Self adjuvanting immunopeptides : design and synthesis

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Chapter 2: Synthesis and evaluation of fluorescent Pam_3Cys peptide conjugates

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Introduction

Conjugated cancer vaccines have attracted much attention as a promising lead for innovative therapeutic interventions[1–5]. A particular flavour of conjugated vaccines, that has been extensively investigated through the years, comprises a structurally defined construct of a Toll-like receptor agonist covalently attached to a synthetic peptide, that contains a T-cell epitope, either model or tumor associated[6]. It has been discovered that a conjugate of this kind show improved T-cell priming and tumor protection when compared to a mixture of the individual antigenic peptide and Toll-like receptor agonist[7,8]. The usefulness of such synthetic peptide based conjugates in tumor vaccination has been demonstrated as well. A commonly used agonist in these studies is a lipopeptide known as Pam_3CysSK_4 that binds to TLR2/TLR1[9–11]. This compound has been derived from the N-terminus of a bacterial lipoprotein of, among others, *E.coli*[12]. Notably, Pam_3CysSK_4 when applied as a component of a vaccine candidate either covalently attached to a longer peptide sequence or simply admixed with a peptide, is often present as a mixture of R- and S-epimers at the glycerol moiety, while it is known that the R-epimer is the biologically active one[13].

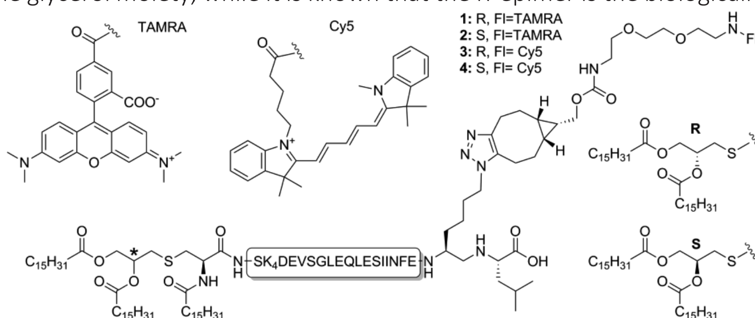
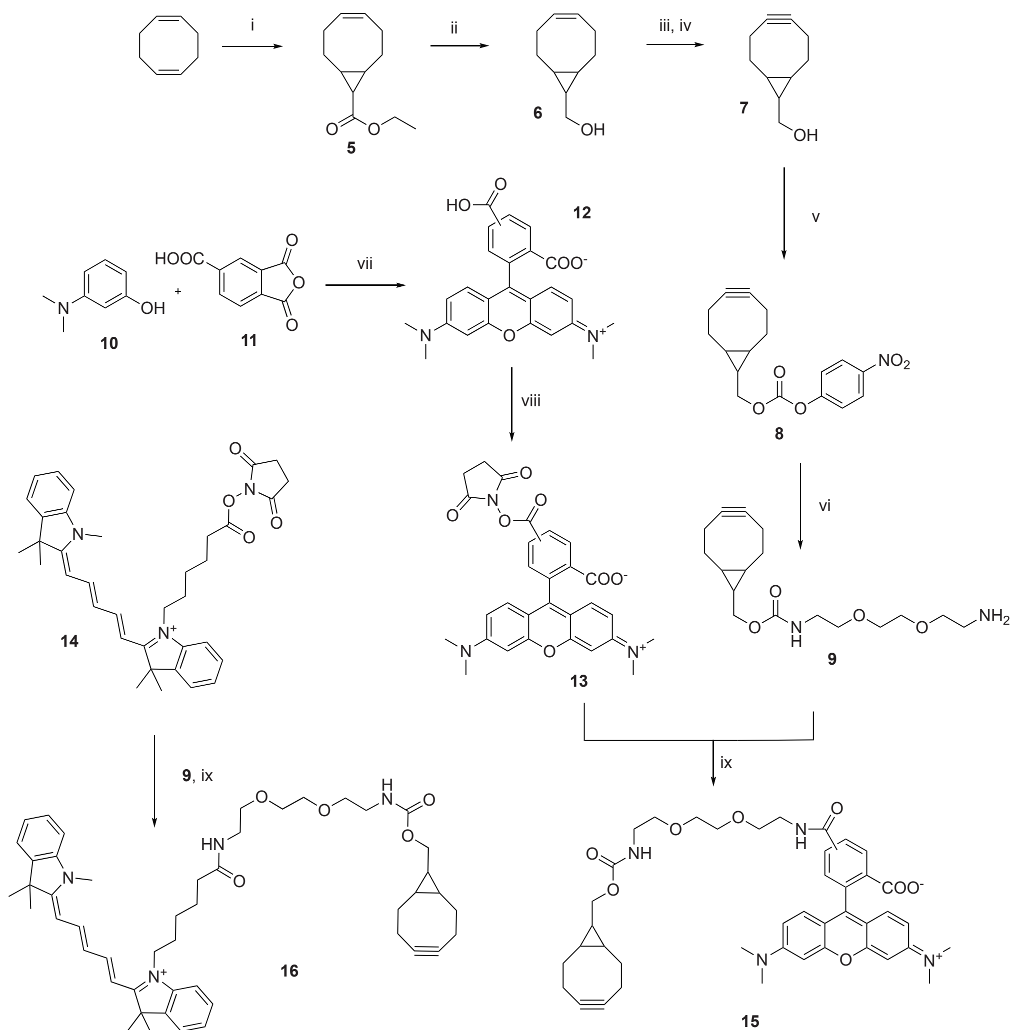


Figure 1. Target labelled Pam_3Cys-lipopeptides



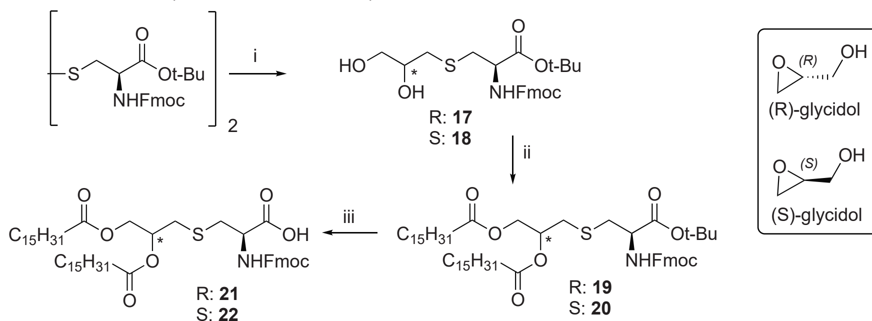
Scheme 1. Synthesis of the reactive dyes **15** and **16** functionalized with a strained alkyne. Reagents and conditions: i) $N_2CH_2C(O)OEt$, $Cu(C_5H_7O_2)_2$, EtOAc,78%, ii) $LiAlH_4$, THF/Et_2O, 91%, iii) Br_2, DCM, iv) KOtBu, THF, 35%, v) p-$NO_2PhOC(O)Cl$, DCM, 59%, vi) 1,8-diamino-3,6-dioxaoctane, NEt_3, DMF, 76%, vii) Cat. H_2SO_4, AcOH, reflux, 30% viii) SuOH, DIC, DMF, ix) D*i*PEA, DMF.

