Synthesis of cyclic peptides as bioconjugation platforms

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4

Design and synthesis of gramicidin S-based scaffolds having three orthogonal handles to append various TLR ligands

A remarkable feat of the yellow fever vaccine is the duration of immunity it provides: up to 80% of vaccinees demonstrate immunity for as long as 40 years.[1] The vaccine was created by Theiler and Smith after the yellow fever virus was first isolated in 1927 by repeatedly propagating the virus over mouse and chicken embryos, stripped of nervous tissue, leading to an attenuated strain termed YF-17D.[2] This virus strain proved to be a safe and effective immunizing agent and since the late 1930s until 2014 has been administered approximately 540 million times.[3] Research into the immunological mechanism of the vaccine revealed the

activation of multiple dendritic cell subsets *via* TLRs 2, 7, 8, and 9 to elicit a broad spectrum of innate and adaptive immune responses leading to long-lasting immunity.[4]

The importance of engaging multiple TLRs is further demonstrated by the group of Berzofsky who found that utilizing a combination of TLR2/6, TLR3 and TLR9 agonists greatly increased the protective efficacy of vaccination with an HIV envelope peptide in mice when compared with using a mix of ligands for only two of these TLRs. Compared to the dual mixtures which amplify T cell responses by increasing the production of T cells, they found that this specific triple combination of TLR agonists augmented the quality of the T cell responses primarily by increasing their functional avidity for the antigen necessary for clearing the virus.[5]

Interestingly, Esser-Kahn and co-workers showed that covalently linking three different TLR ligands substantially alters the resulting immune response compared to the unlinked counterparts. Comparison of five constructs with a different combination of covalently linked triagonists revealed a distinct immune response for each construct with a varying T_H1/T_H2 balance.[6] This was validated in a follow-up study by Gilkes *et al*. with an *in vivo* mouse challenge model of TLR triagonists in combination with a Q fever antigen showing that spatial organization of the triagonist construct can shape immune responses toward desired outcomes.[7]

The research in this Chapter describes the expansion of the dual RAFT platform described in Chapter 4 to a triple RAFT platform. With the ultimate aim to study the effect of spatial orientation of three TLR ligands on the immune response, the three ligation handles are positioned in two manners (*Figure 1*). To achieve this goal, the availability of one additional chemoselective handle, allowing a triple orthogonal ligation strategy, is required. Sequential ligation strategies have been reported before, such as by Willems *et al.* who used a two-step procedure for simultaneous tetrazine/norbornene ligation and Staudinger-Bertozzi ligation, followed by a copper(I)-catalyzed alkyne-azide cycloaddition (CuAAC) to monitor multiple enzymatic activities by activity-based protein profiling.[8] Another ligation strategy, published by Simon *et al*. employed a tetrazine/cyclopropane ligation followed by strain-promoted alkyne-azide cycloaddition (SPAAC) and finally a CuAAC to monitor the formation of plant cell walls.[9] Thomas *et al*. established a cascade methodology for ligating four different glycosides on the RAFT scaffold, introduced by the group of Mutter.[10,11] Functionalized glycosides were ligated on the scaffold by

sequential oxime ligation, photocatalyzed thiol-ene reaction, CuAAC and finally chloroacetamide/thiol coupling.

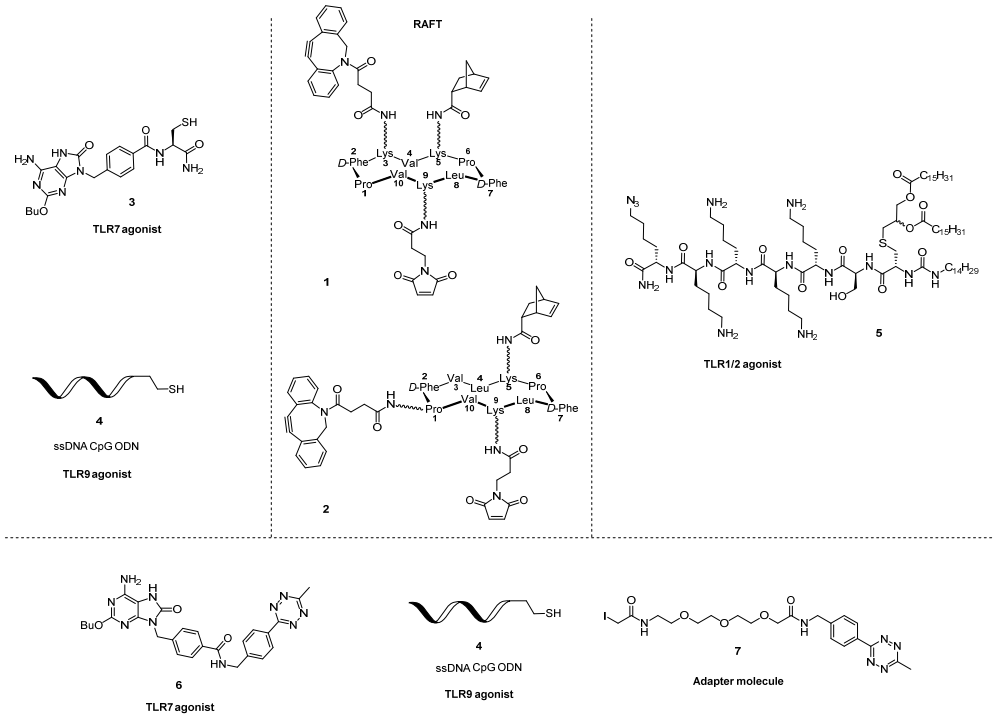


Figure 1. Design of the RAFT scaffold equipped with three orthogonal ligation handles to accommodate for three different TLR agonists.

This Chapter entails the design and synthesis of triple functionalized RAFT platforms with the focus on making the ensuing ligation as streamlined as possible by excluding additional reagents and equipment. Therefore, the choice was made to expand the thiol/maleimide coupling and SPAAC with a tetrazine/norbornene ligation. Since 1,2,4,5-tetrazines are known to react with strained cycloalkynes, the reagents and conditions had to be chosen carefully.[12] Karver and co-workers were able to mitigate this cross-reaction by employing a bulky cycloalkyne and disubstituted tetrazine to create a mutually orthogonal SPAAC and tetrazine/*trans*-cyclooctene ligation pair.[13] Therefore, the bicyclo[6.1.0]nonyne (BCN) group, which was not compatible with tetrazines, was replaced with the more bulky aza-dibenzocyclooctyne (DBCO) (*Figure 1*).[14–16]

On the basis of these considerations, RAFT scaffolds **1** and **2**, equipped with three orthogonal chemoselective handles to create TLR1/2-7-9 triagonists with the ligands in different orientations were selected as targets (*Figure 1*). The cyclic decapeptide gramicidin S will serve as the basis for the scaffold and will be suitably modified to incorporate three different amino-groups for functionalization. Gramicidin S adopts an antiparallel β sheet conformation which is closed by two type II′ β-turns and is highly stabilized by four intramolecular hydrogen bonds involving the backbone.[17] 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 (*Figure 1*).[18] Asano and co-workers showed that the secondary structure is not perturbed by substitution of the ornithine residues with leucine residues proving the conformation is rigid and allows for shuffling of amino acid residues 3-4-5 and 8-9-10 and helps accommodating three functionalization handles in different orientation.[19] The positioning of the handles in scaffold **2** allows for the largest possible distance between the three different ligands. In turn, scaffold **1** has two of the three attachments points at the same face of the macrocycle and one at the opposite face. To attach the TLR-agonists to scaffolds **1** and **2**, TLR7 and TLR9 ligands will be equipped with a tetrazine and in the case of TLR9 agonist this will be achieved by an adapter molecule. These functionalized ligands combined with the ones described in the previous Chapter make possible two different combinations for scaffold **1** with either the TLR7 agonist or the TLR9 ligand being the lone ligand in the plane giving a total of three different scaffolds with the inclusion of scaffold **2**.

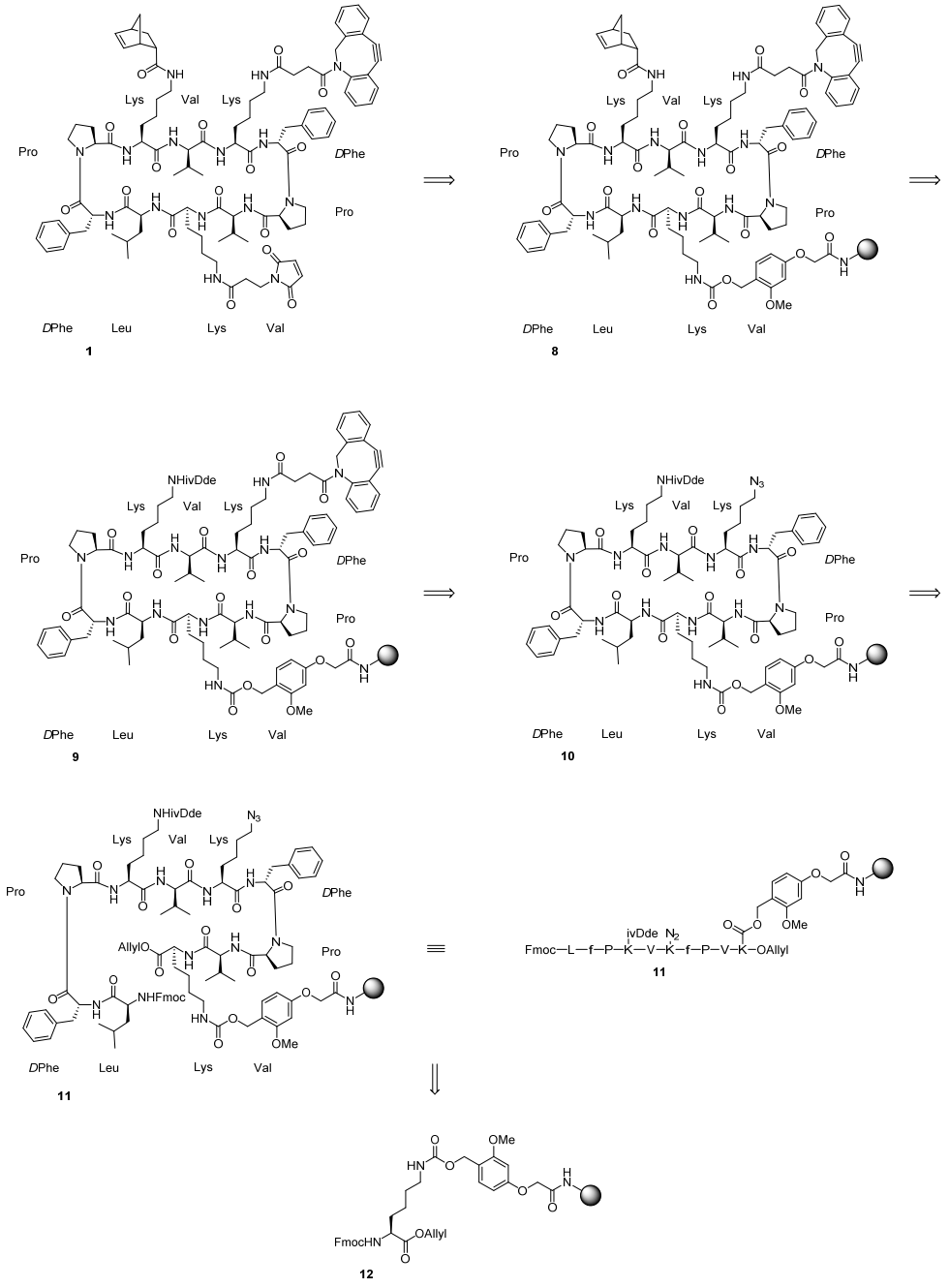
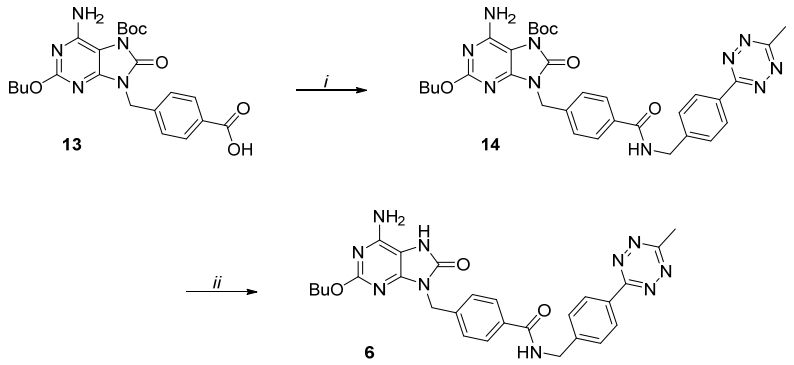


Figure 2. Retrosynthesis of RAFT scaffold **1** with three chemoselective handles.

