Synthesis of Vancomycin Mimics Using Ruthenium-Catalyzed Macrocyclization Chemistries

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Synthesis of Vancomycin Mimics Using Ruthenium-Catalyzed Macrocyclization Chemistries

Synthese van Vancomycine Mimetica met behulp van Ruthenium-Gekatalyseerde Macrocyclisatie Chemie

(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de rector magnificus, prof. dr. G.J. van der Zwaan, ingevolge van het besluit van het college voor promoties in het openbaar te verdedigen op maandag 23 april 2018 des middags te 12.45 uur

door

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geboren op 26 december 1988 te Zhejiang, China

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Copromotor: Dr. ir. D.T.S. Rijkers

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Chapter 1

General Introduction

1.1 Macrocyclic peptide antibiotics

The macrocycle as a structural motif has gained an increased importance in medicinal chemistry and modern drug design.[1] Typical examples of a bioactive macrocycle are cyclic peptides and numerous cyclic peptides have been successfully used in clinical applications within different therapeutic fields as antibiotics,[2] antifungal agents,[3] anticancer agents[4] and immuno-suppressive agents.[5] The most outstanding example of a class of macrocyclic peptide drugs successfully applied in clinical practice is the peptide-based antibiotics, which can be chemically subdivided into the glycopeptides like vancomycin[6], teicoplanin[7] and ramoplanin[8], the polypeptides such as actinomycin[4a] and the lipopeptides, represented here by daptomycin,[9] as shown in Figure 1.

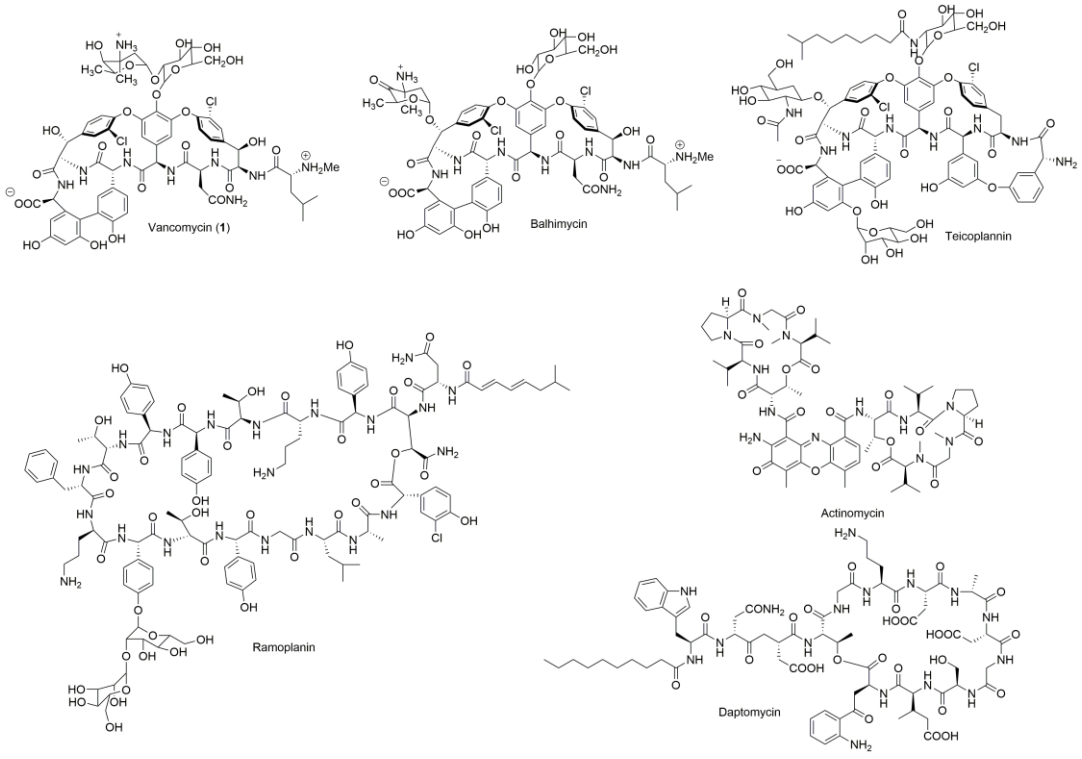


Figure 1. Examples of macrocyclic peptide antibiotics.

1.2 Chemical approaches towards the synthesis of macrocyclic peptides

Macrocyclization enables peptides to adopt a certain degree of structural preorganization and reduces flexibility which in general will result in improved binding affinity and selectivity to their natural targets.[1] Inspired by the intriguingly complexity and synthetically challenging architecture of naturally occurring macrocycles, much effort has been devoted to explore highly efficient synthesis methods for peptide macrocyclization.[10,11] The most often used macrocyclization methodologies are, among others, lactonization,[12] lactamization,[13] transition metal-mediated coupling reactions,[14] ring-closing metathesis (RCM),[15] as well as Cu^{+} and Ru^{2+} catalyzed azide-alkyne cycloadditions (CuAAC and RuAAC, respectively).[16] Besides these, some other special approaches such as, electrostatically controlled macrocyclizations,[17] isocyanide-based multicomponent macrocyclizations,[18] as well as DNA-templated synthesis,[19] provide new synthetic tools toward peptide macrocyclization chemistries.

1.3 Vancomycin and its peptidomimetics

Vancomycin (**1**, as shown in Figure 1) which is the most well-known member of the glycopeptide antibiotics, was first isolated at Eli Lilly Pharmaceutical Company in 1956.[20] Although vancomycin was already introduced into clinical practice in 1958,[20] its full structural assignment as well as its mode of action was not described until 1982 by Harris and co-workers.[21] Since vancomycin has been used as a broad spectrum antibiotic against Gram-positive bacteria, it is nowadays considered as a last resort antibiotic for the treatment of methicillin-resistant *Staphylococcus aureus* (MRSA) infections.[22] Unfortunately, after decades of extensive use, vancomycin-resistant *Enterococci* (VRE)[23] and vancomycin -resistant *Staphylococcus aureus* (VRSA)[24] have emerged and it is expected that the prevalence of vancomycin-resistant bacteria will increase even more in the near future. Therefore, the development of new generations vancomycin-derived analogues against the reported resistant bacterial strains has become an increasingly challenging and important task.

Vancomycin's mechanism of action involves the inhibition of bacterial cell wall biosynthesis[25] by binding to the C-terminal D-Ala-D-Ala dipeptide motif of

precursor peptidoglycans.[26] Extensive NMR studies[27] in combination with X-ray crystallography[28] have identified the strong binding of vancomycin to D-Ala-D-Ala by means of five precisely aligned hydrogen bonds, while the hydrophobic binding pocket, which is formed by the aromatic and aliphatic amino acid side chains, contributes to the enhancement of the hydrogen bonds, as shown in Figure 2. However, in vancomycin-resistant bacteria, the cell wall precursor terminus is remodeled to D-Ala-D-Lac[29]. This replacement, which is an example of an isosteric replacement, results in the loss of a single hydrogen bond and reduces the binding affinity of vancomycin toward D-Ala-D-Lac 1000-fold. Based on this well understood mechanism, much effort has been devoted to the preparation of vancomycin analogues with dual D-Ala-D-Ala/D-Ala-D-Lac binding affinities (vide infra).

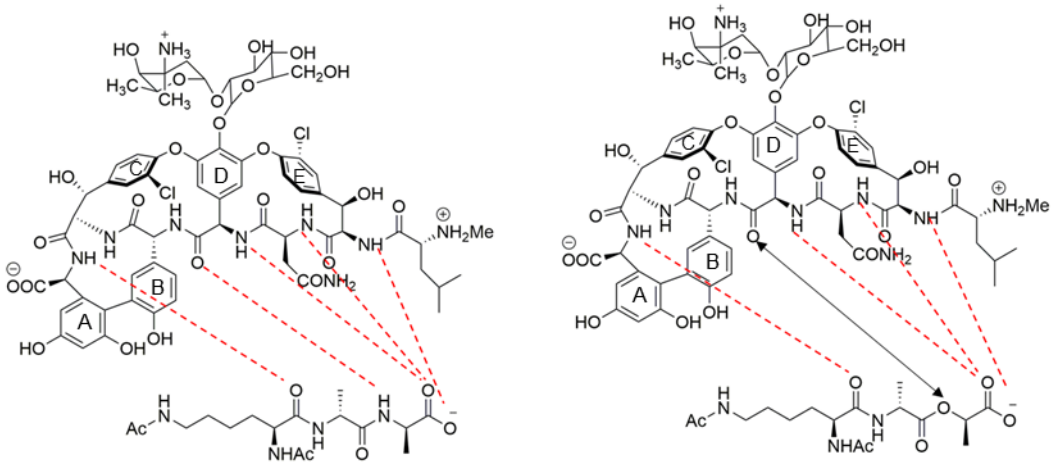


Figure 2. Vancomycin and its binding interactions with Ac-Lys(Ac)-D-Ala-D-Ala-OH and Ac-Lys(Ac)-D-Ala-D-Lac-OH ligands.

Initially, due to the complexity of the total synthesis and structural modification,[30] most vancomycin analogs have been designed and synthesized by simplifying the molecular structure of vancomycin (Figure 3). Such DE-ring mimics are much easier accessible synthetically and some of these were found to act as carboxylate-binding receptor molecules. Hamilton *et al.* designed mimics of the left side of vancomycin as represented by compound **2** with the aim to increase the

number of receptor-substrate interactions.[31] A series of sixteen a-membered DE-ring vancomycin mimics were described by Zhu *et al.*, applying an S_NAr-based macrocyclization protocol. Within this series, compound **3** showed a K_D of 5×10^{-4} M toward Ac-D-lactate as determined by NMR.[32] Ellman *et al.* prepared simplified analogs that mimic the vancomycin DE-ring carboxylate binding pocket. These analogues were C-terminally extended with a short peptide sequence using combinatorial chemistry. Within this series, compound **4** showed a significantly increased binding affinity toward Ac-Lys(Ac)-D-Ala-D-Lac in comparison to vancomycin.[33] Arnusch and Pieters developed a solid phase methodology to obtain vancomcin DE-ring mimics like **5**. In their studies, an on-bead S_NAr cyclization reaction was successfully applied to form the biaryl ether bridge.[34] ten Brink *et al.* reported constrained alkyne-bridged DE-ring mimicking tripeptides like **6** featuring a Pd-catalyzed Sonogashira cross-coupling macrocyclization.[35] The resulting molecule was able to form a cavity-like structure as shown by NMR in combination with molecular dynamics. This might hint toward selective binding toward Ac-Lys(Ac)-D-Ala-D-Ala, which was however, not determined experimentally.

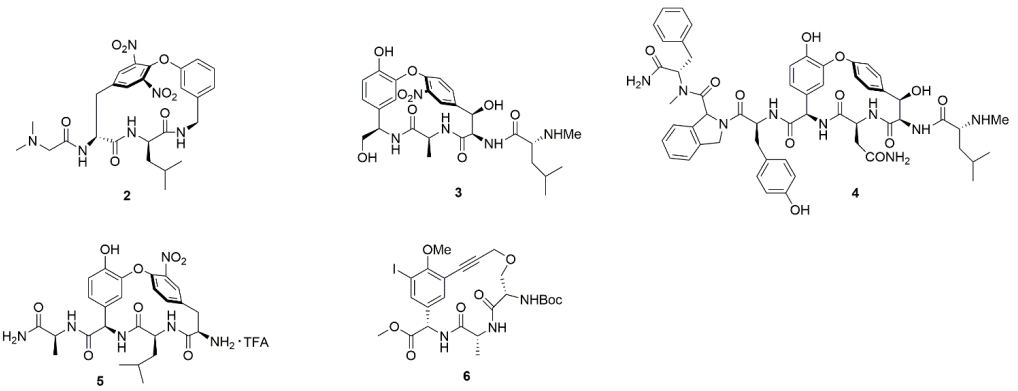


Figure 3. Structures of vancomycin DE-ring peptidomimetics.

Recently, Dale Boger and his coworkers contributed significantly to the design of a new class of second-generation antibiotics based on the vancomycin framework (Figure 4). They reported that vancomycin analogs with modifications at position X (an isosteric replacement of the carbonyl oxygen by S, NH, H_2), forming the carboxylate binding pocket, among which [Ψ[CH_2NH]Tpg^4]-vancomycin **7** and

[Ψ[C(=NH)NH]Tpg^4]-vancomycin **8** represented congeners with the highest antimicrobial potency against vancomycin-resistant bacteria while retaining activity against vancomycin-sensitive bacteria.[36,37] Furthermore, by coupling a (4-chlorobiphenyl)methyl (CBP; R_1, as shown in Figure 4) functionality to the disaccharide of compound **8** the corresponding vancomycin analog **9** showed an additional enhancement in antimicrobial activity.[36,37] Another analogue **10**, which was reported as vancomycin 3.0 was synthesized, improving the minimal inhibitory concentration (MIC) values to the remarkable levels of 0.01-0.005 μg/mL.[38]

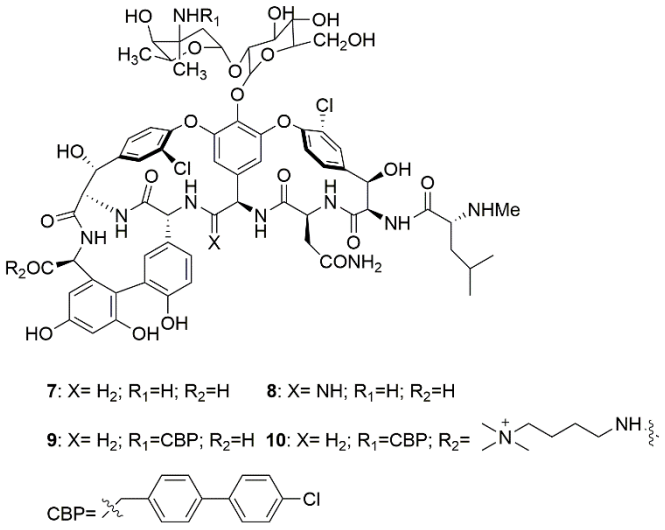


Figure 4. Vancomycin analogues from Boger's group.

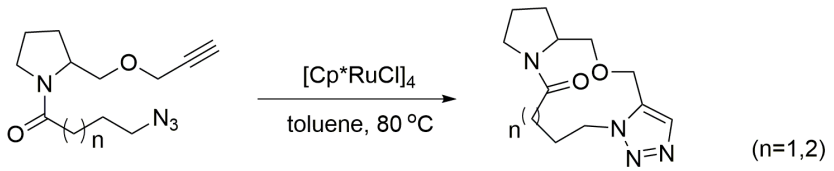
1.4 Azide-alkyne cycloadditions (click chemistry) and their application in peptide macrocyclization

The 1,2,3-triazole heterocyclic motif has been used recently in peptides and peptidomimics since it can serve as an effective isostere for a *trans*- or *cis*-amide bond.[11] The formation of a 1,2,3-triazole moiety from an organic azide and alkyne is a so-called 1,3-dipolar cycloaddition and was described for the first time by Huisgen in 1961. The required reaction conditions were quite harsh and unselective with respect to the 1,4 and 1,5 regioisomers and as a result of this, it did not find

widespread application in bioconjugate chemistry.[39] This, however, changed dramatically, since in 2002, Meldal[16] and Sharpless[40] independently introduced mild and efficient methods to access 1,4-disubstituted 1,2,3-triazoles catalyzed by Cu(I) species. Soon after its discovery, this robust, mild and selective copper catalyzed azide-alkyne cycloaddition (CuAAC) has been widely used as an efficient bioconjugation method in general and more specifically, as a feasible peptide macrocyclization technique.[41]

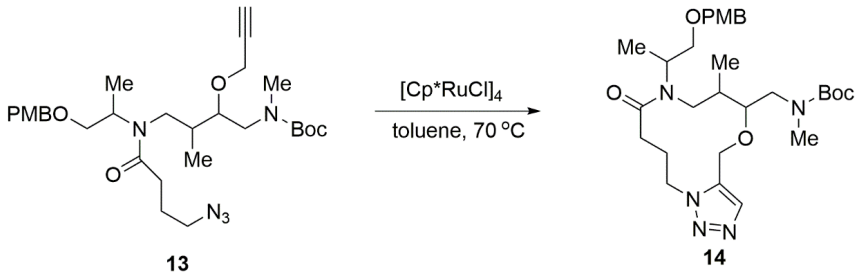
The original Huisgen reaction afforded mixtures of 1,4- and 1,5-disubstituted 1,2,3-triazoles while the CuAAC selectively results in the formation of 1,4-disubstituted 1,2,3-triazoles, which are found to be effective isosteres for the *trans*-amide bond. Alternatively, effective *cis*-amide bond surrogates were found to be 1,5-disubstituted 1,2,3-triazoles, which were accessed by Fokin in 2005,[42] by exchanging the Cu(I) catalyst for a Ru(II) catalyst. So far, this ruthenium-catalyzed azide alkyne cycloaddition (RuAAC) has not been used as commonly as CuAAC in bioconjugation and peptide macrocyclization, its application is, however, of increasing importance and several small, medium and large (peptide) ring-systems have been synthesized successfully.[43]

The first application of RuAAC as the macrocyclization step was reported by Marcaurelle *et al*. A number of 11-, and 12-membered macrocycles containing a 1,5-triazole moiety were successfully synthesized using $[Cp^*RuCl]_4$ as the catalyst, as shown in Scheme 1.[44] A follow-up study by the same group, reported a series of 48 macrocyclic scaffolds, including 1,5-triazole-containing compound **14** as shown in Scheme 2, which was obtained through three different approaches for macrocyclization of the linear precursors.[45] These 48 scaffolds have been used to generate more than 30,000 compounds using SynPhase Lantern technology in an attempt to search for new macrocyclic histone deacetylase inhibitors.[45]



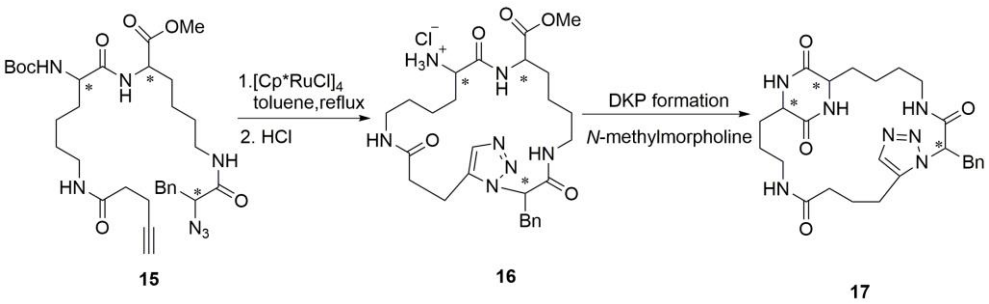
11 **12**

Scheme 1. Synthesis of 1,5-triazole bridged macrocycles using RuAAC.



Scheme 2. Synthesis of 1,5-triazole bridged macrocyclic scaffold using RuAAC.

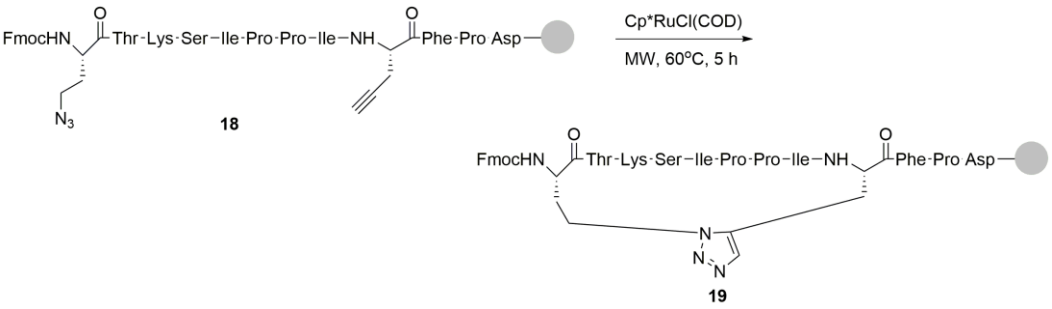
Isidro-Llobet *et al.* described a strategy for the diversity-oriented synthesis (DOS) of a small library of fourteen triazole-bridged macrocyclic peptidomimetics by using CuAAC as well as RuAAC cyclization chemistries (Scheme 3). For further diversification, diketopiperazine (DKP) formation was introduced in these macrocycles featuring solid phase bound reagents, e.g. *N*-methylmorpholine, in combination with microwave irradiation for effective heating.[46]



Scheme 3 Synthesis of 1,5-triazole bridged macrocyclic peptidomimetics using RuAAC cyclization followed by DKP formation

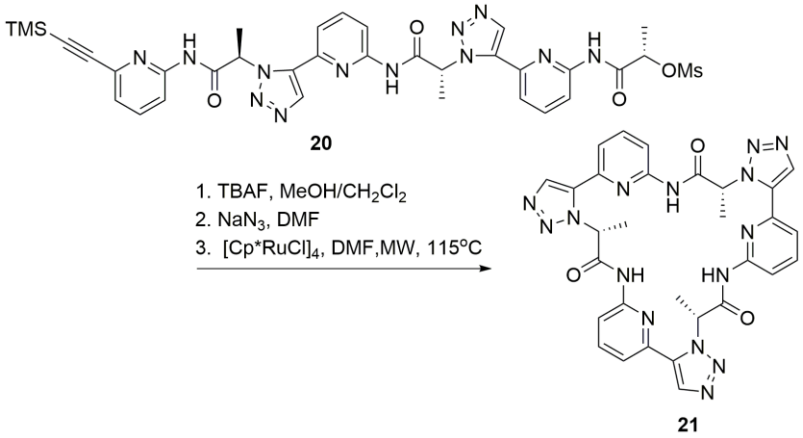
1,5-Disubstituted 1,2,3-triazoles have also been used as a surrogate of a disulfide bond, and this application was first demonstrated by Empting *et al*. (as shown in Scheme 4). RuAAC cyclization was successfully applied on the solid phase to achieve an analogue (**19**) of the sunflower trypsin inhibitor-I. The biological activity of this triazole-containing analogue disulfide mimic was tested, and more or less to the surprise of the authors, their newly designed inhibitor had

the same activity as the original one.[47]



Scheme 4. Synthesis of 1,5-triazole bridged analogue of the sunflower trypsin inhibitor-I using RuAAC cyclization on solid phase.

Another macrocyclic analogue was obtained by replacing the tertiary amide groups by 1,5-disubstituted 1,2,3-triazole functionalities as shown by Krause *et al.* (Scheme 5). In their approach, RuAAC was utilized in the macrocyclization as well as during the synthesis of the linear precursor to obtain a variety of C_3 symmetric macrocyclic pseudohexapeptides.[48]



Scheme 5. Synthesis of 1,5-triazole bridged C_3 symmetric macrocyclic pseudohexapeptides using RuAAC cyclization.

1.5 Ring-closing metathesis and its application in peptide macrocyclization

Ring-closing metathesis (RCM), the most prevalent type of olefin metathesis, has occurred as a powerful synthetic tool for the formation of carbon-carbon double bonds.[49] Its increasing popularity was facilitated by the development of robust metathesis catalysts, as shown in Figure 5, that were compatible with a large diversity of functional groups. One of the first well-defined molybdenum-based metathesis catalysts was introduced by Schrock and coworkers.[50] Subsequently, Grubbs and coworkers reported three generations of ruthenium-based metathesis catalysts (Grubbs' catalyst,[51] Grubbs' GI,[52] and Grubbs' GII[53]) to improve the catalytic activity, thermal stability, and functional group tolerability. The latest improvement was the replacement of the phosphine ligand of Grubbs GII by a bidentate alkylidene to yield the more efficient and robust Hoveyda-Grubbs' GII catalyst.[54] Due to the development of these highly functional-group tolerant ruthenium-based metathesis catalysts, RCM has played a significant role as a synthetic tool to obtain functionalized biomolecules such as constrained cyclic peptides.

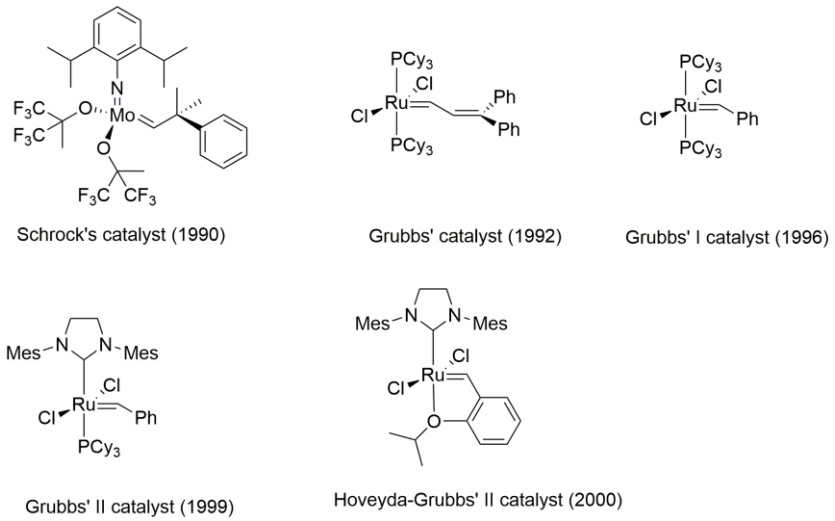
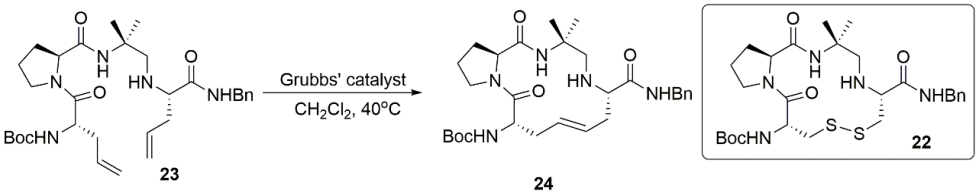


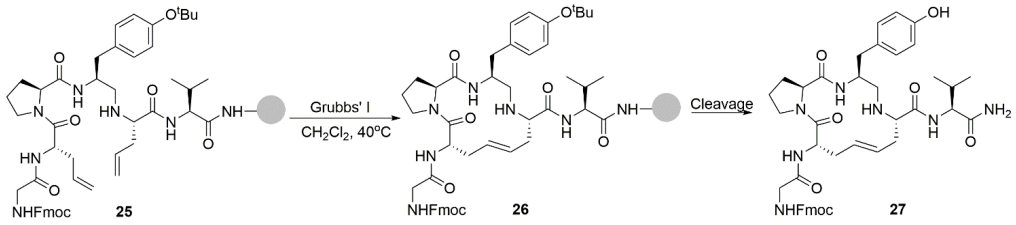
Figure 5. Well-defined olefin metathesis catalysts.

Grubbs and co-workers were the first to apply the strategy of RCM in the

synthesis of conformationally restricted amino acids and peptides.[55] Cyclic amino acid derivatives with different ring sizes were obtained using Grubbs' catalyst. In the meantime native cysteine residues were replaced by allylglycine functionalities to synthesize a constrained tetrapeptide as a functional mimic of a covalently disulfide-stabilized β-turn **22** (Scheme 6). This pioneering work was extended to perform RCM on the solid support as exemplified by the synthesis of cyclic hexapeptide **27**, as shown in Scheme 7.[56]



Scheme 6. Synthesis of a mimic of disulfide-stabilized β-turn though RCM using Grubbs' catalyst.

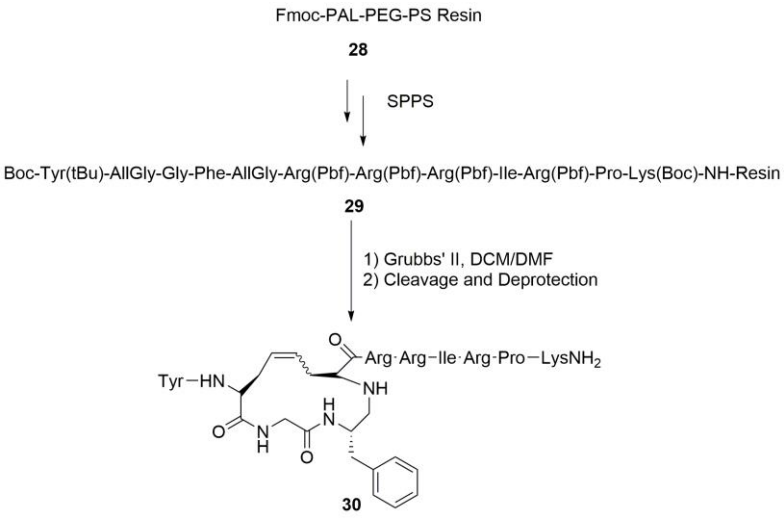


Scheme 7. Synthesis of rigidified cyclic hexapeptide on solid support though RCM using Grubbs' GI catalyst.

Following the pioneering work of Grubbs, many research groups were also successful in discovering bioactive peptides and peptidomimetics applying RCM. Among others, Aldrich *et al.* have reported the use of RCM in the synthesis of potent κ opioid receptor agonists as shown in Scheme 8. Six cyclic dynorphin A-(1-11)-NH_2 analogues were found active agonists (>90% efficacy) at κ opioid receptors, like compound **30**, and as such they represent interesting lead compounds for further optimization.[57]

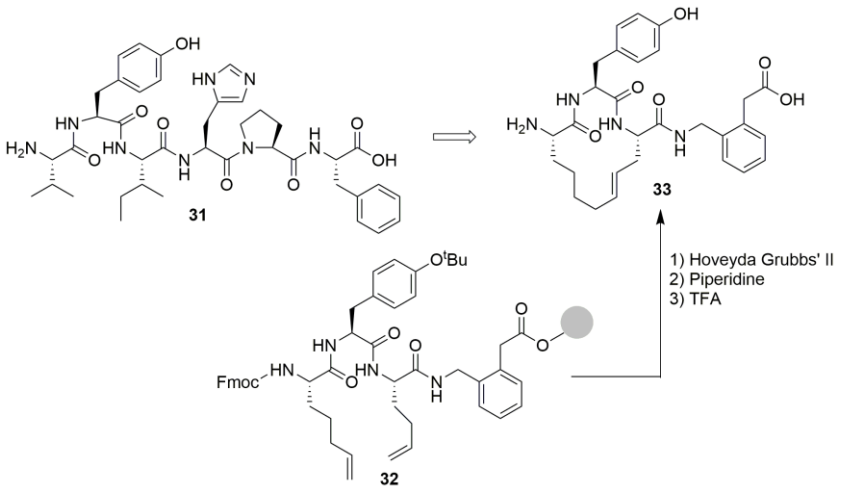
A series of macrocyclic angiotensin IV analogs (H-Val-Tyr-Ile-His-Pro-Phe-OH) have been designed and synthesized by Hallberg *et al.* in order to find drug-like Ang IV peptidomimetics as efficient insulin-regulated aminopeptidase inhibitors

(IRAP). In their approach, a disulfide cyclization scan was first applied to search for efficient and potent cyclic AngIV analogues.[58] Then, these disulfide constraints were replaced by carbon-carbon double bond isosteres featuring RCM, resulting in IRAP-inhibitors that were as effective as their natural congeners however, their stability to degradation by metallopeptidases was increased significantly.[59] One of the newly synthesized dicarba-bridged compounds, represented by macrocycle **33**, as shown in Scheme 9, displayed a low K_i value of 4.1 nM, and confirmed the hypothesis that reducing the conformational freedom will increase the biological activity.

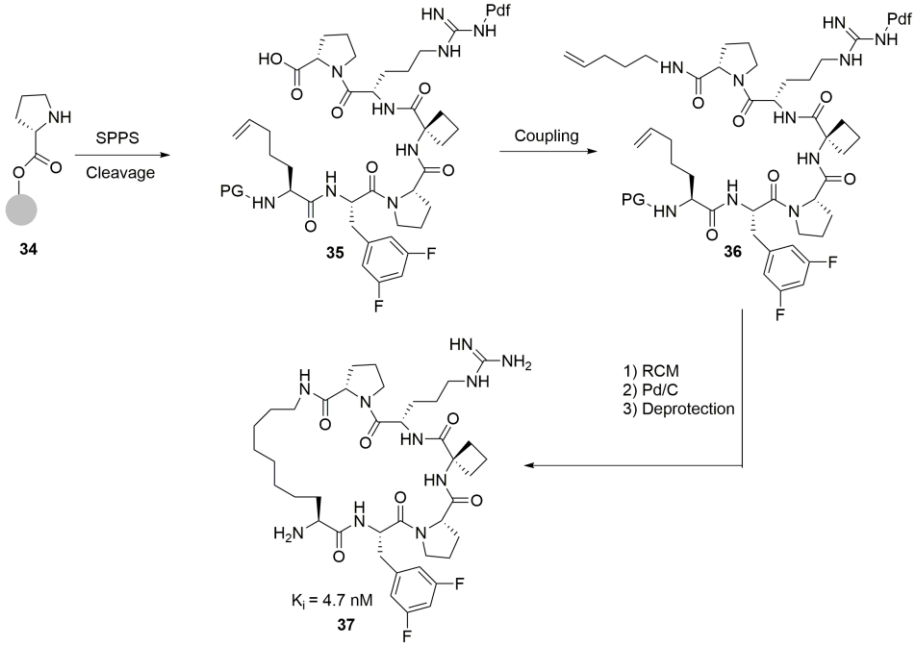


Scheme 8. Synthesis of potent cyclic κ-opioid receptor agonists.

As a final example of successful RCM-based macrocyclization, the design and synthesis of peptidomimetics to inhibit the menin-MLL1 interaction as described by Wang *et al.* will be discussed.[60] Their most promising molecule, as shown in Scheme 10, was found to have a K_i value of 4.7 nM, which was 600 times more potent than the corresponding linear peptide.[60]



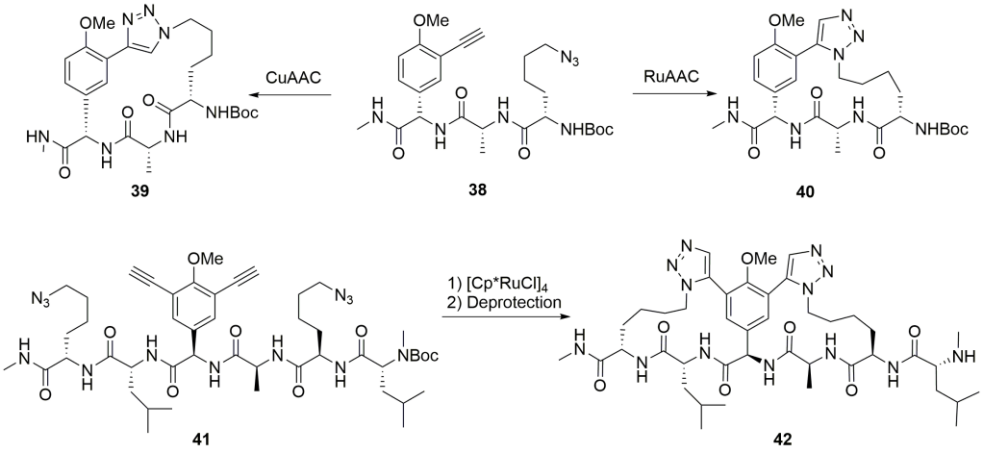
Scheme 9. Design and synthesis of angiotensin IV-derived macrocyclic peptidomimetics.



Scheme 10. The synthesis of a macrocyclic peptidomimetic as an inhibitor of the menin-MLL1 protein-protein interaction.

1.6 Vancomycin mimics accessed by RuAAC macrocyclization and ring-closing metathesis

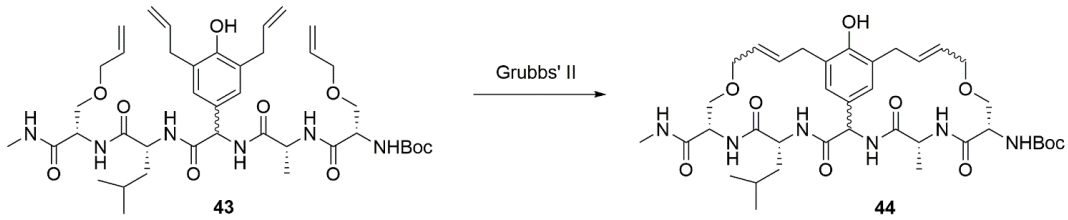
Previously, it was shown by Zhang *et al.* that the biaryl ether bridge in vancomycin could be effectively mimicked by either 1,4- or 1,5-disubstituted triazole moieties using Cu(I)- and Ru(II)-mediated azide-alkyne click cycloaddition chemistries, respectively.[61] Both 1,4- and 1,5-disubstituted triazole-bridged macrocyclic vancomycin DE-ring mimics **39** and **40** showed an excellent structural resemblance to the corresponding part of vancomycin as found by molecular modeling. Encouraged by these results, another newly designed 1,5-triazole-bridged vancomycin CDE-ring mimic **42** was successfully synthesized featuring RuAAC macrocyclization.[62] Bicyclic hexapeptide **42** was found to bind D-Ala-D-Ala albeit with a 100-fold less affinity compared to vancomycin, and also mimic **42** did not display any antibacterial activity.



Scheme 11. Synthesis of vancomycin mimics using click macrocyclization as reported by Zhang.

The strategy of RCM was also applied to arrive at a CDE-ring mimic of vancomycin.[63] The linear pentapeptide **43** with four allyl functionalities was treated with Grubbs' GII as the catalyst and bicyclic pentapeptide **44** was obtained as a complex mixture of eight diastereomers. To continue the search for effective vancomycin mimics a novel approach was designed in which extra rigidification was introduced by combining ruthenium-catalyzed macrocyclization chemistries

(vide infra).



Scheme 12. Synthesis of bicyclic pentapeptide vancomycin mimics via RCM.

1.7 Aim and outline of this thesis

The ultimate aim of the research described in this thesis was to synthesize biologically active vancomycin-inspired macrocyclic peptidomimetics. Previous research showed that both biaryl ether bridges in vancomycin can be mimicked by 1,5-disubstituted triazole moieties using the RuAAC macrocyclization. Although such bicyclic vancomycin CDE-ring mimics, like **45a**, showed a relatively good structural resemblance with vancomycin, their binding affinity toward D-Ala-D-Ala is reduced 100-fold and antibacterial activity is absent. Several approaches were followed to explore possibilities to increase affinity and activity by either amino acid replacements, introduction of an extra cyclic constraint, or by a combination of these two. In a first attempt to improve the binding affinity, **Chapter 2**, describes the design and synthesis of a new CDE-ring mimic **45b** in which the amino acid at position Xaa^1 was phenylglycine instead of leucine while at position Xaa^2 an asparagine moiety was incorporated to foster binding interactions with the amide side chain and the carboxylate of the D-Ala-D-Ala/D-Ala-D-Lac dipeptide. It was assumed that these amino acid substitutions would be more closely resemble the native peptide sequence of vancomycin. The synthesis approach required several optimization steps to cope with the racemization-prone phenylglycine and the polar asparagine side chain. It turned out that this attempt was unsuccessful since bicyclic mimic **45b** displayed almost the same binding affinity as **45a**, and antibacterial activity could not be observed. Although the optimized synthesis route for vancomycin-derived peptides is flexible and robust, it is still rather inefficient and time consuming, therefore the

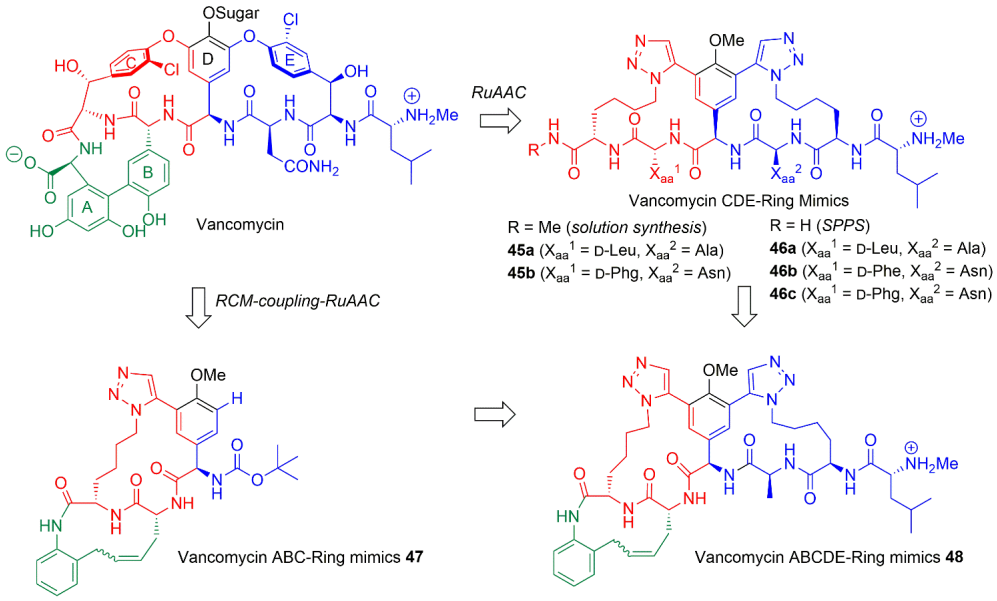
solution phase synthesis was translated into a solid phase approach.

