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# An efficient and scalable synthesis of potent TLR2 agonistic PAM<sub>2</sub>CSK<sub>4</sub>†

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Diacylated PAM<sub>2</sub>CSK<sub>4</sub>, a highly expensive lipopeptide with desirable aqueous solubility and a broad spectrum of cytokine/chemokine induction is a most potent dual (human and murine) Toll-Like Receptor-2 (TLR2) agonist. Besides such thrilling characteristics, its synthetic process is not reported in the literature. The present report describes an efficient and scalable 20 step synthesis of PAM<sub>2</sub>CSK<sub>4</sub> in good yield (all steps > 60%) along with a clear description of the hindrances and easy solutions adopted in each step. Overall, a convergent synthetic approach was adopted involving synthesis of appropriately protected starting materials, synthesis of a key backbone skeleton PAM<sub>2</sub>CS, synthesis of a tetralysine fragment and the final coupling to yield PAM<sub>2</sub>CSK<sub>4</sub>. Tedious column chromatography was avoided on a large scale in many steps.

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## Introduction

The current scenario of a war zone against pathogenic infections demands the enactment of an innate and adaptive immune system and their working as a two-wheel drive. As one of the key players of the innate immune system, Toll-Like Receptors (TLRs) present in the immune cells perceive pathogen associated molecular patterns (PAMPs) conserved in microbes.<sup>1</sup> The recognition of PAMPs through these Pattern Recognition Receptors (PRRs) brings out the induction of a signaling cascade with the production of cytokines and costimulatory molecules.<sup>2</sup> Thereafter, the activation of an adaptive immune system occurs through TLRs, which serve as a bridge between two immune systems. TLRs are the membrane spanning proteins situated on the cell surface of immune cells or in endosomal compartments. Each TLR is amendable to perceive a specific set of pathogens with the variation in inflammatory responses.<sup>3</sup> An overwhelming inflammatory response is expected from the upcoming novel immunotherapeutics or vaccine adjuvants.<sup>4</sup> Therefore, the principle emphasis of numerous specialists in the field of medicinal chemistry is the design and synthesis of ligands that specifically activate TLRs.

Due to the complexity related to mechanistic approaches to combat any particular disease, the choice of appropriate target from total 13 known TLRs (11 in humans and 13 in mice) is

a major concern for immunologists. Natural ligands for all the TLRs have been identified except for TLR10. These ligands include lipopeptides (recognized by TLR2 in combination with TLR1 or TLR6), lipopolysaccharides (LPS) (TLR4), microbial protein components such as flagellin (TLR5), nucleic acids, for example, single-stranded RNA (TLR7 and TLR8) or double-stranded RNA (TLR3) and CpG DNA (TLR9).<sup>5</sup>

Among all the human TLRs, TLR2 is known to sense remarkable array of bacterial, fungal, and viral products as well as inflammatory self-segments.<sup>6</sup> TLR2 is receptor equipped for experiencing heterodimerization with TLR1 (for triacylated lipopeptides) and TLR6 (for diacylated lipopeptides).<sup>7</sup> Additionally, TLR2 has been widely explored as a promising target for drug discovery (Table 1). SPM-105, a TLR2 agonistic autoclaved mycobacteria was considered in preclinical examinations for the treatment of cancer<sup>8</sup> and OM-174, a lipid A derivative with double activity on TLR2 and TLR4, was tried as a chemotherapeutic agent for viability against unmanageable strong tumors.<sup>9</sup> Likewise, bacterial porins showing TLR2 agonistic activity have been utilized in vaccines. Specifically, OmpS1 and OmpS2, the external film proteins from *Salmonella typhi*, are known to actuate TLR2 and TLR4 and are used for immunization in typhoid fever.<sup>10</sup> Among the several TLR2 agonistic bio-macromolecules, the lipopeptides derived from cell-wall components are the most intense ligands.<sup>6</sup> PAM<sub>3</sub>CSK<sub>4</sub> (*N*-palmitoyl-*S*-[2,3-bis(palmitoyloxy)-(2*RS*)-propyl]-*R*-cysteinyl-*S*-seryl-*S*-lysyl-*S*-lysyl-*S*-lysyl-*S*-lysine) as well as PAM<sub>2</sub>CSK<sub>4</sub> (*S*-[2,3-bis(palmitoyloxy)-(2*RS*)-propyl]-*R*-cysteinyl-*S*-seryl-*S*-lysyl-*S*-lysyl-*S*-lysyl-*S*-lysine) are the greatly explored synthetic lipopeptides used against parasitic infections such as leishmania,<sup>11</sup> malaria,<sup>12</sup> influenza<sup>13</sup> and also worked well in suppression of asthma manifestations<sup>14</sup> and melanoma<sup>15</sup> (Table 1).

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† Electronic supplementary information (ESI) available: Activity profiling table for PAM<sub>2</sub>CSK<sub>4</sub> with references; <sup>1</sup>H NMR, <sup>13</sup>C NMR and MS spectra of intermediates and final product PAM<sub>2</sub>CSK<sub>4</sub>. See DOI: 10.1039/c8ra01387j



Table 1 TLR2 agonists as vaccine adjuvant and therapeutic agents in drug discovery

Compound	Target	Indication	Drug class
SMP-105	TLR2 agonist	Bladder cancer	Autoclaved mycobacteria <sup>8</sup>
OM-174	TLR2 & 4 agonist	Cancer chemotherapy	Lipid A derivative <sup>9</sup>
OmpS1 & OmpS2 ( <i>Salmonella typhi</i> )	TLR2 & 4 agonist	Typhoid fever vaccine	Bacterial porins <sup>10</sup>
PAM <sub>3</sub> CSK <sub>4</sub> /PAM <sub>2</sub> CSK <sub>4</sub>	TLR2 agonist	Leishmania infection	Lipoprotein <sup>11</sup>
PAM <sub>3</sub> Cys/PAM <sub>2</sub> Cys	TLR2 agonist	Malaria LbL vaccine	Lipoprotein conjugate <sup>12</sup>
PAM <sub>2</sub> CSK <sub>4</sub>	TLR2 agonist	Influenza vaccine	Lipoprotein <sup>13</sup>
PAM <sub>3</sub> Cys	TLR2 agonist	Suppression of asthma manifestations	Lipoprotein <sup>14</sup>
PAM <sub>3</sub> CSK <sub>4</sub>	TLR2 agonist	Melanoma cancer	Lipoprotein <sup>15</sup>

Several palmitoyl lipopeptides are being explored as TLR2 agonists. Out of the analogs PAM<sub>3</sub>Cys-Ser-Ser-Asn-Ala, PAM<sub>3</sub>-Cys-Ser-(Lys)<sub>4</sub>, PAM<sub>3</sub>Cys-Ala-Gly, and PAM<sub>3</sub>Cys-Ser, PAM<sub>3</sub>CSK<sub>4</sub> was found to be a potent activator due to the presence of hydrophilic lysine residues that enhanced its solubility.<sup>16</sup> On the other hand, the diacylated PAM<sub>2</sub>CSK<sub>4</sub> lipopeptide has better aqueous solubility as compared to triacylated PAM<sub>3</sub>CSK<sub>4</sub>. The vaccines with peptide epitopes and dipalmitoyl lipopeptide constructs are most soluble and effective vaccine candidates.<sup>17</sup> Moreover, dipalmitoyl lipopeptide is more potent immunostimulant than tripalmitoyl lipopeptide for macrophages and splenocytes.<sup>18</sup> It is noteworthy to mention that an extensive SAR investigation on PAM<sub>2</sub>CSK<sub>4</sub> resulted in highly active monoacyl lipopeptides but these molecules being human specific were not further explored in the preclinical investigation.<sup>19</sup>

After the complete review of literature, the diacylated PAM<sub>2</sub>CSK<sub>4</sub> lipopeptide was found to be the most potent dual (human and murine) TLR2 agonist having desirable aqueous solubility showing broad spectrum of cytokine/chemokine inducing property.<sup>20</sup> This synthetic lipopeptide is equipotent (EC<sub>50</sub> = 67 pm)<sup>21</sup> in its NF-κB inducing activity relative to LPS.<sup>22,23</sup> Induction of class I cytokine IL-6, class II cytokine IL-10, IFN-β, IFN-γ, and activation of TNF receptors is reported by this ligand. PAM<sub>2</sub>CSK<sub>4</sub> is also known to induce several chemokines namely, MCP-1, CXCL6, GRO, CXCL8, CCL20, CXCL10 and CCL5 (Table S1, page S3 ESI†).<sup>20–26</sup> PAM<sub>2</sub>CSK<sub>4</sub> is not only a potent TLR2 agonist but also possess property to self assemble at critical aggregation concentration (cac = 0.035 wt%). It self assemble into small spherical micelles distinct from worm-like micelles observed in case of PAM<sub>3</sub>CSK<sub>4</sub>.<sup>27</sup> The distinct mode of self assembly may be related to its bioactivity and could be a useful parameter for the formulation.

PAM<sub>2</sub>CSK<sub>4</sub> is highly expensive TLR2 ligand available from Invivogen<sup>28</sup> but its synthetic process is not yet described in the literature. These observations further drove our interest to optimize the large-scale synthesis of PAM<sub>2</sub>CSK<sub>4</sub> with minimum possible investment. The present report describes an efficient and scalable first complete synthesis of this TLR2 agonistic PAM<sub>2</sub>CSK<sub>4</sub>.

