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

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Chapter 3

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

Introduction

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

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

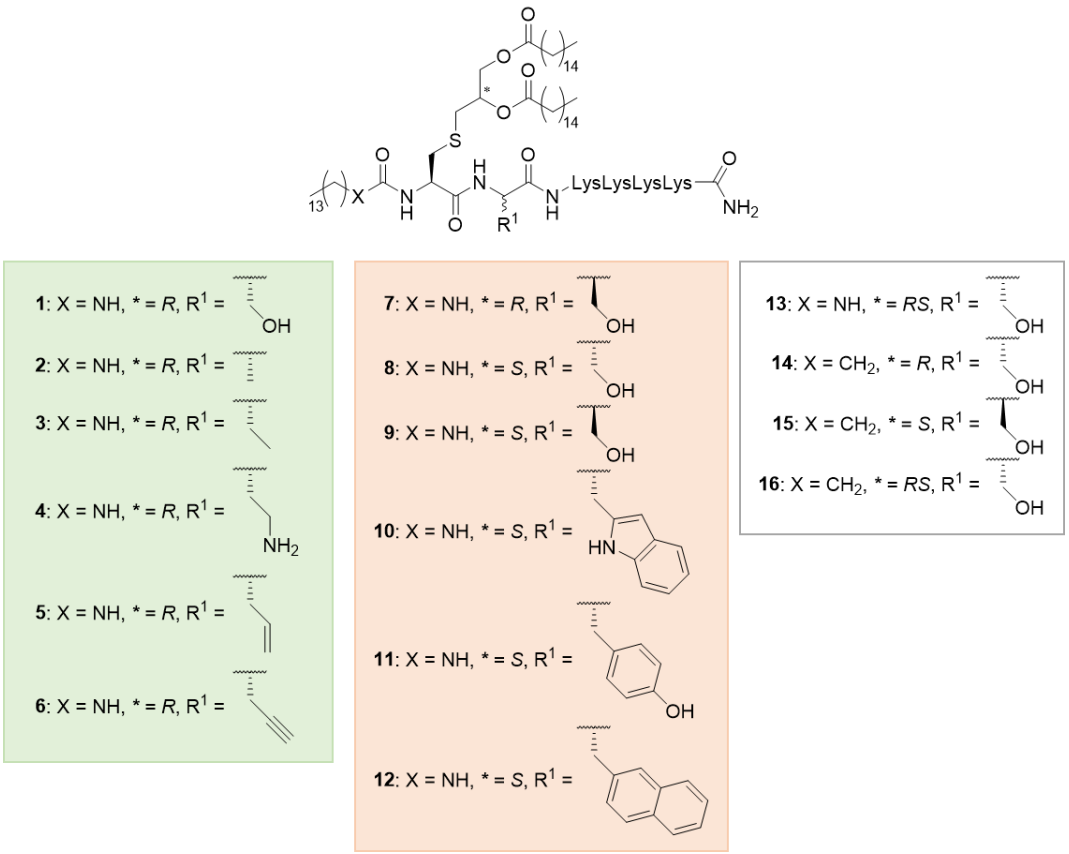
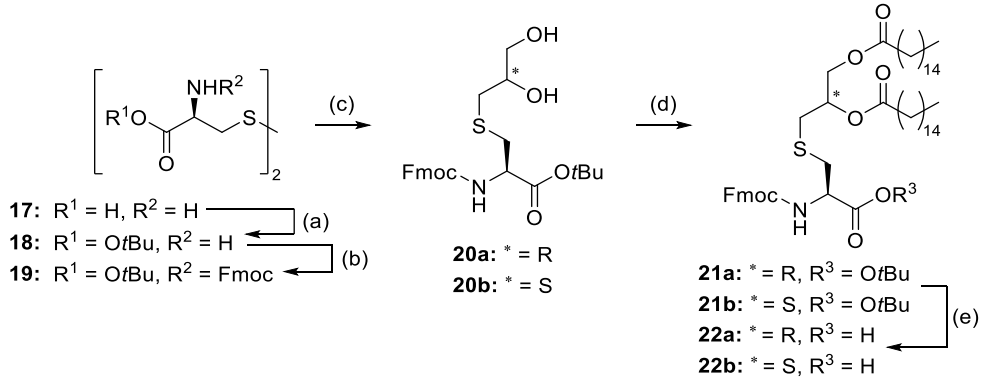


