# Chemical Modification of Peptide Antibiotics

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# Chapter 7

## Conclusions and Perspectives

*This chapter summarizes the most important results obtained in this thesis. Finally, a general conclusion and future outlook will be given.*

## 7.1 Introduction

Antibiotic resistance is becoming a major threat to our modern-day healthcare system. The emergence of multidrug-resistant bacteria is estimated to cause over 10 million deaths annually by the year 2050, and the projected indirect socio-economic consequences are massive.[1] Therefore, the demand for new types of treatments that are less prone to the development of resistance is urgent. Over the years, it is bacteria themselves that have proven to be a rich source of natural products that exhibit antimicrobial activity.[2] Among these, non-ribosomal peptides (NRPs) and Ribosomally synthesized and Post-translationally modified peptides (RiPPs) have become interesting targets in the search for new antibiotics because of their high activity and lower development of resistance compared to conventional antibiotics.[3–5] Yet despite their promising bioactivity profile, clinical application is hampered by their poor pharmacological properties, such as low *in vivo* stability and aqueous solubility, and because a detailed understanding of their mechanism of action is often lacking. The chemical modification of these peptides to make semi-synthetic structural analogs with improved properties is a promising approach to overcome these obstacles. However, the development of methods for the chemo- and site-selective editing of these complex natural products remains a formidable challenge. An increasingly popular strategy is to make use of uniquely reactive structural motifs found exclusively in these compounds as a result of the post-translational machinery involved in their biosynthesis. Dehydroalanine (Dha) residues, which are commonly found in RiPPs, have a distinct reactivity as carbon electrophiles due to their α,β-unsaturation, and they have become versatile orthogonal handles for the selective modification of peptides and proteins.[6–9]

The research described in this thesis aimed at the selective chemical modification of Dha residues in peptide antibiotics with an emphasis on first-row transition metal catalysis. The primary focus of the research was to develop new methods for the late-stage chemical editing of RiPPs via their uniquely reactive Dha residues. The potential of these methods for improving the pharmacological properties of RiPPs and their application in the labeling and targeting of these peptides was also investigated. Another objective was to characterize newly discovered NRPs in order to aid in the development of a method for the systematic discovery and isolation of novel antimicrobial peptides from microorganisms.

Ultimately, three new methodologies for the selective chemical installation of non-natural functionalities onto Dha in RiPPs were developed. The utility of these methods in the labeling and targeting of the peptides was demonstrated, and the

pharmacological properties and biological activity of the resulting semi-synthetic analogs were evaluated. Additionally, several newly discovered antimicrobial NRPs were characterized via NMR spectroscopy. The outcomes of these studies are summarized in the next sections, after which a general conclusion and future outlook is given.

## 7.2 Transition-metal-free Diels-Alder reactions

**Chapter 2** describes how Dha can be employed as a dienophile in the modification of RiPPs via Diels-Alder reactions. Initial screening of the reaction on a protected Dha monomer with cyclopentadiene showed that at room temperature the product could was formed under aqueous conditions, while in organic solvent no product was obtained. This rate acceleration, which is known for Diels-Alder reactions when performed in water instead of organic solvents,[10] showed promise for implementing the reaction on peptides, which require mild and aqueous conditions. Further optimization of the reaction conditions showed that a 1:1 mixture of water with 2,2,2-trifluoroethanol (TFE) as solvent and $Sc(OTf)_3$ as a Lewis acid gave the highest conversions, which was attributed to the activation of Dha as a dienophile by both the scandium catalyst and the mildly Brønsted acidic TFE.