## Results and discussion

PAM<sub>2</sub>CSK<sub>4</sub>, a synthetic analog of MALP-2 consists of two major components, a lipo-dipeptide PAM<sub>2</sub>CS 3 and

a tetrapeptide of lysine K<sub>4</sub> 4 (Fig. 1). The retrosynthetic analysis shows that the constituents necessary for the synthesis are *R*-4-(iodomethyl)-2,2-dimethyl-1,3-dioxalane 5, Boc-Cys-OH 6 and H-Ser(O<sup>t</sup>Bu)-O<sup>t</sup>Bu 7. Based on the commercial availability and the cost of these synthons, a convergent synthetic approach involving synthesis of appropriately protected starting materials (Scheme 1), synthesis of key backbone skeleton (Scheme 2), synthesis of tetralysine fragment (Scheme 3) and final coupling to yield PAM<sub>2</sub>CSK<sub>4</sub> (Scheme 4) was proposed for this synthesis.

The key synthon *R*-4-(iodomethyl)-2,2-dimethyl-1,3-dioxalane 5 (Scheme 1) was synthesized from commercially available *S*-2,2-dimethyl-1,3-dioxolane-4-methanol 12 in good yield. The synthesis involved use of iodine (1.3 eq.), triphenylphosphine (1.2 eq.) and imidazole (3 eq.) reagent in toluene at 90 °C where imidazole acted as general base and an acid scavenger.<sup>29</sup> The excess amount of iodine was necessary to enhance the formation of an alkoxyphosphonium iodide salt which slowly decomposes by S<sub>N</sub>2 mechanism to form product 5.<sup>30</sup> A tedious column chromatography purification was also required to remove the triphenylphosphine oxide (TPPO).

The procedure was improved by replacing the solvent toluene with THF or DCM, where the reaction proceeded at room temperature and TPPO was removed by complexation using zinc chloride (ZnCl<sub>2</sub>) as described by Batesky and coworkers.<sup>31</sup> This protocol was applied to avoid column chromatography at large scale by precipitating out triphenylphosphine oxide as ZnCl<sub>2</sub>(Ph<sub>3</sub>PO)<sub>x</sub> complex.

The high price of H-Ser(O<sup>t</sup>Bu)-O<sup>t</sup>Bu (5 g, Rs. 13 133 from Bachem) 7 required on large scale was another key factor to be considered in this synthesis. Several methods have been reported for the synthesis of *tert*-butyl ethers and esters but the distinct and easy protocol was the protection of both hydroxyl and carboxylic acid functionalities in one pot.<sup>32</sup> The adopted procedure involved use of *tert*-butyl acetate for the protection of hydroxyl and carboxylic acid group of *L*-serine in the presence of 70% HClO<sub>4</sub> (Scheme 1) to yield pure oily product 7 without any need of column chromatography.<sup>33</sup>

A lipopeptide backbone skeleton 3 mainly responsible for the agonistic activity was synthesized in 8 steps (Scheme 2). *L*-Cystine was used as a precursor for the synthesis wherein, the initial step involved the protection of α-amine group of *L*-cystine 14 (Scheme 2) using di-*tert*-butyl dicarbonate (Boc<sub>2</sub>O) to obtain di-Boc-cystine 15.<sup>34</sup> After the completion of reaction,



10% HCl solution (15 mL for 2 g batch) was directly added to the reaction mixture at 0 °C and the crude product **15** was precipitated out and washed with chilled distilled water to remove impurities. No column chromatographic purification was required and the crude product **15** was used as it is for further transformations. The coupling reaction of (*S*)-*tert*-butyl-2-amino-3-*tert*-butoxypropanoate **7** and di-Boc-cystine **15** was carried out using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDCI) and 1-hydroxybenzotriazole (HOBT) in DMF. The column chromatography at this step was also avoided as the addition of crushed ice in the reaction mixture afforded crude product **16** (85% yield) which was found to be good enough to be used as it is. The reaction was carried out at

up to 10 g scale and only a small amount was column purified to record the analytical data.

Various reagents are reported in literature for the cleavage of disulfide bond.<sup>35</sup> Use of Bu<sub>3</sub>P reagent (50% solution in ethyl-acetate) was found to be more cost effective while working on large scale. The crucial point for the reaction was the addition of water (1 mL for 1 g batch). The reaction didn't proceed even after prolong reaction hours if enough quantity of water was not added into the reaction mixture. The solvent was evaporated under reduced pressure and the crude product **17** was used as it is for next step. The crude product **17** on reaction with synthon **5** in presence of K<sub>2</sub>CO<sub>3</sub> in DMF solvent afforded compound **18** in excellent yield (90%). It is noteworthy to mention that no

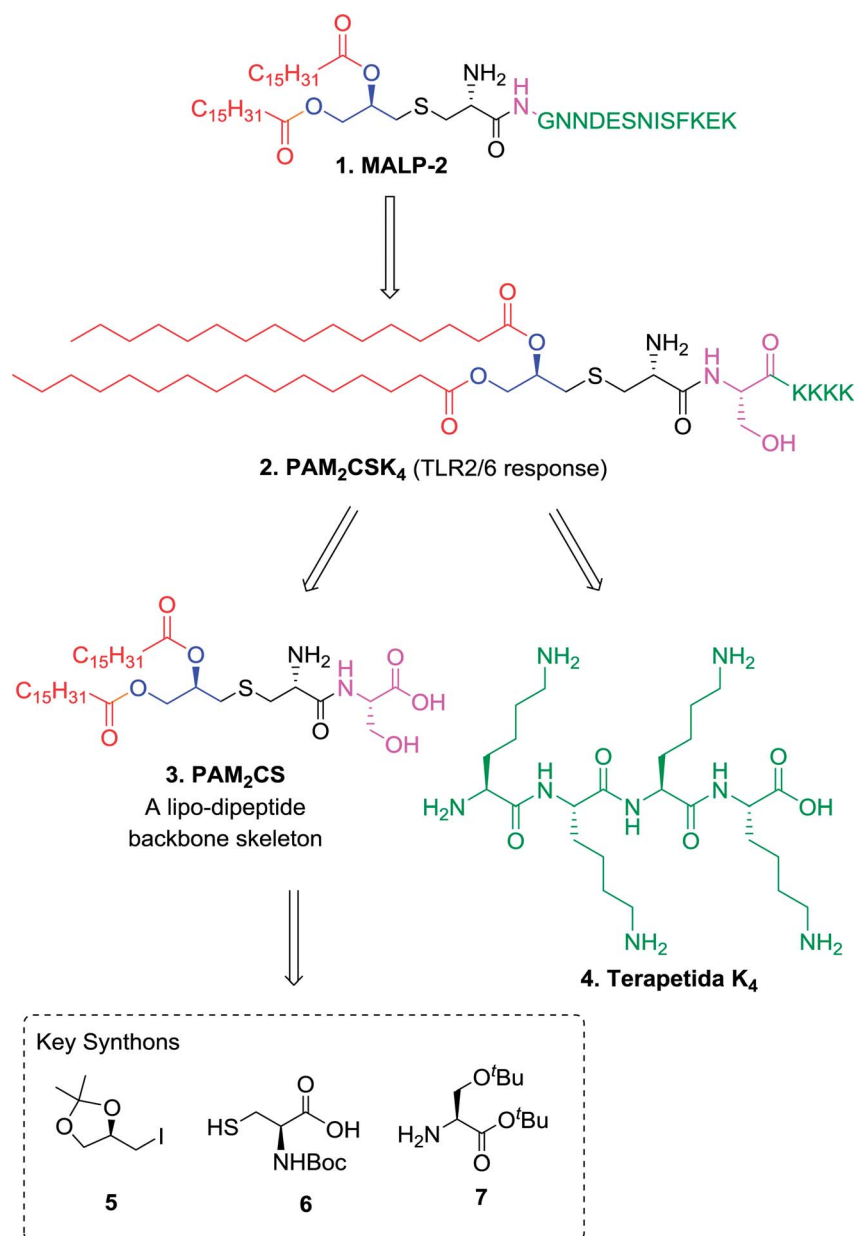
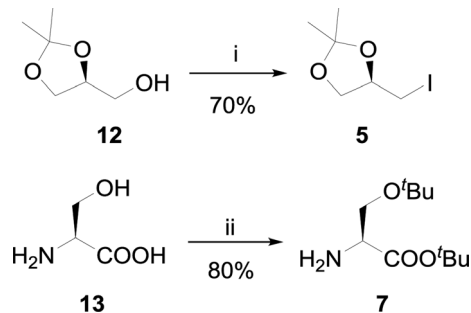


Fig. 1 Retrosynthetic approach for PAM<sub>2</sub>CSK<sub>4</sub>.





Scheme 1 Synthesis of reagents. Conditions: (i)  $\text{PPh}_3$ ,  $\text{I}_2$ , imidazole, THF, rt, 12 h. (ii) *tert*-Butyl acetate, 70%  $\text{HClO}_4$ , 6 h.

column chromatography was required for the purification of product **18** and the addition of crushed ice resulted in the formation of solid product in pure form.