With the aid of non-labelled Pam_3CysSK_4 conjugates it has been shown that R-epimer of Pam_3Cys is indeed the one responsible for dendritic cell (DC) maturation and the S-epimer is inactive while the cellular uptake remained unaffected by the chirality of the glycerol moiety of the Pam_3Cys residue, as judged by the level of the antigen presentation by DC's[13]. In this chapter, it is shown that fluorescently labelled and chirally pure Pam_3Cys-lipopeptides represent useful tools in the studies of antigen processing because these constructs allow a visual evaluation of the antigen uptake irrespective of the DC-maturation status. Towards this end conjugates **1**-**4** (Figure 1) with the fluorescent label covalently attached to the modified side chain of a lysine residue in the commonly used model MHC-I epitope (SIINFEKL) have been synthesized. This design of the

labelled construct proved to be successful in studies preceding this one and that involved the monitoring of the intracellular trafficking of Pam_3Cys-lipopeptides as mixtures of epimers at C-2 of the glycerol moiety[7]. To be able to vary the type of fluorophore more readily a convergent approach based on copper free click chemistry[14–16] has been chosen in the present work. The DC-maturation capacity of the constructs has been evaluated and the uptake of these was studied using confocal microscopy.

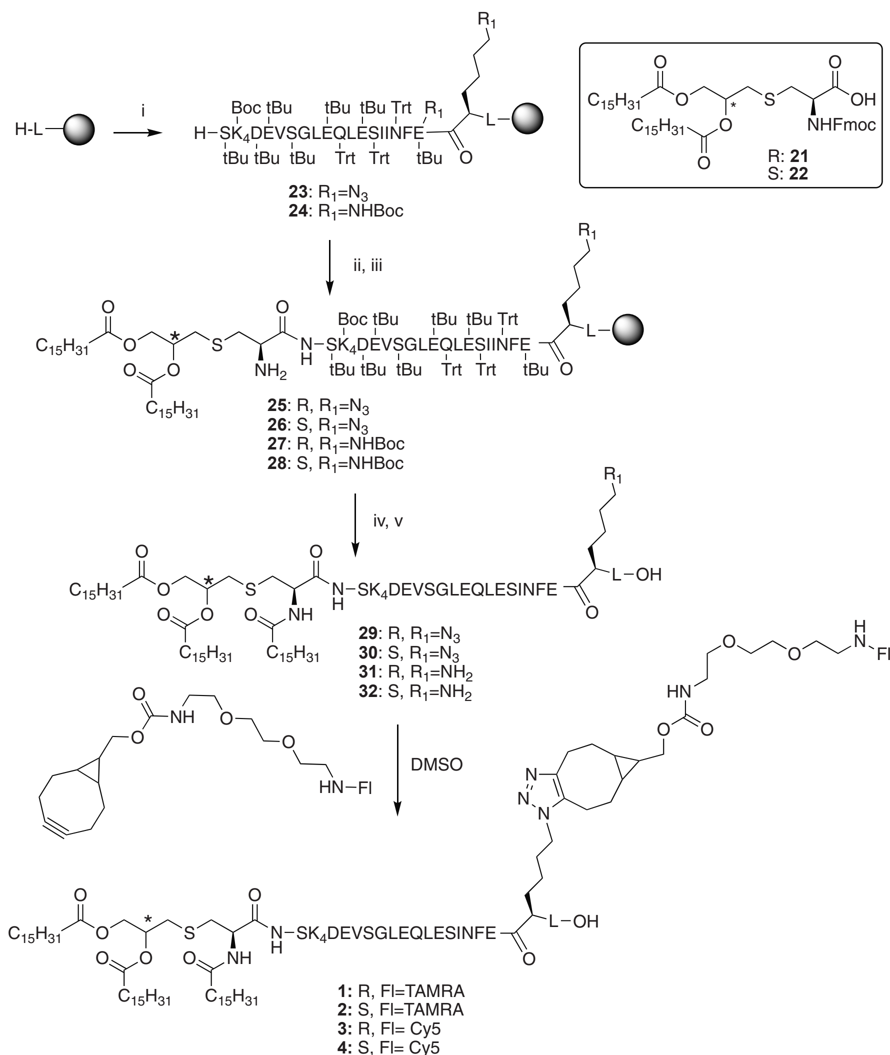
Results and discussion

The key step of the convergent synthesis of conjugates **1** – **4** in which the fluorescent labels are appended to the peptide with the aid of strain promoted [3+2] azide alkyne cycloaddition (Scheme 3) required the availability of azide containing lipopeptides (**29**, **30**) and dyes functionalized with a strained alkyne (**15**, **16**, scheme 1). The lipopeptides **29** and **30** were accessible via standard Fmoc-based solid phase synthesis using chirally pure Fmoc-Pam_2Cys-OH building blocks prepared as described in Scheme 2. The click-reaction prevents the use of a copper catalyst and requires the availability of the bifunctional (1R,8S)-bicyclo[6.1.0]non-4-yn-9-ylmethyl (BCN) linker **9** to which fluorescent labels of choice can be attached via amide bond formation. The synthesis of BCN linker **9** (Scheme 1) is based on the coupling of known BCN 4-nitrophenyl carbonate (**8**) with 1,8-diamino-3,6-dioxaoctane. The reported procedure for the synthesis of BCN alcohol (**6**)[17] commences with cyclopropanation of 1,5-cyclooctadiene through a rhodium tetraacetate mediated Simmons-Smith type reaction to provide exo-**5** (28%) and endo-**5** isomers (58%). Although this step has been reported to be efficient, the cheaper copper acetoacetonate was evaluated as catalyst, in order to facilitate the scaling up of the synthesis. By using ethyl acetate instead of DCM to bring the reaction to higher temperature endo-**5** and exo-**5** could be obtained in 18% and in 58% yield, respectively. A notable difference with the rhodium catalyzed reaction is the appearance of exo-**5** as a major isomer (lowest running spot on TLC). The rest of the synthesis was performed without any major changes with respect to the literature procedure except that the exo-isomer was used to proceed with the synthesis. Ester **5** was reduced using $LiAlH_4$ in a mixture of Et_2O and THF to give BCN alcohol **6**. Subsequent bromination of the double bond in **6** using Br_2 followed by double elimination of bromide from the crude dibromide intermediate generated alkyne **7** in 35% yield. Treatment of **7** with p-nitrophenylchloroformate, followed by addition of 1,8-diamino-3,6-dioxaoctane to the resulting carbonate gave target bifunctional BCN linker **9** in 11% overall yield based on ethyl diazo acetate.



Scheme 2. Synthesis of enantiopure Pam_2Cys building block. Reagents and conditions: i) 1) Zn, H_2SO_4, HCl, MeOH, 2) (R) : (R)-Glycidol, RT, 85%; (S) : (S)-Glycidol, RT, 82%, ii) Palmitic acid, DIC, DMAP, DCM, 89-91%, iii) TFA, RT, 91-96%.

