Synthesis of cyclic peptides as bioconjugation platforms

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Synthesis and application of cyclic peptides

The identification of an adaptive immune system present in microbes has had a profound effect on science. Twenty years after their original discovery, clustered regularly interspaced short palindromic repeats (CRISPR) were recognized to provide acquired immunity against viruses and plasmids.[1–4] Identified in approximately 40% of sequenced bacterial genomes, CRISPR represents a family of DNA repeats separated by stretches of variable sequences termed spacers; short DNA sequences stemming from invading pathogens. Besides this immunological memory, a set of CRISPR-associated (Cas) genes completes the adaptive immune

system which encodes the protein machinery to carry out a directed immune response.[5] The first step in the CRISPR-Cas system is the identification, by Cas proteins, of a new spacer sequence from an invading pathogen which is then inserted into the CRISPR sequence. Expression of this sequence gives an RNA precursor that is subsequently processed so that each unit known as CRISPR RNA (crRNA) contains a single spacer flanked by a part of the repeat sequence. An active CRISPR-Cas complex is formed when this crRNA is combined with one or more Cas proteins. When a foreign nucleic acid is recognized by base-pairing with complementary crRNA sequences, the target is cleaved and degraded.[6]

The CRISPR-Cas system has since been transformed into a simple and efficient genome editing tool, awarded this year (2020) with the Nobel prize in chemistry.[7–10] The CRISPR-Cas9 system has been used to understand mechanisms of genetic diseases, validate disease targets, develop animal disease models, facilitate genetic engineering in plants and allow for more thorough epigenetic studies leading to over 6000 research papers within five years.[11–17] However, CRISPR-Cas does not mark the first time a microbial defense mechanism has had a major impact on science. Secondary metabolites produced by soil microbes have been a vast resource of antibiotics with over 55% of all antibiotics detected between 1945 and 1978 originating from the genus *Streptomyces*.[18] Since the early days of antibiotic research, it was hypothesized that these bioactive compounds act as a defense mechanism to protect the territory and resources from surrounding micro-organisms.[19] More recently, there have been reports that these secondary metabolites may function as signaling molecules as well.[20]

Since their introduction in the clinic, antibiotics have not only been used to treat infectious diseases, but have also found use in the treatment of other diseases, including cancer, and are applied in the context of organ transplants and open-heart surgery. Aided in part by antibiotics, the average human life expectancy has increased with 23 years when compared to 1910.[21] The discoveries of actinomycin, streptothricin and, most notably, streptomycin by the Waksman group in the 1940s kickstarted natural product discovery in the search for novel bioactive compounds. This has resulted in a myriad of bioactive compounds from bacteria, besides antibiotics also anti-cancer drugs, immunosuppressants, antifungals and anthelminthics.[22,23] Amongst these molecules is the class of macrocyclic peptide-based antibiotics, which are predominantly peptidic structures featuring a ring size larger than twelve atoms (*Figure 1*). In recent years, interest in these compounds as

therapeutics has been renewed and several novel macrocyclic peptides have entered clinical phase trials.[24,25]

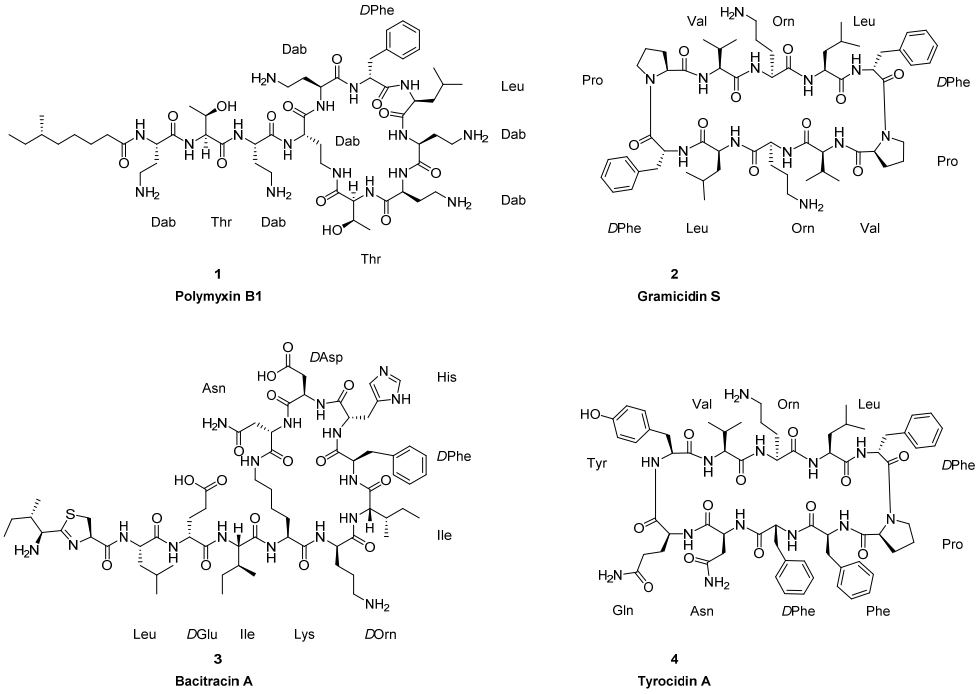

Figure 1. A selection of macrocyclic peptide-based antibiotics.

