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

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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]

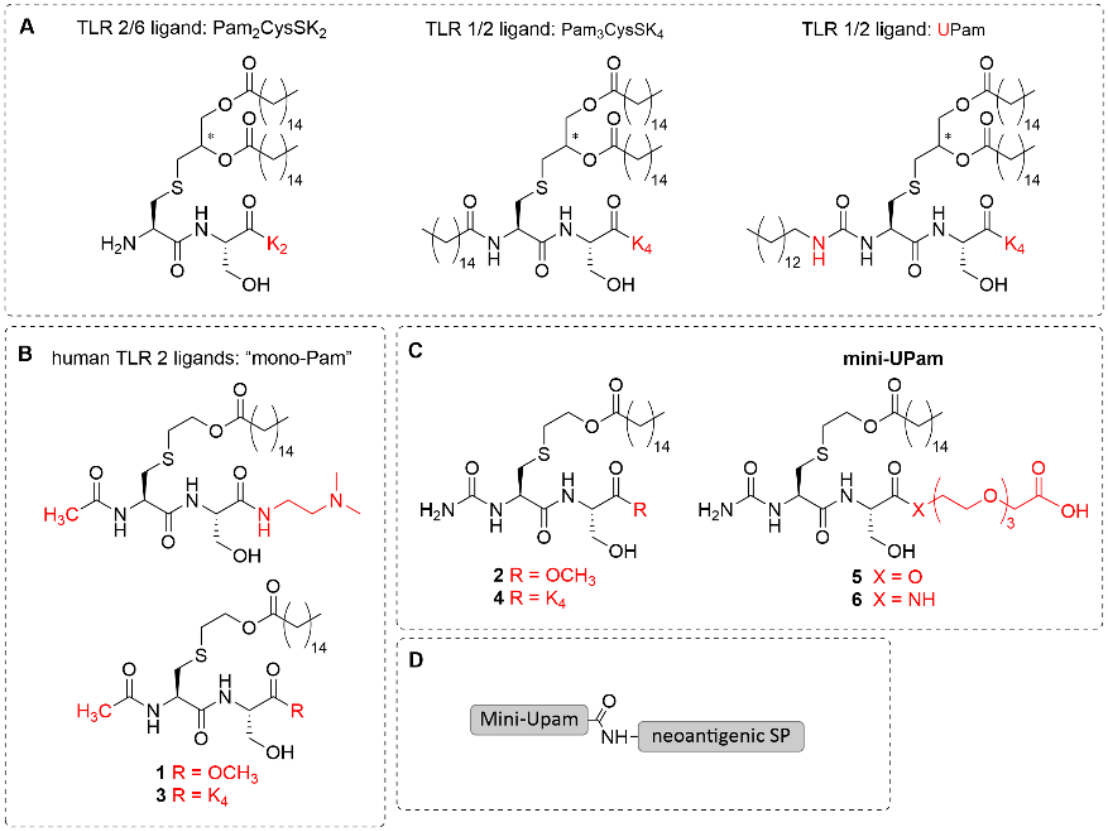


Figure 1. Structures of TLR2 ligands and the projected conjugates. **A**: Highly lipophilic di- and tripalmitoyl TLR2 ligands previously reported; **B**: mono-palmitoyl derivatives with improved solubility and TLR2-activating potency reported previously; **C**: Ureido-monopalmitoyl TLR2 ligands developed 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

