Next generation lipopeptide antibiotics

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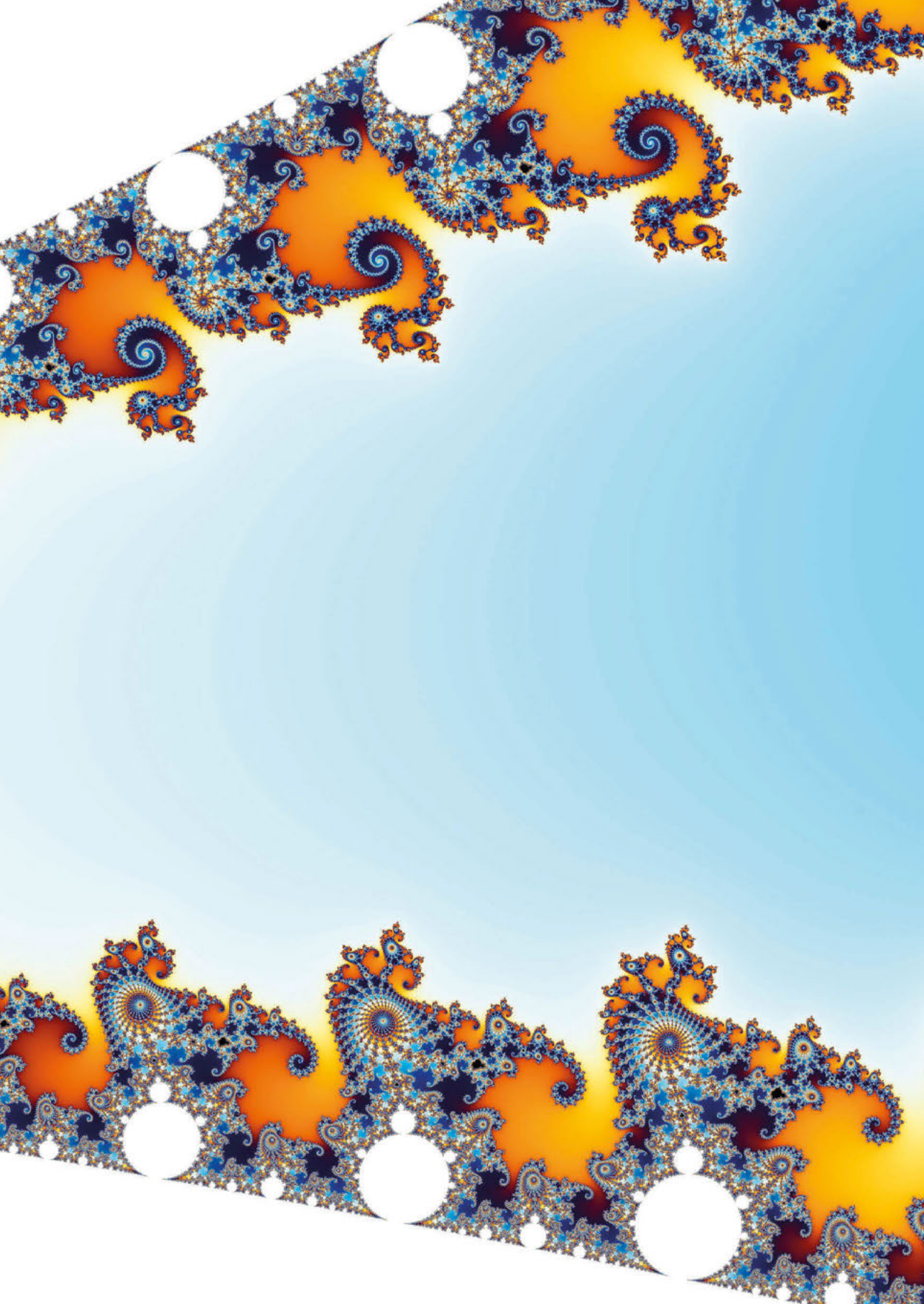

