BIOSYNTHESIS AND MODE OF ACTION OF RIBOSOMALLY SYNTHESIZED AND POST-TRANSLATIONALLY MODIFIED ANTIMICROBIAL PEPTIDES

BY

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DISSERTATION

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ABSTRACT

One of the greatest sources of biologically active compounds is natural products. Often these compounds serve as platforms for the design and development of novel drugs and therapeutics. The overwhelming amount of genomic information acquired in recent years has revealed that ribosomally synthesized and post-translationally modified natural products are much more widespread than originally anticipated. Identified in nearly all forms of life, these natural products display incredible structural diversity and possess a wide range of biological functions that include antimicrobial, antiviral, anti-inflammatory, antitumor, and antiallodynic activities. The unique pathways taken to biosynthesize these compounds offer exciting opportunities for the bioengineering of these complex molecules.

The studies described herein focus on both the mode of action and biosynthesis of antimicrobial peptides. In Chapter 2, it is demonstrated that haloduracin, a recently discovered two-peptide lantibiotic, possesses nanomolar antimicrobial activity against a panel of bacteria strains. The potency of haloduracin rivals that of nisin, an economically and therapeutically relevant lantibiotic, which can be attributed to a similar dual mode of action. Moreover, it was demonstrated that this lantibiotic of alkaliphile origin has better stability at physiological pH than nisin. The molecular target of haloduracin was identified as the cell wall peptidoglycan precursor lipid II. Through the in vitro biosynthesis of haloduracin, several analogues of Halα were prepared and evaluated for their ability to inhibit peptidoglycan biosynthesis as well as bacterial cell growth.

In an effort to overcome the limitations of in vitro biosynthesis strategies, a novel strategy was developed resulting in a constitutively active lantibiotic synthetase enzyme. This methodology, described in Chapter 3, enabled the production of fully-modified lacticin 481
products with proteinogenic and non-proteinogenic amino acid substitutions. A number of lacticin 481 analogues were prepared and their antimicrobial activity and ability to bind lipid II was assessed. Moreover, site-directed mutagenesis of the constitutively active synthetase resulted in a kinase-like enzyme with the ability to phosphorylate a number of peptide substrates.

The hunt for a lantibiotic synthetase enzyme responsible for installing the presumed dehydro amino acids and a thioether ring in the natural product sublancin, led to the identification and characterization of a unique post-translational modification. The studies described in Chapter 4, demonstrate that sublancin is not a lantibiotic, but rather an unusual S-linked glycopeptide. Its structure was revised based on extensive chemical, biochemical, and spectroscopic characterization. In addition to structural investigation, bioinformatic analysis of the sublancin gene cluster led to the identification of an S-glycosyltransferase predicted to be responsible for the post-translational modification of the sublancin precursor peptide. The unprecedented glycosyltransferase was reconstituted in vitro and demonstrated remarkable substrate promiscuity for both the NDP-sugar co-substrate as well as the precursor peptide itself. An in vitro method was developed for the production of sublancin and analogues which were subsequently evaluated in bioactivity assays. Finally, a number of putative biosynthetic gene clusters were identified that appear to harbor the necessary genes for production of an S-glycopeptide. An additional S-glycosyltransferase with more favorable intrinsic properties including better expression, stability, and solubility was reconstituted in vitro and demonstrated robust catalytic abilities.
For my family - Trisha, Liam, and Malia

Philippians 4:13
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CHAPTER 1: BIOSYNTHESIS AND MODE OF ACTION OF RIBOSOMALLY SYNTHESIZED AND POST-TRANSLATIONALLY MODIFIED NATURAL PRODUCTS\(^1\)

1.1. INTRODUCTION

The avalanche of genomic information acquired in the past decade has revealed that natural product biosynthesis using the ribosomal machinery is much more widespread than originally anticipated\(^1\). Sequencing results have provided unparalleled new insights into the genetic capacity of organisms to generate secondary metabolites\(^2\), including a rapid rise in the discovery of natural products that are ribosomally synthesized and extensively post-translationally modified into mature, bioactive forms\(^3\)-\(^{14}\). These tailoring processes release the peptides from the structural and functional constraints imposed on natural ribosomal peptides, while at the same time restricting conformational flexibility to allow better target recognition and increase metabolic and chemical stability. The extent of modification is widely distributed across these natural products, ranging from the modification of just a single amino acid\(^{15}\) to the dense modification in which all but one amino acid is modified\(^{11}\).

There are a number of advantages for bioengineering molecules produced through these pathways. First, the enzymes involved in the post-translational modification process are often multi-functional and a single enzyme can execute a number of catalytic events. As a result, the biosynthetic pathways are relatively short. In addition, there is a direct link between gene sequence and the natural product, therefore non-silent, single nucleotide mutations are directly

translated to a mutated product. Furthermore, many of the biosynthetic enzymes have demonstrated remarkable substrate promiscuity, thus enabling the production of analogues for structure-activity relationship and additional studies.

The number of ribosomally synthesized and post-translationally modified natural products has grown significantly in recent years with the discovery of the biosynthetic origin for many families including lantipeptides/lantibiotics, cytolyisns, thiopeptides, amatoxins, cyanobactins, cyclotides, microviridins, and other bacteriocins.\(^1,16\) These natural products possess a wide variety of biological activities including antimicrobial, antiviral, anti-inflammatory, antitumor, and antiallodynic activities.\(^1,17-19\) Many recent strategies have been explored in efforts to improve the production and pharmacological properties of bioactive peptides. These strategies, in combination with alternative routes of administration, have yielded a growing number of peptide-based drugs and drug candidates.\(^20\) Indeed, there are currently more than 60 therapeutic peptides used commercially and more than 150 in clinical trials.\(^16,20,21\)

In spite of the multitude of producing organisms and the diversity of biological activities, the key unifying theme shared among the ribosomally synthesized natural products is the general pathway by which they are biosynthesized (Figure 1.1). For the vast majority of natural products of ribosomal origin, the initial precursor peptide is much larger than the final product. These precursors typically contain N-terminal leader peptides, and in some cases, C-terminal extensions that are removed in the last step of the maturation process following post-translational modification. Interestingly, a recent comprehensive analysis of the structural motifs generated using these pathways concluded that the types of structures accessible through the ribosomal route are strikingly similar to those produced via nonribosomal biosynthesis (Figure 1.2).\(^22,23\)
The continuing appearance of multi-drug resistant bacteria poses a significant threat to human health and presents an urgent need for the development of new antimicrobial drugs. Lantibiotics are a diverse and promising class of ribosomally synthesized and post-translationally modified antimicrobial peptides produced by a variety of Gram-positive bacteria and display a broad spectrum of activity.\(^\text{24,25}\) A multitude of members have been identified to date, with several displaying potent antimicrobial activity at nanomolar levels against nosocomial pathogens such as methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant enterococci (VRE), and oxacillin-resistant Gram-positives.\(^\text{17,25}\) Importantly, development of significant levels of resistance has not been observed for nisin, the best-studied lantibiotic that has been used world-wide against food-borne pathogens for more than five decades.\(^\text{26}\)
Figure 1.2. Examples of ribosomally synthesized and post-translationally modified peptide natural products highlighting the structural diversity observed in these molecules.
1.2. OVERVIEW OF LANTIBIOTICS

Lantibiotics are small (19-38 amino acids), low molecular weight (<5 kDa) cyclic peptides characterized by extensive post-translational modifications. The post-translational modifications common to all lantibiotics include 2,3-didehydroalanine (Dha) and (Z)-2,3-didehydrobutyrine (Dhb) as well as the cyclized thioether amino acids meso-lanthionine (Lan) and (2S,3R,6R)-3-methylthionine (MeLan) (Figure 1.3). Any ribosomally synthesized peptide with lanthionine and/or methylthionine thioether cross-links can be classified as a lantipeptide; however, peptides with confirmed antimicrobial activity are further defined as a lantibiotic. The post-translational modifications are essential for the biological activities of the molecules. The thioether structures place conformational constraints on the otherwise flexible peptide backbones of these molecules, locking and aligning key interaction residues into optimal orientations to ensure high affinity binding with their specific biological targets. The unsaturated amino acids Dha and Dhb arise from the enzymatic dehydration of serine and threonine residues, respectively. Following dehydration, an enzyme-catalyzed, intramolecular Michael-type addition of cysteine thiols onto the unsaturated amino acids generates Lan and MeLan thioether rings (Figure 1.3). The meso stereochemistry resulting from thioether formation has been determined for a small subset of lantibiotics including nisin, subtilin, epidermin, Pep5, mersacidin, and lactocin S. In addition, meso stereochemistry has been observed in prochlorosins, a family of lantipeptides (lanthionine-containing peptides) with currently unknown biological function. For all other lantibiotics, meso stereochemistry is assumed.

In addition to the characteristic lantibiotic motifs, there are numerous, additional post-translational modifications that introduce key functional groups that demonstrate importance in overall enhancement of metabolic stability and stronger molecular interactions with the
biological target. These post-translational modifications have enabled bacteria to explore greatly expanded chemical space beyond the side-chains of the 20 proteinogenic acids. They include lysinoalanine, β-hydroxy-aspartate, D-alanine, 5-chloro-tryptophan, S-aminovinyl-D-cysteine, 3,4-dihydroxyproline, allo-isoleucine, and N-terminal modifications including 2-oxobutyryl, 2-oxopropionyl, and 2-hydroxypropionyl functionalities (Figure 1.3).17 Recently, additional unique modifications have been discovered that include labionin and N-acetylation.35 Currently there no fewer than 18 unique modifications identified in lantibiotics, although it is likely that additional post-translational modifications will be revealed as new lantibiotics are discovered.

Approximately 60 members of the lantibiotic family have been reported to date, although the number continues to expand as novel lantibiotics are discovered through genome mining efforts.16,24 There is remarkable diversity in the size, structure, extent of modification, and mode of action among the lantibiotics. A representative subset of family members highlighting the diversity of structural topologies and modifications is shown in Figure 1.4. The least modified lantibiotic is lactocin S with 24% modification,36 whereas microbisporicin is the most extensively modified (58%) and includes unusual modifications such as 5-chloro-tryptophan and both mono- and dihydroxylated proline.37 Several classification schemes have been proposed over recent decades to differentiate classes of lantibiotics. The first classification scheme was based on ring topology and biological activity.38 However, as lantibiotics with varied and novel structures and unique biological functions have been revealed, a new classification scheme has been adopted based on the biosynthetic pathway by which maturation of the peptide occurs and the presence or absence of biological activity (discussed in detail in section 1.4.1).17
Figure 1.3. Post-translational modifications found in lantibiotics. (a) A subset of post-translational modifications found in lantibiotics is shown. A shorthand notation commonly used for these modifications is shown in color below.
the chemical structure. (b) The strategies by which lantibiotic synthetases introduce unsaturated amino acids and lanthionine rings in precursor peptides. Following the ribosomal synthesis of the LanA precursor peptide, LanB, LanM, RamC/LabKC, or LanL enzymes catalyze dehydration of Ser/Thr to afford Dha/Dhb. This modification proceeds through a two-step process, phosphorylation of Ser/Thr and subsequent β-elimination of the phosphate to generate Dha/Dhb for class II, III, and IV lantipeptides. LanC, LanM, or LanL enzymes then catalyze intramolecular Michael type addition of Cys thiols onto the unsaturated amino acids in a stereo- and regioselective manner to produce Lan and MeLan rings. It is currently not known how the RamC/LabKC proteins catalyze cyclization. Adapted from Ref. 39.
Figure 1.4. Structural diversity of lantibiotics represented by a subset of structures from classes I, II, and III. There are currently no class IV structures with full structural characterization. The same shorthand notation and color coding is used as in Figure 1.3.
1.3. LANTIBIOTIC MODES OF ACTION

The vast majority of known lanthionine-containing peptides demonstrate bacteriocidal activity\(^{17}\); however, lantipeptides can also act as signaling molecules\(^{40}\) or morphogenetic peptides.\(^{41}\) In addition, some lantibiotics are potent enzyme inhibitors.\(^{42}\) Much of the recent interest in lantibiotics stems from several members displaying nanomolar activity against a wide variety of pathogenic strains.\(^{43-45}\) Historically, it had been assumed that lantibiotics exert their antimicrobial activity through non-specific pore formation mediated by the interaction of the cationic peptide with the negatively charged phospholipids in the bacterial membrane—a mode of action shared among many antimicrobial peptides from a broad range of organisms.\(^{46}\) However, much compelling evidence has accumulated over the past decade demonstrating that a subset of lantibiotics do in fact target a precise biomolecule. The first of many studies on the mode of action of nisin addressed the discrepancy in bactericidal activity between the lantibiotic and other pore-forming peptides such as magainin, ultimately leading to the identification of lipid II as the lantibiotic’s specific target.\(^{43}\)

Lipid II is the monomeric precursor of cell wall peptidoglycan and consists of an undecaprenyl chain, a pyrophosphate group, a disaccharide moiety (MurNAc and GlcNAc), and a pentapeptide chain (Figure 1.5). Bacterial resistance to compounds that bind to lipid II has been slow to develop, possibly because in comparison to other resistance mechanisms such as efflux pumps and enzyme mutations, it is more challenging to change the structure of an advanced intermediate that is biosynthesized in 10 steps.\(^{47,48}\) Peptidoglycan synthesis involves both a cytoplasmic and an extracellular phase.\(^{49,50}\) The construction of the cell-wall subunit begins on the cytoplasmic side of the plasma membrane of the cell, where UDP-MurNAc-
Biosynthesis of cell wall peptidoglycan. Lipid II is polymerized into immature chains of peptidoglycan catalyzed by transglycosylase activity. Transpeptidases catalyze the cross-linking of nearby chains of peptidoglycan thus generating the mesh-like network of disaccharide-pentapeptide units.

Peptidoglycan glycosyltransferases (PGTs) catalyze the processive polymerization of the C_{55} lipid-linked disaccharide to form peptidoglycan, a polymer of carbohydrate chains connected by peptide crosslinks that constitutes the main component of the bacterial cell wall. Layers of cell wall peptidoglycan act as an exoskeleton, forming the first line of defense against...
environmental stresses and enabling the bacteria to withstand high internal osmotic pressures. Although the cell walls of different bacterial strains contain several structural differences, the composition of the peptidoglycan polymer is remarkably similar and the pathway to peptidoglycan biosynthesis is highly conserved. The cell wall in Gram-positive bacteria is approximately 20 layers thick, with each layer containing numerous peptidoglycan subunits. By contrast, a relatively small amount of bactoprenyl-phosphate molecules are present at around 200,000 molecules per cell,\textsuperscript{52,53} so the amount of lipid II synthesized and available for incorporation is limited. As a result, it is a requirement that the lipid II cycle is an extremely dynamic and efficient process, with each lipid II molecule in the cell having a high turnover rate.\textsuperscript{54} The recycling process of lipid phosphate carrier molecule must be tightly controlled and efficient, and is thus regarded as the crux of bacterial cell-wall synthesis. Because an intact cell wall is absolutely essential for bacterial cell survival, peptidoglycan biosynthesis is a key target of many important classes of antibiotics, including penicillins, carbapenems, cephalosporins, the glycopeptides vancomycin\textsuperscript{55} and ramoplanin,\textsuperscript{56-59} host defense peptides,\textsuperscript{46,47} and an increasing number of lantibiotics described in further detail below.

Nisin is the oldest known and most extensively studied lantibiotic.\textsuperscript{60,61} This very potent and economically important class I lantibiotic exerts its antimicrobial activity through a dual mode of action, which involves binding and sequestering lipid II and pore formation in bacterial cell membranes.\textsuperscript{17} Binding studies have elucidated the precise interactions of nisin with lipid II.\textsuperscript{43} A solution NMR structure of nisin bound to a truncated lipid II variant with only three isoprene units demonstrated that the N-terminal A and B rings of nisin form a cage surrounding the pyrophosphate linker region of lipid II.\textsuperscript{62} The remarkably high affinity interaction of nisin and lipid II (approximately $2 \times 10^7$ M$^{-1}$)\textsuperscript{43} effectively sequesters the precursor and prevents
polymerization and integration of peptidoglycan units into the bacterial cell wall. In addition, this halting of peptidoglycan biosynthesis is particularly valuable for inhibitory activity by minimizing the availability of free bactoprenyl-phosphate molecules that must be recycled for further iterative processes in cell wall biogenesis.

In addition to lipid II binding, nisin is also capable of pore formation in the membranes of targeted bacteria. Nisin has been shown to permeabilize membranes by two distinct mechanisms. The first mechanism involves non-specific, electrostatic interactions of nisin with the anionic lipids in the membrane leading to the accumulation and aggregation of nisin monomers, ultimately resulting in the transient formation of pore-like structures. The affinity of nisin for membranes containing anionic lipids is much lower (about 1,800 M$^{-1}$) compared to that for lipid II and as a result, this non-specific mechanism requires micromolar concentrations of the antimicrobial.

The second mechanism involves the specific docking of nisin to lipid II, which facilitates pore formation in the membranes of targeted bacteria. Mutational studies of nisin have shown that the hinge region between the C and D rings provides the required conformational flexibility that allows for the C-terminus to insert in the bacterial membrane. The C-terminus adopts a perpendicular transmembrane orientation relative to the lipid bilayer, producing pores of approximately 2 nm in diameter. Several studies have shown that pores formed by nisin in the presence of lipid II are much more stable than those formed in its absence. The stoichiometry of this pore complex has also been investigated, and it was demonstrated that each pore consists of 8 nisin molecules and 4 lipid II molecules.

The first two rings of several other class I lantibiotics display high homology with those of nisin, suggesting that these peptides interact with lipid II as well. It has been experimentally
determined that epidermin,\textsuperscript{70,71} gallidermin,\textsuperscript{71} and mutacin 1140\textsuperscript{72} also specifically target lipid II. However, epidermin and gallidermin lack the C-terminal tail and are shorter (~30 Å) compared with nisin (50 Å), and these differences in peptide lengths may explain why epidermin and gallidermin are not able to form pores in bacteria that are susceptible to pore formation by nisin.\textsuperscript{71} More recently, an additional class I lantibiotic, clausin, has been reported to bind lipid II in a manner similar to that of nisin.\textsuperscript{73} An additional activity for several lantibiotics including nisin, is the ability to prevent spore outgrowth of Gram-positive bacteria.\textsuperscript{74,75} A recent study suggested that inhibition of outgrowth is directly related to the ability of the lantibiotic to induce damage to the membrane of germinating spores thereby preventing the establishment of oxidative metabolism and a membrane potential.\textsuperscript{76}

Several single component, class II lantibiotics including mersacidin,\textsuperscript{77} actagardine,\textsuperscript{70} plantaricin C,\textsuperscript{78} and lacticin 481 also trigger bacterial cell death through interactions with lipid II.\textsuperscript{79} However, unlike nisin and other class I lantibiotics, these do not display the ability to form pores in bacterial cell membranes. Rather, they exert their antimicrobial activity by disrupting peptidoglycan biosynthesis through inhibition of transglycosylation.\textsuperscript{70,79} Studies on the interactions between lipid II and mersacidin revealed that the lipid binding site includes the terminal GlcNAc sugar moiety and that mersacidin does not bind to lipid I, suggesting a different binding mode compared with nisin and lipid II.\textsuperscript{77} It has been experimentally determined that this alternative binding mechanism results in an overall lower binding affinity between mersacidin and lipid II as compared to nisin’s affinity for this target molecule.\textsuperscript{80} Mersacidin, as well as many other class II lantibiotics, has an overall globular structure. NMR studies have also shown that mersacidin has considerable conformational flexibility and the small hinge region located between the two lanthionine rings at residues 12 and 13 enable this peptide to effectively bind
Comparison of mersacidin with similar lantibiotics reveals a highly conserved thioether bridge pattern comprised of residues 12–18, which suggests that these residues form the core site for lipid II binding. Moreover, the majority of spectroscopic changes were observed in this conserved region upon binding of the antibiotic with lipid II, thus providing additional support of the importance of this motif in lipid II binding. Mutagenesis studies have demonstrated that Dha16 and Glu17 of this ring are required for antimicrobial activity.

There are an increasing number of class II lantibiotics that consists of two-peptide systems. Each peptide of a two-peptide lantibiotic system contributes a unique role in the mechanism of antimicrobial activity. Whereas each of the component peptides displays either weak or no antimicrobial activity, the synergistic interaction of the two peptides often results in nanomolar activity against a wide range of Gram-positive bacteria. A small subset of two-peptide lantibiotics has been demonstrated to have the ability to specifically target lipid II and form pores in the membrane of target cells. These systems are remarkable because they seem to have split the lipid II-targeting and pore forming activities of nisin across two different peptides. A nisin-like model for lacticin 3147 and staphylococcin C55 has been proposed, in which one peptide component (termed the α-peptide) binds to the peptidoglycan precursor to form a α-peptide:lipid II complex. This binding and sequestering of lipid II is often sufficient to provide modest antimicrobial activity through the inhibition of cell wall biosynthesis, much like the mode of action observed for the single component lantibiotic mersacidin. This α-peptide:lipid II complex serves as the docking site to allow for the recruitment of the second peptide (termed the β-peptide) resulting in pore formation. For nearly all reported two-peptide lantibiotics, optimal activity is achieved when the two peptides are present in a 1:1 stoichiometry. In the case of lacticin 3147, it has been proposed that the peptide-lipid II complex consists of 1:1:1...
stoichiometry (lipid II:Ltnα:Ltnβ) and a pore size of 0.6 nm. Haloduracin is a recently discovered two-peptide lantibiotic whose mode of action and lipid II targeting ability has been extensively characterized. These results are discussed in Chapter 2.

Binding to lipid II and pore formation are not the only mechanisms by which lantibiotics exert their activity. Cinnamycin and the related duramycins inhibit phospholipase A2 by sequestering its substrate, phosphatidylethanolamine (PE) with 1:1 stoichiometry. This binding activity prevents the phospholipase-A2-catalyzed release of arachidonic acid from the cell membrane and consequently affects the biosynthesis of leukotrienes and prostaglandins. More recent studies have shown that cinnamycin accesses PE by causing transbilayer lipid movement. Some lantipeptides function as cell signaling molecules. For instance, the Class III lantipeptides SapB and SapT play essential morphological roles in the formation of aerial hyphae in streptomycetes.

1.4. BIOSYNTHESIS

1.4.1. Classification of lantibiotics

Lantibiotics/lantipeptides are classified into four distinct classes based on their biosynthesis, with a particular focus on the steps of dehydration and cyclization (Figure 1.6). The nature and organization of the catalytic domains responsible for these post-translational modifications are unique for each class of lantibiotics. The biosynthesis of class I lantibiotics involves dedicated monofunctional enzymes that carry out distinct steps in lantibiotic maturation. A LanB dehydratase and a LanC cyclase catalyze dehydration and cyclization events, respectively, and secretion and leader peptide removal is performed by an ATP-binding cassette (ABC) transporter LanT and protease LanP, respectively.
In contrast, the remaining three classes involve two multifunctional enzymes to install dehydrated amino acids and thioether cross-links, transport the modified peptide, and remove the leader peptide. Class II lantibiotics are produced by bifunctional LanM enzymes, which are responsible for both dehydration and cyclization steps. The modified precursor peptide is transported and proteolytically cleaved by bifunctional LanT enzymes. A number of class II lantibiotics, termed two-peptide lantibiotics, are composed of two different peptides, and each is synthesized by a dedicated LanM enzyme. Eight two-peptide lantibiotic systems have been reported to date and are further discussed in Chapter 2.

Class III and class IV lantibiotics are less well-characterized. However, additional members are being discovered and an increasing amount of information is becoming available regarding their biosynthesis through comparative genomics and biochemical characterization of the enzymes involved in these pathways. The dehydration and cyclization of class III lantibiotics are produced through the actions of RamC/LabKC multifunctional enzymes. The enzymes
responsible for the transport and leader peptide removal of class III lantibiotics are currently underexplored. However, for the few members that have been studied, two genes with sequence similarity to ABC transporters have been identified in each cluster which may encode for the enzymes responsible for the final steps in class III lantibiotic maturation. More recently, an additional class of lantibiotics was established following the discovery of a novel enzyme capable of installing the characteristic lanthionine post-translational modifications. This class IV subgroup has only a single member to date, but already much has been revealed regarding its biosynthesis mechanism. A multifunctional LanL enzyme catalyzes the dehydration and cyclization processes, and two genes adjacent to the precursor and synthetase genes appear to encode ATP-binding and membrane permease subunits, which may be involved in the export of the modified peptide.

1.4.2. Gene organization

Lantibiotic biosynthetic genes are clustered and designated with the generic locus symbol lan. For each individual lantibiotic, a more specific genotypic designation is used (e.g. nis for nisin, hal for haloduracin). The clustered biosynthetic genes are found in a range of genetic scaffolds including conjugative transposable elements, on the chromosome of the host, or on plasmid replicons (Figure 1.7). Many genes and clusters have been sequenced, and these studies show similar gene organization for the production of various lantibiotics. Recent advances in high-throughput sequencing has lead to the exponential identification of lantibiotic biosynthetic gene clusters, revealing a more widespread distribution of organisms harboring the capacity to produce these complex molecules. In all cases, the cluster includes genes encoding
for one or more precursor peptides (lanA) and the modification enzymes responsible for catalyzing the dehydration and cyclization events including lanB and lanC (class I), lanM (class II), ramC/labKC (class III), or lanL (class IV) genes. In addition, the transporter responsible for excretion of the mature lantibiotic is encoded by genes designated lanT. Class I lantibiotics require a dedicated protease encoded by a lanP gene in addition to the transporter. Some lantibiotics undergo more extensive post-translational modification and the genes encoding for the required enzymes are also typically found within the cluster. Additional genes, such as those that encode for regulation and immunity, may also be found within these clusters or they may be located on closely linked operons.

1.4.3. Precursor peptides

The lanA genes encode precursor peptides (LanA) that contain a core peptide region that will eventually become the mature lantibiotic, as well as an N-terminal leader peptide extension.
that varies in length from 23 to 59 residues. While both the leader and core peptide sequences contain dehydratable serine and threonine residues, only those residues found in the core peptide undergo modification, giving rise to the structural features (Dha, Dhb, Lan, MeLan) shown in Figure 1.3. The role of the leader peptide has been investigated for both class I and class II lantibiotics using *in vivo* mutagenesis studies and *in vitro* reconstitution of the post-translational modification reactions. Much of class I and class II research has focused on the important features of leader peptides required for modification of the core peptide; however, due to their relatively recent discoveries, not much is known regarding leader peptide requirements for class III and class IV lantibiotics. Leader sequences are also present in the precursors of many other ribosomal peptide natural products such as those described in section 1.1., all of which appear to utilize the leader peptide to direct the maturation process.

Several roles have been suggested for the leader peptides of lantibiotics. The role most commonly proposed for the leader peptides is that of a secretion signal. However, the vast majority of leader peptides of natural product biosynthesis have no homology with the peptides of the typical *Sec* and twin-arginine translocation pathways that are used in bacteria, archaea and plants to transport proteins across membranes. A second role that is frequently postulated is that of a recognition motif for the post-translational modification enzymes. It is this role that is most enticing from a natural product engineering perspective as it may allow generation of analogs by attachment of core peptide variants or even very different peptides to the leader peptides. A related proposed task is that of a *cis*-acting chaperone in which the leader peptide actively assists during the PTM process. Taking clues from the roles of leader peptides attached to enzymes, they may also assist in folding of the precursor peptide, stabilizing the precursor against degradation, or keeping the precursor peptide inactive during biosynthesis inside the host until
the appropriate time for secretion and proteolysis. Indeed, many of the proteases involved in ribosomal natural product biosynthesis in the lantibiotics are localized on the outside of the producing cells and in some cases the protease is part of the transporter. Further delineating the function of these peptide sequences will offer exciting insights into basic biological processes as well as provide improved opportunities to reprogram natural product structures by utilizing the leader peptide directed biosynthetic enzymes.

1.4.3.1. Leader peptides of class I lantibiotics

The leader peptides of class I lantibiotics are about 25 amino acids in length and are rich in Asp residues (Figure 1.8). The leader peptide portion often contains a common “FNLD” sequence between −20 to −15 residues (negative numbers for the leader peptide result from counting backwards from the cleavage site, i.e. the first residue N-terminal to the start of the core peptide is −1). Individual mutation of residues of this motif (F−18L, L−16K, D−15A) in the nisin precursor, NisA, resulted in no detection of nisin or its precursors.95 Moreover, the NisA mutants R−1Q and A−4D were still able to be modified and secreted, but the leader peptide was no longer removed. A similar result was observed for Arg−1 mutants of EpiA, the precursor of epidermin.96 Remarkably, mutations of conserved residues Pro−2 and Asp−7 of NisA did not affect the production of nisin.95 More recent studies on substitutions within or outside the FNLD box and with truncated forms of the NisA leader peptide have strongly supported the importance of the leader peptide for efficient processing of the peptide by the nisin biosynthetic machinery.97

NMR studies on fully processed nisin attached to its leader peptide did not reveal any interactions between the leader and the nisin molecule, both in solution and in micelles.98 Moreover, none of the investigated lantibiotic leader peptides displayed secondary structure in
aqueous solution, although they attain $\alpha$-helical conformations in trifluoroethanol$^{99}$ and structure prediction tools anticipate helical character. These observations do not support the hypothesis that the leader peptide acts as a chaperone for folding. However, leader peptides attached to the mature lantibiotics greatly reduce or abolish their antimicrobial activity, consistent with a protective mechanism.$^{95,100}$ In addition, ATP-binding cassette transporters clearly use the class I leader peptides for recognition because various engineered non-lantibiotic cargo attached to these leader sequences are secreted.$^{101,102}$ Similarly, the leader peptide of nisin is also recognized by the dehydratase and cyclase enzymes as demonstrated by \textit{in vivo} processing of non-lantibiotic therapeutic peptides attached to the nisin leader peptide,$^{103,104}$ and by \textit{in vitro} studies on the cyclase enzyme.$^{100}$ The nisin cyclase, NisC, is made up of an $\alpha,\alpha$-barrel that contains the active site and a separate domain proposed to contain the leader peptide binding site.$^{100}$
Figure 1.8. Leader sequence conservation among class I lantibiotics. (a) Sequence alignment of the precursor peptides for selected members of class I lantibiotics. (b) Weblogo representation of the data used in panel (a). The leader peptide is indicated by the blue line above the sequence and the highly conserved “FNLD” sequence is indicated by the orange box.
1.4.3.2. Leader peptides of class II lantibiotics

The leader peptides of class II lantibiotics are typically also rich in Asp and Glu residues, contain an ELXXBXG motif (B = V, L, or I), and usually end in a double Gly motif (Figure 1.9). Originally defined as consecutive Gly residues preceding the proteolytic cleavage site of non-lantibiotic bacteriocins,\textsuperscript{105} this motif now also includes GlyAla and GlySer/Thr sequences. Like the leader peptides of class I lantibiotics, the double Gly leader peptides play several roles that include keeping the modified core peptide inactive when still attached to the leader peptide.\textsuperscript{88} In addition, \textit{in vitro} studies on lacticin 481 have shown that the leader peptide is important for efficient dehydration and cyclization, but not essential. When the leader peptide and the core region of the lacticin 481 precursor peptide were presented \textit{in trans} to lacticin 481 synthetase, dehydration still occurred, albeit with much decreased efficiency.\textsuperscript{106} Unexpectedly, even incubation of the synthetase with just the core peptide resulted in dehydration. Hence, the leader peptide is not a compulsory allosteric element for dehydratase activity. Instead, the enzyme has a low level basal activity in the absence of the leader peptide, suggesting that the leader peptide may influence the equilibrium between an inactive and an active form of the enzyme by binding to the latter.\textsuperscript{107} Upon binding, lacticin 481 synthetase processes its substrate peptide distributively and directionally, moving from N- to C-terminus,\textsuperscript{108} whereas in the absence of the leader peptide, no such directionality was observed.\textsuperscript{106} The unique role of the leader peptide in the biosynthesis of lacticin 481 was exploited in further engineering studies of LctM which are discussed in Chapter 3.

Site-directed mutagenesis studies have shown that the double Gly motif is essential for proteolytic processing,\textsuperscript{109,110} but not for installation of lanthionine rings.\textsuperscript{107} Hence, the
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Figure 1.9. (continued on next page)
Figure 1.9. Leader sequence conservation among class II lantibiotics. (a) Sequence alignment of the precursor peptides for selected members of class II lantibiotics. (b) Weblogo representation of the data used in panel (a). The leader peptide is indicated by the blue line above the sequence. The conserved double-glycine cleavage site and the ELXXBGX motif is indicated by the green and violet boxes, respectively. (c) Sequence alignment of the precursor peptides for bacteriocins that do not undergo post-translational modification.
recognition of the leader peptides is different for the protease and synthetase. It has proven
difficult to delineate the exact factors that are essential for recognition by the synthetase as
nearly all single point mutants of the lacticin 481 leader peptide were still processed.\textsuperscript{107} The only
clear disruption of processing by the synthetase was observed with a series of mutants that
introduced Pro residues, supporting the hypothesis that the leader peptide attains an $\alpha$-helical
conformation when bound to the synthetase.\textsuperscript{107} Interestingly, leader peptides of the double Gly
type with clear homology with lantibiotic leader peptides are also found in many bacteriocins
that do not undergo posttranslational modifications (Figure 1.9). Therefore, these leader peptides
are thought to only be important for secretion and/or for reducing the biological activity of the
bacteriocin.\textsuperscript{111} Hence, the leader peptides of class II lantibiotics may have evolved from a role in
secretion to include additional roles in guiding the enzymes involved in lantibiotic biosynthesis.
However, the hypothesis that non-conserved amino acids in these leader peptides would convey
recognition by the PTM enzymes for each specific lantibiotic is not supported by mutagenesis
studies.\textsuperscript{107}

1.4.4. Lantibiotic synthetases

The formation of unsaturated amino acids and thioether bridges requires both LanB and
LanC proteins for class I lantibiotics (Figure 1.6). LanB proteins are normally very large
(approximately 120 kDa) and have no homology with any other known proteins. The sequence
identity within the LanB protein family is only around 30%. \textit{In silico} analysis of the primary
sequence of LanB proteins reveals overall hydrophilic structures with hydrophobic patches.
Early studies on the dehydration activity of LanB proteins initially demonstrated the ability to
form dehydrated Pep5 peptide after inactivation of \textit{pepC} in a Pep5-producing strain.\textsuperscript{112} A similar
result was obtained in which dehydrated NisA was obtained through the \textit{in vivo} activity of NisB
in a NisC deficient strain. More recently, non-producing *L. lactis* strains were able to heterologously produce dehydrated NisA through expression of *nisABT*. To date, many efforts to reconstitute enzymatic dehydration by a LanB protein in vitro with purified protein or cell-free extracts have not been successful. It was previously hypothesized that this lack of activity may be due to the formation of a membrane-associated multimeric complex with the LanC and LanT proteins. However, two independent studies focused on *nis* gene co-expression systems in *L. lactis* and *E. coli* have demonstrated that complex formation is not required for NisB activity.

LanC proteins catalyze the regio- and stereospecific intramolecular addition of cysteine thiols onto unsaturated amino acids to form the thioether rings found in class I lantibiotics. Initial experiments on Pep5 and nisin biosynthesis verified the role of LanC proteins, where correctly cyclized products were not formed in engineered strains lacking functional LanC proteins. Recently, LanC enzymes involved in nisin and subtilin biosynthesis were cloned, expressed, and purified and were shown to be zinc containing metalloproteins. The Zn atom in these enzymes was proposed to serve a catalytic role in activating the thiol for nucleophilic addition onto the unsaturated residues during lanthionine formation. Through the spectroscopic analysis of SpaC, it was demonstrated that the ligand environment of the metal atom consisted of two Cys residues along with either two His residues or one His and one water molecule. Direct evidence for the identity of the Zn ligands was obtained in a crystal structure of NisC, which showed Cys284, Cys330, His331, and a water molecule serving as the four tetrahedral ligands. Results from the same study demonstrated the in vitro reconstitution of NisC activity using dehydrated NisA obtained from an engineered *L. lactis* strain expressing *nisABT* genes. Later studies, focused on variants of NisC, demonstrated that site-directed mutagenesis of the Zn
binding residues abolished cyclization activity of NisC and decreased the amount of bound metal in the enzyme.\textsuperscript{120}

The \textit{lanM} genes encode for large proteins involved in class II lantibiotic biosynthesis (Figure 1.6). These enzymes consist of an N-terminal domain that displays no sequence homology with the LanB proteins and a C-terminal LanC-like domain.\textsuperscript{24} The first report of the proposed role of LanM proteins came from studies in which lacticin 481 production was halted when the gene encoding LctM was disrupted.\textsuperscript{121,122} Additional supporting evidence was revealed in a genetic study in which introduction of \textit{lctA}, \textit{lctM}, and \textit{lctT} genes and the \textit{lctFEG} immunity genes into a normally non-producing \textit{L. lactis} strain resulted in heterologous production of lacticin 481.\textsuperscript{123} Direct evidence for the role of LanM proteins was provided following the first \textit{in vitro} reconstitution of an active lantibiotic synthetase, LctM.\textsuperscript{88} In this study, treatment of the LctA precursor with LctM resulted in a four-fold dehydrated, fully-cyclized peptide. Following the removal of the leader peptide with a commercially available protease, LysC, bioactive lacticin 481 was obtained. Both Mg\textsuperscript{2+} and ATP were essential for modification by LctM to occur. Later experiments demonstrated that ATP is utilized to activate Ser and Thr hydroxyls as phosphorylated intermediates to facilitate the elimination process during Dha and Dhb formation.\textsuperscript{124,125}

A second example of \textit{in vitro} reconstitution of LanM proteins was reported concerning HalM1 and HalM2 involved in the biosynthesis of the two-peptide lantibiotic, haloduracin.\textsuperscript{4} It has been demonstrated that HalM1 and HalM2 can each modify their substrate HalA1 and HalA2 \textit{in vitro} to produce the bioactive forms of haloduracin \textit{α}- and \textit{β}-peptide. More recently, two additional examples of LanM \textit{in vitro} reconstitution have been reported, including ProcM,\textsuperscript{34}
which is involved in the biosynthesis of prochlorosins, and CinM, which post-translationally modifies the cinnamycin precursor.\textsuperscript{126}

The synthetases responsible for post-translational modification of class III and IV lantibiotics/lantipeptides have not been as thoroughly studied as those involved in class I and II biosynthesis. Synthetases of both class III and IV possess a three domain architecture consisting of a putative phospho-serine/-threonine lyase-like domain, a kinase-like domain, and a cyclase domain (Figure 1.6). This domain composition, in combination with the recently reported \textit{in vitro} reconstitution and analysis of deletion mutants of VenL, a LanL enzyme involved in biosynthesis of venezuelin, suggested a unique dehydration strategy in which two independent domains install Dha and Dhb via a kinase domain through the phosphorylation of Ser and Thr residues, respectively. The subsequent elimination of the phosphate from pSer and pThr is carried out by a putative pSer/pThr lyase domain.\textsuperscript{39,91} Class IV cyclase domains have been shown to possess sequence similarities with LanC enzymes and with the cyclase domain of LanM proteins, suggesting that class I, II, and IV share a similar cyclization strategy, whereby a zinc ion is utilized to activate the cysteines for nucleophilic attack onto Dha and Dhb.\textsuperscript{100,119,120}

The \textit{in vitro} reconstitution of LabKC, identified a class III synthetase with the unusual ability to catalyze C-C bond formation during labyrinthopeptin biosynthesis (Figure 1.6).\textsuperscript{35} This process required the unprecedented use of GTP and Mg\textsuperscript{2+} for the phosphorylation and dehydration of serine residues. Additional experiments have been performed that demonstrate the ability of LabKC to catalyze the consecutive, double Michael addition cyclization reactions required for the formation of lanthionine and labionin motifs. The precise mechanism of cyclization is currently unknown for the LabKC and RamC proteins. In contrast to class I, II, and IV synthetases, class III proteins lack the conserved zinc ligands found in the LanC-like domains.
of the aforementioned classes. As of yet, there has been no experimental evidence identifying the use of a metal ion for cysteine thiol activation.