When thiostrepton, a member of the thiopeptide family of RiPPs, was subjected to the optimized reaction conditions, moderate conversions to singly and doubly modified peptide were observed. However, it was shown that $Sc(OTf)_3$ was detrimental to the overall stability of the peptide and higher and cleaner conversions were obtained when the scandium catalyst was omitted. These transition-metal-free conditions were optimized further and the conversion was increased significantly by heating the reaction mixture in a microwave reactor. This also gave higher conversions than heating at the same temperature in an oil bath, although the exact reason for this remains unclear. The site-selectivity of the reaction on thiostrepton was established via 2D NMR spectroscopy on the purified products. The reaction was shown to be completely selective for the tail region of thiostrepton, which is highly desirable since it is known that modifying this tail region generally has a low impact on the inherent antimicrobial activity of the peptide. Minimum Inhibitory Concentration (MIC) assays of the purified products confirmed that although antimicrobial activities were somewhat lower than that of unmodified thiostrepton, high activities were retained for all derivatives. The thiopeptide nosiheptide and lanthipeptide nisin were also modified efficiently using the optimized conditions, demonstrating that the developed method is broadly applicable for Dha-containing RiPPs.

The Diels-Alder reaction of Dha with cyclopentadiene results in the installation of a norbornene functionality on the peptides. These contain a strained alkene, which can participate in the fast and bio-orthogonal Inverse Electron Demand Diels-Alder (IEDDA) click reaction with tetrazines.[11] The purified Diels-Alder adducts of thiostrepton were labeled successfully using a variety of tetrazines functionalized with fluorescent labels and targeting groups. This shows that the selective installation of a norbornene via the Diels-Alder modification of Dha residues, followed by fast and bio-orthogonal labeling via IEDDA with tetrazines is a powerful method for the labeling and conjugation of RiPPs.

Thiostrepton has a high activity against Gram-positive bacteria due to its ability to block ribosomal protein synthesis by binding to the ribosome.[12,13] Despite that this ribosomal target is also present in Gram-negative bacteria, thiostrepton shows no activity against these strains because it is not able to cross their outer membrane.[12,13] Siderophores, which are used by bacteria for the uptake of Fe(III), are useful targeting groups for antibiotics because they are actively transported into the cells via receptor-mediated uptake.[14] Therefore, the synthesis of antibiotic-siderophore hybrids has become a popular “Trojan horse” strategy for the selective targeting of bacteria.[15,16]

In **chapter 3** the two-step conjugation method described in **chapter 2** was applied to the semi-synthesis of thiostrepton-siderophore hybrids for the targeting of Gram-negative bacteria. Two non-natural siderophore analogs with tetrazine clickable groups were synthesized. To make the existing synthesis compatible with the tetrazine moieties, a new *para*-methoxybenzyl protecting group strategy was developed for the iron-binding catecholate groups, which is complementary to the benzyl protecting group chemistry often used in literature.[17] Ultimately, a convergent synthetic route was developed which led to the isolation of the two tetrazine-siderophore constructs. The norbornene functionality was installed chemically on thiostrepton via the method described in **chapter 2** and the thiostrepton-siderophore hybrids were formed successfully in the IEDDA click reaction with the tetrazine-siderophores. The hybrids were isolated and tested against Gram-negative and Gram-positive bacteria, however they did not display activity against any of the strains tested. The absence of activity even against Gram-positive bacteria, which are normally highly susceptible to thiostrepton, suggests that the covalent attachment of the siderophore is detrimental for the inherent activity of thiostrepton. Although desired activities were not observed, this study clearly demonstrates the potential of this method for the synthesis of antibiotic hybrids. Therefore, further research towards the synthesis of thiostrepton-siderophore hybrids

using this method should continue, but using a linker that facilitates the release of the antibiotic once it is inside the cell.

## 7.3 Cu(II)-catalyzed β-borylations and β-silylations

The modification of Dha in RiPPs has been performed successfully via a variety of different reaction classes.[7–9] However, the focus of these different methods is primarily on C-C-bond formation. The installation of C-N and C-S-bonds has also been reported,[18,19] but efficient methods for the direct chemical incorporation of non-natural bonds on Dha in RiPPs are lacking. The introduction of abiological functionalities such as boronic acids and silyl groups have shown to be effective ways to improve the activity, stability and solubility of pharmaceutical compounds.[20,21] Therefore, such moieties are interesting targets for the modification of RiPPs in order to improve their pharmacological properties. Since boron and silicon are rarely found in biomolecules, introducing them into natural products such as RiPPs also enables the exploration of new types of chemical reactivity that can be used for the conjugation and labeling of these peptides.