The synthesis of RAFT scaffold **1**, and in a similar manner **2**, was envisioned to start from the TentaGel S Ac resin, allowing weakly acidic cleavage mixtures at the end of the assembly (*Figure 2*). With the procedure described in Chapter 2 and expanded in Chapter 3, the ε-amine of lysine will be attached to the resin to give functionalized resin **12**. Using an automated solid-phase peptide synthesis strategy, linear peptide **11** will be synthesized bearing four different protecting groups, which each can be removed orthogonally in the presence of the other three and the linker. To complement the allyl-, Fmoc- and azido-group, the ivDde moiety will be used as the fourth protecting group as it can be removed by treatment with hydrazine and is able to withstand Fmoc deprotection conditions. The α,β-unsaturated ketone present in the ivDde group reacts with hydrazine to form a pyrazole ring liberating the amino-group in the process.[20,21] Of the four protecting groups present, first the allyl-group will be removed using palladium(0) tetrakis(triphenylphosphine) and phenylsilane, after which the Fmoc is deprotected and the peptide cyclized with PyBOP as the condensing agent to furnish cyclic peptide **10**. The azide will then be reduced *via* a Staudinger reduction using trimethylphosphine and functionalized with a DBCO-group. The ivDde moiety will be removed with a solution of hydrazine in DMF to liberate the amine, which will then be functionalized with a norbornene moiety to give peptide **8**. The penultimate step will involve treatment of the resin to a mixture of TFA and DCM (1:199, TFA – DCM) to cleave the peptide off of the resin. Finally, the maleimide moiety will be installed to afford RAFT scaffold **1**.

Results and discussion

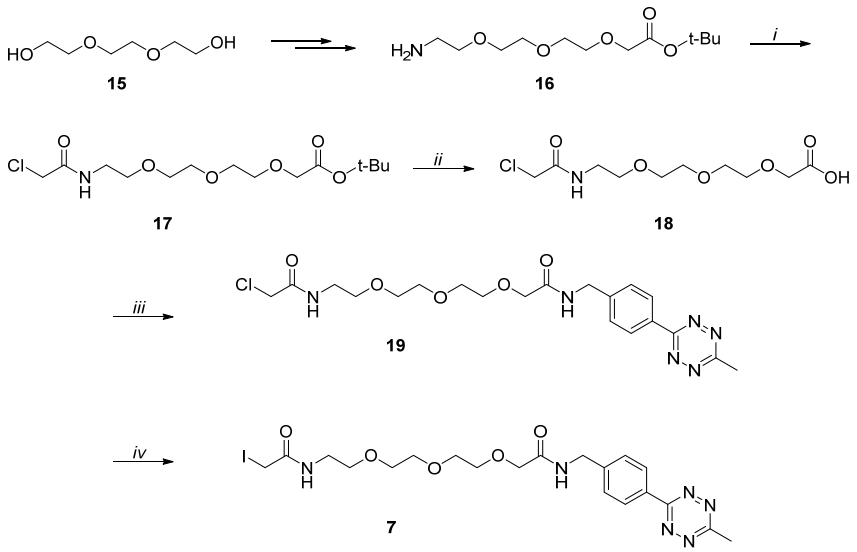
With the inclusion of the tetrazine/norbornene ligation as part of the triple RAFT scaffold, TLR ligands equipped with a tetrazine had to be synthesized. First, the synthesis of functionalized TLR7 ligand **6** was undertaken (*Scheme 1*).



Scheme 1. Reagents and conditions: (*i*) (4-(6-methyl-1,2,4,5-tetrazin-3-yl)phenyl)methanamine hydrochloride, DIC, *N*-methylmorpholine, DCM, DMF, rt, 19 hrs, 18% (*ii*) TFA, DCM, rt, 3 hrs, quant.

The carboxylic acid in known TLR7 ligand derivative **13** (see Chapter 3) was activated by treatment with diisopropylcarbodiimide (DIC) and *N*-methylmorpholine in a DCM for one hour, followed by dropwise addition of a solution of (4-(6-methyl-1,2,4,5-tetrazin-3-yl)phenyl)methanamine hydrochloride in a mixture of DCM and DMF (1:1, DCM – DMF) to afford, after stirring for 19 hours, tetrazine **14** in 18% yield. To remove the Boc-group, a mixture of trifluoroacetic acid and DCM (1:2, TFA – DCM) was added to tetrazine **14** and the reaction was stirred for three hours to give TLR7 ligand **6** equipped with a tetrazine in a quantitative yield.

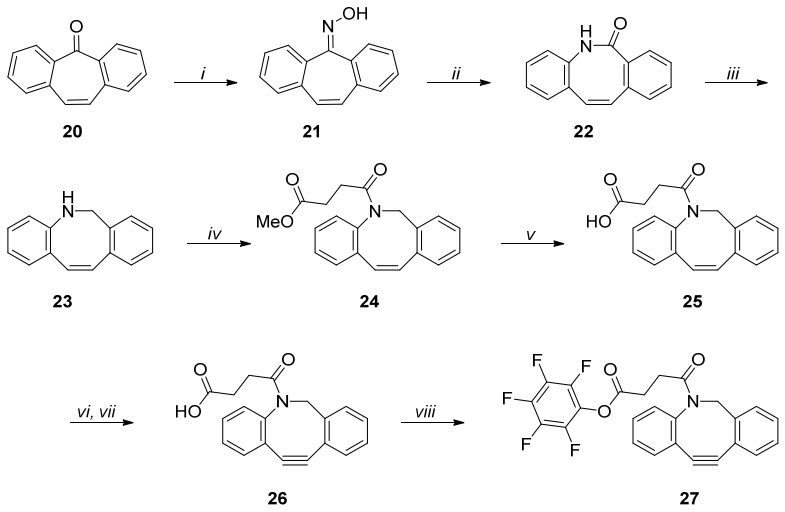
TLR9 ligand is an oligonucleotide provided with a linker functionalized with a thiol-group (*Figure 1*). To equip TLR9 ligand with a tetrazine, adapter molecule **7** was designed featuring an iodoacetamide group complementary with the thiol-group (*Scheme 2*).



Scheme 2. Reagents and conditions: (*i*) chloroacetyl chloride, Et_3N, DCM, 0 °C, 2hrs, 44% (*ii*) TFA, DCM, rt, 17 hrs, quant. (*iii*) (4-(6-methyl-1,2,4,5-tetrazin-3-yl)phenyl)methanamine hydrochloride, DIC, *N*-methylmorpholine, DCM, DMF, rt, 19 hrs, 41% (*iv*) NaI, acetone, rt, 48 hrs, 73%.

To start, triethylene glycol **15** was transformed into amine **16** in four steps as described in Chapter 5. The amine was then dissolved in DCM after which Et_3N was added and the mixture cooled to 0 °C. Chloroacetyl chloride was added dropwise and the reaction was stirred for two hours at 0 °C to give chloroacetamide **17** in a yield of 44%. Next, the *tert*-butyl ester was removed by treatment with 10% (v/v) TFA in DCM for 17 hours to obtain carboxylic acid **18** in a quantitative yield. Carboxylic acid **18** was activated *in situ* with DIC and *N*-methylmorpholine for one hour after which a solution of (4-(6-methyl-1,2,4,5-tetrazin-3-yl)phenyl)methanamine hydrochloride in a mixture of DCM and DMF (1:1, DCM – DMF) was added dropwise to the reaction mixture. The resulting solution was stirred for 19 hours and tetrazine **19** was obtained in a yield of 41%. Finally, chloroacetamide **19** was converted into the corresponding iodoacetamide using the classic Finkelstein reaction with sodium iodide in acetone to give adapter molecule **7** in a 73% yield.[22]

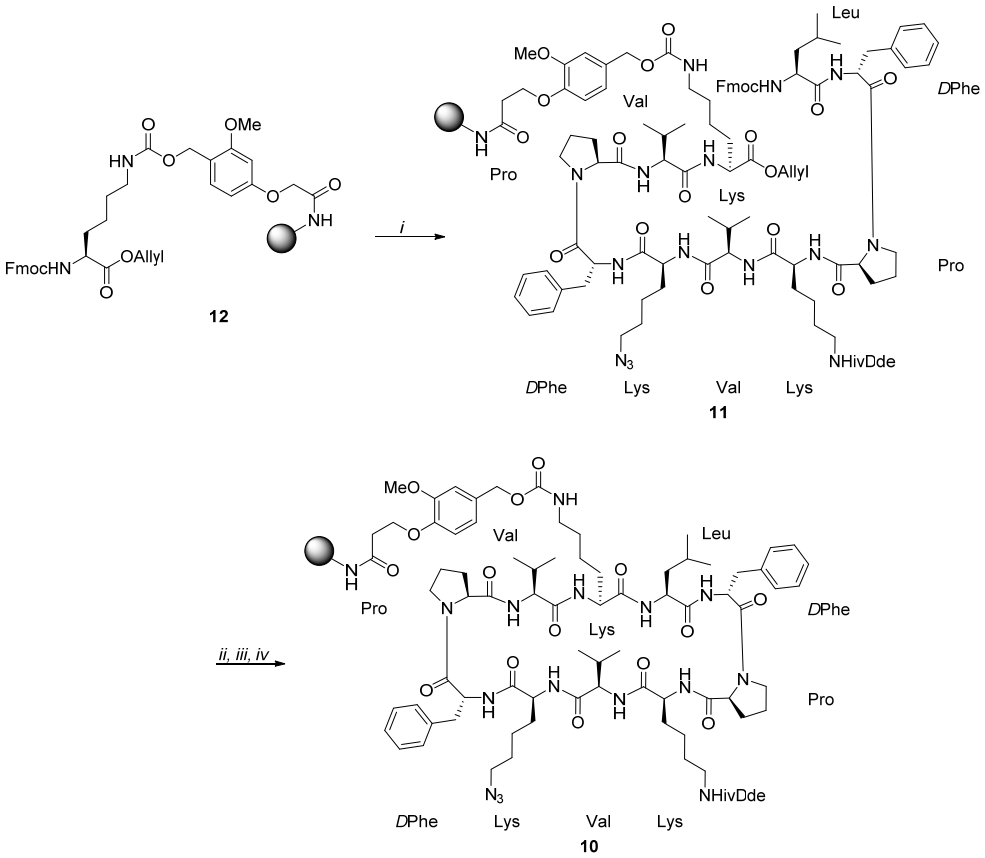
The assembly of RAFT scaffold **1**, having a bulky cyclooctyne moiety incorporated, requires the availability of aza-dibenzocyclooctyne **27**, the synthesis of which is depicted in *Scheme 3*.