Figure 1: All synthesized target UPam derivatives sorted in group based on their expected influence on agonistic activity. Amino acids in the green box should result in a UPam derivative with good or better agonistic activity, whereas the red box represents activity blocking modifications. The white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

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

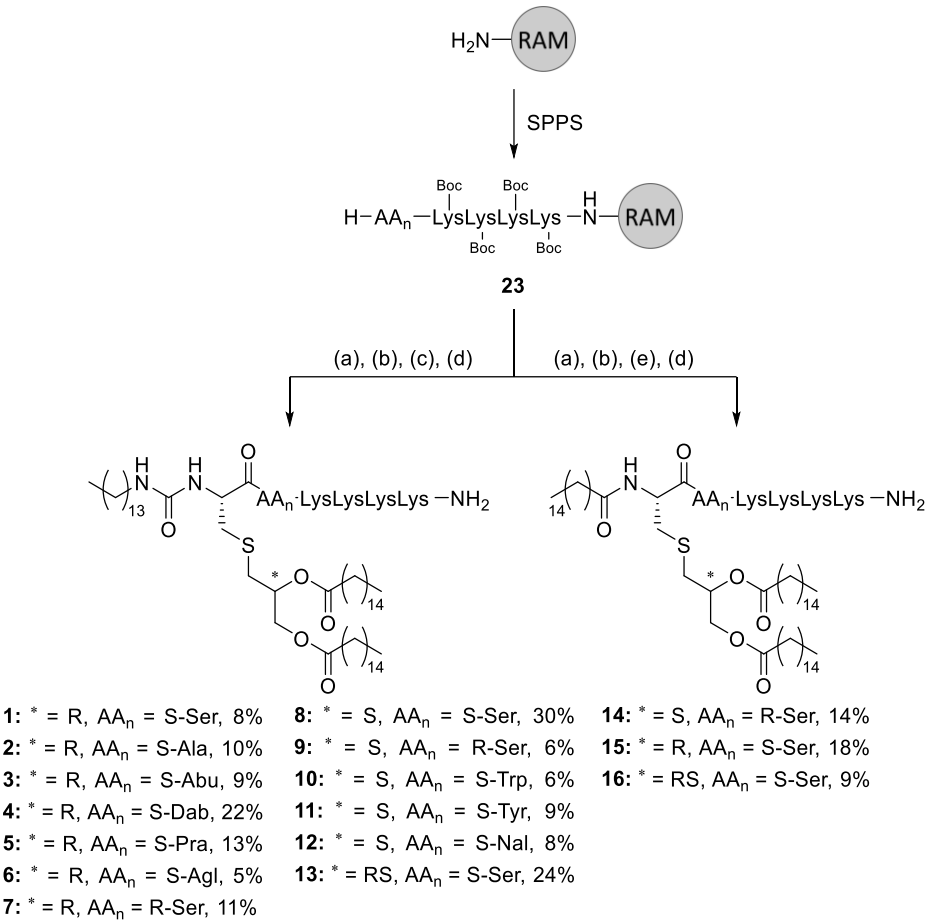
Results and Discussion

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



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

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



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

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

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

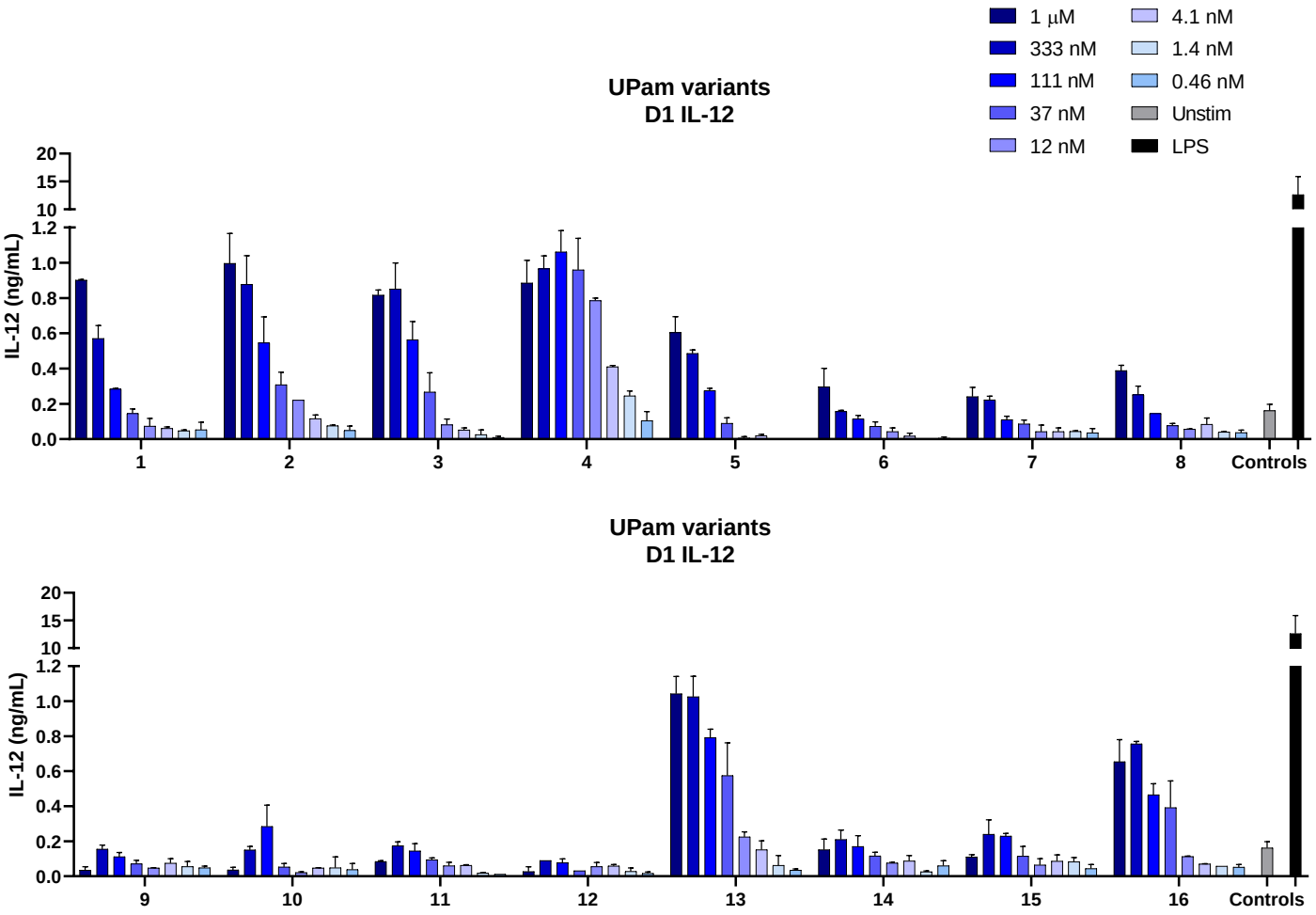


Figure 2: TLR2 activation of the UPam derivatives in D1-DCs, which were incubated with titrated quantities (1 µM – 0.46 nmol, 3-fold titration) of compounds **1**-**16** (Figure 1). LPS concentration was 2µM. After 1 day, supernatants were harvested and the production of IL12-p40 (data shown as mean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

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

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

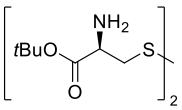
In conclusion, a structure-activity study toward a more active TLR2 ligand has been successfully completed by the synthesis and evaluation of a series UPam derivatives, the design of which was based on observations reported in the literature. The UPam derivatives were prepared by an efficient solid phase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

