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

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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]

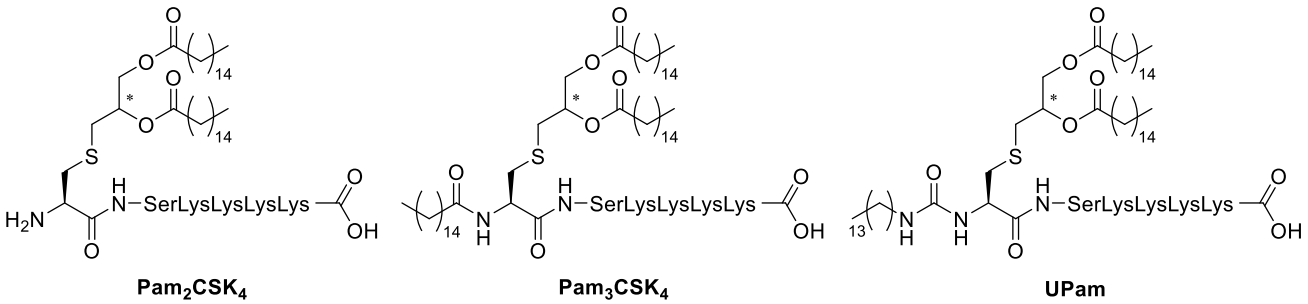


Figure 1: Structures of TLR2 ligands Pam_2CSK_4, Pam_3CSK_4 and UPam

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

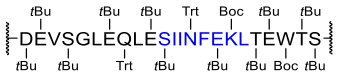
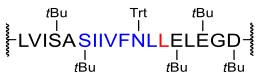
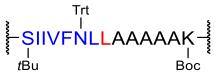
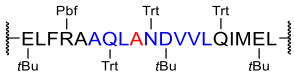
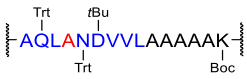
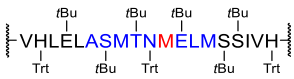
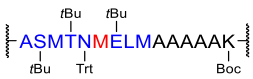
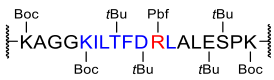
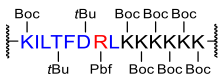
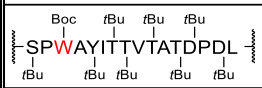
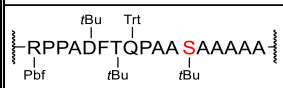
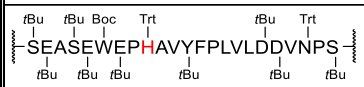
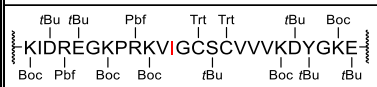
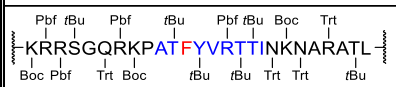
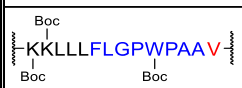
Entry	Sequence	Epitope type
a	DEVSGLEQLESIINFEKLTEWTS	Murine MHC I
b	DEVSGLEQLESIINFEKLAAAAAK	Murine MHC I
c	LVISASIIVFNLLELEGD	Murine MHC I
d	SIIVFNLLAAAAAK	Murine MHC I
e	ELFRAAQLANDVVLQIMEL	Murine MHC I
f	AQLANDVVLAAAAAK	Murine MHC I
g	VHLELASMTNMELMSSIVH	Murine MHC I
h	ASMTNMELMAAAAAK	Murine MHC 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.