With the availability of BCN linker **9** the fluorescent labels TAMRA and Cy5 can be connected to the amine in bifunctional linker **9**. In order to allow optimization of the click reaction sufficient quantities of the relatively stable TAMRA dye should be available. Hence, using a slightly modified procedure from the literature[18] TAMRA was prepared and coupled to BCN linker **9** on mmol scale (Scheme 1). Sulfuric acid mediated condensation of dimethylaminophenol **10** with trimellitic anhydride **11** in acetic acid instead of butyric acid proceeded smoothly to give **12** as a mixture of regioisomers. Crude **12** was precipitated from diethyl ether and the obtained partially purified compound was converted into hydroxysuccinimide ester **13.** Subsequently BCN linker (**9**) was added to the reaction mixture to give fluorescent reagent **15.** After HPLC-purification TAMRA reagent **15** could be obtained as a single isomer in high purity in a low overall yield. The corresponding Cy5 reagent **16** was prepared according to the same procedure using the commercially available hydroxysuccinimide ester of Cy5 **14** and the crude product was immediately used in the ensuing cycloaddition.



Scheme 3. Synthesis of labelled labelled Pam_3Cys-lipopeptides. Reagents and conditions: i) SPPS Fmoc automated synthesis, ii) **21** or **22**, HCTU, D*i*PEA, NMP, iii) 20% piperidine, NMP, iv) PamCl, Pyridine/DCM, v) 95% TFA, 2.5% TIS, 2.5% H_2O. ON = overnight

Synthesis of chirally pure Pam_2Cys building blocks **21**(R) and **22**(S), as shown in Scheme 2, is essentially as reported previously[19]. The disulfide bridge was reduced using activated zinc powder and subsequently enantiopure glycidol (R or S) was added in a one-pot procedure yielding corresponding diols **17** and **18**. Esterification with palmitic acid using carbodiimide as condensating agent was followed by deprotection of the *tert*-butyl ester with neat TFA to give the building blocks **21**(R) and **22**(S) in 57% and 59% overall yield respectively.
Having all building blocks in hand the R- and S-Pam_3CysSK_4 peptide conjugates **29-30** were assembled by standard solid-phase peptide synthesis SPPS using Fmoc-chemistry (Scheme 3). Commercially available suitably protected amino acids were applied while Fmoc-azidonorleucine was prepared based on a published procedure[20]. Automated SPPS was performed until the azide containing peptide **23** was reached. The optically pure R- and S-Pam_3CysSK_4 moieties were appended manually to immobilized peptide fragment **23** using modified cysteine building blocks **21**(R) and **22**(S), respectively and HCTU as a coupling agent. The known[13] lipopeptides **31**-**32** were prepared alongside to be used as controls. This manual coupling saved building blocks as only 1.2 eq **21** and **22** in an overnight reaction instead of the standard 5 eq for 1h could be used. Ensuing, Fmoc deprotection with piperidine was followed by coupling with 10 eq of palmitoyl chloride. Finally, TFA mediated removal of the side chain protecting groups and concomitant cleavage from resin yielded the lipopeptides **29-30**. It is important to note that lipopeptides **29-30** are poorly soluble in both aqueous and organic solvents and pure DMSO is needed for further processing. The use of DMSO brings along precautions as the oxidative power of DMSO together with traces of acid or water may induce oxidation of the thioethers in **29** and **30**. After purification by HPLC the azide containing Pam_3CysSK_4 peptide conjugates **29**(R) and **30**(S) were labelled with TAMRA and Cy-5. The azide containing conjugate (**29-30**) was dissolved in dry DMSO and TAMRA reagent (**15**) was added in 1:1 ratio. After overnight stirring at room temperature, LCMS analysis showed complete conversion of the starting peptides and the untreated reaction mixture was immediately used for purification by preparative RP HPLC, yielding the labelled lipopeptides **1-2**. Introduction of the Cy5-fluorophore with crude reagent **16** using the same procedure, as described for the TAMRA dye (**15**), gave after HPLC purification the labelled lipopeptides **3-4**.

Biological evaluation

Immunological evaluation of labelled conjugates **1-4** started with assessing murine DC-maturation upon exposure to the conjugates as well as relevant reference compounds. DCs were stimulated for 48h with either the R-Pam_3Cys or the S-Pam_3Cys and DC maturation was measured by IL-12 production (Figure 2). Cells treated with R-Pam_3Cys containing lipopeptide (**31**) produced significantly higher amounts of IL-12 compared to the S-Pam_3Cys based counterpart (**32**). Similar results were found with the compounds labelled with Cy5 or TAMRA, showing intact immunogenicity of the fluorophore-labelled conjugates **1** and **3**.

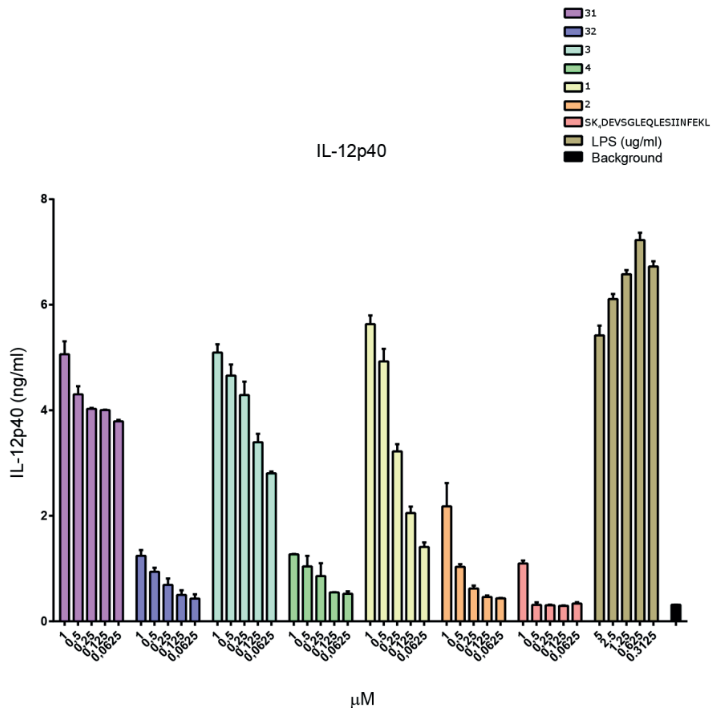


Figure 2. Activation of dendritic cells. DCs were stimulated with titrated amounts of either R-Pam$_3$Cys, S-Pam$_3$Cys (in labelled (**1**-**4**) or non-labelled (**31**-**32**) form; µM), LPS (positive control; µg/ml) or peptide (negative control) for 48h. Supernatants were harvested and analyzed for IL-12 cytokine secretion by ELISA. One representative from three independent experiments is shown.