Syntheses of macrocyclic peptides were first performed to provide structural proof of natural compounds and to create new biologically active analogues.[26] More recently, synthetic cyclic peptides have also been employed to mimic functional domains from proteins and peptides to create peptidomimetic drugs. As an example, the antibiotic protegrin I adopts a well-defined β-hairpin conformation owing to the constraints induced by two disulfide bridges. The absence of one or both bridges leads to loss of the β-hairpin conformation and reduces the membranolytic activity.[27,28] However, significant hemolytic activity hinders the application of protegrin I as an antibiotic. The group of Robinson synthesized a number of cationic head-to-tail cyclic peptides (10 and 14-mers) that feature a β-hairpin structure induced by a L-Pro-D-Pro motif. Optimization of the most promising candidate eventually led to murepavadin lacking the hemolytic

properties of protegrin I but showing *Pseudomonas* specific antimicrobial activity and was projected to enter phase III clinical trials in 2017.[29–31]

The key step in synthetic approaches toward macrocyclic peptides is the macrocyclization reaction. The macrocyclization step most often comprises a lactamization, a lactonization (in case of depsipeptides – macrocyclic peptide-based compounds featuring an ester linkage in the backbone) or the formation of a disulfide bridge. Structurally more diverse macrocycles have been synthesized using other chemical bond forming steps, for instance oxadiazole formation or chemistries involving external stapling agents.[32,33] Depending on its functional groups, cyclization of a peptide can occur in four different ways: head-to-tail (C-terminus to N-terminus), head-to-side-chain, side-chain-to-tail and side-chain-to-side-chain. The most commonly employed strategy is solution-phase cyclization, where the linear peptide is synthesized on the resin and, after cleavage from the resin, is cyclized under dilute conditions (*Figure 2A*). To achieve a selective cyclization a minimum of three levels of orthogonality is required between the linker, the amino acid protecting groups not involved in the cyclization and the N-terminus for elongation. The advantage of this approach is that it enables the synthesis of cyclic peptides on a large scale. However, even under dilute conditions intermolecular reactions may occur to give cyclodimers and cyclooligomers.[34] Performing the cyclization when the peptide is still attached to the solid support remedies this problem owing to pseudo-dilution (*Figure 2B*). Polymer-bound intermediates favor intramolecular reactions over intermolecular ones owing to a low frequency of encounters between intermediates.[34,35] On-resin cyclization starts with the anchoring of an appropriate side-chain (most commonly Asx or Glx) or *via* backbone anchoring. After peptide elongation the sites for cyclization are deprotected orthogonally from the linker and side-chain protecting groups followed by cyclization. Cleavage from the resin and deprotection of applicable side-chains gives the cyclic peptide.

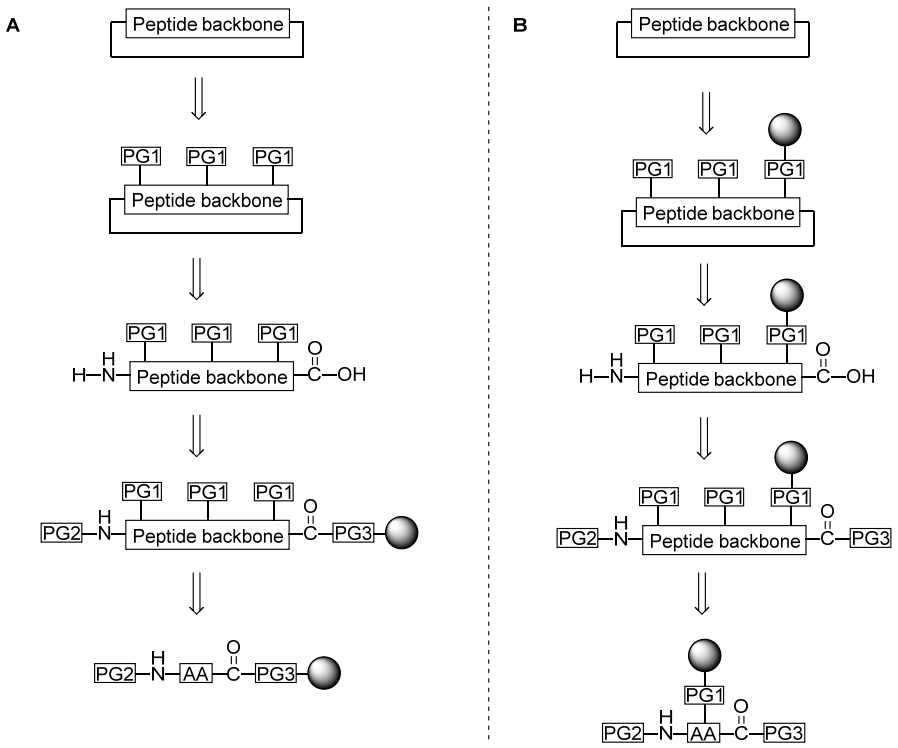

Figure 2. A. Off-resin cyclization strategy for head-to-tail cyclic peptides (PG = protecting group). *B.* The major alternative strategy for head-to-tail cyclic peptides involving on-resin cyclization for which a trifunctional amino acid is required.

Focusing on homodetic cyclic peptides (only amide-linkages connecting the constituent amino acids), both strategies have been employed to synthesize bioactive natural products. For example, the group of Bair utilized a solution-phase cyclization strategy in the synthesis of polymyxin B1 (*Figure 3*).[36] Polymyxin B1 is a side-chain-to-tail cyclic peptide consisting of ten amino acids and a lipophilic moiety at the N-terminus. It is derived from *Bacillus polymyxa* and has been used as a topical antibiotic for over 50 years.[37] Employing a standard Fmoc-based peptide synthesis, Bair and co-workers opted for the sasrin resin, as protected peptides can be liberated from this resin with dilute acid, and protected the Dab that participates in the cyclization with a Dde group. After furnishing linear peptide **6** using HBTU as the condensing agent, the Dde group was removed by three treatments of five minutes each with 2% (v/v) hydrazine in DMF after which the peptide was liberated from the resin using a mixture of TFA and DCM (1:99, TFA – DCM) to obtain compound **7**.

The macrocyclization was carried out with diphenylphosphoryl azide (DPPA) and DIPEA in acetonitrile for 48 hours to obtain protected cyclic peptide **8**. Lastly, global deprotection with 5% (v/v) H_2O in TFA afforded polymyxin B1 in a yield of 20% over 24 steps.