Chapter 3 describes the solid phase synthesis of CDE-ring mimics as a more time-efficient synthesis approach. During the SPPS approach several difficulties were encountered since only three analogues were synthesized successfully. First of all, the high tendency of racemization of the phenylglycine building block during amide bond formation turned out to be problematic and no combination of coupling reagents could be found to avoid racemization. Secondly, after cleavage from the resin, the low solubility of the protected linear peptides hampered efficient purification while protecting group removal proceeded rather sluggishly. Finally, the desired compounds were only identified by mass spectrometry. Unfortunately, the amounts obtained were too small for further analysis and biological evaluation. In hindsight, this chapter was focused to provide a proof-of-principle to access the bicyclic 1,5-triazole-bridged CDE-ring mimics of vancomycin.

In **Chapter 4**, the bicyclic tripeptide **47** as a mimic of the ABC-ring system was successfully synthesized following an RCM-coupling-RuAAC strategy.[64] After RCM, the mixture of double bond isomers could be separated in each individual *E*/*Z* diastereoisomer by preparative HPLC. Compound **47** is an important building block for the synthesis of tricyclic hexapeptide **48** in which the alkene bridge serves as an extra constraint to obtain a peptide backbone topology comparable to vancomycin.

Chapter 5 describes the design and synthesis of a tricyclic hexapeptide **48** in which the olefinic constraint was installed by RCM and both triazole bridges were synthesized featuring RuAAC.[65] Hexapeptide **48** represents a structure in which the ABC- and CDE-ring systems have been combined to adopt the concave-like conformation of the peptide backbone. Based on ITC measurements, hexapeptide **48** was able to bind D-Ala-D-Ala with a comparable affinity as vancomycin: 1.26×10^4 versus 4.23×10^5 M^{-1}, the highest K_a found within this series of vancomycin mimics! This clearly showed the important contribution of the alkene bridge in maintaining the overall conformation of hexapeptide **48** as a vancomycin mimic. Gratifyingly, in line with the results of the ITC measurements, tricyclic mimic **48** displayed antibacterial activity with a MIC value of 37.5 μg/mL while the MIC value of vancomycin was 2 μg/mL. This was the first time that a member of this class of vancomycin-inspired mimics showed a reasonable activity as an

antimicrobial agent.



Scheme 13. Outline of the research described in this thesis.

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Chapter 2

Solution phase synthesis of a D-Phg^{2}/Asn^{4} 1,5-triazole-bridged vancomycin CDE-ring mimic using RuAAC macrocyclization

2.1 Introduction

Macrocyclic peptides become increasingly attractive in medicinal chemistry and modern drug design as they often offer high potency and selectivity.[1] A number of macrocyclic peptides with broad bioactivities have been found in Nature and many of them have been successfully used in clinical applications.[1b-d] The introduction of a cyclic covalent constraint will generally increase the binding affinity of peptides towards their natural ligand which will reduce the conformational flexibility and thus lower the loss of entropy during binding.[2] Vancomycin (**1**) is an outstanding example of a highly constrained peptide in which two biaryl ether bridges stabilize its rigid conformation. Vancomycin is rather difficult to access synthetically, therefore many alternative approaches for peptide cyclization have been explored to obtain vancomycin mimics in which the bioactive conformation is conserved, as discussed in Chapter 1. Among others, we have applied ring-closing metathesis (RCM) to synthesize a bicyclic pentapeptide to mimic the central CDE-ring system of vancomycin[4], and a Pd-catalyzed Sonogashira macrocyclization was used to obtain a series of constrained alkyne-bridged cyclic tripeptides as vancomycin DE-ring mimics.[5] It was also shown that the aryl ether bridge in vancomycin could be mimicked by either 1,4- or 1,5-disubstituted triazole moieties using the Cu(I)- and Ru(II)-catalyzed azide-alkyne cycloaddition reaction, CuAAC and RuAAC, respectively.[6] Encouraged by this result, the complete biaryl ether bridge system was mimicked in the 1,5-triazole-bridged vancomycin mimic (**2a**) and has been successfully synthesized using RuAAC macrocyclization (as shown in Figure 1).[7]

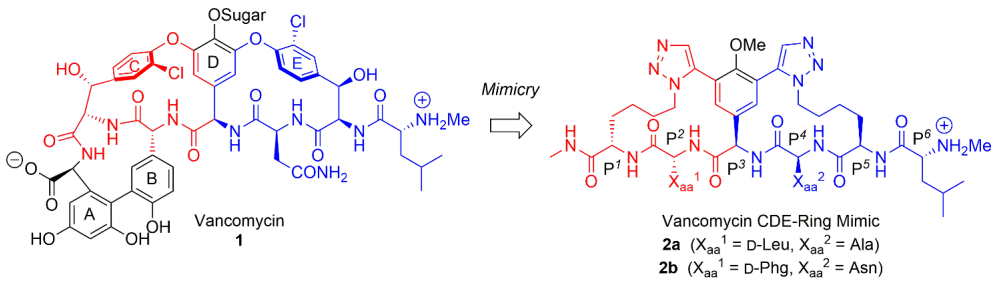
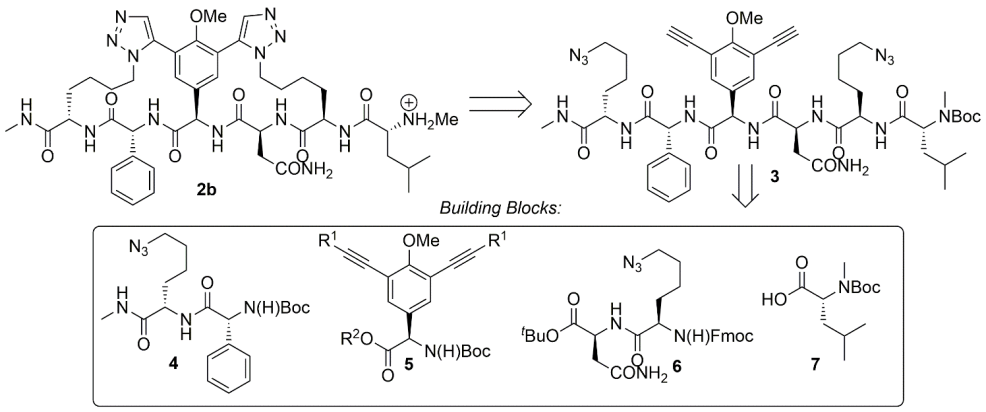


Figure 1. Design of the new 1,5-triazole-bridged bicyclic vancomycin CDE-ring mimics **2a** and **2b**.

Although this bicyclic peptide (**2a**) showed a good structural resemblance with vancomycin, it was found to bind D-Ala-D-Ala with a 100-fold lower affinity, and unfortunately did not show any antibacterial activity in comparison with vancomycin. However, since the synthesis method of the linear hexapeptide as well as the Ru(II)-catalyzed macrocyclization strategy was already optimized, it could be readily used to synthesize derivatives in which the amino acids at positions P^2 and P^4 have been changed into a D-phenylglycine and asparagine, respectively. Both amino acids mimic the natural sequence more closely.[8] Especially the amide moiety in the side chain of asparagine, which is expected to be in proximity of the D-Ala-D-Ala carboxylate to form a hydrogen bond, [8b] might increase the binding affinity. Therefore, CDE-ring mimic (**2b**) was designed and synthesized in an attempt to improve binding affinity as well as antimicrobial activity of this type of bicyclic vancomycin mimics.

2.2 Results and Discussion

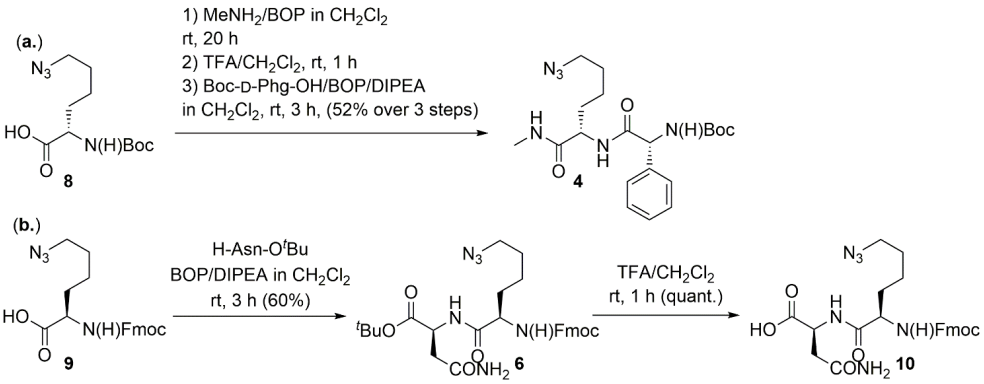
Based on the established synthesis method that was used to obtain mimic **2a**[7], the retrosynthetic analysis of bicyclic vancomycin CDE-ring mimic (**2b**) consisted of two important parts: firstly, the synthesis of the linear peptide **3**, and secondly, a macrocyclization featuring a Ru(II)-catalyzed azide-alkyne cycloadditon strategy, as shown in Scheme 1.



Scheme 1. Retrosynthetic analysis of the bicyclic vancomycin CDE-ring mimics **2b**.

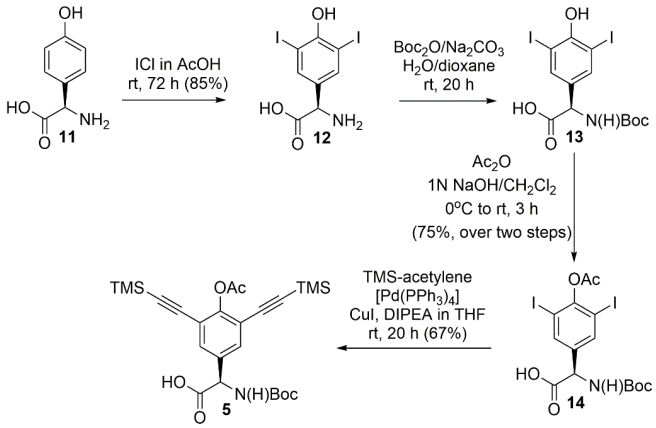
The synthesis of cyclization precursor peptide **3** involved three key stages, firstly, the preparation of the three building blocks, the C-terminal dipeptide **4**, the central phenylglycine functionalized with two alkyne moieties **5**, and the N-terminal dipeptide **6**, while leucine derivative **7** was commercially available (Scheme 1). The second stage was the assembly of the linear pentapeptide by coupling two dipeptide fragments to the central alkyne-functionalized hydroxyphenylglycine residue, followed by the incorporation of *N*-Me-D-leucine to obtain the desired linear hexapeptide. The third and final step was the click reaction featuring RuAAC which was expected to the most important step to afford the desired mimic **2b**.

The synthesis of the linear pentapeptide **3** started by assembly of the three building blocks, as shown in Scheme 2. The synthesis of the dipeptide building blocks, the C-terminal dipeptide Boc-D-Phg-Lys(N_3)-NHMe (**4**) and the N-terminal dipeptide Fmoc-D-Lys(N_3)-Asn-OH (**10**), required BOP/DIPEA-mediated peptide chemistry, while the ε-azido lysine derivatives were obtained by diazotransfer according to the procedure of Goddard-Boger and Stick.[9] Fmoc-D-Lys(N_3)-Asn-O^{t}Bu (**6**) was obtained in a yield of 60%, which was lower compared to Fmoc-D-Lys(N_3)-Ala-O^{t}Bu[7] (80%), mainly due to the low solubility of **6** probably caused by the presence of the unprotected amide of the asparagine moiety. After the removal of *tert*-butyl group by TFA, N-terminal dipeptide acid **10** was afforded quantitatively.



Scheme 2. Synthesis of the C-terminal dipeptide segment **4** and N-terminal dipeptide segment **10**.

The alkyne-functionalized hydroxyphenylglycine derivative **5** (Scheme 1) was a key intermediate in the synthesis of the bicyclic CDE-ring vancomycin mimic. The synthesis of the central alkyne-functionalized hydroxyphenylglycine (as shown in Scheme 3) started with the iodination of D-hydroxyphenylglycine in the presence of ICl dissolved in acetic acid according to a literature procedure.[10] After isolation from the reaction mixture, diiodide **12** could be used without further purification in the next step, that is protection of the amino group leading to **13**.[11] Next, the aromatic hydroxyl group was converted into the corresponding acetyl ester using acetic anhydride to give ester **14** in 75% yield over two steps.[12] Finally, protected amino acid **5** could be obtained from diiodide **14** via a Pd-catalyzed Sonogashira cross-coupling in the presence of TMS-acetylene as the free carboxylate in 67% yield.



Scheme 3. Synthesis of bisalkyne-functionalized phenylglycine derivative **5**.

Since the two dipeptide fragments as well as the central alkyne-functionalized hydroxyphenylglycine derivative were at hand, the stage was set to assemble the linear hexapeptide as shown in Scheme 4. The first step encompassed coupling of Boc-protected amino acid **5** with H-D-Phg-Lys(N_3)-NHMe. Since it is known that phenylglycine derivatives are very sensitive toward racemization under (basic) reaction conditions,[15] four different combinations of coupling reagents, BOP/DIPEA, EDCI/HOBt, DEPBT/$NaHCO_3$,[16] and DCC/HOAt[17] were investigated in this particular coupling step (Table 1). It was found that the lowest

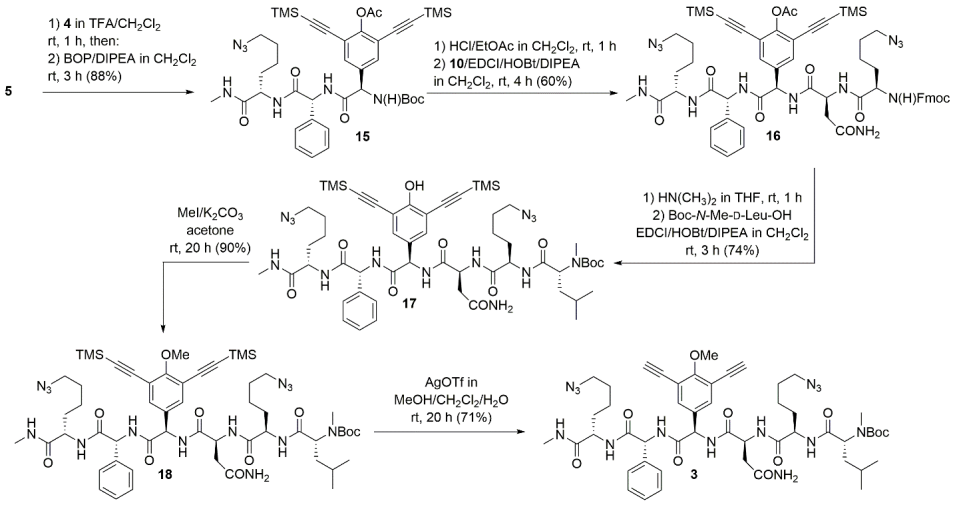
degree of racemization was induced by DCC/HOAt as coupling reagent. Unfortunately, the conversion was found to be very low while only in the presence of BOP/DIPEA a reasonable conversion and yield could be achieved. Gratifyingly, the mixture of both diastereomeric tripeptides could be separated, and the most intense spot on TLC, which was assumed to correlate with the desired diastereoisomer was isolated by column chromatography in 88% yield. The HPLC chromatogram (Figure 2) in combination with the proton NMR spectrum confirmed that a stereochemically pure tripeptide **15** was obtained.

Table 1. Diastereomeric ratios of the tripeptide using different coupling reagents.

coupling reagents	**BOP/DIPEA**	**EDCI/HOBt**	**DEPBT/$NaHCO_3$**	**DCC/HOAt**
Ratios[a] (R,R,S/S,R,S)	4:1	6:1	7:1	8:1

[a] Diastereomeric ratios were determined by NMR.

In the following steps, the Boc group of tripeptide **15** was removed by treatment with anhydrous HCl in EtOAc and Fmoc-D-Lys(N_3)-Asn-OH was coupled in the presence of EDCI/HOBt/DIPEA and pentapeptide **16** was isolated in 60% yield. Then, the Fmoc group was removed by treatment of peptide **16** with dimethylamine, which was also accompanied by the desired aminolysis of the phenolic acetate. Subsequent coupling of Boc-*N*-Me-D-Leu-OH using EDCI/HOBt as coupling reagent in CH_2Cl_2 gave hexapeptide **17** in a good yield of 74%. The phenolic OH was converted into its corresponding methyl ether by treatment with MeI/K_2CO_3 in acetone, which afforded the fully protected hexapeptide **18** in 90% yield. In first instance, the TMS protecting groups were removed by treatment with TBAF. Under these rather basic reaction conditions, it was found that some degree of epimerization of the phenylglycine residue was induced. Therefore, a more selective and mild deprotection agent, AgOTf[14] was used and bisalkyne **3** was obtained in 71% yield as a single diastereomer. The HPLC chromatogram confirmed that stereochemically pure linear hexapeptide **3** (R_t = 28.51 min) was obtained (see, Figure 3a).



Scheme 4. Synthesis of the RuAAC cyclization precursor hexapeptide **3**.

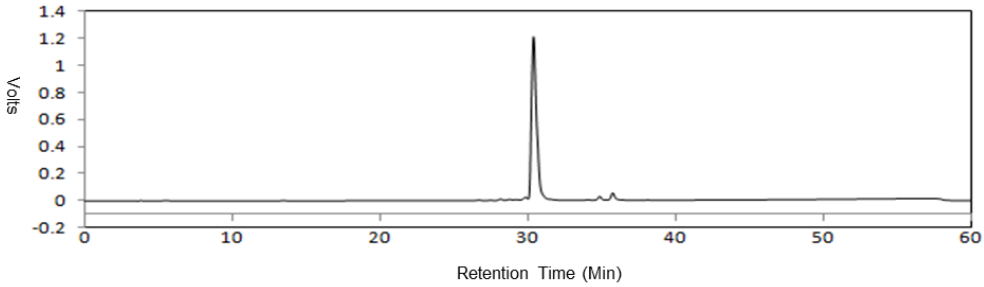
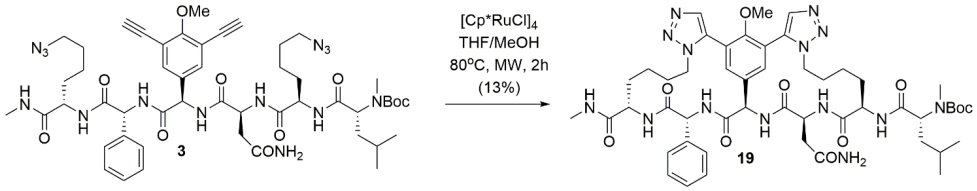


Figure 2. HPLC trace of tripeptide **15** after purification by column chromatography (R_t = 30.28 min).

With the linear hexapeptide in hand, the next and critical step was to develop a reliable and reproducible protocol for the RuAAC macrocyclization using $[Cp^*RuCl]_4$[6,7,18] as the catalyst (Scheme 5).



Scheme 5. RuAAC macrocyclization of the linear hexapeptide **3**.

The previously used reaction conditions: THF/MeOH (19:1 v/v) as the solvent system with a catalyst loading of 30 mol% at 50 ºC for 24 h,[7] did not result in product formation as judged by HPLC analysis, although all starting material had disappeared. Apparently, RuAAC of **3** required further optimization and the results are shown in Table 2.

Entries 1 to 5 dealt with finding the optimal solvent. More importantly was the use of a lower amount of catalyst (5 mol%) and the incorporation of microwave irradiation for an efficient heating of the reaction mixture (entry 6). At these reaction conditions: running the reaction in THF/MeOH (4:1 v/v) with a catalyst loading of 5 mol% at 50 ºC for 1 h under microwave irradiation resulted in product formation since three peaks could be identified by HPLC and LCMS as shown in Figure 3b and 4, respectively. These three new peaks had the same molecular mass as the starting material **3**, which indicated that macrocyclization had occurred while two mono-triazoles had appeared as the monocyclic intermediates. By increasing the reaction temperature as well as reaction time (entries 7 and 8), an increasing amount of the monocyclic intermediates as well as the bicyclic hexapeptide could be identified by HPLC, while the precursor peptide **3** was almost absent (shown in Figure 3c). Increasing the amount of catalyst further (entries 9 and 10) resulted in a decrease of both monocyclic intermediates and an increase of bicyclic hexapeptide, as shown Figure 3d. Finally, at a catalyst loading of 15 mol% and running the reaction in THF/MeOH (4:1 v/v) for 2 h at 80 ºC under microwave irradiation (entry 10), the bicyclization was almost complete and the desired bicyclic compound **19** was isolated by preparative HPLC in a disappointingly low yield of only 13%.

Table 2. Optimization of the RuAAC macrocyclization of precursor peptide **3**.

entry	Catalyst (mol%)	Solvent	Temperature (°C)	Time (h)	Mono1: Mono 2: Bi [a]	Conversion (%)[b]
1	30	THF/MeOH (19:1 v/v)	50	24	0:0:0	100
2	30	THF/MeOH (4:1 v/v)	50	24	0:0:0	100
3	30	THF/MeOH (2:1 v/v)	50	24	0:0:0	100
4	30	toluene/MeOH (19 :1 v/v)	50	24	0:0:0	100
5	30	toluene/MeOH (4 :1 v/v)	50	24	0:0:0	100
6	5	THF/MeOH (4:1 v/v)	50	1 (MW)	1.23:1.53:1	46
7	5	THF/MeOH (4:1 v/v)	80	1 (MW)	1.22:1.51:1	90
8	5	THF/MeOH (4:1 v/v)	80	2 (MW)	1.21:1.48:1	90
9	10	THF/MeOH (4:1 v/v)	80	2 (MW)	0.48:0.59:1	88
10	15	THF/MeOH (4:1 v/v)	80	2 (MW)	0.09:0.11:1	84

[a] Ratio of the monocyclic 1, monocyclic 2 and bicyclic products as determined by HPLC. [b] Conversion was determined by HPLC analysis of the residual starting material in the reaction mixture.

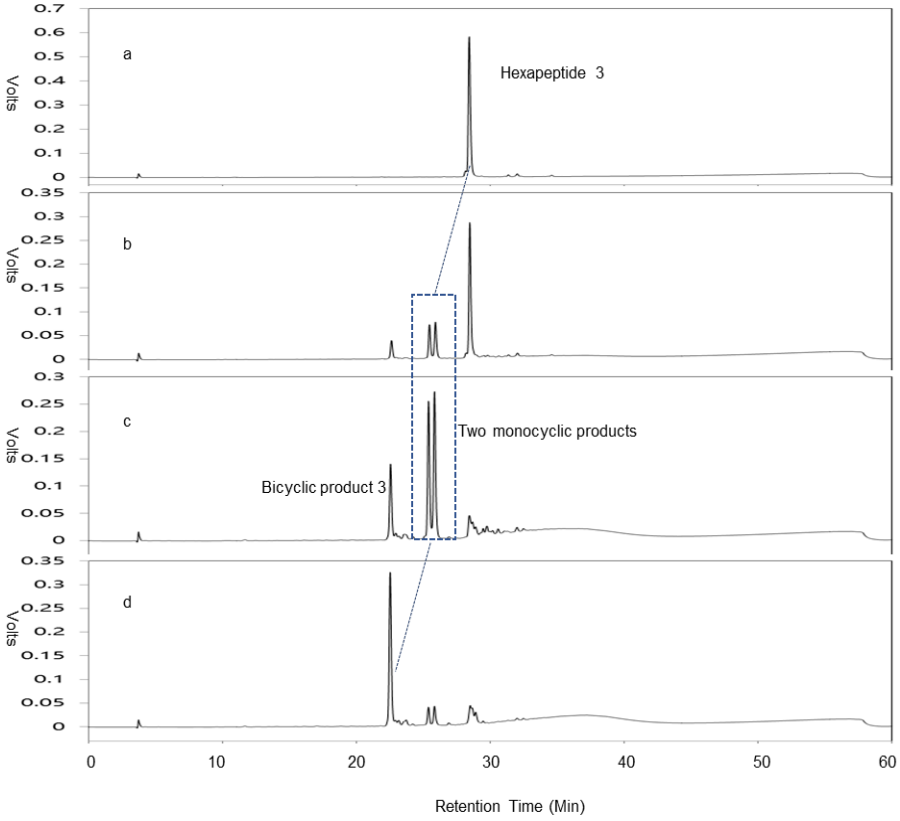


Figure 3. HPLC analysis (100% buffer A to 100% buffer B in 50 min) of the RuAAC macrocyclization of hexapeptide **3**: (a) hexapeptide **3** (b) RuAAC macrocyclization of hexapeptide **3** (entry 6, 5 mol% $[Cp^*RuCl]_4$, 50 ºC, 1 h (MW)) (c) RuAAC macrocyclization of hexapeptide **3** (entry 8, 5 mol% $[Cp^*RuCl]_4$, 80 ºC, 2 h (MW)) (d) RuAAC macrocyclization of hexapeptide **3** (entry 10, 15 mol% $[Cp^*RuCl]_4$, 80 ºC, 2 h (MW).

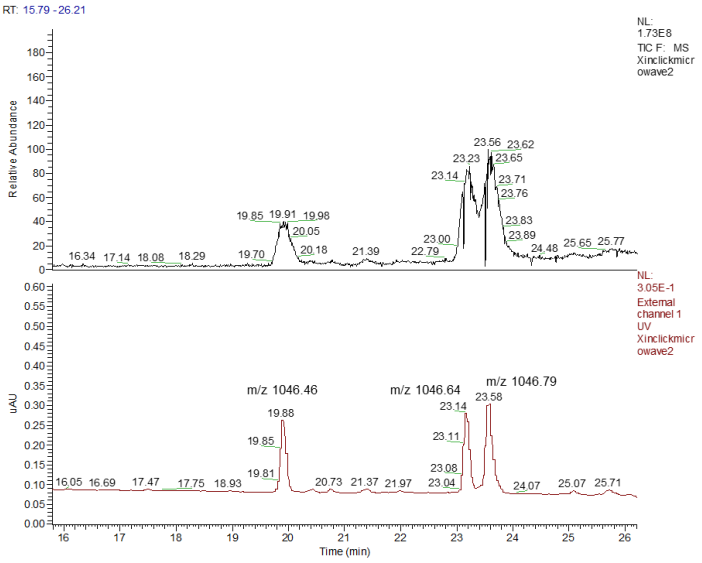
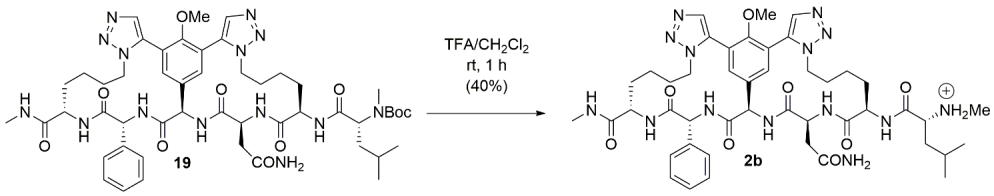


Figure 4. Detail of the LC-MS trace (100% buffer A to 100% buffer B in 40 min) of the RuAAC macrocyclization of hexapeptide **3** with catalyst loading of 5 mol% $[Cp^*RuCl]_4$ at 50 ºC, running for 1 h under microwave irradiation in THF/MeOH 4:1 v/v (entry 6). Top: total ion current, Bottom: HPLC trace: monocyclic intermediates: 23.58 min/1046.79 amu and 23.14 min/1046.64 amu, bicyclic compound: 19.88 min/1046.46 amu.

Further characterization by NMR to identify ^{1}H-^{13}C coupling pattern of both triazole moieties was hampered by the presence of the Boc functionality, since the two conformers of the urethane complicated the analysis. After removal of the Boc functionality by treatment with TFA (Scheme 6), the desired unprotected compound **2b** was obtained in 40% yield after purification by preparative HPLC, as shown in Figure 5. This unprotected bicyclic peptide was characterized by 2D-NMR analysis, and both unique C-H moieties of the triazoles constraint could be assigned in the HSQC spectrum δ_H 7.91ppm/δ_c 133.1 ppm, in agreement with the reported data in the literature.[19]

Isothermal microcalorimetry (ITC)[20] was used to determine the binding affinity of the newly synthesized CDE-ring mimic **2b** toward Ac-Lys(Ac)-D-Ala-D-Ala-OH as a model compound of the natural ligand of vancomycin.[7] As shown in Table 3, the binding affinity of bicyclic peptide **2b** was almost the same as previously

found with compound **2a**, which was still at least 100-fold lower than vancomycin. This clearly implied that replacement of amino acids at position P^2 and P^4 did not lead to significant improvement of binding affinity. To investigate the biological activity of bicyclic mimic **2b**, minimum inhibitory concentration (MIC) was determined using an *in vitro* assay against vancomycin sensitive bacterium *Staphylococcus aureus* (ATCC 49320). Not unexpectedly, inhibition of bacterial growth was absent even at high concentration (300 μg mL^{-1}). These low binding affinities and lack of any antimicrobial activity of mimics like **2** might indicate that the framework of both bicyclic CDE-ring mimics is still too flexible to form a shape persistent cavity-like binding pocket as present in vancomycin. Based on these results it was assumed that the three dimensional shape of the peptide in which the backbone in combination with the side-chain to side-chain connectivity pattern is largely responsible for achieving a high binding affinity rather than the presence of an individual amino acids.



Scheme 6. Deprotection of bicyclic hexapeptide **19**.

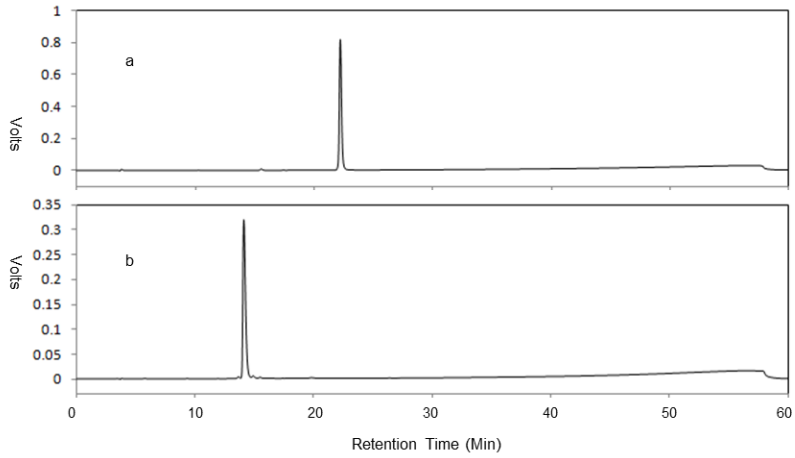


Figure 5. HPLC analysis of protected bicyclic peptide **19** (a) and unprotected bicyclic peptide **2b** (b).

Table 3. The binding affinity as measured using ITC[a]

Compound	Ligand	Ka(M^{-1})
VM	Ac-Lys(Ac)-D-Ala-D-Ala-OH	$(2.97\pm0.17) \times 10^5$
VM	Ac-Lys(Ac)-D-Ala-D-Lac-OH	$(2.07\pm0.03) \times 10^3$
2a	Ac-Lys(Ac)-D-Ala-D-Ala-OH	$(1.91\pm0.02) \times 10^3$
2a	Ac-Lys(Ac)-D-Ala-D-lac-OH	$(1.31\pm0.04) \times 10^3$
2b	Ac-Lys(Ac)-D-Ala-D-Ala-OH	$(1.55\pm0.09) \times 10^3$
2b	Ac-Lys(Ac)-D-Ala-D-Lac-OH	$(1.25\pm0.03) \times 10^3$

[a] Measured in a Na-citrate/citric acid buffer (0.02 M, pH 5.1).

2.3 Conclusion and Outlook

To conclude, a newly designed 1,5-triazole-bridged bicyclic vancomycin CDE-ring mimic using RuAAC macrocyclization was designed and synthesized successfully. The adapted synthesis approach required optimization to cope with the racemization-prone phenylglycine residue as well as with the unprotected amide moiety of asparagine. Unfortunately, bicyclic mimic **2b** did not lead to better binding affinity toward Ac-Lys(Ac)-D-Ala-D-Ala-OH and also no antibacterial affinity could be observed. Although this solution phase synthesis route for linear peptides is flexible and robust, it is rather inefficient and time-consuming which limits the access toward of vancomycin CDE-ring mimics. Therefore, a more efficient alternative such as solid phase peptide synthesis should be considered for the synthesis of a diverse set of linear peptides that mimic the sequence of vancomycin as cyclization precursor with the ultimate goal to improve binding affinity of such vancomycin-inspired peptidomimics.

2.4 Experimental Section

2.4.1 Chemicals and General Experimental Procedures

Chemicals were used as obtained from commercial sources without further purification unless stated otherwise. The solvents were obtained as peptide synthesis grade and stored on molecular sieves (4 Å). Column chromatography was performed using SiliFlash P60 silica gel (particle size 40-63 μm). TLC was performed on precoated silica gel 60F254 glass plates and compound spots were visualized by UV-quenching, ninhydrin, or Cl_2/TDM. Melting points were measured according to Dr. Tottoli and were uncorrected. Optical rotations were measured using a 10 cm cell with a Na 589 nm filter at the specific concentration (in g/100 mL) as indicated. ^{1}H NMR spectra were recorded either on a 400 MHz or a 500 MHz spectrometer in $CDCl_3$, or DMSO-d_6 as solvent. Chemical shifts are reported in delta (δ) units, in parts per million (ppm) relative to TMS (0.00 ppm). Coupling constants (*J*) are reported in Hertz (Hz). ^{13}C NMR analysis was acquired on a 100 MHz or a 125 MHz spectrometer in $CDCl_3$, or DMSO-d_6 as solvent. Chemical shifts are reported in delta (δ) units, in parts per million (ppm) relative to

the solvent residual signal, $CDCl_3$ (77.0 ppm) or DMSO-d_6 (39.0 ppm). Analytical HPLC was performed on an automated HPLC system equipped with a UV/Vis detector operating at 220/254 nm and an evaporative light scattering detector using a C18 column (pore size: 100 Å, particle size: 5 µm; 250 × 4.6 mm) at a flow rate of 1 mL/min using a linear gradient of 100% buffer A (0.1% TFA in CH_3CN/H_2O 5:95 v/v) to 100% buffer B (0.1% TFA in CH_3CN/H_2O 95:5 v/v) in 60 min. Preparative RP-HPLC was performed on an automated preparative HPLC system equipped with a UV/Vis detector operating at 214 nm using a C18 column (pore size: 100 Å, particle size: 10 µm; 250 × 22 mm) at a flow rate of 2.0 mL/min using a linear gradient of 100% buffer A (0.1% TFA in CH_3CN/H_2O 5:95 v/v) to 100% buffer B (0.1% TFA in CH_3CN/H_2O 95:5 v/v) in 60 min. LC-MS(MS) was measured using a C18 column (pore size: 100 Å, particle size: 5 µm; 250 × 4.6 mm) at a flow rate of 1 mL/min using a linear gradient of of 100% buffer A (0.1% TFA in CH_3CN/H_2O 5:95 v/v) to 100% buffer B (0.1% TFA in CH_3CN/H_2O 95:5 v/v) in 60 min. ESI-MS was performed on a bench-top electrospray ionization mass spectrometer. High-resolution mass spectrometry (HRMS) analysis was performed using an ESI-TOF LC/MS instrument.

2.4.2 Syntheses and Compound Analyses

2.4.2.1 Boc-D-Phg-Lys(N_3)-NHMe 4: Azide **8** was dissolved in CH_2Cl_2 (150 mL) in the presence of BOP (9.74 g, 22 mmol) while a solution of $MeNH_2$ (2 M in THF, 30 mL, 60 mmol) was added dropwise during 20 min at 0 °C. The mixture was allowed to warm to room temperature while stirring was continued for 20 h. Then, the solvents were removed by evaporation and the residue was redissolved in EtOAc (300 mL). The resulting solution was washed with 1 N $KHSO_4$ (200 mL, twice), saturated $NaHCO_3$ (200 mL, twice) and brine (100 mL, once), and dried over anhydrous Na_2SO_4. After filtration and removal of the solvent, the residue was purified by column chromatography (EtOAc/hexane, 2:3 to 2:1 v/v). Boc-Lys(N_3)-NHMe was obtained as white solid (4.03 g) in 71% yield. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 6.14 (s, 1H), 4.99 (s, 1H), 4.04 – 4.00 (m, 1H), 3.25 (td, *J* = 6.8 and 1.3 Hz, 2H), 2.80 (d, *J* = 4.8 Hz, 3H), 1.81 – 1.83 (m, 1H), 1.55 – 1.66 (m, 4H), 1.45 – 1.33 (m, 10H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 172.61, 155.77, 79.98, 54.19, 51.14, 32.32, 28.49, 28.27, 26.10, 22.79. In the next step,

Boc-Lys(N_3)-NHMe (2.04 g, 7.15 mmol) was dissolved in CH_2Cl_2 (50 mL) and to this solution TFA (50 mL) was added. The obtained reaction mixture was stirred for 1 h, after which the volatiles were removed by evaporation and any residual TFA was removed by coevaporation with CH_2Cl_2 (3 × 30 mL). After drying for 1 h at high vacuum, the trifluoroacetate was dissolved in CH_2Cl_2 (100 mL) and to this solution Boc-D-Phg-OH (2.16 g, 8.6 mmol), BOP (3.80 g, 8.6 mmol) followed by DIPEA (3.6 mL, 23.6 mmol) were added. The obtained reaction mixture was stirred for 3 h at room temperature. Then, the solvent was removed by evaporation and the residue was redissolved in EtOAc (150 mL). The resulting solution was successively washed with 1 N $KHSO_4$ (100 mL, twice), saturated $NaHCO_3$ (100 mL, twice) and brine (100 mL, once), dried (Na_2SO_4), filtered and evaporated to dryness. The obtained residue was purified by column chromatography (CH_2Cl_2/MeOH, 98:2 v/v) and dipeptide Boc-D-Phg-Lys(N_3)-NHMe **4** was obtained as a white solid (2.20 g, 74%). R_f 0.58 (CH_2Cl_2/MeOH, 9:1 v/v); $[\alpha]_D^{20}$ = –65.8 (*c* = 0.5 CH_2Cl_2); ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.29 – 7.36 (m, 5H), 6.45 – 6.50 (m, 2H), 5.64 – 5.65 (d, *J* = 6.2 Hz, 1H), 5.09 – 5.11 (d, *J* = 6.2 Hz, 1H), 4.35 – 4.40 (td, *J* = 8.3 and 5.3 Hz, 1H), 3.07 – 3.14 (td, *J* = 6.8 and 3.0 Hz, 2H), 2.75 – 2.76 (d, *J* = 4.8 Hz, 3H), 1.81 (s, 1H), 1.41 – 1.57 (m, 12H), 1.15 – 1.11 (m, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 171.71, 170.74, 155.26, 137.86, 128.91, 128.37, 127.03, 80.27, 58.72, 52.91, 50.99, 31.76, 28.25, 28.22, 26.11, 22.41; HRMS, *m*/*z* calcd for $C_{20}H_{31}N_6O_4$ $[M + H]^+$ 419.2362, found 419.2418.

2.4.2.2 Fmoc-D-Lys(N_3)-Asn-O^{t}Bu 6: Fmoc-D-Lys(N_3)-OH (**9**) (2.37g, 6.00 mmol) was dissolved in CH_2Cl_2 (50 mL) and HCl·H-Asn-O^{t}Bu (1.62 g, 7.2 mmol), BOP (3.18 g, 7.2 mmol) followed by DIPEA (2.07 mL, 12 mmol) were added. The reaction mixture was stirred for 3 h at room temperature and subsequently evaporated to dryness after which the residue was redissolved in EtOAc (100 mL). The resulting solution was successively washed with 1 N $KHSO_4$ (100 mL, twice), saturated $NaHCO_3$ (100 mL, twice) followed by brine (100 mL, once) and dried (Na_2SO_4). After filtration the solvent was removed by evaporation. The residue was purified by column chromatography (CH_2Cl_2/MeOH, 96:4 v/v) to afford Fmoc-D-Lys(N_3)-Asn-O^{t}Bu as a white solid (2.03 g, 60%). R_f 0.57 (CH_2Cl_2/MeOH, 9:1 v/v); $[\alpha]_D^{20}$ = +22.0 (*c* = 0.5 CH_2Cl_2/MeOH, 1:1 v/v); ^{1}H NMR (400 MHz,

$CDCl_3$): δ = 7.73 – 7.75 (m, 2H), 7.57 (d, *J* = 7.5 Hz, 2H), 7.35 – 7.40 (m, 2H), 7.27 – 7.31 (m, 2H), 5.63 (s, 1H), 5.47 (d, *J* = 8.3 Hz, 1H), 4.64 (dt, *J* = 9.1 and 4.6 Hz, 1H), 4.36 (q, *J* = 10.3 and 8.8 Hz, 2H), 4.21 (dt, *J* = 13.9 and 6.8 Hz, 1H), 3.24 (t, *J* = 6.7 Hz, 2H), 2.80 (dd, *J* = 16.0 and 4.9 Hz, 2H), 1.83 – 1.88 (m, 1H), 1.52-1.57 (m, 4H), 1.36-1.43 (m, 10H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 171.93, 171.27, 169.35, 155.96, 143.84, 143.68, 141.25, 127.69, 127.05, 125.07, 119.95, 82.64, 67.05, 54.64, 51.10, 49.56, 47.11, 36.94, 32.40, 28.42, 27.84, 22.44; HRMS, *m*/*z* calcd for $C_{29}H_{37}N_6O_6$ $[M + H]^+$ 565.2730, found 565.2794.