1.4.5. Other modifications

In addition to the characteristic (methyl)lanthionine and dehydro-amino acids, many additional post-translational modifications have been observed in lantibiotics (Figure 1.3). These unique modifications bearing unusual functionalities include lysinoalanine, aminovinyl cysteine, D-alanine, mono- and di-hydroxylations, N-terminal modifications, and a number of rare post-translational modifications. These modifications are not installed by the synthetase enzymes, but rather by enzymes often encoded within the same biosynthetic gene cluster. These additional post-translational modifications can contribute significantly to the biological activity of these lantibiotics. Importantly, they provide opportunities for the study of novel lantibiotic enzymes, which may reveal new insights into lantibiotic biosynthesis and result in novel tools for installing new functionalities into peptides and proteins.

A lysinoalanine bridge has been discovered in cinnamycin, ancovenin, and the duramycin family of lantibiotics. A small protein encoded by cinorf7 found within the cinnamycin biosynthetic gene cluster has been proposed to be involved in the formation of the crosslink between the ε-amine of lysine 19 and dehydroalanine 6. In addition to the lysinoalanine modification, L-Asp hydroxylation at the β-carbon to produce erythro-3-hydroxyl-L-aspartic acid has been identified in cinnamycin and the duramycins. The enzyme CinX has recently been identified as the Fe(II)/α-ketoglutarate-dependent hydroxylase responsible for the formation of this modification. The specific recognition unit required for this modification is unknown, as this hydroxylation does not require the leader sequence or a cyclized form of the
core peptide for modification to occur. Both lysinoalanine and β-hydroxylation are critical in mediating the interactions of cinnamycin with its biological target.

The post-translationally installed crosslinks aminovinyl cysteine (AviCys) and aminovinyl methyl cysteine (AviMeCys) have been found at the C-terminus of epidermin, gallidermin, cypemycin, and mutacin III. A group of decarboxylases, designated LanD proteins, are found within the lantibiotic gene clusters and are responsible for this modification. The aminovinyl cysteine residues found within mersacidin and epidermin result from oxidative decarboxylation of a C-terminal cysteine by MrsD and EpiD, respectively. Both enzyme activities have been reconstituted in vitro and the crystal structures of these two flavin-containing decarboxylases have been solved. Whereas EpiD displayed a broad substrate specificity with only a C-terminal Cys as a requirement, MrsD required a more definitive substrate sequence.

The presence of D-alanine has been observed in a small number of lantibiotics. Structural analysis of lactocin S revealed the presence of three D-Ala residues. In addition, this unique modification was also discovered in both peptides of the two-component lantibiotic lacticin 3147. One D-Ala is found in the α-peptide and two D-Ala are found in the β-peptide. The mechanism for D-Ala formation has been shown to involve a two-step process in which an L-serine is dehydrated, followed by the stereospecific hydrogenation of the transiently formed Dha to produce D-Ala. The protein LtnJ is encoded in the lacticin 3147 gene cluster and has been proposed to catalyze this hydrogenation activity based on sequence similarity with known zinc-containing alcohol dehydrogenases.

A number of N-terminal modifications have been identified that are believed to increase peptide stability against bacterial aminopeptidases. The N-terminal modifications 2-
oxopropionyl and 2-oxobutyryl are the products of non-enzymatic hydrolysis of N-terminal Dha and Dhb, respectively, that become exposed following the proteolytic removal of the leader peptide. The 2-oxopropionyl group has been identified in lactocin S, and the 2-oxobutyryl functionality is found at the N-terminus of Pep5. Through NMR spectroscopy, it has been identified that epilancin K7 contains an N-terminal 2-hydroxypropionyl group. In addition, epicidin 280 is believed to bear the same modification. The putative oxidoreductase EciO may be involved, but this has never been confirmed experimentally. This modification is also present on epilancin 15X, and only recently was the stereochemistry of this modification determined via biochemical methods. It was demonstrated that ElxO, an NADP(H)-dependent alcohol dehydrogenase, catalyzes the conversion of an N-terminal pyruvate to D-lactate.

There have been few reports of very rare post-translational modifications. During late stages of cell growth, subtilin can become N-succinylated resulting in a reduction of biological activity. The chlorination of tryptophan has been identified in the recently discovered lantibiotic microbisporicin which likely results from the activity of a flavin-dependent halogenase. An additional post-translational modification of 3,4-dihydroxyproline has also been discovered in the microbisporicin structure. The AviCys containing natural product cypemycin possesses methylations at hydrophobic side chains including a bis-methylation at Ala1 and an L-iso-leucine at position 13. The recent identification and characterization of a structurally related compound, grisemycin, suggests that these compounds are in fact not lantibiotics, but a new class of peptide natural products, the linaridins, which may function as signaling molecules. Finally, actagardine has been reported to contain a sulfoxide as the result of the oxidation of a C-terminal thioether bridge. It is hypothesized that two monooxygenases
identified by sequencing of the producing organisms, ActO and LigO, may catalyze the oxidation.\\(^{150}\)

1.4.6. Proteolysis and export

Nearly all lantibiotic gene clusters code for proteins that are responsible for the enzymatic removal of the leader peptide following post-translational modification and extracellular transport of the mature lantibiotic. Class I and class II lantibiotics utilize different enzymes to catalyze these events. In class I lantibiotics, the proteolytic LanP enzymes vary in size and contain a conserved catalytic triad (Asp, His, and Ser) and an Asn that plays a role in oxyanion hole formation.\\(^{151}\) These proteases display homology with subtilisin-like serine proteases.\\(^{24}\) Peptidase localization is believed to vary widely among class I lantibiotics. These enzymes can be found intracellularly, extracellularly, or anchored to the outside of the cell wall. The protease responsible for leader peptide removal of nisin, NisP, is located outside the cell membrane and is anchored on the cell surface.\\(^{151}\) A study on the \textit{in vivo} activity of NisP and NisT have addressed the substrate specificity of the protease and transporter. On one hand, the leader peptide was only removed from modified NisA after the thioether rings were formed and no protease activity was observed with unprocessed or uncyclized and dehydrated peptides, thus suggesting that NisP is specific toward thioether-containing precursor peptide. On the other hand, the same study demonstrated that the transporter, NisT, is able to export both processed and unprocessed forms of NisA precursor and non-lantibiotic peptides attached to the leader peptide as well.\\(^{102}\) The substrate promiscuity of these enzymes has been exploited in a variety of \textit{in vivo} engineering strategies.\\(^{103,104,152}\) Studies on the activity of EpiP, the protease involved in the biosynthesis of epidermin, demonstrated that this protein is extracellularly located.\\(^{96}\) PepP,\\(^{112}\)
LasP\textsuperscript{151} and ElkP\textsuperscript{144} the proteases for Pep5, lactocin S, and epilancin K7, respectively, lack the N-terminal pro-sequence found in NisP and EpiP and the C-terminal cell wall anchor of NisP. As a result, they are believed to function intracellularly, possibly as part of a membrane-bound complex\textsuperscript{112}. The activity of ElxP involved in the biosynthesis of epilancin 15X was recently reconstituted \textit{in vitro}\textsuperscript{142}.

Class II lantibiotic gene clusters do not contain genes encoding for LanP, but rather for bifunctional LanT transporters. These enzymes consist of an N-terminal protease domain and a C-terminal ATP-binding cassette (ABC) transporter domain, which is composed of a six-helix transmembrane domain with a C-terminal ATP-binding motif. Of the known bifunctional lantibiotic transporters, only the predicted protease domain from LctT comprised of residues 1-150 has been heterologously expressed and reconstituted \textit{in vitro} using expressed LctA and modified LctA as substrates\textsuperscript{110}. The protease domain of Class II LanT proteins share sequence homology with papain-like cysteine proteases and are thought to utilize a conserved Cys and His for catalysis. Mutagenesis studies of LctT demonstrated that the Cys residue was important for proteolytic activity, whereas the His influences regioselectivity\textsuperscript{110}. LctT protease cleaved at the predicted double-glycine motif site, and the formation of lanthionine rings was not required for protease activity.

\textbf{1.5. ENGINEERING OF ANTIMICROBIAL PEPTIDES}

\textbf{1.5.1. \textit{In vivo} approaches}

The unique mode of action of lantibiotics and the long-term use of nisin in commercial applications has led to increased interest in the preparation of lantibiotic analogues with improved therapeutic properties. The gene-encoded nature of lantibiotics has enabled \textit{in vivo}
bioengineering and heterologous expression systems to be developed for a number of lantibiotics including mersacidin, subtilin, Pep5, lacticin 3147, nukacin ISK-1, actagardine, and nisin.

Mutagenesis has yielded enormous insight into the structure-activity relationships of nisin and revealed key portions of the molecule that contribute to antimicrobial activity. To date, six natural variants of nisin have been identified, highlighting the natural diversity and tolerance for mutagenesis of this family of peptides. Remarkably, even subtle changes can have major repercussions on bioactivity. The replacement of Dha5 by Dhb in nisin Z resulted in a 2-10 fold reduction in bioactivity against a panel of strains. In contrast, substitution of Dhb2 for Dha led to a mutant that was twice as active.

Removal of residues that become dehydro amino acids often results in a loss of bioactivity. Mutants of nisin containing a single mutation (Dha33 to Ala) or a double mutation (Dha5 to Ala, Dha33 to Ala) reduced activity to just 1% of native nisin. Mutation or disruption of ring structures often leads to a strong reduction in antimicrobial activity. This effect was observed for the slight alteration of the A-ring via substitution of Ser3 for Thr, resulting in a MeLan instead of a Lan.

Mutagenesis has also been utilized to investigate the effect of alterations at positions not involved in post-translational modifications. The substitution of Asn27 and His31 for Lys residues resulted in a mutant with improved solubility. Mutation of residues within the hinge region of nisin, such as deletion of Asn20 and Met21 led to a dramatic loss in the ability to form pores. Introduction of Pro at these positions had a stronger effect on pore formation, suggesting that this portion of the molecule plays an integral role in providing flexibility that allows for the insertion of the C-terminus into bacterial membranes.
Nearly all \textit{in vivo} mutagenesis studies have resulted in identification of mutants with weaker antimicrobial activity or poorer pharmacological properties. Only a few exceptions exist, such as a three- to four-fold increase of activity observed for subtilin when Glu4 was changed to Ile.\textsuperscript{153} A systematic study focused on the random mutagenesis of the A- and B-rings of nisin led to the identification of A-ring variants with improved bioactivity and a modulated spectrum of target cells.\textsuperscript{165} In a separate \textit{in vivo} mutagenesis study, a large library of nisin analogues was prepared that included several derivatives such as Asn20Pro, Met21Val, and Lys22Ser which demonstrated improved bioactivity against many Gram-positive pathogens including \textit{Listeria monocytogenes} and \textit{Staphylococcus aureus}.\textsuperscript{162} Despite these examples of success, a number of problems can arise when using an \textit{in vivo} mutagenesis approach for the production of novel lantibiotics. These include challenges of identifying the stage at which biosynthesis of a mutant was impaired, potential regulatory and immunity issues, and the limitation of using proteinogenic amino acids.\textsuperscript{166-168}

A particularly exciting approach for the production of lantibiotics \textit{in vivo} is that which involves the co-expression of a lantibiotic LanA precursor in addition to biosynthetic enzymes in \textit{E. coli}. Originally reported for lanthionine formation in the biosynthesis of nukacin ISK-1,\textsuperscript{169} this methodology has now been extended for the production of lantibiotics including nisin, haloduracin \textit{\alpha}-peptide and \textit{\beta}-peptide, cinnamycin,\textsuperscript{126} and several of the recently discovered lantipeptides, prochlorosins.\textsuperscript{118} This strategy displays remarkable tolerance for mutant precursor peptides and enables full modification of the core peptide. Following expression and purification via affinity chromatography, the leader peptide can be removed \textit{in vitro} to yield bioactive core peptides.
1.5.2. *In vitro* approaches

The *in vitro* reconstitution of a number of lantibiotic synthetases has been reported in the last decade representing all four classes of biosynthetic enzymes. The cyclase, NisC, involved in nisin biosynthesis, is the only class I cyclase enzyme to demonstrate activity *in vitro*.100 Neither its dehydratase partner, NisB, nor any other LanB enzymes have been successfully reconstituted *in vitro*. The bifunctional, class II LanM enzymes involved in lacticin 481 (LctM),88 haloduracin (HalM1 and HalM2),4 prochlorosins (ProcM),34 and cinnamycin (CinM)126 biosynthesis have all been successfully reconstituted *in vitro* to produce fully-modified and bioactive products. A single class III enzyme, LabKC,35 and a single class IV enzyme, VenL,91 were recently reported to display activity *in vitro*. The reconstitution of the activities of these enzymes *in vitro* is a significant achievement for understanding lantibiotic biosynthesis and for use in peptide engineering applications.

Site-directed mutagenesis studies focused on alterations within the lantibiotic core peptides have identified key structural features of lacticin 481170 and haloduracin α and β171 that are important for bioactivity. Furthermore, *in vitro* assays of lacticin 481 synthetase has allowed for extensive mechanistic investigations,124,125,172-174 for engineering applications,175,176 and for identification of key structural elements required for substrate recognition.106,107 Recently, an *in vitro* mutasynthesis strategy was utilized to produce lacticin 481 analogues containing proteinogenic and non-proteinogenic substitutions resulting in improved bioactivity compared to a pre-selected lacticin mutant with improved solubility properties (N15R and F21H mutations).176 To date, this is the only report identifying improved variants using an *in vitro* approach; however, as more lantibiotic biosynthetic systems are reconstituted *in vitro*, so should the likelihood of producing and identifying mutants with improved biological activities increase.
1.6. SUMMARY AND OUTLOOK

We have only begun to uncover the remarkable potential that ribosomally synthesized and post-translationally modified peptides offer as novel therapeutics and tools for chemical biology research. Besides the worldwide usage of nisin as a food preservative, many other lantibiotics have relevant applications. The lantibiotics lacticin 3147, haloduracin, and microbisporicin display potent activity against a wide-range of pathogenic organisms. Mersacidin displays similar activity to vancomycin in treating methicillin-resistant \textit{S. aureus} infections in murine models. Lacticin 3147, haloduracin, mutacin 1140, and nisin all display activity against \textit{S. mutans} strains, the causative agents of dental caries. Therefore, these lantibiotics have potential as anti-cavity treatments.

Some therapeutic and beneficial applications go beyond antimicrobial activity. The binding of cinnamycin and duramycin to phosphatidylethanolamine prevents the phospholipase-A2-catalyzed release of arachidonic acid from the cell membrane and consequently affects the biosynthesis of leukotrienes and prostaglandins. Arachidonic acid is a biosynthetic precursor for the prostaglandins, a major class of compounds involved in inflammatory responses, and therefore applications of cinnamycin and the duramycins in treating cardiovascular diseases have been suggested. Duramycin has been shown to increase chloride secretion in lung epithelium, and is currently in clinical trials to evaluate its efficacy in clearing of mucus excretions in patients with cystic fibrosis. Recently, spermicidal activity has been demonstrated for lacticin 3147. Bioactivity assays revealed that the class III lantibiotic, labyrinthopeptin A2, has an excellent efficacy against neuropathic pain in an \textit{in vivo} mouse model.

Our current understanding of lantibiotic biosynthesis and mode of action is the result of synergistic contributions from all areas of chemistry and life science including biochemistry,
microbiology, chemical biology, and genetics. Efforts of the past thirty years have greatly advanced our knowledge and understanding of these complex molecules and as advances in technologies continue to fuel research in this field, we peer with great anticipation of what discoveries lay ahead. This thesis will describe efforts to understand haloduracin mode of action (Chapter 2), leader peptide utilization by LctM (Chapter 3), and investigations of the putative lantibiotic sublancin (Chapter 4).
1.7. REFERENCES


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43


lipid II complex reveals a pyrophosphate cage that provides a blueprint for novel antibiotics".


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(151) van der Meer, J. R.; Polman, J.; Beethuyzen, M. M.; Siezen, R. J.; Kuipers, O. P.; de Vos, W. M. J. Bacteriol. 1993, 175, 2578-88. "Characterization of the Lactococcus lactis nisin A operon genes nisP, encoding a subtilisin-like serine protease involved in precursor processing, and nisR, encoding a regulatory protein involved in nisin biosynthesis".


CHAPTER 2: MODE OF ACTION STUDIES OF THE TWO-PEPTIDE LANTIBIOTIC HALODURACIN\textsuperscript{1,2}

2.1. INTRODUCTION

Two-peptide lantibiotics function through the synergistic activities of two peptides. To date, eight two-peptide lantibiotics have been reported which include haloduracin,\textsuperscript{3-5} lacticin 3147,\textsuperscript{6} staphylococcin C55,\textsuperscript{7} Smb,\textsuperscript{8} plantaricin W,\textsuperscript{9} BHT-A,\textsuperscript{10} cytolysin,\textsuperscript{11} and lichenicidin.\textsuperscript{12} In these systems, two prepeptides are ribosomally synthesized as inactive precursors (LanA\textsubscript{1} and LanA\textsubscript{2}), which are both enzymatically modified to their mature, bioactive forms (Lan\textalpha{} and Lan\textbeta{}) (Figure 2.1). For all currently known two-peptide lantibiotics, the dehydration of serine and threonine residues and subsequent cyclization to form (methyl)lanthionine residues results from the actions of a bifunctional modification enzyme termed LanM. In most two-peptide systems, the homology between the LanA\textsubscript{1} and LanA\textsubscript{2} precursor sequences is low—therefore two different bifunctional enzymes (LanM\textsubscript{1} and LanM\textsubscript{2}) each act specifically on only one of the precursor peptides.\textsuperscript{4,13} The modified LanA\textsubscript{1} and LanA\textsubscript{2} prepeptides are further processed by a LanT protein, which removes the leader peptides and secretes the final products. The mature peptides of several two-peptide systems share structural and sequence homology with known single-peptide lantibiotics. The mature \textalpha{}-peptides resemble the globular lantibiotic mersacidin with several fused thioether rings, whereas the mature \textbeta{}-peptides are typically elongated and more flexible (see Figure 1.4).\textsuperscript{14,15}

\footnotesize{Reproduced in part with permission from:
\textsuperscript{2}“Haloduracin \textalpha{} Binds the Peptidoglycan Precursor Lipid II with 2:1 Stoichiometry.” \textit{J. Am. Chem. Soc.} \textbf{2011}, \textit{ASAP (published online October 17, 2011)}. Copyright 2011 American Chemical Society.}

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Haloduracin, a two-peptide lantibiotic recently discovered by genome mining, is produced by the Gram-positive alkaliphilic bacterium *Bacillus halodurans* C-125. The lantibiotic consists of two post-translationally modified peptides, Halα and Halβ. This chapter describes *in vivo* and *in vitro* studies designed to investigate the mode of action of haloduracin and compares it to nisin, the prototypical lantibiotic. Wild-type haloduracin peptides were isolated, purified to homogeneity, and their independent roles in target binding and antimicrobial activities were assessed. Haloduracin was shown to have high potency against a range of Gram-positive bacteria including pathogens such as methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant *Enterococcus*. The lantibiotic also inhibits spore outgrowth of *Bacillus anthracis*. The two peptides display optimal activity in a 1:1 stoichiometry and have similar efficacy as the commercially used lantibiotic nisin. However, haloduracin is more stable at pH 7 than nisin. The precise roles of the individual haloduracin peptides for interaction with their molecular target, lipid II, have been identified and are discussed. Despite significant structural differences between the two peptides of haloduracin and those of the two-peptide lantibiotic
lacticin 3147, these two systems show similarities in their mode of action. Like Ltnα,15 Halα binds to a target on the surface of Gram-positive bacteria, and like Ltnβ,15 the addition of Halβ results in pore formation and potassium efflux. Using Halα mutants, its B- and C-thioether rings are shown to be important but not required for bioactivity, and a similar observation was made with mutants of Glu22, a residue that is highly conserved amongst several lantibiotics. These results suggest a dual mode of action in which Halα binds to lipid II, consequently preventing peptidoglycan biosynthesis. The Halα:lipid II complex then serves as a docking site for Halβ and once recruited, Halβ facilitates pore formation in the bacterial membrane.

2.2. RESULTS AND DISCUSSION

2.2.1 Haloduracin peptides function synergistically at a 1:1 ratio

Prior to assessing the specific activity of haloduracin against a panel of bacterial strains, the ratio of Halα and Halβ that yields maximum bioactivity was established. For this purpose, the ability of Halα and Halβ to inhibit cell growth at varying concentrations and ratios was examined. As indicated by the resultant isobologram (Figure 2.2), haloduracin is active at nanomolar levels against the indicator strain Lactococcus lactis HP, and Halα and Halβ display optimal activity at a 1:1 ratio. This ratio was determined as the lowest concentration of individual Halα or Halβ peptide that when combined, caused essentially complete growth inhibition (defined as >90%). The optimal synergy of Halα and Halβ at a 1:1 ratio is shared with other two-peptide lantibiotic systems such as staphylococcin C55,7 plantaricin W,9 and lacticin 3147.16

Although the haloduracin peptides are most active in combination, at much higher concentrations both Halα and Halβ displayed independent activity against the highly susceptible L. lactis HP strain. Using liquid media growth assays, Halα and Halβ had independent IC50
Figure 2.2. Isobologram indicating the concentrations of Halα and Halβ required to inhibit the growth of the indicator strain *Lactococcus lactis* HP.

values of $1031 \pm 13$ nM and $3146 \pm 81$ nM, respectively, corresponding to a 50- to 100-fold decrease in efficacy as compared when the peptides were used together (Table 2.1). Though further experiments will be necessary to fully identify how these peptides are able to possess bioactivity in the absence of their counterpart, I speculate based on the structural similarity of Halα with mersacidin, that Halα ability to bind lipid II prevents peptidoglycan biosynthesis (*vide infra*). Halβ may exert its independent activity through a mechanism similar to nisin in the
absence of lipid II, in which the cationic Halβ peptide binds to anionic lipids of the cell membrane and the mass aggregation of Halβ monomers causes the transient formation of pore-like structures in the bacterial cell membrane.\textsuperscript{17}

**2.2.2 Comparison of antimicrobial potency of haloduracin and nisin**

Agar diffusion growth inhibition assays were performed against a panel of Gram-positive and Gram-negative bacteria to obtain qualitative information regarding their sensitivity to treatment with haloduracin and nisin. These preliminary studies provided a binary outcome of either cell growth inhibition or proliferation. Strains that showed susceptibility toward haloduracin and nisin were subjected to growth inhibition assays in liquid media from which quantitative IC\textsubscript{50} values were determined. Haloduracin and nisin shared a similar spectrum of inhibitory activity against a wide range of Gram-positive organisms, but lacked potency against the Gram-negative bacteria tested (Table 2.1). The concentrations of haloduracin and nisin required to inhibit several strains revealed that the effectiveness of the antimicrobial peptides is target strain dependent. For example, \textit{L. lactis} HP was quite sensitive to both lantibiotics with IC\textsubscript{50} values in the low-nanomolar range (29 and 14 nM for haloduracin and nisin, respectively). Some strains were more sensitive to haloduracin than to nisin (vancomycin-resistant \textit{Enterococcus}) and vice versa (\textit{Micrococcus luteus}). However, in most cases, only a two- or three-fold difference was observed in the IC\textsubscript{50} values of haloduracin and nisin against a particular strain and overall, haloduracin and nisin activity was roughly equivalent against the panel of strains examined in this study.
### Specific Activity Assessed by Liquid Growth Inhibition Assay

<table>
<thead>
<tr>
<th>Strain</th>
<th>Source</th>
<th>Haloduracin IC₅₀ (nM)</th>
<th>MIC (nM)</th>
<th>Nisin IC₅₀ (nM)</th>
<th>MIC (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Lactococcus lactis</em> HP</td>
<td>ATCC 11602</td>
<td>29 ± 21</td>
<td>73.4</td>
<td>14 ± 5</td>
<td>32.2</td>
</tr>
<tr>
<td><em>Lactococcus lactis</em> 481</td>
<td>CNRZ 481</td>
<td>63 ± 24</td>
<td>195</td>
<td>68 ± 6</td>
<td>127</td>
</tr>
<tr>
<td><em>Lactococcus lactis</em> 11454</td>
<td>ATCC 11454</td>
<td>308 ± 13</td>
<td>625</td>
<td>1,759 ± 400</td>
<td>2,800</td>
</tr>
<tr>
<td>vancomycin-resistant <em>Enterococcus faecium</em></td>
<td>C33105*</td>
<td>265 ± 15</td>
<td>781</td>
<td>4,953 ± 187</td>
<td>12,500</td>
</tr>
<tr>
<td><em>Bacillus anthracis</em> Sterne 7702</td>
<td>Gut et al.²⁸</td>
<td>363 ± 54</td>
<td>677</td>
<td>311 ± 216</td>
<td>417</td>
</tr>
<tr>
<td><em>Bacillus subtilis</em></td>
<td>ATCC 6633</td>
<td>262 ± 54</td>
<td>469</td>
<td>414 ± 165</td>
<td>820</td>
</tr>
<tr>
<td>methicillin-resistant <em>Staphylococcus aureus</em></td>
<td>C5*</td>
<td>2,616 ± 396</td>
<td>4,690</td>
<td>1,459 ± 7</td>
<td>1,560</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>ATCC 12600</td>
<td>1,144 ± 290</td>
<td>1,560</td>
<td>2,205 ± 523</td>
<td>4,690</td>
</tr>
<tr>
<td><em>Staphylococcus epidermidis</em> 15X</td>
<td>Ekkelenkamp et al.¹⁹</td>
<td>273 ± 23</td>
<td>313</td>
<td>368 ± 152</td>
<td>508</td>
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<tr>
<td><em>Micrococcus luteus</em></td>
<td>ATCC 4698</td>
<td>520 ± 17</td>
<td>1,250</td>
<td>266 ± 92</td>
<td>410</td>
</tr>
<tr>
<td><em>Micrococcus luteus</em></td>
<td>ATCC 25175</td>
<td>1,837 ± 417</td>
<td>2,500</td>
<td>5,394 ± 732</td>
<td>12,500</td>
</tr>
</tbody>
</table>

### Activity Assessed by Agar Diffusion Growth Inhibition Assay

<table>
<thead>
<tr>
<th>Strain</th>
<th>Source</th>
<th>Haloduracin</th>
<th>Nisin</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Lactococcus lactis</em> 117</td>
<td>CNRZ 117</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>Bacillus subtilis</em> DB104</td>
<td>Kawamura et al.²⁹</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>Bacillus subtilis</em> LH45</td>
<td>Liu et al.²¹</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>Bacillus cereus</em> Z4222</td>
<td>INRA Z4222</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>Bacillus cereus</em> TZ415</td>
<td>INRA TZ415</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>Enterobacter cloacae</em> 10-19C</td>
<td>ATCC 29893</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Escherichia coli</em> D158</td>
<td>UIUC-CMF</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Notes: ATCC: American Type Culture Collection; CNRZ: National Centre for Zootechnical Research; INRA: Institut National de la Recherche Agronomique; UIUC-CMF: University of Illinois Urbana-Champaign Cell and Media Facility. An * denotes clinical isolates from Carle Foundation Hospital (Urbana, IL).

### Table 2.1

Haloduracin and nisin share a similar spectrum of activity.

#### 2.2.3 Enhanced stability of haloduracin as compared with nisin

Nisin shows promising activities *in vitro* and in mouse models against several clinically relevant bacterial strains,²² however its inherent chemical instability²³,²⁴ and poor solubility at physiological pH have prevented therapeutic development. Nisin is produced by acidophilic *Lactococcus lactis* strains that grow and produce the compound in highest quantities in an acidic environment. Accordingly, nisin has been shown to exhibit highest solubility and stability at low pH.²⁵ A number of degradation products of nisin have been reported.²³,²⁴,²⁶ In a previous study, nisin stability was systematically monitored in low buffer concentrations over a pH range of 2 – 8. The results showed that nisin is most stable at pH 3, and its stability was drastically compromised at both lower and higher pH values. Moreover, at pH 7 and 8, essentially complete degradation was observed in 10 days.²⁷
Haloduracin was discovered during a search for alkaliphilic lantibiotic producing bacteria. Its producer, *Bacillus halodurans* C-125, flourishes in basic environments with a pH greater than 9.5. For this reason, haloduracin may have improved chemical stability compared to nisin at neutral pH. We monitored the stability of Halα, Halβ, and nisin in a solution of low buffer concentration at pH 7.5 using HPLC and MALDI-TOF MS analysis (Figure 2.3a). Halα displayed remarkable stability with >90% of intact peptide remaining after 36 days. The globular nature of this peptide, in addition to its low number of residues capable of undergoing oxidation may contribute to its overall stability. Halβ showed moderate stability as compared with Halα and the degradation of this linear peptide occurred gradually over the course of this assay. Analysis of the additional peaks that were observed by HPLC show that the products are mostly the result of oxidation (M+16); breakdown of the oligopeptide chain was not observed. Nisin displayed the poorest stability of the three with many oxidation products observed at early time points followed by significant degradation of the peptide chain. The increased susceptibility towards oxidation of both Halβ and nisin may be due to their high number of thioether bridges. Agar diffusion assays were utilized to evaluate the antimicrobial activities of haloduracin and nisin over time, using the same samples that were analyzed by HPLC. As shown in Figure 2.3b, oxidation and degradation of nisin appears to have a much stronger deleterious effect on its bioactivity than oxidation of Halβ has on the antimicrobial activity of haloduracin. The observed activity is not due to solo activity of Halα, because Halα has much weaker activity in the absence of Halβ. It appears, therefore, that the structural requirements for Halβ activity are not critically dependent on unoxidized thioether crosslinks.
Figure 2.3. (a) Assessment of relative stabilities of Halα, Halβ, and nisin at pH 7.5 using HPLC and MS analysis. (b) Bioassay against Lactococcus lactis HP using the same samples as analyzed by HPLC in panel (a). The day of incubation is denoted above each well. Haloduracin: upper left (blue), nisin: lower left (red), nisin standards: right (yellow).

2.2.4 Halα and Halβ act sequentially to affect target cells

To address the potential roles of the individual peptides of haloduracin, the order of binding events required to observe cell growth inhibitory activity was examined. When L. lactis HP target cells were incubated with Halα, followed by stringent washing to remove unbound peptide and subsequent incubation with Halβ, growth inhibition was observed (Figure 2.4). When the order of addition was reversed and cells were first exposed to Halβ, washed, and then treated with Halα, growth inhibition was not observed. When Halα and Halβ peptides were added at the same time, growth inhibition was observed. The described outcome of sequential addition is similar to that observed for the two-peptide lantibiotic lacticin 3147 (Ref.16) and suggests a scenario in which Halα binds a target on the outer surface of the bacterial cell and that the resulting complex is required for Halβ to exert its synergistic effect.
Figure 2.4. Sequential activity of haloduracin peptides against *L. lactis* HP. Growth curves are shown for (a) Halα treatment, washing, and Halβ addition. (b) Halβ treatment, washing, and Halα addition. Haloduracin: 1 μM, 500 nM, 250 nM, 100 nM, 10 nM, 0 nM control, and nisin: 1 μM. When errors bars are not visible, the error was smaller than the size of the symbol used.

2.2.5 Identification of lipid II as the specific molecular target of Halα

Antimicrobial activity at nanomolar concentrations suggests the involvement of a specific target molecule to which haloduracin binds to exert its biological activity. Lipid II has been identified as the docking molecule for several Class I and II lantibiotics (Figure 2.5).\(^{28}\) Based on shared structural features and homology with other lipid II binding lantibiotics, it has been suggested that Halα may serve as the primary mediator of target binding, whereas Halβ plays its major role in pore formation.\(^{15}\) Halα is proposed to inhibit the transglycosylases that form the glycan chains of the peptidoglycan by binding to lipid II on the outer surface of the bacterial membrane. An assay to observe lipid II binding by peptide antibiotic compounds has been recently developed by Professor Suzanne Walker and coworkers.\(^{29}\) In the laboratory of Professor Suzanne Walker, I performed experiments to assess the ability of several lantibiotics and other antimicrobial peptide compounds to bind lipid II. Using the major synthetic transglycosylase in
*E. coli*, PBP1b, and chemo-enzymatically prepared heptaprenyl lipid II substrate, I demonstrated that the Halα peptide does, in fact, inhibit bacterial transglycosylases by binding to lipid II. As anticipated, only the Halα component displayed lipid II binding with an IC$_{50}$ of $9.58 \pm 0.39 \mu\text{M}$ in experiments conducted with 4 µM lipid II. The Halβ component did not show any binding to lipid II even at concentrations greater than 100 µM when analyzed using the same conditions (Figure 2.5).

Figure 2.5. *In vitro* transglycosylase assays using PBP1b, lipid II, and peptide inhibitor. (a) Naturally occurring lipid II and [14C]-GlcNAc labeled heptaprenyl lipid II analogue used for *in vitro* transglycosylase assay. (b) Halα peptide binds lipid II with an IC$_{50}$ of $9.58 \pm 0.39 \mu\text{M}$ (measured with 4 µM lipid II). (c) Halβ does not demonstrate binding at concentrations as high as 100 µM. When errors bars are not visible, the error was smaller than the size of the symbol used. The horizontal axes indicate the concentrations of peptide inhibitor used in reaction. The vertical axes indicate the relative amount of peptidoglycan formed in each reaction. The % PG (relative) is defined by $[\text{polymerized peptidoglycan} / (\text{total free lipid II} + \text{polymerized peptidoglycan})]$ normalized to 100%.
The set of kinetic curves (Figure 2.6) show velocity versus substrate (lipid II) concentration for the PBP1b enzymatic reaction in the presence of 0, 6, and 8 μM Halα. In the absence of Halα, PBP1b displayed typical Michaelis-Menten kinetics. In the presence of Halα at either 6 or 8 μM concentrations, the reaction rate is negligible at low substrate concentrations; however, as soon as the substrate concentration exceeds a critical value, the rate increases and inhibition is largely overcome. These sigmoidal curves are consistent with a mechanism where Halα binds lipid II, effectively sequestering it such that it cannot be processed by PBP1b. Two vital pieces of information regarding this mechanism can be deduced from these inhibition curves. First, the reaction rate rapidly increases when the lipid II concentration exceeds one-half the concentration of Halα, and this result strongly suggests that Halα binds to lipid II in a 2:1 ratio. Second, the transglycosylase reaction at low substrate concentrations was not observable in the presence of Halα, indicating that there is essentially no free lipid II in solution. Therefore, the apparent $K_d$ for the interaction of lipid II and Halα must be very low. A $K_d$ of 14.7 nM for the Halα-lipid II complex was calculated based upon the curve fits (see Figure 2.6 caption for equation) to the kinetic data and assuming a 2:1 binding stoichiometry. The data could not be fit to a 1:1 binding model.
Figure 2.6. The kinetics of transglycosylase inhibition indicates that Halα binds to lipid II with high affinity and in a 2:1 Halα:lipid II ratio. Equation used for fitting: 

\[
\text{rate} = \frac{V_{\text{max}}((0.5[I]-[S]+K_d)^2 + 4*K_d*[S])^{0.5} - (0.5*[I] - [S]+K_d))/2)((K_m+(0.5*[I]-[S]+K_d))2+ 4*K_d*[S])^{0.5}-(0.5*[I]-[S]+K_d))/2). \]

See reference.30

2.2.6 Membrane depolarization by haloduracin

Given the known pore forming activity of several lantibiotics, we examined the effect of haloduracin on membrane potential via flow cytometry with DiOC₂, a membrane potential-sensitive dye. Under physiological conditions, bacteria with intact cytoplasmic membranes maintain a membrane potential on the order of 100 to 200 mV (interior negative with respect to exterior).31 As shown in Figure 2.7, haloduracin rapidly (within 10 min) dissipated the membrane potential of *B. subtilis* cells in a concentration-dependent manner and at levels comparable to or lower than nisin. The extensive dissipation of membrane potential likely reflects damage of the cell membrane.
Figure 2.7. Haloduracin and nisin display comparable membrane depolarization activities. (a) Mean fluorescence intensity (MFI) for treatment of *B. subtilis* 6633 cells with haloduracin (blue) or nisin (red) over a range of concentrations. (b) Representative histogram of cell count vs. DiOC₂ fluorescence intensity for haloduracin. Populations shown are for 0 nM (control, black), 25 nM (red), 250 nM (blue), 2.5 µM (violet), and 25 µM haloduracin (green).

2.2.7 Pore formation by haloduracin and efflux of potassium ions

The effects of haloduracin on membrane potential prompted the investigation of potassium efflux using a cell impermeable, potassium-sensitive dye (PBFI). Treatment of an *M. luteus* cell suspension with Halα and Halβ triggered rapid efflux of intracellular potassium (Figure 2.8). Nearly identical results were observed for other Gram-positive strains such as *Bacillus subtilis* ATCC 6633. Potassium ion release was detected after a short lag time followed by a rapid signal increase that reached a plateau within 4-5 min. The observed potassium release was dose-dependent and efflux was not detected in untreated samples. When cells were treated with Halα or Halβ alone at low micromolar concentrations, no leakage of potassium was observed. These findings, in addition to the results obtained from the sequential binding study described above, prompted us to further investigate the individual roles of the haloduracin peptides in pore formation. When Halα and Halβ were added either together or when cells were
pre-incubated with Halβ for 5 min followed by the addition of Halα, a short lag was observed for 20-30 s followed by rapid K⁺ efflux (Figure 2.9). However, when cells were pre-incubated with Halα followed by the addition of Halβ, rapid onset of efflux was observed and the lag was essentially absent. The combined results of potassium efflux and sequential binding assays suggest that the biological role of Halα involves binding to its target, resulting in a complex that serves as a docking site for Halβ to bind and form pores. A similar mechanism has been proposed for lacticin 3147 (Ref.15), and it may be that this is a general mechanism for two-peptide lantibiotics for which the α-peptide has structural homology with mersacidin.
Figure 2.9. The effects on potassium release by pre-incubating *Micrococcus luteus* cells with either Halα or Halβ prior to addition of its counterpeptide. Shown in blue, (A) 5 μM Halα pre-incubation followed by 5 μM Halβ addition; in green, (B) 5 μM Halβ pre-incubation followed by 5 μM Halα addition; in red, (C) simultaneous addition of 5 μM Halα and 5 μM Halβ; in orange, (D) addition of 5 μM nisin; in violet, (E) addition of 5 μM Halα; in pink, (F) addition of 5 μM Halβ; and in gray (G) a control without haloduracin. Vertical line (black) indicates time of antibiotic addition.

2.2.8 Production and evaluation of Halα analogues

The Halα peptide possesses a conserved CTLTXEC thioether bridge motif (B-ring), which is believed to be important for lipid II binding. The sequence is found in several class II lantibiotics including the single-peptide lantibiotics mersacidin and actagardine, as well as the Ltnα peptide from the two-peptide lantibiotic lacticin 3147. Residing within this ring is a highly conserved glutamate residue, which has been shown for mersacidin to be essential for binding to lipid II.

