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Synthesis and evaluation of fluorescent Pam_3Cys peptide conjugates

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ABSTRACT

Chirally pure *R*- and *S*-epimers of TLR2 ligand Pam_3CysSK_4 were prepared and separately conjugated to an OVA model epitope, in which lysine was replaced by azidonorleucine. The azide function in the conjugate permitted labelling with different fluorophores by use of strain-promoted 3+2 cycloaddition. The *R*-epimer of the labelled conjugates induced TLR2-dependent DC maturation, while *S*-epimer proved to be inactive. Combining the lipophilicity of Pam_3CysSK_4 ligand with fluorophores influenced the solubility of the resulting conjugates in an unpredictable way and only the conjugates labelled with Cy-5 were suitable for confocal fluorescence microscopy experiments. It was shown that both epimers of the Cy-5 labelled lipopeptides were internalized equally well, indicating TLR2-independent cellular uptake. The presented results demonstrate the usefulness of strain-promoted azide-alkyne cycloaddition in the labelling of highly lipophilic lipopeptides without disturbing the in vitro activity of these conjugates with respect to activation of TLR-2.

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 tumour associated.[6] It has been discovered that a conjugate of this kind show improved T-cell priming and tumour 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 tumour 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 bacterial lipoprotein of, among others, *Escherichia 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,[7,10,13–16] 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.[11,17]

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.[17] In this paper we show 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** (Fig. 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 our past studies that involved the monitoring of the intracellular trafficking of Pam_3Cys-lipopeptides as mixtures of epimers at C-2 of the glycerol moiety.[8] To be able to vary the type of fluorophore more readily a convergent approach based on copper free click chemistry has been chosen in the present work.[18–20] The DC-maturation capacity of the constructs has been evaluated and the uptake of these was studied using confocal microscopy.

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 2) 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 Supplementary information. The click-reaction we decided to apply

