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

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

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List of abbreviations

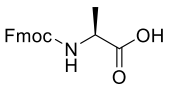
°C	degrees Celsius
2-CTC	2-chlorotrityl chloride
Å	Ångstrom
AA	amino acid
Ac	acetyl
APC	antigen presenting cell
Aq.	aqueous
BMDC	bone marrow-derived dendritic cell
Boc	*tert*-butyloxycarbonyl
BOP	Benzotriazole-1-yl-oxy-tris-(dimethylamino)-phosphonium hexafluorophosphate
Bz	benzyl
CD	cluster of differentiation
CLR	C-type lectin receptor
CMV	cytomegalovirus
conc.	concentrated
CTL	cytotoxic T lymphocyte
CuAAC	copper-catalyzed azide-alkyne cycloaddition
D1-DC	D1 dendritic cells
DAB	diamino butyric acid
DAMP	damage associated molecular pattern
DBU	1,8-diazabicyclo(5.4.0)undec-7-ene
DC	dendritic cell
DCM	dichloromethane
Dde	*N*-(1-(4,4-dimethyl-2,6-dioxocyclohexylidene)ethyl)
DIC	*N*,*N*-diisopropylcarbodiimide
D*i*PEA	*N*,*N*-diisopropylethylamine
DMAP	*N,N*-dimethylpyridin-4-amine
DMF	*N*,*N*-diemethylformamide
DMPA	dimethylol propionic acid
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DODT	2,2'-(ethylenedioxy)diethanethiol
DTT	dithiothreitol
EDC·HCl	*N*-ethyl-*N'*-(3-dimethylaminopropyl)carbodiimide hydrochloric acid
EDT	ethane-1,2-dithiol
ELISA	enzyme-linked immunosorbent assay
ESI	electrospray ionization
Et	ethyl
eq.	equivalent
FACS	fluorescence-activated cell sorting
FMD	Foot-and-mouth
Fmoc	fluorenylmethyloxycarbonyl
GalNAc	2-deoxy-2-acetamido-D-galactose
GM-CSF	granulocyte-macrophage colony-stimulating factor
h	hour
HBTU	*N*,*N*,*N'*,*N'*-tetramethyl-*O*-(1H-benzotriazol-1-yl)uranium hexafluorophosphate
HCTU	*N*,*N*,*N'*,*N'*-tetramethyl-*O*-(6-chloro-1H-benzotriazol-1-yl)uranium hexafluorophosphate
HEK293	Human embryonic kidney 293
HIV	human immunodeficiency virus

HMPB	4-hydroxymethyl-3-methoxyphenoxybutyric acid
HOAt	1-hydroxy-7-azabenzotriazole
HOBt	hydroxybenzotriazole
HPV	Human papillomavirus
HRMS	high resolution mass spectrometry
hν	light
Hz	Hertz
IFNγ	interferon-γ
IgG	immunoglobulin G
IgM	immunoglobulin M
IL	interleukin
IMDM	Iscove's modified Dulbecco's medium
IRF	interferon regulatory factor
J8	GAS B cell antigen
LC-MS	liquid chromatography-mass spectrometry
LCP	lipid-core-peptide
LRR	leucine rich repeats
MALP	macrophage activating lipopeptide
MAPS	multiple antigen peptide system
MBHA	4-methylbenzhydrylamine
MCF	Michigan Cancer Foundation
MCP-1	monocyte chemoattractant protein-1
Me	methyl
MHC	major-histocompatibility complex
min	minute
mini-UPam	(14S,17R) -14-(hyrdoxymethyl)-13,16,23-trioxo-17-ureido-3,6,9,12,22-pentaoxa-19-thia-15-azaoctatriacontanoic acid
MMT	monomethoxytrityl
moDC	monocyte-derived dendritic cell
MPLA	mono-phosphoryl lipid A
MS	mass spectrometry
MUC1	mucin 1
MyD88	myeloid differentiation primary response 88
NF-κB	nuclear factor kappa-light-chain-enhancer of B cells
NLR	NOD-like receptor
NMM	*N*-methylmorpholine
NMP	*N*-methylpyrrolidone
NMR	nuclear magnetic resonance
NO	nitric oxide
NOD	nucleotide-binding and oligomerization domain
OSu	*N*-hydroxysuccinimide
OT-1	ovalbumin-specific $CD8^+$ T cells
PADRE	pan HLA DR-binding epitope
PAMP	pathogen associated molecular pattern
Pbf	2,2,4.6,7-pentamethyldihydrobenzofuran
PBS	phosphate-buffered saline
PHB	4-hydroxybenzylalcohol
PRR	pathogen recognition receptor
PyBOP	benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate
RAM	Rink amide
RNA	ribonucleic acid
RP-HPLC	reverse-phase high performance liquid chromatography
R_t	retention time

RT	room temperature
SAR	structure-activity relationship
sat.	saturated
SLP	synthetic long peptide
SP	synthetic peptide
SPPS	solid-phase peptide synthesis
*t*Bu	*tert*-butyl
TBTU	2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium tetrafluoroborate
TC	tissue-culture
TCR	T-cell receptor
TEA	triethylamine
TFA	trifluoroacetic acid
TFE	tetrafluoroethylene
THF	tetrahydrofuran
TIS	triisopropylsilane
TIR	toll/IL-1 receptor
TLC	thin-layer chromatography
TLR	toll-like receptor
TLR-L	toll-like receptor ligand
TNF-α	tumor necrosis factor alpha
TRIF	TIR—domain-containing adaptor-inducing interferon
Trt	trityl
UPam	1-tetradecyl-urea-Cys((RS)-2,3-di(palmitoyloxy)-propyl)-Ser-Lys-Lys-Lys-Lys
UPLC	ultra-high performance liquid chromatography

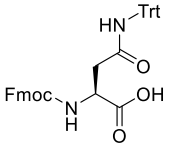
Overview of amino acid nomenclature, standard building blocks and resins

Alanine - Ala - A



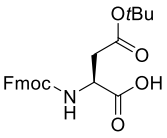
Fmoc-Ala-OH

Asparagine - Asn - N

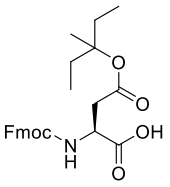


Fmoc-Asn(Trt)-OH

Aspartic acid - Asp - D

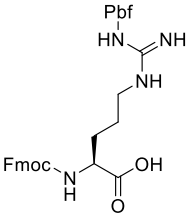


Fmoc-Asp(*t*Bu)-OH



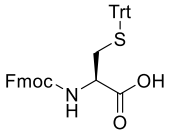
Fmoc-Asp(Mpe)-OH

Arganine - Arg - R



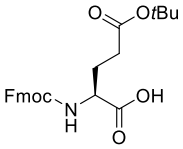
Fmoc-Arg(Pbf)-OH

Cysteine - Cys - C



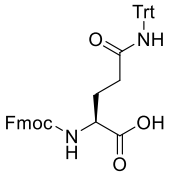
Fmoc-Cys(Trt)-OH

Glutamic acid - Glu - E



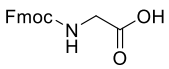
Fmoc-Glu(*t*Bu)-OH

Glutamine - Gln - Q



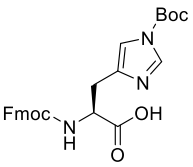
Fmoc-Gln(Trt)-OH

Glycine - Gly - G

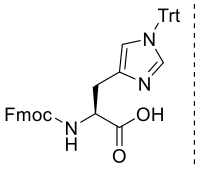


Fmoc-Gly-OH

Histidine - His - H

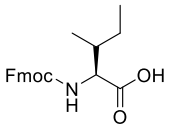


Fmoc-His(Boc)-OH



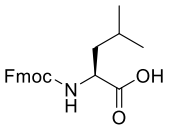
Fmoc-His(Trt)-OH

Isoleucine - Ile - I



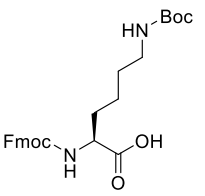
Fmoc-Ile-OH

Leucine - Leu - L



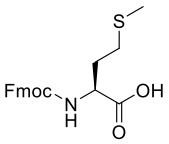
Fmoc-Leu-OH

Lysine - Lys - K



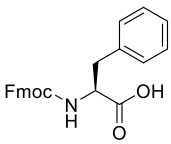
Fmoc-Lys(Boc)-OH

Methionine - Met - M



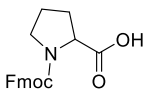
Fmoc-Met-OH

Phenylalanine - Phe - F



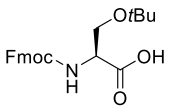
Fmoc-Phe-OH

Proline - Pro - P



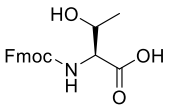
Fmoc-Pro-OH

Serine - Ser - S



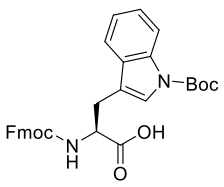
Fmoc-Ser(*t*Bu)-OH

Threonine - Thr - T



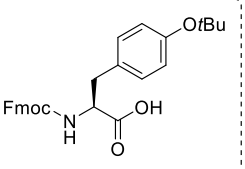
Fmoc-Thr(*t*Bu)-OH

Tryptophan - Trp - W



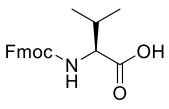
Fmoc-Trp(Boc)-OH

Tyrosine - Tyr - Y

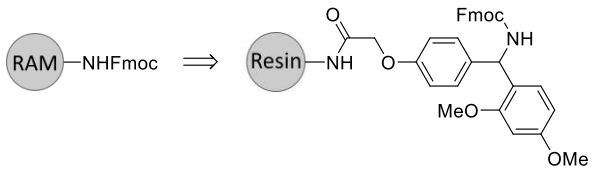


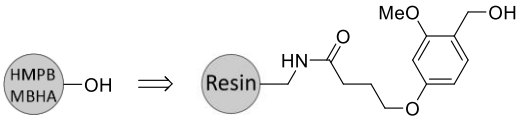
Fmoc-Tyr(*t*Bu)-OH

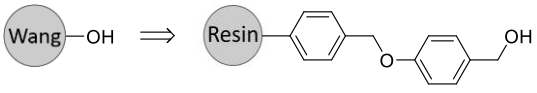
Valine - Val - V

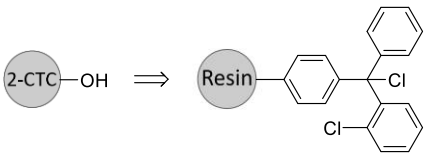


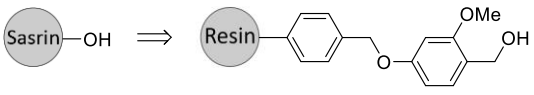
Fmoc-Val-OH











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.

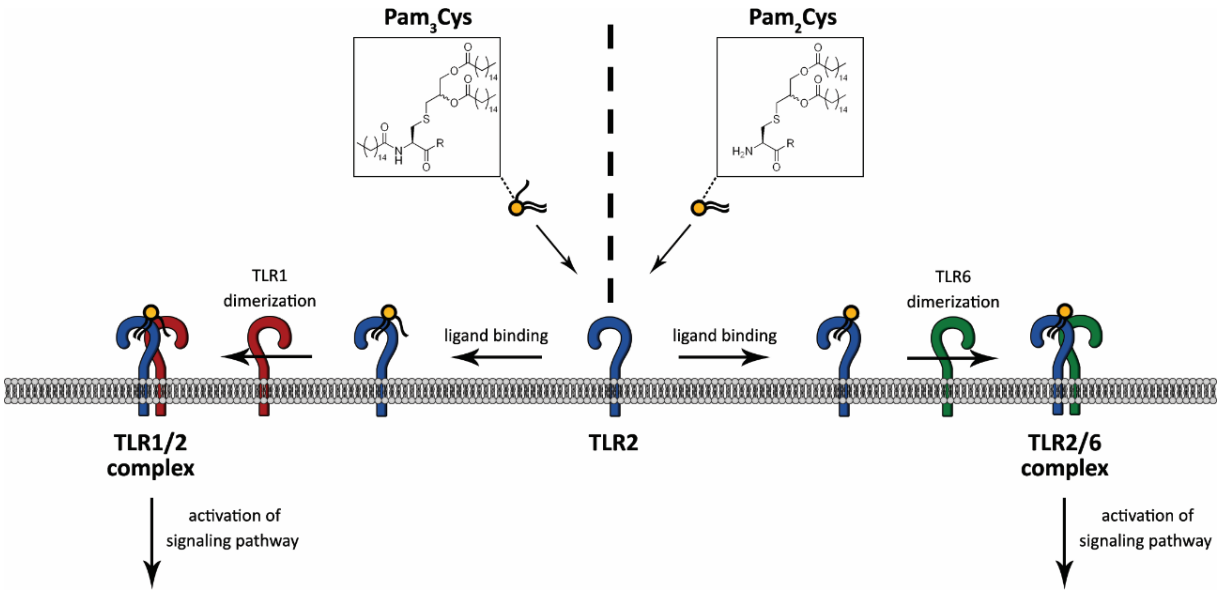


Figure 1: Schematic depiction of TLR1/TLR2 and TLR2/TLR6 dimerization upon association with either Pam_3Cys or Pam_2Cys, respectively.

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]

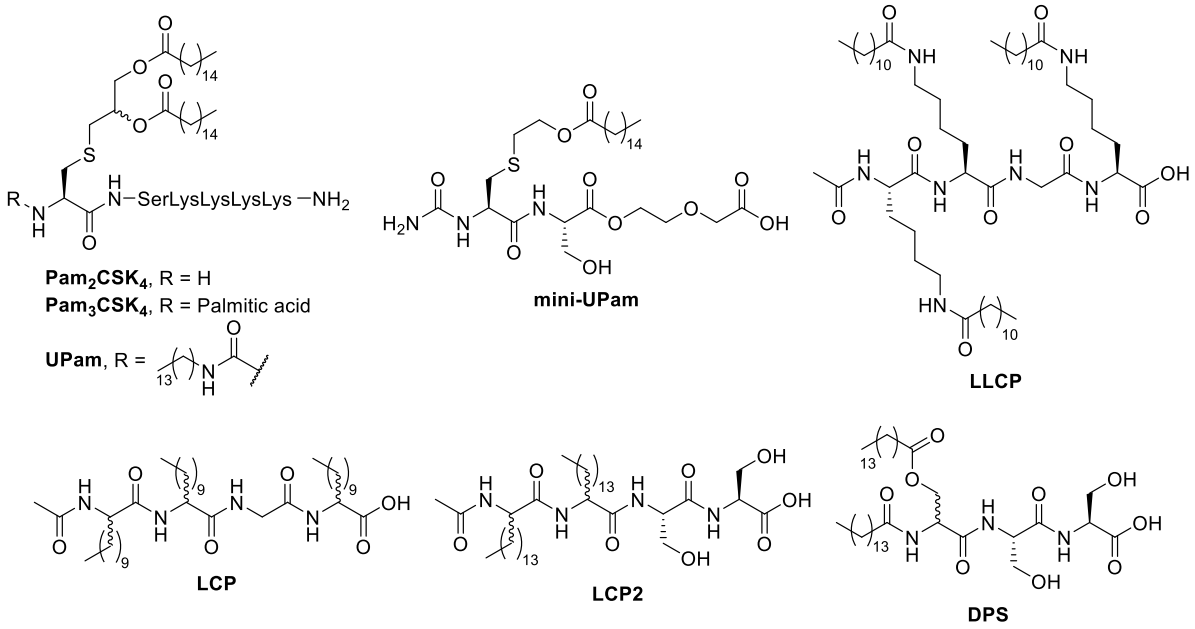


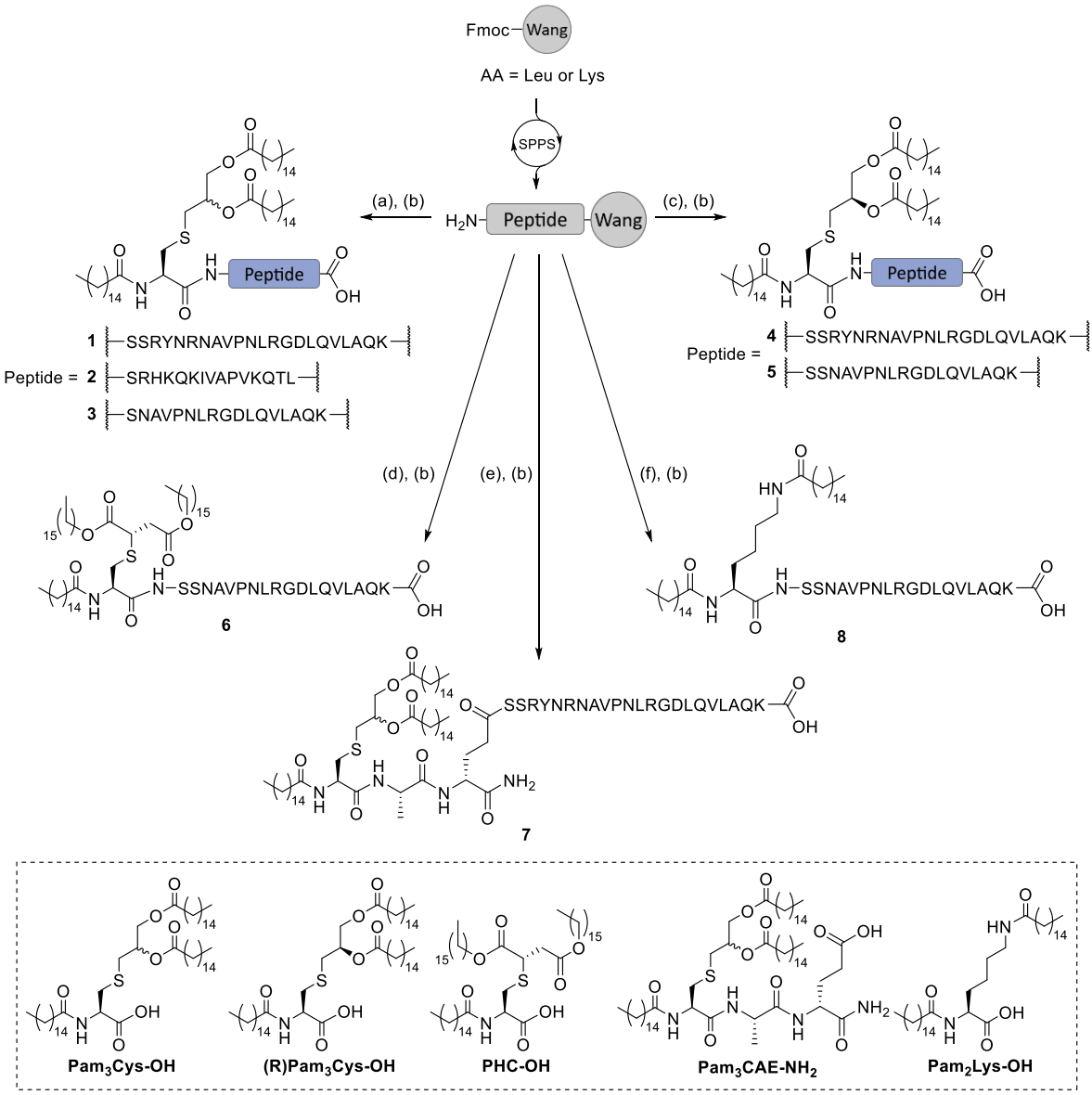
Figure 2: Structures of various lipopeptides determined to be TLR2 agonists and incorporated in future conjugates, namely Pam_2CSK_4, Pam_3CSK_4, LCP, LCP2, LLCP, and DPS.

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.

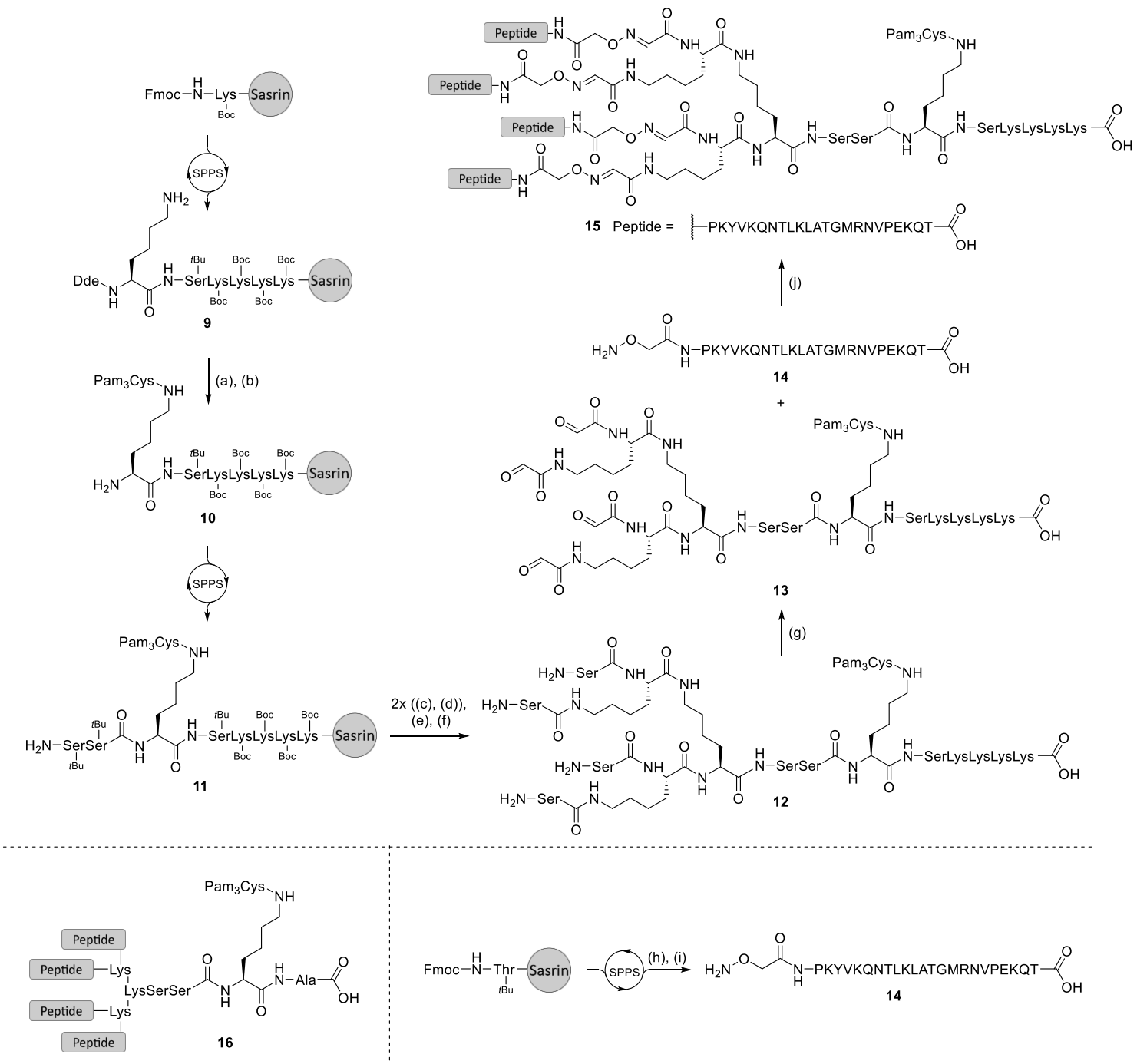


Scheme 1: Synthetic work published by Wiesmüller et al. Reagents and Conditions. **SPPS:** deprotection cycle) 55% piperidine in NMP, 1x 2 min, 1x 5 min; coupling cycle) Fmoc-AA-OH, DIC, HOBt, NMP, 2x 1.5 h; capping cycle) Ac_2O, DiPEA, NMP. **(a)** i) Pam_3Cys-OH, DIC, HOBt, 2:1 DCM:DMF, 4h; ii) NMM, 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.

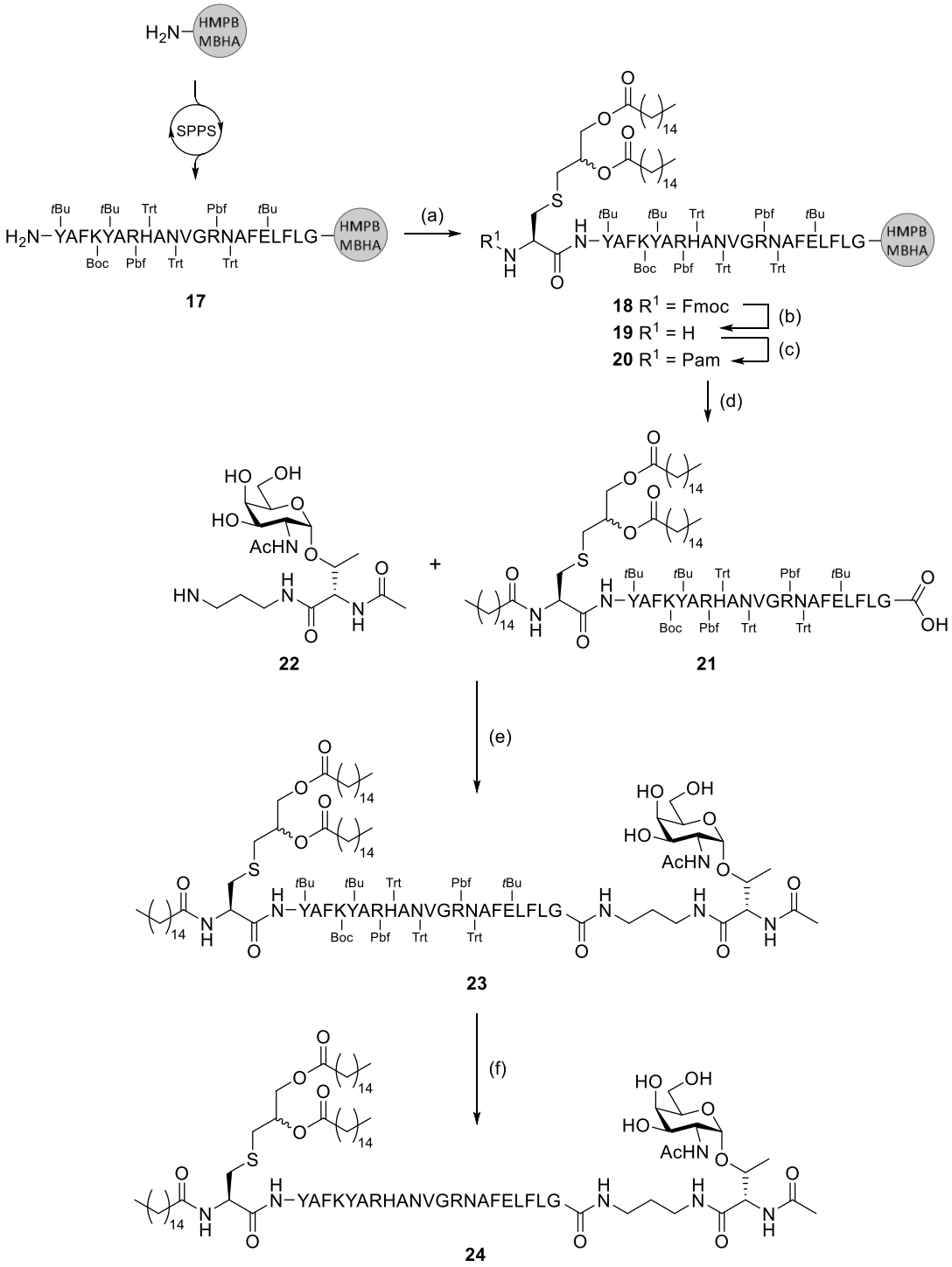
The synthesis of conjugate **15** is outlined in Scheme 2 and started with the manual SPPS of oligopeptide **13** utilizing Fmoc-based chemistry and Sasrin resin as solid support. The following two-step cycle was used for the elongation of the oligopeptide: (i) treatment with 50% piperidine in DMF for 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).



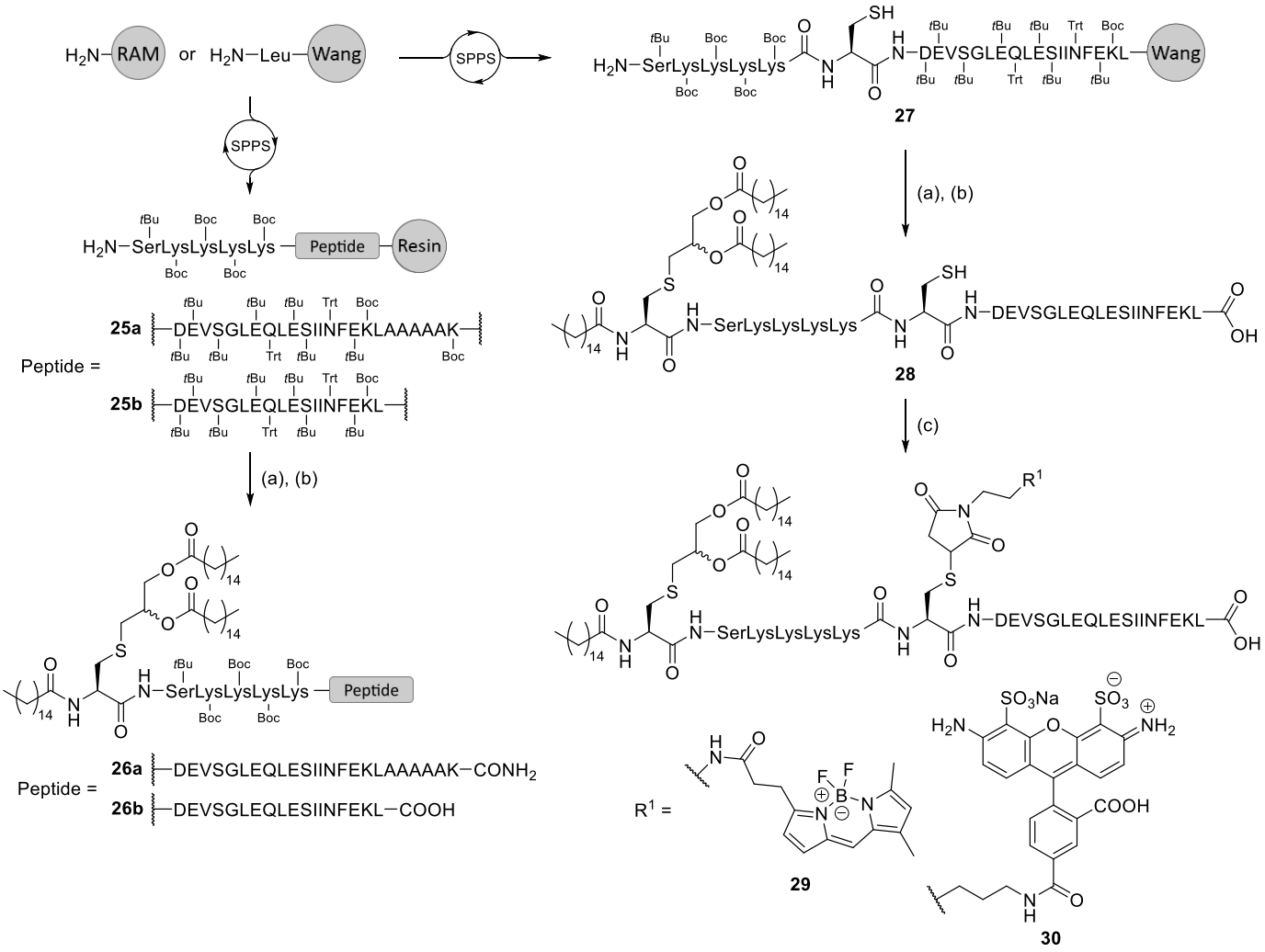
Scheme 3: Synthesis of Tn antigen carrying lipidated glycopeptide by *Buskas et al.* Reactions and conditions: **SPPS**: HBTU/HOBt as activator; **(a)** Pam_2Cys-OH, PyBOP, HOBt, DiPEA, 5:1 DMF:DCM; **(b)** 20% piperidine in DMF; **(c)** Palmitic acid, PyBOP, HOBt, 1:5 DMF:DCM; **(d)** 2% TFA in DCM;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

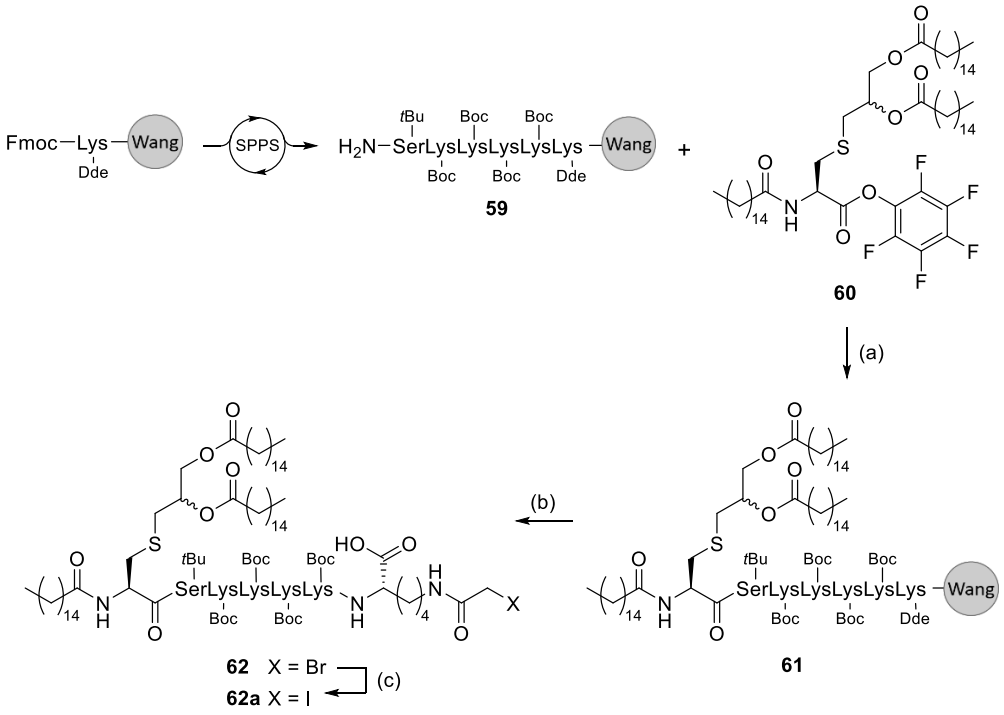
To elucidate the uptake mechanism and intracellular processing of fully synthetic TLR antigen conjugates, *Khan et al*.[36] developed conjugates in which both the ovalbumin model epitope SIINFEKL and a TLR ligand are incorporated (Scheme 4). An additional cysteine for an end-stage labelling was placed 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

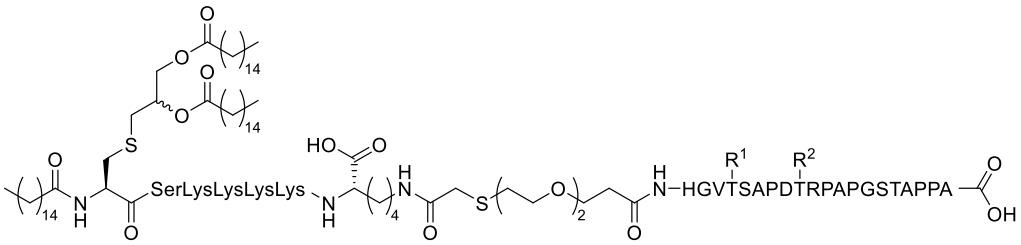
Besides the three-component vaccines of *Wilkinson et al.*[67] and *Lakshminarayanan et al.*[68], *Cai et al.*[69] developed another type of three-component antitumor vaccine that initiates a potent immune response (Scheme 6, page 10). In their design, they linked MUC1 (a glycosylated B-cell epitope) 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.

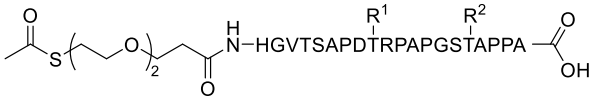
Both the two- and three-component peptide sequences were assembled employing Cl-Trt resin, using similar SPPS conditions as described for lipopeptide **3** (Scheme 1). However, the Tn-antigen carrying building blocks were installed following a slightly different coupling protocol with HATU and HOAt as 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-



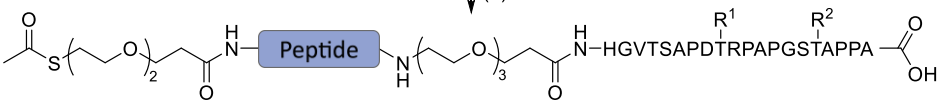
69 R^1 = Tn, R^2 = H **72** R^1 = H, R^2 = Tn
70 R^1 = T, R^2 = H **73** R^1 = H, R^2 = T
71 R^1 = Tn, R^2 = H **74** R^1 = H, R^2 = Tn

(b) or (c), (d)

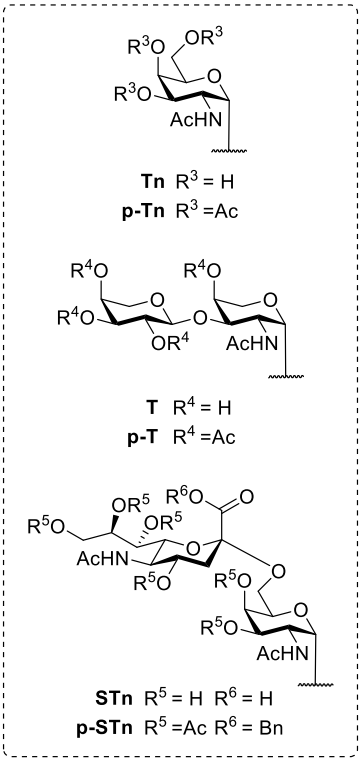


63 R^1 = p-Tn, R^2 = H **66** R^1 = H, R^2 = p-Tn
64 R^1 = p-T, R^2 = H **67** R^1 = H, R^2 = p-T
65 R^1 = p-Tn, R^2 = H **68** R^1 = H, R^2 = p-Tn

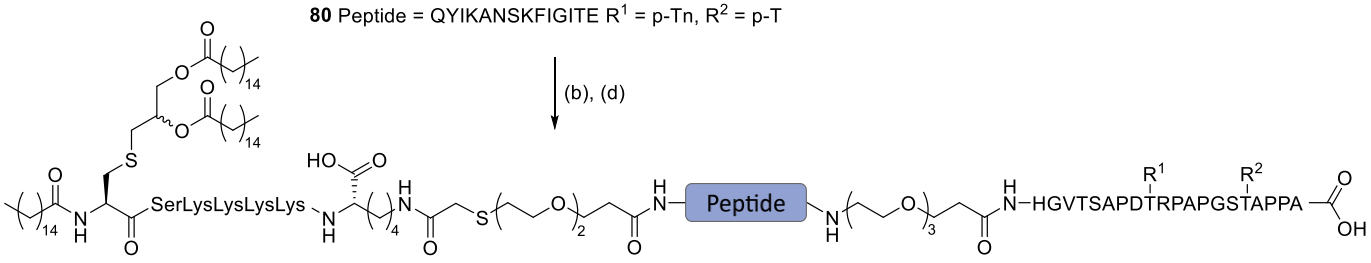
(a)
SPPS
H_2N—Ala—2-CTC
SPPS
(a)



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

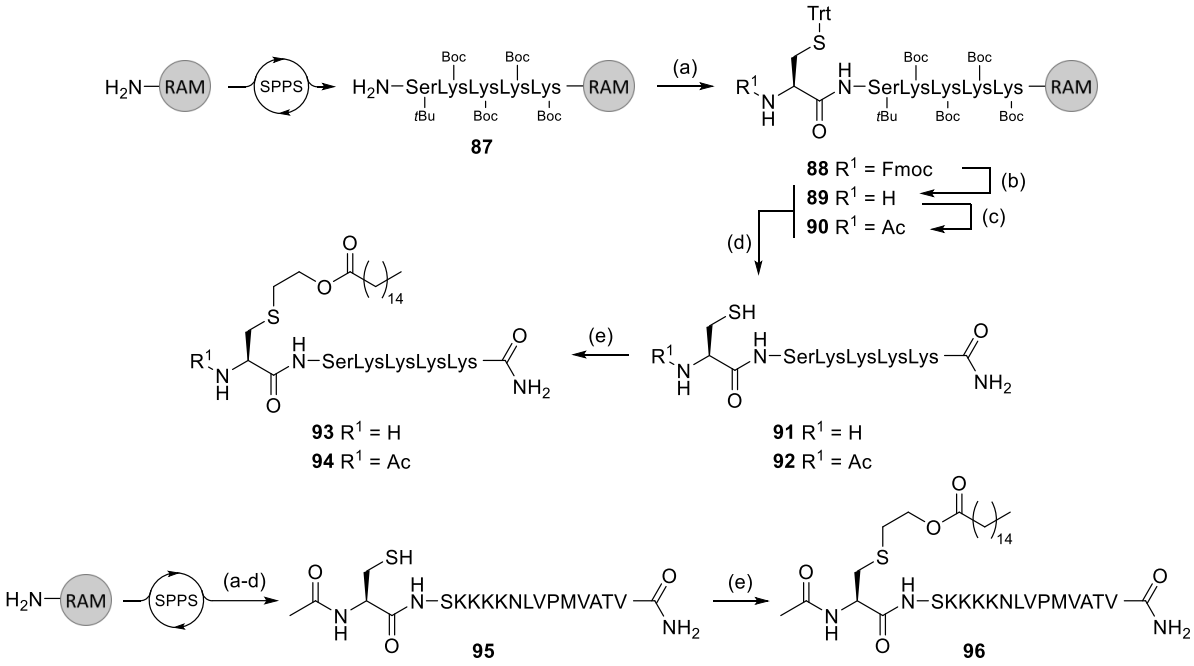


(b), (d)



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.

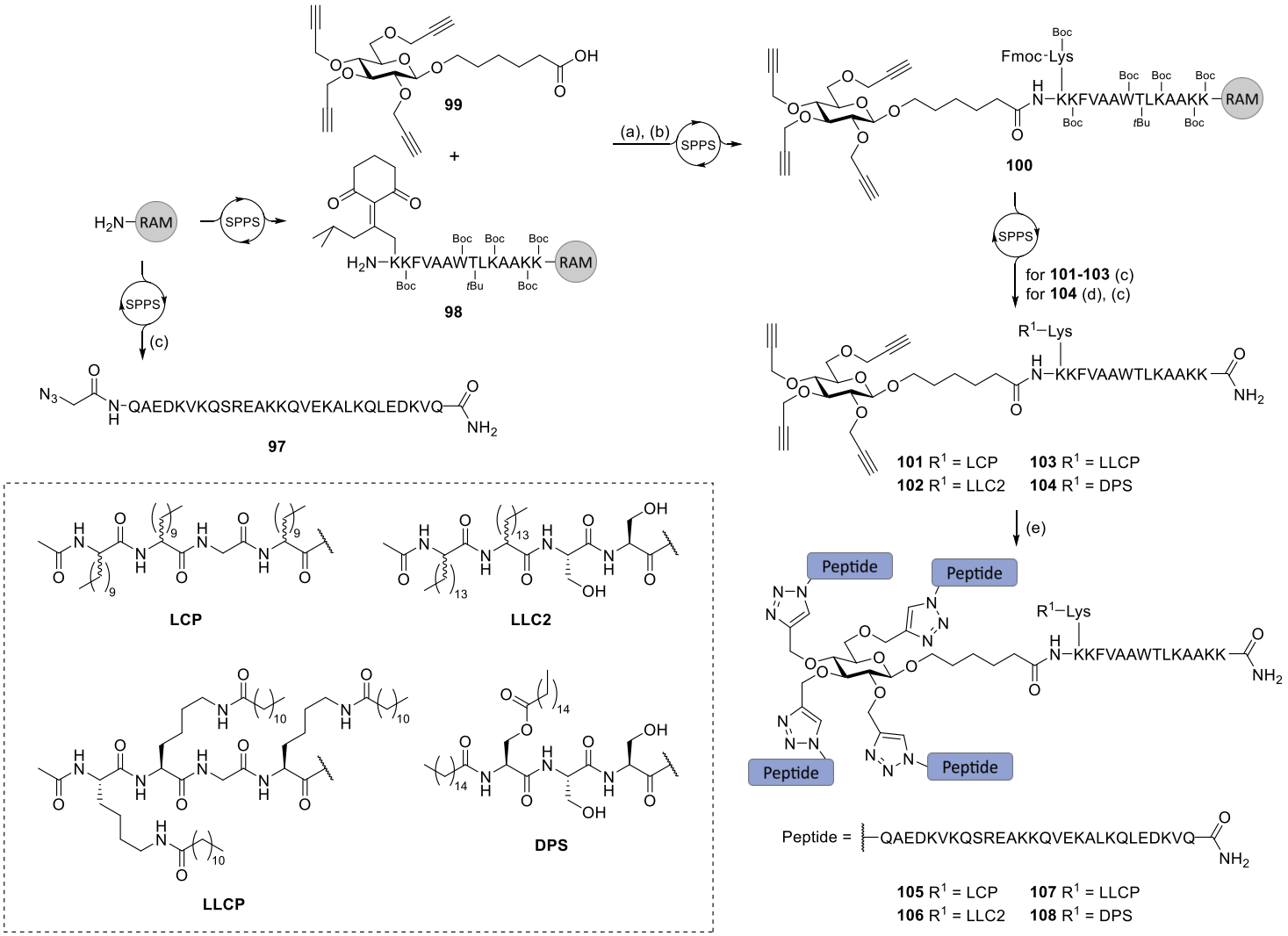
The precursors were obtained using Fmoc based peptide chemistry on a Tribute synthesizer with RAM resin as solid-state and the following three-step cycle: (i) treatment with 20% piperidine in DMF for 2 x 7 min to deprotect the Fmoc group; (ii) double coupling of the

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.

