Natural product antibiotics: synthesis and next generation analogues

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Chapter 6

Summary and Outlook

The escalating incidence of antimicrobial resistance, coupled with the limited economic prospects surrounding the discovery and development of antibacterial drugs, underscores an urgent need for innovative research into new antibiotic strategies.[1-3] In this context, natural product antibiotics continue to present a rich source of structural and mechanistic diversity, making them a key part of the effort to revitalize the currently depleted antibiotic pipeline.[4-6] This thesis aims to address the pressing global issue of antibacterial resistance by focusing on the discovery, synthesis, and modification of antibiotic compounds derived from natural sources.

In **Chapter 1**, an overview of the field of antibacterials is presented, emphasizing the escalating challenge of antibiotic resistance that is increasingly associated with serious infections that contribute to a rise in mortality rates, soaring healthcare expenditures, and extended hospitalization durations.[2,3,7,8] This underscores the urgent need for the development of novel antibiotics, a goal that can be achieved through various approaches, including those outlined in this chapter. Historically, one of the most enduring approaches has been the exploration of natural sources to identify new antibacterial compounds. This method has led to the successful introduction of a diverse array of antimicrobial agents into clinical practice, including penicillin, streptomycin, colistin, chloramphenicol, vancomycin, and daptomycin. However, the initial discoveries of most natural product antimicrobials that reached the market occurred over 50 years ago, during which time bacteria have developed increasing resistance to these agents.[9-11] Advances in the field of organic chemistry have facilitated the total synthesis and modification of natural products.[12] These advancements have significantly contributed to the understanding of the structure-activity relationships of natural antimicrobials, paving the way for the development of novel analogues with enhanced properties. Synthetic approaches played a particularly crucial role in the development of the antitubercular agent rifampicin, as well as multiple generations of penicillins and cephalosporins.[13-16] Of particular interest is cefiderocol, a synthetically modified cephalosporin that has a catechol moiety incorporated into its structure. This catechol enables a “Trojan horse” mechanism, facilitating cefidericol’s entry into bacterial cells by hijacking bacterial iron transport mechanisms. Once within the periplasmic space, the β-lactam warhead of cefiderocol inhibits peptidoglycan biosynthesis, resulting in bacterial cell death.[17-20]

In this thesis, we take a comprehensive approach to addressing the pressing issue of antimicrobial resistance, with a primary focus on the synthesis and modification of natural products to develop innovative antimicrobial agents.

Chapter 2 details the isolation, structural elucidation, and synthesis of a new class of lipopeptides known as paenilipoheptins. Paenilipoheptins are produced by *Paenibacillus* species, bacteria known for their antimicrobial lipopeptides, such as polymyxins, tridecaptins, octapeptins, and paenibacterins.[21,22] The first section of this chapter (**2A**) is dedicated to the identification and isolation of the novel cyclic lipopeptide paenilipoheptin B, alongside the previously reported paenilipoheptin A.[23] Both paenilipoheptins A and B were isolated from fermentation of the natural producer

organism and subjected to detailed analysis through various methods, including genome prediction, MS/MS sequencing, NMR spectroscopy, and Marfey's analysis. This comprehensive investigation resulted in the nearly complete assignment of the natural product and revealed that the previously proposed structure of paenilipoheptin A was incorrect. **Chapter 2B** focuses on the synthesis, further structural elucidation, and confirmation of the paenilipoheptin A structure (**Figure 1**). The data presented in **Chapter 2A** were insufficient to establish the stereochemistry of the unnatural β-amino acid within the structure of paenilipoheptin A. To address this, a synthetic route enabling the preparation of the different stereoisomers of this amino acid was designed, and the synthetic material was compared in Marfey's analysis to that derived from a natural source. Following the determination of the stereochemistry of this center, the total synthesis of paenilipoheptin A was conducted using solid-phase peptide synthesis, with the final cyclization performed in solution. Verification of the synthetic paenilipoheptin A against the natural product was achieved through NMR comparison and HPLC co-injection experiments, thereby establishing the stereochemical framework and confirming the structure of this natural product. Furthermore, the developed synthetic route yielded sufficient material for extensive antimicrobial evaluation of paenilipoheptin A, revealing its specific activity against Gram-positive bacteria.