2.4.2.3 (*R*)-*N*-α-Boc-(4-acetoxy-3,5-diiodo)phenylglycine 14: To a solution of D-4-hydroxyphenylglycine (**11**) (10.5 g, 63.0 mmol) in AcOH (90 mL), ICl (22.5 g, 138.6 mmol) in AcOH (5.0 mL) was added dropwise during 10 min under argon. After stirring for 72 h at room temperature, the reaction mixture was poured into ice water (1 L). The precipitated crystals were filtered off, washed with EtOH (100 mL twice) to provide 3,5-diiodo-D-4-hydroxyphenylglycine (17.4 g, 66%) as light brown crystals. Without further purification, it was used in the next step. A solution of di-tert-butyldicarbonate (2.66 g, 12.2 mmol) in 1,4-dioxane (20 mL) was added to a solution of 3,5-diiodo-D-4-hydroxyphenylglycine (4.2 g, 10 mmol) and Na_2CO_3 (1.32 g, 12.5 mmol) in H_2O (20 mL) at room temperature. After the resulting solution was stirred overnight, the reaction mixture was concentrated under reduced pressure. EtOAc (50 mL) was added to the residue and the resulting mixture was extracted with H_2O (50 mL, three times). The aqueous layer was washed with EtOAc (50 mL, twice), acidified to pH = 3 by the addition of 1N citric acid and extracted with EtOAc (50 mL, three times). The organic layer was washed with brine (100 mL, once) and dried over anhydrous Na_2SO_4. After filtration and removal of the solvent, (*R*)-N-α-Boc-(4-hydroxy-3,5-diiodo) phenylglycine was obtained as a brownish oil and was used in the next step without purification. (*R*)-N-α-Boc-(4-hydroxy-3,5-diiodo)phenylglycine was dissolved in ice-cold aq.1N NaOH/CH_2Cl_2 (80 mL, 1:1 v/v) and to this mixture, Ac_2O (4.36 mL, 46.2 mmol) was added dropwise at 0 °C. Then, the reaction mixture was stirred at room temperature for 3 h. Subsequently, the aqueous solution was acidified with $KHSO_4$ to pH 2-3 and extracted with CH_2Cl_2 (60 mL, twice). The organic layers were combined and dried over anhydrous Na_2SO_4. After filtration and removal of the solvent, the residue was purified by column

chromatography (CH_2Cl_2/MeOH, 98:2 v/v with 0.1% HOAc). (*R*)-N-α-Boc-(4-acetoxy-3,5-diiodo)phenylglycine **14** was obtained as a light yellow solid (4.21 g, 75% over two steps). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.02 (s, 1H), 7.83 (d, *J* = 14.2 Hz, 4H), 5.00 (d, *J* = 4.6 Hz, 1H), 2.38 (s, 3H), 1.25 (s, 9H);^{13}C NMR (100 MHz, $CDCl_3$): δ = 172.19, 167.13, 156.78, 151.26, 139.18, 138.07, 90.22, 82.87, 57.05, 28.29, 28.00, 21.32.

2.4.2.4 *N*-α-Boc-4-acetoxy-3,5-bis((trimethylsilyl)ethynyl)-D-phenylglycine 5: Compound **14** (1.12 g, 2.0 mmol), [$Pd(PPh_3)_4$] (231 mg, 0.2 mmol) and CuI (114 mg, 0.6 mmol) were placed in a flask sealed with a rubber septum. The flask was evacuated and refilled with dry N_2 with was repeated three times. Then, THF (20 mL, which was purged with dry N_2 for 1 h prior to use) was added to the flask via a syringe. The resulting solution was degassed again using three freeze-pump-thaw cycles. Subsequently, DIPEA (0.69 mL, 4.0 mmol) and TMS-acetylene (1.39 mL, 10 mmol) were added to the mixture via a syringe and the obtained reaction mixture was stirred for 20 h at room temperature under a continuous flow of N_2. The resulting suspension was filtered through a path of Celite and the filtrate was evaporated to dryness. The obtained residue was redissolved in EtOAc (50 mL) and the solution was successively washed with 1 N $KHSO_4$ (50 mL, twice), followed by saturated $NaHCO_3$ (50 mL, twice) and brine (50 mL, once). Finally, the organic layer was dried (Na_2SO_4), filtered and evaporated to dryness. The residue was purified by column chromatography (EtOAc/hexane, 1:3 v/v with 0.1% HOAc) and TMS-protected bis-alkyne **5** was obtained as a yellowish solid (740 mg, 67%). R_f 0.50 (EtOAc/hexane, 1:1 v/v with 0.1% HOAc); $[\alpha]_D^{20}$ = –44.3 (*c* = 0.5 CH_2Cl_2); ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.84 (broad s, 1H), 7.48 (d, *J* = 13.8 Hz, 2H), 5.02 (s, 1H), 2.30 (s, 3H), 1.29 (s, 9H), 0.21 (s, 18H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 172.40, 167.44, 156.81, 152.78, 135.78, 131.72, 118.20, 100.24, 98.58, 82.52, 57.88, 28.25, 27.98, 20.35, 0.22; HRMS, *m*/*z* calcd for $C_{25}H_{35}NO_6Si_2Na$ [*M* + Na]$^+$ 524.1901, found 524.1884.

2.4.2.5 *N*-α-Boc-4-acetoxy-3,5-bis((trimethylsilyl)ethynyl)-D-phenylglycyl-D-Phg-Lys(N_3)-NHMe 15: The dipeptide Boc-D-Phg-Lys(N_3)-NHMe **4** (703 mg, 1.68 mmol) was dissolved in CH_2Cl_2 (15 mL) and to this solution TFA (15 mL) was added. The reaction mixture was stirred for 1 h at room temperature after

which the volatiles were removed and any residual TFA was removed by coevaporation with CH_2Cl_2 (20 mL, twice). After drying for 1 h at high vacuum, the trifluoroacetate was dissolved in CH_2Cl_2 (50 mL) and to this solution bis-alkyne **5** (600 mg, 1.2 mmol), BOP (743 mg, 1.68 mmol) followed by DIPEA (1.02 mL, 4.24 mmol) were added. The obtained reaction mixture was stirred for 3 h at room temperature and the solvent was removed *in vacuo* and the residue was redissolved in EtOAc (100 mL). This solution was successively washed with 1 N $KHSO_4$ (50 mL, twice), saturated $NaHCO_3$ (50 mL, twice) and brine (50 mL, once), dried (Na_2SO_4) filtered and evaporated to dryness. The residue was purified by column chromatography (CH_2Cl_2/MeOH, 98:2 v/v). *N*-α-Boc-4-acetoxy-3,5-bis ((trimethylsilyl)ethynyl)-D-phenylglycyl-D-Phg-Lys(N_3)-NHMe **15** was obtained as a yellowish solid (840 mg, 88%). R_f 0.76 (CH_2Cl_2/MeOH, 9:1 v/v); $[\alpha]_D^{20}$ = – 48.3 (*c* = 0.5 CH_2Cl_2); ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.27 (s, 1H), 7.45 – 7.44 (m, 2H), 7.41 – 7.31 (m, 5H), 6.78 (s, 2H), 5.96 (s, 1H), 5.48 (d, *J* = 6.8 Hz, 1H), 5.35 (s, 1H), 4.49 (s, 1H), 3.01 – 2.92 (m, 2H), 2.64 (d, *J* = 4.6 Hz, 3H), 2.30 (d, *J* = 1.6 Hz, 3H), 1.72 (s, 2H), 1.66 – 1.49 (m,1H), 1.34 (s, 9H), 1.34 – 1.19 (m, 2H), 0.89 – 1.04 (m, 1H), 0.21 (d, *J* = 1.6 Hz, 18H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 171.23, 169.50, 168.57, 167.54, 155.04, 152.93, 138.21, 136.29, 131.82, 128.99, 128.52, 127.06, 118.36, 100.33, 98.59, 80.41, 77.19, 57.62, 56.86, 52.77, 50.90, 32.28, 30.89, 28.43, 28.24, 28.16, 26.01, 22.14, 20.36, 0.21; HRMS, *m*/*z* calcd for $C_{40}H_{55}N_7O_7Si_2$ $[M + H]^+$ 802.3735, found 802.3799, *m*/*z* calcd for $C_{40}H_{55}N_7O_7Si_2Na$ $[M + Na]^+$ 824.3599, found 824.3609.

2.4.2.6 Fmoc-D-Lys(N_3)-Asn-(4-acetoxy-3,5-bis((trimethylsilyl)ethynyl)-D-phenylglycyl)-D-Phg-Lys(N_3)- NHMe 16: *N*-α-Boc-4-acetoxy-3,5-bis((trimethyl -silyl)ethynyl)-D-phenylglycyl-D-Phg-Lys(N_3)-NHMe **15** (540 mg, 0.67mmol) was dissolved in CH_2Cl_2 (10 mL) and to this solution 3 M HCl in EtOAc (15 mL) was added. The obtained reaction mixture was stirred for 1 h at room temperature and the volatiles were subsequently removed by evaporation and the residue was further dried for 1 h at high vacuum. The hydrochloride was suspended in CH_2Cl_2 (300 mL) and Fmoc-D-Lys(N_3)-Asn-OH **10** (which was obtained in quantitative yield by treatment of dipeptide ester **6** (497 mg, 0.88 mmol) with TFA (10 mL)), EDCI (168 mg, 0.88 mmol), HOBt (119 mg, 0.88 mmol) followed by DIPEA (482 μL, 2.01 mmol) were added and the obtained reaction mixture was stirred at room

temperature for 16 h. Subsequently, the solvent was removed by evaporation and the residue was redissolved in EtOAc (100 mL). The resulting solution was successively washed with 1 N $KHSO_4$ (50 mL, twice), saturated $NaHCO_3$ (50 mL, twice) followed by brine (50 mL, once), dried (Na_2SO_4). After filtration and removal of the solvent, the residue was purified by column chromatography (CH_2Cl_2/MeOH, 97:3 v/v). Pentapeptide **16** was obtained as a white solid (480 mg, 60%). R_f 0.49 (CH_2Cl_2/MeOH, 9:1 v/v); $[\alpha]_D^{20} = -65.8$ (c = 0.5 CH_2Cl_2); ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.87 (s,1H), 7.72 (d, J = 7.5 Hz, 2H), 7.55 (d, J = 7.8 Hz, 1H), 7.49 – 7.16 (m, 13H), 6.53 (s, 1H), 6.36 (s, 1H), 6.09 (s, 1H), 5.56 (d, J = 7.5 Hz, 1H), 5.38 (d, J = 5.6 Hz, 1H), 4.90 (s, 1H), 4.78 – 4.71 (m, 1H), 4.41 – 4.26 (m, 2H), 4.08 – 3.92 (m, 2H), 3.54 (d, J = 6.6 Hz, 1H), 3.28 – 3.10 (m, 5H), 2.71 (d, J = 4.0 Hz, 3H), 2.61 – 2.66 (m, 2H), 2.25 (s, 3H), 1.91 – 1.75 (m, 2H), 1.64 – 1.51 (m, 6H), 1.53 – 1.45 (m, 3H), 1.30 – 1.15 (m, 2H), 0.19 (s, 18H); ^{13}C NMR (100 MHz, DMSO-d_6): δ = 172.16, 171.79, 170.88, 169.57, 168.76, 167.82, 162.72, 156.31, 144.33, 144.11, 141.13, 138.72, 137.42, 132.36, 128.64, 128.01, 127.59, 127.46, 125.67, 120.48, 117.61, 100.81, 99.13, 65.96, 55.32, 50.91, 50.81, 47.12, 40.26, 36.21, 31.75, 31.20, 28.24, 27.94, 25.91, 22.61, 20.47, 0.05; HRMS, m/z calcd for $C_{60}H_{73}N_{13}O_{10}Si_2$ $[M + H]^+$ 1192.5175, found 1192.5238.

2.4.2.7 Boc-*N*-α-Me-D-Leu-D-Lys(N_3)-Asn-(3,5-bis((trimethylsilyl)ethynyl)-D-4-hydroxyphenylglycyl)- D-Phg-Lys(N_3)-NHMe 17: Pentapeptide **16** (480 mg, 0.403 mmol) was dissolved in THF (10 mL) and to this solution $(CH_3)_2NH$ (2M in THF; 4.0 mL, 8.0 mmol) was added and the resulting reaction mixture was stirred for 1 h at room temperature. The solvents were removed under reduced pressure and the free peptide amine was further dried for 1 h at high vacuum. Subsequently, the peptide amine was dissolved in CH_2Cl_2 (20 mL), and to this solution Boc-*N*-Me-D-Leu-OH (109 mg, 0.443 mmol), EDCI (85 mg, 0.443 mmol), HOBt (60 mg, 0.443 mmol) followed by DIPEA (141 μL, 0.806 mmol) were added. The obtained reaction mixture was stirred for 3 h after which the solvent was removed by evaporation and the residue was redissolved in EtOAc (100 mL). The resulting solution was successively washed with 1 N $KHSO_4$ (50 mL, twice), saturated $NaHCO_3$ (50 mL, twice) followed by brine (50 mL, once) and dried (Na_2SO_4). After filtration and removal of the solvent, the residue was purified by column chromatography (CH_2Cl_2/MeOH, 96:4 v/v). The linear hexapeptide, **17** was

obtained as a white solid (350 mg) in 75% yield. R_f 0.48 (CH_2Cl_2/MeOH, 9:1 v/v); $[\alpha]_D^{20}$ = –66.3 (*c* = 0.5 CH_2Cl_2); ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.09 (s, 1H), 7.57 (s, 1H), 7.51 (d, *J* = 7.8 Hz, 2H), 7.43 – 7.22 (m, 5H), 6.74 (d, *J* = 5.2 Hz, 1H), 6.49 (s, 1H), 6.23 (s, 1H), 5.97 (s, 1H), 5.56 (d, *J* = 7.3 Hz, 1H), 5.21 (d, *J* = 4.9 Hz, 1H), 4.78 (s, 1H), 4.36 – 4.28 (m, 2H), 3.72 (s, 1H), 3.23 (t, *J* = 6.8 Hz, 4H), 2.72 (d, *J* = 4.7 Hz, 3H), 2.48 (s, 3H), 1.84 – 1.81(m, 2H), 1.68 (s, 4H), 1.55 – 1.51 (m, 5H), 1.42 (s, 9H), 1.39 – 1.33 (m, 3H), 1.04 – 1.14 (m, 1H), 0.80 (d, *J* = 6.7 Hz, 3H), 0.71 (d, *J* = 6.5 Hz, 3H), 0.23 (s, 18H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 174.20, 172.77, 172.35, 170.70, 169.55, 157.91, 132.59, 128.67, 128.28, 128.03, 110.38, 101.90, 98.44, 57.67, 53.45, 51.10, 50.96, 36.38, 35.13, 30.34, 28.37, 28.29, 26.16, 23.11, 22.84, 21.52, 0.16; HRMS, *m*/*z* calcd for $C_{60}H_{73}N_{13}O_{10}Si_2$ [*M* + H]$^+$ 1192.5175, found 1192.5238.

2.4.2.8 Boc-*N*-α-Me-D-Leu-D-Lys(N_3)-Asn-(3,5-bis((trimethylsilyl)ethynyl)-D-4-methoxyphenylglycyl)- D-Phg-Lys(N_3)-NHMe 18: Hexapeptide **17** (350 mg, 0.303 mmol) was dissolved in acetone (20 mL) and MeI (42 μL, 0.606 mmol) followed by K_2CO_3 (168 mg, 1.21 mmol) were added. The obtained reaction mixture was stirred for 20 h at room temperature after which the reaction went to completion as judged by ESI-MS. The solvent was removed by evaporation and the residue was redissolved in EtOAc (100 mL). The resulting solution was successively washed with 1 N $KHSO_4$ (50 mL, once), saturated $NaHCO_3$ (50 mL, once) followed by brine (50 mL, once) and dried (Na_2SO_4). After filtration and removal of the solvent, the residue was purified by column chromatography (CH_2Cl_2/MeOH, 97:3 to 96:4 v/v). The fully protected hexapeptide Boc-*N*-α-Me-D-Leu-D-Lys(N_3)-Asn-(3,5-bis((trimethylsilyl)ethynyl)-D-4-methoxyphenylglycyl)-D-Phg-Lys(N_3)-NHMe (**18**) was obtained as a white solid (320 mg, 90%). R_f 0.48 (CH_2Cl_2/MeOH, 9:1 v/v); $[\alpha]_D^{20}$ = –33.8 (*c* = 0.5 CH_2Cl_2); ^{1}H NMR (400 MHz, DMSO-d_6): δ = 8.92 (d, *J* = 7.4 Hz, 1H), 8.27 (d, *J* = 8.5 Hz, 1H), 8.21 – 8.15 (m, 1H), 7.79 (q, *J* = 5.2 and 4.7 Hz, 1H), 7.69 (d, *J* = 7.7 Hz, 1H), 7.49 – 7.39 (m, 3H), 7.32 – 7.22 (m, 4H), 6.80 (s, 1H), 5.49 – 5.39 (m, 2H), 4.55 (s, 1H), 4.42 (s, 1H), 4.17 (s, 1H), 4.06 (td, *J* = 8.8 and 4.9 Hz, 1H), 3.88 (s, 3H), 3.23 (td, *J* = 6.9 and 1.7 Hz, 2H), 3.03 (tt, *J* = 8.3 and 4.3 Hz, 2H), 2.62 (s, 3H), 2.53 (dd, *J* = 4.6 and 1.3 Hz, 3H), 2.31 – 2.26 (m, 2H), 1.53 – 1.36 (m, 6H), 1.36 (s, 9H), 1.31 – 1.18 (m, 3H), 1.00 – 0.81 (m, 9H), 0.21 (s, 18H); ^{13}C NMR (100 MHz,

DMSO-d_6): δ = 171.84, 171.63, 171.26, 170.77, 169.65, 169.04, 161.96, 155.91, 138.82, 134.46, 133.54, 128.61, 128.01, 127.60, 127.57, 116.72, 100.94, 99.80, 79.42, 60.96, 57.08, 56.72, 55.30, 52.84, 52.43, 50.94, 50.82, 49.98, 49.01, 39.89,37.62, 37.08, 31.82, 30.15, 28.38, 28.22, 27.94, 25.89, 24.87, 23.55, 22.66, 22.60, 21.86, 21.56, 0.09; HRMS, *m*/*z* calcd for $C_{56}H_{84}N_{14}O_{10}Si_2$ [*M* + H]$^+$ 1169.6016, found 1169.6143.

2.4.2.9 Boc-*N*-α-Me-D-Leu-D-Lys(N_3)-Asn-(3,5-bis(ethynyl)-D-4-methoxy -phenylglycyl)-D-Phg-Lys(N_3)-NHMe 3: The fully protected hexapeptide **18** (280 mg, 0.24 mmol) was dissolved in a mixture of MeOH/CH_2Cl_2/H_2O (24 mL, 4:7:1 v/v/v) and to this solution AgOTf (25 mg, 0.096 mmol) was added and the obtained reaction mixture was stirred for 20 h at room temperature. Then, the solvents were removed *in vacuo* and the residue was redissolved in EtOAc (50mL). The resulting solution was washed with H_2O (50 mL, once) followed by brine (50 mL, once), and dried (Na_2SO_4). After filtration and removal of the solvent, the residue was purified by column chromatography (CH_2Cl_2/MeOH, 96:4 v/v) to afford Boc-*N*-α-Me-D-Leu-D-Lys(N_3)-Asn-(3,5-bis(ethynyl)-D-4-methoxyphenyl -glycyl)-D-Phg-Lys(N_3)-NHMe (**3**) as a white solid (175 mg, 71%). R_f 0.47 (CH_2Cl_2/MeOH, 9:1 v/v); $[\alpha]_D^{20}$ = –44.0 (*c* = 0.5 CH_2Cl_2/MeOH, 1:1 v/v); ^{1}H NMR (400 MHz, DMSO-d_6): δ = 8.94 (t, *J* = 6.9 Hz, 1H), 8.28 (dd, *J* = 17.8 and 7.7 Hz, 1H), 8.17 (d, *J* = 7.6 Hz, 1H), 7.81 (q, *J* = 4.8 Hz, 1H), 7.68 (s, 1H), 7.52 – 7.38 (m, 3H), 7.35 – 7.20 (m, 4H), 6.87 – 6.79 (m, 1H), 5.56 – 5.40 (m, 2H), 4.56 (s, 1H), 4.45 (s, 1H), 4.38 (s, 2H), 4.19 (s, 1H), 4.07 (td, *J* = 8.9 and 4.9 Hz, 1H), 3.87 (d, *J* = 0.7 Hz, 3H), 3.29 – 3.15 (m, 2H), 3.04 (tt, *J* = 6.9 and 3.6 Hz, 2H), 2.63 (s, 3H), 2.52 (d, *J* = 4.6 Hz, 3H), 2.45 – 2.24 (m, 2H), 1.53 – 1.36 (m, 6H), 1.36 (s, 9H), 1.32 – 1.20 (m, 3H)), 0.97 – 0.82 (m, 9H); ^{13}C NMR (100 MHz, DMSO-d_6): δ = 171.84, 171.59, 171.27, 170.75, 169.62, 168.92, 162.10, 138.85, 134.78, 133.55, 128.63, 128.03, 127.60, 127.56, 116.36, 85.88, 79.45, 79.39, 61.34, 57.02, 55.06, 52.77, 52.41, 50.94, 50.81, 49.99, 37.66, 31.89, 30.19, 28.42, 28.21, 27.94, 25.92, 24.90, 23.58, 22.67, 22.59; HRMS, *m*/*z* calcd for $C_{50}H_{68}N_{14}O_{10}$ [*M* + H]$^+$ 1025.5276, found 1025.5254.

2.4.2.10 *N*-Boc protected bicyclic hexapeptide 19: Boc-*N*-α-Me-D-Leu-D-Lys(N_3)-Asn- (3,5-bis(ethynyl)-D-4-methoxyphenylglycyl)-D-Phg-Lys(N_3)-NHMe

(**3**) (30 mg, 0.029 mmol) and $[Cp^*RuCl]_4$ (4.7 mg, 4.4 μmol) were dissolved in THF/MeOH (5 mL, 4:1 v/v). The obtained reaction mixture was heated by microwave irradiation at 80 °C for 2 h after which the solvents were removed under reduced pressure and the residue was purified by column chromatography (CH_2Cl_2/MeOH, 9:1 v/v). A product fraction was obtained which was further purified by preparative RP-HPLC, finally *N*-Boc-protected bicyclic hexapeptide **19** was obtained as a white fluffy solid after lyophilization (4 mg, 13%). R_f 0.25 (CH_2Cl_2/MeOH, 9:1 v/v); HRMS, *m*/*z* calcd for $C_{50}H_{68}N_{14}O_{10}$ $[M + H]^+$ 1025.5276, found 1025.5320, *m*/*z* calcd for $C_{50}H_{68}N_{14}O_{10}Na$ $[M + Na]^+$ 1047.5141, found 1047.5144.

2.4.2.11 Bicyclic hexapeptide 2b: *N*-Boc-protected bicyclic hexapeptide **19** (10 mg, 0.0098 mmol) was treated with TFA (1 mL) in CH_2Cl_2 (1 mL) for 1 h, after which the volatiles were removed under reduced pressure. The crude unprotected bicyclic peptide was purified by preparative RP-HPLC. Bicyclic hexapeptide **2b** was obtained as a white fluffy solid after lyophilization (4 mg, 40%). ^{1}H NMR (500 MHz, DMSO-d_6): δ = 9.11 (d, *J* = 9.4 Hz, 1H), 8.87 – 8.74 (m, 2H), 8.39 (d, *J* = 7.2 Hz, 1H), 8.25 (s, 1H), 7.91 (d, *J* = 3.6 Hz, 2H), 7.84 (d, *J* = 5.1 Hz, 1H), 7.70 – 7.62 (m, 2H), 7.53 (s, 1H), 7.36 – 7.29 (m, 5H), 6.86 (s, 1H), 6.49 (s, 1H), 5.56 (d, *J* = 7.5 Hz, 1H), 5.34 (d, *J* = 7.5 Hz, 1H), 4.42 (d, *J* = 14.0 Hz, 1H), 4.30 – 4.27 (m, 3H), 4.04 – 3.94 (m, 3H), 3.72 (s, 1H), 3.10 (s, 3H), 2.51 (s, 3H), 1.89 – 1.82 (m, 2H), 1.72 – 1.64 (m, 4H), 1.56 – 1.47 (m, 3H), 1.42 – 1.34 (m, 2H), 1.20 (s, 2H), 1.17 – 1.06 (s, 3H), 0.89 – 0.75 (m, 8H); ^{13}C HSQC NMR (125 MHz, DMSO-d_6): δ = 133.10, 132.8, 130.9, 128.4, 127.6, 126.8, 60.50, 58.80, 57.22, 55.53, 53.03, 52.28, 51.74, 47.65, 46.85, 38.23, 35.31, 31.05, 30.80, 30.42, 29.47, 29.09, 25.19, 21.90, 21.72, 21.09; HRMS, *m*/*z* calcd for $C_{45}H_{60}N_{14}O_8$ $[M + H]^+$ 925.4752, found 925.4829 and $[M + 2H]^{2+}$ 463.2475.

2.4.3 Binding affinity measurements

Binding affinity measurements were determined by using microcalorimetry, which was performed on automated MicroCal Auto-iTC200 equipment. ITC (isothermal titration calorimetry) experiment was carried out by injection the ligand solution (10-15 mM) into the cell containing the solution of the synthesized mimics or

vancomycin (0.1-0.3 mM) dissolved in a 0.02 M Na-citrate/citric acid buffer (pH 5.1). The typical experiment contains 16 injections in 40 min and the resulting data was analyzed by non-linear fitting in Origin software.

2.5 References

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Chapter 3

Synthesis of a series of 1,5-triazole-bridged vancomycin CDE-ring mimics using solid phase peptide synthesis and RuAAC macrocyclization

3.1 Introduction

Previously, it has been shown that a convenient synthetic approach toward 1,5-triazole-bridged vancomycin CDE-ring mimics could be achieved.[1] The optimized solution phase synthesis method of the linear peptide as well as the Ru(II)-catalyzed macrocyclization strategy as described in Chapter 2 led to the possibility of synthesizing a series of vancomycin CDE-ring analogues in which the amino acid residues at position P^2 and position P^4 were varied. However, this synthesis approach limited the number of analogues. To address this shortcoming, an approach that combined the speed of solid phase peptide synthesis (SPPS) and the efficient RuAAC macrocyclization in solution was designed for the synthesis of bicyclic vancomycin mimics. This chapter aims to explore this alternative method for fast and reliable access of a diverse set of several vancomycin mimics.

3.2 Results and Discussion

Three bicyclic analogues were designed as shown in Figure 1. In first instance, D-leucine (Leu) and L-alanine (Ala) were chosen to be incorporated at position P^2 and P^4, respectively, to translate the optimized synthesis in solution to conditions compatible with SPPS. The replacement at P^2 with either D-phenylalanine (Phe) or D-phenylglycine (Phg) and at P^4 with naturally occurring L-asparagine (Asn) resulted in two additional analogues.[2] The resin that was used resulted in a C-terminal amide while the molecules accessed by solution phase synthesis were functionalized as the corresponding methylamide.

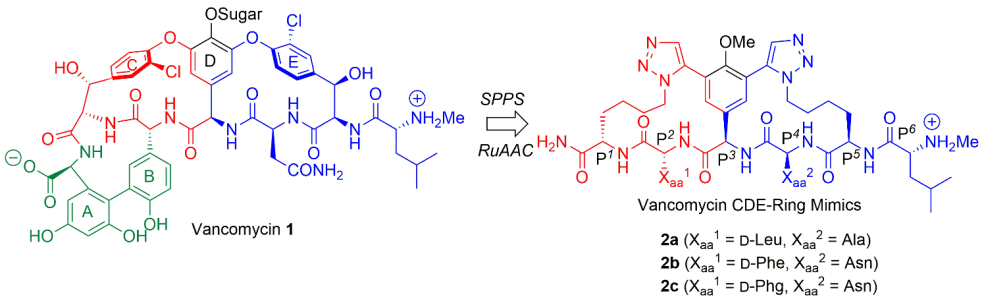
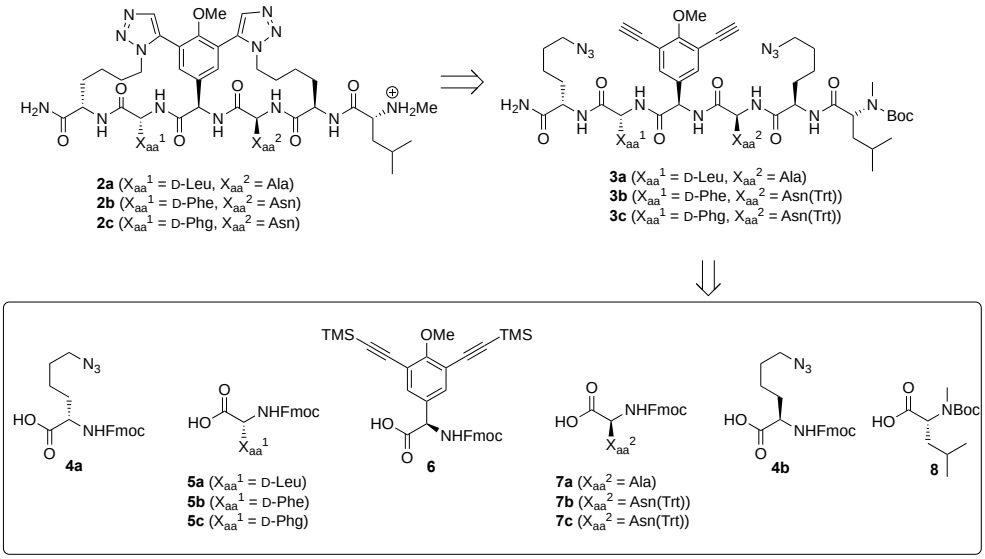


Figure 1. Design of the bicyclic vancomycin CDE-ring mimics

The synthesis of these three analogues involved three key stages and was designed based on the retrosynthesis as shown in Scheme 1. The first stage was the preparation of building blocks which were suitable for Fmoc/^{t}Bu-based solid phase peptide synthesis. As the second stage, stepwise C to N assembly of the linear peptide by SPPS, applying repetitive coupling and deprotection steps, was used. The final and most important stage was the click reaction using RuAAC to afford the bicyclic hexapeptides CDE-ring mimics of vancomycin.

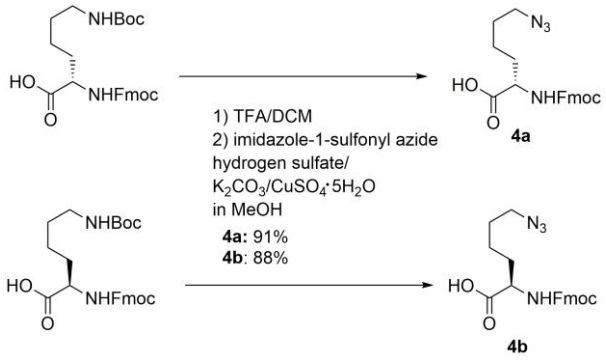


Scheme 1. Retrosynthetic analysis of the bicyclic vancomycin CDE-ring mimics.

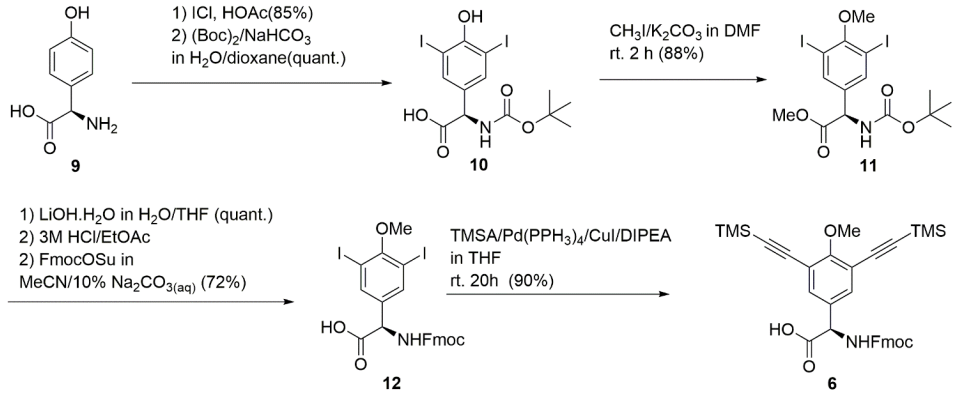
The Fmoc-protected azidoamino acids **4a** and **4b** (as shown in Scheme 2) were easily synthesized by first removing the Boc protecting group by treatment of commercially available Fmoc-(D/L)-Lys(Boc)-OH with TFA, followed by the conversion of the ε-amine into an azide moiety[3] using the diazotransfer reagent imidazole-1-sulfonyl azide hydrogen sulfate[4], obtaining **4a** or **4b** in ~90% yield.

The synthesis of building block **6** started with the iodination of commercially available D-hydroxyphenylglycine following a procedure from the literature (Scheme 3).[5] In the next step, the free amine was protected by a Boc group to give compound **10** in a quantitative yield.[6] Subsequent methylation of the carboxylic

acid moiety along with the phenolic hydroxy group in the presence of MeI/K_2CO_3 in DMF gave the fully protected diiodide **11** in a yield of 88%. After saponification of the methyl ester and removal of the Boc group, the free amine was reprotected by an Fmoc group using FmocOSu in an aqueous basic solution to give compound **12**.[7] Finally, both alkyne moieties were introduced via Pd-catalyzed Sonogashira cross-coupling in presence ousingf TMS-acetylene/CuI to afford bis-alkyne **6** in a yield of 90%.



Scheme 2. Synthesis of azidoamino acids **4a** and **4b**.

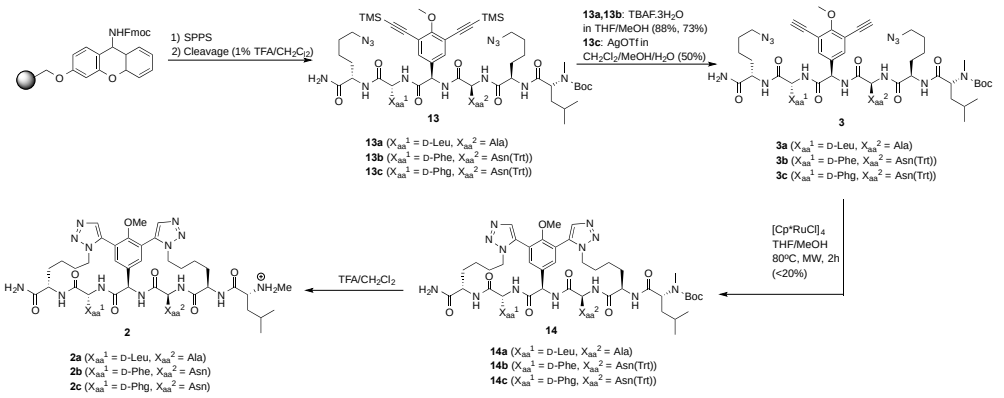


Scheme 3. Synthesis of central building block **6**.

With all building blocks at hand, assembly of linear peptides **13a-c** using SPPS could be started, however, the right choice of the resin-linker combination had still to be made. Initially, the peptide synthesis was attempted with the Rink Amide

TentaGel S Resin, which required at least 50% TFA in CH_2Cl_2 for the cleavage, which resulted in the loss of the indispensable N-terminal Boc protecting group and also in a partially removal of the TMS functionalities. Indeed, RuAAC macrocyclization turned out to be possible only with a protected amine. Therefore, Sieber Amide Resin[8] became the resin of choice as it allowed resin cleavage of the linear peptides with 1% TFA/CH_2Cl_2, conserving all the important acid labile protecting groups[9]. The peptide couplings were performed using Fmoc protected amino acid (4.0 equiv), BOP (4.0 equiv) and DIPEA (8.0 equiv) in NMP (1 mL/100 mg of resin).

During the coupling of the hydroxphenylglycine building block, the major issue was its high sensitivity toward racemization under basic conditions.[10] To suppress the racemization of this amino acid during SPPS, different combinations of coupling reagents for coupling of hydroxyphenylglycine derivative **6** were attempted to determine which combination gave the highest coupling yield at the least racemization.[11] As shown in Table 1, the degree of racemization of the hydroxyphenylglycine residue which was identified by LC-MS was roughly the same with all coupling reagents tested. A BOP/DIPEA-mediated coupling in NMP resulted in a diastereomeric ratio of 1.16:1 (this means *SRR* versus *SR**S***), while in the presence of EDCI/HOBt, somewhat surprisingly in view of the slightly acidic conditions during coupling, the racemization was almost complete and the ratio was found to be 1.07:1. It was reported in the literature that the coupling reagents DEPBT[12] or COMU in combination with the weaker base 2,4,6-trimethylpyridine (TMP) strongly suppressed racemization. However, applying DEPBT/TMP and COMU/TMP in NMP, a slightly lower percentage of racemization was found, with ratios of 1.3:1 and 1.42:1, respectively. Although COMU/TMP in NMP resulted in the lowest amount of racemization, coupling reaction conversion was unfortunately incomplete. Therefore, the original conditions, a BOP/DIPEA-mediated coupling in NMP, were used for SPPS.



Scheme 4. Synthesis of 1,5-triazole-bridged vancomycin CDE-ring mimics.

Table 1. Diastereomeric ratios of the tripeptide Fmoc-D-Phg(4-OMe-3,5-bis-TMS-alkyne) -D-Leu-Lys(N_3)-NH_2 using different coupling reagents and solvents.

Conditions	**BOP/ DIPEA/ NMP**	**BOP/ DIPEA/ CH_2Cl_2**	**EDCI/ HOBt/ NMP**	**HATU/ TMP/ NMP**	**DEPBT/ TMP/ NMP**	**COMU/ TMP/ NMP**
Ratio D/L	1.16:1	1:1	1.07:1	1.2:1	1.3:1	1.42:1

Upon completion of SPPS, each linear peptide was cleaved from the resin by the treatment with 1% TFA/CH_2Cl_2. After isolation by HPLC purification, the linear hexapeptides **13a** and **13b** were treated with TBAF, while hexapeptide **13c** was treated with AgOTf[13] since racemization of phenylglycine will be minimal due to the mild reaction conditions, to remove both TMS protecting groups (Scheme 4). The next and most critical step was bicyclization of the linear hexapeptides featuring RuAAC macrocyclization.[1,14] This intramolecular cyclization reaction did not result in a shift of molecular weight, a complicating factor to determine whether the hexapeptides have fully or only partially cyclized. In order to be certain that the bicyclic compounds were obtained, different amounts of catalyst were used and the reaction progress was monitored by LC-MS. As is shown in Figure 2, at a catalyst loading of 10 mol%, three new peaks were identified, based on a major shift in retention time, that corresponded to the same mass as the linear hexapeptide **3a**. It was concluded that macrocyclization had occurred and two

monocyclic products appeared as intermediates. Increasing of the amount of catalyst resulted in a decrease of the monocyclic compounds while the amount of bicyclic **14a** was increased . This trend could be observed for all three analogues and the desired bicyclic compounds were isolated and purified by preparative RP-HPLC. As the final step, the removal of protecting groups with TFA was carried out. It turned out that this deprotection was very sluggish and the desired compounds could not be isolated from the reaction mixture. The presence and identity, however, could only be confirmed by HR-MS. The obtained compounds were analyzed by HR-MS, confirming the formation of final products **2**.