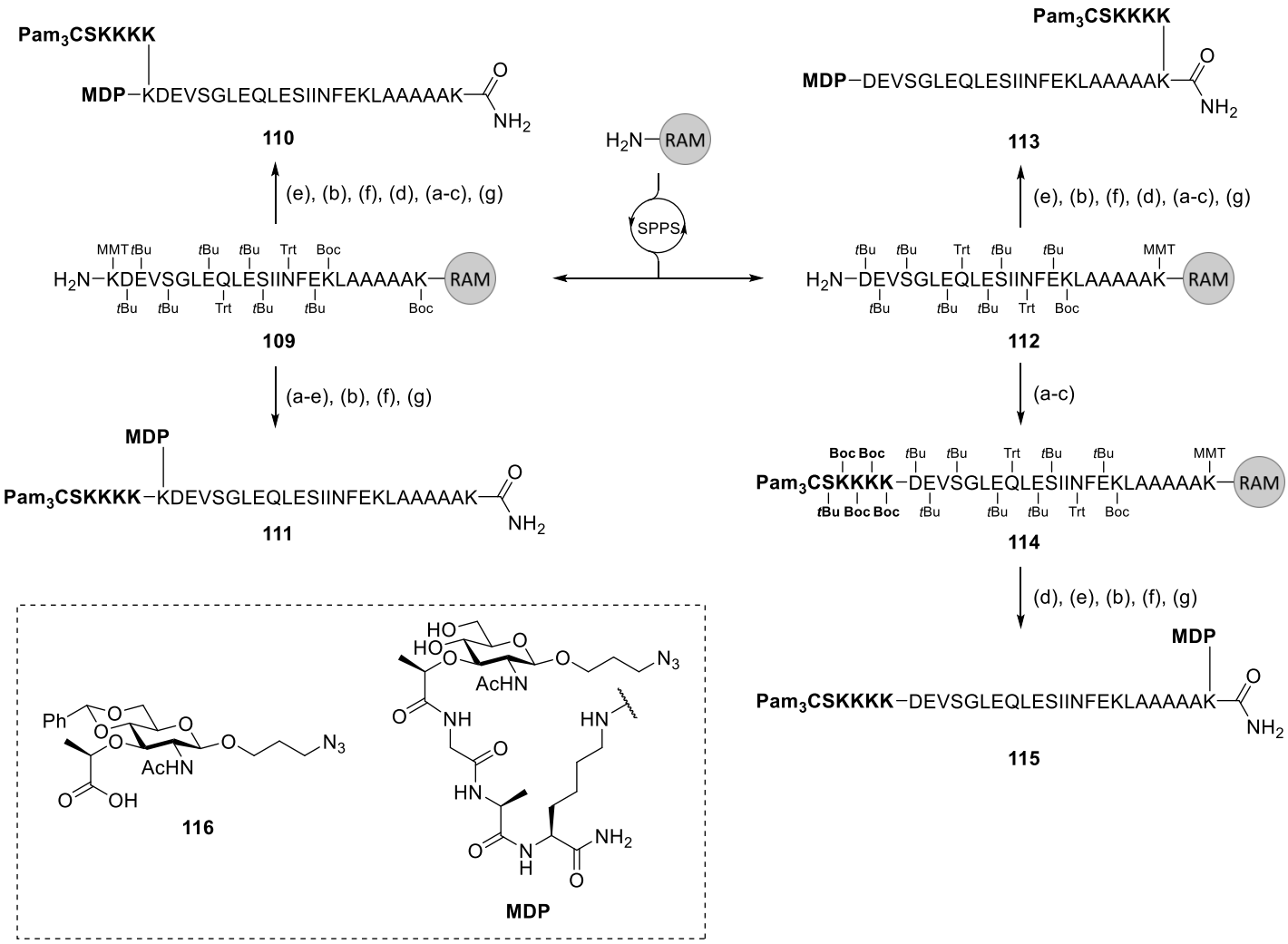
min to deprotect the Fmoc group; (ii) coupling of the protected amino acids by treating the resin twice with preactivated Fmoc-AA-OH (4.2 eq.) in DMF with HATU (4 eq.) and D*i*PEA (6.2 eq.) for 5 min at 70 °C; (iii) masking the remaining free amino functions by treatment with a 2.5% Ac_2O and 2.5% D*i*PEA 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**.



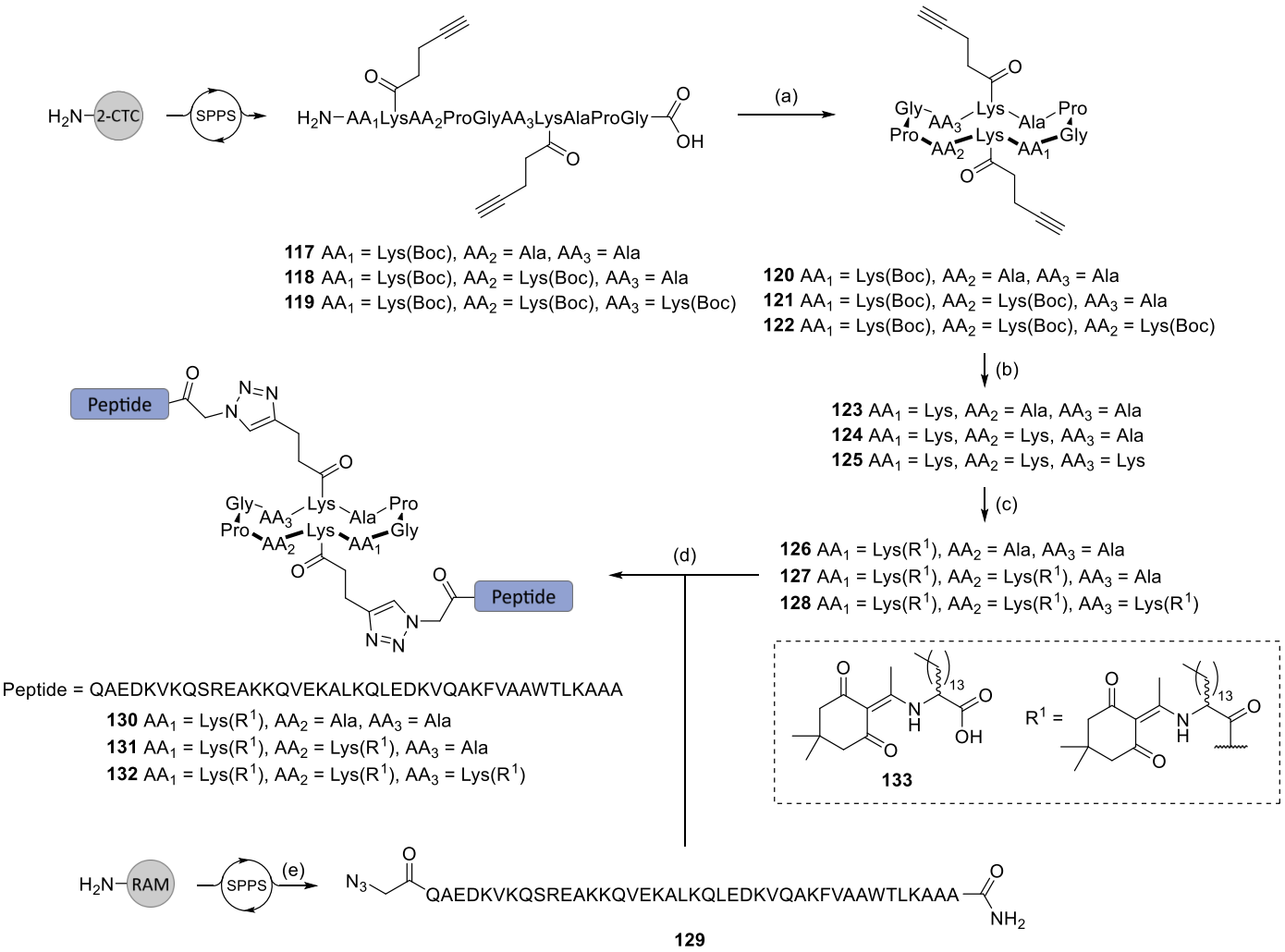
Scheme 9: Synthesis of bis-functionalized SIINFEKL containing peptides by *Zom et al.* Reagents and conditions: **SPPS**: deprotection cycle) 20% piperidine in NMP, 15 min; coupling cycle) Fmoc-AA-OH, HCTU, DiPEA, NMP, 30 min; capping cycle) Ac_2O, D*i*PEA, NMP; **(a)** Fmoc SPPS cycles for SK_4; **(b)** 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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Chapter 2

Synthesis of UPam functionalized murine and human neoantigenic peptides and their initial immunological evaluation

Introduction

A promising approach for invoking specific immunologically induced tumour regression is the treatment of patients with so called "neoantigens"[1]. Neoantigens are tumour specific antigens that are the product of somatic DNA mutations in a tumour cell, which consequently could manifest itself as an amino acid substitution in the peptides translated from the mutanome of the malignant cell. This results in a new peptide sequence that can be loaded onto major histocompatibility complexes (MHCs) on antigen presenting cells (APCs) such as dendritic cells (DCs). Above all, these altered sequences sufficiently differ from the original sequence to pass as a "non-self" epitope and, therefore, can be used as a tool for eliciting a highly specific immune response. Additionally, neoantigens should only be expressed by malignant cells, thus offering theoretically a more specific and superior therapy compared to more traditional approaches such as radio- and chemotherapy. That T-cells are capable of recognizing peptide based neoepitopes was first demonstrated by *Wölfel et al.*[2] In this publication it is demonstrated that SK29-C, a cytolytic T lymphocyte cell line from a melanoma patient, recognizes a synthetic peptide based on the mutanome of the related tumour which contains an arginine to cysteine mutation. Following this discovery, more evidence emerged of neoantigen driven T-cell responses in a variety of different human tumour types, including ovarian cancer[3] and various types of carcinomas.[4-6] However, interest in the clinical application of neoantigens remained limited due to technical complications such as mutanome sequencing and peptide production. With the rise of high-throughput genome sequencing and further development of automated peptide synthesis, neoantigens became a truly viable tool for cancer immunotherapy.

Despite T-cells recognizing neoantigens loaded onto MHCs, neoepitopes itself remain a very weak immunological species. It was speculated that, due to direct loading onto MHCs, these peptide fragments evade intracellular DC processing and missed co-stimulation of matured DCs. Administrating full-length recombinant protein circumvents this problem, however, brings its own set of limitations to the table. A far more efficient and practical strategy[7-9], is the integration of a well-defined neoantigen into a longer peptide fragment, referred to as synthetic peptides (SPs), and have been used for vaccine development against HPV,[10-11] influenza,[12] malaria,[13] and various cancer types.[7, 14-15] Generally, synthetic peptides are defined to be 25-35 amino acids long and are usually embedded usually with a single defined epitope. The epitope's position in the sequence seems to be irrelevant, although a carboxylic C-terminus is required when the epitope is located at the C-terminal end of the peptide. SPs are not limited to a single epitope per sequence, various studies have embedded simultaneously both $CD8^+$ and $CD4^+$ epitopes or both T-cell and B-cell epitopes[16-19] in the SP sequence[7, 20-22]

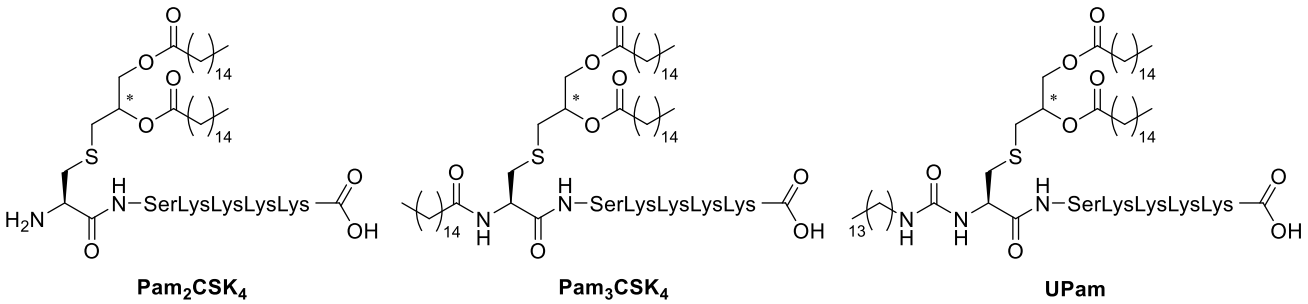


Figure 1: Structures of TLR2 ligands Pam_2CSK_4, Pam_3CSK_4 and UPam

While SPs address the issues of internalization and processing by DCs, they are not capable of initiating DC maturation by themself. 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 a pathogen associated molecular pattern (PAMP) to a SP offers an opportunity to deliver both signals to a single DC with one construct.[23-26] Additionally, conjugated constructs have been reported to out-perform their non-conjugated counterparts.[15, 27] Several PAMPs, originating from TLR, NOD-like and C-type lectin ligands, have been intensively studied and incorporated in covalently linked conjugates as vaccine modalities.[12, 14-15, 17-20, 27-34] For the research described in this chapter a TLR2-ligand (TLR-L) was chosen to be conjugated with a set of neoantigen containing peptides, since TLR2-L-SP conjugates already have been demonstrated to induce functional T-cell responses[29] and increased survival rates of tumour bearing mice.[35] TLR2 is able to bind bacterial lipopeptides and upon stimulation with a PAMP, TLR2 dimerizes with either TLR1 or TLR6 forming heterodimers capable of initiating DC maturation. TLR2 ligands have been extensively researched leading to the discovery of two small well-defined TLR2 agonists, namely ligand Pam_3CSK_4 for TLR1/TLR2[36-37] and ligand Pam_2CSK_4 for heterodimer TLR2/TLR6 [38-40] (Figure 1). Further optimization of Pam_3CSK_4 by *Willems et al.* resulted in 1-tetradecyl-urea-Cys((RS)-2,3-di(palmitoyloxy)-propyl)-Ser-Lys-Lys-Lys-Lys,[30] otherwise known as UPam (Figure 1), and was

selected for its well-defined and small structure, high potency and synthetic accessibility. Furthermore, UPam already has been successfully conjugated with antigens associated with oncogenic HPV[11] and is capable of inducing T-cell responses.[14]

This chapter presents, the solid-phase synthesis of a collection conjugates of SPs, incorporating either a murine (**a-n**) or a human (neo)epitope (**o-s**), that are covalently linked to UPam (See Table 1). Of the murine SPs, sequences **c-n** were identified by a combination of whole-exome sequencing and mass spectrometry of MHC-1 peptides eluted from the MC38 murine tumour cells.[41] This tumour was developed to serve as a suitable model for chemotherapy.[42] Furthermore, the reference synthetic peptides for sequences **a**, **b**, **i**, and **l-s** were also synthesized. The murine sequences **a-k** are all MHC-I epitope containing SPs[43-44], where sequences **d**, **f**, **h**, **j**, and **k** are epitopes outfitted with minimal flanking regions. In these sequences, the amino acids on the N-terminal end of the antigen were removed, connecting the epitope directly to the UPam ligand with Lys_4 serving as both flanking region and spacer for the TLR ligand. Furthermore, the C-terminal flanking region in these conjugates was substituted with Ala_5Lys, which is an artificial extension regularly incorporated in ovalbumin epitope containing peptides and conjugates embedded with a well-studied murine MHC-I epitope from ovalbumin (SIINFEKL). The Ala_5 sequence is known to be efficiently processed by APCs, whereas the lysine was added for future functionalization of the C-terminal end of these conjugates.[45] The aim of these adaptations is to find alternative sequences for regularly occurring synthetically inaccessible peptide conjugates to increase their synthetic accessibility while retaining efficient epitope presentation. To investigate this, conjugates **c-k** were compared to known ovalbumin derived peptides **a** and **b**, which served as controls.[29, 46] SPs **l-n** are sequences endowed with multiple MHC-II murine neoepitopes discovered by eluting them from MHC-II and are hitherto unpublished. Peptides **o** and **p** contain human neoantigens discovered from a melanoma patient successfully treated with adaptive cell transfer[47-48] and finally **q-s**, where **s** is a T-cell epitope associated with impaired peptide processing (TEIPP) and two mutated versions of the latter (**q** and **r**).[49]

Table 1: The target peptide sequences including with epitopes and mutations shown

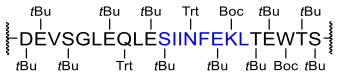
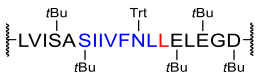
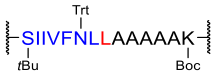
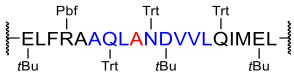
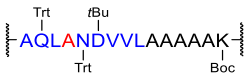
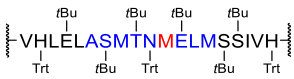
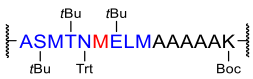
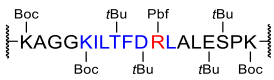
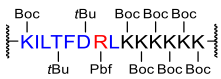
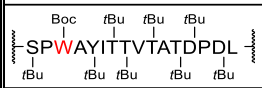
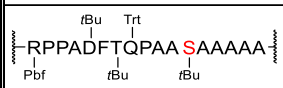
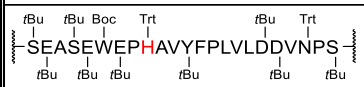
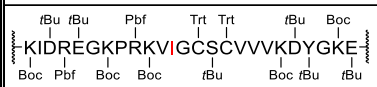
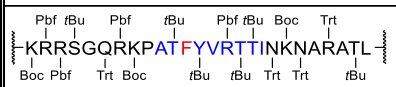
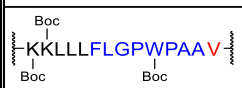
Entry	Sequence	Epitope type
a	DEVSGLEQLESIINFEKLTEWTS	Murine MHC I
b	DEVSGLEQLESIINFEKLAAAAAK	Murine MHC I
c	LVISASIIVFNLLELEGD	Murine MHC I
d	SIIVFNLLAAAAAK	Murine MHC I
e	ELFRAAQLANDVVLQIMEL	Murine MHC I
f	AQLANDVVLAAAAAK	Murine MHC I
g	VHLELASMTNMELMSSIVH	Murine MHC I
h	ASMTNMELMAAAAAK	Murine MHC I
i	KAGGKILTFDRLALESPK	Murine MHC I
j	KILTFDRLAAAAAK	Murine MHC I
k	KILTFDRLKKKKKK	Murine MHC I
l	SPWAYITTVTATDPDL	Murine MHC II
m	RPPADFTQPAASAAAAA	Murine MHC II
n	SEASEWEPHAVYFPLVLDDVNPS	Murine MHC II
o	KIDREGKPRKVIGCSCVVVKDYGKE	Human MHC II
p	KRRSGQRKPATFYVRTTINKNARATL	Human MHC I
q	RGLPALLLLLFLGPWPAAV	Human MHC I
r	KKLLLFLGPWPAAV	Human MHC I
s	KKLLLFLGPWPAAS	Human MHC I

When the peptide is embedded with a single defined epitope, the epitope is colored blue. The mutated amino acid is colored red.

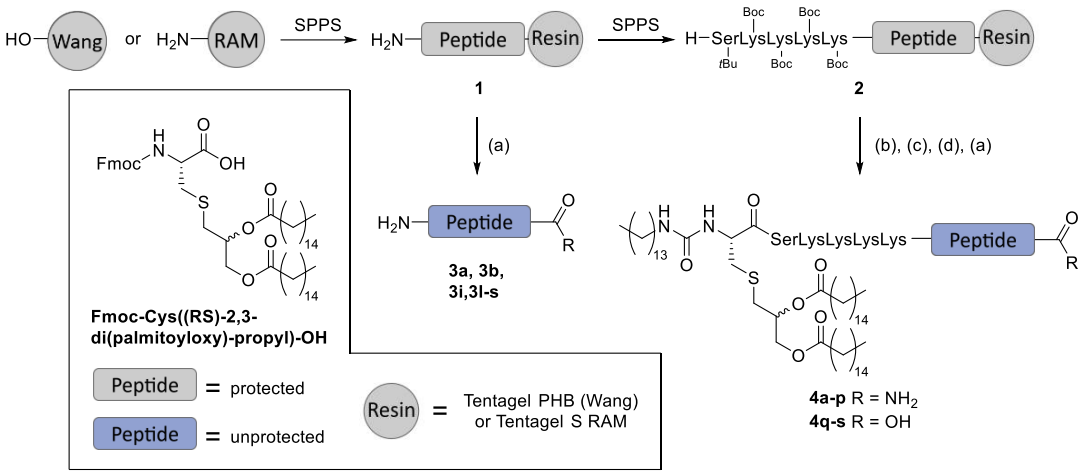
Results and Discussion

The set of peptides containing murine MHC I neoantigens, reference peptides, and the associated UPam conjugates (**a-k**) were synthesized, using Fmoc-based peptide chemistry in combination with TRIBUTE® Peptide Synthesizer (See Scheme 1). Tentagel S Ram (Rink amide) resin was applied to obtain C-terminal amides and commercially available protected amino acid building blocks were used in a single coupling event while the syntheses were executed on a 100 µmol scales. The three-step elongation cycle of the oligopeptide synthesis entailed**: (i)** treatment with 20% piperidine in NMP for 3 x 3 min to deprotect the Fmoc group, (ii) coupling of the protected amino acids (5 eq.) under influence of HCTU (5 eq.) with D*i*PEA (10 eq.) as base for 1 h at room temperature and (iii) masking the remaining free amino functions by treatment with a solution of 10% Ac_2O with 5% NMM in NMP for 3 min. From the batches of immobilized peptides **1** (See Scheme 1), two separate portions of 25 µmol were taken to prepare respectively the target peptides and the associated UPam conjugates. To acquire the target oligopeptides, resin **1** was treated with 95:2.5:2.5 TFA:H_2O:TIS to give the released and deprotected peptides, that were precipitated with a 1:1 mixture of ether and pentane, cooled to -40 °C for 1 h, and subsequently purified by HPLC on a C4 column to give pure homogenous peptides **3a**, **3b** and **3i**. The assembly of the corresponding UPam conjugates **4a-k** started with the automated elongation of immobilized oligopeptides **1** with four lysines and one serine, using the same elongation cycle as described above. Next, the Upam moiety was appended manually by condensing the free terminal amine in the thus obtained immobilized peptide **2** with Fmoc-Cys((RS)-2,3-di(palmitoyloxy)-propyl)-OH using HCTU as activation reagent and D*i*PEA as base. The ensuing removal of the Fmoc group was followed by reaction of the freed amine with tetradecyl isocyanate to give the protected and immobilized Upam conjugates. The conjugates were deprotected and cleaved from the resin using the same 95:2.5:2.5 TFA:H_2O:TIS cocktail, precipitated and purified by RP-HPLC on either a C4 or diphenyl column to give the pure, homogenous Upam conjugates **4a-4k**, with exception of conjugate **4c**. All pure constructs were stored under N_2 to suppress the possible oxidation of the sulphur atoms in cysteine and methionine over time. As depicted in Table 2 (**a-k**), the overall yields of UPam conjugates mostly exceed those of the associated peptides except for reference peptide **3b**. Although LCMS analysis of a test cleavage of the SIIVFNL sequence conjugate (**4c**) did indicate product formation, HPLC purification did not led to the isolation of pure conjugate **4c**. Based on these results, the protocol of the assembly of the set of murine MHC II neoantigen containing peptides **3l-3n** and the associated UPam conjugates **4l-4n** was adapted by using a double coupling in an otherwise unchanged Fmoc based synthesis protocol. Fortunately, with this adaptation the synthesis of all peptides and conjugates proceeded effortlessly with no clear relation between the obtained yields.

Table 2: Conjugates (4) and associated oligopeptides (3) synthesized on solid phase

Entry	Peptide	Resin	Coupling method	R	Yield	
					3	4
a		Rink-Amide	single	NH_2	4%	8%
b		Rink-Amide	single	NH_2	7%	2%
c		Rink-Amide	single	NH_2	ns	x
d		Rink-Amide	single	NH_2	ns	6%
e		Rink-Amide	single	NH_2	ns	2%
f		Rink-Amide	single	NH_2	ns	7%
g		Rink-Amide	single	NH_2	ns	5%
h		Rink-Amide	single	NH_2	ns	11%
i		Rink-Amide	single	NH_2	9%	10%
j		Rink-Amide	single	NH_2	ns	13%
k		Rink-Amide	single	NH_2	ns	6%
l		Rink-Amide	double	NH_2	8%	3%
m		Rink-Amide	double	NH_2	5%	16%
n		Rink-Amide	double	NH_2	9%	5%
o		Rink-Amide	double	NH_2	5%	9%
p		Rink-Amide	double	NH_2	5%	4%
q		Wang	double	OH	36%	8%
r		Wang	double	OH	39%	26%
s		Wang	double	OH	21%	13%

When the peptide is embedded with a single defined epitope, the epitope is colored blue. The mutated amino acid is colored red. A failure to synthesize a product is indicated with an X, while ns stands for not synthesized. The applied side chain protecting groups are indicated.



Scheme 1: Solid phase synthesis of the target UPam functionalized antigen conjugates **4a-s** and the associated references peptides **3a, 3b, 3i, 3l-s,** of which the sequences are shown in Table 1 Entries **a-s**. Protected peptides are depicted in grey and deprotected peptides in blue. Table 2 presents the protected peptide sequences **a-s** and other relevant solid phase synthesis data. Reagents and conditions: (a) TFA:TIS:H_2O 95:2,5:2,5, RT, 105 min; (b) Fmoc-Cys((RS)-2,3-di(palmitoyloxy)-propyl)-OH, HCTU, DiPEA, 1:1 DCM:NMP, overnight; (c) 20% piperidine in NMP, RT, 3x 5 min; (d) tetradecyl isocyanate, 1:1 DCM:DMF overnight, 105 min.

For the assembly of the set of human neoantigen embedded conjugates (Table 2 and Figure 1, **o-s**) either Tentagel RAM, for sequences **o** and **p,** or Tentagel PHB (Wang), for sequences **q-s** was used as solid support. Tentagel PHB (Wang) resin gave peptides with a carboxylic acid C-terminus, as carboxamides might suppress the peptide loading onto MHCs. The assemblage of these peptides and conjugates was not without difficulties and several procedures were tried. For instance, attempts to acquire peptides **3o** and **3p,** using the same elongation procedure with a single or double coupling step in combination with TRIBUTE Peptide Synthesizer, as described above, proved to be unsuccessful. Likewise, the application of commercially available Cys-Ser and Thr-Thr pseudo-prolines[50] and an IG back-bone[51] protected building block as a possible solution for the preparation of 'difficult' oligopeptides did not lead to substantial improvement in terms of yield and quality. Fortunately, the use of Liberty Blue™ Automated Microwaved Peptide Synthesizer, suitable for higher reaction temperatures, in combination with a double coupling protocol provided significant quantities of the target peptides. The elongation cycle, that lacks a capping step, comprised; (i) 2x 20% piperidine in DMF at 90 °C for 40 seconds to deprotect the Fmoc (ii) 5 eq. of amino acid building, 5 eq. of DIC as activator ad 5 eq. Oxyma-Pure as activator-base at 90 °C for 4 min to couple the protected amino acid. All target peptides (**o-s**) were synthesized using these optimized conditions, at a 100 µmol scale and the intermediate immobilized peptides **1** were split in separate portions of 25 µmol. These portions of resin **1** were directly subjected to the standard deprotection protocol (vide supra) and the obtained crude products were purified by HPLC on a C18 column to provide peptides **3o-s**. The 25 µmol portions of resin **1** were also used for the installation of the UPam ligand, using the procedure described above, to give the immobilized fully protected conjugates. Finally, removal of the protecting groups and concomitant cleavage of the conjugates from the resin with 95:2.5:2.5 TFA:H_2O:TIS was

followed by purification by HPLC on either a C4 or diphenyl column to furnish homogeneous conjugates **4o-s**.

Each MC38 antigen conjugate (**4e-n**) was analyzed for its capacity to induce the expression markers on D1 APC by serial dilution in comparison to UPam only. Interestingly, some conjugates seem to have failed to induce any maturation, namely the Rpl18-A_5K (**4j**) and full Adpgk (**4g**) conjugates. In addition, the Zmiz1 (**4m**), Ddr2 (**4n**), and full Reps1 (**4l**) conjugates required a 5x higher concentration for equal stimulation of D1s. All other conjugates were equal or better, with a 25x lower concentration for Adpgk-A_5K (**4h**) and PcdH18 (**4l**) conjugates for equal stimulation. We couldn't observe any correlation towards the type of conjugate and its potential to mature D1 cells. Most modifications of conjugates are therefore equally capable in stimulating APCs compared to ligand only or full-length peptide conjugates, with some exceptions due to unknown variables.

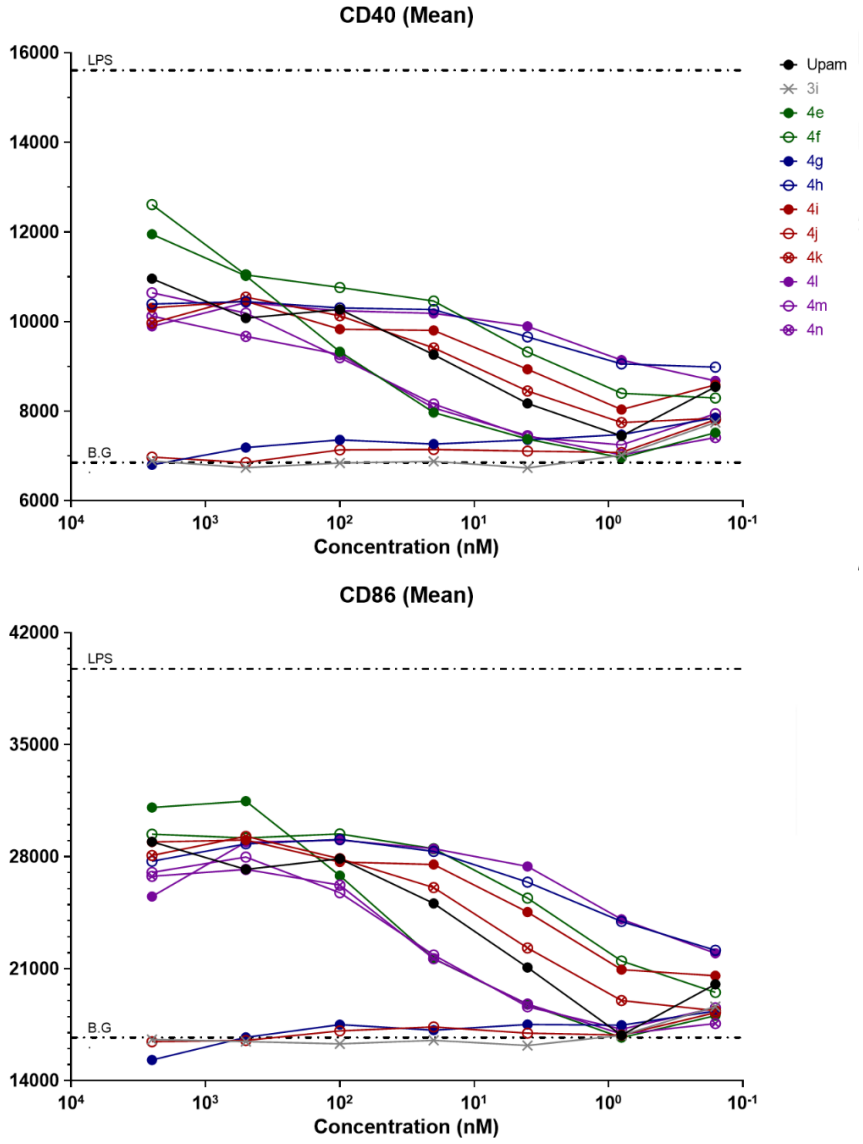


Figure 2: CD40 and CD86 expression determined with flow cytometry on DC1 cells, grown on factor-supplemented supernatant, incubated overnight with either UPam, peptide **3i**, or a UPam conjugates **4e-n** (concentration range: 2.5 µM, 1.25 µM, 0.625 µM, 0.313 µM, and 0.162 µM).

Concluding, UPam was successfully linked to 19 different SPs embedding either a human or murine neoantigen. Furthermore, replacing the natural flanking motifs with artificial ones in conjugates **4d, 4f, 4h, 4j** and **4k** did not indicate a clear synthetic advantage, but did produce considerable quantities of product in the case of conjugate **4d** where its natural counterpart could not be isolated. As expected, preliminary test results indicated that the artificial flanking regions do not significantly (measured by CD40 and CD86 upregulation) suppress the activity of TLR ligand UPam. For the future, the conjugates should be tested in MHC presentation assays to establish if the modifications influence neoantigen loading. It is assumed that the Ala_5Lys linker will largely be tolerated, but the effect of $SerLys_4$ as flanking region remains unknown. With the rise of algorithm based neoantigen prediction,[52] artificial constructs as flanking regions could make viable targets of epitopes embedded in natural sequences which are not processed properly by APCs. Additionally, a well-thought-out design could ensure synthetic availability of a SP and/or benefit practical handling of a conjugate. For example, one could incorporate a hydrophilic region in the peptide sequence to counterbalance the lipophilic nature of UPam.

Experimental

General Information

All reagents and solvents used in the solid phase peptide synthesis were purchased from Biosolve (Netherlands). Fmoc amino acids building blocks were purchased from Sigma Aldrich or Novabiochem. Tentagel based resins were purchased from Rapp Polymere GmbH (Germany). The solid-phase peptide synthesis was performed on a TRIBUTE™ Peptide Synthesiser (Gyros Protein Technologies AB, Arizona, USA) or Liberty Blue™ Automated Microwaved Peptide Synthesizer (CEM, North Caroline, USA) applying Fmoc based protocol starting with Tentagel S RAM resin (0.22-0.25 mmol/g) on a 100 µmol scale using established Fmoc protocols. LC-MS analysis was performed on one of the following LC-MS systems: A Thermo Finnigan LCQ Advantage MAX ion-trap mass spectrometer with an electrospray ion source coupled to Surveyor HPLC system (Thermo Finnegan), A Thermo Finnigan LCQ Fleet MAX ion-trap mass spectrometer with an electrospray ion source coupled to a Vanquish UHPLC system (Thermo Finnegan) or an Agilent Technologies 1260 Infinity LC system (detection simultaneously at 214 and 254 nm) coupled to a Agilent Technologies 6120 Quadrupole MS. Using an analytical Phenomenex Gemini® (3 µm C18 110 Å 50x4.6 mm), Vydac 219TP 5 µm (150x4.6mm (Phenomex, 50 x 4.60 mm, 3 microns) or Cosmosil $5C_4$-MS (5 µm particle size, 150x4.6 mm) in combination with eluents A: H_2O; B: ACN and C: 1% TFA (aq.) as the solvent system, in which the gradient was modified by changing the ratio of A in B in combination with 10% C. High resolution mass spectra were recorded on a Q-Exactive HF Orbitrap (Thermo Scientific) equipped with an electrospray ion source (ESI), injection of 2 µL of a 1 µM solution via Ultimate 3000 nano UPLC (Dionex) system, with an external calibration (Thermo Scientific). Source voltage of 3.5 kV, capillary temperature 275 °C, no sheath gas, Resolution = 240.000 at m/z=400. Mass range m/z=160-2000 or to a maximum of 6000. Eluents used: MeCN:H_2O (1:1 v/v) supplemented with 0.1% formic acid.

General procedure for TRIBUTE™ peptide synthesizer

1. Deprotection of the Fmoc protecting group with 3 x 4 mL 20% piperidine in NMP for 3 min;
2. Wash, 3 x 4 mL NMP;
3. Coupling of the appropriate amino acid applying a five-fold excess. The Fmoc amino acid (0.5 mmol, 5 eq.) building block was dissolved 0.2 M HCTU in NMP (2.5 mL, 5 eq.) in its loading cartridge and the resulting solution was transferred to the reaction vessel. Next, the cartridge was washed with a 0.5 M D*i*PEA in NMP (2 mL, 10 eq) and subsequently the solution was transferred to the reaction vessel. The reaction vessel was shaken for 1 h at RT;
4. Wash, 3 x 4 mL NMP;
5. Capping of unreacted peptide with 1 x 5 mL 10% Ac_2O and 5% NMM in NMP solution for 3 min;
6. Wash, 3 x 4 mL NMP;

After the last coupling cycle, the final Fmoc group was deprotected with 3 x 4 mL 20% piperidine in NMP for 3 min. Finally, the resin was washed with NMP (3x) and DCM (3x) and dried using a N_2 flow.

The following amino acid building blocks were used for oligopeptide assembly: Fmoc-Ala-OH, Fmoc-Asn(Trt)-OH, Fmoc-Asp(*t*Bu)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Cys(Trt)-OH, Fmoc-Gln(Trt)-OH, Fmoc-Glu(*t*Bu)-OH, Fmoc-Gly-OH, Fmoc-His(Boc)-OH, Fmoc-Ile-OH, Fmoc-Leu-OH, Fmoc-Lys(Boc)-OH, Fmoc-Met-OH, Fmoc-Phe-OH, Fmoc-Pro-OH, Fmoc-Ser(*t*Bu)-OH, Fmoc-Thr(*t*Bu), Fmoc-Trp(Boc)-OH Fmoc-Tyr(*t*Bu)-OH, Fmoc-Val-OH.

General procedure for Liberty Blue™ Automated Microwaved Peptide Synthesizer

1. Deprotection of the Fmoc protecting group with 2 x 4 mL 20% piperidine in DMF for 3 min;
2. Wash, 3 x 4 mL DMF;
3. First coupling of the appropriate amino acid applying a five-fold excess. First, a 0.2 M solution of the Fmoc amino acid in DMF (2.5 mL) was added to the resin in the reaction vessel. Next, a 0.5 M solution of DIC in DMF (1 mL) and a 1 M solution of Oxyma Pure in DMF (0.5 mL) was added to the reaction vessel. The reaction vessel was shaken for 4 min. at 90°;
4. Wash, 3 x 4 mL DMF;
5. Second coupling of the appropriate amino acid applying a five-fold excess. First, a 0.2 M solution of the Fmoc amino acid in DMF (2.5 mL) was added to the resin in the reaction vessel. Next, a 0.5 M solution of DIC in DMF (1 mL) and a 1 M solution of Oxyma Pure in DMF (0.5 mL) was added to the reaction vessel. The reaction vessel was shaken for 4 min at 90°;
6. Wash, 3 x 4 mL DMF;

After the last coupling cycle, the final Fmoc group was deprotected with 3 x 4 mL 20% piperidine in DMF for 3 min Finally, the resin was washed with DMF (3x) and DCM (3x) and dried using a N_2 flow.

The following amino acid building blocks were used for oligopeptide assembly: Fmoc-Ala-OH, Fmoc-Asn(Trt)-OH, Fmoc-Asp(OMpe)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Cys(Trt)-OH, Fmoc-Gln(Trt)-OH, Fmoc-Glu(*t*Bu)-OH, Fmoc-Gly-OH, Fmoc-His(Trt)-OH, Fmoc-Ile-OH, Fmoc-Leu-OH, Fmoc-Lys(Boc)-OH, Fmoc-Met-OH, Fmoc-Phe-OH, Fmoc-Pro-OH, Fmoc-Ser(*t*Bu)-OH, Fmoc-Thr(*t*Bu), Fmoc-Trp(Boc)-OH Fmoc-Tyr(*t*Bu)-OH, Fmoc-Val-OH.

General Procedure UPam functionalization

The peptide sequences were prepared using a Peptide Synthesiser applying Fmoc chemistry for 100 µmol with the appropriate resin. 25 µmol dried resin was taken from the crude batch (unless mentioned otherwise) and a solution of N-(((9H-fluoren-9-yl)methoxy)carbonyl)-S-(2,3-bis(palmitoyloxy)propyl)-L-cysteine (50 µmol, 44.7 mg, 2 eq.) and HCTU (50 µmol, 20.9 mg, 2 eq.) in 1:1 DCM:NMP (0.75 mL) was added to the resin. D*i*PEA (50 µmol, 8.7 µL, 2 eq.) was added and the reaction syringe was shaken. After 15 min a second portion of D*i*PEA (50 µmol, 8.7 µL, 2 eq.) was added and the reaction syringe was shaken overnight. The next morning the resin was washed with NMP(3x) and DCM(3x) and dried under a N_2 flow. Next, a solution of tetradecyl isocyanate (225 µmol, 62.5 µL, 9 eq.) in 1:1 DCM:NMP (2.5mL) was added to the resin and the syringe was shaken overnight. The resin was washed with NMP (3x), DCM (3x) and dried using a N_2 flow. The conjugate was cleaved using a 95:2.5:2.5 TFA:H_2O:TIS solution for 105 min and the cleavage solution was split over two centrifuge tubes filled with a mixture of 1:1 Et_2O:Pentane (2 x 48 mL). Both tubes were stored at -40˚C for 1 h. The tubes were centrifuged, and the solution decanted, leaving a wet white pallet inside the centrifuge tubes. These pellets were dried with a N_2 flow and dissolved for purification. The crude products were purified using HPLC after which the appropriate fractions were collected and concentrated *in vacuo*. Reference peptides were immediately deprotected and purified following the same procedures as for the conjugates.

(((2S,5R)-5-amino-6-((2,3-bis(palmitoyloxy)propyl)thio)-1-hydroxy-3,4-dioxohexan-2-yl)amino)-Ser-Lys-Lys-Lys-Lys-Asp-Glu-Val-Ser-Gly-Lue-Glu-Gln-Leu-Glu-Ser-Ile-Ile-Asn-Phe-Glu-Lys-Leu-Ala-Ala-Ala-Ala-Ala-Lys-NH_2 (3a)

Synthesizer: TRIBUTE™; **Resin:** Tentagel S Ram; **HPLC solution:** *t*BuOH:H_2O:MECN (1:1:1); 4% yield (5.91 mg, 2.22 µmol). **LC-MS:** R_t = 12.221 (Vydac 219TP 5 µm (150x4.6mm) Diphenyl, 10-90% MeCN, 21 min); **ESI-MS:** m/z 1347.2 $[M+2H^+]^{2+}$; **HRMS [M+7H]$^{7+}$:** $[C_{118}H_{189}N_{28}O_{42}]^{5+}$ 538.88357 (measured), 538.88212 (calculated).

H-Asp-Glu-Val-Ser-Gly-Lue-Glu-Gln-Leu-Glu-Ser-Ile-Ile-Asn-Phe-Glu-Lys-Leu-Ala-Ala-Ala-Ala-Ala-Lys-NH_2 (3b)

Performed on a 50 µmol scale; **Synthesizer:** TRIBUTE™; **Resin:** Tentagel S RAM; **HPLC solution**: *t*BuOH:H_2O:MECN (1:1:1); 7% yield (9.11 mg, 3.58 µmol). **LC-MS:** R_t = 8.533 (Vydac 219TP 5 µm (150x4.6mm) Diphenyl, 10-90% MeCN, 21 min); **ESI-MS:** m/z 1273.8 $[M+2H^+]^{2+}$; **HRMS [M+3H]$^{3+}$:** $[C_{112}H_{185}N_{29}O_{38}]^{3+}$ 849.12193 (measured), 849.12179 (calculated).

H-Lys-Ala-Gly-Gly-Lys-Ile-Leu-Thr-Phe-Asp-Arg-Leu-Ala-Leu-Glu-Ser-Pro-Lys-NH_2 (3i)

Performed on a 100 µmol scale; **Synthesizer:** TRIBUTE™; **Resin:** Tentagel S RAM; **HPLC solution:** *t*BuOH:H_2O:MECN (1:1:1); 9% yield (17.01 mg, 8.75 µmol). **LC-MS:** R_t = 3.90 (Phenomenex Gemini® 3 µm C18 110 Å 50x4.6 mm, 10-90% MeCN, 10 min); **ESI-MS:** m/z 1944.1 $[M+H^+]^+$; **HRMS [M+3H]$^{3+}$:** $[C_{88}H_{154}N_{25}O_{24}]^{3+}$ 648.38586 (measured), 648.38607 (calculated).

H-Ser-Pro-Trp-Ala-Tyr-Ile-Thr-Thr-Val-Thr-Ala-Thr-Asp-Pro-Asp-Leu-NH_2 (3l)

Synthesizer: TRIBUTE™; **Resin:** Tentagel S Ram; **HPLC solution:** *t*BuOH:H_2O:MECN (1:1:1); 8% yield (3.4 mg, 1.94 µmol). **LC-MS:** R_t = 7.48 (Vydac 219TP 5 µm (150x4.6mm) Diphenyl, 10-90% MeCN, 21 min); **ESI-MS:** m/z 1750.3 $[M+H^+]^+$; **HRMS [M+2H]$^{2+}$:** $[C_{80}H_{122}N_{18}O_{26}]^{2+}$ 875.43791 (measured), 875.43833 (calculated).

H-Arg-Pro-Pro-Ala-Asp-Phe-Thr-Gln-Pro-Ala-Ala-Ser-Ala-Ala-Ala-Ala-Ala-NH_2 (3m)

Synthesizer: TRIBUTE™; **Resin:** Tentagel S RAM; **HPLC solution:** *t*BuOH:H_2O:MECN (1:1:1); 5% yield (2.16 mg, 1.34 µmol). **LC-MS:** R_t = 3.75 (Phenomenex Gemini® 3 µm C18 110 Å 50x4.6 mm, 10-90% MeCN, 10 min); **ESI-MS:** m/z 1612.1 $[M+H^+]^+$; **HRMS [M+2H]$^{2+}$:** $[C_{70}H_{112}N_{22}O_{22}]^{2+}$ 806.41480 (measured), 806.41553 (calculated).

H-Ser-Glu-Ala-Ser-Glu-Trp-Glu-Pro-Hys-Ala-Val-Tyr-Phe-Leu-Val-Leu-Asp-Asp-Val-Asn-Pro-Ser-NH_2 (3n)

Synthesizer: TRIBUTE™; **Resin:** Tentagel S RAM; **HPLC solution:** *t*BuOH:H_2O:MECN (1:1:1); 9% yield (5.66 mg, 2.18 µmol). **LC-MS:** R_t = 5.15 (Phenomenex Gemini® 3 µm C18 110 Å 50x4.6 mm, 10-90% MeCN, 10 min); **ESI-MS:** m/z 1301.3 $[M+2H^+]^{2+}$; **HRMS [M+2H]$^{2+}$:** $[C_{119}H_{172}N_{28}O_{38}S]^{2+}$ 1300.61909 (measured), 1300.61882 (calculated).

H-Lys-Ile-Asp-Arg-Glu-Gly-Lys-Pro-Arg-Lys-Val-Ile-Gly-Cys-Ser-Cys-Val-Val-Val-Lys-Asp-Tyr-Gly-Lys-Glu-NH_2 (3o)

Synthesizer: TRIBUTE™; **Resin:** Tentagel S RAM; **HPLC solution**: *t*BuOH:H_2O:MECN (1:1:1); 5% yield (3.38 mg, 1.26 µmol). **LC-MS:** R_t = 4.307 (Phenomenex Gemini® 3 µm C18 110 Å 50x4.6 mm, 10-90% MeCN, 10 min); **ESI-MS:** *m/z* 1404.3 $[M+2H^+]^{2+}$; **HRMS [M+5H]$^{5+}$:** $[C_{121}H_{212}N_{37}O_{35}S_2]^{5+}$ 561.50687 (measured), 561.50721 (calculated).

H-Lys-Arg-Arg-Ser-Gly-Gln-Arg-Lys-Pro-Ala-Thr-Phe-Tyr-Val-Arg-Thr-Thr-Ile-Asn-Lys-Asn-Ala-Arg-Ala-Thr-Leu-NH_2 (3p)

Synthesizer: TRIBUTE™; **Resin:** Tentagel S RAM; **HPLC solution:** *t*BuOH:H_2O:MECN (1:1:1); 18% yield (13.62 mg, 4.49 µmol). **LC-MS:** Rt = 3.479 (Phenomenex Gemini® 3 µm C18 110 Å 50x4.6 mm, 10-90% MeCN, 10 min); **ESI-MS:** *m/z* 1012.1 $[M+3H^+]^{3+}$; **HRMS [M+6H]$^{6+}$:** $[C_{131}H_{232}N_{48}O_{35}]^{6+}$ 506.29643 (measured), 506.29687 (calculated).

