Synthesis of cyclic peptides as bioconjugation platforms

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Protocol for side-chain anchoring of the ornithine δ-amine and the lysine ε-amine in the synthesis of macrocyclic peptides

Macrocycles are defined as organic compounds featuring twelve or more atoms in their cyclic structure. Macrocycles are abundant in nature and have received considerable interest from the organic synthesis community both because of their synthetic challenge as well as their promising biological properties.[1–3] Compared to their linear counterparts, macrocycles are conformationally much more constrained. This may be of considerable biological and biomedical advantage, for instance when the conformational preference in solution matches that of the bioactive conformation when bound to a pharmacological target.[4] Macrocycles are

relatively large, and natural ones often quite polar, features that do not comply with Lipinski's rule of five traditionally used to evaluate the chance of success of potential clinical candidates.[5] Recent years however have witnessed the development of several compounds that notwithstanding the fact that they fall short of these benchmarks, have reached the clinic.[6,7] Many of these are macrocycles, further spurning academic interest in these compounds, as well as their synthesis.[8–12]

Cyclic peptides encompass a major class of macrocyclic compounds, varying widely in structure, ring size, functional group patterns and biological activities. Cyclic peptides, and indeed macrocycles in general, have found wide application as antibiotics, ever since the discovery of gramicidin S and tyrothricin in the 1940's. Tyrothricin is a mixture of peptides first isolated from the species *Aneurinibacillus migulanus* (formerly known as *Bacillus brevis*) and contains the cyclic decapeptide tyrocidine. In an effort to isolate tyrothricin from Russian soil *B. brevis*, Gause *et al.* discovered gramicidin S, which already for decades is prescribed as antibiotic for topical infections.[13,14] Both tyrocidine and gramicidin S belong to the so-called head-to-tail cyclic peptide compounds, also subject of the synthesis studies described in this chapter, in which the macrocycle is exclusively made up from the peptide backbone. Cyclic peptides exist as well in which amino acid side chain functionalities partake in macrocyclic structure.

Compared to the synthesis of linear peptides, the synthesis of head-to-tail cyclic peptides is inherently more complicated, predominantly due to the need to condense, at one stage or another in the synthetic procedure, the N-terminal amine with the C-terminal carboxylate to form an amide. This cyclization may occur either on-resin (whence a solid phase peptide synthesis procedure is followed, which is often the case) or off-resin, and both procedures require additional functional (protective) group manipulations compared to the solid phase synthesis of standard, linear peptides (*Figure 1*). On-resin cyclisation for instance requires immobilization of the first amino acid building block through a side chain functionality, rather than the carboxylate, as is standard practice in the solid phase synthesis of linear peptides. Off-resin cyclisation requires the use of side chain amine/carboxylate protective groups orthogonal to the N-terminal one and that can withstand conditions to cleave the linear precursor from the resin. A major intrinsic advantage of on-resin cyclization is that due to the inherent pseudo-dilution effect, the occurrence of intermolecular condensations is diminished compared to that of in solution procedures.[15] Of note as well are simultaneous cyclization/cleavage procedures that

have seen some usage but that appear limited in application to relatively simple macrocyclic peptides.[16,17]

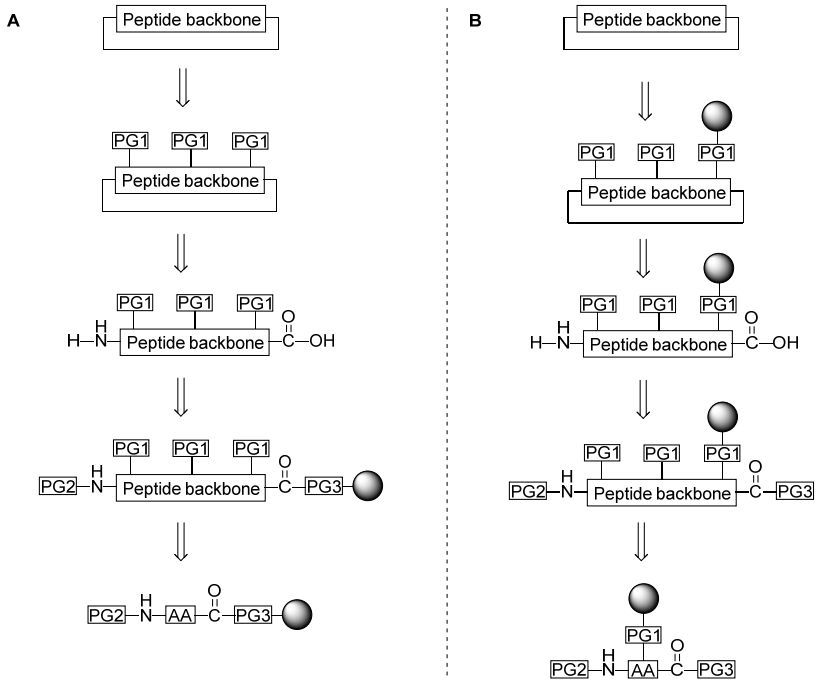


Figure 1. A. Off-resin cyclization strategy for the synthesis of head-to-tail cyclic peptides. *B.* The major alternative strategy for head-to-tail cyclic peptides involving on-resin cyclization for which a trifunctional amino acid is required. (PG = protecting group, AA = amino acid)

The research described in this Chapter entails the development of optimized protocols for the synthesis of head-to-tail cyclic peptides through on-resin cyclization, with particular focus on the nature of the amino acid selected for attachment to the resin through its side chain, the nature of the connecting functionality and the chemistry used to bring the linkage about. Such a chain anchoring methodology requires amino acids with three functional groups that can be addressed individually, and for which new chemistries may need to be developed. Anchoring through carboxamide or carboxylic acid side-chain functionalities (leading to aspartate/glutamate/asparagine/glutamine residues in the final products) can be achieved using established peptide coupling methodologies.[18,19] Other amino acids that have been attached to a resin *via* their side-chain and for which new chemistries have been developed include cysteine[20],

histidine[21,22], serine[23,24], threonine[23,24], tyrosine[25], lysine[26,27], tryptophan[28], arginine[29] and phenylalanine.[30]

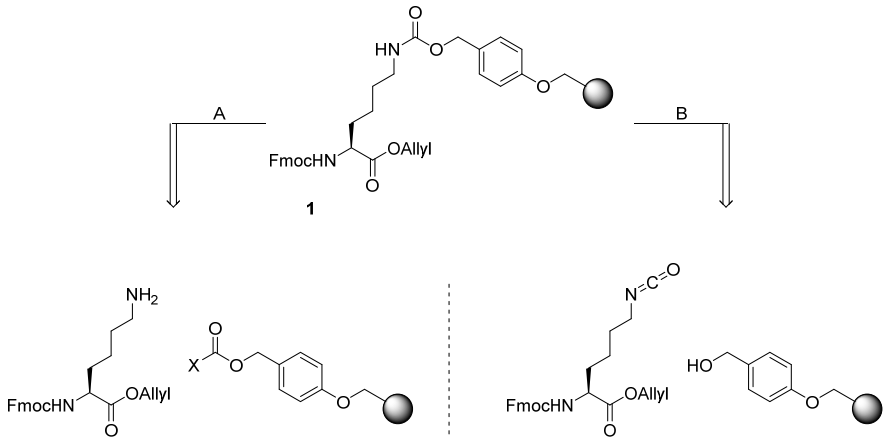


Figure 2. A. Conventional approach of lysine side-chain anchoring that involves an electrophilic resin. *B.* Strategy with a nucleophilic resin resulting in the same linkage.

The work presented in this Chapter entails utilizing the ornithine δ-amine as well as the lysine ε-amine for anchoring to a solid support through an acid-labile functionality. For this purpose, the para-hydroxymethylphenyloxy (Wang) linker in combination with a carbamate as connecting functionality was selected (*Figure 2*). This side chain anchoring system features in several literature studies, in which it was created through nucleophilic attack of the free lysine ε-amine onto an activated carbonate as depicted in *Figure 2, route A*.[26,27] Carbamates can however also be generated by reacting an alcohol with an isocyanate (*Figure 2 route B*) and, since this strategy has not been implemented in the generation of neither anchored lysine ε-amine **1** nor anchored ornithine δ-amine **2** exploration of this route became subject of studies presented in this Chapter (*Figure 2 & 3*). Thus, results here entail the preparation of **1** and **2** following *route B* in efficiencies at least equal to those reported in the literature based on *route A* as well as implementation of anchored ornithine δ-amine **2** in the synthesis of four representative head-to-tail cyclic peptide antibiotics, including gramicidin S and tyrocidine (*Figure 3*).