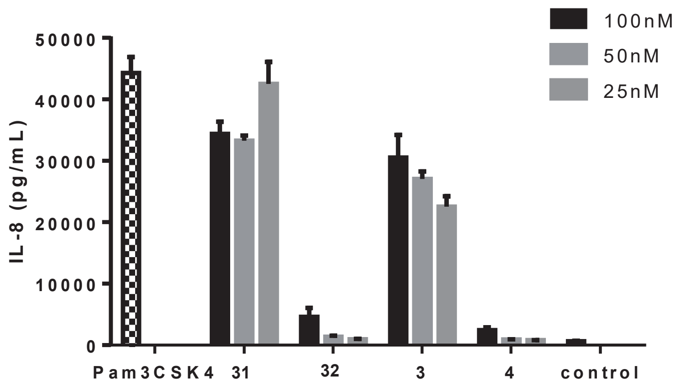


Figure 3. Ability of immunogenic lipopeptides in triggering human IL-8 production via TLR-2. (a) HEK TLR-2 cells were incubated with compounds **31**, **32**, **3** and **4** (100-25nM) or 100ng/mL Pam$_3$CysSK$_4$ for 24 h. Error bars represent SD.

To corroborate the TLR-2 dependent activation of DC's by the fluorescent conjugates the compounds were next assessed using HEK-cells transfected with TLR2. The level of IL-8 produced in the assay reflects the capacity of the conjugates to activate the receptor. The results (Figure 3) show the ability of compounds **31** and **3** to trigger human TLR-2. Compound **31** showed a similar behaviour to the natural TLR-2 ligand Pam_3CysSK_4 while compound **3** showed a lower ability in triggering TLR-2 especially at lower concentration (25nM). Compounds **32** and **4** showed no ability in triggering human TLR-2. To control the receptor specificity of immunogenic lipopeptides for TLR-2, HEK cells expressing TLR-4 were stimulated with compounds **31**, **32**, **3** and **4** (Figure 4). None of the compounds were able to trigger human TLR-4 showing not only the high specificity of the immunogenic lipopeptides for TLR-2 but also the absence of any inadvertent LPS contamination in the samples of the TLR-2 activating conjugates of this study (**3**, **4**, **31**, **32**).

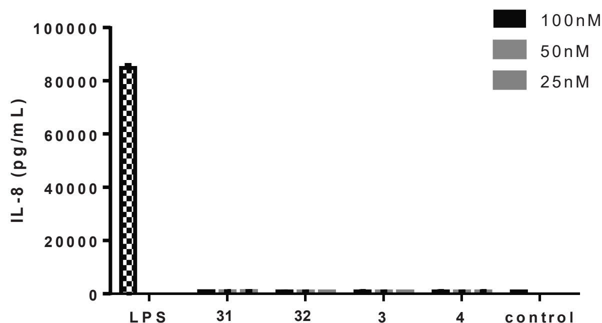


Figure 4. Pam-conjugates do not activate TLR-4. HEK TLR-4 cells were incubated with compounds **31**, **32**, **3** and **4** (100-25nM) or 10 ng/mL LPS for 24 h. Untreated cells were used as control. Supernatants were subsequently analyzed for IL-8 production by ELISA. The graphs are representative of two different independent experiments performed in duplicate.

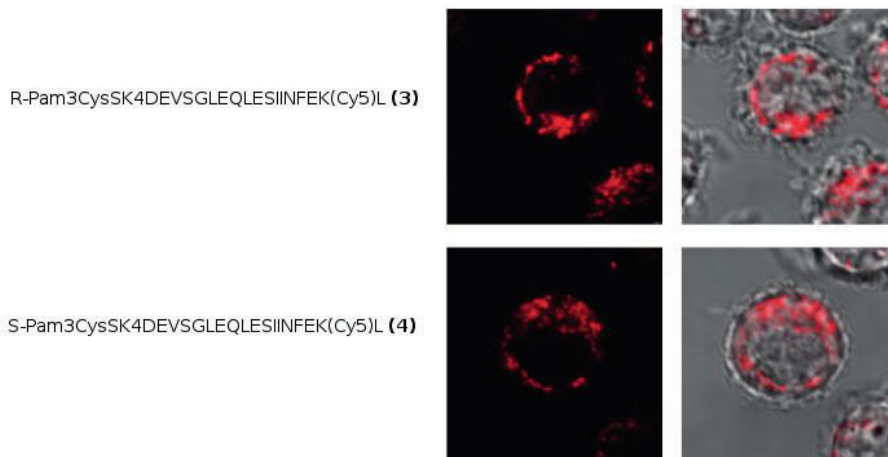


Figure 5. Uptake of Pam-conjugates by dendritic cells. DCs were incubated for 15 min with compounds 3 or 4 (1µM). The uptake and localization of the compounds were analyzed with confocal laser scanning microscopy with Leica system settings as described.[20] The images are representative for multiple cells in at least 3 experiments.

The uptake of **3** and the **4** was measured with confocal microscopy. After 15 min, both compounds were efficiently internalized by murine DCs (shown in red and overlay with DC) and accumulated in hot spots surrounding the nucleus (Figure 5). Similar as have already been reported, no differences in localization or uptake intensity were observed[7,13].

Conclusion

Summarizing, using strain-promoted [3+2]cycloaddition a small set of fluorescent Pam_3Cys-based lipopeptides (**1-4**) has been successfully synthesized and compared to known immunogenic compounds (LPS, **31**, **32**). The R- and S-epimer of Pam_3Cys in the prepared fluorescent lipopeptides triggered DCs maturation in TLR-2-dependent manner and at approximately the same level as their unlabelled analogues. However, the poor aqueous solubility of the conjugates containing TAMRA (**1** and **2**) precluded the use of those for microscopy studies. This indicates that attaining sufficient solubility remains a major challenge in the synthesis of Pam_3Cys-based constructs labelled with fluorophores. Nevertheless, conjugate **3** (R-epimer) and conjugate **4** (S-epimer), both labelled with Cy-5, could be successfully used for confocal microscopy and were taken up by dendritic cells to the same extent. This result corroborates previous findings that suggested a TLR-independent uptake of the peptides conjugated to a TLR-ligand.

Experimental

General methods: All reactions were carried out in oven-dried (110 °C) glassware. Solvents were removed under reduced pressure using standard rotary evaporator. “Dry solvents” were dried over activated 4A° molecular sieves for at least 15 hours before use. All other chemicals were used as received. Thin layer chromatography analysis was performed on pre-coated silica gel 60 plates (Merck) and irradiated with UV light (λ=254 nm), sprayed with a staining solution of $KMnO_4$ (5 g), K_2CO_3 (25 g) in distilled water (1 L) followed by heating at around 150 °C. TLC-MS analyses were executed on a CAMAG TLC interface

connected to an Agilent 6130 Quadrupole or API 165 mass spectrometer. One- and two-dimensional ^{1}H and ^{13}C NMR spectra were recorded on a Bruker AV-400 (400 MHz and 100 MHz for respectively ^{1}H and ^{13}C nuclei) instrument, with chemical shift (δ) in ppm relative to tetramethylsilane (TMS: ^{1}H, δ: 0 ppm). Spectra were recorded at room temperature. Optical rotations were measured on a Propol automatic polarimeter (Sodium D-line, λ: 589 nm). Infrared spectra were recorded on a Shimadzu FTIR-8300 and absorbance bands are reported in cm^{-1}. LC-MS measurements were done on an API 3000 Alltech 3300 with a Grace Vydac 214TP 4,6 mm x 50 mm C4 column and preparative high pressure liquid chromatography was conducted on a Gilson GX281 with an automatic fraction collector and Grace Vydac 214TP 10 mm x 250 mm C4 column or Gemini 5u C18 110A 250x10.0 mm. Buffer A: 0.1% TFA in MilliQ water, Buffer B: ACN. Solid phase peptide synthesis (SPPS) was carried out with an ABI 433A peptide synthesizer.