Haloduracin biosynthesis was recently reconstituted *in vitro* providing a method for the production of haloduracin mutants. The effects of individually disrupting the B- and C-thioether rings of Halα were probed by the mutation of Cys23 and Cys27 to alanines,
Figure 2.10. General method for the *in vitro* production of Halα mutant peptides. The HalA1-Xa wt peptide was mutated at specific residues of the core peptide. Treatment of expressed and purified peptide with HalM1, followed by treatment with Factor Xa to proteolytically remove the leader peptide and HPLC purification resulted in the Halα mutant peptides as indicated. Shaded circles of Halα peptides indicate residues that were mutated in this study.

respectively. Additionally, the conserved glutamate residing in the conserved B-ring was mutated to the isosteric glutamine (Figure 2.10). It has been shown previously that these mutations do not affect the formation of the other ring structures.\(^{32}\)

All Halα mutants retained the ability to inhibit *L. lactis* HP cell growth in liquid medium when incubated in the presence of wild type Halβ (Figure 2.11a). Quantitative IC\(_{50}\) values highlight the importance of the thioether rings of Halα in the antimicrobial activity of haloduracin with IC\(_{50}\) values of 335 ± 29 nM (10-fold loss) and 1180 ± 48 nM (30-fold loss) for wt Halβ combined with Halα C23A and C27A, respectively. The highly conserved glutamate residue residing in the B-ring of Halα also was important but not required for activity and displayed an IC\(_{50}\) of 964 ± 76 nM for Halα E22Q in combination with wt Halβ.
Maintaining biological activity at all in spite of the absence of conserved thioether rings present in Halα was unanticipated as these motifs are generally considered to be the basis of lantibiotic bioactivity, and disruption of a ring has often resulted in abolished activity. The relatively modest reduction of bioactivity of the highly conserved B-ring mutant (10-fold) was therefore most unexpected. The retention of activity for the C27A and E22Q mutants were also unexpected as these mutants had no activity in agar diffusion assays. Increased activity of two-peptide lantibiotics in liquid media compared to solid agar media has been previously observed. Additional studies were used to investigate whether the reduced potency of Halα mutants in combination with wild type Halβ is reflective of the ability of Halα mutants to bind lipid II or to effectively recruit Halβ for synergistic activity, or both.

As a comparison to the above growth inhibition study, these mutants were also subjected to analysis using the described PBP1b transglycosylase assay. These peptides were analyzed using identical reaction conditions as used for evaluation of Halα and Halβ. Loss of lipid II binding ability corresponded well to the diminished activity observed for the cell-based growth inhibition assays. Overall, all mutants displayed weaker ability to prevent peptidoglycan formation (Figure 2.11b). Of the mutations investigated, the E22Q proved most impactful. Not only was antimicrobial activity significantly reduced, but lipid II binding was essentially abolished. This mutant did not display any binding to lipid II even at high concentrations up to 100 μM, thus corroborating the critical role of this glutamate residue in lipid II binding. The IC\textsubscript{50} values for lipid II binding with C23A and C27A were 50.7 ± 1.7 μM and 29.5 ± 3.5 μM with 4 μM lipid II, respectively, corresponding to approximately 5- and 3-fold weaker inhibitory activity as compared to the \textit{in vivo} isolated Halα peptide (IC\textsubscript{50} of 9.58 ± 0.39 μM). The IC\textsubscript{50} values for Halα C23A and C27A lipid II binding were surprisingly reversed as compared with
the observed growth inhibition values. This observation may be due to different degrees to which lipid II binding occurs and the ability to recruit Halβ peptide. Introduction of the C23A mutation opened the B-ring which resembles the highly conserved CTLTEXC lipid II binding motif, thereby greatly diminishing its ability to bind lipid II. However, the C-terminal C-ring remained intact and this mutant remained quite effective in antimicrobial activity when used in combination with Halβ. The C27A mutation opened the C-ring of the peptide resulting in a flexible C-terminal tail. This mutant was still quite effective in lipid II binding; however, of the three mutants evaluated, this peptide displayed the weakest antimicrobial activity. It is likely that the C-ring may serve as the critical surface for Halα-Halβ interactions and the disruption of C-terminal structure rigidity may result in the inability to effectively recruit Halβ. Further experiments such as solution NMR, isothermal calorimetry, or other techniques to investigate peptide-peptide interactions would greatly enhance our understanding of how two-peptide lantibiotic interact with each other.

**Figure 2.11.** Deleterious effects of disrupting conserved thioether bridges and mutating conserved residues of the Halα peptide. (a) Impacts on antimicrobial activity against *L. lactis* HP cells. Treatment included Halβ wt peptide at a 1:1 Halα mutant:Halβ ratio. (b) Effects on lipid II binding ability using *in vitro* transglycosylase assay. For both panels: Halα E22Q (red), Halα C23A (green), Halα C27A (blue).
2.2.9 Influence of the leader peptide on the biological activity of Halα

The Halα mutants used in this work were made using a previously described in vitro reconstituted biosynthesis. In this process, the lanthionine synthetase HalM1 carries out a series of post-translational modifications on the HalA1 precursor peptide that result in the thioether crosslinks shown in Figure 2.1. The precursor peptide has an additional N-terminal extension of 41 amino acids called the leader peptide that is important for recognition by HalM1. In addition, the leader peptides of lantibiotic precursor peptides are generally believed to keep their products inactive while they are synthesized in the cytoplasm. For haloduracin, the bifunctional protease/transporter HalT removes the leader peptide. HalT has not been investigated to date, but in a related system for the lantibiotic lacticin 481, the dedicated protease domain of the transporter LctT removes the leader peptide of modified LctA precursor peptide and secretes the final product. Given the common belief that lantibiotics with their leader peptide still attached are inactive, it was surprising to find that Halα with its leader peptide intact (Leader-Halα) displayed antimicrobial activity against L. lactis HP when combined with Halβ (Figure 2.12a). It may be possible that the indicator strain secretes a protease that removes all or part of the leader peptide from a small subset of Halα molecules, resulting in the observed activity. Alternatively, Halα with its leader peptide attached may still engage bind to lipid II. To test the latter explanation, the peptidoglycan polymerization assay was conducted in the presence of leader-Halα. Indeed, this peptide proved a potent inhibitor of lipid II polymerization (Figure 2.12b) with an IC₅₀ of 7.1 ± 0.2 μM (with 4 μM lipid II). This potency was similar to the activity of wt-Halα (compare Figure 2.5b). The weaker antimicrobial activity of leader-Halα with wt Halβ when compared to Halα combined with Halβ (Figure 2.12a) is likely the consequence of less optimal synergy between the two peptides when the leader peptide is still attached to Halα.
Figure 2.12. Evaluation of the antimicrobial activity and enzyme inhibitory activity of Halα with its leader peptide attached. (a) Agar diffusion growth inhibition assay against *L. lactis* HP. Inhibitory activity was assessed using individual peptides (50 μM Halα, 50 μM Leader-Halα, and 50 μM Halβ, upper row) and in combination (at 50 μM) with 50 μM Halβ (lower row left and center). Nisin was used as a control at a 50 μM concentration (lower right). (b) Inhibition of the PBP1b-catalyzed formation of peptidoglycan (PG) by leader-Halα.

2.2.10 Inhibition of spore outgrowth

Nisin inhibits the outgrowth of spores from several pathogenic Gram-positive bacteria including *Bacillus* and *Clostridium.*18,44,45 To determine if haloduracin is capable of preventing spore outgrowth, *Bacillus anthracis* (Sterne 7702) was selected as a model. Spores were germinated in Brain Heart Infusion (BHI) medium in the presence of a range of haloduracin or nisin concentrations (Figure 2.13a and b). For cultures containing low amounts of haloduracin (0.1 μM), differential interference contrast (DIC) microscopy revealed chains of vegetative bacilli after 5 and 10 h. However, no bacilli were present within cultures supplemented with 1, 10, or 100 μM haloduracin. This dose dependent response shows that haloduracin was equally effective as nisin in the inhibition of *B. anthracis* spore outgrowth. Halα and Halβ inhibited spore outgrowth only at 100 μM when incubated independently, whereas vegetative bacilli were observed in cultures containing 10 μM or less of either peptide (Figure 2.13c and d). Hence, the
Figure 2.13. Inhibition of *Bacillus anthracis* spore outgrowth by haloduracin peptides in combination and separately. (a) Spore outgrowth inhibition by haloduracin (Halα and Halβ in combination). (b) Spore outgrowth inhibition by nisin (control). (c) Spore outgrowth inhibition by Halα. (d) Spore outgrowth inhibition by Halβ. White scale bars equal 5.0 μm for all images.

spore outgrowth inhibition depicted in Figure 2.13a is the result of a synergistic mechanism of both peptides.

2.3. SUMMARY

This study illustrates the potent antimicrobial activity displayed in a synergistic manner by the two peptides of haloduracin. The activity of this two-peptide lantibiotic rivals that of nisin, which has been used worldwide as an effective preservative against food-borne pathogens. Haloduracin displayed higher stability than nisin at pH 7, which bodes well for potential commercial use. Despite significant differences in the structures of both the α- and β-peptides of
lacticin 3147 and haloduracin, these two compounds appear to operate by a similar mechanism that involves recognition of lipid II by the $\alpha$-peptides of a target at the cell surface, namely lipid II, and utilization of the $\beta$-peptides for membrane permeabilization. As such, these compounds combine the two activities of nisin into separate peptides.$^{17}$

Lipid II plays an essential role in bacterial cell wall biosynthesis by serving as the substrate for the bacterial cell wall biosynthetic enzymes. The transport of the fundamental disaccharide-pentapeptide building blocks of peptidoglycan across the plasma membrane where they can be incorporated into the growing cell wall is necessary for bacterial viability. Due to its critical role in this process, lipid II is the target for several antibiotics which include glycopeptides (teicoplanin, ramoplanin, and vancomycin) and both class I and class II lantibiotics.$^{28}$ Lipid II is utilized in at least two different modes of action by these lantibiotic classes.$^{17}$ Class II lantibiotics bind to lipid II and thereby prevent its use by cell wall biosynthetic enzymes to effectively inhibit peptidoglycan formation. Class I lantibiotics have also been shown to bind lipid II to inhibit peptidoglycan biosynthesis and additionally use this molecule to form pores in the membrane of target cells.

Haloduracin is a member of the growing subclass of two-component lantibiotics which fall into the Class II family. The antimicrobial activity at nanomolar concentrations displayed by haloduracin implies the involvement of a specific target molecule, which was predicted to be lipid II based on previous studies with other lipid II-binding lantibiotics.$^{3}$ Sequence alignment of both Hal$\alpha$ and Hal$\beta$ peptides with other known two-component systems that target lipid II reveal significant similarities in lanthionine bridging patterns.$^{4}$ In addition to homologies in ring patterns, Hal$\alpha$ contains a remarkably conserved CTLTXEC motif which is believed to be essential for lipid II binding. This sequence is shared between several Class II members
including the single-component lantibiotics mersacidin\textsuperscript{33} and actagardine,\textsuperscript{34} as well as the Ltn\(\alpha\) peptide from the two-component lantibiotic lacticin 3147.\textsuperscript{6}

Our initial bioactivity studies suggested that Hal\(\alpha\) and Hal\(\beta\) peptides play two very different roles in the antimicrobial activity of haloduracin. The isobolographic analysis and specific activity studies indicate that both peptides are absolutely required to exert optimal antibiotic activity against sensitive strains. Though this contributes to the understanding of the stoichiometry of the bioactive complex, no information regarding the order of binding events can be inferred. Several lines of evidence from the cell-based assays suggest that the initial contact between haloduracin and the cell involves the Hal\(\alpha\) peptide. The outcome of the sequential addition of haloduracin peptides suggests a situation in which Hal\(\alpha\) binds lipid II on the outer surface of the bacterial cell. Upon the subsequent addition of Hal\(\beta\) to cells pre-treated with Hal\(\alpha\) inhibition of cell growth was observed. This inhibitory effect was not observed when the order of peptide addition was reversed, thus the peptides appear to operate in a sequential manner, a conclusion that is strengthened by the lipid II polymerization assays.

Binding of lipid II by the Hal\(\alpha\) peptide was confirmed by \textit{in vitro} analysis of enzymatic conversion of lipid II into immature peptidoglycan by PBP1b. Hal\(\alpha\) was shown to bind tightly to its target with a nanomolar dissociation constant, whereas Hal\(\beta\) displayed no binding to lipid II even at high micromolar levels. The disruption of conserved thioether rings and binding motifs of Hal\(\alpha\) resulted in loss of binding efficiency to its lipid II target. This loss in binding due to disruption of conserved features was particularly evident for the highly conserved Glu22 residue whereby mutation to Gln resulted in complete abolishment of binding ability. Trends observed in the diminished binding to lipid II correlated well with \textit{in vivo} growth inhibition assays where Hal\(\alpha\) mutants displayed much poorer antimicrobial activities than the wild-type \(\alpha\)-peptide.
The results of *in vivo* and *in vitro* experiments have enabled the first proposal of the mode of action of a two-peptide lantibiotic that takes into consideration the stoichiometry of the event (Figure 2.13). This three-step model of antibiotic activity of haloduracin begins with the (i) Halα peptide associating with the cell membrane and binding lipid II. (ii) The binding of lipid II induces or stabilizes a specific conformation of Halα resulting in a lipid II-Halα complex that facilitates the recruitment of Halβ. (ii) When bound to the lipid II-Halα complex, Halβ is able to adopt a transmembrane conformation which enables the formation of a defined pore. Haloduracin is a two-component lantibiotic which has now been shown to function in a 1:1 Halα:Halβ ratio and bind lipid II in a 2:1 Halα:lipid II ratio. Collectively, the proposed ratio of components in the pore structure is 2:2:1 Halα:Halβ:lipid II.

![Diagram of proposed dual mode of action of haloduracin.](image)

**Figure 2.14.** Proposed dual mode of action of haloduracin.

This work provides additional insights into lipid II binding by the peptides of a two-component lantibiotic and reveals the stoichiometry of the haloduracin-lipid II pore. These results open the door for future investigations of the haloduracin-lipid II interaction including characterization of the complex via solution NMR spectroscopy and further transglycosylase
experiments—both of which may lead to the discovery of key motifs of lipid II that are critical for recognition by Halα and determination of the residues important for Halα-Halβ interactions.

The widespread prevalence of antibiotic resistance and the paucity of recent efforts towards discovery and development of antibiotics by the pharmaceutical industry have resulted in a great need for new antimicrobials. Members of the lantibiotic family of bacteriocins, and particularly those such as haloduracin which display greater stability and solubility at physiological pH, hold promising potential to help alleviate this problem and by understanding how these unusual peptides work, we may be able to rationally design highly effective antibiotics for future use.
2.4. EXPERIMENTAL

2.4.1. Materials, cultures, and conditions

All chemicals and HPLC grade solvents were purchased from Sigma-Aldrich, unless otherwise stated. Tris, MOPS, and HEPES buffers were obtained from Fisher and α-cyano-4-hydroxy-cinnamic acid from Fluka. All media including Luria Bertoni (LB), Brain Heart Infusion (BHI), Todd Hewitt (TH), Mueller Hinton Broth (MHB), and M17 supplemented with 0.5% glucose (w/v) (GM17) was purchased from BD Biosciences. Factor Xa was purchased from New England Biolabs. Table 2.2 describes the bacteria strains, source, and culture conditions for all bacteria used for this study. All Halα mutants were prepared as described previously.\textsuperscript{4,32}

<table>
<thead>
<tr>
<th>Strain</th>
<th>Source</th>
<th>Media</th>
<th>Conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacillus halodurans C-125</td>
<td>ATCC BAA-125</td>
<td>LB</td>
<td>30 °C, aerobic</td>
</tr>
<tr>
<td>Lactococcus lactis HP</td>
<td>ATCC 11602</td>
<td>GM17</td>
<td>30 °C, anaerobic</td>
</tr>
<tr>
<td>Lactococcus lactis 481</td>
<td>CNRZ 481</td>
<td>GM17</td>
<td>30 °C, anaerobic</td>
</tr>
<tr>
<td>Lactococcus lactis 11454</td>
<td>ATCC 11454</td>
<td>GM17</td>
<td>30 °C, anaerobic</td>
</tr>
<tr>
<td>Vancomycin-resistant Enterococcus faecium</td>
<td>C33105*</td>
<td>BHI</td>
<td>37 °C, aerobic</td>
</tr>
<tr>
<td>Bacillus anthracis Sterne 7702</td>
<td>Gut, I. M. \textit{et al.}\textsuperscript{18}</td>
<td>BHI</td>
<td>30 °C, aerobic</td>
</tr>
<tr>
<td>Bacillus subtilis</td>
<td>ATCC 6633</td>
<td>LB</td>
<td>30 °C, aerobic</td>
</tr>
<tr>
<td>Methicillin-resistant Staphylococcus aureus</td>
<td>C5*</td>
<td>BHI</td>
<td>37 °C, aerobic</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>ATCC 12600</td>
<td>BHI</td>
<td>37 °C, aerobic</td>
</tr>
<tr>
<td>Staphylococcus epidermidis 15X</td>
<td>Ekkelenkamp. \textit{et al.}\textsuperscript{19}</td>
<td>MHB</td>
<td>37 °C, aerobic</td>
</tr>
<tr>
<td>Micrococcus luteus</td>
<td>ATCC 4698</td>
<td>LB</td>
<td>37 °C, aerobic</td>
</tr>
<tr>
<td>Streptococcus mutans</td>
<td>ATCC 25175</td>
<td>TH</td>
<td>37 °C, anaerobic</td>
</tr>
<tr>
<td>Lactococcus lactis 117</td>
<td>CNRZ 117</td>
<td>GM17</td>
<td>30 °C, anaerobic</td>
</tr>
<tr>
<td>Bacillus subtilis DB104</td>
<td>Kawamura, F. \textit{et al.}\textsuperscript{20}</td>
<td>LB</td>
<td>30 °C, aerobic</td>
</tr>
<tr>
<td>Bacillus subtilis LH45</td>
<td>Liu, W. \textit{et al.}\textsuperscript{21}</td>
<td>LB</td>
<td>30 °C, aerobic</td>
</tr>
<tr>
<td>Bacillus cereus Z4222</td>
<td>INRA Z4222</td>
<td>LB</td>
<td>30 °C, aerobic</td>
</tr>
<tr>
<td>Bacillus cereus TZ415</td>
<td>INRA TZ415</td>
<td>LB</td>
<td>30 °C, aerobic</td>
</tr>
<tr>
<td>Enterobacter cloacae 10-19C</td>
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<td>LB</td>
<td>37 °C, aerobic</td>
</tr>
<tr>
<td>Escherichia coli DH5α</td>
<td>UIUC-CMF</td>
<td>LB</td>
<td>37 °C, aerobic</td>
</tr>
</tbody>
</table>

Notes: ATCC: American Type Culture Collection, CNRZ: National Centre for Zootechnical Research, INRA: Institut National de la Recherche Agronomique, UIUC-CMF: University of Illinois Urbana-Champaign Cell and Media Facility. An * denotes clinical isolates from Carle Foundation Hospital (Urbana, IL).

Table 2.2. Growth conditions for strains used in this study.
2.4.2. Purification of haloduracin

A culture of *Bacillus halodurans* C-125 was grown in LB under aerobic conditions for 15 h at 37 °C. Aliquots of the culture (300 μL) were removed and plated on Brain Heart Infusion agar plates (15 x 150 mm) and bacteria were grown for 96 h at 30 °C. Agar plates were gently washed with 70% isopropanol in sterile deionized water (v/v) to harvest cells from plates. The cell suspension was incubated for 24 h at 30 °C with vigorous agitation. Cells and debris were removed by centrifugation at 10k x g for 30 min at 4 °C and the haloduracin containing supernatant was concentrated via rotary evaporation. The resultant preparation was filtered using a 0.45 μm syringe filter to remove any residual cells or spores. Haloduracin peptides were purified by preparative HPLC performed on a Waters Delta 600 instrument equipped with a Phenomenex Jupiter Proteo C12 column (10 μm, 90 Å, 250 mm x 15 mm) equilibrated in 10% B (A: 0.1% TFA in water, B: 0.0866% TFA in 80% ACN/20% water (v/v)). The crude material was applied to the column and haloduracin peptides were eluted by maintaining the mobile phase at 10% B for 5 min, followed by an increase to 100% B over 50 min with a flow rate of 10.0 mL min⁻¹. Under these conditions, Halα and Halβ eluted at 30.5 and 32 min, respectively. Halα fractions containing trace amounts of Halβ were further purified by lyophilization, resuspension of the peptide mixture in 50 mM TCEP and incubating at room temperature for 3 h. This material was re-applied to the preparative HPLC column and further purified using the conditions described above. The reduced Halα peptide retention time shifted to 27 min whereas the Halβ retention time remained unchanged at 32 min. All fractions were analyzed by mass spectrometry performed on a matrix-assisted laser desorption/ionization time-of-flight mass spectrometer (Applied Biosystems Voyager DE-STR). A 1 μL aliquot of sample was combined with 1 μL of matrix consisting of saturated α-cyano-4-hydroxy-cinnamic acid matrix in 50% ACN/50%
water with 0.1% TFA, and the total volume was spotted onto a MALDI target and dried under ambient conditions prior to MS analysis. The purified peptides were lyophilized to dryness and stored under N\textsubscript{2} at \(-80 \text{ °C}\) until further use (Figure 2.14). Typical yields for the peptides were 2 – 3 mg Hal\textsubscript{α} and 1.5 – 2 mg Hal\textsubscript{β} per liter of agar.

Figure 2.15. MALDI-TOF mass spectra for purified Hal\textsubscript{α} (blue) and Hal\textsubscript{β} (red) peptides. Hal\textsubscript{α} expected [M+H]: 3047.61, observed: 3046.48. Hal\textsubscript{β} expected [M+H]: 2332.88, observed: 2332.27.

2.4.3. Purification of Nisin

A 500 mg sample of Nisaplin (Danisco) was suspended in 5 mL of 25% acetonitrile with 0.1% TFA and was sonicated for 30 min, followed by centrifugation at 15,000 x g for 10 min to remove insoluble material. Nisin was purified by preparative HPLC using a Waters Delta 600 instrument equipped with a Waters Delta-Pak C4 column (15 \(\mu\text{m}\), 100 Å, 100 mm x 25 mm) equilibrated in 2% B (A: 0.1% TFA in water, B: 0.0866% TFA in 80% ACN/20% water (v/v)).
The soluble material was applied to the column and nisin was eluted by increasing to 100% B over 45 min with a flow rate of 8.0 mL min\(^{-1}\). Nisin eluted at 28 min. All fractions were analyzed by MALDI-TOF mass spectrometry as described above. The purified peptide was lyophilized to dryness and stored under N\(_2\) at \(-80\) °C until further use (Figure 2.15).

**Figure 2.16.** MALDI-TOF mass spectra for purified nisin. Expected [M+H]: 3355.20, observed: 3355.09.

### 2.4.4. Preparation of Hal\(\alpha\) mutants

Purified HalA1-Xa peptides\(^{32}\) were dissolved (final concentration 0.3 mg mL\(^{-1}\)) in HalM1 assay buffer (50 mM Tris, pH 8.3) containing 10 mM MgCl\(_2\), 2.5 mM ATP, and 1 mM TCEP. HalM1 enzyme was added (10 \(\mu\)M final concentration) and reactions were incubated at 25 °C for 3.5 h. At 3.5 h, complete conversion to dehydrated and cyclized product was observed in the soluble fraction as determined by MALDI-TOF MS. Insoluble material (unmodified peptide) was pelleted via centrifugation, and to the supernatant was added NaCl, CaCl\(_2\), and Factor Xa protease (final concentrations of 100 mM, 2 mM, and 1.5 \(\mu\)g mL\(^{-1}\), respectively). Cleavage
reactions were incubated at 25 °C for 6 h and were quenched with 1% TFA. Reactions were lyophilized to dryness and resuspended in 10% acetonitrile in water (v/v). Halα mutant peptides were purified using preparative HPLC and analyzed by MALDI-TOF MS as described above (Figures 2.17 – 2.19). IC₅₀ values for Halα mutants were determined against the L. lactis HP as described below in combination with wild type Halβ peptide obtained from the producing strain. Both Halα and Halβ wild type peptides (in combination) and nisin standards were used as controls.

Modified HalA1-Xa peptide was prepared by Xiao Yang (Wilfred van der Donk Laboratory) via co-expression strategy described previously (Figure 2.20).⁴⁶

![Graph](image)

**Figure 2.17.** MALDI-TOF mass spectra for purified Halα C23A analogue. Expected [M+H]: 3016.55, observed: 3017.96.
Figure 2.18. MALDI-TOF mass spectra for purified Halα C27A analogue. Expected [M+H]: 3016.55, observed: 3017.83.

Figure 2.19. MALDI-TOF mass spectra for purified Halα E22Q analogue. Expected [M+H]: 3047.63, observed: 3049.71.
2.4.5. Construction of the isobologram

Individual peptides were incubated at the concentrations indicated in Figure 2.2 with the indicator strain \textit{L. lactis} HP and nisin standards were used as controls. Ninety-six-well microtiter plates were used for analysis. Serial dilutions of Hal\textalpha{} and Hal\textbeta{} peptides were prepared in sterile deionized water (SDW). For assay plates, the total volume in each well was 200 μL; the experimental wells contained 50 μL of diluted Hal\textalpha{} and Hal\textbeta{} peptides at defined concentrations and 150 μL of a 1-in-10 dilution (approximately 1 x 10^{8} CFU mL^{-1}) of a culture of indicator strain diluted in fresh growth medium. In addition, each plate contained several blank (150 μL fresh growth medium and 50 μL SDW) and control wells (150 μL of untreated 1-in-10 diluted culture and 50 μL SDW). The optical density at 600 nm (OD\textsubscript{600}) was recorded at hourly intervals from 0 to 5 h with an additional measurement at 18 h. Plates were incubated at 30 °C under
anaerobic conditions. The triplicate readings were averaged, and blanks (growth medium and SDW only) were subtracted from these readings.

### 2.4.6. Agar diffusion growth inhibition assays

Solid agar diffusion assays were used to assess antimicrobial activity. An overnight culture of indicator strain was grown in appropriate media and under optimal conditions (Table 2.2). Ninety-six well agar plates were prepared by combining 20 mL of molten media agar (cooled to 42 °C) with 200 μL of dense overnight culture (approx. $10^8$-$10^9$ CFU mL$^{-1}$). The seeded agar was allowed to solidify at 25 °C in a sterile Nunc OmniTray. An additional 20 mL of cooled molten media was combined with 200 μL of culture and poured over the lower solidified agar layer. A sterile 96-well PCR plate was placed in the molten agar upper layer, which was allowed to solidify at 25 °C. After sufficient solidification, the 96-well PCR plate was removed, and 15 μL of peptide solutions at a range of concentrations (10 μM, 5 μM, 1 μM, 500 nM, and 0 nM) were separately dispensed into the newly formed wells. For assays with haloduracin peptides, a 1:1 ratio of Halα:Halβ was used. Plates were left at optimal growth temperature (strain dependent) for 15 h and antibacterial activity was qualitatively determined by the presence or absence of growth inhibition.

### 2.4.7. Specific activity determination

Microtiter plates were used to determine the IC$_{50}$ values (the concentration at which 50% growth inhibition is observed) of indicator strains. For assays with haloduracin, a 1:1 ratio of Halα:Halβ was used. Serial dilutions of Halα and Halβ peptides were prepared in sterile deionized water (SDW). Ninety-six well and forty-eight well microtiter plates (Corning Costar)
were utilized for anaerobic and aerobic strains, respectively (see Materials, Cultures, and Conditions). For 96-well plates, the total volume of culture in each well was 200 μL; the experimental wells contained 50 μL of diluted Halα and Halβ peptides at defined concentrations and 150 μL of a 1-in-10 dilution (approximately 1 x 10^8 CFU mL^-1) of a culture of indicator strain diluted in fresh growth medium. In addition, each plate contained several blank (150 μL fresh growth medium and 50 μL SDW) and control wells (150 μL of untreated 1-in-10 diluted culture and 50 μL SDW). For 48-well plates, the total volume in each well was 300 μL; the experimental wells contained 75 μL of diluted Halα and Halβ peptides at defined concentrations and 225 μL of a 1-in-10 dilution (approximately 1 x 10^8 CFU mL^-1) of a culture of indicator strain diluted in fresh growth medium. In addition, each plate contained several blank (225 μL fresh growth medium and 75 μL SDW) and control wells (225 μL of untreated 1-in-10 diluted culture and 75 μL SDW). The optical density at 600 nm (OD₆₀₀) was recorded at hourly intervals from 0 to 5 h with an additional measurement at 18 h using a BioTek Synergy 2 plate reader. Plates were incubated according to optimal growth conditions (see Materials, Cultures, and Conditions). The triplicate readings were averaged, and blanks (growth medium and SDW only) were subtracted from these readings. Growth curves were developed using control (culture and SDW only) readings to ensure sufficient OD changes for accurate inhibition assessment. Curve fits for IC₅₀ determination were produced by fitting the data with Origin8 software using the dose-response curve with the equation: \( y = A_1 + (A_2 - A_1) / (1 + 10^{(\log x_0 - x)p}) \), with \( p = \) variable Hill slope. A 50% growth inhibition was determined as half the final OD₆₀₀ ± 0.05 of control culture. Nisin IC₅₀ values were determined using identical procedures as described above.
Specific activities for individual peptides were determined essentially as described above, except that peptides were evaluated independently. Specific activity was assessed against the indicator strain *L. lactis* HP and nisin standards were used as controls.

### 2.4.8. Stability tests

Stock solutions of nisin, Halα, and Halβ were prepared in 50 mM MOPS, 100 mM NaCl at pH 7.5 with final peptide concentrations of 25 μM. These stocks were divided into 1 mL aliquots in sterile microcentrifuge tubes and were incubated at 25 °C. At the times indicated, an aliquot was analyzed by reverse-phase HPLC (Beckman Coulter System Gold) and MALDI-TOF MS (Applied Biosystems Voyager DE-STR). Peptide aliquots were applied to a Phenomenex Jupiter Proteo C12 column (10 μm, 90 Å, 250 mm x 4.6 mm) equilibrated in 10% B (A: 0.1% TFA in water, B: 0.0866% TFA in 80% ACN/20% water (v/v)). Separation of intact peptide from degradation products was achieved by maintaining the mobile phase at 10% B for 5 min, followed by an increase to 100% B over 50 min with a flow rate of 1.0 mL min⁻¹. Fractions were collected and analyzed by MALDI-TOF MS as described above. Peak area corresponding to the intact peptide was determined via integration and plotted as a percentage relative to the peak area of peptide of day 0 (peak area of intact peptide day *n* / peak area of intact peptide day 0). Agar diffusion growth assays were performed against *L. lactis* HP as described above. For haloduracin samples, 7.5 μL of Halα (day *n*) was combined with 7.5 μL of Halβ (day *n*) and the total 15 μL was dispensed in the well. For nisin samples, 7.5 μL of nisin (day *n*) was combined with 7.5 μL of sterile 50 mM MOPS, 100 mM NaCl at pH 7.5 and the total 15 μL was dispensed in the well. Positive controls included 15 μL of nisin at 20 μM, 10 μM, 1 μM, and 100 nM in sterile buffer.
2.4.9. Sequential binding assays with Halα and Halβ

*Lactococcus lactis* HP cultures (in triplicate) were diluted 1-in-10 (approximately 1 x 10^8 CFU mL^-1), and 150 μL of each culture was added to 0.6 mL sterile microcentrifuge tubes which contained 50 μL of Halα or Halβ alone (at concentrations of 1 μM, 500 nM, 250 nM, 100 nM, 10 nM, or 0 nM). Tubes were incubated at 25 °C for 20 min prior to centrifugation at 13,000 x g for 1 min. The supernatants were removed from each tube and cell pellets were washed twice in fresh GM17 media. Following the second wash, cell pellets were resuspended in 150 μL of fresh GM17. Cells treated with Halα alone were added to microtiter wells, which contained 50 μL of Halβ, and cells treated with Halβ alone were added to microtiter wells that contained 50 μL of Halα (at concentrations of 1 μM, 500 nM, 250 nM, 100 nM, 10 nM, or 0 nM). As controls, Halα and Halβ were used in combination and nisin was used at 1 μM concentrations. The microtiter plates were incubated at 30 °C and the optical density at 600 nm was recorded at hourly intervals from 0 to 5 h with an additional measurement at 18 h using a BioTek Synergy 2. The triplicate readings were averaged, and blanks (150 μL of GM17 media and 50 μL SDW) were subtracted from these readings.

2.4.10. Transglycosylase assays for lipid II binding studies

The gene encoding *E. coli* PBP1b was previously PCR amplified from MG1655 genomic DNA and cloned into pET21b vector (Novagen) as a C-terminal hexa-histidine (His₆) fusion gene. The enzyme was expressed and purified by Tsung-Shing Andrew Wang or Tania Lupoli (Dan Kahne and Suzanne Walker Laboratory, Harvard University) as previously published.
[14C]GlcNAc-labeled heptaprenyl lipid II analogue was chemo-enzymatically prepared by Dr. Yuto Sumida and Dr. Hiro Tsukamoto (Dan Kahne Laboratory) as previously described.48

For IC₅₀ determinations, assays were carried out by separately incubating [14C]GlcNAc-labeled heptaprenyl lipid II analogue (final concentration = 4 μM, typical specific activity = 288 μCi/μmol) and peptide inhibitors (concentrations are indicated in the figure legends) in low-binding microcentrifuge tubes (VWR) containing 9 μL of buffer consisting of 50 mM HEPES (pH 7.5), 10 mM CaCl₂, 1,000 units/mL penicillin G, 0.2 mM octaethylene glycol monodecyl ether (decyl-PEG; Anatrace, Maumee, OH), and 11% DMSO (v/v) for 30 min at room temperature prior to PBP1b addition (to allow binding to occur). Reactions were started by adding 1 μL of PBP1b (from a solution freshly prepared by diluting the 50% glycerol stock 20-fold into PBP1b dilution buffer consisting of 5 mM Tris (pH 8.0), 8 mM decyl-PEG) to the reaction mixture (final PBP1b concentration = 20-100 nM). Reactions were typically stopped after 30-45 min by adding 10 μL of ice-cold 10 mM Tris (pH 8.0) containing 10% Triton X-100. Quenched reactions were stored on ice until they were spotted on cellulose chromatography paper strips (3MM Whatman chromatography paper #3030-861, cut 1 cm width x 20 cm height) Products and starting material were separated using chromatography (isobutyric acid/1 M NH₄OH, 5:3). The lipid II starting material migrates on the strip, while polymerized product remains at the origin. The paper strips were removed from chromatography chambers, allowed to dry, cut, and added to separate scintillation vials containing EcoLite(+) liquid scintillation fluid (MP Biomedical). Samples were analyzed using a LS6500 scintillation counter (Beckman Coulter). The percent radioactivity of the polymer product was calculated by comparing the amount of radioactivity that remains at the origin (peptidoglycan) to the total amount of radioactivity on the strip.
For kinetics assays, reactions were carried out by separately incubating various concentrations (indicated in the figure legends) of [14C]GlcNAc-labeled heptaprenyl lipid II analogue (typical specific activity = 273 cpm/pmol) and Halα inhibitor (at 0, 6, or 8 μM) in low-binding microcentrifuge tubes (VWR) containing 9 μL of buffer consisting of 50 mM HEPES (pH 7.5), 10 mM CaCl₂, 1,000 units/mL penicillin G, 0.2 mM decyl-PEG, and 11% DMSO (v/v) for 30 min at room temperature prior to PBP1b addition (to allow binding to occur). Reactions were started by adding 1 μL of PBP1b (from a solution freshly prepared by diluting the 50% glycerol stock 20-fold into PBP1b dilution buffer consisting of 5 mM Tris (pH 8.0), 8 mM decyl-PEG) to the reaction mixture (final PBP1b concentration = 20-100 nM). Reactions were typically stopped after 15 min by adding 10 μL of ice-cold 10 mM Tris (pH 8.0) containing 10% Triton X-100. Quenched reactions were stored on ice until analyzed using paper chromatography and scintillation counting as described above. Equation used for fitting: rate = \( V_{\text{max}} \times \frac{((0.5[I]-[S]+K_d)^2+4K_d[S])^{0.5}-(0.5[I]-[S]+K_d)}{2/(K_m+((0.5[I]-[S]+K_d)^2+4K_d[S])^{0.5}-(0.5[I]-[S]+K_d))/2}) \). See reference.30

2.4.11. Membrane potential assays

Membrane potential was measured using the membrane potential-sensitive dye, 3-3’ diethyloxacarbocyanine iodide (DiOC₂, Molecular Probes/Invitrogen). *B. subtilis* ATCC 6633 cultures (in triplicate) were grown to a density of 4 x 10⁶ cells mL⁻¹ and aliquots were transferred to tubes containing DiOC₂ (final concentration of 300 nM). Cells were incubated with the dye for 20 min at 30 °C under aeration conditions prior to the addition of either haloduracin or nisin at 25 μM, 2.5 μM, 250 nM, 25 nM, and 0 nM. Following addition of peptides, cultures were incubated at 30 °C with aeration for an additional 10 min prior to analysis. The membrane
potential was assessed by measuring the *B. subtilis*-associated DiOC2 fluorescence by flow cytometry (BD Biosciences LSR II flow cytometer) with excitation at 488 nm with an argon laser and measurement of emission through a band-pass filter at 530/30 nm. A minimum of 50,000 events were detected for each sample and the data was analyzed using the FCS Express 3.00.0311 V Lite Stand-alone software. The data were plotted as the geometric mean of the fluorescence intensity (MFI).

### 2.4.12. Potassium ion release assays

Potassium efflux was measured using the K⁺-sensitive fluorescent dye 1,3-benzenedicarboxylic acid, 4,4’-(1,4,10,13-tetraoxa-7,16-diazacyclooctadecane-7,16-diylibis(5-methoxy-6,2-benzofurandiyli))bis (PBFI, Molecular Probes/Invitrogen). A culture of *Micrococcus luteus* was grown to an OD₆₀₀ of 1.0 and washed five times with assay buffer (5 mM HEPES, 5 mM glucose, pH 7.2). Washed cells were resuspended in one-fifth the volume of assay buffer containing PBFI (final concentration of 10 μM) and aliquots were added to UV-transparent microtiter plates (Nunc). Sample fluorescence (excitation = 346 nm, emission = 505 nm) background data was collected (BioTek Synergy 2 plate reader, excitation filter 360/40, emission filter 508/20) until baseline signal was achieved, followed by the addition of haloduracin (Halα and Halβ in a 1:1 ratio) or nisin at 10 μM, 5 μM, 1 μM, 250 nM, and 0 nM. For pre-incubation studies of the haloduracin peptides, baseline signal was achieved followed by the addition of either Halα or Halβ to wells at the concentrations indicated and incubated for 5 min prior to the addition of Halβ or Halα, respectively.
2.4.13. Inhibition of *Bacillus anthracis* spore outgrowth

*B. anthracis* spores at a concentration of 4.0 x 10^6 spores mL^-1 were incubated in Brain Heart Infusion (BHI) medium supplemented with haloduracin (0.1, 1, 10, 100 μM at a Halα:Halβ ratio of 1:1) or 0.1 M MOPS (pH 6.8) as a control. For assessment of Halα and Halβ used independently, spores were incubated in BHI medium supplemented with the peptide at 0.1, 1, 10, 100 μM or with 0.1 M MOPS (pH 6.8) as a control. As a positive control, spores were treated with nisin under the same conditions and concentrations as used for Halα and Halβ. Cultures were incubated at 37 °C under aeration conditions and ambient CO₂ (0.03% CO₂). At 0, 5, and 10 h, samples were removed from the *B. anthracis* cultures and fixed by incubation in 3% formaldehyde (v/v) for 30 min at 37 °C. Samples were mounted on glass microscope slides in 20% glycerol (v/v). Differential interference contrast (DIC) microscopy images were collected with an Applied Precision assembled DeltaVision epifluorescence microscope equipped with an Olympus Plan Apo x100 oil objective with a numerical aperture of 1.42 and a working distance of 0.15 mm. Images were processed with SoftWoRX Explorer Suite.
2.5. REFERENCES


(10) Hyink, O.; Balakrishnan, M.; Tagg, J. R. *FEMS Microbiol. Lett.* **2005**, *252*, 235-41. "*Streptococcus rattus* strain BHT produces both a class I two-component lantibiotic and a class II bacteriocin".