H-Arg-Gly-Leu-Pro-Ala-Leu-Leu-Leu-Leu-Leu-Phe-Leu-Gly-Pro-Trp-Pro-Ala-Ala-Val-OH (3q)

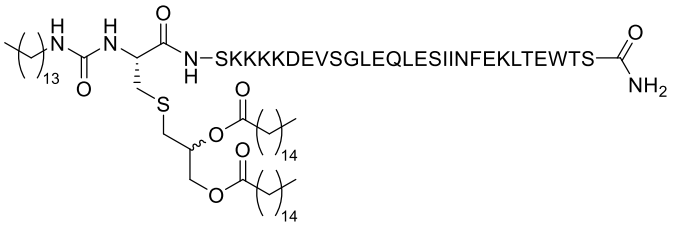
Synthesizer: Liberty Blue™; **Resin:** Tentagel PHB resin; **HPLC solution:** DMSO; 36% yield (13.62 mg, 9.00 µmol). **LC-MS:** R_t = 6.05 (Phenomenex Gemini® 3 µm C18 110 Å 50x4.6 mm, 10-90% MeCN, 10 min); **ESI-MS:** *m/z* 1009.5 $[M+2H^+]^{2+}$; **HRMS [M+2H]$^{2+}$:** $[C_{101}H_{163}N_{23}O_{20}]^{2+}$ 1009.12150 (measured), 1009.12169 (calculated).

H-Lys-Lys-Leu-Leu-Leu-Phe-Leu-Gly-Pro-Trp-Pro-Ala-Ala-Val-OH (3r)

Synthesizer: Liberty Blue™; **Resin:** Tentagel PHB resin; **HPLC solution:** DMSO; 39% yield (15.16 mg, 9.76 µmol). **LC-MS:** R_t = 4.48 (Phenomenex Gemini® 3 µm C18 110 Å 50x4.6 mm, 10-90% MeCN, 10 min); **ESI-MS:** *m/z* 1552.9 $[M+H^+]^+$; **HRMS [M+3H]$^{3+}$:** $[C_{79}H_{125}N_{17}O_{15}]^{3+}$ 518.32418 (measured), 518.532531 (calculated).

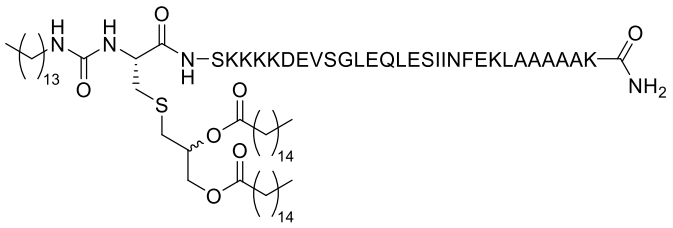
H-Lys-Lys-Leu-Leu-Leu-Phe-Leu-Gly-Pro-Trp-Pro-Ala-Ala-Ser-OH (3s)

Synthesizer: Liberty Blue™; **Resin:** Tentagel PHB resin; **HPLC solution:** DMSO; 21% yield (7.9 mg, 5.13 µmol). **LC-MS:** R_t = 4.26 (Phenomenex Gemini® 3 µm C18 110 Å 50x4.6 mm, 10-90% MeCN, 10 min); **ESI-MS:** *m/z* 1541.8 $[M+H^+]^+$; **HRMS [M+3H]$^{3+}$:** $[C_{77}H_{124}N_{17}O_{16}]^{3+}$ 514.31312 (measured), 514.31318 (calculated).



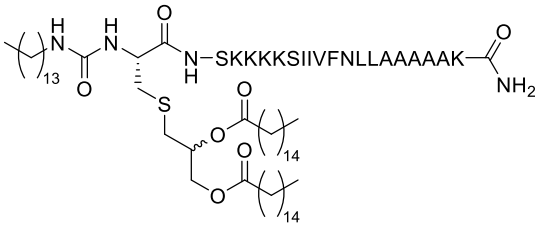
(((2S,5R)-5-amino-6-((2,3-bis(palmitoyloxy)propyl)thio)-1-hydroxy-3,4-dioxohexan-2-yl)amino)-Ser-Lys-Lys-Lys-Lys-Ala-Gln-Leu-Ala-Asn-Asp-Val-Val-Leu-Ala-Ala-Ala-Ala-Ala-Lys-NH_2 (4a)

Resin: Tentagel S RAM; **HPLC solution:** *t*BuOH:H_2O:MECN (1:1:1); 8% yield (8.48 mg, 2.04 µmol). **LC-MS:** R_t = 12.44 (Vydac 219TP 5 µm (150x4.6mm) Diphenyl, 10-90% MeCN, 21 min); **ESI-MS:** m/z 1387.7 $[M+3H^+]^{3+}$; **HRMS [M+4H]$^{4+}$:** $[C_{198}H_{341}N_{39}O_{52}S]^{4+}$ 1040.36987 (measured), 1040.37087 (calculated).



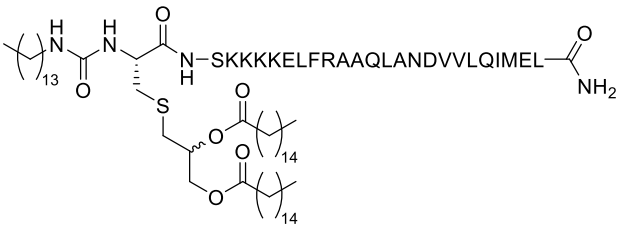
(((2S,5R)-5-amino-6-((2,3-bis(palmitoyloxy)propyl)thio)-1-hydroxy-3,4-dioxohexan-2-yl)amino)-Ser-Lys-Lys-Lys-Lys-Ala-Gln-Leu-Ala-Asn-Asp-Val-Val-Leu-Ala-Ala-Ala-Ala-Ala-Lys-NH_2 (4b)

Resin: Tentagel S RAM; **HPLC solution**: *t*BuOH:H_2O:MECN (1:1:1); 2% yield (2.45 mg, 0.61 µmol). **LC-MS:** R_t = 12.221 (Vydac 219TP 5 µm (150x4.6mm) Diphenyl, 10-90% MeCN, 21 min); **ESI-MS:** m/z 1347.2 $[M+3H^+]^{3+}$; **HRMS [M+4H]$^{4+}$:** $[C_{192}H_{342}N_{40}O_{50}S]^{4+}$ 1010.12769 (measured), 1010.12865 (calculated).



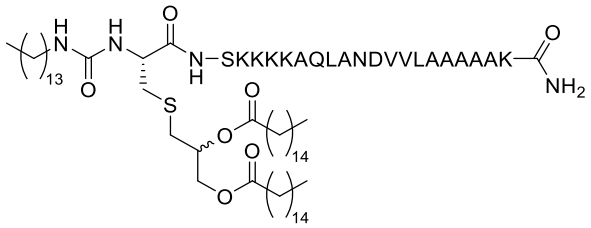
(((2S,5R)-5-amino-6-((2,3-bis(palmitoyloxy)propyl)thio)-1-hydroxy-3,4-dioxohexan-2-yl)amino)-Ser-Lys-Lys-Lys-Lys-Ser-Ile-Ile-Val-Phe-Asn-Leu-Leu-Ala-Ala-Ala-Ala-Ala-Lys-NH_2 (4d)

Performed on a 50 µmol scale. **Resin:** Tentagel S RAM; **HPLC solution:** *t*BuOH:H_2O:MECN (1:1:1); 6% yield (9.09 mg, 3.14 µmol). **LC-MS:** R_t = 12.63 (Vydac 219TP 5 µm (150x4.6mm) Diphenyl, 10-90% MeCN, 21 min); **ESI-MS:** m/z 1447.6 $[M+2H^+]^{2+}$; **HRMS [M+3H]$^{3+}$:** $[C_{146}H_{269}N_{28}O_{28}S]^{3+}$ 965.00604 (measured), 965.00635 (calculated).



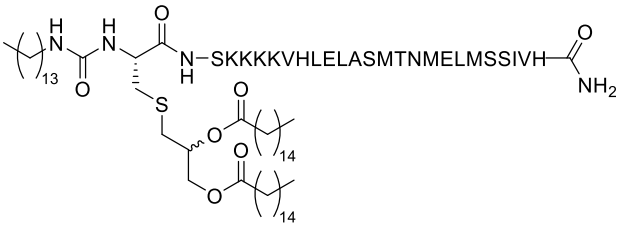
(((2S,5R)-5-amino-6-((2,3-bis(palmitoyloxy)propyl)thio)-1-hydroxy-3,4-dioxohexan-2-yl)amino)-Ser-Lys-Lys-Lys-Lys-Glu-Leu-Phe-Arg-Ala-Ala-Gln-Leu-Ala-Asn-Asp-Val-Val-Leu-Gln-Ile-Met-Glu-Leu-NH_2 (4e)

Resin: Tentagel S RAM; **HPLC solution**: *t*BuOH:H_2O:MECN (1:1:1); 2% yield (3.52 mg, 0.95 µmol). **LC-MS:** R_t = 12.33 (Vydac 219TP 5 µm (150x4.6mm) Diphenyl, 10-90% MeCN, 21 min); **ESI-MS:** m/z 1222.3 $[M+3H^+]^{3+}$; **HRMS [M+7H]$^{7+}$:** $[C_{177}H_{322}N_{37}O_{40}S_2]^{7+}$ 524.33121 (measured), 524.33133 (calculated).



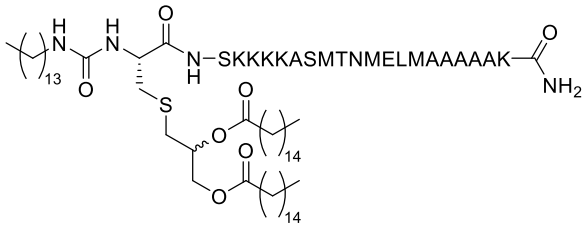
(((2S,5R)-5-amino-6-((2,3-bis(palmitoyloxy)propyl)thio)-1-hydroxy-3,4-dioxohexan-2-yl)amino)-Ser-Lys-Lys-Lys-Lys-Ala-Gln-Leu-Ala-Asn-Asp-Val-Val-Leu-Ala-Ala-Ala-Ala-Ala-Lys-NH_2 (4f)

Resin: Tentagel S RAM; **HPLC solution:** *t*BuOH:H_2O:MECN (1:1:1); 7% yield (5.3 mg, 1.82 µmol). **LC-MS:** R_t = 12.33 (Vydac 219TP 5 µm (150x4.6mm) Diphenyl, 10-90% MeCN, 21 min); **ESI-MS:** m/z 1459.6 $[M+2H^+]^{2+}$; **HRMS [M+5H]$^{5+}$:** $[C_{142}H_{267}N_{30}O_{31}S]^{5+}$ 584.19122 (measured), 584.19136 (calculated).



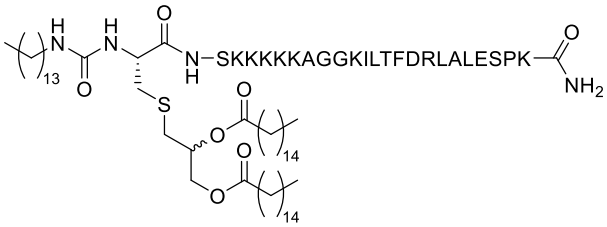
(((2S,5R)-5-amino-6-((2,3-bis(palmitoyloxy)propyl)thio)-1-hydroxy-3,4-dioxohexan-2-yl)amino)-Ser-Lys-Lys-Lys-Lys-Val-His-Leu-Glu-Leu-Ala-Ser-Met-Thr-Asn-Met-Glu-Leu-Met-Ser-Ser-Ile-Val-His-NH_2 (4g)

Resin: Tentagel S RAM; **HPLC solution:** *t*BuOH:H_2O:MECN (1:1:1); 5% yield (4.52 mg, 1.24 µmol). **LC-MS:** R_t = 12.57 (Vydac 219TP 5 µm (150x4.6mm) Diphenyl, 10-90% MeCN, 21 min); **ESI-MS:** m/z 1818.7 $[M+2H^+]^{2+}$; **HRMS [M+7H]$^{7+}$:** $[C_{171}H_{313}N_{36}O_{40}S_4]^{7+}$ 519.88409 (measured), 519.88428 (calculated).



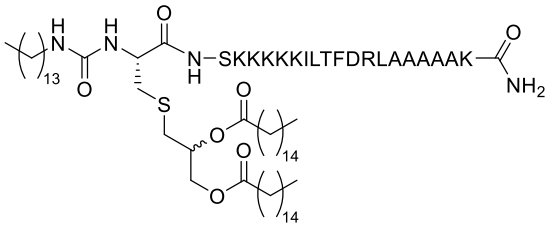
(((2S,5R)-5-amino-6-((2,3-bis(palmitoyloxy)propyl)thio)-1-hydroxy-3,4-dioxohexan-2-yl)amino)-Ser-Lys-Lys-Lys-Lys-Ala-Ser-Met-Thr-Asn-Met-Glu-Leu-Met-Ala-Ala-Ala-Ala-Ala-Lys-NH_2 (4h)

Resin: Tentagel S RAM; **HPLC solution:** *t*BuOH:H_2O:MECN (1:1:1); 11% yield (8.1 mg, 2.70 µmol). **LC-MS:** R_t = 12.50 (Vydac 219TP 5 µm (150x4.6mm) Diphenyl, 10-90% MeCN, 21 min); **ESI-MS:** m/z 1502.1 $[M+2H^+]^{2+}$; **HRMS [M+3H]$^{3+}$:** $[C_{141}H_{264}N_{29}O_{32}S_4]^{3+}$ 1001.29297 (measured), 1001.29295 (calculated).



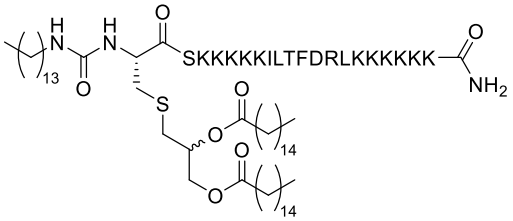
(((2S,5R)-5-amino-6-((2,3-bis(palmitoyloxy)propyl)thio)-1-hydroxy-3,4-dioxohexan-2-yl)amino)-Ser-Lys-Lys-Lys-Lys-Lys-Ala-Gly-Gly-Lys-Ile-Leu-Thr-Phe-Asp-Arg-Leu-Ala-Leu-Glu-Ser-Pro-Lys-NH_2 (4i)

Performed on a 100 µmol scale. **Resin**: Tentagel S RAM; **HPLC solution:** *t*BuOH:H_2O:MECN (1:1:1); 10% yield (33.8 mg, 9.83 µmol).**LC-MS:** R_t = 12.33 (Vydac 219TP 5 µm (150x4.6mm) Diphenyl, 10-90% MeCN, 21 min); **ESI-MS:** m/z 1459.6 $[M+3H^+]^{3+}$; **HRMS [M+5H]$^{5+}$:** $[C_{168}H_{309}N_{36}O_{36}S]^{5+}$ 687.86306 (measured), 687.86297 (calculated).



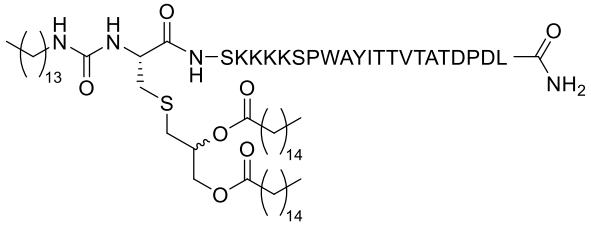
(((2S,5R)-5-amino-6-((2,3-bis(palmitoyloxy)propyl)thio)-1-hydroxy-3,4-dioxohexan-2-yl)amino)-Ser-Lys-Lys-Lys-Lys-Lys-Ile-Leu-Thr-Phe-Asp-Arg-Leu-Ala-Ala-Ala-Ala-Ala-Lys-NH_2 (4j)

Resin: Tentagel S RAM; **HPLC solution**: *t*BuOH:H_2O:MECN (1:1:1); 13% yield (9.85 mg, 3.30 µmol). **LC-MS:** R_t = 12.08 (Vydac 219TP 5 µm (150x4.6mm) Diphenyl, 10-90% MeCN, 21 min); **ESI-MS:** m/z 1491.1 $[M+2H^+]^{2+}$; **HRMS [M+3H]$^{3+}$:** $[C_{148}H_{274}N_{31}O_{29}S]^{3+}$ 994.01980 (measured), 994.02077 (calculated).



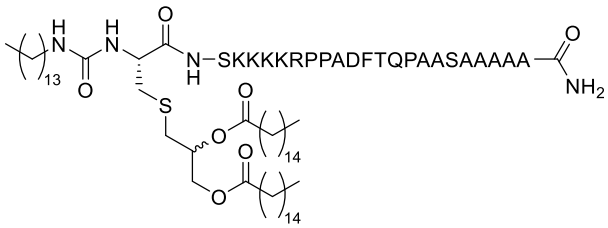
(((2S,5R)-5-amino-6-((2,3-bis(palmitoyloxy)propyl)thio)-1-hydroxy-3,4-dioxohexan-2-yl)amino)-Ser-Lys-Lys-Lys-Lys-Lys-Ile-Leu-Thr-Phe-Asp-Arg-Leu-Lys-Lys-Lys-Lys-Lys-Lys-NH_2 (4k)

Resin: Tentagel S RAM; **HPLC solution:** *t*BuOH:H_2O:MECN (1:1:1); 6% yield (4.83 mg, 1.48 µmol). **LC-MS:** R_t = 11.70 (Vydac 219TP 5 µm (150x4.6mm) Diphenyl, 10-90% MeCN, 21 min); **ESI-MS:** m/z 1633.7 $[M+2H^+]^{2+}$; **HRMS [M+6H]$^{6+}$:** $[C_{163}H_{312}N_{36}O_{29}S]^{6+}$ 545.06245 (measured), 545.06223 (calculated).



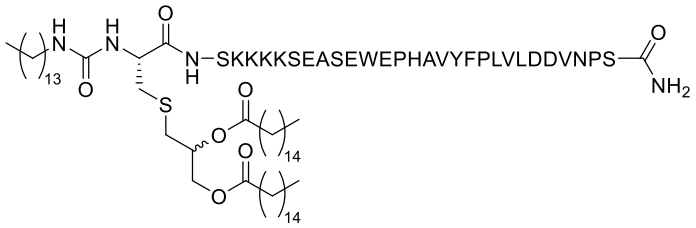
(((2S,5R)-5-amino-6-((2,3-bis(palmitoyloxy)propyl)thio)-1-hydroxy-3,4-dioxohexan-2-yl)amino)-Ser-Lys-Lys-Lys-Lys-Ser-Pro-Trp-Ala-Tyr-Ile-Thr-Thr-Val-Thr-Ala-Thr-Asp-Pro-Asp-Leu-NH_2 (4l)

Resin: Tentagel S RAM; **HPLC solution:** *t*BuOH:H_2O:MECN (1:1:1); 3% yield (2.66 mg, 0.82 µmol). **LC-MS:** R_t = 12.88 (Vydac 219TP 5 µm (150x4.6mm) Diphenyl, 10-90% MeCN, 21 min); **ESI-MS:** m/z 1621.9 $[M+2H^+]^{2+}$; **HRMS [M+4H]$^{4+}$:** $[C_{160}H_{277}N_{29}O_{38}S]^{4+}$ 811.25812 (measured), 811.258.33 (calculated).



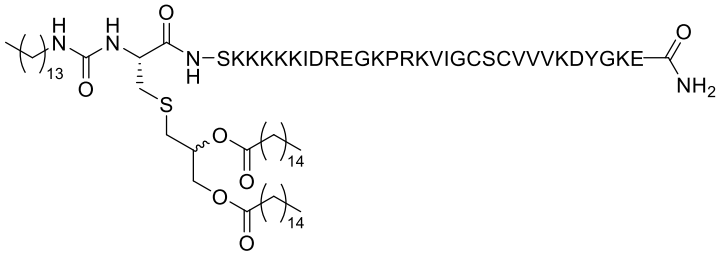
(((2S,5R)-5-amino-6-((2,3-bis(palmitoyloxy)propyl)thio)-1-hydroxy-3,4-dioxohexan-2-yl)amino)-Ser-Lys-Lys-Lys-Lys-Arg-Pro-Pro-Ala-Asp-Phe-Thr-Gln-Pro-Ala-Ala-Ser-Ala-Ala-Ala-Ala-Ala-NH_2 (4m)

Resin: Tentagel S RAM; **HPLC solution:** *t*BuOH:H_2O:MECN (1:1:1); 16% yield (12.54 mg, 4.04 µmol). **LC-MS:** R_t = 18.53 (Cosmosil 5C_4-MS (5 µm particle size, 150x4.6 mm), 10-90% MeCN, 21 min); **ESI-MS:** m/z 1622.2 $[M+2H^+]^{2+}$; **HRMS [M+4H]$^{4+}$:** $[C_{150}H_{267}N_{33}O_{34}S]^{4+}$ 776.74633 (measured), 776.74692 (calculated).



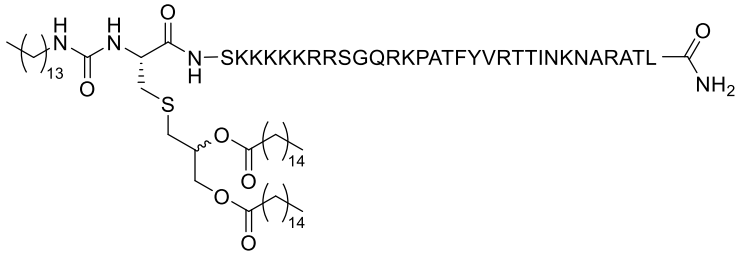
(((2S,5R)-5-amino-6-((2,3-bis(palmitoyloxy)propyl)thio)-1-hydroxy-3,4-dioxohexan-2-yl)amino)-Ser-Lys-Lys-Lys-Lys-Ser-Glu-Ala-Ser-Glu-Trp-Glu-Pro-Hys-Ala-Val-Tyr-Phe-Leu-Val-Leu-Asp-Asp-Val-Asn-Pro-Ser-NH_2 (4n)

Resin: Tentagel S RAM; **HPLC solution:** *t*BuOH:H_2O:MECN (1:1:1); 5% yield (4.88 mg, 1.19 µmol). **LC-MS:** R_t = 12.47 (Vydac 219TP 5 µm (150x4.6mm) Diphenyl, 10-90% MeCN, 21 min); **ESI-MS:** m/z 1365.4 $[M+3H^+]^{3+}$; **HRMS [M+4H]$^{4+}$:** $[C_{199}H_{327}N_{39}O_{50}S]^{4+}$ 1023.84824 (measured), 1023.84857 (calculated).



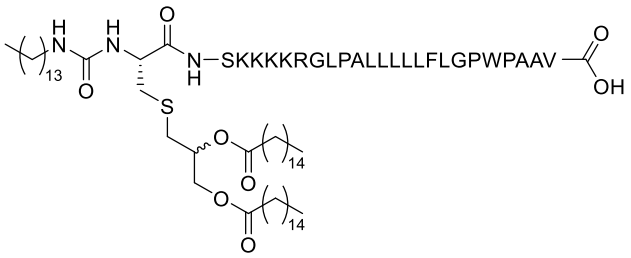
(((2S,5R)-5-amino-6-((2,3-bis(palmitoyloxy)propyl)thio)-1-hydroxy-3,4-dioxohexan-2-yl)amino)-Ser-Lys-Lys-Lys-Lys-Lys-Ile-Asp-Arg-Glu-Gly-Lys-Pro-Arg-Lys-Val-Ile-Gly-Cys-Ser-Cys-Val-Val-Val-Lys-Asp-Tyr-Gly-Lys-Glu-NH_2 (4o)

Resin: Tentagel S RAM; **HPLC solution:** *t*BuOH:H_2O:MECN (1:1:1); 9% yield (9.75 mg, 2.34 µmol). **LC-MS:** R_t = 6.01 (Phenomenex Gemini® 3 µm C18 110 Å 50x4.6 mm, 10-90% MeCN, 10 min); **ESI-MS:** m/z 1247.3 $[M+3H^+]^{3+}$; **HRMS [M+7H]$^{7+}$:** [C164H292N51O46S]$^{7+}$ 534.88357 (measured), 534.88212 (calculated).



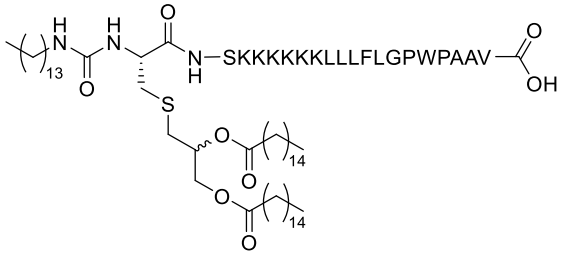
(((2S,5R)-5-amino-6-((2,3-bis(palmitoyloxy)propyl)thio)-1-hydroxy-3,4-dioxohexan-2-yl)amino)-Ser-Lys-Lys-Lys-Lys-Lys-Arg-Arg-Ser-Gly-Gln-Arg-Lys-Pro-Ala-Thr-Phe-Tyr-Val-Arg-Thr-Thr-Ile-Asn-Lys-Asn-Ala-Arg-Ala-Thr-Leu-NH_2 (4p)

Resin: Tentagel S RAM; **HPLC solution:** *t*BuOH:H_2O:MECN (1:1:1); 4% yield (4.65 mg, 1.03 µmol). **LC-MS:** R_t = 11.676 (Vydac 219TP 5 µm (150x4.6mm) Diphenyl, 10-90% MeCN, 21 min); **ESI-MS:** m/z 1132.5 $[M+3H^+]^{3+}$; **HRMS [M+8H]$^{8+}$:** $[C_{211}H_{379}N_{59}O_{47}S]^{8+}$ 566.49241 (measured), 566.49241 (calculated).



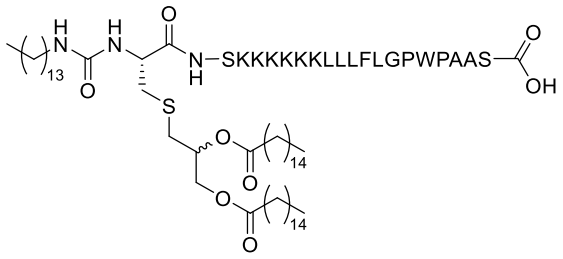
(((2S,5R)-5-amino-6-((2,3-bis(palmitoyloxy)propyl)thio)-1-hydroxy-3,4-dioxohexan-2-yl)amino)-Ser-Lys-Lys-Lys-Lys Arg-Gly-Leu-Pro-Ala-Leu-Leu-Leu-Leu-Leu-Phe-Leu-Gly-Pro-Trp-Pro-Ala-Ala-Val-OH (4q)

Resin: Tentagel PHB resin; **HPLC solution:** DMSO; 8% yield (7.33 mg, 2.09 µmol). **LC-MS:** R_t = 18.596 (Vydac 219TP 5 µm (150x4.6mm) Diphenyl, 10-90% MeCN, 21 min); **ESI-MS:** m/z 1756.2 $[M+2H^+]^{2+}$; **HRMS $[M+4H]^{4+}$:** $[C_{181}H_{319}N_{35}O_{31}S]^{4+}$ 877.85358 (measured), 877.85400 (calculated).



(((2S,5R)-5-amino-6-((2,3-bis(palmitoyloxy)propyl)thio)-1-hydroxy-3,4-dioxohexan-2-yl)amino)-Ser-Lys-Lys-Lys-Lys-Lys-Lys-Leu-Leu-Leu-Phe-Leu-Gly-Pro-Trp-Pro-Ala-Ala-Val-OH (4r)

Resin: Tentagel PHB resin; **HPLC solution:** DMSO; 26% yield (19.71 mg, 6.47 µmol). **LC-MS:** R_t = 16.731 (Vydac 219TP 5 µm (150x4.6mm) Diphenyl, 10-90% MeCN, 21 min); **ESI-MS:** m/z 1523.7 $[M+2H^+]^{2+}$; **HRMS $[M+3H]^{3+}$:** $[C_{160}H_{279}N_{27}O_{27}S]^{3+}$ 1015.70495 (measured), 1015.70601 (calculated).



(((2S,5R)-5-amino-6-((2,3-bis(palmitoyloxy)propyl)thio)-1-hydroxy-3,4-dioxohexan-2-yl)amino)-Ser-Lys-Lys-Lys-Lys-Lys-Lys-Leu-Leu-Leu-Phe-Leu-Gly-Pro-Trp-Pro-Ala-Ala-Ser-OH (4s)

Resin: Tentagel PHB resin; **HPLC solution:** DMSO; 13% yield (9.89 mg, 3.26 µmol). **LC-MS:** R_t = 16.610 (Phenomenex Gemini® 3 µm C18 110 Å 50x4.6 mm, 10-90% MeCN, 10 min); **ESI-MS:** m/z 1517.7 $[M+2H^+]^{2+}$; **HRMS $[M+3H]^{3+}$:** $[C_{158}H_{278}N_{27}O_{28}S]^{3+}$ 1011.69175 (measured), 1011.63988 (calculated).

UPam D1 Titration (work of B.J. Hos)

D1 cells at 80% confluency in growth factor-supplemented supernatant were harvested and used for serial dilution cultures. 5 x 10^4 D1 cells were incubated in complete medium supplemented with UPam and UPam-conjugates, starting at 2,5 µM and in 5x dilution steps. After overnight incubation, cells

were harvested and fluorescently stained for flowcytometry. Expression levels of CD40 and CD86 was analyzed by FlowJo vX.

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Chapter 3

Synthesis and evaluation of stereo-chemically defined UPam derivatives as novel TLR2 ligands

Introduction

Upon the recognition of PAMPs, TLR2 forms heterodimers with co-receptors TLR1 and TLR6 on the cell surface. Among the array of these PAMPs are fragments of lipoproteins and the specificity of their recognition by one of the heterodimers is dependent on the degree of acylation. While diacylated ligands binds to TLR2/TLR6, TLR2/TLR1 heterodimers recognize the corresponding triacylated ones with, respectively, Pam_2Cys[1-4] and Pam_3Cys[5-6] as the most often pursued agonists. Although the native form of Pam_3Cys (or 2,3-tris(palmitoyloxy)propyl]cysteine) contains a glycerol moiety of R-configuration, most research employs diastereomeric mixtures of Pam_3Cys. However, already in 1983 *Wiesmüller et al.*[5] suggested that Pam_3Cys with a R-configured glycerol moiety displayed a higher agonistic activity than its S-configured counterpart. Later, in 2000 *Takeuchi et al.*[7] demonstrated by quantifying the release of TNF-α, NO and IL-6 in murine macrophages and TNF-α, MCP-1 and IL-8 in human monocytes, an approximately 100-fold difference in activity between the Pam_2Cys epimers. Most of the agonistic activity shown by (S)-Pam_2Cys-MALP in the experiments was explained by a trace level contamination with the active R-epimer, implying an inability of the S-epimer to activate TLR2/TLR6 at all. *Khan et al.*[8] made similar observations when stimulating DCs with Pam_3Cys-peptide epitope conjugates and monitoring upregulation of CD40, CD86 and IL-12 production. Furthermore, priming T-cell with S-configured conjugate resulted in significantly less INF-γ and tetrameric positive $CD8^+$ T-cells.

After these publications R-configured Pam_2- or Pam_3Cys starts appearing more frequently in literature as the ligand of choice.[9-14] Structure activity studies based on Pam_3CSK_4[15] led to 1-tetradecyl-urea-Cys((RS)-2,3-di(palmitoyloxy)-propyl)-Ser-Lys-Lys-Lys-Lys, otherwise known as UPam, as the next generation TLR2 ligand, in which the methylene at the α position of the carboxyl in the N-terminal palmitoyl chain is replaced with an amine, while the glycerol moiety was left racemic.[16-17] The design of UPam was inspired by the crystal structure of the TLR2/TLR1 co-crystallized with Pam_3CSK_4,[18] by assuming that the introduction of a NH hydrogen-bond donor could lead to the formation of an additional hydrogen bridge with the Phe312 residue in the peptide backbone of TLR1. Consequently, this could increase binding affinity of the ligand which translates into a higher potency, providing a more potent ligand for the TLR2/TLR1 heterodimer. Additionally, substitution of the serine with other natural or unnatural amino acids was investigated. Activity was determined by following the upregulation of the biomarkers IL12p40, CD40, CD86 and MHC class II as a representation of DC maturation. Results indicated that substitutions by diaminobutyric acid (Dab) or by amino acid residues having small side chains were beneficial to activity while bulky side chains largely suppressed upregulation of the biomarkers. It was postulated that the favorable side chains fitted in a small cavity of the TLR2 subunit, thereby improving binding activity of the ligands. Furthermore, incorporation of D-serine (D-Ser) diminished the potency of the UPam ligand.

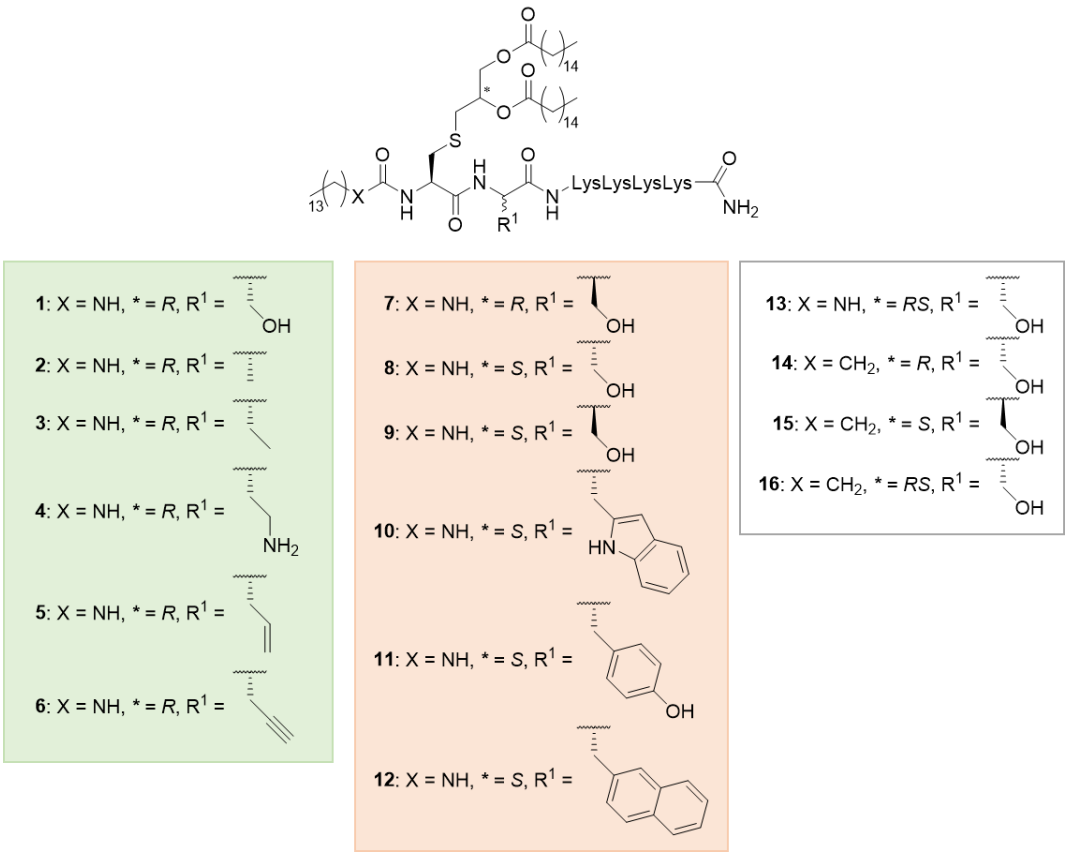
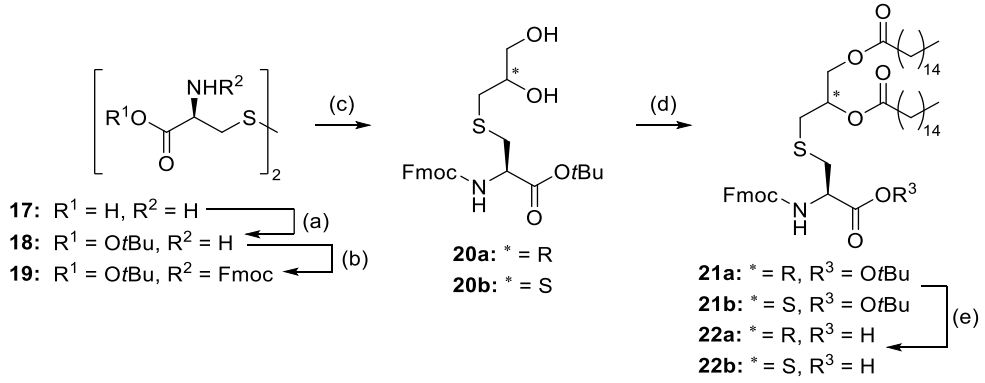


Figure 1: All synthesized target UPam derivatives sorted in group based on their expected influence on agonistic activity. Amino acids in the green box should result in a UPam derivative with good or better agonistic activity, whereas the red box represents activity blocking modifications. The white box depicts all reference compounds that were synthesized.

With the objective to improve the potency of the UPam ligand, a small library of chiral pure derivatives was designed (See Figure 1). To test the current paradigm, three groups of modifications can be discerned based on their expected influence on the agonistic activity: beneficial, detrimental and controls. Beneficial modifications include different small side chain amino acids substituting for Ser in the original UPam (**1**) with **DAB** as the most promising substitution (in compound **4**). The group of potentially less active candidates contains amino acid residues with bulky side chains, such as tryptophan **10,** tyrosine **11** and 1-naphtyl alanine **12**, but also D-serine **7** and **9.** As relevant positive controls *R*-$Pam_3CysCSK_4$ (**14**) was used together with UPam and Pam_3CSK_4 prepared as the mixtures of the *R*- and *S*-configuration at the glyceryl moiety (compounds **13** and **16**, respectively) and the "double epimer" of Pam_3CSK_4 with *S*-configuration at both the glyceryl moiety and the serine residue was synthesized as a negative control.

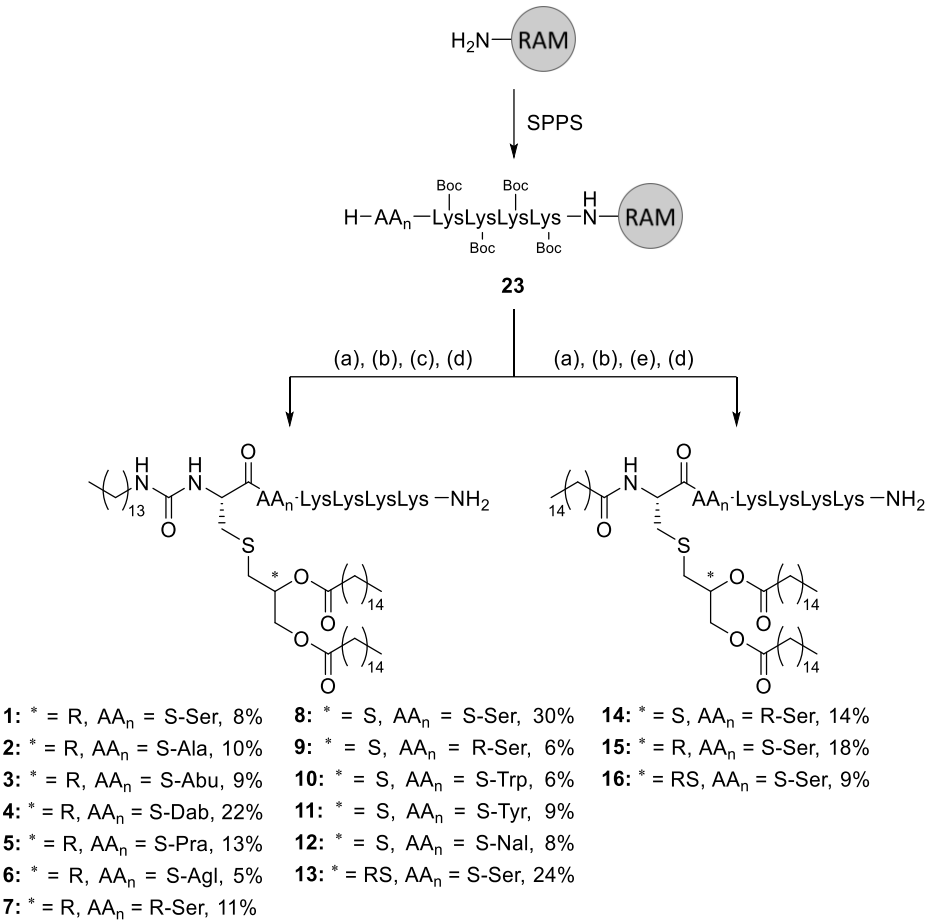
Results and Discussion

Fmoc based SPPS chemistry was chosen as synthesis strategy to enable assembly of all UPam derivatives. First, the synthesis of both the *R*- and *S*-configured dipalmitoyl glycerol functionalized cysteine building blocks **22a** and **22b** from commercially available L-cystine (**17**) was undertaken (See Scheme 1). The synthesis started with the protection of the free carboxylic acid in **17** as *tert*-butyl ester **18** using *tert*-butylacetate and perchloric acid. Reacting amine **18** with Fmoc-OSu in the presence of NMM as a base provided fully protected cystine **19** in a 56% yield over two steps.



Scheme 1: Synthesis of building blocks **22a** and **22b**. Reagents and conditions: (a) *t*BuOAc, $HClO_4$, RT, 48h, 62%; (b) Fmoc-OSu, NMM, DCM, RT, overnight, 90%; (c) Zn, MeOH, H_2SO_4, HCl, (R)-glycidol or (S)-glycidol, RT, overnight, **20a**: 80%, **20b**: 87%; (d) palmitic acid, EDC·HCl, DMAP, DCM, RT, overnight, **21a**: 89%, **21b**: 70%; (e) TFA/DCM, RT, 1h, **22a**: 84% , **22b**: 81%.

Next, the disulphide bond was reduced under influence of Zn and the resulting thiol was immediately alkylated with either *R*- or *S*-glycidol, following the reported procedures in literature,[20-22] to give **20a** and **20b**, respectively in excellent yields. Subsequent EDC mediated condensation of the alcohol functions with palmitic acid gave fully protected building blocks **21a** and **21b**. Deprotection of the *tert*-butyl ester with 1:1 mixture of TFA and DCM yielded blocking blocks **22a** and **22b** in an overall yield of 33% and 28%, respectively.



Scheme 2: Synthesis of UPam derivatives. Reagents and conditions: (a) Fmoc-Cys((RS)-2,3-di(palmitoyloxy)-propyl)-OH (**22a** or **b** or as a mixture of epimers) or Palmitoyl-Cys((RS)-2,3-di(palmitoyloxy)-propyl)-OH, HCTU, DiPEA, RT, overnight (b) 20% piperidine in DMF, RT, 3x 3 min; (c) tetradecyl isocyanate, DCM, RT, overnight; (d) TFA:TIS:H_2O 95:2.5:2.5, RT, 105 min; (e) palmitoyl chloride, DCM, RT, overnight.

With the Fmoc protected building blocks **22a**, **22b** and the suitably protected commercially available amino acids available, the target library of chiral pure UPam derivatives (See Figure 1) was assembled using Tentagel S Rink amide as solid support. The Fmoc based SPPS elongation cycle consisted of the use of HCTU in presence of D*i*PEA as coupling reagent and 20% piperidine in DMF as deprotection solution. Elongation of the resin with four lysines to give the H-Lys(Boc)-Lys(Boc)-Lys(Boc)-Lys(Boc)-Tentagel S RAM resin was performed fully automatically with a peptide synthesizer on 250 µmol scale. The assembly of the target UPam derivatives was continued manually and in the next step the amino acids of choice were incorporated on 25 or 50 µmol scale to give the array of immobilized peptides **23** that can be used to obtain derivatives of both Pam_3CysSK_4 and UPam targets. After removal of the Fmoc group by 20% piperidine in DMF, each resin **23** was condensed with the appropriate cysteine building block, that is **22a**, **22b** or **22** as epimeric mixture, under the influence of HCTU and D*i*PEA overnight at room temperature. Caution was needed as the dipalmitoyl glycerol moiety can be eliminated when the activated palmitoyl-Cys((RS)-2,3-di(palmitoyloxy)-propyl)-OH building block is exposed to an excess of D*i*PEA.[23-24] To prevent this, D*i*PEA (2 equivalents) was added sequentially in two equal portions with an interval of 15 minutes. Removal of the Fmoc

protecting group for all compounds, except for **16** since it uses Palmitoyl-Cys((RS)-2,3-di(palmitoyloxy)-propyl)-OH as commercially available building block, was conducted by treating the resin three times with a 20% solution of piperidine in DMF for 3 min. Reacting the liberated amine of thus obtained immobilized Pam_2Cys-peptides with palmitoyl chloride provided the immobilized and fully protected chiral pure Pam_3CysSK_4 **14** and **15**, whereas the immobilized UPam derivatives **1**-**13** were obtained by treatment of the corresponding Pam_2Cys-resins with tetradecyl isocyanate. Finally, all target TLR2 ligands **1**-**16** were obtained by deprotection and release from the resin using 95:2.5:2.5 TFA:H_2O:TIS as a final deprotection cocktail, precipitation and purification by RP-HPLC with either a C4 or diphenyl column.