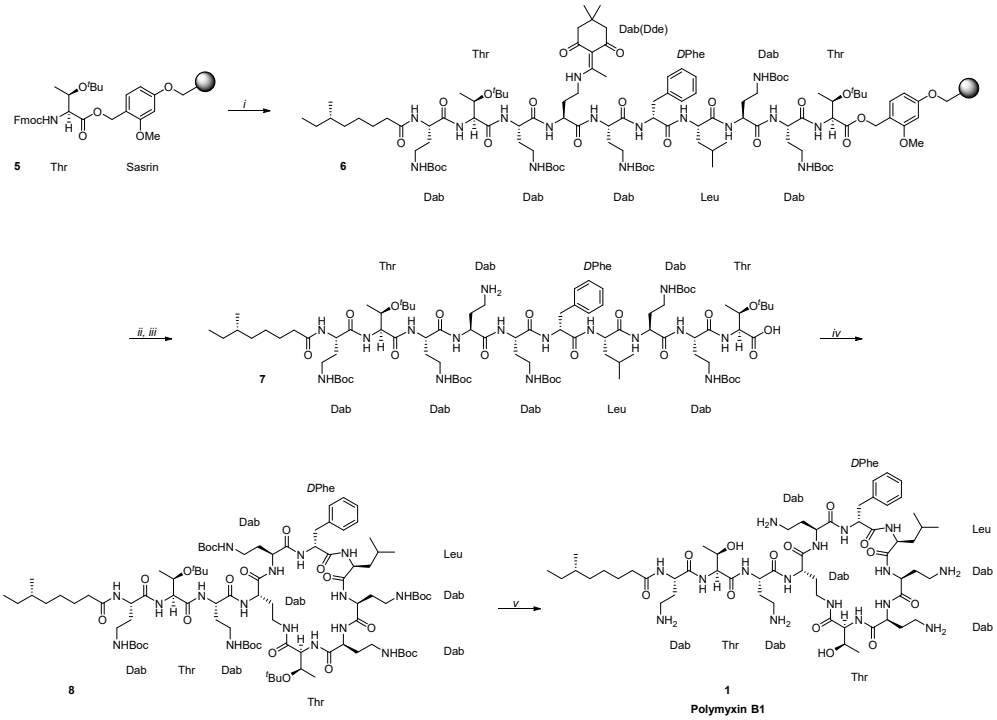

Figure 3. Solution-phase cyclization strategy for the synthesis of polymyxin B1. Reagents and conditions: (*i*) SPPS: (a) piperidine, DMF, rt, 2x, 5 and 15 min. (b) Fmoc-AA, HBTU, DIPEA, NMP, rt, 21 min. (*ii*) hydrazine – DMF (1:49), rt, 3x5 min. (*iii*) TFA – DCM (1:99), rt, 3x15 min. (*iv*) DPPA, DIPEA, MeCN, rt, 48 hrs (*v*) TFA – H_2O (95:5), rt, 1 hr, 20%.

Owing to several appealing characteristics as an antibiotic, gramicidin S has been the subject of numerous studies. Isolated from Russian soil *Bacillus brevis*, this head-to-tail cyclic decapeptide is active against both Gram-positive and Gram-negative bacteria with no observed resistance since its introduction into the clinic in the 40's of the 20th century.[38] Unfortunately, toxicity toward human red blood cells has limited its use to topical infections.[39–41] A range of analogues have been synthesized in an effort to mitigate the hemolysis caused by the parent compound.[42] As a consequence, both solution-phase cyclization strategies as well as on-resin cyclization approaches have been utilized to synthesize gramicidin S and its

analogues. The group of Ulrich employed a solution-phase cyclization strategy utilizing an Fmoc-based SPPS approach (*Figure 4*).[43] Orthogonality between linker and side-chain protecting groups was achieved by using a 2-chlorotrityl resin cleavable with acid and Dde moieties to protect the ornithine δ-amines that can be cleaved with hydrazine. The appropriate Fmoc amino acids were pre-activated with HCTU, 6-Cl-HOBt and DIPEA in DMF for two minutes after which it was coupled to the resin for two hours. After obtaining polymer-bound linear peptide **10**, the resin was subjected to a cleavage cocktail (185:10:5, TFA – TIPS – H_2O) giving decapeptide **11**. The macrocyclization was carried out with PyBOP acting as the condensing agent, HOBt as an additive, DIPEA as the base and DCM as the solvent. The solution was stirred for 20 hours after which the solvent was removed and the crude oil treated with 2% (v/v) hydrazine in THF for 16 hours to liberate the ornithine δ-amines. Ultimately, gramicidin S **2** was obtained in a yield of 69% over 23 steps.