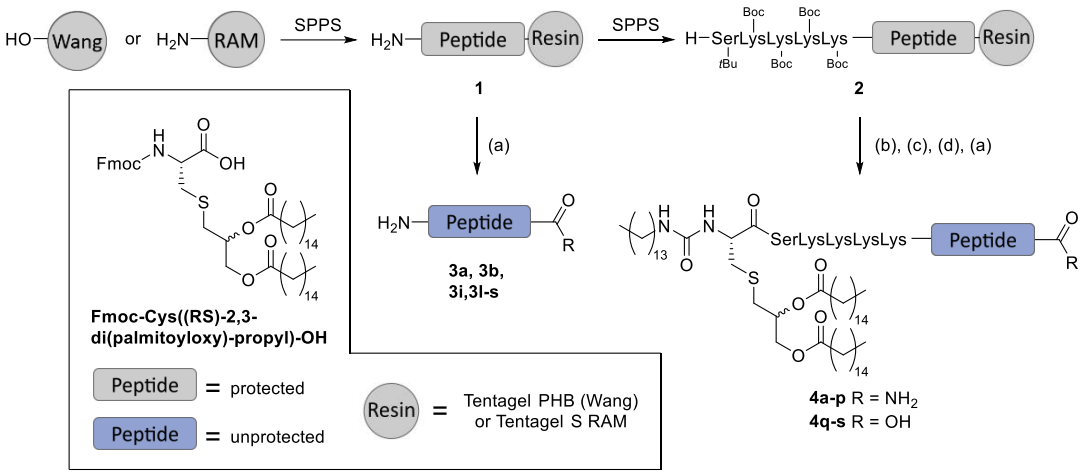
Results and Discussion

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.

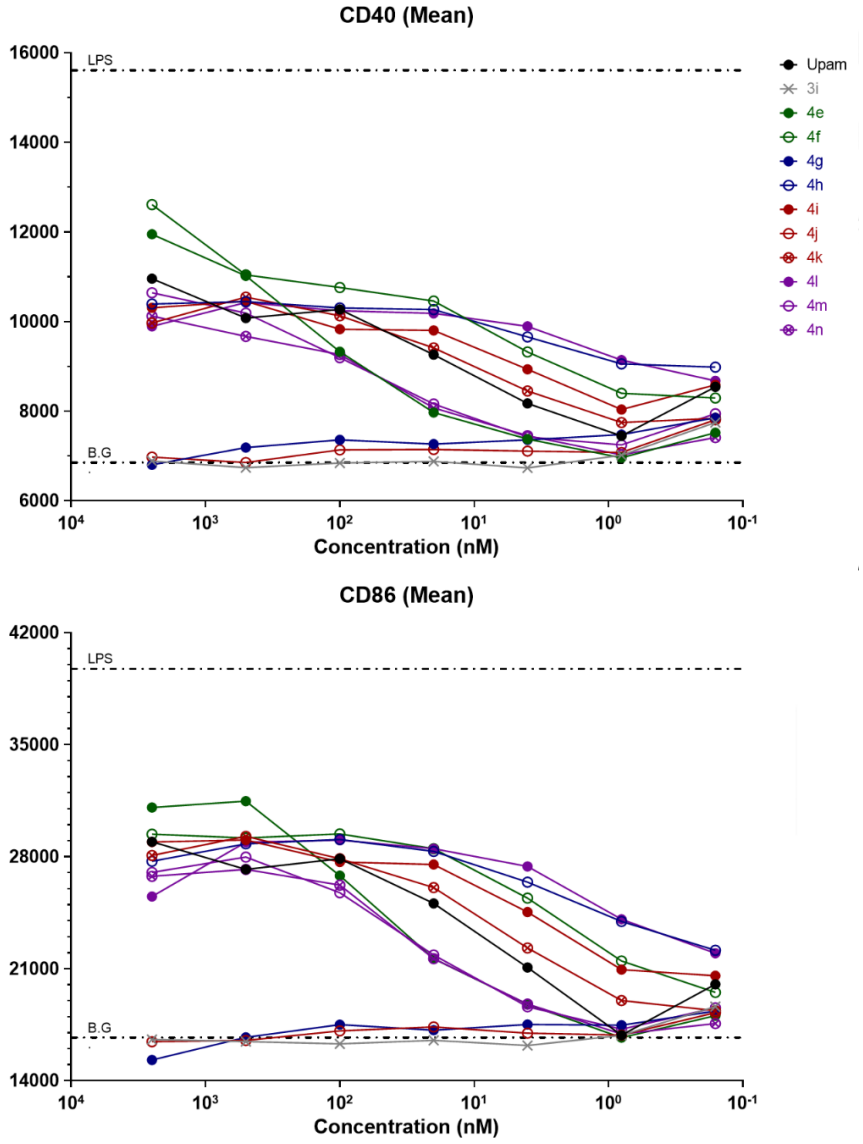


Figure 2: CD40 and CD86 expression determined with flow cytometry on DC1 cells, grown on factor-supplemented supernatant, incubated overnight with either UPam, peptide **3i**, or a UPam conjugates **4e-n** (concentration range: 2.5 µM, 1.25 µM, 0.625 µM, 0.313 µM, and 0.162 µM).