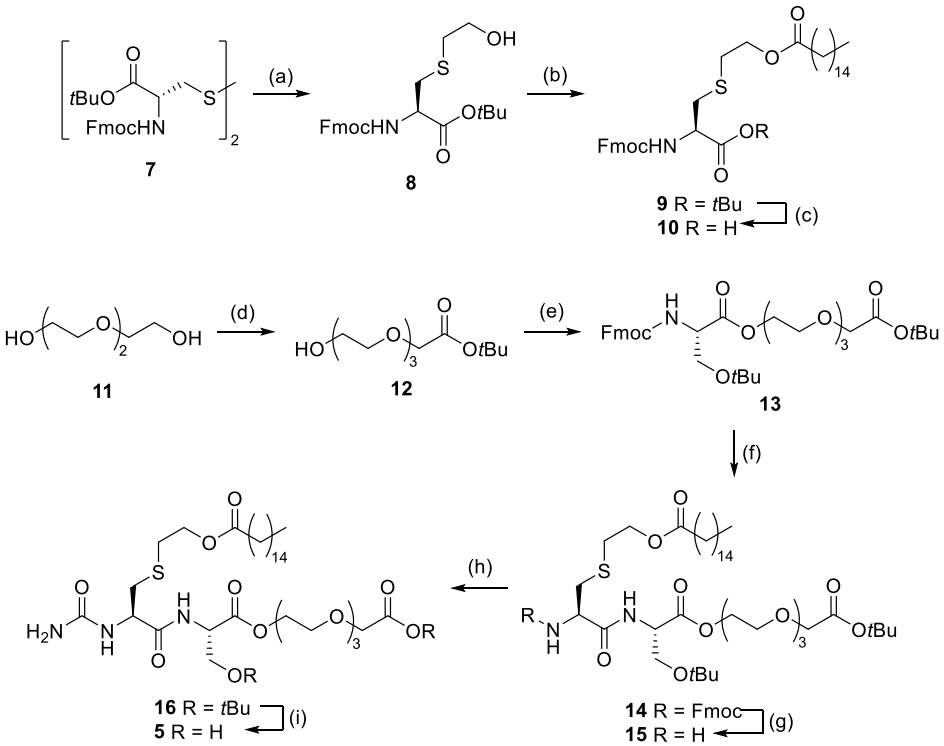
When the peptide is embedded with a single defined epitope, the epitope is colored blue. The mutated amino acid is colored red.

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.

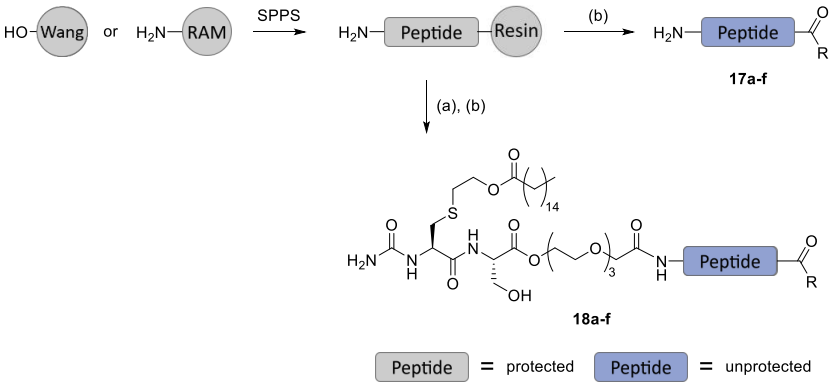
Results and Discussion

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.

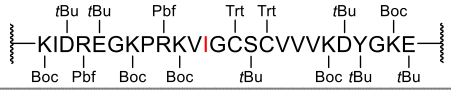
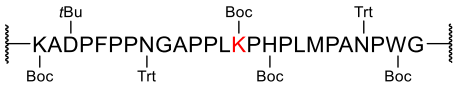


Scheme 1: Synthesis of palmitoyl functionalized cysteine **10** and the subsequent synthesis of mini-UPam **5**. Reaction conditions: (a) i) Zn, H_2SO_4 (aq), HCl (aq), MeOH, RT, 15 min; ii) Oxirane, RT, overnight, 89%; (b) EDC·HCl, DiPEA, DMAP, Palmitic acid, DCM, RT, overnight, 76%; (c) 1:1 DCM:TFA, 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.