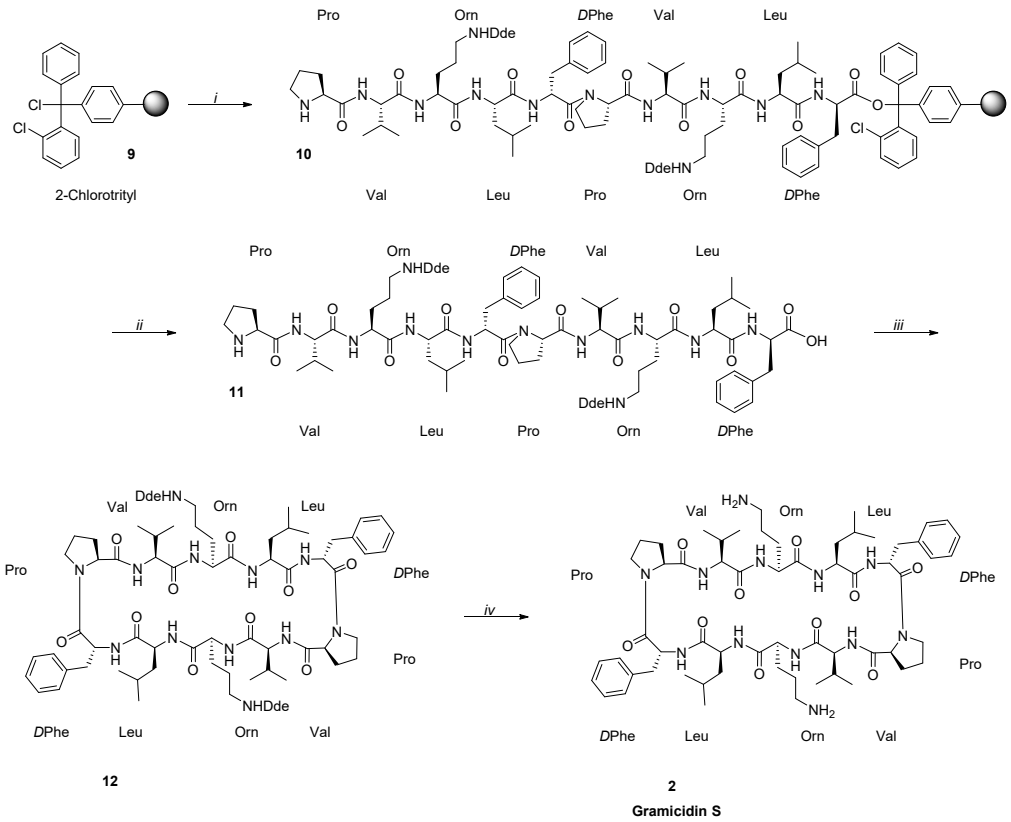

Figure 4. Solution-phase strategy for the synthesis of gramicidin S. Reagents and conditions: (*i*) SPPS: (a) piperidine, DMF, rt, 30 min. (b) Fmoc-AA, 6-Cl-HOBt, HCTU, DIPEA, NMP, rt, 2 hrs (*ii*) TFA – TIPS – H_2O (185:10:5), rt, 3.5 hrs (*iii*) PyBOP, HOBt, DIPEA, DCM, DMF, rt, 20 hrs (*iv*) hydrazine, THF, rt, 16 hrs, 69%.

Alternatively, an on-resin cyclization strategy to synthesize gramicidin S was utilized by the group of González-Muñiz (*Figure 5*).[44] Employing a resin functionalized with a para-hydroxybenzyl alcohol (PHB) linker, the ornithine ε-amine was anchored onto the solid support in two steps. First, the benzyl alcohol was activated with *N*,*N*′-disuccinimidyl carbonate and DMAP for two hours to give carbonate **14**. The resin was then reacted with the trifluoroacetate salt of Fmoc-Orn-OAll and DIPEA for four hours to afford anchored ornithine **15** in a 71% yield. After elongation of the peptide using solid-phase synthesis, the C-terminal allyl ester was deprotected with $Pd(PPh_3)_4$ and morpholine as the allyl acceptor followed by Fmoc deprotection with piperidine. With the N- and C-termini liberated, the cyclization was carried out with PyAOT, HOAt and DIPEA in DMF for two hours at which

point ninhydrin analysis showed no remaining free amino groups. Simultaneous cleavage from the resin and side-chain deprotection with 5% (v/v) H_2O in TFA furnished gramicidin S **2** in a yield of 10% over 22 steps.