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)

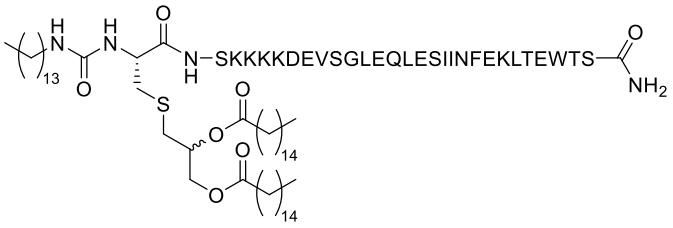
Synthesizer: Liberty Blue™; **Resin:** Tentagel PHB resin; **HPLC solution:** DMSO; 36% yield (13.62 mg, 9.00 µmol). **LC-MS:** R_t = 6.05 (Phenomenex Gemini® 3 µm C18 110 Å 50x4.6 mm, 10-90% MeCN, 10 min); **ESI-MS:** *m/z* 1009.5 $[M+2H^+]^{2+}$; **HRMS [M+2H]$^{2+}$:** $[C_{101}H_{163}N_{23}O_{20}]^{2+}$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

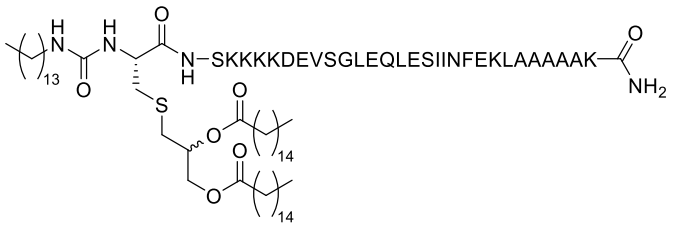
H-Lys-Lys-Leu-Leu-Leu-Phe-Leu-Gly-Pro-Trp-Pro-Ala-Ala-Ser-OH (3s)

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).



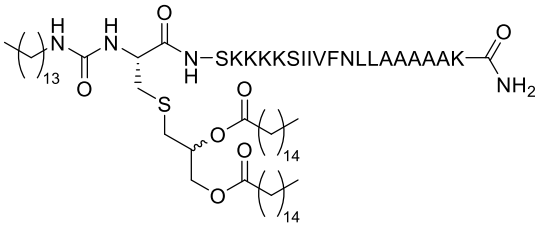
(((2S,5R)-5-amino-6-((2,3-bis(palmitoyloxy)propyl)thio)-1-hydroxy-3,4-dioxohexan-2-yl)amino)-Ser-Lys-Lys-Lys-Lys-Ala-Gln-Leu-Ala-Asn-Asp-Val-Val-Leu-Ala-Ala-Ala-Ala-Ala-Lys-NH_2 (4a)

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).



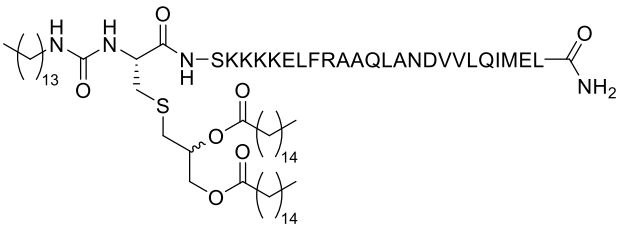
(((2S,5R)-5-amino-6-((2,3-bis(palmitoyloxy)propyl)thio)-1-hydroxy-3,4-dioxohexan-2-yl)amino)-Ser-Lys-Lys-Lys-Lys-Ala-Gln-Leu-Ala-Asn-Asp-Val-Val-Leu-Ala-Ala-Ala-Ala-Ala-Lys-NH_2 (4b)

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).