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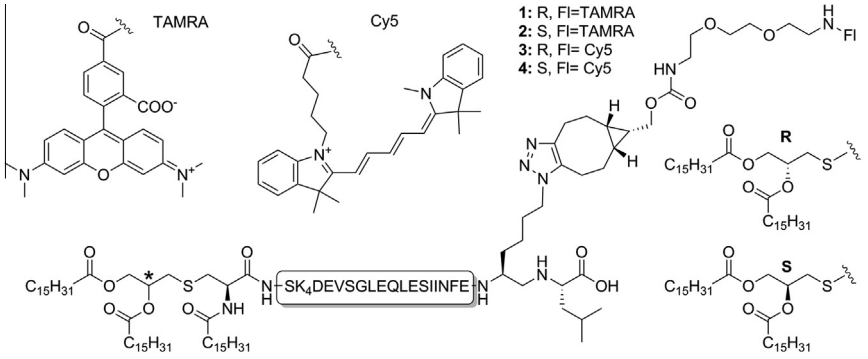
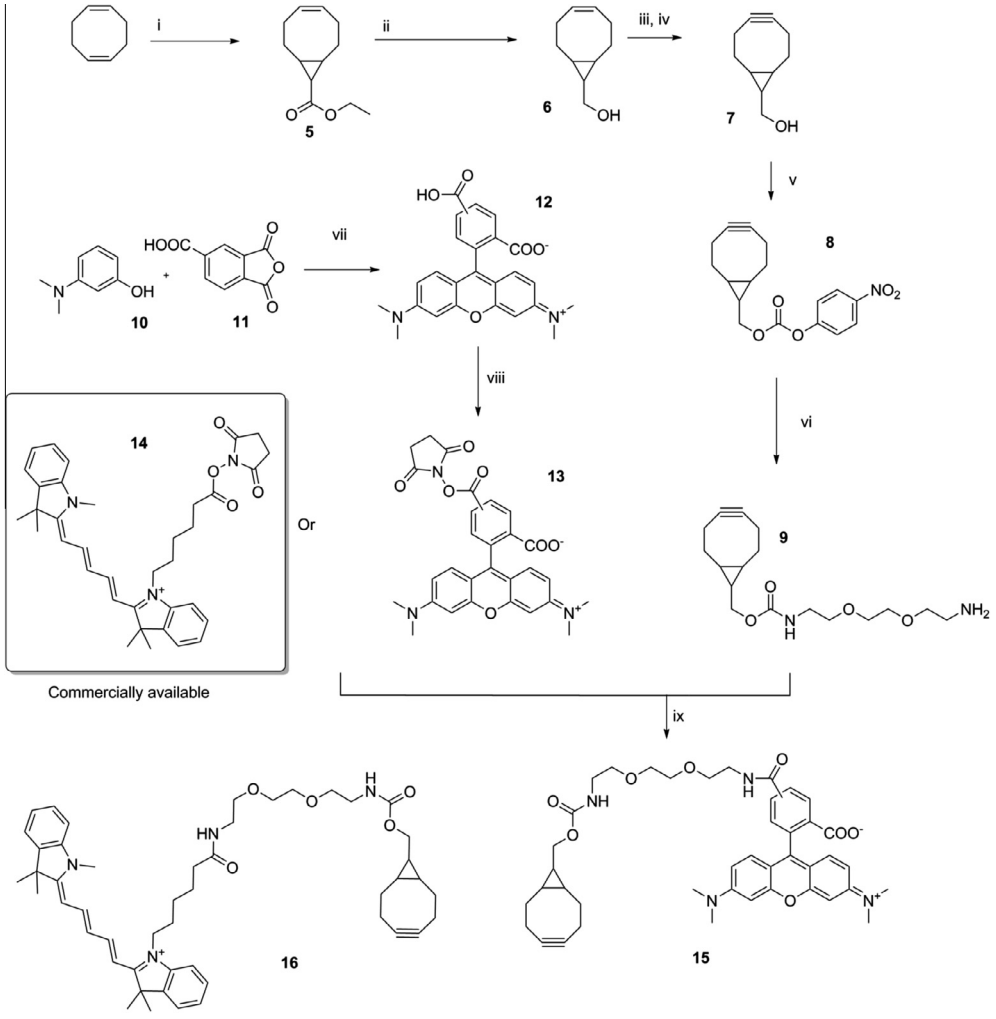
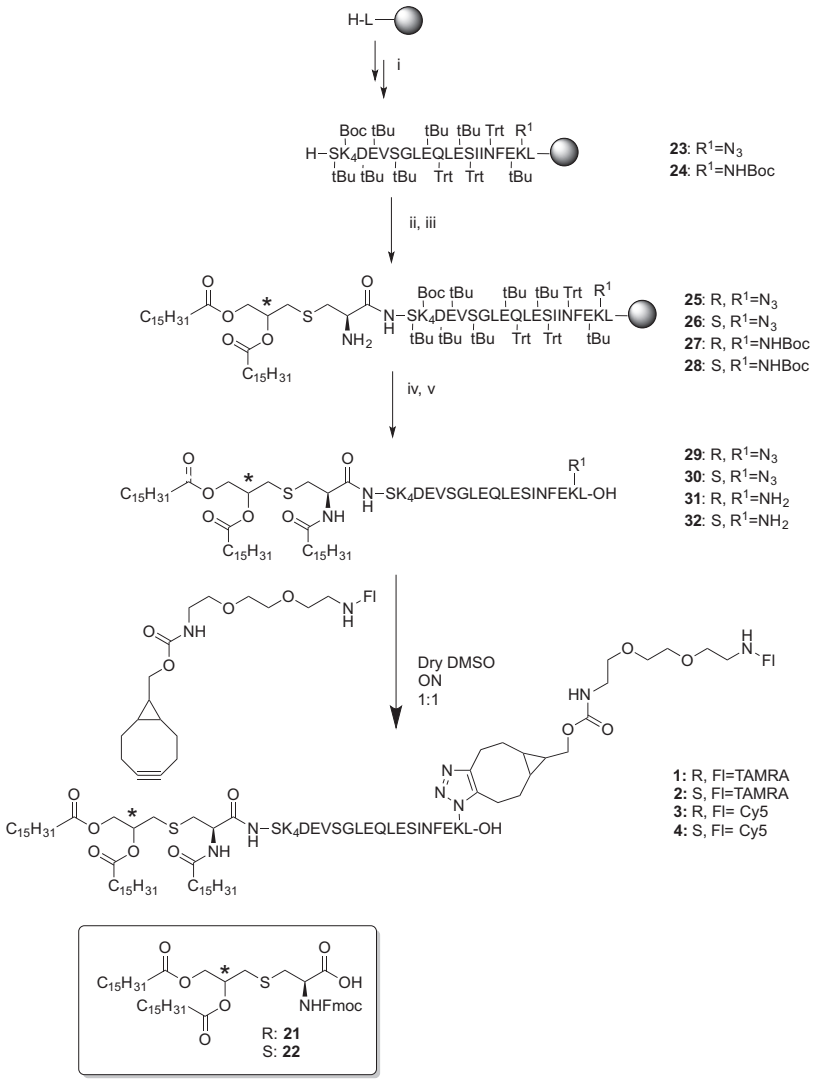


Figure 1. Target labelled Pam_3Cys-lipopeptides.