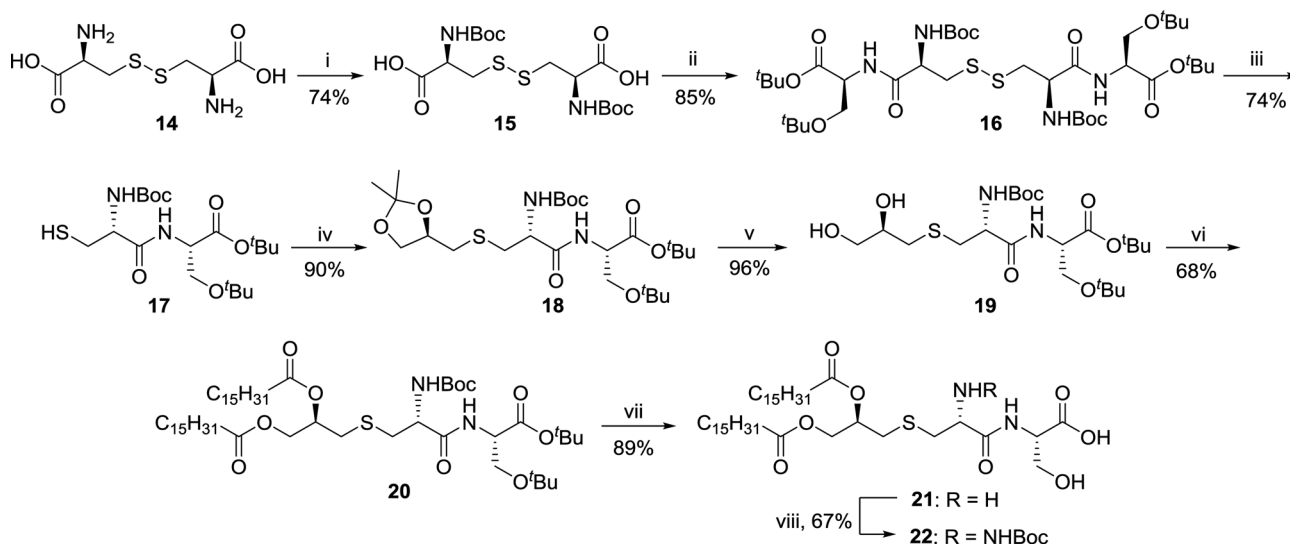
Compound **18** was further treated with 70% acetic acid and stirred at room temperature for 16 h to furnish compound **19** in quantitative yield (96%).<sup>36</sup> Compound **19** on *O*-palmitoylation using palmitoyl chloride and  $\text{Et}_3\text{N}$  in the presence of catalytic amount of 4-dimethylaminopyridine (DMAP) afforded compound **20** which was purified by column chromatography.<sup>21</sup> The global deprotection of Boc and *tert*-butyl ether functionalities was done by stirring the solution of compound **20** in TFA for 30 minutes at 25 °C. Evaporation of TFA in order to get pure product **21** was crucial and it was achieved by co-evaporation of the crude product with chloroform and dichloromethane till a white solid product was obtained which was confirmed by mass spectrometry. The solid TFA salt of amine was further treated with excess of di-*tert*-butyl dicarbonate and  $\text{Et}_3\text{N}$  to furnish compound **22** ready for final coupling with tetralysine residue.<sup>37</sup>

The tripeptide  $\text{H}[\text{Lys}(\text{Boc})]_4\text{-O}^t\text{Bu}$  **31** was synthesized in eight steps (Scheme 3) starting from commercially available

carboxylic acid precursor Fmoc-Lys(Boc)-OH **23**. The amine precursor of the appropriately protected lysine was synthesized in two steps as described by Pratesi *et al.*<sup>38</sup> The general method for protection of carboxylic acid as *tert*-butyl esters involved the use of coupling reagents (EDCI and HOBT). In the present approach, a mixed anhydride was prepared using di-*tert*-butyl dicarbonate which on reaction with *tert*-butanol resulted in the formation of product **24**.<sup>38</sup> This protocol was found to be clean and high yielding. Further, compound **25** was obtained by treating the DMF solution of Fmoc-[Lys(Boc)]-O<sup>t</sup>Bu **24** with 20% piperidine. The synthesis of  $\text{H}[\text{Lys}(\text{Boc})]_2\text{-O}^t\text{Bu}$  **27** was obtained by the coupling reaction of reagent **23** and  $\text{H}[\text{Lys}(\text{Boc})]\text{-O}^t\text{Bu}$  **25** followed by the deprotection of Fmoc group as described. The formation of product **27** was confirmed by ESI-MS and  $^1\text{H}$  NMR.<sup>39</sup>

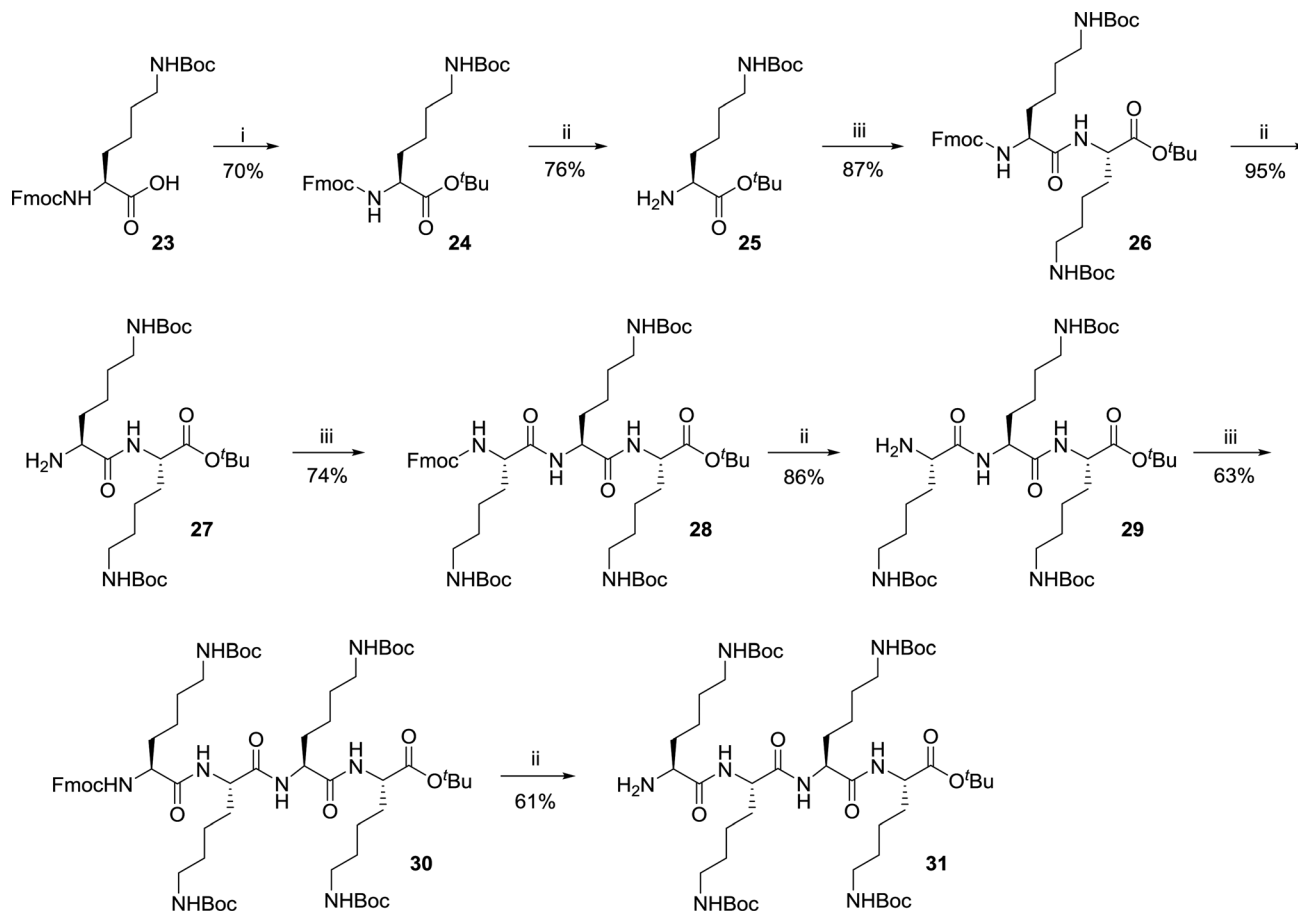
The formation of tripeptide  $\text{H}[\text{Lys}(\text{Boc})]_3\text{-O}^t\text{Bu}$  **29** was achieved as mentioned above by using coupling reagents in DMF and following deprotection of Fmoc group. These reactions were reproducible and scalable in grams (2 g) with 74% and 86% yields, respectively. Similarly, the tetrapeptide  $\text{H}[\text{Lys}(\text{Boc})]_4\text{-O}^t\text{Bu}$  **31** was synthesized through the coupling of starting precursor **23** with intermediate **29** and subsequent treatment with 20% piperidine with acceptable yields. The formation of tetrapeptide  $\text{H}[\text{Lys}(\text{Boc})]_4\text{-O}^t\text{Bu}$  **31** was confirmed through mass spectrometric analysis. Ionic liquid mediated Fmoc removal<sup>40</sup> as well as use of polymer-bound piperazine<sup>49</sup> were the probable alternatives to avoid the column chromatography to remove the side products during this deprotection step but the column chromatography was found to be cost effective as well as simple during this process optimization.

The penultimate coupling of compound **22** with  $\text{H}[\text{Lys}(\text{Boc})]_4\text{-O}^t\text{Bu}$  **31** (Scheme 4) using EDCI and HOBT coupling reagents in DMF resulted in the formation of an adduct **32** which on treatment with HCl in dioxane yielded the desired  $\text{PAM}_2\text{CSK}_4$  **2** in quantitative yield.