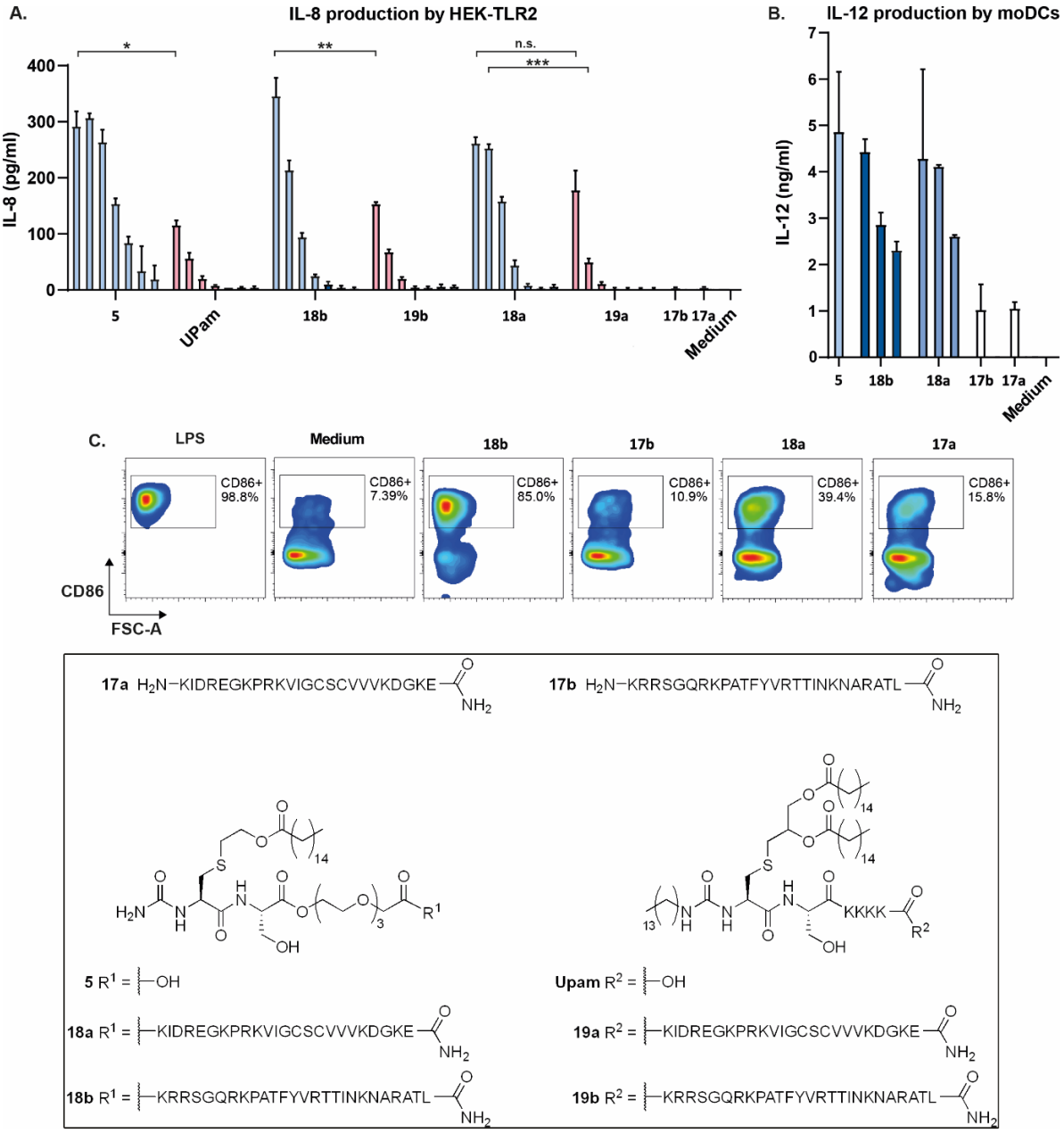


Figure 3. SP-mini-UPam conjugates efficiently target the human TLR2 receptor resulting in moDC activation and maturation. The synthesis of compounds **19a** and **19b** can be found in Chapter 2 **(A)** IL-8 production determined in the supernatant of HEK-TLR2 cells after 48 h incubation with compound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