Scheme 3. Reagents and conditions: (*i*) hydroxylamine hydrochloride, pyridine, EtOH, reflux, 17 hrs, 98% (*ii*) Eaton's reagent, 100 °C, 30 min, 87% (*iii*) $LiAlH_4$, Et_2O, reflux, 19 hrs, 75% (*iv*) methyl 4-chloro-4-oxobutyrate, Et_3N, DCM, 0 °C to rt, 90 min, 68% (*v*) lithium hydroxide monohydrate, MeOH, H_2O, reflux, 20 hrs, 98% (*vi*) Br_2, DCM, 0 °C, 2 hrs (*vii*) KO^{t}Bu, THF, -45 °C, 4 hrs (*viii*) pentafluorophenol, DIC, *N*-methylmorpholine, DCM, rt, 18 hrs, 33% over 3 steps.

Although several syntheses have been reported for carboxylic acid **26**, it was decided to employ the procedure described by the group of Adronov who optimized the synthetic route for scaling up.[14,23,24] The synthesis started from 5-dibenzosuberenone **20** which was converted into oxime **21** by refluxing a solution of the ketone and hydroxylamine hydrochloride in a mixture of pyridine and ethanol. A Beckmann rearrangement was then performed at 100 °C promoted by Eaton's reagent (7.7 wt% P_2O_5 in methanesulfonic acid) to furnish lactam **22** in 87% yield. The amide was then reduced using lithium aluminium hydride in refluxing ether to give amine **23**, which was then acylated with methyl-4-chloro-4-oxobutyrate to obtain amide **24**. Afterwards, saponification of methyl ester **24** was achieved in near quantitative yield by refluxing the ester with lithium hydroxide monohydrate in a mixture of methanol and water for 20 hours. The synthesis of alkyne **26** was then realized by bromination of alkene **25** at 0 °C for two hours followed by a double elimination with potassium *tert*-butoxide at -45 °C for four hours. Lastly, carboxylic acid **26** was condensed with pentafluorophenol using DIC as the activator and *N*-methylmorpholine as the base to give activated ester **27** in 33% yield over three steps.

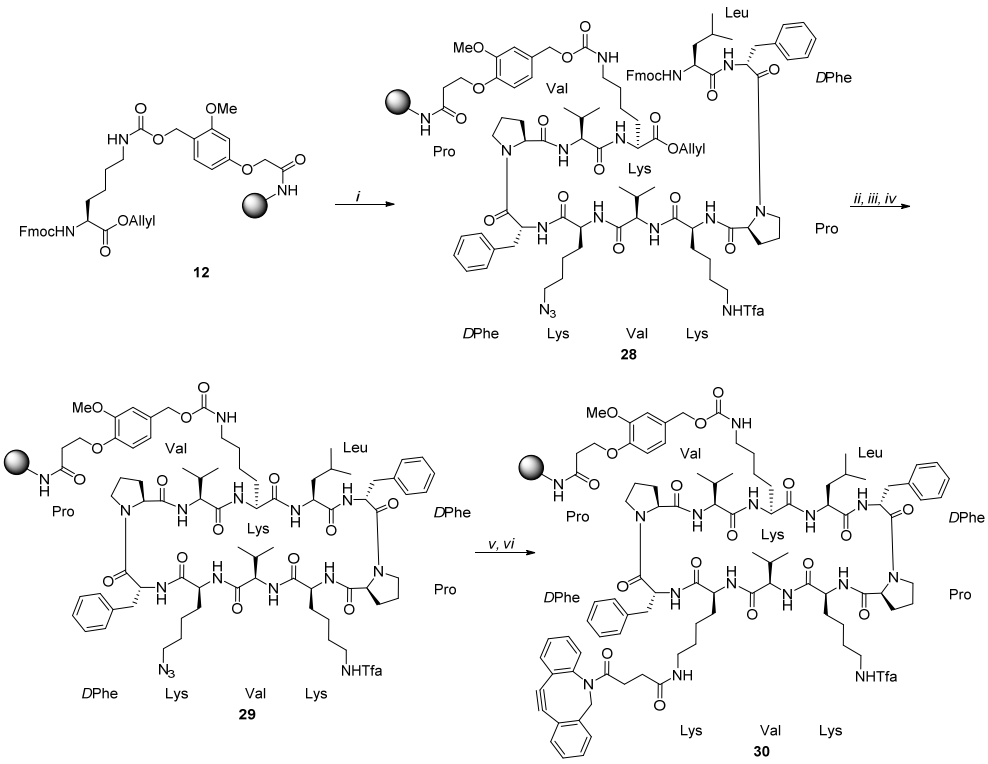
With the necessary building blocks in hand, the synthesis of RAFT scaffold **1** was explored (*Scheme 4*).



Scheme 4. 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.

After synthesizing side-chain functionalized resin **12**, as described in Chapter 3, the peptide chain was elongated using a Protein Technologies Tribute automated peptide synthesizer, entailing 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 lysine residues which were equipped with the 1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)-3-methylbutyl (ivDde) group or with the ε-amine masked as an azide. For the coupling the resin was shaken 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 **11** was obtained after which the synthesis was continued manually. Deallylation was achieved by treatment with $Pd(PPh_3)_4$ and phenylsilane for 90 minutes which was followed by deprotection of the Fmoc group using 20% (v/v) piperidine in DMF for 2x10 minutes. With the N- and C-termini liberated, the peptide was cyclized with PyBOP acting as the condensing agent and *N*-methylmorpholine as base to afford cyclic decapeptide **10**. Cleavage of the crude peptide off the resin and subsequent analysis by LC-MS showed the formation of the cyclic decapeptide **10** alongside substantial amount of side products, indicating a low overall yield as the deprotection and functionalization stage have yet to follow. Although ivDde is more resistant towards piperidine than the original Dde group, side product formation with the ivDde group has been reported before.[25] Even with the deliberate choice to introduce the ivDde group at the last possible position to limit the exposure to piperidine proved not to be sufficient enough to suppress the side-reactions. Therefore, it was decided to substitute the ivDde group and protect the amine as the trifluoroacetamide (*Scheme 5*).

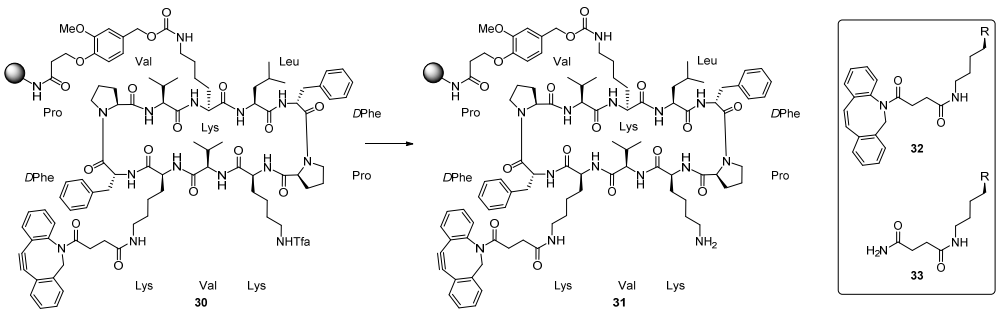


Scheme 5. Reagents and conditions: (*i*) SPPS: (a) piperidine, DMF, 90 °C, 2x90 sec (b) Fmoc-AA-OH, DIC, Oxyma Pure, DMF, 90 °C, 2 min. (*ii*) $Pd(PPh_3)_4$, $PhSiH_3$, DCM, DMF, rt, 1.5 hrs (*iii*) piperidine, DMF, rt, 2x10 min. (*iv*) benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate, 1-hydroxybenzotriazole hydrate, *N*-methylmorpholine, DMF, rt, 2.5 hrs (*v*) PMe_3, toluene, 1,4-dioxane, rt, 2 hrs, then H_2O, rt, 4 hrs (*vi*) **27**, DIPEA, DMF, rt, 72 hrs.

This time elongation of the peptide was performed using a CEM Liberty Blue microwave peptide synthesizer with nine peptide coupling cycles. First, the Fmoc was deprotected with two treatments of 20% (v/v) piperidine in DMF at 90 °C for 90 seconds. The resin was then washed twice with DMF before the liberated N-terminus was coupled to the appropriate standard Fmoc building block using DIC and Oxyma Pure, except for the lysine residues which were equipped with the trifluoroacetyl (Tfa) group or with the ε-amine masked as an azide. The coupling was conducted at 90 °C for two minutes. Nine such cycles furnished linear peptide **28** after which the synthesis was continued manually as before to afford cyclized peptide **29**. LC-MS analysis of the crude peptide after cleavage showed the presence of the desired cyclic peptide without significant side products. Next, the azide was reduced by treating the resin 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 and the mixture shaken for an additional four hours. The liberated amine was then functionalized with building block **27** using DIPEA to afford peptide **30** after 72 hours as observed by LC-MS analysis of the crude peptide after cleavage. Next the deprotection of the trifluoroacetamide was explored using various conditions (*Table 1*).

Table 1. Screening of conditions for the deprotection of the trifluoroacetamide group.[a]

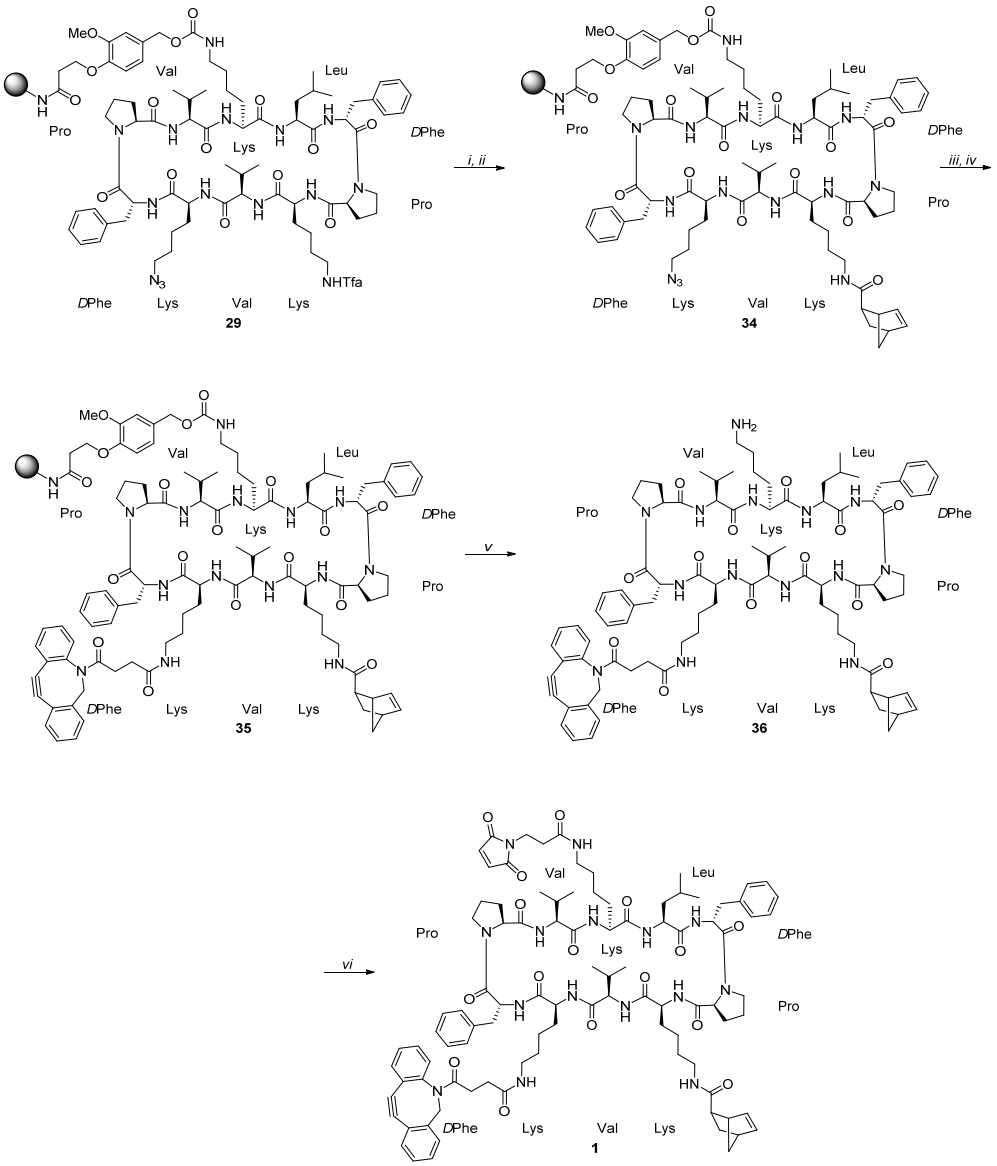


Entry	Reagent	Equivalents	Solvent	Reaction time	Product
1	–	–	0.50 M NH_3 in 1,4-dioxane	17 hours	**30**
2	25 wt% NaOMe in MeOH	5	DCM	1 hour	–[b]
3	$NaBH_4$	10	1:1, THF – EtOH –	30 min.	**32**
4	–	–	7.0 M NH_3 in MeOH	17 hours	**30/31/33**

[a] Reactions were performed at a 25 μmol scale after which the peptide was cleaved from the resin and analyzed by LC-MS. [b] No significant product could be detected.