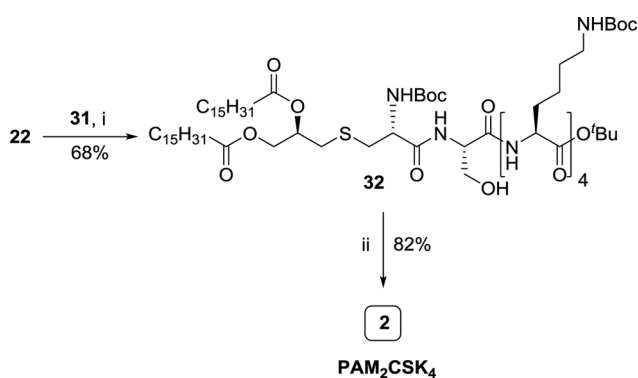


Scheme 2 Synthesis of  $\text{PAM}_2\text{CS}$ . Conditions: (i)  $\text{Boc}_2\text{O}$ ,  $\text{Et}_3\text{N}$ ,  $\text{H}_2\text{O}$ , rt, 12 h. (ii) *H*-Ser(O<sup>t</sup>Bu)O<sup>t</sup>Bu-HCl (**7**), EDCI, HOBT,  $\text{Et}_3\text{N}$ , DMF, rt, 5 h. (iii)  $\text{Bu}_3\text{P}$ , DCM, rt, 30 min. (iv) *R*-4-(Iodomethyl)-2,2-dimethyl-1,3-dioxalane (**5**),  $\text{K}_2\text{CO}_3$ , DMF, rt, 2 h. (v) 70% AcOH in  $\text{H}_2\text{O}$ , rt, 12 h. (vi)  $\text{C}_{15}\text{H}_{31}\text{COCl}$ ,  $\text{Et}_3\text{N}$ , DMAP, DCM, rt, 12 h. (vii) TFA, rt, 30 min. (viii)  $\text{Boc}_2\text{O}$ ,  $\text{Et}_3\text{N}$ , DCM, 4 h.





Scheme 3 Synthesis of tetrapeptide  $K_4$ . Conditions: (i) Di-*tert*-butyl dicarbonate, DMAP, *tert*-butyl alcohol, rt, overnight. (ii) 20% piperidine, DMF, rt, 1 h. (iii) Fmoc-L-Lys(Boc)-OH (23), EDCI, HOBT, Et<sub>3</sub>N, DMF, rt, 6 h.



Scheme 4 Synthesis of PAM<sub>2</sub>CSK<sub>4</sub>. Conditions: (i) H-[Lys(Boc)]<sub>4</sub>-O<sup>t</sup>Bu, EDCI, HOBT, Et<sub>3</sub>N, DMF, 6 h. (ii) HCl, dioxane, 2 h.

## Conclusions

Overall, with a systematic reaction optimization and appropriate synthetic planning we have demonstrated a twenty step first complete synthesis of PAM<sub>2</sub>CSK<sub>4</sub> which is scalable and will give an easy access to pharmacologically important TLR2 agonist capable of eliciting immune responses in both human and mice models. The complete procedure involved synthesis

of two moieties, PAM<sub>2</sub>CS 3 (10 steps) and  $K_4$  tetrapeptide 4 (8 steps) and the final coupling and global deprotection to furnish PAM<sub>2</sub>CSK<sub>4</sub> with 0.55% overall yield. For the cost effectiveness, the appropriately protected amino acids were synthesized by one step procedure from cheaply available cystine and serine. The present synthetic route will provide an easy access of PAM<sub>2</sub>CSK<sub>4</sub> to explore its usefulness in the preparation of novel adjuvant formulations.

## Experimental section

### General

All the reactions were performed with commercially available reagents. The organic solvents were dried by standard procedures wherever necessary. The bulk solvents (hexane, DCM) were distilled before use. All reactions were monitored by thin-layer chromatography (TLC) carried out on Merck silica gel F<sub>254</sub> aluminium sheets and visualized with different stains such as phosphomolybdic acid and ninhydrin solutions. All starting amino acid precursors were L enantiomers and purchased from Sigma aldrich. <sup>1</sup>H NMR was recorded on Bruker Advance II 400 or 500 MHz or Jeol FT NMR 300 MHz. Mass analysis was performed with Waters, Q-TOF-Micromass (ESI-MS) Spectrometer.



**(R)-4-(Iodomethyl)-2,2-dimethyl-1,3-dioxolane (5)**

To a solution of *S*-2,2-dimethyl-1,3-dioxolane-4-methanol **12** (1.0 g, 7.6 mmol) in DCM (10 mL) was added triphenylphosphine (2.4 g, 9.1 mmol), imidazole (1.5 g, 22.7 mmol), and iodine (2.5 g, 9.8 mmol). The reaction mixture was stirred at room temperature for 12 h. After the completion of reaction, DCM (100 mL) was added and the organic layer was washed with saturated sodium thiosulphate Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub> (100 mL × 2), brine (100 mL × 2), and dried over Na<sub>2</sub>SO<sub>4</sub> and filtered. The organic solvent was then removed under vacuum and the residue was purified by flash column chromatography (2% EtOAc/hexane) to obtain compound (1.1 g, 63%). <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ 1.34 (s, 3H), 1.45 (s, 3H), 3.14 (m, 1H), 3.25 (dd, *J* = 9.8 Hz, 4.7 Hz, 1H), 3.78 (dd, *J* = 8.5 Hz, 5.4 Hz, 1H), 4.14 (dd, *J* = 8.6 Hz, 5.9 Hz, 1H), 4.27 (m, 1H). The <sup>1</sup>H NMR was found to be consistent with that reported in the literature.<sup>28</sup> For large scale (10 g), column purification was avoided by addition of warm 1.8 M methanolic solution of ZnCl<sub>2</sub> (50 mL) into a DCM solution (10 mL) of the crude product which resulted in the formation of white shining crystals of ZnCl<sub>2</sub>(Ph<sub>3</sub>PO)<sub>x</sub><sup>31</sup> after keeping the reaction mixture at room temperature for overnight. The reaction mixture was filtered and concentrated to yield pure product which was used as it for further transformations.

***tert*-Butyl-*O*-(*tert*-butyl)-*L*-serinate (7)**

To a suspension of *L*-serine (5 g, 0.047 mol) in *tert*-butylacetate (121 mL, 0.36 mol), 70% HClO<sub>4</sub> (4.6 mL, 76 mol) was added slowly by dropping funnel at 0 °C. After stirring at room temperature for 6 h, 2 N dil HCl (50 mL) was added to reaction mixture and further, basified to pH 9 by the addition of K<sub>2</sub>CO<sub>3</sub> solution. The product was extracted using DCM (300 mL) and the organic layer was washed with, water (200 mL × 2), brine (200 mL × 2) and dried over Na<sub>2</sub>SO<sub>4</sub>, filtered and concentrated to afford (*S*)-*tert*-butyl 2-amino-3-*tert*-butoxypropanoate as colorless oil (8.26 g, 80%). <sup>1</sup>H NMR (CDCl<sub>3</sub>, 300 MHz) δ 3.58 (m, 2H), 3.44 (m, 1H), 1.74 (s, 3H), 1.47 (s, 9H), 1.16 (s, 9H). <sup>13</sup>C NMR (CDCl<sub>3</sub>, 100 MHz) δ 173.4, 80.9, 72.8, 63.9, 55.6, 28.0, 27.4. The <sup>1</sup>H NMR and <sup>13</sup>C NMR spectra were consistent with that reported in the literature.<sup>32</sup>

**(2*R*,2'*R*)-3,3'-Disulfanediybis(2-((*tert*-butoxycarbonyl)amino)propanoic acid) (15)**

Compound **15** was synthesized by a modified procedure as reported in the literature to avoid column chromatography on large scale. To a solution of *L*-cystine **14** (2 g, 8.32 mmol) in water (80 mL), triethylamine (3.5 mL, 24.97 mmol) was added followed by di-*tert*-butyl-dicarbonate (5.44 g, 24.97 mmol) and it was stirred at room temperature for 24 h. The reaction was quenched by adding 10% HCl solution (15 mL) at 0 °C. The product was precipitated out as a white solid which was filtered and washed with cold distilled water to obtain pure compound **15** (2.7 g, 74%). <sup>1</sup>H NMR (CDCl<sub>3</sub>, 300 MHz) δ 5.6 (m, 1H), 4.5 (m, *J* = 4.8 Hz, 2H), 3.24 (m, 4H), 1.45 (s, 18H). The <sup>1</sup>H NMR was consistent with that reported in the literature.<sup>33</sup>

**Di-*tert*-butyl-2,2'-(((2*R*,2'*R*)-3,3'-disulfanediybis(2-((*tert*-butoxycarbonyl)amino)propanoyl))bis(azanediy)))(2*S*,2'*S*)-bis(3-(*tert*-butoxy)propanoate) (16)**