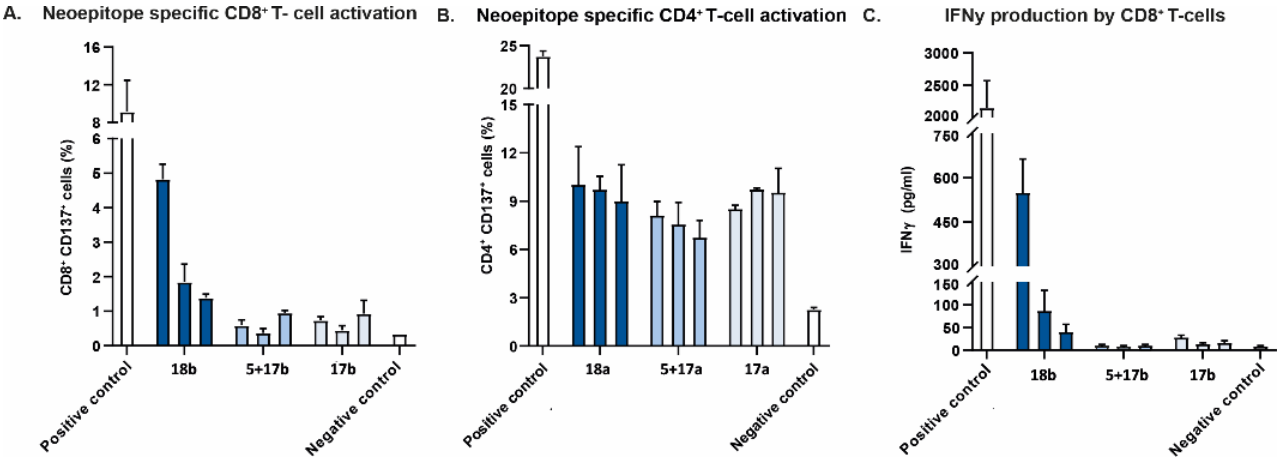


Figure 4. Activation of neoepitope-specific human $CD8^+$ and $CD4^+$ T-cells by the SP-mini-UPam conjugates. Percentages of total $CD8^+$ **(A)** and $CD4^+$ T-cells **(B)** expressing the activation marker CD137 after overnight co-culture with monocytes loaded with conjugate **18b** or **18b,** free **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]

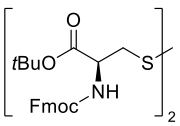
Experimental

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}.

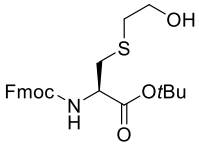
di-*tert*-butyl 3,3'-disulfanediyl(2R,2'R)-bis(2-((((9H-fluoren-9-yl)methoxy)carbonyl)amino) propanoate) (7)



(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)**

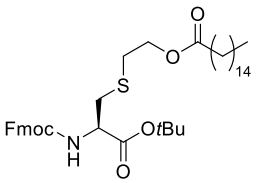


Compound **7** (4.86 mmol, 3.88 g, 1 eq.) was dissolved in THF (50 mL). Zinc powder (37.9 mmol, 2.48 g, 7.7 eq.) and a 100:7:1 solution of MeOH:37% HCl:98% H_2SO_4 (15 mL) were added to the reaction mixture and the mixture was stirred for 15 min at RT. Oxirane (50 mmol, 2.5 mL, 10.3 eq.) was added at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

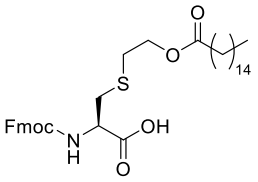
(R)-2-((2-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)-3-(*tert*-butoxy)-3-oxopropyl)thio)ethylpalmitate (9)



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.

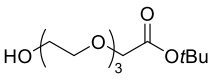
***tert*-butyl *N*-(((9H-fluoren-9-yl)methoxy)carbonyl)-S-(2-plamiroyloxy)ethyl)-L-cysteine (10)**



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.

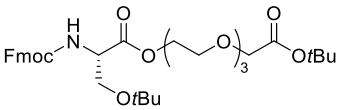
***tert*-butyl 2-(2-(2-(2-hydroxyethoxy)ethoxy)ethoxy)acetate (12)**



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.

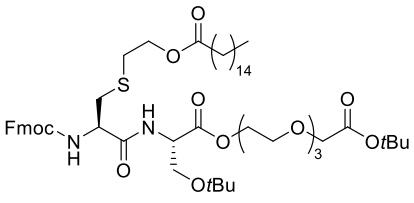
13,13-dimethyl-11-oxo-3,6,9,12-tetraoxatetradecyl *N*-(((9H-fluoren-9-yl)methoxy)carbonyl)-O-(*t*-butyl)-L-serinate (13)



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)

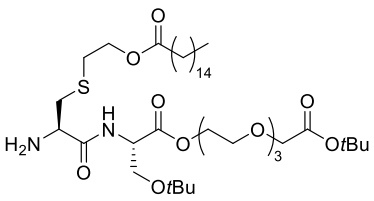


Compound **13** (10.6 mmol, 6.66 g, 1 eq.) was dissolved in dry THF (100 mL) under a N_2 atmosphere. Next, DBU (10.1 mmol, 1.51 mL, 0.95 eq.) was added and the solution was stirred at RT for 5 min, after which HOBt (21.3 mmol, 3.26 g, 2 eq.) was added to the reaction mixture. Then, compound **4** (10.6 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.

