprotection of the oligopeptide moiety with 95:2.5:2.5 TFA:H_2O:EDT for 1 h, and purification by HPLC yielding lipidated glycopeptide **24**.

It was demonstrated that conjugate **24** could function as self-adjuvanting tricomponent vaccine, by immunizing five female BALB/c mice with freshly prepared liposomes incorporated with conjugate **24**. The antibody responses in the mice were determined using bovine serum albumin Tn conjugate coated microtitre plates, which themselves could be labelled with anti-mouse IgM and IgG antibodies. The average results of both IgM and IgG titres confirmed that the conjugate was capable of eliciting antibodies against the Tn antigen. Follow-up studies of which were directed to the optimization of the degree activation of cytotoxic T-lymphocytes as part of the adaptive immune system.

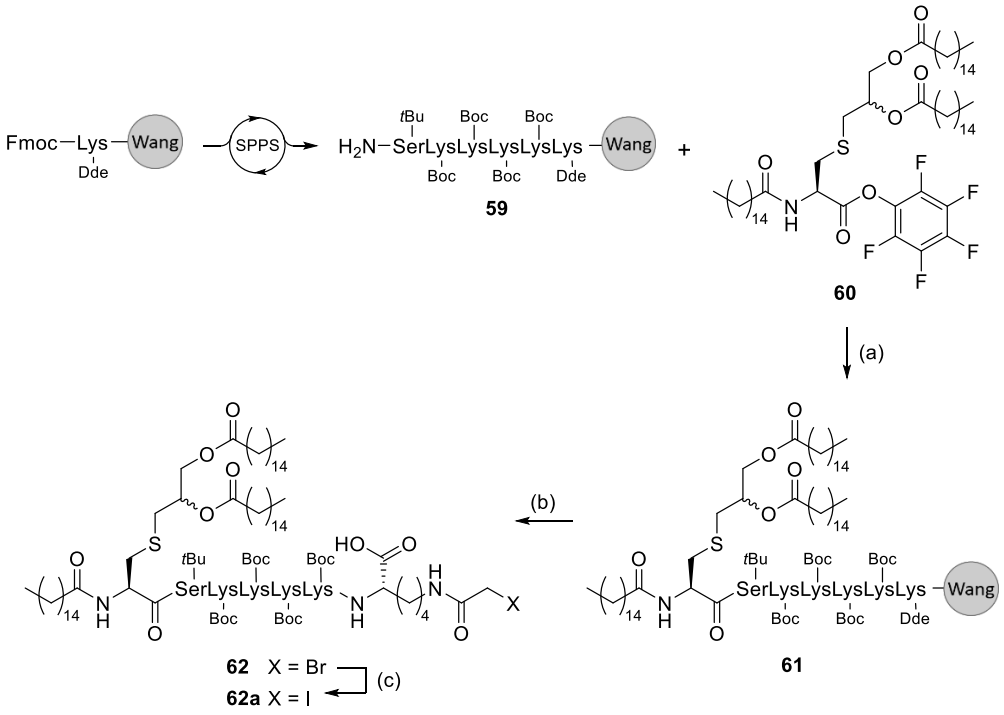
To elucidate the uptake mechanism and intracellular processing of fully synthetic TLR antigen conjugates, *Khan et al*.[36] developed conjugates in which both the ovalbumin model epitope SIINFEKL and a TLR ligand are incorporated (Scheme 4). An additional cysteine for an end-stage labelling was placed between the TLR ligand and the antigen. Although it was not specified in the article, the fluorophore should be released from the epitope by proteasomal processing to prevent hampering the loading of the antigen onto MHCs. Two maleimide modified fluorophores (Alexa488 and Bodipy-FL), two agonists (a TLR2 ligand and a TLR9 ligand) and two different ovalbumin derived peptide sequences were selected to be incorporated into the self-adjuvanting vaccines. The article describes four different conjugates which contain Pam_3Cys as a TLR2 agonist, namely conjugate **26a**, **26b**, **29**, and **30**. Both conjugate **26a** and **26b** miss the additional cysteine for end-stage labelling, where conjugate **26a** contains a peptide that needs both C-terminal and N-terminal proteasomal processing by DCs, whereas **26b** only needs the latter. For labelled conjugates **29** and **30**, only DEVSGLEQLESIINFEKL was incorporated as antigen and linked to Bodipy-FL and Alexa488, respectively.



Scheme 4: The solid-phase assembly of three Pam_3CysSK_4 functionalized ovalbumin peptides published by *Khan et al.* Additionally conjugate **29** and **30** were covalently bound to either a Bodipy or Alexa reporter group through a maleimide coupling. Reagents and conditions: **SPPS**: deprotection cycle) 20% piperidine in NMP, 15 min, 1x 5 min; coupling cycle) Fmoc-AA-OH, DIC, HCTU, D*i*PEA, NMP, 45 min; capping cycle) Ac_2O, D*i*PEA, NMP; **(a)** Pam_3-Cys-OH, PyBOP, D*i*PEA, overnight; **(b)** TFA:TIS:H_2O 95:2.5:2.5, 2 h; **(c)** Bodipy-FL-maleimide or Alexa-FL-C_5-maleimide, phosphate buffer, 72 h.

As depicted in Scheme 4 for the synthesis of conjugates with the Pam_3Cys ligand, *Khan et al*. used either unloaded or leucine preloaded PHB resin as a solid support and Fmoc-based peptide elongation cycle performed on a CS Bio 336 automated peptide synthesizer. After completion of the peptide sequence (antigen and SK_4 spacer included) the Pam_3Cys moiety was installed on the resulting immobilized peptides **25a** and **25b**, using a protocol similar to *Wiesmüller et al.* Finally, simultaneous removal of all protecting groups and cleavage from the solid support using a 95:2.5:2.5 TFA:H_2O:TIS solution, gave after HPLC purification conjugates **26a** and **26b**. A similar method was used to obtain immobilized peptide **27** and subsequently conjugate **28**. The fluorophores were appended via conjugate addition through dissolving the pure conjugate and the appropriate reactive dye (Bodipy-FL-maleimide or Alexa-FL-C_5-maleimide) in a phosphate buffer and sonicate the solution for 60 h under argon. Afterwards the reaction mixture was diluted and directly purified by HPLC yielding reporter conjugates **29** and **30**.