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3.1. INTRODUCTION

One of the greatest sources of biologically active compounds is natural products. Often these compounds serve as platforms for the design and development of novel drugs and therapeutics. Microbes unknowingly provide scientists with numerous compounds showing promising activities and raising an increased focus on the discovery of novel natural products from these microscopic sources. However, while these compounds often display potent activity toward specific biological targets, additional pharmacological properties including solubility, stability, bioavailability, toxicity, and metabolism often necessitate improvement. Whereas small molecule pharmacophores are amenable to modifications via synthetic approaches, the structural complexity and often chemical sensitivity of natural products often precludes them from synthetic alterations required for further investigations such as structure-activity relationship studies. To overcome this barrier, a number of biotechnological and semi-synthetic approaches have been utilized in natural product research. Using these approaches, entire biosynthetic pathways or individual enzymes involved in the natural product biosynthesis are manipulated and utilized to afford diversified natural products with desired properties.

Historically, most peptide-based natural products with therapeutic properties are biosynthetically assembled via non-ribosomal peptide synthetase (NRPS) pathways.\textsuperscript{1,2} However, in recent years, it has become evident that there is a rapidly expanding class of peptide-based natural products with a wide range of biological functions that are produced by the ribosomal machinery.\textsuperscript{3-9} These types of compounds are ribosomally synthesized and post-translationally
modified to become molecules with a variety of activities including antimicrobial, antiviral, anti-inflammatory, antitumor, and antiallodynic activities. These post-translational modifications release the peptides from the structural and functional constraints imposed on natural ribosomal peptides, while at the same time restricting conformational flexibility to allow better target recognition and increase metabolic and chemical stability.

The potent activity of lantibiotics and their unique modes of action make them attractive candidates as antimicrobial therapeutics. This class of polycyclic peptide natural products exhibit activity against a variety of clinically relevant bacterial pathogens including methicillin-resistant *Staphylococcus aureus*, vancomycin-resistant *Enterococci*, *Listeria monocytogenes*, and *Clostridium difficile*. Some lantibiotics have been shown to antagonize Gram-negative gastrointestinal pathogens such as *Helicobacter pylori* and *Neisseria* strains. Most of the biological activity assays and potency evaluations have been conducted *in vitro*, and there is little information available regarding their efficacy *in vivo*. Despite the evident promise lantibiotics hold for the treatment of these types of infections in the clinic, much development and optimization is necessary for the compounds to reach their full potential.

Lantibiotics possess remarkable structural complexity (see Figure 1.4). The synthesis of lantibiotic analogues to improve the pharmacological qualities of naturally occurring lantibiotics is typically not feasible. There have been a few reports of efforts toward lantibiotic total synthesis. Although the structure of nisin was established in 1971, the first total synthesis of the peptide was finally completed over 15 years later. In this report, 5 peptide segments of nisin were first prepared via solution-phase synthesis followed by the condensation of the independently prepared segments to afford full-length nisin in less than 1% total yield. Nonetheless, this synthetic feat has inspired attempts toward lantibiotic total synthesis in the past
decade including lactocin $S^{17}$ and lacticin 3147 $\beta$ peptide.$^{18}$ In addition, reports on the synthesis of lantibiotic fragments have focused on construction of preformed thioether rings and other post-translational building blocks.$^{19-28}$

In spite of the advances in synthetic approaches, the majority of efforts for lantibiotic production have focused on \textit{in vivo} and \textit{in vitro} bioengineering. These strategies take advantage of the ability and tolerance of the biosynthetic machinery to perform their catalysis in a variety of backgrounds including within heterologous hosts and in buffered solutions. In addition, these approaches capitalize on the promiscuity of the enzymes and their acceptance of mutagenized precursor peptide substrates. The gene-encoded nature of the lantibiotic precursor offers a key advantage over synthetic manipulation strategies in that simple, non-silent changes at the nucleotide level have a direct impact on precursor sequence and ultimately, the final lantibiotic compound. Therefore, the design and construction of analogues with improved therapeutic potential or for potential use as biochemical tools is feasible through genetic strategies. Additionally, the biosynthetic enzyme catalysts themselves are genetic in origin and are amenable to engineering and evolution strategies to improve their efficiencies and range of acceptable substrates.

\textit{In vivo} bioengineering approaches and heterologous expression systems for the production of analogues have been developed for a number of lantibiotics including mersacidin,$^{29}$ subtilin,$^{30}$ Pep5,$^{31}$ and nisin,$^{32-36}$ and are discussed in detail in Chapter 1. Despite its widespread use and success, only a handful of analogues produced through \textit{in vivo} bioengineering have displayed improved biological activity (Figure 3.1). The \textit{in vitro} reconstitution of lantibiotic biosynthetic enzymes offers several key advantages over \textit{in vivo} techniques, including the capacity to prepare combinatorial libraries of mutant LanA precursor
peptides, the ability to produce bioactive peptides in a cell-free environment to avoid complications arising from producer (or host) regulation and immunity, and the additional benefit to incorporate non-proteinogenic amino acids into the lantibiotic product.

Figure 3.1. Lantibiotic analogues with improved specific activity. The dashed arrows indicate increased activity arising from the substitution of a single amino acid residue (light blue spheres), a combination of amino acid substitutions (red spheres), or non-proteinogenic amino acids (light green spheres). Adapted from Ref. 37.
To date, the activities of several lanthionine synthetase enzymes representing several lanthionine-producing enzyme classes have been reconstituted \textit{in vitro} (described in detail in Chapter 1.4.2.). This list includes enzymes of the LanM class: lacticin 481 synthetase (LctM) involved in lacticin 481 biosynthesis,\textsuperscript{38} cinnamycin synthetase (CinM) involved in the modification of cinnamycin,\textsuperscript{39} and HalM1 and HalM2 synthetases involved in the biosynthesis of haloduracin $\alpha$ and $\beta$ peptides, respectively.\textsuperscript{40} ProcM synthetase is an additional member of the LanM family and is involved in the biosynthesis of prochlorosins, a class of lantipeptides with currently undefined biological functions.\textsuperscript{41} The activities of a single member of the LanL class, VenL involved in the biosynthesis of venezuelin,\textsuperscript{42} and the LanKC class, LabKC involved in the biosynthesis of labyrinthopeptin,\textsuperscript{43} have also been successfully reconstituted \textit{in vitro}. Finally, a monofunctional LanC cyclase enzyme, NisC, involved in maturation of nisin has demonstrated activity \textit{in vitro};\textsuperscript{44} however, \textit{in vitro} reconstitution of its dehydratase partner, NisB, or any other enzymes in the LanB class, has not been successful.

Lacticin 481 is a lantibiotic produced by \textit{Lactococcus lactis} CNRZ 481 that contains three Me(Lan) rings and one Dhb residue (Figure 3.2).\textsuperscript{45} The lacticin 481 precursor, LctA, is a 51-amino acid peptide consisting of a C-terminal structural peptide that undergoes post-translational modification, as well as an N-terminal leader peptide that is required for efficient processing by LctM.\textsuperscript{46,47} LctM is a bifunctional enzyme that introduces the post-translational modifications found in lacticin 481 by catalyzing both dehydration and cyclization steps.\textsuperscript{38} LctM was the first lantibiotic synthetase to be reconstituted \textit{in vitro} and has since served as a model system for \textit{in vitro} characterization of lantibiotic biosynthesis. Although its activity was reconstituted nearly a decade ago, very little is understood regarding the biological activity and
Figure 3.2. Biosynthesis of lacticin 481, a class II lantibiotic. The lacticin 481 precursor peptide (LctA) containing an N-terminal leader and C-terminal core peptide is transformed into a polycyclic thioether product through the action of a bifunctional enzyme (LctM) that dehydrates Ser to dehydroalanine and Thr to dehydrobutyrine, and subsequently catalyzes the Michael-type addition of Cys residues to these unsaturated amino acids. The leader is proteolytically removed from the modified core peptide by a bifunctional protease/transporter enzyme (LctT). Although the process is drawn as complete dehydration before the commencement of cyclization, recent studies suggest the dehydration and cyclization events may be alternating.48,49

chemical properties of lacticin 481. In the past several years, several reports on LctM have focused on the characterization of the promiscuous substrate specificity of the enzyme towards truncated LctA mutant substrates.50-52

Only in more recent years have studies focused on engineering of the full length lacticin 481 product.53 A significant challenge for the introduction of non-proteinogenic amino acids into lacticin 481 is the preparation of full length LctA prepeptides. To overcome this obstacle, a strategy was recently devised where a triazole-linked LctA peptide analogue was prepared via Cu(I)-catalyzed 1,3-dipolar cycloaddition of an alkyne-functionalized LctA leader peptide and an
azide-modified LctA structural region.\textsuperscript{52,54} By employing this technology, triazole-linked LctA substrates were prepared that contained mutations in the structural peptide with several non-proteinogenic amino acids, including $\beta$-amino acids, D-amino acids, and N-alkyl glycine (peptoid) residues. An additional two mutations, Asn15Arg and Phe21His, were introduced to improve solubility of the final product.\textsuperscript{52,54,55} Starting with this more soluble version of the LctA precursor, a number of additional analogues were produced via an \textit{in vitro} mutagenesis strategy (Figure 3.3).\textsuperscript{54} The bioactivity of these analogues were evaluated and it was demonstrated that many of the analogues had increased bioactivity compared to the parent compound.\textsuperscript{54} These efforts represented the first preparation of lantibiotic analogues containing non-proteinogenic amino acids.

Despite the invaluable potential of this approach, several shortcomings exist. Synthesis of the precursor peptide involves multiple reaction and purification steps following completion of SPPS, limiting process throughput and overall yield. Most class II single component lantibiotic precursor peptides are hydrophobic in nature, especially the residues of the leader peptide.\textsuperscript{10} As such, solubility of the full-length lantibiotic precursor peptide whether the leader and core peptide is joined via an amide bond or a triazole moiety, is often a major issue, especially for LctA. Furthermore, the process of first appending and then removing the 23-residue leader peptide is poorly atom efficient, and challenges the scalability of the reactions. Finally, leader peptide cleavage by LysC commercial protease is very low yielding and comes with the additional expense of losing the N-terminal lysine found in the natural product, thus producing lacticin 481 $\Delta l$.\textsuperscript{53,54}
Figure 3.3. In vitro mutasynthesis of lacticin 481 analogues. Synthetic substrate analogues were prepared using copper-catalyzed [2+3] cycloaddition of the leader peptide and core peptide fragments. The resulting LctA analogue was treated with LctM, which dehydrated the underlined Ser and Thr residues and incorporated the thioether rings shown. Endoproteinase LysC was then used to remove the leader peptide, but it also removes the first residue of native lacticin 481, Lys1. Adapted from Ref. 54.

Despite the ubiquitous nature of leader sequences in directing post-translational modifications in precursor peptides, the precise role of the leader, as well as the nature of its interaction with modifying enzymes, is not well understood (see Chapter 1.2.2 for additional information). The leader peptide was previously thought to be a recognition sequence necessary for substrate processing; however, studies on LctM have demonstrated that the core peptide is modified (albeit only one of four expected dehydrations) when the leader is absent. Interestingly, providing the leader peptide in trans allowed for further modification of the core peptide. 
peptide substrate in a distributive and directional fashion including dehydration and cyclization, though the extent of processing was still lower than the naturally occurring reactions with full-length LctA substrate.\textsuperscript{47} Prolonged incubation times or higher concentrations of LctM did not afford fully-modified product. These data fit a previously proposed model in which an equilibrium population of LctM containing mostly inactive species in the absence of leader binding is shifted towards a population consisting more of an active conformation when the leader peptide binds (Figure 3.4).\textsuperscript{46}

\textbf{Figure 3.4.} The proposed role of the leader peptide in LctM activity based on several studies involving lacticin 481 biosynthesis. It is proposed that the leader peptide shifts the equilibrium between inactive and active enzyme towards the active enzyme species. Adapted from Ref.\textsuperscript{46}. 
This chapter is focused on the design, development, and implementation of a novel strategy for the production of lantibiotics and lantibiotic analogues. This strategy capitalizes on the substrate promiscuity of the LanM synthetases enzyme, LctM, and its ability to catalyze dehydration and cyclization events \textit{in vitro}. By the generation of a “constitutively active” enzyme, a number of analogues of lacticin 481 were prepared and the mutations to this single component lantibiotic were evaluated in live cell and \textit{in vitro} assays. This novel fusion enzyme displays overall better enzymatic properties with core peptide alone than wild-type LctM when provided with leader peptide and core peptide \textit{in trans}. Single amino acid mutations were identified that resulted in improved bioactivity and inhibition of peptidoglycan biogenesis. In addition, the introduction of a single point mutation in the constitutively active LctM enzyme resulted in generation of a kinase-like enzyme with the potential to phosphorylate biologically relevant peptides at select serine or threonine residues, thus demonstrating potential for use in the preparation of a phosphopeptide library.

3.2 RESULTS AND DISCUSSION

3.2.1 Design and development of constitutively-active fusion enzymes

The unanticipated ability of LctM to partially process the lacticin 481 core peptide in the absence of the leader peptide prompted additional studies of the unique role of the leader peptide during lantibiotic biosynthesis. The enhanced processing of the core peptide facilitated by the addition of leader peptide \textit{in trans} suggested that the presence of the leader peptide conduces a shift in the equilibrium population from inactive to active enzyme species, thus leading to further, albeit incomplete, processing of the core peptide. An \textit{in trans} approach for \textit{in vitro} production of lantibiotics is advantageous from multiple aspects. As previously demonstrated,
non-proteinogenic amino acids could be incorporated into the core peptide for use in the production of unnatural amino-acid containing lantibiotic analogues. However, these studies required a triazole linkage to physically join the core and leader peptide together, whereas the \textit{in trans} approach renders the challenging click chemistry unnecessary. An additional key benefit using the \textit{in trans} strategy is that leader peptide removal via proteolysis would be avoided; thereby reducing the overall steps reported in traditional methods of \textit{in vitro} lantibiotic production. Unfortunately, LctM was only capable of dehydrating the core peptide at 3 of 4 possible sites under \textit{in trans} reaction conditions and was not able to fully process the core peptide to generate bioactive material. Attempts at reaction optimization including longer incubation times or higher concentrations of LctM did not result in fully-processed, bioactive product.

The importance of the leader peptide for promoting efficient core peptide processing demonstrates its essential role as a vital element in the \textit{in trans} synthetase reaction. As such, a novel strategy was developed to provide the leader peptide to the reaction without having to synthetically prepare and exogenously introduce it to the reaction. Rather than chemical synthesis and exogenous supplementation, the leader peptide would be provided to the reaction through genetic manipulation of the synthetase enzyme. The LctA leader peptide would be ribosomally synthesized as a fusion with the LctM synthetase enzyme joined together by a flexible linker. This effort to develop a constitutively active synthetase enzyme would take advantage of the demonstrated effects of the leader peptide on shifting the equilibrium population toward greater concentrations of active synthetase species. This approach would
enable the leader peptide to be provided in a “one-to-one” stoichiometry with the synthetase and promote a high local concentration of the leader peptide due to its physical connection with the enzyme. Additionally, this tethered approach would greatly simplify the components of the reaction as the leader peptide would not have to be exogenously added to or purified from the reaction mixture.

It has been demonstrated in previous studies that modification of a lantibiotic precursor by its LanM synthetase enzyme is possible regardless of the presence of additional amino acid sequences at the N-termini of both the precursor substrate and the enzyme. These sequences include many peptides, such as affinity tags utilized for purification, and it has been demonstrated that these terminal appendages are generally well-tolerated and do not adversely affect catalytic activity in either *in vivo* and *in vitro* backgrounds.\textsuperscript{38-42,53,56} Taking these results into consideration, a constitutively-active fusion enzyme, herein referred to as “ConFusion enzyme”, was designed with the LctA leader peptide fused at the N-terminal end of the LctM synthetase enzyme, joined by a small, flexible proteogenic linker consisting of glycine-serine repeating units (Figure 3.5a-b). The gene sequence encoding for the ConFusion enzyme was designed and constructed from naturally occurring nucleotide sequences (LctA leader and LctM synthetase portions) as well as codon-optimized, synthetic sequences (linker portion). The newly constructed hybrid gene, *lctCE-GSG*, was subcloned into pET28b vector (Figure 3.5c). With anticipation that the length of the linker between leader peptide and the synthetase enzyme would have an effect on the constitutively active properties of the designed enzyme, the gene encoding this fusion was constructed with unique restriction sites that would allow for the excision of the sequence encoding for the linker and subsequent replacement with longer encoding sequences to produce progressively longer glycine-serine repeat linkers (Figure 3.5c).
The *lctCE-GSG* gene, encoding for His$_6$-LctCE-GSG was overexpressed in *E. coli* and purified via IMAC affinity chromatography. Synthetically prepared LctA(1-27) N15R F21H core peptide (prepared by Patrick Knerr) was incubated with purified His$_6$-LctCE-GSG in the presence of Mg$^{2+}$, ATP, and BSA in a Tris buffer (pH 7.5) and modification of the core peptide was observed (Figure 3.6). A fully modified product with four dehydrations was observed as a minor product. As observed with the previously published *in trans* assays, intermediates that underwent 1, 2, and 3 dehydrations were also present in the mixture, providing further evidence of a distributive mechanism by which lacticin 481 synthetase processes its substrate. The major product as determined by mass spectrometry was an intermediate with 3 dehydrations and one phosphorylation. Importantly, the mixture of material contained fully-modified −4 H$_2$O product that demonstrated inhibitory activity against *Lactococcus lactis* HP indicator strain (Figure 3.6b).
Figure 3.5. The design and construction of a constitutively active lacticin 481 synthetase enzyme, “LctCE-GSₙ”. (a) Current model by which the modification enzyme LctM interacts with the full-length LctA precursor. It is proposed that the leader peptide shifts the equilibrium between inactive and active enzyme toward the active enzyme species thereby facilitating the post-translational modification of LctA core peptide. (b) The proposed model of a constitutively active lacticin 481 synthetase enzyme. LctA leader peptide is physically fused to the synthetase via a flexible proteinogenic linker. As with the model in panel a, it is proposed that the leader peptide shifts the equilibrium population toward the active enzyme species thereby enabling the modification of LctA core peptide without the need of leader peptide and core peptide being physically attached. (c) The genetic strategy for increasing the linker length between leader peptide and synthetase enzyme.
3.2.2 Effects of linker length on substrate processing

In an effort to create a better and more active lacticin 481 ConFusion enzyme, the linker region between the two components was expanded and the resulting enzymes were evaluated. Expansion of the linker was made possible via restriction endonuclease excision of the nucleotide sequence encoding for the GSG linker followed by ligation of a synthetically prepared double-stranded DNA (dsDNA) insert. The dsDNA insert consisted of oligonucleotides designed to self-hybridize with “preformed” overhangs for ligation at the vector restriction sites between the sequences encoding for the leader peptide and the synthetase (Figure 3.5c). The newly formed and expanded fusion genes were independently expressed in E. coli, purified, and utilized for modification of LctA (1-27) core peptide using the previously described assay conditions.
The products of the reactions and the relative activity of the enzymes were analyzed by MALDI-TOF mass spectrometry (Figure 3.7). An enzyme with a linker encoding for 30 amino acids was able to convert the material to include a $-4 \text{H}_2\text{O}$ product as the dominant peak with a low amount of intermediates. As the linker length was decreased, so was the extent of processing of the core peptide. With enzymes possessing shorter linkers, a lower amount of $-4 \text{H}_2\text{O}$ product was observed and products of intermediate processing were present. Collectively, the results demonstrate that linker length has a positive correlation with the extent of processing. That is, as the linker length was extended, the enzyme was able to more completely modify the core peptide.

![Figure 3.7](image)

**Figure 3.7.** Effects of linker length extension on the *in vitro* processing of LctA(1-27) N15R F21H core peptide by a series of lacticin 481 ConFusion enzymes. Shown are MALDI-TOF mass spectra of LctA(1-27) N15R F21H core following the termination of *in vitro* reactions after 1 h. Indicated to the right of the spectra is the enzyme utilized for the reaction. The final concentrations of substrate and enzyme was 20 µM and 2 µM, respectively. Numbers above the peaks indicate the number of dehydrations. *Asterisk indicates presence of phosphorylation.
In each reaction, a $-3 \text{ H}_2\text{O} + \text{PO}_3^{2-}$ product was detected as a highly abundant intermediate species. It is not known why this intermediate is present in such high amounts, but it may reflect an overall low affinity of LctCE-GS30 for the nearly fully modified core peptide, thus leading to an incompletely modified product. It is evident in an LctCE-GS30 time course assay monitored by mass spectrometry, that the initial dehydration events occur more rapidly compared to the final event (Figure 3.8). It has been previously demonstrated that HalM2 synthetase also displays an overall faster rate of reaction for early dehydration events and a slower rate as the modification of its substrate approaches completeness.\textsuperscript{48} It is necessary to point out that this apparent change in the rate of processing was observed even in a case with the use of full length substrate (leader attached to core). The C-terminal end of lacticin 481 core peptide is remarkably complex, with several overlapping thioether rings. The final dehydration of lacticin 481 core peptide is Thr24 which is adjacent to Cys25 and Cys26—two residues involved in the overlapping B and C-rings.\textsuperscript{48} As observed with HalM2 synthetase, it is likely that dehydration and cyclization events catalyzed by LctCE-GS30 also occur at the same time. In the ConFusion reaction, there is the additional hurdle of a leader-less substrate. It is has been proposed that the physical attachment of the leader peptide and core peptide is important for anchoring the core peptide near the synthetase active site during the modification process. Therefore, it is not surprising that the ConFusion enzyme system, with a leader-less substrate may have a challenged ability to catalyze the final dehydration event. Importantly, the unsaturated amino acid arising from Thr24 is not involved in a thioether ring—a modification that is generally essential for effective antimicrobial activity.\textsuperscript{8,11,57,58}
Figure 3.8. Time course assay of LctA(1-27) N15R F21H core peptide with LctCE-GS30. Shown are MALDI-TOF mass spectra of LctA(1-27) N15R F21H core following the termination of *in vitro* reactions at the indicated time points. The final concentrations of substrate and enzyme was 20 µM and 2 µM, respectively. Numbers above the peaks indicate the number of dehydrations. *Asterisk indicates presence of phosphorylation.

3.2.3 Comparison of LctCE-GS30:core and LctM:leader:core (*in trans*) activities

The relative abilities of LctCE-GS30 and LctM with leader provided *in trans* to catalyze the modification of LctA core peptide were evaluated using a quantitative HPLC assay. For this evaluation, the synthetases were first subjected to IMAC separation followed by gel filtration to yield highly uniform and purified enzymes (Figure 3.17). His$_6$-LctCE-GS30 and His$_6$-LctM were then utilized in the modification assays at a 10:1 substrate:enzyme ratio (final concentrations 20 µM substrate and 2 µM enzyme). In the LctM reaction, the LctA leader peptide was supplemented at the same concentration as synthetase enzyme, thus mimicking the ConFusion enzyme where the leader and synthetase enzyme by design are present in stoichiometric equivalents. Under these conditions, there was no fully modified lacticin core peptide observed
in the LctM-catalyzed \textit{in trans} reaction. Rather, products with $-2$ and $-3$ dehydrations were observed (Figure 3.9a). However, in the ConFusion enzyme reaction containing LctCE-GS30 synthetase and core peptide, fully modified material was observed in as little as 30 min with the $-4 \text{ H}_2\text{O}$ material as the major product within 180 min (Figure 3.9b). It should be noted that the disconnect between the extent of modification as determined by MALDI-TOF MS versus HPLC analysis is explained by ionization bias. In general, when using MALDI-TOF MS-based evaluation, modified precursor peptides tend to ionize better and falsely appear to be in higher abundance compared to linear peptide molecules. Similarly, phosphorylated intermediates tend to ionize better than non-phosphorylated intermediates. This bias is not observed with HPLC analysis where a more accurate representation of fully-modified, partially-modified, and starting material populations is achieved.
Figure 3.9. Comparison of the activities of LctM versus LctCE-GS30 by a quantitative HPLC time course assay. LctA(-1 - -24) leader peptide was provided *in trans* with LctA(1-27) N15R F21H core peptide for the LctM assay. LctA(1-27) N15R F21H core peptide was provided alone for the assay with LctCE-GS30. Shown are the retention time windows corresponding to the analytes detected and identified via mass spectrometry. Numbers above the peaks indicate the number of dehydrations. *Asterisk indicates presence of phosphorylation.
3.2.4 Preparation of Lacticin 481 analogues

The ultimate goal of *in vitro* and *in vivo* reconstitution of lantibiotic biosynthesis is the production and identification of lantibiotic analogues with improved pharmacological properties. In a previous report, lacticin 481 wt produced by *L. lactis* CRNZ 481 was isolated and was demonstrated to possess high nanomolar inhibitory activity (IC$_{50}$ = 750 nM) against *L. lactis* HP.$^{54}$ This level of activity is modest compared to nisin,$^{59}$ haloduracin,$^{11}$ microbisporicin,$^{9,60}$ and lacticin 3147$^{61}$ which all possess MICs in the low-nanomolar to high-picomolar range. As such, lacticin 481 serves as an ideal candidate for optimization and further improvement. Previous reports have demonstrated that lacticin 481 Δ1 (lacticin 481 lacking N-terminal Lys)$^{38}$ and several analogues$^{53,54}$ could be prepared via mutagenesis approaches to include proteinogenic and non-proteinogenic amino acid substitutions. Although analogues with improved bioactivity were identified, none of these analogues displayed greater potency in bacteria growth inhibition assays compared to *in vivo* isolated lacticin 481, thus suggesting the importance of the N-terminal Lys residue.$^{54}$ It is noteworthy that it has been previously demonstrated that reducing the overall cationic charge results in diminished activities of lantibiotics and other cationic peptides.$^{29,33,53,62-72}$

The newly developed and optimized LctCE-GS30 ConFusion enzyme can enable for the first time, construction of full-length lacticin 481 analogues. Taking the results described previously,$^{54}$ a number of full-length lacticin 481 analogues were prepared including N15R F21H, N15R F21Pal, N15R W19Nal F21H, and N15R W19Nal F21H (Pal = pyridynyl alanine, Nal = naphthyl alanine, Figure 3.10). The LctA (1-27) core peptide analogues were synthetically prepared by Patrick Knerr and the precursors were independently modified using purified His$_6$-LctCE-GS30 enzyme in the presence of Mg$^{2+}$, ATP, and BSA in Tris buffer (pH 7.5). Fully-
modified product with 4 dehydration and 3 cyclization events was observed in each reaction. Partially-processed intermediates were readily separated from the desired product via reversed-phase HPLC (Figure 3.11). Authentic lacticin 481 (produced by *L. lactis* CNRZ 481) was evaluated along with the above analogues (*vide infra*) and was previously isolated from culture and purified by Juan Velasquez.

**Figure 3.10.** Strategy for the production of lacticin 481 analogues containing proteinogenic and non-proteinogenic substitutions. Indicated at the top are primary sequences of the core peptides and chemical structures surrounding the lantibiotic indicate the type and location of amino acid substitutions.
3.2.5 Evaluation of \textit{in vivo} and \textit{in vitro} activities of lacticin 481 analogues

The biological activities of fully-modified, full-length lacticin 481 analogues were evaluated using cell-based and \textit{in vitro} inhibition assays. The inhibitory activity of each analogue was assessed against \textit{L. lactis} HP indicator strain (Figure 3.12). The IC$_{50}$ for lacticin 481 was 785 nM, and aligns well with the previously reported IC$_{50}$ (750 nM).$^{54}$ Excitingly, two lacticin analogues, lacticin 481 N15R F21Pal and lacticin 481 N15R F21H, displayed greater inhibitory activity compared to \textit{in vivo} lacticin 481 with IC$_{50}$ values of 213 and 428 nM, respectively. The triply substituted analogues were not as active as \textit{in vivo} lacticin 481 under the conditions tested.
Figure 3.12. Cell growth inhibition curves for lacticin 481 wild-type and analogues tested against *L. lactis* HP. Inset table indicates the calculated IC$_{50}$ value (defined as the concentration of peptide required to inhibit 50% of cell growth) and the MIC (minimal inhibitory concentration) for all peptides tested.

An *in vitro* transglycosylase inhibition assay was used to further characterize the impact of the amino acid substitutions on biological function as described in Chapter 2 for haloduracin. As observed with the cell growth inhibition assays against *L. lactis* HP, the same two analogues (N15R F21Pal and N15R F21H) displayed an enhanced ability to inhibit the transglycosylase reaction. A summary of the results is displayed in Figure 3.13. Overall, similar trends of efficacy were observed in both the assay against whole bacterial cells and in the transglycosylase *in vitro* assay. The combined results and the correlations shared between the two types of assays described above highly suggest that inhibition of peptidoglycan biosynthesis is the primary pathway by which lacticin 481 exerts its antimicrobial activity, and that improvement of the antimicrobial activity is correlated with increased binding affinity with lipid II.
Previous studies have demonstrated that all three thioether rings are essential for antimicrobial activity of lacticin 481; though the precise partner composition of the conformationally-stabilizing bridges is not critical (i.e. Lan could be substituted for MeLan and vice versa).\textsuperscript{53} It is currently not known at the molecular level which thioether rings and the residues within those rings are important for lacticin 481 and lipid II interactions. The studies described in this chapter have focused rather on substitution of amino acids at specific sites that do not affect the overall topology of the overlapping rings. The introduction of an additional positive charge into the lacticin 481 compound by the Asn to Arg substitution at position 15 resulted in greater inhibitory activity against whole cells and in the transglycosylase assay. This result was not unanticipated provided the influence of positive charges on the activity of lantibiotics\textsuperscript{62} and other cationic peptides.\textsuperscript{65} In addition, the substitution of the aromatic Phe residue at position 21 for amino acids with basic aromatic side-chains (His or Pal) resulted in analogues with further enhancement in specific activity, suggesting that electrostatic interactions at this site may play a key role in the ability of these analogues to effectively bind their target. Furthermore, substitution of the non-basic aromatic Trp at position 19 with an amino acid bearing non-heterocyclic aromatic side-chain (Nal) adversely impacted the specific activity of the compound. When both non-proteinogenic amino acids Nal and Pal were incorporated at positions 19 and 21, respectively, an analogue was produced with the weakest inhibitory properties. Further experiments including analyses to investigate precise molecular interactions (i.e. NMR or crystallography) will be required to further distinguish all contributing factors of the bioactivity of lacticin 481.
Figure 3.13. Inhibition curves for lacticin 481 wild-type and analogues tested in an \textit{in vitro} transglycosylase inhibition assay. Inset table indicates the calculated IC$_{50}$ value (defined as the concentration of peptide required to inhibit 50\% of peptidoglycan formation (PG). Lipid II concentration was 4 \mu M.

3.2.6 Mutagenesis to produce mutant kinase-like enzymes

The promiscuous activities of several lantibiotic synthetases have been exploited for the production of mutants with correct dehydration and cyclization along the modified core peptide. Recently, key active site residues of LctM shown to be responsible for elimination of the phosphorylated intermediate during the dehydration process were identified in an enzyme mutagenesis study.\textsuperscript{55} It was demonstrated that mutations at either positions Arg399 or Thr405 led to the production of a mutant synthetase enzyme with phosphorylation capability, but little subsequent elimination activity. Peptides attached to the C-terminus of the LctA leader peptide were utilized to probe the phosphorylation substrate scope of the T405A kinase-like enzyme and
it was demonstrated that LctM T405A possesses a remarkable ability to phosphorylate Ser residues in many diverse substrates.\textsuperscript{73} However, just as in previous studies involving LctM-catalyzed modifications, substrates required the physical attachment to the LctA leader peptide, which must be subsequently cleaved to yield a useful phosphopeptide product.

The T405A and R399M mutations were independently introduced into the LctCE-GS30 enzyme and the resulting enzymes were evaluated for their phosphorylation activity. Incubation of either enzyme with LctA (1-27) N15R F21H core peptide in the presence of Mg\textsuperscript{2+}, ATP, and BSA in Tris buffer (pH 7.5) resulted in a multiply phosphorylated product (Figure 3.14). Up to 3 phosphorylations were observed, and the major product was that which was doubly phosphorylated. The promiscuous nature of LctCE ConFusion synthetase in combination with the additional benefit of not requiring the physical attachment to the leader peptide prompted further investigations to explore the utility of the enzyme for the production of a phosphopeptide library.

### 3.2.7 Efforts toward creating phosphopeptides

Diverse peptide substrates were designed based on biologically relevant and commercially available phosphopeptides that have been previously utilized in studies focused on kinase/phosphatase signaling pathways or that have been shown to be substrates for phosphorylation by LctM T405A when attached to the C-terminal end of the LctA leader peptide (Table 3.1).\textsuperscript{73} The substrates were prepared by Patrick Knerr via SPPS methodology and the ability of LctCE-GS30 T405A to phosphorylate the substrates was assessed by subjecting the substrates to the reaction conditions described above and monitoring the reactions by MALDI-
Figure 3.14. Phosphorylation of LctA(1-27) N15R F21H by mutated ConFusion enzyme. Shown are MALDI-TOF mass spectra before (black) and after (red) treatment with LctCE-GS30 T405A enzyme in the presence of Mg$^{2+}$ and ATP.

TOF MS. Unfortunately, only three of ten substrates tested were phosphorylated by the mutant LctCE-GS30 enzyme (Table 3.1). The best substrate among the ten selected was the AKT/PKB/RAC peptide, which was phosphorylated at a single site (Figure 3.15). Although the promiscuity of the LctCE-GS30 T405A or R399M enzymes was not at the level of the LctM T405A or R399M counterpart, the kinase-like ConFusion enzyme methodology does have the key benefit of not needing leader peptide attachment or genetic manipulation to fuse the substrate of interest to the C-terminal end of the LctA leader peptide.\textsuperscript{73} Moreover, the phosphorylation reaction with mutant LctCE-GS30 T405A has not been optimized and may be better tuned to promote greater extent of phosphorylation and enable a broader peptide substrate scope.
Table 3.1. Ten potential substrates for phosphorylation by LctCE-GS30 T405A were prepared and tested. The primary peptide sequence for each substrate is shown. Sites of potential phosphorylation are bolded and underlined.

The ability to introduce and remove protein phosphorylations is a powerful means to investigate and control signaling events and cascades in many organisms and model systems. The unique kinase-like activity of LctCE-GS30 T405A and the elimination activity of wild-type LctCE-GS30 open the door of opportunity to control the presence and absence of protein phosphorylation in model systems. The non-phosphorylated peptide “ARKRERTYSFGHHA” is a substrate for AKT/PKB/RAC-protein kinases which have been identified as proto-oncogenes. Previous studies have revealed that AKT regulates a number of cellular functions including cell survival, cell growth, cell differentiation, and cell cycle progression.⁷⁴
Figure 3.15. Phosphorylation of the AKT/PKB/RAC peptide by LctCE-GS30 T405A enzyme. Shown are MALDI-TOF mass spectra before (black) and after (red) completion of reaction. An 80 Da mass increase indicates a single phosphorylation of the peptide.

Through the use of LctCE-GS30 wild-type, the phosphorylation of the AKT/PKB/RAC peptide was rapidly removed via the elimination activity of the enzyme, thereby generating the dehydrated fragment of AKT/PKB/RAC (Figure 3.16). Thus a strategy to phosphorylate and eliminate peptides without the need to append a leader sequence or supply one exogenously has been demonstrated. A key consideration and limitation of this technique is the concomitant dehydration that occurs with elimination of the phosphate group which may introduce a non-natural, electrophilic amino acid at a critical regulatory site. On the other hand, the electrophilicity of the dehydro amino acid thus generated may provide a handle to localize phosphorylations as demonstrated previously.80,81
Figure 3.16. Elimination of the phosphate group of phosphorylated AKT/PKB/RAC peptide by LctCE-GS30 wild-type enzyme. Shown are MALDI-TOF mass spectra before (black) and after (red) completion of reaction. An 98 Da mass decrease indicates elimination of phosphorylation and concomitant dehydration of the peptide.

3.3. SUMMARY

Two key limitations of traditional \textit{in vitro} biosynthesis of lantibiotics are the difficulty to incorporate non-proteinogenic amino acids and the challenges associated with leader peptide removal during the final steps of lantibiotic maturation. The latter limitation has been historically addressed by two methods. The first involves mutagenesis of the precursor peptide to include commercial protease sites, which can have deleterious implications including reduced solubility and heterologous expression levels, diminished precursor processing, and non-specific proteolytic cleavage after modification of the core peptide. Alternatively, the precursor leader-core peptide junction can be left unaltered, but a commercial protease must be selected to remove as much leader peptide as possible without excision of core peptide residues. Unfortunately, as
demonstrated in the case previously described, LysC protease removes a critical N-terminal Lys residue during this final step in lacticin 481 preparation.

Taking the current model of LctA leader peptide and LctM synthetase interactions, a novel strategy was developed that utilized a hybrid protein consisting of the LctA leader peptide fused to LctM synthetase via a flexible, proteinogenic linker. The constitutively active properties of this enzyme were demonstrated and its ability to catalyze full modification of lacticin 481 core peptide was shown. In addition, this novel enzyme was able to process the core peptide faster and further to completion compared to an in trans reaction utilizing LctM and LctA leader peptide in a one-to-one ratio. A number of analogues of lacticin 481 were prepared and the mutations were evaluated for antimicrobial activity against L. lactis HP cells and for their ability to inhibit transglycosylation in a lipid II polymerization assay. Two analogues displayed improved activities over authentic lacticin 481 isolated from the producing organisms. In addition, the introduction of a single point mutation in the constitutively active LctM enzyme resulted in the generation of a kinase-like enzyme with modest ability to phosphorylate biologically relevant peptides. To date, a ConFusion enzyme with a linker length of 30 amino acids has demonstrated remarkable ability to modify synthetic core peptides. Further optimization of the enzyme to include longer linker lengths as well as optimized positioning (i.e. N-terminal versus C-terminal) and fine-tuning of reaction conditions open opportunities for further development in this area of protein engineering.
3.4. EXPERIMENTAL

3.4.1. Materials, strains and plasmids, and general methods

Materials.

All oligonucleotides were purchased from Integrated DNA Technologies. Restriction endonucleases, DNA polymerases, and T4 DNA ligase were purchased from New England Biolabs or Invitrogen. Media for bacterial culture and chemicals were purchased from Fisher Scientific, Sigma Aldrich, or CalBiochem, unless noted otherwise, and used without further purification.

Strains and Plasmids.

The indicator strain, *Lactococcus lactis* HP ATCC 11602 was obtained from American Type Culture Collection, Manassas, VA. *Escherichia coli* DH5α (UIUC Cell Media Facility) was used as host for cloning and plasmid propagation, and *E. coli* Rosetta 2 (DE3) (Novagen) was used as a host for protein expression. Cloning vectors were obtained from Novagen.

General Methods.