To a solution of *Nα,Nα'*-di-Boc-cystine **15** (0.69 g, 1.58 mmol) in anhydrous DMF were added H-Ser(<sup>t</sup>Bu)-O<sup>t</sup>Bu·HCl **7** (0.84 g, 3.31 mmol), HOBT (0.513 g, 3.31 mmol), and Et<sub>3</sub>N (0.46 mL, 3.31 mmol). The reaction mixture was stirred at 0 °C for 30 min, followed by addition of EDCI (0.24 g, 1.58 mmol) at 0 °C. The reaction mixture was allowed to come to room temperature and stirred for 6 h. After completion of reaction, the solvent was evaporated under reduced pressure and residue obtained was dissolved in ethyl acetate (250 mL). It was washed with water (150 mL × 2), brine (150 mL × 1). The organic layer was dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>, filtered, and evaporated under reduced pressure to obtain crude product which was purified using column chromatography (50% EtOAc/hexane) to obtain compound **16** (1.12 g, 85%). <sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>) δ 7.35 (s, 2H), 5.69 (s, 2H), 4.71–4.50 (m, 4H), 3.77 (dd, *J* = 8.8, 3.4 Hz, 2H), 3.55 (dd, *J* = 8.8, 3.4 Hz, 2H), 3.18–3.06 (m, 4H), 1.46 (s, 36H), 1.14 (s, 9H). <sup>13</sup>C NMR (126 MHz, CDCl<sub>3</sub>) δ 170.03, 168.95, 155.39, 81.77, 80.16, 73.35, 62.08, 53.54, 43.22, 28.42, 28.02, 27.35. MS (ESI) calculated for [M + Na]<sup>+</sup> C<sub>38</sub>H<sub>70</sub>N<sub>4</sub>NaO<sub>12</sub>S<sub>2</sub><sup>+</sup> *m/z* 861.43, found 861.44. For large scale, compound **16** was used without column chromatography after precipitation as discussed in the result and discussion section.

***tert*-Butyl-*N*-(*N*-(*tert*-butoxycarbonyl)-*S*-(((*R*)-2,2-dimethyl-1,3-dioxolan-4-yl)methyl)-*L*-cysteinyl)-*O*-(*tert*-butyl)-*L*-serinate (18)**

To a solution of compound **16** (1 g, 1.29 mmol) in DCM (20 mL) and water (1 mL), tributylphosphine (50% solution in EtOAc, 964 μL, 3.86 mmol) was added and the reaction mixture was stirred at room temperature for 30 minutes. After the completion of the reaction, the solvent was removed under reduced pressure to obtain the crude product **17** (720 mg, 74%). The small amount of crude product **17** was column purified for the analysis. <sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>) δ 6.90 (d, *J* = 7.9 Hz, 1H), 5.50 (d, *J* = 6.8 Hz, 1H), 4.54 (dt, *J* = 8.0, 2.8 Hz, 1H), 4.37 (s, 1H), 3.79 (dd, *J* = 8.8, 2.9 Hz, 1H), 3.55 (dd, *J* = 8.8, 3.0 Hz, 1H), 3.08 (ddd, *J* = 13.9, 8.0, 4.2 Hz, 1H), 2.75 (ddd, *J* = 13.9, 10.2, 6.3 Hz, 1H), 1.77 (t, *J* = 8.8 Hz, 1H), 1.46 (s, 18H), 1.14 (s, 9H). <sup>13</sup>C NMR (126 MHz, CDCl<sub>3</sub>) δ 169.55, 168.95, 155.20, 82.11, 80.32, 73.22, 61.81, 55.57, 53.52, 28.30, 28.01, 27.47, 27.35. To the solution of crude compound **17** (700 mg, 1.67 mmol) in DMF (10 mL) were added K<sub>2</sub>CO<sub>3</sub> (346 mg, 2.51 mmol) and compound **5** (1.01 g, 4.17 mmol). The reaction mixture was stirred at room temperature for 2 h. After completion of the reaction, reaction mixture was poured onto crushed ice with stirring and product was extracted with DCM (250 mL), followed by washings with water (150 mL × 2), brine (150 mL × 2). The organic layer was finally concentrated to obtain crude product which was purified using column chromatography (25% EtOAc/hexane) to obtain compound **18** (800 mg, 90%). <sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>) δ 7.06 (d, *J* = 8.0 Hz, 1H), 5.50 (s, 1H), 4.54 (dt, *J* = 8.0, 2.9 Hz, 1H), 4.35–4.26 (m, 2H), 4.10 (dd,



$J = 8.3, 6.1$  Hz, 1H), 3.79 (dd,  $J = 8.8, 2.9$  Hz, 1H), 3.70 (dd,  $J = 8.3, 6.6$  Hz, 1H), 3.53 (dd,  $J = 8.8, 3.0$  Hz, 1H), 3.05 (dd,  $J = 13.9, 5.9$  Hz, 1H), 2.94 (dd,  $J = 13.9, 6.3$  Hz, 1H), 2.82 (dd,  $J = 13.5, 6.2$  Hz, 1H), 2.71 (dd,  $J = 13.5, 6.1$  Hz, 1H), 1.46 (d,  $J = 1.7$  Hz, 18H), 1.44 (s, 3H), 1.36 (s, 3H), 1.15 (s, 9H).  $^{13}\text{C}$  NMR (126 MHz,  $\text{CDCl}_3$ )  $\delta$  170.18, 168.91, 155.28, 109.67, 81.94, 80.17, 75.59, 73.13, 68.74, 61.99, 54.07, 53.49, 35.56, 35.32, 28.30, 28.01, 27.35, 26.87, 25.56. MS (ESI) calculated for  $[\text{M} + \text{Na}]^+ \text{C}_{25}\text{H}_{46}\text{N}_2\text{NaO}_8\text{S}^+$   $m/z$  557.28, found 558.26.

***tert*-Butyl-*N*-(*N*-(*tert*-butoxycarbonyl)-*S*-((*R*)-2,3-dihydroxypropyl)-*L*-cysteinyl)-*O*-(*tert*-butyl)-*L*-serinate (19)**

The compound **18** (2.9 g) was dissolved in 70% acetic acid (30 mL, AcOH : water = 7 : 3) and the reaction mixture was stirred at room temperature for 16 h. After completion of the reaction, the reaction mixture was diluted with EtOAc (350 mL) and washed with 10% sodium bicarbonate (200 mL  $\times$  2), water (200 mL  $\times$  2) and brine (200 mL) and organic layer was concentrated under reduced pressure to yield crude product which was purified using column chromatography (5% MeOH/DCM) to obtain compound **19** (2.5 g, 96%).  $^1\text{H}$  NMR (500 MHz,  $\text{CDCl}_3$ )  $\delta$  7.23 (d,  $J = 7.7$  Hz, 1H), 5.63 (d,  $J = 8.1$  Hz, 1H), 4.57 (dt,  $J = 8.1, 2.9$  Hz, 1H), 4.42 (d,  $J = 5.8$  Hz, 1H), 3.89–3.83 (m, 1H), 3.81–3.78 (m, 1H), 3.70 (dd,  $J = 11.3, 3.6$  Hz, 2H), 3.60 (dd,  $J = 11.3, 6.2$  Hz, 1H), 3.55 (dd,  $J = 8.9, 3.0$  Hz, 1H), 3.00 (dd,  $J = 14.1, 5.6$  Hz, 1H), 2.91 (dd,  $J = 14.1, 6.7$  Hz, 1H), 2.78 (m, 2H), 1.47 (s, 9H), 1.45 (s, 9H), 1.16 (s, 9H).  $^{13}\text{C}$  NMR (126 MHz,  $\text{CDCl}_3$ )  $\delta$  170.23, 169.20, 155.56, 82.26, 80.36, 73.34, 71.10, 65.23, 62.02, 53.49, 35.93, 28.30, 28.02, 27.34. MS (ESI) calculated for  $[\text{M} + \text{Na}]^+ \text{C}_{22}\text{H}_{37}\text{N}_2\text{NaO}_8\text{S}^+$   $m/z$  517.25, found 517.23. For large scale, compound **19** was used further without any column purification.

**((*R*)-3-((*R*)-2-(*tert*-Butoxycarbonylamino)-3-((*S*)-1,3-di-*tert*-butoxy-1-oxopropan-2-ylamino)-3-oxopropylthio)propane-1,2-diyl) dipalmitate (20)**

To a solution of compound **19** (650 mg, 1.31 mmol) in anhydrous DCM (15 mL) were added  $\text{Et}_3\text{N}$  (733  $\mu\text{L}$ , 5.26 mmol) and a catalytic amount of DMAP (20 mol%). The reaction mixture was stirred at 0  $^\circ\text{C}$  for 30 minutes. Palmitoyl chloride (1.2 mL, 3.94 mmol) was then added at 0  $^\circ\text{C}$ , followed by stirring at room temperature for 12 h. After completion of the reaction, the reaction mixture was thoroughly washed with water (250 mL  $\times$  2), saturated sodium bicarbonate solution (250 mL  $\times$  2), brine (200 mL  $\times$  1). The organic layer was dried over anhydrous  $\text{Na}_2\text{SO}_4$ , filtered and, evaporated under reduced pressure to obtain crude product which was purified using column chromatography (20% EtOAc/hexane) to obtain compound **20** (880 mg, 68%).  $^1\text{H}$  NMR (500 MHz,  $\text{CDCl}_3$ )  $\delta$  7.04 (d,  $J = 8.0$  Hz, 1H), 5.40 (d,  $J = 6.1$  Hz, 1H), 5.18 (dd,  $J = 5.7, 3.7$  Hz, 1H), 4.53 (dt,  $J = 8.1, 2.9$  Hz, 1H), 4.33 (dd,  $J = 11.9, 3.5$  Hz, 2H), 4.17 (dd,  $J = 11.9, 5.9$  Hz, 1H), 3.79 (dd,  $J = 8.8, 2.9$  Hz, 1H), 3.53 (dd,  $J = 8.8, 3.0$  Hz, 1H), 2.96 (d,  $J = 6.3$  Hz, 2H), 2.82 (d,  $J = 6.0$  Hz, 2H), 2.32 (ddd,  $J = 12.3, 6.7, 3.0$  Hz, 4H), 1.67–1.56 (m, 5H), 1.46 (d,  $J = 3.5$  Hz, 18H), 1.27 (d,  $J = 15.1$  Hz, 48H), 1.15 (s, 9H), 0.88 (t,  $J = 7.0$  Hz, 6H).  $^{13}\text{C}$  NMR

(126 MHz,  $\text{CDCl}_3$ )  $\delta$  173.50, 173.23, 170.19, 169.03, 155.36, 82.06, 80.28, 73.26, 70.45, 63.63, 62.09, 54.12, 53.64, 35.77, 34.43, 34.23, 33.28, 32.07, 29.85, 29.81, 29.66, 29.51, 29.46, 29.45, 29.29, 29.28, 28.43, 27.48, 25.04, 25.02, 22.84, 14.27. MS (ESI) calculated for  $[\text{M} + \text{Na}]^+ \text{C}_{54}\text{H}_{102}\text{N}_2\text{NaO}_{10}\text{S}^+$ ,  $m/z$  993.71, found 993.19.