To determine the antigenicity of the conjugates, both their ability to elicit $CD8^+$ T-cells and DC maturation were investigated. Strong $CD8^+$ specific T-cell responses were observed by analysing the spleen cells 10 days after immunization of C75/B6 mice with either TLR2 and TLR9 conjugated antigens. DC maturation was determined by following the expression of the cell surface markers CD40, CD86, MHCI, and MHCII in BMDCs. Increased upregulation of the cell surface markers was observed in DCs incubated with conjugates compared to a mixture of separate ligand and antigen. The presence of the appended fluorophores did not alter the ability of the conjugates to activates DCs. *Kahn et al.* were able to demonstrate the beneficial effect of self-adjuvanting candidate vaccines on DC maturation and MHC peptide presentation, which in turn results in strong T-cell responses. Interestingly, the uptake mechanisms proved to be independent of the expression of TLR2 and TLR9 while similar trafficking and intracellular processing pathways seem to be followed.



Scheme 5: Reagents and conditions: **SPPS**: deprotection cycle) 20% piperidine:DMF, coupling cycle) Fmoc-AA-OH, HBTU, HOBt, DiPEA, DMF, capping cycle) Ac_2O, HOBt, D*i*PEA, DMF, Threonine building blocks were manually installed with HATU and HOAt; **(a)** HOBt, NMP; **(b)** i) 3% H_2N-NH_2, DMF, 3 min, ii) pentafluorophenyl bromoacetate, HOBt, DMF, iii) 90:5:5 TFA:TIS:H_2O; **(c)** KI, 8M urea, 0.1M NaOAc

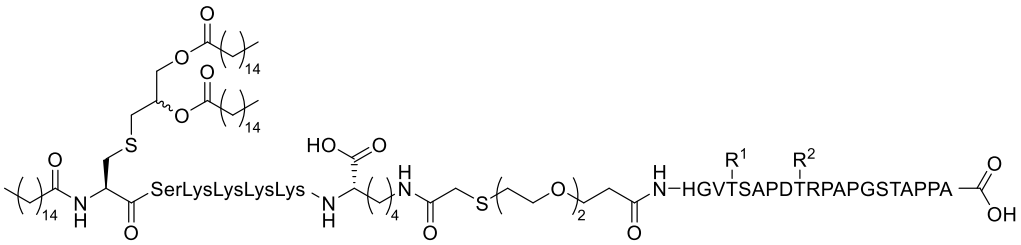
Besides the three-component vaccines of *Wilkinson et al.*[67] and *Lakshminarayanan et al.*[68], *Cai et al.*[69] developed another type of three-component antitumor vaccine that initiates a potent immune response (Scheme 6, page 10). In their design, they linked MUC1 (a glycosylated B-cell epitope) with either P2 or P4 (both universal T-cell epitopes) via a triethylene glycol spacer creating a two-component linear peptide sequence. This strategy required the availability of Pam_3CysSK_4 moiety equipped with an iodoacetyl. This was accomplished by appending an additional C-terminal lysine to Pam_3CysSK_4 and installing the iodoacetyl on its side chain by means of orthogonal protection with a hydrazine sensitive Dde-group. Outfitting the two-

component peptide with a C-terminal thiol containing spacer group, accommodates the installation of Pam_3CysSK_4 as the third component of the vaccine. Additionally, a set of Tn-, STn- and T-antigen functionalized threonine or serine building blocks were incorporated by SPPS in the MUC glycopeptide structure.

As shown in Scheme 5 the synthesis of iodoacetyl Pam_3CysSK_5 **62a** begins with Wang resin preloaded with a lysine, of which the side chain is protected with a Dde group. Elongation of this resin with SK_4 using Fmoc-based peptide chemistry on a CEM Liberty peptide synthesizer gave immobilized peptide **59**. The following elongation cycle was used; (i) 20% piperidine:DMF mediated Fmoc deprotection (ii) coupling of the protected amino acids by treating the resin twice with a solution of Fmoc-AA-OH (6 eq.) in DMF with HBTU (6 eq.), HOBt (6 eq.) and D*i*PEA (12 eq.) for 15 min at 50 °C; (iii) masking the remaining free amino functions by treatment with a 13 mM solution of HOBt in a solution of 4.75:2.25:93 Ac_2O:D*i*PEA:DMF at 50 °C for 3 min. Next, peptide **59** was reacted with pentafluorophenyl activated Pam_3Cys ester **60** resulting in protected immobilized Pam_3CysSK_5 **61**. Removal of the Dde protection with 3% hydrazine in DMF was followed by coupling of the released amine with pentafluorophenyl bromoacetate. The immobilized ligand was released and deprotected using a standard deprotection TFA cocktail and stored as bromoacetyl **62,** since the corresponding iodoacetyl groups are unstable in the presence of free amines and thioethers.

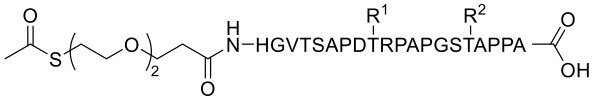
Both the two- and three-component peptide sequences were assembled employing Cl-Trt resin, using similar SPPS conditions as described for lipopeptide **3** (Scheme 1). However, the Tn-antigen carrying building blocks were installed following a slightly different coupling protocol with HATU and HOAt as condensation reagents and a coupling duration of 25 min. After cleaving the peptides from the resin using TFA:TIS:H_2O, the crude products were purified by HPLC yielding pure glycopeptides **63**-**68** and **75**-**80**. Next, the remaining protecting group on thiol group and the Tn-, STn- and T antigens were removed. Glycopeptides containing Tn and T saccharides were treated with NaOMe in MeOH at pH 10, whereas STn saccharides were deprotected with aqueous NaOH a pH 11.4. Then, Iodoacetyl **62a** was prepared by treating bromoacetyl **62** with an excess of KI, quickly purified and dissolved with a single deprotected glycopeptide in DMF with TEA to promote the ligation. After 16 h, the solution was acidified to pH 6 and purified by HPLC to give two-component vaccines **69**-**74** and three-component vaccines **81**-**86**.