All molecular biology manipulations were carried out using standard techniques. Polymerase chain reaction (PCR) amplifications were carried out using an automated thermocycler (C1000, BioRAD). DNA sequencing was performed using appropriate primers by the Biotechnology Center (University of Illinois at Urbana-Champaign, UIUC). GC-MS analysis was performed at the Roy J. Carver Metabolomics Center (UIUC). LC-ESI-Q/TOF MS analyses were conducted using a Synapt MS system equipped with an Acquity UPLC (Waters). MALDI-TOF MS analyses were conducted at the Mass Spectrometry Facility (UIUC) using a Voyager
DE-STR (Applied Biosystems). For MALDI-TOF MS analysis of salt-free samples, a 1 μL aliquot of analyte was combined with 1 μL of matrix (saturated α-cyano-4-hydroxy-cinnamic acid matrix in 50% ACN/50% water with 0.1% TFA), and the total volume was spotted onto a MALDI target and dried under ambient conditions prior to analysis. Salt containing samples were desalted using ZipTipC18 (Millipore), eluted with 4 μL of matrix, and 2 μL was spotted onto a MALDI target and dried as described above.

3.4.2. Construction of LctCE-GSG expression plasmid

The plasmids pET15b LctA and the pET28b LctM were isolated from E. coli DH5α cells previously transformed with the constructs. The partial gene encoding for LctA leader, GSG linker, and the 5'-end of LctM was prepared via overlap extension PCR (Figure 3.5). The forward megaprimer (FMP) was PCR amplified by 30 cycles of denaturing (94 °C for 1 min), annealing (50 °C for 1 min), and extending (72 °C for 1 min) using His6 NheI Ldr FP (5'-CGCGGCAGCGCTAGCAAAGAAAACAAAC -3’ and LctCE-GSG RP: 5'-TTGGTAAGTCTTTTTTTTATCTGCAGACCAGAACCACCCTAGTTGCACCTAAAATAAGGTCCAA -3’ as primers and pET15b LctA as template (bold indicates NheI restriction site, underlined indicates SpeI and PstI restriction sites). The reverse megaprimer (RMP) was PCR amplified by 30 cycles of denaturing (94 °C for 1 min), annealing (50 °C for 1 min), and extending (72 °C for 1 min) using LctCE-GSG FP: 5'-TTGGACCTTATTTTAGGTGCAACTAGTGTTCTGGTCTGCAGATGAAAAAAAAGACGT TACCAA -3’ and LctM HindIII RP: 5’- ATCTTTTATTTTCTAAAAGCTTTTACCTGTACTCT -3’ and pET28b LctM as template (bold indicates HindIII restriction site, underlined indicates SpeI and PstI restriction sites). The PCR mixtures included 1X PCR Buffer (Invitrogen), dNTPs
(0.25 mM each), MgCl₂ (1.5 mM), Platinum Taq DNA polymerase (0.05 U/μL), and primers (0.5 μM each). Amplifications were confirmed by 2% agarose gel electrophoresis, and the PCR products were purified using a QIAQuick PCR Purification Kit (QIAGEN). The overlapping products from the FMP and RMP reactions were combined in equal amounts and extended by seven cycles of denaturing, annealing and extending using the same PCR conditions. Following the extension, the His₆ NheI Ldr FP and LctM HindIII RP primers were added (final concentration 2 μM) and the mixture was subjected to another 25 cycles of denaturing, annealing and extending. Amplification of the final PCR product was confirmed and purified by 2% agarose gel electrophoresis. The resulting partial gene DNA insert and the original pET28b LctM vector were digested with NheI and HindIII at 37 °C for 5 h. The digested insert and vector products were purified by 2% and 1% agarose gel electrophoresis, respectively, and gel extracted using a QIAquick Gel Extraction Kit (QIAGEN). The resulting DNA products were ligated at 25 °C for 3 h in 1X T4 DNA Ligase buffer with T4 DNA Ligase (0.7 U/μL). E. coli DH5α cells were transformed with 2.5 μL of the ligation product by heat shock, and cells were plated on LB-kanamycin agar plates and grown for 15 h at 37 °C. Several colonies were picked and used to inoculate separate 5 mL cultures of LB-kanamycin medium. The cultures were grown at 37 °C for 12 h, and plasmids were isolated using a QIAprep Spin Miniprep Kit (QIAGEN). The sequences of the resulting plasmid products were confirmed by DNA sequencing.

3.4.3. Expression and purification of His₆-LctCE-GSG

E. coli Rosetta 2 (DE3) cells were transformed with the pET28b LctCE-GSG construct via electroporation. A single colony transformant was used to inoculate a 30 mL culture of LB supplemented with 50 μg/mL kanamycin. The culture was grown at 37 °C for 12 h and was used to inoculate 3 L of LB containing 50 μg/mL kanamycin, and cells were grown at 37 °C to OD₆₀₀
≈ 0.6. The culture was incubated at 4 °C on ice for 20 min, then IPTG was added to a final concentration of 0.5 mM and the culture was incubated at 18 °C for an additional 16-20 h. Cells were harvested by centrifugation at 12,000 ×g for 15 min at 4 °C, and the pellet was resuspended in 30 mL of start buffer (20 mM Tris (pH 7.6), 500 mM NaCl, 10% glycerol) and stored at −80 °C.

All protein purification steps were performed at 4 °C. The cell paste was suspended in start buffer and the cells were lysed using a high pressure homogenizer (Avestin, Inc.). Cell debris was pelleted via centrifugation at 23,700 ×g for 20 min at 4 °C. The supernatant was injected via a superloop onto a fast protein liquid chromatography (FPLC) system (ÄKTA, GE Heathcare Life Sciences) equipped with a 5 mL HisTrap HP IMAC column previously charged with Ni²⁺ and equilibrated in start buffer. The column was washed with 50 mL of buffer A (30 mM imidazole, 20 mM Tris (pH 7.6), 500 mM NaCl, 10% glycerol) and the protein was eluted using a linear gradient of 0-100% B (buffer B = 200 mM imidazole, 20 mM Tris (pH 7.6), 500 mM NaCl, 10% glycerol) over 40 min at a 2 mL/min flow rate. UV absorbance (280 nm) was monitored and fractions were collected and analyzed by SDS-PAGE (4-20% Tris-glycine READY gel, BioRAD). The fractions containing LctCE-GSG were combined and concentrated using an Amicon Ultra-15 Centrifugal Filter Unit (30 kDa MWCO, Millipore). Gel filtration chromatography was used to further purify the enzyme. The concentrated protein sample was injected onto an FPLC system (ÄKTA) equipped with an XK16 16/60 (GE Healthcare Life Sciences) column packed with SuperDex 75 resin previously equilibrated in 20 mM HEPES (pH 8.0) and 100 mM KCl. The protein was eluted with a flow rate of 0.9 mL/min. Both UV absorbance (280 nm) and conductance were monitored and fractions were collected. Misfolded/aggregated protein was efficiently separated from soluble, correctly folded protein.
and the desired fractions were combined and concentrated using an Amicon Ultra-15 Centrifugal Filter Unit. The resulting protein sample was stored at −80 °C. Protein concentration was determined using a Bradford Assay Kit (Pierce) and typical yields were 20-30 mg His<sub>6</sub>-LctCE-GSG from 3 L of cell culture.

### 3.4.4. In vitro reconstitution of ConFusion enzymes

LctA(1-27) N15R F21H core peptide (and analogues thereof) were prepared via solid-phase peptide synthesis and purified to homogeneity by Patrick Knerr. Core peptide (final concentration 20 µM) was incubated with purified His<sub>6</sub>-LctCE-GSG (final concentration 2 µM) in the presence of 10 mM MgCl<sub>2</sub>, 2 mM ATP, and 25 µg/mL BSA in a 50 mM Tris buffer (pH 7.5) and was incubated at 25 °C for 3.5 h. At 3.5 h, reactions were quenched by the addition of TFA to 0.5% final concentration (pH 1-2) and the reactions were desalted by ZipTip<sub>C18</sub> and analyzed by MALDI-TOF MS.

### 3.4.5. Antimicrobial activity assays of in vitro prepared lacticin 481 and analogues

A non-acidified 50 µL in vitro modification reaction was concentrated to 20 µL via vacuum centrifugation. An overnight culture of <i>L. lactis</i> HP ATCC 11602 (indicator strain) was grown in GM17 media with 0.5% glucose (v/v) under anaerobic conditions at 30 °C for 15 h. Ninety-six well agar plates were prepared by combining 20 mL of molten GM17 with 0.5% glucose (v/v) medium agar (cooled to 42 °C) with 50 µL of dense overnight culture (approx 10<sup>8</sup>-10<sup>9</sup> CFU/mL). The seeded agar was poured into a sterile OmniTray (Nunc) and allowed to solidify at 25 °C for 30 min. An additional 30 mL of molten GM17 with 0.5% glucose (v/v) medium was cooled to 42 °C, combined with 75 µL of culture, and poured over the lower
solidified agar layer. A sterile 96-well PCR plate was placed in the molten agar upper layer and was allowed to solidify at 25 °C for 45 min. After sufficient solidification, the 96-well PCR plate was removed. The total 20 μL volume of concentrated in vitro reaction was dispensed into separate newly formed wells. Authentic nisin standards were spotted in 15 μL volumes at varied concentrations. Plates were left at 30 °C for 15 h and antibacterial activity was qualitatively determined by the presence or absence of growth inhibition.

3.4.6. Expansion of the linker of lacticin 481 ConFusion construct

The length of the linker between the LctA leader and the LctM synthetase sequences was expanded (Figure 3.5) via restriction endonuclease digestion of the pET28b LctCE-GSG construct with SpeI and PstI at 37 °C for 5 h. The excised insert byproduct was removed from the digested plasmid by purification via 1% agarose gel electrophoresis. The desired digested plasmid was subsequently gel extracted using a QIAquick Gel Extraction Kit (QIAGEN).

Double-stranded DNA (dsDNA) inserts encoding for linkers of repetitious Gly-Ser residues were designed to self-hybridize and generate “preformed” overhangs for ligation at the vector restriction sites between the sequences encoding for the leader peptide and the synthetase. These inserts of several lengths were prepared by combining 100 nM each of synthetic, self-complementary DNA oligos (see below for pairs and resulting linker) in PCR tubes and subjecting them to denaturing conditions (94 °C for 5 min) following by slow temperature gradient return to 25 °C over 30 min. The resulting dsDNA linker and cut pET28b LctCE-GSG plasmid products were ligated at 25 °C for 3 h in 1X T4 DNA Ligase buffer with T4 DNA Ligase (0.7 U/μL). E. coli DH5α cells were transformed with 2.5 μL of the ligation product by heat shock, and cells were plated on LB-kanamycin agar plates and grown for 15 h at 37 °C. Several colonies were picked and used to inoculate separate 5 mL cultures of LB-kanamycin medium. The cultures
were grown at 37 °C for 12 h, and plasmids were isolated using a QIAprep Spin Miniprep Kit
(QIAGEN). The sequences of the resulting plasmid products were confirmed by DNA sequencing.
SpeI recognition site (encodes for Thr Ser)

PstI recognition site (encodes for Leu Gln)

Target: LctCE-GS10 wt

5’
3’

1 2 3 4 5 6 7 8 9 10
“ThrSerGlySerGlySerGlySerGlySerGlySerLeuGln”
CTAGTGGTTCTGGTTCTGGTTCTGGTTCTGGTTCTCTGCA
ACCAAGACCAAGACCAAGACCAAGACCAAGAG

3’
5’

Target: LctCE-GS20 wt

5’
3’

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
“ThrSerGlySerGlySerGlySerGlySerGlySerGlySerGlySerGlySerGlySerGlySerLeuGln”
CTAGTGGTTCTGGTTCTGGTTCTGGTTCTGGTTCTGGTTCTGGTTCTGGTTCTGGTTCTGGTTCTCTGCA
ACCAAGACCAAGACCAAGACCAAGACCAAGACCAAGACCAAGACCAAGACCAAGACCAAGAG

3’
5’

Target: LctCE-GS30 wt

5’
3’

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30
CTAGTGGTTCCGGCTCTGGTAGCGGCTCTGGTTCCGGCTCTGGTAGCGGCTCTGGTTCCGGCTCTGGTAGCGGCTCTGGTTCCGGCTCTGGTAGCCTGCA
ACCAAGGCCGAGACCATCGCCGAGACCAAGGCCGAGACCATCGCCGAGACCAAGGCCGAGACCATCGCCGAGACCAAGGCCGAGACCATCGG

3’
5’

3.4.7. Expression and purification of His6-LctCE-GSn and LctM enzymes
E. coli Rosetta 2 (DE3) cells were used as the host for expression of pET28b LctCEGS10, pET28b LctCE-GS20, pET28b LctCE-GS30, and pET28b LctM. These enzymes were
expressed and purified as described in Experimental 3.4.3. The resulting protein samples were
stored at −80 °C. Protein concentration was determined using a Bradford Assay Kit (Pierce) and
typical yields were 20-30 mg protein from 3 L of cell culture.

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3.4.8. Evaluation of linker length expansion on extent of core peptide processing

The influence of the length of the linker between the LctA leader and the LctM synthetase sequences was assessed using the *in vitro* modification reactions described in Experimental 3.4.4. Briefly, core peptide (final concentration 20 µM) was incubated with purified His₆-LctCE-GS₆ (ConFusion enzyme with defined linker length, final concentration 2 µM) in the presence of 10 mM MgCl₂, 2 mM ATP, and 25 µg/mL BSA in a 50 mM Tris buffer (pH 7.5) and was incubated at 25 °C for 1 h. At 1 h, reactions were quenched by the addition of TFA to 0.5% final concentration (pH 1-2) and the reactions were desalted by ZipTip<sub>C18</sub> and analyzed by MALDI-TOF MS.
3.4.9. Comparison of processing efficiencies between LctCE-GS30 and LctM

Quantitative end-point assays were used to evaluate the efficiency of core peptide processing between LctCE-GS30 ConFusion enzyme with core peptide alone versus LctM with leader and core peptide provided in trans. For ConFusion assays, LctA(1-27) N15R F21H core peptide (final concentration 20 µM) was incubated with purified His$_6$-LctCE-GS30 (final concentration 2 µM) in the presence of 10 mM MgCl$_2$, 2 mM ATP, and 25 µg/mL BSA in a 50 mM Tris buffer (pH 7.5) at 25 °C. At 0, 5, 15, 30, 60, and 180 min, a 550 µL volume of reaction was removed and quenched by the addition of TFA to 0.5% final concentration (pH 1-2). A 500 µL volume of each reaction was subjected to HPLC analysis (see conditions below) and the remainder of each reaction was desalted by ZipTip$_{C18}$ and analyzed by MALDI-TOF MS. For LctM in trans assays, LctA(1-27) N15R F21H core peptide (final concentration 20 µM) and LctA(−1 - −24) leader peptide (final concentration 2 µM) was incubated with purified His$_6$-LctCE-GS30 (final concentration 2 µM) in the presence of 10 mM MgCl$_2$, 2 mM ATP, and 25 µg/mL BSA in a 50 mM Tris buffer (pH 7.5) and was incubated at 25 °C. At 0, 5, 15, 30, 60, and 180 min, a 550 µL volume of reaction was removed and quenched by the addition of TFA to 0.5% final concentration (pH 1-2). A 500 µL volume of each reaction was subjected to HPLC analysis (see conditions below) and the remainder of each reaction was desalted by ZipTip$_{C18}$ and analyzed by MALDI-TOF MS.

The above reaction sets were analyzed using analytical HPLC. HPLC was performed using a Beckman Coulter System Gold HPLC equipped with a Phenomenex Jupiter Proteo C12 column (10 µm, 90 Å, 250 mm x 4.6 mm) equilibrated in 2% solvent B (solvent A = 0.1% TFA in water, solvent B = 0.0866% TFA in 80% ACN/20% water). The quenched reactions were centrifuged at 15 krpm in a microcentrifuge for 2 min and the resulting supernatant was applied
to the column. The mixture of products and intermediates were resolved and eluted by maintaining the mobile phase at 2% B for 1 min, followed by an increase to 100% B over 45 min with a flow rate of 1.0 mL/min. Under these conditions, the fully modified $\sim 4 \text{H}_2\text{O}$ product eluted at 24.6 min. The $\sim 3 \text{H}_2\text{O}$ product eluted at 25.1 and a mixture of phosphorylated $\sim 2 \text{H}_2\text{O}$ and $\sim 3 \text{H}_2\text{O} + \text{PO}_3^{2-}$ intermediates eluted at 26.2 min. All fractions were analyzed by MALDI-TOF MS as described above.

**3.4.10. Preparation of lacticin 481 analogues**

A number of LctA 1-27 core peptides were prepared by Patrick Knerr. These included LctA 1-27 N15R F21H, LctA 1-27 N15R F21Pal, LctA 1-27 N15R W19Nal F21H, and LctA 1-27 N15R W19Nal F21H. The reactions were uniformly scaled as compared to those previously described. Core peptide (final concentration 20 µM) was incubated with purified His$_6$-LctCE-GS30 (final concentration 2 µM) in the presence of 10 mM MgCl$_2$, 2 mM ATP, and 25 µg/mL BSA in a 50 mM Tris buffer (pH 7.5) at 25 °C for 5 h. The extent of the reaction was monitored by mass spectrometry by periodically removing aliquots of the reaction and acidifying them with TFA, desalting the samples via ZipTip$_{C18}$, and analyzing by MALDI-TOF MS. By 5 h, the maximum extent of reaction was complete and reactions were quenched by the addition of TFA to 0.5% final concentration (pH 1-2). The quenched reaction containing a mixture of $\sim 4 \text{H}_2\text{O}$, $\sim 3 \text{H}_2\text{O} + \text{PO}_3^{2-}$, and a minor amount of $\sim 3 \text{H}_2\text{O}$ and $\sim 2 \text{H}_2\text{O}$ products was concentrated by lyophilization to one-fifth of the original reaction volume.

The individual analogues were then purified using preparative HPLC. HPLC was performed on a Waters Delta 600 instrument equipped with a Phenomenex Jupiter Proteo C12 column (10 µm, 90 Å, 250 mm x 15 mm) equilibrated in 10% B (A: 0.1% TFA in water, B:
0.0866% TFA in 80% ACN/20% water (v/v)). The quenched and concentrated reaction was centrifuged at 15 krpm in a microcentrifuge for 2 min to remove any insoluble material and the resulting supernatant was applied to the column. The products were eluted by maintaining the mobile phase at 10% B for 5 min, followed by an increase to 100% B over 50 min with a flow rate of 10.0 mL min⁻¹. Under these conditions, lacticin 481 analogues eluted between 23.0 and 24.5 min. All fractions were analyzed by MALDI-TOF MS as described above. Purified material was lyophilized to dryness and stored under N₂ at −80 °C until further use.

3.4.11. Specific activity determination against L. lactis HP cells

Microtiter plates were used to determine the IC₅₀ values (the concentration at which 50% growth inhibition is observed) of indicator strains. Serial dilutions of lacticin 481 peptides were prepared in sterile deionized water (SDW). Ninety-six well microtiter plates (Corning Costar) were utilized for testing against the anaerobic L. lactis HP strain. The total volume of culture in each well was 200 μL; the experimental wells contained 50 μL of diluted lacticin 481 peptides at defined concentrations and 150 μL of a 1-in-10 dilution (approximately 1 x 10⁸ CFU mL⁻¹) of an indicator strain culture diluted in fresh growth medium. In addition, each plate contained several blank (150 μL fresh growth medium and 50 μL SDW) and control wells (150 μL of untreated 1-in-10 diluted culture and 50 μL SDW). The optical density at 600 nm (OD₆₀₀) was recorded at hourly intervals from 0 to 5 h with an additional measurement at 18 h using a BioTek Synergy 4 Hybrid plate reader. Plates were incubated at 30 °C between measurements. The triplicate readings were averaged, and blanks (growth medium and SDW only) were subtracted from these readings. Growth curves were developed using control (culture and SDW only) readings to ensure sufficient OD changes for accurate inhibition assessment. Curve fits for IC₅₀
determination were produced by fitting the data with Origin8 software using the dose-response curve with the equation: \[ y = A1 + \frac{(A2 - A1)}{\left(1 + 10\left(\frac{\log(10^0 - x)}{p}\right)\right)} \], with \( p \) = variable Hill slope. A 50% growth inhibition was determined as half the final OD\(_{600} \) ± 0.05 of control culture. IC\(_{50} \) values for authentic lacticin 481 and nisin were determined using identical procedures as described above.

3.4.12. Inhibition of peptidoglycan formation by lacticin 481 and analogues

The gene encoding \( E. coli \) PBP1b was previously PCR amplified from MG1655 genomic DNA and cloned into pET21b vector (Novagen) as a C-terminal hexa-histidine (His\(_6\)) fusion gene.\(^82\) The enzyme was expressed and purified by Tsung-Shing Andrew Wang or Tania Lupoli (Dan Kahne and Suzanne Walker Laboratory, Harvard University) as previously published.\(^82\) \( ^{14}\text{C}\)GlcNAc-labeled heptaprenyl lipid II analogue was chemo-enzymatically prepared by Dr. Yuto Sumida and Dr. Hiro Tsukamoto (Dan Kahne Laboratory) as previously described.\(^83\)

For IC\(_{50} \) determinations, assays were carried out by separately incubating \( ^{14}\text{C}\)GlcNAc-labeled heptaprenyl lipid II analogue (final concentration = 4 \( \mu \)M, typical specific activity = 288 \( \mu \)Ci/\( \mu \)mol) and peptide inhibitors (concentrations are indicated in the figure legends) in low-binding microcentrifuge tubes (VWR) containing 9 \( \mu \)L of buffer consisting of 50 mM HEPES (pH 7.5), 10 mM CaCl\(_2\), 1,000 units/mL penicillin G, 0.2 mM octaethylene glycol monodecyl ether (decyl-PEG; Anatrace, Maumee, OH), and 11% DMSO (v/v) for 30 min at room temperature prior to PBP1b addition (to allow binding to occur). Reactions were started by adding 1 \( \mu \)L of PBP1b (from a solution freshly prepared by diluting the 50% glycerol stock 20-fold into PBP1b dilution buffer consisting of 5 mM Tris (pH 8.0), 8 mM decyl-PEG) to the reaction mixture (final PBP1b concentration = 20-100 nM). Reactions were typically stopped
after 30-45 min by adding 10 μL of ice-cold 10 mM Tris (pH 8.0) containing 10% Triton X-100. Quenched reactions were stored on ice until they were spotted on cellulose chromatography paper strips (3MM Whatman chromatography paper #3030-861, cut 1 cm width x 20 cm height) Products and starting material were separated using chromatography (isobutyric acid/1 M NH₄OH, 5:3). The lipid II starting material migrates on the strip, while polymerized product remains at the origin. The paper strips were removed from chromatography chambers, allowed to dry, cut, and added to separate scintillation vials containing EcoLite(+) liquid scintillation fluid (MP Biomedical). Samples were analyzed using a LS6500 scintillation counter (Beckman Coulter). The percent radioactivity of the polymer product was calculated by comparing the amount of radioactivity that remains at the origin (peptidoglycan) to the total amount of radioactivity on the strip.

3.4.13. Construction and expression of His₆-LctCE-GS30 T405A and R399M enzymes

The engineered plasmid pET28b LctCE-GS30 wt and the native plasmids pET28b LctM T405A and pET28b LctM R399M were isolated from *E. coli* DH5α cells previously transformed with the constructs. The partial gene of pET28b LctCE-GS30 wt encoding for LctA leader, GS30 linker, and the 5’-end of LctM was excised via restriction digestion with *Nhe*I and *Hind*III at 37 °C for 5 h. The digested partial gene and vector products were purified by 2% and 1% agarose gel electrophoresis, respectively, and gel extracted using a QIAquick Gel Extraction Kit (QIAGEN). The plasmids pET28b LctM T405A and pET28b LctM R399M were also digested with *Nhe*I and *Hind*III at 37 °C for 5 h, gel purified, and gel extracted using the same technique. The partial gene and cut pET28b LctM T405A vector were ligated at 25 °C for 3 h in 1X T4 DNA Ligase buffer with T4 DNA Ligase (0.7 U/μL). The same ligation procedure was completed using cut pET28b LctM R399M. *E. coli* DH5α cells were transformed with 2.5 μL of the ligation products by heat shock, and
cells were plated on LB-kanamycin agar plates and grown for 15 h at 37 °C. Several colonies were picked and used to inoculate separate 5 mL cultures of LB-kanamycin medium. The cultures were grown at 37 °C for 12 h, and plasmids were isolated using a QIAprep Spin Miniprep Kit (QIAGEN). The sequences of the resulting plasmid products were confirmed by DNA sequencing.

*E. coli* Rosetta 2 (DE3) cells were used as the host for expression of pET28b LctCE-GS30 T405A and pET28b LctCE-GS30 R399M. These enzymes were expressed and purified as described in Experimental 3.4.3. The resulting protein samples were stored at −80 °C. Protein concentration was determined using a Bradford Assay Kit (Pierce) and typical yields were 15-20 mg protein from 3 L of cell culture.

### 3.4.14. *In vitro* production and elimination of phosphopeptides

**Phosphorylation of peptides.**

A number of biologically relevant peptides were designed based on known information from Anaspec, a commercial peptide synthesis company ([www.anaspec.com](http://www.anaspec.com)). The peptides selected have been experimentally examined and have demonstrated the ability to serve as surrogate substrates for protein kinases/phosphatases involved in phosphorylation pathways. These partial fragments of larger protein substrates are of sufficient length for interaction and recognition by the upstream kinase and have the Ser, Thr, or Tyr residue(s) that serves as the specific receptor of the phosphate group. The peptides were designed by myself and Patrick Knerr and were prepared by Patrick Knerr. These included those listed in Table 3.1.

Peptide substrate (final concentration 20 µM) was incubated with purified His<sub>6</sub>-LctCE-GS30 T405A (final concentration 2 µM) in the presence of 10 mM MgCl<sub>2</sub>, 2 mM ATP, and 25 µg/mL BSA in a 50 mM Tris buffer (pH 7.5) and was incubated at 25 °C for 3 h. At 3 h, reactions were quenched by the addition of TFA to 0.5% final concentration (pH 1-2) and the
reactions were desalted by ZipTipC18 and analyzed by MALDI-TOF MS. Additional analysis included LC-ESI-q/TOF MS and MSMS to identify the specific site of phosphorylation of the AKT/PKB/RAC peptide.

The phosphorylated and non-phosphorylated peptides were purified using HPLC. HPLC was performed using a Beckman Coulter System Gold HPLC equipped with a Phenomenex Jupiter Proteo C12 column (10 μm, 90 Å, 250 mm x 4.6 mm) equilibrated in 2% solvent B (solvent A = 0.1% TFA in water, solvent B = 0.0866% TFA in 80% ACN/20% water). The quenched reactions were centrifuged at 15 krpm in a microcentrifuge for 2 min and the resulting supernatant was applied to the column. The phosphorylated and non-phosphorylated peptides were eluted by maintaining the mobile phase at 2% B for 1 min, followed by an increase to 100% B over 45 min with a flow rate of 1.0 mL/min. Under these conditions, the phosphorylated peptide and non-phosphorylated peptide eluted within 22-26 min, respectively and typically had a retention time separation of 1.5 – 2.0 min. All fractions were analyzed by MALDI-TOF MS as described above. Purified material was lyophilized to dryness and stored under N₂ at −80 °C until further use.

**Elimination of peptide phosphorylation.**

Elimination of peptide phosphorylation was studied in two ways: with purified phosphopeptide and with phosphorylated/non-phosphorylated peptide reaction mixture. For assays with purified phosphopeptide, phosphopeptide substrate (final concentration ~20 μM) was incubated with purified His₆-LctCE-GS30 wt (final concentration 2 μM) in the presence of 10 mM MgCl₂, 2 mM ATP, and 25 μg/mL BSA in a 50 mM Tris buffer (pH 7.5) and was incubated at 25 °C for 3 h. At 3 h, reactions were quenched by the addition of TFA to 0.5% final
concentration (pH 1-2) and the reactions were desalted by ZipTip_{C18} and analyzed by MALDI-TOF MS.

For assays with phosphorylated/non-phosphorylated peptide reaction mixtures, a phosphorylation reaction was first run in which peptide substrate (final concentration 20 µM) was incubated with purified His\textsubscript{6}-LctCE-GS30 T405A (final concentration 2 µM) in the presence of 10 mM MgCl\textsubscript{2}, 2 mM ATP, and 25 µg/mL BSA in a 50 mM Tris buffer (pH 7.5) and was incubated at 25 °C for 3 h. At 3 h, a small aliquot of the reaction was removed, desalted using ZipTipC18, and analyzed by MALDI-TOF MS to verify that phosphorylation of the peptide had occurred. Without any further manipulation, His\textsubscript{6}-LctCE-GS30 wt was added to 2 µM and the reaction was returned to 25 °C for an additional 3 h. At 3 h, reactions were quenched by the addition of TFA to 0.5% final concentration (pH 1-2) and the reactions were desalted by ZipTip_{C18} and analyzed by MALDI-TOF MS.
3.5. REFERENCES


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CHAPTER 4: STRUCTURAL CHARACTERIZATION AND BIOSYNTHESIS OF SUBLANCIN AND OTHER S-LINKED GLYCOPEPTIDE NATURAL PRODUCTS

4.1. INTRODUCTION

Post-translationally modified peptide natural products are a rapidly expanding class of compounds with a wide variety of biological activities. One such peptide, sublancin, is produced by *Bacillus subtilis* 168, the laboratory strain that was one of the first bacterial strains for which the genome was sequenced. Sublancin is encoded by the SPβ-prophage in strains that are lysogenic for the SPβ bacteriophage, and inhibits the growth of non-lysogenic strains. The reported structure of sublancin contains a dehydroalanine and one of the thioether crosslinks that are characteristic of lantibiotics (Figure 4.1a). Like other lantibiotics, sublancin is biosynthesized as a precursor peptide bearing an N-terminal leader peptide of the double glycine-type (19 amino acids, Figure 4.1b) and a C-terminal core peptide (37 amino acids) that is converted to the mature compound.

Interestingly, a search of the *B. subtilis* 168 genome did not reveal genes for any of the four known classes of lantibiotic biosynthetic enzymes (see Figure 1.6). Intrigued by the possibility of a fifth pathway to lanthionine-containing peptides, the biosynthesis of sublancin was investigated. It is demonstrated here that this compound, initially reported as a lantibiotic is in fact not a lantibiotic, but rather a very unusual S-linked glycopeptide. This chapter describes efforts toward the structure revision of sublancin, the reconstitution of the *in vitro* activity

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Figure 4.1. (a) Originally reported structure of sublancin proposed to contain a dehydroalanine at position 16 and a methyllanthionine bridge arising from dehydration of Thr19 to Dhb19 and subsequent attack of Cys22 on Dhb19 to form the thioether linkage. (b) Primary sequence of the SunA precursor peptide containing a 19 amino acid N-terminal leader peptide (blue) and a 37 amino acid C-terminal core peptide (black). A double glycine-type proteolytic cleavage site is underlined in red.

of the S-glycosyltransferase responsible for installing its unique post-translational modification, and the in vitro reconstitution of its antimicrobial activity. It addition, it is demonstrated how the versatility of the S-glycosyltransferase can be utilized to generate other bioactive S-linked products. This discovery of an S-linked glycopeptide and the enzyme responsible for installation of this unique post-translational modification is the first of its kind, and has opened the door for the identification and characterization of additional S-glycopeptide precursors and their biosynthetic enzymes. Indeed, a search of the protein databases suggests that sublancin is a member of a larger family of novel S-linked glycopeptides, further expanding the already impressive structural diversity of post-translationally modified peptide natural products. One example of an additional S-glycopeptide and glycosyltransferase pair was examined and is reported in this chapter.
4.2. RESULTS AND DISCUSSION

4.2.1 Structural characterization of in vivo produced sublancin

Sublancin was purified as described previously and analyzed by mass spectrometry. The mass of the unmodified core peptide predicted from the sunA gene sequence is 3717.72 Da whereas the observed mass of purified sublancin is 3875.74 Da (Figure 4.2). The core peptide contains five Cys residues (Figure 4.1b), but native sublancin does not react with iodoacetamide demonstrating it does not contain free thiols. However, treatment of sublancin with the reducing agent TCEP led to a mass increase of exactly four Da (Figure 4.3). Following treatment of the peptide with both TCEP and iodoacetamide, four out of five cysteines were alkylated (Figure 4.4). Thus, one cysteine is modified such that it is not available for alkylation.

Sublancin was further analyzed by tandem mass spectrometry. When native sublancin with intact disulfide bridges was subjected to collision induced dissociation, fragmentation of the peptide amide bonds did not occur. Rather, the post-translational modification with a mass of 162 Da, later identified as an S-linked glucosylation (vide infra), was lost from the parent ion (Figure 4.5). To allow fragmentation, the disulfide bonds were first reduced and the reduced peptide was treated with chymotrypsin, providing a series of peptide digest fragments as determined by MALDI-TOF mass spectrometry (Figure 4.6). The fragment spanning residues 12-32 of sublancin was analyzed by electrospray ionization-quadrupole/time-of-flight mass spectrometry. The observed fragmentation pattern combined with the sequence of the precursor
Figure 4.2. ESI-Q/TOF MS analysis of native sublancin. Pure sublancin isolated from *B. subtilis* 168 without any further manipulation was analyzed by LC-ESI-Q/TOF MS. Expected [M+H]: 3876.74, observed: 3876.75.

Figure 4.3. ESI-Q/TOF MS analysis of reduced sublancin. Pure sublancin was reduced using TCEP and analyzed by LC-ESI-Q/TOF MS. The mass of reduced sublancin is 4 mass units larger than native sublancin, suggesting that two disulfide linkages exist in the native structure of sublancin. Expected [M+H]: 3880.77, observed: 3880.78.
Figure 4.4. ESI-Q/TOF MS analysis of reduced and alkylated sublancin. Pure sublancin was reduced and alkylated using TCEP and iodoacetamide and analyzed by LC-ESI-Q/TOF MS. Expected [M+H] (for four alkylations): 4107.86, observed: 4107.83.

Figure 4.5. ESI-Q/TOF MSMS analysis of native sublancin. Pure sublancin isolated from B. subtilis 168 was analyzed by LC-ESI-Q/TOF MSMS.
Figure 4.6. MALDI-TOF MS analysis of sublancin digested with chymotrypsin under reducing conditions. Sublancin was digested with chymotrypsin under reducing conditions and analyzed by MALDI-TOF MS. The resulting mass spectrum is shown. Chymotrypsin cleavage sites (F, W, and Y residues) are indicated in the peptide sequence above the spectrum. The masses of the observed ions and their corresponding digest fragments are assigned. The results are consistent with Ser16 and Thr19 remaining unmodified and the peptide spanning residues Leu12-Tyr32 bearing a +162 Da post-translational modification.

gene sunA indicated that residue 16 is a Ser (Figure 4.7) and not a dehydroalanine as previously reported. Furthermore, the observed fragments indicate that Thr19 is intact and therefore not engaged in a methyllanthionine ring. The data further suggest that Cys22 is carrying a post-translational modification with a mass of 162 Da (Figure 4.8). This mass is consistent with a hexose conjugated to the Cys thiol.
Figure 4.7. ESI-Q/TOF MSMS analysis of residues Leu12-Tyr32 of sublancin. Sublancin was digested with chymotrypsin under reducing conditions and analyzed using LC-ESI-Q/TOF MSMS. The digest mixture was fractionated using LC and the multiply charged ion corresponding to the peptide spanning residues Leu12-Tyr32 was selected for MSMS analysis. (a) Fragmentation patterns are indicated for the full b ion series and (b) zoom of the spectra showing unmodified Ser and Thr residues.
Figure 4.8. ESI-Q/TOF MSMS analysis of residues Leu12-Tyr32 of sublancin. Sublancin was digested with chymotrypsin under reducing conditions and analyzed using LC-ESI-Q/TOF MSMS. The digest mixture was fractionated using LC and the multiply charged ion corresponding to the peptide spanning residues Leu12-Tyr32 was selected for MSMS analysis. (a) Fragmentation patterns are indicated for the full y" ion series and (b) zoom of the spectra showing modified Cys residue.
4.2.2 Identification of the natural sugar modification of sublancin

To determine the identity of the hexose, sublancin was subjected to acidic hydrolysis to release the sugar. Conversion of the acetal to a mixture of two stereoisomer oximes and subsequent chemical modification to a trimethylsilylated derivative allowed analysis by GC-MS. Comparison with a series of authentic standards treated in the same way demonstrated that the hexose of sublancin is glucose (Figure 4.9. See additional supporting data in Experimental 4.4.6).

To the best of our knowledge, reports of S-linked glycopeptides are extremely rare. A small number of S-linked glycopeptides have been disclosed, but little in depth characterization has been done on those discovered. Forty years ago in 1971, two different S-linked glycopeptides were reported. The more well-studied peptide, found in human urine, was identified to contain an S-glycosidic bond between an N-terminal Cys and a digalactosyl moiety. This octapeptide was partially purified and subjected to dansyl-Edman degradation to determine amino acid content and hydrolysis/derivatization followed by gas chromatography analysis to identify the bound sugar. Ultimately, it was determined that for each 2 mol of galactose, amino acid analysis revealed 2 mol of His and 1 mol each of Asp, Ser, Cys, Glu, Gly, and Ala. Sequential analysis indicated a peptide with the sequence “Cys-Glu-His-Ser-His-Asp-Gly-Ala” and it was proposed that the digalactose was appended to the Cys residue via an S-glycosidic linkage.

That year, the same group identified and reported a second S-glycopeptide in human erythrocyte membrane. The novel peptide was characterized using similar techniques as described for the peptide found in human urine, and for this peptide, a triglucosyl group was identified as the Cys-bound modification. The human erythrocyte membrane glycopeptide shared remarkable sequence homology with that of the urine derived peptide and consisted of the amino
acid sequence, “Cys-Glu-Gly-His-Ser-His-Asp-His-Gly-Ala” with the S-linked trisaccharide also bound to the N-terminal Cys residue. The biosynthesis and function of these peptides has not been reported and it is currently unknown if the modifications are enzymatically installed. Furthermore, the sequences of these peptides are not found in the predicted human proteome and hence their origins are unclear.

A third S-linked glycosylation reported is Allergen M, an acidic protein isolated from the white muscle albumin of cod. The complete primary structure of the protein was elucidated via dansyl-Edman degradation and gas chromatography analyses. Combined with previously reported information on the C-terminal portion of the protein, the intact allergen consists of 113 amino acids (12,328 Da MW) and a single glucosylation at Cys18. Finally, a fourth S-glycoprotein reported is inter-α-inhibitor, a serine protease inhibitor found in high concentrations in human plasma. This protein is composed of a light inhibitory chain and two heavy chains of unknown function. The heavy chains are heavily post-translationally modified possessing several N-linked and O-linked glycosylations. In addition, the heavy chains contain nine Cys residues, one of which, Cys26, is proposed to be modified by dihexosylation while the remaining eight Cys are involved in disulfide pairs.

The conjugation of sugars to Cys through an S-glycosidic linkage results in more stable products than the O-glycosidic linkage to Ser, both at low and high pH. As a result, S-linked glycopeptides have been the topic of several recent synthetic investigations. Indeed, it has been demonstrated that sublancin is a remarkably stable peptide that tolerates both low and high pH.
Figure 4.9. Gas chromatography-mass spectrometry analysis of sublancin and hexose standards. (a) Hexose standards were derivatized and analyzed by GC-MS. (b) The naturally occurring sugar of sublancin was released from the peptide natural product via acid hydrolysis, derivatized, and analyzed by GC-MS. The peak corresponding to the derivatized sugar of sublancin had a similar retention time as glucose. (c) Sublancin sample was spiked with authentic derivatized glucose standard and the spiked mixture was analyzed by GC-MS. (d) The electron-impact mass spectrum obtained for the sublancin sugar peak at 10.984 min. Spectra comparison with standards and databases confirmed the presence of a TMS-derivatized glucose.