Experimental

General experimental procedures building block synthesis

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

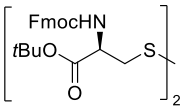
L-cystine bis(*tert* butyl ester) (18)



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

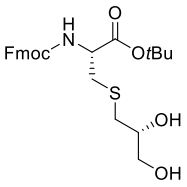
^{1}H NMR (300 MHz, $CDCl_3$) δ 3.69 (dd, *J* = 7.9, 4.6 Hz, 2H), 3.13 (dd, *J* = 13.4, 4.6 Hz, 2H), 2.88 (dd, *J* = 13.4, 7.9 Hz, 2H), 1.73 (s, 4H), 1.48 (s, 18H). **^{13}C NMR (75 MHz, $CDCl_3$)** δ 172.82, 81.75, 54.28, 44.04, 27.99.

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



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

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

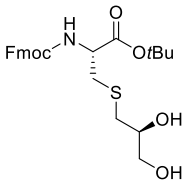


***N*-Fmoc-S-((R)-2,3-dihydroxypropyl)-L-cysteine *tert* butyl ester (20a)**

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

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

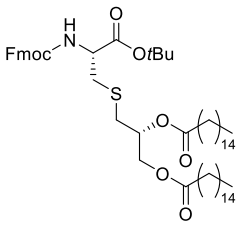
***N*-Fmoc-S-((S)-2,3-dihydroxypropyl)-L-cysteine *tert* butyl ester (20b)**



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

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

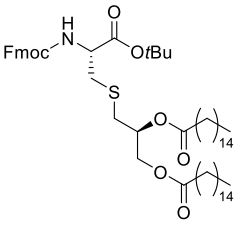
***N*-Fmoc-S-((R)-2,3-bis(palmitoyloxy)propyl)-L-cysteine *tert* butyl ester (21a)**



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

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

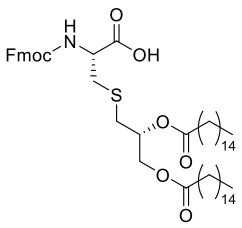
***N*-Fmoc-S-((S)-2,3-bis(palmitoyloxy)propyl)-L-cysteine *tert* butyl ester (21b)**



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

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

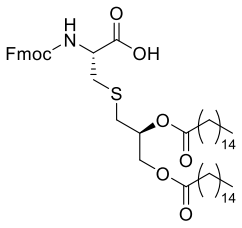
***N*-Fmoc-S-((R)-2,3-bis(palmitoyloxy)propyl)-L-cysteine (22a)**



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

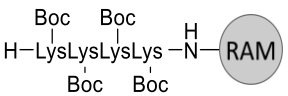
^{1}H NMR (300 MHz, $CDCl_3$) δ 8.00 (s, 1H), 7.75 (d, *J* = 7.4 Hz, 2H), 7.60 (d, *J* = 7.3 Hz, 1H), 7.39 (t, *J* = 7.4 Hz, 2H), 7.30 (td, *J* = 7.4, 0.8 Hz, 2H), 5.83 (d, *J* = 7.7 Hz, 1H), 5.16 (d, *J* = 3.4 Hz, 1H), 4.65 (d, *J* = 6.2 Hz, 1H), 4.46 – 4.28 (m, 3H), 4.23 (t, *J* = 7.0 Hz, 1H), 4.15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

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



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

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



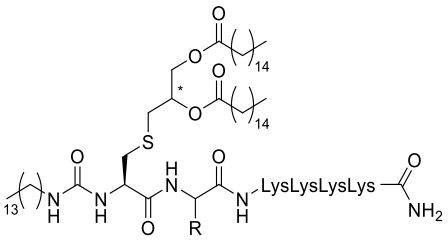
H-Lys(Boc)-Lys(Boc)-Lys(Boc)-Lys(Boc)-Tentagel S Ram