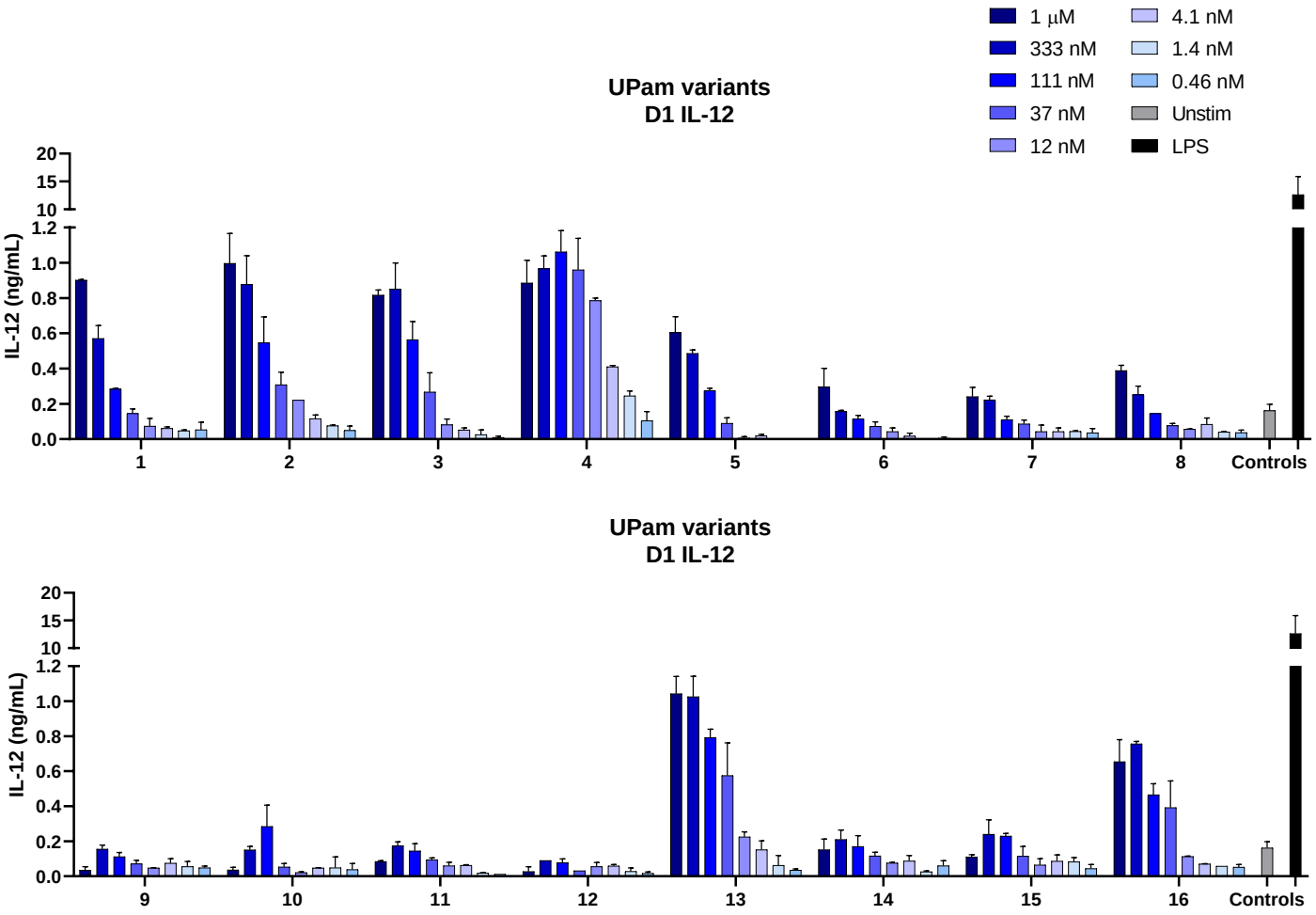


Figure 2: TLR2 activation of the UPam derivatives in D1-DCs, which were incubated with titrated quantities (1 µM – 0.46 nmol, 3-fold titration) of compounds **1**-**16** (Figure 1). LPS concentration was 2µM. After 1 day, supernatants were harvested and the production of IL12-p40 (data shown as mean ± SD, n = 2) was determined by specific ELISA.

The capability of all UPam derivatives to induce DC maturation was measured by quantifying immunostimulatory cytokine IL-12-p40 in murine D1 dendritic cells (D1-DCs). D1-DCs were selected for their close resemblance to freshly isolated bone marrow derived DCs plus their behaviour has been well characterized. The DCs were incubated for 24 h with a single UPam

derivative, after which the supernatant was harvested and analysed by ELISA (see Figure 2). The results largely confirm the hypothesis (*vide supra*, Figure 1) and follow the observations made by *Willems et al.*[12] and *Khan et al.*[4] R-configured derivatives elicit higher levels of IL12-p40 production, where compounds **2**, **3** and **4** surpass traditional chiral pure UPam **1**. As expected, R-UPam-Dab **4** had the highest agonistic activity. The introduction of the R-configured serine into the ligand instead of the native one reduced activity, as shown by derivative **7** and when additionally, the chirality of the glycerol is inverted, as in compound **9**, the agonistic activity is completely abolished. The importance of chirality is further underscored by the activity of reference compounds **14** and **15**, whose production of IL-12 is greatly diminished. Furthermore, combining an inverted glycerol moiety with bulky side chain groups kills the activity as demonstrated by UPam **10**, **11**, and **12**.

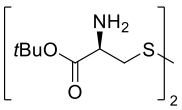
In conclusion, a structure-activity study toward a more active TLR2 ligand has been successfully completed by the synthesis and evaluation of a series UPam derivatives, the design of which was based on observations reported in the literature. The UPam derivatives were prepared by an efficient solid phase procedure and their ability to induce DC maturation was assessed by quantifying IL12-p40 production with ELISA. A more potent UPam derivative, namely compound R-UPam-(S)DabSK$_4$ **4**, was discovered whilst the outcome of the study also supported reported structure-activity relationships among the Pam$_3$CysSK$_4$ analogues. First, chirality of the glycerol moiety is critical for ligand activity and inverting this chiral centre abolishes activity. Second, replacement of the first serine at the N-terminal end by an amino acid with a small side chain is favorable while a bulky side chain is not tolerated and finally the *S*-configuration of this second amino acid after the cysteine head is essential.

Experimental

General experimental procedures building block synthesis

All reactions were performed at room temperature. All solvents used under anhydrous conditions were stored over 4Å molecular sieves. Solvents used for workup and column chromatography were of technical grade from Sigma Aldrich and used directly. Unless stated otherwise, solvents were removed by rotary evaporation under reduced pressure at 40°C. TLC monitoring was performed using Machery-Nagel DC-Fertigfolien ALUGRAM® Xtra SIL G/UV$_{254}$. Compounds were visualized by UV detection if applicable at 254 nm and by spraying with 1% $KMNO_4$ (aq.), followed by charring. Column chromatography was performed on Screening Devices Silica Gel 40 – 63 µm. Analytical LC-MS was conducted on an Agilent Technologies 6120 Quadrupple LC-MS system using a Vydac 219 TP diphenyl (5 µm particle size, 150x4.6 mm dimensions), Cosmosil 5C$_4$-MS (5 µm particle size, 150x4.6 mm dimensions) and Cosmosil 5C$_4$-MS (5 µm particle size, 50x4.6 mm dimensions) column. Solvent system for LC-MS: A: 100% H_2O, B: 100% MECN, C: 1% TFA. ^{1}H and ^{13}C NMR spectra were recorded with a Brüker AV-300(300/75 MHz), Brüker AV-400 (400/100 MHz) or Brüker AV-500 UltraShield™ (500/125 MHz). Chemical shifts (δ) are given in ppm relative to tetramethylsilane or residual solvent as internal standard and coupling constants are given in Hz.

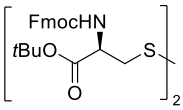
L-cystine bis(*tert* butyl ester) (18)



L-cystine **17** (101 mmol, 24.3 g, 1 eq.) was suspended in *tert*-butyl acetate (3.7 mol, 500 mL, 37 eq.). 70% perchloric acid aq. (57 mL) was added dropwise to the mixture at 0°C over 1 h. The resulting mixture was heated to room temperature and stirred for 2 days. A white solid crystallised out and was redissolved in 10% $NaHCO_3$ aq. (100 mL). $NaHKCO_3$ (s) was added until the mixture reached a pH of 9. The water layer was extracted with DCM (2x). The combined organic layers were dried with $MgSO_4$, filtered, and concentrated *in vacuo* yielding compound **2** (62.2 mmol, 21.9 g, 62%) as a yellow oil.

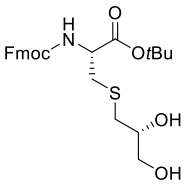
^{1}H NMR (300 MHz, $CDCl_3$) δ 3.69 (dd, *J* = 7.9, 4.6 Hz, 2H), 3.13 (dd, *J* = 13.4, 4.6 Hz, 2H), 2.88 (dd, *J* = 13.4, 7.9 Hz, 2H), 1.73 (s, 4H), 1.48 (s, 18H). **^{13}C NMR (75 MHz, $CDCl_3$)** δ 172.82, 81.75, 54.28, 44.04, 27.99.

***N,N*-bis-Fmoc-L-cystine bis(*tert* butyl ester) (19)**



A solution of NMM (11.6 mmol, 1.27 mL, 4.1 eq.) and compound **18** (2.82 mmol, 0.993 g, 1 eq.) in dry THF (20 mL) was brought under a N_2 atmosphere. Fmoc-OSu (6.82 mmol, 2.30 g, 2.4 eq.) was dissolved in 20 mL dry THF. This solution was added dropwise to the reaction mixture and then stirred overnight. Next, the reaction mixture was concentrated *in vacuo* and redissolved in a mixture of EtOAc/H_2O (60 mL, 5:1). The organic layer was separated from the water layer and was washed with H_2O (3x) and Brine (1x). The organic layer was dried with $MgSO_4$, filtered, and concentrated *in vacuo*. The crude product was purified by silica gel chromatography (0:1 → 2:3 EtOAc:pentane), yielding compound **19** (2.55 mmol, 2.03 g, 90%) as a white solid.

^{1}H NMR (300 MHz, $CDCl_3$) δ 7.73 (d, *J* = 7.4 Hz, 4H), 7.58 (d, *J* = 7.4 Hz, 4H), 7.37 (t, *J* = 7.4 Hz, 4H), 7.27 (t, *J* = 7.4 Hz, 4H), 5.77 (d, *J* = 7.6 Hz, 2H), 4.58 (dd, *J* = 12.7, 5.3 Hz, 2H), 4.34 (t, *J* = 9.2 Hz, 4H), 4.19 (t, *J* = 7.0 Hz, 2H), 3.31 – 3.10 (m, 4H), 1.47 (s, 18H). **^{13}C NMR (75 MHz, $CDCl_3$)** δ 169.43, 155.81, 143.87, 141.37, 127.79, 127.16, 125.25, 120.06, 83.21, 67.32, 54.26, 47.20, 41.99, 28.10.

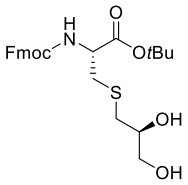


***N*-Fmoc-S-((R)-2,3-dihydroxypropyl)-L-cysteine *tert* butyl ester (20a)**

To a solution of compound **19** (8.37 mmol, 6.67 g, 1 eq.) in 67 mL DCM was added zinc powder (58.7 mmol, 3.84 g, 7 eq., <10µm). The solution was diluted with a mixture of MeOH/37% HCl aq./conc. H_2SO_4 aq. (29 mL, 100:7:1) and stirred for 15 min. Next, (R)-glycidol (20.9 mmol, 1.4 mL, 2.5 eq.) was added to the solution, which was stirred overnight. The progress of the reaction was followed by TLC analysis (EtOAc:pentane 6:4, R_f = 0.3). The reaction mixture was filtered and concentrated *in vacuo* to about a third of the original volume. Next, the reaction mixture was diluted with a mixture of 10% $KHSO_4$ aq./DCM (550 mL, 1:10) and washed with H_2O (1x). The organic layer was dried with $MgSO_4$, filtered, and concentrated *in vacuo*. The crude product was purified by column chromatography (1:9 → 6:4 EtOAc:pentane) yielding compound **20a** (13.4 mmol, 6.36 g, 80%) as a white solid.

^{1}H NMR (300 MHz, $CDCl_3$) δ 7.69 (d, *J* = 7.4 Hz, 2H), 7.57 (d, *J* = 7.1 Hz, 2H), 7.33 (t, *J* = 7.3 Hz, 2H), 7.26 (t, *J* = 7.5 Hz, 2H), 6.16 (d, *J* = 8.0 Hz, 1H), 4.48 (dd, *J* = 12.5, 5.6 Hz, 1H), 4.34 (d, *J* = 7.1 Hz, 2H), 4.17 (t, *J* = 6.9 Hz, 1H), 3.78 (s, 1H), 3.63 (dd, *J* = 11.1, 7.2 Hz, 1H), 3.51 (dd, *J* = 11.2, 6.2 Hz, 1H), 3.07 – 2.80 (m, 2H), 2.67 (ddd, *J* = 21.0, 13.6, 6.1 Hz, 2H), 1.44 (s, 9H). **^{13}C NMR (75 MHz, $CDCl_3$)** δ 169.78, 156.01, 143.58, 140.99, 127.57, 126.94, 124.99, 119.83, 82.76, 71.01, 67.04, 65.05, 54.44, 46.90, 36.13, 35.23, 27.82.

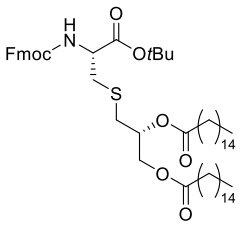
***N*-Fmoc-S-((S)-2,3-dihydroxypropyl)-L-cysteine *tert* butyl ester (20b)**



To a solution of compound **19** (1.94 mmol, 1.55 g, 1 eq.) in 67 mL DCM was added zinc powder (14.55 mmol, 0.95 g, 7.5 eq., <10 µm). The solution was diluted with a mixture of MeOH/37% HCl aq. /conc. H_2SO_4 aq. (7 mL, 100:7:1) and stirred for 15 min. Next, (S)-glycidol (5.0 mmol, 0.33 mL, 2.5 eq.) was added to the solution, which was stirred overnight. The progress of the reaction was followed by TLC analysis (EtOAc:pentane 1:1, R_f = 0.2). The reaction mixture was filtered and concentrated *in vacuo* to about a third of the original volume. Next, the reaction mixture was diluted with 0.9% $KHSO_4$ aq. (220 mL) and extracted with DCM (1x). The organic layer was dried with $MgSO_4$, filtered, and concentrated *in vacuo*. The crude product was purified by column chromatography (3:7 → 5:5, EtOAc:pentane) yielding compound **20b** (3.38 mmol, 1.66 g, 87%) as a white solid.

^{1}H NMR (300 MHz, $CDCl_3$) δ 7.70 (d, *J* = 7.3 Hz, 2H), 7.58 (d, *J* = 7.3 Hz, 2H), 7.34 (t, *J* = 7.1 Hz, 2H), 7.26 (t, *J* = 7.2 Hz, 2H), 6.21 (d, *J* = 8.1 Hz, 1H), 4.48 (dd, *J* = 13.3, 5.7 Hz, 1H), 4.35 (d, *J* = 7.2 Hz, 2H), 4.18 (t, *J* = 7.0 Hz, 1H), 3.94 (d, *J* = 4.3 Hz, 1H), 3.80 (d, *J* = 2.7 Hz, 1H), 3.59 (ddd, *J* = 14.2, 11.6, 6.1 Hz, 3H), 3.08 – 2.86 (m, 2H), 2.68 (ddd, *J* = 20.8, 13.6, 6.2 Hz, 2H), 1.45 (s, 9H). **^{13}C NMR (75 MHz, $CDCl_3$)** δ 169.69, 156.01, 143.58, 141.04, 127.51, 126.89, 124.96, 119.77, 82.59, 70.92, 66.99, 64.90, 54.43, 46.88, 36.05, 35.26, 27.76.

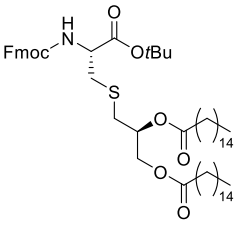
***N*-Fmoc-S-((R)-2,3-bis(palmitoyloxy)propyl)-L-cysteine *tert* butyl ester (21a)**



A solution of compound **20a** (13.4 mmol, 6.36 g, 1 eq.), palmitic acid (29.8 mmol, 7.63 g, 2.2 eq.), DMAP (3.22 mmol, 0.39 g, 0.24 eq.) and EDC·HCl (29.7 mmol, 5.69 g, 2.2 eq.) in 130 mL dry DCM was prepared under a N_2 atmosphere. The solution was stirred overnight. The progress of the reaction was followed by TLC analysis (Et_2O:pentane 4:6 R_f = 0.8). The reaction mixture was washed with 1M HCl aq. (1x), sat. $NaHCO_3$ aq. (3x) and Brine (1x). The organic layer was dried with $MgSO_4$, filtered, and concentrated *in vacuo*. The crude product was purified by column chromatography (5:95 → 3:7 Et_2O:pentane) yielding compound **21a** (12.0 mmol, 11.4 g, 89%).

^{1}H NMR (500 MHz, $CDCl_3$) δ 7.72 (d, *J* = 7.5 Hz, 2H), 7.60 (d, *J* = 7.4 Hz, 2H), 7.35 (t, *J* = 7.4 Hz, 2H), 7.28 (t, *J* = 7.4 Hz, 2H), 5.88 (d, *J* = 7.7 Hz, 1H), 5.17 (dd, *J* = 5.8, 3.6 Hz, 1H), 4.52 (dd, *J* = 12.4, 5.1 Hz, 1H), 4.43 – 4.28 (m, 3H), 4.21 (dd, *J* = 14.8, 7.6 Hz, 1H), 4.15 (dd, *J* = 11.9, 6.0 Hz, 1H), 3.05 (ddd, *J* = 19.3, 13.8, 5.0 Hz, 2H), 2.83 – 2.72 (m, 2H), 2.32 – 2.18 (m, 4H), 1.60 (ddd, *J* = 18.1, 14.1, 6.9 Hz, 4H), 1.53 – 1.40 (s, 9H), 1.37 – 1.16 (s, 48H), 0.88 (t, *J* = 6.9 Hz, 6H). **^{13}C NMR (125 MHz, $CDCl_3$)** δ 173.11, 172.85, 169.49, 155.72, 143.79, 141.22, 127.61, 126.98, 125.13, 125.09, 119.87, 82.73, 70.24, 67.17, 63.42, 54.37, 47.08, 35.21, 34.18, 34.10, 33.99, 33.88, 33.15, 31.92, 29.70, 29.66, 29.63, 29.48, 29.36, 29.28, 29.10, 29.08, 27.89, 24.86, 24.84, 24.77, 22.68, 22.32, 14.09, 14.02.

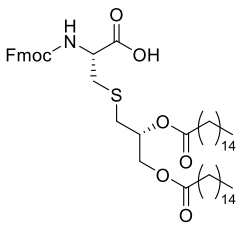
***N*-Fmoc-S-((S)-2,3-bis(palmitoyloxy)propyl)-L-cysteine *tert* butyl ester (21b)**



A solution of compound **20b** (3.38 mmol, 1.60 g, 1 eq.), palmitic acid (7.45 mmol, 1.91 g, 2.2 eq.), DMAP (0.80 mmol, 0.10 g, 0.24 eq.) and EDC·HCl (7.48 mmol, 1.44 g, 2.2 eq.) in 33 mL dry DCM was prepared under a N_2 atmosphere. The solution was stirred overnight. The progress of the reaction was followed by TLC analysis (Et_2O:pentane 4:6 R_f = 0.8). The reaction mixture was washed with 1M HCl aq. (1x), sat. $NaHCO_3$ aq. (3x) and Brine (1x). The combined organic layers were dried with $MgSO_4$, filtered, and concentrated *in vacuo*. The crude product was purified by column chromatography (5:95 → 3:7 Et_2O:pentane) yielding compound **21b** (1.42 mmol, 1.35 g, 42%).

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.71 (d, *J* = 7.5 Hz, 2H), 7.58 (dt, *J* = 30.2, 15.2 Hz, 2H), 7.35 (t, *J* = 7.4 Hz, 2H), 7.27 (t, *J* = 7.4 Hz, 2H), 5.93 (d, *J* = 7.8 Hz, 1H), 5.17 (dd, *J* = 5.8, 3.6 Hz, 1H), 4.53 (dd, *J* = 12.5, 5.1 Hz, 1H), 4.43 – 4.28 (m, 3H), 4.21 (t, *J* = 7.2 Hz, 1H), 4.13 (dd, *J* = 11.9, 6.0 Hz, 1H), 3.04 (ddd, *J* = 36.1, 13.8, 5.0 Hz, 2H), 2.76 (d, *J* = 6.4 Hz, 2H), 2.28 – 2.18 (m, 4H), 1.66 – 1.51 (m, 4H), 1.45 (d, *J* = 23.2 Hz, 9H), 1.36 – 1.16 (m, 48H), 0.88 (t, *J* = 6.8 Hz, 6H). **^{13}C NMR (100 MHz, $CDCl_3$)** δ 173.05, 172.77, 169.48, 155.68, 143.74, 141.17, 127.56, 126.94, 125.09, 119.82, 82.66, 70.27, 67.15, 63.37, 54.31, 47.03, 35.15, 34.13, 33.94, 33.87, 33.01, 31.89, 29.67, 29.63, 29.60, 29.45, 29.34, 29.25, 29.06, 29.04, 27.85, 24.80, 24.72, 22.65, 14.07.

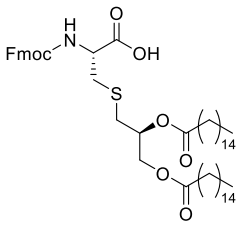
***N*-Fmoc-S-((R)-2,3-bis(palmitoyloxy)propyl)-L-cysteine (22a)**



A solution of compound **21a** (12.0 mmol, 11.4 g) in a mixture of DCM:TFA (14 mL, 2:5) was prepared and the reaction mixture was stirred for 60 min. The mixture was concentrated *in vacuo* and co-evaporated with toluene (2x). The crude product was purified by column chromatography (15:85 Et_2O:pentane → 3:1 Et_2O:pentane + 1% acetic acid) yielding compound **22a** (10.1 mmol, 9.02 g, 84%) as a white solid.

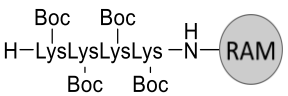
^{1}H NMR (300 MHz, $CDCl_3$) δ 8.00 (s, 1H), 7.75 (d, *J* = 7.4 Hz, 2H), 7.60 (d, *J* = 7.3 Hz, 1H), 7.39 (t, *J* = 7.4 Hz, 2H), 7.30 (td, *J* = 7.4, 0.8 Hz, 2H), 5.83 (d, *J* = 7.7 Hz, 1H), 5.16 (d, *J* = 3.4 Hz, 1H), 4.65 (d, *J* = 6.2 Hz, 1H), 4.46 – 4.28 (m, 3H), 4.23 (t, *J* = 7.0 Hz, 1H), 4.15 (dd, *J* =11.9, 6.1 Hz, 1H), 3.11 (ddd, *J* = 19.8, 13.9, 5.2 Hz, 2H), 2.73 (t, *J* = 19.0 Hz, 2H), 2.30 (dd, *J* = 13.4, 7.4 Hz, 4H), 1.70 – 1.49 (m, 4H), 1.28 (s, 48H), 0.88 (t, *J* = 6.7 Hz, 6H). **^{13}C NMR (75 MHz, $CDCl_3$)** δ 174.13, 173.71, 173.60, 156.15, 143.80, 141.41, 127.88, 127.23, 125.27, 120.11, 70.41, 67.60, 63.71, 53.82, 47.21, 34.77, 34.44, 34.23, 33.13, 32.06, 29.85, 29.81, 29.65, 29.50, 29.43, 29.25, 25.04, 25.00, 22.82, 14.25.

***N*-Fmoc-S-((S)-2,3-bis(palmitoyloxy)propyl)-L-cysteine (22b)**



A solution of compound **21b** (2.36 mmol, 2.24 g) in a mixture of DCM:TFA (13 mL, 2:3) was prepared and the reaction was stirred for 60 min. The mixture was concentrated *in vacuo* and co-evaporated with toluene (2x). The crude product was purified by column chromatography (15:85 Et_2O:pentane → 3:1 Et_2O:pentane + 1% acetic acid) yielding compound **22b** (1.92 mmol, 1.72 g, 81%) as a white solid.

^{1}H NMR (300 MHz, $CDCl_3$) δ 8.10 (s, 1H), 7.75 (d, *J* = 7.4 Hz, 2H), 7.66 – 7.52 (m, 2H), 7.39 (t, *J* = 7.2 Hz, 2H), 7.30 (td, *J* = 7.4, 1.1 Hz, 2H), 5.84 (d, *J* = 7.8 Hz, 1H), 5.23 – 5.08 (m, 1H), 4.72 – 4.61 (m, 1H), 4.36 (dd, *J* = 15.7, 5.0 Hz, 3H), 4.23 (t, *J* = 7.0 Hz, 1H), 4.14 (dd, *J* = 11.9, 6.2 Hz, 1H), 3.12 (dd, *J* = 14.1, 5.1 Hz, 2H), 2.89 – 2.64 (m, 2H), 2.29 (td, *J* = 7.6, 3.3 Hz, 4H), 1.56 (dd, *J* = 21.8, 16.1 Hz, 4H), 1.27 (s, 48H), 0.88 (t, *J* = 6.7 Hz, 6H. **^{13}C NMR (75 MHz, $CDCl_3$)** δ 174.11, 173.76, 173.52, 156.11, 143.79, 141.41, 127.87, 127.22, 125.26, 120.11, 70.39, 67.60, 63.75, 53.67, 47.20, 34.85, 34.43, 34.23, 32.97, 32.06, 29.84, 29.81, 29.65, 29.50, 29.43, 29.27, 25.03, 24.99, 22.82, 14.25.



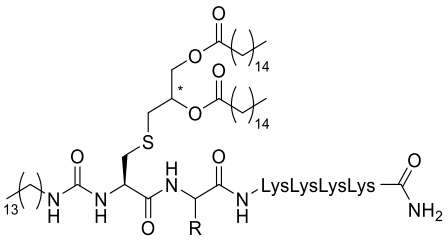
H-Lys(Boc)-Lys(Boc)-Lys(Boc)-Lys(Boc)-Tentagel S Ram

H-Lys(Boc)-Lys(Boc)-Lys(Boc)-Lys(Boc)-Tentagel S RAM resin was prepared using a TRIBUTE® Peptide Synthesiser applying Fmoc chemistry for 250 µmol Tentagel S RAM resin. The resin was deprotected by treating the resin three times with 20% piperidine in NMP (4 mL) for 3 min and afterwards washed

with NMP (3 x 5 mL). Then, four lysines were coupled onto the resin repeating for four times the following cycle:

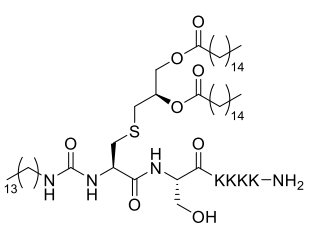
(1) Treating the resin with a solution of Fmoc-Lys(Boc)-OH (1 mmol, 468.35 mg, 4 eq.), HCTU (1 mmol, 413.69 mg, 4 eq.) and D*i*PEA (2 mmol, 348 µmol, 8 eq.) in NMP (4 mL) for 1 h. Afterwards the resin was washed with NMP (3 x 5 mL);
(2) The resin was capped by treating the resin to a solution of 10% Ac_2O and 5% NMM in NMP (5 mL) for 3 min. Afterwards the resin was washed with NMP (3 x 5 mL);
(3) The Fmoc-group was cleaved by treating the resin three times with a solution of 20% piperidine in NMP (4 mL) for 3 min**.** Afterwards the resin was washed with NMP (3 x 5 mL);

After these four cycles the resin was washed with DMF (3 x 4 mL) and DCM (3 x 4 mL). Finally, the resin was dried under a N_2 flow and stored dry in a closed reaction syringe.



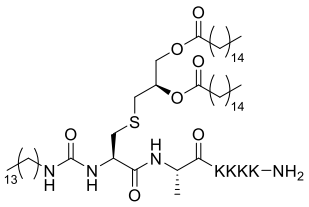
General procedure for the synthesis of 1-tetradecyl-urea-Cys((R)-2,3-di(palmitoyloxy)-propyl)-AA-Lys-Lys-Lys-Lys-NH_2

A reaction syringe was charged with 25 µmol of H-Lys(Boc)-Lys(Boc)-Lys(Boc)-Lys(Boc)-Tentagel-S-RAM resin and a solution of HCTU (50 µmol, 20.7 mg, 2 eq.), D*i*PEA (100 µmol, 17,4 µL, 4 eq.) and the appropriate amino acid (50 µmol, 2 eq.) in DMF (0.75 mL). The syringe was shaken for 2 h, drained, washed with DMF (3x), DCM (3x) and dried using a N_2 flow. Next, the resin was treated three times with 20% piperidine in DMF for 5 min and afterwards washed with DMF (3x) and DCM (3x). A solution of HCTU (50 µmol, 20.7 mg, 2 eq.), D*i*PEA (50 µmol, 17,4 µL, 2 eq.) and with the appropriate cysteine building block (50 µmol, 2 eq.) in DMF (0.75 mL) was added to the dried resin and shaken for 15 min. A second portion of D*i*PEA (50 µmol, 17,4 µL, 2 eq.) was added to the reaction syringe and left shaking overnight. The following morning the reaction syringe was drained and the resin was washed with DMF (3x), DCM (3x) and dried using a N_2 flow. Finally, a solution of tetradecyl isocyanate (225 µmol, 62.5 µL, 9 eq.) in 1:1 DCM:NMP (2.5 mL) was added to the resin and the syringe was shaken overnight. The resin was washed with DMF (3x), DCM (3x) and dried using a N_2 flow. The lipopeptide was cleaved and deprotected by addition of a 95:2.5:2.5 TFA:TIS:H_2O solution (3 mL) and shaking it for 105 min. The deprotected lipopeptide was precipitated by adding the cleavage cocktail to a 1:1 Et_2O:pentane solution (46 mL). TFA (1 mL) was used to wash the resin and also added to the Et_2O:pentane solution, which was stored at -40 ˚C. The precipitate was spun down, the organic mixture decanted, and the remaining pallet was dried with a N_2 flow. The dry pallet was dissolved in 1.5 mL of 1:1:1 HO*t*Bu:H_2O:MeCN and purified by HPLC.



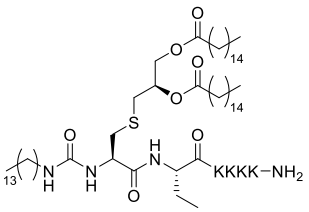
1-tetradecyl-urea-Cys((R)-2,3-di(palmitoyloxy)-propyl)-(S)Ser-Lys-Lys-Lys-Lys-NH_2 (1)

Building block: **22a**. 2.91 mg (1.93 µmol, 8%); **LC-MS:** R_t = 12.72 (Vydac 219TP 5 µm (150x4.6mm) Diphenyl, 10-90% MeCN, 15 min); **ESI-MS:** *m/z* 1509.83 $[M+H]^+$; **HRMS [M+3H]$^{3+}$:** $[C_{80}H_{161}N_{12}O_{12}S]^{3+}$ 504.06353 (measured), 504.06349 (calculated).



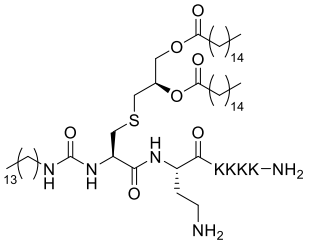
1-tetradecyl-urea-Cys((R)-2,3-di(palmitoyloxy)-propyl)-(S)Ala-Lys-Lys-Lys-Lys-NH_2 (2)

Building block: **22a**. 3.91 mg (2.62 µmol, 10%); **LC-MS:** R_t = 12.79 (Vydac 219TP 5 µm (150x4.6mm) Diphenyl, 10-90% MeCN, 15 min); **ESI-MS:** *m/z* 1494.92 $[M+H^+]$; **HRMS $[M+3H]^{3+}$:** $[C_{80}H_{156}N_{12}O_{11}S]^{3+}$ 498.73199 (measured), 498.73185 (calculated).



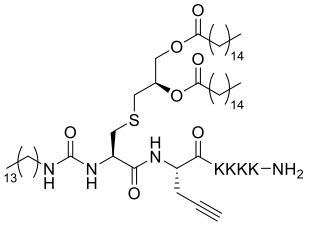
1-tetradecyl-urea-Cys((R)-2,3-di(palmitoyloxy)-propyl)-(S)Abu-Lys-Lys-Lys-Lys-NH_2 (3)

Building block: **22a**. 3.36 mg (2.23 µmol, 9%); **LC-MS:** R_t = 12.88 (Vydac 219TP 5 µm (150x4.6mm) Diphenyl, 10-90% MeCN, 15 min); **ESI-MS:** *m/z* 1508.83 $[M+H^+]$; **HRMS $[M+3H]^{3+}$:** $[C_{81}H_{158}N_{12}O_{11}S]^{3+}$ 503.40391 (measured), 503.40373 (calculated).



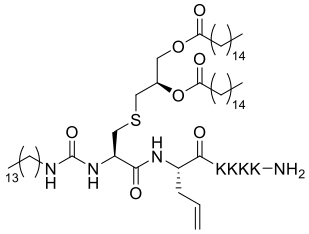
1-tetradecyl-urea-Cys((R)-2,3-di(palmitoyloxy)-propyl)-(S)Dab-Lys-Lys-Lys-Lys-NH_2 (4)

Building block: **22a**. 8.35 mg (5.48 µmol, 22%); **LC-MS:** R_t = 17.01 (Cosmosil 5C_4-MS (5 µm particle size, 150x4.6 mm), 10-90% MeCN, 15 min); **ESI-MS:** *m/z* 1524.2 $[M+H^+]$; **HRMS $[M+3H]^{3+}$:** $[C_{81}H_{159}N_{13}O_{11}S]^{3+}$ 508.40705 (measured), 508.40737 (calculated).



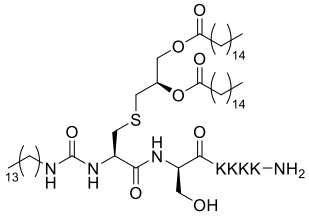
1-tetradecyl-urea-Cys((R)-2,3-di(palmitoyloxy)-propyl)- (S)Pra-Lys-Lys-Lys-Lys-NH_2 (5)

Building block: **22a**. 4.82 mg (3.17 µmol, 13%); **LC-MS:** R_t = 12.76 (Vydac 219TP 5 µm (150x4.6mm) Diphenyl, 10-90% MeCN, 15 min); **ESI-MS:** *m/z* 1517.83 $[M+H^+]$; **HRMS $[M+2H]^{2+}$:** $[C_{82}H_{156}N_{12}O_{11}S]^{2+}$ 759.59327 (measured), 759.59414 (calculated).



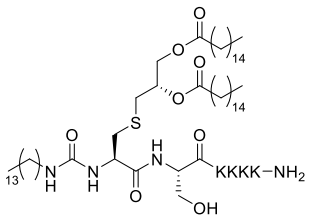
1-tetradecyl-urea-Cys((R)-2,3-di(palmitoyloxy)-propyl)-(S)Agl-Lys-Lys-Lys-Lys-NH_2 (6)

Building block: **22a**. 1.93 mg (1.27 µmol, 5%); **LC-MS:** R_t = 12.77 (Vydac 219TP 5 µm (150x4.6mm) Diphenyl, 10-90% MeCN, 15 min); **ESI-MS:** *m/z* 1521.2 $[M+H^+]$; **HRMS $[M+3H]^{3+}$:** $[C_{164}H_{292}N_{51}O_{46}S]^{7+}$ 534.88357 (measured), 534.88212 (calculated).



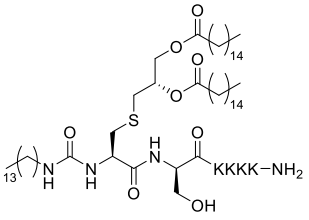
1-tetradecyl-urea-Cys((R)-2,3-di(palmitoyloxy)-propyl)- (R)Ser-Lys-Lys-Lys-Lys-NH_2 (7)

Building block: **22a**. 4.00 mg (2.65 µmol, 11%); **LC-MS:** R_t = 12.63 (Vydac 219TP 5 µm (150x4.6mm) Diphenyl, 10-90% MeCN, 15 min); **ESI-MS:** *m/z* 1509.83 $[M+H^+]$; **HRMS $[M+3H]^{3+}$:** $[C_{80}H_{161}N_{12}O_{12}S]^{7+}$ 504.06345 (measured), 504.06349 (calculated).



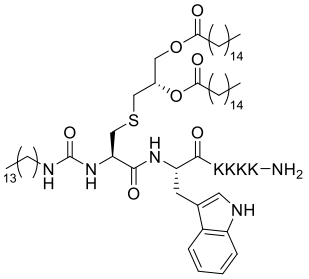
1-tetradecyl-urea-Cys((S)-2,3-di(palmitoyloxy)-propyl)-(S)Ser-Lys-Lys-Lys-Lys-NH_2 (8)

Building block: **22b**. 11.21 mg (7.42 µmol, 30%); **LC-MS:** R_t = 17.57 (Cosmosil 5C_4-MS (5 µm particle size, 150x4.6 mm), 10-90% MeCN, 15 min); **ESI-MS:** *m/z* 1510.2 [M+H$^+$]; **HRMS [M+3H]$^{3+}$:** [$C_{80}H_{161}N_{12}O_{12}S$]$^{3+}$ 504.06334 (measured), 504.06349 (calculated).



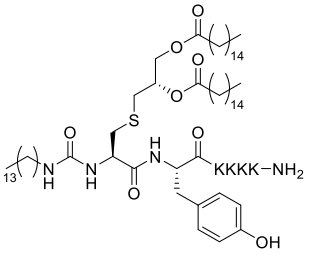
1-tetradecyl-urea-Cys((S)-2,3-di(palmitoyloxy)-propyl)-(R)Ser-Lys-Lys-Lys-Lys-NH_2 (9)

Building block: **22b**. 2.34 mg (1.55 µmol, 6%); **LC-MS:** R_t = 12.57 (Vydac 219TP 5 µm (150x4.6mm) Diphenyl, 10-90% MeCN, 15 min); **ESI-MS:** *m/z* 1510.2 [M+H$^+$]; **HRMS [M+3H]$^{3+}$:** [$C_{80}H_{161}N_{12}O_{12}S$]$^{3+}$ 504.06353 (measured), 504.06349 (calculated).



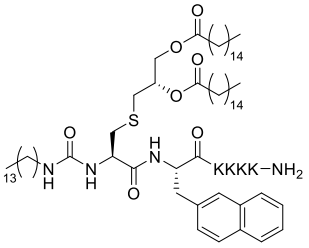
1-tetradecyl-urea-Cys((S)-2,3-di(palmitoyloxy)-propyl)-(S)Trp-Lys-Lys-Lys-Lys-NH_2 (10)

Building block: **22b**. 2.58 mg (1.60 µmol, 6%); **LC-MS:** R_t = 12.12 (Vydac 219TP 5 µm (150x4.6mm) Diphenyl, 10-90% MeCN, 15 min); **ESI-MS:** 1609.2 *m/z* [M+H$^+$]; **HRMS [M+3H]$^{3+}$:** [$C_{88}H_{161}N_{13}O_{11}S$]$^{3+}$ 537.07929 (measured), 537.07925 (calculated).



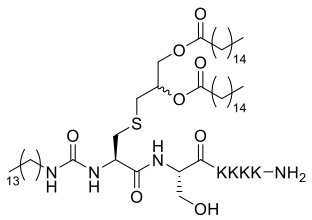
1-tetradecyl-urea-Cys((S)-2,3-di(palmitoyloxy)-propyl)-(S)Tyr-Lys-Lys-Lys-Lys-NH_2 (11)

Building block: **22b**. 3.69 mg (2.33 µmol, 9%); **LC-MS:** Rt = 12.00 (Vydac 219TP 5 µm (150x4.6mm) Diphenyl, 10-90% MeCN, 15 min); **ESI-MS:** *m/z* 1586.2 [M+H$^+$]; **HRMS [M+3H]$^{3+}$:** [$C_{86}H_{160}N_{12}O_{12}S$]$^{3+}$ 529.40701 (measured), 529.40726 (calculated).



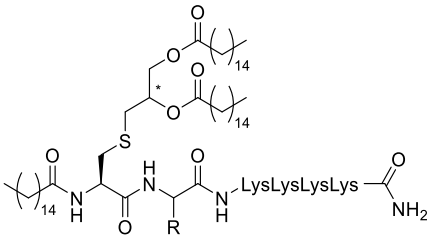
1-tetradecyl-urea-Cys((S)-2,3-di(palmitoyloxy)-propyl)-(S)Nal-Lys-Lys-Lys-Lys-NH_2 (12)

Building block: **22b**. 3.37 mg (2.08 µmol, 8%); **LC-MS:** R_t = 12.25 (Vydac 219TP 5 µm (150x4.6mm) Diphenyl, 10-90% MeCN, 15 min); **ESI-MS:** *m/z* 1621.3 [M+H$^+$]; **HRMS [M+3H]$^{3+}$:** [$C_{90}H_{162}N_{12}O_{11}S$]$^{3+}$ 540.74760 (measured), 540.74760 (calculated).



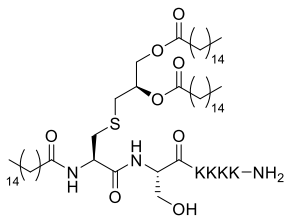
1-tetradecyl-urea-Cys((RS)-2,3-di(palmitoyloxy)- propyl)-(S)Ser-Lys-Lys-Lys-Lys-NH_2 (13)

Building block: Fmoc-Cys((RS)-2,3-di(palmitoyloxy)-propyl)-OH. 8.88 mg (5.88 µmol, 24%); **LC-MS:** R_t = 17.53 (Cosmosil 5C_4-MS (5 µm particle size, 150x4.6 mm), 10-90% MeCN, 15 min); **ESI-MS:** *m/z* 1510.2 [M+H^+]; **HRMS [M+3H]$^{3+}$:** [$C_{80}H_{161}N_{12}O_{12}S$]$^{3+}$ 504.06331 (measured), 504.06349 (calculated).



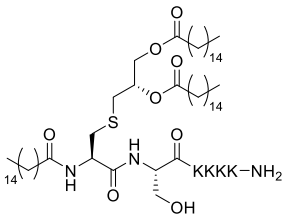
General procedure for the synthesis palmitoyl-Cys((R)-2,3-di(palmitoyloxy)-propyl)-AA-Lys-Lys-Lys-Lys-NH_2

A reaction syringe was charged with 25 µmol of H-Lys(Boc)-Lys(Boc)-Lys(Boc)-Lys(Boc)-Tentagel-S-RAM resin and a solution of HCTU (50 µmol, 20.7 mg, 2 eq.), D*i*PEA (100 µmol, 17,4 µL, 4 eq.) and the appropriate amino acid (50 µmol, 2 eq.) in DMF (0.75 mL). The syringe was shaken for 2 h, drained, washed with DMF (3x), DCM (3x) and dried using a N_2 flow. Next, the resin was treated three times with 20% piperidine in DMF for 5 min and afterwards washed with DMF (3x) and DCM (3x). A solution of HCTU (50 µmol, 20.7 mg, 2 eq.), D*i*PEA (50 µmol, 17,4 µL, 2 eq.) and with the appropriate cysteine building block (50 µmol, 2 eq.) in DMF (0.75 mL) was added to the dried resin and shaken for 15 min. A second portion of D*i*PEA (50 µmol, 17,4 µL, 2 eq.) was added to the reaction syringe and left shaking overnight. The following morning, the resin of compound **15** and **16** was three times treated with 20% piperidine in DMF for 5 min and afterwards washed with DMF (3x) and DCM (3x). After the deprotection, the resin of **15** and **16** was treated with palmitoyl chloride (225 µmol, 62.5 µL, 9 eq.) in DCM (2.5mL) was added to the resin and the syringe was shaken overnight. Finally, all lipopeptides were cleaved and deprotected by addition of a 95:2.5:2.5 TFA:TIS:H_2O solution (3 mL) and shaking it for 105 min. The deprotected lipopeptide was precipitated by adding the cleavage cocktail to a 1:1 Et_2O:pentane solution (46 mL). TFA (1 mL) was used to wash the resin and also added to the Et_2O:pentane solution, which was stored at -40 ˚C. The precipitate was spun down, the organic mixture decanted, and the remaining pallet was dried with a N_2 flow. The dry pallet was dissolved in 1.5 mL of 1:1:1 HO*t*Bu:H_2O:MeCN and purified by HPLC.



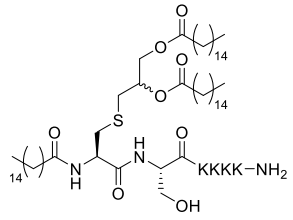
Palmitoyl-Cys((R)-2,3-di(palmitoyloxy)-propyl)-(S)Ser-Lys-Lys-Lys-Lys-NH_2 (14)

Building block: **22a**. 6.98 mg (4.62 µmol, 18%); **LC-MS:** R_t = 18.95 (Cosmosil 5C_4-MS (5 µm particle size, 150x4.6 mm), 10-90% MeCN, 15 min); **ESI-MS:** *m/z* 1509.2 [M+H^+]; **HRMS [M+3H]$^{3+}$:** [$C_{81}H_{157}N_{11}O_{12}S$]$^{3+}$ 503.73190 (measured), 503.73174 (calculated).



Palmitoyl-Cys((S)-2,3-di(palmitoyloxy)-propyl)-(R)Ser-Lys-Lys-Lys-Lys-NH_2 (15)

Building block: **22b**. 5.47 mg (3.62 µmol, 14%); **LC-MS:** R_t = 18.73 (Cosmosil 5C_4-MS (5 µm particle size, 150x4.6 mm), 10-90% MeCN, 15 min); **ESI-MS:** *m/z* [M+H^+]; **HRMS [M+3H]$^{3+}$:** [$C_{81}H_{157}N_{11}O_{12}S$]$^{3+}$ 503.73165 (measured), 503.73174 (calculated).



Palmitoyl-Cys((RS)-2,3-di(palmitoyloxy)-propyl)-(S)Ser-Lys-Lys-Lys-Lys-NH_2 (16)

Building block: Palmitoyl-Cys((RS)-2,3-di(palmitoyloxy)-propyl)-OH. 3.47 mg (2.30 µmol, 9%); **LC-MS:** R_t = 9.51 (Cosmosil 5C_4-MS (5 µm particle size, 50x4.6 mm), 10-90% MeCN, 15 min); **ESI-MS:** *m/z* 1509.2 [M+H^+]; **HRMS [M+3H]$^{3+}$:** [$C_{81}H_{157}N_{11}O_{12}S$]$^{3+}$ 503.73207 (measured), 503.73174 (calculated).

In vitro D1 maturation assay (work of M.G.M. Camps)

The growth factor dependent dendritic cell line D1 was maintained in non-TC treated culture dishes (Greiner Bio-One) in complete IMDM (Lonza) containing 50 µM β-mercaptoethanol (Sigma-Aldrich), 2mM GlutaMAX (Gibco), 80 IU/mL penicillin, and 10% FCS (Sigma-Aldrich), supplemented with 30% conditioned supernatant derived from murine GM-CSF producing NIH/3T3 cells. UPam derivatives were titrated in 96-well flat bottom plates (Corning, Amsterdam, The Netherlands).[25] D1 cells were seeded on top of the derivatives at 50.000 cells/well. The plate was subsequently incubated for 24 h at 37 °C. The supernatant was taken from the wells for IL12p40 ELISA analysis in Nunc Maxisorb ELISA plates (Thermo Scientific) using Purified anti-mouse IL-12/IL-23 p40 clone C15.6 (Biolegend) as capture antibody and Biotin anti-mouse IL-12/IL-23 p40 clone C17.8 (Biolegend) as detection antibody.