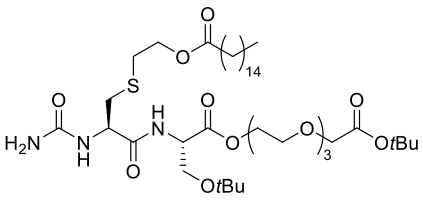
(17S,20R)-20-amino-17-(*tert*-butoxymethyl)-2,2-dimethyl-4,16,19-trioxo-3,6,9,12,15-pentaoxa-22-thia-18-azateracosan-24-yl palmitate (15)



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.

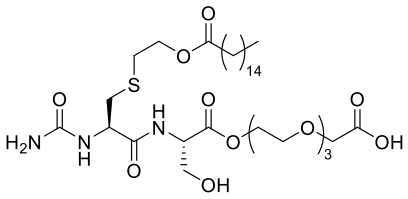
(17S,20R)-20-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)-17-(*tert*-butoxymethyl)-2,2-dimethyl-4,16,19-trioxo-3,6,9,12,15-pentaoxa-22-thia-18-azatetracosan-24-yl palmitate (16)



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).

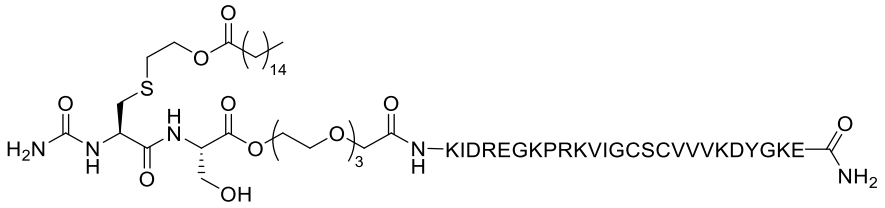
H-Lys-Lys-Leu-Leu-Leu-Phe-Leu-Gly-Pro-Trp-Pro-Ala-Ala-Ser-OH (17f)

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)

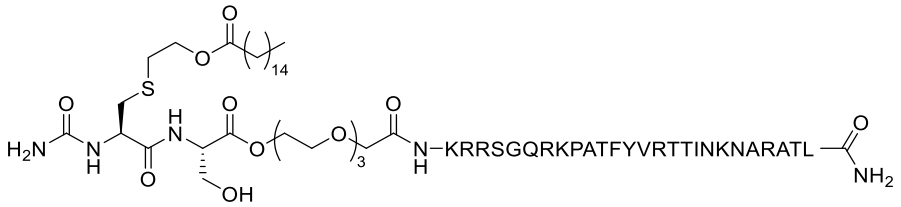
H-Arg(Pbf)-Gly-Leu-Pro-Ala-Leu-Leu-Leu-Leu-Leu-Phe-Leu-Gly-Pro-Trp(Trt)-Pro-Ala-Ala-Val-Tentagel S RAM resin was prepared using a TRIBUTE® Peptide Synthesiser applying Fmoc chemistry for 100 µmol Tentagel RAM resin. 25 µmol was taken from the crude batch and treated with a standard deprotection cocktail 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)

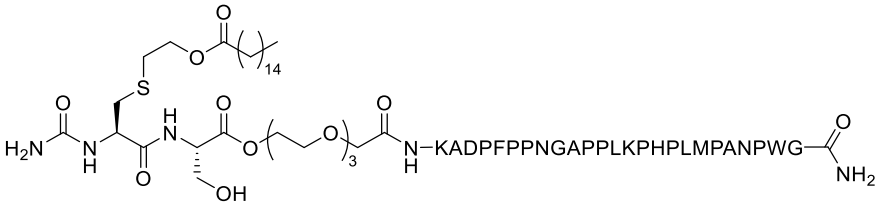
Compound **5** (50 µmol, 36 mg, 2 eq.), HCTU (50 µmol, 21 mg, 2 eq.) and D*i*PEA (100 µmol, 17.4 µL, 4 eq.) were dissolved in NMP (0.75 mL, 0.07 M). This solution was added to dry H-Lys(Boc)-Ile-Asp(*t*Bu)-Arg(Pbf)-Glu(*t*Bu)-Gly-Lys(Boc)-Pro-Arg(Pbf)-Lys(Boc)-Val-Ile-Gly-Cys(Trt)-Ser(*t*Bu)-Cys(Trt)-Val-Val-Val-Lys(Boc)-Asp(*t*Bu)-Tyr(*t*Bu)-Gly-Lys(Boc)-Glu(*t*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)