IL-12p40 ELISA

D1 dendritic cells (immature splenic DCs line derived from B6(H-2^b) mice were plated in a 96-wells plate and incubated with the compounds for 48h as indicated in the figure legends[21]. Supernatants were collected and tested with ELISA for IL-12p40 using a standard sandwich ELISA. Coating Ab: rat anti-mouse IL-12p40 mAb (clone C15.6, Biolegend). Detection Ab: biotinylated rat anti-mouse IL-12p40 mAb (clone C17.8, Biolegend). Streptavidin-Poly-HRP (Sanquin) and 3,3',5,5' Tetramethylbenzidine (Sigma-Aldrich) were used as enzyme and substrate, respectively.

Confocal microscopy

D1 DCs were incubated with 1 µM **3** or **4** for 15min at 37 ˚C and washed with culture medium. The cells were plated out into glass-bottom Petri dishes (MatTek) and imaged using the Leica SP5-STED with a 63x objective lens. Differential interference contrast (DIC) was used to image cell contrast. Images were acquired in 10x magnification and processed with Leica LAS AF Lite software.

Activity assay on transfected TLR-2/4 HEK cells assay

Human TLR-expressing HEK cells were cultured in DMEM medium enriched with Penicillin/Streptomycin/Glutamine and 1% FCS. HEK TLR-2 and HEK TLR-4 cells were cultured in the presence of G418 (Geneticin, 0.5 mg/mL). Suspensions of 100 µL cells (1.10^6 cells/mL) were stimulated for 24h with compounds **31**, **32**, **3** and **4** or appropriate control TLR ligands Pam_3CysSK_4 (100 ng/mL) for TLR-2, LPS, 10 ng/mL for TLR-4. Supernatants were subsequently analyzed for IL-8 production by ELISA.

(1*R*,8*S*,9*r*)-bicyclo[6.1.0]non-4-yn-9-ylmethyl 3,6,9-trioxa-12-azadodecylcarbamate (9)

To a solution of 1,5-cyclooctadiene (100 mL, 0.816 mol) and $Cu(C_5H_7O_2)_2$ (525 mg, 2 mmol) in EtOAc (50 mL) was added dropwise in 3 h a solution of ethyl diazoacetate (10.5 mL, 100 mmol) in EtOAc (50 mL). This solution was stirred overnightunder reflux. EtOAc was evaporated and the excess of cyclooctadiene was removed by filtration over a glass filter filled with silica and elution with EtOAc:heptane, 1:200. The filtrate was concentrated in vacuo and the residue was purified by column chromatography on silica gel to afford ***endo*-5** (3.5 g, 18 %) and ***exo*-5** (10.1 g, 58%) and mixed isomers (1.6g, 8.1%) as colorless oils. The rest of the synthesis was performed starting with ***exo*-5** as published.[17]

^{1}H-NMR (400 MHz): ($CDCl_3$) δ: 5.53 (m,1H), 3.90-3.88 (d, 2H), 3.55-3.44 (m, 8H), 3.30 (m, 2H), 2.33-2.05 (m, 8H), 1.28 (m, 2H), 0.67-0.58 (m, 3H)

^{13}C-NMR (100 MHz): ($CDCl_3$) δ: 156.88, 98.75, 73.00, 70.23, 70.14, 70.12, 68.93, 41.49, 40.72, 33.26, 23.73, 22.79, 21.36

HRMS: $[M+H]^+$: 325.21218 found: 325.21157

5-carboxy-2-(6-(dimethylamino)-3-(dimethyliminio)-3H-xanthen-9-yl)benzoate (12)

Dimethyl aminophenol (6.9 g, 50 mmol) and trimellitic anhydride (4.8 g, 25 mmol) were dissolved in AcOH (400 ml). After adding a catalytic amount of conc. H_2SO_4 (0.5 mL) the mixture was refluxed overnight. Reaction mixture was concentrated to a small volume and diethyl ether (200 mL) was added. Filtration of the precipitate yielded 2.7 g (30 %) of a mixture containing desired of regio-isomers **12**.

LCMS: RT (C_{18} column, 10%B-90%B, 13min grad): 5.28 min, 5.46 min

$[M+H]^+$:431.7

((1*R*,8*S*,9*r*)-bicyclo[6.1.0]non-4-yn-9-yl)methyl (2-(2-(2-(3',6'-bis(dimethylamino)-3-oxo-3*H*-spiro[isobenzofuran-1,9'-xanthene]-5-carboxamido)ethoxy)ethoxy)ethyl)carbamate (15)

Regioisomeric mixture of TAMRA **12** (0.1 mmol, 0.043 g) was suspended in DMF(1 mL). N-hydroxy succinimide (0.1 mmol, 0.011 g) and DIC (0.1 mmol, 0.015 mL) was added and the reaction was stirred overnight at room temperature. Mixture was flushed over silica filter a concentrated. Regioisomeric mixture of TAMRA-OSu (0.056 mmol, 0.03 g) was dissolved in DMF (0.5 mL). D*i*PEA (0.056 mmol, 0.01 mL) and **9** (0.05 mmol, 0.0138 g) were added and the mixture was stirred overnight at room temperature. The crude product was directly purified by HPLC (C_{18} column, 20%-55%B, 30 min grad) yielding pure **15**.

LCMS: RT: (C_{18} column, 10%B-90%B, 13min grad): 5.28 min
$[M+H]^+$: 737.4

1-(1-((1*R*,8*S*,9*r*)-bicyclo[6.1.0]non-4-yn-9-yl)-3,14-dioxo-2,7,10-trioxa-4,13-diazanonadecan-19-yl)-3,3-dimethyl-2-((1*E*,3*E*)-5-((*E*)-1,3,3-trimethylindolin-2-ylidene)penta-1,3-dien-1-yl)-3*H*-indol-1-ium (16)

Cy5-OSu (1.6 µmol, 1 mg) was dissolved in DMF (1.5 mL). D*i*PEA (as 0.1M solution in DMF, 1.6 µmol, 16 µL) and **9** (0.015 mmol, 0.0041 g) were added and the mixture was stirred overnight at room temperature. The remaining of Cy5-OSu was quenched using 1,8-diamino-3,6-dioxaoctane (0.015 µmol, 2 µL) for 2h. The crude product was directly used without further purification.
LCMS: RT: (C_{18} column, 10%B-90%B, 13 min grad): 4.10 min
$[M+H]^+$: 789.6

N-Fluorenylmethoxycarbonyl-S-[2,3-dihydroxy-(2R)-propyl]-(R)-cysteine tert-butyl ester (17)