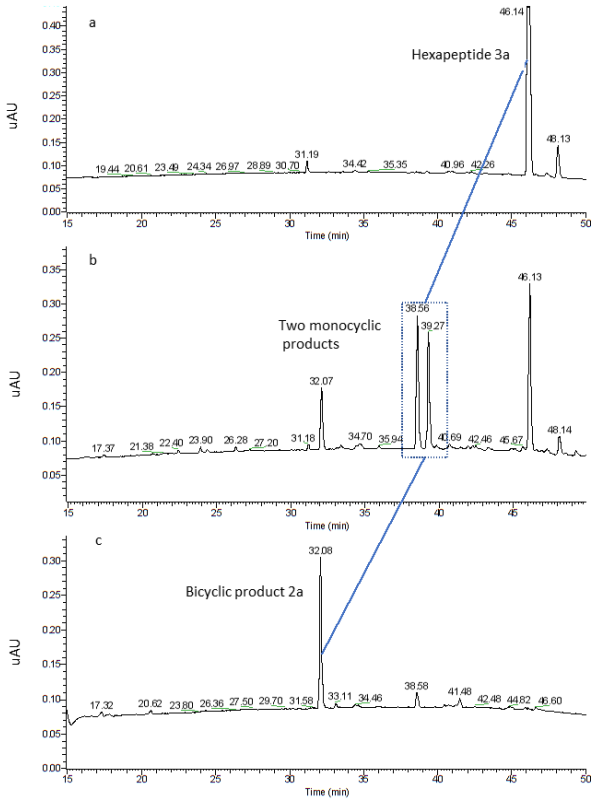


Figure 2. LC-MS analysis of the RuAAC macrocyclization of hexapeptide **3a**: (a) hexapeptide **3a**, (b) RuAAC macrocyclization of hexapeptide **3a** (10 mol% $[Cp^*RuCl]_4$, 80 ºC, 2 h (MW)) and (c) RuAAC macrocyclization of hexapeptide **3** (30 mol% $[Cp^*RuCl]_4$, 80 ºC, 2 h (MW)). R_t 46.14 min represents the linear precursor peptide; R_t 38.56/39.27 min represents the monocyclic intermediate; R_t 32.08 min represents the bicyclic compound.

3.3 Conclusion and Outlook

Three 1,5-triazole-bridged vancomycin CDE-ring mimics were designed and synthesized by a combination of SPPS and solution-based RuAAC macrocyclization. During the SPPS approach several difficulties were encountered. First of all, the high tendency of racemization of the phenylglycine building block during amide bond formation. Secondly, after cleavage from the resin, the low solubility of the protected linear peptides hampered efficient purification while protecting group removal proceeded rather sluggishly. Finally, the desired compounds were only identified by mass spectrometry. Unfortunately, the amounts obtained were too small for further analysis and biological evaluation.

In hindsight, this chapter was focused to provide a proof-of-principle to access the bicyclic 1,5-triazole-bridged CDE-ring mimics of vancomycin. To the best of our knowledge, three papers deal with RuAAC on the solid support. Kee *et al.*[15] described the reaction of solid phase bound azidoalanine with diethyl ethynylphosphonate to provide phosphohistidine analogs, while Horne *et al.*[16] found that reacting resin-bound azidoalanine with isoleucine-derived propargylic amine resulted in a new class of HDAC inhibitors. Resin-bound RuAAC macrocyclization was only reported once in the literature. Empting *et al.*[17] described that a 1,5-triazole bridge could replace a disulfide bond in sunflower trypsin inhibitor to arrive at redox stable derivatives. Based on these results, it is assumed that after the linear synthesis of a hexapeptide like **13**, selective removal of TMS followed by RuAAC bicyclization and subsequently global deprotection and detachment from the resin might be a real option to improve the efficiency of an SPPS approach toward bicyclic peptides like **2**. This improved synthetic methodology will pave the way to perform SAR studies to optimize binding affinity toward the natural ligand, D-Ala-D-Ala as well as the modified D-Ala-D-Lac depsipeptide motif.

3.4 Experimental Section

3.4.1 Chemicals and General Experimental Procedures.

Chemicals were used as obtained from commercial sources without further

purification unless stated otherwise. The solvents were obtained as peptide synthesis grade and stored on molecular sieves (4 Å). Column chromatography was performed using SiliFlash P60 silica gel (particle size 40-63 μm). TLC was performed on precoated silica gel 60F254 glass plates and compound spots were visualized by UV-quenching, ninhydrin, or Cl_2/TDM. Melting points were measured according to Dr. Tottoli and were uncorrected. Optical rotations were measured using a 10 cm cell with a Na 589 nm filter at the specific concentrations (in g/100 mL) as indicated. ^{1}H NMR spectra were recorded either on a 400 MHz or a 500 MHz spectrometer in $CDCl_3$, or DMSO-d_6 as solvent. Chemical shifts are reported in delta (δ) units, in parts per million (ppm) relative to TMS (0.00 ppm). Coupling constants (*J*) are reported in Hertz (Hz). ^{13}C NMR analysis was acquired on a 100 MHz or a 125 MHz spectrometer in $CDCl_3$, or DMSO-d_6 as solvent. Chemical shifts are reported in delta (δ) units, in parts per million (ppm) relative to the solvent residual signal, $CDCl_3$ (77.0 ppm) or DMSO-d_6 (39.0 ppm). Analytical HPLC was performed on an automated HPLC system equipped with a UV/Vis detector operating at 220/254 nm and an evaporative light scattering detector using a C18 column (pore size: 100 Å, particle size: 5 μm; 250 × 4.6 mm) at a flow rate of 1 mL/min using a linear gradient of 100% buffer A (0.1% TFA in CH_3CN/H_2O 5:95 v/v) to 100% buffer B (0.1% TFA in CH_3CN/H_2O 95:5 v/v) in 60 min. Preparative RP-HPLC was performed on an automated preparative HPLC system equipped with a UV/Vis detector operating at 214 nm using a C18 column (pore size: 100 Å, particle size: 10 μm; 250 × 22 mm) at a flow rate of 2.0 mL/min using a linear gradient of 100% buffer A (0.1% TFA in CH_3CN/H_2O 5:95 v/v) to 100% buffer B (0.1% TFA in CH_3CN/H_2O 95:5 v/v) in 60 min. LC-MS(MS) was measured using a C18 column (pore size: 100 Å, particle size: 5 μm; 250 × 4.6 mm) at a flow rate of 1 mL/min using a linear gradient of of 100% buffer A (0.1% TFA in CH_3CN/H_2O 5:95 v/v) to 100% buffer B (0.1% TFA in CH_3CN/H_2O 95:5 v/v) in 60 min. ESI-MS was performed on a bench-top electrospray ionization mass spectrometer. High-resolution mass spectrometry (HRMS) analysis was performed using an ESI-TOF LC/MS instrument.

3.4.2 Syntheses and Compound Analyses

3.4.2.1 Compound 4a: Fmoc-Lys(Boc)-OH (6 g, 12.6 mmol) was dissolved in

CH_2Cl_2 (75 mL) and to this solution TFA (75 mL) was added The obtained reaction mixture was stirred for 1 h, after which the volatiles were removed by evaporation and any residual TFA was removed by coevaporation with CH_2Cl_2 (3 × 50 mL). The resulting product was redissolved in methanol (150 mL). To this solution, K_2CO_3 (4.46 g, 25.6 mmol), $CuSO_4{\cdot}5H_2O$ (50 mg, 0.1 mmol) and imidazole-1-sulfonyl azide hydrogen sulfate (4.17 g, 15.4 mmol) were added and stirred overnight at room temperature. The mixture was then concentrated, redissolved in H_2O (150 mL) and acidified with 1N $KHSO_4$ to pH 2-3. The resulting solution was then extracted with EtOAc (3 × 100 mL). The combined organic layer was dried (Na_2SO_4), filtered and concentrated. The residue was purified by column chromatography (CH_2Cl_2/MeOH 99:1 v/v with 0.1% AcOH) to give compound **4a** as a white solid (4.62 g, 91%). R_f 0.38 (CH_2Cl_2/MeOH 9:1 v/v); $[\alpha]_D^{20}$ = +6.7 (c = 1, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$) δ = 7.75 (d, *J* = 7.5 Hz, 2H), 7.59 (d, *J* = 6.1 Hz, 2H), 7.39 (t, *J* = 7.4 Hz, 2H), 7.30 (t, *J* = 7.4 Hz, 2H), 5.43 (d, *J* = 8.0 Hz, 1H), 4.41 (d, *J* = 6.9 Hz, 2H), 4.21 (t, *J* = 6.8 Hz, 1H), 3.26 (t, *J* = 6.6 Hz, 1H), 1.97-1.88 (m, 1H), 1.77-1.68 (m, 1H), 1.65-1.59 (m, 2H), 1.51-142 (m, 2H); ^{13}C NMR (400 MHz, $CDCl_3$) δ = 175.92, 163.41, 156.07, 143.64, 141.28, 127.71, 127.04, 125.03, 119.98, 77.33, 77.01, 76.69, 67.06, 53.53, 51.05, 47.11, 36.89, 31.88, 28.34, 22.38; HR-MS (ESI) *m/z* calcd for $C_{21}H_{22}N_4O_4$ $[M + Na]^+$ 417.1539, found 417.1528.

3.4.2.2 Compound 4b: Fmoc-D-Lys(Boc)-OH (8.24 g, 17.6 mmol) was dissolved in dissolved in CH_2Cl_2 (100 mL) and to this solution TFA (100 mL) was added. The obtained reaction mixture was stirred for 1 h, after which the volatiles were removed by evaporation and any residual TFA was removed by coevaporation with CH_2Cl_2 (3 × 50 mL). The resulting product was redissolved in methanol (200 mL). To this solution, K_2CO_3 (6.13 g, 35.2 mmol), $CuSO_4{\cdot}5H_2O$ (50 mg, 0.1 mmol) and imidazole-1-sulfonyl azide hydrogen sulfate (4.43 g, 21.1 mmol) were added and stirred overnight at room temperature. The mixture was then concentrated, redissolved in H_2O (150 mL) and acidified with 1N $KHSO_4$ to pH 2-3. The resulting solution was then extracted with EtOAc (3 × 100 mL). The combined organic layer was dried (Na_2SO_4), filtered and concentrated. The residue was purified by column chromatography (CH_2Cl_2/MeOH 99:1 v/v with 0.1% AcOH) to give compound **4a** as a white solid (6.21 g, 90%). R_f 0.36 (CH_2Cl_2/MeOH 9:1 v/v);

$[\alpha]_D^{20}$ = -5.9 (c = 1, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$) δ = 7.76 (d, *J* = 7.5 Hz, 2H), 7.59 (d, *J* = 6.7 Hz, 2H), 7.40 (t, *J* = 7.5 Hz, 2H), 7.31 (t, *J* = 7.4 Hz, 2H), 7.30 (d, *J* = 8.1 Hz, 1H), 4.42 (d, *J* = 6.8 Hz, 2H), 4.22 (t, *J* = 6.8 Hz, 1H), 3.28 (t, *J* = 6.5 Hz, 2H), 1.97-1.89 (m, 1H), 1.78-1.71 (m, 1H), 1.67-1.58 (m, 2H), 1.50-1.43 (m, 2H); ^{13}C NMR (400 MHz, $CDCl_3$) δ = 176.53, 156.34, 143.76, 143.61, 141.30, 127.79, 127.11, 125.06, 120.04, 77.47, 77.15, 76.83, 67.20, 56.78, 53.59, 51.03, 47.09, 31.71, 28.30, 22.48, HR-MS (ESI) *m/z* calcd $C_{21}H_{22}N_4O_4$ [*M* + Na]$^+$ 417.1539, found 417.1529.

3.4.2.3 Compound 11: Compound **10** (17.63 g, 34 mmol) was dissolved in DMF (500 mL). Potassium carbonate (10.50 g, 76 mmol) and iodomethane (4.76 mL, 76 mmol) were added to the solution. The solution was left to stir for 2 h before diluting it with H_2O (500 mL) and extracted with EtOAc (3 × 200 mL). The combined organic layers were washed with H_2O (2 × 300 mL) and brine (200 mL), dried over Na_2SO_4 and concentrated. The residue was purified by column chromatography (PE/EtOAc 8:1 v/v) and compound **11** was obtained as a white solid (16.31g, 88%). R_f 0.62 (PE/EtOAc, 3:1 v/v); $[\alpha]_D^{20}$ = –46.8 (*c* 1.0, $CHCl_3$); ^{1}H-NMR (400 MHz, $CDCl_3$) δ = 7.72 (s, 2H), 5.60 (d, *J* = 7.0 Hz, 1H), 5.17 (d, *J* = 7.0 Hz, 1H), 3.83 (s, 3H), 3.73 (s, 3H), 1.42 (s, 9H). ^{13}C-NMR (100 MHz, $CDCl_3$) δ = 171.1, 170.5, 158.9, 154.5, 141.8, 138.3, 136.7, 129.5, 90.8, 80.6, 60.6, 55.6, 53.1, 28.3, 21.0, 14.2; HRMS *m/z* calcd for $C_{15}H_{19}I_2NNaO_5$ [*M* + Na]$^+$ 569.9250, found 569.9243.

3.4.2.4 Compound 12: Compound **11** (16.31 g, 30 mmol) was dissolved in a mixture of THF/H_2O (400 mL, 1:1 v/v) and the solution was cooled to 0 ˚C in an ice bath. To this solution, Lithium hydroxide monohydrate (1.90 g, 45 mmol) was added. After stirring for 0.5 h at room temperature, a solution of 1N $KHSO_4$ was added until pH 2-3. Then the aqueous phase was extracted with EtOAc (3 × 100 mL). The combined organic layers were washed with water (100 mL) and brine (100 mL) then dried over Na_2SO_4 and concentrated to give the free acid compound quantitatively as a white solid. Without further purification, it was used for the following step. The resulting acid was dissolved in 3M HCl/EtOAc (150 mL). The obtained reaction mixture was left to stir for 2 h. Then, the volatiles were removed by evaporation. After drying for 1 h at high vacuum, the resulting fee amine was

used directly without further purification. This intermediate was then dissolved in a solution of MeCN /10% Na_2CO_3 (200 mL, 1:1 v/v). A solution of FmocOSu (8.7 g, 25.7 mmol) in MeCN (100 mL) was then added dropwise over 30 min and left to stir overnight at room temperature. Then the reaction mixture was diluted with water (100 mL) and extracted with diethyl ether (2 × 100 mL). The aqueous layer was acidified to pH 3 with 1N $KHSO_4$ and extracted with EtOAc (3 × 100 mL). The combined organic layers were dried over Na_2SO_4, filtered and concentrated. The resulting residue was then left to stir for 1 h in a solution of PE/EtOAc (500 mL, 4:1 v/v). After filtration, compound **12** was obtained as a white powder (12.20 g, 72%); R_f 0.20 (CH_2Cl_2/MeOH 9:1 v/v); $[\alpha]_D^{20}$ = -95.5 (c = 1, $CHCl_3$); ^{1}H NMR (400 MHz, DMSO-d_6) δ = 8.24 (d, *J* = 8.3 Hz, 1H), 7.86 (d, *J* = 10.6 Hz, 4H), 7.69 (d, *J* = 7.4 Hz, 2H), 7.37 (t, *J* = 7.4 Hz, 2H), 7.29 (q, *J* = 7.2 Hz, 2H), 8.14 (d, *J* = 8.2 Hz, 1H), 4.27-4.22 (m, 3H), 3.71 (s, 3H); ^{13}C NMR (100 MHz, DMSO-d_6) δ = 158.51, 156.14, 144.20, 144.06, 141.12, 139.28, 137.83, 128.09, 127.51, 125.72, 120.53, 91.66, 66.43, 60.70, 56.37, 47.0; HR-MS (ESI) *m/z* calcd $C_{24}H_{19}I_2NO_5$ [*M* + Na]$^+$ 677.9250, found 677.9243.

3.4.2.5 Compound 6: Compound **12** (0.940 g, 1.43 mmol), CuI (82 mg, 0.43 mmol) and [$Pd(PPh_3)_4$] (0.165 g, 0.143 mmol), were placed in a schlenk flask sealed with a rubber septum. The flask was evacuated and refilled with dry N_2 (repeated for three times). Then, THF (20 mL, which was purged with dry N_2 for 1 h prior to use) was added to the flask via a syringe. The resulting solution was degassed again using three freeze-pump-thaw cycles. Subsequently, TMS-acetylene (0.99 mL, 7.18 mmol) and DIPEA (0.5 mL, 2.87 mmol) were added to the mixture via a syringe and the obtained reaction mixture was stirred for 20 h at room temperature under a continuous flow of N_2. The resulting suspension was filtered through a path of Celite and the filtrate was evaporated to dryness. The obtained residue was redissolved in EtOAc (50 mL) and the solution was successively washed with 1 N $KHSO_4$ (2 × 50 mL), followed by saturated $NaHCO_3$ (2 × 50 mL) and brine (50 mL). Finally, the organic layer was dried over Na_2SO_4, filtered and evaporated to dryness. The residue was purified by column chromatography (EtOAc/PE 1:3 v/v with 0.1% AcOH) and TMS-protected bis-alkyne **6** was obtained as a yellowish solid (0.778 g, 91%). R_f 0.59 (EtOAc/PE, 1/3, v/v with 0.1% AcOH); $[\alpha]_D^{20}$ = -12.3 (c = 1, $CHCl_3$); ^{1}H NMR (400 MHz,

DMSO-d_6) δ = 8.21 (d, *J* = 8.2 Hz, 1H), 7.84 (d, *J*= 7.5 Hz, 2H), 7.70 (d, *J* = 7.4 Hz, 2H), 7.49 (s, 2H), 7.39-7.34 (m, 2H), 7.28 (dd, *J* = 12.6, 7.1 Hz, 2H), 5.15 (d, *J* = 8.2 Hz, 1H), 4.28-4.16 (m, 3H), 3.90 (s, 3H), 0.21 (s, 18H); ^{13}C NMR (100 MHz, DMSO-d_6) δ = 162.08, 156.12, 144.24, 144.06, 141.11, 133.92, 133.72, 128.03, 127.48, 127.44, 125.73, 120.51, 116.81, 100.79, 100.03, 79.99, 66.39, 61.10, 57.05, 46.99; HR-MS (ESI) *m/z* calcd $C_{34}H_{37}NO_5Si_2$ [*M* + Na]$^+$ 618.2108, found 618.2750.

3.4.2.6 General procedure for solid phase peptide synthesis: The linear TMS protected hexapeptides **13ac** were synthesized on Sieber Amide Resin (1.0 g, 0.3 mmol) using standard Fmoc solid phase peptide synthesis protocols. Peptide couplings were performed by using Fmoc protected amino acid (4.0 equiv), BOP (4.0 equiv) and DIPEA (8.0 equiv) in NMP (10 mL/1 g of resin). Upon completion of SPPS, each linear peptide was cleaved from the resin by the treatment with 1% TFA/CH_2Cl_2 (5 × 5min, 1ml/100mg of resin). Then, the volatiles were removed by evaporation and the resulting residue was purified by column chromatography (CH_2Cl_2/MeOH, 96:4 v/v).

3.4.2.7 Linear TMS protected hexapeptide 13a (38 mg, 12%); R_f 0.74 (CH_2Cl_2/MeOH 9:1 v/v); $[\alpha]_D^{20}$= +24.6 (c = 1.0, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$) δ = 8.12 (d, *J* = 8.5 Hz, 1H), 7.42 (d, *J* = 7.1 Hz, 1H), 7.35 (s, 2H), 7.00 (s, 1H), 6.90 (s, 1H), 6.56 (s, 1H), 6.12 (s, 1H), 5.12 (s, 1H), 4.69 (s, 1H), 4.55-4.39 (m, 3H), 4.03 (s, 4H), 3.30-3.22 (m, 4H), 2.80 (s, 3H), 2.04-1.77 (m, 8H), 1.62-1.56 (m, 6H), 1.45 (s, 12H), 1.39 (d, *J* = 6.8 Hz, 5H), 1.01-0.94 (m, 15H), 0.25 (s, 18H); ^{13}C NMR (100 MHz $CDCl_3$) δ = 174.74, 173.70, 173.32, 172.24, 169.13, 162.87, 156.25, 33.40, 130.52, 118.06, 100.38, 99.68, 80.42, 77.19, 60.93, 58.93, 56.27, 52.48, 52.11, 51.14, 51.02, 50.25, 39.37, 36.73, 31.36, 30.29, 29.64, 28.34, 28.22, 25.20, 25.02, 23.46, 22.81, 22.21, 20.65, 16.32; HR-MS (ESI) *m/z* calcd for $C_{52}H_{85}N_{13}O_9Si_2$ [*M* + H]$^+$ 1092.6210, found 1092.6204.

3.4.2.8 Linear TMS protected hexapeptide 13b (65 mg, 15%); R_f 0.35 (CH_2Cl_2/MeOH 95:5 v/v); $[\alpha]_D^{20}$ = +25.4 (c = 1.0, $CHCl_3$); ^{1}H NMR (400 MHz, DMSO-d_6) δ = 8.53 (s, 1H), 8.44 (d, *J* = 8.0 Hz, 1H), 8.21 (d, *J* = 7.8 Hz, 1H), 8.16 (d, *J* = 8.5 *Hz*, 1H), 7.59 (d, *J* = 7.9 Hz, 1H), 7.39 (s, 2H), 7.34 (s, 1H), 7.19 (dd, *J*

= 15.4, 7.7 Hz, 9H), 7.13 (t, *J* = 7.7 Hz, 9H), 7.01-6.96 (m, 4H), 6.87-6.85 (m, 2H), 5.35 (d, *J* = 7.7 Hz, 1H), 4.56 (d, *J* = 7.5 Hz, 2H), 4.43 (dd, *J* = 13.8, 8.3 Hz, 2H), 4.26 (s, 1H), 4.15 (q, *J* = 13.3, 8.5 Hz, 1H), 3.88 (s, 3H), 3.23 (t, *J* = 6.8 Hz, 2H), 3.14 (t, *J* = 6.9 Hz, 2H), 2.63 (s, 4H), 2.56 (d, *J* = 6.9 Hz, 2H), 1.62-1.39 (m, 10H), 1.36-1.30 (m, 13H), 1.26-1.15 (m, 7H), 0.81-0.76 (m, 5H), 0.22 (s, 18H); ^{13}C NMR (100 MHz, DMSO-d_6) δ = 173.79, 171.29, 170.85, 170.73, 169.15, 161.98, 145.18, 137.47, 133.65, 129.44, 128.98, 128.02, 127.83, 126.73, 116.68, 101.09, 99.60, 79.42, 69.83, 60.91, 54.38, 52.34, 50.92, 40.16, 39.95, 39.74, 31.96, 28.40, 28.25, 28.14, 0.18; HR-MS (ESI) *m/z* calcd for $C_{75}H_{98}N_{14}O_{10}Si_2$ [*M* + H]$^+$ 1411.7207, found 1411.7209.

3.4.2.9 Linear TMS protected hexapeptide 13c (84 mg, 20%); R_f 0.81 (CH_2Cl_2/MeOH 9:1 v/v); $[\alpha]_D^{20}$= -31.8 (c = 1.0, $CHCl_3$)**;** ^{1}H NMR (400 MHz, DMSO-d_6) δ = 8.93 (s, 1H), 8.51 (s, 1H), 8.26 (d, *J* = 8.0 Hz, 1H), 8.08 (d, *J* = 8.2 Hz, 1H), 7.66 (d, *J* = 8.1 Hz, 1H), 7.49 (s, 2H), 7.42 (d, *J* = 7.1 Hz, 2H), 7.35 (s, 1H), 7.31-7.09 (m, 20H), 6.96 (s, 1H), 5.49 (d, *J* = 7.3 Hz, 1H), 5.45 (d, *J* = 7.5 Hz, 1H), 4.57 (s, 2H), 4.24 (s, 1H), 4.13-4.08 (m, 1H), 3.88 (s, 2H), 3.19-3.13 (m, 2H), 3.06-2.98 (m, 2H), 2.61 (s, 3H), 2.47 (dt, *J* = 3.6, 1.8 Hz, 4H), 1.61-1.42 (m, 6H), 1.37 (s, 4H), 1.30-1.20 (m, 8H), 0.81 (s, 7H), 0.20 (s, 18H); ^{13}C NMR (100 MHz, DMSO-d_6) δ = 173.85, 172.29, 169,85, 169,35, 162.29, 145.56, 139.41, 133.98, 129.33, 128.98, 128.19, 127.99, 127.06, 117.07, 101.36, 100.19, 79.81, 70.14, 61.39, 57.37, 52.30, 51.29, 51.20, 40.53, 40.32, 40.11, 32.36, 28.79, 28.53, 28.28, 25.20, 23.14, 22.81; HR-MS (ESI) *m/z* calcd for $C_{74}H_{96}N_{14}O_{10}Si_2$ [*M* + H]$^+$ 1397.7051, found 1397.7037.

3.4.2.10 Linear hexapeptide 3a: Compound **13a** (22 mg, 0.020 mmol) was dissolved in THF/MeOH (2 mL, 19:1 v/v) and to this solution TBAF·3H_2O (19 mg, 0.060 mmol) was added. The obtained reaction mixture was stirred for 1 h. Based on TLC analysis, the reaction was complete. Subsequent removal of the solvent, the residue was directly purified by column chromatography (CH_2Cl_2/MeOH 97:3 v/v) and compound **3a** was obtained as a grey solid (18 mg, 93%). R_f 0.67 (CH_2Cl_2/MeOH 9:1 v/v); $[\alpha]_D^{20}$= +21.8 (c = 1.0, $CHCl_3$); ^{1}H NMR (400 MHz, CD_3OD) δ = 7.50 (s, 2H), 5.19 (s, 1H), 4.55 (s, 1H), 4.31 (dd, *J* = 9.9, 4.5 Hz, 4H), 3.98 (s, 3H), 3.78 (s, 2H), 2.73 (s, 3H), 1.89-1.56 (m, 14H), 1.45 (s, 11H),

1.41-1.27 (m, 9H), 0.96-0.87 (m, 14H); ^{13}C NMR (400 MHz, CD_3OD) δ = 175.22, 172.95, 170.93, 162.87, 133.92, 131.58, 116.95, 82.96, 80.22, 78.22, 50.83, 50.81, 48.19, 47.98, 47.77, 47.56, 47.34, 47.13, 46.92, 39.18, 36.53, 31.32, 30.66, 29.45, 28.04, 27.93, 27.27, 24.66, 22.76, 22.14, 20.73, 19.98, 16.17; HR-MS (ESI) *m/z* calcd for $C_{46}H_{69}N_{13}O_9$ $[M + H]^+$ 948.5419, found 948.5404.

3.4.2.11 Linear hexapeptide 3b: Compound **13b** (38 mg, 0.029 mmol) was dissolved in THF/MeOH (3 mL, 19:1 v/v) and to this solution TBAF·$3H_2O$ (25 mg, 0.087 mmol) was added. The obtained reaction mixture was stirred for 1 h. Based on TLC analysis, the reaction was complete. Subsequent removal of the solvent, the residue was directly purified by column chromatography (CH_2Cl_2/MeOH 98:2 v/v) and compound **3a** was obtained as a grey solid (27 mg, 73%); R_f 0.26 (CH_2Cl_2/MeOH 95:5 v/v); $[\alpha]_D^{20}$ = +4,8 (c = 1.0, $CHCl_3$); ^{1}H NMR (400 MHz, DMSO-d_6) δ = 8.54 (s, 1H), 8.52 (d, *J* = 8.1 Hz, 1H), 8.26 (s, 2H), 8.19 (d, *J* = 8.2 Hz, 1H), 7.60 (s, 1H), 7.43 (s, 2H), 7.34 (s, 1H), 7.21-7.11 (m, 18H), 7.00-6.91 (m, 7H), 5.39 (d, *J* = 7.7 Hz, 1H), 4.57 (d, *J* = 6.0 Hz, 2H), 4.46-4.41 (m, 2H), 4.37 (s, 1H), 4.27 (s, 1H), 4.19-4.13 (m, 1H), 3.87 (s, 3H), 3.23 (t, *J* = 6.9 Hz, 2H), 3.15 (t, *J* = 6.9 Hz, 2H), 2.82 (dd, *J* = 13.6, 4.7 Hz, 1H), 2.63 (s, 4H), 2.58 (s, 2H), 1.64-1.53 (m, 4H), 1.50-1.40 (m, 7H), 1.36-1.30 (m,14H), 1.26-1.15 (m, 7H), 0.79 (d, *J* = 6.3 Hz, 8H); ^{13}C NMR (100 MHz, DMSO-d_6) δ = 174.55, 174.16, 172.29, 171.70, 171.21, 170.62, 169.51, 162.45, 145.55, 137.82, 134.09, 129.77, 129.35, 128.39, 128.20, 127.11, 116.66, 86.07, 79.80, 70.20, 61.62, 55.69, 52.71, 51.28, 49.28, 40.53, 40.32, 40.11, 32.35, 30.90, 30.69, 29.79, 28.77, 28.62, 25.19, 23.95, 23.22, 23.06, 18.02; HR-MS (ESI) *m/z* calcd for $C_{69}H_{82}N_{14}O_{10}$ $[M + H]^+$ 1267.6417, found 1267.6392.

3.4.2.12 Linear hexapeptide 3c: Compound **13c** (50 mg, 0.036 mmol) was dissolved in a mixture of MeOH/CH_2Cl_2/H_2O (1.2 mL, 4:7:1 v/v/v) and to this solution AgOTf (2.76 mg, 0.096 mmol) was added and the obtained reaction mixture was stirred for 20 h at room temperature. A solution saturated aqueous ammoniumchloride was added (1 mL) and the mixture was extracted with CH_2Cl_2 (3 × 50 mL). The combined organic layers were dried over Na_2SO_4 and concentrated. The residue was purified by column chromatography (DCM/MeOH 96:4 v/v) and compound **3c** was obtained as a white solid (23 mg, 50%). R_f 0.73

(CH_2Cl_2/MeOH 9:1 v/v); $[\alpha]_D^{20}$= -27.7 (c = 1.0, $CHCl_3$); ^{1}H NMR (400 MHz, DMSO-d_6) δ = 8.96 (d, *J* = 6.7 Hz, 1H), 8.53 (s, 1H), 8.34-8.27 (m, 2H), 8.15 (d, *J* = 8.4 Hz, 1H), 7.66 (d, *J* = 7.0 Hz, 2H), 7.51 (s, 2H), 7.43 (d, *J* = 7.4 Hz, 2H), 7.35-7.10 (m, 22H), 6.98 (s, 1H), 5.57 (d, *J* = 7.5 Hz, 1H), 5.46 (d, *J* = 7.4 Hz, 1H), 4.58 (s, 2H), 4.38 (s, 1H), 4.26 (s, 1H), 4.14-4.08 (m, 1H), 3.86 (s, 3H), 3.16 (t, *J* = 6.3 Hz, 2H), 3.02 (t, *J* = 6.7 Hz, 2H), 2.66 (s, 3H), 2.62 (s, 3H), 2.14 (t, *J* = 8.0 Hz, 3H), 2.00-1.82 (m, 4H), 1.59-1.44 (m, 7H), 1.37 (s, 14H), 1.28-1.20 (m, 12H), 0.98-0.91 (m, 4H), 0.80 (s, 8H); ^{13}C NMR (100 MHz, DMSO-d_6) δ = 173.51, 172.01, 169.49, 168.99, 162.10, 145.18, 138.98, 134.78, 133.61, 128.96, 128.61, 127.83, 127.63, 126.71, 116.38, 85.87, 79.40, 69.79, 61.34, 50.93, 50.82, 48.91, 39.95, 39.74, 31.98, 30.53, 29.43, 28.42, 28.14, 27.93, 24.82, 22.46, 17.65; HR-MS (ESI) *m/z* calcd for $C_{68}H_{80}N_{14}O_{10}$ $[M + H]^+$ 1253.6260, found 1253.6252.

3.4.2.13 Bicyclic hexapeptide 14a: Compound **3a** (7.7 mg, 0.0081 mmol) and $[Cp^*RuCl]_4$ (2.6 mg, 0.0024 mmol) were dissolved in THF/MeOH (1.5 mL, 19:1 v/v). The obtained reaction mixture was heated by microwave irradiation at 80 °C for 2 h after which the solvents were removed under reduced pressure and the residue was purified by preparative RP-HPLC, and Boc-protected bicyclic hexapeptide **14a** was obtained as a white fluffy solid after lyophilization (1.3 mg, 17%). ^{1}H NMR (500 MHz, CD_3OD) δ = 8.02 (s, 1H), 7.86 (s, 1H), 7.82 (s, 1H), 7.53 (s, 1H), 5.72 (s, 1H), 4.42-4.31 (m, 3H), 4.25-4.17 (m, 5H), 3.94-3.91 (m, 1H), 3.19 (s, 1H), 2.89 (s, 3H), 2.03 (s, 1H), 1.91-1.82 (m, 4H), 1.74-1.68 (m, 4H), 1.65-1.50 (m, 6H), 1.46 (s, 11H), 1.42 (d, *J* = 7.4 Hz, 4H), 1.30 (s, 1H), 1.15-1.08 (m, 2H), 0.96 (d, *J* = 6.6 Hz, 12H), 0.90 (d, *J* = 6.4 Hz, 6H).

3.4.2.14 Bicyclic hexapeptide 14b: Compound **3b** (11 mg, 0.0086 mmol) and $[Cp^*RuCl]_4$ (2.83 mg, 0.0026 mmol) were dissolved in THF/MeOH (3 mL, 19:1 v/v). The obtained reaction mixture was heated by microwave irradiation at 80 °C for 2 h after which the solvents were removed under reduced pressure and the residue was purified by preparative RP-HPLC, and Boc/Trt-protected bicyclic hexapeptide **14b** was obtained as a white fluffy solid after lyophilization (1.2 mg, 11%).

3.4.2.15 Bicyclic hexapeptide 14c: Compound **3c** (5 mg, 0.004 mmol) and $[Cp^*RuCl]_4$ (0.87 mg, 0.008 mmol) were dissolved in THF/MeOH (2 mL, 19:1 v/v).

The obtained reaction mixture was heated by microwave irradiation at 80 °C for 2 h after which the solvents were removed under reduced pressure and the residue was purified by preparative RP-HPLC, and Boc/Trt-protected bicyclic hexapeptide **14c** was obtained as a white fluffy solid after lyophilization (0.7 mg, 14%).

3.4.2.16 Deprotected bicyclic hexapeptide 2a: Compound **14a** (1.0 mg, 0.001 mmol) was treated with TFA/CH_2Cl_2 (1 mL, 1:9 v/v) for 1 h, after which the volatiles were removed under reduced pressure. Deprotected bicyclic hexapeptide **2a** was obtained and its identity was confirmed by HR-MS. HR-MS (ESI) *m/z* calcd for $C_{41}H_{61}N_{13}O_7$ $[M + H]^+$ 848.4895, found 848.4892.

3.4.2.17 Deprotected bicyclic hexapeptide 2b: Compound **14b** (1.2 mg, 0.0012 mmol) was treated with TFA/CH_2Cl_2 (1 mL, 1:1 v/v) for 1 h, after which the volatiles were removed under reduced pressure. Deprotected bicyclic hexapeptide **2b** was obtained and its identity was confirmed by HR-MS. HR-MS (ESI) *m/z* calcd for $C_{45}H_{60}N_{14}O_8$ $[M + H]^+$ 925.4797, found 925.4809.

3.4.2.18 Deprotected bicyclic hexapeptide 2c: Compound **14c** (0.7 mg, 0.0012 mmol) was treated with TFA/CH_2Cl_2 (1 mL, 1:1 v/v) for 1 h, after which the volatiles were removed under reduced pressure. Deprotected bicyclic hexapeptide **2c** was obtained and its identity was confirmed by HR-MS. HR-MS (ESI) *m/z* calcd for $C_{44}H_{58}N_{14}O_8$ $[M + H]^+$ 911.4640, found 911.4643.

3.5 References

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Chapter 4

Synthesis of bicyclic tripeptides inspired by the ABC ring system of vancomycin through ruthenium-based cyclization chemistries

This chapter is based on: Xin Yang, Lucas P. Beroske, Johan Kemmink, Dirk T. S. Rijkers, Rob M. J. Liskamp. 'Synthesis of bicyclic tripeptides inspired by the ABC-ring system of vancomycin through ruthenium-based cyclization chemistries', in: *Tetrahedron Letters* **2017**, *58*, 4542 - 4546.

4.1 Introduction

Cyclic peptides are increasingly important and serve as relevant lead structures and bioactive molecules in medicinal chemistry and drug design since they offer a plethora of biological activities and are especially interesting for the modulation of protein-protein interactions.[1] Their (total) synthesis, however, is still a highly challenging task and the development of novel and efficient cyclization approaches is an active field of research.[1f] Within this class, multicyclic as well as side-chain knotted cyclic peptides, including the conotoxins and cyclotides,[2] lantibiotics,[3] and glycopeptide antibiotics,[4] are of special interest since they combine extreme potency with shape persistent folding of the peptide backbone. The most classical and outstanding example of the effects of macrocyclization and side-chain knotting is found in the heptapeptide vancomycin.[5] These lead to an almost absolute control of shape and folding of this glycopeptide antibiotic and allow for a very strong binding of the rather flexible natural target ~Lys-D-Ala-D-Ala-OH.[6]

Over the years we have been inspired by cyclized peptides such as vancomycin as well as nisin and have explored alternative approaches for peptide cyclization, among others, Sonogashira cross-coupling,[7] ring closing metathesis,[8] and Cu^{+} as well as Ru^{2+} catalyzed azide-alkyne cycloadditions,[9] to obtain highly constrained side-chain to side-chain knotted peptides. Previously, we have shown that alkyne-, alkene- and triazole-tethered cyclic tri-, hexa- and heptapeptides could be synthesized that mimic vancomycin by binding ~Lys-D-Ala-D-Ala-OH and ~Lys-D-Ala-D-Lac-OH. To further increase the rigidity of these mimics, we were looking for complementary cyclization approaches for introducing an additional cyclic constraint to control the sequence of cyclization and thereby the folding topology of the peptide backbone. So far, most vancomycin mimics, including our own, have focused mainly on the CDE-ring system.[10] However, the ABC-ring system probably provides the additional needed rigidity in the form of a lid, making vancomycin more of a clam to hold ~Lys-D-Ala-D-Ala-OH more permanently by reducing the off-rate. Herein, we describe our efforts towards effective mimicry of the ABC-ring system,[10h, 11, 12] which ultimately combined with DE-mimicry should lead to potent vancomycin mimics.

4.2 Results and Discussion

Previously, we have reported the synthesis of triazole-containing vancomycin mimics like **2** (Figure 1).[9b] Despite their bicyclic framework these structures are still relatively flexible, since mimics such as **2** do bind Ac-Lys(Ac)-D-Ala-D-Ala-OH albeit with a lower affinity than vancomycin as judged by isothermal microcalorimetry (ITC). In order to include the AB-ring system,[11] we wished to apply RCM, which we and others have successfully reported on several occassions.[13] This led to the target bicyclic tripeptide **3**. Retrosynthesis showed that bicycle **3** might be accessible through two consecutive macrocyclization steps starting from **4** (Figure 1). Since the preferred order of the macrocyclization steps, by RCM or RuAAC,[14] was not known (*vide infra*), precursor dipeptide **5** was proposed, in order to optimize the RCM in the presence of an azide moiety (Figure 1).

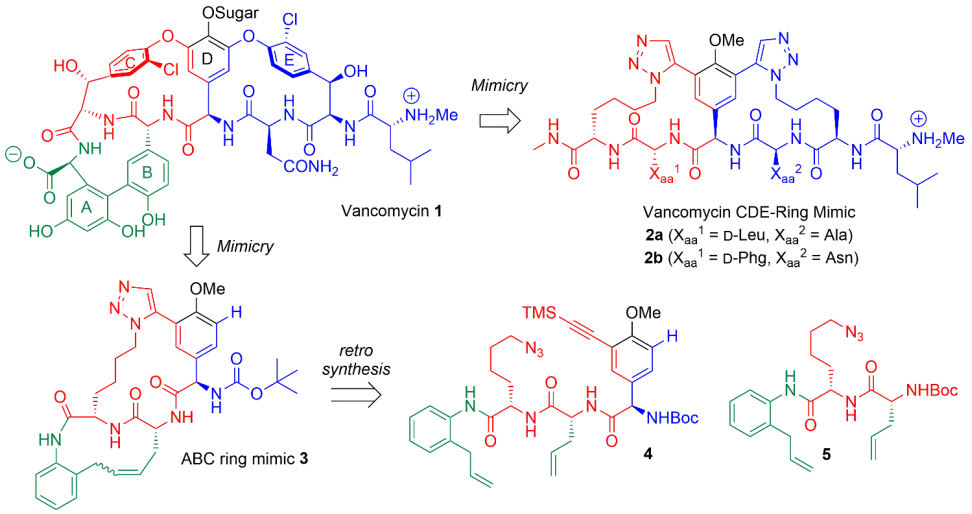
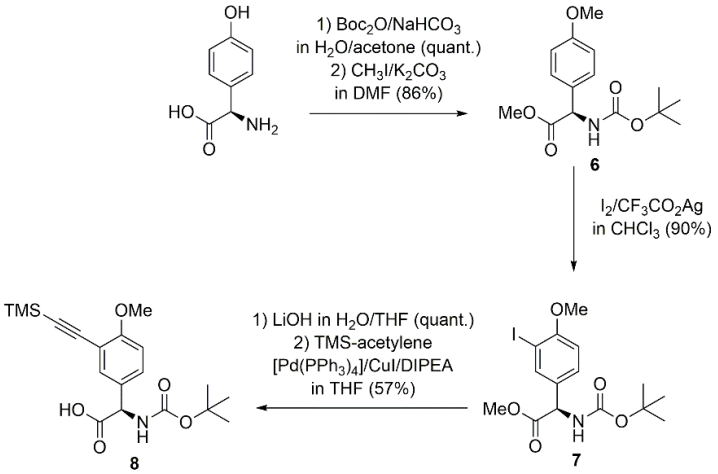


Figure. 1. Design of vancomycin mimics.