In **chapter 4** the β-borylation of Dha in RiPPs via Cu(II)-catalysis is described. The β-borylation of small α,β-unsaturated carbonyl substrates has been shown to proceed efficiently using $B_2(OH)_4$ as the boron source and $CuSO_4$ as a catalyst under mild and aqueous conditions, providing an excellent starting point for the modification of Dha in peptides and proteins. First, the β-borylation of a protected Dha monomer was screened using previously reported conditions. Full conversion was achieved within 1 hour of reaction time at room temperature, showing that Dha is indeed a good substrate for this reaction. The reaction was tested next on thiostrepton, and after optimization of the reaction conditions complete consumption of the starting peptide was achieved within 1 hour at room temperature. Full conversion to a single major product was observed, which was proven to be the double borylated peptide. This implied that the reaction is both rapid and highly selective, especially when considering the high structural complexity of the natural product. NMR studies on the purified product showed a double β-borylation of the two terminal Dha residues in the tail region of thiostrepton, confirming the high chemo- and site-selectivity of the method. The approach has a broad scope, since the thiopeptide nosiheptide, the lanthipeptide nisin Z and the protein Small Ubiquitin-like Modifier (SUMO) that had a dehydroalanine residue incorporated were all modified efficiently.

The effect of the boronic acid modification on the aqueous solubility of thiostrepton and nosiheptide was also evaluated. The poor solubility of thiopeptides

in water is one of their greatest limitations that hamper their clinical application. Solubility assays of the purified borylated analogs showed that for thiostrepton the aqueous solubility was indeed increased up to 84-fold compared to the unmodified peptide. Antimicrobial activity assays of both analogs showed that although the activity was decreased compared to the wild-type peptides, high effectivity against their target strains was retained. Collectively, these results demonstrate that using this approach the poor aqueous solubility of RiPPs can be improved, while maintaining antimicrobial activities that compare well with conventional antibiotics.

Boronic acids are also useful intermediates for a variety of chemical transformations, which is a highly useful attribute for the further modification of RiPPs via chemical mutagenesis. In **chapter 4**, it was also shown that the boronic acids installed on thiostrepton were oxidized efficiently to hydroxyl functionalities using $NaBO_3$ as a mild oxidant. This borylation-oxidation of dehydroalanines provides a fast and simple procedure for the chemical installation of serines, which are uncommon residues in RiPPs. Moreover, the reversible boronate ester formation of boronic acids with diols and triols was demonstrated on the borylated thiostrepton derivative. The pH-controlled formation of boronate-triol complexes has shown to be an efficient strategy for the reversible labeling of RiPPs. These results highlight the versatility of boronic acid modification as a platform for the structural diversification of these peptides, although its full potential has not yet been reached. Boronic acids are intermediates for a multitude of functional group transformations and cross-coupling reactions such as Suzuki and Chan-Lam couplings. Therefore, research should continue to focus on the exploration of such opportunities for the chemical mutagenesis of RiPPs. An interesting practical application would be to immobilize adamantane triols on resin and use the reversible boronate-triol coupling for the isolation and purification of borylated peptides via pH-triggered capture and release.

**Chapter 5** describes how the Cu(II)-catalysis presented in **chapter 4** can also be employed for the selective β-silylation of dehydroalanine residues in peptides and proteins. It has been shown that when $PinBSiMe_2Ph$, also known as Suginome's Reagent, is used instead of a bis-boron reagent, a β-silylation can be performed selectively instead of a β-borylation. The reaction was initially studied on thiostrepton using Suginome's Reagent instead of $B_2(OH)_4$, while the rest of the reaction conditions were kept identical to those of the β-borylation. It was found that the silylation proceeds rapidly, showing full conversion of thiostrepton to the double silylated analog within 1 hour at room temperature. NMR studies confirmed that the β-silylation is selective for the Dha residues in the tail region of thiostrepton. Moreover, the reaction is broadly applicable, since nosiheptide, nisin Z and SUMO

were also silylated efficiently. The study demonstrates that this approach is a powerful method for the silylation of Dha in RiPPs, yet the effects of silylation on the properties and function of these natural products are still unclear. In reversed-phase chromatography a decrease in polarity is observed for the silylated products compared to the unmodified peptides, in contrast to the drastic increase in polarity found for the borylated derivatives. This implies a lower aqueous solubility for the silylated variants, although this remains to be assessed quantitatively. On the other hand, the increased lipophilicity can be advantageous for the ability of peptides to cross cellular membranes.[20] Therefore, further research should be conducted to investigate how these effects are reflected in the antimicrobial activity of silylated RiPPs.