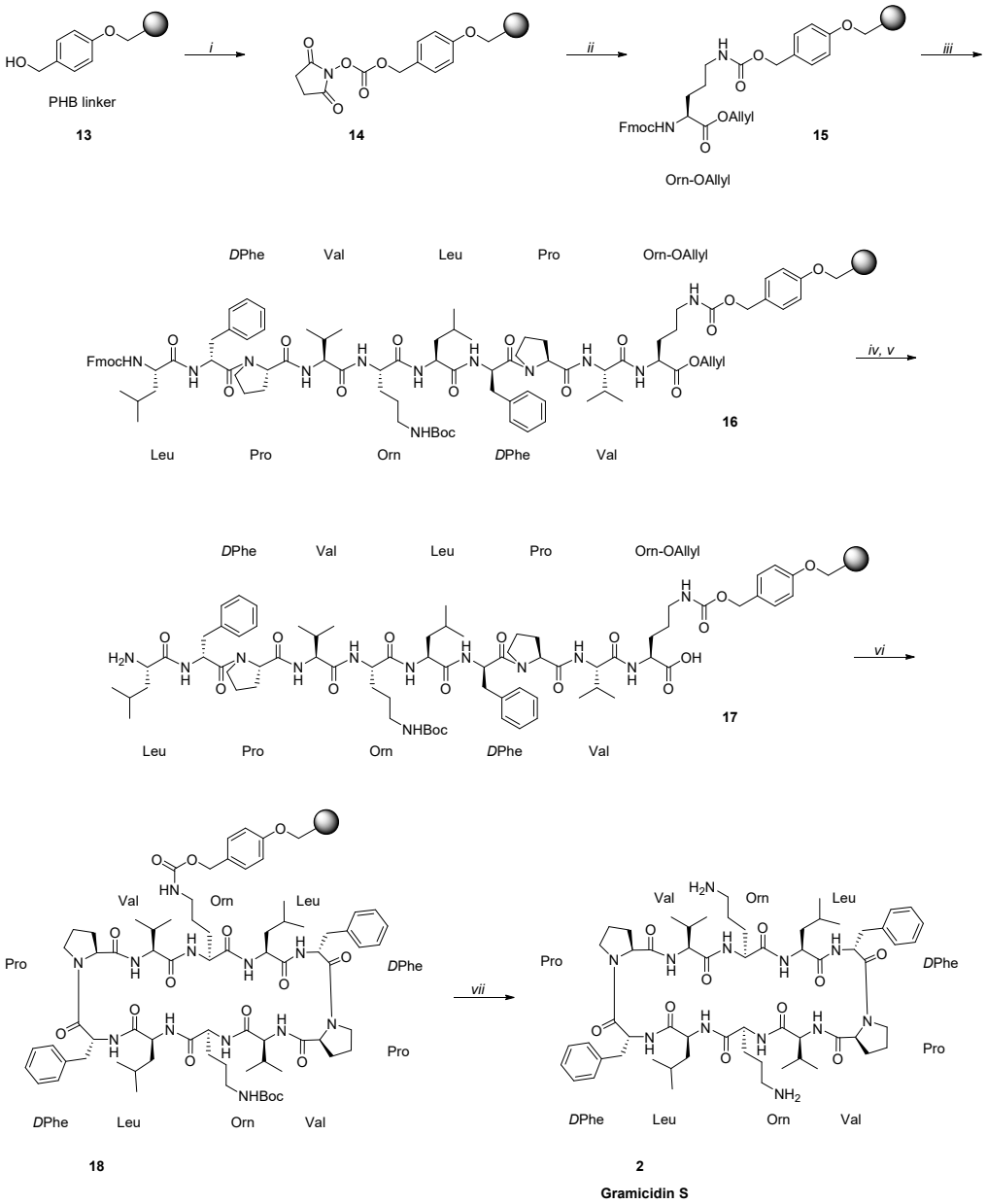

Figure 5. On-resin cyclization strategy for the synthesis of gramicidin S. Reagents and conditions: (*i*) *N*,*N*′-disuccinimidyl carbonate, DMAP, DMF, rt , 2 hrs (*ii*) Fmoc-Orn-OAll trifluoroacetate, DIPEA, DMF, rt, 4 hrs, 71% (*iii*) SPPS: (a) piperidine, DMF, rt, 2x1 min + 1x10 min (b) Fmoc-AA, TBTU, DIPEA, DMF, rt, 1 hr (*iv*) $Pd(PPh_3)_4$, DMSO, THF, 0.5 M aq. HCl, morpholine, rt, 2.5 hrs (*v*) piperidine, DMF, rt, 2x1 min + 1x10 min (*vi*) PyAOP, HOAt, DIPEA, DMF, rt, 2 hrs (*vii*) TFA – H_2O (19:1), rt, 2 hrs, 10%.

The synthesis of the antibiotic bacitracin A by the group of Griffin is another example of utilizing an on-resin cyclization approach (*Figure 6*).[45] Bacitracin A is a side-chain-to-tail cyclic peptide produced by *Bacillus subtilis* and is widely used as a component in topical antibacterial ointments.[46,47] For the synthesis of bacitracin A, Griffin and co-workers anchored the side-chain of Fmoc-Asp-OAll onto an acid-labile PAL resin, which gives an asparagine residue upon cleavage. Elongation of the peptide was performed using SPPS with HBTU and DIPEA as the coupling agents furnishing linear peptide **21** after ten cycles. The lysine ε-amine involved in the cyclization was protected with an alloc group, which was deprotected together with the C-terminal allyl ester with two treatments of $Pd(PPh_3)_4$. The cyclization was then performed with PyBOP, HOBt and DIPEA over 24 hours to give cyclic peptide **23**. After the N-terminal Fmoc was removed and further modified, bacitracin A **3** was liberated from the solid support with a cleavage cocktail (93:5:2, TFA – phenol – TIPS) in an overall yield of 24% over 24 steps.

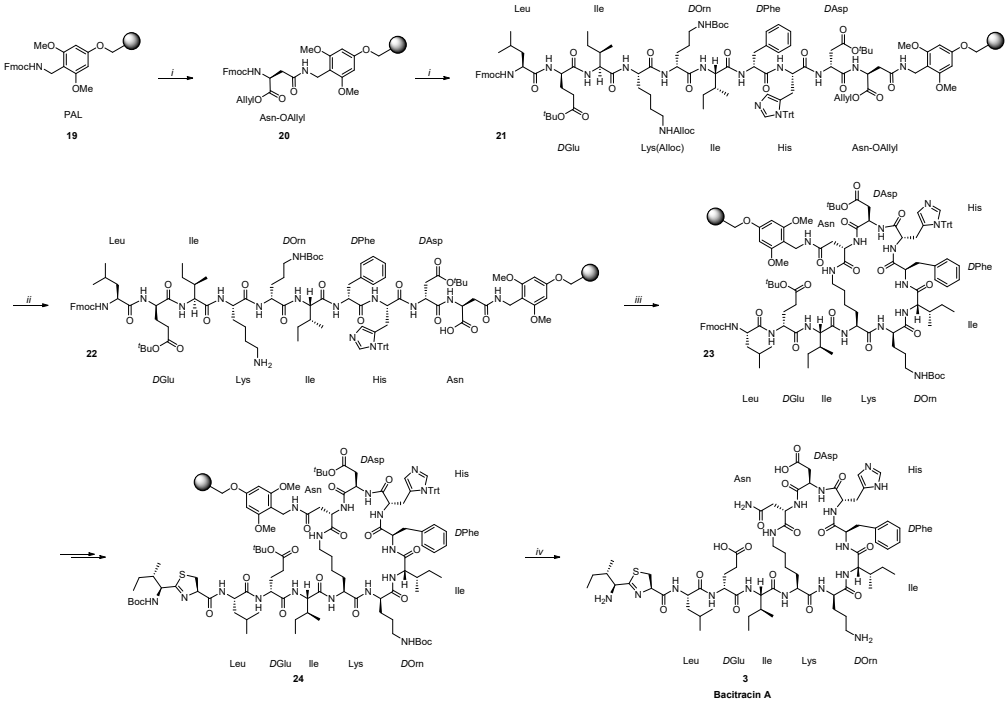

Figure 6. On-resin cyclization strategy for the synthesis of bacitracin A. Reagents and conditions: (*i*) SPPS: (a) HBTU, DIPEA, NMP, rt, 21 min (b) piperidine, DMF, rt, 1x5 min + 1x15 min (*ii*) $Pd(PPh_3)_4$, acetic acid, *N*-methylmorpholine, $CHCl_3$, rt, 1x4 hrs + 1x12 hrs (*iii*) PyBOP, HOBt, DIPEA, NMP, rt, 24 hrs (*iv*) TFA – phenol – TIPS (93:5:2), rt, 1 hr, 24%.