Of all two- and three-component vaccines, only conjugates **69**, **81**, **84**, **85** and **86** were used to investigate their potency. Immunogenicity was determined by following MUC1 specific antibody production in sera isolated from mice and subsequently examining the binding ability of the sera to MCF-7 tumor cells by FACS. First, four mice were immunized five times with one of the respective conjugates either dissolved in PBS buffer or loaded in liposomes. Afterwards, sera were isolated from the mice and analyzed for MUC1 specific antibodies using ELISA. All five conjugates could induce potent MUC1 specific antibody production, where conjugates **84** and **85** in PBS buffer displayed the strongest antibody reactions. The sera isolated from the mice administered with conjugate **84** and **85** also showed the strongest binding towards MCF-



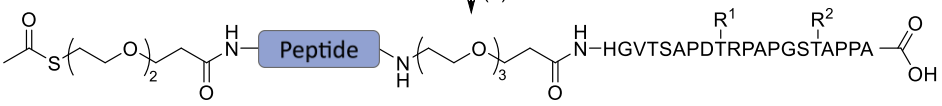
69 R^1 = Tn, R^2 = H **72** R^1 = H, R^2 = Tn
70 R^1 = T, R^2 = H **73** R^1 = H, R^2 = T
71 R^1 = Tn, R^2 = H **74** R^1 = H, R^2 = Tn

(b) or (c), (d)

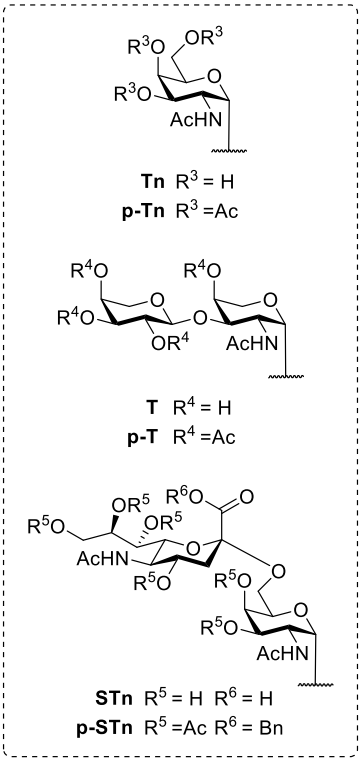


63 R^1 = p-Tn, R^2 = H **66** R^1 = H, R^2 = p-Tn
64 R^1 = p-T, R^2 = H **67** R^1 = H, R^2 = p-T
65 R^1 = p-Tn, R^2 = H **68** R^1 = H, R^2 = p-Tn

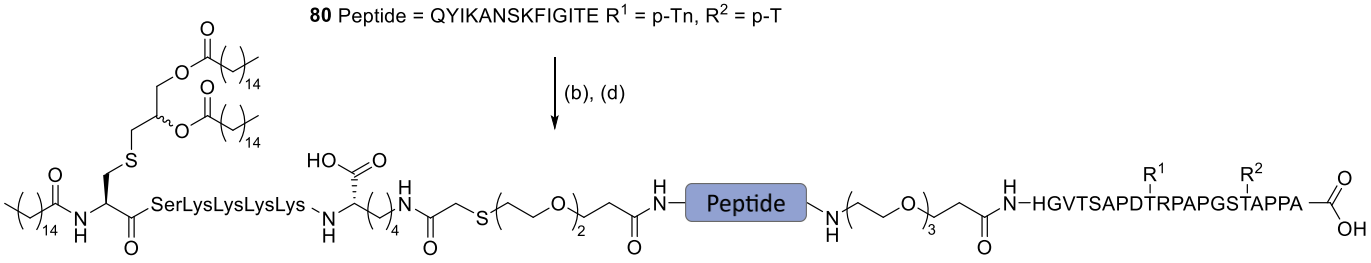
(a)
SPPS
H_2N—Ala—2-CTC
SPPS
(a)



75 Peptide = GQIGNDPNRDIL R^1 = p-Tn, R^2 = H
76 Peptide = GQIGNDPNRDIL R^1 = p-Tn, R^2 = p-Tn
77 Peptide = GQIGNDPNRDIL R^1 = p-Tn, R^2 = p-T
78 Peptide = QYIKANSKFIGITE R^1 = p-Tn, R^2 = H
79 Peptide = QYIKANSKFIGITE R^1 = p-Tn, R^2 = p-Tn
80 Peptide = QYIKANSKFIGITE R^1 = p-Tn, R^2 = p-T

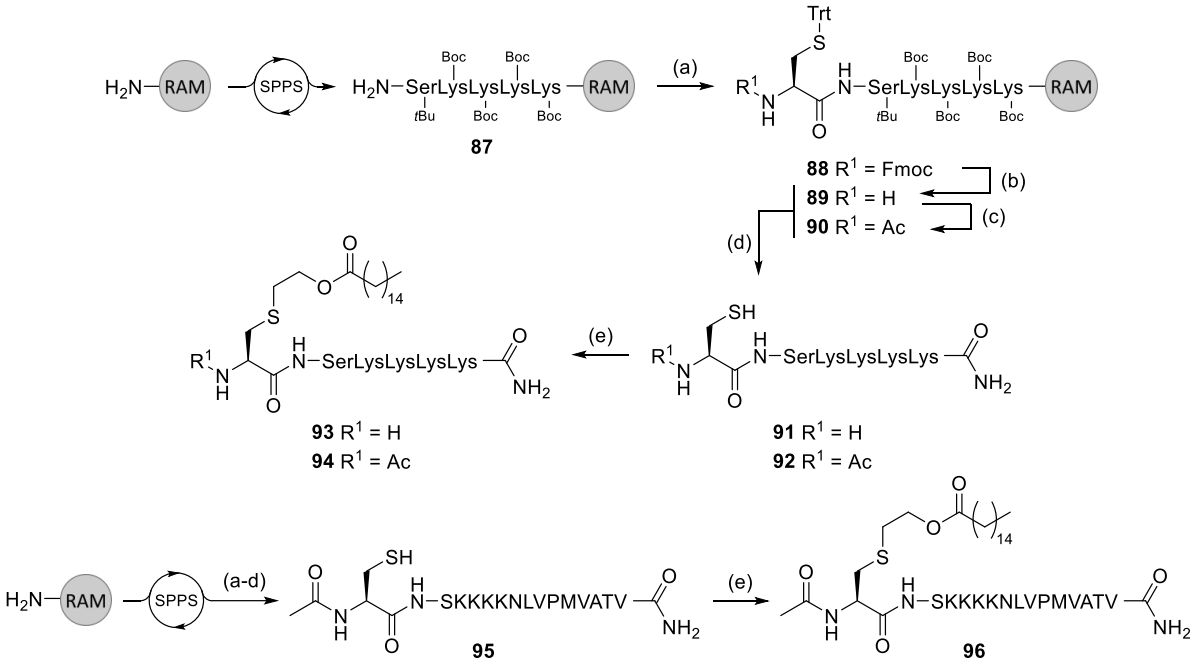


(b), (d)