***S*-((*R*)-2,3-Bis(palmitoyloxy)propyl)-*N*-(*tert*-butoxycarbonyl)-*L*-cysteinyl-*L*-serine (22)**

The compound **20** (500 mg, 0.53 mmol) was stirred with trifluoroacetic acid (TFA, 8 mL) for 30 minutes at 25  $^\circ\text{C}$ , followed by purging nitrogen gas. After that remaining TFA was co-evaporated with  $\text{CHCl}_3$  (5 mL  $\times$  3) and DCM (5 mL  $\times$  2) in order to get solid crude compound which was purified by column chromatography (10% MeOH/DCM) to get compound **21** (350 mg, 89%). MS (ESI) calculated for  $[\text{M} + \text{H}]^+ \text{C}_{41}\text{H}_{78}\text{N}_2\text{O}_8\text{S}$   $m/z$  758.54, found 758.28. To a solution of compound **21** (286 mg, 0.32 mmol) in DCM, di-*tert*-butyl dicarbonate (246 mg, 1.31 mmol)  $\text{Et}_3\text{N}$  (133  $\mu\text{L}$ , 0.92 mmol) were added and the reaction mixture was stirred for 4 h. The reaction mixture was diluted with DCM (300 mL) and organic layer was sequentially washed with water (250 mL  $\times$  2), saturated sodium bicarbonate solution (250 mL  $\times$  2), brine (200 mL  $\times$  1). The organic layer was dried over anhydrous sodium sulfate, filtered and evaporated under reduced pressure to obtain the crude product which was purified using column chromatography (5% MeOH/DCM) to afford compound **22** (188 mg, 67%).  $^1\text{H}$  NMR (500 MHz,  $\text{CDCl}_3$ )  $\delta$  12.17–11.77 (m, 1H), 7.59–7.48 (m, 1H), 5.67–5.54 (m, 1H), 5.20–5.13 (m, 1H), 4.63–4.53 (m, 1H), 4.44–4.36 (m, 1H), 4.32 (s, 1H), 4.14 (s, 1H), 4.09–4.01 (m, 1H), 3.96–3.82 (m, 1H), 3.10 (s, 1H), 3.06–2.88 (m, 2H), 2.76 (s, 2H), 2.29 (d,  $J = 7.4$  Hz, 4H), 1.65–1.55 (m, 4H), 1.43 (s, 9H), 1.21 (s, 48H), 0.86 (s, 6H).  $^{13}\text{C}$  NMR (126 MHz,  $\text{CDCl}_3$ )  $\delta$  173.89, 173.74, 171.04, 156.02, 80.96, 77.41, 77.16, 76.91, 70.53, 63.84, 62.82, 55.31, 54.04, 45.82, 35.16, 34.50, 34.25, 32.91, 32.08, 29.87, 29.83, 29.70, 29.68, 29.53, 29.48, 29.32, 29.29, 28.42, 25.06, 25.02, 22.85, 14.28, 8.69. MS (ESI) calculated for  $[\text{M} + \text{Na}]^+ \text{C}_{46}\text{H}_{86}\text{N}_2\text{NaO}_{10}\text{S}^+$   $m/z$  881.58, found 881.58.

***tert*-Butyl-*N*^2-(((9*H*-fluoren-9-yl)methoxy)carbonyl)-*N*^6-(*tert*-butoxycarbonyl)-*L*-lysinate (Fmoc-Lys(Boc)-*O*^tBu) (24)**

The compound **24** was synthesized using precursor Fmoc-Lys(Boc)-OH **23** (5.0 g, 10.7 mmol) using a reported protocol.<sup>38</sup> Yield (3.91 g, 70%).  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ )  $\delta$  7.76 (d,  $J = 7.5$  Hz, 2H), 7.60 (d,  $J = 7.3$  Hz, 2H), 7.40 (t,  $J = 7.5$  Hz, 2H), 7.31 (t,  $J = 7.5$  Hz, 2H), 5.36 (d,  $J = 8$  Hz, 1H), 4.37–4.42 (m, 2H), 4.20–4.27 (m, 2H), 3.10 (t,  $J = 6.6$  Hz, 2H), 1.77–1.86 (m, 4H), 1.47 (s, 9H), 1.43 (s, 11H).

***tert*-Butyl-*N*^6-(*tert*-butoxycarbonyl)-*L*-lysinate (H-Lys(Boc)-*O*^tBu) (25)**

The compound **25** (4.0 g, 7.62 mmol) was synthesized as reported in the literature<sup>38</sup> and confirmed by mass spectrometric analysis. Yield (1.75 g, 76%). MS (ESI) calculated for  $[\text{M} + \text{H}]^+ \text{C}_{15}\text{H}_{31}\text{N}_2\text{O}_4^+$   $m/z$  303.22, found 303.53.



***tert*-Butyl-*N*<sup>2</sup>-(*N*<sup>2</sup>-(((9*H*-fluoren-9-yl)methoxy)carbonyl)-*N*<sup>6</sup>-(*tert*-butoxycarbonyl)-*L*-lysyl)-*N*<sup>6</sup>-(*tert*-butoxycarbonyl)-*L*-lysinate (Fmoc-Lys(Boc)-Lys(Boc)-O<sup>t</sup>Bu) (26)**

The compound **26** (1.6 g, 5.2 mmol) was obtained using similar procedure as reported and by using pyridine instead of *N,N*-diisopropylethylamine (DIPEA).<sup>39</sup> Yield (3.08 g, 87%). <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ 7.75 (d, *J* = 7.5 Hz, 2H), 7.59 (d, *J* = 7.4 Hz, 2H), 7.39 (t, *J* = 7.4 Hz, 2H), 7.30 (m, 2H), 6.57 (bs, 1H), 5.59 (bs, 1H), 4.67 (bs, 2H), 4.37–4.46 (m, 3H), 4.11–4.23 (m, 2H), 3.05–3.11 (m, 4H), 1.83 (m, 2H), 1.71 (m, 4H), 1.43 (m, 32H), 1.25 (m, 2H), 1.19–1.21 (m, 2H).

***tert*-Butyl-*N*<sup>6</sup>-(*tert*-butoxycarbonyl)-*N*<sup>2</sup>-(*N*<sup>6</sup>-(*tert*-butoxycarbonyl)-*L*-lysyl)-*L*-lysinate (H-Lys(Boc)-Lys(Boc)-O<sup>t</sup>Bu) (27)**

The deprotection of Fmoc-Lys(Boc)-Lys(Boc)-O<sup>t</sup>Bu **26** (500 mg, 0.66 mmol) was carried out as described for compound **24** to afford compound **27**.<sup>39</sup> Yield (334.8 mg, 95%). <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ 7.71 (d, *J* = 7.0 Hz, 1H), 4.69 (bs, 2H), 4.42–4.47 (m, 1H), 3.37–3.40 (m, 1H), 3.09–3.12 (m, 4H), 1.80 (m, 4H), 1.45–1.75 (m, 8H), 1.45 (s, 9H), 1.42 (s, 18H). MS (ESI) calculated for [M + H]<sup>+</sup> C<sub>26</sub>H<sub>51</sub>N<sub>4</sub>O<sub>7</sub> *m/z* 531.37, found 531.40.

