Synthesis of cyclic peptides as bioconjugation platforms

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Design and synthesis of gramicidin S derivatives bearing chemoselective handles in different orientations

For a vaccine to elicit an effective immune response, an antigen alone is not sufficient. Immunostimulatory compounds or adjuvants improve the immunogenicity of vaccines and such agents have been added in most formulations either through accidental means owing to the material used or deliberately.[1,2] The first hint for adjuvanticity occurred in 1926 when Glenny *et al.* reported that an antigen that was precipitated onto insoluble particles of aluminum potassium sulphate before immunization induced better antibody responses in guinea pigs than the soluble antigen alone.[3] Since this discovery aluminum salts have been used

in clinical vaccines worldwide as the principle adjuvants.[4] The research on adjuvants took another step forward starting from 1989 when Janeway hypothesized the existence of pattern recognition receptors (PRRs) that would recognize certain pathogen-associated molecular patterns (PAMPs) not found in the host.[5] This hypothesis was validated in the 1990s with the discovery of Toll-like receptors (TLRs), which upon pathogen detection induce the production of cytokines and type I interferons in the host.[6]

Extensive research has been conducted on Toll-like receptors, which is the first discovered family of PRRs with ten receptors being identified in humans (TLR1-TLR10). TLRs are transmembrane proteins either located on the plasma membrane or the endosome of dendritic cells, macrophages, lung epithelial cells and B cells (*Table 1*).[7] The extracellular domain is shaped like a horseshoe bearing leucine-rich repeats that surrounds variable binding regions capable of recognizing various PAMPs. TLRs bind their agonist, as a homo- or heterodimer along with a co-receptor or accessory molecule, initiating a signalling cascade leading to the expression of inflammatory cytokines and triggering an adaptive immune response.[8] TLR10 is the only exception and has been shown to exhibit immunosuppressive properties on B cells.[9]

Table 1. Overview of the family of immunostimulatory Toll-like receptors in humans.[6,10]

Entry	Receptor	Location	Agonist	Synthetic analog
1	TLR1/2	Plasma membrane	Triacylated lipopeptides	Pam_3CSK_4
2	TLR2/6	Plasma membrane	Diacylated lipopeptides	Pam_2CGDPKHPKSF (FSL-1)
3	TLR3	Endosome	Double-stranded RNA	Poly(I:C)
4	TLR4	Plasma membrane	Lipopolysaccharide	Monophosphoryl lipid A (MPL)
5	TLR5	Plasma membrane	Flagellin	–
6	TLR7/8	Endosome	Single-stranded RNA	Resiquimod
7	TLR9	Endosome	CpG-rich hypomethylated DNA	CpG oligodeoxynucleotide

Considerable efforts have gone into the synthesis of analogues and the isolation of fragments of the native agonists of TLRs to identify compounds capable of binding to the receptor and stimulating an immune response.[10] A noteworthy example is monophosphoryl lipid A (MPL) which is obtained through a series of hydrolysis steps from lipopolysaccharide (LPS) of *Salmonella minnesota* R595 and has been shown to have an immunostimulatory effect. Compared to LPS, MPL exhibits not only reduced potencies, but also reduced the toxicity, allowing MPL to be used safely in humans at correct doses.[11] MPL has since found its way as an adjuvant into the human papilloma virus (HPV) vaccine as part of adjuvant system AS04.[2] Another example is the use of TLR2/6 agonist lipoteichoic acid (LTA) from *Staphylococcus aureus* and variants by Stadelmaier *et al.* in an effort to assess the active component of LTA stimulation of the immune system.[12]

With the availability of known TLR agonists, research on cross-talk between the receptors was intensified as the triggering of a single TLR is rarely sufficient to stimulate an effective immune response to protect the host from infection.[13] As a result of these research efforts both synergistic and antagonistic effects have been shown for soluble, conjugated as well as encapsulated combinations of PRR ligands.[14–17] Recently, the group of Esser-Kahn reported on the significance of the linker length between the covalently connected pairs of different TLR-agonists for the ability of such a conjugate to trigger NF-κB pathway and to induce IL-6 production. It turned out that different agonist pairs preferred different spatial presentation.[18] Much research is, however, needed to establish the effect of the orientation of TLR agonists in their ability to stimulate an immune response. The work described in this Chapter aims to design and synthesize a suitable scaffold, to which different TLR ligands can be attached, in order to further probe the influence of their spatial positioning on the immunogenicity of the covalent cluster of TLR-agonists. Inspiration was drawn from a concept introduced by the group of Mutter and termed regioselective addressable functionalized template (RAFT) which was

employed as a tertiary structure-inducing device in *de novo* protein design (*Figure 1*).[19,20]

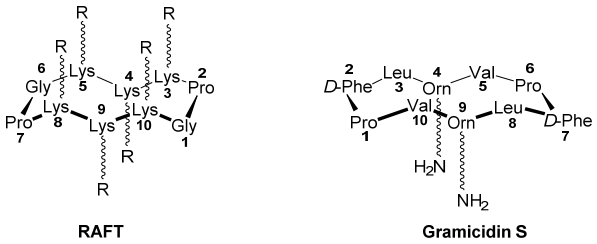


Figure 1. Comparison of the amino acid composition of the regioselective addressable functionalized template platform introduced by the group of Mutter and gramicidin S with lysines instead of the naturally occurring ornithines.

RAFT is a cyclic decapeptide platform composed of two adjacent proline-glycine motifs as type II β-turn-inducers that constrain the backbone conformation in an antiparallel β sheet. This conformational restraint presents two separate spatial domains with residues 3-5-8-10 oriented in the lower plane and residues 4-9 in the opposite plane. Up to six lysine residues can be incorporated and are made regioselectively addressable by way of orthogonal protecting groups.[21] In recent years, the RAFT platform has received interest for its potential as a vaccine scaffold offering improved stability against degradation compared to linear peptides and the ease of introducing a multivalent carbohydrate presentation.[22,23]

The work presented in this Chapter entails the synthesis of a RAFT platform, with a pair of major alterations over the original, as a scaffold for TLR agonist ligation in different orientations. First, a derivative of the cyclic decapeptide gramicidin S is used as the basis for the scaffold bearing two lysine groups (instead of the naturally occurring ornithine groups) which can be used for functionalization with two TLR substrates (*Figure 1*). Gramicidin S adopts the same antiparallel β sheet conformation, which is closed by two type II′ β-turns and is highly stabilized by four intramolecular hydrogens bonds involving the backbone as confirmed by crystal structure analysis.[24] Asano and co-workers showed that the secondary structure is not perturbed by substitution of the ornithine moieties with leucine residues proving the conformation is rigid and allows for shuffling of amino acid residues 3-4-5 and 8-9-10.[25] The second alteration is the way of regioselectively addressing the lysine side-chains by replacing the orthogonal protecting groups with orthogonal

ligation handles, opting for a maleimido-group and the strained cyclooctyne bicyclo[6.1.0]non-4-yne (BCN) (**1**, *Figure 2*).

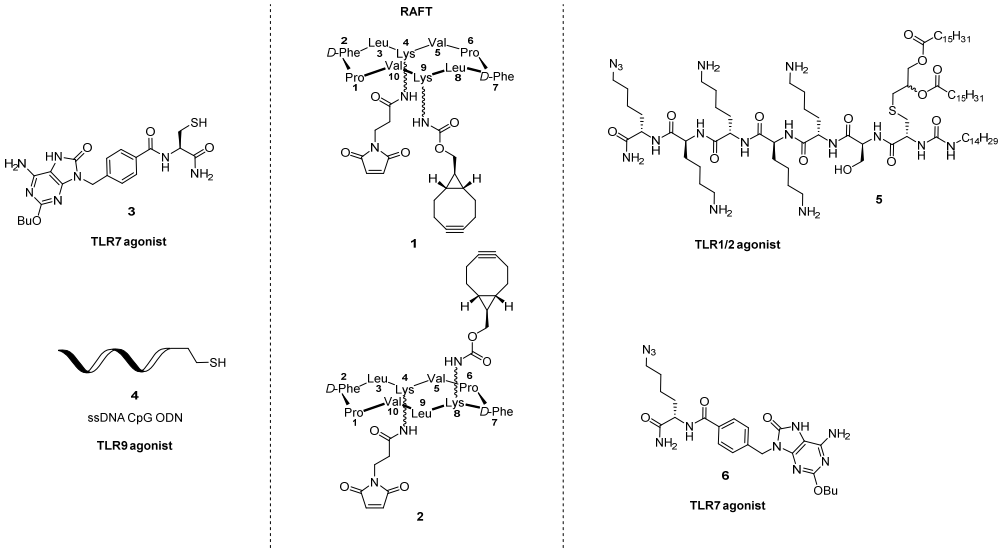


Figure 2. Design of the RAFT scaffold bearing two chemoselective groups and various TLR agonists functionalized with their chemoselective counterpart.

With these design choices RAFT scaffolds **1** and **2** were envisioned bearing two chemoselective handles in different orientations. Scaffold **1** has the ligation handles on the same face of the macrocycle while RAFT **2** has them on the opposite faces. TLR7 and TLR9 agonists were designed with a free thiol as the ligation partner for the maleimido-group, while TLR1/2 and TLR7 agonists were equipped with an azide as the complementary group for the strained alkyne. Overall, this gives rise to the possibility of obtaining dual TLR1/2-7, TLR1/2-9 and TLR7-9 agonists on two scaffolds for a total of six different entities. For the synthesis of the ligands, suitably modified to include a free thiol group or an azide moiety, the known syntheses previously developed in-house for the preparation of TLR1/2 agonist UPam and TLR7 agonist 9-benzyl-8-oxo-2-butoxy-adenine could be used as the starting point.[26,27]