81 Peptide = GQIGNDPNRDIL R^1 = Tn, R^2 = H
82 Peptide = GQIGNDPNRDIL R^1 = Tn, R^2 = Tn
83 Peptide = GQIGNDPNRDIL R^1 = Tn, R^2 = T
84 Peptide = QYIKANSKFIGITE R^1 = Tn, R^2 = H
85 Peptide = QYIKANSKFIGITE R^1 = Tn, R^2 = Tn
86 Peptide = QYIKANSKFIGITE R^1 = Tn, R^2 = T

Scheme 6: Synthesis of two component vaccine **69-74** containing Pam_3CysSK_4 and a glycosylated MUC1 peptide; three component vaccine **81-83** containing Pam_3CysSK_4, a P4 tetanus toxoid and a glycosylated MUC1 peptide; and three component vaccine **84-86** containing Pam_3CysSK_4, a P2 tetanus toxoid and a glycosylated MUC1 peptide.; **(a)** 88:6:6 TFA:TIS:H_2O; **(b)** NaOMe:MeOH, pH 10.0 for compound **81-83**; **(c)** NaOH:H_2O, pH 11.4, for compound **84-86**; **(d)** Compound **62a**, TEA, DMF, 40 °C, 16 h.



Scheme 7: Direct lipidation of a cleaved peptide resulting in a PamCSK$_4$ self-adjuvanting vaccine candidate by *Wright et al.* Reagents and conditions. **SPPS**: deprotection cycle) 20% piperidine in DMF, 20 min; coupling cycle) Fmoc-AA-OH, HBTU, NMM, DMF, 1 h; capping cycle) Ac_2O, D*i*PEA, DMF, 20 min; **(a)** Fmoc-Cys(Trt)-OH, BOP, HOBt·H_2O, D*i*PEA, 1:1 DCM:DMF, 1 h; **(b)** 20% piperidine in DMF, 20 min; **(c)** Ac_2O; **(d)** 94:2.5:2.5:1 TFA:H_2O:DODT:TIS, 2 h; **(e)** vinyl palmitate, DMPA, DTT, DMSO, 365 nm, hv, 15 min.

7 tumor cells. Interestingly, these sera could not only bind MCF-7 cells but could induce the killing of the bound tumor cells by activation of complement-dependent cytotoxicity.

The discovery of mono-acylated lipopeptides as novel human specific TLR2 ligands[20] was implemented in the development of an alternative method for manufacture TLR2 peptide conjugates by *Wright et al.*[70] Instead of a modular approach where the peptide and ligand are synthesized separately on solid phase and connected in the final stage of the synthesis, *Wright et al.* assembled the whole conjugate on solid phase starting from an immobilized peptide sequence that was extended at the N-terminus with a cysteine followed by lipidation of the cysteine side chain through a thiol-alkene ligation. This method offers an interesting alternative for difficult to synthesize peptides and circumvents the construction of intricate building blocks but requires more complicated protection strategies when dealing with sequences containing multiple cysteines. For validation, the thiol-alkene ligation was first performed by either thermal- or photoinitiation. Where thermal initiation resulted in a complex mixture of products, photoinitiation by stirring a solution of Fmoc-Cys-OH, vinyl palmitate and DMPA in DCM for 1 h while irradiated by UV light yielded 44% product. Next, peptides **91** and **92** (Scheme 7) were targeted to investigate the application of the thio-alkene ligation on more intricate structures.

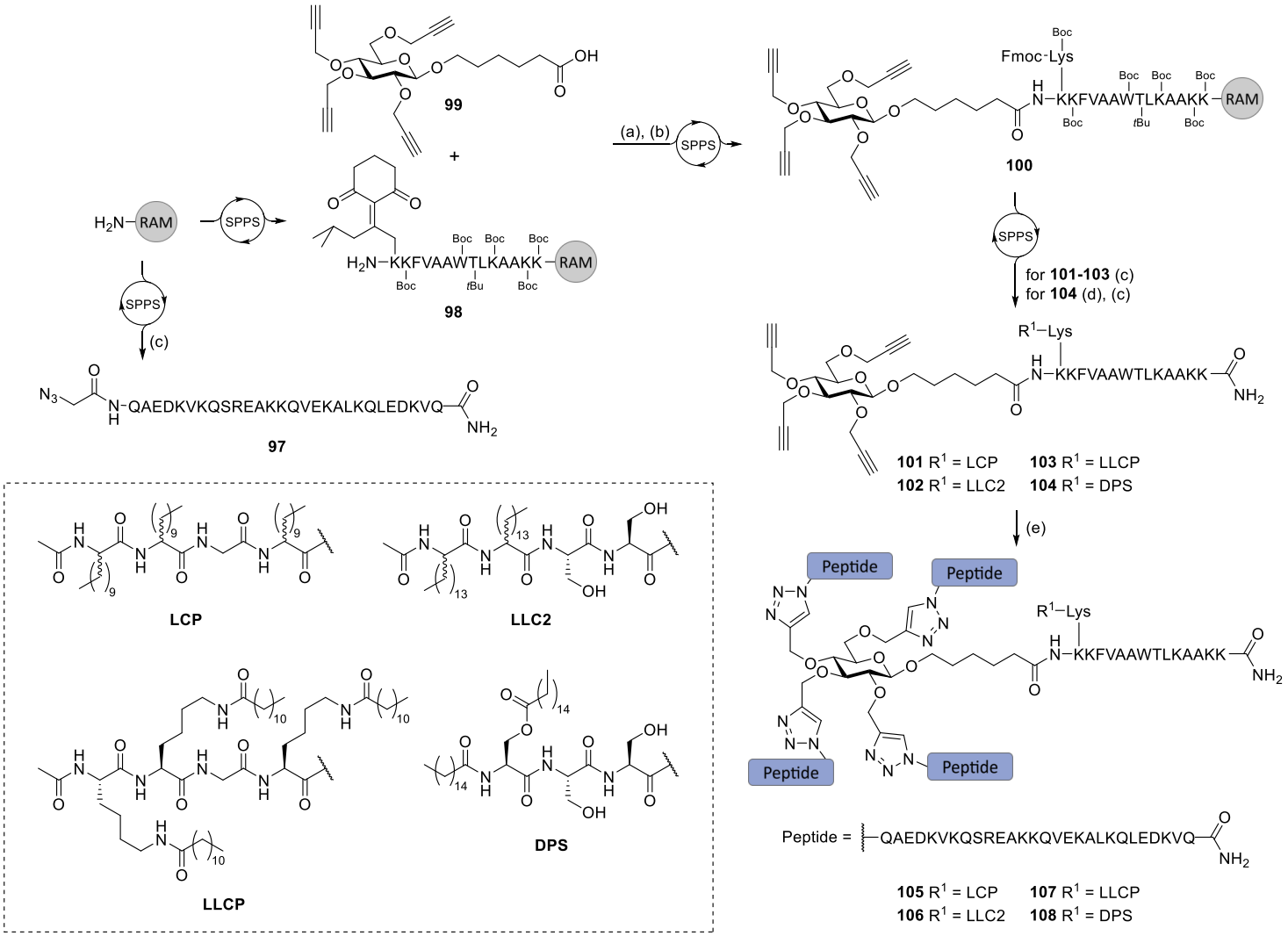
The precursors were obtained using Fmoc based peptide chemistry on a Tribute synthesizer with RAM resin as solid-state and the following three-step cycle: (i) treatment with 20% piperidine in DMF for 2 x 7 min to deprotect the Fmoc group; (ii) double coupling of the