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

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

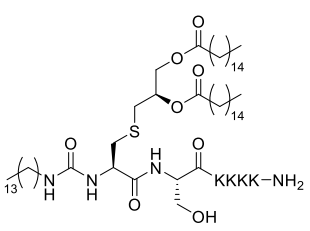
(1) Treating the resin with a solution of Fmoc-Lys(Boc)-OH (1 mmol, 468.35 mg, 4 eq.), HCTU (1 mmol, 413.69 mg, 4 eq.) and D*i*PEA (2 mmol, 348 µmol, 8 eq.) in NMP (4 mL) for 1 h. Afterwards the resin was washed with NMP (3 x 5 mL);
(2) The resin was capped by treating the resin to a solution of 10%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

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



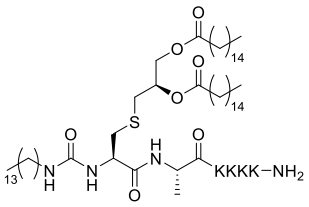
General procedure for the synthesis of 1-tetradecyl-urea-Cys((R)-2,3-di(palmitoyloxy)-propyl)-AA-Lys-Lys-Lys-Lys-NH_2

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



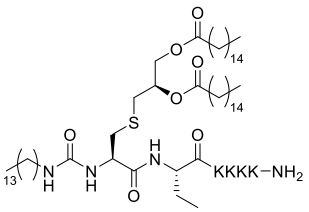
1-tetradecyl-urea-Cys((R)-2,3-di(palmitoyloxy)-propyl)-(S)Ser-Lys-Lys-Lys-Lys-NH_2 (1)

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



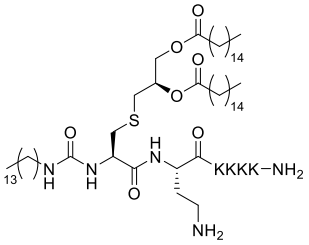
1-tetradecyl-urea-Cys((R)-2,3-di(palmitoyloxy)-propyl)-(S)Ala-Lys-Lys-Lys-Lys-NH_2 (2)

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



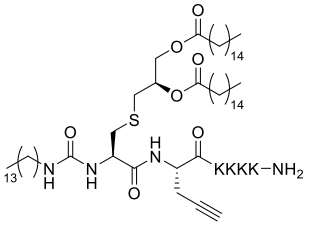
1-tetradecyl-urea-Cys((R)-2,3-di(palmitoyloxy)-propyl)-(S)Abu-Lys-Lys-Lys-Lys-NH_2 (3)

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



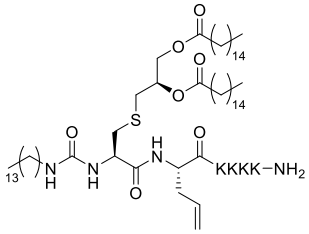
1-tetradecyl-urea-Cys((R)-2,3-di(palmitoyloxy)-propyl)-(S)Dab-Lys-Lys-Lys-Lys-NH_2 (4)

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



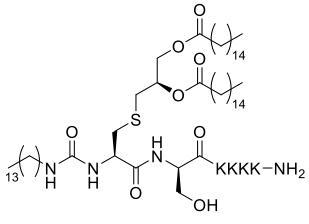
1-tetradecyl-urea-Cys((R)-2,3-di(palmitoyloxy)-propyl)- (S)Pra-Lys-Lys-Lys-Lys-NH_2 (5)

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



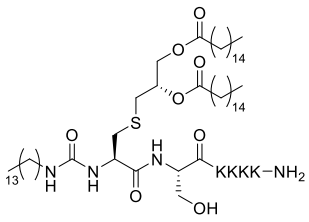
1-tetradecyl-urea-Cys((R)-2,3-di(palmitoyloxy)-propyl)-(S)Agl-Lys-Lys-Lys-Lys-NH_2 (6)