An interesting cleavage by cyclization approach toward cyclic peptides was reported by Yang and Moriello and applied to tyrocidine A by the group of Guo (*Figure 7*).[48,49] For this approach to be high-yielding a pre-organized conformation of the linear peptide is required.[50] Tyrocidine A is a cyclic decapeptide with antibacterial properties produced by *Bacillus brevis* and possesses a conformational preference to self-cyclize making it an ideal candidate for this approach.[47,51,52] First, Kenner's safety-catch resin was reacted with Fmoc-Leu-OH and DIC in the presence of 1-methylimidazole for 24 hours to give compound **26**. The peptide was elongated using SPPS with a combination of DIC and HOBt as the coupling reagents. The safety-catch linker was then activated by treatment with iodoacetonitrile and DIPEA in NMP for 24 hours. After activation, the linker could be cleaved with a nucleophile which was generated by global deprotection with a cleavage cocktail (88:5:5:2, TFA – phenol – TIPS – H_2O). Treatment with DIPEA stimulated cyclization and simultaneous liberation from the solid support giving tyrocidine A **4** in a yield of 25% over 22 steps.

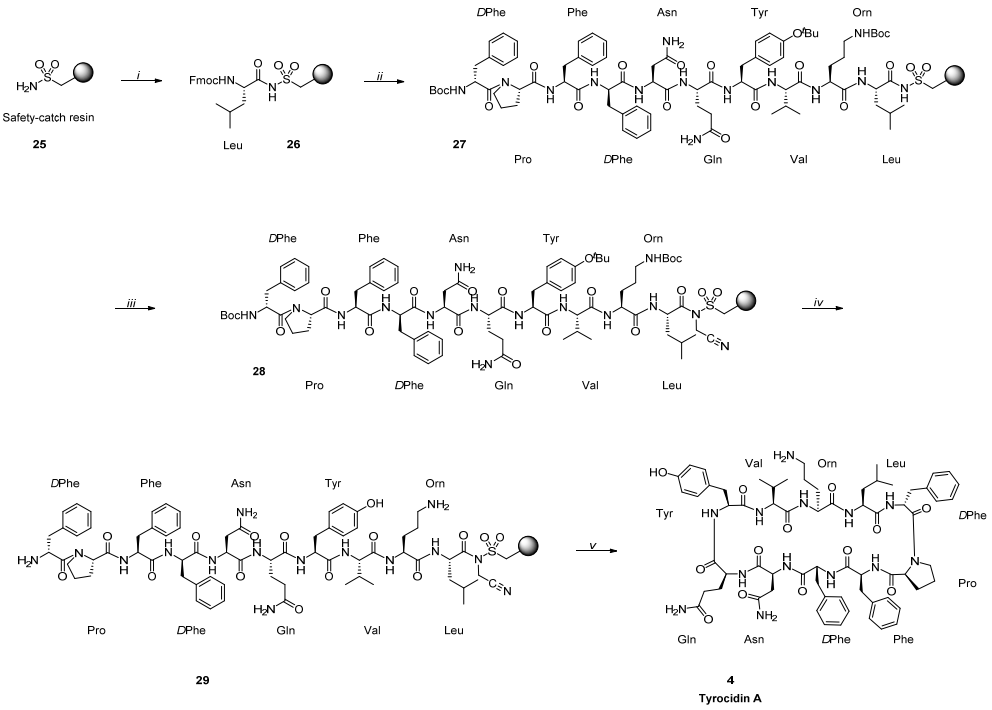

Figure 7. Strategy for synthesis of tyrocidin A involving simultaneous cyclization and cleavage from the resin. Reagents and conditions: (*i*) Fmoc-Leu-OH, DIC, 1-methylimidazole, DCM, DMF, rt, 24 hrs (*ii*) SPPS: (a) piperidine, DMF, rt, 30 min (b) Fmoc-AA (Boc-AA for last residue), DIC, HOBt, DMF, rt, 4 hrs (*iii*) iodoacetonitrile, DIPEA, NMP, rt, 24 hrs, (*iv*) TFA – phenol – TIPS – H_2O (88:5:5:2), rt, 2 hrs (*v*) DIPEA, THF, rt, 6 hrs, 25%.

With the available methods to synthesize them, exemplified by the syntheses described above, in combination with their reduced conformational mobility, cyclic peptides have seen a widespread usage as a multifunctional platform. A peptide template that serves as a structural motif was introduced by the group of Mutter and was termed regioselectively addressable functionalized template (RAFT) has seen considerable usage over the years (*Figure 8*).[53,54] 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 upper 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.[55]

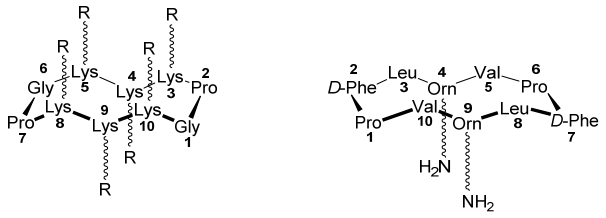

Figure 8. The regioselectively addressable functionalized template **30** as termed by Mutter and co-workers and gramicidin S **31** sharing structural similarities.

Similar to RAFT scaffold **30**, gramicidin S **31** adopts an antiparallel β sheet conformation which is closed by two type II′ β-turns. As shown by the crystal structure, the two ornithine residues present in gramicidin S are both oriented in the same plane making it suitable for the introduction of various functional groups.[56] For example, Martin-Pastor and co-workers functionalized gramicidin S by reacting the ornithine δ-amines with activated esters of achiral phosphines to create a chiral ligand for transition metal catalysis (*Figure 9*).[57] Rhodium(I) complexes were formed with phosphine-containing *para*-substituted gramicidin S **32** as well as the *meta*-variant. These complexes were found to be active in Rh-catalyzed asymmetric hydrogenation with up to 52% enantiomeric excess. The group of Kawai synthesized dinuclear Zn(II) complex **33** by reacting gramicidin S with 2-formylpyridine and $NaBH_3CN$ followed by addition of zinc chloride.[58] This dinuclear complex markedly accelerated the cleavage of the phosphodiester bond of RNA model substrate 2-hydroxypropyl *p*-nitrophenyl phosphate.

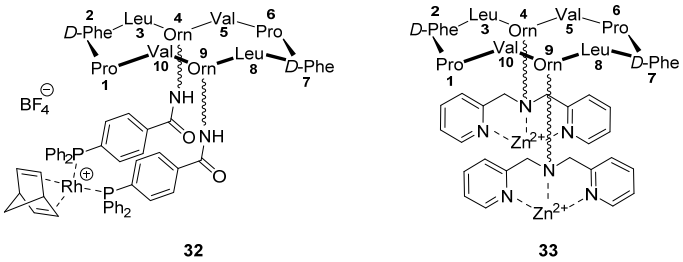

Figure 9. Examples of mono-functionalized cyclic peptides.