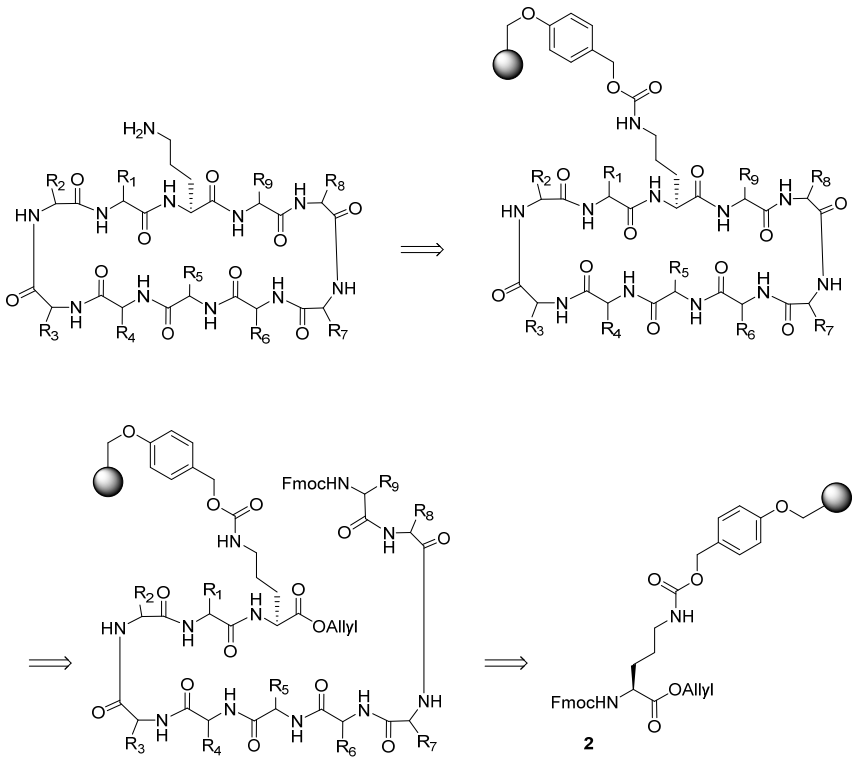


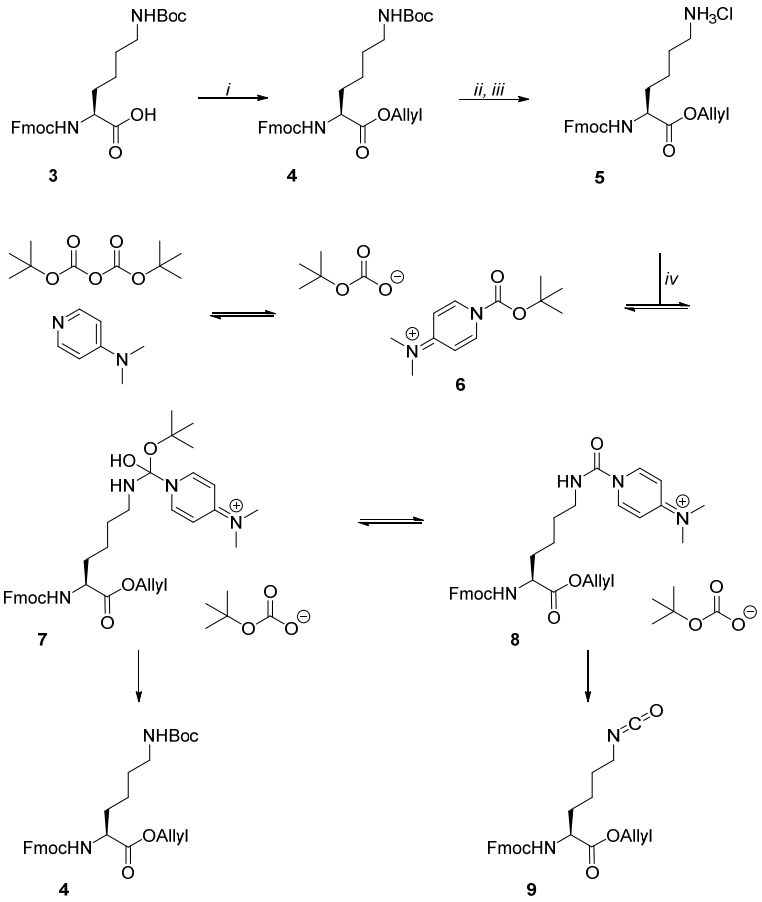
Figure 3. Retrosynthesis of head-to-tail cyclic peptides using an on-resin cyclization strategy.

The target cyclic peptides were selected to allow for comparison of the efficiency (yield, purity) of the investigated synthesis procedure to that of published literature syntheses. As the initial testing will be performed on lysine as opposed to ornithine, due to its availability at cheaper cost, investigating whether the conditions used for the formation of **1** hold true for the formation of anchored ornithine δ-amine **2** is necessary. If true, the construction of the target peptides follows the strategy outlined for on-resin cyclization (*Figure 1B & 3*). Starting from **2** equipped with an N-terminal Fmoc group and a C-terminal allyl protecting group, the peptide is elongated following Fmoc-based solid phase peptide synthesis (SPPS) procedures using amino acids with the appropriate acid-labile protecting groups. After deprotection of the N- and C-terminal protecting groups, the peptide is cyclized in an on-resin fashion. Final global deprotection is envisaged under acidic conditions that also result in removal of the para-alkoxybenzyl group and subsequent

decarboxylation – liberation, in other words, of the ornithine-containing cyclic peptide from the resin.

Results and discussion

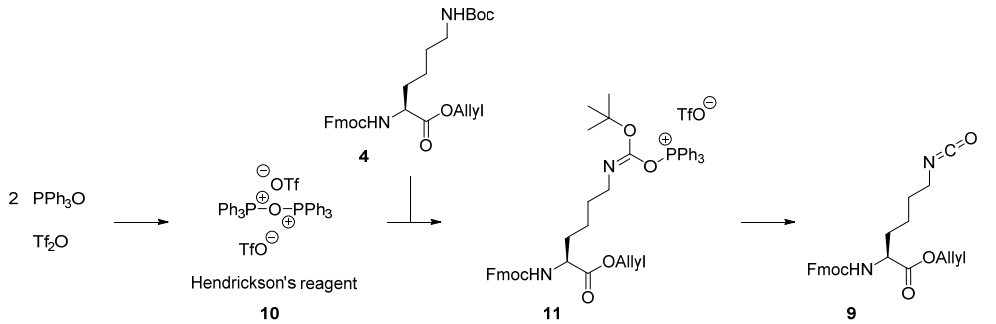
As the first research objective, the synthesis of a suitably and orthogonally protected lysine residue for ensuing side chain anchoring to resins was undertaken. Starting from *N*-α-Fmoc-*N*-ε-Boc lysine **3** (the standard lysine building block commonly applied in Fmoc-based solid phase peptide synthesis), alkylation of the carboxylate with allyl bromide and silver carbonate as the base afforded fully and orthogonally protected lysine **4** (*Scheme 1*).



Scheme 1. Postulated mechanism by Knölker and co-workers to convert a free amine to the isocyanate applied to starting material **5**.[31] Reagents and conditions: (*i*) Ag_2CO_3, allyl bromide, DMF, 0 °C to rt, 2.5 hrs, 95% (*ii*) $SnCl_4$, DCM, EA, rt, 1 hr, 88% (*iii*) DMAP, MeCN, rt, 5 min. (*iv*) Boc_2O, DMAP, MeCN, rt, 10 min., 68% (**3**).

With orthogonally protected lysine **4** in hand, the synthesis of the isocyanate was envisioned *via* the free amine followed by conversion to the isocyanate using the procedure originally reported by Knölker *et al.*[31] In this procedure, a free amine is added to a mixture of 4-dimethylaminopyridine and di-*tert*-butyldicarbonate in which *N*-acylpyridinium ion **6** is postulated to be transiently formed. The mechanism postulated by the authors involves the formation of tetrahedral intermediate **7**. At this stage, loss of the pyridinium ion may occur resulting in the formation of *N*-Boc protected amine **3**. However, the authors propose, based on the products they observed, that *tert*-butanol is liberated leading to intermediate **8**.

Finally, loss of the pyridinium ion generates isocyanate **9** together with regeneration of DMAP. In order to investigate this method, *tert*-butyl carbamate **4** was first deprotected using tin(IV) chloride to furnish ammonium chloride **5** in 88% yield. The free amine was generated by treatment with DMAP in MeCN and the solution was subsequently added to a mixture of DMAP and di-*tert*-butyldicarbonate (1:1.4, DMAP – Boc_2O). The solution was stirred for 10 minutes, concentrated and the residue purified by silica gel column chromatography. However, instead of isocyanate **9** urethane **4** was found to be formed as the major product (68% isolated yield).



Scheme 2. Postulated mechanism by Cho and co-workers to convert *N*-Boc urethane to isocyanate applied to starting material **4.** Reagents and conditions: (*i*) PPh_3O, Tf_2O, DCM, 0 °C, 30 min, then **4**, DCM, 0 °C to rt, 3 hrs, 19%.

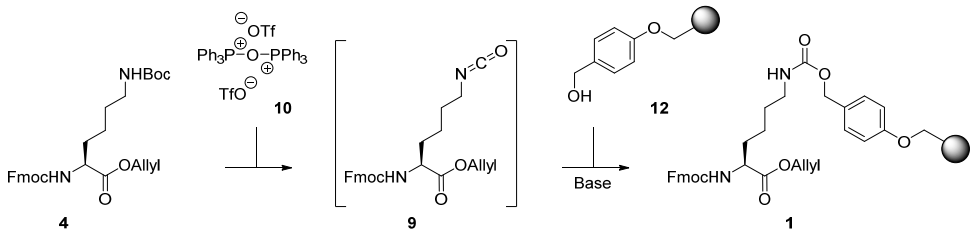
In an alternative procedure, carbamates themselves can be directly transformed into isocyanates following a variety of literature procedures.[32–36] As lysine **4** encompasses two carbamates (N-α-Fmoc and N-ε-Boc), any successful method to turn **4** into **9** should transform the latter carbamate into the isocyanate while leaving the former untouched. Cho and co-workers described a method to convert an *N*-Boc carbamate to an isocyanate (*Scheme 2*).[37] The procedure involves the use of Hendrickson's dehydrating reagent **10**, which is formed by reacting two equivalents of triphenylphosphine oxide with one equivalent of triflic anhydride.[38,39] From this work, in which they investigated a number of carbamates varying in the nature of the oxygen alkyl substituent it could be concluded that the efficiency of the transformation correlates with the acid lability of the carbamate. The authors suggest that the triflic acid that is formed during the reaction aids the conversion of **11** into isocyanate **9** by removing the acid-labile *tert*-butyl group. It was therefore hypothesized that the methodology should be applicable to convert lysine **4** into isocyanate **9**, thus to transform the acid-labile *O*-^{t}Bu carbamate while leaving the acid-stabile *O*-fluorenylmethyl carbamate intact.

To test this hypothesis, Hendrickson reagent was prepared *in situ* by mixing triphenylphosphine oxide and triflic anhydride after which a solution of *N*-Boc carbamate **4** in dichloromethane was added. The reaction proceeded in a clean fashion and after three hours all starting material had been converted and the formation of a more apolar compound was observed by TLC analysis. A sample of the crude reaction mixture was subjected to IR spectroscopy which revealed a strong peak at 2262 cm^{-1} strongly suggesting the formation of an isocyanate. For further examination, this compound was isolated by silica gel column chromatography by directly applying the crude solution onto the column affording an orange oil in a 19% yield. Characterization by ^{1}H NMR spectroscopy showed the presence of the fluorenylmethyl group as well as the absence of the *tert*-butyl group. The ^{13}C NMR spectrum displayed a new peak at 122 ppm characteristic for an isocyanate carbon. Conclusive evidence was obtained using heteronuclear multiple-bond correlation spectroscopy (HMBC) showing correlation between the isocyanate carbon and the ε-protons of the lysine, while no correlation was observed with the α-proton indicating that the Fmoc-carbamate was intact. During column chromatography, besides isolation of isocyanate **9**, a significant amount of the corresponding amine was obtained. Since no amine was formed during the reaction according to TLC, it is likely that the isocyanate degraded on the column. Therefore, it was decided to forego the purification step and add the isocyanate as a crude solution to Wang-type resin **12** (*Table 1*).