References

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Chapter 4

Synthetic PamCys-peptide conjugates as self-adjuvanting vaccines for future personalized immunotherapies

Introduction

Cancer vaccines aim to induce specific immune responses directed against patients' tumours. Activation of specific T-cells is crucial since they detect and destroy malignant cells by recognition of tumour-expressed antigens.[1-3] A highly specific class of tumour antigens are neoantigens[1-2] which are the result of somatic DNA mutations in tumour cells, translating into amino acid residue changes and antigenic peptides. Since these antigens are uniquely expressed on the tumour cells of a patient, these neoepitopes offer an ideal target for personalized cancer immunotherapy and have shown to be more immunogenic than widely expressed tumour antigens.[1, 3] The induction of anti-tumour T-cell responses has been reported by vaccination with synthetic peptides (SPs) encoding defined amino acid sequences

of various tumour antigens.[1-5] However, SPs are only weakly immunogenic and require an adequate adjuvant to provoke a potent immune response that is able to clear tumours.[1] It was reported that conjugation of a SP with an optimized Toll-like receptor (TLR) 2 ligand is an effective strategy to induce functional T-cell responses. Therapeutic vaccination with TLR2 ligands conjugated to SPs, in which the peptide sequence embedded either model or oncoviral tumour antigens, resulted in tumour clearance and increased survival in multiple tumour-bearing mouse models.[6-7] The TLR2 ligand UPam has also been conjugated to SPs containing the oncogenic antigen sequences of the human papillomavirus (HPV). These constructs were able to efficiently activate *ex vivo* human T-cells derived from patients with HPV positive cervical cancer.[8]

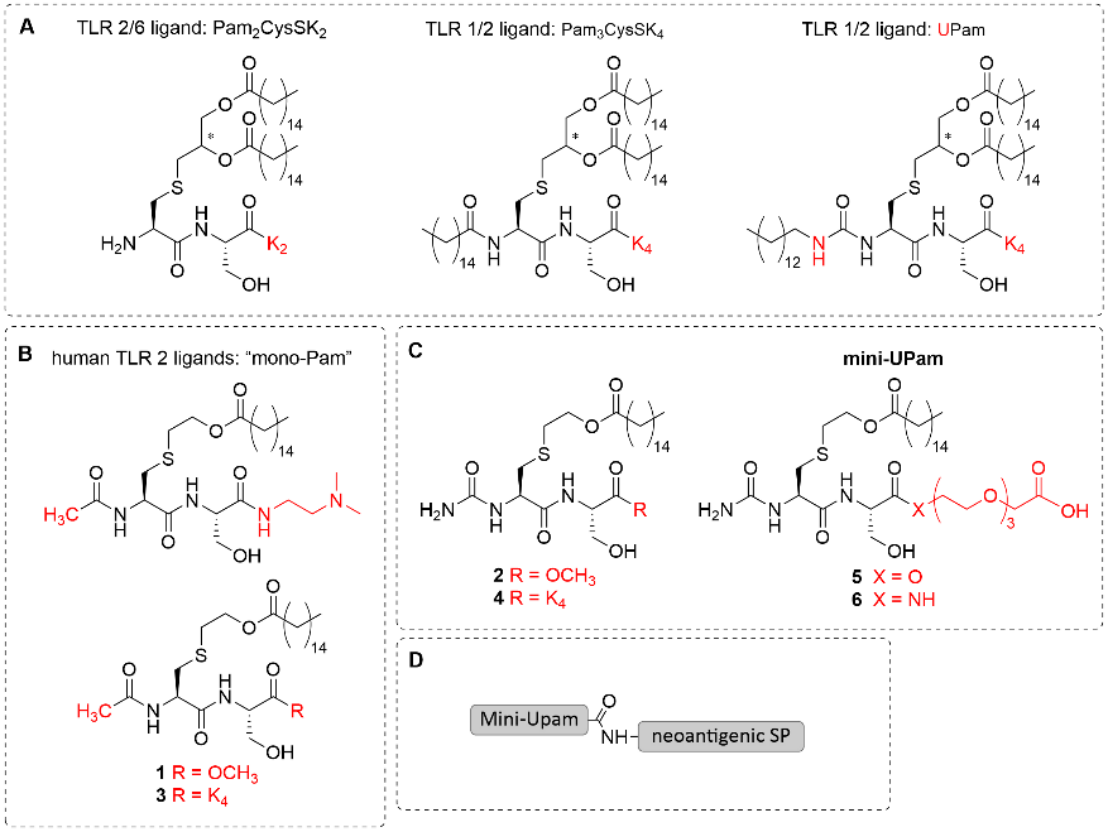


Figure 1. Structures of TLR2 ligands and the projected conjugates. **A**: Highly lipophilic di- and tripalmitoyl TLR2 ligands previously reported; **B**: mono-palmitoyl derivatives with improved solubility and TLR2-activating potency reported previously; **C**: Ureido-monopalmitoyl TLR2 ligands developed in this work; **D**: Design of the mini-UPam-synthetic peptide conjugates.

For the development of new adjuvants with known and improved properties, agonists of pattern recognition receptors (PRRs) such as TLRs, NOD-like receptors (NLRs) and C-type lectin receptors (CLRs) are intensively investigated.[9-12] For some PRRs, synthetic structurally well-defined agonistic ligands with a relatively small molecular weight have been discovered, which have been used to elicit defined innate immune responses for several purposes.[13] In particular, the ligands for TLR2 have been extensively subjected to structure-activity relationship (SAR) studies, resulting in the often applied Pam_3CSK_4 and Pam_2CSK_4 ligands (Figure 1A) respectively for the TLR2/TLR1 and TLR2/TLR6 heterodimer combinations.[14-18]

Interestingly, small molecule ligands prove to be suitable for incorporation in conjugates, in which a peptide epitope is covalently connected to a structurally defined TLR ligand.[19-24] These conjugates outperformed, in terms of immunological properties, mixtures of the non-covalently linked components.[7,25] Thus, the widely used TLR2 ligand Pam_3CysSK_4, which contains a tetralysine linker (K_4), combined with an antigenic peptide in a conjugate (TLR2L-SP) has shown to induce functional T-cell responses.[21] Therapeutic vaccination with a TLR2L-SP, in which the SP encoded a model tumour antigen, resulted in tumour clearance and increased survival of tumour-bearing mice in several mouse models.[7, 22] In the context of these studies a new TLR2 ligand (UPam, Figure 1A) was found, which upon incorporation in a conjugate with an antigenic SP induced functional T-cell responses.[7] Upam was conjugated to SPs containing antigen sequences of the oncogenic HPV. These constructs were able to efficiently activate human T-cells derived from HPV positive tumour-draining lymph nodes *ex vivo* and are currently used in a phase I clinical vaccination study in $HPV16^+$ cancer patients.[8, 26] These favourable properties were an incentive to prepare and evaluate TLR2L-neoantigen conjugates. However, covalent linking of a great variety of antigenic peptides to a lipophilic TLR2 ligand is not always feasible due to solubility problems during synthesis and final preparation as a vaccine. This could hamper the production of personalized cancer vaccines in which a short production time is essential.

To tackle the solubility issue, attention was directed to a new design of the conjugates in which the lipophilicity of the TLR2 ligand is minimized while its linker to the antigenic peptide would further improve solubility. Importantly, these modifications should not be detrimental for the TLR2-activating activity of the ligand. SAR studies by David and co-workers resulted in a relatively simple TLR2 ligand (Figure 1B) which contains only one lipophilic tail and is not only water soluble but also human TLR2 (hTLR2) specific.[27-30] SAR studies by Filippov and co-workers on TLR2 ligands showed that the replacement of the amide by a urea moiety at the N-terminal amine of the Cys residue led to the more potent ligand $UPam_3CysSK_4$.[6] It was decided to combine structural features of mono-palmitoyl hTLR2 ligand with the outcome of these studies, resulting in the replacement of the acetyl group at the N-terminal amine of the cysteine in the hTLR2 ligand by a urea moiety, to give a new ureido TLR2 ligand **2** (Figure 1C). To ultimately obtain effective conjugates, three linkers were tested to allow covalent attachment of this new ureido TLR2 ligand to the neoantigen peptides. The conventional tetralysine linker K_4 (resulting in **4**) and a triethylene glycol linker, connected to new TLR2 ligand **2** via an ester (resulting in **5**) or amide bond (resulting in **6**) were selected.[31] Triethylene glycol was selected as a linker to limit the complexity and to minimize the size of the conjugate. It is known that such a linker could be inserted between $Pam_3CysSer$ and an antigenic peptide without detriment to the immunogenicity of the construct.[32] Ligand **1**, previously reported by David and co-workers and its derivative **3** provided with tetra lysine linker K_4 were taken as relevant references. DC maturation by ligands **1-6** was assessed by quantifying IL-8 and hIL-12 production of TLR2 transfected human embryonic kidney cells (HEK293), which was presented in the thesis of G.P.P. Gential (Leiden, 2018). Based solely on

IL-8 production, tetralysine was the worst candidate of the three spacers, and an ureido group as N-terminal modification was preferred over an acetyl. Both triethylene glycol functionalized $Pam_3CysSer$ outperformed the others, and linking the spacer (i.e. **5**) through an ester group gave slightly higher IL-8 and hIL-12 production.

Table 1: The target peptide sequences including their assigned epitopes and mutations

Entry	Sequence	Epitope type
a	KIDREGKPRKVIGCSCVVVKDYGKE	Human MHC II
b	KRRSGQRKPATFYVRTTINKNARATL	Human MHC I
c	KADPFPPNGAPPLKPHPLMPANPWG	Human MHC I
d	RGLPALLLLLFLGPWPAAV	Human MHC I
e	KKLLLFLGPWPAAV	Human MHC I
f	KKLLLFLGPWPAAS	Human MHC I

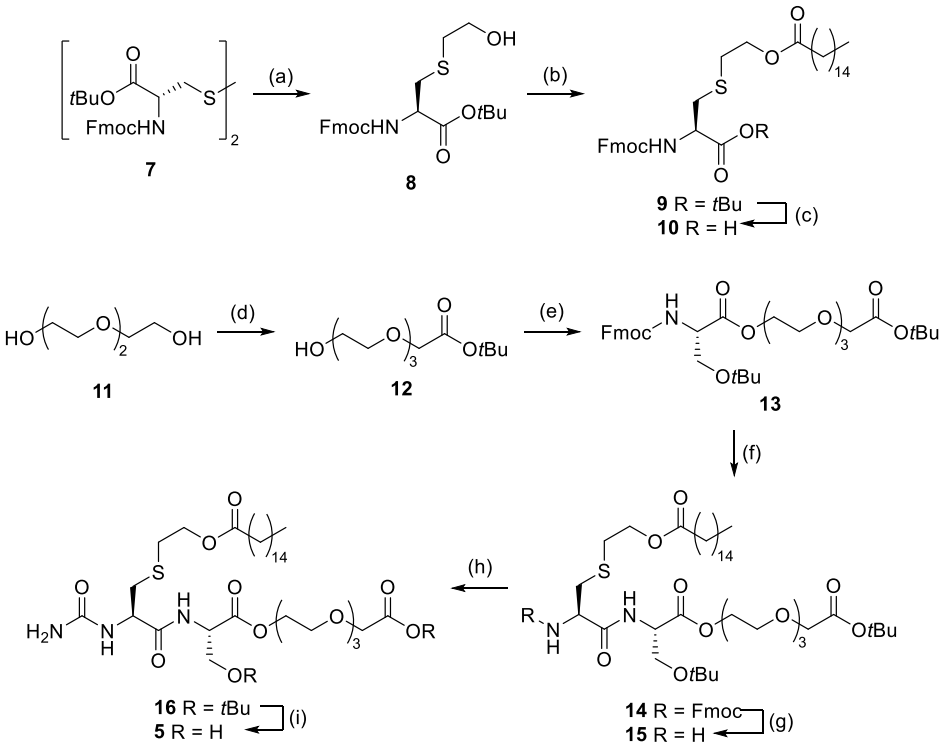
When the peptide is embedded with a single defined epitope, the epitope is colored blue. The mutated amino acid is colored red.

This chapter presents the synthesis of two sets of conjugates in which human antigen containing peptides are covalently connected to TLR2 ligand **5**, named Mini-Upam. The first set (**a-c**, Table 1) contain three neoepitopes originating from the successful treatment of a melanoma patient using adaptive cell transfer.[33-34] The other set (**d-f**, Table 1) consist out of a three peptide sequences containing two T-cell neoepitopes associated with peptide processing.[35] Two of the peptides sequences of the first set of conjugates where compared with their Upam conjugate counterparts and a mixture of the free peptide and free compound **5** by following IL-8 production in HEK-TLR2 cells and IL-12 production in monocyte-derived DCs (moDCs). These novel conjugates demonstrated to be superior in induction of DC maturation compared to the lipophilic UPam lipopeptides and elicit effective neoepitope-specific human $CD8^+$ T-cell as well as $CD4^+$ T-cells activation.

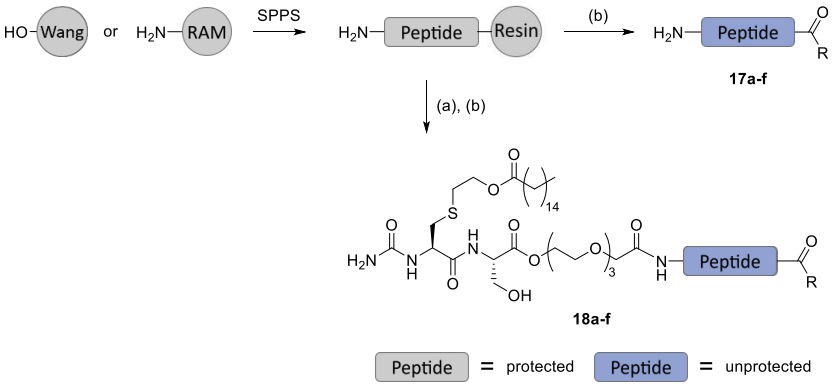
Results and Discussion

The synthesis of novel TLR2 ligand **5** is depicted in Scheme 1 using triethylene glycol **11** and cystine **7** as starting compounds. Fully protected cystine **7** was converted to palmitoylated cysteine **10** by the following sequence of reactions. First, the disulfide bond in cystine **7** was quickly reduced with Zn and strong acid in MeOH, after which oxirane was added to the reaction mixture and stirred overnight giving alcohol **8** in 89% yield. Next, the free alcohol in **8** was condensed with palmitic acid under influence of EDC·HCl, D*i*PEA and DMAP as nucleophilic catalyst. Deprotection of the *tert*-butyl with a 1:1 mixture TFA and DCM yielded palmitoylated cysteine **10** in an overall yield of 64%. The next building block (**13**) was prepared by alkylation of triethylene glycol **11** with tert-butyl bromoacetate and subsequent condensation with Fmoc-Ser(*t*Bu)-OH. With building blocks **10** and **13** in hand, PamCysSer derivative **5** could be synthesized. In a one pot reaction, the Fmoc group in **13** was cleaved with DBU in DMF, quenched with an equal amount of HOBt and directly condensed with building block **10** using EDC·HCl and HOBt as coupling reagents producing protected PamCys **14** in 95% yield. The cleavage of the Fmoc group with DBU and 1-octanethiol as scavenger gave free amine **15**. Functionalization of the amine with trimethylsilyl isocyanate gave partially protected intermediate **16**

in a yield of 74%. Finally, removal of the remaining tert-butyl group with TFA in the presence of TIS and purification by silica gel column chromatography yielded water-soluble urea ligand **5** in an overall yield of 11% starting from triethylene glycol.

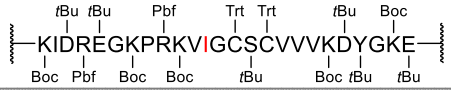
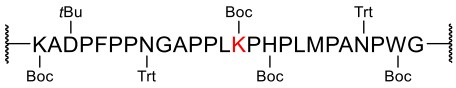


Scheme 1: Synthesis of palmitoyl functionalized cysteine **10** and the subsequent synthesis of mini-UPam **5**. Reaction conditions: (a) i) Zn, H_2SO_4 (aq), HCl (aq), MeOH, RT, 15 min; ii) Oxirane, RT, overnight, 89%; (b) EDC·HCl, DiPEA, DMAP, Palmitic acid, DCM, RT, overnight, 76%; (c) 1:1 DCM:TFA, RT, 30 min, 95%; (d) *t*-butyl bromoacetate, NaH, TBAI, THF, overnight, 40%; (e) Fmoc-Ser(*t*Bu)-OH, EDC·HCl, DMAP, DCM, RT, overnight 97%; (f) i) DBU, THF, RT; ii) Compound **10**, HOBt, EDC·HCl, RT, 95%; (g) DBU, 1-octanethiol, DCM, RT, 2 h, 99%; (h) TMS isocyanate, *i*-propanol, DCM, RT, 40 h, 74%; (i) TFA:TIS:H2O 95:2.5:2.5, RT, 55%.



Scheme 2. Synthesis of neoantigen-TLR2 ligand conjugates using SPPS conditions. Reaction conditions: (a) Compound **5**, HCTU, D*i*PEA, NMP or DMF, overnight; (b) 95:2.5:2.5 TFA:TIS:H_2O, 105 min.

Table 2: Structures of all protected peptides and the resin used for their assembly. Yields for both peptides and mini-Upam conjugates are given after the sequence.

	Peptide	Resin	R	Yield	
				17	18
a		Rink-Amide	NH_2	5%	2%
b		Rink-Amide	NH_2	5%	3%
c		Rink-Amide	NH_2	6%	7%
d		Wang	OH	36%	4%
e		Wang	OH	39%	9%
f		Wang	OH	21%	5%
g		Rink-Amide	NH_2	8%	10%

When the peptide is embedded with a single defined epitope, the epitope is colored blue. The mutated amino acid is colored red.

Neoepitope carrying peptides **17a-g** were selected for conjugation to mini-Upam **5** to give mini-UPam conjugates **18a-g** (for sequences see Table 2). As shown in Scheme 2, all conjugates **18a-g** and the associate reference peptides **17a-g** were assembled with SPPS using either Wang or Rink amide resin and a standard Fmoc-based protocol. After completion of the SPPS, part of the resin was subjected to the standard deprotection cocktail (TFA:TIS:H_2O) and the released crude peptides were purified by HPLC to give peptides **17a-g.** Another part of the resin was used for the installation of Mini-UPam at the N-terminal end of the immobilized peptide by HCTU mediated condensation of building block **5** with the free amino group of the peptide. The conjugates were deprotected and released from the solid support by treatment with the standard deprotection (TFA:TIS:H_2O) cocktail and finally purified by RP-HPLC to give mini-UPam conjugates **18a-g**.

The TLR2-stimulating potency of the SP-mini-UPam conjugates **18a** and **18b** together with Upam conjugates **19a** and **19b** as references (their synthesis is described in Chapter 2) was first evaluated in HEK293 cells that were stably transfected with the human TLR2 gene (HEK-TLR2). Titration of the free ligands (**5** and Upam) as well as the $CD8^+$ (**18a**, **19a**) and $CD4^+$ (**18b**, **19b**) T-cell epitope conjugates resulted in the production of IL-8 (Figure 3A), whereas no IL-8 production was observed in HEK293 cells that lacked hTLR2 expression, indicating retained biological activity of mini-UPam upon conjugation to a SP. Both free mini-UPam ligand **5** and

mini-UPam containing conjugates (**18a**,**b**) outperformed their UPam counterparts (**19a**,**b**), demonstrating mini-Upam as superior ligand as adjuvant for vaccines. Next, the ability to mature human DCs was tested by titration of both conjugated neoepitopes and incubation of moDCs. Prior to their use, the upregulation of CD11c and loss of CD14 expression was

determined to ensure successful differentiation of the monocytes into moDCs. After 36 hours of incubation the production of IL-12p40, a Th1-inducing cytokine (Figure 3B), and the upregulation of the maturation markers CD86 was determined (Figure 3C). Both the conjugate **18b** and conjugate **18a** were able to efficiently mature moDCs and the conjugation did not compromise the bioactivity of the UPam.

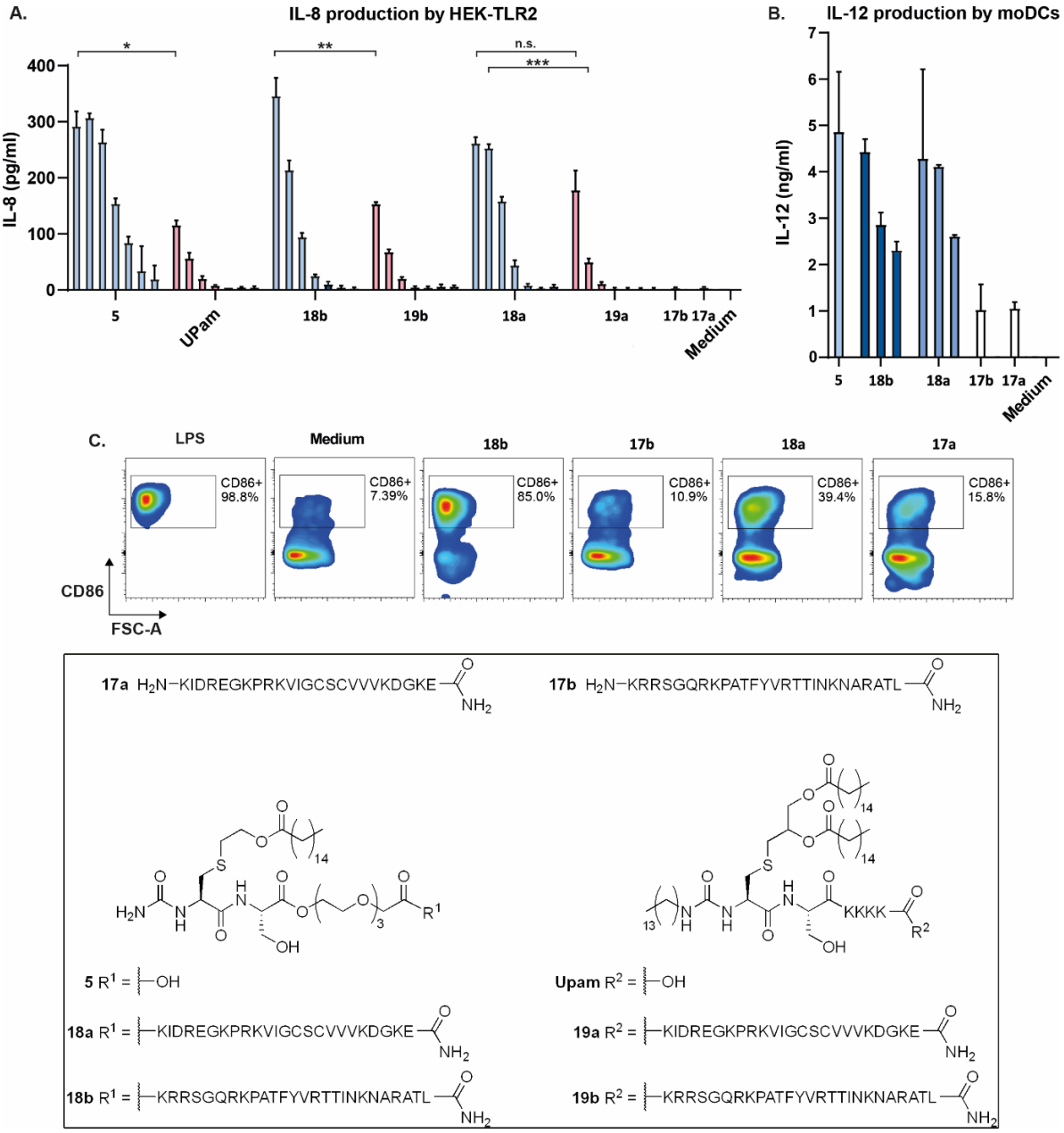


Figure 3. SP-mini-UPam conjugates efficiently target the human TLR2 receptor resulting in moDC activation and maturation. The synthesis of compounds **19a** and **19b** can be found in Chapter 2 **(A)** IL-8 production determined in the supernatant of HEK-TLR2 cells after 48 h incubation with compound **5**, UPam, **18a**, **18b**,

19a and **19b** (concentration range: 1 µM; 200 nM; 40 nM; 8 nM; 1.6 nM; 320 pM; 64 pM, 5-fold titration) and SPs **17a** and **17b** 1 µM concentration. **(B)** IL-12p40 production by moDCs. Concentration **5** 16 nM; concentration conjugates **18a** and **18b** 10 µM; 400 nM; 16 nM and SP **17a** and **17b** 10 µM. **(C)** Upregulation of the maturation marker CD86 by human moDCs after 36 h of incubation with the indicated compounds (concentration LPS 1 µg/mL; conjugates and SP 10 µM) (data shown as mean ± SD, n = 3). ** p < 0.01, ***p<0.001, determined by multiple T-test with Bonferroni-Dunn correction for multiple comparison.

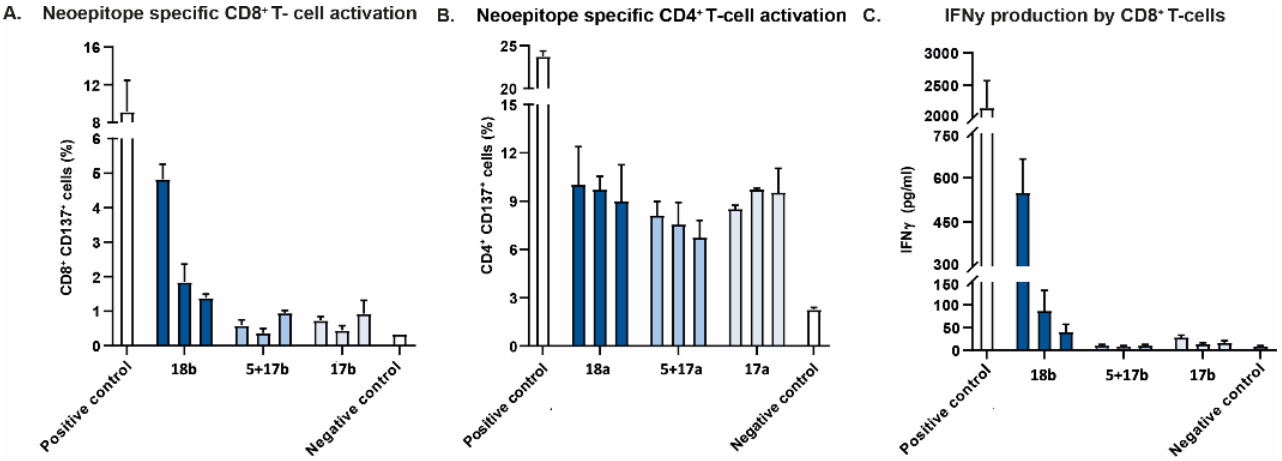


Figure 4. Activation of neoepitope-specific human $CD8^+$ and $CD4^+$ T-cells by the SP-mini-UPam conjugates. Percentages of total $CD8^+$ **(A)** and $CD4^+$ T-cells **(B)** expressing the activation marker CD137 after overnight co-culture with monocytes loaded with conjugate **18b** or **18b,** free **5** with either SP **17a** or **17b** mixed or the SP **17a** or **17b** alone (concentration range: 2; 1; 0.5 µM). Positive controls were a SP containing the minimal $CD8^+$ T-cell epitope (1 µg/mL) **(A)** or a SP containing the $CD4^+$ T-cell epitope with the natural flanking amino acids (10 µg/mL) **(B).** Negative controls were unloaded monocytes. Data from a representative experiment out of three independent experiments is shown. **(C)** IFNγ production determined in the supernatant of the activated $CD8^+$ T-cells. Positive control is a SP containing the minimal $CD8^+$ T-cell epitope (1 µg/mL). Representative experiment is shown as mean ± SD (n = 4) out of three independent experiments.

Finally, the uptake, processing, and subsequent antigen presentation and T cell stimulation of the conjugated $CD8^+$ and $CD4^+$ T-cell epitopes (**18a** and **18b**) by antigen-presenting cells (APCs) were tested by making use of human moDCs and neoepitope-specific human T-cells obtained from the melanoma patient who was successfully treated with these neoepitope-specific T-cells. The tumour-reactive T-cell cultures used for this successful treatment were established and stored in the LUMC (Leiden) and used for this study.[33-34, 36] The APCs used in these experiments were derived from HLA-matched donors or, if available, autologous cells were used. After differentiation, the APCs were loaded overnight with titrated amounts of the $CD8^+$ T-cell epitope-containing SP (**18b**) or $CD4^+$ T-cell epitope-containing SP (**18a**) conjugates. The patient's T-cells, containing the neoepitope-specific $CD8^+$ and $CD4^+$ T-cells, were added, followed by overnight incubation of the APCs. T-cell activation was determined by the expression of the activation marker CD137 and the production of the cytokine IFNγ. 5D9-mini-UPam conjugate **18b** was able to significantly activate the 5D9-specific $CD8^+$ T-cells, whereas the equimolar mix of the peptide and free mini-UPam only marginally resulted in T-cell activation (Figure 4A). This indicates adequate processing and MHC class I presentation of the conjugated epitope and the relevance of the conjugation of a TLR2 ligand to an antigenic peptide to improve its immunogenicity.[7, 37] The activated $CD8^+$ T-cells were capable of

producing IFNγ upon stimulation with the 5D9-mini-UPam (**18b**) conjugate, indicating their functionality/activation of their effector function (Figure 4C). The APCs loaded with the 4H7-mini-UPam (**18a**) conjugate were also able to activate neoepitope-specific $CD4^+$ T-cells, showing that the conjugated $CD4^+$ T-cell epitope was properly processed and correctly loaded into the MHC class II complex followed by presentation on the cell surface. However, as it was observed previously for $CD4^+$ T-cell epitopes conjugated to a TLR2 ligand,[8] the 4H7-mini-UPam **18a** conjugates do not outperform free SP or mixtures in T-cell activation in *in vitro* settings (Figure 4B).

In conclusion, the synthesis of seven conjugates, comprising a mini-UPam TLR2 ligand that is covalently linked to the N-terminus of oligopeptides containing a human cancer neoepitope, was successfully completed. Mini-UPam is much less lipophilic than the corresponding UPam TLR2 ligand and the covalent attachment of mini-UPam to an oligopeptide should have a lesser impact on the physicochemical properties of the resulting conjugate. It turned out that the synthesis and the chromatographic purification of the projected seven mini-UPam conjugates match with those of the incorporated individual peptides. The immunological properties of two conjugates (**18a** and **18b**) were evaluated, showing that the ability to trigger TLR2 signalling was preserved. Both conjugates (**18a** and **18b**) were able to functionally stimulate human DCs and activate neoepitope-specific human T-cells. Conjugate **18b** with a CTL epitope showed a strongly enhanced activation of neoepitope-specific $CD8^+$ T-cells. The equimolar mix of the free mini-UPam with the corresponding SP did not result in activation of the specific $CD8^+$ T-cells, which is consistent with earlier observations. The CD4 mini-UPam-SP conjugate **18a** with a T-helper epitope, however, was not superior in antigen presentation and $CD4^+$ T-cell activation as compared to the mix of free SP and TLR2-ligand or free SP alone. This was observed before in studies with mouse and human $CD4^+$ T-cell epitope-containing TLR-L-SPs and can be explained by the different uptake and routing of MHC class II presented peptides as compared to MHC class I processing routes[7-8]. Apparently, *in vitro* the continuous presence of SP in the DC culture allows endosomal uptake and processing of antigenic peptides in the MHC class II processing route. Based on the results this process cannot be improved *in vitro* by conjugation of the antigenic peptide to a TLR-targeting adjuvant. This is in contrast to the MHC class I processing route which requires uptake and endosomal escape to a cytosolic route which may be improved by TLR-ligand targeting and signaling.[38] Importantly, TLR2 ligand adjuvanting for MHC class II presented peptide in the synthetic vaccine is still preferred, since it was shown that *in vivo* not only $CD8^+$ but also $CD4^+$ T-cell activation and tumour control obtained by conjugates is superior to that obtained by physical mixtures of free TLR2 ligands and SPs.[6-7]

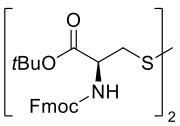
Experimental

General Information

All solvents used under anhydrous conditions were stored over 4Å molecular sieves, except for methanol, which was stored over 3Å molecular sieves. Solvents used for workup and

column chromatography were of technical grade from Sigma Aldrich and used directly. Unless stated otherwise, solvents were removed by rotary evaporation under reduced pressure at 40 °C. Reactions were monitored by TLC-analysis using Merck 25 DC plastikfolien 60 F254 with detection by spraying with 1% $KMnO_4$, 10% Na_2CO_3 (aq.) (unless stated otherwise) followed by charring at approx. 150°C. Column chromatography was performed on Fluka silicagel (0.04 – 0.063mm). LC-MS analysis was performed on one of the following LC-MS systems: A Thermo Finnigan LCQ Advantage MAX ion-trap mass spectrometer with an electrospray ion source coupled to Surveyor HPLC system (Thermo Finnegan), A Thermo Finnigan LCQ Fleet MAX ion-trap mass spectrometer with an electrospray ion source coupled to Vanquish UPLC system (Thermo Finnegan) or an Agilent Technologies 1260 Infinity LC system (detection simultaneously at 214 and 254 nm) coupled to a Agilent Technologies 6120 Quadrupole MS. Using an analytical Phenomenex Gemini® 3 µm C18 110 Å 50x4.6 mm in combination with eluents A: H_2O; B: MeCN and C: 1% TFA (aq.) as the solvent system, in which the gradient was modified by changing the ratio of A in B in combination with 10% C. High resolution mass spectra were recorded on an Q-Exactive HF Orbitrap (Thermo Scientific) equipped with an electrospray ion source (ESI), injection of 2 µL of a 1 uM solution via Ultimate 3000 nano UPLC (Dionex) system, with an external calibration (Thermo Scientific); Source voltage of 3.5 kV, capillary temperature 275 °C, no sheath gas, resolution = 240.000 at m/z=400. Mass range m/z=160-2000 or to a maximum of 6000. Eluents used: MeCN:H_2O (1:1 v/v) supplemented with 0.1% formic acid. ^1H and ^{13}C NMR spectra were recorded with a Brüker AV 400 (400/100 MHz). Chemical shifts are given in ppm (δ) relative to TMS (0 ppm) and coupling constants are given in Hz.Optical rotations were measured in $CHCl_3$. IR spectra were recorded on a Shimadzu FTIR-8300 and are reported in cm^{-1}.

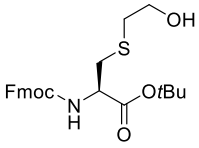
di-*tert*-butyl 3,3'-disulfanediyl(2R,2'R)-bis(2-((((9H-fluoren-9-yl)methoxy)carbonyl)amino) propanoate) (7)



(H-Cys-O*t*Bu)$_2$ (5 mmol, 2.16 g, 1 eq.) and NMM (21 mmol, 1.1 mL, 4.1 eq.) were dissolved in dry THF (50 mL) under a N_2 atmosphere. A solution of Fmoc-OSu (12 mmol, 4.05 g, 2.4 eq.) in dry THF (50 mL) added dropwise to the reaction mixture whilst stirring. The reaction mixture was stirred overnight at RT and subsequently concentrated *in vacuo*. The residue was dissolved in EtOAc and the solution was washed with H_2O (3x) and Brine (1x). Next, the organic layer was dried with $MgSO_4$, filtrated, and concentrated *in vacuo*. The crude product was purified by silica gel column chromatography (5% → 40% EtOAc:Pentane), yielding compound **7** as a white solid (4.86 mmol, 3.88 g, 96%).

$[\alpha]_D$: -7.4°. **HRMS [M+H]$^+$:** $[C_{44}H_{49}N_2O_8S_2]^+$ 797.2944 (measured), 797.2925 (calculated). **^{1}H NMR (400 MHZ, $CDCl_3$):** δ 7.74 (d, *J* = 9.0 Hz, 2H), 7.58, (d, *J* = 9.5 Hz, 2H), 7.38 (t, *J* = 9.0 Hz, 2H) , 7.28 (t, *J* = 9.5 Hz, 2H), 5.74 (d, *J* = 9.5 Hz, 1H), 4.59-4.57 (m, 1H), 4.37-4.35 (m, 2H), 4.19 (t, *J* = 9 Hz, 1H, C_6), 3.22-3.19 (m, 2H), 1.48 (s, 9H). **^{13}C NMR (100 MHz, $CDCl_3$):** δ 169.46, 155.83, 143.89, 141.40, 127.83, 127.30, 125.30 , 120.10, 83.29, 67.35, 54.26, 47.21, 42.02, 28.13.

***tert*-butyl *N*-(((9H-fluoren-9-yl)methoxy)carbonyl)-S-(2-hydroxyethyl)-L-cyseinate (8)**

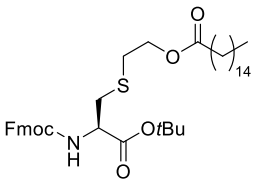


Compound **7** (4.86 mmol, 3.88 g, 1 eq.) was dissolved in THF (50 mL). Zinc powder (37.9 mmol, 2.48 g, 7.7 eq.) and a 100:7:1 solution of MeOH:37% HCl:98% H_2SO_4 (15 mL) were added to the reaction mixture and the mixture was stirred for 15 min at RT. Oxirane (50 mmol, 2.5 mL, 10.3 eq.) was added at 0 ˚C and the reaction mixture was stirred overnight at RT. The reaction mixture was filtrated and directly

concentrated *in vacuo*. The crude product was purified by silica gel column chromatography (20% → 40% EtOAc:Pentane), yielding compound **8** (8.64 mmol, 3.83 g, 89%) as a white solid.

$[\alpha]_D$: +0.3°. **HRMS [M+Na]$^+$:** $[C_{24}H_{29}NNaO_5S]^+$ 466.1666 (measured), 466.1659 (calculated). **^{1}H NMR (400 MHz, $CDCl_3$):** δ 7.77 (d, *J* = 9.5 Hz, 2H), 7.61 (d, *J* = 9 Hz, 2H), 7.40 (t, *J* = 9.3 Hz, 2H), 7.32 (t, *J* = 9.4 Hz, 2H), 5.77 (d, *J* = 9.5 Hz, 1H), 4.52-4.51 (m, 1H), 4.41 (m, 2H), 4.24 (t, *J* = 8.7, 1H), 3.73 (m, 2H), 3.00-2.97 (m, 2H), 2.78-2.74 (m, 2H), 1.50 (s, 9H). **^{13}C NMR (100 MHz, $CDCl_3$):** δ 169.77, 156.04, 143.94 ,143.80, 141.43, 127.86, 127.21, 125.24, 120.13, 83.24, 67.25, 60.85, 54.69, 47.23, 36.62, 35.21, 28.12.

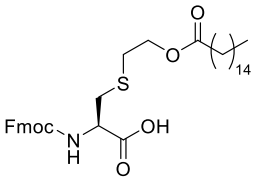
(R)-2-((2-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)-3-(*tert*-butoxy)-3-oxopropyl)thio)ethylpalmitate (9)



Compound **8** (20.5 mmol, 9.14 g, 1 eq.), palmitic acid (22.6 mmol, 5.80 g, 1.1 eq.), and DMAP (2.26 mmol, 276 mg, 0.11 eq.) were dissolved in dry DCM (200 mL) under a N_2 atmosphere. Next, EDC·HCl (22.6 mmol, 4.33 g, 1.1 eq.) was added and the reaction mixture was stirred overnight at RT. Next, the reaction mixture was washed with 1 M HCl aq. (1x), sat. $NaHCO_3$ aq. (3x) and Brine (1x). The organic layer was dried with $MgSO_4$, filtrated, and concentrated *in vacuo*. The crude product was purified by silica gel column chromatography (2% → 12% EtOAc:Pentane), yielding compound **9** as a clear thick oil (15.5 mmol, 10.6 g, 76%).

$[\alpha]_D$: -1.0°. **HRMS [M+Na]$^+$:** $[C_{40}H_{59}NNaO_6S]^+$ 703.3964 (measured), 703.3955 (calculated). **^{1}H NMR (400 MHz, $CDCl_3$):** δ 7.77 (d, *J* = 9.5 Hz, 2H), 7.61 (d, *J* = 9.5 Hz, 2H), 7.40 (t, *J* = 9.5 Hz, 2H), 7.32 (t, *J* = 9.3 Hz, 2H), 5.68 (d, *J* = 9.5 Hz, 1H), 4.54-4.49 (m, 1H), 4.45-4.35 (m, 2H), 4.26-4.19 (m, 3H), 3.09-3.00 (m, 2H), 2.79-2.76 (m, 2H), 2.30-2.27 (m, 2H), 1.64 (m, 2H), 1.56 (s, 9H) 1.24 (m, 24H), 0.88 (t, *J* = 8.5 Hz, 3H). **^{13}C NMR (100 MHz, $CDCl_3$):** δ 173.75, 169.69, 155.82, 143.91, 141.42, 127.85, 127.20, 125.27, 120.12, 83.16, 67.27, 63.21, 54.43, 47.24, 34.04, 34.22, 32.06, 31.54, 29.83, 29.79, 29.75, 29.60, 29.50, 29.40, 29.25, 28.12, 25.01, 22.83, 14.27.

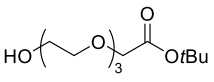
***tert*-butyl *N*-(((9H-fluoren-9-yl)methoxy)carbonyl)-S-(2-plamiroyloxy)ethyl)-L-cysteine (10)**



Compound **9** (28.3 mmol, 19.3 g, 1 eq.) was dissolved in a 1:1 DCM:TFA (30 mL) solution and the mixture was stirred for 30 min. The reaction mixture was concentrated *in vacuo* and co-evaporated with toluene (2x). The crude product was purified by silica gel column chromatography (1% → 5% DCM:MeOH), yielding compound **10** as a clear viscous oil (26.9 mmol, 16.8 g, 95%).

$[\alpha]_D$: +5.4°. **HRMS [M+H]$^+$:** $[C_{36}H_{52}NO_6S]^+$ 626.3618 (measured), 626.3610 (calculated). **^{1}H NMR (400 MHz, $CDCl_3$):** δ 7.76 (d, *J* = 9.5 Hz, 2H), 7.61 (d, *J* = 9 Hz, 2H), 7.40 (t, *J* = 9.5 Hz, 2H), 7.31 (t, *J* = 9.3 Hz, 2H), 5.75 (d, *J* = 9.5 Hz, 1H), 4.67-4.63 (m, 1H), 4.43-4.41 (m, 2H), 4.26-4.20 (m, 3H), 3.16-3.05 (m, 2H), 2.80-2.77 (m, 2H), 2.31-2.28 (m, 2H), 1.61-1.56 (m, 2H), 1.24 (m, 24H), 0.88 (t, *J* = 8.5 Hz, 3H). **^{13}C NMR (100 MHz, $CDCl_3$):** δ 174.15, 173.77, 156.09, 143.84, 143.76, 141.44, 127.91, 127.24, 125.23, 120.15, 67.51, 63.26, 53.65, 47.21, 34.53, 34.32, 32.07, 31.34, 29.84, 29.80, 29.78, 29.62, 29.51, 29.41, 29.27, 25.00, 22.84, 14.28.

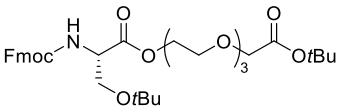
***tert*-butyl 2-(2-(2-(2-hydroxyethoxy)ethoxy)ethoxy)acetate (12)**



Triethylene glycol (19.9 mL, 150 mmol, 2 eq.) was dissolved in dry THF (750 mL) under a N_2 atmosphere at 0 ˚C. First, NaH (60% in mineral oil, 77.3 mmol, 3.15 g, 1.05 eq.) was added and the solution was stirred for 5 min at 0 ˚C. Then, TBAI (7.5 mmol, 2.77 g, 0.1 eq.) and *t*-butyl bromoacetate (75 mmol, 11.1 mL, 1 eq.) were added whilst stirring at 0 ˚C and the reaction mixture was stirred overnight whilst warming to RT. The mixture was filtrated and concentrated *in vacuo*. The crude product was purified by silica gel column chromatography (80% → 100% EtOAc:Pentane), yielding compound **12** a yellow oil (29.6 mmol, 7.85 g, 40%).

HRMS [M+Na]⁺: $[C_{12}H_{24}NaO_6]^+$ 287.1473 (measured), 287.1465 (calculated). **^{1}H NMR (400 MHz, $CDCl_3$)** : δ 4.02 (s, 2H), 3.79-3.68 (m, 10H), 3.56 (m, 2H), 1.47 (s, 9H). **^{13}C NMR (100 MHz, $CDCl_3$)**: δ 168.88, 80.59, 72.02, 70.53, 69.91, 69.84, 69.79, 69.60, 68.48, 60.69, 27.35.

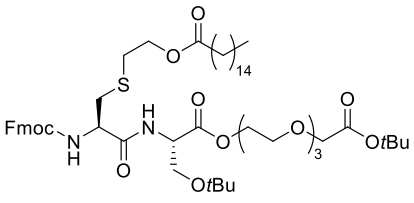
13,13-dimethyl-11-oxo-3,6,9,12-tetraoxatetradecyl *N*-(((9H-fluoren-9-yl)methoxy)carbonyl)-O-(*t*-butyl)-L-serinate (13)



Compound **12** (16 mmol, 4.23 g, 1 eq.), Fmoc-Ser(tBu)-OH (17.6 mmol, 6.75 g, 1.1 eq.) and DMAP (1.76 mmol, 220 mg, 0.11 eq.) were dissolved in dry DCM (200 mL) under a N_2 atmosphere. EDC·HCl (17.6 mmol, 3.37 g, 1.1 eq.) was added whilst stirring and the reaction mixture was stirred overnight at RT. Next, the reaction mixture was washed with 1 M HCl aq. (3x), sat. $NaHCO_3$ aq., and Brine (1x). The organic layer was dried with $MgSO_4$, filtered, and concentrated *in vacuo*. The crude product was purified by silica gel column chromatography (20% → 50% EtOA:Pentane), yielding compound **13** as a yellow solid (15.5 mmol, 9.73 g, 97%).

$[\alpha]_D$: +8.5°. **HRMS [M+Na]⁺:** $[C_{34}H_{47}NNaO_{10}]^+$ 652.3106 (measured), 652.3092 (calculated). **^{1}H NMR (400 MHz, $CDCl_3$):** δ 7.77 (d, *J* = 9.5 Hz, 2H), 7.62 (d, *J* = 9 Hz, 2H), 7.40 (t, *J* = 9.3 Hz, 2H), 7.32 (t, *J* = 9 Hz, 2H), 5.74 (d, *J* = 11 Hz, 1H), 4.51 (d, *J* = 11 Hz, 1H), 4.40-4.28 (m, 5H), 4.01 (s, 2H), 3.85 (m, 1H), 3.72-3.60 (m, 11H), 1.47 (s, 9H), 1.16 (s, 9H). **^{13}C NMR (100 MHz, $CDCl_3$):** δ 170.76, 169.78, 156.24, 144.12, 143.94, 141.40, 127.82, 127.21, 125.34, 125.30, 112.10, 81.71, 73.60, 70.82, 70.75, 70.70, 69.13, 67.30, 64.66, 62.23, 54.78, 47.26, 28.23, 27.46.