## 7.4 NMR characterization of novel antimicrobial NRPs

In **chapter 6** the characterization of novel antimicrobial NRPs via NMR spectroscopy is described. 10 new peptides belonging to 3 different classes of NRPs were isolated from the soil bacterium *Brevibacillus laterosporus* MG64 by the group of Oscar P. Kuipers. Initially, several new mutants of the bogorol family of NRPs were isolated. This included a number of analogs that contained a succinylation modification, which were therefore recognized as a new class of NRPs that was given the name succilins. Additionally, 2 relacidines, a novel class of cyclic lipopeptides, were isolated and characterized. The purified peptides were analyzed by $^{1}$H NMR and several 2D NMR techniques. The amino acid sequence was identified using NOESY NMR, while amino acid side chains and important structural features such as mutations and modifications were characterized via NOESY and TOCSY NMR. Ultimately, the structures predicted using tandem MS were confirmed for all compounds. The complete characterization of these peptides has been a significant contribution to a larger study that was aimed at investigating the potential of these peptides as antibiotics and their producing organism as a biocontrol strain.[22,23] Detailed knowledge of their chemical structure is crucial for providing a comprehensive understanding of their biosynthesis and mechanism of action. Collectively, this research forms a solid base for the clinical application of these peptide antibiotics and the development of methodologies for the systematic discovery of novel antimicrobial peptides in the future.

## 7.5 Conclusion and outlook

In conclusion, the methodologies presented in this thesis demonstrate that the first-row transition metal catalyzed modification of Dha residues is a powerful

approach for the late-stage chemical editing of RiPPs. The methods are valuable additions to the field, since the chemical installation of non-natural bonds and completely abiological functionalities has significantly expanded the chemical space that can be explored via Dha modification. The developed modifications enabled the semi-synthesis of analogs with improved pharmacological properties and conjugates that are useful for the labeling and targeting of RiPPs, via types of chemical bonding that are completely novel to these natural products. Biologically active derivatives were obtained, which differ greatly in polarity and aqueous solubility compared to the unmodified peptides. This complementarity provides a promising starting point for the fine-tuning of these pharmacological properties, which is crucial for the *in vivo* effectivity of these antibiotic candidates and their clinical application in the future.

The reactions proceeded efficiently at low temperatures in water, while the use of co-solvents is also tolerated. Because of this flexibility the methods are broadly applicable, even though every RiPP natural product has specific requirements in terms of solubility and stability. Moreover, none of the methodologies require the rigorous removal of oxygen, which makes setting up the reactions and scale-up to preparative scale facile. Collectively, these results show that the procedures presented here perform better compared to most existing methods in terms of versatility and practicality.

The first-row transition metal salts used as catalysts in these studies are known to have a lower inherent toxicity compared to heavy metal complexes. Additionally, these catalysts were easily separated from the modified peptides during purification, in contrast to the platinum-group metals, which can prove to be incredibly difficult to remove from crude mixtures. This is an important advantage from a microbiology perspective, since background toxicity of metal catalyst are a major challenge for evaluating the antimicrobial activity of the reaction products, and could be detrimental for their therapeutic use. The reaction scope of first-row transition metal catalysts for the modification of Dha might still be relatively limited, but their distinct features such as Lewis acidity and redox properties are promising attributes for further exploration of new types of chemical reactivity. Therefore, the development of new methodologies should continue in order to reach the full potential of this approach.