Usage of the crude isocyanate solution to furnish side-chain anchored resin **1** necessitated neutralization of the triflic acid that is generated during the reaction. Fortunately, tertiary amines act as a catalyst in the reaction between isocyanates and alcohols so it was envisioned to catch two birds with one stone.[40] The catalytic activity of tertiary amines generally increases as basicity increases. However, the presence of the base-labile Fmoc group limits the choice of catalysts. As the first attempt *N*-methylmorpholine was chosen because of its use as catalyst in the polyurethane industry as well as base in Fmoc-based SPPS.[41] Thus, isocyanate **9** was synthesized as before and 8.5 equivalents *N*-methylmorpholine were added to the solution (7.2 equivalents to neutralize the triflic acid and the remaining 1.3 equivalents to act as catalyst). TentaGel S PHB resin **12** was co-evaporated three times with 1,4-dioxane to remove traces of water. The solution containing isocyanate **9** was transferred to a reaction vessel containing resin **12** and the suspension was shaken for 17 hours. The suspension was filtered and washed with dichloromethane

followed by diethyl ether to shrink the resin. The resin was dried under vacuum and two samples (5 – 10 mg) were taken to determine the loading. Each sample was swollen in DMF (0.80 mL) for 20 minutes followed by addition of piperidine (0.20 mL) and the resulting suspension was shaken for another 20 minutes. The sample was filtrated into a volumetric flask, the resin washed with additional 20% (v/v) piperidine in DMF and subsequently diluted with 20% (v/v) piperidine in DMF to a total volume of 10 mL. The absorption of this solution at 301.0 nm was measured against a blank 20% (v/v) piperidine in DMF solution using a Shimadzu UV-1601 UV-VIS spectrometer with a Quartz cuvette. The loading was determined with the formula described by Eissler *et al.* and the yield was calculated by multiplying the loading substitution with the dry weight of the resin.[42] Less than 5% loading yield was obtained in this way (*Table 1, entry 1*) and alternative conditions involving a variety of catalysts were explored next.

Table 1. Screening of catalysts in the condensation of isocyanate 9 and resin-bound alcohol 12.[a]



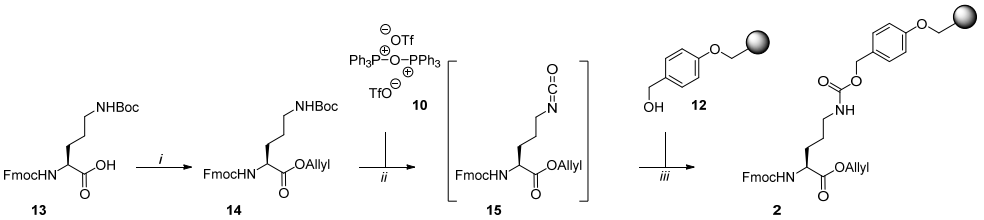
Entry	Catalyst	Equivalents	Base	Excess[b]	Yield
1	–	–	*N*-methylmorpholine	1.3	<5%
2	–	–	1-methylimidazole	1.3	<5%
3	–	–	DMAP	1.3	Fmoc cleavage
4	$Ti(O^tBu)_4$	1.0	*N*-methylmorpholine	0.3	55%
5	$Ti(O^tBu)_4$	3.0	*N*-methylmorpholine	0.3	57%
6	Zr(IV)acac	1.0	*N*-methylmorpholine	0.3	94%
7	Dibutyltindilaurate	1.0	*N*-methylmorpholine	0.3	>99%
8	Dibutyltindilaurate	1.0	1-methylimidazole	0.3	44%

[a] Reactions were performed at a 0.1 mmol scale with 3.0 equivalents of **4** compared to resin **12** over 17 hrs. [b] Excess in equivalents compared to resin **12**.

Yoganathan *et al.* described the use of nucleophilic catalysts 1-methylimidazole and 4-dimethylaminopyridine (DMAP) in the condensation of alcohols with isocyanates.[43] Inspired by these literature precedents, both catalysts were assessed in their efficacy to condense **4** with resin **12** to provide **1**. Concerning the compatibility with the Fmoc group, 1-methylimidazole was expected to cause no issues as it has been used in the presence of an Fmoc group previously.[44] With respect to DMAP it had been shown that a 10% (w/v) solution of this base in DMF can cleave the Fmoc group with a half-life time of 85 minutes.[45] A redeeming quality was that Yoganathan and co-workers observed complete conversion in the condensation with 10 mol% DMAP in 60 minutes. Using 1-methylimidazole as the catalyst and base furnished anchored lysine **1** in less than a 5% yield (*Entry 2*) whereas using DMAP led to partial Fmoc cleavage (*Entry 3*). Attention was then shifted to a procedure by the group of Arbour, who applied titanium(IV) *tert*-butoxide to catalyze the condensation of alcohols with isocyanates.[46] By separating the function of base and catalyst, *N*-methylmorpholine was chosen as the base as it apparently did not catalyze the transformation. In the first instance, 55% yield of resin-bound lysine was obtained with this catalyst (*Entry 4*). Increasing the amount of catalyst to 3.0 equivalents did not lead to a significant increase of the yield affording side-chain anchored lysine **1** in a 57% yield (*Entry 5*).

Two catalysts were finally attempted that originate from the field of polyurethane chemistry, namely zirconium(IV) acetylacetonate and dibutyltin dilaurate.[47,48] With *N*-methylmorpholine as the base, both compounds proved highly effective in catalyzing the desired transformation, giving anchored lysine **1** in a 94% yield for zirconium(IV) acetylacetonate and >99% yield for dibutyltin dilaurate (*Entry 6 & 7)*. Comparing the two catalysts, dibutyltin dilaurate is more efficient in the synthesis of the urethane while zirconium(IV) acetylacetonate is considerably less toxic.[49,50] To assess the influence of the base, *N*-methylmorpholine was substituted for 1-methylimidazole while using dibutyltin dilaurate as the catalyst. This substitution proved detrimental giving urethane **1** in 44% yield (*Entry 8*). This result highlights the influence of the base and further research is necessary to determine the most effective base-catalyst combination.

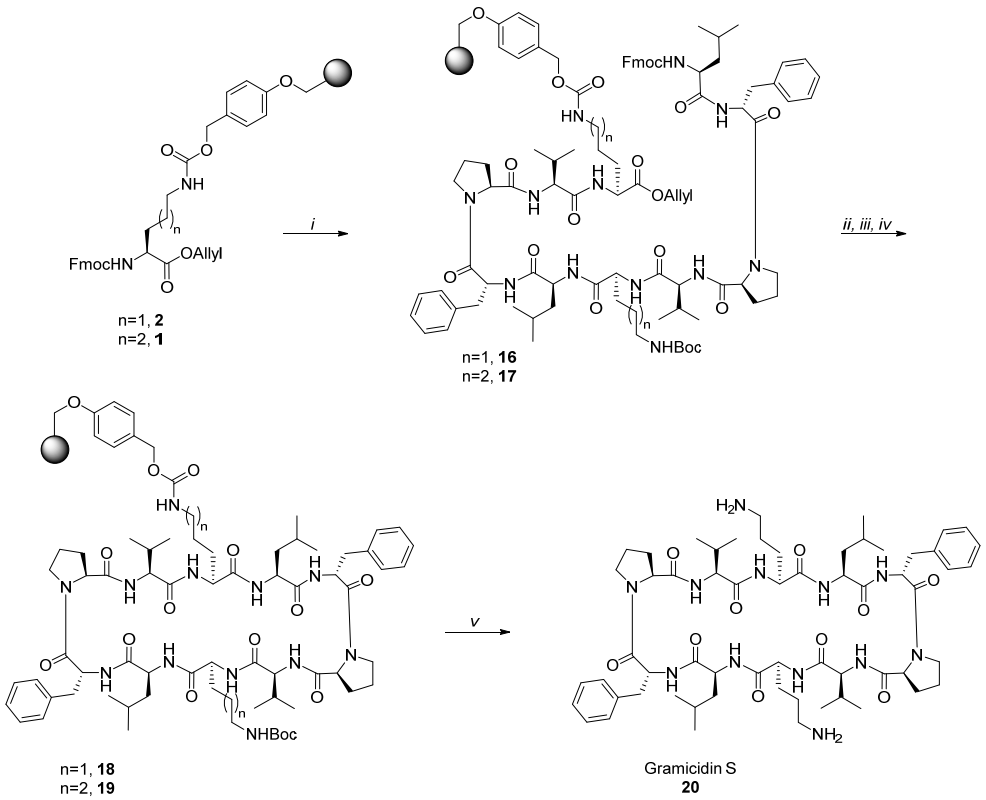
For the synthesis of the targeted head-to-tail cyclic peptides, first the method had to be applied to ornithine. Therefore, the C-terminus of *N*-α-Fmoc-*N*-δ-Boc ornithine **13** was protected as the allyl ester using allyl bromide and silver carbonate to furnish fully protected ornithine **14** in a 91% yield (*Scheme 3*).



Scheme 3. Reagents and conditions: (*i*) Ag_2CO_3, allyl bromide, DMF, 0 °C to rt, 2.5 hrs, 91% (*ii*) DCM, rt, 15 min. (*iii*) dibutyltin dilaurate, *N*-methylmorpholine, DCM, rt, 24 hrs, 94%.

Using the same procedure as for lysines, *N*-Boc carbamate **14** was converted into isocyanate **15** by Hendrickson's reagent **10**. To the crude solution containing the isocyanate was added *N*-methylmorpholine and dibutyltin dilaurate and the mixture was transferred to a vessel containing TentaGel S PHB resin **12**. After shaking for 24 hours, ornithine-bound resin **2** was obtained in 94% yield. With both resin **2** and lysine-bound resin **1** in hand, the synthesis of the head-to-tail cyclic peptides was started. Methods for allyl deprotection and on-resin cyclization were first conducted on lysine-bound resin **1** and the peptide fragments examined by LC-MS after a sample of the resin was treated to a cleavage cocktail. Suitable conditions were then applied to resin **2** to minimize byproduct formation and incomplete conversions during the synthesis of the first target, gramicidin S.

The synthesis of gramicidin S **20** started with nine peptide coupling cycles on a Protein Technologies Tribute automated peptide synthesizer (*Scheme 4*). Each cycle started with two treatments of 20% (v/v) piperidine in DMF for three minutes to remove the Fmoc group. After a series of washing steps, condensation was achieved by reacting the appropriate partially protected amino acid with deprotected resin-bound peptide using HCTU as the activator and Hünig's base as the base for one hour at room temperature. Unreacted amines were capped by two treatments of 10% (v/v) Ac_2O in DMF for three minutes. After nine such cycles, fully protected linear peptide **16** was obtained.



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 (*v*) TFA – TIPS – H_2O (38:1:1), rt, 3 hrs, 43%.