TMS-protected alkyne **8** was conveniently accessible from commercially available 4-hydroxy-D-phenylglycine (Scheme 1). After protection of the amine group, conversion of the phenolic hydroxy group to a methyl ether and preparation of the methyl ester, amino acid derivative **6** was subjected to iodination, according to Nicolaou and co-workers,[15] to give mono-iodo compound **7** in high yield (90%).

After saponification of the methyl ester, the alkyne was installed by a Pd-catalyzed Sonogashira cross-coupling reaction to give protected phenylglycine building block **8** in an acceptable yield of 57%.[16]

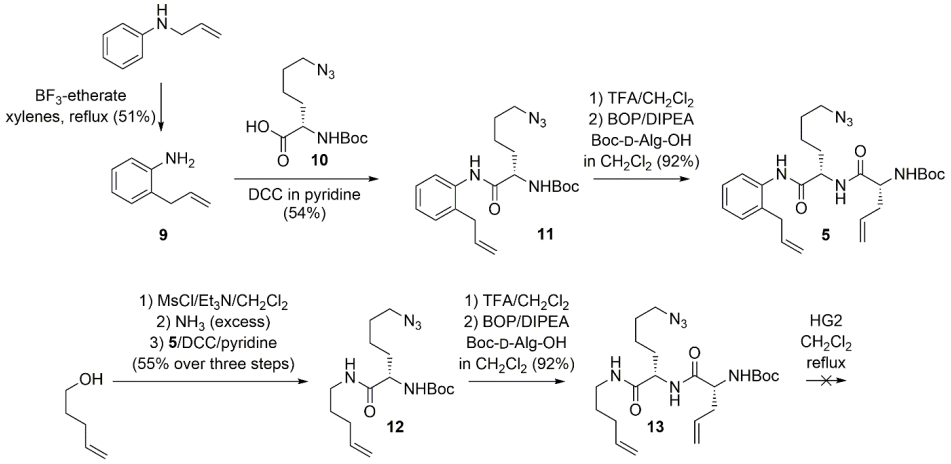


Scheme 1. Synthesis of phenylglycine building block **8**.

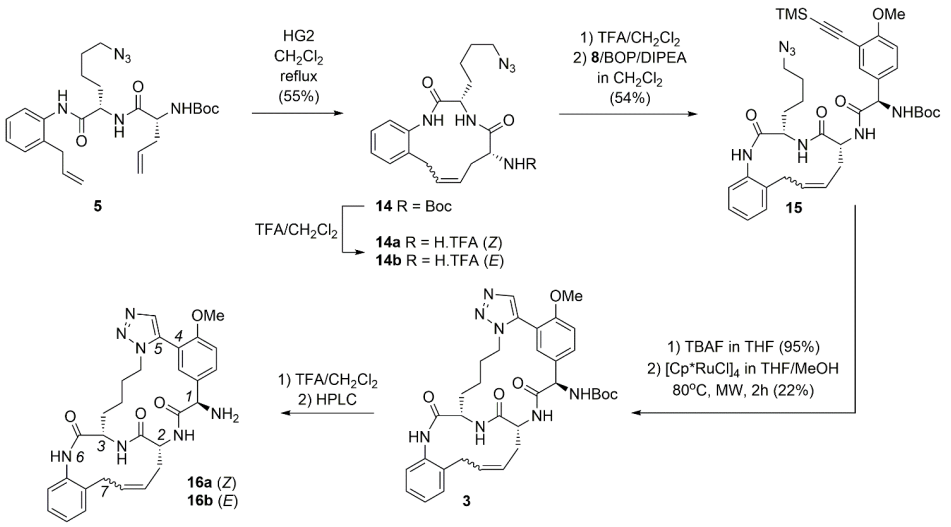
The synthesis of the required RCM-precursor **5** is shown in Scheme 2. To this end 2-allylaniline **9** was obtained by a Claisen rearrangement according to Brucelle and Renaud.[17] In a first attempt to couple aniline **9** to azido acid **10**,[18] BOP/DIPEA as a coupling reagent did not afford anilide **11**. Therefore, DCC in pyridine was used to form the anilide **11** in 54% yield since this combination of coupling reagent/solvent was effective in the coupling of the poor nucleophile 4-nitroaniline with amino acids.[19] After deprotection of anilide **11** by treatment with TFA, Boc-D-Alg-OH was coupled using BOP/DIPEA, and bisalkene dipeptide **5** was obtained in 92% yield over two steps. To anticipate on the rigidity of precursor **5** that might hamper macrocyclization by RCM, the more flexible congener **13** was also synthesized using pent-4-en-1-amine as the alkene (Scheme 2). The latter amine was prepared from the corresponding alcohol featuring a mesylation followed by aminolysis with ammonia.[20]

As RCM precursor peptide **5** contained an azide moiety, the first[21] and second[22] generation Grubbs catalysts could not be used since the tricyclohexylphosphine

ligands would likely reduce the azide into an amine *via* a Staudinger reduction.[23] Therefore, the second generation Hoveyda-Grubbs catalyst[24] was used in refluxing CH_2Cl_2 and macrocyclic peptide **8** was isolated in 55% yield as a mixture of the *E*/*Z* diastereoisomers, as shown in Scheme 3 (**14a** (*Z*):**14b** (*E*) = 1:2.3). After Boc removal both diastereoisomers **14a** and **14b** could be separated by preparative HPLC and were characterized by NMR and LC-(HR)MS.



Scheme 2. Synthesis of RCM precursor dipeptide **5** and **13**.



Scheme 3. RCM–coupling–RuAAC strategy for the synthesis of bicyclic tripeptide **3**.

In its protected form, both diastereoisomers (R_t 32.97 and 33.36 min) of macrocycle **14** could not be separated (Figure 2a). However, when an aliquot of **14** was treated with TFA to remove the Boc functionality, this resulted in a base line separation of both diastereoisomers **14ab** (R_t 22.24 and 22.98 min, respectively, Figure 2b). The ^{1}H NMR spectrum (as measured in DMSO-d_6) of diastereoisomer **14a** gave broad peaks at 25 ˚C, fortunately at higher temperature, peak splitting was observed and at 80 ˚C a well-defined *J* coupling of the olefinic protons could be derived (Figure 3). This value, ~8 Hz, corresponded to the *Z*-configuration of the double bond. The proton spectrum of diastereoisomer **14b** had resulted already at 25 ˚C in a well-defined *J* coupling of ~13 Hz for the alkene bond indicating an *E*-geometry of **14b** (Figure 4).[25]

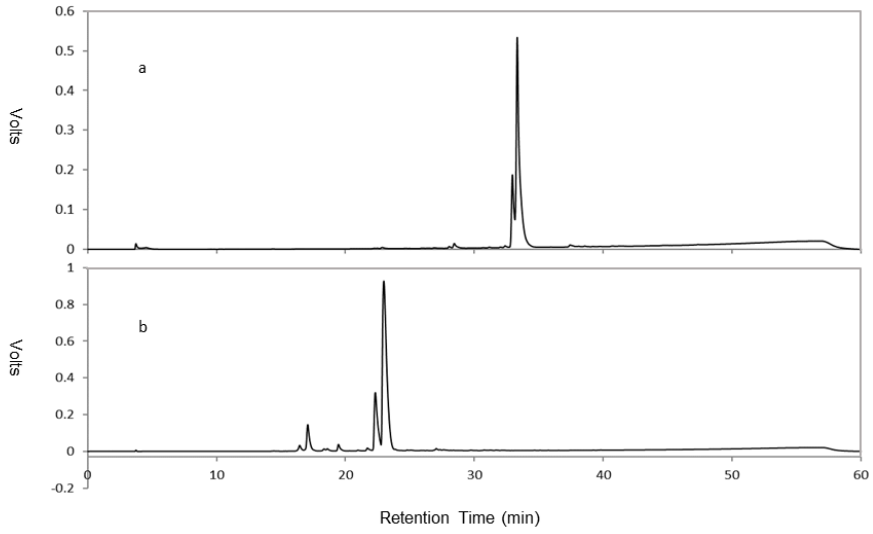


Figure 2. Analytical HPLC chromatogram of compound **14** (Figure 2a) and compound **14ab (**Figure 2b).

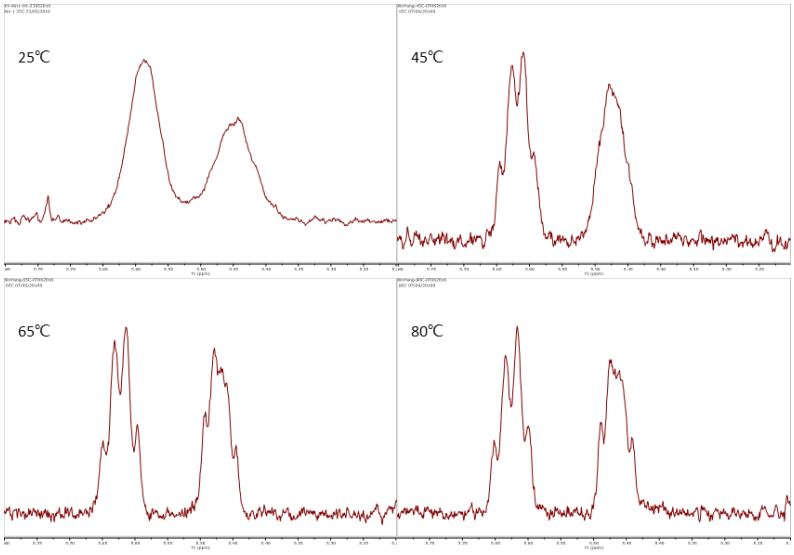


Figure 3. Detail of the proton NMR spectrum of **14a** indicating the chemical shift of the two *Z*-olefinic protons as function of the temperature.

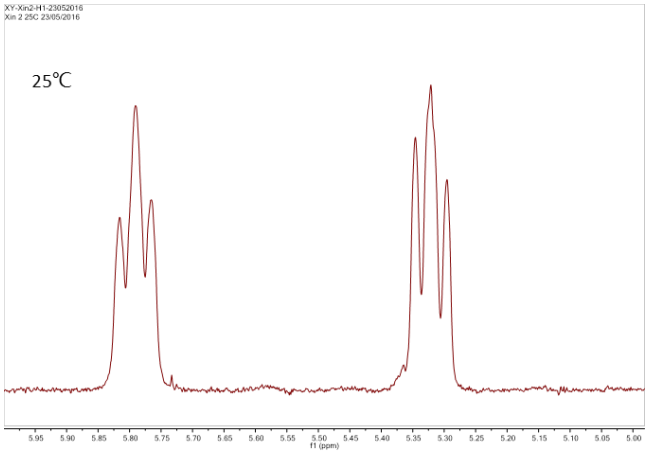


Figure 4. Detail of the proton NMR of **14b** indicating the chemical shift of the two *E*-olefinic protons at 25 ℃.

Thus, both diastereoisomers of macrocycle **14** were treated with TFA and the resulting free α-amine coupled to alkyne derivative **8** in the presence of

BOP/DIPEA to afford the protected click precursor **15** in 54% overall yield (Scheme 3). Then, tripeptide **15** was treated with TBAF to remove the TMS functionality, and the unprotected alkyne which was isolated in 95% yield after column chromatography, was subjected to RuAAC in THF/MeOH at 80 ˚C under microwave irradiation in the presence of 10 mol% $[Cp^*RuCl]_4$. Bicyclic tripeptide **3** was isolated in 22% yield after column chromatography as a mixture of *E*/*Z* diastereoisomers. Heating at 80 ˚C under microwave irradiation in the absence of $[Cp^*RuCl]_4$ did not lead to any conversion. To improve the yield of cyclization, lower and higher catalyst loadings (5 and 15 mol%, respectively) were used which turned out to be ineffective since incomplete conversion of the starting material (at 5 mol%) or extensive formation of baseline compounds (at 15 mol%) were observed. As a control experiment, the unprotected alkyne was also subjected to regular CuAAC[26] in the presence of either CuI or $[Cu(CH_3CN)_4]PF_6$ as the Cu^+ species, these reaction conditions did not lead to the formation of a bicyclic 1,4-triazole bridged tripeptide. This experiment showed that a 1,5-triazole moiety with a curved geometry was essential for ring closure since the more linear geometry of the 1,4-triazole was clearly incompatible with the topology of the bicyclic framework of tripeptide **3**. Similar to macrocycle **14**, the individual diastereoisomers of bicyclic tripeptide **16** (*Z*-**16a** and *E*-**16b**, respectively) could be obtained after Boc removal of **3** and purification by preparative HPLC. It is interesting to note that the *Z*:*E* ratio during the conversion of **14a**, **b** into **16a**, **b** shifted from 1:2.3 to 2:1, an indication that the *Z*-geometry of the alkene was favored in the bicyclic tripeptide topology.

Since bicyclic tripeptide **16** can be used as a versatile building block in the synthesis of tricyclic heptapeptides to mimic the side chain to side-chain connectivity pattern of vancomycin, hydrogenation of the double bond to an alkane bridge would be desirable to obtain a single isomer instead of an *E*/*Z* mixture of diastereoisomers. Therefore, several hydrogenation conditions in the presence of Pd/C, Raney Ni, and $Pd(OH)_2$ were investigated, unfortunately all were unsuccessful.

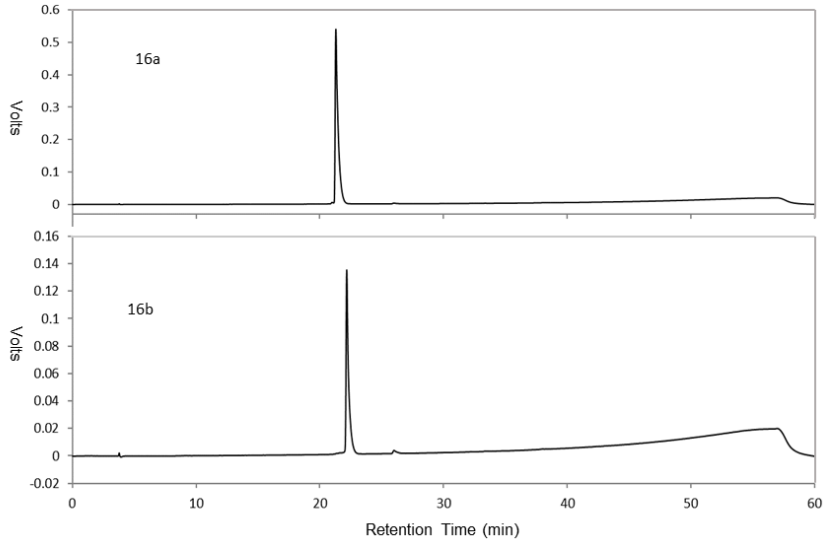
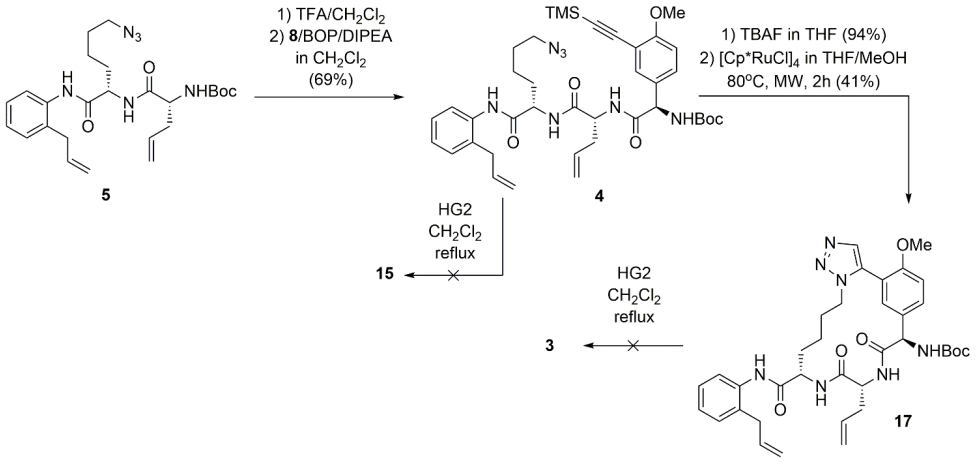


Figure 5. Analytical HPLC chromatogram of compound **16a** (R_t = 21.28 min) and **16b** (R_t =22.17 min) after purification by preparative RP-HPLC.

Two reverse reaction sequences to arrive at the desired bicyclic tripeptide **3** were also investigated, both starting from linear dipeptide **5** (Scheme 4). As the first step, Boc-protected **5** was treated with TFA and the resulting amine coupled to alkyne derivative **8** in the presence of BOP/DIPEA to afford tripeptide **4** in 69% yield after purification by column chromatography. RCM of precursor peptide **4** in the presence of second generation Hoveyda-Grubbs catalyst in CH_2Cl_2 or 1,2-dichloroethane did not result in the formation of alkene-bridged macrocycle **15**. The progress of the reaction was monitored by LCMS, and although starting material had disappeared, the desired bicyclic peptide **15** could not be observed. In hindsight, it was assumed that the desired ene-ene RCM pathway was possibly overrun by the thermodynamically favored ene-yne reactivity to yield 2-silyl-substituted 1,3-dienes, which, however, were not identified.[27] Alternatively, TMS-protected precursor peptide **4** was treated with TBAF and subsequently subjected to RuAAC to install the triazole moiety as the cyclic constraint. Via this route, macrocycle **17** was obtained in an improved 41% yield compared to the above preparation of **3** (Scheme 3). Unfortunately, RCM of **17** did not result in the successful isolation of bicyclic tripeptide **3**. TLC analysis indicated that conversion

of the starting compound was incomplete and some baseline material was present, additionally the formation of bicyclic tripeptide **3** could not be observed by LCMS.



Scheme 4. Alternative RCM–coupling–RuAAC strategies for the synthesis of bicyclic tripeptide **3**.

The structures of bicyclic tripeptides **16a** (as the *Z*-diastereoisomer) and **16b** (as the *E*- diastereoisomer) were energy minimized using the simulated annealing protocol employing the AMBER99 force field using the YASARA Structure 10.5.2.1 software package.[28] The peptides were superimposed with the left half of the vancomycin-related balhimycin antibiotic comprising the ABC-ring system.[29] An RMSD of 0.76 and 0.58 Å over seven atoms was calculated (see Scheme 3 for atom numbering) of the superimpositions of **16a** and **16b**, respectively. To evaluate if this structural resemblance correlates with binding affinity toward Ac-Lys(Ac)-D-Ala-D-Ala-OH and Ac-Lys(Ac)-D-Ala-D-Lac-OH, isothermal microcalorimetry (ITC) was performed, as shown in Table 1.[30,31] Based on these data, mimics **16a** and **16b** still bind Ac-Lys(Ac)-D-Ala-D-Ala-OH appreciably, considering that a large part of the 'clam' is missing, albeit at least 100-fold less compared to vancomycin. Bicycle **16b** is somewhat more active than **16a**, while binding toward Ac-Lys(Ac)-D-Ala-D-Lac-OH was comparable for all three receptor molecules. This was in line with the MIC-values obtained from a growth inhibition assay[32] of the *Staphylococcus aureus* ATCC 49320 strain, where values of 300 μg/mL (**16b**), >300 μg/mL (**16a**) and 2 μg/mL (VM) were found, respectively. Although a reasonable structural resemblance was found, not

unexpectedly, for efficient binding (and activity) some extra factors need to be addressed such as the proper alignment of hydrogen bonding and further rigidification, possibly in attempts to combine the ABC and CDE-ring systems.[9b]

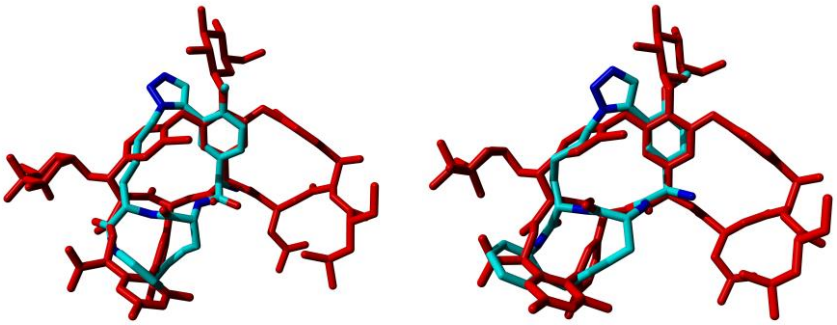


Figure 5. Superimposition of balhimycin (in red) with bicyclic tripeptide **16a** (left) and **16b** (right), respectively (see Scheme 3 for atom numbering). The carbon atoms αC^1, αC^2, αC^3, arom-C^4, triazole-C^5, N^6, benzylic-C^7 have been used as fixed coordinates for superimposition.

Table 1. The binding affinity as measured using ITC.

compound	ligand	K_a (M^{-1})[a]
VM	Ac-Lys(Ac)-D-Ala-D-Ala-OH	$(3.95\pm0.41)\times10^5$
VM	Ac-Lys(Ac)-D-Ala-D-Lac-OH	$(2.38\pm0.23)\times10^3$
16a	Ac-Lys(Ac)-D-Ala-D-Ala-OH	$(2.35\pm0.36)\times10^3$
16a	Ac-Lys(Ac)-D-Ala-D-Lac-OH	$(2.17\pm0.30)\times10^3$
16b	Ac-Lys(Ac)-D-Ala-D-Ala-OH	$(4.06\pm0.84)\times10^3$
16b	Ac-Lys(Ac)-D-Ala-D-Lac-OH	$(1.21\pm0.86)\times10^3$

[a]Measured in a Na-citrate/citric acid buffer (0.02 M, pH 5.1), VM: vancomycin.

4.3 Conclusion

In conclusion, bicyclic tripeptide **16** as a mimic of the ABC-ring system was successfully synthesized starting from precursor dipeptide **5** following an RCM-coupling-RuAAC strategy. Mimics of this part of the vancomycin structure

are less explored as only a single hydrogen bond contributes to the binding of Ac-Lys(Ac)-D-Ala-D-Ala-OH via the carbonyl oxygen of the lysine residue. The mixture of double bond isomers could be separated by HPLC to give each individual *E*/*Z* diastereoisomer as the free amine as judged by NMR and LC-MS. Bicyclic tripeptide **16** represents an important building block to ultimately arrive at a series of tricyclic heptapeptides for possible effective mimicry of vancomycin in which ruthenium-based cyclization approaches will be used to control the topology and rigidity of the peptide backbone.

4.4 Experimental Section

4.4.1 Chemicals and General Experimental Procedures

Chemicals were used as obtained from commercial sources without further purification unless stated otherwise. The solvents were obtained as peptide synthesis grade and stored on molecular sieves (4 Å). Column chromatography was performed using SiliFlash P60 silica gel (particle size 40-63 μm). TLC was performed on precoated silica gel 60F254 glass plates and compound spots were visualized by UV-quenching, ninhydrin, or Cl_2/TDM. The petrol ether (PE) fraction 40-60˚C was used as the eluent. Optical rotations were measured using a 10 cm cell with a Na 589 nm filter at the specific concentrations (in g/100 mL) as indicated. ^{1}H NMR spectra were recorded either on a 400 MHz or a 500 MHz spectrometer in $CDCl_3$, or DMSO-d_6 as solvent. Chemical shifts are reported in delta (δ) units, in parts per million (ppm) relative to TMS (0.00 ppm). Coupling constants (*J*) are reported in Hertz (Hz). ^{13}C NMR analysis was acquired on a 100 MHz or a 125 MHz spectrometer in $CDCl_3$, or DMSO-d_6 as solvent. Chemical shifts are reported in delta (δ) units, in parts per million (ppm) relative to the solvent residual signal, $CDCl_3$ (77.0 ppm) or DMSO-d_6 (39.0 ppm). Microwave reactions were performed in a pressure and temperature controlled microwave reactor at 300W. Analytical HPLC was performed on an automated HPLC system equipped with a UV/Vis detector operating at 220/254 nm and an evaporative light scattering detector using a C18 column (pore size: 100 Å, particle size: 5 μm; 250 × 4.6 mm) at a flow rate of 1 mL/min using a linear gradient of 100% buffer A (0.1% TFA in CH_3CN/H_2O 5:95 v/v) to 100% buffer B (0.1% TFA in CH_3CN/H_2O 95:5

v/v) in 50 min. Preparative RP-HPLC was performed on an automated preparative HPLC system equipped with a UV/Vis detector operating at 214 nm using a C18 column (pore size: 100 Å, particle size: 10 µm; 250 × 22 mm) at a flow rate of 12.0 mL/min using a linear gradient of 100% buffer A (0.1% TFA in CH_3CN/H_2O 5:95 v/v) to 100% buffer B (0.1% TFA in CH_3CN/H_2O 95:5 v/v) in 50 min. LC-MS(MS) was performed using a C18 column (pore size: 100 Å, particle size: 5 µm; 250 × 4.6 mm) at a flow rate of 1 mL/min using a linear gradient of of 100% buffer A (0.1% TFA in CH_3CN/H_2O 5:95 v/v) to 100% buffer B (0.1% TFA in CH_3CN/H_2O 95:5 v/v) in 60 min. ESI-MS was performed on a bench-top electrospray ionization mass spectrometer. High-resolution mass spectrometry (HRMS) analysis was performed using an ESI-TOF LC/MS instrument.

4.4.2 Syntheses and Compound Analyses

Compounds 2-allylaniline (**9**) and Boc-Lys(N_3)-OH (**10**) were synthesized according to Brucelle and Renaud[17] and Zhang et al.[9a], respectively.

4.4.2.1 *N*-α-Boc-4-methoxy-D-phenylglycine methyl ester (6): To a solution of 4-hydroxy-D-phenylglycine (8.4 g, 50 mmol) in a mixture of acetone /H_2O (1:1 v/v, 500 mL), solid $NaHCO_3$ (6.3 g, 75 mmol) and di-*tert*-butyl dicarbonate (Boc_2O, 12 g, 55 mmol) were added successively and the obtained reaction mixture was stirred overnight at room temperature. Then, the reaction mixture was concentrated to the half of its original volume by evaporation and the aqueous solution was acidified to pH 2 by adding 1N $KHSO_4$ and subsequently extracted with EtOAc (2 × 200 mL). The combined organic layers were dried (Na_2SO_4), filtered and concentrated in vacuo to obtain *N*-α-Boc-4-hydroxy-D-phenylglycine quantitatively. In the next step, *N*-α-Boc-4- hydroxy-D-phenylglycine (5.0 g, 18.7 mmol) was dissolved in DMF (250 mL) and to this solution, K_2CO_3 (5.8 g, 42 mmol, 2.25 equiv) followed by CH_3I (2.6 mL, 42 mmol, 2.25 equiv) were added. The obtained reaction mixture was stirred for 1.5 h at room temperature after which it was poured into water (500 mL) under vigorous stirring. The aqueous solution was extracted with EtOAc (3 × 300 mL) and the combined organic layers were subsequently washed with H_2O (2 × 200 mL) and brine (200 mL), dried (Na_2SO_4), filtered and evaporated under reduced pressure. The residue was purified by column chromatography (PE/EtOAc,

6:1 v/v) to give compound **6** as a colorless liquid in 86% yield (4.75 g). R_f 0.50 (PE/EtOAc, 3:1 v/v); $[\alpha]^{D}_{20}$ = –112.9 (*c* 1.0, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$) δ = 7.26-7.25 (m, 2H), 6.86-6.82 (m, 2H), 5.49 (d, *J* = 7.2 Hz, 1H), 5.23 (d, *J* = 7.4 Hz, 1H), 3.76 (s, 3H), 3.68 (s, 3H), 1.40 (s, 9H); ^{13}C NMR (100 MHz, $CDCl_3$) δ = 171.8, 159.6, 154.8, 128.9, 128.3, 114.2, 80.1, 58.3, 57.0, 55.2, 52.6, 28.3; HRMS *m/z* calcd for $C_{15}H_{21}NNaO_5$ $[M + Na]^+$ 318.1317, found 318.1353.

4.4.2.2 *N*-α-Boc-3-iodo-4-methoxy-D-phenylglycine methyl ester (7): This synthesis was performed according to a procedure described by Nicolaou et al.[15] Methyl ester **6** (1.5 g, 5.0 mmol) was dissolved in $CHCl_3$ (100 mL) and to this solution, silver trifluoroacetate (6.2 g, 11.2 mmol) followed by I_2 (1.52 g, 6.0 mmol) were added. The reaction mixture was stirred for 20 h at room temperature, and subsequently quenched by adding a solution of sat. aq. $Na_2S_2O_3$ (100 mL). After phase separation, the resulting aqueous phase was extracted with $CHCl_3$ (2 × 100mL) and the combined organic layers were dried (Na_2SO_4), filtered and concentrated by evaporation. The residue was purified by column chromatography (PE/EtOAc, 6:1 v/v) to give *N*-α-Boc-3-iodo-4-methoxy- D-phenylglycine methyl ester **7** as a yellowish solid in 90% yield (1.9 g). R_f 0.45 (0.1% AcOH in PE/EtOAc, 1:1 v/v); $[\alpha]^{D}_{20}$ = –116.2 (*c* 1.0, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$) δ = 7.72 (d, *J* = 2.19 Hz, 1H), 7.28 (dd, *J* = 8.51 and 2.22 Hz, 1H), 6.74 (d, *J* = 8.49 Hz, 1H), 5.55 (d, *J* = 7.22 Hz, 1H), 5.18 (d, *J* = 7.25 Hz, 1H), 3.83 (s, 3H), 3.69 (s, 3H), 1.38 (s, 9H); ^{13}C NMR (100 MHz, $CDCl_3$) δ = 171.3, 158.1, 137.9, 131.0, 128.5, 110.9, 86.3, 80.2, 56.4, 56.3, 52.8, 28.3; HRMS *m/z* calcd for $C_{15}H_{20}INNaO_5$ $[M + Na]^+$ 444.0284, found 444.0341.

4.4.2.3 *N*-α-Boc-3-((trimethylsilyl)ethynyl)-4-methoxy-D-phenylglycine (8): Methyl ester **7** (1.2 g, 2.85 mmol) was dissolved in a mixture of THF/H_2O (60 mL, 1:1 v/v) and the solution was cooled to 0˚C and LiOH·H_2O (179 mg, 4.27 mmol) was added as a single portion. After stirring for 30 min at room temperature, the reaction mixture was acidified by a solution of citric acid (10 wt-%) until pH 2-3 was reached. Subsequently, the aqueous phase was extracted with EtOAc (3 × 100 mL) and the combined organic layers were washed with H_2O (100 mL) and brine (100 mL), dried (Na_2SO_4), filtered and evaporated to dryness to give the corresponding acid as a white solid in quantitative yield. ^{1}H NMR (400 MHz,

$CDCl_3$) δ = 7.98 (s, 1H), 7.85-7.77 (m, 1H), 7.35 (d, *J* = 8.55 Hz, 1H), 6.77 (d, *J* = 8.55 Hz, 1H), 5.00 (d, *J* = 4.79 Hz, 1H), 3.85 (s, 3H), 1.23 (s, 9H); ^{13}C NMR (100 MHz, $CDCl_3$) δ = 173.3, 157.8, 156.8, 138.3, 132.4, 128.3, 110.6, 85.6, 82.0, 57.5, 56.4, 28.3, 28.2, 28.0. In the next step, a dry flask was loaded with *N*-α-Boc-3-iodo-4-methoxy-D-phenyl-glycine (1.1 g, 2.7 mmol), [$Pd(PPh_3)_4$] (317 mg, 0.27 mmol, 0.10 equiv) and CuI (157 mg, 0.82 mmol, 0.30 equiv) and sealed with a rubber septum. The flask was evacuated and refilled with dry N_2 (repeated three times). Then, THF (40 mL) (purged with dry N_2 for 1 h prior to use) was added to the flask via a syringe and the resulting solution was degassed by three freeze-pump-thaw cycles. Subsequently, DIPEA (0.95 mL, 5.5 mmol) and TMS-acetylene (1.14 mL, 8.23 mmol, 3.0 equiv) were added to the mixture via a syringe. The obtained reaction mixture was stirred for 20 h at room temperature under N_2 and the resulting suspension was filtered through a path of celite and the filtrate was evaporated to dryness. The residue was redissolved in EtOAc (50 mL) and this solution was successively washed with 1N $KHSO_4$ (2 × 50 mL), sat. $NaHCO_3$ (2 × 50 mL) and brine (50 mL), and dried over anhydrous Na_2SO_4. After filtration and removal of the solvent, the residue was purified by column chromatography (CH_2Cl_2/MeOH/AcOH, 98:2:0.1 v/v/v). *N*-α-Boc-3-((trimethylsilyl)ethynyl)-4-methoxy- D-phenylglycine was obtained as a yellowish solid in 57% yield (580 mg). R_f 0.45 (0.1% AcOH in PE/EtOAc, 1:1 v/v); $[\alpha]^{D}_{20}$ = –56.4 (*c* 1.0, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$) δ = 7.96 (s, 1H), 7.49-7.43 (m, 1H), 7.33-7.27 (m, 1H), 6.79 (d, *J* = 8.6 Hz, 1H), 5.00 (d, *J* = 5.0 Hz, 1H), 3.84 (s, 3H), 1.23 (s, 9H), 0.24 (s, 9H); ^{13}C NMR (100 MHz, $CDCl_3$) δ = 173.4, 160.0, 156.9, 133.2, 130.2, 128.7, 112.3, 111.0, 81.8, 80.4, 57.9, 55.9, 28.0, 0.03; HRMS *m/z* calcd for $C_{19}H_{27}NNaO_5Si$ [*M* + Na]$^+$ 400.1556, found 400.1615.

4.4.2.4 Boc-Lys(N_3)-(2-allyl)anilide (11): This synthesis was performed according to a procedure described by Rijkers et al.[19] Boc-Lys(N_3)-OH (**5**) (3.6 g, 13.2 mmol) and 2-allylaniline (**9**) (1.46 g, 11 mmol) were dissolved in pyridine (80 mL). To this solution DCC (2.72 g, 13.2 mmol) was added and the reaction mixture was stirred for 20 h at room temperature. Then, pyridine was removed under reduce pressure and the residue was redissolved in EtOAc (100 mL). This solution was successively washed with $KHSO_4$ (3 × 100 mL), sat. $NaHCO_3$ (3 × 100 mL) and brine (100 mL). The organic layer was dried (Na_2SO_4), filtered and concentrated in

vacuo. The residue was purified by column chromatography (CH_2Cl_2/MeOH, 99:1 v/v) to give anilide **11** as a yellowish oil in 54% yield (2.3 g). R_f 0.47 (CH_2Cl_2/MeOH, 98:2 v/v); $[\alpha]^D_{20}$ = –28.1 (*c* 1.0, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$) δ = 7.96 (s, 1H), 7.86 (d, *J* = 8.05 Hz, 1H), 7.25-7.21 (m, 1H), 7.17-7.07 (m, 2H), 6.00-5.90 (m, 1H), 5.16-5.15 (m 2H), 5.00 (s, 1H), 4.18 (s, 1H), 3.36-3.34 (m, 2H), 3.30-3.23 (m, 2H), 2.01-1.93 (m, 2H), 1.73-1.58 (m, 4H), 1.45 (s, 9H); ^{13}C NMR (100 MHz, $CDCl_3$) δ = 170.2, 155.8, 153.4, 135.9, 135.4, 130.4, 130.1, 127.3, 125.4, 123.4, 116.6, 80.4, 55.0, 51.1, 36.3, 31.6, 28.5, 22.9; HRMS *m/z* calcd for $C_{20}H_{29}N_5NaO_3$ [*M* + Na]$^+$ 410.2168, found 410.2189.

4.4.2.5 Boc-Alg-Lys(N_3)-(2-allyl)anilide (5): Compound **11** (2.0 g, 5.16 mmol) was dissolved in CH_2Cl_2 (50 mL) and TFA (50 mL) was added. The reaction mixture was stirred for 1 h at room temperature, after which TFA was evaporated and the obtained residue was coevaporated with CH_2Cl_2 (4 × 50 mL) to remove any residual TFA. After drying for 1 h at high vacuum, the amine was redissolved in CH_2Cl_2 and to this solution, Boc-Alg-OH (1.56 g, 7.22 mmol, 1.40 equiv), BOP (3.2 g, 7.22 mmol, 1.40 equiv) and DIPEA (2.24 mL, 12.9 mmol, 2.50 equiv) were added. The obtained reaction mixture was stirred for 20 h at room temperature. Then, CH_2Cl_2 was evaporated and the residue was redissolved in EtOAc (150 mL) and this solution was successively washed with 1N $KHSO_4$ (3 × 150 mL), sat. $NaHCO_3$ (3 × 150 mL) and brine (150 mL). The organic layer was dried over Na_2SO_4 followed by filtration and evaporated to dryness. The residue was purified column chromatography (CH_2Cl_2/MeOH, 98:2 v/v) to give dipeptide **5** as a colorless oil in 92% yield (2.3 g). R_f 0.44 (CH_2Cl_2/MeOH, 98:2 v/v); $[\alpha]^D_{20}$ = –4.2 (*c* 1.0, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$) δ = 8.08 (s, 1H), 7.70 (d, *J* = 8.0 Hz, 1H), 7.21-7.08 (m, 2H), 6.98 (d, *J* = 8.0 Hz, 1H), 5.98-5.88 (m, 1H), 5.75-5.64 (m, 1H), 5.15-4.99 (m, 5H), 4.49 (dt, *J* = 7.9 and 5.8 Hz, 1H), 4.17 (q, *J* = 6.7 Hz, 1H), 3.33 (t, *J* = 4.0 Hz, 2H), 3.26-3.23 (m, 2H), 2.56-2.41 (m, 2H), 1.83-1.46 (m, 5H), 1.40 (s, 9H); ^{13}C NMR (100 MHz, $CDCl_3$) δ = 172.0, 169.5, 155.5, 135.9, 135.2, 132.8, 130.4, 127.2, 125.7, 123.9, 119.3, 116.6, 80.5, 56.2, 53.6, 51.1, 36.7, 36.1, 31.1, 28.2, 22.8; HRMS *m/z* calcd for $C_{25}H_{36}N_6NaO_4$ [*M* + Na]$^+$ 507.2696, found 507.2728.

4.4.2.6 Boc-Lys(N_3)-*N*-(pent-4-en-1-yl) amide (12): Pent-4-en-1-ol (5.00 g, 58 mmol) was dissolved in CH_2Cl_2 (100 mL) and to this solution Et_3N (9.7 mL, 69.6 mmol, 1.2 equiv) was added and the mixture was subsequently cooled on ice. Then, methanesulfonyl chloride (5.4 mL, 69.6 mmol, 1.2 equiv) was added dropwise and the reaction mixture was stirred for 2 h at room temperature. After completion of the reaction, the reaction mixture was quenched with saturated $NaHCO_3$ (50 mL) and the aqueous phase was extracted with CH_2Cl_2 (2 × 50 mL) and the combined organic layers were dried (Na_2SO_4), filtrated and concentrated in vacuo. The residue was purified by column chromatography (PE/EtOAc, 1:1 v/v) to give pent-4-en-1-yl methanesulfonate as a white solid in high yield (94%, 8.90 g). In the next step the mesylate, (1.00 g, 6.10 mmol) was dissolved in MeOH (10 mL) and 25% aqueous ammonia (10 mL) was added, and the obtained mixture was stirred for 18 h at room temperature. Subsequently, the volatiles were removed by coevaporation with toluene (3 × 20 mL) and crude pent-4-en-1-amine was directly used in the next step by adding pyridine (60 mL) followed by Boc-Lys(N_3)-OH (1.70 g, 6.10 mmol) and DCC (1.50 g, 7.30 mmol, 1.2 equiv). The obtained reaction mixture was stirred for 20 h at room temperature and finally evaporated to dryness. The residue was redissolved in EtOAc (100 mL) and this solution was subsequently washed with $KHSO_4$ (3 × 100 mL), $NaHCO_3$ (3 × 100 mL) and brine (100 mL). The organic layer was dried (Na_2SO_4) filtered and concentrated in vacuo. The residue was purified by column chromatography (CH_2Cl_2/MeOH, 99:1 v/v) to give Boc-Lys(N_3)-*N*-(pent-4-en-1-yl) amide as a yellowish oil in 58% yield (1.20 g) over two steps. R_f 0.78 (CH_2Cl_2/MeOH, 95:5 v/v); $[\alpha]^D_{20}$ = –19.2 (*c* 1.0, $CHCl_3$); ^{1}H-NMR (400 MHz, $CDCl_3$) δ = 6.74 (s, 1H), 5.76-5.66 (m, 1H), 5.36 (d, *J* = 6.7 Hz, 1H), 4.97-4.89 (m, 2H), 4.05 (t, *J* = 6.0 Hz, 1H), 3.25-3.09 (m, 4H), 2.06-1.94 (m, 2H), 1.81-1.68 (m, 1H), 1.60-1.50 (m, 7H), 1.49 (s, 9H); ^{13}C-NMR (100 MHz, $CDCl_3$) δ = 172.0, 155.8, 153.4, 137.5, 115.1, 79.8, 54.2, 51.1, 38.9, 32.2, 30.9, 28.6, 28.5, 28.3, 22.8; HRMS *m/z* calcd for $C_{16}H_{29}N_5NaO_3$ [*M* + Na]$^+$ 362.2168, found 362.2141.