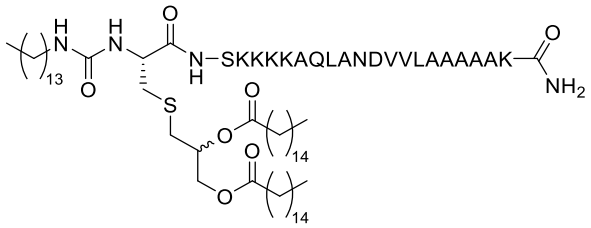
(((2S,5R)-5-amino-6-((2,3-bis(palmitoyloxy)propyl)thio)-1-hydroxy-3,4-dioxohexan-2-yl)amino)-Ser-Lys-Lys-Lys-Lys-Ser-Ile-Ile-Val-Phe-Asn-Leu-Leu-Ala-Ala-Ala-Ala-Ala-Lys-NH_2 (4d)

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).



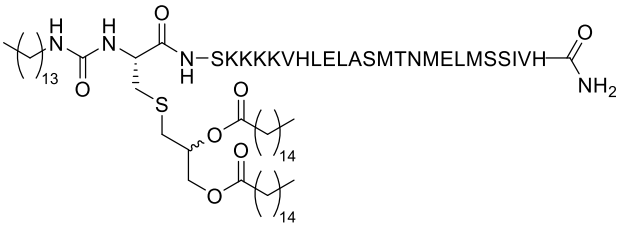
(((2S,5R)-5-amino-6-((2,3-bis(palmitoyloxy)propyl)thio)-1-hydroxy-3,4-dioxohexan-2-yl)amino)-Ser-Lys-Lys-Lys-Lys-Glu-Leu-Phe-Arg-Ala-Ala-Gln-Leu-Ala-Asn-Asp-Val-Val-Leu-Gln-Ile-Met-Glu-Leu-NH_2 (4e)

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).



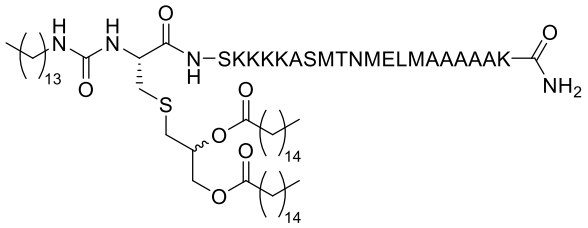
(((2S,5R)-5-amino-6-((2,3-bis(palmitoyloxy)propyl)thio)-1-hydroxy-3,4-dioxohexan-2-yl)amino)-Ser-Lys-Lys-Lys-Lys-Ala-Gln-Leu-Ala-Asn-Asp-Val-Val-Leu-Ala-Ala-Ala-Ala-Ala-Lys-NH_2 (4f)

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).



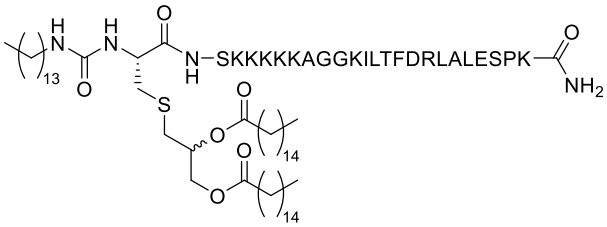
(((2S,5R)-5-amino-6-((2,3-bis(palmitoyloxy)propyl)thio)-1-hydroxy-3,4-dioxohexan-2-yl)amino)-Ser-Lys-Lys-Lys-Lys-Val-His-Leu-Glu-Leu-Ala-Ser-Met-Thr-Asn-Met-Glu-Leu-Met-Ser-Ser-Ile-Val-His-NH_2 (4g)

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).



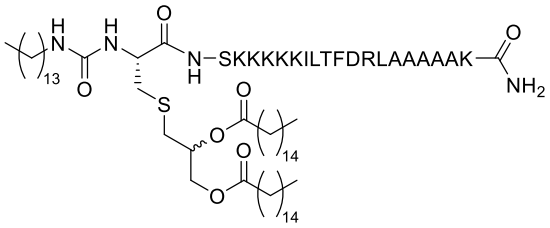
(((2S,5R)-5-amino-6-((2,3-bis(palmitoyloxy)propyl)thio)-1-hydroxy-3,4-dioxohexan-2-yl)amino)-Ser-Lys-Lys-Lys-Lys-Ala-Ser-Met-Thr-Asn-Met-Glu-Leu-Met-Ala-Ala-Ala-Ala-Ala-Lys-NH_2 (4h)

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).