The synthesis of gramicidin S was continued manually by deprotecting the allyl group. The allyl ester was deprotected before cleavage of the Fmoc-group since the free amine would poison the palladium catalyst.[51] Complete removal of the allyl was observed (as established by cleavage from the resin of a fraction of reacted immobilized peptide **17**) after treatment with palladium tetrakis(triphenylphosphine) with phenylsilane as the allyl group scavenger for 90 minutes. Two treatments with 20% (v/v) piperidine in DMF for ten minutes furnished the liberated N-terminus. For the key on-resin cyclization step, the phosphonium salt benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate was chosen as the coupling reagent over uronium- and aminium-based salts as phosphonium-based coupling reagents do not suffer from

the formation of guanidine derivatives as has been reported for the uronium- and aminium-based reagents.[52,53] 1-Hydroxybenzotriazole was also added to provide more efficient coupling and to lower racemization of the activated amino acid.[54] Cyclization under these conditions plus *N*-methylmorpholine as the base was applied to resin **16** and the suspension was shaken for 2.5 hours. Liberation of gramicidin S **20** from the resin was achieved by treating resin **18** with a cleavage cocktail (38:1:1, TFA – TIPS – H_2O) for three hours. The suspension was filtered and the resin was washed with additional cleavage cocktail. The crude peptide was obtained by evaporation and subsequent purification by size exclusion chromatography afforded gramicidin S in 43% yield.

Analysis by analytical reversed-phase HPLC revealed gramicidin S was obtained with a purity of 96% (*Table 2, entry 1*). In comparison, an on-resin cyclization approach by Andreu and co-workers furnished gramicidin S in a 24% yield with a purity of *ca.* 90%.[55] The major difference in their approach is the method of resin anchoring of the ornithine residue which involved transforming the resin into an activated carbonate species before treatment with ornithine δ-amine (*Figure 2A*). Extensive research investigating an off-resin cyclization strategy in the synthesis of gramicidin S was conducted by Wadhwani *et al.*[56] The highest yield and purity were obtained when the peptide synthesis was started from the D-phenylalanine residue affording gramicidin S in a 69% overall yield with a 95% purity. When the solid-phase synthesis started with Fmoc-Orn(Dde)-OH the yield and purity dropped to 17% and 70% respectively.

Table 2. Synthesis of cyclic peptides using side-chain anchoring.

Entry	Peptide	Yield[a]	Purity[b]
1	*cyclo*(-Leu-*D*Phe-Pro-Val-Orn-)$_2$ Gramicidin S	43%	96%
2	*cyclo*(-Leu-*D*Phe-Pro-Phe-*D*Phe-Asn-Gln-Tyr-Val-Orn-) Tyrocidin A	66%	73%
3	*cyclo*(-Leu-*D*Tyr-Pro-Phe-*D*Phe-Asn-Asp-Tyr-Val-Orn-) Loloatin A	59%	93%
4	*cyclo*(-Leu-*D*Phe-Pro-Leu-*D*Trp-Asn-Gln-Tyr-Val-Orn-) Streptocidin A	40%	53%

[a] Yield after size exclusion chromatography, [b] Chromatographic purity based on area percentage.

To investigate the scope of the procedure described for the synthesis of gramicidin S, three additional natural head-to-tail cyclic peptides that also feature ornithine residues, namely tyrocidin A, loloatin A and streptocidin A, were synthesized in the same manner.[14,57–59] Tyrocidin A was obtained in a 66% yield with a purity of 73% (*Entry 2*). In the synthesis of tyrocidin A, a concurrent cyclization and cleavage approach was employed by Ösapay and co-workers using the Kaiser oxime resin furnishing tyrocidin A in a yield of 55% with a >95% purity.[60] This strategy has also been employed by the group of Guo in the syntheses of gramicidin S (25% yield, 87% purity), tyrocidin A (25% yield, >95% purity), loloatin A (28% yield, 90% purity) and streptocidin A (16% yield, 94% purity).[61–64] The syntheses of loloatin A and streptocidin A were achieved in a 59% and 40% yield with a purity of 93% and 53% respectively (*Entry 3&4*). A reported synthesis of loloatin A by Scherkenbeck *et al.* also employed an on-resin cyclization strategy but here the side-chain of the asparagine residue was used to anchor the growing peptide to the resin.[65] This strategy gave loloatin A in an overall yield of 31% with a 97% purity. Tyrocidin and streptocidin were obtained in a lower purity, which based on the observed masses of the formed product mixtures may be because in both cases incomplete detritylation of the -Asn-Gln- motif had occurred. Perhaps longer exposure to trifluoroacetic acid could alleviate this problem. The synthesis of streptocidin A in turn appeared accompanied by formation of other minor byproducts as well.

Conclusion

In the synthesis of cyclic peptides, the methods for side-chain anchoring of amino acids are limited. In this Chapter, the δ- and ε-amine of ornithine and lysine respectively was successfully anchored to a Wang-type resin. With this method, the properties of the linker remain unaffected compared to coupling to the respective C-terminus as exposure to trifluoroacetic acid liberates the amine accompanied by formation of carbon dioxide. The method involves the formation of an isocyanate which was readily achieved from the *N*-Boc carbamate using Hendrickson's reagent following a procedure by Cho *et al*.[37] In the coupling of the isocyanate to a TentaGel Wang-type resin, dibutyltin dilaurate and zirconium(IV) acetylacetonate were found to be efficient in catalyzing this transformation with *N*-methylmorpholine acting as the base. The choice of base proved to be influential as 1-methylimidazole hampered the catalysis of dibutyltin dilaurate. The side-chain anchoring procedure was then applied to the synthesis of several natural head-to-tail cyclic peptides including gramicidin S and tyrocidin A. The peptides were synthesized in 40-66%

yields with a purity ranging from 53-96%, results that compare well to those reported in the literature on alternative synthesis procedures. A lower purity was observed for the synthesis of tyrocidine A and streptocidin A and was believed to be due to incomplete detritylation of the -Asn-Gln- motif. Longer treatment with trifluoroacetic acid might achieve full deprotection.

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. Fmoc-D-Tyr(^{t}Bu)-OH, Fmoc-D-Trp(Boc)-OH were acquired from Iris Biotech. The resins TentaGel S PHB (0.27 mmol/g) and TentaGel S AC (0.23 mmol/g) were 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} column (250 x 10.0 mm, 3 μm particle size) with a flow rate of 5 mL/min and solvent gradients as described for each compound. All HPLC solvents were filtered with a Millipore filtration system equipped with a 0.22 μm nylon membrane filter prior to use. HPLC solvent compositions: solvent A is H_2O; solvent B is MeCN; solvent C is 1.0% TFA in H_2O at a continuous 10% of the volume throughout the run.

Characterization

Nuclear magnetic resonance (^{1}H 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

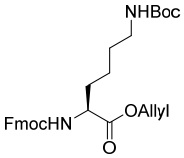
Resin was dried before loading calculation by washing with DCM (3x 30 sec.) and Et_2O (3x 30 sec.) followed by purging with N_2. A small amount of resin (5 – 10 mg) was weighed and DMF (0.80 mL) was added and the resin was swollen for 20 min. Piperidine (0.20 mL) was then added and shaken for 20 min. 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[42]:

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

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

Fmoc-Lys(Boc)-OAllyl



4

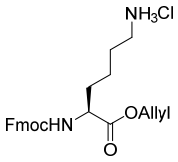
Standard Fmoc-protected lysine building block **3** (4.7 g, 10 mmol, 1.0 equiv.) was dissolved in DMF (40 mL, 0.25 M) and the solution was cooled to 0 °C. Silver carbonate (3.6 g, 13 mmol, 1.3 equiv.) was added and the reaction was stirred for 15 min. The cooling bath was removed, allyl bromide (4.0 mL, 46 mmol, 4.6 equiv.) was added and the mixture was stirred for an additional 2.5 hrs. The suspension was then filtered, diluted with EA and subsequently washed with 10% (w/v) aq. $KHSO_4$ and H_2O. The organic layer was dried ($MgSO_4$), filtered and the volatiles were removed under reduced pressure. Purification by silica gel column chromatography (1:4, EA – Pentane to 3:2, EA – Pentane) furnished allyl ester **4** (4.8 g, 9.5 mmol, 95%) as a white solid.

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.77 (d, *J* = 7.5 Hz, 2H, C*H*-arom), 7.65 – 7.52 (m, 2H, C*H*-arom), 7.40 (t, *J* = 7.5 Hz, 2H, C*H*-arom), 7.32 (tt, *J* = 7.5, 1.3 Hz, 2H, C*H*-arom), 5.91 (ddt, *J* = 16.4, 10.3, 5.8 Hz, 1H, OCH_2CH=CH_2), 5.45 – 5.23 (m, 3H, N*H*Fmoc, OCH_2CH=CH_2), 4.68 – 4.62 (m, 2H, OCH_2CH=CH_2), 4.57 (s, 1H, N*H*Boc), 4.47 – 4.33 (m, 3H, CH_2-Fmoc, α-Lys), 4.23 (t, *J* = 7.0 Hz, 1H, C*H*-Fmoc), 3.16 – 3.07 (m, 2H, ε-Lys), 1.95 – 1.82 (m, 1H, β-Lys), 1.79 – 1.67 (m, 1H, β-Lys), 1.43 (s, 13H, δ-Lys, CH_3-Boc, γ-Lys).

^{13}C NMR (101 MHz, $CDCl_3$) δ 172.3 (*C*OOAllyl), 156.2 (*C*=O-Boc), 156.1 (*C*=O-Fmoc), 144.0 (*C*q-arom), 143.9 (*C*q-arom), 141.4 (*C*q-arom), 131.6 (OCH_2CH=CH_2), 127.8 (CH-arom), 127.2 (CH-arom), 125.2 (CH-arom), 120.1 (CH-arom), 120.1 (CH-arom), 119.2 (OCH_2CH=CH_2), 83.0 (*C*(CH_3)$_3$), 67.2 (CH_2-Fmoc), 66.2 (OCH_2CH=CH_2), 53.9 (α-Lys), 47.3 (*C*H-Fmoc), 40.2 (ε-Lys), 32.3 (β-Lys), 29.7 (δ-Lys), 28.5 (CH_3-Boc), 22.5 (γ-Lys).

HRMS (ESI-Orbitrap) calcd. for $C_{29}H_{36}N_2O_6Na$ $[M+Na]^+$ 531.24656, found 531.24641.

Fmoc-Lys-OAllyl



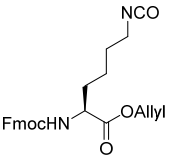
5

Boc-protected amine **4** (50 mg, 98 μmol, 1.0 equiv.) was dissolved in EA (0.98 mL, 0.10 M) and $SnCl_4$ (1.0 M in DCM, 0.39 mL, 0.39 mmol, 4.0 equiv.) was added. The reaction was stirred at room temperature for 1 hr. Afterwards, the reaction mixture was evaporated and dissolved in a small amount of MeOH. Product crashed out upon addition of Et_2O and was collected by filtration affording amine **5** (38 mg, 86 μmol, 88%) as the hydrochloric acid salt.