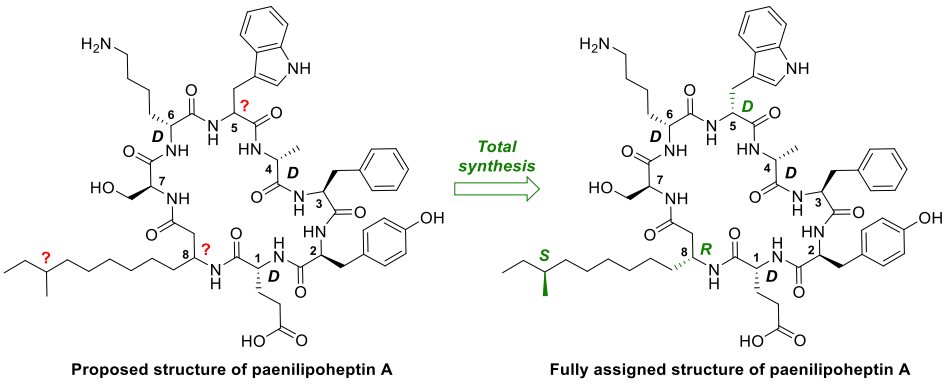


Figure 1. The proposed and fully assigned structures of paenilipoheptin A.

The discovery and structural elucidation of the paenilipoheptin class of natural products further enhances the understanding of the antimicrobial peptides produced by *Paenibacillus* spp. and may hold implications for refining the accuracy of genome mining predictions for other nonribosomally synthesized peptides produced by bacteria. A comprehensive investigation into the mechanism of action of the paenilipoheptins is also warranted. An approach to doing this could involve the cultivation of resistance mutants to identify the target of the paenilipoheptins. Additionally, structure-activity relationship (SAR) studies could also be undertaken to explore the potential for modifying paenilipoheptins. Such investigations may pave the way for improvements in the solubility and antibacterial activity of this unique class of lipopeptides.

In **Chapter 3**, the synthesis and structural elucidation of the natural product evybactin (**Figure 2**) are presented. Evybactin is a cyclic depsipeptide produced by *Photorhabdus nonneputensis*, which has been demonstrated to selectively inhibit the growth of *Mycobacterium tuberculosis* (*Mtb*). The compound targets the highly conserved DNA gyrase of the bacteria, with its anti-*Mtb* selectivity attributed primarily to its capacity to penetrate the bacterial cell via the unique mycobacterial transporter, BacA.[24] Given that no synthetic routes had been previously reported, we decided to synthesize the compound in order to confirm the proposed structure and to address issues associated with its limited yield obtained via isolation from the producing microorganism. Two routes were employed for the synthesis of the proposed evybactin structure, utilizing a combined solid and solution-phase approach. The most robust route involved on-resin esterification and final cyclization through amide formation. However, the NMR spectra of the prepared molecule did not correspond to those of the natural product, necessitating additional structure elucidation experiments. With the help of the Lewis group, the discoverers of evybactin, detailed MS/MS and Marfey's analyses were performed on naturally isolated evybactin. These investigations revealed that the stereochemistry of the His(3-Me) residue within the evybactin structure had been originally misassigned, leading to our revision of the configuration of this amino acid as D. Armed with this information, a streamlined approach for the synthesis of the Fmoc-protected version of this unnatural amino acid was also developed, enabling the subsequent synthesis of evybactin with the correct structure. Our synthetic material exhibited identical biological activity to the natural product and matched natural evybactin in NMR and HPLC co-injection experiments, thereby allowing for the unequivocal establishment of evybactin's structure. The synthesis method also culminated in the preparation of evybactin at a multi-hundred milligram scale, resolving production issues faced when trying to isolate the natural product from the producing organism.