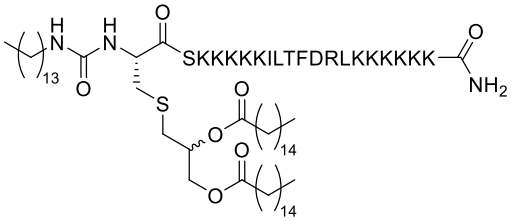
(((2S,5R)-5-amino-6-((2,3-bis(palmitoyloxy)propyl)thio)-1-hydroxy-3,4-dioxohexan-2-yl)amino)-Ser-Lys-Lys-Lys-Lys-Lys-Ala-Gly-Gly-Lys-Ile-Leu-Thr-Phe-Asp-Arg-Leu-Ala-Leu-Glu-Ser-Pro-Lys-NH_2 (4i)

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).



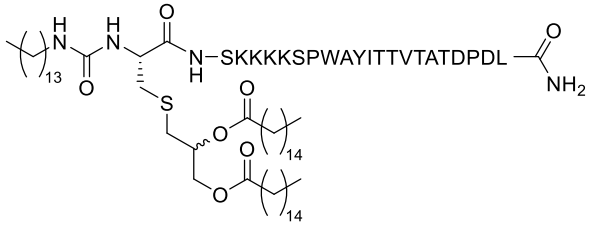
(((2S,5R)-5-amino-6-((2,3-bis(palmitoyloxy)propyl)thio)-1-hydroxy-3,4-dioxohexan-2-yl)amino)-Ser-Lys-Lys-Lys-Lys-Lys-Ile-Leu-Thr-Phe-Asp-Arg-Leu-Ala-Ala-Ala-Ala-Ala-Lys-NH_2 (4j)

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).



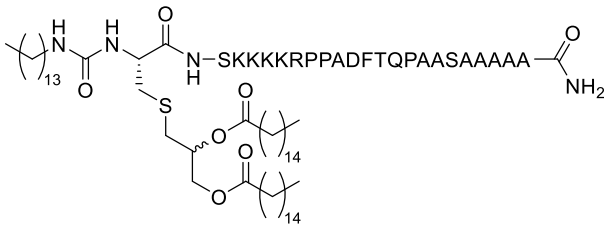
(((2S,5R)-5-amino-6-((2,3-bis(palmitoyloxy)propyl)thio)-1-hydroxy-3,4-dioxohexan-2-yl)amino)-Ser-Lys-Lys-Lys-Lys-Lys-Ile-Leu-Thr-Phe-Asp-Arg-Leu-Lys-Lys-Lys-Lys-Lys-Lys-NH_2 (4k)

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).



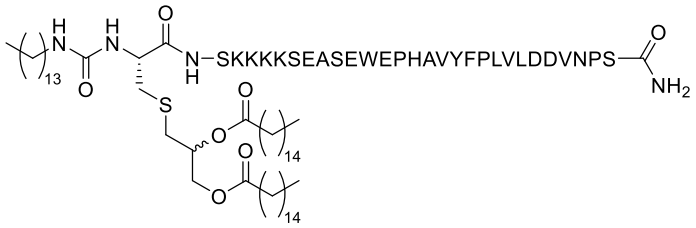
(((2S,5R)-5-amino-6-((2,3-bis(palmitoyloxy)propyl)thio)-1-hydroxy-3,4-dioxohexan-2-yl)amino)-Ser-Lys-Lys-Lys-Lys-Ser-Pro-Trp-Ala-Tyr-Ile-Thr-Thr-Val-Thr-Ala-Thr-Asp-Pro-Asp-Leu-NH_2 (4l)

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).