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) DiPEA, DMF.

prevents the use of a copper catalyst and requires the availability of the bifunctional (1*R*,8*S*,9*s*)-bicyclo[6.1.0]non-4-yn-9-yl methyl (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 2,2′-(ethylenedioxy)bis(ethylamine). The reported procedure[21] to BCN alcohol (**6**) 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 is arguably 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 catalysed reaction is the appearance of exo-**5** as a major isomer (lowest running spot on



Scheme 2. Convergent synthesis of 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. The designators R and S refer to the configuration of the chiral centre marked with an asterisk.

TLC). The rest of the synthesis was performed without any major changes except that the exo-isomer was used to proceed with the synthesis. Ester **5** was reduced using a $LiAlH_4$ 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*-nitrophenyl chloroformate, followed by addition of 2,2′-(ethylenedioxy)bis(ethylamine) to the resulting carbonate gave target bifunctional BCN linker **9** in 11% overall yield based on 1,5-cyclooctadiene.

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 optimisation of the click reaction sufficient quantities of the relatively stable TAMRA dye should be available. Hence, using a slightly modified procedure from the literature[22] 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 and 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 projected copper free labelling step.

Having all building blocks at our disposal the *R*- and *S*-Pam_3-$CysSK_4$ peptide conjugates **31**–**32** were assembled by standard solid-phase peptide synthesis SPPS using Fmoc-chemistry (Scheme 2). Commercially available suitably protected amino acids were applied while Fmoc-azidonorleucine was prepared based on a published procedure.[23] 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. This manual coupling saved building blocks as only 1.2 equiv **21** and **22** overnight instead of the standard 5 equiv for 1 h could be used. Ensuing Fmoc deprotection with piperidine was followed by coupling with 10 equiv of palmitoyl chloride. Finally, TFA mediated removal of the side chain protecting groups and concomitant cleavage from resin yielded the lipopeptides **29**–**30** in 12% and 11% overall yield, respectively. It is important to recognise that

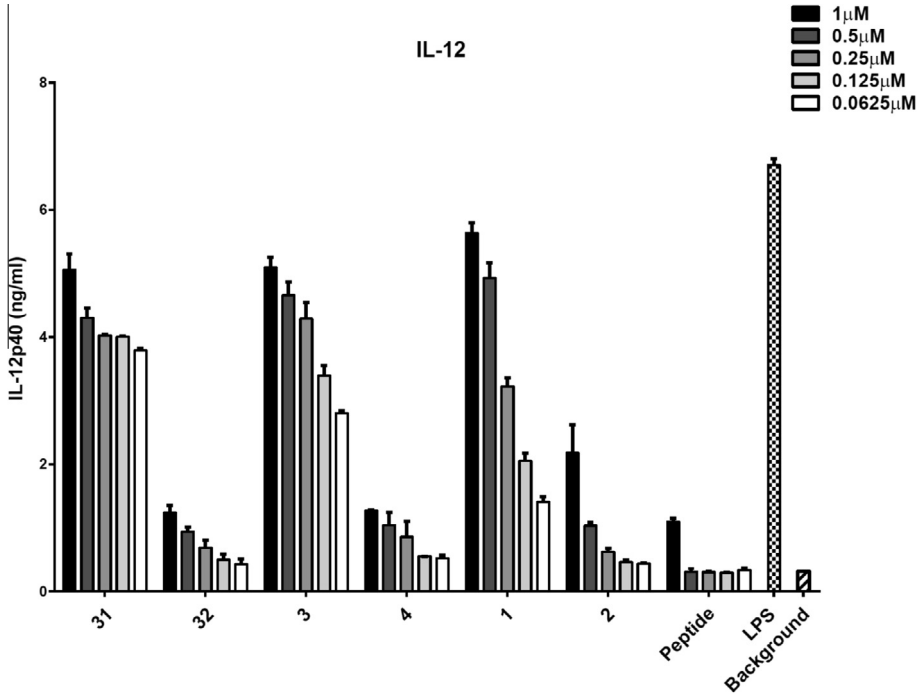


Figure 2. Activation of dendritic cells. DCs were stimulated with titrated amounts of either *R*-Pam3Cys, *S*-Pam3Cys [in the labelled (**1**–**4**) or the unlabelled (**31**–**32**) form; μM], LPS (positive control; 1.25 μg/ml) or peptide (SK_4DEVSGLEQLESIINFEKL; negative control) for 48 h. Supernatants were harvested and analysed for IL-12 cytokine secretion by ELISA. One representative from three independent experiments is shown.