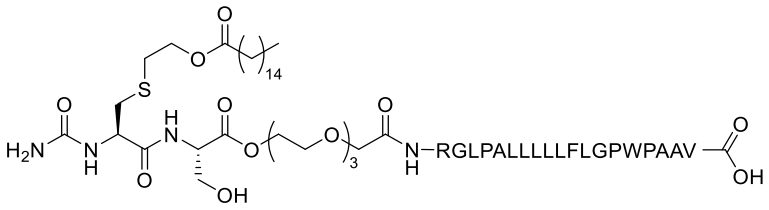
Compound **5** (50 µmol, 36 mg, 2 eq.), HCTU (50 µmol, 21 mg, 2 eq.) and D*i*PEA (100 µmol, 17.4 µL, 4 eq.) were dissolved in NMP (0.75 mL, 0.07 M). This solution was added to dry H-Lys(Boc)-Arg(Pbf)-Arg(Pbf)-Ser(*t*Bu)-Gly-Gln(Trt)-Arg(Pbf)-Lys(Boc)-Pro-Ala-Thr(*t*Bu)-Phe-Tyr(*t*Bu)-Val-Arg(Pbf)-Thr(*t*Bu)-Thr(*t*Bu)-Ile-Asn(Trt)-Lys(Boc)-Asn(Trt)-Ala-Arg(Pbf)-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).



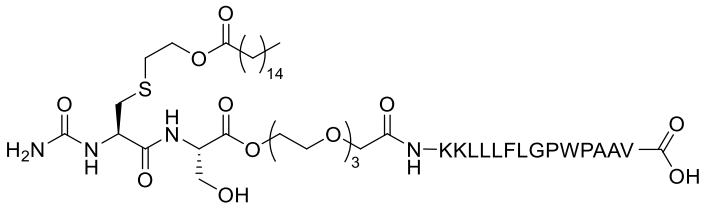
((S)-15-hydroxy-13-oxo-14-((R)-3-((2-(palmitoyloxy)ethyl)thio)-2-ureidopropanamido)-3,6,9,12-tetraoxapentadecanoyl)-Lys-Ala-Asp-Pro-Phe-Pro-Pro-Asn-Gly-Ala-Pro-Pro-Leu-Lys-Pro-His-Pro-Leu-Met-Pro-Ala-Asn-Pro-Trp-Gly-NH_2 (18c)

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).



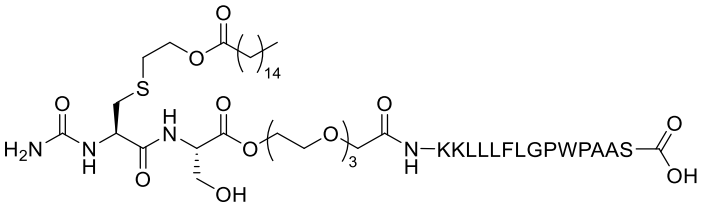
((S)-15-hydroxy-13-oxo-14-((R)-3-((2-(palmitoyloxy)ethyl)thio)-2-ureidopropanamido)-3,6,9,12-tetraoxapentadecanoyl)-Arg-Gly-Leu-Pro-Ala-Leu-Leu-Leu-Leu-Leu-Phe-Leu-Gly-Pro-Trp-Pro-Ala-Ala-Val-OH (18d)

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).



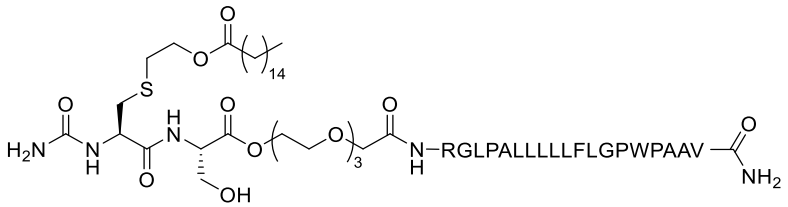
((S)-15-hydroxy-13-oxo-14-((R)-3-((2-(palmitoyloxy)ethyl)thio)-2-ureidopropanamido)-3,6,9,12-tetraoxapentadecanoyl)-Lys-Lys-Leu-Leu-Leu-Phe-Leu-Gly-Pro-Trp-Pro-Ala-Ala-Val-OH (18e)

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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