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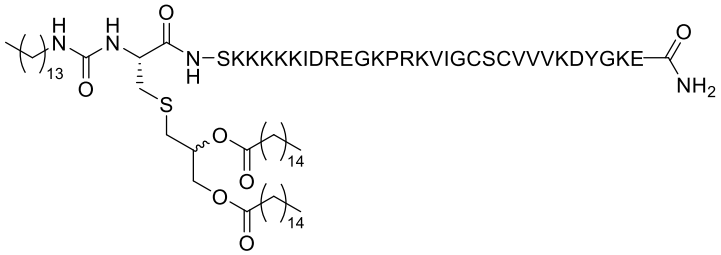
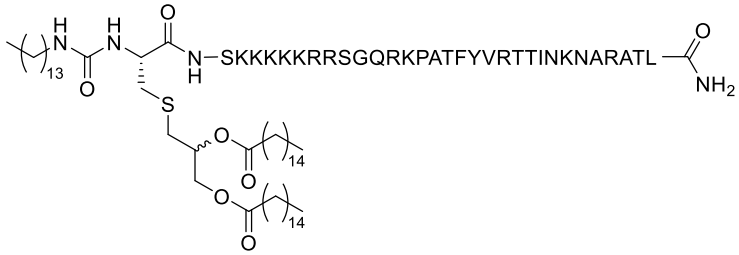
Resin: Tentagel S RAM; **HPLC solution:** *t*BuOH:H_2O:MECN (1:1:1); 5% yield (4.88 mg, 1.19 µmol). **LC-MS:** R_t = 12.47 (Vydac 219TP 5 µm (150x4.6mm) Diphenyl, 10-90% MeCN, 21 min); **ESI-MS:** m/z 1365.4 $[M+3H^+]^{3+}$; **HRMS [M+4H]$^{4+}$:** $[C_{199}H_{327}N_{39}O_{50}S]^{4+}$ 1023.84824 (measured),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



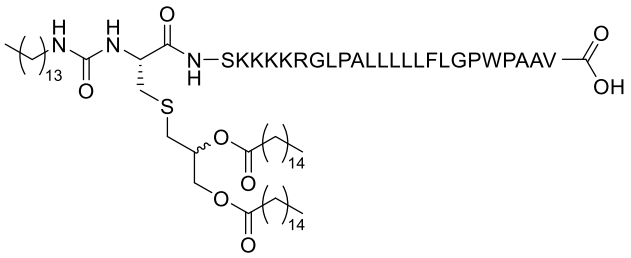
(((2S,5R)-5-amino-6-((2,3-bis(palmitoyloxy)propyl)thio)-1-hydroxy-3,4-dioxohexan-2-yl)amino)-Ser-Lys-Lys-Lys-Lys-Lys-Ile-Asp-Arg-Glu-Gly-Lys-Pro-Arg-Lys-Val-Ile-Gly-Cys-Ser-Cys-Val-Val-Val-Lys-Asp-Tyr-Gly-Lys-Glu-NH_2 (4o)

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).



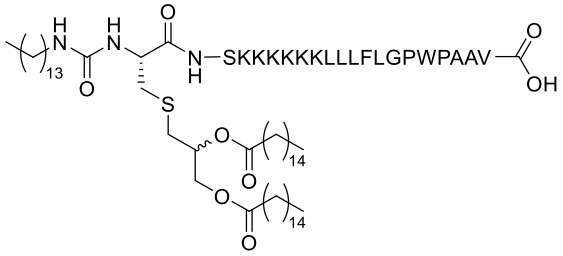
(((2S,5R)-5-amino-6-((2,3-bis(palmitoyloxy)propyl)thio)-1-hydroxy-3,4-dioxohexan-2-yl)amino)-Ser-Lys-Lys-Lys-Lys-Lys-Arg-Arg-Ser-Gly-Gln-Arg-Lys-Pro-Ala-Thr-Phe-Tyr-Val-Arg-Thr-Thr-Ile-Asn-Lys-Asn-Ala-Arg-Ala-Thr-Leu-NH_2 (4p)

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).



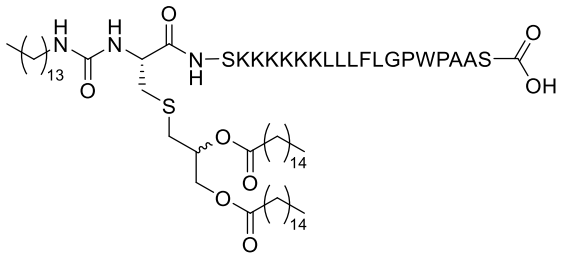
(((2S,5R)-5-amino-6-((2,3-bis(palmitoyloxy)propyl)thio)-1-hydroxy-3,4-dioxohexan-2-yl)amino)-Ser-Lys-Lys-Lys-Lys Arg-Gly-Leu-Pro-Ala-Leu-Leu-Leu-Leu-Leu-Phe-Leu-Gly-Pro-Trp-Pro-Ala-Ala-Val-OH (4q)

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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