$(Fmoc\text{-}Cys\text{-}OtBu)_2$ (1.64 mmol, 1.31 g) was dissolved in DCM (12.9 mL). Zinc dust (11.37 mmol, 0.74 g) and H_2SO_4/HCl/MeOH (5.5 mL, 1/7/100) were added and the reaction mixture was stirred at RT. After 15 min, (R)-Glycidol (16.56 mmol, 1.11 mL) was added, the resulting mixture was stirred for 5 h at 40°C. The reaction mixture was filtered and then concentrated under vacuum until half of the volume. The crude was diluted (EtOAc) and washed (10% aq. $KHSO_4$). The aqueous layer was back extracted with EtOAc three times. The combined organic layers were dried over $MgSO_4$, filtered and concentrated. Silica gel column chromatography (50-80 % EtOAc in PE) yielded compound **17**(2.01 mmol, 0.9544 g, 61.3%).
^{1}H-NMR (400 MHz): ($CDCl_3$) δ: 7.76 (d, *J* = 7.5 Hz, 2H),7.69 – 7.58 (m, 2H), 7.40 (t, *J* = 7.4 Hz, 2H), 7.32 (t, *J* = 7.3 Hz, 2H), 6.20 (d, *J* = 8.1 Hz, 1H), 4.61 – 4.48 (m, 1H), 4.40 (d, *J* = 7.3 Hz, 2H), 4.24 (t, *J* = 7.2 Hz, 2H), 3.90 – 3.78 (m, 1H), 3.70 - 3.57 (m, 2H), 3.01 (qd, *J* = 14.0, 5.8 Hz, 2H), 2.83 – 2.56 (m, 2H), 1.50 (s, 9H).
^{13}C-NMR (100 MHz): ($CDCl_3$) δ:170.00, 156.24, 143.83, 141.28, 127.79, 127.15, 125.20, 120.05, 83.04, 71.18, 67.25, 65.24, 54.62, 47.07, 36.29, 35.44, 28.02
IR:3360, 1732.08, 1699.29, 1527.62, 1220.94, 758.02
α_D: -1.6°

N-Fluorenylmethoxycarbonyl-S-[2,3-dihydroxy-(2S)-propyl]-(R)-cysteine tert-butyl ester (18)

$(Fmoc\text{-}Cys\text{-}OtBu)_2$ (1 mmol, 0.797 g) was dissolved in DCM (7.9 mL). Zinc dust (6.92 mmol, 0.45 g) and a solution of H_2SO_4/HCl/MeOH (1/7/100) (3.37 mL) was added and the reaction mixture was stirred at RT. After 15 min (S)-glycidol (10.1 mmol, 0.75 g, 0.7 mL) was added to the flask and the mixture was stirred for 5 h under reflux (40 °C). The reaction mixture was then filtered, concentrated and diluted with EtOAc. The solution was washed (10% aq. $KHSO_4$) and the aqueous layer was back extracted 3 times (EtOAc). The organic layer was then dried ($MgSO_4$), filtered and concentrated. The crude was purified by column chromatography (50-80 % EtOAc/PE), compound **18** (1 mmol, 0.478 g, 50 %) was obtained.
^{1}H-NMR (400 MHz): ($CDCl_3$) δ: 7.78 (d, *J* = 7.4 Hz, 2H), 7.64 (d, J = 7.4 Hz, 2H), 7.42 (t, *J* = 7.4 Hz, 2H), 7.33 (t, *J* = 7.3 Hz, 2H), 6.02 (d, *J* = 8.1 Hz, 1H), 4.56 – 4.47 (m, 1H), 4.41 (d, *J* = 7.3 Hz, 2H), 4.25 (t, *J* = 7.2 Hz, 2H), 3.84 (m, 1H), 3.70 – 3.58 (m, 2H), 3.01 (qd, *J* = 14.0, 5.8 Hz, 2H), 2.84 – 2.57 (m, 2H), 1.51 (s, 9H).
^{13}C-NMR (100 MHz): ($CDCl_3$) δ:169.8, 156.2, 143.8, 141.3, 127.8, 127.1, 125.2, 120.0, 83.1, 70.8, 67.3, 65.1, 54.6, 47.1, 36.4, 35.7, 28.0
IR:3350.35, 2933.73, 1697.36, 1149.57, 758.02.
α_D: +3°

N-Fluorenylmethoxycarbonyl-S-[2,3-bis(palmitoyloxy)-(2R)-propyl]-(R)-cysteine tert-Butyl ester (19)

Compound **17** (11.5 mmol, 5.44 g) was dissolved in dry DCM (150 mL) under argon condition. Consecutively palmitic acid (36.8 mmol, 9.42 g), DIC (44.85 mmol, 5.65 g, 7 mL) and DMAP (4.6 mmol, 0.56 g) were added. The reaction mixture was stirred over the weekend at RT. Glacial acetic acid (86.25 mmol, 5.2 g, 5 mL) was added and the solution was stirred for 15 min, then filtered and concentrated. Crystallization in DCM/MeOH (1/19) yielded compound **19** (8.9 mmol, 8.46 g, 77 %).

^{1}H-NMR (400 MHz): ($CDCl_3$) δ: 7.80 (d, *J* = 7.5 Hz, 2H), 7.65 (d, *J* = 7.5 Hz, 2H,), 7.43 (t, *J* = 7.4 Hz, 2H), 7.35 (t, *J* = 7.4 Hz, 2H), 5.74 (d, *J* = 7.6 Hz, 1H), 5.19 (dd, *J* = 6.4, 3.7 Hz, 1H), 4.54 (dt, *J* = 7.7, 5.1 Hz, 1H), 4.46 – 4.34 (m, 2H), 4.27 (t, *J* = 7.2 Hz, 2H), 4.19 (dd, *J* = 12.0, 6.0 Hz, 1H), 3.08 (qd, *J*= 13.7, 5.0 Hz, 2H), 2.80 (d, *J* = 6.4 Hz, 2H), 2.33 (q, *J* = 7.3 Hz, 4H), 1.70 – 1.55 (m, 4H), 1.52 (s, 9H), 1.28 (s, 48H), 0.91 (t, *J* = 6.7 Hz, 6H).

^{13}C-NMR (100 MHz): ($CDCl_3$) δ:173.4,173.1,169.5, 155.7, 143.8, 141.3, 127.7, 127.1, 125.2, 120.0, 83.0, 70.2, 67.2, 63.5, 54.3, 47.1, 35.4, 34.3, 33.3, 32.0,29.7, 29.7, 28.0, 24.9, 22.7, 14.2

IR:3356.14, 2916.37, 2848.86, 1732.08, 1699.29, 1527.62, 1157.29, 736.81

α_D: -1.2°

N-Fluorenylmethoxycarbonyl-S-[2,3-bis(palmitoyloxy)-(2S)-propyl]-(R)-cysteine tert-Butyl ester (20)

18 (1 mmol, 0.478 g) was dissolved in dry THF (15 mL). Palmitic acid (4 mmol, 1.024 g), DIC (4.5mmol, 0.567 g ,0.7 mL) and DMAP (0.40 mmol, 0.05 g) were added. The mixture was stirred at RT for 5h. Then glacial acetic acid was added (8 mmol, 0.48 g, 0.5 mL) and the reaction mixture was stirred for 15 min at RT. The reaction mixture was then filtered and concentrated. Purification of the crude by crystallization (DCM/MeOH (1/19)) yielded **20** (1 mmol, 1.236 g, quant).