Chapter 6

Summary and Outlook

Chapter 1 briefly describes the history of infectious disease research giving examples of key discoveries recorded throughout written history. Building on the work of many generations of researchers, society today has a understanding of infectious diseases that only recently adopted the germ theory of disease over the miasma theory. The chapter also discusses the discovery of the first synthetic antibiotic Salvarsan and the first naturally derived antibiotic penicillin. Penicillin and antibiotics in general have helped transform the medical field but the threat of antibiotic resistance (AMR) looms over our society and the supply of efficacious antibiotics in the future is not a guarantee. The AMR threat was recently described eloquently by Cook and Wright as a kinetics problem.[1] The equilibrium can be shifted by either slowing the spread of resistance or increasing the rate of discovery. While antibiotic stewardship programs have been gaining traction, investment in antibiotic discovery is severely lacking with most research and development efforts currently led by academic institutions. The low cost per treatment, inevitable appearance of resistance, and the need to show superiority over existing treatments currently make antibiotic development too risky for most pharmaceutical companies.[2] In a perfect scenario a company could easily develop a drug that is potent and safe enough to treat an infection and that drug would then be saved for future use when other treatments have become ineffective. In such a scenario the rate of resistance spread is minimized and the rate of discovery optimized. In the real world, however, companies that develop a new antibiotic do not generate enough revenue to stay afloat, as currently there exist multiple cheap generic alternatives already in the clinic. Clearly a shift is needed in how the development of these lifesaving drugs is being funded in the current economic system. Luckily some things are changing as pilot initiatives are being explored involving new forms of financial incentives, such as those based on market entry awards which guarantee a return on investment for the developer, effectively decoupling revenue from the number of prescriptions written.[3] Recently people have also become more interested in alternative therapeutic strategies such as phage therapies,[4] endolysins,[5] vaccines,[6] antibody-antibiotic conjugates[7] and even bacterial proteolysis targeting chimeras.[8] While such strategies could hold immense therapeutic potential, until they are clinically validated classical approaches such as those described in this thesis will remain central to the fight against bacterial infections.

Chapter 2 describes the first total synthesis of brevicidine and laterocidine (Fig. 1), which we, together with the group of Stephen Cochrane (Belfast) have proposed to constitute a new family of lipopeptide antibiotics we've termed the ornicidine class of antibiotics. Brevicidine and laterocidine were originally discovered by Li and coworkers using a genome mining strategy and shown to target Gram-negative bacterial via interaction with lipopolysaccharide (LPS). The subsequent isolation of both lipopeptides from fermentation of the producing organism led to limited amount of material and in the case of laterocidine only *in vitro* activity against Gram-negative pathogens could be shown due to sub-milligram per liter fermentation yields.[9] The approach described in this chapter made it possible to synthesize enough material for further biological evaluation and is currently being used to facilitate more elaborate mechanism of action (MOA) studies. This chapter also describes the synthesis of a number of analogues of both brevicidine and laterocidine, such as the enantiomers of the parent compounds, that were shown to be less active. Interestingly, the activities of the enantiomeric species were also shown to be antagonized by LPS. This finding raises the question if another biomolecular target might be involved in the MOA of these antibiotics. Given that the tridecaptins, which are structurally similar to the ornicidines were shown to bind Gram-negative Lipid II, it

might be fruitful to explore if the ornicidines display a similar MOA.[10] It can, however, be challenging to find a supply of Gram-negative Lipid II as it is typically made through labor intensive multi-step synthesis on a small scale. A small structure-activity relationship (SAR) study evaluating the importance of Thr9 is also described in **Chapter 2**. In doing so, Thr9 was substituted to Ser to study the effect of the side chain methyl group which resulted in analogues with comparable activity to the natural products. We also explored ester-to-amide modifications in the C-terminal macrocycles of brevicidine and laterocidine by replacing Thr9 with (S)-2,3-diaminopropanoic acid (Dap) or (2S,3R)-2,3-diaminobutanoic acid (MeDap). Interesting, while the amide analogues of brevicidine showed diminished activity, in the case of laterocidinethe same substitutions were well tolerated and both the Dap9 and MeDap9 analogues retained activity against most of the tested bacterial strains. This discrepancy might arise from the difference in macrocycle size and/or conformation between the two scaffolds. An investigation into the folding of these peptides, with techniques such as X-ray diffraction or nuclear magnetic resonance (NMR) could shed light into these observed differences. Also of note is the recently finding reported by the group of Oscar Kuipers (Groningen) who discovered that a natural analogue of brevicidine named brevicidine B, possessing Phe instead of Tyr at position 2, shows broad spectrum activity against both Gram-negative and Gram-positive bacteria. Given these findings it would be interesting to ascertain if the effect could be replicated on the laterocidine scaffold.[11] Finally, efforts into increasing the potency and improving pharmacokinetic and pharmacodynamic properties of the ornicidines need to be taken before they can be considered proper drug candidates.