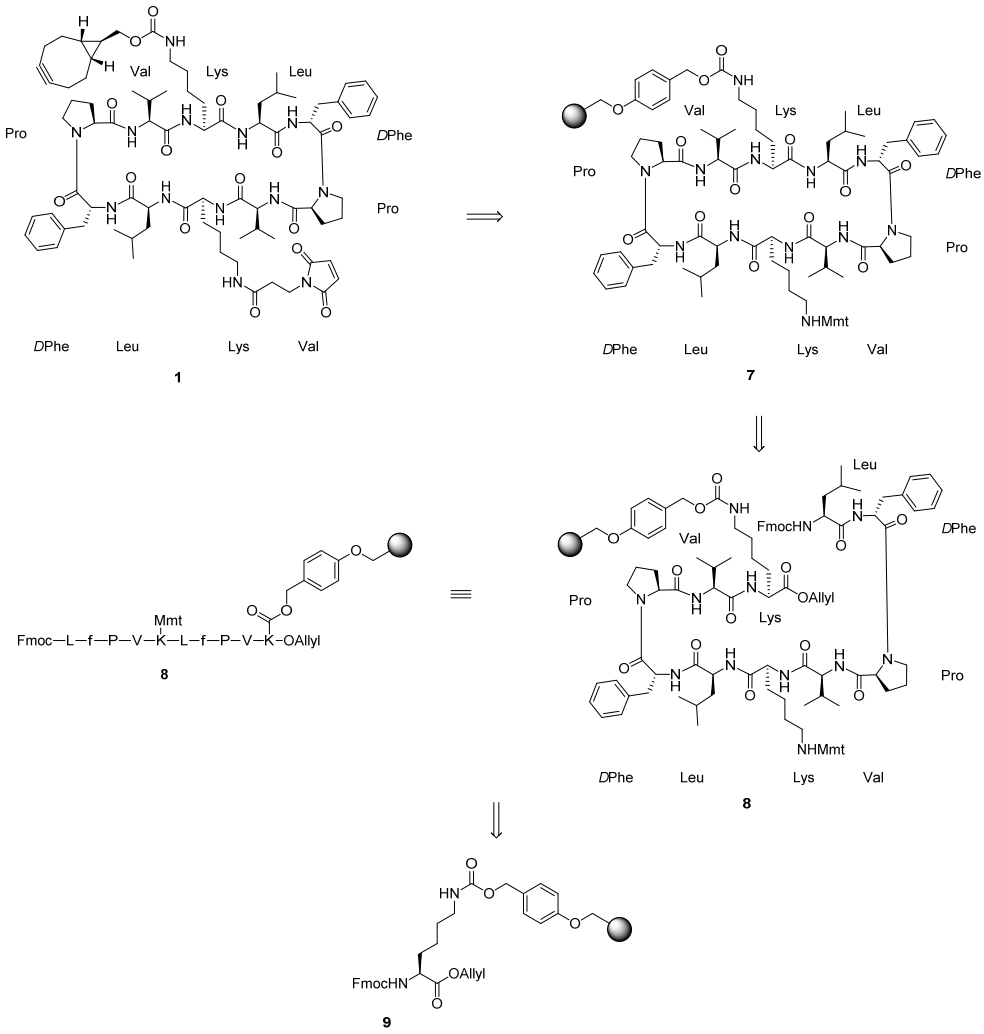


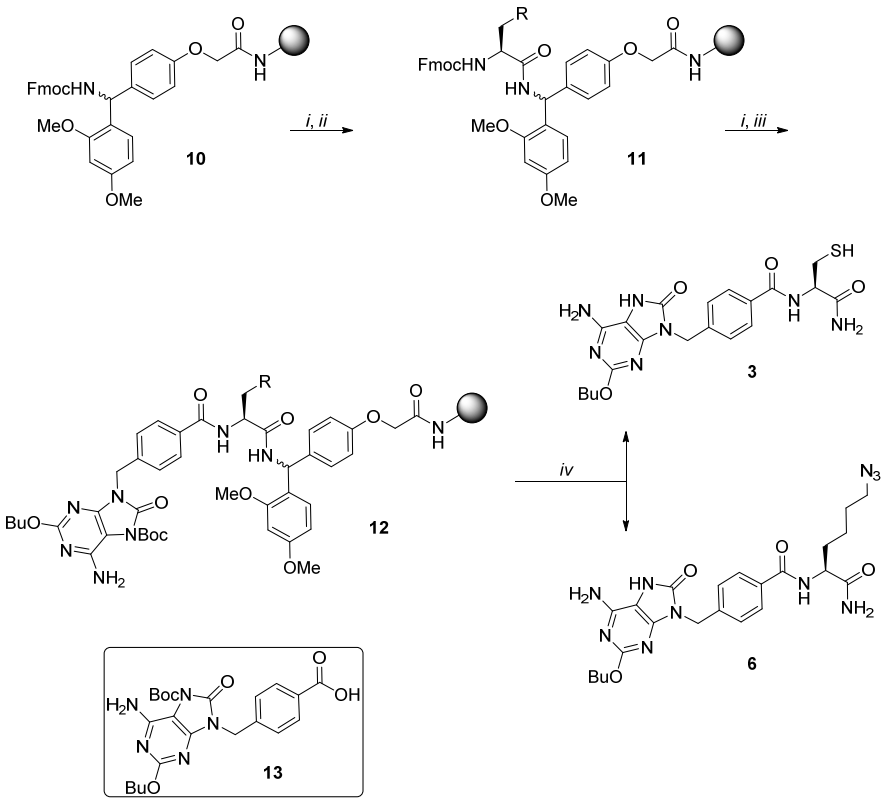
Figure 3. Retrosynthesis of regioselectively addressable template **1** based on gramicidin S.

The synthesis of RAFT scaffold **1**, and in a similar manner scaffold **2**, was envisioned *via* an on-resin cyclization strategy employing the procedure for anchoring the lysine ε-amine to the resin as described in the previous Chapter (*Figure 3*). The added benefit of using this procedure is the ease of functionalizing the side-chain of the non-anchored lysine. Starting from lysine functionalized resin **9**, elongation will be achieved by Fmoc-based automated solid-phase peptide synthesis (SPPS) to furnish linear peptide **8** bearing three orthogonal protecting groups and the linker to the

resin. Palladium-catalyzed deprotection of the C-terminal allyl group and N-terminal Fmoc deprotection with piperidine will allow for on-resin cyclization which will afford cyclic peptide **7**. The first orthogonal ligation handle will be installed by first deprotecting the monomethoxytrityl (Mmt) group using weak acidic conditions followed by functionalization of the lysine ε-amine with a maleimido-group. The cyclic peptide will be cleaved off the resin by treatment with a strong acid simultaneously liberating the second lysine ε-amine which will then be functionalized with a BCN moiety giving scaffold **1**.

Results and discussion

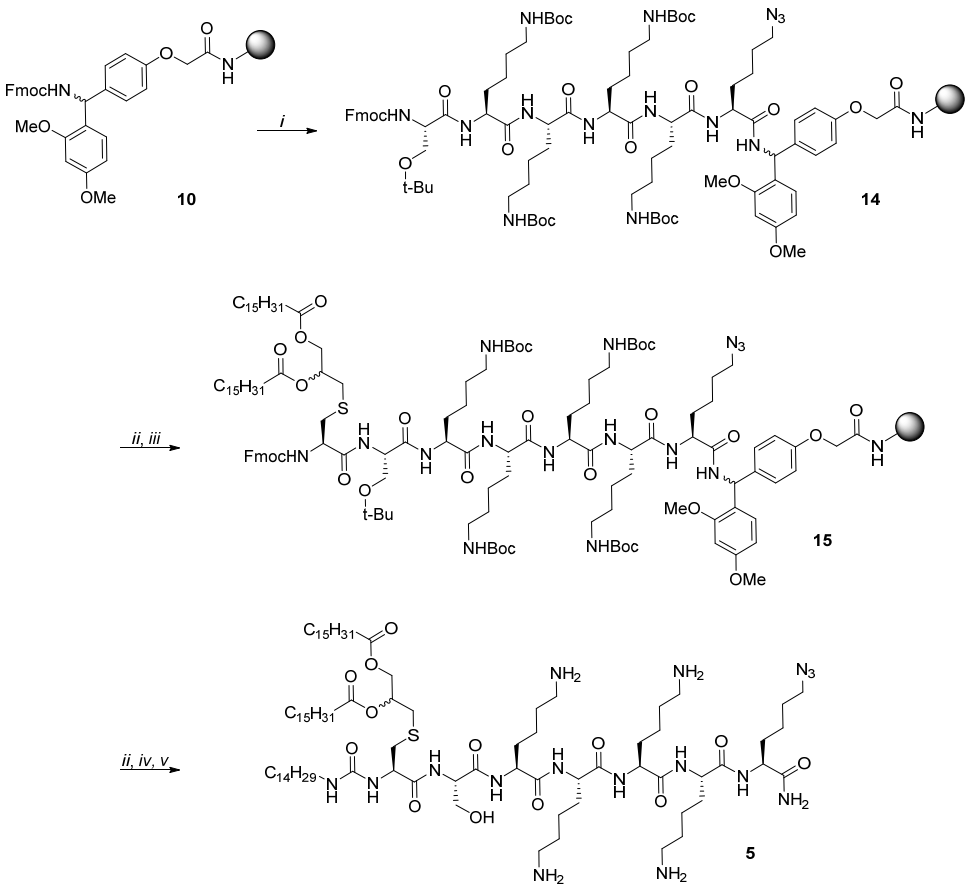
The construction of scaffolds bearing chemoselective handles in different orientations is preceded by the synthesis of the TLR agonists bearing complementary handles and in particular TLR7 ligands **3** and **6** (*Scheme 1*).



Scheme 1. Reagents and conditions: (*i*) piperidine, DMF, rt, 2x10 min (*ii*) Fmoc-AA-OH, HCTU, DIPEA, DMF, rt, 1 hr (*iii*) 4-((6-amino-2-butoxy-7-(tert-butoxycarbonyl)-8-oxo-7,8-dihydro-9*H*-purin-9-yl)methyl)benzoic acid, HCTU, DIPEA, DMF, rt, 17 hrs (*iv*) TFA – H_2O – TIPS (190:5:5), rt, 3 hrs, 39% (**3**), 61% (**6**).

Starting from TentaGel S RAM **10**, the Fmoc-group was deprotected with two treatments of 20% (v/v) piperidine in DMF. The liberated amine was then condensed with either Fmoc-Lys(N_3)-OH or Fmoc-Cys(Trt)-OH, using HCTU and DIPEA in DMF for one hour. After another cycle of Fmoc deprotection, adenine derivative **13** was introduced by treatment with peptide coupling reagent HCTU and DIPEA for 17 hours. Compound **13** bears a Boc protecting group to increase its solubility and make it suitable for SPPS.[27] Liberation of the target molecules **3** and **6** from the resin was achieved by treating the resin with a cleavage cocktail (190:5:5, TFA – TIPS – H_2O) for three hours. The suspension was filtered and the resin was washed with an additional treatment with the cleavage cocktail. The crude compounds were obtained by evaporation and subsequent purification by reversed-phase HPLC delivering TLR7 ligands **3** and **6** in a yield of 39% and 61% respectively.

Next, the attention was shifted to the synthesis of TLR1/2 agonist UPam **5** bearing an azido group for ligation purposes (*Scheme 2*).

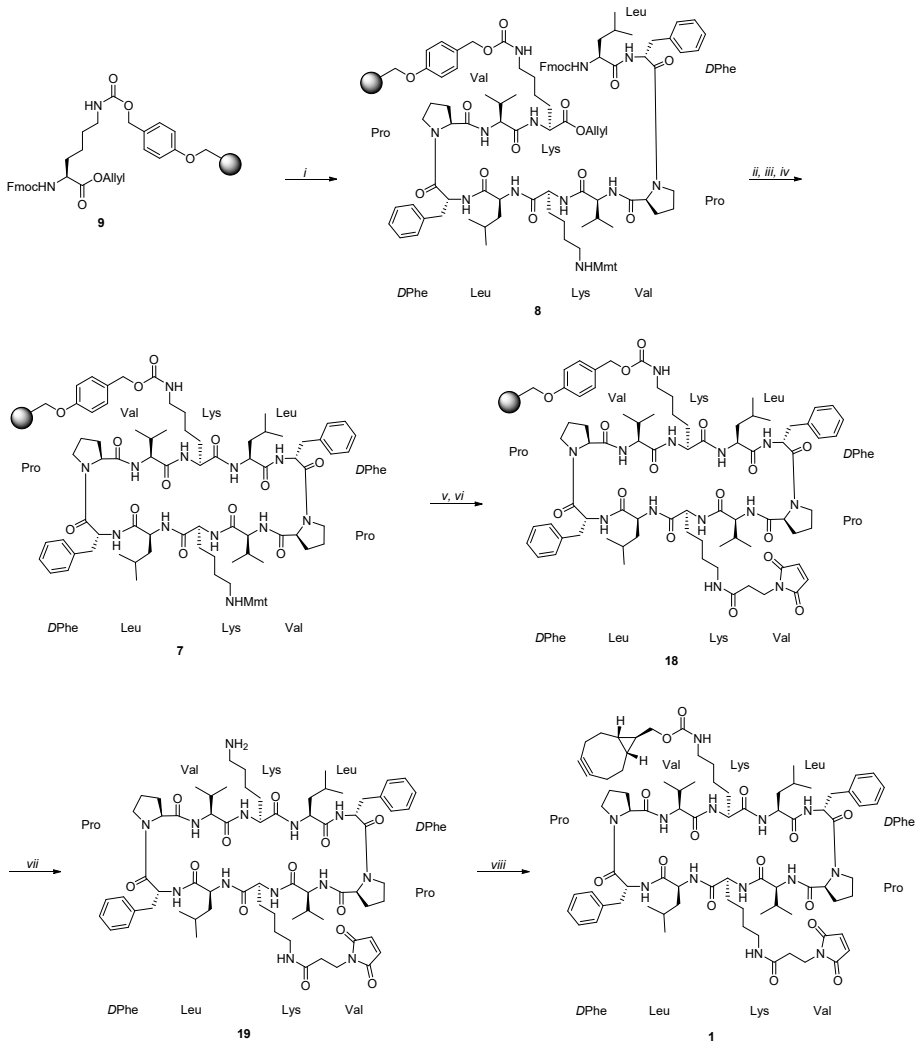


Scheme 2. Reagents and conditions: (*i*) SPPS: (a) piperidine, DMF, rt, 2x3 min. (b) Fmoc-AA-OH, HCTU, DIPEA, DMF, rt, 1 hr (c) Ac_2O, DMF, rt, 2x3 min. (*ii*) piperidine, DMF, rt, 2x10 min (*iii*) Fmoc-Cys((*RS*)-2,3-di(palmitoyloxy)-propyl)-OH, HCTU, DIPEA, DMF, DCM, rt, 24 hrs (*iv*) tetradecylisocyanate, DMF, DCM, rt, 24 hrs (*v*) TFA – H_2O – TIPS (190:5:5), rt , 3 hrs, 7.2%.

First, TentaGel S RAM **10** was elongated using six peptide coupling cycles on a Protein Technologies Tribute automated peptide synthesizer to afford resin-bound peptide **14**. Each cycle started with two treatments of 20% (v/v) piperidine in DMF for three minutes to deprotect the Fmoc group. After a series of washing steps, condensation was achieved by reacting the side-chain protected Fmoc amino acid with deprotected resin-bound peptide using HCTU as the activator and DIPEA as

the base for one hour at room temperature. Unreacted amines were capped by two treatments of 10% (v/v) Ac_2O in DMF for three minutes. The synthesis was then continued manually and the resin was subjected to two treatments of 20% (v/v) piperidine in DMF to liberate the N-terminus. Afterwards, the resin-bound peptide was coupled with Fmoc-Cys((*RS*)-2,3-di(palmitoyloxy)-propyl)-OH using HCTU as the coupling reagent and DIPEA as the base in a mixture of DMF and DCM (1:1, DMF – DCM). The resin was shaken for 24 hours and subsequently filtered and washed with DMF and DCM to give peptide **15**. After another manual Fmoc deprotection cycle, the N-terminus was reacted with tetradecylisocyanate in a mixture of DMF and DCM (1:1, DMF – DCM) for 24 hours. The resin was washed with DMF and DCM followed by treatment with a cleavage cocktail (190:5:5, TFA – TIPS – H_2O) for three hours. The suspension was filtered and the resin was washed with additional cleavage cocktail. Evaporation and subsequent purification by reversed phase HPLC afforded UPam **5** bearing an azide moiety in a yield of 7.2%.