First, 0.50 M NH_3 in dioxane was added and the resin was shaken for 17 hours. The suspension was filtered and the resin was washed with 1,4-dioxane, MeOH and DCM. The crude peptide was then cleaved off of the resin with ten treatments of 0.5% (v/v) TFA in DCM for two minutes with a filtration after each treatment. Analysis of the filtrate by LC-MS showed that the trifluoroacetamide was still intact (*Entry 1*). Next, a solution-phase procedure for Tfa deprotection reported by Snider *et al.* was tried (*Entry 2*).[26] This reaction employs sodium methoxide in methanol for the deprotection in combination with DCM as the solvent which would swell the resin significantly compared to the more polar dioxane and methanol. However, after treatment of the resin with NaOMe in DCM for one-hour LC-MS spectroscopic analysis showed apart from numerous byproducts no significant peak for either the product or starting material. Lokey and co-workers reported the quantitative

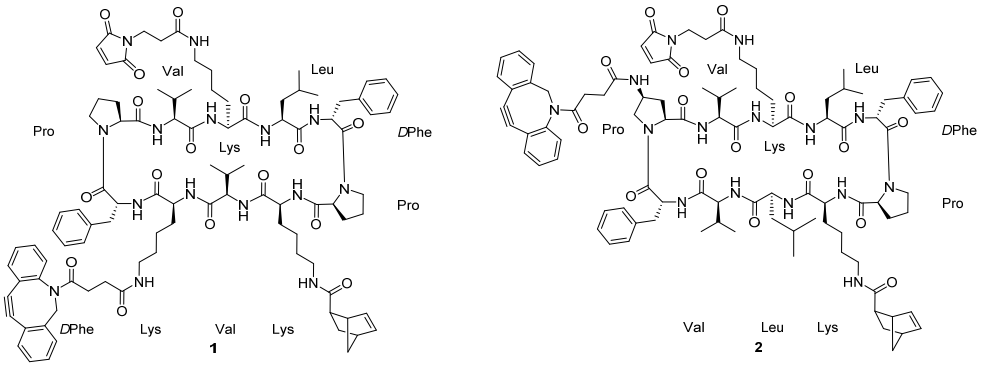
removal of the Tfa group on-resin in 30 minutes using sodium borohydride in a mixture of THF and ethanol (1:1, THF – EtOH).[27] However, employing this method did not lead to removal of the Tfa group (*Entry 3*). Instead, LC-MS analysis showed a peak with a mass two dalton higher than the mass corresponding to starting material **30** as the major product suggesting a partial reduction of the alkyne to alkene **32** had taken place. Alcohols have been shown to reduce strained alkynes.[28,29] Lastly, a solution of 7.0 M NH_3 in methanol was tried hoping that with a higher concentration of ammonia the trifluoroacetamide could be removed (*Entry 4*). After 17 hours, the crude peptide was analyzed and showed that starting material **30** and product **31** were present on the resin indicating that a partial Tfa removal had occurred. However, two other major products were detected that corresponded with the substitution of the strained cycoalkyne with ammonia (**33**) with and without Tfa deprotection. The incompatibility of the cyclooctyne with NH_3-mediated Tfa removal, urged to find out whether the order of deprotection could be reversed (Tfa removal before azide reduction) and the Tfa removal could be driven to completion with longer reaction times and/or microwave irradiation. Indeed, when peptide **29** was treated with 7.0 M NH_3 in methanol for 12 hours using microwave irradiation complete deprotection was observed (*Scheme 6*).



Scheme 6. Reagents and conditions: (*i*) NH_3, MeOH, μw, 90 °C, 12 hrs (*ii*) 5-norbornene carboxylic acid NHS ester, DIPEA, DMF, rt, 19 hrs (*iii*) PMe_3, toluene, 1,4-dioxane, rt, 2 hrs, then H_2O, rt, 4 hrs (*iv*) **27**, DIPEA, DMF, rt, 72 hrs (*v*) TFA – DCM (1:199), rt, 10x2 min (*vi*) 3-maleimidopropionic acid NHS ester, DIPEA, DMF, rt, 4 hrs.

After deprotection of the Tfa protection, the liberated amine was functionalized using 5-norbornene carboxylic acid NHS ester (4:1, endo – exo) and DIPEA. After 19 hours the peptide was cleaved off of the resin and analyzed by LC-MS to indicate

complete conversion to peptide **34** and the minor exo-stereoisomer. Afterwards, 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 and the resin shaken for four hours. Cleavage of the peptide and LC-MS analysis showed a complete and clean conversion to the amine. The amine was then reacted with DBCO building block **27** and DIPEA for 72 hours giving peptide **35** with no observable starting material. The peptide was cleaved from the resin with ten treatments of 0.5% (v/v) TFA in DCM for two minutes with a filtration after each treatment. The TFA present in the filtrate was immediately neutralized with the basic ion-exchange resin Amberlyst A-21. Filtration and subsequent concentration afforded crude peptide **36**. The crude peptide was taken up in DMF and reacted with 3-maleimidopropionic acid NHS ester and DIPEA for four hours. The reaction mixture was concentrated *in vacuo* and purified by RP-HPLC. Lyophilization of the pure fractions afforded RAFT scaffold **1** in 2.3% yield bearing three different chemoselective handles.



Scheme 7. RAFT scaffolds **1** and **2** bearing three chemoselective handles in different orientations.

Employing the cis-Fmoc-(2*S*, 4*S*)-4-azidoproline in the linear peptide synthesis instead of a regular proline allowed for the synthesis of RAFT scaffold **2**. RAFT scaffold **2** was synthesized in 3.3% yield using the same procedure as described for scaffold **1**. These two scaffolds in combination with the TLR ligands synthesized give rise to three different orientations of TLR ligands depending on the manner of attachment. The synthesis of these TLR1/2-7-9 constructs should be the focus going forward.

Conclusion

Recent studies reveal the importance of engaging multiple TLRs simultaneously to the immune response. Gilkes *et al.* showed that the spatial organization of TLR constructs can shape immune responses toward desired outcomes. The research outlined in this Chapter was aimed toward synthesizing a RAFT scaffold, able to accommodate three different TLR ligands in different orientations, to study the effect on the immune response. The scaffolds, allowing the adhering of two ligands, as described in Chapter 3 were expanded with a norbornene/tetrazine ligation to accommodate a third TLR ligand. The inclusion of this ligation method prompted a change in cyclooctyne from BCN to DBCO to achieve mutual orthogonality. Therefore, a DBCO building block was synthesized. The synthesis of the RAFT scaffolds was first attempted with ivDde as the additional amino protecting group. However, the purity of the cyclic peptide carrying this group was insufficient and a switch was made to trifluoroacetamide (Tfa) as the protecting group of choice. Various conditions were tried to deprotect this group on-resin and a clean conversion was shown by treating the resin with 7.0 M NH_3 in methanol under microwave irradiation for 12 hours. RAFT scaffolds **1** and **2** were ultimately obtained bearing three different chemoselective handles. To functionalize these scaffolds, a TLR7 ligand equipped with a tetrazine was synthesized as well as an adapter molecule to fit the TLR9 ligand with a tetrazine. The ligation of these TLR ligands to the scaffolds should be the focus in the future.

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. (4-(6-Methyl-1,2,4,5-tetrazin-3-yl)phenyl)methanamine hydrochloride was purchased from Click Chemistry Tools. Fmoc-L-Lys(N_2)-OH and Fmoc L-Lys(TFA)-OH were 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, 2-(6-methyl-1,2,4,5-tetrazin-3-yl)ethan-1-amine hydrochloride and 3-maleimidopropionic acid *N*-hydroxysuccinimidyl ester were available in-house.The resin TentaGel S AC (0.23 mmol/g) was bought from Rapp Polymere. 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. 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:

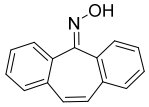
$$\text{Loading}_{\text{resin}} = \frac{A_{301.0\text{ nm}} * 10^6\ \text{mmol mol}^{-1}\ \text{mg g}^{-1} * V * D}{\varepsilon_{301.0\text{ nm}} *\ m_{\text{resin}} * l}$$

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

5*H*-dibenzo[*a,d*][7]annulen-5-one oxime

To a solution of dibenzosuberenone **20** (5.2 g, 25 mmol, 1.0 equiv.) in ethanol (63 mL, 0.40 M) was added hydroxylamine hydrochloride (8.7 g, 0.13 mol, 5.0 equiv.) and pyridine (14 mL, 0.17 mol, 6.7 equiv.). The mixture was heated to reflux and stirred for 17 hrs. DCM was added and the organic layer was washed with 1.0 M aq. HCl (3x) and brine. The organic phase was dried ($MgSO_4$), filtered and concentrated *in vacuo* to afford **21** (5.4 g, 24 mmol, 98%) as a light brown solid.



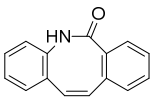
21

^{1}H NMR (400 MHz, $CDCl_3$) δ 9.07 (br s, 1H, O*H*), 7.70 – 7.62 (m, 1H, C*H*-arom), 7.62 – 7.53 (m, 1H, C*H*-arom), 7.47 – 7.35 (m, 6H, C*H*-arom), 6.90 (d, *J* = 2.9 Hz, 2H, C*H*-cycloheptene).

^{13}C NMR (101 MHz, $CDCl_3$) δ 156.7 (*C*q-oxime), 135.4 (*C*q-arom), 134.6 (*C*q-arom), 133.8 (*C*q-arom), 130.8 (*C*H-cycloheptene), 130.7 (*C*H-cycloheptene), 130.5 (*C*q-arom), 129.5 (*C*H-arom), 129.2 (*C*H-arom), 129.1 (*C*H-arom), 129.0 (*C*H-arom), 128.9 (*C*H-arom), 127.8 (*C*H-arom), 127.7 (*C*H-arom).

HRMS (ESI-Orbitrap) calcd. for $C_{15}H_{12}NO$ $[M+H]^+$ 222.09134, found 222.09112.