protected amino acids by pre-activation of Fmoc-AA-OH (5 eq.) in DMF with HBTU (5 eq.) and NMM (20 eq.), followed by coupling for 1 h at RT; (iii) masking the remaining free amino functions by treatment with a solution of Ac_2O with D*i*PEA in DMF. SPPS gave immobilized pentapeptide **87**, which was followed by manually condensing the free amine in **87** with Fmoc-Cys(Trt)-OH, BOP, and HOBt as coupling reagents and D*i*PEA as base. Treating the resulting resin **88** with 20% piperidine in DMF liberated the amine to give **89**, that was converted using two procedures into target **93** or **94**. Direct cleavage of **89** with a mixture of 1:2.5:2.5:2.5 TIS:DODT:H_2O:TFA yielded **91**, while first acetylation and subsequent cleavage of **90** gave N-acetylated **92**. Again, thermal initiation was unsuccessful, but product formation was confirmed by MS for both compounds with photoinitiation. After optimization, a solution of vinyl palmitate with 2,2-dimethoxy-2-phenylacetophenone (DMPA) as photo-initiator and DTT as additive to prevent vinyl palmitate telomerization and mixed disulfides formation were founded to be the best conditions for the ligation (with **92** as substrate). Finally, CMV epitope containing peptide **95** was synthesized using the previous described conditions and successfully lipidated with the optimized reaction conditions to give after purification by HPLC, self-adjuvanting peptide **96**.

The activity of **93**, **94**, and **96** was investigated by following the upregulation of CD80 of monocytes isolated from fresh blood samples. The monocytes were identified by using specific cell surface markers, after which the CD80 expression on these cells was determined before and after exposing the blood samples to one of the three mono-acylated lipopeptides or Pam_3CSK_4, which was used as a reference. All lipopeptides displayed a similar upregulation of CD80 compared to the reference, with compound **94** showing the lowest potency. The introduction of the antigenic peptide in lipopeptide **96** did not seem to affect the agonistic potency of the TLR2 ligand.

The self-adjuvanting vaccines (**105**-**108**, Scheme 8), developed by *Fagan et al.*[72] feature four copies of group A streptococcal B cell epitope J8 (sequence; QAEDKVKQSREAKKQVEKALKQLEDKVQ)[73] that are linked to a tetramethyl triazolyl glucose scaffold originating from the Huisgen copper-catalyzed azide-alkyne cycloaddition (CuAAC). The construct further includes a single PADRE epitope, a universal peptide that activates antigen specific-$CD4^+$ T cells (sequence; KKFVAAWTLKAA) attached to anomeric centre of the glucose scaffold via a 6-oxyhexanoyl spacer. All vaccine candidates contain a single copy of a TLR2 ligand that is attached to the extra lysine branching in the PADRE sequence similar to *Defoort et al.*[58] and *Zeng et al.*[60] Except for the CuAAC ligation and the synthesis of the glucose scaffold **99**, the synthesis of the target synthetic vaccines was done using solid phase procedures.

With the aid of a Discover Bio System manual peptide synthesizer, Fmoc chemistry and RAM resin, the SPPS of the PADRE epitope, extended with a N-terminal lysine orthogonally protected with ivDde group for later installation of a TLR2 ligand, provided immobilized peptide **98** (Scheme 8). The following three-step cycle was used for the elongation of the peptide carrier: (i) treatment 20% piperidine in DMF at 70 °C once for 2 min and once for 5



Scheme 8: Assembly of four different group A streptococcal glycolipopeptides containing the J8 and the PADRE epitopes by *Fagan et Al.* Reagents and conditions: **SPPS**: deprotection cycle) 20% piperidine in DMF, 70 °C , 1x 2 min, 1x 5 min; coupling cycle) Fmoc-AA-OH, HATU, D*i*PEA, DMF, 70 °C, 2x 10 min; capping cycle) 5% Ac_2O, 5% D*i*PEA, DMF, 70 °C, 10 min (only after first coupling cycle)[71] **(a)** HBTU, D*i*PEA, DMF, 70 °C, 5 min; **(b)** 2% hydrazine in DMF, 4 h; **(c)** 95:2.5:2.5 TFA:H_2O:TIS, 3 h **(d)** Palmitic acid, DMAP, DIC, overnight; **(e)** Copper wire, DMF, 50 °C, 1-2 h.