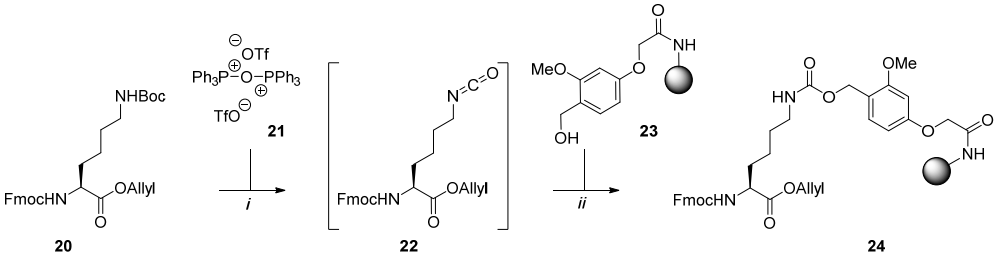
With the TLR ligands in hand, the synthesis of RAFT scaffold **1** was attempted (*Scheme 3*). Using the procedure described in Chapter 3, the lysine ε-amine was attached to TentaGel S PHB resin with a urethane linkage to afford resin **9**. The peptide was subsequently elongated using a Protein Technologies Tribute automated peptide synthesizer with nine peptide coupling cycles. Each cycle started with two treatments of 20% (v/v) piperidine in DMF for three minutes followed by a series of washing steps to remove residual amounts of piperidine. The liberated N-terminus was then coupled to the appropriate standard Fmoc building block using HCTU and DIPEA, except for the second lysine residue, whose side chain was equipped with the monomethoxytrityl (Mmt) group. The resin was shaken for an hour at room temperature which was followed by capping of the unreacted amines by subjecting the resin to two treatments of 10% (v/v) Ac_2O in DMF for three minutes. After nine cycles, resin-bound linear peptide **8** was obtained.



Scheme 3. Reagents and conditions: (*i*) SPPS: (a) piperidine, DMF, rt, 2x3 min. (b) Fmoc-AA-OH, HCTU, DIPEA, DMF, rt, 1 hr (c) Ac_2O, DMF, rt, 2x3 min. (*ii*) $Pd(PPh_3)_4$, $PhSiH_3$, DCM, DMF, rt, 1.5 hrs (*iii*) piperidine, DMF, rt, 2x10 min. (*iv*) benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate, 1-hydroxybenzotriazole hydrate, *N*-methylmorpholine, DMF, rt, 2.5 hrs (*v*) TFA – DCM (1:49), rt, 10x2 min. **(*vi*)** 3-maleimidopropionic acid NHS ester, DIPEA, DMF, rt, 4 hrs (*vii*) TFA – TIPS – H_2O (190:5:5), rt, 3 hrs (*viii*) BCN PNP ester, DIPEA, DMF.

The synthesis was continued manually by deprotecting the C-terminal allyl ester using tetrakis(triphenylphosphine) palladium(0) and phenyl silane in a mixture of DCM and DMF (1:1, DCM – DMF) for 90 minutes. After the Fmoc group was deprotected with two treatments of 20% (v/v) piperidine in DMF for ten minutes,

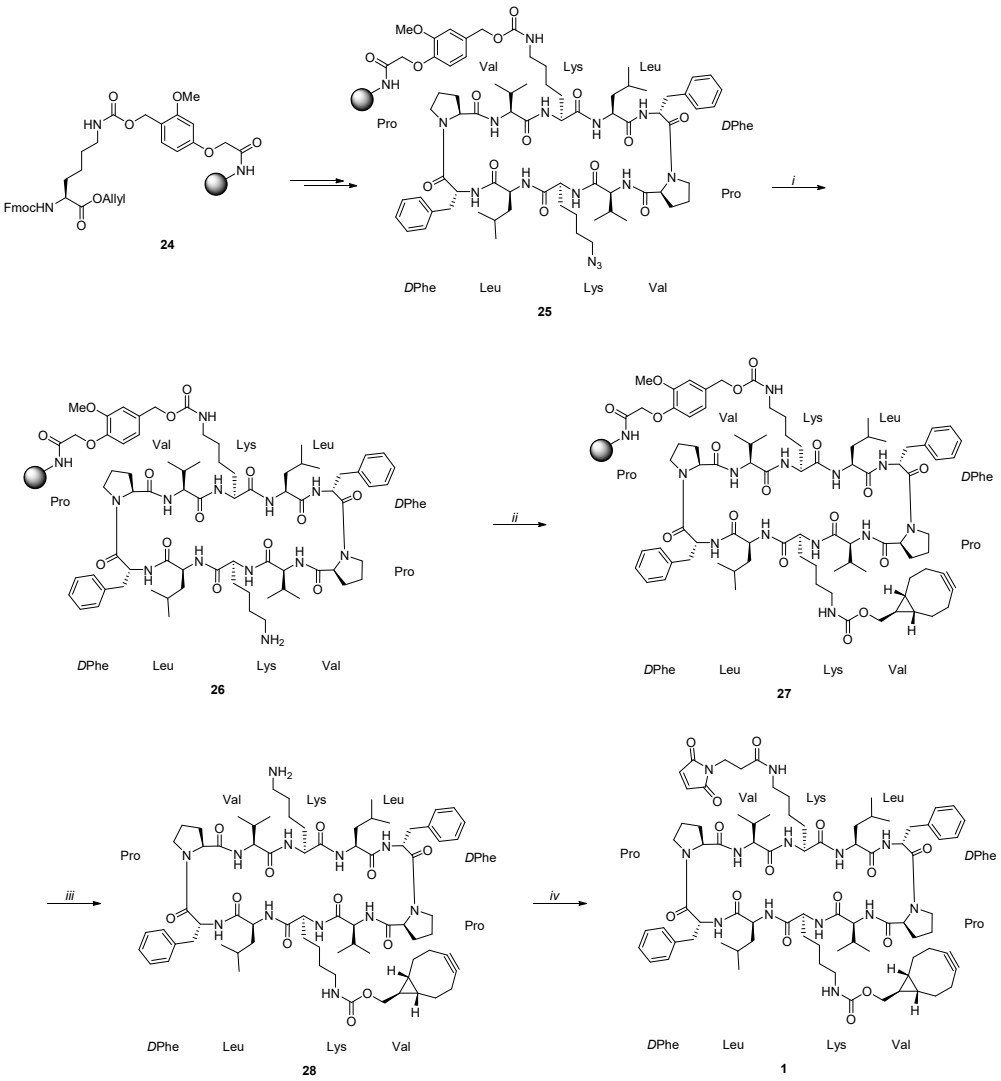
the resin-bound peptide was cyclized using PyBOP as the condensing agent, 1-hydroxybenzotriazole as additive and *N*-methylmorpholine as the base for 2.5 hours. Next, the removal of the Mmt group from immobilized peptide **7** was attempted. Several methods were tried, namely, treatment with TFA mixture (1:99, TFA – DCM) for 10 x 2 minutes with and without a neutralization wash with 0.5 M DIPEA in DMF afterwards as well as treatment with TFA (1:49, TFA – DCM) until no trityl cations were detected based on color.[28] However, these methods proved to be insufficiently reproducible giving fluctuating yields of crude peptide after cleavage. Therefore, a different synthetic strategy to **1** was adopted as an alternative to the exploitation of the subtle differences in the sensitivity of Mmt group and Wang linker to diluted TFA (*Scheme 3*, from **7** to **1** via **18** and **19**). Instead of utilizing two acid-sensitive functionalities in the same molecule, a change from the Mmt group to an azide was made as a means to mask the lysine ε-amine. Simultaneously, it was decided to introduce the BCN moiety first and leave the option available in a late stage to choose between a maleimide and an iodoacetamide moiety. As a consequence the more acid-labile resin TentaGel S Ac was necessary as strained octynes are more prone to decomposition at higher trifluoroacetic acid concentration.[29,30] The first step was verifying if the method used for side-chain anchoring as described in Chapter 3 was still applicable (*Scheme 4*).



Scheme 4. Reagents and conditions: (*i*) DCM, rt, 5 min (*ii*) dibutyltin dilaurate, *N*-methylmorpholine, DCM, rt, 72 hrs, 89%.

First, Hendrickson's reagent **21** was created *in situ* by reacting two equivalents of triphenylphosphine oxide with one equivalent of triflic anhydride at 0 °C for 30 minutes. Fully protected lysine **20** was added to the mixture and stirred for five minutes to generate isocyanate **22**. Dibutyltin dilaurate and *N*-methylmorpholine were then added to the solution and the mixture was transferred to a shaker containing TentaGel S Ac **23**, which carries an additional methoxy group on the linker compared to TentaGel S PHB. The resin was shaken for 24 hours after which

the mixture was filtered and the resin washed with DCM and Et_2O. After drying the resin over N_2, loading and yield were determined in the same manner as described in Chapter 3. With a reaction time of 24 hours, side-chain anchored lysine **24** was obtained in a yield of 59% on a 0.1 mmol scale. Increasing the reaction time to 48 hours yielded product **24** in 91% yield proving that the method is applicable to TentaGel S Ac as well. The longer reaction time is likely caused by the increased steric hindrance stemming from the additional methoxy group compared to the TentaGel S PHB resin. When scaling up the synthesis to 0.5 mmol, the reaction time had to be increased to 72 hours in order to obtain resin **24** in 89% yield. With resin **24** in hand, the synthesis of RAFT scaffold **1** was attempted once more (*Scheme 5*).



Scheme 5. Reagents and conditions: (*i*) PMe_3, toluene, 1,4-dioxane, rt, 2 hrs, then H_2O, rt, 4 hrs (*ii*) BCN PNP ester, DIPEA, DMF, rt, 1 week (*iii*) TFA – DCM (1:199), rt, 10x2 min (*iv*) 3-maleimidopropionic acid NHS ester, DIPEA, DMF, rt, 4 hrs.

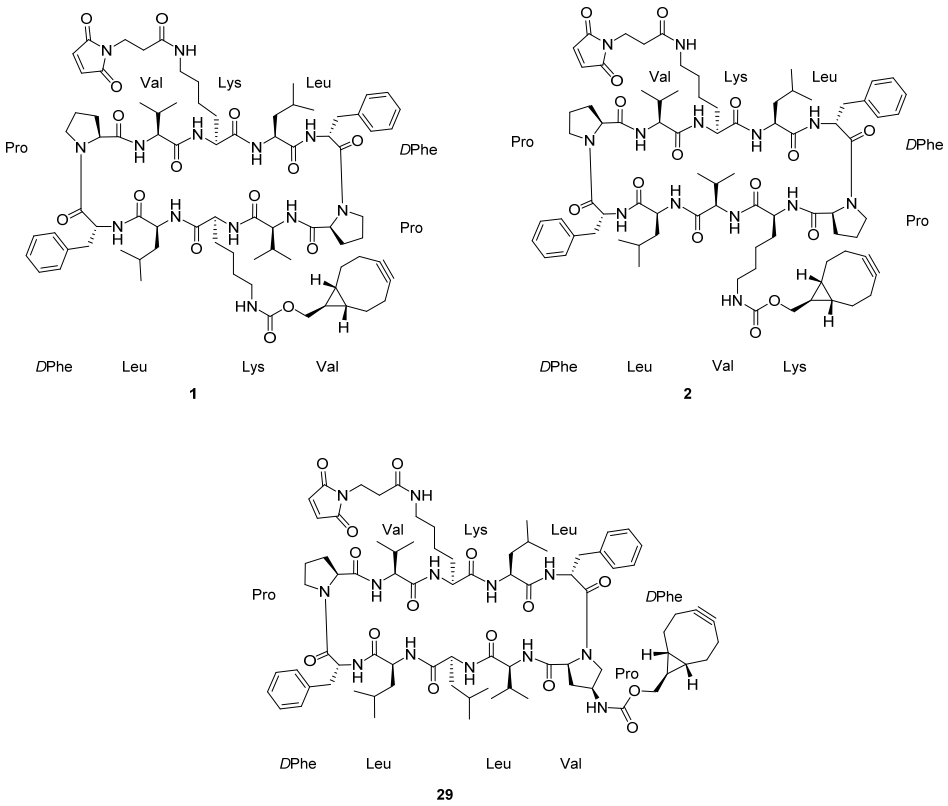
Starting from functionalized resin **24**, cyclized peptide **25** was synthesized employing the same standard conditions as described above. For the reduction of the azide, the resin was treated with an excess of PMe_3 (1.0 M in toluene) diluted in 1,4-dioxane for two hours after which H_2O was added. The mixture was shaken for an additional four hours and the suspension was filtered. After rinsing the resin with

1,4-dioxane and DCM a small amount of resin (~5 mg) was treated with a cleavage cocktail (190:5:5, TFA – H_2O – TIPS) for one hour followed by filtration. Analysis of the filtrate with LC-MS indicated a clean and complete reduction of the azide to the amine. Having successfully reduced the azide on-resin, the strained cyclooctyne BCN was introduced by reacting the newly formed amine in **26** with BCN *para*-nitrophenyl carbonate and DIPEA to furnish resin **27**. Analysis of the cleavage products with LC-MS at different reaction times revealed that full conversion was achieved after one week of shaking the suspension. For the cleavage of the peptide off the resin, a mixture of TFA and DCM (1:199, TFA – DCM) was added and the suspension was shaken for two minutes. After filtration, the filtrate containing trifluoroacetic acid was immediately neutralized by a solution of pyridine in methanol. These steps were repeated ten times. To verify complete cleavage of the peptide, the resin was then treated with a higher concentration of TFA (1:99, TFA – DCM) using the same procedure followed by an even higher concentration (1:49, TFA – DCM). No additional cleavage products could be detected using LC-MS at these concentrations, indicating ten treatments with 0.5% (v/v) TFA in DCM for two minutes is sufficient for complete cleavage.