(*Z*)-dibenzo[*b,f*]azocin-6(5*H*)-one



22

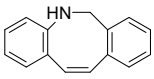
To oxime **21** (5.3 g, 24 mmol, 1.0 equiv.) was added Eaton's reagent (32 mL, 0.75 M). The solution turned dark red and was heated to 100 °C. After 30 min of stirring, the reaction was quenched by the addition of H_2O. After the mixture was cooled to ca. 70 °C, the product was extracted with hot EtOAc (5x). The pooled organic fractions was evaporated at 70 °C to a volume of ca. 20 mL and cooled to rt. After filtration, amide **22** (4.6 g, 21 mmol, 87%) was obtained as a brown solid.

^{1}H NMR (500 MHz, $CDCl_3$) δ 8.07 (s, 1H, N*H*), 7.48 (dd, *J* = 7.7, 1.5 Hz, 1H, C*H*-arom), 7.32 (td, *J* = 7.5, 1.5 Hz, 1H, C*H*-arom), 7.28 – 7.24 (m, 1H, C*H*-arom), 7.23 – 7.11 (m, 4H, C*H*-arom), 7.09 – 7.04 (m, 1H, C*H*-arom), 6.96 (d, *J* = 11.7 Hz, 1H, C*H*-cyclooctene), 6.85 (d, *J* = 11.6 Hz, 1H, C*H*-cyclooctene).

^{13}C NMR (126 MHz, $CDCl_3$) δ 174.0 (NHC=O), 135.2 (*C*q-arom), 135.1 (*C*q-arom), 134.3 (*C*q-arom), 134.3 (*C*q-arom), 133.1 (*C*H-cyclooctene), 129.9 (*C*H-cyclooctene), 129.9 (*C*H-arom), 129.3 (*C*H-arom), 128.4 (*C*H-arom), 128.3 (*C*H-arom), 127.8 (*C*H-arom), 127.3 (*C*H-arom), 126.4 (*C*H-arom).

HRMS (ESI-Orbitrap) calcd. for $C_{15}H_{12}NO$ $[M+H]^+$ 222.09134, found 222.09125.

(*Z*)-5,6-dihydrodibenzo[*b,f*]azocine



23

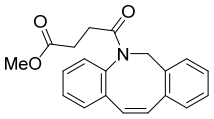
Et_2O (35 mL, 0.39 M) was slowly added to a mixture of amide **22** (3.0 g, 14 mmol, 1.0 equiv.) and lithium aluminium hydride (10 g, 0.27 mol, 20 equiv.). The mixture was heated to reflux and stirred for 19 hrs. DCM was added and the mixture was cooled to 0 °C. H_2O was added dropwise until the lithium aluminium hydride was quenched. The suspension was filtered over Celite and washed with DCM and H_2O. The organic layer was separated, dried ($MgSO_4$), filtered and concentrated *in vacuo* to give amine **23** (2.1 g, 10 mmol, 75%) as a yellow foam.

^{1}H NMR (500 MHz, $CDCl_3$) δ 7.26 – 7.23 (m, 1H, C*H*-arom), 7.20 – 7.11 (m, 3H, C*H*-arom), 6.96 (dd, *J* = 7.9, 1.6 Hz, 1H, C*H*-arom), 6.87 (ddd, *J* = 8.5, 7.1, 1.6 Hz, 1H, C*H*-arom), 6.59 (ddd, *J* = 7.7, 7.1, 1.2 Hz, 1H, C*H*-arom), 6.53 (d, *J* = 13.1 Hz, 1H, C*H*-cyclooctene), 6.45 (dd, *J* = 8.0, 1.2 Hz, 1H, C*H*-arom), 6.35 (d, *J* = 13.1 Hz, 1H, C*H*-cyclooctene), 4.57 (s, 2H, CH_2), 4.23 (br s, 1H, N*H*).

^{13}C NMR (126 MHz, $CDCl_3$) δ 147.2 (*C*q-arom), 139.3 (*C*q-arom), 138.3 (*C*q-arom), 134.8 (*C*H-arom), 132.9 (*C*H-cyclooctene), 130.3 (*C*H-arom), 129.0 (*C*H-arom), 128.1 (*C*H-arom), 127.8 (*C*H-arom), 127.5 (*C*H-arom), 127.5 (*C*H-cyclooctene), 121.9 (*C*q-arom), 118.1 (*C*H-arom), 117.8 (*C*H-arom), 49.7 (CH_2).

HRMS (ESI-Orbitrap) calcd. for $C_{15}H_{14}N$ $[M+H]^+$ 208.11208, found 208.11210.

methyl (*Z*)-4-(dibenzo[*b,f*]azocin-5(6*H*)-yl)-4-oxobutanoate



24

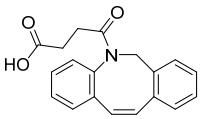
Amine **23** (2.0 g, 9.8 mmol, 1.0 equiv.) was dissolved in DCM (67 mL, 0.15 M). Et_3N (2.7 mL, 20 mmol, 2.0 equiv.) was added and the mixture was cooled to 0 °C. Methyl 4-chloro-4-oxobutyrate (1.8 mL, 15 mmol, 1.5 equiv.) was added dropwise over 30 min. The cooling bath was removed and the reaction was stirred at room temperature for 90 min. The solution was washed with 2.0 M aq. NaOH (3x), 2.0 M aq. HCl (3x) and brine. The organic fraction was dried ($MgSO_4$), filtered and concentrated under reduced pressure. Purification by silica gel column chromatography (1:4, EA – Pentane to 1:3, EA – Pentane) afforded methyl ester **24** (2.1 g, 6.7 mmol, 68%) as a white amorphous solid.

^{1}H NMR (500 MHz, $CDCl_3$) δ 7.28 – 7.25 (m, 5H, C*H*-arom), 7.20 – 7.08 (m, 3H, C*H*-arom), 6.79 (d, *J* = 12.9 Hz, 1H, C*H*-cyclooctene), 6.62 (d, *J* = 13.0 Hz, 1H, C*H*-cyclooctene), 5.52 (d, *J* = 15.1 Hz, 1H, CH_2), 4.26 (d, *J* = 15.1 Hz, 1H, CH_2), 3.62 (s, 3H, (C=O)OCH_3), 2.66 – 2.56 (m, 1H, CH_2(C=O)N), 2.51 – 2.38 (m, 2H, CH_2(C=O)N, CH_2(C=O)OMe), 2.06 – 1.96 (m, 1H, CH_2(C=O)OMe).

^{13}C NMR (126 MHz, $CDCl_3$) δ 173.6 (*C*=O), 171.0 (*C*=O), 140.7 (*C*q-arom), 136.6 (*C*q-arom), 136.0 (*C*q-arom), 134.7 (*C*q-arom), 132.8 (*C*H-cyclooctene), 132.0 (*C*H-arom), 131.0 (*C*H-arom), 130.3 (*C*H-arom), 128.7 (*C*H-arom), 128.4 (*C*H-arom), 128.2 (*C*H-arom), 127.5 (*C*H-arom), 127.4 (*C*H-arom), 127.1 (*C*H-cyclooctene), 54.6 (*C*H_2), 51.8 ((C=O)O*C*H_3), 29.7 (*C*H_2(C=O)OMe), 29.2 (*C*H_2(C=O)N).

HRMS (ESI-Orbitrap) calcd. for $C_{20}H_{20}NO_3$ $[M+H]^+$ 322.14377, found 322.14369.

(*Z*)-4-(dibenzo[*b*,*f*]azocin-5(6*H*)-yl)-4-oxobutanoic acid



25

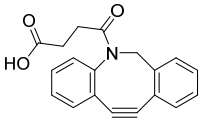
Methyl ester **24** (2.1 g, 6.6 mmol, 1.0 equiv.) was dissolved in a mixture of H_2O and methanol (2:1, MeOH – H_2O, 66 mL, 0.10 M). Lithium hydroxide monohydrate (1.7 g, 39 mmol, 6.0 equiv.) was added and the mixture was heated to reflux. The reaction was stirred for 20 hrs. 1.0 M aq. $NaHSO_4$ was added and the product was extracted with DCM (3x). The organic fractions were combined and washed with H_2O and brine. The organic layer was dried ($MgSO_4$), filtered and evaporated under reduced pressure to give carboxylic acid **25** (2.0 g, 6.4 mmol, 98%) as a white solid.

^{1}H NMR (500 MHz, $CDCl_3$) δ 7.32 – 7.21 (m, 5H, C*H*-arom), 7.20 – 7.09 (m, 3H, C*H*-arom), 6.80 (d, *J* = 12.8 Hz, 1H, C*H*-cyclooctene), 6.61 (d, *J* = 12.8 Hz, 1H, C*H*-cyclooctene), 5.52 (d, *J* = 15.1 Hz, 1H, CH_2), 4.29 (d, *J* = 15.1 Hz, 1H, CH_2), 2.61 (ddd, *J* = 16.8, 8.3, 5.4 Hz, 1H, CH_2(C=O)N), 2.51 (ddd, *J* = 16.8, 7.1, 5.3 Hz, 1H, CH_2(C=O)N), 2.41 (ddd, *J* = 17.0, 8.3, 5.3 Hz, 1H, CH_2(C=O)OH), 2.04 (ddd, *J* = 17.0, 7.1, 5.4 Hz, 1H, CH_2(C=O)OH).

^{13}C NMR (126 MHz, $CDCl_3$) δ 176.8 (*C*=O), 171.9 (*C*=O), 140.2 (*C*q-arom), 136.6 (*C*q-arom), 136.0 (*C*q-arom), 134.2 (*C*q-arom), 133.0 (*C*H-cyclooctene), 131.9 (*C*H-arom), 131.0 (*C*H-arom), 130.3 (*C*H-arom), 128.8 (*C*H-arom), 128.5 (*C*H-arom), 128.2 (*C*H-arom), 127.5 (*C*H-arom), 127.4 (*C*H-arom), 127.3 (*C*H-cyclooctene), 54.7 (*C*H_2), 29.8 (*C*H_2(C=O)OH), 29.7 (*C*H_2(C=O)N).

HRMS (ESI-Orbitrap) calcd. for $C_{19}H_{18}NO_3$ $[M+H]^+$ 308.12812, found 308.12801.

DIBAC-4-oxobutanoic acid



26

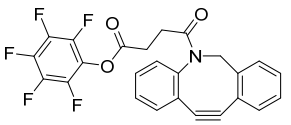
A solution of carboxylic acid **25** (0.92 g, 3.0 mmol, 1.0 equiv.) in DCM (40 mL, 75 mM) was cooled to 0 °C. Bromine (0.49 mL, 9.0 mmol, 3.0 equiv.) was added dropwise and the reaction was stirred at 0 °C for 2 hrs. The cooling bath was removed and bromine was quenched by addition of sat. aq. $Na_2S_2O_5$. Additional DCM was added and the layers were separated. The organic layer was washed with sat. aq. $Na_2S_2O_3$ and brine. The organic phase was dried ($MgSO_4$), filtered and concentrated *in vacuo*. The crude product was taken up in THF (50 mL, 60 mM) and cooled to -45 °C. Potassium *tert*-butoxide (1.0 M in THF, 9.0 mL, 9.0 mmol, 3.0 equiv.) was added dropwise to the solution. After 90 min, additional potassium *tert*-butoxide (1.0 M in THF, 4.0 mL, 4.0 mmol, 1.3 equiv.) was added followed by extra potassium *tert*-butoxide (1.0 M in THF, 3.0 mL, 3.0 mmol, 1.0 equiv.) after 3 hrs of stirring. The reaction was stirred in total for 4 hrs at -45 °C. The mixture was warmed to room temperature and quenched with 1.0 M aq. $NaHSO_4$ until the pH was 1. The aqueous layer was extracted with DCM (3x). The pooled organic fractions were washed with H_2O and brine. The organic phase was dried (Na_2SO_4), filtered and the volatiles were removed under reduced pressure to furnish alkyne **26** which was used immediately in the next step without further purification.