4.4.2.7 Boc-D-Alg-Lys(N_3)-*N*-(pent-4-en-1-yl) amide (13): Compound **12** (0.50 g, 1.47 mmol) was dissolved in CH_2Cl_2/TFA (20 mL, 1:1 v/v) and the reaction mixture was stirred for 1 h at room temperature, after which the volatiles were evaporated followed by coevaporation with CH_2Cl_2 (4 × 10 mL) to remove any

residual TFA and drying for 1 h at high vacuum. In the next step, the trifluoroacetate salt was redissolved in CH_2Cl_2 and to this solution, Boc-D-Alg-OH (0.44 g, 2.06 mmol), BOP (0.91 g, 2.06 mmol) and DIPEA (0.64 mL, 3.68 mmol, 1.80 equiv) were added. This reaction mixture was stirred for 20 h at room temperature. Subsequently, the solvent was removed by evaporation and the residue was redissolved in EtOAc (50 mL) and this solution was washed with $KHSO_4$ (3 × 50 mL), $NaHCO_3$ (3 × 50 mL) and brine (50 mL), dried (Na_2SO_4) filtered and concentrated to dryness. The residue was purified by column chromatography (CH_2Cl_2/MeOH, 98:2 v/v) to give dipeptide **13** as a colorless oil in 73% yield (0.47 g). R_f 0.73 (CH_2Cl_2/MeOH, 95:5 v/v); $[\alpha]^D_{20}$ = –8.2 (*c* 1.0, $CHCl_3$); ^{1}H-NMR (400 MHz, $CDCl_3$) δ = 6.87 (d, *J* = 8.4 Hz, 1H), 6.75 (s, 1H), 5.80-5.64 (m, 2H), 5.16-5.08 (m, 3H), 5.02-4.93 (m, 2H), 4.39 (double t, *J* = 8.1 Hz, *J* = 5.7 Hz, 1H), 4.09 (q, *J* = 6.5 Hz, 1H), 3.26-3.18 (m, 4H), 2.53-2.38 (m, 2H), 2.06-2.01 (m, 2H), 1.91-1.83 (m, 1H), 1.69-1.44 (m, 7H), 1.40 (s, 9H); ^{13}C-NMR (100 MHz, $CDCl_3$) δ = 171.7, 171.0, 155.8, 137.5, 132.7, 119.2, 115.2, 80.3, 54.3, 53.0, 51.1, 49.1, 39.1, 36.6, 33.9, 31.6, 31.0, 28.6, 28.5, 28.2, 25.6, 24.9, 22.7; HRMS *m/z* calcd for $C_{21}H_{36}N_6NaO_4$ $[M + Na]^+$ 459.2696, found 459.2704.

4.4.2.8 Cyclic dipeptide (14): Bisalkene **5** (760 mg, 1.57 mmol) was dissolved in CH_2Cl_2 (100 mL) and the solution was purged for 25 min with dry N_2. Then, a single portion of Hoveyda-Grubbs 2G catalyst (98.3 mg, 0.157 mmol) was added, and the reaction mixture was purged for an additional 10 min with N_2 and subsequently stirred under reflux for 20 h. Then, the solvent was removed under reduced pressure and the residue was purified by column chromatography (CH_2Cl_2/MeOH, 98.5:1.5 v/v) to give cyclic dipeptide **8** as a grey solid (390 mg) as a mixture of *E*/*Z* isomers in a yield of 55%. An aliquot of Boc-protected cyclic dipeptide **14** (15 mg) was treated with TFA/CH_2Cl_2 (2 mL) to obtain the corresponding amine. After 1 h of stirring, all volatiles were removed under reduced pressure and the residue was coevaporated with CH_2Cl_2 (3 × 5 mL) to remove any residual TFA. The remaining solid was purified by preparative RP-HPLC which resulted in the isolation of two major fractions, cyclic dipeptide amine **14a** and **14b**, and could be identified as the *Z* and *E* isomer, respectively. Cyclic amine **14a** (1.8 mg, R_t 22.24 min); $[\alpha]^D_{20}$ = –122.2 (*c* 1.0, $CHCl_3$); ^{1}H NMR (500 MHz, DMSO-d_6) δ = 8.37 (s, 1H), 7.91 (d, *J* = 8.0 Hz, 1H), 7.27-7.23 (m, 2H),

7.07 (t, *J* = 7.4 Hz, 1H), 5.62 (q, *J* = 8.0 Hz, 1H), 5.49-5.45 (m, 1H), 3.95-3.86 (m, 2H), 3.44-3.34 (m, 4H), 2.86-2.80 (m, 1H), 2.70-2.64 (m, 1H), 2.12-1.96 (m, 2H), 1.64-1.56 (m, 2H), 1.47-1.40 (m, 2H); ^{13}C NMR (125 MHz, DMSO-d_6) δ = 129.6, 126.1, 123.2, 120.6, 54.9, 50.8, 48.1, 39.0, 38.9, 38.2, 36.9, 27.1, 25.7, 25.4, 21.1; HRMS *m/z* calcd for $C_{18}H_{24}N_6NaO_2$ [*M* + Na]$^+$ 379.1858, found 379.1891. Cyclic amine **14b** (2.5 mg, R_t 22.98 min); $[\alpha]^D_{20}$ = –92.4 (*c* 1.0, $CHCl_3$); ^{1}H NMR (500 MHz, DMSO-d_6) δ = 8.98 (d, *J* = 6.9 Hz, 1H), 8.47 (s, 1H), 8.26-8.24 (m, 2H), 7.21-7.17 (m, 2H), 6.98 (t, *J* = 7.4 Hz, 1H), 5.79 (t, *J* = 12.9 Hz, 1H), 5.37-5.28 (m, 1H), 3.92 (dd, *J* = 10.9 Hz, 5.6 Hz, 1H), 3.78-3.74 (m, 1H), 3.62-3.57 (m, 2H), 2.63-2.60 (m, 1H), 2.22-2.15 (m, 2H), 2.01-1.94 (m, 3H), 1.60-1.48 (m, 2H), 1.45-1.38 (m, 1H), 1.36-1.30 (m, 1H); ^{13}C NMR (125 MHz, DMSO-d_6) δ = 132.4, 129.7, 126.3, 125.3, 123.7, 118.8, 54.8, 50.3, 39.0, 38.9, 36.5, 36.4, 31.0, 30.2, 29.7, 27.5, 26.1; HRMS *m/z* calcd for $C_{18}H_{24}N_6NaO_2$ [*M* + Na]$^+$ 379.1858, found 379.1876.

4.4.2.9 Cyclic tripeptide (15): Cyclic dipeptide **14** (360 mg, 0.788 mmol; as the *E*/*Z* mixture) was dissolved in CH_2Cl_2/TFA (40 mL, 1:1 v/v) and the reaction mixture was stirred for 1 h, after which the volatiles were removed under reduced pressure. The residue was coevaporated with CH_2Cl_2 (3 × 3 mL) to remove any residual TFA and subsequently dried for 1 h at high vacuum. Then, the amine as the trifluoroacetate was dissolved in CH_2Cl_2 (30 mL) and to this solution, acid **8** (416 mg, 1.1 mmol) followed by BOP (488 mg, 1.1 mmol) and DIPEA (344 μL, 1.97 mmol) were added. The obtained reaction mixture was stirred overnight at room temperature. Subsequently, the solvent was removed under vacuo and the residue was redissolved in EtOAc (50 mL). This solution was successively washed with 1N $KHSO_4$ (2 × 50 mL), saturated $NaHCO_3$ (2 × 50 mL), brine (50 mL) and dried (Na_2SO_4). After filtration and removal of the solvent, the residue was purified by column chromatography (CH_2Cl_2/MeOH, 98.5:1.5 v/v). Compound **15** (304 mg) was obtained as a yellowish solid in 54% yield as a mixture of *E*/*Z* diastereoisomers.

4.4.2.10 Boc-protected bicyclic tripeptide (3): Cyclic tripeptide **15** (210 mg, 0.294 mmol) was dissolved in THF (25 mL) and to this solution TBAF·3H_2O (93 mg, 0.294 mmol) was added. The obtained reaction mixture was stirred for 1 h as

the reaction was complete, based on TLC. Subsequently, the reaction mixture was diluted with EtOAc (30 mL) and the resulting solution was successively washed with 1N $KHSO_4$ (30 mL), saturated $NaHCO_3$ (30 mL) and brine (30 mL), and dried (Na_2SO_4). After filtration and removal of the solvent, the residue was purified by column chromatography (CH_2Cl_2/MeOH, 98:2 v/v) and the unprotected alkyne was obtained in 95% yield as a grey solid (180 mg, as a mixture of *E*/*Z* diastereoisomers). In the next step, the unprotected alkyne (70 mg, 0.11 mmol) and $[Cp^*RuCl]_4$ (12 mg, 0.011 mmol) were dissolved in THF/MeOH (14 mL, 4:1 v/v) and the resulting reaction mixture was heated by microwave irradiation at 80 °C for 2 h, after which the solvent was removed under reduced pressure and the residue was purified by column chromatography (CH_2Cl_2/MeOH, 98:2 v/v) affording bicyclic tripeptide **3** as a grey solid in 22% yield (15.5 mg, as a mixture of *E*/*Z* diastereoisomers). Based on LC-MS, *Z* diastereoisomer (R_t 23.54 min) calcd for $C_{34}H_{41}N_7O_6$ $[M]^+$ 643.31, found 643.19; *E* diastereoisomer (R_t 24.22 min) calcd for $C_{34}H_{41}N_7O_6$ $[M]^+$ 643.31, found 643.20.

4.4.2.11 Unprotected Bicyclic tripeptide (16): Boc-protected bicyclic tripeptide **3** (15.5 mg) was treated with CH_2Cl_2/TFA (40 mL, 1:1 v/v) for 1 h, after which the volatiles were removed under reduced pressure. The residue was purified by preparative RP-HPLC and the individual diastereoisomers (**16a** as the *Z* and **16b** as the *E* diastereoisomer, respectively) could be isolated and characterized. Compound **16a**: (1.3 mg, R_t 21.28 min); $[\alpha]^D_{20}$ = –119.5 (*c* 1.0, $CHCl_3$); ^{1}H-NMR (500 MHz, DMSO-d_6) δ = 8.80 (d, *J* = 7.1 Hz, 1H), 8.57 (s, 1H), 8.11 (s, 1H), 7.90 (d, *J* = 8.1 Hz, 1H), 7.72-7.68 (m, 1H), 7.65 (s, 1H), 7.38 (d, *J* = 2.3 Hz, 1H), 7.32 (d, J = 8.7 Hz, 1H), 7.27-7.18 (m, 2H), 7.04 (t, *J* = 7.5 Hz, 1H), 5.57-5.52 (m, 1H), 5.34-5.30 (m, 1H), 5.09 (s, 1H), 4.41 (s, 1H), 4.35-4.30 (m, 1H), 4.22-4.17 (m, 1H), 3.85 (s, 3H), 3.75-3.70 (m, 1H), 3.62-3.51 (m, 1H), 2.97-2.88 (m, 1H), 1.90-1.81 (m, 1H), 1.71-1.46 (m, 4H), 1.21-1.09 (m, 1H); ^{13}C-NMR (125 MHz, DMSO-d_6) δ = 131.4, 131.2, 129.4, 129.0, 128.9, 126.1, 124.7, 123.2, 120.8, 111.9, 55.4, 53.5, 46.8, 38.6, 29.8, 29.7, 28.2, 28.1, 28.0; HRMS *m/z* calcd for $C_{29}H_{34}N_7O_4$ $[M + H]^+$ 544.2672, found 544.2668; $C_{29}H_{33}N_7NaO_4$ $[M + Na]^+$ 566.2492, found 566.2476. Compound **16b**: (2.4 mg, R_t = 22.17 min); $[\alpha]^D_{20}$ = –124.5 (*c* 1.0, $CHCl_3$); ^{1}H-NMR (500 MHz, DMSO-d_6) δ = 8.87 (d, *J* = 7.8 Hz, 1H), 8.54 (s, 1H), 8.33 (s, 1H), 8.32 (s, 1H), 7.68 (m, 1H), 7.67 (s, 1H), 7.40 (d, *J* = 2.3 Hz, 1H), 7.31 (d, *J* =

8.6 Hz, 1H), 7.21-7.16 (m, 2H), 6.99 (d, *J* = 7.4 Hz, 1H), 5.96-5.91 (m, 1H), 5.42-5.37 (m, 1H), 5.04 (s, 1H), 4.51 (q, *J* = 7.7 Hz, 1H), 4.34-4.29 (m, 1H), 4.21-4.16 (m, 1H), 3.85 (s, 1H), 3.70-3.60 (m, 1H), 3.48 (d, *J* = 6.5 Hz, 1H), 2.33-2.27 (m, 1H), 1.88-1.58 (m, 3H), 1.50-1.42 (m, 1H), 0.98-0.75 (m, 2H); ^{13}C-NMR (125 MHz, DMSO-d_6) δ = 132.3, 131.6, 131.3, 129.7, 128.6, 126.5, 126.2, 122.3, 117.8, 111.1, 55.5, 55.1, 54.8, 52.9, 52.1, 46.8, 39.1, 38.7, 38.5, 25.3, 24.7, 20.7, 20.8; HRMS *m/z* calcd for $C_{29}H_{34}N_7O_4$ $[M + H]^+$ 544.2672, found 544.2673; $C_{29}H_{33}N_7NaO_4$ $[M + Na]^+$ 566.2492, found 566.2474.

4.4.2.12 Linear tripeptide (4): Boc-protected dipeptide **5** (240 mg, 0.495 mmol) was dissolved in CH_2Cl_2/TFA (40 mL, 1:1 v/v) and the reaction mixture was stirred for 1 h, after which the volatiles were removed and the residual TFA was removed by coevaporation with CH_2Cl_2 (3 × 30 mL) and dried for 1 h at high vacuum. Next, the amine as the trifluoroacetate was dissolved in CH_2Cl_2 (20 mL) and to this solution, compound **5** (217 mg, 0.693 mmol) and BOP (307 mg, 0.693 mmol) followed by DIPEA (216 µL, 1.24 mmol) were added, and the obtained reaction mixture was stirred overnight. Then, the solvent was removed in vacuo and the residue was redissolved in EtOAc (50 mL). The resulting solution was successively washed with 1N $KHSO_4$ (2 × 50 mL), saturated $NaHCO_3$ (2 × 50 mL) and brine (50 mL), and dried (Na_2SO_4). After filtration and removal of the solvent, the residue was purified by column chromatography (CH_2Cl_2/MeOH, 98:2 v/v). Linear tripeptide **4** was obtained as a yellowish solid in a yield of 69% (254 mg). R_f 0.70 (CH_2Cl_2/MeOH, 96:4 v/v); $[\alpha]^D_{20}$ = –43.1 (*c* 1.0, $CHCl_3$); ^{1}H-NMR (400 MHz, $CDCl_3$) δ = 8.30 (s, 1H), 7.58 (d, *J* = 8.0 Hz, 1H), 7.38 (d, *J* = 2.3 Hz, 1H), 7.29-7.02 (m, 6H), 6.62 (d, *J* = 8.6Hz, 1H), 5.87-5.78 (m, 1H), 5.75-5.64 (m, 1H), 5.19-5.12 (m, 3H), 5.04-4.97 (m, 2H), 4.55 (q, *J* = 6.7 Hz, 2H), 3.86-3.80 (m, 1H), 3.75 (s, 3H), 3.27-3.16 (m, 4H), 2.56-2.50 (m, 2H), 1.98-1.84 (m, 1H), 1.76-1.48 (m, 4H), 1.38 (s, 9H), 0.21 (s, 9H); ^{13}C-NMR (100 MHz, $CDCl_3$) δ = 171.2, 169.8, 169.5, 160.4, 155.8, 135.8, 135.2, 132.7, 132.3, 129.9, 129.1, 127.0, 125.5, 123.9, 119.4, 116.4, 113.1, 111.1, 100.5, 99.3, 81.1, 58.6, 55.9, 53.8, 52.3, 51.1, 36.8, 35.7, 31.0, 22.9, 0.02; HRMS *m/z* calcd for $C_{39}H_{53}N_7NaO_6Si$ $[M + Na]^+$ 766.3724, found 766.3751.

4.4.2.13 Triazole-cyclized tripeptide (17): TMS-alkyne **4** (235 mg, 0.316 mmol) was dissolved in THF (25 mL) and to this solution TBAF·$3H_2O$ (99 mg, 0.316 mmol) was added and the obtained reaction mixture was stirred for 1 h after which the deprotection was completed, as judged by TLC. Subsequently, the reaction mixture was diluted with EtOAc (30 mL) and the resulting solution was successively washed with 1N $KHSO_4$ (30 mL), saturated $NaHCO_3$ (30 mL) and brine (30 mL), and dried (Na_2SO_4). After filtration and removal of the solvent, the residue was purified by column chromatography (CH_2Cl_2/MeOH, 98:2 v/v) to afford the alkyne precursor peptide as a grey solid (200 mg, 94%). $[\alpha]^{D}_{20}$ = –59.6 (*c* 1.0, $CHCl_3$); ^{1}H-NMR (400 MHz, $CDCl_3$) δ = 8.19 (s, 1H), 7.69-7.62 (m, 2H), 7.55-7.44 (m, 2H), 7.23-7.06 (m, 4H), 6.74 (d, *J* = 8.6 Hz, 1H), 5.91-5.82 (m, 1H), 5.74-5.64 (m, 1H), 5.17-4.97 (m, 5H), 4.53-4.45 (m, 2H), 3.89-3.85 (m, 1H), 3.81 (s, 3H), 3.32-3.20 (m, 5H), 2.60 (t, *J* = 6.6 Hz, 2H), 1.99-1.90 (m, 1H), 1.82-1.60 (m, 4H), 1.40 (s, 9H); ^{13}C-NMR (100 MHz, $CDCl_3$) δ = 171.2, 169.8, 169.4, 160.7, 155.9, 135.9, 135.3, 132.7, 132.5, 132.1, 132.0, 131.9, 129.9, 129.4, 128.5, 128.4, 127.0, 125.5, 123.7, 123.6, 119.5, 116.5, 112.0, 111.2, 81.9, 81.2, 79.3, 58.7, 55.9, 53.9, 52.3, 51.1, 36.5, 35.8, 30.7, 28.5, 28.3, 22.9; HRMS *m/z* calcd for $C_{36}H_{45}N_7NaO_6$ [*M* + Na]$^+$ 694.3329, found 694.3306. In the next step, the precursor peptide (20 mg, 0.03 mmol) and $[Cp^*RuCl]_4$ (3.2 mg, 0.003 mmol) were dissolved in THF/MeOH (4 mL, 4:1 v/v) and the resulting solution was heated by microwave irradiation at 80°C for 2 h. Subsequently, the solvent was removed under reduced pressure and the residue was purified by column chromatography (CH_2Cl_2/MeOH, 98:2 v/v) and monocyclic tripeptide **17** was obtained as a grey solid in 41% yield (8.2 mg). R_f 0.58 (CH_2Cl_2/MeOH, 96:4 v/v); $[\alpha]^{D}_{20}$ = +4.7 (*c* 1.0, $CHCl_3$); ^{1}H-NMR (400 MHz, $CDCl_3$) δ = 8.11 (s, 1H), 7.55-7.41 (m, 3H), 7.32 (m, 2H), 7.16-7.04 (m, 3H), 6.96 (d, *J* = 8.6Hz, 1H), 6.64 (d, *J* = 7.1Hz, 1H), 6.02 (s, 1H), 5.92-5.85 (m, 1H), 5.76-5.66 (m, 1H), 5.20 (s, 1H), 5.16-4.95 (m, 4H), 4.49-4.41 (m, 2H), 4.32-4.21 (m, 2H), 3.80 (s, 3H), 3.26 (double q, *J* = 16.3, *J* = 6.3 Hz, 2H), 2.67-2.60 (m, 2H), 2.56-2.48 (m, 2H), 1.60-1.52 (m, 2H), 1.42 (s, 9H), 0.99-0.84 (m, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ = 171.5, 171.0, 169.7, 156.3, 154.9, 136.0, 135.4, 135.1, 133.4, 133.0, 130.9, 130.0, 129.4, 126.9, 125.7, 124.0, 119.2, 116.6, 116.5, 111.9, 80.4, 55.8, 54.1, 53.5, 48.6, 35.9, 35.4, 30.3, 30.0, 28.3, 22.1, 18.4; HRMS *m/z* calcd for $C_{36}H_{45}N_7NaO_6$ [*M* + Na]$^+$ 694.3329, found 694.3305.

4.4.3 Binding affinity measurements

Binding affinity measurements were determined by using microcalorimetry, which was performed on automated MicroCal Auto-iTC200 equipment. ITC (isothermal titration calorimetry) experiment was carried out by injection the ligand solution (10-15 mM) into the cell containing the solution of the synthesized mimics or vancomycin (0.1-0.3 mM) dissolved in a 0.02 M Na-citrate/citric acid buffer (pH 5.1). The typical experiment contains 16 injections in 40 min and the resulting data was analyzed by non-linear fitting in Origin software.

4.5 References

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Chapter 5

Synthesis of a Vancomycin-inspired Tricyclic Hexapeptide Using Ruthenium-based Macrocyclization

5.1 Introduction

Macrocyclic peptides are increasingly attractive as therapeutic agents due to their outstanding biological affinity, selectivity and other drug-like properties, among others, good solubility, lipophilicity, metabolic stability and bioavailability.[1] Their common structural motif, the macrocyclic ring, provides a certain degree of structural preorganization as well and reduced flexibility,[1d] which results in an improved binding affinity to their natural receptors as compared to their linear counterparts.[2] One of the most outstanding examples of such a preorganized peptide is vancomycin,[3] which possesses a unique molecular geometry that consists of a heptapeptide framework and a rigid cavity-like macrocycle formed by two biaryl ether bridges through a side-chain to side-chain connectivity pattern. However, structural modification of vancomycin is quite challenging due to its synthetic complexity. Thus, much effort has been devoted to exploring molecules that mimic the cavity-like shape of vancomycin which are much easier to synthesize.[4]

Over the years, ring-closing metathesis[4f] (RCM), Sonogashira cross-coupling[4g], Cu(I)- or Ru(II)-catalyzed azide alkyne cycloaddition[4h,4i] (CuAAC and RuAAC, respectively) have been successfully applied to synthesize promising mono- as well as bicyclic vancomycin mimics, in which the biaryl ether bridge has been replaced by alkene, alkyne and triazole moieties, respectively. Although the resulting DE- and CDE-ring mimics showed an excellent structural resemblance to the corresponding part of vancomycin, the loss of binding affinity in combination with the reduced antimicrobial activity indicated that these frameworks, including the bicyclic congeners, still too flexible to adopt a cavity-like structure. To increase the rigidity of this class of peptidomimetics any further, structural optimization, especially the incorporation of an extra constraint, was required. In addition to the (C)DE-ring, incorporation of the AB(C) cyclic constraint would probably enhance the formation of a clam-like structure of the newly designed vancomycin mimic. This chapter describes an optimized synthesis approach toward a tricyclic hexapeptide in an effort to obtain a molecule that -mimics vancomycin in a better way than the previously described analogs.

5.2 Results and Discussion

Previously, the synthesis of a bicyclic tripeptide, compound **3**, that represented the ABC-ring system of vancomycin , as shown in Figure 1, was reported that followed a RCM-coupling-RuAAC strategy.[5] Herein, RCM[6] was applied to install the alkene-bridge as the macrocyclic constraint while the triazole was incorporated featuring RuAAC since it was previously shown that the bended 1,5-triazole moiety could effectively mimic/replace a biaryl ether bridge.[6] As described in the previous chapters, a single AB(C)- as well as a single (C)DE-ring mimic was not able to display the binding affinity or antimicrobial activity compared to vancomycin. Therefore, tricyclic hexapeptide **1** was designed in which the AB(C)-ring was introduced by RCM while the (C)DE-ring systems were installed by RuAAC, as shown in Figure 1.

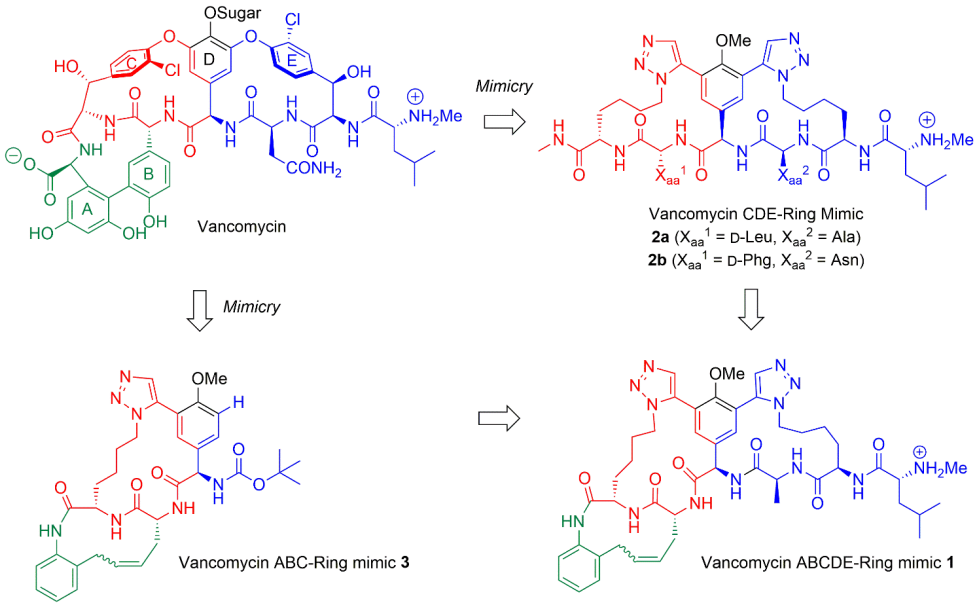
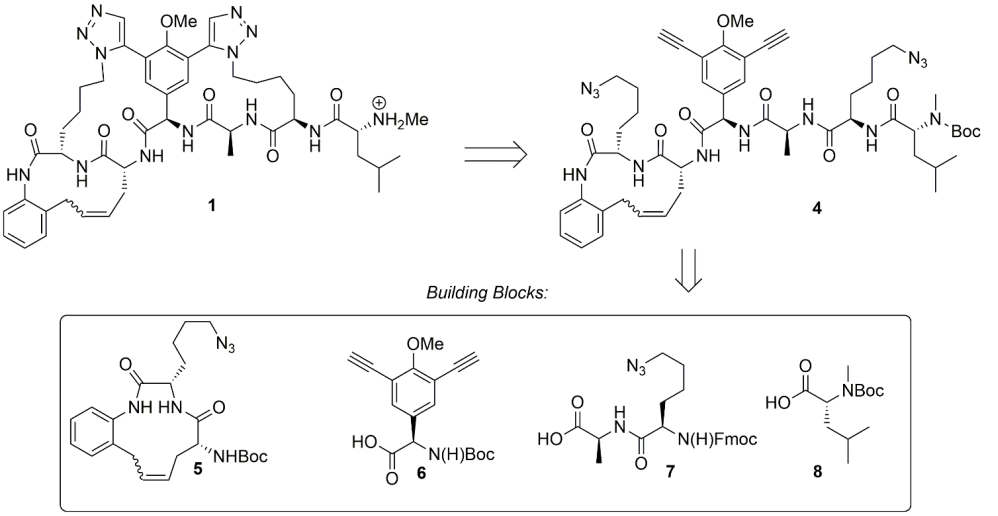


Figure 1. Design of vancomycin mimics.

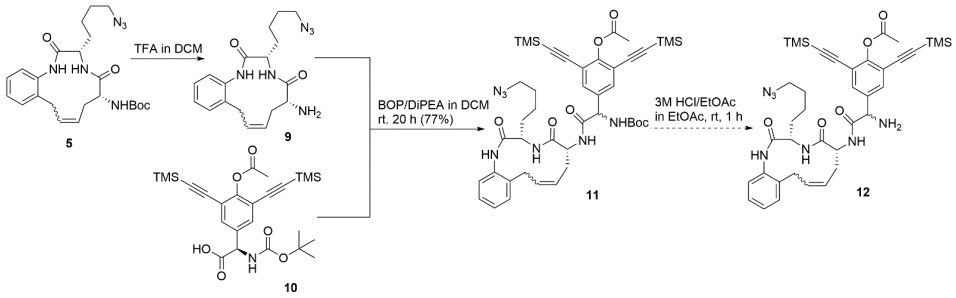
Retrosynthetic analysis showed that tricyclic hexapeptide **1** involved three key stages (see Scheme 1): the first stage was the synthesis of the required building blocks that contained the desired alkyne and azide functionalities, here already, cyclic peptide **5** was chosen as one of the most important building blocks since RCM could not be performed in presence of protected alkyne moieties as discussed

in chapter 4.[7] The second stage was assembly of Boc-protected hexapeptide **4** by sequential peptide coupling steps. Finally, the third stage was the RuAAC macrocyclization step to afford the bistriazole-bridged tricyclic hexapeptide **1**. This chapter describes an optimized synthetic approach toward tricyclic hexapeptide **1** including the synthesis of the required building blocks.



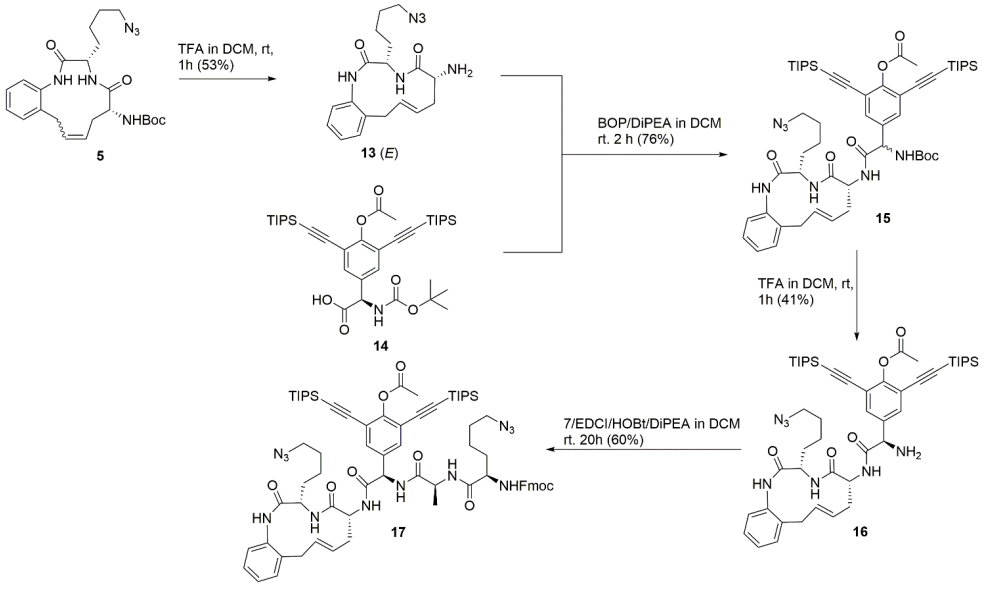
Scheme 1. Retrosynthetic analysis of the tricyclic hexapeptide **1**.

The synthesis of the required dipeptide segments **5**[5] and **7**[4i] has been described previously. Cyclic dipeptide **5**, was obtained as a mixture of *E/Z* diastereoisomers by RCM using the second generation of Hoveyda-Grubbs catalyst.[9] This mixture of diastereoisomers was used directly in the peptide elongation steps. The right choice of the central D-hydroxyphenylglycine derivative, however, required several optimization steps before a suitable building block was at hand. In first instance, the TMS-protected building block **10** was used (as shown in Scheme 2) and tripeptide **11** was obtained as a complex mixture of stereoisomers that could not be separated in each individual stereoisomer. Moreover, the TMS functionalities were not stable under the acidic conditions required for Boc-removal, so this route was abandoned (Scheme 2).



Scheme 2. Synthesis of tripeptide **12** starting from hydroxyphenylglycine derivative **10**.

It was noticed that in the absence of the Boc-functionality the corresponding amine dipeptide **9** could be separated by column chromatography (eluent: CH_2Cl_2/MeOH 95:5 v/v in the presence of 0.2% Et_3N) in the individual *E*/*Z* isomers. Since the *E*-isomer was isolated (assigned as **13**) in a pure form and high yield (53%), it was decided to continue the syntheses with this derivative.

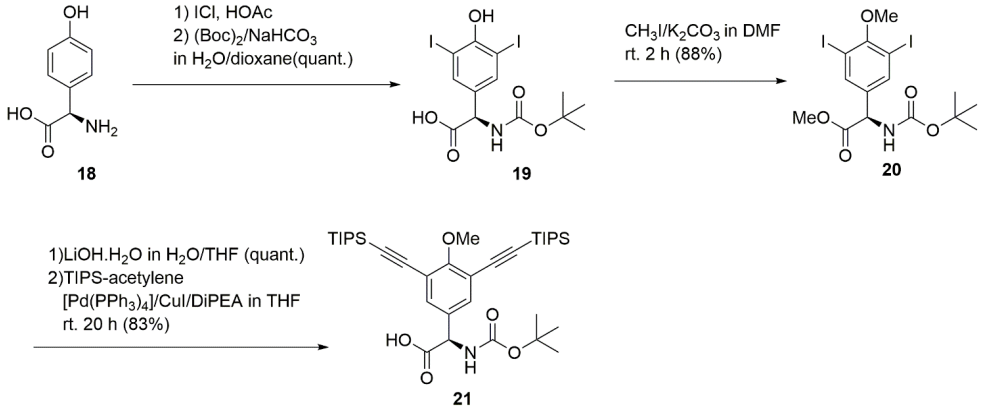


Scheme 3. Synthesis of pentapeptide 17 starting from hydroxyphenylglycine derivative **14**.

An alternative was probably found employing building block **14** (Scheme 3) in

which both alkyne moieties were protected by a TIPS functionality since this protecting group is known to be less acid sensitive. Acid **14** was coupled to amine **9** in the presence of BOP/DIPEA and tripeptide **15** was isolated in 76% yield as a mixture of diastereoisomers since the phenylglycine partially racemized under these basic reaction conditions. Gratifyingly, after Boc-group removal the amine **16** could be isolated as a single stereoisomer, since the most intensive spot, which was assumed to represent the desired *R*-isomer, could be isolated by column chromatography (eluent: CH_2Cl_2/MeOH 97:3 v/v in the presence of 0.1% AcOH) in 41% yield. In the next step, Fmoc-D-Lys(N_3)-Ala-OH was coupled in the presence of EDCI/HOBt/DIPEA to give pentapeptide **17** in 60% yield. Unexpectedly, two spots on TLC were observed, an indication that racemization occurred, probably again at the αC atom of phenylglycine, since based on previous coupling at a C-terminal alanyl carboxylate, racemization of alanine, could be excluded. At this stage, separation into individual stereoisomers did not work, and it was decided to replace the mild electron-donating acetoxy group by the strong electron-donating methoxy functionality to decrease the acidity of the αH atom of phenylglycine to avoid racemization by base-induced proton abstraction. Based on these considerations, building block **21** was designed and its synthesis is shown in Scheme 4.

The synthesis of phenylglycine derivative **21** began with the iodination of commercially available D-hydroxyphenylglycine **18** according to a literature procedure.[9] In the next step, the amino functionality was protected with a Boc group to give acid **19** in a quantitative yield.[10] Subsequent methylation of the carboxylic acid along with the phenolic hydroxyl functionality by treatment with MeI/K_2CO_3 in DMF gave the fully protected amino acid derivative **20** in a yield of 88%. Finally, after saponification of the methyl ester, both alkyne moieties were introduced using TIPS-protected acetylene via a Pd-catalyzed Sonogashira cross-coupling to give bis-alkyne **21** in high yield (83%).

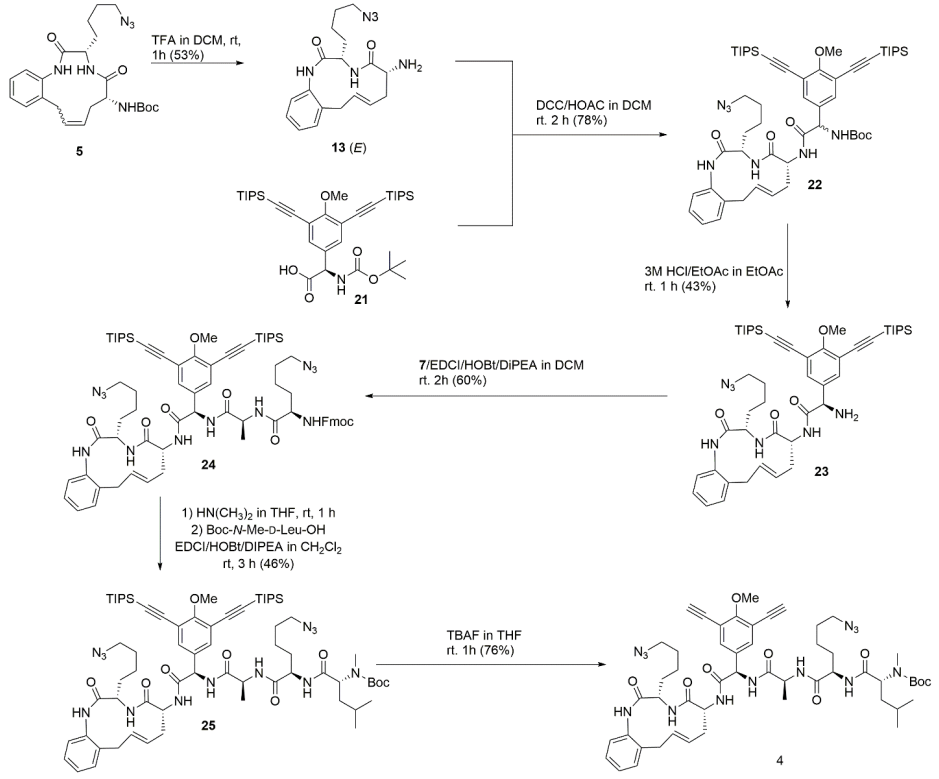


Scheme 4. Synthesis of the suitably functionalized phenylglycine derivative **21**.

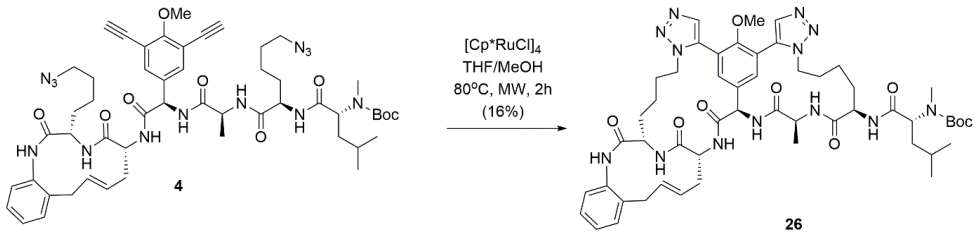
With these building blocks in hand, the linear assembly of peptide **4**, as shown in Scheme 5, was started. In an attempt to minimize racemization of the phenylglycine derivative during the coupling with amine **13** three different combinations of coupling reagents were investigated.[11] A BOP/DIPEA-mediated coupling in CH_2Cl_2 resulted in a diastereomeric ratio of 1.3:1 as determined by LC-MS, while by using of DCC/HOAt,[12] this ratio could be slightly improved to 1.4:1. The coupling reagent 3-(diethoxyphosphoryloxy)-1,2,3-benzotriazin-4(3*H*)-one (DEPBT)[13] was reported to be superior to typical phosphonium and uronium-based coupling reagents to suppress racemization during peptide fragment coupling. However, applying DEPBT/TEA in the coupling reaction of **21**, a reasonable conversion could not be achieved. Consequently, tripeptide **22** was isolated as a mixture of diastereoisomers and after treatment with 3 M HCl in EtOAc to remove the Boc group, the desired diastereoisomer **23** was isolated with 43% yield by column chromatography. In the next step, unprotected tripeptide **23** was coupled with Fmoc-D-Lys(N_3)-Ala-OH in presence of EDCI/HOBt/DIPEA and pentapeptide **24** was obtained in 60% yield. Gratifyingly, racemization of phenylglycine was absent likely due to the presence of the methoxy functionality as judged by LC-MS, which indicated that pentapeptide **24** consisted of a single diastereoisomer. Next, the Fmoc group was removed by treatment with dimethylamine, which was followed by an EDCI/HOBt-mediated coupling of Boc-*N*-Me-D-Leu-OH in CH_2Cl_2 to give fully protected hexapeptide **25** in 46% yield over two steps. Finally, both TIPS groups were removed by treatment with

TBAF in THF and unprotected bisalkyne **4** was obtained in 76% yield.