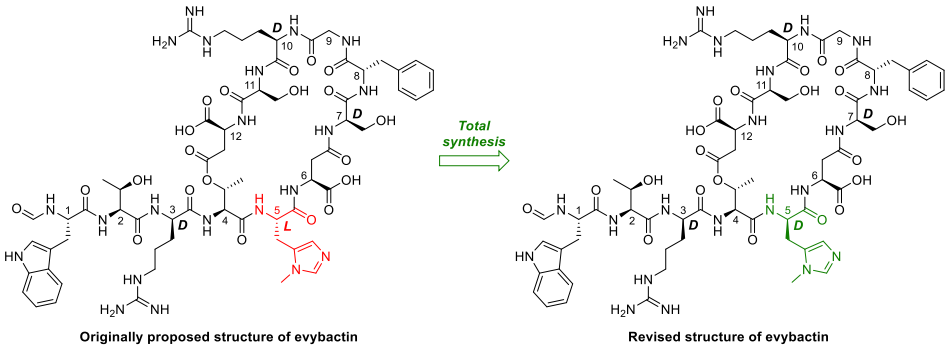


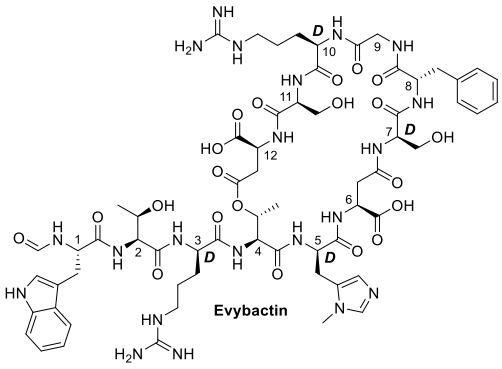
Figure 2. The proposed and revised structures of evybactin

The studies performed in this chapter have led to the correction of the previously published structure of evybactin. The establishment of the structure, combined with the robust synthesis methods we developed, enables further studies aimed at establishing the

therapeutic potential of evybactin, and also paves the way for detailed SAR studies of this promising natural product.

SAR studies with evybactin are outlined in **Chapter 4** of this thesis, wherein our previously developed robust method for the synthesis of evybactin was employed to conduct a comprehensive assessment of the structural features that underscore its activity (**Table 1**). Beginning with an alanine scan, specific positions in the evybactin sequence were found to have a lower degree of influence on antibacterial activity, including Thr^{2}, Ser^{7}, and D-Ser^{11}. In contrast, substitutions at other residues resulted in a notable reduction in potency. Subsequently, the contribution of charged amino acids within the molecule was examined, revealing that the positive charge at the arginine positions is favored. Furthermore, it was determined that the His(3-Me) residue is critical for the compound's activity against *Mtb*, as even minor alterations, such as substituting this residue with a standard histidine, significantly impacted the biological activity. The significance of both the exo- and endocyclic components on evybactin's activity was also investigated. It was found that minimal modifications in the cyclic structure, such as the substitution of Thr^{4} with Ser^{4}, resulted in a reduction in activity, while the incorporation of the 2,3-diaminopropionic acid (Dap) residue at this position entirely diminished the compound's activity. An examination of the N-terminus for potential modifications indicated that the formyl group is non-essential for evybactin's activity, allowing for substitution with a more stable and readily incorporated acetyl group without a significant loss in functionality.

Table 1. Highlights of the most significant findings from the SAR studies of evybactin. Minimum inhibitory concentrations (MICs) measured against *M. tuberculosis* H37Rv mc^{2}6020 mCherry.



Modification	MIC (µg/mL) against *Mtb*
Original evybactin	0.063
Thr^{2} => Ala^{2}	0.125
D-Ser^{7} => D-Ala^{7}	0.063
Ser^{11} => Ala^{11}	0.25
D-Arg^{3} => D-Lys^{3}	0.5
D-Arg^{3} => D-Cit^{3}	4
D-Arg^{10} => D-Lys^{10}	0.25
D-Arg^{10} => D-Cit^{10}	4
D-His(3-Me)^{5} => D-His^{5}	>16
Thr^{4} => Ser^{4}	4
Thr^{4} => Dap^{4}	>16
Formyl-Trp^{1} => Ac-Trp^{1}	0.25

Collectively, these insights into structure-activity relationships provide a valuable foundation for future investigations aimed at further optimizing evybactin and harnessing its potential as a promising antibacterial agent. The N-terminus of evybactin has been identified as a highly promising site for future optimization, as modifications at this position can be introduced with relative ease. This characteristic, along with the broad availability of various carboxylic acid building blocks, allows for the incorporation of a diverse array of N-terminal substituents. The initial approach should involve assessing whether lipidic modifications at this position lead to enhancements in antibacterial activity, given that many antimicrobials include N-terminal hydrophobic groups. In addition to the N-terminus, positions Thr^{2}, Ser^{7}, and D-Ser^{11} might also be utilized for implementing various conjugation strategies aimed at improving the properties of evybactin. As demonstrated, the activity of evybactin against Gram-negative bacteria is significantly affected by their outer membrane. This suggests that conjugating this natural product with known membrane disruptors, such as polymyxin B nonapeptide,[25] might enhance its membrane penetration properties, thereby improving antimicrobial activity.