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.70 (dd, *J* = 7.5, 1.3 Hz, 1H, C*H*-arom), 7.48 – 7.34 (m, 5H, C*H*-arom), 7.33 – 7.25 (m, 1H, C*H*-arom), 7.21 (dd, *J* = 7.5, 1.5 Hz, 1H, C*H*-arom), 5.19 (d, *J* = 13.8 Hz, 1H, CH_2), 3.71 (d, *J* = 13.8 Hz, 1H, CH_2), 3.02 – 2.89 (m, 1H, CH_2(C=O)N)), 2.87 – 2.69 (m, 2H, CH_2(C=O)N), CH_2(C=O)OH), 2.18 – 2.07 (m, 1H, CH_2(C=O)OH).

^{13}C NMR (101 MHz, $CDCl_3$) δ 170.9 (*C*=O), 169.0 (*C*=O), 151.2 (*C*q-arom), 147.9 (*C*q-arom), 132.4 (*C*H-arom), 129.2 (*C*H-arom), 128.7 (*C*H-arom), 128.5 (*C*H-arom), 128.5 (*C*H-arom), 127.9 (*C*H-arom), 127.4 (*C*H-arom), 125.6 (*C*H-arom), 123.1 (*C*q-arom), 122.9 (*C*q-arom), 115.1 (*C*≡*C*), 107.6 (*C*≡*C*), 55.7 (*C*H_2), 29.5 (*C*H_2(C=O)OH), 28.7 (*C*H_2(C=O)N).

HRMS (ESI-Orbitrap) calcd. for $C_{19}H_{16}NO_3$ $[M+H]^+$ 306.11247, found 306.11212.

DIBAC-4-oxobutanoic acid pentafluorophenyl ester



27

Crude alkyne **26** was dissolved in DCM (30 mL, 0.10 M). Pentafluorophenol (0.61 g, 3.3 mmol, 1.1 equiv.), DIC (0.51 mL, 3.3 mmol, 1.1 equiv.) and *N*-methylmorpholine (0.73 mL, 6.6 mmol, 2.2 equiv.) were added. The reaction was stirred for 18 hrs. The volatiles were removed under reduced pressure and the crude was purified by silica gel column chromatography (1:9, EA – Pentane to 1:4, EA – Pentane) to give pentaflurophenyl ester **27** (0.47 g, 0.99 mmol, 33% over 3 steps) as a white amorphous solid.

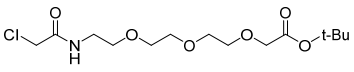
^{1}H NMR (400 MHz, $CDCl_3$) δ 7.70 – 7.65 (m, 1H, C*H*-arom), 7.48 – 7.37 (m, 4H, C*H*-arom), 7.33 (td, *J* = 7.5, 1.6 Hz, 1H, C*H*-arom), 7.30 – 7.25 (m, 1H, C*H*-arom), 7.20 (dd, *J* = 7.5, 1.6 Hz, 1H, C*H*-arom), 5.18 (d, *J* = 13.9 Hz, 1H, CH_2), 3.72 (d, *J* = 14.0 Hz, 1H, CH_2), 3.04 – 2.89 (m, 1H, CH_2(C=O)N)), 2.88 – 2.69 (m, 2H, CH_2(C=O)N), CH_2(C=O)OPfp), 2.22 – 2.10 (m, 1H, CH_2(C=O)OPfp).

^{13}C NMR (101 MHz, $CDCl_3$) δ 171.5 (*C*=O), 169.0 (*C*=O), 151.0 (*C*q-arom), 147.6 (*C*q-arom), 132.4 (*C*H-arom), 129.2 (*C*H-arom), 128.8 (*C*H-arom), 128.7 (*C*H-arom), 128.4 (*C*H-arom), 127.9 (*C*H-arom), 127.4 (*C*H-arom), 125.6 (*C*H-arom), 123.1 (*C*q-arom), 122.9 (*C*q-arom), 115.1 (*C*≡*C*), 107.5 (*C*≡*C*), 55.8 (CH_2), 29.5 (*C*H_2(C=O)OPfp), 28.7 (CH_2(C=O)N).

^{19}F NMR (471 MHz, $CDCl_3$) δ -151.38 – -153.05 (m, C*F*-arom), -158.28 (t, *J* = 21.7 Hz, C*F*-arom), -162.54 (td, *J* = 22.3, 4.8 Hz, C*F*-arom).

HRMS (ESI-Orbitrap) calcd. for $C_{25}H_{15}F_5NO_3$ [M+H]$^+$ 472.09666, found 472.09686.

***tert*-butyl 1-chloro-2-oxo-6,9,12-trioxa-3-azatetradecan-14-oate**



17

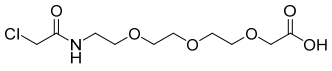
A solution of *tert*-butyl 2-(2-(2-(2-aminoethoxy)ethoxy)ethoxy)acetate **16** (0.47g, 1.8 mmol, 1.0 equiv.) and Et_3N (0.32 mL, 2.3 mmol, 1.3 equiv.) in DCM (18 mL, 0.10 M) was cooled to 0 °C and chloroacetyl chloride (0.18 mL, 2.3 mmol, 1.3 equiv.) was added dropwise. The reaction was stirred at 0 °C for 2 hrs. The reaction mixture was diluted with DCM and washed with sat. aq. $NaHCO_3$, H_2O and brine. The organic layer was dried ($MgSO_4$), filtered and concentrated *in vacuo*. Purification by silica gel column chromatography (1:49, MeOH – DCM to 1:19, MeOH – DCM) afforded chloroacetamide **17** (0.27 g, 0.78 mmol, 44%) as a colorless oil.

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.09 (br s, 1H, N*H*), 3.97 (s, 2H, CH_2Cl), 3.94 (s, 2H, CH_2C(C=O)O^{t}Bu), 3.66 – 3.55 (m, 8H, OCH_2), 3.54 – 3.49 (m, 2H, CH_2CH$_2$NHClAc), 3.42 (q, *J* = 5.5 Hz, 2H, CH_2NHClAc), 1.42 – 1.36 (m, 9H, (C=O)O^t*Bu*).

^{13}C NMR (101 MHz, $CDCl_3$) δ 169.6 ((*C*=O)O^{t}Bu), 166.1 (NH(*C*=O)), 81.5 (*C*CH_3), 70.6 (OCH_2), 70.5 (OCH_2), 70.4 (OCH_2), 70.2 (OCH_2), 69.3 (*C*H_2CH$_2$NHClAc), 68.9 (*C*H_2(C=O)O^{t}Bu), 42.6 (*C*H_2Cl), 39.5 (*C*H_2NHClAc), 28.0 (*C*H_3).

HRMS (ESI-Orbitrap) calcd. for $C_{14}H_{26}ClNO_6Na$ [M+Na]$^+$ 362.13409, found 362.13391.

1-chloro-2-oxo-6,9,12-trioxa-3-azatetradecan-14-oic acid



18

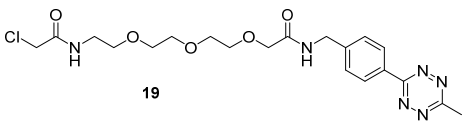
tert-Butyl ester **17** (71 mg, 0.21 mmol, 1.0 equiv.) was dissolved in a mixture of TFA and DCM (1:9, TFA – DCM, 2.1 mL, 0.10 M). The reaction was stirred for 17 hrs. Toluene was added and the solution was evaporated to a volume of ca. 2 mL (3x). The mixture was evaporated to dryness to obtain carboxylic acid **18** (59 mg, 0.21 mmol, quant.) as a colorless oil.

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.97 (br s, 1H, COO*H*), 7.23 (br s, 1H, N*H*), 4.18 (s, 2H, CH_2(C=O)OH), 4.10 (s, 2H, CH_2Cl), 3.81 – 3.74 (m, 2H, OCH_2), 3.73 – 3.64 (m, 6H, OCH_2), 3.63 – 3.58 (m, 2H, CH_2CH$_2$NHClAc), 3.52 (q, *J* = 5.1 Hz, 2H, CH_2NHClAc).

^{13}C NMR (101 MHz, $CDCl_3$) δ 172.7 ((*C*=O)OH), 166.9 (NH(*C*=O)), 71.3 (OCH_2), 70.6 (OCH_2), 70.3 (OCH_2), 70.1 (OCH_2), 69.5 (*C*H_2CH$_2$NHClAc), 68.7 (*C*H_2(C=O)OH), 42.7 (*C*H_2Cl), 39.7 (*C*H_2NHClAc).

HRMS (ESI-Orbitrap) calcd. for $C_{10}H_{18}ClNO_6Na$ [M+Na]$^+$ 306.07149, found 306.07120.

2-chloro-*N*-(1-(4-(6-methyl-1,2,4,5-tetrazin-3-yl)phenyl)-3-oxo-5,8,11-trioxa-2-azatridecan-13-yl)acetamide



19

Carboxylic acid **18** (14 mg, 50 μmol, 1.0 equiv.) was dissolved in DCM (0.50 mL, 0.10 M) and DIC (14 μL, 0.10 mmol, 2.0 equiv.) and *N*-methylmorpholine (11 μL, 0.10 mmol, 2.0 equiv.) and the reaction was stirred. After 1 hr, a solution of (4-(6-methyl-1,2,4,5-tetrazin-3-yl)phenyl)methanamine

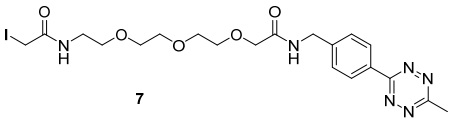
hydrochloride (12 mg, 50 μmol, 1.0 equiv.) and *N*-methylmorpholine (11 μL, 0.10 mmol, 2.0 equiv.) in a mixture of DCM and DMF (1:1, DCM – DMF, 1.0 mL, 50 mM) was added dropwise and the reaction was stirred for 19 hrs. The reaction mixture was washed with H_2O (5x) and the organic layer was dried ($MgSO_4$), filtered and concentrated under reduced pressure. Purification by silica gel column chromatography (1:39, MeOH – DCM) afforded tetrazine **19** (9.5 mg, 20 μmol, 41%) as a pink oil.

^{1}H NMR (500 MHz, $CDCl_3$) δ 8.56 (d, *J* = 8.0 Hz, 2H, C*H*-arom), 7.53 (d, *J* = 8.1 Hz, 3H, C*H*-arom, N*H*Bn), 7.00 (s, 1H, N*H*ClAc), 4.62 (d, *J* = 5.7 Hz, 2H, CH_2-benzyl), 4.14 (s, 2H, CH_2(C=O)NHBn), 4.03 (s, 2H, CH_2Cl), 3.74 (dd, *J* = 5.8, 2.6 Hz, 2H, OCH_2), 3.68 (dd, *J* = 5.7, 2.5 Hz, 2H, OCH_2), 3.60 (dd, *J* = 5.8, 3.1 Hz, 2H, OCH_2), 3.54 (dd, *J* = 5.8, 3.1 Hz, 2H, OCH_2), 3.50 (t, *J* = 4.9 Hz, 2H, CH_2CH$_2$NHClAc), 3.43 (q, *J* = 5.2 Hz, 2H, CH_2NHClAc), 3.10 (s, 3H, CH_3).

^{13}C NMR (126 MHz, $CDCl_3$) δ 170.4 (*C*q-tetrazine), 167.4 ((*C*=O)NHBn), 166.1 (*C*q-tetrazine), 164.0 ((*C*=O)CH$_2$Cl), 143.3 (*C*q-arom), 131.1 (*C*q-arom), 128.5 (*C*H-arom), 128.4 (*C*H-arom), 71.2 (OCH$_2$), 70.7 (CH$_2$(C=O)NHBn), 70.6 (OCH$_2$), 70.5 (OCH$_2$), 70.4 (OCH$_2$), 69.5 (CH$_2$CH$_2$NHClAc), 42.8 (CH$_2$Cl), 42.6 (CH$_2$-benzyl), 39.6 (CH$_2$NHClAc), 21.3 (CH$_3$).