(17S,20R)-20--((((9H-fluoren-9-yl)methoxy)carbonyl)amino)-17-(*tert*-butoxymethyl)-2,2-dimethyl-4,16,19-trioxo-3,6,9,12,15-pentaoxa-22-thia-18-azateracosan-24-yl palmitate (14)

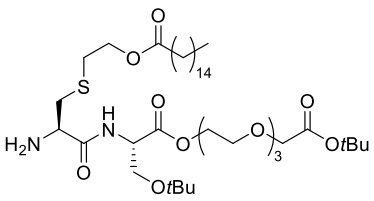


Compound **13** (10.6 mmol, 6.66 g, 1 eq.) was dissolved in dry THF (100 mL) under a N_2 atmosphere. Next, DBU (10.1 mmol, 1.51 mL, 0.95 eq.) was added and the solution was stirred at RT for 5 min, after which HOBt (21.3 mmol, 3.26 g, 2 eq.) was added to the reaction mixture. Then, compound **4** (10.6 mmol, 6.66 g, 1 eq.) and EDC·HCl (10.6 mmol, 2.04 g, 1 eq.) were added and the reaction mixture was stirred at RT for 2.5 h. The reaction mixture was washed with 1 M HCl aq. (2x), sat. $NaHCO_3$ aq. (2x), and Brine (1x). The organic layer was dried with $MgSO_4$, filtrated, and concentrated *in vacuo*. The crude product was purified by silica gel column chromatography (20% → 50% EtOAc:Pentane), yielding compound **14** as a yellow oil (10.2 mmol, 10.3 g, 95%).

$[\alpha]_D$: +5.0°. **HRMS [M+H]⁺:** $[C_{55}H_{87}N_2O_{13}S]^+$ 1015.5924 (measured), 1015.5923 (calculated). **^{1}H NMR (400 MHz, $CDCl_3$):** δ 7.76 (d, *J* = 9 Hz, 2H), 7.60 (d, *J* = 9 Hz, 2H), 7.39 (t, *J* = 9.3 Hz, 2H), 7.31 (t, *J* = 9.3 Hz, 2H), 7.26 (m, 1H), 5.91 (m, 1H), 4.70 (d, *J* = 10, 1H), 4.42-4.35 (m, 3H), 4.35-4.24 (m, 5H), 4.02 (s,

2H), 3.86 (m, 1H), 3.72-3.64 (m, 10H), 3.60 (m, 1H), 2.99 (m, 2H), 2.86 (m, 2H), 2.30 (t, *J* = 9.5 Hz, 2H), 1.62 (m, 2H), 1.47 (s, 9H), 1.30-1.24 (m, 24H), 1.14 (s, 9H), 0.88 (t, *J* = 8.8 Hz, 3H). **^{13}C NMR (100 MHz, $CDCl_3$):** δ 173.71, 170.11, 169.95, 169.67, 155.89, 143.80, 143.76, 141.31, 127.77, 127.12, 125.20, 125.14, 120.02, 81.60, 73.59, 70.72, 70.63, 70.59, 70.56, 69.00, 68.96, 67.27, 64.61, 62.93, 61.64, 54.12, 53.26, 47.11, 34.93, 34.20, 31.96, 30.98, 29.73, 29.69, 29.65, 29.51, 29.40, 29.32, 29.18, 28.14, 27.34, 24.91, 22.73, 14.18.

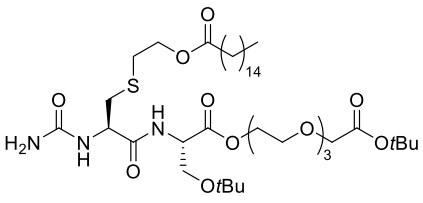
(17S,20R)-20-amino-17-(*tert*-butoxymethyl)-2,2-dimethyl-4,16,19-trioxo-3,6,9,12,15-pentaoxa-22-thia-18-azateracosan-24-yl palmitate (15)



Compound **14** (5.3 mmol, 5.38 g, 1 eq.) was dissolved in dry DCM (53 mL). 1-Octanethiol (28.3 mmol, 4.1 mL, 5 eq.) was added followed by DBU (0.53 mmol, 79 µL, 0.1 eq.) and the reaction mixture was stirred for 2 h RT. The reaction mixture was absorbed on Celite and the solvent was evaporated *in vacuo*. The crude product was purified by silica gel column chromatography (0% → 1% Et_3N:EtOAc), yielding compound **15** as a clear oil (5.26 mmol, 4.18 g, 99%).

$[\alpha]_D$: -19.5°. **HRMS [M+H]$^+$:** $[C_{40}H_{76}N_2O_{11}S]^+$ 793.5260 (measured), 793.5243 (calculated). **^{1}H NMR (400 MHz, $CDCl_3$):** δ 8.07 (d, *J* = 10.5 Hz, 1H), 4.69 (d, *J* = 10.5 Hz, 1H), 4.30 (m, 2H), 4.22 (t, *J* = 8.5 Hz, 2H), 4.02 (m, 2H), 3.86 (d, *J* = 11 Hz), 3.73-3.68 (m, 10H), 3.59-5.54 (m, 2H), 3.09 (m, 1H), 2.78 (m, 3H), 2.32 (t, *J* = 9 Hz, 2H), 1.62 (m, 2H), 1.48 (s, 9H), 1.28-1.26 (m, 24H), 1.15 (s, 9H), 0.88 (t, *J* = 8 Hz, 3H). **^{13}C NMR (100 MHz, $CDCl_3$):** δ 173.73, 173.30, 170.43, 169.73, 81.66, 73.45, 72.65, 70.79, 70.72, 70.68, 70.67, 69.09, 64.58, 63.12, 62.08, 54.21, 52.77, 37.91, 34.27, 32.01, 30.67, 29.78, 29.75, 29.71, 29.56, 29.46, 29.37, 29.23, 29.20, 28.20, 27.44, 25.00, 22.79, 14.16.

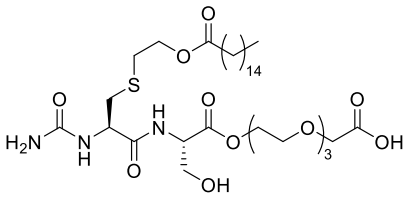
(17S,20R)-20-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)-17-(*tert*-butoxymethyl)-2,2-dimethyl-4,16,19-trioxo-3,6,9,12,15-pentaoxa-22-thia-18-azatetracosan-24-yl palmitate (16)



Compound **15** (7.15 mmol, 5.67 g, 1 eq.) was dissolved in dry DCM (500 mL) under a N_2 atmosphere. *i*-PrOH (152 mmol, 11.7 mL) was added, followed by (trimethylsilyl)isocyanate (76.2 mmol, 10.3 mL) and the reaction mixture was stirred for 40 h at RT. Celite was added to the solution and the solvent was evaporated *in vacuo*. The crude product was purified by silica gel column chromatography (1%:60%:39% Et_3N:EtOAc:Pentane → 1%:80%:19% Et_3N:EtOAc:Pentane), yielding compound **16** as a clear oil (5.30 mmol, 4.43 g, 74%).

$[\alpha]_D$: +7.1°. **HRMS [M+H]$^+$:** $[C_{41}H_{78}N_3O_{12}S]^+$ 836.5327 (measured), 836.5301 (calculated). **^{1}H NMR (400 MHz, $CDCl_3$):** δ 7.55 (d, *J* = 10 Hz, 1H), 6.47 (d, *J* = 10 Hz, 1H), 5.27 (s, 2H), 4.65 (m, 1H), 4.55 (m, 1H), 4.31-4.22 (m, 2H), 4.03 (s, 2H), 3.83 (dd, *J* = 11.3, 4.3 Hz, 1H), 3.74-3.66 (m, 10H), 3.59 (dd, *J* = 11.3, 4.3 Hz, 1H), 2.97-2.94 (m, 1H), 2.86-2.82 (m, 3H), 2.31 (t, *J* = 9.5 Hz, 2H), 1.60 (m, 2H), 1.48 (s, 9H), 1.28 (m, 24H), 1.14 (s, 9H), 0.88 (t, *J* = 8.5 Hz, 3H). **^{13}C NMR (100 MHz, $CDCl_3$):** δ 173.76, 171.52, 170.00, 169.69, 158.60, 81.66, 73.52, 70.64, 70.49, 70.45, 68.89, 64.50, 62.95, 61.60, 53.36 ,53.26, 35.06, 34.15, 31.88, 30.83, 29.65, 29.62, 29.60, 29.46, 29.32, 29.27, 29.14, 28.07, 27.27, 24.85, 22.75, 14.16.

(14S,17R) -14-(hyrdoxymethyl)-13,16,23-trioxo-17-ureido-3,6,9,12,22-pentaoxa-19-thia-15-azaoctatriacontanoic acid (5)



Compound **16** (5.30 mmol, 4.43 g, 1 eq.) was dissolved in 95:2.5:2.5 solution of TFA:TIS:H_2O (100 mL) and was stirred for 1 h at RT. The reaction mixture was added in portions of 5 mL to centrifuge tubes containing Et_2O (45 mL) and the tubes were stored for 90 h at -40 ˚C. The tubes were centrifuged, the Et_2O solution was decanted, and the remaining pallet was dried with a N_2 flow. The pallets were redissolved in a **HO*t*Bu:H_2O:MeCN** solution (2:1:1, total volume of 50 mL) and lyophilised. Compound **5** was isolated as a white powder (2.95 mmol, 2.14 g, 55%).

$[\alpha]_D$: - 8.8°. **HRMS $[M+H]^+$:** 724.4053 (measured), 724.4049 (calculated). **^{1}H NMR (400 MHz, $CDCl_3$):** δ 7.96 (d, *J* = 8 Hz, 1H), 6.51 (s, 1H), 5.42 (s, 2H), 4.69 (m, 1H), 4.65 (m, 1H), 4.49 (m, 1H), 4.24-4.22 (m, 3H), 4.02 (s, 2H), 3.89 (dd, 1H), 3.72-3.65 (m, 10H), 3.00-2.99 (m, 2H), 2.79 (m, 2H), 2.31 (t, j = 9.5 Hz, 2H), 1.60 (m, 2H), 1.28 (m, 24H), 0.88 (t, j = 8.5 Hz, 3H). **^{13}C NMR (100 MHz, $CDCl_3$):** δ 174.07, 173.44, 171.88, 170.17, 160.05, 70.70, 70.50, 70.40, 68.92, 68.54, 64.45, 63.16, 62.57, 55.11 ,53.67, 34.86, 34.31, 32.05, 30.99, 29.83, 29.79, 29.65, 29.49, 29.46, 29.32, 25.02, 22.81, 14.26.

Synthesis of simplified TLR2 ligand conjugated neo-epitope containing synthetic long peptides

General procedure for Tribute™ automated solid phase synthesis

All reagents and solvents used in the solid phase peptide synthesis were purchased from Biosolve (Netherlands). Fmoc amino acids building blocks were purchased from Sigma Aldrich or Novabiochem. Tentagel based resins were purchased from Rapp Polymere GmbH (Germany). The solid-phase peptide synthesis was performed on a TRIBUTE® Peptide Synthesiser (Gyros Protein Technologies AB, Arizona, USA) applying Fmoc based protocol starting with Tentagel S RAM resin (0.22-0.25 mmol/g) on a 100 µmol scale using established Fmoc protocols. **T**he consecutive steps preformed during each cycle were:

1. Deprotection of the Fmoc protecting group with 3 x 4 mL 20% piperidine in NMP for 3 min;
2. Wash, 3 x 4 mL NMP;
3. First coupling of the appropriate amino acid applying a five-fold excess. The Fmoc amino acid building block (0.5 mmol, 5 eq.) was dissolved 0.2 M HCTU in NMP (2.5 mL, 5 eq.) in its loading cartridge and the resulting solution was transferred to the reaction vessel. Next, the cartridge was washed with a 0.5 M D*i*PEA in NMP (2 mL, 10 eq.) and subsequently the solution was transferred to the reaction vessel. The reaction vessel was shaken for 1 h at RT;
4. Wash, 3 x 4 mL NMP;
5. Capping of unreacted peptide with 1 x 10% Ac_2O and 5% NMM in NMP solution (5 mL) for 3 min;
6. Wash, 3 x 4 mL NMP;

After the last coupling cycle, the final Fmoc group was deprotected with 3 x 4 mL 20% piperidine in DMF for 3 min. Finally, the resin was washed with DMF (3x) and DCM (3x) and dried using a N_2 flow.The following amino acid building blocks were used for the synthesis: Fmoc-Ala-OH, Fmoc-Asn(Trt)-OH, Fmoc-Asp(*t*Bu)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Cys(Trt)-OH, Fmoc-Gln(Trt)-OH, Fmoc-Glu(*t*Bu)-OH, Fmoc-Gly-OH, Fmoc-His(Trt)-OH, Fmoc-Ile-OH, Fmoc-Leu-OH, Fmoc-Lys(Boc)-OH, Fmoc-Met-OH,

Fmoc-Phe-OH, Fmoc-Pro-OH, Fmoc-Ser(*t*Bu)-OH, Fmoc-Thr(*t*Bu), Fmoc-Trp(Boc)-OH Fmoc-Tyr(*t*Bu)-OH, Fmoc-Val-OH.

General procedure for Liberty Blue™ Automated Microwaved Peptide Synthesizer

1. Deprotection of the Fmoc protecting group with 2 x 4 mL 20% piperidine in DMF for 3 min;
2. Wash, 3 x 4 mL DMF;
3. First coupling of the appropriate amino acid applying a five-fold excess. First, a 0.2 M solution of the Fmoc amino acid in DMF (2.5 mL) was added to the resin in the reaction vessel. Next, a 0.5 M solution of DIC in DMF (1 mL) and a 1 M solution of Oxyma Pure in DMF (0.5 mL) was added to the reaction vessel. The reaction vessel was shaken for 4 min. at 90°C;
4. Wash, 3 x 4 mL DMF;
5. Second coupling of the appropriate amino acid applying a five-fold excess. First, a 0.2 M solution of the Fmoc amino acid in DMF (2.5 mL) was added to the resin in the reaction vessel. Next, a 0.5 M solution of DIC in DMF (1 mL) and a 1 M solution of Oxyma Pure in DMF (0.5 mL) was added to the reaction vessel. The reaction vessel was shaken for 4 min. at 90°C;
6. Wash, 3 x 4 mL DMF;

After the last coupling cycle, the final Fmoc group was deprotected with 3 x 4 mL 20% piperidine in DMF for 3 min. Finally, the resin was washed with DMF (3x) and DCM (3x) and dried using a N_2 flow.

The following amino acid building blocks were used for the synthesis: Fmoc-Ala-OH, Fmoc-Asn(Trt)-OH, Fmoc-Asp(OMpe)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Cys(Trt)-OH, Fmoc-Gln(Trt)-OH, Fmoc-Glu(*t*Bu)-OH, Fmoc-Gly-OH, Fmoc-His(Trt)-OH, Fmoc-Ile-OH, Fmoc-Leu-OH, Fmoc-Lys(Boc)-OH, Fmoc-Met-OH, Fmoc-Phe-OH, Fmoc-Pro-OH, Fmoc-Ser(*t*Bu)-OH, Fmoc-Thr(*t*Bu), Fmoc-Trp(Boc)-OH Fmoc-Tyr(*t*Bu)-OH, Fmoc-Val-OH.

H-Lys-Ile-Asp-Arg-Glu-Gly-Lys-Pro-Arg-Lys-Val-Ile-Gly-Cys-Ser-Cys-Val-Val-Val-Lys-Asp-Tyr-Gly-Lys-Glu-NH_2 (17a)

H-Lys(Boc)-Ile-Asp(*t*Bu)-Arg(Pbf)-Glu(*t*Bu)-Gly-Lys(Boc)-Pro-Arg(Pbf)-Lys(Boc)-Val-Ile-Gly-Cys(Trt)-Ser(*t*Bu)-Cys(Trt)-Val-Val-Val-Lys(Boc)-Asp(*t*Bu)-Tyr(*t*Bu)-Gly-Lys(Boc)-Glu(tBu)-Tentagel S RAM resin was prepared using a TRIBUTE® Peptide Synthesiser applying Fmoc chemistry for 100 µmol Tentagel S RAM resin. 25 µmol was taken from the crude batch and treated with a standard deprotection cocktail 95:2.5:2.5 TFA:TIS:H_2O (3 mL) for 105 min. The resin was filtered off and the filtrate was collected in a 1:1 Et_2O:pentane solution (46 mL). The resin was washed with TFA (1 mL) and was collected in the same Et_2O:pentane solution. The solution containing the crude peptide was cooled to -20°C and centrifuged after which the organic layer was decanted. The remaining pallet was dried with a N_2 flow after it was dissolved in NMP. The solution was further diluted with an equal volume of a 1:1:1 HO*t*Bu:H_2O:MeCN solution. Purification by HPLC provided compound **17a** in a 5% yield (3.38 mg, 1.26 µmol). **LC-MS:** R_t = 4.307 (Phenomenex Gemini® 3 µm C_{18} 110 Å 50x4.6 mm, 10-90% MeCN, 10 min); **ESI-MS:** *m/z* 1404.3 $[M+2H^+]^{2+}$; **HRMS [M+5H]$^{5+}$:** $[C_{121}H_{212}N_{37}O_{35}S_2]^{5+}$ 561.50687 (measured), 561.50721 (calculated).

H-Lys-Arg-Arg-Ser-Gly-Gln-Arg-Lys-Pro-Ala-Thr-Phe-Tyr-Val-Arg-Thr-Thr-Ile-Asn-Lys-Asn-Ala-Arg-Ala-Thr-Leu-NH_2 (17b)

H-Lys(Boc)-Arg(Pbf)-Arg(Pbf)-Ser(*t*Bu)-Gly-Gln(Trt)-Arg(Pbf)-Lys(Boc)-Pro-Ala-Thr(*t*Bu)-Phe-Tyr(*t*Bu)-Val-Arg(Pbf)-Thr(*t*Bu)-Thr(*t*Bu)-Ile-Asn(Trt)-Lys(Boc)-Asn(Trt)-Ala-Arg(Pbf)-Ala-Thr(*t*Bu)-Leu-Tentagel S RAM resin was prepared using a TRIBUTE® Peptide Synthesiser applying Fmoc chemistry for 100 µmol Tentagel S RAM resin. 50 µmol was taken from the crude batch and treated with a standard deprotection cocktail 95:2.5:2.5 TFA:TIS:H_2O (3 mL) for 105 min. The resin was filtered off and the filtrate was collected in a 1:1 Et_2O:Pentane solution (46 mL). The resin was washed with TFA (1 mL) and was collected in the same Et_2O:Pentane solution. The solution containing the crude peptide was cooled to -20°C and centrifuged after which the organic layer was decanted. The remaining pallet was dried with a N_2 flow after it was dissolved in NMP. The solution was further diluted with an equal volume of a 1:1:1 HO*t*Bu:H_2O:MeCN solution. Purification by HPLC provided compound **17b** in a 18% yield (13.62 mg, 4.49 µmol). **LC-MS:** R_t = 3.479 (Phenomenex Gemini® 3 µm C_{18} 110 Å 50x4.6 mm, 10-90% MeCN, 10 min); **ESI-MS:** *m/z* 1012.1 $[M+3H^+]^{3+}$; **HRMS [M+6H]$^{6+}$:** $[C_{131}H_{232}N_{48}O_{35}]^{6+}$ 506.29643 (measured), 506.29687 (calculated).

H-Lys-Ala-Asp-Pro-Phe-Pro-Pro-Asn-Gly-Ala-Pro-Pro-Leu-Lys-Pro-His-Pro-Leu-Met-Pro-Ala-Asn-Pro-Trp-Gly-NH_2 (17c)

H-Lys(Boc)-Ala-Asp(*t*Bu)-Pro-Phe-Pro-Pro-Asn(Trt)-Gly-Ala-Pro-Pro-Leu-Lys(Boc)-Pro-His(Trt)-Pro-Leu-Met-Pro-Ala-Asn(Trt)-Pro-Trp(Boc)-Gly-Tentagel S RAM resin was prepared using a TRIBUTE® Peptide Synthesiser applying Fmoc chemistry for 100 µmol Tentagel S RAM resin. 25 µmol was taken from the crude batch and treated with a standard deprotection cocktail 95:2.5:2.5 TFA:TIS:H_2O (3 mL) for 105 min. The resin was filtered off and the filtrate was collected in a 1:1 Et_2O:Pentane solution (46 mL). The resin was washed with TFA (1 mL) and was collected in the same Et_2O:Pentane solution. The solution containing the crude peptide was cooled to -20°C and centrifuged after which the organic layer was decanted. The remaining pallet was dried with a N_2 flow after it was dissolved in NMP. The solution was further diluted with an equal volume of a 1:1:1 HO*t*Bu:H_2O:MeCN solution. Purification by HPLC provided compound **17c** in a 18% yield (13.62 mg, 4.49 µmol). **LC-MS:** R_t = 4.21 (Phenomenex Gemini® 3 µm C_{18} 110 Å 50x4.6 mm, 10-90% MeCN, 10 min); **ESI-MS:** *m/z* 1323.53 $[M+2H^+]^{+2}$; **HRMS [M+3H]$^{3+}$:** $[C_{125}H_{188}N_{33}O_{29}S]^{3+}$ 882.44366 (measured), 882.463087 calculated).

H-Arg-Gly-Leu-Pro-Ala-Leu-Leu-Leu-Leu-Leu-Phe-Leu-Gly-Pro-Trp-Pro-Ala-Ala-Val-OH (17d)

H-Arg(Pbf)-Gly-Leu-Pro-Ala-Leu-Leu-Leu-Leu-Leu-Phe-Leu-Gly-Pro-Trp(Trt)-Pro-Ala-Ala-Val-Tentagel-Wang resin was prepared using a TRIBUTE® Peptide Synthesiser applying Fmoc chemistry for 100 µmol Tentagel Wang resin. 25 µmol was taken from the crude batch and treated with a standard deprotection cocktail 95:2.5:2.5 TFA:TIS:H_2O (3 mL) for 105 min. The resin was filtered off and the filtrate was collected in a 1:1 Et_2O:Pentane solution (46 mL). The resin was washed with TFA (1 mL) and was collected in the same Et_2O:Pentane solution. The solution containing the crude peptide was

cooled to -20°C and centrifuged after which the organic layer was decanted. The remaining pallet was dried with a N_2 flow after it was dissolved in DMSO. Purification by HPLC provided compound **17d** in a 36% yield (13.62 mg, 9.00 µmol). **LC-MS:** R_t = 6.05 (Phenomenex Gemini® 3 µm C_{18} 110 Å 50x4.6 mm, 10-90% MeCN, 10 min); **ESI-MS:** *m/z* 1009.5 $[M+2H^+]^{2+}$; **HRMS [M+2H]$^{2+}$:** $[C_{101}H_{163}N_{23}O_{20}]^{2+}$ 1009.12150 (measured), 1009.12169 (calculated).

H-Lys-Lys-Leu-Leu-Leu-Phe-Leu-Gly-Pro-Trp-Pro-Ala-Ala-Val-OH (17e)

H-Lys(Boc)-Lys(Boc)-Leu-Leu-Leu-Phe-Leu-Gly-Pro-Trp(Trt)-Pro-Ala-Ala-Val-Tentagel Wang resin was prepared using a TRIBUTE® Peptide Synthesiser applying Fmoc chemistry for 100 µmol Tentagel Wang resin. 25 µmol was taken from the crude batch and treated with a standard deprotection cocktail 95:2.5:2.5 TFA:TIS:H_2O (3 mL) for 105 min. The resin was filtered off and the filtrate was collected in a 1:1 Et_2O:Pentane solution (46 mL). The resin was washed with TFA (1 mL) and was collected in the same Et_2O:Pentane solution. The solution containing the crude peptide was cooled to -20°C and centrifuged after which the organic layer was decanted. The remaining pallet was dried with a N_2 flow after it was dissolved in DMSO. Purification by HPLC provided compound **17e** in a 39% yield (15.16 mg, 9.76 µmol). **LC-MS:** R_t = 4.48 (Phenomenex Gemini® 3 µm C_{18} 110 Å 50x4.6 mm, 10-90% MeCN, 10 min); **ESI-MS:** *m/z* 1552.9 $[M+H^+]^+$; **HRMS [M+3H]$^{3+}$:** $[C_{79}H_{125}N_{17}O_{15}]^{3+}$ 518.32418 (measured), 518.532531 (calculated).

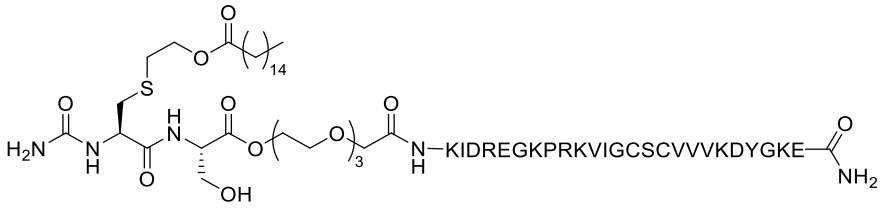
H-Lys-Lys-Leu-Leu-Leu-Phe-Leu-Gly-Pro-Trp-Pro-Ala-Ala-Ser-OH (17f)

H-Lys(Boc)-Lys(Boc)-Leu-Leu-Leu-Phe-Leu-Gly-Pro-Trp(Trt)-Pro-Ala-Ala-Ser(*t*Bu)-Tentagel Wang resin was prepared using a TRIBUTE® Peptide Synthesiser applying Fmoc chemistry for 100 µmol Tentagel Wang resin. 25 µmol was taken from the crude batch and treated with a standard deprotection cocktail 95:2.5:2.5 TFA:TIS:H_2O (3 mL) for 105 min. The resin was filtered off and the filtrate was collected in a 1:1 Et_2O:Pentane solution (46 mL). The resin was washed with TFA (1 mL) and was collected in the same Et_2O:Pentane solution. The solution containing the crude peptide was cooled to -20°C and centrifuged after which the organic layer was decanted. The remaining pallet was dried with a N_2 flow after it was dissolved in DMSO. Purification by HPLC provided compound **17f** in a 21% yield (7.9 mg, 5.13 µmol). **LC-MS:** R_t = 4.26 (Phenomenex Gemini® 3 µm C_{18} 110 Å 50x4.6 mm, 10-90% MeCN, 10 min); **ESI-MS:** *m/z* 1541.8 $[M+H^+]^+$; **HRMS [M+3H]$^{3+}$:** $[C_{77}H_{124}N_{17}O_{16}]^{3+}$ 514.31312 (measured), 514.31318 (calculated).

H-Arg-Gly-Leu-Pro-Ala-Leu-Leu-Leu-Leu-Leu-Phe-Leu-Gly-Pro-Trp-Pro-Ala-Ala-Val-NH_2 (17g)

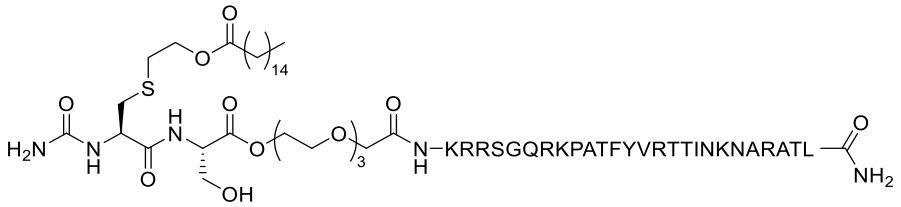
H-Arg(Pbf)-Gly-Leu-Pro-Ala-Leu-Leu-Leu-Leu-Leu-Phe-Leu-Gly-Pro-Trp(Trt)-Pro-Ala-Ala-Val-Tentagel S RAM resin was prepared using a TRIBUTE® Peptide Synthesiser applying Fmoc chemistry for 100 µmol Tentagel RAM resin. 25 µmol was taken from the crude batch and treated with a standard deprotection cocktail 95:2.5:2.5 TFA:TIS:H_2O (3 mL) for 105 min. The resin was filtered off and the filtrate was collected in a 1:1 Et_2O:Pentane solution (46 mL). The resin was washed with TFA (1 mL)

and was collected in the same Et_2O:Pentane solution. The solution containing the crude peptide was cooled to -20°C and centrifuged after which the organic layer was decanted. The remaining pallet was dried with a N_2 flow after it was dissolved in DMSO. Purification by HPLC provided compound **17g** in an 8% yield (4.11 mg, 2.04 µmol). **LC-MS:** R_t = 7.19 (Phenomenex Gemini® 3 µm C_{18} 110 Å 50x4.6 mm, 10-90% MeCN, 10 min); **ESI-MS:** *m/z* 1009.3 $[M+2H^+]^{2+}$; **HRMS [M+2H]$^{2+}$:** $[C_{101}H_{164}N_{24}O_{19}]^{2+}$ 1009.12150 (measured), 1009.12169 (calculated).



((S)-15-hydroxy-13-oxo-14-((R)-3-((2-(palmitoyloxy)ethyl)thio)-2-ureidopropanamido)-3,6,9,12-tetraoxapentadecanoyl)-Lys-Ile-Asp-Arg-Glu-Gly-Lys-Pro-Arg-Lys-Val-Ile-Gly-Cys-Ser-Cys-Val-Val-Val-Lys-Asp-Tyr-Gly-Lys-Glu-NH_2 (18a)

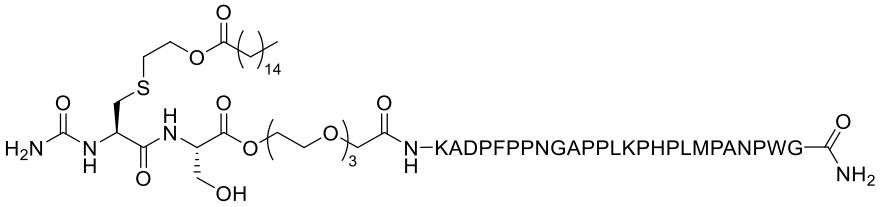
Compound **5** (50 µmol, 36 mg, 2 eq.), HCTU (50 µmol, 21 mg, 2 eq.) and D*i*PEA (100 µmol, 17.4 µL, 4 eq.) were dissolved in NMP (0.75 mL, 0.07 M). This solution was added to dry H-Lys(Boc)-Ile-Asp(*t*Bu)-Arg(Pbf)-Glu(*t*Bu)-Gly-Lys(Boc)-Pro-Arg(Pbf)-Lys(Boc)-Val-Ile-Gly-Cys(Trt)-Ser(*t*Bu)-Cys(Trt)-Val-Val-Val-Lys(Boc)-Asp(*t*Bu)-Tyr(*t*Bu)-Gly-Lys(Boc)-Glu(*t*Bu)-Tentagel S RAM resin (25 µmol) and the reaction vessel was shaken overnight. The resin was washed with 3 x NMP and 6 x DCM and dried under a N_2 flow. Next, the resin was treated with a standard deprotection cocktail 95:2.5:2.5 TFA:TIS:H_2O (3 mL) for 105 min. The resin was filtered off and the filtrate was collected in a 1:1 Et_2O:Pentane solution (46 mL). The resin was washed with TFA (1 mL) and was collected in the same Et_2O:Pentane solution. The solution containing the crude peptide was cooled to -20°C and centrifuged after which the organic layer was decanted. The remaining pallet was dried with a N_2 flow after it was dissolved in NMP. The solution was further diluted with an equal volume of a 1:1:1 HO*t*Bu:H_2O:MeCN solution. Purification by RP-HPLC provided compound **18a** in a 2% yield (1.36 mg, 0.40 µmol). **LC-MS:** R_t = 5.784 (Phenomenex Gemini® 3 µm C_{18} 110 Å 50x4.6 mm, 10-90% MeCN, 10 min); **ESI-MS:** *m/z* 1171.1 $[M+3H^+]^{3+}$; **HRMS [M+4H]$^{4+}$:** $[C_{154}H_{270}N_{40}O_{46}S_3]^{4+}$ 877.97584 (measured), 877.97895 (calculated).



((S)-15-hydroxy-13-oxo-14-((R)-3-((2-(palmitoyloxy)ethyl)thio)-2-ureidopropanamido)-3,6,9,12-tetraoxapentadecanoyl)-Lys-Arg-Arg-Ser-Gly-Gln-Arg-Lys-Pro-Ala-Thr-Phe-Tyr-Val-Arg-Thr-Thr-Ile-Asn-Lys-Asn-Ala-Arg-Ala-Thr-Leu-NH_2 (18b)

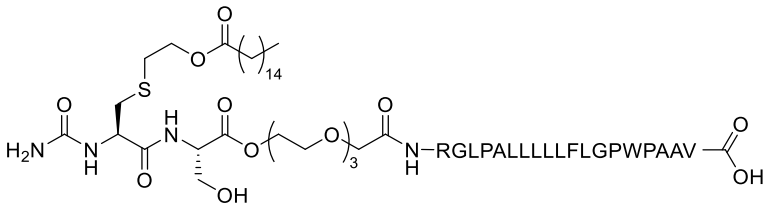
Compound **5** (50 µmol, 36 mg, 2 eq.), HCTU (50 µmol, 21 mg, 2 eq.) and D*i*PEA (100 µmol, 17.4 µL, 4 eq.) were dissolved in NMP (0.75 mL, 0.07 M). This solution was added to dry H-Lys(Boc)-Arg(Pbf)-Arg(Pbf)-Ser(*t*Bu)-Gly-Gln(Trt)-Arg(Pbf)-Lys(Boc)-Pro-Ala-Thr(*t*Bu)-Phe-Tyr(*t*Bu)-Val-Arg(Pbf)-Thr(*t*Bu)-Thr(*t*Bu)-Ile-Asn(Trt)-Lys(Boc)-Asn(Trt)-Ala-Arg(Pbf)-Ala-Thr(*t*Bu)-Leu-Tentagel S RAM resin (25 µmol) and the reaction vessel was shaken overnight. The resin was washed with 3 x NMP and 6 x

DCM and dried under a N_2 flow. Next, the resin was treated with a standard deprotection cocktail 95:2.5:2.5 TFA:TIS:H_2O (3 mL) for 105 min. The resin was filtered off and the filtrate was collected in a 1:1 Et_2O:Pentane solution (46 mL). The resin was washed with TFA (1 mL) and was collected in the same Et_2O:Pentane solution. The solution containing the crude peptide was cooled to -20°C and centrifuged after which the organic layer was decanted. The remaining pallet was dried with a N_2 flow after it was dissolved in NMP. The solution was further diluted with an equal volume of a 1:1:1 HO*t*Bu:H_2O:MeCN solution. Purification by HPLC provided compound **18b** in a 3% yield (3.04 mg, 0.81 µmol). **LC-MS:** R_t = 6.007 (Phenomenex Gemini® 3 µm C_{18} 110 Å 50x4.6 mm, 10-90% MeCN, 10 min); **ESI-MS:** m/z 1247.3 $[M+3H^+]^{3+}$; **HRMS [M+7H]$^{7+}$:** $[C_{164}H_{292}N_{51}O_{46}S]^{7+}$ 534.88360 (measured), 534.88212 (calculated).



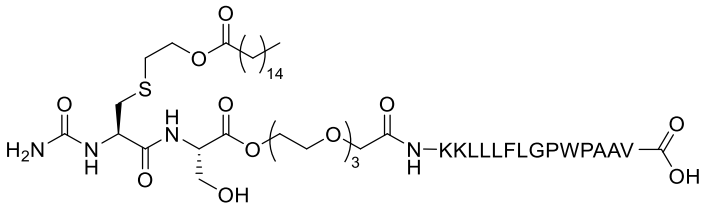
((S)-15-hydroxy-13-oxo-14-((R)-3-((2-(palmitoyloxy)ethyl)thio)-2-ureidopropanamido)-3,6,9,12-tetraoxapentadecanoyl)-Lys-Ala-Asp-Pro-Phe-Pro-Pro-Asn-Gly-Ala-Pro-Pro-Leu-Lys-Pro-His-Pro-Leu-Met-Pro-Ala-Asn-Pro-Trp-Gly-NH_2 (18c)

Compound **5** (100 µmol, 72 mg, 2 eq.), HCTU (100 µmol, 42 mg, 2 eq.) and D*i*PEA (200 µmol, 34.8 µL, 4 eq.) were dissolved in NMP (1.5 mL, 0.07 M). This solution was added to dry H-Lys(Boc)-Ala-Asp(*t*Bu)-Pro-Phe-Pro-Pro-Asn(Trt)-Gly-Ala-Pro-Pro-Leu-Lys(Boc)-Pro-His(Trt)-Pro-Leu-Met-Pro-Ala-Asn(Trt)-Pro-Trp(Boc)-Gly-Tentagel S RAM resin (50 µmol) and the reaction vessel was shaken overnight. The resin was washed with 3 x NMP and 6 x DCM and dried under a N_2 flow. Next, the resin was treated with a standard deprotection cocktail 95:2.5:2.5 TFA:TIS:H_2O (6 mL) for 105 min. The resin was filtered off and the filtrate was collected in a 1:1 Et_2O:Pentane solution (2x 46 mL). The resin was washed with TFA (1 mL) and was collected in the same Et_2O:Pentane solution. The solution containing the crude peptide was cooled to -20°C and centrifuged after which the organic layer was decanted. The remaining pallet was dried with a N_2 flow after it was dissolved in NMP. The solution was further diluted with an equal volume of a 1:1:1 HO*t*Bu:H_2O:MeCN solution. Purification by HPLC provided compound **18c** in a 7% yield (11.22 mg, 3.35 µmol). **LC-MS:** R_t = 6.499 (Phenomenex Gemini® 3 µm C_{18} 110 Å 50x4.6 mm, 10-90% MeCN, 10 min); **ESI-MS:** m/z 1676.5 $[M+2H^+]^{2+}$; **HRMS [M+3H]$^{3+}$:** $[C_{158}H_{247}N_{36}O_{40}S_2]^{3+}$ 1117.59471 (measured), 1117.59418 (calculated).



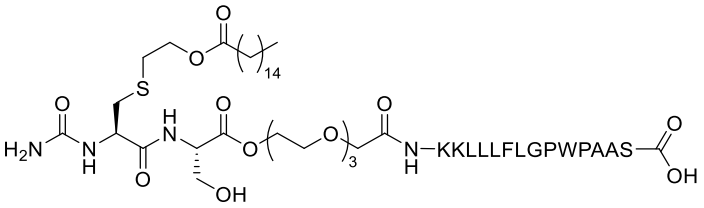
((S)-15-hydroxy-13-oxo-14-((R)-3-((2-(palmitoyloxy)ethyl)thio)-2-ureidopropanamido)-3,6,9,12-tetraoxapentadecanoyl)-Arg-Gly-Leu-Pro-Ala-Leu-Leu-Leu-Leu-Leu-Phe-Leu-Gly-Pro-Trp-Pro-Ala-Ala-Val-OH (18d)

Compound **5** (100 µmol, 72 mg, 2 eq.), HCTU (100 µmol, 42 mg, 2 eq.) and D*i*PEA (200 µmol, 34.8 µL, 4 eq.) were dissolved in NMP (1.5 mL, 0.07 M). This solution was added to dry H-Arg(Pbf)-Gly-Leu-Pro-Ala-Leu-Leu-Leu-Leu-Leu-Phe-Leu-Gly-Pro-Trp(Trt)-Pro-Ala-Ala-Val-Tentagel Wang resin (50 µmol) and the reaction vessel was shaken overnight. The resin was washed with 3 x NMP and 6 x DCM and dried under a N_2 flow. Next, the resin was treated with a standard deprotection cocktail 95:2.5:2.5 TFA:TIS:H_2O (6 mL) for 105 min. The resin was filtered off and the filtrate was collected in a 1:1 Et_2O:Pentane solution (2x 46 mL). The resin was washed with TFA (1 mL) and was collected in the same Et_2O:Pentane solution. The solution containing the crude peptide was cooled to -20°C and centrifuged after which the organic layer was decanted. The remaining pallet was dried with a N_2 flow after it was dissolved in DMSO. Purification by HPLC provided compound **18d** in a 4% yield (5.57 mg, 2.05 µmol). **LC-MS:** R_t = 8.72 (Phenomenex Gemini® 3 µm C_{18} 110 Å 50x4.6 mm, 10-90% MeCN, 10 min); **ESI-MS:** m/z 1362.3 $[M+2H^+]^{2+}$; **HRMS [M+2H]$^{2+}$:** $[C_{134}H_{222}N_{26}O_{31}S]^{2+}$ 1361.81541 (measured), 1361.81520 (calculated).



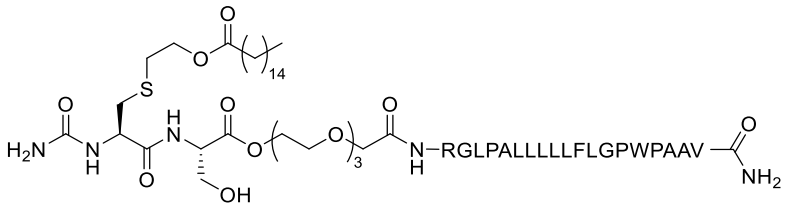
((S)-15-hydroxy-13-oxo-14-((R)-3-((2-(palmitoyloxy)ethyl)thio)-2-ureidopropanamido)-3,6,9,12-tetraoxapentadecanoyl)-Lys-Lys-Leu-Leu-Leu-Phe-Leu-Gly-Pro-Trp-Pro-Ala-Ala-Val-OH (18e)

Compound **5** (100 µmol, 72 mg, 2 eq.), HCTU (100 µmol, 42 mg, 2 eq.) and D*i*PEA (200 µmol, 34.8 µL, 4 eq.) were dissolved in NMP (1.5 mL, 0.07 M). This solution was added to dry H-Ser(Boc)-Lys(Boc)-Lys(Boc)-Lys(Boc)-Lys(Boc)-Lys(Boc)-Lys(Boc)-Leu-Leu-Leu-Phe-Leu-Gly-Pro-Trp(Trt)-Pro-Ala-Ala-Val-Tentagel-Wang resin (50 µmol) and the reaction vessel was shaken overnight. The resin was washed with 3 x NMP and 6 x DCM and dried under a N_2 flow. Next, the resin was treated with a standard deprotection cocktail 95:2.5:2.5 TFA:TIS:H_2O (6 mL) for 105 min. The resin was filtered off and the filtrate was collected in a 1:1 Et_2O:Pentane solution (2x 46 mL). The resin was washed with TFA (1 mL) and was collected in the same Et_2O:Pentane solution. The solution containing the crude peptide was cooled to -20°C and centrifuged after which the organic layer was decanted. The remaining pallet was dried with a N_2 flow after it was dissolved in DMSO. Purification by HPLC provided compound **18e** in a 9% yield (9.63 mg, 4.26 µmol). **LC-MS:** R_t = 4.54 (Phenomenex Gemini® 3 µm C_{18} 110 Å 50x4.6 mm, 10-90% MeCN, 10 min); **ESI-MS:** m/z 1130.3 $[M+2H^+]^{2+}$; **HRMS [M+2H]$^{2+}$:** $[C_{112}H_{186}N_{20}O_{26}S]^{2+}$ 1123.65925 (measured), 1123.65965 (calculated).



((S)-15-hydroxy-13-oxo-14-((R)-3-((2-(palmitoyloxy)ethyl)thio)-2-ureidopropanamido)-3,6,9,12-tetraoxapentadecanoyl)-Lys-Lys-Leu-Leu-Leu-Phe-Leu-Gly-Pro-Trp-Pro-Ala-Ala-Ser-OH (18f)

Compound **5** (100 µmol, 72 mg, 2 eq.), HCTU (100 µmol, 42 mg, 2 eq.) and D*i*PEA (200 µmol, 34.8 µL, 4 eq.) were dissolved in NMP (1.5 mL, 0.07 M). This solution was added to dry H-Ser(Boc)-Lys(Boc)-Lys(Boc)-Lys(Boc)-Lys(Boc)-Lys(Boc)-Lys(Boc)-Leu-Leu-Leu-Phe-Leu-Gly-Pro-Trp(Trt)-Pro-Ala-Ala-Ser(*t*Bu)l-Tentagel Wang resin (50 µmol) and the reaction vessel was shaken overnight. The resin was washed with 3 x NMP and 6 x DCM and dried under a N_2 flow. Next, the resin was treated with a standard deprotection cocktail 95:2.5:2.5 TFA:TIS:H_2O (6 mL) for 105 min. The resin was filtered off and the filtrate was collected in a 1:1 Et_2O:Pentane solution (2x 46 mL). The resin was washed with TFA (1 mL) and was collected in the same Et_2O:Pentane solution. The solution containing the crude peptide was cooled to -20°C and centrifuged after which the organic layer was decanted. The remaining pallet was dried with a N_2 flow after it was dissolved in DMSO. Purification by HPLC provided compound **18f** in a 5% yield (5.93 mg, 2.64 µmol). **LC-MS:** R_t = 3.91 (Phenomenex Gemini® 3 µm C_{18} 110 Å 50x4.6 mm, 10-90% MeCN, 10 min); **ESI-MS:** m/z 1124.2 $[M+2H^+]^{2+}$; **HRMS $[M+2H]^{2+}$:** $[C_{110}H_{182}N_{20}O_{27}S]^{7+}$ 1117.59418 (measured), 1117.59445 (calculated).



((S)-15-hydroxy-13-oxo-14-((R)-3-((2-(palmitoyloxy)ethyl)thio)-2-ureidopropanamido)-3,6,9,12-tetraoxapentadecanoyl)-Arg-Gly-Leu-Pro-Ala-Leu-Leu-Leu-Leu-Leu-Phe-Leu-Gly-Pro-Trp-Pro-Ala-Ala-Val-OH (18d)

Compound **5** (50 µmol, 36 mg, 2 eq.), HCTU (50 µmol, 21 mg, 2 eq.) and D*i*PEA (100 µmol, 17.4 µL, 4 eq.) were dissolved in NMP (1.5 mL, 0.07 M). This solution was added to dry H-Arg(Pbf)-Gly-Leu-Pro-Ala-Leu-Leu-Leu-Leu-Leu-Phe-Leu-Gly-Pro-Trp(Trt)-Pro-Ala-Ala-Val-Tentagel S RAM resin (25 µmol) and the reaction vessel was shaken overnight. The resin was washed with 3 x NMP and 6 x DCM and dried under a N_2 flow. Next, the resin was treated with a standard deprotection cocktail 95:2.5:2.5 TFA:TIS:H_2O (3 mL) for 105 min. The resin was filtered off and the filtrate was collected in a 1:1 Et_2O:Pentane solution (2x 46 mL). The resin was washed with TFA (1 mL) and was collected in the same Et_2O:Pentane solution. The solution containing the crude peptide was cooled to -20°C and centrifuged after which the organic layer was decanted. The remaining pallet was dried with a N_2 flow after it was dissolved in DMSO. Purification by HPLC provided compound **18d** in a 10% yield (6.99 mg, 2.57 µmol). **LC-MS:** R_t = 8.72 (Phenomenex Gemini® 3 µm C_{18} 110 Å 50x4.6 mm, 10-90% MeCN, 10 min); **ESI-MS:** m/z 1362.3 $[M+2H^+]^{2+}$; **HRMS $[M+3H]^{3+}$:** $[C_{134}H_{224}N_{27}O_{30}S]^{3+}$ 907.88495 (measured), 907.88456 (calculated).