^{1}H-NMR (400 MHz): ($CDCl_3$) δ: 7.79 (d, *J* = 7.5 Hz, 2H), 7.65 (d, *J* = 7.5 Hz, 2H,), 7.43 (t, *J* = 7.4 Hz, 2H), 7.34 (t, *J* = 7.4 Hz, 2H), 5.75 (d, *J* = 7.6 Hz, 1H), 5.19 (dd, *J* = 6.4, 3.7 Hz, 1H,), 4.56 (dt, *J* = 7.7, 5.1 Hz, 1H), 4.39 (m, 2H), 4.27 (t, *J* = 7.2 Hz, 2H), 4.18 (dd, *J* = 12.0, 6.0 Hz, 1H), 3.08 (qd, *J* = 13.7, 5.0 Hz, 2H), 2.80 (d, *J* = 6.4 Hz, 2H), 2.31 (q, *J* = 7.3 Hz, 4H), 1.65 – 1.55 (m, 4H,), 1.53 (s, 9H), 1.28 (s, 48H), 0.91 (t, *J* = 6.7 Hz, 6H).

^{13}C-NMR (100 MHz): ($CDCl_3$) δ:173.4, 173.0, 169.5, 155.7, 143.8, 141.3, 127.7, 127.1, 125.2, 120.0, 83.0, 70.3, 67.3, 63.4, 54.3, 47.1, 35.4, 34.3, 33.2, 32.0, 29.7, 29.7, 28.0 24.9, 22.7, 14.2

IR:3398, 2916.37, 2848.86,1737.86, 1701.22, 1531.48,1155.36, 736.81

α_D: -2.6°

N-Fluorenylmethoxycarbonyl-S-[2,3-bis(palmitoyloxy)-(2R)-propyl]-(R)-cysteine (21)

TFA (130.7 mmol, 14.9 g, 9.1 mL) was added to compound **7** (1.2 mmol, 1.1 g) and the solution was stirred for 1h at RT. Co-evaporation of TFA with toluene (3 x 10 mL) resulted in a crude compound. Purification of the crude by column chromatography (15%-20% EtOAc in PE/ 1% AcOH) yielded the final building block **21**, (R)-Pam_2CysOH (1.03 mmol, 0.9195 g, 86 %).

^{1}H-NMR (400 MHz): ($CDCl_3$) δ: 7.79 (d, *J* = 7.5 Hz, 2H), 7.64 (d, *J* = 7.5 Hz, 2H), 7.42 (t, *J* = 7.4 Hz, 2H), 7.34 (t, *J* = 7.4 Hz, 2H), 5.88 (d, *J* = 7.9 Hz, 1H), 5.21 (dd, *J* = 6.3, 3.7 Hz, 1H,), 4.70 (q, *J* = 6.1 Hz, 1H), 4.48 – 4.34 (m, 2H), 4.27 (t, *J* = 7.1 Hz, 2H), 4.19 (dd, *J* = 11.9, 6.1 Hz, 1H) 3.15 (ddd, *J* = 46.3, 14.0, 5.5 Hz, 2H), 2.81 (d, *J* = 6.4 Hz, 2H), 2.33 (q, *J* = 7.7 Hz, 4H), 1.62 (d, *J* = 7.1 Hz, 4H), 1.29 (s, 48H), 0.91 (t, *J* = 6.7 Hz, 6H).

^{13}C-NMR (100 MHz): ($CDCl_3$) δ:174.3, 173.6, 173.5, 156.0, 143.7, 141.3, 127.8, 127.1, 125.2, 120.0, 70.3, 67.5, 63.6, 53.7, 47.1, 34.1, 33.0, 32.0, 29.8, 29.7, 24.9, 22.7, 14.2.

IR: 2916.37, 2848.86, 1730.15, 1521.84, 1168.86, 763.81

α_D: +8.2°

N-Fluorenylmethoxycarbonyl-S-[2,3-bis(palmitoyloxy)-(2S)-propyl]-(R)-cysteine (22)

TFA (1146.68 mmol, 130.72 g, 85.6 mL) was added to compound **20** (10.52 mmol, 10 g) under stirring. After 1 h the reaction mixture was co-evaporated with toluene (3x 90 mL). Purification was done via column chromatography (10% EtOAc/pentane, glacial AcOH 1%) and yielded compound **22** (S)-Pam_2CysOH (8.38 mmol, 7.49 g, 80%).

^{1}H-NMR (400 MHz): ($CDCl_3$) δ: 7.79 (d, *J* = 7.5 Hz, 2H), 7.64 (d, *J* = 7.5 Hz, 2H), 7.43 (t, *J* = 7.4 Hz, 2H), 7.34 (t, *J* = 7.4 Hz, 2H), 5.84 (d, *J* = 7.9 Hz, 1H), 5.21 (dd, *J* = 6.3, 3.7 Hz, 1H,), 4.71 (q, *J* = 6.1 Hz, 1H), 4.46 – 4.35 (m, 2H), 4.27 (t, *J* = 7.1 Hz, 2H), 4.17 (dd, *J* = 11.9, 6.1 Hz, 1H), 3.16 (ddd, *J* = 46.3, 14.0, 5.5 Hz, 2H), 2.88 – 2.72 (m, 2H), 2.33 (q, *J* = 7.7 Hz, 4H), 1.62 (d, *J* = 7.1 Hz, 4H), 1.28 (s, 48H), 0.91 (t, *J* = 6.7 Hz, 6H).

^{13}C-NMR (100 MHz): ($CDCl_3$) δ:174.4, 173.7, 173.4, 155.9, 143.7, 141.3, 127.8, 127.1, 125.2, 120.0, 70.3, 67.5, 63.6, 53.5, 47.1, 34.1, 32.9, 32.0, 29.8, 29.7, 24.9, 22.7, 14.2
IR:2916.37, 2848.86, 1701.22, 1521.84, 1151.50, 736.81
α_D: +10.6°

Imidazole-1-sulfonyl azide hydrochloride[22]
Sulfuryl chloride (16.1 mL, 200 mol) was added drop-wise to an ice-cooled suspension of NaN_3 (13.0 g, 200 mmol) in MeCN (200 mL) and the mixture stirred overnight at room temperature. Imidazole (25.9 g, 380 mmol) was added portion-wise to the ice-cooled mixture and the resulting slurry stirred for 3 h at room temperature. The mixture was diluted with EtOAc (400 mL), washed with H_2O (2 × 400 mL) then saturated aqueous $NaHCO_3$ (2 × 400 mL), dried over $MgSO_4$ and filtered. A solution of HCl in EtOH (obtained by the drop-wise addition of AcCl (21.3 mL, 300 mmol) to ice-cooled dry ethanol (75 mL)] was added drop-wise to the filtrate with stirring, the mixture chilled in an ice-bath, filtered and the filter cake washed with EtOAc (3 × 100 mL) to give Imidazole-1-sulfonyl azide.HCl as colorless needles (24.9 g, 59 %).