HRMS (ESI-Orbitrap) calcd. for $C_{20}H_{28}ClN_6O_5$ $[M+H]^+$ 467.18042, found 467.18018.

2-iodo-*N*-(1-(4-(6-methyl-1,2,4,5-tetrazin-3-yl)phenyl)-3-oxo-5,8,11-trioxa-2-azatridecan-13-yl)acetamide



7

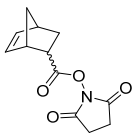
Chloroacetamide **19** (9.5 mg, 20 μmol, 1.0 equiv.) was dissolved in acetone (0.50 mL, 40 mM) and sodium iodide (9.0 mg, 60 μmol, 3.0 equiv.) was added. The reaction was stirred for 48 hrs and subsequently concentrated *in vacuo* and purified by silica gel column chromatography (1:39, MeOH – DCM) to give iodoacetamide **7** (8.1 mg, 15 μmol, 73%) as a pink oil.

^{1}H NMR (500 MHz, $CDCl_3$) δ 8.57 (d, *J* = 7.8 Hz, 2H, C*H*-arom), 7.54 (d, *J* = 8.0 Hz, 2H, C*H*-arom), 7.49 (s, 1H, N*H*Bn), 6.90 (s, 1H, N*H*CH$_2$I), 4.63 (d, *J* = 4.3 Hz, 2H, CH_2-benzyl), 4.22 (s, 2H, CH_2(C=O)NHBn), 3.80 – 3.65 (m, 6H, OCH_2, CH_2I), 3.63 – 3.59 (m, 2H, OCH_2), 3.57 – 3.53 (m, 2H, OCH_2), 3.51 – 3.47 (m, 2H, CH_2CH$_2$NHIAc), 3.42 – 3.38 (m, 2H, CH_2NHIAc), 3.11 (s, 3H, CH_3).

^{13}C NMR (126 MHz, $CDCl_3$) δ 170.7 (*C*q-tetrazine), 167.5 ((*C*=O)NHBn), 166.0 (*C*q-tetrazine), 164.0 ((*C*=O)CH$_2$I), 143.2 (*C*q-arom), 131.1 (*C*q-arom), 128.6 (*C*H-arom), 128.4 (*C*H-arom), 71.0 (OCH$_2$), 70.9 (CH$_2$(C=O)NHBn), 70.9 (OCH$_2$), 70.4 (OCH$_2$), 70.3 (OCH$_2$), 69.6 (CH$_2$CH$_2$NHIAc), 42.7 (CH$_2$-benzyl), 40.3 (CH$_2$NHIAc), 21.3 (CH$_3$), -0.3 (CH$_2$I).

HRMS (ESI-Orbitrap) calcd. for $C_{20}H_{28}IN_6O_5$ $[M+H]^+$ 559.11604, found 559.11582.

2,5-dioxopyrrolidin-1-yl (1*S*,4*S*)-bicyclo[2.2.1]hept-5-ene-2-carboxylate



37

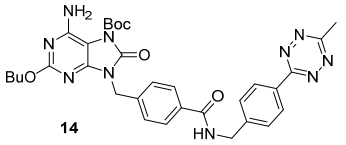
5-Norbornene-2-carboxylic acid (1.3 mL, 11 mmol, 1.0 equiv.) was dissolved in DCM (50 mL, 0.21 M). *N*-Hydroxysuccinimide (4.6 g, 40 mmol, 3.8 equiv.) and EDC hydrochloride (7.7 g, 40 mmol, 3.8 equiv.) were added and the reaction was stirred for 15 hrs. The reaction mixture was washed with 1.0 M aq. HCl (3x). The organic layer was dried ($MgSO_4$), filtered and concentrated *in vacuo*. Purification by silica gel column chromatography (1:9, EA – Pentane to 7:13, EA – Pentane) afforded activated ester **37** (2.5 g, 11 mmol, 99%) as a white solid.

^{1}H NMR (400 MHz, $CDCl_3$, both isomers) δ 6.28 – 6.18 (m, 1H, C*H*=C*H*), 6.18 – 6.09 (m, 1H, C*H*=C*H*), 3.44 – 3.37 (m, 0.8H, C*H*-bridgehead-endo), 3.30 – 3.20 (m, 1H, C*H*-bridghead-exo, C*H*(C=O)O-endo), 3.03 – 2.96 (m, 1H, C*H*-bridgehead-exo, C*H*-bridghead-endo), 2.89 – 2.74 (m, 4H, CH_2(C=O)N), 2.51 (ddd, *J* = 9.0, 4.5, 1.5 Hz, 0.2H, C*H*(C=O)O-exo), 2.09 – 1.97 (m, 1H, CH_2CH(C=O)O-exo, CH_2CH(C=O)O-endo), 1.58 – 1.41 (m, 2.2H, CH_2CH(C=O)O-exo, CH_2CH(C=O)O-endo, CH_2-bridge exo, CH_2-bridge endo), 1.38 – 1.31 (m, 0.8H, CH_2-bridge endo).

^{13}C NMR (101 MHz, $CDCl_3$, both isomers) δ 171.7 (*C*=O), 170.0 (C=O), 169.5 (*C*=O), 169.3 (C=O), 138.6 (CH=CH-exo), 138.2 (CH=CH-endo), 135.3 (CH=CH-exo), 132.2 (CH=CH-endo), 49.7 (CH$_2$-bridge-endo), 47.2 (CH-bridgehead-exo), 46.5 (CH-bridgehead-endo), 46.5 (CH$_2$-bridge-exo), 42.6 (CH-bridghead-endo), 41.8 (CH-bridgehead-exo), 40.7 (CH(C=O)O-endo), 40.3 (CH(C=O)O-exo), 31.0 (CH$_2$CH(C=O)O-exo), 29.6 (CH$_2$CH(C=O)O-endo), 25.7 (CH$_2$(C=O)N-exo), 25.6 (CH$_2$(C=O)N-endo).

HRMS (ESI-Orbitrap) calcd. for $C_{12}H_{14}NO_4$ $[M+H]^+$ 236.09173, found 236.04506.

***tert*-butyl 6-amino-2-butoxy-9-(4-((4-(6-methyl-1,2,4,5-tetrazin-3-yl)benzyl)carbamoyl)benzyl)-8-oxo-8,9-dihydro-7*H*-purine-7-carboxylate**



14

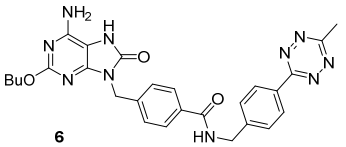
4-((6-amino-2-butoxy-7-(tert-butoxycarbonyl)-8-oxo-7,8-dihydro-9*H*-purin-9-yl)methyl)benzoic acid **13** (23 mg, 50 μmol, 1.0 equiv.) was dissolved in DCM (0.50 mL, 0.10 M) and DIC (14 μL, 0.10 mmol, 2.0 equiv.) and *N*-methylmorpholine (11 μL, 0.10 mmol, 2.0 equiv.) and the reaction was stirred. After 1 hr, a solution of (4-(6-methyl-1,2,4,5-tetrazin-3-yl)phenyl)methanamine hydrochloride (12 mg, 50 μmol, 1.0 equiv.) and *N*-methylmorpholine (11 μL, 0.10 mmol, 2.0 equiv.) in a mixture of DCM and DMF (1:1, DCM – DMF, 1.0 mL, 50 mM) was added dropwise and the reaction was stirred for 19 hrs. The reaction mixture was washed with H_2O (5x) and the organic layer was dried ($MgSO_4$), filtered and concentrated under reduced pressure. Purification by silica gel column chromatography (1:39, MeOH – DCM) afforded tetrazine **14** (5.6 mg, 8.9 μmol, 18%) as a pink solid.

^{1}H NMR (500 MHz, $CDCl_3$) δ 8.59 – 8.53 (m, 2H, C*H*-arom), 7.81 – 7.75 (m, 2H, C*H*-arom), 7.57 – 7.50 (m, 4H, C*H*-arom), 6.55 (t, *J* = 5.9 Hz, 1H, N*H*), 5.02 (s, 2H, CH_2N), 4.75 (d, *J* = 5.9 Hz, 2H, CH_2NH), 4.27 (t, *J* = 6.7 Hz, 2H, OCH_2), 3.10 (s, 3H, CH_3), 1.79 – 1.70 (m, 2H, OCH$_2$CH_2), 1.62 (s, 9H, N(C=O)O^t*Bu*), 1.53 – 1.42 (m, 2H, CH_2CH$_3$), 0.96 (t, *J* = 7.4 Hz, 3H, CH$_2$CH_3).

^{13}C NMR (126 MHz, $CDCl_3$) δ 167.4 (NH(*C*=O), 167.2 (*C*q-tetrazine), 164.0 (*C*q-tetrazine), 162.0 (NH$_2$*C*q), 150.9 (BuO*C*q), 150.4 (N(*C*=O)N), 150.2 (N(*C*=O)O^{t}Bu), 149.7 (N*C*qN), 143.2 (*C*q-arom), 139.7 (*C*q-arom), 133.8 (*C*q-arom), 131.2 (*C*q-arom), 129.1 (*C*H-arom), 128.6 (*C*H-arom), 128.5 (*C*H-arom), 127.5 (*C*H-arom), 97.2 (*C*qNBoc), 86.4 (*C*CH$_3$), 67.5 (O*C*H$_2$), 43.9 (*C*H$_2$NH), 43.4 (*C*H$_2$N), 31.1 (OCH$_2$*C*H$_2$), 28.1 (N(C=O)O^t*Bu*), 21.3 (*C*H$_3$), 19.3 (CH$_2$*C*H$_3$), 14.0 (CH$_2$*C*H$_3$).

HRMS (ESI-Orbitrap) calcd. for $C_{32}H_{37}N_{10}O_5$ $[M+H]^+$ 641.29429, found 641.29470.

4-((6-amino-2-butoxy-8-oxo-7,8-dihydro-9*H*-purin-9-yl)methyl)-N-(4-(6-methyl-1,2,4,5-tetrazin-3-yl)benzyl)benzamide



6

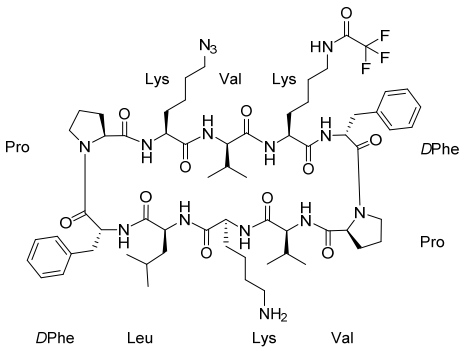
Tetrazine **14** (5.6 mg, 8.9 μmol, 1.0 equiv.) was dissolved in a mixture of TFA and DCM (1:2, TFA – DCM, 0.50 mL, 18 mM). The reaction was stirred for 3 hrs. Toluene was added and the solution was evaporated to a volume of ca. 1 mL (3x). The mixture was evaporated to dryness to obtain tetrazine **6** (4.8 mg, 8.9 μmol, quant.) as a pink solid.