The RAFT template has been used by the group of Haehnel for the *de novo* synthesis of an antiparallel four helix bundle protein that is able to accommodate two bis-histidine ligated heme groups. Utilizing amphiphilic helices, Rau and Haehnel

synthesized water-soluble model **34** of the cytochrome *b* core consisting of two parallel heme-binding helices H1 alternated with two antiparallel helices H2 to shield the two hydrophobic heme binding sites (*Figure 10*).[59] The spectroscopic properties of the bound heme groups were found to resemble natural *b*-type cytochromes. For the synthesis, the cyclic decapeptide template was equipped with four protected cysteine residues with one level of orthogonality allowing for stepwise installation of the helices using a bromoacetyl-thiol coupling. Pifferi *et al.* designed and synthesized anticancer vaccines employing a RAFT scaffold with one plane creating a multivalent display of Tn antigen analogues and the opposite domain presenting an ovalbumin peptide containing T helper $CD4^+$ and $CD8^+$ epitopes, such as tetravalent scaffold **35**.[60] These constructs were designed to boost B cell activation and antibody production by effective clustering of the B-cell receptor through high-valency targeting. The synthesis was achieved by conjugating the GalNAc moieties to the scaffold through oxime ligation. A three-step procedure was then performed on the remaining lysine ε-amine (cysteine introduction, deprotection, heterodisulfide synthesis) to attach the peptide *via* disulfide bridge formation. Immunological evaluation showed the hexadecavalent construct to be the most promising generating potent and functional humoral and cellular immune responses with no observed toxicity.

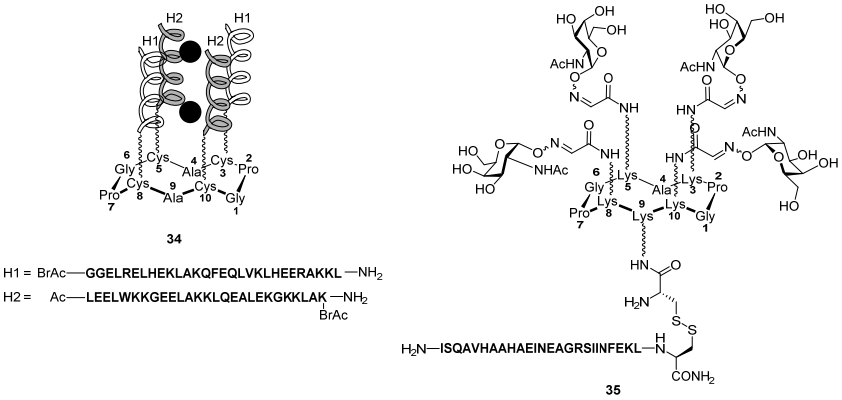

Figure 10. Examples of double functionalized cyclic peptides.

Delangle and co-workers designed a cyclodecapeptide scaffold to lower copper concentration in hepatocytes which is important in Wilson's disease.[61] Wilson's disease is a genetic disorder impairing copper transport in hepatocytes leading to cytosolic accumulation of copper and ultimately necrosis. This causes the release of

large amounts of copper into the blood stream damaging the membrane of red blood cells leading to hemolytic anemia.[62] The group of Delangle designed RAFT scaffold **36** with one plane decorated with GalNAc moieties to target the asialoglycoprotein receptor uniquely expressed on the surface of hepatocytes (*Figure 11*). The lower face of the RAFT scaffold is dedicated to soft metal ion complexation and is under normal conditions protected as a disulfide. Upon entering the reductive medium of the hepatic cells, the disulfide is reduced restoring the ability for copper chelation. The scaffold is also equipped with the fluorophore TRITC to visualize the uptake of the compound in hepatic cell lines. Its synthesis was performed by SPPS on a 2-chlorotrityl chloride resin. On-resin oxidation of the linear peptide furnished the disulfide after which the peptide was cleaved from the resin and subsequently cyclized. The GalNAc moieties were installed by oxime ligation after which the D-Lys ε-amine was reacted with tetramethylrhodamine isothiocyanate to give peptide **36**. Five different bioorthogonal conjugations were used by the group of Renaudet to synthesize well-defined scaffold **37**.[63] A heteroglycocluster of four different carbohydrates comprises one plane of the scaffold while presenting the TLR9 ligand CpG oligodeoxynucleotide (CpG ODN) on the other side. First, β-Glc hydroxylamine was conjugated to the RAFT scaffold by oxime ligation followed by attachment of a GlcNAc thiol to an alloc group by a photo-induced thiol-ene coupling. A copper-catalyzed alkyne-azide cycloaddition (CuAAC) was then performed between α-GalNAc propargyl and azidolysine after which α-Man thiol was coupled to chloroacetamide in an S_N2 reaction to complete the heteroglycocluster. Next, the lysine ε-amine facing the opposite side was functionalized with a hydroxylamine which is then coupled to CpG ODN carrying an aldehyde at the 5′-end obtaining RAFT scaffold **37**.

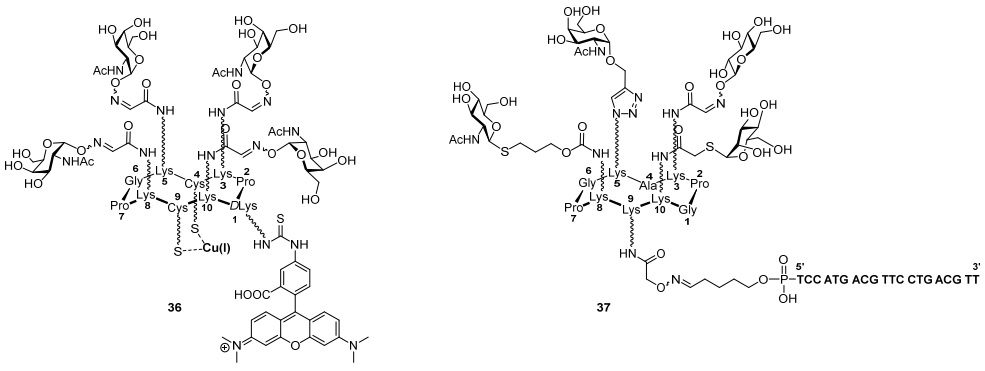

Figure 11. Examples of higher-order functionalized cyclic peptides.