Removal of the pyridinium trifluoroacetate proved to be more troublesome however as attempts to precipitate the peptide were of no avail. To simplify the work-up and circumvent the formation of salts, a method reported by Srinivasan *et al.* was employed where the basic ion-exchange resin Amberlyst A-21 was used to neutralize TFA.[31] Before use, the Amberlyst A-21 resin was washed with methanol, dry THF and DCM and dried under high-vacuum to give a free-flowing solid. A suspension was then prepared of the Amberlyst resin (14 g per mL TFA) in DCM to which the filtrate containing the cleavage products were added. After addition of the tenth and final filtrate solution, the suspension was vigorously stirred for an additional 30 minutes followed by filtration. Evaporation of the filtrate afforded crude cleavage product **28**, which was then taken up in DMF and reacted with 3-maleimidopropionic acid NHS ester and DIPEA for four hours. Evaporation followed by purification using RP-HPLC afforded RAFT scaffold **1**. However, after evaporation of the pure fractions a byproduct was observed with LC-MS corresponding to a mass of $[M+18+H]^+$ corresponding to the addition of water. Lyophilization of the fractions using a rotor wherein the temperature increases to 35 °C saw the formation of the same byproduct. A recent report by Spangler *et al.* has shown that concentration of HPLC fractions of BCN conjugates containing 0.1% TFA

buffer under reduced pressure at 40 °C induces alkyne degradation.[32] Under these conditions, hydration of the triple bond occurs giving the vinyl alcohol which tautomerizes to the corresponding ketone. Freeze-drying the fractions without the rotor and the inherent temperature increase saw no significant by-product formation and pure scaffold **1** could ultimately be obtained in 3.7% yield.

With the procedure to generate RAFT scaffold **1** in hand, the synthesis route was then applied to afford RAFT scaffold **2** in 3.3% yield (*Scheme 6*).



Scheme 6. RAFT scaffolds **1**, **2** and **29** all bearing the chemoselective handles in different orientations.

An advantage of masking the lysine ε-amine with an azido group is that it opens up the possibility of employing the commercially available 4-azidoproline amino acid as an additional means of inducing a different orientation of the chemoselective handles. When employing the cis-Fmoc-(2*S*, 4*S*)-4-azidoproline in place of a regular proline, RAFT scaffold **29** was obtained in 3.3% yield giving a third RAFT scaffold

with a different orientation. With these scaffolds in hand together with the synthesized TLR2, 7 and 9 ligands nine different combinations can be synthesized, which should be the focus going forward.

Conclusion

Complementing recent research efforts into synergistic effects of various TLR ligands, this Chapter is focused on synthesizing a system to study the effect of the orientation on the synergy between different TLR agonists. Inspiration was drawn from a concept introduced by Mutter termed regioselectively addressable functionalized template (RAFT) and was applied on the cyclic decapeptide gramicidin S, bearing lysine groups.[19,20] With the method described in Chapter 3 and here expanded to the TentaGel S AC resin, it was possible to differentiate between the two lysine ε-amines to install the strained cyclooctyne BCN and a maleimido moiety. The first attempted synthesis was carried out on the TentaGel S PHB resin and the Mmt group was used as the temporary lysine ε-amine protecting group, but deprotection of this group resulted in fluctuating yields. The switch to TentaGel S AC resin was made to accompany the on-resin introduction of the moderately acid-labile cyclooctyne. Simultaneously, the azide was chosen as the masking group for the lysine ε-amine and resulted in clean on-resin reduction. The cleavage procedure was simplified by using the Amberlyst A-21 basic ion-exchange resin to neutralize the trifluoroacetic acid. With this procedure three different RAFT scaffolds were synthesized. TLR2, 7 and 9 ligands were synthesized bearing complementary groups to those present on the scaffolds giving rise to nine different possible combinations of ligands and orientations which in the future could be used to study the effects of orientation of the TLR combinations on their ability to stimulate an immune response.

General information

Materials, reactions and purification

Standard Fmoc-amino acids and resins for solid-phase peptide synthesis (SPPS), amino acids for solution-phase synthesis and peptide coupling reagents 2-(6-chloro-1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HCTU), *N*,*N*'-diisopropylcarbodiimide (DIC), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), ethyl cyano(hydroxyimino)acetate (Oxyma Pure) and 1-hydroxybenzotriazole (HOBt) were purchased from Novabiochem or Sigma-Aldrich. The resins TentaGel S PHB (0.27 mmol/g), TentaGel S AC (0.23 mmol/g) and TentaGel S RAM (0.25 mmol/g) were bought from Rapp Polymere. Fmoc-Cys((*RS*)-2,3-di(palmitoyloxy)-propyl)-OH was obtained from Bachem. Fmoc-L-Lys(N_2)-OH was purchased from Iris Biotech. 4-((6-Amino-2-butoxy-7-(tert-butoxycarbonyl)-8-oxo-7,8-dihydro-9*H*-purin-9-yl)methyl)benzoic acid and 3-maleimidopropionic acid *N*-hydroxysuccinimidyl ester were available in-house. All other chemicals were purchased from Acros, Sigma Aldrich, VWR, Fluka, Merck and Fisher Scientific and used as received unless stated otherwise. Tetrahydrofuran (THF), *N*,*N*-dimethylformamide (DMF), dichloromethane (DCM), 1,4-dioxane and toluene were stored over molecular sieves before use. Commercially available ACS grade solvents were used for column chromatography without any further purification, except for toluene and ethyl acetate which were distilled prior to use. All reactions were carried out under a nitrogen atmosphere, unless indicated otherwise. Reaction progress and chromatography fractions were monitored by thin layer chromatography (TLC) on silica-gel-coated aluminium sheets with a F254 fluorescent indicator purchased from Merck (Silica gel 60 F_{254}). Visualization was achieved by UV absorption by fluorescence quenching, permanganate stain (4 g $KMnO_4$ and 2 g K_2CO_3 in 200 mL of H_2O), ninhydrin stain (0.6 g ninhydrin and 10 mL acetic acid in 200 mL ethanol). Silica gel column chromatography was performed using Screening Devices silica gel 60 (particle size of 40 – 63 μm, pore diameter of 60 Å) with the indicated eluent. Analytical reversed-phase high-performance liquid chromatography (RP-HPLC) was performed on a Thermo Finnigan Surveyor HPLC system with a Phenomenex Gemini C_{18} column (4.6 mm x 50 mm, 3 μm particle size) with a flow rate of 1 mL/min and a solvent gradient of 10-90% solvent B over 8 min coupled to a LCQ Advantage Max (Thermo Finnigan) ion-trap spectrometer (ESI^+). Preparative RP-HPLC was performed with a GX-281 Liquid Handler and a 331 and 332-H2 primary and secondary solvent pump respectively with a Phenomenex Gemini C_{18} or C_4 column (250 x 10.0 mm, 3 μm particle size) with a flow rate of 5 mL/min and solvent gradients as described for each compound. All HPLC solvents were filtered with a Millipore filtration system equipped with a 0.22 μm nylon membrane filter prior to use. HPLC solvent compositions: solvent A is 0.1% (v/v) TFA in H_2O; solvent B is MeCN. Preparative RP-HPLC was also performed on an Agilent 1200 HPLC system coupled to a 6130 Quadrupole Mass Spectrometer using a Nucleodur C_{18} Gravity column (250 x 10.0 mm, 5 μm particle size) with a flow rate of 5 mL/min and a gradient over 12 min. as described for each compound. HPLC solvent composition: solvent A is 0.2% (v/v) TFA in H_2O and solvent B is MeCN. All HPLC solvents were filtered with a Millipore filtration system equipped with a 0.22 μm nylon membrane filter prior to use.

Characterization

Nuclear magnetic resonance (^1H and ^{13}C APT NMR) spectra were recorded on a Brüker DPX-300, Brüker AV-400, Brüker DMX-400, Brüker AV-500 or Brüker DMX-600 in the given solvent. Chemical shifts are reported in parts per million (ppm) with the residual solvent or tetramethylsilane (0 ppm) as reference. High-resolution mass spectrometry (HRMS) analysis was performed with a Thermo Finnigan LTQ Orbitrap mass spectrometer equipped with an electronspray ion source in positive mode (source voltage 3.5 kV, sheath gas flow 10 ml/min, capillary temperature 250 °C) with resolution R = 60000 at m/z 400 (mass range m/z = 150 – 2000) and dioctyl phthalate (m/z = 391.28428) as a "lock mass". The high-resolution mass spectrometer was calibrated prior to measurements with a Thermo Finnigan calibration mixture. Nominal and exact m/z values are reported in daltons.

Solid-phase peptide synthesis

General methodology

Manual solid-phase peptide synthesis

Manual amino acid couplings were carried out using a fritted reaction syringe equipped with a plunger and syringe cap or a manual reaction vessel (SHG-20260-PI, 60 mL) purchased from Peptides International. The syringe was shaken using

either a Heidolph Multi Reax vortexer set at 1000 rpm or a St. John Associates 180° Flask Shaker (model no. A5-6027). Fmoc deprotection was achieved by agitating the resin with 20% (v/v) piperidine in DMF (2 x 10 min.). After draining the reaction vessel, the resin was washed with DMF (6 x 30 sec.). The appropriately side-chain protected Fmoc-amino acid (5.0 equiv.) in DMF (5.0 mL) was pre-activated with HCTU (5.0 equiv.) and DIPEA (10 equiv.) for 5 min, then added to resin and agitated for 60 min. After draining the reaction vessel, the resin was washed with DMF (4 x 30 sec.). The completion of all couplings was assessed by a Kaiser test and double coupling was performed as needed.

Automated solid-phase peptide synthesis

The automated peptide coupling was performed on a CEM Liberty Blue microwave peptide synthesizer or a Protein Technologies Tribute peptide synthesizer using standard Fmoc protected amino acids. For the Tribute peptide synthesizer, amino acids were presented as solids and 0.20 M HCTU in DMF was used as activator, 0.50 M DIPEA in DMF as the activator base, 20% (v/v) piperidine in DMF as the deprotection agent and a 90:10, DMF – Ac_2O mixture as the capping agent. Coupling of each amino acid occurred at room temperature for 1 hr followed by a capping step (2x 3 min.) betwixt two washing steps. Subsequently, Fmoc was deprotected using the deprotection agent (2x 3 min.) followed by two more washing steps. For the Liberty Blue microwave synthesizer, amino acids were presented as a solution (0.20 M in DMF) and 0.50 M DIC in DMF was used as activator, 1.0 M Oxyma Pure in DMF as additive and 20% (v/v) piperidine in DMF as the deprotection agent. Amino acid coupling in the microwave synthesizer occurred at 90 °C for 2 min. followed by Fmoc deprotection at 90 °C using the aforementioned deprotection agent (2x 90 sec.) and two washing steps.