^{1}H NMR (400 MHz, MeOD) δ 7.79 (d, *J* = 7.5 Hz, 2H, C*H*-arom), 7.66 (t, *J* = 7.5 Hz, 2H, C*H*-arom), 7.39 (t, *J* = 7.4 Hz, 2H, C*H*-arom), 7.30 (t, *J* = 7.4 Hz, 2H, C*H*-arom), 5.93 (ddt, *J* = 16.1, 10.8, 5.6 Hz, 1H, OCH_2CH=CH_2), 5.32 (dq, *J* = 17.2, 1.7 Hz, 1H, OCH_2CH=CH_2), 5.21 (dq, *J* = 10.5, 1.4 Hz, 1H, OCH_2CH=CH_2), 4.62 (dt, *J* = 5.6, 1.5 Hz, 2H, OCH_2CH=CH_2), 4.43 – 4.28 (m, 2H, CH_2-Fmoc), 4.25 – 4.16 (m, 2H, C*H*-Fmoc, α-Lys), 2.97 – 2.85 (m, 2H, ε-Lys), 1.95 – 1.82 (m, 1H, β-Lys), 1.80 – 1.62 (m, 3H, β-Lys, δ-Lys), 1.56 – 1.38 (m, 2H, γ-Lys).

^{13}C NMR (101 MHz, MeOD) δ 173.5 (*C*OOAllyl), 158.7 (*C*=O-Fmoc), 145.2 (*C*q-arom), 142.5 (*C*q-arom), 133.3 (OCH_2CH=CH_2), 128.8 (*C*H-arom), 128.1 (*C*H-arom), 126.2 (*C*H-arom), 126.1 (*C*H-arom), 120.9 (*C*H-arom), 118.7 (OCH_2CH=CH_2), 67.9 (CH_2-Fmoc), 66.7 OCH_2CH=CH_2), 55.2 (α-Lys), 48.3 (*C*H-Fmoc), 40.5 (ε-Lys), 31.9 (β-Lys), 27.9 (δ-Lys), 23.8 (γ-Lys).

Fmoc-Lys(CO)-OAllyl



9

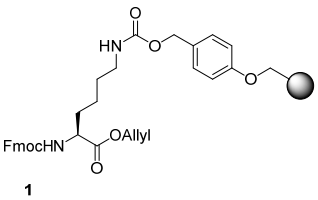
Triphenylphosphine oxide (0.16 g, 0.58 mmol, 2.4 equiv.) was dissolved in DCM (8.0 mL, 30 mM) and the solution was cooled to 0 °C. Triflic anhydride (48 μL, 0.29 mmol, 1.2 equiv.) was added and the reaction was stirred at 0 °C for 30 min. during which time a white precipitate was formed. Lysine building block **4** (0.12 g, 0.24 mmol, 1.0 equiv.) was added to the suspension and the reaction was stirred for 3 hrs allowing the mixture to gradually warm to room temperature. The solution was loaded onto a silica gel column and purified by silica gel column chromatography (1:4, EA – Pentane) to give isocyanate **9** (20 mg, 46 μmol, 19%) as an orange oil.

^{1}H NMR (500 MHz, $CDCl_3$) δ 7.79 – 7.73 (m, 2H, C*H*-arom), 7.63 – 7.54 (m, 2H, C*H*-arom), 7.40 (tt, *J* = 7.5, 1.5 Hz, 2H, C*H*-arom), 7.31 (tt, *J* = 7.5, 1.1 Hz, 2H, C*H*-arom), 5.91 (ddt, *J* = 16.6, 11.0, 5.9 Hz, 1H, OCH_2CH=CH_2), 5.39 – 5.22 (m, 3H, N*H*Fmoc, OCH_2CH=CH_2), 4.68 – 4.61 (m, 2H, OCH_2CH=CH_2), 4.45 – 4.34 (m, 3H, CH_2-Fmoc, α-Lys), 4.22 (t, *J* = 7.0 Hz, 1H, C*H*-Fmoc), 3.31 (t, *J* = 6.6 Hz, 2H, ε-Lys), 1.93 – 1.85 (m, 1H, β-Lys), 1.76 – 1.57 (m, 3H, β-Lys, δ-Lys), 1.55 – 1.36 (m, 2H, γ-Lys).

^{13}C NMR (126 MHz, $CDCl_3$) δ 172.1 (*C*OOAllyl), 156.0 (*C*=O-Fmoc), 144.0 (*C*q-arom), 143.8 (*C*q-arom), 141.4 (*C*q-arom), 131.5 (OCH_2CH=CH_2), 127.9 (*C*H-arom), 127.2 (*C*H-arom), 125.2 (*C*H-arom), 122.1 (N*C*O), 120.1 (*C*H-arom), 120.1 (*C*H-arom), 119.3 (OCH_2CH=CH_2), 67.1 (CH_2-Fmoc), 66.3 (OCH_2CH=CH_2), 53.7 (α-Lys), 47.3 (*C*H-Fmoc), 42.8 (ε-Lys), 32.2 (β-Lys), 30.7 (δ-Lys), 22.3 (γ-Lys).

IR (thin film) ν (cm^{-1}) 2262, 1719, 1700, 1451, 1183, 1085, 759, 741.

Fmoc-Lys(TentaGel S PHB)-OAllyl

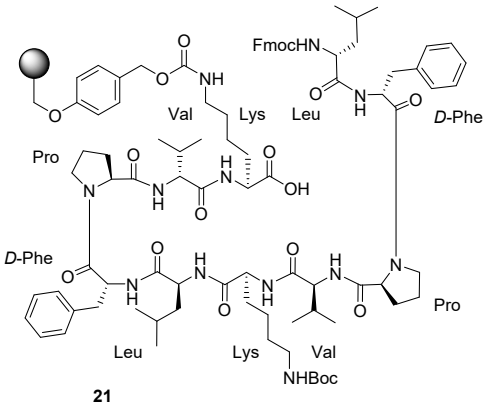


1

A solution containing triphenylphosphine oxide (0.20 g, 0.72 mmol, 7.2 equiv.) in DCM (3.0 mL, 0.12 M) was cooled to 0 °C and triflic anhydride (1.0 M in DCM, 0.36 mL, 0.36 mmol, 3.6 equiv.). The reaction was stirred at 0 °C for 30 min. during which a white precipitate was formed. A solution of *N*-Boc protected lysine **4** (0.15 g, 0.30 mmol, 3.0 equiv.) in DCM (0.34 mL, 0.88 M) was then added to the suspension and the cooling bath was removed. The reaction was stirred for 5 min. followed by the addition of *N*-methylmorpholine (83 μL, 0.75 mmol, 7.5 equiv.) and dibutyltin dilaurate (59 μL, 0.10 mmol, 1.0 equiv.). The solution was transferred to TentaGel S PHB resin (0.27 mmol/g, 0.37 g, 0.10 mmol, 1.0 equiv.) which was co-evaporated previously with 1,4-dioxane (3x) and the suspension was shaken for 18 hrs. The suspension was filtered and the resin was washed with DCM (4x) and Et_2O (4x). Drying the resin over N_2 afforded functionalized resin **1** (0.43 g, 0.10 mmol, >99%) with a loading of 0.24 mmol/g. A fraction of the resin (5.0 mg) was subjected to a cleavage cocktail (190:5:5, TFA – H_2O – TIPS) for 2 hrs and analyzed by LC-MS.

LC-MS (ESI^+) calcd. for $C_{24}H_{29}N_2O_4$ $[M+H]^+$ 409.21, observed 409.25 with a retention time of 5.34 min.

Fmoc-Leu-*D*Phe-Pro-Val-Lys(Boc)-Leu-*D*Phe-Pro-Val-Lys(TentaGel S PHB)-OH

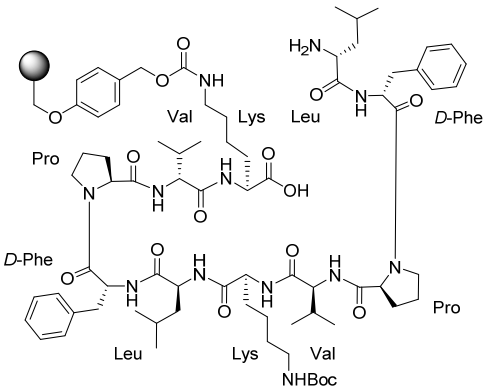


21

Functionalized resin **1** (0.43 g, 0.10 mmol, 1.0 equiv.) was elongated using the Tribute peptide synthesizer. Afterwards, the resin was washed with DCM (4x), Et_2O (4x) and dried over N_2. Resin was suspended in a mixture of DCM and DMF (1:1, DCM – DMF, 4.0 mL, 25 mM) and swollen for 20 min. Phenylsilane (31 µL, 0.25 mmol, 2.5 equiv.) and $Pd(PPh_3)_4$ (29 mg, 25 µmol, 25 mol%) were added and the resin was shaken for 90 min. while being protected from light. The suspension was filtered and the resin was washed with DCM (3x), 0.50% (w/v) sodium diethyldithiocarbamate in DMF (2x) and DMF (3x). A small amount of resin (5.0 mg) was subjected to a cleavage cocktail (190:5:5, TFA – H_2O – TIPS) for 2 hrs and analyzed by LC-MS.

LC-MS (ESI^+) calcd. for $C_{77}H_{108}N_{12}O_{13}$ $[M+H]^+$ 1408.82, observed 1409.73 with a retention time of 6.96 min.

H-Leu-*D*Phe-Pro-Val-Lys(Boc)-Leu-*D*Phe-Pro-Val-Lys(TentaGel S PHB)-OH

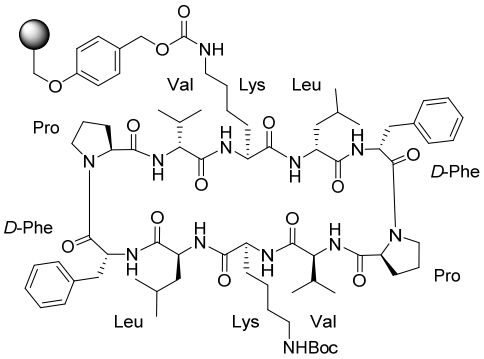


22

To decapeptide **21** was added 20% (v/v) piperidine in DMF (5.0 mL, 20 mM) and the resin was shaken for 10 min. Resin was filtered and 20% (v/v) piperidine in DMF (5.0 mL, 20 mM) was added. Resin was shaken for 10 min. Afterwards, the resin was filtered and washed with DMF (6x). A small amount of resin (5.0 mg) was subjected to a cleavage cocktail (190:5:5, TFA – H_2O – TIPS) for 2 hrs and analyzed by LC-MS.

LC-MS (ESI^+) calcd. for $C_{62}H_{99}N_{12}O_{11}$ $[M+H]^+$ 1187.76, observed 1187.60 with a retention time of 5.31 min.