4.2.3 Addressing the disulfide connectivities of native sublancin

The bridging patterns of the two disulfide bonds of the peptide were investigated as well. Four cysteine residues are available for disulfide pairing resulting in 3 possible connectivity arrangements. Minimal structural information was obtained by using tandem mass spectrometry to examine native sublancin, therefore, proteolytic degradation followed by peptide mass fingerprint analysis of sublancin was used to address disulfide bond connectivities. Sublancin was digested with trypsin in the presence or absence of a reducing agent. The resulting mass spectra indicated that one disulfide bond is shared between Cys7 and Cys35, and the other
Figure 4.10. ESI-Q/TOF MS analysis of sublancin digested with chymotrypsin under non-reducing and reducing conditions. (a) Sublancin was digested with chymotrypsin under non-reducing conditions and analyzed using LC-ESI-Q/TOF MS to investigate disulfide bridge connectivity. Chymotrypsin cleavage sites (F, W, and Y residues) and the proposed disulfide bridges are indicated in the peptide sequence above the spectrum. The masses of the observed ions and their corresponding digest fragments are assigned. The results support the conclusion that a disulfide bond exists between Cys7-Cys36 and between Cys14-Cys29. The results are also consistent with Ser16 and Thr19 remaining unmodified and the peptide spanning residues Leu12-Tyr32 bearing a +162 Da post-translational modification. (b) Sublancin was digested with chymotrypsin under reducing conditions and analyzed using LC-ESI-Q/TOF MS. Chymotrypsin cleavage sites (F, W, and Y residues) are indicated in the peptide sequence above the spectrum. The masses of the observed ions and their corresponding digest fragments are assigned. The results support that the disulfide bond existing between Cys7-Cys35 was reduced as the peak corresponding to the ion with a mass of 1392.62 Da (native sublancin) was no longer observed. Rather, a peak corresponding to the peptides spanning residues Gly1-Trp11 with a mass of 1117.53 Da was observed. In addition, the ion corresponding to Leu12-Tyr32 had a 2 Da mass increase compared to the Leu12-Tyr32 ion found in the native sublancin digest. These results further suggest that a disulfide bond exists between Cys14-Cys29 in native sublancin. The results are also consistent with Ser16 and Thr19 remaining unmodified and residues Leu12-Tyr32 bearing a +162 Da post-translational modification.

is shared between Cys14 and Cys29 (Figure 4.10). It has been demonstrated for many conformationally constrained, bioactive peptides that proper connectivity of disulfides is essential for biological activity.27-29 Indeed, reduction of the disulfides of sublancin resulted in complete loss of antimicrobial activity against *B. subtilis* ATCC 6633 in an agar diffusion assay, showing their importance for activity (Figure 4.11).
Figure 4.11. Antimicrobial activity assay of native, reduced, and reduced and alkylated sublancin. The importance of intact disulfides for the bioactivity of sublancin was assessed by testing the activities of native, reduced, and reduced and alkylated sublancin against *B. subtilis* ATCC 6633. Native sublancin samples were prepared by incubation in Tris buffer at pH 7.5 and pH 8.3. Reduced sublancin was prepared by incubation in Tris buffer (pH 8.3) in the presence of TCEP. Reduced and alkylated sublancin was prepared by incubation in Tris buffer (pH 8.3) in the presence of TCEP and iodoacetamide. All samples were analyzed by MS. The antimicrobial properties of each sample were assessed by its ability to inhibit the growth of *B. subtilis* ATCC 6633. Authentic sublancin and nisin standards were tested as positive controls. As indicated below, both samples of native sublancin (pH 7.5 and pH 8.3) retained the ability to inhibit the growth of the indicator strain. Both reduced and reduced and alkylated samples did not display antimicrobial activities.
4.2.4 NMR analysis of sublancin

Provided the known inversion of stereochemistry of the GT2 family of GT-A glycosyl transferases, a $\beta$-linkage was expected and NMR studies confirmed this assumption. Isotopically labeled sublancin was prepared and purified by culturing *B. subtilis* 168 in minimal media enriched with $^{15}$NH$_4$Cl and $^{13}$C-glucose followed by purification via preparative reversed-phase chromatography. Milligram quantities of both $^{13}$C-sublancin as well as double labeled $^{13}$C/$^{15}$N-sublancin was obtained (see Figures 4.42 and 4.43 in Experimental 4.4.14). With the support of Dr. John Boettcher (a former graduate student of Prof. Chad Rienstra), several 1D and 2D data sets were collected (Figure 4.12). From the 2D data, the unique sugar ring spin system was identified with chemical shifts between 3.2 – 4.6 ppm ($^1$H) and 69 – 85 ppm ($^{13}$C). After establishing the assignments of the conjugated glucose we used the NMR spectra to address the stereochemistry of the $S$-linkage. By measuring the $^3$J$_{H-H}$ coupling constant between the anomeric proton (proton 1) and the neighboring proton (proton 2) the dihedral angle was calculated using the Karplus relationship.$^{30}$ Using an NMR data fitting program, the cross peak of 1-2 was fit with a $^3$J$_{H-H}$ coupling constant of 9.965 Hz. This results in a dihedral angle of $171^\circ \pm 10^\circ$ based on the Karplus equation and indicates a diaxial orientation of the two protons. Combined with the knowledge that the sugar molecule is glucose, the stereochemistry of conjugation to Cys22 must be through a $\beta$-linkage. The revised structure of sublancin based on our data is shown in Figure 4.13. In addition, many 3D experiments have been completed and data analysis for full 3D-structure elucidation is currently underway. This revised structure also explains the discrepancy noted in the original report between the mass of its proposed structure and the actual mass.$^5$
Figure 4.12. (a) Expanded region of a one-dimensional $^1$H NMR spectrum of sublancin in 100% D$_2$O. (b) A two-dimensional $^{13}$C-HSQC spectrum of [U-$^{13}$C]-sublancin in 100% D$_2$O. Labels are shown for the aromatic and sugar resonances as well as the single threonine resonance (Thr19). (c) (top) Correlations for the proton spin system in the COSY spectrum for which an expanded region of the $^{13}$C-HSQC is also depicted (bottom). The assignments of the sugar resonances are shown. All spectra were acquired at 500 MHz at 25 °C. The spectra were processed with zero filling to double the original size and apodized with a sine bell function.
4.2.5 Bioinformatic analysis of the sublancin biosynthetic gene cluster

The sublancin biosynthetic gene cluster is shown in (Figure 4.14). It contains the precursor gene \textit{sunA} and genes encoding two thiol-disulfide oxidoreductases, BdbA and BdbB. In addition, it contains two orfs of unkown function, \textit{yolJ} and \textit{yolF}. YolF was recently suggested to be important for immunity of the producing strain and renamed SunI, the function of YolJ has not yet been reported. Bioinformatic analysis showed that YolJ has sequence similarity to the GT-A glycosyltransferases of the GT2 family (Figure 4.15), consistent with the observed glucose attached to Cys22. Heretoforth we will refer to the enzyme as SunS, a sublancin biosynthetic enzyme.

Figure 4.15. (continued on next page)
Figure 4.15. Sequence alignment of SunS with glycosyltransferases sharing high homology. BLAST analysis resulted in several glycosyltransferases (hypothetical and experimentally defined) sharing sequence homology with SunS and located in a cluster with a putative peptide substrate (see Figure 4.26b). Shown is the sequence alignment of SunS and the top hits from the BLAST search in August 2010. The asterisks (*) above the sequences indicate the putative divalent metal (Mg²⁺, Mn²⁺) binding site. The C-termini of these proteins appear less conserved and may confer upon SunS the unique ability to glycosylate a Cys. The C-terminus of SunS also has homology with proteins involved in lectin binding.

4.2.6 In vitro reconstitution of SunS glycosyltransferase activity

To confirm its function, sunS was cloned and expressed in Escherichia coli as an N-terminal fusion protein with a hexa-histidine tag (His₆-SunS, Figure 4.16). Upon purification by immobilized metal affinity chromatography, His₆-SunS was incubated with the purified precursor peptide His₆-SunA, also expressed in E. coli. Addition of uridine diphosphate α-D-glucose (UDP-Glc) and Mg²⁺ resulted in conversion of SunA to a product consistent with glucosylation (Figure 4.17). Subsequent chymotrypsin digest and ESI-MSⁿ analysis verified that...
glucose was attached to Cys22 (Figure 4.18), demonstrating the strong regioselectivity of the glycosyltransferase as the SunA substrate contains five Cys residues. Because His$_6$-SunS glycosylated reduced His$_6$-SunA, the presence of the disulfides is not required for substrate recognition.

Figure 4.16. Purification of SunS glycosyltransferase. The gene sunS was cloned and expressed in *Escherichia coli* as an N-terminal fusion protein with a hexa-histidine tag (His$_6$-SunS). His$_6$-SunS consists of 442 amino acids and has a predicted molecular weight of 51,928 Da. Shown is an SDS-PAGE (BioRAD Ready Gel 4-20%) analysis of purified SunS protein.

Figure 4.17. *In vitro* reconstitution of SunS activity. MALDI-TOF mass spectra of His$_6$-SunA before (black) and after (red) incubation with His$_6$-SunS, UDP-glucose, and Mg$^{2+}$. 

SunA wt [M+H]$^+$ 8015.57
SunA wt + Glc [M+H]$^+$ 8178.25
Figure 4.18. Determination of the site of \textit{in vitro} modification of His\textsubscript{c}-SunA by His\textsubscript{c}-SunS. (a) ESI-Q/TOF MS analysis of glucose-modified His\textsubscript{c}-SunA digested with chymotrypsin under reducing conditions. His\textsubscript{c}-SunA was modified with glucose, digested with chymotrypsin under reducing conditions, and analyzed by using LC-ESI-
Chymotrypsin cleavage sites (F, W, and Y residues) are indicated in the peptide sequence above the spectrum. The masses of the observed ions and their corresponding digest fragments are assigned. The results are consistent with Ser54 and Thr57 remaining unmodified and the peptide spanning residues Leu50-Tyr70 bearing a +162 Da post-translational modification. (b) ESI-Q/TOF MSMS analysis of residues Leu50-Tyr70 of glucose-modified His$_6$-SunA. His$_6$-SunA was modified with glucose, digested with chymotrypsin under reducing conditions, and analyzed by using LC-ESI-Q/TOF MSMS. The digest mixture was fractionated using LC and the multiply charged ion corresponding to residues Leu50-Tyr70 was selected for MSMS analysis. Fragmentation patterns are indicated for the y’’ ion series (upper) and the b ion series (lower). The results confirm that Ser54 and Thr57 remain unmodified and that Cys60 has a +162 Da post-translational modification.

4.2.7 Probing the precursor peptide and NDP-sugar specificity of SunS

Interestingly, the enzyme displays strong chemoselectivity for glycosylation of a thiol because the His$_6$-SunA mutant C22S (subcloned and expressed by Dr. Huan Wang of Prof. Wilfred van der Donk group) was not a substrate (Figure 4.19). The inability of SunS to modify the SunA C22S mutant peptide demonstrates its exclusive reactivity toward thiol-containing amino acid acceptors.

**Figure 4.19.** Modification of His$_6$-SunA C22S with glucose was attempted by incubation with His$_6$-SunS in the presence of UDP-Glc, MgCl$_2$, and TCEP in Tris (pH 7.5) buffer. The reaction was analyzed by MALDI-TOF MS.
To further probe the substrate specificity of the peptide, four mutant SunA peptides were prepared and analyzed by Dr. Huan Wang. Interestingly, three of the four Cys residues in SunA that are not glucosylated are flanked by Gln and Ala, whereas Cys22 that is glucosylated has two flanking Gly residues. When these two glycines were mutated to Gln and Ala, the resulting SunA-G21Q/G23A peptide was still glucosylated by SunS (Figure 4.20a). Mutation of these flanking glycines to charged residues (Lys or Glu) also did not prevent glucosylation (Figure 4.20b-d) demonstrating that the flanking residues do not provide the basis of substrate recognition.

**Figure 4.20.** Modification of (a) His<sub>6</sub>-SunA G21Q G23A, (b) His<sub>6</sub>-SunA G21E, (c) His<sub>6</sub>-SunA G23E, and (d) His<sub>6</sub>-SunA G23K with glucose was achieved by incubation with His<sub>6</sub>-SunS in the presence of UDP-Glc, MgCl<sub>2</sub>, and TCEP in Tris (pH 7.5) buffer. The reactions were analyzed by MALDI-TOF MS.

To evaluate whether the leader peptide guides SunS activity, I engineered a Factor Xa cleavage site into the SunA substrate at the junction of the leader and core peptides (Figure
Expression of His₆-SunA-Xa and proteolytic cleavage with Factor Xa allowed purification of the 37-residue core peptide. Incubation with His₆-SunS and UDP-Glc resulted in full glucosylation of Cys22, demonstrating that the leader peptide is not required for enzyme activity (Figure 4.21b).

Figure 4.21. (a) Sequences of His₆-SunA and His₆-SunA Xa peptides. His₆ tag (red), leader peptide (blue), and core peptide (black) are indicated. Factor Xa cleavage site is underlined. (b) SunA core peptide was modified with glucose by incubation with His₆-SunS in the presence of UDP-Glc, MgCl₂, and TCEP in Tris (pH 7.5) buffer. The leader peptide was not added to the reaction. The reaction was analyzed by MALDI-TOF MS. The overlay of mass spectra obtained by MALDI-TOF MS analysis of the mixture before (black) and after SunS reaction (red) is shown.
To determine the substrate selectivity of SunS, the enzyme was incubated with His₆-SunA and Mg²⁺ in the presence of UDP-α-D-N-acetylglucosamine (UDP-GlcNAc) and UDP-α-D-galactose (UDP-Gal) resulting in glycosylation of SunA at Cys22 (Figure 4.22a-b). GlcNAc-modified His₆-SunA peptide was digested with chymotrypsin to afford digest fragments indicating glycosylation at the expected Cys residue. Tandem MS analysis of the peptide of interest confirmed N-acetylglucosaminylation at Cys22 (see Experimental 4.4.11). Surprisingly, even guanosine diphosphate α-D-mannose (GDP-Man) and UDP-α-D-xylose (UDP-Xyl) proved to be substrates (Figure 4.22c-d). In the absence of SunS, no activity was detectable.

Figure 4.22. (a) MALDI-MS data for modification of His₆-SunA with various sugars. His₆-SunA was modified with various sugars by incubation with His₆-SunS in the presence of respective NDP-sugar, MgCl₂, and TCEP in Tris (pH 7.5) buffer. The reaction was analyzed by MALDI-TOF MS. Modification of SunA with (a) N-acetylglucosamine, (b) galactose, (c) mannose, and (d) xylose.
Analysis of the glycosylation reactions with UDP-GlcNAc, UDP-Gal, GDP-Man, and UDP-Xyl demonstrated that the reactivity of these substrates was somewhat less efficient than the corresponding reaction with UDP-glucose but all reactions could be coerced to high conversion (Table 4.1. See Experimental 4.4.14). Thus, SunS is highly promiscuous with respect to its nucleotide-sugar donor co-substrate. These findings provide a new tool for glycodiversification of peptide natural products\(^{34,35}\) and enables the addition of rationally designed S-linked structures to the natural product repertoire.

<table>
<thead>
<tr>
<th>Sugar</th>
<th>Concentration of NDP-Sugar</th>
<th>Observed % Conversion</th>
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</thead>
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<tr>
<td>Glc</td>
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<td>95.2</td>
</tr>
<tr>
<td></td>
<td>50 μM</td>
<td>92.6</td>
</tr>
<tr>
<td></td>
<td>500 nM</td>
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<tr>
<td>Gal</td>
<td>5 mM</td>
<td>91.6</td>
</tr>
<tr>
<td></td>
<td>50 μM</td>
<td>69.9</td>
</tr>
<tr>
<td></td>
<td>500 nM</td>
<td>6.2</td>
</tr>
<tr>
<td>Man</td>
<td>5 mM</td>
<td>90.6</td>
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<td>50 μM</td>
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</tr>
<tr>
<td></td>
<td>500 nM</td>
<td>4.2</td>
</tr>
<tr>
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</tr>
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<td></td>
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<td>57.9</td>
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<td>50 μM</td>
<td>39.4</td>
</tr>
<tr>
<td></td>
<td>500 nM</td>
<td>1.4</td>
</tr>
</tbody>
</table>

UDP-Glc > UDP-Gal > GDP-Man > UDP-GlcNAc > UDP-Xyl

**Table 4.1.** Summary of results for the kinetics of SunS glycosyltransferase activity with NDP-sugars. The relative amounts of sugar-modified and unmodified His\(_6\)-SunA peptide was determined by quantifying the HPLC peak area corresponding to each form of the peptide by integration. Percent conversion was calculated by dividing the peak area of sugar-modified peptide by the sum of the peak areas corresponding to sugar-modified and unmodified peptide. As indicated in the table, the observed conversions suggest that SunS displays preference for NDP-sugars in the order from most preferred to least preferred substrate.
4.2.8 In vitro reconstitution of sublancin biosynthesis

Reduction of the disulfides of sublancin resulted in complete loss of antimicrobial activity against *B. subtilis* ATCC 6633 in an agar diffusion assay, showing their importance for activity (Figure 4.12). An *in vitro* oxidative folding protocol was therefore developed to investigate the effect of glycosylation on the antimicrobial activity of sublancin. His₆-SunA-Xa glucosylated by His₆-SunS was treated with Factor Xa, followed by oxidative folding through the addition of a mixture of oxidized and reduced glutathione and EDTA. Subsequent analysis by antimicrobial assays, proteolytic digests, and ESI-MSⁿ analysis verified the complete *in vitro* reconstitution of sublancin biosynthesis with the correct disulfide connectivities (see supporting data in Experimental 4.4.18). Leaving out any of the three steps (glucosylation, proteolysis or oxidative refolding) resulted in inactive material (Figure 4.23). These observations show that glucosylation is required for bioactivity and that the leader peptide inhibits bioactivity. It was not possible to investigate whether SunS can also glycosylate oxidatively folded SunA because the enzyme required the presence of reducing agent for activity.
Figure 4.23. Antimicrobial activity assay of *in vitro* prepared sublancin with omission of single steps. The importance of each step (glycosylation, leader peptide removal, and disulfide formation) in the *in vitro* preparation of sublancin was demonstrated through an antimicrobial activity assay against *B. subtilis* ATCC 6633. Sublancin samples were prepared in parallel whereby a different step of the preparation process was omitted for each of the samples. Glucose was used as the modifying sugar. The antimicrobial properties of each sample were assessed by its ability to inhibit the growth of *B. subtilis* ATCC 6633. Authentic sublancin standards were tested as positive controls. The steps that were used are indicated below. The sample in which all three steps (glycosylation, leader peptide removal, and disulfide formation) were used displayed a zone of inhibition (far left). The samples which did not undergo glucosylation (middle left), had the leader peptide intact (middle right), or did not undergo oxidative folding (far right) did not show inhibitory activity.

### 4.2.9 *In vitro* production of sublancin analogues

Interestingly, the SunA core peptides with different sugar moieties attached to Cys22 were also amenable to oxidative folding resulting in various sublancin analogues with the correct disulfide connectivities as determined by proteolytic digests and ESI-MS" analysis (see Experimental 4.4.18). Bioassays demonstrated that the native (glucosylated) sublancin was
slightly more active than these analogues (Figure 4.24), but it cannot be ruled out that small differences observed in the inhibition zones may be the result of small amounts of co-eluting peptides with alternative disulfide topologies that may be present in the final material. Regardless, although glycosylation is required for bioactivity, the stereochemistry of the hexose appears to be less critical.

**Figure 4.24.** Antimicrobial activity assays of *in vitro* produced sublancin and sublancin analogues against *B. subtilis* 6633. The type of sugar attached to Cys22 is indicated. Compounds were produced by incubating His6-SunA-Xa with His6-SunS and NDP-sugar, followed by proteolysis with Factor Xa to remove the leader peptide, and subsequent oxidative folding to generate the disulfides. Authentic sublancin standards produced and purified from *B. subtilis* 168 were used as positive controls.
4.2.10 Efforts toward identifying the specific molecular target of sublancin

It is unknown if sublancin has a precise molecular target which it utilizes to affect sensitive bacteria cells. In a previous report, the bioactivity of sublancin was evaluated against a panel of Gram-positive and Gram-negative bacteria. Sublancin was active against only a subset of Gram-positive species. Inhibitory activity was strongest against related bacilli including \textit{B. megaterium} and \textit{B. subtilis} 6633 with low micromolar MICs, and relatively weaker against cocci including \textit{Staphylococcus aureus} and \textit{Streptococcus pyogenes} (mid to high micromolar MICs). This overall narrow spectrum and weaker inhibitory activity may be an indication that sublancin does not bind to a precise target or may do so, but with weak affinity.

Lipid II has been identified as the molecular target for many peptide-based antibiotics including members of the post-translationally modified lantibiotics family\textsuperscript{37-40} and several glycopeptides.\textsuperscript{41-46} A recently developed transglycosylase assay\textsuperscript{47} was utilized to determine if sublancin binds to lipid II (see Section 2.2.6). Sublancin did not demonstrate any ability to bind lipid II to inhibit the transglycosylase reaction even at high concentrations up to 200 μM (Figure 4.25).
Figure 4.25. *In vitro* transglycosylase assays using PBP1b, lipid II, and sublancin peptide. Sublancin does not demonstrate inhibition at concentrations as high as 200 μM. When errors bars are not visible, the error was smaller than the size of the symbol used. The horizontal axes indicate the concentrations of sublancin used in the reaction. The vertical axes indicate the relative amount of peptidoglycan formed in each reaction. The %PG (relative) is defined by \([\text{polymerized peptidoglycan} / (\text{total free lipid II} + \text{polymerized peptidoglycan})]\) normalized to 100%.

4.2.11 Discovery of biosynthetic gene clusters for putative S-linked glycopeptides

A search of the databases revealed several gene clusters in diverse host organisms that are similar to the sublancin cluster, including genes for putative precursor peptides and putative S-glycosyltransferases (Figure 2.26). The precursor peptides have low sequence identity but all are rich in Cys residues in the predicted core peptide. Thus, S-linked glycopeptides may be more common than currently appreciated. Many putative precursor peptides have 5 Cys residues within their predicted core peptide sequences, and therefore may share structural features with sublancin including two disulfide bonds and a single modified Cys residue.
Figure 4.26. (a) Representative gene clusters containing both a short open reading frame that may encode a substrate peptide and a glycosyl transferase with homology to SunS. Glycosyl transferase genes shown in green; genes encoding substrate peptide in black; genes encoding transporters shown in red; genes encoding thiol/disulfide isomerases shown in yellow, and genes encoding putative immunity proteins shown in light blue. (b) Sequence alignments of the sublancin precursor peptide with other peptides. Database mining of sequenced genomes revealed several clusters that could potentially harbor genes that encode for glycopeptide precursor peptides. These precursor peptide genes were found clustered near genes encoding for putative glycosyltransferase enzymes and were found among phylogenetically diverse bacteria. Fully conserved residues are in red font. Overall the sequence conservation is low, except for among the four variants in *B. cereus* where the conservation is moderate in the predicted core peptides (the peptide following the predicted GG or GS protease cleavage site). All leader peptides are rich in Glu, as previously noted as a general observation in leader peptides involved in natural product peptide biosynthesis. All cysteines are highlighted in green. Like sublancin, the precursor peptides from all organisms except *B. cereus* and *E.*
faecalis contain five cysteine residues. The cysteine that is glycosylated in sublancin is indicated with an asterisk and is conserved in all peptides. The precursor peptides from B. cereus and E. faecalis have an even number of cysteine residues. If they are involved in two disulfides as in sublancin, then they cannot be glycosylated. The glycosyltransferases in their gene clusters may then function as O-glycosyl transferases.

Each identified cluster contains a single gene encoding for a putative glycosyltransferase enzyme which is predicted to install a sugar post-translational modification onto the precursor core peptide (Figure 4.27). There is high sequence variability at the N-termini of the selected set of glycosyltransferases; however, from residues 100 to 300, there is remarkable sequence conservation. Finally, the sequences of the C-terminal tail amongst the putative glycosyltransferases are again highly variable. Analysis of the SunS amino acid sequence by I-TASSER, a de novo protein structure prediction program, identified two distinct domains that included an N-terminal catalytic domain of approximately 370 residues and a C-terminal tail of approximately 50 residues (Figure 4.28). The N-terminal 370 amino acids shares high sequence identity with human UDP-GalNAc polypeptide alpha-N-acetylgalactosaminyltransferase-2 catalytic domain, whereas the C-terminal tail does not share significant identity with other known proteins. Interestingly, this polypeptide alpha-N-acetylgalactosaminyltransferase-2 homolog has a disposable C-terminal domain in addition to its catalytic N-terminal domain. In an effort to determine if SunS also possesses a disposable C-terminal domain, Dr. Huan Wang introduced a stop codon just 3’ of the 370 amino acid of SunS and subsequently expressed and purified the predicted N-terminal catalytic domain of the glycosyltransferase. Remarkably, the truncated protein still possessed glycosyltransferase activity, albeit its high specificity for exclusive modification of Cys22 was lost. More than one site of glycosylation along the SunA precursor sequence was observed (H. Wang and W. van der Donk, unpublished results).
Figure 4.27. (continued on next page)
Figure 4.27. Sequence alignments of SunS with other putative glycosyltransferases located near genes encoding short peptides. Database mining of sequenced genomes revealed several clusters containing a gene for a putative glycosyltransferase. These glycosyltransferase genes were found clustered near genes encoding for potential for glycopeptide precursor peptides. The putative glycosyltransferase enzymes were found among phylogenetically diverse bacteria. The asterisks (*) above the sequences indicate the putative divalent metal (Mg$^{2+}$, Mn$^{2+}$) binding site.

Figure 4.28. De novo structure prediction results for SunS. N-Terminal domain shown on left side of image, C-terminal domain on right side.
In addition to the presence of a substrate peptide and glycosyltransferase, many of the identified biosynthetic clusters contain two genes encoding for putative thiol-disulfide oxidoreductase/isomerase or thioredoxin-like proteins with sequence homology to \( bdbA \) and \( bdbB \) genes. Each cluster also contains a gene encoding for a putative ATP-dependent transporter much like that of \( sunT \) which encodes for the dedicated bi-functional transporter and protease responsible for export and leader peptide removal in the final steps of sublancin maturation.

### 4.2.12 Identification and characterization of thuringiencin

One of the many putative \( S \)-linked glycopeptide biosynthetic gene clusters identified through bioinformatics analysis was further characterized. The biosynthetic gene cluster found in \textit{Bacillus thuringiensis serovar andalouisiensis} BGSC 4AW1 (Figure 4.29) contains a putative precursor gene (termed \( thuA \), NCBI: bthur0009_56250, ZP_04099938) encoding for 80 residues including the N-terminal Met. Based on sequence homology with other known bacteriocins and the glycopeptide sublancin, it is predicted that this precursor sequence contains an N-terminal leader peptide of 38 residues and a C-terminal core peptide of 42 amino acids (Figure 4.30a). The C-terminal region of the leader peptide is remarkably glycine rich and its leader sequence

![Figure 4.29. Putative biosynthetic gene cluster identified via genome mining for the production of the \( S \)-glycopeptide, thuringiencin. The gene encoding for the glycosyltransferase shown in green; gene encoding substrate peptide in black; genes encoding transporter shown in red; and genes encoding thiol/disulfide isomerases shown in yellow.](image)
resembles that of the double glycine type. Overall, the ThuA leader peptide is significantly longer than that of SunA (19 residues). In consideration of the dispensability of the SunA leader peptide, this two-fold increase in length may suggest that the ThuA leader sequence will have an influence on core peptide modification. The predicted thuringiencin core peptide displays high sequence similarity with sublancin. Both peptides share a GXGXAQCA motif at their core peptide N-termini as well as a CXSGG(T/S) sequence just N-terminal to their expected Cys glycosylation site (Figure 4.30b).

**Figure 4.30.** (a) Primary sequence of the full length precursor peptide ThuA. Leader is shown in blue, core peptide in black, and predicted cleavage site is underlined in red. (b) Sequence alignment of SunA and ThuA precursor peptides. Fully conserved residues text in red and highlighted in yellow. Similar residues highlighted in green. Fully conserved Cys residues of the core peptide bolded and underlined.

Like the gene cluster for the biosynthesis of sublancin, genes encoding for enzymes of similar function were also found in the thuringiencin biosynthetic gene cluster including two putative thiol-disulfide oxidoreductases (NCBI: bthur0009_56260 and unannotated gene immediately downstream to bthur0009_56280), an ATP-dependent transporter with an N-terminal transport domain and C-terminal protease domain (NCBI: bthur0009_56260), and a putative glycosyltransferase (NCBI: bthur0009_56280) sharing 39.3% amino acid sequence identity with SunS. To date, none of these genes or their gene products have been characterized.
4.2.13 *In vitro* reconstitution of ThuS glycosyltransferase activity

To confirm its predicted function, the gene encoding for the putative glycosyltransferase (termed *thuS*) was cloned from isolated *Bacillus thuringiensis serovar andalousiensis* BGSC 4AW1 genomic DNA and was expressed in *Escherichia coli* as an N-terminal fusion protein with a hexa-histidine tag (His$_6$-ThuS, Figure 4.31). Upon purification by immobilized metal affinity chromatography, His$_6$-ThuS was incubated with the putative precursor peptide His$_6$-ThuA, also cloned and expressed in *E. coli*. Addition of uridine diphosphate $\alpha$-D-glucose (UDP-Glc) and Mg$^{2+}$ resulted in conversion of ThuA to a product consistent with glucosylation at two sites (Figure 4.32). Subsequent trypsin digest and ESI-q/TOF MS analysis led to the identification of a digest fragment spanning residues Ala69-Arg96 that contained the two glucose modifications (Figure 4.33a), numbering relative to predicted start site of full-length His$_6$-ThuA peptide). Tandem mass spectrometry analysis of this digest fragment verified that single glucose modifications were installed at residues Cys85 and Ser76 (Figure 4.33b).

![Figure 4.31. Purification of ThuS glycosyltransferase. The gene *thuS* was cloned and expressed in *Escherichia coli* as an N-terminal fusion protein with a hexa-histidine tag (His$_6$-ThuS). His$_6$-ThuS consists of 447 amino acids and has a predicted molecular weight of 52,240 Da. Shown is an SDS-PAGE (BioRAD Ready Gel 4-20%) analysis of purified ThuS protein.](image)
The ability of ThuS to modify its precursor peptide at two different sites was not anticipated provided that SunS was only able to modify its substrate at a single site. Moreover, each site of modification by ThuS is chemically different as well. A total of 5 Cys reside within the ThuA core peptide. Despite its promiscuous chemoselectivity, ThuS shares similar regioselective properties as SunS and catalyzes modification of residues in which the residue to be modified is flanked by small hydrophobic residues located approximately halfway along the core peptide backbone. Mutagenesis studies of the ThuA precursor sequence are necessary to fully identify the specific “rules” ThuS follows to preferentially modify the core peptide at a limited number of sites.

![Figure 4.32](image)

**Figure 4.32.** *In vitro* reconstitution of ThuS activity. MALDI-TOF MS spectra of His<sub>6</sub>-ThuA before (black) and after (red) incubation with His<sub>6</sub>-ThuS, UDP-glucose, and Mg<sup>2+</sup>. 
Figure 4.33. Determination of the site of in vitro modification of His₆-ThuA by His₆-ThuS. (a) His₆-ThuA was modified with glucose, digested with trypsin under reducing conditions, and analyzed by using LC-ESI-Q/TOF MS.
Trypsin cleavage sites (K and R residues) are indicated in the peptide sequence above the spectrum. The masses of the observed ions and their corresponding digest fragments are assigned. The results are consistent with residues Ala69-Arg96 bearing two +162 Da post-translational modifications. (b) The digest mixture was fractionated using LC and the multiply charged ion corresponding to doubly modified Ala69-Arg96 (3177.3232 Da) was selected for MSMS analysis. Fragmentation pattern is indicated for the y” ion series. The results confirm that both Cys85 and Ser76 have a +162 Da post-translational modification.

4.2.14 ThuS displays the ability to modify unnatural substrate peptides

The successful *in vitro* reconstitution of ThuS glycosyltransferase activity confirms an additional substrate-enzyme pair involved in the biosynthesis of an S-linked glycopeptide. Though many other pairs are likely to exist, ThuA-ThuS and SunA-SunS represent the only substrate-enzyme cognate pairs identified and characterized to date for S-glycosylation. To determine the ability of the glycosyltransferases to act upon unnatural, but homologous substrates, a precursor peptide (SunA or ThuA) was incubated with either glycosyltransferase (SunS or ThuS) in the presence of UDP-Glc and Mg$^{2+}$. Remarkably, ThuS was able to doubly modify SunA peptide (Figure 4.34). Subsequent chymotrypsin treatment of ThuS-modified SunA peptide generated a series of digest fragments (Figure 4.35). The digest fragment corresponding to residues Leu12-Tyr32 contained both sugar modifications. Tandem mass spectrometry analysis of this digest fragment clearly identified a glucosylation at Cys22 (Figure 4.36). The second glucosylation occurred on either Ser16, Thr19, or Cys29 in a mutually exclusive manner (Figure 4.37). Thus, a mixture of modified ThuA products was obtained bearing modifications at Cys22 and Ser16, modifications at Cys22 and Thr19, or modifications at Cys22 and Cys29. SunS glycosyltransferase was not able to modify ThuA peptide.
Figure 4.34. Modification of His6-SunA with glucose was achieved by incubation with His6-ThuS in the presence of UDP-Glc, MgCl₂, and TCEP in Tris (pH 7.5) buffer. The reaction was analyzed by MALDI-TOF MS. The overlay of mass spectra obtained by MALDI-TOF MS analysis of the mixture before (black) and after ThuS reaction (red) is shown.
Figure 4.35. His\textsubscript{6}-SunA was double'ly modified with glucose by His\textsubscript{6}-ThuS, digested with chymotrypsin under reducing conditions, and analyzed by using LC-ESI-Q/TOF MS. Chymotrypsin cleavage sites (F, W, and Y residues) are indicated in the peptide sequence above the spectrum. The masses of the observed ions and their corresponding digest fragments are assigned. The results are consistent with the peptide spanning residues Leu50-Tyr70 bearing two +162 Da post-translational modifications. "(Glc)" indicates that one of the three sites is modified on any given peptide sample (see Figure 4.37)
Figure 4.36. ESI-Q/TOF MSMS analysis of residues Leu50-Tyr70 of doubly glucose modified His$_6$-SunA by His$_6$ ThuS. His$_6$-SunA was double modified with glucose, digested with chymotrypsin under reducing conditions, and analyzed by using LC-ESI-Q/TOF MSMS. The digest mixture was fractionated using LC and the multiply charged ion corresponding to residues Leu50-Tyr70 + 2 Glc (2253.9150 Da) was selected for MSMS analysis. Fragmentation patterns are indicated for the y$^n$ ion series. The results confirm that Cys60 (Cys22 for core peptide numbering) has a +162 Da post-translational modification.
Figure 4.37. (continued on next page)
Based on the sequence homology shared between ThuA and SunA, the known topology of sublancin, and the results of the in vitro reconstitution of ThuS glycosyltransferase activity, a structure for the final product of the S-glycopeptide from Bacillus thuringiensis 4AW1, termed thuringienicin, has been proposed (Figure 4.38). Considering the activity of ThuS, two post-translational modifications are likely to exist on this natural product at positions Cys28 and Ser19. However it may be possible that only one glycosylation is present on the in vivo produced thuringienicin at Cys28, and that the double modification is in fact an artifact of an overactive
glycosyltransferase in the *in vitro* activity assays. However, the recently reported structure of the antimicrobial peptide glycocin F demonstrates that two glycosylations, at a Cys and Ser residue, can exist on a single S-glycopeptide natural product.\(^{49,50}\) It may then be possible that a single glycosylation, as observed with sublancin, is an anomaly. Furthermore, the sugar on thuringiencin may not be glucose. The isolation and purification of the natural product from the producing organism will greatly enhance our understanding of the structure, post-translational modification, and biosynthesis of this novel product.

![Proposed structure for the novel S-glycopeptide thuringienicin based on *in vitro* studies. Glycosylations were identified at residues Ser19 and Cys 28 of the core peptide.](image)

**Figure 4.38.** Proposed structure for the novel S-glycopeptide thuringienicin based on *in vitro* studies. Glycosylations were identified at residues Ser19 and Cys 28 of the core peptide.

### 4.3. SUMMARY

In an effort to identify a fifth pathway to produce lanthionine-containing peptides, a novel and unusual class of S-linked glycopeptides were discovered and examined. The work presented in this chapter represents a significant breakthrough in the structural characterization and biosynthesis of S-linked glycopeptides and opens the opportunity for the discovery of additional natural products of this type. The structure of *in vivo* produced sublancin was studied in depth.
using various approaches and the results of these studies offer a long-awaited answer to the questionable mass discrepancies noted in the original report describing sublancin. The revised structure aligns with the original reported mass of the natural product and establishes S-glucosylation as a new post-translational modification.

Examination of the sublancin biosynthetic gene cluster found in the *B. subtilis* 168 genome lead to the identification of a gene with sequence homology with known glycosyltransferases. The gene was cloned and heterologously expressed in *E. coli*. The recombinant protein was remarkably tolerant to variability of the NDP-sugar co-substrate and was able to glycosylate the sublancin precursor peptide with not only its native hexose sugar, glucose, but also a number of sugars including N-acetylglucosamine, galactose, mannose, and xylose. The SunS glycosyltransferase enzyme displayed remarkable chemo- and regio-selectivity for modification of only a single Cys among five available residues. Mutagenesis studies of the SunA precursor peptide demonstrated that flanking residues are not the recognition units utilized for regiospecificity of the glycosyltransferase enzyme. Unlike the lantibiotics and several other ribosomal peptide natural products made via leader peptide-directed biosynthesis, the leader peptide of SunA precursor was completely dispensible and is not a required element for the post-translational modification of sublancin.

Using an *in vitro* production strategy, a number of sublancin analogues were produced to possess different sugar modifications at Cys22. Bioactivity assays against *B. subtilis* 6633 demonstrated that the native (glucosylated) sublancin was slightly more active than the other analogues. In addition, glycosylation, leader peptide removal, and correct disulfide connectivity all were required for antimicrobial activity.
Bioinformatic analysis of sequenced genomes led to the identification of several biosynthetic gene clusters that may produce S-linked glycopeptides in a diverse range of bacteria. Each identified biosynthetic gene cluster harbors genes encoding for a putative precursor peptide, glycosyltransferase, transporter, and additional tailoring enzymes. One cluster from \textit{Bacillus thuringiensis serovar andalouiensis} BGSC 4AW1 was studied further. Its precursor peptide and glycosyltransferase were cloned, expressed, and characterized. The glycosyltransferase, ThuS, was found to function as an S- and O-glycosyltransferase and was able to catalyze the addition of glucose to a Cys and Ser residue of its native precursor peptide in a highly specific fashion. In addition, ThuS could modify Cys, Thr, and Ser residues along the core peptide of SunA. This glycosyltransferase demonstrates remarkable catalytic capabilities and is the second example of an enzyme of this type. Its unique catalytic properties, in combination with its good stability and solubility, make it an attractive target for crystallographic studies. Given the increased chemical stability of S-linked glycopeptides, SunS and ThuS have great potential for use in preparation of such compounds and provide a new tool for the glycodiversification of peptide natural products.
4.4. EXPERIMENTAL

4.4.1. Materials, strains and plasmids, and general methods

Materials.