***In vitro* experiments**[39] (work of J.M.M. Heuts)

Activation TLR-2 transfected HEK cells

HEK-TLR2 cells were cultured in DMEM F12 medium (Gibco, Bleiswijk, The Netherlands) supplemented with 8% fetal calf serum (FCS) (Greiner Biosciece, Alphen a/d Rijn, the Netherlands), 100 IU/mL penicillin/streptomycin, 2 mM L-glutamin and the antibiotic G418 (Gibco) as an selection agent. For the described experiments the cells were seeded in a 96-well plate, 3x104 cells per well, in DMEM F12 medium and left overnight at 37 °C and 5% CO2 to ensure proper attachment. The following day the different compounds were titrated, in DMEM F12, and added to the seeded HEK-TLR2 c ells. After 48 h of incubation the supernatant was harvested and the amount of human IL-8 was determined by a sandwich ELISA (Biolegend, Amsterdam, The Netherlands).

Monocyte derived dendritic cell maturation

Peripheral blood mononuclear cells (PBMCs) were obtained from healthy donors or the melanoma patient, when available, by the centrifugation of buffy coats (Sanquin Blood bank, Leiden, The Netherlands) over a ficoll gradient in Leucosep tubes (Biogreiner, Alphen a/d Rijn, The Netherlands). The CD14+ monocytes were isolated from the PBMC fraction by making use of magnetic CD14+ microbeads (Miltenyi Biotec, Leiden, The Netherlands). The monocytes were cultured in RPMI supplemented (Gibco) with 500 U/mL of human IL-4 and 800 U/mL human GMCSF at 37 °C and 5% CO_2. After 3 days fresh RPMI was added, similar volume as in which the cells were cultured, with 1000 U/mL of human IL-4 and 1600 U/mL human GM-CSF. On day 5 the percentage of CD11c+ monocyte derived dendritic cells (moDCs) was determined by Flow-cytometry (BD, Vianen, The Netherlands) to ensure successful differentiation. For the maturation experiments 3x104 cells/well were seeded after which the different compounds were added in titrated amounts. After 48 h of incubation the supernatant was harvested to determine the produced amount of IL-12p40 by a sandwhich ELISA (Biolegend). The moDCs were harvested and fluorescently labeled with antibodies for CD83 and CD86 (Biolegend) and expression was determined by using a LSR II flow cytometer (BD, Vianen, The Netherlands). Data processing was performed with Flowjo software (Flowjo LLC).

Neoepitope specific T-cell activation

The tumor specific T-cells used in this study were previously established in the laboratory of Medical Oncology (LUMC, Leiden, The Netherlands) as previously described. s1, s2 The cell line was obtained after informed consent of the patient in the context of clinical trial (P04.085) that was approved by the Medical Ethical Committee of the Leiden University Medical Center and conducted in accordance with the Declaration of Helsinki. In brief, the melanoma tissue was surgically obtained after which mechanical dissociation was performed to obtain single cell suspensions. Cells were cultured in DMEM medium (Life Technologies, Breda, the Netherlands) supplementend with 8% FCS, penicillin (50 U/mL), streptomycin (50 µg/mL), and L-glutamine (4mM) (Life Technologies). The tumor specific T-cells were generated by a mixed lymphocyte-tumor culture (MLTC). In brief, PBMCs were incubated with irradiated autologous tumor cells in T-cell medium, (Iscoves Modified Dulbecco's Medium (IMDM) with penicillin (100 IU/mL), streptomycin (100 µg/mL) and L-glutamine (4mM) (all from Life Technologies, Breda, the Netherlands), and 7.5% heat inactivated pooled human serum (Sanquin, Bloodbank, Amsterdam, the Netherlands) supplemented with interleukin-2 (IL-2) 150 IU/ml (Aldesleukin, Novartis, Arnhem, The Netherlands). Medium was refreshed every 2 to 3 days, after 4 weeks of culture the T-cells were stored by cryopreservation. For the antigen presentation

experiments HLA-matched monocytes or, if available, autologous monocytes were used as antigen presenting cells. On the first day 5x105 monocytes per well, X-Vivo medium (Life Technologies), of a 48-well plate. Monocytes were left two hours to adhere at 37 °C and 5% CO2 after which the non-adherent cells were removed. Subsequently, the attached monocytes were washed. X-vivo medium supplemented with 800 U/ml of GM-CSF was added after which the cells were cultured for 2 days at 37 °C and 5% CO2. On day 3 the monocytes were gently washed to remove additional non-adherent cells. The different S5 compounds were titrated (2 – 0.5 µM) in X-vivo medium and added to the monocytes. The following day the medium was removed and 2x105 T-cells per well were added to the monocytes. After overnight incubation the supernatant was harvested for IFNγ quantification by a sandwhich ELISA (Sanquin, Amsterdam, The Netherlands). The monocytes were stained with fluorescent antibodies for CD3, CD4, CD8, CD137 (Biolegend) and Yellow Arc (Thermofisher, Bleiswijk, The Netherlands) was used as the live/dead marker. Cells were analyzed by using a Fortessa flow cytometer (BD). Data processing was performed with Flowjo software (Flowjo LLC).

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Chapter 5

Synthesis and evaluation of Ovalbumin derived peptides functionalized with a TLR2 and TLR7 agonist

Introduction

Pattern recognition receptors (PRRs) belong to the first line of defense of vertebrates for the identification of pathogen invasions. In particular, 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 specific highly conserved molecular moieties, that are termed pathogen associated molecular patterns (PAMPs) or damage associated molecular patterns (DAMPs). When these molecular patterns bind to the 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), C-type lectin receptors (CLRs) and Toll-like receptors (TLRs). 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. The human TLR family consists of ten members, each recognizing specific PAMPs.[1] TLRs are transmembrane proteins whose structure consists out of a leucine rich region that harbors the PAMP binding pocket, a transmembrane domain and a cytoplasmic tail called the TIL domain. Upon association with a PAMP, a TLR forms either a homo- or a heterodimer, which drives conformational adjustments in both receptors bringing both TIL-

domains together. This activates intracellular signalling cascades, leading amongst other to, cell-maturation, up-regulation of the cell- surface expression of co-stimulatory (such as CD80 and CD86) molecules and the induction of the expression of cytokines, (such as IL-12). TLRs are situated at different cellular locations, while TLR1, 2, 4, 5, and 6 occur at the cell surface, TLR3, 7, 8, and 9 reside in intracellular endosomal compartments. Synthetic ligands of relatively low molecular weight have been discovered for the TLR1/2 and TLR 2/6 combinations, for TLR4, and either selective or not for TLR7 and TLR8. Furthermore, structure-activity studies have culminated in several active agonists with the TLR4 ligand mono-phosphoryl lipid A (MPLA) as the first approved agonist for application as adjuvant in vaccines. Although much can be expected from the immunological properties of individual TLRs, multiple PRRs are activated upon a pathogen attack, making it an incentive to investigate the potential synergistic effect of combinations of synthetically accessible PRR ligands. Many data have been obtained with the immunological evaluation of mixtures of separate PAMPs.[2-4] Apart from this, the approach of the group of Esser-Kahn stands out, who show the great potential of covalently linked bi- and tri-agonists.[5-7] The design of such multi-agonists is challenging as their activity depends on the nature of the composing PAMPS, the position of attachment and the linker that interconnects them.

In line with the possible synergistic activity of covalently connected PAMPS[5-11] is the evolvement of the completely synthetic vaccines in which one structurally defined TLR or other PRR ligand has been covalently fused with a synthetic oligopeptide, containing a specific epitope. The favorable immunological properties of these vaccine modalities have been demonstrated many times. Synthetic peptides, containing epitopes of different origin have mostly been covalently connected to TLR2 ligands.[12-30] However, other PRR ligands[31] have also been incorporated into conjugates with various antigens such as a TLR4 (Lipid A analogue[32-33]), a TLR7/8 (2-alkoxy-8-oxo-adenine,[34-36] 6-(4-amino-2-butyl-imidazoquinolyl)-norleucine[37]), a TLR9 (CpG oligonucleotide[12]) and a NOD ligand (muramyl dipeptide[17]). With the expectation that peptide conjugates incorporating more than one PRR ligand may exhibit an improved immunological profile, some of these types of conjugates have been designed, synthesized and immunologically evaluated, such as conjugates incorporating ligands for both NOD2 and TLR2 together with a class-I OVA epitope,[20] a conjugate harbouring ligands for the mannose-6-phosphate receptor and TLR7 together with a class-I OVA epitope[36] and conjugates having a combination of DC-SIGN ligands, a TLR7 agonist and a gp100 antigen.[38] This chapter describes the synthesis, and immunological evaluation of conjugates, in which the TLR7 ligand [4-((6-amino-2-butoxy-8-oxo-7,8-dihydro-9H-purin-9-yl)methyl)benzoic acid] and the TLR2 ligand [Pam_3CSK_4] are covalently attached to ovalbumin derived peptides, provided with $DEVA_5K$ (**1a**, **2a**) and HAAHA (**1b**, **2b**) as model peptides, containing an MHC I or MHC II epitope respectively (Figure 1). From now on in this chapter, all $DEVA_5K$ or HAAHA containing constructs will be referred to with an **a** or **b** affix, respectively.

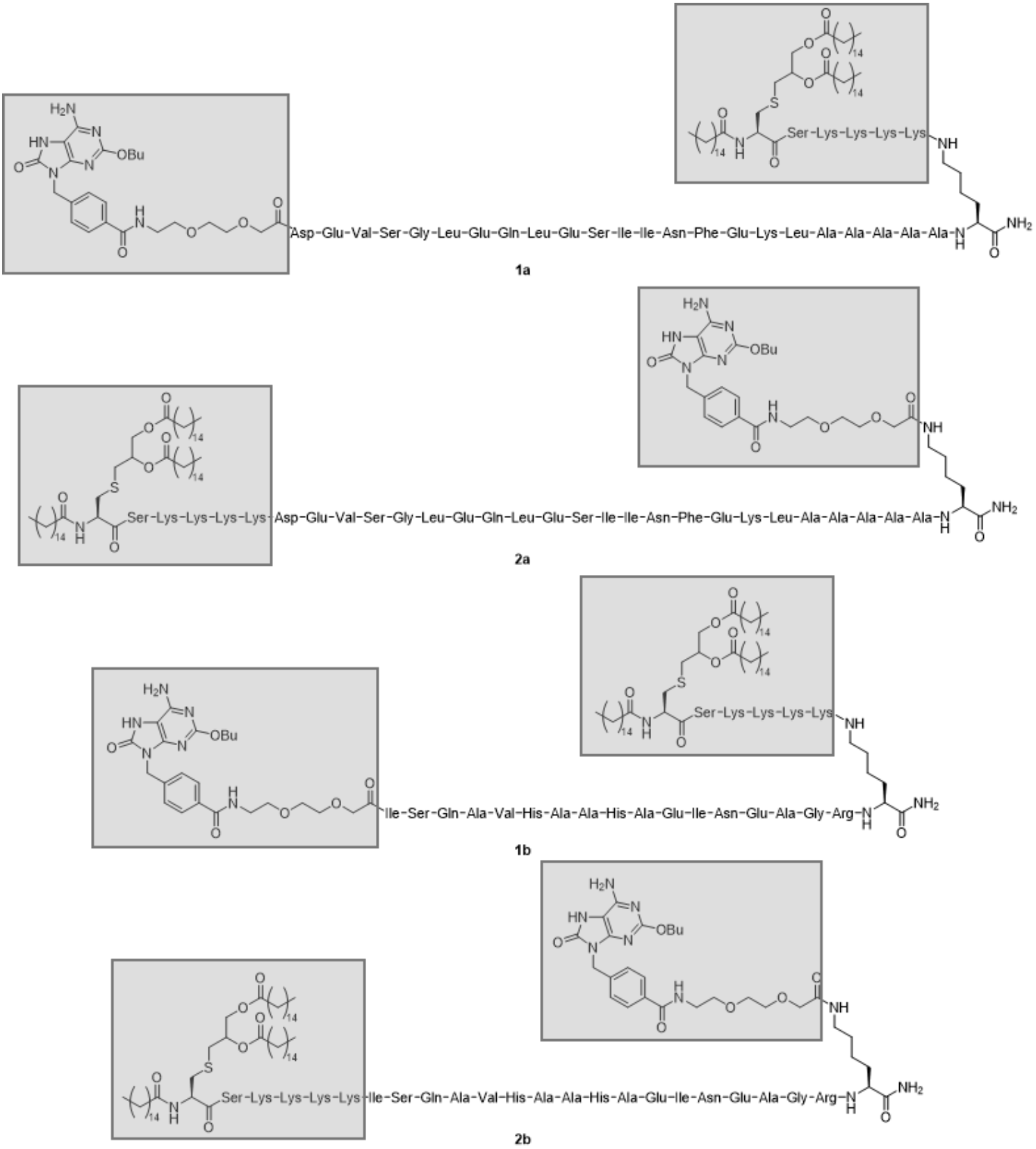
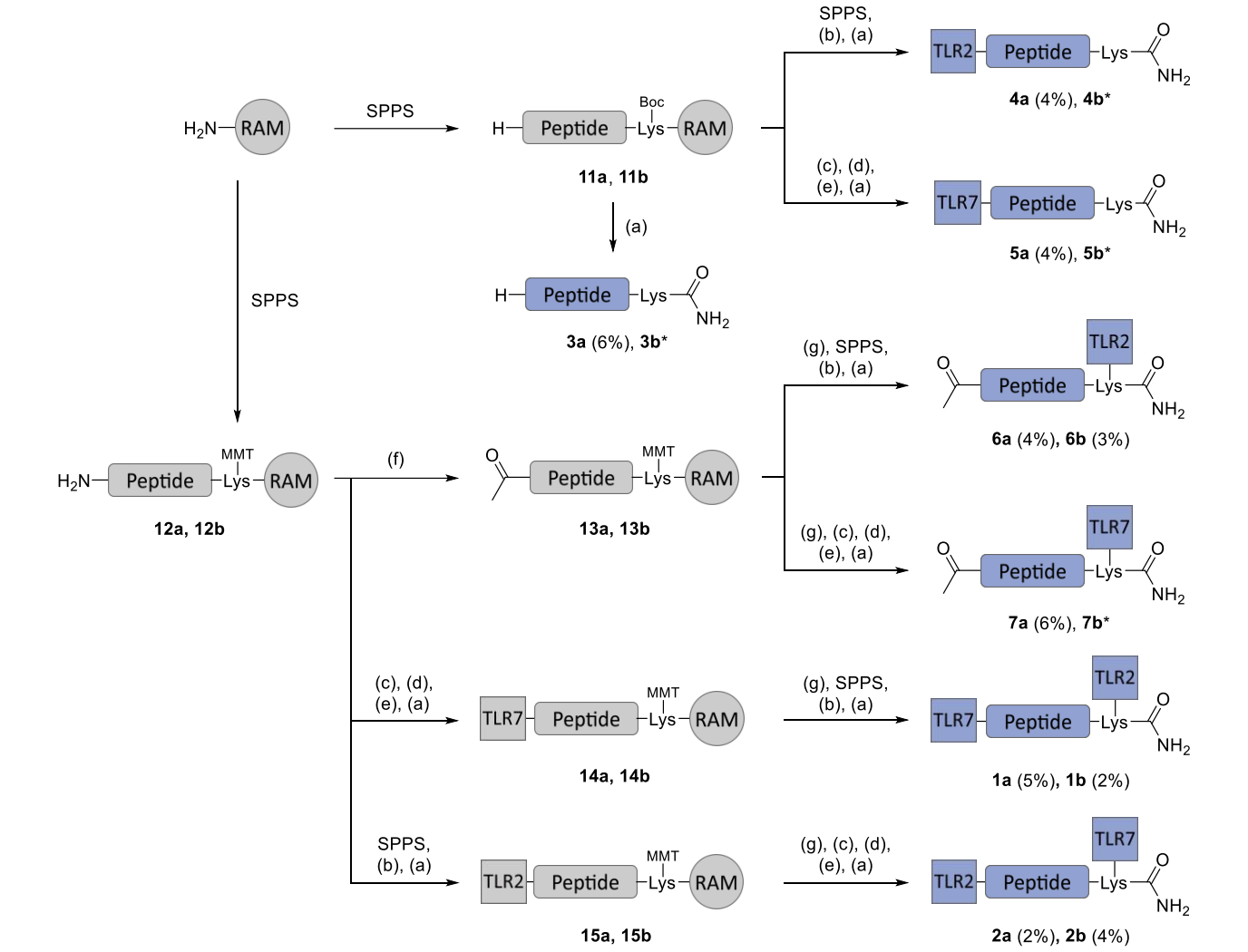


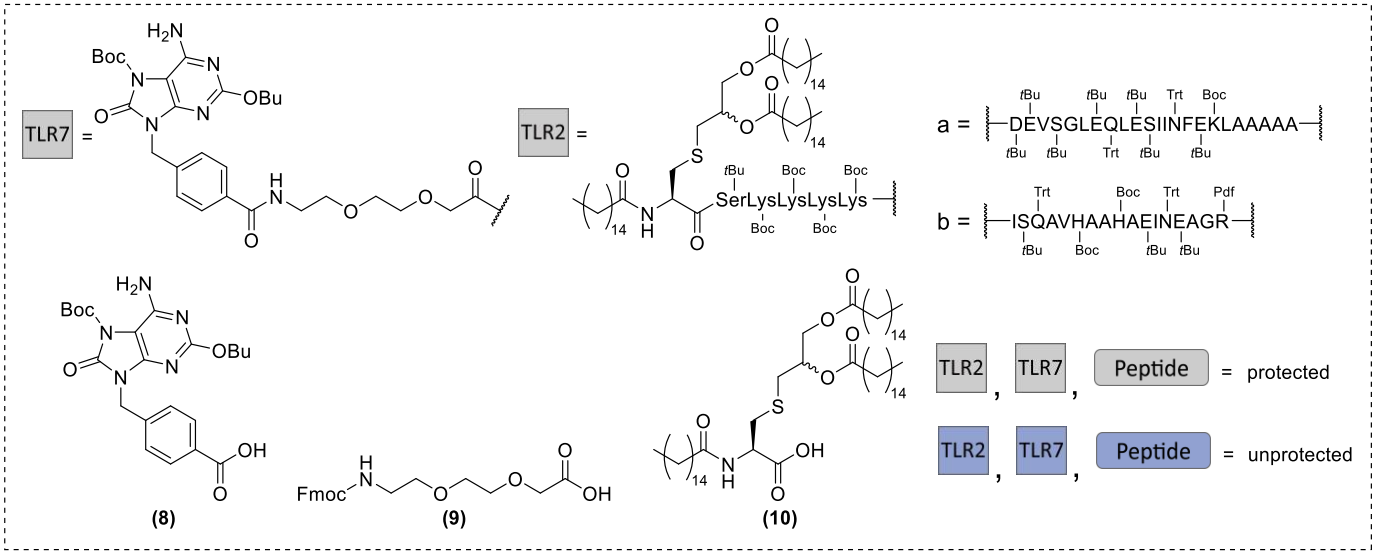
Figure 1: Structures of the target TLR2/TLR7 dual-functionalized ovalbumin antigen conjugates, where the individual structures of the ligands, including the connecting spacer are marked with a grey box. Conjugates **1a** and **2a** contain the $DEVA_5K$ sequence, and conjugates **1b** and **2b** contain the HAAHA sequence.

Results and Discussion

As shown in Figure 1, conjugate **1a** comprises an ovalbumin peptide, provided with MHC class I $DEVA_5K$ epitope that is connected at its N-terminal end to the TLR7 ligand, 4-((6-amino-2-butoxy-8-oxo-7,8-dihydro-9H-purin-9-yl)methyl)benzoic acid via the 8-amino-3,6-dioxaoctanoic acid spacer while the side chain of its C-terminal lysine is linked to the TLR2 ligand Pam_3CSK_4 via the Lys_4 spacer. Conjugate **2a** comprises the same constituents as **1a** while the TLR7 ligand and the TLR2 ligand have switched position. Conjugate **1b** corresponds with **1a** and conjugate **2b** corresponds with **2a** but in both cases the $DEVA_5K$ (**1a, 2a**) is replaced by HAAHA (**1b, 2b**) epitope. For the immunological evaluation of these target dual conjugates the following control compounds are needed; (i) the individual peptides **3a**, **b** (ii) the corresponding single functionalized conjugates linked at the N-terminal end with either a TLR2 **4a**, **b** or TLR7 ligand **5a**, **b** (iii) the corresponding single functionalized conjugates linked at the C-terminal end with either a TLR2 **6a, b** or TLR7 ligand **7a**, **b** (see Scheme 1).

All compounds were assembled using a TRIBUTE® Peptide Synthesizer and Tentagel S RAM as solid support, with the aid of a Fmoc chemistry elongation protocol, in which all amino acids were coupled twice under influence of HCTU as condensing agent. The thus automatically produced immobilized peptides were subsequently manually functionalized at the selected N- or C- ends with either one (TLR2L and TLR7L) or with consecutively both ligands (Scheme 1). The synthesis of the protected TLR7 ligand (**8**) is described elsewhere,[35] whereas spacer (**9**), TLR 2 ligand (**10**) and all amino acids are commercially available. Peptide **3b** and conjugates **4b**, **5b** and **7b** were synthesized by Niels Reintjes as described in his thesis.[39] Since the unfunctionalized peptide **3a** and the N-functionalized conjugates (either with a TLR2 **4a** or TLR7 ligand **5a**) do not require an orthogonal protecting group at the side chain of the C-terminal lysine, only standard amino acid building blocks were used for the SPPS of the immobilized precursor peptide **11a** (Scheme 1). Direct deprotection using TFA:TIS:H_2O and precipitation with a mixture 1:1 Et_2O:pentane was followed by centrifugation to give a pellet of the crude peptide. Isolation of the target peptides was attained by dissolving the crude material in 1:1:1 HO*t*Bu:H_2O:MeCN and subsequent RP-HPLC purification with a C18 column, providing $DEVA_5K$ peptide **3a** in 6% yield. The assembly of mono-conjugate **4a** with a N-terminal TLR2 ligand entailed the installation of the Pam_3SK_4 ligand at the N-terminal end of **11a**. Elongation of immobilized peptide **11a** with the spacer SK_4 was followed by the HCTU mediated condensation of palmitoyl-Cys((RS)-2,3-di(palmitoyloxy)-propyl)-OH **10** to give the immobilized precursors of mono-conjugate **4a**. Similar to individual peptide **3a** all protecting groups were removed with concomitant release from the resin, precipitation and centrifugation to give the crude conjugate **4a** as a pellet. Finally, purification by HPLC, using a diphenyl column, yielded Pam_3CSK_4 functionalized $DEVA_5K$ conjugate **4a** in an overall yield of 4%. The assembly of the monoconjugate **5a** with a N-terminal TLR7 ligand started with applying the linker by condensation of immobilized and protected peptide **11a** with the commercially available 8-(Fmoc-amino)-3,6-dioxaoctanoic acid (**9**) under influence of HCTU. After the removal of the Fmoc group by treating the obtained resin three times with 20%





Scheme 1: Assembly of reference compounds **3a-7a**, **6b-7b** and dual conjugates **1a**, **1b**, **2a**, and **2b**. Below the scheme the structure of both TLR ligands and peptide sequences are given. Reaction Conditions: (a) TFA:TIS:H_2O 95:2.5:2.5, RT, 105 min; (b) Compound **10**, HCTU, DiPEA, 1:1 DCM:NMP, overnight; (c) Compound **9**, HCTU, D*i*PEA, DMF, overnight; (d) 20% piperidine in DMF, 3x 5 min; (e) compound **8**, HCTU, D*i*PEA, DMF, overnight; (f) Ac_2O, NMM, DMF, 5 min; (g) 1% TFA in DCM, 10x 30 sec then 2x 5 min.
*Peptide **3b** and conjugates **4b**, **5b** and **7b** were synthesized by Niels Reintjes.[39]

piperidine in DMF for 5 minutes, the released amine was coupled with TLR7 building block **8** using HCTU as condensing agent overnight to give the protected and immobilized progenitor of mono-conjugate **5a**. Removal of the protecting groups, cleavage from the solid support and HPLC purification, according to a similar procedure as described for **4a** resulted in the isolation of N-terminal TLR7 functionalized conjugate **5a** in 4% yield.

To obtain C-terminal mono- and dual functionalized conjugates, immobilized peptides (**12a, b**), containing the orthogonal MMT protecting group at the side chain of the C-terminal lysine were assembled using the same SPPS protocol (Scheme 1). En route to monofunctionalized TLR2 conjugates (**6a, b**) and TLR7 conjugate (**7a**), the SPPS of **12a, b** was followed by acetylation of the N-terminal amine with Ac_2O and NMM to give the capped peptide **13a, b**. Selective removal of the MMT group with 1% TFA in DCM, allowed for the introduction of each TLR ligand on the C-terminal end, using the same sequence of reactions and conditions, as described above for the appendage of TLR2 and TLR7 ligands at the N-terminal end (i.e. **4a** and **5a** respectively). It is important to mention, that after the MMT deprotection, the freed amine is protonated and requires quenching with a base to react it further or store immobilized peptide for longer periods of time. Like **4a** and **5a**, the immobilized progenitors were subjected to the same sequence of events leading to the isolation of the C-terminal monofunctionalized TLR2 conjugates (**6a, b**) and TLR7 conjugates (**7a**) in satisfactory yields.

Now that the stage is reached where all mono-conjugates are synthesized and the needed experimental protocols for ligand incorporation are available, the assemblage of the four target dual conjugates can be undertaken using immobilized protected peptides (**12a, b**). Both the TLR2 and the TLR7 were appended at the N-terminal end of **12a** and **12b** to give the monofunctionalized resins (**14a, b**) and (**15a, b**) respectively. The TLR2 ligand was installed at the C-terminal end of resin (**14a, b**) and the TLR7 ligand was installed at the C-terminal end of resin (**15a, b**) using the same protocols as described above. Finally, the same isolation used for the monoconjugates was successful for the isolation of all dual conjugates to give $DEVA_5K$ dual conjugates **1a** and **2a** in a yield of 5% and 2% respectively, and HAAHA dual conjugates **1b** and **2b** in a yield of 2% and 4% respectively.

Immunological evaluation

Until now, only conjugates **1a** and **1b** were tested for their ability to induce DC maturation by determining the expression of surface markers CD40 CD70, CD80 and CD86 and IL-12 cytokine production in bone marrow-derived dendritic cells (BMDCs). Conjugates **2a** and **2b** were left out this evaluation, since their immunological relevance was questionable after *Gential et al.* published that C-terminal TLR7 functionalized conjugates failed to induce any significant T-cell responses.[35] Dual conjugates were compared with its reference compounds to investigate any synergistic interactions between TLR2 and TLR7. In addition, mixtures containing TLR7 ligand **8**, TLR2 ligand **11**, both **8** and **11**, and reference conjugates **5a, b** and **6a, b** with TLR2 ligand **11** and TLR7 **8** respectively were incubated with BMDCs. Finally, peptides **3a** and **3b** served as a

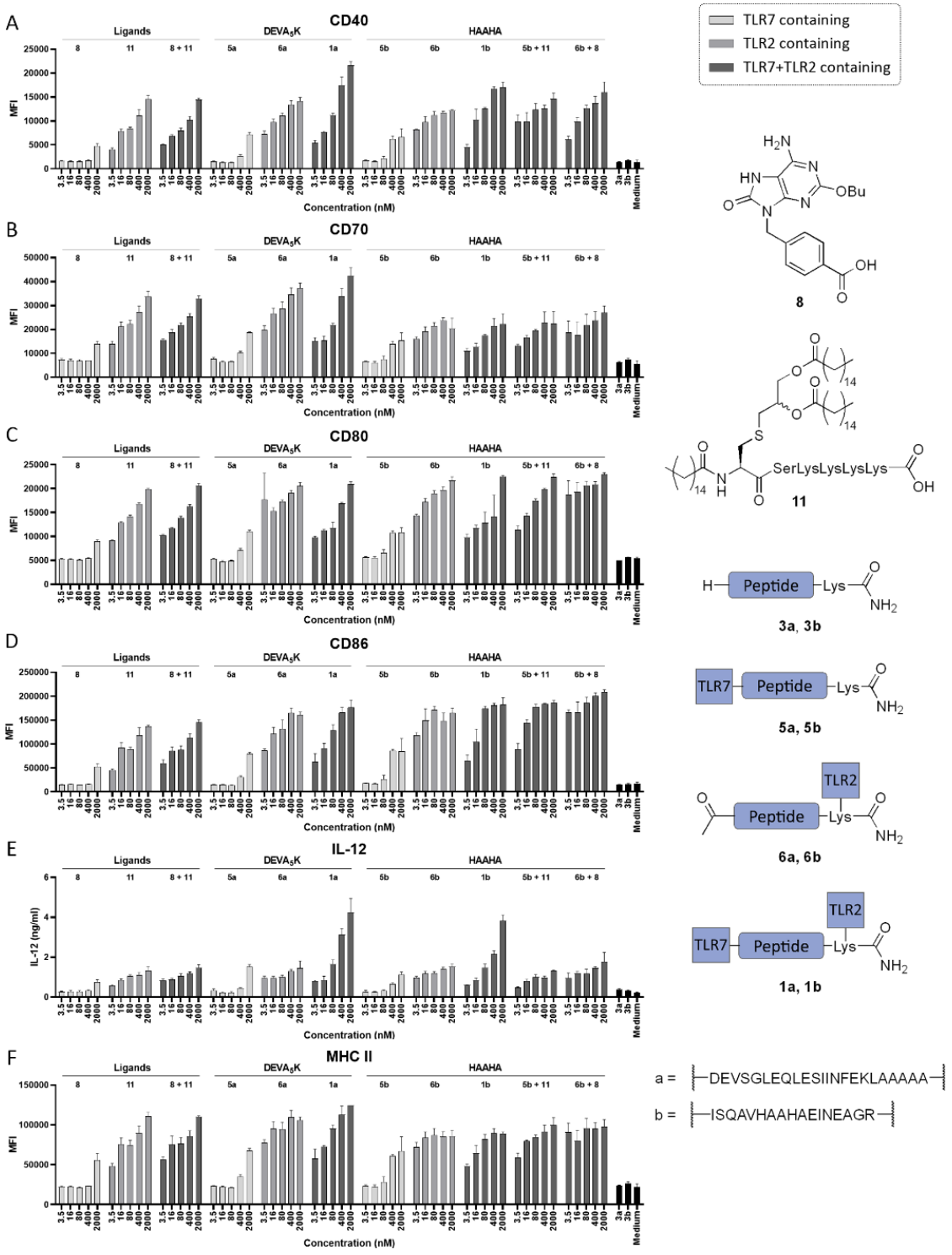


Figure 2: Induction of BMDC maturation by TLR2-TLR7 functionalized conjugates **1a** and **1b** with reference compounds **3a**, **3b**, **5a**, **5b**, **6a**, **6b**, **8** and **11** as measured by the expression of CD40 (A), CD70 (B), CD80 (C), CD86 (D), MHC II (F) and the production of IL-12 (E). BMDCs were incubated with either ligand, conjugate, or ligand plus conjugate of interested. After 3 h, the cells were washed and incubated with fresh medium. After another 16 h, the supernatant was harvested and analysed for IL-12 production by ELISA analysis. Additionally, the cells were harvested and stained with an appropriate fluorescent antibody for CD40, CD70, CD80, CD86 and MHC II. Expression levels could then be determined by flow cytometry. Sequences for MHC I epitope containing peptide DEVA$_5$K (**a**) and MHC II epitope containing peptide HAAHA (**b**) are given in the bottom right of the figure.

negative control. By comparing the data of these control experiments with those of the dual conjugates, the effect of the conjugation of both ligands to the antigenic peptides and potential synergistic effects of the ligands could be determined.

Overall, biological evaluation of dual conjugates **1a** and **1b** show upregulation of surface markers comparable to all other conditions where the TLR2 ligand is present, either as free ligand or as a monoconjugate (see Figure 2A-D and F). This is consistent with the fact that significantly less upregulation is observed when the dendritic cells are exposed to the TLR7 or the TLR7 monoconjugate. Monoconjugates of ligand **8** and **11** with both antigens display slightly higher activities as does co-stimulation of both TLR2 and TLR7. The same trend is observed for IL-12 production, however treatment with **1a** and **1b** elicited by far the strongest IL-12 productions out of all incubation conditions (see Figure 2E). Interestingly, as the conjugation of either TLR2 or TLR7 ligands to the antigen or their synergistic interaction show only small increases in cytokine production, the nature for its increase should find its origin elsewhere. It could be that activation of both TLR2 and TLR7 in proximity induces stronger synergistic activation where in this case the antigen facilitates the clustering of receptors as it serves as a bridge between both agonists. Though TLR2 primarily resides on the cell membrane and TLR7 inside endosomes, it has been reported that TLR2 is internalized through endosomes[40-41] and thus being in proximity. Furthermore, *Mancini et al.*[8] reported a similar increase in activity when covalent coupling a TLR2 and TLR9 ligand together with an PEG based linker.

In conclusion, the newly designed dual conjugates comprising either model peptide $DEVA_5K$ (**1a, b**) or HAAHA (**2a, b**) and functionalized at the C- and N-terminal ends with a TLR2 and TLR7 ligand were successfully assembled via a combination of automatic and manual SPPS procedures, utilizing Fmoc-chemistry elongation protocol. Functionalization of the C-terminal end was attained using a lysine building block having its side chain orthogonally protected with the very acid sensitive MMT group. Biological evaluation of the dual conjugates **1a** and **1b** showed an upregulation of surface markers CD40, CD70, CD80, CD87 comparable to mono Pam_3 functionalized conjugates but elicited a superior IL-12 production. This increase could be explained by the clustering of activated TLR2 and TLR7 as the mixtures of the mono-conjugates and simultaneous activation of the receptors do not produce similar results. To determine whether the receptor clustering is the driving force in the increased agonistic activity, a similar experiment could be performed using a construct where both ligands are connected with a polyethylene glycol spacer, similarly to the approach of *Esser-Kahn et al.*[8] In the case that purely the conjugation drives increased activity, one could also investigate what the optimal length of the spacer would be and use this information to optimize self-adjuvating vaccine designs.

Experimental

Synthesis of simplified TLR2 ligand conjugated neoepitope containing synthetic long peptides

General Information

All reagents and solvents used in the solid phase peptide synthesis were purchased from Biosolve (Netherlands). Fmoc amino acids building blocks were purchased from Sigma Aldrich or Novabiochem. Tentagel based resins were purchased from Rapp Polymere GmbH (Germany). The solid-phase peptide synthesis was performed on a TRIBUTE™ Peptide Synthesiser (Gyros Protein Technologies AB, Arizona, USA) applying Fmoc based protocol starting with Tentagel S RAM resin (0.22-0.25 mmol/g) on a 100 µmol scale using established Fmoc protocols. LC-MS analysis was performed on one of the following LC-MS systems: A Thermo Finnigan LCQ Advantage MAX ion-trap mass spectrometer with an electrospray ion source coupled to Surveyor HPLC system (Thermo Finnegan), A Thermo Finnigan LCQ Fleet MAX ion-trap mass spectrometer with an electrospray ion source coupled to Vanquish UPLC system (Thermo Finnegan) or an Agilent Technologies 1260 Infinity LC system (detection simultaneously at 214 and 254 nm) coupled to a Agilent Technologies 6120 Quadrupole MS. Using an analytical Phenomenex Gemini® (3 µm C_{18} 110 Å 50x4.6 mm), Vydac 219TP 5 µm (150x4.6mm (Phenomex, 50 x 4.60 mm, 3 microns) or Cosmosil 5C_4-MS (5 µm particle size, 150x4.6 mm) in combination with eluents A: H_2O; B: MeCN and C: 1% TFA (aq.) as the solvent system, in which the gradient was modified by changing the ratio of A in B in combination with 10% C. High resolution mass spectra were recorded on an Q-Exactive HF Orbitrap (Thermo Scientific) equipped with an electrospray ion source (ESI), injection of 2 µL of a 1 µM solution via Ultimate 3000 nano UPLC (Dionex) system, with an external calibration (Thermo Scientific); Source voltage of 3.5 kV, capillary temperature 275 °C, no sheath gas, resolution = 240.000 at m/z = 400. Mass range m/z = 160-2000 or to a maximum of 6000. Eluents used: MeCN:H_2O (1:1 v/v) supplemented with 0.1% formic acid.

General procedure for automated solid phase synthesis

All reagents and solvents used in the solid phase peptide synthesis were purchased from Biosolve (Netherlands). Fmoc amino acids building blocks were purchased from Sigma Aldrich or Novabiochem. Tentagel based resins were purchased from Rapp Polymere GmbH (Germany). The solid-phase peptide synthesis was performed on a TRIBUTE® Peptide Synthesiser (Gyros Protein Technologies AB, Arizona, USA) applying Fmoc based protocol starting with Tentagel S-RAM resin (0.22-0.25 mmol/g) on a 100 µmol scale using established Fmoc protocols. The consecutive steps preformed during each cycle were:

1. Deprotection of the Fmoc protecting group with 3 x 4 mL 20% piperidine in DMF for 3 min;
2. Wash, 3 x 4 mL DMF;
3. First coupling of the appropriate amino acid applying a five-fold excess. Generally, the Fmoc amino acid (0.5 mmol, 5 eq.) building block was dissolved 0.2 M HCTU in DMF (2.5 mL, 5 eq.) in its loading cartridge and the resulting solution was transferred to the reaction vessel. Next, the cartridge was washed with a 0.5 M D*i*PEA in DMF (2 mL, 10 eq.) and subsequently the solution was transferred to the reaction vessel. The reaction vessel was shaken for 1 h at RT;
4. Wash, 3 x 4 mL DMF;
5. Second coupling of the appropriate amino acid applying a five-fold excess. Generally, the Fmoc amino acid (0.5 mmol, 5 eq.) building block was dissolved 0.2 M HCTU in DMF (2.5 mL, 5 eq.) in its loading cartridge and the resulting solution was transferred to the reaction vessel. Next, the

cartridge was washed with a 0.5 M D*i*PEA in DMF (2 mL, 10 eq.) and subsequently the solution was transferred to the reaction vessel. The reaction vessel was shaken for 1 h at RT;

6. Wash, 3 x 4 mL DMF;
7. Capping of unreacted peptide with 1 x 10% Ac_2O in DMF solution (5 mL) for 3 min;
8. Wash, 3 x 4 mL DMF;

After the last coupling cycle, the final Fmoc group was deprotected with 3 x 4 mL 20% piperidine in DMF for 3 min. Finally, the resin was washed with DMF (3x) and DCM (3x) and dried using a N_2 flow.

The following amino acid building blocks were used for the synthesis: Fmoc-Ala-OH, Fmoc-Asn(Trt)-OH, Fmoc-Asp(*t*Bu)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Cys(Trt)-OH, Fmoc-Gln(Trt)-OH, Fmoc-Glu(*t*Bu)-OH, Fmoc-Gly-OH, Fmoc-His(Trt)-OH, Fmoc-Ile-OH, Fmoc-Leu-OH, Fmoc-Lys(Boc)-OH, Fmoc-Met-OH, Fmoc-Phe-OH, Fmoc-Pro-OH, Fmoc-Ser(*t*Bu)-OH, Fmoc-Thr(*t*Bu), Fmoc-Trp(Boc)-OH Fmoc-Tyr(*t*Bu)-OH, Fmoc-Val-OH, Fmoc-Abu-OH.

General procedure capping of N-terminal end peptide 12a and 12b

25 µmol of immobilized peptide was taken and treated with a solution of 10% Ac_2O and 5% NMM in DMF for shaken for 5 min in a reaction syringe. Afterwards, the reaction syringe was drained, and the resin was washed with DMF (3x) and DCM (6x) and dried under a N_2 flow.

General procedure palmitoyl-Cys((*RS*)-2,3-di(palmitoyloxy)-propyl)-OH installation

25 µmol of SK_4 elongated peptide or conjugate was taken and treated with a solution of Palmitoyl-N-(2,3-bis(palmitoyloxy)propyl)-L-cysteine (50 µmol, 44.7 mg, 2 eq.) and HCTU (50 µmol, 20.9 mg, 2 eq.) in 1:1 DCM:DMF (0.75 mL). D*i*PEA (50 µmol, 8.7 µL, 2 eq.) was added and the reaction syringe was shaken for 15 min. A second portion of D*i*PEA (50 µmol, 8.7 µL, 2 eq.) was added and the reaction syringe was shaken overnight. The next morning the resin was washed with DMF (3x) and DCM (6x) and dried under a N_2 flow.

General procedure MMT removal

25 µmol of MMT protect peptide or conjugate was taken and treated multiple times, first 10x 30 seconds and second 2x 5 minutes, with a 1% TFA in DCM solution (4 mL). After each treatment, the resin was washed with DCM and finally wash 6x with DCM. Important! Before coupling the next moiety, the freed side chain, the resin must be washed with 20% D*i*PEA in NMP.

General procedure (2-(2-(Fmoc-amino)ethoxy)ethoxy)acetic acid installation

25 µmol of peptide or deprotected amine was treated with a solution of (2-(2-(Fmoc-amino)ethoxy)ethoxy)acetic acid (50 µmol, 19.3 mg, 2 eq.), HCTU (50 µmol, 20.9 mg, 2 eq.), D*i*PEA (100 µmol, 34.8µL, 4 eq.) in DMF (0.75 mL) and shaken overnight. The next morning the resin was washed with DMF (3x) and DCM(6x) and dried under a N_2 flow.

General procedure 1-(4-((6-amino-2-butoxy-8-oxo-7,8-dihydro-9H-purin-9-yl)methyl)phenyl)-1-oxo-5,8-dioxa-2-azadecan-10-acid installation

25 µmol of peptide or deprotected amine was treated with a solution of 1-(4-((6-amino-2-butoxy-8-oxo-7,8-dihydro-9H-purin-9-yl)methyl)phenyl)-1-oxo-5,8-dioxa-2-azadecan-10-acid (50 µmol, 22.9 mg, 2 eq.), HCTU (50 µmol, 20.9 mg, 2 eq.), D*i*PEA (100 µmol, 34.8µL, 4 eq.) in DMF (0.75 mL) and

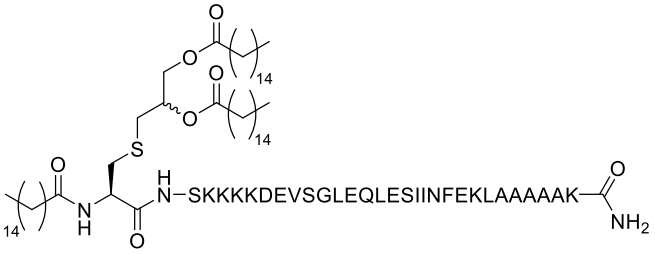
shaken overnight. The next morning the resin was washed with DMF (3x) and DCM (6x) and dried under a N_2 flow.

General procedure final deprotection and purification

25 µmol of peptide or conjugate containing resin was deprotected using a solution of 95:2.5:2.5 TFA:H_2O:TIS (3 mL) for 105 min. The deprotection solution was added to a centrifuge tube containing 46 mL of 1:1 Et_2O:Pentane and cooled at -20 ˚C. The tube was centrifuged for 15 min, the organic solution was decanted, and the remaining pallet was dried using a N_2 flow. Sequences containing a histidine moiety were redissolved in a solution of 1:1:1 HO*t*Bu:H_2O:MeCN and lyophilized overnight. The crude products were purified using HPLC.

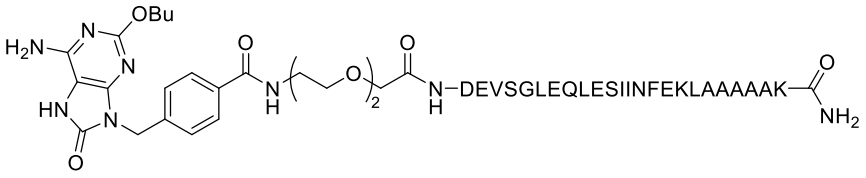
H-Asp-Glu-Val-Ser-Gly-Lue-Glu-Gln-Leu-Glu-Ser-Ile-Ile-Asn-Phe-Glu-Lys-Leu-Ala-Ala-Ala-Ala-Ala-Lys-NH_2 (3a)

6% yield (3.96 mg, 1.56 µmol). **LC-MS:** R_t = 8.533 (Vydac 219TP 5 µm (150x4.6mm) Diphenyl, 10-90% MeCN, 21 min); **ESI-MS:** m/z 1273.8 $[M+2H^+]^{2+}$; **HRMS [M+3H]$^{3+}$:** $[C_{112}H_{185}N_{29}O_{38}]^{3+}$ 849.12193 (measured), 849.12179 (calculated).



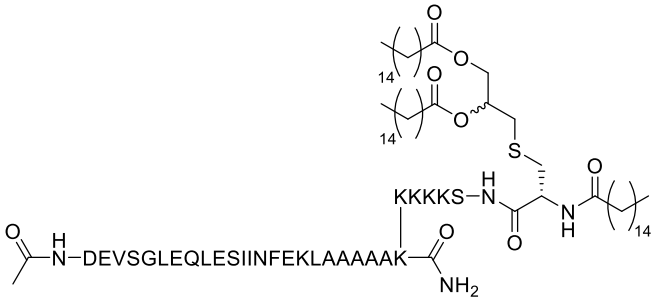
Palmitoyl-Cys((RS)-2,3-di(palmitoyloxy)-propyl)-Ser-Lys-Lys-Lys-Lys-Asp-Glu-Val-Ser-Gly-Lue-Glu-Gln-Leu-Glu-Ser-Ile-Ile-Asn-Phe-Glu-Lys-Leu-Ala-Ala-Ala-Ala-Ala-Lys-NH_2 (4a)

4% yield (5.91 mg, 2.22 µmol). **LC-MS:** R_t = 12.221 (Vydac 219TP 5 µm (150x4.6mm) Diphenyl, 10-90% MeCN, 21 min); **ESI-MS:** m/z 1347.2 $[M+3H^+]^{3+}$;



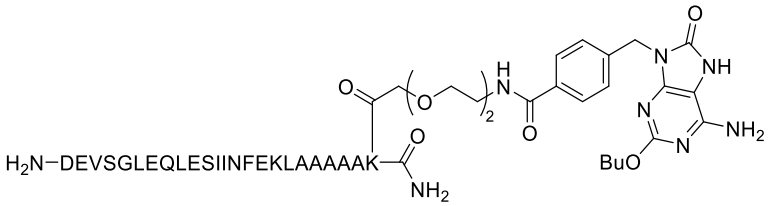
1-(4-((6-amino-2-butoxy-8-oxo-7,8-dihydro-9H-purin-9-yl)methyl)phenyl)-1-oxo-5,8-dioxa-2-azadecan-10-amide-Asp-Glu-Val-Ser-Gly-Lue-Glu-Gln-Leu-Glu-Ser-Ile-Ile-Asn-Phe-Glu-Lys-Leu-Ala-Ala-Ala-Ala-Ala-Lys-NH_2 (5a)

2% yield (1.28 mg, 0.49 µmol). **LC-MS:** R_t = 5.25 (Phenomenex Gemini® 3 µm C_{18} 110 Å 50x4.6 mm, 10-90% MeCN, 10 min); **ESI-MS:** m/z 1515.8 $[M+2H^+]^{2+}$; **HRMS [M+3H]$^{3+}$:** $[C_{135}H_{216}N_{35}O_{44}]^{3+}$ 1010.52322 (measured), 1010.52413 (calculated).