***cyclo*(-Leu-*D*Phe-Pro-Val-Lys(Boc)-Leu-*D*Phe-Pro-Val-Lys(TentaGel S PHB)-)**

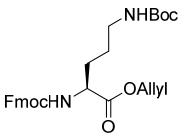


19

To decapeptide **22** was added DMF (4.0 mL, 25 mM). Subsequently, 1-hydroxybenzotriazole hydrate (68 mg, 0.50 mmol, 5.0 equiv.), benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate (0.26 g, 0.50 mmol, 5.0 equiv.) and *N*-methylmorpholine (0.11 mL, 1.0 mmol, 10 equiv.) were added to the suspension and the reaction was stirred for 2.5 hrs. The suspension was filtered and the residue was washed with DMF (3x) and DCM (3x). A cleavage mixture (190:5:5, TFA – H_2O – TIPS) was added to a small amount of resin (5.0 mg) and shaken for 2 hrs. The suspension was filtered and the filtrate was analyzed by LC-MS.

LC-MS (ESI$^+$) calcd. for $C_{62}H_{97}N_{12}O_{10}$ $[M+H]^+$ 1169.74, observed 1169.67 with a retention time of 7.13 min.

Fmoc-Orn(Boc)-OAllyl



14

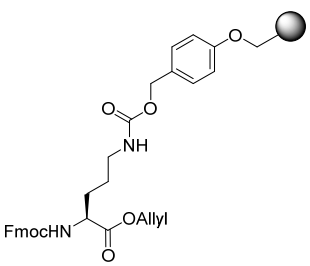
Fmoc-Orn(Boc)-OH **13** (2.0 g, 4.4 mmol, 1.0 equiv.) was dissolved in DMF (18 mL, 0.25 M) and cooled to 0 °C. Silver carbonate (1.6 g, 5.7 mmol, 1.3 equiv.) was added and the reaction was stirred for 15 min. Allyl bromide (1.8 mL, 20 mmol, 4.6 equiv.) was added, cooling bath was removed and the mixture was stirred at room temperature for 3 hrs. The suspension was filtered and the filtrate was diluted with DCM and subsequently washed with 10% (w/v) aq. $KHSO_4$. The organic phase was dried ($MgSO_4$), filtered and the volatiles were removed under reduced pressure. Purification by silica gel column chromatography (1:4, EA – Pentane to 2:3, EA – Pentane) afforded allyl ester **14** (2.0 g, 4.1 mmol, 91%) as a white solid.

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.77 (dq, *J* = 7.7, 1.0 Hz, 2H, C*H*-arom), 7.64 – 7.57 (m, 2H, C*H*-arom), 7.41 (tq, *J* = 7.5, 1.0 Hz, 2H, C*H*-arom), 7.32 (tt, *J* = 7.4, 1.2 Hz, 2H, C*H*-arom), 5.91 (ddt, *J* = 16.4, 10.9, 5.8 Hz, 1H, OCH_2CH=CH_2), 5.45 (d, *J* = 8.3 Hz, 1H, N*H*Fmoc), 5.37 – 5.24 (m, 2H, OCH_2CH=CH_2), 4.65 (d, *J* = 5.8 Hz, 2H, OCH_2CH=CH_2), 4.57 (s, 1H, N*H*Boc), 4.45 – 4.36 (m, 3H, CH_2-Fmoc, α-Orn), 4.22 (t, *J* = 6.9 Hz, 1H, C*H*-Fmoc), 3.22 – 3.10 (m, 2H, δ-Orn), 1.96 – 1.84 (m, 1H, β-Orn), 1.77 – 1.49 (m, 3H, β-Orn, γ-Orn), 1.44 (s, 9H, CH_3-Boc).

^{13}C NMR (101 MHz, $CDCl_3$) δ 172.1 (*C*OOAllyl), 156.1 (*C*=O-Boc), 156.1 (*C*=O-Fmoc), 143.9 (*C*q-arom), 141.5 (*C*q-arom), 131.6 (OCH_2CH=CH_2), 127.9 (*C*H-arom), 127.2 (*C*H-arom), 125.2 (*C*H-arom), 120.1 (*C*H-arom), 120.1 (*C*H-arom), 119.3 (OCH_2CH=CH_2), 67.1 (CH_2-Fmoc), 66.2 (OCH_2CH=CH_2), 53.8 (α-Orn), 47.3 (*C*H-Fmoc), 40.1 (δ-Orn), 30.0 (β-Orn), 28.5 (CH_3-Boc), 26.3 (γ-Orn).

HRMS (ESI-Orbitrap) calcd. for $C_{28}H_{34}N_2O_6Na$ $[M+Na]^+$ 517.23091, found 517.23091.

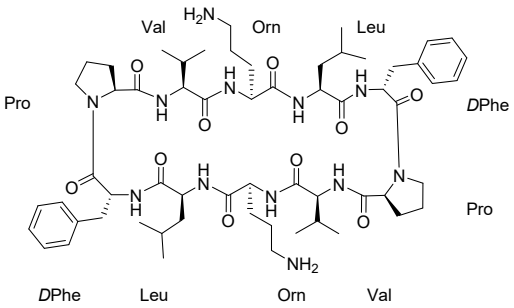
Fmoc-Orn(TentaGel S PHB)-OAllyl



2

A solution containing triphenyl phosphine oxide (1.0 g, 3.6 mmol, 7.2 equiv.) in DCM (14 mL, 0.26 M) was cooled to 0 °C and triflic anhydride (1.0 M in DCM, 1.8 mL, 1.8 mmol, 3.6 equiv.) was added. The reaction was stirred at 0 °C for 30 min. forming a white precipitate. A solution of allyl ester **8** (0.74 g, 1.5 mmol, 3.0 equiv.) in DCM (1.7 mL, 0.88 M) was added and the cooling bath was removed. The reaction was stirred at room temperature for 15 min. followed by the addition of *N*-methylmorpholine (0.41 mL, 3.8 mmol, 7.5 equiv.) and dibutyltin dilaurate (0.30 mL, 0.5 mmol, 1.0 equiv.). The solution was transferred to TentaGel S PHB resin (0.27 mmol/g, 1.9 g, 0.50 mmol, 1.0 equiv.) which was previously co-evaporated with 1,4-dioxane (3x) and the suspension was shaken for 24 hrs. The reaction mixture was filtered and the resin was washed with DCM (4x) and Et_2O (4x). Drying the resin over N_2 furnished functionalized resin **2** (2.1 g, 0.47 mmol, 94%) with a loading of 0.23 mmol/g.

Gramicidin S
***cyclo*(-Leu-*D*Phe-Pro-Val-Orn-)$_2$**

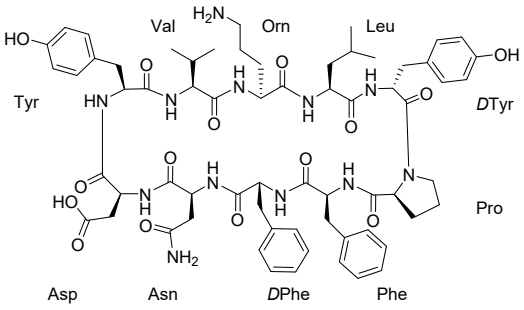


20

Functionalized resin **2** (0.44 g, 0.10 mmol, 1.0 equiv.) was elongated using the Tribute peptide synthesizer. Afterwards, the resin was washed with DMF (4x) and DCM (4x). Resin was suspended in a mixture of DCM and DMF (1:1, DCM – DMF, 4.0 mL, 25 mM) and phenylsilane (31 μL, 0.25 mmol, 2.5 equiv.) and $Pd(PPh_3)_4$ (29 mg, 25 μmol, 25 mol%) were added. 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 resin was shaken for 10 min. Suspension was filtered and 20% (v/v) piperidine in DMF (5.0 mL, 20 mM) was added to the residue. Resin was shaken for 10 min. followed by filtration and washing with DMF (6x). To the resin was added DMF (4.0 mL, 25 mM). 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 (6x). A cleavage mixture (190:5:5, TFA – H_2O – TIPS, 10 mL, 10 mM) was then added to the resin and the resulting suspension was shaken for 3 hrs. The suspension was filtered and the volatiles of the filtrate were removed under a stream of N_2. Residue was dissolved in a mixture of DCM and MeOH (1:1, MeOH – DCM) and purified by size exclusion chromatography (Sephadex LH-20, 1:1, MeOH – DCM) to give gramicidin S **20** (49 mg, 43 μmol, 43%) as a white solid.

^{1}H NMR (400 MHz, CD_3OH, 4.94 ppm suppressed) δ 8.94 (br s, 2H, N*H*-*D*Phe), 8.72 (dd, *J* = 12.0, 9.4 Hz, 4H, N*H*-Leu, N*H*-Orn), 7.71 (d, *J* = 9.0 Hz, 2H, N*H*-Val), 7.37 – 7.20 (m, 10H, C*H*-arom-*D*Phe), 4.66 (q, *J* = 7.6 Hz, 2H, α-Leu), 4.54 – 4.45 (m, 2H, α-*D*Phe), 4.37 – 4.32 (m, 2H, α-Pro), 4.15 (t, *J* = 8.8 Hz, 2H, α-Val), 3.73 (t, *J* = 9.5 Hz, 2H, δ-Pro), 3.15 – 2.82 (m, 8H, β-*D*Phe, δ-Orn, β-*D*Phe, δ-Orn), 2.50 – 2.42 (m, 2H, δ-Pro), 2.32 – 2.22 (m, 2H, β-Val), 2.09 – 1.96 (m, 4H, β-Pro, β-Orn), 1.81 – 1.34 (m, 18H, β-Orn, γ-Pro, β-Pro, γ-Pro, γ-Orn, β-Leu, γ-Leu, β-Leu), 0.98 – 0.84 (m, 24H, γ-Val, δ-Leu).
Note: α-Orn is in the suppressed region of 4.94 ppm.
^{13}C NMR (101 MHz, CD_3OH) δ 173.5 (*C*ONH), 173.5 (*C*ONH), 173.4 (*C*ONH), 172.8 (*C*ONH), 172.4 (*C*ONH), 136.8 (*C*q-arom-*D*Phe), 130.3 (*C*H-arom-*D*Phe), 129.6 (*C*H-arom-*D*Phe), 128.5 (*C*H-arom-*D*Phe), 61.9 (α-Pro), 60.3 (α-Val), 55.9 (α-*D*Phe), 52.4 (α-Orn), 51.4 (α-Leu), 47.9 (δ-Pro), 42.0 (β-Leu), 40.5 (δ-Orn), 31.9 (β-Val), 30.2 (β-Orn), 28.0 (β-Pro), 26.8 (γ-Orn), 25.6 (γ-Val), 24.4 (γ-Pro), 23.1 (δ-Leu), 23.0 (δ-Leu), 19.6 (γ-Val), 19.4 (γ-Val).
HRMS (ESI-Orbitrap) calcd. for $C_{60}H_{93}N_{12}O_{10}$ $[M+H]^+$ 1141.71321, found 1141.68680.