All oligonucleotides were purchased from Integrated DNA Technologies. Restriction endonucleases, DNA polymerases, and T4 DNA ligase were purchased from New England Biolabs or Invitrogen. Factor Xa was purchased from New England Biolabs and chymotrypsin was purchased from Worthington Biochemical Corporation. Media for bacterial culture and chemicals were purchased from Fisher Scientific, Sigma Aldrich, or CalBiochem, unless noted otherwise, and used without further purification. Isotopically labeled media components were purchased from ISOTEC - Sigma Aldrich.

Strains and Plasmids.

Bacillus subtilis 168 (sublancin producing organism) was obtained from Bacillus Genetics Stock Center, Ohio State University, Columbus, OH. The indicator strain, Bacillus subtilis ATCC 6633 was obtained from American Type Culture Collection, Manassas, VA. Escherichia coli DH5α (UIUC Cell Media Facility) was used as host for cloning and plasmid propagation, and E. coli BL21 (DE3) and E. coli Rosetta 2 (DE3) (Novagen) were used as hosts for protein expression. Cloning vectors (pET15b and pET28b) were obtained from Novagen.

General Methods.

All molecular biology manipulations were carried out using standard techniques. Polymerase chain reaction (PCR) amplifications were carried out using an automated thermocycler (C1000, BioRAD). DNA sequencing was performed using appropriate primers by
the Biotechnology Center (University of Illinois at Urbana-Champaign, UIUC). GC-MS analysis was performed at the Roy J. Carver Metabolomics Center (UIUC). LC-ESI-Q/TOF MS analyses were conducted using a Synapt MS system equipped with an Acquity UPLC (Waters). MALDI-TOF MS analyses were conducted at the Mass Spectrometry Facility (UIUC) using a Voyager DE-STR (Applied Biosystems). For MALDI-TOF MS analysis of salt-free samples, a 1 μL aliquot of analyte was combined with 1 μL of matrix (saturated α-cyano-4-hydroxy-cinnamic acid matrix in 50% ACN/50% water with 0.1% TFA), and the total volume was spotted onto a MALDI target and dried under ambient conditions prior to analysis. Salt containing samples were desalted using ZipTipC18 (Millipore), eluted with 4 μL of matrix, and 2 μL was spotted onto a MALDI target and dried as described above.

**LC-ESI-Q/TOF MS and MSMS analyses.**

A 5 μL volume of sample was injected on a Waters Acquity UPLC system equipped with a BEH C8 column (1.7 um, 100 mm x 1.0 mm) equilibrated in 3% B (solvent A = 0.1% formic acid in water, solvent B = 0.1% formic in methanol). The sample was fractionated by employing a gradient of 3%-97% B over 12 min and directly subjected to ESI-Q/TOF MS using a Waters Synapt mass spectrometer. Data was acquired in ESI positive mode with the capillary voltage set to 3.0-3.5 kV. Nitrogen was used as cone gas (0 L/h) and desolvation gas (600 L/h). The ionization source and desolvation gas were heated to 120 °C and 300 °C, respectively. The transfer collision energy was set to 4 V for both MS and MSMS analyses. The trap collision energy was set to 6 V (constant) for MS. For MSMS analysis, multiply charged parent ions were selected and fragmented using collision induced dissociation (nitrogen) with a trap collision energy ramp ranging from 20-60 V or a constant value between 25-45 V. The applied collision
energies for MSMS were analyte dependent. [Glu$^1$]-Fibrinopeptide B (Sigma) was directly infused as lock mass with lock spray sampling at minute intervals. The acquired spectra were processed using MaxEnt3 software and analyzed by Protein/Peptide Editor in BioLynx 4.1 (Waters).

4.4.2. Isolation and purification of sublancin

A culture of *B. subtilis* 168 was grown in LB media under aerobic conditions at 37 °C for 12-15 h. The overnight culture was used to inoculate (at 1%) 500 mL volumes of Medium A in 2 L flasks. Medium A consisted of 900 mL of Medium A nutrient broth combined with 100 mL of 10X Medium A salts. Medium A nutrient broth was prepared by dissolving 20 g sucrose, 11.7 g citric acid, 4 g Na$_2$SO$_4$, 4.2 g (NH$_4$)$_2$HPO$_4$, and 5 g yeast extract in 900 mL of millipore water. The pH was adjusted to 6.8-6.9 using NaOH and the medium was autoclaved. Medium A salts (10X) were prepared by dissolving 7.62 g KCl, 4.18 g MgCl$_2$-6 H$_2$O, 0.543 g MnCl$_2$-4 H$_2$O, 0.49 g FeCl$_3$-6 H$_2$O, and 0.208 g ZnCl$_2$ in 1 L of millipore water followed by sterilization via 0.22 μm filtration. The Medium A cultures were grown under aerobic condition at 37 °C for 28-48 h with vigorous agitation. A color change to pinkish-brown was observed and the pH of cultures had lowered to 6-6.5. Sublancin production was consistently observed when these events occurred.

Cultures were acidified to pH 2.5 with concentrated phosphoric acid (85% in water) and centrifuged to remove cells and insoluble material. Sublancin was isolated by ammonium sulfate precipitation. Briefly, a 500 mL volume of sublancin-containing culture supernatant was combined with 245 g (NH$_4$)$_2$SO$_4$ in a 1 L glass bottle to provide a 75% ammonium sulfate saturation at 4 °C. The precipitation mixture was stirred at 4 °C for 24 h and precipitated sublancin was isolated from the solution by centrifugation. The pelleted peptide was re-
solubilized in 50/50 ACN/water with 0.1% TFA and was analyzed by MALDI-TOF MS. A 1 μL aliquot of sample was combined with 1 μL of matrix consisting of saturated α-cyano-4-hydroxycinnamic acid matrix in 50% ACN/50% water with 0.1% TFA, and the total volume was spotted onto a MALDI target and dried under ambient conditions prior to analysis. The sublancin containing product were combined, lyophilized to dryness, and stored under N₂ at −80 °C until purification by preparative HPLC.

Preparative HPLC was performed using a Waters Delta 600 instrument equipped with a Phenomenex Jupiter Proteo C12 column (10 μm, 90 Å, 250 mm x 15 mm) equilibrated in 2% B (solvent A = 0.1% TFA in water, solvent B = 0.0866% TFA in 80% ACN/20% water). Dry sublancin material was resuspended in 2% B and was applied to the column. Sublancin was eluted by maintaining the mobile phase at 2% B for 1 min, followed by an increase to 100% B over 45 min with a flow rate of 10.0 mL/min. Under these conditions, sublancin eluted at 22.7 min. All fractions were analyzed by MALDI-TOF MS as described above. Purified sublancin was lyophilized to dryness and stored under N₂ at −80 °C until further use. Typical yields were 10-15 mg sublancin per liter of processed Medium A culture.

4.4.3. Bioinformatics analysis of gene clusters

Analysis of the biosynthetic gene cluster of sublancin

Information on the biosynthetic gene cluster responsible for in vivo production of sublancin containing genes sunI, sunA, sunT, bdbA, yolJ (sunS), and bdbB was accessed via the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/) with the following accession numbers: RefSeq = NC_000964 and GenBank = AL009126. The gene
The gene cluster is 5080 nucleotides in length, is of chromosomal origin, and is found between base pairs 2,270,305 – 2,265,225 (complement).

**Analysis of the biosynthetic gene cluster of thuringiencin.**

Information on the biosynthetic gene cluster responsible for production of thuringiencin containing genes: *thuA* (bthur0009_56250), *sunT* homolog (bthur0009_56260), *bdbA* homolog (bthur0009_56270), *thuS* (bthur0009_56280), and *bdbB* homolog (unannotated, immediately downstream of bthur0009_56280) was accessed via the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/) with the following accession numbers: RefSeq = NZ_ACNG00000000 and GenBank = ACNG01000000. The gene cluster is 4713 nucleotides in length, is of chromosomal origin, and is found between base pairs 5,424,818 – 5,429,531.

**4.4.4. Iodoacetamide assays for detection of free cysteines**

To detect the presence of free cysteine thiols in native and reduced sublancin, an iodoacetamide assay was used. For the detection of free Cys residues in native sublancin, reactions contained 50 mM Tris (pH 8.3), 10 mM iodoacetamide, and 25 μM sublancin. For the detection of free Cys residues in reduced sublancin, reactions contained 50 mM Tris (pH 8.3), 5 mM TCEP, 10 mM iodoacetamide, and 25 μM sublancin (50 μL total volume). All reactions were incubated for 2.5 h at 25 °C in the dark. At 2.5 h, 10 μL of reaction was quenched with 5% TFA to pH 1-2. Samples were desalted using Zip-Tip<sub>C18</sub> prior to MALDI-TOF MS analysis. Samples evaluated by LC-ESI-Q/TOF MS were analyzed without further manipulation. The
detection of free cysteines was determined by the presence or absence of carboxyamidomethyl (CAM) thiol modifications.

4.4.5. Chymotrypsin digests of sublancin

For analysis of native sublancin, the peptide was digested under non-reducing conditions in order to maintain the integrity of the in vivo formed disulfides. The digestion sample contained 100 mM Tris (pH 7.5), 0.05 mg/mL chymotrypsin (Worthington), and 0.5 mg/mL sublancin. For analysis of the reduced peptide, 0.5 mg/mL sublancin was digested in 100 mM Tris (pH 7.5), 5 mM TCEP, 0.05 mg/mL chymotrypsin. All reactions were incubated at 25 °C for 5 h and then quenched with 5% TFA to pH 1-2. Quenched samples were desalted with a ZipTipC18 prior to analysis by MALDI-TOF MS or by LC-ESI-Q/TOF MS.

4.4.6. Determination of the natural sugar modification of sublancin

The presence of glucose on Cys22 of sublancin isolated from B. subtilis 168 was confirmed via acid-catalyzed hydrolysis and derivatization of the sugar, analysis by gas chromatography-mass spectrometry (GC-MS), and comparison to derivatized hexose standards. Lyophilized sublancin (approximately 0.5-1.0 mg) was dissolved in 1 mL of 0.5 M HCl in water and heated at 75 °C in a high-pressure, sealed vessel for 5 h. The reaction was cooled, transferred to a microcentrifuge tube with the aid of millipore water, and quenched by addition of aqueous NaOH to pH 5. The total volume was concentrated via vacuum centrifugation. After complete drying, 50 μL of 20 mg/mL O-methylhydroxylamine hydrochloride (Fluka) in pyridine was added to the sample. The sample was vortexed for 1 min and was incubated at 50 °C for 1 h. Next, 50 μL of N-trimethylsilyl-N-methyl trifluoroacetamide (Fluka) was added. Again, the
sample was vortexed for 1 min and incubated at 50 °C for 1 h. The sample was cooled at 25 °C for 5 min prior to vortexing for 1 min followed by centrifugation at 15 krpm in a microcentrifuge for 2 min. Hexose sugar standards D-(+)-glucose, D-(+)-galactose, D-(+)-mannose, and D-(−)-fructose were also treated and derivatized using the conditions described above.

The derivatized hexose of sublancin and sugar standards were analyzed individually and in combinations using an Agilent GC-MS system (6890N GC, HP 5973 mass selective detector) equipped with a Phenomenex ZB-1MS column (30 m x 0.32 mm i.d., 0.25 μm df) (Figure 4.39a-h). Samples were introduced via split injection (3:1 split ratio). The temperature gradient used was 150 °C for 2 min, then 150 °C to 185 °C over 3.5 min (10 °C/min ramp), then 185 °C to 210 °C over 12.5 min (2 °C/min ramp), then 210 °C to 300 °C over 9 min (10 °C/min ramp). The carrier gas was helium and was set at a 3.0 mL/min flow rate. The mass selective detector was operated in positive ion scan mode (25-800 m/z). Analyses were performed using electron impact (EI) mode and to preserve the filament of the ionization source, MS records were performed 4 min after injection.
Figure 4.39a. GC-MS analysis of glucose. D-(+)-Glucose standard was derivatized as described in methods and analyzed by GC-MS. The total ion chromatogram (TIC) is shown with the retention times as indicated (upper). Below the chromatogram are EI mass spectra corresponding to the peaks at 11.017 min (lower left) and 11.399 min (lower right) that correspond to the two isomers of the sugar generated during derivatization. The characteristic ions for TMS derivatized glucose are annotated within the spectra (319, 205, 147, 103, and 73 m/z).
Figure 4.39b. GC-MS analysis of galactose. D-(+)-Galactose standard was derivatized and analyzed by GC-MS. The TIC is shown with the retention times as indicated (upper). Below the chromatogram EI mass spectra corresponding to the peaks at 10.890 min (lower left) and 11.332 min (lower right) are shown that correspond to the two isomers of the sugar generated during derivatization. The characteristic ions for TMS derivatized galactose are annotated within the spectra (319, 205, 147, 103, and 73 m/z).
Figure 4.39c. GC-MS analysis of mannose. D- (+)-Mannose standard was derivatized and analyzed by GC-MS. The TIC is shown with the retention times as indicated (upper). Below the chromatogram, EI mass spectra corresponding to the peaks at 10.811 min (lower left) and 11.143 min (lower right) are shown that correspond to the two isomers of the sugar generated during derivatization. The characteristic ions for TMS derivatized mannose are annotated within the spectra (319, 205, 147, 103, and 73 m/z).
Figure 4.39d. GC-MS analysis of fructose. D-(-)-Fructose standard was derivatized and analyzed by GC-MS. The TIC is shown with the retention times as indicated (upper). Below the chromatogram, EI mass spectra corresponding to the peaks at 10.489 min (lower left) and 10.675 min (lower right) are shown that correspond to the two isomers of the sugar generated during derivatization. The characteristic ions for TMS derivatized fructose are annotated within the spectra (307, 217, 147, 103, and 73 m/z).
Figure 4.39e. Resolution of derivatized standards by GC-MS. A mixture of derivatized D-(+)-glucose, D-(+)-galactose, D-(+)-mannose, and D-(−)-fructose standards was analyzed by GC-MS. Shown are the TIC for the mixture (upper) and the overlay of the TIC for the mixture and the total ion chromatograms from each individually analyzed sugar (lower). The EI mass spectra corresponding to the individual peaks of the mixture were essentially identical to the spectra obtained when each sugar standard was analyzed individually (Figure 4.39a-d).
Figure 4.39f. GC-MS analysis of the sugar attached to sublancin. The S-linked hexose sugar conjugated to Cys22 of *in vivo* isolated sublancin was chemically released via acid hydrolysis, derivatized, and analyzed by GC-MS. The TIC is shown with the retention times as indicated (upper). EI mass spectra corresponding to the peaks at 11.023 min (lower left) and 11.395 min (lower right) are shown that correspond to the two isomers of the sugar generated during derivatization. The hexose sugar was identified as glucose, as determined by retention time and comparison of the mass spectra with the glucose standard. The characteristic ions for TMS derivatized glucose are annotated within the spectra (319, 205, 147, 103, and 73 m/z).
Figure 4.39g. Identification of the sugar in sublancin as glucose. An overlay of the total ion chromatograms for the sublancin-derived and silylated sugar sample and for the mixture of silylated sugar standards is shown (upper: full chromatogram, lower: zoom to peaks of interest).
Figure 4.39h. Confirmation of glucose as the sugar in sublancin by spiking with a standard. To confirm the presence of S-linked glucose conjugated to Cys22 of in vivo isolated sublancin, the sublancin derived sugar sample was spiked with glucose standard and analyzed by GC-MS. An overlay of the total ion chromatograms for the sublancin derived sugar sample and for the sublancin sample with glucose spike is shown (upper: full chromatogram, lower: zoom to peaks of interest). The EI-MS mass spectra corresponding to the sublancin sample with glucose spike were similar to the spectra obtained when the sublancin derived sugar sample (without added glucose) was analyzed. The characteristic ions for TMS derivatized glucose were observed.
4.4.7. Molecular cloning of precursor and glycosyltransferase genes

Molecular cloning of \textit{sunA} and \textit{sunS} genes.

Genomic DNA was isolated from an overnight culture of \textit{B. subtilis} 168 using a Microbial DNA Isolation Kit (MO-BIO) according to the manufacturer’s protocol. The gene \textit{sunA} was PCR amplified by 30 cycles of denaturing (94 °C for 1 min), annealing (50 °C for 1 min), and extending (72 °C for 1 min) using SunA NdeI FP (5’-CGGCAGCCATATGGAAAAAGCTATTAAAGA -3’) and SunA XhoI RP (5’-GATCCTCGAGTTATCTGCAGAATTGACGATAG -3’) as primers and \textit{B. subtilis} 168 genomic DNA as template (\textbf{bold} indicates restriction sites). The PCR mixture included 1X PCR Buffer (Invitrogen), dNTPs (0.25 mM each), MgCl\textsubscript{2} (1.5 mM), Platinum Taq DNA polymerase (0.05 U/μL), and primers (0.5 μM each). Amplifications were confirmed by 2% agarose gel electrophoresis, and the PCR products were purified using a QIAQuick PCR Purification Kit (QIAGEN). The insert DNA fragment and pET15b vector were double digested in separate reactions containing 1X NEBuffer 4 (New England Biolabs) with \textit{NdeI} and \textit{XhoI} for 15 h at 37 °C. The \textit{sunA} and pET15b digests were purified by 2% and 1% agarose gel electrophoresis, respectively, and gel extracted using a QIAquick Gel Extraction Kit (QIAGEN). The resulting DNA products were ligated at 25 °C for 5 h in 1X T4 DNA Ligase buffer with T4 DNA Ligase (0.7 U/μL). \textit{E. coli} DH5α cells were transformed with 2.5 μL of the ligation product by heat shock, and cells were plated on LB-ampicillin agar plates and grown for 15 h at 37 °C. Several colonies were picked and used to inoculate separate 5 mL cultures of LB-ampicillin medium. The cultures were grown at 37 °C for 12 h, and plasmids were isolated using a QIAprep Spin Miniprep Kit (QIAGEN). The sequences of the resulting plasmid products were confirmed by DNA sequencing.
The gene *sunS* (previously designated as *yolJ*) was PCR amplified by 30 cycles of denaturing (94 °C for 1 min), annealing (50 °C for 1 min), and extending (72 °C for 2 min) using SunS NdeI FP (5’- GCGGCAGCCATATGAAACTGAGTGATATTTA -3’) and SunS XhoI RP (5’- GTGCTCGAGTCATACTTCAATTCCTTTACAGG -3’) as primers and *B. subtilis* 168 genomic DNA as template (bold indicates restriction sites). The PCR mixture included 1X PCR Buffer (Invitrogen), dNTPs (0.25 mM each), MgCl₂ (1.5 mM), Platinum *Taq* DNA polymerase (0.05 U/μL), and primers (0.5 μM each). Amplifications were confirmed by 2% agarose gel electrophoresis, and the PCR products were purified using a QIAQuick PCR Purification Kit (QIAGEN). The insert DNA fragment and pET28b vector were double digested in separate reactions containing 1X NEBuffer 4 (New England Biolabs) with *Ndel* and *XhoI* for 15 h at 37 °C. The *sunS* and pET28b digests were purified by 2% and 1% agarose gel electrophoresis, respectively, and gel extracted using a QIAquick Gel Extraction Kit (QIAGEN). The resulting DNA products were ligated at 25 °C for 5 h in 1X T4 DNA Ligase buffer with T4 DNA Ligase (0.7 U/μL). *E. coli* DH5α cells were transformed with 2.5 μL of the ligation product by heat shock, and cells were plated on LB-kanamycin agar plates and grown for 15 h at 37 °C. Several colonies were picked and used to inoculate separate 5 mL cultures of LB-kanamycin medium. The cultures were grown at 37 °C for 12 h, and plasmids were isolated using a QIAprep Spin Miniprep Kit (QIAGEN). The sequences of the resulting plasmid products were confirmed by DNA sequencing.
Molecular cloning of \textit{thuA} and \textit{thuS} genes.

Genomic DNA was isolated from an overnight culture of \textit{B. thuringiensis serovar andalousiensis} BGSC 4AW1 using a Microbial DNA Isolation Kit (MO-BIO) according to the manufacturer’s protocol. The gene \textit{thuA} was PCR amplified by 30 cycles of denaturing (94 °C for 1 min), annealing (50 °C for 1 min), and extending (72 °C for 1 min) using ThuA NdeI FP (5’- CGGCAGCCATATGAAAGAACTAATCAAAGA -3’) and ThuA XhoI RP (5’- GATCCTCGAGTTAACAGTATTTTCTTGCTAATTCCAC -3’) as primers and \textit{B. subtilis} 168 genomic DNA as template (bold indicates restriction sites). The PCR mixture included 1X PCR Buffer (Invitrogen), dNTPs (0.25 mM each), MgCl$_2$ (1.5 mM), Platinum Taq DNA polymerase (0.05 U/μL), and primers (0.5 μM each). Amplifications were confirmed by 2% agarose gel electrophoresis, and the PCR products were purified using a QIAQuick PCR Purification Kit (QIAGEN). The insert DNA fragment and pET15b vector were double digested in separate reactions containing 1X NEBuffer 4 (New England Biolabs) with \textit{NdeI} and \textit{XhoI} for 15 h at 37 °C. The \textit{thuA} and pET15b digests were purified by 2% and 1% agarose gel electrophoresis, respectively, and gel extracted using a QIAquick Gel Extraction Kit (QIAGEN). The resulting DNA products were ligated at 25 °C for 5 h in 1X T4 DNA Ligase buffer with T4 DNA Ligase (0.7 U/μL). \textit{E. coli} DH5α cells were transformed with 2.5 μL of the ligation product by heat shock, and cells were plated on LB-ampicillin agar plates and grown for 15 h at 37 °C. Several colonies were picked and used to inoculate separate 5 mL cultures of LB-ampicillin medium. The cultures were grown at 37 °C for 12 h, and plasmids were isolated using a QIAprep Spin Miniprep Kit (QIAGEN). The sequences of the resulting plasmid products were confirmed by DNA sequencing.
The gene *thuS* was PCR amplified by 30 cycles of denaturing (94 °C for 1 min), annealing (50 °C for 1 min), and extending (72 °C for 2 min) using ThuS NheI FP (5’-CCATATGGCTAGCGTGGAACATTAAC -3’) and ThuS XhoI RP (5’-GTGCTCGAGTTATATTTTTTGCTGATATGAGTATTTATTG -3’) as primers and *B. subtilis* 168 genomic DNA as template (*bold* indicates restriction sites). The PCR mixture included 1X PCR Buffer (Invitrogen), dNTPs (0.25 mM each), MgCl₂ (1.5 mM), Platinum *Taq* DNA polymerase (0.05 U/μL), and primers (0.5 μM each). Amplifications were confirmed by 2% agarose gel electrophoresis, and the PCR products were purified using a QIAquick PCR Purification Kit (QIAGEN). The insert DNA fragment and pET28b vector were double digested in separate reactions containing 1X NEBuffer 4 (New England Biolabs) with *Ndel* and *XhoI* for 15 h at 37 °C. The *sunS* and pET28b digests were purified by 2% and 1% agarose gel electrophoresis, respectively, and gel extracted using a QIAquick Gel Extraction Kit (QIAGEN). The resulting DNA products were ligated at 25 °C for 5 h in 1X T4 DNA Ligase buffer with T4 DNA Ligase (0.7 U/μL). *E. coli* DH5α cells were transformed with 2.5 μL of the ligation product by heat shock, and cells were plated on LB-kanamycin agar plates and grown for 15 h at 37 °C. Several colonies were picked and used to inoculate separate 5 mL cultures of LB-kanamycin medium. The cultures were grown at 37 °C for 12 h, and plasmids were isolated using a QIAprep Spin Miniprep Kit (QIAGEN). The sequences of the resulting plasmid products were confirmed by DNA sequencing.
4.4.8. Molecular engineering and mutagenesis of precursor genes

Molecular engineering of a Factor Xa cleavage site into the sunA gene.

A site-directed mutagenesis approach was used to install a proteolytic cleavage site directly N-terminal to the core peptide of SunA. The primers were designed to contain nucleotide sequences necessary to encode the amino acids IEGR in place of four wild-type peptide residues (QKGS). The plasmid pET15b sunA Xa was PCR amplified by 30 cycles of denaturing (94 °C for 1 min), annealing (55 °C for 1 min), and extending (72 °C for 6 min) using SunA Xa FP (5'-GAGGAACCTGAAAACATCGAAGGTCGGATTAGGAAAGCT -3') and SunA Xa RP (5'-AGCTTTTCCTAATCCACGACCTTCGATTTTTCGAGTTTCCTC -3') as primers and pET15b sunA as template (underline indicates nucleotide change). The PCR mixture included 2X Pfx Amp Buffer (Invitrogen), dNTPs (2.5 mM each), MgSO$_4$ (1 mM), Platinum Pfx DNA polymerase (0.05 U/μL), and primers (1.5 μL each). Amplifications were confirmed by 1% agarose gel electrophoresis. To digest the methylated template prior to transformation, 1X REACT 4 buffer and 1 μL of DpnI (Invitrogen) was added to the PCR product and the digest was incubated at 37 °C for 3 h. E. coli DH5α cells were transformed with 2.5 μL of the digest product by heat shock, and cells were plated on LB-ampicillin agar plates and grown for 15 h at 37 °C. Several colonies were picked and used to inoculate separate 5 mL cultures of LB-ampicillin medium. The cultures were grown at 37 °C for 12 h, and plasmids were isolated using a QIAprep Spin Miniprep Kit (QIAGEN). The sequences of the resulting plasmid products were confirmed by DNA sequencing.
Mutagenesis of the *sunA* and *sunA* Xa genes to produce various mutant precursor peptides.

Site-directed mutagenesis of pET15b *sunA* and pET15b *sunA* Xa constructs was performed by either site-directed mutagenesis or multi-step overlap extension PCR. These constructs were designed in collaboration with Dr. Huan Wang and mutagenesis was performed by Dr. Huan Wang.

4.4.9. Overexpression and purification of His$_6$-SunA precursor peptides.

*E. coli* BL21 (DE3) cells or *E. coli* Rosetta 2 (DE3) cells were transformed via electroporation with a pET15b SunA construct (BL21 used for pET15b SunA; Rosetta 2 was used for all pET15b SunA constructs containing mutations: pET15b SunA Xa, pET15b SunA C22S, pET15b SunA G21E, pET15b SunA G23E, pET15b SunA G23K, and pET15b SunA Xa G21Q/G23A). A single colony transformant was used to inoculate a 30 mL culture of LB supplemented with 100 $\mu$g/mL ampicillin (and 25 $\mu$g/mL chloramphenicol for Rosetta 2 cells). The culture was grown at 37 °C for 12 h and was used to inoculate 3 L of LB containing 100 $\mu$g/mL ampicillin (and 25 $\mu$g/mL chloramphenicol for Rosetta 2 cells), and cells were grown at 37 °C to OD$_{600}$ ≈ 0.6-0.8. IPTG was added to a final concentration of 1 mM and the culture was incubated at 37 °C for an additional 3 h. His$_6$-SunA peptides were expressed as insoluble peptides. Cells were harvested by centrifugation at 12,000 $\times g$ for 15 min at 4 °C, and the pellet was resuspended in 30 mL of start buffer (20 mM NaH$_2$PO$_4$ (pH 7.5), 500 mM NaCl, 0.5 mM imidazole, 20% glycerol) and stored at −80 °C.

The cell paste was suspended in start buffer and the suspension was sonicated on ice for 20 min to lyse the cells. Cell debris was removed by centrifugation at 23,700 $\times g$ for 30 min at 4 °C. The supernatant was discarded and the pellet containing the insoluble peptide was
resuspended in 30 mL of start buffer. The sonication and centrifugation steps were repeated. Again the supernatant was discarded and the pellet was resuspended in 30 mL of buffer 1 (6 M guanidine HCl, 20 mM NaH$_2$PO$_4$ (pH 7.5), 500 mM NaCl, 0.5 mM imidazole). The sample was sonicated and insoluble material was removed by centrifugation at 23,700 ×g for 30 min at 4 °C, followed by filtration of the supernatant through a 0.45 μm filter. The filtered sample was applied to a 5 mL HisTrap HP (GE Healthcare Life Sciences) immobilized metal affinity chromatography (IMAC) column previously charged with NiSO$_4$ and equilibrated in buffer 1. The column was washed with two column volumes of buffer 1, followed by two column volumes of buffer 2 (4 M guanidine HCl, 20 mM NaH$_2$PO$_4$ (pH 7.5), 500 mM NaCl, 30 mM imidazole). The peptide was eluted with 1-2 column volumes of elution buffer (4 M guanidine HCl, 20 mM NaH$_2$PO$_4$ (pH 7.5), 500 mM NaCl, 1 M imidazole). The fractions were desalted using a ZipTipC$_{18}$ and analyzed by MALDI-TOF MS. The fractions containing the desired peptide were pooled and purified using preparative HPLC.

Preparative HPLC was performed using a Waters Delta 600 instrument equipped with a Waters Delta-Pak C4 column (15 μm, 300 Å, 100 mm x 25 mm) equilibrated in 2% B (solvent A = 0.1% TFA in water, solvent B = 0.0866% TFA in 80% ACN/20% water). Peptide containing material was applied to the column and was fractionated using a gradient from 2-100% B over 45 min with a flow rate of 8.0 mL/min. All fractions were analyzed by MALDI-TOF MS as described above. Purified peptide was lyophilized to dryness and stored under N$_2$ at −80 °C until further use. Typical yields from 3 L of cell culture were 15-20 mg His$_6$-SunA, 5-8 mg His$_6$-SunA Xa, 3-5 mg of each of His$_6$-SunA C22S, His$_6$-SunA G21E, His$_6$-SunA G23E, His$_6$-SunA G23K, and 1-1.5 mg of His$_6$-SunA Xa G21Q/G23A.
4.4.10. Overexpression and purification of His$_6$-SunS

_E. coli_ Rosetta 2 (DE3) cells were transformed with the pET28b SunS construct via electroporation. A single colony transformant was used to inoculate a 30 mL culture of LB supplemented with 50 μg/mL kanamycin. The culture was grown at 37 °C for 12 h and was used to inoculate 3 L of LB containing 50 μg/mL kanamycin, and cells were grown at 37 °C to OD$_{600}$ ≈ 0.6. The culture was incubated at 4 °C on ice for 20 min, then IPTG was added to a final concentration of 0.5 mM and the culture was incubated at 18 °C for an additional 16-20 h. Cells were harvested by centrifugation at 12,000 ×g for 15 min at 4 °C, and the pellet was resuspended in 30 mL of start buffer (20 mM Tris (pH 8.0), 500 mM NaCl, 1 mM TCEP, 10% glycerol) and stored at −80 °C.

All protein purification steps were performed at 4 °C. The cell paste was suspended in start buffer and the cells were lysed using a high pressure homogenizer (Avestin, Inc.). Cell debris was pelleted via centrifugation at 23,700 ×g for 20 min at 4 °C. The supernatant was injected via a superloop onto a fast protein liquid chromatography (FPLC) system (ÄKTA, GE Heathcare Life Sciences) equipped with a 5 mL HisTrap HP IMAC column previously charged with Ni$^{2+}$ and equilibrated in start buffer. The column was washed with 50 mL of buffer A (30 mM imidazole, 20 mM Tris, pH 8.0, 1 M NaCl) and the protein was eluted using a linear gradient of 0-100% B (buffer B = 200 mM imidazole, 20 mM Tris, pH 8.0, 1 M NaCl) over 40 min at a 2 mL/min flow rate. UV absorbance (280 nm) was monitored and fractions were collected and analyzed by SDS-PAGE (4-20% Tris-glycine READY gel, BioRAD). The fractions containing SunS were combined and concentrated using an Amicon Ultra-15 Centrifugal Filter Unit (10 kDa MWCO, Millipore). Gel filtration purification was used to further purify SunS. The concentrated protein sample was injected onto an FPLC system.
(ÄKTA) equipped with an XK16 16/60 (GE Healthcare Life Sciences) column packed with SuperDex 75 resin previously equilibrated in 20 mM HEPES (pH 7.5), 100 mM KCl, and 1 mM TCEP. The protein was eluted with a flow rate of 0.9 mL/min. Both UV absorbance (280 nm) and conductance were monitored and fractions were collected. Misfolded/aggregated protein was efficiently separated from soluble, correctly folded protein and the desired fractions were combined and concentrated using an Amicon Ultra-15 Centrifugal Filter Unit. The resulting protein sample was stored at −80 °C. Protein concentration was determined using a Bradford Assay Kit (Pierce) and typically yields were 15-18 mg His6-SunS from 3 L of cell culture.

4.4.11. Enzymatic in vitro sugar modification of SunA peptides by SunS

His6-SunA (50 μM) was incubated with His6-SunS (2 μM) in a reaction buffer containing 50 mM Tris (pH 7.5), 1 mM MgCl2, 1 mM TCEP, and 5 mM NDP-sugar (final concentrations). Five NDP-sugars were independently evaluated: uridine-5′-diphosphate-α-D-glucose (UDP-Glc, CalBiochem), uridine-5′-diphosphate-α-D-galactose (UDP-Gal, CalBiochem), uridine-5′-diphosphate-α-D-N-acetylglucosamine (UDP-GlcNAc, Sigma), guanosine-5′-diphosphate-α-D-mannose (GDP-Man, CalBiochem), and uridine-5′-diphosphate-α-D-xylose (UDP-Xyl, Complex Carbohydrate Research Center, University of Georgia). The reactions were incubated at 25 °C for various durations of time (1-12 h depending on future use). Reactions were either immediately used for subsequent proteolytic leader peptide removal reactions and oxidative folding or quenched with 5% TFA to pH 1-2 for MALDI-TOF MS analysis. For MALDI-TOF MS analysis, samples were desalted using ZipTipC18 and analyzed as described above. For modification of His6-SunA mutant peptides (His6-SunA Xa, His6-SunA C22S, His6-SunA G21E,
His$_6$-SunA G23E, His$_6$-SunA G23K, and His$_6$-SunA Xa G21Q/G23A, reactions were conducted under identical conditions as described above.

4.4.12. Chymotrypsin digests of sugar modified His$_6$-SunA peptides

Modified His$_6$-SunA (taken directly from the SunS reaction) was digested in 100 mM Tris (pH 7.5), 5 mM TCEP, and 0.1 mg/mL chymotrypsin (Worthington). All reactions were incubated at 25 °C for 5 h and then quenched with 5% TFA to pH 1-2. Quenched samples were desalted with a ZipTip$_{C18}$ prior to analysis by MALDI-TOF MS or analyzed by LC-ESI-Q/TOF MS without further manipulation. Representative data for GlcNAc labeled His6-SunA are shown in Figure 4.40a and b.

![Figure 4.40a. ESI-Q/TOF MS analysis of N-acetylglucosamine modified His$_6$-SunA digested with chymotrypsin under reducing conditions. His$_6$-SunA was modified with N-acetylglucosamine, digested with chymotrypsin under reducing conditions, and analyzed by using LC-ESI-Q/TOF MS. Chymotrypsin cleavage sites (F, W, and Y residues) are indicated in the peptide sequence above the spectrum. The masses of the observed ions and their corresponding digest fragments are assigned. The results are consistent with Ser54 and Thr57 remaining unmodified and the peptide spanning residues Leu50-Tyr70 bearing a +203 Da post-translational modification.](image-url)
Figure 4.40b. ESI-Q/TOF MSMS analysis of the peptide spanning residues Leu50-Tyr70 of N-acetylglucosamine-modified His$_6$-SunA. His$_6$-SunA was modified with N-acetylglucosamine, digested with chymotrypsin under reducing conditions, and analyzed by using LC-ESI-Q/TOF MSMS. The digest mixture was fractionated using LC and the multiply charged ion corresponding to residues Leu50-Tyr70 was selected for MSMS analysis. Fragmentation patterns are indicated for the y" ion series (upper) and the b ion series (lower). The results confirm that Ser54 and Thr57 remain unmodified and Cys60 has a +203 Da post-translational modification.
4.4.13. Base-catalyzed β-elimination of glucose from Cys22 of sublancin

Two different strong bases were used to evaluate the ability to β-eliminate the sugar from \textit{in vivo} isolated sublancin peptide (Figure 4.41). For elimination with a strong base under reducing conditions, pure sublancin was dissolved to a final concentration of 25 μM in a solution containing 0.3 M NaBH$_4$ and 0.1 M NaOH and was incubated at 4 °C for 5 h followed by quenching the reaction with 5% TFA to pH 1-2. The sample was desalted using a ZipTip$_{C18}$ and analyzed by MALDI-TOF and ESI-Q/TOF MS (Figure 4.41a, c, and d). For elimination with a strong base under non-reducing conditions, pure sublancin was dissolved to a final concentration of 25 μM in a solution containing 0.1 M 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) in n-PrOH and was incubated at 25 °C for 5 h followed by quenching the reaction with 5% TFA to pH 1-2. The sample was desalted using a ZipTip$_{C18}$ and analyzed by MALDI-TOF MS (Figure 4.41b). Sublancin samples that were subjected to base-catalyzed β-elimination were digested under reducing conditions with chymotrypsin in a similar manner as described for modified His$_6$-SunA peptides and analyzed by ESI-Q/TOF MSMS (Figure 4.41c and d).
Figure 4.41a. Investigation of the site of modification on Cys22 by chemical degradation. MALDI-TOF MS analysis of base-catalyzed β-elimination of the sugar of sublancin. The sugar of sublancin was β-eliminated by treating native sublancin with NaBH₄ under basic conditions and analyzed by MALDI-TOF MS. Elimination to form an alkene followed by reduction of the alkene was observed. The overlay of mass spectra obtained by MALDI-TOF MS analysis of sublancin before (blue) and after treatment with NaBH₄ (red) is shown.

Figure 4.41b. Non-reductive elimination of the sugar of sublancin. The sugar of sublancin was β-eliminated by treating native sublancin with DBU and analyzed by MALDI-TOF MS. Elimination to form an alkene was observed. The overlay of mass spectra obtained by MALDI-TOF MS analysis of sublancin before (blue) and after treatment with DBU (magenta) is shown.
Figure 4.41c. ESI-Q/TOF MS analysis of base-catalyzed β-elimination of the sugar and chymotrypsin digest of sublancin. The sugar of sublancin was β-eliminated by treating native sublancin with NaBH₄ under basic conditions, digested with chymotrypsin under reducing conditions, and analyzed by LC-ESI-Q/TOF MS. The resulting mass spectrum is shown. Chymotrypsin cleavage sites (F, W, and Y residues) are indicated in the peptide sequence above the spectrum. The masses of the observed ions and their corresponding digest fragments are assigned. The results are consistent with Ser54 and Thr57 remaining unmodified and the chemical conversion from Cys22 bearing a +162 Da post-translational modification to form an Ala at position 22.
Figure 4.41d. ESI-Q/TOF MSMS analysis of the peptide spanning residues Leu12-Tyr32 after base-catalyzed β-elimination of the sugar and chymotrypsin digest of sublancin. The sugar of sublancin was β-eliminated by treating native sublancin with NaBH$_4$ under basic conditions, the product was digested with chymotrypsin under reducing conditions, and analyzed using LC-ESI-Q/TOF MSMS. The digest mixture was fractionated using LC and the multiply charged ion corresponding to the peptide spanning residues Leu12-Tyr32 was selected for MSMS analysis. Fragmentation patterns are indicated for the y' ion series (upper) and the b ion series (lower). The results support that Ser16 and Thr19 remain unmodified and Cys22 has lost its post-translational modification and was converted to an Ala residue.
4.4.14. Production of isotopically labeled sublancin and NMR analysis

Four samples of sublancin were prepared for NMR analysis. For a sample in 100% D$_2$O, lyophilized sublancin was dissolved in 100% D$_2$O (Cambridge Isotope Laboratories) to exchange amide protons and simplify the NMR spectrum in the down field region. The sample was lyophilized, the procedure was repeated twice, and the final sample was dissolved in D$_2$O to a final concentration of approximately 2.5 mM. For a sample in 90% H$_2$O/10% D$_2$O, lyophilized sublancin was dissolved in 90% H$_2$O/10% D$_2$O to a final concentration of approximately 2.5 mM.