^{1}H NMR (500 MHz, DMF) δ 10.21 (s, 1H, N*H*(C=O)N), 9.15 (t, *J* = 6.0 Hz, 1H, N*H*Bn), 8.51 – 8.46 (m, 2H, C*H*-arom), 8.02 – 7.99 (m, 2H, C*H*-arom), 7.70 – 7.65 (m, 2H, C*H*-arom), 7.54 – 7.48 (m, 2H, C*H*-arom), 6.67 (br s, 2H, NH_2), 5.04 (s, 2H, CH_2N), 4.72 (d, *J* = 6.0 Hz, 2H, CH_2NH), 4.19 (t, *J* = 6.6 Hz, 2H, OCH_2), 3.04 (s, 3H, CH_3), 1.71 – 1.62 (m, 2H, OCH$_2$CH_2), 1.47 – 1.36 (m, 2H, CH_2CH$_3$), 0.92 (t, *J* = 7.4 Hz, 3H, CH$_2$CH_3).

^{13}C NMR (126 MHz, DMF) δ 168.5 NH(*C*=O)Ph, 167.4 (*C*q-tetrazine), 164.7 (*C*q-tetrazine), 161.7 (NH$_2$*C*q), 153.9 (BuO*C*q), 150.9 (NH(*C*=O)N), 149.2 (N*C*qN), 145.8 (*C*q-arom), 142.0 (*C*q-arom), 134.9 (*C*q-arom), 131.9 (*C*q-arom), 129.3 (*C*H-arom), 128.6 (*C*H-arom), 128.6 (*C*H-arom), 128.5 (*C*H-arom), 99.7 (NH*C*q), 67.2 (O*C*H$_2$), 43.9 (NH*C*H$_2$), 43.4 (N*C*H$_2$), 32.0 (OCH$_2$*C*H$_2$), 21.4 (*C*H$_3$), 20.1 (*C*H$_2$CH$_3$), 14.4 (CH$_2$*C*H$_3$).

HRMS (ESI-Orbitrap) calcd. for $C_{27}H_{29}N_{10}O_3$ $[M+H]^+$ 541.24186, found 541.24211.

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

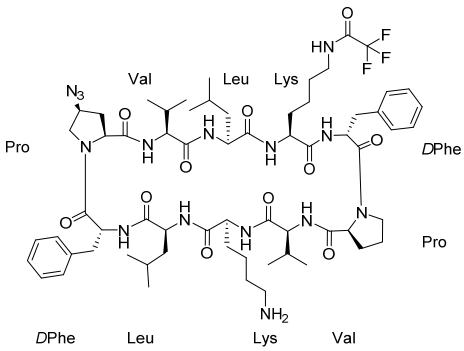


38

Functionalized resin **12** (0.53 g, 0.10 mmol, 1.0 equiv.) was elongated using the Liberty Blue 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 $C_{64}H_{95}F_3N_{15}O_{11}$ $[M+H]^+$ 1306.73, observed 1306.67 with a retention time of 7.90 min.

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

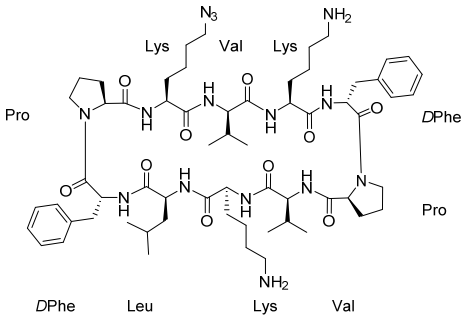


39

Functionalized resin **12** (0.53 g, 0.10 mmol, 1.0 equiv.) was elongated using the Liberty Blue 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 $C_{64}H_{95}F_3N_{15}O_{11}$ $[M+H]^+$ 1306.73, observed 1306.87 with a retention time of 7.74 min.

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

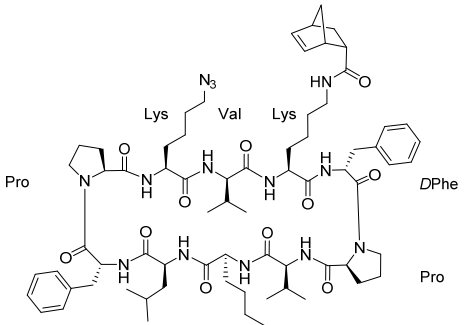


40

Resin **29** (0.10 mmol, 1.0 equiv.) was suspended in 7.0 M NH_3 in MeOH (8.0 mL, 13 mM). The suspension was irradiated in a microwave to 90 °C and stirred at 90 °C for 12 hrs. The suspension was filtered and the resin was washed with MeOH (4x), 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 $C_{62}H_{96}F_3N_{15}O_{10}$ $[M+H]^+$ 1210.75, observed 1210.67 with a retention time of 6.38 min.

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

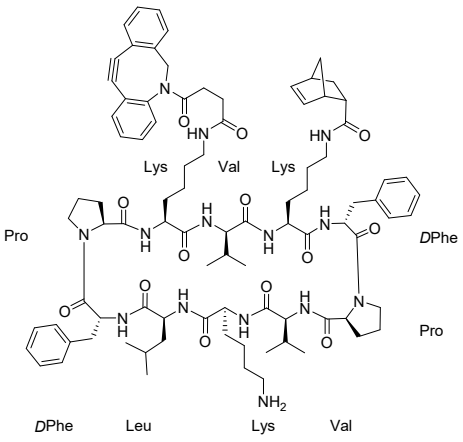


41

To the resin with the free lysine ε-amine (0.10 mmol, 1.0 equiv.) was added activated ester **37** (47 mg, 0.20 mmol, 2.0 equiv.) and DMF (4.0 mL, 25 mM) followed by DIPEA (70 μL, 0.40 mmol, 4.0 equiv.). The suspension was shaken for 19 hrs followed by filtration. The resin was washed with DMF (4x) and DCM (4x). A cleavage mixture (1:199, TFA – DCM) was added to a small amount of resin (5.0 mg) and shaken for 2 min followed by filtration (10x). The filtrate was analyzed by LC-MS.

LC-MS (ESI^+) calcd. for $C_{70}H_{104}N_{15}O_{11}$ $[M+H]^+$ 1330.80, observed 1330.93 with a retention time of 8.21 min.

***cyclo*(-Leu-*D*Phe-Pro-Lys(DIBAC)-Val-Lys(Norb)-*D*Phe-Pro-Val-Lys-)**

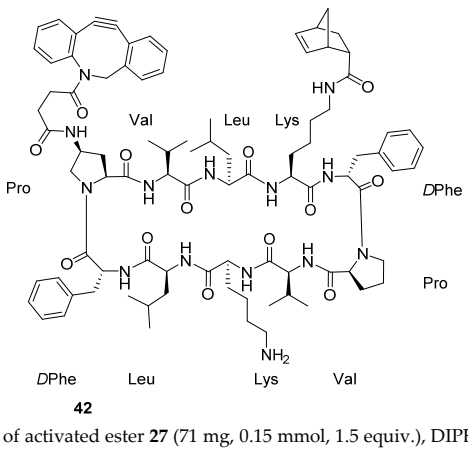


36

To functionalized resin **34** (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 activated ester **27** (71 mg, 0.15 mmol, 1.5 equiv.), DIPEA (52 μL, 0.30 mmol. 3.0 equiv.) in DMF (4.0 mL, 25 mM) was added to the resin and was subsequently shaken for 72 hrs. 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, 53% to 59% solvent B) to obtain alkyne **36** (4.0 mg, 2.5 μmol, 2.5%) as a white solid.

HRMS (ESI-Orbitrap) calcd. for $C_{89}H_{120}N_{14}O_{13}$ $[M+2H]^{2+}$ 796.45742, found 796.45703.

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

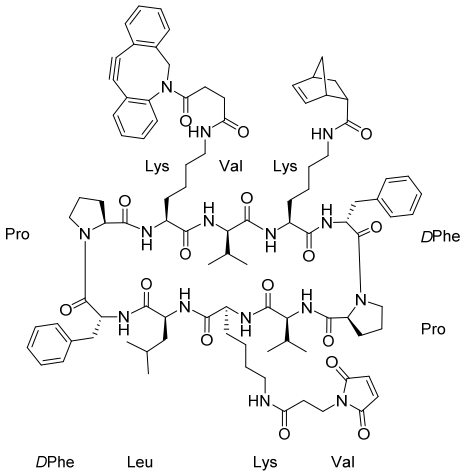


42

The resin bearing the cyclopeptide (0.10 mmol, 1.0 equiv.) was suspended in 7.0 M NH_3 in MeOH (8.0 mL, 13 mM). The suspension was irradiated in a microwave to 90 °C and stirred at 90 °C for 12 hrs. The suspension was filtered and the resin was washed with MeOH (4x), DMF (3x) and DCM (3x). To the resin was then added activated ester **37** (47 mg, 0.20 mmol, 2.0 equiv.) and DMF (4.0 mL, 25 mM) followed by DIPEA (70 μL, 0.40 mmol, 4.0 equiv.). The suspension was shaken for 19 hrs followed by filtration. The resin was washed with DMF (4x) and DCM (4x) and to the resin 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 activated ester **27** (71 mg, 0.15 mmol, 1.5 equiv.), DIPEA (52 μL, 0.30 mmol. 3.0 equiv.) in DMF (4.0 mL, 25 mM) was added to the resin and was subsequently shaken for 72 hrs. 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, 55% to 61% solvent B) to obtain alkyne **42** (6.2 mg, 3.9 μmol, 3.9%) as a white solid.

HRMS (ESI-Orbitrap) calcd. for $C_{89}H_{120}N_{14}O_{13}$ $[M+2H]^{2+}$ 796.45742, found 796.45711.

***cyclo*(-Leu-*D*Phe-Pro-Lys(DIBAC)-Val-Lys(Norb)-*D*Phe-Pro-Val-Lys(Mal)-)**

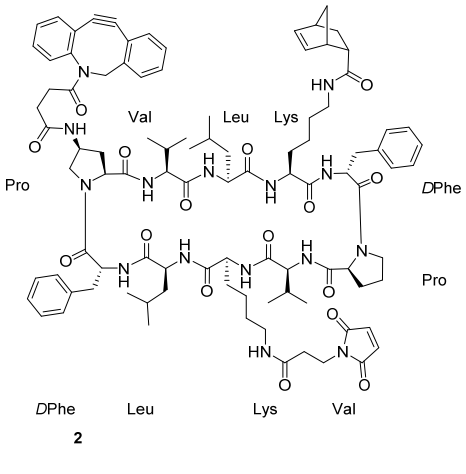


1

Crude alkyne **36** (30 mg, 17 μmol, 1.0 equiv.) was dissolved in DMF (0.50 mL, 34 mM). 3-Maleimidopropionic acid NHS ester (40 mg, 0.15 mmol, 8.8 equiv.) and *N*-methylmorpholine (17 μL, 0.15 mmol, 8.8 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, 69% to 71% solvent B) and subsequent lyophilization furnished maleimide **1** (4.0 mg, 2.3 μmol, 2.3%) as a white solid.

HRMS (ESI-Orbitrap) calcd. for $C_{96}H_{125}N_{15}O_{16}$ $[M+2H]^{2+}$ 871.97089, found 871.97109.

***cyclo*(-Leu-*D*Phe-Pro((4*S*)-4-NHDIBAC)-Val-Leu-Lys(Norb)-*D*Phe-Pro-Val-Lys(Mal)-)**



2

Crude alkyne **42** (30 mg, 17 μmol, 1.0 equiv.) was dissolved in DMF (0.50 mL, 34 mM). 3-Maleimidopropionic acid NHS ester (40 mg, 0.15 mmol, 8.8 equiv.) and *N*-methylmorpholine (17 μL, 0.15 mmol, 8.8 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, 68% to 70% solvent B) and subsequent lyophilization furnished maleimide **2** (5.8 mg, 3.3 μmol, 3.3%) as a white solid.

HRMS (ESI-Orbitrap) calcd. for $C_{96}H_{125}N_{15}O_{16}$ $[M+2H]^{2+}$ 871.97089, found 871.97098.

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