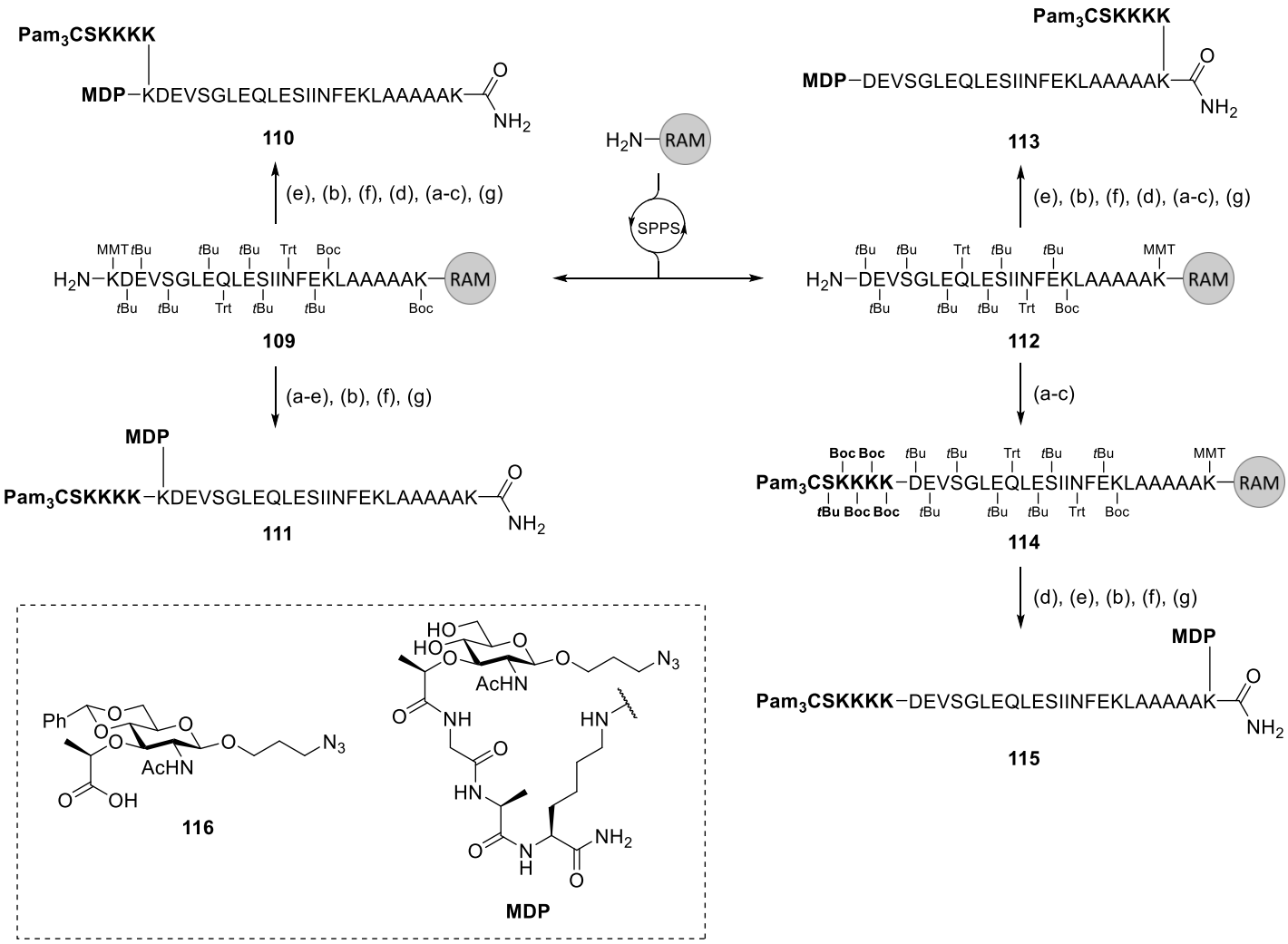
min to deprotect the Fmoc group; (ii) coupling of the protected amino acids by treating the resin twice with preactivated Fmoc-AA-OH (4.2 eq.) in DMF with HATU (4 eq.) and D*i*PEA (6.2 eq.) for 5 min at 70 °C; (iii) masking the remaining free amino functions by treatment with a 2.5% Ac_2O and 2.5% D*i*PEA in DMF at 70 °C for 2 x 2 min. The carboxylic acid in the anomeric spacer of glucose scaffold **99** was condensed with the terminal amine in immobilized peptide **98** using HBTU as an activator, ensued by the selective removal of the ivDde group using 2% hydrazine in DMF and coupling of the released amine with Fmoc-Lys(Boc)-OH according to the elongation protocol giving peptide carrier **100**. From here, four different TLR2 ligands (LCP, LLC2, LLCP an DPS) were installed by SPPS, where compound **104** demanded an extra SPPS cycle to install the N-terminal palmitoyl tail, after which the lipidated peptides could be deprotected and released from the resin by standard SPPS cleavage yielding the propargylated precursors **101**-**104**. Azido-acetyl **97** was prepared by SPPS and purified by HPLC in advance of

the CuAAC ligation. The cycloaddition was performed in accordance with the publication of *Urbani et al.*[74] yielding pure three-component vaccines **105**-**108** were isolated by preparative HPLC.

To determine the activity of compounds **105-108**, groups of five C57BL/6 mice were immunized with one of four three-component vaccines. The mice received two boosts at day 21 and day 28 and samples were collected at day 39 after the primary immunization. Isolated sera were analyzed for J8-specific IgG antibodies by ELISA. Vaccines **106-108** induced strong antibody production while vaccines **105** elicited a 100-fold weaker reaction.

The first synthetic peptide conjugate in which two different PAMPS are incorporated was published by *Zom et al.* (Scheme 9).[43] Their self-adjuvanting vaccines consist out of Pam_3CSK_4 and MDP as TLR2 and NOD ligand, respectively, with $DEVA_5K$ (DEVSGLEQLESIINFEKLAAAAAK) as a model antigen peptide. To determine the optimal position of each ligand at the termini of the peptide epitope, *Zom et al.* designed two types of vaccines of which the first one bears both ligands on the C-terminal end of the peptide (**110** and **111**) while, in the second type one ligand is positioned at the C-terminal and the other is at the N-terminal end (**113** and **115**). SPPS with incorporation of an additional lysine provided with an orthogonal MMT protected side chain at a predetermined position in the peptide chain allowed installing one of the selected ligands.

Immobilized peptide **109** and **112** (Scheme 9), the respective precursors of both types of dual conjugates were assembled using a Applied Biosystems ABI 433A, Tentagel RAM resin, Fmoc-chemistry and the following three-step elongation cycle : (i) treatment with 20% piperidine in NMP for 15 min to deprotect the Fmoc group; (ii) coupling of the protected amino acids by treating the resin with a solution of Fmoc-AA-OH (5 eq.) in NMP with HCTU (5 eq.) and D*i*PEA (10 eq.), for 30 min at RT; (iii) masking the remaining free amino functions by treatment with a 0.5 M solution of Ac_2O with D*i*PEA (10 eq.) in NMP. With immobilized peptide **109** in hand, installation of both ligands could commence. Synthesis of dual conjugates **111** and **113** begins by elongating the immobilized peptide with SK_4 using the previously described SPPS conditions, after which the final Fmoc group was removed with 20% piperidine in NMP. The liberated amine was condensed with Pam_3Cys-OH under the influence of PyBOP as coupling reagent and D*i*PEA as base, followed by the selective cleavage of the MMT protecting group could be by treating the resin with 3% TFA in DCM (exposure time was not mentioned). The liberated side chain was subsequently elongated with Fmoc-Ala-*i*Glu(NH_2)-OH using a single SPPS cycle followed by one more deprotection cycle. Finally, the freed amine was manually reacted with prepared building block **116** in the presence of HATU and D*i*PEA in NMP. Dual conjugates **113** and **115** were synthesized in a similar fashion however by inversing the reaction steps taken before and after the MMT removal gave immobilized precursors **112** and **114**. All deprotections and cleavages from the respective resins were attained using a mixture of 95:2.5:2.5 TFA:H_2O:TIS, precipitation in Et_2O, and purification of the crude products by RP-HPLC yielding dual conjugates **111-114**.