Fmoc-azidonorleucine
Fmoc-lysine.HCl (19.7 mmol, 7.98 g) was dissolved in 8:2 MeOH:H_2O (150 mL). $CuSO_4$ (0.5 mmol, 80 mg), $NaHCO_3$ (90 mmol, 7.56 g) and HCl.imidazole-1-sulfonyl azide (24 mmol, 5.0 g) was added. After overnight stirring, 3M HCl (aq) (30 mL) was added to acidify the solution to pH=2. The methanol was evaporated *in vacuo* and the mixture was diluted with EtOAc and consequently washed with 10% $KHSO_4$ (aq) (3x) and brine (1x). The solution was dried ($MgSO_4$), filtrated and concentrated. The crude was adsorbed on Celite and purified by silica gel column chromatography (From 9:1 Pentane :EtOAc to 5:5 Pentane :EtOAc, Δ=5%). Fmoc-azidonorleucine (14.8 mmol, 5.84 g) was obtained with an 75% yield.
HRMS: $[M+H]^+$: 395.17120 (calculated 395.17138)
^{1}H-NMR (400 MHz): ($CDCl_3$) δ: 9.38 (s, 1H), 7.74 (d, *J* = 7.5 Hz, 2H), 7.59 (d, *J* = 5.8 Hz, 2H), 7.38 (t, *J* = 7.4 Hz, 2H), 7.29 (m, 2H), 5.47 (d, *J* = 33.8 Hz, 1H), 4.51 (m, 1H), 4.39 (m, 2H), 4.20 (t, *J* = 6.8 Hz, 1H), 3.22 (m, 2H), 1.99 – 1.82 (m, 0.5H), 1.80 – 1.65 (m, 0.5H), 1.58 (m, 2H), 1.52 – 1.38 (m, 2H).
^{13}C-NMR (100 MHz): ($CDCl_3$) δ:176.38, 156.31, 143.82, 141.35, 127.83, 127.15, 125.12,120.09, 67.19, 53.62, 51.09, 47.14, 31.81,28.38, 22.51
IR: 2949, 2093,1694

R- Pam_3CysSK_4DEVSGLEQLESIINFEK(N_3)L (29)
The SK_4DEVSGLEQLESIINFEK(N_3)L (**23**) peptide was prepared by applying Fmoc-protocol starting from Tentagel S RAM resin (loading 0.23 mmol/g) on a ABI-433 peptide synthesizer. To a mixture of resin bound peptide (0.05 mmol,0.228 g) in NMP/DCM (1:1) was added (R,R)-Fmoc-Pam_2Cys-OH (**21**) (0.125 mmol, 0.111 g), PyBOP (0.175 mmol, 0.091 g) and D*i*PEA 1M (0.25 mmol, 0.032 g, 250 µL). The D*i*PEA was added in two times, first an amount of 125 µL, after 10 min another amount of 125 µL. The reaction mixture was then stirred overnight on the orbital shaker at rt. The resin was washed three times with DCM and the Fmoc was cleaved with 20% piperidine/DMF (3 times, 5 min each). The free amine was palmitoylated by adding palmitic chloride (0.3 mmol, 0.137 g, 151 µL) in pyridine/DCM (1:1), the mixture was stirred for 2.5 h. The solution was washed with DCM three times and the resulting conjugate was cleaved off the resin by adding TFA/TIS/H_2O (95/2.5/2.5) (2h). Purification of the conjugate was done by adding the crude to cold diethyl ether/n-pentane (1:1) (14 mL) and centrifugation (4000 rpm, 5min) was performed. The precipitate was dissolved in dry DMSO. This solution was then subjected to semi-preparative HPLC, pure lipopeptide fractions were collected and concentrated by freeze-drying. This yielded conjugate **29** in 12% yield.
LC:RT: (C_4 column, 50%B-90%B, 13min grad): 8.4min
HRMS: $[M+3H]^{3+}$: 755.09364 (calculated :755.09397)

S-Pam_3CysSK_4DEVSGLEQLESIINFEK(N_3)L (30)
Compound **30** was synthesized (11% yield) using method described above using (S,R)-Fmoc-Pam_2Cys-OH **22** instead.
LC:RT: (C_4 column, 50%B-90%B, 13min grad): 8.3min
HRMS: $[M+2H]^{2+}$: 1791.60431 (calculated :1791.60281)

R- Pam_3CysSK_4DEVSGLEQLESIINFEKL (31)
Compound **31**(15% yield) was synthesized using method described above for **29** using Fmoc-Lys(Boc)-OH instead of Fmoc-Nle(N_3)-OH.
LC:RT: (C_4 column, 50%B-90%B, 13min grad): 8.4min
HRMS: $[M+2H]^{2+}$: 1778.61622 (calculated :1778.60756)

S-Pam_3CysSK_4DEVSGLEQLESIINFEKL (32)
Compound **32** (13% yield) was synthesized using method described above for **30** using Fmoc-Lys(Boc)-OH instead of Fmoc-Nle(N_3)-OH.
LC:RT: (C_4 column, 50%B-90%B, 13min grad): 8.4min
HRMS: $[M+2H]^{2+}$: 1778.61283 (calculated :1778.60756)

R-Pam_3CysSK_4DEVSGLEQLESIINFEK(TAMRA)L (1)
Compound **29** (0.22 µmol, 0.8 mg) was dissolved in dry DMSO (100 µL). TAMRA-BCN **15** (0.3 µmol, 0.22 mg) in dry DMSO (100 µL) was added to the solution. The reaction mixture was stirred at RT for 1 week. After HPLC purification, **1** (0.1 µmol, 0.43 mg) was obtained in 45% yield.
LCMS: RT: (C_4 column, 50%B->90%B, 13min grad): 7.9min
$[M+3H]^{3+}$: 1441.0

S- Pam_3CysSK_4DEVSGLEQLESIINFEK(TAMRA)L (2)
Compound **2** was synthesized (37% yield) using method described above starting with **30** instead of **29**.
LCMS: RT: (C_4 column, 50%B->90%B, 13min grad): 7.9min
$[M+3H]^{3+}$: 1441.3

R- Pam_3CysSK_4DEVSGLEQLESIINFEK(Cy5)L (3)
Compound **3** was synthesized as **1** using method described above using Cy5-BCN **16** instead of TAMRA-BCN **15** (52% yield).
LCMS:RT: (C_4 column, 50%B->90%B, 13min grad): 8.3min
$[M+3H]^{3+}$: 1458.0

S- Pam_3CysSK_4DEVSGLEQLESIINFEK(Cy5)L (4)
Compound **4** was synthesized using method described above starting with **30** instead of **29** (48% yield).
LCMS:RT: (C_4 column, 50%B->90%B, 13min grad): 8.3min
$[M+3H]^{3+}$: 1457.4

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