The last crucial step was bicyclization of linear hexapeptide **4** by RuAAC reaction, as shown in Scheme 6. As the first attempt the intramolecular RuAAC macrocyclization was performed with 5 mol% $[Cp^*RuCl]_4$[4i,14] in THF/MeOH (4:1 v/v) for 2 h at 80 ºC under microwave irradiation. Three new peaks were identified by HPLC (Figure 2b), and according to LC-MS these three peaks had the same mass as the linear hexapeptide **4** which indicated that macrocyclization into the desired tricyclic compound had occurred and that two bicyclic compounds appeared as cyclization intermediates. By increasing the loading of catalyst to 10 mol%, an increasing amount of tricyclic hexapeptide was identified by HPLC, while the cyclization precursor **4** and one of the bicyclic intermediates were almost absent (Figure 2c). At a catalyst loading of 20 mol% (Figure 2d), the bicyclization was almost complete and the desired tricyclic compound **26** was isolated by preparative RP-HPLC in a yield of 16% (see Figure 3a).



Scheme 5. Synthesis of hexapeptide **4** starting with phenylglycine derivative **21**.



Scheme 6. RuAAC macrocyclization of precursor **4**.

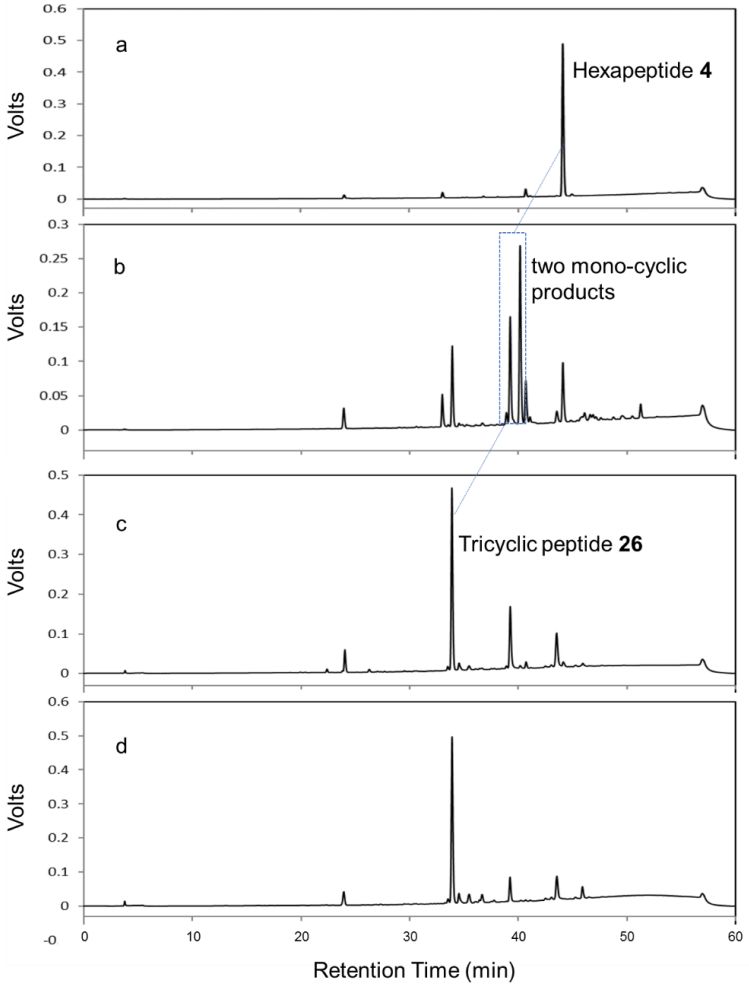
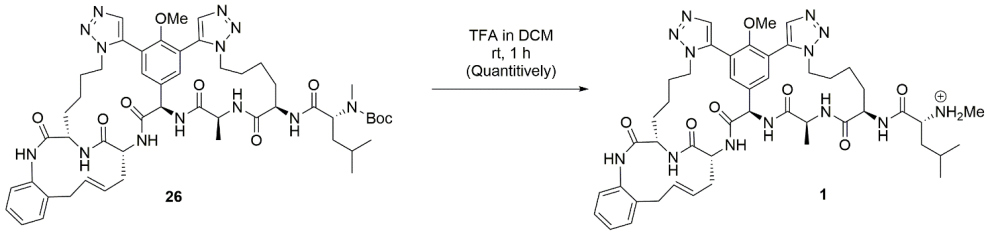


Figure 2. HPLC analysis of the RuAAC macrocyclization of hexapeptide **4**: (a) hexapeptide **4**, (b) RuAAC macrocyclization of hexapeptide **4** (5 mol% $[Cp^*RuCl]_4$, 80 ºC, 2 h (MW)), (c) RuAAC

macrocyclization of hexapeptide **4** with catalyst (10 mol% $[Cp^*RuCl]_4$, 80 ºC, 2 h (MW)), and (d) RuAAC macrocyclization of hexapeptide **4** with catalyst (20 mol% $[Cp^*RuCl]_4$, 80 ºC, 2 h (MW).

After the removal of the Boc-group by treatment with TFA (Scheme 7), the desired unprotected tricyclic hexapeptide **1** was obtained quantitatively in high purity (Figure 3b). This unprotected tricyclic peptide was characterized by ^1H, ^1H-^1H COSY, ^1H-^{13}C HSQC NMR spectroscopy in combination with HRMS, and was identified as the desired tricyclic peptide **1**. Both unique protons of the 1,5-disubstituted-1*H*-1,2,3-triazoles could be assigned as δ_H 7.93 and δ_H 7.89 ppm with corresponding ^{13}C shifts δ_C 134.26 ppm and δ_C 133.80 ppm, which was in agree with the literature,[15] as shown in Figure 4.



Scheme 7. Deprotection of tricyclic peptide **26**.

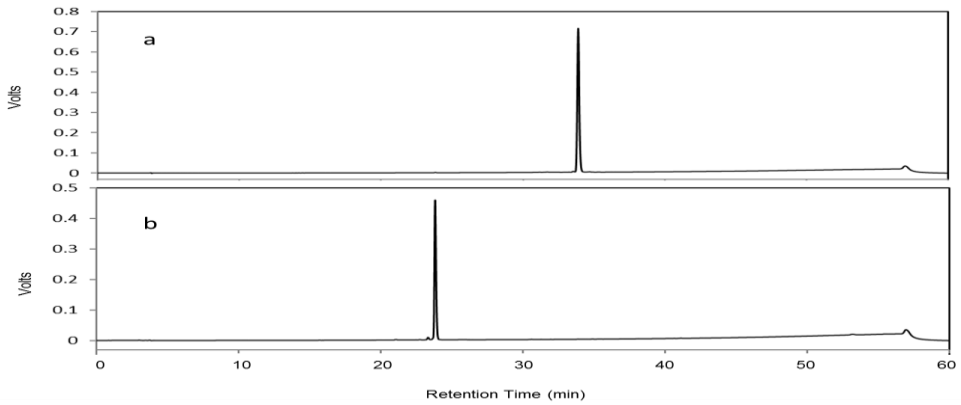


Figure 3. HPLC analysis of (a) protected tricyclic peptide **26** and (b) unprotected tricyclic peptide **1**.

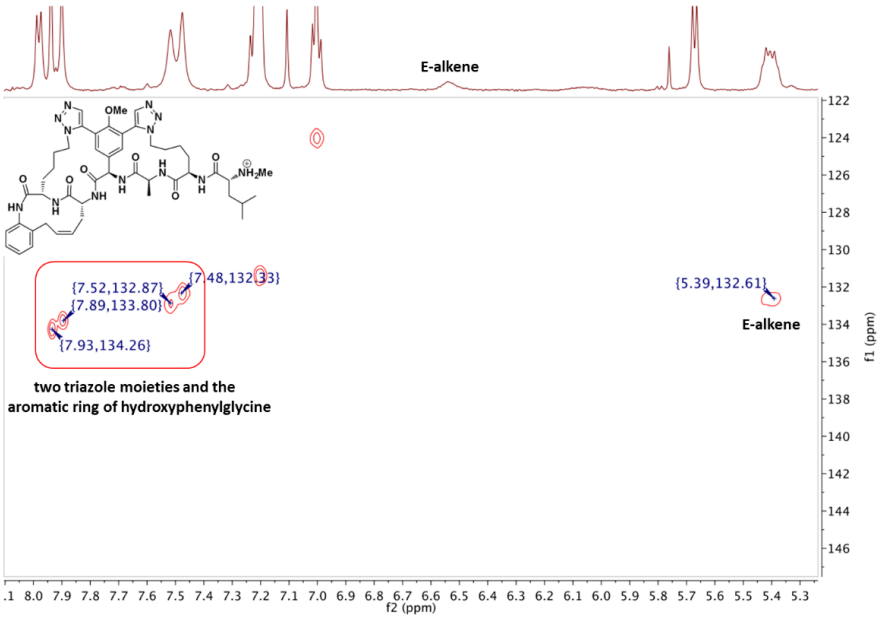


Figure 4. Detail of the HSQC NMR spectrum of the tricyclic peptide **1**.

After energy minimization using the simulated annealing protocol employing the AMBER99 force field using the YASARA Structure 10.5.2.1 software package, the tricyclic peptide **1** was superimposed on the whole crystal structure of vancomycin-related balhimycin antibiotic.[16] This superimposition indicated a high structure resemblance, with a RMSD of 1.0074 Å over eight atoms, between the tricyclic peptide backbone and balhimycin (Figure 5).

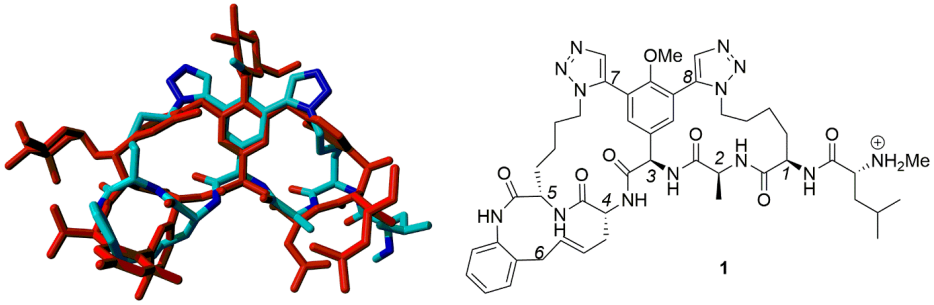


Figure 5. Superimposition of balhimycin (in red) with tricyclic peptide **1**. The carbon atoms αC^1, αC^2, αC^3, αC^4, αC^5, benzylic-C^6, triazole-C^7 and C^8, have been used as fixed coordinates for superimposition.

Isothermal microcalorimetry (ITC)[17] was used to determine the binding affinities toward the natural ligand and to evaluate to what extent tricyclic peptide **1** was able to mimic vancomycin. As shown in Table 1, the binding affinities of this tricyclic peptide toward Ac-Lys(Ac)-D-Ala-D-Ala-OH was around 30-fold less when compared with vancomycin while the CDE and ABC-ring mimics were 100-fold less than vancomycin. The improvement of binding affinity confirmed the increased rigidity of the newly designed mimic.

Table 1. The binding affinity as measured using ITC[a]

Compound	Ligand	$K_a(M^{-1})$
VM	Ac-Lys(Ac)-D-Ala-D-Ala-OH	$(4.23\pm0.46) \times 10^5$
VM	Ac-Lys(Ac)-D-Ala-D-Lac-OH	$(2.59\pm0.41) \times 10^3$
1	Ac-Lys(Ac)-D-Ala-D-Ala-OH	$(1.26\pm0.24) \times 10^4$
1	Ac-Lys(Ac)-D-Ala-D-Lac-OH	$(3.28\pm0.31) \times 10^3$

[a] Measured in a Na-citrate/citric acid buffer (0.02 M, pH 5.1) with 5% DMSO.

To investigate the bioactivity of tricyclic mimic **1**, the minimum inhibitory concentration (MIC) was evaluated against vancomycin sensitive bacterium *Staphylococcus aureus* (ATCC 49320) using an *in vitro* assay.[18] Fortunately, in line with the result of the ITC measurements, the tricyclic mimic **1** displayed a promising antibacterial activity corresponding to a MIC value of 37.5 μg mL^{-1} (the MIC value of vancomycin was 2 μg mL^{-1} as reference), a strong indication that the all three rings contribute to the antimicrobial activity.

In conclusion, the tricyclic hexapeptide **1** as designed to mimic vancomycin was successfully synthesized following an RCM-coupling-RuAAC strategy. This newly synthesized mimic showed a good structural resemblance compared to balhymicin, a structural analog of vancomycin. Based on the binding affinity and antibacterial activity measurements, tricyclic mimic **1** was able to bind the D-Ala-D-Ala dipeptide motif by hydrogen bond formation and the highest antimicrobial activity

was observed within this series of vancomycin mimics. Via this RCM-coupling-RuAAC strategy, it is expected that the synthesis of a number of tricyclic analogues will be possible in which the overall rigidity can be increased to form a more constrained shell-like topology as found in native vancomycin molecule.

5.3 Experimental Section

5.3.1 Chemicals and General Experimental Procedures

Chemicals were used as obtained from commercial sources without further purification unless stated otherwise. The solvents were obtained as peptide synthesis grade and stored on molecular sieves (4 Å). Column chromatography was performed using SiliFlash P60 silica gel (particle size 40-63 μm). TLC was performed on precoated silica gel 60F254 glass plates and compound spots were visualized by UV-quenching, ninhydrin or Cl_2/TDM. Optical rotations were measured using a 10 cm cell with a Na 589 nm filter at the specific concentration (in g/100 mL) as indicated. ^{1}H NMR spectra were recorded either on a 400 MHz or a 500 MHz spectrometer in $CDCl_3$, or DMSO-d_6 as solvent. Chemical shifts are reported in delta (δ) units in parts per million (ppm) relative to TMS (0.00 ppm). Coupling constants (*J*) are reported in Hertz (Hz). ^{13}C NMR analysis was acquired on a 100 MHz or a 125 MHz spectrometer in $CDCl_3$, or DMSO-d_6 as solvent. Chemical shifts are reported in delta (δ) units in parts per million (ppm) relative to the solvent residual signal, $CDCl_3$ (77.0 ppm) or DMSO-d_6 (39.0 ppm). Microwave reactions were performed in a pressure and temperature controlled microwave reactor at 300 W. Analytical HPLC was performed on an automated HPLC system equipped with a UV/Vis detector operating at 220/254 nm and an evaporative light scattering detector using a C18 column (pore size: 100 Å, particle size: 5 μm; 250 × 4.6 mm) at a flow rate of 1 mL/min using a linear gradient of 100% buffer A (0.1% TFA in CH_3CN/H_2O 5:95 v/v) to 100% buffer B (0.1% TFA in CH_3CN/H_2O 95:5 v/v) in 50 min. Preparative RP-HPLC was performed on an automated preparative HPLC system equipped with a UV/Vis detector operating at 214 nm using a C18 column (pore size: 100 Å, particle size: 10 μm; 250 × 22 mm) at a flow rate of 12.0 mL/min using a linear gradient of 100% buffer A (0.1% TFA in CH_3CN/H_2O 5:95

v/v) to 100% buffer B (0.1% TFA in CH_3CN/H_2O 95:5 v/v) in 50 min. LC-MS(MS) was performed using a C18 column (pore size: 100 Å, particle size: 5 μm; 250 × 4.6 mm) at a flow rate of 1 mL/min. ESI-MS was performed on a bench-top electrospray ionization mass spectrometer. High-resolution mass spectrometry (HRMS) analysis was performed using an ESI-TOF LC/MS instrument.

5.3.2 Syntheses and Compound Analyses

The syntheses of compounds **5**, **10**, **14**, **19** and Fmoc-D-Lys(N_3)-Ala-OH were performed as described previously.[4i, 5]

5.3.2.1 Compound (11): *N*-Boc-protected cyclic dipeptide compound **5** (56.8 mg, 0.124 mmol; as a mixture of *E/Z* diastereoisomers) was dissolved in CH_2Cl_2/TFA (4 mL, 1:1 v/v) and the reaction mixture was stirred for 1 h, after which the volatiles were removed under reduced pressure. The residual TFA was removed by coevaporation with CH_2Cl_2 (3 × 2 mL) and dried for 1 h at high vacuum. Next, the amine, as the corresponding trifluoroacetate, was dissolved in CH_2Cl_2 (5 mL) and to this solution compound **10** (52 mg, 0.104 mmol), BOP (55 mg, 0.124 mmol) and DIPEA (50 μL, 0.208 mmol) were added. This reaction mixture was stirred overnight. Then, the solvent was removed in vacuo and the residue was redissolved in EtOAc (10 mL). The resulting solution was successively washed with 1 N $KHSO_4$ (2 × 10 mL), saturated $NaHCO_3$ (2 × 10 mL) brine (10 mL), and dried over anhydrous Na_2SO_4. After filtration and removal of the solvent, the residue was purified by column chromatography (CH_2Cl_2/MeOH, 99:1 v/v). Compound **11** was obtained as a yellowish solid in 77% yield as a mixture of four stereoisomers.

5.3.2.2 Cyclic dipeptide (13): *N*-Boc-protected cyclic dipeptide compound **5** (1.0 g, 2.2 mmol) was treated with CH_2Cl_2/TFA (40 mL, 1:1 v/v) for 1 h, after which the volatiles were removed under reduced pressure. The residual TFA was removed by coevaporation with CH_2Cl_2 (3 × 20 mL) and then the residue was purified by column chromatography (CH_2Cl_2/MeOH, 98:2 v/v with 0.2% TEA). Compound **13** (as the *E*-diastereoisomer) was obtained as a white solid (410 mg, 53%). R_f 0.57 (CH_2Cl_2/MeOH, 95:5 v/v with 0.2% TEA). $[\alpha]^{D}_{20} = -92.4$ (*c* 1.0, $CHCl_3$); ^{1}H NMR (500 MHz, DMSO-d_6) δ = 8.98 (d, *J* = 6.9 Hz, 1H), 8.47 (s, 1H),

8.26-8.24 (m, 2H), 7.21-7.17 (m, 2H), 6.98 (t, *J* = 7.4 Hz, 1H), 5.79 (t, *J* = 12.9 Hz, 1H), 5.37-5.28 (m, 1H), 3.92 (dd, *J* = 10.9 Hz, 5.6 Hz, 1H), 3.78-3.74 (m, 1H), 3.62-3.57 (m, 2H), 2.63-2.60 (m, 1H), 2.22-2.15 (m, 2H), 2.01-1.94 (m, 3H), 1.60-1.48 (m, 2H), 1.45-1.38 (m, 1H), 1.36-1.30 (m, 1H); ^{13}C NMR (125 MHz, DMSO-d_6) δ = 156.4, 153.7, 150.3, 149.3, 147.7, 142.8, 84.2, 74.3, 63.0, 62.9, 60.5, 55.0, 54.2, 53.7, 51.5, 50.1; HRMS *m/z* calcd for $C_{18}H_{24}N_6NaO_2$ $[M + Na]^+$ 379.1858, found 379.1876.

5.3.2.3 Compound 15: Cyclic dipeptide **13** (25 mg, 0.07 mmol) was dissolved in CH_2Cl_2 (2 mL) and to this solution compound **14** (56.3 mg, 0.084 mmol), BOP (37.2 mg, 0.084 mmol) and DIPEA (33.8 μL, 0.14 mmol) were added. This reaction mixture was stirred for 2 h. Then, the solvent was removed under reduced pressure and the residue was redissolved in EtOAc (10 mL). The resulting solution was successively washed with 1 N $KHSO_4$ (2 × 10 mL), saturated $NaHCO_3$ (2 × 10 mL) and brine (10 mL), and dried over anhydrous Na_2SO_4. After filtration and removal of the solvent, the residue was purified by column chromatography (CH_2Cl_2/MeOH, 98:2 v/v). Compound **15** (as a mixture of two diastereoisomers) was obtained as a yellowish solid in 76% yield (54 mg).

5.3.2.4 Compound 16: Boc-protected compound **15** (54 mg, 0.67 mmol) was treated with CH_2Cl_2/TFA (10 mL, 1:1 v/v) for 1 h, after which the volatiles were removed under reduced pressure. The residual TFA was removed by coevaporation with CH_2Cl_2 (3 × 5 mL) and the residue was purified by column chromatography (CH_2Cl_2/MeOH, 97:3 v/v with 0.1% AcOH). Compound **16** was isolated as a single stereoisomer and was obtained as a white solid (20 mg, 41%). R_f 0.38 (CH_2Cl_2/MeOH, 96:4 v/v with 0.3% AcOH); $[\alpha]^{D}_{20}$ = –31.0 (*c* 1.0, $CHCl_3$); ^{1}H-NMR (400 MHz, $CDCl_3$) δ= 8.46 (s, 1H), 8.33 (d, *J* = 8.2 Hz, 1H), 8.15 (s, 1H), 7.47 (s, 2H), 7.21-7.17 (m, 1H), 7.07-6.96 (m, 2H), 5.86-5.78 (m, 1H), 5.64-5.57 (m, 1H), 4.65 (s, 1H), 4.39 (q, *J* = 4.9 Hz, 1H), 4.11-4.07 (m, 1H), 3.47-3.33 (m, 2H), 3.21-3.17 (m, 2H), 2.67-2.62 (m, 1H), 2.49-2.41 (m, 1H), 2.28 (s, 3H), 1.94-1.82 (m, 1H), 1.61-1.48 (m, 1H), 1.41-1.31(m, 3H), 1.09 (s, 42H); ^{13}C-NMR (100 MHz, $CDCl_3$) δ = 171.4, 168.9, 167.9, 153.0, 137.3, 133.9, 131.5, 130.6, 127.8, 127.3, 126.7, 124.1, 120.9, 119.2, 100.1, 97.8, 58.1, 55.6, 54.7, 53.4, 51.1, 37.9, 35.6, 29.0, 28.1, 23.1, 20.6, 18.5, 11.3; HRMS *m/z* calcd for $C_{50}H_{74}N_7O_5Si_2$

$[M + H]^+$ 908.5290, found 908.5284.

5.3.2.5 Compound 17: Amine **16**, as the corresponding trifluoroacetate, (20 mg, 0.022 mmol) was dissolved in CH_2Cl_2 (3mL). To this solution, Fmoc-D-Lys(N_3)-Ala-OH (11.1 mg, 0.024 mmol), EDCI (4.6 mg, 0.024 mmol) and HOBt (3.2 mg, 0.024 mmol) were added, followed by the addition of DIPEA (11.5 µL, 0.066 mmol) and the reaction mixture was stirred overnight. Subsequently, the solvent was removed by evaporation and the residue was redissolved in EtOAc (10 mL). The resulting solution was successively washed with 1 N $KHSO_4$ (2 × 10 mL), saturated $NaHCO_3$ (2 × 10 mL) and brine (10 mL) and dried over anhydrous Na_2SO_4. After filtration and removal of the solvent, the residue was purified by column chromatography (CH_2Cl_2/ MeOH, 99:1 v/v). Compound **17** was obtained as a white solid (18 mg, 60%) as a mixture of two diastereoisomers, due to epimerization of the phenylglycine residue.

5.3.2.6 Compound 20: Compound **19** (17.63 g, 34 mmol) was dissolved in DMF (500 mL) and to this solution, K_2CO_3 (10.50 g, 76 mmol) followed by iodomethane (4.76 mL, 76 mmol) were added to the solution. The solution was left to stir for 2 h after which H_2O (500 mL) was added to quench the reaction mixture, followed by extraction with EtOAc (3 × 200 mL). The combined organic layers were washed with H_2O (2 × 300 mL) and brine (200 mL), dried over Na_2SO_4 and concentrated under reduced pressure. The residue was purified by column chromatography (PE/EtOAc, 8:1 v/v) and compound **20** was obtained as a white solid in 88% yield (16.31 g). R_f 0.62 (PE/EtOAc, 3:1 v/v); $[\alpha]^D_{20} = -46.8$ (*c* 1.0, $CHCl_3$); ^{1}H-NMR (400 MHz, $CDCl_3$) δ= 7.72 (s, 2H), 5.60 (d, *J* = 7.0 Hz, 1H), 5.17 (d, *J* = 7.0 Hz, 1H), 3.83 (s, 3H), 3.73 (s, 3H), 1.42 (s, 9H); ^{13}C-NMR (100 MHz, $CDCl_3$) δ = 171.1, 170.5, 158.9, 154.5, 141.8, 138.3, 136.7, 129.5, 90.8, 80.6, 60.6, 55.6, 53.1, 28.3, 21.0, 14.2; HRMS *m/z* calcd for $C_{15}H_{19}I_2NNaO_5$ $[M + Na]^+$ 569.9250, found 569.9243.

5.3.2.7 Compound 21: Methyl ester **20** (16.31 g, 30 mmol) was dissolved in a mixture of THF/H_2O (400 mL, 1:1 v/v) and the solution was cooled to 0 ˚C on ice. To this solution, lithium hydroxide monohydrate (1.90 g, 45 mmol) was added and the obtained reaction mixture was stirred for 30 min at room temperature. Then, a

solution of 1N $KHSO_4$ was added until a pH between 2 and 3 was reached. Subsequently, the aqueous phase was extracted with EtOAc (3 × 100 mL) and the combined organic layers were washed with water (100 mL) and brine (100 mL), dried (Na_2SO_4) and concentrated in vacuo to afford the corresponding acid as a white solid in quantitative yield. Without further purification, the acid was subjected to a Sonogashira reaction to install both TIPS-protected acetylene functionalities. For this, the acid (1.06 g, 2.0 mmol) in combination with [$Pd(PPh_3)_4$] (231 mg, 0.2 mmol) and CuI (114 mg, 0.6 mmol) were placed in a flask sealed with a rubber septum. The flask was evacuated and refilled with dry N_2 (repeated three times). Then, THF (40 mL) (purged with dry N_2 for 1 h prior to use) was added to the flask via a syringe and the resulting solution was degassed again by using a freeze-pump-thaw procedure (repeated three times). Subsequently, DIPEA (0.70 mL, 4.0 mmol) and TIPS-acetylene (2.68 mL, 12 mmol) were added to the mixture via a syringe and the obtained reaction mixture was stirred for 20 h at room temperature under N_2. The resulting suspension was filtered through a path of celite and the filtrate was evaporated to dryness. The residue was redissolved in EtOAc (150 mL) and this solution was successively washed with 1N $KHSO_4$ (2 × 150 mL), saturated $NaHCO_3$ (2 × 150 mL) and brine (150mL) and dried over anhydrous Na_2SO_4. After filtration and removal of the solvent, the residue was purified by column chromatography (PE/EtOAc, 3:1 v/v with 0.1% AcOH). Bis-alkyne **21** was obtained as a yellowish solid in 83% yield (1.1 g). R_f 0.48 (PE/EtOAc, 1:1 v/v with 0.5% AcOH); $[\alpha]^D_{20} = -10.7$ (*c* 1.0, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$) δ = 7.79 (s, 1H), 7.41 (s, 2H), 5.48 (d, *J* = 5.6 Hz, 0.5H (NH)), 5.22 (d, *J* = 5.6 Hz, 0.5H (NH)), 5.00 (d, *J* = 5.6 Hz, 1H), 3.99 (s, 3H), 1.42 (s, 9H), 1.11 (s, 42H); ^{13}C-NMR (100 MHz, $CDCl_3$) δ = 173.0, 162.3, 157.0, 133.5, 133.4, 133.1, 117.9, 101.8, 95.9, 82.4, 61.1, 58.1, 28.0, 18.6, 11.3; HRMS *m/z* calcd for $C_{36}H_{59}NNaO_5Si_2$ [*M* + Na]$^+$ 664.3829, found 664.3825.

5.3.2.8 Compound 22: Unprotected cyclic dipeptide **13** (300 mg, 0.84 mmol) was dissolved in CH_2Cl_2 (20 mL) and to this solution compound **21** (756 mg, 1.18 mmol), HOAt (161 mg, 1.18 mmol) and DCC (243 mg, 1.18 mmol) were added. This reaction mixture was stirred for 3 h. Then, the solvent was removed in vacuo and the residue was redissolved in EtOAc (100 mL). The resulting solution was successively washed with 1 N $KHSO_4$ (2 × 50 mL), saturated $NaHCO_3$ (2 × 50 mL) and brine (50

mL) and dried over anhydrous Na_2SO_4. After filtration and removal of the solvent, the residue was purified by column chromatography (CH_2Cl_2/MeOH, 98:2 v/v). Compound **22** was obtained in 78% yield (640 mg) as a mixture of two diastereoisomers due to epimerization of the phenylglycine residue.

5.3.2.9 Compound 23: Boc-protected compound **22** (600 mg, 0.68 mmol) was dissolved in 3M HCl/EtOAc (25 mL) and the reaction mixture was stirred for 1 h. Then, the volatiles were removed by evaporation. After drying for 1 h at high vacuum, the hydrochloride was purified by column chromatography (CH_2Cl_2/MeOH, 97:3 v/v with 0.1% AcOH). Compound **23** was obtained as a single stereoisomer as a yellowish solid in 43% yield (230 mg). R_f 0.5 (CH_2Cl_2/MeOH, 95:5 v/v with 0.3% AcOH); $[\alpha]^D_{20}$ = –62.3 (*c* 1.0, $CHCl_3$); ^{1}H-NMR (400 MHz, $CDCl_3$) δ = 8.42 (s, 1H), 8.38 (d, *J* = 8.0 Hz, 1H), 8.07 (s, 1H), 7.48 (s, 1H), 7.20 (td, *J* = 7.9 Hz, *J* = 1.7 Hz, 1H), 7.06-6.96 (m, 2H), 6.27 (s, 1H), 5.98-5.91 (m, 1H), 5.71-5.64 (m, 1H), 4.63 (s, 1H), 4.31 (q, *J* = 4.9 Hz, 1H), 4.23-4.17 (m, 1H), 3.99 (s, 3H), 3.48 (d, *J* = 7.1 Hz, 2H), 3.12-3.09 (m, 2H), 2.80-2.74 (m, 1H), 2.54-2.50 (m, 1H), 2.06-1.98 (m, 2H), 1.50-1.31 (m, 4H), 1.07 (s. 42H); ^{13}C-NMR (100 MHz, $CDCl_3$) δ = 172.6, 171.2, 168.9, 162.9, 137.5, 134.2, 131.8, 130.5, 127.9, 126.4, 124.0, 120.6, 118.8, 101.3, 97.4, 61.1, 60.4, 58.4, 54.8, 51.1, 38.3, 34.8, 29.8, 27.8, 23.0, 21.0, 18.6, 18.2, 18.0, 14.2, 11.7, 11.5, 11.3, 11.0; HRMS *m/z* calcd for $C_{49}H_{74}N_7O_4Si_2$ $[M + H]^+$ 880.5341, found 880.5332.

5.3.2.10 Compound 24: The hydrochloride **23** (140 mg, 0.16 mmol) was dissolved in CH_2Cl_2 (20 mL) and to this solution, Fmoc-D-Lys(N_3)-Ala-OH (82 mg, 0.18 mmol), EDCI (33.4 mg, 0.18 mmol) and HOBt (23.6 mg, 0.18 mmol) followed by DIPEA (84 μL, 0.48mmol) were added, and the reaction mixture was stirred for 2 h at room temperature. Subsequently, the solvent was removed by evaporation and the residue was redissolved in EtOAc (30 mL). The resulting solution was successively washed with 1 N $KHSO_4$ (2 × 20 mL), saturated $NaHCO_3$ (2 × 20 mL) and brine (20 mL) and dried over anhydrous Na_2SO_4. After filtration and removal of the solvent, the residue was purified by column chromatography (CH_2Cl_2/MeOH, 99:1 to 98:2 v/v). Compound **24** was obtained as a white solid in 60% yield (142 mg). R_f 0.47 (CH_2Cl_2/MeOH, 95:5 v/v); $[\alpha]^D_{20}$ = –23.8 (*c* 1.0, $CHCl_3$); ^{1}H-NMR (400 MHz, DMSO-d_6) δ = 8.66 (d, *J* = 5.0 Hz, 1H), 8.58 (d, *J* =

8.0 Hz, 1H), 8.54 (s, 1H), 8.30 (d, *J* = 8.3 Hz, 1H), 8.11 (s, 1H), 7.97 (d, *J* = 7.4 Hz, 1H), 7.83 (d, *J* = 8.1 Hz, 2H), 7.58-7.54 (m, 4H), 7.41-7.33 (m, 3H), 7.26-7.15 (m, 4H), 6.96 (td, *J* = 7.4 Hz, *J* = 1.3 Hz, 1H), 6.06-5.99 (m, 1H), 5.48 (d, *J* = 7.8 Hz, 1H), 5.38-5.31 (m, 1H), 4.42 (q, *J* = 7.0 Hz, 1H), 4.26-4.14 (m, 3H), 4.04 (d, *J* = 5.2 Hz, 1H), 3.99-3.90 (m, 2H), 3.84 (s, 3H), 3.47 (d, *J* = 7.4, 2H), 3.27-3.22 (m, 4H), 2.40-2.22 (m, 2H), 1.94-1.85 (m, 2H), 1.80-1.78(m, 2H), 1.59-1.40 (m, 6H), 1.35-1.22 (m, 2H), 1.18 (d, *J* = 7.0 Hz, 3H), 1.04 (s. 42H); ^{13}C-NMR (100 MHz, DMSO-d_6) δ = 172.1, 171.8, 171.6, 170.0, 169.9, 162.3, 156.4, 144.4, 144.1, 141.1, 138.0, 134.7, 133.7, 131.9, 131.2, 128.0, 127.8, 127.7, 127.4, 125.7, 125.6, 123.9, 120.5, 120.0, 117.4, 105.0, 102.4, 96.0, 66.1, 61.3, 55.4, 54.9, 50.9, 48.2, 47.1, 31.8, 28.3, 27.9, 23.1, 18.9, 11.1; HRMS *m/z* calcd for $C_{73}H_{99}N_{12}O_8Si_2$ $[M + H]^+$ 1327.7247, found 1327.7242; calcd for $C_{73}H_{98}N_{12}NaO_8Si_2$ $[M + Na]^+$ 1349.7069, found 1349.7060.

5.3.2.11 Compound 25: Compound **24** (130 mg, 0.1 mmol) was dissolved in THF (10 mL) and to this mixture a solution of 2M $(CH_3)_2NH$ in THF (4.0 mL, 8.0 mmol) was added. The resulting reaction mixture was stirred for 1 h. After removal of the volatiles under reduced pressure, the free amine was obtained and after drying for 1 h at high vacuum, the amine was dissolved in CH_2Cl_2 (20 mL) and to this solution Boc-*N*-Me-D-Leu-OH (27 mg, 011 mmol), EDCI (21 mg, 0.11 mmol) and HOBt (15 mg, 0.11mmol) were added, followed by the addition of DIPEA (35 µL, 0.2 mmol). The obtained reaction mixture was stirred for 3 h. Then, the solvent was removed by evaporation and the residue was redissolved in EtOAc (30 mL). The resulting solution was successively washed with 1 N $KHSO_4$ (2 × 20 mL), saturated $NaHCO_3$ (2 × 20 mL) and brine (50 mL) and dried over anhydrous Na_2SO_4. After filtration and removal of the solvent, the residue was purified by column chromatography (CH_2Cl_2/MeOH, 99:1 v/v). Hexapeptide **25** was obtained as a white solid in 46% yield (60 mg); R_f 0.6 (CH_2Cl_2/MeOH, 95:5 v/v); $[\alpha]^D_{20}$ = – 87.5 (*c* 1.0, $CHCl_3$); ^{1}H-NMR (400 MHz, DMSO-d_6) δ = 8.90 (d, *J* = 3.2 Hz, 1H), 8.78 (d, *J* = 12.6 Hz, 1H), 8.55 (s, 1H), 8.32 (d, *J* = 8.1 Hz, 1H), 8.01 (d, *J* = 7.6 Hz, 1H), 7.86 (s, 1H), 7.67 (s, 1H), 7.50 (s, 2H), 7.20-7.16 (m, 2H), 6.96 (t, *J* = 7.5 Hz, 1H), 6.10-6.04 (m, 1H), 5.46 (d, *J* = 6.9 Hz, 1H), 5.42-5.35 (m, 1H), 4.60-4.56 (m, 1H), 4.45-4.43 (m, 2H), 4.23-4.19 (m, 2H), 3.87 (s, 3H), 3.48-3.47 (m, 2H), 3.22 (t, *J* = 6.6 Hz, 2H), 3.05-2.92 (m, 3H), 2.64 (s, 3H), 2.41-2.36 (m, 2H),

1.83-1.79 (m, 2H), 1.77-1.43 (m, 11H), 1.37 (s, 9H), 1.15 (d, J = 6.9 Hz, 3H), 0.95-0.85 (m, 8H); ^{13}C-NMR (100 MHz, DMSO-d_6) δ = 172.5, 171.6, 171.5, 171.2, 170.7, 169.7, 169.6, 166.4, 162.0, 138.0, 134.7, 133.1, 132.0, 131.2, 128.9, 127.8, 123.9, 119.7, 117.3, 102.5, 95.8, 79.5, 61.2, 60.2, 55.3, 54.8, 52.8, 51.0, 50.9, 37.6, 31.9, 28.4, 28.2, 27.7, 24.9, 22.8, 21.2, 18.9, 14.5, 11.2; HRMS *m/z* calcd for $C_{70}H_{110}N_{13}O_9Si_2$ [M + H]$^+$ 1332.8088, found 1332.8097; calcd for $C_{70}H_{109}N_{13}NaO_9Si_2$ [M + Na]$^+$ 1354.7909, found 1354.7917.

5.3.2.12 Compound 4: TIPS-protected hexapeptide **25** (60 mg, 0.045 mmol) was dissolved in THF (10 mL), and to this solution TBAF·3H_2O (28.4 mg, 0.09 mmol) was added. The obtained reaction mixture was stirred for 1 h and according to TLC analysis, the reaction was complete. Subsequently, the reaction mixture was diluted with EtOAc (10 mL) and this solution was successively washed with 1N $KHSO_4$ (10 mL), saturated $NaHCO_3$ (10 mL) and brine (10 mL) and dried over anhydrous Na_2SO_4. After filtration and removal of the solvent, the residue was purified by column chromatography (CH_2Cl_2/MeOH, 98:2 v/v), and bis-alkyne hexapeptide **4** was obtained in 76% yield as a grey solid (35 mg). R_f 0.45 (CH_2Cl_2/MeOH, 95:5 v/v); $[\alpha]^D_{20}$ = –57.3 (*c* 1.0, $CHCl_3$/MeOH); ^{1}H-NMR (400 MHz, DMSO-d_6) δ = 8.86 (s, 1H), 8.67 (s, 1H), 8.55 (s, 1H), 8.29 (d, J = 8.1 Hz, 1H), 8.05 (s, 1H), 7.90 (s, 1H), 7.70-7.66 (m, 1H), 7.51 (s, 2H), 7.20-7.15 (m, 2H), 6.96 (t, J = 7.5 Hz, 1H), 6.10-6.04 (m, 1H), 5.46 (d, J = 6.9 Hz, 1H), 5.39-5.32 (m, 1H), 4.62-4.56 (m, 1H), 4.46-4.37 (m, 4H), 4.21-4.17 (m, 2H), 3.84 (s, 3H), 3.57-3.42 (m, 2H), 3.22 (t, J = 6.6 Hz, 2H), 3.14-3.07 (m, 2H), 2.63 (s, 3H), 2.40-2.29 (m, 2H), 1.83-1.73 (m, 2H), 1.66-1.41 (m, 7H), 1.37 (s, 9H), 1.15 (d, J = 6.9 Hz, 3H), 1.05-0.85 (m. 8H); ^{13}C-NMR (100 MHz, DMSO-d_6) δ = 172.5, 171.7, 171.6, 170.1, 169.8, 162.1, 138.0, 134.4, 133.6, 131.9, 131.2, 128.7, 127.9, 127.7, 124.0, 123.9, 111.9, 116.4, 86.0, 79.4, 61.3, 55.3, 52.8, 50.9, 49.0, 37.4, 34.9, 31.9, 30.2, 28.4, 28.2, 27.8, 25.0, 22.9; HRMS *m/z* calcd for $C_{52}H_{70}N_{13}O_9$ [M + H]$^+$ 1020.5419, found 1020.5416.