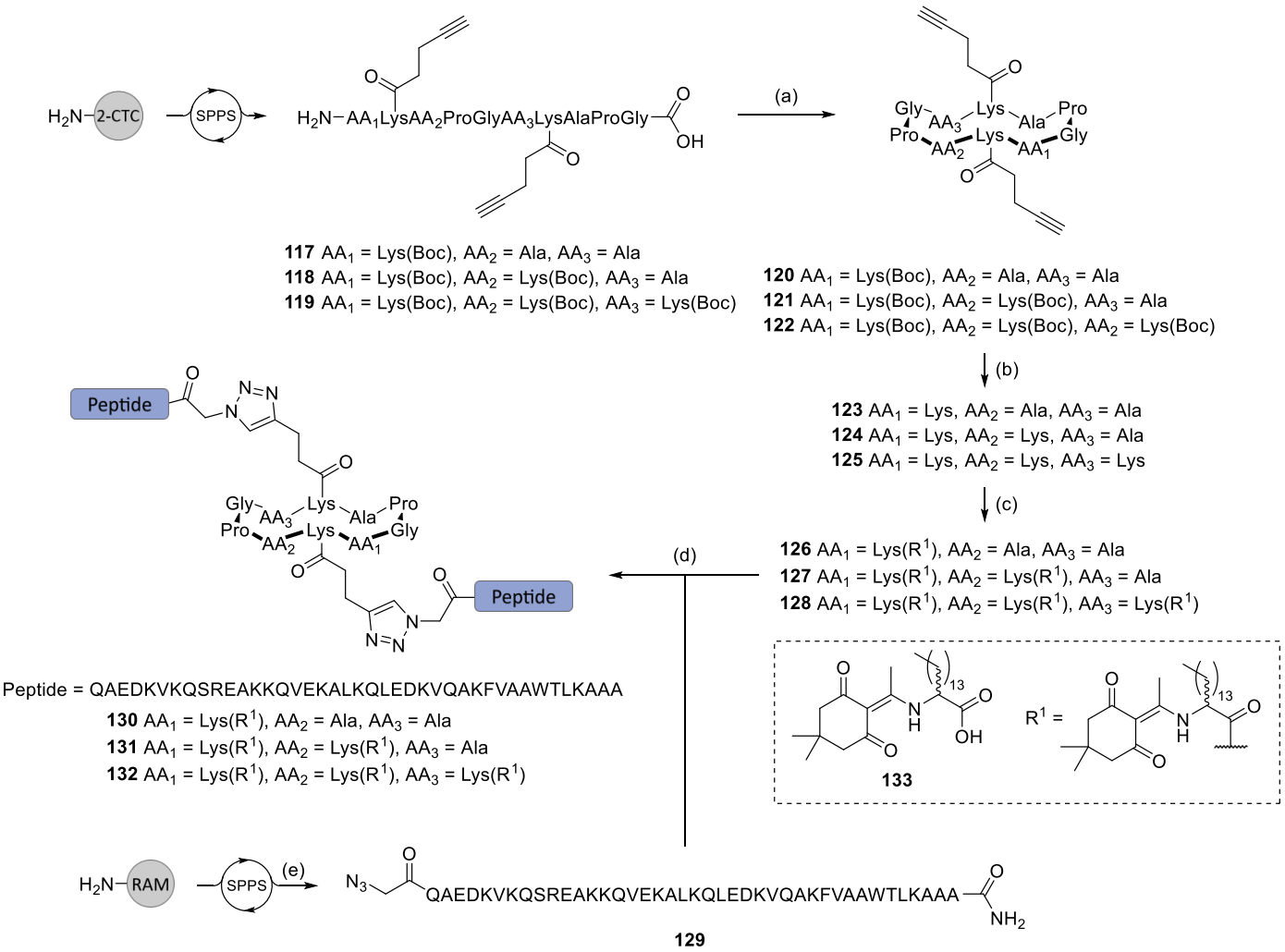
Scheme 9: Synthesis of bis-functionalized SIINFEKL containing peptides by *Zom et al.* Reagents and conditions: **SPPS**: deprotection cycle) 20% piperidine in NMP, 15 min; coupling cycle) Fmoc-AA-OH, HCTU, DiPEA, NMP, 30 min; capping cycle) Ac_2O, D*i*PEA, NMP; **(a)** Fmoc SPPS cycles for SK_4; **(b)** 20% piperidine in NMP, 15 min; **(c)** Pam_3Cys-OH, PyBOP, D*i*PEA; **(d)** 3% TFA in DCM; **(e)** Fmoc SPPS cycle with Fmoc-Ala-*i*Glu(NH_2)-OH; **(f)** Compound **116**, HATU, D*i*PEA, NMP; **(g)** 95:2.5:2.5 TFA:H_2O:TIS.

The bioactivity of the dual conjugates was determined by assessing the ability to activate the DCs and T-cells through a series of experiments. First, it was demonstrated that all conjugates retain their capacity to activate both TLR2 and NOD by following IL-12p40 production on DCs, IL-8 production on either TLR2 or hNOD2 transfected HEK293 cells, and B3Z activation (able to recognize DCs loaded with SIINFEKL) by murine DCs treated with the dual conjugates. All these experiments indicate a superior response to conjugate **113**, which was subsequently used for all follow up experiments. An increase in cytokine and chemokine production was observed when comparing dual conjugate **113** with its respective mono-functionalized conjugates when examining CCL4, TNF-α, MCP-1, IL-6, IL-8, and IFN-γ production in moDCs. Finally, it was shown that murine DCs incubated with **113** were able to activate OT1 specific T cells measured by the accumulation frequency of TNF-α, IL-2, and IFN-γ inside the T-cells and the concentration of IL-2, and IFN-γ in the supernatant.