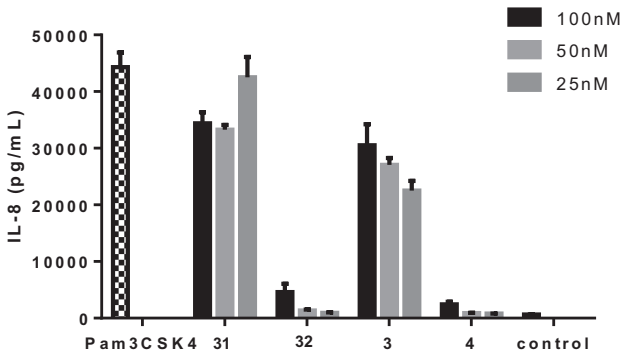


Figure 3. Ability of immunogenic lipopeptides in triggering human IL-8 production via TLR-2. (a) HEK TLR-2 cells were incubated with compounds **31**, **32**, **3** and **4** (100-25nM) or 100 ng/mL Pam_3CysSK_4 for 24 h. Error bars represent SD.

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 aqueous acid may induce oxidation of the thioethers[24] in **29** and **30**. We did not observe such an oxidation when pure dry DMSO was use to dissolve the Pam_3Cys-containing conjugates. 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 de starting peptides and the untreated reaction mixture was used for purification by preparative RP HPLC, yielding the labelled lipopeptides **1**–**2** in 45% and 37% yield, respectively. Introduction of the Cy5-fluorophore with crude reagent **16** using the same procedure, as described for the

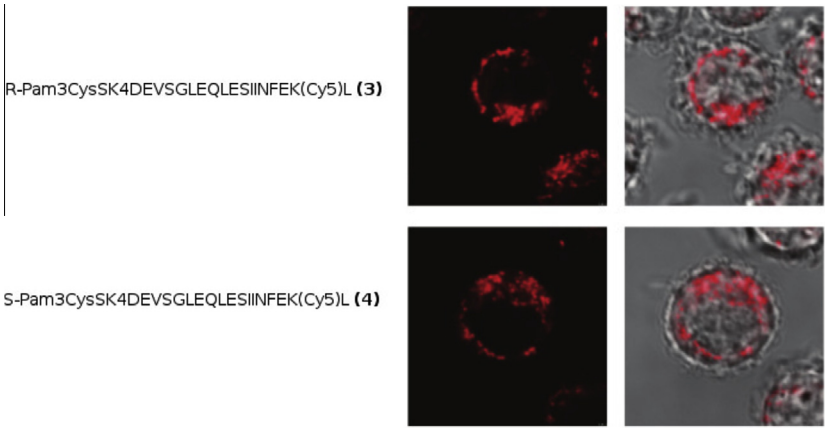


Figure 4. Uptake of Pam-conjugates by dendritic cells. DCs were incubated for 15 min with compounds **3** or **4** (1 μM). The uptake and localisation of the compounds were analysed with confocal laser scanning microscopy with Leica system settings as described.[25] The images are representative for multiple cells in at least 3 experiments.

TAMRA dye (**15**), gave after HPLC purification the labelled lipopeptides **3** (52% yield) and **4** (48% yield).

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 48 h with either the *R*-Pam_3Cys or the *S*-Pam_3Cys and DC maturation was measured by IL-12 production (Fig. 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**.

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 (Fig. 3) show the ability of compounds **31** and **3** to trigger human TLR-2. Compound **31** showed a behaviour similar to the natural TLR-2 ligand Pam_3CysSK_4 while compound **3** showed a lower ability in triggering TLR-2 especially at lower concentration (25 nM). 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** (Fig. 1S, Supporting information). 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**).

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 (Fig. 4). Similar as we have previously reported, no differences in localisation or uptake were observed.[8,17]

Summarising, 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*- not *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 reminds that the 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.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2016.05.094.

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