5.3.2.13 Tricyclic hexapeptide 26: Bis-alkyne hexapeptide **4** (25 mg, 0.025 mmol) and [Cp*RuCl]$_4$ (5.1 mg, 0.005 mmol) were dissolved in THF/MeOH (10 mL, 4:1 v/v). The obtained reaction mixture was heated by microwave irradiation at 80 °C for 2 h after which the solvents were removed under reduced pressure and the residue was purified by preparative RP-HPLC Boc-protected tricyclic hexapeptide

26 was obtained as a white fluffy solid after lyophilization in 16% yield (4 mg). HRMS *m/z* calcd for $C_{52}H_{70}N_{13}O_9$ [*M* + H]$^+$ 1020.5419, found 1020.5415; calcd for $C_{52}H_{69}N_{13}NaO_9$ [*M* + Na]$^+$ 1042.5239, found 1042.5238.

5.3.2.14 Unprotected tricyclic hexapeptide 1: *N*-Boc-protected tricyclic hexapeptide **26** (4 mg, 0.0039 mmol) was treated with TFA (0.2 mL) in CH_2Cl_2 (1 mL) for 1 h, after which the volatiles were removed under reduced pressure. Unprotected tricyclic hexapeptide **1** was obtained quantitavely as a white fluffy solid after lyophilization. ^{1}H-NMR (500 MHz, DMSO-d_6) δ = 8.94 (s, 1H), 8.92 (s, 1H), 8.83 (s, 1H), 8.82 (s, 1H), 8.43 (s, 1H), 8.38(d, *J* = 7.9 Hz, 1H), 7.98 (d, *J* = 7.2 Hz, 1H), 7.94 (s, 1H), 7.90 (s, 1H), 7.52 (s, 1H), 7.48 (s, 1H), 7.24-7.20 (m, 2H), 7.00 (t, *J* = 7.5 Hz, 1H), 5.67 (d, *J* = 7.9 Hz, 1H), 5.43-5.39 (m, 1H), 4.44 (s, 1H), 4.17-4.04 (m, 6H), 3.77 (s, 1H), 3.50 (s, 2H), 3.18 (m, 3H), 3.02 (s, 1H), 2.48 (s, 3H), 2.37-2.08 (m, 2H), 1.79-1.49 (m, 8H), 1.30 (d, *J* = 6.9 Hz, 3H), 1.24-1.11 (m. 5H), 0.99-0.82 (m, 8H); ^{13}C-NMR (125 MHz, DMSO-d_6) δ = 134.3, 133.8, 132.9, 132.6, 132.3, 131.4, 128.0, 124.0, 119.8, 61.5, 59.6, 56.4, 55.6, 55.1, 54.2, 51.6, 48.2, 48.1, 44.4, 40.4, 39.3, 37.5, 34.3, 31.8, 31.1, 30.4, 30.2, 29.5, 29.2, 25.1, 24.4, 23.3, 22.9, 22.4, 22.8, 22.3,17.5, 14.1; HRMS *m/z* calcd for $C_{47}H_{62}N_{13}O_7$ [*M* + H]$^+$ 920.4890, found 920.4904.

5.3.3 Binding affinity measurements

Binding affinity measurements were determined by using microcalorimetry, which was performed on automated MicroCal Auto-iTC200 equipment. ITC (isothermal titration calorimetry) experiment was carried out by injection the ligand solution (10-15 mM) into the cell containing the solution of the synthesized mimics or vancomycin (0.1-0.3 mM) dissolved in a 0.02 M Na-citrate/citric acid buffer (pH 5.1). The typical experiment contains 16 injections in 40 min and the resulting data was analyzed by non-linear fitting in Origin software.

5.3.4 Antimicrobial assay

A *Staphylococcus aureus* strain (ATCC 49230) was obtained from Medische

Microbiologie/AMC on bloodagar. TSB (Tryptic Soy Broth) culture medium was made in house. Bloodagar plates were purchased from FischerSci Oxoid (art PB5008A). Sterile flatbottom microplate (96 wells) was used for incubation. Antibacterial activity of vancomycin and the tricyclic hexapeptide **1** was evaluated employing an LC99.9 assay, which can determine the lowest sample concentration to give 99.9% bacterial death. One colony of bacteria (*S. aureus*, ATCC 49230) was first incubated in 5 mL of TSB culture at 37 °C and 150 rpm for 18 h. Then this bacterial culture was diluted 100-fold and incubated for 3 h at 37 °C and 150 rpm. The CFU/mL of this bacterial culture was determined by measurement of the OD_{630} and this bacterial culture was diluted to 2×10^6 CFU/mL. A certain amount of compound **1** was dissolved in DMSO and subsequently diluted in TSB to make a stock solution. This stock solution of compound **1** was diluted to different concentration in the microplate well with a final volume of 50 µL. To each well, 50 µL of bacterial culture (2×10^6 CFU/mL) was added. The microplate was incubated for 24 h at 37°C and 120 rpm. The CFU/mL of each well was determined by measurement of the OD_{630} and the LC99.9 was defined as the lowest concentration of the sample at which less than 0.1% of an inoculums of 10^6 CFU/mL survived. The bacteria survival was examined by adding 10 µL of the sample on bloodagar plate and the plate was incubated for 24 h at 37°C.

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Chapter 6

Summary

Samenvatting in het Nederlands

全文总结

Summary

Cyclic peptides are increasingly important in medicinal chemistry and modern drug design as they demonstrate significant biological activities and selectivities while interacting with challenging targets. However, due to the fascinating architecture of naturally occurring macrocycles their synthetic accessibility as well as the approaches for efficient peptide macrocyclization are rather limited. Therefore, much effort has been devoted to exploring highly efficient synthesis methods and macrocyclization chemistries toward cyclic peptides.

Vancomycin, which belongs to the class of glycopeptide antibiotics, has been used as a broad-spectrum antibiotic against Gram-positive bacteria and is considered as a last resort antibiotic for the treatment of methicillin-resistant *Staphylococcus aureus* (MRSA) infections. Unfortunately, after decades of excessive clinical use, the reported cases of infections caused by resistant bacteria have increased dramatically over the recent years. Therefore, the development of new generations vancomycin-derived analogues to treat infections caused by the resistant bacteria is a growing need for the near future. Due to the complexity of the total synthesis and the difficulty to incorporate structural modifications, most vancomycin analogs have been designed and synthesized by simply mimicking the cavity-like shape of vancomycin. Previously, it has been shown that promising mono- as well as bicyclic vancomycin mimics, in which alkene, alkyne and triazole moieties were alternatives for the biaryl ether bridge in vancomycin, could be synthesized by following different effective macrocyclization approaches. In this thesis, the focus was to develop applications of ruthenium-catalyzed macrocyclization chemistries for the synthesis of bi- as well as tricyclic vancomycin mimics.

In **Chapter 1**, a general introduction to vancomycin **1** and chemical approaches to synthesize cyclic peptides is described. The reported applications of ruthenium-catalyzed macrocyclization (RuAAC) and ring-closing metathesis (RCM) in the synthesis of cyclic peptides, provide several examples to use ruthenium-based macrocyclization strategies in peptide synthesis for the design and synthesis of vancomycin-inspired mimics, as shown in Figure 1.

Based on the previously described successful application of RuAAC

macrocyclization for the synthesis of the 1,5-triazole-bridged bicyclic vancomycin CDE-ring peptidomimetics **2a**, a new 1,5-triazole-bridged bicyclic vancomycin CDE-ring mimic **2b** was designed and synthesized by RuAAC which is described in **Chapter 2**. The synthesis of the newly designed CDE-ring mimic **2b** required further optimization since the molecule contained the racemization-prone phenylglycine residue as well as the unprotected amide moiety of asparagine. After the successful synthesis, unfortunately, it turned out that the obtained bicyclic CDE-ring mimic **2b** did not result in an improved binding affinity toward Ac-Lys(Ac)-D-Ala-D-Ala-OH and in line with this observation no antibacterial activity could be measured. Although the optimized solution phase synthesis route for vancomycin-derived peptides is flexible and robust, it is still rather inefficient and time consuming, therefore the solution phase synthesis was translated into a solid phase synthesis approach in an attempt to improve the synthetic efficiency of this class of vancomycin mimics.

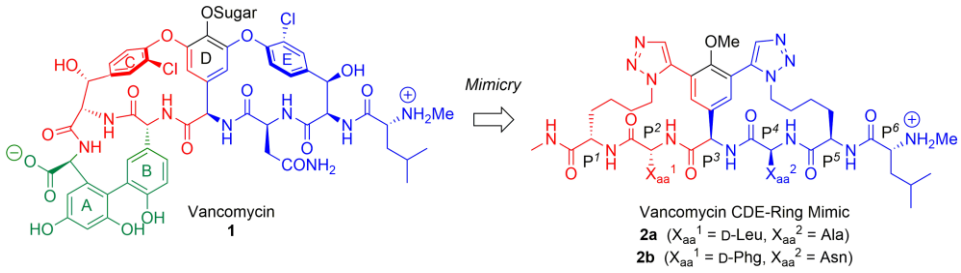


Figure 1. Design of the new 1,5-triazole-bridged bicyclic vancomycin CDE-ring mimics**.**

Chapter 3, describes the design and synthesis of three 1,5-triazole-bridged vancomycin CDE-ring mimics **3abc** accessed by a combination of SPPS and solution-based RuAAC macrocyclization (Figure 2).

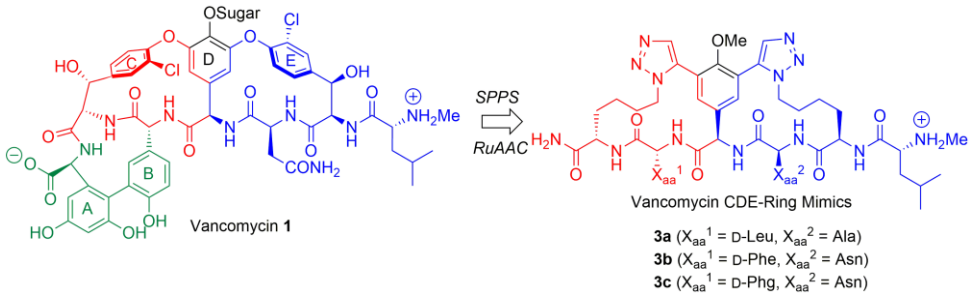


Figure 2. Design of the bicyclic vancomycin CDE-ring mimics using Solid phase peptide synthesis and RuAAC macrocyclization.

Due to the high tendency of racemization of the phenylglycine building block during amide bond formation and the low solubility of the semi-protected linear peptides after cleavage from the resin, the amounts obtained were too small for further NMR analysis and biological evaluation and the desired compounds could only be identified by mass spectrometry. In hindsight, this chapter was focused to provide a proof-of-principle to access the bicyclic 1,5-triazole-bridged CDE-ring mimics of vancomycin using SPPS and RuAAC macrocyclization.

Chapter 4, describes the design of a bicyclic tripeptide as a mimic **4** of the ABC-ring system and its successful synthesis consisting of an RCM-coupling-RuAAC strategy (as shown in Figure 3). The mixture of double bond isomers could be separated by preparative HPLC to give each individual *E*/*Z* diastereoisomer as the unprotected α-amine as characterized and identified by NMR and LC-MS. This bicyclic tripeptide represents an important building block to arrive at a tricyclic hexapeptide as the ultimate mimic of vancomycin in which ruthenium-based cyclization approaches will be used to control the topology and rigidity of the peptide backbone.

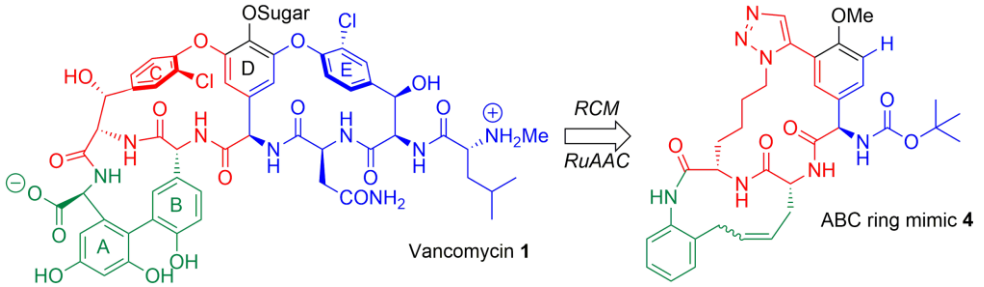


Figure 3. Design of vancomycin ABC-ring mimics using RCM and RuAAC macrocyclization.

Chapter 5 describes the design and synthesis of a tricyclic hexapeptide **5** in which the olefinic constraint was installed by RCM and both triazole bridges were synthesized featuring RuAAC (as shown in Figure 4). Molecular modeling indicated that the structure of the tricyclic hexapeptide showed a high resemblance with the crystal structure of balhimycin (a structural analog of vancomycin). The binding affinity measured by ITC also indicated that the tricyclic hexapeptide was able to bind D-Ala-D-Ala ligand with a comparable affinity as vancomycin, 1.26×10^4 and 4.23×10^5 M^{-1}, respectively. Gratifyingly, in line with the result of the ITC measurements, this tricyclic mimic displayed promising antibacterial activity with a MIC value of 37.5 μg/mL while the MIC value of vancomycin was 2 μg/mL. This was the first time that a member of this class of vancomycin-inspired mimics showed a reasonable activity as an antimicrobial agent.

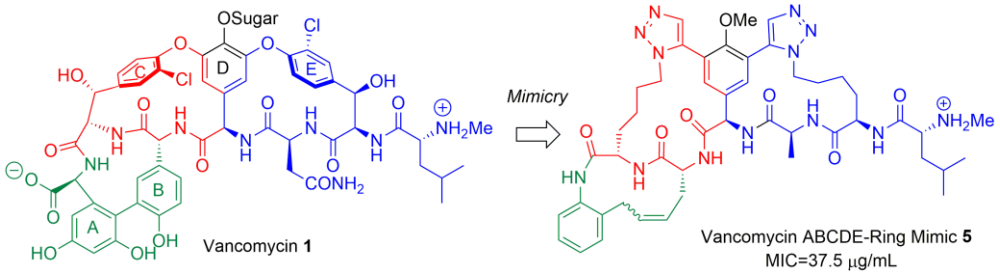


Figure 4. Design of vancomycin ABCDE-ring mimics.

In conclusion, this thesis describes several successful synthesis approaches toward vancomycin mimics, in which ruthenium-catalyzed macrocyclization chemistries have been used for peptide rigidification. Furthermore, inspired by the

(preliminary) results of Chapter 5, it is expected that the synthesis of a set of tricyclic hexapeptide analogues will be possible in which the overall rigidity and shell-like topology of these peptides will lead to biologically active vancomycin mimics.

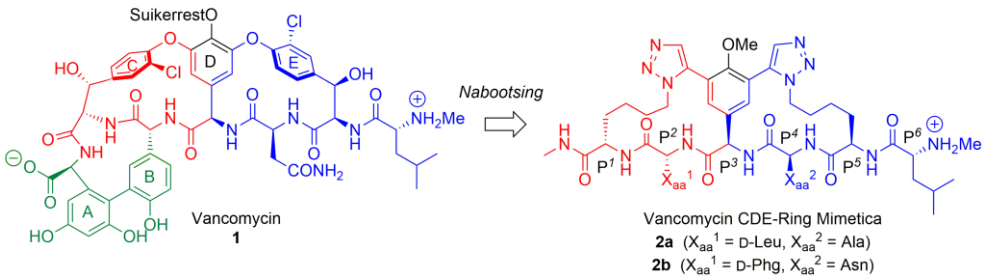
Samenvatting in het Nederlands

Cyclische peptiden zijn van toenemend belang in de medicinale chemie en in de moderne geneesmiddelontwikkeling omdat deze moleculen een belangrijke biologische activiteit en selectiviteit vertonen op een divers aantal receptor- en doelmoleculen. Echter, als gevolg van de fascinerende en complexe structuur van in de natuur voorkomende macrocyclische peptiden is zowel de beschikbaarheid via chemische synthese als ook methoden voor efficiënte ringsluitingsbenaderingen in het algemeen beperkt. Als gevolg hiervan wordt veel onderzoek verricht naar verbeterde synthesemethoden en cyclisatie-strategieën om cyclische peptiden op een rendabele manier te verkrijgen.

Vancomycine is een multicyclisch peptide en behoort tot de klasse van glycopeptide-antibiotica, en vindt toepassing als een breedspectrum antibioticum ter bestrijding van infecties welke veroorzaakt worden door Gram-positieve bacteriën. Vancomycine geldt als laatste redmiddel tegen infecties veroorzaakt door zogenaamde methicilline-resistente *Stapylococcus aureus* (MRSA) bacteriën. Echter, door veelvuldig gebruik (en misbruik) van antibiotica gedurende de afgelopen decennia wordt steeds vaker melding gemaakt van bacteriën die antibiotica-resistentie verworven hebben, zelfs tegen vancomycine. Deze dramatische ontwikkeling nodigt uit te zoeken naar nieuwe klassen van antibiotica of bestaande antibiotica te verbeteren die hun werkzaamheid tegen resistente bacteriën opnieuw verkregen hebben. Vancomycine kan hiervoor als startpunt gebruikt worden, echter de totaalsynthese van vancomycine en ook het aanbrengen van subtiele structuurvariaties in vancomycine zijn zeer uitdagend en daarom is een toevlucht gezocht naar moleculen die de structuur en werking van vancomycine nabootsen en die daarnaast ook eenvoudig toegankelijk zijn via chemische synthese. Uit eerder onderzoek is gebleken dat monocyclische en bicyclische vancomycine analoga gesynthetiseerd kunnen worden waarin de gangbare biaryl-ether brug vervangen kan worden door een alkeen-, of een alkyn-, of een triazool-eenheid waarbij gebruik gemaakt wordt van een drietal verschillende ringsluitingsbenaderingen. In dit proefschrift ligt de nadruk om twee, door het metaal ruthenium gekatalyseerde, cyclisatie-methoden te combineren om

hiermee bi- en tricyclische, van vancomycine afgeleide hexapeptiden, te synthetiseren.

Hoofdstuk 1 voorziet in een algemene inleiding van het proefschrift waarin vancomycine (**1**) en chemische synthesemethoden voor cyclische peptiden nader worden toegelicht. Daarnaast worden specifieke voorbeelden uit de literatuur besproken van succesvolle ruthenium-gekatalyseerde macrocyclisaties, zoals ring-sluitingsmetathese (ring-closing metathesis RCM) en de ruthenium-gekatalyseerde azide-alkyn cycloadditiereactie (ruthenium-catalyzed azide-alkyne cycloaddition reaction, RuAAC), ter verkrijging van cyclische peptiden welke ook in dit proefschrift gebruikt zijn voor de synthese van vancomycine-afgeleide peptidomimetica, zoals afgebeeld in Figuur 1.

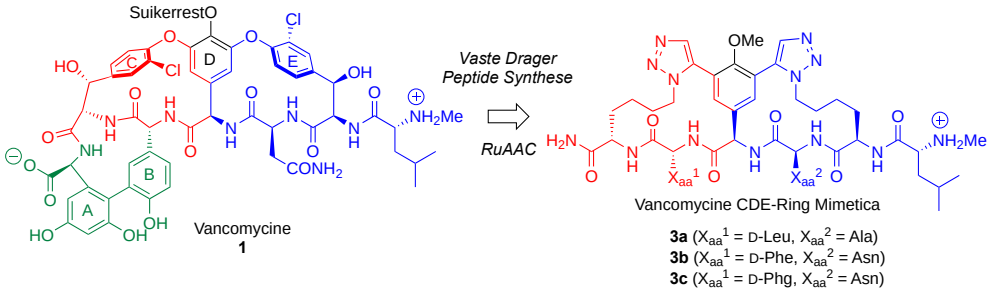


Figuur 1. Het ontwerp van nieuwe 1,5-triazool-gebrugde bicyclische vancomycine CDE-ring mimetica**.**

Afgeleid van een eerdere succesvol toegepaste RuAAC-gebaseerde syntheseroute voor een vancomycine CDE-ring mimeticum **2a** is een nieuw derivaat **2b** ontworpen en gesynthetiseerd en de resultaten daarvan staan in **Hoofdstuk 2** beschreven. In dit nieuwe vancomycine-analogon **2b** zijn de aminozuren phenylglycine en asparagine ingebouwd die om verschillende redenen, racemisatie-gevoeligheid respectievelijk oplosbaarheid, belangrijke aanpassingen in de originele syntheseroute vereisten. Na synthese bleek het nieuw ontworpen molecuul geen verbeterde affiniteit voor het natuurlijke ligand, Ac-Lys(Ac)-D-Ala-D-Ala-OH, te hebben en overeenkomstig dit resultaat bleek het molecuul niet actief als bacteriedodend middel. Niettemin was er nog steeds behoefte aan een geoptimaliseerde synthesemethode voor dit soort vancomycine-analoga die de versnelde synthese van structuurvariaties in het

basisskelet van vancomycine mogelijk maakte.

In **Hoofdstuk 3** wordt deze aangepaste synthesebenadering beschreven. De tot nu toe gebruikte oplossings-synthese is vertaald naar een vaste-drager-synthese methodologie (Figuur 2).

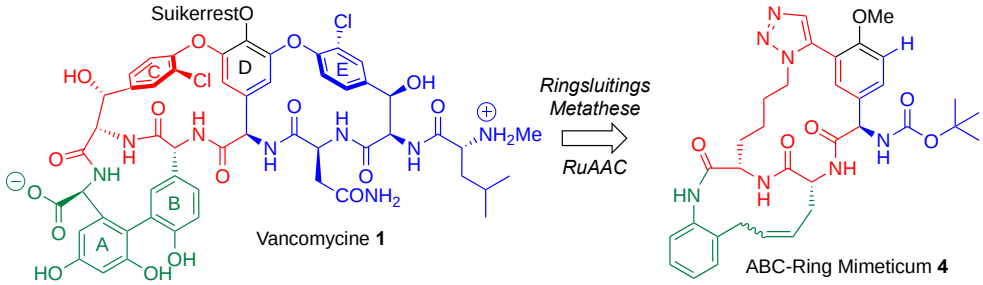


Figuur 2. Het ontwerp van bicyclische vancomycine CDE-ring mimetica verkregen door combinatie vaste drager peptide synthese en RuAAC-gebaseerde macrocyclisatie.

De synthese van peptiden op de vast-drager is een beproefd concept en leent zich zeer goed voor een versnelde toegankelijkheid van vancomycine-analoga **3abc** waarin een of meerdere aminozuren gewijzigd zijn om hiermee inzicht te verkrijgen in de bijdrage van elk individueel aminozuurresidu in de biologische activiteit als antimicrobieel middel. Als gevolg van de extreme gevoeligheid voor racemisatie van het phenylglycine-residu tijdens de vorming van de amidebinding en de verlaagde oplosbaarheid van de gedeeltelijk ontschermde lineaire peptiden bleek opzuivering met behulp van kolomchromatografie zeer lastig. Dit alles resulteerde in een zeer lage opbrengst van een drietal vancomycine-analoga en als gevolg van de minimale geisoleerde hoeveelheden kon slechts met massaspectrometrie de identiteit worden vastgesteld. Verdere analyses met behulp van kernspinresonantie ter bevestiging van de gewenste structuur als ook biologische activiteitsbepalingen konden door deze kleine hoeveelheden niet betrouwbaar uitgevoerd worden. Achteraf beschouwd is Hoofdstuk 3 een 'proof-of-principle' gebleken van de vertaling van de oplossings-synthese naar de vaste-drager terwijl de vast-drager-synthese (nog) niet betrouwbaar is om in relatief korte tijd een groot aantal structuur-variaties gebaseerd op vancomycine te realiseren.

Hoofdstuk 4 beschrijft het ontwerp en de succesvolle synthese van een

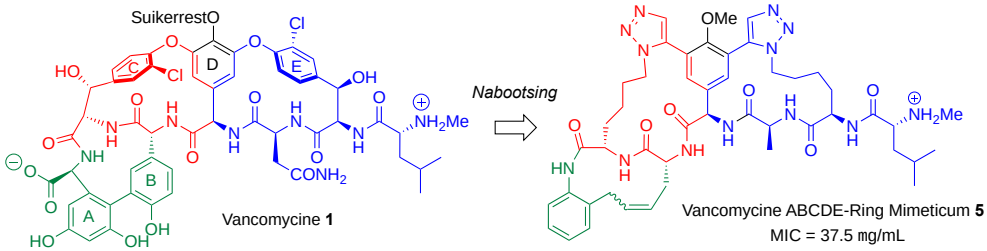
bicyclisch tripeptide **4** dat het ABC-ring systeem van vancomycine nabootst (zoals afgebeeld in Figuur 3). De gevolgde syntheseroute is gebaseerd op een sequentie van de volgende reactiestappen: RCM - amidevorming - RuAAC. Het is gebleken dat de mengsel van alkenen, verkregen na de RCM stap, alleen als het vrije α-amine in de afzonderlijke diastereoisomeren met kolomchromatografie te scheiden was, waarna toekenning van de stereochemie van de dubbele binding in *E* en *Z* met behulp van kernspinresonantie en massaspectrometrie mogelijk bleek. Het verkregen bicyclische tripeptide vertegenwoordigt een belangrijke bouwsteen in de synthese van een tricyclisch hexapeptide, welke als het ultieme mimeticum van vancomycine beschouwd kan worden omdat hiermee de gewenste topologie en rigiditeit van de peptideketen verkregen wordt.



Figuur 3. Het ontwerp van vancomycine-afgeleide ABC-ring mimetica verkregen via een combinatie van ringsluitings-metathese and RuAAC-gebaseerde macrocyclisatie.

Tot slot beschrijft **Hoofdstuk 5** het ontwerp en synthese van het eerder genoemde tricyclische hexapeptide **5**, waarin de alkeen-eenheid (verkregen via RCM) in combinatie met twee triazool-eenheden (verkregen via RuAAC) het molecuul de gewenste drie-dimensionele structuur verleend om de biologische activiteit van vancomycine na te bootsen (zoals afgebeeld in Figuur 4). Computerberekeningen hebben aangetoond dat de structuur van het tricyclische hexapeptide in grote mate overeenkomt met een kristalstructuur van balhimycin (een structuuranalogon van vancomycine). De bindingsaffiniteit tussen het tricyclische hexapeptide en het D-Ala-D-Ala ligand, gemeten met isotherme titratie calorimetrie, gaf een waarde die te vergelijken is met vancomycine 1.26×10^4 respectievelijk 4.23×10^5 M^{-1}. In overeenstemming met dit resultaat was de gemeten antimicrobiële activiteit uitgedrukt als de minimale inhibitie concentratie

van 37,5 µg/mL, terwijl voor vancomycine deze waarde 2 µg/mL bedraagt. Zowel de bindingsaffiniteit als de antimicrobiële activiteit bleek de hoogste te zijn (van de tot nu toe gemeten waarden) voor deze klasse van vancomycine mimetica waaruit nog maar eens blijkt hoe belangrijk de ruimtelijke structuur van de peptideketen is voor de biologische activiteit.



Figuur 4. Het ontwerp van vancomycine-afgeleide ABCDE-ring mimetica.

De samenvattende conclusie luidt, dat dit proefschrift verschillende synthesebenaderingen voor vancomycine-mimetica beschrijft waarin ruthenium-gekatalyseerde ringsluitings-strategieën een sleutelrol spelen het gewenste hexapeptide te voorzien van de gewenste rigiditeit om biologisch actief te zijn. Het is de verwachting dat de bemoedigende resultaten van hoofdstuk 5 verder uitgewerkt kunnen worden door een serie van analoga te synthetiseren waarin de peptideketen is opgebouwd uit verschillende aminozuurresiduen die echter wel de biologisch actieve conformatie aannemen overeenkomstig met die van vancomycine.

全文总结

环肽类化合物在和其相对应的靶点相互作用时，常常表现出非常显著的生物活性和选择性，因此在药物化学以及现代药物设计领域中占据了越来越重要的地位。然而，由于其结构的复杂性，该类化合物往往无法通过有效的多肽大环化来进行合成。因此，探索高效的环肽类化合物合成方法显得极其重要。

万古霉素属于糖肽类抗生素的一种广谱抗生素，对革兰式阳性菌具有良好的抑制作用，在临床上一直被认为是治疗耐甲氧苯青霉素的金黄色葡萄球菌感染的最后一道防线。然而，由于过去几十年对万古霉素的过度使用，临床上已经报道了大量耐药菌产生的感染病例。因此，研究新一代的万古霉素类似物来治疗耐药菌产生的感染越来越重要。为了克服万古霉素结构复杂性导致的改造困难，很多万古霉素类似物在设计之初就只通过模拟活性部分来将其结构简化。我们课题组已通过有效的大环化方法成功合成了一系列单环以及双环的万古霉素类似物，用双键，三键以及三氮唑环来替代万古霉素结构中的芳联醚桥。在本论文中，我们着重探索了钌催化的大环化方法来合成二环以及三环万古霉素类似物。

第一章主要是对万古霉素（**1**）的背景以及目前主要的大环化合成方法进行了综述。其中详细列举了钌催化的叠氮-炔加成反应(RuAAC)以及关环复分解反应(RCM)在大环化中的应用，为我们之后通过钌催化的大环化方法来合成万古霉素类似物提供了方向（图 1）。

基于之前成功地运用了 RuAAC 大环化合成了具有 1,5-三氮唑结构的万古霉素二环类似物 **2a**，**第二章**设计并合成了新的具有 1,5-三氮唑结构的万古霉素 CDE 二环类似物 **2b**。相较于之前的 CDE 二环类似物，本章所设计的类似物 **2b** 含有较容易消旋的苯甘氨酸残基以及天冬酰胺残基中的游离氨基，因此需要对其合成方法进行进一步的优化。然而，活性结果表示该类似物 **2b** 和之前的二环类似物对于 Ac-Lys(Ac)-D-Ala-D-Ala-OH 受体的结合力并没有提高，而且也没有观察到明显的抗菌活性。虽然经过优化后的合成方法具有很大的灵活性和重现性，但还是相对低效和费时，因此我们引入了一个新的合成方法——固相合成，来提高此类类似物的合成效率。

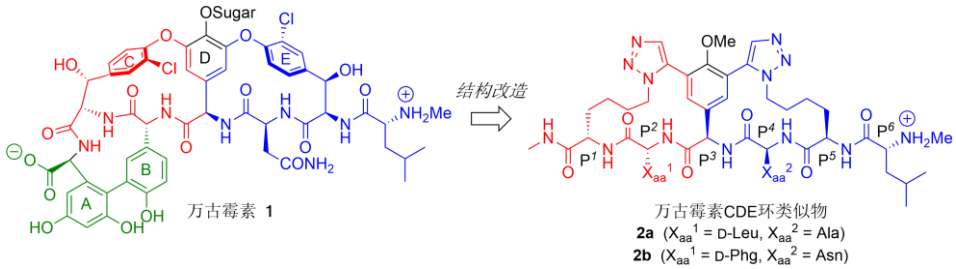


图 1. 新的万古霉素 CDE 环类似物的设计和合成

第三章主要描述了通过固相合成和液相合成相结合的方法合成三个具有 1,5-三氮唑结构的万古霉素 CDE 二环类似物 **3abc**。其中由苯甘氨酸残基在多肽的固相合成中引入的消旋，以及从树脂上切割下来的链状多肽的低溶解性，导致只得到足够进行质谱分析的化合物，无法进行后期的核磁结构确证以及生物活性评价。但是，本章主旨只在提供通过固相合成和液相合成相结合的方法合成此类万古霉素 CDE 二环类似物的可能性。

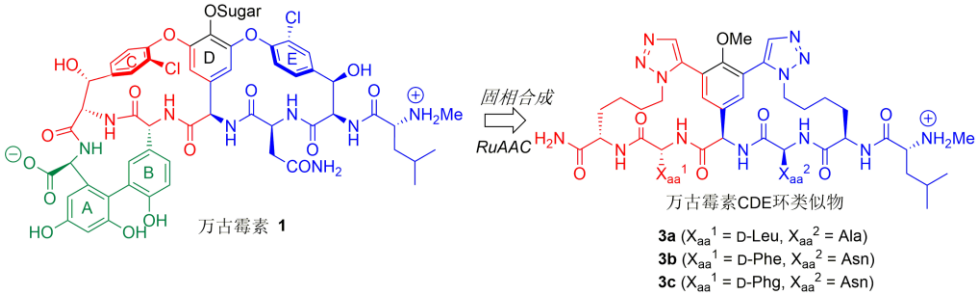


图 2. 固相合成和 RuAAC 大环化相结合设计和合成新的万古霉素 CDE 环类似物

第四章主要设计和合成了万古霉素 ABC 二环类似物 **4**。该类似物可以通过 RCM-缩合-RuAAC 的步骤顺利合成。其中由于双键引入的 E/Z 异构体可以通过制备液相分离，核磁以及液质确证分离得到了具有单一构型的化合物。该双环三肽结构可以作为之后用钌催化的大环化合成方法合成三环六肽万古霉素类似物过程中一个非常重要的片段。

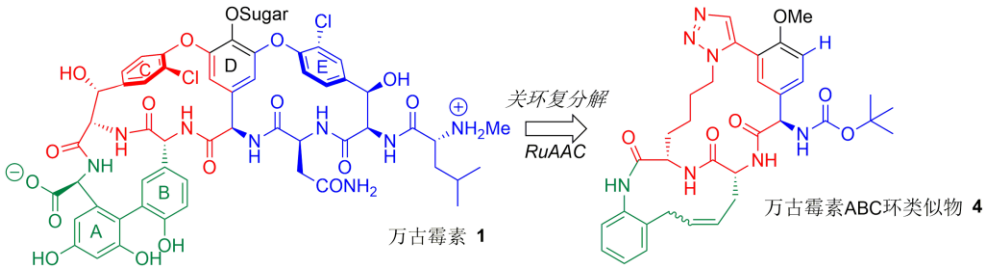


图 3. 设计和合成万古霉素 ABC 环类似物

第五章主要设计和合成了万古霉素三环类似物 **5**，其中双键结构以及两个三氮唑结构分别由 RCM 反应和 RuAAC 反应引入。分子模拟结果表明该三环类似物的结构和 balhimycin（万古霉素的结构类似物）的晶体结构具有很高的相似性。ITC 结果也证明该化合物可以和 D-Ala-D-Ala 受体结合，其和万古霉素测得的结合力分别为 1.26×10^{4} 和 4.23×10^{5} M^{-1}。和 ITC 结果相对应的抗菌实验表明，该化合物具有非常高潜力的抗菌活性，它和万古霉素的 MIC 值分别为 37.5 μg/mL 和 2 μg/mL。该三环类似物也是该类万古霉素类似物中第一个具有抗菌活性的分子。

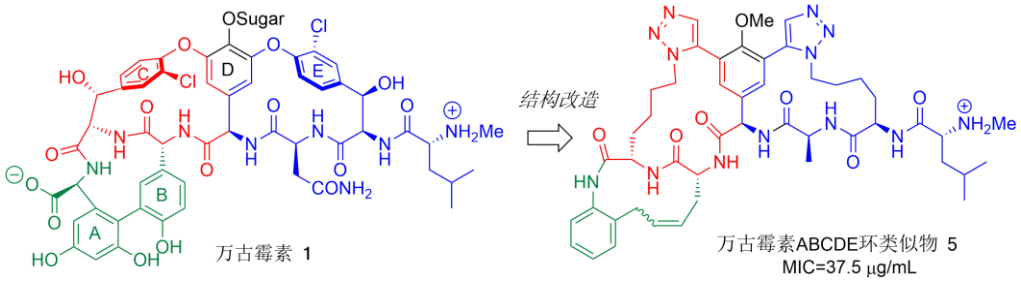


图 4. 设计和合成万古霉素 ABCDE 环类似物

综上所述，本论文主要描述了通过不同合成方法，尤其是不同的钌催化的大环化方法，来合成万古霉素类似物。基于第五章中万古霉素类似物的初步活性测试结果，我们希望能进一步合成一系列具有刚性、贝壳状结构，以及具有潜在活性的三环万古霉素类似物。

Appendices

Curriculum Vitae

Xin Yang, was born in Zhejiang, China on December 26, 1988. After graduating from Shengzhou No. 1 Middle School, he started with his education at the College of Pharmaceutical Sciences, Zhejiang University, China, where he received his Bachelor's degree in Pharmaceutical Sciences in 2010. Then he was recommended to study (without entrance examination) at the Shanghai Institute of Pharmaceutical Industry, China, for his Master's degree. During his three years Master's studies, he worked in the State Key Lab of New Drug and Pharmaceutical Process and focused on the synthetic industrialization studies of sorafenib tosylate and pazopanib hydrochloride under the supervision of Prof. Junda Cen. After receiving his Master's degree in 2013, he was awarded with a scholarship from the Chinese Scholarship Council and moved to the Division of Chemical Biology & Drug Discovery at the Department of Pharmaceutical Sciences at Utrecht University in the Netherlands, in November 2013. He was a PhD candidate from November 2013 until April 2018 under supervision of Prof. dr. Rob Liskamp and dr. Dirk Rijkers. During his PhD studies, he worked on synthesizing vancomycin mimics using ruthenium-catalyzed macrocyclization chemistries and the results of this research are described in this thesis. After finishing his PhD studies, he will return to China and will start his new job as manager of the Department of Drug Discovery at Shanghai Dude Medical Science & Technology Co. LTD.

List of Abbreviations

Ac	acetyl
Ac_2O	acetic anhydride
AcOH	acetic acid
Ala	alanine
allGly	allylglycine
Alg	allylglycine
AngIV	angiotensin IV
Asn	asparagine
Asp	aspartic acid
Arg	arginine
Bn	benzyl
Boc	*tert*-butyloxycarbonyl
BOP	benzotriazol-1-yloxy-tris-(dimethylamino)-phosphonium hexafluorophosphate
*t*Bu	*tert*-butyl
CBP	(4-chlorobiphenyl)methyl
COD	1,5-cyclooctadiene
COMU	(1- cyano-2-ethoxy-2-oxoethylidenaminooxy) dimethylaminomorpholinocarbenium hexafluorophosphate
COSY	correlation spectroscopy
Cp	cyclopentadienyl
Cy	cyclohexyl
CuAAC	copper-catalyzed azide alkyne cycloaddition
DCC	dicyclohexylcarbodiimide

DCM	dichloromethane
DEPBT	3-(diethoxyphosphoryloxy)-1,2,3-benzotriazin-4(3H)-one
DIPEA	diisopropylethylamine
DKP	diketopiperazine
DMF	*N, N*-dimethylformamide
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DOS	diversity-oriented synthesis
EDCI	1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride
ESI-MS	electrospray ionization mass spectrometry
ESI-TOF MS	electrospray ionization time-of-flight mass spectrometry
EtOAc	ethyl acetate
Et_2O	diethyl ether
Fmoc	9-fluorenylmethyloxycarbonyl
HATU	1-[bis(dimethylamino)methylene]-1H-1,2,3-triazolo-[4,5-b] pyridinium 3-oxid hexafluorophosphate
HDAC	histone deacetylase
HG2	second generation Hoveyda-Grubbs catalyst
His	histidine
HOAt	7-aza-1-hydroxybenzotriazole
HOBt	1-hydroxybenzotriazole
HPLC	high performance liquid chromatography
HSQC	heteronuclear single quantum coherence
Ile	isoleucine
IRAP	insulin-regulated aminopeptidase inhibitors
ITC	isothermal titration calorimetry

Lac	lactic acid
LC-MS	liquid chromatography mass spectrometry
Leu	leucine
Lys	lysine
Me	methyl
Mes	mesityl
MIC	minimum inhibition concentration
MLL	mixed lineage leukemia
MRSA	methicillin-resistant *Staphylococcus aureus*
Ms	methanesulfonyl
MW	microwave
NMR	nuclear magnetic resonance
NMP	*N*-methyl pyrrolidone
PAL-PEG-PS	peptide amide linker-poly(ethylene glycol)-polystyrene
Pbf	2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl
Ph	phenyl
Phe	phenylalanine
Phg	phenylglycine
PMB	p-methoxybenzyl
Pro	proline
RMSD	root-mean-square-deviation
RCM	ring-closing metathesis
RP	reverse phase
RuAAC	ruthenium-catalyzed azide alkyne cycloaddition
Ser	serine
S_NAr	nucleophilic aromatic substitution
SPPS	solid phase peptide synthesis

TBAF	tetrabutylammonium fluoride
TFA	trifluoroacetic acid
Trt	trityl
THF	tetrahydrofuran
Thr	threonine
TLC	thin layer chromatography
TIPS	triisopropylsilyl
TMS	trimethylsilyl
Tyr	tyrosine
Val	valine
VRE	vancomycin-resistant *Enterococci*
VRSA	vancomycin-resistant *Staphylococcus aureus*
Xaa	amino acid

List of Publications

Publications and patents:

Xin Yang, Lucas P. Beroske, Johan Kemmink, Dirk T. S. Rijkers, Rob M. J. Liskamp. Synthesis of bicyclic tripeptides inspired by the ABC-ring system of vancomycin through ruthenium-based cyclization chemistries, *Tetrahedron Letters* **2017**, *58*, 4542-4546.

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Xin Yang, Dirk T. S. Rijkers, Rob M. J. Liskamp. Ruthenium-based cyclization chemistries to access tricyclic heptapeptides as mimics of vancomycin.

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