A $^{13}$C-labeled sublancin sample was obtained by growing *B. subtilis* 168 on minimal medium containing uniformly $^{13}$C-labeled glucose.$^{51}$ An culture of *B. subtilis* 168 was grown in LB medium for 15 h with aeration. This culture was used to inoculate a 500 mL volume of minimal media in a 2 L flask. The minimal media contained the following components per 500 mL total volume: 25 mL of 20% $^{13}$C-glucose, 100 mL of 5X M9 salts, 5 mL of 4 mg/mL Leucine, 500 $\mu$L of 20 mg/mL CaCl$_2$, 500 $\mu$L of 120 mg/mL MgSO$_4$, 100 $\mu$L of 5 mg/mL D-biotin, 100 $\mu$L of 5 mg/mL thiamine-HCl, 500 $\mu$L of 1000X heavy metal stock solution, and 368.3 mL of millipore water to bring total volume to 500 mL. The 5X M9 salts solution consisted of 64 g Na$_2$HPO$_4$ 7H$_2$O, 15 g KH$_2$PO$_4$, 2.5 g NaCl, 5 g NH$_4$Cl, and 1 L millipore water. The 1000X heavy metal stock solution was prepared by combining the following components in 1 M HCl, stirred overnight at 25° C, and filter sterilized to remove insoluble material.
<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>MoNa$_2$SO$_4$•2H$_2$O</td>
<td>500 mg</td>
</tr>
<tr>
<td>CoCl$_2$</td>
<td>250 mg</td>
</tr>
<tr>
<td>CuSO$_4$•5H$_2$O</td>
<td>175 mg</td>
</tr>
<tr>
<td>MnSO$_4$•H$_2$O</td>
<td>1 g</td>
</tr>
<tr>
<td>MgSO$_4$•7H$_2$O</td>
<td>8.75 g</td>
</tr>
<tr>
<td>ZnSO$_4$•7H$_2$O</td>
<td>1.25 g</td>
</tr>
<tr>
<td>FeCl$_2$•4H$_2$O</td>
<td>1.25 g</td>
</tr>
<tr>
<td>CaCl$_2$•2H$_2$O</td>
<td>2.5 g</td>
</tr>
<tr>
<td>H$_3$BO$_3$</td>
<td>1 g</td>
</tr>
<tr>
<td>1 M HCl</td>
<td>to 1 L</td>
</tr>
</tbody>
</table>

A $^{13}$C/$^{15}$N-labeled sublancin sample was obtained by growing $B$. subtilis 168 on minimal medium containing uniformly $^{13}$C-labeled glucose and $^{15}$NH$_4$Cl.$^{51}$ The minimal media contained the following components per 500 mL total volume: 25 mL of 20% $^{13}$C-glucose, 100 mL of 5X M9 salts, 5 mL of 4 mg/mL leucine, 500 µL of 20 mg/mL CaCl$_2$, 500 µL of 120 mg/mL MgSO$_4$, 100 µL of 5 mg/mL D-biotin, 100 µL of 5 mg/mL thiamine-HCl, 500 µL of 1000X heavy metal stock solution, and 368.3 mL of millipore water to bring the total volume to 500 mL. The 5X M9 salts solution consisted of 64 g Na$_2$HPO$_4$ 7H$_2$O, 15 g KH$_2$PO$_4$, 2.5 g NaCl, 5 g $^{15}$NH$_4$Cl, and 1 L millipore water. The 1000X heavy metal stock solution were prepared as described above.

A culture of $B$. subtilis 168 was grown in LB medium for 15 h with aeration. This culture was used to inoculate a 500 mL volume of minimal media in a 2 L flask and was grown at 37°C with aeration for up to 96 hrs. The production of labeled sublancin was monitored by periodic sampling of the growing culture and analysis via MALDI-TOF mass spectrometry. Briefly, a 1 mL aliquot of $B$. subtilis 168 minimal media culture was acidified to pH 2 with concentrated phosphoric acid (85% in water). The sample was centrifuged to remove cells and the supernatant was ZipTip$_{C18}$ desalted, and analyzed by mass spectrometry. Typically, isolation and purification process was started 24 h after the first detection of labeled product. Labeled sublancin was generally observed within 48-72 h post-inoculation. Isotopically labeled sublancin was purified.
using identical conditions as described above for natural abundance sample (acidification, cell harvest, ammonium sulfate precipitation, and preparative HPLC). A natural abundance sublancin sample was prepared during the preparation of the labeled peptides as a control.

**Figure 4.42.** MALDI-TOF mass spectrum of purified $^{13}$C-labeled sublancin. Expected [M+H]: 4040.38, observed: 4038.96.
Figure 4.43. MALDI-TOF mass spectrum of purified $^{13}\text{C}^{15}\text{N}$-labeled sublancin. Expected [M+H]: 4090.38, observed: 4086.41.

Figure 4.44. MALDI-TOF mass spectrum of purified natural abundance sublancin that was produced in parallel with the isotopic labeling preparations except using natural abundance culture growth medium. Expected [M+H]: 3876.74, observed: 3880.76.
All solution NMR spectra were acquired by Dr. John Boettcher at the NMR Facility (School of Chemical Sciences, University of Illinois at Urbana-Champaign) on a Varian INOVA 500 MHz spectrometer equipped with a 5 mm triple resonance ($^1$H−$^{13}$C−$^{15}$N) triaxial gradient probe, using VNMRJ version 2.1B with the BioPack suite of pulse programs released in early 2006. One and two-dimensional $^1$H homonuclear spectra were acquired on both unlabeled sublancin samples at 25° C. Two-dimensional COSY, TOCSY and NOESY spectra were measured for an average of 2 h per spectrum, digitizing 1024 points in the indirect $^1$H dimension ($t_{1,\text{max}} = 128$ ms) and were utilized by Dr. Boettcher to establish correlations among backbone resonances and the conjugated glucose ring of the sublancin sample. A $^{13}$C-HSQC spectrum was acquired on a $^{13}$C-labeled sublancin sample digitizing 256 points in the $^{13}$C dimension ($t_{1,\text{max}} = 15$ ms). These spectra were utilized to establish correlations between carbon and proton resonances of the conjugated glucose of sublancin. An additional COSY spectra with a long pulse delay (9 ms) was acquired with the unlabeled sublancin sample in 100% D$_2$O for measurement of $^3$J$_{\text{H-H}}$ couplings. Spectra were processed with NMRPipe$^{52}$, and analyzed in Sparky$^{53}$. The $^3$J$_{\text{H-H}}$ couplings were measured using the ACME program supplied as a feature of the NMRPipe software.$^{54}$

Initial efforts focused on the region of the spectrum from 6.0 ppm to 7.5 ppm that was previously suggested to contain resonances from the Dha residue, as reported by Paik et al.$^5$ For this specific investigation the sample in 100% D$_2$O was used to avoid overlap with amide proton resonances as was also done by Paik et al.. The previous study relied on one-dimensional $^1$H spectra and chemical shift analysis to propose that the resonance at 6.2 ppm belonged to the Dha residue and the remaining resonances were attributed to aromatic side chain protons. However, whereas the resonance at 6.2 ppm was seen in the proton spectrum no correlations to carbon
atoms were seen in the $^{13}$C-HSQC spectrum. The $^{13}$C-HSQC spectrum does show correlations of the protons downfield of 6.0 ppm with carbon atoms with chemical shifts between ~112-133 ppm. These observations are consistent with the aromatic side chains found in sublancin. The resonance at 6.2 ppm can at present not be assigned but is likely arising from a proton attached to a heteroatom that is resistant to exchange with solvent. The data do rule out, however, that this resonance is associated with a Dha residue, which agrees with the mass spectrometric analysis.

With a $^{13}$C-HSQC spectrum in hand, the focus was shifted onto the unique chemical shifts of the carbon atoms found in sugars. All the carbons in the glucose group were predicted to have chemical shifts between ~70 and 90 ppm. Chemical shifts in this range are not commonly found for amino acids except for the beta carbon of Thr. Only a single Thr is present in sublancin, whereas 7 carbon resonances can be seen in this region of the HSQC spectrum. Combining this information with the connectivity observed in the COSY spectrum, assignments were made for the resonances arising from the sugar protons and carbons. Unique assignments were possible for positions 1, 2 and 6, relying on the downfield shift of the anomeric carbon and proton resonances to assign C1/H1, and using the COSY correlations to subsequently assign C2/H2. C6/H6 was also assigned based on both chemical shift and COSY correlations. The 3, 4 and 5 carbon chemical shifts and the associated proton chemical shifts could not be assigned site-specifically due to degeneracy in the proton chemical shifts. A tentative assignment of the resonance of the beta carbon of threonine 19 was also made.

After establishing the assignments of the conjugated glucose the NMR spectra were used by Dr. Boettcher to determine the stereochemistry of the glycosylation site. By measuring the $^3J_{H-H}$ coupling constant between the anomeric proton (proton 1) and the neighboring proton (proton 2) the dihedral angle was calculated using the Karplus relationship.$^{30}$ A COSY spectrum
acquired with a long pulse delay was used to determine the $^{3}J_{HH}$ coupling constant between these two protons. Using the ACME software the cross peak of 1-2 was fit with a $^{3}J_{HH}$ coupling constant of 9.965 Hz. This results in a dihedral angle of $171^\circ \pm 10^\circ$ based on the Karplus equation$^{30}$ and indicates a diaxial orientation of the two protons. Combined with the knowledge that the sugar molecule is a glucose, the stereochemistry of conjugation to Cys22 must be through a $\beta$-linkage.

4.4.15. Kinetics of SunS glycosyltransferase activity using end-point assays

To evaluate the NDP-sugar substrate preference of His$_6$-SunS, His$_6$-SunA was modified with different NDP-sugars at varied NDP-sugar concentrations and products were quantified using HPLC. His$_6$-SunA (5 $\mu$M) was incubated with His$_6$-SunS (2 $\mu$M) in a 250 $\mu$L reaction buffer containing 50 mM Tris (pH 7.5), 1 mM MgCl$_2$, 1 mM TCEP, and varying concentrations of NDP-sugar (final concentrations). NDP-sugar concentrations were 5 mM, 50 $\mu$M, or 500 nM. All five NDP-sugars were independently evaluated: UDP-Glc, UDP-Gal, UDP-GlcNAc, GDP-Man, and UDP-Xyl. The reactions were incubated at 25 °C for 1 h. Reactions were quenched with TFA to pH 1-2 and analyzed by MALDI-TOF MS and the extent of glycosylation was quantified by analytical HPLC. For MALDI-TOF MS analysis, samples were desalted using ZipTip$_{C18}$ and analyzed as described above (Figure 4.45a-e upper).

HPLC analysis was performed using a Beckman Coulter System Gold HPLC equipped with a Grace-Vydac Protein C4 column (5 $\mu$m, 300 Å, 250 mm x 4.6 mm) equilibrated in 2% solvent B (solvent A = 0.1% TFA in water, solvent B = 0.0866% TFA in 80% ACN/20% water). A 200 $\mu$L volume of quenched SunS reaction was applied to the column. Sugar-modified and unmodified His$_6$-SunA wt peptide were eluted by maintaining the mobile phase at 2% B for
1 min, followed by an increase to 100% B over 45 min with a flow rate of 1.0 mL/min. Under these conditions, sugar-modified His6-SunA wt peptide and unmodified His6-SunA wt peptide eluted at 24.3 and 24.8 min, respectively (Figure 4.44a-e lower). All fractions were analyzed by MALDI-TOF MS as described above. The relative amounts of sugar-modified and unmodified His6-SunA peptide was determined by quantifying the peak area corresponding to each form of the peptide by integration. Percent conversion was calculated by dividing the peak area of sugar-modified peptide by the sum of the sugar-modified and unmodified peak areas.
Figure 4.45a-e. Kinetics of SunS glycosyltransferase activity using end-point assays. To identify the NDP-sugar substrate preference of SunS, His$_6$-SunA was modified with different NDP-sugars at varied NDP-sugar concentrations and products were analyzed by MALDI-TOF MS and quantified using HPLC. The glycosyltransferase reactions were run for 1 h, quenched, and the reaction mixture was analyzed by MALDI-TOF MS. The reaction mixture, containing unmodified starting material and glycosylated product, was fractionated by HPLC and relative amounts of unmodified and modified His$_6$-SunA peptide were quantified by peak integration. For each NDP-sugar tested, an overlay of MALDI-TOF mass spectra (upper) and an overlay of analytical HPLC chromatograms (lower) are shown.

Figure 4.45a. SunS glycosyltransferase activity with UDP-glucose.
Figure 4.45b. SunS glycosyltransferase activity with UDP-galactose.
Figure 4.45c. SunS glycosyltransferase activity with GDP-mannose.
Figure 4.45d. SunS glycosyltransferase activity with UDP-N-acetylglucosamine.
Figure 4.45e. SunS glycosyltransferase activity with UDP-xylose.
**4.4.16. Proteolysis of SunA Xa precursor and purification of leader and core peptides**

To assess whether the leader peptide of SunA is required for SunS-catalyzed core peptide modification, His₆-SunA Xa precursor peptide was proteolytically cleaved using Factor Xa protease to generate leader peptide and unmodified core peptide. His₆-SunA Xa (1.0 mg/mL) was digested in 50 mM Tris (pH 7.5), 100 mM NaCl, 2 mM CaCl₂, and 0.1 mg/mL Factor Xa (New England Biolabs). The reaction was incubated at 25 °C for 2.5 h and an aliquot of the reaction was quenched with 5% TFA to pH 1-2 prior to desalting by ZipTip C₁₈ and analysis by MALDI-TOF MS. At 2.5 h, no starting material was observed by MS (Figure 4.46a), and the remainder of reaction was quenched with 5% TFA to pH 1-2. The leader and core peptide were then purified using HPLC. HPLC was performed using a Beckman Coulter System Gold HPLC equipped with a Phenomenex Jupiter Proteo C₁₂ column (10 μm, 90 Å, 250 mm x 4.6 mm) equilibrated in 2% solvent B (solvent A = 0.1% TFA in water, solvent B = 0.0866% TFA in 80% ACN/20% water). The quenched proteolysis reaction containing a mixture of core and leader peptide was centrifuged at 15 krpm in a microcentrifuge for 2 min and the resulting supernatant was applied to the column. The core and leader peptides were eluted by maintaining the mobile phase at 2% B for 1 min, followed by an increase to 100% B over 45 min with a flow rate of 1.0 mL/min. Under these conditions, the leader peptide and core peptide eluted at 22.7 and 25.5 min, respectively. All fractions were analyzed by MALDI-TOF MS as described above. Purified material was lyophilized to dryness and stored under N₂ at −80 °C until further use. Typical yields were 0.2 mg of leader and 0.15 mg of core peptide per mg of His₆-SunA Xa (Figure 4.46b-c).
Figure 4.46a. His6-SunA Xa was digested with Factor Xa protease and analyzed by MALDI-TOF MS. The cleavage products (leader and core peptide) were then purified using reversed-phase HPLC and analyzed by MALDI-TOF MS. The resulting mass spectrum of crude cleavage mixture is shown. Factor Xa cleavage occurred C-terminal to Arg38 resulting in leader peptide (Gly1-Arg38) and core peptide (Gly39-Arg75). Non-specific cleavage occurred C-terminal to Arg71 to produce a truncated core peptide (Gly39-Arg71). The masses of the observed ions and their corresponding digest fragments are assigned.
Figure 4.46b. The mass spectrum of HPLC purified leader peptide (Gly1-Arg38) is shown. The masses of the observed ions and their corresponding digest fragments are assigned. Expected [M+H]: 4367.84, observed: 4369.14.
4.4.17. Evaluation of the requirement of leader peptide for core peptide modification by SunS

Purified leader and core peptide were incubated in combination (in trans) and independently in the presence of SunS reaction components. For the in trans reaction, leader peptide (10 μM) and core peptide (10 μM) were incubated with His₆-SunS (2 μM) in a reaction buffer containing 50 mM Tris (pH 7.5), 1 mM MgCl₂, 1 mM TCEP, and 5 mM UDP-glucose (all final concentrations). For the independent reactions, leader peptide (10 μM) or core peptide (10 μM) was incubated with His₆-SunS (2 μM) in a reaction buffer containing 50 mM Tris (pH 7.5), 1 mM MgCl₂, 1 mM TCEP, and 5 mM UDP-glucose. All reactions were incubated at 25 °C for 1
h and then quenched with 5% TFA to pH 1-2. Quenched samples were desalted using a ZipTipC18 prior to analysis by MALDI-TOF MS (Figure 4.47a-b).

**Figure 4.47a.** SunA core peptide was modified with glucose by incubation with His<sub>6</sub>-SunS in the presence of UDP-Glc, MgCl<sub>2</sub>, and TCEP in Tris (pH 7.5) buffer. The leader peptide was not added to the reaction. The reaction was analyzed by MALDI-TOF MS. The overlay of mass spectra obtained by MALDI-TOF MS analysis of the mixture before (black) and after SunS reaction (red) is shown. Expected [M+H] (with glycosylation): 3882.41, observed: 3883.91.
Figure 4.47b. SunA leader peptide was not modified with glucose by incubation with His$_6$-SunS in the presence of UDP-Glc, MgCl$_2$, and TCEP in Tris (pH 7.5) buffer. The core peptide was not added to the reaction. The reaction was analyzed by MALDI-TOF MS. The overlay of mass spectra obtained by MALDI-TOF MS analysis of the mixture before (black) and after SunS reaction (red) is shown. Expected [M+H] (without glycosylation): 4367.84, observed: 4368.81.
Figure 4.47c. SunA core peptide was modified with glucose by incubation with His$_6$-SunS in the presence of SunA leader peptide, UDP-Glc, MgCl$_2$, and TCEP in Tris (pH 7.5) buffer. SunA core peptide and leader peptide were present in equimolar concentrations. The reaction was analyzed by MALDI-TOF MS. The overlay of mass spectra obtained by MALDI-TOF MS analysis of the mixture before (black) and after SunS reaction (red) is shown. Expected [M+H]$^+$ (core peptide with glycosylation): 3882.41, observed: 3882.88.

4.4.18. In vitro preparation of sublancin and sublancin analogues for bioactivity assays and LC-ESI-Q/TOF MS" analysis

Sublancin and sublancin analogues containing non-natural sugars were prepared by modifying His$_6$-SunA Xa peptide with a sugar (SunS reaction), proteolytic removal of the leader peptide (Factor Xa reaction), and oxidative folding of sublancin to afford the disulfide linkages (oxidative folding reaction). Sugar modified His$_6$-SunA Xa was prepared in 250 μL of 50 mM Tris (pH 7.5), 1 mM MgCl$_2$, 1 mM TCEP, 5 mM NDP-sugar, 50 μM His$_6$-SunA Xa, and 2 μM...
His\textsubscript{6}-SunS. The reaction was incubated at 25 °C for 12 h. The extent of sugar modification was verified by removing a 5 μL aliquot of the reaction, quenching with 5% TFA to pH 1-2, desalting using a ZipTip\textsubscript{C18}, and analysis by MALDI-TOF and ESI Q/TOF MS. Following analysis, the leader peptide of sugar modified His\textsubscript{6}-SunA Xa was proteolytically cleaved by the addition of NaCl and CaCl\textsubscript{2} to 100 mM and 2 mM, respectively, and the addition of Factor Xa to 0.075 mg/mL (final concentrations). The reaction was incubated at 25 °C for 4-6 h and the extent of cleavage was monitored by MALDI-TOF MS as stated above. Following analysis, the disulfides of the modified sublancin core peptide were formed by addition of Tris (pH 7.5), oxidized glutathione (GSSG), reduced glutathione (GSH), and EDTA to final concentrations of 50 mM, 2 mM, 2 mM, and 0.1 mM, respectively. The total volume of the oxidative folding reaction was 500 μL and the reaction was incubated at 25 °C for an additional 12 h. The extent of disulfide formation was monitored by removing a 5 μL aliquot of the reaction, quenching with 5% TFA to pH 1-2, desalting using a ZipTip\textsubscript{C18}, and analyzing by MALDI-TOF MS. Disulfide formation was observed as a peak with a −4 Da mass difference compared with material that was not subjected to oxidative folding.

4.4.19. Evaluation of \textit{in vitro} prepared sublancin and sublancin analogues

The \textit{in vitro} preparation of sublancin and sublancin analogues was evaluated in three ways. The sugar modification, leader peptide removal, and oxidative folding are all required to obtain bioactivity against the indicator strain \textit{B. subtilis} ATCC 6633. As such, the \textit{in vitro} prepared peptides were assessed using the antimicrobial activity assay described below.

In addition to bioactivity assays, sublancin and analogues were structurally characterized via LC-ESI-Q/TOF tandem MS and the resulting fragmentation patterns of the acquired mass
spectra were compared to native and reduced authentic sublancin analyzed under identical conditions (Figure 4.48a-f). For additional confirmation of correct disulfide bond formation of in vitro prepared sublancin and sublancin analogues, the fully modified peptides were independently proteolytically digested with chymotrypsin and the reactions were analyzed by LC-ESI-Q/TOF MS. Briefly, in vitro prepared peptide (SunS treated, Factor Xa treated, and oxidatively folded) was digested under non-reducing conditions in 100 mM Tris (pH 7.5) and 0.05 mg/mL chymotrypsin. All reactions were incubated at 25 °C for 3 h and then quenched with 5% TFA to pH 1-2. Quenched samples were analyzed by LC-ESI-Q/TOF MS without further manipulation (Figure 4.49a-e).
**Figure 4.48a-f.** ESI-Q/TOF MSMS analysis of in vitro prepared sublancin and sublancin analogues. His$_6$-SunA Xa was modified with sugar, digested with Factor Xa, oxidatively folded, and analyzed by using LC-ESI-Q/TOF MSMS. Material for all four sugars and aglycon material was analyzed. If disulfide bonds were correctly formed during the oxidative folding process, then fragmentation of the peptide backbone was not expected to occur. Rather, the post-translation modification (S-linked glycosylation) was expected to be lost from the parent ion (loss of 162 Da). As controls, an authentic sublancin sample (isolated and purified from B. subtilis 168) and a reduced sublancin sample (authentic sublancin treated with TCEP) were independently analyzed using identical instrument settings as with in vitro prepared samples.

**Figure 4.48a.** His$_6$-SunA Xa was modified with glucose, digested with Factor Xa, oxidatively folded, and analyzed by using LC-ESI-Q/TOF MSMS. Fragmentation of the amide bonds of sublancin (Glc) did not occur, rather, the glucosylation was lost from the parent ion (-162 Da).
Figure 4.48b. His₆-SunA Xa was modified with galactose, digested with Factor Xa, oxidatively folded, and analyzed by using LC-ESI-Q/TOF MSMS. Fragmentation of the amide bonds of sublancin (Gal) did not occur, rather, the galactosylation was lost from the parent ion (-162 Da).

Figure 4.48c. His₆-SunA Xa was modified with mannose, digested with Factor Xa, oxidatively folded, and analyzed by using LC-ESI-Q/TOF MSMS. Fragmentation of the amide bonds of sublancin (Man) did not occur, rather, the mannosylation was lost from the parent ion (-162 Da).
Figure 4.48d. His$_6$-SunA Xa was modified with $N$-acetylglucosamine, digested with Factor Xa, oxidatively folded, and analyzed by using LC-ESI-Q/TOF MSMS. Fragmentation of the amide bonds of sublancin (GlcNAc) did not occur, rather, the $N$-acetylglucosaminylation was lost from the parent ion (-203 Da).

Figure 4.48e. His$_6$-SunA Xa was unmodified, digested with Factor Xa, oxidatively folded, and analyzed by using LC-ESI-Q/TOF MSMS. Fragmentation of the amide bonds of sublancin (aglycon) did not occur.
Figure 4.48f. As a control, pure sublancin isolated from *B. subtilis* 168 was analyzed using LC-ESI-Q/TOF MSMS. Fragmentation of the amide bonds of sublancin did not occur, rather, the glycosylation was lost from the parent ion (-162 Da).
Figure 4.49a-e. ESI-Q/TOF MS analysis of *in vitro* prepared sublancin and sublancin analogues digested with chymotrypsin under non-reducing conditions. His$_6$-SunA Xa was modified with sugar, digested with Factor Xa, oxidatively folded, and digested with chymotrypsin under non-reducing conditions. The reactions were analyzed by LC-ESI-Q/TOF MS to investigate extent of glycosylation and correct disulfide bridge connectivity. Chymotrypsin cleavage sites (F, W, and Y residues) and the proposed disulfide bridges are indicated in the peptide sequence above the spectrum. The masses of the observed ions and their corresponding digest fragments are assigned. The results support that a disulfide bond exists between Cys$_7$-Cys$_{36}$ and between Cys$_{14}$-Cys$_{29}$. The results are consistent with residues Leu$_{12}$-Tyr$_{32}$ bearing a glycosylation in reactions which His$_6$-SunA Xa peptide was modified with a sugar.

Figure 4.49a. ESI-Q/TOF MS analysis of *in vitro* prepared sublancin (Glc) digested with chymotrypsin under non-reducing conditions. His$_6$-SunA Xa was modified with glucose, digested with Factor Xa, oxidatively folded, digested with chymotrypsin under non-reducing conditions, and analyzed by using LC-ESI-Q/TOF MS.
Figure 4.49b. ESI-Q/TOF MS analysis of *in vitro* prepared sublancin (Gal) digested with chymotrypsin under non-reducing conditions. His6-SunA Xa was modified with galactose, digested with Factor Xa, oxidatively folded, digested with chymotrypsin under non-reducing conditions, and analyzed by using LC-ESI-Q/TOF MS.
Figure 4.49c. ESI-Q/TOF MS analysis of *in vitro* prepared sublancin (Man) digested with chymotrypsin under non-reducing conditions. His$_6$-SunA Xa was modified with mannose, digested with Factor Xa, oxidatively folded, digested with chymotrypsin under non-reducing conditions, and analyzed by using LC-ESI-Q/TOF MS.
**Figure 4.49d.** ESI-Q/TOF MS analysis of *in vitro* prepared sublancin (GlcNAc) digested with chymotrypsin under non-reducing conditions. His$_6$-SunA Xa was modified with N-acetylglucosamine, digested with Factor Xa, oxidatively folded, digested with chymotrypsin under non-reducing conditions, and analyzed by using LC-ESI-Q/TOF MS.
Figure 4.49e. ESI-Q/TOF MS analysis of in vitro prepared sublancin (aglycon) digested with chymotrypsin under non-reducing conditions. His$_6$-SunA Xa was not modified with a sugar, digested with Factor Xa, oxidatively folded, digested with chymotrypsin under non-reducing conditions, and analyzed by using LC-ESI-Q/TOF MS.
4.4.20. Antimicrobial activity assays of in vitro prepared sublancin and sublancin analogues

The 500 μL reactions described above (SunS reaction, Factor Xa reaction, folding reaction) were concentrated to 20 μL via vacuum centrifugation. An overnight culture of *B. subtilis* ATCC 6633 (indicator strain) was grown in LB media under aerobic conditions at 37 °C for 12 h. Ninety-six well agar plates were prepared by combining 20 mL of molten LB medium agar (cooled to 42 °C) with 50 μL of dense overnight culture (approx 10^8-10^9 CFU/mL). The seeded agar was poured into a sterile OmniTray (Nunc) and allowed to solidify at 25 °C for 30 min. An additional 30 mL of molten LB medium was cooled to 42 °C, combined with 75 μL of culture, and poured over the lower solidified agar layer. A sterile 96-well PCR plate was placed in the molten agar upper layer and was allowed to solidify at 25 °C for 45 min. After sufficient solidification, the 96-well PCR plate was removed. The total 20 μL volume of each concentrated in vitro reaction was dispensed into separate newly formed wells. Authentic sublancin standards were spotted in 15 μL volumes at the concentrations indicated. Plates were left at 25 °C for 15 h and antibacterial activity was qualitatively determined by the presence or absence of growth inhibition.

4.4.21. Antimicrobial activity assay with native, reduced, and reduced and alkylated sublancin

The importance of intact disulfides for the bioactivity of sublancin was assessed by testing the activities of native, reduced, and reduced and alkylated sublancin against *B. subtilis* ATCC 6633. Native sublancin was prepared by dissolving pure sublancin in 50 mM Tris (pH 7.5 or pH 8.3). Reduced sublancin was prepared by dissolving pure sublancin in 50 mM Tris (pH 8.3) with 5 mM TCEP. Reduced and alkylated sublancin was prepared by dissolving pure
sublancin in 50 mM Tris (pH 8.3), 5 mM TCEP, and 10 mM iodoacetamide. All reactions contained sublancin at a final concentration of 50 μM and were prepared on a 50 μL scale. All reactions were incubated at 25 °C for 3 h in the dark, then concentrated to 20 μL prior to dispensing into separate wells of a bioactivity plate (prepared as described above) seeded with *B. subtilis* ATCC 6633. Authentic sublancin and nisin standards were spotted in 15 μL volumes at the concentrations indicated. Plates were left at 25 °C for 15 h and antibacterial activity was qualitatively determined by the presence or absence of growth inhibition.

### 4.4.22. Antimicrobial activity assay of *in vitro* prepared sublancin with omission of single steps

The importance of each step (glycosylation, leader peptide removal, and disulfide formation) in the *in vitro* preparation of sublancin was demonstrated through an antimicrobial activity assay against *B. subtilis* ATCC 6633. Sublancin samples were prepared in parallel whereby one step of the preparation process was omitted.

To prepare non-glucosylated sublancin, leader peptide of unmodified His6-SunA Xa was first removed by combining 50 mM Tris (pH 7.5), 100 mM NaCl, 2 mM CaCl2, 0.075 mg/mL Factor Xa, and 50 μM His6-SunA Xa in a 250 μL reaction. The reaction was incubated at 25 °C for 6 h. The disulfides were formed by addition of Tris (pH 7.5), oxidized glutathione (GSSG), reduced glutathione (GSH), and EDTA to final concentrations of 50 mM, 2 mM, 2 mM, and 0.1 mM, respectively. The 500 μL total volume reaction was incubated at 25 °C for 12 h.

To prepare sublancin with the leader peptide intact, His6-SunA Xa was first glucosylated by combining 50 mM Tris (pH 7.5), 1 mM MgCl2, 1 mM TCEP, 5 mM UDP-glucose, 50 μM His6-SunA Xa, and 2 μM His6-SunS in 250 μL. The reaction was incubated at 25 °C for 12 h.
The disulfides of the modified His6-SunA Xa peptide were formed by addition of Tris (pH 7.5), oxidized glutathione (GSSG), reduced glutathione (GSH), and EDTA to final concentrations of 50 mM, 2 mM, 2 mM, and 0.1 mM, respectively. The 500 μL total volume reaction was incubated at 25 °C for 12 h.

To prepare unfolded sublancin, His6-SunA Xa was first glucosylated by combining 50 mM Tris (pH 7.5), 1 mM MgCl₂, 1 mM TCEP, 5 mM UDP-glucose, 50 μM His6-SunA Xa, and 2 μM His6-SunS in 250 μL. The reaction was incubated at 25 °C for 12 h. The leader peptide of glucosylated His6-SunA Xa was then cleaved by addition of 50 mM Tris (pH 7.5), 100 mM NaCl, 2 mM CaCl₂, 0.075 mg/mL Factor Xa (final concentrations) to the 250 μL reaction. The reaction was incubated at 25 °C for 6 h.

As a positive control, all three steps were performed in the following order: SunS glucosylation reaction, Factor Xa leader peptide cleavage reaction, and oxidative folding reaction. All of the above reactions were concentrated to 20 μL via vacuum centrifugation and the total 20 μL volumes were separately dispensed into separate wells of a bioactivity plate (prepared as described above) seeded with B. subtilis ATCC 6633. Authentic sublancin standards were spotted in 15 μL volumes at the concentrations indicated. Plates were left at 25 °C for 15 h and antibacterial activity was qualitatively determined by the presence or absence of growth inhibition.

**4.4.23. Efforts toward identifying the molecular target of sublancin**

The gene encoding *E. coli* PBP1b was previously PCR amplified from MG1655 genomic DNA and cloned into pET21b vector (Novagen) as a C-terminal hexa-histidine (His6) fusion gene. The enzyme was expressed and purified by Tsung-Shing Andrew Wang or Tania Lupoli
[14C]GlcNAc-labeled heptaprenyl lipid II analogue was chemo-enzymatically prepared by Dr. Yuto Sumida and Dr. Hiro Tsukamoto (Dan Kahne Laboratory) as previously described.55

For IC$_{50}$ determinations, assays were carried out by separately incubating [14C]GlcNAc-labeled heptaprenyl lipid II analogue (final concentration = 4 μM, typical specific activity = 288 μCi/μmol) and peptide inhibitors (concentrations are indicated in the figure legends) in low-binding microcentrifuge tubes (VWR) containing 9 μL of buffer consisting of 50 mM HEPES (pH 7.5), 10 mM CaCl$_2$, 1,000 units/mL penicillin G, 0.2 mM octaethylene glycol monodecyl ether (decyl-PEG; Anatrace, Maumee, OH), and 11% DMSO (v/v) for 30 min at room temperature prior to PBP1b addition (to allow binding to occur). Reactions were started by adding 1 μL of PBP1b (from a solution freshly prepared by diluting the 50% glycerol stock 20-fold into PBP1b dilution buffer consisting of 5 mM Tris (pH 8.0), 8 mM decyl-PEG) to the reaction mixture (final PBP1b concentration = 20-100 nM). Reactions were typically stopped after 30-45 min by adding 10 μL of ice-cold 10 mM Tris (pH 8.0) containing 10% Triton X-100. Quenched reactions were stored on ice until they were spotted on cellulose chromatography paper strips (3MM Whatman chromatography paper #3030-861, cut 1 cm width x 20 cm height). Products and starting material were separated using chromatography (isobutyric acid/1 M NH$_4$OH, 5:3). The lipid II starting material migrates on the strip, while polymerized product remains at the origin. The paper strips were removed from chromatography chambers, allowed to dry, cut, and added to separate scintillation vials containing EcoLite(+) liquid scintillation fluid (MP Biomedical). Samples were analyzed using a LS6500 scintillation counter (Beckman Coulter). The percent radioactivity of the polymer product was calculated by comparing the
amount of radioactivity that remains at the origin (peptidoglycan) to the total amount of radioactivity on the strip.

4.4.24. Overexpression and purification of His\textsubscript{6}-ThuA precursor peptide

His\textsubscript{6}-ThuA was overexpressed and purified as described for His\textsubscript{6}-SunA peptides (see Experimental 4.4.8)

4.4.25. Overexpression and purification of His\textsubscript{6}-ThuS

\textit{E. coli} Rosetta 2 (DE3) cells were transformed with the pET28b ThuS construct via electroporation. A single colony transformant was used to inoculate a 30 mL culture of LB supplemented with 50 \(\mu\)g/mL kanamycin. The culture was grown at 37 °C for 12 h and was used to inoculate 3 L of LB containing 50 \(\mu\)g/mL kanamycin, and cells were grown at 37 °C to OD\textsubscript{600} \(\approx 0.6\). The culture was incubated at 4 °C on ice for 20 min, then IPTG was added to a final concentration of 0.5 mM and the culture was incubated at 18 °C for an additional 16-20 h. Cells were harvested by centrifugation at 12,000 \(\times\)g for 15 min at 4 °C, and the pellet was resuspended in 30 mL of start buffer (20 mM Tris (pH 8.0), 500 mM NaCl, 1 mM TCEP, 10% glycerol) and stored at \(-80\) °C.

All protein purification steps were performed at 4 °C. The cell paste was suspended in start buffer and the cells were lysed using a high pressure homogenizer (Avestin, Inc.). Cell debris was pelleted via centrifugation at 23,700 \(\times\)g for 20 min at 4 °C. The supernatant was injected via a superloop onto a fast protein liquid chromatography (FPLC) system (ÄKTA, GE Heathcare Life Sciences) equipped with a 5 mL HisTrap HP IMAC column previously charged with Ni\textsuperscript{2+} and equilibrated in start buffer. The column was washed with 50 mL of buffer A (30
mM imidazole, 20 mM Tris, pH 8.0, 1 M NaCl) and the protein was eluted using a linear gradient of 0-100% B (buffer B = 200 mM imidazole, 20 mM Tris, pH 8.0, 1 M NaCl) over 40 min at a 2 mL/min flow rate. UV (280 nm) was monitored and fractions were collected and analyzed by SDS-PAGE (4-20% Tris-glycine READY gel, BioRAD). The fractions containing SunS were combined and concentrated using an Amicon Ultra-15 Centrifugal Filter Unit (10 kDa MWCO, Millipore). Gel filtration purification was used to further purify ThuS. The concentrated protein sample was injected onto an FPLC system (ÄKTA) equipped with an XK16 16/60 (GE Healthcare Life Sciences) column packed with SuperDex 75 resin previously equilibrated in 20 mM HEPES (pH 7.5), 100 mM KCl, and 1 mM TCEP. The protein was eluted with a flow rate of 0.9 mL/min. Both UV (280 nm) and conductance were monitored and fractions were collected. Misfolded/aggregated protein was efficiently separated from soluble, correctly folded protein and the desired fractions were combined and concentrated using an Amicon Ultra-15 Centrifugal Filter Unit. The resulting protein sample was stored at −80 °C. Protein concentration was determined using a Bradford Assay Kit (Pierce) and typically yields were 100-140 mg His6-ThuS from 3 L of cell culture.

4.4.26. Enzymatic in vitro sugar modification of ThuA and SunA peptides by ThuS or SunS

His6-precursor peptide (50 μM) was incubated with His6-glycosyltransferase (2 μM) in a reaction buffer containing 50 mM Tris (pH 7.5), 1 mM MgCl2, 1 mM TCEP, and 5 mM uridine-5′-diphosphate-α-D-glucose (UDP-Glc, CalBiochem). The reactions were incubated at 25 °C for various durations of time (1-12 h depending on future use). Reactions were either immediately used for subsequent proteolysis studies or quenched with 5% TFA to pH 1-2 for MALDI-TOF
MS analysis. For MALDI-TOF MS analysis, samples were desalted using ZipTip_{C18} and analyzed as described above.

4.4.27. Proteolytic digests of multi-sugar modified His_{6}-precursor peptides

Modified His_{6}-SunA peptide (taken directly from the glycosyltransferase reaction) was digested in 100 mM Tris (pH 7.5), 5 mM TCEP, and 0.1 mg/mL chymotrypsin (Worthington). Modified His_{6}-ThuA peptide (taken directly from the glycosyltransferase reaction) was digested in 100 mM Tris (pH 7.5), 5 mM TCEP, 1 mM CaCl_{2}, and 0.1 mg/mL trypsin (Worthington). All reactions were incubated at 25 °C for 5 h and then quenched with 5% TFA to pH 1-2. Quenched samples were desalted with a ZipTip_{C18} prior to analysis by MALDI-TOF MS or analyzed by LC-ESI-Q/TOF MS" without further manipulation.
4.5. REFERENCES


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