Loading calculation

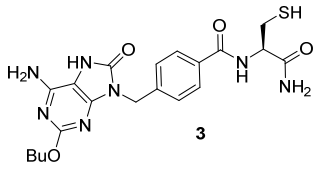
Resin was dried before loading calculation by washing with DCM (3x 30 sec.) and Et_2O (3x 30 sec.) followed by purging with N_2. A small amount of resin (5 – 10 mg) was weighed and DMF (0.80 mL) was added and the resin was swollen for 20 min. Piperidine (0.20 mL) was then added and shaken for 20 min. Following the deprotection, the suspension was filtered and diluted with 20% (v/v) piperidine in DMF to a total volume of 10 mL in a volumetric flask. The absorption of this solution was measured against a blank 20% (v/v) piperidine in DMF solution using a Shimadzu UV-1601 UV-VIS spectrometer with a Quartz cuvette (optical pathway = 1 cm). The loading was then calculated using the following equation:

$$\text{Loading}_{\text{resin}} = \frac{A_{301.0\,\text{nm}} * 10^6\ \text{mmol mol}^{-1}\ \text{mg g}^{-1} * V * D}{\varepsilon_{301.0\,\text{nm}} * m_{\text{resin}} * l}$$

where:

$\text{Loading}_{\text{resin}}$ = Fmoc substitution in mmol/g
$A_{301.0\,\text{nm}}$ = Absorption of sample at 301.0 nm
10^6 mmol mol^{-1} mg g^{-1} = Conversion factor of mmol to mol and mg^{-1} to g^{-1}
V = Total volume in L
D = Dilution factor
$\varepsilon_{301.0\,\text{nm}}$ = Molar absorption coefficient at 301.0 nm (8021 L mol^{-1} cm^{-1})
m_{resin} = sample weight of the resin in mg
l = optical path length of the cell in cm

(*R*)-4-((6-amino-2-butoxy-8-oxo-7,8-dihydro-9*H*-purin-9-yl)methyl)-*N*-(1-amino-3-mercapto-1-oxopropan-2-yl)benzamide



3

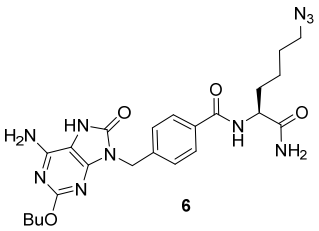
TentaGel S RAM (0.25 mmol/g, 0.40 g, 0.10 mmol, 1.0 equiv.) was elongated with standard cysteine building block (Fmoc-Cys(Trt)-OH) using manual peptide synthesis procedure. After Fmoc-deprotection and subsequent washing steps, a solution of 4-((6-amino-2-butoxy-7-(tert-butoxycarbonyl)-8-oxo-7,8-dihydro-9*H*-purin-9-yl)methyl)benzoic acid (69 mg, 0.15 mmol, 1.5 equiv.), HCTU (62 mg, 0.15 mmol, 1.5 equiv.) and DIPEA (52 μL, 0.30 mmol, 3.0 equiv.) in DMF (4.0 mL, 25 mM) was added to the resin and the mixture was shaken for 17 hrs. The resin was washed with DMF (4x) and DCM (4x) and a cleavage cocktail (190:5:5, TFA – H_2O – TIPS, 10 mL, 10 mM) was added and the resin was shaken for 3 hrs. The suspension was filtered and the filtrate was concentrated over a stream of N_2. Purification by RP-HPLC (GX-281, C_{18} column, 25% to 45% solvent B) followed by lyophilization afforded thiol **3** (18 mg, 39 μmol, 39%) as a white solid.

^{1}H NMR (500 MHz, DMF) δ 10.25 (s, 1H, N*H*(C=O)N), 8.47 (d, *J* = 8.0 Hz, 1H, N*H*-Cys), 8.00 – 7.93 (m, 2H, C*H*-arom), 7.69 (s, 1H, N*H*$_2$-Cys), 7.52 – 7.47 (m, 2H, C*H*-arom), 7.23 (s, 1H, N*H*$_2$-Cys), 6.70 (br s, 2H, N*H*$_2$), 5.04 (s, 2H, C*H*$_2$-benzyl), 4.69 (td, *J* = 8.2, 4.7 Hz, 1H, α-Cys), 4.21 (t, *J* = 6.6 Hz, 2H, OC*H*$_2$), 3.13 – 3.06 (m, 1H, β-Cys), 3.00 – 2.95 (m, 1H, β-Cys), 2.33 (t, *J* = 8.5 Hz, 1H, S*H*), 1.72 – 1.63 (m, 2H, OCH$_2$C*H*$_2$), 1.48 – 1.37 (m, 2H, C*H*$_2$CH$_3$), 0.93 (t, *J* = 7.4 Hz, 3H, C*H*$_3$).

^{13}C NMR (126 MHz, DMF) δ 175.0 (NH$_2$C=O), 167.6 (Ph(*C*=O)NH), 161.7 (NH$_2$*C*q), 153.9 (BuO*C*q), 150.9 (NH(*C*=O)N), 149.2 (N*C*qN), 142.0 (*C*q-arom), 134.7 (*C*q-arom), 128.8 (*C*H-arom), 128.5 (*C*H-arom), 99.8 (NH*C*q), 67.2 (O*C*H$_2$), 57.4 (α-Cys), 43.4 (*C*H$_2$-benzyl), 32.0 (OCH$_2$*C*H$_2$), 27.2 (β-Cys), 20.1 (*C*H$_2$CH$_3$), 14.4 (*C*H$_3$).

HRMS (ESI-Orbitrap) as disulfide calcd. for $C_{40}H_{49}N_{14}O_8S_2$ [M+H]$^+$ 917.32937, found 917.32989.

(*S*)-4-((6-amino-2-butoxy-8-oxo-7,8-dihydro-9*H*-purin-9-yl)methyl)-*N*-(1-amino-6-azido-1-oxohexan-2-yl)benzamide



6

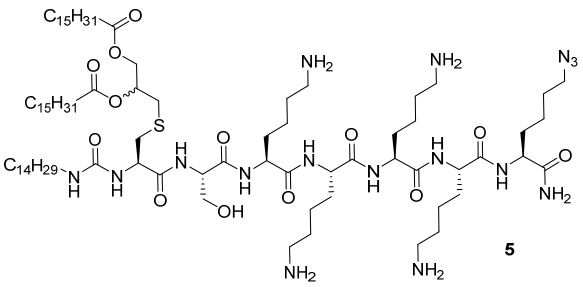
TentaGel S RAM (0.25 mmol/g, 0.40 g, 0.10 mmol, 1.0 equiv.) was elongated with Fmoc-L-Lys(N$_2$)-OH using manual peptide synthesis procedure. After Fmoc-deprotection and subsequent washing steps, a solution of 4-((6-amino-2-butoxy-7-(tert-butoxycarbonyl)-8-oxo-7,8-dihydro-9*H*-purin-9-yl)methyl)benzoic acid (69 mg, 0.15 mmol, 1.5 equiv.), HCTU (62 mg, 0.15 mmol, 1.5 equiv.) and DIPEA (52 μL, 0.30 mmol, 3.0 equiv.) in DMF (4.0mL, 25 mM) was added to the resin and the mixture was shaken for 17 hrs. The resin was washed with DMF (4x) and DCM (4x) and a cleavage cocktail (190:5:5, TFA – H_2O – TIPS, 10 mL, 10 mM) was added and the resin was shaken for 3 hrs. The suspension was filtered and the filtrate was concentrated over a stream of N_2. Purification by RP-HPLC (GX-281, C_{18} column, 25% to 40% solvent B) followed by lyophilization afforded azide **6** (31 mg, 61 μmol, 61%) as a white solid.

^{1}H NMR (500 MHz, DMF) δ 10.21 (s, 1H, N*H*(C=O)N), 8.36 (d, *J* = 8.1 Hz, 1H, N*H*-AzLys), 7.99 – 7.94 (m, 2H, C*H*-arom), 7.61 (s, 1H, N*H*$_2$-AzLys), 7.52 – 7.46 (m, 2H, C*H*-arom), 7.07 (s, 1H, N*H*$_2$-AzLys), 6.73 (br s, 2H, N*H*$_2$), 5.04 (s, 2H, C*H*$_2$-benzyl), 4.58 (ddd, *J* = 9.5, 8.0, 4.7 Hz, 1H, α-AzLys), 4.22 (t, *J* = 6.6 Hz, 2H, OC*H*$_2$), 3.37 (t, *J* = 6.8 Hz, 2H, ε-AzLys), 2.01 – 1.89 (m, 1H, β-AzLys), 1.89 – 1.76 (m, 1H, β-AzLys), 1.72 – 1.38 (m, 8H, OCH$_2$C*H*$_2$, δ-AzLys, γ-AzLys, C*H*$_2$CH$_3$), 0.93 (t, *J* = 7.4 Hz, 3H, C*H*$_3$).

^{13}C NMR (126 MHz, DMF) δ 175.2 (NH$_2$C=O), 167.4 (Ph(*C*=O)NH), 161.4 (NH$_2$*C*q), 153.8 (BuO*C*q), 150.9 (NH(*C*=O)N), 149.0 (N*C*qN), 141.8 (*C*q-arom), 134.9 (*C*q-arom), 128.8 (*C*H-arom), 128.5 (*C*H-arom), 99.7 (NH*C*q), 67.3 (O*C*H$_2$), 54.5 (α-AzLys), 52.0 (ε-AzLys), 43.4 (*C*H$_2$-benzyl), 32.6 (β-AzLys), 32.0 (OCH$_2$*C*H$_2$), 29.3 (δ-AzLys), 24.3 (γ-AzLys), 20.0 (*C*H$_2$CH$_3$), 14.4 (*C*H$_3$).

HRMS (ESI-Orbitrap) calcd. for $C_{23}H_{31}N_{10}O_4$ [M+H]$^+$ 511.25243, found 511.25237.

(6*R*,9*S*,12*S*,15*S*,18*S*,21*S*,24*S*)-12,15,18,21-tetrakis(4-aminobutyl)-28-azido-24-carbamoyl-9-(hydroxymethyl)-7,10,13,16,19,22-hexaoxo-6-(3-tetradecylureido)-4-thia-8,11,14,17,20,23-hexaazaoctacosane-1,2-diyl dipalmitate



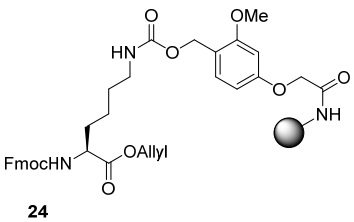
TentaGel S RAM (0.25 mmol/g, 0.40 g, 0.10 mmol, 1.0 equiv.) was elongated using the Tribute peptide synthesizer to obtain **14**. Using manual peptide chemistry, the resin was treated with piperidine in DMF (2 x 10 min) and washed with DMF (6x). A solution of Fmoc-Cys((*RS*)-2,3-di(palmitoyloxy)-propyl)-OH (0.13 g, 0.15 mmol, 1.5 equiv.), HCTU (62 mg, 0.15 mmol, 1.5 equiv.) and DIPEA (26 μL, 0.15 mmol, 1.5 equiv.) in a mixture of DCM and DMF (1:1, DMF – DCM, 4.0 mL, 25 mM) was added to the resin and the resin was shaken for 15 min. Additional DIPEA ((26 μL, 0.15 mmol, 1.5 equiv.) was added and the resin was shaken for another 24 hrs. The resin was washed with DMF (4x) and DCM (4x). After Fmoc-deprotection and subsequent washing steps, tetradecylisocyanate (0.25 mL, 0.74 mmol, 7.4 equiv.) and a mixture of DMF and DCM (1:1, DMF – DCM, 10 mL, 10 mM) were added and the mixutre was shaken for 24 hrs. The resin was washed with DMF (4x) and DCM (4x). A cleavage cocktail (190:5:5, TFA – H_2O – TIPS, 10 mL, 10 mM) was added and the resin was shaken for 3 hrs. The suspension was filtered and the filtrate was conctentrated under a stream of N_2 Purification by RP-HPLC (GX-281, C_4 column, 40% to 90% solvent B) and lyophilization afforded azide **5** (12 mg, 7.2 μmol, 7.2%) as a white solid.

^{1}H NMR (500 MHz, DMF) δ 8.49 (t, *J* = 6.9 Hz, 1H, N*H*-Lys), 8.39 (t, *J* = 7.0 Hz, 1H, N*H*-Ser), 8.16 – 8.10 (m, 1H, N*H*-Lys), 8.03 – 7.98 (m, 2H, N*H*-Lys), 7.81 (d, *J* = 7.9 Hz, 1H, N*H*-AzLys), 7.48 (s, 1H, NH_2C=O), 7.20 (s, 1H, NH_2C=O), 6.88 – 6.81 (m, 1H, N*H*-Cys), 6.79 (t, *J* = 5.8 Hz, 1H, $C_{14}H_{29}$N*H*), 5.26 – 5.15 (m, 1H, $C_{15}H_{31}$O(C=O)C*H*), 4.43 – 4.37 (m, 3H, α-Ser, α-Cys, $C_{15}H_{31}$(C=O)OCH_2), 4.37 – 4.17 (m, 6H, α-Lys, α-AzLys, $C_{15}H_{31}$(C=O)OCH_2), 3.95 (dd, *J* = 11.1, 5.3 Hz, 1H, β-Ser), 3.79 – 3.74 (m, 1H, β-Ser), 3.35 (t, *J* = 6.8 Hz, 2H, ε-AzLys), 3.21 – 3.13 (m, 2H, CH_2NH(C=O)NH), 3.13 – 2.99 (m, 10H, β-Cys, ε-Lys, β-Cys), 2.98 – 2.94 (m, 1H, CH_2S), 2.89 – 2.80 (m, 1H, CH_2S), 2.40 – 2.31 (m, 4H, CH_2(C=O)O), 1.96 – 1.70 (m, 18H, β-Lys, β-AzLys, δ-Lys), 1.64 – 1.43 (m, 18H, δ-AzLys, CH_2CH_2(C=O)O, CH_2CH_2NH(C=O)NH, γ-Lys, γ-AzLys), 1.37 – 1.22 (m, 70H, CH_2-alkyl), 0.91 – 0.85 (m, 9H, CH_3).