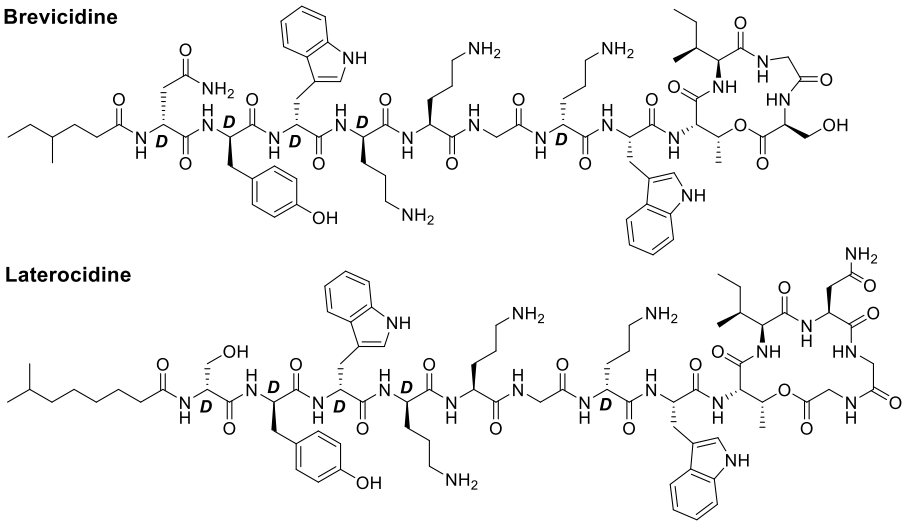

Figure 1. Structures of brevicidine and laterocidine. D-amino acids labeled D.

Chapter 3 reports on the SAR study performed on the brevicidine and laterocidine scaffold in which the branched lipids found in the natural products were substituted for simpler linear saturated lipids of varying length. Although the exact function of the lipid has not been studied, removal of the lipid resulted in complete loss of antibacterial activity as observed

with other lipopeptides. For example, the polymyxins are thought to destabilize the outer membrane by binding LPS and eventually lead to the lysis of the cell.[12] The polymyxin B nonapeptide, the macrocyclic part of polymyxin B devoid of the lipid, however shows no considerable activity against most bacterial strains.[13] Although more data is needed to confirm this, the lipid tails of brevicidine and laterocidine are likely also necessary to insert into the bacterial membrane. Furthermore, the data presented in **Chapter 3** clearly shows that there exists a somewhat optimal lipid length of eight carbon atoms that closely approximates the lipid length of the natural products. Increasing the lipid length did not produce more active compounds. It did however result in more lysis of red blood cells, a parameter indicating non-selective membrane disruption.