The RAFT scaffolds described above all employ a solution-phase cyclization strategy with a representative example shown in *Figure 12*. The synthesis of antiparallel four-helix bundle protein **34** by Rau and Haehnel started from the extremely acid-sensitive 4-carboxytrityl resin which was preloaded with a glycine residue and the peptide was elongated using Fmoc-based solid-phase synthesis. Special care was taken to avoid racemization of the cysteine residues by introducing them as the symmetrical anhydrides under neutral conditions. The cysteine residues were alternately protected with the trityl (Trt) and acetamidomethyl (Acm) groups that serve as selectively addressable functional groups. The linear peptide was then cleaved from the resin with a cleavage cocktail (5:1:4, AcOH – MeOH – DCM) leaving the side-chain protections intact. Cyclization was performed with TBTU and DIPEA in DMF to give cyclic peptide **41**. After the trityl groups were removed with strong acid, the free thiols were conjugated with the bromoacetamide group present on helix H1 in an S_N2 fashion to obtain functionalized peptide **44**. Deprotection of the Acm groups were achieved with mercury(II) acetate and the free thiols were subsequently reacted with the bromoacetamide group present on helix H2 to give four-helix bundle protein **45**. Finally, incorporation of the heme groups furnished bis-heme binding protein **34** with the spectral properties resembling the natural cytochrome *b*.

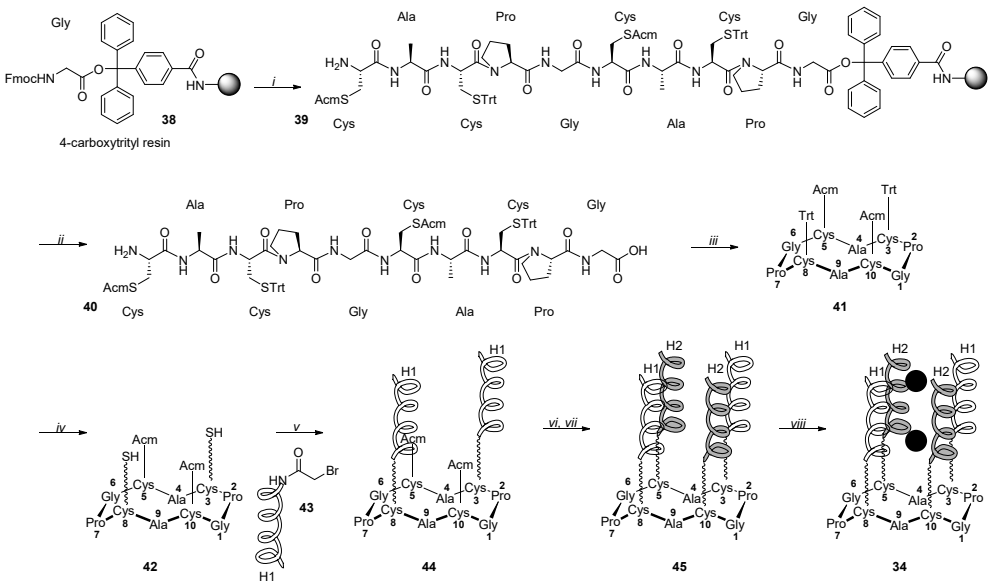

Figure 12. Synthesis of water-soluble model **34** of the cytochrome *b* core. Reagents and conditions: (*i*) SPPS: (a) piperidine, DMF, rt, 7 min. (b) Fmoc-AA, TBTU, DIPEA, DMF, rt, 30 min. or Fmoc-Cys symmetrical anhydride, DMF, rt, 30 min. (*ii*) AcOH – MeOH – DCM (5:1:4), rt, 2 hrs (*iii*) TBTU, DIPEA, DMF, rt, 8 hrs (*iv*) TFA – dithiothreitol (19:1), rt, 30 min. (*v*) 3:2 (v/v) 0.15 M sodium phosphate buffer (pH 7.5) – MeCN, rt, 3 hrs (*vi*) Hg(II)OAc, ammonium acetate buffer (pH 4), rt, 1 hr, then dithiothreitol, rt, 4 hrs (*vii*) H2, 3:2 (v/v) 0.15 M sodium phosphate buffer (pH 7.5) – MeCN, rt, 4 hrs (*viii*) Fe(III)-protoporphyrin IX (heme), DMSO, 50 mM Tris-HCl buffer (pH 8), rt, 30 min.

Thesis outline

This thesis features an on-resin cyclization strategy to synthesize several RAFT scaffolds based on gramicidin S. In **Chapter 2** a method for anchoring lysine ε- and ornithine δ-amines to a nucleophilic solid support is described. The method is used in the synthesis of several head-to-tail cyclic peptides and compared to other reported syntheses. Utilizing this method, **Chapter 3** describes the synthesis of various RAFT scaffolds equipped with two chemoselective handles in differing orientations. Several TLR ligands are synthesized with their chemoselective counterparts to study the effect of orientation on synergy between these ligands. Expanding upon these scaffolds, **Chapter 4** entails the synthesis of gramicidin-based constructs bearing three orthogonal chemoselective handles in various orientation. TLR constructs bearing the additional chemoselective counterpart are also synthesized. The synthesis of a well-defined fusion protein through employing a chemical ligation strategy is the subject in **Chapter 5**. The emphasis is put on late-stage derivatization of the individual proteins for which a two-component linker

system is designed. The linker system is then applied to conjugate two camelid antibodies together. Finally, **Chapter 6** summarizes the content of this Thesis followed by future prospects.

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