^{13}C NMR (126 MHz, DMF) δ 175.3 (*C*=O), 174.1 (*C*=O), 173.9 (*C*=O), 173.6 (*C*=O), 173.3 (*C*=O), 172.8 (*C*=O), 172.7 (*C*=O), 159.8 (NH(*C*=O)NH), 71.5 ($C_{15}H_{31}$(C=O)OCH), 64.7 ($C_{15}H_{31}$(C=O)OCH_2), 62.8 (β-Ser), 57.6 (α-Ser), 55.8 (α-Cys), 55.3 (α-Lys), 55.2 (α-Lys), 54.9 (α-Lys), 54.6 (α-Lys), 54.0 (α-AzLys), 52.1 (ε-AzLys), 40.9 (*C*H_2NH(C=O)NH), 40.6 (ε-Lys), 40.5 (ε-Lys), 40.5 (ε-Lys), 35.8 (β-Cys), 34.9 (*C*H_2(C=O)O), 34.6 (*C*H_2(C=O)O), 33.3 (*C*H_2S), 32.8 (*C*H_2-alkyl), 32.1 (β-Lys), 31.9 (β-Lys), 31.7 (β-Lys), 31.5 (β-Lys), 31.2 (β-AzLys), 30.6 (*C*H_2-alkyl), 30.6 (*C*H_2-alkyl), 30.5 (*C*H_2-alkyl), 30.4 (*C*H_2-alkyl), 30.3 (*C*H_2-alkyl), 30.2 (*C*H_2-alkyl), 30.2 (*C*H_2-alkyl), 30.0 (*C*H_2-alkyl), 30.0 (*C*H_2-alkyl), 29.3 (δ-AzLys), 28.0 (δ-Lys), 28.0 (δ-Lys), 27.9 (δ-Lys, *C*H_2-alkyl), 27.9 (δ-Lys, *C*H_2-alkyl), 25.9 (CH_2CH_2(C=O)O), 25.8 (CH_2CH_2(C=O)O), 24.0 (γ-AzLys), 23.8 (γ-Lys), 23.7 (γ-Lys), 23.7 (γ-Lys), 23.6 (γ-Lys), 23.5 (*C*H_2CH$_3$), 14.7 (*C*H_3).

HRMS (ESI-Orbitrap) calcd. for $C_{86}H_{169}N_{16}O_{13}S$ $[M+3H]^{3+}$ 555.42531, found 555.42457.

Fmoc-Lys(Tentagel S Ac)-OAllyl

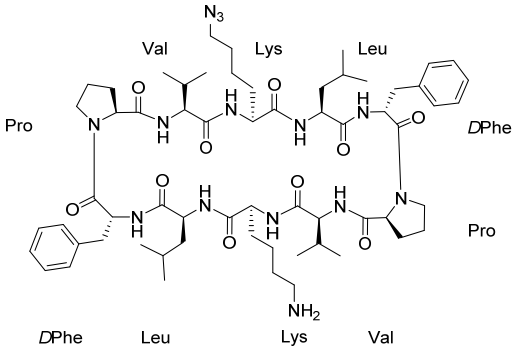


24

A solution containing triphenylphosphine oxide (1.0 g, 3.6 mmol, 7.2 equiv.) in DCM (15 mL, 0.12 M) was cooled to 0 °C and triflic anhydride (1.0 M in DCM, 1.8 mL, 1.8 mmol, 3.6 equiv.). The reaction was stirred at 0 °C for 30 min. during which a white precipitate was formed. A solution of *N*-Boc protected lysine **20** (0.77 g, 1.5 mmol, 3.0 equiv.) in DCM (2.0 mL, 0.75 M) was then added to the suspension and the cooling bath was removed. The reaction was stirred for 5 min. followed by the addition of *N*-methylmorpholine (0.41 mL, 3.8 mmol, 7.5 equiv.) and dibutyltin dilaurate (0.30 mL, 0.50 mmol, 1.0 equiv.). The solution was transferred to TentaGel S Ac resin (0.23 mmol/g, 2.2 g, 0.50 mmol, 1.0 equiv.) which was co-evaporated previously with 1,4-dioxane (3x) and the suspension was shaken for 72 hrs. The suspension was filtered and the resin was washed with DCM (4x)

and Et_2O (4x). Drying the resin over N_2 afforded functionalized resin **24** (2.3 g, 0.44 mmol, 89%) with a loading of 0.19 mmol/g.

***cyclo*(-Leu-*D*Phe-Pro-Val-Lys(N_2)-Leu-*D*Phe-Pro-Val-Lys-)**



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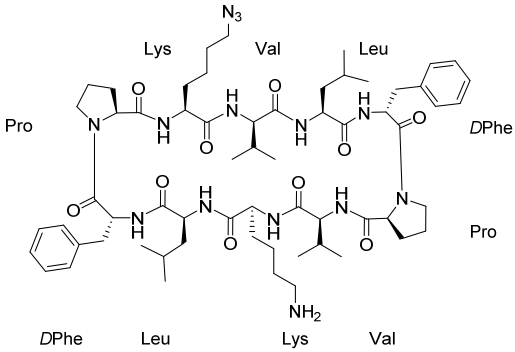
Functionalized resin **24** (0.53 g, 0.10 mmol, 1.0 equiv.) was elongated using the Tribute peptide synthesizer. Afterwards, the resin was washed with DCM (4x), Et_2O (4x) and dried over N_2. Resin was suspended in a mixture of DCM and DMF (1:1, DCM – DMF, 4.0 mL, 25 mM) and swollen for 20 min. Phenylsilane (31 µL, 0.25 mmol, 2.5 equiv.) and $Pd(PPh_3)_4$ (29 mg, 25 µmol, 25 mol%) were added and the resin was shaken for 90 min. while being protected from light. The suspension was filtered and the resin was washed with DCM (3x), 0.50% (w/v) sodium diethyldithiocarbamate in DMF (2x) and DMF (3x). To the resin was added 20% (v/v) piperidine in DMF (5.0 mL, 20 mM) and the mixture was shaken for 10 min. Resin was filtered and 20% (v/v) piperidine in DMF (5.0 mL, 20 mM) was added. The suspension was shaken for 10 min. Afterwards, the resin was filtered and washed with DMF (6x). DMF (4.0 mL, 25 mM) was added to the resin. Subsequently, 1-hydroxybenzotriazole hydrate (68 mg, 0.50 mmol, 5.0 equiv.), benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate (0.26 g, 0.50 mmol, 5.0 equiv.) and *N*-methylmorpholine (0.11 mL, 1.0 mmol, 10 equiv.) were added to the suspension and the reaction was stirred for 2.5 hrs. The suspension was filtered and the residue was washed with DMF (3x) and DCM (3x). A cleavage mixture (190:5:5, TFA – H_2O – TIPS) was added to a small amount of resin (5.0 mg) and shaken for 2 hrs. The suspension was filtered and the filtrate was analyzed by LC-MS.

LC-MS (ESI^+) calcd. for $C_{62}H_{95}N_{14}O_{10}$ $[M+H]^+$ 1195.74, observed 1195.87 with a retention time of 8.91 min.

***cyclo*(-Leu-*D*Phe-Pro-Lys(N_2)-Val-Leu-*D*Phe-Pro-Val-Lys-)**

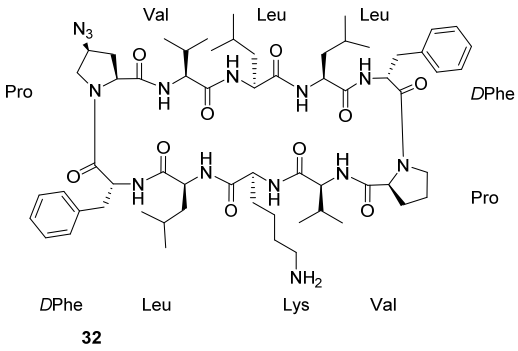


31

Functionalized resin **24** (0.53 g, 0.10 mmol, 1.0 equiv.) was elongated using the Tribute peptide synthesizer. Afterwards, the resin was washed with DCM (4x), Et_2O (4x) and dried over N_2. Resin was suspended in a mixture of DCM and DMF (1:1, DCM – DMF, 4.0 mL, 25 mM) and swollen for 20 min. Phenylsilane (31 µL, 0.25 mmol, 2.5 equiv.) and $Pd(PPh_3)_4$ (29 mg, 25 µmol, 25 mol%) were added and the resin was shaken for 90 min. while being protected from light. The suspension was filtered and the resin was washed with DCM (3x), 0.50% (w/v) sodium diethyldithiocarbamate in DMF (2x) and DMF (3x). To the resin was added 20% (v/v) piperidine in DMF (5.0 mL, 20 mM) and the mixture was shaken for 10 min. Resin was filtered and 20% (v/v) piperidine in DMF (5.0 mL, 20 mM) was added. The suspension was shaken for 10 min. Afterwards, the resin was filtered and washed with DMF (6x). DMF (4.0 mL, 25 mM) was added to the resin. Subsequently, 1-hydroxybenzotriazole hydrate (68 mg, 0.50 mmol, 5.0 equiv.), benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate (0.26 g, 0.50 mmol, 5.0 equiv.) and *N*-methylmorpholine (0.11 mL, 1.0 mmol, 10 equiv.) were added to the suspension and the reaction was stirred for 2.5 hrs. The suspension was filtered and the residue was washed with DMF (3x) and DCM (3x). A cleavage mixture (190:5:5, TFA – H_2O – TIPS) was added to a small amount of resin (5.0 mg) and shaken for 2 hrs. The suspension was filtered and the filtrate was analyzed by LC-MS.

LC-MS (ESI$^+$) calculated for $C_{62}H_{95}N_{14}O_{10}$ [M+H]$^+$ 1195.74, observed 1195.87 with a retention time of 8.71 min.

***cyclo*(-Leu-*D*Phe-Pro((4*S*)-4-azido)-Val-Leu-Leu-*D*Phe-Pro-Val-Lys-)**

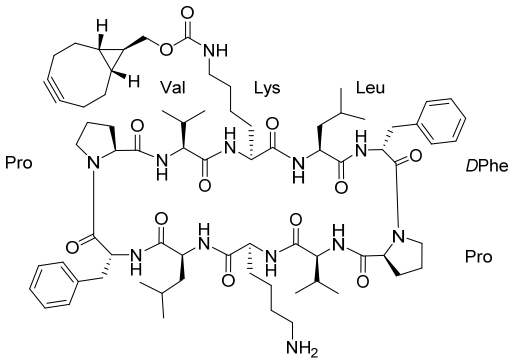


32

Functionalized resin **24** (0.53 g, 0.10 mmol, 1.0 equiv.) was elongated using the Tribute peptide synthesizer. Afterwards, the resin was washed with DCM (4x), Et_2O (4x) and dried over N_2. Resin was suspended in a mixture of DCM and DMF (1:1, DCM – DMF, 4.0 mL, 25 mM) and swollen for 20 min. Phenylsilane (31 µL, 0.25 mmol, 2.5 equiv.) and $Pd(PPh_3)_4$ (29 mg, 25 µmol, 25 mol%) were added and the resin was shaken for 90 min. while being protected from light. The suspension was filtered and the resin was washed with DCM (3x), 0.50% (w/v) sodium diethyldithiocarbamate in DMF (2x) and DMF (3x). To the resin was added 20% (v/v) piperidine in DMF (5.0 mL, 20 mM) and the mixture was shaken for 10 min. Resin was filtered and 20% (v/v) piperidine in DMF (5.0 mL, 20 mM) was added. The suspension was shaken for 10 min. Afterwards, the resin was filtered and washed with DMF (6x). DMF (4.0 mL, 25 mM) was added to the resin. Subsequently, 1-hydroxybenzotriazole hydrate (68 mg, 0.50 mmol, 5.0 equiv.), benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate (0.26 g, 0.50 mmol, 5.0 equiv.) and *N*-methylmorpholine (0.11 mL, 1.0 mmol, 10 equiv.) were added to the suspension and the reaction was stirred for 2.5 hrs. The suspension was filtered and the residue was washed with DMF (3x) and DCM (3x). A cleavage mixture (190:5:5, TFA – H_2O – TIPS) was added to a small amount of resin (5.0 mg) and shaken for 2 hrs. The suspension was filtered and the filtrate was analyzed by LC-MS.

LC-MS (ESI$^+$) calculated for $C_{62}H_{95}N_{14}O_{10}$ [M+H]$^+$ 1195.74, observed 1195.87 with a retention time of 8.80 min.