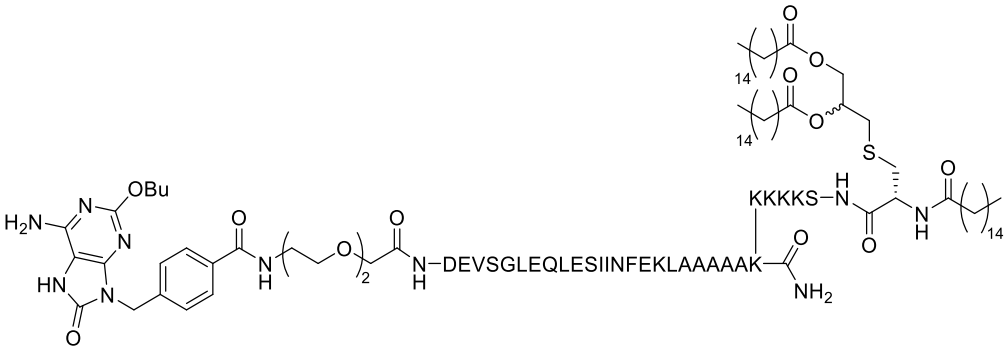
Acetyl-Asp-Glu-Val-Ser-Gly-Lue-Glu-Gln-Leu-Glu-Ser-Ile-Ile-Asn-Phe-Glu-Lys-Leu-Ala-Ala-Ala-Ala-Ala-Lys(Palmitoyl- Cys((RS)-2,3-di(palmitoyloxy)-propyl)-Ser-Lys-Lys-Lys-Lys)-NH_2 (6a)

4% yield (4.53 mg, 1.11 µmol). **LC-MS:** R_t = 12.221 (Vydac 219TP 5 µm (150x4.6mm) Diphenyl, 10-90% MeCN, 21 min); **ESI-MS:** m/z 1360 $[M+3H^+]^{3+}$; **HRMS [M+4H]$^{4+}$:** $[C_{195}H_{341}N_{39}O_{51}S]^{4+}$ 1020.38177 (measured), 1020.38251 (calculated).



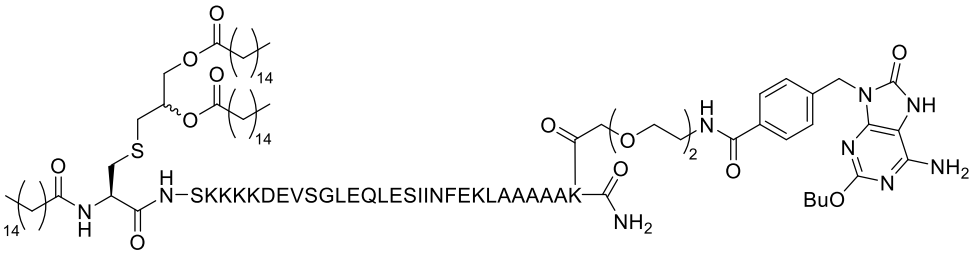
Acetyl-Asp-Glu-Val-Ser-Gly-Lue-Glu-Gln-Leu-Glu-Ser-Ile-Ile-Asn-Phe-Glu-Lys-Leu-Ala-Ala-Ala-Ala-Ala-Lys(1-(4-((6-amino-2-butoxy-8-oxo-7,8-dihydro-9H-purin-9-yl)methyl)phenyl)-1-oxo-5,8-dioxa-2-azadecan-10-amide)-NH_2 (7a)

4% yield (2.97 mg, 0.97 µmol). **LC-MS:** R_t = 5.43 (Phenomenex Gemini® 3 µm C_{18} 110 Å 50x4.6 mm, 10-90% MeCN, 10 min); **ESI-MS:** m/z 1536.9 $[M+2H^+]^{2+}$; **HRMS [M+3H]$^{3+}$:** $[C_{137}H_{218}N_{35}O_{45}]^{3+}$ 1024.52767 (measured), 1024.52765 (calculated).



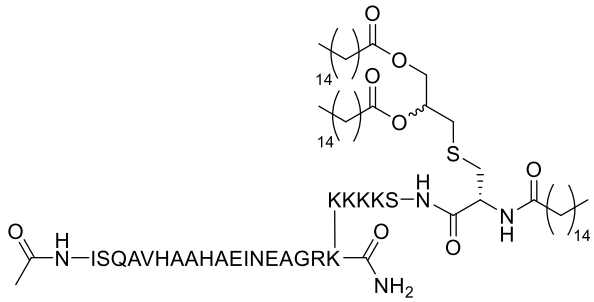
1-(4-((6-amino-2-butoxy-8-oxo-7,8-dihydro-9H-purin-9-yl)methyl)phenyl)-1-oxo-5,8-dioxa-2-azadecan-10-amide-Asp-Glu-Val-Ser-Gly-Lue-Glu-Gln-Leu-Glu-Ser-Ile-Ile-Asn-Phe-Glu-Lys-Leu-Ala-Ala-Ala-Ala-Ala-Lys(Palmitoyl- Cys((RS)-2,3-di(palmitoyloxy)-propyl)-Ser-Lys-Lys-Lys-Lys)-NH_2 (1a)

5% yield (5.18 mg, 1.15 µmol). **LC-MS:** R_t = 12.48 (Vydac 219TP 5 µm (150x4.6mm) Diphenyl, 10-90% MeCN, 21 min); **ESI-MS:** m/z 1508.2 $[M+3H^+]^{3+}$; **HRMS [M+5H]$^{5+}$:** $[C_{216}H_{372}N_{45}O_{56}S]^{7+}$ 904.94672 (measured), 904.94676 (calculated).



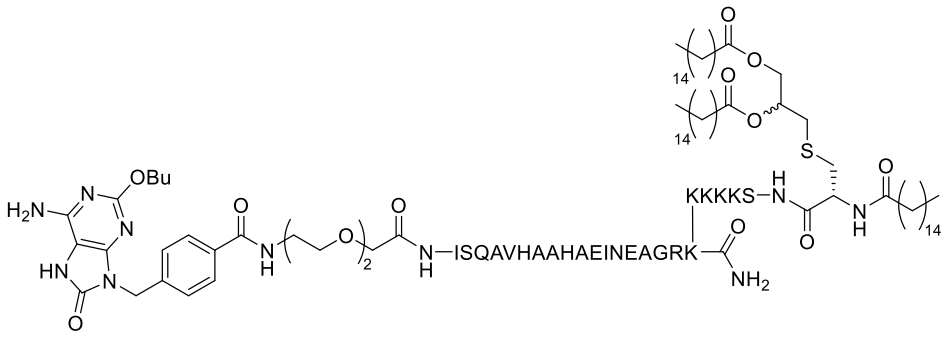
Palmitoyl-Cys((RS)-2,3-di(palmitoyloxy)-propyl)-Ser-Lys-Lys-Lys-Lys-Asp-Glu-Val-Ser-Gly-Lue-Glu-Gln-Leu-Glu-Ser-Ile-Ile-Asn-Phe-Glu-Lys-Leu-Ala-Ala-Ala-Ala-Ala-Lys(1-(4-((6-amino-2-butoxy-8-oxo-7,8-dihydro-9H-purin-9-yl)methyl)phenyl)-1-oxo-5,8-dioxa-2-azadecan-10-amide)-NH_2 (2a)

2% yield (2.81 mg, 0.62 µmol). **LC-MS:** R_t = 13.09 (Vydac 219TP 5 µm (150x4.6mm) Diphenyl, 10-90% MeCN, 21 min); **ESI-MS:** m/z 1508.3 $[M+3H^+]^{3+}$; **HRMS [M+6H]$^{6+}$:** $[C_{216}H_{373}N_{45}O_{56}S]^{6+}$ 754.29018 (measured), 754.29011 (calculated).



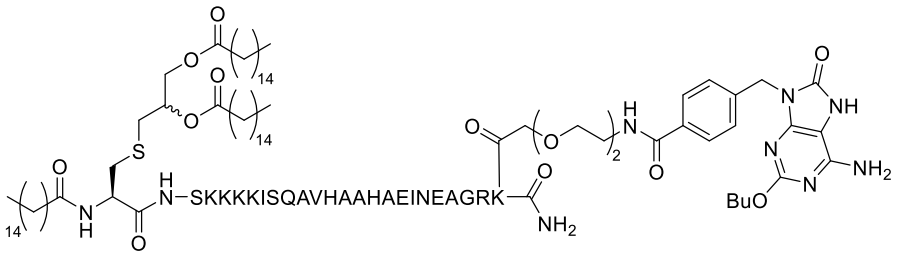
Acetyl-Ile-Ser-Gln-Ala-Val-His-Ala-Ala-Hys-Ala-Glu-Ile-Asn-Glu-Ala-Gly-Arg-Lys(Palmitoyl- Cys((RS)-2,3-di(palmitoyloxy)-propyl)-Ser-Lys-Lys-Lys-Lys)-NH_2 (6b)

3% yield (2.54 mg, 0.74 µmol). **LC-MS:** R_t = 8.54 (Phenomenex Gemini® 3 µm C_4 110 Å 50x4.6 mm, 10-90% MeCN, 10 min); **ESI-MS:** m/z 1718.3 $[M+2H^+]^{2+}$; **HRMS [M+5H]$^{5+}$:** $[C_{163}H_{294}N_{39}O_{38}S]^{5+}$ 687.63900 (measured), 687.63931 (calculated).



1-(4-((6-amino-2-butoxy-8-oxo-7,8-dihydro-9H-purin-9-yl)methyl)phenyl)-1-oxo-5,8-dioxa-2-azadecan-10-amide-Ile-Ser-Gln-Ala-Val-His-Ala-Ala-Hys-Ala-Glu-Ile-Asn-Glu-Ala-Gly-Arg-Lys(Palmitoyl- Cys((RS)-2,3-di(palmitoyloxy)-propyl)-Ser-Lys-Lys-Lys-Lys)-NH_2 (1b)

Performed on a 50 µmol. 2% yield (3.31 mg, 0.85 µmol). **LC-MS:** R_t = 8.54 (Phenomenex Gemini® 3 µm C_4 110 Å 50x4.6 mm, 10-90% MeCN, 10 min); **ESI-MS:** m/z 1939.2 $[M+2H^+]^{2+}$; **HRMS [M+6H]$^{6+}$:** $[C_{184}H_{321}N_{45}O_{43}S]^{6+}$ 646.90049 (measured), 646.90005 (calculated).



Palmitoyl-Cys((RS)-2,3-di(palmitoyloxy)-propyl)-Ser-Lys-Lys-Lys-Lys-Ile-Ser-Gln-Ala-Val-His-Ala-Ala-Hys-Ala-Glu-Ile-Asn-Glu-Ala-Gly-Arg-Lys(1-(4-((6-amino-2-butoxy-8-oxo-7,8-dihydro-9H-purin-9-yl)methyl)phenyl)-1-oxo-5,8-dioxa-2-azadecan-10-amide)-NH_2 (2b)

Performed on a 50 µmol. 4% yield (7.77 mg, 2.00 µmol). **LC-MS:** R_t = 8.39 (Phenomenex Gemini® 3 µm C_4 110 Å 50x4.6 mm, 10-90% MeCN, 10 min); **ESI-MS:** m/z 1939.8 $[M+2H^+]^{2+}$; **HRMS $[M+5H]^{5+}$:** $[C_{184}H_{320}N_{45}O_{43}S]^{5+}$ 717.18531 (measured), 717.18533 (calculated).

In vitro experiment[42] (work of E. Tondini)

Cell culture.

Cell culture. Bone marrow-derived dendritic cells were differentiated from bone marrow stem cells that were harvested from the femurus and tibia of C57BL/6 mice and cultured in IMDM (Lonza) supplemented with FCS (Greiner) , Glutamax (Gibco), penicillin (Gibco), β-mercaptoethanol (Merck) and R1 supernatant. R1 supernatant was obtained by culturing NIH3T3 fibroblasts transfected with GM-CSF. The B3Z and OTIIZ cell lines were cultured in IMDM medium (Lonza supplemented with 8% FCS (Greiner), penicillin and streptomycin, glutamine (Gibco) β-mercaptoethanol (Merck), and hygromycin B (AG Scientific Inc, San Diego, CA, USA) to maintain expression of the beta-galactosidase reporter gene

In vitro DC maturation assay

The test compounds were dissolved in DMSO at a concentration of 1 mM and sonicated in water bath for 15 min. Murine bone marrow-derived dendritic cells were seeded in 96-well plates at a density of 50.000 cells/well and incubated with titrated amounts of compounds. After 3 h of incubation, the cells were washed once and incubated with fresh medium. After 16 h, supernatant was harvested for ELISA analysis (Biolegend) to measure the amount of produced IL-12p40. The cells were harvested and stained with fluorescently labeled antibodies directed against CD40, CD70, CD80, CD86 and MHCII, after which expression levels were determined by flow cytometry.

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Chapter 6

Summary and future prospects

Pattern recognition receptors (PRRs) belong to the first line of defense of vertebrates for the identification of pathogen invasions and are the first involved in the initiation of the adaptive immune system. PRRs are expressed by cells of the innate immune system such as antigen presenting cells (APCs) and each type of receptor has evolved to detect specific highly conserved molecular moieties, which are termed pathogen associated molecular patterns (PAMPs) or damage associated molecular patterns (DAMPs). When PAMPs or DAMPs bind to their respective associated PRR on an APC, the APC matures by upregulating the cytokine production and increasing endocytosis to scavenge the environment for pathogenic material which can be processed into epitopes. Generally, epitopes are small oligopeptides that can be mounted onto major histocompatibility complexes (MHCs), which is required for initiating an adaptive immune response. Once matured and presenting epitopes on their MHCs, APCs migrate towards a lymph node where complementary T-cells capable of binding the presented epitope are activated, and thus initiating an adaptive immune response. This process can be manipulated and employed to attack a target of choice if one is able to activate a PRR and to provide the APC an appropriate epitope. By combining both a PAMP and an epitope into a single entity, a self-adjuvating vaccine, able to activate relevant T-cell can potentially be created. **Chapter 1** presents advances in the design of self-adjuvanting vaccines by discussing nine publications dealing with various conjugates of antigenic peptides and TLR2

ligands.[1-9] The research chapters of this thesis describe the synthesis of such self-adjuvanting vaccines embedding a TLR2 agonist and the outcome of the accompanying immunological investigations.

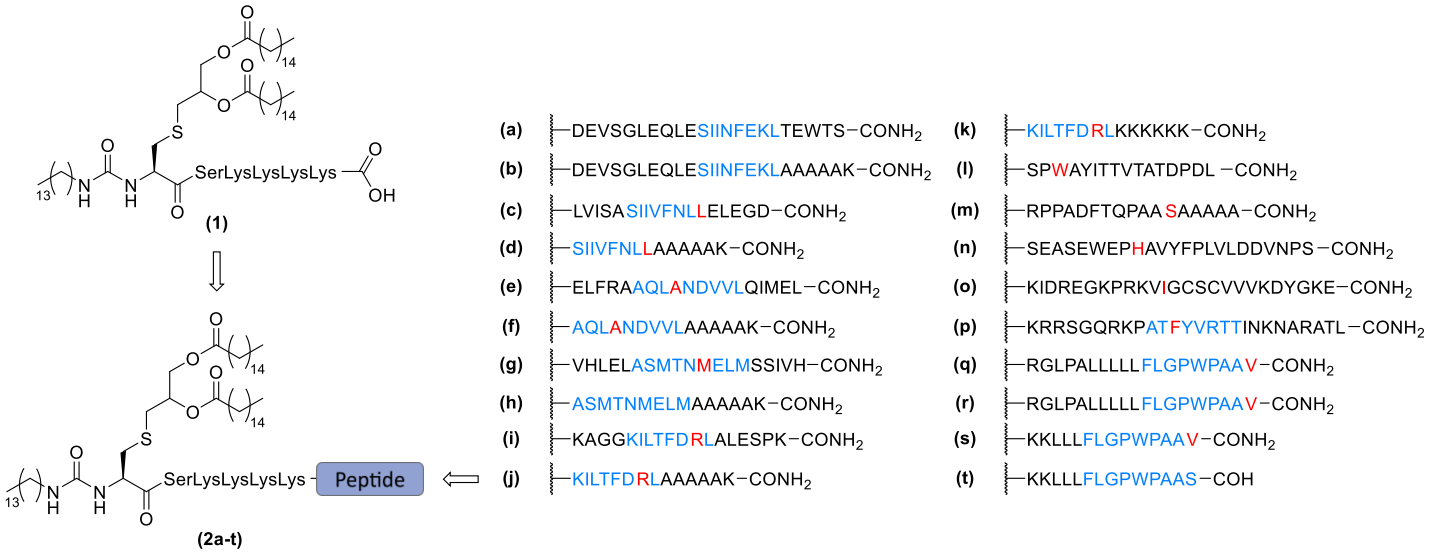

Figure 1: Structures of UPam (**1**) and synthesized UPam functionalized antigens (**2a-t**). When the peptide is endowed with a single defined epitope, the epitope is colored blue. The mutated amino acid is colored red.

In the past, it has been demonstrated that the conjugation of antigens with TLR ligands improves antigen loading and elicits superior T-cell responses, whilst retaining agonist activity, compared to mixtures that consist of the separated constituents of the conjugate.[8, 10] **Chapter 2** describes the assembly of a set of nineteen conjugates , in which antigen containing synthetic peptides are covalently linked with 1-tetradecyl-urea-Cys((RS)-2,3-di(palmitoyloxy)-propyl)-Ser-Lys-Lys-Lys-Lys, otherwise referred to as UPam[11] (compound **1**, Figure 1). The peptides can be divided into three different groups based on the epitope embedded in the peptide, namely human neoepitopes (**2o**-**p**), murine epitopes (**2a-n**) or T-cell epitopes associated with peptide processing (**2q-t**, TEIPP). Additionally, the natural flanking regions in some of the murine epitope containing peptides were either removed on the N-terminal end or replaced with Ala_5Lys or Lys_6 on the C-terminal end (compound **2b**, **2d**, **2f**, **2h**, **2i**, and **2k**; Figure 1). This was done to investigate the flexibility of antigen processing in DCs, which could lead to new strategies for the vaccine design. For now, the conjugates **2a-o** containing murine epitopes were tested for the ability to induce DC maturation measured by the upregulation of CD40 and CD86 on D1 cells, showing an equal capacity to induce maturation. In the future, T-cell proliferation essays should be performed where epitopes with altered flanking regions are compared with the natural counterparts to establish whether these variations are truly tolerated when inducing an adaptive immune response. Currently, only Ala_5Lys and Lys_6 were incorporated but other options could also be considered. The conjugates of human neoantigens, **2o** and **2p** have also been tested for the immunogenicity, and the results of these experiments are discussed in **Chapter 4**, while the conjugates **2q-t** based on TEIPP antigens are still under investigation.

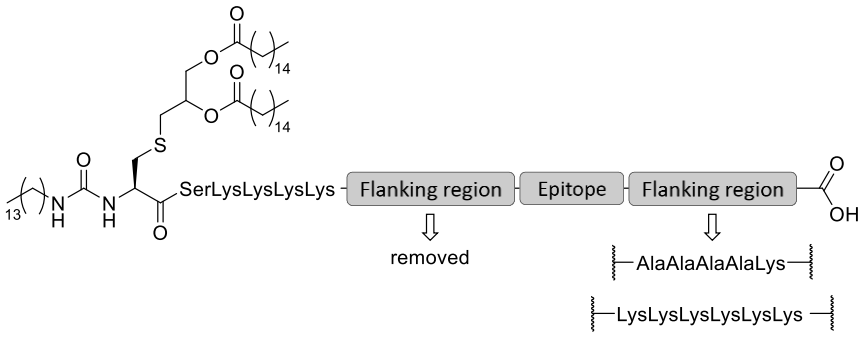

Figure 2: Schematic representation for replacing the natural flanking regions with artificial ones.

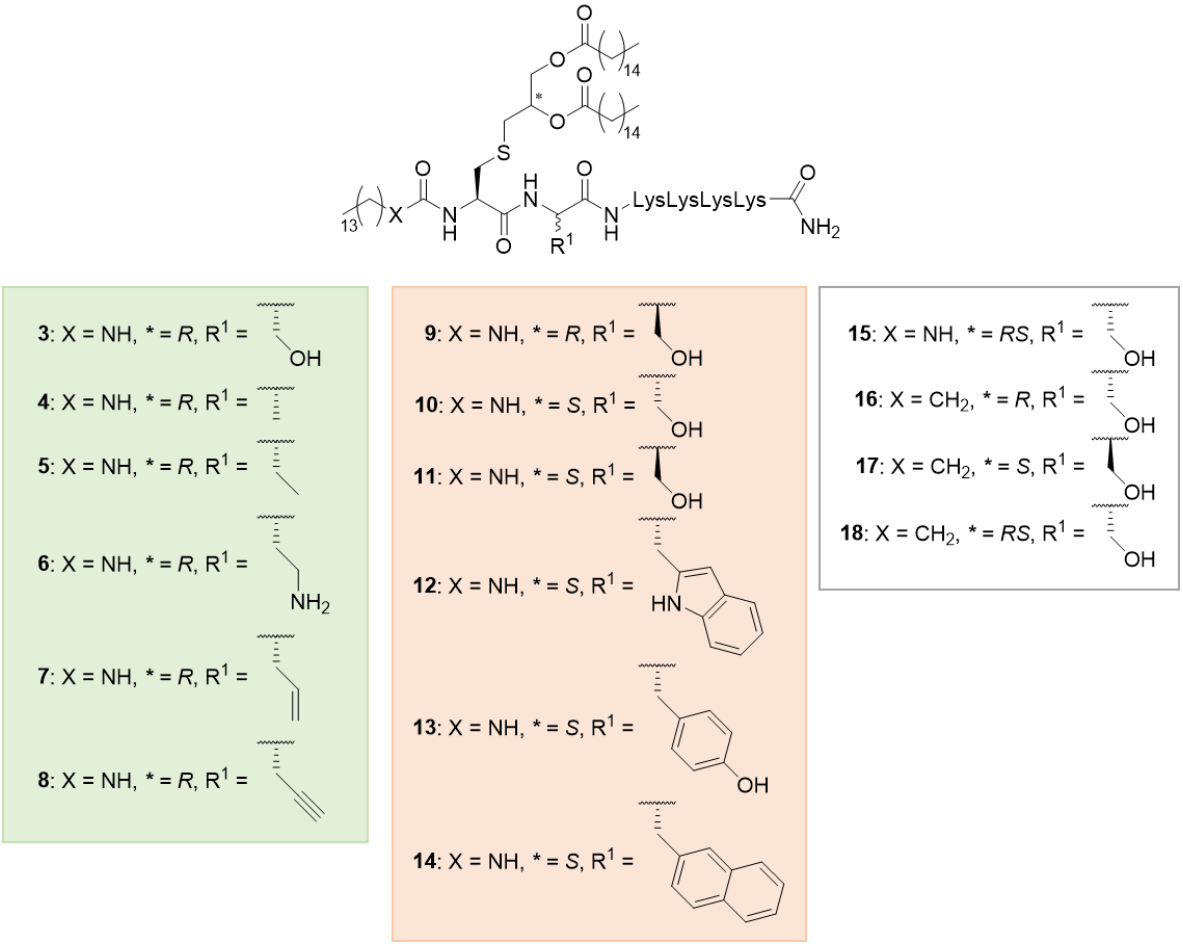

Figure 3: All synthesized UPam derivatives sorted in three group based on their expected influence on agonistic activity. Amino acids in the green box should result in a UPam derivative with good or better agonistic activity, whereas the red box represents activity blocking modifications. The white box depicts all reference compounds that were synthesized.

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 (Figure 3). Currently, it is postulated that the amino acid following the lipidated cysteine should fit a small cavity in the TLR2 subunit, thereby improving binding activity of the ligand.[11] Also, an R-configured glycerol moiety linking the lipid chains to the cysteine should favor the activity.[12-13] In the projected library, three groups of modifications can be discerned based on their expected influence on the

agonistic activity: beneficial, detrimental and controls. All target conjugates were successfully synthesized by employing solid-phase peptide synthesis and isolation by HPLC. The entire library was tested for their capability to induce DC maturation as measured by the concentration of IL-12 in the supernatant of D1 DCs incubated overnight with one of the members. Results followed the current paradigms, where the R-orientation of the glycerol moiety was essential for activity, small side chains had favorable activity and larger aromatic groups completely stopped IL-12 production. The best candidate **6** contained 2,4-D-diaminobutyric acid as a substitute for L-Serine in **3**.

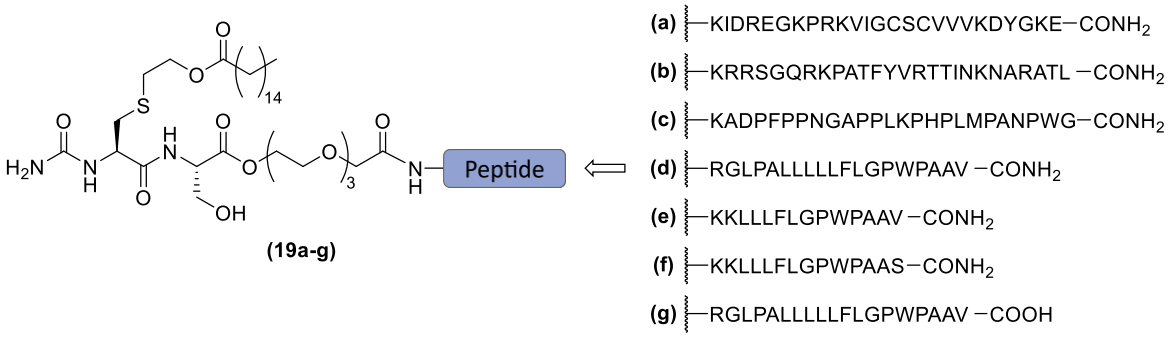

Figure 4: Human neoantigenic peptides connected to mini-UPam from Chapter 4.

Chapter 4 describes the design, synthesis, and evaluation of human neoantigenic peptides, which were functionalized with mini-UPam, a simplified mono-palmitoylated derivative of UPam (Figure 4). With these conjugates, the synthetic accessibility of such self-adjuvanting vaccines was investigated and their capacity to elicit T cell proliferation was assessed. Furthermore, mini-UPam only contains a single palmitoyl tail and a triethylene glycol spacer instead of a tetralysyl spacer, offering a simplified structure with reduced lipophilicity that displays a higher affinity for TLR2 while being less lipophilic compared to UPam. This offers a great opportunity to overcome experimental difficulties encountered with the production of self-adjuvanting vaccines. Seven targets (**19a-g**, Figure 4) were successfully synthesized, the immunological properties of two of them (**19a**, **19b**) were compared with their UPam counterparts (see **Chapter 2**) and the corresponding individual constituents. DC maturation was measured by following IL-8 production in HEK cells transfected with TLR2 and the upregulation of IL-12 and CD86 in moDCs, showing a superior response for mini-UPam linked peptides. T-cell activation was determined by the percentage of CD4 and CD137 positive T-cells after exposure to moDCs incubated with either mini-UPam conjugate, the individual peptides, the mini-UPam ligand, or mixtures thereof. Only self-adjuvanting conjugate **19b** with a MHC class I epitope induced significantly more T-cell activation as compared to the mixture of mini-UPam and the peptide. Self-adjuvanting vaccine **19a**, with an embedded MHC class II epitope was equally active as either the mixture of the components or the antigenic peptide alone. This contrast in potency is attributed to the different uptake pathways for the two different classes of MHC antigens.

To further optimize the activity of mini-UPam conjugates, one could replace the serine residue with 2,4-D-diaminobutyric acid. Since this substitution was found to increase activity for

parent UPam (see Chapter 3), it is likely to work similarly for mini-UPam. These optimization studies could be combined with an alternate synthetic approach where both the ligand and the peptide epitope are assembled on resin. Such an approach only requires the synthesis of two small building blocks, namely ethylene glycol spacer (**20**) and lipidated cysteine (**24**), and procurement of two commercially available building blocks, namely Fmoc-Dab-OH and isocyanate (**26**) (see Figure 5). Synthesis of lipidated cysteine (**24**) has been described in Chapter 4 (compound **5**), while glycol spacer (**20**) can be synthesized by reacting triethylene glycol with *tert*-butyl bromoacetate, protecting the second alcohol with Fmoc-chloride and removing the *tert*-butyl group. Standard SPPS of an antigenic peptide, followed by the correct sequence of reactions with building blocks (**20**, **23**, **24**, and **26**), will result in self-adjuvanting vaccine (**27**).

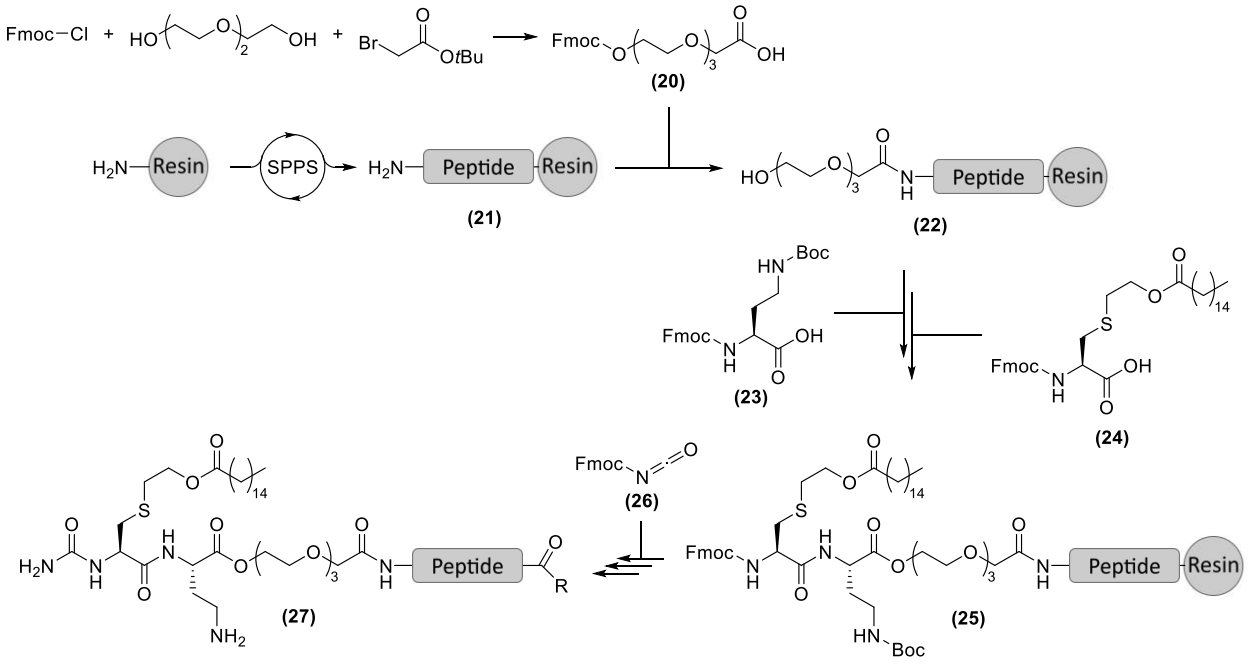

Figure 5: A proposal for an alternative approach to prepare (optimized) mini-UPam conjugates, exemplified by the modular installation of a new mini-UPam, having 2,4-D-diaminobutyric acid as a replacement for serine. Building blocks **20**, **23**, **24**, and **26** will be necessary for the modular assembly, using (automated) SPPS.

Combinations of various classes of TLR ligands have potential synergistic agonistic effects when administered as a mixture or when incorporated in a single molecular entity.[14-16] To combine this with improved antigen delivery and T-cell activation, four dual functionalized conjugates, in which murine antigens are linked to both a TLR2-L [Pam_3CSK_4] and a TLR7-L [4-((6-amino-2-butoxy-8-oxo-7,8-dihydro-9H-purin-9-yl)methyl)benzoic acid][17] were designed, synthesized and evaluated, as described in **Chapter 5**. The dual conjugates were successfully constructed by automated SPPS, followed by the on-resin installation of both ligands and finally purification by HPLC. After incubation of D1 DCs with either the dual conjugates or combinations of their respective components, the upregulation of CD40, CD70, CD80, CD86, IL-12, and MHCII was measured to investigate synergistic activities between TLR2 and TLR7. The results demonstrate increased production of IL-12 when the DCs are exposed to the dual conjugates, however no significant upregulation of the other surface markers was observed.

The increase in IL-12 production could be attributed to enhanced TLR2/TLR7 clustering facilitated by the conjugation of both ligands, as described by *Mancini et al.*[14] From here, different TLR2 ligands could be incorporated in these types of constructs, such as UPam or mini-UPam.

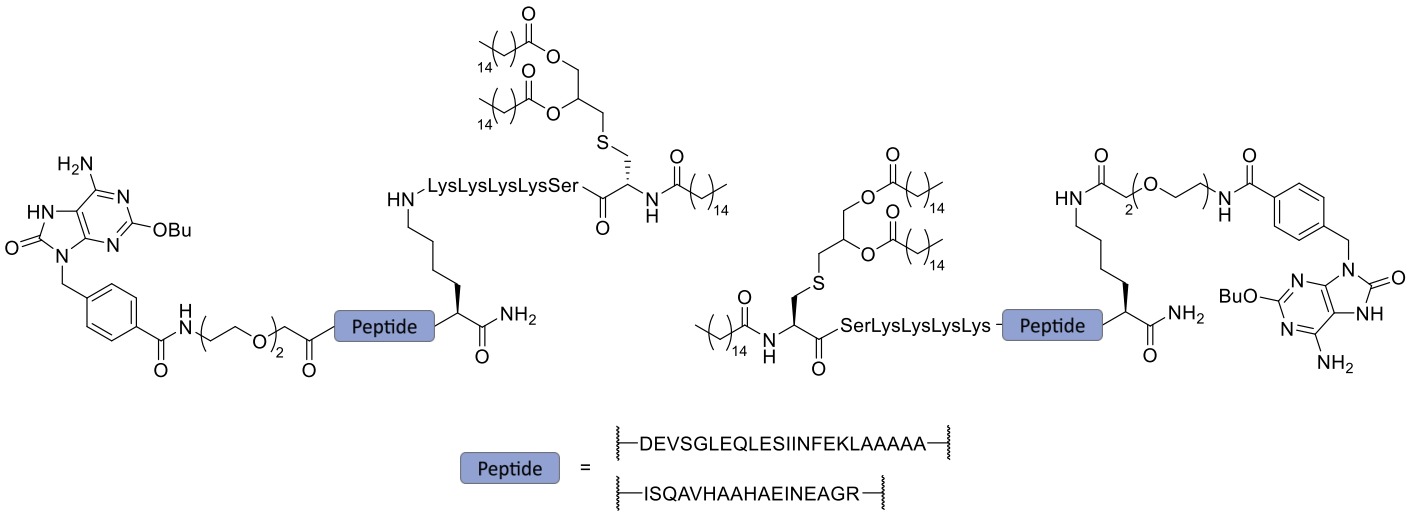

Figure 6: Structures of the synthesized dual conjugates for the assessment of the synergistic interaction between TLR2 and TLR7**.**

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Nederlandse samenvatting

Pathogeen herkennende receptoren (PRRs) behoren tot de eerste verdedigingslinie van gewervelde dieren ter bestrijding van invasies van pathogenen door de initiatie van het aangeboren immuunsysteem. De PRRs van antigeen-presenterende cellen (APCs) spelen een belangrijke rol bij het herkennen van pathogenen wanneer die het lichaam binnendringen. Er bestaan verschillende typen PRRs en elk type receptor is geëvolueerd om een pathogeen specifiek moleculair patroon (PAMP) te herkennen en te binden. Wanneer een PAMP bindt aan de bijbehorende receptor op een APC, stimuleert dit de APC om te matureren en zijn cytokineproductie te verhogen. Daarnaast begint de APC meer materiaal uit de omgeving op te nemen doormiddel van endocytose. Binnenin de APCs wordt dit materiaal afgebroken waarbij uit eiwitten korte oligopeptiden worden gevormd die als epitoop kunnen dienen. Na maturatie begint de APC epitopen te presenteren op de major histocompatibiliteit complexen (MHCs) op de buitenkant van deze cellen. Vervolgens migreren de APCs richting de dichtstbijzijnde lymfeklier waar ze aan een complementaire receptor van een T-cel binden waardoor het aangeboren immuunsysteem wordt geactiveerd. Er wordt veel onderzoek verricht naar de mogelijkheid om met behulp van specifieke epitopen en gedefinieerde PAMPs voor specifieke PRRs het immuunsysteem zodanig te manipuleren dat nieuwe vaccins kunnen worden ontwikkeld. Wanneer zowel een epitoop als een PRR-ligand in een enkele moleculaire entiteit aanwezig is kan dit leiden naar een zogenoemd zelf-adjuverend vaccin dat mogelijk ziekten kan bestrijden waarvoor nog geen vaccin beschikbaar is. **Hoofdstuk 1** illustreert de recente vooruitgang op het gebied van zelf-adjuverende vaccins door de bespreking van negen artikelen over dit ontwerp en de synthese van conjugaten, bestaande uit één (of meerdere) peptide antigen(en) en één (of meerdere) Toll-like receptor 2 (TLR 2) ligand(en).

Het onderzoek dat in dit proefschrift wordt gepresenteerd omvat het ontwerp en de synthese van verschillende zelf-adjuverende vaccins en het onderzoek naar hun immunologische potentie. Onderzoekresultaten uit het verleden hebben aangetoond dat wanneer antigenen covalent gebonden worden aan een TLR-ligand, zowel de antigeen presentatie als de T-cel activatie van zo'n conjugaat verbeterd in vergelijking met een mengsel van de losse moleculaire onderdelen. **Hoofdstuk 2** presenteert de synthese van een groep van negentien conjugaten, waarin een antigeen bevattende peptide covalent gekoppeld is aan 1-tetradecyl-urea-Cys((RS)-2,3-di(palmitoyloxy)-propyl)-Ser-Lys-Lys-Lys-Lys, een artificieel TLR2 ligand, UPam genaamd. De antigenen kunnen in drie groepen verdeeld worden op basis van het type epitoop dat ze bevatten namelijk humane neoantigenen (MHC I), muizen neoantigenen (MHC I en MHC II) en T-cel epitopen relateert aan peptide verwerking. Daarnaast is in een deel van de muizen neoantigenen het natuurlijke C-terminale einde van het peptide verwijderd terwijl bij anderen het natuurlijke N-terminale einde is vervangen door Ala_5Lys of Lys_6. Met deze modificaties kan men onderzoeken of verschillende peptiden die eenzelfde epitoop bevatten, in staat zijn om vergelijkbare presentatie op MHCs en proliferatie van T-cellen te induceren. Op het ogenblik zijn deze op muizen gerichte conjugaten alleen getest op hun vermogen DC maturatie te induceren, waarbij het vervangen van de natuurlijke flankerende regio's weinig

tot geen effect bleek te hebben op de activiteit van UPam. In de toekomst moeten de verschillende conjugaten getest en vergeleken worden op hun vermogen om T-cellen te activeren om vast te kunnen stellen of de aanpassingen getolereerd worden door het immuunsysteem. De resultaten die verkregen zijn voor de conjugaten met een humane epitoop bevattende peptide worden in **Hoofdstuk 4** besproken.

Hoofdstuk 3 beschrijft de synthese van een bibliotheek van zestien chiraal zuivere UPam derivaten. Deze zijn getest op hun TLR-2 activiteit om de huidige consensus omtrent het ontwerpen van lipopeptides als ligand voor TLR2 te bevestigen en UPam verder te optimaliseren. Volgens de huidige theorie past het aminozuur serine van UPam in een kleine holte van de TLR2 receptor en zou het vervangen van serine door een aminozuur met een groter zijketen de activiteit van Upam moeten verminderen. Tevens is een R-geconfigureerde glycerol noodzakelijk voor activiteit van het ligand. De bibliotheek kan opgesplitst worden in 3 groepen UPam derivaten op basis van het verwachtte effect van de modificatie op de activiteit en de functie, namelijk positief, negatief en controle verbindingen. Alle derivaten zijn succesvol verkregen doormiddel van vaste fase peptide synthese en zuivering met HPLC. De gehele bibliotheek aan derivaten is getest op hun vermogen om dendritische cellen (DCs) te matureren door de IL-12 productie te bepalen. De resultaten kwamen overeen met de huidige paradigma's. Conjugaten die een S-georienteerde glycerol of een aminozuur met een grote zijgroep bevatten vertoonde praktisch geen activiteit. Over het algemeen waren de UPam derivaten die kleine aminozuren bevatten actiever met als beste kandidaat 2,4-L-diaminoboterzuur.

Hoofdstuk 4 beschrijft het ontwerp, de synthese en de evaluatie van humane neoantigenen bevattende peptiden die gefunctionaliseerd zijn met mini-UPam, een vereenvoudigde versie van UPam. Ondanks een versimpelde structuur met maar één enkele lipide staart en een glycerol linker vertoont mini-UPam een superieure TLR2 activiteit ten opzichte van Upam. Dit hoofdstuk behandelt de procedure om dit soort conjugaten te synthetiseren en hun vermogen om een T-cel proliferatie te induceren. Zeven van de acht doelverbindingen zijn succesvol gesynthetiseerd door middel van vaste fase peptide synthese, waarvan twee conjugaten vergeleken zijn met hun Upam tegenhanger en het mengsel met hun individuele onderdelen. DCs activatie is vastgesteld aan de hand van IL-8 productie in HEK cellen en IL-12 productie en CD86 expressie in myeloïde afgeleide dendritische cellen. Alle mini-Upam conjugaten toonde superieure TLR-2 activiteit ten opzichte van hun UPam tegenhanger op basis van de gemeten factoren. Het vermogen van de conjugaten om T-cellen te activeren is onderzocht door de expressie van CD4 en CD137 te volgen op T-cellen. Alleen het zelf-adjuverende vaccin waarin een MHC-I epitoop verwerkt is, liet verbeterde T-cel proliferatie zien. Het verschil in proliferatie ten opzichte van de MHC-II bevattende conjugaten wordt verklaard aan de hand van de verschillende wegen in de cel die beide conjugaten volgen voordat het epitoop op een MHC geladen wordt.

Het verwerken van verschillende klasse TLR-liganden in een enkele moleculaire entiteit heeft de potentie om synergistische effect te hebben op de immunologische eigenschappen. Vier duaal gefunctionaliseerde conjugaten zijn ontworpen om dit potentieel synergistische effect vast te stellen met verbeterde antigeen presentatie en T-cel activatie. Alle conjugaten

bevatten een TLR-2 ligand, een TLR-7 ligand en een peptide waarin een muizen epitoop is verwerkt. Het ontwerp, de synthese en de evaluatie van deze conjugaten wordt beschreven in **Hoofdstuk 5**. Door eerst de peptiden te synthetiseren door middel van geautomatiseerde vaste drager peptide synthese, beide liganden te installeren, de conjugaten van de drager af te splitsen en tenslotte zuivering met HPLC, zijn alle vier de duale conjugaten succesvol verkregen. Door de CD40, CD70, CD80, CD86 en MHCII-expressie en de productie van IL-12 van de duale conjugaten in vergelijking met referentie conjugaten te meten in D1 DCs is het effect van de aanwezigheid van deze twee liganden bepaald. De resultaten laten zien dat DCs meer IL-12 produceren wanneer ze blootgesteld worden aan een duaal conjugaat. Echter, er wordt geen significant verschil geobserveerd in de expressie van de CD40, CD70, CD80, CD86 en MHCII. In **Hoofdstuk 6** tenslotte worden de onderzoeksresultaten samengevat en aanbevelingen gegeven voor toekomstig onderzoek.

List of Publications

Reaction Rates of Various *N*-Acylenamines in the Inverse-Electron-Demand Diels-Alder Reaction

Sander B. Engelsma, Thomas C. van den Ende, Hermen S. Overkleeft, Gijsbert A. van der Marel, Dmitri V. Filippov

European Journal of Organic Chemistry, 2018, 2587-2591

https://doi.org/10.1002/ejoc.201800094

Simplified Monopalmitoyl Toll-like Receptor 2 Ligand Mini-UPam for Self-Adjuvanting Neoantigen-Based Synthetic Cancer Vaccines

Thomas C. van den Ende, Jeroen M. M. Heuts, Geoffroy P. P. Gential, Marten Visser, Michel J. van de Graaff, Nataschja I. Ho, Wim Jiskoot, A. Rob P.M. Valentijn, Nico J. Meeuwenoord, Herman S. Overkleeft, Jeroen D. C. Codée, Sjoerd H. van der Burg, Els M. E. Verdegaal, Gijsbert A. van der Marel, Ferry Ossendorp, Dmitri V. Filippov

ChemBioChem, 2021, 22, 1215-1222

https://doi.org/10.1002/cbic.202000687

Identification of a neo-epitope dominating endogenous CD8 T cell responses to MC-38 colorectal cancer

Brett J. Hos, Marcel G.M. Camps, Jitske van den Bulk, Elena Tondini, Thomas C. van den Ende, Dina Ruano, Kees Franken, George M.C. Janssen, Arnoud Ru, Dmitri V. Filippov, Ramon Arens, Peter A van Veelen, Noel Miranda & Ferry Ossendorp

OncoImmunology, 2020, 9 (1), 1215-1222

https://doi.org/10.1080.2162402X.2019.1673125

Curriculum Vitae

Thomas van den Ende was born on the 8th of July 1991 in Delft, the Netherlands. He obtained his high school diploma at Stanislas College Westplantsoen (VWO, specialization 'Natuur en Techniek') in 2009, after which he started studying for his bachelor's degree in Molecular Science and Technology at Leiden University and the Technical University of Delft. During his bachelor, he did multiple short internships with various groups at both Universities, such as Bio-organic Synthesis (BIOSYN), Medicinal Chemistry, and Metals in Catalysis Biomimetics & Inorganic materials and followed a minor in Drug Discovery. He completed his bachelor studies with a final research internship on the synthesis of 6-phosphate mannopyranosides at BIOSYN under the supervision of Dr. Chung Sing Wong and prof. Dr. Gijs van der Marel. In 2013, he received his bachelor's degree with a major in Chemistry. During the last year of his bachelor, he also functioned as Assessor Onderwijs on the board of the chemistry study association, Chemisch Dispuut Leiden.

He continued his education by pursuing a Master Chemistry degree at Leiden University with an emphasis on Research and Organic Chemistry. In 2016, he finalized his master by performing a final internship at the Biosyn group under the supervision of Dr. Sander Engelsma, Dr. Dimitri Filippov, and Prof. Dr. Gijs van der Marel. His master thesis was entitled: "Further development of N-acyl azetine as dieneophile for tetrazine ligation".

After obtaining his master's degree, he started the research described in this Thesis under the supervision of Dr. Dimitri Filippov, Prof. Dr. Gijs van der Marel, and Prof. Dr. Ferry Ossendorp in the BIOSYN group of Leiden Institute of Chemistry at Leiden University in close collaboration with the Tumor Immunology Group at Leiden University Medical Center. Part of this work was orally presented at NWO Chains 2020 in Veldhoven, the Netherlands.

In November 2021, Thomas started as a Project Manager at the TIM company in Zeist.