***tert*-Butyl-*N*<sup>2</sup>-(*N*<sup>2</sup>-(*N*<sup>2</sup>-(((9*H*-fluoren-9-yl)methoxy)carbonyl)-*N*<sup>6</sup>-(*tert*-butoxycarbonyl)-*L*-lysyl)-*N*<sup>6</sup>-(*tert*-butoxycarbonyl)-*L*-lysyl)-*N*<sup>6</sup>-(*tert*-butoxycarbonyl)-*L*-lysinate (Fmoc-Lys(Boc)-Lys(Boc)-Lys(Boc)-O<sup>t</sup>Bu) (28)**

H-Lys(Boc)-Lys(Boc)-O<sup>t</sup>Bu **27** (400 mg, 0.75 mmol) and Fmoc-Lys-OH **23** (317.8 mg, 0.678 mmol) were dissolved in 5 mL of DMF at 0 °C at inert atmosphere. HOBt (51 mg, 0.376 mmol) and pyridine (0.12 mL, 1.5 mmol) were further added to the reaction mixture at 0 °C. After 10 minutes stirring, EDCI (216.5 mg, 1.13 mmol) was added to the reaction mixture and reaction was allowed to warm to room temperature and stirred for 6 h. The completion of the reaction was monitored by TLC. The reaction mixture was dissolved in water (100 mL) and EtOAc (200 mL) and the organic phase was washed successively with 5% citric acid (50 mL × 2), NaHCO<sub>3</sub> (50 mL × 2) and brine (50 mL × 2). The crude product obtained was purified by column chromatography (2% MeOH/DCM) to obtain compound **28** (547 mg, 74%). <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ 7.74–7.76 (d, *J* = 7.5 Hz, 2H), 7.59–7.60 (d, *J* = 7.0 Hz, 2H), 7.38 (t, *J* = 7.4 Hz, 2H), 7.30 (t, *J* = 7.2 Hz, 2H), 6.94 (m, 1H), 6.83 (m, 1H), 5.81 (m, 1H), 4.93 (m, 1H), 4.83 (m, 1H), 4.39 (m, 3H), 4.20 (t, *J* = 7.0 Hz, 2H), 3.05 (m, 5H), 2.06 (m, 2H), 1.84 (m, 3H), 1.65 (m, 3H), 1.42 (m, 44H). <sup>13</sup>C NMR δ 171.23, 156.31, 156.19, 141.26, 127.72, 127.11, 125.14, 119.96, 82.10, 79.10, 77.25, 67.17, 54.85, 52.68, 47.10, 40.11, 39.94, 39.78, 32.06, 31.76, 29.48, 28.47, 28.01, 22.53, 22.32. MS (ESI) calculated for [M + Na]<sup>+</sup> C<sub>52</sub>H<sub>80</sub>N<sub>6</sub>NaO<sub>12</sub> *m/z* 1003.57, found 1003.54.

***tert*-Butyl-*N*<sup>6</sup>-(*tert*-butoxycarbonyl)-*N*<sup>2</sup>-(*N*<sup>6</sup>-(*tert*-butoxycarbonyl)-*L*-lysyl)-*L*-lysyl)-*L*-lysinate (H-Lys(Boc)-Lys(Boc)-Lys(Boc)-O<sup>t</sup>Bu) (29)**

Fmoc-Lys(Boc)-Lys(Boc)-Lys(Boc)-O<sup>t</sup>Bu **28** (450 mg, 0.458 mmol) was dissolved in a solution of 20% of piperidine in DMF (3 mL) and stirred for 1 h. After completion of reaction, the solvent was

evaporated and the crude product obtained was purified by column chromatography (10% MeOH/DCM) to obtain compound **29** (300 mg, 86%). MS (ESI) calculated for [M + H]<sup>+</sup> C<sub>37</sub>H<sub>71</sub>N<sub>6</sub>O<sub>10</sub> *m/z* 759.52, found 759.24.

***tert*-Butyl-(2*R*,5*S*)-5-((2*R*,5*S*)-5-(((9*H*-fluoren-9-yl)methoxy)carbonyl)amino)-9-((*tert*-butoxycarbonyl)amino)-2-(4-((*tert*-butoxycarbonyl)amino)butyl)-4-oxononanamido)-9-((*tert*-butoxycarbonyl)amino)-2-(4-((*tert*-butoxycarbonyl)amino)butyl)-4-oxononanoate (Fmoc-Lys(Boc)-Lys(Boc)-Lys(Boc)-Lys(Boc)-O<sup>t</sup>Bu) (30)**

NH<sub>2</sub>-Lys(Boc)-Lys(Boc)-Lys(Boc)-O<sup>t</sup>Bu **29** (300 mg, 0.40 mmol) and Fmoc-Lys-OH **23** (166.7 mg, 0.36 mmol) were dissolved in 10 mL of DMF at 0 °C under nitrogen atmosphere. HOBt (26.7 mg, 0.197 mmol) and pyridine (63.7 μL, 0.79 mmol) were further added to the reaction mixture under cooling condition. After 10 minutes stirring, EDCI (113.6 mg, 0.59 mmol) was added to the reaction mixture and reaction was allowed to warm to room temperature and stirred for 6 h. The completion of the reaction was monitored by TLC. The reaction mixture was dissolved in water (150 mL) and EtOAc (200 mL) and the organic phase was washed successively with 5% citric acid (50 mL × 2), NaHCO<sub>3</sub> (50 mL × 2) and brine (50 mL × 2). The crude product obtained was purified by column chromatography on 60–120 mesh silica gel in 4% MeOH/DCM to obtain compound **30** (301 mg, 63%). <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ 9.08 (bs, 1H), 8.54 (bs, 1H), 7.75 (d, *J* = 7.5 Hz, 2H), 7.59–7.61 (d, *J* = 6.4 Hz, 2H), 7.38 (t, *J* = 7.4 Hz, 2H), 7.29 (m, 2H), 6.98 (m, 1H), 6.76 (m, 1H), 5.93–6.08 (m, 2H), 5.37 (m, 1H), 4.96 (m, 1H), 4.82–4.86 (m, 1H), 4.32–4.56 (m, 6H), 4.19–4.22 (m, 2H), 2.88–3.07 (m, 9H), 1.85 (m, 6H), 1.59–1.71 (m, 7H), 1.43 (m, 67H). <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>) δ 171.34, 156.44, 141.39, 127.78, 127.19, 125.37, 120.0, 60.55, 47.32, 28.62, 28.13, 22.83, 21.21, 14.34. MS (ESI) calculated for [M + Na]<sup>+</sup> C<sub>63</sub>H<sub>100</sub>N<sub>8</sub>NaO<sub>15</sub> *m/z* 1231.72, found 1231.63.

***tert*-Butyl-(2*R*,5*S*)-5-((2*R*,5*S*)-5-amino-9-((*tert*-butoxycarbonyl)amino)-2-(4-((*tert*-butoxycarbonyl)amino)butyl)-4-oxononanamido)-9-((*tert*-butoxycarbonyl)amino)-2-(4-((*tert*-butoxycarbonyl)amino)butyl)-4-oxononanoate (H-Lys(Boc)-Lys(Boc)-Lys(Boc)-O<sup>t</sup>Bu) (31)**

Fmoc-Lys(Boc)-Lys(Boc)-Lys(Boc)-Lys(Boc)-O<sup>t</sup>Bu **30** (300 mg, 0.248 mmol) was dissolved in a solution of 20% of piperidine in DMF (2 mL) and stirred for 1 h. The solvent was evaporated and the crude product obtained was purified by column chromatography on 60–120 mesh silica gel in 15% MeOH/DCM to obtain compound **31** (150 mg, 61%). MS (ESI) calculated for [M + H]<sup>+</sup> C<sub>48</sub>H<sub>91</sub>N<sub>8</sub>O<sub>13</sub> *m/z* 987.67, found 987.65.

***S*-(2,3-Bis(palmitoyloxy)propyl)-cysteinyl-seryl-lysyl-lysyl-lysyl-lysine (2)**

To a solution of compound **22** (100 mg, 0.12 mmol) in anhydrous DMF were added H-[Lys(Boc)]<sub>4</sub>-O<sup>t</sup>Bu **31** (126 mg, 0.13 mmol), HOBt (16 mg, 0.12 mmol), and Et<sub>3</sub>N (34 μL, 0.24 mmol). The reaction mixture was stirred at 0 °C for 30 min, followed by addition of EDCI (69.0 mg, 0.36 mmol) at 0 °C. The reaction



mixture was further stirred at room temperature for 6 h, followed by evaporation of the solvent under reduced pressure. The residue was then dissolved in ethyl acetate and washed with 5% citric acid (100 mL × 2), NaHCO<sub>3</sub> (100 mL × 2) and brine (100 mL × 2). The organic solvent was dried over anhydrous sodium sulfate, filtered, and evaporated under reduced pressure to obtain the crude product which was purified using column chromatography (50% EtOAc/hexane) to obtain compound 32 (145 mg, 68%). The compound 32 (50 mg) was treated with HCl in dioxane solution and stirred for 2 h to obtain compound 2 (28.5 mg, 82%). <sup>1</sup>H NMR (500 MHz, MeOD) δ 5.30 (ddd, *J* = 8.0, 6.5, 2.8 Hz, 2H), 4.57 (t, *J* = 5.7 Hz, 2H), 4.47 (dd, *J* = 12.0, 2.7 Hz, 2H), 4.37 (dddd, *J* = 19.6, 14.4, 9.3, 5.0 Hz, 8H), 4.24 (dd, *J* = 9.0, 5.1 Hz, 2H), 4.17 (dd, *J* = 12.0, 6.7 Hz, 2H), 3.96 (dd, *J* = 11.0, 5.4 Hz, 2H), 3.84 (dd, *J* = 11.0, 6.0 Hz, 2H), 3.75–3.73 (m, 1H), 3.69–3.65 (m, 2H), 3.60–3.57 (m, 1H), 3.28 (d, *J* = 5.1 Hz, 2H), 2.99 (dt, *J* = 13.8, 5.8 Hz, 18H), 2.89 (dd, *J* = 14.6, 9.0 Hz, 2H), 2.82 (dd, *J* = 14.3, 8.3 Hz, 2H), 2.35 (dt, *J* = 13.3, 7.4 Hz, 8H), 1.98–1.84 (m, 9H), 1.83–1.66 (m, 24H), 1.66–1.44 (m, 25H), 1.39–1.15 (m, 91H), 0.90 (t, *J* = 7.0 Hz, 12H). <sup>13</sup>C NMR (126 MHz, MeOD) δ 175.02, 174.98, 174.91, 174.21, 174.12, 174.06, 172.33, 168.94, 73.57, 72.45, 71.33, 65.23, 63.19, 62.17, 56.77, 55.07, 54.68, 54.52, 53.41, 53.30, 40.60, 40.58, 40.52, 40.47, 35.23, 34.99, 34.00, 33.11, 33.00, 32.29, 32.17, 31.78, 30.85, 30.81, 30.75, 30.72, 30.55, 30.52, 30.27, 30.26, 27.99, 27.90, 27.85, 26.14, 26.07, 23.89, 23.86, 23.76, 23.64, 14.47. MS (ESI) calculated for [M + H]<sup>+</sup> C<sub>65</sub>H<sub>127</sub>N<sub>10</sub>O<sub>12</sub>S<sup>+</sup> *m/z* 1271.93, found 1271.92.