***cyclo*(-Leu-*D*Phe-Pro-Val-Lys(BCN)-Leu-*D*Phe-Pro-Val-Lys-)**

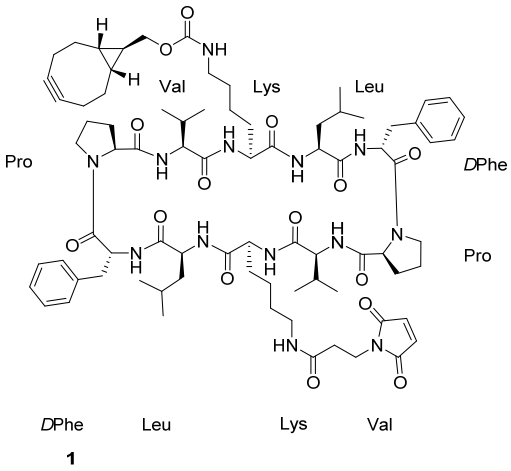


28

To functionalized resin **25** (0.10 mmol, 1.0 equiv.) was added 1,4-dioxane (10 mL, 10 mM) followed by trimethylphosphine (1.0 M in toluene, 1.6 mL, 16 equiv.) and the suspension was stirred for 2 hrs. H_2O (1.0 mL, 55 mmol, 5.5 x 10^2 equiv.) was added and the resin was shaken for an additional 4 hrs. The suspension was filtered and the resin was washed with 1,4-dioxane (3x) and DCM (3x). A solution of BCN PNP ester (63 mg, 0.20 mmol, 2.0 equiv.), DIPEA (70 µL, 0.40 mmol. 4.0 equiv.) in DMF (4.0 mL, 25 mM) was added to the resin and was subsequently shaken for 1 week. The suspension was filtered and the resin was washed with DMF (4x) and DCM (4x). The resin was treated with a cleavage cocktail (TFA – DCM, 1:199, 10 mL) for 2 min. The suspension was filtered into a vigorously stirred mixture of Amberlyst A-21 (7.0 g, previously rinsed with MeOH, THF and DCM) in DCM (20 mL) to neutralize the TFA. This procedure was repeated ten times. The Amberlyst A-21 resin was separated by filtration and rinsed with additional DCM. The filtrate was concentrated *in vacuo* and purified by RP-HPLC (Agilent 1200, 63% to 69% solvent B) to obtain alkyne **28** (4.4 mg, 3.3 µmol, 3.3%) as a white solid.

LC-MS (ESI$^+$) calcd. for $C_{73}H_{109}N_{12}O_{12}$ [M+H]$^+$ 1345.83, found 1345.87 with a retention time of 9.49 min.

***cyclo*(-Leu-*D*Phe-Pro-Val-Lys(BCN)-Leu-*D*Phe-Pro-Val-Lys(Maleimido)-)**

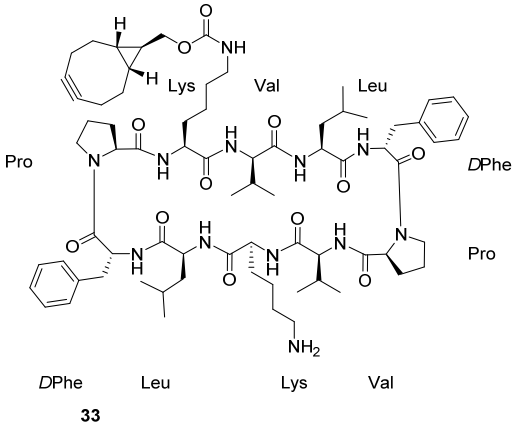


1

Crude alkyne **28** (40 mg, 30 μmol, 1.0 equiv.) was dissolved in DMF (0.50 mL, 60 mM). 3-Maleimidopropionic acid NHS ester (40 mg, 0.15 mmol, 5.0 equiv.) and *N*-methylmorpholine (17 μL, 0.15 mmol, 5.0 equiv.) were added and the reaction was stirred for 4 hrs. The mixture was concentrated *in vacuo* at room temperature. Purification by RP-HPLC (Agilent 1200, 78% to 84% solvent B) and subsequent lyophilization furnished maleimide **1** (5.6 mg, 3.7 μmol, 3.7%) as a white solid.

HRMS (ESI-Orbitrap) calcd. for $C_{80}H_{114}N_{13}O_{15}$ $[M+H]^+$ 1496.85519, found 1496.85720.

***cyclo*(-Leu-*D*Phe-Pro-Lys(BCN)-Val-Leu-*D*Phe-Pro-Val-Lys-)**

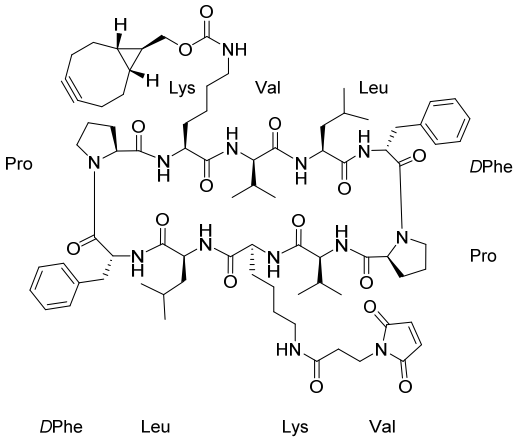


33

To functionalized resin (0.10 mmol, 1.0 equiv.) was added 1,4-dioxane (10 mL, 10 mM) followed by trimethylphosphine (1.0 M in toluene, 1.6 mL, 16 equiv.) and the suspension was stirred for 2 hrs. H_2O (1.0 mL, 55 mmol, 5.5 x 10^2 equiv.) was added and the resin was shaken for an additional 4 hrs. The suspension was filtered and the resin was washed with 1,4-dioxane (3x) and DCM (3x). A solution of BCN PNP ester (63 mg, 0.20 mmol, 2.0 equiv.), DIPEA (70 μL, 0.40 mmol. 4.0 equiv.) in DMF (4.0 mL, 25 mM) was added to the resin and was subsequently shaken for 1 week. The suspension was filtered and the resin was washed with DMF (4x) and DCM (4x). The resin was treated with a cleavage cocktail (TFA – DCM, 1:199, 10 mL) for 2 min. The suspension was filtered into a vigorously stirred mixture of Amberlyst A-21 (7.0 g, previously rinsed with MeOH, THF and DCM) in DCM (20 mL) to neutralize the TFA. This procedure was repeated ten times. The Amberlyst A-21 resin was separated by filtration and rinsed with additional DCM. The filtrate was concentrated *in vacuo* and purified by RP-HPLC (Agilent 1200, 57% to 63% solvent B) to obtain alkyne **33** (6.4 mg, 4.8 μmol, 4.8%) as a white solid.

HRMS (ESI-Orbitrap) calcd. for $C_{73}H_{109}N_{12}O_{12}$ $[M+H]^+$ 1345.82824, found 1345.82947.

***cyclo*(-Leu-*D*Phe-Pro-Lys(BCN)-Val-Leu-*D*Phe-Pro-Val-Lys(Maleimido)-)**

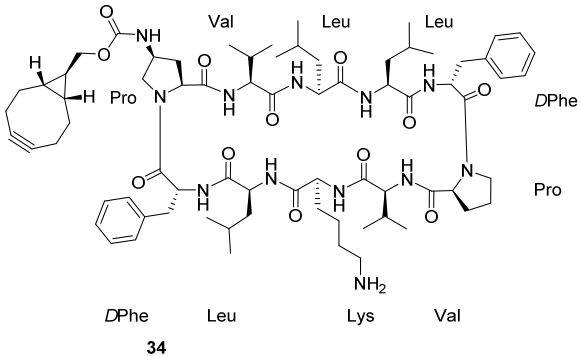


2

Crude alkyne **33** (33 mg, 25 μmol, 1.0 equiv.) was dissolved in DMF (0.50 mL, 50 mM). 3-Maleimidopropionic acid NHS ester (40 mg, 0.15 mmol, 6.0 equiv.) and *N*-methylmorpholine (17 μL, 0.15 mmol, 6.0 equiv.) were added and the reaction was stirred for 4 hrs. The mixture was concentrated *in vacuo* at room temperature. Purification by RP-HPLC (Agilent 1200, 74% to 77% solvent B) and subsequent lyophilization furnished maleimide **2** (5.0 mg, 3.3 μmol, 3.3%) as a white solid.

HRMS (ESI-Orbitrap) calcd. for $C_{88}H_{113}N_{13}O_{15}Na$ $[M+Na]^+$ 1518.83713, found 1518.83894.

***cyclo*(-Leu-*D*Phe-Pro((4*S*)-4-NHBCN)-Val-Leu-Leu-*D*Phe-Pro-Val-Lys-)**



34

To functionalized resin (0.10 mmol, 1.0 equiv.) was added 1,4-dioxane (10 mL, 10 mM) followed by trimethylphosphine (1.0 M in toluene, 1.6 mL, 16 equiv.) and the suspension was stirred for 2 hrs. H_2O (1.0 mL, 55 mmol, 5.5 x 10^2 equiv.) was added and the resin was shaken for an additional 4 hrs. The suspension was filtered and the resin was washed with 1,4-dioxane (3x) and DCM (3x). A solution of BCN PNP ester (63 mg, 0.20 mmol, 2.0 equiv.), DIPEA (70 μL, 0.40 mmol. 4.0 equiv.) in DMF (4.0 mL, 25 mM) was added to the resin and was subsequently shaken for 1 week. The suspension was filtered and the resin was washed with DMF (4x) and DCM (4x). The resin was treated with a cleavage cocktail (TFA – DCM, 1:199, 10 mL) for 2 min. The suspension was filtered into a vigorously stirred mixture of Amberlyst A-21 (7.0 g, previously rinsed with MeOH, THF and DCM) in DCM (20 mL) to neutralize the TFA. This procedure was repeated ten times. The Amberlyst A-21 resin was separated by filtration and rinsed with additional DCM. The filtrate was concentrated *in vacuo* and purified by RP-HPLC (Agilent 1200, 59% to 65% solvent B) to obtain alkyne **34** (4.6 mg, 3.4 μmol, 3.4%) as a white solid.

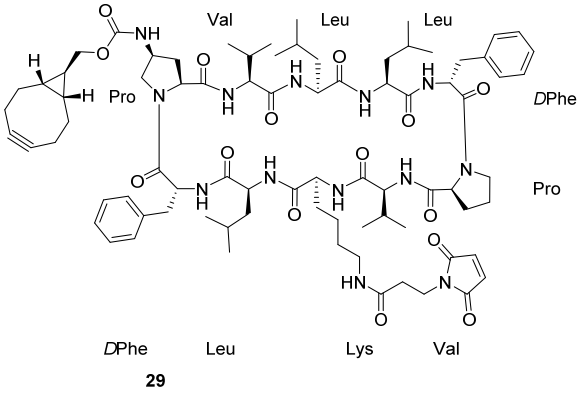
^{1}H NMR (500 MHz, DMF) δ 8.91 (d, *J* = 4.4 Hz, 1H, N*H*-*D*Phe), 8.58 (d, *J* = 9.2 Hz, 1H, N*H*-Lys), 8.53 – 8.45 (m, 3H, N*H*-Leu), 8.40 (d, *J* = 5.8 Hz, 1H, N*H*-*D*Phe), 8.23 (br s, 3H, NH_2-Lys), 7.41 – 7.25 (m, 11H, N*H*-Val, C*H*-arom), 7.20 (d, *J* = 9.1 Hz, 1H, N*H*-Val), 6.93 (d, *J* = 13.4 Hz, 1H, N*H*BCN), 5.01 – 4.92 (m, 1H, α-Lys), 4.82 (q, *J* = 8.0 Hz, 1H, α-Leu), 4.71 – 4.56 (m, 4H, α-*D*Phe, α-Leu), 4.50 (dd, *J* = 9.2, 6.9 Hz, 1H, α-Val), 4.48 – 4.39 (m, 3H, α-Val, α-Pro, α-AmPro), 3.93 – 3.85 (m, 2H, NH(C=O)OCH_2), 3.77 – 3.71 (m, 1H, δ-Pro), 3.45 – 3.31 (m, 3H, γ-AmPro, δ-AmPro), 3.22 – 3.09 (m, 3H, ε-Lys, β-*D*Phe), 3.06 – 2.98 (m, 3H, β-*D*Phe), 2.88 – 2.81 (m, 1H, δ-Pro), 2.39 – 2.08 (m, 9H, CH_2Cp, β-AmPro, CH_2C≡C, β-Val), 2.05 – 2.02 (m, 1H, β-Pro), 2.00 – 1.92 (m, 1H, β-AmPro), 1.90 – 1.64 (m, 8H, β-Lys, δ-Lys, γ-Pro, β-Pro, γ-Leu, β-Leu), 1.62 – 1.31 (m, 12H, β-Leu, γ-Leu, β-Lys, γ-Lys, β-Leu, CH_2Cp), 0.99 – 0.81 (m, 30H, γ-Val, δ-Leu), 0.75 – 0.68 (m, 2H, C*H*-bridgehead), 0.64 – 0.60 (m, 1H, NH(C=O)OCH$_2$C*H*).

^{13}C NMR (126 MHz, DMF) δ 173.4 (NH(*C*=O)), 172.8 (NH(*C*=O)), 172.5 (NH(*C*=O)), 172.3 (NH(*C*=O)), 172.0 (NH(*C*=O)), 172.0 (NH(*C*=O)), 171.9 (NH(*C*=O)), 171.6 (NH(*C*=O)), 171.5 (NH(*C*=O)), 156.7 (NH(*C*=O)O), 138.2 (*C*q-arom), 137.7 (*C*q-

arom), 130.5 (CH-arom), 129.7 (CH-arom), 129.5 (CH-arom), 128.2 (CH-arom), 127.9 (CH-arom), 99.7 (C≡C), 69.2 (NH(C=O)OCH_2), 61.2 (α-Pro) , 60.0 (α-AmPro), 59.0 (α-Val), 58.4 (α-Val), 55.1 (α-*D*Phe), 54.8 (α-*D*Phe), 53.4 (α-Lys), 52.7 (δ-AmPro), 52.5 (α-Leu), 51.5 (α-Leu), 51.2 (α-Leu), 50.7 (γ-AmPro), 47.3 (δ-Pro), 42.4 (β-Leu), 42.3 (β-Leu), 41.9 (β-Leu), 41.1 (ε-Lys), 37.6 (β-*D*Phe), 37.3 (β-*D*Phe), 35.9 (β-AmPro), 34.2 (CH_2Cp), 34.1 (β-Lys), 32.9 (β-Val), 32.6 (β-Val), 30.3 (β-Pro), 28.8 (δ-Lys), 25.8 (γ-Leu), 25.6 (γ-Leu), 24.7 (NH(C=O)OCH_2CH), 24.7 (γ-Pro), 23.7 (δ-Leu), 23.7 (CH-bridgehead), 23.6 (δ-Leu), 23.5 (γ-Lys), 23.5 (δ-Leu), 23.5 (δ-Leu), 23.4 (δ-Leu), 23.2 (δ-Leu), 21.9 (CH_2C≡C), 20.1 (γ-Val), 20.0 (γ-Val), 19.6 (γ-Val), 19.1 (γ-Val).