Chapter 4 outlines the discovery of linear brevicidine and laterocidine analogues that retain antibacterial activity. Previously, Li et al. found that linear analogue of brevicidine and laterocidine were less active than the natural products.[14] In this chapter, however, we were able to show that linear analogue of brevicidine and laterocidine can exhibit activity comparable to the parent compounds, provided they are prepared as the C-terminal amides. Because the linear analogues prepared by Li et al. bear a carboxylic acid at the C-terminus, at physiologic pH their overall charge is lower than their cyclic counterparts. The overall charge of antibacterial peptide is known to be of critical importance for activity due to binding interactions with charged phospholipids found throughout the bacterial membrane.[15] By masking the negative charge at the C-terminus through substitution of the carboxylic acid moiety to a neutrally charged amide we were able to produce analogues rivalling the natural products in potency. Furthermore, a full alanine scan of both linear peptide scaffolds was performed as well as truncation studies which gave further insight into residues that are key for activity. This revealed that for both peptides, the residues (D-Tyr2, D-Trp3, D-Orn4, Orn5, D-Orn7, and Trp8) are essential for bioactivity. These residues are either cationic or aromatic (hydrophobic) and are likely key for target binding and maintaining an active conformation to cross the bacterial cell membrane. Further MOA studies will be required to confirm how these peptides elicit their activity, as linearization might have impacted the MOA. Similarly, the proteolytic stability of these peptides needs to be assessed if they are to be taken further into development. The compounds, however, may be expected to be stable to proteolytic degradation due to the large number of D-amino acids in their sequences. Furthermore, the stability studies on brevicidine and laterocidine detailed in **Chapter 2** showed that the linear peptides were identified as the degradation products and these did not degrade further after 24 h incubation in human serum.

Chapter 5 reports the total synthesis of the recently discovered lipodepsipeptide antibiotics relacidine A and B (Fig. 2), which was guided by bioinformatic analysis of the biosynthetic gene cluster (BGC). Like the brevicidines and laterocidines, relacidine A and B belong to the ornicidine class of antibiotics and were recently discovered by the Kuipers group. Notably, the stereochemical configurations of the amino acids comprising relacidine A and B had not been determined in the initial report leading us to pursue a full structural assignment.[16] In doing so we were able to first predict these configurations by examining the position of the epimerization domains within the relacidine BGC. In addition the examination of the BGC led to the discovery of the potential cause of the promiscuity of the adenylation domain that incorporates the 13th amino acid in the relacidines, leading to production of two isoform.

It is likely that a tryptophan to a tyrosine mutation in this domain allows for the slightly larger alanine residue to be incorporated alongside the glycine incorporated at other positions in the ornicidine family. Following this, we also synthesized relacidine A and B, as well as various analogues and in doing so validated our bioinformatic prediction by HPLC co-elution experiments and comparison with the NMR data reported by the Kuipers group. The serum stability of the synthesized lipopeptides was assessed, showing that an amide analogue containing a Thr9 to Dap9 substitution, exhibits increased stability while retaining potent activity. The amide analogue was therefore chosen for evaluation in a *Galleria mellonella* larvae infection model due to its high serum stability, low hemolytic activity, ease of synthesis and promising *in vitro* activity.

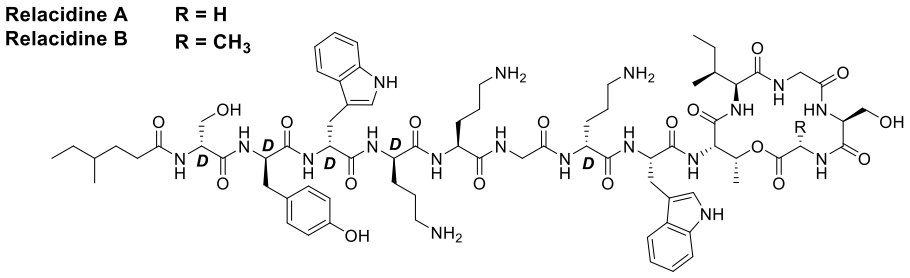

Figure 2. Structures of relacidine A and B. D-amino acids labeled D.

To conclude, the research described in this thesis has improved our understanding of the ornicidine class of lipopeptide antibiotics. As genetic engineering of non-ribosomal peptide synthetases is still in its infancy, development of synthetic approaches to produce these natural products and their analogs has the power to increase access to these promising compounds and enable further study of their mechanisms of action. The data presented in this thesis has likewise shed light on some of the compelling characteristics of the ornicidines and their analogues, such as their potency against drug-resistant strains of pathogenic bacteria *in vitro* as well as *in vivo*. Conversely, characteristics that pose a hurdle for further development have also been identified. Some of them, such as the proteolytic stability of the natural products has been already been addressed in this thesis but others, such as the toxicity and pharmacokinetics/pharmacodynamics profile need to be thoroughly optimized. Clearly, significant preclinical development will be required to advance these compounds further down the pipeline.

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