Loloatin A
***cyclo*(-Leu-*D*Tyr-Pro-Phe-*D*Phe-Asn-Asp-Tyr-Val-Orn-)**



23

Functionalized resin **2** (0.44 g, 0.10 mmol, 1.0 equiv.) was elongated using the Tribute peptide synthesizer. Afterwards, the resin was washed with DMF (4x) and DCM (4x). Resin was suspended in a mixture of DCM and DMF (1:1, DCM – DMF, 4.0 mL, 25 mM) and phenylsilane (31 μL, 0.25 mmol, 2.5 equiv.) and $Pd(PPh_3)_4$ (29 mg, 25 μmol, 25 mol%) were added. 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 resin was shaken for 10 min. Suspension was filtered and 20% (v/v) piperidine in DMF (5.0 mL, 20 mM) was added to the residue. Resin was shaken for 10 min. followed by filtration and washing with DMF (6x). To the resin was added DMF (4.0 mL, 25 mM). 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 (6x). A cleavage mixture (190:5:5, TFA – H_2O – TIPS, 10 mL, 10 mM) was then added to the resin and the resulting suspension was shaken for 3 hrs. The suspension was filtered and the volatiles of the filtrate were removed under a stream of N_2. Residue was dissolved in a mixture of DCM and MeOH (1:1, MeOH – DCM) and purified by size exclusion chromatography (Sephadex LH-20, 1:1, MeOH – DCM) to give loloatin A **23** (76 mg, 59 μmol, 59%) as a white solid.

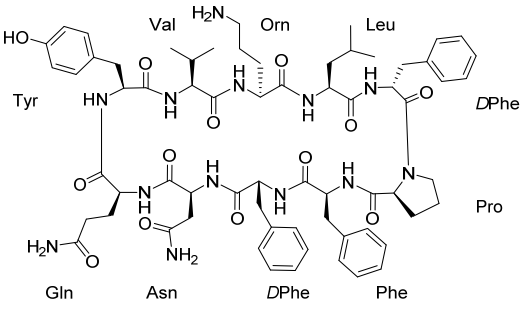
^{1}H NMR (400 MHz, CD_3OH, 4.94 ppm suppressed) δ 9.52 – 9.33 (m, 2H, N*H*-Asn, N*H*-*D*Tyr), 9.23 (s, 1H, N*H*-Orn), 9.00 (br s, 1H, N*H*-*D*Phe), 8.86 (d, *J* = 9.4 Hz, 1H, N*H*-Tyr), 8.63 (d, *J* = 8.7 Hz, 1H, N*H*-Leu), 8.43 (d, *J* = 4.3 Hz, 1H, N*H*-Asp), 8.14 (s, 1H, δ-Asn), 7.93 (d, *J* = 8.9 Hz, 1H, N*H*-Val), 7.64 – 7.49 (m, 2H, δ-Asn, N*H*-Phe), 7.26 – 7.11 (m, 10H, C*H*-arom-Phe, C*H*-arom-*D*Phe), 7.05 (d, *J* = 8.1 Hz, 2H, C*H*-arom-*D*Tyr), 6.81 (d, *J* = 8.1 Hz, 2H, C*H*-arom-Tyr), 6.69 (d, *J* = 8.1 Hz, 2H, C*H*-arom-*D*Tyr), 6.46 (d, *J* = 8.0 Hz, 2H, C*H*-arom-Tyr), 5.94 – 5.83 (m, 1H, α-*D*Phe), 5.53 – 5.45 (m, 1H, α-Orn), 4.68 (s, 1H, α-Asn), 4.60 – 4.51 (m, 2H, α-Tyr, α-Phe), 4.47 – 4.38 (m, 2H, α-*D*Tyr, α-Asp), 4.15 (d, *J* = 8.1 Hz, 1H, α-Pro), 3.42 – 3.33 (m, 3H, β-Asn, β-*D*Phe, δ-Pro), 3.27 – 2.75 (m, 8H, β-Asn, β-*D*Tyr, β-Tyr, δ-Orn, β-*D*Phe, δ-Orn), 2.58 – 2.49 (m, 1H, β-Asp), 2.46 – 2.14 (m, 7H, β-Asp, β-Phe, δ-Pro, β-Orn, β-Phe, β-Val), 1.89 – 1.43 (m, 6H, γ-Orn, β-Leu, γ-Leu, β-Leu, β-Pro), 1.38 – 1.32 (m, 1H, β-Pro), 1.19 – 1.04 (m, 13H, γ-Val, γ-Pro, δ-Leu), 0.46 (s, 1H, γ-Pro).

Note: α-Leu and α-Val are in the suppressed region of 4.94 ppm.

^{13}C NMR (101 MHz, CD_3OH) δ 175.2 (*C*OOH), 173.8 (*C*ONH_2), 173.7 (*C*ONH), 173.7 (*C*ONH), 173.5 (*C*ONH), 173.4 (*C*ONH), 173.3 (*C*ONH), 172.9 (*C*ONH), 172.8 (*C*ONH), 172.1 (*C*ONH), 172.1 (*C*ONH), 172.0 (*C*ONH), 157.9 (*C*qOH-Tyr), 157.2 (*C*qOH-Tyr), 138.9 (*C*q-Phe), 138.8 (*C*q-Phe), 131.6 (*C*H-arom-Tyr), 130.8 (*C*H-arom-Phe), 130.6 (*C*H-arom-Phe), 130.0 (*C*H-arom-Phe), 129.2 (*C*H-arom-Phe), 129.0 (*C*q-arom-Tyr), 127.6 (*C*H-arom-Phe), 127.2 (*C*q-arom-Tyr), 116.2 (*C*H-arom-Tyr), 116.1 (*C*H-arom-Tyr), 61.4 (α-Pro), 59.5 (α-Tyr), 58.5 (α-Val), 56.3 (α-*D*Tyr), 55.3 (α-Phe), 54.7 (α-*D*Phe), 54.1 (α-Asp), 52.6 (α-Orn), 52.3 (α-Leu), 50.9 (α-Asn), 47.6 (δ-Pro), 43.1 (β-Leu), 41.3 (β-*D*Phe), 40.6 (δ-Orn), 38.7 (β-Phe), 38.2 (β-Tyr), 37.3 (β-*D*Tyr), 36.6 (β-Asn), 36.5 (β-Asp), 33.2 (β-Val), 32.9 (β-Orn), 29.7 (β-Pro), 26.2 (γ-Leu), 24.4 (γ-Orn), 23.8 (δ-Leu), 23.3 (γ-Pro), 22.9 (δ-Leu), 19.6 (γ-Val), 19.1 (γ-Val).

HRMS (ESI-Orbitrap) calcd. for $C_{65}H_{88}N_{12}O_{15}$ $[M+H]^+$ 1273.62519, found 1273.62505.

Tyrocidin A
***cyclo*(-Leu-*D*Phe-Pro-Phe-*D*Phe-Asn-Gln-Tyr-Val-Orn-)**



24

Functionalized resin **2** (0.40 g, 90 μmol, 1.0 equiv.) was elongated using the Tribute peptide synthesizer. Afterwards, the resin was washed with DMF (4x) and DCM (4x). Resin was suspended in a mixture of DCM and DMF (1:1, DCM – DMF, 4.0 mL, 23 mM) and phenylsilane (31 μL, 0.25 mmol, 2.8 equiv.) and $Pd(PPh_3)_4$ (29 mg, 25 μmol, 28 mol%) were added. 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 resin was shaken for 10 min. Suspension was filtered and 20% (v/v) piperidine in DMF (5.0 mL, 18 mM) was added to the residue. Resin was shaken for 10 min. followed by filtration and washing with DMF (6x). To the resin was added DMF (4.0 mL, 23 mM). Subsequently, 1-hydroxybenzotriazole hydrate (68 mg, 0.50 mmol, 5.6 equiv.), benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate (0.26 g, 0.50 mmol, 5.6 equiv.) and *N*-methylmorpholine (0.11 mL, 1.0 mmol, 11 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 (6x). A cleavage mixture (190:5:5, TFA – H_2O – TIPS, 10 mL, 9.0 mM) was then added to the resin and the resulting suspension was shaken for 3 hrs. The suspension was filtered and the volatiles of the filtrate were removed under a stream of N_2. Residue was dissolved in a mixture of DCM and MeOH (1:1, MeOH – DCM) and purified by size exclusion chromatography (Sephadex LH-20, 1:1, MeOH – DCM) to give tyrocidin A- **24** (75 mg, 59 μmol, 66%) as a white solid.

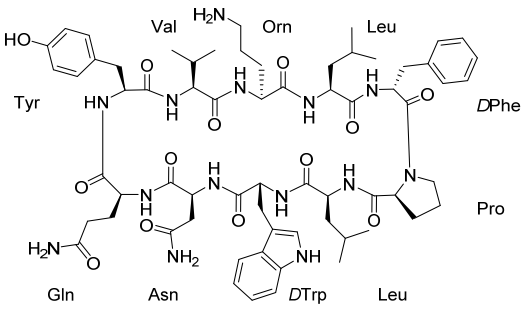
^{1}H NMR (400 MHz, CD_3OH, 4.94 ppm suppressed) δ 9.40 – 9.27 (m, 2H, N*H*-Asn, N*H*-*D*Phe), 9.15 – 9.02 (m, 2H, N*H*-Orn, N*H*-Gln), 8.92 (d, *J* = 9.7 Hz, 1H, N*H*-*D*Phe), 8.74 (d, *J* = 9.8 Hz, 1H, N*H*-Tyr), 8.50 (br s, 1H, N*H*-Leu), 8.10 (s, 1H, δ-Asn), 7.88 (d, *J* = 9.1 Hz, 1H, N*H*-Val), 7.57 (s, 1H, δ-Asn), 7.52 (d, *J* = 9.0 Hz, 1H, N*H*-Phe), 7.37 (d, *J* = 2.4 Hz, 1H, ε-Gln), 7.34 – 7.10 (m, 15H, C*H*-arom-Phe, C*H*-arom-*D*Phe), 6.91 (d, *J* = 2.3 Hz, 1H, ε-Gln), 6.86 (d, *J* = 8.0 Hz, 2H, C*H*-arom-Tyr), 6.50 (d, *J* = 7.8 Hz, 2H, C*H*-arom-Tyr), 5.87 – 5.77 (m, 1H, α-*D*Phe), 5.47 (q, *J* = 8.2 Hz, 1H, α-Orn), 4.70 – 4.51 (m, 3H, α-Asn, α-Tyr, α-Phe), 4.49 – 4.43 (m, 1H, α-*D*Phe), 4.13 (d, *J* = 8.0 Hz, 1H, α-Pro), 4.04 (q, *J* = 5.8, 5.1 Hz, 1H, α-Gln), 3.39 – 3.32 (m, 2H, δ-Pro, β-Asn), 3.30 – 3.04 (m, 6H, β-*D*Phe, β-Asn, β-*D*Phe, β-Tyr), 3.01 – 2.79 (m, 3H, δ-Orn, β-*D*Phe, δ-Orn), 2.41 (t, *J* = 13.2 Hz, 1H, β-Phe), 2.33 – 1.87 (m, 7H, β-Phe, δ-Pro, β-Val, β-Orn, γ-Gln, β-Orn), 1.85 – 1.55 (m, 6H, γ-Orn, β-Leu, β-Gln, γ-Leu), 1.52 – 1.41 (m, 2H, β-Pro, β-Leu), 1.37 – 1.33 (m, 1H, β-Pro), 1.15 – 1.00 (m, 13H, γ-Val, γ-Pro, δ-Leu), 0.48 – 0.34 (m, 1H, γ-Pro).