## Conflicts of interest

There are no conflicts to declare.

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## References

- 1 T. H. Mogensen, *Clin. Microbiol. Rev.*, 2009, **22**, 240.
- 2 T. Kawasaki and T. Kawai, *Front. Immunol.*, 2014, **5**, 1.
- 3 D. Werling and T. W. Jungi, *Vet. Immunol. Immunopathol.*, 2003, **91**, 1.
- 4 A. G. Dalgleish, *Hum. Vaccines Immunother.*, 2014, **10**, 3369.
- 5 T. Kawai and S. Akira, *Nat. Immunol.*, 2010, **11**, 373.
- 6 A. O. Aliprantis, R.-B. Yang, M. R. Mark, S. Suggett, B. Devaux, J. D. Redolf, G. R. Klimpel, P. Godowski and A. Zychlinsky, *Science*, 1999, **285**, 736.
- 7 (a) M. S. Jin, S. E. Kim, J. Y. Heo, M. E. Lee, H. M. Kim, S. G. Paik, H. Lee and J. O. Lee, *Cell*, 2007, **130**, 1071; (b) J. Y. Kang, X. Nan, M. S. Jin, S. J. Youn, Y. H. Ryu, S. Mah, S. H. Han, H. Lee, S. G. Paik and J. O. Lee, *Immunity*, 2009, **31**, 873.
- 8 M. Murata, *Cancer Sci.*, 2008, **99**, 1435.
- 9 N. Isambert, P. Fumoleau, C. Paul, C. Ferrand, S. Zanetta, J. Bauer, K. Ragot, G. Lizard, J. Jeanin and M. Bardou, *BMC Cancer*, 2013, **13**, 172.
- 10 M. A. Moreno-Eutimio, A. Tenorio-Calvo, R. Pastelin-Palacios, C. Perez-Shibayama, C. Gil-Cruz, R. Lopez-Santiago, I. Baeza, M. Fernandez-Mora, L. Bonifaz, A. Isibasi, E. Calva and C. Lopez-Macias, *Immunology*, 2013, **139**, 459.
- 11 A. Halliday, J. D. Turner, A. Guimarães, P. A. Bates and M. J. Taylor, *Parasites Vectors*, 2016, **9**, 96.
- 12 T. J. Powell, WO Pat., 2013148426A1, 2013.
- 13 A. Maruyama, H. Shime, Y. Takeda, M. Azuma, M. Matsumoto and T. Seya, *Biochem. Biophys. Res. Commun.*, 2015, **457**, 445.
- 14 M. C. Nawijn, A. C. Motta, R. Gras, S. Shirinbak, H. Maazi and A. J. M. Oosterhout, *PLoS One*, 2013, **8**, e55307.
- 15 J. R. Slingluff, L. Craig and I. S. Mauldin, WO Pat., 201507003A1, 2015.
- 16 U. Buwitt-Beckmann, H. Heine, K. H. Wiesmuller, G. Jung, R. Brock and A. J. Ulmer, *FEBS J.*, 2005, **272**, 6354.
- 17 W. Zeng, S. Ghosh, Y. F. Lau, L. E. Brown and D. C. Jackson, *J. Immunol.*, 2002, **169**, 4905.
- 18 (a) J. W. Metzger, A. G. Beck-Sickinger, M. Loleit, M. Eckert, W. G. Bessler and G. J. Jung, *Pept. Sci.*, 1995, **3**, 184; (b) P. F. Muhlrardt, M. Kiess, H. Meyer, R. Sussmuth and G. Jung, *J. Exp. Med.*, 1997, **185**, 1951; (c) P. F. Muhlrardt, M. Kiess, H. Meyer, R. Sussmuth and G. Jung, *Infect. Immun.*, 1998, **66**, 4804.
- 19 D. B. Salunke, S. W. Connelly, N. M. Shukla, A. R. Hermanson, L. M. Fox and S. A. David, *J. Med. Chem.*, 2013, **56**, 5885.
- 20 D. B. Salunke, X. Guo and S. A. David, *US Pat.*, 20150337009, 2015.
- 21 W. Wu, R. Li, S. S. Malladi, H. J. Warshakoon, M. R. Kimbrell, M. W. Amolins, R. Ukani, A. Datta and S. A. David, *J. Med. Chem.*, 2010, **53**, 3198.
- 22 M. R. Kimbrell, H. Warshakoon, J. R. Cromer, S. Malladi, J. D. Hood, R. Balakrishna, T. A. Scholdberg and S. A. David, *Immunol. Lett.*, 2008, **118**, 132.
- 23 J. D. Hood, H. J. Warshakoon, M. R. Kimbrell, N. M. Shukla, S. S. Malladi, X. Wang and S. A. David, *Hum. Vaccines*, 2010, **6**, 322.
- 24 M. Azuma, R. Sawahata, Y. Akao, E. Takashi, S. Yamazaki, M. Matsumoto, M. Hashimoto, K. Fukase, Y. Fujimoto and S. Teya, *PLoS One*, 2010, **5**, e12550.
- 25 J. C. Farges, F. Carrouela, J.-F. Keller, C. Baudouin, P. Msika, F. Bleicher and M.-J. Staquet, *Immunobiology*, 2011, **216**, 513.
- 26 A. C. D. Salyer, G. Caruso, K. K. Khetani, L. M. Fox, S. S. Malladi and S. A. David, *PLoS One*, 2016, **11**, e0149848.
- 27 L. W. Hamley, S. Kirkham, A. Dehsorkhi, V. Castelletto, M. Reza and J. Ruokolainen, *Chem. Commun.*, 2014, **50**, 15948.
- 28 <http://www.invivogen.com/pam2csk4>.



- 29 N. Martin and E. J. Thomas, *Org. Biomol. Chem.*, 2012, **10**, 7952.
- 30 P. J. Garegg, T. Regberg, J. Stawinski and R. Strornberg, *J. Chem. Soc., Perkin Trans. 2*, 1987, **11**, 271.
- 31 D. C. Batesky, M. J. Goldfogel and D. J. Weix, *J. Org. Chem.*, 2017, **82**, 9931.
- 32 L. Sambri, G. Bartoli, G. Bencivenni and R. Dalpozzo, *Curr. Org. Synth.*, 2012, **9**, 137.
- 33 P. Plastina, A. Fazio, M. Attya, G. Sindona and B. Gabriele, *Nat. Prod. Res.*, 2012, **26**, 1799.
- 34 D. B. Salunke, N. M. Sukhla, Y. Euna, B. M. Crall, R. Balakrishna, S. S. Malladi and S. A. David, *J. Med. Chem.*, 2012, **55**, 3353.
- 35 (a) Y. Hao, M. Zhang, J. He and P. Ni, *Langmuir*, 2012, **28**, 6448; (b) S. H. Lee and H. Kohn, *Heterocycles*, 2003, **60**, 47; (c) R. D. Besten, S. Hardeer and L. J. Brandsma, *Organomet. Chem.*, 1990, **385**, 153.
- 36 G. Agnihotri, B. M. Crall, T. C. Lewis, T. P. Day, R. Balakrishna, H. J. Warshakoon, S. S. Malladi and S. A. David, *J. Med. Chem.*, 2011, **54**, 8148.
- 37 S. A. David and N. M. Shukla, *US Pat.*, 201202944885, 2012.
- 38 (a) A. Pratesi, M. Ginanneschi, F. Melani, M. Chinol, A. Carollo, G. Paganelli, M. Lumini, M. Bartoli, M. Frediani, L. Rosi, G. Petrucci, L. Messori and A. M. Papini, *Org. Biomol. Chem.*, 2015, **13**, 3988; (b) S. Banerjee, W. J. Wiggins, J. L. Geoghegan, C. T. Anthony, E. A. Woltering and D. S. Masterson, *Org. Biomol. Chem.*, 2013, **11**, 6307; (c) C. Kim, T. Young, Y. Cao, J. Ma, M. Kim, S. Pinkerton and P. G. Schultz, *WO Pat.*, 2015057852, 2015.
- 39 J. T. Herpt, M. C. A. Stuart, W. R. Browne and B. L. Feringa, *Chem. - Eur. J.*, 2014, **20**, 3077.
- 40 M. L. Di Gioia, P. Costanzo, A. De Nino, L. Maiuolo, M. Nardi, F. Olivitod and A. Procopiod, *RSC Adv.*, 2017, **7**, 36482.