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



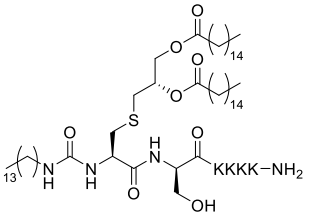
1-tetradecyl-urea-Cys((R)-2,3-di(palmitoyloxy)-propyl)- (R)Ser-Lys-Lys-Lys-Lys-NH_2 (7)

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



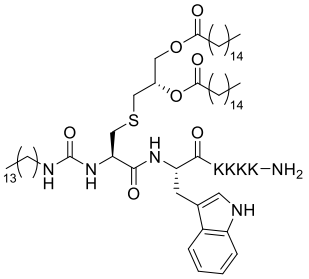
1-tetradecyl-urea-Cys((S)-2,3-di(palmitoyloxy)-propyl)-(S)Ser-Lys-Lys-Lys-Lys-NH_2 (8)

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



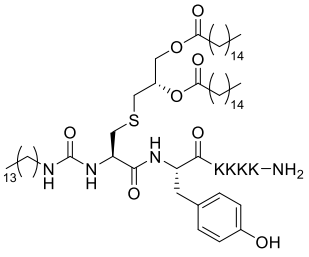
1-tetradecyl-urea-Cys((S)-2,3-di(palmitoyloxy)-propyl)-(R)Ser-Lys-Lys-Lys-Lys-NH_2 (9)

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



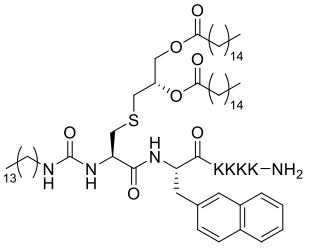
1-tetradecyl-urea-Cys((S)-2,3-di(palmitoyloxy)-propyl)-(S)Trp-Lys-Lys-Lys-Lys-NH_2 (10)

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



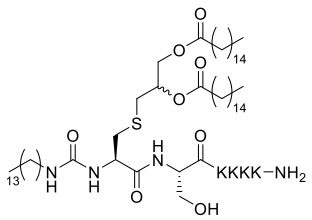
1-tetradecyl-urea-Cys((S)-2,3-di(palmitoyloxy)-propyl)-(S)Tyr-Lys-Lys-Lys-Lys-NH_2 (11)

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



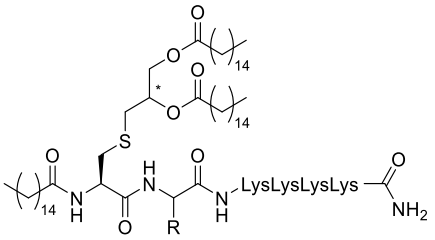
1-tetradecyl-urea-Cys((S)-2,3-di(palmitoyloxy)-propyl)-(S)Nal-Lys-Lys-Lys-Lys-NH_2 (12)

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



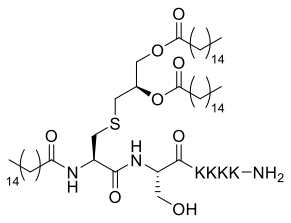
1-tetradecyl-urea-Cys((RS)-2,3-di(palmitoyloxy)- propyl)-(S)Ser-Lys-Lys-Lys-Lys-NH_2 (13)

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



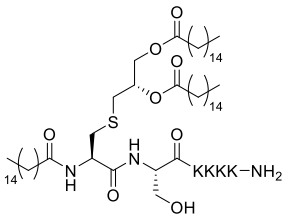
General procedure for the synthesis palmitoyl-Cys((R)-2,3-di(palmitoyloxy)-propyl)-AA-Lys-Lys-Lys-Lys-NH_2

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



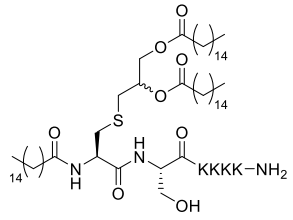
Palmitoyl-Cys((R)-2,3-di(palmitoyloxy)-propyl)-(S)Ser-Lys-Lys-Lys-Lys-NH_2 (14)

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



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

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



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

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

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

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

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