Note: α-Leu and α-Val are in the suppressed region of 4.94 ppm.

^{13}C NMR (101 MHz, CD_3OH) δ 178.4 (*C*ONH_2-Gln), 175.1 (*C*ONH_2-Asn), 173.9 (*C*ONH), 173.8 (*C*ONH), 173.4 (*C*ONH), 173.4 (*C*ONH), 173.3 (*C*ONH), 173.1 (*C*ONH), 173.0 (*C*ONH), 172.9 (*C*ONH), 172.2 (*C*ONH), 172.0 (*C*ONH), 157.2 (*C*qOH-Tyr), 138.9 (*C*q-*D*Phe), 138.7 (*C*q-*D*Phe), 136.8 (*C*q-Phe), 130.9 (*C*H-arom-Tyr), 130.8 (*C*H-arom-Phe), 130.5 (*C*H-arom-Phe), 130.0 (*C*H-arom-Phe), 129.5 (*C*H-arom-Phe), 129.3 (*C*H-arom-Phe), 129.2 (*C*H-arom-Phe), 129.1 (*C*q-arom-Tyr), 128.4 (*C*H-arom-Phe), 127.7 (*C*H-arom-Phe), 127.6 (*C*H-arom-Phe), 116.3 (*C*H-arom-Tyr), 61.4 (α-Pro), 59.6 (α-Val), 58.3 (α-Tyr), 56.7 (α-Gln), 56.0 (α-*D*Phe), 55.4 (α-Phe), 54.6 (α-*D*Phe), 52.4 (α-Orn), 52.3 (α-Leu), 50.9 (α-Asn), 47.6 (δ-Pro), 42.9 (β-Leu), 41.4 (β-*D*Phe), 40.6 (δ-Orn), 38.7 (β-Phe), 38.3 (β-Tyr), 37.3 (β-*D*Phe), 36.5 (β-Asn), 33.2 (β-Val), 32.6 (β-Orn), 31.6 (γ-Gln), 29.9 (β-Pro), 26.8 (β-Gln), 26.3 (γ-Leu), 24.4 (γ-Orn), 23.8 (δ-Leu), 23.3 (γ-Pro), 22.7 (δ-Leu), 19.5 (γ-Val), 19.1 (γ-Val).

HRMS (ESI-Orbitrap) calcd. for $C_{66}H_{88}N_{13}O_{13}$ $[M+H]^+$ 1270.66191, found 1270.66186.

Streptocidin A
***cyclo*(-Leu-*D*Phe-Pro-Leu-*D*Trp-Asn-Gln-Tyr-Val-Orn-)**



25

Functionalized resin **2** (0.40 g, 90 μmol, 1.0 equiv.) was elongated using the Tribute peptide synthesizer. Afterwards, the resin was washed with DMF (4x) and DCM (4x). Resin was suspended in a mixture of DCM and DMF (1:1, DCM – DMF, 4.0 mL, 23 mM) and phenylsilane (31 μL, 0.25 mmol, 2.8 equiv.) and $Pd(PPh_3)_4$ (29 mg, 25 μmol, 28 mol%) were added. 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 resin was shaken for 10 min. Suspension was filtered and 20% (v/v) piperidine in DMF (5.0 mL, 18 mM) was added to the residue. Resin was shaken for 10 min. followed by filtration and washing with DMF (6x). To the resin was added DMF (4.0 mL, 23 mM). Subsequently, 1-hydroxybenzotriazole hydrate (68 mg, 0.50 mmol, 5.6 equiv.), benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate (0.26 g, 0.50 mmol, 5.6 equiv.) and *N*-methylmorpholine (0.11 mL, 1.0 mmol, 11 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 (6x). A cleavage mixture (190:5:5, TFA – H_2O – TIPS, 10 mL, 9.0 mM) was then added to the resin and the resulting suspension was shaken for 3 hrs. The suspension was filtered and the volatiles of the filtrate were removed under a stream of N_2. Residue was dissolved in a mixture of DCM and MeOH (1:1, MeOH – DCM) and purified by size exclusion chromatography (Sephadex LH-20, 1:1, MeOH – DCM) to give streptocidin A- **25** (45 mg, 35 μmol, 40%) as a white solid.

^{1}H NMR (400 MHz, CD_3OH, 4.94 ppm suppressed) δ 10.40 (s, 1H, N*H*-arom-Trp), 9.45 (d, *J* = 7.5 Hz, 1H, N*H*-Asn), 9.22 (br s, 1H, N*H*-*D*Phe), 9.07 (s, 1H, N*H*-Gln), 8.98 (br s, 1H, N*H*-Orn), 8.80 (d, *J* = 9.7 Hz, 1H, N*H*-Tyr), 8.38 – 8.27 (m, 1H, N*H*-Leu), 8.22 (d, *J* = 9.5 Hz, 1H, N*H*-*D*Trp), 8.08 (s, 1H, δ-Asn), 7.89 (d, *J* = 9.2 Hz, 1H, N*H*-Val), 7.60 (d, *J* = 8.0 Hz, 1H, C*H*-arom-*D*Trp), 7.54 (s, 1H, δ-Asn), 7.43 – 7.39 (m, N*H*-Leu), 7.39 (s, 1H, ε-Gln)), 7.34 – 7.20 (m, 6H, C*H*-arom-*D*Phe, C*H*-arom-*D*Trp), 7.09 – 6.96 (m, 2H, C*H*-arom-*D*Trp), 6.93 (s, 1H, ε-Gln), 6.90 – 6.82 (m, 3H, C*H*-arom-*D*Trp, C*H*-arom-Tyr), 6.55 – 6.44 (m, 2H, C*H*-arom-Tyr), 5.87 – 5.74 (m, 1H, α-*D*Trp), 5.55 – 5.45 (m, 1H, α-Orn), 4.75 – 4.64 (m, 1H, α-Asn), 4.63 – 4.55 (m, 1H, α-Tyr), 4.47 – 4.39 (m, 1H, α-*D*Phe), 4.26 – 4.20 (m, 1H, α-Pro), 4.17 – 4.08 (m, 1H, α-Leu), 4.05 (q, *J* = 6.1, 5.2 Hz, 1H, α-Gln), 3.62 – 3.56 (m, 1H, δ-Pro), 3.29 – 3.21 (m, 2H, β-Asn, β-*D*Trp), 3.19 – 2.96 (m, 7H, β-Asn, β-*D*Trp, β-Tyr, β-*D*Phe, δ-Orn), 2.91 – 2.78 (m, 1H, δ-Orn), 2.35 (q, *J* = 8.6 Hz, 1H, δ-Pro), 2.27 – 2.21 (m, 1H, β-Val), 2.07 – 1.96 (m, 4H, β-Orn, γ-Gln, β-Orn), 1.92 – 1.86 (m, 1H, β-Pro), 1.84 – 1.70 (m, 4H, γ-Orn, β-Gln), 1.65 – 1.35 (m, 5H, β-Pro, γ-Pro, γ-Leu, β-Leu), 1.28 – 1.17 (m, 2H, β-Leu, γ-Leu), 1.14 (d, *J* = 6.7 Hz, 3H, γ-Val), 1.11 (d, *J* = 6.8 Hz, 3H, γ-Val), 1.02 – 0.92 (m, 7H, δ-Leu, β-Leu), 0.63 (d, *J* = 6.6 Hz, 6H, δ-Leu), -0.15 (s, 1H, β-Leu).

Note: α-Leu and α-Val are in the suppressed region of 4.94 ppm.

^{13}C NMR (101 MHz, CD_3OH) δ 179.4 ($CONH_2$-Gln), 178.4 ($CONH_2$-Asn), 175.1 (*C*ONH), 174.7 (*C*ONH), 174.5 (*C*ONH), 174.0 (*C*ONH), 173.9 (*C*ONH), 173.3 (*C*ONH), 173.1 (*C*ONH), 173.0 (*C*ONH), 172.1 (*C*ONH), 172.0 (*C*ONH), 157.2 (*C*q-OH-Tyr), 138.2 (*C*q-arom-*D*Phe), 136.8 (*C*q-arom-*D*Trp), 130.9 (*C*H-arom-Tyr), 130.7 (*C*H-arom-*D*Phe), 130.5 (*C*H-arom-*D*Phe), 129.5 (*C*H-arom-*D*Phe), 129.1 (*C*q-arom-Tyr), 128.4 (*C*q-arom-*D*Trp), 125.3 (*C*H-arom-*D*Trp), 122.5 (*C*H-arom-*D*Trp), 120.1 (*C*H-arom-*D*Trp), 120.0 (*C*H-arom-*D*Trp), 116.2 (*C*H-arom-Tyr), 112.2 (*C*H-arom-*D*Trp), 111.1 (*C*q-arom-*D*Trp), 61.7 (α-Pro), 59.6 (α-Val), 58.3 (α-Tyr), 56.6 (α-Gln), 56.0 (α-*D*Phe), 53.2 (α-*D*Trp), 52.6 (α-Orn), 52.4 (α-Leu), 52.4 (α-Leu), 50.9 (α-Asn), 47.7 (δ-Pro), 42.3 (β-Leu), 40.6 (δ-Orn), 40.4 (β-Leu), 38.2 (β-Tyr), 37.8 (β-*D*Phe), 36.5 (β-Asn), 33.0 (β-Val), 32.9 (β-Orn), 31.6 (γ-Gln), 31.6 (β-Trp), 30.1 (β-Pro), 26.7 (β-Gln), 26.2 (γ-Leu), 25.5 (γ-Leu), 24.3 (γ-Orn), 24.0 (δ-Leu), 23.6 (γ-Pro), 23.4 (δ-Leu)), 22.2 (δ-Leu), 20.7 (δ-Leu), 19.5 (γ-Val), 19.3 (γ-Val).

HRMS (ESI-Orbitrap) calcd. for $C_{65}H_{91}N_{14}O_{13}$ $[M+H]^+$ 1275.68846, found 1275.68889.

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