HRMS (ESI-Orbitrap) calcd. for $C_{73}H_{108}N_{12}O_{12}Na$ $[M+Na]^+$ 1367.81019, found 1367.81108.

***cyclo*(-Leu-*D*Phe-Pro((4*S*)-4-NHBCN)-Val-Leu-Leu-*D*Phe-Pro-Val-Lys(Maleimido)-)**



29

Crude alkyne **34** (30 mg, 22 μmol, 1.0 equiv.) was dissolved in DMF (0.50 mL, 44 mM). 3-Maleimidopropionic acid NHS ester (40 mg, 0.15 mmol, 6.8 equiv.) and *N*-methylmorpholine (17 μL, 0.15 mmol, 6.8 equiv.) were added and the reaction was stirred for 4 hrs. The mixture was concentrated *in vacuo* at room temperature. Purification by RP-HPLC (Agilent 1200, 77% to 80% solvent B) and subsequent lyophilization furnished maleimide **29** (5.0 mg, 3.3 μmol, 3.3%) as a white solid.

HRMS (ESI-Orbitrap) calcd. for $C_{73}H_{108}N_{12}O_{12}Na$ $[M+Na]^+$ 1518.83713, found 1518.83842.

References

(1) Del Giudice, G.; Rappuoli, R.; Didierlaurent, A. M. Correlates of Adjuvanticity: A Review on Adjuvants in Licensed Vaccines. *Semin. Immunol.* **2018**, *39*, 14–21. https://doi.org/10.1016/j.smim.2018.05.001.

(2) McKee, A. S.; Marrack, P. Old and New Adjuvants. *Curr. Opin. Immunol.* **2017**, *47*, 44–51. https://doi.org/10.1016/j.coi.2017.06.005.

(3) Glenny, A. T.; Pope, C. G.; Waddington, H.; Wallace, U. Immunological Notes. XVII–XXIV. *J. Pathol. Bacteriol.* **1926**, *29* (1), 31–40. https://doi.org/10.1002/path.1700290106.

(4) Marrack, P.; McKee, A. S.; Munks, M. W. Towards an Understanding of the Adjuvant Action of Aluminium. *Nat. Rev. Immunol.* **2009**, *9* (4), 287–293. https://doi.org/10.1038/nri2510.

(5) Janeway, C. A. Approaching the Asymptote? Evolution and Revolution in Immunology. *Cold Spring Harb. Symp. Quant. Biol.* **1989**, *54 Pt 1*, 1–13. https://doi.org/10.1101/sqb.1989.054.01.003.

(6) O'Neill, L. A. J.; Golenbock, D.; Bowie, A. G. The History of Toll-like Receptors - Redefining Innate Immunity. *Nat. Rev. Immunol.* **2013**, *13* (6), 453–460. https://doi.org/10.1038/nri3446.

(7) Applequist, S. E.; Wallin, R. P. A.; Ljunggren, H.-G. Variable Expression of Toll-like Receptor in Murine Innate and Adaptive Immune Cell Lines. *Int. Immunol.* **2002**, *14* (9), 1065–1074. https://doi.org/10.1093/intimm/dxf069.

(8) Botos, I.; Segal, D. M.; Davies, D. R. The Structural Biology of Toll-like Receptors. *Structure* **2011**, *19* (4), 447–459. https://doi.org/10.1016/j.str.2011.02.004.

(9) Hess, N. J.; Jiang, S.; Li, X.; Guan, Y.; Tapping, R. I. TLR10 Is a B Cell Intrinsic Suppressor of Adaptive Immune Responses. *J. Immunol.* **2017**, *198* (2), 699–707. https://doi.org/10.4049/jimmunol.1601335.

(10) Mancini, R. J.; Stutts, L.; Ryu, K. A.; Tom, J. K.; Esser-Kahn, A. P. Directing the Immune System with Chemical Compounds. *ACS Chem. Biol.* **2014**, *9* (5), 1075–1085. https://doi.org/10.1021/cb500079s.

(11) Ulrich, J. T.; Myers, K. R. Monophosphoryl Lipid A as an Adjuvant. In *Vaccine Design: The Subunit and Adjuvant Approach*; Powell, M. F., Newman, M. J., Eds.; Pharmaceutical Biotechnology; Springer US: Boston, MA, 1995; pp 495–524. https://doi.org/10.1007/978-1-4615-1823-5_21.

(12) Stadelmaier, A.; Morath, S.; Hartung, T.; Schmidt, R. R. Synthesis of the First Fully Active Lipoteichoic Acid. *Angew. Chem. Int. Ed.* **2003**, *42* (8), 916–920. https://doi.org/10.1002/anie.200390243.

(13) Trinchieri, G.; Sher, A. Cooperation of Toll-like Receptor Signals in Innate Immune Defence. *Nat. Rev. Immunol.* **2007**, *7* (3), 179–190. https://doi.org/10.1038/nri2038.

(14) Garcia-Cordero, J. L.; Nembrini, C.; Stano, A.; Hubbell, J. A.; Maerkl, S. J. A High-Throughput Nanoimmunoassay Chip Applied to Large-Scale Vaccine Adjuvant Screening. *Integr. Biol.* **2013**, *5* (4), 650–658. https://doi.org/10.1039/C3IB20263A.

(15) Kasturi, S. P.; Skountzou, I.; Albrecht, R. A.; Koutsonanos, D.; Hua, T.; Nakaya, H. I.; Ravindran, R.; Stewart, S.; Alam, M.; Kwissa, M.; Villinger, F.; Murthy, N.; Steel, J.; Jacob, J.; Hogan, R. J.; García-Sastre, A.; Compans, R.; Pulendran, B. Programming the Magnitude and Persistence of Antibody Responses with Innate Immunity. *Nature* **2011**, *470* (7335), 543–547. https://doi.org/10.1038/nature09737.

(16) Mancini, R. J.; Tom, J. K.; Esser-Kahn, A. P. Covalently Coupled Immunostimulant Heterodimers. *Angew. Chem. Int. Ed.* **2014**, *53* (1), 189–192. https://doi.org/10.1002/anie.201306551.

(17) Shukla, N. M.; Mutz, C. A.; Malladi, S. S.; Warshakoon, H. J.; Balakrishna, R.; David, S. A. Toll-Like Receptor (TLR)-7 and -8 Modulatory Activities of Dimeric Imidazoquinolines. *J. Med. Chem.* **2012**, *55* (3), 1106–1116. https://doi.org/10.1021/jm2010207.

(18) Ryu, K. A.; Slowinska, K.; Moore, T.; Esser-Kahn, A. Immune Response Modulation of Conjugated Agonists with Changing Linker Length. *ACS Chem. Biol.* **2016**, *11* (12), 3347–3352. https://doi.org/10.1021/acschembio.6b00895.

(19) Silla, U.; Mutter, M. Topological Templates as Tool in Molecular Recognition and Peptide Mimicry: Synthesis of a TASK Library. *J. Mol. Recognit.* **1995**, *8* (1–2), 29–34. https://doi.org/10.1002/jmr.300080105.

(20) Dumy, P.; Eggleston, I. M.; Cervigni, S.; Sila, U.; Sun, X.; Mutter, M. A Convenient Synthesis of Cyclic Peptides as Regioselectively Addressable Functionalized Templates (RAFT). *Tetrahedron Lett.* **1995**, *36* (8), 1255–1258. https://doi.org/10.1016/0040-4039(94)02481-P.

(21) Dumy, P.; Eggleston, I. M.; Esposito, G.; Nicula, S.; Mutter, M. Solution Structure of Regioselectively Addressable Functionalized Templates: An NMR and Restrained Molecular Dynamics Investigation. *Biopolymers* **1996**, *39* (3), 297–308. https://doi.org/10.1002/(sici)1097-0282(199609)39:3<297::aid-bip3>3.0.co;2-j.

(22) Pifferi, C.; Berthet, N.; Renaudet, O. Cyclopeptide Scaffolds in Carbohydrate-Based Synthetic Vaccines. *Biomater. Sci.* **2017**, *5* (5), 953–965. https://doi.org/10.1039/C7BM00072C.

(23) Madge, H. Y. R.; Sharma, H.; Hussein, W. M.; Khalil, Z. G.; Capon, R. J.; Toth, I.; Stephenson, R. J. Structure–Activity Analysis of Cyclic Multicomponent Lipopeptide Self-Adjuvanting Vaccine Candidates Presenting

Group A Streptococcus Antigens. *J. Med. Chem.* **2020**, *63* (10), 5387–5397. https://doi.org/10.1021/acs.jmedchem.0c00203.

(24) Llamas-Saiz, A. L.; Grotenbreg, G. M.; Overhand, M.; van Raaij, M. J. Double-Stranded Helical Twisted β-Sheet Channels in Crystals of Gramicidin S Grown in the Presence of Trifluoroacetic and Hydrochloric Acids. *Acta Crystallogr. D Biol. Crystallogr.* **2007**, *63* (3), 401–407. https://doi.org/10.1107/S0907444906056435.

(25) Asano, A.; Matsuoka, S.; Minami, C.; Kato, T.; Doi, M. [Leu2]Gramicidin S Preserves the Structural Properties of Its Parent Peptide and Forms Helically Aligned β-Sheets. *Acta Crystallogr. Sect. C Struct. Chem.* **2019**, *75* (10), 1336–1343. https://doi.org/10.1107/S2053229619011872.

(26) Willems, M. M. J. H. P.; Zom, G. G.; Khan, S.; Meeuwenoord, N.; Melief, C. J. M.; van der Stelt, M.; Overkleeft, H. S.; Codée, J. D. C.; van der Marel, G. A.; Ossendorp, F.; Filippov, D. V. N-Tetradecylcarbamyl Lipopeptides as Novel Agonists for Toll-like Receptor 2. *J. Med. Chem.* **2014**, *57* (15), 6873–6878. https://doi.org/10.1021/jm500722p.

(27) Gential, G. P. P.; Hogervorst, T. P.; Tondini, E.; van de Graaff, M. J.; Overkleeft, H. S.; Codée, J. D. C.; van der Marel, G. A.; Ossendorp, F.; Filippov, D. V. Peptides Conjugated to 2-Alkoxy-8-Oxo-Adenine as Potential Synthetic Vaccines Triggering TLR7. *Bioorg. Med. Chem. Lett.* **2019**, *29* (11), 1340–1344. https://doi.org/10.1016/j.bmcl.2019.03.048.

(28) Matysiak, S.; Böldicke, T.; Tegge, W.; Frank, R. Evaluation of Monomethoxytrityl and Dimethoxytrityl as Orthogonal Amino Protecting Groups in Fmoc Solid Phase Peptide Synthesis. *Tetrahedron Lett.* **1998**, *39* (13), 1733–1734. https://doi.org/10.1016/S0040-4039(98)00055-0.

(29) Wang, X.; Gobbo, P.; Suchy, M.; Workentin, M. S.; Hudson, R. H. E. Peptide-Decorated Gold Nanoparticles via Strain-Promoted Azide–Alkyne Cycloaddition and Post Assembly Deprotection. *RSC Adv.* **2014**, *4* (81), 43087–43091. https://doi.org/10.1039/C4RA07574A.

(30) Chigrinova, M.; McKay, C. S.; Beaulieu, L.-P. B.; Udachin, K. A.; Beauchemin, A. M.; Pezacki, J. P. Rearrangements and Addition Reactions of Biarylazacyclooctynones and the Implications to Copper-Free Click Chemistry. *Org. Biomol. Chem.* **2013**, *11* (21), 3436–3441. https://doi.org/10.1039/C3OB40683K.

(31) Srinivasan, N.; Yurek-George, A.; Ganesan, A. Rapid Deprotection of N-Boc Amines by TFA Combined with Freebase Generation Using Basic Ion-Exchange Resins. *Mol. Divers.* **2005**, *9* (4), 291–293. https://doi.org/10.1007/s11030-005-4386-8.

(32) Spangler, B.; Yang, S.; Baxter Rath, C. M.; Reck, F.; Feng, B. Y. A Unified Framework for the Incorporation of Bioorthogonal Compound Exposure Probes within Biological Compartments. *ACS Chem. Biol.* **2019**, *14* (4), 725–734. https://doi.org/10.1021/acschembio.9b00008.