The methods presented here display outstanding chemoselectivities, and even high site-selectivities are observed. However, the relative reactivities of the different dehydroalanine residues within a peptide generally dictate the regioselectivity, and the field still lacks methods that give chemists true control of

site-selectivity. Moreover, the stereochemical outcome of reactions on Dha is also difficult to regulate. In most studies, including those presented here, products are obtained as mixtures of diastereomers. The work in this thesis and previous literature highlight that the site-selectivity and stereochemical outcome of the chemical modification of dehydroalanines can have a significant impact on the biological activity of RiPPs.[18,24] Overcoming these challenges would therefore be major advancement of the field. The use of chiral ligands for the stereoselective modification of Dha residues could be a suitable approach for fist-row transition metal catalysts. However, achieving stereoselectivity is particularly difficult in this case, since in most types of reactions on Dha the new bonds are made at the β-carbon, while the chirality in the product is formed at the α-carbon during the final protonation step. The use of molecular templates, which can recognize a desired site of modification in a peptide and bring it in close proximity to reactants also bound in the template, could be a suitable strategy to achieve site-selective transformations. Enzymes involved in the post-translational machinery of RiPPs can assume this role due to their moderate substrate specificity, and it has been shown that they can be repurposed to perform non-natural reactions on a wide range of RiPPs.[25] However, the palette of organic reactions that such enzymes can perform is limited, and tailor-made solutions will remain the standard for the time being. Controlling the regioselectivity remains challenging, since a single, universal approach seems impractical due to the structural diversity of RiPPs. Nevertheless, the extraordinary chemoselectivity associated with Dha modification does make up for this. As long as improved and active products are obtained, Dha residues will continue to be a versatile platform for the chemical modification of RiPPs.

A wide range of reactions have been established on Dha so far, yet investigating the utility of such methods for useful applications often gets neglected. Shifting the focus of the field from developing new methodologies towards employing them in the semi-synthesis of active hybrids with improved properties is an important step towards the therapeutic use of RiPPs. The work in this thesis was aimed at addressing this challenge, and it was shown that the developed methods could indeed be applied in the conjugation and labeling of RiPPs. Most of these approaches were demonstrated as a proof-of-concept, but the successful conjugation of siderophores to thiostrepton truly proved that Dha modification is a powerful platform for the semi-synthesis of antibiotic hybrids. Unfortunately, these hybrids did not display the desired antimicrobial activities due to the loss of inherent activity. A logical continuation of this project would be to design hybrids with a linker that selectively gets cleaved inside the target organism in order to facilitate release of the active antibiotic. Collectively, these results underline once more that the effect of

modifications on the function of natural products have to be taken into account in the rational design of such conjugates. Therefore, interdisciplinary collaboration between chemists and microbiologists is essential for the success of such endeavors and should be stimulated.

The NMR studies conducted in this thesis are part of a larger field of research that shows that non-ribosomal peptides are also a rich source of antibiotic candidates.[22,23] However, NRPs often suffer from the same shortcomings as RiPPs in terms of their poor pharmacological properties, which limit their clinical use. Expression and engineering of their intricate biosynthesis is difficult, which is a bottleneck for the generation of improved variants. A recent study showed that the ribosomal synthesis and post-translational modification machinery of RiPPs can be programmed genetically to make peptides that mimic the molecular structure of existing NRPs.[26] This enables the genetic encoding of libraries of functional mimics of NRPs that have improved properties and novel activities.[26] The post-translational machinery of RiPPs can perform a multitude of modifications with high chemo-, site- and stereoselectivity. However, their reaction scope is limited, and there are modifications that simply cannot be performed biosynthetically. This is where organic chemists come into play. An interesting extension of the research in this thesis would be to take advantage of the low toxicity of first-row transition metals, and explore the integration of the developed methods into the programmable RiPP biosynthesis inside living organisms. In this way, genetic encoding and post-translational modifications can be combined with selective chemical editing, which could lead to therapeutically relevant variants of both NRPs and RiPPs in the future.

Altogether, the research presented here successfully addresses several challenges in the chemical modification RiPPs via their dehydroalanine residues. The studies demonstrate that the use of low-toxic first-row transition metal catalysts is a mild and effective approach, and paves the road for useful applications in the semi-synthesis of clinically relevant antimicrobial peptides.

## 7.6 References

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