To improve pharmacokinetic properties and enhance the presentation of antigens, *Madge et al.*[45] designed a cyclic peptide carrier linked to two copies of a synthetic long peptide (SLP) embedded with B cell epitope J8 (QAEDKVKQSREAKKQVEKALKQLEDKVQ) and universal T helper epitope PADRE (AKFVAAWTLKAA) and to one, two or three lipids functioning as TLR2 ligand (Scheme 10). The cyclic peptide features two glycine-proline sub-units, which push the sequence into a β-sheet-like configuration, two lysines pre-functionalized with alkyne, and one, two or three Boc-protected lysines for post-resin modification. All leftover positions in the ten amino acid long sequence were filled in with alanine residues. The Boc-protected lysines could be used to install the lipids after the cyclic carrier was cleaved from the resin. The synthesis ended with the additions of the alkynes in peptide carriers **126**-**128** to the azide acetyl functionalized J8-PADRE SLPs (**129**) using a CuAAC, giving self-adjuvanting vaccines **130-132**.

Peptides **117**-**119** were prepared using Fmoc-chemistry on a CEM Discovery microwave synthesizer and 2-chlorotrityl chloride (2-CTC) as solid support (Scheme 10). The following two-step cycle elongation protocol was used to construct the peptides: (i) treatment with 20% piperidine in DMF for 2 x 10 min at RT to deprotect the Fmoc group; (ii) coupling of the protected amino acids by threating the resin with a solution of Fmoc-AA-OH (4.2 eq.) in DMF with HATU (4 eq.) and D*i*PEA (5 eq.), for 30 min at RT. The immobilized peptides were directly deprotected and cleaved by exposing the resin to a mixture of 1:1:4 AcOH:TFE:DCM for 30 min, yielding liberated peptides **117-119**. The crude products were deemed sufficiently pure to continue with the cyclization without further purification. The peptide was cyclized through condensation of the C- and N-terminus with HATU as a coupling reagent and D*i*PEA as a base giving cyclic peptide carriers **120-122**. Removal of the Boc-protecting groups with a mixture of 95:5 TFA:H_2O liberated the side-chain amines of the lysine residues and subsequently coupled with pre-prepared lipidated building block **133** to provide lipidated cyclic peptides **126-128.** Parallel to **117**-**119**, peptide **129** was synthesized using the same synthesizer following an alternative two step protocol: (i) treatment with 20% piperidine in DMF for 2x 5 min at 70 °C to deprotect the Fmoc group; (ii) coupling of the protected amino acids by threating the resin with a solution of Fmoc-AA-OH (4.2 eq.) in DMF with HATU (4 eq.) and D*i*PEA (5 eq.), for 2x 5 min at 70 °C. The immobilized peptide was deprotected and release using standard deprotection conditions, after which the crude product was purified by RP-HPLC, yielding peptide **129**. Finally, lipidated cyclic peptide carriers **126**-**128** were linked via a CuAAC with azido peptide **129** in a mixture of methanol and pentanol at 36 °C. Purification by RP-HPLC yielded self-adjuvanting vaccines **130**-**132.**

The capacity of the conjugates to induce a functional immune response was assessed by immunizing five C57BL/6 mice with one of the three candidates. Besides the primary immunization, three boosts were given 20, 27 and 34 days after the first immunization. 41 days after the start of the experiment sera of the mice was collected and analyzed for the presence of J8 specific antibodies. All cyclic conjugates elicited significant antibody production, while the degree of lipidation did not seem to affect antibody production at all.



Scheme 10: Synthesis of lipidated cyclic peptides, which were covalently linked to two peptides containing the T helper Pan DR epitope (PADRE), and a known B cell GAS epitope (J8) using the copper-catalyzed alkyne-azide cycloaddition by *Madge et al.* Reagents and conditions: **SPPS:** deprotection cycle) 20% piperidine in DMF, 70 °C , 1x 2 min, 1x 5 min; coupling cycle) Fmoc-AA-OH, HATU, DiPEA, DMF, 70 °C, 2x 10 min; capping cycle) 5% Ac_2O, 5% DiPEA, DMF, 70 °C, 10 min (only after first coupling cycle)[71]; **(a)** HATU, DiPEA, DMF, 4 h; **(b)** 95:5 TFA:H_2O, 3 h; **(c)** Compound **133**, HATU, DiPEA, DMF; **(d)** $CuSO_4$, NaAsc, MeOH, Pentanol, 36 °C **(e)** 95:2.5:2.5 TFA:TIS:H_2O, 3 h.

Summarizing, this introductory chapter has concisely discussed the current approaches to obtain new and improved conjugate vaccines, namely the development of a single molecule that is obtained by organic synthesis and featuring all properties to induce the desired immune responses, including T-cell activation. Such self-adjuvanting vaccine modalities comprise one or more copies of an oligopeptide antigen containing relevant epitopes. Because single oligopeptides are generally non-immunogenic, these SLPs are covalently linked to an adjuvant, a synthetically accessible, well-defined, and relatively small molecule mimicking a PAMP, able to activate the innate immune system through its binding to a PRR. SAR studies of ligands of TLR receptors, which are prominent members of PRR family, have led to the discovery of synthetically accessible agonists of TLR2,[20, 75-76] TLR4,[77-78] TLR7 and TLR8.[79-81] Several of these ligands that mimic different PAMP's have been applied for the design, synthesis, and evaluation of vaccine modalities, in which peptide antigens and TLR

agonists are incorporated. In this chapter, selected examples of these constructs are discussed in terms of preparation and immunological properties. Most promising agonists are ligands of TLR2 and the research described in this thesis is centred around this type of ligands. **Chapter 2** describes the assembly of a set of nineteen conjugates, in which antigen-containing synthetic peptides are covalently linked to UPam. UPam is an artificial ligand to TLR 2 and a close isostere of Pam_3CysSK_4, in which the N-palmitoyl group of the native Pam_3Cys moiety is replaced with tetradecylurea. **Chapter 3** describes the synthesis of a small library of sixteen conjugates, consisting of chiral pure derivatives of the UPam agonist, which were used to test the paradigms concerning the design of lipidated cysteines as TLR2 agonists. **Chapter 4** describes the design, synthesis, and evaluation of human neoantigen containing peptides functionalized with mini-Upam, a novel, less lipophilic derivative of UPam. **Chapter 5** describes the synthesis of TLR2 and TLR7 functionalized synthetic peptide conjugates which are embedded with a model murine antigen to test the possible synergistic actions of TLR2 and TLR7 when simultaneously activate by the same construct.

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