Finally, **Chapter 5** focuses on the application of the "Trojan horse strategy"[26-28] to enhance the antibacterial profile of rifampicin, a drug utilized in the treatment of tuberculosis and infections caused by other Gram-positive bacteria. Although rifampicin binds to a highly conserved bacterial RNA subunit, its activity is generally poor against Gram-negative bacteria due to the presence of their outer membrane. With the aim of expanding rifampicin's efficacy, we designed and synthesized several rifampicin-siderophore conjugates. An initial attempt to employ click chemistry for attaching a catechol moiety to rifampicin resulted in an inactive molecule. This led us to further investigate the nature of the linkers used and to characterize their influence on the activity of the conjugates. With this knowledge, an improved linker strategy was developed, leading to the synthesis of rifampicin-siderophore conjugates that exhibit enhanced anti-Gram-negative activity compared to rifampicin. The findings indicate that the structural characteristics of both the linker and the catechol contribute to the activity of the conjugates. Notably, compound **33** (**Figure 3**), which links rifampicin to a chlorocatechol unit via a short ester linker, demonstrates the greatest increase in potency. Utilizing this compound, further investigations were conducted to elucidate the mechanism by which the molecule is transported inside the cell. Although the exact transporter responsible for the increased activity of the conjugate could not be identified, it was demonstrated that the activity of compound **33** is iron-dependent and can be diminished by the supplementation of the medium with the natural siderophore enterobactin. This suggests that conjugate **33** utilizes a siderophore mechanism for entry into bacterial cells. Additionally, this compound was tested against a diverse selection of Gram-negative pathogens, including several resistant isolates. In nearly all cases, compound **33** showed enhanced activity compared to unmodified rifampicin, highlighting the potential of the "Trojan horse" strategy.

The investigation into the linker strategies described in this chapter provides significant insights into the design of antibiotic-siderophore conjugates. It has been demonstrated that the linker used has an impact comparable to that of the antimicrobial and siderophore components of the molecule, with an inappropriate linker selection having the potential to completely undermine the activity of such conjugates. Furthermore, it has been established that the "Trojan horse" strategy can enhance the properties of the typically Gram-positive antibiotic rifampicin, thereby enabling it to exhibit potent activity against Gram-negative bacteria. A greater number of Gram-positive antibiotics need to be investigated within the context of this strategy to gain a more comprehensive understanding of its limitations. One such antibacterial agent that warrants consideration is evybactin, as delineated in **Chapters 3** and **4**. This compound, which also encounters challenges in penetrating the outer membrane of Gram-negative bacteria, targets DNA gyrase, which is conserved across various bacterial species, indicating that its efficacy may potentially be improved through the application of the "Trojan horse" strategy.

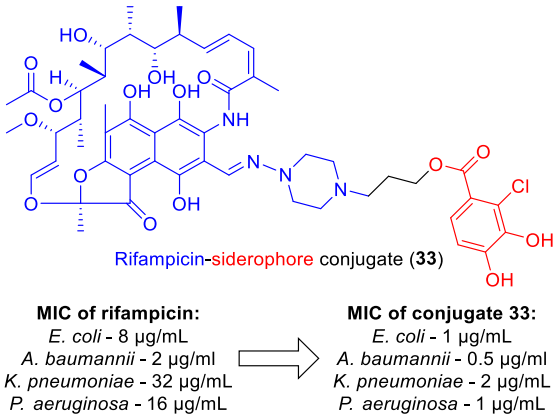


Figure 3. The structure and MICs of rifampicin-siderophore conjugate **33** compared to those of rifampicin. Rifampicin is highlighted in blue, the linker part in black, and the siderophore moiety in red.

In conclusion, the results presented within this thesis collectively contribute to the ongoing investigation of various antibacterial strategies in the fight against multidrug-resistant pathogens. The focus on natural products, which serves as the primary emphasis of the research reported in this thesis, is expected to continue playing a pivotal role in the discovery and design of novel antimicrobials. Traditional methodologies, which start with the isolation of active compounds followed by structure elucidation and activity assessment, are complemented by modern techniques such as genome mining, which offer the potential to expand the collection of accessible natural compounds. Furthermore, alternative approaches that rely on synthetic methodologies to enable the introduction of various modifications can enhance the properties of natural products. The combination of traditional and innovative strategies explored in this thesis underscores the remarkable potential of natural products as effective antibiotics, highlighting their vital role in the future of antimicrobial therapy.

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